Hybridization in North Island Tree Weta

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Abstract

Hybridization has important implications for species concepts, understanding the speciation process, as well as the issue of sister species coexistence and conservation. Tree weta (genus *Hemideina*) are an interesting natural system for studying hybridization, as some species have multiple chromosome races that can interbreed, and all are broadly parapatric with at least one other species in the wild, so there are many opportunities for natural hybridization to occur. It is not known in many cases whether species pairs are hybridizing in the wild, or whether introgression occurs. This study focused on the interactions between *H. thoracica* and its two neighbouring species; *H. crassidens* and *H. trewicki.*

Surveys of one area of sympatry between *H. thoracica* and *H. crassidens,* and one between *H. thoracica* and *H. trewicki*, were conducted to see if the parent species and putative hybrids could be easily distinguished, and to find out the relative ratios of the parents and putative hybrid individuals. Weta from the parent populations were studied in areas where they are sympatric as well as allopatric to look for evidence of possible divergence and/or introgression in sympatry. These studies showed that where these species pairs are sympatric, parent forms were predominant, with few morphological intermediates, despite parent species existing in similar proportions. *Hemideina thoracica* and *H. trewicki* differed in sympatry regarding both size and possibly life history, with *H. thoracica* females being larger and both sexes maturing later than *H. trewicki. Hemideina thoracica* and *H. crassidens* showed possible evidence of introgression, but no evidence of divergence in sympatry.

Karyotypes, a mitochondrial locus, and eight nuclear loci were examined for evidence of introgression between the species pairs in sympatry. All putative hybrids (morphological intermediates) from both species pairs were found to be genetic hybrids, with strong evidence of being F_1 hybrids. No evidence was found for introgression of karyotypes or of mitochondrial haplotypes in either case. No evidence of introgression was found at nuclear loci for *H. thoracica* and *H. trewicki.* However, *H. thoracica* and *H. crassidens* showed some overlap at nuclear markers in sympatry, suggesting a low level of introgression. There was also a sex-bias in the production of F_1 hybrids, with most having a *H. crassidens* mother.

Hemideina thoracica appears to interact differently with its two neighbouring species; *H. crassidens* and *H. trewicki. Hemideina thoracica* and *H. trewicki* appear to be reproductively isolated, and are possibly exploiting different niches. *Hemideina thoracica* and *H. crassidens* by contrast, showed no evidence of divergence and are presumably dealing with strong interspecific competition, as well as introgression where they meet. These two species are unusual in maintaining a bimodal hybrid zone in the apparent absence of assortative mating. They also contrast with Haldane's rule, as F_1 males have some level of fertility, while females are likely infertile. A sex-bias in the production of F_1 hybrids may be due to 'sexual exclusion', and so could possibly provide an explanation of how *H. thoracica* has managed to displace *H. crassidens* from much of its former range.

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Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1.1 Hybridization

Hybridization is the production of offspring between genetically distinct populations (Harrison, 1993). In plants hybridization has long been recognised as an important source of genetic diversity, occasionally leading to the formation of new species, often via allopolyploidy (Rensch, 1959; Rieseburg, 1987; Rieseburg & Wendel, 1993; Arnold, 1997). Many microorganisms and viruses acquire adaptive traits via hybridization (Ochman, 2000; Gogarten & Townsend, 2005; Smith et al. 2009; etc.). In animals it is much less common, probably because individual animals generally require a greater degree of genomic compatibility than plants to be viable. Around 10% of animals are estimated to produce F_1 hybrids with at least one other species, with potential introgression of genetic material (Mallet, 2005, and references therein). Despite hybridization in animals historically being regarded as wasted genetic material, occurring only when there was a breakdown in assortative mating, it is now generally recognised as an important aspect of evolution in many lineages (Dowling & Secor, 1997; Arnold et al. 1999; Mallet, 2005; etc.). However, despite its acceptance as a natural and potentially beneficial process, hybridization still presents problems for species concepts, and there is much that is not yet known about its frequency and precise role in the evolutionary history of animals. An understanding of hybridization and its consequences is important for understanding current biodiversity patterns as well as the speciation process. Hybridisation also has practical implications for conservation efforts and approaches where endangered species are concerned (Allendorf et al. 2001).

For most species, assortative mating prevents hybridization with related species (Price & Bouvier, 2002; Coyne & Orr, 2004). Time since divergence of lineages appears to be one of the most important factors in determining if species are able to produce hybrids (Coyne & Orr, 1997; Arnold, 1997; Edmunds, 2002; Price & Bouvier, 2002; Bolnick & Near, 2005). This has led to the general conclusion that genetic divergence will usually prevent successful hybridization once enough time has passed to accumulate genetic differences. The Dobzansky-Muller model provides a good explanation for how these incompatibilities arise (Dobzhansky, 1937; Muller, 1942). F_1 hybrids often suffer from some form of hybrid disadvantage, due to genomic incompatibilities that lead to low survival and/or reduced fertility. Genomic incompatibilities arise because certain alleles are co-adapted, particularly in loci that are involved in the same biochemical pathway. Often alleles from one genetic background will fail to function adaptively in another genetic background. An example of this is the LHR gene alleles that differ between *Drosophila simulans* and *Drosophila melanogaster;* the combination of which cause hybrid lethality (Brideau et al. 2006). Many hybrids are viable, but are infertile 'mules', which cannot reproduce. An example is the two species of tree weta *Hemideina femorata* and *H. ricta* (Morgan-Richards, 1995). Infertility or limited fertility is a common problem in hybrids. It is often due to meiotic non-disjunction which produces unbalanced gametes, particularly if there are large cytological differences (chromosome number and morphology) between populations. An example is the *Pygaera* moth genus, where cytological differences were first related to infertility in male hybrids (Federley, 1913). Infertility can also be due to incompatibility between reproductive genes, which can prevent proper formation and/or function of reproductive organs, as shown in a study of hybridization between *Drosophila pseudoobscura and Drosophila persimilis* (Orr,

1987). There can also be reproductive problems if the parent populations do not recognise the hybrid as a potential mate due to morphological differences, an example of which is found in some *Heliconius* butterflies where specific wing patterns are very important for mate recognition (Mavárez et al. 2006).

1.1.2 Hybrid zones

In some cases of hybridization, F_1 hybrids will have limited fertility. In these cases, backcrossing with parent species can occur, leading to introgression of alleles between the parent populations. These second and later generation hybrids may or may not suffer from some form of hybrid disadvantage. However, they generally have higher fertility than the F_1 hybrids (Mallet et al. 1998; Mallet, 2005; Descimon & Mallet, 2009). If there is some disadvantage suffered by later generation hybrids, a tension zone may form where the two species meet, with the zone width being determined by dispersal versus the disadvantage suffered by hybrids (Baton & Gale, 1983; Barton & Hewitt, 1985). Many cases of tension zones are found in animals. An example is the tension zones formed where the nine chromosome races of the Auckland tree weta (*H. thoracica*) meet one another in different locations in the north island (Morgan-Richards, 1997). Another example of this situation is found in *Heliconius erato* where frequency-dependant selection on warning colouration limits the width of hybrid zones between races (Mallet & Barton, 1989). In some cases hybrid zones may be found on marginal habitat that is not ideally suited to either parent population (i.e. where parent populations are adapted to different biomes) (Harrison & Rand, 1989). One example is found in Carrion (*Coronus corone corone*) and Hooded magpies (*Coronus corone cornix*) that inhabit either alpine valleys or extensively cultivated plains, with hybrids suffering no distinguishable disadvantage when found in ecotones (Saino & Villa, 1992).

In rare situations hybrids will experience hybrid vigour and be better suited to the environment than either parent species. This is usually due to novel combinations of alleles that increase fitness, or the immediate increase in genetic diversity experienced by hybrids. An example of the former is found in the freshwater snail *Melanoides tuberculata* where hybrid individuals have managed to outcompete parent forms (Facon et al. 2005). In other cases a lack of hybrid disadvantage may result in the two populations freely interbreeding and homogenising, as has been found in many cases where formerly allopatric species have been brought into contact via human habitat modification. Mallard (*Anas superciliosa*) and Grey ducks (*Anus platyrynchos*) are an example; they show some level of assortative mating when in contact, but have lost a lot of their distinct genetic identity (Hitchmough et al. 1990; Muller, 2012). In some cases introgression will lead to genetic swamping of one population, so that it eventually becomes incorporated into the other population and loses its own genetic identity. There is also evidence for the hybrid origin of some animal species, such as the parthenogenic stick insect genus *Acanthoxyla* in New Zealand (Morgan-Richards & Trewick, 2005), and a cichlid fish species in Lake Malawi (Smith et al. 2003). As hybrid origin of a species is difficult to distinguish genetically from ancestral polymorphism, there may be more animal species that have arisen this way than are currently recognised.

1.1.3 Selection for divergence

Hybridization sometimes represents an intermediate stage in the speciation process. Populations that speciate in sympatry will most likely be responding to divergent selection pressures (Dieckman & Doebeli, 1999; Mallet et al. 2009). In some cases, this split may lead to an immediate cessation of gene flow, such as when seasonal flowering times diverge (Devaux & Lande, 2010). In other cases hybridization would likely be a natural occurrence until F_1 hybrids started to suffer from heterozygote disadvantage (if they were not ideally adapted to either of the parent populations' habitats). Populations that speciate in allopatry may also be prone to hybridization if they come back into contact. Species that diverge in allopatric situations would have little need to develop mate recognition systems to exclude their sister species, and any hybrid disadvantage would result from pleiotropic effects of genes that had diverged for other reasons (Darwin, 1859; Mayr, 1963; Cohn & Orr, 2004). Once populations resume contact, if there is little to no hybrid disadvantage, they may eventually homogenise back into a single population, as selective pressures that aim to remove the disadvantage suffered by hybrids will likely be higher than selective pressures that would separate the populations (Arnold, 1997). If hybrid disadvantage is high, then the process of reinforcement may help to reproductively isolate populations by selecting against the production of hybrids (Ridely, 2003; Ollerton, 2005). However, this selection pressure only exists where the two species occur in sympatry; allopatric populations are not expected to show evidence of reinforcement. Reinforcement may be followed by reproductive character displacement, which is the divergence of reproductive traits between the species to increase assortative mating. This process can happen in populations that have diverged in sympatry or allopatry (Dieckmann & Doebeli, 1999). There is also evidence that some hybrid zones may be stable over long periods of time, including many bird species that hybridize across an east-west distribution in the Great Plains (Rising, 1983). Cases of hybridization seen today likely represent snapshots in the dynamic speciation process, and may lead to insights about how the process works.

1.1.4 Species concepts

Hybridization also has implications for species concepts. The lack of clear boundaries separating varieties from species, and some of the issues this can cause when delimiting species, has been long been recognised (Darwin, 1859). Many different species concepts have been created, although there appears to be none that hold true in every situation, especially where situations such as asexual reproduction and horizontal gene transfer are taken into account (Mishler & Donoghue, 1982; Nixon et al. 1990; Agapow et al. 2004). The widely used Biological Species Concept (Mayr) defines a species as a population of individuals that can freely interbreed, but are reproductively isolated from other such groups. Populations that form infertile F_1 hybrids fit into this species category. However, this species concept (along with many others) presents problems in cases where limited introgression occurs. As there appears to be a gradation between full fertility and complete infertility among different cases, the limit on how much gene flow is acceptable between populations before they lose their species status is still debated (Agapow et al. 2004). Other species concepts such as the evolutionary species concept (Simpson, Wiley), cohesion species concept (Ghiselin, Hull), concordance species concept (Avise, Ball), and genotypic cluster species concept (Mallet) are more lenient, and allow for some gene flow as long as the populations retain their genetic identity and

follow their own evolutionary trajectories. There have also been suggestions that the definition of a species should stay separate from the issue of species delimitation (i.e. the processes or methods that keep species apart) (Qeuiroz, 2007), although the debate about the nature of species will likely continue for some time.

1.1.5 Conservation

As well as helping us understand the process of speciation and the nature of species, hybridization between populations/species has become a recognised problem for conservation. Human modification of habitat has caused many formerly allopatric species to regain contact, leading to many instances of hybridization between related species, such as the example above with Grey and Mallard ducks. Although commonly seen in response to human habitat modification, hybridization between related taxa appears to be a common natural occurrence as well, with many examples cited above. Problems arise where endangered species are involved. Hybridization with more common taxa may lead to genetic swamping, where the endangered species loses its own genetic identity and becomes incorporated into the common species (the current concern with Grey and Mallard ducks). In some cases, it could be argued that they were not proper species and so not worthy of conservation efforts, but it will depend on the conservation approach, and whether the aim is to retain the greatest amount of genetic diversity or to preserve 'species' (Brien & Mayr, 1991; Dowling & Secor, 1997; Allendorf et al. 2001). In some cases, the increase in genetic diversity gained via hybridization may be seen as a positive event, enhancing the survival chances of endangered populations.

1.1.6 Tree weta

New Zealand Tree Weta (genus *Hemideina*) are a group of seven species that fall into the order Orthoptera, family: Anostostomatidae (Morgan-Richards & Gibbs, 2001). They are all large (adults can weigh up to 10g), flightless and nocturnal (Trewick & Morgan-Richard, 1996). They are an important part of native New Zealand ecosystems, sometimes being labelled 'invertebrate mice', supposedly adopting this niche in the absence of native land mammals (Fleming 1973; Southern 1979; Duthie et al. 2006) although this role has recently been disputed (Griffin et al. 2011). They may also have a role in the dispersal of seeds for two native plant species (Duthie et al. 2006; but see Wyman at al, 2010). Most of the *Hemideina* species are widespread and abundant in native forest habitat, with only one species considered endangered (*Hemideina ricta*) (Trewick & Morgan-Richards, 1996).They have likely escaped the endangered status of most members of the closely related giant weta (genus *Deinacrida)* due to their more aggressive behaviour and preference for hiding in holes in tree branches during the day where they are safe from introduced predators.

New Zealand weta are an interesting group for evolutionary studies, as there are many known hybrid zones within species. Two of the *Hemideina* species contain multiple chromosome races. *Hemideina thoracica* has nine distinct chromosome races identifiable by gross karyotype morphology. These chromosome races can interbreed and introgression occurs in zones of overlap, shown by cytological, allozyme and mitochondrial data (Morgan-Richards, 1997; Morgan-Richards &

Wallis, 2003). The different chromosome races studied form tension zones where they meet, with chromosome or other genomic differences appearing to prevent populations from fully homogenising (Morgan-Richards & Wallis, 2003; Morgan-Richards et al. 2000). *Hemideina crassidens* has two known chromosome races that can interbreed (Morgan-Richards, 2000; Morgan-Richards, 2002). In contrast, the four South Island species show no intra- or interspecific difference in the gross morphology of their karyotypes (Morgan-Richards & Gibbs, 2001). *Hemideina maori* contains at least one hybrid zone distinguishable by colour variation and mtDNA haplotype, although no chromosome differences are known in this species (King et al. 1996; King et al. 2003). Two members of the closely related giant weta genus; *Deinacrida connectens* and *D. elegans,* also have multiple chromosome races (Morgan-Richards & Gibbs, 2001), which shows that karyotype differences are not enough to reproductively isolate populations in this group. All of the tree weta species are parapatric with at least one other species at the border of their range (Morgan-Richards & Gibbs 2001). In some of these zones of overlap, putative F_1 hybrids have been identified based on colouration and cytogenetics (M. Morgan-Richards, personal communication, 20 January, 2012). One case of hybridization has been proven using genetic data (Morgan-Richard & Townsend, 1995). This small study looked at hybridization between the widespread *H. femorata* and the endangered Banks Peninsula tree weta *H. ricta*. There was concern that the more numerous *H. femorata* would genetically swamp the Banks Peninsula Weta and hinder conservation efforts. However, although the study showed strong evidence that F_1 hybrids were being produced, there was no evidence of later generation hybrids or introgression, and infertility of the F_1 hybrids was inferred (Morgan-Richard & Townsend, 1995).

The existence of hybridization and/or introgression between other species pairs is so far unclear. The three North Island species are thought to be reproductively isolated and are all morphologically distinguishable (Field & Bigelow, 2001). The 17 chromosome race of *H. thoracica* meets both *Hemideina trewicki* and the 15 chromosome race of *Hemideina crassidens* at its southern borders (Figure 1.1). There is also contact between *H. thoracica* and *H. crassidens* on three mountainous regions in the central North Island. The range of *H. crassidens* appears to be restricted to higher altitudes in these regions, where they are completely surrounded by *H. thoracica.* On Mt Taranaki there is only a very narrow region of overlap, and no evidence of hybridization has been found so far (Jacobson, 2009; Bulgarella et al. 2014). However, around the Manawatu region there is a large area where the two species are found in sympatry, and they have often been found sharing the same daytime roosts (P. M. Wehi, personal communication, 10 January, 2012). Individuals that appear morphologically intermediate between the two species have been found and are assumed to be hybrids (pers. obs.), although it is currently unknown if these putative hybrids between *H. thoracica* and *H. crassidens* are hybrids, and if so, whether they are fertile and capable of backcrossing with the parent species leading to introgression. A similar situation has been found between *H. thoracica* and *H. trewicki*, where they exist in sympatry in some areas of the Hawke's Bay region. One individual from this region was classified as a hybrid using karyotype and allozyme data, and considered to be a F_1 hybrid. The detection of only one F_1 hybrid suggested infertility, as later generation hybrids and backcrosses would be expected to be more common than F_1 hybrids if they suffered little hybrid disadvantage (Morgan-Richards, 1995). While introgression between these two species pairs has been considered unlikely, it has not been extensively searched for. Hybrids may also be cryptic, so cytological and genetic data are needed to understand the extent of hybridization (if any) between these species pairs

1.1.7 Aims of study

The overall aim of this thesis is find out how the species pairs interact in areas of sympatry. The ratio of the two species will be examined to determine that the study regions truly represent areas of sympatry. Evidence for the divergence of traits and/or introgression in regions of sympatry will be sought by comparing individuals from allopatric and sympatric populations (Chapter 2). Genetic and cytological evidence will be used to determine whether the 17 chromosome race of *H. thoracica* does produce F_1 hybrids with its two neighbouring species, *H. crassidens* and *H. trewicki,* and whether there is a detectable level of genetic introgression in these regions (Chapter 3). Preliminary data will also be presented on the potential fertility of hybrid individuals (Appendix A).

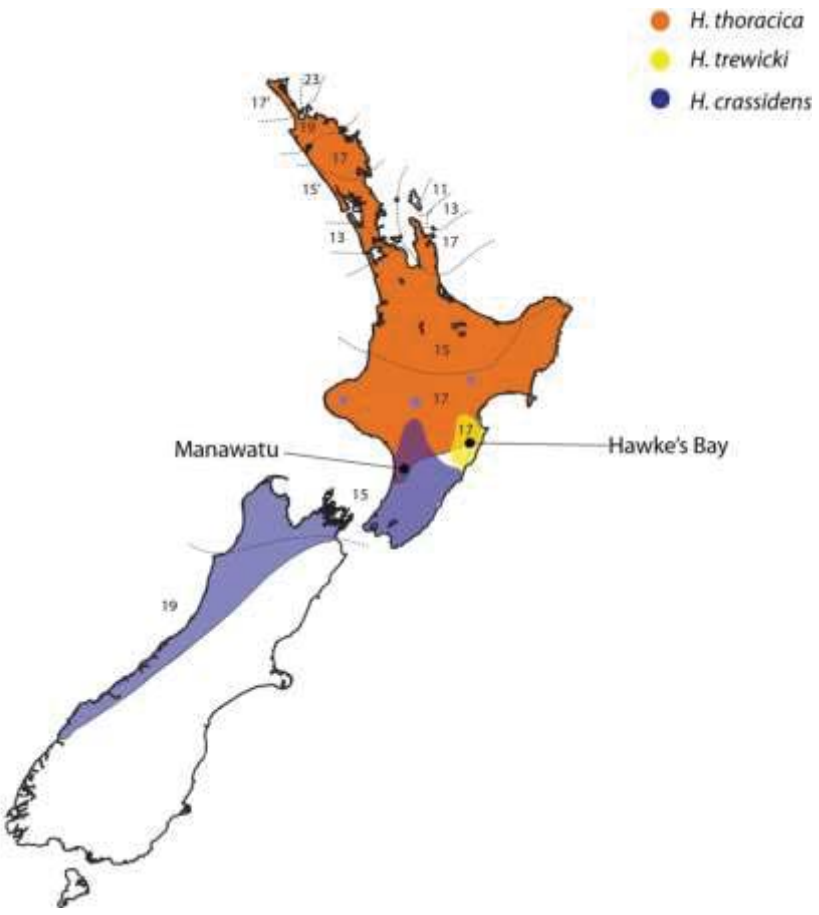

Figure 1.1: Distribution of three New Zealand species of tree weta (*Hemideina*). Distributions of chromosome races within species are delineated by dotted lines, with number representing chromosome number of males (XO). The distributions of the *H. thoracica* and *H. crassidens* chromosome races were taken from Morgan-Richards & Wallis (2003) and Morgan-Richards (2000) respectively. The two areas of sympatry that are the focus of this work are indicated.

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Chapter 2: Tree weta in sympatry

2.1 Introduction

2.1.1 Issues with sympatry

One of the fundamental laws of ecology is Gause's competitive exclusion principle, which states that two species cannot coexist indefinitely if they share the same ecological niche (Hardin, 1960). This is because niche sharing causes competition for resources between species, and even the slightest advantage in one species means that it should eventually displace its competitor. Sister species that diverge from one another in sympatry are likely responding to some kind of disruptive selection, and thus their origin and use of different niches probably occur simultaneously, allowing them to coexist (Bolnik et al. 2007). However, species that diverge in allopatry may be primarily the result of genetic drift, with or without divergent adaptive responses to the environment (Dobzansky, 1937; Mayr, 1963; Coyne & Orr, 2004). This means that the speciation process can produce sister species that share the same ecological niche, as long as they are geographically isolated. Theoretically, if species of this origin come into contact at a later time, one should either displace the other, or competition in sympatry should cause them to diverge to the point where competitive pressure is limited and allows the species to coexist. For species that have not diverged completely, another factor is hybridization with sister species. This can either lead to gene flow and an eventual breakdown in the barriers that separate species, or if hybrid disadvantage is strong enough, reinforcement to prevent interbreeding (Hoskin et al. 2005). If the F_1 hybrids are inviable or infertile then selection would favour reproductive character displacement to increase assortative mating (Dieckmann & Doebeli, 1999).

2.1.2 Patterns in sympatric populations

Comparison of allopatric and sympatric populations of two species can show whether they are diverging in sympatry or likely to be competing with one another. Species that are diverging in sympatry should show character displacement in traits that pertain to their ecological role in sympatric, but not allopatric, populations. This is a common phenomenon seen in natural populations, where in some cases sympatric populations are immediately distinguishable, but allopatric populations of different species are almost identical and difficult to tell apart (Brown & Wilson, 1956). Reproductive character displacement is a particular type of character displacement that is relevant if hybridization is an issue. This is a divergence in reproductive traits that helps increase assortative mating and decrease interspecific mating, if interspecific mating leads to offspring with low or no fitness (Dieckmann & Doebeli, 1999). If the species are hybridizing and introgression is occurring, the opposite pattern is generally found, with greater morphological overlap in sympatry than in allopatry. In contrast, a number of fixed differences in morphological or ecological characters in sympatry show that the species are maintaining strict boundaries. If both species show very little difference in sympatric and allopatric populations, this would imply that there is no divergence and competition is likely to be an issue (unless they are similar enough genetically to merge into a single population where they meet). All of these potentialities can lead to

insights about the species' ecological roles and be used to make predictions about the future outcome of the interaction.

2.1.3 Tree weta

The tree weta genus *Hemideina* contains seven species with mostly parapatric distributions (with the exception of widespread overlap of *H. broughi* and *H. crassidens*). All of the species have distributions that meet at least one other tree weta species at the border of their distribution (Pratt et al. 2008). In the North Island there are three species; *H. thoracica* which covers most of the northern North Island, meeting *H. crassidens* in the western part of the Island around the Manawatu region, and *H. trewicki* in Hawke's Bay on the eastern side of the Island. In this study, *H. thoracica* populations will be compared to sympatric *H. crassidens* and *H. trewicki* populations.

Hemideina thoracica and H. crassidens

H*emideina crassidens* has a distribution that covers most of the southern part of the Island and extends into the South Island. However, *H. crassidens* is also found at higher altitudes on Mt Taranaki, Mt Ruapehu and Whirinaki Park in the central North Island completely surrounded by *H. thoracica.* This odd distribution suggests that at one time *H. crassidens* may have occupied most of the North Island, and was subsequently pushed south by an expanding *H. thoracica* population (Trewick & Morgan-Richards, 1995; Bulgarella et al. 2014). As the temperature has been increasing since the last glacial maxim (LGM) 15,000 years ago, it has been hypothesised that *H. crassidens* has a better tolerance for colder climates, while *H. thoracica* is probably better adapted for warmer climates (Jacobsen, 2009; Minnards, 2011; Bulgarella et al. 2014). This hypothesised adaptation explains how *H. crassidens* has managed to keep its former distribution only at altitudes in which it can successfully exclude *H. thoracica.* As global warming continues, it also predicts that *H. crassidens* will be pushed further south and higher up on the mountains in central North Island where it currently lives (Bulgarella et al. 2014), restricting its range even further. Competitive exclusion between these species is also supported by the fact that in most areas of contact, the zone of contact is narrow, as on Mt Taranaki. Habitat modelling by Bullgarella *et al.* (2014) shows that based on the current distribution, *H. thoracica* and *H. crassidens* should be able to maintain larger distributions than they currently hold, also supporting the idea of competitive exclusion between the species. Studies looking at the dietary preferences of the two species revealed no differences in the type or number of native plants consumed (Dewhurst 2012; Wehi et al. 2014). There are, however, some narrow regions where *H. thoracica* lives in sympatry with *H. crassidens,* such as on the northwestern side of the Tararua ranges. In this location they coexist without any apparent difference in ecological niche (Wehi et al. 2014) or any apparent ability to recognise members of their own species and exclude sister species as potential mates. The weta share the same types of daytime refuges and mixed species harems are commonly found in this zone of sympatry (Figure 2.1). These two species will mate in captivity, and weta have been found in the wild that appear morphologically intermediate between the parent species (pers. obs.).The extent of hybridisation in this area is currently unknown, but is important for understanding how these two species interact in the wild.

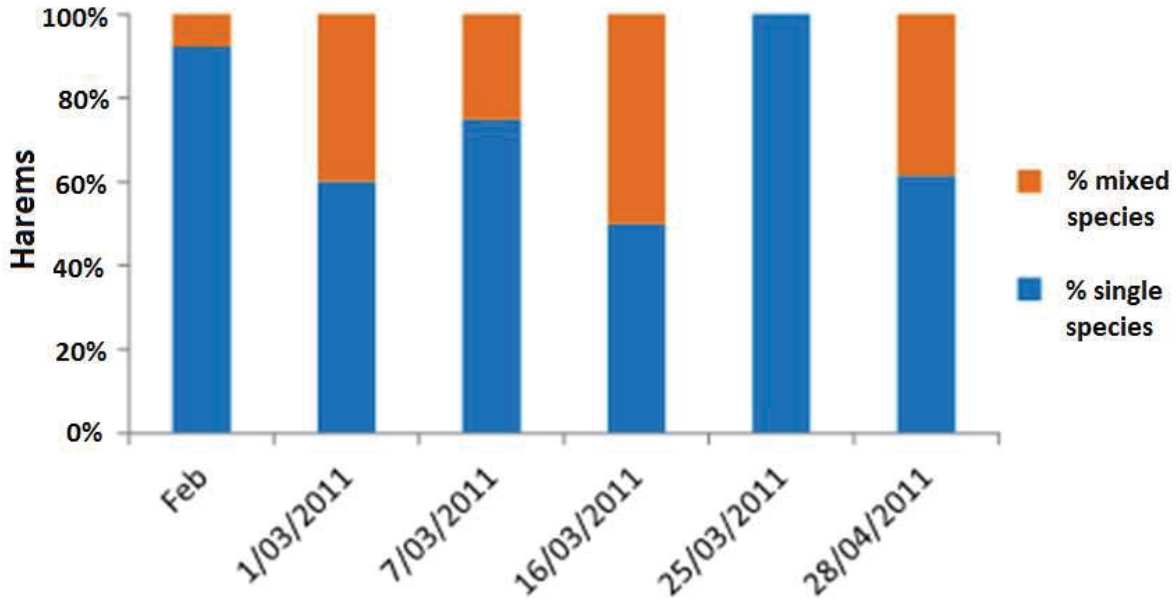

Figure 2.1: Proportions of mixed and single-species harems in the Turitea Valley over a breeding season, where *H. Thoracica* and *H. crassidens* occur in sympatry. (Proportions of the two species in this area are currently unknown, but the data does show that mixed species harems are in any case fairly common). Taken from Wehi et al. (2014).

Hemideina thoracica and H. trewicki

Hemideina trewicki has the smallest distribution of the three North Island species. Its known distribution extends throughout the Hawke's Bay region, where it meets *H. thoracica* in the northern part of its range and *H. crassidens* from the south (Trewick & Morgan-Richards, 1995). *H. thoracica* and *H. trewicki* have been found living in sympatry in several remnants of native bush in the Hawkes bay region (Trewicki & Morgan-Richards, 1995), which shows that the upper part of the *H. trewicki* distribution is probably fully sympatric with *H. thoracica,* and likely was in the past before most of the native forest in this region was cleared for farmland. The contact zone between *H. trewicki* and *H. thoracica* differs from that of *H. thoracica* and *H. crassidens,* in that the zone of sympatry with *H. trewicki* is much larger. This may mean that the ecological niches of these species have diverged and allow them to live in the same region without excluding each other, although little is currently known about the ecology of *H. trewicki.* A single confirmed F_1 hybrid has been found (Morgan-Richards, 1995), although again, the extent of hybridization is unknown.

2.1.4 Aims

The aim of this chapter was to seek evidence of divergence or overlap in traits where *H. thoracica* meets the other two species in zones of sympatry. First, an approximate ratio of the parent species and putative hybrids in two well-known areas of sympatry was used to establish whether these species pairs exhibit a bimodal zone of overlap (i.e. mostly parental forms), rather than forming a tension zone or freely interbreeding (in which the distribution of the parent species' diagnostic traits would exist as a cline). Next, an analysis of a number of morphological characters will be used to determine if there is any divergence or overlap in sympatric populations relative to allopatric populations. Stridulatory ridges are a structure involved in weta communication and differ between other species pairs of weta that contact each other in the wild (e.g. *H. femorata* and *H. ricta* (Field, 1993)). Although the stridulatory ridges are known to be similar in number and structure between the three North Island species, variation within species and populations has been found (Field, chapter 15, 2001). It is therefore possible that they may differ between *H. thoracica* and its neighbouring species in areas of sympatry to allow them the ability to exclude sister species as mates, so this possibility was examined.

2.2 Methods for Chapter 2: Tree weta in sympatry

2.2.1 Study sites

For *H. thoracica/ H. trewicki* Mohi Bush (GPS: S 39.45, E 176.88333) was chosen as surveys have been conducted previously in this area (Trewick & Morgan-Richards, 2000). For *H. thoracica/ H. crassidens* weta were surveyed and collected from the Kahutawera Valley (GPS: S 40.47184, E 175.60943) in the Manawatu. Specimens from the adjacent Turitea Valley in the Manawatu (GPS: S40.431725, E 175.674595) was included when looking at morphological characters. These areas were chosen as they have both been sampled in previous studies and both species of the pair are known to occur in these areas naturally (Trewick & Morgan-Richards, 1995).

An extra allopatric population was added for both *H. thoracica* and *H. crassidens* for the purpose of clarifying the results. Allopatric populations will make it possible to distinguish differences and similarities that are the result of ancestral polymorphism from introgression, as the latter will show fixed differences in allopatric populations but not in sympatric ones. The allopatric *H. thoracica* population was in the Taupo region, and for *H. crassidens* was in the Wellington region. These two populations were chosen because they are geographically well removed from the populations at the contact zones while still belonging to the same chromosome race (Morgan-Richards, 1997) (Figure 2.2). An allopatric population of *H. trewicki* was not added as it was not possible to find an appropriate one. This species has a small distribution relative to the other two, and it potentially overlaps with *H. thoracica* and *H. crassidens* throughout most of its range (Trewick and Morgan-Richards, 1995).

2.2.2 Identification of species

Species were identified based on the colour and pattern of the abdomen and the colour of the pronotum for *H. thoracica* and *H. crassidens* (Ramsey and Bigelow, 1978; Morgan-Richards, 1995), as well as the number of prolateral hind tibia spines (Table 2.1). *H. crassidens* and *H. trewicki* have alternating black and yellow bands on the abdomen, while *H. thoracica* has a mottled brown abdomen. *Hemideina thoracica* and *H. trewicki* have pale pronotums with black hieroglyphic type markings, while *H. crassidens* has a more uniform brown or black pronotum. *H. thoracica* can have either three or four spines, while the other two species have four (Morgan-Richards & Gibbs, 2001). Individuals identified as putative hybrids were those that had intermediate abdominal colouration; they appeared to have an orange coloured abdomen with only light banding, which did not closely resemble either of the parent species.

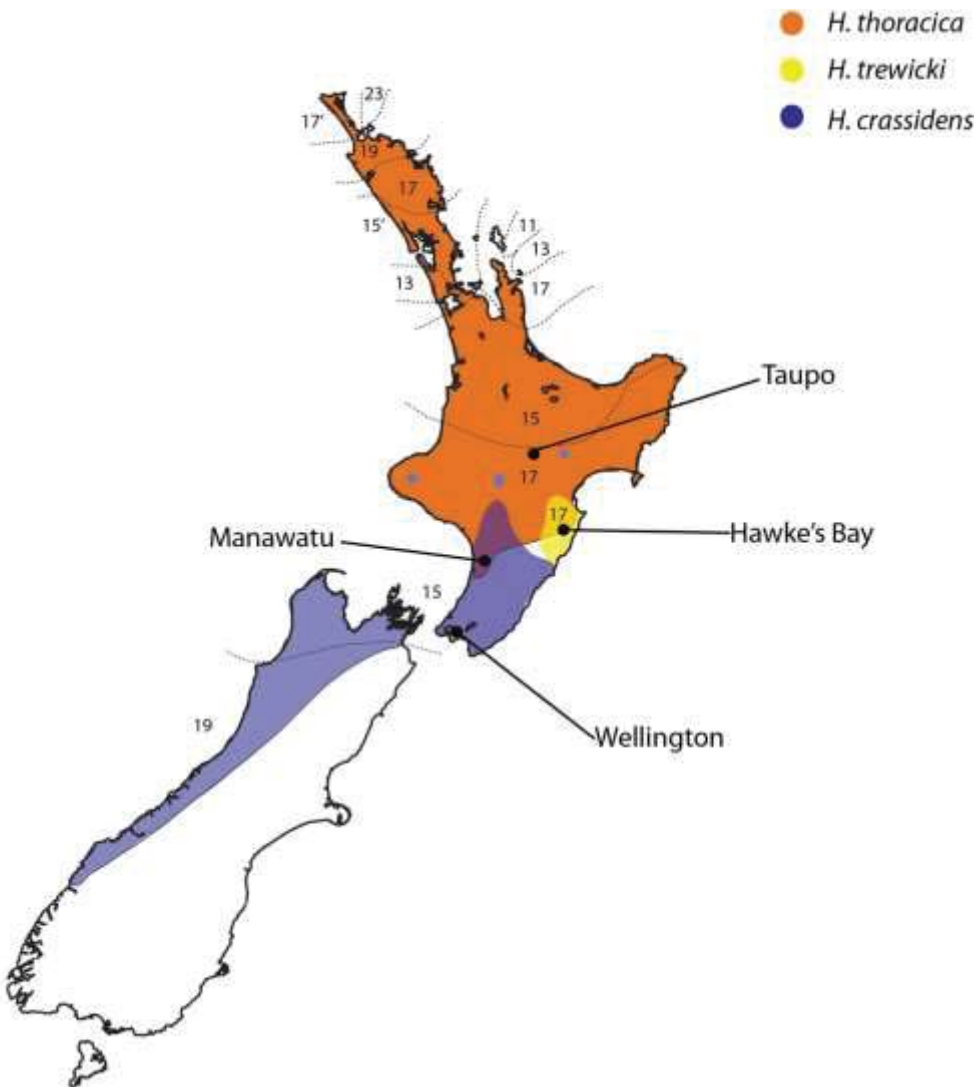

Figure 2.2: Distribution of three New Zealand species of tree weta (*Hemideina*). Distributions of chromosome races within species are delineated by dotted lines, with number representing chromosome number of males (XO). The distributions of the *H. thoracica* and *H. crassidens* chromosome races were taken from Morgan-Richards & Wallis (2003) and Morgan-Richards (2000) respectively. The four sympatric and two allopatric populations sampled in this chapter are indicated.

Table 2.1: Combination of characters that are known to differ between the three North Island *Hemideina* species, which were used to identify weta from parent populations and putative hybrids.

	Pronotum	Abdominal Colour	Abdominal Bands	Prolateral Hind Tibia Spines
H. thoracica	Pale	Brown	No	3/4
H. crassidens	Dark	Yellow	Yes	4
H. trewicki	Pale	Yellow	Yes	4

2.2.3 Ratio of parent species and hybrids

The Kahutawera Valley *H. thoracica/H. crassidens* populations were surveyed on three days over five weeks during August and September 2013. A total of 105 tree weta were identified over these days. For *H. thoracica/ H. trewicki* the sample of 101 weta were recorded over two consecutive days in August 2013.

The weta were surveyed by finding suitable roost habitats in dry dead wood, as weta are nocturnal and hide in holes in trees during the day, usually adopting holes bored by other insects such as the pururi moth (Gosset, 1878; Hudson, 1885). Weta also live in live trees but these were not included as this would have involved damaging or killing or the tree. Most of the dead wood was still standing and included holes of comparable size to those found in live trees, and so should have contained plenty of optimum roost cavities. Most of the standing dead wood consisted of rangiora *(Brachyglottis repanda)* in the Kahutawera Valley and putaputaweta/marbleleaf (*Carpodetus serratus*) at Mohi Bush, as these are both abundant in roost cavities. Other dead wood was found on the forest floor, and although damp or rotting wood has been known to contain tree weta (Trewick & Morgan-Richards, 1995), this is generally considered to be marginal habitat, so was not extensively searched. The roost cavities were opened using a saw to cut branches into a suitable length and then split open with an axe. Care was taken not to injure weta which were checked for species. The sex and approximate age of the weta (juvenile, subadult or adult) was also recorded. The sex was easily noted for all but the smallest weta by the presence or absence of an ovipositor or elongated cerci. Adult weta are distinguishable by the width at the base of the ovipositor in females and the length and shape of the cerci in males. The demarcation between subadult and adult females was based on ovipositor length (nearly adult length for subadults and very short or only visible when the ventral part of the weta was examined for juveniles). For male weta the difference was not as easily defined and was based on both relative body and head size, with larger immature males with elongated heads being considered subadults (although male weta can mature at three different instars, so not all weta pass through this artificially named subadult phase). Data was recorded in the field and then the weta were released and covered with leaves, to lower predation risk until nightfall.

The data was used to calculate ratios for the parent species and hybrids. Sex and age ratios were also calculated, although age ratios for the Kahutawera Valley are not comparable with Hawke's Bay, as sampling took place over five weeks (which is long enough for a weta to pass through an instar in captivity). A Chi Square test was used to look for differences in age distributions, and the Hardy-Weinberg calculation used to calculate expected numbers of hybrids should the species pairs be freely interbreeding.

2.2.4 Morphological Characters

Specimens

For colour characters, tibia spines, and stridulatory ridges, a total of 156 tree weta were studied. The size data used a slightly different sample (see below), and included a total of 65 weta (Table 2.2). Individuals of both sexes were included, although the weta used in this section were all large juveniles or older, due to the difficulty of differentiating early instar specimens for both species and

Table 2.2: Sample sizes for each of the parent populations and the number of adult females available that were measured from e

	Wellington *H. crassidens*	Manawatu *H. crassidens*	Manawatu hybrids	Manawatu *H. thoracica*	Taupo *H. thoracica*	Hawke's Bay *H. thoracica*	
Total sample size	26	26	6	22	22	26	
Adult females	14	19	NA	15	9	8	

southeastern location of the original Taupo population), and another dataset for the Wellington location (same location, different specimens) was taken from Wehi (2014). All other adult females measured belonged to the same samples used to collect the other morphological data. The specimens used were all dead and were stored in 99% ethanol, with the exception of five *H. trewicki* adult females that were recorded in the field (and then released) to increase sample size for this group.

Colour characters

All of the colour characters mentioned above in the *Identification of species* section (also see table 2.1) were recorded in the weta sampled. Other colour characters that were recorded were colouration of the mesonotum and metanotum. For the three thoracic segments the colour and presence or absence of bands (seen on the abdomen of *H. crassidens* and *H. trewicki*) and hieroglyphic type markings (as seen on the pronotum of *H. thoracica* and *H. trewicki*) were recorded. The presence of a dorsal stripe on the abdomen, as found on both *H. crassidens* and *H. trewicki* was also noted.

Leg spines

The number of prolateral spines on each hind tibia was counted for each individual. Extra spines that were present in unusual places (such as between the 2nd and 3rd typical spines on only one leg, or

growing out of another spine) were considered to be the result of minor deformities or accidents during the moulting process and were not counted for the purpose of this study. Small bumps in the position usually occupied by prolateral spine VI in *H. crassidens/H. trewicki* were counted as half spines (Figure 2.3).

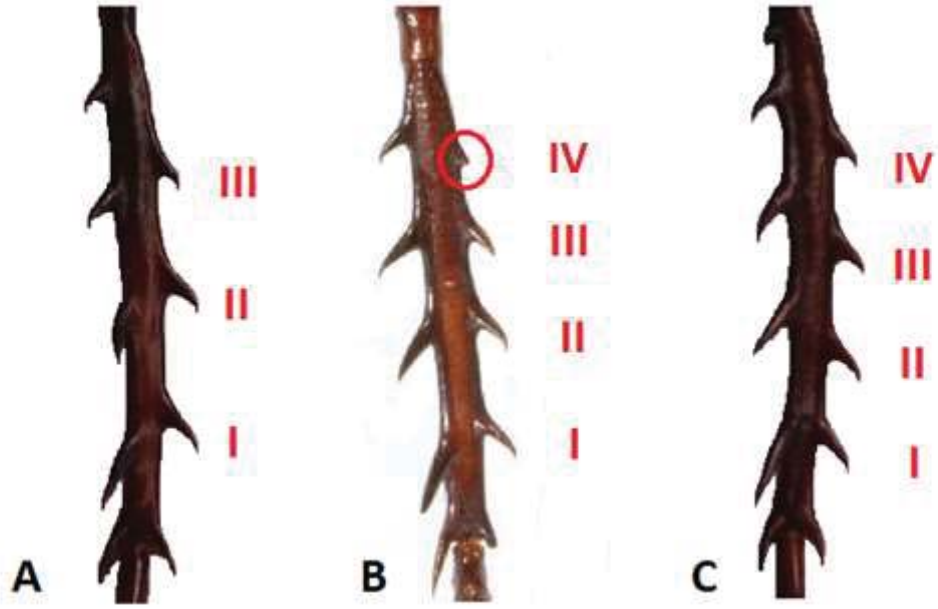

Figure 2.3: Prolateral leg spines on the right hind tibia for three weta found in the Manawatu. A: *Hemideina thoracica* specimen with 3 spines. B: Putative hybrid with '3.5' spines. C: *Hemideina crassidens* with 4 spines.

Size

Size was measured for adult females in the parent populations, using electronic callipers to measure the length of the left hind tibia (to the nearest 0.01mm), which is a good indicator of overall body size (Spencer, 1993). In cases where the left tibia was missing the right was used instead. Only adult females were measured because male weta can mature at three different instars, while females only mature at the tenth (Spencer, 1993; Kelly & Adams, 2010). The data was analysed in Minitab 16 Statistical software. As the numbers of some datasets were different, tests were performed to make sure that the data fit the assumptions for an ANOVA. This statistical test usually requires that data points be normally distributed, independent, and that variance is homoscedastic. Normal distribution of each sample was tested, after which Bartlett's test was used to check for significant difference among the variances. An ANOVA was then performed, along with Tukeys test to check for significant differences between species pairs.

Stridulatory ridges

The number of stridulatory ridges (on the base of the second tergite) on each side of the abdomen was counted under a Leica L2 dissecting microscope and added together to give a total number per individual. The data was analysed in Minitab 16 Statistical software. As the numbers of some datasets were different, tests were performed to make sure that the data fit the assumptions for an ANOVA. This statistical test usually requires that data points be normally distributed, independent, and that variance is homoscedastic. Normal distribution of each sample was tested, after which Bartlett's test was used to check for significant difference among the variances. An ANOVA was then performed, along with Tukey's test to check for significant differences between species pairs.

2.3 Results for Chapter 2: Tree weta in sympatry

2.3.1 Ratio of Parent Species and Hybrids in Sympatry

For the region of sympatry between *H. thoracica* and *H. crassidens* in the Kahutawera Valley the ratio of parent species was similar, with 45% of the sample consisting of *H. thoracica* (n=47) and 52% *H. crassidens* (n=55). 3% of the sample consisted of putative hybrid weta (n=3), which were confirmed as F_1 hybrids (Chapter 3). The observed and expected frequencies of putative hybrid to parent forms deviated significantly from Hardy-Wienburg equilibrium with a Chi Square P-value of <0.001. If these two species were freely interbreeding, just under half of the weta collected would have had hybrid morphology (Table 2.3).

Table 2.3: Observed numbers of parent and hybrid forms in the Kahutawera Valley, with calculated expected numbers (if the weta were in Hardy-Weinberg equilibrium).

	Observed	Expected
H. crassidens	55	30.4
Hybrids	3	52.2
H. thoracica	47	22.4

Sex ratios for *H. thoracica* were very similar (Table 2.4), but differed more for *H. crassidens* with more males being seen in this species. The overall sex ratio for both species was about even. The three hybrids consisted of one male and two females. The age ranges between the two species in the Kahutawera Valley are significantly different, with a chi square P-value of 0.002. Relatively more adult *H. thoracica* were found, and relatively more juvenile *H. crassidens* (Figure 2.4).

Table 2.4: Sex differences within and between *H. thoracica* and *H. crassidens* in the Kahutawera Valley.

	Male	Female	Total
H. thoracica	n=21 (54%)	n=26 (55%)	47
H. crassidens	n=38 (69%)	n=17 (31%)	55
Total	n=59 (58%)	n=43 (42%)	102

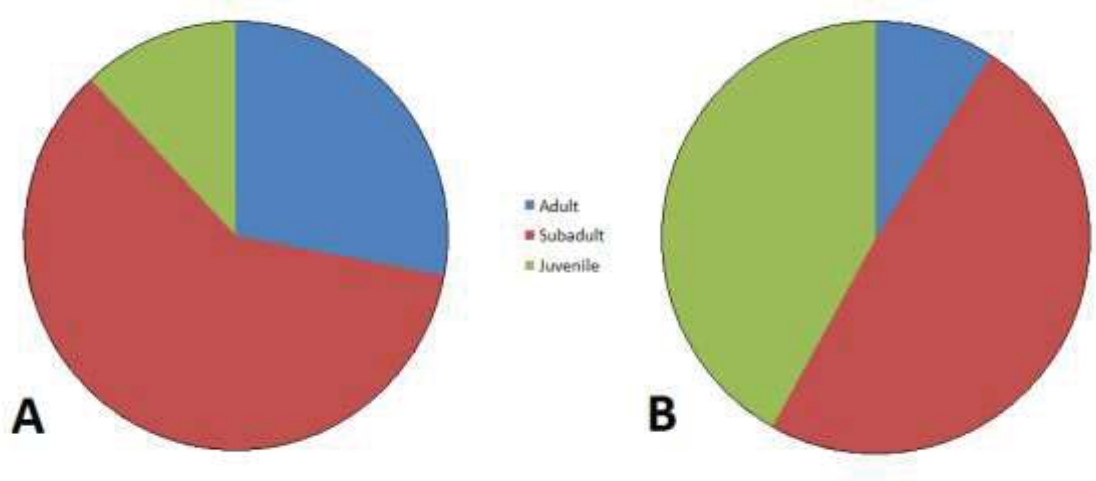

Figure 2.4: The relative proportions of weta at different ages for the two species where they overlap in the Kahutawera Valley in the Manawatu region. A: *H. thoracica,* B: *H. crassidens.*

For the region of sympatry between *H. thoracica* and *H. trewicki* at Mohi Bush the ratio of parent species was similar, with 45% of weta belonging to *H. thoracica* (n=45) and 54% belonging to *H. trewicki* (n=54). There was only one putative hybrid which made up just under 1% of the sample (n=1), which was confirmed as a hybrid in Chapter 3. The ratio of putative hybrid and parent weta deviated significantly from Hardy-Weinberg equlilbrium (Table 2.5). There are significantly fewer putative hybrids than expected if the two parent species were freely interbreeding (Chi square, P-value <0.001).

Table 2.5: Observed numbers of parent and hybrid forms for Mohi Bush Scenic Reserve, with calculated expected numbers (if the weta were in Hardy-Weinberg equilibrium).

	Observed ratio	Expected Ratio
H. trewicki	54	29.7
Hybrids	1	49.6
H. thoracica	45	20.7

Sex ratios for Mohi Bush were similar for each species (Table 2.6).The one hybrid was male. Age ratios differed significantly between the two species (Chi Square P-value = 0.014). 16% of the *H. trewicki* sample consisted of adult weta (n=9), while no adults were found in the *H. thoracica* sample (Figure 2.5). The age proportions of weta was also significantly different between the two samples of *H. thoracica* collected from Mohi Bush and the Kahutawera Valley in August and September (Chi Square P-value <0.001).

Table 2.6: Sex differences within and between *H. thoracica* and *H. trewicki* at Mohi bush Scenic Reserve.

	Male	Female	Unknown	Total
H. thoracica	n=21 (47%)	n=24 (53%)	-	45
H. trewicki	n=22 (41%)	n=30 (56%)	n=2 (3%)	54
Total	n=43 (43%)	n=54 (55%)	n=2 (2%)	99

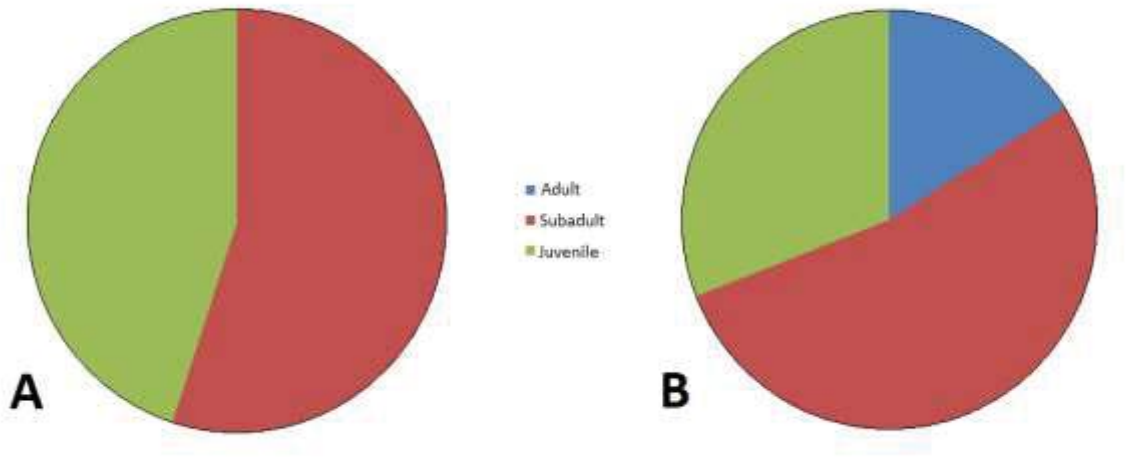

Figure 2.5: Proportions of weta at different ages where they exist in sympatry at Mohi Bush Scenic Reserve. A: *H. thoracica;* B: *H. trewicki.*

2.3.2 Colouration

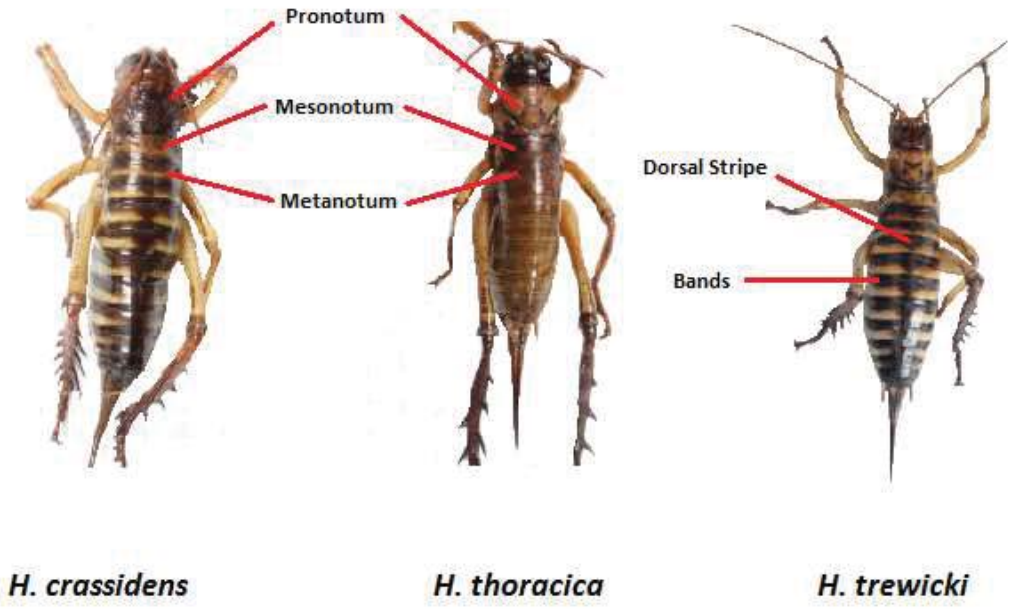

Figure 2.6: Adult female specimens from the three North Island species of tree weta (genus *Hemideina*), showing typical colouration. Colour characters that were used in this study are indicated.

All specimens morphologically examined and assigned to one of the three parent species showed the typical abdominal colouration and banding pattern (Appendices B-H); all *H. thoracica* had a brown abdomen which showed very light banding (rather than no banding), and all *H. crassidens* and *H. trewicki* had a yellow coloured abdomen with distinctive black bands (Figure 2.6). All specimens from *H. crassidens* and *H. trewicki* also contained a black dorsal stripe, which no *H. thoracica* specimen had. There appeared to be a slight difference in the colour of the abdomen between *H. crassidens* and *H. trewicki.* *Hemideina trewicki* generally had a lighter yellow colour than *H. crassidens,* almost white in some specimens. The pronotum in both *H. thoracica* populations and the *H. trewicki* population was pale with black hieroglyphic-type markings, as expected. *Hemideina crassidens* specimens showed more diversity, ranging from pale in one individual through tan coloured to black. Two specimens in the sample had pronotums which were predominantly black, but had white edges, and in a number of cases the colour of the pronotum varied, being lighter on the anterior half. The mesonotum of all *H. trewicki* specimens was pale with black hieroglyphic-type markings, as seen in the pronotum of this species. The metanotum in *H. trewicki* resembled the abdominal tergites, containing a yellow colouration with a posterior black band. The mesonotum in *H. crassidens* was somewhat similar, although with more diversity similar to that seen in the pronotum of this species. Some specimens had mesonotums that were pale with hieroglyphic-type markings, while others were banded similar to abdominal tergites. A few specimens had both hieroglyphic-type markings and a band. The metanotum in *H. crassidens* was banded similar to *H. trewicki.* *Hemideina thoracica* specimens nearly all had dark brown mesonotums and metanotums, which resembled the abdominal tergites, although the mesonotum was generally darker than the

metanotum and abdomen. There were a few cases where *H. thoracica* specimens had pale mesonotums with hieroglyphic-type markings and banded metanotums resembling the other two species. In these four cases, the specimens were juveniles (Appendix C). Putative hybrid individuals (confirmed as hybrids in Chapter 3) from both species pair's had an orange-coloured abdomen with bands that were obvious but fainter than those of *H. crassidens* and *H. trewicki* (Appendix F). These specimens all lacked the distinctive dorsal stripe of *H. crassidens* and *H. trewicki,* although most hybrid specimens from the Manawatu and the one Hawke's Bay hybrid had a row of dark spots in the middle of each tergite, running down the dorsal midline. The pronotum was pale with hieroglyphic-type markings in all hybrid specimens except one (to be expected in the Hawkes Bay hybrid) similar to *H. thoracica*, while the mesonotums and metanotums of these specimens showed a variety of patterns which most resembled the typical *H. crassidens/H. trewicki* pattern (Figure 2.7).

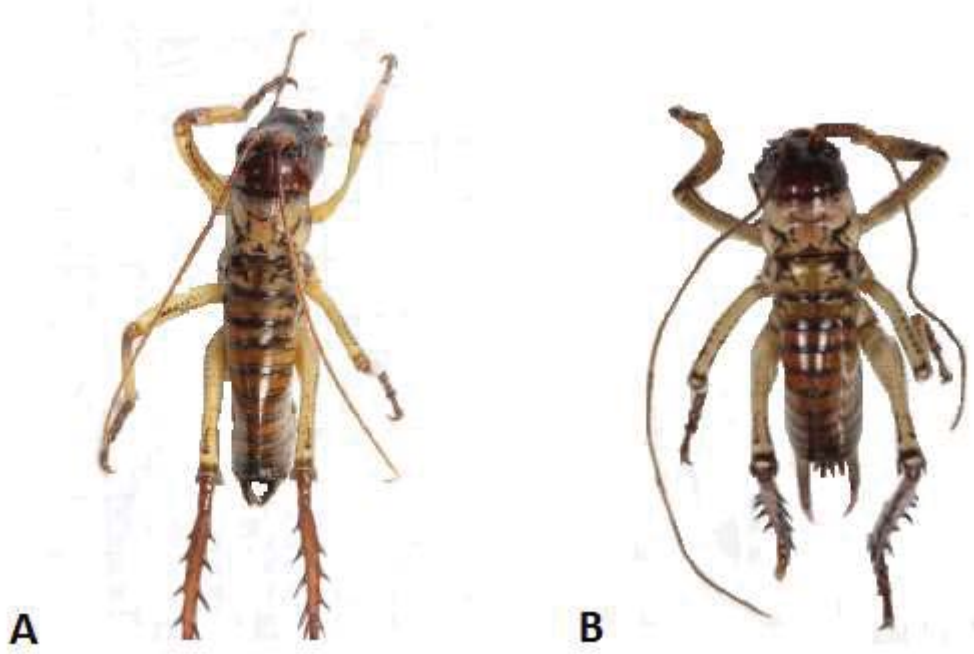

Figure 2.7: A: Juvenile male hybrid of *H. thoracica* and *H. crassidens.* B: Adult male hybrid of *H. thoracica* and *H. trewicki.* Photographs not to scale.

Table 2.7: Number of prolateral hind tibia spines on each leg for the four parent populations, putative hybrids, and two allopatric

Spine No.	Wellington *H. crassidens*	Manawatu *H. crassidens*	Manawatu hybrids	Manawatu *H. thoracica*	Taupo *H. thoracica*	Hawk *H. tho*
2.5, 3	-	-	-	1	-	
3	-	1	1	20	13	2
3, 3.5	-	-	-	1	1	
3.5	-	-	1	-		
3, 4	-	1	2	-	2	
3.5, 4	-	-	1	-	1	
4	26	24	1	-	1	
4, 4.5	-	-	-	-		
4.5	-	-	-	-	-	

with all *H. thoracica* individuals having three spines on each leg, and all *H. trewicki* having four or more. The hybrid weta for the Manawatu showed varying numbers of spines, with some having three or four on both legs and resembling one parent species, and others having a number that fell in between (i.e. three and four, three and a half on each etc.). The one hybrid from Hawke's bay had four spines on each leg and resembled its *H. trewicki* parent. The allopatric population at Taupo for *H. thoracica* shows that a small number of this species have a fourth spine where they live in allopatric populations, while the allopatric for *H. crassidens* from Wellington shows that in this species, allopatric populations may be monomorphic for four spines.

2.3.4 Size differences

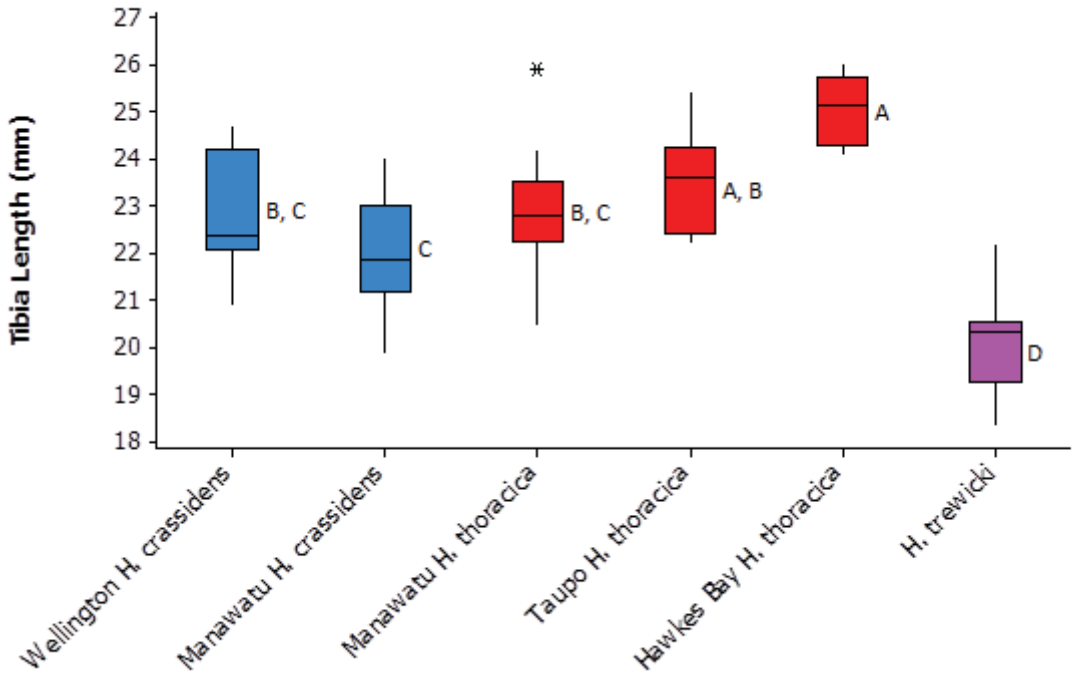

Figure 2.8: Size distribution (hind tibia length) of adult female North Island tree weta (genus *Hemideina*) collected from the wild for the four sympatric populations and allopatric population of *H. thoracica* and *H. crassidens*. Different letters represent significantly different pairs of populations as given by the Tukey's test.

All datasets were normally distributed, and variance was not significantly different, so the assumptions for ANOVA were met. There was a significant difference among samples, with an ANOVA p-value of <0.001 (Figure 2.8). Significant differences were found between *H. trewicki* and all other samples when tukeys test was implemented. There was no difference between the sympatric *H. thoracica* and *H. crassidens* in the Manawatu, or between the two allopatric samples for these species, however, the Hawke's Bay *H. thoracica* population, which can also be regarded as allopatric with respect to the Manawatu *H. thoracica,* was significantly different from the Manawatu populations and the allopatric *H. crassidens* population from Wellington.

2.3.5 Stridulatory ridges

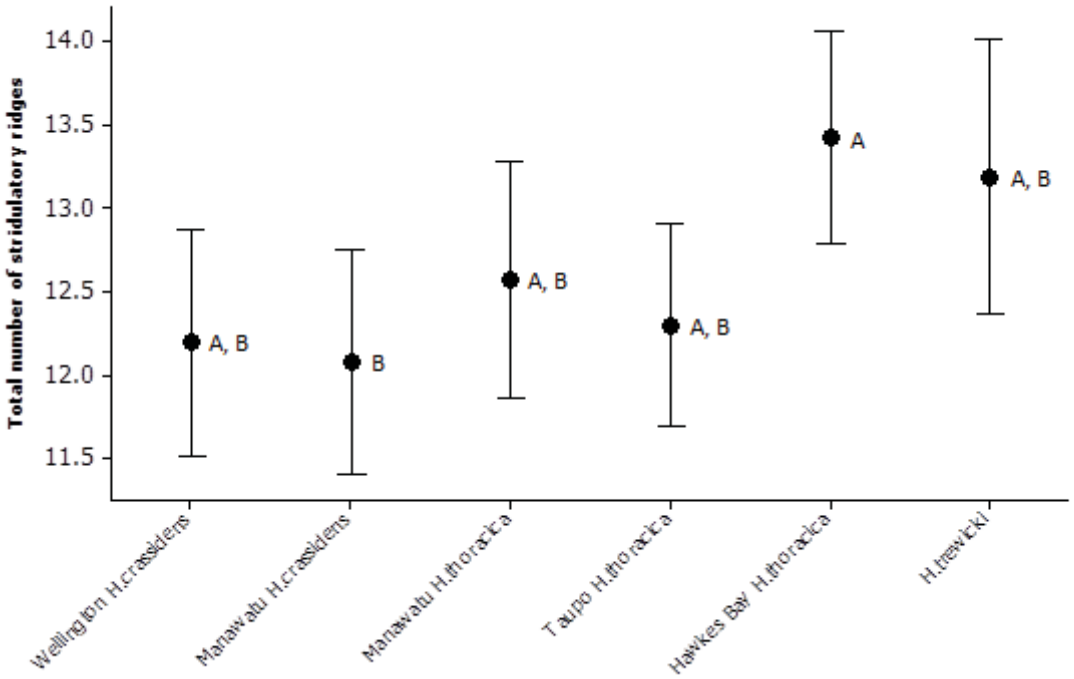

Figure 2.9: 95% CI for the means of the total number stridulatory ridges for each population of the three species examined in this study (genus *Hemideina*). Different letters represent significantly different pairs of populations as given by the Tukey's test.

All datasets were normally distributed, and variance between populations was not significantly different, so the assumptions for ANOVA were met. There was a significant difference among samples, with an ANOVA p-value of 0.015. The two Hawke's Bay populations had higher means than the other populations (Figure 2.9), however, significant differences were only found between Hawke's Bay *H. thoracica* and Manawatu *H. crassidens* when Tukey's test was implemented.

2.4 Discussion for Chapter 2: Tree weta in sympatry

The aim of this chapter was to find out the approximate ratio of the parent species and putative hybrids where they exist in two areas of sympatry, to find out if the species show a bimodal distribution, as had been suggested by previous studies. A range of colouration/ morphological characters were then used to look for possible evidence of divergence and/or introgression in areas of sympatry.

2.4.1 Morphological characters for the parent species and putative hybrids

There were a number of morphological characters that differed between the species. Colouration and banding pattern of the abdomen as well as the dorsal stripe were found to be good characters for distinguishing parent species. The colour of the pronotum proved to be more difficult. This character does not differ between *H. thoracica* and *H. trewicki;* however it is commonly used to distinguish *H. thoracica* and *H. crassidens.* This study showed that nearly all of the parent weta including those in the allopatric populations conformed to this typical pattern, with one exception from the Manawatu region. This *H. crassidens* specimen had a very pale pronotum and resembled *H. trewicki.* As introgression is possible here, this character may still be a reliable indicator of species. The mesonotum and metanotum differed between parent species pairs, so these two characters may be useful in future for identifying and differentiating species, although only adults should be used as *H. thoracica* resembles *H. crassidens* and *H. trewicki* in the colouration of these two characters until around the subadult stage. *Hemideina thoracica* also contains abdominal bands and a dorsal stripe in very early instars (pers. obs.) (although specimens of this age were not included in this study), and only later develops its species specific colouration. This suggests that the *H. crassidens/ H. trewicki* colouration is the ancestral form. As hybrids mostly resembled the *H. crassidens/ H. trewicki* species in the colouration of the mesonotum and metanotum, they will probably not be of much use by themselves for identifying hybrids, although taken together with other morphological markers, they may be helpful. Hybrids did have a distinctive abdominal colour not found in any parent individuals, although it appeared intermediate between the yellow *H. crassidens/ H. trewicki* colouration and the mottled brown of *H. thoracica.* They also had abdominal bands that were not as dark as *H. crassidens/ H. trewicki,* and all lacked a dorsal stripe, although most appeared to have the remnants of one, which consisted of a line of spots in the centre of each tergite running down the dorsal midline (also a distinctive feature). Only one hybrid from the Manawatu had a light tan coloured pronotum with black hieroglyphic markings still visible, with the rest all pale coloured like *H. thoracica.* Taken together, hybrids do appear to have a distinctive colouration that makes them easily identifiable in the field, although it is not certain whether these hybrids represent only F_1 hybrids, or whether some were backcrosses with either parent species (or what these backcrosses typically look like if they exist).

2.4.2 Ratio of parent species

The results showed that in both the Kahutawera Valley and in Mohi Bush, the ratio of parent species was similar. Both samples included putative hybrid individuals, with one in 35 individuals from the Manawatu and one in 100 from Hawke's Bay showing intermediate morphology. These estimates agree with previous anecdotal evidence collected from both areas, although greater sampling is needed to infer whether there is a difference in the number of hybrids produced between the different species pairs. The sex ratios were also about even in all but the *H. crassidens* population, although this may be due to small sample size rather than an actual sex bias in this species, or due to the problems noted by Wehi et al. (2011). Their study used a meta-analysis to determine if there was sex-biased predation in tree weta due to the high level of sexual dimorphism and conspicuous male weaponry. However, their study found no significant bias in any of the six tree weta studied, including in populations in the Manawatu region, so the bias found in this comparatively small study is unlikely to reflect a real difference. Only a subset of holes used by weta could be opened because the methods used did not involve cutting down living trees. As adult tree weta females form harems during the summer (Wehi et al. 2013) they need large cavities, so the methods used here might result in sex bias. Another problem with the Kahutawera sample is that the sampling was done on three separate days over a five week period, meaning that seasonal affects may also have played a part, although the type of seasonal responses displayed by weta are largely unknown. This may also make interpretation of the age range data for this area problematic, as if one species is maturing earlier than the other, the time of year samples are taken is crucial to understanding species specific differences in life cycle. The sampling of Mohi Bush was done over two consecutive days, so this data provides a more robust comparison between the two species. It was interesting that no adult *H. thoracica* was found in this study which targeted mostly larger holes in dry dead wood which should have represented optimum roost cavities. As 16% of the *H. trewicki* sample consisted of adults, it is possible that this species typically matures earlier in the year than does *H. thoracica* at this location. As the exact time of year that weta mature is still unknown and generally considered to be at least partly variable within weta populations, this aspect of weta ecology probably needs further investigation.

2.4.3 *Hemideina thoracica* and *Hemideina trewicki*

All colour characters and the number of tibia spines were found to be non-overlapping. A non-overlapping size difference was also found in adult females between *H. thoracica* and *H. trewicki* for tibia length. Spencer (1993) showed that leg length and weight are highly correlated so longer tibia reflects larger total mass. *H. thoracica* females were larger than individual *H. trewicki,* the smallest species in any of the populations examined. It is not known whether this size difference only exists where *H. trewicki* exists in sympatry with *H. thoracica* as it was hard to find a suitable allopatric population for *H. trewicki* due to its relatively narrow distributions and possible contact or sympatry with *H. thoracica* and/or *H. crassidens* throughout much of its range. It has been suggested that a hybrid zone may exist between *H. trewicki* and *H. crassidens,* due to the presence of some genetically intermediate individuals found in one location (M. Morgan-Richards, unpublished data, 2010). The width of this putative hybrid zone is unknown, and in many areas of Hawke's Bay it is unknown whether there is sympatry with both species or whether populations contain only one

species. In this case introgression with another species or divergent selection could alter its size distribution, as appears to occur where it contacts *H. thoracica.* Lack of these factors would need to be established before these populations could be considered as suitable allopatric populations. Whether the size distribution of *H. trewicki* is similar throughout its range or not, the difference found in sympatry with *H. thoracica* suggests that it may be exploiting a different niche from *H. thoracica.* As the size range of the Hawke's Bay *H. thoracica* was significantly larger than the Manawatu *H. thoracica* population, but not the Taupo *H. thoracica* (although the mean for this population was still smaller), it is difficult to infer whether the Hawke's Bay population has diverged where it lives in sympatry with *H. trewicki*. One issue with *H. trewicki* being much smaller is potential interspecies competition for mates among males. It is therefore possible that the two species have adequate mate recognition systems in the wild (or at least in areas of sympatry) despite findings in captivity which show that the two species will readily mate. It is also possible that the data collected about age distribution in weta could partly explain how *H. trewicki* get around the issue of fighting off much larger interspecific rivals. If the weta are maturing earlier in the year than *H. thoracica,* they may be able to avoid the problem of interspecies mating. This possible shift in the life history of along with the size difference in sympatry, suggests that the two species may have diverged in sympatry.

2.4.4 *Hemideina thoracica* and *Hemideina crassidens*

One *H. crassidens* specimen had a very pale pronotum and resembled *H. trewicki.* However, there are no *H. trewicki* in this region and genetic data later revealed that this individual belonged to *H. crassidens* (Chapter 3). There are only two other recorded specimen of *H. crassidens* with a very pale pronotum, one of which was found at Mt Ruapehu (Trewicki & Morgan-Richards, 1995). The other is a population of pale-pronotum *H. crassidens* in Taihape (Morgan-Richards, 2000). As all of these weta belong to populations that are sympatric with or adjacent to *H. thoracica,* it is difficult to rule out introgression between these species as a cause. It is unclear what the ancestral pronotum colour is in tree weta, as *H. femorata* has a pale pronotum similar to *H. thoracica* and *H. trewicki*, while *H. maori* and *H. ricta* have dark pronotums. *Hemideina crassidens* may have retained some pale-pronotum individuals at very low frequency, or pale pronotums may arise easily through *de novo* mutations. To confirm either of these last two hypotheses, weta from outside the contact zones would need to be found which also have pale pronotums, as none were found in the allopatric population used in this study, making introgression the most likely explanation.

The size situation between *H. thoracica* and *H. crassidens* appears to be different from that of *H. thoracica* and *H. trewicki*, as no significant difference was found in sympatry or allopatry. These two species have been reared in captivity in a previous study where *H. crasssidens* adults were found to be heavier although tibia length was similar to *H. thoracica* (Minards, 2012). The data for this species pair may also be complicated by the fact that some of these weta were raised for part of their lifecycle in captivity, and as protein levels in the diet and temperature can alter adult size (Griffin, 2011) the data collected here may not be a reliable indicator of size in the wild. However, only some of the *H. crassidens* females in this study were raised in captivity and were fed protein, so this should mean that this species was larger overall, which is not the case. Allopatric populations were not shown to be significantly different for *H. thoracica* or *H. crassidens* in the Taupo or Wellington

populations studied here, but the average size of both these species is already known to vary across their range (Minards et al. in prep), so the fact that they are similar where they meet in the Manawatu is unusual, given that weta would be expected to diverge rather than converge in areas of sympatry. It is possible that weta are responding to the local environment in this regard, or that they have not been living in sympatry for long enough to show strong divergence. The similarity in size removes the problem possibly encountered by the *H. thoracica/ H. trewicki* species pair, as male weta should theoretically be on equal terms where competition for mates is concerned in the Manawatu regions of sympatry, and although only females were measured, the data suggests that average weta size is similar between sexes.

Hemideina thoracica is polymorphic for prolateral hind tibia spines, given that the Taupo population contained a small number of weta with four spines. This polymorphism has been noted before, although not in southern populations of *H. thoracica.* The fact that four-spined *H. thoracica* were not found in Hawke's Bay, and only one weta with an extra half spine was found in the Manawatu, means that this morph is either very rare in the southernmost *H. thoracica* or that it has introgessed into these populations from *H. crassidens.* The former explanation is more likely, and the low numbers of four-spined morphs in the southern regions can probably be explained as the result of expansion and resultant loss of diversity (also shown by loss of mtDNA diversity in southern populations compared to northern population of this species; Bulgarella et al. 2014). However, two *H. crassidens* weta from the Manawatu had three spines, and this morph has not been noted in this species before. It is very likely that the four-spined morph is the ancestral morph, as all other tree weta species have four spines. The allopatric population at wellington was monomorphic for four spines, so this is either ancestral polymorphism at very low frequencies or introgression with neighbouring *H. thoracica.*

2.4.5 Reproductive character displacement

The data collected for stridulatory ridges did not show the pattern expected, with weta appearing to be more similar in sympatry rather than less, although this conclusion is tentative, as the Tukey's test only gave a significant difference between the Manawatu *H. crassidens* and Hawke's Bay *H. thoracica.* However, the means for both Hawke's Bay weta were higher than other populations, and the ANOVA showed a significant difference overall, so it is possible that a larger sample or a less conservative test would identify larger significance here. It is also possible that the data represents random fluctuations between populations and does not have any biological significance. Whether the total number of stridulatory ridges has converged in sympatry, or simply represents a lack of change between allopatric and sympatric populations' remains to be determined. The allopatric populations for *H. thoracica* and *H. crassidens* did not differ, so it is likely that in these two species, the similarities are ancestral and that there has been little reason for change (or constraints on change). However, it appears that the Hawke's Bay *H. thoracica* may have altered their stridulatory morphology to match that of *H. trewicki* where they live in sympatry (although a larger dataset would be needed to state this conclusively). It is difficult to explain this possible convergence via environmental affects, as these ridges are only used by weta to communicate and defend themselves. Weta are facing the same species of predators in most of New Zealand (eg. rats, moreporks), so there should not be any reason for this to cause local differences in sound

production by weta. Differences between allopatric populations were only average differences, with a large amount of overlap. This situation differs from the *H. femorata/ H. ricta* species pair in which the difference found is non-overlapping throughout their distribution. A non-overlapping difference would presumably be a useful way to distinguish between conspecific and interspecific weta, while an average difference would only be a guideline, although this was found in neither of the species pairs in sympatry. As it does not seem likely that many hybrids are produced, or that there is extensive gene flow, it may be that auditory communication is not involved in species recognition in North Island weta. Weta share the same roost cavities in the wild (in both species pairs), so it is possible that any convergence in sympatry is the result of the need for aggressive communication. Male weta presumably recognise interspecies males as rivals for resources (optimum roosts) and mates, so it may be advantageous to both species if they can interpret aggressive signals from rival interspecies males, reducing the need for actual physical combat. It is unclear whether females direct aggressive signals toward conspecifics, or whether females are only using this form of noise for predator defence, so the potential role of females in shaping this behaviour is unknown.

2.4.6 Conclusion

The survey of the species pairs where they are known to live in sympatry shows that the species appear to be retaining their own identity with a bimodal distribution, as most individuals examined fit the description of one species or the other. A few individuals were found to have an intermediate morphology, but not the large number that would be expected if the species were freely interbreeding. Putative hybrids were found to have a distinctive combination of parent characters as well as their own unique markers so should be easy to identify in future in the field. A few morphological characters were found to differ between the species pairs in addition to the typical ones used in the field. These were the colouration of the mesonotum and metanotum, and the presence/absence of a dorsal stripe. There was a large non-overlapping size difference between *H. thoracica* and *H. trewicki*, and a putative overlap of the average total number of stridulatory ridges in sympatry, which suggests divergent and convergent selection pressures respectively. The Hawke's Bay weta showed no overlap in any of the morphological or colour characters studied here (excluding the putative the F_1 hybrid) except in the average number of stridulatory ridges. A few unexpected findings in the *H. thoracica*/ *H. trewicki* species pair, such as the possible difference in lifecycle, size difference, and the possible convergence in numbers of stridulatory ridges in sympatry probably needs further study to clarify what they mean for the ecological niches of this pair. All of these traits may have a significant effect on their ability to either coexist or displace each other. There was some overlap in morphological and colour characters in the Manawatu between *H. thoracica* and *H. crassidens*, and it is not clear whether this is the result of introgression or ancestral polymorphism, so genetic studies are needed confirm or disprove these hypotheses. There did not appear to be as many characters or traits separating this pair. This may mean that this species pair experiences stronger competition where they exist in the wild, although the slight overlap in some characters means that limited introgression between the species cannot be ruled out.

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Chapter 3: Introgression

3.1 Introduction

3.1.1 Introgression

Introgression is “the permanent incorporation of genes [alleles] from one set of differentiated populations into another” (Reisburg & Wendel, 1993). Introgression can be viewed as a dilution of a species gene pool, or as a positive event that increases genetic diversity. With rapidly changing molecular genetic tools available many new studies have been able to detect introgression, leading to a greater acceptance that hybridisation is common within an evolutionary time frame. For example, neanderthal alleles were recently discovered in the human genome (Noonan, 2010; Sankararaman, 2014). Introgression is the signal of past hybridisation (see chapter 1), and the ability to successfully hybridise might be of fundamental importance to the future of a species, while climates and environments continue to change (Becker et al. 2013).

Many viruses incorporate DNA from their environment, leading to the formation of new strains that can take advantage of novel environments (Smith et al. 2009). Many microorganisms will use various methods of horizontal gene transfer to spread alleles rapidly, such as antibiotics resistance (Ochman, 2000). There are also reports of the benefits of introgression in the animal lineage, such as the Melanocortin 1 receptor allele that has introgressed from domestic dogs into the Gray Wolf (*Canus lupus*) and has experienced positive selection (Anderson et al. 2009). Darwin’s finches were shown to have experienced extensive introgression, which likely helped with the adaptive radiation seen in this group (Lowe, 1936; Grant et al. 2004). In the Orthopteran order, hybridization resulting in introgression appears to be a relatively common phenomenon (Hewitt, 1987). One relatively well studied example is introgression between *Chorthippus parallelus* subspecies which retain karyotype differences (Vazquez et al. 1994; Ferris, 1993). Introgression is often viewed as a problem where endangered species are concerned, especially if introgression is occurring between common introduced species and rare endemics, or due to human modification of habitat. An example is the endangered Forbes’ parakeet (*Cyanoramphus forbesi*) on Mangere Island (New Zealand) that began hybridizing with the neighbouring Chatham Island Red-crowned parakeet (*Cyanoramphus novaezelandiae chathamensis*) after forest clearance for farmland, threatening the survival of the original gene pool (Chan et al. 2006). If introgression threatens to swamp an endangered species, conservation efforts may need to focus on preventing interbreeding. Debate about how common introgression is, and its short and long term consequences will continue while we have few well-studied examples. Estimates of introgression in natural systems (where humans are not implicated) provide important data about the frequency of the phenomenon and its natural consequences for biodiversity (Grant et al. 2005).

3.1.2 Tree Weta

The three species of tree weta in North Island New Zealand each have distinct chromosome complements (Morgan-Richards, 1995, 1997, 2000). Karyotype differences are generally viewed as a

barrier to gene flow. However, some species are able to contain multiple chromosome races and retain gene flow, often through tension zones (Baton & Gale, 1983; Barton & Hewitt, 1985). In particular, small mammals (Searle, 1993) and orthoptera (White, 1978) show relatively high levels of intraspecific karyotype variation. In the *Hemideina* genus, *H. thoracica* has nine known chromosome races, while *H. crassidens* has two (Morgan-Richards, 2000; Morgan-Richards & Wallis, 2003). These races have been shown to interbreed (intraspecific hybridisation) in many areas (Morgan-Richards & Wallis, 2003), and allozyme and DNA sequence data show that the populations, although differentiated, are still exchanging alleles, and that the different chromosome races are unlikely to qualify as separate species (Morgan-Richards, 1997; Morgan-Richards & Wallis 2003). A study of one hybrid zone in *H. thoracica* around Lake Taupo showed that nuclear markers introgressed further than chromosome markers, suggesting that chromosome differences are limiting introgression in this species. A comparison of five hybrid zones within *H. thoracica* showed that counter to expectations, degree of karyotype difference (determined by number of chromosome alterations and percentage of the total genome involved) did not determine zone width (Morgan-Richards & Wallis 2003). Whole-genome factors may be more important in limiting introgression than chromosome differences in *H. thoracica* (Morgan-Richards & Wallis 2003). The two chromosome races of *H. crassidens* can produce F_1 hybrids in laboratory conditions. The difference in karyotype between these two races is probably the result of two roberstonian fusions, as the 19 chromosome race forms a monophyletic clade sister to the two 15 chromosome race clades (Bulgarella et al. 2014). Six chromosomes line up to form two trivalents during meiosis in males, which means that there may be little reduction in fertility where races meet, and suggests chromosome differences might not be a barrier to gene flow in this species (Morgan-Richards, 2002). The mountain scree weta in the closely related giant weta genus (*Deinacrida connectens*) has at least seven chromosome races, with higher levels of heterozygosity than observed in *H. thoracica,* indicating that in *D. connectens* karyotype differences may create less of a barrier to gene flow in comparison (Morgan-Richards & Gibbs, 1996). Altogether, these studies show that chromosome differences alone are not enough to reproductively isolate populations within this lineage. However, there are a number of differences in the gross morphology of karyotypes between *H. thoracica* and its two neighbouring species. While the difference between the karyotypes of *H. trewicki* and *H. crassidens* can be explained as a result of two roberstonian fissions/fusions, the differences between these two species and *H. thoracica* is much greater. It may be that karyotype differences of this size are enough to isolate species within this family, but this has not been examined in detail.

3.1.3 Potential outcomes of hybridization

There are a number of possible outcomes if two species are able to hybridize (Jiggins & Mallet, 2000; Morgan-Richards et al. 2009). High levels of gene flow at one end of the spectrum will lead to the species losing their genetic identity, and if this is the case, it may be more appropriate to label them as part of the same species or as subspecies, although this will depend on the species concept employed (Fig 3.1, far left). If there is some restriction to gene flow, either due to genetics or assortative mating (or both), the level of restriction will show in the pattern of the hybrid zone. Moderate levels of gene flow will lead to tension (or unimodal) zones, assuming that hybrids suffer a selective disadvantage. In certain areas of contact between *H. thoracica* and *H. crassidens* the environment is known to differ in temperature and elevation, and this has been linked to different

adaptations in these species (Bullgarella et al. 2014). However, in the Manawatu the environment is fairly uniform, and as the species do not appear to show preferences for different habitats, intrinsic genetic or behavioural factors are probably the only factors preventing hybridization and determining hybrid disadvantage. *Hemideina thoracica* and *H. trewicki* are likewise found living in identical habitat. Tension zones form semi-permeable barriers to gene flow but are usually geographically constrained. Although the populations outside the contact zone retain most of their own species' genetic identity, nearly all individuals within the contact zone show mixed ancestry. In contrast, in a bimodal hybrid zone gene flow is lower, allowing parent forms to predominate. In this case both species will be found in the same geographic area, with the occasional individual who has mixed ancestry. At the far end of the spectrum (Fig 3.1, far right), there may be no gene flow (F_1 hybrids may be produced, but will be infertile). In this situation, both species may be found in the same geographic area, but this will be determined by factors such as competitive exclusion and habitat availability rather than introgression. *Hemideina thoracica* appears to be differentiated in colour pattern, morphology and karyotype from *H. trewicki*, and although only differentiated in some colour characters and karyotype from *H. crassidens*, these differences form a bimodal zone (Chapter 2). Thus introgression is predicted to be non-existent, or at the very low end of the spectrum should it occur.

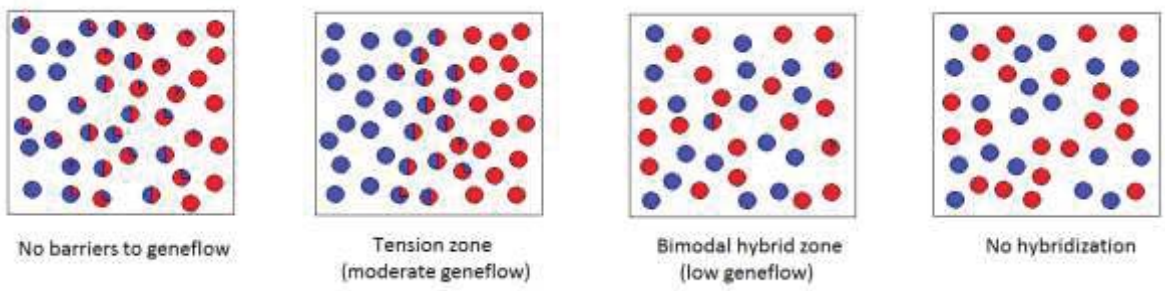


Figure 3.1: The range of potential genetic results of sister species meeting. Different colours represent two hypothetical species and hybrids suffer selective disadvantage from very low (left) to high (right).

3.1.4 Range expansion and genetic patterns

Hemideina thoracica has been hypothesised to have expanded its range southward since the LGM about 15,000 years ago (Morgan-Richards et al. 2001; Bulgarella et al. 2014). Range expansions result in particular genetic patterns (Charlesworth, 2009; Excoffier, 2009). Spatial expansion will lead to low genetic diversity in the expanding population (as seen in *H. thoracica*), as well as a smaller population size compared to local species in the leading edge. If there is introgression between an expanding species and a local species, the expanding species is more likely to contain introgressed alleles. This is because high levels of intraspecific gene flow and a larger population size will make fixation of new alleles less likely in a local population than in a smaller expanding population (Petit & Excoffier, 2009). These findings are backed up by evidence from simulation models (Excoffier et al. 2009), which show that surfing of rare alleles is much more likely on the wave-fronts of expanding

species. If there is any introgression in the species' pairs compared in this study, *H. thoracica* would be expected to have more foreign alleles than the species it hybridizes with, particularly in comparison to *H. crassidens* which has been displaced from much of its former range by *H. thoracica*. If one sex is the dispersing sex in a lineage, this should also leave a particular genetic signature in cases where introgression occurs. For example, if dispersal is male-biased, then mitochondrial genomes should introgress more frequently into the colonising species, while any male specific elements present in the genome would have a much higher chance of introgressing into the local species (Petit & Excoffier 2009). For tree weta, it is unclear whether dispersal is male or female biased (e.g. Kelly 2006; c.f. Wehi et al. 2012). If introgression does occur in either of the species pairs, it may be able to give insights about sex-related dispersal patterns in tree weta.

3.1.5 Aims

The aim of this research was to confirm the identity of putative hybrids and look for evidence of introgression between *H. thoracica* and the two species it meets and at the southern border of its range. Sampling included both allopatric and sympatric populations to establish alleles unique to each species. A range of characters were assessed for evidence of introgression; chromosomes, mtDNA and nuclear loci (gene sequences and microsatellite genotyping). Putative hybrids were genetically tested for heterozygosity and evidence of introgression (i.e. F_2 or later backcross hybrids) was sought. Statistical tools for estimating gene flow allow inference about direction as well as frequency (Wilson & Rannala, 2003), so were employed to examine these factors.

Table 3.1: Sample sizes for populations of tree weta (genus *Hemideina*) from North Island New Zealand used to obtain genetic da
were collected from a subset of the total sample (nuclear data was collected for all individuals). *Data obtained in previous studie
et al 2014).

	Akitio *H. crassidens*	Otaki Forks *H. crassidens*	Manawatu *H. crassidens*	Manawatu hybrids	Manawatu *H. thoracica*	Taupo *H. thoracica*	Hawke's *H. thora*
Karyotype information	-	-	11	1	10	-	12
mtDNA	10*	10*	13 (+9*)	9	5 (+10*)	10*	8
Total sample	10	10	23	9	22	10	12

twenty-three *H. crassidens*, and nine putative hybrids were collected in the Manawatu from the Kahutawera Valley (GPS: S 40.47184, E 175.60943) and the Turitea Valley (GPS: S40.431725, E 175.674595) where the two species are sympatric. The two valleys are 7km apart, on the western side of the Tararua mountain range, and are assumed to provide identical habitats with weta from the same gene-pool.

Karyotype data was obtained from fresh animals. Mitochondrial fragments had been sequenced for some weta as part of an earlier study (Genebank accession number: KC913540–KC913542 cytochrome oxidase I for *H. thoracica*, and KC913276–KC913283 cytochrome b for *H. crassidens*).

Ten *H. thoracica* were included from an allopatric population at Hinemaiaia Reserve in the Taupo District (GPS: S 38.892269, E 176.095022) (Figure 3.2). These weta are well removed form *H. crassidens*, and are the furthest population (so far identified) from the zones of sympatry that still belongs to the same 17 chromosome race of *H. thoracica*. The specimens were previously used in a study by Morgan-Richards et al. (2000), which showed that karyotype and a mitochondrial locus (small ribosomal subunit (12 S) gene) from these specimens were all typical of the 17 chromosome race of *H. thoracica*. As the Hawke's Bay and the Manawatu *H. thoracica* populations are separated geographically by approximately 157km, they can be considered allopatric populations with respect to each other, meaning that each sympatric population of this species effectively had two allopatric populations for comparison. For *H. crassidens*, two allopatric populations were selected,

one at Otaki Forks on the west coast (GPS: S 40.87467517, E 175.2351446), the other at Akitio in the Tararua District, east coast (GPS: S 40.61463, E 176.41338). These two populations are well outside the zones of sympatry and relatively far from one another, being located on opposite sides of the Tararua ranges (Figure 3.2). Mitochondrial data was available (cytochrome b) Genbank accession number: KC913379–KC913388 for Akitio, and KC913234–KC913235 for Otaki Forks (Bulgarella et al. 2014). Karyotypes from a sample of these populations had already been determined (Morgan-Richards 2000; Bulgarella et al. 2014), and all specimens belonged to the 15 chromosome race. It was not possible to include an allopatric population for *H. trewicki* which has a narrow range broadly overlapping with *H. thoracica* and *H. crassidens* (Trewick & Morgan-Richards, 1995).

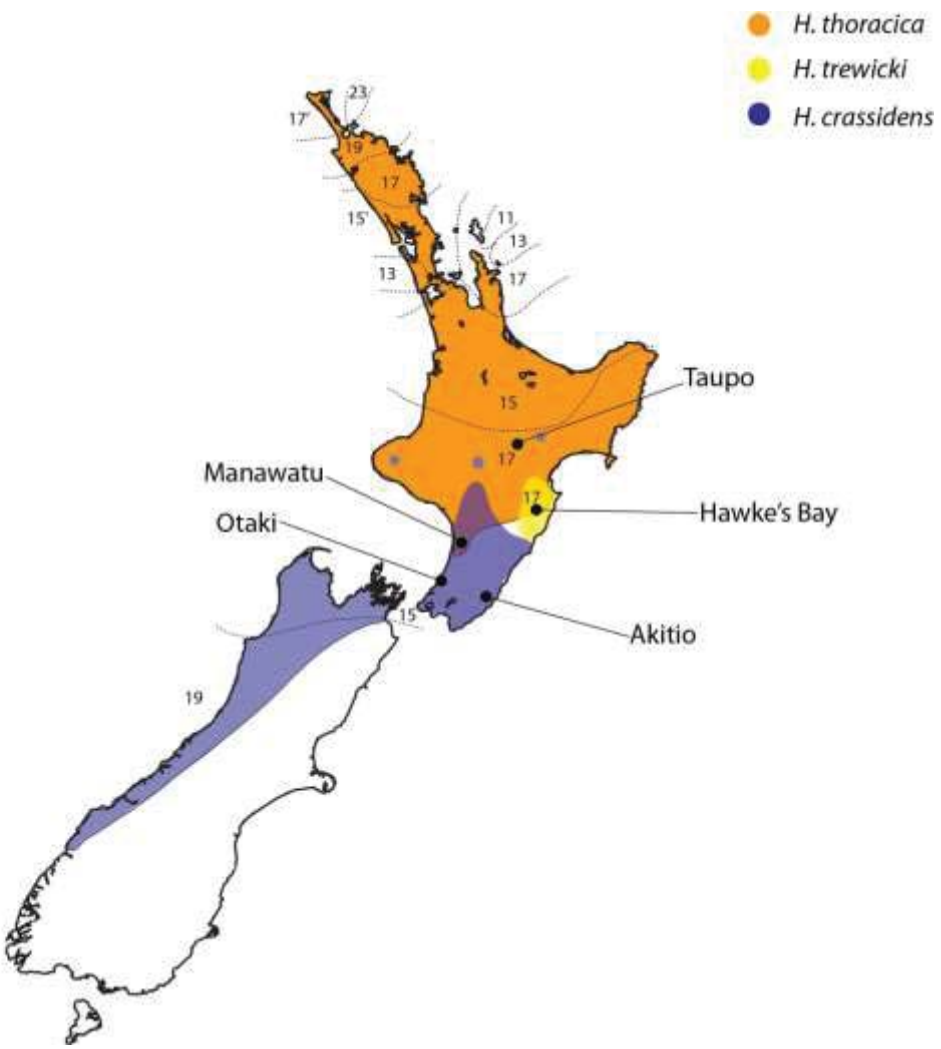


Figure 3.2: Distribution of three New Zealand species of tree weta (*Hemideina*). Distributions of chromosome races within species are delineated by dotted lines, with number representing chromosome number of males (XO). The distributions of the *H. thoracica* and *H. crassidens* chromosome races were taken from Morgan-Richards & Wallis (2003) and Morgan-Richards (2000) respectively. The four sympatric and three allopatric populations sampled in this chapter are indicated.

3.2.2 Karyotype

Most of the weta specimens used in this part of the study had been karyotyped as part of a previous study (Mckean et al. submitted) examining karyotype differences among the three species. Six weta were added to the dataset (three Hawke's Bay *H. thoracica,* one Manawatu *H. crassidens,* and two *H. trewicki*) to increase sample size. No putative hybrids were karyotyped in the aforementioned study, so karyotypes for the putative Hawke's Bay (*H. thoracica* x *H. trewicki)* and a putative Manawatu hybrid (*H. thoracica* x *H. crassidens*) were also analysed.

The chromosome preparations were based on the method of Cokendolpher & Brown (1985). Weta were anesthetised with ethyl acetate, and then their testes or ovaries were removed and placed in hypotonic solution (insect saline: water, 1:4) for 15min. They were then removed and stored in fixative (methanol: acetic acid, 3:1) for a minimum of 1 hour. Between three and six ovarioles or tubules from the testes were placed on a 25mm x 75mm x 1mm pre-cleaned frosted slide (LabSev) with a few drops of acetic acid, and the cell dispersed by tapping with the blunt end of a metal rod. Slides were air dried at 50^{o}C and then labelled on the frosted end. They were then then stained for 15min in a 5% Geimsa staining solution, which consisted of 5ml Geimsa stain in 95ml sodium-phosphate buffer (1g of KH_2PO_4, 0.92g of $Na_2HPO_4.H_2O$, dissolved in 1000ml H_2O) at pH 6.8. After drying, coverslips were added with a few drops of DPX mountant and left to dry. Slides were then examined under an Olympus BX51 Compound Microscope at 800x magnification, or under oil immersion at 1200x, if chromosomes were too close for clear identification at the lower magnification. An Olympus SC30 camera and Olympus CellSense Dimension 1.6 software was used to photograph clear mitotic metaphase or anaphase spreads. Chromosomes were then counted and checked to see if they matched the size/centromere position for the parent karyotype (McKean et al. submitted) of the species they had been assigned to, based on morphological characteristics. For putative hybrids, F_1 karyotypes were determined based on parent karyotypes and then checked against actual karyotype from putative hybrids. For the Hawke's Bay species pair (*H. thoracica* and *H. trewicki*), total chromosome number is the same, so F_1 hybrids should have 17/18 chromosomes depending on sex (weta have an XO sex-determination system; females have two copies of the sex chromosome, males have only one). F_1 hybrids from this species pair were also expected to have karyotypes with five small acrocentric/submetacentric chromosomes, four intermediate-sized metacentric chromosomes and eight or nine large metacentric chromosomes depending on sex. For the Manawatu species pair (*H. thoracica* and *H. crassidens*), the total chromosome number differs, with 15/16 for *H. crassidens* and 17/18 for *H. thoracica.* This means that the hybrid number is 16/17, leading to a situation where F_1 males would have an even number of chromosomes, and F_1 females an uneven number (in contrast to what is usually found in an XO system). The expected karyotype for a Manawatu hybrid consisted of five small acrocentric/submetacentric chromosomes, one metacentric intermediate-sized chromosome and either ten or eleven large metacentric chromosomes depending on sex.

3.2.3 DNA Extraction

Weta specimens were stored in 99% ethanol prior to DNA extraction which used a standard salting out method (Sunnucks & Hale, 1996). Tissue from the hind femur was removed and added to 10µl of

Protinase K (10ng/µl) and 600 µl TNES Buffer (10 mM Tris (pH 7.5), 400 mM NaCl, 100mM EDTA, 0.6% SDS) for 3 hours at 55^{o}C in a Total Lab Systems LTD Infors Minitron shaker at approximately 200rpm, or left overnight in a Biolab Scientific LTD Boekel heat-block. Proteins and cell debris were then removed by adding 170 µl of 5M NaCl, shaking well and spinning in centrifuge at 14,000rpm for 5min, and then pipetting supernatant into a clean tube. DNA was precipitated using 600µl of ice-cold ethanol, mixed via inversion and left in freezer for 5min. After spinning at 14,000rpm for 10min, the supernatant was poured out, and then the pellet was rinsed with 400µl of ethanol and spun at the same speed for 5min. The ethanol was poured off, and after drying, the DNA was re-suspended in 50µl of H_2O for storage. Concentration and quality of DNA was checked using a Thermoscientific nanodrop spectrophotometer and ND-1000 V3.6.0 software. Concentration and quality were also checked by electrophoresing DNA on a 1% agarose gel (Amesco agarose powder and 1x TAE Buffer: 40 mM Tris acetate, 1mM EDTA pH 8.0) alongside a 1Kb+ DNA Ladder (Life Technologies). The gel was run at 90V for one hour. DNA was visualised and photographed under U.V. light using a BioRad gel-doc with BioRad QualityOne 4.4.0 software. Concentration information was used to dilute DNA for each specimen to an approximately 10ng^{-1} µl working solution.

3.2.4 Mitochondrial data

A fragment of the CO1 mtDNA gene was amplified using the same reverse primer for all weta; 12WetaR (ATT GCA CTT ATC TGC CAT ATT AG (Bulgarella et al. 2014)). However, due to differences between *H. thoracica* and the other two species at this locus, the forward primer 1490thor (AAC TAA TCA CAA GGA TAT TGG (Bulgarella et al. 2014)) was used for *H .thoracica,* giving an approximately 1200bp fragment, while 10MTDcrass (AAC ACT TAT TTT GAT TCT TTG G (Bulgarella et al. 2014)) was used for both *H. crassidens* and *H. trewicki* and gave an approximately 800bp fragment. Standard polymerase chain reaction (PCR) conditions were used (Trewick & Morgan-Richards, 2005). An initial denaturation temperature of 94°C for 3mins was followed by 38 cycles of: 94°C for 30sec (denaturation); 55°C for 15sec (annealing); 72°C for 1:30sec (extension). This was followed by a final extension at 72°C for 15min. MacLab Taq and Buffer (containing 1.5mM Mg^{+})were used for the PCR, with Mg^{+} concentration increased from 1.5mM to 2mM for the 1490thor/12WetaR primer pair reactions. PCR products were electrophoresed on a 1% agarose gel made from Amesco agarose powder and 1x TAE Buffer (40 mM Tris acetate, 1mM EDTA pH 8.0) at 110V for 40min, alongside a 1Kb+ DNA Ladder (Life Technologies). PCR products were then visualised and photographed under U.V. light using a BioRad gel-doc with BioRad QualityOne 4.4.0 sotware. Samples were checked for the quality and quantity of the DNA fragment, and were also checked against a 1Kb+ DNA Ladder (Life Technologies) to make sure that they belonged to the expected size class. Appropriate PCR products were then sequenced at the Massey Genome Service using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc).

For putative hybrids, both primer pairs were used. As only one pair gave a PCR product and/or a clean sequence in each individual hybrid, this was assumed to represent the mitochondrial genome for that particular individual (mitochondrial genomes are maternally inherited, so each weta was expected to have a copy from only one species). There were some problems with nuclear copies of these gene fragments, although in most cases the ratio of nuclear to mitochondrial genomes was sufficient to obtain a clean sequence. For some individuals it was impossible to obtain a clean

sequence (particularly for *H. thoracica* specimens). In this case, these individuals were left out of the mtDNA analysis, after first attempting to amplify the locus using the other primer set (in case they had foreign mitochondria). Notably, all putative hybrids gave a clean sequence with one primer pair (as these presumably have nuclear copies from both parent species).

Software Geneious 6.1.7 (Boimatters LTD) was used to visualise and align sequences and trim ends. A haplotype network was created in PopArt (Allan Wilson Centre Imaging Evolution Initiative; http://popart.otago.ac.nz), using an integer neighbour-joining method with reticulation tolerance set to zero. This allowed unequivocal assignment of each haplotype to species clades. A chi square test was use to check if the hybrid haplotypes suggested a sex-bias (i.e mothers of one species being more common). As phylogenetic relationships have already been inferred for these species using mitochondrial DNA sequences (Morgan-Richards et al. 2001; Trewick & Morgan-Richards, 2004, 2005; Bulgarella et al. 2014), and as identifying potential introgression and maternity/paternity of the hybrids was the objective of this section, the data was not analysed further.

3.2.5 Nuclear Sequences

Eleven nuclear primer pairs developed for either tree or ground weta were tested with all three species in this study. Two primer combinations gave single products and clean sequences, and also showed variation among the three species. Variation was important as allelic differences were needed to differentiate the species pairs. A 400bp fragment of the nuclear gene Sperm flagallar protein (Sflag), was amplified with forward primer TWnucSflagF (Twort 2012; 5´TCGCCAGTTCAGACCTAGGATGAGG3´), and reverse primer TWnucSflagR (Twort 2012; 5´TGGCTCTGTACAAGGCTGGGA3´). An approximately 500bp fragment of the nuclear gene Testis kinase 1 (Testis_kin_1), was amplified with forward primer TWnucTestis_kin_1F (Twort 2012; 5'CGGAAGTAGTAAGTGGGACGCCG3´) and reverse primer TWnucTestis_kin_1R (Twort 2012; 5´TGTCGATCAGCACCTTTCACATCC3'). All reactions were carried out with Roche Taq, Buffer (1.5mM Mg+) and dNTPs. PCR conditions were as follows: 95°C (initial denaturation), followed by 35 cycles of: 95°C for 15sec (denaturation); 59°C for Sflag or 67°C for Testis_kin_1 for 30sec (annealing); 72°C for 30sec (primer extension). This was followed by a final extension period at 72°C for 5min. PCR products were then electrophoresed through a 1% agarose gel (Amesco agarose powder and 1x TAE Buffer: 40 mM Tris acetate, 1mM EDTA pH 8.0) at 110V for 40min alongside a 1Kb+ DNA Ladder (Life Technologies). They were then then visualised and photographed under U.V. light using a BioRad gel-doc with BioRad QualityOne 4.4.0 software. Photographs of samples were used to check the quality and quantity of the DNA fragment, and were also checked against the ladder to make sure that they belonged to the expected size class. Appropriate PCR products were then sequenced at the Massey Genome Service using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc).

Software Geneious 6.1.7 (Boimatters LTD) was used to visualise and align the sequences. The ends of the sequences were then trimmed to remove ambiguities that arose at the ends for some sequences. Alleles were then scored for each locus by looking at the homozygous sequences for reference. Genotypes of weta that were heterozygous at either of the two nuclear loci were resolved by determining which combination of alleles would result in the observed pattern of nucleotide heterozygosity in their DNA sequences. Given the low number of variable sites, this

method provided unambiguous genotypes at both loci without the need to clone. As these two loci represented coding regions from their respective genes, single nucleotide polymorphisms are not necessarily neutral. Twort (2012) found a small number of non-synonymous substitutions in these gene fragments, but no evidence of positive or constraining selection at either loci.

A minimum spanning network for each of the two loci was created in software PopArt (Allan Wilson Centre), to visualise the data for the parent populations. Fixed differences between parent populations were compared with putative hybrid genotypes to determine if these individuals could represent F_1 or later generation hybrids. F_1 hybrids should be heterozygous at every locus that differs between species (excluding sex-linked loci), while later generation hybrids should be heterozygous at some loci but not others.

3.2.6 Microsatellite loci

Sixteen microsatellite primer pairs that have been successful in genotyping the South Island species of tree weta (*H. femorata, H. ricta* & *H. maori*) were trialled (King et al. 1997; Hale et al. 2010). Eight markers consistently amplified fragments in all three North Island species. One locus, Hma02, amplified a fragment that was the same size across all specimens from all three species, so was discarded as monomorphic. Mendelian inheritance was tested by genotyping parents and offspring from a captive cross. Locus Hma01 reliably amplified one or two fragments (putative alleles) per specimen, and showed species differentiation, although it was determined that the products of Hma01 were not inherited in a typical mendelian fashion, and so this "loci" was excluded from further analysis. The non-typical inheritance for Hma01 could not be explained in terms of sex-linkage, PCR artefacts or scoring errors. In total, six microsatellite loci were suitable for genetic analysis, and used to genotype all specimens in this study (Table 3.2).

Amplification of DNA (PCR reactions) were carried out with the following conditions: 95°C for 1:30min (initial denaturation), 40 cycles of: 94°C for 15sec (denaturation); 54°C or 55°C for 15sec (annealing – see Table 3.2); 72°C for 30sec (extension). This was followed by a final extension at 72°C for 10min. All reactions were carried out with Roche Taq, Buffer (1.5mM Mg+) and DNTPs. Mg^+ concentration was increased from 1.5mM to 2mM for all reactions. One primer of each pair was pre-labelled with 6FAM, VIC, NED or PET fluorescent tags. PCR products were pooled for genotyping, which was done by the Massey Genome Service using an ABI3730 Genetic Analyzer (Applied Biosystems Inc).

Fixed allelic differences between parent populations were identified, and putative F_1 hybrids were expected to be heterozygote at these loci.

Table 3.2: Microsatellite loci used in this study for the three North Island *Hemideina* species, along primer information and source. * Fluorescently labelled primer.

Locus	Forward Primer	Reverse Primer	Primer sequences	Annealing temperature	Source
HR12	HR12F*	HR12R	5'CCAATTCGCTACCTTCTTTCC3' 5'CCCTAGGGAATAAGCGATGA3'	55	Hale et al. 2010
HR13A	HR13AF	HR13AR*	5'CTTAGCGTAGGGCGATTTTT3' 5'TTTCCGTGAACTGCTAGGTG3'	55	Hale et al. 2010
HR14	HR14F*	HR14R	5'TTTTGACTCTGTTCAGAATGACC3' 5'TACAGAGCCTGGGGAAGAAA3'	55	Hale et al. 2010
HR35	HR35F	HR35R*	5'CAACTGGGGATCAATTCCTG3' 5'GGAGGGAAATGGAAGAGTCC3'	55	Hale et al. 2010
HR43	HR43F*	HR43R	5'GGGTGGTGAGGGATACAGGT3' 5'ACATTGAGGCAATCGACAGG3'	55	Hale et al. 2010
Hma04	Hma04F*	Hma04R	5'CACGAAACTAGACAGAGTTACA3' 5'CCAACCTTCAGGTTATACAC3'	54	King et al. 1997

3.2.7 Validity of Nuclear Loci

The DNA sequences of the two nuclear genes were coded as alleles so that they could be included in this part of the analysis with the microsatellite data. Microchecker software V2.2.3 (The University of Hull) was used to check for scoring errors, the presence of null alleles, and large allele dropout. Any allele that presented problems in one but not the other two species was excluded for comparison for the problematic species only. As errors detected by microchecker may be due to the small size of some samples, this was taken into consideration before discarding data.
Genepop 3.4 (Raymond & Rousset, 1995) online software was used to look for linkage disequilibrium among loci. Linkage disequilibrium would imply that two or more of the nuclear loci were physically connected on a chromosome, and so make one of the loci redundant. All pairs of loci were tested, with 10,000 dememorisations and iterations, and with 1000 batches used.

3.2.8 Population structure

The fixation index F_{ST} is used to see if there is evidence of reproductive isolation between populations. In this case, it is assumed that in populations from different species, there will be low or no interbreeding, while populations within species from different locations may show some differentiation due to isolation by distance. Pairwise F_{ST} values were generated in Arlequin V3.5.1.3 (Excoffier & Lischer, 2010). All eight loci were used. Populations were compared to see if the predicted population differentiation matched relative F_{ST} values. Populations were expected to be more similar (have lower F_{ST} values) to other populations within their own species, and less similar

(have higher F_{ST} values) to populations from other species. Although an F_{ST} of 1 indicates total differentiation and a lack of interbreeding, this value would not be reached if any loci retained ancestral alleles.

To recreate population structure using only the genotypes of individual weta and a specified number of populations (K Value) as a guide, the Software Structure V2.3.4 (Falush et al. 2007) was used. This tested whether sympatric species pairs could be classed as different populations according to their genotype, and determined where hybrid genotypes fit within the genetic structure of the sympatric species pairs. Before this was done, all seven parent populations (without hybrid individuals) were run through structure to see where the strongest demarcations were. It was expected that the three species would be more strongly differentiated from one another (e.g. give tighter clusters when run though Structure) than would individual populations. If this was not the case, it would have suggested that the three – or possibly two – of the species were perhaps not separate species, but populations of the same species. The two nuclear sequences were converted to microsatellite genotypes (arbitrary numbers) for analysis. The Manawatu dataset excluded locus HR13A, as it was shown to have a strong possibility of null alleles in the Manawatu *H. crassidens.* Each of these three datasets was run through Structure separately, each with 100,000 burnin runs discarded, and a further 100,000 MCMC reps used after the burnin runs. The admixture model was used. For the seven parent populations, K was set from 2-6, with higher values unnecessary, as the quality of clusters decreased from K=4 onward. For the sympatric species pairs and their respective hybrids, K was set at 2-3. For each K value for each dataset, 10 iterations were run. This was because different runs of the same data can sometimes give slightly different results, due to inherent stochastic simulations in the software's algorithms. This potential problem was removed by amalgamating all 10 iterations using Clumpp V1.1.2 (Jakobsson & Rosenberg, 2007) and *distruct* V1.1 (Rosenberg, 2004) software programmes. Output data from Structure was converted into a suitable file formats via Structure Harvester online software (Earl et al. 2005). This data was then run through Clumpp which merges the individual data for each weta and for each population over the 10 iterations. The FullSearch method was used for all K=2 and K=3 iterations, but due to the large number of comparisons needed for higher K values along with time constraints, the Greedy Search method was used instead, with random input orders and 100,000 repeats. The search was weighed by number of individuals in the population, as some populations in the dataset had different sample numbers. The output files from Clumpp were then run through *distruct*, which used the two data files to create a graph depicting the results.

The averaged K values within each of the datasets was compared using the Evanno method (Evanno et al. 2005) in Structure Harvester online software, to find out which K values had the most support and reflected the most likely natural groupings. K values of 1 for each dataset, and K values of 4 for the sympatric pairs, were created for the comparison (also with 10 iterations). As the dataset with all seven populations represented multiple levels of population structure - including both multiple species and populations within species – this was expected to show in the results. The important issue (for this study) was whether the three species could be reliably separated into groups or not, as this shows both whether they are indeed separate species, and if there is moderate or extensive introgression between sympatric populations of different species.

3.2.9 Estimating introgression

To estimate gene flow between species pairs two tools were used; a model-based method for identifying hybrids using multilocus genetic data and a bayesian inference of recent migration using multilocus genotypes. Data sets included all eight nuclear loci but not mtDNA or karyotypes. As in the above section, microsatellite locus HR13A was excluded from the *H. thoracica/H. crassidens* dataset due to evidence of null alleles in *H. crassidens.*

NewHybrid 1.1 (Anderson & Thompson, 2002) was used to identify parent classes and different classes of hybrids. The classes given were pure species 1, pure species 2, F_1 hybrid, B_1 (first generation backcross) with species 1, B_1 with species 2. Later generation backcrosses (B_2 with species 1, B_2 with species 2) were used for *H. thoracica*/*H. crassidens,* but did not appear to affect the results for *H. thoracica*/*H. trewicki* so were removed for this pair. Allopatric populations of *H. thoracica* and *H. crassidens* were labelled as such, but all Manawatu and Hawke's Bay individuals including F_1 hybrids were left unidentified for their respective runs. For the Hawke's Bay weta no allopatric populations were added as original results were straightforward and did not require extra information. Both allopatric populations of each species were included for the Manawatu sample. Multiple runs of the software with different random starting seeds were very similar, always converging on the same pattern, so multiple runs were not averaged. 100,000 MCMC iterations were used with 10,000 discarded burnin.

BayesAss 3.0 (Wilson & Rannala, 2003) was used to estimate migration rates between the sympatric species only, and excluded F_1 hybrids as these may be infertile (detecting introgression rather than putatively infertile F_1 was the aim of this analysis). As all population samples were well separated geographically, and as weta are slow-moving flightless insects, it would be very unlikely that they are able to migrate between the populations in this study, unless moved by human intervention, so analysing allopatric populations was unnecessary. Measurements of migration between sympatric populations were technically looking at rate of gene flow, rather than physical migration, as the species already share the same geographic space and often share the same daytime refuges. 5,000,000 MCMC iterations were used and 1,000,000 burnin discarded. Multiple runs with different random starting seeds gave similar results, so as with the previous software, they were not averaged.

3.3 Results

3.3.1 Karyotype

Karyotype data was obtained or available for 45 weta (Table 3.3). All weta identified morphologically as one of the three parent species had the expected karyotype for that species. *Hemideina thoracica* from both Manawatu and Hawkes Bay samples showed 17/18 chromosomes, with four pairs of very small chromosomes characteristic of their species (Figure 3.3, B). *H. crassidens* had 15/16 chromosomes and only one pair of small chromosomes, and one intermediate sized pair (Figure 3.3, A). *Hemideina trewicki* had one small pair and four intermediate sized pairs of chromosomes (Figure 3.3, C). No evidence was found of unusual karyotypes that would have suggested F_2 or later generation hybrids and no spreads gave evidence of cryptic F_1 hybrids.

Two putative hybrid weta provided high quality chromosome spreads for karyotype analysis. Cell division from two putative hybrids (Figure 3.3, D & E; Table 3.3) matched the expected karyotype for an F_1 hybrid between their respective parent species. The putative hybrid from Hawke's Bay had a karyotype that was morphologically intermediate between *H. thoracica* and *H. trewicki* (Figure 3.3, E). The putative F_1 hybrid from the Manawatu had a karyotype morphologically intermediate between its *H. thoracica* and *H. crassidens* parent species (Figure 3.3, D).

Table 3.3: Sample size and karyotype for each sympatric population and putative hybrids from the two species pairs of North Island tree weta (genus *Hemideina*).

	Sample size	Species + chromosome number
Manawatu *H. crassidens*	**11**	All typical *H. crassidens* (15)
Putative hybrids	**1**	F_1 *H. thoracica* x *H. crassidens* (16)
Manawatu *H. thoracica*	**10**	All typical *H. thoracica* (17)
Hawke's Bay *H. thoracica*	**12**	All typical *H. thoracica* (17)
Putative hybrids	**1**	F_1 *H. thoracica* x *H. trewicki* (17)
Hawke's Bay *H. trewicki*	**10**	All typical *H. trewicki* (17)

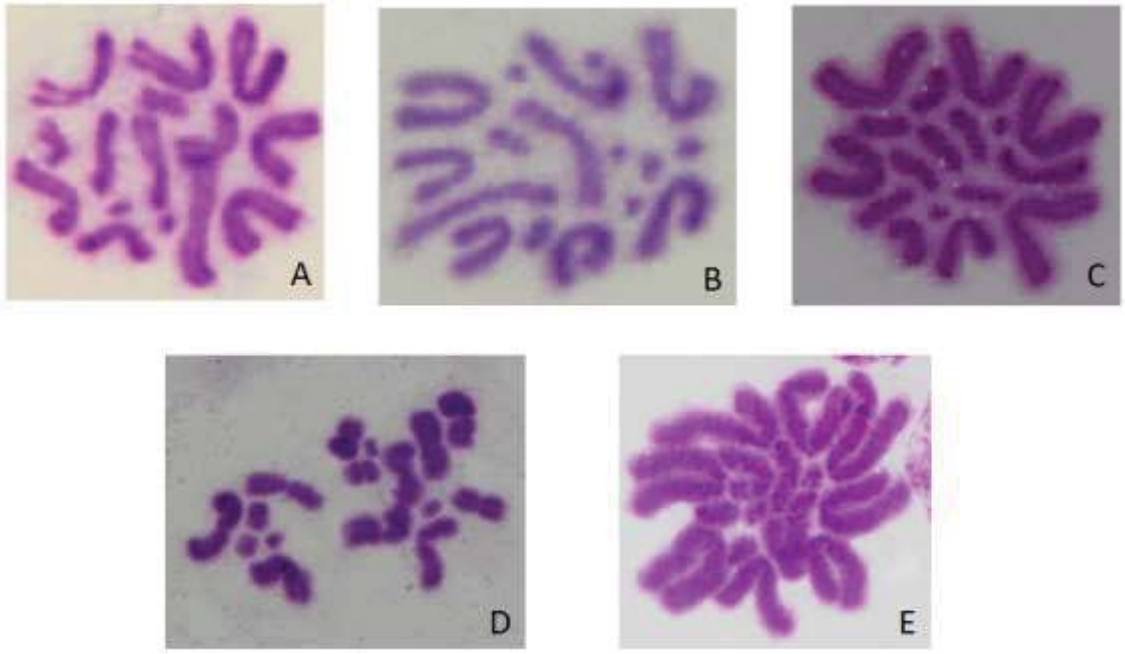


Figure 3.3: A = Karyotype for an *H. crassidens* specimen, B = karyotype for an *H. thoracica* specimen, C = karyotype for an *H. trewicki* specimen. D = F_1 hybrid from *H. thoracica* and *H. crassidens.* E = F_1 hybrid from *H. thoracica* and *H. trewicki.* All mitotic spreads belong to male weta.

3.3.2 Mitochondrial sequences

In total, CO1 mitochondrial sequences were obtained from 43 weta, which included the nine putative Manawatu hybrids. The trimmed sequences were 645bp long (Appendix J), and twelve unique haplotypes were identified, which fall into four clusters that correspond with the three species with *H. crassidens* having two distinct mitochondrial lineages as previously identified (Bulgarella et al. 2014) (Figure 3.4). *Hemideina thoracica* has the least mtDNA sequence diversity with each population being monomorphic for a single haplotype. As there is no overlap between the three species, there is no evidence in this dataset for introgression of the mitochondrial genome. For the Manawatu putative hybrids (Table 3.4) 8/9 had a mitochondrial genome that fell within the *H. crassidens* lineage, from which it can be inferred that they had a *H. crassidens* mother and a *H. thoracica* father. There was only one putative hybrid individual who had the opposite parentage. This differs from expectations of equal likelihood of the two parent taxa being the mother (chi square test; p-value = 0.0196).

Figure 3.4: The three North Island tree weta species (genus *Hemideina*) are polymorphic for a 645bp sequence from the mtDNA CO1 gene. Integer Neighbour-Joining network showing genetic distances between haplotypes from the four sympatric populations of North Island tree weta (genus *Hemideina*), with the four clades separating into the three species (with *H. crassidens* having two clades), and the Manawatu hybrids (putative *H. thoracica* x *H. crassidens*) nearly all belonging to one *H. crassidens* clade. Colours represent sampling locations/species of weta, size of circles scaled by sample size.

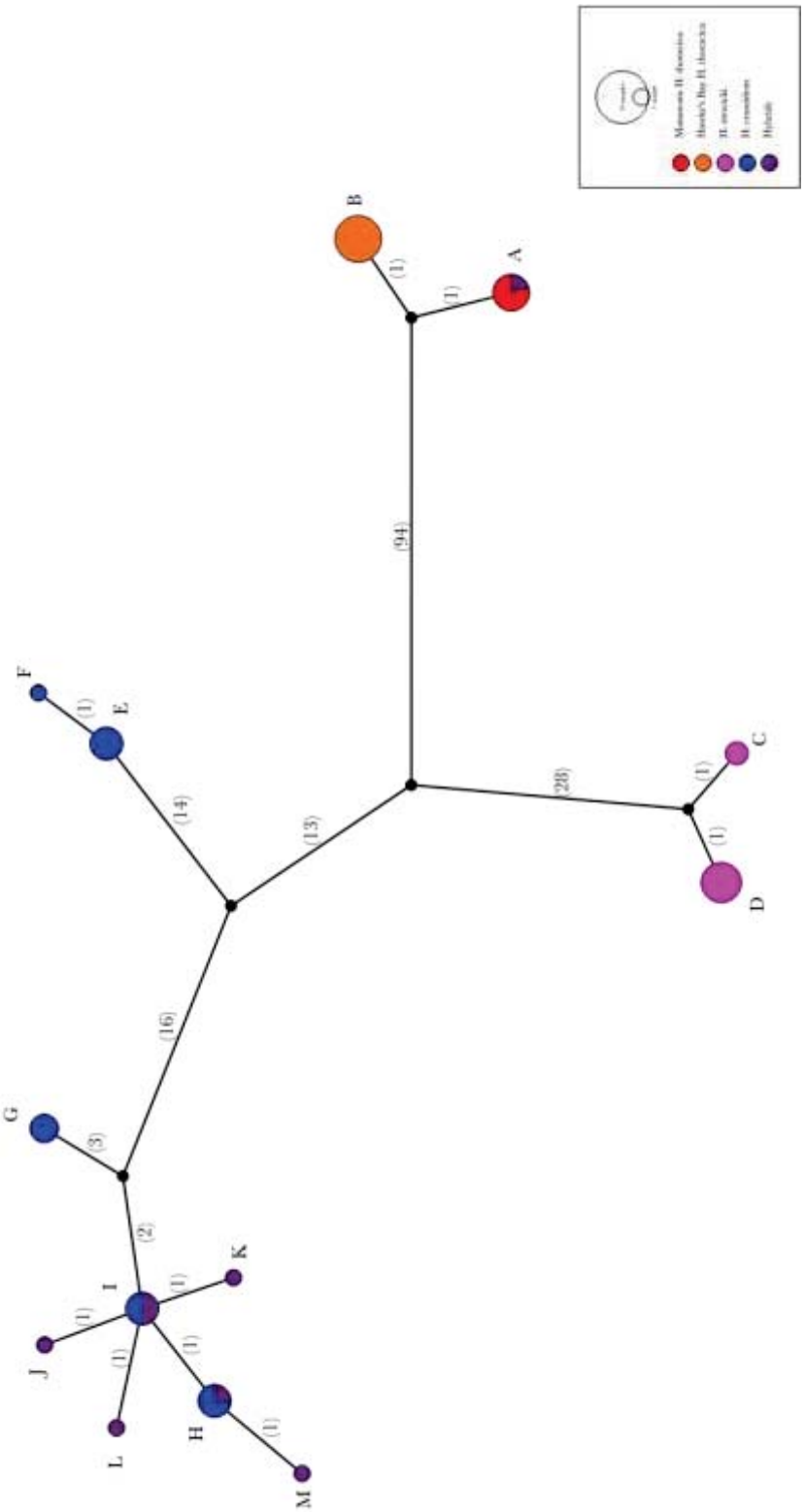


Table 3.4: MtDNA haplotypes observed in each population and putative hybrids from three tree weta species (*Hemideina* spp.) from North Island New Zealand, including sample size. Colours represent species of origin.

	Sample size	mtDNA haplotype
Manawatu *H. crassidens*	**13**	E, F, G, H, I
Putative hybrids	**9**	A, H, I, J, K, L, M
Manawatu *H. thoracica*	**5**	A
Hawke's Bay *H. thoracica*	**8**	B
Hawke's Bay *H. trewicki*	**8**	C, D

3.3.3 Nuclear Loci

103 weta produced unambiguous sequences for each locus. The trimmed sperm flagella protein sequences were 250bp long and eight alleles were identified (Figure 3.5; Appendix K). The testis kinase 1 sequences were 269bp, and eleven alleles were identified at this locus (Figure 3.6; Appendix K). For sperm flagella protein, *H. thoracica* and *H. crassidens* shared the common allele D (Figure 2.5). This allele was found in both allopatric *H. thoracica* populations and one allopatric *H. crassidens* population, as expected of ancestral polymorphism. There was a fixed difference between *H. trewicki* and *H. thoracica*, so sperm flagella protein is a suitable locus to differentiate these two species. In contrast, at testis kinase 1, *H. thoracica* and *H. trewicki* share the common allele A. As all *H. thoracica* populations contained allele A, and *H. trewicki* was nearly monomorphic for it this might be explained by ancestral polymorphism. However, testis kinase 1 alleles differentiate *H. crassidens* from the other two weta species (Figure 3.6), making it useful for studying hybridization between *H. thoracica* and *H. crassidens* where they live in sympatry. Fixed differences at these two loci suggest low or no gene flow. Putative hybrid weta were all heterozygous at the locus that differentiated their respective parent species (Table 3.7), supporting the inference that they are hybrids.

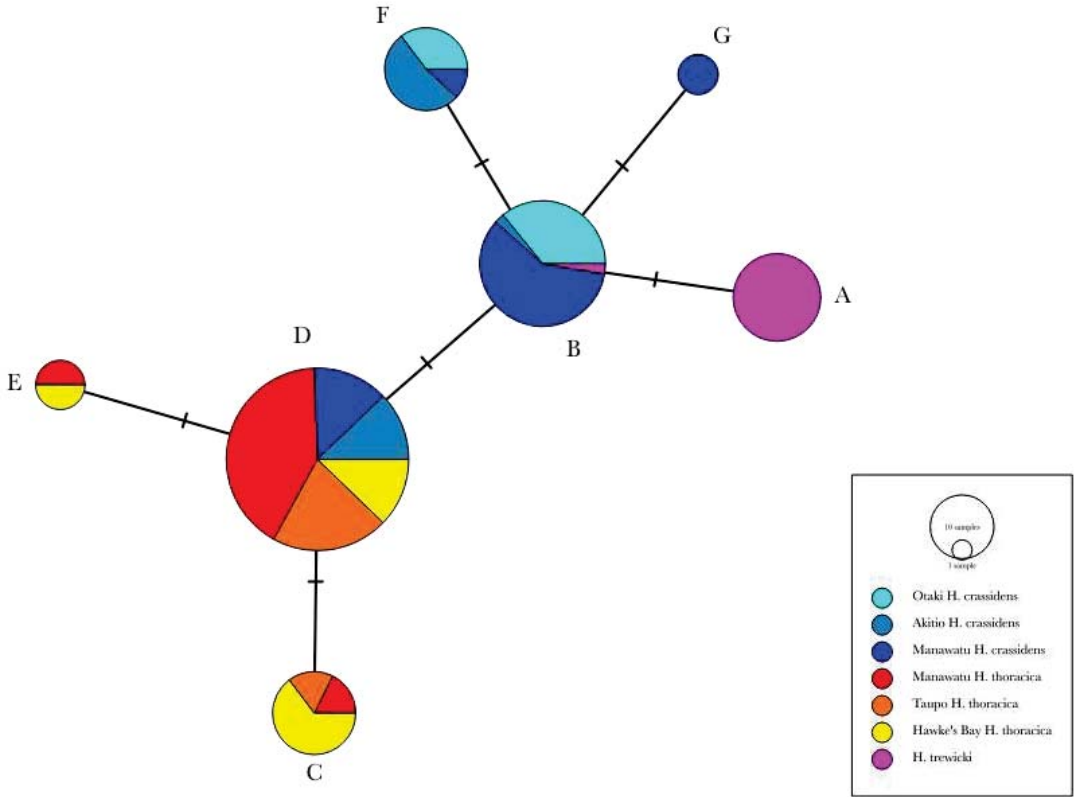


Figure 3.5: The three North Island tree weta (*Hemideina* spp.) are polymorphic for the nuclear gene Sperm flagella protein as revealed by sequencing 250 bp. Integer neighbour joining network showing relationships of 7 alleles identified at the Sperm flagella protein locus. Allele H is not shown here due to unresolved ambiguities. Colours represent sampling locations/species of weta, size of circles scaled by sample size.

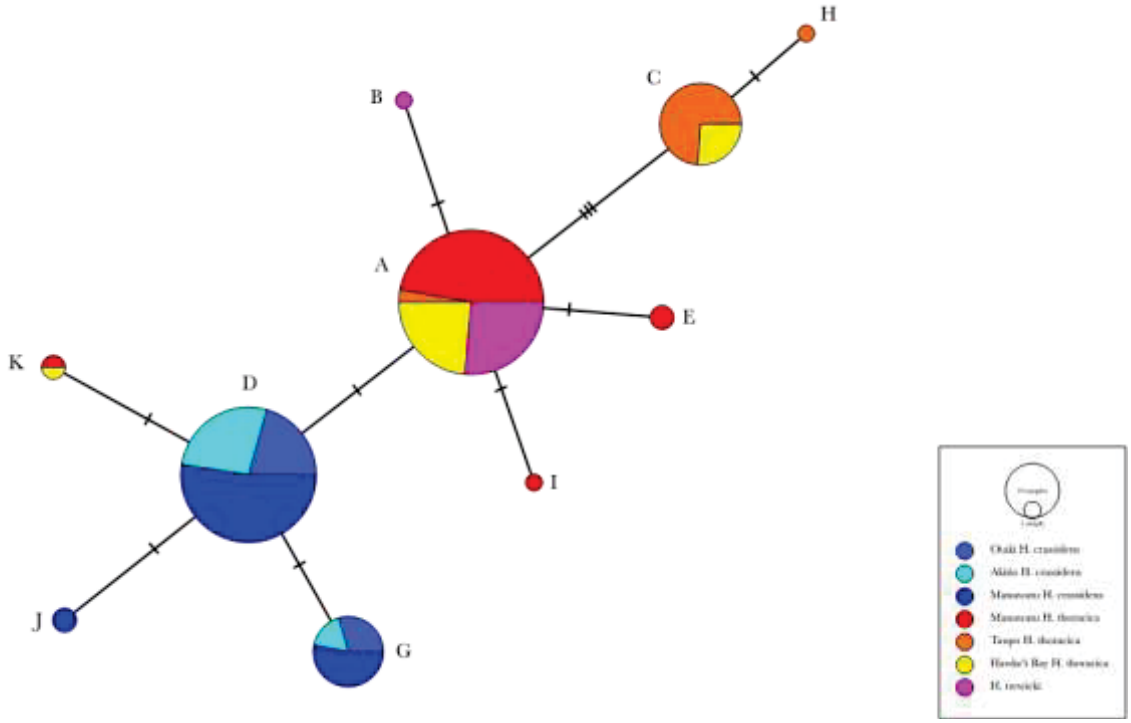


Figure 3.6: The three North Island tree weta (*Hemideina* spp.) are polymorphic for the nuclear gene Testis kinase 1 as revealed by DNA sequencing 269 bp. Integer neighbour joining network showing relationships of 10 Testis kinase 1 alleles identified. Allele F is not shown here due to unresolved ambiguities. Colours represent sampling locations/species of weta, size of circles scaled by sample size.

3.3.4 Microsatellites

Validity of Nuclear loci

There was no evidence of scoring error due to stuttering, or of large allele dropout shown by the results of micro-checker analysis. There was, however, evidence of null alleles in four population samples. Evidence of null alleles at HR12 in the Akitio population and HR35 in the Otaki population are most likely due to small sample size (n=10 for both populations), as there was no evidence of null alleles in other populations within the same species. Null alleles at HR13A were found in the Manawatu *H. crassidens* population. As 12 weta from the Manawatu and Otaki *H. crassidens* populations would not amplify any microsatellite fragment (despite attempts to repeat the PCR reaction, and all weta from other populations giving a result) the chances of a null allele are high. As this seemed to be only an *H. crassidens* issue, this locus was not included in further analysis for this species. The Taupo *H. thoracica* population also gave evidence for null alleles when analysed by micro-checker. For this locus, every weta in the Taupo population was homozygous for one of two alleles. The reason for this is unclear, as both of the other *H. thoracica* populations did not give evidence of null alleles. Again, small sample size (n=10) may be a factor. As *H. thoracica* has many examples of chromosome rearrangements throughout its distribution, sex-linkage in this population (but not others) cannot be ruled out. No evidence of linkage disequilibrium between any pair of loci was found.

Genotypes

Genotypes for all 107 weta at all six microsatellite loci were obtained, with the exception of two weta at the HR14 locus and the above mentioned HR13A samples (Appendix L). The six microsatellite loci had between 3 and 24 alleles (Table 3.5). All loci gave evidence of private alleles for at least one species (Table 3.6). Overall, *H. crassidens* showed the highest genetic diversity with 42 alleles of which 24 were private. Diversity in the *H. thoracica* sample (n = 42 from 3 locations) was the same as observed in the 10 *H. trewicki* (from one location).

Table 3.5: Size range of alleles for six microsatellite loci and number of shared and private alleles observed in each tree weta species (*Hemideina* spp.). (Number of private alleles are given in brackets).

Locus	Size range	*H. thoracica*	*H. crassidens*	H. trewicki	Total
HR12	166-196	4(4)	3(2)	2(1)	7
HR13A	162-187	6(5)	8(5)	2	11
HR14	166-183	1	3(1)	2	3
HR35	224-274	4	20(12)	7(1)	25
HR43	103-127	1	4(2)	2	4
Hma04	80-95	1(1)	4(2)	2	5
Total		**17**	**42**	**17**	**55**

Table 3.6: Eight nuclear markers provide evidence of species specific alleles within each tree weta species in North Island New Ze
all populations, without putative hybrids. Coloured alleles/genotypes are private to that species. Putatively introgressed alleles a
origin.

	Sample size	Sperm Flagella Protein	Testis Kinase 1	HR12	HR13A	HR14	HR35
Akito *H. crassidens*	**10**	B, D, F	D, G	179, 184, 187	170, 177	166, 183	224, 231, 233, 240, 242, 244,
Otaki *H. crassidens*	**10**	B, F	D, G	179, 184	166, 187	168, 183	224, 227, 236, 240, 242, 244,
Manawatu *H. crassidens*	**22**	B, D, F, G, H	D, G, J	179, 184, 186, 187	162, 168, 187	168, 183	224, 227, 228, 230, 233, 240, 242, 244, 247, 250,
Manawatu *H. thoracica*	**21**	C, D, E	A, E, I, K	166, 186, 188	172, 174, 177, 179	183	242, 244, 247, 250
Taupo *H. thoracica*	**10**	C, D	A, C, H	186, 188	174, 182	183	244, 247
Hawke's Bay *H. thoracica*	**12**	C, D, E	A, C, K	174, 186, 188	174, 176, 179	183	244
Hawke's Bay *H. trewicki*	**10**	A, B	A, B	184, 196	162, 170	168, 183	233, 235, 244, 247, 253,

3.3.5 Putative F_1 hybrids and backcrosses

Heterozygosity at each of the two differentiated microsatellite loci (Table 3.6) was seen in all nine putative hybrids from the Manawatu region (Table 3.7). Along with sequence data from the testis kinase 1 locus, which also shows species-heterozygosity, there is good evidence that all of these nine putative hybrids are F_1 *H. thoracica x H. crassidens* hybrids, rather than later generation back-crosses. Three microsatellite loci showed fixed differences between *H. thoracica* and *H. trewicki* (Table 3.6). The single putative hybrid was heterozygous at all four loci that showed fixed differences between species (Table 3.8), agreeing with karyotype data, thus there is strong evidence to class this individual as an F_1 *H. thoracica x H. trewicki* hybrid.

Table 3.7: Genetic data for the putative hybrids from the Manawatu region where *H. thoracica* and *H. crassidens* are sympatric. The three loci in the table all showed fixed differences between the two putative parent species. Red alleles belong to *H. thoracica* and blue alleles belong to *H. crassidens.* *Determined in previous study.

Putative hybrid	Sex	mtDNA	Testis_kin_1	HR12	Hma04
1	F	I	A/D	184/186	80/88
2	M	A	A/D	184/186	80/88
3	M	L	A/D	184/186	80/88
4	F	H	A/D	184/186	80/88
5	M	M	A/D	184/186	80/88
6	M	K	A/D	184/189	80/88
7	M	H. crassidens*	A/D	184/186	80/88
8	F	J	A/D	184/186	80/88
9	M	I	A/D	184/186	80/88

Table 3.8: Genetic data for the putative hybrid from the Hawke's Bay region where *H. thoracica* and *H. trewicki* are sympatric. The four loci in the table showed fixed difference between the putative parent species. Red alleles belong to *H. thoracica* and purple alleles belong to *H. trewicki.*

Putative hybrid	Sex	Sflag	HR12	HR13A	Hma04
1	M	A/D	186/196	170/179	80/88

Hemideina thoracica and *H. crassidens*

There were three nuclear loci that showed fixed differences between the H. *thoracica* and *H. crassidens* samples (Figure 3.6; Table 3.6). However, HR12 showed possible evidence of introgression, with a common *H. thoracica* allele found in a single *H. crassidens* individual. As this allele was not seen in either of the allopatric *H. crassidens* populations, ancestral polymorphism is not a good explanation for the presence of this allele in *H. crassidens.* HR35 also gave possible evidence of introgression (Table 3.6; Figure 3.7). Although this locus did not show fixed differences between any of the species, it did show that in the Manawatu, *H. thoracica* has two alleles that are also found in *H. crassidens*. These alleles were not found in the allopatric populations of *H. thoracica,* which have only two alleles at this locus compared to the 20 found in *H. crassidens*. All *H. crassidens* populations contained the two common *H. thoracica* alleles, so they are easily explained as ancestral polymorphism. This also means that it is not possible to know whether introgression may have been going in the opposite direction from *H. thoracica* to *H. crassidens,* as there are no suitable marker alleles to obtain this information. Three loci showed fixed differences between *H. thoracica* and *H. trewicki* (Table 3.6), with no evidence of introgression at any loci.

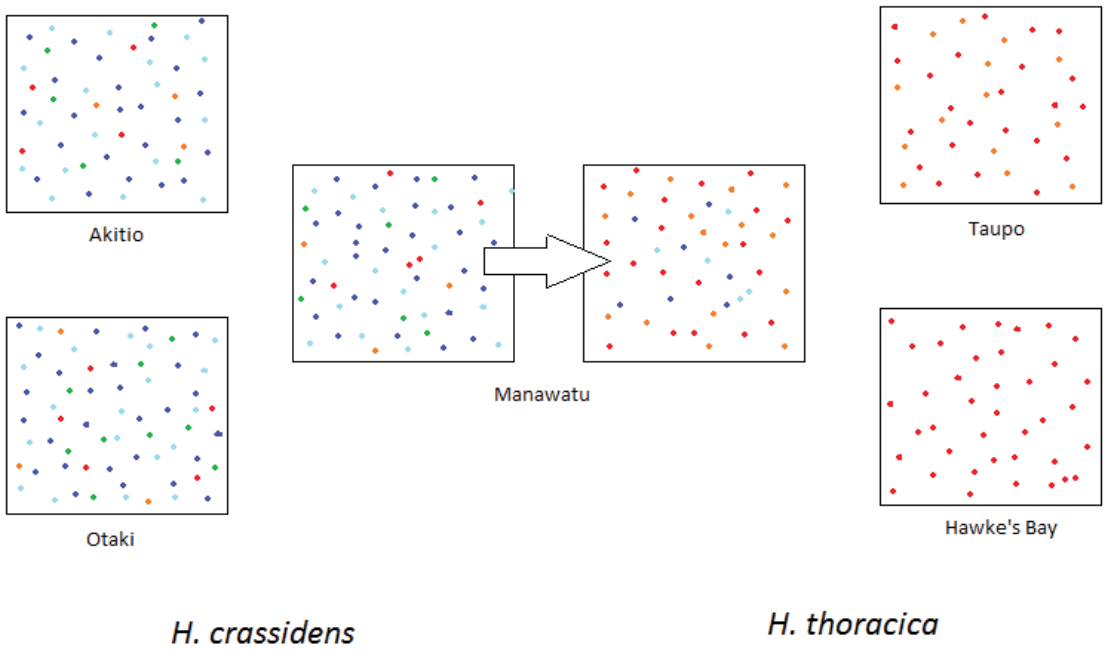


Figure 3.7: Diagram showing an approximation of the results for microsatellite locus HR35, with possible introgression from *H. crassidens* to *H. thoracica* in the Manawatu. *Hemideina crassidens* has more alleles than shown here (n=20), so blue alleles are not as frequent as depicted in the figure.

3.3.6 Population Structure

Population pairwise F_{ST} estimates (Table 3.9) were all greater than zero (p-values < 0.001). As was expected, population pairs within a species had lower F_{ST} values than population pairs between species. Although F_{ST} estimates between species were all less than 1, populations in sympatry have substantial differentiation and little allelic exchange; F_{ST} = 0.606 (Hawkes Bay) and 0.665 (Manawatu).

Table 3.9: Pairwise F_{ST} values for all parent populations examined in the three North Island tree weta (genus *Hemideina*), showing higher differentiation between species than populations within species.

	Akitio ***H. crassidens***	**Otaki** ***H. crassidens***	**Manawatu** ***H. crassidens***	**Manawatu** ***H. thoracica***	**Taupo** ***H. thoracica***	**Hawke's Bay** ***H. thoracica***	**Hawke's Bay** ***H. trewicki***
Akitio *H. crassidens*	0						
Otaki *H. crassidens*	0.2871	0					
Manawatu *H. crassidens*	0.36865	0.20849	0				
Manawatu *H. thoracica*	0.7078	0.6178	0.66525	0			
Taupo *H. thoracica*	0.68443	0.5874	0.64588	0.35945	0		
Hawke's Bay *H. thoracica*	0.66953	0.5651	0.63366	0.17132	0.30068	0	
Hawke's Bay *H. trewicki*	0.59893	0.46131	0.54326	0.65409	0.70091	0.60617	0

Population structure for K values 2 and 3 matched species identification based on morphology (Figure 3.8). At K = 2 *H. crassidens* and *H. trewicki* were combined, which is as expected given the closer similarity of these two species compared with *H. thoracica* (Morgan-Richards, 1995). At K = 3 the three species were separated. At K = 4 and K = 5 the three *H. crassidens* populations were broken up, although these population demarcations were not as clean as those separating species. At K = 6, no further population structure could be detected. Structure failed to differentiate the three *H. thoracica* populations, due to the low diversity in this part of its range. When *H. thoracica* populations were run separately (not shown here), only limited population structure could be seen, with the Taupo population somewhat differentiated from the other two at K = 2 or K = 3.

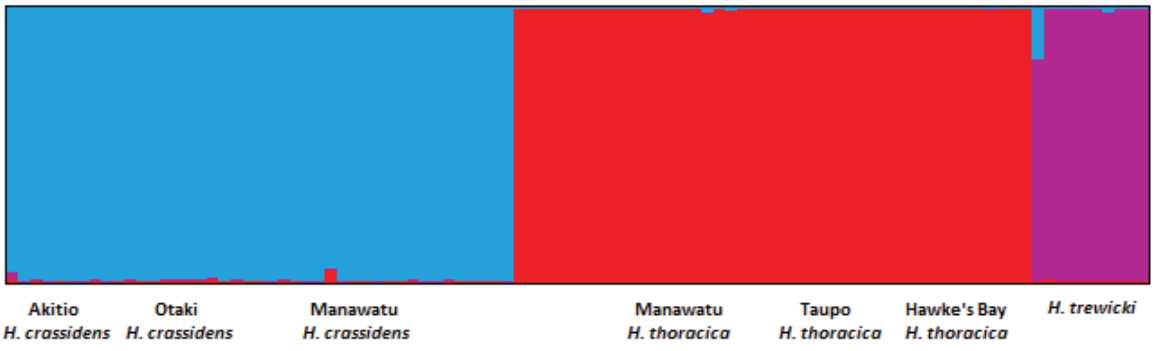
The highest support was found for K = 2, which probably supports the deepest divergence, between the putative ancestor of *H. crassidens/H. trewicki* and the ancestor of *H. thoracica.* The next highest support was for K = 3, which separates the three species, followed by K = 5, which separates out the three *H. crassidens* populations (Table 3.10).

In all cases the sympatric populations fell out into the two species as identified by morphology with low levels of possible introgression visualised (Figure 3.8).

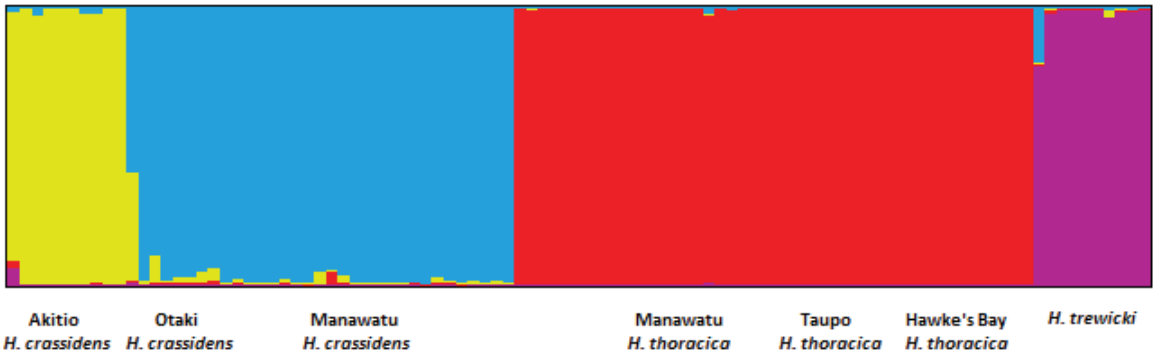
K=2


Akitio
H. crassidens
Otaki
H. crassidens
Manawatu
H. crassidens
Manawatu
H. thoracica
Taupo
H. thoracica
Hawke's Bay
H. thoracica
H. trewicki

K=3


Akitio
H. crassidens
Otaki
H. crassidens
Manawatu
H. crassidens
Manawatu
H. thoracica
Taupo
H. thoracica
Hawke's Bay
H. thoracica
H. trewicki

K=4


Akitio
H. crassidens
Otaki
H. crassidens
Manawatu
H. crassidens
Manawatu
H. thoracica
Taupo
H. thoracica
Hawke's Bay
H. thoracica
H. trewicki

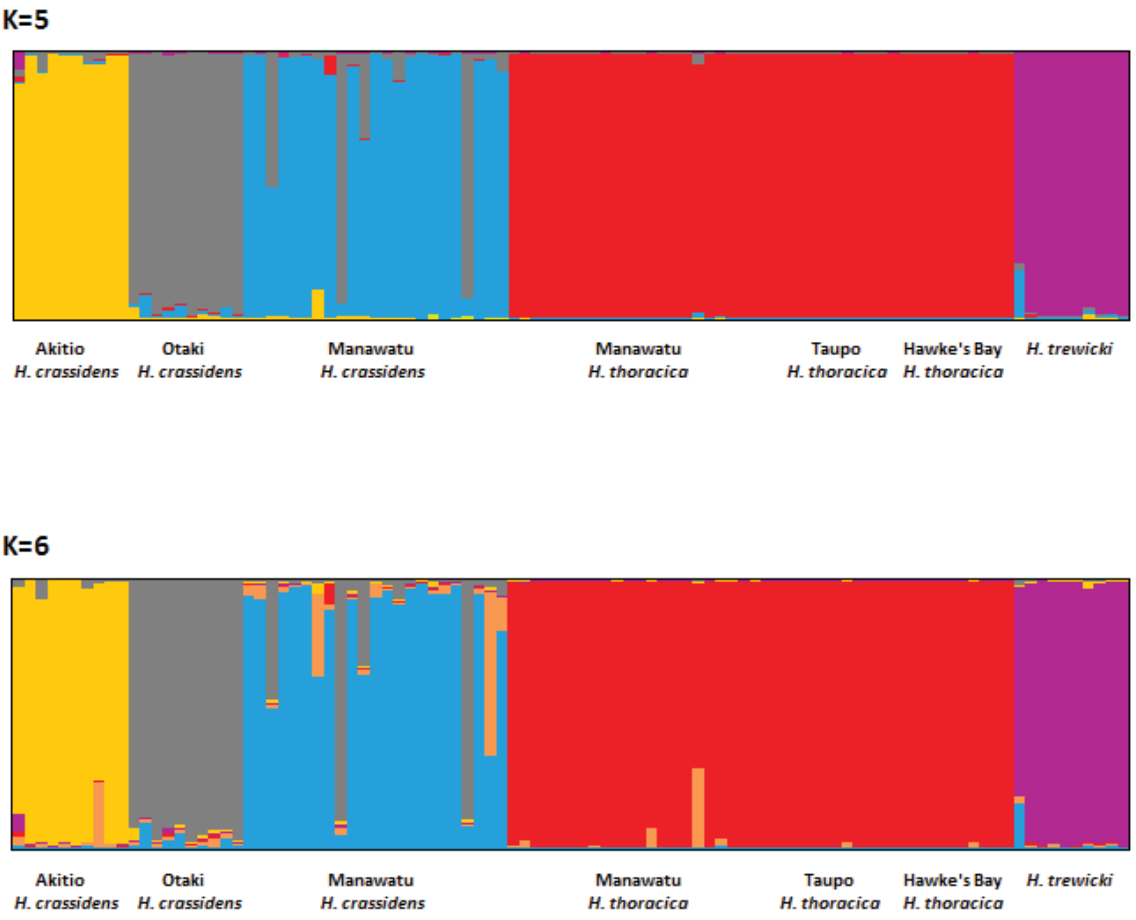


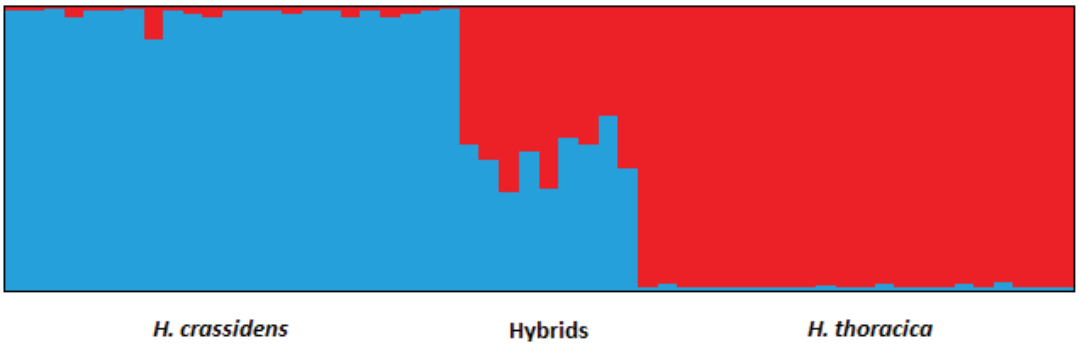
Figure 3.8: Population structure for the seven parent populations of North Island tree weta (genus *Hemideina*) examined in this study without F_1 hybrids. Graphs show average results of 10 structure iterations for K=2 to K=6, showing that populations structure could be inferred for all K values up to K = 5.

Table 3.10: Results from the Evanno method comparing K values 2-6, showing that K = 2 has the most support, followed by K = 3, and then K = 5.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-2118.2	0.46679	—	—	—
2	10	-1464.7	0.55468	653.54	455.56	821.307
3	10	-1266.7	0.51088	197.98	77.48	151.659
4	10	-1146.2	0.71562	120.5	59.68	83.3963
5	10	-1085.4	0.749	60.82	70.87	94.6196
6	10	-1095.4	1.8283	-10.05	8.82	4.82416
7	10	-1096.7	37.1523	-1.23	—	—

K=2 gave the best population demarcations for the sympatric species pairs with their respective hybrids, showing that in both cases, the F_1 hybrid individuals showed mixed ancestry (Figure 3.9 & 3.10). Increasing the population number to K=3 did not help to clarify the situation. The *H. crassidens* individual that showed possible evidence of introgression at the HR12 locus can be seen with the largest red proportion in the *H. crassidens* population (Structure results suggested that this individual belonged 92% to the *H. crassidens,* and 8% to the *H. thoracica* population), although Structure does not give estimates of gene flow. The results of the comparison of K values also gave the highest support for K=2 in both sympatric pairs (Table 3.11; Table 3.12).

K=2



K=3

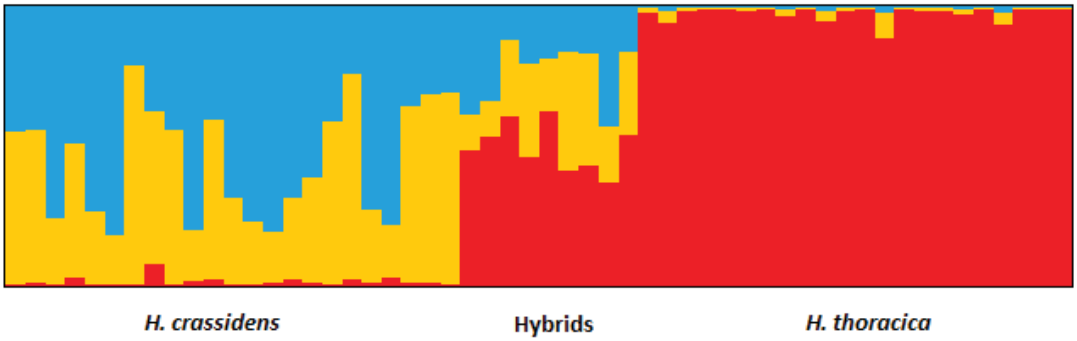


Figure 3.9: Genetic structure of *Hemideina thoracica* and *H. crassidens* and F_1 hybrids from the sympatric Manawatu population. Average results of 10 Structure iterations for K=2 and K=3, showing that K= 2 gives a much cleaner distinction between the two species.

Table 3.11: Results for the Evanno method of comparison for the *H. thoracica* and *H. crassidens* sympatric populations in the Manawatu, with 10 iterations for each K value, showing that K = 2 has the most support.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-318.43	0.573585	—	—	—
2	10	-190.66	0.189737	127.77	131.18	691.3793
3	10	-194.07	1.000056	-3.41	4.99	4.989723
4	10	-202.47	3.590744	-8.4	—	—

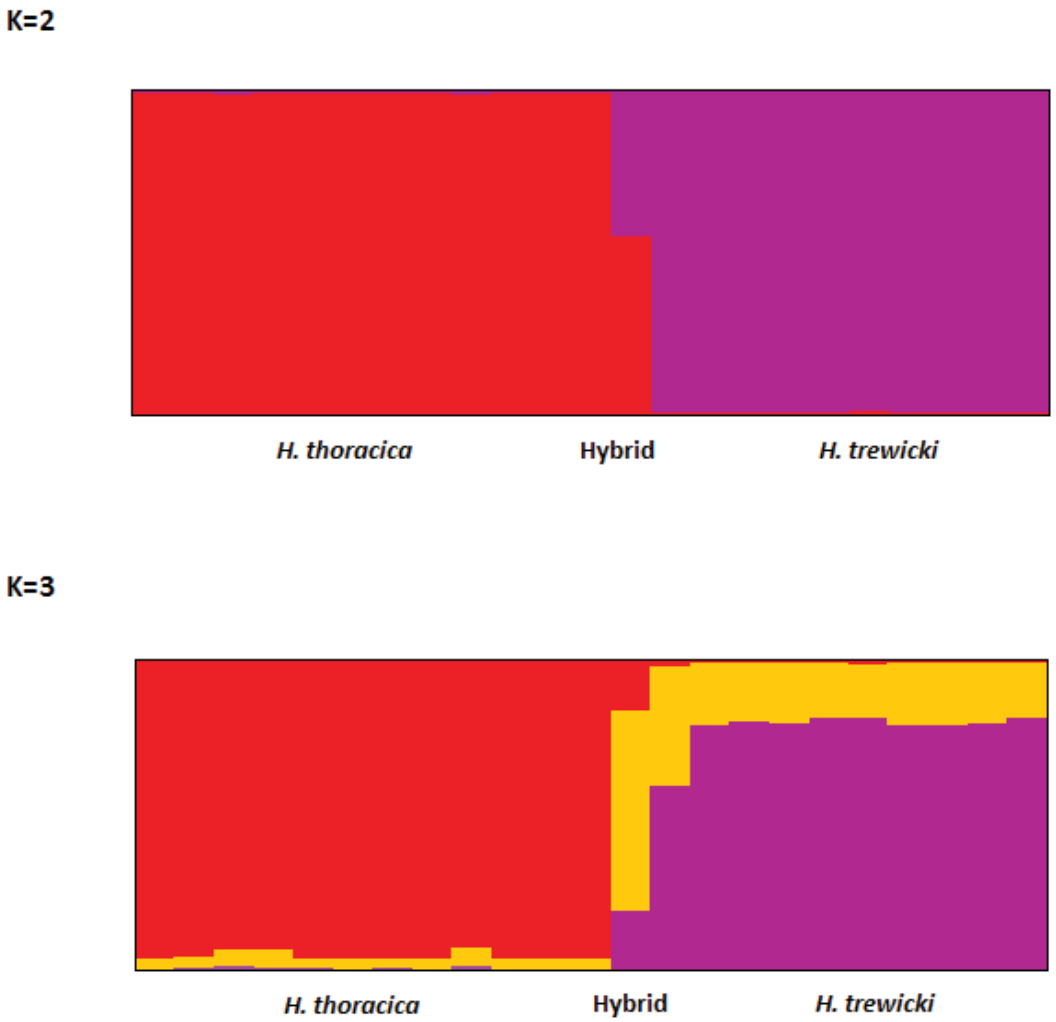


Figure 3.10: Genetic structure of *Hemideina thoracica* and *H. trewicki* and F_1 hybrid from the sympatric Hawke's Bay population. Average results of 10 Structure iterations for K=2 and K=3, showing that K= 2 gives a much cleaner distinction between the two species.

Table 3.12: Results for the Evanno method of comparison for the *H. thoracica* and *H. trewicki* sympatric populations in the Hawke's Bay, with 10 iterations for each K value, showing that K = 2 has the most support.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-318.43	0.573585	—	—	—
2	10	-190.66	0.189737	127.77	131.18	691.3793
3	10	-194.07	1.000056	-3.41	4.99	4.989723
4	10	-202.47	3.590744	-8.4	—	—

3.3.7 Estimates of introgression

All putative F_1 hybrids were given strong support for their F_1 classification by NewHybrids, with at least a 90% probability (Figure 3.11 & 3.12), while the one *H. crassidens* individual with a potentially introgressed allele at locus HR12 was given a 29% chance of being either a first or second generation backcross (B_1 or B_2) with *H. crassidens*. All other weta were classed as having a high probability of belonging to the parent species they had been previously identified as.

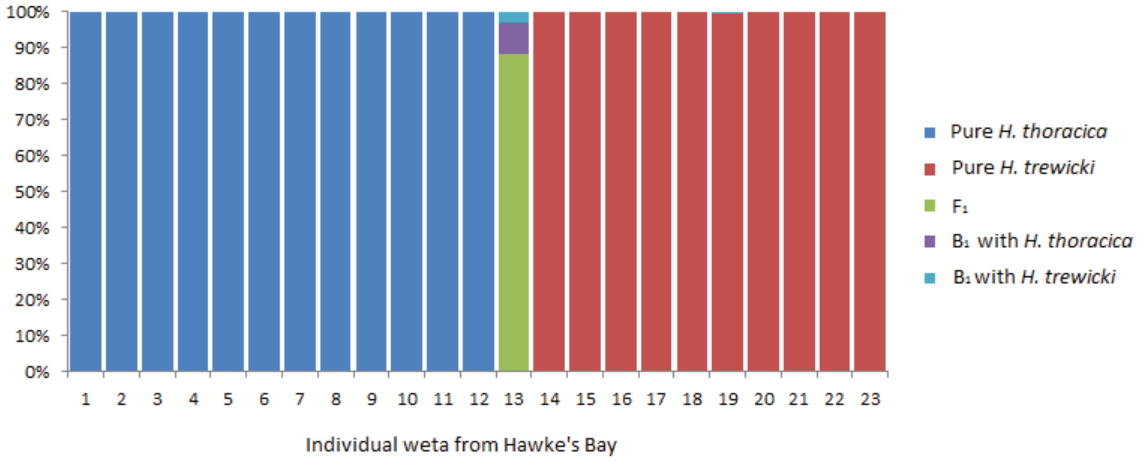


Figure 3.11: Probability that individuals belong to a particular parent or hybrid class for the sympatric *H. thoracica* and *H. trewicki* weta from Hawke's Bay, showing strong support for the original classifications of individuals in this species pair.

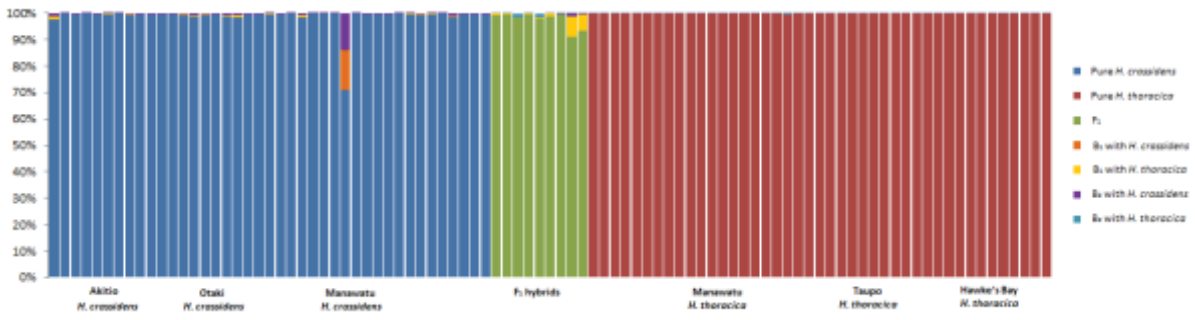


Figure 3.12: Probability that individuals belong to a particular parent or hybrid class for the sympatric *H. thoracica* and *H. crassidens* weta from the Manawatu, showing strong support for the original classifications of individuals in this species pair, with the possible exception of one *H. crassidens* individual.

The inferred gene flow as calculated by BayesAss was very low (Figure 3.13). As the confidence interval for both species pairs includes 0, estimated gene flow by BasyesAss is between low and non-existent in both species pairs.

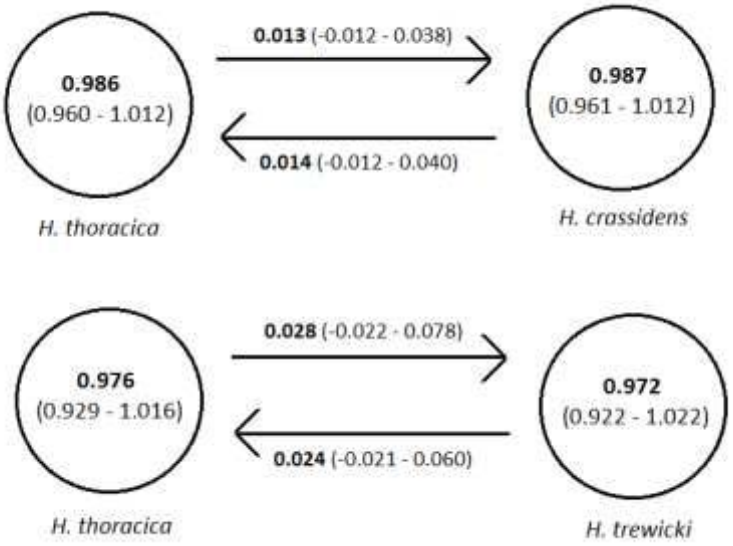


Figure 3.13: BayesAss estimates of gene flow between the sympatric species pairs with 95% confidence intervals, showing that gene-flow is between low and non-existent in both cases.

3.4 Discussion

Eight nuclear loci have been examined, as well as karyotype and a mitochondrial marker, where *Hemideina thoracica*'s range overlaps with *H. crassidens* and *H. trewicki*. Evidence that morphological hybrids between *H. thoracica* and its two neighbouring species are genetically F_1 hybrids was unequivocal; however, estimates of introgression between these species provide mixed inferences.

3.4.1 *Hemideina thoracica* and *H. trewicki*

Six fixed differences were found between sympatric *H. thoracica* and *H. trewicki* population samples from Hawkes Bay (karyotype, mitochondria and four nuclear loci). The putative hybrid identified by morphology was confirmed as a genetic hybrid. The hybrid had a karyotype that was intermediate between its two parent species, which is expected for an F_1 hybrid. The putative hybrid was also heterozygous at all four nuclear loci that showed fixed differences between the species, making it very likely that this weta was in fact an F_1 hybrid. Unfortunately, mitochondrial data was not obtained so it is unknown which species the mother was from. There was no evidence of introgression in the Hawke's Bay population sample suggesting that the F_1 hybrids are infertile, or if they are not, backcrossing with the parent species is very rare; a similar situation to that seen with *H. femorata* and *H. ricta* where they meet on Banks Peninsula (Morgan-Richards, 1995). This means that the boundaries between *H. thoracica* and *H. trewicki* may be impermeable. Competition is therefore expected to result in selection for niche divergence between the species. Chapter 2 showed that these two species are already differentiated in terms of size and it is possible that a combination of timing of maturity and body size has already reduced interspecific competition.

3.4.2 *Hemideina thoracica* and *H. crassidens*

Karyotype and mitochondrial data revealed fixed difference between a small sample of *H. thoracica* and *H. crassidens* (n = 21) where they are sympatric in the Manawatu. Three nuclear loci in a larger sample (n = 45) also showed fixed differences between these species. The putative hybrids identified by morphology were confirmed as genetic hybrids, as all nine putative hybrids were heterozygous at all loci. Only one of the Manawatu hybrids was karyotyped, and this confirmed that this individual was an F_1 hybrid. There was little evidence that any of these individuals were later generation hybrids when analysed with the model-based methods for identifying species hybrids using multilocus genetic data (NewHybrids and BayesAss), and no evidence of cryptic hybrids was found. Therefore, it appears that any weta that looks like a hybrid between these two species is most likely an F_1 hybrid. The apparent lack of backcross individuals as shown by model-based analysis and the five fixed differences suggest very low or no gene flow between these two species in the Manawatu.

As F_1 females are likely infertile (Appendix A), the lack of mitochondrial introgression is to be expected. As mitochondria are only inherited through the maternal line, no bridge exists in this species pair for the transfer of mitochondrial haplotypes. As tree weta also have an XO sex-

determination system, there is no male specific element in the genome to pass on, so sex-related dispersal bias cannot be inferred from introgression in this species pair.

Mitochondrial data was collected for all nine hybrid weta, and revealed that eight had a *H. crassidens* mother, and only one had a *H. thoracica* mother. The sample size here was not large, but is still of interest, as it suggests that hybridization may be more likely to occur between male *H. thoracica* and female *H. crassidens* (although not exclusively). A sex bias in hybridization could be due to post-mating constraints (as mating in captivity occurs in both directions) caused by asymmetric postmating prezygotic barriers (e.g. Larson, et al. 2012) or asymmetric genetic constraints (Welch, 2004 and references therein), or it may mean that *H. thoracica* males are outcompeting *H. crassidens* males in the wild through sexual exclusion (Hochkirch, et al. 2007).

3.4.3 Introgression?

Estimates of gene flow between the species pairs used a bayesian inference of recent migration, using multilocus genotypes. This test was not able to exclude the possibility that there is no gene flow between either species pairs. The difference between no gene flow and low levels of introgression is of importance when inferring potential for selection to either reduce the production of F_1 hybrids or increase the fertility of F_1 hybrids in the future. The theoretical difference between low gene flow and no gene flow is also crucial in understanding the potential role of introgression in adaptation during range expansion. However, visually examining the data and constructing a simple model (as in the results section) provided a slightly different view from relying on the Bayesian estimates. There were two loci that gave evidence of potential introgression when allele distribution was inspected in detail, and these are concordant with inferences from morphology. The patterns observed are concordant with no gene flow between the two species in Hawkes Bay, but limited gene flow in the Manawatu between *H. thoracica* and *H. crassidens*.

At the locus HR12, one weta from the Manawatu had an allele that was identified as having hybrid ancestry (although another study looking at the same loci, to examine interspecific diversity in *H. crassidens,* identified the same common *H. thoracica* allele in another population of *H. crassidens* that borders *H. thoracica* in Rangiwahia – where 20% of alleles were of putatively *H. thoracica* origin in a sample of 29 weta (L. Sivyer, unpublished data, 2014). None of the eight allopatric populations in this study contained this allele, supporting the case for introgression at this locus). The other was in HR35, which did not show a fixed difference, but gave evidence that the *H. thoracica* population may have incorporated two *H. crassidens* alleles in the Manawatu, as no allopatric *H. thoracica* populations contained them. Ten of the *H. thoracica* from the Manawatu contained one of the two alleles, although all but one were heterozygous with another *H. thoracica* allele. Genetic diversity in *H. thoracica* at this locus is twice (or more) that seen in allopatric population samples (Hawke's Bay *H. thoracica* had one allele, and the Taupo population had two), and as the alleles were found at a frequency higher than the *H. crassidens* population that putatively supplied them, this may suggest that *H. thoracica* is retaining introgressed alleles, which agrees with the model developed by Excoffier et al. (2009), which shows that expanding species have a higher chance than local species of retaining introgressed alleles. Other species pairs that hybridize have shown a resultant increase

in genetic diversity (e.g. mosquitoes in the *Anopheles* genus (Besansky, 2003)), and increased genetic diversity has been connected with past adaptive radiations (Lowe, 1936; Seehausen, 2004; etc.).

The lack of mitochondrial introgression is likely explained by female infertility (Appendix A), although fixed karyotype differences suggest that the relatively large karyotype differences may serve to limit introgression. This limitation is seen between chromosome races of *H. thoracica* (Morgan-Richards & Wallis, 2003), although to a lesser degree that probably reflects the closer similarities of many karyotypes within *H. thoracica*.

Genetic evidence suggests that the sympatric populations of *H. thoracica* and *H. crassidens* in the Manawatu might be a hybrid zone with a bimodal distribution; with most of the sample containing parent forms and few weta of hybrid ancestry (frequency in a random sample was about 3% F_1 hybrids, see chapter 2). About 30% of the parent sample showed evidence of being possible backcross individuals when morphological/colouration data was also taken into consideration, however, the fact that most weta only showed possible hybrid ancestry in one character (out of 18 in total), shows that they were likely not early generation backcrosses, but might represent historical introgression. This is in contrast to the single weta identified as having a 30% chance of being a backcross hybrid using a Bayesian model-based approach with the multilocus genetic data (NewHybrids). Fixed differences were found between these two species, indicating little evidence for a tension zone in this location, and the weta are clearly not in the process of merging into a single population. Species with bimodal hybrid zones are generally regarded as being at the tail end of the speciation process; differentiated enough to maintain separate evolutionary trajectories, but able to exchange genes at low frequencies (Jiggins & Mallet, 2000).

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Chapter 4: Discussion

4.1.1 Summary of findings

This study has examined evidence of niche divergence in sympatry, and putative hybridization and introgression between the common tree weta *Hemideina thoracica* and its two neighbouring species, *H. crassidens* and *H. trewicki*. The findings suggest that *H. thoracica* and *H. trewicki* may have diverged where their ranges overlap, with *H. thoracica* adult females having longer hind tibia and maturing later than *H. trewicki*. An F_1 hybrid demonstrates that these species will at least occasionally interbreed in sympatry, however, no evidence for introgression was detected.

In contrast, in the Manawatu *H. thoracica* and *H. crassidens* showed no evidence of divergence in sympatry. This morphological similarity adds weight to studies of growth rate (Minards et al. in press); diet (Dewhurst, 2012); and nutritional studies (P.C. Wehi, personal communication, June 2012) that fail to find significant differences between *H. thoracica* and *H. crassidens* in the Manawatu. All morphological intermediates in this study were genetically identified as F_1 hybrids. The detection of just first generation hybrids could be an indication that they are sterile but preliminary data suggests this might be the case for females only (Appendix A). Five lines of evidence suggest limited introgression is possible in the Manawatu. Two of these factors consisted of overlap in spine numbers and pronotum colour in sympatry but not allopatry (Chapter 2), two of allele sharing at genetic loci in sympatry (Chapter 3), and one F_1 hybrid male produced four offspring in captivity (Appendix A). However, the level of introgression must be low, and a bimodal hybrid zone appears to be the best description for this region of contact. There is also a possibility of sex-biased production of hybrids, with F_1 hybrids being significantly more likely to have an *H. thoracica* father and an *H. crassidens* mother than vice versa.

4.1.2 Reproductive barriers and weta behaviour

These three species of tree weta do not appear to have any pre-mating barriers to reproduction. As bimodal hybrid zones are typically associated with pre-mating rather than post-mating barriers (Jiggins & Mallet, 2000 and references therein), the situation here is somewhat unusual. If there is no assortative mating between these species pairs, it suggests that barriers are more likely to be the result of genetic constraints. It is not known at what stage the production of F_1 hybrids is limited; but as intermediate forms are far less common than would be expected if the species were freely interbreeding (Chapter 2) there must be some reproductive barriers. Barriers are hypothesised to be at the post-mating pre-zygotic stage or else very early in development (Appendix A). It is also possible that the weta are using unknown behavioural mechanisms to avoid interbreeding to some extent in the wild. However, a study looking at a bimodal hybrid zone in two species of chrysomelidae beetles (*Chyrsochus cobaltinus* and *C. auratus*) also found that postzygotic barriers were stronger than prezygotic barriers (Peterson et al. 2005), so the association of assortative mating and bimodal hybrid zones has exceptions. A later study of these same beetles also showed a significant sex-bias in the production of offspring (most had mtDNA haplotypes and hence mothers from one species), despite mating occurring in both directions in the wild, and offspring in both sex-pairings being produced in equal numbers and being equally viable in laboratory crosses at the first

instar (Monsen et al. 2007). The proposed explanation was asymmetric post-mating pre-zygotic barriers, or possibly asymmetric inviability later in development. As *H. thoracica* and *H. crassidens* appear to exhibit both a bimodal hybrid zone in the apparent absence of pre-mating barriers, and also a sex-biased production of F_1 offspring there may be some similarities in the mechanisms causing reproductive isolation in these disparate species pairs. Examples such as these may give insights into how bimodal hybrid zones are typically formed and maintained.

The sex-bias in the production of F_1 hybrids between *H. thoracica* and *H. crassidens* could have a number of explanations. The sample may have been too small (n=9), even though a chi square test gave a significant difference. It may also be that reciprocal crosses are not equally viable for an unknown reason. For example, in the sunfish family (Centrarchidae), hybridization in 17 out of 18 cases between different species resulted in significantly different viability in F_1 offspring, depending on which species the mother came from (Bolnick & Near, 2005 and references therin). As dobzansky-muller incompatibilities often arise in one species first, they probably have a role in explaining non-reciprocal viability differences (Welch, 2004 and references therein). It is also possible that postmating prezygotic mechanisms are restricting hybridization in an asymmetric fashion, as seen in the example above with two *Chyrsochus* species (Monsen et al. 2007), and also in some orthopteran species pairs (e.g. Larson et al. 2012). Both of these last two hypotheses could be tested via captive breeding experiments.

Another possible explanation is the interactions of the two species in the wild. Most F_1 hybrids examined here had a *H. thoracica* father (n=8/9), which may suggest that *H. thoracica* males are outcompeting *H. crassidens* for mates. Interbreeding results in competition among males for harems of females, as male weta have a resource-based polygynous mating system (Wehi et al. 2013; c.f. Kelly, 2006). Where sympatric, females of both species will congregate together in roost cavities, so any large harem guarded by a male will be likely to contain both species. This means that any advantage by males of one species to gain and hold a harem will have a significant effect on the relative fitness of both species in sympatry. One obvious implication of *H. trewicki* being much smaller than *H. thoracica* where they live in sympatry is that *H. thoracica* males may have a strong advantage in defending harems. If this sample is indicative of the population as a whole then maturing early in the season by *H. trewicki* with respect to *H. thoracica* (Chapter 2) may mean that they partially avoid this problem, as has been seen in other closely related species pairs (e.g. Blondheim, 1990; Fergus et al. 2011; etc.). By contrast *H. thoracica* and *H. crassidens* do not show any evidence of niche divergence where they live in sympatry, and are presumably dealing with strong interspecific competition as are many other hybridizing species (Huxel, 1999 and references therein).

Competition between these species may be focused on obtaining roost cavities, as their native plant food source is abundant and does not appear to be a limiting factor (Field & Sandlant, 2001; Wehi, 2014). Competition for roosts and competition for mates by males are probably not separate factors, as males of the same species appear to avoid each other and will usually not cohabitate (Wehi et al. 2013). This means that any competition for daytime refuges will be stronger among males than females. ‘Sexual exclusion’ or reproductive interference that has the same outcome as competitive exclusion has been shown previously by Hochkirch et al. (2007) in two orthopteran species, and was suggested to be a common occurrence, particularly where herbivorous insects are concerned, as it is

often difficult to define a limited resource that these species are competing for (Strong et al. 1984). *Hemideina thoracica* may have a slightly faster metabolic rate (Minards et al. in press), and be the more aggressive of the two species, as they bite and show defensive displays more often in captivity, as well a seemingly higher level of activity (personal observation, 2012 - 2014; Jacobsen, 2009). This may provide an explanation for the bias in F_1 hybrid production, as well as an explanation of how *H. thoracica* has managed to displace *H. crassidens* in the past, so behavioural differences between the two species may be worth further study.

4.1.3 Introgression

The statistical approaches used in this study were not able to distinguish between the hypotheses of low gene flow or no gene flow. However, visual examination of the genetic and morphological/colouration data and construction of simple models suggested that while there is no introgression in Hawke's Bay between *H. thoracica* and *H. trewicki* (similar to the situation seen between *H. femorata* and *H. ricta* in Banks Peninsula (Morgan-Richards, 1995)), there probably is a low level in the Manawatu between *H. thoracica* and *H. crassidens.* The difference between low and no introgression is biologically important. Species that produce infertile F_1 offspring theoretically face selective pressures that aim to prevent interbreeding in sympatry completely, as infertile F_1 offspring constitute a strong fitness disadvantage to the parents who are producing them. For species which are exchanging genes, the situation is more complicated with a range of potential outcomes. Selection pressures can favour both increase and decrease in reproductive isolation, depending on the relative advantages/disadvantages to the fitness of the individual, or they may lead to a stable situation where multiple factors have opposing outcomes (such as the maintenance of a tension zone, where dispersal in to the area and hybrid disadvantage determine the width of the zone, e.g. hybrid zones between *H. thoracica* chromosome races (Morgan-Richards, 1997; Morgan-Richards & Wallis, 2003)).

There are possible implications of exchanging alleles that would make the interaction of these two species more difficult to predict than if they were reproductively isolated. For example, *H. crassidens* has been hypothesised to have a better tolerance for cold climates, which explains how it has managed to retain its original distribution at high altitudes. If this allele or combination of alleles were to introgress into *H. thoracica,* it would give this species strong advantage over *H. crassidens,* presumably removing any current advantage that *H. crassidens* holds in certain climates. This could also go the other way, with *H. crassidens* managing to obtain *H. thoracica's* advantage in warmer climates. With only low levels of introgression, it is unlikely that selection pressure would be high to remove barriers to reproduction; rather that selection should reinforce them. Over a long enough timescale, full reproductive isolation may be achieved, unless the relatively low gene flow currently experienced proves beneficial in some way.

The exact level of gene flow will also depend on the relative fitness of hybrid individuals. As F_1 hybrid females are likely infertile (in contrast to Haldane's rule), F_1 hybrids have at least a 50% reduction in fertility overall compared to parent forms. However, as F_1 males appear to mature at a large size and late instar (Appendix A), it is possible that they have a reproductive advantage, if they are able to produce offspring in the wild. Again, the possible bimodal hybrid zone found here in the absence of

assortative mating suggests strong genetic barriers, but future research is required to determine where these barriers lie.

4.1.5 Applying species concepts to North Island tree weta

According to the widespread biological species concept (Mayr, 1942), which allows no gene flow, *H. thoracica* and *H. crassidens* would have to be regarded as subspecies with respect to each other, while *H. thoracica* and *H. trewicki* fit the definition for good species. Presumably, this would imply that the three north island species constitute a ring species (two reproductively isolated forms connected by a chain of interbreeding populations), if predictions about a hybrid zone between *H. crassidens* and *H. trewicki* prove correct. Many species concepts do allow for some level of gene flow, and in this case *H. thoracica* and *H. crassidens* do not have any problem retaining their species status. The genotypic cluster species concept (Mallet, 1995) provides a good example, by allowing introgression as long as species largely retain their own distinctive cluster of traits (morphological and/or genetic) where they exist in sympatry. As these species display a bimodal hybrid zone with low levels of gene flow, they can retain their species status under any species concept that allows for some level of gene flow.

4.1.6 Comparison of species pairs and future interaction

It was expected, given the genetic similarity of all three North Island species and particularly that of *H. crassidens* and *H. trewicki,* that the interaction of *H. thoracica* with its two neighbouring species would be similar. However, as this study shows, the two species pairs, *H. thoracica*/*H. crassidens* and *H. thoracica*/*H. trewicki*, are clearly interacting in different ways in their areas of sympatry. It appears that *H. thoracica* and *H. trewicki* have adapted to living in sympatry, and that their ecological niches have diverged to the point where competition may already be limited. This bodes well for their ability to coexist in the future, and suggests that sympatry will probably not result in one species being forced to restrict its range. Presumably these weta have not always been in contact; genetic diversity within *H. thoracica* suggests recent range expansion from northland (since the LGM ~15,000 years ago) (Trewicki & Morgan-Richards, 2005). As warmer temperatures appear to explain this pattern (Bulgarella et al. 2014), global warming would predict continued range expansion of *H. thoracica* into *H. trewicki* territory in the future (it now shares approximately the upper half of *H. trewicki*'s territory), but this will likely not be a major problem for *H. trewicki.* In contrast, *H. thoracica* and *H. crassidens* gave very little evidence of divergence where they meet, a situation which has been noted before (Minards et al. in press; Dewhurst, 2012; P.C. Wehi, personal communication, 10 June 2012). The low level of gene flow may have some part in explaining this, by preventing any divergent adaptation that arises in sympatry from staying exclusively in one species. However, the level of gene flow seen in this study suggested a bimodal form of hybridization. Bimodal hybridization usually means that there is already significant isolation between the two species (e.g. Jiggins & Mallet, 2000 and references therein), and that they are thus retaining separate evolutionary trajectories, so theoretically they should be able to diverge in sympatry. *Hemideina crassidens* appears to have already lost much of its former north island range to *H.*

thoracica (Trewick & Morgan-Richards, 1995; Bulgarella et al. 2014), which supports the hypothesis of lack of divergence with competition between these two species. Global warming would thus predict that *H. thoracica* will continue to displace *H. crassidens* in future (the same prediction was reached in Bulgarella et al. 2014), although some level of *H. crassidens* alleles may be preserved in the *H. thoracica* gene-pool as a result of introgression.

The situation may not be the same where different chromosome races of *H. thoracica* meet *H. crassidens.* The 15 chromosome race of *H. thoracica* meets *H. crassidens* at Mt Taranaki, Mt Ruapehu and Whirinaki Forest Park, all areas where islands of *H. crassidens* are found surrounded by *H. thoracica* (Trewicki & Morgan-Richards, 1995) (Figure 1.1, 2.2, 3.2). The contact zone on Mt Taranaki is known to be narrow, with the species appearing to maintain a more parapatric rather than sympatric contact zone (Minnards, 2012). As environment may have a strong part to play in some of these areas, as well as karyotype, these contact zones would make a good comparison for the data collected in this study.

4.1.7 Conclusion

The species pairs examined in this study give two examples of hybridization in similar natural systems along with their genetic outcomes. The situation with *H. thoracica* and *H. trewicki* was as expected, with reproductive isolation being complete despite the presence of F_1 hybrids. Along with possible niche divergence, these two species constitute “good species”. The situation in *H. thoracica* and *H. crassidens* is somewhat unusual, both because F_1 hybrids contrast with Haldane’s rule, and because they appear to show a bimodal hybrid zone in the absence of assortative mating. Both of these factors may prove interesting for future research and may be able to give insights as to how/when these general rules apply to natural systems. There was also a possible sex bias in the production of F_1 offspring which may suggest sexual exclusion and could help explain patterns of range expansion and displacement by *H. thoracica.*

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Appendix A: Preliminary data on hybrid viability and fertility

5.1 Introduction

5.1.1 Hybrid viability and fertility

Hybrid animals commonly have lower fitness than the parent species that produced them. This can sometimes be caused by incompatible allele combinations but may also involve chromosome differences between the parent taxa, and a range of incompatibilities involving cellular organelles (mitochnodrial) and intracellular bacteria (see examples and references in Chapter 1). If hybrids are not inviable then they may be infertile or have limited fertility. If fertility of F_1 hybrids is very low, fertility levels usually improve in the next generation of backcross hybrids (Mallet, 2005; Mallet et al. 1998; Descimon & Mallet, 2009). Meiotic non-disjunction is a common cause of hybrid infertility, as the chromosomes of F_1 hybrids often do not line up properly on the metaphase plate, leading to gametes with abnormal chromosome complements that usually lack vital genetic material or contain too much (leading to over-activity of certain genes) (Federley, 1913). Tree weta are known to be particularly tolerant of chromosome rearrangements, as the presence of multiple chromosome races in some species clearly shows (Morgan-Richards, 1997; Morgan-Richards & Wallis, 2003), so it is not known to what extent karyotype differences may affect fertility between hybrids of tree weta species. Haldane's rule states that if one sex is absent, rare or infertile in F_1 hybrids, it will be the heterogametic sex (Haldane, 1922). In the case of tree weta, this would be the males, as tree weta have an XO sex determination system (females have two copies of the sex chromosome, males have one).

5.1.2 *Wolbachia*

Wolbachia is an endosymbiotic intracellular bacteria that infects a large proportion of the nematode and arthropod phylums (Werren et al. 2008). In arthropods it is estimated to infect about 65% of species (Hilgenboecker, 2008), and is known to manipulate the reproductive biology of many of its hosts. While mostly appearing to have a mutualistic relationship with its nematode hosts, it appears to function parasitically in insects, altering the mating system of its host in a number of ways to its own evolutionary advantage (Werren et al. 2008). Some of the currently known host-reproductive manipulations include male killing, induction of parthenogenesis, feminization of genetic males, forced production of haploid individuals in haplodiploid systems and cytoplasmic incompatibility (Werren, 1997). Cytoplasmic incompatibility prevents interbreeding among individuals of differing strains (or between individuals that do and do not contain *Wolbachia*), via an unknown host modification-rescue mechanism (Poinsot et al. 2003). Cytoplasmic incompatibility is the most common parasitic modification of insect groups known to result from *Wolbachia* infection, and has been seen in eight arthropod orders including orthoptera (Werren et al. 2008). *Wolbachia* are known to be transferred vertically to offspring though infection of germline tissue (Kose & Karr, 1995). However, unlike in nematodes, parasite and host phylogenies are not concordant in arthropods, indicating extensive lateral transfer among species (Funk et al. 2000). It is currently unknown whether the weta lineage contains this intracellular parasite. As *Wolbachia* is hypothesised

to have a role in arthropod speciation via induction of cytoplasmic incompatibility, if present, it may be able to explain any infertility between the tree weta species in this study. If this is the case, it may also give an explanation as to why most tree weta species appear to be still sharing the same ecological niche, a fact currently hypothesised to be the result of allopatric speciation and recent contact.

This appendix aims to collect together all information that could be obtained about hybrid viability and fertility between the species pairs, including an investigation to determine whether weta contain the common intracellular parasite *Wolbachia*.

5.2 Methods

5.2.1 Captive Conditions

The live weta were all kept in individual containers at a constant temperature of 14°C. They were given a hollow flax stalk about 15cm long for a roost to sleep in. They were fed leaves from at least three native plant species each week along with 80% soy protein pellets for food. The native plant species used for food were Puriri (*Vitex lucens*), *Coprosma repens,* Miro (*Prumnopitys ferruginea*), Karaka/New Zealand Laurel (*Corynocarpus laevigatus*), and Mahoe/Whitey wood (*Melicytus ramiflorus*). Disturbance outside of weekly feeding/ cleaning of container and experiments was kept to a minimum, so as not to stress the weta. Some weta were used in more than one experiment (Table 5.1).

Table 5.1: Information for each hybrid weta, along with the experiments that each weta was involved in.

Code	Sex	Location	Confirmed Hybrid	Mating Behaviour	Breeding	Examination of Ovaries
Hybrid 1	M	Mohi Bush	Yes	Yes	No	NA
Hybrid 2	M	Kahutawera valley	Yes	Yes	Yes	NA
Hybrid 3	M	Turitea valley	Yes	Yes	Yes	NA
Hybrid 4	M	Turitea valley	Yes	Yes	Yes	NA
Hybrid 5	F	Kahutawera valley	Yes	Yes	NA	Yes
Hybrid 6	F	Kahutawera valley	No	Yes	NA	Yes
Hybrid 7	F	Kahutawera valley	No	Yes	NA	Yes
Hybrid 8	F	Kahutawera valley	Yes	No	NA	Yes
Hybrid 9	F	Kahutawera valley	No	No	NA	Yes

5.2.2 Size of F_1 Hybrids

The age and sex of all F_1 and putative F_1 hybrids was recorded to look for any potential biases in these two factors that might suggest inviability of some hybrids. The left hind tibia (or right, if the left was missing or deformed) of each weta was measured using electronic callipers. The sex of hybrids and instar at maturity for male weta was noted. Males are known to mature at any of the eighth, ninth or tenth instars, while females all mature at the tenth. If the instar at maturity was unclear - there is some overlap between larger males of one instar and smaller males of the next (Spencer, 1995) - this was noted as well.

5.2.3 Mating Behaviour

There were seven live hybrid weta available for this part of the experiment. All weta were adults (this can be determined by the shape and size of the ovipositor in females and cerci in males). All seven live weta were given one mate of each of the parent species in a Perspex Tank 60cm x 60cm x 60cm, except the *H. thoracica* x *H. trewicki* hybrid, who was only given a *H. trewicki* female due to time constraints. These trials were done in the evening so as to be close to the time that weta are generally active. Male weta were given half an hour from the time that the male noticed the presence of the female (males will rapidly twitch their palps upon detecting the scent of a female).Typically, males in this situation will attempt to mate with the female once locating her, so attempts to mate and actual mating by hybrid males was recorded. Attempts to mate were defined as the male curling up his abdomen and trying to position himself for mating. Females sometimes actively resist mating, although they will usually allow it to occur (personal observation, 2012-2014). Females do not appear to actively choose or approach male weta, so only female acceptance or active resistance was recorded for the hybrid female weta. As little is known about weta mating behaviour, resistance was defined as any behaviour that appeared to obstruct mating attempts by the males. These behaviours were; running away, stridulating by kicking the legs downward against the abdomen (a defensive/ aggressive gesture in tree weta), kicking the male to dislodge him, biting the male, and pulling the ovipositor downward to prevent the male positioning himself. Willingness to mate was defined as the female staying in one place and allowing copulation to be initiated and completed.

5.2.4 Egg Production

As initial attempts to get a female hybrid to lay eggs were unsuccessful (despite parent females of either species laying eggs in the same conditions), the female and two others were dissected to find out if they were producing eggs. Ovaries that had been removed from two other adult putative hybrid females were also obtained from the Massey University (Ecology group) collection, and all were examined under a dissecting microscope. The presence or absence of eggs was noted, as was any unusual ovarian morphology. Females begin producing eggs as soon as they reach maturity (mature females can be easily identified due to the unique shape and size of the ovipositor at this stage), but the age since maturity was calculated or estimated from information stored on a Massey University catalogue, in case it proved relevant. Eggs inside the ovarioles of a mature female typically vary in developmental stage and range from very small undeveloped yellow eggs right through to large black eggs with a thick outer casing.

5.2.5 Male Fertility

One of the hybrid males was given three adult virgin females to mate with (two *H. crassidens*, one *H. thoracica*), and the other male hybrid was given two females, one of each species (the *H. crassidens* died soon after without laying eggs).

The weta were placed together in a Perspex Tank 60cm x 60 cm x 60cm. They were then observed until a mating occurred, and afterwards left together in the tank overnight, so multiple matings would have a chance to occur. It was also hoped that during the night the weta would be more active and more likely to display typical behaviour, as these species are nocturnal. Female weta were removed the next morning and placed into a container with a layer of soil slightly higher than the length of the ovipositor. After a number of eggs had been laid, the female was removed, and the eggs counted and then placed back into the soil. The boxes of eggs were placed outside so that they could experience normal temperature fluctuations through the year. A *H. crassidens* x *H. crassidens* mating was set up as a positive control in the same manner as the hybrid mating experiments. Noticeable growth and hatching of the eggs, if it occurred, was recorded.

5.2.6 *Wolbachia*

Evidence of the presence of *Wolbachia* was sought in two ways. *Wolbachia*-specific primers (Table 5.2) were used to amplify weta DNA. DNA was extracted from all three tree weta species from different tissues. For *H. thoracica* tissue from the hind femur was extracted; for *H. crassidens* tissue was taken from testes of a male weta; from *H. trewicki* tissue was taken from ovaries of a female specimen. The standard salting out method detailed in chapter 3 was used to extract the DNA. A DNA sample from an introduced gregarious parisitiod wasp was used as a positive control. PCR conditions were: initial denaturation temperature of 94°C for 3mins was followed by 38 cycles of: denaturation at 94°C for 30sec; annealing at 55°C for 15sec; and an extension at 72°C for 1:30sec. This was followed by a final extension at 72°C for 15min. PCR conditions were adjusted according to the results produced by individual primers by increasing or decreasing annealing temperature. Absence of PCR products in contrast to the positive control was considered a negative result. DNA fragments of the wrong size or smears on the gel were also considered a negative result, although these were repeated to rule out PCR problems. In the case of CoxA, a clear band of the wrong size appeared in all three weta samples (approximately 650bp compared to 450bp positive control band). This band was isolated from other faint bands by increasing the annealing temperature. One PCR product was sequenced and then visualised and trimmed on Geneious 6.1.7 (Boimatters LTD) software. The remaining clean 269bp sequence was then run through Basic Local Alignment Search Tool (BLAST) on the NCBI website to see if it was a match for *Wolbachia*.

Table 5.2: Information for loci and primer pairs used in this study for the detection of *Wolbachia* in the three *Hemideina* species.

Locus	Forward Primer	Reverse Primer	Primer sequences	Source
Wolbachia surface protein (wsp)	Wsp81F	Wsp691R	5'TGGTCCAATAAGTGATGAAGAAAC3' 5'AAAAATTAAACGCTACTCCA3'	Braig et al. 1998
Fructose-bisphosphate aldolase (fbpA)	fbpAF1	fbpAR1	5'CTTACATTCGAAGAATCTGACT3' 5'TCTTTTAACATATTCAATTCTTTTAGG3'	Baldo et al. 2006
Cytochrome c oxidase, subunit I (coxA)	CoxAF1	CoxAR1	5'CGAAAGTATGAAAAGGTGGTGG3' 5'GGTGGAGCGATTGAATTGAAG3'	Baldo et al. 2006
Wolbachia specific portion of 16S ribosomal RNA gene (wol16S)	Wol16SF	Wol16SR	5' CGGGGGAAAAATTTATTGCT 3' 5' AGCTGTAATACAGAAAGTAAA 3'	Chiel et al. 2007

The second method involved mapping next-generation sequences obtained from two different weta specimens against a complete *Wolbachia* genome. As *Wolbachia* is an intracellular parasite, its DNA would be extracted alongside weta DNA during the extraction process. This method has been used previously to discover *Wolbachia* within potential hosts (e.g. Salzburg et al. (2005) used a similar method to discover *Wolbachia* infections in three *Drosophila* species). The next-generation sequences were obtained during a previous study by Dowle (2013) with the aim of constructing the entire mitochondrial genome for two species of tree weta (*H. thoracica* and *H. crassidens*). The *H. thoracica* weta had been collected from the Kahutawera Valley, and the *H. crassidens* weta was from a South Island population. There were 5,191,884 paired-end sequences available for the *H. thoracica* specimen and 17,434,429 paired-end sequences available for the *H. crassidens*. Tissue from the testes of the male weta was used to the extract the DNA, and as *Wolbachia* is typically passed on vertically from host to host, reproductive cells should contain high numbers of this parasite. Even if only one *Wolbachia* parasite is present per cell, this would still represent 0.5% of sequence reads in an average-sized host genome (Salzburg et al. 2005). The *Wolbachia* genome was downloaded from the New England Biolabs website (http://tools.neb.com/*Wolbachia*) which contained a complete *Wolbachia* genome from *Brugia malayi* (Foster et al. 2005). The next-generation sequences were aligned with the *Wolbachia* genome with software Bowtie 2 (John Hopkins University). Software Tablet V1.7.0_35 from the James Hudson Institute (Milne et al. 2013) was used to visualise the results. For any sequence matches, one aligned weta sequence for each location on the *Wolbachia* genome was run through Basic Local Alignment Search Tool (BLAST) (National Library of Medicine) on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to see if *Wolbachia* was the most likely origin of the sequence. This was to check if these sequences were a closer match to *Wolbachia* than to any related bacteria species.

5.3 Results

5.3.1 Size of Hybrid Weta

All *H. thoracica* x *H. crassidens* hybrids fell within the normal size range of both parent species (Chapter 2; Figure 2.8). However, the *H. thoracica* x *H. trewicki* hybrid was smaller than the lower end of the range for *H. trewicki*, and much smaller than the *H. thoracica* range. This specimen was a male, and as the individuals measured in Chapter 2 were female, some allowances can be made for the fact that males can mature at earlier instars than females, causing them to have slightly smaller body size. The fact that most hybrid weta in this study were adults (or reached adulthood in captivity – two weta) suggests there is no hybrid inviability that prevents F_1 hybrids of either species pair from reaching maturity. The sex ratio for the Manawatu *H. thoracica* x *H. crassidens* hybrids was about even, with 5 females and 6 males, so there does not appear to be a sex-bias in F_1 hybrid offspring. The *H. thoracica* x *H. trewicki* hybrid appeared to have matured at the eighth instar, or possible earlier, given its' very small head relative to body size as well as its small overall size. The five adult male *H. thoracica* x *H. crassidens* hybrids had all matured at the tenth instar (as was evident by their very large head and mandibles relative to their body size). There was one weta who was not yet mature (Hybrid 12; Table 5.3), but was clearly a ninth instar weta given its large head relative to body size, and so would have had no choice but to mature at the tenth instar, as the ninth instar is the last instar that can be reached before maturity (Spencer, 1995).

Table 5.3: Sex, age and size data for each hybrid weta (*Hemideina* spp), along with instar at maturity.

Weta	Sex	Age	Tibia Length	Instar at Maturity
Hybrid 1	M	Adult	15.18	7/8
Hybrid 2	M	Adult	23.63	10
Hybrid 3	M	Adult	24.01	10
Hybrid 4	M	Adult	23.62	10
Hybrid 5	F	Adult	22.92	10
Hybrid 6	F	Adult	21.26	10
Hybrid 7	F	Adult	23.76	10
Hybrid 8	F	Adult	21.26	10
Hybrid 10	F	Adult	22.29	10
Hybrid 11	M	Juvenile	16.00	10
Hybrid 12	M	Sub-adult	18.42	10
Hybrid 13	M	Adult	21.11	10

5.3.2 Mating Behaviour

The *H. thoracica* x *H. trewicki* hybrid attempted to mate with the female weta, but actual mating did not occur. The hybrid showed some interest, as was evident by the rapid twitching of its palps upon discovering the female, and curing up its abdomen in attempt to mate. However, the hybrid seemed to be only partially interested, and did not bother to follow the female when she started to walk away. All three *H. thoracica* x *H. crassidens* males mated with females of both species (Table 5.4). They exhibited normal mating behaviour, and were accepted by the females. No abnormal behaviour was noted during these trials.

In contrast, two of the hybrid females actively resisted mating. The third female hybrid responded differently to the two males; she allowed the *H. thoracica* male to begin copulation several times, but then dislodged him and proceeded to bite him and display other resistance behaviours. She did mate once with the *H. crassidens* male, and then resisted subsequent mating attempts by this male.

Table 5.4: Summary of mating behaviour displayed for each hybrid weta.

Code	Sex	Behaviour
Hybrid 1	M	Attempted to mate
Hybrid 2	M	Normal; mated
Hybrid 3	M	Normal; mated
Hybrid 4	M	Normal; mated
Hybrid 5	F	Resisted Mating
Hybrid 6	F	Resisted Mating
Hybrid 7	F	Partial Resistance

5.3.3 Egg production

All five female hybrids dissected contained no eggs, in any stage of development (Table 5.5). A lack of eggs was never observed in more than 50 mature parent females of either species (personal observation, 2012 - 2013; M. Morgan-Richards, personal communication, 20 February 2013).

Table 5.5: Age and presence or absence of eggs in the ovarian tissue of female F_1 hybrids between *H. crassidens* and *H. thoracica.*

Code	Age from Maturity	Eggs
Hybrid 5	6 months	No
Hybrid 6	4 months	No
Hybrid 7	3 months	No
Hybrid 8	approx. 1 year	No
Hybrid 9	Unknown	No

5.3.4 Male fertility

Four weta fathered by one of the hybrid males and an *H. thoracica* mother hatched. No other eggs hatched (n = 37), although some laid by all females showed signs of growth, with many eggs increasing in size and changing colour from black to light brown or yellow (Table 5.6). The backcross hybrids were anesthetised for genetic analysis within two days of hatching.

Table 5.6: Results for captive breeding experiments with F_1 hybrid *H. thoracica* x *H. crassidens* males.

Male	Female	No. Eggs Laid	Growth	Hatched
Hybrid 2	*H. crassidens*	50	Yes	0
	H. crassidens	35	Yes	0
	H. thoracica	111	Yes	0
Hybrid 3	*H. thoracica*	37	Yes	4
	H. crassidens	-	-	-
H. crassidens	*H. crassidens*	122	Yes	0

5.3.5 *Wolbachia*

Both fbpA and Wol16S primer pairs failed to give a PCR product from the weta DNA. Wsp and CoxA primer pairs gave a series of faint bands of the wrong size class (Table 5.7). The larger than control PCR product amplified using the CoxA primers was sequenced from tree weta but no similar match was found when compared to sequences on Genbank using BLAST. As *Wolbachia* sequences amplified with the CoxA primer pair used here are available on Genbank, this sequence was most likely a randomly amplified fragment of the weta genome. The weak amplification using Wsp primers were different sizes in each weta sample. Fragments of the wrong size were regarded as a negative result.

Table 5.7: Results for weta DNA amplification with *Wolbachia*-specific PCR primers. *Bands of the wrong size class were present.

Locus	wsp	fbpA	CoxA	Wol16S
Result	-ve*	-ve	-ve*	-ve

None of the *H. crassidens* next-generation sequences aligned with the *Wolbachia* genome, however, eight 100bp sequence matches were found in the *H. thoracica* sequences. Six of the sequences mapped to one location, all with 10 mismatches. The other two mapped to a different location on the *Wolbachia* genome and contained 9 mismatches each. The paired-end for all eight of these sequence reads (which is approximately 100 bp downstream from the matching sequence) was unable to be mapped onto the *Wolbachia* genome. Results from BLAST for the Genbank database identified the first location sequence as a 93% match for 26 sequences that belonged to the 16S rRNA gene from various members of the Chlamydiae phylum, with six of these matches belonging to the *Rhabdochlamydia* genus. The second sequence was a 93% match for three 28S gene fragments from *Simkania negevensis*, which also belongs to the Chlamydiae phylum. As no close *Wolbachia* sequences came up in either case, it is likely that the *H. thoracica* weta was infected with a bacteria species from the chlamydia family, but not *Wolbachia*. Both the 16S and 23S rRNA genes are highly conserved among bacteria, and there were no matches to *Wolbachia*-specific regions of the *Wolbachia* genome.

5.4 Discussion

5.4.1 Hybrid viability and fertility

Although first generation hybrids are produced where *H. thoracica* overlaps with *H. trewicki and H. crassidens*, genetic data (Chapter 3) suggests these hybrids are either sterile or have very low reproductive success. Reproduction requires reaching sexual maturity, successful mating and production of viable zygotes. This chapter presents preliminary data on hybrid viability and fertility. Most of the datasets used were small, due to the difficulty obtaining large numbers of hybrids, as they make up the minority of weta collected from sympatric areas the wild (and captive breeding of tree weta is difficult). However, the results are biologically significant, and suggest potential areas of future research.

As the size of *H. thoracica* x *H. crassidens* hybrids fell within the normal range expected for the parent species, and as many were found as adults as well as juveniles in the wild, there was no evidence found of hybrid inviability or stunted growth. There may of course, be a level of inviability early in development, during the pre-hatching or early instar phase, but it appears that at least by the time F_1 hybrids have reached the larger juvenile phase (approximately 5^{th} to 7^{th} instar), they are as viable as a typical weta of either parent species. All F_1 males matured at a late instar, which may be important in understanding their reproductive success (if any). None of the *H. thoracica* x *H. crassidens* F_1 hybrids showed obvious signs of being unwell or slow in development. The two F_1 hybrids that were captured as large juveniles appeared to moult at a rate that was as fast, or faster than other weta kept in the lab (once a month until maturity), although this is anecdotal evidence and would have depended on many factors. The *H. thoracica* x *H. trewicki* F_1 hybrid was different in these respects. It was very small at maturity (which it reached after only one moult in captivity). Its small size suggested that it may have matured unusually early (possibly at the seventh instar), but data for typical size at maturity in males of the parent species and more extensive research on male maturation in general would be needed to confirm this. Its general behaviour seemed normal (if somewhat aggressive), but it appeared to have only a partial interest in mating, so it is not known whether it would have mated successfully in the wild. Again, other F_1 hybrids from this species pair would be needed for comparison.

Mating behaviour appeared to be normal for hybrid males between *H. thoracica* and *H. crassidens.* Also of interest is that one of the males was found in the wild with a harem consisting of two adult *H. crassidens* females. This male produced offspring in captivity; hence it is likely that this male, along with others, are behaving in a manner typical of the parent species males in the wild. In contrast, the female F_1 hybrids did not show typical mating behaviour, but this may be irrelevant to fertility if they cannot produce eggs. The lack of egg production in all five female hybrids is probably biologically important, despite the small sample, because it contrasts with that seen in all parent female weta. Approximately 50 mature females were dissected in the process of collecting data for this thesis (not all were included in results for various reasons), and all contained visible eggs in various stages of development. This lack of eggs in all hybrid females suggests that F_1 hybrid females are typically infertile. In contrast, some male hybrids do have at least some fertility, as was evident from offspring produced in captivity. Male weta being partially fertile while females are infertile

would contradict Haldane's rule and may be of interest for future research. Haldane's rule applies across a great number of taxa, including animals with an XO sex determination system (Haldane, 1922).

5.4.2 *Wolbachia*

Neither of the two methods employed here provided evidence of *Wolbachia* infection. The primer pairs here should have been able to pick up on the common *Wolbachia* supergroups that infect arthropods (Simoes et al. 2011), and the mapping software was sensitive enough to detect infection by another bacterial parasite, so it is highly likely that weta do not contain this intracellular parasite. Another factor was that cytoplasmic incompatibility between different strains usually prevents the production of F_1 hybrids, by killing such embryos before they have a chance to develop. As F_1 hybrids were found in this study, and possibly backcrosses between *H. thoracica* and *H. crassidens*, it seems unlikely that cytoplasmic incompatibility is a factor in weta reproductive biology. Other effects of *Wolbachia* infection are also not apparent; factors such as parthenogenesis and male-killing are not seen in tree weta.

A chlamydia infection was detected in one of the weta in this study however, and as this bacteria functions as an intracellular parasite (Wyrick, 2000), it may be of interest. Weta do make good candidates for sexually transmitted diseases. These diseases generally require some level of promiscuity, as well as overlapping adult generations (Knell & Webberley, 2004), both phenomena seen in tree weta. However, very little is known about the prevalence or role of bacterial infections of this kind in insects, despite being well-studied in mammals and birds (Knell & Webberley, 2004).

5.4.3 Conclusion

In summary, both male and female F_1 hybrids are capable of reaching maturity, and although *Wolbachia* is not involved in limiting hybridization, there is at least a 50% (probably higher) reduction in F_1 hybrid fertility, which likely has a strong effect on limiting introgression in the wild. There does seem to be a contrast between complete failure by female F_1 hybrids to produce eggs and the clear evidence of at least partial fertility of some male hybrids, which also contrasts with Haldane's rule.

5.5 References

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Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Prol S
1	F	Adult	6	6	19.87	Yes	Yes	Tan/Dark	Brown-B	Brown-B	
2	F	Adult	5	6	22.23	Yes	Yes	Dark/Black	Brown-H/B	Brown-B	
3	F	Adult	7	7	20.15	Yes	Yes	Dark/Black	Brown-B	Brown-B	
4	F	Adult	4	6	22.71	Yes	Yes	Dark	Brown-B	Brown-B	
5	F	Adult	7	5	19.91	Yes	Yes	Dark/Black	Brown-B	Brown-B	
6	F	Adult	6	6	24.02	Yes	Yes	Tan/Dark	Brown-H	Brown-B	
7	F	Adult	6	6	22.49	Yes	Yes	Tan/Dark	Brown-H	Brown-B	
8	F	Adult	5	5	23.09	Yes	Yes	Tan-H	Brown-H	Brown-B	
9	F	Adult	5	4	21.71	Yes	Yes	Tan/Black	Brown-B	Brown-B	
10	F	Sub	6	6	20.45	Yes	Yes	Dark	Brown-B	Brown-B	
11	F	Sub	7	7	19.33	Yes	Yes	Dark	Brown-B	Brown-B	
12	M	Adult	6	6	17.3	Yes	Yes	Black	Pale-H	Brown-B	
13	M	Adult	5	7	17.32	Yes	Yes	Black	Brown-B	Brown-B	
14	M	Adult	8	8	18.15	Yes	Yes	Black (White E)	Pale-H	Brown-B	
15	M	Adult	6	6	19.3	Yes	Yes	Black	Pale/Brown	Brown-B	
16	M	Adult	7	6	18.84	Yes	Yes	Pale-H	Brown-H	Brown-B	
17	M	Adult	5	6	18.5	Yes	Yes	Black	Brown-H	Brown-B	
18	M	Adult	7	7	20.29	Yes	Yes	Black	Brown-H	Brown-B	
19	M	Adult	6	5	22.92	Yes	Yes	Black	Brown-H	Brown-B	
20	M	Adult	7	6	23.15	Yes	Yes	Black (White E)	Paleish-H	Brown-B	
21	M	Adult	7	7	20.01	Yes	Yes	Tan/Dark	Brown/Pale	Brown-B	
22	M	Adult	6	6	22.17	Yes	Yes	Tan/Black	Brown/Pale	Brown-B	
23	M	Sub	5	7	15.17	Yes	Yes	Dark	Brown-B	Brown-B	
24	M	Sub	5	4	17.08	Yes	Yes	Tan/Black	Brown-H/B	Brown-B	
25	M	Sub	7	6	14.22	Yes	Yes	Brown	Dark/Brown-B	Brown-B	
26	M	Sub	-	7	16.72	Yes	Yes	Brown	Brown-B	Brown-B	

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum
1	F	Adult	7	7	23.04	Very Light	No	Pale-H	Dark	Dark
2	M	Adult	5	6	18.22	Very Light	No	Pale-H	Dark	Dark
3	M	Adult	6	8	18.8	Very Light	No	Pale-H	Dark	Dark
4	M	Adult	6	7	20.34	Very Light	No	Pale-H	Dark	Dark
5	M	Adult	6	5	20.9	Very Light	No	Pale-H	Dark	Dark
6	M	Sub	6	7	7.32	Very Light	No	Pale-H	Pale	Pale
7	M	Adult	7	7	15.07	Very Light	No	Pale-H	Dark	Dark
8	M	Sub	9	7	18.12	Very Light	No	Pale-H	Grey	Grey
9	M	Sub	7	7	17.81	Very Light	No	Pale-H	Dark	Dark
10	M	Juv	6	6	15.49	Very Light	No	Pale-H	Pale	Pale
11	M	Sub	6	5	20.31	Very Light	No	Pale-H	Dark	Dark
12	M	Juv	7	7	12.45	Very Light	No	Pale-H	Pale-H	Pale-B
13	M	Juv	7	6	15.5	Very Light	No	Pale-H	Pale	Pale
14	F	Adult	6	7	24.19	Very Light	No	Pale-H	Dark	Dark
15	F	Adult	7	6	21.95	Very Light	No	Pale-H	Dark	Dark
16	F	Sub	-	-	17.65	Very Light	No	Pale-H	Dark	Dark
17	M	Adult	7	6	19.43	Very Light	No	Pale-H	Dark	Dark
18	F	Adult	5	5	22.23	Very Light	No	Pale-H	Dark	Dark
19	F	Adult	5	5	25.9	Very Light	No	Pale-H	Dark	Dark
20	F	Adult	6	5	23.65	Very Light	No	Pale-H	Dark	Dark
21	F	Adult	6	7	23.51	Very Light	No	Pale-H	Dark	Dark
22	F	Adult	6	5	22.45	Very Light	No	Pale-H	Dark	Dark

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Pr
1	F	Adult	7	7	24.1	Very Light	No	Pale-H	Dark	Dark	
2	F	Adult	7	6	25.2	Very Light	No	Pale-H	Dark	Dark	
3	F	Adult	7	7	25.11	Very Light	No	Pale-H	Dark	Dark	
4	F	Adult	6	5	24.18	Very Light	No	Pale-H	Dark	Dark	
5	F	Adult	6	6	25.86	Very Light	No	Pale-H	Dark	Dark	
6	F	Adult	6	6	25.43	Very Light	No	Pale-H	Dark	Dark	
7	F	Adult	6	6	26.06	Very Light	No	Pale-H	Dark	Dark	
8	F	Adult	7	6	24.59	Very Light	No	Pale-H	Dark	Dark	
9	F	Sub	6	7	22.15	Very Light	No	Pale-H	Dark	Dark	
10	F	Sub	6	6	21.01	Very Light	No	Pale-H	Dark	Dark	
11	F	Sub	7	8	21.43	Very Light	No	Pale-H	Dark	Dark	
12	F	Sub	9	6	20.31	Very Light	No	Pale-H	Dark	Dark	
13	F	Sub	7	6	21.03	Very Light	No	Pale-H	Dark	Dark	
14	F	Sub	7	8	19.82	Very Light	No	Pale-H	Dark	Dark	
15	F	Sub	6	7	19.24	Very Light	No	Pale-H	Dark	Dark	
16	M	Adult	6	7	21.35	Very Light	No	Pale-H	Dark	Dark	
17	M	Adult	7	8	20.28	Very Light	No	Pale-H	Dark	Dark	
18	M	Adult	6	6	19.47	Very Light	No	Pale-H	Dark	Dark	
19	M	Adult	8	8	20.74	Very Light	No	Pale-H	Dark	Dark	
20	M	Adult	5	7	20.11	Very Light	No	Pale-H	Dark	Dark	
21	M	Sub	9	9	16.04	Very Light	No	Pale-H	Dark	Dark	
22	M	Sub	6	6	17.82	Very Light	No	Pale-H	Dark	Dark	
23	F	Sub	6	8	9.27	Very Light	No	Pale-H	Dark	Dark	
24	M	Juv	7	7	16.45	Very Light	No	Pale-H	Dark	Dark	
25	M	Juv	7	7	12.51	Very Light	No	Pale-H	Dark	Dark	
26	M	Juv	6	6	12.53	Very Light	No	Pale-H	Pale-H	Pale-B	

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	F
1	F	Adult	7	7	20.45	Yes	Yes	Pale-H	Pale-H	Brown B	
2	F	Adult	7	7	18.33	Yes	Yes	Pale-H	Pale-H	Brown B	
3	F	Adult	8	6	20.44	Yes	Yes	Pale-H	Pale-H	Brown B	
4	F	Adult	6	7	19.44	Yes	Yes	Pale-H	Pale-H	Brown B	
5	F	Adult	7	7	20.65	Yes	Yes	Pale-H	Pale-H	Brown B	
6	F	Adult	5	6	20.08	Yes	Yes	Pale-H	Pale-H	Brown B	
7	F	Adult	6	7	18.61	Yes	Yes	Pale-H	Pale-H	Brown B	
8	F	Adult	4	5	22.19	Yes	Yes	Pale-H	Pale-H	Brown B	
9	F	Juv	5	5	14.72	Yes	Yes	Pale-H	Pale-H	Pale B	
10	M	Adult	6	7	18.91	Yes	Yes	Pale-H	Pale-H	Brown B	
11	M	Adult	5	7	19.84	Yes	Yes	Pale-H	Pale-H	Brown B	
12	M	Adult	8	7	8.18	Yes	Yes	Pale-H	Pale-H	Brown B	
13	M	Sub	8	7	15.96	Yes	Yes	Pale-H	Pale-H	Brown B	
14	M	Sub	9	7	16.7	Yes	Yes	Pale-H	Pale-H	Brown B	
15	M	Sub	6	6	13.51	Yes	Yes	Pale-H	Pale-H	Brown B	
16	M	Sub	6	7	13.67	Yes	Yes	Pale-H	Pale-H	Brown B	
17	M	Sub	6	8	15.57	Yes	Yes	Pale-H	Pale-H	Brown B	
18	M	Adult	8	8	17.2	Yes	Yes	Pale-H	Pale-H	Brown B	
19	M	Sub	6	7	12.38	Yes	Yes	Pale-H	Pale-H	Brown B	
20	M	Adult	7	7	18.52	Yes	Yes	Pale-H	Pale-H	Brown B	
21	M	Sub	7	6	13.61	Yes	Yes	Pale-H	Pale-H	Brown B	
22	M	Adult	6	5	17.89	Yes	Yes	Pale-H	Pale-H	Brown B	
23	F	Adult	-	-	20.4	Yes	Yes	Pale-H	Pale-H	Brown B	
24	F	Adult	-	-	20.79	Yes	Yes	Pale-H	Pale-H	Brown B	
25	F	Adult	-	-	20.32	Yes	Yes	Pale-H	Pale-H	Brown B	
26	F	Adult	-	-	19.07	Yes	Yes	Pale-H	Pale-H	Brown B	
27	F	Adult	-	-	19.93	Yes	Yes	Pale-H	Pale-H	Brown B	

Table 10.1: Putative Hybrids between *H. thoracica* and *H. crassidens* from the Manawatu.

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Prolateral Tib Spines (L)
1	M	Adult	6	6	23.63	Yes	No	Pale-H	Brown-H	Brown-B	4
2	F	Adult	8	8	22.92	Yes	No	Paleish-H	Brown-B	Brown-B	3
3	M	Adult	7	6	24.01	Yes	No	Paleish-H	Brown-H	Brown-B	4
4	M	Adult	6	8	23.62	Yes	No	Pale-H	Paleish-H	Paleish-B	3.5
5	F	Adult	7	8	22.29	Yes	No	Paleish-H	Brown-B	Brown-B	3.5
6	M	Juv	6	7	16	Yes	No	Pale-H	Brown-B	Brown-B	4

Table 10.2: Putative Hybrids between *H. thoracica* and *H. trewicki* from Hawke's Bay.

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Prolateral Tib Spines (L)
1	M	Adult	-	-	15.18	Yes	No	Paleish-H	Brown-B	Brown-B	4

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Prolater Spine
1	F	Subadult	6	7	18.6	Yes	Yes	Brown	Brown-H	Brown-B	4
2	F	Adult	7	6	21.75	Yes	Yes	Brown	Brown-H	Brown-B	4
3	-	Juvenile	5	6	-	Yes	Yes	Brown	Brown-H	Brown-B	-
4	F	Adult	5	5	20.81	Yes	Yes	Brown	Brown-H	Brown-B	4
5	F	Adult	5	5	24.72	Yes	Yes	Brown	Brown-H	Brown-B	4
6	F	Adult	6	7	23.82	Yes	Yes	Brown	Brown-H	Brown-B	4
7	F	Subadult	5	6	17.84	Yes	Yes	Brown	Brown-H	Brown-B	4
8	F	Juvenile	7	7	11.23	Yes	Yes	Brown	Brown-H	Brown-B	4
9	M	Adult	5	6	17.66	Yes	Yes	Brown	Brown-H	Brown-B	4
10	F	Juvenile	7	5	13.65	Yes	Yes	Brown	Brown-H	Brown-B	4
11	F	Juvenile	6	7	10.52	Yes	Yes	Brown	Brown-H	Brown-B	4
12	M	Subadult	6	7	16.04	Yes	Yes	Brown	Brown-H	Brown-B	4
13	M	Subadult	7	7	17.98	Yes	Yes	Brown	Brown-H	Brown-B	4
14	F	Subadult	5	7	16.91	Yes	Yes	Brown	Brown-H	Brown-B	4
15	F	Adult	7	5	22.33	Yes	Yes	Brown	Brown-H	Brown-B	4
16	M	Juvenile	7	6	10.3	Yes	Yes	Brown	Brown-H	Brown-B	4
17	M	Subadult	7	6	15.65	Yes	Yes	Brown	Brown-H	Brown-B	4
18	F	Subadult	8	7	17.93	Yes	Yes	Brown	Brown-H	Brown-B	4
19	F	Adult	5	5	20.93	Yes	Yes	Tan	Brown-H	Brown-B	4
20	F	Juvenile	6	6	13.19	Yes	Yes	Brown	Brown-H	Brown-B	4
21	M	Juvenile	5	5	10.57	Yes	Yes	Brown	Brown-H	Brown-B	4
22	M	Juvenile	5	6	9.68	Yes	Yes	Brown	Brown-H	Brown-B	4
23	M	Adult	5	7	22.77	Yes	Yes	Brown	Brown-H	Brown-B	4
24	F	Subadult	6	5	19.5	Yes	Yes	Brown	Brown-H	Brown-B	4
25	F	Juvenile	6	5	10.08	Yes	Yes	Brown	Brown-H	Brown-B	4
26	M	Subadult	9	8	14.75	Yes	Yes	Brown	Brown-H	Brown-B	4

Code	Sex	Age	Stridulatory Ridges (L)	Stridulatory Ridges (R)	Tibia length (mm)	Bands	Stripe	Pronotum	Mesonotum	Metanotum	Prolateral Ti Spines (L)
1	F	Adult	5	6	25.71	Very light	No	Pale-H	Dark	Dark	3
2	M	Subadult	7	5	15.93	Very light	No	Pale-H	Dark	Dark	3
3	M	Subadult	7	6	20.14	Very light	No	Pale-H	Dark	Dark	3.5
4	M	Subadult	6	7	20.27	Very light	No	Pale-H	Dark	Dark	3
5	M	Subadult	6	6	17.73	Very light	No	Pale-H	Dark	Dark	-
6	M	Subadult	6	6	16.31	Very light	No	Pale-H	Dark	Dark	-
7	M	Subadult	6	7	-	Very light	No	Pale-H	Dark	Dark	-
8	M	Subadult	5	6	16.3	Very light	No	Pale-H	Dark	Dark	-
9	M	Juvenile	7	5	16.67	Very light	No	Pale-H	Tan	Tan	3
10	F	Subadult	7	7	21.43	Very light	No	Pale-H	Dark	Dark	3
11	F	Subadult	6	7	20.93	Very light	No	Pale-H	Dark	Dark	3
12	M	Juvenile	7	7	17.76	Very light	No	Pale-H	Tan	Tan	3
13	M	Adult	6	7	20.79	Very light	No	Pale-H	Dark	Dark	3
14	M	Adult	7	6	19.13	Very light	No	Pale-H	Dark	Dark	3
15	F	Juvenile	6	6	17.79	Very light	No	Pale-H	Dark	Dark	4
16	F	Juvenile	5	6	15.75	Very light	No	Pale-H	Dark	Dark	3
17	M	Adult	8	7	22.29	Very light	No	Pale-H	Dark	Dark	3
18	F	Adult	6	6	23.42	Very light	No	Pale-H	Dark	Dark	3
19	F	Juvenile	-	-	12.07	Very light	No	Pale-H	Tan	Tan	3.5
20	F	Juvenile	5	5	13.29	Very light	No	Pale-H	Dark	Dark	3
21	M	Juvenile	-	-	9.46	Very light	No	Pale-H	Dark	Dark	3
22	F	Juvenile	5	5	13.84	Very light	No	Pale-H	Dark	Dark	3

Appendix I: Extra tibia length data for Manawatu and allopatric populations (Chapter 2).

Wellington *H. crassidens*	*Manawatu* *H.crassidens*	Manawatu *H.thoracica*	Taupo *H. thoracica*
24.71	23.01	20.44	24.28
24.72	21.56	23.31	25.43
24.68	21.86	21.33	22.36
24.04	21.63	23.44	24.2
20.86	23.67	22.79	23.62
22.17	21.19	22.78	22.22
22.42		22.19	22.47
22.79		23.44	23.88
21.92		22.76	22.55
22.31		22.35	
21.02		23.63	
22.84			
22.14			
22.23			

	1	4	10	12	13	16	21	22	28	30	31	33	34	40	42	46	49	55	58	61	64	67	70	73
A	G	G	T	G	T	A	G	G	G	T	T	T	G	A	C	G	G	A	G	G	A	A	A	A
B	G	G	T	G	T	A	G	G	G	T	T	T	G	A	C	G	G	A	G	G	A	A	A	A
C	A	A	T	G	A	C	A	T	T	A	G	T	A	T	A	G	T	T	G	A	A	G	G	G
D	A	A	T	G	A	C	A	T	T	A	G	T	A	T	A	G	T	T	G	A	A	G	G	G
E	A	A	T	A	A	C	A	T	A	A	T	C	A	T	A	G	T	T	A	G	A	G	A	G
F	A	A	T	A	A	C	A	T	A	A	T	C	A	T	A	G	T	T	A	G	A	G	A	G
G	A	A	C	A	A	C	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G
H	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G
I	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G
J	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G
K	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G
L	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	A	T	T	A	G	G	G	A	G
M	A	A	C	A	A	T	A	T	A	A	C	C	A	T	A	G	T	T	A	G	G	G	A	G

106	109	124	127	132	133	142	143	144	151	159	166	169	172	175	178	180	181	184	192	196	205	208	210	211
G	G	A	A	A	G	G	G	C	G	G	A	G	G	A	A	G	C	G	G	A	A	A	G	G
G	G	A	A	A	G	G	G	C	G	G	A	G	G	A	A	G	C	G	G	A	A	A	G	G
A	A	T	T	G	G	G	G	T	A	A	G	A	A	G	T	A	T	T	A	A	A	C	A	A
A	A	T	T	G	G	G	G	T	A	A	G	A	A	G	T	A	T	T	A	A	A	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	G	A	A	G	C	A	C
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	G	A	A	G	C	A	C
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	T	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A
A	G	T	T	G	A	A	A	T	A	A	A	A	A	A	T	A	C	C	A	C	G	C	A	A

247	250	256	262	268	271	280	286	289	292	295	300	301	307	310	315	316	319	322	325	330	331	334	34
G	G	G	A	G	G	G	A	A	G	G	G	A	A	G	G	G	C	G	A	G	T	T	T
G	G	G	A	G	G	G	A	A	G	G	G	A	A	G	G	G	C	G	A	G	T	T	T
T	A	A	G	T	A	A	G	A	A	A	T	T	G	G	A	C	T	T	G	G	C	C	C
T	A	A	G	T	A	A	G	A	A	A	T	T	G	G	A	C	T	T	G	G	C	C	C
T	G	A	G	T	A	A	A	G	A	A	T	T	G	A	A	T	T	T	G	A	T	C	C
T	G	A	G	T	A	A	A	G	A	A	T	T	G	A	A	T	T	T	G	A	T	C	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	C	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C
C	A	A	G	T	A	G	A	A	A	A	T	T	A	A	A	T	T	T	G	A	T	T	C

367	375	376	382	388	393	394	397	400	403	409	412	417	427	439	442	445	448	454	460	469	472	475	478
C	G	A	G	G	G	C	T	T	G	T	T	G	A	T	A	G	A	G	A	G	G	A	G
C	G	A	G	G	G	C	T	T	G	T	T	G	A	T	A	G	A	G	A	G	G	A	G
C	A	G	A	G	A	T	C	A	A	A	G	A	G	C	T	T	T	A	T	T	A	G	A
T	A	G	A	G	A	T	C	A	A	A	G	A	G	C	T	T	T	A	T	T	A	G	A
C	A	G	A	A	A	T	T	T	A	G	G	G	G	T	T	T	T	A	T	T	A	A	A
C	A	G	A	A	A	T	T	T	A	G	G	G	G	T	T	T	T	A	T	T	A	A	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A
T	A	G	G	A	A	T	C	T	A	A	G	G	G	T	T	T	T	A	T	T	A	G	A

508	514	517	520	526	529	532	533	535	538	540	544	550	553	555	559	562	571	574	576	577	580	586	595
G	G	G	T	T	A	G	A	T	T	G	T	G	T	G	T	A	A	A	G	T	G	G	T
G	G	G	T	T	A	G	A	T	T	G	T	G	T	G	T	A	A	A	G	T	G	G	T
A	G	G	G	T	G	A	A	T	T	A	A	T	T	A	A	A	T	T	A	A	G	A	T
A	G	G	G	T	G	A	A	T	T	A	A	T	T	A	A	A	T	T	A	A	G	A	T
A	G	A	G	C	G	A	A	C	T	A	A	G	C	A	A	A	T	T	G	G	A	A	C
A	G	A	G	C	G	A	A	C	T	A	A	G	C	A	A	A	T	T	G	G	A	G	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	T	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C
A	A	A	G	T	G	A	A	T	C	A	A	G	T	A	A	G	T	T	G	A	A	A	C

Appendix K: Alleles for nuclear sequences (Chapter 3).

Table 15.1: Alleles for sperm flagella protein locus.

	34	74	86	155	157	187	206
A	T	A	G	T	C	G	G
B	T	T	G	T	C	G	G
C	T	T	T	C	C	G	G
D	T	T	G	C	C	G	G
E	T	T	G	C	C	T	G
F	T	T	G	T	T	G	G
G	C	T	G	T	C	G	G
H	T	T	G	T	C/T	G	T

Table 15.2: Alleles for testis kinase 1 locus.

	31	40	62	78	98	156	194	195	200	206	234
A	A	G	G	T	T	A	T	T	A	C	A
B	A	G	G	T	T	A	G	T	A	C	A
C	A	G	G	T	A	A	T	T	G	C	G
D	A	G	G	G	T	A	T	T	A	C	A
E	A	G	G	T	T	C	T	T	A	C	A
F	A	A	G	T	T/A	A/C	T	T	A/G	C	A/G
G	A	G	A	G	T	A	T	T	A	C	A
H	A	G	G	T	A	A	T	G	G	C	G
I	G	G	G	T	T	A	T	T	A	C	A
J	A	G	G	G	T	A	T	T	A	T	A

Appendix L: Karyotype and genetic information for all individuals and populations found in Chapter 3.

Code	Location	Karyotype	mtDNA	Sflag	Testis_kin_1	HR12	HR13A	HR35	Hma04	HR14	HR43
Ak1	Akitio	NA	*H. crassidens**	D	D/G	184	170	233/244	88	183	103
AK2	Akitio	NA	*H. crassidens**	D/F	D/G	184	170	253	88	166	103
AK3	Akitio	NA	*H. crassidens**	D	D	184	177/170	224/240	88	183	103
AK4	Akitio	NA	*H. crassidens**	F	D	184	170	224/253	88	166	103
AK5	Akitio	NA	*H. crassidens**	D/F	D	184	177/170	231/250	88	166/183	103
AK6	Akitio	NA	*H. crassidens**	D/F	D	184	170	231	88	166/183	103
AK7	Akitio	NA	*H. crassidens**	B/F	D	179	177/170	224/253	88	166/183	103
AK8	Akitio	NA	*H. crassidens**	D/F	D/G	184/187	177/170	233/242	88	183	103
AK9	Akitio	NA	*H. crassidens**	D/F	D	179	170	224	88/95	166/183	103
AK10	Akitio	NA	*H. crassidens**	D/F	D	184	170	224	88	183	103
Ot1	Otaki	NA	*H. crassidens**	F	G	184	?	224	88	183	114
Ot2	Otaki	NA	*H. crassidens**	B	D	184	166/187	224/227	88	183	114
Ot3	Otaki	NA	*H. crassidens**	B/F	D/G	179	166	224/236	88	183	114
Ot4	Otaki	NA	*H. crassidens**	B	D	179/184	187	240/244	88/90	168/183	114/127
Ot5	Otaki	NA	*H. crassidens**	B	?	184	?	224	88	183	114
Ot6	Otaki	NA	*H. crassidens**	B/F	D/G	184	166/187	240	90	183	114/127
Ot7	Otaki	NA	*H. crassidens**	B/F	D	184	187	240/242	88	183	114
Ot8	Otaki	NA	*H. crassidens**	B/F	D	179	187	247	88	183	114/127
Ot9	Otaki	NA	*H. crassidens**	B	D	184	187	236/262	88/90	168/183	114
Ot10	Otaki	NA	*H. crassidens**	B	D/G	184	?	240	88	183	114
C1	Manawatu	Crass	I	B/D	D	184	?	227/274	88/92	168	114/127
C2	Manawatu	Crass	G	D/G	D/G	184	?	258/270	88	168	114
C3	Manawatu	Crass	F	B	D/G	179/184	?	224	88	168	114/127
C4	Manawatu	Crass	E	D/G	D	184/187	168	224/244	88	168	114
C5	Manawatu	Crass	I	B	D	184	?	227	88	168	114
C6	Manawatu	NA	G	B/D	D	184	168	224	88	168	114/127
C7	Manawatu	Crass	H	B/F	D/G	184/186	168	227/250	88	168	114/125
C8	Manawatu	Crass	H	B/D	D	184	168	230/242	88	168	114
C9	Manawatu	Crass	E	B/F	D/G	179/184	187	224/233	88	168	114/127
C10	Manawatu	Crass	E	B/D	D	184	168	224	88	168/183	114/127
C11	Manawatu	Crass	E	B	D	184	168	240/242	88	168/183	114/127
C12	Manawatu	Crass	G	D/G	D	184	162/168	224/247	88	168	114/127
C13	Manawatu	NA	H	B/H or F/H	D/G	184	?	224/227	88	168	114/127
C14	Manawatu	NA	*H. crassidens**	B	D	184	?	224	88	168	114
C15	Manawatu	NA	*H. crassidens**	B	D	184	?	227/244	88	168	114/127
C16	Manawatu	NA	*H. crassidens**	B/D	D	184	168	230/258	88	168	114/127
C17	Manawatu	NA	*H. crassidens**	B/G	G/J	184	168	224/253	88	168	114/127

C18	Manawatu	NA	*H. crassidens**	B/D	G/J	184	168	228/244	88	168	114
C19	Manawatu	NA	*H. crassidens**	D	D/G	184	168	224/258	88	168	114/127
C20	Manawatu	NA	*H. crassidens**	B	D	184	?	224	88	183	114/127
C21	Manawatu	NA	*H. crassidens**	B	D/G	184	168	247/265	88/92	168	114
C22	Manawatu	NA	*H. crassidens**	?	D	184	162/164	228/254	88	?	114/127
C23	Manawatu	NA	*H. crassidens**	B	D/J	184	?	230/262	88	168	114/127
Hybrid 1	Manawatu	NA	I	B/D	A/D	184/186	174	224/242	80/88	183	114/127
Hybrid 2	Manawatu	NA	A	D	A/D	184/186	168/177	224/247	80/88	168/183	114
Hybrid 3	Manawatu	F1	L	D	A/D	184/186	177	239/247	80/88	183	114
Hybrid 4	Manawatu	?	H	B/C	A/D	184/186	177	244/270	80/88	183	114/127
Hybrid 5	Manawatu	?	M	D	A/D	184/186	168/177	244/246	80/88	183	114
Hybrid 6	Manawatu	?	K	D/F	A/D	184/189	174	233/242	80/88	168/183	114
Hybrid 7	Manawatu	NA	*H. crassidens**	B/D	A/D	184/186	168/172	242/250	80/88	168/183	114
Hybrid 8	Manawatu	NA	J	B/D	A/D	184/186	177	224/244	80/88	168	114
Hybrid 9	Manawatu	NA	I	B/D	A/D	184/186	168/174	244/260	80/88	183	114
Mt1	Manawatu	Thor	A	D	C/F or E/F	186/188	172	244	80	183	114
Mt2	Manawatu	Thor	?	D	A	186	174/177	242	80	183	114
Mt3	Manawatu	Thor	A	D/E	A	186	174/177	242/244	80	183	114
Mt4	Manawatu	Thor	A	D	A	186?	174	244	80	183	114
Mt5	Manawatu	Thor	A	D	A	186	177	244/247	80	183	114
Mt6	Manawatu	Thor	?	C/E	A	186	174/177	242/247	80	183	114
Mt7	Manawatu	Thor	A	D	A	186	172/174	244	80	183	114
Mt8	Manawatu	Thor	?	C/D	A/E	186	174	244/247	80	183	114
Mt9	Manawatu	Thor	?	D	A	186	174/177	244	80	183	114
Mt10	Manawatu	Thor	?	D	A/K	186	177	244/247	80	183	114
Mt11	Manawatu	NA	?	D	A	186/188	174	242/244	80	183	114
Mt12	Manawatu	NA	*H. thoracica**	D	A	186	179	244	80	183	114
Mt13	Manawatu	NA	*H. thoracica**	D/E	A/E	166/188	172/174	244	80	183	114
Mt14	Manawatu	NA	*H. thoracica**	D	A	186	177/179	244	80	183	114
Mt15	Manawatu	NA	*H. thoracica**	D	A	186	174/177	244/250	80	183	114
Mt16	Manawatu	NA	*H. thoracica**	D	A	186	174/177	244/250	80	183	114
Mt17	Manawatu	NA	*H. thoracica**	?	?	186	156/184?	242/247	80	?	114
Mt18	Manawatu	NA	*H. thoracica**	D	A	186	174	244	80	183	114
Mt19	Manawatu	NA	*H. thoracica**	C/D	A/I	186	174/177	242/244	80	183	114
Mt20	Manawatu	NA	*H. thoracica**	D	A	186	177	242/244	80	183	114
Mt21	Manawatu	NA	*H. thoracica**	D	A	186	174	244	80	183	114
Mt22	Manawatu	NA	*H. thoracica**	D	A	186	177	242/244	80	183	114
Hin1	Taupo	NA	*H. thoracica**	C/D	C	186	182	244/247	80	183	114
Hin2	Taupo	NA	*H. thoracica**	C/D	C	186	182	244	80	183	114
Hin3	Taupo	NA	*H. thoracica**	D	A/C	186	182	244	80	183	114
Hin4	Taupo	NA	*H. thoracica**	D	C	186/188	174	244/247	80	183	114
Hin5	Taupo	NA	*H. thoracica**	D	A/C	186	174	244/247	80	183	114
Hin6	Taupo	NA	*H. thoracica**	D	C	186	182	244	80	183	114

Hin7	Taupo	NA	*H. thoracica**	D	C	186	182	244/247	80	183	114
Hin8	Taupo	NA	*H. thoracica**	C/D	C/H	186	182	244	80	183	114
Hin9	Taupo	NA	*H. thoracica**	D	C	186	174	244	80	183	114
Hin10	Taupo	NA	*H. thoracica**	D	C	186	182	244/247	80	183	114
Ht1	Hawke's Bay	Thor	B	C/D	A/C	186	174	244	80	183	114
Ht2	Hawke's Bay	Thor	B	E	A	186	174	244	80	183	114
Ht3	Hawke's Bay	Thor	B	D	A	186/188	174/176	244	80	183	114
Ht4	Hawke's Bay	Thor	?	D	C/K	186	174/179	244	80	183	114
Ht5	Hawke's Bay	Thor	B	C/D	A	186	174/179	244	80	183	114
Ht6	Hawke's Bay	Thor	?	C/D	A/C	186	174	244	80	183	114
Ht7	Hawke's Bay	Thor	?	C/D	A	186/188	174/179	244	80	183	114
Ht8	Hawke's Bay	Thor	B	C/D	A/C	186/188	174	244	80	183	114
Ht9	Hawke's Bay	Thor	B	D/E	A	174/186	174	244	80	183	114
Ht10	Hawke's Bay	Thor	?	C	A	186	174	244	80	183	114
Ht11	Hawke's Bay	Thor	B	C	A/C	186	174	244	80	183	114
Ht12	Hawke's Bay	Thor	B	C	A/C	186/188	174/179	244	80	183	114
Hybrid 1	Hawke's Bay	F1	?	A/D	A/C	186/196	170/179	244/250	80/88	183	114
K1	Hawke's Bay	Tre	C	A/B	A	184/196	170	244/258	88	183	114
K2	Hawke's Bay	Tre	D	A	A	196	170	247/255	88/90	183	114
K3	Hawke's Bay	Tre	C	A	A	196	162/170	235/244	90	168/183	114
K4	Hawke's Bay	Tre	C	A	A/B	196	170	244/255	88	183	114
K5	Hawke's Bay	Tre	D	A	A	196	170	235/244	88	168/183	114
K6	Hawke's Bay	Tre	?	A	A	196	170	244	88	168/183	114
K7	Hawke's Bay	Tre	C	A	A	184/196	170	235/253	88	168/183	114
K8	Hawke's Bay	Tre	C	A	A	196	162/170	233/255	88	168/183	114
K9	Hawke's Bay	Tre	C	A	A	196	170	244/253	88/90	168	114
K10	Hawke's Bay	Tre	?	A	A	196	162/170	235/244	88	183	114

*Determined in a previous study.