Concerted Evolution of the rDNA ITS-1 in Two Caddisfly Species

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Abstract

The ribosomal RNA (rRNA) gene family shows high levels of within-species homogeneity and between-species diversity. This pattern of mutation distribution among gene family members is termed concerted evolution. Molecular drive is a process by which mutations spread through a gene family (homogenization) and through a population (fixation) as a consequence of non-Mendelian DNA transfer within and between chromosomes. The fast evolving internal transcribed spacers (ITS) of the rDNA are considered to be homogenized within species and within genetically isolated populations. However, recent studies question the dogma of homogenization and caution the use of ITS in phylogenetic and population studies.

Two presumed sister species (*Triplectides obsoleta* and *T. dolichos*) were chosen for an investigation of ITS-1 variation. ITS-specific PCR primers were developed that amplified products homologous to a range of other insects. In a pilot study, variation in a single individual *T. obsoleta* was found among eight ITS-1 clones but not in ITS-2. Therefore, ITS-1 was chosen for a survey of the nature of the variation, testing the extent of heterogeneity in these species, the types of coevolutionary changes taking place in this region, and its usefulness as a population genetic marker.

This survey required an hierarchical approach, sampling multiple ITS-1 copies from within and among individuals. 203 ITS-1 clones were screened for conformational differences by single-strand conformation polymorphism (SSCP) analysis of restriction digested fragments. Some ITS-1 variants were present at a higher frequency than others in all 17 individuals tested. All variants derived from independent PCR reactions were sequenced. The type of DNA sequence variation was very similar in both species though *T. dolichos* was

more variable. A majority of the variation appears to have resulted from replication slippage and the remainder by point mutation. Repeat DNA and an A+T bias were found in the ITS-1; a characteristic of cryptically simple non-coding DNA. Extensive ITS-1 heterogeneity was present and parsimony analysis for both species showed the absence of population-specific variation. ITS-1 variants common in some individuals were rare in others, even within the same population. Neither complete homogenization nor fixation of the ITS-1 occurs in these species. This precludes its use as a population genetic marker in these species and cautions against the assumption of homogenization.

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1.1 Introduction

Concerted evolution

Eukaryotic (and some archaebacterial) genomes contain substantial numbers of multiple-copy families of genes and non-coding sequences. It has been apparent for some years that many such families exhibit unexpected sequence homogeneity within and between individuals of a species (Dover, 1982; 1986). These repeat DNA families have high levels of within-species homogeneity and between-species heterogeneity irrespective of their copy-number, function and chromosomal distribution. This pattern of sequence variation is known as concerted evolution (Polanco et al., 1998; Zimmer et al., 1980), although it has previously been called horizontal evolution (Brown et al., 1972) and coincidental evolution (Hood et al., 1975). The importance of concerted evolution lies primarily with our understanding of how the great many repeated DNA sequences of the eukaryotic genome evolve.

Concerted evolution was first demonstrated in the ribosomal RNA gene family of two species of African toad, *Xenopus*, by Brown et al. (1972). This, and subsequent studies (e.g., Coen et al., 1982b; Williams et al., 1985; Bowen & Dover, 1995) demonstrated that the individual repeat members of a multigene family evolve in concert rather than independently. On the surface this suggests a process of parallel evolution for all members of a gene family. However, Coen et al. (1982b) state that, "concerted evolution is an observation of family

homogeneity that is an outcome of processes in the genome that are able to progressively fix a variant member in the family." Through genetic interactions among its members, multiple-copy families of genes and non-coding sequences evolve in a concerted fashion, seemingly as a single unit or locus.

Concerted evolution challenges both conventional neutralist and selectionist evolutionary explanations. Neither the data nor their implications can be squeezed into the classical mould of a two-party (selection versus drift) state (Dover, 1986). Under genetic drift and selection, individual gene family members are expected to evolve independently by mutation, gradually diverging from one another over time. If each member of a family is free to accumulate independent mutations over time then the coefficient of identity between any two members within a species would equal that of any two members chosen at random from two different species (Dover, 1982; Ridley, 1993). However, mutations do not occur independently in the different members of a family in neither the gene-coding nor, more unexpectedly, non-coding regions of gene families and the coefficient of identity is higher within than between species.

Negative (purifying) selection is thought to impede the spread of new mutations in the functionally important (gene-coding) regions of a gene family, because of their potentially negative effect on an individual's fitness (Wheeler & Honeycutt, 1988). This argument though does not explain within-species homogeneity found in the non-coding regions of a gene family. Intraspecific homogeneity is maintained not by selective constraints but by processes which can spread mutations in a repeat unit 'horizontally' to other members of the family (Li, 1997).

DNA turnover processes and molecular drive

Molecular drive is the process where mutations spread through a repeat family within an individual (homogenization) and through a sexually reproducing population (fixation) as a consequence of non-Mendelian DNA transfer within and between chromosomes. Homogenization and fixation are inextricably linked because chromosomes are continually being shuffled and assorted into new combinations at each generation through sex (Dover, 1986). The generative processes involved include; gene conversion (Eldeman & Gally, 1970; Nagylaki & Petes, 1982), unequal crossing-over (UCO) (Smith, 1973; Ohta, 1976), replication slippage (slipped-strand mispairing), transposition and RNA-mediated exchange (Dover, 1982; 1986). Of these, gene conversion, UCO and replication slippage are considered to be the most important. For recent reviews see Elder and Turner (1995) and Li (1997).

Gene conversion is a non-reciprocal recombination process where one allele is converted to the other (Li, 1997). It is thought gene conversion results from DNA repair after invasion of a double helix of one allele by a single strand of the helix of another, immediately after chromosome replication. Gene conversion changes allele frequencies and any bias in the DNA repair leads to the progressive and accelerated fixation of an allele (variant) (Dover, 1982; Li & Graur, 1991). UCO is a reciprocal recombination process that creates a sequence duplication in one chromatid or chromosome and a corresponding deletion in the other (Li & Graur, 1991). It is an interhelical event that can only take place during chromosome alignment in cell division (Levinson & Gutman, 1987). Continual UCO has the effect of expanding and contracting the copy number of a family and can lead to the stochastic fixation of any one variant. Replication slippage involves local denaturation and displacement of DNA strands (of a single DNA duplex) followed by base mispairing at a site containing a tandem repeat sequence. The consequence of mispairing, when followed by replication or DNA repair, is an insertion or deletion of one or several of the tandem

repeats (Levinson & Gutman, 1987). Slipped-strand mispairing is perhaps a better term because it also occurs prior to cell division, in non-replicating DNA.

These processes of DNA turnover are analogous to the turnover of new for old banknotes (Dover, 1982). They cause a continual non-Mendelian gain or loss of genetic material during mitosis and meiosis, involving 'old' variation (nucleotide sequences already present in the genome) and/or 'new' variation, such as point mutation. Although it defies the paradigm that cell division is the necessary event for point mutation and DNA turnover, both have been demonstrated in resting cells (Bridges, 1997). DNA turnover is a larger mutational event but does not involve the creation of new variation, just changes in its frequency. DNA turnover spreads variation at a significantly higher rate than can be generated by point mutations alone (Hoelzel & Dover, 1991). The balance between the spreading consequences of DNA turnover and the accumulation of point mutations is manifest as a degree of homogeneity found among the multiple copies of repeat DNA sequences.

C-value paradox and selfish DNA

The lack of correspondence between the amount of DNA in the haploid genome (C value) and the amount of gene-coding DNA present has become known as the C-value paradox. Solving the C-value paradox and accounting for the repeated structure of the eukaryotic genome requires an evolutionary mechanism for the long-term maintenance of vast quantities of nongenic (non-coding), seemingly superfluous DNA (Li & Graur, 1991). This functionless non-coding DNA, often called 'junk DNA' (Ohno, 1972) or 'selfish DNA' (Orgel & Crick, 1980; Doolittle & Sapienza, 1980), has the property of forming additional copies of itself while making no contribution to the phenotype. This lack of phenotypic expression means that normal phenotypic (Darwinian) selection cannot explain its continued presence, as junk DNA contributes little or nothing to an organism's fitness.

Dover and Doolittle (1980) suggested there are constraints that impose a type of selection on these sequences, not necessarily as a result of their phenotypic effects but as a consequence of the molecular processes of replication and recombination. The generative processes of molecular drive, that is, DNA turnover, provide an explanation for the presence of repeated sequences (regardless of their function) and hence much of the structure of the eukaryotic genome.

1.2 The nuclear ribosomal RNA gene family

Structure and function

The ribosomal RNA (rRNA) genes, and their associated spacer regions, are collectively called ribosomal DNA (rDNA) (Hillis & Dixon, 1991). They exhibit the pattern of concerted evolution typical of other gene families. Ribosomal RNA has a primary functional role in most if not all stages of protein synthesis (Dahlberg, 1989). The rDNA is composed of multiple copies of a repeating unit. This includes the highly conserved gene-coding rRNA molecules which are synthesized by RNA polymerase I, before being processed to yield mature rRNA, and several fast evolving non-coding spacers (see figure 1.1). These rRNAs combine with ribosomal proteins to form ribosomes, the organelles that direct protein synthesis from messenger RNA. The entire rDNA intergenic spacer (IGS) acts as an enhancer for ribosomal transcription by polymerase I (Mougey et al., 1996), and the other spacers (ETS, ITS-1 and ITS-2) are necessary for pre-rRNA processing (Musters et al., 1990).

The rDNA of a eukaryote nuclear genome is typically clustered in one or a few tandem arrays per haploid chromosome set. The length of the transcribed unit for most eukaryotes is about 8 kb, while the length of the intervening IGS can vary considerably, e.g., from 3.5 to >20 kb in a hybrid *Neotyphodium* fungi (Ganley & Scott, 1998) and 7.2 to 20.5 kb in the greenbug, *Schizaphis graminum*

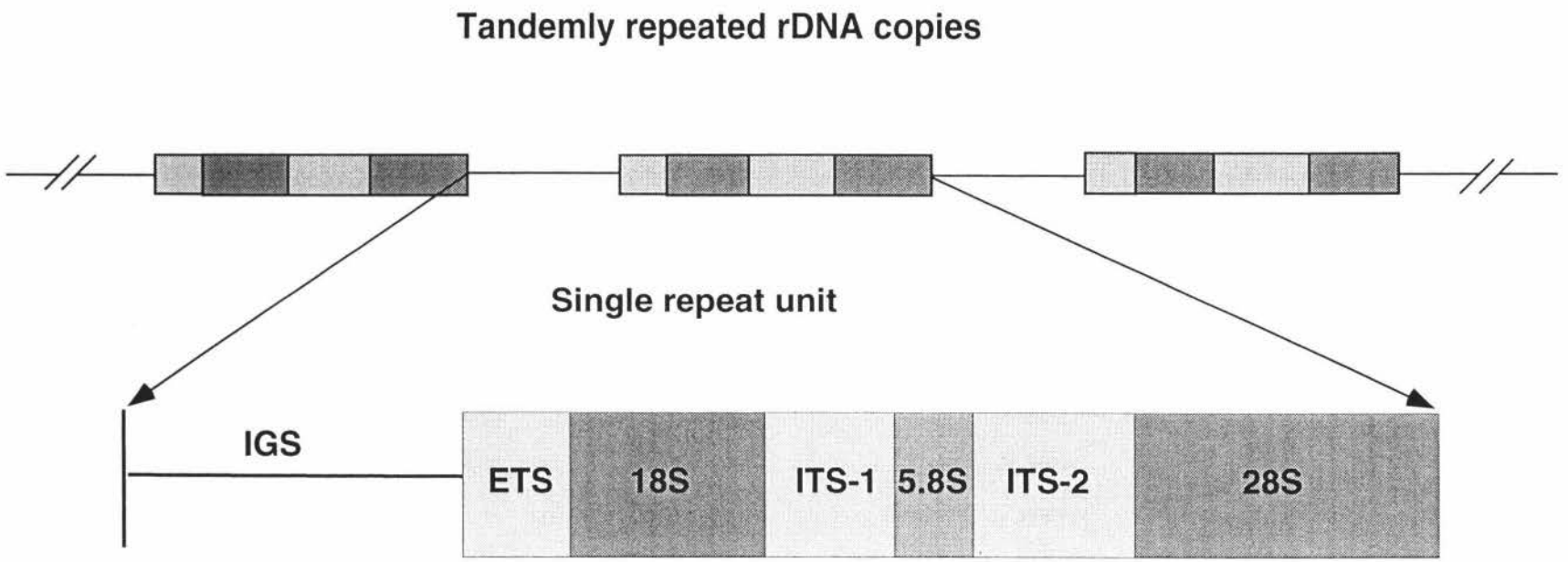

Figure 1.1 The ribosomal RNA gene family is clustered into a group of tandemly repeated units (copies) in a head-to-tail fashion. Three of these are pictured above and a single unit below it. A single unit contains both gene-coding and non-coding regions: IGS = intergenic spacer, ETS = external transcribed spacer, 18S, 5.8S and 28S = rRNA genes, and ITS-1 and ITS-2 = the first and second transcribed spacers, respectively.

(Shufran et al., 1997). The number of gene copies also varies considerably among taxa. Within insects it is common to find several hundred copies present per haploid genome and several thousand in plants (Long & Dawid, 1980).

Variable regions

The terms 'variable region' and 'expansion segment' are both used to describe regions varying between species in both length and content, which lie between regions of conserved sequences (Wesson et al., 1992). In general, the conserved regions of the rDNA are gene-coding and share greater amounts of sequence homology between species than do the variable regions. Variable regions include the non-coding spacers and the expansion segments of 18S and 28S rDNA. Expansion segments are separated by conserved 'core' regions, and may be functionless structures which are tolerated only because they do not interfere with ribosome function (Gerbi, 1985).

The redundancy of rDNA spacers

The rates of change within the different regions of the rDNA unit are thought to differ as a consequence of functional constraints. Non-coding spacers such as the internal transcribed spacer (ITS) of the rDNA are generally considered to be under low functional constraint (Schlotterer et al., 1994), and therefore free to accumulate mutations. These spacers could be viewed as 'junk DNA', perhaps redundant entities hitch-hiking their way in the genome aboard the gene-coding rDNA. However, studies involving DNA sequence comparisons and secondary structure analyses have shown that variation in these regions is non-random, perhaps indicating some functional constraint (see Schlotterer et al., 1994; Wesson et al., 1992; Linares et al., 1994). These variable regions provide an opportunity to study the types of coevolutionary changes taking place in gene families, and the dynamics of their establishment (Tautz et al., 1988).

1.3 "Variation is Evolution"

The idea of variation as random

> "Variation, whatever may be its cause, and however it may be limited, is the essential phenomenon of evolution. Variation, in fact, is evolution. The readiest way, then, of solving the problem of evolution is to study the facts of variation" (Bateson, 1894).

It is commonly thought that the action of an external force (natural selection) and the random fluctuation of gene frequencies (genetic drift) segregates variation in both space and time. The dynamics of natural selection and genetic drift are modelled on the premise that mutations are unitary and passive events (Dover, 1982). Without the sifting mechanism of selection acting on what is assumed to be random variation, we reason there would be nothing but incoherent disorder (Kauffman, 1995). This 'sifting mechanism' is thought to create order from what would otherwise be an unstructured world. Unfortunately, the neo-Darwinian concept of random variation carries with it the major fallacy that everything conceivable is possible (Ho & Saunders, 1984). It is important to understand just how entrenched the idea of chance and random variation is in biology.

> "We say that these events [mutations] are accidental, due to chance. And since they constitute the *only* possible source of modifications in the genetic text, itself the *sole* repository of the organism's hereditary structures, it necessarily follows that chance *alone* is at the source of every innovation, of all creation in the biosphere. Pure chance, absolutely free but blind, at the very root of the stupendous edifice of evolution: this central concept of modern biology is no longer one among other possible or even conceivable hypotheses. It is today the *sole* conceivable hypothesis, the only one compatible with observed and tested fact. And nothing warrants the supposition (or the hope) that conceptions about this should, or even could, be revised" (Monod, 1972). [emphasis in original.]

Chance; the happening of events *without any cause* that can be seen or understood (Hornby, 1970). Monod's dogmatic quote is the expression of a neo-Darwinian view that evolution, that is, the origin and maintenance of variation is solely a chance driven process, immune to any causal explanation. Chance and randomness are concepts regularly used in neo-Darwinism to cover ignorance (Lima-de-Faria, 1988). Although contingency plays an important role, our understanding of evolution need not be restricted to probabilistic models. A great deal of variation is non-randomly distributed in time and space, and can only be understood in the light of real demonstrable biological processes (generative processes) which order and segregate genetic variation.

Cairns et al. (1988) were able to generate gene mutations in cultures of bacteria in a seemingly directed fashion. The main purpose of their paper was to show how insecure our belief is in the spontaneity (randomness) of most mutations, it seems to be a doctrine that has never been properly put to the test (Cairns et al., 1988). The phenomenon of compensatory mutations that preserve base pairing in RNA molecules provides another example of the existence of a process that can generate non-random mutation (variation). Given that an RNA sequence can be copied back into DNA, a proportion of DNA mutations must also arise through natural error-prone copying involving RNA intermediates rather than in a haphazard, mysterious fashion (Steele et al., 1998).

1.4 Interpreting the variation

A problem of explanation

It is the task of science to discover which hypothetical or imagined processes are not imaginary but real; or, to put it the other way, to discover what the processes are, and to produce an adequate account of them (Bhaskar, 1978). From an idealist (e.g. neo-Darwinian) perspective, there is an inclination that building a plausible narrative explanation into a model, which is then

empirically tested, is a sufficient explanation. Lambert (1995) contends "by this we create a kind of 'taxonomy of knowledge,' that is, a system for the organization of what we know [and in] using this procedure we can potentially explain any example."

From a realist perspective a distinction is made between observable patterns and the generative processes responsible for their production (Webster, 1984). The failure of neo-Darwinism to make this distinction perhaps brought about the necessity for a new evolutionary theory to understand the intraspecific homogeneity of repeat DNA families. In the theory of molecular drive, as outlined by Dover (1982) and Ohta and Dover (1984), the distinction is made between the observed pattern of concerted evolution and the DNA turnover processes that generate repeat sequence homogeneity.

Processes and models

Genetics, and in turn, population genetics, forms the conceptual basis of neo-Darwinism. Through population genetics, neo-Darwinism claims a monopoly in explaining the processes of evolution (Lovtrup, 1976, 1977). Despite the sophistication of the mathematics of natural selection and genetic drift, there are relatively few experimental proofs of the genetic processes inherent in these models (Dover, 1982). Underlying this may be a confusion between the value of a model of evolution over generative processes. From an idealist perspective however, it is only necessary to establish that such processes might exist. For the idealist, a process is imaginary, for the realist it may be real, and come to be established as such (Bhaskar, 1978).

It is considered that population genetic models greatly sharpen our understanding of the population dynamics of concerted evolution (Li, 1997). The main aim of these models is to obtain probabilities of identity by descent (i.e., genetic identities) under a variety of assumptions and constraints.

However, the genealogies produced by such models restrict our interpretation of evolution to that of a historical narrative. This is further restricted because the evolutionary genetics of molecular drive, like that of Mendelian populations under selection and drift, can be modelled only in general terms (Ohta & Dover, 1984). The models themselves do not provide an adequate rationale for the use of laws to explain phenomena in open systems or the experimental establishment of that knowledge in the first place (Bhaskar, 1978). This can only be achieved by investigation of the underlying generative processes.

Homology

Phylogenies for systematics and population studies require a model of evolution that contains an inherent assumption of identity by descent. A common ancestor and independent character change are necessary conditions for reconstructing phylogenies. Concerted evolution is considered advantageous for phylogeny reconstruction as it produces almost completely homogenized gene families (Sanderson & Doyle, 1992). However, multigene families arise by gene duplication from a single 'ancestral gene', and this duplication creates a set of unique problems regarding the accuracy and reconstruction of organismal phylogenies.

Homology in gene families is complicated by gene duplication events. This necessitates the elaboration of the concept of homology to include orthology (derivation by speciation; shared by descent) and paralogy (derivation by duplication) (Patterson, 1988). If one is interested in reconstructing the phylogenetic history of taxa by inferring relationships among genes contained in those taxa, then it is usually necessary to examine orthologous genes (Hillis, 1994). Paralogy is not considered a problem with multigene families because the mechanisms of DNA turnover are thought to homogenize all members within a family, such that each member has a nearly identical sequence. The whole family is treated as a single character and the non-independence of the

individual members is nullified by concerted evolution.

The dogma of homogenization

Williams et al. (1988), and more recently, Rich et al. (1997) suggest that the dogma of rDNA gene family homogenization is overstated. The assumption of Hillis and Davis (1988) that a high level of gene family homogenization within species is reached in only short periods of evolutionary time, may have tempted workers to under-sample the amount of variation present. Recent studies however, (Wesson et al., 1992; Volger & DeSalle, 1994; Tang et al., 1996; Rich et al., 1997) have shown that rDNA homogeneity can no longer be assumed. This is especially important for population studies, where intra-individual variation can be as high as variation in the entire species (e.g., Volger & DeSalle, 1994).

1.5 A study of ITS-1 variation in two caddisflies

Caddisflies

The Trichoptera (caddisflies) are a holometabolous order of insects, with egg, larval, and pupal stages generally living in freshwater habitats and with adults in terrestrial habitats (Morse, 1997). Caddisflies are ubiquitous in both still and running freshwater environments. They are of considerable ecological importance and form a major part of freshwater communities. They are also used as bio-indicators in water quality monitoring because many caddisfly species tolerate only a narrow range of abiotic conditions.

Triplectides

The stick caddis; *T. obsoleta* (McLachan, 1862), *T. dolichos* (McFarlane & Cowie, 1981), and *T. cephalotes* (Walker, 1852) constitute the three endemic *Triplectides* species of New Zealand. They are common aquatic insects and are found throughout New Zealand. The common name refers to the habit of larvae to use a hollowed-out twig as a portable case. The larvae eat detritus (mainly leaves

and wood) and appear as slowly moving sticks in freshwater habitats. These species have distinct larval habitat preferences: *T. obsoleta* prefers slow flowing waters of lowland streams and rivers, *T. dolichos* prefers pools in small mountain streams (headwaters) and seepages, while *T. cephalotes* prefers lakes and backwaters of larger rivers.

T. obsoleta and *T. dolichos* are presumed sister species. Both species are morphologically very similar, a fact that only lead to the 'discovery' of *T. dolichos* in 1981. Both are large insects as adults (~2 cm in length) that can be easily recognised in the field among a mass of other nocturnal insects that are attracted to UV-light traps. Trap records of these species provide good data of their geographic distribution. Although common, there are several indications that their dispersal away from water is restricted and hence, they may be likely to have genetically structured populations (Collier & Smith, 1996; Jackson & Resh, 1992).

Panmixus

If the dispersal ability of a species is extreme, a state of reproductive panmixus may result and genetic variation would not be restricted geographically. *T. dolichos*, in particular, is not expected to be panmictic because its habitat (headwaters and seepages) is disconnected spatially, whereas the river habitat of *T. obsoleta* is connected, at least to the level of a drainage basin. However, the Cook Strait that separates the North and South Islands of New Zealand is likely to be a barrier to dispersal for both species, as it is for many presumptive sister species pairs of caddisflies that are isolated from each other by the strait (Henderson, pers. comm).

The results of several studies (Jackson & Resh, 1992; Hughes et al., 1995, 1996; Bunn & Hughes, 1997) indicate that many aquatic invertebrate species do not represent single panmictic populations throughout their entire range, but are

subdivided regionally and sometimes even locally into a number of genetically distinct populations. Analysis of the genetic structure of larval populations of some caddisfly species suggests that in-stream movement is also limited to a small spatial scale (Bunn & Hughes, 1997). Limited inland dispersal may be due, impart, to the reproductive behaviour of individuals. Freshly emerged female caddisflies frequently fly inland following mating where they may rest until eggs mature (Ross, 1944), though after egg maturation, females always return to the water to oviposit (Kovats et al., 1996).

Collier and Smith (1996) used several kinds of traps to investigate distances travelled by adult aquatic insects into riparian zones. In their study *T. obsoleta* were caught up to 30 m and *T.* dolichos up to 80 m from the edge of three different streams. The distance measure for *T. dolichos* may be confounded by the presence of other suitable habitat (small seepages) away from these streams. These direct estimates suggest both species do not disperse widely despite having large wings. The possession of large wings does not necessarily mean that their function is for long distance dispersal. For example, Liebherr (1988) found no correlation with the degree of flight-wing development and dispersal ability among five beetle species (*Platynus spp.*). One fully winged species exhibited genetic heterogeneity of the same order as a vestigally winged species (Liebherr, 1988).

ITS-1 variation in Triplectides

Many studies of ITS-1 variation in insects have been carried out on flies (Diptera) (e.g. fruit flies: Strachan et al., 1985; mosquitoes: Wesson et al., 1992; and black flies: Tang et al., 1996), which are close relatives to caddisflies. As a result of these studies, there are now many rDNA sequences published in GenBank which enable comparisons of conserved gene-coding regions for identifying PCR products and designing PCR primers. The position of the spacer region between the rRNA molecules can also be estimated by

comparison to the rRNA secondary structure analysis of the fruit fly, *Drosophila melanogaster* (Hancock et al., 1988).

In this study, *T. obsoleta* and *T. dolichos* were assayed for ITS-1 variation in the rRNA gene family at the intra-individual and intra-population level. The purpose of the assay was to determine the extent of homogeneity in the spacer molecule, the frequency of ITS-1 variants within individuals, and the presence of any population-specific variation. This study therefore, constitutes a further test of the assumption of gene family homogenization and a test of a prediction of molecular drive, that populations should be evolving in a cohesive manner.