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The evaluation of morphological and molecular techniques for discrimination among and verification of lucerne (*Medicago sativa*) cultivars

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Plant Science

(Seed Technology)

at Massey University

New Zealand

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1996

ABSTRACT

The stability and usefulness of plant morphological characters for discrimination among lucerne (*Medicago sativa* L.) cultivars was investigated under three sets of field and glasshouse conditions, using two New Zealand cultivars and four Iranian ecotypic cultivars representing diverse geographical adaptations. Following the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV) and the requirements of the Organization for Economic Co-operation and Development (OECD) seed certification scheme for testing distinctness, homogeneity and stability of lucerne cultivars in field test plots, morphological data were recorded from four replicates of 18 spaced plants per cultivar in the field in the establishment year (1993) and the following year. Flower colour was recorded from a further 150 one-and two-year old plants of each cultivar in the field. In a glasshouse, where the minimum and maximum temperatures were set at 16°C and 22°C respectively, morphological characters were recorded from three replicates of 22 one-year old plants of each cultivar. The majority of the 12 morphological characters recommended by UPOV and OECD for discrimination and verification of lucerne cultivars were not independent of the environment. Only three characters, number of plant stems immediately above the ground, plant recovery height, and leaflet width/length ratio were stable, and thus could be used as reliable morphological descriptors for lucerne cultivars. However, none of the individual characters, or any combination of these characters, were sufficient to differentiate all of the cultivars at $P<0.01$, the standard required by UPOV to detect differences among lucerne cultivars. Morphologically based methods are therefore not effective for discrimination among lucerne cultivars, and there is a need to find more precise and effective techniques for assessing whether cultivars are actually different (UPOV) or whether individual seed lots of a cultivar do not differ from the cultivar standard (OECD).

A digital image processing algorithm (VIPS) was used for image processing of 150 individual seeds of 17 seed lots of eight lucerne cultivars and also for image analysis of 66 leaflets from individual glasshouse grown plants of six cultivars. Of the 21 morphological characters and derived measurements recorded from individual seeds of

the cultivars, 10 were useful for cultivar verification and discrimination. Among individual characters, red colour/total intensity, and blue/total intensity of seed had the highest, while actual area of seed/convex area had the lowest ability (64 % vs 18%) to discriminate among cultivars. A better cultivar discrimination (86%) was obtained on the basis of a combined value from the 10 selected characters using Canonical Discriminant Analysis. This was further improved (to 94%) using the result from the 10 individual characters plus the combination of the selected seed characters. Using image analysis of leaflets of individual plants, only 67% of the cultivars could be discriminated.

Discontinuous SDS-polyacrylamide electrophoresis was used for protein analysis of both bulked and single seeds of 14 seed lots from six Iranian and two New Zealand cultivars. A series of preliminary experiments was first undertaken to determine the optimal protein loading volume for bulk, and also single seed samples of the cultivars, to enable production of clear, sharp and therefore easily evaluated bands. Of the many protein bands produced as a result of SDS-PAGE analysis of single seeds of the cultivars, the seventeen most distinctive bands were selected for analysis. Their intensities were assessed using a Vision Image Processing System (VIPS), and the data were used for discrimination among the cultivars. Electrophoresis of protein extracted from the bulked seed samples failed to differentiate the majority of the cultivars. However, 82% of the cultivars could be differentiated using the combined results from intensities of the 17 selected protein bands from single seeds of the cultivars. Since all eight cultivars could not be discriminated using SDS-PAGE of individual seeds, two other molecular techniques (Restriction Fragment Length Polymorphism (RFLP) and Random Amplified DNA Polymorphism (RAPD) were employed to determine if better discrimination among lucerne cultivars was possible using DNA techniques.

DNA samples were extracted from 4-5 frozen leaves (at -70° C) from 40 individual 45 day old seedlings of six Iranian, two New Zealand, and two internationally recognised cultivars. Following purification and quantification, bulked DNA samples of the cultivars, plus those from individual seedlings were digested with four common restriction endonucleases to cleave the DNA into smaller fragments. The digested DNA

fragments were separated by agarose gel electrophoresis and were then transferred to a nylon membrane. This membrane was hybridised with nine probes *i.e.* six lucerne cDNA probes (I013, 492, 281, 328, 473, 457), two apple probes (ADH, cDNA and ribosomal DNA (rDNA)) and one clover probe (ADH) to determine the best combination between restriction enzymes and probes to optimize the number of polymorphic bands. A non-radioactive method was used for probe labelling.

Of the six lucerne probes screened with the restriction endonucleases (EcoR *I*, Bam H*I*, Hind *III* and Xba *I*) all except I013 produced extremely faint bands from the bulked DNA samples of the cultivars. Although a combination of I013 and Hind *III* gave the highest number of RFLP fragments in initial experiment, the result was not reproducible.

No distinct RFLPs were detected using the clover ADH probe from the bulked DNA samples of the cultivars, and the apple rDNA probe detected polymorphism between only some of the cultivars. This result suggested that DNA fingerprinting of lucerne cultivars may not be feasible on the basis of the RFLPs from bulk DNA samples of the cultivars. It was therefore hypothesised that better discrimination among the cultivars might be possible on a population basis *i.e.* by examining the percentage of plants within each cultivar containing particular fragments. This hypothesis was tested by analysing scoring data based on the presence (1) and absence (0) of the RFLP fragments from 40 individual seedlings of each cultivar using Canonical Discriminant Analysis. RFLP analysis of individual seedlings of the cultivar using each of the rDNA and the ADH probes produced distinct but highly polymorphic RFLPs among the seedlings which was an indication of great genetic diversity within each of the cultivars. Seventy percent of cultivar pairs could be discriminated using the RFLPs detected by apple rDNA compared with 56% for the clover ADH probe at $P<0.05$. Some pairs of cultivars which could not be discriminated using the rDNA, were discriminated on the basis of RFLPs detected by the ADH probe. When the results from these two probes were combined, 91% of the cultivars could be discriminated.

Using the PCR based RAPD technique, bulk DNA extracts from 10 lucerne cultivars plus DNA from 40 individual 45 day old seedlings of each cultivar were analyzed.

Twenty-six 10-base arbitrary primers were screened using bulk DNA samples of the cultivars to select those which were able to generate clear RAPD bands. Of these, four (*i.e.* OPA08, OPB13 OPO19 and OPC10) produced sharp and distinctive RAPD bands. OPB19 produced the highest number of distinct RAPD fragments and all of the cultivars, even those which were closely related, could be discriminated. Although all of the cultivars could not be discriminated using the RAPD profiles generated by OPA08, OPB13 and OPC10 individually, a combination of the results from these primers provided sufficient information for discrimination among all of the 10 cultivars. The number of distinct RAPD fragments generated by a combination of the primers OPB19 and OPC10 was less than those produced by individual primers alone. To check the reproducibility of the RAPD results, replicated reactions were carried out using two primers (OPB19 and OPC10) alone and in combination with the standard reaction mixture using different batches of enzyme from the same manufacturer. This demonstrated that a high degree of reproducibility of results obtained by the RAPD technique is possible.

To assess genetic variation within and among the cultivars, nine distinct RAPD bands generated by primer OPO8 were scored. The RAPD products from 40 individual seedlings of the each cultivars produced distinct but very diverse profiles, indicating genetic diversity within each individual population. Pairwise comparison of the LSMEANS of the RAPD profiles illustrated that 86% of the cultivars were significantly different.

These molecular techniques and seed image analysis were also assessed for their ability to test uniformity, and detect genetic relationships among the cultivars. For seed protein and DNA analysis, uniformity was assessed using similarity of protein and DNA banding profiles from individual seeds and seedlings from each of the seed lots of the lucerne cultivars. For seed image analysis, uniformity of the cultivars was assessed on the basis of the proportion of uniform seeds in each of the seed lots. Genetic relatedness among the cultivars was detected using the squared Mahalanobis distances (D^2) of the cultivars.

The genetic relatedness established among the cultivars was almost the same irrespective of whether the three molecular or the seed image analysis techniques were used, despite the different nature of each analysis. The results were consistent with the genetic background, autumn-dormancy and geographical adaptation of the cultivars examined. However, these techniques did not produced the same results for testing of uniformity. Analysis of a larger sample size from a number of cultivars with known genetic background would be needed before drawing any final conclusion as to the relative merits of these techniques for estimation of relatedness and uniformity of lucerne cultivars.

Comparisons between protein banding profiles and also seed morphological characters of seed lots from the same cultivar illustrated significant differences between the seed lots of some of the cultivars. This suggested that genetic shift had occurred during seed multiplication. As all of the seed lots studied had been certified under the OECD scheme, the scheme is apparently not capable of detecting this degree of genetic shift.

Of all the techniques investigated, image analysis is regarded as the most suitable alterative to plot testing for discrimination among, and verification of lucerne cultivars, and for detection of genetic shift. The reasons for this are the speed of analysis, lower running costs, lack of need for chemicals and ease of operation of the equipment. However, this will be dependent on access to image analysis facilities. For genetic analysis of lucerne cultivars the RAPD technique was considered the best among the molecular techniques used.

In the name of God the most compassionate the most merciful

ACKNOWLEDGMENTS

It is a pleasure to thank the many people who have each contributed in their own way to the completion of this thesis.

Firstly, I would like to thank God for his love, blessing and his guidance enabling me to complete my study.

My sincerest thanks to my chief supervisor, Professor John G. Hampton of the Seed Technology Centre, Massey University, for his guidance and wise supervision through the conducting of experiments and writing of the manuscript. Appreciation and thanks are extended to my co-supervisor, Dr. Sue E. Gardiner of the Molecular Genetic Group, HortResearch, CRI, Palmerston North for her direct supervision and extremely valuable guidance and encouragement for genetic analysis of lucerne cultivars.

My sincere thanks also extended to my other co-supervisors *i.e.*

Prof. M. J. Hill, Director of the Seed Technology Centre Massey University for his support, and valuable guidance and parental care during this study.

Assoc. Prof. S. J. Haslett, Director of the Applied Statistics Consulting Centre, Massey University, Palmerston North, New Zealand, for his advice on the statistical analysis.
Prof. R.G. Thomas of Plant Biology and Biotechnology, Massey University, New Zealand for his guidance in the plant physiological study.

Dr. D. Woodfield of Agricultural research, CRI, Palmerston North, New Zealand, for his advise and guidance

I would like to express my gratitude to my wife, Tahereh, for her support, patience, encouragement and help in many ways for running the experiments, and also to my son,

Mahdi, and my daughter, Zeinab, for their valuable assistance for data collection and analysis and my youngest son, Mohammad-Hossian whose smile and sweetness was great encouragement for continuation of this study.

I would like to thank Dr. D.G. Bailey of the Image Analysis Unit, Massey University for his guidance and writing the required image analysis programs in this study and also Dr. M.A. Naderi-Shahab; Dr. Bob Skip; Mr. B. Show; Dr. M.R. Ghannaha; Dr. S.R. Mirhosseini; Dr. H. Mirzaie and Dr. M. Askarian for their help and guidance.

I would like express my sincere thanks to Mr. Ray Johnston; Mr. Robert Southward; Mr Hugh Neilson; Mr. Charlie H. Forbes; Mr Criag McGill; Mr. Naba R. Devkota; Ms. Colette Gwynne; Ms Kathy Hamilton; Ms Karen Hill; Ms Ruth G. Morrison of the Plant Science Department, Massey University for providing technical assistance during this study.

Ms Heather Bassett and Ms Charolotte Madie of the Molecular Genetic Group, Horticultural Research, CRI, Palmerston North for their technical assistance.

I am grateful for the grant awarded to me by the Ministry of Agriculture through the Sugar Beet Research Institute and the Agricultural Research Organization, and by the Ministry of Culture and Higher Education of the Islamic Republic of Iran.

I thank the Seed and Plant Improvement Institute, Ministry of Agriculture, Karadj, Islamic Republic of Iran for the supply of seed of the Iranian cultivars, and the Margot Forde Germplasm Centre, New Zealand Pastoral Agriculture Research Institute Ltd., Palmerston North, New Zealand for supply of seed of other cultivars.

To all these people, Thank you.

Majid Dehghan-Shoar

March 1996

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CHAPTER 1

GENERAL INTRODUCTION

The word 'cultivar' was created by combining the words 'cultivated' and 'variety' (Kelly, 1989) and is defined in Article 10 of the International Code of Nomenclature for Cultivated Plants (1980), as "an assemblage of cultivated individuals which is distinguished by any characters (morphological, physiological, cytological, chemical or others) and which when reproduced (sexually or asexually), retains its distinguishing features". This term is used throughout this thesis, rather than the term 'variety', the only exception being the term Plant Variety Rights (PVR).

According to Articles 6.1a, 6.1c and 6.1.d of the Convention for the Protection of New Varieties of Plants (1978) " Whatever may be the origin, artificial or natural, of the initial variation from which it has resulted, the new cultivar must be clearly distinguishable by one or more important characteristics from any other cultivar whose existence is a matter of common knowledge at the time when protection is applied for ..... A new cultivar may be defined and distinguished by morphological or physiological characteristics. In all cases such characteristics must be capable of precise description and recognition. The new cultivar must be sufficiently homogenous, having regard to the particular features of its sexual reproduction or vegetative propagation, and must be stable in its essential characteristics; that is to say, it must remain true to its description after repeated reproduction or repropagation".

The granting of plant variety rights (PVR) to the breeder of a specific cultivar provides protection from other people who could otherwise freely multiply or reproduce the material. The International Union for the Protection of New Varieties of Plants (UPOV) was established to provide the regulatory requirements and the testing techniques required for registration of new cultivars. According to UPOV (1988), before a new cultivar can be granted PVR, it must be evaluated in growing trials to determine its distinctness, uniformity, and stability. This process is commonly termed DUS testing.

Chapter 1

Seed certification schemes such as that of the Organization for Economic Co-operation and Development (OECD, 1988) have been developed to ensure that a cultivar remains genetically the same after being multiplied by seed growers, or a breeding agency, and also to give a guarantee to the farmer that the seed which he purchased is that which it is supposed to be, and retains those beneficial characters claimed by the breeders (Hampton and Scott, 1990). Initially, field inspection was the only plant morphological based method for the check of trueness to cultivar and by inference cultivar purity (Esbo, 1980). However, two main problems were associated with the development of seed certification *i.e.* the development of a satisfactory standard for cultivar purity at field inspection of the growing seed crop, and the difficulty of identifying new cultivars as they were released (Coolbear and Hill, 1987; Kelly and Bowring, 1990). As a result it became virtually impossible to require an inspector to make a positive identification of a cultivar during a field inspection (Kelly and Bowring, 1990) and pre and post control plot testing was introduced to provide a solution to these problems (Kelly and Bowring, 1990). Therefore, in the OECD seed certification scheme for example, plot testing is used to check whether all the procedures required for seed certification have been carried out correctly (Kelly, 1989).

Plot testing largely forms the basis for testing of distinctness of a cultivar prior to cultivar registration (Cooke, 1995b). However, there are problems associated with assessing the distinctness of cultivars of cross-pollinating species, such as lucerne (*Medicago sativa* L), as a cultivar of this species comprises a population of plants containing many genotypes which can express a range of morphological characters. In lucerne, it is likely that the number of stable and environmentally independent morphological descripters is limited and therefore plot testing may fail to provide adequate morphological data for distinctness testing. This hypothesis is explored in Chapter 2. There are also other serious problems associated with plot testing (Hampton, 1991; Gardiner and Forde, 1992; Cooke, 1995b), and these are also discussed in Chapter 2. As the trend is for a steady increase in the number of lucerne cultivars, many of which are closely related (Steiner *et al.*, 1992), there is a need to develop accurate, fast,

repeatable and cost effective techniques for verification and identification of lucerne, both for DUS testing and seed certification. In the remaining chapters of this thesis the usefulness of some alternatives to plot testing have been investigated. In addition, the usefulness of these techniques for estimation of genetic relatedness among cultivars was also assessed.

Automated vision systems, commonly termed image analysis, have been used for a wide range of tasks in agriculture. For example for quantification of seed and leaf shape and leaf colour of a wide range of different plant species (eg. strawberry, Engels and Petry, 1991; barley, Goto *et.al.*, 1991; conifers, Kershaw and Larsen, 1992), root length and diameter (eg. ryegrass, Cochrane *et al.*, 1990; small-seeded field vegetables, McCormac *et.al.*, 1990); as an alternative to visual monitoring for determining of the extent of foliar diseases in a wide range of plant species (Blaich and Blaich, 1990; Dreier *et.al.*, 1991); for determination of chlorophyll fluorescence in leaves and assessment of area of photosynthetic inhibition of sunflower (Omasa and Shimazaki, 1990); for assessment of skinning injury in sweet potato roots (Bonet *et. al.*, 1993); and also for determining uniformity of tomato seedlings (Ruzhitsky and Ling, 1992).

This technique has been shown to be very cost effective (Blaich and Blaich, 1990) with reproducible results (Lebowitz, 1989; Dreier *et.al.*, 1991), and processing is rapid (Keefe, 1992; Cooke, 1995b). This latter feature suggests the technique could be very promising for verification and identification of cultivars of cross-pollinating species, as a large sample size is required to ensure that the gene pool of a cultivar is represented. Despite this interesting possibility, no reports were found in the literature regarding the use of this technique for analysis of seed or leaves of cultivars of cross-pollinating species. Therefore using single seeds and plant leaflets from eight lucerne cultivars, the usefulness of image analysis for discrimination among and verification of lucerne cultivars was assessed. In addition, the usefulness of this technique for estimation of genetic relatedness among lucerne cultivars was also assessed. This work is reported in Chapter 3.

Extensive reports in the literature (eg. Clapham and Almgard, 1976; Wright *et.al.*, 1983; Nielsen, 1985; Waltman *et al.*, 1988; Brummer *et al.*, 1991; Gardiner and Forde, 1992; Kiss *et al.*, 1993; Steiner, 1993; Vieritz, 1993; Kidwell *et al.*, 1994; Cooke, 1995a, and Faville *et al.*, 1995) suggest that electrophoretic techniques could provide alternatives for plot testing. Among cross-pollinating (allogamous) species, ryegrass (*Lolium*) is the only one for which the electrophoresis of seed storage protein has been accepted by the International Seed Testing Association for cultivar verification (ISTA, 1992; Cooke, 1995a). The possible use of isozyme electrophoresis for registration of new cultivars of this species has been also considered (UPOV, 1995). However, little work has been reported in the literature about the potential of molecular techniques for discrimination among lucerne cultivars.

Reports by Fahmi *et al.* (1990), and Gardiner and Forde (1992), suggested that electrophoresis of protein extract from bulk seed was not feasible for discrimination among lucerne cultivars. However, no published report was found as to whether discrimination among lucerne cultivars is possible using single seeds. Analysing single seed of various wheat cultivars, Cooke (1984) suggested that electrophoresis can be used to assess the uniformity of cultivars of this species. To investigate as to whether both DUS testing and verification of lucerne cultivars would be possible by means of protein electrophoresis using the SDS-PAGE method (*i.e.* polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate; Gardiner and Forde, 1992) individual seeds and also bulk seed samples of eight lucerne cultivars were analyzed. The possible application of this method for estimation of genetic relatedness among lucerne cultivars was also investigated. This is reported in Chapter 4.

Restriction fragment length polymorphism (RFLP), is the first DNA profiling technique to be widely applied in the study of genetic variation among plants of different species (Saghai-Maroof *et al.*, 1984; Gebhardt *et al.*, 1989; Brummer *et. al.*, 1991; Kidwell *et al.*, 1994; Pupilli *et al.*, 1995). RFLPs are inherited according to Mendelian genetics (Lewin, 1990; Watson *et al.*, 1992) and the individual chromosome segments close to

the RFLPs being studied can be followed as they pass from generation to generation by tracing the inheritance of the markers. This suggests that the RFLP technique could be used as a powerful tool for genetic analysis of genotypes as well as detection of polymorphism within and among cultivars. The majority of the reports in the literature refer to the application of this technique for genome mapping in a number of plant species including maize (Helentjaris *et al.*, 1988); rice (McCouch *et al.*, 1988); potato (Gebhardt *et al.*, 1989); sugar beet (Hallde'n and Tuvesson, 1991); tomato (Helentjaris *et al.*, 1986; Bernatzky and Tanksley, 1986b); and also lucerne (Brummer *et. al.*, 1991). Despite its importance as a forage crop, genetic characterization of lucerne lagged behind other major crops until 1991 (Brummer *et al.*, 1991). However, several studies on RFLP analysis of lucerne have been reported in recent years (Brummer *et al.*, 1991 and 1993; Blondon *et al.* , 1993; Kiss *et al.*, 1993; Kidwell *et al.*, 1994; Pupilli *et al.*, 1995). There is however, very little information on discrimination among lucerne cultivars. The only relevant report in the literature is that by Brummer *et al.* (1993) who used three genetically divergent cultivars, and found that discrimination among those cultivars was possible. However, it is not clear as to whether this technique could be employed for discrimination among genetically related cultivars. This question was addressed in this study by RFLP analysis of DNA extract from individual seedlings of six Iranian ecotypic cultivars (some of which are closely related), two New Zealand and two internationally recognised lucerne cultivars. Genetic relatedness among the cultivars was also investigated. These reports are discussed in Chapter 5.

Random Amplified Polymorphic DNA (RAPD, Williams *et al.*, 1990; Welsh and McClelland, 1990) are commonly used as markers in genome mapping in a wide range of plant species including lucerne (Echt *et al.*, 1992; Kiss *et al.*, 1993), and are now being increasingly employed for studies of genetic relatedness among cultivars of species which are predominantly autogamous or self-pollinating (Yu and Nguyen, 1994). This technique has also been used for discrimination among cultivars of some self-pollinating species (eg. wheat and barley, Francisco-Ortega *et al.*, 1993; broccoli, Hu and Quiros, 1991; celery, Yang and Quiros, 1993). However, reports on the use of RAPDs for

genetic analysis and discrimination among cultivars of cross-pollinating (allogamous) species are more limited. No report was found in the literature on the use of this technique for discrimination among lucerne cultivars. Using bulked DNA extracts from 10 cultivars, the usefulness of this technique for DNA finger printing of lucerne cultivars was assessed. In addition, the use of RAPD profiles from individual seedlings of the cultivars were tested for the ability to discriminate among the cultivars, as well as to provide genetic analysis of the cultivars. This work is reported in Chapter 6.

The last chapter in this study (Chapter 7) comprises a general discussion and conclusions, and recommendations for further research.

CHAPTER 2

VERIFICATION OF STABILITY, AND USEFULNESS OF PLANT MORPHOLOGICAL CHARACTERS FOR DISCRIMINATION AMONG LUCERNE (*MEDICAGO SATIVA* L.) CULTIVARS

2.1 LITERATURE REVIEW

2.1.1 Introduction

A cultivar is a group of cultivated plants, distinguishable from other groups of plants belonging to the same species by distinctive, inherited characteristics (eg. morphological, physiological or agronomic features). When reproduced, the offspring retain these distinctive characteristics.

In the early part of cultivar improvement, problems were created by frequent changing of the names of cultivars. There were no regulations with respect to cultivar names and as a result, many names could be used for one cultivar (Davidson, 1950). Seed adulteration and misrepresentation were indulged in, and to prevent fraud through admixtures of worthless materials and substitution of inferior cultivars, rules and regulations were set up for registration of new cultivars to prevent cultivar misrepresentation, and for seed certification to safeguard the genetic quality of the seed being produced. As plant morphological characters are the direct expression of the genetic construction of a genotype, field-based morphological methods, such as plot testing, were developed to fulfil the requirement for registration of new cultivars, and also for certification of the seed being produced.

2.1.2 Cultivar registration and the requirement for plot testing

According to the International Union for the Protection of New Varieties of Plants (UPOV, 1988) the three basic criteria *i.e.* distinctness, homogeneity or uniformity and stability (DUS, as defined in chapter.1) should be fulfilled for registration of

a new cultivar (termed by UPOV as 'variety').

According to UPOV (1988, 1995), a new cultivar must be clearly differentiated from other cultivars by one or more important morphological or physiological characteristics. The cultivar must be sufficiently homogenous, and stable in its essential characteristics, *i.e.* it must remain true to its description.

Unlike cultivars of vegetatively propagated (eg. potato) and inbreeding species (eg. wheat) where individual plants are almost identical, a cultivar of an out-breeding species, such as lucerne, is a heterogeneous population. Therefore, cultivars of these species must be kept, within limits, true to description by careful initial selection and description of their founder population within a specific range of variation for a number of characters. Assessment of uniformity of cultivars of out-breeding species involves plant-by-plant examination and the acceptance that distinctness can be established among cultivars with different but stable proportions of genotypes. Although assessment of uniformity is to ensure that any cultivar of such a species which shows a distinctive characteristic for registration will maintain that feature during multiplication and commercialisation, it could be claimed that it is the stability of the characters rather than absolute uniformity of the population of plants, which is essential for out-breeding species (UPOV, 1995).

Guidelines and the requirements for plot testing, as a conventional method, have been published by UPOV for different species. The DUS test requirements for lucerne cultivars are discussed in UPOV (1988). According to this guideline the minimum duration of tests should be two similar growing periods. Each test should consist of 60 single-spaced plants per cultivar arranged in three to six replicates. Morphological characters (such as plant height, leaflet length and width, time of flowering, flower colour, disease resistance) should be recorded from individual plants in the trial so that the mean value per plot can be obtained.

According to UPOV (1988), when distinctness depends on measured characteristics the differences have to be considered clear if they occur at one percent probability of an error, for example, on the basis of Least Significant Difference.

2.1.3 Seed certification and the requirement for plot testing

Seed certification schemes, controlled pedigree systems and rules and regulations for seed growing and distribution are all aimed at maintaining cultivar authenticity and purity of the seed (Hampton, 1991). The determination of cultivar purity was developed to assess the quality of the cultivar before the seed is sown (Baekgaard, 1964; ISTA, 1993).

The word "quality" when linked with seed has several meanings. According to Kelly (1973), these can best be made clear by posing three questions:

1. Is the seed of the right cultivar?
2. Is the seed healthy and capable of vigorous germination?
3. Is the seed unadulterated by weed seed, other crop seeds and inert matter?

Seed quality was defined by Esbo (1980) as "a collection of seed properties which are considered to be important for the value of seed for sowing purposes". One of these is cultivar purity. A high standard of cultivar purity can be assured by controlling the production of seed. Although absolute control over all of the operations involved is not possible, control is exercised as far as is practical in two ways (Thompson, 1979):

1. By ensuring that seed is multiplied in such a way as to minimize the risk of mechanical and genetic contamination.

2. By setting standards and checking each seed lot against these standards at a stage

that the cultivar can be identified. This can be done by checking plants from which the seed is to be harvested.

Seed certification is primarily for the purpose of assuring trueness to cultivar and purity. The first certification schemes were started to improve the assurance of trueness to cultivar, and were based on field inspection only. For the check of trueness to cultivar and cultivar purity, field inspection was complemented by check plot tests (Esbo, 1980).

Seed certification under the OECD scheme is based on trueness to cultivar (i.e. lots are certified true to the cultivar's characters, including any variation as described by the breeder), and require post control plot tests (Thompson 1979). The main object of the post control plot test is to ascertain that the scheme is operating satisfactorily, and to determine that the cultivar's characters have remained unchanged (OECD, 1968). According to the OECD scheme the plot tests are conducted for the following main purposes (OECD, 1968):

1. To check that a breeder's or basic seed lot is true to name, and that as a result of maintenance over a period of years, the cultivar is not showing any "shift" in expression of its distinguishing characters. This type of test requires objective measurement of individual plants in a spaced plant test in comparison with the standard sample (which may be from certified seed or blend of such seed, basic seed or even breeders' seed as appropriate).

2. To check that a particular certified seed lot is true to the cultivar name and has not been mixed with other cultivars or otherwise altered during multiplication. Such tests can normally be performed in row or sward plots in comparison with basic seed lots of known satisfactory cultivar authenticity.

Under this scheme all certified seed produced must be related directly through one

or more generations to authenticated basic seed of the cultivar. Satisfactory conditions for the production and processing of basic seed and certified seed must be ensured and verified by field inspection and post control plot test testing.

Post control testing is normally conducted within an independent organisation such as a research station or agricultural college, where there is suitable land and adequate equipment. Therefore, characteristics of growing plants can be observed by skilled workers at the optimum time. This provides unbiased opinion of the quality of the seed under test, and also access to skilled personnel and equipment in such organizations (Hampton, 1991). Plants in check plots are planted in such a way that the cultivar characters are emphasised (eg. by spacing the plants).

The characters recommended by OECD (1968; 1988) for assessment of trueness of cultivars varies from species to species. The following characters are recommended to be recorded from individual plants in the plot test for cultivar certification of lucerne seed lots moving in international trade:

1. Height of plant in the spring, when the first sign of growth appears on the earliest cultivar in the trial.
2. Height of plants one month later.
3. Growth during the month (1-2).
4. Length and width of leaflet in mm (at early flower bud stage on 4th leaf below bud, both measurements on the terminal leaflet). The combination of leaflet length and leaflet width will sometimes serve to distinguish between cultivars having the same or similar leaflet length.
5. Angle of growth at early flower bud stage.
6. Date of flowering, when three florets are open.
7. Height of longest stem at flowering to top of inflorescence.
8. Flower colour; each plant can be classified according to the colour: blue, violet, variegated, white or yellow.

Note: Each cultivar is cut after all plants of the latest cultivar in the trial have flowered.

9. Recovery height, three weeks after cutting.

10. Recovery height, six weeks after cutting.

Note: All cultivars are cut again six weeks after the cutting at 10.

11. Recovery height , three weeks after second cutting

12. Disease observations (eg. resistance to *Pseudopeziza medicaginis).*

13. Percentage of plants killed in winter.

In addition, other characters such as plant colour and the growth habit (erect, semi erect, medium), are also recommended by UPOV (1988) for testing uniformity, distinctness and stability (DUS test) of lucerne cultivars.

As discussed in the following sections, some of the morphological characters and physiological behaviour of lucerne plants may be influenced by environmental factors and also by management systems. This suggests that two populations of plants which are, for example, different for a morphological character at one site, may not be necessarily different if the plants are grown under different sets of environmental conditions. This appears to be a potential problem associated with DUS testing on the basis of morphological data from plot testing .

In addition, as the OECD recommended characters for lucerne plants are based on experiments in England, it has been suggested by OECD that where climatic conditions are different, it is necessary to determine which characters should be used for plot testing (OECD, 1968). This implies that the usefulness of a particular morphological character for identification of cultivars might vary from country to country, or even within a country. This suggests that finding the most stable and highly discriminating morphological and physiological characters is the key for performing successful plot tests for certification of the seeds being produced.

2.1.4 Leaf morphology

Leaf size is one of the morphological characters recommended for cultivar identification. However, there is evidence that this character can be influenced by the environment and thus may not be very useful for cultivar identification. For example, the effect of temperature on growth and development of lucerne plants has been studied by Bula (1972). This study was conducted using 40 individual plants from each of three cultivars (4 replicates of 10 plants/cultivar), representing a wide range of environmental adaptation (cv. African, DuPuits, and Culver). Plants were grown in controlled environment chambers under a regime that provided in excess of 35Klux illumination from fluorescent and filament lamps, 16-hour photoperiod, 50% relative humidity, and constant air temperature of 20° C, 25° C, 30° C, and 35° C respectively. The aerial portions of the plants were harvested twice *i.e.* when flowering started (25 days after the seed was sown) and 22 days later. Leaf area, (cm^2/plant), and also terminal leaflet size (width multiplied by length, cm^2), were recorded from each individual harvested plant. Plants grown at the highest temperature (35° C) had smaller cells, which in turn resulted in smaller leaves and a lower leaf area per plant, compared to the more optimum temperature of 25° C. Generally, leaf area per plant was highest for plants at 25° C and lowest at 35° C. As this research was conducted only to study growth and development in lucerne plants in general, there was no emphasis on the leaf area variations among the cultivars in response to the different temperature regimes. Data in Table 2.1.1., adapted from Bula (1972), clearly demonstrate interactions between cultivar x temperature and also the age of the plants for leaf area. For example, despite little variation in the average leaf area of cvs. African and DuPuits (Table 2.1.1), variation was larger for cv. Culver, where there was a significant difference between leaf area of plants grown at 20° and 25° C; 20° and 35° C, and also among 25°, 30°, and 35° C. This suggests that temperature sensitivity response can vary from cultivar to cultivar.

Comparison between leaf areas of 22 and 47 day old plants suggests that age of plants also interacted with temperature (Table 2.1.1). For example the large variation in leaf area of the 22 day old plants of cv. Culver was reduced for the 47 day old plants, when only plants grown at 25° C had a larger leaf area than those grown at 35° (Table 2.1.1). This temperature response pattern, however, was unlike that for cv. DuPuits, in which older plants showed more sensitivity to different temperature regimes than younger plants.

Table 2.1.1 Leaf area of three lucerne cultivars grown under four temperature regimes, 22 and 47 days after seed was sown (adapted from Bula, 1972).

Cultivar	Leaf area, cm^2/plant (22 days after sowing)				Leaf area, cm^2/plant (47 days after sowing)			
	African	DuPuits	Culver	Mean	African	DuPuits	Culver	Mean
20° C	85 a	99 a	87 b	90 a	145 a	95 b	76 ab	106 ab
25° C	86 a	85 a	116 a	95 a	156 a	152 a	122 a	143 a
30° C	62 ab	90 a	67 bc	73 ab	88 b	91 b	95 ab	92 ab
35° C	46 b	43 b	57 c	49 b	62 b	59 b	62 b	61 b

* Means for each cultivar at the four temperatures (columns) followed by the same letter do not differ at $P< 0.05$ according to Duncan's Multiple Range Test.

Bula (1972) also found that size of the fully developed central leaflet, as indicated by width x length measurement, was largest for plants grown at 20° C, whereas plants grown at 30° and 35° C had considerably smaller leaves than those grown at 20° and 25° C, and plants grown at 25° C were intermediate to those grown at 20° and 30° or 35° C (Table 2.1.2).

Unlike leaf area, which was influenced by temperature, age of the plants, cultivar and also probable interactions between these factors, the temperature response pattern for leaf size was unique among the cultivars and, interestingly, this was

almost consistent for plants at the two different growth development stages (Table 2.1.2). This suggests that unlike leaf area, the size of the central leaflet can be used as a stable character for identification of lucerne cultivars.

Table 2.1.2 Size (width multiplied by length) of fully developed centre leaflets, sampled from 22 and 47 day old plants grown under four temperature regimes as presented by Bula, 1972).

Cultivar	Leaflet size, cm^2 (22 days after sowing)				Leaflet size, cm^2 (47 days after sowing)			
	African	DuPuits	Culver	Mean	African	DuPuits	Culver	Mean
20° C	1.56	2.14	2.61	2.10	3.90	4.59	2.70	3.73
25° C	1.51	1.77	1.13	1.47	2.42	2.78	2.25	2.48
30° C	0.94	0.94	0.87	0.92	1.48	1.59	0.80	1.29
35° C	0.75	1.18	0.85	0.93	1.68	1.37	1.33	1.46

No literature was found regarding the influence of other environmental factors or management systems on leaf morphology of lucerne plants. However, some information is available for the influence of these factors on leaf morphology of white clover, another out-crossing species from the Leguminosae.

A comparison among white clover populations from areas of less than 760 mm rainfall and from more than 1400 mm rainfall has shown that when grown in a uniform environment, dry-land populations are larger leaved, more cyanogenic, taller and have a wider taproot diameter than populations from wet environments (Caradus, 1994). Similarly, analysis of a collection of white clover populations from Southwest Europe grown in a uniform environment has shown that high latitude populations were smaller leaved, later flowering and had poorer spring growth but better persistence in New Zealand than low latitude populations. Most of these differences are possibly due to the effect of temperature and day length

with changing latitude; high latitude populations being subjected to lower temperature and longer days (Caradus, 1994). Exposure to frequent frosting for example, results in natural selection of plants with small leaves, a prostrate habit and low cyanogenic reaction (Caradus *et al.*, 1989).

Leaf size in white clover is associated with intensity and frequency of defoliation, with larger leaf size being expressed under lax infrequent defoliation. Much of this variation is due to phenotypic plasticity; however, there is also a genetic component (Caradus, 1994). Caradus (1984) reported that six populations of white clover were collected from areas that had been cut at different frequencies and intensities, or had been subjected to heavy treading. Plants from the area that had received infrequent lax cutting were larger leaved and tall with long internodes, while the population collected from the area that had been cut frequently had small leaves and short internodes. These differences persisted after these genotype had been grown in uniform conditions for two years ($r=0.90$ for leaflet width, $df=4$) although the range of leaflet widths had reduced.

Populations of white clover vary morphologically from prostrate , small leaved, densely-stoloniferous types, apparently adapted to close grazing, through to upright, large leaved, sparsely-stoloniferous types, apparently adapted to lax grazing or cutting. Much of this variation is known to be genetic (Caradus, 1994), but considerable variation in the morphological characteristics in white clover is attributed to phenotypic plasticity i.e. plants of a given genotype may have different morphological attributes depending upon the environment in which they are grown (Caradus, 1994). Genetic variation in the extent of phenotypic plasticity has also been reported (Caradus, 1993).

Genetic variation within white clover had been documented for phosphorus (P) response in a glasshouse (Snaydon and Bradshaw, 1962; Caradus and Snaydon, 1986), aluminium tolerance (Caradus *et al.*, 1991), response to calcium,

magnesium and potassium (Snaydon and Bradshaw, 1969), and salinity tolerance (Rogers *et.al.*, 1993). In a glasshouse study, Caradus and Snaydon (1986) showed that a white clover population adapted to a low-P soil tended to have more fine roots (<1mm diameter) and less very coarse roots (<2mm diameter) than a population adapted to high-P soils, when grown in a uniform environment. The population from low-P soils had a slower rate of leaf senescence but exported less P from senescencing leaves than populations from high-P soils. In another glasshouse study Caradus and Snaydon (1987) found that populations from low-P soils accumulated more inorganic-P in their leaf tissue when grown at high-P, and were able to reduce these inorganic P levels to lower concentrations when the P supply was deficient, illustrating an interaction between soil fertility and physiological behaviour of the plants.

These reports clearly suggest that results from plot testing from one environment might not necessarily be reproducible in another environment, and that this is a potential problem associated with this method for checking the authenticity and genetic purity of a seed lot.

2.1.5 Plant growth and development

Although the physiological basis of the autumn dormancy response (winter hardiness) of lucerne is poorly understood (Heichel and Henjum, 1990), it has long been recognized that lucerne cultivars differ in their response to day length and thus, they can be classified as being non-dormant, intermediate, or dormant. Plants from non-dormant cultivars continue to grow after being cut in autumn, while those from dormant cultivars make little recovery growth. The amount of regrowth of intermediate cultivars lies between that of non-dormant and dormant cultivars (Nittler and Gibbs, 1959). This suggests that identification and discrimination of lucerne cultivars on the basis of plant regrowth or recovery height after cutting in autumn is feasible.

Schornhorst *et.al.* (1957) examined the recovery height of nine cultivars of lucerne and a mixture of cv. Ranger (an autumn dormant cultivar; Nittler and Gibbs, 1959) and an African cultivar (a very non dormant cultivar). In this study the plants were grown under three different day lengths at 15.5, 21, and 27° C for 8 weeks, and then placed in a hardening chamber for two weeks. After all cultivars had been cut back, the plants were returned to their respective controlled temperature chambers and their height recorded three weeks later. The results of this study illustrated that a 12 h photoperiod and 15.5° C temperature was the best among the regimes tested. However this regime was able to classify the cultivars only into three groups. The failure of the authors to achieve better discrimination among the cultivars might have been because the day length or temperature was not suitable to reveal regrowth differences among the cultivars. This was supported by Coffindoffer and Burger (1958), who compared the effect of three photoperiods (10, 12, and 14 h) on the growth of three autumn dormant cultivars (Narragansett Williamsburge, Vernal and Coffindoffer), and found that at a 14 h photoperiod, Vernal made less growth than either of the other two cultivars.

The effect of day length on growth of lucerne seedlings, both in the field and glasshouse, was studied by Oakley and Westover (1921) who found that when seedlings were grown in a glasshouse for six weeks with a 7 h photoperiod, Southern USA adapted cultivars could be readily distinguished from Northern USA cultivars due to the taller growth of the Southern cultivars. Nittler (1954), grew six lucerne cultivars in a glasshouse with a photoperiod of 8.5 h at temperatures varying from 21-23° C, and found that after eight weeks the stems of Chilean (a non-dormant cultivar) plants were on average twice as long as those of cvs Ranger, Narragansett, and Grimm which are dormant cultivars. Nittler (1954) also reported a highly significant difference between cv. Narragansett and a Chilean-Narraganset mixture containing 40% of the Chilean cultivar. However, the average stem length of a mixture containing 20% of the Chilean cultivar was not significantly different from cv. Narragansett. This result suggests there may be

problems in discriminating among cultivars with intermediate autumn dormancy on the basis of their autumn growth rate.

2.1.6 Time of flowering

Nittler and Kenny (1964) showed that cultivars, temperature, and the temperature x spacing interaction all significantly affected the percentage of flowering plants, and also the average number of inflorescences per plant. Data in Table 2.1.3 (adapted from the results reported by these authors), suggest that a wide plant spacing (37.5 cm^2 /plant) produced more plants with blooms than a close spacing (10.1 cm^2/plant) in nearly every case. However, these data clearly illustrate that the response of different cultivars to temperature and plant spacings was not unique, suggesting that the distinctness of a lucerne cultivar on the basis of flowering time might be influenced by change in temperature, plant spacing, and also interaction between these factors. For example at the wide plant spacing under the four temperature treatments (Table 2.1.3) cvs. DuPuits and Vernal had similar percentages of flowering plants, whereas cv. Williams Burge showed a negative response to the first temperature regime (Treatment A, Table 2.1.3), as fewer plants flowered than in the other three treatments (67% in treatment A vs 93% in treatment B, and 82% in C and D, Table 2.1.3). In the close spacing, however, all cultivars showed a negative response to Treatment A.

2.1.7 Number of vegetative nodes to the first flower

In lucerne, the number of vegetative nodes formed before the onset of the reproductive phase, as measured from the base of a stem to the first-formed raceme, varies with cultivar, time of year and age of plant. Dobrenz *et.al.* (1965) found that the average number of nodes to the first inflorescence of cv. Moapa in Tucson, Arizona, ranged from 6.9 after cutting back in August to 10.5 after cutting back in April.

Between lucerne cultivars the number of vegetative nodes before the onset of the reproductive phase is not generally correlated with the earliness of flowering in the spring (Zaleski, 1954). This author found however, that in England the amount of overall spring growth was correlated with flowering time, in so far as the earliest flowering cultivars, such as Chartrain-villiers, DuPuits, New Zealand Strain B and New Zealand Marlborough, made more spring growth than late flowering cultivars, such as Grimm, Rhizoma and Argentine. Thus it would be quite possible for cultivars with the same growth rate to flower at different times depending on the date growth commenced in the spring, but for them to flower at the same node. The lack of correlation between the number of nodes to flowering and flowering time would not, perhaps, be expected within a given cultivar.

Zaleski (1954) reported that the flowering time of early and late flowering cultivars in spring differed by only three weeks. The difference between the number of nodes to flowering in different cultivars is thus probably related to the growth rate. Within the same flowering time a rapid growing cultivar would produce many more vegetative nodes to flowering than a slow growing one. The negative correlation between spring growth and date of flowering in the spring also implies that even with a similar growth rate, early and late flowering cultivars flower at the same node.

2.1.8 Seed yield

A significant interaction between seed yield of lucerne and location has been reported from Eucarpia trials by Rod *et al.* (1986), when seed yields from 14 cultivars from different European origins were compared in six countries. There were significant differences in seed yield of the same cultivar under different environmental conditions, suggesting that the seed yield components *i.e.* number of stems/plant, racemes/stem, pods/raceme, seeds/pod and seed weight are influenced by the environment.

Table 2.1.3 Percentage of five week old lucerne plants with blooms as affected by temperature and spacing (data from Nittler and Kenny ,1964)

Temperature treatment	Wide spacing (37.5 cm^2 /plant)			Close spacing (10.1 cm^2/plant)		
	DuPuits	Vernal	Williams Burge	DuPuits	Vernal	Williams Burge
(A) 30° C 20 h;27°C 4 h	100	89	67	59	48	30
(B) 30° C 12 h;27°C 12 h	100	93	93	95	92	69
(C) 30° C 4 h;27°C 20 h	96	93	82	97	76	78
(D) 27° C 20 h;30°C 4 h	100	93	82	95	92	86

2.1.9 Seed morphology

The seed characters commonly considered in an ordinary laboratory test are what Oomen (1969) termed as geometrical differences; length, width, and thickness. Wood *et.al.* (1977) stated that these characters are affected by environmental factors.

The influence of the environment on rice grains was highlighted by Quero (1980), who investigated the possibility of differentiating rice cultivars on the basis of grain length, one of the techniques recommended by Rosta (1975). He showed that seed produced in New Zealand was in all cases significantly smaller than that of the same cultivars produced in the Philippines, and that the relative changes were not always the same. The influence of environment on morphology of oat and wheat seed was also shown by Symons and Fulcher (1988). However in *Poa pratensis*, Olsen (1975) and Niemyski and Grzelak (1975) have shown that although the physical measurements (length, width, and thickness) are influenced by environmental conditions, the relative differences between cultivars remain

stable.

Wierzbicki and Rawa (1977) and Wiseman and Koszykowski (1978) stated that for cereal grains, length and width measurements, if properly taken, could be used to distinguished cultivars by classifying them into groups. According to Webb *et. al.* (1968), among 4400 rice cultivars in the USDA collection, the correlation coefficients between kernel length and kernel width and length/width ratio were significant. No reports in the literature were found about the possible effects of the environment on morphological characters of seed of out-breeding species such as lucerne, and the application of seed size and shape properties for cultivar identification of such species.

As no published work was found in the literature about the stability and usefulness of plant morphological characters for DUS testing and also verification of lucerne cultivars, this study was carried out with the following objectives:

1. To determine which morphological and physiological characters of lucerne plants were able to produce the discrimination needed for DUS testing and seed certification.

2. To verify the stability and environmental dependency of plant morphological characters which UPOV and OECD recommend for DUS testing and seed certification.

2.2 MATERIALS AND METHODS

2.2.1 Experimental field

The field experiments were established at the Seed Technology Centre, Massey University, New Zealand (40°S, 175°E). The soil, an Ohakea silt loam (Cowie, 1974) is classified as an aeric fraguiaqualf (gleyed yellow-grey earth; Cowie,1974), part of the rolling hills at the foot of the western Tararua ranges (Barker, 1983).

2.2.2 Cultivars used in this study

Certified seed lots (1st generation) of two New Zealand cultivars (Grasslands Oranga and Wairau produced in 1991) plus four Iranian ecotypic cultivars (Gharahuonjeh or Azari, Hamedani, Bami and Esfahani) originating from contrasting climatic regions of Iran and produced in 1990 were used in this study. The New Zealand cultivars were provided by Wrightson Seeds Ltd., and the Iranian cultivars by the Seed and Plant Improvement Institute of Iran (S.P.I.I.). The latitudes and altitude, and also air temperature and rainfall for 1991-1992 of the areas in which the Iranian cultivars were produced are illustrated in Appendices 2.2 and 2.3.

2.2.3 Land preparation

On 10th Oct. 1992 the land was cultivated out of glyphosate desiccated perennial ryegrass and white clover pasture, and was thoroughly prepared by firstly ploughing and secondly twice harrowing until a good tilth was attained. Before the last harrowing, 3.2 t/ha of lime was uniformly applied by hand to the experimental area to increase pH from 5.3 to a satisfactory optimum level for lucerne growth (6.2-7.8; Lanyon and Griffith, 1983). The soil analysis results are shown in Appendix 2.1a.

2.2.4 Experimental design

2.2.4.1 Spaced plants trial

Following the recommendation by UPOV (1988) a randomised block design with four replicates was used in this study to compare the morphological characteristics and physiological behaviour of the six cultivars (Plate 2.1). In each replicate, 18 plants of each cultivar were planted at 1m square spacings.

2.2.4.2 Flowering trial

To compare flower colour of the six lucerne cultivars, 150 plants of each cultivar were planted in two rows (i.e. 75x2) where intra-plant and inter-plant distances were 80 cm and 100 cm respectively (Plate 2.2). The rows of each cultivar were randomly arranged.

The air temperature, rainfall for 1992-1994 and also the 60 year average temperature data, all recorded at AgResearch Grasslands (1.5 km from the trial site) are presented in Appendix 2.4.

2.2.5 Transplanting of seedlings into the field

Seeds of the six cultivars were coated following manufacturers instructions with a prepared rhizobium inoculated peat (Coated Seed Ltd). The rhizobium for this inoculation was *Rhizobium meliloti* strain Nitrobug. On 19th November 1992, the coated seeds were sown in peat pellet No.7 Jiffy pots (Jiffy Products, Ltd, Norway) which were then placed in a glasshouse located at the Plant Growth Unit, Massey University. The minimum and maximum glasshouse temperatures were set at 16°C and 22°C respectively.

Twenty seven days after emergence (21.12.93) when seedlings had three to four

true leaves, they were transplanted into the field. To obtain a satisfactory plant establishment, water was applied to the transplanted seedlings once a week for a period of four hours during the first two weeks after transplanting. The irrigation was performed via sprinkler, using a hose with an internal diameter of 34mm which delivered 7800 litre/ha. However, because rain subsequently fell regularly (Appendix 2.4), no further irrigation was required.

Plate 2.1 Field spaced plant trial

Plate 2.2 Field flowering trial

2.2.6 Morphological and physiological characters recorded from first and second year plants

Following the OECD Scheme for the Varietal Certification of Herbage Seed (OECD, 1988), and UPOV's guidelines for the conduct of tests for distinctness, homogeneity and stability of lucerne cultivars (UPOV, 1988), the following morphological characters and agronomic behaviour of the six lucerne cultivars were recorded from individual plants in the establishment and following year.

1. Height of the plants in the spring at the first sign of growth in the earliest cultivar in the trial (mid September).
2. Height of the plants one month later (mid October).
3. Width and length of one terminal leaflet from individual plants at flowering : this and other characteristics of the leaflet were recorded using an automatic machine

vision system (see section 3.4).

4. Length and width ratio of the leaflet.

5. Assessment of plant angle at the early flower bud stage and after cutting. Growing plants in the field were classified into five categories ranging from prostrate to very erect plants (Plate 2.3).

6. Date of first flowering defined as the time when the first three florets were open.

7. Height of the longest stem of each plant at first flowering, to the top of the inflorescence.

8. Flower colour.

   Flower colour in lucerne usually ranges from very light to very dark purple (Plate 2.4), although there can be variegated (a mixture of several colours, plate 2.5) and cream flower coloured plants in a population. In this study the flower colours were classified into cream, three categories of purple (light, medium and dark, plate 2.6) and variegated, and flower colour assessment was performed by matching flower colour of the individual plants of the cultivars with this photograph at the time when the first three florets had opened. Flower colour was also reassessed at peak flowering of each cultivar.

9. Recovery height after cutting.

   According to the OECD Scheme, plants should be cut above the ground when all cultivars in the trial have open flowers, and six weeks later a second cutting should be performed. The Scheme requires that three cultivar recovery heights should be recorded from second year plants (three and six weeks after the first cutting and three weeks after the second cutting). However, in this present work nine recovery heights were recorded from the first year plants (six weekly recordings after the first cutting (29.3.93) and three weekly recordings after the second cutting (17.5.93) but in the second year, the OECD recommendation was followed. In this year, the first and second cutting were performed on 24.12.94 and 7.2.94 respectively. As the height at which the plants should be cut at the

first and second cutting is not mentioned in the OECD scheme, in this present work the plants were cut 5 cm above the ground to study the recovery height.

10. Disease observation

Pathogens were identified by the Plant Health Group of the Plant Science Department, Massey University. A manual of assessment keys for plant diseases (James, 1971) was used as a guideline for scoring the cultivars based on the level of leaf and stem infection on the plants. The scoring was performed on a whole plant basis, using a range from one (no infection) to five (severe damage).

Scoring of the plants for infection with downy mildew (*Peronospora trifoliorum* L) was performed on 29.7.93 on 72 plants grown in the spaced plants trial and scoring of plants for infection by leaf spot (*Pseudopeziza medicaginis* Lib) was performed on 7.12.93 on 150 plants grown in the flowering trial.

In this experiment key No. 2.2 of the manual was used to score the plants for leaf spot and key No. 2.3 for downy mildew.

11. Plant growth habit i.e. erect, semi-erect and prostrate growth

12. Number of stems immediately above the ground at flowering

13. Number of stems 5 cm above the ground at flowering

14. Thickness of longest stem (mm) 5 cm above the crown at flowering

15. Number of nodes to the first open raceme at flowering

16. Assessment of foliage production potential of the cultivars via oven-drying of the vegetative material from the first cutting at 80°C for 48 hr.

2.2.7 Weed control

The herbicide 2,4-DB is recommended for the control of broad leaf weeds in lucerne crops (New Zealand Agrochemical and Plant Protection Manual, 1993). However, despite using the recommended rate of this herbicide (2.4 kg a.i/ha),

when it was applied to established plants on 2nd January 1993 it resulted in an increased leaflet thickness and decreased leaflet area. Thus no further herbicide was subsequently directly applied to the plants, and weeds were controlled by either rotaryhoeing the inter-row space and hand pulling within-row weeds, or hand hoeing the weeds within the rows and applying herbicides such as Simazine (1.0 kg a.i/ha) and paraquat (0.6 kg a.i/ha) between the rows.

2.2.8 Pest and disease control

The names, date of applications and the rates of fungicides and pesticides applied to field grown plants are presented in Table 2.2.1.

2.2.9 Application of fertiliser

To support the newly established plants, 100 kg N/ha (as potassium nitrate) was distributed by hand onto the experimental site on 15th January 93. No further fertiliser was applied.

2.2.10 Statistical analysis

Statistical analysis was performed using SAS (1989). Least significant differences at the 5% probability level ($P<0.05$) were used to differentiate treatment means where analyses of variance (ANOVA) or the general linear model (GLM) were significant at the 0.05% or 0.01% level of confidence. Using the Duncan's grouping test results, data mean values of all the pairs of the cultivars were grouped as either differing significantly or not differing significantly. These results are presented as the percentage of pair wise comparisons which differed significantly. This calculation was carried out for all data sets.

Plate 2.3 Classification of growing plants in the field according to the angle of their outer stems in relation to the ground, ranging from prostrate (Pr.) to very erect plants (5).

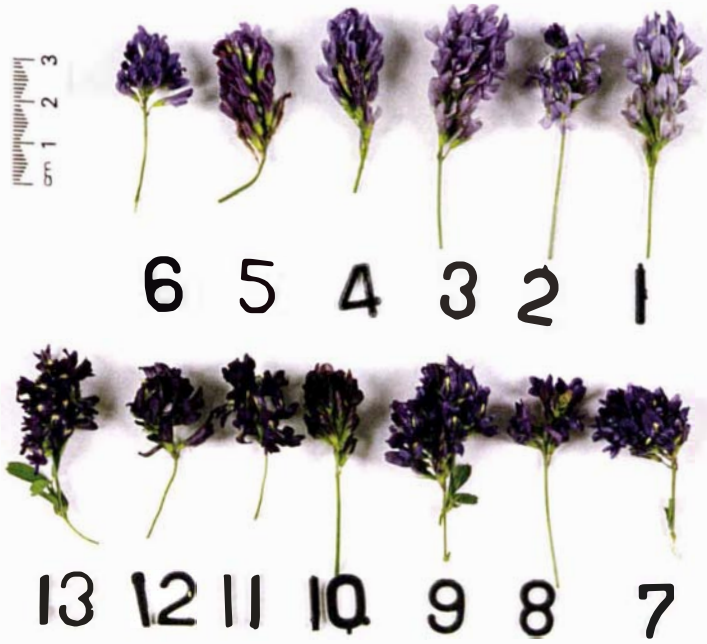


Plate 2.4 Illustration of a wide range of purple flower colour in lucerne

Plate 2.5 Different types of variegated flower colour in lucerne

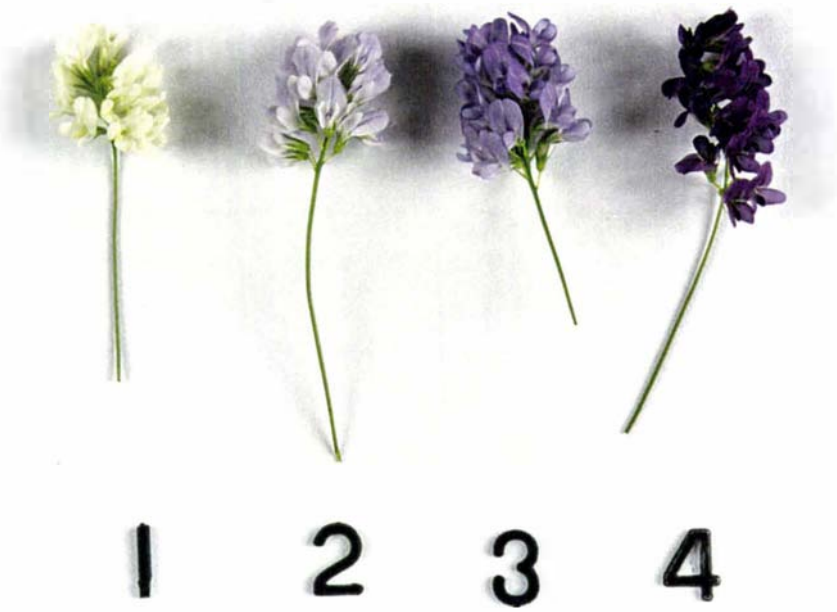


Plate 2.6 **Cream and the three purple flower colours used in this study as the standard pattern for classification of flower colour in the field and in the glasshouse**

Table 2.2.1 Pesticides used during the establishment and following year of the field experiment

NAME	FOR CONTROLLING	DATE OF APPLICATION	RATE (a.i./ha)
Methiocarb (*Molluscide*)	snails and slugs	11.12.92	52 kg/ha
Taufluvalinate (insecticide)	aphids and caterpillar	22.1.93	170 g/ha
Esfenvalerate (insecticide) Benomyl (fungicide)	caterpillars leaf spot	22.2.93	35 g/ha 350 g/ha
Taufluvalinate (insecticide) Captan (fungicide)	aphids and caterpillars downy mildew	11.3.93	170 g/ha 640 g/ha
Pirimiphos-methyl & Permethrin (insecticides) Chlorothalonil (fungicide)	lucerne flea downy mildew	28.4.93	30 g/ha 900 g/ha
Methiocarb (*Molluscide*)	snails & slugs	21.6.93	52 kg/ha
Pirimiphos-methyl & Permethrin (insecticides) Benomyl (fungicide)	lucerne flea leaf spot	5.7.93	30 g/ha 350 g/ha
Metalaxyl & Mancozeb (fungicide) Pirimiphos- methyl & Permethrin (insecticide)	downy mildew lucerne flea	30.7.93	30 g/ha 400 g/ha 12.5 g/ha
Metalaxyl (fungicide) Mancozeb (fungicide) Benomyl (fungicide)	leaf spot leaf spot downy mildew	16.11.93	71 g/ha 570 g/ha 450 g/ha
Metalaxyl (fungicide) Mancozeb (fungicide) Benomyl (fungicide)	leaf spot leaf spot downy mildew	30.11.93	71 g/ha 570 g/ha 450 g/ha

2.2.11 Examination of plant morphological characteristics in controlled glasshouse conditions

2.2.11.1 Growth environment

The glasshouse temperatures were set at 22°C maximum and 16°C minimum. The actual temperature of the glasshouse during plant growth was recorded

(Appendix 2.4) using a Squirrel meter/Logger (Grant; type Sq.-4U, -10°C -90°C). Heating of the glasshouse was achieved by forced air, hot water and a heat exchanger. Louvred vents (multi-blade opening and closing system) and fans plus a spray mist were used for evaporative cooling.

2.2.11.2 Growing media

Using the same seedling growing procedure as described in section 2.2.5, 27 day old seedlings of six lucerne cultivars were transplanted into 2cm x 22cm polythene pots (P.B. No. 12), filled with a long term potting mixture of pH 6.0. The growing medium consisted of 80% peat and 20% pumice (v:v), to which the following were added/m^3.

1. Agronomic lime 1000 grams
2. Dolomite 3000 grams
3. Micromax 1000 grams
4. Osmocote (14 - 6.1 - 11.6, 3-4 months) 1500 grams

The Micromax component of the fertiliser comprised:iron 12%,manganese 2.5%,zinc 1.0%,copper 0.5%,boron,0.1% molybdenum 0.005% and sulphur 15%.

2.2.11.3 Experimental design

Using a randomised block design with three replications of 22 plants for each cultivar, 396 plants from the six cultivars were placed on the benches arranged in three rows (Plate 2.7). The plants were labelled and the morphological characteristics of individual plants recorded (see section 2.2.5). The statistical analysis of this experiment was as described in section 2.1.10.

2.2.11.4 Watering system

A capillary watering system for irrigation of the plants was arranged on the

benches (Plate 2.7). To keep the moisture of the potting mix at field capacity, plants were watered for 20 minutes one to four times a day as the plants developed. Top watering was also applied once a week by adding 600-700 ml of water to each pot to flush out salts accumulated at the top of the pot. As shown in Plate 2.7, wicks were placed along the edges of the benches to remove excess water from the benches.

2.2.11.5 Morphological characteristics recorded from the plants grown in the glasshouse

1. Number of stems immediately above the ground at flowering.
2. Number of stems 5 cm above the ground at flowering.
3. Thickness of the longest stem (mm) 5 cm above the crown at flowering.
4. Number of nodes to the first opened flower.
5. Length and width of one terminal leaflet as described in section 2.2.6.
6. Date of first flowering (blossoming) defined as the time when the first three florets were open.
7. Height of the longest stems at flowering, to the top of the inflorescence.
8. Flower colour. This character was assessed as described in section 2.2.6.
9. Recovery height. Following the same technique as described in section 2.2.6, the recovery height of the plants was recorded weekly for six weeks after the first cutting, and for three weeks after the second cutting.
10. Foliage production. Assessment of foliage production potential of the cultivars by oven-drying the cut material at 80°C for 48 h.

2.2.11.6 Experimental management

Bamboo sticks and twist ties were used to support the growing plants (Plate 2.7). A broad spectrum insecticide (Attack, containing 25 g/litre permethrin plus 475 g/litre pirimiphos-methyl, 30g a.i./ha) was sprayed at 5 weekly intervals to

control whitefly, aphids and caterpillars. In addition, every 2 months Mesurol (containing 750 g/kg methiocarb) was distributed in the glasshouse (3-4 pellets /pot) to control slugs and snails.

Potting mix and plant tissue were analyzed six weeks after transplanting by the Soil Science Dept., Massey University to check for nutrient deficiencies in the potting mix. As no deficiencies were found in the media or the plant tissues (Appendix 2.1b), no extra fertiliser was applied.

2.2.12 Definitions

As there are varying interpretations for the concepts of cultivar identification and distinctness, the definitions by Cooke (1995b), will be followed in this work; identification means: what cultivar is this?; and distinctness reveals: is the cultivar different from others?.

'Verification' in this thesis, is as defined by ISTA (1993), *ie.* that a particular seed lot is true to the cultivar name, and that it has not been mixed with other cultivars or otherwise altered during multiplication. In this test a submitted seed lot and an authenticated standard seed lot of the named cultivar are compared.

Plate 2.7 **Three replicates of 22 plants of each cultivar grown on different benches which were arranged in three rows in the glasshouse.**

2.3 RESULTS

2.3.1 Stem number

No significant difference was found between the number of stems immediately above the ground (stem.1) of the plants from cvs. Esfahani and Bami (Iran) grown in the field, but this character did differ significantly ($P<0.05$) when plants of these cultivars were grown under glasshouse conditions (Table 2.3.1). In contrast, the number of stem.1 of cvs. G.Oranga and Wairau (New Zealand) did not differ in the glasshouse, but were significantly different ($P<0.05$) when the plants were grown under field conditions. However, there was no significant difference between these two cultivars at a significance level of $P<0.01$ (data not shown). No significant difference was found between stem.1/plant of cvs. Hamedani and Esfahani under field or glasshouse conditions. According to the Duncan's grouping test results (Table 2.3.1), 10 out of 15 pairs of cultivars (66%) had a different number of stem.1 in the field, and therefore could be discriminated from each other, while only 6 pairs of cultivars (40%) had a different number of stem.1 when the plants were grown in the glasshouse. No significant difference was found between the number of stem.2 (main stems plus secondary branches at 5 cm above the ground) of plants of cvs. G.Oranga and Wairau in the glasshouse, whereas they did differ significantly ($P<0.05$) in the field (Table 2.3.1). Unlike these two cultivars, cvs. Bami and Esfahani which had the same numbers of stem.2 in the field, had a significantly different number of stem.2 when the plants were grown in the glasshouse. No significant difference was found between the number of stem.2 of the plants of cvs. Azari and Hamedani grown in the field or the glasshouse (Table 2.3.1).

2.3.2 Thickness of the main stem

A significant difference was found between some pairs of cultivars for main stem

thickness in plants grown in the glasshouse (Table 2.3.1). As a result, the cultivars were classified into three groups. However, no significant difference for this character was found among cultivars when plants were grown in the field (Table 2.3.1).

2.3.3 Plant height at flowering

There were significant differences between plant height of some pairs of cultivars as recorded from individual plants from the same field trial in two successive years (1993 and 1994), and also in the glasshouse (Table 2.3.1). Pairwise comparisons between the cultivars using data in this Table show, however, that the response of cultivars to different environmental conditions was not unique. For example, 50% of the pairs of cultivars had a significantly different plant height when grown in the glasshouse. However, only 25% (1993), and 11% (1994) of pairs of cultivars had significantly different plant height in the field. As shown in Table 2.3.1. cvs. Wairau and G.Oranga, the two New Zealand cultivars, had the same plant height in the field in both years, but they differed significantly when grown in the glasshouse. The environmental response of cvs. Azari and Bami (Iran) was different from that of the two New Zealand cultivars. While there was no significant difference between these two cultivars in the field in 1993, they did differ significantly in the field in 1994, and also in the glasshouse. No significant difference was found between cvs. Esfahani and Hamedani in either the field or glasshouse (Table 2.3.1).

2.3.4 Leaflet size

No significant difference ($P<0.05$) was found between leaflet length and width of the plants of the four Iranian cultivars (Azari, Bami, Esfahani, and Hamedani) grown in the field. However, significant differences were found between leaf length and width of some pairs of these cultivars when the plants were grown

in the glasshouse (Table 2.3.2). Despite there being a significant difference in width/length ratio of some of the cultivars in the glasshouse, they did not differ significantly in the field. No significant difference was found between leaflet length, leaflet width, and width/length ratio of plants of cvs. G. Oranga and Wairau (New Zealand) when grown in either the field or the glasshouse (Table 2.3.2).

2.3.5 Number of nodes on the main stem

The node numbers of all of the cultivars in the glasshouse were less than those recorded in the field in two successive years (1993 and 1994). For example, overall mean node numbers for cv. Wairau in the field were 12.2 and 13.4 in 1993 and 1994 respectively, compared with 8.5 in the glasshouse (Table 2.3.3). Number of nodes for cv. Bami in the field were 11.9 and 13.4 in 1993 and 1994 respectively, but only 8.4 in the glasshouse. No significant difference was found between number of nodes of some pairs of cultivars in either the field (both 1993 and 1994), and glasshouse (eg. Esfahani, Hamedani and G.Oranga; Table 2.3.3), unlike cvs. Azari and Esfahani which did differ significantly ($P<0.05$) in all three conditions. Interestingly, cvs Wairau and G.Oranga which were not different in the field in the two successive years, did differ significantly in the glasshouse.

2.3.6 Foliage dry weight

A significant difference was found between foliage production of some pairs of the cultivars in the field in 1993 and 1994 (at the time of cut.1), and also in the glasshouse. However, they did not differ in the field after cut.2. (Table 2.3.3). Pairwise comparisons between the cultivars from this table indicate that more discrimination among cultivars was possible in the field (both in 1993, and 1994 at cut.1) than in the glasshouse. Forty-four percent of pairs of cultivars did differ significantly ($P<0.05$) from the field data, whereas in the glasshouse only

11% of pairs of cultivars were significantly different. For example, there was a significant difference between cvs. Azari and Wairau, and also between cvs. Hamedani and G.Oranga in the field in two successive years (1993, and 1994 at cut.1, Table 2.3.3), but they did not differ when grown in the glasshouse.

2.3.7 Time of flowering

Data for time of flowering, defined as the number of days required for a proportion (10%, 25%, 50%, 75%, and 100%) of individual plants within each cultivar to produce the first three open florets, are presented in Table 2.3.4. No significant difference ($P<0.05$) was found between cvs. Azari and Bami for 10% flowering time in the field. However, they did differ significantly in the glasshouse. Conversely, cvs. Hamedani and Esfahani which did not differ for 10% flowering in the glasshouse, did differ significantly in the field. Unlike these four cultivars, no significant difference was found for 10% flowering of cvs. G. Oranga and Wairau in either the field or glasshouse.

No significant difference was found for 50% flowering of cultivars in the field, despite a significant difference ($P<0.05$) between some pairs of cultivars in the glasshouse. For example cv. G.Oranga differed significantly from cv. Wairua, and cv. Azari from the other three Iranian cultivars (Bami, Esfahani, and Hamedani, Table 2.3.4).

A significant difference was also found between some pairs of cultivars for 75% flowering in the field and in the glasshouse. No significant difference was found among the four Iranian cultivars in the field. However, they did differ when plants were grown in the glasshouse (Table 2.3.4). A similar result was also found for cvs G.Oranga and Wairau, in that despite there being no difference between these cultivars in the field, they did differ significantly ($P<0.05$) in the glasshouse. Full flowering (100%) was also influenced by the environmental

conditions of the field and glasshouse (Table 2.3.4). For example, despite there being no significant difference for 100% flowering of cvs. Azari and Hamedani in the glasshouse, they differed significantly in the field.

2.3.8 Flower colour

Data recorded for flower colour of individual plants of cultivars grown in the two separate field experiments, and also in the glasshouse are presented in Table 2.3.5. In most cases, the percentages of plants with identical flower colour from each of the cultivars in the two field experiments (spaced plant, and flowering trials) and under glasshouse conditions were different (standard errors for identical flower colour are presented in Table 2.3.5). For example, 18% of the plants from cv. Azari in the spaced plant trial had dark purple flowers, while this was 23% in the field flowering trial. However, only 5 % of the plants of this cultivar in the glasshouse were classified as having dark purple flowers. For cv. Esfahani, 25% and 24% of the plants in the spaced plant trial and in the glasshouse were respectively scored as being light purple flowering, but only 7 % of the plants of this cultivar in the flowering trial were classified as being light purple flowering.

Overall means of cultivars for identical flower colours (Table 2.3.4) showed that cv. Azari had the highest, whereas cv. Bami had the lowest percentage of light purple flowering plants (36.3% vs 12.7%). On the other hand, cv. Bami had the highest, while cv. Wairau had the lowest number of dark purple flowering plants. G. Oranga and Wairau, the two New Zealand cultivars, could be easily discriminated from all of the four Iranian cultivars because they had a higher proportion of variegated flowering plants in their populations (Table 2.3.5). The number of variegated flowering plants of cv. Azari was about 3% which was enough to differentiate this cultivar from the other three Iranian cultivars.

Table 2.3.1 **Characters recorded from individual plants grown under both glasshouse and field conditions. Character means for cultivars with the same letter are not significantly different at P<0.05.**

Cultivar	Number of stems immediately above the ground (stem.1)		Number of stems 5cm above the ground (stem.2)		Height of plants at flowering (cm)			Thickness of the main stem at flowering (mm)	
	Field (1993)	Glass-house	Field (1993)	Glass-house	Field (1993)	Field (1994)	Glass-house	Field (1993)	Glass-house (1993)
Azari	4.74c	4.36bc	12.53c	6.71d	51.20a	52.53b	92.35a	4.61 a	2.71 b
Bami	8.72a	8.32a	16.69b	10.89a	45.90abc	58.66a	75.89c	3.97 a	2.39 d
Esfahani	7.89ab	3.64c	16.50b	6.12d	48.38ab	55.55a	82.46b	4.32 a	2.71 b
Hamedani	6.15b	5.04bc	13.63bc	7.26cd	46.05abc	55.19ab	87.43ab	5.45 a	2.67 bc
G.Oranga	4.93c	5.18bc	13.81bc	8.86bc	40.91c	56.21ab	85.41b	4.96 a	3.09 a
Wairau	7.00b	6.10b	21.01a	9.32ab	44.19bc	56.65ab	75.06c	5.03 a	2.8 b
LSD(5%)	1.56	1.84	3.57	1.75	5.75	5.39	6.35	1.62	0.18

Table 2.3.2 **Length, width, and width/length ratio of the terminal leaflet of individual plants of the cultivars grown under field and glasshouse conditions. Character means for cultivars with the same letter are not significantly different at P<0.05.**

Cultivar	Length of leaflet (mm)		Width of leaflet (mm)		Width/length ratio	
	Field	Glasshouse	Field	Glasshouse	Field	Glasshouse
Azari	29.71b	20.41c	13.39b	10.37bc	0.448a	0.52a
Bami	29.46b	25.66a	14.13ab	12.01a	0.474a	0.47ab
Esfahani	30.10b	23.22b	14.49ab	9.66c	0.481a	0.43b
Hamedani	29.80b	24.15ab	14.10ab	10.68b	0.468a	0.45b
G. Oranga	34.00a	25.01ab	15.56a	11.19ab	0.471a	0.45b
Wairau	34.06a	25.27ab	14.84ab	11.00b	0.445a	0.44b
LSD (5%)	2.81	2.17	1.77	0.89	0.045	0.049

Table 2.3.3 Dry weight and number of nodes recorded from individual plants grown under both glasshouse and field conditions. Character means for cultivars with the same letter are not significantly different at P<0.05.

Cultivar	Dry weight/plant (gr.)				Number of nodes to the first flower bud		
	Field			Glass-house	Field		Glass-house
	1993	1994					
	Cut.1	Cut.1	Cut.2	Cut.1	1993	1994	
Azari	45.4c	196.9b	44.9a	31.0ab	12.7a	15.2a	10.1a
Bami	53.2cb	150.0bc	53.2a	32.8ab	11.9ab	13.4b	8.4c
Esfahani	66.8a	190.6b	69.9a	27.8b	11.6b	13.0b	9.0bc
Hamedani	46.9c	127.0c	113.3a	33.0ab	11.8b	14.0ab	9.2abc
G.Oranga	61.7ab	196.3b	61.0a	39.6a	12.1ab	13.8ab	9.6ab
Wairau	66.8a	247.4a	65.9a	37.5a	12.2ab	13.4b	8.5c
LSD(5%)	9.6	61.6	86.6	8.2	0.7	1.6	0.98

Table 2.3.4 Days required for flowering of 10% (DRFFT.10%), 25% (DRFFT.25%), 50 % (DRFFT.50%), 75% (DRFFT.75%), and 100 % (DRFFT.100%) of the individual plants in each of the cultivars. The means for cultivars with the same letter are not significantly different at P<0.05.

Cultivar	DRFFT.10%		DRFFT.25%		DRFFT.50%		DRFFT.75%		DRFFT.100%	
	Field	Glasshouse	Field	Glasshouse	Field	Glasshouse	Field	Glasshouse	Field	Glasshouse
Azari	99.5 a	59.3 a	103.25 a	62.7 a	106.2 a	65.3 a	108.9 ab	68.7 a	115.2 c	76.0 a
Bami	93.7 ab	56.7 b	98.2 ab	58.3 bc	104.7 a	60.3 b	109.1 ab	63.7 c	124.0 ab	68.7 b
Esfahani	94.5 ab	56.7 b	98.6 ab	57.6 c	105.0 a	59.3 b	108.6 ab	63.7 c	118.2 bc	73.7 ab
Hamedani	85.5 c	56.3 b	96.25 b	57.67 c	104.0 a	61.0 b	110.7 a	64.7 cb	129.7 a	74.33 ab
G.Oranga	95.2 cb	58.0 ab	98.4 ab	59.7 b	103.7 a	64.0 a	107.37 b	66.7 ab	115.7 c	75.33 ab
Wairau	88.5 cb	56.7 b	98.7 ab	57.7 c	103.6 a	59.3 b	108.5 ab	63.0 c	119.2 cb	69.7 ab
LSD (5%)	7.76	2.43	5.04	1.98	3.61	2.48	3.00	2.29	6.34	7.27

Table 2.3.5 **Comparison between percentages of plants of cultivars with identical flower colour in two field and one glasshouse experiments. Flowers were classified into light purple (LP), purple (P), dark purple (DP) and variegated (VAR) colours.**

Cultivar	Flower Colour	Field		Glasshouse (%)	Overall cultivar mean (%)	Standard Error
		Spaced plant (%)	Flowering trial (%)			
Azari	LP	41	30	38	36.3	2.68
	P	38	46	53	45.7	3.57
	DP	18	23	5	15.3	4.38
	VAR	3	1	4	2.7	0.72
Bami	LP	12	13	13	12.7	0.27
	P	53	49	64	55.3	3.66
	DP	35	38	23	32.0	3.75
	VAR	0	0	0	0	-
Esfahani	LP	25	7	24	18.6	4.77
	P	53	52	56	53.7	0.98
	DP	22	41	20	27.7	5.47
	VAR	0	0	0	0	-
Hamedani	LP	21	15	36	24	5.10
	P	56	56	52	54.6	1.09
	DP	23	28	12	21	3.86
	VAR	0	1	0	0.33	-
G.Oranga	LP	21	18	19	19.3	0.72
	P	19	26	26	23.7	1.91
	DP	34	34	23	30.3	2.99
	VAR	25	22	30	25.7	1.91
Wairau	LP	26	17	32	25.0	3.56
	P	22	26	12	20.0	3.40
	DP	13	15	5	11.0	2.50
	VAR	39	41	51	43.7	3.03

2.3.9 Plant recovery height

Plant recovery height after the first and second cuts in 1993 (see Material and Methods) was significantly different ($P<0.05$) for some pairs of cultivars at all of the recording times (Table 2.3.6). Cultivars Bami and Esfahani had the highest plant recovery height in the field. Cultivar Azari, with the exception of the second week after the first cut in which it had the same recovery height as G.Oranga, had the shortest recovery height of all cultivars. However, results for plant recovery height of cultivars in the glasshouse were not consistent with those in the field (Table 2.3.6). For example, despite a significant difference ($P<0.05$) between cvs. Esfahani and Hamedani in all of the recordings in the field, they did not differ significantly in the glasshouse. No significant difference was also found between the recovery height of cvs. G. Oranga, and Wairau (Table 2.3.6). Pairwise comparisons between plant recovery height of cultivars from data in Table 2.3.6 showed that more differences among cultivars were found in the field than in the glasshouse (Table 2.3.7). Accordingly, greater discrimination amongst cultivars was possible using field recovery height of plants three weeks after the first cut, than from the other recordings. Plate 2.8 clearly illustrates the superiority of plant recovery of cv. Bami (2) when compared with that of cv. Azari (4) three weeks after the first cut.

Plate 2.8 **Comparison between plant recovery height of cvs. Azari (4) and Bami (2) at three weeks after the first cut.**

Table 2.3.6 **Weekly recovery height in the field and glasshouse up to 6 weeks after the first cut (CUT1.W2-CUT1.W6), and three weeks after the second cut (CUT2.W3). Character means for cultivars with the same letter are not significantly different at P<0.05**

Cultivar	CUT1.W2		CUT1.W3		CUT1.W4		CUT1.W5		CUT1.W6		CUT2.W1	
	Field	Glass-house	Field	Glass-house	Field	Glass-house	Field	Glass-house	Field	Glass-house	Field	Glass-house
Azari	12.17 d	14.04 c	17.71 d	34.51 c	19.28 d	61.67 cb	24.48 c	108.19 a	28.01 c	125.56 a	19.79 c	27.51 b
Bami	20.98 a	22.15 a	29.66 a	44.26 a	33.20 a	67.35 ab	41.25 a	102.64 ab	44.73 a	117.42 ab	43.63 a	38.38 a
Esfahani	21.96 a	19.37 ab	31.10 a	43.57 a	34.53 a	67.96 a	42.17 a	106.47 ab	44.57 a	124.78 a	46.18 a	38.51 a
Hamedani	18.48 b	18.49 b	25.28 b	41.51 ab	27.95 b	68.86 a	32.68 b	106.91 ab	36.80 b	123.05 a	30.63 b	35.44 a
G.Oranga	13.49 cd	16.79 bc	20.78 c	34.58 c	22.74 c	57.59 c	32.15 b	96.21 ab	36.95 b	112.87 ab	31.95 b	27.86 b
Wairau	14.28 c	15.91 bc	22.04 c	34.64 cb	24.65 c	58.17 cb	31.92 b	93.23 b	36.66 b	105.79 b	29.97 b	27.23 b
LSD(5%)	1.91	3.56	2.42	6.58	2.91	9.24	4.30	14.66	4.54	17.11	9.04	5.43

Table 2.3.7 Pairwise comparisons between mean values of recovery height of cultivars two (CUT1.W2), three (CUT1.W3), four (CUT1.W4), five (CUT1.W5), and six (CUT1.W6) weeks after the first cut, and also three weeks (CUT2.W3) after the second cut in the field and glasshouse.

Recording time	Percentage of pairs of cultivars which were significantly different (P<0.05)	
	Field (%)	Glasshouse (%)
CUT1.W2	80	40
CUT1.W3	87	53
CUT1.W4	80	46
CUT1.W5	73	7
CUT1.W6	73	20
CUT2.W3	73	60

2.3.10 Discrimination among cultivars using plant morphological characters which could only be recorded from the field trials

2.3.10.1 Angle of plants at flowering and after cutting

Results from scoring of plants on the basis of their angle (see Materials and Methods) showed there was a significant difference between some cultivars (Table 2.3.8). For example, in all three scorings the four Iranian cultivars (Azari, Bami, Esfahani, and Hamedani) differed significantly from each other. However, no significant difference was found between the two New Zealand cultivars (G.Oranga and Wairau), or between these cultivars and Hamedani (Iran).

2.3.10.2 Disease observation

Results from scoring of plants for infection with downy mildew (*Peronospora trifoliorum* L) in field spaced plants indicated that cultivars were not equally resistant to the pathogen (Table 2.3.8). Cultivar G. Oranga was more resistant to downy mildew than cvs. Bami, Esfahani and Hamedani. No significant difference was found between cvs. Wairau, Azari, and Hamedani. No significant difference was also found between cvs. G.Oranga and Wairau (Table 2.3.8).

Results from scoring cultivars in the flowering plant trial for infection of leaves and stems of individual plants by leaf spot (*Pseudopeziza medicaginis*) demonstrated a significant difference between some pairs of cultivars grown in both the flowering plant, and spaced plant trials (Table 2.3.8), and G.Oranga and Bami were respectively the most and the least resistant cultivars to the pathogen (Plate 2.9).

In the flowering trial, cv. G. Oranga (New Zealand) was more resistant to the pathogen then any of the Iranian cultivars. However, no significant difference was found between the level of infection of this cultivar and Wairau, the other New Zealand cultivar. No significant difference was also found between cvs. Wairau and Azari (Iran). The result from the spaced plant trial (Table 2.3.8) was not consistent with that found in the flowering trial. For example, cvs. Bami and Esfahani which did not differ in the flowering trial, did differ significantly in the spaced plant trial. No significant difference was found between cv. G Oranga and Azari, despite the fact that they did differ in the flowering trial.

2.3.10.3 Plant growth habit

There was no significant difference between the majority of cultivars in the percentage of erect, semi-erect and prostrate plants. The only cultivar which did

differ from the others was Azari which had significantly ($P<0.05$) more prostrate plants than the other cultivars.

Plate 2.9 **Illustration of the difference in tolerance to leaf spot (*Pseudopeziza medicaginis*) between cv. Bami (2) and G.Oranga (5), grown in the flowering trial.**

Table 2.3.8 Morphological data and disease resistance of individual plants of four Iranian and two New Zealand cultivars grown in the field. Character means for cultivars with the same letter are not significantly different at P<0.05

Cultivar	Recovery height of plants in autumn (cutting on 16 Jun. and then recording on 16 Jul. 1994)	Scoring of plants according to the level of infection			Angle of plant 2 years after establishment		
		Downy Mildew (Spaced plants)	Leaf spot		At flowering time	Four weeks after the first cut	Five weeks after the first cut
			Flowering plant trial	Spaced plant trial			
Azari	14.02 e	1.43 abc	2.0 cb	2.74 c	1.34 d	1.28 d	1.09 d
Bami	31.66 a	1.89 a	2.70 a	4.17 a	3.16 a	3.58 a	3.92 a
Esfahani	28.41 b	1.93 a	2.69 a	3.61 b	2.59 b	3.05 b	3.20 b
Hamedani	19.56 d	1.59 ab	2.02 ab	3.35 b	2.11 c	2.43 c	2.46 c
G.Oranga	23.61 c	1.03 c	1.20 d	2.53 c	1.80 c	2.58 c	2.54 c
Wairau	19.97 d	1.12 cb	1.43 cd	2.48 c	1.77 c	2.32 c	2.31 c
LSD(5%)	1.37	0.49	0.60	0.36	0.37	0.44	0.47

2.4 DISCUSSION

Morphological data from individual plants of Iranian and New Zealand cultivars grown in the field and the glasshouse (see Materials and Methods) suggested that the majority of the recorded characters, including those which are recommended by OECD and UPOV for discrimination and verification of lucerne cultivars were influenced by growing conditions. However, the extent of this influence varied for different characters. Although there are some physiological explanations for character variation under different environments, these are not addressed in this discussion, because the issue is beyond the scope of this thesis. In this section only the usefulness of plant morphological characters for lucerne cultivar discrimination is considered.

2.4.1 Stem number

Although number of stems is not among the OECD and UPOV recommended plant morphological characters (OECD 1968, 1988; UPOV, 1988), the number of plant stems immediately above the ground (stem.1) appears to be a stable and environmentally independent character in lucerne cultivars. The overall stem.1 mean of the cultivars did not differ significantly between the field and the glasshouse (6.6 vs 5.4, Table 2.3.1), and with the exception of cv. Esfahani, the classification of the cultivars on the basis of this character was unique for all of the cultivars. However, because of the result for cv. Esfahani, Table 2.3.1), an examination of more cultivars is required before making any final conclusion as to the usefulness of this character for discrimination among lucerne cultivars.

Unlike stem.1, the number of the stems/plant 5cm above the ground (stem.2) was influenced by the growth conditions, because the overall mean of stem.2 of the cultivars in the field was nearly twice that in the glasshouse (15.5 vs 8.2 ,Table 2.3.1). In addition, the classification of the cultivars on the basis of stem.2 was

not unique for any of the cultivars in the two environmental conditions (Table 2.3.1), suggesting that stem.2 is not a useful character for lucerne cultivar verification.

2.4.2 Thickness of the main stem

Despite there being no significant difference between the cultivars for main stem thickness of plants in the field, there was a significant difference between some pairs of cultivars in the glasshouse (Table 2.3.1) suggesting that thickness of the main stem is influenced by the growing conditions. Fifty percent of the pairs of cultivars could be discriminated using thickness of the main stem of plants in the glasshouse (Table 2.3.1). This suggests that stem thickness might be useful for differentiation among lucerne cultivars if the test is conducted under environmentally controlled conditions. Variation was observed among some of cultivars for thickness of the main stem of plants at the seedling stage (45 days old), but these data were not collected. This suggests it may be possible that lucerne cultivar discrimination might be achieved using seedlings rather than a second year crop. This is yet to be confirmed.

2.4.3 Plant height at flowering

Plant response to the environmental conditions in the field in 1993 and 1994, and also in the glasshouse, as expressed by the height of plants at flowering varied from cultivar to cultivar. For example, cvs. G. Oranga and Wairau (New Zealand), and Bami and Esfahani (Iran) which had similar plant height in the field in both 1993 and 1994, differed significantly in the glasshouse. This clearly shows that plant height data from one cultivar may not be reproducible under another set of conditions.

2.4.4 Time of flowering, and the number of nodes on the stem to the first flower buds

Time of flowering was also influenced by plant growth environment. In addition, the response of cultivars to the different environments was not unique (see 2.3.7). This result was consistent with Nittler and Kenny (1964) who showed that cultivars, temperature, and the temperature x spacing interaction all significantly affected the percentage of flowering plants (see 2.2.2.3). These results suggest that time of flowering is not a stable character and may not be useful for verification of lucerne cultivars.

For this character, more discrimination among cultivars was achieved in the glasshouse than in the field. For example, despite there being no significant difference among cultivars for 50% flowering, 44% of cultivars in the glasshouse could be discriminated on the basis of 50% flowering. A similar result was also found for 75% flowering (*i.e.* 6% vs 39% of cultivars which could be differentiated in the field and glasshouse respectively). The larger variation among cultivars for the time of flowering in the glasshouse suggests that this character might be useful for cultivar discrimination if the test is conducted under controlled conditions.

2.4.5 Flower colour

Flower colour, as a genetically controlled character in lucerne (Barnes and Hanson, 1967; Teuber and Brick, 1988) usually ranges from very light to very dark purple. There can also be variegated and cream flower coloured plants in the population of a hybrid cultivar. Unlike cream and variegated flowers which could be easily differentiated from each other and also from the purple colour, classification of purple flowers into different categories, even three classes of light, medium, and dark as used in this study, is a subjective assessment and

there can be confusion in recording of flower colour and also interpretation of the data. This is clear from the difference in scoring results for flower colour in the two field (spaced plant, and flowering trials) and glasshouse experiments.

Differentiation of G. Oranga and Wairau, the two New Zealand cultivars, from all of the four Iranian cultivars on the basis of the proportions of variegated flower colour (Table 2.3.5) suggested that discrimination among lucerne cultivars could be achieved successfully, when comparing hybrid variegated colour flower with purple colour flower cultivars. However, because of the requirement for subjective assessment, the scoring result may not be reproducible for cultivars with a similar proportion of variegated colour flowers.

2.4.6 Leaflet size

Leaflet width and length were smaller in the glasshouse than in the field (the overall mean of cultivars for leaflet length and width was 31.2 vs 23.9 mm for leaflet length and 14.1 vs 10.8mm for leaflet width in the field and glasshouse respectively, Table 2.3.2), suggesting that leaflet width and length are influenced by growing conditions. However, the influence of the environment on the leaflet size was not the same for all cultivars. For example, the mean leaflet length for cv. Azari in the glasshouse was 31% less than for in the field, while for cv. Bami the reduction was only 13%. Different leaflet area responses of three lucerne cultivars to three different temperature regimes have been also reported by Bula (1972, see 2.2.2.1). This suggests that it would be unlikely to have a unique response for each cultivar in different conditions.

Unlike leaflet length and width, the overall mean of leaflet width/length ratio of all of six cultivars was the same in the field and glasshouse (0.464 vs 0.460, Table 2.3.2) which indicates that this ratio is stable and thus, useful for verification of lucerne cultivars. However, cv. Azari did have a larger leaflet

in the glasshouse than in the field. This shows that leaflet width and length are not dependent characters, and thus may change differently under dissimilar environmental conditions. Therefore, the result from plot testing may not always be reproducible, even for those characters which appear to be stable.

2.4.7 Plant recovery height

Although the physiological basis for variation in regrowth or recovery height of lucerne plants in cool temperature and short days is poorly understood (Heichel and Henjum, 1990), it has long been used for classification of lucerne cultivars into three groups *i.e.* autumn non-dormant, intermediate and dormant (Nittler and Gibbs, 1959). This character has also been found to be useful for lucerne cultivar verification when recorded from the flowering plant, three and six weeks after cutting (OECD, 1988).

Recovery height of plants in the field and the glasshouse up to six weeks after the first cut and three weeks after the second cut (see Materials and Methods) was able to differentiate some of cultivars. However, the percentage of discrimination among the pairs of cultivars in the glasshouse was much smaller than in the field (eg. 72% vs 44% for field and glasshouse respectively, see Table 2.3.6). More discrimination among cultivars in the field was most likely due to cooler temperature, as this condition facilitates the expression of differences in the level of autumn dormancy of lucerne cultivars (M.Dehghan-Shoar unpublished data). Data from the recovery height of plants four, five, and six weeks after the first cut suggest that the differences among cultivars gradually levelled off in both sets of conditions, but were much faster in the glasshouse, indicating that the differences among lucerne cultivars would be more consistent in a cooler environment.

The comparison between plant recovery height of cultivars two and three weeks after the first cut showed that in both conditions cvs. Azari, G. Oranga, and Wairau had the shorter, Hamedani the intermediate, and Bami and Esefhani had the higher recovery height. This suggest that plant recovery height is a stable and environmentally independent character, which is therefore useful for lucerne cultivar verification. However as already mentioned, this character failed to discriminate more than 87% of the pairs of cultivars.

2.4.8 Foliage dry weight

Classification pattern of cultivars on the basis of foliage dry weight in the field in the first and second year was not unique (Table 2.3.3), suggesting that this character is most likely influenced by plant age, and possibly by different growth environments. This pattern was also influenced by the stage of plant growth. Despite there being no difference among cultivars at the second cut, they were able to be classified into three cultivar groups for the first cut (Table 2.3.3). Different classification patterns for cultivars in the glasshouse and in the field (Table 2.3.3) also suggested that foliage production cannot be used as a reliable measure for distinctness of a given cultivar.

2.4.9 Discrimination among cultivars using plant morphological characters which could only be recorded from the field trials

2.4.9.1 Plant angle

Scoring of cultivars on the basis of plant angle resulted in a unique cultivar classification pattern for all three recordings in the field (*i.e.* at flowering time, and four and five weeks after the first cut), by which 80% of pairs of cultivars could be discriminated (Table 2.3.8). Although this suggests a reasonable discrimination value for plant angle, there are two problems associated with

discrimination of lucerne cultivars on the basis of this character; firstly, plant angle might be altered by environmental factors, in particular wind; and secondly lucerne cultivar verification on the basis of plant angle requires subjective assessment, and accordingly the result may not be always reproducible.

2.4.9.2 Disease resistance

Scoring of plants in the field on the basis of the level of leaf spot (*Pseudopeziza medicaginis* Lib.)infection, resulted in a differentiation of 50% and 61% of pairs of cultivars in the two field trials. The percentage of pairs of cultivars which could be differentiated by infection with downy mildew (*Peronospora trifoli* L), was however, less than that for leaf spot. Although the discrimination results on the basis of disease resistance of cultivars were promising, the two problems already discussed for plant angle are also associated with discrimination of lucerne cultivars on the basis of disease resistance.

2.4.10 Problems associated with the conventional test plot method for registration of lucerne cultivars

1. According to UPOV (1988, 1995) a cultivar of a plant species must be clearly distinguishable from any other cultivar by one or more characters, and the distinctive features of cultivar should be maintained during multiplication and commercialisation (see 2.1.2). Among the characters recorded in this study only three *i.e.* the number of plant stems immediately above the ground, plant recovery height, and leaflet width/length ratio were likely to be stable, and independent of the environment. The remaining characters, including the other UPOV (1988) recommended plant morphological characters for lucerne (*i.e.* plant height at flowering, width and length of terminal leaflet), were influenced by the growth environment, and possibly soil type. Therefore due to the lack of

stability in various environments, these characters can not be considered reliable for distinguishing among lucerne cultivars.

2. According to UPOV (1988) when distinctness depends on measured characteristics such as plant height at flowering, any differences are significant if they occur with a one percent probability of an error ($P<0.01$). Results from this study demonstrated that even at $P<0.05$, discrimination among all of the cultivars was not possible using any of the plant morphological characters. This clearly illustrates the deficiency of the test plot for registration of lucerne cultivars. For example, two lucerne cultivars might be considered distinct at one site, but may not be under a different set of environmental conditions.

3. Morphological data for registration of new cultivars should be recorded from individual plants in two growing periods (UPOV, 1988). This is a time consuming practice which can require a substantial area of land.

4. Discrimination among lucerne cultivars using plot testing requires skilled workers for a long period of assessment time, and therefore is an expensive practice.

5. Some of the UPOV (1988) recommended morphological characters for discrimination among lucerne cultivars require subjective assessment (e.g. flower colour, angle of plants, growth habit, and disease resistance) and therefore, the results of the test plot may not always be reproducible.

2.4.11 Problems associated with the conventional plot testing method for lucerne seed certification

1. Seed certification requires plot testing (OECD, 1988). This is to make sure a particular certified seed lot is true to the named cultivar, and has not been

mixed with other cultivars or otherwise altered during multiplication. In this type of test morphological characters of individual plants of the seed lots under test are compared with those for the standard seed lot of the cultivars (eg. breeders seed, OECD, 1988, see 2.1.3). Therefore, theoretically, any possible influence of growing conditions on plant morphological characters should be the same for all of the seed lots of a cultivar. Thus, lack of plant morphological character stability may not be so important for seed certification. However, no report was found in the literature to confirm this hypothesis. Thus morphological data from the same seed lots of lucerne cultivars grown in various environmental conditions are needed before drawing any final conclusion as to the validity of plot testing for certification of lucerne cultivars.

2. If no genetic shift occurs in seed lots of a cultivar during successive multiplications, the seed lots should be the same as the standard seed lot of the cultivar. But as a result of out-breeding mechanisms, genetic shift may continue with each succeeding generation (see 4.4.7) and seed lots of a lucerne cultivar may therefore not be the same as the standard seed lot of the cultivar for an identical morphological character. No report was found in the literature as to whether this shift can be detected by plot testing using morphological characters.

3. Morphological data for seed certification should be recorded from second year crops (OECD, 1988). This requires two years from sowing of the seed up to the final recording. Therefore, any results from a plot test are often obtained when it is too late to down grade or reject a seed lot from certification, as the seed lot under test has usually already been sold and sown. In addition to these three mentioned issues, problems number three, four, and five which were addressed in section 2.4.10 are also relevant for plot testing for lucerne seed certification.

Because of the problems identified with the required plot testing for lucerne cultivar registration and certification of the seed being produced, it is necessary to look at other possible alternatives; for example laboratory techniques such as image analysis of seed and plant leaflets; and also molecular techniques for verification of, and discrimination among, lucerne cultivars. These are discussed in the following chapters of this thesis.

CHAPTER 3
IMAGE ANALYSIS OF PLANT LEAFLETS AND INDIVIDUAL SEEDS FOR IDENTIFICATION OF, AND DISCRIMINATION AMONG, LUCERNE CULTIVARS

3. 1 LITERATURE REVIEW

3.1.1 Introduction

The identification, verification and/or characterisation of cultivars is essential for the successful operation of national seed certification schemes, for the award and implementation of plant breeders rights, and for control of seed trading in markets dependent on specific quality attributes determined by, or linked to, genotype. Traditionally, cultivar identification has been accomplished by means of what Cooke (1995b) termed the classical taxonomic approach. This is largely achieved by the characterization of submitted genotypes through morphological observations, and measurements on both seeds and growing plants (Keefe and Draper, 1986).

Morphological descriptions are widely used, and standardized combinations of descriptions for particular species have been defined by the International Board of Plant Genetic Resources (Keefe and Draper, 1988). For example, in ground nut (*Arachis* spp.) leaflet length and width are recorded together as shape. Records are also taken of seed shape, defined as either angular, irregular-rounded or pea-shaped. Other examples of seed shape descriptors occur in cereals and pulses. Many of the morphological characters are continuously variable, and this has been a special problem in the past, particularly for parameters such as leaf or root shape (Keefe and Draper, 1988). However in practice, such an approach has been extremely successful, and largely forms the basis, for example, of the distinctness testing procedure prior to cultivar registration. However, as already discussed in chapter 2, the recording of morphological characters is time consuming and expensive, and requires large areas of land and highly skilled personnel making what are often subjective judgments. Many of the

morphological descriptors used are multigenic, quantitative and their expression can be influenced by environmental factors (Symons and Fulcher, 1988; Cooke, 1995b, see also section 2.2.2.6 of this thesis); this requires replicated measurements in order to obtain statistical means for discriminating among individuals.

Possible alternatives to the time consuming and costly manual measurements of the morphological descriptors are automated vision systems (machine vision). The term "image analysis" has also been used by many authors in this context. However, as mentioned by Cooke (1995b), the latter more strictly refers to extraction of numerical data for an acquired image. Apart from providing an automated means of obtaining measurements, a great advantage of machine vision is that it is possible, given suitable computer software, to provide detailed comparisons of a set of data, *i.e.* to operate a pattern recognition system. This makes the system very attractive for both cultivar description and identification (Cooke, 1995b). This technique has been shown to be very cost effective (Blaich and Blaich, 1990) with reproducible results (Lebowitz, 1989; Dreier *et.al.,* 1991).

3.1.2 Principle of image analysis

Image analysis by computer, following data acquisition by a video camera coupled with image processing and analysis is provided by a commercially available image analysis unit, computer and a custom-built sample presentation device in the form of a computer-controlled motorized camera gantry. The system can acquire images from samples varying in size (5-300 mm^2) and will accept input from photographic negatives and prints. Samples can be arranged over an area of 500 x 1000 mm and viewed from above or from the side and in transmitted or reflected light. The camera can be programmed to move automatically to the next field of view. The software can quantify colour, area, length, width and angle together with a range of mathematically more complex

shape descriptor (Keefe and Draper, 1988). See also section 3.3.1 of this thesis for more information on the description, and also the basic elements of the system.

No report in the literature was found regarding the application of image analysis techniques for the measurement of lucerne seed and leaf size and shape, and most published reports are about other species, in particular wheat and barley. In the following sections some of these reports are reviewed.

3.1.3 Application of the image analysis technique for determination of leaf size and shape properties

Image analysis was used by Goto *et.al.* (1991) to determine morphological and anatomical variations among barley cultivars which may affect straw degradability. The morphological composition (leaf blade, leaf sheath and stem content) of straw from three barley cultivars was determined and various quantitative anatomical measures, including the proportion of internode area occupied by specific tissues, cell number per unit area and cell-wall thickness, were made on stem sections using scanning electron microscopy (SEM) coupled with image analysis. The proportion of tissue area was similar for all cultivars, although there were differences among cultivars in the number of ground parenchyma and sclerenchyma cells per unit area. Differences among some of the cultivars were observed in the thickness of epidermis cell walls.

Engels and Petry (1990) used true-colour quantitative image analysis for the non-destructive measurement of leaf and plant growth of strawberry plants from different origins. In this study, no significant difference was found among the cultivars.

An optical planimetric technique was used by Kershaw and Larsen (1992) for

measuring the projected leaf area of conifer needle samples. This technique combined a rapid field recording method with an inexpensive hand-held optical scanner. The field recording method utilized strips (10X28 cm) of diazo paper (blueprint paper), to make positive images of the needle samples. An analysis program calculated leaf area from the output files produced by the hand-held scanner. Results from this system were compared with those from a Li-Cor 3100 leaf area meter and from an image analysis system using both standard samples of known area and needle samples from three conifer species. All three systems produced similar results in terms of means and variances. The digital camera system typically had the lowest variances between successive measurements of the same sample. These reports clearly suggest that image analysis, as a high speed and easily operated system, can be employed for measurement of leaf size and leaf shape as a means of identifying or characterizing cultivars.

Image processing of morphological traits of grape germplasm was developed by Shiraishi *et.al.* (1994) to make descriptions of morphological traits more accurate and effective. For seven grape accessions, a plant image was taken with a still video camera and displayed through a digital to analogue conversion. A high-quality image was obtained by 500 TV pieces in horizontal resolution, allowing the discrimination of, in particular, the density of prostrate hairs between mature leaf veins (lower surface). The analogue image was stored on an optical disc for semipermanent preservation without loss of quality. However, the analogue processed image proved inadequate for retrieval and image analysis, and hence revision of the data management system is required.

3.1.4 Application of image analysis techniques for determination of seed size and shape properties

Using a low cost image capture and processing system, Draper and Travis (1984)

demonstrated that it was possible to analyze the shape of seeds. The measurements included length, width, area, and perimeter, and also two derived variates, namely aspect ratio (width/length) and shape factor (4π area/perimeter^2). Using seeds of 49 crop and weed species, they further demonstrated that most species could be differentiated on the basis of the shape factor in combination with seed length (Draper and Travis, 1984). This result clearly demonstrated the taxonomic potential of machine vision, and encouraged the extension of the work to cultivar identification.

The potential of image analysis for identifying or characterizing wheat cultivars on the basis of differences in grain morphology was investigated by Keefe and Draper (1986). Using transmitted light, individual grains resting horizontally, adaxial surface lowermost, were viewed in side elevation. Nine parameters describing grain shape together with the area of the grain silhouette were measured for each grain. These authors found that out of the nine shape descriptors measured, seven had potential for use as taxonomic characters. The use of samples of well filled grains, as opposed to a completely random selection, appeared to give little consistent benefit in terms of resolution of cultivar distinctness.

Neuman *et.al.* (1987) demonstrated that a correct classification of four durum wheat cultivars in admixture with 10 common wheats, representing a broad range of grain types, was quite possible by means of image analysis of grain size and shape. Samples of each of five Canada Western Red Spring (CWRS) cultivars were all correctly allocated to the CWRS class; none of 432 grains of other types was misclassified as CWRS. Discrimination among four other cultivar classes was less satisfactory. Discrimination within classes among the 14 cultivars gave inconclusive results, correct classification scores ranging from 15 to 96%. These authors suggested that although the method was useful for screening breeding material to identify lines with undesirable atypical grain characteristics, it needed

further improvement for use in cultivar and class identification.

Digitized images of whole grain samples of hard red spring wheat, barley, rye and oats were taken by Sapirstein *et.al.* (1987) using a charge-coupled device (CCD) video camera interfaced to a custom-built data-acquisition system. Computed grain features included seed length, width, area, aspect and thinness ratios, contour length and normalized central moments. Size and shape parameters were evaluated with regard to discrimination ability by stepwise discriminant analysis. In this study Canonical Discriminant Analysis was applied to visualize cereal class differences and a linear discriminant model was derived. The result of this study was promising, as only approximately 1% of the over 1100 seeds tested were incorrectly classified among wheat, oats, barley and rye in a four-way admixture.

Symons and Fulcher (1988) reported that variation in whole grain morphology within the eastern Canadian soft white winter wheats was difficult to assess by visual inspection, although functional tests have shown that differences exist among cultivars. Digital image analysis was used by these authors to evaluate the morphological variation of five of these cultivars. Whole grain, crease and bran features were measured quantitatively. Whole grain features alone were inadequate for cultivar classification, while the inclusion of crease or bran features improved cultivar classification, suggesting that a combination of more features increased the chance of cultivar discrimination. From a six-cultivar discriminant model, the classification of cvs Augusta and Fredrick (from four test sites) demonstrated that environmental influences greatly affected grain morphology and subsequent classification. However, in a two-way model, Augusta and Fredrick grains were correctly identified irrespective of environmental influence.

Using digital image analysis, twelve morphological features of the grains of nine

cultivars of milling quality wheats grown in eastern Canada were determined by Symons and Fulcher (1988). Four soft white winter (SWW; biscuit/pastry quality), five bread making, three hard red spring (HRS) and two hard red winter (HRW) cultivars were examined. The HRW cultivars were grown at two different locations. Stepwise discriminant analysis selected seven variables which clustered cultivars by class and the HRS wheats by origin. A four-way linear discriminant model, separating bread and biscuit quality wheats, correctly assigned (>80%) of the HRS and HRW classes and totally segregated the SWW wheats. While grains could be assigned to classes using external morphology, cultivars within the SWW class could not be clearly distinguished.

Zayas *et.al.* (1989) used image analysis to discriminate between wheat and non-wheat components in grain samples. Morphometrical data were extracted and stored on a computer disk, and multivariate discriminant analysis was used to distinguish between wheat and non-wheat components and between weed seeds. These authors were able to distinguish between wheat and non-wheat components and weed seeds.

Variation in oat seed morphology was assessed by Symons and Fulcher (1988) using digital image analysis. In this study the features of the grain of six cultivars were measured. Grain area was highly correlated ($r \simeq 0.98$) with grain weight. Width/length ratio differed both within and between the six populations, suggesting that differentiation of oat cultivars is feasible using the image analysis technique.

Grain shape variation in five cultivars of wheat of UK origin was studied by Keefe (1990) using image analysis. Grains were viewed sitting crease down, as silhouettes, in side profile and quantitative measures of area, angles and distances were taken using purpose-written software. Examination of individual grain measurements from samples taken from harvested bulks indicated that for

most parameters in all cultivars, the data points were not normally distributed. The majority of distributions appeared multimodal. For any given parameter, marked differences in variance were observed between cultivars and these differences were consistent when 10 samples of each cultivar from separate bulks were examined. Assessment of the relationship between the grain's position of origin in the inflorescence and its shape was also determined, using dissected wheat heads. Grain shape was strongly associated with position of origin (Keefe, 1990).

A semi-automatic, dedicated wheat grain image analyzer was constructed by Keefe (1992) for rapid processing of wheat grain samples. For each grain the analyzer takes 33 measurements and calculates 36 derived parameters to describe its outline shape. The instrument was tested on 20 wheat cultivars. For a 50 grain sample the time taken from receiving the sample to having the data ready for analysis was about five minutes. This clearly demonstrates the functional speed of the system. Results by Keefe (1992) showed that for all measured and derived parameters, there was some overlap between all cultivars. This appears, at least for wheat, to be a serious deficiency of the system for discrimination among cultivars on the basis of seed size and shape. Estimation of the Bayes error rate (32.9-65.8%) by Nearest Neighbour analysis suggested that it would not be possible to positively identify every grain by cultivar, although the instrument was considered to be adequate for research purposes and for use in National Variety Registration Schemes (Keefe, 1992).

Development of more sophisticated image analysing systems, such as the three-dimensional image analysis used by Thomson and Pomeranz (1991) for differentiation of two wheat cultivars, could offer a better opportunity for researchers to look at the surface features more closely, and may increase the chances of successful cultivar identification. In the three dimensional system a computer-controlled laser scanning system was developed to acquire

three-dimensional images of the surface of cereal grains. Each picture element represents the elevation of the seed surface above the reference plane. In addition, a separate image is acquired in which each picture element represents the intensity of the light reflected from the seed surface. The images are then used for feature extraction. The system was used to distinguish between seeds of the wheat cultivars Daws (soft white winter) and Tyee (club). A combination of 14 features based on nine topographic images and five intensity images permitted discriminate analysis to correctly classify 92-94% of the seeds. This system was also used by these authors to identify sprout damage in harvested wheat seeds. A topographical image of the seed surface was used to differentiate among sprouted and unsprouted seeds. A discriminant model based on four features correctly identified 89% of the sprouted seeds and 83% of the unsprouted seeds when applied to an independent set of test seeds.

A three- dimensional image analysis was used by Sakai and Yonekawa (1991) for determining the shape of soya bean seed; its axial length, surface area, volume, particle density, compactness and sphericity were measured. From the analysis on a reduced sample of the seed population, the typical size and shape of seeds was calculated. The 3-D properties of surface area and volume were expressed as functions of seed mass. In general, these authors found that soya bean seeds appeared to be uniform in shape over a wide range of sizes, and thus this property could not be used as a means for cultivar identification of this species.

Reports discussed in this brief review indicate the potential of the image analysis technique for cultivar identification of plant species. However, the majority of the species used in these studies were self pollinating crops, in which genetic variation is much smaller than those of cross pollinating species. The latter often produce overlap results from different cultivars. Therefore this technique may not be suitable for cultivar identification of these species.

Chapter 3

A cultivar of a cross pollinating species, such as lucerne, is a population of plants, comprising many genotypes. As a result, the statistical errors which result from data analysis of these crops are large, and thus a large sample size is required to reduce the error (Mead and Curnow, 1983). Although this may cause serious problems for cultivar identification of such species when using the time consuming manual measurement of seed and leaf morphology, image analysis is a very fast technique which can provide computed data in a short period of time (Keefe, 1992). This promising feature makes the investigation of this technique as a means for cultivar identification and discrimination of cross pollinating species a worthwhile study.

3.2 MATERIALS AND METHODS

Vision Image Processing System version 4.1 (VIPS, 1992), a digital image processing algorithm development environment, was used for image processing of seeds of six Iranian and two New Zealand cultivars (Table 3.2.1) and also leaflets from the plants of four Iranian and two New Zealand cultivars grown both in the field and in the glasshouse. This system has been developed by the Image Analysis Unit, Massey University and works under Microsoft Windows. A detailed description of the system is provided in Bailey and Hodgson (1988).

3.2.1 Descriptive summary of the process

The components of the VIPS hardware are illustrated in Figure 3.2.1 and the following is a brief explanation of their functions.

Camera - converts the light intensity pattern into an electrical (video) signal

Frame grabber - samples and digitises the video signal, converting it into a two dimensional array of numbers, where each element in the array (pixel) corresponds to a different position on the object (seed or leaflet), and the pixel value represents the intensity of light at that point.

Computer - processes this array of numbers, or digital image, to extract the required data which are then transferred to a result file for later statistical analysis

3.2.2 The configuration of the VIPS used in this study consisted of the following:

i) The host computers used for image processing of the seed and the leaves were a 486DX50 IBM compatible P.C. (16Mb Ram) and a 486DX33 IBM compatible P.C. (16MbRam), both with Imaging Technology frame grabber cards installed.

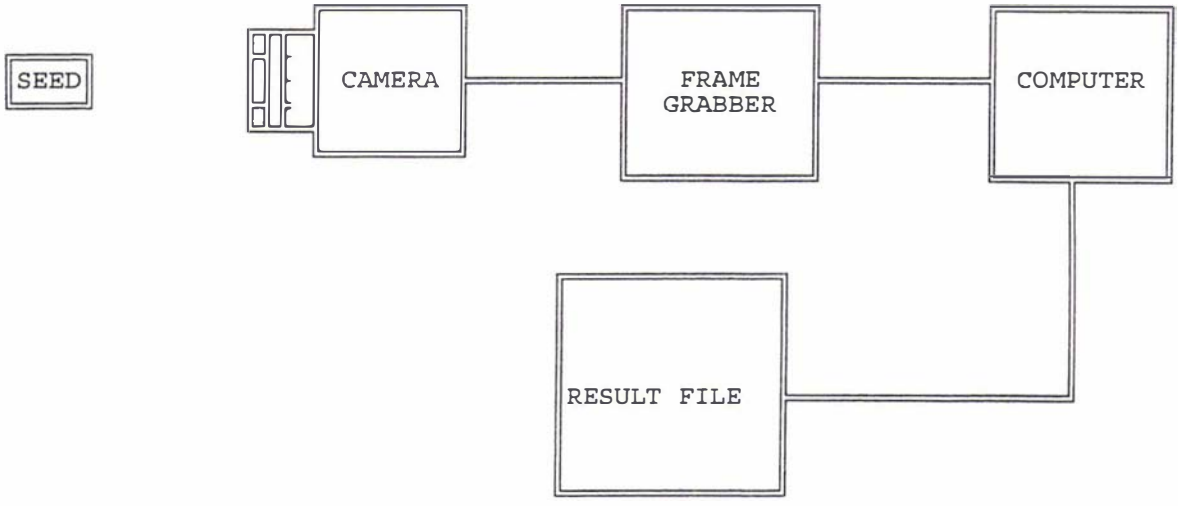
ii) The frame grabber for the seed analysis was a Vision Plus At Colour Frame Grabber and that for the leaves was a PC Vision Plus frame grabber

iii) A poloroid MP4 Copy stand using transmitted light was used for the leaves, and reflected light for the seeds.

iv) A Sony DXC- 3000P colour CCD camera was used to capture the images of the seeds and the leaves.

v) A 10mm-120mm Macro Fujinon VCL-1012 lens with close-up filter was used on the camera.

Figure 3.2.1 The components of the Vision Image Processing System (VIPS)



3.2.3 Computer commands used for image analysis of seeds and leaflets

Programs for the image analysis of individual seeds and leaflets of the cultivars examined in this study were written by Dr. D.G. Bailey of the Image Analysis Unit, Massey University, Palmerston North, New Zealand. The commands were written using the VIPS language (Bailey and Hodgson, 1988; VIPS, 1992). The systems analysis and software design are not presented in this thesis, but this information is available from the Image Analysis Unit, Massey University.

3.2.4 Image processing of individual seeds and plant leaflets of the cultivars

Morphological characters were recorded from 66 (three replicates of 22) terminal leaflets of individual one year old plants of four Iranian (Azari, Bami, Esfahani, and Hamedani), and two New Zealand cultivars (G.Oranga and Wairau) grown in a glasshouse (see 2.2.2). For seed image analysis, three randomly drawn (ISTA, 1993) replicates of 50 seeds from the pure seed fraction (ISTA, 1993) of 17 seed lots of six Iranian and two New Zealand cultivars (Table 3.2.1) were used for image processing.

Image processing of the terminal leaflets was carried out by arranging a set of 6-8 leaflets from each replicate in rows within a 100mm square on a light table, using four rows of two leaflets at a distance of about 5 mm within and between rows. The leaflets were lit from behind using a light table to give good contrast of the leaflet edges. The images were thresholded (*ie.* each pixel was compared with a pre specified grey level, with pixel values less than this level classified as belonging to a leaflet, and values greater than the level classified as background pixels; Russ, 1995) to detect the leaflet from the background. The required measurements (see section 3.2.5) were made from the boundary of each individual leaflet (defined as a contiguous group of pixels classified as belonging to a leaflet). For seed image analysis, 50 seeds from each of three replicates were placed in rows

in the configuration shown in Figure 3.2.3 within a 50-70 mm square on a copy stand, with 10 rows of five seeds at a distance of 5-7 mm within and between rows. The seeds were lit from above to enable colour measurements of the seed surface to be made. The image was thresholded (as already described) to detect the seeds from the background. Each individual seed (again defined as a group of contiguous seed pixels) was processed to determine the colour (from the original image) and the shape characters which are listed in section 3.2.6.

To enable the measurement to be provided in mm rather than pixels, the imaging system was calibrated by using a circular object of known size using the procedure described by Bailey (1995).

3.2.5 Morphological characters of terminal leaflets

Morphological characters were recorded from terminal leaflet of individual two years old plants of four Iranian (Azari, Bami, Esfahani, and Hamedani), and two New Zealand (G.Oranga and Wairau) cultivars. The characters recorded were as as follows:

1. Actual leaf area (mm^2, Area.1, Fig.3.2.2a)
2. Area of leaf (mm^2) after performing a convex hull (Area 2 Fig.3.2.2b).
3. Perimeter of the leaf(mm, perimeter 1, Fig.3.2.2a).
4. Perimeter of leaf after convex hull (mm, perimeter 2, Fig.3.2.2b).
5. Length of leaf (mm, Fig.3.2.2c).
6. Width of leaf (mm, Fig.3.2.2c)
7. Shape balance factor (termed in the thesis as leaflet shape):
   This was measured using the formula: distance between the centre of leaflet and the centre of gravity (weighted on the area, X in Fig.3.2.2c), divided by the total leaf length multiplied by 100. Thus the greater the difference between the centre of gravity and the centre of the leaf, the more the leaf is concentrated at one end.

8. Angle of leaf: The angle from the end of the leaf to points 15 pixel along the boundary on either side (Fig. 3.2.2c)
9. Roughness.1 of the leaflet, using the formula: area2- area1)/area1 x 100% (Bailey 1994, pers.comm.)
10. Roughness.2 of the leaflet, using the formula: perimeter 1 - perimeter 2)/perimeter2.
11. Elongation.1=length/ width.
12. Elongation.2=width/length.

All these characteristics were recorded from terminal leaflets of 66 one year old plants of each of the cultivars grown in the glasshouse.

3.2.6 Seed Properties measured

1. Total reflectance or colour intensity of the seed: The sum of pixel values seen by the camera system for the red, green, and blue colour components averaged over the whole seed. These values are dependant on the light quality, camera spectral characteristics (sensitivity to red, green and blue colour), white balance, and gamma (Bailey 1994, pers.comm). As all of the seed samples were analyzed under the same conditions, it was assumed that all of these characters except seeds, were constant over the set of measurements.

2. Red/Green colour: When the value of 1.0 represents the colour yellow, a value more than 1.0 means that the seed is more red, while a value of less than 1.0 indicates that the seed is more green.

3. Red colour /total intensity.

4. Green colour/total intensity.

5. Blue colour/total intensity.

6. Length of seed (Fig. 3.2.3a).

7. Width of seed (Fig.3.2.3a).

To measure width and length of the seed, the smallest area rectangle surrounding the seed was defined by the computer and the width and length of the rectangle recorded as the length and the width of the seed (Fig. 3.2.3a)

8. Width/length,
9. Length/width.
10. Concavity of seed: This factor expresses the depth of concavity of the seed. This was recorded by measuring the distances between the convex hull of the seed and the deepest point on the seed coat (X, Fig.3.2.3b).
11. Curve position (i.e. distance of sharpest curve along the length of the seed from the centre, Fig.3.2.3b). When the sharpest curve is at the middle of the seed length, the value of the curve position is zero.
12. Actual area : Area of projection of seed under camera (Fig.3.2.3Ctd.c).
13. Perimeter (Fig.3.2.3Ctd.c).
14. Curvature of the seed which was assessed by measuring the sharpest internal angle in the concave region (Fig.3.2.3 Ctd.e). The angle was measured by looking at the angle between point 5 pixel on either side along the boundary.
15. Convex area : The area of the convex hull (Fig.3.2.3Ctd.d).
16. Convex perimeter: The perimeter of the smallest convex hull (Fig.3.2.3Ctd.d).
17. Average width of seed, calculated from the formula: Area of the seed/length.
18. Concavity /width : Represents the depth of the concavity as a fraction of the width of the seed.
19. Actual area of the seed /convex area.
20. Convex perimeter of the seed /actual perimeter.
21. $4\prod$ area/perimeter^2. This property of the seed can indicate the circularity of the seed and when the value is 1.0, it means that the seed has a circular shape.

Table 3.2.1 Seed lots of the cultivars used in the seed image analysis study

Cultivars	Seed lot number	Abbreviation of the names of the seed lots which are used in the result and discussion sections
Azari	No.1	Aza
Bami	No.1	Ba-1
Bami	No.2	Ba-2
Hamedani	No.1	Ha-1
Hamedani	No.2	Ha-2
Esfahani	No.1	Esf
Nikshahri	No.1	Nik
G. Oranga	Basic seed-B86	Or-1
G. Oranga	1st gen.seed-B294	Or-2
G. Oranga	Basic seed-B486B	Or-3
G. Oranga	1st gen. seed	Or-4
G. Oranga	Breeders seed-AF2401	Or-5
Wairau	Nucleus seed	Wa-1
Wairau	1st gen. seed-B525	Wa-2
Wairau	1st gen. seed-B52	Wa-3
Wairau	1st gen. seed	Wai-4
Yazdi	No.1	Yazd

Figure 3.2.2 Illustration of plant leaflet morphological characters recorded using the Vision Image Processing System (VIPS)

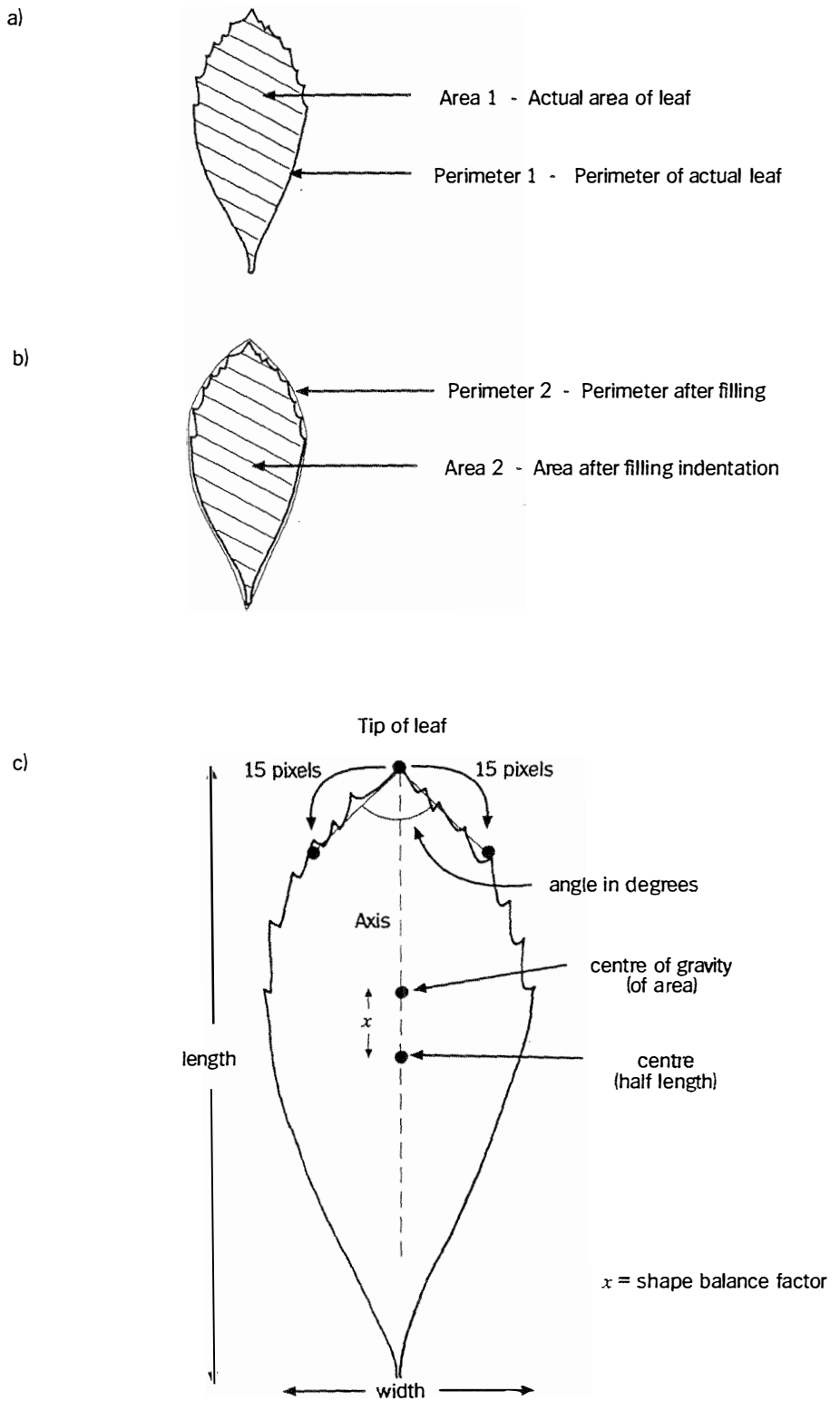
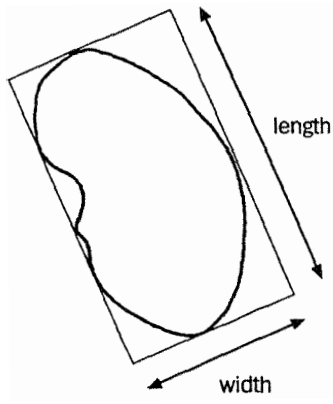


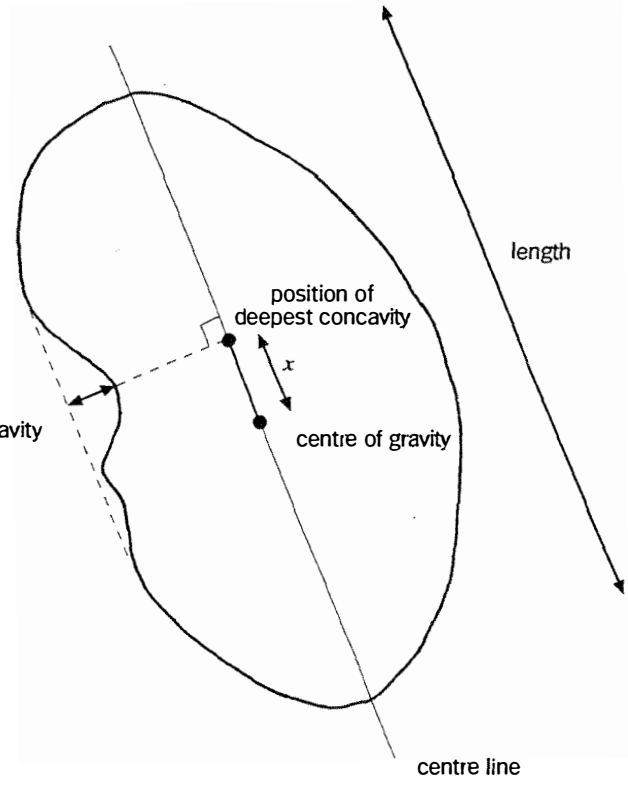
Figure 3.2.3 Illustration of seed morphological characters recorded using the Vision Image Processing System (VIPS)

a)



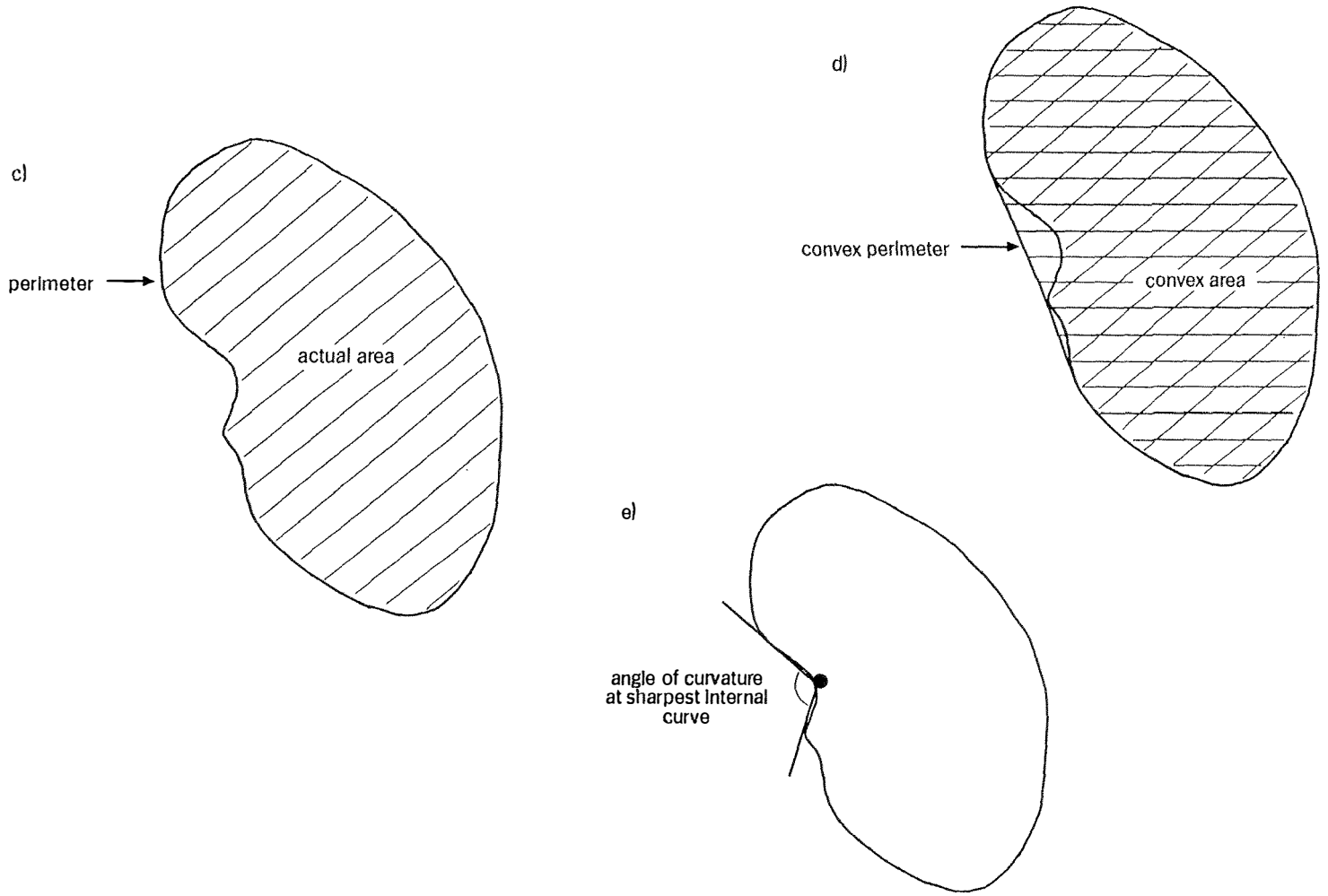
Smallest area rectangle

b)



x = distance of deepest concavity along the length of the seed from centre of gravity.

Figure 3.2.3 Ctd. Illustration of seed morphological characters recorded using Vision Image Processing System (VIPS)



3.2.7 Statistical analysis

3.2.7.1 Comparison between cultivars using each morphological characters recorded from individual seeds and leaflets of the seed lots of the cultivars

Analyses of Variance or the general linear model (ANOVA, SAS 1989) was used in this study to discriminate among the cultivars using each of the seed and leaflet morphological characters. Whenever ANOVA results indicated that these differences were significant, overall pairwise comparison tests (SAS, 1989) were employed for each character to further assess the significance of these differences.

3.2.7.2 Comparison between cultivars on the basis of a combination value from morphological characters recorded from individual seeds and leaflets of the seed lots of the cultivars

Canonical Discriminant Analysis (CDA, SAS 1989) was also used in this study for discrimination among, verification of and also estimation of genetic relatedness as well as uniformity of the cultivars. CDA was employed to find a linear function of the variables (eg. seed or leaflet morphological characters) that maximally discriminated the cultivars. This approach distinguished several uncorrelated canonical discriminant functions (CDF). These are linear combinations of the original values that provide the best separation of the means of the groups of observations relative to within-group separations (Renchar, 1992).

The canonical coefficients of each CDF provide information about the joint contribution of the variables to that CDF. The first CDF (CDF1) yields the maximum possible variation between groups with respect to within-group variation, reflecting group differences to the greatest degree possible. CDF2 reflects group differences not displayed by CDF1, and so on (Manly ,1986). Thus in the data

analysis for this study, the weighted averages of different numbers of variables (ie. seed and also leaflet morphological characters) were calculated using the following formula and used to discriminate between the seed lots of the cultivars:

$$DS= RCCS1*(S1-\bar{S}1)+ RCCS2*(S2-\bar{S}2)+..... +RCCS21*(S21-\bar{S}21)$$

where RCC is the Raw Canonical Coefficient of the variables (eg. seed morphological characters, S1-S21) and $\bar{S}1$-$\bar{S}21$ are the average values of each of the variables.

Determination of the pairwise squared Mahalanobis distances (Mahalanobis, 1936; Weatherup, 1994) between the mean values of seed and leaflet characters for each of the cultivars enabled comparison of the degree of relatedness between them. Calculations were according to the formula:

$$D^2 (\bar{X}i:\bar{X}j)= (\bar{X}i-\bar{X}j)^T Cov^{-1} (\bar{X}i-\bar{X}j)$$

where D^2 is the squared distance or Mahalanobis distance between mean values for the two cultivars (i and j), Cov^{-1} is the inverse of the covariance matrix and $\bar{X}i$ and $\bar{X}j$ are the means for the group or population, and T is the transpose of the vector of difference of mean values.

As the mean vectors for the samples can be regarded as estimates of the true mean vector of the groups, the Mahalanobis distances of individuals to group centres can then be calculated, and each individual can be allocated to the group that it is closest to. The percentage of correct allocations is clearly an indication of how well the groups can be separated using the available variables (Manly, 1988). Whenever required, tests of difference of two proportions were used to check whether the probability of membership in different seed lots from the same cultivar are significantly different.

The formula used in this test was as follows:

$$T = \frac{\hat{P}_1 - \hat{P}_2}{[\hat{P}_1(1 - \hat{P}_1)/n_1 + \hat{P}_2(1 - \hat{P}_2)/n_2]^{1/2}}$$

where '$\hat{P}$' denotes the proportion of the seeds classified into each of the seed lots 1 and 2 and 'n' denotes the corresponding sample sizes.

Pairwise comparisons of the least squared means (LSMEANS or means adjusted for unequal sample size) and the GLM (General Linear Model) or ANOVA (Analysis Of Variance) procedure for the variable CDF.1 (the first canonical discriminant function, or the combination of S1 to S21 variables (eg. seed characters) is that best discriminated among the seed lots of the cultivars were employed in this study to check if the differences among the seed lots of the cultivars were significant. Whenever the one way ANOVA indicated that these differences were significant, overall pairwise comparison tests (SAS, 1989) were employed for variable CDF.1 to further assess the significance of these differences.

3.3 RESULTS

3.3.1 Discrimination among lucerne cultivars on the basis of seed morphological characters recorded using image analysis

3.3.1.1 Assessment of the most useful seed morphological characters for identification of, and discrimination among, lucerne cultivars

The ability of the 21 seed morphological characters recorded from individual seeds of five seed lots of cv. Grasslands Oranga, and also four seed lots of cv. Wairau (see Materials and Methods) to place seed lots into either of the two groups varied (Appendix 3.1). Eleven characters (*i.e.* total red, green, and blue colour components averaged over the whole seed; seed length; seed width; average width of seed; seed area; seed perimeter; curvature of the seed; curve position; convex area; convex perimeter; convex perimeter of the seed /actual perimeter) were not considered useful for the purpose because the mean value for identical characters meant that seed lots of the cultivars were often interspersed (Appendix 3.1). However, 10 characters (*i.e.* red/green colour, red/total intensity, blue/total intensity, width/length, length/width, $4\prod$ area/perimeter^2, concavity of seed, concavity/width, seed curvature and actual area/convex area) were able to place the seed lots into two cultivar groups, even though the mean values were not always significantly different for the identical characters (Appendix 3.1). For example, the mean values of the ratio of red/green colour of the certified seed lots from cv. G. Oranga ranged from 1.24-1.29, and the mean values of all four seed lots of this cv. were mostly nearer to that of the standard seed lot of this cultivar (G. Oranga.5, Breeders seed) than that of the seed lots of cv. Wairau (Appendix 3.1). Similarly, the mean red/green values of three seed lots of cv. Wairau (which ranged from 1.22-1.24) were mostly grouped more closely to the nucleus seed lot of this cultivar (Wairau.1) than those of the seed lots of cv. G. Oranga (Appendix 3.1). A highly significant difference ($P<0.0001$) for all of the identical characters was found between the two cultivars when data for seed lots of each of the cultivars were used as replicates of the cultivars and analyzed using the GLM procedure (SAS, 1989, see Appendix 3.2).

Canonical Discriminant Analysis (CDA) was used to compare the value of a combination (CDF1) of the 10 selected characters, the 11 characters which were considered to be not useful, and that for all of the 21 recorded characters (see Materials and Methods) for discrimination among 17 seed lots of six Iranian, and two New Zealand cultivars. Pairwise comparison between least squared means (LSMEANS) of the seed lots using all 21 characters showed that 83% of the comparisons were significantly different ($P<0.05$, Table 3.3.2), while 87% of the comparisons were significantly different for the 10 selected characters (Table 3.3.3). For the remaining 11 characters only 68% of the comparisons were significantly different (Table 3.3.4). The percentage of comparisons which differed significantly was not improved by either increasing or further reducing the number of characters. For example, the same discrimination value for that of the 21 characters (83%) was obtained using a combination of the 10 selected characters and two of the characters which were considered to be not useful (*i.e.* total intensity, and green/total intensity). Also, a combination of four characters (red/green colour, red/total intensity, width/length, and $4\prod$ area/perimeter^2) and two of them (width/length, and $4\prod$ area/perimeter^2) had lower discrimination values than that of either the 10 or 21 characters (*i.e.* 80% for four characters, and 38% for two characters of the pairwise comparisons between LSMEANS of the seed lots were significantly different at $P<0.05$ (data not presented). The canonical coefficients for the CDF1 (the first canonical discriminant function or the combination of the ten characters that best discriminated among the seed lots of the cultivars, see section 3.2.7), for the colour components of red/total intensity, blue/total intensity, and red/green were 783.5, 323.7, and 99.9 respectively. The coefficients of the remaining characters were: 39.6 for seed concavity/width; 23.1 for concavity; 8.4 for actual area of the seed /convex area; 6.7 for circularity of seed (*i.e.*$4\prod$ area/perimeter^2); 2.0 for width/length and length/width ratio, and 0.02 for seed curvature. In the following sections only the results from application of the most useful characters for identification and discrimination among the cultivars are presented.

3.3.1.2 Discrimination among eight lucerne cultivars using ten individual morphological characters of single seeds of the standard seed lots of the cultivars

The GLM or ANOVA (Analysis Of Variance) procedure for the ten individual morphological characters of single seeds from the standard seed lots of the cultivars illustrated that there were highly significant differences ($P<0.0001$) among some of the cultivars. Duncan's Multiple range test was therefore employed for the individual characters, to further assess the significance of these differences. There were significant differences among some of the cultivars for each of the given characters (Table 3.3.1). Cultivars could be classified into four overall groups when each of the five characters of red/green colour, red/total intensity, blue/total intensity, concavity, and curvature of seed were used for discrimination among the cultivars. However, using each of the four characters width/length, length/width, $4\prod$ area/perimeter^2, concavity/width resulted in classification of the cultivars into only three groups. The character of actual area/convex area was the only one among the ten characters which failed to classify the cultivars into more than two groups. G. Oranga and Azari were the only cultivars in this study which could be discriminated from each other, and all others, on the basis of some of the individual morphological characters (Table 3.3.1). For cv. Azari this was as a result of having distinct width/length, $4\prod$ area/perimeter^2, concavity, concavity/width and curvature characters, while for cv. G. Oranga it was because of the high ratio of red/green colour, red/total intensity and blue/total intensity of the seed.

Pairwise comparisons between the cultivars using the data in Table 3.3.1 demonstrated that the seed characters of red/total intensity, and blue/total intensity were of the best discriminant functions among the ten characters examined in this study, as 64% of the pairs of cultivars differed significantly ($P<0.05$) using these characters. Seed curvature was the next best character for discrimination among the cultivars, as 53% of the pairs of the cultivars could be discriminated on the basis of this character.

The percentages of significance of the pairwise comparisons between the cultivars using each of the other characters ranged from 18%, for actual area/convex area, to 35% for the width/length and concavity of the seeds. As none of the individual seed morphological characters were sufficient for differentiation among all of the cultivars, it was hypothesised that a combination of these characters might be useful for achieving a better discrimination among the cultivars. This is discussed in section 3.3.2.

Table 3.3.1 Duncan's Multiple range test for discrimination among six Iranian and two New Zealand lucerne cultivars on the basis of selected individual morphological characters of single seeds of the cultivars recorded using image analysis. The percentage of pairs of the cultivars which differed significantly at P<0.05 for any of the given characters is calculated on the basis of the Duncan's grouping result, and these are also cited in this table (see POSDC). Seed lots linked by the same line do not differ significantly for the characters at P<0.05 (for cultivar codes see Material and Methods).

1	Red/green colour	Cultivar	Or-5	Ba-1	Ha-1	Wa-1	Yazd	Aza	Esf	Nik
		Mean	1.297	1.254	1.247	1.235	1.231	1.24	1.224	1.223
		Duncan's grouping (POSDC = 32%)								
2	Red/total intensity	Cultivar	Or-5	Ba-1	Ha-1	Wa-1	Aza	Yazd	Nik	Esf
		Mean	0.4177	0.4076	0.4071	0.4067	0.4053	0.4020	0.4001	0.3996
		Duncan's grouping (POSDC = 64%)								
3	Blue/total intensity	Cultivar	Esf	Nik	Yazd	Ba-1	Ha-1	Aza	Wa-1	Or-5
		Mean	0.2661	0.2639	0.2636	0.2613	0.2604	0.2606	0.2606	0.2601
		Duncan's grouping (POSDC =64%)								
4	Width/length	Cultivar	Aza	Nik	Ha-1	Esf	Wa-1	Or-5	Ba-1	Yazd
		Mean	0.720	0.655	0.647	0.646	0.645	0.642	0.632	0.631
		Duncan's grouping (POSDC =35%)								
5	4π area/perimeter^2	Cultivar	Aza	Esf	Nik	Wa-1	Ha-1	Yazd	Ba-1	Or-5
		Mean	0.859	0.840	0.839	0.835	0.834	0.832	0.831	0.828
		Duncan's grouping (POSDC =28%)								

Table 3.3.1 Ctd Duncan's Multiple range test for discrimination among 6 Iranian and 2 New Zealand lucerne cultivars on the basis of selected seed morphological characters determined by image analysis. The percentage of pairs of the cultivars which differed significantly at P< 0.05 for any of the given characters is calculated on the basis of the Duncan's grouping result, and these are also cited in this table (see POSDC). Seed lots linked by the same line do not differ significantly for the characters at P<0.05 (for cultivar codes see Material and Methods).

6	Concavity of seed	Cultivar	Or-5	Ba-1	Yazd	Wa-1	Nik	Ha-1	Esf	Aza
		Mean	0.121	0.118	0.111	0.111	0.107	0.103	0.1001	0.074
		Duncan's grouping (POSDC =35%)								
7	Concavity/Width	Cultivar	Or-5	Ba-1	Yazd	Wa-1	Ha-1	Nik	Esf	Aza
		Mean	0.0779	0.0772	0.0745	0.0724	0.07178	0.0704	0.0666	0.0055
		Duncan's grouping (POSDC =19%)								
8	Curvature of seed	Cultivar	Aza	Ha-1	Esf	Yazd	Ba-1	Wa-1	Nik	Or-5
		Mean	158.90	152.49	151.49	150.43	149.71	149.03	148.91	145.63
		Duncan's grouping (POSDC =53%)								
9	Actual area of seed/Convex area	Cultivar	Aza	Esf	Nik	Wa-1	Ha-1	Yazd	Or-5	Ba-1
		Mean	0.9930	0.9898	0.9897	0.9879	0.9872	0.9865	0.9863	0.9859
		Duncan's grouping (POSDC =18%)								

Table 3.3.2 Probabilities that pairs of LSMEANS of 21 morphological characters of individual seeds from a given cultivar are equal.

From To	Aza	Ba-1	Ba-2	Esf	Ha-1	Ha-2	Nik	Or-1	Or-2	Or-3	Or-4	Or-5	Wa-1	Wa-2	Wa-3	Wa-4	Yazd
Aza		0.0001	0.0001	0.1164	0.0007	0.0001	0.0564	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0126
Ba-1	***		0.0001	0.0001	0.0052	0.9891	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Ba-2	***	***		0.0001	0.0001	0.0001	0.0001	0.1603	0.0001	0.9302	0.6482	0.0001	0.0010	0.0077	0.4346	0.0001	0.0001
Esf	ns	***	***		0.0001	0.0001	0.7304	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.3544
Ha-1	**	*	***	***		0.0063	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Ha-2	***	ns	***	***	*		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.1335	0.0001
Nik	ns	***	***	ns	***	***		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0564	0.5634
Or-1	***	***	ns	***	***	***	***		0.0001	0.1880	0.0629	0.0001	0.0574	0.2057	0.5334	0.0001	0.0001
Or-2	***	***	***	***	***	***	***	***		0.0001	0.0001	0.9712	0.0001	0.0001	0.0001	0.0001	0.0001
Or-3	***	***	ns	***	***	***	***	ns	***		0.5865	0.0001	0.0014	0.0100	0.4878	0.0001	0.0001
Or-4	***	***	ns	***	***	***	***	ns	***	ns		0.0001	0.0002	0.0018	0.2159	0.0001	0.0001
Or-5	***	***	***	***	***	***	***	***	ns	***	***		0.0001	0.0001	0.0001	0.0001	0.0001
Wa-1	***	***	**	***	***	***	***	ns	***	**	**	***		0.5210	0.0119	0.0003	0.0001
Wa-2	***	***	*	***	***	***	***	ns	***	*	*	***	ns		0.05793	0.0001	0.0001
Wa-3	***	***	ns	***	***	***	***	ns	***	ns	ns	***	ns	ns		0.0001	0.0001
Wa-4	***	ns	***	***	***	ns	***	***	***	***	***	***	**	***	***		0.0001
Yazd	ns	***	***	ns	***	***	ns	***	***	***	***	***	***	***	***	***	

As some apparently significant results cited in this table may be due to chance, the significance probability for the LSMEANS was set at 0.01 for this test. The level of 0.01 has been chosen because of the need to control the overall test error rate. For P=0.01 the probability for 100 tests is that one apparently significant result is due to chance. The significant results are indicated by stars and not significant by 'ns'. The corresponding probability can be found in the upper right hand entries. '*' 0.01> P> 0.001, '**' 0.001 >P>0.0001, '***'P<0.0001 between the cultivars.

Table 3.3.3 Probabilities that pairs of LSMEANS of 10 morphological characters of individual seeds from seed lots of the same cultivar are equal.

From To	Aza	Ba-1	Ba-2	Esf	Ha-1	Ha-2	Nik	Or-1	Or-2	Or-3	Or-4	Or-5	Wa-1	Wa-2	Wa-3	Wa-4	Yazd
Aza		0.8377	0.0001	0.0001	0.7420	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0003	0.0001	0.5211	0.0001
Ba-1	ns		0.0001	0.0001	0.9006	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0019	0.0006	0.0001	0.3961	0.0001
Ba-2	***	***		0.0001	0.0001	0.0053	0.0001	0.3102	0.0001	0.0298	0.0455	0.0001	0.0001	0.0003	0.3720	0.0001	0.0001
Esf	***	***	***		0.0001	0.0001	0.1592	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0009
Ha-1	ns	ns	***	***		0.0029	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0023	0.0010	0.0001	0.3309	0.0001
Ha-2	***	***	*	***	***		0.0001	0.0002	0.0001	0.0001	0.0001	0.0001	0.3109	0.4607	0.0003	0.0001	0.0001
Nik	***	***	***	ns	***	***		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0567
Or-1	***	***	ns	***	***	**	***		0.2468	0.3244	0.0001	0.0001	0.0001	0.0001	0.9028	0.0001	0.0001
Or-2	***	***	***	***	***	***	***	***		0.0036	0.021	0.8975	0.0001	0.0001	0.0001	0.0001	0.0001
Or-3	***	***	ns	***	***	***	***	ns	*		0.8629	0.0058	0.0001	0.0001	0.2005	0.0001	0.0001
Or-4	***	***	ns	***	***	***	***	ns	*	ns		0.0034	0.0001	0.0001	0.2681	0.0001	0.0001
Or-5	***	***	***	***	***	***	***	***	ns	*	*		0.0001	0.0001	0.0001	0.0001	0.0001
Wa-1	*	*	***	***	*	ns	***	***	***	***	***	***		0.7731	0.0001	0.0001	0.0001
Wa-2	**	**	**	***	**	ns	***	***	***	***	***	***	ns		0.0001	0.0001	0.0001
Wa-3	***	***	ns	***	***	**	***	ns	***	ns	ns	***	***	***		0.0001	0.0001
Wa-4	ns	ns	***	***	ns	***	***	***	***	***	***	***	***	***	***		0.0001
Yazd	***	***	***	**	***	***	ns	***	***	***	***	***	***	***	***	***	

As some apparently significant results cited in this table may be due to chance, the significance probability for the LSMEANS was set at 0.01 for this test. The level of 0.01 has been chosen because of the need to control the overall test error rate. For P=0.01 the probability for 100 tests is that one apparently significant result is due to chance. The significant results are indicated by stars and not significant by 'ns'. The corresponding probability can be found in the upper right hand entries . '*' 0.01> P> 0.001, '**' 0.001 >P>0.0001, '***'P<0.0001 between the cultivars.

Table 3.3.4 Probabilities that pairs of LSMEANS of 11 morphological characters of individual seeds from a given cultivar are equal.

From To	Aza	Ba-1	Ba-2	Esf	Ha-1	Ha-2	Nik	Or-1	Or-2	Or-3	Or-4	Or-5	Wa-1	Wa-2	Wa-3	Wa-4	Yazd
Aza		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Ba-1	***		0.0001	0.5520	0.0001	0.0001	0.3627	0.0046	0.7085	0.0754	0.0234	0.0001	0.4711	0.0014	0.0546	0.0021	0.0016
Ba-2	***	ns		0.0073	0.0001	0.0001	0.0073	0.0001	0.0866	0.0001	0.8527	0.0711	0.0053	0.2626	0.0001	0.0001	0.0001
Esf	***	ns	*		0.0001	0.0001	0.7505	0.0248	0.3315	0.2362	0.0042	0.0001	0.8956	0.0002	0.1838	0.0131	0.0105
Ha-1	***	***	***	***		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0002	0.0003
Ha-2	***	***	***	***	***		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Nik	***	ns	*	ns	***	***		0.0550	0.1983	0.3886	0.0015	0.0001	0.8536	0.0001	00314	0.0311	0.0257
Or-1	***	*	***	ns	***	***	ns		0.0013	0.2880	0.0001	0.0001	0.0363	0.0001	0.3589	0.8117	0.7529
Or-2	***	ns	ns	ns	***	***	ns	*		0.0309	0.0567	0.0005	0.2738	0.0047	0.0213	0.0005	0.0004
Or-3	***	ns	***	ns	***	***	ns	ns	ns		0.0001	0.0001	0.2970	0.0001	0.8847	0.1934	0.1685
Or-4	***	ns	ns	*	***	***	*	***	ns	***		0.1051	0.0030	0.3496	0.0001	0.0001	0.0001
Or-5	***	***	ns	***	***	***	***	***	**	***	ns		0.0001	0.4924	0.0001	0.0001	0.0001
Wa-1	***	ns	*	ns	***	***	ns	ns	ns	ns	*	***		0.0001	0.2355	0.0198	0.0162
Wa-2	***	*	ns	**	***	***	***	***	*	***	ns	ns	***		0.0001	0.0001	0.0001
Wa-3	***	ns	***	ns	***	***	ns	ns	ns	ns	***	***	ns	***		0.2478	0.2179
Wa-4	***	*	***	*	**	***	ns	ns	**	ns	***	***	ns	***	ns		0.9390
Yazd	***	*	***	ns	**	***	ns	ns	**	ns	***	***	ns	***	ns	ns	

As some apparently significant results cited in this table may be due to chance, the significance probability for the LSMEANS was set at 0.01 for this test. The level of 0.01 has been chosen because of the need to control the overall test error rate. For P=0.01 the probability for 100 tests is that one apparently significant result is due to chance. The significant results are indicated by stars and not significant by 'ns'. The corresponding probability can be found in the upper right hand entries. '*' 0.01> P> 0.001, '**' 0.001 >P>0.0001, '***' P<0.0001 between the cultivars.

3.3.2 Identification of, and discrimination among the cultivars using a combination of ten useful morphological characters of individual seeds

3.3.2.1 Pairwise comparison between squared Mahalanobis distances between the standard seed lots of the cultivars using ten morphological characters of individual seeds that best discriminate among seed lots of the cultivars

Pairwise comparisons between seed morphological characters of the standard[1] seed lots of six Iranian and two New Zealand cultivars indicated that with a squared Mahalanobis distance (D^2 , see section 3.2.7) of 0.23, Esfahani and Yazdi, two Iranian cultivars, were closest, whereas cvs. Esfahani and the standard seed lot of cv. G. Oranga (Oranga-5) were the most distinct among the cultivars tested (D^2 =7.48, Table 3.3.5). Azari was the most distinct from all the other Iranian cultivars, as the D^2 between this cultivar and the others ranged from 1.77 for cv. Hamedani, to 4.40 for cv. Esfahani. D^2 existing between most of the other Iranian cultivars were much smaller (D^2 between cvs. Nikshahri and Eesfahani = 0.25, and for cvs. Yazdi and Nikshahri = 0.39) . The squared distance between G. Oranga and Wairau, the two New Zealand cultivars, was 2.39. Interestingly, the D^2 between cv. Wairau and all of the Iranian cultivars were much smaller than those between cv. G. Oranga and all of the Iranian cultivars (Table 3.3.5).

A comparison between squared Mahalanobis distances of seed morphological characters of different seed lots from the same cultivars (Table 3.3.6) illustrated that the D^2 between the nucleus seed lot of cv. Wairau (Wa-1) and the other seed lots of this cultivar were much smaller than those between Wa-1 and any of the seed lots from the other cultivars. Similarly, with the exception of Ba-2, the D^2 between the Breeders seed lot of cv. G. Oranga (Or-5) and the other seed lots of this cultivar were smaller than those existing between Or-5 and other seed lots from the other cultivars.

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[1] This definition is correct for cvs. Wairau and G.Oranga (standard seed lots for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'

Table 3.3.5 Pairwise comparison between squared Mahalanobis distances between cultivars using the ten selected morphological characters defined using image analysis

From To	Aza	Ba-1	Esf	Ha-1	Nik	Or-5	Wa-1	Yazd
Aza		3.53	4.40	1.77	4.02	6.45	2.89	3.74
Ba-1	3.53		1.70	0.52	1.46	2.39	0.68	0.81
Esf	4.40	1.70		1.84	0.25	7.48	2.73	0.23
Ha-1	1.77	0.52	1.84		1.55	2.89	0.65	0.99
Nik	4.02	1.46	0.25	1.56		6.64	2.06	0.39
Or-5	6.45	2.38	7.48	2.89	6.64		2.39	5.60
Wa-1	2.89	0.68	2.73	0.65	2.06	2.39		1.74
Yazd	3.74	0.81	0.23	0.99	0.39	5.60	1.74	

Table 3.3.6 Pairwise comparison between squared distances of certified seed lots from two Iranian and two New Zealand cultivars

From To	Aza	Ba-1	Ba-2	Esf	Ha-1	Ha-2	Nik	Or-1	Or-2	Or-3	Or-4	Or-5	Wa-1	Wa-2	Wa-3	Wa-4	Yazd
Ba-1	3.53		0.94	1.70	0.52	3.06	1.46	1.72	2.64	2.00	1.66	2.39	0.68	1.26	1.89	1.33	0.81
Ba-2	4.25	0.94		4.40	1.26	3.02	3.80	1.14	1.36	1.16	0.84	0.65	0.75	1.14	1.01	2.01	3.03
Ha-1	1.77	0.52	1.26	1.84		1.31	1.55	1.20	2.29	1.52	1.95	2.89	0.65	1.55	1.37	0.69	0.99
Ha-2	0.59	3.07	3.02	4.98	1.31		4.57	1.50	2.29	1.70	3.01	4.61	2.08	3.15	1.58	1.61	3.84
Or-1	2.99	1.72	1.14	4.65	1.21	1.50	4.22		0.39	0.07	0.53	1.94	0.93	1.34	0.26	1.64	3.28
Or-2	4.30	2.64	1.36	6.92	2.29	2.29	6.20	0.39		0.31	0.56	1.74	1.38	1.83	0.50	2.73	5.04
Or-3	3.23	2.00	1.16	5.25	1.52	1.70	4.63	0.07	0.31		0.39	1.82	1.02	1.36	0.27	1.84	3.73
Or-4	4.50	1.66	0.84	5.16	1.95	3.01	4.47	0.53	0.56	0.39		1.67	0.79	0.74	0.51	2.02	3.69
Or-5	6.45	2.38	0.65	7.48	2.89	4.61	6.64	1.94	1.74	1.82	1.66		2.39	3.03	2.12	4.38	5.60
Wa-1	2.89	0.68	0.75	2.73	0.65	2.07	2.06	0.92	1.38	1.02	0.79	2.39		0.30	0.57	0.49	1.74
Wa-2	4.00	1.26	1.14	3.09	1.55	3.15	2.43	1.34	1.83	1.36	0.74	3.03	0.30		0.74	0.80	2.28
Wa-3	2.96	1.89	1.01	4.75	1.37	1.58	4.04	0.27	0.50	0.28	0.51	2.12	0.57	0.74		1.15	3.51
Wa-4	1.75	1.32	2.01	1.91	0.69	1.61	1.29	1.64	2.73	1.84	2.02	4.38	0.49	0.80	1.15		1.45

3.3.2.2 Assessment of cultivar uniformity using cross validation data for each of the seed lots of the cultivars

From image analysis of individual seeds of the standard seed lot of the cultivar, the cross validation, defined as the probability of seeds within the seed lot being classified as from each individual cultivar, is presented in Table 3.3.7. For example for cv. G.Oranga, 71% of the seeds were classified by the discriminant analysis as belonging to this cultivar, while the remaining 29% of the seeds were classified as belonging to other cultivars. For cv. Yazdi, only 14% of the seeds were classified as belonging to this cultivar, suggesting that cv. G.Oranga seed was more uniform than that of cv. Yazdi.

3.3.2.3 Pairwise comparisons of least squared means (LSMEANS) of six Iranian and two New Zealand cultivars on the basis of the CDF1, the combination value of ten morphological characters of individual seeds

Data in Table 3.3.5 show how close or distinct a pair of the cultivars are in terms of similarity of seed morphological characters. However, it is not clear from this table whether the differences between the cultivars are significant. The presence or absence of significant differences in seed morphological character pairs of the seed lots is illustrated in Table 3.3.8, where the pairwise least squared means of the seed lots are compared. As shown in this table, 24 out of 28 (86%) of the pairwise comparisons between the LSMEANS of seed characters of the cultivars were significantly different ($P<0.0001$), and as a result, the majority of the cultivars could be discriminated from each other on the basis of this result. For example there was a highly significant difference between seed characters of G. Oranga and Wairau, the two New Zealand cultivars and also between cvs. Esfahani and Hamedani. This result also illustrated that there were significant differences between seed characters of the two New Zealand cultivars and all of the Iranian cultivars.

Table 3.3.7 Cross validation of seeds by cultivar

discriminant classifications true[2] cultivar	Aza	Ba.1	Ha.1	Esf	Nik	Or.5	Wa.1	Yazdi
Azari	62.0	1.0	15.0	5.0	1.0	5.0	9.0	1.0
Bami	1.0	15.0	10.0	14.0	7.0	20.0	17.0	11.0
Hamedani	15.0	10.0	19.0	9.0	5.0	14.0	24.0	6.0
Esfahani	5.0	14.0	9.0	39.0	19.0	11.0	7.0	11.0
Nikshahri	1.0	7.0	5.0	19.0	31.0	4.0	14.0	8.0
G.Oranga-5	5.0	20.0	14.0	11.0	4.0	71.0	9.0	1.0
Wairau.1	9.0	17.0	24.0	7.0	14.0	9.0	17.0	4.0
Yazdi	1.0	11.0	6.0	11.0	8.0	1.0	4.0	14.0

Table 3.3.8 Probabilities that pairs of LSMEANS of the morphological characters of individual seeds from a given cultivar are equal.

From To	Aza	Ba-1	Esf	Ha-1	Nik	Or-5	Wa-1	Yazd
Aza		0.0001	0.0001	0.7420	0.0001	0.0001	0.0010	0.0001
Ba-1	***		0.0001	0.9006	0.0001	0.0001	0.0019	0.0001
Efs	***	***		0.0001	0.1516	0.0001	0.0001	0.0009
Ha-1	ns	ns	***		0.0001	0.0001	0.0001	0.0001
Nik	***	***	ns	***		0.0001	0.0001	0.0567
Or-5	***	***	***	***	***		0.0001	0.0001
Wa-1	**	**	***	***	***	***		0.0001
Yazd	***	***	***	***	ns	***	***	

The significant results are indicated by stars and not significant by 'ns'. The corresponding probability can be found in the upper right hand entries . '*' 0.01> P> 0.001, '**' 0.001 >P>0.0001, '***'P<0.0001

[2] This definition is correct for cvs. Wairau and G.Oranga (standard seed lots for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'

3.3.2.4 General linear models (GLM) procedure for dependent variable CDF1.

The GLM or ANOVA (Analysis Of Variance) procedure for the variable CDF1 (the first canonical discriminant function or the combination of the ten seed morphological characters that best discriminated among the seed lots of the cultivars, see section 3.2.7) in this study confirmed that there were highly significant differences (P<0.0001) between the seed characters of the cultivars. The summary result of this procedure is presented in the following ANOVA table.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	16	1498.20	93.68	93.68	0.0001
Error	2492	2492.20	1.00		
Corrected total	2508	39.91.16			

R-Square	C.V.	RootMSC	CAN1M
0.3755	9.90	1.000	0.00097

3.3.2.5 Duncan's Multiple range test for discrimination between seed lots of the cultivars

Given that there were significant differences between the seed characters of the cultivars (the GLM result), the Duncan's Multiple range test was employed for variable CDF1 to further assess the significance of these differences. There were highly significant differences (P<0.01) between the seed characters of some of the pairs of cultivars (Table 3.3.9), and as a result, the eight cultivars were categorised into five groups, and the Iranian cultivars into three groups. A significant difference was found between the seed characters of G. Oranga and Wairau, the two New Zealand cultivars. There was also a significant difference between these two

cultivars and all of the six Iranian cultivars (Azari, Barni, Esfahani, and Hamedani, Nikshahri, and Yazdi). No significant difference was found between cvs. Hamedani (Hamedani-1), Bami (Bami-1), and Azari, but they differed significantly ($P<0.01$) from cvs. Yazdi, Nikshahri, and Esfahani. No significant difference was found between pairs of cvs. Nikshahri and Yazdi, and Nikshahri and Esfahani. However, the seed character of cv. Esfahani differed significantly from cv. Yazdi.

Duncan's Multiple range test of seed characters of seed lots from the same cultivar in this study (Table 3.3.10) illustrated a significant difference ($P<0.01$) between seed lots of cv. Bami (Iran). However there was no significant difference between seed lots of cv. Hamedani, the other Iranian cultivar. A highly significant ($P<0.01$) difference was found between the Breeders seed lot (Oranga-5) of cv G.Oranga (New Zealand) and the other seed lots of this cultivar (Table 3.3.10). However, no significant difference was found between the two Basic seed lots of this cultivar (Oranga-3 and Oranga-1). No significant difference was also found between Oranga-3 (Basic seed), and the two 1st generation seed lots of this cultivar tested (Oranga-2 and Oranga-4). Among the seed lots of cv. Wairau, Wairau-2 (1st generation) was the only seed lot which did not differ from the nucleus seed lot of this cultivar for seed morphological characters. A significant difference was found between Wairau-3 and Wairau-4, the other two 1st generation seed lots of cv. Wairau.

Table 3.3.9 Duncan's Multiple range test for discrimination among the six Iranian and two New Zealand cultivars on the basis of the ten selected morphological characters of individual seeds of the cultivars. The calculated means in the table are the means for CDF1, the combination of values from the ten characters that best discriminate among the cultivars (see section 3.2.7). Cultivars linked by the same line do not differ significantly at P<0.01.

		Mean	Duncan's grouping		
1	G. Oranga-5	1.551			
2	Wairau.1	0.520			
3	Hamedani-1	0.172			
4	Bami-1	0.158			
5	Azari	0.133			
6	Yazdi	-0.623			
7	Nikshari	-0.844			
8	Esfahani	-1.008			

Table 3.3.10 Duncan's Multiple range test of data from morphological characters of individual seeds of the seed lots recorded by image analysis. The calculated means in the table are the means for CDF1, a combination of ten variables (seed characters) that best discriminate among the seed lots (see section 3.2.7). Seed lots linked by the same line do not differ significantly at P<0.01.

		Mean	Duncan's grouping
1	G. Oranga-5	1.053	
2	G. Oranga-2	0.633	
3	G. Oranga-4	0.436	
4	Bami-2	0.357	
5	G. Oranga-3	0.329	
6	G.Oranga-1	0.209	
7	Wairau-3	0.111	
8	Wairau-2	-0.256	
9	Wairau-1	-0.316	
10	Bami-1	-0.408	
11	Hamedani-2	-0.610	
12	Hamedani-1	-0.637	
13	Wairau-4	-0.942	

3.3.3 Discrimination among four Iranian and two New Zealand lucerne cultivars on the basis of twelve morphological characters of plant leaflets recorded using image analysis

3.3.3.1 Cultivar discrimination on the basis of the individual characters

The result from Duncan's Multiple range test of data from 12 morphological characters of terminal leaflets of individual plants of the cultivars (see Materials and Methods) demonstrated that four of these characters (*i.e.* leaflet shape, angle, roughness-1 and roughness-2) did not differ significantly among the cultivars. However, significant differences ($P<0.05$) were found among the cultivars using the other recorded characters (leaflet area-1 and area-2, perimeter-1, perimeter-2, length, length/width and width/length). However, these characters did not have the same discrimination values for classifying the cultivars into different cultivar groups *i.e.* using either leaflet area-1 or area-2 classified the cultivars into four cultivar groups; using perimeter-1, perimeter-2, length, and width of leaflets classified the cultivars into three cultivar groups, and using length/width and width/length ratio of the leaflets classified the cultivars into only two cultivar groups (Table 3.3.11).

A similar cultivar grouping was obtained using either area-1 or area-2 (Table 3.3.11). Pairwise comparisons between mean values of the cultivars from this Table showed that 44% of the pairs of the cultivars could be discriminated using either of these characters. For example, with the exception of cv. Esfahani, cv. Azari was significantly different from all of the other Iranian and also from the New Zealand cultivars. There were no significant differences between cvs. Hamedani and Esfahani, but these two cultivars did differ from Bami, the other Iranian cultivar. No difference was found between G. Oranga and Wairau, the two New Zealand cultivars.

A unique cultivar grouping pattern was also obtained using either perimeter-1 or perimeter-2 of the leaflets of individual plants of the cultivars. Cultivar Azari had

a smaller perimeter-1 and perimeter-2 than that of the other cultivars, making this cultivar distinct from all of the other Iranian cultivars. A significant difference was also found between leaflet perimeter-1 and perimeter-2 for cvs. Bami and Esfahani. Data in Table 3.3.11 also illustrate that Azari and Esfahani were the only Iranian cultivars which were significantly different from the two New Zealand cultivars. Data in this table also suggest that plants from cv. Bami had the widest leaflets among the Iranian cultivars.

As shown in Table 3.3.11, none of the individual morphological characters were able to differentiate all of the cultivars. Therefore, a combination value from all 12 leaflet characters (CDF1) was used for discrimination among the cultivars. The results are presented in the following sections.

3.3.3.2 Cultivar discrimination using a combination value from 12 morphological characters of leaflets of individual plants of the cultivars (CDF1, see section 3.2.7).

3.3.3.2.1 Pairwise comparison between squared distances (Mahalanobis distances) of the cultivars on the basis of CDF1, a combination value from the 12 morphological characters of leaflets of individual plants of the cultivars grown under glasshouse conditions

Pairwise comparisons between squared distances (D^2) of the leaf characters of four Iranian and two New Zealand cultivars indicated that despite the fact that leaflet characters of some pairs of the cultivars were closely related, the others were distinct from each other. For example with a D^2 of 0.41, 0.53, 0.59, and 0.60 pairs of the cultivars G. Oranga (New Zealand) and Hamedani (Iran), Bami and Hamedani (Iran), Bami and G. Oranga, and G. Oranga and Wairau (New Zealand) respectively had similar leaflet characters. However with a D^2 of 1.89 and 1.82, pairs of cvs. Bami and Azari, and Bami and Esfahani (all from Iran), were the most distinct among the cultivars tested in this study (Table 3.3.12).

Table 3.3.11 **Duncan's Multiple range test of data from five morphological characters of leaflets of individual plants of the cultivars. Cultivars linked by the same line do not differ significantly for the characters at P<0.05.**

Leaflet characters	Cultivar name	Bami	Wairau	G. Oranga	Hamedani	Esfahani	Azari
1. Area-1 (mm^2)	Mean	214.65	198.00	193.20	179.64	155.43	150.63
	Duncan's grouping						
2. Area (mm^2)	Mean	218.46	201.21	197.03	183.22	158.87	153.37
	Duncan's grouping						
3. Perimeter-1 (mm)	Mean	65.05	62.34	61.91	59.52	56.96	52.38
	Duncan's grouping						
4. Perimeter-2 (mm)	Mean	62.27	60.37	60.17	57.93	55.17	50.85
	Duncan's grouping						
5. Length (mm)	Mean	25.66	25.27	25.012	24.15	23.22	20.41
	Duncan's grouping						

Table 3.3.11 Ctd. **Duncau's Multiple range test of data from the individual morphological characters of plant leaflets of the cultivars recorded using image analysis. Cultivars linked by the same line do not differ significantly for the characters at P<0.05.**

6. Width	Cultivar name	Bami	G. Oranga	Wairau	Hamedan	Azari	Esfahani
	Mean	12.01	11.19	11.00	10.68	10.37	9.66
	Duncan's grouping						
7. Length/width	Name of cultivar	Esfahani	Wairau	Hamedani	G. Oranga	Bami	Azari
	Mean	2.43	2.33	2.30	2.27	2.18	2.01
	Duncan's grouping						
8. Width/length	Mean	Azari	Bami	G. Oranga	Hamedani	Wairau	Esfahani
		0.52	0.47	0.45	0.45	0.44	0.43
	Duncan's grouping						

Table 3.3.12 **Pairwise comparisons between squared distances of the cultivars using CDF1, a combination value of the twelve morphological characters of the leaflets of the individual plants of the cultivars grown under glasshouse conditions**

From To	Azari	Bami	Esfahani	Hamedani	G. Oranga	Wairau
Azari		1.89	1.02	1.29	1.54	1.33
Bami	1.89		1.82	0.53	0.59	0.87
Esfahani	1.03	1.82		0.86	1.02	1.17
Hamedani	1.29	0.53	0.86		0.41	0.81
G.Oranga	1.54	0.59	1.02	0.41		0.60
Wairau	1.33	0.87	1.17	0.81	0.60	

3.3.3.2.3 Pairwise comparison of least squared means (LSMEANS) of four Iranian and two New Zealand cultivars on the basis of CDF1, a combination value from the twelve morphological characters of leaflets of the individual plants grown under glasshouse conditions

The presence or absence of significant differences between pairs of LSMEANS of the cultivars are presented in Table 3.3.13. As shown in this Table, 20 out of 30 (67%) pairs of cultivars had significantly different LSMEANS ($P<0.05$), and thus could be discriminated from each other using a combination value of the twelve morphological characters of the plant leaflet. For example leaflet characters of cvs. Esfahani and Azari did not differ, but leaflet characters of these two cultivars did differ significantly from all of the other cultivars (Table 3.3.13). However, no significant difference was found between leaflet characters of Wairau and G.Oranga, the two New Zealand cultivars or between either of these two cultivars and cv. Hamedani (Iran). No difference was also found between leaflet characters of cv. Bami (Iran) and cv G. Oranga, despite there being a significant difference between this cultivar and Wairau.

Table 3.3.13 Probabilities that pairs of LSMEANS of the morphological characters of the leaflets of the given cultivars are equal.

From To	Azari	Bami	Esfahani	Hamedani	G. Oranga	Wairau
Azari		0.0001	0.2618	0.0001	0.0001	0.0001
Bami	***		0.0001	0.0213	0.0981	0.0118
Esfahani	ns	***		0.0002	0.0001	0.0004
Hamedani	***	*	**		0.5127	0.8287
G. Oranga	***	ns	***	ns		0.3839
Wairau	***	*	**	ns	ns	

The significant results are indicated by stars and not significant by 'ns'. The corresponding probability can be found in the upper right hand entries . '*' 0.05 P> 0.01, '**' 0.01 >P>0.001, '***'P<0.001

3.3.3.2.3 General linear models (GLM) procedure, and Duncan's Multiple range test for variable CDF1

The GLM or ANOVA (Analysis Of Variance) procedure applied to the variable CDF1 (the first canonical discriminant function or combination of the variables that best discriminates among the cultivars, see section 3.2.7) for the morphological characters of the leaflets of individual plants from the cultivars grown in the glasshouse showed that there was a very highly significant difference (P<0.0001) between the cultivars.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Cultivar	5	76.89	15.38	15.38	0.0001
Error	352	352.00	1.00		
Corrected total	357	428.89			

Given that there were significant differences for leaflet characters (the GLM

result), the Duncan's Multiple range test was employed for variable CDF1 to further assess the significance of these differences. There were significant differences ($P<0.05$) between leaflet characters of some of the pairs of seed lots (Table 3.3.14) and as a result, the cultivars were classified into three cultivar groups. No significant difference was found between leaflet characters of individual plants of cvs. Azari and Esfahani; however, they did differ significantly from the other cultivars (Table 3.3.14). A significant difference was also found between pairs of cvs. Wairau and cvs Bami and also Hamedani. However, no significant difference was found between pairs of Wairau and G. Oranga, the two New Zealand cultivars, and between these two and Hamedani.

Table 3.3.14 Duncan's Multiple range test of data from leaflet characters of the cultivars recorded using image analysis. The calculated means in the table are the means for CDF1, a combination of twelve variables (leaflet characters) that best discriminate among seed lots of the cultivars (see section 3.2.7)

	Cultivar	Mean	Duncan's grouping		
1	Bami	0.602			
2	G. Oranga	0.299			
3	Hamedani	0.179			
4	Wairau	0.140			
5	Esfahani	- 0.515			
6	Azari	- 0.722			

3.4 DISCUSSION

3.4.1 Assessment of the most useful seed morphological characters for identification of lucerne cultivars

From the 10 individual characters and 11 combinations of characters (see Materials and Methods), only two of the individual characters (*i.e.* seed concavity and seed curvature) and eight of the combinations of the characters (*i.e.* red/green colour, red/total intensity, blue/total intensity, width/length, length/width, $4\prod$ area/perimeter^2, actual area/convex area, and seed concavity/width) were able to place the certified seed lots of the two New Zealand cultivars (G.Oranga and Wairau) into their cultivar groups, even though the mean values were not always significantly different for identical characters (Appendix 3.1). This suggested that these characters are potentially valuable for lucerne cultivar verification. This was confirmed when data from seed lots of each of the two New Zealand cultivars were used as replicates of the cultivars and analyzed using the GLM procedure (SAS, 1989; Appendix 3.2). As a result, a highly significant difference ($P<0.0001$) between the two cultivars was found for all of the above characters. As the seed lots of cvs. G.Oranga and Wairau were grown on different farms and in different years, the correct classification of the seed lots into their cultivar group by means of the 10 chosen characters suggests that these characters are, most likely, independent of the environment, and therefore useful for the verification of lucerne cultivars.

Analysis of the data from the 10 chosen seed characters by means of canonical discriminant analysis (CDA; SAS, 1989) showed that the values of these characters for classification of seed lots into their cultivar groups were different (see 3.3.1.1). The colour components of red/total intensity, blue/total intensity, and red/green had the highest discrimination values (canonical coefficients of 783.5, 323.7, and 99.9 respectively), whereas seed curvature had the lowest

discrimination value (a canonical coefficient of 0.02). The high values for the seed colour components are most likely due to the fact that seed colour is known to be genetically controlled in lucerne species (Barnes and Hanson, 1967; Teuber and Brick, 1988) and therefore is independent of the environment. A larger canonical coefficient for seed concavity in combination with width (concavity/width) than for seed concavity alone (39.6 vs 23.1, see 3.3.1.1), probably demonstrates that a more effective classification of seed lots of lucerne cultivars and cultivar identification can be achieved using a combination value of certain seed characters. This is consistent with Draper and Travis (1984) who found that discrimination among 49 crop and weed species was more effective using seed shape factors *i.e.* $4\prod$ area/perimeter^2 in combination with seed length than individual seed characters. This might be due to the fact that individual characters are more liable to be influenced by the seed production environment than the ratios of these characters.

3.4.2 Assessment of the most useful seed morphological characters for discrimination among lucerne cultivars

Pairwise comparison between least squared means of 17 seed lots of six Iranian and two New Zealand cultivars using CDA showed that 83% of the comparisons among the seed lots were significantly different (Table 3.3.2) when all of the 21 seed characters were used for discrimination among the seed lots, while 87% of the comparisons were significantly different for the ten selected characters (Table 3.3.3). For twelve characters (the ten selected characters and two of the other characters (the total seed intensity, and green/total intensity)), the discrimination value was 83%. For the remaining 11 characters only 68% of the comparisons were significantly different (Table 3.3.4). This suggests that the 10 characters which were useful for identification of the cultivars are also valuable for discrimination among lucerne cultivars. However, the percentage of the pairwise comparisons between least squared means of the seed lots which were

significantly different was smaller for some of the useful characters than for all of the 10 selected characters (see 3.3.1.1). This suggests that the discrimination power of a combination of these characters was not enough to show the maximum possible variation between seed lots.

3.4.3 Discrimination among eight lucerne cultivars using each of the ten seed morphological characters

There were significant differences between some pairs of the cultivars for some of the ten recorded seed morphological characters (Table 3.3.1). For example, there were significant differences between seed character of cvs. G.Oranga and Wairau, the two New Zealand cultivars. This result is consistent with the divergent genetic background of these two cultivars. Cultivar Wairau was produced from 20 foundation plants from an old New Zealand ecotype, Marlborough, plus two of cv. Greem (introduced from Germany, Barnes and Hanson, 1967), plus two of Ontario variegated (a Canadian cultivar derived from *Medicago varia,* Barnes and Hanson, 1967), and two from American commercial (unknown origin, Palmer, 1967). It is not certain from what sources Marlborough lucerne originated. Hadfield and Calder (1936) suggested that the centre of origin of this cultivar was Argentina but gave no authority for this. Palmer (1967) suggested that this cultivar is somewhat more winter dormant than Hunter River or Provence in New Zealand, and may have arisen by natural selection from these strains. Cultivar G.Oranga was the result of a simple mass selection from cultivar WL311 (Easton and Cornege, 1984). The latter cultivar originated from WL-synthetics and WL215, produced from four American germplasms *i.e.* Atlantic, Buffalo, Ranger, and Vernal (Barnes and Hanson, 1967). A significant difference was also found between the seed characters of cv. G.Oranga and all of the Iranian cultivars (Table 3.3.1), which again is consistent with the genetic diversity of these cultivars (see 6.3.4.3). These results clearly demonstrate the potential of seed image analysis for discrimination among cultivars of a cross-pollinating

species such as lucerne, despite the fact that there is tremendous variation within each cultivar (see 6.3.4.1).

Classification of the cultivars into four overall groups using each of the five characters: red/green colour, red/total intensity, blue/total intensity, concavity, and seed curvature, in comparison with three cultivar groups using each of the four characters: width/length, length/width, 4π area/perimeter^2, concavity/width and only two cultivar groups using actual area/convex area of the seed illustrated that the discrimination values of these seed characters are not the same. Otherwise, all should have produced the same classification pattern.

Pairwise comparisons between the cultivars (Tables 3.3.1) demonstrated that the seed characters of red/total intensity, and blue/total intensity had the highest, whereas actual area/convex area had the lowest cultivar discrimination function among the ten characters (64% vs 18% of pairs of the cultivars could be differentiated respectively on this basis). This shows seed colour, as a genetically controlled character (Barnes and Hanson, 1967; Teuber and Brick, 1988), offers better cultivar discrimination value than the other physical characters. However, colour assessment by means of image analysis is more complex than for other physical properties of seed (Russ, 1995). Different factors are involved for colour assessment, such as light intensity and quality; camera spectral characteristics or sensitivity of the camera to red, green and blue colour; the Frame grabber; and the white balance (Bailey , 1994 pers.comm). Therefore, the system should be standardized (Russ, 1995) and colour calibration would be necessary prior to each set of experiments. Otherwise as a result of differences in the light intensity of the laboratory for example, the results may not be reproducible (Bailey, 1994 pers. comm).

None of the seed characters determined in this study were sufficient for discrimination among all of the cultivars. Therefore, using canonical discriminant analysis (see 3.2.7), a combination of ten characters was used in this study to

examine if any further improvement in discrimination among the cultivars could be achieved.

3.4.4 Discrimination among lucerne cultivars on the basis of a combination value of ten morphological characters of individual seeds defined using image analysis

Pairwise comparisons between least squared means of CDF1, the combination value of ten morphological characters of individual seeds of the cultivars (see 3.2.7) demonstrated a highly significant difference between the majority of the cultivars (Table 3.3.8). Out of the 28 pairs of cultivars 24 pairs (86%) could be discriminated from each other using the LSMEANS of the CDF1 of the ten selected seed morphological characters. The four pairs of cultivars which could not be discriminated were Iranian (see Table 3.3.8). This might be due to the genetic similarity of these Iranian ecotypic cultivars as expressed by seed colour. As the highest percentage of discrimination between pairs of cultivars using individual characters was 56%, this result suggests that a better discrimination among cultivars can be achieved on the basis of a combination value (CDF1) of seed morphological characters.

A comparison between the results from the canonical discriminant analysis of seed morphological data on the basis of the CDF1 of the characters (Table 3.3.8) and that from the Duncan's Multiple range test of the cultivars on the basis of the individual seed characters (Table 3.3.1), suggests that out of the four pairs of Iranian cultivars which did not differ significantly from each other for CDF1 (Table 3.3.8), two pairs (*i.e.* Hamedani and Azari; Yazdi and Nikshahri) could be discriminated using the result from individual seed characters (Table 3.3.1). This indicates that about 94% of the pairs of the cultivars tested in this study could be discriminated using data from individual and/or combinations of the seed morphological characters. This clearly demonstrates that image analysis of seed

coupled with canonical discriminant analysis, is not only able to illustrate differences between genetically divergent cultivars (eg. G.Oranga and Wairau, Easton 1995, pers. comm., see also 3.4.3), but also has sufficient discrimination power to differentiate closely related cultivars (eg. Hamedani and Esfahani, Vaezzadeh, 1993, pers. comm.). Even in an inbreeding species such as wheat, which has limited within-cultivar variation, not all cultivars could be discriminated using seed image analysis (eg. Neuman *et.al.*, 1987; Thomson and Pomeranz, 1991; Keefe, 1992). However, the results of this study suggest that this technique, coupled with canonical discriminant analysis is nevertheless a very useful test of distinctness as required for registration of new cultivars (UPOV, 1995).

The lack of significant differences between seed characters of two pairs of the Iranian cultivars (Hamedani and Bami; Nikshahri and Esfahani) does not necessarily mean that these cultivars are the same. This result may be because of a larger within-population variation for these cultivars, which masks differences. In this case, the discrimination power of the technique could be improved by analysing a larger sample size (Mead and Curnow, 1983). However, this requires further study.

3.4.5 Test of uniformity of cultivars using a combination value from the 10 selected morphological characters of individual seeds

A new cultivar must be clearly distinguishable from any other cultivars, sufficiently uniform, having regard to the particular feature of its sexual reproduction or vegetative propagation, and must be stable in its essential characteristics (UPOV, 1988 and 1995; see also 2.1.1). Therefore along with distinctness and uniformity, stability of a new cultivar should also be tested before registration. Considering the possible use of certain electrophoretic methods for registration of a cultivar of a cross-pollinating crop, UPOV has already established the principle of linking distinctness and uniformity for each characteristic,

indicating that uniformity of a cultivar of such species must be established on the basis of examination of individuals plants (UPOV, 1995). As seed and plant characters are both expressions of the genetic construction of a cultivar, results from image analysis of individual seeds, as performed in this study, could be used for registration of new cultivars.

The proportion of individual seeds with uniform morphological characters varied amongst the cultivars tested in this study. This ranged from 14-71%. There were clear differences between seed uniformity of cvs. G.Oranga and Wairua, the two New Zealand cultivars, and also between some of the Iranian cultivars (see 3.3.2.2). This suggested that image analysis of individual seeds could also be used for testing of uniformity, as well as for distinctness.

A new cultivar must be stable in its essential characteristics, *i.e.* it must remain true to its description after repeated reproduction, or where a breeder has defined a particular cycle of reproduction or multiplication, at the end of each cycle (UPOV, 1988). As found in this study (see 3.4.1) some of the seed characters can be used as stable descriptors for lucerne cultivars. Thus a comparison between these characters from seed lots of a given cultivar after a number of multiplications could most likely provide information on the stability of the cultivars under test. However, this is yet to be confirmed.

3.4.6 Pairwise comparison between squared Mahalanobis distances of seed lots of the cultivars

Besides discrimination of cultivars on the basis of seed morphological characters, canonical discriminant analysis can provide some other useful information about the degree of relatedness among cultivars and also the uniformity of the cultivar. This information is valuable for plant improvement, in descriptions of new cultivars and also when assessing cultivar purity in seed certification programmes.

In the following section the more relevant results for discriminating among, and identifying, lucerne cultivars *i.e.* relatedness among lucerne cultivars using pairwise comparisons between squared Mahalanobis distances (D^2, see section 3.2.7), is discussed.

3.4.7 Test of relatedness among eight Iranian and New Zealand lucerne cultivars on the basis of a combination value from the 10 selected morphological characters of individual seeds of the cultivars

With a squared Mahalanobis distance (D^2) of 0.23, two Iranian cultivars, Esfahani and Yazdi, were the closest, whereas cvs. Esfahani and G. Oranga were the most distinct among the cultivars (D^2 =7.48, Table 3.3.5) on the basis of seed morphological characters. Interestingly, despite the New Zealand cultivars being produced under similar environmental conditions, the overall D^2 between the standard seed lot of cv. Wairau and the Iranian cultivars was much smaller than that between this seed lot and cv. G.Oranga (Table 3.3.5). The suggests two possibilities: firstly, as lucerne originated in the Caucasus, northeastern Turkey, Turkmenistan and northwestern Iran (Michaud *et al.*, 1988; Brummer *et al.*, 1991, Appendix 2.2), the genotypes used to produce cv. Wairau may well have originated from Iranian genotypes. However, as the origin of cv. Marlborough (the major genetic contributor to cv. Wairau) is not known (see 3.4.3), it is not possible to prove this is the case. Secondly, the recorded seed characters are, most likely, independent of the seed production environment, as environmental conditions for seed production of the seed lots of the two New Zealand cultivars studied are more similar than are the Iranian cultivars (see Appendices 2.3 and 2.4).

D^2 between cvs. Hamedani and Bami was 0.52, compared with 1.77 between cvs. Hamedani and Azari. Cultivar Azari is derived from an area within the centre of origin for lucerne, in the province of Azerbaijan, which has a mild summer and

a cold winter (latitude 32.5-34°N, altitude 1362m). Hamedani is from the province of Hamedan in the west of Iran, which has a mild summer and a very cold winter (latitude 35-36°N, altitude 1646m), and Bami from the province of Bam in the south of Iran, with a very warm summer and a mild winter (latitude 29°N, altitude 1062m, Appendix 2.2). This suggests that since geographical adaptation and temperature during seed production of cvs. Hamedani and Azari are more similar than that for Hamedani and Bami, the D^2 between the two latter cultivars, theoretically, should be less than that between cvs. Hamedani and Bami, if seed characters are influenced by environment. However, the data in Table 3.3.5 suggest this is not the case, again suggesting that seed morphological characters are, more likely, independent of geographical adaptation and environmental conditions during seed production.

3.4.8 Test of relatedness of seed lots from the same cultivar to the standard seed lots of the cultivar using a combination value from the 10 selected morphological characters of individual seeds of the seed lots

A comparison between squared Mahalanobis distances (D^2) of seed morphological characters of different seed lots of the same cultivar (Table 3.3.6) illustrated that the D^2 between the standard seed lot of cv. Wairau (Nucleus seed, Wa-1) and the other seed lots of this cultivar (which ranged from 0.30-0.57) were much smaller than that between Wa-1 and any of the seed lots from the other cultivars. Similarly, with the exception of Ba-2, the D^2 between the standard seed lot of G.Oranga (Breeders seed, Or-5) and the other seed lots of this cultivar (which ranged from 1.66-1.74, Table 3.3.6) were smaller than that between Or-5 and other seed lots from the other cultivars. This result clearly suggests that the image analysis of seed can be used for estimation of relatedness among seed lots of cultivars and thus for cultivar identification.

The mean value of the D^2 between the standard seed lots (Breeders seed) of cv. G. Oranga and the other seed lots of this cultivar was more than that for cv.

Wairau (1.79 vs 0.45, Table 3.3.6). This is most likely related to a larger heterogeneity within the population of cv. G.Oranga than exists in cv. Wairau (see 4.3.3.1, and 5.3.4.1). This demonstrates that the result from seed image analysis is consistent with findings from the analysis of the cultivars using molecular techniques (see 4.3.3.1, and 5.3.4.1). However, as seed lots of only two cultivars (G.Oranga, and Wairau) were examined in this study, image analysis of seed lots from different generations (Nucleus seed, Breeders seed, Basic seed and certified seed lots) of a sufficient number of cultivars is required before making any final conclusions as to the applicability of the technique for identification, verification and/or characterisation of cultivars. This would seem to be essential before the technique could be confidently used in the successful operation of national seed certification schemes, for the award of plant breeders rights, and the registration of new cultivars of lucerne.

3.4.9 Assessment of genetic shift in seed lots of lucerne cultivars using a combination value from the 10 selected morphological characters of individual seeds

It could be expected that all seed lots from the same cultivar would have similar seed morphological characters. However, results from this study (Table 3.3.10) suggest that this may not always be the case. Despite there being no significant difference between seed characters of the two seed lots of cv. Hamedani (Iran), there was a highly significant difference between the seed characters of the standard and the other seed lots of cvs. Bami (Iran), G.Oranga, and Wairau (both from New Zealand). For example, the standard seed lot of cv. G.Oranga (Breeders seed lot, Oranga-5) differed significantly from the two Basic seed lots of this cultivar (Oranga-1 and Oranga-3), and also from the two 1st generation seed lots (Oranga-2 and Oranga-4, Table 3.3.10). However, no significant difference was found between the two Basic seed lots of this cultivar. One of the Basic seed lots (Oranga-3), did not differ from the two 1st generation seed lots

(Oranga-2 and Oranga-4), although these latter two differed significantly from each other, and also from the other Basic seed lot of this cultivar (Oranga-1). Several factors might be associated with differences between the seed characters of the seed lots (see also section 4.5.5 of this thesis for a more detailed discussion). For example, error in the sampling of the seed lot being analyzed; out-crossing between seed lots from different cultivars; contamination of the seed lots at harvest, or during seed cleaning and processing, and/or genetic shift within the cultivar, because of some factors other than out-crossing between seed lots of different cultivars. Only if the first three of these factors are known not to have occurred, could seed image analysis be used to check if any genetic shift has occurred in a lucerne cultivar during seed multiplication.

3.4.5 Discrimination among four Iranian and two New Zealand lucerne cultivars on the basis of 12 recorded characters from leaflets of individual plants grown under glasshouse conditions

Of the 12 morphological characters of leaflets of individual plants recorded from cultivars grown in the glasshouse, four (*i.e.* leaflet shape, angle, roughness-1 and roughness-2) did not differ significantly among the cultivars. However, significant differences were found between some pairs of cultivars using each of the characters: leaflet area-1 and area-2, perimeter-1, perimeter-2, leaflet length and width, length/width and width/length. Despite this, the discrimination value of these characters was not the same (Table 3.3.11). Leaflet area-1 and area-2 were the best in that 44% of pairs of the cultivars could be discriminated, whereas leaflet length/width, and width/length were the worst in terms of differentiating cultivars, since only 22% of cultivars could be discriminated (Table 3.3.11).

Analysis of data from the combination value of the leaflet characters by means of canonical discriminant analysis (CDA, see 3.2.7) resulted in better discrimination among cultivars than that obtained for individual characters. Sixty-seven percent

of pairs of the cultivars could be differentiated on the basis of the pairwise comparisons of least squared means of the combination of leaflet characters (Table 3.3.13). However, because there was an overlap in the results for some of the cultivars (eg. G.Oranga and Wairau, Esfahani and Azari), these cultivars could not be differentiated. It is not clear whether the overlap results are because no differences occurred between cultivars, or whether glasshouse conditions were not appropriate for cultivars to express these differences. This overlap in the results might also have occurred because of the small number of leaflets analyzed from each cultivar (three replicates of 20 leaflets), despite that fact that this sampling intensity is recommended by UPOV (1988). Although the involvement of the first two factors in producing overlap results can not be excluded, the effect of the latter factor is commonly known to be a highly pronounced factor when analysing morphological data from out-crossing species. Presumably, since cultivars of lucerne are usually mixed populations of different genotypes, often with a tremendous variation within the cultivar, the cultivars may have been able to be separated by analysing a larger number of leaflets. However, this requires verification.

Morphological data should be recorded from leaflets of individual second year plants growing in plots (OECD, 1988; UPOV 1988). However, 28% of the cultivars could not be discriminated using leaflet characters. Therefore, image analysis of plant leaflets is not considered an effective method for registration of lucerne cultivars. However, seed morphological data can be recorded quickly from a submitted sample (six seconds/seed or possibly less, using fast computer technology). These important features, coupled with the promising results already discussed in this chapter, makes seed image analysis a powerful tool for identification of, and discrimination among cultivars, as well as for estimating relatedness between the cultivars.

FUTURE STUDY

1. Although the results of this study indicate that some of the 21 seed characters examined were likely to be independent of the seed production environment, image analysis of seed lots from different cultivars multiplied in different production environments may provide clearer information about the most stable characters. This would be very useful for tests of distinctness, uniformity, and stability as required for registration of new cultivars of lucerne.

2. As within cultivar variation in lucerne is likely to be the most significant factor producing an overlap in cultivar classification results, analysing different cultivars with known genetic background would be very useful for developing a standard seed image analysis method for verification of, and discrimination among, lucerne cultivars. This approach may also provide useful information for the verification of cultivars from other out-crossing species.

3. Image analysis of seed samples from different generations (Nucleus seed, Breeders seed, Basic seed and certified seed lots) of a sufficient number of cultivars would be required to determine the overall range of the squared Mahalabios distances between the standard seed lot and seed lots from other multiplication generations of a cultivar. In practice, this range could be used as a scale for cultivar verification, and to check whether a seed lot is 'true' to a given cultivar description.

CHAPTER 4

SDS-PAGE OF SEED STORAGE PROTEINS

4.1 LITERATURE REVIEW

4.1.1 Introduction

Proteins are the direct products of gene translation and transcription. A specific sequence of nucleotides in the DNA is transcribed to mRNA which is then translated to synthesise a specific protein or polypeptide. Thus, analysis of protein composition can be considered to be an analysis of gene expression, and comparison among the composition of different proteins can be used as a comparison of the genetic variation between individuals (Cooke, 1995a).

Gel electrophoresis of proteins can provide information about the difference among the proteins synthesised under the control of DNA, the material of heredity in the chromosomes. Therefore it is possible to define differences among individuals and populations of plants using biophysical analytical techniques (eg. electrophoresis) that utilise macromolecular biochemical markers such as seed storage protein and isozymes, as well as by more conventional morphological and physiological characteristics.

The use of protein electrophoresis began with Tiselius (1937). Although biochemical genetics had been used in biological research for many years, the breakthrough in the application of electrophoresis as a tool for studying population genetics came in the middle of the 1960s with the development of a range of different gel electrophoresis techniques (Ferguson, 1980). Further development has given an indirect measure of genome variability by exposing structural variation in enzymes or other protein gene products.

4.1.2 Advantages of protein electrophoresis for cultivar identification and discrimination

The advantages of the electrophoretic approach to cultivar identification include the speed of the test (it is possible to obtain information about the cultivar identity in 24-48 hours) and the reproducibility of the protein banding pattern (ISTA, 1992). Because of their proximity to primary genetic information (DNA), the protein patterns are for the most part unaffected by environmental factors. However, care must be taken when dealing with certain enzymes, particularly those expressed in the vegetative tissue (ISTA, 1992). In general though, protein composition is strictly a function of a plant's genotype, and can thus be employed for identification purposes with more confidence than many morphological descriptors.

Other advantages of the electrophoresis technique which have been described by different authors (eg. Gardiner and Forde, 1992) are the freedom from the influence of environment or management practices, freedom from subjective assessment of the results (such as that which may occur when scoring morphological characteristics of plants by the traditional plot test method), as well as elimination or reduction of requirement for land or plant growth facilities, ease of storage of the seed, and cost effectiveness.

4.1.3 Methods for protein electrophoresis

All plant cultivars are different from one another, and the variation must be genetically based. Therefore, protein composition is a suitable means of cultivar discrimination (Cooke, 1995a).

It has been suggested by several authors (Larsen *et al.,* 1968; Larsen, 1967; Clapham and Almgard, 1976; Nielsen, 1985; Gardiner and Forde, 1992; Vieritz

1993; Cooke, 1995a, and Faville *et al.,* 1995) that electrophoretic differences in proteins could provide a valuable means of distinguishing and identifying plant cultivars. However, electrophoresis techniques do vary in their suitability for differentiation of plant species (Stegemann, 1983). Gel electrophoresis techniques have been classified by Cooke (1995a) into the four following groups:

1. Gel electrophoresis methods for analysis of "native" or non-denatured proteins

This group includes those methods in which no dissociating agent such as sodium dodecylsulphate (SDS) is present. Accordingly, protein charge is not affected, and the biological activity of the proteins is largely preserved. These include methods which have been used for analysis of proteins, including enzymes.

Gel electrophoretic techniques for "native" proteins include acid polyacrylamide gel electrophoresis (acid PAGE) of cereal prolamins, which has been reported as being an extremely useful method for cultivar identification of self-pollinated cereals (Cooke, 1995a), and acid and alkaline PAGE analysis of other proteins. However, PAGE at alkaline pH is more commonly used .

2. Gel electrophoresis method for analysis of denatured proteins

Electrophoresis of proteins in this method (referred to as SDS-PAGE) is accomplished in the presence of sodium dodecylsulphate (SDS). SDS-PAGE fractionates proteins on the basis of their molecular weight. SDS-PAGE can be applied for analysis of both total and specific protein fractions and has been extensively used for cultivar identification of both self- and cross-pollinating species (see 4.1.4).

3. Gel electrophoresis method using isoelectric focusing

The separation of proteins by this technique is on the basis of the isoelectric point of proteins. Although IEF has long been known as an extremely powerful technique for providing a very high degree of protein resolution, it has not been widely used for identification of cultivars. However, this situation is beginning to change, as this technique evolves and becomes more reliable (and also cheaper), and more attention is paid to crops other than self-pollinating crops.

4. Two-dimensional gel electrophoresis

Two-dimensional protein electrophoresis (2D) methods have, in theory, several advantages over the three above mentioned one-dimensional techniques. In 2D dimensional methods, proteins are separated on the basis of two independent characteristics (commonly charge and molecular mass). Therefore a number of products can be analyzed in one experiment. The large range of proteins which can be analyzed and the use of autoradiography coupled with automated gel analysis, make it possible to assess quantitative and qualitative variation.

4.1.4 SDS-PAGE analysis of seed storage proteins for identification and discrimination among cultivars of plant species

Seed storage proteins are highly polymorphic (Cooke, 1995a), and less conserved than other plant proteins in evolution (Konarev *et al.*, 1987). They therefore may be better discriminators than the other plant proteins. These proteins provide many polymorphic markers which can be used for classification of germplasm collections (Forde and Gardiner, 1991; Abernethy *et al.*, 1989; Vieritz, 1993; Cooke, 1995a), and also for discriminating among cultivars from a range of species (Gardiner and Forde, 1992; see also the Proceedings of an ISTA Symposium on Biochemical Tests for Cultivar Identification, 1983, and

also the review by Cooke, 1995a).

4.1.4.1 SDS-PAGE analysis of seed storage proteins for discrimination and identification of autogamous (inbreeding) species

The banding profiles produced following polyacrylamide gel electrophoresis of total seed storage protein in the presence of sodium dodecylsulphate (SDS), can be an effective technique for the identification of species (Wrigley *et al.*, 1982; Cooke, 1984; Gardiner and Forde, 1987; Cooke, 1988; Cooke 1989; Gilliland, 1989; Vieritz, 1993). However, the value of the SDS-PAGE profiles of seed proteins as a cultivar descriptor varies both between and within different genera (Gardiner and Forde, 1992). This suggests that modification of the basic technique may be required for analysis of some species.

Results by Curtis and Chadwick (1984) have illustrated that SDS-PAGE analysis of a total protein extract could be used to differentiate cultivars of the three cereal species wheat, barley and oats. The discriminating power for wheat, using glutenins alone, was lower than that provided by acid-SGE or acid-PAGE of gliadin proteins. The results suggested that the banding patterns of the more mobile fractions (gliadins) coupled with differences in band intensity, may allow cultivars with even very similar protein patterns (eg. those in glutenin group 6) to be distinguished. Such differences in band intensity are used in the system of Clydesdale and Draper (1982) which is said to allow differentiation of all cultivars.

4.1.4.2 SDS-PAGE analysis of seed storage proteins for discrimination and identification of allogamous (out-breeding) species

There have been limited applications of this approach to allogamous (cross-

breeding) species such as forage and amenity grasses (Wright *et al.*, 1983; Gilliland, 1989; Ferguson and Grabe, 1984; Ferguson and Grabe, 1986; Gardiner *et al.,* 1986; Gardiner and Forde, 1987; Gardiner and Forde, 1988a). Cultivars of out-breeding species are usually mixed populations comprising plants of a range of different genotypes, since new cultivars of such species are often formed by intercrossing a limited number of plants from registered cultivars. With selection of progeny with specific attributes from the segregating progeny, a large number of alleles present at a species level tend to be represented within each cultivar, and it is rare for a given allele to be totally absent from the gene pool of one cultivar and present in that of other (Wright *et al.*, 1983). This may suggest that there are serious problems associated with identification of out-breeding species with the electrophoresis technique. However, work by Gardiner and Forde (1987, 1988a, 1988b, 1992) demonstrated that some variations to the basic SDS-PAGE technique produced good results for a number of out-breeding species in terms of discrimination among the cultivars of such species. This technique is now accepted by the International Seed Testing Association (ISTA) for identifying commercial seed lots of *Lolium* cultivars (ISTA, 1992).

SDS-PAGE has also been used to analyze storage proteins extracted from single seeds and/or bulk seed of some other out-breeding forage species. The results are promising in terms of discrimination of cultivars. The species included in these studies were: brassica (*Brassica* spp, Woods and Thurman, 1976; Willis *et al,* 1979), clovers (Gardiner and Forde, 1988a); cocksfoot (*Dactylis glomerata,* Lumaret *et al.,* 1981; Gardiner and Forde, 1987); fescues (*Festuca* spp., Hicks *et al.,* 1982; Villamill *et al.,* 1982; Gardiner and Forde, 1987), ryegrass (*Lolium* spp., Hayward and McAdam, 1977; Gilliland *et al.,* 1982; Ferguson and Grabe 1986; Nielsen *et al.*, 1985; Gardiner *et al.*, 1986) and browntop (*Agrostis capillaris*, Forde and Gardiner 1990; Faville *et al.*, 1995).

4.1.5 Practical application of protein electrophoresis techniques in discrimination and identification of lucerne cultivars

Despite its importance as a forage crop, very few workers have reported the application of protein and enzyme electrophoretic techniques for the genetic study of lucerne. Bingham and Yeh (1971) found that basic gels gave a better electrophoretic field for separation of seed storage protein in lucerne than acid gel, and used a 7% acrylamide disc electrophoresis system at pH 9.5 for analysis of storage protein extracted from bulk seed samples of 31 lucerne cultivars. They classified these cultivars into four groups and although each did not have a definite qualitative 'fingerprint', most cultivars differed more in the electrophoretic pattern of seed protein than they did in gross morphology.

Bingham and Yeh (1971) also noted that further separation of cultivars within each group was possible on the basis of visually detectable density differences in the common bands. Since density of each of the protein bands appeared to be independent of the other bands, these authors suggested that in a further study it would be desirable to measure density differences using a densitometer. In addition, as serological testing of cultivar seed proteins was reported to be definitive enough to distinguish nine cultivars (Esposito *et al.*, 1966), Bingham and Yeh (1971) suggested that there is enough electrophoretic and/or serological variation in seed protein to warrant further investigation of the these techniques for cultivar identification.

Using starch gel electrophoresis and isozyme fingerprinting of 21 mother plants of lucerne preserved in field nursery pots for breeding and genetic studies, Quiros (1980) reported that anodal isozymes of peroxidases, esterases, and acid phosphatases from leaf tissue were very useful for the identification of individual plants. Despite being genetically closely related to each other, most of these plants had distinct zymograms for acid phosphatases.

Fahmi *et al.* (1990) carried out a series of experiments to compare the usefulness of the banding patterns provided by three electrophoretic analysis systems [ie. saline PAGE of seed protein fractions (albumin and globulin); esterase and peroxidase isozymes] for differentiation of lucerne cultivars. Using bulked seed samples of 25 lucerne cultivars, they found that saline PAGE and esterase isozymes were the most effective for cultivar classification. However, they were unable to differentiate all cultivars even using combined results of the three systems.

Gardiner and Forde (1992) examined the SDS-PAGE technique for identification of lucerne cultivars. As the protein banding profiles from the bulk seed samples were identical for most of the cultivars tested, they concluded that cultivar differentiation by SDS-PAGE was not feasible for lucerne.

Gilliland (1989) suggested that an electrophoretic descripter in an allogamous species can not usually be obtained by identifying the total presence or absence of specific bands from cultivar to cultivar, but rather by examining possible differences in the numbers of plants that express electrophoretic profiles which are common to each cultivar examined. SDS-PAGE analysis of single seeds could therefore provide a useful data base for identification of lucerne cultivars.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

As impurities, particularly in the polyacrylamide, can cause poor resolution of protein bands and lack of reproducibility (Anon., 1987), all chemicals used for protein electrophoresis were of analytical grade.

4.2.2 Seed samples

In this experiment protein samples were extracted both from bulk and single seed samples of 14 seed lots from six Iranian and two New Zealand cultivars (Table 4.2.1). A precision seed divider (Dean Gamet, M.F.G. Company, Minneapolis, USA) was used to randomly select about 1.2 g of seed (700-1000 seeds) from a 200 g sample of each of the seed lots. As the seed lot samples were gifted by the providers of the seed (Table 4.2.1), no information is available as to how the subsamples of the cultivars were taken from the seed lots, but it is assumed they were randomly drawn originally. The bulk protein samples were extracted from 20 mg of the ground seed meal from the 1.2 g bulk (see section 4.2.3). The single seed protein samples were extracted individually from 38 randomly drawn single seeds from each seed lot.

Seed lots of the Iranian cultivars were provided by the Seed and Plant Improvement Institute, Agricultural Research Organisation, Ministry of Agriculture, Karadj, Iran and seed lots of the New Zealand cultivars by the Margot Forde Germplasm Centre, N.Z. Pastoral Research Institute Ltd, Palmerston North, New Zealand and Wrightson Seeds Ltd., Christchurch, New Zealand.

Table 4.2.1 Cultivar name, seed lot number and origin of the seed lots used for protein electrophoresis

	Name of cultivar	Seed lot No.	Origin
1	Azari	1	Iran
2	Bami	1	Iran
3	Bami	2	Iran
4	Hamedani	1	Iran
5	Hamedani	2	Iran
6	Esfahani	1	Iran
7	Nikshahry	1	Iran
8	Yazdi	1	Iran
9	G.Oranga	AF 2401 (Breeders seed)	New Zealand
10	G.Oranga	B 486 (Basic seed)	New Zealand
11	G.Oranga	1st generation seed	New Zealand
12	Wairau	269 A(1st generation seed)	New Zealand
13	Wairau	Nucleus seed	New Zealand
14	Wairau	1st generation seed	New Zealand

4.2.3 Grinding of the seed samples

Following the technique of Gardiner and Forde (1992), the bulk seed samples (1.2 g) were ground using an household electric coffee grinder with a lid modified by the addition of flanges to direct the seeds onto the rotor blades. The single seeds

were crushed individually using flat-nose pliers with a smooth gripping surface, and the entire crushed seed was then transferred to the extraction tube.

4.2.4 Protein extraction procedure

The extraction buffer used was that of Smith and Payne (1984). This consisted of: 12.5 ml of 0.75 M Tris-HCL, pH6.8; 19.8 ml water; 20 ml glycerol; 4 g sodium dodecylsulphate and 12 mg bromophenol blue. The prepared solution was stored at 4°C. Just before use, the stock solution was mixed with 2-mercaptoethanol, dimethylformamide and water in the ratio of 3:1.06:1.76:3 and added to 20 mg of ground seed in 1.5 ml stoppered plastic microcentrifuge tubes. Routinely, 20 mg of seed meal was extracted with 0.5 ml of the extraction buffer (Gardiner and Forde, 1992). However, for ground single seeds (which weighed ca. 1.5-2mg), 100 µl of the extraction buffer was used.

Extraction followed the protocol used by Gardiner and Forde (1992). The samples were mixed and left at room temperature for 1 h and then were thoroughly mixed again with a motor-driven pestle constructed to fit the centrifuge tube closely. The samples were left at room temperature overnight and then resuspended using a vortex mixer. After heating at 85°C for 10 minutes in a water bath they were mixed again for 1 minute with a vortex mixer. This procedure has been shown to give reproducible results (Gardiner and Forde, 1992). The extracts were centrifuged for 5 minutes in a microcentrifuge to remove all of the particulate matter and volumes of the aliquot of the supernatant were then used for electrophoresis as specified in section 4.2.6.

4.2.5 Optimising loading volume for sharpest resolution of protein bands

The effect of protein loading on band interpretation is known to be a problem associated with polypeptide electrophoresis (Gardiner and Forde, 1992).

Overloading the extracted protein onto the gel causes poor resolution of the protein in the electric field, and as a result, produces broad and badly resolved bands. Conversely, under-loading of the samples results in the production of faint bands. Both of these cause problems during evaluation of the results (Steiner, 1993).

A series of preliminary experiments was undertaken in this study to determine the optimal protein loading volume for bulk, and single seed samples of the cultivars, to enable production of clear and sharp easily evaluated bands. In these experiments loading volumes of 4, 6, 8 and 10 µl were tested.

4.2.6 Electrophoresis

Discontinuous SDS-polyacrylamide electrophoresis was performed following the method of Laemmli (1970). However, the concentration of the buffer was increased to give a better resolution (Fling and Gregerson, 1986). The running gel contained 0.56 M Tris-HCL (pH 8.8), 0.1% SDS and 12.5 % polyacrylamide and the stacking gel comprised 0.19 M Tris-PO_4 (pH 6.8), 0.1% SDS and 5% polyacrylamide (Tables 4.2.2 and 4.2.3, Gardiner and Forde (1992)).

The upper electrode buffer was 0.03817 M Tris, 0.29 M Glycine and SDS 1% (W/V). A dilution of 2:1 of this buffer was used for the lower tank buffer.

A Biorad Protean II electrophoresis apparatus which allows control of the heat generated during electrophoresis was used in this study. This system allows two gels to be used for protein electrophoresis. The running gels were each 13 cm in length, 16.2 cm wide and 1.5mm thick. The width of the wells for loading the sample was 5mm. In each gel 19 protein samples and one broad range SDS-PAGE protein molecular weight standard (Biorad product) were loaded.

Water at 4±1°C was circulated through the central core of the apparatus and the level of the lower buffer was extended to the top of the running gel to enable even cooling and obtain the best possible resolution. The lower buffer was stirred using a magnetic stirrer, and the whole apparatus was placed in an ice/water bath. The protocol for running gels described by Gardiner and Forde (1992) was used in this study (i.e. 25 mA/gel for 1 h then 15 watts/gel for about 4h until the tracking dye reached the bottom of the gel).

Table 4.2.2 Volume of the components used for making the running gel polyacrylamide (12.5 % w/v), sufficient for making two protein electrophoresis gels.

	Component	Volume
1	H_2O	17.0 ml
2	1.5 M Tris-HCL buffer (pH 8.8)	37.5 ml
3	10% SDS (sodium dodecylsulphate)	1.0 ml
4	TEMED (N,N,N',N'-tetramethylethylenediamine)	50.0 µl
5	2% ammonium persulphate (prepared daily)	2.5 ml
6	30% acrylamide	42.0 ml

Table 4.2.3 Volume of the components used for making the stacking gel (20 % polyacrylamide, % w/v) sufficient for making two protein electrophoresis gels.

	Component	Volume
1	H_2O	12.2 ml
2	0.75 M Tris-PO4 buffer (pH 6.8)	6.2 ml
3	10% SDS (sodium dodecylsulphate)	0.26 ml
4	TEMED (N,N,N^1-tetramethylethylenediamine)	20.0 µl
5	2% ammonium persulphate (prepared daily)	1.0 ml
6	20% acrylamide	6.2 ml

4.2.7 Protein fixing procedure

After electrophoresis,the gels were first soaked in methanol:water:acetic acid (5:5:1,v/v/v) for 30 minutes to precipitate the proteins, and were then washed with three changes of distilled water for a total of 30 minutes (i.e.3 x10 minutes).The washings were performed on a rocker with slow agitation (17 rpm).

4.2.8 Staining

Following the fixing procedure, the protein was stained via a two step staining procedure:

4.2.8.1 First staining

The gels were immersed in a solution containing 0.02 % Coomassie Blue R (dissolved in 5% ethanol), 6% trichloroacetic acid and 25 % methanol. Although bands were clearly visible after overnight staining, to achieve a maximum staining of the protein the gels were left in the solution for three days.

4.2.8.2 Second staining

Following rinsing of the stained gels in distilled water for 2 h (two changes of water), a second staining of the proteins was performed prior to photography of the gels. This was both to reduce the background colour of the gels and intensify the staining of some of the bands which were not readily visible after the first staining. The Coomassie Blue G stain described by Blakesley and Boezi (1977) was used for this second staining. The gels were stored in this solution for two days in the dark to intensify before photography, as the blue-stained bands fade if the gels are left in daylight (Gardiner and Forde, 1992).

4.2.9 Photography and evaluation of the results

The double-stained gels were photographed on a light box (i.e. a 50 x 40 cm metal box with opaque perpex on top which was illuminated with fluorescent light at a day light balance at 5500 Kelvin), using a red filter and Ilford Pan F film. The films were developed following standard manufacturers conditions with Ilford ID 11 developer and printed on Ilfospeed paper (3.1 M grade).

Reports by Curtis and Chadwick (1983) from SDS-PAGE of protein electrophoresis of wheat, barely and oat species suggested that analysis of the banding patterns of the more mobile fractions (gliadins) coupled with differences in band intensity is a very sensitive method which may allow cultivars with even the most similar protein patterns to be distinguished. Differences in the band intensity is also used in the system of Clydesdale and Draper (1982) which are said to allow differentiation of all of the cultivars examined. Therefore in this present study, the intensity difference of identical protein bands from the cultivars was used for discrimination.

Assessment of the protein profiles was based on the relative intensity of a selection of the bands measured using the formula: 100 x intensity of a specific band/total intensities of the bands. As many bands were produced as a result of protein electrophoresis of single seeds (Plate 4.2.1), and some were faint and unreliable, 17 distinctive bands were selected to compare the seed lots. The bands (and their molecular weight) which were used as the variables in the analysis of the results are illustrated in Plate 4.2.1.

As visual comparison of the bands between lanes is subjective, a Vision Image Processing System version four (VIPS; Bailey and Hodgson, 1988, see description of the process in Chapter.3) was used for image processing of gel photographs (detail of the technique is discussed in section 3.3.1). This was employed for

both estimation of molecular weight of the bands (i.e. by comparison of the position of the seed protein bands with the bands of molecular weight references) and for quantification of the relative amount of protein per band, via counting the number of pixels in each band. The software was programmed in such a way as to be able to estimate and eliminate by subtraction the background fog in the image which affects the results (Bailey, 1994, pers. comm.).

In practice, lanes towards the centre of the gel often tend to migrate faster than lanes towards the side of the gel. This problem often causes a slight bowing in the band pattern across the width of the gel (Plate 4.2.2) which usually causes a problem for scoring (Steiner, 1993). The software was programmed to eliminate the bowing or "smiling" of the images (Plate 4.2.3, Bailey, 1994, pers. comm.). Details of this program can be obtained from the Image Analysis unit Massey University, Palmerston North New Zealand.

4.2.10 Statistical analysis

Using intensities data of the 17 protein bands scored (Plate 4.2.1) as variables, Canonical Discriminant Analysis (CDA, SAS 1989) was used in this study to discriminate among and also to assess variation within each seed lot. The details of the statistical analysis procedure for discrimination among, and verification of the cultivars are presented in section 3.2.7.

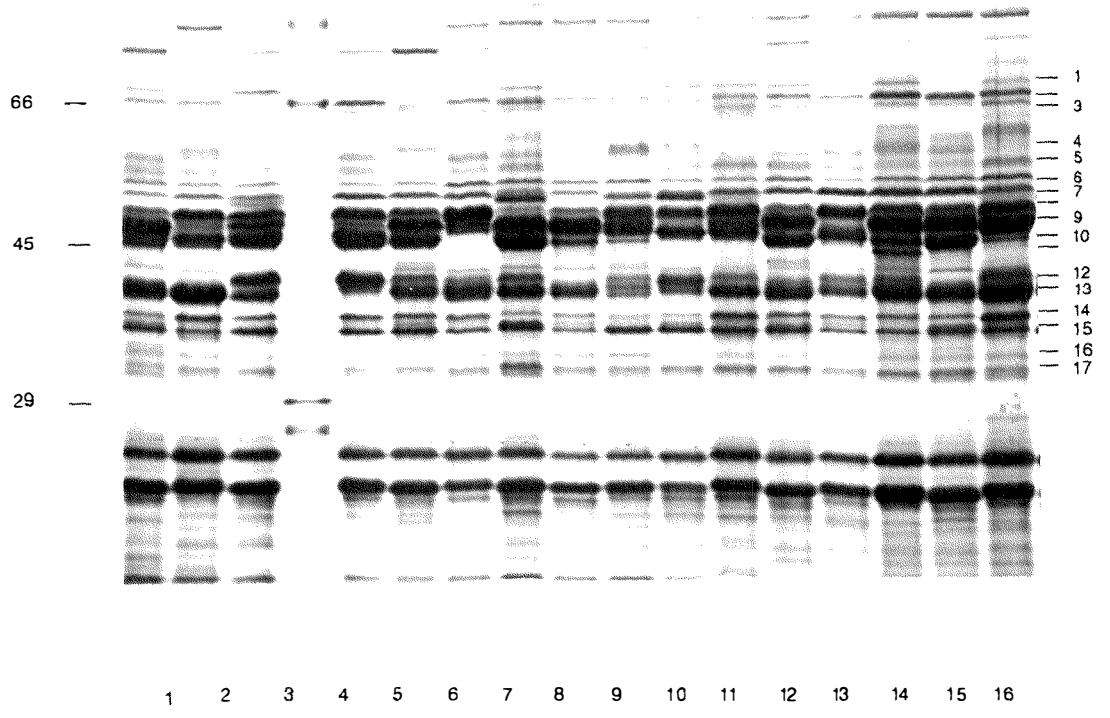

Plate 4.2.1 Protein bands of single seeds of cv. Azari for which intensity values were used as variables in the statistical analysis of this study. The lane three from the left is the protein molecular weight standard. The numbers on the left hand side of the photograph are the molecular weight of the standard ladder.

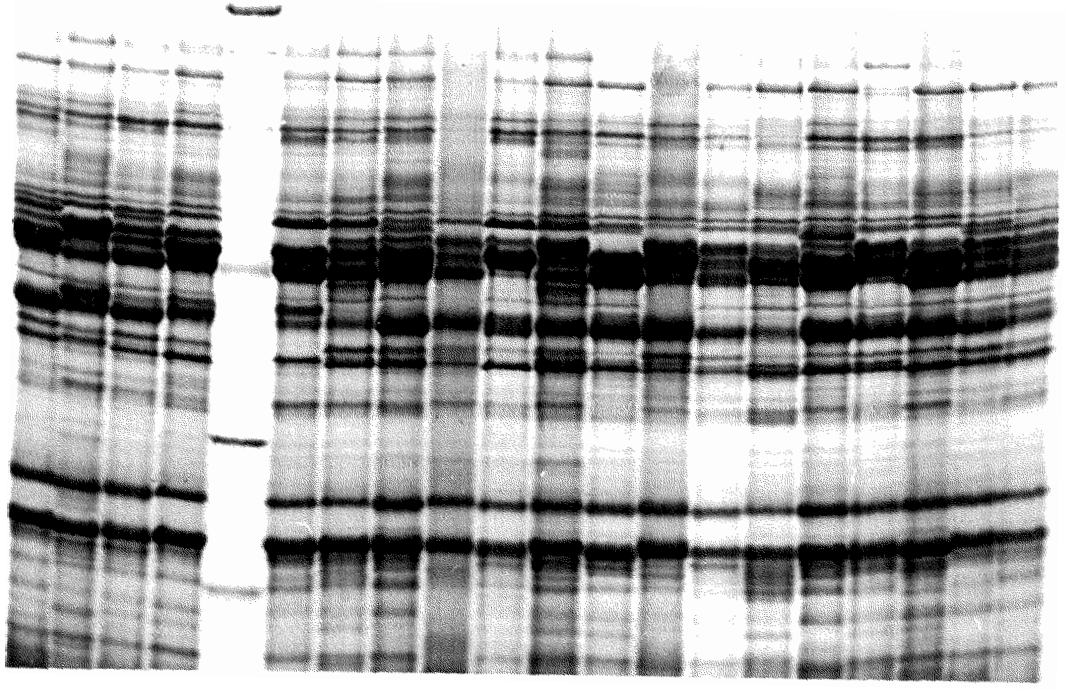

Plate 4.2.2 A sample of protein profiles demonstrating the slight bowing in the band profile across the width of the gel

a

b

Plate 4.2 3 Protein profiles of individual seeds from a Breeders seed lot of cv. G.Oranga before (a) and after (b) elimination of the bowing or "smiling" across the width of the gel .

4.3 RESULTS

4.3.1 Optimising loading volume of extract for the best resolution of protein bands

The loading of 6 µl of the extracted protein of bulk seed samples provided the most distinct protein banding profiles (Plate 4.3.1), whereas 4 µl of protein extract give rise to faint bands (picture not presented). Loading 8 and 10 µl of the sample produced broad, badly resolved bands (Plate 4.3.2) which were distorted at the edge of the lanes.

Assessment of the optimal protein loading volume for single seeds of the cultivars also revealed that the appropriate loading volume for the seed lots was dependent on cultivar. When the same volume (16µl) of the protein extract from single seeds of cultivars Azari and Wairau was loaded, cultivar Wairau produced broader and less clear protein bands than Azari (plates 4.3.3 and 4.3.4). To assess the optimal protein loading volume for the individual cultivars, protein banding profiles which resulted from loading 16 µl of protein extract from each of 5 randomly selected single seeds of the cultivars were examined, and the appropriate loading volume for each of the cultivars was determined based on the sharpness of the resulting bands. Therefore, the loading volumes of the cultivars in the main experiments varied from 8-16µl.

4.3.2 Discrimination among lucerne cultivars using the protein extracted from bulk seed samples of the cultivars

The seed protein banding pattern produced by electrophoresis of extracted protein from bulk samples of 10 Iranian and New Zealand seed lots showed that all cultivars had an identical protein banding profile except for cultivar Wairau. The only visible difference between protein banding profiles for cultivar Wairau and the others was an extra band with a molecular weight of approximately 40 in Wairau (Plate 4.3.5).

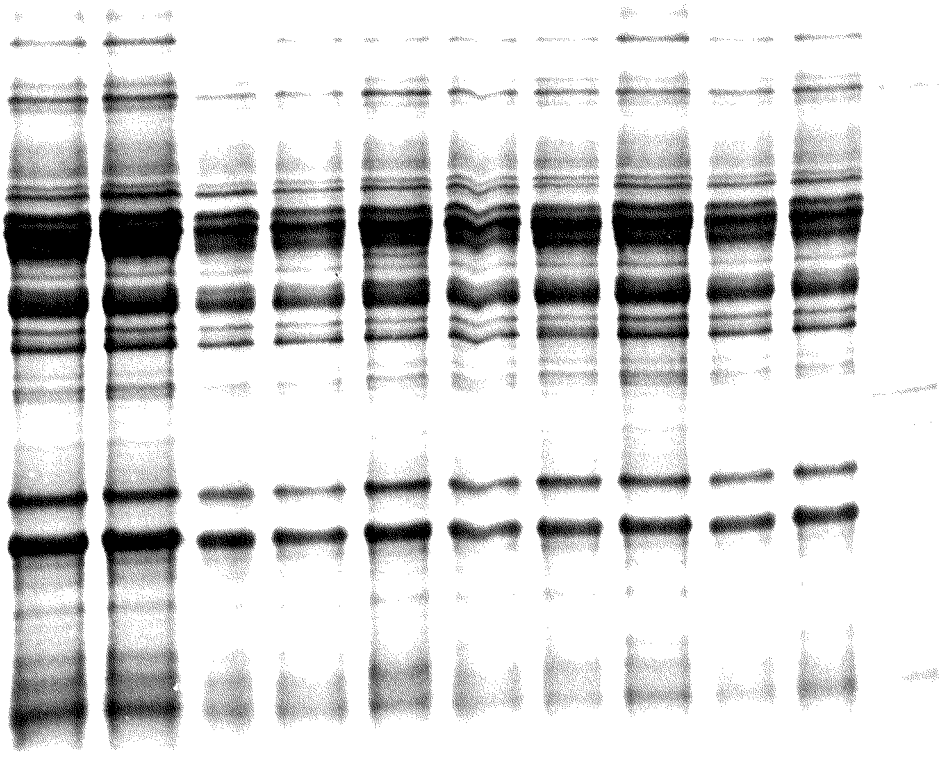

Plate 4.3.1 **Protein banding profile resulting from SDS-PAGE of 6 µl of the extracted protein from bulk seed samples of the cultivars. The profiles (left to right) belong to cultivars: Yazdi (1); Nikshahri (2); Bami 1 (3); Bami 2 (4); Wairau (5); G.Oranga (6); Azari (7); Esfahani (8); Hamedani 1 (9) Hamedani 2 (10). Lane number 11 is the broad range SDS-PAGE protein molecular weight standard.**

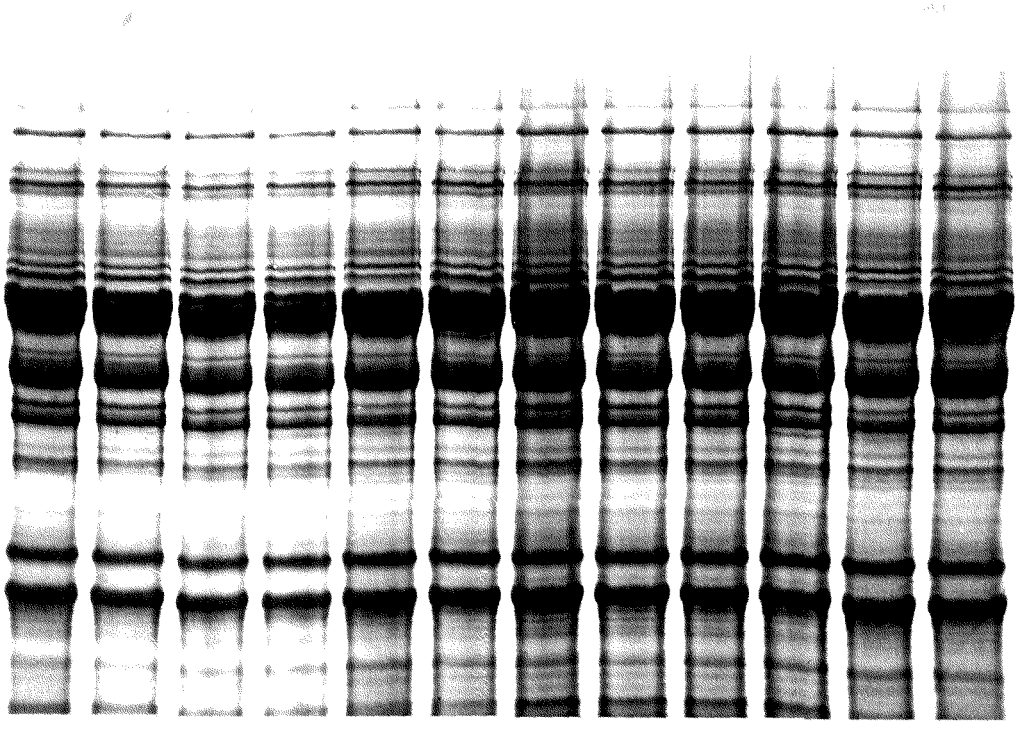

Plate 4.3.2 Protein banding profile resulting from SDS-PAGE of 8 µl (6 lanes at the left) and 10 µl (adjacent 6 lanes at the right of the plate) of the extracted protein from bulk seed samples of six cultivars.

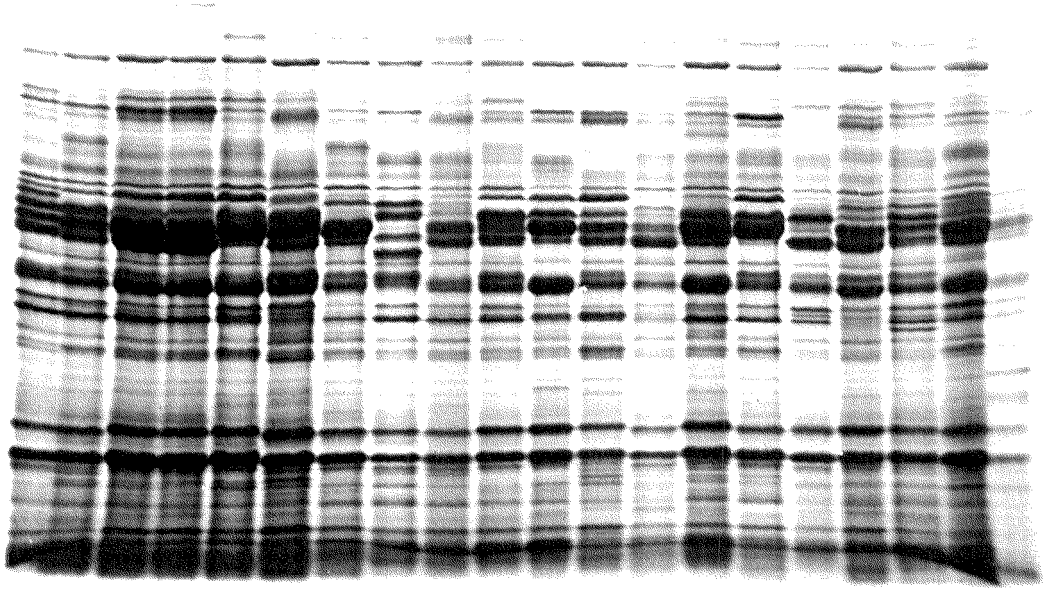

Plate 4.3.3 Protein banding profile resulting from SDS-PAGE of 16 µl of the extracted protein of single seeds of cultivar Azari.

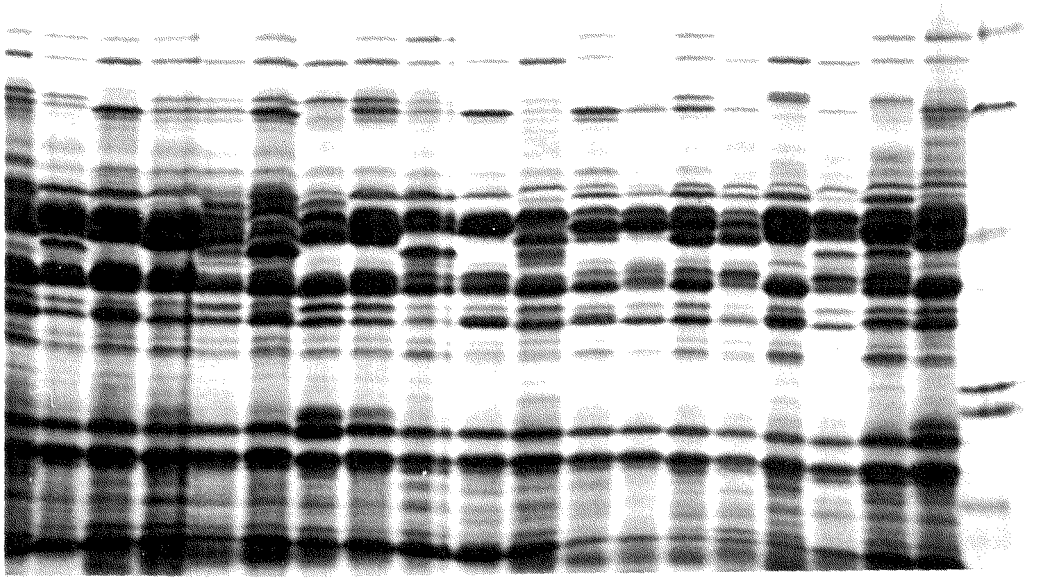

Plate 4.3.4 Protein banding profile resulting from SDS-PAGE of 16 µl of the extracted protein of single seeds of cultivar Wairau

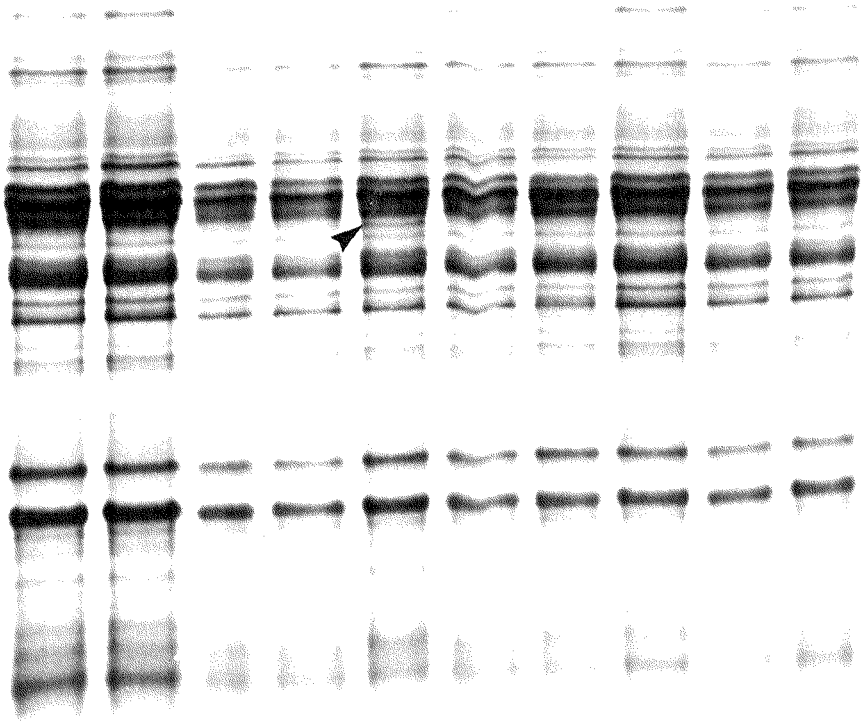

Plate 4.3.5 **Protein banding profile resulting from SDS-PAGE of the extracted protein from bulk seed samples of the cultivars. The profiles (left to right) belong to cultivars: Yazdi (1); Nikshahri (2); Bami 1 (3); Bami 2 (4); Wairau (5); G.Oranga (6); Azari (7); Esfahani (8); Hamedani 1 (9) Hamedani 2 (10). Lane number 11 is the broad range SDS-PAGE protein molecular weight standard. The protein band specific to cv. Wairau is shown by an arrow.**

4.3.3 Discrimination among seed lots of six Iranian and two New Zealand lucerne cultivars by electrophoresis of proteins from a number of single seeds from each cultivar

4.3.3.1 Cross validation for each of the seed lots of the cultivars

From the analysis of 38 single seeds of each cultivar, the cross validation, defined by probability of seeds within the seed lot being classified as from each individual cultivar, is presented in Table 4.3.1. For example for Hamedani, 70.6% of the seeds produced protein banding patterns which led to them being classified by the discriminant analysis as belonging to this cultivar, while the remaining 29.4% of the seeds gave protein banding profiles which corresponded to profiles found in the seed lots of cultivars other than Hamedani.

Of the eight cultivars, the seed lot of cultivar Yazdi produced the most uniform protein pattern (78.8% of the seeds being correctly classified) while Nikshahri was the least uniform (50% of the seeds being correctly classified).

There were also differences in the protein banding profile among seed lots of the same cultivar (Table 4.3.2). For example the Breeders seed lot of cv. G.Oranga had 40.6% of the seeds with protein banding patterns which classified them as this cultivar, while the Basic seed lot of this cultivar had 70.6% of seeds correctly classified and the 1st generation seed lot of this cultivar had 62.9 % of seeds correctly classified .

The differences between protein banding profile of seed lots of cultivar Wairau were smaller than those for seed lots of cultivar G.Oranga (ie 57.6% of seeds correctly classified for the Nucleus seed lot, and 65.6% and 55.9% correctly classified for the two 1st generation seed lots of this cultivar).

Tests of difference between two proportions (see 3.2.7) were performed in this study to check whether the differences between protein banding profiles of the seed lots of the same cultivar (Table 4.3.2) were significant. The results of this test indicated that there was a significant difference ($P<0.05$) between the protein banding pattern of the two seed lots of cv. Hamedani. There was also a significant difference between protein banding profiles of the Breeders seed and Basic seed of cv. G.Oranga, although the difference between the profiles of Breeders and 1st generation seed of this cultivar was not significant. No significant differences were found between protein banding patterns of seed lots of cv. Wairau or cv. Bami.

4.3.3.2 Pairwise comparison between squared distances (Mahalanobis distances) of protein banding profiles of seed lots from the cultivars

Pairwise comparisons between protein banding profiles of seed lots from six Iranian and two New Zealand cultivars (Table 4.3.3) indicated that with a squared distance of 2.9, Esfahani and Bami 2 produced protein banding patterns which were the closest among the seed lots tested in this study, while Hamedani 1 and Nikshahri gave protein banding patterns which were the most distinct among the Iranian seed lots (squared distance= 15.4). Of the Iranian seed lots, Hamedani 1 produced protein banding profiles which were closest to those of the New Zealand seed lots (Table 4.3.3). The squared distance between the protein profiles of this seed lot and G.Oranga 1 (Breeders seed) was 7.4 and with Wairau 1 (Nucleus seed) it was 4.3. However the most distinct Iranian and New Zealand seed lots were Esfahani and Wairau 3 with a squared distance of 18.9.

A comparison between Mahalanobis distances of the protein banding profiles of different seed lots from the same cultivars (Table 4.3.4) showed that the distance between protein profiles of Wairau 2 (1st generation seed) and Wairau 1 (Nucleus seed) was 13.3 and for Wairau 2 and Wairau 3 (1st generation seed) was 14.2. However, the distance between Wairau 1 and Wairau 3 was 5.8. The squared

distance between protein banding patterns of G.Oranga 3 (1st generation seed) and G.Oranga 1 (Breeders seed) was 10.3, which for G.Oranga 3 and G.Oranga 2 it was 4.8 and for G.Oranga 1 and 2 was 4.9. These squared distances between the Nucleus and 1st generation seeds of cv. Wairau, the Breeders and the Basic seeds of G.Oranga and those between the seed lots of cultivars Hamedani and Bami suggest major differences in protein banding patterns among seed lots of the same cultivar.

4.3.3.3 Pairwise comparisons of least squared means (LSMEANS) of protein banding patterns of the seed lots

Tables 4.3.3 and 4.3.4 indicate how close or distinct the cultivars are in terms of similarity of protein banding patterns. However, it is not clear from these tables whether the differences between the protein banding profiles of seed lots are significant. The presence or absence of significant differences in protein banding profiles between pairs of the seed lots is illustrated in Table 4.3.5, where the pairwise least squared means of the seed lots are compared.

There were significant differences between LSMEANS of protein profiles of seed lots of some of the cultivars (Table 4.3.5). For example despite there being no significant difference between protein banding patterns of Wairau 1 (Nucleus seed) and Wairau 3 (1st generation seed), a highly significant ($P<0.0001$) difference was found between the profiles of these two seed lots and that of Wairau 2, the other 1st generation seed lot of this cultivar. Significant differences also were found between protein profiles of seed lots from cultivars Bami ($P<0.0024$) and Hamedani ($P<0.0001$). There was no significant difference between the protein pattern of Breeders seed and the 1st generation seed lot of cultivar G.Oranga (G.Oranga 1 and G.Oranga 3). However, a highly significant difference was found between the profiles from these two seed lots and that of the Basic seed lot of this cultivar.

Table 4.3.1 Cross validation of seeds by cultivar

discriminant classifications true cultivar[1]	Azari	Bami 1	Bami 2	Hamedani 1	Hamedani 2	Esfahani	Nikshahri	G.Oranga 1 Breeders seed	G.Oranga 2 Basic seed	G.Oranga 3 1st generation seed	Wairau 1 Nucleus seed	Wairau 2 1st generation seed	Wairau 3 1st generation seed	Yazdi
Azari	75.0	3.1	0.0	3.1	0.0	3.1	0.0	3.1	0.0	0.0	0.0	0.0	12.5	0.0
Bami	0.0	70.6	0.0	0.0	5.9	2.9	2.9	0.0	17.7	0.0	0.0	0.0	0.0	0.0
Hamedani	2.9	0.0	0.0	70.6	2.9	0.0	0.0	2.9	2.9	2.9	5.9	0.0	8.8	0.0
Esfahani	0.0	0.0	11.8	0.0	11.8	55.9	0.0	0.0	5.9	0.0	0.0	11.8	0.0	2.9
Nikshahri	0.0	5.9	14.7	0.0	2.9	5.9	50.0	0.0	2.9	5.9	0.0	0.0	0.0	11.8
G.Oranga 1 Breeders seed	6.2	0.0	0.0	9.4	3.1	0.0	0.0	40.6	15.6	6.2	3.1	0.0	12.5	3.1
Wairau 1 Nucleus seed	6.1	0.0	0.0	6.0	0.0	0.0	0.0	12.1	0.0	6.0	57.6	0.0	12.1	0.0
Yazdi	0.0	9.0	3.0	0.0	0.0	0.0	3.0	0.0	6.0	0.0	0.0	0.0	0.0	78.8

[1]This definition is correct for cvs. G.Oranga and Wairau (standard seed lot for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'

Table 4.3.2 Cross validation of seeds from different seed lots of the same cultivar.

From To	Azari	Bami 1	Bami 2	Hamedani 1	Hamedani 2	Esfahani	Nikshahri	G.Oranga 1 Breeder seed	G.Oranga 2 Basic seed	G.Oranga 3 1st generation seed	Wairau 1 Nucleus seed	Wairau 2 1st generation seed	Wairau 3 1st generation seed	Yazdi
Bami 1	0.0	70.6	0.0	0.0	5.9	2.9	2.9	0.0	17.6	0.0	0.0	0.0	0.0	0.0
Bami 2	0.0	0.0	50.0	0.0	8.8	14.7	8.8	0.0	8.8	2.9	0.0	2.9	0.0	0.0
Hamedani 1	2.9	0.0	0.0	70.6	2.9	0.0	0.0	2.9	2.9	2.9	5.9	0.0	8.8	0.0
Hamedani 2	2.9	0.0	11.8	2.9	47.0	5.9	2.9	2.9	11.8	2.9	0.0	2.9	0.0	5.9
G.Oranga 1 Breeders seed	6.2	0.0	0.0	9.4	3.1	0.0	0.0	40.6	15.6	6.2	3.1	0.0	12.5	3.1
G.Oranga 2 Basic seed	0.0	8.8	2.9	0.0	2.9	5.9	0.0	2.9	70.6	2.9	0.0	5.9	2.9	0.0
G.Oranga3 1st generation seed	2.9	2.9	0.0	11.4	0.0	0.0	2.9	0.0	14.3	62.9	0.0	0.0	2.9	0.0
Wairau 1 Nucleus seed	6.0	0.0	0.0	6.0	0.0	0.0	0.0	12.1	0.0	6.0	57.6	0.0	12.1	0.0
Wairau 2 1st generation seed	2.9	0.0	8.6	0.0	5.8	5.7	0.0	0.0	5.7	0.0	0.0	65.7	0.0	5.7
Wairau 3 1st generation seed	8.8	2.9	2.9	0.0	0.0	2.9	0.0	5.9	0.0	5.9	8.8	5.9	55.9	0.0

Table 4.3.3 Pairwise comparison between squared distances between certified seed lots from six Iranian and two New Zealand cultivars.

From To	Azari	Bami 1	Bami 2	Hamedani 1	Hamedani 2	Esfahani	Nikshahri	G.Oranga 1 Breeders seed	G.Oranga 2 Basic seed	G.Oranga 3 1st generation seed	Wairau 1 Nucleus seed	Wairau 2 1st generation seed	Wairau 3 1st generation seed	Yazdi
Azari	0.0	7.3	10.0	8.1	8.8	12.7	11.3	8.2	11.1	15.5	9.5	14.1	6.6	11.2
Bami 1	7.3	0.0	4.9	11.3	5.8	7.7	3.9	9.1	5.0	10.0	11.7	12.6	10.8	7.3
Hamedani 1	8.1	11.3	12.1	0.0	10.1	12.4	15.4	7.4	8.2	6.4	4.3	15.3	6.8	14.9
Esfahani	12.7	7.7	2.9	12.4	2.8	0.0	5.1	12.5	7.9	11.4	13.0	6.0	18.9	8.1
Nikshahri	11.3	3.9	2.9	15.4	6.3	5.1	0.0	14.1	10.4	14.4	14.3	10.6	16.1	4.3
G.Oranga 1 breeder seed	8.2	9.1	9.7	7.4	9.0	12.5	14.1	0.0	4.9	10.3	5.5	11.8	6.7	10.3
Wairau 1 Nucleus seed	9.5	11.7	11.7	4.3	8.9	13.1	14.3	5.6	8.7	7.0	0.0	13.3	5.8	9.6
Yazdi	11.2	7.3	4.6	14.9	7.3	8.1	4.3	10.3	10.4	12.0	9.6	9.8	11.1	0.0

[1]This definition is correct for cvs. G.Oranga and Wairau (standard seed lot for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'

Table 4.3.4 Pairwise comparison between squared distances between certified seed lots from two Iranian and two New Zealand cultivars

From To	Azari	Bami 1	Bami 2	Hamedani 1	Hamedani 2	Esfahani	Nikshahri	G.Oranga 1 Breeders seed	G.Oranga 2 Basic seed	G.Oranga 3 1st generation seed	Wairau 1 Nucleus seed	Wairau 2 1st generation seed	Wairau 3 1st generation seed	Yazdi
Bami 1	7.3	0.0	4.9	11.3	5.8	7.7	3.9	9.1	5.9	10.0	11.7	12.6	10.8	7.3
Bami 2	10.0	4.9	0.0	12.1	3.3	2.5	2.9	9.7	6.6	9.1	11.7	5.6	12.3	4.6
Hamedani 1	8.1	11.3	12.1	0.0	10.1	12.4	15.2	7.4	8.2	6.4	4.3	15.3	6.8	14.9
Hamedani 2	8.8	5.8	3.3	10.1	0.0	2.7	6.3	9.0	4.9	9.1	8.9	7.6	13.6	7.3
G.Oranga 1 Breeders seed	8.2	9.1	9.8	7.4	9.0	12.5	14.1	0.0	4.9	10.3	5.5	11.8	6.7	10.3
G.Oranga 2 Basic seed	11.1	5.1	6.6	8.2	4.9	7.9	10.4	4.9	0.0	4.8	8.7	12.2	10.6	10.4
G.Oranga 3 1st generation seed	15.5	10.0	9.1	6.4	9.1	11.4	14.4	10.3	4.8	0.0	7.0	14.8	8.9	12.0
Wairau 1 Nucleus seed	9.5	11.7	11.7	4.3	8.9	13.1	14.3	5.6	8.7	7.1	0.0	13.3	5.8	9.6
Wairau 2 1st generation seed	14.1	12.6	5.6	15.3	7.6	6.0	10.5	11.9	12.2	14.8	13.3	0.0	14.2	9.8
Wairau 3 1st generation seed	6.6	10.8	12.3	6.8	13.6	18.9	16.1	6.7	10.6	8.9	5.8	14.2	0.0	11.1

Table 4.3.5 Probabilities that pairs of LSMEANS are equal.

From To	Azari	Bami 1	Bami 2	Hamedani 1	Hamedani 2	Esfahani	Nikshahri	G.Oranga 1	G.Oranga 2	G.Oranga 3	Wairau 1	Wairau 2	Wairau 3	Yazdi
Azari		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0780	0.1034	0.2244	0.0009	0.0001	0.0001	0.0001
Bami 1	***		0.0024	0.0001	0.2329	0.0001	0.0001	0.0001	0.0005	0.0001	0.0001	0.0219	0.0001	0.1791
Bami 2	***	*		0.0001	0.0629	0.1645	0.0814	0.0001	0.0001	0.0001	0.0001	0.4354	0.0001	0.0917
Hamedani 1	***	***	***		0.0001	0.0001	0.0001	0.0219	0.0001	0.0033	0.4731	0.0001	0.4913	0.0001
Hamedani 2	***	ns	ns	***		0.0012	0.0003	0.0001	0.0001	0.0001	0.0001	0.2732	0.0001	0.8727
Esfahani	***	***	ns	***	*		0.7235	0.0001	0.0001	0.0001	0.0001	0.0295	0.0001	0.0023
Nikshahri	***	***	ns	***	**	ns		0.0001	0.0001	0.0001	0.0001	0.0114	0.0001	0.0007
G.Oranga 1	ns	**	***	ns	***	***	***		0.007	0.5563	0.1158	0.0001	0.0031	0.0001
G.Oranga 2	ns	***	***	***	***	***	***	**		0.0039	0.0001	0.0001	0.0001	0.0001
G.Oranga 3	ns	***	***	*	***	***	***	ns	*		0.0280	0.0001	0.0003	0.0001
Wairau 1	**	***	***	ns	***	***	***	ns	***	ns		0.0001	0.1617	0.0001
Wairau 2	***	ns	ns	***	ns	ns	ns	***	***	***	***		0.0001	0.3543
Wairau 3	***	***	***	ns	***	***	***	*	***	**	ns	***		0.0001
Yazdi	***	ns	ns	***	ns	*	**	***	***	***	***	ns	***	

As some apparently significant results may be due to chance, the significance probability for the LSMEANS was set at 0.01 for this test. The level of 0.01 has been chosen because of the need to control the overall test error rate. For P=0.01 the probability for 100 tests is that one apparently significant result is due to chance. The significant results are indicated by stars and non significant by 'ns'. The corresponding probability can be found in the upper right hand entries . '*' 0.01> P> 0.001, '**' 0.001 >P>0.0001, '***'P<0.0001

4.3.3.4 General linear models (GLM) procedure for dependent variable: CDF1

The GLM or ANOVA procedure for the variable CDF1AN.1 (the first canonical discriminant function or the combination of I1 to I17 variables that best discriminates among the seed lots of the cultivars (see section 3.2.7) in this study confirmed that there were highly significant differences ($P<0.0001$) between the protein banding patterns of the cultivars. The summary result of this procedure is presented in the following ANOVA table.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	13	739.20	56.86	56.86	0.0001
Error	458	457.99	0.99		
Corrected-Total	471	1197.20			

4.3.3.5 Duncan's Multiple range test for discrimination between seed lots of the cultivars

Given that there were significant differences between protein banding patterns of the cultivars (the GLM result), the Duncan's Multiple range test was employed for variable CDF1 to further assess the significance of these differences. There were significant differences ($P<0.05$) between protein banding profiles of some of the pairs of seed lots (Table 4.3.6), and as a result, the seed lots are categorised into eight groups. Although protein banding patterns of Wairau 1 (Nucleus seed) and Wairau 2 (1st generation seed) did not differ, there was a significant difference between the profiles of these two seed lots and that of Wairau 3, the other 1st generation seed lot of this cultivar. Significant differences were also found among protein patterns of different seed lots of cultivars Bami, Hamedani and G.Oranga. There was a significant difference between protein banding patterns of the Basic and Breeders

seed lot of cultivar G.Oranga, but the protein pattern of the 1st generation seed lot did not differ from that of the Breeders seed lot.

Table 4.3.6 Duncan's Multiple range test for discrimination of seed lots. The calculated means in the table are the means for CDF1, the combination of I1 to I17 variables that best discriminates among seed lots of the cultivars (see section 3.2.7)

	Cultivar	Stage of production	Origin	Mean	Duncan's grouping
1	Wairau 3	1st generation	New Zealand	1.86	
2	Hamedani 1	Certified seed	Iran	1.69	
3	Wairau 1	Nucleus seed	New Zealand	1.52	
4	G.Oranga 3	1st generation	New Zealand	1.13	
5	G.Oranga 1	Breeders seed	New Zealand	0.98	
6	Azari	Certified seed	Iran	0.69	
7	G.Oranga 2	Basic seed	New Zealand	0.29	
8	Bami 1	Certified seed	Iran	-0.56	
9	Hamedani 2	Certified seed	Iran	-0.85	
10	Yazdi	Certified seed	Iran	-0.88	
11	Wairau 2	1st generation	New Zealand	-1.11	
12	Bami 2	Certified seed	Iran	-1.29	
13	Esfahani	Certified seed	Iran	-1.64	
14	Nikshahri	Certified seed	Iran	-1.72	

4.4 DISCUSSION

4.4.1 Optimising loading volume of protein extracts from single and bulk seed samples

The volume of the protein extract loaded onto the gel prior to electrophoresis has a great influence on protein resolution and hence band interpretation (Gardiner and Forde, 1992). Overloading causes poor resolution of the protein which results in broad and badly resolved bands. Conversely, under-loading of the samples results in the production of faint bands. Both of these cause problems during evaluation of the results (Steiner, 1993).

Assessment of the protein loading volume for single seeds in this study demonstrated that the appropriate loading volume was dependent on the seed lots (see 4.3.1). This was likely related to protein content of single seeds of the different seed lots, which could be affected by interactions between different environments during seed production, field management, soil type and nutrient content of the seeds. It would be reasonable, before any protein electrophoresis study, to optimize loading for each seed lot by testing protein extracts from a few randomly selected single seeds of each seed lot.

Differences in resolution of protein extracts were also found in electrophoresis of the same loading volume of some of the single seeds from the same seed lot (Plate 4.3.3). This was likely due to variation in seed size and/or protein content of single seeds within each of the seed lots. This variation, which is a result of the out-crossing nature of lucerne, is one of the problems associated with protein electrophoresis of single seeds in such crops.

Loading of 6 µl of protein extract from the bulked samples of the seed lots produced the clearest and most easily evaluated bands, while the other loading

volumes (4 and 8 and 10 µl) produced either faint, or broad, badly resolved bands. This result was in agreement with that obtained by Gardiner and Forde (1992), although they used different cultivars. This may suggest that there is no variation between the required loading volumes of protein from bulked seed of different cultivars, perhaps because differences in seed size do not have an effect on bulks.

4.4.2 Discrimination among lucerne cultivars on the basis of protein profiles resulting from SDS-PAGE of bulk seed of the cultivars

Electrophoresis of protein extract from bulk seed of the 10 seed lots in this study gave rise to identical protein banding profiles for all of the cultivars, except for cv. Wairau. This, and the result obtained by Gardiner and Forde (1992), suggests that SDS-PAGE of protein extract from bulked seed may not be a feasible method for lucerne cultivar identification. Gilliland (1989) suggested that in out-crossing species, polymorphic protein bands can not usually be obtained by identifying the presence or absence of specific bands in the protein profile from cultivars. Differences are more likely quantitative. Cultivars of out-crossing species (such as lucerne) are usually mixed populations of different genotypes. As new cultivars of such species are formed by intercrossing of registered cultivars, with selection of progeny with specific characteristics from the segregating generation, many alleles present in one species tend to be represented within other cultivars, and it is rare for given alleles to be totally absent from the gene pool of one cultivar and present in that of another cultivar (Wright *et al.*, 1983).

4.4.3 Discrimination among lucerne cultivars on the basis of protein profiles resulting from SDS-PAGE of a number of single seeds within each seed lot

SDS-PAGE analysis of protein extract from 38 individual seeds from each of the

seed lots of the eight lucerne cultivars produced distinct but very diverse protein banding patterns. This type of profile has also been found for individual seeds of other cross-fertilized species (eg. *Trifolium pratense* L.; Gardiner and Forde, 1992). There were also differences in density of the protein bands in common between individual seeds within and among seed lots of the cultivars.

Gilliland (1989) hypothesised that polymorphism among the protein banding patterns from out-crossing cultivars might arise from differences in the number of plants that express electrophoretic profiles which are common to each cultivar. In addition Bingham and Yeh (1971) noted that discrimination of lucerne cultivars might be possible on the basis of visual density differences among the protein bands in common. These hypotheses were tested in this study by statistical analysis (Duncan's Multiple range test, and pairwise comparison between least squared means, LSMEANS) of intensity of the protein bands obtained by image analysis of the pictures resulting from gel electrophoresis of individual seeds from the six Iranian and two New Zealand cultivars. Significant ($P<0.05$) differences were found between protein banding profiles of some of the pairs of the cultivars (Table 4.3.6). Pairwise comparison of least squared means of protein banding patterns of the standard seed lots of the cultivars, plus six extra seed lots from some of the cultivars (ie. 14 seed lots) illustrated that 75% of the seed lots had protein banding profiles which were significantly different ($P<0.01$, Table 4.3.5). The two New Zealand cultivars (G.Oranga and Wairau) had distinct protein profiles. The Iranian cultivars produced protein profiles which allowed classification into four groups.

The results of this study were promising in terms of discrimination among lucerne cultivars which have tremendous inter-cultivar variation, as even in inbreeding species such as wheat, barley and oats, which have very limited variation within cultivars, all cultivars could not be discriminated using the SDS-PAGE analysis technique (Curtis and Chadwick, 1983).

4.4.4 Assessment of the genetic uniformity of the cultivars based on the similarity of the protein banding profile from individual seeds within each seed lot

Cross validation for each of the seed lots of the cultivars, defined by the probability of the seeds within the seed lot being classified as being from each individual cultivar, was used to obtain information on the genetic uniformity of the cultivars (as proposed by Cooke *et al.*, 1983). There were significant differences in the genetic uniformity of the cultivars. Cultivar Yazdi, an Iranian cultivar, was the most uniform (78.8% of the seeds being classified into the cultivar), while cv. G.Oranga, a New Zealand cultivar, was the least uniform (only 40.6% the seeds being classified into the cultivar).

As the specific sequence of nucleotides in a gene results in synthesis of a specific protein, low uniformity in protein profiles of individual seeds within a cultivar may express the presence of more genetic heterozygosity of the cultivars. Heterozygosity is known to be highly correlated with yields of tetraploid lucerne plants (Kidwell *et al.*, 1994). Thus information by SDS-PAGE analysis of individual seeds of lucerne cultivars might be useful in plant breeding and plant improvement programs.

4.4.5 Comparisons among protein banding profiles of different seed lots from the same cultivar

It might be expected that all seed lots from the same cultivar would have similar protein profiles. However, the comparison between Mahalanobis squared distances (D^2) of the protein profiles of the seed lots demonstrated differences between protein profiles of seed lots from the same cultivar. For example, the D^2 between Nucleus seed of cv. Wairau and one of the first generation seed lots of this cultivar was 5.8, whereas this distance for Nucleus seed and the other first

generation seed lot of this cultivar was 14.2 (Table 4.3.4). There were also significant differences between genetic uniformity of seed lots from some of the other cultivars (eg. seed lots from cv. Hamedani and cv. G.Oranga, Table 4.3.2).

Pairwise comparisons between least squared means of protein patterns of seed lots (Table 4.3.5), and the result from the Duncan's Multiple range test (Table 4.3.6), demonstrated significant differences (at $P<0.05$ and $P<0.01$) between protein banding profiles from different seed lots of the same cultivar.

The differences among protein profiles of Nucleus seed of cv. Wairau and one of the first generation seed lots of this cultivar, and also those among the seed lots from within the other cultivars found in this study raise an interesting issue concerning protein electrophoresis studies in lucerne. Any of the following might be associated with the differences in protein banding profiles of seed lots of the same cultivar:

i) Out-crossing between seed lots from different cultivars
ii) Contamination of seed lots at harvest, or during seed cleaning and processing
iii) Sampling error of the seed lot being analyzed
iv) Genetic shift of the cultivars, because of some factor other than out-crossing between seed lots of different cultivars

Through the requirements of seed certification schemes and seed supply protocols, there is sufficient knowledge about the effects of the first three of these factors on genetic purity of a lucerne cultivar amongst seeds producers and seed companies, and by applying correct and careful management during seed production, using the required isolation distances, as well as employing correct and effective sampling methods, these factors can be controlled. However, there is relatively poor understanding of the mechanisms involved in genetic shift of

lucerne cultivars. The following are factors which might be associated with genetic shift.

4.4.6 Genetic shift in cultivars as a result of removal of a proportion of a seed lot due to shedding and shattering of seed, and also exclusion of immature seeds from the seed lot

In lucerne, as an out-crossing legume, each cultivar comprises plants of different genotypes. Some are early flowering and some late flowering plants. In practice, seed harvesting is usually performed when the majority (around 80%) of plants have pods which are brown/black in colour (Hill, 1975). At harvest, some of the seeds produced by early flowering plants have already been shed, and are therefore excluded from the harvested population. Conversely some of the seeds produced on late flowering plants are immature, and will also be excluded from the seed lot during harvesting and processing. If they are included in the seed lot, it is possible that because of their retarded physiological development, they will not give rise to vigorous plants capable of producing seeds in the next generation.

As a result of the removal of a proportion of the seeds from the seed lot, the population will be more uniform, and the genetic range of the cultivar will be narrowed, which causes some genetic shift in a cultivar. This genetic change may continue with each succeeding generation.

4.4.7 Preference of pollinators for visiting flowers of a particular colour

Lucerne plants must be cross-pollinated to produce commercial amounts of good quality seed (Rincker *et al.,* 1988). Pollinators of lucerne plants generally prefer blue or purple flower colour to white or yellow (Loper and Waller, 1970; Kehr, 1973; Goplen and Brandt, 1975; Steiner *et al.*, 1992). Purple is a wide spectrum

flower colour in lucerne (including a broad range from very light to very dark purple) and hence, some differences in attraction of the pollinator might be present even in a population of plants with 'purple' flowers. Fixation of pollinators on certain flower colours has been reported (Clement, 1965; Hagemannand, 1968) and would reduce pollination of the remaining plants.

In summary more seed could be expected from attractive plants than non-attractive plants. This, in practice, may result in some genetic shift in the succeeding generations of a cultivar. Steiner *et al.* (1992) demonstrated the reality of directional genetic shift for flower colour, resulting from differential crossing by insect pollinators, which did not happen when the plants were cross pollinated by hand. Honey bee attraction by plants may also be stable (Kouffeld *et al.,* 1969, Lopper and Waller, 1970) or may vary (Kouffeld *et al.,* 1969) with location and years. This indicates that the preference of the pollinator for a particular flower colour, and therefore the possible genetic change, might interact with other factors such as plant genotype and environmental factors during seed production. Therefore, continued maintenance of genetic lines may be difficult to achieve due to pollinator preference for flower colour and the resulting genetic shift (Bula *et al.,* 1972; Goplen and Brandt, 1975).

4.4.8 Other plant genotype effects on attraction of the pollinator

Different genotypes of lucerne are not equally attractive for bees for reasons other than flower colour (Boren *et al.,* 1962; Kauffeld *et al.*, 1969). This might be due to the genotype's potential for pollen production (Tysdal and Crandall, 1948) which is important for attraction of pollen collecting leafcutter bees (Kehr, 1973); nectar production for nectar collecting honey bees (Pedersen, 1953; Kehr, 1973) and canopy type *i.e.* ease of access for the pollinators to visit and trip the flowers.

As there are different genotypes within each lucerne population or cultivar, there will be variation among plants in their ability to attract pollinators. This may also result in production of more seeds by the more attractive plants, which may cause genetic change in the cultivar.

4.4.9 Relationship between protein electrophoresis pattern and morphological characteristics of lucerne cultivars

In cross-pollinating cultivars, considerable erosion of any genetic characteristic is likely to occur. The characteristic disuniformity which exists in these cultivars means that electrophoresis can identify differences among cultivars and also between plants of the same cultivar at a similar level of magnitude. Therefore, a cultivar can easily be segregated into a number of mutually distinct sub-groups, each of which may not differ in performance or morphological appearance from the complete cultivar (Gilliland, 1989). This possibility has been shown by Quaite and Camlin (1986) who selected perennial ryegrass plants of the same homozygous genotype from a cultivar containing several genotypes. A new 'cultivar' was synthesized and shown to be identical phenotypically to the original but significantly distinct by electrophoresis. This has been explained by Larsen (1969) who stated that "all inherent morphological manifestation of cultivar differences must ultimately have a biochemical difference but not all biochemical differences are necessarily reflected morphologically".

4.4.10 Usefulness of the SDS-PAGE technique for discrimination and identification of lucerne cultivars

Seventy-five percent of pairwise comparisons between the protein banding profiles of the seed lots in this study were significantly different ($P<0.01$, Table 4.3.5). This demonstrated that SDS-PAGE analysis of individual seeds of the cultivars is a useful technique for discrimination among lucerne cultivars.

This study has therefore demonstrated the potential of the SDS-PAGE technique for identification of lucerne cultivars. For example the Nucleus seed of cv. Wairau and one of the 1st generation seed lots of this cultivar showed similar protein profiles. However, the other 1st generation seed lot of this cultivar gave a profile distinctly different from that for the Nucleus seed lot. As the standard seed lots were analyzed for only two cultivars (G.Oranga and Wairau), further research will be needed before any final conclusions about the value of the SDS-PAGE technique for identification of lucerne cultivars can be made *ie.* protein banding profiles from individual seeds of the Nucleus seed lot (the standard), along with the succeeding generations within the seed certification scheme (Breeders, Basic and 1st generation). Nevertheless, the differences between protein profiles from one 1st generation seed lot and the Nucleus seed lot of cultivar Wairau, in particular, and those between seed lots from the same certification class (Tables 4.3.5 and 4.3.6.) were an indication of some genetic change in this 1st generation seed lot. Although this change may not be able to be defined on the basis of morphological characteristics of the seed lot (Quaite and Camlin, 1986; Gilliland, 1989), SDS-PAGE analysis of individual seeds of the population is most likely able to indicate genetic changes in seed lots of the cultivars. In summary, the ability to produce information about the variation within and between cultivars, detection of genetic shift plus the advantages mentioned in section 4.2.2, make the SDS-PAGE technique a powerful tool for protein electrophoresis studies in lucerne cultivars.

4.4.11 Practical advantages of the Canonical Discriminant Analysis (CDA)

Despite tremendous within-cultivar variation, Canonical Discriminant Analysis (CDA, SAS 1989) was demonstrated to have a good potential for discrimination among lucerne cultivars. This technique was also of value for determining variation within each seed lot, which is useful for plant breeding and improvement programs (see section 4.4.3).

The success of CDA for discrimination among the seed lots, and for exploring the genetic uniformity of the cultivars, was most likely due to the power of this technique in finding a linear function of the variables (intensity of 17 protein bands) that maximally discriminate the seed lots. This approach could distinguish several uncorrelated canonical discriminant functions (CDF). These are linear combinations of the original values (intensity of the bands) that separate the mean of the groups of observations relative to within-group separations (see section 3.2.7).

CHAPTER 5

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLP)

5.1 LITERATURE REVIEW

5.1.1 Principle of the restriction fragment length polymorphism (RFLP) technique

Until recently, genetic polymorphism could be detected only if it was expressed by phenotypic differences of plants, differences in enzyme activity, and their electrophoretic mobility. As a result, the scope for genetic analysis was limited, but now polymorphisms are detectable at the DNA level.

The first method for examining polymorphisms at the genome level is RFLP (restriction fragment length polymorphism) analysis. Restriction fragment length polymorphism depends on inherited differences in recognition sites for restriction enzymes (*i.e.* caused by base changes in the target site) that result in differences in the length of the fragments produced by cleavage with the relevant restriction enzyme (Lewin, 1990; Watson *et al.*, 1992). The RFLP technique is the basis for DNA fingerprinting which has become a powerful tool for the detection of genetic polymorphism between individuals and of heterozygosity within individuals.

A restriction endonuclease enzyme cuts a DNA molecule only at a limited number of nucleotide sequences specific to that enzyme. For example, the restriction endonuclease *Bgl II* cuts DNA molecules at the following 6 base sequence (Watson *et al.*,1992):

```
... A |G A T C T...          ...A              G A T C T...
      --------      ──→                   +
...T C T A G |A...           ...T C T A G      A...
```

If one or more nucleotides in a sequence are changed, the enzyme no longer

recognizes its restriction site, does not cut the DNA there, and therefore longer fragments of DNA are produced. When a given restriction enzyme site is present in the DNA molecule of one chromosome but absent from that of the other homologous chromosome, a shorter DNA fragment is produced at this position from the chromosome with the site than the other chromosome. It is therefore possible to distinguish the two chromosomes in such an individual on the basis of restriction fragment length polymorphism (RFLP). The individual is heterozygous for this RFLP.

To perform RFLP analysis, DNA extracted from leaf or other plant tissues is digested with a commonly available restriction endonuclease to cleave it into smaller fragments (Hillis and Moritz, 1990). These DNA fragment are then size-separated in agarose gels by electrophoresis and blotted onto nylon membranes by the method of Southern (1975), prior to probe hybridisation.

The DNA bound to the membrane is hybridised with a DNA probe, derived from cloned fragment of DNA, usually from the same or a related genus, which is complementary to the region of interest. Probes for RFLP analysis are labelled either radioactively, usually with ^{32}P (Nybom, 1990b) or, less commonly, nonradioactively (eg. with peroxidase as in the Amersham ECL method, Amersham International plc UK).

Autoradiographs (from the radioactive labelling method), or lumigrams (from chemiluminescent methods) are produced by exposing an X-ray film with the hybridised membrane and then developing the film to reveal sites of probe hybridisation. Identically sized restriction fragments in different plants represent similarity, and different sized fragments are interpreted as genetic differences.

5.1.2 Applications of the RFLP technique for genetic analysis of plant species

RFLPs are inherited according to Mendelian genetics (Lewin, 1990; Watson *et al.*, 1992) and the individual chromosome segments close to the RFLPs being studied can be followed as they pass from generation to generation by tracing the inheritance of the markers. This can be used by plant breeders for selection of progeny carrying specific genes linked to these markers (marker assisted selection, Paran *et al.*, 1991)

RFLPs are able to reveal genetic homozygosity or heterozygosity of an individual on the basis of the presence or absence of polymorphic sites for restriction binding on homologous chromosomes (Watson *et al.*, 1992). A particular polymorphic site may be present or absent from DNA of both homologous chromosomes. In this case the individual is homozygous for that RFLP. However, if the polymorphic site is present on one and absent from the other homologous chromosome, the individual is heterozygous at that site.

Combining the information derived from several probe/enzyme combinations can give valuable information on the comparative heterozygosity of individuals (Gebhardt *et al.,* 1989; Brummer *et. al.*, 1991; Steiner, 1993), detection of genetic polymorphism within and between populations, as well as determining the relationship among species and populations (Saghai-Maroof *et al.*, 1984).

RFLPs can be useful for distinguishing germplasm sources (Walton *et al.*,1988; Kidwell *et al.*, 1994; Steiner, 1993), determining the genetic contribution of different germplasm sources to new cultivars (Kidwell *et al.*, 1994) and as an estimation of relatedness among different accessions in germplasm collections. It is also possible to estimate the phylogeny of different accessions in such collections (Swofford, 1993).

RFLPs have been used for genome mapping in a number of plant species including maize (Helentjaris *et al.*, 1988); rice (McCouch *et al.*, 1988); potato (Gebhardt *et al.*, 1989); sugar beet (Hallden and Tuvesson, 1991) and tomato (Helentjaris *et al.*, 1986; Bernatzky and Tanksley, 1986). This technique is also used for marker assisted selection.

Despite being an important forage crop, molecular genetic characterization of lucerne lagged behind other major crops until 1991 (Brummer *et al.* 1991). However, several studies on RFLP analysis of lucerne have been reported in recent years (Brummer *et al.*, 1991; 1993; Blondon *et al.*, 1993; Kiss *et al.*, 1993; Kidwell *et al.*, 1994a and b; Pupilli*et al.*, 1995).

Brummer *et al.* (1993) constructed a RFLP linkage map for diploid lucerne. Another genetic map has been constructed by Kiss *et al.* (1993) using a combination of RFLPs, RAPDs (random amplified polymorphic DNA), isozymes and morphological markers.

5.1.3 Applications of the RFLP technique in cultivar identification

RFLPs have been used for cultivar identification in some crops such as rice (Dallas, 1988), beets (Nagamine *et al.*, 1989), maize (Smith and Smith, 1991); potato (Görg *et al.*, 1992); barley (Pecchioni *et al.*, 1993) and wheat (Vaccino *et al.*, 1993). However, despite there being many reports in the literature about the application of the RFLP technique in cultivar identification of such inbreeding crops, there are only limited numbers of reports on the use of this technique for detection of variation within and between cultivars of out-crossing species.

RFLP profiles from seven individual plants from each of two sugar beet (*Beta vulgaris*) cultivars of different ploidy level (*ie.* one triploid and one tetraploid cultivar), plus two wild species of beet were studied by Nagamine *et al.* (1989).

The results of this study demonstrated that out of three probes tested, one was able to reveal genetic polymorphisms among the populations. The results also illustrated that one of the cultivars was more polymorphic than the other. Although the number of the plants per cultivar and species may not have been sufficient for genetic study at a population level (Cliff, 1987), particularly in an out-crossing species such as beet, these results demonstrate that the RFLP technique is able to discriminate among genetically divergent populations of out-breeding species. However, it is not clear whether closely related cultivars of this species can be distinguished using RFLPs.

RFLP variation in diploid and tetraploid lucerne was studied by Brummer *et al.* (1991) using 20 plants for each of three cultivars of very diverse genetic background and 19 RFLP probes. As could be expected, more complex RFLP banding patterns were found for tetraploid plants than for diploid. Since no fragments unique to a particular population were detected in all plants of the populations, they suggested that DNA fingerprinting of lucerne cultivars or subspecies (as has been done in rice using a human minisatellite probe (Dallas, 1988)) was unlikely to be successful. They suggested that alternatively, it might be possible to discriminate among lucerne cultivars on a population basis by examining the percentages of plants containing particular fragments. In addition fingerprinting individual plants might be possible using several probes that produce a highly complex and unique RFLP banding profile for each plant. However, it is not clear from their work whether more closely related cultivars of lucerne could be identified using RFLP analysis.

In a second study by Kidwell *et al.* (1994), genetic diversity of nine *Medicago* accessions, representing original germplasm sources for North American lucerne cultivars, was assessed. The diversity was estimated by screening DNAs from 12 individual plants of each accession, along with bulk samples containing equal amounts of DNA from four, six or 12 individuals, for nuclear RFLPs by

hybridization to 35 cloned lucerne sequences. The results of their study revealed that individuals within accessions were highly polymorphic, but very few accession-specific polymorphisms were identified. Although individuals from two sources, *Medicago falcata* and a Peruvian cultivar, formed distinct clusters, genotypes from the other seven accessions were not clearly discriminated. Kidwell *et al.* (1994) suggested that this non-distinctness among most of the accessions could be due to a similar genetic composition of the initial introductions, intercrossing of different sources during maintenance, or genetic shift during maintenance.

As reported by these authors the individuals within accessions were highly polymorphic, and thus analysing 12 plants per accession might not be expected to be sufficient to reveal actual genetic distances among the accessions (see also Cliff, 1987). In addition, the probes used might not have been suitable for revealing polymorphisms among the accessions, although 35 cDNA clones is a reasonable probe number to screen.

5.1.4 Importance of probe selection in revealing polymorphisms among populations and cultivars

Determining the most suitable probe for hybridisation to the Southern-blots of genomic DNA is a critical factor in revealing polymorphisms among cultivars. Dallas (1988) used a human minisatellite probe, highly polymorphic in men and various animals, to detect polymorphisms in rice cultivars. Liu *et al.* (1992) have found a repeated, and highly variable genomic sequence of wheat by which 56 common cultivars were discriminated .

Vaccino *et al.* (1993) proposed a method for wheat cultivar identification which combined the results from two restriction enzymes and two probes, specific for high molecular weight glutenins and γ-gliadins respectively. Although none of

the single probe/enzyme combinations was able to discriminate among all of the wheat cultivars, a complete fingerprinting of 54 cultivars was obtained using a combination of two DNA probes and two restriction enzymes.

A ribosomal DNA probe [(rDNA, derived from an rDNA spacer region of the 'White Angel' crab apple (Simon *et al.*, 1992)] was used by Nybom *et al.* (1992) to hybridise Southern-blots containing *Rsa*I-digested DNA samples of three cultivars (Golden Delicious, Jonathan and Red Delicious) and DNA samples from 16 apple seedlings. The high number of copies and the highly conserved coding sequences of the rDNA genes permitted the use of this probe to reveal polymorphism within and among the populations. However, despite their utility, ribosomal genes have not been used by other workers as a source of polymorphisms for genetic relatedness studies of plant species (Nybom *et al.*, 1992).

5.1.5 Advantages and disadvantages of the RFLP technique

RFLP data can be collected efficiently as the Southern blots can be hybridised with many different probes in succession (Thormann and Osborn, 1993). This advantage coupled with the valuable information obtained as discussed as in the last sections of this review, make the RFLP technique a powerful tool for genetic studies both at an individual as well as at a population level. However, the procedure for nucleic acid isolation and purification, electrophoresis, blotting and hybridisation for detection of RFLPs by Southern-blot hybridization is relatively time consuming and technically complex (Kangfu and Pauls, 1993). A suitable library of DNA clones must be available to produce the probes that are needed to detect polymorphisms. Library construction is time consuming and requires considerable technical expertise. Few commercial libraries are available for forage species, but it is often possible to obtain libraries from other scientists who are conducting related research (Steiner, 1993). Some expertise is also required for isolation and purification of cloned DNA fragments to prepare probes.

Special licences must be obtained to use ^{32}P and very careful procedures are required to ensure safety. There are currently non-radioactive methods that can be used to label probes, providing a possible alternative to the more commonly used radioactive methods (Steiner, 1993) but it is generally acknowledged that skill is required to use this technique successfully.

5.2 MATERIALS AND METHODS

5.2.1 Plant Material

In this study certified (stages in the certification scheme not known) seed lots from six Iranian cultivars (Azari, Bami.1, Hamedani.1, Esfahani, Nikshaheri and Yazdi) and seed lots from two New Zealand cultivars (Wairau, Nucleus seed), and Oranga (Breeders seed); and basic seed lots from two internationally recognized cultivars from USA (Saranac and Moapa) were tested.

The Iranian cultivars were provided by the Seed and Plant Improvement Institute, Ministry of Agriculture, Karadj, Iran and the others by the Margot Forde Germplasm Centre, Pastoral Research Institute Ltd, Palmerston North, New Zealand and by Wrightson Seeds Ltd, Christchurch, New Zealand

5.2.2 DNA extraction

The DNA extraction buffer used in this study was one modified by Dr. S. Gardiner (1993, pers. comm.) and was made as specified in Table 5.2.1

Table 5.2.1 Volume of components used for making 50 ml of DNA extraction buffer

	Chemicals	
1	Sorbitol (2.0 M)	3.5 ml
2	Tris pH 7.5 (1.5 M)	11.0 ml
3	EDTA pH 8.0 (0.5M)	2.2 ml
4	NaCl (5.0 M)	8.0 ml
5	Sterile H_2O	25.0 ml
6	CTAB (Hexadecyltri-methylammonium bromide)	0.4 g
7	N-Lauroylsarcosine	0.5 g

DNA samples were extracted from the fresh leaves of 40 young (45 day old) single seedlings grown in a growth chamber at the Plant Growth Unit, Plant Science Department, Massey University, Palmerston North. The temperature and relative humidity of the cabinet were 20 ±2°C and 60 ± 5% respectively.

RFLP analysis was performed on DNA from individual seedlings and also on bulked DNA samples of the cultivars. Because of the cross pollinating nature of lucerne, there were variations in leaf size and accordingly in the quantity of DNA extracted from different seedlings. Therefore, the bulk DNA sample for each cultivar was prepared by combining equivalent amounts of DNA from individual seedlings following DNA quantification.

For the DNA extraction, four to five leaves from each seedling was placed in a labelled plastic bag (approx. 15x20 cm) and stored temporarily in a thermos of liquid air. Two ml of the DNA extraction buffer [consisting of: 280 mM

Sorbitol; 660 mM Tris (pH 7.5); 44 mM EDTA (pH 8.0); 160 M NaCl; 32 mg CTAB and 40 mg N-Lauroylsarcosine] plus 1% PVP (Polyvinyl-Pyrrolidone, MW 40000) were added to each bag. The buffer was made fresh daily from the stocks (Table 5.2.1) which were kept in a refrigerator at 4° C). The bag was closed using an electric bag sealer. The leaves were ground using a wooden roller on a firm surface to produce a homogenous pulp which was then removed into a 2.0 ml Eppendorf tube. 400 µl of chloroform + octanol (24:1 v/v) was added, and vigorously mixed with the pulp. A small slit was made in the cap of the Eppendorf tube to release pressure and the sample was incubated at 65°C in a water bath for 30 mins. The samples were then placed on ice for about 10 mins to cool and centrifuged at 15000 rpm for 10 mins using a Sorvall centrifuge . After centrifuging, the supernatant was removed into 1.0 ml ice cold isopropanol in a 2.0ml Eppendorf tube and then mixed gently by inversion to initiate DNA precipitation. Although a period of 5-15 mins below room temperature is recommended for precipitation (Dr. S.E. Gardiner, 1994, Pers. Comm.), the samples were stored in the refrigerator overnight for this study, as it was found that yield was higher.

The tube was then centrifuged at 12000 rpm for 5 mins to precipitate the DNA and the supernatant was removed with a sterile Pasteur pipette. The precipitated DNA pellet was washed twice with 1 ml of ice cold 70% ethanol. The ethanol was drained off and DNA pellets were dried for 15-20 mins in a vacuum dryer prior to resuspension in 100µl of distilled water.

5.2.3 DNA quantification

To determine the concentration of the extracted DNA, a dilution of 1:5 (V/V) of the concentrated DNA was made and from this, dilutions of 1:2; 1:5 and 1:10 were prepared. To each of these dilutions 2 µl of 10 x loading buffer (Appendix 5.1b) and sterile water were added to make a total of 20 µl. After mixing and centrifugation, the samples were heated to 65°C in a water bath and after a brief

centrifugation were loaded into a 0.9% agarose gel in TAE. Along with these samples, two 50 ng samples of bacteriophase lambda DNA (undigested, BRL) were also loaded into separate lanes of each row as standards for estimation of DNA concentration. The gels were electrophoresised at 60 Volts (4 V/cm) for 1.5 h in TAE buffer+ ethidium bromide (see Appendix 5.1b) and were then photographed in black and white on a UV-transilluminator (312 nm) using a Polaroid camera (Plate 5.2.1)

5.2.4 Optimising loading concentration of the digested DNA into the gel for production of sharp, easily evaluated RFLPs

Bulked DNA samples from eight cultivars were used in this study to carry out a series of experiments to optimise DNA loading. One, two, three and five μg DNA were tested. The findings are presented in the Results.

5.2.5 General procedure for DNA digestion, preparation of the Southern blots, probe hybridisation and signal detection, plus determination of best probe/enzyme combinations

In an initial experiment, bulked DNA samples of 10 cultivars were digested with a variety of common restriction endonucleases (Eco R1, Bam H1, Hind III and Xba1) to cleave the DNA into smaller fragments (Hillis and Moritz, 1990). The digested DNA fragments were separated using a 0.9% agarose gel electrophoresis (20 x 20 x 0.9 cm, 43 slots/gel, the dimension of each slot being 3 mm x 1.5 mm) and transferred to a Hybond-N^+ nylon blotting membrane (Amersham International PLC, Amesham UK). A non-radioactive labelling method (Amersham ECL) was used for probe labelling and the membrane was incubated in a Hybaid oven at 42°C to provide the controlled conditions required for probe binding. The hybridised membrane was washed with a primary wash buffer (6 M urea, 0.4% SDS (sodium dodecylsulphate), and 0.5 x SSC (sodium citrate +

NaCl, Sambrook, *et al.*, 1989) and then used to expose an X-ray film (2-4h depending on expected probe reaction).

This membrane was hybridised with a number of probes to determine the best combination between restriction enzyme and probe to optimize the number of polymorphic bands. These combinations were then used for digestion and probing of the DNA samples from single seedlings of the cultivars (see the details for this procedure in the following sections).

In this study six lucerne cDNA probes (I013, 492, 281, 328, 473, 457), two apple probes [ADH cDNA and ribosomal DNA (rDNA)] and one clover probe (ADH) were screened. The lucerne probes were supplied by courtesy of Drs. E.C. Brummer, G. Kochert and J.H. Bouton, Dept. of Agronomy, University of Georgia, USA. The apple DNA probe was a gift from Dr. C.J. Simon, 215 Johnson Hall, Washington State University, Pullman, WA 99164; the apple ADH probe was by courtesy of Dr. G.Ross, HortResearch, Auckland, New Zealand and the clover DNA probe was a gift from Dr. N. Ellison, AgResearch, Palmerston North, New Zealand.

5.2.5.1 DNA Digestion

5µg of DNA (for all of the probes screened except for apple rDNA probing for which 0.2µg was sufficient) was placed in a 1.5 ml Eppendorf tube together with 20-40 units of the required restriction enzyme (proportionally less for apple rDNA probing) plus 2-3 µl of enzyme buffer (10% of the total volume) and water to produce a final volume of 20-30 µl. The samples were mixed and centrifuged briefly and then incubated in a 37°C water bath overnight following the digestion. 10xSB buffer was added to the tube (10% of total volume) and this was then heated at 65°C for 10 mins. The samples, including positive controls (BRL 1 kb ladder, conc. 1µg/µl), were loaded onto the gel (1 control at each end of the gel)

and were electrophoresed overnight on an 0.9% agarose gel at 51 Volts (about 2.5 Volt/cm) in TAE buffer (Sambrook, *et al.* 1989).

5.2.5.2 Processing of the gel

When the bromophenol blue dye had reached the end of the gel tray, the gel was rinsed in TAE buffer (1mg/ml) plus ethidium bromide (2% of X50 stock) for 30 mins and then routinely photographed on a UV transilluminator using black and white Polaroid film (ISO 3000) to check the success of the digestion. Plate 5.2.2 is a colour photograph (using Kodak Ekta-chrome 64 T colour slide film) of a gel on which DNA samples from cultivar Moapa digested with Xba *I* were run.

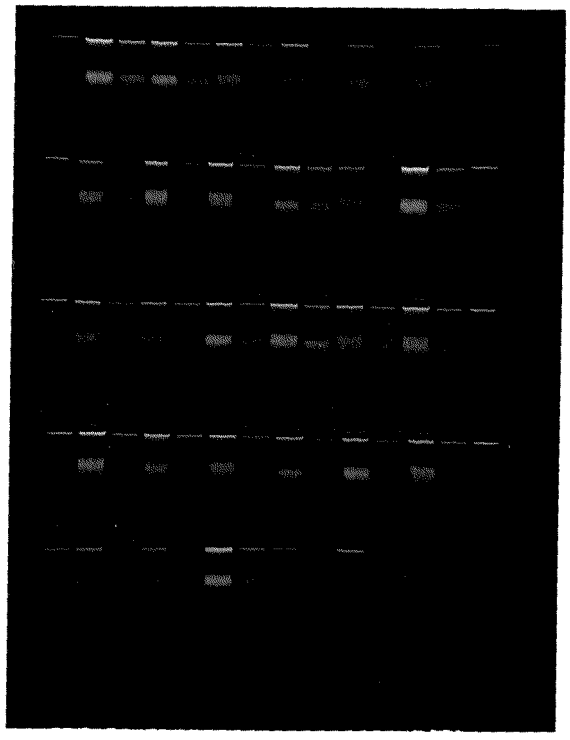

Plate 5.2.1 **A sample photograph of a DNA quantification gel in this study. The DNA samples at the first and the last lane of each row are two standard samples of 50 ng of bacteriophase lambda DNA (undigested, BRL) which were loaded for estimation of concentration of DNA extract of lucerne seedlings loaded next to these standard samples.**

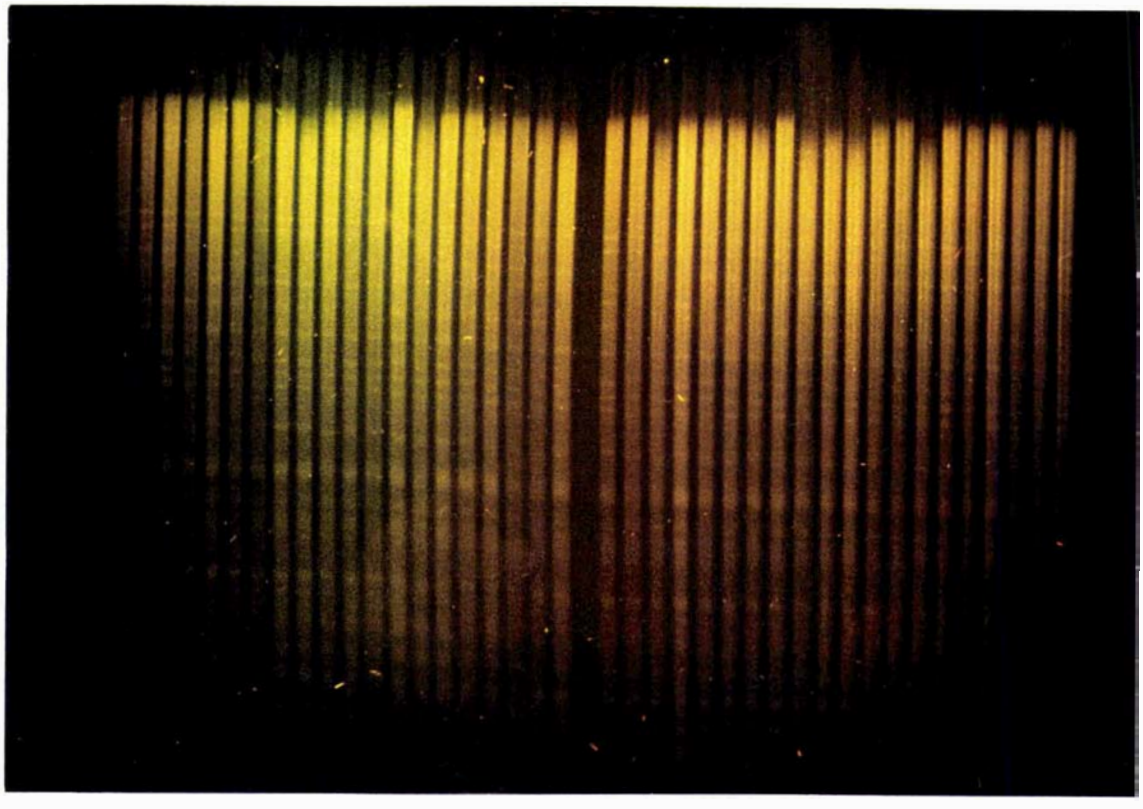

Plate 5.2.2 **A colour photograph of Xba *I*-digested DNA samples of cultivar Moapa run in a 0.9 % agarose gel in TAE buffer and stained with ethidium bromide**

5.2.5.3 Capillary blotting

Prior to capillary blotting, the photographed gels were washed (agitation provided by an rocking platform, 15 strokes/min) with the following solutions.

1. 7 mins in 0.25 N HCl (depurination solution) followed by washing with water purified by reverse osmosis (3 times).

2. 30 mins in 1.5 M NaCl / 0.5 M NaOH (denaturation solution) followed by washing with water purified by reverse osmosis (3 times).

3. 30 mins in 1.5 M NaCl / 0.5 M Tris pH 7.5 (neutralisation solution)

The capillary blotting was performed using a plastic tray (40 x 40 cm and 7 cm depth) filled with 0.4 M NaOH, on top of which a piece of glass was placed as a supporting platform. The glass was covered with three pieces of filter paper (Whatman No.3 MM), with the edges touching the NaOH solution in the tray.

The washed gel was carefully placed on the filter papers and a pre-wetted nylon membrane (first with H_2O then 0.4 M NaOH) carefully layered on top. Parafilm strips were placed around the gel to prevent evaporation of NaOH. The membrane was covered with three pieces of damp Whatman No.3MM filter paper (wetted with 0.4 M NaOH), then two Wettex cloths squeezed dry, and then a stack of paper towels of about 15cm thickness. On top of this was placed a glass plate, and finally a weight of approximately 400 g. The blotting stack was left overnight to facilitate a complete capillary transfer of DNA from the gel to the contact surface of the membrane. After blotting, the membrane was rinsed in 5 x SSC for 1 min (with agitation) and after air drying was baked at 80°C for 2h and stored at 4°C until used.

5.2.5.4 Probing

5.2.5.4.1 Prehybridisation

The membrane was wetted in a plastic tray with 2x SSC and then rolled up and placed in a hybridisation bottle (Hybaid) with 15 ml of 2x SSC to facilitate unrolling of the membrane so that it was lying against the glass walls of the tube. The 2x SSC in the tubes was then discarded. Each tube was rinsed with 3 ml of hybridisation solution (ECL, Amersham). This was discarded prior to adding 15 ml of this solution. The tube was then placed in a Hybaid oven at 42°C for 1 h for prehybridisation.

The hybridisation solution was prepared from 100 ml of the hybridisation solution and 5 g of blocking agent (both from the ECL Kit from Amersham) plus 2.92g of NaCl. This was heated gently in a microwave oven to dissolve and was stored in a freezer until use.

5.2.5.4.2 Probe preparation and hybridisation

The probe was prepared using the Amersham ECL kit immediately prior to hybridisation as follows:

A volume of the DNA probe to give 400 ng and 2 µl of 1:200 dilution of 1 kb ladder and water (to make the total volume of sample to 40 µl) from the kit were placed in a 1.5 ml Eppendorf tube. The tube was placed in a boiling water bath for 10 mins to denature the double stranded DNA probe.

To prevent reannealing, the sample was quickly frozen in a -70°C freezer for 5 mins and then thawed prior to centrifugation to collect the sample at the base of the tube. An equal volume of the DNA labelling reagent and then gluteraldehyde reagent (i.e. 40 µl of each from the ECL kit) were added. The tube was then

incubated at 37°C for 10 mins after gentle mixing.

One ml of hybridisation solution was added to the probe in each tube and after mixing, all of the contents of the tube were removed to the hybridisation bottle and distributed over the membrane by turning the bottle gently and thoroughly. The tube was returned to the Hybaid oven for hybridisation of the DNA probe to the DNA blot overnight at 42°C.

5.2.5.4.3 Washing the membrane

Following the hybridisation, each membrane was removed to a clean tray and pre-rinsed with a small amount of primary wash buffer (6 M urea, 0.4% SDS and 0.5 x SSC) at 42°C. The wash buffer was poured off and the membrane was then covered with excess fresh wash buffer at 42°C and incubated with agitation for a total time of 40 mins (2 x20 mins) at 42°C.

After the second wash, the membrane was transferred to a clean plastic container, pre-rinsed with a small amount of 2 x SSC, then incubated (x2) at room temperature on a rocking platform for 5 mins with fresh 2 x SSC, and finally air dried for 10 mins.

5.2.5.4.4 Signal detection (modified from the Amersham technique)

Prior to signal detection one sheet of Whatman No.3 MM filter paper (22x18 cm) was placed in a plastic folder prepared from ducting grade plastic (a little larger all around than the filter). As 3.5 ml of the ECL reagents (ie. 1.75ml of each of A and B) is required for signal detection per 100 cm^2 of the membrane plus 1 ml of the mixed reagent on top of the membrane; 7.5ml from each of the complementary reagents A and B (as supplied by Amersham) were mixed and then 14 ml was evenly poured over the filter paper so that it soaked in evenly.

The hybridised membrane was layered on this filter paper (DNA side up) and 1 ml of the mixed reagents was then pipetted onto the DNA face of the membrane. Lastly the plastic folder was rolled down and its edges sealed with a bag sealer to prevent leakage. At all stages care was taken to prevent formation of air bubbles between surfaces.

The membrane sandwich was placed against the X-ray film in an X-ray type film cassette with the upper (DNA face) to the X-ray (RX Fuji Medical) film. Exposure was 30-60 min for the apple rDNA and 2-4 h depending on expected probe reaction. The X-Ray film was developed using the recommendations by the supplier of the chemicals, Fuji Hunt Photographic Chemicals, PTE LTD 13/15 TUAS Av. 7. Singapore 2263. The procedure was as follows:

1. Rinsing in the developer for 6 mins
2. Rinsing in a 3% acetic acid to stop developing
3. Rinsing in the fixer for 5 mins

5.2.5.4.5 Probe stripping

It is suggested that membranes which have been probed using the ECL gene detection system can be reprobed up to 10 times without stripping of the previous probe (Amersham ECL booklet). However, in this study it was found that reprobing of the hybridised membranes immediately after initial probing caused a black background on the X-ray film which led to difficulties in scoring results. This problem was assumed to be somehow related to the presence of the previously labelled probes on the membrane.

In the probe/enzyme screening experiment of this study (see 5.2.5) it was found that the storage of hybridised membrane in a refrigerator even for 10-15 days could not eliminate this black background on the X-ray film, whereas probe

stripping was shown to be a good solution to this problem. For stripping, the probed membrane was placed in a clean plastic container (DNA face up) and covered with 200 ml of heated (100 °C) stripping solution (0.1% SDS + 0.1% SSC). The container was then placed on a rocking platform for about 20 mins until the solution cooled. The membrane was then pre-rinsed with 2 x SSC for 1 min, washed for 5 mins (x2) with fresh 2 x SSC (with agitation) and then air dried.

5.2.6 Evaluation of the results and statistical analysis

Using scoring values based on the presence (1) or absence (0) of the RFLPs, Canonical Discriminant Analysis (CDA, SAS 1989) was used in this study to discriminate the seed lots and also to assess variation within each seed lot. The details of the statistical analysis procedure for discimination of the cultivars are presented in section 3.2.7. Plate 5.2.3 demonstrates the 17 RFLP fragments which were scored from membranes probed with clover ADH and Plate 5.2.4 illustrates the nine RFLP bands for the membranes which were probed with the apple rDNA probe.

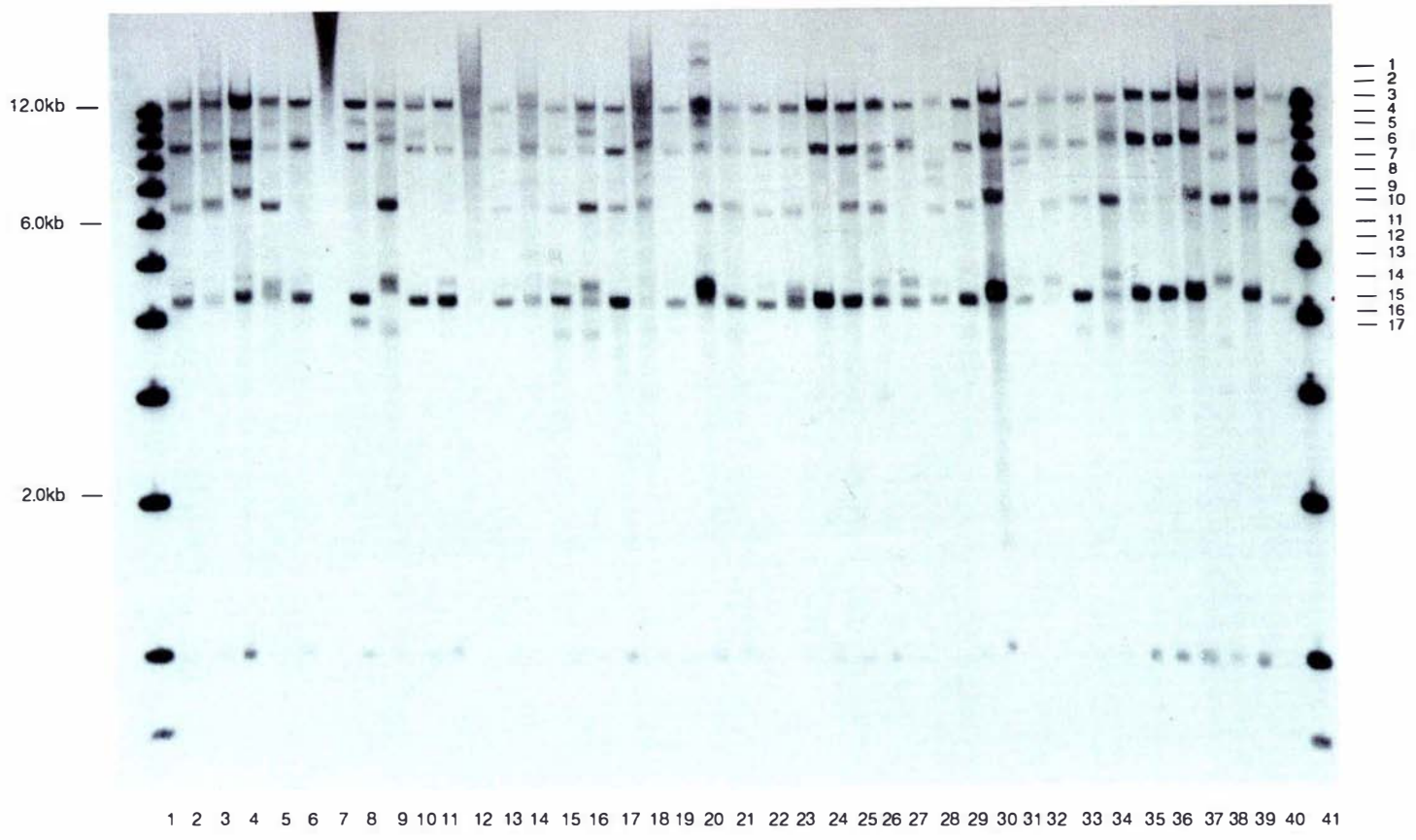

Plate 5.2.3 **Illustration of RFLP bands resulting from signal detection of membranes probed with the clover ADH probe (exposure time 4 h). The marked DNA fragments are RFLPs which were scored for genetic analysis of the cultivars.**

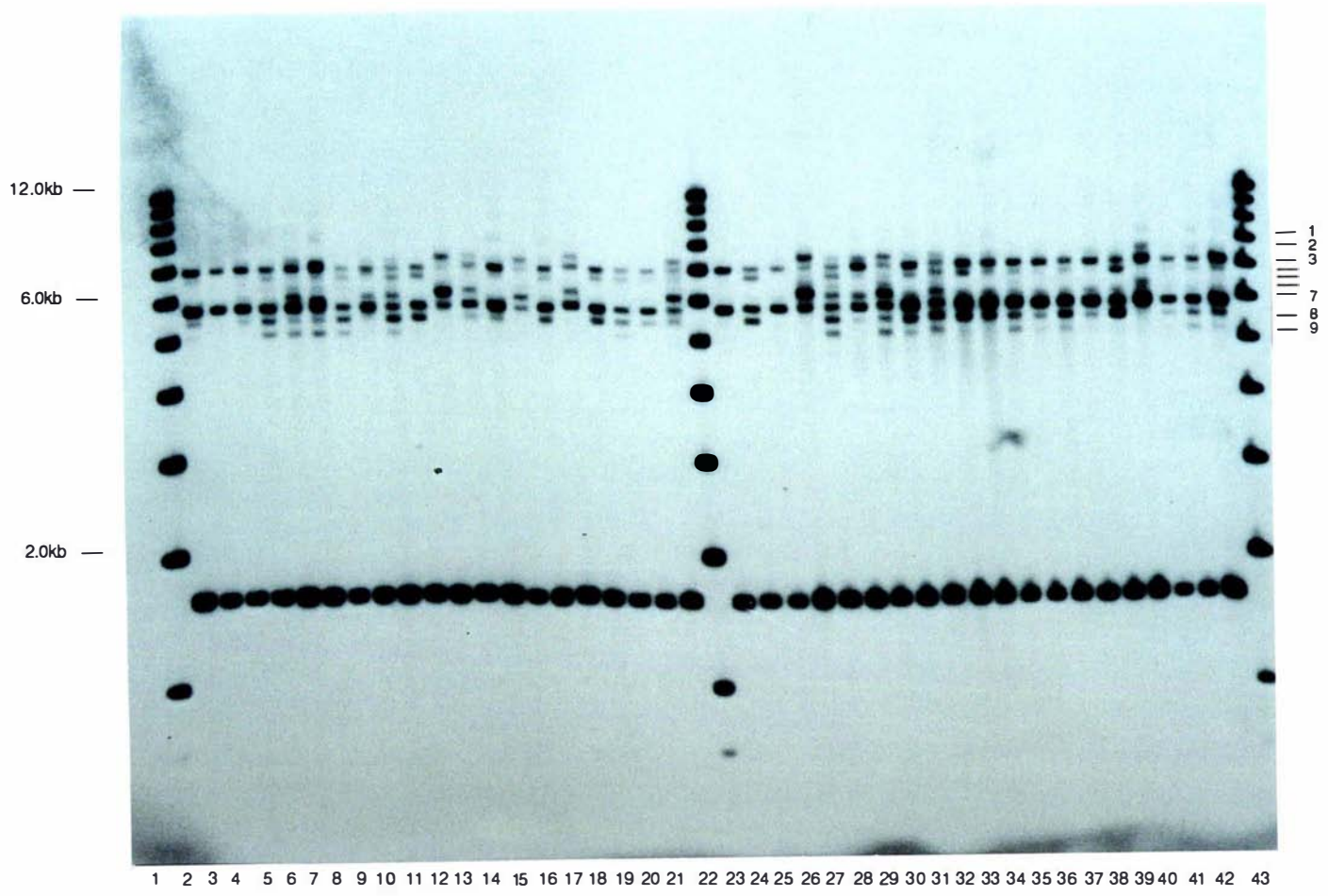

Plate 5.2.4 **Illustration of RFLP fragments resulting from signal detection of membranes probed with the apple ribosomal DNA probe (exposure time 30 mins.). The marked DNA fragments are RFLPs which were scored for genetic analysis of the cultivars.**

5.3 RESULTS

5.3.1 Optimising the loading of the digested DNA onto the gel to produce clear, easily evaluated restriction fragment length polymorphism (RFLP) banding profiles

Loading 5μg of digested lucerne DNA onto the gel produced the clearest RFLP bands in comparison with the other amounts tested in this study (i.e 1μg which is routinely used for RFLP analysis in apple (*Malus*, Gardiner, 1994, pers. comm.) and 2.5μg, plate 5.3.1).

5.3.2 Screening of endonuclease restriction enzymes and DNA probes for determining the best combinations for production of clear and easily evaluated RFLP bands

From the screening of four common restriction endonuclease enzymes (EcoR *I*, Bam H*I*, Hind *III* and Xba *I*) with nine DNA probes (see section 5.3.5), the following results were obtained:

5.3.2.1 Lucerne probes

Of the six lucerne probes screened in this study, I013 gave clear RFLP fragments. Plate 5.3.2 is a sample result from Xba *I*-digested DNA samples of 40 single seedlings of cv. Wairau hybridised with this probe. In combination with I013 endonuclease restriction enzyme Hind *III* gave the highest number of distinct RFLP bands, and Eco R*I* gave more RFLP bands than Xba *I* (Plate 5.3.3) whereas Bam H*I* produced large, and badly resolved DNA fragments (Plate not shown) which were not polymorphic. However, the results from I013 were not reproducible. Therefore, this probe was not used further in the study.

5.3.2.2 Clover probe

For the enzyme digests screened with the clover ADH probe, restriction endonuclease Xba *I* produced the highest number of clear and easily evaluated RFLP bands (Plate 5.3.4). This combination was chosen for use and gave consistently clear and easily evaluated results throughout this study. Plate 5.3.5 is a sample result from Xba *I*-digested DNA samples of 40 single seedlings of cv. Nikshahri hybridised with the ADH probe. The combination of Bam H *I* with the ADH probe gave the fewest polymorphic bands (results not shown).

5.3.2.3 Apple probes

In combinations between the restriction endonucleases and the apple ribosomal DNA probe (rDNA), BamH *I* produced the most polymorphic and easily evaluated fragments. The other enzyme digests gave very few RFLP bands and Hind *III* produced very large DNA fragments (Plate 5.3.6). The combination between BamH- *I* digestion and the rDNA probe produced consistently clear results and was therefore chosen for use in this study. Plate 5.3.7 is a sample result from Bam H*I*-digested DNA of 40 single seedlings of cv. Moapa, hybridised with apple rDNA. An apple ADH probe produced very faint RFLP bands (result not shown).

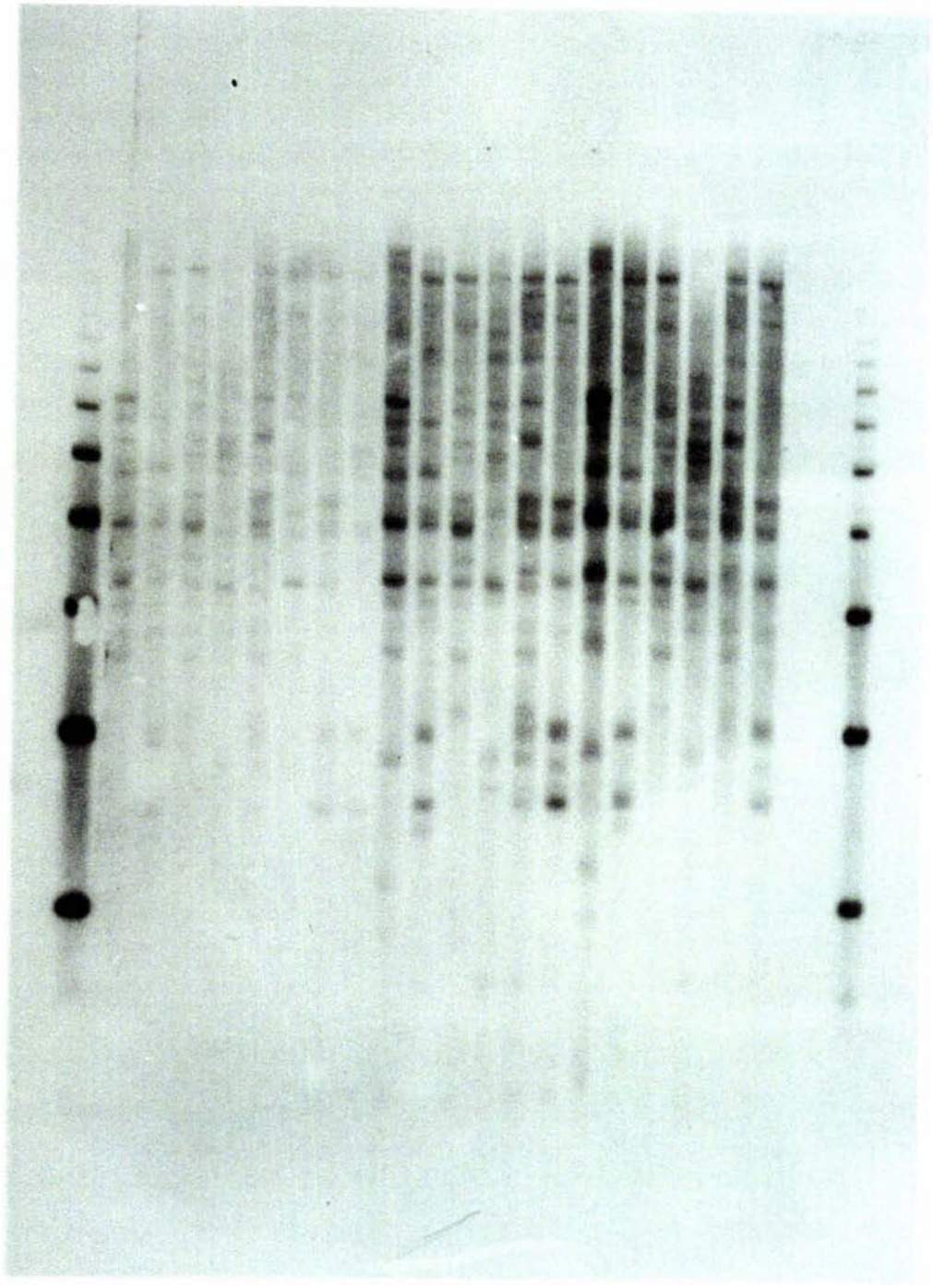

Plate 5.3.1 **Lumigram produced by hybridisation of a Southern blot with 1.0, 2.5 and 5µg (left to right) of the Xba *I*-digested DNA samples of single seedlings of cultivar G.Oranga with I013 lucerne probe (exposure time was 4 h).**

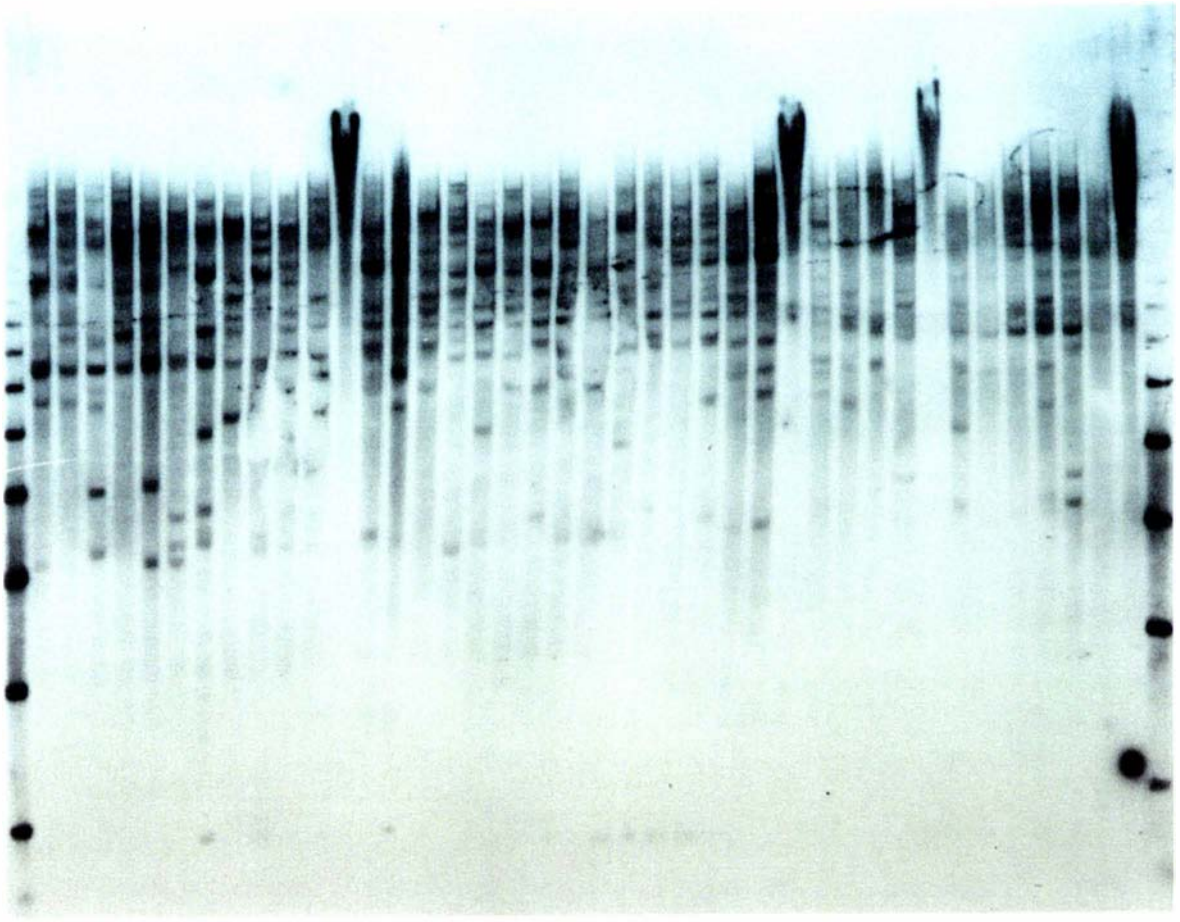

Plate 5.3.2 **Lumigram derived from hybridisation of a Southern blot with Xba *I*-digested DNA samples from 40 single seedlings of cv Wairau with the I013 lucerne probe (exposure time was 4 h)**

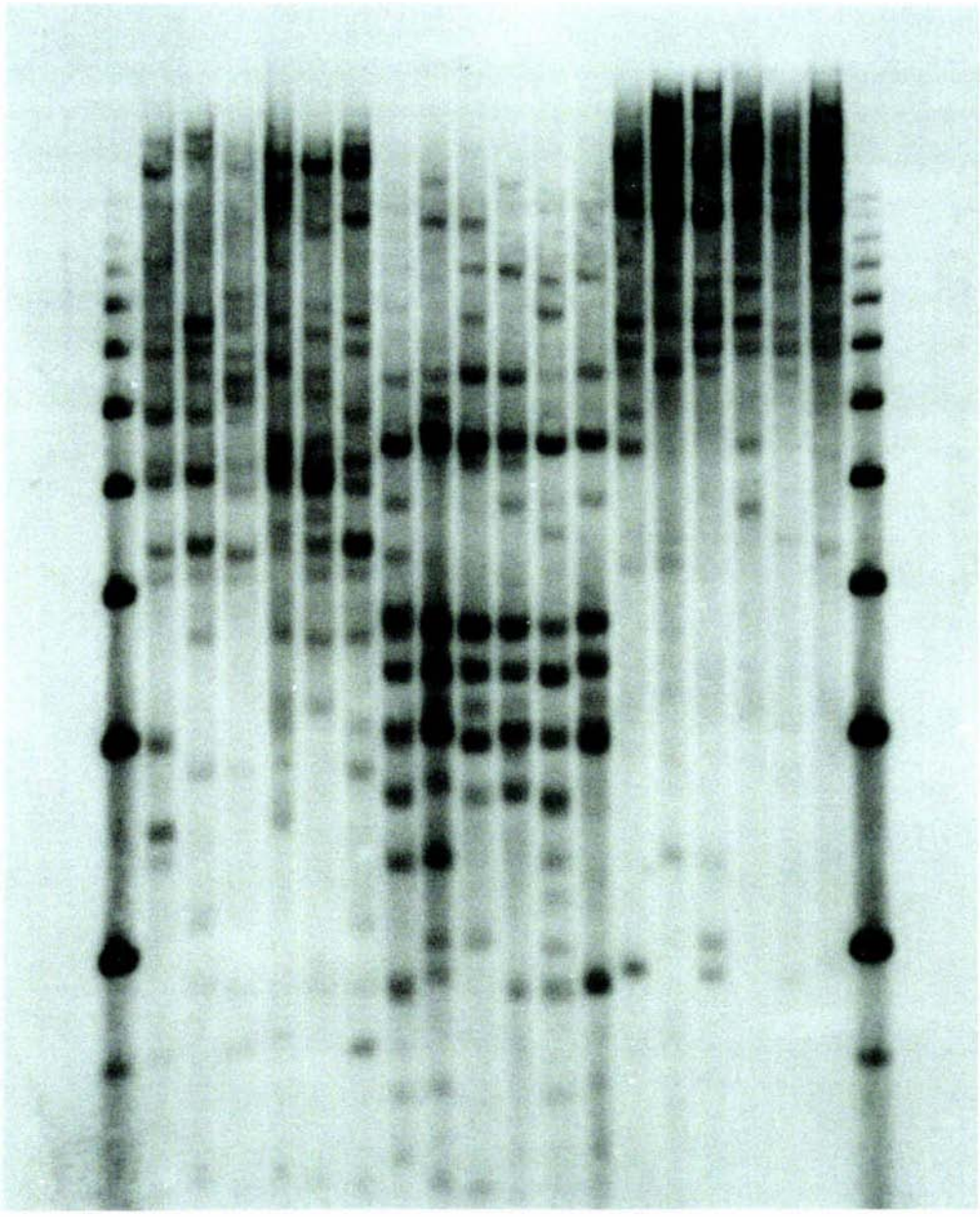

Plate 5.3.3 **Lumigram derived from probing a Southern blot of Eco R*I* (six lanes at the left), Hind *III* (six lanes in the middle) and Xba *I* (adjacent six lanes at the right of the plate) digested DNA samples of single seedlings of cv. G.Oranga with I013, a lucerne DNA probe (exposure time was 4 h).**

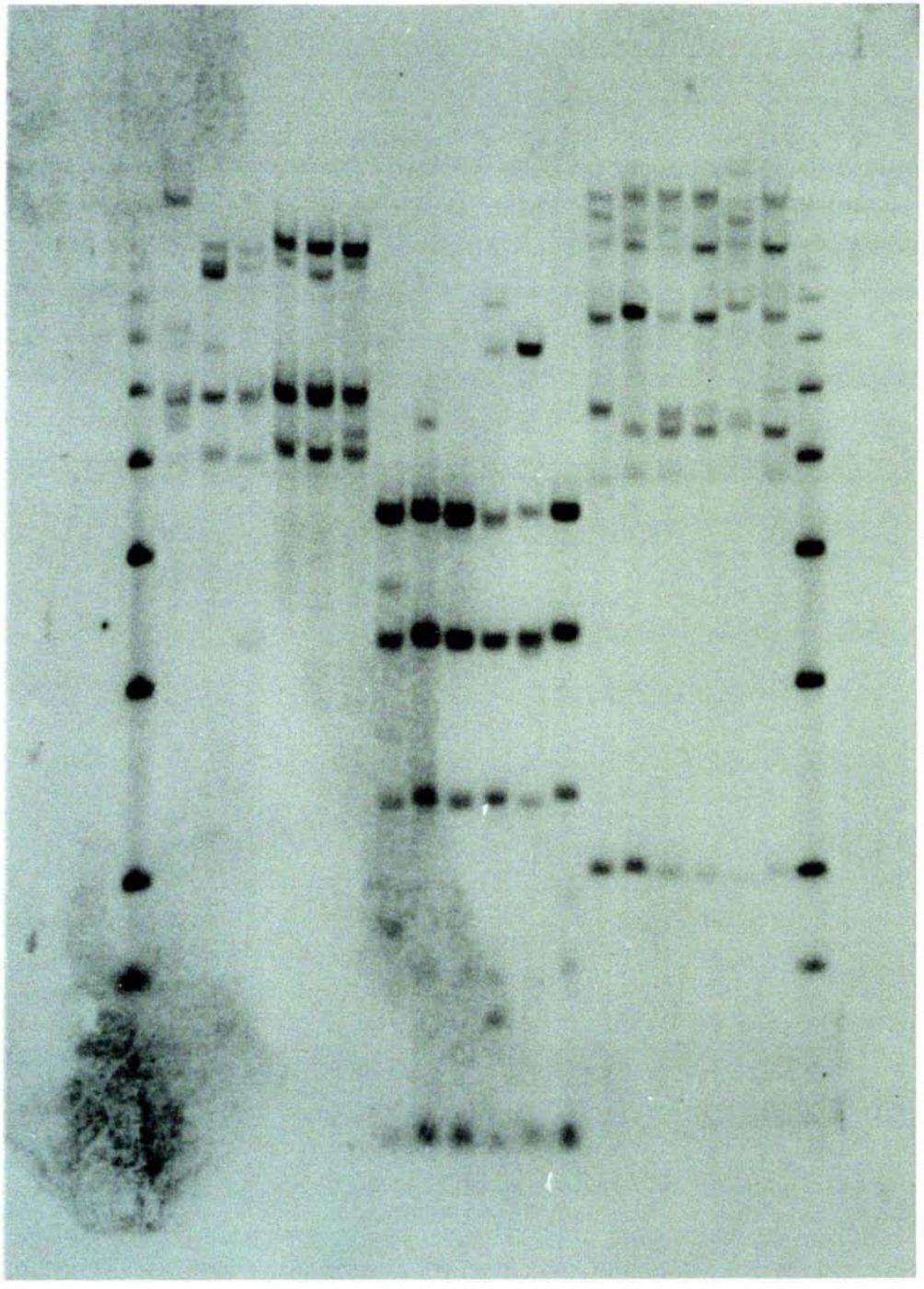

Plate 5.3.4 **Lumigram derived from hybridisation of a Southern blot of 5µg digested DNA samples of six single seedlings of cv. G.Oranga, restricted with the clover ADH probe. Enzymes in blocks of six lanes left to right are Eco R*I*, Hind *III* and Xba *I* (exposure time was 4 h).**

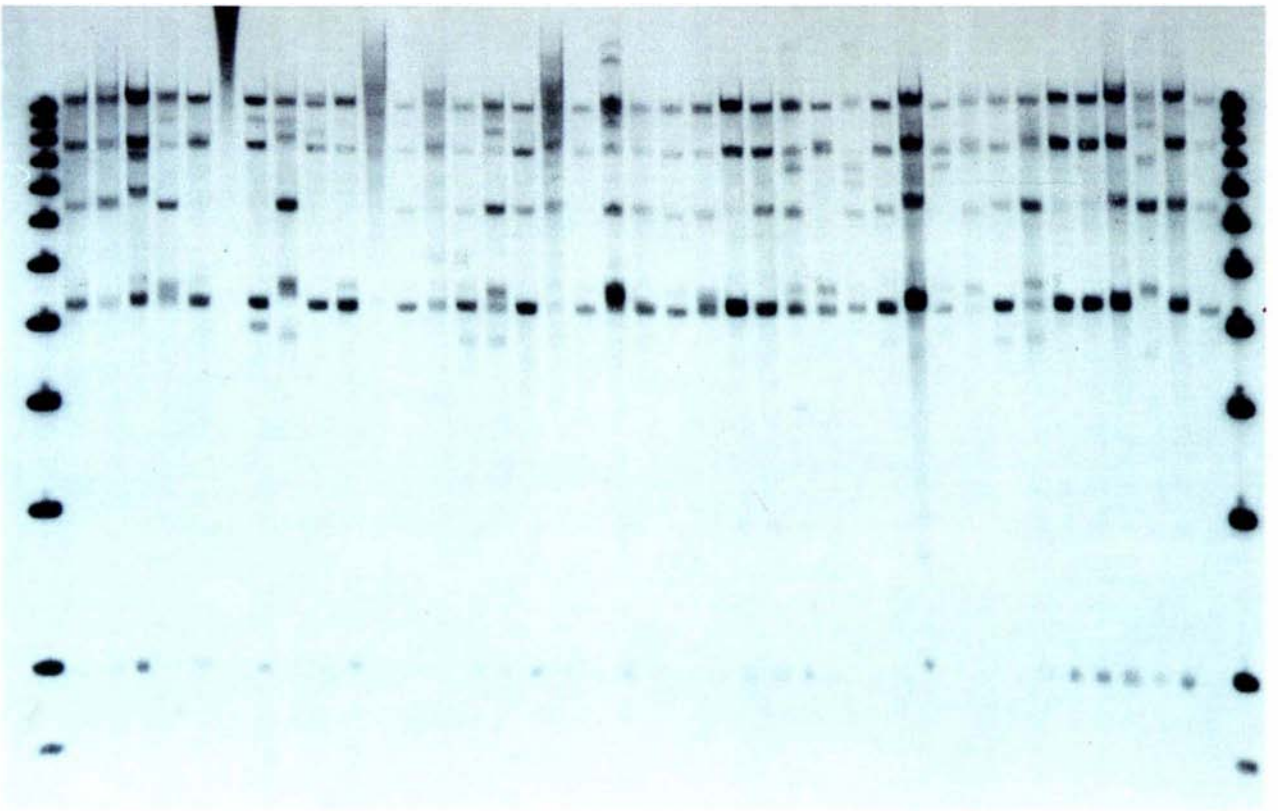

Plate 5.3.5 **Lumigram derived from hybridisation of a Southern blot of Xba *I*-digested DNA samples of 40 single seedlings of cv. Nikshahri with the clover ADH probe (exposure time was 4 h).**

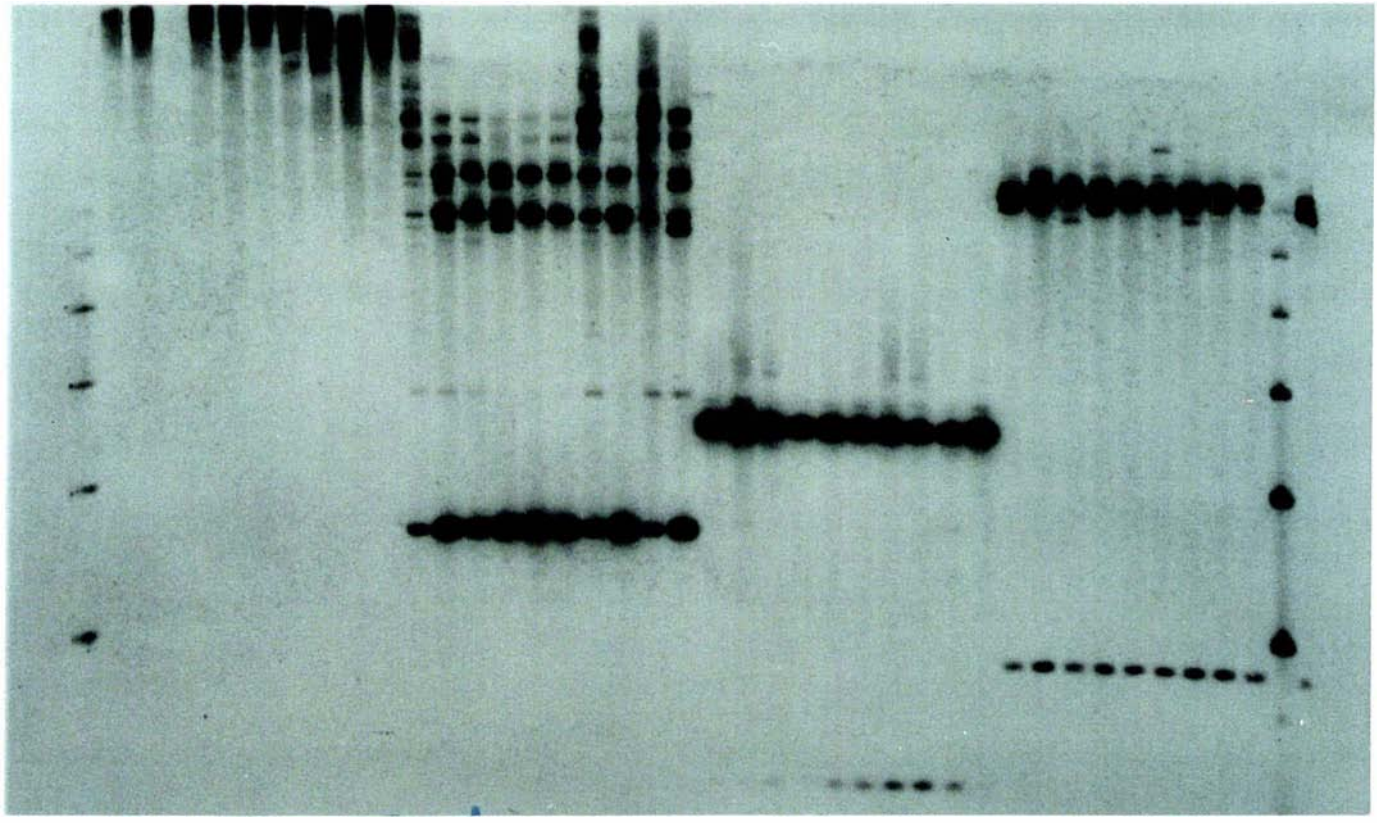

Plate 5.3.6 **Lumigram derived from hybridisation of a Southern blot of Hind *III*, Bam H*I*, Eco R*I* and Xba *I*-digested DNA samples of the cv. G.Oranga (in blocks of six lanes left to right) with the apple ribosomal DNA (rDNA) probe (exposure time was 1 h).**

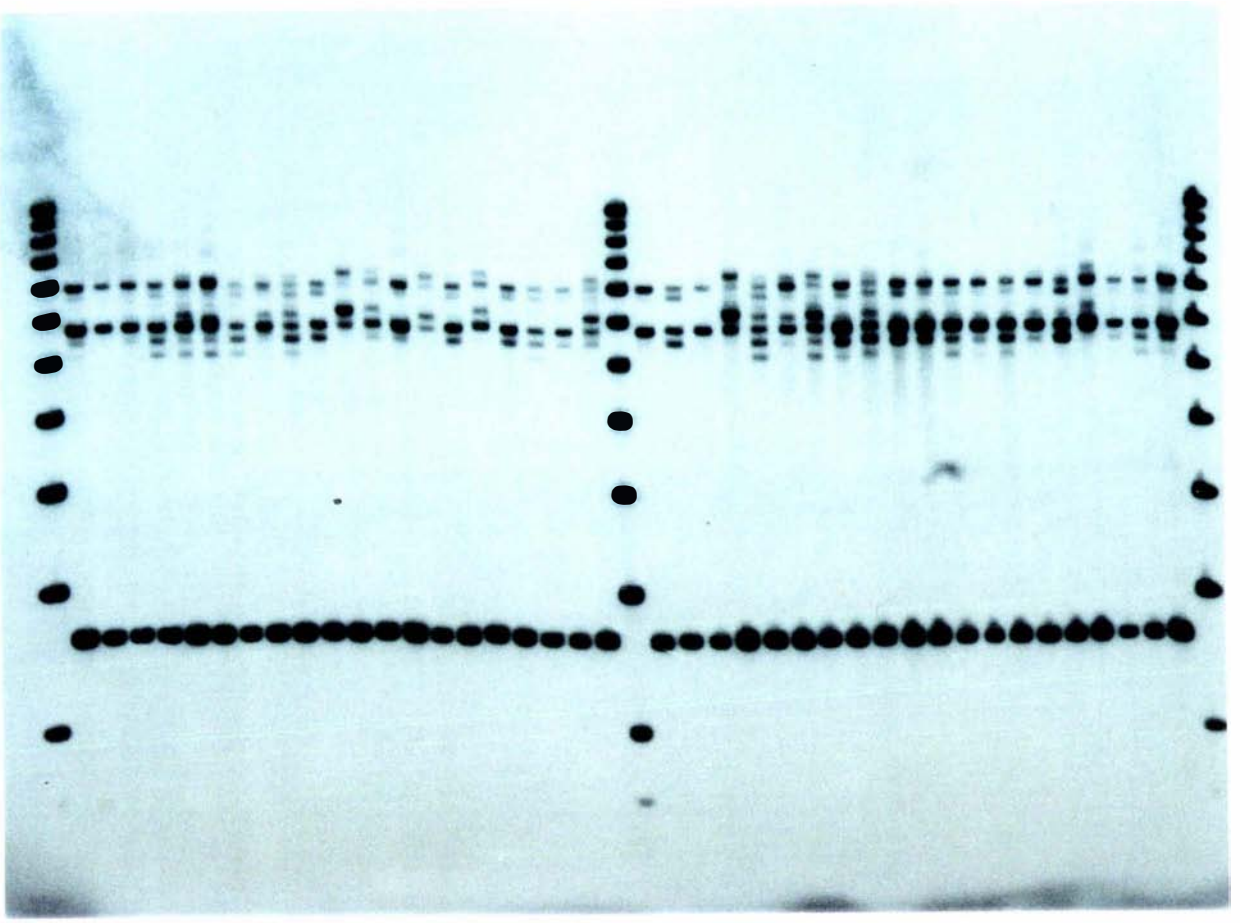

Plate 5.3.7 **Lumigram derived from hybridisation of a Southern blot with BamH *I*-digested DNA samples from 40 single seedlings from cv. Moapa with the apple ribosomal DNA probe (exposure time was 1 h)**

5.3.3 Use of apple rDNA probe for discrimination among lucerne cultivars based on the restriction fragments from probing of Southern blot of BamH *I*-digested bulked DNA samples from the cultivars.

Some differences were found between RFLP profiles from bulked DNA samples of seed lots from the 10 cultivars tested (Plate 5.3.8). For example an extra band was consistently found in the profile from cv. G.Oranga DNA (band No. 7, indicated by an arrow on the plate) which was not present in the RFLP profiles of the other cultivars. There was also one band (No. 1) in the profiles from Azari, Bami, Esfahani, G.Oranga and Saranac which was absent in the profiles of other cultivars. Band Number 4 (Plate 5.3.8) was present in the profile of cvs. Azari, Bami, Hamedani, Esfahani, G.Oranga and Saranac but was not present in the profiles from other cultivars.

Pairwise comparisons between RFLP bands from bulked samples of the cultivars, detected with the apple rDNA probe (Table 5.3.1), summarise the differences in the restriction fragment length polymorphism profiles from some cultivars. Cultivars Hamedani and G.Oranga gave RFLP profiles which were different from each other, as well as those from the other cultivars. The profiles from the two internationally recognized cultivars, Saranac and Moapa, were different (in two polymorphic bands No. 1 and 4). A difference was also seen between the RFLP profiles from G.Oranga and Wairau, the two New Zealand cultivars (in polymorphic bands No. 1, 4 and 7). Differences were also found between RFLP profiles of some of the Iranian cultivars.

5.3.4 Use of apple rDNA probe for discrimination among lucerne cultivars on the basis of the frequencies of RFLP bands detected by the apple rDNA probe on Southern blots of the Bam *HI*-digested DNA from 40 single seedlings of each of the cultivars

5.3.4.1 Cross validation for each of the seed lots of the cultivars

From the restriction fragment analysis of the genomic DNA of 40 single seedlings from each of the seed lots of the cultivars, the cross validation, defined by the probability of seedlings within the seed lot being classified as from each individual cultivar, is presented in Table 5.3.2. For instance for cultivar Moapa, 35% of the seedlings had a restriction fragment profile which led to them being classified by the discriminate analysis as belonging to this cultivar, whereas the remaining 65% of the seedlings had a RFLP profile which corresponded to the profiles found in the other cultivars. Wairau had the most uniform restriction fragment profile (62.5% of the seedlings being correctly classified into this cultivar), whereas Saranac had the least uniform RFLP profile (2.7% of the seedlings being correctly classified into this cultivar). With 37.8% of the seedlings being correctly classified into the cultivar, the remaining 62.2% of the seedlings of cv. Hamedani had restriction fragment profiles which corresponded to the profiles found in the other cultivars.

A test of difference between two proportions (see 3.2.7) was performed on data from this experiment to check whether the proportions of seedlings which were correctly classified into the cultivars were significantly different. The results of this test indicated that there was a significant difference between the restriction fragment profiles of some of the cultivars (Table 5.3.3). For example, highly significant differences were found between the proportions of the seedlings which were correctly classified into cv. Saranac and Moapa and also between G.Oranga and Wairau. However, no significant differences were found between the

proportion of the seedlings of cv. Bami and other Iranian cultivars. The proportion of cv. Azari was significantly different from those for Yazdi, Nikshahri and Esfahani. There were also significant differences between the proportion of the seedlings correctly classified into cvs Hamedani and Nikshahri, Esfahani and Yazdi.

5.3.4.2 Pairwise comparison between Mahalanobis squared distances of RFLP profiles of the cultivars resulting from probing Southern blots with the BamH *I*-digested DNA extracted from 40 single seedlings of each of the cultivars with apple rDNA

Pairwise comparisons between the Mahalanobis squared distances (D^2) of the cultivars (Table 5.3.4) indicate that with a squared distance of 0.1, cv. Nikshahri and Wairau had restriction fragment profiles which were the closest among the cultivars tested in this study, while Hamedani and Moapa had restriction fragment profiles which were the most distinct among the cultivars tested in this study (D^2 = 4.4). Among the Iranian cultivars, Nikshahri and Bami had RFLP profiles which were the closest (D^2 = 0.2) whereas Hamedani and Yazdi had RFLP profiles which were the most distinct (D^2 = 3.6) of the cultivars. The D^2 between the restriction fragment profiles of the two New Zealand cultivars (G.Oranga and Wairau), and that for cv. Saranac and Moapa were 1.9 and 2.0 respectively. This suggests that the diversity among RFLP profiles of some of the Iranian cultivars was much higher than that for two internationally recognized cultivars and also the two New Zealand cultivars.

Table 5.3.1 **Pairwise comparison between DNA fragments hybridising to the apple rDNA probe (bands No. 1-7 shown in Plate 5.3.8) using bulked samples of DNA from 40 seedlings of each of the cultivars. The figures in this table indicate the RFLP fragments (Plate 5.3.8) by which a pair of cultivars can be distinguished.**

	Azari	Barni	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		-	1	-	1,4	1,4	7	-	1,4	1,4
Barni	-		1	-	1,4	1,4	7	-	1,4	1,4
Hamedani	1	1		1	4	4	1,7	1	4	4
Esfahani	-	-	1		1,4	1,4	7	-	1,4	1,4
Moapa	1,4	1,4	4	1,4		-	1,4,7	1,4	-	-
Nikshahri	1,4	1,4	4	1,4	-		1,4,7	1,4	-	-
G.Oranga	7	7	1,7	7	1,4,7	1,4,7		7	1,4,7	1,4,7
Saranac	-	-	1	-	1,4	1,4	7		1,4	1,4
Wairau	1,4	1,4	4	1,4	-	-	1,4,7	1,4		-
Yazdi	1,4	1,4	4	1,4	-	-	1,4,7	1,4	-	

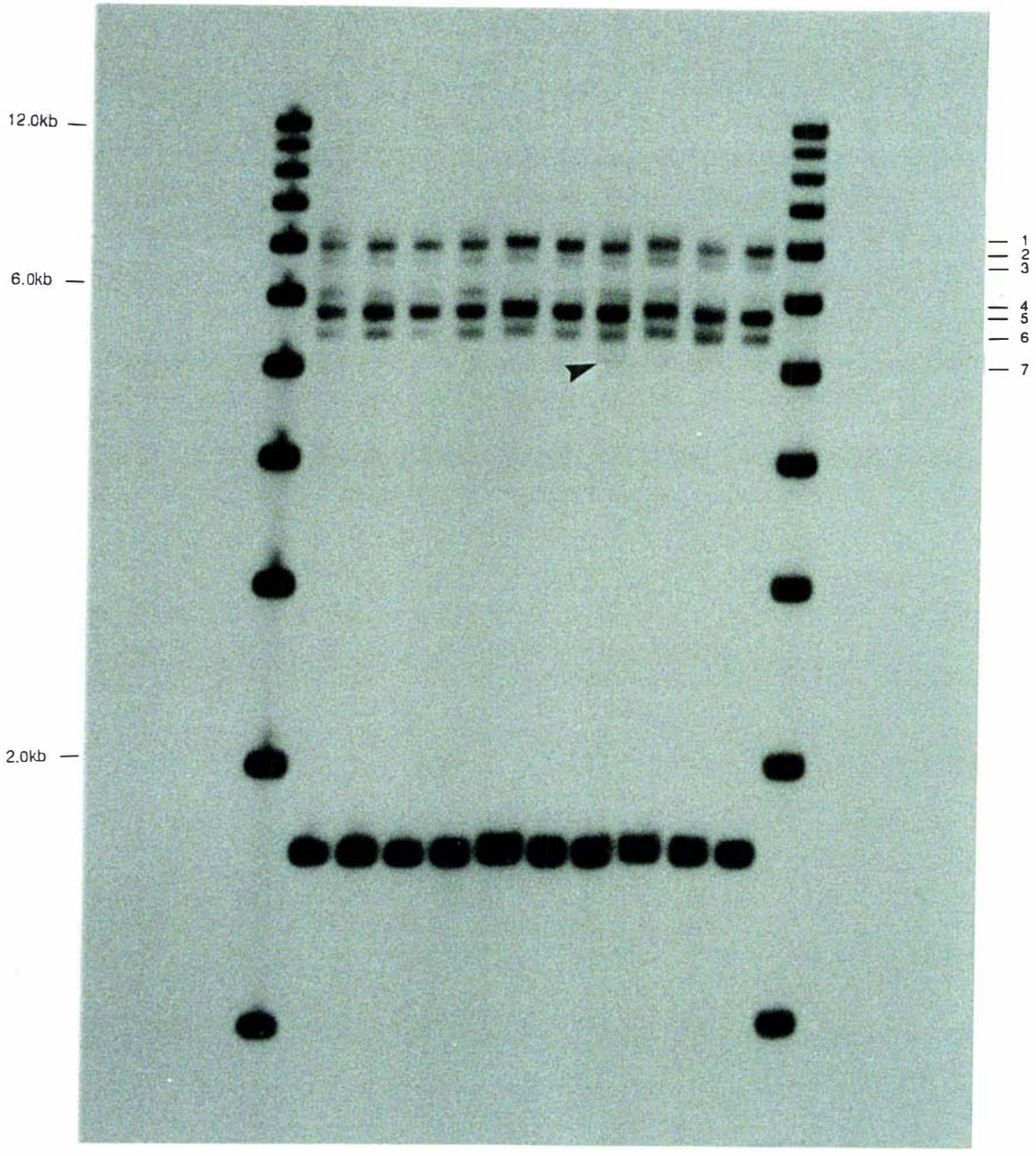

Plate 5.3.8 **DNA fragments hybridising to the apple rDNA probe (exposure of Lumigram time was 1 h). Bam H*I* was used to digest bulked DNA from the 10 cultivars (40 seedling of each): Azari, Bami, Hamedani, Esfahani, Moapa, Nikshahri, G.Oranga, Saranac, Wairau and Yazdi (left to right). Scored bands were visible on the X-ray film but could not be clearly reproduced as a photograph.**

Table 5.3.2 Cross validation of seedlings by cultivar based on frequencies of the RFLP bands resulting from probing Southern blots of the Bam HI-digested DNA extracted from 40 single seedlings of each of the cultivars with apple rDNA

discriminant classifications True cultivar[1]	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari	**35.1**	21.9	4.9	4.9	4.9	7.3	0.0	2.4	17.0	2.4
Bami	7.5	**22.5**	0.0	2.5	12.5	10.0	10.0	0.0	32.5	2.5
Hamedani	16.2	10.8	**37.8**	2.7	2.7	2.7	10.8	5.4	8.1	2.7
Esfahani	15.4	20.5	2.7	**10.2**	5.1	2.5	7.7	5.1	25.6	0.0
Moapa	0.0	10.0	0.0	0.0	**35.0**	2.5	12.5	0.0	35.0	5.0
Nikshahri	10.3	10.3	0.0	2.5	12.8	**10.0**	10.0	0.0	43.6	7.7
G.Oranga	7.5	7.5	17.5	2.5	12.5	10.0	**22.5**	0.0	20.0	0.0
Saranac	10.8	10.8	8.1	8.1	2.7	0.0	18.9	**2.7**	37.8	0.0
Wairau	12.5	7.5	0.0	0.0	5.0	10.0	0.0	0.0	**62.5**	2.5
Yazdi	17.5	5.0	0.0	0.0	22.5	10.0	0.0	0.0	37.5	**7.5**

[1]This definition is correct for cvs. G.Oranga and Wairau (standard seed lot for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'.

Table 5.3.3 Tests of significance between the proportions of the seedlings from different cultivars which were correctly classified by the discriminant analysis as belonging to each of the cultivars. The significant (P<0.05) results are indicated by stars ,*, and not significant by 'ns'

From To	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		ns	ns	*	ns	*	ns	*	*	*
Bami	ns		ns	ns	ns	ns	ns	*	*	ns
Hamedani	ns	ns		*	ns	*	ns	*	*	*
Esfahani	*	ns	*		*	ns	ns	ns	*	ns
Moapa	ns	ns	ns	*		*	ns	*	*	*
Nikshahri	*	ns	*	ns	*		ns	ns	*	ns
G.Oranga	ns	ns	ns	ns	ns	ns		*	*	ns
Saranac	*	*	*	ns	*	ns	*		*	ns
Wairau	*	*	*	*	*	*	*	*		*
Yazdi	*	ns	*	ns	*	ns	ns	ns	*	

Table 5.3.4 **Pairwise comparison between squared distances of the RFLP profiles of the cultivars based on frequencies of the restriction fragments resulting from probing the Bam *HI*-digested DNA extracted from 40 single seedlings of each of the cultivars with apple rDNA**

From To	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari	0.0	1.2	2.5	1.1	3.2	1.2	2.1	1.3	1.7	1.4
Bami	1.2	0.0	2.8	1.1	1.1	0.2	1.0	0.5	0.3	0.5
Hamedani	2.5	2.8	0.0	1.6	4.4	3.3	1.4	1.4	3.8	3.6
Esfahani	1.1	1.1	1.6	0.0	3.1	1.2	1.9	0.6	1.4	2.0
Moapa	3.2	1.1	4.4	3.1	0.0	1.2	1.8	2.0	1.2	0.9
Nikshahri	1.2	0.3	3.3	1.2	1.2	0.0	1.8	0.8	0.1	0.3
G.Oranga	2.1	1.0	1.4	1.9	1.8	1.8	0.0	0.6	1.9	1.7
Saranac	1.3	0.5	1.4	0.6	2.0	0.8	0.6	0.0	0.8	1.3
Wairau	1.7	0.3	3.8	1.4	1.2	0.1	1.9	0.8	0.0	0.5
Yazdi	1.4	0.5	3.6	2.0	0.9	0.3	1.7	1.3	0.5	0.0

5.3.4.3 Pairwise comparisons of least squared means (LSMEANS) of RFLPs of the cultivar, resulting from hybridisation of Southern blots of Bam *HI*-digested DNA from 40 single seedlings of each of the cultivars with an apple rDNA probe

There were highly significant differences between LSMEANS of the RFLP profiles for some of the cultivars (Table 5.3.5). For instance the RFLP profile of cv. Hamedani differed significantly from those for all the other cultivars. There was a very highly significant difference between the RFLP profiles of the two internationally recognised cultivars (Moapa and Saranac), and that between the RFLP profiles of Wairau and G.Oranga, the two New Zealand cultivars. The RFLP profile of cv. Azari differed significantly from those for the other Iranian cultivars, except for cv. Esfahani. However, no significant difference was found between LSMEANS of the restriction fragment profiles of cv. Bami, Nikshahri and Yazdi.

5.3.4.4 General linear models (GLM) procedure for dependent variable: CDF1

There were highly significant differences ($P<0.0001$) between the profiles of restriction fragments revealed by the rDNA probe for the cultivars. This was shown using the GLM or ANOVA (Analysis Of Variance) procedure for the variable CDF1 (the first canonical discriminant function or combination of the variables R1-R9 that best discriminates among the cultivars, see section 3.2.7). The ANOVA table is as follows:

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Cultivar	9	138.07	15.34	15.34	0.0001
Error	383	383.02	1.0		
Corrected-Total	392	521.09			

5.3.4.5 Duncan's Multiple range test for discrimination among seed lots of the cultivars based on the frequencies of the restriction fragments resulting from hybridisation of Southern blots of Bam H*I*-digested DNA extracted from 40 single seedlings of each of the cultivars with apple rDNA

Since there were significant differences between RFLP profiles of the cultivars (the GLM result), the Duncan's Multiple range test was employed for variable CDF1 to further assess the significance of these differences.

There were significant differences ($P<0.05$) between restriction fragment profiles of some of the cultivars. As a result, cultivars could be classified into four groups (Table 5.3.6). The two internationally recognized cultivars (Moapa and Saranac) are classified into different groups. The New Zealand cultivars, Wairau and G.Oranga, also fall into different groups. In addition the six Iranian cultivars are separated among three groups. This suggests that there was genetic divergence among some of the seed lots of the cultivars tested in this study.

Table 5.3.5 Probabilities that pairs of LSMEANS are equal. The significance probability for LSMEANS was set at 0.05 for this test

From cultivar	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		0.0224	0.0001	0.2637	0.0001	0.0026	0.6004	0.8155	0.0004	0.0002
Bami	*		0.0001	0.0008	0.0257	0.4548	0.0054	0.0141	0.2003	0.1595
Hamedani	*	*		0.0010	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Esfahani	ns	*	*		0.0001	0.0001	0.5523	0.3902	0.0001	0.0001
Moapa	*	*	*	*		0.1404	0.0001	0.0001	0.3392	0.4069
Nikshahri	*	ns	*	*	ns		0.0005	0.0016	0.5988	0.5145
G.Oranga	ns	*	*	ns	*	*		0.7806	0.0001	0.0001
Saranac	ns	*	*	ns	*	*	ns		0.0002	0.0001
Wairau	*	ns	*	*	ns	ns	*	*		0.8993
Yazdi	*	ns	*	*	ns	ns	*	*	ns	

The significant results are indicated by stars (*) and not significant by 'ns', and the corresponding probability can be found in the upper right hand entries .

Table 5.3.6 **Duncan's Multiple range test for discrimination among the cultivars for the rDNA probe. The calculated means in this table are the mean value for CDF1, the combination of R1-R9 variables that best discriminate among the cultivars (see section 3.2.7). Cultivars linked by the same line do not differ significantly for the character at P<0.01.**

	Name of cultivar	Mean	Duncan's grouping		
1	Hamedani	1.26			
2	Esfahani	0.50			
3	G.Oranga	0.37			
4	Saranac	0.30			
5	Azari	0.25			
6	Bami	-0.26			
7	Nikshahri	-0.43			
8	Wairau	-0.55			
9	Yazdi	-0.57			
10	Moapa	-0.76			

5.3.5 Use of clover ADH probe for discrimination of lucerne cultivars based on the RFLP fragments resulting from hybridisation of Southern blot of Xba*I*-digested bulked DNA samples of the cultivars

Hybridisation of the Xba *I*-digested DNA samples of the cultivars with the clover ADH probe gave some polymorphic restriction fragments in the banding profile of some cultivars (Figure not presented). An extra restriction fragment was present in the profiles of cv. Azari, Esfahani and Saranac which was not present in the profiles of other cultivars. However, because difficulty was experienced in obtaining clear intense bands, ADH probing of the bulked DNA samples was not judged to be reliable enough to be used as the basis for discrimination among the cultivars.

5.3.6 Use of clover ADH probe for discrimination among the cultivars based on frequencies of the restriction fragments produced by hybridisation of Southern blots of Xba *I*-digested DNA samples from 40 single seedlings from each of the cultivars

5.3.6.1 Cross validation for each of the seed lots of the cultivars

From the restriction fragment analysis of DNA of 40 single seedlings from each of the cultivars, the cross validation, defined by the probability of the seedlings within the seed lot being classified as from each individual cultivar, is presented in Table 5.3.7. For example for cultivar Wairau, 33.3% of the seedlings gave restriction fragment profiles which led to them being classified by the discriminate analysis as belonging to this cultivar, whereas the remaining 66.7% of the seedlings produced RFLP bands which corresponded to profiles found in other cultivars.

Among the cultivars tested in this study, cv. G.Oranga and Nikshahri were the

most homogeneous (56.4% of the seedlings from G.Oranga and 53% of the seedlings from cv. Nikshahri being correctly classified into the cultivar) while Esfahani was the least heterogenous cultivar (21.9% of the seedlings being correctly classified into this cultivar) in respect of their restriction fragment profile.

Tests of difference between two proportions (see 3.2.7) illustrated that there were significant differences ($P<0.05$ and $P<0.01$) between uniformity of the restriction fragment profiles of some of the cultivars which were demonstrated by the ADH probing (Table 5.3.8). Unlike significant differences between uniformity of 23 pairs of cultivars detected by the rDNA (see section 5.3.4.1, Tables 5.3.2 and 5.3.3), only six pairs of the cultivars had different uniformity using the ADH probe.

The uniformity of the profile for cv. G.Oranga was significantly different from that for cv. Wairau, Azari, Hamedani and Esfahani. The uniformity of the restriction fragment profile for cv. Nishahri differed significantly from Hamedani and Esfahani (Table 5.3.8).

5.3.6.2 Pairwise comparison between squared distances (Mahalanobis distances) of restriction fragment profiles of the cultivars resulting from probing the Southern blot with the Xba *I*-digested DNA extracted from 40 single seedlings of each of the cultivars with the ADH probe

Pairwise comparisons between the squared distances of the cultivars (Table 5.3.9) indicate that with a D^2 of 1.5, cv. Hamedani and Yazdi had restriction fragment profiles which were the closest, while with a D^2 of 4.2,cv. Nikshahri and Wairau had profiles which were the most distinct among the cultivars tested in this study. The D^2 between restriction fragment profiles for cv. Hamedani and Bami (3.4),and that between the New Zealand cultivars (Wairau and G.Oranga,2.4) show more genetic diversity between some of the Iranian than in the New Zealand cultivars.

5.3.6.3 Pairwise comparisons of least squared means (LSMEANS) of the RFLP profiles of the cultivars based on the frequencies of the restriction fragments resulting from hybridisation of Southern blot with Xba *I*-digested DNA extracted from 40 single seedlings of each of the cultivars with the ADH probe

There were significant differences between least squared means (LSMEANS) of the restriction fragment profiles of some cultivars (Table 5.3.10). The restriction fragment profile of cv. G.Oranga did not differ from that of cv. Wairau, although they were both significantly different from the Iranian cultivars. No significant differences were found between the profiles of cv. Azari, Bami, Esfahani and Yazdi. However, the RFLP profiles for cv. Azari, Hamedani and Esfahani were significantly different from cv. Nikshahri.

Table 5.3.7 Cross validation of seedlings by cultivar based on frequencies of the restriction fragments resulting from hybridisation of Southern blots, with Xba *I*-digested DNA extracted from 40 single seedlings of each of the cultivars with the clover ADH probe

discriminant classifications True cultivar[1]	Azari	Bami	Hamedani	Esfahani	Nikshahri	G.Oranga	Wairau	Yazdi
Azari	**30.0**	13.3	6.7	3.3	16.7	0.0	16.6	13.3
Bami	5.7	**37.1**	2.8	2.8	14.3	8.6	5.7	22.8
Hamedani	6.1	0.0	**27.3**	3.0	12.1	9.1	12.1	30.3
Esfahani	3.1	6.2	12.5	**21.9**	18.7	21.9	6.2	9.4
Nikshahri	0.0	11.8	0.0	5.9	**53.0**	5.9	5.9	17.6
G.Oranga	7.7	2.6	5.1	0.0	5.13	**56.4**	12.8	10.3
Wairau	2.8	13.9	2.8	5.6	5.6	22.2	**33.3**	13.9
Yazdi	8.3	8.3	11.1	2.8	16.7	2.8	13.9	**36.1**

[1]This definition is correct for cvs. G.Oranga and Wairau (standard seed lot for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'.

Table 5.3.8 **Tests of significance between the proportions of the seedlings from different cultivars which were correctly classified by the discriminant analysis as belonging to each of the cultivars. The significant (P<0.05) results are indicated by stars '*' and not significant by 'ns'**

From To	Azari	Bami	Hamedani	Esfahani	Nikshahri	G.Oranga	Wairau	Yazdi
Azari		ns	ns	ns	ns	*	ns	ns
Bami	ns		ns	ns	ns	ns	ns	ns
Hamedani	ns	ns		ns	*	*	ns	ns
Esfahani	ns	ns	ns		*	*	ns	ns
Nikshahri	ns	ns	*	*		ns	ns	ns
G.Oranga	*	ns	*	*	ns		*	ns
Wairau	ns	ns	ns	ns	ns	*		ns
Yazdi	ns	ns	ns	ns	ns	ns	ns	

Table 5.3.9 **Pairwise comparison between squared distances of RFLP profiles of the cultivars based on frequencies of the restriction fragments resulting from hybridisation of Southern blot with Xba *I*-digested DNA from 40 single seedlings of each of the cultivars with the ADH probe**

From To	Azari	Bami	Hamedani	Esfahani	Nikshahri	G.Oranga	Wairau	Yazdi
Azari	0.0	2.6	1.9	2.1	3.0	3.2	2.6	1.6
Bami	2.6	0.0	3.4	2.9	2.0	3.6	2.5	1.6
Hamedani	1.9	3.4	0.0	1.7	2.9	3.3	2.2	1.5
Esfahani	2.1	2.9	1.7	0.0	1.6	2.2	3.5	1.9
Nikshahri	3.0	2.0	2.9	1.6	0.0	3.4	4.2	1.6
G.Oranga	3.2	3.6	3.3	2.2	3.4	0.0	2.4	3.7
Wairau	2.6	2.5	2.2	3.5	4.2	2.4	0.0	3.7
Yazdi	1.6	1.6	1.5	1.9	1.6	3.7	2.6	0.0

Table 5.3.10 Probabilities that pairs of LSMEANS are equal. The significance probability for LSMEANS was set at 0.05 for this test

From cultivar	Azari	Bami	Hamedani	Esfahani	Nikshahri	G.Oranga	Wairau	Yazdi
Azari		0.1636	0.5858	0.2886	0.0010	0.0069	0.0005	0.0532
Bami	ns		0.0465	0.7520	0.0433	0.0001	0.0001	0.5772
Hamedani	ns	*		0.1014	0.0001	0.0047	0.0025	0.0109
Esfahani	ns	ns	ns		0.0223	0.0001	0.0001	0.3884
Nikshahri	*	*	*	*		0.0001	0.0001	0.1373
G.Oranga	*	*	*	*	*		0.7902	0.0001
Wairau	*	*	*	*	*	ns		0.0001
Yazdi	ns	ns	*	ns	ns	*	*	

The significant results are indicated by stars and not significant by 'ns', and the corresponding probability can be found in the upper right hand entries.

5.3.6.4 General linear models (GLM) procedure for dependent variable: CDF1

A very highly significant difference (P<0.0001) was found between the profile of restriction fragments revealed by the ADH probe for the cultivars. This was confirmed by applying the GLM or ANOVA procedure for the variable CDF1 (the first canonical discriminant function or combination of the variables R1-R17 that best discriminates among the cultivars, see section 3.2.7). The ANOVA table is as follows:

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	7	66.30	9.47	13.21	0.0001
Error		267	191.41	0.71	
Corrected-Total		274	257 .71		

5.3.6.5 Duncan's Multiple range test for discrimination among seed lots of the cultivars based on the frequencies of the restriction fragments resulting from probing Southern blot with Xba *I*-digested DNA extracted from 40 single seedlings of each of the cultivars with the ADH probe

Since there were highly significant differences between RFLP profiles for the cultivars (the GLM result), the Duncan's Multiple range test was used for variable CDF1 to further assess the significance of these differences between the frequency of the restriction fragments of the cultivars.

Significant differences were found between restriction fragment profiles of some cultivars (Table 5.3.11). As a result, the cultivars were categorised into four groups. Although RFLP profiles of the two New Zealand cultivars (G.Oranga and Wairau) did not differ from each other, they were significantly different (P<0.05) from the Iranian cultivars. Hamedani had a restriction fragment profile which was

significantly different from cvs Yazdi and Nikshahri. No significant difference, however, was found between the profiles of cultivars Azari and Esfahani. However, the restriction fragment profiles of these cultivars did differ from cv. Nikshahri.

Table 5.3.11 **Duncan's Multiple range test for discrimination among the seed lots of the cultivars for the ADH probe. The calculated means in the table are the mean value for CDF1, the combination of R1-R17 variables that best discriminates among the cultivars (see section 3.2.7). Cultivars linked by the same line do not differ significantly for the character at P<0.01.**

	Name of cultivar	Mean	Duncan's grouping			
1	Wairau	0.736				
2	G.Oranga	0.684				
3	Hamedani	0.113				
4	Azari	-0.004				
5	Esfahani	-0.232				
6	Bami	-0.298				
7	Yazdi	-0.410				
8	Nikshahri	-0.712				

5.4 DISCUSSION

5.4.1 Importance of probe selection in revealing polymorphisms among lucerne cultivars

5.4.1.1 Lucerne probes

Of the six lucerne probes screened against lucerne DNA digested with the four common restriction endonucleases (EcoR *I*, Bam H*I*, Hind *III* and Xba *I*) all except probe I013 produced extremely faint bands. This might have been due to the low copy number of the complementary DNA sequences in the plant genome. However, further work is required to determine this.

A combination of probe I013 and Hind *III* initially gave the highest number of RFLP fragments. This was in agreement with results reported by Brummer *et al.* (1991). However, this result was not reproducible, as in further runs only faint fragments were revealed. Such non-reproducibility of the results from this lucerne probe set has also been experienced by other workers (Bouton, 1994 pers. comm).

5.4.1.2 Clover probe

Of the four common restriction endonuclease enzymes screened with the clover ADH probe, a combination of the endonuclease Xba *I* with this probe produced the highest number of clear and easily evaluated RFLP fragments from DNA extracted from individual seedlings of the cultivars. Despite following the same protocol as that used for RFLP analysis of the eight other cultivars, the combination of the Xba *I* /ADH clover probes failed to produce any distinct RFLPs from genomic DNA of the two cvs Moapa and Saranac. The failure of the technique for detection of RFLPs from these cultivars was experienced in three signal detections using the same membrane, and also in three signal detections on

two new Southern blots with genomic DNA of these two cultivars.

An RFLP analysis comprises several steps, *ie.* preparation of the probe, including amplification and purification of cloned DNA; DNA isolation from plant tissue and purification; electrophoresis and blotting; hybridisation and subsequent washing of the membrane; and signal detection. Many factors, in particular freshness of the chemiluminescent signal detection reagents (Gardiner, 1995, pers. comm.) might affect the results during the procedure. Therefore it is extremely difficult to point out the actual factors associated with the present failure of the technique, and time was not available for the investigation. The non-reproducibility of RFLP detection using the lucerne and clover probes, as found in this study and observed by other workers (Bouton, 1994; Gardiner, 1994, pers. comm.), appears to be an important disadvantage of their use for the genetic analysis of plant species.

5.4.1.3 Apple probes

Among the combinations between the apple ribosomal DNA probe (rDNA) and the four restriction endonucleases, Bam HI/rDNA produced the most polymorphic and easily evaluated RFLPs from DNA from the individual seedlings of the cultivars. This combination consistently produced clear results and was therefore chosen for use for RFLP analysis of the 10 cultivars in this study.

The ribosomal DNA as a broad spectrum probe offered several advantages which suggested that it is extremely useful for detection of polymorphisms within and between cultivars of plant species, *i.e.*

1. The RFLP profile produced by the rDNA was reproducible for all 10 cultivars for different membranes. Reproducible results were also obtained by repeated signal detection on the same membranes.

2. The required digested genomic DNA/plant for the rDNA probe was significantly

less than that required for the other probes used in this study (0.2 μg vs 5 μg). Nevertheless, the fragments detected by this probe were darker than those detected by other probes. This is likely to be due to the high copy number of ribosomal DNA in the plant genome (Watson *et al.*, 1992)

3. The maximum exposure time for signal detection of RFLPs on the Southern-blots hybridised by the rDNA was 1h, as compared with 4 h for other probes screened in this study.

5.4.2 Discrimination among lucerne cultivars on the basis of RFLPs from bulked DNA samples of the cultivars using clover ADH and apple rDNA probes

No distinctive polymorphic fragments were detected by the clover ADH probe from the bulked DNA samples of the 10 cultivars. The apple rDNA probe detected only polymorphism between some cultivars (Table 5.3.1). Similarly no fragments unique to a particular lucerne population were reported by Brummer *et al.* (1991). This suggests that DNA fingerprinting of lucerne cultivars, as has been done in self-pollinating rice using a human minisatellite probe (Dallas, 1988), does not appear feasible on the basis of the RFLPs from bulk DNA samples of the cultivars.

5.4.3 Discrimination among lucerne cultivars on the basis of frequencies of RFLPs from single seedlings of the cultivars detected by the apple rDNA and the clover ADH probes

A sample size of 40 individual seedlings from each of 10 cultivars was used in this study for RFLP analysis. This sample size was larger than the 12 plants per accession previously used by Kidwell *et al.* (1994) to estimate genetic diversity of nine *Medicago* accessions. These authors failed to find polymorphism for all accessions, with the exception of two with very diverse genetic backgrounds (see Literature Review, section 5.1.3), despite using 35 cloned lucerne sequences. This

sample size was also larger than the 20 plants per population used by Brummer *et al.* (1991) for RFLP variation in three lucerne cultivars with very diverse genetic backgrounds.

RFLP analysis of 40 seedlings from each cultivar in this study was an attempt to achieve better discrimination among lucerne cultivars than these previous workers by reducing statistical errors relating to within-cultivar variation. Two non-lucerne probes (*ie* a cloned DNA sequence from clover species (ADH probe) and an apple ribosomal DNA (rDNA) probe which had not been used by other workers for genetic analysis of lucerne were used to detect polymorphisms among the cultivars. As a result of these experiments, not only cultivars with a divergent background were discriminated from each other, but cultivars which are closely related were also distinguished on the basis of their restriction fragment length polymorphism (see later).

RFLP analysis of the individual seedlings of a cultivar using each of the rDNA and the ADH probes produced distinct but highly polymorphic fragments,which was an indication of great genetic diversity within each of the cultivars. This type of profile has also been reported by other workers (Brummer *et al.*, 1991; Kidwell *et al.*, 1994).

Brummer *et al.* (1991) suggested that it might be possible to discriminate among lucerne cultivars on a population basis by examining the percentage of plants containing particular fragments. This hypothesis was tested in this study using Canonical Discriminant Analysis (SAS, 1989) to discriminate cultivars on the basis of the frequencies of the RFLP fragments from the 40 individual seedlings of the cultivars detected by the rDNA and the ADH probes.

The Multiple Range test demonstrated significant differences ($P<0.05$) among RFLP profiles produced using the ADH and the rDNA probe from some cultivars. Pairwise comparison between the least squared means (LSMEANS) of the RFLP

profiles detected by rDNA from DNA samples of the seedlings of the cultivars demonstrated that 70% of the pairwise comparisons of RFLP profiles from the cultivars were significantly different ($P<0.05$, Table 5.3.5), whereas 56% of the pairwise comparisons of the profiles from cultivars detected by the ADH probe differed significantly (Table 5.3.10). The superiority of the apple rDNA over the clover ADH probe is likely due to the high number of copies of rDNA genes in the genome which permits detection of more polymorphism among the populations (Watson *et al.*, 1992). Use of this heterologous probe between unrelated species is possibly due to the highly conserved coding sequences of the rDNA. However, some pairs of cultivars which were not discriminated using rDNA were differentiated on the basis of RFLPs detected by the ADH probe. If the results from these two probes are combined, 91% of the pairwise comparisons between the LSMEANs of the cultivars were significantly different ($P<0.05$).

The results of this study were promising, since not only cultivars with a divergent background (eg. Moapa and Saranac) were discriminated from each other, but cultivars which are closely related (eg. Esfahani and Hamedani) were also distinguished on the basis of their restriction fragment length polymorphisms.

5.4.4 Assessment of genetic uniformity of lucerne cultivars on the basis of similarity of the RFLP profiles from the individual seedlings

Assessment of the genetic uniformity level of the cultivars, as defined by the probability of the seedlings within the seed lots being classified as being from each individual cultivar, demonstrated that there were significant differences in the estimated genetic uniformity level of the cultivars on the basis of RFLPs detected by either of the rDNA and the ADH probes (Tables 5.3.2 and 5.3.7).

5.4.4.1 Genetic uniformity of the cultivars on the basis of frequencies of the RFLPs detected by the apple rDNA probe

Cultivar Wairau had the most uniform, whereas Saranac had the least uniform

RFLP profiles (62.5% vs 2.7%, Table 5.3.2). This is likely to be an accurate estimation of the genetic uniformity of the cultivars, as cv Wairau is a 26 clone synthetic derived from selected plants of Marlborough, Grimm, Ontario variegated and American commercial (see 3.4.3), whereas Saranac is a 500 clone synthetic derived from selected plants of A225 Alfa, DuPuits and Flamance (MAF, 1981). The genetic uniformity of cv. G.Oranga was significantly less than that of cv. Wairau (22.5 vs 62.5%). This appears to be a reasonable estimation of the genetic uniformity as cv. G.Oranga resulted from a simple mass selection from 40000 plants of cv. WL311 (Easton and Cornege, 1984).

5.4.4.2 Genetic uniformity of the cultivars on the basis of frequencies of the RFLPs detected by the clover ADH probe

Unlike the wide range of differences between the uniformity of the RFLP profiles from the cultivars detected by the rDNA probe, differences were limited to a few cultivars for the ADH probe. In addition, some cultivars (eg. Azari and Yazdi) which had different genetic uniformity on the basis of the RFLPs detected by the rDNA, did not differ when RFLPs were detected by the ADH probe (Tables 5.3.3 and 5.3.8). On the other hand, cv Wairau which from the rDNA probe was more uniform than cv. G.Oranga, was of lower uniformity when the RFLPs were detected by the ADH probe. In an RFLP analysis, each probe only reveals genetic information about small regions within the total genome that happen to hybridize with the particular probe. A population of plants might be uniform in a particular portion of the genome, but not in other parts. Therefore, information from an individual probe can not be used as an absolute indication of genetic uniformity of a cultivar. Hybridisation of the Southern blots with different probes would cover more portions of the genome, and hence better reveal the overall genetic uniformity of a cultivar.

A cultivar of lucerne is a heterogenous population, and can suffer severe inbreeding depression, resulting in a reduction in forage yield of the inbred

population (Rumbaugh *et. al.,* 1988). The uniformity revealed by RFLP analysis can illustrate the level of genetic uniformity and thus could be very useful in plant breeding and plant improvement programs. The number of RFLPs is associated with the heterozygosity level of each individual plant (see Literature Review, section 5.2.1). Therefore, the number of fragments/plant can be used as an indication of heterozygosity of the cultivars, and this has been shown to be highly correlated with yield in tetraploid lucerne plants (Kidwell *et al.*, 1994). There were significant differences between average numbers of restriction fragments/ plant detected by the rDNA and ADH probes for some pairs of cultivars (Table 5.4.1), suggesting that the cultivars were of different heterozygosity. This clearly demonstrates that the RFLP technique can provide useful information for plant breeding and plant improvement programs. Cultivars Hamedani, Azari and Esfahani were more heterozygous than the other cultivars, whereas Nikshahri and Yazdi and Bami were the least heterozygous cultivars when the results from the rDNA and the ADH probes were combined (Table 5.4.1). This supports the proposal that the latter cultivars originated in the Northwestern area of Iran and have been moved to the Southwest of this country (see Chapter.7). The hypothesis is that because of migration to drier and warmer, or in the case of Nikshahri hot climatic condition, the genetic range of these cultivars was narrowed as a result of natural selection of plants in the new locations, which gradually lowered heterozygosity of the cultivars. These results also suggest that increased forage yield of these cultivars is possible, by introducing genetically divergent cultivars suitable for these areas to increase heterozygosity, and therefore forage yield as suggested by Kidwell *et. al.* (1994).

5.4.5 Estimation of genetic relatedness among lucerne cultivars on the basis of RFLP profiles from individual seedlings detected by rDNA and the ADH probe

A wide range of genetic similarity among the cultivars was demonstrated by pairwise comparisons between the squared distances of the RFLP profiles from the

cultivars, detected using the rDNA and the ADH probes. Although, a similar D^2 was found between RFLP profiles from some of the cultivars produced by these two probes (eg. the D^2 between RFLP profiles from cvs Hamedani and Esfahani were 1.6 and 1.7 for the rDNA and ADH probe respectively, Tables 5.3.4 and 5.3.9), there was a big difference between the D^2 of the RFLP profiles from some of the other cultivars (eg. the D^2 between RFLP profiles from cvs Wairau and Nikshahri was 0.1 using the rDNA, while it was 4.2 for those produced by the ADH probe, Tables 5.3.4 and 5.3.9). As only small parts of the plant genome were revealed via hybridisation of the Southern blots with rDNA and ADH probes (see section 5.4.4), it is difficult to draw any conclusion about the disagreement between these results. However, the results do suggest that use of further probes is needed for obtaining a reliable estimation of relatedness among lucerne cultivars using RFLPs.

In summary, RFLP can be used as a powerful technique for discrimination among lucerne cultivars, assessment of genetic heterozygosity within each cultivar, and also detection of genetic relatedness among lucerne cultivars, which is very important for lucerne breeding programs.

Table 5.4.1 Average number of RFLP bands for the cultivars detected by the rDNA and AHD probes

Cultivar	RFLPs/plant
Hamedani	11.93
Azari	11.64
Esfahani	11.53
G.Oranga	10.65
Wairau	10.50
Bami	10.10
Yazdi	9.82
Nikshahri	9.55
Standard error	0.29

CHAPTER 6

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

6.1 LITERATURE

6.1.1 Polymerase chain reaction (PCR)

The best studied and most widely used target amplification technique is the polymerase chain reaction (PCR, Mullis *et al.,* 1986; 1987; Persing and Landry, 1989; Saiki *et al.*, 1988; Arnheim *et al.,* 1994; Wolcott, 1992). This method uses repeated cycles of oligonucleotide-directed DNA synthesis to perform *in vitro* replication of target nucleic acid sequences. The PCR technique was first published in 1985 (Mullis *et al.*, 1986) and its development has already resulted in the award of a Nobel Prize (Steiner, 1993).

The polymerase chain reaction is an enzymatic method for *in vitro* amplification of a specific DNA fragment. It involves two oligonucleotide primers that flank the DNA fragment to be amplified, repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primer with DNA polymerase. These primers hybridize to opposite strands of the target sequence and are directed so that DNA synthesis by the polymerase proceeds across the region between the primers. Because the PCR extension products themselves are also complementary to and able to bind to the same primers, successive cycles of amplification double the amount of the target DNA synthesized into the previous cycle. The result is an exponential accumulation of the specific target fragments, approximately 2^n, where n is the number of cycles of amplification performed (Saiki, 1992).

PCR requires information on the target DNA sequence for the design of primers. The time and cost for this information is prohibitive for many large scale genetic mapping applications. However, a newer PCR based method, "RAPD" (random amplified DNA polymorphism) has been described by Williams *et al.* (1990), and by Welsh and McClelland (1990), which does not require the target DNA sequence information.

6.1.2 Random amplified polymorphic DNA (RAPD)

The use of arbitrary oligonucleotide primers for DNA amplification by PCR, was co-developed independently by Williams *et al.* (1990), to identify DNA polymorphisms which could be used as genetic markers in gene mapping studies, and by Welsh and McClelland (1990), to produce simple and reproducible genomic fingerprints which could be used in strain identification.

Williams *et al.* (1990) proposed the term random amplified polymorphic DNA (RAPD) for this class of genetic marker, and this acronym is now widely used. However there are disadvantages in using the acronym "RAPD", and it can be misleading in two ways (Morell *et al.* 1993): Firstly it implies that primers are chosen randomly. This is not the case as the primers are of arbitrary sequence, but are carefully chosen to have a 50% or more 'GC' content with no internal complementarity. Second, the acronym may be taken to imply that the technique is random, unreproducible and unreliable. In section 6.1.5.1 of this review the reproducibility of the technique is discussed.

The DNA amplification reaction used in the RAPD method is a variant of the basic polymerase chain reaction (PCR) protocol (Saiki *et al.* 1988), with two important distinguishing features: 1) The RAPD method uses only a single short (10-20 bp) oligonucleotide primer; and 2) the priming site targets are unknown, so that different arbitrary primers detect polymorphisms in the absence of primer-specific nucleotide sequence information. In the RAPD procedure, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other (approximately 5000 bp) a discrete DNA product is produced through thermocycle amplification (Tingey and Del Tufo 1993).

In the Williams *et al.* (1990) method, RAPD banding profiles are visualized by staining with ethidium bromide, whereas in the Welsh and McClelland (1990) method, bands are detected by autoradiography. The ethidium bromide detection method is less sensitive, but it has the advantages that it is faster, easier, and does not use radioactivity. For these reasons, the Williams *et al.* (1990) protocol has become the most commonly used PCR based technique *ie*, the amplified polymorphic DNA fragments are size fractionated by agarose gel electrophoresis, stained with ethidium bromide, and visualised under ultraviolet light. For further information on the principles of the RAPD technique see review paper by Tingey and Del Tufo (1993).

6.1.3 Application of RAPD markers for genetic research and cultivar identification

The detection and exploration of naturally occurring DNA sequence polymorphisms represents one of the most significant recent developments in plant breeding (Waugh and Powell, 1992). Among new molecular techniques, RAPDs have already proved to be of great value in variations in DNA sequence and genetic linkage mapping (Tulsieram *et al.*, 1992; Echt *et al.*, 1992).

The RAPD markers can be used for identification in plant and animal breeding and also marker-assisted selection of polymorphisms associated with genetic traits (Yu and Pauls, 1993). Examples for successful application of this technique are the identification of markers linked to disease resistance genes in several plant species (eg. *Pseudomonas* resistance genes in tomato, Martin *et al.*, 1991; downy mildew resistance genes in lettuce, Paran *et al.*, 1991; Paran and Michelmore, 1993).

Since a single base change in the arbitrary primer can generate completely different and reproducible patterns of polymorphisms, RAPD markers are powerful tools for discrimination among populations and individuals (Anon.,

1992; Barcaccia, 1994; Mailer *et al.*, 1994). The technique has also been used for studies in population genetics, and for estimation of genetic relationships among populations (Hedrik, 1992), as well as for estimation of the out-crossing rate in some plant species (eg. *Dastica glomerata*, Fritsch and Riesenberg, 1992).

There are several commercially available kits of different oligonucleotide primers which could be used. A large number of primers are required to find ones that are suitable for discrimination among particular groups of plants. The more closely related the two groups are, the more extensive is the screening required to detect differences (Steiner, 1993).

Morell *et al.* (1994) showed that RAPDs can distinguish among cultivars of *Citrus* effectively and reliably. They also presented a summary of results obtained by other authors about the success of the RAPD technique in cultivar identification of some other self pollinating plant species, for example: barley (Francisco-Ortega *et al.*, 1993); broccoli, (Hu and Quiros, 1991); celery (Yang and Quiros, 1993); and wheat, (Francisco-Ortega *et al.*, 1993). All of these authors reported that the relationship revealed by the RAPD analysis was generally consistent with other types of evidence, such as allozyme or protein markers, thus validating the value of the RAPD technique.

Genetic variation among nine upland and four lowland rice cultivars was investigated by Yu and Nguyen (1994) who used forty-two random primers. As a result, 260 RAPD fragments were obtained, 208 of which (80%) were polymorphic. Using these bands a dendrogram indicating the genetic distance of the cultivars was constructed by the authors and they demonstrated that the RAPD analysis is a useful tool in determining genetic relationships among rice cultivars. Although there are many other publications about the usefulness of the RAPD technique for genetic analysis of self-pollinating crops, fewer reports have been published about RAPD analysis of populations within cross-pollinating crops.

RAPD variation within and among four natural populations of outcrossing buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) was studied by Huff *et al.* (1993). The DNA extracts from 12 individual plants from each of the four populations (Mexico-A and B; Texas A and B) were analyzed in their study. There was considerable variation within each of the four populations, and every individual was genetically unique. Despite this, and the small number of individual plants studied from each of the populations, there was sufficient divergence to be used for discrimination among the populations. Although this was very different from the result with inbred strains of other species, the result did reveal the great potential of the RAPD technique for identification of discrimination among outcrossing populations.

The RAPD technique was also employed by Yu and Paul (1993) to estimate genetic relatedness among three lucerne cultivars (Anik, 100% *Medicago falcata*; Dupuits, 100% *Medicago sativa* and Peace with *M. falcata* and *M. sativa* in its background). In two separate experiments genomic DNA from five and seven individual plants respectively of each of the cultivars and also the bulked DNA samples of the cultivars (the same number of plants/bulk) was amplified using four primers individually in the first and 10 primers in the second experiment, and the genetic distances between the three cultivars was calculated using the resulting RAPD fragments.

Results of the first experiment using five plants per cultivar and four primers, revealed that cv. Anik was the most distinct among the three cultivars tested (genetic distance between cv. Anik and cv. Dupuits, and Anik and Peace were 0.60 and 0.71 respectively). The genetic distance between cv. Peace and Dupuits was 0.20. This result is not surprising as cv. Anik is 100% *Medicago falcata* whereas cv. Dupuits is 100% Flemish *Medicago sativa.* Surprisingly, despite sharing both *M.falcata* and *M.sativa* in its background,cv. Peace had a greater genetic distance from cv. Anik than existed between cv. Dupuits and cv. Anik

(0.71 vs 0.60).

In the second experiment Yu and Pauls (1993) used seven individual plants per cultivar and the amplification was directed by 10 primers. In contrast with the results found in the first experiment, the genetic distance between cv. Anik and cv. Peace was greater than that between cv. Anik and cv. Dupuits (0.60 vs 0.93) and the genetic distance between cv. Peace and cv. Dupuits was 0.35. However, a sample of 10 plants/population is stated as the smallest possible sample size for genetic studies of plant cultivars with a sample size of 30 plants being proposed as a reasonable number of samples to be tested (Cliff, 1987). Therefore, the contradiction between the results obtained by Yu and Pauls (1993) in their two experiments could well be related to the small sample size used in their study.

As found by Huff *et al.* (1993) in buffalograss there are certainly variations in the RAPD profiles of members within out-crossing populations. This suggests that sample size should be relatively large when performing RAPD analysis so that the sample is representative of the genetic construction of the population under study, thus minimizing the likelihood of unrealistic results.

6.1.4 Advantages of RAPD analysis

According to several review and research publications (Arnheim, 1994; Weber, 1990; Tingey and Del Tufo, 1993; Steiner, 1993; Torres *et al.*, 1993) the RAPD technique offers several important advantages which make it the most efficient and probably the most cost effective DNA profiling technology which has been developed for plant species.

1. There is no need for the isolation of cloned DNA probes such as is required for RFLP analysis, as libraries of arbitrary 10-base primers are now commercially available (eg. Operon Technologies Inc., Alameda, CA 94501,

currently offers 1,000). This provides, at a minimal cost, a vast range of potential primer sequences that give the technique great diagnostic power.

2. PCR based methods require only small amounts of DNA and often small miniprep procedures yield sufficient quantity and quality.

3. The technique involves fewer steps than RFLP analysis and is faster to perform.

4. PCR based techniques do not require the use of radioactivity to visualize polymorphisms (except the protocol of Welsh and McClland, 1990).

6.1.5 Problems associated with the RAPD technique

6.1.5.1 Assessment of reproducibility of results

Steiner (1993) suggested that the RAPD technique is very sensitive to variations in the concentration of the components in the PCR reaction mixture and temperature profiles during the reaction. He noted that even small variations in any of these parameters may result in variation in the RAPD products that are observed. This implies that the RAPD result may not be reproducible.

The reproducibility of the RAPD profiles was investigated by Weeden *et al.* (1992) using different concentrations of reaction components, and two genetically defined systems in which at least a portion of the amplification or scoring errors could be identified. Although template DNA of high purity was found to be crucial for reproducible results, the concentration of the DNA could be varied ten-fold without seriously affecting the RAPD profile. They also found that oligonucleotide primer and Mg^{+2} concentration could be changed 2-to 5-fold while still maintaining the profile of RAPD products. Identical RAPD profiles were

also obtained when they used different batches of Tag polymerase, supplied by three different companies (Perkin-Elmer, Promega, and Boehringer), for RAPD analysis of several grape species.

To summarize their analysis of the effects of reaction conditions on the accuracy and reproducibility of the results of the RAPD technique, Weeden *et al.* (1992) stated that "we don't find that the amplification processes is so sensitive to one or more of the parameters tested to seriously affect the reproducibility of the technique". However, in contrast, Barcaccia (1994) did detect a different performance of these enzymes for RAPD analysis of lucerne plants, so care should be taken to check the reproducibility when the enzyme is changed.

The reproducibility of the RAPD results has also been studied by Penner *et al.* (1993), who compared RAPD results among laboratories for two oat cultivars. Some differences in the DNA banding profiles were found among the laboratories. However they concluded that if the temperature profiles during the PCR reactions are identical among laboratories, then RAPD fragments are reproducible for appropriately chosen primers.

Working with citrus, Morell *et al.* (1994) found reproducible RAPD results for 10 primers: 1) for DNA extracted by three different methods, 2) among multiple PCR reactions (at least 5), 3) between two operators working in separate laboratories in different organisations. Reproducible RAPD results were also obtained by Yu and Pauls (1993) who amplified genomic DNA from three lucerne cultivars with two primers. The results were consistent in all three to five replicates.

As the amplification of RAPD fragments is performed under conditions of low stringency, some of the products formed are the result of mismatching of oligonucleotide primers to the target DNA, and this can result in poor

reproducibility for some of primers and bands. Reproducible RAPD fragment profiles can be obtained by a careful selection of primers, optimisation of PCR conditions for the target species, and replication to ensure that only the reproducible bands are scored.

6.1.5.2 Presence of a low incidence of non-inherited bands in RAPD products

A second problem reported for RAPDs is a low incidence of non-inherited bands which are stated to be probably PCR artifacts (Morell *et al.*, 1994). For example Heun and Helentjaris (1993) reported this problem for a small RAPD fragment in maize and have mentioned that similar profiles have been found in other plants. Although it has been stated that the great majority of RAPD bands are inherited as Mendelian markers (Morell *et al.*, 1994), it is clear that care needs to be taken when drawing conclusions based on a small number of band differences.

6.2 MATERIALS AND METHODS

6.2.1 DNA samples and optimisation of target DNA concentration

The polymerase chain reaction (PCR) was used in this study to amplify the lucerne plant DNA for RAPD (Random Amplified Polymorphic DNA) analysis. DNA samples from the 10 cultivars used for this analysis were dilutions of those used for the RFLP study (section 5.3.1). As no evidence in the literature was found concerning the concentration of lucerne genomic DNA required for RAPD analysis, optimisation was required. Therefore, bulked DNA samples of 3 different concentrations were used in preliminary RAPD experiments. The concentrations used in this study were 1ng /12.5 µl reaction (as used for apple *(Malus)* DNA, Gardiner, 1994, pers. comm.), 5ng/12.5µl and 10 ng/12.5 µl reaction. The result of this experiment showed that the 5 ng/12.5 µl reaction provided the clearest banding patterns for the PCR products, and therefore this

concentration was used in subsequent RAPD experiments.

6.2.2 Reaction mixture and amplification conditions

The protocol follows that which is currently used by Dr. S. Gardiner (Apple Gene Mapping Laboratory, HortResearch, Palmerston North, New Zealand). The 12.5 µl reaction mixture used for each of the PCR reactions contained the following: Buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin], 1.5 % formamide, 0.1 mM of each dNTP, 16 ng primer, 5 ng lucerne DNA and 1.25 units of Taq polymerase (Stratagene). The reaction was overlaid with one drop of liquid paraffin.

Amplification reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 40 cycles with the following temperature profile: 1 minute at 94°C (5 minutes at the first cycle), 1 minute at 40°C and 2 minutes at 72°C. Cycling ended with a final extension at 72°C for 10 minutes.

To separate the RAPD products, the reaction mixture was loaded on a 2% [1% BRL Ultra pure, 1% Nu-Sieve GTG (FMC)] agarose gel and electrophoresed in TAE buffer at 80 Volts (4 Volt/cm) for approximately 5 h. 100 bp ladders [1 µl of 100 bp ladder (conc. 1µg/µl), 3 µl of 10xSB and 23 µl water sufficient for two ladders) were loaded in the first and the last lanes of the gel.

After electrophoresis the gels were stained with 0.35 µg/ml ethidium bromide (with agitation) for 1 h, rinsed in water for 1-1.5 h, and then visualised under ultraviolet light. Photography was performed using a red filter (Mamiya 77 Ø) colour slide film (Kodak, Ektachrome 6.4T). The film was developed commercially as colour negatives.

6.2.3 Oligonucleotide Primers

Twenty-six 10-base arbitrary primers (Table 6.2.1) were screened using bulk DNA samples (from 40 single seedlings) of the cultivars and consistent amplification conditions to select the primers which gave rise to the clearest polymorphisms between cultivars (see Results). The identification number and sequence of these oligonucleotide primers (Operon Technologies, INC, 1000 Atlantic Ave., Alameda CA 9401) are cited in Table 6.2.1.

6.2.4 Evaluation of the results and statistical analysis

RAPD analysis of 40 individual seedlings from each of the 10 cultivars was performed based on nine distinctive polymorphic DNA bands generated using primer OA08 (Plate 6.2.1). The presence (1) and absence (0), of the bands were scored. Canonical Discriminant Analysis (CDA, SAS, 1989) was employed to discriminate the seed lots and also to assess variation within each seed lot. The details of the statistical analysis procedures are the same as those discussed in section 3.2.7.

Table 6.2.1 Sequence of the 26 oligonucleotide primers screened (based on information supplied by OPERON). A, G, C, and T in the table are the abbreviations for the nucleotides Adenine, Guanine, Cytosine, and Thymine respectively.

No.	Primer ID	Sequence (5' to 3')
1	OPA-08	G T G A C G T A G G
2	OPA-16	A G C C A G C G A A
3	OPA-17	G A C C G C T T G T
4	OPB-07	G G T G A C G C A G
5	OPB-08	G T C C A C A C G G
6	OPB-13	T T C C C C C G C T
7	OPB-17	A G G G A A C G A G
8	OPB-18	C C A C A G C A G T
9	OPB-19	A C C C C C G A A G
10	OPB-20	G G A C C C T T A C
11	OPC-01	T T C G A G C C A G
12	OPC-03	G G G G G T C T T T
13	OPC-04	C C G C A T C T A C
14	OPC-05	G A T G A C C G C C
15	OPC-06	G A A C G G A C T C
16	OPC-10	T G T C T G G G T G
17	OPC-13	A A G C C T C G T C
18	OPC-15	G A C G G A T C A G
19	OPC-18	T G A G T G G G T G
20	OPC-20	A C T T C G C C A C
21	OPD-01	A C C G C G A A G G
22	OPD-02	G A C C C C A A C C
23	OPD-03	G T C G C C G T C A
24	OPD-04	T C T G T G A G G G
25	OPD-05	T G A G C G G A C A
26	OP-D10	G G T C T A C A C C

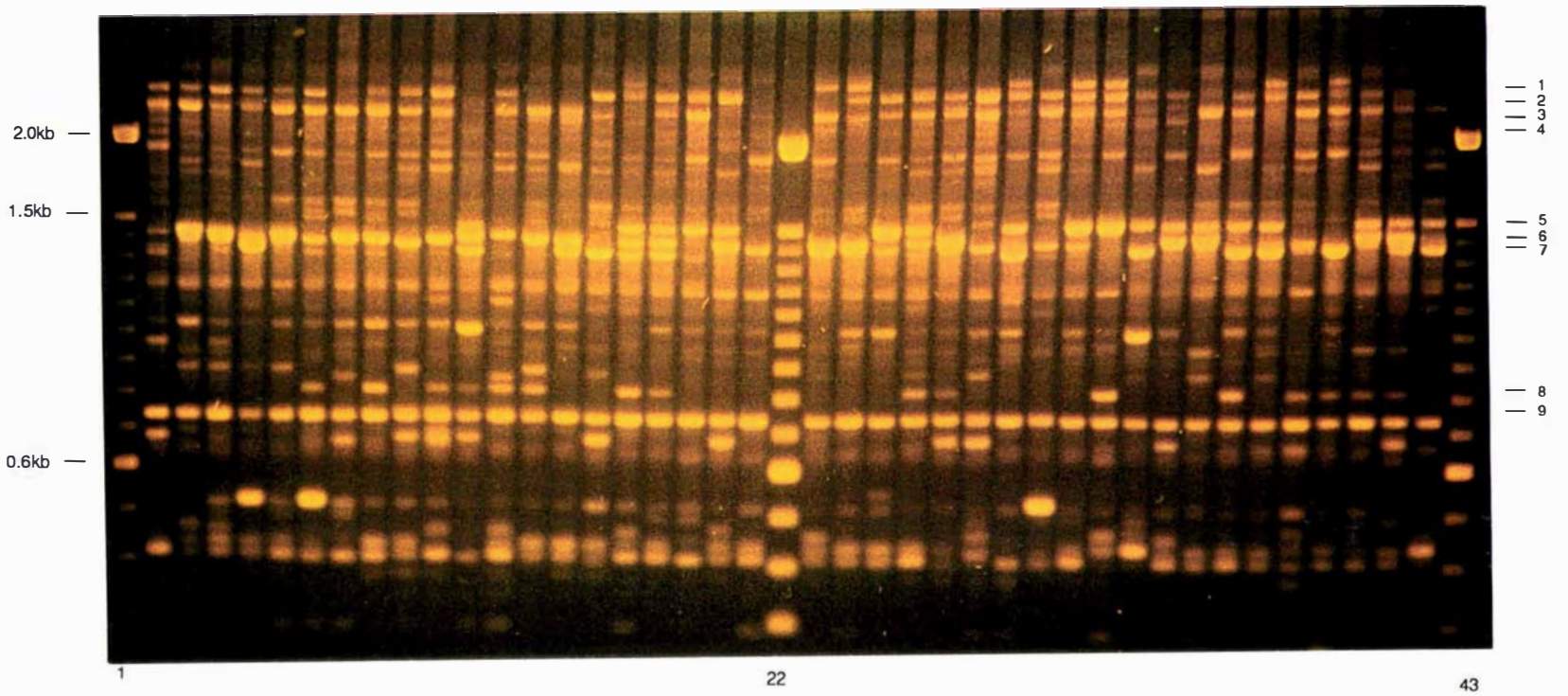

Plate 6.2.1 RAPD bands which were scored for individual seedlings. These fragments were generated using primer OA08 from genomic DNA extracted from 40 individual seedlings of each of the 10 cultivars (result for cv. Bami). Lanes 1, 22 and 43 are 100 bp standard DNA ladders.

6.3 RESULTS

6.3.1 Results from combination of two oligonucleotides in a single PCR reaction

Of the twenty-six 10-base oligonucleotides tested individually to amplify bulk DNA samples from the 10 lucerne cultivars, 10 individual primers generated polymorphic fragments. Four of these (OP0A8, OPB13, OPB19 and OPC10), gave distinctive RAPD bands. From these results it was suggested that a combination of two primers might generate more polymorphisms than one primer in a single reaction.

This hypothesis was tested by using the primers OPB19 and OPC10 alone and in combination to amplify bulk DNA samples of the 10 cultivars. Results of this study demonstrated that the number of distinct RAPD fragments generated by a combination of these two primers was fewer than those produced by each individual primer alone (Plate 6.3.1). Thus for discrimination among cultivars, primers were used individually in the reaction mixture.

6.3.2 Assessment of reproducibility of the results of the RAPD technique

To check the reproducibility of the RAPD results obtained in this study, replicated PCR reactions were carried out using two primers (OPB19 and OPC10) alone and in combination with the standard reaction mixture (section 6.2.2) using different batches of enzyme from the same manufacturer. The findings of this experiment (Plates 6.3.1, 6.3.2) demonstrated that a high degree of reproducibility of the results obtained by the RAPD technique is possible.

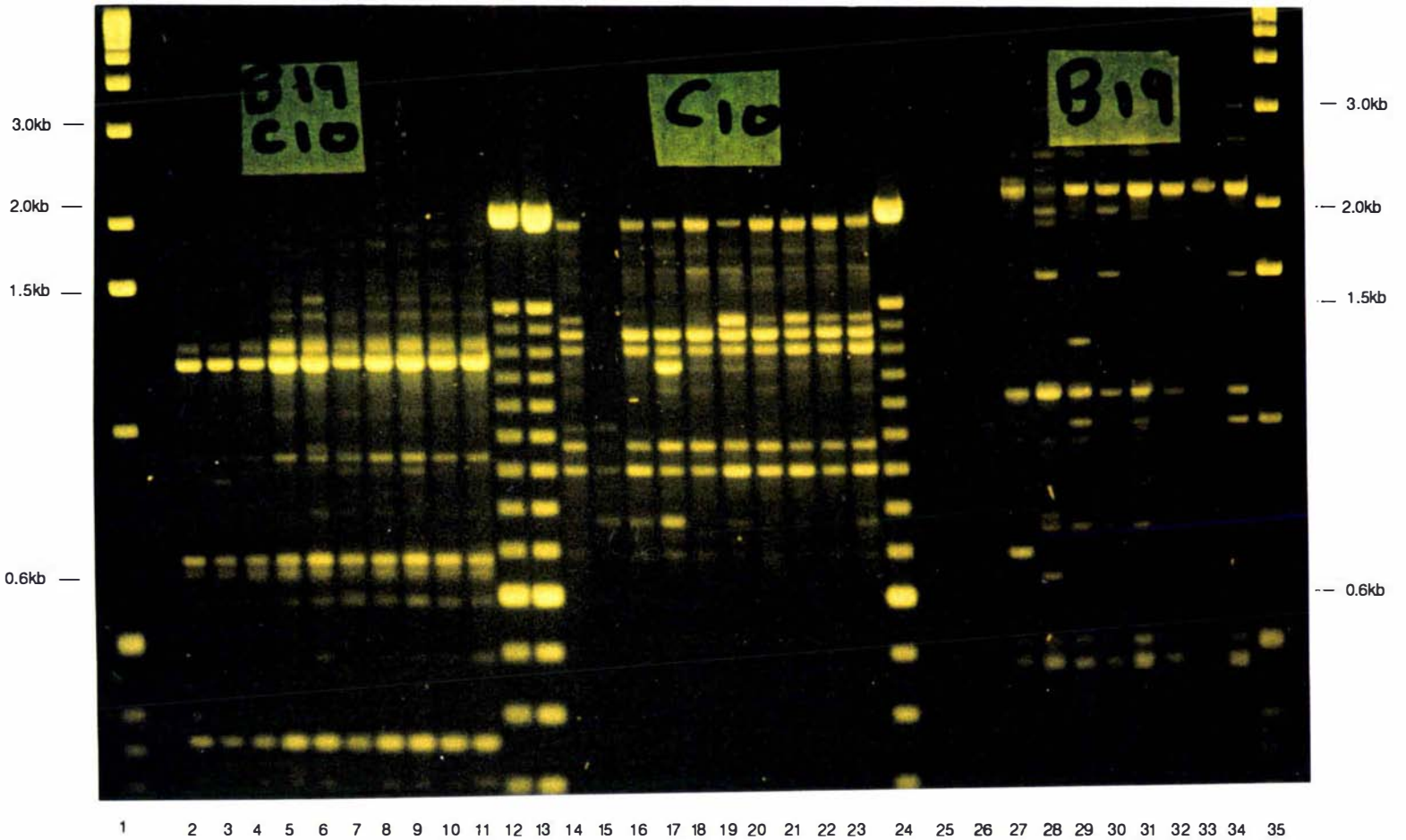

Plate 6.3.1 **Amplified DNA fragments from bulked DNA samples from the cultivars generated using the 10-base oligonucleotides OPC10 and OPB19 alone, and in combination. The profiles are from cvs. Yazdi; Wairau; Saranac; G.Oranga; Nikshahri; Moapa; Esfahani; Hamedani; Bami and Azari. Lanes number 1, 12, 13, 24, and 35 are 100 bp and 1 kbp standard DNA ladders.**

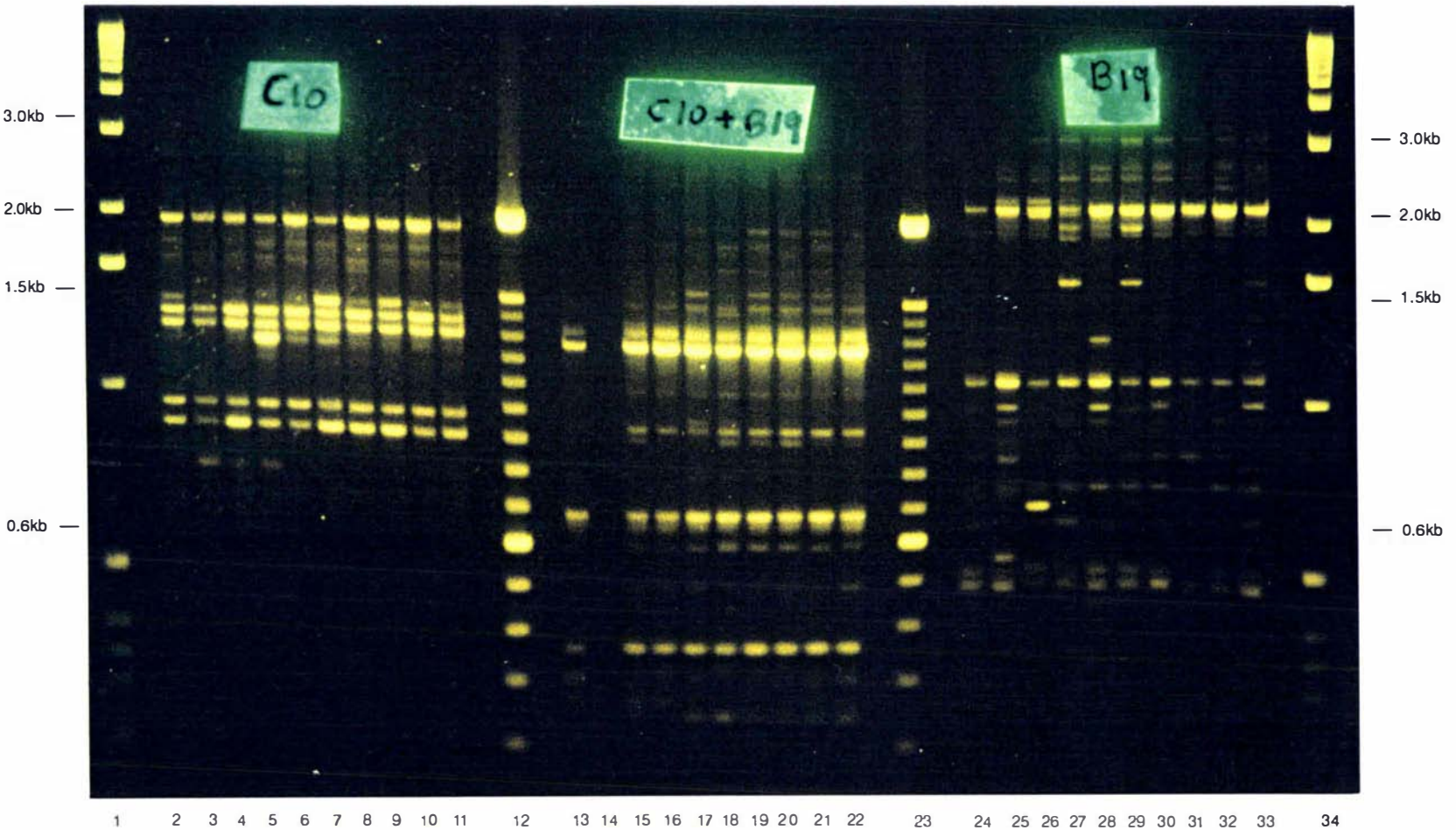

Plate 6.3.2 The RAPD profiles from bulked DNA samples from the cultivars using 10-base oligonucleotides OPC10 and OPB19 alone, and in combination. The profiles (left to right) belong to cultivars: Yazdi; Wairau; Saranac; G.Oranga; Nikshahri; Moapa; Esfahani; Hamedani; Bami and Azari. Lanes number 1, 12, 23, and 34 are 100 bp and 1 kbp standard DNA ladders.

6.3.3 Screening of twenty-six 10-base arbitrary primers for their power of discrimination

Among the twenty-six 10-base arbitrary primers screened individually in this study to generate RAPD profiles from bulk DNA samples of the 10 cultivars (section 6.2.3), primer OPB19 produced the highest number of clear and easily evaluated polymorphic DNA fragments from the bulked DNA samples of the cultivars (Plate 6.3.3). Each of the oligonucleutides OPA08, OPB13, OPC10 also gave rise to clear and easily evaluated RAPD profiles (Plate 6.3.4-6.3.6). The other primers screened in this study produced either very faint RAPD profiles, or gave rise to RAPD profiles with little polymorphism among the cultivars (eg. oligonucleotide OPB18, Plate 6.3.7).

Pairwise comparisons between the RAPD fragments from the bulked samples of the cultivars demonstrated that all ten of the cultivars could be discriminated using the RAPD profiles produced by primer OPB19 alone (Table 6.3.1). The second best primer (OPB13) produced polymorphic DNA profiles (Table 6.3.2) by which the majority of the cultivars, except Azari and Moapa, could be distinguished. Although all of the cultivars could not be discriminated using the RAPD profiles produced by primers OP08, OPB13 and OPC10 used individually, a combination of the results from OPA08, OPC10 and OPB13 (Tables 6.3.2-6.3.4) provided sufficient information for discrimination among all 10 of the cultivars.

Pairwise comparison between polymorphisms from the cultivars (Tables 6.3.1-6.3.4) demonstrated that the two internationally recognised cultivars (Moapa and Saranac) could be discriminated from each other using the RAPDs fragments generated by any of the primers OP08, OPB13, OPB19 and OPC10 (Table 6.3.1-6.3.4). Likewise, the two New Zealand cultivars (G.Oranga and Wairau) could also be differentiated using the results obtained from any of these primers.

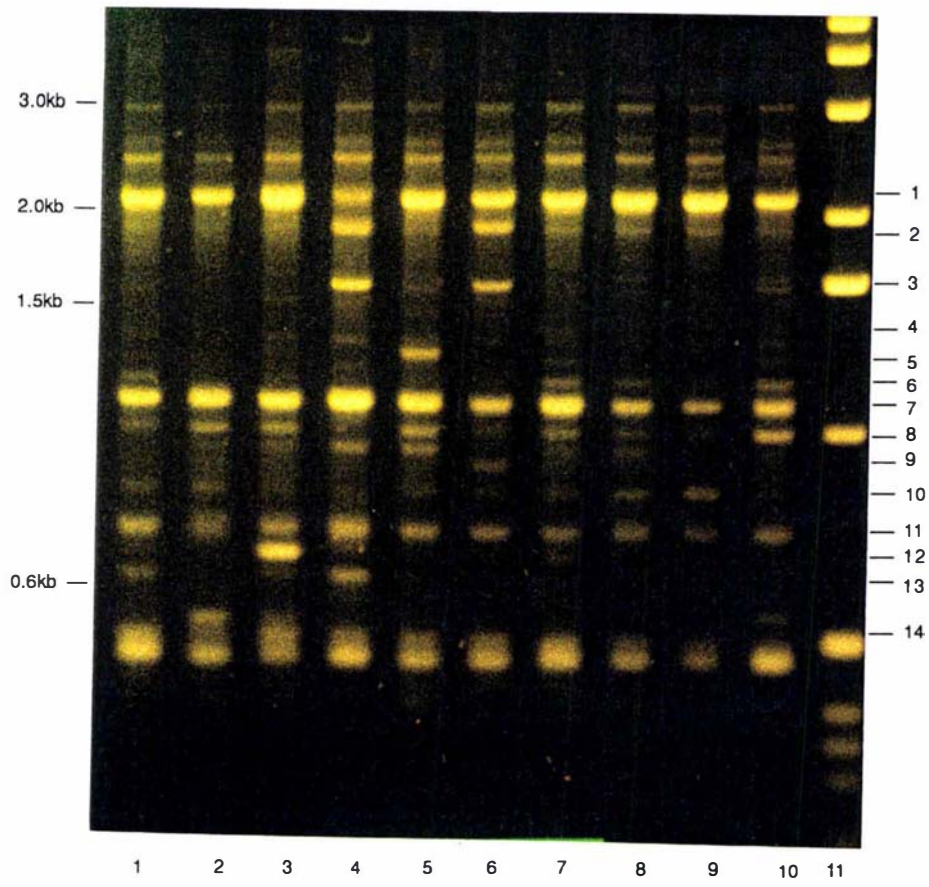

Plate 6.3.3 **The RAPD profiles generated by 10-base oligonucleotide OPB19 from bulked DNA samples from the cultivars. The profiles belong to cultivars: Yazdi (1); Wairau (2); Saranac (3); G.Oranga (4); Nikshahri (5); Moapa (6); Esfahani (7); Hamedani (8); Bami (9) and Azari (10). Lane number 11 is 1 kbp standard DNA ladder.**

Table 6.3.1 Pairwise comparison between the polymorphic fragments from bulked DNA samples (40 single seedlings) from the cultivars generated using the 10-base oligonucleotide OPB19. The figures in this table indicate the RAPD fragments (Plate 6.3.3) by which a pair of cultivars can be distinguished.

	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		3,4,5,6,7,8,9	3,4,5,9	3, 12	2,4	3,5,6,9	2,5,6,8,13	3,4,5,6,12	3,4,5,6	3,4,5,13
Bami	3,4,5,6,7,8,9		3,4,5,9	5, 6, 8, 12	2,3,10	4,8,9	3,9,13	8,12	8	6,13
Hamedani	3,4,5,9	5,6,8		5,9,12	2,3,6.8,9,10	4,6	2,3,4,6,8,13	6,9	6,9	9,13
Esfahani	3,12	5,6,8,12	5,9,12		2,3,6,8,10,12	4,6,9,12	2,3,5,6,9,13	5,6	5,6,12	12,13
Moapa	2, 4	2,3,10	2,3,6,8,9,10	2,3,6,8,10,12		2,3,4,8,9,10	4,9,10,13	2,3,8,10	2,3,8,10	2,3,4,6,13
Nikshahri	3,5,6,9	4,8,9	4,6	4,6,9,12	2,3,4,8,9,10		2,3,8,13	4,8,10,12	4,9	4,6,8,9
G.Oragna	2,5,6,8,13	3,9,13	2,3,4,6,9,13	2,3,5,6,9,13	4,9,10,13	2,3,8,13		2,3,4,8,9,12,13	2,3,4,8,9,13	2,3,4,6,9
Saranac	3,4,5,6,12	8,12	6,9	5,6	2,3,8,10	4,8,10,12	2,3,4,9,9,12,13		12	6,13
Wairau	3,4,5,6	8	6,9	5,6,12	2,3,8,10	4,9	2,3,4,8,,9,13	12		6,13
Yazdi	3,4,5,13	6,13	9,13	12,13	2,3,4,6,13	4,6,8,9	2,3,4,9	6,13	6,13	

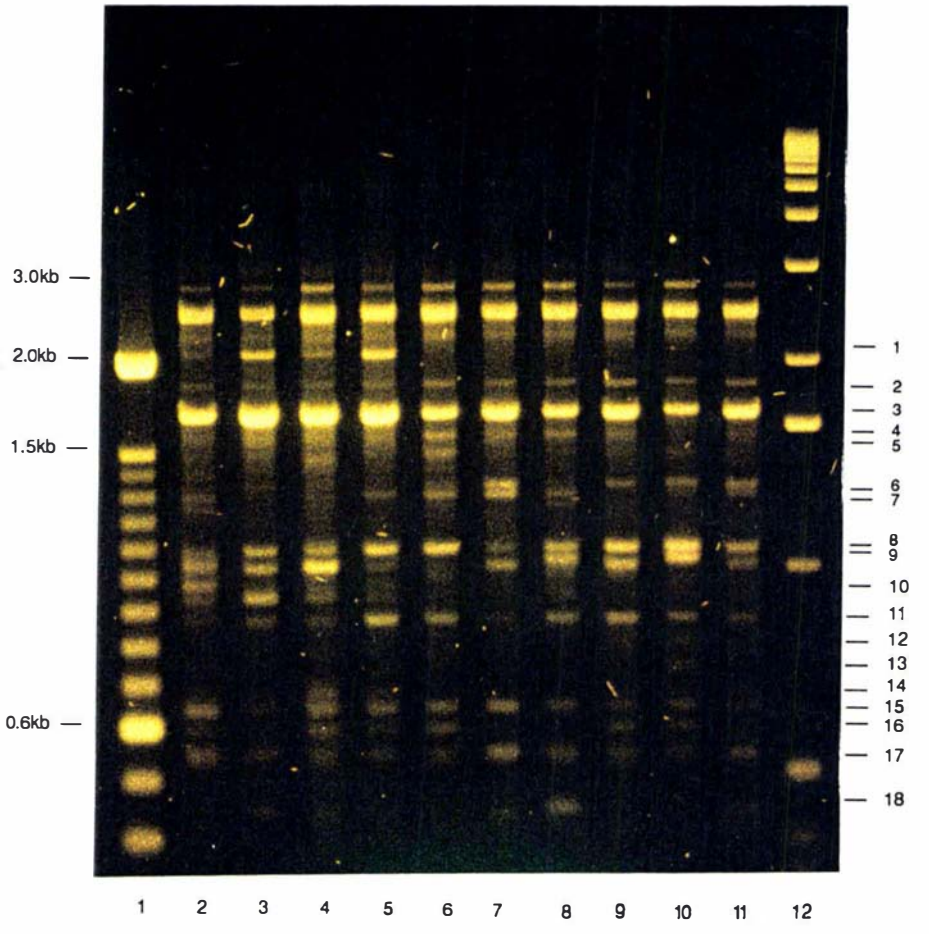

Plate 6.3.4 **The RAPD profiles from bulked DNA samples from the cultivars generated using 10-base oligonucleotide OPB13. The profiles are from cvs. Yazdi (2); Wairau (3); Saranac (4); G.Oranga (5); Nikshahri (6); Moapa (7); Esfahani (8); Hamedani (9); Bami (10) and Azari (11). Lanes number 1, 12, are 100bp and 1 kbp standard DNA ladders.**

Table 6.3.2 Pairwise comparison between amplified polymorphic bands from bulked DNA samples (40 single seedlings) from the cultivars, amplified using the 10-base oligonucleotide OPB13. The figures in this table indicate the RAPD fragments (Plate 6.3.4) by which a pair of cultivars can be distinguished. The stars indicate there was no difference between the RAPD profiles from the pair of cultivars

OPB13	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		13,14,16	4,7,16	4,6	**	4,5,6,9,16	1,6,9	1,6,16	1,6,11	1,10
Bami	13,14,16		4,13,14	4,6,7,13,16,18	7,13,14,16	4,5,6,7,9,13,14	1,6,7,9,13,14	1,6,13,14	1,6,11,16	10,13,14,16
Hamedani	4,7,16	4,13,14		6,7,16,18	7,16	5,6,7,9,	1,4,6,7,9	1,4,6	1,4,6,11,16	4,10,16
Esfahani	4,6	4,6,7,13	6,7,16,18		6	5,9,16,18	1,4,9,10	1,4,7,16,18	1,4,7,11	4,10,18
Moapa	**	7,13,14,16	7,16	6		5,6,9,16	1,6,9	1,6,7,16	1,6,7,11	10
Nikshahri	4,5,6,9,16	4,5,6,7,9,13,14	5,6,7,9	5,9,16,18	5,6,9,16		1,4,5	1,4,7,9	1,4,5,7,9,11,16	4,5,9,10,16
G.Oragna	1,6,9	1,6,7,9,13,14	1,4,6,7,9	1,4,9,10	1,6,9	1,4,5		7,9	7,9,11	9,10
Saranac	1,6,16	1,6,13,14	1,4,6	1,4,7,16,18	1,6,7,16	1,4,7,9	7,9		16	10,16
Wairau	1,6,11	1,6,11,16	1,4,6,11,16	1,4,7,11	1,6,7,11	1,4,5,7,9,11,18	7,9,11	16		10
Yazdi	1,10	10,13,14,16	4,10,16	4,10,18	10	4,5,9,10,16	9,10	10,16	10	

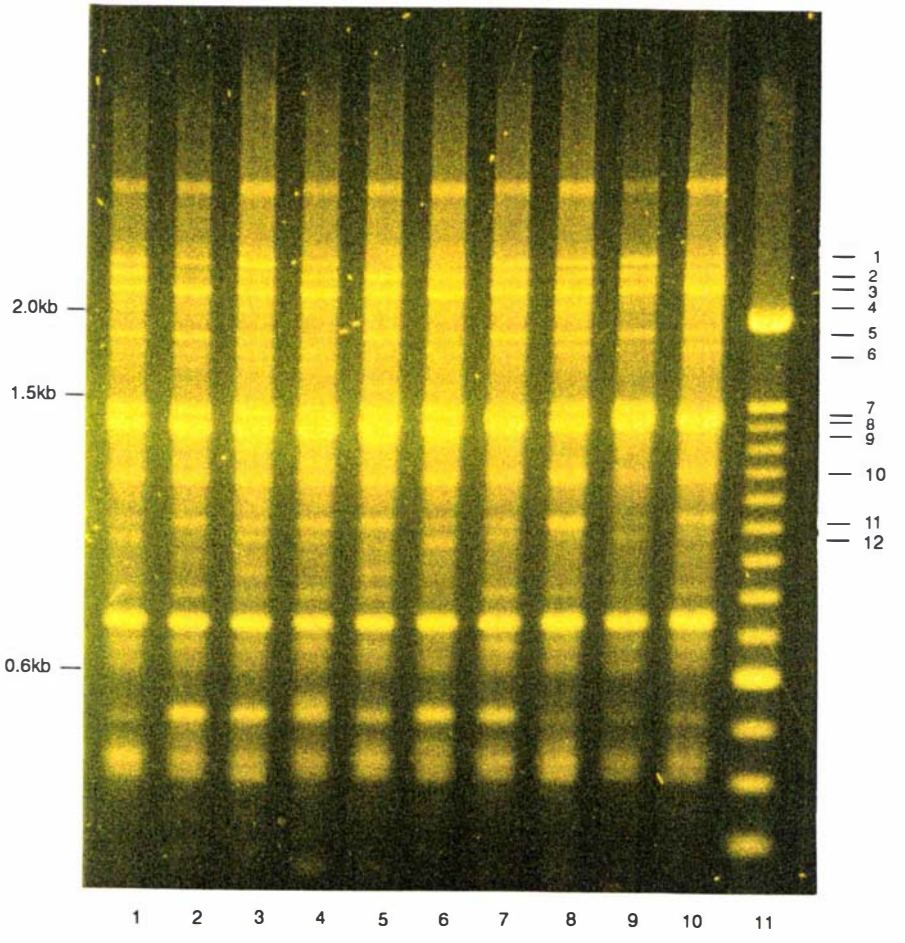

Plate 6.3.5 **The RAPD profiles from the bulked DNA samples from the cultivars generated using 10-base oligonucleotide OPA08. The profiles are from cvs. Yazdi (1); Wairau (2); Saranac (3); G.Oranga (4); Nikshahri (5); Moapa (6); Esfahani (7); Hamedani (8); Bami (9) and Azari (10). Lane number 11 is 100bp standard DNA ladder.**

Table 6.3.3 Pairwise comparison between polymorphic bands from bulked DNA samples (40 single seedlings) of the cultivars, amplified using the 10-base oligonucleotide OPA08. The figures in this table indicate the RAPD fragments (Plate 6.3.5) by which a pair of cultivars can be distinguished. The stars indicate there was no difference between the RAPD profiles from the pairs of cultivars

	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		6, 10	6	**	1, 6	1,6	6	2, 6, 8	2, 6	6
Bami	6, 10		5, 9, 10	10, 11	1, 11	1	5, 10	5, 8, 10	2, 5, 10	5, 11
Hamedani	5, 6	5, 9, 10		11	1, 11	1	**	2, 11	2	**
Esfahani	**	10, 11	11		1	1	**	2, 8	2	**
Moapa	1, 6	1, 11	1, 11	1		11	1, 11	1, 2, 8	1, 2. 11	1
Nikshahri	1, 6	1	1	1	11		1	1, 2, 8, 11	1, 2	1, 11
Oragna	6	5, 10	**	**	1, 11	1		2, 8	2	**
Saranac	2, 6, 8	5, 10, 8	2, 11	2, 8	1, 2, 11	1, 2, 8, 11	2, 8		2, 8	2, 8
Wairau	2, 6	2, 5, 10	2	2	1, 2, 11	1, 2	2	2, 8		2, 11
Yazdi	6	5, 11	**	**	1	1, 11	**	2, 8	2, 11	

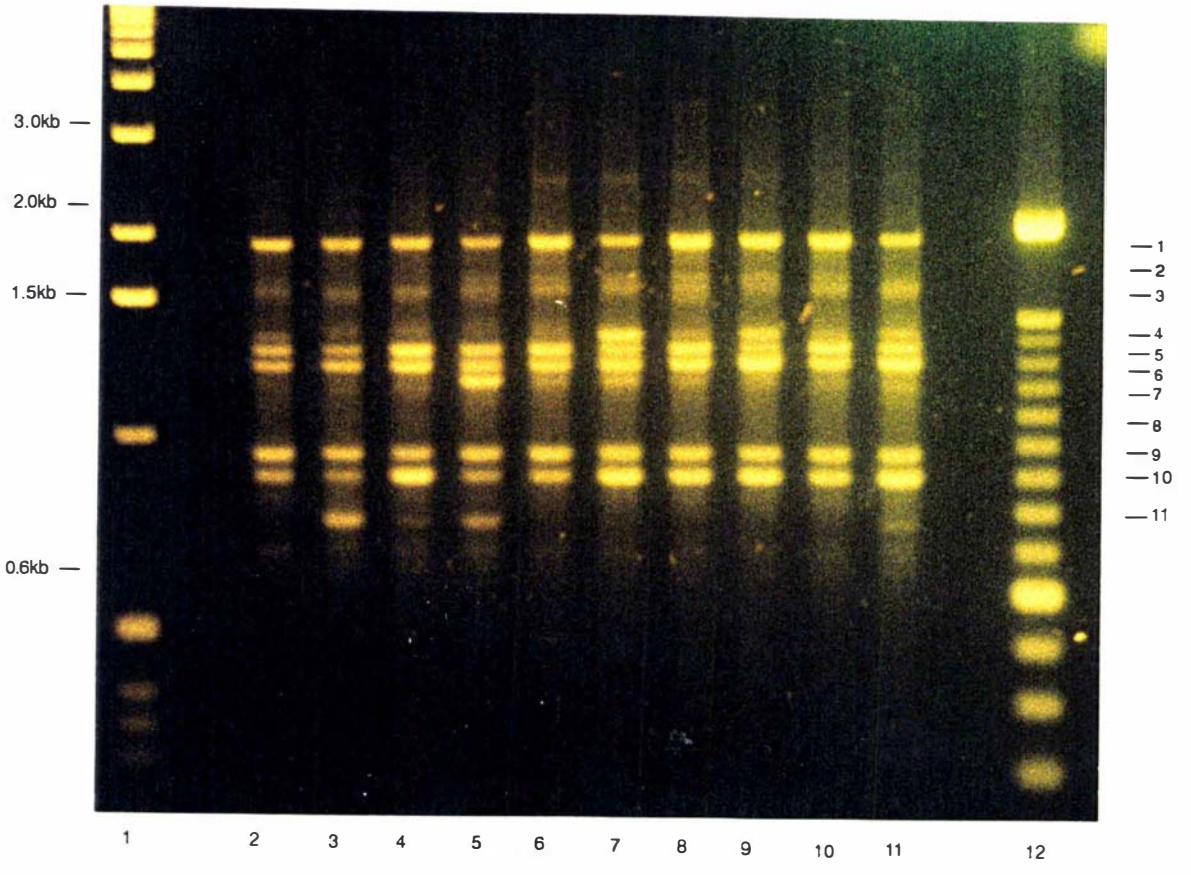

Plate 6.3.6 **The RAPD profiles from the bulked DNA samples from the cultivars amplified using the 10-base oligonucleotide OPC10. The profiles are from cvs. Yazdi (2); Wairau (3); Saranac (4); G.Oranga (5); Nikshahri (6); Moapa (7); Esfahani (8); Hamedani (9); Bami (10) and Azari (11). Lanes number 1, 12, are 1 kbp and 100bp standard DNA ladders.**

Table 6.3.4 Pairwise comparison between the RAPD fragments from bulked DNA samples (40 single seedlings) from the cultivars amplified using the 10-base oligonucleotide OPC10. The figures in this Table indicate the RAPD fragments (Plate 6.3.6) by which a pair of cultivars can be distinguished. The stars indicate there was no difference between the RAPD profiles from the pairs of cultivars

OPC10	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		**	**	**	7	4,7	4,7	4,11	4,11	11
Bami	**		**	**	7	4,7	4,7,11	4,11	4,11	**
Hamedani	**	**		**	7	4,7	4,7,11	4,11	4,11	**
Esfahani	**	**	**		7	4,7	4,7,11	4,11	4,11	**
Moapa	7	7	7	7		4,7	4,11	4,11	4,7,11	7
Nikshahri	4,7	4,7	4,7	4,7	4		11	7,11	7,11	4,7
G.Oragna	4,7,11	4,7,11	4,7,11	4,7,11	4,11	11		7	7	4,7,11
Saranac	4	4,11	4,11	4,11	4,11	4,11	7		**	4,7,11
Wairau	4,11	4,11	4,11	4,11	4,11	11	7	**		4,7,11
Yazdi	11	**	**	**	7	4	4,7,11	4,7,11	4,7,11	

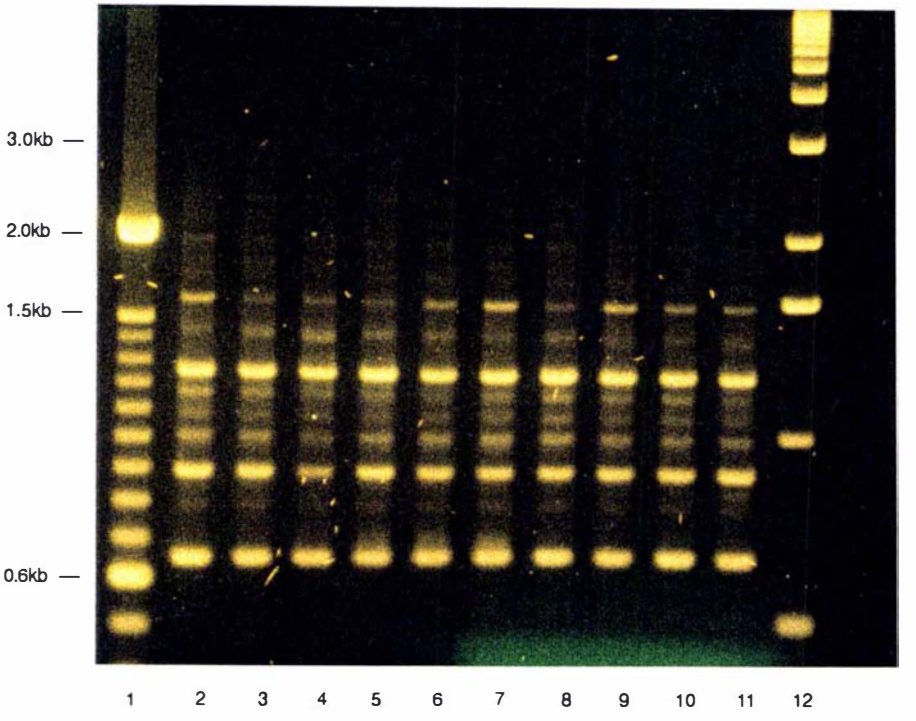

Plate 6.3.7 **The RAPD profiles from the bulked DNA samples from the cultivars amplified using the 10-base oligonucleotide OPB18. The profiles (left to right) are from: Yazdi; Wairau; Saranac; G.Oranga; Nikshahri; Moapa; Esfahani; Hamedani; Bami and Azari.**

6.3.4 Discrimination among the cultivars based on the frequencies of the amplified polymorphic DNA fragments from 40 single seedlings of the cultivars generated using the primer OPA08

Canonical Discriminant analysis (CDA) was used for analysis of the data, obtained by scoring the frequencies of the RAPD fragments generated using primer OP08 from 40 individual seedlings of each of the 10 cultivars. Samples of the RAPD profiles from individual seedlings from three of the cultivars are presented in Plates 6.3.8-6.3.10.

6.3.4.1 Cross validation for each of the seed lots of the cultivars

The cross validation, defined by the probability of seedlings within the seed lot being classified as from each individual cultivar from the RAPD analysis of DNA from 40 single seedlings of each of the cultivars, is presented in Table 6.3.5. For example for Moapa, 67.6% of the seedlings had RAPD profiles which led to them being classified by the discriminant analysis as belonging to this cultivar, whereas the remaining 32.4% of the seedlings had RAPD profiles which corresponded to the profiles found from cultivars other than Moapa. The percentage of seedlings correctly classified into cv. Wairau (26.5%) and G.Oranga (32.4%) was less than that for Moapa.

Among the cultivars tested in this study, Yazdi had the least variability in respect of RAPD profile (91.9% of the seedlings being correctly classified into the cultivar), whereas Esfahani had the most variability (21.9% of the seedlings being correctly classified into the cultivar).

RAPD analysis of the cultivars using primer OPA08 (Table 6.3.5) also suggested that genomic DNA of cv. Azari gave a RAPD profile (Plate 6.3.9) which was the closest to the profile from cv. Hamedani (35% of the seedlings gave RAPD

profiles which corresponded to the polymorphic DNA profile from cv. Hamedani, Plate 6.3.8). However, the RAPD profile from cv. Bami (Plate 6.3.10), the other Iranian cultivar, was distinct from that for cv. Hamedani (only 5% of the seedlings gave a polymorphic DNA profile which corresponded to the profile from Hamedani).

The Test of Difference between two proportions (section 3.2.7) showed that there were significant differences ($P<0.05$) between the uniformity of RAPD profiles from some of the cultivars (Table 6.3.6). No significant differences were found among the uniformity of RAPD profiles from cv. Azari, Bami and Hamedani and the other cultivars. However, a highly significant difference was found between the uniformity of cultivar Yazdi and other cultivars.

No significant difference was found between the uniformity of the RAPD profiles from the two internationally recognized cultivars, Moapa and Saranac, and between the two New Zealand cultivars, G.Oranga and Wairau. Although the uniformity of RAPD profiles from cv. G.Oranga did differ from Moapa, no significant difference was found between the uniformity of the RAPD profile of this cultivar and that for cv. Saranac. However, the uniformity of cv. Wairau was significantly different from Saranac and Moapa.

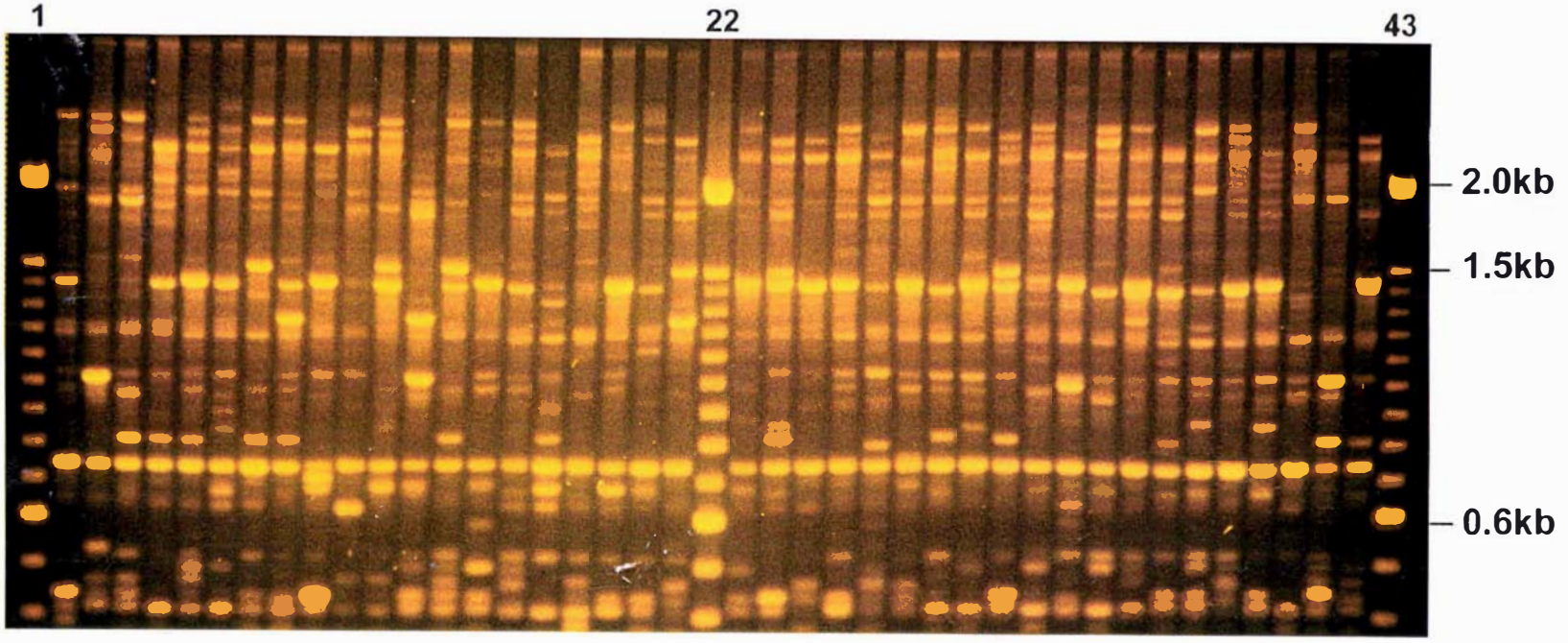

Plate 6.3.8 RAPD profiles from 40 single seedlings of cv. Hamedani generated using the 10-base oligonucleotide OPA08.

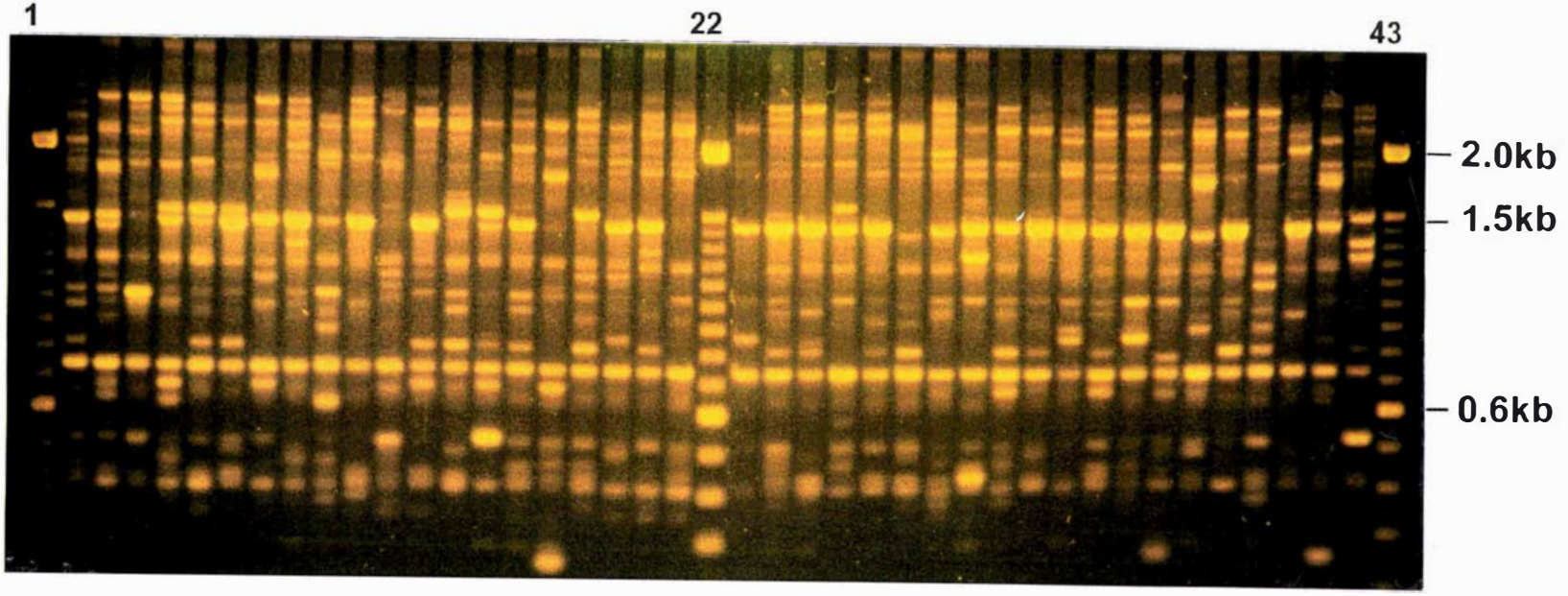

Plate 6.3.9 Amplified polymorphic DNA profiles from 40 single seedlings of cv. Azari generated using the 10-base oligonucleotide OPA08.

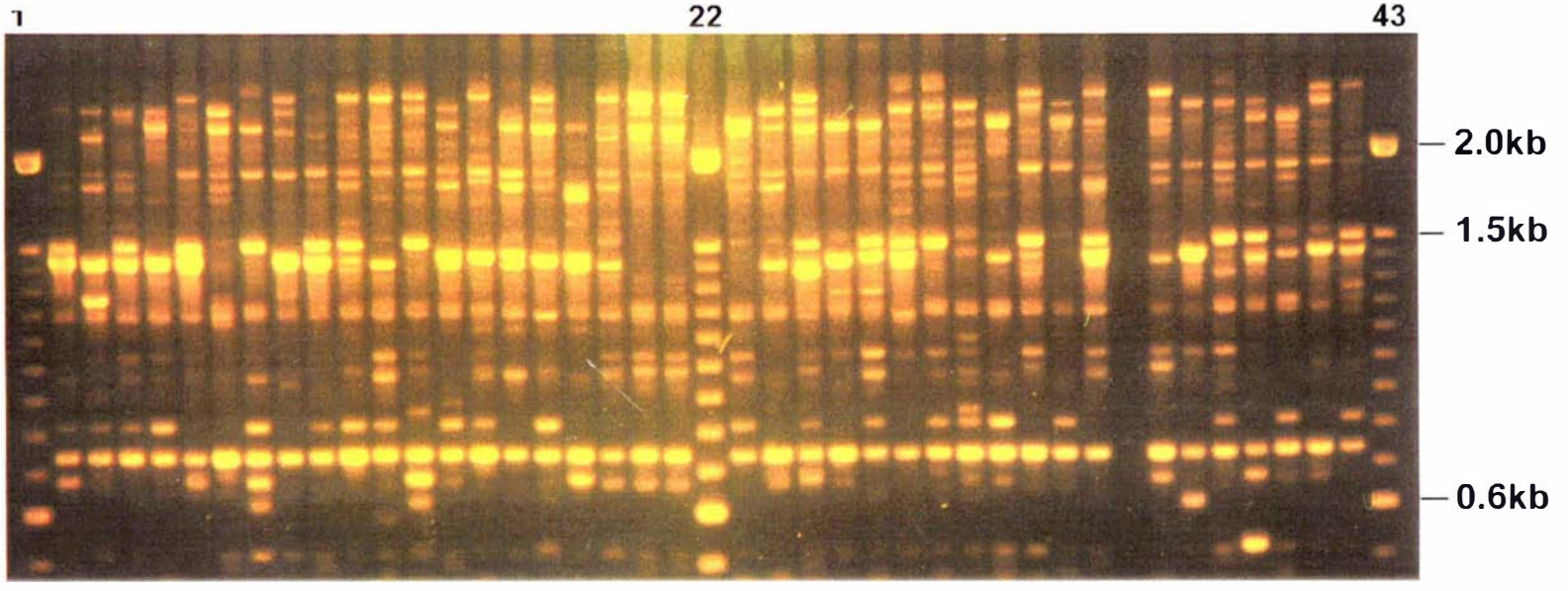

Plate 6.3.10 RAPD profiles from 40 single seedlings of cv. Bami generated using the 10-base oligonucleotide OPA08.

6.3.4.2 Pairwise comparison between the squared Mahalanobis distances of the RAPD profiles from the cultivars amplified by primer OPA08

Pairwise comparisons between the RAPD profiles from the cultivars (Table 6.3.7) indicated that with a squared Mahalanobis distance (D^2) of 0.9, the DNA from the seedlings of cv. Hamedani and Esfahani and cv. Wairau and Saranac gave RAPD profiles which were the closest, while cv. Moapa and Yazdi generated RAPD profiles which were the most distinct (D^2 = 20.6) among the cultivars tested in this study. The results of this study also demonstrated that cv. G.Oranga and Wairau had very close RAPD profiles (D^2= 1.0).

6.3.4.3 Pairwise comparisons of least squared means (LSMEANS) of RAPD profiles from the cultivars amplified using the primer OPA08

Pairwise comparisons between least squared means of the cultivars (Table 6.3.8) indicated that there were significant differences between RAPD profiles for some of the cultivars. There was a very highly significant difference ($p<0.0001$) between the RAPD profile from cv. Yazdi and those from the other cultivars. A significant difference was also found between RAPD profiles from cultivars Saranac and Moapa. Unlike cultivar G.Oranga, the RAPD profile from cv. Wairau was not significantly different from that of cv. Saranac. However a very highly significant difference was found between the RAPD profiles from two New Zealand cultivars and cv. Moapa. A highly significant difference was also found between the RAPD profile of cultivar Saranac and all of the Iranian cultivars.

Table 6.3.5 Uniformity of cultivars: Cross validation of seedlings by cultivar based of the frequencies of the RAPDs amplified using the primer OPA08

Discriminant classifications True cultivar[1]	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari	**30.0**	12.5	35.0	5.0	5.0	0.0	5.0	2.5	5.0	0.0
Bami	7.7	**33.3**	5.1	7.7	12.8	12.8	0.0	5.1	15.4	0.0
Hamedani	7.5	0.0	**40.0**	5.0	15.0	10.0	10.0	7.5	5.0	0.0
Esfahani	2.7	13.5	21.6	**21.6**	13.5	13.5	2.7	2.7	8.1	0.0
Moapa	0.0	0.0	5.4	5.4	**67.6**	5.4	13.5	2.7	0.0	0.0
Nikshahri	5.0	7.5	10.0	5.0	20.0	**50.0**	0.0	2.5	0.0	0.0
G.Oranga	2.7	5.4	8.1	2.7	2.7	2.7	**32.4**	18.9	8.1	16.2
Saranac	0.0	0.0	13.5	5.4	0.0	5.4	8.1	**51.3**	16.2	0.0
Wairau	2.9	11.8	14.7	2.9	0.0	5.9	20.6	8.8	**26.5**	5.8
Yazdi	0.0	0.0	0.0	0.0	0.0	0.0	5.4	0.0	2.7	**91.9**

[1]This definition is correct for cvs. Wairau and G.Oranga (standard seed lots for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'

Table 6.3.6 Tests of significance between the proportions of the seedlings from different cultivars which were correctly classified by the discriminant analysis of the RAPD results as belonging to each of the cultivars. The significant (P<0.05) results are indicated by stars '*' and not significant by 'ns'

From cultivar	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		ns	ns	ns	*	ns	ns	ns	ns	*
Bami	ns		ns	ns	*	ns	ns	ns	ns	*
Hamedani	ns	ns		ns	*	ns	ns	ns	ns	*
Esfahani	ns	ns	ns		*	*	ns	*	ns	*
Moapa	*	*	*	*		ns	*	ns	*	*
Nikshahri	ns	ns	ns	*	ns		ns	ns	*	*
G.Oranga	ns	ns	ns	ns	*	ns		ns	ns	*
Saranac	ns	ns	ns	*	ns	ns	ns		*	*
Wairau	ns	ns	ns	ns	*	*	ns	*		*
Yazdi	*	*	*	*	*	*	*	*	*	

Table 6.3.7 Pairwise comparisons between squared distances for the RAPD profiles from the cultivars amplified using the primer OPA08

From To	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari	0.0	1.5	1.2	1.9	5.5	4.6	4.0	4.2	2.7	12.5
Bami	1.5	0.0	2.2	1.5	4.5	2.8	5.1	3.2	2.6	13.9
Hamedani	1.2	2.2	0.0	0.9	3.7	3.5	3.2	2.4	2.2	13.0
Esfahani	1.9	1.5	0.9	0.0	2.7	3.0	4.6	2.9	3.0	16.5
Moapa	5.5	4.5	3.8	2.7	0.0	3.1	6.8	6.1	5.8	20.6
Nikshahri	4.6	2.8	3.5	3.0	3.1	0.0	7.6	6.2	5.6	16.7
G.Oranga	4.0	5.0	3.2	4.6	6.8	7.6	0.0	1.7	1.0	5.2
Saranac	4.2	3.2	2.4	2.9	6.1	6.2	1.7	0.0	0.9	9.2
Wairau	2.7	2.6	2.2	3.0	5.8	5.6	1.0	0.9	0.0	7.6
Yazdi	12.5	13.9	13.0	16.5	20.6	16.7	5.2	9.2	7.6	0.0

Table 6.3.8 Probabilities that pairs of LSMEANS of the RAPD profiles (directed by primer OPA08) of the given cultivars are equal.

	Azari	Bami	Hamedani	Esfahani	Moapa	Nikshahri	G.Oranga	Saranac	Wairau	Yazdi
Azari		0.1903	0.5304	0.0056	0.0001	0.0007	0.0001	0.0022	0.0004	0.0001
Bami	ns		0.4918	0.1368	0.0005	0.0396	0.0001	0.0001	0.0001	0.0001
Hamedani	ns	ns		0.0305	0.0001	0.0059	0.0001	0.0002	0.0001	0.0001
Esfahani	*	ns	*		0.0432	0.5863	0.0001	0.0001	0.0001	0.0001
Moapa	*	*	*	*		0.1285	0.0001	0.0001	0.0001	0.0001
Nikshahri	*	*	*	ns	ns		0.0001	0.0001	0.0001	0.0001
G.Oranga	*	*	*	*	*	*		0.0074	0.0355	0.0001
Saranac	*	*	*	*	*	*	*		0.6007	0.0001
Wairau	*	*	*	*	*	*	*	ns		0.0001
Yazdi	*	*	*	*	*	*	*	*	*	

The significant ($P<0.05$) results are indicated by stars (*)and not significant by 'ns', and the corresponding probability can be found in the upper right hand entries .

6.3.4.4 General linear models (GLM) procedure for dependent variable: CDF1

The GLM or ANOVA (Analysis Of Variance) procedure for the variable CDF1 (the first canonical discriminant function or combination of the variables that best discriminates among the cultivars, see section 3.2.7) in the RAPD study illustrated that there was a very highly significant difference ($P<0.0001$) between the RAPD profiles for the cultivars. The ANOVA table is as follows:

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	9	552.6	61.4	61.40	0.0001
Error	368	367.9	0.99		
Corrected-Total	337	920.6			

6.3.4.5 Duncan's Multiple range test for discrimination among seed lots of the cultivar based on the frequencies of RAPDs amplified using the primer OPA08

As there were significant differences among the RAPD profiles from the cultivars (the GLM result), the Duncan's Multiple range test was employed for variable CDF1 to further assess the significance of these differences. There were significant differences among the RAPD profiles for most of the cultivars (Table 6.3.9). As a result, the 10 cultivars were categorised into six groups overall, and the six Iranian cultivars were classified into four groups.

Cultivars Yazdi and G.Oranga gave RAPD profiles which were significantly different from each other and those for all of the other cultivars. There was also a significant difference between the RAPD profiles for the two internationally recognized cultivars, Saranac and Moapa, and also between G.Oranga and Wairau, the two New Zealand cultivars. Although there was no difference between the

RAPD profiles for cv. Azari and Hamedani, they differed significantly from the profiles for cv. Esfahani, Nikshahri and Yazdi. The RAPD profile of Azari was significantly different from that of cv. Bami.

Table 6.3.9 Duncan's Multiple range test for discrimination among the seed lots based on the frequencies of the RAPDs amplified using the primer OPA08. The calculated means in this table are the mean value for CDF1, the combination of the variables that best discriminates between the cultivars (see section 3.2.7). Cultivars linked by the same line do not differ significantly for the character at P<0.01.

	Name of cultivar	Mean	Duncan's grouping
1	Moapa	1.40	
2	Nikshahri	1.05	
3	Esfahani	0.93	
4	Bami	0.59	
5	Hamedani	0.44	
6	Azari	0.29	
7	Saranac	-0.41	
8	Wairau	-0.53	
9	G.Oranga	-1.03	
10	Yazdi	-2.95	

6.4 DISCUSSION

6.4.1 Genomic fingerprinting of lucerne cultivars using a combination of oligonucleotide primers

In RAPD analysis of animal genomes (eg. rat), the combination of two oligonucleotide primers in a PCR reaction mixture generated more distinct and reproducible RAPD bands than those produced using individual primers (Micheil *et al.*, 1993). This suggests that modifying the RAPD technique to use two primers together in a PCR reaction instead of one individual primer as originally used by Williams *et al.* (1990) and Welsh and McClelland (1990) might increase the chance of detecting more polymorphisms within, between and among cultivars of plant species.

The result from RAPD analysis of DNA from the 10 lucerne cultivars in this study was not consistent with that found by Micheil *et al.* (1993), as the number of distinct RAPD fragments generated by a combination of two primers was less than those produced by each used individually (Plate 6.3.1). This indicates there is no advantage from using a combination of two primers for RAPD analysis of plant species, a result also reported for lucerne by Barcaccia (1994), who demonstrated that a combination of two primers in the PCR reaction mixture inhibited the amplification of some major DNA bands and enhanced the production of several minor bands, characterised by low molecular weight. As the target DNA in both studies was from lucerne plants, further RAPD analysis of a number of different plant species with different primers could be necessary before making any final conclusion as to the advantage or disadvantage of using a combination of oligonucleotide primers for RAPD analysis of plant species in general.

6.4.2 Reproducibility of RAPD results

Since the development of the RAPD technique by Williams *et al.* (1990) and Welsh and McClelland (1990), the reproducibility of the RAPD fragments generated has been a major concern of many research workers (see Literature Review).

Variations in concentration of any of the components in the PCR reaction mixture (eg. Taq polymerase enzyme, deoxynucleotide triphosphates (dNTPs) and primers), may result in mismatching of the primers to non-complementary sequences of the template DNA (Innis *et al.*, 1990), and thus affect the reproducibility of the results. Mg^{+2} concentration for instance, may affect strand dissociation temperature of both template and PCR product specificity, formation of a template-independent artefact (termed primer-dimer), enzyme activity and fidelity (Innis *et al.*, 1990).

Barcaccia (1994) showed that Mg^{+2} concentration was critical for amplifying DNA from two different genotypes of lucerne. There was evidence that a concentration of magnesium chloride above or below 3.5 mM in the RAPD reaction could change the efficiency of the amplification and/or result in the complete lack of amplification products. Consistent RAPD results were found in this study (eg. Plates 6.3.3 and 6.3.10) with a concentration of 1.5 mM of magnesium chloride in the RAPD reaction mixture.

This lack of precise agreement between the results of the two studies for Mg^{+2} optimum concentration may well be related to differences in the temperature profiles (i.e. 93° C for 2 min followed by 40 cycles of 92° C (denaturation) for 45s, 37° C (annealing) for 30 s, 72° C (extension) for 1 min and 45 s and ending with 5 min at 72°C as used by Barcaccia (1994), vs 1 min at 94°C (5 min at the first cycle), 1 min at 40°C and 2 min at 72°C ending with a final extension at

72°C for 10 min as used in this study), or to the differences in the concentration of the PCR components used (i.e. Barcaccia (1994) used three times the genomic DNA used in this study (5ng vs 15 ng per 12.5 µl PCR reaction). He used Taq polymerase from Perkin/Elmer Cetus, while a similar concentration of Taq polymerase from a different manufacturer (Stratagene) was used in this study.

A high degree of reproducibility of the RAPD profiles was obtained in this work using two primers individually, and in combination, in replicated PCR reactions (Plate 6.3.1-6.3.3 and 6.3.6). This experiment was carried out using the same reaction mixture (section 6.3.2), but with different batches of Taq polymerase from the same supplier (Stratagene) for RAPD analysis of bulk DNA samples of the 10 lucerne cultivars. The results agree with those of Yu and Pauls (1993) who found reproducible RAPD results using genomic DNA from three lucerne cultivars with two primers for all three-five replicated reactions.

In summary, when experimental variables, for example: Taq polymerase brands; temperature profiles during DNA amplification; and the type of thermal cycler are not the same, it may not always be possible to generate identical RAPD profiles from a given genotype. Thus optimization of the PCR conditions by individual laboratories are necessary for obtaining reproducible results by each labratory, and standardization of the technique among laboratories will be necessary for comparison of results.

6.4.3 Application of the RAPD technique for genetic analysis

RAPDs have been shown by many workers to be a very efficient technique for DNA fingerprinting and genetic analysis of plant species, as it is a relatively simple and quick method for generating a substantial body of data on DNA polymorphisms. Despite numerous reports in the literature on the application of this technique for fingerprinting and genetic analysis of self-pollinating crops,

there are a very limited number of reports on the use of this technique for genetic analysis of out-crossing species, among which only a few reports are available for lucerne. This might be related to the genetic complexity of this species. A cultivar of lucerne is a heterogenous population comprising many genotypes. Therefore, analysis of a number of individuals is needed to describe the genetic structure of a cultivar. Although this is a time consuming process, such an analysis provides useful genetic information about variation among, and within cultivars.

6.4.3.1 Discrimination among 10 lucerne cultivars on the basis of frequencies of the RAPD fragments from single seedlings generated using a single primer (OPA08)

Random amplification of DNA from 40 individual seedlings from each of the 10 cultivars using primer OPA08 produced distinct, but very diverse RAPD profiles, clearly indicating genetic diversity within each individual population. This diversity of RAPD profiles was consistent with that reported for individual seedlings of another cross pollinating species, buffalograss, (Huff *et al.*, 1993).

Pairwise comparison of least squared means of the RAPD profiles from the cultivars illustrated that 86% of the comparisons for pairs of cultivars were significantly different ($P<0.05$, Table 6.3.8). The Duncan's Multiple range test also demonstrated significant differences ($P<0.01$) among the RAPD profiles of some of the cultivars, and as a result, the 10 cultivars were categorised into six groups overall. The six Iranian cultivars were classified into four groups (Table 6.3.9).

The results from RAPD analysis of the individual seedlings were not only able to demonstrate the polymorphism between genetically divergent cultivars (eg. Moapa and Saranac), but also had sufficient discriminatory power to detect

differences between closely related cultivars (eg. Hamedani and Esfahani).

The lack of significant differences between RAPD profiles from some of the cultivars (eg. Hamedani and Bami) does not necessarily mean that these cultivars are not genetically different. Each primer targets genomes at specific sequences, complementary to the sequence of the primer. The most suitable primers for generating polymorphisms are those which bind to more variable portions of the genome (i.e. are subject to base change resulting in addition or deletion of primer binding sites and hence result in synthesis or non-synthesis respectively of RAPD fragments). Although primer OPA08 was good, it might not be the best one to differentiate all of the cultivars on the basis of RAPDs from single seedlings of the cultivars. This is supported by the result from RAPD analysis of the bulk DNA samples of the cultivars (see 6.4.4), where, unlike primer OPA08, primer OPB19 was able to discriminate all 10 cultivars. This suggests that primer OPB19 might have been a better choice for the single seed analysis. As suitability of OPB19 was demonstrated later in the study from a further screening of oligonucleotide primers, this primer was not used for RAPD analysis of single seedlings of the cultivars.

6.4.3.2 Assessment of the genetic uniformity of lucerne cultivars on the basis of similarity of the RAPD profiles from individual seedlings generated using primer OPA08

Assessment of the genetic uniformity of the cultivars, as defined by the probability of seedlings within the seed lot being classified as from each individual cultivar, illustrated that there were significant differences in the uniformity of the cultivars (Table 6.3.5). For example Yazdi, an Iranian cultivar, was the most uniform (92% of the seedlings were classified as belonging to the cultivar), whereas Esfahani, another Iranian cultivar, was the least uniform in respect of the RAPD profile (21.9% of the seedlings being correctly classified

into the cultivar). The high genetic uniformity for cv. Yazdi is possibly due to the narrowing of the genetic range of the original population. This cultivar was most likely imported from the North West of Iran, which has a cold temperature (*i.e.* Azarbaijan, the proposed mother land for lucerne at about 38° N; Michaud *et al.*, 1988; Brummer *et al.*, 1991, Appendix 2.2), to the dry and warm area of Yazd Province (32°N) via natural selection. This uniformity might have been enhanced by inbreeding of the plants within the populations growing in these areas. The reason for the low genetic uniformity for cv. Esfahani is possibly due to the closeness of this cultivar and Hamedani (the other low genetic uniformity Iranian cultivar, Vaez Zadeh, 1994, pers. comm.), and possibly also the continuous use of seed lots of cv Hamedani by some farmers in the Esfahan area (Nadery Shahab, 1995, pers. comm.). Cross pollination among these imported seed lots and those locally adapted cultivars, which can happen as a result of lack of control of out-crossing among the cultivars, most likely increases the heterogeneity of the seed lots produced. For more explanation about the genetic uniformity of the cultivars, see Chapter 7.

A cultivar of lucerne is a heterogenous population, and can suffer severe inbreeding depression, resulting in a reduction in the forage yield of the inbred population (Rumbaugh *et. al.*, 1988). Tests of Busbice's predictive for assessment of inbreeding level in lucerne cultivars, on the basis of forage yield over multiple years and locations and generations, has been shown to be effective and accurate (Rowe and Gurgis, 1982), but this is a cost restrictive procedure. However, the inbreeding level of lucerne cultivars can be estimated on the basis of the uniformity of data from a RAPD analysis of individual seedlings, which in comparison with field evaluations can be performed in a short period of time, and is therefore more cost effective.

6.4.3.3 Estimation of genetic similarity among lucerne cultivars on the basis of RAPD profiles of individual seedlings generated using primer OPA08

Genetic relatedness among the 10 lucerne cultivars, determined by a comparison between squared distances (D^2) of the RAPD profiles of 40 seedlings from each of the cultivars, demonstrated that there was a wide range of relatedness among the cultivars. With a D^2 of 0.9, RAPD profiles from the Iranian cvs Hamedani and Esfahani (two known related cultivars, A. Vaezzadeh, 1994, pers. comm.) were the closest, while cv. Moapa (from USA) and Yazdi (from Iran) generated RAPD profiles which were the most distinct (D^2 = 20.6, Table 6.3.7). The D^2 between the four Iranian ecotypic cultivars Azari, Bami, Hamedani and Esfahani were low, ranging from 0.9 to 2.2, suggesting a close genetic origin. This result clearly demonstrates the potential of RAPD analysis coupled with the statistical technique of Canonical Discriminant Analysis for estimation of relatedness among the cultivars. However, as each primer only reveals genetic information about specific regions within the total genome that anneal with that primer, further RAPD analysis of the genomic DNA with other primers would be necessary to confirm the accuracy of this relatedness among lucerne cultivars.

The earlier genetic analyses of lucerne cultivars all involved a smaller number of plants per cultivar. The study by Yu and Pauls (1993) involved RAPD analysis of lucerne cultivars with a widely divergent genetic background released from *Medicago falcata* and *Medicago sativa.* They used DNA samples from up to seven individual plants from each of the cultivars and four and 10 individual oligonucletide primers in two separate experiments. The disagreement between the results from the two was interpreted by the authors as being due to differences in the number of the primers and plants used in these experiments (*ie.* five plants and four primers in the first, and seven plants and 10 primers in the second experiment), but the disagreement was more likely to be due to the small number of plants analyzed in their study. This sample size most likely cannot represent

the genetic construction of the cultivar of an outcrossing species such as lucerne. These results indicate that care must be taken in the sample sizes utilized when analysing heterogenous populations such as lucerne cultivars.

A sample size of 20 individual plants per population was chosen by Brummer *et al.* (1991) for genetic analysis of lucerne cultivars using RFLPs, while Kidwell *et al.* (1994) used 12 plants per population. In the present work genomic DNA samples from 40 individual seedlings from each of the cultivars were analyzed. Although analysing a larger sample size of lucerne plants might reduce statistical error relating to the within cultivar variation, a practical approach to the genetic analysis also needs to be taken into account. This sample size was the maximum number of the PCR products samples which could be loaded onto a single 20 cm wide electrophoresis gel with narrow wells. This sample size was more than that suggested by Cliff (1987) to be adequate for comparison among populations using multivariate analysis (ie. a sample size of 30). In addition to the sample size, selection of an appropriate sampling method also has a critical influence on the accuracy of the results. Irrespective of the sample size, if the sample size is not randomly selected, it may not represent the gene pool of a cultivar, particularly in the case of a cross pollinating species such as lucerne, in which a cultivar is a heterogenous population.

6.4.4 Genomic fingerprinting of lucerne cultivars on the basis of the RAPD profiles from bulk DNA samples of the cultivars generated by an individual primer (OPA08)

Discrimination of lucerne cultivars by RAPD analysis of individual seedlings is time consuming and, most likely is not a cost effective method for the purpose of discrimination among cultivars. One approach to overcome this problem is to bulk genomic DNA samples prior to the RAPD analysis. This was first practised by Michelmore *et al.* (1991) for finger printing of peanut cultivars and, among

others, later on by Yu and Pauls (1993) for RAPD analysis of three heterogenous populations of lucerne and Mailer *et.al.* (1994), for discrimination among rapeseed cultivars (see Literature Review).

In this study twenty six oligonucleotide primers were screened for amplification of RAPD fragments from the bulk DNA samples of the cultivars (made up from equal amounts of DNA from 40 individual seedlings from each of the cultivars). Among these primers, four of them generated distinct and easily evaluated polymorphic DNA fragments (OPB19, OPB13, OPC10, OPA08, Plates 6.3.3-6.3.6).

Primer OPB19 generated the greatest number of RAPD fragments and allowed all of the 10 cultivars to be discriminated (see Plate 6.3.3 and Table 6.3.1). This suggests that the technique is powerful enough not only to discriminate cultivars with divergent genetic background (eg. Moapa and Saranac), but also to illustrate genetic polymorphisms between closely related cultivars (eg. Hamedani and Esfahani, the two Iranian cultivars).

The second most useful primer of this study (OPB13), produced RAPD fragments which allowed all except two cultivars (Azari and Moapa), to be discriminated. However, this and the other primers (*ie.* OPA08, and OPC10) provided polymorphisms which were sufficient for discrimination among all of the cultivars (see Table 6.3.2-6.3.4) if the results for these primers were combined. This indicated that if one individual primer could not generate sufficiently polymorphic profiles to enable discrimination among cultivars for all of the cultivars, differentiation could rapidly be obtained by using the results from two or more primers.

6.4.5 Summary

Primer OPA08 generated RAPD profiles from individual seedlings which enabled 86% of the pairwise comparisons between LSMEANS of RAPD profiles from the cultivars to be significantly different (Table 6.3.8). This primer also generated RAPD fragments from the bulk DNA samples of the cultivars by which 88% of the pairwise comparisons were different (Table 6.3.3). This suggests that RAPD analysis of the bulk DNA samples of cultivars offers similar discriminatory power to that of individual plant analysis.

The great potential for discrimination among the cultivars (even those cultivars which are closely related), the genetic information provided about variation within and among the cultivars, and the reproducibility of the results make the RAPD technique a powerful tool for DNA finger printing and also genetic analysis of lucerne cultivars because:

1. There is no need for the isolation of cloned DNA probes, such as is required for RFLP analysis. Libraries of arbitrary 10-base primers are now commercially available. This provides at a minimal cost a vast range of potential primer sequences that give the technique great diagnostic power.

2. The RAPD method requires only small amounts of DNA, and often small miniprep procedures yield a sufficient quantity and quality.

3. The technique involves fewer steps than RFLP analysis and is faster to perform.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

With the development of plant breeding there has been a parallel development of programs for safeguarding the genetic quality of the seed produced. Seed certification schemes generally ensure that the seed sold to farmers is true to the cultivar's characteristics, and therefore reproduces the characteristics of new and improved cultivars. Organisations such as UPOV were also established to provide the testing techniques required to ensure that any new cultivar released by plant breeders is in fact a new cultivar, and not a previously registered one. Since the start of both seed certification and DUS testing, field-based plot testing, using plant morphological and physiological characters, has been used for verification, identification and/or characterisation of cultivars of different species. However, this method is a time-consuming and expensive process, requiring substantial areas of land and highly skilled personnel. Because of the steady increase in the number of new cultivars, many of which are closely related, there will be a growing need for finding rapid, precise, and cost-effective techniques for cultivar verification and identification.

The potential of new techniques (eg. electrophoresis) for the verification and description of cultivars, has been investigated over a considerable period of time. As a result, for some self-pollinating or autogamous crop species (eg. wheat, barley, and maize) standard electrophoretic methods for registration of new cultivars have been accepted (UPOV, 1995). Electrophoretic techniques have also been accepted by the International Seed Testing Association for verification of commercial seed lots of cultivars from several self-pollinating species (eg. wheat, barley and pea).

Unlike cultivars of self-pollinating species, a cultivar of a cross-pollinating species is a population of plants comprising many genotypes expressing a range of plant morphological characters. A cultivar of such a species contains different

combinations of homozygous and heterozygous genes, including those encoding seed storage proteins or isozymes (Cooke, 1995a). Thus, there are major problems associated with the verification and identification of cultivars of these species (Wright, *et.al.*, 1983). Extensive work has been directed towards finding an effective technique for discriminating among and identifying cultivars of ryegrass (eg. Hayward and McAdam, 1977; Gilliland *et al.*, 1982; Gilliland, 1982; Gilliland, 1983; Ferguson and Grabe, 1986; Nielsen *et al.*, 1985; Gardiner *et al.*, 1986; Gardiner and Forde, 1987; Gardiner and Forde, 1988a 1988b). This species is the only cross-pollinating or allogamous species for which the electrophoresis of seed storage protein has been accepted by the International Seed Testing Association for verifying commercial seed lots of cultivars (ISTA, 1992; Cooke, 1995b). The possible application of isozyme electrophoresis for registration of new cultivars of this species has been also considered (UPOV, 1995). However, little work has been reported in the literature about the potential of molecular techniques for discrimination among cultivars of cross-pollinating lucerne species. Also no literature was found on the application of machine vision for discrimination among, and/or verification of lucerne cultivars. In this study, along with evaluation of the reproducibility of results from the plot testing method, the usefulness of alternative laboratory techniques, such as image analysis of seed and plant leaflets, and molecular techniques for discrimination among and verification of lucerne cultivars, was assessed. An overview of the results of this study is presented in this chapter.

7.2 Evaluation of the plot testing, molecular and image analysis techniques for registration of lucerne cultivars

7.2.1 Test of distinctness

7.2.1.1 Use of plot testing for cultivar discrimination among lucerne cultivars

Most of the morphological characters recommended by UPOV (1988) and OECD

(1988) for verification of lucerne cultivars were affected by environmental factors. Only three characters *i.e.* number of plant stems immediately above the ground, plant recovery height, and leaflet width/length ratio were stable, and thus could be used as reliable morphological discriptors for lucerne cultivars. However, none of the individual characters, or any combination of characters, was sufficient to differentiate all of the cultivars at $P<0.01$, which is the significance level required by UPOV (1988) for any apparent differences among lucerne cultivars. These results clearly show that plot testing is not an effective method for discriminating among lucerne cultivars, and suggests there is a need for more precise and effective techniques.

For the majority of plant characters recorded, better cultivar discrimination was obtained in the glasshouse than in the field. However, none of the characters were able to discriminate all of the cultivars (see 2.4.10). Improved discrimination in the glasshouse was most likely due to reduced environmental variation compared with conditions in the field. Variation in the field environment produced larger standard errors which produced more overlapping results. This suggests that distinctness testing of lucerne cultivars using morphological characters of growing plants under controlled conditions would be more effective than plot testing, and supports the recommendation (ISTA, 1993) for the use of growth chamber methods for lucerne cultivar differentiation. ISTA (1993) recommend that plants be grown in a growth chamber under a 24-hour photoperiod at 25° C, and for time of flowering and flower colour (scored as purple, variegated or yellow) to be recorded from the growing plants. It has also suggested (ISTA, 1993) that for recording plant height and growth habit (to be scored as upright or rosette forms), plants could be grown under a 12-hour photoperiod with cool temperature. This method is much quicker than the required plot testing method, as all of the morphological characters are recorded from seedlings and therefore, data collection is completed within five weeks. However, there are some problems associated with this method. Firstly it may not be cost effective, as it requires the maintenance of a controlled environment,

in terms of both light intensity and temperature. Secondly while it may be useful for cultivar verification, for which plant morphological characters of seed lots from the same cultivar are compared, it may not, as found in the glasshouse (see 2.3), be effective for discriminating among the cultivars under test. Reports in the literature (eg. Clapham and Almgard, 1976; Wright *et.al.*, 1983; Nielsen, 1985; Walton *et al.*, 1988; Brummer *et al.*, 1991; Gardiner and Forde, 1992; Kiss *et al.*, 1993; Steiner, 1993; Vieritz, 1993; Steiner, 1993; Kidwell *et al.*,1994; Cooke, 1995a; Faville *et al.*, 1995) suggest that electrophoretic techniques could be very good alternatives to plot testing.

7.2.1.2 Use of electrophoresis of seed storage protein for discrimination among lucerne cultivars

Electrophoresis of protein extracted from bulked seed of the seed lots of eight Iranian and two New Zealand cultivars failed to differentiate the majority of cultivars. This, and the result obtained by Gardiner and Forde (1992), suggests that SDS-PAGE of protein extract from bulked seed is not a feasible method for lucerne cultivar discrimination. However, when a pairwise comparison was made between the standard seed lots of the eight cultivars, 82% of the cultivars could be differentiated ($P<0.01$) using a combination of the results from intensity of the selected protein bands which resulted from SDS-PAGE of individual seeds of the cultivars (see Table 4.3.5). Since in self-pollinating species such as wheat, barley and oats, which have limited variation within cultivars, all cultivars could not be discriminated using SDS-PAGE analysis of seeds (Curtis and Chadwick, 1983), this result is very promising for discrimination among cross-pollinating lucerne cultivars, particularly as some of them are closely related. Besides offering a better discrimination value than plot testing, SDS-PAGE of lucerne seed coupled with densitometric analysis of results also has the following advantages:

1. Proteins are the direct product of gene translation and transcription and therefore

can be considered as markers for the structural genes that encode them (Cooke, 1995a).

2. The speed of the test (it is possible to obtain information about the cultivar identity in 24-48 hours) and the reproducibility of the protein banding pattern (ISTA, 1992).

3. Freedom from subjective assessment of the results, such as that which occurs when scoring some morphological characteristics of plants.

4. Elimination or reduction of requirement for land or plant growth facilities

5. Ease of storage of the seed.

As all of the cultivars could not be discriminated using SDS-PAGE of individual seeds of the cultivars, two more modern molecular techniques (Restriction Fragment Length Polymorphism (RFLP) and Random Amplified DNA Polymorphisms (RAPD), which have already been shown to be extremely powerful for detection of DNA polymorphism among individuals and populations of plants of some crop species, were employed to determine if a better discrimination among lucerne cultivars could be achieved.

7.2.1.3 Use of the RFLP technique for discrimination among lucerne cultivars

No clear DNA polymorphism was detected among the 10 cultivars using bulked DNA samples of the cultivars and the apple rDNA and clover ADH probes. Similarly no fragments unique to a particular lucerne population were reported by Brummer *et al.* (1991). This suggests therefore that DNA fingerprinting of lucerne cultivars, as has been done in inbreeding rice using a human minisatellite probe (Dallas 1988), does not appear feasible on the basis of the RFLPs from

bulk DNA samples of the cultivars. However, 91% of pairs of the cultivars were differentiated ($P<0.05$) solely on the basis of DNA polymorphism detected by two probes from DNA extracted from 40 individual seedlings of each of the cultivars. The results of this study were promising, as not only cultivars with a divergent genetic background (eg. Moapa and Saranac) were discriminated from each other, but cultivars which are closely related (eg. Esfahani and Hamedani) were distinguished on the basis of the restriction fragment length polymorphism detected. Since Southern blots from cultivars could be hybridised in succession with other different probes this provides a further possibility for detecting even more polymorphism among the cultivars under examination. In addition, a combination of different restriction enzymes and probes would add even more discrimination value to the RFLP technique. Despite being an extremely powerful tool for detection of polymorphism among lucerne cultivars, there were problems associated with the reproducibility of RFLP results from the hybridisation of the majority of the cloned DNA probes, including all of the lucerne and clover probes screened in this study. Lack of reproducibility at low levels of detection sensitivity with the RFLP technique has also been observed by other workers (Bouton, 1994; Gardiner, 1994, pers. comm.). RFLP analysis comprises several steps *ie.* preparation of the probe (amplification and purification of cloned DNA), DNA isolation from plant tissue and purification, electrophoresis and blotting, hybridisation and washing the membrane, and signal detection. Many factors, in particular purity of the chemicals, activity power and freshness of the signal detection reagents for chemiluminescent detection (Gardiner, 1995 pers. comm) may affect the results during these procedures. Therefore it is extremely difficult to specify the precise factors associated with any failures with this technique, and the lack of reproducibility would be a major concern for the employment of this technique for the test of distinctness for registration of new cultivars. However, the reproducibility of the result from one of the probes screened in this study (apple ribosomal DNA, rDNA) suggested that this problem could be overcome, providing more suitable high copy number probes could be obtained.

7.2.1.4 Use of the RAPD technique for discrimination among lucerne cultivars

Of the twenty-six 10-base oligonucleotide primers tested individually to amplify RAPDs from bulk DNA samples of the 10 lucerne cultivars, four generated distinct RAPD bands and interestingly, one generated distinctive RAPD profiles for all 10 cultivars. This was a very promising result, as the bulked DNA technique was not only able to demonstrate the polymorphism between genetically divergent cultivars (eg. Moapa and Saranac), but also had sufficient discriminatory power to detect differences between closely related cultivars (eg. Hamedani and Esfahani). This study also demonstrated that a high degree of reproducibility of the results obtained by the RAPD technique is possible. However, care should be taken in the selection of primers, and optimisation of PCR conditions for the target species including replication analysis, to ensure that only reproducible bands are scored.

RAPD analysis of the bulk sample of cultivars fails to meet the two basic UPOV requirements for registration of new cultivars, as firstly uniformity of the cultivars is not examined and secondly, there is no genetic interpretation of the differences (UPOV, 1995). In considering the possible application of electrophoretic methods, UPOV has already firmly established the principle of linking distinctness and uniformity for each characteristic. Therefore, uniformity of a cultivar must be confirmed by analysing individual plants (UPOV, 1995).
To fulfil the UPOV requirements for registration of new cultivars, the RAPD technique was used for genetic analysis of individual plants of the 10 cultivars. Pairwise comparison between least squared means of the 10 cultivars on the basis of frequencies of the RAPD fragments from the DNA extracted from individual seedlings of the cultivars which were amplified using primer OPA08 demonstrated that 86% of the cultivars could be discriminated. The lack of differences between RAPD profiles from some of the cultivars does not necessarily mean that these cultivars are not genetically different, or that the technique is not powerful

enough to differentiate lucerne cultivars. Each primer targets the genome at specific sequences, complimentary to the sequence of the primer. The most suitable primers are those which bind to more variable portions of the genome. Therefore, primer OPA08 might not be the best one to differentiate all of the cultivars on the basis of RAPDs from single seedlings. Results from assessment of a number of primers for their ability to reveal genetic diversity by screening them over DNA bulked from a number of seedlings from each cultivar demonstrated that primers do vary in their power to generate fragments which are polymorphic between lucerne cultivars and that primers OPB13 and OPB19 could reveal more polymorphism than OPAO8 among cultivars on the basis of RAPDs of individual seedlings. RAPD analysis offers several advantages for the genetic analysis of lucerne cultivars:

1. There is no need for the isolation of cloned DNA probes, such as is required for RFLP analysis. Libraries of arbitrary 10-base primers are commercially available. This provides, at a minimal cost, a vast range of potential primer sequences that give the technique great diagnostic power.

2. The RAPD method requires only small amounts of genomic DNA, and often small miniprep procedures yield sufficient quantity and quality.

3. The technique involves fewer steps than RFLP analysis and is faster to perform.

4. The great potential for discrimination among the lucerne cultivars (even those cultivars which are closely related), the genetic information provided about variation within and among the cultivars (see later), the reproducibility of the results, and also the already mentioned advantages over the other RFLP technique, make RAPD an excellent alternative to plot testing.

7.2.1.5 Use of image analysis for discrimination among lucerne cultivars

To date, the majority of the research on the application of image analysis for classification of cultivars of crop species has been conducted for discrimination among cultivars of wheat and barley on the basis of seed morphological characters and derived parameters (see 3.1.4). However, no report was found in the literature on the use of this technique for verification of, and discrimination among cultivars of cross-pollinating species, such as lucerne. The published results with other crop species suggest that image analysis can serve as a powerful automated tool for discrimination among cultivars of plant species using seed or leaves (see 3.1.4). However, reports in the literature (eg. Draper and Travis, 1984) suggest that the success of this technique relies on the selection of useful morphological characters and the derived parameters. The success is also dependent on the application of an effective statistical analysis process in order to obtain maximum advantage of output data (Cooke, 1995b). The results of this present work illustrate that image analysis can provide an extremely useful data base for both discrimination among, and verification of lucerne cultivars.

From the 21 morphological characters and derived measurements recorded from individual seeds of eight Iranian and two New Zealand cultivars, 10 were useful for verification of (see 3.4.1) and discrimination among the cultivars (see 3.4.2). Analysis of variance (SAS, 1989) of the data from seed morphological characters showed that the characters red/total intensity, and blue/total intensity had the highest discriminant function. Sixty-four percent of the cultivars could be differentiated using each of these characters. A better discrimination (86%) among the cultivars was obtained when a combined value of the 10 most useful characters from the Canonical Discriminant Analysis (CDA, SAS, 1989) was used for differentiation among the cultivars (see 3.4.4). This clearly demonstrates the benefit of an effective method of statistical analysis for discrimination among cultivars of a crop species. Interestingly, a further discrimination improvement

(to 94%) was achieved using the result from the 10 selected individual characters and the combination of the useful seed characters (see 3.4.4). The failure of discrimination among the rest of the cultivars might be simply a reflection of within-cultivar variation. In this case, the discrimination power of the technique might be improved by analysing a larger sample size (Mead and Curnow, 1983), although this was not determined in this study.

This result clearly demonstrates the potential of image analysis coupled with canonical discriminant analysis. The highest discrimination potential plus other advantages over the other techniques used in this study *i.e.* speed of analysis (a maximum of five minutes for 50 individual seeds timed from receiving the sample to having the data ready for analysis), lower running cost; no need for chemicals; ease of operation of equipment, make seed image analysis the best alternative to plot testing for testing of distinctness and registration of lucerne cultivars. Unlike seed image analysis, only 67% of the cultivars were differentiated using image analysis of leaflets of individual plants (see 3.4.10). This suggests that image analysis of the plant leaflet is not an effective method for DUS testing. In addition, morphological data should be recorded from leaflets of individual second year plants growing in plots (OECD, 1988; UPOV 1988). This is a time consuming practice.

7.2.2 Application of molecular techniques and image analysis for testing the uniformity or homogeneity of lucerne cultivars

For seed protein and DNA analysis, uniformity was assessed using similarity of protein and DNA banding profiles from individual seeds and seedlings from each of the seed lots of the lucerne cultivars (see 4.4.4, 5.4.4 and 6.4.3.2). For seed image analysis, uniformity of the cultivars was assessed on the basis of the proportion of uniform seeds in each seed lot (see 3.4.5).

The majority of the cultivars tested in this study were apparently more uniform on the basis of protein banding pattern than for the DNA profiles (from both RAPD and RFLP analysis of seedlings, see Table 7.1). Cultivars had very divergent uniformity on the basis of seed morphological characters; uniformity ranging from 14-71% (Table. 7.1). When the molecular results are compared for the cvs. excluding G. Oranga and Yazdi, the only methods showing comparable trends of cultivars from high to low uniformity were RAPDs and the RFLP markers using the ADH probe. There appeared to be no consistency in these results with the seed morphological results. Further investigation would be needed to find the most reliable method for testing cultivar uniformity, preferably using a larger sample size from seed lots of successive generations from a number of cultivars. One might expect the method examining the largest number of unrelated loci in the genome to be the most reliable.

A gene mapping study in apple (Hemmat *et.al.*, 1994) showed that most of the time the RAPD fragments generated by a single primer are assorted independently over the plant genome. As a result of being dominant (Hemmat *et.al.*, 1994), each RAPD fragment represents one locus on the plant genome. Therefore, the nine RAPD fragments scored in this study (see 6.2.4), could be distributed in up to nine regions of the lucerne genome. Use of extra primers would certainly provide more genetic information about the uniformity of the cultivars.

7.3 Application of molecular and image analysis techniques for verification and certification of lucerne cultivars

To determine whether the requirements of the OECD scheme for seed certification (see 2.1.3) could be fulfilled using seed image analysis and SDS-PAGE of seed storage proteins, the results using seed characters and protein profiles from

individual seeds of seed lots of each of the cultivars and the standard[1] seed lots of the cultivar were compared.

Of the 21 seed morphological characters recorded from individual seeds of seed lots of the cultivars, 10 characters were able to place the certified seed lots of the two New Zealand cultivars (G.Oranga and Wairau) into their cultivar groups (see 3.4.1), suggesting that these characters are potentially valuable for lucerne cultivar verification.

It might be expected that all certified seed lots of a cultivar should have characters which do not differ from the standard seed lot of the cultivar. However, results from this study for both types of characters (Table 3.3.10) suggest that this may not be always the case. Unlike cultivar Hamedani, for which the standard and the other seed lot of this cultivar had similar seed characters, there were significant differences between seed lots of cv. G.Oranga and the standard seed lot of this cultivar (see 3.3.2.3.5). For example, despite there being no differences between seed characters of the two Basic seed lots of this cultivar, they differed significantly from the standard seed lot (Breeders seed lot) of this cultivar (see 3.3.2.3.5).

Comparisons between protein banding profiles of seed lots from the same cultivars (see section 4.3.3.5) also illustrated a significant difference between seed lots of some of the cultivars including G.Oranga. For example, the protein banding profile of seed lots of this cultivar differed from the Breeders seed lot of this cultivar.

A number of factors, such as contamination of the seed lots at harvest, or during

[1] This definition is correct for cvs. Wairau and G.Oranga (standard seed lots for the cultivars), but this may not be the case for the other cultivars. However, they were all supplied as being 'true to cultivar'.

seed cleaning and processing; error in the sampling of the seed lot being analyzed; out-crossing between seed lots from different cultivars; and genetic shift within the cultivar because of some factors other than out-crossing between seed lots of different cultivars (see section 4.4.6), might be associated with differences between seed characters and protein profiles of seed lots of the same cultivar.

Steiner *et al.* (1992) suggest that genetic shift is highly likely to occur in a lucerne cultivar, as a cultivar of this species comprises different genotypes, expressing various morphological characters. For example, flower colour in lucerne usually ranges from very light to very dark purple, although there can be variegated and cream flower coloured plants in a population. There is also inter-cultivar variation for canopy type of the plants, and time of flowering.

Lucerne plants must be cross-pollinated to produce good quality seed, and tripping of blossoms by bees is necessary for cross-pollination (Carlson, 1935; Rincker *et al.*, 1988). Pollinators of lucerne plants generally prefer the purple flower colour (Loper and Waller, 1970; Kehr, 1973; Goplen and Brandt, 1975; Steiner *et al.*, 1992). In addition, purple is a wide spectrum flower colour in lucerne, including a broad range from very light to very dark purple, and hence, some differences in attraction for the pollinator might be present even in a population of plants with 'purple' flowers. Boren *et al.* (1962) and Kauffeld *et al.* (1969) have reported that different genotypes of lucerne are not equally attractive for pollinators for reasons other than flower colour. This might be as result of the genotype's potential for pollen production (Tysdal and Crandall, 1948) which is important for attraction of pollen collecting leafcutter bees (Kehr, 1973); nectar production for nectar collecting honey bees (Pedersen, 1953 and Kehr, 1973), and canopy structure *i.e.* ease of access for the pollinators to visit the flowers.

Assessment of flower colour for some of the cultivars in the field showed for

example that 21% of the plants of cv G.Oranga had light purple, 19% purple, 34% dark purple, and 25% variegated flowers, and this result was similar to that in the glasshouse (see 2.4.5). It is possible that because of pollinator preference, there would have been more chance for the purple flowered plants in the population of the Breeders seed lot of cv. G.Oranga to set quality seed than the others, and therefore the characteristics of the following generation seed lot (*i.e.* Basic seed) would have shifted toward the genotype with purple flowers. However, this has not been substantiated.

Observations of flowering plants in the field indicated a considerable difference among the flowering time of individual plants within each of the cultivars in the trials. For example, this was 22 days for cv. G.Oranga. This suggests that genetic shift might also happen because of the removal of very early genotypes due to shedding of mature seed prior to harvest, and also the exclusion of immature seeds from late genotypes, because at harvest they were too small to be collected by the harvester, or were screened out during seed cleaning.

The seed lots of cv. G. Oranga tested in this study were certified under the OECD scheme, and therefore passed through all of the required processes, including plot testing. This suggests that the scheme was not able to detect this small genetic shift, and indeed it was not set up to do so. Its major role is to check the possibility of major shift, and/or gross contamination in seed lots.

These results clearly show that SDS-PAGE of individual seeds and seed image analysis are very useful for lucerne cultivar verification. However, further work is needed before drawing any conclusions as to the possibility of using these modern techniques to replace the time consuming and costly plot testing *i.e.*

1. To check whether differences among certified seed lots of a cultivar as revealed by seed image analysis can be detected by plot testing, or whether the former

technique is too sensitive to be used as a practical method for seed certification.

2. If seed image analysis can be used, it will be necessary to determine what probability level for the differences between the seed lots of a given cultivar may be appropriate for lucerne certification.

3. It is usual practice in a plot test to make a direct comparison between a seed lot and the standard seed lot of the named cultivar. No report was found in the literature to suggest whether successive generations of a cultivar which have been certified are morphologically the same. A comparison between results from seed image analysis and/or SDS-PAGE of seed with morphological data from different seed lots of a number of cultivars in a plot test could illustrate whether plot testing would be able to reveal any genetic shift in a cultivar during seed multiplication.

4. There is certainly need for further work to test the usefulness of RFLP and RAPD analysis for verification of lucerne cultivars.

7.4 Genetic analysis among and within cultivars of lucerne using three molecular methods and seed image analysis

Lucerne is outcrossing (allogamous) and is characterized by tetrasomic inheritance, multiple allelelism and inbreeding depression (Barcaccia, 1994). The breeding of new cultivars is by intermating a number of selected genotypes (up to 100 in modern cultivars) followed by the selection of a population of superior individuals based on phenotypic characters such as vigour and yield (Yu and Pauls, 1993; Barcaccia, 1994). Seed of a cultivar is derived initially from interbreeding within this nucleus population and later from further multiplication for agricultural use. Because of the pronounced effects of inbreeding depression

in this crop, breeders need to be able to estimate both the degree of relatedness between existing parental material (cultivars or ecotypic cultivars) and also the degree of uniformity among the individuals in these populations in order to breed for maximum heterosis.

Along with evaluation of the four laboratory techniques (*i.e.* RFLP, RAPD, SDS-PAGE of seed, and seed image analysis) for tests of distinctness, uniformity and verification of the cultivars, the usefulness of these techniques for detection of genetic relationship among the cultivars, defined by the squared Mahalanobis distances (D^2, see 3.2.7), between pairs of cultivars was also assessed. It was hypothesised that the greater the genetic similarity between two cultivars, the smaller the D^2. To test this hypothesis, a comparison of the D^2 for each of the methods was made between two Iranian ecotypic cvs., Azari and Bami, which are likely to be genetically related, and two cvs. which are unlikely to be related, cv. Azari and a New Zealand cultivar (G.Oranga). For all methods the D^2 was less between cvs. Azari and Bami (Iran) than between cvs. Azari and G. Oranga (Table 7.2) which suggests confirmation of the above hypothesis. This relationship was explored for other cultivar pairs (Table 7.2), and was found to hold for the majority of these comparisons. For example, with the exception of the same genetic distance between the cultivar pairs Nikshahri/Esfahani, and Nikshahri/Azari which were calculated using RFLPs from the rDNA probe, D^2 detected by all other techniques between cvs. Nikshari and Esfahani was less than between cvs. Nikshahri and Azari (Table 7.2). This is consistent with the geographical adaptation, and dormancy level, of these cultivars. Azari is an autumn-dormant ecotypic cultivar derived from an area within the centre of origin for lucerne (Michaud *et al.*, 1988), in the province of Azerbaijan (1362m and 37.5-38.5°N, Appendix 2.2). All other Iranian ecotypic cultivars including Esfahani and Nikshahri, have likely moved out from this region, and become adapted in new areas. Nikshahri is a very non-dormant cultivar from the region of lowest altitude in southern Iran (Nikshahr, 566m, 26-27°N, see Appendix 2.2)

which has a hot summer and warm winter (see Appendix 2.3). Cv. Esfahani is an autumn non-dormant ecotypic cultivar from the province of Esfahan, a high altitude region in the centre of Iran (1575m, 32.5-34°N) with a warm summer and relatively cold winter.

Both RFLP analysis of individual seedlings of the cultivars using clover ADH and rDNA probes, and RAPD analysis using primer OPAO8 showed that the genetic distance between cvs. Hamedani and Esfahani was less than that for cvs. Hamedani and Nikshahri (Table 7.2). This result was consistent with the known genetic background and the geographic adaptation of these ecotypic cultivars. A large proportion of the genotypes of cv. Esfahani have been derived from Hamedani, a semi autumn-dormant cultivar (Vaezzadeh, 1994, pers. comm.) as they come from adjacent regions (latitudes 32.5-34°N vs 35-36°N) and very similar altitudes (1575m and 1646m) respectively (see Appendix 2.2). However, the result from seed image analysis for these cultivars was not in agreement with that from SDS-PAGE, RFLP and RAPD analysis. This could well be related to differences in the nature of the techniques for genetic analysis of the cultivars. Plant morphological data (Dehghan-Shoar, unpublished data) and the result from genetic analysis of cultivars (see 5.4.4.2 and 6.4.3.2) suggests that Esfahani and Hamedani were the least uniform among the Iranian cultivars. The differing result from these two techniques may also be simply related to inadequate sample size. However, this needs to be confirmed.

The genetic distance detected using seed image analysis showed a greater similarity between the pairs of cvs. Hamedani (Iran) and Wairau (New Zealand) than that between the pairs of cvs. Hamedani and G.Oranga, the other New Zealand cultivar (Table 7.2). This was consistent with the D^2 detected by SDS-PAGE of seed storage protein, RFLP (using the clover ADH probe) and RAPD analysis. However, it was not in agreement with the result from RFLPs detected by the rDNA probe. This might be related to differences in the nature of these

techniques for the detection of genetic relatedness among the cultivars. For example, different regions of the lucerne genome would be targeted by each of the ADH and rDNA probes and RAPD primers. However, as already mentioned in section 7.2, the disagreement between some of the results might be simply related to the small sample size used in this study. Further work is required to test whether this is the case.

This study has produced some promising results in terms of consistency for genetic relatedness from the three molecular methods and seed image analysis, despite the different nature of these analyses. This suggests that these techniques could be useful for the detection of parentage and origin, which is important in plant breeding (Barcaccia, 1994). More accurate estimation for both genetic relatedness among cultivars and cultivar homogeneity might be possible using a larger sample size. This would be most practical using seed image analysis, the most rapid, simple and the cheapest technique amongst those used in this study. However, analysis of a larger sample size from a number of cultivars with known genetic background would be needed before drawing any final conclusion as to the application of these techniques for estimation of relatedness and uniformity of lucerne cultivars.

Table 7.1 Uniformity (percentage of individuals within a cultivar with similar characters) within lucerne cultivars using three molecular methods plus seed image analysis.

Technique / Cultivar	RAPD using primer OPAO8	RFLP		SDS-PAGE of seed storage protein	Seed image analysis
		using ADH probe	using rDNA probe		
Azari	30.0	30.0	35.0	75.0	62.0
Bami	33.3	37.0	22.5	70.6	15.0
Esfahani	21.0	22.0	10.2	56.0	39.0
Hamedani	40.0	27.0	38.0	70.0	19.0
Nikshahri	50.0	53.0	10.0	50.0	31.0
G. Oranga	32.0	56.0	22.4	41.0	71.0
Wairau	26.5	33.0	62.5	58.0	17.0
Yazdi	91.0	36.0	7.5	79.0	14.0

Table 7.2 Comparisons between genetic relatedness (D^2) of pairs of cultivars detected using three molecular methods and seed image analysis

Technique / Cultivars	Seed image analysis	SDS-PAGE of seed storage protein	RFLP		RAPD using primer OPAO8
			using ADH probe	using rDNA probe	
Azari vs G. Oranga	6.45	8.2	3.2	2.1	4.0
Azari vs Bami	3.53	7.3	2.6	1.2	1.5
Hamedani vs G. Oranga	2.89	7.4	3.3	1.4	3.2
Hamedani vs Esfahani	1.84	12.4	1.7	1.6	0.9
Hamedani vs Wairau	0.65	4.3	2.2	3.8	2.2
Hamedani vs G.Oranga	2.89	7.4	3.3	1.4	3.2
G.Oranga vs Bami	2.39	9.1	3.6	1.0	5.1
G.Oranga vs Nikshahri	6.64	14.1	3.4	1.8	7.6
Nikshahri vs Esfahani	0.25	5.1	1.6	1.2	3.0
Nikshahri vs Azari	4.02	11.3	3.0	1.2	4.6
Wairau vs Azari	2.89	9.5	2.6	1.7	2.7
Wairau vs Nikshahri	2.06	14.3	4.2	0.1	5.6
Hamedani vs Esfahani	1.84	12.4	1.7	1.6	0.9
Hamedani vs Nikshahri	1.55	15.4	2.9	3.3	3.5
Yazdi vs G.Oranga	5.60	10.3	3.7	1.7	5.2
Yazdi vs Wairau	1.74	9.6	3.7	0.5	7.6

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Appendices

Appendix 2.1

a): Soil analysis data from field trials

pH	Olsen P	SO_4	Exch. K	Exch. Ca	Exch. Mg	Cond.	% SS
6.4	29.0	8.5	0.34	11.0	1.1	1982	0.001

Phosphate and sulphate values are expressed as µg/g (air-dry). Exchangeable (Exch.) potassium, calcium and magnesium values are expressed as meq/100 g (air-dry). conductivity (Cond.) measurement were determined in saturated calcium sulphate, corrected for temperature and expressed as µS/ Cm. Percent soluble salt (% SS) was calculated from conductivity.

b): Analysis data from three randomly selected tissue samples of the plants grown in the glasshouse

Sample	N (mg/g)	P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)
1	32.5	3.85	31.9	11.0	3.56
2	29.4	3.68	24.5	12.8	4.35
3	28.2	3.06	28.0	11.4	3.41

Appendix 2.2 **The latitudes and altitude of the areas in which the Iranian cultivars were produced**

Appendix 2.3 Air temperature and rainfall for the areas in which the Iranian cultivars were produced in 1991. Data provided by the Seed and Plant Improvement Institute of Iran (S.P.I.I.).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Azarbaijan	Rainfall	13.4	17.8	39.7	42.4	32.8	41.4	21.2	28.4	0	0	6.0	33.5
	Min Temp	-2.0	-6.5	-3.0	3.3	8.5	10.3	17.3	19.5	22.3	15.3	11.5	3.1
	Max Temp	4.0	3.3	6.3	12.5	19.5	22.0	29.0	33.0	34.6	29.3	22.8	12.1
	Mean Temp	-2.5	-1.3	1.5	6.8	11.3	17.3	21.8	26.0	26.0	20.3	17.0	7.1
Bam	Rainfall	12.4	11.2	5.8	52.6	0	0	0	0	0	0	0	0
	Min Temp	4.3	8.3	7.0	13.8	19.3	23.0	26.8	28.5	27.3	24.0	18.3	14.0
	Max Temp	15.3	18.3	17.8	23.5	31.0	36.0	38.8	40.3	39.6	36.6	28.8	25.0
	Mean Temp	10.0	11.0	14.5	19.0	22.8	27.8	32.3	32.8	31.6	29.0	25.3	17.5
Hamedan	Rainfall	18.3	35.7	30.2	32.8	13.7	3.0	0	0	0	0	5 .5	32.8
	Min Temp	-7.8	-4.3	-4.8	2.0	5.5	7.0	11.3	13.8	13.6	9.3	5.5	0.0
	Max Temp	2.0	2.3	4.5	13.3	20.5	23.3	31.0	33.8	35.6	31.3	22.3	13.8
	Mean Temp	-3.3	-1.8	2.3	6.3	10.0	15.5	20.5	24.3	24.6	19.6	14.8	6.0
Nikshar	Rainfall	9.1	40.1	7.2	37.3	0	0	0	2.0	0	0	0	6.7
	Min Temp	7.5	12.0	10.0	16.3	21.8	25.0	28.5	29.5	29.0	26.0	20.3	16.3
	Max Temp	20.5	23.5	21.5	29.0	36.3	39.3	43.8	44.3	44.3	40.6	35.3	29.0
	Mean Temp	14.0	14.3	18.5	23.0	27.0	33.0	36.8	37.5	36.6	33.0	29.0	20.5
Yazd	Rainfall	18.2	14.7	18.1	6.0	1.3	0	0	0	0	0	2.1	27.3
	Min Temp	-1.75	1.0	2.0	9.3	14.8	15.8	22.5	24.5	23.0	21.6	19.0	7.0
	Max Temp	10.5	9.5	14.5	20.8	28.3	31.8	34.3	39.5	39.3	36.0	27.3	21.4
	Mean Temp	4.8	5.3	10.0	14.3	18.8	24.8	29.3	31.3	30.3	26.0	20.8	12.0

Appendix 2.4 **Air temperature (maximum and minimum) and rainfall for 1992-1994 in the field and the 60 year average, plus air temperature in the glasshouse in 1993. Data recorded at AgResearch Grasslands, 1.5 km from the trial site.**

		Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
60 year Average	Rainfall	79.0	67.0	69.0	81.0	89.0	97.0	89.0	89.0	75.0	88.0	78.0	94.0
	Min Temp	12.8	12.8	11.7	9.6	6.8	4.7	4.0	5.0	6.6	8.3	9.8	11.6
	Max Temp	21.9	22.3	20.9	18.2	15.0	12.6	11.9	13.1	14.7	16.6	18.5	20.6
Field (1992)	Rainfall	77.2	155.2	88.9	44.4	27.6	74.8	141.6	110.2	87.7	85.7	60.8	167.3
	Min Temp	12.9	12.4	9.5	6.8	5.8	4.9	5.4	4.4	6.3	7.6	11.0	10.8
	Max Temp	21.9	20.9	18.6	15.4	12.7	12.2	12.2	12.0	13.3	16.0	18.3	19.3
Field (1993)	Rainfall	53.9	43.7	79.8	53.7	78.4	102.6	11.5	63.1	60.3	60.0	138.2	81.3
	Min Temp	11.5	11.7	10.0	8.4	7.2	7.1	4.0	6.3	6.0	8.7	7.6	11.4
	Max Temp	19.9	20.8	19.2	16.7	15.5	13.9	12.5	12.6	13.0	17.1	16.9	19.1
Field (1994)	Rainfall	33.3	30.3	58.3	43.8	121.9	91.8	73.8	82.0	172.7	69.8	179.7	45.5
	Min Temp	13.5	13.7	9.6	8.6	8.4	4.6	3.5	5.7	6.2	7.6	10.1	11.3
	Max Temp	22.7	24.4	20.1	19.0	15.9	12.4	12.3	13.6	14.2	16.1	17.3	20.5
Glasshouse	Min Temp	18.0	17.2	17.0	15.6	16.0	15.6	13.6	16.7	16.0	16.8	16.2	17.0
	Max Temp	22.0	24.0	26.4	26.8	24.0	26.0	18.4	22.8	23.6	23.6	23.5	24.0

Appendix 3.1 **Discrimination among nine seed lots of cultivars G. Oranga and Wairau, on the basis of 20 individual seed morphological characters. Seed lots linked by the same line do not differ significantly for the characters at P<0.05.**

	Characters	Seed lot	Wairau-4	Wairau-1	Wairau-2	Oranga-2	Wairau3	Oranga-4	Oranga-3	Oranga-1	Oranga-5
1	total of red, green, and blue colour	Mean	356.57	355.54	355.24	352.35	347.90	341.81	341.73	340.90	324.61
		Duncan's grouping									
2	red/green colour	Seed lot	Oranga-5	Oranga-2	Oranga-3	Oranga-1	Oranga-4	Wairau-3	Wairau-1	Wairau-2	Wairau-4
		Mean	1.29	1.25	1.25	1.25	1.24	1.24	1.23	1.23	1.22
		Duncan's grouping									
3	red/total intensity	Seed lot	Oranga-5	Oranga-2	Oranga-3	Oranga-4	Oranga-1	Wairau-3	Wairau-1	Wairau-2	Wairau-4
		Mean	0.417	0.412	0.412	0.410	0.41	0.408	0.406	0.405	0.403
		Duncan's grouping									
4	green/total intensity	Seed lot	Wairua-2	Wairua-4	Wairua-3	Oranga-2	Wairua-1	Oranga-4	Oranga-3	Oranga-1	Oranga-5
		Mean	0.3308	0.3306	0.3302	0.3299	0.3294	0.3294	0.3209	0.3288	0.3222
		Duncan's grouping									
5	blue/total intensity	Seed lot	Wairau-4	Wairau-1	Wairau-2	Oranga-1	Wairau-3	Oranga-3	Oranga-4	Oranga-5	Oranga-2
		Mean	0.2661	0.2639	0.2636	0.2613	0.2604	0.2606	0.2606	0.2601	0.2582
		Duncan's grouping									

Appendix 3.1

	Characters	Seed lot	Oranga-4	Wairau-2	Oranga-5	Oranga-2	Wairau-1	Oranga-3	Oranga-1	Wairau-3	Wairau-4
6	seed length	Mean	2.491	2.469	2.457	2.436	2.379	2.360	2.346	2.343	2.249
		Duncan's grouping									
		Seed lot	Wairau-2	Oranga-3	Oranga-5	Wairau-1	Wairau-3	Wairau-4	Oranga-3	Oranga-2	Oranga-1
7	seed width	Mean	1.599	1.565	1.559	1.518	1.502	1.495	1.486	1.484	1.459
		Duncan's grouping									
		Seed lot	Wairau-4	Wairau-2	Wairau-3	Wairau-1	Oranga-5	Oranga-3	Oranga-4	Oranga-1	Oranga-2
8	width/length	Mean	0.6732	0.6564	0.6483	0.6456	0.6419	0.6364	0.6349	0.6273	0.6134
		Duncan's grouping									
		Seed lot	Oranga-2	Oranga-1	Oranga-4	Oranga-3	Oranga-5	Wairau-1	Wairau-3	Wairau-2	Wairau-4
9	length/width	Mean	1.6486	1.6172	1.5996	1.5984	1.5891	1.5748	1.5724	1.5534	1.5160
		Duncan's grouping									
		Seed lot	Wairau-2	Oranga-4	Oranga-5	Wairau-1	Oranga-2	Wairau-3	Oranga-3	Oranga-1	Wairau-4
10	area	Mean	2.9828	2.9586	2.8887	2.7364	2.7290	2.6629	2.6549	2.5892	2.5428
		Duncan's grouping									

	Characters	Seed lot	Oranga-4	Wairau-2	Oranga-5	Oranga-2	Wairau-1	Oranga-3	Wairau-3	Oranga-1	Wairau-4
11	perimeter	Mean	6.6872	6.6761	6.6131	6.4554	6.3977	6.3376	6.3101	6.2513	6.1342
		Duncan's grouping									
		Seed lot	Wairau-4	Wairau-2	Wairau-3	Wairau-1	Oranga-1	Oranga-4	Oranga-3	Oranga-5	Oranga-2
12	4∏ area/perimeter^2	Mean	0.846	0.8386	0.8368	0.8348	0.8300	0.8290	0.8281	0.8280	0.8209
		Duncan's grouping									
		Seed lot	Wairau-2	Oranga-4	Oranga-5	Wairau-1	Wairau-3	Wairau-4	Oranga-3	Oranga-2	Oranga-1
13	average width of seed (area of the seed/length)	Mean	1.208	1.186	1.175	1.144	1.135	1.130	1.142	1.183	1.1021
		Duncan's grouping									
		Seed lot	Orang-4	Oranga-5	Oranga-1	Oranga-3	Wairau-2	Wairau-1	Oranga-1	Wairau-3	Wairau-4
14	concavity of seed	Mean	0.1294	0.1212	0.1190	0.1168	0.1145	0.1109	0.1077	0.1013	0.0938
		Duncan's grouping									
		Seed lot	Oranga-4	Oranga-2	Orang-3	Oranga-5	Oranga-1	Wairau-1	Wairau-2	Wairau-3	Wairau-4
15	concavity /width	Mean	0.0829	0.0805	0.0789	0.0779	0.07370	0.07243	0.07198	0.06779	0.06284
		Duncan's grouping									

Appendix 3.1

	Characters	Seed lot	Wairau-4	Wairau-1	Wairau-3	Oranga-1	Oranga-2	Wairau-2	Oranga-5	Oranga-3	Oranga-4
16	curvature of seed	Mean	151.49	149.03	148.22	147.70	147.03	146.22	145.63	145.27	143.82
		Duncan's grouping									
17	curve position	Seed lot	Wairau-4	Wairau-3	Oranga-5	Oranga-3	Oranga-1	Oranga-2	Wairau-1	Wairau-2	Oranga-4
		Mean	0.1564	0.1519	0.1508	0.1459	0.1394	0.1350	0.1338	0.1270	0.1246
		Duncan's grouping									
18	convex area (area o f the convex hull)	Seed lot	Wairau-1	Oranga-4	Oranga-5	Wairau-1	Oranga-2	Wairau-3	Oranga-3	Oranga-1	Wairau-4
		Mean	3.019	3.001	2.930	2.773	2.770	2.6900	2.6898	2.6197	2.5650
		Duncan's grouping									
19	convex perimeter	Seed lot	Oranga-4	Wairau-4	Oranga-5	Oranga-2	Wairau-1	Oranga-3	Wairau-3	Oranga-1	Wairau-4
		Mean	6.6485	6.6439	6.5795	6.4175	6.3695	6.3021	6.2843	6.2252	6.1148
		Duncan's grouping									
20	actual area of the seed /convex area	Seed lot	Wairau-4	Waira-3	Oranga-1	Wairau-2	Wairau-1	Oranga-3	Oranga-5	Oranga-4	Oranga-2
		Mean	0.9930	0.9990	0.9887	0.9883	0.9879	0.9874	0.9863	0.9860	0.9855
		Duncan's grouping									

Appendix 3.2 Discrimination between cvs. G.Oranga and Wairau on the basis of the 10 selected seed morphological characters (variables) recorded from seed lots of the cultivars.

Variable 2 Red/Green colour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.2348	0.2348	146.70	0.0001
Seed lot	7	0.3307	0.0472	29.51	0.0001
Error	1329	2.1274	0.0016		
Corrected Total	1337	2.6929			

R-Square	C.V.	MSE Root	V2Mean
0.2100	3.21	0.040	1.2450

Variable V3 Red colour /total intensity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0111	0.0111	248.54	0.0001
Seed lot	7	0.0090	0.0013	28.71	0.0001
Error	1329	0.0595	0.00004		
Corrected Total	1337	0.0796			

R-Square	C.V.	MSE Root	V3 Mean
0.2527	1.63	0.0067	0.4093

Variable 4 Green colour/total intensity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0019	0.0019	50.65	0.0001
Seed lot	7	0.0062	0.0009	23.96	0.0001
Error	1329	0.0490	0.0004		
Corrected Total	1337	0.0570			

R-Square	C.V.	MSE Root	V4 Mean
0.1411	1.84	0.0061	0.3290

Variable 5 Blue colour/total intensity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0039	0.0039	134.02	0.0001

Seed lot	7	0.0030	0.0004	15.08	0.0001
Error	1329	0.0384	0.0003		
Corrected Total	1337	0.0453			

R-Square	C.V.	MSE Root	V5 Mean
0.1527	2.05	0.0053	0.2617

Variable.8 Width/length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.2103	0.2103	26.16	0.0001
Seed lot	7	0.1416	0.0202	2.52	0.0143
Error	1329	10.6847	0.0080		
Corrected Total	1337	11.0366			

R-Square	C.V.	MSE Root	V8 Mean
0.0319	13.96	0.0897	0.6419

Variable 9 Length/width

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	1.0614	1.0614	27.53	0.0001
Seed lot	7	0.6609	0.0944	2.45	0.0170
Error	1329	51.247	0.0386		
Corrected Total	1337	52.970			

R-Square	C.V.	MSE Root	V9 Mean
0.0325	12.38	0.1963	1.58559

Variable 12 $4\prod$ area/perimetr^2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0475	0.0475	39.17	0.0001
Seed lot	7	0.0195	0.0028	2.29	0.0253
Error	1329	1.6128	0.0012		
Corrected Total	1337	1.6798			

R-Square	C.V.	MSE Root	V12 Mean

Appendix 3.2

Variable 14 Concavity of seed

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0623	0.0623	23.04	0.0001
Seed lot	7	0.0758	0.0108	4.00	0.0002
Error	1329	3.5957	0.0027		
Corrected Total	1337	3.7339			

R-Square	C.V.	MSE Root	V14 Mean
0.0370	46.13	0.0520	0.1128

Variable 15 Concavity /width

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0337	0.0337	29.11	0.0001
Seed lot	7	0.0159	0.0023	1.96	0.0571
Error	1329	1.5384	0.0012		
Corrected Total	1337	1.5886			

R-Square	C.V.	MSE Root	V15 Mean
0.0312	45.76	0.0340	0.0743

Variable 20 Actual area of the seed /convex area

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Cultivar	1	0.0024	0.0024	19.50	0.0001
Seed lot	7	0.0023	0.0003	2.67	0.0096
Error	1329	0.1654	0.0001		
Corrected Total	1337	0.1702			

R-Square	C.V.	MSE Root	V20 Mean
0.0279	1.12	0.0112	0.9880

Appendix 5.1

a): Gel-loading buffer (storage temperature 4°C)

0.25 % bromophenol blue

0.25 % xylene cyanol FF

30.0 % glycerol in water

b): TAE buffer

Buffer	Working solution	Concentrated stock solution (per litre)
Tris-acetate (TAE)	1 x : 0.04 M Tris-acetate 0.001 M EDTA	50 x: 242 g Tris-base 57.1 ml glacial acetate acid 100 ml 0.5M EDTA (pH 8.0)

From : Sambrook *et al.* (1989)