Biogeography and Systematics of the Tasmanian Mountain Shrimps of the Family Anaspididae (Crustacea: Syncarida)

Jane Andrew

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This thesis is submitted in fulfilment of the requirements for the degree of Master of Science, University of Tasmania, Hobart.

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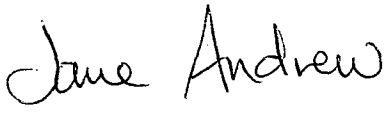

Jane Andrew, October 2004.

ABSTRACT

The family Anaspididae is a relictual Gondwanan group of malacostracan crustaceans now surviving only in cool waters and caves of Tasmania. Its most widespread species, *Anaspides tasmaniae*, is also the most morphologically plesiomorphic of the order Anaspidacea, and shows very little development since Triassic fossil anaspid forms. A number of issues relating to the systematics and taxonomy of the family Anaspididae have been raised since the first discovery of the extant species. In particular, the separate species status of *Anaspides spinulae* has been questioned. The level of genetic differentiation among populations of *Anaspides tasmaniae* has never been ascertained, but has been presumed to be high due to the long periods of isolation between populations. The relationships between the three anaspid genera are also of interest as they elucidate the early evolution of the family in Tasmania.

In order to clarify the systematics of the genus *Anaspides*, an allozyme and mitochondrial DNA study of populations of *Anaspides tasmaniae*, *A. spinulae*, *Paranaspides lacustris*, *Allanaspides helonomus* and *Allanaspides hickmani* was undertaken. *Anaspides* populations were sampled over the entire geographic range of the genus and a wide variety of habitats, including caves. Genetic affinities ascertained from the allozyme and 16S mtDNA analyses were largely congruent. The allozyme study also provided information on within-population processes and the mtDNA analysis provided confidence levels on phylogenies and molecular clock estimates for divergence times. The results showed that:

- *Anaspides spinulae* was not supported as a species separate from *A. tasmaniae.*
- Cave populations do not form a distinct genetic group despite the common loss of pigmentation. All cave populations sampled shared genotypes with the nearest epigean populations.
- Populations of *Anaspides tasmaniae* (including *A. spinulae*) were characterised by generally low levels of within-population genetic variation, with many instances of fixed differences and private alleles, and often high levels of among-population genetic differentiation.
- The genus *Anaspides* contains three distinct geographical groups all of which were supported genetically as separate species. These are found in the south (Huon area), the southwest and the Central Plateau / Derwent areas. It is proposed that these groups be considered as separate species requiring detailed description.
- Differentiation among *Anaspides* populations within the Central Plateau was significantly less than that within the southern and southwestern group. Speciation appears to be continuing in isolated populations, particularly in the southwest.

- The Central Plateau group has a core group of 8 populations, and 5 populations on the fringe of the Plateau that are more distant. An isolation by distance test found a significant correlation between inter-population structure and geographical distance.
- The southern group of *Anaspides* showed the same high level of differentiation from the other two *Anaspides* groups as from *Paranaspides*. This level of differentiation is similar to genus-level divergence in other crustacean groups. As a result, it is recommended that the southern *Anaspides* group be described as a new genus. A thorough examination of morphological characters should be undertaken in order to establish the southern *Anaspides* as a new genus.
- The emergence of *Allanaspides* was found to be the earliest divergence from the anaspid lineage, estimated at about 43 million years ago by molecular clock calculations. The separation of *Paranaspides lacustris* and the southern group of *Anaspides*, and the divergence of the two *Allanaspides* species is likely to have occurred between about 20 and 25 million years ago. The Central Plateau and southwest groups appear to have diverged approximately 10 million years ago. The estimated time at which the last common ancestor occurred within geographical groups is approximately 6 my for the southwestern group, 3.5 my for the southern group and 2 my for the Central Plateau.
- Biogeographical explanations for divergences in the family Anaspididae are suggested. These include climatic warming in the Eocene, increasing aridity in the late Oligocene and Miocene, various geological faulting and tectonic events in the Tertiary and the invasion of Tasmanian freshwaters by fish predators. Vicariant speciation in the Tertiary appears to have been the major influence on evolution in the Anaspididae, with Pleistocene glaciation and/or recent warming possibly forcing populations into cave refugia. There is evidence that glacial meltwater may have allowed some populations to mix, having a homogenising effect on genotypes, and possibly overlaying more ancient relationships between populations.

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I would like to express my gratitude to Dr Roy Swain for his excellent supervision and advice, his endless patience and cheerfulness, and for sharing with me his depth of knowledge of Tasmanian crustaceans, which ultimately helped me gain some understanding of the links between genetic processes, ecology and the long history of the Tasmanian landscape. Dr Bob Ward of CSIRO Marine Research s Genetics Laboratory also helped immensely with this project in many ways over the years, and contributed valuable comments on the technical aspects of the thesis - for his help I am especially grateful. Many thanks are also due to Dr Simon Jarman for the time he spent teaching me laboratory and data handling techniques for the molecular study, and for making samples and data available. I am grateful too to Bronwyn Innes of CSIRO, who was typically generous with her support and assistance in the laboratory.

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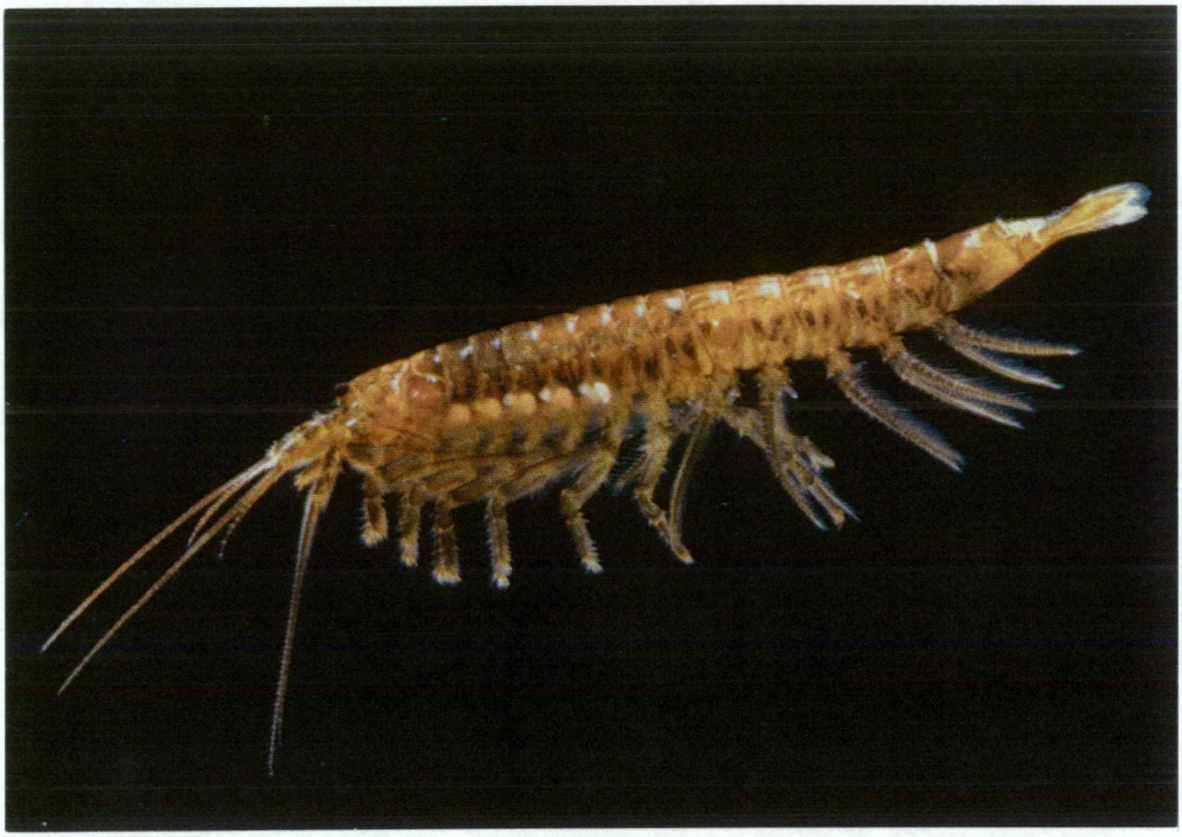

'Goethe somewhere remarks that the most insignificant natural object is, as it were, a window through which we can look into infinity. And certainly when I first saw the Mountain Shrimp walking quietly about in its crystal-clear habitations, as if nothing of any great consequence had happened since its ancestors walked in a sea peopled with strange reptiles, by a shore on which none but cold-blooded creatures plashed among the rank forests of fern-like trees, before ever bird flew or youngling was suckled with milk, time for me was annihilated and the imposing kingdom of man shrunk indeed to a little measure.'

Geoffrey Smith, *A Naturalist in Tasmania*, 1909.

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Chapter 1. Introduction

The fauna and flora of Tasmania exhibit a degree of isolation, discreteness, relative simplicity and relictual elements which make it an area of great interest for biological study (Williams, 1974). Tasmania is unique in that it contrasts in climate and geological history with its nearest continental landmass, mainland Australia; Tasmania is more temperate and well-watered, with many permanent rivers, streams and freshwater lakes, and unlike mainland Australia has large areas that were glaciated during Pleistocene times.

The influence of Tasmania s past conjunctions and disjunctions with other land masses can still be seen in the distributions of many of its plant and animal species. Tasmania s connection with the Antarctic continent persisted for about 60 million years after the separation of mainland Australia from Antarctica, finally being severed about 30 million years ago (Veevers, 1991). This was the latest separation from Antarctica of all the former Gondwanan land masses. Tasmania s cool climate and the intermittent marine barrier in Bass Strait have afforded protection for some of the ancient Gondwanan biota, many of which have high endemicity and diversity within Tasmania. It is also climatically and geographically most distant from the invasion routes into Australia of biota from South-East Asia (Williams, 1974).

Tasmania is well-known for its abundant and diverse marsupial fauna, whose existence has been attributed largely to the lack of placental mammals which have become established on mainland Australia since Quaternary times. The persistence and abundance of marsupial species such as the Tasmanian devil, bettong, potoroo, pademelon and quoll, which are now extinct or uncommon on mainland Australia, and the presence of different forms of mainland species, indicating continuing divergence of mammalian lines, make Tasmania an important faunal area for mammalian studies (Green, 1974). With the increasing recognition of the uniqueness of our Gondwanan faunal heritage, more interest has recently been shown in the associated Tasmanian invertebrate fauna, such as the onychophoran velvet worm, the ptunurra brown butterfly, endemic stoneflies and caddis-flies, and especially crustaceans. Presumably due to the abundance and diversity of freshwater habitats, Tasmanian freshwater crustaceans are particularly well-represented among the relictual invertebrate groups; these include members of the isopod suborder Phreatoicidea, the freshwater crayfish genera *Spinastacoides and Ombrastacoides* (formerly *Parastacoides*; Hansen, 2001), *Engaeus* and *Astacopsis*, the amphipod family Gammaridae and the syncarid order Anaspidacea, of which the family Anaspididae is the subject of this thesis.

The Division Syncarida is thought to have arisen in marine environments in the Early Carboniferous, about 350 million years ago (Schminke, 1982; Schram, 1982). The group is believed to have undergone a radiation around the Paleozoic continent of Laurentia during the Carboniferous and a more extensive radiation to other regions with the formation of the supercontinent Pangea in the Permian. Syncarids appear to have been common and widespread throughout brackish and near-shore marine environments in the later Paleozoic. A number of syncarid fossils have been identified from North America and Europe from this period, from both marine and brackish water assemblages (Brooks, 1962; Schram, 1984).

The Syncarida today contains three orders, the extinct Paleocaridacea, the extant Anaspidacea and the extant Bathynellacea (Brooks, 1962; Schram, 1984). Apart from 2 species in Lake Baikal, all the Bathynellacea are interstitial or groundwater forms. In adapting to subterranean life, they have tended towards paedomorphosis, being greatly reduced in size (0.5 - 3.5 mm) and complexity of form. They are found on all continents except Antarctica, but are not found in recently glaciated areas of the Northern Hemisphere (Schminke, 1982).

Of fossil forms, the majority have been placed in the Paleocaridacea, and are of Paleozoic age (Schram, 1984). The earliest examples are very small, Lower Carboniferous syncarids of the family Minicarididae, which apparently lacked abdominal appendage and are therefore considered to be a possible origin of the Bathynellacea (Schram, 1984). The great interest shown in the living Anaspidacea is largely because they share a generalised form with the larger Paleocaridacea. The shared characters include the lack of a carapace, the number of free segments of the body and the general form of the antennae, antennules, pleopods and tail fan (Calman, 1896; Schram, 1982). The family Anaspidacea is considered to be the most primitive of the extant syncarids (Schram and Hessler, 1984).The earliest descriptions of living anaspidaceans by Thomson (1893, 1894) did not recognise their affinities with fossil forms, and placed them in a now defunct group, the Schizopoda. It was not until 1896 that Calman (1896) first aligned them with their fossil relatives and established a new division, Syncarida, to accommodate both the living Tasmanian and the fossil animals.

In the following century, a small number of South American and Australian fossil syncarids were found and allocated to the Anaspidacea, as they are morphologically more similar to living anaspidaceans than are the Paleocaridacea. These include *Clarkecaris brasilicus* from the Brazilian Permian (Schram, 1982), *Anaspidites antiquus* from Triassic deposits near Sydney, NSW (Chilton, 1929), and *Koonaspides indistinctus* from the Cretaceous in southeastern Victoria (Jell and Duncan, 1986). All inhabited swamps or shallow lakes, but there is disagreement as to whether *Clarkecaris* was from fresh or saline water; the latter two were freshwater species (Brooks, 1962; Schram, 1982; Jell

and Duncan, 1986). *Anaspidites antiquus* reached a length of 42 mm, and *Clarkecaris brasilicus* and *Koonaspides indistinctus* 21 — 30 mm and 23 mm respectively (Brooks, 1962; Schram, 1984; Jell and Duncan, 1986). The extant members of the anaspidacean family Anaspididae are morphologically almost indistinguishable from the 200 million year old fossil *Anaspidites antiquus*.

The order Anaspidacea has been found only in Australia, South America and New Zealand, indicating a Gondwanan history. There are four extant families, the Stygocarididae, Psammaspididae, Koonungidae and Anaspididae, all of which occur in Tasmania. Only the Stygocarididae have been found outside Australia (Williams, 1980). These are small (1.4 - 4.2 mm) interstitial forms, and are similar to the Bathynellacae in their habitat and general reduction in form; for example, they have no eyes, tail fan, antennal exopodite, mandibular palp or pleopods except for the uropod and petasma (Schminke, 1982).

The Psammaspididae occupy interstitial water in coarse substrates and are known from New England, NSW, and Devonport, Tasmania. The family contains two genera and two species, *Psammaspides williamsi* Schminke (1974) and *Eucrenonaspides oinotheke* Knott and Lake (1980). These are both medium-sized (7 - 14 mm), eyeless and have somewhat reduced appendages, particularly on the abdomen where they are very rudimentary (Schminke, 1982). Their adaptation to coarse interstitial habitats has obviously led to some paedomorphosis, although not as extreme as that seen in the Bathynellacae or the Stygocarididae.

The family Koonungidae contains three species in two genera: *Koonunga cursor* Sayce (1908) and *K. crenarum* Zeidler (1985) from north-western Tasmania, Victoria and South Australia, and *Micraspides calmani* Nicholls (1931) from the west coast of Tasmania. Drummond (1959) and Zeidler (1985) suggest that undescribed species of *Koonunga* exist on the Australian mainland and Bass Strait islands. *Koonunga cursor* is about 10 mm in length, has small sessile eyes and is found in crayfish (*Engaeus* species) burrows and neighbouring surface pools and swamps after rainfall. It appears that this species has adapted to arid conditions by using crayfish burrows to allow access to the water table in times of drought (Drummond, 1959), and its size has reduced accordingly. *Koonunga crenarum* is found in sinkholes and caves, lacks eyes and is about twice as large as *K. cursor*, at over 20 mm (Zeidler, 1985). *Micraspides calmani* occurs in waters associated with freshwater crayfish (*Parastacoides* species) burrows in swampy areas in the western half of Tasmania. It lacks eyes, attains lengths of no more than 8 mm and has an elongate, rounded body ideally suited to subterranean habitats, with more reduced appendages than those of *Koonunga* (Nicholls, 1931). No members of the Koonungidae swim strongly (Drummond, 1959; Zeidler, 1985), although

Micraspides is able to swim and may also move by a wriggling motion (Nicholls, 1931). *Koonunga cursor* is reportedly able to burrow in soft substrates (Drummond, 1952).

All three of the anaspidacean families discussed so far are adapted to a subterranean existence through reduction in size, number and type of appendages and restriction of sight and locomotory function. The least hypogean species, *K. cursor*, which sometimes occurs in surface water, is the largest and the only species with eyes. All the permanently subterranean anaspidacean species lack eyes. The interstitial forms are the smallest and most paedomorphic and in these features resemble the Bathynellacea. It appears that in the Syncarida, particular morphological characters have a certain amount of plasticity and have responded to different subterranean environments, such as caves, crayfish burrows and interstitial water, in similar ways, indicating convergent evolution of the reduced features of subterranean syncarid species. An alternative hypothesis is that at some time in the past all syncarids were subterranean and the family Anaspididae has since colonised surface water. This scenario is possible, but unlikely to have occurred any later than the Paleozoic, for a number of reasons, including evidence from the fossil record of early syncarids showing them to have been surface-water dwellers (from their size and ecological assemblages; Schram, 1984) and the accepted plesiomorphy of extant surface-water anaspidaceans. Whether it was anaspidaceans or paleocaridaceans which first colonised surface waters, and when this occurred, cannot be answered without further fossil data, and is beyond the scope of this work.

The family Anaspididae is endemic to Tasmania and is the only extant syncarid taxon commonly found in surface waters. There are two fossil genera, *Anaspidites* and *Koonaspides*, containing the 2 species described above, and three extant genera containing 5 named species, *Anaspides tasmaniae* Thomson 1893, *Anaspides spinulae* Williams 1965, *Paranaspides lacustris* Smith 1908, *Allanaspides helonomus* Swain, Wilson, Hickman and Ong 1970 and *Allanaspides hickmani* Swain, Wilson and Ong 1971. All the Anaspididae have pedunculate eyes.

Knott (1975) compared the relative lengths of segments and tagma of the fossil *Anaspidites antiquus* with those of the extant anaspid genera. He concluded that *Anaspides* was most similar to *Anaspidites* on the basis of similar proportions of the segments within the head and thorax and of the tagma over the whole body; *Allanaspides* and *Paranaspides* have a reduced thorax and elongated abdomen. *Paranaspides* shared with *Anaspidites* similar proportions of the abdomenal segments, specifically the proportionally greater length of the uropod when compared with *Allanaspides* and *Anaspides* (Knott, 1975).

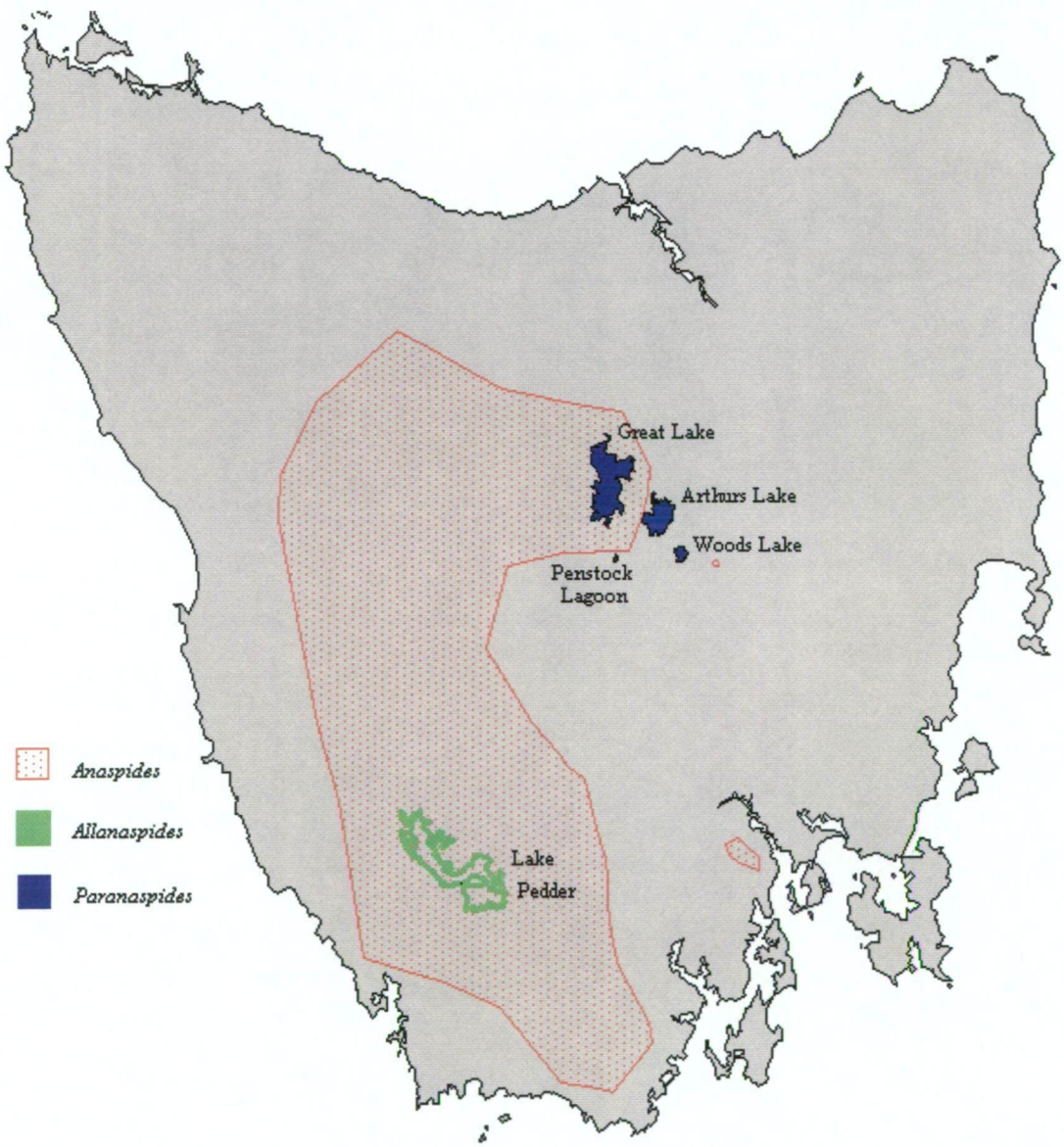
Paranaspides lacustris is the only pelagic syncarid and occurs in association with patches of aquatic vegetation in Great Lake, Penstock Lagoon, Shannon Lagoon, Arthurs Lake and Woods Lake on the southeastern side of the Central Plateau (Fulton, 1982) (Figure 1.1). It is usually found at depths of between 1 and 8 m, although will occur in shallower water (0.2 — 1.0 m) when the weed-beds are more exposed at low lake levels (Fulton, 1982). It is up to 25 mm in length, is the most shrimp-like in appearance, due to a pronounced dorsal flexure between the thorax and abdomen, and is the strongest swimmer of the anaspids (Wells *et al.*, 1983).

The genus *Allanaspides* has been found to be abundant at a number of sites around the shores of Lake Pedder (*Allanaspides helonomus*) and a small area at McPartlans Pass between Lake Pedder and Lake Gordon (*A. helonomus* and *A. hickmani*) (Figure 1.1). It has been assumed that the two species originated in different drainage systems, although one specimen of *A. hickmani* has been found on the western shore of Lake Pedder (Horwitz, 1988). It is likely that the damming of these two lakes has restricted the distribution of the genus considerably (Swain *et al.*, 1970), but may also have created new opportunities for the two species to cohabit. Both species occur in button-grass plains, in the burrows of freshwater crayfish (*Ombrastacoides* spp.) and associated surface water. They are the smallest anaspids (7 — 12 mm; Swain *et al.*, 1970, 1971) and are not strong swimmers. The initial description of the genus by Swain *et al.* (1970) reports many features of its morphology to be intermediate between *Paranaspides* and *Anaspides,* and describes four new characters to justify its generic status. The most significant of these is the presence of a unique structure, the *fenestra dorsalis*, on the dorsal surface of the cephalothorax. This is believed to be a deeply invaginated active ion transport system with numerous inner and outer mitochondrial pumps (Lake *et al.*, 1974; McConnell, 1987). It is possible that the structure developed in response to the low ionic content of the waters of southwestern Tasmania, coupled with the inhibitory effect on ion absorption of high acidity from an excess of organic matter in buttongrass swamps (Swain *et al.*, 1970). The size, shape and colour of the *fenestra dorsalis* differs between *Allanaspides* species (Swain *et al.*, 1971).

Anaspides species are the largest living syncarids, growing to 40 — 50 mm in most environments, although in many areas its growth is restricted to about 25 mm; the largest specimen recorded is 63 mm and females attain a considerably larger size than males (Swain and Reid, 1983). *Anaspides* can swim effectively, but is usually found walking among stones or submerged vegetation. Its walking and swimming activity are similar, and are apparently mediated by the same coordination of the nervous system, a feature that is presumed to be primitive (Macmillan *et al.*, 1981). It has no dorsal flexure, but has a well-developed caridoid reflex (the flicking movement used by decapod crayfish as an escape mechanism), which in *Anaspides* can be maintained for longer than is seen in crustaceans with carapaces, and is probably its only defence against predators (Silvey and Wilson, 1979). It sometimes

emerges from the water and has been found to survive and remain active for up to several days out of the water if humidity is high (Swain and Reid, 1983). It is omnivorous and prone to cannibalism, but usually feeds on detritus, algae, mosses, small invertebrates and worms (Manton, 1930; Smith, 1908). It does not brood its eggs, but sheds them freely into the water, and lacks a larval stage (Schminke, 1982; Hickman, 1937).

Figure 1.1 Distribution of three genera of the family Anaspididae. *Anaspides* distributional range based on O'Brien (1990).



Anaspides is an ecological generalist and is locally abundant in a wide range of alpine and subalpine habitats throughout central, western and southern Tasmania, including small streams, rivers, small alpine tarns, lakes, moorland and sedgeland pools and sphagnum runnels. It is also found in streams and pools in a many limestone caves throughout Tasmania, including the Ida Bay, Hastings, Junee-Florentine and Mole Creek systems (O Brien, 1990). Figure 1.1 shows the extent of the distribution of the genus. A population of unusually large individuals (about 60 mm) occurs in Wet Cave, in the north of its range. It is not found in still, shallow, surface waters, presumably because it is not resistant to desiccation and is sensitive to water temperatures over about 22 °C (Swain and Reid, 1983). It appears to survive in smaller water bodies such as small tarns, runnels and highland streams only where there is access to deep water or shelter from overhanging rocks, banks or bolster vegetation.In an electrophoretic study, Andrew (1999) showed that there was no genetic differentiation between *Anaspides* populations from flowing and still water. The distribution of the genus has probably diminished over the last 150 years through predation by the introduced brown trout, *Salmo trutta,* and climatic warming. There is evidence that one large population, in Hartz Lake in the far southeast of its range, has disappeared entirely since trout were introduced about 30 years ago. *Anaspides* and trout co-exist in the highland Clarence Lagoon and Lake St Clair, possibly as there is a variety of habitats available to *Anaspides* in these large lakes which afford some protection, such as weed beds (*Isoetes* sp.), exfoliating rock sheets in Lake St Clair, and stoney outcrops in Clarence Lagoon (Ron Mawbey pers. comm.; O Brien, 1990). There is some doubt whether *Anaspides* are preyed upon by native galaxiid fish, but it seems likely that large galaxiids would prey on them. There are few, if any, waters where these species cohabit (Fulton and Horwitz, 1987), but the influence of predation by trout on both native fish and anaspids confuses this argument (O Brien, 1990).

Many physiological and morphological studies of *Anaspides* have been undertaken because the genus is considered likely to display plesiomorphic character states. The morphology, physiology and embryology of *Anaspides* are seen as potentially informative on the nature of the early malacostracan line, despite the fact that some derived characters will probably be found in any extant form. Studies of the giant lateral neurone (Silvey and Wilson, 1979), foregut morphology (Wallis and Macmillan, 1998), mouthparts and feeding (Cannon and Manton, 1928; Manton, 1930a; Gordon, 1961), transport tissues (McConnell, 1987), organ of Bellonci (Kauri and Lake, 1972), maxillary glands (Manton, 1930b), embryology (Hickman, 1937), locomotory function (Macmillan *et al.*, 1981), spermiogenesis (Jespersen, 1983) and heart ultrastructure (Tjonneland *et al.*, 1984) have all contributed to a relatively high level of understanding of the genus and the evolution of crustacean structure and function.

The genus contains two described species, *Anaspides tasmaniae* Thomson 1893 and *Anaspides spinulae* Williams 1965, but its taxonomy has been questioned by a number of authors. The spination and serrations on the margins of the epimera, abdominal segments and telson were used by Williams (1965) to describe *Anaspides spinulae*, but these characters have been found to be variable in populations of *A. tasmaniae*, with intermediate forms in a number of locations on the Central Plateau (O Brien, 1990). Cave forms have also been suggested as warranting taxonomic investigation. All cave forms are unpigmented and Lake and Coleman (1977) described a third pattern of telson spination and anophthalmia in the Wolfe Hole population. O Brien (1990) presents data showing the occurrence of this cave type of telson spination in *Anaspides* from some 16 caves in the south of the state, but it is by no means clear that this character is phylogenetically useful rather than merely highly variable, and it is not always fixed within populations (S. Eberhard pers. comm.). Knott (1975), the IUCN Invertebrate Red Data Book (Wells *et al.* 1983), O Brien (1990), Andrew (1999) and Jarman and Elliot (2000) suggest a need to revise the taxonomy of the genus and Swain *et al.* (1970) called for a redescription of the entire family, as the original characters used by Thomson to describe it became invalid with the discovery of *Allanaspides.*

Numerous studies of isolated populations of freshwater crustaceans have found morphological stasis coupled with genetic divergence (Penton *et al.*, 2004; Witt *et al.*, 2003; Lee and Frost, 2002; Grandjean *et al.*, 1998; Daniels, 2003; Vainola, *et al.*, 1994; Witt and Hebert, 2000; Gomez *et al.*, 2002; Muller, 2000; Hansen, 2001). Among-population genetic divergence is obviously related to genetic drift and the limited dispersal capability of most freshwater crustaceans, but morphological conservatism is harder to explain; maybe it is the result of past selection pressures, whereby a limited number of crustacean body plans that are well-suited to their particular niches while still being able to accommodate some environmental fluctuation became fixed. The converse, morphological variability in certain characters in genetically homogeneous populations, is well known in most animal taxa.

For these reasons we could argue that in the genus *Anaspides* a single character (spination) which has variable rather than discrete states is not enough to delineate species, while at the same time predicting that the numerous isolated populations throughout the state have probably accumulated genetic differences, through selection or genetic drift. It is highly likely that the extraordinarily long history of the Anaspididae within Australia, and the geological, climatic and biotic changes during that time, would have led to the accumulation of phylogenetically significant amounts of genetic differentiation between isolated populations, or groups of populations. Examining these questions is the subject of the current work.

1.1 Project objectives

The primary aim of this thesis is to assess the genetic relationships among populations of the genus *Anaspides*, to establish a reliable phylogeny for the family Anaspididae, and to attempt to account for the phylogenetic relationships biogeographically. Allozyme and 16S mt DNA markers are used to examine genetic differentiation within populations, among populations, among species, and among genera. The phylogenetic relationships are used to suggest a valid taxonomy. All species in the family have been included in the study and *A. tasmaniae* was sampled across its entire geographical range.

The thesis consists of 5 chapters:
Chapter 1 summarises background information on the evolutionary history and taxonomy of the syncarids, their history and present-day distribution in Australia, and includes a more detailed examination of the family Anaspididae.
Chapter 2 provides details of the sample populations used in the study, their habitats and locations, and a map showing sampling sites.
Chapter 3 reports on the allozyme electrophoresis study, which establishes relationships among populations, levels of differentiation within populations and a preliminary phylogeny in the form of a cluster analysis.
In Chapter 4 mitochondrial DNA sequencing provides further phylogenetic information to validate the allozyme study, resulting in three phylogenies produced by different analytical methodologies. It includes a discussion of how to ensure a valid and useful phylogenetic analysis of sequence data. A molecular clock is applied to the results to estimate times of divergence of anaspid lineages.
Chapter 5 consolidates the phylogenetic information from the allozyme and mt DNA studies, and suggests geological, biotic and climatic causes for the relationships within the family and the current distribution. Changes to the taxonomy and further work needed are also suggested.

Specific questions which will be addressed are:

1. Are there genetically distinct geographical groups within the genus *Anaspides* and if so what are the relationships between them?
2. Is *Anaspides spinulae* supported as a genetically distinct taxon?
3. Is the cave form a monophyletic group?
4. How might any geographical groups have originated?
5. Are there any differences in the levels of divergence within geographical groups?
6. What are the phylogenetic relationships between the three genera *Anaspides, Paranaspides* and *Allanaspides*? Which is the most basal?

7. What are the estimated times that each of the genera, species and populations diverged from each other?
8. What geological, climatic or biotic causes can be established for the reported levels of divergence and time estimates?

Chapter 2. Material

Specimens of *Anaspides tasmaniae* were collected by hand net from eighteen sites, including both lacustrine and riverine habitats. Sites were chosen to represent much of the geographical distribution of the species. Three of these populations were from subterranean waters within limestone cave systems. Specimens of *A. spinulae* were collected from Lake St Clair and Clarence Lagoon on the Central Plateau by diving and electroshocking respectively.

The remaining three species in the family Anaspididae were sampled to be used as outgroups, in order to relate intrageneric levels of genetic diversity to those found between genera. Specimens of *Paranaspides lacustris* were collected from Woods Lake on the eastern side of the Central Plateau by hand net. Populations of *Allanaspides hickmani* and *Allanaspides helonomus* were sampled, also by hand net, at McPartlans Pass near Lake Gordon, and further specimens of *Allanaspides helonomus* were collected in the Harlequin Hill region near Lake Pedder.

The location and details of the collection sites can be found in Figure 2.1 and Table 2.1. Details of people who collected material used in this study can be found in Appendix 3. Type localities were sampled for all species, except for *P. lacustris,* and are noted in Table 2.1. *P. lacustris* was not able to be sampled from its type locality, Great Lake, so was taken from nearby Woods Lake.

Animals collected during this study were transported live to the laboratory where they were frozen in liquid nitrogen, except those from Clarence Lagoon and Woods Lake, which were frozen in liquid nitrogen immediately on collection. Samples were stored at -80 iC at the CSIRO Division of Marine Research Genetics laboratory for the duration of the study.

Frozen homogenates of specimens of *A. tasmaniae*, collected by S. Jarman from five southwestern populations, were included in the DNA study. Additional sequence data originating from work by Jarman and Elliot (2000) were obtained from Genbank for a further five populations and added to the sequence database. These populations are noted in Table 2.1. Only one population of *Allanaspides helonomus* (from the Harlequin Hill area) was used in the DNA study, otherwise all populations from the allozyme work were also represented in the DNA study.

Table 2.1 Details of collection sites of members of Anaspididae used in this study

Location	Number	Habitat description	Grid reference #
***Anaspides tasmaniae* - surface populations**			
Central Plateau - near Lake Ball	15	Small deep pools among and under sphagnum, *Richea* spp. and pencil pine, almost no exposed surface water	4416 53668
Central Plateau - near Powena Creek	16	Deep open pools, overhanging sphagnum and coral fern banks	4483 53634
Central Plateau - near Olive Lagoon	15	Slow-flowing deep open runnels, overhanging sphagnum banks	4458 53550
Central Plateau - Hydro Creek	16	Deep pool in fast flowing rocky creek in tea-tree and heathland	4877 5358
Central Plateau - Lonely Lake	9	Shaded boulders in alpine lake	4588 53787
Central Plateau - Jacks Lagoon outflow	14	On boulders in fast flowing alpine creek	4454 53835
Central Plateau - tributary of Clarence Lagoon	7	Deep open runnels with overhanging sphagnum banks	4416 53401
Mount Wellington **(type locality)**	9	Pool with boulder and cement substrate in small rocky stream	5191 52512
Hartz Mountains	17	Rocky substrate in alpine tarn	4812 52127
Adamsons Peak	19	Fast-flowing alpine creek with gravelly substrate; partitioning of size-classes — small and large individuals separated by fast-flowing water over a steep gradient.	4863 52004
Weld River	21	On logs in fast-flowing river in wet sclerophyll forest	4536 52599
Mount Field	16	Alpine tarn with silty substrate	4653 52741
Mount Anne	22	Alpine tarns	4526 52438
Frenchmans Cap	14	Alpine lake	4040 53199
Western Arthur Range - Lake Oberon	15	Cobble substrate around shore of lake in alpine heathland	4405 52224
*Western Arthur Range - Square Lake	2	Cobble substrate around shore of lake in alpine heathland	4405 52221
*Western Arthur Range - Haven Lake	2	Alpine lake	4457 52198
*Coronation Peak	2	Alpine tarn	4193 52483
*Lake Picton	1	Alpine lake	4705 52214
*Lake Rhona	1	Alpine lake	4414 52885

Table 2.1 cont.

[α]Snowy North	1	Stream flowing into Styx River	4718 52574
[α]Sandbanks Tier	1	Stream flowing from beneath boulders	4878 53678
[α]Zion Hill	1	Stream at base of hill	4433 53705
[α]Mt Rufus	1	Stream flowing into Lake St Clair	4262 53365
[α]Mt Ossa	1	Ridge between Mt Ossa and Mt Doris	4236 53643
***Anaspides tasmaniae* - limestone cave populations**			
Wolfe Hole	9	Underground lake (Lake Pluto) with silty substrate	4866 51963
Newdegate Cave	5	Small underground stream, intermittent flow	4876 51966
Wet Cave	13	Stream fed by three surface creeks - 0.15 to 6 km underground before Wet Cave site	4502 53944
Anaspides spinulae			
Lake St Clair, south of Pumphouse Point **(type locality)**	7	Weedbeds at depths of about 2 metres in large highland lake	4340 53381
Clarence Lagoon	15	Rocky substrate about 1.5 metres deep in lake in alpine heathland	4437 53403
Paranaspides lacustris			
Woods Lake	20	Lake in wet sclerophyll forest	5010 53430
Allanaspides hickmani			
McPartlans Pass **(type locality)**	8	Small shallow pools with crayfish burrows in buttongrass plains	4344 52542
Allanaspides helonomus			
Harlequin Hill area **(type locality)**	8	Extensive pools in buttongrass plains	4476 52434
McPartlans Pass	2	Small shallow pools with crayfish burrows in buttongrass plains	4344 52542

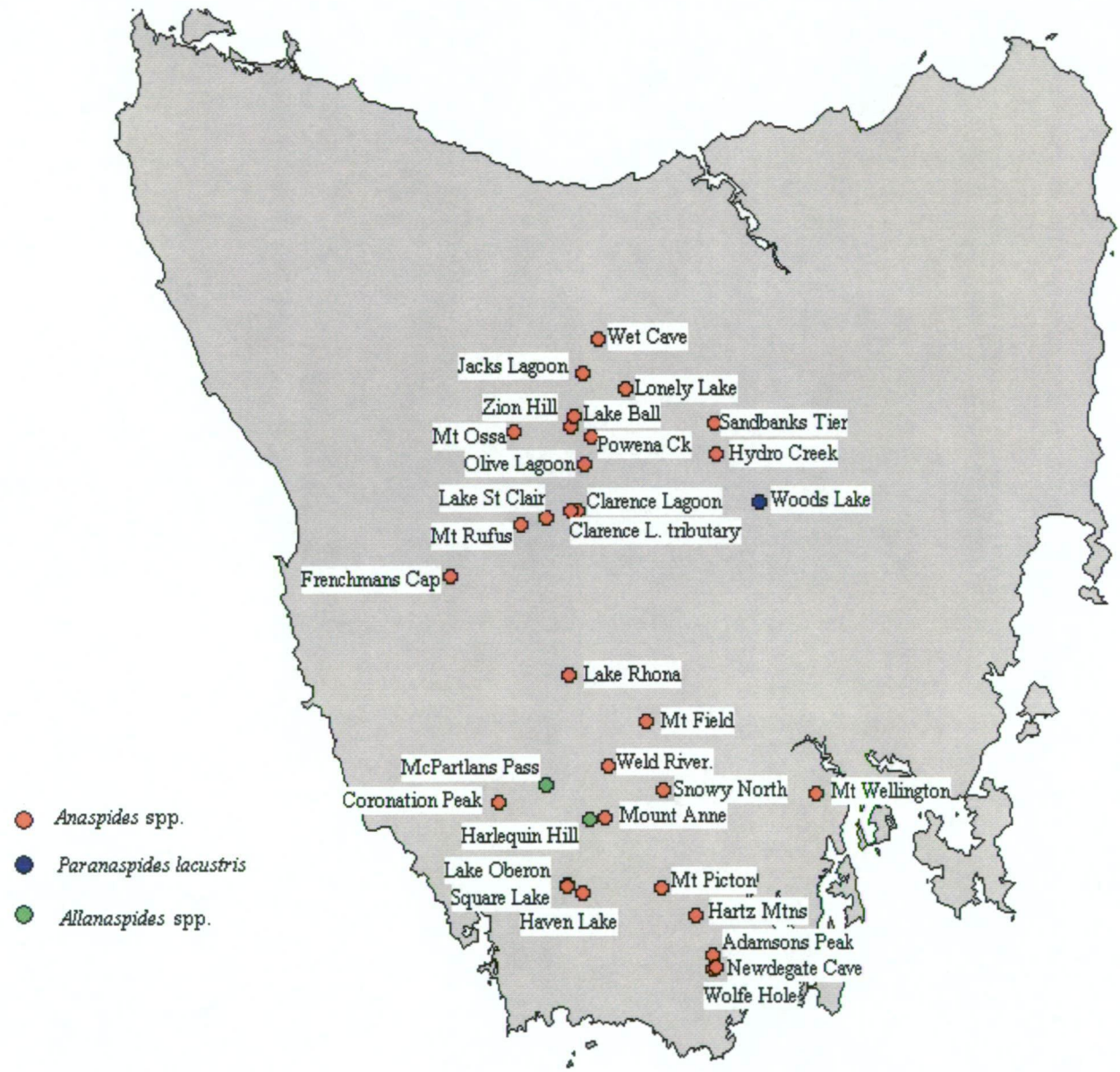
[#] All grid references are based on Australian Geodetic Datum 1966 (to nearest 100 m).

* Provided as homogenates for DNA analysis only; not collected in this study.

[α] DNA sequence data from work reported in Jarman and Elliot (2000); not collected or sequenced in this study.

To summarise, a total of 24 populations were collected and underwent allozyme analysis, 28 underwent DNA sequencing during this work, and 33 populations were available for DNA sequence analysis.

Figure 2.1 Locations of populations used in this study



Chapter 3. Allozyme differentiation among and between populations

3.1 Introduction

Protein electrophoresis is the differential migration of proteins through a gel matrix under the influence of an electrical field. Homogenised samples are applied to a gel matrix of starch, acrylamide or cellulose acetate which has been infused with a buffer solution in order to avoid the denaturing of proteins by pH changes during electrophoresis. Differences in migration rate between samples are indicative of a different electrical charge, size or shape of the protein molecules. As these molecular features are dependent on the amino acid sequence of the protein, which is itself determined by the genetic code, it can be assumed that differences in migration rate between samples indicate genetic differences. Electrophoresis of both enzymes and non-enzymatic proteins has been used to visualise differences among the underlying genetic codes of proteins which are functionally similar, by using specific stains for each protein. Allozymes are variant forms of an enzyme which represent different alleles at the same gene locus.

Since its inception in the 1960s, enzyme electrophoresis has been widely used in taxonomic and phylogenetic studies of a vast range of organisms and at different taxonomic levels. Results may also be applied in conservation programs where species boundaries (geographic and taxonomic) need to be established or where protection of genetic diversity itself is an objective. Although it has been partially replaced by the various DNA approaches, allozyme electrophoresis remains a valid genetic analysis technique, with many advantages over DNA sequencing in terms of cost and ease of operation.

3.1.1 Issues arising from the use of allozyme electrophoresis

Problems with the use of allozyme electrophoresis in systematics may arise as a result of different substitution rates at different loci, too much or too little variability, the presence of null (unexpressed) alleles, non-genetic sources of variability, and an inability to resolve electrophoretically all genetic differences at loci through coincidental similarity in molecular structure between allozymes, leading to the potential for underestimating the amount of variation (Thorpe, 1982; Hillis *et al.*, 1996). The impact of these factors can be diminished to a large extent by the use of many loci. Confidence in results can be increased through the use of bootstrapping techniques and consideration of standard errors during statistical analysis.

A general area of debate in systematics has been the relative influence of the various forces driving evolutionary change in phenotypic character traits — selection, drift, mutation and migration. For any species a combination of these forces has determined its present-day genotype, phenotype, distribution and population structure; however large-scale selection decreases the reliability of phylogenetic inferences from systematic studies. With the advent of the use of allozyme data in such studies, the origin of protein polymorphism became the focus of the debate, and the two opposing theories of neutralism and selectionism were proposed. The neutral theory holds that most protein polymorphisms are explained by random genetic drift of mutant genes that are functionally equivalent. Under this theory selection is largely negative, acting to remove deleterious mutants, although some positive adaptive genetic change may also occur, albeit slowly over geological time. By contrast, under the selectionist theory molecular evolution is caused largely by selection for variants and their subsequent preservation in populations. Selective mechanisms are seen to act similarly on genotypes as they do on phenotypes, where certain conditions may favour one variant over another. Spatially or temporally diverse environments are believed by selectionists to be responsible for maintaining a large proportion of protein polymorphism (Nevo, 1983).

In trying to substantiate either theory, reviews of the levels of polymorphism in the same loci over different taxa have confirmed that certain loci are more subject to selection than others (Nevo, 1983; Hedgecock, Tracey and Nelson, 1982). Furthermore, modelling of predicted levels of heterozygosity in the absence of selection in crustaceans found that expected levels were much higher than actual levels, possibly implying that selection was occurring or had occurred (Hedgecock, Tracey and Nelson, 1982). Reservations about this method included the inability to accurately estimate population sizes or the number of generations since divergence, possibly resulting in unreliable estimates of the number of accumulated mutations (Hedgecock, Tracey and Nelson, 1982).

Other studies have found examples of selection for certain alleles occurring under particular environmental conditions. Rodriguez *et al.* (2002) describe significant differences in allele frequency and heterozygosity among samples of a single population of *Artemia franciscana* which had been held at different temperatures and salinities. Likewise, environmental stress in intertidal barnacles (Schmidt and Rand, 2001) and amphipods (Duan *et al.*, 2001) was found to be associated with between-population polymorphism at from one to four loci. A similar study of the freshwater fish *Hoplias malabaricus* found four out of 24 loci varying between habitats (Peres *et al.*, 2002). The effect of the environment on multiallelic systems, and

epistatic processes in general, is more likely to be the source of selective pressure, but harder to verify.

Gillespie and Kojima (1968) proposed that enzymes used in allozyme electrophoresis be grouped according to their metabolic functioning. Enzymes using substrates produced within the organism (Group 1 enzymes) were compared to those utilising substrates provided by the environment (Group 2 enzymes). Evidence of lower heterozygosity in the former group, which would imply selective pressure, has not been widely established, and the allocation of enzymes to either group is not always obvious (Ward, Skibinski and Woodwark, 1992).

A novel approach by Nelson and Hedgecock (1980) established differences between Group 1 and Group 2 enzymes in their relationships with environmental heterogeneity, trophic state and other ecological descriptors, using data pooled from diverse decapod crustacean taxa. Although there was considerable taxon- and enzyme-dependent variation, their general conclusions were that for the decapods heterozygosities in Group 1 enzymes are positively correlated with environmental variability and negatively correlated with trophic state, whereas heterozygosities in Group 2 enzymes are positively correlated with trophic state and negatively correlated with environmental variability. The environmental heterogeneity — trophic diversity model was proposed to explain the results, by contrasting Group1 and 2 heterozygosities found in decapod species with coarse grain adaptive strategies against those found in species on a fine-grain path (Nelson and Hedgecock, 1980).

The difficulty of distinguishing between the effects of genetic drift and selection in long-isolated populations remains as much a problem for electrophoretic studies as for morphological ones. The conservative option is that neutrality should be assumed in the absence of evidence for selection, and therefore that mutation and drift are assumed to be the likely sources of genetic variation (Allendorf and Phelps, 1981; Varvio-Aho, 1983). A large number of electrophoretic loci is normally used to ensure that if selection has occurred at one or more loci its influence will be minimised.

3.1.2 The interpretation of allozyme data for taxonomic and systematic purposes

Relationships between taxonomic status, genetic variation and population structure have been established for many organisms using allele frequency data from electrophoretic studies. Statistical analyses can be grouped broadly as measures of the amount of genetic variation within a taxonomic unit (usually a population or species) and the distribution of genetic variation among units.

Measures of the amount of genetic variation within a taxonomic unit include heterozygosity (H, the proportion of heterozygotes observed or expected from allele frequencies), the mean proportion of polymorphic loci per individual (P) and the number of alleles per locus (N_a). The proportion of polymorphic loci per individual and the number of alleles per locus may vary with sample size and number and choice of loci. Heterozygosity is an independent measure, but if it is being calculated as observed rather than expected H for a species, the possibility of additional interpopulation variation must be considered. Expected heterozygosity is considered more accurate as it is estimated from allele rather than genotype sample frequencies.

Mean values of P and H in 242 mostly animal species were calculated using data from studies using over 14 loci at P = 0.26 ± s.d. 0.15 and H = 0.07 ± s.d. 0.05 (Nevo, 1983). Ward *et al.* (1992) reviewed levels of heterozygosity of more than one thousand species over all major taxonomic groupings and compared them with results of a later review by Nevo *et al.* (1984, cited in Ward *et al.*, 1992). Both studies found crustaceans to have by far the lowest heterozygosity of the invertebrates (Table 3.1).

Table 3.1 Estimates of heterozygosity in major taxonomic groupings (adapted from Ward *et al.*, 1992)

	Ward *et al.* (1992)		**Nevo *et al.* (1984)**	
	Mean H (± SE)	**No of species**	**Mean H (± SE)**	**No of species**
Invertebrates - total	0.122 ± 0.004	370	0.100 ± 0.005	361
-crustaceans	0.052 ± 0.005	80	0.082 ± 0.007	122
Vertebrates-total	0.071 ± 0.02	648	0.054 ± 0.003	551

Note that Ward *et al.* (1992) used H_e (Hardy-Weinberg expected) values calculated from the pooled allele frequency data for all populations of each species, while Nevo *et al.* (1984) used mean observed H values, which are the mean of values from sampled populations, ignoring possible between-population differentiation. Thus Ward s values should be higher than those of Nevo *et al.* where interpopulation differentiation occurs. The aberrant low value of H_e for crustaceans is explained as an artifact caused by one large study of twelve species of prawn which had very low overall heterozygosity. Further analysis by Ward *et al.* (1992) revealed

that partitioning the H_e between total and subpopulation heterozygosity resulted in mean subpopulation values which were more similar to those of Nevo *et al.* (1984).

A review of electrophoretic studies of crustaceans by Hedgecock, Tracey and Nelson (1982) summarised allozyme variation in ten taxonomic subgroups and in the Crustacea as a whole. The average number of alleles per locus, N_a, across 97 species cited was 1.5 (S.D. = 0.39, range 1 — 3.2), mean proportion of loci polymorphic per individual 0.305 (0.99 criterion, S.D.= 0.177, range 0.00 — 0.92) and expected proportion of loci heterozygous per individual 0.073 (S.D.= 0.052, range 0.000 — 0.241). Table 3.2 gives a detailed summary of the same parameters for crustacean subgroups.

More recent allozyme studies of genetic variation in crustaceans report values falling broadly within the ranges cited above. Studies include work by Sugama *et al.* (2002) on tiger prawns (N_a = 1.3 - 1.5; H_o = 0.018 - 0.047); by de la Rosa-Velez *et al.*. (2000) on two *Penaeus* species (P = 0.125 - 0.313; H_e = 0.023 - 0.086); by Daniels (2003) on a freshwater crab (N_a = 1 - 1.5, P = 0 - 0.2 and H_e = 0.0 - 0.035); by Balakirev and Fedoseev (2000) on the red king crab (P = 0.065 and $H_e = 0.027 \pm 0.008$); by Wang and Schreiber (1999) on an isopod woodlouse (N_a = 1.72, P = 0.329 and H_o = 0.155); and by Coelho *et al.* (2002) on the amphipod *Gammarus locusta* (P = 0.116; H_e = 0.023).

The distribution of genetic variation among taxonomic units is measured as pairwise genetic distance (or identity), which is based on the presence of alleles in common. Distance or identity matrices are used in clustering algorithms to demonstrate levels of relatedness and displayed as dendrograms, or in tests of correlation, most commonly with geographical distance as a test of genetic isolation by distance.

Population structure (genetic variation among subpopulations) is analysed using F- or G-statistics (shown by Swofford and Selander, 1989, to be theoretically identical), which separate the total heterozygosity into that found within populations and that found between populations. Significant results from these tests are used to demonstrate spatial population differentiation; if a higher proportion of the total heterozygosity occurs between samples than within samples then significant substructure is indicated and the assumption that all samples came from one population is disproved. Within-population structure is indicated where the test for conformity with Hardy-Weinberg equilibrium shows a heterozygote deficit, and in a sexually reproducing population generally means a restriction of gene flow among components of the population.

Table 3.2 Summary of allozyme variation in various taxonomic subgroups and in the Crustacea as a whole[a] (from Hedgecock, Tracey and Nelson, 1982).

Taxonomic Group	No of species	Mean number of loci per species (SD) (range)	Mean number of alleles per locus (SD) (range)	Mean proportion[b] loci polymorphic per species (SD) (range)	Mean expected proportion of loci heterozygous per individual (SD) (range)
Branchiopoda					
Diplostraca	3	14.3 (2.1) (12-16)	1.56 (1.50-1.62)	0.385 (0.140) (0.333-0.530)	0.135 (0.044) (0.100-0.185)
Maxillopoda					
Copepoda	5	19.0 (2.8) (15-23)	1.71 (0.41) (1.16-2.16)	0.414 (0.220) (0.105-0.600)	0.142 (0.078) (0.054-0.241)
Cirripedia	15	19.2 (5.1) (12-27)	1.90 (0.34) (1.54-2.46)	0.552 (0.156) (0.389-0.92)	0.120[c] (0.059) (0.048-0.253)
Hoplocarida	2	22.5 (33, 12)[d]	1.21 (1.33, 1.08)[d]	0.193 (0.303, 0.083)[d]	0.034 (0.045, 0.022)[d]
Eumalacostraca					
Peracarida	4	18.5 (4.7) (13-24)	1.47 (0.24) (1.24-1.81)	0.214 (0.075) (0.118-0.300)	0.073 (0.067) (0.057-0.104)
Eucarida					
Euphausiacea	3	31.3 (4.2) (28-36)	2.52 (0.69) (1.81-3.20)	0.625 (0.233) (0.361-0.800)	0.137 (0.078) (0.057-0.213)
Decapoda					
Penaeoidea + Caridea	12	24.9 (5.1) (15-30)	1.64 (0.39) (1.18-2.17)	0.267 (0.099) (0.107-0.458)	0.051[c] (0.027) (0.008-0.089)
Astacidea + Palinura	11	25.6 (9.4) (15-43)	1.24 (0.06) (1.11-1.49)	0.185 (0.053) (0.100-0.276)	0.050 (0.014) (0.025-0.066)
Anomura	19	23.3 (6.6) (12-38)	1.38 (0.16) (1.11-1.77)	0.271 (0.074) (0.105-0.455)	0.068 (0.031) (0.009-0.125)
Brachyura	23	24.0 (4.9) (13-32)	1.26 (0.17) (1.00-1.70)	0.200 (0.105) (0.000-0.455)	0.039 (0.031) (0.000-0.128)
Totals for Crustacea	97	22.8 (6.9) (12-43)	1.50 (0.39) (1.00-3.20)	0.305 (0.177) (0.000-0.92)	0.073 (0.052) (0.000-0.241)

[a] Means are arithmetic averages;

[b] Polymorphism defined as frequency of most common allele no greater than 0.99;

[c] Observed H used when expected values not given: Cirripedia (5); Penaeoidea + Caridea (1);

[d] Actual values for the two species.

In a major review by Thorpe (1983), over eight thousand estimates of genetic identity between units at the same taxonomic level were compiled from electrophoretic studies of all major taxa (mammals, birds, reptiles, amphibians, fish, invertebrates and plants). With the exception of birds, levels of electrophoretically detectable genetic divergence at the family, genus and species levels were found to show very little difference between the major taxa. Nei s genetic distance D (Nei, 1972) was found to be below 0.3 for conspecific populations,

between 0.3 and 0.8 for congeneric species and above 0.8 for confamilial genera (Thorpe, 1983).

The results of a similar literature review of crustaceans by Hedgecock, Tracey and Nelson (1982) are shown in Table 3.3. The levels of genetic identity (I; I = 1 — Nei s D) for congeneric species fall within Thorpe s reported range, with the exception of species in four decapod genera, in which the genetic identity values between species are higher and fall within Thorpe s range for conspecific populations (I > 0.7; D < 0.3). The identity values between three confamilial crayfish genera are likewise larger than Thorpe s quoted levels for confamilial genera (I < 0.2; D > 0.8).

Table 3.3 Heterozygosities within and genic similarities between species and genera in the Crustacea. (Hedgecock *et al.*, 1982)

Comparisons	**Number of species**	**Heterozygosity H_e**	**Nei s Identity I**	**Number of loci**
Between species				
Cirripedia				
Chthamalus spp.	5	0.049 — 0.104	0.20 — 0.61	12 — 16
Hoplocarida				
Squilla spp.	2	0.028 — 0.035	0.54	30
Peracarida				
Isopoda				
Excirolana spp.	2	0.065 — 0.086	0.45	15
Eucarida				
Euphausiacea				
Euphausia spp.	3	0.058 — 0.211	0.33 — 0.51	28 — 30
Decapoda				
Pandalus spp.	3	0.025 — 0.082	0.50 — 0.68	21 — 24
Crangon spp.	2	0.037 — 0.056	0.83	29
Homarus spp.	2	0.033 — 0.039	0.90	30
Orconectes spp.	3	0.029 — 0.060	0.67 — 0.74	12 — 14
Cambarus spp.	3	0.040 — 0.083	0.50 — 0.53	14 — 17
Procambarus spp.	2	0.032 — 0.051	0.68	15
Callianassa spp.	2	0.105 — 0.126	0.36	19
Calcinus spp.	2	0.046 — 0.048	0.74	18
Clibanarius spp.	3	0.017 — 0.094	0.41 — 0.67	14 — 16
Coenobita spp.	2	0.094 — 0.111	0.58	9
Matuta spp.	2	0.0005 — 0.001	0.81	20

Table 3.3 cont.

Comparisons	Number of species	Heterozygosity H_e	Nei s Identity I	Number of loci
Cancer spp.	2	0.017 — 0.054	0.65	21
Callinectes spp.	2	0.085 — 0.113	0.55	20
Charybdis spp.	2	0.032 — 0.054	0.74	17
Pachygrapsus spp.	2	0.025 — 0.026	0.68	21
Ocypode spp.	3	0.012 — 0.120	0.83 — 0.92	22
Uca spp.	4 (2 and 2)	0.029 — 0.075	0.50 ; 0.62	16 ; 19
	Total = 53		**Mean = 0.59**	
Between genera				
Orconectes and *Cambarus*			0.41	12
Orconectes and *Procambarus*			0.74	12
Cambarus and *Procambarus*			0.46	14

3.1.3 Allozyme electrophoresis in phylogenetic and biogeographic studies

As biochemical genetic distance is correlated with taxonomic distance and both are functions of evolutionary time, the phylogenetic and systematic implications of electrophoretic data can also be considered (Avise, 1974, 1983). Freshwater crustaceans often lack morphological differentiation between species and populations whilst showing substantial genetic differentiation (Hedgecock *et al.,* 1982). Morphological conservatism, relatively low heterozygosity and high levels of between-population differentiation within an ancient lineage distinguish systematic and evolutionary studies of crustaceans.

Numerous taxonomic, systematic or phylogenetic electrophoretic studies of crustaceans have been undertaken, including the systematics of Australian *Daphnia* (Benzie, 1988), population structure in the American lobster *Homarus americanus* (Tracey *et al.*, 1975), phylogeography of Mediterranean *Proasellus* isopods (Ketmaier, 2002), genetic zoogeography and systematics of *Mysis relicta* in north America and Europe (Vainola *et al.*,1994), taxonomy and evolution in brine shrimp (Beardmore and Abreu-Grobois, 1983), taxonomic relations of *Atyid* prawns (Benzie and de Silva, 1984), systematics and affinities within Western Australian shrimp species (Boulton and Knott, 1984) and speciation in the *Jaera albifrons* isopod complex (Carvalho, 1988).

Biogeographic studies using allozyme data have reported relationships between genetic differentiation and isolation by distance (Arnaud, 2003; Jordaens *et al.*, 2001), past sea-level changes (Stevens and Hogg, 2004), glaciation (Vainola *et al.*, 1994; Alexandrino *et al.*, 2000),

climatic change, vicariance (Planes and Fauvelot, 2002) or combinations of these factors (Ketmaier, 2002).

Previous electrophoretic work on freshwater Tasmanian crustaceans includes a taxonomic and biogeographic review of the freshwater crayfish genus *Parastacoides* (Hansen, 2000), systematics and taxonomy of the freshwater crayfish *Engaeus* (Horwitz, 1990) and an unpublished systematics study of janirid isopods (Horwitz and Andrew, 1997). Low levels of genetic variability were found in the two freshwater crayfish genera, *Engaeus* (P = 0 - 0.15; H_e = 0 - 0.048; Horwitz, 1990) and *Parastacoides* (mean H_o = 0.026, range 0 - 0.114; Hansen, 2001). Low heterozygosity is a common feature of freshwater crayfish in Australia (Horwitz, 1990; Austin, 1996; Austin and Knott, 1996; Avery and Austin, 1997) and elsewhere (Nemeth and Tracey, 1979).

3.1.4 The genetics of *Anaspides tasmaniae* : aims of this study

There are two possible genetic bases for the survival of *Anaspides* species. Its survival for long periods through greatly differing climatic regimes and in various habitat types may be due to high levels of genetic diversity within populations. This would have provided the genetic flexibility to enable populations to adapt to different environmental conditions. It would be expected that eventually morphological differences would also arise, especially in isolated populations where genetic drift and adaptation have a greater impact. The second possibility is that although the genus may have limited genetic diversity it could have evolved, through selection, a specific genome that allows it to inhabit varied habitats with little phenotypic variation. Such a species would thus be a genetic as well as an ecological generalist, but would present low genetic diversity. As *Anaspides* is morphologically conservative one could predict the latter to be the case, implying low levels of genetic diversity within populations. In a study of marine fish, Smith and Fujio (1982) found high levels of variability in habitat specialists and low variability in habitat generalists.

The aim of this study is to clarify taxonomic relationships within the family Anaspididae by using allozyme electrophoresis to assess the amount of genetic diversity within and between isolated populations of *Anaspides* species. Hypotheses may then be established regarding their evolutionary history and the biogeographic forces which have shaped the distribution of the genus.

Information on the number of species of *Anaspides* and their distribution is needed (Swain *et al.*, 1970; Wells *et al.*, 1983; Horwitz, 1989; O'Brien, 1990) in order that the requirements for

conservation management of the genus can be identified. The resolution of the status of *Anaspides spinulae* as a separate species or as a synonym for *A. tasmaniae* is a priority. Although there appear to be distinct morphological differences in the spination on the posterior segments of the abdomen of the Lake St Clair and Clarence Lagoon *Anaspides* populations, doubt has been expressed about the specific status of these animals following the discovery of two intermediate forms at a number of Central Plateau sites (O'Brien, 1990) and a mitochondrial DNA study of the two groups (Jarman and Elliot, 2000). If the distinctive spination on the putative *A. spinulae* group justifies separate species status, *A. spinulae* would form a monophyletic branch separate from all *A. tasmaniae* populations in phylogenetic analyses.

3.2 Methods

3.2.1 Laboratory procedures

An approximately 0.5 cm x 0.5 cm portion of tissue was dissected from each animal for electrophoresis. Most specimens were too small to dissect different tissues separately so samples were taken from the abdomen which is predominantly muscle tissue. Sample preparation was carried out at 4 ¡C and samples were kept on ice or frozen at all times. Each sample was ground in a 1.5 ml microcentrifuge tube with an approximately equal volume of a homogenizing solution, which consisted of 100 ml distilled water, 100 microlitres of §-mercaptoethanol and 10 mg NADP (Richardson, Baverstock and Adams, 1986). Ground samples were spun at 10 000g for 3 minutes and the supernatant was used for electrophoresis.

Electrophoretic runs were carried out on Helena Titan III cellulose acetate plates, using either 200 volts through a tris-glycine buffer (0.02 M tris, 0.192 M glycine, pH 8.5) at room temperature, or 150 v through a tris-citrate buffer (75 mM tris, 25 mM citrate, pH 7.0) at 4 ¡C.

Thirty-three enzyme systems were examined, eighteen of which were able to be stained and scored reliably. Three of these encoded two loci, so a total of twenty-one loci was examined in the study. Electrophoretic conditions and quaternary structure for all loci used are given in Table 3.4. For those enzyme systems in which two loci were scored, the suffix 1 is used for the more anodally migrating enzyme and the suffix 2 for the more slowly migrating enzyme. Staining procedures followed those given in Hebert and Beaton (1989) or Richardson *et al.* (1986). Banding on gels was scored and interpreted conservatively. Differences in allozyme mobility between populations were always verified from more than one gel.

Table 3.4 Enzyme names and structure and electrophoretic conditions.

Enzyme	EC number	Locus	Buffer[#]	Run time (mins)	Subunit number*
Aspartate aminotransferase	2.6.1.1	*Aat-1*	B	65	2
		Aat-2	B	65	2
Alcohol dehydrogenase	1.1.1.1	*Adh*	A	25	unknown
Aldolase	4.1.2.13	*Ald*	A	25	unknown
Aldehyde oxidase	1.2.3.1	*Ao*	A	30	2
Arginine phosphokinase	2.7.3.3	*Apk*	A	45	unknown
Fumarase	4.2.1.2	*Fum*	B	65	unknown
Glyceraldhyde-3-phosphate dehydrogenase	1.2.1.12	*G3pdh*	A	30	3
Isocitrate dehydrogenase	1.1.1.42	*Idh*	B	70	unknown
Lactate dehydrogenase	1.1.1.27	*Ldh*	A	30	2
Malic dehydrogenase	1.1.1.37	*Mdh-1*	B	65	2
		Mdh-2	B	65	unknown
Malic enzyme	1.1.1.40	*Me*	A	25	unknown
Mannosephosphate isomerase	5.3.1.8	*Mpi*	A	20	1
Peptidase (leucine-tyrosine)	3.4.11.-	*Pep-lt-1*	B	70	2
		Pep-lt-2	B	70	uncertain
6-phosphogluconate dehydrogenase	1.1.1.44	*6pgdh*	B	50	2
Glucosephosphate isomerase	5.3.1.9	*Pgi*	A	20	2
Phosphoglucomutase	5.4.2.2	*Pgm*	A	20	1
Sorbitol dehydrogenase	1.1.1.14	*Sdh*	A	40	uncertain
Unknown dehydrogenase		*Nildh*	B	70	unknown

[#] Buffers : A=Tris-glycine ; B=Tris citrate.

* Although all loci in this study were polymorphic, no within-site variation was found at some loci. Where all variation was between sites (i.e. no heterozygotes were found), the sub-unit number is unknown.

3.2.2 Statistical analysis

Individual genotype data were analysed using statistical software Biosys-1 (Swofford and Selander, 1989) and Genepop v1.2 (Raymond and Rousset, 1995).

For all populations, including outgroups, allele frequencies, and indices of genetic variability (mean number of alleles per locus, percentage of loci polymorphic and mean heterozygosity) were calculated. Tests of Hardy Weinberg equilibrium were performed by Markov chain iterations with 1000 dememorisations in 100 batches and 1000 iterations, and chi-squared tests using Fisher s exact method (Genepop v1.2; Raymond and Rousset, 1995).

Nei s unbiased genetic distance (Nei, 1978) and the modified Rogers distance (Wright, 1978, cited in Swofford and Selander, 1989) were calculated for each pair of populations including outgroups by Biosys-1 (Swofford and Selander, 1989). Genetic distances were then subjected to cluster analysis to show relationships between populations. UPGMA (unweighted pair group method with averaging) cluster analysis and the related dendrogram of relationships between the sites were derived. This method assumes that all lineages have evolved at a constant rate. The use of the Wagner method to derive a dendrogram rooted by outgroups was also included as this method does not assume a constant rate of evolution and relates development within *Anaspides* to that between *Anaspides* and outgroup genera.

Genetic substructure across the geographical range was assessed through calculation of F-statistics (Raymond and Rousset, 1995) to further examine the variance revealed by the cluster analysis. F_{IS} and F_{IT} are fixation indices which characterise the distribution of genotypes within (F_{IS}) and between (F_{IT}) populations. F_{ST} is the proportion of the total variation which is found between subpopulations and is thus a measure of spatial differentiation :

$$(1 - F_{IT}) = (1 - F_{IS}) (1 - F_{ST})$$

In practical terms, $F_{ST} = \frac{H_T - H_S}{H_T}$ (= Nei s G_{ST}, Swofford and Selander, 1989), where H is the Hardy-Weinberg expectation of heterozygosity, S relates to the subpopulation and T relates to the total across all subpopulations, that is :

$H_S = 1 - (p^2 + q^2)$ for each subpopulation, and

$H_T = 1 - ([\text{mean } p]^2 + [\text{mean } q]^2)$, where p and q are allele frequencies.

F_{ST} ranges from 0 to |1|.

F-statistics were calculated for individual loci across all *Anaspides* populations and then combined over loci. Matrices of F_{ST} values between pairs of populations were also calculated over all loci in order to determine the spatial distribution of genetic variance. The methods for combining values from multiallelic loci and over many loci were as given by Genepop (Raymond and Rousset, 1995), from Weir and Cockerham (1984).

An isolation by distance model was used to test correlations of pairwise genetic differences between subpopulations (expressed as F_{ST} / 1 - F_{ST}) with physical distance, both direct and along watercourses. These calculations were performed using Genepop version 1.2 (Raymond and Rousset, 1995), incorporating Mantel tests, a permutation procedure which tests the null hypothesis of independence between genotype and distance. In this study, 1000 permutations were performed for each analysis. Direct geographical distance was calculated arithmetically using grid references and distances along waterways were measured using MapInfo Geographical Information Systems software with data of 1:25,000 resolution. Where populations were separated by sea, distances in marine waters were measured by the shortest possible route.

Each of the groups of populations revealed by the previous analyses was analysed separately using F-statistics and isolation by distance to examine more detailed affinities and processes within the groups.

To test the hypothesis that adaptation to the two basic habitat types, flowing and still water, had led to selection, the heterozygosity of the two groups was compared. Differentiation between the two groups was also tested using F-statistics applied to two datasets containing all the individuals in each habitat type.

3.3 Results

Allele frequencies in all samples are shown in Table 3.5. All loci were variable across all genera, but two loci, *Adh* and *Idh*, were monomorphic within *Anaspides*. Fixed differences among *Anaspides* samples (where there were no alleles in common) were found at all loci except *Adh*, *Idh*, *G3pdh*, *Mdh-1* and *Pep-1*, and appear to indicate considerable clustering of alleles within groups of populations over many loci.

Private alleles among *Anaspides* samples (where alleles were present in only one sample) were found at seven loci in seven samples (Table 3.6). Private alleles among all three genera were found at three loci in four *Anaspides* samples. The Wolfe Hole sample was monomorphic for private alleles at two loci, and the Mount Wellington and Hydro Creek samples were monomorphic for private alleles at one locus each. An allele at *Sdh* which was monomorphic in both southern cave populations was also unique across genera (Table 3.5). This allele ran cathodally, could be seen clearly on gels, and was scored as the slowest allele in that locus.

Table 3.5 Allele frequencies in all populations

	Population																						
Locus	**B**	**P**	**O**	**HC**	**L**	**J**	**WC**	**HM**	**WH**	**NC**	**AP**	**W**	**MF**	**MA**	**FC**	**WA**	**MW**	**SC**	**C**	**Ct**	***Pl***	***Ahi***	***Ah*H**	***Ah*P**
AAT-1																								
(N)	15	16	12	16	9	14	13	17	9	5	16	20	16	22	14	15	9	7	14	7	18	8	8	2
A	0.067	0.031	-	-	0.111	0.036	-	-	-	-	-	-	0.156	-	-	-	-	0.286	0.071	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.389	-	-	-
C	0.933	0.969	1	0.969	0.889	0.964	1	-	-	-	0.063	1	0.844	1	1	1	1	0.714	0.929	1	-	1	1	1
D	-	-	-	0.031	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	1	1	1	0.938	-	-	-	-	-	-	-	-	-	0.611	-	-	-
AAT-2																								
(N)	15	16	12	16	9	14	13	17	9	5	16	19	16	22	14	15	9	7	15	7	18	8	8	2
A	-	-	-	-	-	-	-	0.059	-	0.5	0.563	-	-	-	-	-	-	-	-	-	0.056	-	-	-
B	-	-	-	-	-	-	-	0.941	1	0.5	0.438	-	-	-	0.929	-	-	-	-	-	0.944	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	1	1	1	0.071	1	1	-	-	-	-	0.063	-	1
D	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	1	1	1	-	0.938	1	-
ADH																								
(N)	15	16	12	13	9	14	13	17	9	3	14	20	16	19	14	13	9	7	13	5	20	8	8	2
A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
ALD																								
(N)	15	15	12	16	9	14	13	14	9	5	16	20	16	18	14	15	9	7	15	7	18	4	4	2
A	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
C	1	1	1	1	1	1	0.269	-	-	-	-	1	0.031	1	-	1	1	1	1	1	-	-	1	1
D	-	-	-	-	-	-	0.731	-	-	-	-	-	0.969	-	1	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.972	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.028	-	-	-
AO																								
(N)	15	16	10	16	9	14	13	15	9	5	16	20	16	22	14	15	9	7	15	7	18	8	8	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
C	1	1	1	1	1	1	1	1	1	1	1	-	1	-	-	-	1	1	1	1	-	-	1	1
D	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	1	-	-	-	-	-	-	-	-

Locus	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C	Ct	*Pl*	*Ahi*	*Ah*H	*Ah*P
APK																								
(N)	15	16	10	16	9	14	13	15	9	5	16	20	12	14	14	15	9	7	15	7	20	8	8	2
A	-	-	-	-	-	-	-	-	0.889	1	0.313	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	1	0.111	-	0.688	-	0.958	-	-	-	0.722	-	-	-	-	1	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.125	-	0.938	1
D	0.267	0.781	0.15	-	-	0.179	0.346	-	-	-	-	0.825	0.042	0.929	0.286	0.733	0.278	0.143	0.267	0.429	0.8	-	-	-
E	0.733	0.219	0.85	1	1	0.821	0.654	-	-	-	-	0.175	-	0.071	0.714	0.267	-	0.857	0.733	0.571	0.075	-	0.063	-
FUM																								
(N)	14	16	12	16	9	14	13	15	9	5	15	20	16	22	14	12	9	7	15	7	20	8	8	2
A	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	1	-	1	1	-	-	-	-	-	-	-	-	-	1	-	-	-
C	1	1	1	1	1	1	1	-	-	-	-	-	1	-	-	-	1	1	1	1	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1
E	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	0.625	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.375	-	-	-	-	-	-	-	-
G3PD																								
(N)	11	16	12	15	9	14	13	15	9	5	16	20	12	21	14	15	9	7	14	5	17	8	8	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1
B	-	-	-	-	0.111	-	-	-	-	0.1	0.219	-	0.292	0.071	0.107	-	0.167	-	-	-	-	-	-	-
C	0.636	1	1	0.967	0.889	1	1	1	1	0.9	0.781	1	0.708	0.929	0.893	1	0.833	1	1	1	1	-	-	-
D	0.364	-	-	0.033	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IDH																								
(N)	15	14	10	16	9	14	13	10	9	5	16	15	16	22	14	15	9	7	15	7	20	8	8	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-
LDH																								
(N)	15	13	10	16	9	14	13	15	9	5	13	20	14	21	13	15	9	7	15	7	19	8	8	2
A	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	0.033	-	-	-	-	0.053	-	-	-
B	1	1	1	1	1	1	-	-	-	-	-	-	1	1	-	-	-	1	1	1	-	-	-	-
C	-	-	-	-	-	-	1	-	-	-	-	1	-	-	1	0.967	-	-	-	-	0.921	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	0.026	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1

Locus	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C	Ct	*Pl*	*Ahi*	*Ah*H	*Ah*P
MDH-1																								
(N)	15	16	15	13	9	14	13	15	9	5	16	20	16	15	14	15	9	7	15	7	20	8	8	2
A	1	1	1	1	1	1	1	1	1	1	0.969	0.975	1	1	1	1	1	1	1	1	1	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.938	-	-
C	-	-	-	-	-	-	-	-	-	-	0.031	0.025	-	-	-	-	-	-	-	-	-	0.063	0.25	1
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.75	-
MDH-2																								
(N)	14	16	15	12	9	14	13	15	9	5	16	20	12	14	14	14	9	5	15	6	20	8	8	2
A	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-
B	-	-	-	-	-	-	-	1	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
C	1	1	1	-	1	1	1	-	-	-	-	1	1	1	1	1	1	1	1	1	0.975	-	-	-
D	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
ME																								
(N)	14	13	12	13	9	14	13	15	9	5	11	20	16	22	14	15	9	7	15	6	20	6	6	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	1	-	-	-	-	-	1	1	1
C	1	1	1	1	1	1	1	1	1	1	1	-	1	-	-	-	1	1	1	1	-	-	-	-
MPI																								
(N)	15	16	12	16	9	14	13	15	9	5	16	20	16	22	14	15	9	7	15	7	20	8	7	2
A	-	-	-	-	-	-	-	0.8	1	0.9	1	-	-	-	-	-	-	-	-	-	-	-	0.929	1
B	-	-	-	-	-	-	-	0.2	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	0.967	1	1	1	0.889	0.893	1	-	-	-	-	-	1	-	-	-	-	0.929	0.6	0.857	-	1	0.071	-
D	-	-	-	-	-	-	-	-	-	-	-	0.075	-	0.023	-	-	-	-	-	-	-	-	-	-
E	0.033	-	-	-	0.111	0.107	-	-	-	-	-	0.925	-	0.977	1	1	1	0.071	0.2	0.071	0.075	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.071	0.825	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-
PEP-1																								
(N)	15	16	15	16	9	14	13	15	9	5	16	20	16	22	14	15	9	7	15	7	20	8	8	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.071	-	-	-	-	0.125	-
B	1	1	0.933	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.929	1	1	0.1	-	0.438	1
C	-	-	0.067	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.438	-

Locus	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C	Ct	*Pl*	*Ahi*	*Ah*H	*Ah*P
PEP-2																								
(N)	15	16	15	16	9	14	13	15	9	5	16	20	16	22	14	15	9	7	15	7	20	8	8	2
A	-	0.031	-	-	-	-	-	-	-	-	-	1	-	1	1	1	-	-	-	-	-	-	-	-
B	1	0.969	1	1	1	1	-	-	-	-	-	-	1	-	-	-	-	0.643	1	1	-	-	-	-
C	-	-	-	-	-	-	1	1	-	1	1	-	-	-	-	-	-	0.357	-	-	-	-	-	-
D	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1
6PGDH																								
(N)	15	16	12	16	9	14	13	15	9	5	16	20	16	22	14	15	9	5	11	4	18	8	8	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
C	0.133	0.625	0.125	-	0.111	0.179	-	-	-	-	-	0.025	-	-	-	-	-	0.2	0.227	-	0.028	-	-	-
D	0.1	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-	0.2	-	-	0.917	-	-	-
E	0.767	0.375	0.875	1	0.889	0.821	0.923	-	-	-	-	0.975	0.875	0.977	1	1	1	0.6	0.773	1	0.056	-	-	-
F	-	-	-	-	-	-	0.077	-	-	-	-	-	0.125	0.023	-	-	-	-	-	-	-	-	-	-
PGI																								
(N)	15	16	12	16	9	14	13	15	9	5	14	21	16	22	14	15	9	7	15	7	20	8	6	2
A	-	-	-	-	-	-	-	-	-	-	0.071	-	-	-	-	-	-	-	-	-	-	-	0.667	1
B	-	-	-	0.25	-	-	-	-	-	1	0.107	-	-	-	-	-	-	-	-	-	-	1	-	-
C	1	1	1	0.156	1	1	1	1	1	-	0.821	-	1	1	-	1	1	1	0.833	1	-	-	0.333	-
D	-	-	-	0.594	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	0.167	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.975	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-
PGM																								
(N)	15	16	12	16	9	14	13	15	9	5	16	20	16	22	14	15	9	7	15	7	20	8	8	2
A	-	-	-	-	-	-	-	-	-	-	0.156	-	-	-	-	-	-	-	-	-	-	-	-	-
B	0.967	0.813	0.875	1	0.833	0.893	1	0.3	-	-	0.594	0.975	1	1	0.714	0.967	-	1	1	1	-	1	-	-
C	-	0.188	0.125	-	0.167	0.107	-	0.433	1	1	0.25	0.025	-	-	0.286	0.033	1	-	-	-	-	-	-	-
D	0.033	-	-	-	-	-	-	0.267	-	-	-	-	-	-	-	-	-	-	-	-	0.075	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.675	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.25	-	0.313	0.5
G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.688	0.5

Locus	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C	Ct	*Pl*	*Ahi*	*Ah*H	*Ah*P
SDH																								
(N)	15	16	10	13	9	14	13	15	9	5	16	20	7	10	14	12	9	6	15	6	17	3	5	2
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1
B	1	1	1	1	1	1	1	0.067	-	-	-	-	1	-	-	-	-	1	1	1	-	-	-	-
C	-	-	-	-	-	-	-	0.933	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	1	1	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
F	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NILDH																								
(N)	15	16	12	16	9	14	13	15	9	5	16	21	16	22	11	15	9	7	15	7	17	6	4	1
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
C	1	1	1	1	1	1	1	1	1	1	1	-	1	-	-	-	1	1	1	1	-	-	1	1
D	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-

Key to populations

P	Lake Ball	**WH**	Wolfe Hole	**MW**	Mt Wellington
P	Powena Ck	**NC**	Newdegate Cave	**SC**	Lake St Clair (*A. spinulae*)
O	Olive Lagoon	**AP**	Adamsons Peak	**C**	Clarence Lagoon (*A. spinulae*)
HC	Hydro Ck	**W**	Weld River	**Ct**	Clarence Lagoon tributary
L	Lonely Lake	**MF**	Mount Field	***Pl***	*Paranaspides lacustris*
J	Jacks Lagoon	**MA**	Mount Anne	***Ahi***	*Allanaspides hickmani*
WC	Wet Cave	**FC**	Frenchmans Cap	***Ah*H**	*Allanaspides helonomus* Harlequin Hill
HM	Hartz Mtns	**WA**	Western Arthurs	***Ah*P**	*Allanaspides helonomus* McPartlans Pass

Table 3.6 Private alleles among samples of the *Anaspides* genus and three anaspid genera.

Locus	Allele	Population	Monomorphic	Private among *Anaspides* species	Private among all genera
Aat-1	D	Hydro Creek	-	x	x
Fum	A	Wolfe Hole	x	x	x
Fum	F	Western Arthurs	-	x	x
Ldh	D	Mount Wellington	x	x	-
Mdh-2	A	Hydro Creek	x	x	-
Mdh-2	D	Wolfe Hole	x	x	-
Pep-1	A	Lake St Clair	-	x	-
Pep-1	C	Olive Lagoon	-	x	-
Pgi	A	Adamsons Peak	-	x	-
Pgm	A	Adamsons Peak	-	x	x

Hardy-Weinberg tests confirmed that all samples are likely to have come from randomly mating populations. Probability values for all loci and all populations are given in Table 3.7, with no p-values indicating significant deviation from Hardy-Weinberg equilibrium. An anomaly was found at the *Mpi* locus for the Clarence Lagoon tributary population of *A. tasmaniae* ($p = 0.070$), as one heterozygote was found to have alleles not present in the remaining samples, which were homozygous for a third allele. Interestingly, the two alleles in the heterozygote were both found in the downstream Clarence Lagoon population of *A. spinulae*, indicating possible gene flow between the two populations. Further sampling of the Clarence Lagoon tributary population may reveal more widespread variation as only seven animals were sampled.

Measures of genetic variability for eighteen populations of *A. tasmaniae,* two populations of *A. spinulae* and three outgroup species of Anaspididae are shown in Table 3.8. Mean expected heterozygosity for eighteen *A. tasmaniae* populations was 0.048, with a range of 0.010 to 0.114. The Mount Anne and Wolfe Hole samples had the lowest H_e values (< 0.02), and the Adamsons Peak sample had the highest value of 0.114. The *A. spinulae* populations had somewhat higher variability ($H_e = 0.100$ and 0.085; $H_o = 0.113$ and 0.082), as did *Paranaspides lacustris* with $H_e = 0.114$ and proportion of polymorphic loci of 52.4%. *Allanaspides helonomus* was much more heterozygous than *Allanaspides hickmani* (H_e of 0.107 compared to 0.012).

Table 3.7 Tests for Hardy-Weinberg equilibrium and chi-squared tests

Population & Locus	P-value	S.E.	F_{IS} *	All loci ** chi-squared	d.f.	P-value
Anaspides species						
Lake Ball				5.358	8	0.716
AAT-1	1	0	-0.037			
AO	1	0	+0.012			
G3PDH	0.537	0.002	+0.259			
6PGDH	0.126	0.004	+0.337			
Powena Creek				3.973	6	0.680
AO	1	0	+0.118			
6PGDH	0.317	0.002	-0.304			
PGM	0.433	0.002	+0.211			
Olive Lagoon				0	8	1.00
AO	1	0	-0.125			
PEP-1	1	0	-0.037			
6PGDH	1	0	-0.100			
PGM	1	0	-0.100			
Hydro Creek						
PGI	0.653	0.004	+0.140			
Lonely Lake				0	10	1.00
AAT-1	1	0	-0.067			
G3PD	1	0	-0.067			
MPI	1	0	-0.067			
6PGDH	1	0	-0.067			
PGM	1	0	-0.143			
Jacks Lagoon				8.598	8	0.377
AO	0.346	0.002	+0.304			
MPI	1	0	-0.083			
6PGDH	0.348	0.002	+0.304			
PGM	0.113	0.002	+0.649			
Wet Cave				4.664	6	0.588
ALD	0.168	0.002	+0.446			
AO	0.578	0.002	+0.189			
6PGDH	1	0	-0.043			
Hartz Mtns				2.745	8	0.949
AAT-2	1	0	-0.032			
MPI	0.458	0.002	+0.200			
PGM	0.554	0.004	+0.113			
SDH	1	0	-0.037			
Wolfe Hole						
AO	1	0	-0.067			
Newdegate Cave						
AAT-2	1	0	-0.091			

Table 3.7 (cont.)

Population & Locus	P-value	S.E.	F_{IS} *	All loci ** chi-squared	d.f.	P-value
Adamsons Peak				5.216	12	0.950
AAT-1	1	0	-0.034			
AAT-2	0.348	0.002	+0.268			
AO	0.594	0.002	+0.159			
G3PDH	0.543	0.002	-0.250			
PGI	1	0	-0.121			
PGM	0.656	0.004	+0.140			
Weld River				1.537	4	0.820
AO	0.464	0.002	+0.159			
MPI	1	0	-0.056			
Mount Field				4.770	6	0.574
AAT-1	1	0	-0.154			
G3PDH	0.487	0.002	-0.375			
6PGDH	0.189	0.002	+0.455			
Mount Anne				0	4	1
AO	1	0	-0.040			
G3PDH	1	0	-0.053			
Frenchmans Cap				2.694	8	0.952
AAT-2	1	0	-0.040			
AO	0.260	0.002	+0.333			
G3PDH	1	0	-0.083			
PGM	1	0	-0.013			
Western Arthurs				0	4	1
AO	1	0	+0.012			
FUM	1	0	+0.154			
Mt Wellington				0	4	1
AO	1	0	-0.333			
G3PDH	1	0	-0.143			
Lake St Clair				0	8	1
AAT-1	1	0	-0.333			
AO	1	0	-0.091			
PEP-2	1	0	+0.143			
6PGDH	1	0	-0.333			
Clarence Lagoon				3.595	10	0.964
AAT-1	1	0	-0.040			
AO	1	0	+0.012			
MPI	0.509	0.004	+0.082			
6PGDH	1	0	-0.250			
PGI	0.326	0.002	+0.311			
Clarence Lagoon Tributary				5.249	4	0.263
AO	1	0	-0.091			
MPI	0.073	0.003	+0.500			

Table 3.7 (cont.)

Population & Locus	P-value	S.E.	F_{IS} *	All loci ** chi-squared	d.f.	P-value
Paranaspides lacustris				5.662	16	0.991
AAT-1	0.646	0.002	-0.141			
AAT-2	1	0	-0.030			
AO	0.589	0.006	-0.008			
LDH	1	0	-0.038			
MPI	0.459	0.006	+0.038			
PEP-1	1	0	-0.086			
6PGDH	1	0	-0.041			
PGM	0.338	0.004	+0.081			
Allanaspides hickmani						
Allanaspides helonomus **(Harlequin Hill)**				5.848	8	0.664
MDH-1	0.381	0.002	+0.391			
PEP-1	0.288	0.004	-0.400			
PGI	1	0	-0.429			
PGM	0.490	0.002	-0.400			
Allanaspides helonomus **(McPartlans Pass)**						
PGM	1	0	-1			

* Weir & Cockerham, 1984
** Fisher s Method

Mean observed heterozygosity for *Anaspides tasmaniae* was 0.046 (range 0.011 - 0.106) and the mean percentage polymorphic loci (95% criterion for polymorphism, where the most common allele does not have a frequency of over 95%) was 19% (range 4.8 - 33.3%). The mean number of alleles per locus was 1.2 (range 1.0 - 1.4).

Five loci used here were categorised according to substrate source by Hedgecock *et al.*. (1982) as Goup II enzymes, *Adh, Ao, Pep-1, Pep-2* and *Sdh.* These loci have mean H_e values across all *Anaspides* populations of 0, 0.271, 0.014, 0.028 and 0.006 respectively (mean 0.064), and mean number of alleles (N_a) of 3.2. The remaining loci belong to Group I, with the exception of *Nildh* which was excluded as its substrate is unknown, and have H_e values ranging from 0 to 0.154 (mean 0.053) and mean N_a of 3.5. A t-test revealed no significant difference between the two groups ($p = 0.84$).

Table 3.8 Genetic variability at 21 loci in all populations [*Unbiased estimate (Nei, 1978)]

Population	Mean sample size per locus (S.E.)	Mean no. of alleles per locus (S.E.)	% loci polymorphic: 95% (100%) criterion	Mean heterozygosity Direct-count (S.E.)	Mean heterozygosity HW expected* (S.E.)
***Anaspides tasmaniae* - surface populations**					
Central Plateau - near Lake Ball	14.7 (0.2)	1.33 (0.1)	19.0 (28.6)	0.062 (0.027)	0.074 (0.033)
Central Plateau - near Powena Creek	15.6 (0.2)	1.2 (0.1)	14.3 (23.8)	0.063 (0.034)	0.061 (0.030)
Central Plateau - near Olive Lagoon	12.1 (0.4)	1.2 (0.1)	19.0 (19.0)	0.044 (0.021)	0.041 (0.019)
Central Plateau - Hydro Creek	15.2 (0.3)	1.2 (0.1)	4.8 (14.3)	0.030 (0.024)	0.034 (0.028)
Central Plateau - Lonely Lake	9.0 (0.0)	1.2 (0.1)	23.8 (23.8)	0.058 (0.024)	0.054 (0.022)
Central Plateau - Jacks Lagoon outflow	14.0 (0.0)	1.2 (0.1)	19.0 (23.8)	0.037 (0.017)	0.051 (0.023)
Central Plateau - Clarence Lagoon tributary	6.5 (0.2)	1.1 (0.1)	9.5 (9.5)	0.034 (0.028)	0.038 (0.028)
Mount Wellington	9.0 (0.0)	1.1 (0.1)	9.5 (9.5)	0.042 (0.030)	0.034 (0.024)
Hartz Mountains	15.0 (0.3)	1.2 (0.1)	19.0 (19.0)	0.053 (0.031)	0.059 (0.035)
Adamsons Peak	15.4 (0.3)	1.4 (0.1)	28.6 (33.3)	0.106 (0.039)	0.114 (0.043)
Weld River	19.8 (0.3)	1.2 (0.1)	9.5 (23.8)	0.026 (0.014)	0.028 (0.015)
Mount Field	14.9 (0.5)	1.2 (0.1)	14.3 (23.8)	0.056 (0.031)	0.051 (0.025)
Mount Anne	19.9 (0.8)	1.2 (0.1)	9.5 (19.0)	0.018 (0.010)	0.017 (0.009)
Frenchmans Cap	13.8 (0.1)	1.2 (0.1)	19.0 (19.0)	0.051 (0.026)	0.056 (0.029)
Western Arthur Range - Lake Oberon	14.6 (0.2)	1.2 (0.1)	9.5 (19.0)	0.045 (0.027)	0.049 (0.029)
***Anaspides tasmaniae* - cave populations**					
Wolfe Hole	9.0 (0.0)	1.0 (0.0)	4.8 (4.8)	0.011 (0.011)	0.010 (0.010)
Newdegate Cave	4.9 (0.1)	1.1 (0.1)	14.3 (14.3)	0.048 (0.031)	0.046 (0.029)
Wet Cave	13.0 (0.0)	1.1 (0.1)	14.3 (14.3)	0.037 (0.022)	0.049 (0.029)
Mean *A. tasmaniae*	**13.1**	**1.19**	**14.5**	**0.046**	**0.048**

Table 3.8 Genetic variability (cont.)

Population	Mean sample size per locus (S.E.)	Mean no. of alleles per locus (S.E.)	Percentage of loci polymorphic: 95% (100%) criterion	Mean heterozygosity Direct-count (S.E.)	Mean heterozygosity Hardy-Weinberg expected* (S.E.)
Anaspides spinulae					
Lake St Clair	6.8 (0.1)	1.3 (0.1)	28.6 (28.6)	0.113 (0.049)	0.100 (0.042)
Clarence Lagoon	14.6 (0.2)	1.3 (0.1)	23.8 (23.8)	0.082 (0.037)	0.085 (0.037)
Mean *A. spinulae*	**10.7**	**1.3**	**26.2**	**0.098**	**0.093**
Paranaspides lacustris					
Woods Lake	19.0 (0.3)	1.8 (0.2)	38.1 (52.4)	0.117 (0.036)	0.114 (0.035)
Allanaspides hickmani					
McPartlans Pass	7.4 (0.3)	1.1 (0.1)	9.5 (9.5)	0.012 (0.008)	0.012 (0.008)
Allanaspides helonomus					
Harlequin Hill area	7.2 (0.3)	1.3 (0.1)	28.6 (28.6)	0.128 (0.057)	0.107 (0.044)
McPartlans Pass	2.0 (0.0)	1.0 (0.0)	4.8 (4.8)	0.048 (0.048)	0.032 (0.032)
Mean *Allan. helonomus*	**4.6**	**1.2**	**16.7**	**0.088**	**0.07**

Values of Nei s unbiased genetic distance D (Nei, 1978) for intraspecific *Anaspides* populations varied widely, from 0.001 to 1.435, indicating significant genetic similarities among some populations and differentiation between others (Table 3.9). The highest intraspecific genetic distances were those between Newdegate Cave and other *A. tasmaniae*, except for the three neighbouring populations in the Huon area (range 0.810 - 1.435). The lowest intraspecific values were those amongst the six Central Plateau sites, Lake Ball, Powena Creek, Olive Lagoon, Lonely Lake, Jacks Lagoon and Clarence Lagoon tributary, and between the two *A. spinulae* populations. Distances between Jacks Lagoon and the other Central Plateau populations consistently gave the lowest distance values in the study.

Genetic distances between *A. spinulae* and *A. tasmaniae* populations fell within the lower end of the intraspecific range, from 0.003 to 0.935. The genetic distance between the two *Allanaspides* species was a high interspecific value at 0.899.

Table 3.9 Genetic distance matrices — Nei s unbiased D (1978) below diagonal; modified Rogers D above diagonal. A. All populations and taxa.

	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C	Ct	*Pl*	*Ahi*	*Ah*H	*Ah*P
B		0.174	0.092	0.292	0.102	0.088	0.359	0.709	0.737	0.761	0.693	0.702	0.368	0.636	0.727	0.657	0.560	0.129	0.114	0.101	0.844	0.882	0.798	0.847
P	0.030		0.177	0.352	0.208	0.166	0.383	0.703	0.727	0.752	0.692	0.699	0.388	0.631	0.741	0.658	0.555	0.192	0.170	0.164	0.830	0.888	0.802	0.850
O	0.007	0.032		0.278	0.056	0.032	0.352	0.713	0.738	0.762	0.701	0.704	0.372	0.641	0.724	0.658	0.554	0.119	0.097	0.078	0.847	0.891	0.807	0.857
HC	0.092	0.137	0.082		0.279	0.281	0.449	0.742	0.770	0.759	0.723	0.715	0.467	0.704	0.750	0.718	0.632	0.302	0.284	0.290	0.880	0.883	0.817	0.857
L	0.008	0.044	0.001	0.083		0.053	0.360	0.710	0.734	0.758	0.697	0.706	0.377	0.643	0.719	0.659	0.548	0.118	0.102	0.110	0.851	0.894	0.806	0.854
J	0.006	0.028	0.000	0.084	0.000		0.353	0.707	0.733	0.757	0.695	0.696	0.371	0.632	0.718	0.650	0.546	0.110	0.075	0.073	0.844	0.891	0.804	0.853
WC	0.145	0.166	0.137	0.234	0.144	0.138		0.674	0.737	0.730	0.659	0.680	0.430	0.685	0.659	0.635	0.579	0.322	0.361	0.349	0.815	0.885	0.825	0.873
HM	0.767	0.743	0.761	0.858	0.759	0.749	0.650		0.489	0.402	0.166	0.840	0.685	0.816	0.808	0.804	0.692	0.675	0.702	0.712	0.776	0.951	0.868	0.882
WH	0.834	0.790	0.815	0.928	0.808	0.804	0.817	0.282		0.452	0.497	0.862	0.742	0.839	0.823	0.826	0.678	0.717	0.731	0.741	0.790	0.968	0.877	0.890
NC	0.945	0.900	0.926	0.910	0.917	0.915	0.810	0.183	0.232		0.359	0.856	0.768	0.861	0.829	0.848	0.739	0.731	0.750	0.765	0.796	0.962	0.880	0.884
AP	0.746	0.738	0.753	0.823	0.748	0.740	0.634	0.027	0.302	0.145		0.822	0.677	0.801	0.805	0.790	0.698	0.661	0.687	0.698	0.782	0.933	0.846	0.857
W	0.728	0.713	0.718	0.748	0.729	0.701	0.652	1.336	1.416	1.418	1.288		0.716	0.379	0.449	0.235	0.633	0.698	0.679	0.686	0.810	0.914	0.879	0.857
MF	0.153	0.171	0.155	0.256	0.160	0.155	0.214	0.682	0.837	0.958	0.684	0.761		0.649	0.716	0.674	0.537	0.384	0.376	0.364	0.858	0.886	0.865	0.855
MA	0.549	0.532	0.548	0.709	0.557	0.531	0.662	1.176	1.251	1.435	1.153	0.158	0.571		0.456	0.320	0.597	0.634	0.618	0.619	0.839	0.917	0.873	0.861
FC	0.826	0.870	0.796	0.887	0.785	0.782	0.608	1.176	1.201	1.276	1.223	0.235	0.776	0.241		0.451	0.686	0.720	0.700	0.719	0.789	0.915	0.897	0.903
WA	0.611	0.609	0.601	0.770	0.607	0.585	0.547	1.142	1.212	1.393	1.127	0.058	0.646	0.111	0.240		0.581	0.652	0.638	0.641	0.806	0.909	0.864	0.852
MW	0.398	0.387	0.381	0.531	0.374	0.370	0.428	0.695	0.632	0.833	0.738	0.531	0.355	0.454	0.676	0.432		0.557	0.542	0.546	0.822	0.945	0.841	0.828
SC	0.012	0.035	0.009	0.097	0.009	0.007	0.112	0.673	0.775	0.842	0.660	0.724	0.167	0.547	0.814	0.604	0.395		0.127	0.142	0.828	0.882	0.799	0.849
C	0.011	0.029	0.007	0.087	0.008	0.003	0.147	0.750	0.817	0.910	0.733	0.667	0.161	0.512	0.744	0.569	0.370	0.012		0.087	0.828	0.888	0.798	0.844
Ct	0.008	0.026	0.004	0.089	0.010	0.003	0.134	0.757	0.823	0.935	0.743	0.665	0.146	0.497	0.776	0.557	0.367	0.015	0.005		0.842	0.892	0.808	0.856
Pl	1.529	1.393	1.490	1.797	1.545	1.490	1.277	1.067	1.088	1.156	1.159	1.217	1.606	1.390	1.134	1.218	1.301	1.433	1.420	1.443		0.944	0.914	0.932
Ahi	1.664	1.694	1.679	1.597	1.744	1.707	1.643	2.746	2.928	3.015	2.605	1.913	1.660	1.911	2.009	1.905	2.442	1.710	1.751	1.685	2.978		0.750	0.802
***Ah*H**	1.188	1.194	1.198	1.249	1.205	1.195	1.323	1.700	1.675	1.779	1.604	1.734	1.649	1.651	2.050	1.631	1.408	1.215	1.197	1.199	2.709	0.899		0.307
***Ah*P**	1.394	1.394	1.412	1.408	1.414	1.400	1.552	1.663	1.637	1.644	1.541	1.400	1.418	1.410	1.877	1.394	1.219	1.440	1.389	1.399	2.668	1.059	0.097	

Table 3.9 (cont.)

B. Central Plateau / Derwent catchment populations — *A.tasmaniae*and *A. spinulae*

	B	P	O	HC	L	J	WC	MF	MW	SC	C	Ct
B		0.174	0.092	0.292	0.102	0.088	0.359	0.368	0.560	0.129	0.114	0.101
P	0.030		0.177	0.352	0.208	0.166	0.383	0.388	0.555	0.192	0.170	0.164
O	0.007	0.032		0.278	0.056	0.032	0.352	0.372	0.554	0.119	0.097	0.078
HC	0.092	0.137	0.082		0.279	0.281	0.449	0.467	0.632	0.302	0.284	0.290
L	0.008	0.044	0.001	0.083		0.053	0.360	0.377	0.548	0.118	0.102	0.110
J	0.006	0.028	0.000	0.084	0.000		0.353	0.371	0.546	0.110	0.075	0.073
WC	0.145	0.166	0.137	0.234	0.144	0.138		0.430	0.579	0.322	0.361	0.349
MF	0.153	0.171	0.155	0.256	0.160	0.155	0.214		0.537	0.384	0.376	0.364
MW	0.398	0.387	0.381	0.531	0.374	0.370	0.428	0.355		0.557	0.542	0.546
SC	0.012	0.035	0.009	0.097	0.009	0.007	0.112	0.167	0.395		0.127	0.142
C	0.011	0.029	0.007	0.087	0.008	0.003	0.147	0.161	0.370	0.012		0.087
Ct	0.008	0.026	0.004	0.089	0.010	0.003	0.134	0.146	0.367	0.015	0.005	

C. Southern populations - *A. tasmaniae*

	HM	WH	NC	AP
HM		0.489	0.402	0.166
WH	0.282		0.452	0.497
NC	0.183	0.232		0.359
AP	0.027	0.302	0.145	

D. Southwestern populations - *A. tasmaniae*

	W	MA	FC	WA
W		0.379	0.449	0.235
MA	0.158		0.456	0.320
FC	0.235	0.241		0.451
WA	0.058	0.111	0.240	

Population

B Lake Ball
P Powena Creek
O Olive Lagoon
HC Hydro Creek
L Lonely Lake
J Jacks Lagoon
WC Wet Cave
HM Hartz Mtns
WH Wolfe Hole
NC Newdegate Cave
AP Adamsons Peak
W Weld River
MF Mount Field
MA Mount Anne
FC Frenchmans Cap
WA Western Arthurs
MW Mount Wellington
SC Lake St Clair (*A. spinulae*)
C Clarence Lagoon (*A. spinulae*)
Ct Clarence Tributary
Pl *Paranaspides lacustris*
Ahe *Allanaspides hickmani*
AhH Allanaspides *helonomus* Harlequin Hill
AhP *Allanaspides helonomus* McPartlans Pass

Intergeneric values ranged from 1.067 to 3.015 and were generally highest between *Paranaspides* and *Allanaspides*. In some instances intraspecific comparisons of Nei s D values, between populations of *Anaspides tasmaniae*, exceeded those between different genera (Table 3.9).

The cluster analyses and dendrograms from both the Nei s and modified Rogers distance values revealed each of the three genera to be monophyletic but the unrooted Wagner tree (using modified Rogers D) grouped *Paranaspides lacustris* with *Allanaspides* whereas the UPGMA tree (using Nei s D) placed it closer to the *Anaspides*. No differentiation between the two named species of *Anaspides, A. spinulae* and *A. tasmaniae,* was found (Figures 3.1 and 3.2).

Very high levels of differentiation were revealed between three geographically separate groups of populations in the *Anaspides* genus, based in the Central Plateau / Derwent catchment, the southern Huon region, and the southwest.

The Central Plateau group included the Lake Ball, Olive Lagoon, Jacks Lagoon, Lonely Lake, Clarence Lagoon tributary, Powena Creek, Hydro Creek, Wet Cave and Mt Field populations of *A. tasmaniae* as well as both the *A. spinulae* populations of Clarence Lagoon and Lake St Clair. Hydro Creek, Wet Cave and Mount Field, in that order, appeared increasingly genetically distant from the other populations in the group. Mt Wellington was considerably more distant; it clustered with the Central Plateau group in the UPGMA tree and with the southwest group in the Wagner tree. Using Thorpe s (1983) guidelines, all the Central Plateau group belongs to the same species (Nei s unbiased $D < 0.3$); however, the Mount Wellington population did not join this group (D values with Central Plateau populations of 0.355 - 0.531). Whether Mt Wellington should be considered as the same species as the Central Plateau group may be elucidated by further morphological and molecular comparisons.

The southwest group consisted of populations from Mount Anne, the Western Arthur Range, Weld River and Frenchmans Cap and appeared to have greater affinities with the Central Plateau group than with the southern group, despite the fact that Mount Anne and the Weld River are both in the Huon catchment.

The southern group consists of Adamsons Peak, Hartz Mountains and the two cave populations, Wolfe Hole and Newdegate Cave. The surface populations are very closely

Figure 3.1 UPGMA dendrogram

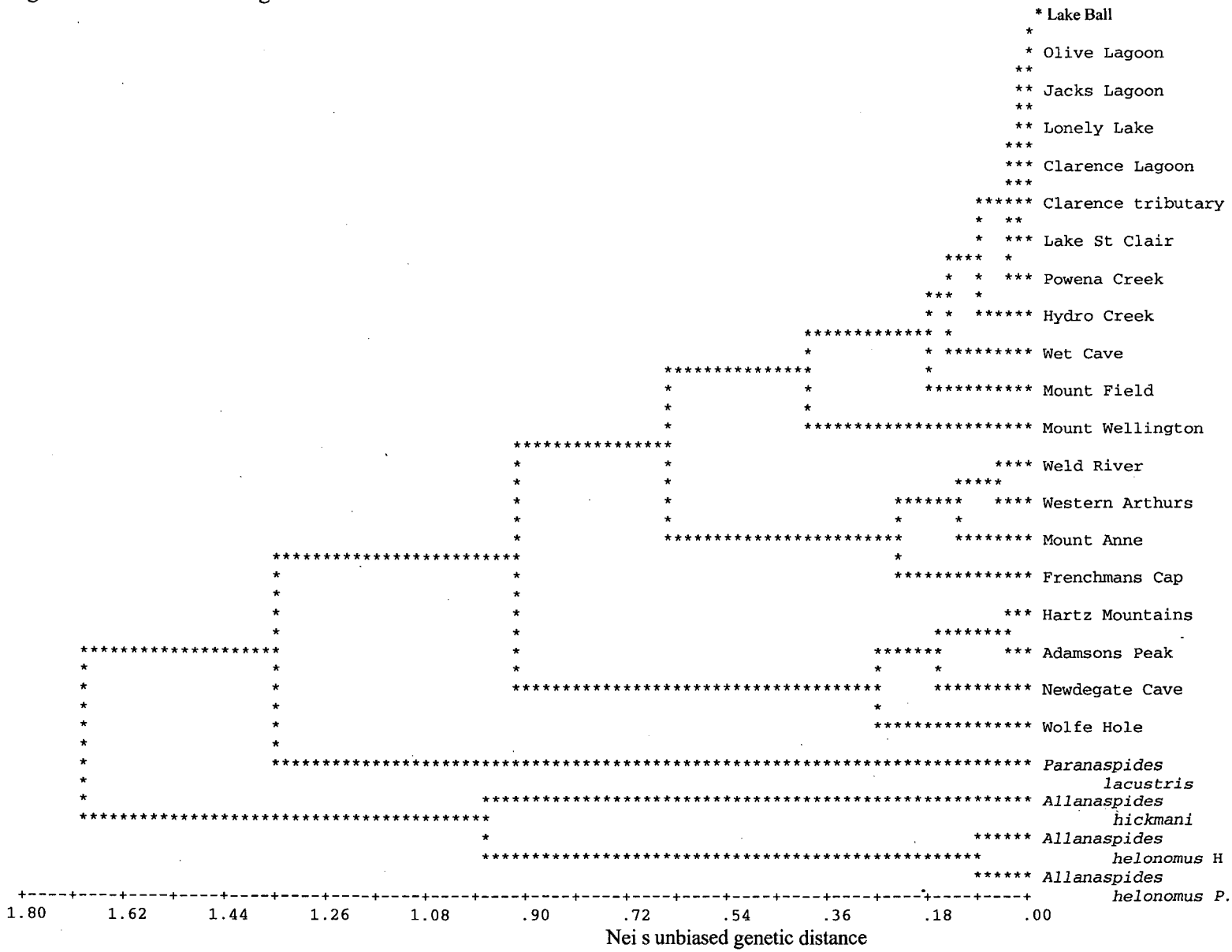
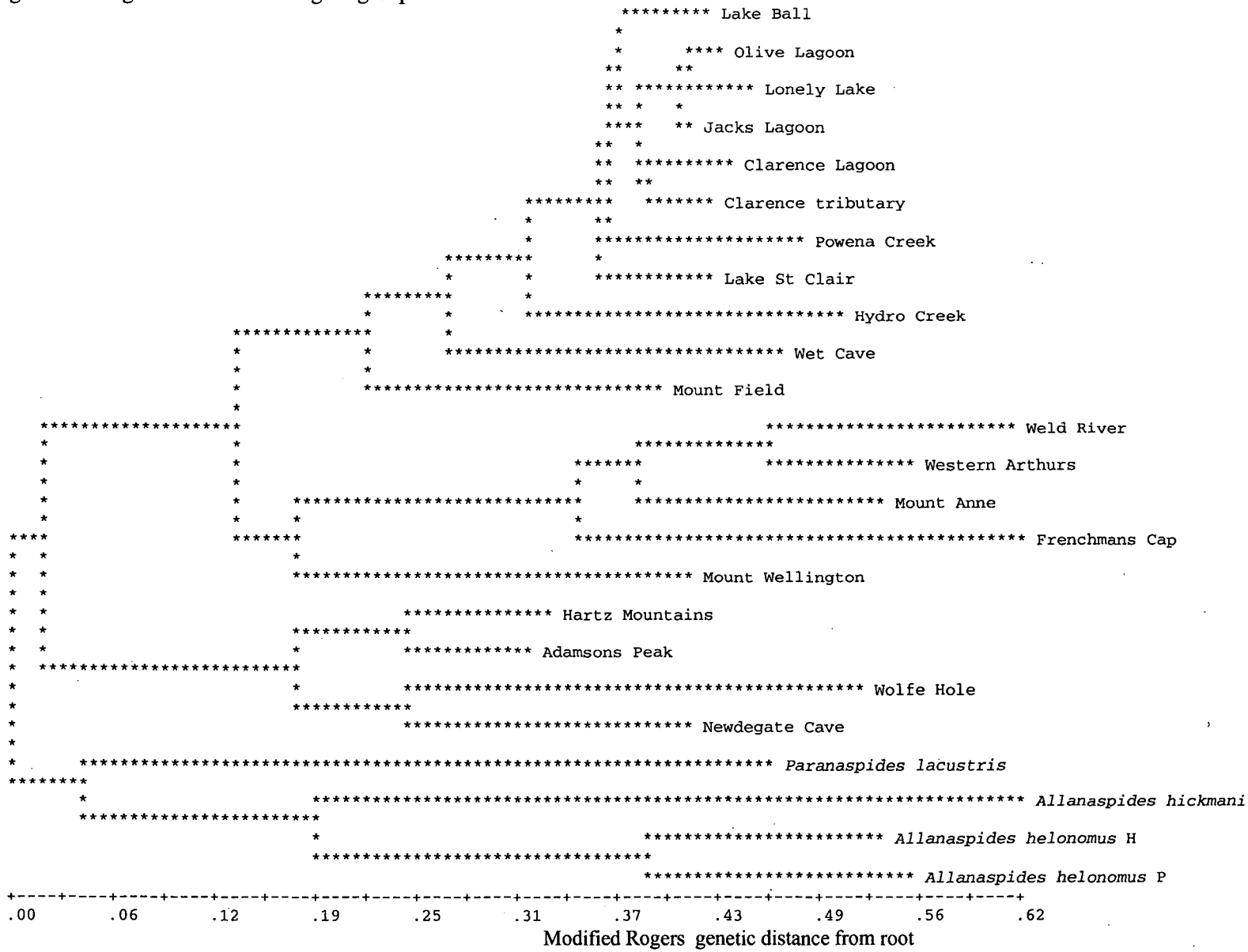


Figure 3.2 Wagner tree rooted using outgroup method



related, and appear to be somewhat distinct from both cave samples, which are also distinct from each other. The Wolfe Hole population appears to be the most differentiated within this group. By Thorpe s (1983) levels of genetic distances for taxonomic categories, the entire southern group displays generic level distance from other *A. tasmaniae*, with the largest distances between the southern and southwestern groups (Nei s unbiased D ranging from 1.127-1.435).

The only major differences in the topology of the two dendrograms were in the position of Mount Wellington in relation to the Central Plateau and southwestern groups, the position of *Paranaspides* in relation to *Anaspides* and *Allanaspides* and the position of Wolfe Hole in relation to Newdegate Cave.

It is clear from the dendrograms that no parallel genetic development has occurred among the three cave populations, which cluster with the geographically neighbouring surface populations rather than each other. However, genetic differences between all three cave populations and the nearby surface populations were greater than those among the surface populations themselves, probably as a result of the fixed allelic differences, so some differentiation in cave populations has occurred.

F-statistics for all *Anaspides* populations were calculated at $F_{IS} = 0.0530$, $F_{ST} = 0.8791$ and $F_{IT} = 0.8855$ over all loci, confirming substantial spatial genetic heterogeneity (Table 3.10). Of 21 loci, all but 3 (*G3pdh, Pep-1* and *Mdh-1)* had substantially more variation between populations than within populations. The matrix of pairwise F_{ST} values for population pairs (Table 3.9) shows that there is a wide range of levels of differentiation between population pairs. F_{ST} values of over 0.8 for most population pairs indicate large spatial disjunctions between most populations. The lowest F_{ST} value of 0.0008 was between the Lonely Lake and Jacks Lagoon populations.

When calculated within the groupings identified by the cluster analysis, F-statistics revealed that there was still spatial differentiation at moderate to high levels within all three groups, with F_{ST} values of between 0.657 and 0.823 over all loci (Table 3.10). However, on removing populations sequentially which were in the Central Plateau cluster but genetically distinct from it, F_{ST} values fell to 0.1738 for the Central Plateau group without Mount Wellington, Mount Field, Wet Cave and Hydro Creek (Table 3.11). The core Central Plateau group displayed the least geographic differentiation in the study. The proportion of variable loci for which the difference between F_{IT} and F_{ST} was less than 0.05 also indicated that very little heterogeneity was found between the group of 8 Central Plateau populations (Table 3.11).

Table 3.10 Fst estimates over all loci for population pairs. A. All *Anaspides*

	B	P	O	HC	L	J	WC	HM	WH	NC	AP	W	MF	MA	FC	WA	MW	SC	C
P	0.298																		
O	0.094	0.360																	
HC	0.615	0.722	0.676																
L	0.098	0.412	0.024	0.650															
J	0.077	0.312	-0.024	0.646	0.001														
WC	0.675	0.721	0.726	0.831	0.713	0.706													
HM	0.885	0.891	0.908	0.922	0.897	0.899	0.891												
WH	0.917	0.925	0.952	0.959	0.944	0.937	0.942	0.851											
NC	0.896	0.907	0.933	0.940	0.918	0.919	0.916	0.738	0.901										
AP	0.836	0.846	0.855	0.874	0.840	0.850	0.836	0.222	0.761	0.555									
W	0.915	0.920	0.938	0.944	0.933	0.928	0.927	0.944	0.971	0.959	0.911								
MF	0.677	0.729	0.750	0.840	0.734	0.728	0.785	0.896	0.939	0.923	0.846	0.931							
MA	0.909	0.916	0.940	0.955	0.939	0.927	0.936	0.950	0.978	0.972	0.915	0.859	0.932						
FC	0.891	0.903	0.913	0.927	0.903	0.905	0.890	0.918	0.946	0.927	0.882	0.835	0.904	0.858					
WA	0.880	0.889	0.906	0.928	0.897	0.896	0.893	0.923	0.953	0.938	0.884	0.602	0.903	0.771	0.797				
MW	0.841	0.857	0.889	0.921	0.872	0.868	0.885	0.905	0.955	0.935	0.850	0.930	0.863	0.938	0.908	0.889			
SC	0.130	0.316	0.157	0.634	0.119	0.107	0.606	0.867	0.917	0.877	0.801	0.916	0.695	0.919	0.883	0.873	0.837		
C	0.112	0.268	0.095	0.581	0.089	0.040	0.654	0.874	0.905	0.882	0.826	0.901	0.675	0.895	0.875	0.864	0.818	0.113	
Ct	0.073	0.296	0.078	0.698	0.149	0.031	0.718	0.904	0.961	0.932	0.839	0.938	0.720	0.940	0.909	0.902	0.889	0.175	0.046

Table 3.10 (cont.) Fst estimates over all loci in population pairs

B. In pairs of Central Plateau / Derwent catchment populations

	B	P	O	HC	L	J	WC	MF	SC	C
P	0.298									
O	0.094	0.360								
HC	0.615	0.722	0.676							
L	0.098	0.412	0.024	0.650						
J	0.077	0.312	-0.024	0.646	0.001					
WC	0.675	0.721	0.726	0.831	0.713	0.706				
MF	0.677	0.729	0.750	0.840	0.734	0.728	0.785			
SC	0.130	0.316	0.157	0.634	0.119	0.107	0.606	0.695		
C	0.112	0.268	0.095	0.581	0.089	0.040	0.654	0.675	0.113	
Ct	0.073	0.296	0.078	0.698	0.149	0.031	0.718	0.720	0.175	0.046

C. In pairs of southern populations

	HM	WH	NC
WH	0.851		
NC	0.738	0.901	
AP	0.222	0.761	0.555

D. In pairs of southwestern populations

	MA	FC	WA
FC	0.858		
WA	0.771	0.797	
W	0.859	0.835	0.602

B	Lake Ball
P	Powena Creek
O	Olive Lagoon
HC	Hydro Creek
L	Lonely Lake
J	Jacks Lagoon
WC	Wet Cave
MF	Mount Field
SC	Lake St Clair
C	Clarence Lagoon
Ct	Clarence Lagoon tributary
HM	Hartz Mountains
WH	Wolfe Hole
NC	Newdegate Cave
AP	Adamsons Peak
MA	Mount Anne
FC	Frenchmans Cap
WA	Western Arthurs
W	Weld River

Table 3.11 Summary of F-statistics and isolation by distance tests over all loci, for all populations of Anaspides together and for geographically-grouped populations.

Group	Number of populations	$F_{(IS)}$	$F_{(ST)}$	$F_{(IT)}$	Proportion of variable loci where $F_{(IT)} - F_{(ST)} <= 0.05$	Isolation by distance test p-values	
						linear	waterway
All *Anaspides*	20	0.053	0.879	0.886	0.95	0.000	0.395
Central Plateau all	12	0.046	0.657	0.673	0.86	0.000	0.557
Central Plateau excluding MW	11	0.057	0.570	0.595	0.77	0.010	0.555
Central Plateau excluding MW,MF	10	0.069	0.484	0.519	0.58	0.056	0.241
Central Plateau excluding MW,MF,WC	9	0.050	0.350	0.383	0.5	0.129	0.145
Central Plateau excluding MW,MF,WC,HC	8	0.045	0.174	0.211	0.44	0.795	0.702
Southern	4	0.075	0.682	0.705	0.75	0.610	0.467
Southwestern	4	0.066	0.823	0.835	0.83	0.655	0.553

The pairwise F_{ST} matrix showed that the Central Plateau group of populations (including *A. spinulae*) had F_{ST} values ranging from 0.001 to 0.840, the southern group had values ranging from 0.222 to 0.901 and the southwestern group s values ranged from 0.602 to 0.859 (Table 3.10 B to D). Mt Wellington showed high levels of structural variation when compared with all other sites, with values between 0.818 and 0.955 (Table 3.10 A).

Isolation by distance was tested by correlating physical distance (direct geographical and along watercourses) between pairs of populations with the pairwise F_{ST} / 1 - F_{ST} values. Calculation of

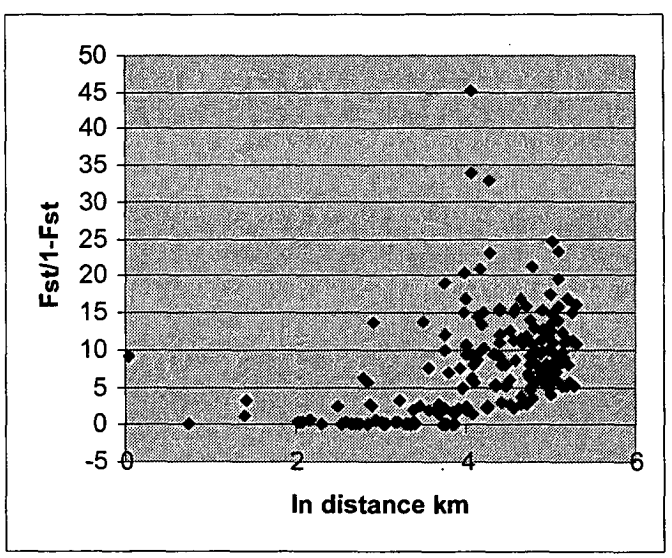
distances along watercourses was problematic in that, at current sea levels, only 14% of population pairs are linked by freshwater and a further 4% are linked by estuarine water. Between-population distances by sea are probably meaningless when discussing isolation by distance for an animal whose fossil record supports a freshwater history of at least 200 million years (Banarescu, 1995).

Over all population pairs, the correlation of F_{ST} / 1 - F_{ST} with direct geographic distance was found to be highly significant, with none of the values from 1000 permutations under the Mantel test greater than the observed value (Fig. 3.3 A). It is likely that the geographical separation of groups of populations rather than of individual populations dominated this analysis. No correlations with watercourse distance were found to be significant.

Tests of isolation by distance within the three *Anaspides* groups revealed highly significant correlations for the Central Plateau group (Fig. 3.3 B). Significance decreased as populations were removed from the test until the results were no longer significant for the core group of 8 populations (as described above), it appears that the Mount Wellington, Mount Field and to a lesser extent Wet Cave populations were the main contributors to the significant result (Table 3.11). Tests of isolation by distance within the southern and southwestern groups were not significant (Table 3.11).

Figure 3.3 Isolation by distance

A. All *Anaspides* (20 populations)



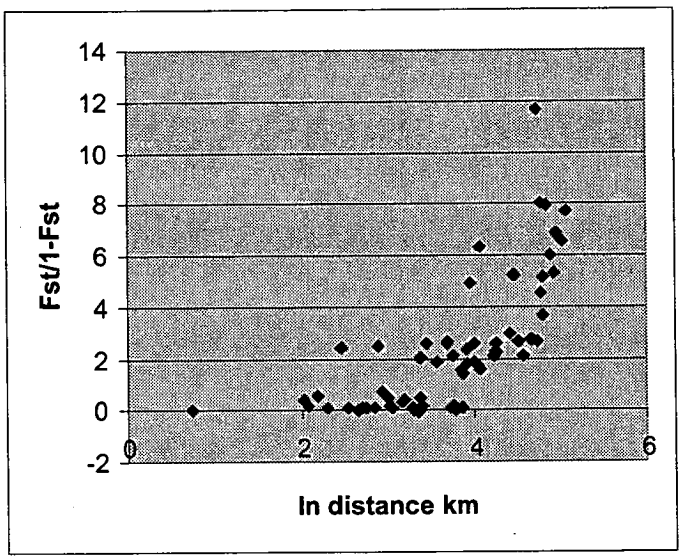
Fitting Fst/(1-Fst) to a + b ln(distance)
a = -6.2077736, b = 3.36392170

1000 permutations (statistic: Spearman Rank correlation coefficient):

Test of isolation by distance (One tailed P-value):
P(correlation > observed correlation) =0.00000
under null hypothesis

Figure 3.3 (cont.) Isolation by distance

B. Central Plateau / Derwent catchment *Anaspides* (12 populations)



Fitting Fst/(1-Fst) to a + b ln(distance)
a = -5.1612999, b = 2.00732722

1000 permutations (statistic: Spearman Rank correlation coefficient):

Test of isolation by distance (One tailed P-value):
P(correlation > observed correlation) =0.00000
under null hypothesis

A t-test found no significant difference between the heterozygosity (H_e) values of populations from running (n=7) and still (n=10) surface water habitats (p = 0.385), although excluding the Adamsons Peak population resulted in a significant result (p = 0.012). F-statistics using *Anaspides* data assigned to groups according to these habitat types showed that almost all of the total variation was within the two groups rather than between them (F_{IS} = 0.8724; F_{ST} = 0.0339; F_{IT} = 0.8768).

3.4 Discussion

Fossil evidence shows that anaspid syncarids have existed in Australian freshwater environments for at least the last 200 million years (Brooks, 1962; Schminke, 1982) and have survived enormous climatic and geological change over that period. It is likely that the distribution of genetic variation among extant anaspid populations reflects this sequence of influences, but its length and complexity make substantial gaps in our understanding of anaspid biogeography inevitable. Despite this, patterns of allozyme variation can give an indication of evolutionary processes, without providing any certainty of their timing or duration.

The genus *Anaspides* is characterised by very high levels of genetic variation across populations, many fixed differences between populations, generally low levels of variation within populations with a

prevalence of single-allele fixation, and the frequent occurrence of private alleles. Populations appear to have had a long history of genetic isolation during which differences have accumulated to the point of fixation, probably exacerbated by small population sizes at some time during their history. It is likely that the more extreme forms of genetic drift (founder effect and bottlenecks) are largely responsible for the low heterozygosity and high levels of population differentiation.

Where populations are monomorphic for rare or private alleles, such as the Wolfe Hole, Mount Wellington and Hydro Creek populations, selection for a rare or newly mutated allele at some time cannot be ruled out. These three sites represent extreme habitats (an underground lake and two small surface streams respectively) for *Anaspides* populations, which may have been reduced to low numbers and / or had to adapt to unusual conditions. The low heterozygosity over all loci in these populations indicates organismal processes rather than impacts on particular loci, so selection is unlikely. The overall similarity of heterozygosity levels for loci grouped according to enzyme function also uphold this conclusion. However, the fixation of rare alleles, whatever its original cause, does indicate a long history of isolation as even one migrant per generation from a neighbouring population would have resulted in greater polymorphism (Allendorf and Phelps, 1981).

Instances of geographically related populations monomorphic for an allele absent in other populations are common. If corresponding patterns of environmental discontinuities also occurred, selection could be considered a factor. However, no evidence of current assortative mating or active selection in the form of deviance from genotype equilibrium was found in any study population, so selection followed by fixation would have had to have occurred in the order of hundreds of generations ago, depending on population size (Futuyma, 1998).

Genetic variation in the study populations falls at the very low end of the range for crustaceans cited by Hedgecock *et al.* (1982) and is slightly lower than that reported by Ward *et al.* (1992). *Anaspides* shows levels of variation comparable to that seen in the hoplocarids and some decapods, including the astacid and *Palinura* group of freshwater crayfish and lobsters. The genus appears to have somewhat higher heterozygosity than the two Tasmanian freshwater crayfish genera, *Engaeus* and *Parastacoides*, perhaps reflecting its potential for maintaining larger populations.

There seems to be no genetic differentiation associated with survival in flowing or still water. In general, still surface water populations have higher heterozygosity than those in flowing surface water, with the exception of the Adamsons Peak creek population. This trend could reflect the limitations on habitat availability within streams, which would impact on population sizes and thus on heterozygosity.

Although individual animals do not seem to actively avoid areas with high flow rates, reproductive success may be reduced by high flows in the winter when eggs are developing. Two of the flowing water sites, the tributaries of Olive and Clarence Lagoons, are runnels between deep sphagnum moss beds and do not reach the high rates of flow of the true creek sites - Hydro Creek, Jacks Lagoon outflow, Mount Wellington, Weld River and Adamsons Peak. The combination of high flows with rock or boulder substrates might have caused occasional reproductive failure at these sites, with the exception of the Adamsons Peak site which has a gravel bed in which eggs might be protected from flows.

Alternatively, smaller surface creeks are more vulnerable to desiccation and raised water temperatures in hot dry periods, and populations without access to deep water may be adversely impacted at these times. Those populations in lakes, deep pools, deep runnels beneath sphagnum, the deeper creek sites (such as the Jacks Lagoon outflow) or gravel beds (such as the Adamsons Peak site) would have an improved chance of survival by using deeper water as a refuge from high temperatures. The same refuges would also be available for protection from low temperatures. Swain and Reid (1983) suggested that some animals in the Mount Field population of *A. tasmaniae* may survive winter in a relatively shallow alpine tarn by burrowing into the silty substrate.

Considering that syncarids generally have a tendency towards interstitial or hypogean life (the entire Order Bathynellacea, and anaspidaceans in the Psammaspididae, Koonungidae and Stygocaridae, as well as cave populations of *Anaspides* demonstrate this tendency), the higher heterozygosity of those populations with access to either very deep water (such as in the larger lakes, St Clair and Clarence Lagoon, or beneath sphagnum beds such as the Lake Ball site) or a gravel substrate (such as Adamsons Peak) should not be surprising. Suitable habitat, particularly where refuge from adverse conditions is provided, helps maintain a consistently high population size, resulting in the retention of rare alleles.

The partitioning of size classes in the Adamsons Creek population is an interesting phenomenon not noted elsewhere, and may also contribute to higher juvenile survival rates in this population, as cannibalism of juveniles occurs in this genus (personal observation).

In general the loss of genetic variability within populations can be seen as the result of genetic drift in two forms - a long history of population isolation leading to the fixation of single alleles, and reductions in population sizes to varying degrees, probably as a result of environmental pressures. The possibilities of past mutation, selection and founder effects also cannot be ruled out.

Genetic distances between populations of *Anaspides* show clear geographic differentiation between three groups - a southern, a southwestern, and a Central Plateau / Derwent catchment group, which includes *A. spinulae*. Using Thorpe s (1983) guidelines, pairwise genetic distances within each of these groups indicate conspecific relationships. Between these groups the genetic distance levels are in the congeneric (interspecific) range, although the southern group is rather more differentiated, with the cave populations in particular showing confamilial (intergeneric) levels of genetic distance with *Anaspides* from other areas. Between the three genera, differentiation levels are appropriate for confamilial taxa. Very high levels of population substructure are also common across the whole study, with only a few pairs of populations on the Central Plateau, and the two southern surface water populations, showing genetic homogeneity.

The southern group includes populations from two caves (Wolfe Hole and Newdegate Cave) and two surface water bodies, on the Hartz Mountains and Adamsons Peak. These populations are from waters which flow eastward into the D Entrecasteaux Channel south of the Huon River mouth. The creeks draining Adamsons Peak and Ladies Tarn on the Hartz Mountains both flow into the Esperance River estuary, while the Newdegate Cave creek flows into Lune River, about 12 km south. It is not clear whether Lake Pluto in the Wolfe Hole ever connects with surface waters as it appears to be part of the groundwater system.

Genetic relationships within the southern group indicate close affinities between the two surface water populations. The cave populations may have differentiated by adaptation to subterranean habitats, or the surface populations may have greater genetic similarity because they shared the same freshwater catchment when sea levels were lower during glaciations. The former is unlikely as the Newdegate Cave population shows closer affinities to the surface populations and the Wolfe Hole population is greatly differentiated from all three. The Wolfe Hole population is also more highly morphologically differentiated than any other population, through its lack of eyes as well as body pigmentation (as in other cave populations), and so may represent a much older, relict population. These relationships probably reflect the timing of separation of each population from an ancestral group which would have been centred in lowland freshwater bodies either during wetter climatic conditions or during glaciation. At the height of the Pleistocene glaciations, the Esperance, Lune and Huon Rivers were tributaries of the same river system, which ran into the sea south-east of Bruny Island.

The southwestern group includes the Weld River, Mount Anne, Western Arthur Range (Lake Oberon) and Frenchmans Cap populations. The Weld River and Mount Anne waterways are part of the Huon River catchment which flows eastward, the Western Arthurs drain into Port Davey to the southwest and

Frenchmans Cap drains into Macquarie Harbour, to the west. The Frenchmans Cap population is slightly differentiated from the other three populations, and the closest affinities are between the Weld River and Western Arthurs populations. Although these four populations are found in very mountainous terrain and are more widely separated than populations in the other two groups, they are nevertheless genetically similar, and may indicate relatively recent gene flow. It is interesting to note that while the southern group appears to be related on the basis of sharing a catchment, the south-western group shows no such tendency. The Mount Anne and Weld River sites would also have shared the ancient Huon catchment but exhibit intergeneric levels of genetic distance from the southern group, by Thorpe s (1983) criteria.

The Central Plateau / Derwent catchment group of twelve populations includes those described by Williams (1962?) as *A. spinulae*. My data throw further doubt on the current taxonomy, originally questioned by O Brien (1990) after the discovery of intermediate morphotypes. The conclusions of Jarman and Elliot (2000), namely that the two morphological forms are phylogenetically a single species, are reinforced by the addition of this allozyme data to their smaller study of mitochondrial DNA sequences. The two putative *A. spinulae* populations fall within the core group of 8 populations which show a homogeneous structure. There has clearly been substantial mixing of genetic material within this core group (possibly somewhere near the north-western edge of the Plateau) more recently than within the other two geographical groupings.

The outlying populations in the Central Plateau / Derwent catchment group are an intriguing feature of this study, occurring in a cave to the north, an isolated creek to the east, and two mountains to the south. Fixation of alleles private within the group are the main contributor to the differentiation of these populations from the core group of 8 Central Plateau populations. This, together with the increasing genetic distance with geographical isolation, indicates that these populations may have been colonised from a single Central Plateau population by a limited number of individuals. Further reductions in genetic variability may then have been imposed by environmental conditions (through bottlenecking or selection). It appears that the Mount Wellington population has diverged considerably from the Central Plateau group, and is likely to constitute a species in its own right.

The response of populations to Pleistocene glaciation has often been proposed as the likely mechanism by which distributions of freshwater species have been determined. With their vagility restricted to waterways, dispersal pathways should be more easily traceable, at least in the recent past. However, the influences of other geologically recent factors, including the invasion by other species (in this case particularly the galaxiid freshwater fish), climatic changes and changes to drainages, such as river

capture and reversal, need to be considered. Earlier geological episodes also leave their mark on today s landscape and biota, but the ability to distinguish deep from shallow history in biogeographical studies is methodologically difficult and requires evidence from many taxa (Cracraft, 1988). Molecular evidence points to speciation within the Anaspididae occurring between 7 and 25 million years ago (Jarman and Elliot, 2000). Little is known of the biogeography of other central Tasmanian biota in the intervening period.

Stevens and Hogg (2003) review the theoretical framework of the role of Pleistocene glaciation in genetic differentiation, describing two opposing views. Divergence caused by habitat fragmentation during glacial advances (vicariance) is contrasted with the homogenising effect of cycles of population expansion and contraction. It is possible that the Central Plateau *Anaspides* populations were subject to mixing in one or more postglacial phases when waterways were connected by meltwater, or in interglacial periods when the climate was warmer and wetter.

In summary, the allozyme data identify three distinct geographical groupings of populations that are characterised by large between-group genetic differentiation and low within-group and within-population differentiation. The three groups display interspecific levels of genetic distance. There is some evidence for lower variability in flowing water populations and for the earlier differentiation of cave populations.

Chapter 4 Mitochondrial DNA phylogeny

4.1 Introduction

In crustaceans mitochondrial DNA (mtDNA) is typically a single circular molecule consisting of up to 17,000 bases (Raimond *et al.*, 1999). As in other animals it is characterised by maternal inheritance and so has an effectively haploid nature. This mode of inheritance affects the pattern of genetic variation within species by increasing the effect of drift and the rate of turnover within a population, and thus increases variation among populations (Moore, 1995; Hillis *et al.*, 1996). Combined with a higher rate of base substitution, sorting of ancestral alleles within and between species is then considerably more rapid than in nuclear genes (Hillis *et al.*, 1996), resulting in an abundance of genotypes.

Mitochondrial genes, particularly the small and large subunit ribosomal DNA (12S and 16S rDNA respectively) and the protein-coding genes cytochrome oxidase 1 (CO1) and cytochrome b (cyt *b*), are routinely used for phylogenetic and population genetic studies, due to their relatively rapid rate of base substitution and interspecific polymorphism (Hillis *et al.*, 1996; Raimond *et al.*, 1999). The two ribosomal genes appear to be relatively conserved in sequence and secondary structure among taxa, but 16S has regions with enough variability to be phylogenetically informative at the species and genus level, and at a population level in some taxa (Hillis *et al.*, 1996).

In this study, a 479 bp sequence of 16S mtDNA from a total of 58 samples of all three genera of the family *Anaspididae,* from 33 sites across Tasmania, was compared in order to assess the phylogenetic affinities suggested by the allozyme study and to establish confidence levels in the groupings. The absolute times of divergence of genera, species, geographical groups and populations were estimated by the application of a molecular clock.

4.1.1 16S mitochondrial rDNA phylogenetic studies of crustaceans

Many phylogenetic studies of crustaceans have used 16S mtDNA sequence data, in conjunction with other DNA sequences (Giessler *et al.,* 1999; Maggioni *et al.*, 2001; Matthews *et al.,* 2002; Muller *et al.*, 2002; Wetzer, 2001; Munasinghe *et al.*, 2003; Cristescu and Hebert, 2002; Crandall *et al.*, 2000a; Taylor *et al.*, 1998), in conjunction with allozymes (Giessler *et al.,* 1999; Muller, 2000; Maggioni *et al.*, 2001; Muller *et al.*, 2002; Grandjean *et al.*, 2002; Munasinghe *et al.*, 2003; Taylor *et al.*, 1998), and alone (de Bruyn *et al.*, 2004; Jarman and Elliot, 2000; Crandall *et al.*, 1999; Crandall *et al.*,

2000b; Camacho *et al.*, 2002). These studies have typically been used to elucidate a species or genus taxonomy and/or biogeography.

In the above studies, where 16S mtDNA was used alongside other DNA sequences, seven out of nine studies reported phylogenetic congruence between the markers, and five out of seven studies combining 16S mtDNA and allozymes reported congruence. The fact that many of the combined DNA studies used other mitochondrial genes may have contributed to the high rate of congruence, as different mitochondrial genes might be subject to similar functional constraints and possibly coevolution (Flook and Rowell, 1997).

These findings appear to indicate that problems arising from deep coalescence and secondary structure are not particularly widespread in crustacean phylogenetics, the former possibly because lineage sorting should not be a problem with relatively ancient organisms. A protocol involving ensuring that separate data sets are homogeneous before combining them for phylogenetic analysis is often adopted (Cristescu and Hebert, 2002; Crandall *et al.*, 2000a).

Two previous studies of Tasmanian crustaceans have used 16S mitochondrial rDNA sequences. Hansen (2000) combined allozyme and 16S mitochondrial data in a major systematic review of the freshwater crayfish *Parastacoides*. Jarman and Elliot (2000) sequenced the 16S rDNA of 28 *Anaspides* individuals from 13 sites, and one each of *Paranaspides lacustris, Allanaspides hickmani* and *Allanaspides helonomus,* in a study of evolutionary rates and cryptic speciation in the Anaspididae. They found a stochastically constant rate of evolution within the Anaspididae and used a relative rate methodology to calibrate a molecular clock at 0.2871 % ± 0.1344 % SE change per million years.

The use of molecular clocks to estimate absolute time is controversial. Hillis *et al.*(1996) have summarised many sources of inaccuracy and recommend the use of externally calibrated evolutionary rates and confidence intervals. The work by Jarman and Elliot (2000) used a 16S clock for the malacostracan family Lithodidae calibrated through vicariant divergence associated with three separate well-supported dates of geological events from fossil evidence (Cunningham *et al.*, 1992), and supplied 95% confidence limits for the estimates.

The present study of *Anaspides* greatly increases the numbers of individuals and populations sampled and the breadth of geographical coverage in the central, western, southwestern and subterranean environments, and seeks to emphasise biogeography rather than species evolution.

4.1.2 Comparison of analytical methods for molecular phylogenies

The many analytical methods available for the construction of phylogenetic trees can be grouped broadly into three categories based on character state, distance and maximum likelihood (Sherry and Batzer, 1997).

Character state analysis assesses all possible trees using the maximum parsimony (MP) criterion. Under this criterion a score representing the minimum number of base substitutions needed to produce the current data is calculated for each tree and the tree with the lowest score is chosen as the most parsimonious tree. If many trees with the same score are found, it is possible to form a consensus tree, although multiple most-parsimonious trees are sometimes taken to indicate the inappropriateness of the method for that data set (Hillis and Huelsenbeck, 1992; Sourdis and Nei, 1988). Farris (1970) proposed the first algorithm for MP searching, but the methods have since been substantially modified and many variations of parsimony are now used (Hillis *et al.*, 1996).

Distance-based methods express data as measures of pairwise evolutionary distance (analogous to the genetic distance measures used in allozyme analysis) and construct trees using either UPGMA (chapter 3) or neighbour-joining (NJ, Saitou and Nei, 1987). NJ is a stepwise additive-distance method which groups taxa as neighbours so as to minimise the total length of the tree. Unlike the previous methods, NJ is an algorithmic method which builds a single tree rather than assessing possible trees using optimality criteria. Variations may include the use of different distance measures and incorporation of among-site rate differences and character weighting (Hillis *et al.*, 1996).

The maximum likelihood method (ML, Felsenstein, 1981) assesses trees by comparing probability scores (the sum of the likelihoods of a certain series of substitutions resulting in the data set). The tree with the highest probability is selected as the preferred tree. As in MP, it uses an evolutionary model to examine randomly constructed trees and assigns a score to each, but unlike MP, among site rate variation is able to be incorporated into the model for ML (Yang, 1993).

The most commonly used methods are maximum parsimony and neighbour-joining, with maximum likelihood also favoured but considered computationally intensive for large data sets. No one method

is appropriate for all data sets as all have problems with efficiency, consistency or robustness in certain circumstances (Hasegawa and Fujiwara, 1993). A number of authors have compared the accuracy and robustness of common phylogenetic methods through simulations of varying complexity.

All methods rely on assumptions concerning the evolutionary history of the organism and, since these are often violated (Yang, 1993; Huelsenbeck, 1995a; Fukami-Kobayashi and Tateno, 1991), the robustness of a method to the violation of assumptions is important. Assumptions likely to be unrealistic are that the rate of substitution is constant at different nucleotide sites, that the rate of evolution is constant for different taxa, and that the ratio of transitions to transversions is constant across taxa (Yang, 1993; Kuhner and Felsenstein, 1994; Huelsenbeck, 1995a).

Shortcomings of the maximum parsimony method appeared to be common to all simulations that have tested it. Situations where MP was outperformed by neighbour joining and /or maximum likelihood included: when sequences were long (Saitou and Imanishi, 1989); when substitution rates varied among branches (Felsenstein, 1978; Jin and Nei, 1990; Kuhner and Felsenstein, 1994) and among nucleotide sites (Jin and Nei, 1990); when rates of substitution were high (Sourdis and Nei, 1988; Saitou and Imanishi, 1989); and when the ratio of transitions to transversions was biased (Jin and Nei, 1990). No simulations found MP to be more accurate or robust than NJ or ML.

Comparisons of methods including maximum likelihood and neighbour joining showed that both approaches are very likely to recover the correct tree; however, maximum likelihood was found to be generally more robust to the violation of assumptions (Fukami-Kobayashi and Tateno, 1991; Saitou and Imanishi, 1989; Kuhner and Felsenstein, 1994; Huelsenbeck, 1995a and 1995b). The violation of the assumption that evolutionary rates are constant among nucleotide sites caused the most inaccuracy and bias for both methods (Kuhner and Felsenstein, 1994). All these studies found that ML is most likely to retrieve the correct tree, but recommended using NJ with care where computation time is a constraint. Saitou and Nei (1987) showed that NJ was preferable to other additive-distance methods and Huelsenbeck (1995a and 1995b) and Jin and Nei (1990) showed that gamma correction for rate heterogeneity of distance values greatly improved the accuracy of tree topology under NJ. Yang (1993) also showed that variation in substitution rates among sites could be corrected by using the gamma distribution in ML for greater accuracy.

The above studies typically simulated phylogenies for between 4 and 8 taxa and the effect of adding more taxa is unknown, although Huelsenbeck (1995b) and Kuhner and Felsenstein (1994) expected less accuracy in all methods with more taxa. Saitou and Imanishi (1989) found that increasing the sequence length from 300 to 600 bp doubled the number of correct trees recovered.

Confidence in tree accuracy may be tested by resampling methods such as bootstrapping (Felsenstein, 1985) or jacknifing. Hillis and Bull (1993) showed that bootstrap values are highly conservative estimates of correct inference under most conditions. However, for large data sets bootstrapping can be prohibitively time-consuming, particularly with the more intensive maximum likelihood analysis. Mort *et al.* (2000) found that for data sets of over 25 taxa, fast bootstrapping methods, which omit branch-swapping in order to reduce the computational intensity of the normal exhaustive methods, result in bootstrap support levels that are similar to branch-swapping methods, provided moderate (between 500 and 1000) numbers of replicates are used. These support values were shown to be typically slightly lower and have lower standard deviations than full bootstrap values, and are thus reliable, if conservative, estimates of nodal support. Fast jacknife analysis is similar to the fast bootstrap, but is even faster as the size of the data set is reduced at each resampling. Although it is not often used as it is considered to contain less phylogenetic information, Mort *et al.* (2000) found that with fewer replicates and 50% deletion, the fast jacknife produced support levels which were generally closer to or lower than those of a full bootstrap than did the fast bootstrap. In this work, a fast bootstrap support value of 70% or over is considered moderate to strong support for a node and between 50% and 70% is considered weak support.

An ingenious approach described by Hillis and Huelsenbeck (1992) addresses the problem of separating phylogenetic signal from the random noise which results from saturation by change at variable sites, particularly in older lineages. A frequency distribution of branch lengths is produced from the original data, randomised but with base frequencies maintained for each site. The authors show that increased hierarchical structure (true signal) causes a skew to the left in the branch length distribution. A skew to the right indicates increased noise. The g_1 skewness statistic is calculated, which becomes more negative with increasing left skew. A table of critical values ($p=0.05$ and 0.01) of g_1 with number of taxa and characters was presented (Hillis and Huelsenbeck, 1992). Swofford *et al.* (1996) expressed some reservations about this method as strong skewness can be related to very localised structure, such as the duplication of taxa, which would create very short branches, so extreme values of g_1 should probably be disregarded.

4.2 Methods

4.2.1 DNA extraction and amplification

DNA was extracted from 2 individuals from each of the populations of *Anaspides* species listed in Table 2.1 (except for the 10 asterisked populations), and from specimens of *Allanaspides hickmani, All. helonomus* and *Paranaspides lacustris.* Approximately 1.5 mg of tissue from each individual was macerated and digested in a QIAGEN™ Dneasy Tissue Kit using Proteinase K. All procedures followed the manufacturer s instructions. The resulting total DNA was stored in 1.5 ml centrifuge tubes at -20 ^{0}C to be used as templates in amplification by polymerase chain reaction (PCR).

Synthetic oligonucleotide primers 16Sar-L (5 -cgcctgtttatcaaaaacat-3) and 16Sbr-H (5 - ccggtctgaactcagatcacgt-3) were used to PCR amplify a region of 16S mitochondrial rDNA of approximately 542 base pairs. Initial reactions were 50 μL, containing 25 μL Promega™ Master Mix (50 units mL^{-1} *Taq* DNA polymerase; 400 μM each dNTP; 3mM $MgCl_2$; pH 8.5 buffer), 0.5 μL each primer, 12.5 μL DNA template and distilled water. These quantities were corrected when test runs showed that optimal results were achieved with 5 μL DNA, additional $MgCl_2$ (1 mM) and additional *Taq* DNA polymerase (0.25 units).

All PCR runs contained both positive (krill, *Euphausia* sp., DNA) and negative (distilled water) controls. An annealing gradient run was performed to determine the optimum annealing temperature. Cycle conditions were denaturation at 94 ¡C (30 seconds), annealing at 54 ¡C (1 minute) and extension at 72 ¡C (1 minute 30 seconds) for 40 cycles, followed by 72°¡C (6 minutes).

PCR product was electrophoresed through a 1.2% agarose, 0.5 μg mL^{-1} ethidium bromide gel for 60 minutes at 90 V. The bands of DNA were excised from the gel under ultra-violet light and stored at -20 ^{0}C until extraction using a Qiaquick Gel Extraction kit (Qiagen ™). The manufacturer s instructions were followed with the exception of step 5 (addition of isopropanol). DNA concentration was measured in the product using a Picoflour flourimeter and Big Dye Terminator sequencing PCR reactions were run using 10 μL reactions with 13 ng sample DNA and the forward primer 16Sar only.

4.2.2 Sequencing and sequence alignment

Sequence analysis was undertaken at CSIRO Marine Research laboratories (Hobart) using an ABI 377 automated DNA sequencer (Applied Biosystems).Sequences were first checked and manually aligned using Sequence Navigator software (ABI) and then transferred to ClustalX (Thompson *et al.*,

Table 4.1 Numbers and source of sequences used in the mtDNA study. Refer to Table 2.1 for further details.

Population	**n**	**Allozyme study**	**Genbank***	**New samples#**
Anaspides tasmaniae				
Wolfe Hole	3	WH7, WH9	AF133694	-
Newdegate Cave	1	NC1	-	-
Wet Cave	2	WC7, WC10	-	-
Adamsons Peak	3	AP5, AP9	AF133693	-
Hartz Mountains	3	-	AF133692	a,b
Square Lake (Western Arthurs)	2	-	-	a, b
Haven Tarn (Western Arthurs)	2	-	-	a, b
Snowy North	1	-	AF133691	-
Lake Picton	1	-	-	a
Weld River	2	W16, W18	-	-
Lake Oberon (Western Arthurs)	2	WA4, WA8	-	-
Coronation Peak	2	-	-	a, b
Mount Anne	3	MA8, MA10	AF133689	-
Lake Rhona	1	-	-	a
Frenchmans Cap	2	FC3, FC11	-	-
Near Lake Ball	2	-	-	a, b
Zion Hill	1	-	AF133688	-
Jacks Lagoon	2	-	-	a, b
Mt Rufus	1	-	AF133687	-
Mt Ossa	1	-	AF133686	-
Sandbank Tier	1	-	AF133685	-
Mt Field	3	MF6	AF133684	b
Powena Creek	1	-	-	a
Lonely Lake	2	L1, L5	-	-
Hydro Creek	2	-	-	a, b
Olive Lagoon	1	-	-	a
Mt Wellington	1	-	AF133683	-
Clarence Lagoon tributary	1	Ct3	-	-
A.spinulae Clarence Lagoon	2	C9	-	a
Lake St Clair	2	SC1	AF133679	-
Allanaspides hickmani	3	Ahi5, Ahi6	AF133681	-
Allanaspides helonomus	1	-	AF133680	-
Paranaspides lacustris	1	-	AF133682	-
Total	58	23	15	20

* Sequences from study by Jarman and Elliot (2000), with permission from the authors.
\# Samples not used previously, designated a and b to distinguish them.

1997) for full alignment. The initial sequence of 542 nucleotides was trimmed to 479 by removing sites at both ends where data was missing or ambiguous in some samples. Samples without useful sequences were discarded, and Genbank sequences (from Jarman and Elliot (2000)) were added to provide additional data, including sequences from five new populations - Snowy North, Sandbank Tier, Zion Hill, Mt Rufus and Mt Ossa.

4.2.3 Phylogenetic analysis

Analysis of 16S mtDNA sequence data was performed using *PAUP** v4.0 b10 for Macintosh (Swofford, 1996) on a Macintosh G3 PowerBook. Maximum parsimony, neighbour joining and maximum likelihood analyses were applied to a total of 58 sequences. Of these, 35 sequences were from populations previously used in the allozyme study, 8 were from individuals representing new populations and 15 were sequences deposited in Genbank by Jarman and Elliot (2000). The data set included sequences from 3 samples of *Allanaspides hickmani*, 1 from each of *Allanaspides helonomus* and *Paranaspides lacustris*, 4 from *Anaspides spinulae* and the remaining 49 from *Anaspides tasmaniae*. Further details of samples used in the mtDNA analysis are shown in Table 4.1.

The HKY85 + Γ (discrete gamma) model of nucleotide evolution (Hasegawa *et al.*, 1985; Yang, 1993) was used in the maximum likelihood and neighbour joining analyses to correct for rate heterogeneity among taxa, unequal equilibrium base frequencies, and different transition and transversion rates. An initital assessment of the rates of nucleotide substitutions among sites was undertaken by maximum likelihood analysis with minimum evolution as the objective function, in order to estimate the shape parameter (α) for discrete-gamma correction of rate heterogeneity. This value was subsequently used in the HKY85 + Γ model for calculation of the pairwise distance matrix (for neighbour joining) and the maximum likelihood phylogeny.

The same initial analysis also yielded base frequencies, the number of variable and parsimony-informative characters and the ratio of transitions to transversions. The assumption of equal base frequencies was tested using a χ^2 - test.

The g_1 skewness statistic was calculated from 100,000 random trees, for the whole data set, for the data set without duplicate samples from the same population, and for the *Anaspides* samples only, without duplicates.

Maximum parsimony

Character data were weighted using a stepmatrix assumption set, under which gaps and transversions were defined as 5 and 2 times as significant as transitions respectively. Gaps were treated as a 5th base . Brown (1982) showed that transitions are more likely than transversions; therefore transversions are less likely to be homoplasious and should be treated as more significant. The same reasoning applies to insertion and deletion event which cause gaps.

Maximum parsimony analysis was undertaken by heuristic searches with stepwise addition and branch-swapping using the TBR (tree-bisection-reconnection) algorithm. Branches of effectively zero length were collapsed. The initial number of trees to be saved was set to 100 and increased automatically, but a maximum of 100 trees with scores over 250 was saved from each replication, as the number of sequences was large and the number of trees able to be stored was limited. A strict consensus tree of all saved trees, which maintains only groupings present in all the most-parsimonious trees, was found.

Use of outgroups followed the revised outgroup method for simultaneous analysis proposed by Nixon and Carpenter (1993) and justified theoretically by Smith (1994). Unrooted trees were produced initially, as ingroup relationships are more likely to be correctly resolved without the confounding influence of unknown changes in the outgroup since the divergence of the ingroup (Nixon and Carpenter, 1993). In this stage the two sister genera of *Anaspides* (two *Allanaspides* and one *Paranaspides* species) were included as though they were part of the ingroup (simultaneous outgroup analysis as described by Nixon and Carpenter, 1993). The consensus tree was then rooted using the four *Allanaspides* sequences as the outgroup, as they are a single close sister clade as recommended by Smith (1994). Using two non-anaspidacean outgroups (an anostracan and a stomatopod), Jarman and Elliot (2000) had determined that the most basal split among the three anaspid genera lay where *Allanaspides* diverged, a result supported in the current work.

Confidence in the consensus tree was assessed by resampling the data using the bootstrap technique (Felsenstein, 1985) with 5,000 pseudoreplicates in *PAUP** s fast stepwise option.

Neighbour-joining

The distance matrix of pairwise nucleotide sequence divergence was calculated using the HKY85 + Γ nucleotide evolution model (Hasegawa *et al.*, 1985) and used to construct a neighbour-joining

phylogram. All substitutions were included, minimum evolution was selected as the objective function, and negative branch lengths were set to zero.

The 16S mtDNA evolutionary rate of Jarman and Elliot (2000) was applied to the distance data averaged over the major clades, to give estimated times (with 95% confidence limits) of divergence within and among the geographical groups.

Maximum likelihood

Data were weighted using the stepmatrix described previously, but the likelihood method does not consider gaps so these were not weighted. The default settings in *PAUP** were retained for all likelihood settings, with the exception that the gamma distribution was invoked to take variable rates among sites into account. The shape parameter α was set to the value found in the initial analysis. Heuristic searches were conducted as described above, but the number of trees to be saved was not constrained by a score value. The procedure used for rooting the maximum likelihood tree was the same as that described for maximum parsimony.

Bootstrap resampling (Felsenstein, 1985) by fast stepwise search was performed with 100 pseudoreplicates, due to computational constraints. As fast bootstrapping with only 100 replicates has been found to overestimate support values at up to 30% of nodes (Mort *et al.*, 2000). A fast jacknife with 50% deletion and 300 replicates was also performed, which should give support values equal to or less than a full bootstrap at approximately 85% to 90% of nodes (Mort *et al.*, 2000). As this is a more conservative measure than bootstrapping, 50% jacknife support is considered to be significant.

4.3 Results

4.3.1 DNA sequencing and alignment

Of 56 samples from which DNA was extracted and amplified, 12 did not have enough DNA to proceed to sequencing and another one did not produce an interpretable sequence. However, with the addition of 15 sequences from Genbank, all populations were represented by at least one individual (Table 4.1).

Alignment of 58 sequences revealed two deletions and three insertions. These consisted of a one-character deletion in sequences from all 10 southwestern populations and all *Allanaspides*, a one-

character deletion in all sequences from the 4 southern populations, a four-character insertion in *Allanaspides hickmani*, a two-character insertion at the same site in *Allanaspides helonomus*, and single-character insertions in *Allanaspides helonomus, A. hickmani,* and in one Frenchmans Cap sample. Appendix 1 contains the full data set of 58 taxa and 479 bp after alignment.

4.3.2 Phylogenetic analysis

Of 479 characters, 363 were constant, 21 were variable but parsimony-uninformative and 95 were parsimony-informative. Frequencies of bases averaged over all sequences, including outgroups, were A - 0.393, C - 0.114, G - 0.173 and T - 0.321, indicating a strong A - T bias of 71.7%. A χ^2 - test confirmed the consistency of base frequencies across taxa ($p = 1.000$). The ratio of transitions to transversions was 2.203.

The shape parameter (α) for use in discrete-gamma correction for rate heterogeneity was estimated at 0.1666, indicating that most nucleotide sites have evolved slowly, but a small number has evolved quite rapidly (Hillis *et al.*, 1996). The sequence data show three relatively more variable regions of approximately 40 to 50 bp (Appendix 1).

The g_1 skewness statistic was calculated at -1.128 for the whole data set, -1.063 for the whole data set without duplicate samples and -0.714 for *Anaspides* samples only, without duplicates. All values were highly significant ($p < 0.05$) indicating that the performance of parsimony in detecting the true phylogeny should be excellent (Hillis and Huelsenbeck, 1992). Interestingly, the statistic became less extreme when the more distant samples, *Paranaspides* and *Allanaspides,* were removed (relevant critical values for $p = 0.05$ of $g_1 = -0.10$). This appears to indicate that localised structure among the *Anaspides* sequences was probably not increasing skewness.

Maximum parsimony

A large number (2,778) of equally parsimonious trees were found, with a tree length score of 196. Although such a high number of equal-scoring trees might cast doubt on the value of the analysis, the evidence from the skewness test described above, the levels of bootstrap support and the moderately low homoplasy index of 0.245 all indicate that it should not be discounted. It is possible that the large number of closely related samples within the three major clades contributed to the high number of equally parsimonious trees. The strict consensus tree with bootstrap values, rooted with the *Allanaspides* clade, is shown in Figure 4.1.

Maximum parsimony analysis grouped populations into three major geographical clades which corresponded with those found in the allozyme analysis (chapter 3). Of the populations not included in the allozyme study, Square Lake, Haven Tarn, Snowy North, Lake Picton, Coronation Peak and Lake Rhona were included in the southwestern group; Zion Hill, Mount Rufus, Mount Ossa and Sandbanks Tier were included in the Central Plateau group (Figure 4.1).

Bootstrap values strongly supported the basal split of *Allanaspides* and the divergence of *Paranaspides,* the southern clade, the southwestern clade and the Central Plateau clade (including Mt Field and Mount Wellington). The divergence of the Mount Wellington population from the Central Plateau group was weakly supported.

Within the southern and southwestern clades, populations showed significant levels of divergence from each other, although Lake Rhona and Frenchmans Cap together formed a well-supported clade. Square Lake and Haven Tarn, both on the Western Arthur Range showed some similarity to each other, but were distinct from Lake Oberon, the third Western Arthur population. In contrast, the Central Plateau populations displayed little inter-population divergence. Only Mount Field and Hydro Creek were found to be significantly distinct and weak support was shown for the Clarence Lagoon population of *A. spinulae.*

Within-population structure in *Allanaspides hickmani* and the Lake St Clair *Anaspides spinulae* population was suggested by moderate support values.

Neighbour-joining

The HKY85 + Γ distance matrix shows average distances within and between the three geographical groupings of *Anaspides* and the three outgroup species. It is attached as Appendix 2 and summarised in Table 4.2.

The distance between the Central Plateau and southwestern groups is significantly lower than other comparisons (Table 4.2). These groups clearly share an affinity. The southern group is highly divergent from both these groups and shows approximately the same amount of divergence from *Paranaspides* as from other *Anaspides.* Both *Allanaspides* and *Paranaspides* are more similar to the southern than to the southwest and central groups. *Allanaspides hickmani* is consistently the most differentiated anaspid species. Differentiation among populations within the same geographical area is highest in the southwestern group.

Table 4.2 Mean 16S mtDNA distances within and among *Anaspides* population groups and outgroup species, summarised from the full pairwise HKY85+Γ distance matrix (Appendix 2).

Mean distance (HKY85+Γ measure)	**n**	**Southern group**	**Southwest group**	**Central Plateau group**	***P. lacustris***	***All. hickmani***
Southern group	10	0.010				
Southwest group	18	0.065	0.018			
Central Plateau group	25	0.056	0.030	0.006		
Paranaspides lacustris	1	0.058	0.070	0.066	-	
Allanaspides hickmani	3	0.115	0.138	0.131	0.130	0.001
Allanaspides helonomus	1	0.102	0.122	0.103	0.112	0.072

HKY85 distances were used to construct the neighbour-joining phenogram (minimum evolution score 0.5009) shown in Figure 4.2. The phenogram was not rooted and the *Allanaspides* clade was positioned within the southern group, with very long branches due to the large number of substitutions separating the clades. Otherwise the topology was very similar to the maximum parsimony tree and had the same major groupings, although slightly more structure is shown within these groups. The Mount Wellington population was placed outside all three of the geographical groups. Populations which were clearly differentiated within their geographical groups were Hartz Mountains in the south; Haven Tarn, Snowy North, Lake Picton, the Lake Rhona / Frenchmans Cap clade, Lake Oberon and Coronation Peak in the southwest, and Sandbanks Tier, Hydro Creek and Mount Field in the central group.

The 16S rDNA evolutionary rates of Jarman and Elliot (2000) applied to the distance data gave estimated times of divergence within and among geographical groups (Table 4.3). The 95% confidence intervals are large, so these values can only be considered to suggest a broad range of times. The last common ancestor of *Allanaspides* and the other two anaspid genera existed around 43 million years ago (between 30 and 82mya). The divergence of *Paranaspides* and the southern clade of *Anaspides* both occurred at about 20 mya (13 to 45 mya) and the southwest and Central Plateau clade diverged at about 10 mya (7 to 20 mya). The estimated time since the last common ancestor of populations within geographical groups ranges from 350,000 years for

populations of *Allanaspides hickmani* to approximately 6 million years for the southwestern *Anaspides tasmaniae.*

Table 4.3 Estimated times of divergence (million years) calculated using the HKY+Γ evolution model and an absolute rate of 16S rDNA evolution of 0.002871 ±0.001344 distance units per million years. Times of divergence between populations within groups are italicised.

Million years (95% confidence limits)	**Southern group**	**Southwest group**	**Central Plateau group**	***Paranaspides lacustris***
Southern group	*3.48 (2.37-6.55)*			
Southwest group	22.64 (15.42-42.57)	*6.27 (4.27-11.79)*		
Central Plateau group	19.51 (13.29-36.67)	10.45 (7.12-19.65)	*2.09 (1.42-3.93)*	
Paranaspides lacustris	20.20 (13.76-37.98)	24.38 (16.61-45.84)	22.99 (15.66-43.22)	
All *Allanaspides*	**all *Anaspides*** 43.54 (29.66-81.86)			43.54 (29.66-81.86)
Divergence of *Allanaspides helonomus* from *All. hickmani*	25.08 (17.08-47.15)			
Divergence of populations within *Allanaspides hickmani*	*0.35 (0.24-0.65)*			

Maximum likelihood

The maximum likelihood phylogeny, rooted with *Allanaspides* and showing bootstrap and jacknife support values, is displayed in Figure 4.3. The tree has a log likelihood score (-ln L) of 1654 and a topology which is similar to the previous analyses, differing only in that the Central Plateau and southwestern groups form a single clade. The southwestern populations nevertheless form a well-supported clade within that group. Jacknife values are more conservative than bootstrap values, as expected.

The southern and southwestern groups again contained many populations which were clearly differentiated. Well-supported distinct clades within these geographical groups include the southern cave populations, Hartz Mountains, Lake Oberon, Coronation Peak, Mount Anne, Lake Rhona / Frenchmans Cap and Haven Tarn. Mount Field and Hydro Creek are the only well-differentiated Central Plateau populations. Mount Wellington was included in the Central Plateau / southwestern group in this phylogeny.

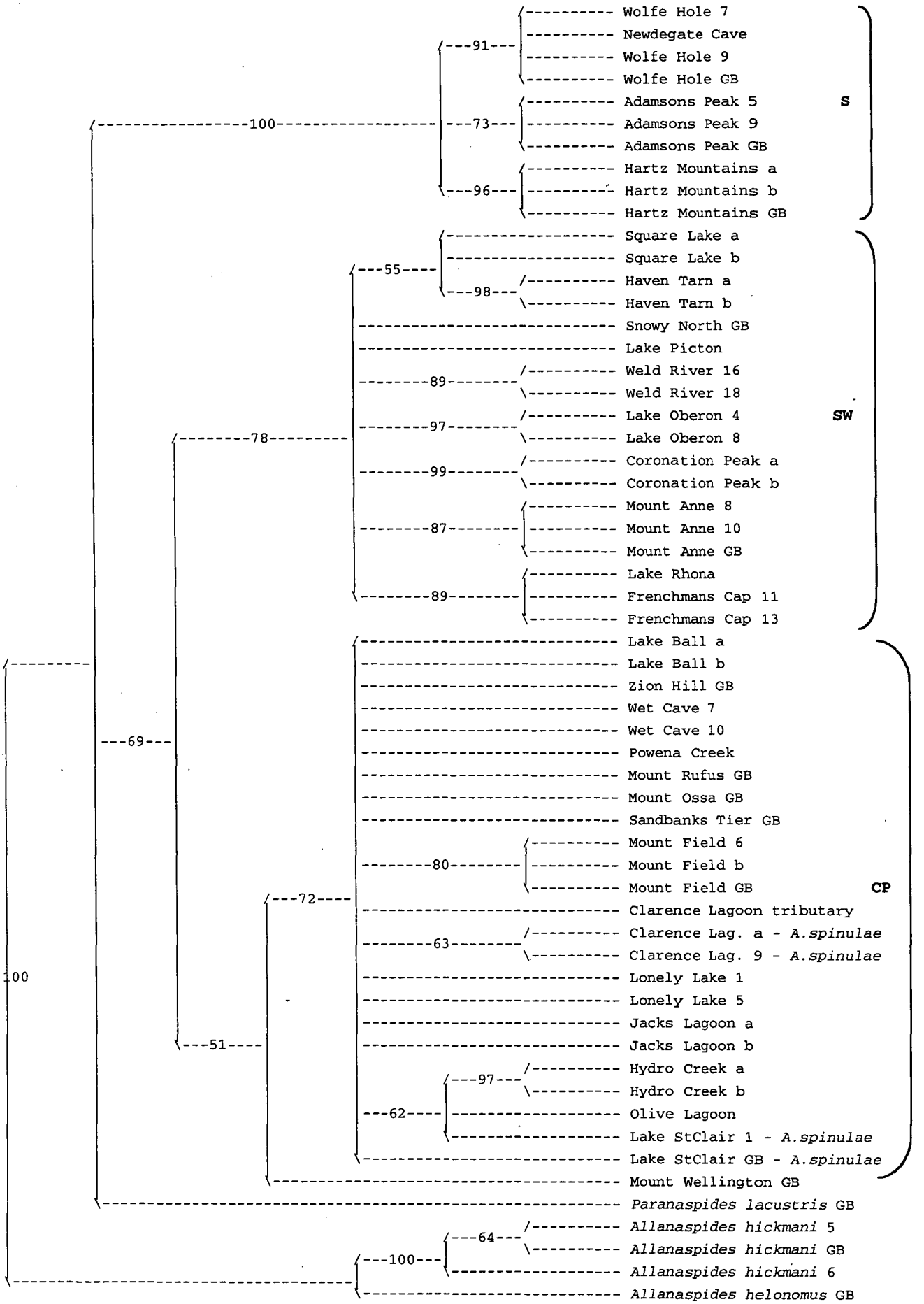


Figure 4.1 Maximum parsimony phylogeny (strict consensus of 2,778 equally parsimonious trees). Bootstrap support values over 50% are shown (fast stepwise method with 5,000 pseudoreplicates). Major clades are shown within brackets —**S** southern, **SW** southwestern, **CP** Central Plateau.

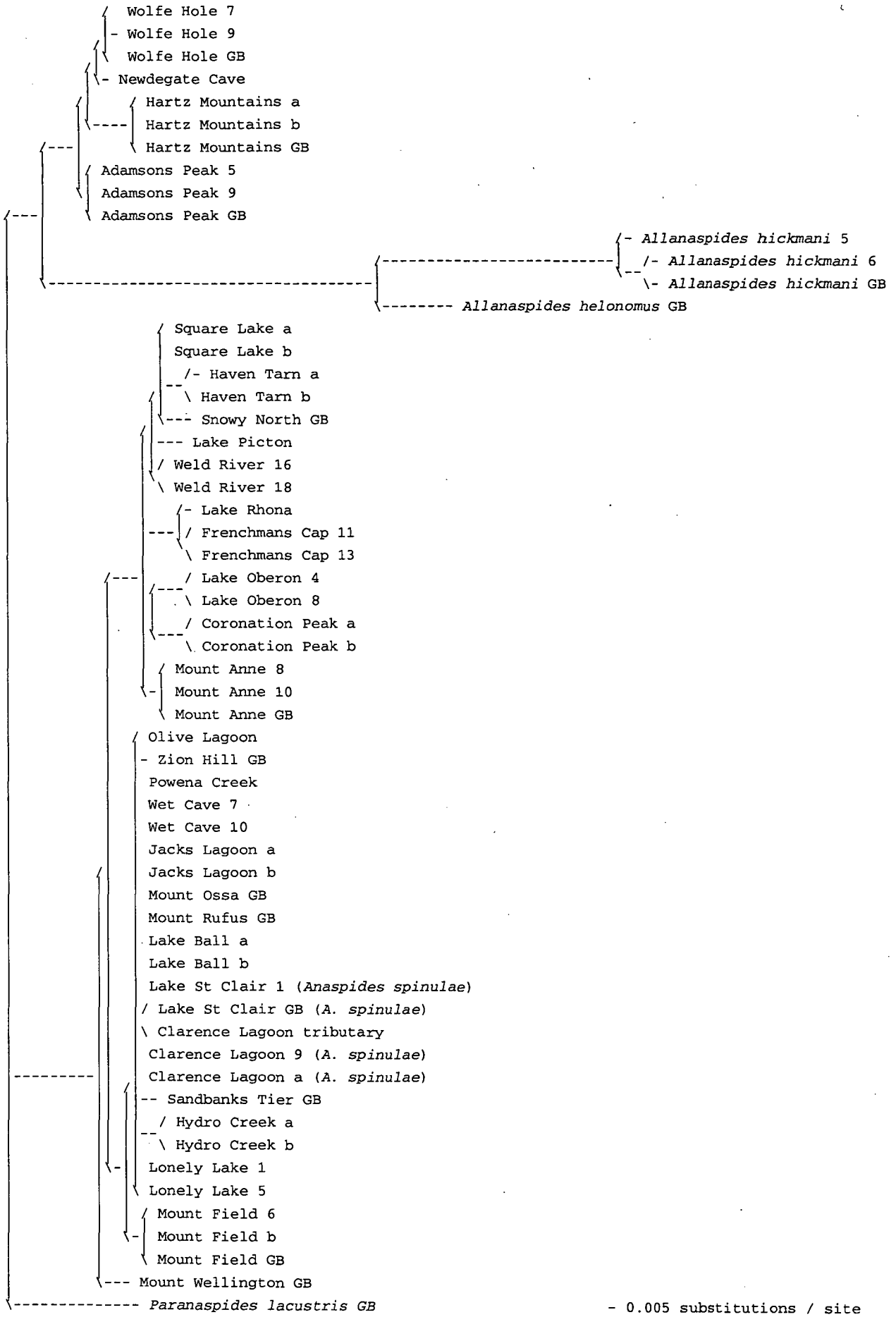


Figure 4.2 Unrooted neighbour-joining phenogram. Distance measure was HKY85 with gamma correction for among site rate variation.

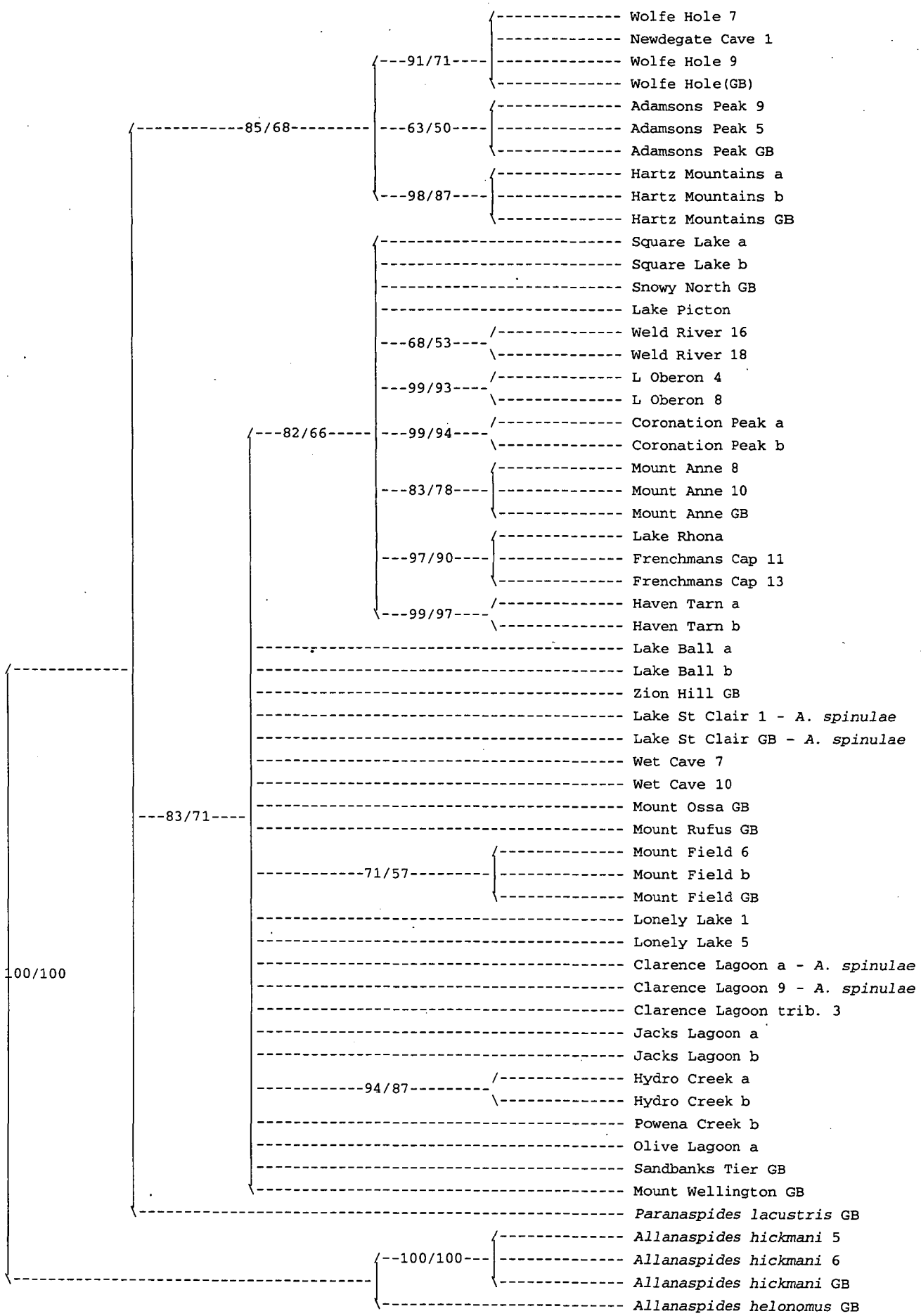


Figure 4.3 Maximum likelihood phylogeny with fast bootstrap / fast jacknife support values (100 and 300 pseudoreplicates respectively).

4.4 Discussion

The application of molecular data to phylogenetic analysis is characterised by the enormous amount of information potentially available from large data sets balanced by the need to manage the limitations of the data and various methodologies. This discussion begins with the consideration of some of the limitations on the use of molecular data. This balance can be attained by defining and testing assumptions, choosing analytical techniques appropriate for the assumptions, using more than one technique and assessing confidence in results statistically.

4.4.1 Possible limitations on the use of molecular data

Evolutionary time for which mtDNA phylogenies are appropriate

There is considerable difference among taxa with regard to the level of evolutionary relatedness for which particular genes are useful. The amount of genetic variability of the marker molecule must be appropriate for the level of relationship being studied but has been found to vary among taxa depending on factors such as mutation rate, molecular structure and function (Hillis *et al.*, 1996). In general nuclear genes are used for clarifying ancient relationships and mitochondrial genes for more recently separated taxa. Over sufficiently long periods, the rapid rate of mitochondrial evolution means that early base substitutions are overwritten by later ones, so mitochondrial genes tend to increasingly underestimate the number of substitutions with time (Futuyma, 1998). Consequently rates of evolution (numbers of base substitutions with time) along branches of mtDNA phylogenies are probably only linear for recently evolved groups (Hillis *et al.*, 1996), although exactly how recently this applies varies among taxa (Futuyma, 1998). Assumptions of linear rates of evolution in the phylogeny of organisms with a long history may not be reliable, and are not easily tested.

Lineage sorting

As mitochondrial DNA is a single linkage group, lacking recombination, it is analogous to a single allozyme locus with many alleles, providing only one independent estimate of a species phylogeny, regardless of how many variable sites are found. The phylogeny of the alleles within a mitochondrial sequence therefore may not represent that of the species itself. Allelic lineages are sorted stochastically over time, and paraphyly or polyphyly is likely in the short term as each

allele follows an individual pathway, with monophyly developing after a specific length of time as some alleles become extinct. If populations are sampled while paraphyly or polyphyly is still occurring, the gene tree may not be congruent with the species tree (Avise and Wollenberg, 1997), and any conclusions drawn from such a phylogeny would be unreliable.

The length of time since a particular extant allele was found in only one line has significance for whether the allelic tree will correspond with the species tree. Only if this time of coalescence falls between the first and second node of the species tree (that is, at some point after the common ancestor first diverged but before any further mutation events (Slowinski and Page, 1999)) will congruence of the species and gene trees occur. A large body of literature has considered the problems and various theoretical and statistical solutions to the application of coalescence theory to phylogeny. The methods proposed in these studies agree that a single mitochondrial gene sequence is insufficient evidence on which to base a phylogeny, but differ in the recommended treatment of multiple data sets (Avise and Wollenberg,1997; Slowinski and Page, 1999; Hillis *et al.*, 1996; Futuyma, 1998).

Moore (1995) showed that the drawbacks arising from so-called deep coalescence are somewhat less for mitochondrial genes than for nuclear genes. It has been agreed that, under neutral theory, the smaller the effective population size the more likely that coalescence occurred in the appropriate phylogenetic window. The uniparental and haploid nature of mitochondrial inheritance means that the effective population size for the genetics of the mitochondrial genome is only one-quarter that for a nuclear-autosomal sequence with the same number of genes. Thus it is more likely that a mtDNA sequence phylogeny will provide an accurate species tree than a nuclear sequence, and furthermore a larger number of nuclear than mitochondrial gene trees is needed to attain the same confidence level in the species tree (Moore, 1995). This does not negate the need for more than one marker when using mtDNA, however.

Secondary structure

A recent area of discussion is the relative rates of molecular evolution of nucleotides in stems and loops of the mitochondrial genome (Hillis *et al.*, 1996). Where models of the secondary structure of mitochondrial genes have been prepared, a statistical approach comparing functional regions has shown that loops can be more variable than stems or connecting regions and complex compensatory mechanisms may confound unambiguous sequence alignment. Smith and Bond (2003) found that down-weighting or excluding regions of greater variability in 16S in spiders

improved the phylogeny, leading to a tree which was congruent with that produced by combined analysis of other DNA markers and morphology.

Incorporating knowledge of secondary structure into mtDNA phylogenetic studies would be useful, but such knowledge is not always available. This area of molecular phylogenetics is in its infancy, with structural models continuing to improve but as yet available for few organisms (Flook and Rowell, 1997). It is unclear in which genes secondary structure is conserved across taxa.

4.4.2 Conclusions from this study

The general problems of saturation by change, lineage sorting and secondary structure have been addressed as far as possible by statistical resampling to assess confidence levels and by the test for skewed branch length distribution, which tests specifically for random signal. The high bootstrap values supporting the most basal node indicate that this region of the 16S molecule is probably not yet saturated with change. The presence of short areas of fast-evolving nucleotides offers potential for the future study of secondary structure of 16S in the Anaspididae but does not appear to have unduly influenced the results.

Rate variation and substitution type bias are seen as potentially problematic assumptions in phylogenetic reconstruction. Rate variation among nucleotide sites was accommodated in the current study by discrete-gamma correction of distance and likelihood calculations. Rate variation among populations of anaspid taxa has previously been ruled out by log likelihood test (Jarman and Elliot, 2000).

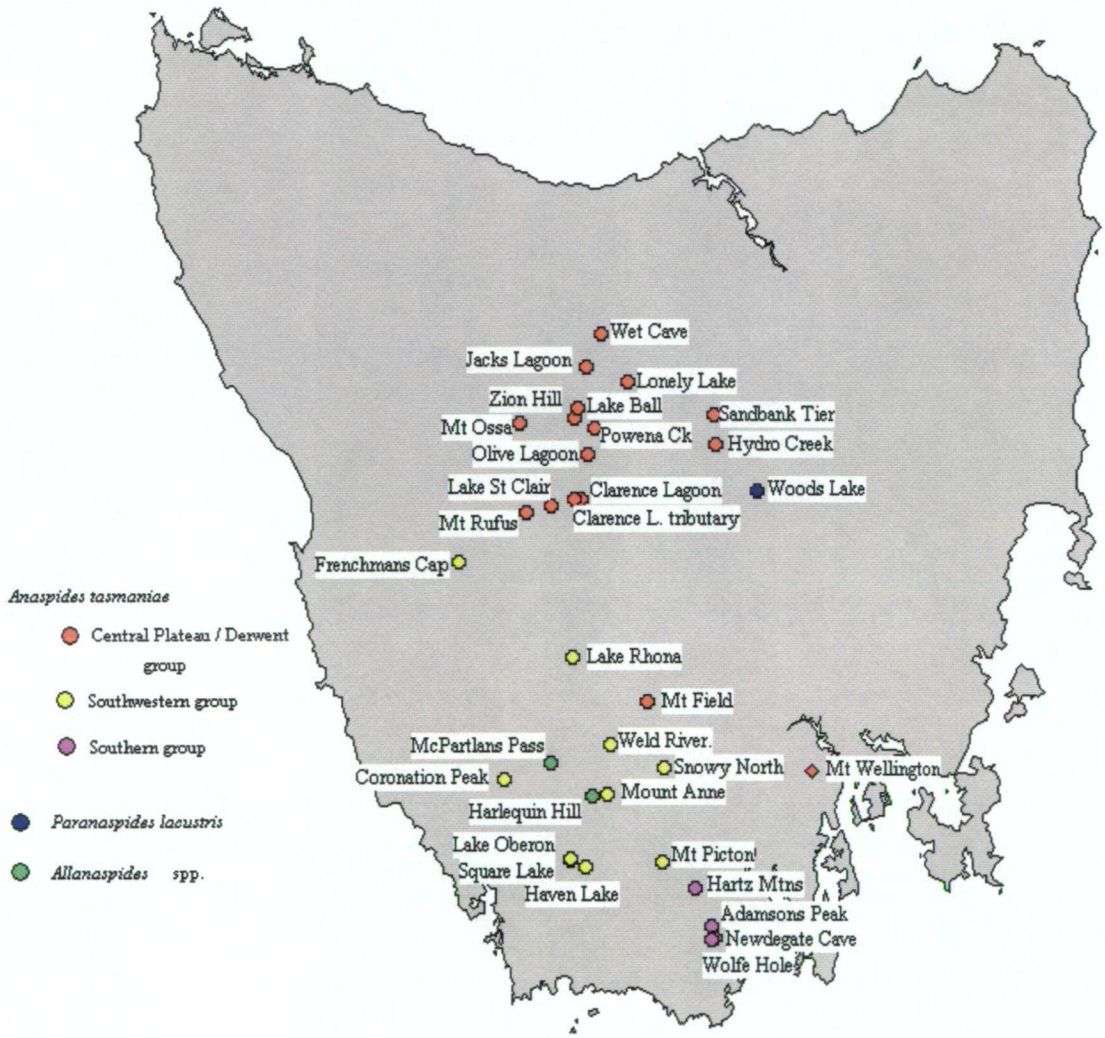
The ratio of transitions to transversions was not high (~2:1), possibly due to the bias towards A and T across all taxa. Substitution types were weighted by 2 towards transversions to compensate for the possible randomness of transitional change. Crozier and Crozier (1993) suggested that a high frequency of A and T nucleotides means that transitions are unlikely to predominate over transversions to any great extent, unless some mechanism for rapid reversal of transitions were to occur. Predominance of A and T is not unusual in arthropods (Stevens and Hogg, 2003). Crozier and Crozier (1993) found a combined A-T frequency of 84.9% in the whole mitochondrial genome (with 85.1% in 16S alone) of the honeybee *Apis mellifera* and quoted previous data for *Drosophila yakuba* of 78.6%. The tendency for A and T to act as a resting state may somehow function as a means of avoiding saturation by change.

Three phylogenetic methods produced topologically very similar trees. High bootstrap confidence levels confirm that *Allanaspides* was the earliest group to diverge from the anaspid lineage. The genera *Anaspides* and *Paranaspides* together form a monophyletic clade, casting doubt on the generic status of the southern group of *Anaspides*, which is as distant from the other *Anaspides* populations as is *Paranaspides*. There is no evidence of greater genetic similarity between the two populations of *Anaspides spinulae* than between either population and the remaining Central Plateau populations, so its separate species status is not supported here.

In all trees, the southern and southwestern groups form distinct clades and the Central Plateau group is related to the southwestern group. The three phylogenies differ in whether the southwest group is included within or placed as a sister group to the Central Plateau group. Maximum likelihood analysis, considered the most likely to produce the correct phylogeny, places the southwestern clade within the Central Plateau group, while the neighbour-joining analysis, also considered highly accurate, separates these two groups by a small distance only. Maximum parsimony shows bootstrap support for the Central Plateau and southwest groups as distinct clades. It appears likely that these two groups have been part of a single population or have undergone mixing, and are now in the process of differentiating. The divergence of the southern group is far greater. Figure 4.4 shows the geographic groups of genetically distinct populations.

The genus *Anaspides* appears to be undergoing continued speciation. As well as the evolution of genetically distinct broad geographical groups, almost all populations within the southern and southwestern groups are accumulating genetic changes that are causing them to become differentiated. The populations from Square Lake and Adamsons Peak are exceptions, with the latter weakly supported as a distinct clade. The southern cave populations together form a distinct group. All phylogenies found a strong association between the Lake Rhona and Frenchmans Cap populations. These lie close to the southern edge of the Central Plateau and are separated by about 20 km. As only one sample from Snowy North and Lake Picton was included, it is not possible to comment on the differentiation of these populations, although the distance measures indicate a large separation in both cases.

Figure 4.4 Genetically distinct geographical groups as determined by molecular analysis.



In contrast, populations in the Central Plateau group appear to have relatively similar 16S sequences. All phylogenies support only the Mount Field and Hydro Creek populations as well-differentiated populations. The northern cave population (Wet Cave) is not supported as a distinct clade. Both parsimony and neighbour joining separate the Mount Wellington population from the major geographical groups, whereas likelihood analysis places it within the Central Plateau group. The single sequence from the Sandbanks Tier population appears from the distance data (and consequently the neighbour joining phenogram) to be substantially differentiated. It is interesting that both this and the Hydro Creek populations are the most highly differentiated of the Central Plateau populations and show no similarity to each other despite being separated by only about 5 km.

The timing of all divergences within the anaspid lineage have been estimated by robust molecular clock methodology as occurring since the early to mid Tertiary. The divergence of the genus *Allanaspides* was estimated at about 43 million years ago (mya), that of *Paranaspides* and the southern group of *Anaspides* at about 20 mya and that of the southwestern group at about 10 mya. The differentiation of populations within the geographical groupings apparently occurred about 6 mya for populations in the southwest group, about 3.5 mya for populations in the southern group, about 2 mya for populations in the Central Plateau group and about 350,000 years ago for *Allanaspides hickmani* populations. (The latter differentiation was based on a small sample size so should be regarded as inconclusive). It is interesting that the separation of populations in the southwest group appears either to have occurred earlier or to have been more complete than that of other *Anaspides* populations.

Jarman and Elliot (2000) proposed that the three groups identified by the 16S phylogeny be recognised as three phylogenetic species on the basis of exclusive monophyly diagnosed by unique synapomorphies (Jarman and Elliot, 2000). From the results of the current study, it is arguable whether the speciation of the southwestern group from the Central Plateau group is complete, and whether applying the same phylogenetic criteria should lead us to consider the southern group for status as a separate genus as well as a separate species. The use of other genetic markers is critical in evaluating the systematics of the group, as a single gene phylogeny may not reflect the organismal phylogeny. A comparison of the allozyme and mt DNA analyses will be made in chapter 5. Morphological markers will also be essential for the description of new taxa.

Considering the long evolutionary history of the anaspidacean lineage (at least 200 million years from the fossil record), the development of the Tasmanian Anaspididae is relatively recent, occurring since the mid-Eocene. For a morphologically highly conservative group, the amount of genetic evolution occurring over a relatively short time-span is remarkable. It would probably be wise to confirm the estimated evolutionary rate of the Anaspididae by using the same molecular clock methodology with another malacostracan crustacean the evolution of which has also been confidently dated.

From molecular evidence it appears that anaspid evolution in Tasmania has been dominated largely by vicariance events and that this geography-mediated divergence is continuing. The biogeographical factors influencing the current distributions and relationships of *Anaspides* will be considered in the following chapter.

Chapter 5 General Discussion

5.1 Early syncarid biogeography

The earliest recorded syncarids are marine Carboniferous fossils of the order Paleocaridacea from the tropical northern continent of Laurasia (Brooks, 1962). By the late Carboniferous era, these syncarids were occupying warm, brackish and fresh lagoonal habitats in North America and Europe (Schram and Schram, 1974; Schram, 1981). A radiation from Laurasia and the invasion of freshwater habitats apparently both occurred in the late Paleozoic and coincided with the formation of Pangea and the marine radiation of peracarid and eucarid malacostracans. These seem to have replaced marine paleocaridacean syncarids in the fossil record by the late Paleozoic, leading to the hypothesis that Paleozoic syncarids moved into freshwater as a result of competition with later, more efficient crustaceans (Schram and Schram, 1974). However, the recent discovery of freshwater crayfish fossils from the early Permian in Antarctica indicates that eucarid decapod crustaceans had also radiated into freshwater in the late Paleozoic (Babcock *et al.*, 1998) and does not support the competition hypothesis. The loss of much of the near-shore habitat when Pangea formed in the Permian (Campbell, 1993) is likely to have put pressure on both syncarids and decapods to adapt to terrestrial or freshwater habitats. No records of marine syncarids later than the Permian occur, and nearly all extant syncarids inhabit freshwater (Brooks, 1962). It is accepted that syncarids have been almost exclusively freshwater since the Triassic (Banarescu, 1990).

The Permian occurrence in South America of *Clarkecaris*, in what is presumed to have been brackish water, is the earliest record of the order Anaspidacea (Brooks, 1962). The significance of this early South American fossil is confounded by the apomorphic nature of the current South American anaspidaceans (stygocarids), and by the plesiomorphic form and diversity of the exclusively Tasmanian family Anaspididae. The origin, direction and path of the radiation between continents is therefore a matter of conjecture, but must have included an occupation of Antarctica, as the present distribution of anaspidaceans in South America, New Zealand and Australia is classically circum-Antarctic (Banarescu, 1990). The very sparse fossil record indicates that anaspidaceans were occupying shallow freshwater lakes in mainland Australia in the Triassic and the Cretaceous (Brooks, 1962; Jell and Duncan, 1986). Unlike much of the world, the uplifted terrain of south-east Australia was experiencing cool to cold conditions with extreme seasonality throughout much of the Mesozoic (Frakes and Barron, 2001), so aquatic fauna with low vagility would have adapted to cool conditions at that time.

Banarescu (1995) lists a number of freshwater taxa which today share the same general distribution as the Anaspidacea, including the bathynellacean syncarid group *Chilibathynella*. Many other combinations of Gondwanan continental affinities exist among Tasmanian crustaceans, such as the New Zealand / Australia / South Africa (East Gondwanan) pattern shown by the phreatoicid isopods and chiltoniine amphipods, and the pan-Gondwanan distribution of the parastacid crayfish and notonemourid stoneflies (Banarescu, 1995).

5.2 Family-level divergence in the order Anaspidacea

The early records of syncarid distribution, the restriction of all extant families but one to hypogean habitats, and the current distribution of the primitive Anaspididae all reflect a refugial history. Like many other Gondwanan relicts, anaspidaceans are cold-adapted and are not found in seasonally warm waters or at latitudes lower than about 30°; in addition they may have been unable to compete with more recent fauna (Banarescu, 1990) or have been vulnerable to predation (Schram and Hessler, 1984). The four extant anaspidacean families probably evolved as the result of the development of different survival strategies in the face of these susceptibilities. For example, the family Stygocarididae probably developed in the late Paleozoic / early Mesozoic as an adaptation to hypogean habitats, possibly as a refuge from warm temperatures or predators such as decapod crustaceans. In the case of the Anaspididae, competition from more recent fauna is an unlikely cause of divergence, as their persistence in similar habitats over tens of millions of years alongside a variety of other ancient crustaceans and aquatic insects is proof of successful survival strategies (Knott, 1975).

This study aims to shed light on the evolution and biogeography of the most plesiomorphic extant anaspidacean family, the Tasmanian Anaspididae. The sequence of influences that has led the Anaspididae from the shallow lakes of Mesozoic mainland Australia to their present refugial Tasmanian distribution is necessarily a matter of conjecture, but the island s geological and climatic history (as far as is known), the use of a molecular clock and the biogeography of other Tasmanian freshwater animals of Gondwanan lineage, can all contribute to a conceivable if necessarily somewhat speculative history of the family.

Fossil evidence shows that the early Anaspididae such as *Anaspidites* and *Koonaspides*, were occupying shallow, open, freshwater lakes in southeastern Australia up to at least 100 million years ago (mya) and possibly longer. The climate across southern Australia by that time had changed from the wetter Triassic and Jurassic, and become dry and very cold in the Cretaceous, with estimated average annual temperatures in Victoria of between —3 and 5 °C

(Frakes and Barron, 2001; Veevers, 1991). The closely related family Koonungidae possibly diverged and became adapted to opportunistic use of refugial subterranean habitats in response to the drying of the climate in the Cretaceous, but no fossil evidence exists to suggest the time of divergence. From comparisons of segmental proportions, Knott (1975) suggested that the Koonungidae evolved directly from *Anaspidites antiquus* or a close relative. It is perhaps significant that the distribution of the modern Koonungidae includes Victoria, South Australia, Bass Strait islands and the northern Tasmanian lowlands, up to the bottom of the Great Western Tiers escarpment, while the range of the Anaspididae is bounded to the north by this escarpment, with the exception of the caves lying at its foot.

A molecular study including all four anaspidacean families would reveal much about the phylogenetic relationships and evolutionary history of the order, and may help clarify the origin of the syncarids as a whole, including whether they originated as a hypogean group and repeatedly emerged into surface waters, or, as is more commonly accepted, originated as a surface form and repeatedly retreated to refugial hypogean environments. Riddle (1996) advocates the use of molecular phylogeography to try to separate recent from older patterns of diversity and distribution. He suggests that biotic responses to recent glacial / interglacial events are not large enough to erase biogeographical structure produced by earlier episodes of isolation and divergence.

5.3 Origin of currently recognised anaspid genera

5.3.1 Mid-to-late Eocene — *Allanaspides*

Mitochondrial DNA sequence analysis in this study indicates that the first divergence from the anaspid lineage in Tasmania, that of the genus *Allanaspides*, occurred between 30 and 80 mya, probably about 43 mya, in the mid to late Eocene. Possible causes of the divergence of *Allanaspides* at this time include climatic change and geological upheaval.

The Paleocene-Eocene transitional period at 55 mya marked the change from a homogeneous, moist, warm greenhouse world to a more climatically varied icehouse world (Crouch, 2002). Global warming began at ~59 mya, peaked at 50 — 52 mya and then diminished, and was associated with major biotic perturbations not unlike the effects of current global warming (Crouch, 2002). It is possible that the relatively sudden increase in temperatures and the unprecedented high temperatures at the maximum caused a sympatric speciation through anaspids inhabiting crayfish burrows around the edges of lakes for shelter from high water temperature. This scenario relies on a slight inaccuracy of the molecular clock, but within the 95% confidence limits. By 38 mya, in the late Eocene, the annual mean temperature in Victoria was still about 18 °C (Frakes and Barron, 2001). The temperature

difference between Victoria and Tasmania was probably not as marked as it is today, as the circum-Antarctic current had not yet become established (Veevers, 1991), so Tasmania can be assumed to have been quite warm throughout the period during which the divergence of *Allanaspides* occurred.

The late Eocene was also characterised by intense geological activity in Tasmania, as tensions associated with the break-up of Gondwana caused extensive faulting. Rifting uplifted plateaus, reactivated older fault systems and created fault troughs in the Bass Basin, Tamar Valley, Midlands, Macquarie Harbour and Derwent Valley (Sutherland, 1980). The area in which *Allanaspides* is now found is home to a group of endemic freshwater species, which includes other Gondwanan types such as phreatoicid isopods, parastacid crayfish and ceinid amphipods. Knott (1975) speculated that these are relicts of the fauna of four shallow lakes in the region, including one in the Serpentine-Huon drainage and one straddling the Wedge and Gordon Rivers. Carey (1961) discovered faulting which had created these extinct lakes, and assigned them a Quaternary age because Lake Edgar (in the same vicinity and also created by faulting) appears to be relatively young. However, faulting was much more widespread during the Tertiary than the Quaternary, and the lakes in question were almost completely filled in by the time the area was flooded for hydro-electric development in the early 1970s, so a synchronous development with Lake Edgar may not be valid. The age of the fauna, including *Allanaspides,* of the button-grass plains which now occupy the area of the ancient lakes, suggests that the lakes may have originated through Tertiary faulting (Knott, 1975) and thus *Allanaspides* probably adapted to a marshland existence after the lakes began filling with sediment.

Knott (1975) also suggested that *A. helonomus* evolved in lakes of the Serpentine system and *A. hickmani* in lakes of the Gordon system. Their current distribution has probably been influenced by anthropogenic cross-catchment flooding and cannot be used to validate this assertion, but it appears likely from the earliest records. The present molecular clock study found that a general form of *Allanaspides* existed for about 20 million years before the two species became differentiated (see below). The climate in the southwest was probably wet through the Miocene (Frakes and Barron, 2001), so that the populations in the Serpentine/Huon and Gordon/Wedge lakes would probably have been genetically similar through migration or mixing of the drainages across low-lying areas or in downstream river stretches.

Horwitz (1988) undertook a detailed study of subterranean syncarid distribution in southwest Tasmania and found no anomalies with Knott s suggested origin for *Allanaspides*, pointing out that the genus is now not a lake dweller but relies on buttongrass plains and crayfish

burrows, implying a long history of divergence from the generalised anaspid type and adaptation to life in shallow weedy marshland.

If the anaspid ancestor was widespread in Tasmania as well as southern Australia, as we have no reason to doubt (Bass Strait was not flooded prior to the Oligocene), then it seems clear that *Allanaspides* could well have evolved *in situ* in the Tasmanian southwest. Vicariant speciation as suggested by Knott s model fits with the molecular clock estimate of a Tertiary divergence and climatic warming may have determined *Allanaspides* dependence on crayfish burrows as refuges from high water temperatures.

5.3.2 Late Oligocene - Early Miocene — *Paranaspides* and southern *Anaspides*

The genetic distances between the southern and the central / southwestern groups of *Anaspides* were found to be at the intergeneric level for most population comparisons in both the allozyme and mtDNA studies, by comparison of distances between *Anaspides* and *Paranaspides* and by Thorpe s (1983) estimations using allozyme distances. As a result, either *Paranaspides* should be reclassified as a species of *Anaspides* (as suggested by Knott, 1975) or the southern *Anaspides* group should be described as a new genus. The former is not warranted due to the generic level of morphological differentiation displayed by *Paranaspides* (Swain *et al.*, 1970) and the extremely high genetic distances between *Anaspides* and *Paranaspides* (using the criteria of Thorpe, 1983). Doubt about the status of the southern group as a new genus should be addressed through morphometric or further molecular studies, but for the purpose of this discussion the level of phylogenetic separation will be taken to indicate putative generic status.

Between 20 and 25 million years ago, the southern group of *Anaspides* and *Paranaspides* both diverged from the anaspid lineage. This node on the phylogenetic tree is a three-way polytomy, with one clade containing the southern *Anaspides*, another containing *Paranaspides* and the third containing the central and southwestern *Anaspides*. Speciation within the genus *Allanaspides* occurred at about the same time (see **5.4.1**). Whether the same mechanisms influenced all three speciation events is unknown, so in the following discussion I consider first separate and then general possible causes.

Southern *Anaspides*

The two most significant environmental events of this time were the rising sea level and the volcanic activity in parts of the state. The sea level rose in the late Oligocene and remained high from then until the end of the Miocene (~25 - 5 mya) (Davies, 1965). The rise in sea level may have caused the separation of the southern populations from remaining anaspid

populations, which probably occupied lowland as well as highland waterways. As the D Entrecasteaux Channel was progressively flooded from the south, anaspids in the Hastings area would have been isolated first, followed by the Adamsons Peak and Hartz Mountains populations. The slightly greater genetic distances between the Hastings caves populations and the Esperance catchment populations appear to reflect this progression. This hypothesis proposes vicariance as the mode of speciation (allopatric Type 1 as defined by Bush, 1975) and is supported by the restriction of southern genotypes to east-flowing waterways between the Huon River and the sea; geographically close populations in the Huon catchment such as Mt Picton and Snowy North are members of the southwest group.

Paranaspides

The genus *Paranaspides* probably evolved as an adaptation to lacustrine conditions. The timing of its emergence can probably elucidate the origin of Great Lake, apparently the centre of its dispersal. The age of Great Lake (or more accurately, the 2 lakes existing prior to the creation for hydro-electric purposes of the current single Great Lake) has been something of a mystery, with the most common estimates ranging from quite recent (as a result of tilting of the plateau; Davies, 1965) to an Oligocene origin (Sutherland *et al.*, 1973). The molecular clock in this work supports the latter suggestion.

Geologically, Tasmania was structurally stable throughout the Oligocene and Miocene, except for a number of basalt eruptions that occurred across the state (Banks, 1965). Sutherland (1980) shows 9 extinct volcanoes within about 15 km of Great Lake, and extensive basalt lava fields adjacent to the western shore and to the south of the lake. Sutherland *et al.* (1973) aged the basalt surrounding the Ouse River 3 km west of Great Lake at 23.6 million years. It is highly likely that shallow lakes were formed by the damming of watercourses running into the Ouse River after this or other eruptions to the west of Great Lake. The continuity of water bodies in the Great Lake area since the start of the Miocene is likely to have been maintained through a series of minor lakes rather than one or two larger lakes (Sutherland *et al.*, 1973). The ancestors of *Paranaspides* adopted a pelagic existence, relying on weedbeds for food and shelter, while the remaining anaspids either retreated upstream in tributary creeks where they still persist, or possibly coexisted with *Paranaspides* in the lakes for an indeterminate period before succumbing to predation or unsuitable environmental conditions. There were no obvious genetic affinities between populations of *A. tasmaniae* from tributaries of Great Lake and *P. lacustris*, adding weight to the older age estimates for Great Lake. The agreement between the age of the lava flows and the molecular clock for *Paranaspides* is striking, and adds considerable support to this hypothesis. Speciation in this case would have been sympatric according to the definition of Bush (1975).

Widespread effects

The icehouse conditions that developed in the mid-Eocene continued through the late Oligocene and early Miocene (20 — 25 mya), which was increasingly cold and arid as moisture became bound in the polar ice-caps. By about 30 mya, the separation of Tasmania and Antarctica was sufficiently wide for the circumpolar ocean current to be established (Veevers, 1991) and Tasmania then became much cooler. By the early Miocene, mean temperatures in central Tasmania were 6-8 ¡C cooler than Victorian temperatures, but rainfall may not yet have significantly diminished (Frakes and Barron, 2001; Hill *et al.*, 1999). The desiccation of water bodies may have brought about the separation of the southern *Anaspides* from the anaspid line,but is unlikely to have contributed to the divergence of the *Paranaspides* line. Climatic variation across Tasmania at the time (Frakes and Barron, 2001) is likely to have kept the central and southwestern areas wetter than the south-east coast, so desiccation could have been severe enough to cut off the southern from the central and southwest groups without isolating these two from each other, or isolating populations within groups, at this time. Discussion of this issue with relation to intra-generic differentiation can be found in section **5.4**.

A further possible consequence of the rise of sea levels in the Oligocene — Miocene transitional period is its effect on the fauna of lowland water bodies, which would probably have moved upstream as much as ecological constraints would allow, increasing the likelihood of predation on resident freshwater animals. In this way, predation might have forced resident anaspids to move into more marginal habitats at this time. The presence of freshwater galaxiid fish in Tasmanian waters during this period is plausible, as their basic distribution is Gondwanan and a number of authors have indicated an ancient origin for these fish, at least predating the Pleistocene (McDowall, 2002; Waters and Burridge, 1999; Waters and Wallis, 2001). White (1997) proposed southeast Australia as the Darwinian centre of origin of local species prior to a Quaternary marine dispersal to New Zealand. Fossil evidence of a galaxiid from the Miocene in New Zealand (McDowall, 2002) and a molecular clock estimate by Waters and Burridge (1999) indicating that galaxiids were already in the west Pacific region during the Miocene add some credence to this scenario, but the timing of the first invasion of Tasmanian freshwater bodies by these fish remains unknown. The majority of extant galaxiid species are found predominantly at low altitudes (Frankenburg, 1974), so if they did in fact occur in Tasmania in the early Miocene, they would probably have been present in coastal streams and lagoons, such as the large lagoon that occupied the Macquarie Harbour basin (Knott, 1975). If true, these events may explain the concurrent separation of the southern group of *Anaspides,* the speciation within the genus *Allanaspides* and, more improbably, the disappearance of benthic anaspids from Great Lake. Fulton and Horwitz (1987) report that predation by freshwater fish has influenced the distribution of *Anaspides*

in Tasmania, as populations are only abundant where native and introduced fish are absent. The coexistence of galaxiids and *Allanaspides* in the Lake Pedder region can probably be explained by the reliance of the latter on refugial habitat, particularly the subterranean.

5.4 Intra-generic divergence

5.4.1 Speciation within the genus *Allanaspides*

The divergence of *Allanaspides hickmani* and *A. helonomus* occurred at the end of the Oligocene around 25 mya, at approximately the same time as the divergence events described in the previous section. The most likely cause of separation is the infilling of lakes and the increasing climatic dryness that would have contributed to a reduction in the size of the lakes, isolating the populations and bringing about speciation by vicariance.

Knott (1975) suggests parallel speciations of *Allanaspides hickmani* and the fish *Galaxias parvus* in the ancient Gordon lake and *A. helonomus* and *G. pedderensis* in the ancient Serpentine lake, as their current distributions would suggest. The influence of predation by these freshwater fish (or their ancestral species) on speciation in *Allanaspides*, by isolating populations in the marshy verges of the two lakes and preventing mixing between lakes could be suggested, although no fossil or molecular evidence exists for the time of emergence of these fish. A combination of different factors is possible.

5.4.2 Divergence of Central Plateau and southwest *Anaspides*

The split between the southwestern and Central Plateau / Derwent catchment populations constitutes an interspecific divergence and occurred approximately 10 mya, in the mid-Miocene. The Miocene has been identified as a time of great environmental change and speciation in Australia, caused by increasing aridity, decreasing atmospheric CO_2, vulcanism, the separation of Tasmania from Australia and the increasing dominance of C4 plants (Vickers-Rich and Rich, 1993; Hill *et al.*, 1999; Hansen, 2001). There has also been speculation about the possibility of widespread glaciation in Tasmania in the Oligocene and Miocene epochs (Vickers-Rich and Rich, 1993; Hill *et al.*, 1999), with the discovery of palynological evidence of an Oligocene glaciation in the upper Forth Valley (Macphail *et al.*, 1993).

Dry conditions became more severe globally during the mid to late Miocene (Vickers-Rich and Rich, 1993; Hill *et al.*, 1999) and may have had substantial impacts on Tasmanian freshwater fauna, limiting the availability of suitable habitat and isolating populations. The progressive isolation of all *Anaspides* groups, and populations within groups can most parsimoniously be explained by ongoing climatic drying since the Miocene.

It is conceivable that the populations of *Anaspides* in the Central Plateau and southwest had already been isolated by the drying conditions in the early Miocene, and were then able to mix during the melting phase of an Oligocene / Miocene glaciation, before finally separating again due to the greatly increasing aridity of the mid-Miocene. This mechanism has been shown in freshwater fauna in Northern Hemisphere Pleistocene glaciations (in mysids by Vainola *et al.*, 1994, and in gudgeons by Schreiber, 2002). Stevens and Hogg (2003) have examined the possibility that as well as causing speciation through habitat fragmentation, glaciation can also cause cycles of population contraction and expansion that act as a homogenising force. This would be especially applicable to freshwater fauna as large volumes of glacial meltwater are released into the environment over relatively long timespans. The times of isolation given by the molecular clock may in fact be times of the most recent separation, overlaid on older relationships. Waters and Burridge (1999) and McDowall (2002) describe similar effects on genetic affinities of recent marine dispersals over ancient Gondwanan relationships. The use of molecular markers with different rates of evolution would be needed to separate these associations.

The invasion of freshwater habitats by galaxiid fish may also have occurred during the mid-Miocene and isolated *Anaspides* populations in headwaters and other refugial habitats.

The southwest group is wholly found to the west of the major faunal discontinuity known as Tyler s Line. This break was first described by Shiel *et al.* (1989) on the basis of rotifer assemblages, but is now known to apply to a large range of other animals, including frogs, grasshoppers, landhoppers, land snails, millipedes, velvet worms, stoneflies, centipedes and freshwater crayfish (Mesibov, 1996). The line repesents a marked ecotone, with steep environmental gradients for climate, rainfall, geology, vegetation and ionic concentrations in water bodies (Mesibov, 1994; Tyler, 1974; Buckney and Tyler, 1973). *Paranaspides* and the southern and Central Plateau / Derwent *Anaspides* lie to the east of the line, and *Allanaspides* and the southwestern *Anaspides* lie to the west, adding support to separate species status for the latter group.

5.4.3 Divergence within geographical groups

The divergence times of populations within groups were estimated at 6 mya within the southwest group, at 3.5 mya within the southern group and at 2 mya within the Central Plateau. These figures represent an average for all pairs of populations within each group, so should be accepted with caution.

The core group of Central Plateau populations (Jacks Lagoon, Lonely Lake, Clarence Lagoon, Clarence Lagoon tributary, Lake St Clair, Lake Ball, Powena Creek, Olive Lagoon, Mt Rufus, Mt Ossa and Zion Hill) are genetically indistinguishable, and the Wet Cave population is also similar (although more divergent in allozymes than mt DNA). Hydro Creek, Sandbanks Tier, Mount Field and Mount Wellington are increasingly divergent, the latter possibly at species level. The acceptance of an isolation by distance model for the Central Plateau may indicate the frequent partial mixing of populations on the Plateau that would have occurred after each Pleistocene glaciation, hence the more recent time of divergence. Meltwater lakes were common features of periglacial regions during and after glaciations (Davies, 1965). Clearly, the four non-core populations are found in areas where periglacial mixing did not occur.

The differentiation of populations in the southern and southwestern groups probably reflects ongoing climatic drying and the isolating influence of predation by native and introduced fish. The steepness of the terrain in the southwest may have caused earlier fragmentation of populations as migration between populations would have been more difficult and smaller streams are at greater risk of drying up. On the other hand, the steepness and colder temperatures may also have protected the fauna of highland waters from both galaxiids and trout, as well as providing invertebrate fauna with more habitat in tarns through higher precipitation and lower evaporation.

The clade formed by Frenchmans Cap and Lake Rhona in the southwest may be the remnants of a widespread population throughout the Franklin and Gordon drainages. However, they clearly show a closer affinity than that between other southwest populations and the same factors (aridity, climatic warming, predation) should have led to similar levels of interpopulation divergence. Periglacial mixing may have caused recent genetic congruence. Kiernan (1990, 1999) reports Pleistocene glaciers flowing from the Central Plateau ice cap into the Franklin and Derwent Valleys, with the latter bifurcating above Lake St Clair and spreading through the Cuvier Valley. This and/or the Franklin Valley glacier probably formed periglacial lakes in the vicinity of the Navarre and King William Plains, which would have fed into the Derwent, Franklin and Gordon Rivers. Although this reasoning satisfactorily accounts for the close affinities between the Lake Rhona and Frenchmans Cap populations, their much more distant relationship with the Lake St Clair and other western Central Plateau populations is puzzling.

The most significant feature of the southern populations is the use of caves as refugia, most likely from climatic extremes during and since the last glaciations, as caves offer more stable conditions than surface environments. (This probably partially explains the extremely large

individuals found in Wet Cave.) There are no separate cave genetic types, so the use of caves is likely to have been relatively recent. Some authors have described morphological differences in cave populations in addition to a lack of pigmentation, but none of these are constant across all cave populations, and most are not constant within populations (O Brien, 1990; S. Eberhard, pers. comm.). All three cave populations in this study exhibit similar genotypes to the closest epigean populations, although the Newdegate Cave and Wolfe Hole populations are the most genetically divergent populations from *Anaspides* elsewhere in Tasmania.

Allanaspides hickmani appears to be actively differentiating. Unfortunately, not enough information on the source population of each animal sampled was available to indicate the reasons for divergence in this species.

5.4.5 *Anaspides* genetics — general features

The genus is characterised by a large proportion of fixed differences, many private alleles and low heterozygosity, reflecting an ancient lineage that has probably been serially affected by bottlenecking and founder effects. This is not surprising considering the range of climatic conditions since the Oligocene, particularly those since the last glaciation that have resulted in increasingly high water temperatures, and the threats posed by invading predators.

Populations with low heterozygosity and large interpopulation distances, such as those from the southern caves, Mount Anne, Mount Wellington and Hydro Creek, appear to have been reduced in numbers quite often. Founder effects and/or historically low numbers are likely to have exaggerated the effects of vicariance, and may well explain the large genetic distances between some populations within the same geographical group, such as mountain populations in the southwest. Conversely, the higher heterozygosity found in the Adamsons Peak and Lake St Clair (previously *Anaspides spinulae*) populations (and *Paranaspides lacustris*) indicate that these populations have been able to maintain high numbers.

5.5 Taxonomic implications

This work clearly contains implications for the taxonomy of the family Anaspididae. It is recommended that:

- the genus *Anaspides* needs to be redefined, based on the type locality (Mt Wellington) population,
- *A. tasmaniae* needs to be redescribed, based on the type locality (Mt Wellington) population,

- southwestern samples should be examined morphologically and redescribed as a new species of *Anaspides,*
- morphological characters which define the southern group of *Anaspides tasmaniae* should be investigated, leading to its description as a new genus and new species,
- morphological examination of the Central Plateau / Derwent catchment group is needed to assess whether it should be retained in *A. tasmaniae* or redescribed as a new species of *Anaspides*,
- *Anaspides spinulae* should be discarded and the Lake St Clair and Clarence Lagoon populations included with the Central Plateau group. Although it would be normal procedure to call this group by the older name (*A. spinulae*), this name refers to the spination, which is variable across the Central Plateau populations. The name of this new species should be decided when it is formally described.

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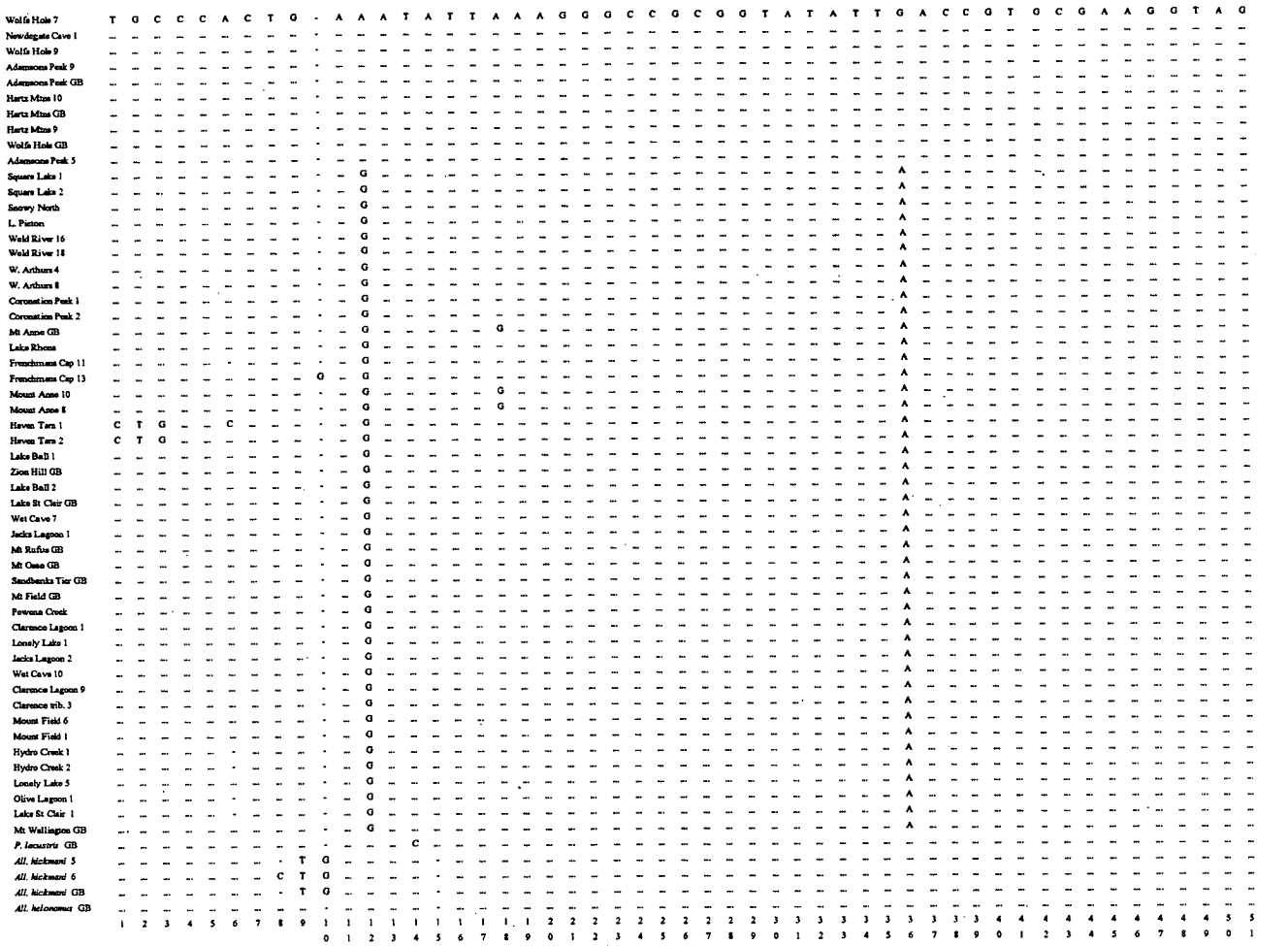
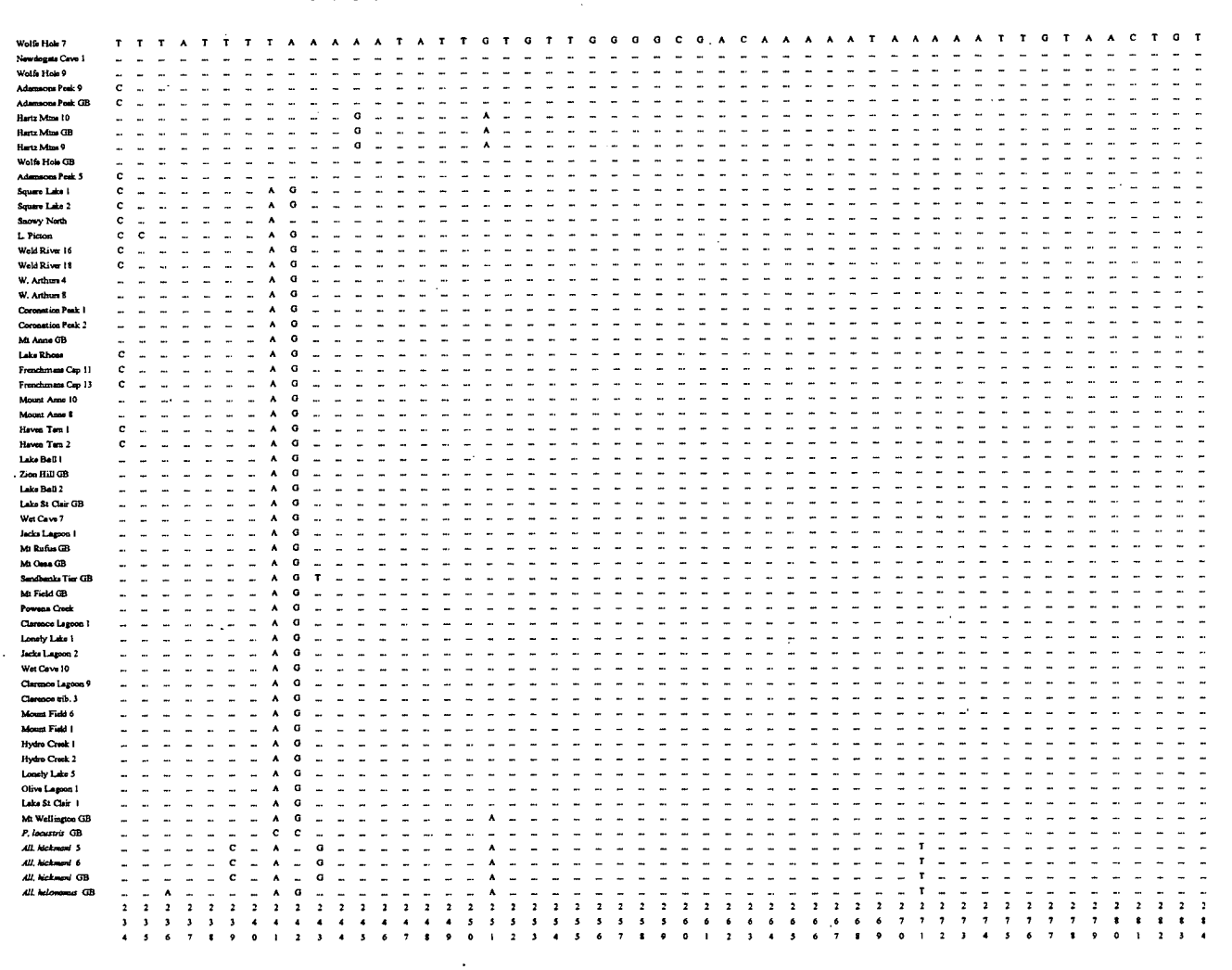
Appendix 1

16S mtDNA sequence data from 58 samples of three genera of the Family Anaspididae


Wolfs Hole 7
Newdegate Cave 1
Wolfs Hole 9
Adamsons Peak 9
Adamsons Peak GB
Hartz Mtns 10
Hartz Mtns GB
Hartz Mtns 9
Wolfs Hole GB
Adamsons Peak 5
Square Lake 1
Square Lake 2
Snowy North
L. Picton
Weld River 16
Weld River 18
W. Arthurs 4
W. Arthurs 8
Coronation Peak 1
Coronation Peak 2
Mt Anne GB
Lake Rhona
Frenchmans Cap 11
Frenchmans Cap 13
Mount Anne 10
Mount Anne 8
Haven Tarn 1
Haven Tarn 2
Lake Ball 1
Zion Hill GB
Lake Ball 2
Lake St Clair GB
Wet Cave 7
Jacks Lagoon 1
Mt Rufus GB
Mt Oasa GB
Sandbanks Tier GB
Mt Field GB
Pawona Creek
Clarence Lagoon 1
Lonely Lake 1
Jacks Lagoon 2
Wet Cave 10
Clarence Lagoon 9
Clarence trib. 3
Mount Field 6
Mount Field 1
Hydro Creek 1
Hydro Creek 2
Lonely Lake 5
Olive Lagoon 1
Lake St Clair 1
Mt Wellington GB
P. lacustris GB
All. hickmani 5
All. hickmani 6
All. hickmani GB
All. helonomus GB


Wolfs Hole 7
Newdegate Cave 1
Wolfs Hole 9
Adamsons Peak 9
Adamsons Peak GB
Hartz Mtns 10
Hartz Mtns GB
Hartz Mtns 9
Wolfs Hole GB
Adamsons Peak 5
Square Lake 1
Square Lake 2
Snowy North
L. Picton
Weld River 16
Weld River 18
W. Arthurs 4
W. Arthurs 8
Coronation Peak 1
Coronation Peak 2
Mt Anne GB
Lake Rhona
Frenchmans Cap 11
Frenchmans Cap 13
Mount Anne 10
Mount Anne 8
Haven Tarn 1
Haven Tarn 2
Lake Ball 1
Zion Hill GB
Lake Ball 2
Lake St Clair GB
Wet Cave 7
Jacks Lagoon 1
Mt Rufus GB
Mt Oasa GB
Sandbanks Tier GB
Mt Field GB
Pawona Creek
Clarence Lagoon 1
Lonely Lake 1
Jacks Lagoon 2
Wet Cave 10
Clarence Lagoon 9
Clarence trib. 3
Mount Field 6
Mount Field 1
Hydro Creek 1
Hydro Creek 2
Lonely Lake 5
Olive Lagoon 1
Lake St Clair 1
Mt Wellington GB
P. lacustris GB
All. hickmani 5
All. hickmani 6
All. hickmani GB
All. helonomus GB

	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
Wolfs Hole 7	C	A	T	A	A	T	C	A	T	T	A	G	T	C	T	T	T	T	A	A	T	T	G	A	A	G	G	C	T	G	G	A	A	T	G	A	A	T	G	G	T	T	G	A	A	C	A	A	A	A	A
Newdegate Cave 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335
Wolfs Hole 7	T	T	A	T	T	T	A	T	T	A	A	A	A	A	C	T	-	A	T	A	A	T	A	A	A	A	G	A	-	-	-	-	A	A	A	A	G	T	G	A	T	C	C	T	T	T	A	G	T	A	A
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Wolfs Hole 7	A	T	A	T	C	C	T	G	T	C	T	C	A	C	T	A	T	T	A	A	A	A	T	T	A	A	A	A	T	T	T	G	A	C	T	T	T	T	A	A	G	T	A	A	A	A	A	G	G	C	T
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Zion Hill GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lake Ball 2	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lake St Clair GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wet Cave 7	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jacks Lagoon 1	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mt Rufus GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mt Ossa GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sandbanks Tier GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mt Field GB	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Powena Creek	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarence Lagoon 1	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lonely Lake 1	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Clarence Lagoon 9	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarence trib. 3	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mount Field 6	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mount Field 1	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydro Creek 1	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydro Creek 2	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lonely Lake 5	-	-	-	G	T	-	-	-	-	-	-	-	G	T	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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P. lacustris GB	-	-	-	G	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
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All. helonomus GB	-	-	-	G	T	-	-	-	-	-	-	-	-	A	-	G	C	-	-	-	-	-	-	-	-	G	-	-	A	-	-	A	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-

	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386
Wolfs Hole 7	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	T	A	C	T	T	T	A	G	G	G	A	T	A	A	C	A	G	C	G	T	A	A	T	C	T	T	T	T	T	T	A	A	G	A
Newdegate Cave 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wolfs Hole 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Adamsons Peak 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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L. Picton	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Weld River 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Weld River 18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Coronation Peak 1	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coronation Peak 2	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Frenchmans Cap 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Frenchmans Cap 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Lake St Clair GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
Wet Cave 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
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Mt Ossa GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
Sandbanks Tier GB	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
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Powena Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
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	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	9	0	0	0	0	0
	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4

Wolfe Hole 7	G	T	T	C	A	T	A	T	C	G	A	C	A	A	A	A	A	G	A	C	T	T	G	C	G	A	C	C	T	C	G	A	T	G	T	T	G	A	A	T	T	A	A	G	A	A	G	T	C	C	T
Newdegate Cave 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wolfe Hole 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hartz Mtns 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Hartz Mtns 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wolfe Hole GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Square Lake 1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Square Lake 2	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Snowy North	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Weld River 16	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Weld River 18	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Coronation Peak 1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Coronation Peak 2	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Frenchmans Cap 11	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Frenchmans Cap 13	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Lake St Clair GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Wet Cave 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Jacks Lagoon 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Mt Ossa GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Sandbanks Tier GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
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Powena Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Clarence Lagoon 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Lonely Lake 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Jacks Lagoon 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Wet Cave 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Clarence Lagoon 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Clarence trib. 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Mount Field 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Mount Field 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Hydro Creek 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Hydro Creek 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Lonely Lake 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Olive Lagoon 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Lake St Clair 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Mt Wellington GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
P. lacustris GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
All. hickmani 5	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All. hickmani 6	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All. hickmani GB	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All. helonomus GB	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	8	8	8	9	9	9	9	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7

Wolfe Hole 7	A	T	A	A	C	T	A	A	T	A	A	G	T	T	T	G	A	A	T	T	T	A	A	A	A	A	T	T	A
Newdegate Cave 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wolfe Hole 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak 9	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak GB	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hartz Mtns 10	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
Hartz Mtns GB	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
Hartz Mtns 9	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
Wolfe Hole GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adamsons Peak 5	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Square Lake 1	-	-	-	-	G	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
Square Lake 2	-	-	-	-	G	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
Snowy North	-	-	-	-	T	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Weld River 16	-	-	-	-	T	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Coronation Peak 2	-	-	-	-	T	-	-	-	-	-	-	.	-	-	G	A	-	-	-	-	-	-	-	-	-	-	-	-	G
Mt Anne GB	-	-	-	-	T	-	-	-	-	-	-	.	-	C	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Frenchmans Cap 11	-	-	-	-	T	-	-	-	-	-	-	.	C	-	A	A	-	-	-	-	A	-	-	-	-	-	-	-	G
Frenchmans Cap 13	-	-	-	-	T	-	-	-	-	-	-	.	C	-	A	A	-	-	-	-	A	-	-	-	-	-	-	-	G
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Mt Rufus GB	-	-	-	-	-	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Sandbanks Tier GB	-	-	-	-	T	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Hydro Creek 1	-	-	-	-	T	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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Lonely Lake 5	-	-	-	-	T	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
Olive Lagoon 1	-	-	-	-	-	-	-	-	-	-	-	.	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	G
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All. helonomus GB	-	-	-	-	A	-	-	-	-	-	-	.	.	.	.	.	G	-	A	-	-	-	-	-	-	-	-	-	-
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	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3

Wolfe Hole 7	T	T	T	G	G	T	G	T	A	G	C	A	G	T	T	A	A	A	A	A	G	G	A	A	G	G	T	C	T	G	T	T	C	G	A	C	C	T	T	T	A	A
Newdegate Cave 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wolfe Hole 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Adamsons Peak 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Square Lake 2	-	-	-	-	-	A	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Snowy North	-	-	-	-	A	A	-	C	-	-	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
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Frenchmans Cap 13	-	-	-	-	-	-	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Haven Tarn 2	-	-	-	-	-	A	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Wet Cave 10	-	-	-	-	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarence Lagoon 9	-	-	-	-	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarence trib. 3	-	-	-	-	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mount Field 6	-	-	-	-	-	-	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Hydro Creek 1	-	-	-	-	-	-	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydro Creek 2	-	-	-	-	-	-	-	C	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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P. lacustris GB	-	-	-	A	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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All. hickmani 6	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All. hickmani GB	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
All. helonomus GB	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7
	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9

Appendix 2

HKY85 distance matrix from 16S mtDNA sequence data

Appendix 2
HKY85 distance matrix from 16S mtDNA sequence data

HKY85 distance	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
1 Wolfe Hole 7	-																																																										
2 Newdegate Cave 1	0.002	-																																																									
3 Wolfe Hole 9	0.000	0.002	-																																																								
4 Adamsons Peak 9	0.011	0.013	0.011	-																																																						
5 Adamsons Peak GB	0.011	0.013	0.011	0.000	-																																																				
6 Hartz Mountains b	0.015	0.017	0.015	0.013	0.013	-																																																			
7 Hartz Mountains GB	0.015	0.017	0.015	0.013	0.013	0.000	-																																																	
8 Hartz Mountains a	0.015	0.017	0.015	0.013	0.013	0.000	0.000	-																																														
9 Wolfe Hole GB	0.002	0.004	0.002	0.013	0.013	0.017	0.017	0.017	-																																													
10 Adamsons Peak 5	0.011	0.013	0.011	0.000	0.000	0.013	0.013	0.013	0.013	-																																											
11 Square Lake a	0.063	0.063	0.063	0.056	0.056	0.068	0.068	0.068	0.068	0.056	-																																										
12 Square Lake b	0.063	0.063	0.063	0.056	0.056	0.068	0.068	0.068	0.068	0.056	0.000	-																																									
13 Snowy North GB	0.065	0.065	0.065	0.056	0.056	0.068	0.068	0.068	0.068	0.056	0.011	0.011	-																																									
14 Lake Picton	0.066	0.066	0.066	0.061	0.061	0.073	0.073	0.073	0.068	0.061	0.013	0.013	0.020	-																																								
15 Weld River 16	0.064	0.064	0.064	0.054	0.054	0.066	0.066	0.066	0.066	0.054	0.011	0.011	0.017	0.011	-																																								
16 Weld River 18	0.064	0.064	0.064	0.054	0.054	0.066	0.066	0.066	0.066	0.054	0.011	0.011	0.017	0.011	0.000	-																																								
17 Western Arthurs 4	0.066	0.066	0.066	0.061	0.061	0.068	0.068	0.068	0.068	0.061	0.017	0.017	0.024	0.026	0.020	0.020	-																																							
18 Western Arthurs 6	0.066	0.066	0.066	0.061	0.061	0.068	0.068	0.068	0.068	0.061	0.017	0.017	0.024	0.026	0.020	0.020	0.000	-																																							
19 Coronation Peak a	0.065	0.065	0.065	0.061	0.061	0.068	0.068	0.068	0.068	0.061	0.019	0.019	0.026	0.028	0.022	0.022	0.022	0.022	-																																							
20 Coronation Peak b	0.065	0.065	0.065	0.061	0.061	0.068	0.068	0.068	0.068	0.061	0.019	0.019	0.026	0.028	0.022	0.022	0.022	0.022	0.000	-																																							
21 Mt Anne GB	0.063	0.063	0.063	0.058	0.058	0.066	0.066	0.066	0.066	0.058	0.011	0.011	0.017	0.020	0.013	0.013	0.015	0.015	0.017	0.017	-																																							
22 Lake Rhona	0.066	0.066	0.066	0.056	0.056	0.068	0.068	0.068	0.068	0.056	0.022	0.022	0.028	0.026	0.020	0.020	0.026	0.026	0.028	0.028	0.024	-																																						
23 Frenchmans Cap 11	0.066	0.066	0.066	0.056	0.056	0.068	0.068	0.068	0.068	0.056	0.017	0.017	0.024	0.022	0.015	0.015	0.022	0.022	0.024	0.024	0.020	0.004	-																																					
24 Frenchmans Cap 13	0.066	0.066	0.066	0.056	0.056	0.068	0.068	0.068	0.068	0.056	0.017	0.017	0.024	0.022	0.015	0.015	0.022	0.022	0.024	0.024	0.019	0.004	0.000	-																																					
25 Mt Anne 10	0.063	0.063	0.063	0.058	0.058	0.066	0.066	0.066	0.066	0.058	0.011	0.011	0.017	0.020	0.013	0.013	0.015	0.015	0.017	0.017	0.000	0.024	0.020	0.019	-																																					
26 Mt Anne 8	0.063	0.063	0.063	0.058	0.058	0.066	0.066	0.066	0.066	0.058	0.011	0.011	0.017	0.020	0.013	0.013	0.015	0.015	0.017	0.017	0.000	0.024	0.020	0.019	0.000	-																																					
27 Haven Tarn a	0.070	0.070	0.070	0.068	0.068	0.080	0.080	0.080	0.073	0.068	0.013	0.013	0.022	0.019	0.022	0.022	0.028	0.028	0.030	0.030	0.022	0.033	0.026	0.028	0.022	0.022	-																																					
28 Haven Tarn b	0.068	0.068	0.068	0.065	0.065	0.078	0.078	0.078	0.070	0.065	0.011	0.011	0.019	0.017	0.019	0.019	0.026	0.026	0.028	0.028	0.019	0.031	0.026	0.026	0.019	0.019	0.002	-																																					
29 Lake Ball a	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	-																																					
30 Zion Hill GB	0.054	0.054	0.054	0.056	0.056	0.063	0.063	0.063	0.056	0.056	0.028	0.028	0.035	0.033	0.028	0.028	0.035	0.035	0.035	0.035	0.024	0.035	0.035	0.035	0.024	0.024	0.037	0.035	0.002	-																																					
31 Lake Ball b	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	-																																				
32 Lake St Clair GB	0.049	0.051	0.049	0.051	0.051	0.059	0.059	0.059	0.051	0.051	0.028	0.028	0.035	0.033	0.028	0.028	0.035	0.035	0.035	0.035	0.028	0.035	0.035	0.035	0.028	0.028	0.037	0.035	0.002	0.004	0.002	-																																				
33 Wet Cave 7	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	-																																			
34 Jacks Lagoon b	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	-																																			
35 Mt Rufus GB	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	-																																		
36 Mt Ossa GB	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	-																																	
37 Sandbanks Tier GB	0.061	0.061	0.061	0.058	0.058	0.063	0.063	0.063	0.063	0.058	0.033	0.033	0.037	0.035	0.031	0.031	0.037	0.037	0.037	0.037	0.030	0.037	0.037	0.037	0.030	0.030	0.042	0.039	0.009	0.011	0.009	0.011	0.009	0.009	0.009	0.009	-																																	
38 Mt Field GB	0.058	0.058	0.058	0.061	0.061	0.065	0.065	0.065	0.061	0.061	0.024	0.024	0.033	0.028	0.024	0.024	0.030	0.030	0.030	0.030	0.024	0.030	0.031	0.030	0.024	0.024	0.033	0.030	0.006	0.009	0.006	0.009	0.006	0.006	0.006	0.006	0.013	-																																	
39 Powena Ck	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.006	-																																	
40 Clarence Lagoon a	0.051	0.051	0.051	0.054	0.054	0.058	0.058	0.058	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.006	0.004	0.000	-																																	
41 Lonely Lake 1	0.054	0.054	0.054	0.051	0.051	0.058	0.058	0.058	0.056	0.051	0.026	0.026	0.030	0.028	0.024	0.024	0.030	0.030	0.030	0.030	0.024	0.030	0.031	0.030	0.024	0.024	0.035	0.033	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.006	0.009	0.002	0.002	-																																	
42 Jacks Lagoon a	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.006	0.000	0.000	0.002	-																																	
43 Wet Cave 10	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.006	0.000	0.000	0.002	0.000	-																																	
44 Clarence Lagoon 9	0.051	0.051	0.051	0.054	0.054	0.058	0.058	0.058	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.035	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.008	0.004	0.000	0.000	0.002	0.000	0.000	-																																	
45 Clarence tributary	0.049	0.051	0.049	0.051	0.051	0.059	0.059	0.059	0.051	0.051	0.028	0.028	0.035	0.033	0.028	0.028	0.035	0.035	0.035	0.035	0.028	0.035	0.035	0.035	0.028	0.028	0.037	0.035	0.002	0.004	0.002	0.000	0.002	0.002	0.002	0.002	0.011	0.009	0.002	0.002	0.004	0.002	0.002	0.002	-																																	
46 Mt Field 6	0.058	0.058	0.058	0.061	0.061	0.065	0.065	0.065	0.061	0.061	0.024	0.024	0.033	0.028	0.024	0.024	0.030	0.030	0.030	0.030	0.024	0.030	0.031	0.030	0.024	0.024	0.033	0.030	0.006	0.009	0.006	0.009	0.006	0.006	0.006	0.006	0.013	0.009	0.006	0.006	0.009	0.006	0.006	0.004	0.009	-																																	
47 Mt Field b	0.058	0.058	0.058	0.061	0.061	0.065	0.065	0.065	0.061	0.061	0.024	0.024	0.033	0.028	0.024	0.024	0.030	0.030	0.030	0.030	0.024	0.030	0.031	0.030	0.024	0.024	0.033	0.030	0.006	0.009	0.006	0.009	0.006	0.006	0.006	0.006	0.013	0.009	0.006	0.006	0.009	0.006	0.006	0.004	0.009	0.000	-																																	
48 Hydro Ck a	0.063	0.063	0.063	0.061	0.061	0.068	0.068	0.068	0.066	0.061	0.031	0.031	0.037	0.033	0.028	0.028	0.035	0.035	0.035	0.035	0.028	0.035	0.035	0.035	0.028	0.028	0.037	0.037	0.011	0.013	0.011	0.013	0.011	0.011	0.011	0.011	0.015	0.013	0.011	0.011	0.013	0.011	0.011	0.011	0.013	0.013	0.013	-																																	
49 Hydro Ck b	0.063	0.063	0.063	0.061	0.061	0.068	0.068	0.068	0.066	0.061	0.031	0.031	0.037	0.033	0.028	0.028	0.035	0.035	0.035	0.035	0.028	0.035	0.035	0.035	0.028	0.028	0.037	0.037	0.011	0.013	0.011	0.013	0.011	0.011	0.011	0.011	0.015	0.013	0.011	0.011	0.009	0.011	0.011	0.011	0.013	0.013	0.013	0.000	-																																	
50 Lonely Lake 5	0.054	0.054	0.054	0.051	0.051	0.058	0.058	0.058	0.056	0.051	0.026	0.026	0.030	0.028	0.024	0.024	0.030	0.030	0.030	0.030	0.024	0.030	0.031	0.030	0.024	0.024	0.035	0.033	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.006	0.009	0.002	0.002	0.000	0.002	0.002	0.002	0.004	0.009	0.009	0.009	0.009	-																																	
51 Olive Lagoon	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.033	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.006	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.006	0.006	0.011	0.011	0.002	-																																	
52 Lake St Clair 1	0.051	0.051	0.051	0.054	0.054	0.061	0.061	0.061	0.054	0.054	0.026	0.026	0.033	0.031	0.026	0.026	0.033	0.033	0.033	0.033	0.026	0.033	0.033	0.033	0.026	0.026	0.033	0.033	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.006	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.006	0.006	0.011	0.011	0.002	0.000	-																																	
53 Mt Wellington GB	0.054	0.054	0.054	0.049	0.049	0.051	0.051	0.051	0.056	0.049	0.026	0.026	0.033	0.031	0.024	0.024	0.031	0.031	0.028	0.028	0.022	0.031	0.031	0.031	0.022	0.022	0.037	0.035	0.019	0.019	0.019	0.022	0.019	0.019	0.019	0.019	0.024	0.022	0.019	0.019	0.017	0.019	0.019	0.019	0.022	0.022	0.022	0.022	0.022	0.017	0.019	0.019	-																																	
54 *Paranaspides lacustri*	0.058	0.061	0.058	0.056	0.056	0.058	0.058	0.058	0.061	0.056	0.070	0.070	0.072	0.070	0.063	0.063	0.067	0.067	0.070	0.070	0.068	0.070	0.070	0.070	0.068	0.068	0.082	0.079	0.065	0.067	0.065	0.063	0.065	0.065	0.065	0.065	0.067	0.069	0.065	0.063	0.062	0.065	0.065	0.063	0.063	0.069	0.069	0.072	0.072	0.062	0.065	0.065	0.063	-																																	
55 *Ali. hickmani 5*	0.115	0.118	0.115	0.115	0.115	0.115	0.115	0.115	0.118	0.115	0.134	0.134	0.139	0.139	0.134	0.134	0.128	0.128	0.136	0.136	0.133	0.133	0.131	0.130	0.133	0.133	0.150	0.147	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.131	0.128	0.128	0.126	0.128	0.128	0.128	0.126	0.126	0.128	0.128	0.134	0.134	0.128	0.128	0.128	0.123	0.131	-																																
56 *Ali. hickmani 6*	0.115	0.117	0.115	0.115	0.115	0.115	0.115	0.115	0.117	0.115	0.139	0.139	0.144	0.144	0.139	0.139	0.133	0.133	0.141	0.141	0.136	0.138	0.136	0.135	0.138	0.138	0.155	0.152	0.133	0.133	0.133	0.131	0.133	0.133	0.133	0.133	0.136	0.133	0.133	0.131	0.133	0.133	0.133	0.131	0.131	0.133	0.133	0.139	0.139	0.133	0.133	0.133	0.128	0.130	0.002	-																																
57 *Ali. hickmani* GB	0.113	0.115	0.113	0.113	0.113	0.113	0.113	0.113	0.115	0.113	0.136	0.136	0.141	0.142	0.137	0.137	0.131	0.131	0.139	0.139	0.136	0.136	0.134	0.133	0.136	0.136	0.162	0.150	0.131	0.131	0.131	0.128	0.131	0.131	0.131	0.131	0.134	0.131	0.131	0.129	0.131	0.131	0.131	0.129	0.128	0.131	0.131	0.137	0.137	0.131	0.131	0.131	0.126	0.128	0.002	0.000	-																																
58 *Ali. helonomus* GB	0.100	0.102	0.100	0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.123	0.123	0.130	0.117	0.118	0.118	0.120	0.120	0.120	0.120	0.120	0.125	0.123	0.122	0.120	0.120	0.130	0.128	0.102	0.102	0.102	0.100	0.102	0.102	0.102	0.102	0.107	0.105	0.102	0.100	0.102	0.102	0.102	0.100	0.100	0.105	0.105	0.113	0.113	0.102	0.102	0.102	0.107	0.112	0.073	0.073	0.070	-																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57

Appendix 3

People who collected samples of anaspids used in this study

Location	Collectors	Organisation
Central Plateau - near Lake Ball	author	University of Tasmania (UT)
Central Plateau - near Powena Creek	"	"
Central Plateau - near Olive Lagoon	"	"
Central Plateau - Hydro Creek	"	"
Central Plateau - Lonely Lake	"	"
Central Plateau - Jacks Lagoon outflow	"	"
Central Plateau - Clarence Lagoon trib.	"	"
Mount Wellington	"	"
Hartz Mountains - Ladies Tarn	"	"
Adamsons Peak	"	"
Weld River	"	"
Mount Field	"	"
Mount Anne	Simon Jarman	UT (now Aust. Antarctic Division)
Frenchmans Cap	Laurie Cook	Freshwater Systems
Western Arthur Range - Lake Oberon	Jean Jackson Brett Mawbey	Inland Fisheries Service (IFS) "
Western Arthur Range - Square Lake	Simon Jarman	UT (now Aust. Antarctic Division)
Western Arthur Range - Haven Lake	"	"
Coronation Peak	"	"
Lake Picton	"	"
Lake Rhona	"	"
Snowy North	"	"
Sandbanks Tier	"	"
Zion Hill	"	"
Mt Rufus	"	"
Mt Ossa	"	"
Wolfe Hole	author	UT
Newdegate Cave	"	"
Wet Cave	Stefan Eberhard	Tas. Parks and Wildlife Service (now unknown)
Lake St Clair	Mike Driessen	Nature Conservation Branch, DPIWE
Clarence Lagoon	author Adam Stevens	UT "
Woods Lake	David Bluhdorn	IFS (now Hydro Tasmania)
McPartlans Pass	author Pierre Horwitz Mike Driessen	UT Edith Cowan University, WA Nature Conservation Branch, DPIWE
Harlequin Hill area	author Pierre Horwitz	UT Edith Cowan University, WA