A COMPARATIVE PHENETIC AND CLADISTIC ANALYSIS OF THE GENUS *HOLCASPIS* CHAUDOIR (COLEOPTERA: CARABIDAE)

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A comparative phenetic and cladistic analysis of the genus *Holcaspis* Chaudoir (Coleoptera: Carabidae)

by Yupa Hanboonsong

The systematics of the endemic New Zealand carabid genus *Holcaspis* are investigated, using phenetic and cladistic methods, to construct phenetic and phylogenetic relationships. Three different character data sets: morphological, allozyme and random amplified polymorphic DNA (RAPD) based on the polymerase chain reaction (PCR), are used to estimate the relationships.

Cladistic and morphometric analyses are undertaken on adult morphological characters. Twenty six external morphological characters, including male and female genitalia, are used for cladistic analysis. The results from the cladistic analysis are strongly congruent with previous publications.

The morphometric analysis uses multivariate discriminant functions, with 18 morphometric variables, to derive a phenogram by clustering from Mahalanobis distances (D^2) of the discrimination analysis using the unweighted pair-group method with arithmetical averages (UPGMA). In contrast to the cladistic analysis, the phenetic clustering results in a less useful estimation of affinities of the genus. However, this analysis reveals a method with a relatively high probability of assigning an individual to the correct species (70%-100%). Therefore morphometric analysis is shown to be useful for species identification.

Allozyme data are derived by electrophoresis using a cellulose acetate medium. A total of 42 alleles of 13 presumptive loci from 10 enzyme systems are used for cladistic and phenetic analysis of 13 *Holcaspis* species. A phenogram is generated by UPGMA clustering using a genetic distance matrix. Cladograms are constructed using both independent alleles and loci as characters. The cladograms from both allele and locus data are highly congruent with the phenogram derived from the genetic similarity matrix data.

Intraspecific allozyme variation is also investigated with a limited number of populations and a relatively confined range of sample sites. A high degree of heterozygosity is revealed in *H. oedicnema*. The mean genetic similarity among the *Holcaspis* species is I= 0.382±0.142 and the mean genetic distance is D= 1.055±0.143.

Molecular data are used in the intraspecific variation study and to estmate species relationships of *Holcaspis*. Optimal RAPD-PCR conditions such as primer concentration, magnesium chloride concentration and RAPD-PCR programme, are established for reproducible and informative amplifying of banding patterns of *Holcaspis* species. A total of 271 band positions are scored for all individuals studied and are subjected to both cladistic and phenetic analysis to estimate the species relationships. Phenograms using UPGMA are generated from both simple matching similarity coefficients and Jaccard's similarity coefficients. The resulting two phenograms are identical. Principal coordinate analysis is also used to demonstrate the relationships among species. The results are congruent with the phenograms. However, the pattern of species relationships is indistinct. The cladogram generated from cladistic analysis shows relatively high congruence with the phenogram. In addition, the results from RAPD-PCR are much more congruent with the results from allozyme data than with the morphological data. The RAPD-PCR technique is, therefore, promising as a new tool for estimating phylogenetic relationships. In addition, the results show that the RAPD-PCR technique is a constructive, quick method for species grouping. From both RAPD-PCR and allozyme data, *H. oedicnema* shows extreme intraspecific variation that suggests that *H. oedicnema* is a species complex.

To assess the best fit of phylogenetic relationships of the *Holcaspis* species, three character data sets: morphological, allozyme, and RAPD-PCR, are tested for congruence using both character congruence and taxonomic congruence method. The result indicates that the character congruence method of all character data sets combined produced a more informative result than the taxonomic congruence method. This study confirms the previous indication that *H. punctigera* and *H. mordax* are closely related both morphologically and genetically. The study suggests that *H. ovatella* is most genetically distinct from the rest of the *Holcaspis* species and that *H. oedicnema* is a genetically diverse species.

Keywords: *Holcaspis*; Carabidae; morphometrics; morphological; allozymes; random amplified polymorphic DNA; cladistic; cladogram; phenetic; phenogram; phylogenetic.

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CHAPTER 1

Introduction

1.1 Background

Systematics is the science of the relationships of living things. It is absolutely fundamental to all biology and seeks to provide a framework within which to order biological knowledge. Usually it is combined with evolutionary theory in order to help provide this framework and can be used to develop a series of testable hypotheses about the relationships of living things.

Over the last 25-30 years, there has been a revolution in the way that biologists study systematics. The single most significant idea or group of ideas driving the revolution has been the work of the German entomologist Willi Hennig that has come to be known as '*Phylogenetic Systematics*' (Hennig 1966). His ideas led directly to the development of the method of systematics known as '*cladistics*'.

The same period saw the main development of a different, less evolutionary based style of systematics known as '*phenetics*' , which relied heavily on the newly available power of computers to investigate the relationships of organisms based on hundreds of characters in ways that had not been possible previously. The traditional evolutionary taxonomists have not been left out of this revolution but, rather, have been forced to examine their methodologies more critically in the light of other developments.

While the revolution in systematics over the last 25-30 years has been driven by exciting new ideas, it has also been aided by the rapid development of several powerful pieces of technology. It is probably no coincidence that this revolution of ideas and their ready acceptance, has developed as electronic computers first became readily available. Most of the techniques for cladistics and phenetics would not be possible, for realistic data sets, without recourse to substantial computing power.

Another technology that has had a profound effect on the recent development of biological systematics is the development of ready means of analysis of biological compounds. This has been an ongoing process, but can be thought of as the successive

development of techniques for handling the analysis of ever more complex biological compounds. It started with plant and animal secretions and secondary metabolites, moved into proteins, particularly enzymes and, most recently, the development of techniques for routine analysis and handling of nucleic acids. It has become known as molecular biology.

Each of the molecular biological techniques can provide substantial amounts of biological information about organisms, broadly distinct from the traditional morphological data available to traditional systematics. Another feature of these data that is appealing to many people is that they seem to get closer and closer to the underlying genetic makeup of organisms, which presumably lies at the heart of the evolutionary process.

1.2 The propositions

While each new idea and technology has been avidly seized by practitioners of systematics and analyzed, debated, modified, developed, and used, there have been relatively few comparisons of techniques across a wide range of what is available. The basic idea of this thesis was therefore to take a group of organisms and compare a range of different available methodologies and technologies to investigate their different strengths and weaknesses for systematics.

A secondary aim of this work was to investigate the usefulness of a relatively novel molecular technique, random amplified polymorphic DNA based on ploymerase chain reaction (RAPD-PCR), as a possibly more accessible alternative to DNA sequencing for routine systematics. The whole field of the application of molecular techniques to systematics is changing so rapidly that consensus on the most useful techniques for routine systematics has not yet emerged.

1.3 Why the genus *Holcaspis* ?

This study, or one like it, could in theory be carried out on any group of organisms, but *Holcaspis*, a genus of ground beetles, seems to be a useful model group. There are several reasons for this claim.

First, there is a great deal of work going on at both ends of the spectrum of biological diversity, from studies investigating the higher classification of organisms (Michener and Sakagami 1990; Urbani *et al.* 1992; Rodrigo *et al.* 1993) down to populational differences (Cann *et al.* 1987; Kambhampati *et al.* 1992) and studies on closely related sibling and cryptic species (MacArthur 1986; Daugherty *et al.* 1990; Pape 1992). However, in general, everyday systematics between the species and genus level seems to be getting less attention.

Also, the *Holcaspis* species are relatively well known. There is a recent taxonomic treatment of the group (Butcher 1984) and there was some expertise in their taxonomy within the Department of Entomology and Animal Ecology at Lincoln University. A purely practical consideration, but it was felt an important one. At the same time, while the species, as entities, in the genus *Holcaspis* were relatively well known, their relationships, one to another and, particularly, the relationships of groups of species within the genus, were entirely unknown.

Thirdly, *Holcaspis* is a manageable sized group of species, with some 30 distinct species. They are relatively large (10-26 mm long) and robust, making problems of handling easier and the extraction of enzymes and DNA from individuals practical.

Finally, almost all the *Holcaspis* species tend to be restricted to relatively narrow habitats, and some species, particularly *H. stewartensis*, are located in restricted geographical areas. According to The International Union for the Conservation of Nature and Natural Resources (IUCN) (Groombridge 1992), rare species with small populations located within restricted areas may be categorised as threatened and may face a significant risk of extinction. It is believed that some New Zealand *Holcaspis* species are in danger of becoming threatened and probably have the potential to become extinct. In these circumstances, a good understanding of their systematics can be most helpful in devising a conservation strategy. While that is not the main aim of this study, it is potentially, a very useful practical application.

1.4 Objectives

The objectives of this investigation were:

1. To investigate the systematic relationships of the genus *Holcaspis*, using a variety of methodologies and techniques, including:
   (a) their cladistic relationships based on morphological data;
   (b) the use of quantitative data, including a morphometric analysis, to provide a comparison of phenetic and cladistics techniques;
   (c) their cladistic relationships based on allozyme analysis of a subset of species; and
   (d) their phenetic relationships based on the genetic distance of allozyme data of a subset of species.
2. To evaluate the innovative molecular technique of random amplified polymorphic DNA based on polymerase chain reaction as a means of investigating systematics using *Holcaspis* species as the test group. (The data from the random amplified polymorphic DNA analysis will be used to further investigate the cladistic and phenetic relationships of the *Holcaspis* species).
3. To investigate congruency based on cladistic analysis among the three independent data sets: morphological, allozyme and random amplified polymorphic DNA based on polymerase chain reaction
4. To provide a better understanding of the relationships of the species of *Holcaspis* for conservation and other purposes.

1.5 Format of the thesis

The thesis has been written as independent chapters. It begins with a general introduction to the concepts and framework of systematics complemented with the history of the genus *Holcaspis*. The following three chapters report three independent approaches to the investigation of relationships in the genus *Holcaspis*. Each has been formatted as a scientific paper with introduction, materials and methods, results and discussion. The first

approach is based on morphological data. It is presented in Chapter 3. In that chapter, external morphological characters were used for a cladistic study as well as a morphometric analysis that involved multivariate analysis. This is employed to provide a comparison with the cladistic analysis. Chapter 4 describes the results of an investigation using allozyme electrophoresis. Both allele and locus data are analyzed to investigate phylogenetic relationships. Results using cladistic analysis and genetic distance data are then compared. Chapter 5 is an evaluation of the application of the novel RAPD technique to the systematics of the genus *Holcaspis*. Two analyses of the cladistics as well as cluster analysis are explored to determine the species relationships. Chapter 6 involves the standardization and testing for congruence of the phylogenetic trees from the three different character data sets (Chapters 3-5); comparisons of both the taxonomic congruence and character congruence approaches are made. The final chapter is a general discussion of the two different methods, cladistics and phenetics, for constructing phylogenetic and phenetic relationships and the three independent approaches to the investigation of the relationships of *Holcaspis*. Also in this final chapter, the need for further studies is discussed and the overall conclusions of the study are presented.

CHAPTER 2

Literature review

2.1 Introduction

A general literature review is provided for two areas of study: (a) the principles of systematics and (b) the history of the genus *Holcaspis* (Carabidae: Pterostichini).

2.2 Basic systematics concepts

Mayr (1969) stated that the field of systematics can be thought of as a continuum of study. He designated three different levels of study within the continuum as alpha, beta and gamma systematics. Alpha, or the analytical stage, is the beginning process of analysis and description of unknown species. The outcome from this process is the construction of a specific description to enable the recognition of each species. The synthetic stage, classification or beta systematics, is the next stage in the process. The species should be characterized and arranged in orderly categories. Classification, in the definition of Mayr, means a list of taxa names that are allocated to categories. The question which follows after grouping the taxa in an order is how to determine whether similarities of two or more species are due to convergence responses to environment or are due to close ancestor-descendant relationships. This is the third level of study, which Mayr called gamma systematics. The ultimate aim of gamma systematics is to determine the pattern of evolutionary history of the taxa and how they are related to each other.

Scott-Ram (1990) stated that activities in the structure of systematics can be separated into two aspects: microtaxonomy and macrotaxonomy. Microtaxonomy is concerned with delimitation of species, with analysis of the genetical and ecological barriers between populations and with the investigation of the ranges of morphological and genetic variability of populations in relation to their environments. In contrast to microtaxonomy, macrotaxonomy deals specifically with the arranging and grouping of species into a classification system.

2.2.1 Systematics

Over recent years, three contemporary schools of thought in systematics have generally been recognised. They are described as follows:

2.2.1.1 Evolutionary systematics

Evolutionary systematics, or orthodox taxonomy, is generally regarded as '*traditional taxonomy*'. The classical works on evolutionary systematics are Simpson's (1961) *Principles of Animal Taxonomy*, Mayr's (1969) *Principles of Systematic Zoology* and Ross's (1974) *Biological Systematics*. The basic hypothesis of orthodox systematists is based on the general evolutionary theory by natural selection of Darwin and Wallace (Hawksworth and Bisby 1988; Mayr 1988; Brusca 1990).

The main idea of the theory is that the cause and effect relationship between organisms and environment will result in changes in lineages with time. Therefore, the aim of evolutionary systematics is to discover groups of organisms in nature that are the product of evolution. The elucidation of relationships among organisms should then be based on genealogy and overall similarity. Evolutionary systematists give significance to the rate of evolution. The different appearance of the same character in various taxa is the result of different rates of evolution in different lineages; for example, the mouthparts of Hemiptera are of a remarkably specialized piercing sucking type whereas the mouthparts of Psocoptera are of a primitive piercing sucking type. Both Hemiptera and Psocoptera evolved from a common ancestor. After the divergence of these two groups from their common ancestor, the Psocoptera mouthparts changed relatively slowly but the Hemiptera mouthparts changed at a significantly faster rate. Weighting of characters is also one of the assumptions that is accepted by evolutionary systematists. Cain and Harrison (1960) stated that rare characters should receive more weight because they are better indicators of evolutionary relationships between taxa, or groups of organisms. Mayr also believed that different characters contain very different amounts of information concerning their ancestors and evolutionary changes along a lineage.

Simpson (1961), on the other hand, did not emphasise character weighting according

to supposed ancestral character distributions as information for phylogeny. He believed that fossils should play a significant role in determining evolutionary relationships among groups of organisms. This is fine in principle but, in practice, it is quite difficult to apply to the reconstruction of phylogenetic relationships. Simpson was a mammalian and penguin systematist, and therefore worked with groups that often had relatively good fossil records. Fossils are less useful in insect systematics.

Evolutionary systematics has been criticized for relying too much on subjective judgement and intuitive methodology to produce classifications, and also on ambiguously expressed hypotheses of evolutionary process. For example, the determination of how characters change in evolution, which characters are more conservative than the others and which characters should be used and which should not, relies on value judgments by the individual specialist. Simpson regarded the methodology of evolutionary systematics as an art being based on '*human contrivance and ingenuity*' (Simpson 1961). As a result of this highly subjective process, it can be said that evolutionary systematics is not really a scientific method since it is unlikely to produce repeatable results and strictly testable hypotheses. For these reasons, the school of evolutionary systematics in classification received strong criticism from other systematists and its shortcomings led later to the development of the other schools of thought like cladistics and phenetics.

2.2.1.2 Phenetics

Phenetic classification was first suggested by the French botanist Michel Adanson in 1757 (Scott-Ram 1990). Much later, in 1937, J.S.L. Gilmour developed the ideas of Adanson and proposed the idea of using naturalness as a means for evaluating classifications (Scott-Ram 1990). The ideas of these two workers were later followed by the pheneticists. The classical work on phenetics can be found in Sokal and Sneath's (1963) *Principles of Numerical Taxonomy*, Jardine and Sibson's (1971) *Mathematical Taxonomy* and Sneath and Sokal's (1973) *Numerical Taxonomy*. The term phenetics is now synonymous with numerical phenetics. The term numerical taxonomy does not only mean phenetics any more but has combined two areas of methodology: numerical

phenetics and numerical cladistics (Scott-Ram 1990).

The basic idea of phenetic classification is to group or arrange the organisms based on overall similarity and using all available characters, without any weighting (Sokal and Camin 1965). Unlike the evolutionary systematists, the pheneticists reject grouping taxa on the basis of *a priori* weighting. They believe there is no character or any sort of feature that is inherently more significant than the rest of the available characters. Such pheneticists agree that every feature or character is of equal weight in creating natural taxa (Sneath 1961). Pheneticists also claim that the character weighting of evolutionary taxonomy is a subjective method that cannot be the basis of a precise science. The pheneticists claim that phenetics is a truly scientific method because it is a repeatable and objective technique. The whole procedure of phenetics is exactly specified. It can be repeated by any taxonomist who, starting from the same set of characters, would come up with the same classification. Moreover, it is an objective method because it is empirically based on many observations of characters and experimental studies. Pheneticists argue that, in practice, the search for the true or most perfect phylogenetic relationship for a group of organisms is an impossible dream. Therefore, the most useful means of classification should be based on convenience, simplicity, and use the fewest assumptions.

The most remarkable assumption in phenetic methodology is the complete rejection of phylogenetic assumptions, particularly information about evolutionary events, such as the evolutionary rate, or the origins of resemblance of characters in the taxa. In other words, the pheneticists emphasise the distinction between the process of evolution and the process of classification. However, the basic assumption of phenetics is that organisms that are very similar are likely to be closely related. In contrast, organisms that are dissimilar tend to have remote ancestors. Moreover, it is sometimes stated that phenetic similarity, which is based on overall similarity of taxonomic characters, can be assumed to reflect genetic similarity. This statement of phenetics looks contradictory and has been criticised on the basis that the overall phenotypic likeness of organisms is unable to characterize the genetic paradigm of such organisms (Sokal and Sneath 1963; Jardine and Sibson 1971; Sneath and Sokal 1973; Farris 1977).

2.2.1.3 Cladistic systematics (Phylogenetic systematics)

Since the publication of Willi Hennig's *Phylogenetic Systematics* in 1966, the methodology for reconstruction of phylogenetic relationships has undergone many changes and has developed more diversity. For example, the statistical method of maximum likelihood, which has been applied to gene frequencies and nucleic acid sequence data, the compatibility and distance matrices, and the most popular parsimony methods have all been introduced since that time (Felsenstein 1983). However, it still has the same basic assumptions. This review focuses only on the cladistics method, which is based on the parsimony principle of phylogenetic reconstruction.

Cladistics developed from Hennig's phylogenetic systematics and has become a more and should more popular approach for understanding the relationships of various organisms. The cladistics method contrasts with phenetics, which is based on overall similarity. Like evolutionary systematics, proponents of cladistics believe that evolutionary theory should have an essential role in taxonomy and that biological classification should have a systematic relationship to phylogeny (Scott-Ram 1990). Therefore, the goal of cladistic systematics is to produce testable hypotheses of genealogical relationships among organisms, based on the possession of shared derived characters, which pass from recent common ancestors, through genealogical descent.

Cladists believe that inferences of evolutionary history should be based on studies of recent taxa, because evolution is an ongoing process of development, and should also involve the study of successive changes in characters. Characters that remain unchanged from their ancestral state contain no information about genealogical relationships, in contrast to characters that have changed (derived states) and can signal evolutionary events (Hennig 1966). Cladism is dependent, therefore, on the status of primitive and derived character states. One of the key questions for cladistic analysis is how to determine which character similarities, within a group, are evolutionary novelties and reflect genealogical relationships. This can be accomplished by a process referred to as character state polarity analysis in which decisions are made about which character states, in the taxa being examined, are derived (apomorphic) and which are primitive (plesiomorphic). Several criteria are used to assess the evolutionary polarity of character states. These have been

well discussed by many authors, such as Steven (1980), Watrous and Wheeler (1981), Maddison *et al.* (1984) and Nelson (1985).

Cladistics is also based on the Camin-Sokal '*principle of minimum-steps evolution*' (Camin and Sokal 1965). According to this method, the best hypothetical phylogeny is the one that requires the smallest number of character changes across all characters; there should be as few reversals and convergences of characters as possible. This principle is also called '*the principle of parsimony*' (Bonde 1977; Sober 1983). Therefore, if there is a conflict of character changes in a phylogenetic construction, for cladistic analysis the maximum parsimony criterion, which only allows the fewest steps of change, determines the most suitable phylogenetic tree. Cladistic methodology and concepts have been widely discussed and treatments can be found in many publications, such as Eldredge and Cracraft (1980), Ridley (1985) and Scott-Ram (1990).

2.2.2 Data for systematics studies

Systematics studies always involve the consideration of a number of taxonomic characters. Mayr (1969) defined a taxonomic character as '*any attribute of a member of a taxon by which it differs from a member of a different taxon*'. It is implied that characters can be any internal or external morphological, physiological, behavioural, biochemical, molecular or any other kind of feature of the organism.

There has been considerable controversy over whether biochemical and molecular, or morphological characters are inherently better sources of information for estimating phylogeny (Berlocher 1984; Hillis 1987; Patterson 1987; Moritz and Hillis 1990). Some have declared that morphological characters are likely to be misleading (Frelin and Vuilleumier 1979), whereas others have argued that molecular characters are relatively weak (Kluge 1983). The advantages and disadvantages of these two sources of information were well documented by Hillis (1987) and Moritz and Hillis (1990).

2.3 The carabid genus *Holcaspis* Chaudoir

The ground beetle genus *Holcaspis* Chaudoir (family Carabidae) comprises a group

of New Zealand endemic species and has been known since late last century. *Holcaspis* is a genus rich in species with more than 30 recognised entities (Butcher 1984). The systematics of the genus have not been well studied, only the initial aspect of *Holcaspis* species delimitation has been attempted. The phylogeny of the genus *Holcaspis* is still unknown.

2.3.1 History of the carabid genus *Holcaspis*

It is over 120 years since the genus *Holcaspis* was originally described and placed in the tribe Pterostichini (Feroniini) by the great French entomologist Baron Maximillien de Chaudoir. *Holcaspis* was originally described as a subgenus of *Feronia* and distinguished by Chaudoir (1865) on the basis of its plurisulcate scutellum and the absence of grooves on the tarsi. He originally included three species, *H. angustula*, *H. intermittens* and *H. ovatella*, in the genus.

Fifteen years later, Captain Thomas Broun published his '*Manual of the New Zealand Coleoptera*, Part 1' (Broun 1880) where two further species of *Holcaspis* were described (*H. dentifera* and *H. hispidula*, a synonym of *H. hispida* that he had briefly described three years earlier). The genus name *Holcaspis* was later synonymised with *Pterostichus* by Sharp (1886) and subsequently abandoned by Broun (1893). The preference for *Pterostichus* was on the grounds that the taxonomic characters that Chaudoir used to characterise the genus *Holcaspis* were insufficient to distinguish it, because these characters would also apply to species in several groups of New Zealand pterostichines (Britton 1940). Broun was an early New Zealand entomologist who collected and described a large number of species that are now included in the genus *Holcaspis*. During the years 1880-1921, many works including *Holcaspis* species were published by Broun. However, Broun's publications have been severely criticised because they were not of good quality, even by the standards of the time (Sharp 1886). Broun never constructed a key, nor illustrated his publications, except with a few pages of poor photographs, none of which were of Carabidae (Watt 1982).

Britton (1940) revised the New Zealand Pterostichini from material held in the collections of the British Museum (Natural History). This included the Broun collection

and many species described by White (1846) and by Sharp. He also examined the type specimens of species described by Dejean, Bates (1874) and Chaudoir in the Oberthur Collection, now housed in the Muséum National d'Histoire Naturelle, Paris. Britton resurrected the name *Holcaspis* to distinguish an endemic group of New Zealand species from the Northern Hemisphere *Pterostichus*. His publication (Britton 1940) included a key to the 22 species and one subspecies of *Holcaspis* known then. Apart from constructing a key to the species and establishing much synonymy, Britton also demonstrated the very high value of illustrations of the pronotum and male genitalia for the distinction of *Holcaspis* species.

Apart from a key to the Pterostichinae of Australia and New Zealand by Moore (1965), which indicated some possible relatives of *Holcaspis*, there was no subsequent taxonomic work on *Holcaspis* until Butcher (1984). Thirty one *Holcaspis* species, including seven new species, were distinguished by Butcher. He also removed *H. edax* Chaudoir from the genus to *Neoferonia* Britton. *Neoferonia* may be a sister group to *Holcaspis*. Butcher also constructed a valuable key to *Holcaspis* species and included drawings of male and female genitalia. He also grouped the *Holcaspis* species into nine species groups, based on morphological similarity.

2.3.2 Taxonomic characters of *Holcaspis* species

The genus *Holcaspis* includes nearly half of the New Zealand indigenous species of Pterostichini (Britton 1940). A generalised description is as follows:

> *All species in the genus are entirely black and body size varies between 10 and 26 mm. Mentum with a deep pit on each side towards the base, third elytral interval often with one or more setiferous punctures. Hind wings absent, elytra joined together along the suture. Dorsal surface of all tarsal segments smooth. Basal segment of the antennae rounded, posterior tibiae in male without apical prolongation. The seventh elytral interval without setiferous punctures. Right paramere with a highly modified stylus, falcate or contorted apically, left paramere conchoid with rounded apex and orifice of aedeagus strictly dorsal. Males and females can be distinguished*

by the foretarsal segments. In the male, the three basal tarsal segments on the forelegs are obviously expanded compared with the female, where these segments are similar to those of other legs (Modified after Britton 1940; Moore 1965).

Several typical characters can be used to distinguish *Holcaspis* species from other similar New Zealand Pterostichini. For example, in *Holcaspis* species, the mentum has a deep pit on each side towards the base, a character not present in *Neoferonia*. Another significant character of *Holcaspis* is the lack of setiferous punctures on the seventh elytral interval that are found in *Megadromus*.

According to Britton (1940) and Butcher (1984), who both constructed keys to *Holcaspis* species, several important morphological features of the adult are useful in classification. For example, the shape and setation of the pronotum was extensively used. Britton (1940) presented illustrations of the *Holcaspis* pronotum in his review of the genus. Another key taxonomic character is the presence or absence of dorsal setiferous punctures and strial grooves on the elytra. Genitalia structure is also a prominent character used by previous taxonomists working with *Holcaspis*. The male genitalia have been generally used, but female genitalia were also considered by Butcher (1984). He illustrated the female gonostyli of *Holcaspis* species for the first time. All of our knowledge of *Holcaspis* taxonomy is based on the morphology of the adult stage. Larvae have not been described.

2.3.3 Geographic distribution

The distribution of *Holcaspis* species was well covered by Butcher (1984). *Holcaspis* species are found throughout the North and South Islands of New Zealand and one species (*H. stewartensis*) is confined to Stewart Island. All of the species are restricted to either the North or South Island, except *H. oedicnema,* which is found on both islands, and *H. punctigera*, which is found on both the South and Stewart Islands (Emberson, *pers. com.*). Three quarters of the species are found only in the South Island.

2.3.4 Bionomics of the genus *Holcaspis*

There has been very little work done on the biology of New Zealand *Holcaspis*. The only work, Butcher and Emberson (1981), covered some aspects of the biology of several *Holcaspis* species found on Banks Peninsula.

2.3.4.1 Habitat preference

Holcaspis is a genus of ground dwelling, carnivorous beetles. During the day, they can be found hiding under stones or logs. Butcher and Emberson (1981) observed that the preferred habitat of four species of *Holcaspis* (*H. angustula*, *H. elongella, H. subaenea* and *H. suteri*) mostly tended to be close to native forest or in clearings in the bush where ground cover included a mat of leaf litter. *Holcaspis suteri* was the most numerous species found in the study. Both *H. elongella* and *H. suteri* seemed to prefer dense, bush covered areas. *Holcaspis subaenea* was able to tolerate grass sward cover more readily than *H. suteri*. From personal observations, *H. intermittens* appears to favour pasture cover to bush cover. At this stage, with the limitation of work in this genus, the habitat preference of the other *Holcaspis* species is still unknown.

2.3.4.2 Phenology

Not all carabid beetles are active as adults throughout the year, even under favourable conditions (Thiele 1977). Each carabid species has a characteristic annual activity cycle. Temperate zone species are usually divided into spring or autumn breeders, corresponding mainly to adult and larval overwinterers (Luff 1973). The activity and life cycles of carabids are influenced by seasonal changes. Temperature and day length are also known to influence carabid growth.

In the Northern Hemisphere, Larochelle (1979) observed that some *Pterostichus* species have an autumn breeding period and overwintering larva. Kirk (1971) suggested that, in the U.S.A., some *Pterostichus* are spring and summer breeders. The adult stage may overwinter after breeding and has been known to live for up to three years.

In New Zealand, *Holcaspis*, which is related to *Pterostichus*, is reproductively active during the summer (Butcher and Emberson 1981). It is probable that New Zealand *Holcaspis* species are spring and summer breeders.

2.3.4.3 Sex Ratio

The sex ratios of four *Holcaspis* species were determined by Butcher and Emberson (1981). They caught more males than females in pitfall traps for three of the four species. Only with *H. suteri* were fewer males than females caught. A similar occurrence of males predominating has been noted with the species *Pterostichus madidus* (Fabricius) in Europe (Luff 1973). Muller (1970 cited by Luff 1987) noted that such imbalances presumably arise from differences in the activity of each sex during the activity cycle, but also depend on environmental factors and on the response of each sex to the type of trap used. Other aspects of biology such egg, larval stage or life cycle of New Zealand *Holcaspis* are still unknown.

2.3.5 Classification of the genus *Holcaspis*

The most recent classification of the genus *Holcaspis* is by Butcher (1984). He classified the genus into 31 species arranged in 9 species groups and 7 species complexes as follows :

1. *ovatella* group

H. ovatella Chaudoir 1865

2. *punctigera* group

H. punctigera Broun 1882

H. mordax Broun 1886

3. *angustula* group

3.1 *impiger* complex

H. impiger Broun 1886

H. stewartensis Butcher 1984

3.2 *angustula* complex

H. angustula Chaudoir 1865

H. bathana Butcher 1984

3.3 *algida* complex

H. algida Britton 1940

H. brevicula Butcher 1984

H. ohauensis Butcher 1984

***angustula* complex** (cont.)

H. falcis Butcher 1984

H. implica Butcher 1984

H. placida Broun 1881

4. *catenulata* group

H. catenulata Broun 1882

5. *brouniana* group

H. brouniana (Sharp 1886)

H. elongella (White 1846)

H. tripunctata Butcher 1984

6. *egregialis* group

H. egregialis (Broun 1917)

7. *hudsoni* group

H. hudsoni Britton 1940

H. suteri Broun 1893

8. *odontella* group

H. odontella (Broun 1908)

9. *oedicnema* group

9.1 *oedicnema* complex

H. dentifera (Broun 1880)

H. oedicnema Bates 1874

H. subaenea (Guérin-Ménéville 1841)

9.2 *mucronata* complex

H. mucronata Broun 1886

H. hispida (Broun 1876)

H. vexata (Broun, 1908)

9.3 *delator* complex

H. delator (Broun, 1908)

H. intermittens Chaudoir, 1865

9.4 *vagepunctata* complex

H. sinuiventris (Broun, 1908)

H. vagepunctata (White,1846)

2.3.6 The phylogenetic position of the genus *Holcaspis*

Recent studies recognize *Holcaspis* as a genus in the tribe Pterostichini, which is the largest tribe in the family Carabidae. Britton (1940) reviewed the Pterostichini in New Zealand and stated that there were nine genera in this tribe (*Megadromus*, *Zeopoecilus*,

Aulacopodus, *Holcaspis*, *Plocamostethus*, *Neoferonia*, *Rhytisternus*, *Psegmatopterus* and *Laemostenus*). Both *Rhytisternus* and *Laemostenus* are introduced, *Rhytisternus* from Australia and *Laemostenus* from Europe or North Africa. From Britton's work, *Holcaspis* has been grouped closely with *Aulacopodus*, *Plocamostethus* and *Neoferonia.* The most distant groups from *Holcaspis* were *Psegmatopterus* and *Laemostenus*; this latter genus is often now included in the Platynini or Sphodrini (Lindroth 1974).

In Moore's (1965) work on Australian and New Zealand Pterostichini, it was suggested that *Holcaspis* was a sister group to *Neoferonia*, both having styloid left parameres. *Holcaspis* is distinguished from *Neoferonia* by having a deep pit on each side of the mentum. These two genera form a very closely related group that may also include *Aulacopodus*. One *Holcaspis* species not seen by Britton (1940) (*H. edax*) was removed from the genus to *Neoferonia* (Butcher 1984).

2.4 Concluding remarks

The biological discipline known as systematics basically deals with the theory and practice of describing and categorizing organisms. Three current schools of thought: evolutionary systematics, phenetics, and cladistics, are well known in the science of systematics. The basic ideas and controversy among these major schools of thought in systematics were discussed. This review has attempted to provide the basic concepts and theories in systematics in order to apply them to the genus *Holcaspis*. Since the genus *Holcaspis* has been known work on the systematics of the genus has focused only on species delimitation. The phylogeny of the genus is still unexplored. In addition, knowledge of other aspects of the biology and ecology of this New Zealand endemic insect genus is still poor.

CHAPTER 3

A cladistic and morphometric analysis of the genus *Holcaspis* based on morphological data

3.1 Introduction

Since *Holcaspis* was first described by Chaudoir in 1865, much work has been done on the systematics of the genus, but work on phylogenetic relationships among the *Holcaspis* species has never been published. Butcher (1984), in a revision of the genus *Holcaspis*, grouped the 31 species into nine species groups, based on similarity of morphological characters. However, he did not consider the relationships of the species groups distinguished, nor the phylogenetic value of the characters used.

The purpose of this part of the study was to investigate the phylogenetic relationships of *Holcaspis* species based on external morphological characters of the adult stage using a cladistic approach. A morphometric analysis was also used for comparison with the cladistic analysis.

3.2 Materials and methods

In order to facilitate the cladistic analysis of a relatively large number of *Holcaspis* species and characters (30 and 26, respectively), a computerized phylogenetic analysis was used. The selection of taxa, including outgroup and ingroup taxa, the characters selected for analysis, and the various procedures undertaken for phylogenetic construction are discussed below.

3.2.1 Ingroup species

Thirty of the 31 *Holcaspis* species recognized by Butcher (1984) were used as the ingroup taxa. *Holcaspis vexata* was omitted from this study because specimens were unavailable and there was not enough morphological information from the published

species description for cladistic analysis.

Material of 23 ingroup taxa were available as specimens in the Entomology Museum of the Department of Entomology and Animal Ecology, Lincoln University. Morphological characters were recorded from these specimens. Specimens of the other seven *Holcaspis* species (*H. bathana, H. brevicula, H. odontella, H. sinuiventris, H. subaenea, H. tripunctata* and *H. vagepunctata*) were unavailable, but some morphological detail was obtained from species descriptions by Broun (1886), Britton (1940) and Butcher (1984).

3.2.2 Outgroup species

As discussed earlier (Chapter 2), the polarity of characters can be traced by using an outgroup comparison. In order to estimate the polarization of the 26 characters used in the analysis, it was necessary to select an appropriate outgroup. For this purpose, a multi-species outgroup, including three species, was used. The genus *Neoferonia* was selected as one of the outgroups on the assumption that it is a possible sister genus and represents the closest genus within the Pterostichini. *Neoferonia integreta* (Bates) was used as the representative species for the genus. In addition, two phylogenetically more distant outgroup species were used. The species *Megadromus antarcticus* (Chaudoir) from the tribe Pterostichini and *Laemostenus complanatus* (Dejean) from the tribe Platynini, were used as distant outgroups.

3.2.3 Character analysis

There has been a lot of criticism of the choice of outgroups for character analysis. To overcome this criticism, the polarities of each character ,therefore, have been hypothesized based on two options: (1) outgroup comparison and (2) the groundplan character state comparison of Wagner (1980). In this study, the choice of outgroups was difficult due to the present uncertainty about the phylogenetic relationships of *Holcaspis* to other genera within the Pterostichini. Therefore the multi-species outgroup comparison method of Maddison *et al.* (1984) was used. When the outgroup is heterogeneous (multi-

species outgroup), the most parsimonious assignment of an ancestral condition for the ingroup depends upon how the outgroup taxa are related to each other (Swofford 1992).

The alternative method to determine character polarity is the groundplan character state comparison method based on the examination of taxa outside the genus *Holcaspis* and a survey of literature in order to resolve the primitive and derived state of characters.

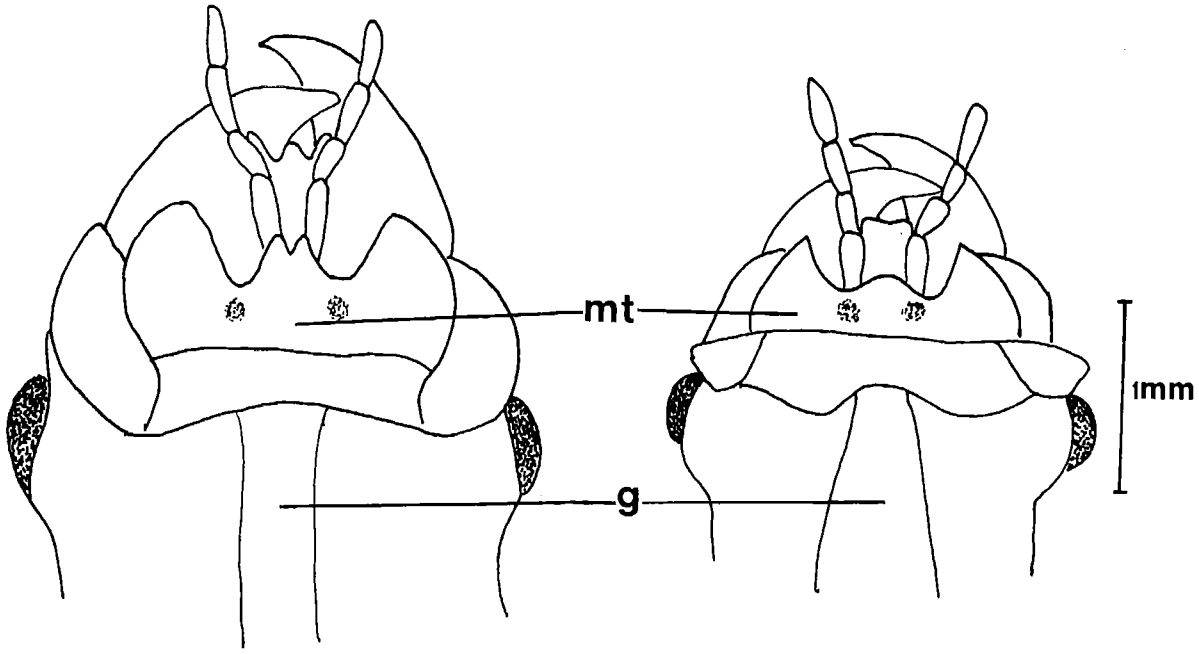
The following list describes the 26 external morphological characters used in the study. The characters include most of those that have been used for distinguishing the species and groups of species. Some additional characters, which may be important in resolving the relationships among *Holcaspis*, were included.

Five characters are from the male genitalia, four from the female genitalia and 17 from external structures. The numbers in parentheses in each description indicate how the states for each character were coded in the data matrix. All characters were coded by a whole number '0-3'. Eight characters (1, 3, 6, 8, 14, 18, 21 and 25) were coded as non-additive because they were multistate characters. The character transformation series were coded as undirected when their polarity could not be determined *a priori*. The characters used (some are shown in Figures 3.1-3.4) were:

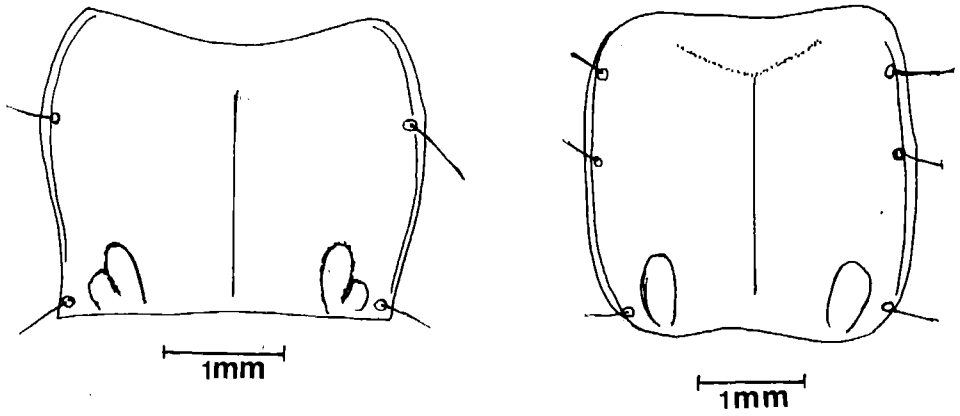
1. **Mentum tooth:** The anterior edge of the mentum often bears a unidentate tooth (0). This tooth may be absent (1) (Figure 3.1B), or the mentum tooth may be bidentate, due to a median invagination (2) (Figure 3.1A). Most Carabidae, in widely divergent groups, have a mentum with a unidentate tooth (Liebherr 1986). Therefore, a unidentate toothed mentum is presumed to be a plesiomorphic character.
2. **Shape of gula:** The gula shape is in two character states. In some species, it appears to be long and parallel shaped (0) (Figure 3.1A); in other species, the gula narrows towards the apex and gradually becomes wider at the base. Also, the maximum width of the gula is nearly as great as the length (1) (Figure 3.1B). Character state (1) does not occur in the outgroup species and is therefore inferred to be an apomorphic character.
3. **Prothoracic shape:** Most *Holcaspis* species, and the outgroup species, have a cordiform prothoracic shape; the lateral margins of the prothorax are inwardly sinuate

Figure 3.1 Ventral view of the mouthparts of *Holcaspis* species (mentum (mt) and gula (g)): **(A)** *H. elongella*, **(B)** *H. mordax.*

Figure 3.2 Dorsal view of the pronotum of *Holcaspis* species: **(A)** *H. falcis*, **(B)** *H. tripunctata.*



A



B

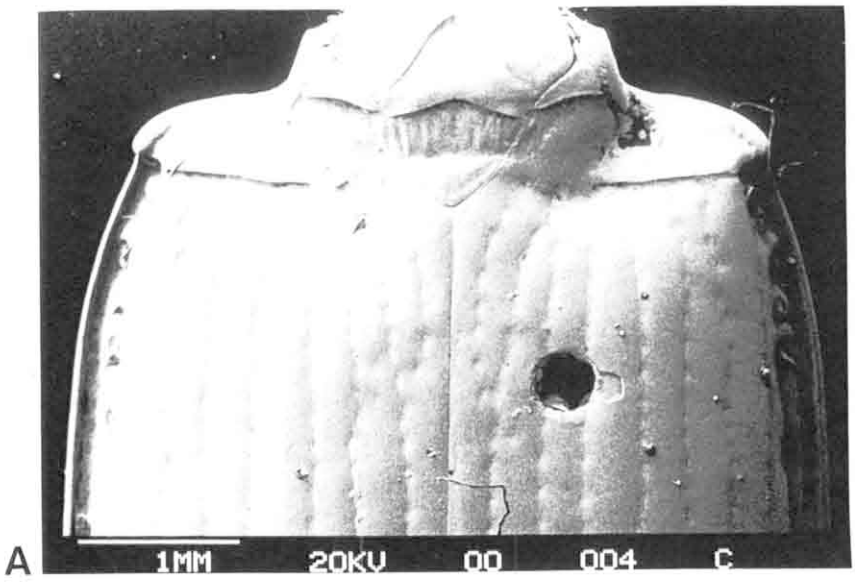
before the hind angle (0) (Figure 3.2A). A few of the ingroup species have a noncordate prothoracic shape but still have basal lateral areas with distinct hind angles (1). This area may be rounded off and without distinct hind angles (2) (Figure 3.2B). Since a cordiform prothoracic shape is common to both ingroup and outgroup taxa and is widespread in many lineages of carabids, it is inferred that this is a plesiomorphic character.

4. **The latero-basal impression of the pronotum:** The latero-basal impression of the pronotum is often single and appears only as a small scar without a distinct marginal groove (0) (Figure 3.2B). This character state is present in a majority of *Holcaspis* species. In some species, the latero-basal pronotal impression is well impressed with separated, deep, double, basal impressions, or joined together medially (1) (Figure 3.2A).
5. **Microsculpture on the dorsal surface of the pronotum:** Meshes of microsculpture on the pronotal dorsal surface of adults are isodiametric (0). Isodiametric microsculpture is a generalized character in the Carabidae. The microsculpture in some taxa is in the form of transverse meshes (1); this is an apomorphic character.
6. **Setae on the lateral margin of the prothorax:** Two setae are most commonly present on the lateral margins of carabid prothorax. The two-setae character is usually expressed as one seta at the basal angle and the other at, or near, the middle of the lateral margin (0) (Figure 3.2A). Some *Holcaspis* species may possess three setae (1) (Figure 3.2B) but in the majority of *Holcaspis* species there are four or more setae on the lateral margins (2). The number, if four or more, is often variable within a species.
7. **Microsculpture on the dorsal surface of the elytra:** In general, the dorsal elytral microsculpture in Carabidae, including *Holcaspis*, is an isodiametric reticulated pattern (0). A few *Holcaspis* species possess transverse microsculpture (1).
8. **Setae on the third elytral interval:** Three setae are most commonly associated with the third elytral interval in many carabid lineages (0). The number of seta may be 1 or 2 in some species (1). In other taxa, there are more than three setae (2), but in others there may be no setiferous punctures on the third elytral interval (3). In *H. catenulata*, 1-3 setae can be found, so it is coded as 'a', which implies it is an

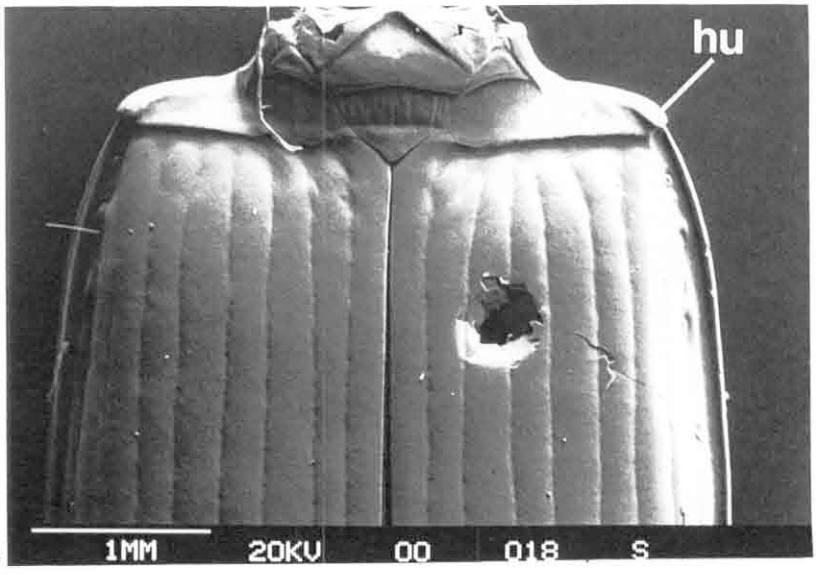
uncertain character.

9. **Strial impression:** The striae on the dorsal elytral surface may be distinctly impressed and continuous, with punctures (0) (Figures 3.3B and 3.3C). The striae may be shallow and discontinuous without punctures, appearing as broken lines (1). In a few taxa, the striae are faint, irregular and form wavy lines (2) (Figure 3.3A).
10. **Basal margin of the elytron:** The basal margin of the elytron forms a complete line from shoulder to scutellar striole (0). Sometimes the basal margin line is discontinuous, the line being incomplete from shoulder to scutella striole (1).
11. **Shape of the elytral shoulder (humeral angle):** Wing development in insects is often correlated with the shape of the humeral angle. All species in the genus *Holcaspis* are unable to fly and the humeral angle is not well developed. Even so, they may have a square angle, with a tooth-shaped protruberance on the elytral shoulder (0). In some taxa, the elytral shoulder is rounded and without a tooth (1).
12. **Shape of elytral intervals:** Pterostichini generally have convex dorsal elytral intervals (0). In some taxa, the elytral intervals are flat (1).
13. **Width of elytral intervals:** Elytral intervals are usually approximately even (0) (Figure 3.3C). In some taxa, the elytral intervals are uneven, the third and fifth intervals being distinctly wider than the other intervals (1) (Figures 3.3A and 3.3C).
14. **The scutellar striole:** The scutellar striole is present and separated at its end since it does not connect with the first stria (0). The second state is that the striole is present and continuous with the first stria (1) (Figures 3.3A-3.3C). Thirdly, the scutellar striole is absent or not joined to the first stria (2). In *H. hudsoni*, the striole can either be present and continuous with the first stria or it may be absent or not joined to the first stria. Therefore, for *H. hudsoni*, the state of this character is an uncertain, and it is coded as 'b'.
15. **First and second elytral striae:** The first and second elytral striae are independently formed and do not intersect the basal margin together (0) (Figure 3.3B). The first and second elytral striae may unite at the basal margin (1) (Figures 3.3A and 3.3C).
16. **Tooth on femur:** The hind femur of males is generally smooth, without dentations (0). In some taxa, a tooth is present on each male hind femur (1).

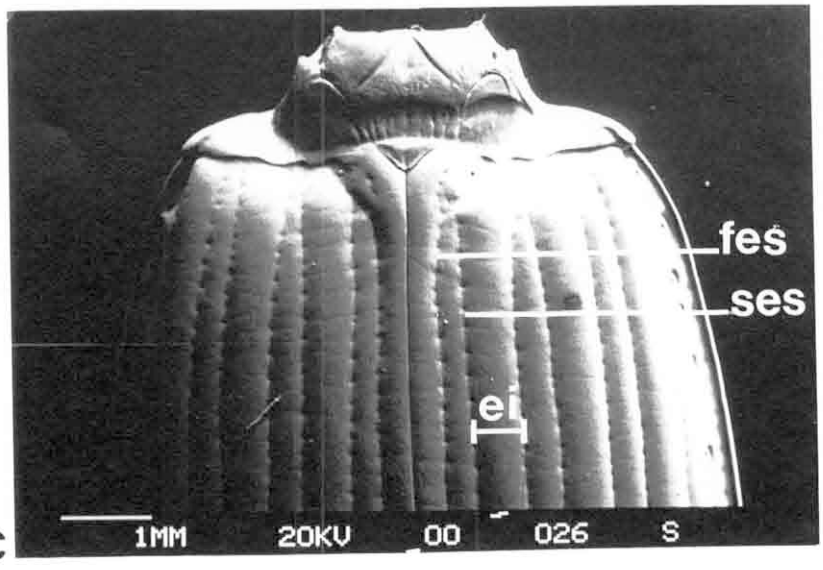
Figure 3.3 Scanning electron micrographs of the basal quarter of the elytra viewed dorsally, showing the humeral angle (hu), first elytral stria (fes), second elytral stria (ses) and elytral interval (ei): **(A)** *Holcaspis stewartensis*, **(B)** *H. algida*, **(C)** *H. dentifera.*



A



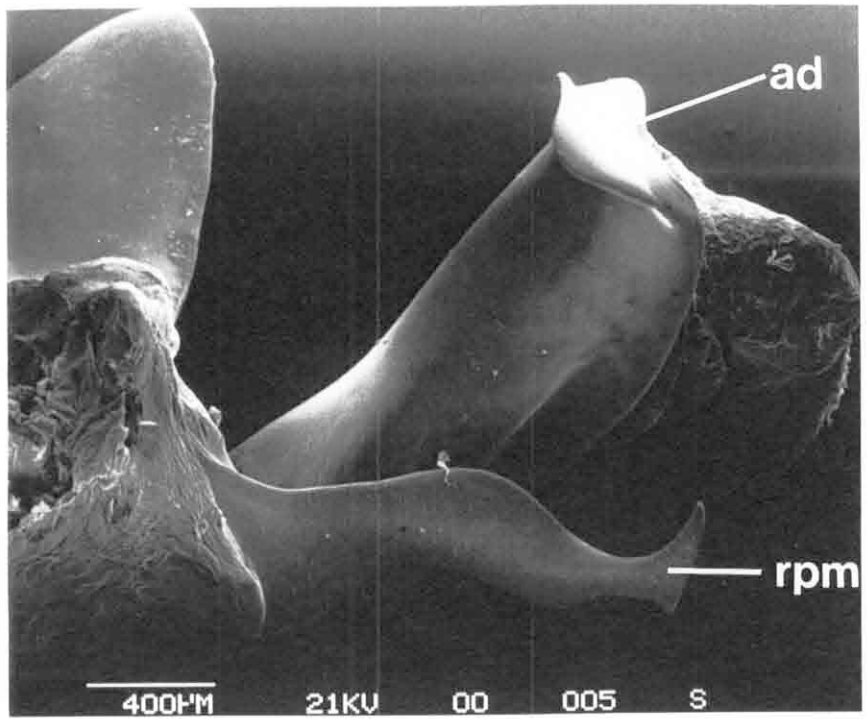
B



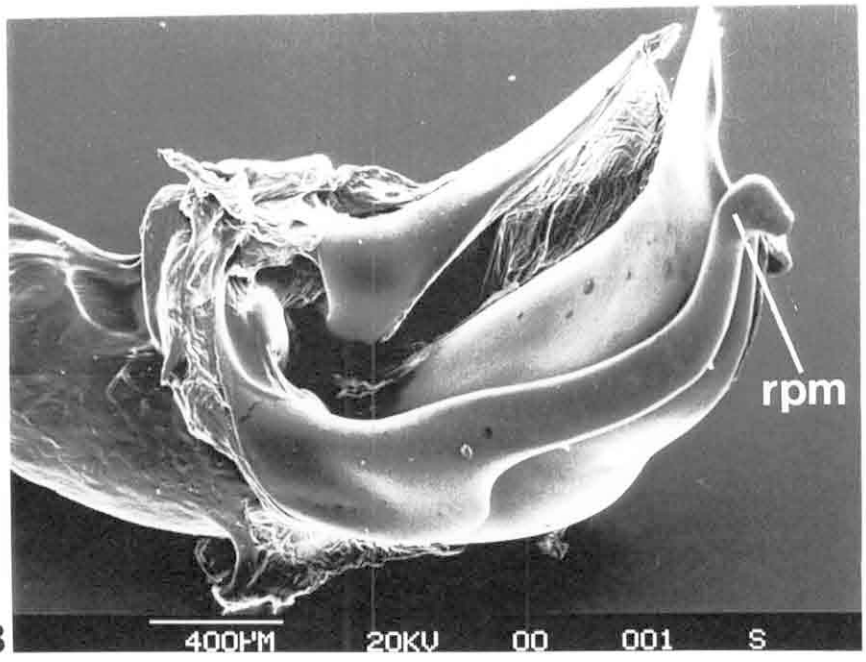
C

17. **Proepisternal surface:** The proepisternal surface is usually smooth, without any pits (0). The surface is rough with small pits (1).
18. **Apex of median lobe of aedeagus:** In most carabids, the tip of the median lobe of the aedeagus is acuminate, without a hooklike modification (0). The median lobe tip may be modified to form a more or less straight spatulate shape (1) (Figure 3.4D). The spatulate tip may curve to the right (2) (Figure 3.4C). In other taxa, the spatulate tip may appear as a hook to the right (3).
19. **Internal sac of the aedeagus median lobe (membranous endophallus):** The generalized condition of the aedeagal internal sac is unornamented without sclerites (0). The aedeagal internal sac may be modified to have a spinose sclerite on the sac (1).
20. **Right paramere shaft:** The right paramere shaft is more or less straight (0). Alternatively, the right paramere shaft is twisted (1).
21. **Right paramere apex:** The right paramere apex is a non-twisted hammerhead shape (0) (Figure 3.4A). In some taxa, the right paramere apex is modified to form a single hook-like structure (1) (Figure 3.4B). Finally, the right paramere apex is sometimes modified to form a double, hooked shape (2) (Figure 3.4C).
22. **Left paramere apex:** The left paramere apex is rounded (0). In some taxa, a mucron is present at the apex of the left paramere (1).
23. **The penultimate segment of the gonostylus:** The penultimate segment of the gonostylus has an apical fringe of setae on its ventral surface (0). Alternatively, the segment is glabrous without any setae (1).
24. **The apical segment of the gonostylus:** The apical segment of the gonostylus bears a seta on its dorsal surface (0). Alternatively, the seta is absent in some taxa (1).
25. **The seta on the lateral apical segment of the gonostylus:** The lateral apical segments of the gonostylus generally bear two setae (0), but there may be more than two lateral setae on the segment (1), there may be one setae (2), or the lateral setae may be absent (3).
26. **The seta on apical segment of the gonostylus:** The apical segment of the gonostylus generally bears two fine setae at its tip (0). Alternatively, these setae may be absent (1).

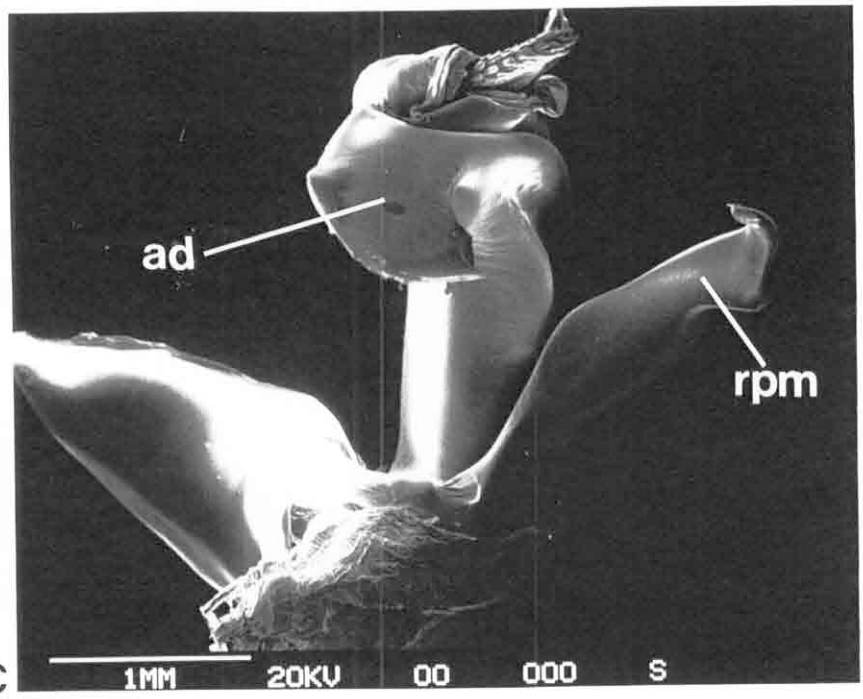
Figure 3.4 Scanning electron micrographs of the male genitalia showing the aedeagal tip (ad) and right paramere shaft (rpm): **(A)** *Holcaspis angustula*: aedeagal tip viewed dorsally, right paramere shaft viewed laterally from right; **(B)** *H. mordax*: right paramere shaft viewed laterally from right; **(C)** *H. mucronata*: aedeagal tip viewed dorsally, right paramere shaft viewed laterally from right; **(D)** *H. mordax* aedeagal tip viewed dorsally.



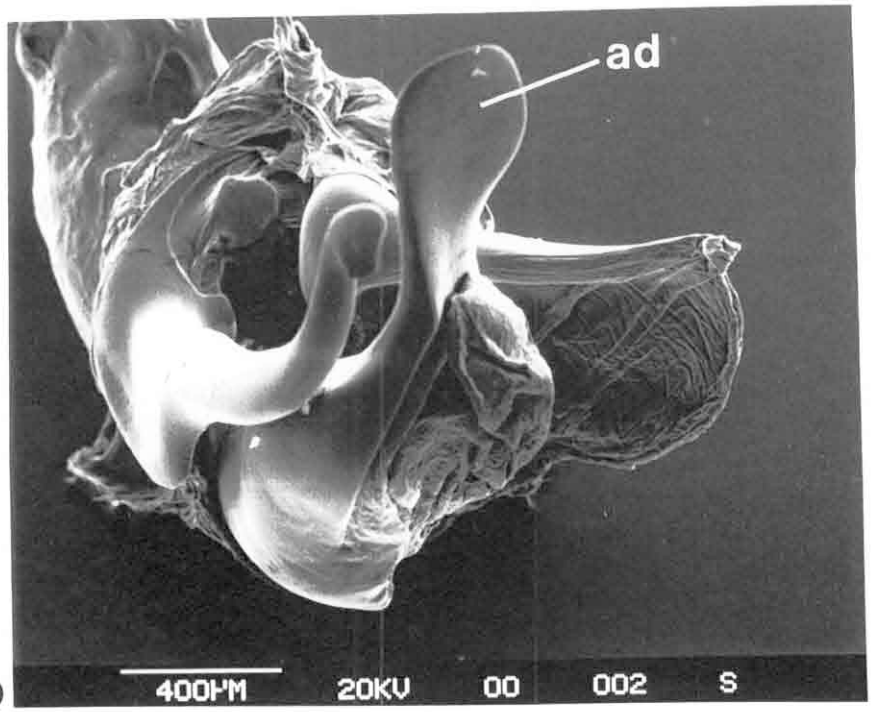
A



B



C



D

3.2.4 Cladistic method

3.2.4.1 Determination of character states' polarity

A major problem for phylogenetic analysis is to recognize whether a character is apomorphic or plesiomorphic. In this study, the polarity of the characters was inferred by using a three species outgroup comparison and a hypothetical ancestor exhibiting the groundplan character states within the Carabidae.

3.2.4.1.1 *Using the outgroup comparison.* The basic assumption in using an outgroup comparison is that the extensive distribution of any character is the result of inheritance from a shared common ancestor; it does not result from independent evolutionary development. In an outgroup comparison, therefore, it is hypothesized that if the state of a character expressed in some members of the taxon studied also occurs in taxa of at least the next higher category (outgroup taxon) then this character is likely to be an ancestral trait.

3.2.4.1.2 *Using the hypothetical ancestor.* Primitive and derived states for the 26 characters were determined using the adult Carabidae groundplan character state comparison. The hypothetical ancestor codes with all the characters of this analysis scored as plesiomorphic. Characters believed to be ancestral were entered as '0' and derived or apomorphic characters were entered as '1-3', depending on the number of different derived states.

3.2.4.2 Cladistic analysis

The character states were entered into a character matrix (Table 3.1). The cladogram was based on the principle of maximum parsimony analysis. For the analysis, the computer package '*Phylogenetic Analysis Using Parsimony*' (PAUP) version 3.0 (Swofford 1992) was used. The search for the shortest tree(s) was made by heuristic methods (since the number of taxa studied was large). The tree bisection reconnection in

the branch-swapping algorithm was selected. No weight assumptions were involved in the analysis; all characters were treated as being equally important (unweighted). The binary and multistate characters were determined as unordered changes. The multistate characters were coded as non-additive. Therefore no transformation character series were formed.

The cladograms were rooted in the PAUP analysis by using both a coalition of the three outgroup species (*N. integrata*, *L. complanatus*, and *M. antarcticus*) and a hypothetical ancestor (all'0').

Table 3.1 Character state matrix for the cladistic analysis of 30 *Holcaspis* species.(A '?' in the data matrix indicates that the character is unknown for that taxon, 'a' and 'b' in the data indicate that the character exhibits more than one state for that taxon and the groundplan state for the taxon is uncertain, respectively).

Taxon	**Character**
	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 (10 = 1 0; 20 = 2 0)
An ancestor	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
M. antarcticus	2 0 0 0 0 0 0 2 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 2 0
L. complanatus	0 0 0 0 0 0 0 3 0 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0
N. integrata	2 0 0 0 0 0 0 3 0 0 0 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0
H. algida	2 0 0 1 0 0 0 3 0 0 0 1 0 1 0 0 1 3 1 0 1 0 0 1 3 1
H. angustula	2 0 0 1 0 0 0 3 0 0 0 1 0 1 0 1 1 1 0 0 0 0 0 1 0 0
H. bathana	? ? 0 1 ? 0 ? 3 0 1 0 1 0 1 0 1 ? 3 0 1 1 0 0 1 3 1
H. brevicula	? ? 0 1 ? 0 ? 3 0 1 0 1 0 0 0 0 ? 1 0 0 1 0 ? ? ? ?
H. brouniana	2 0 2 0 0 2 0 3 1 1 0 0 0 1 1 1 0 2 1 1 2 0 1 0 0 1
H. catenulata	2 0 2 0 0 2 0 a 1 1 0 1 1 0 1 0 0 2 1 1 0 0 1 0 0 1
H. delator	2 0 0 0 0 2 0 3 1 1 0 0 1 0 1 0 1 2 1 1 2 0 1 0 0 1
H. dentifera	2 0 2 0 0 2 0 3 0 0 0 0 1 1 1 1 0 2 1 0 1 1 1 0 1 0
H. egregialis	2 0 0 0 0 2 0 1 0 0 0 1 0 2 1 0 0 3 0 0 0 0 1 0 0 1
H. elongella	2 0 2 0 0 2 0 3 1 1 1 0 1 2 1 0 0 1 1 0 0 1 1 0 0 1
H. falcis	2 0 0 1 0 0 0 3 0 0 0 1 0 1 0 0 1 3 0 0 0 0 0 1 3 0
H. hispida	2 0 0 0 0 2 0 3 0 1 0 0 1 2 1 0 0 2 1 1 1 0 1 0 0 1

Taxon	Character 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
H. hudsoni	2 0 0 1 0 2 0 2 0 1 0 0 0 b 1 1 0 3 1 0 2 0 1 0 1 1
H. impiger	2 1 0 1 0 0 0 3 2 1 0 1 1 1 1 0 1 2 1 0 0 0 0 0 0 0
H. implica	2 0 0 1 0 0 0 3 0 1 0 1 0 1 0 1 1 3 0 1 1 0 0 1 0 0
H. intermittens	2 0 0 0 0 2 0 3 1 1 0 0 1 0 1 0 0 2 1 1 2 0 1 0 1 0
H. mordax	1 1 0 0 1 0 1 1 0 0 0 0 0 2 0 0 1 1 1 0 1 1 0 0 1 0
H. mucronata	2 0 0 0 0 2 0 3 0 0 0 0 1 0 1 1 0 2 1 0 2 0 1 0 1 0
H. odontella	2 0 1 0 ? 2 ? 3 1 1 0 0 ? ? ? 0 ? 1 ? 1 2 0 ? ? ? ?
H. oedicnema	2 0 1 0 0 2 0 3 1 1 0 0 0 1 1 1 1 2 1 1 1 0 1 0 1 0
H. ohauensis	2 0 0 1 ? 0 ? 3 1 1 0 1 0 1 0 0 0 3 0 0 1 0 0 1 3 0
H. ovatella	2 0 2 0 0 0 0 0 0 0 1 0 0 0 1 0 0 3 1 1 0 0 0 1 3 1
H. placida	2 0 0 1 0 0 0 3 0 0 0 1 0 1 0 1 1 3 0 0 0 0 0 1 0 0
H. punctigera	1 1 0 0 0 0 0 1 0 0 0 1 0 2 0 0 1 1 1 0 1 1 1 0 1 0
H. sinuiventris	? ? 0 0 ? 2 ? 3 0 0 0 0 ? ? ? 0 ? 2 1 1 2 0 0 1 0 1
H. stewartensis	2 0 0 1 1 0 1 3 2 1 0 1 1 1 1 0 1 2 1 0 0 0 1 0 1 0
H. subaenea	? 0 0 0 ? 2 ? 3 1 1 0 0 1 ? ? 1 ? ? ? ? ? ? ? ? ? ?
H. suteri	2 0 0 0 0 2 0 2 0 0 0 0 0 0 1 0 0 3 1 0 0 0 0 0 0 0
H. tripunctata	2 ? 2 0 ? 1 ? 3 1 1 1 1 1 0 1 0 ? 2 1 1 2 0 ? ? ? ?
H. vagepunctata	? ? 0 0 ? 2 ? 3 1 0 0 0 1 ? ? 0 ? 2 1 1 2 0 1 0 0 1

3.2.5 Morphometric method

The reason for using morphometric analysis was to discover reliable taxonomic characters and to estimate the species relationships of *Holcaspis* species from quantitative morphological data.

3.2.5.1 Examination of specimens

The specimens for morphometric measurement were from 21 of the 31 species used

for the cladistic analysis (Table 3.2). The specimens had been collected from a variety of localities throughout the North and South Islands. In all, 392 adult specimens, both male and female, of 21 *Holcaspis* species were examined.

3.2.5.2 Character measurement

Nineteen external morphological characters were measured for each specimen. Measurement was done using a calibrated ocular micrometer in a binocular dissecting microscope. The micrometer measurements were converted and recorded in millimetres. The measurements taken are shown in Figure 3.5. The 19 characters were as follows:

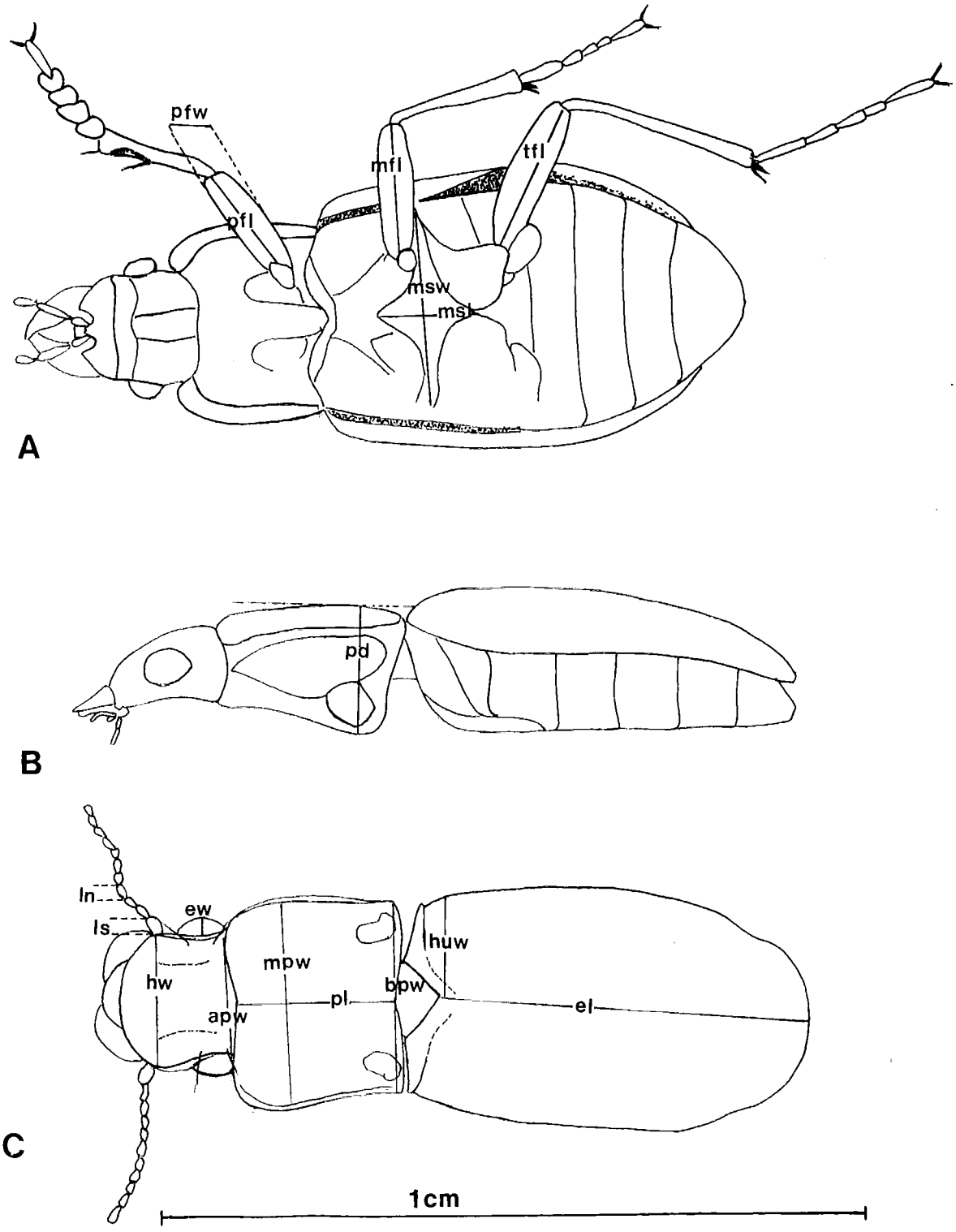
1. **Head width (hw):** The width across the head, measured from the outer supraorbital ridges.
2. **Eye width (ew):** The greatest width of an eye measured from the dorsal surface.
3. **Length of scape (ls):** Measured from base to tip of scape.
4. **Length of fourth antennomere (ln):** Measured from the base to the tip of the antennal segment.
5. **Apical prothoracic width (apw):** The width measured by joining the two most anterior points of the pronotal front angles.
6. **Maximum prothoracic width (mpw):** The maximum width across the prothorax.
7. **Basal prothoracic width (bpw):** The width across the base of the prothorax measured at the posterior angles.
8. **Prothoracic length (pl):** The midline length of the pronotum.
9. **Prothoracic depth (pd):** The distance from the convexity of the pronotum to the convexity of the prosternal process.
10. **Humeral width (huw):** The width measured from the tip of scutellum to the elytral margin at the humeral angle.
11. **Elytral length (el):** The distance measured from the tip of scutellum to the apex of the elytron.
12. **Profemur length (pfl):** The distance along the median, ventral, femoral surface from the apex of the trochanter to the apex of the femur.

Table 3.2 *Holcaspis* species material examined for the morphometric analysis.

Species	Locality	Number of specimens examined	
		Male	Female
H. algida	Mid Canterbury: South Island	3	3
H. angustula	Canterbury area: South Island	20	20
H. brouniana	North east of the South Island	2	4
H. delator	Mid and South Canterbury	10	6
H. egregialis	Otago, Dunedin: South Island	12	12
H. elongella	Canterbury area: South Island	20	20
H. falcis	MacKenzie Basin: South Island	3	1
H. hispida	Throughout the North Island	6	5
H. hudsoni	North Canterbury: South Island	20	10
H. impiger	Otago, Dunedin: South Island	5	3
H. implica	Mid & South Canterbury, Otago	3	3
H. intermittens	Mid & South Canterbury	20	20
H. mordax	Throughout the North Island	8	3
H. mucronata	Throughout the North Island	7	5
H. oedicnema	Western North Island, north-west South Island	21	15
H. ohauensis	MacKenzie Basin: South Island	2	1
H. ovatella	Southern, eastern South Island	12	11
H. placida	South east of the South Island	4	6
H. punctigera	Southern South Island	8	13
H. stewartensis	Stewart Island	2	6
H. suteri	Mid Canterbury: South Island	20	20

13. **Profemur width (pfw):** The maximum width of the femur perpendicular to the profemur length.
14. **Mesofemur length (mfl):** The distance along the median, ventral, mid femoral surface from the apex of the trochanter to the apex of the mesofemur.

Figure 3.5 The 19 morphological characters used in the morphometric analysis of *Holcaspis* species: **(A)** ventral view; **(B)** lateral view; **(C)** dorsal view. (Character abbreviations as in text).


pfw
pfl
mfl
tfl
msw
msl
A
pd
B
ln
ls
ew
hw
apw
mpw
pl
bpw
huw
el
C
1cm

15. **Mesofemur width (mfw):** The maximum width of the mesofemur perpendicular to the mesofemur length.
16. **Metafemur length (tfl):** The distance along the median, ventral, hind, femoral surface from the apex of the trochanter to the apex of the metafemur.
17. **Metafemur width (tfw):** The maximum width of the metafemur perpendicular to the metafemur length.
18. **Metasternum length (msl):** The distance along the median ventral surface of the metathorax.
19. **Metasternum width (msw):** The maximum width of the ventral surface of the metathorax.

3.2.5.3 Morphometric analysis

Male and female data sets were analyzed separately using multivariate discriminant functions. All 19 characters were tested by using stepwise multiple discriminant analysis in the SAS computer programme. A stepwise procedure was chosen to select for only discriminating variables that contributed to separate morphologically similar taxa. Only those characters that showed significant discrimination among the species were selected for further analysis. From the selected variables, a canonical discriminant analysis, which maximizes among group variation relative to within group variation, was conducted to test for discrimination between species. The discrimination analysis provided the Mahalanobis distance (D^2), which is a measurement of a square distance among species. The phenogram was calculated from the Mahalanobis distance using cluster analysis with unweighted pair group method with arithmetic averages (UPGMA) in the SPSS statistical package.

3.3 Results and discussion

3.3.1 Cladistic analysis

At least 100 equally parsimonious trees with tree lengths of 114 and 109 character

changes, and consistency indexes of 0.333 and 0.339 were obtained using a coalition of the three outgroup species (*N. integrata*, *M. antarcticus* and *L. complanatus*) and the hypothetical ancestor respectively. The majority rule consensus trees based on these 100 equally parsimonious trees from both outgroup taxa are shown in Figures 3.6 and 3.7. The cladogram produced using the three species outgroup showed fewer unresolved phylogenetic relationships than the cladogram produced using the hypothetical ancestor as an outgroup. However, the cladograms produced using the three outgroup taxa (Figures 3.6 and 3.8) and the hypothetical ancestor (Figures 3.7 and 3.9) show many clades of *Holcaspis* species in common. The analyses both show that the *punctigera* group and *angustula* group are closely related. This strong congruency of the cladograms for the *punctigera* group and *angustula* group is based on all species sharing three synapomorphies: flattened elytral intervals, the presence and continuous suture of the scutellar striole, and the rough surface of the proepisternum (characters 12, 14 and 17). Within this clade, *H. punctigera* and *H. mordax* are closely related species, characterized by sharing a number of uniquely derived characters: the mentum without a tooth, gula not parallel, and three setae on the third elytral interval (characters 1, 2 and 8). The phylogenetic relationship of these two species, from this cladistic analysis, supports Butcher (1984). He placed these two species together in the *punctigera* group and indicated the relationship of this group to the *angustula* group.

The cladistic analysis also suggests, like Butcher (1984), that the most closely related group to the *punctigera* group is the *angustula* group. All species in the *angustula* group possess a synapomorphic character of having a deep double impression in the latero-basal region of the pronotum. Within the *angustula* clade, the analysis shows that *H. impiger* and *H. stewartensis* are united. This was recognised by Butcher as the *impiger* complex, which is characterised by the possession of irregular wavy striae (character 9). The remainder of *Holcaspis* species within *angustula* group (the *algida* complex and the *angustula* complex) are characterized by sharing the characters of bearing unornamented aedeagal internal sacs and a single hook-like apex to the right paramere (characters 19 and 24). However, *H. algida* (a species placed by Butcher in the *algida* complex) possesses an aedeagal internal sac with a spinose sclerite (character 24). It is possible that this character is a reversal to the ancestral lineage of the *Holcaspis* group. The cladistic

Figure 3.6 Majority rule consensus tree for 100 equally parsimonious trees of the genus *Holcaspis* rooted with three outgroup species. (The percentage of the 100 shortest trees that contain that component are shown along each branch.)

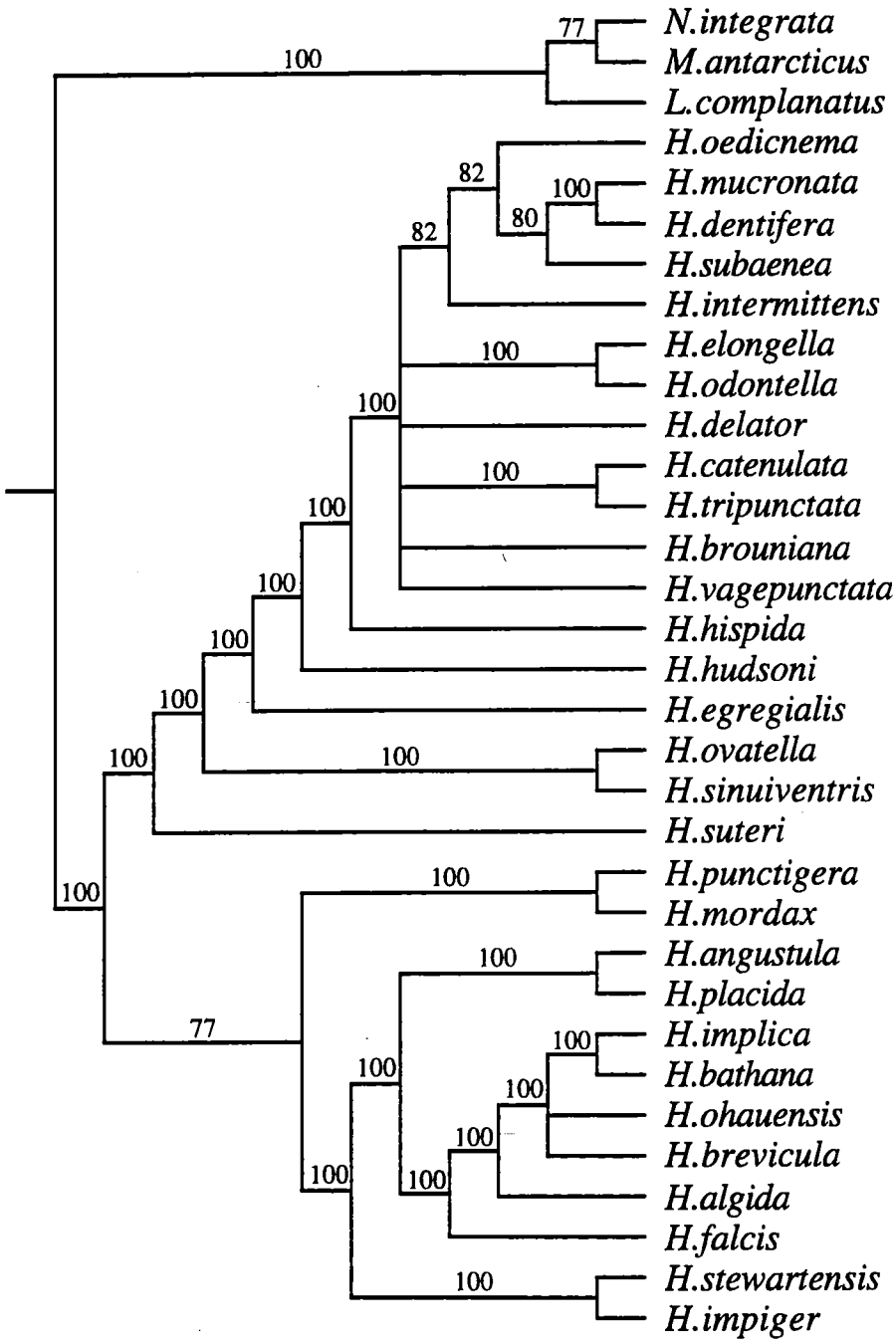

N.integrata
M.antarcticus
L.complanatus
H.oedicnema
H.mucronata
H.dentifera
H.subaenea
H.intermittens
H.elongella
H.odontella
H.delator
H.catenulata
H.tripunctata
H.brouniana
H.vagepunctata
H.hispida
H.hudsoni
H.egregialis
H.ovatella
H.sinuiventris
H.suteri
H.punctigera
H.mordax
H.angustula
H.placida
H.implica
H.bathana
H.ohauensis
H.brevicula
H.algida
H.falcis
H.stewartensis
H.impiger
77
100
82
100
80
82
100
100
100
100
100
100
100
100
100
77
100
100
100
100
100
100
100
100
100
100

Figure 3.7 Majority rule consensus tree for 100 equally parsimonious trees of the genus *Holcaspis* rooted with the hypothetical ancestor. (The percentage of 100 shortest trees that contain that component are shown along each branch.)

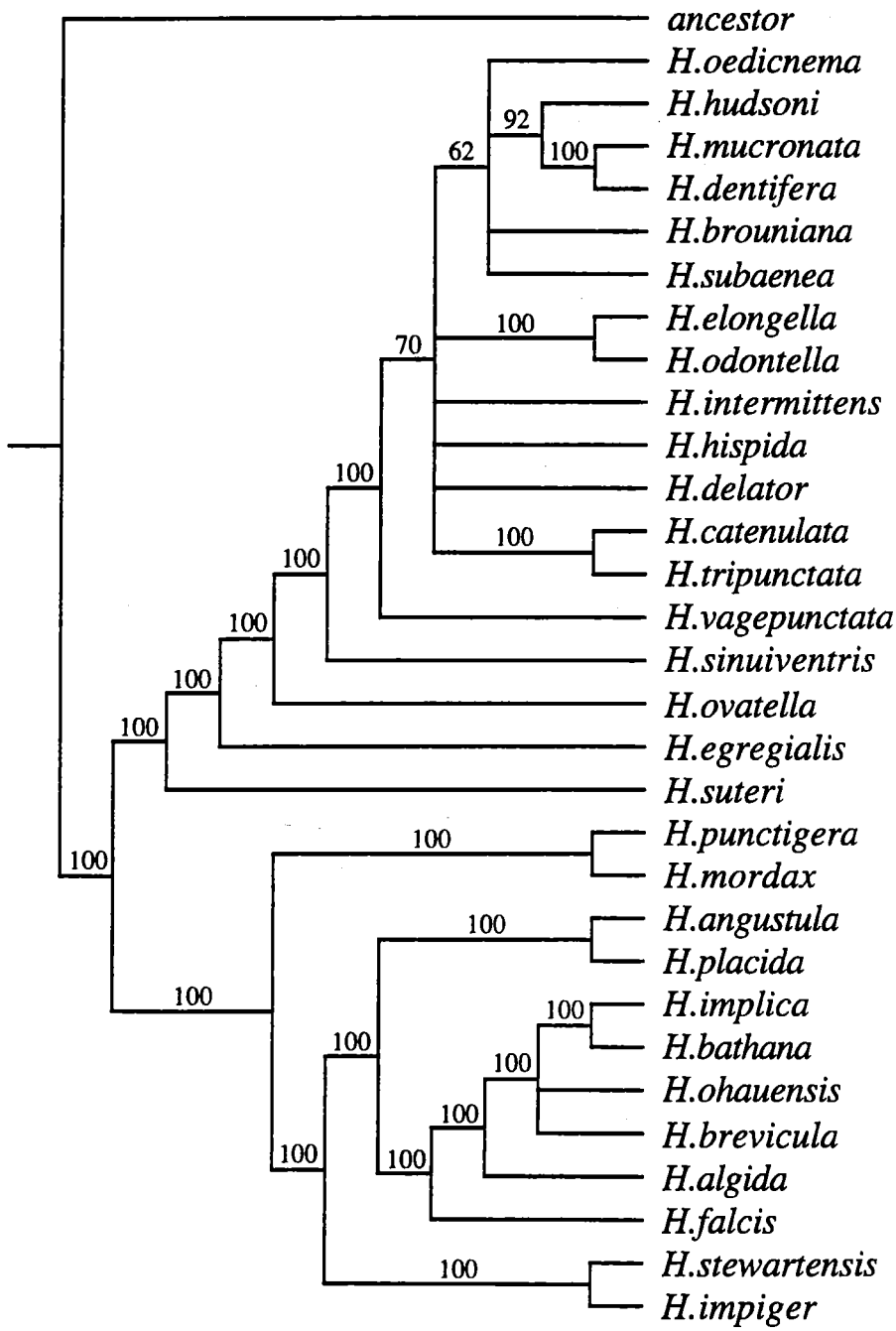

ancestor
H.oedicnema
H.hudsoni
H.mucronata
H.dentifera
H.brouniana
H.subaenea
H.elongella
H.odontella
H.intermittens
H.hispida
H.delator
H.catenulata
H.tripunctata
H.vagepunctata
H.sinuiventris
H.ovatella
H.egregialis
H.suteri
H.punctigera
H.mordax
H.angustula
H.placida
H.implica
H.bathana
H.ohauensis
H.brevicula
H.algida
H.falcis
H.stewartensis
H.impiger
92
62
100
100
70
100
100
100
100
100
100
100
100
100
100
100
100
100
100
100
100
100
100
100

Figure 3.8 One of the most parsimonious trees of the genus *Holcaspis* rooted with the three outgroup species.

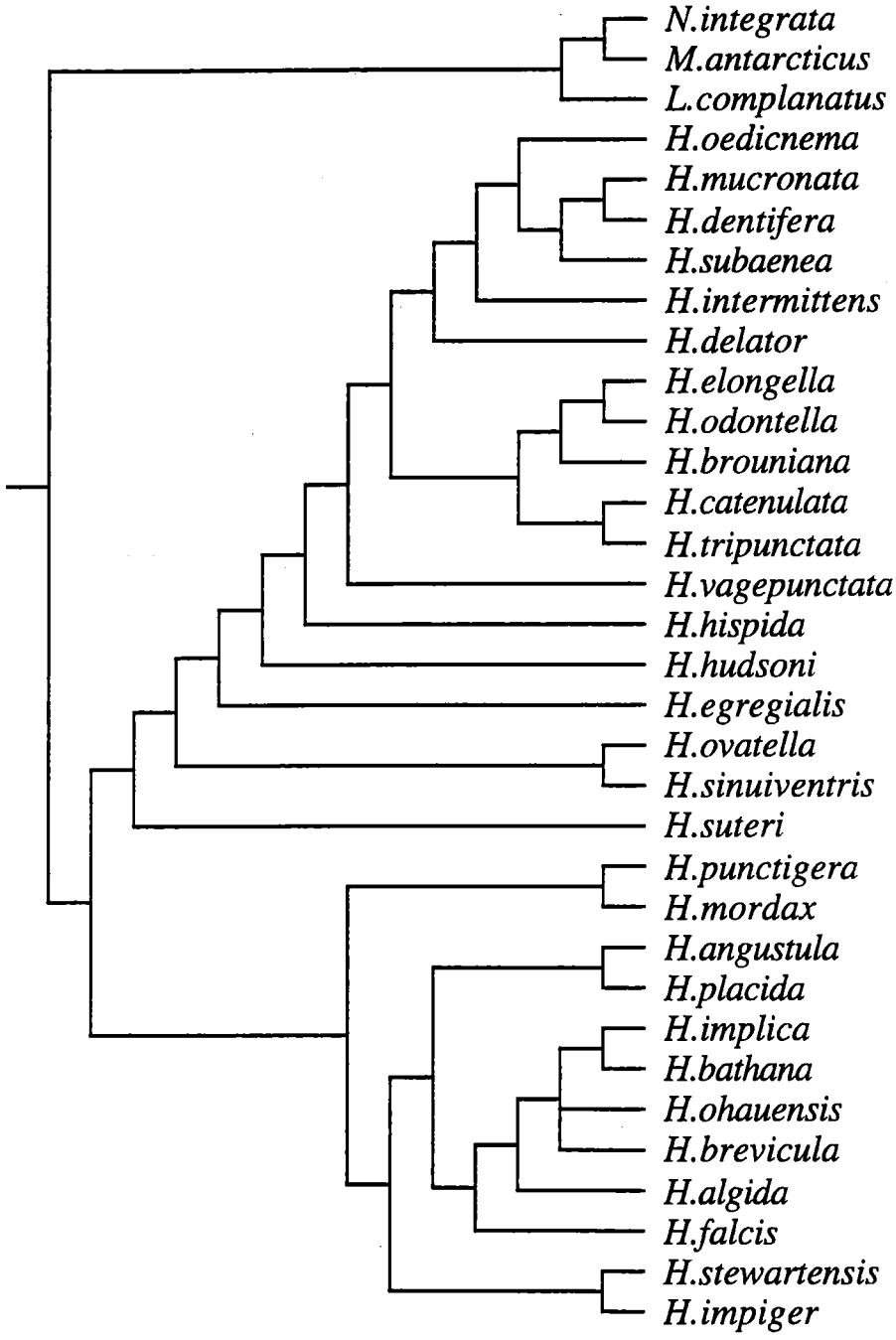
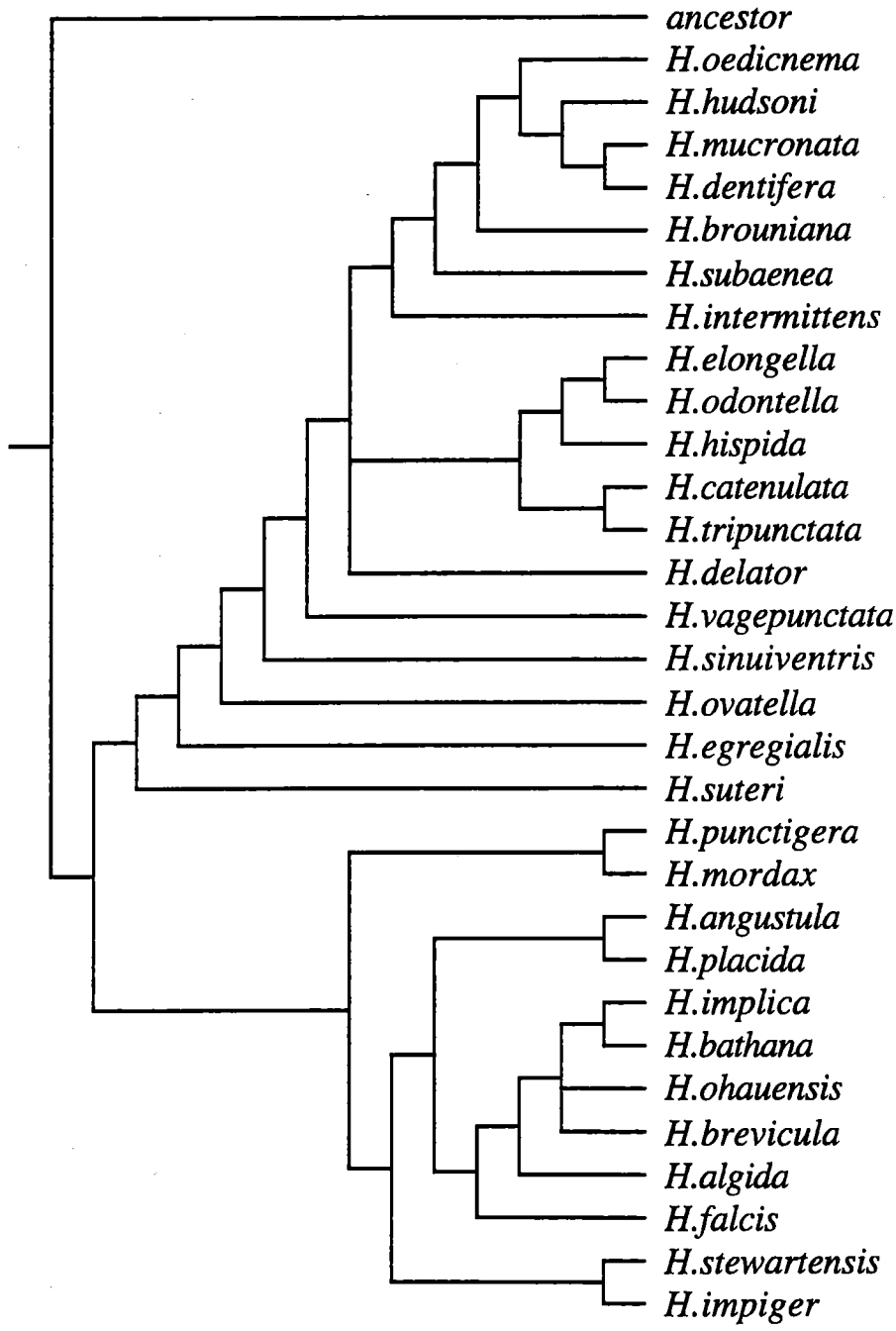

N.integrata
M.antarcticus
L.complanatus
H.oedicnema
H.mucronata
H.dentifera
H.subaenea
H.intermittens
H.delator
H.elongella
H.odontella
H.brouniana
H.catenulata
H.tripunctata
H.vagepunctata
H.hispida
H.hudsoni
H.egregialis
H.ovatella
H.sinuiventris
H.suteri
H.punctigera
H.mordax
H.angustula
H.placida
H.implica
H.bathana
H.ohauensis
H.brevicula
H.algida
H.falcis
H.stewartensis
H.impiger

Figure 3.9 One of the most parsimonious trees of the genus *Holcaspis* rooted with the hypothetical ancestor.


ancestor
H.oedicnema
H.hudsoni
H.mucronata
H.dentifera
H.brouniana
H.subaenea
H.intermittens
H.elongella
H.odontella
H.hispida
H.catenulata
H.tripunctata
H.delator
H.vagepunctata
H.sinuiventris
H.ovatella
H.egregialis
H.suteri
H.punctigera
H.mordax
H.angustula
H.placida
H.implica
H.bathana
H.ohauensis
H.brevicula
H.algida
H.falcis
H.stewartensis
H.impiger

analysis shows that two *Holcaspis* species (*H. angustula* and *H. placida*) placed in the *angustula* complex in the previous classification are grouped separately from the rest of the *angustula* complex, being the sister group to the next monophyletic group of *falcis*, *algida*, *brevicula*, *ohauensis*, *bathana* and *implica*. The affinity of *H. angustula* and *H. placida* from this analysis supports Britton's (1940) classification based on the possession of a prominent tooth on the hind femur in the male.

The rest of the *Holcaspis* species, which form a sister group to the clade *punctigera-angustula,* are placed together, based on the possession of having more than four setae on the lateral margin of prothorax and the unification of the first and second elytral striae at the basal margin (characters 6 and 15). The cladograms, using both the association of three outgroup species and the hypothetical ancestor as the tree rooting outgroup, show internal relationships of this clade that do not differ greatly. Only *H. hudsoni* is placed in different phylogenetic relationships by the two treatments.

The clade including the *catenulata*, *brouniana* and *odontella* groups is consistent in both cladograms (Figures 3.8 and 3.9). The clustering of the *catenulata* group with the *brouniana* group is congruent with Butcher's work. The cladistic analysis indicates, however, that within this clade *H. odontella* is closely related to *H. elongella*, based on two shared synapomorphic characters: the absence of the striole scutellar on the elytra and having a spatulate apex to the medium lobe of the aedeagus (characters 14 and 18). These two characters are possibly character reversals and may indicate parallel evolution in these lineages. The grouping of *H. odontella* with *H. catenulata* is likely to be arbitrary since the grouping of this clade is based on the rounded shape of basal-latero pronotum. However, the character does not occur in *H. odontella.* Both cladograms (Figures 3.8 and 3.9) also show more or less similar grouping of the *oedicnema-delator* clade, which was grouped by Butcher (1984) as the *oedicnema* group. Within this clade, the results show that *H. mucronata* and *H. dentifera* are closely related species based on sharing the characters of distinct strial impression and the complete line on the basal margin of the elytral (characters 9 and 10). The grouping of *H. mucronata* and *H. dentifera* disagrees with Butcher's work. He separated them into different species complexes though he still placed them in the same species group.

In conclusion, cladistic analysis of the genus *Holcaspis* based on morphological

features shows strong correspondence with earlier classifications among some of the species groups. The analysis shows that a large amount of homoplasy exists in the cladogram. It implies that certain derived characters in the genus have arisen independently many times in the evolution of the lineage. However, the phylogeny is not completely resolved. The number of characters used needs to be larger considering the large number of species in the group.

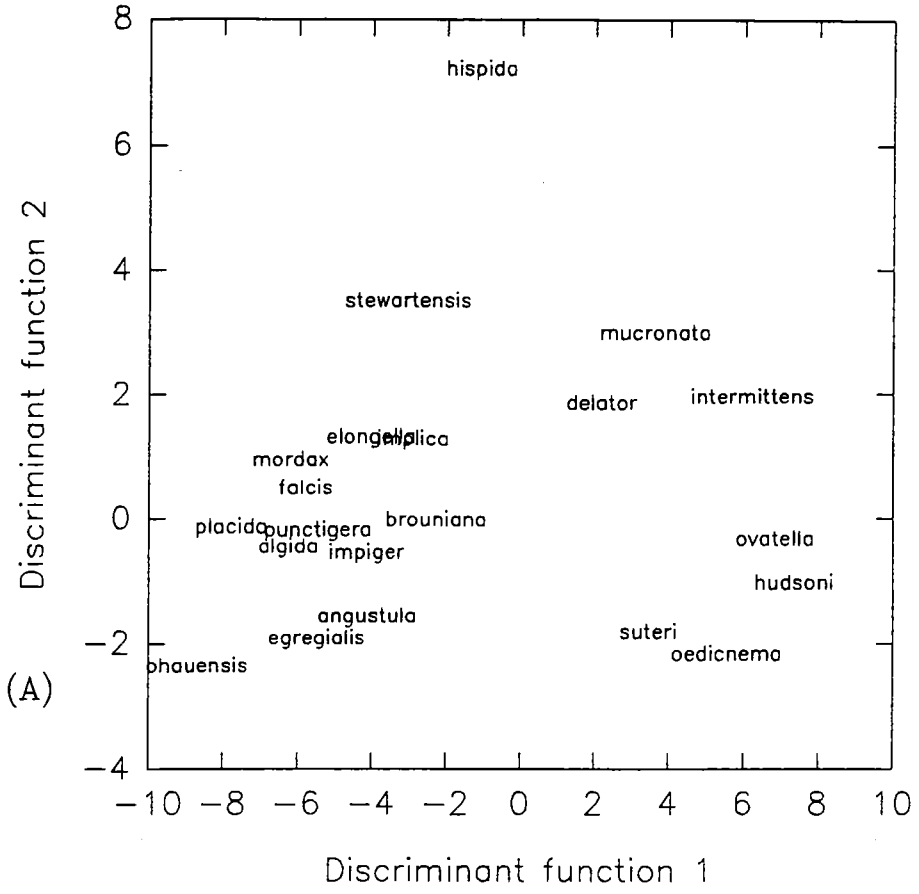
3.3.2 Morphometric analysis

Eighteen of 19 variables measured were selected as containing useful information by the stepwise discriminant analysis. The variable length of the scape segment of the antenna was excluded from the further analysis because of its inability to contribute to the discrimination. The results of multivariate discriminant analysis were similar for the female and male data sets (Figures 3.10A and 3.10B). This result is not unexpected because *Holcaspis* species are not sexually dimorphic in size. Butcher (1984) pointed out that the shape of the three terminal tarsal segments on the forelegs is a character that shows strong sexual dimorphism. The two data sets were, therefore, combined and discriminant analysis applied to the 18 selected variables.

The standardized canonical discriminant function coefficients from both males and females are given in Table 3.3. These coefficients indicate the relative effect on the discriminant function of each variable used. The results show that the variables contributing most to discrimination along discriminant function 1 were: metasternum length, profemur length, and hind femur width and, along discriminant function 2, were: length of fourth antenna, head width, and metasternum width.

The analysis of probability of assigning an individual to the correct species was 70-100 %. The probability of correctly classifying most species was 100 %. However, there was a high probability of misclassification among individuals of *H. hispida* (30 %) and *H. egregialis* (29 %). There is much evidence to show that discriminant analysis generally has a high confidence of correctly identifying species (Sluss *et al.* 1982; Burne 1987; Kambhampati and Rai 1991).

Figure 3.10 Discriminant function scatterplots of *Holcaspis* species for functions 1 and 2: **(A)** females and **(B)** males


hispida
stewartensis
mucronata
delator
intermittens
elongella
implica
mordax
falcis
placida
punctigera
brouniana
algida
impiger
ovatella
hudsoni
angustula
egregialis
suteri
oedicnema
ohauensis
(A)
Discriminant function 2
Discriminant function 1
8
6
4
2
0
−2
−4
−10 −8 −6 −4 −2 0 2 4 6 8 10

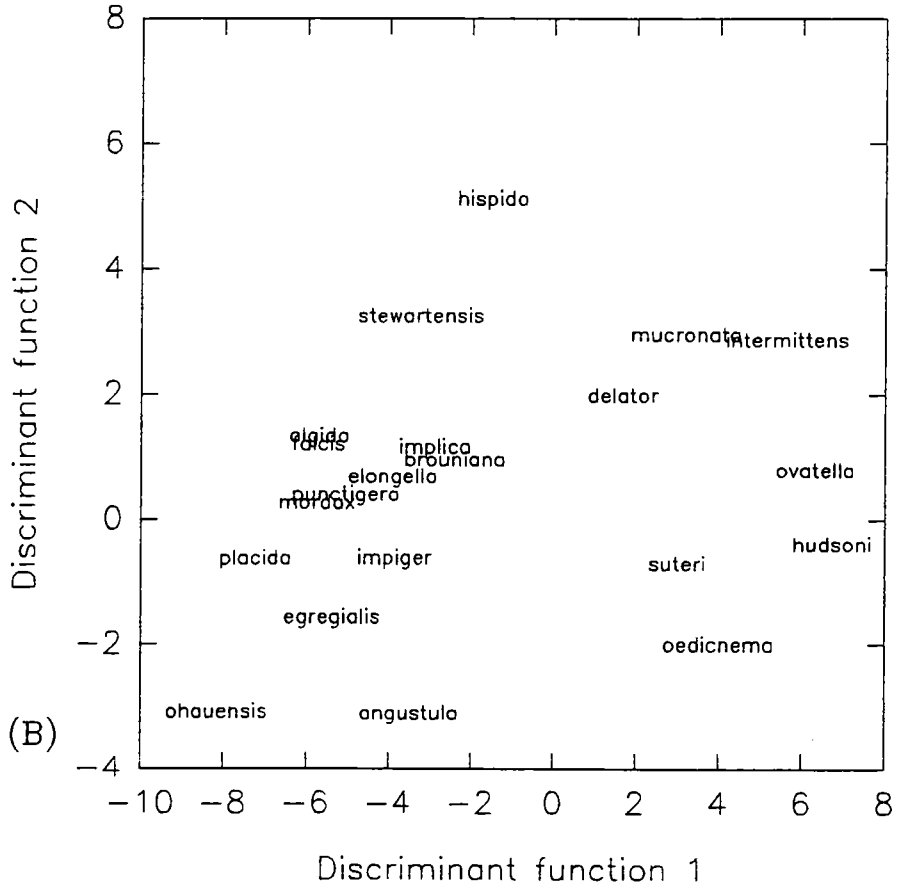

hispida
stewartensis
mucronata
intermittens
delator
algida
falcis
implica
brouniana
elongella
ovatella
punctigera
mordax
hudsoni
placida
impiger
suteri
egregialis
oedicnema
ohauensis
angustula
(B)
Discriminant function 2
Discriminant function 1
8
6
4
2
0
−2
−4
−10 −8 −6 −4 −2 0 2 4 6 8

Table 3.3 Standardized canonical discrimination function coefficients for morphometric analysis of 18 selected morphological characters from *Holcaspis* species.

Variable	Discriminant function 1	Discriminant function 2
Middle femur length	-0.033	0.041
Middle femur width	0.124	-0.038
Maximum prothoracic width	0.155	0.268
Head width	0.151	-0.436
Metasternum width	-0.048	-0.397
Apical prothoracic width	0.078	0.236
Metasternum length	0.513	-0.113
Basal prothoracic width	0.044	-0.374
Length of fourth antenna	-0.024	0.840
Hind femur width	-0.421	-0.387
Hind femur length	0.232	0.080
Prothoracic length	-0.211	-0.215
Profemur length	0.425	0.369
Profemur width	0.267	0.121
Elytral length	0.383	-0.202
Prothoracic depth	0.064	0.291
Humeral width	0.094	0.279
Eye diameter	0.120	0.040

The Mahalanobis matrix distances among pairs of the 21 *Holcaspis* species from discriminant analysis are given in Table 3.4. The phenogram of the relationships of 21 *Holcaspis* species derived from these distances using cluster analysis is shown in Figure 3.11A. It seems that the results from the canonical discriminant function analysis based only on overall body size measurements are likely to cluster the species according to size. Therefore, in order to minimize the body size effect, each measured character of each individual was divided by a standard estimate of body size (prothoracic length plus elytral

length). These particular measurements were used because they had already been measured and, when it was decided to calculate this ratio, the specimens had all been returned. It was therefore not possible to measure the total size and be sure that the same individuals as had already been measured were being measured. The ratios from the 19 characters (as listed before) were then used in a second discriminant function analysis. The stepwise multiple discriminant analysis was again used to test these characters in order to define their validity for taxonomic separation. Stepwise analysis showed that four characters: elytral length, mesofemur length, metafemur length, and length of scape, did not contribute significantly to the discriminant analysis. The phenogram from the rest of the characters (Figure 3.11B) was generated from the Mahalanobis distance matrix from Table 3.5. The *Holcaspis* species clustered together except for one group of three species: *H. algida*, *H. hispida* and *H. ohauensis*, which were relatively highly distinct from the rest. However, the phenogram showed some clades with unresolved relationships and the relationship pattern among *Holcaspis* species was unclear in comparison with the previous phenogram (Figure 3.11A). These results indicate that the ratio values for characters to minimize the effects of body size were relatively constant and therefore, could not unambiguously separate them in the discriminant analysis; the species are likely to overlap to each other.

Overall, the phenogram from morphometric analysis (Figure 3.11) gave a pattern of species relationships incongruent with the cladograms (Figures 3.6 and 3.8) from cladistic analysis based on morphology and the previous classification of Butcher (1984). This study showed that morphometric analysis was unable to produce informative results about the relationships among *Holcaspis* species. It is probable that the morphometric analysis, based only on characters which are related to overall body size, does not reflect phylogenetic relationships. There is strong evidence that body size is influenced by ecological parameters (Andersen 1985). It is suggested that trying to determine the relationships among species from morphometric analysis is difficult because it is subject to size-related changes rather than their inheritance. In addition, the incongruence between the phenogram and cladograms is due to the fact that the characters traditionally used in *Holcaspis* species identification are based on qualitative features rather than quantitative ones.

Table 3.4 Data matrix of Mahalanobis distances (D^2) of 21 *Holcaspis* species from the first discriminant analysis.

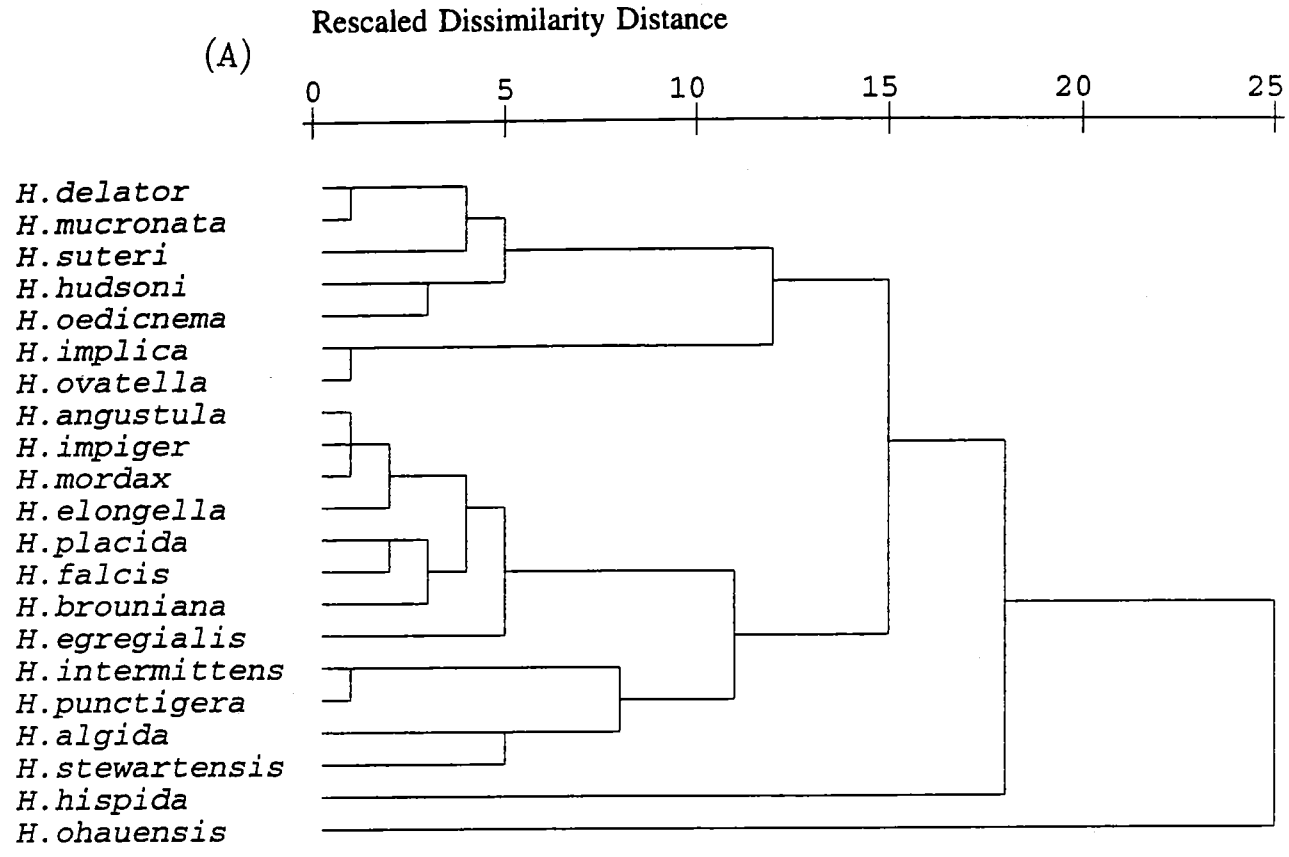
	Species																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *H. brouniana*	0																				
2 *H. ohauensis*	153	0																			
3 *H. angustula*	30	97	0																		
4 *H. placida*	38	87	18	0																	
5 *H. delator*	43	181	57	99	0																
6 *H. egregialis*	47	110	30	33	84	0															
7 *H. elongella*	28	103	23	23	46	41	0														
8 *H. hispida*	117	245	106	144	71	145	94	0													
9 *H. hudsoni*	91	299	126	190	36	158	115	158	0												
10 *H. impiger*	29	94	15	22	51	30	19	118	112	0											
11 *H. implica*	45	97	27	41	33	41	17	84	93	18	0										
12 *H. intermittens*	82	308	125	182	24	161	109	128	15	111	91	0									
13 *H. mordax*	50	61	20	23	63	37	24	99	159	16	151	93	0								
14 *H. mucronata*	60	232	91	130	13	124	65	92	27	81	15	101	40	0							
15 *H. oedicnema*	102	279	112	185	61	157	124	186	29	130	59	171	64	41	0						
16 *H. falcis*	21	101	24	19	75	41	38	121	154	27	164	28	101	158	94	0					
17 *H. ovatella*	86	290	124	195	338	166	117	138	16	119	16	158	35	39	135	25	0				
18 *H. algida*	47	112	52	45	103	62	48	156	165	42	169	47	125	183	38	154	107	0			
19 *H. punctigera*	43	77	24	32	66	41	35	113	149	14	137	14	107	170	31	135	38	72	0		
20 *H. stewartensis*	62	119	43	63	44	67	45	66	123	37	98	39	63	148	47	116	90	40	75	0	
21 *H. suteri*	53	189	69	120	22	94	70	118	30	64	51	38	93	40	41	94	25	107	72	75	0

Table 3.5 Data matrix of Mahalanobis distances (D^2) of 21 *Holcaspis* species from the second discriminant analysis.

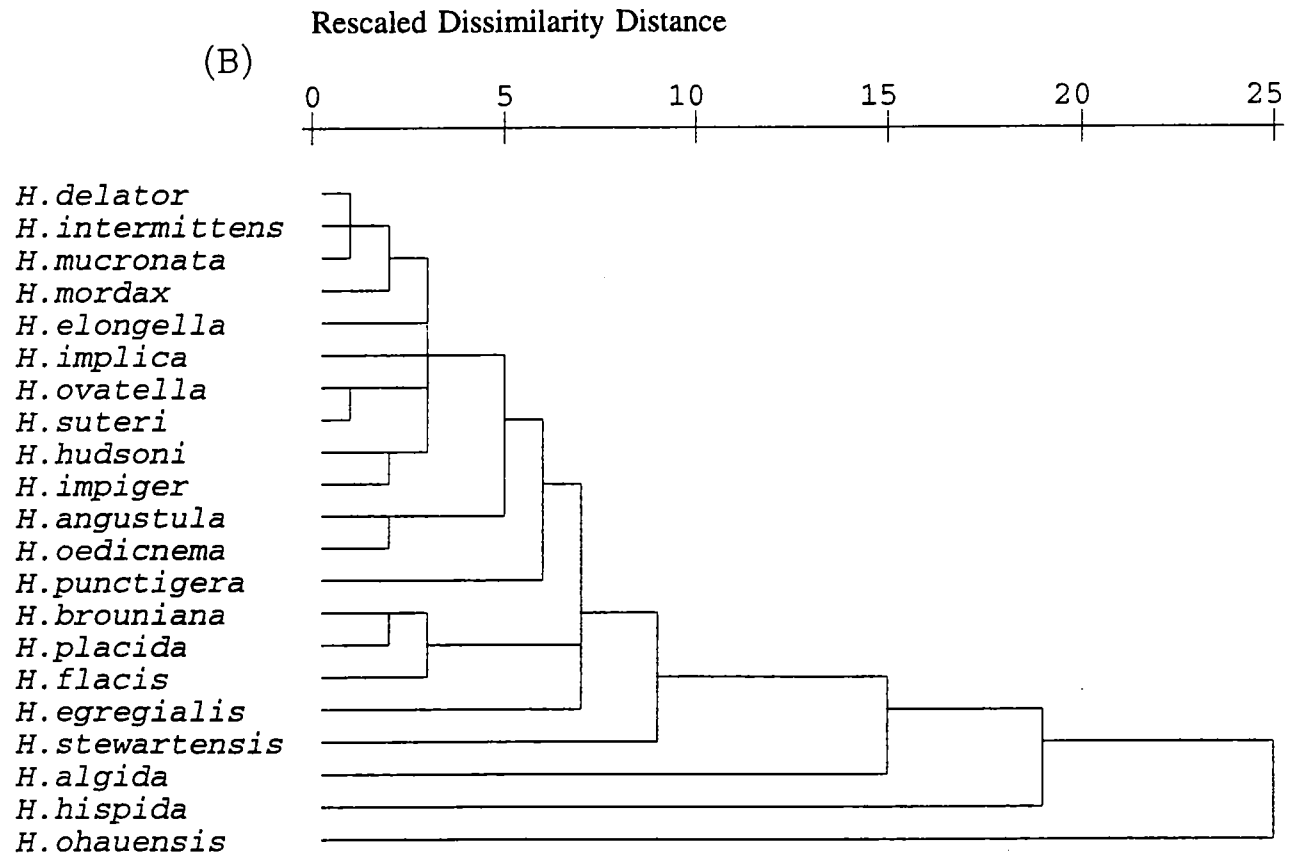
	Species																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *H. brouniana*	0																				
2 *H. ohauensis*	158	0																			
3 *H. angustula*	26	128	0																		
4 *H. placida*	13	154	18	0																	
5 *H. delator*	29	117	24	33	0																
6 *H. egregialis*	34	149	31	38	36	0															
7 *H. elongella*	25	141	31	25	14	45	0														
8 *H. hispida*	114	237	97	113	66	125	80	0													
9 *H. hudsoni*	32	117	21	33	13	33	17	108	0												
10 *H. impiger*	25	126	19	22	20	33	27	110	12	0											
11 *H. implica*	48	106	33	36	16	41	20	80	20	23	0										
12 *H. intermittens*	24	139	25	26	5	38	13	85	11	13	20	0									
13 *H. mordax*	35	97	24	34	6	43	27	67	18	20	16	14	0								
14 *H. mucronata*	33	128	34	30	8	48	10	75	15	24	14	7	13	0							
15 *H. oedicnema*	45	106	15	38	33	38	33	133	17	34	37	38	36	42	0						
16 *H. falcis*	16	139	34	19	43	47	50	112	49	36	49	44	33	43	62	0					
17 *H. ovatella*	27	110	20	37	10	38	18	91	11	18	27	11	16	22	24	49	0				
18 *H. algida*	61	180	89	77	89	95	83	165	74	73	91	79	77	83	101	62	59	0			
19 *H. punctigera*	38	111	33	45	27	50	48	100	33	21	37	26	20	42	59	43	19	64	0		
20 *H. stewartensis*	67	122	51	54	35	67	51	65	57	43	22	39	31	35	84	50	53	120	42	0	
21 *H. suteri*	32	95	20	36	16	30	27	103	14	19	25	21	19	32	22	49	10	78	16	56	0

Figure 3.11 The phenograms generated from the Mahalanobis distances (D^2) of 21 *Holcaspis* species using cluster analysis with unweighted pair group method with arithmetic averages (UPGMA): **(A)** from the first discrimination analysis, **(B)** from the second discrimination analysis.

(A)


Rescaled Dissimilarity Distance
0
5
10
15
20
25
H.delator
H.mucronata
H.suteri
H.hudsoni
H.oedicnema
H.implica
H.ovatella
H.angustula
H.impiger
H.mordax
H.elongella
H.placida
H.falcis
H.brouniana
H.egregialis
H.intermittens
H.punctigera
H.algida
H.stewartensis
H.hispida
H.ohauensis

(B)


Rescaled Dissimilarity Distance
0
5
10
15
20
25
H.delator
H.intermittens
H.mucronata
H.mordax
H.elongella
H.implica
H.ovatella
H.suteri
H.hudsoni
H.impiger
H.angustula
H.oedicnema
H.punctigera
H.brouniana
H.placida
H.flacis
H.egregialis
H.stewartensis
H.algida
H.hispida
H.ohauensis

However, the results of the discriminant function analysis showed that the morphometric measurements of *Holcaspis* species allowed a high probability of correctly classifying individuals belonging to these species. The selected measurements (18 characters: Table 3.3) were therefore reliable taxonomic characters.

It is concluded that the morphometric approach that was applied to *Holcaspis* species can be used as a rapid indicator to species identification but it fails to adequately reflect the species phylogenetic relationships.

3.4 Summary

Cladistic and morphometric methods were applied to the genus *Holcaspis* using morphological characters of adult beetles. The cladistic method using a three different species outgroup and the hypothetical ancestor as an outgroup resulted in similar topologies. The cladistic results showed highly congruent relationships with the previous species grouping of Butcher (1984). In contrast, the morphometric approach was unable to reflect the hypothesized phylogenetic relationships among *Holcaspis* species. However, the morphometric analysis which yielded similar results for males and females, was found to be a powerful method for identifying *Holcaspis* species.

CHAPTER 4

Holcaspis relationships inferred from allozyme variation

4.1 Introduction

Since the enzyme electrophoretic technique was developed, it has been increasingly used to provide useful information in a wide range of biological situations. This chapter focuses on the application of the enzyme electrophoresis technique for systematic studies as part of an investigation of the relationships of the genus *Holcaspis* using both phenetic and cladistic methods. It also includes a short review of the principles involved in the application of the enzyme electrophoresis technique.

4.2 Literature review

4.2.1 Electrophoresis and systematics

The advantages of electrophoretic techniques in different aspects of biology are well known and have been discussed by many authors. For example, Thorpe (1983) suggested that electrophoretic techniques could be usefully applied to solve many problems in agricultural entomology, such as identifying the immature stages of different species when it is not possible to use morphological characters. Powell and Walton (1989) pointed out that success in biological control and integrated pest management programmes depends on recognition, not only of the target pest, but also of the natural enemies. Electrophoresis can be applied to monitor the presence and population levels of natural enemies in pest populations. Electrophoresis can also be used in ecological studies of predator/prey relationships (Murray *et al.* 1989). Bartlett (1981) and Thorpe (1983) suggested that, in mass reared laboratory populations of insects, electrophoretic techniques are useful for monitoring genetic changes. Electrophoresis is also an appropriate method for detecting insecticide resistance in individual insects and monitoring accurately changes in

resistant gene frequency (Devonshire 1989).

For systematic purposes, the value of electrophoresis as a tool is being increasingly recognised by taxonomists and the field has been reviewed by many authors (Avise 1975; Ferguson 1980; Thorpe 1983; Menken and Ulenberg 1987; Powell and Walton 1989). The use of electrophoresis as a source of characters for systematic studies has been considered for many years (Avise 1983; Richardson *et al.* 1986). Any characters that are valuable as taxonomic characters should satisfy two important criteria: the character should be genetically stable and not vary with environmental conditions; and, secondly, each character should be independent of other taxonomic characters used. Electrophoresis satisfies both criteria (Richardson *et al.* 1986). In many instances, electrophoretic data have contributed greatly to insect systematics. Electrophoresis can be a powerful aid to discriminate between and identify taxa at all levels and to produce a hierarchical classification of taxa (Berlocher 1984; Menken and Ulenberg 1987; Brown 1990). This ability is based on the fact that taxa at different levels of systematic divergence will have different probabilities of genetic identity (Thorpe 1983). Thorpe (1979) suggested that, in conspecific populations, the probability of genetic identity would be above about 0.9, but between congeneric species or species from different but related genera, the genetic identity probability would be between about 0.25 and 0.85. Populations and individuals can also be differentiated by electrophoresis. Furthermore, electrophoresis can overcome the problems of intraspecific variation when variation is present within species. Various factors cause variation within species such as genetic variation, age, sex, nutritional condition, and environment (Ferguson 1980; Thorpe 1983; Richardson *et al.* 1986). Even though these factors influence electrophoretic studies of protein only the genetic variation factor, which is qualitative (protein mobility), is valuable in systematic studies. In contrast, the non-genetic changes, like environmental factors, cause quantitative variation, which affects the amount of a particular protein rather than in its mobility. This kind of variation is not significant in systematics (Ferguson 1980) and can be ignored.

An additional advantage of electrophoretic data over conventional morphological data is the ability to clarify and distinguish between ambiguous species where the only morphological characters are nearly, or completely, indistinguishable, such as in sibling species, cryptic species and species complexes (Thorpe 1983; Hillis 1987). Avise (1975)

pointed out that closely related species are likely to share homologous proteins differing in amino acid sequence, which can produce differences in the net charge of proteins and so mobility. Thus, by using electrophoresis, it is possible to detect dissimilar proteins.

In general, individuals comprising a species are capable of interbreeding to produce viable, fertile offspring but, due to geographical barriers, they may not have chance to do so. However, individuals of one species are reproductively isolated from individuals of all other species. It is often quite difficult to decide whether two geographically isolated populations belong to the same or to different, allopatric species. Two isolated populations may have morphological similarity even though they live in mutually exclusive geographical areas. In contrast, two populations that are sympatric may be separate species. When such species are morphologically distinct, they can be recognised as different species. Often, however, sympatric species are not obviously distinguishable at the morphological level. Enzyme electrophoresis can be a most powerful tool to clarify the taxonomic status of dubious species that occur sympatrically or allopatrically. When sympatric populations are compared by electrophoresis, allele frequencies are used as an indicator to distinguish between populations. If such populations have different frequencies at a series of loci, or especially if they have fixed allelic differences, then clearly they are reproductively isolated (Thorpe 1983; Richardson *et al.* 1986). With allopatric populations, the populations accumulate genetic changes slowly over a long time as a result of environmental influences. Interpretation of allelic differences is not clear cut because reproductive isolation of allopatric populations may not be completely distinguishable from the effect of physical isolation (Bush and Hoy 1984; Ferguson 1988). However, the determination of taxonomic status for allopatric populations can be done using genetic distances from closely related taxa (Ayala 1983; Thorpe 1983).

Electrophoresis is useful not only for identifying and discriminating between species but also for determining the degree of relationship among species and other taxonomic levels, as well as for analysis of systematic relationships (Prager and Wilson 1978; Baverstock *et al.* 1979; Ferguson 1980; Buth 1984; Menken and Ulenberg 1987; Murphy 1993).

Traditionally, the assessment of species relationships has been based mainly on morphological characters. However, the determination of species relationships from

macromolecular data such as isozyme electrophoresis is increasingly being practised (Prager and Wilson 1978). Nevertheless, there is a divergence of opinion on the treatment of enzyme electrophoresis data for studies of systematic relationships. Basically, two preferred treatments of electrophoresis data are used. These treatments are distance data and character state data (Buth 1984; Murphy 1993). From distance data, genetic similarity or distance coefficients are calculated from allele frequencies at various loci and then can be clustered by several clustering algorithms such as the unweighted pair group method (UPGMA), the F.M. procedure (Fitch and Margoliash 1967) and the Wagner distance method (Farris 1972). The other approach, character state data, can be used with both allele and locus coded as characters (Mickevich and Mitter 1981; Buth 1984). However, the controversy over coding electrophoresis data as independent alleles (presence/absence) or loci still continues (Mickevich and Mitter 1981; Buth 1984; Swofford and Berlocher 1987; Murphy 1993).

4.2.2 The electrophoresis technique

The practical details of electrophoresis as well as the theoretical background have been discussed by many authors such as Brewer and Sing (1970), Harris and Hopkinson (1976), Richardson *et al.* (1986) and Pasteur *et al.* (1988).

The principles of electrophoresis have been known since the end of the nineteenth century. In general, the term electrophoresis may be defined as the migration of particles in a supporting medium, such as starch agar, cellulose acetate or polyacrylamide gel, under the influence of an electric field (Ferguson 1980; Richardson *et al.* 1986). Electrophoresis was developed as an analytical technique for chemical and biological research. It has been used with considerable success on a number of different groups of animals (Ferguson 1980; Richardson *et al.* 1986). Electrophoresis is able to separate all types of charged compounds. One of the most widespread uses of the technique is to separate of proteins that contain both acidic and basic groups. Proteins consist of one or more polypeptide chains or subunits. Each polypeptide chain is composed of a series of amino acids joined together by covalent or peptide bonds. The order and nature of these amino acids determines the primary structure of a given polypeptide. The side chains of

amino acids that make up a protein molecule may have charged acidic (COO^-) or basic (NH^+) groups. The protein may carry a nett negative charge, a nett positive charge or no nett charge. The charge on protein molecules causes them to migrate in an electric field towards the oppositely charged terminal. The amino acid sequence is directly dependent on the information contained in the DNA (deoxyribonucleic acid) of the gene that codes for the particular polypeptide (Ferguson 1980; Pasteur *et al.* 1988).

4.2.3 Isozyme and allozyme electrophoresis

Electrophoresis has been used extensively to solve the problem of inter- and intraspecific variation of organisms using enzymes (which are one sort of protein), particularly isozymes (Market and Moller 1959, cited by Ferguson 1988), and allozymes (Prakash *et al.* 1969, cited by Buth 1984). Isozymes or isoenzymes are multiple molecular forms of an enzyme occurring within a single species because of the presence of more than one structural gene (genes code for proteins). Such multiple genes may be due to multiple gene loci or multiple alleles at a single locus. An allozyme is a variant protein produced by multiple alleles of the same locus. In other words, an allozyme is a subset of isozymes. Insects and plants have considerably higher amounts of allozyme variation among and within taxonomic groups than vertebrates, which tend to have relatively low levels (Menken and Ulenberg 1987).

The electrophoretic patterns (or bands) of isozymes and allozymes are very useful and valuable characteristics in systematics, and in the population and evolutionary genetics of organisms. The band patterns are an expression of the genes of the organism. Electrophoretic band patterns can be interpreted in different ways, such as the number of bands, presence or absence of bands, the density and width of bands as well as their position and relative mobility. However, the qualitative character of relatively mobility of banding has proved to be highly useful in determining taxonomic relationships. As differences in enzyme electrophoretic mobility are the result of proteins that were encoded by segments of DNA differing in at least one base pair, electrophoretic band mobility of protein provides indirect information about the DNA or gene (Anderson *et al.* 1979; Richardson *et al.* 1986).

4.2.4 The detection of banding patterns

Since most proteins are colourless, in order to identify the position of the protein products for a particular locus, specific stains have been developed. Staining can be carried out either by using a non-specific dye such as amino black or coomassie brilliant blue, which are general protein stains, or by more specific stains.

Each enzyme catalyses a specific reaction and breaks down a specific substrate. This property can be used to detect an enzyme's position after electrophoresis. For example, the enzyme being stained may convert the substrate into a visible product. Alternatively, the substrate can be converted into a product that is not visible itself, but can be made visible by the addition of other histochemicals. Enzymes can also be detected by coupling the reaction. In this case, the appropriate linking enzymes are added to the staining reagents to convert the first product into a second product. It can then be made visible by using additional histochemicals. The coloured bands appear on the gel wherever the product is formed by the action of the relevant enzyme. The techniques and recipes for staining in enzyme electrophoresis are well presented by many authors such as Harris and Hopkinson (1976), Richardson *et al.* (1986) and Pasteur *et al.* (1988).

4.2.5 Cellulose acetate electrophoresis

A variety of supporting media have been employed for enzyme separation. Differences in the natural properties of supporting media may cause differences in the resolution of electrophoretic patterns. There is no single medium that is routinely superior to all others for isozyme electrophoresis; each has its own particular advantages and disadvantages.

Cellulose acetate or cellogel for electrophoresis has been available commercially for a number of years. The gel is available in a ready-to-use form. It is supplied as strips from 25 mm to 150 mm wide and up to 200 mm long. The gel has a porous texture so it can separate protein particles by differences in the net charge but with little or no separation due to molecular weight or size differences (Harris and Hopkinson 1976; Richardson *et al.* 1986). The advantages, disadvantages and the techniques of cellulose

acetate electrophoresis have been discussed thoroughly by Richardson *et al.* (1986).

For cellulose acetate electrophoresis, the amount of sample required is a very small; 0.5-2.0 µl of sample is used for each analysis. This is particularly useful for electrophoresis that involves only small samples, e.g., from microorganisms or single individuals of larger organisms. It is also useful for detection of a large number of enzymes within a single sample. Only a small volume, 50-100 ml for each staining, of the staining solution is required. Cellulose acetate electrophoresis is very quick with each run taking 30-120 min.

Electrophoresis produces heat in proportion to the power (volts x amperes) applied to the gel. If too much power is applied, excess heat will cause enzyme denaturation. With cellulose acetate electrophoresis, only low voltages are required and this avoids the problem of protein denaturation. Some criticisms of the technique have also been made. The main disadvantage is that the ability to separate protein is less than with other supporting media like polyacryamide gel. There would appear to be more advantages to cellulose acetate electrophoresis than disadvantages (Harris and Hopkinson 1976; Richardson *et al.* 1986).

4.2.6 Allozyme electrophoresis as applied to *Holcaspis* species

Electrophoresis can assess genetic diversity and help to define intra- and interspecific genetic variability. It has also been useful in resolving taxonomic problems in many insect groups. Electrophoresis has not been carried out before on the genus *Holcaspis*. However, a large number of electrophoretic analyses have been done on many other species of Coleoptera for both intra- and interspecific systematics (Menken and Ulenberg 1987).

The objective of this part of the study was to investigate the relationships of *Holcaspis* species with both qualitative and quantitative electrophoretic data, using cladistic and phenetic analysis.

4.3 Materials and methods

4.3.1 Specimens

The 13 *Holcaspis* species used in this study were field collected from various locations. The localities are shown in Figure 4.1.

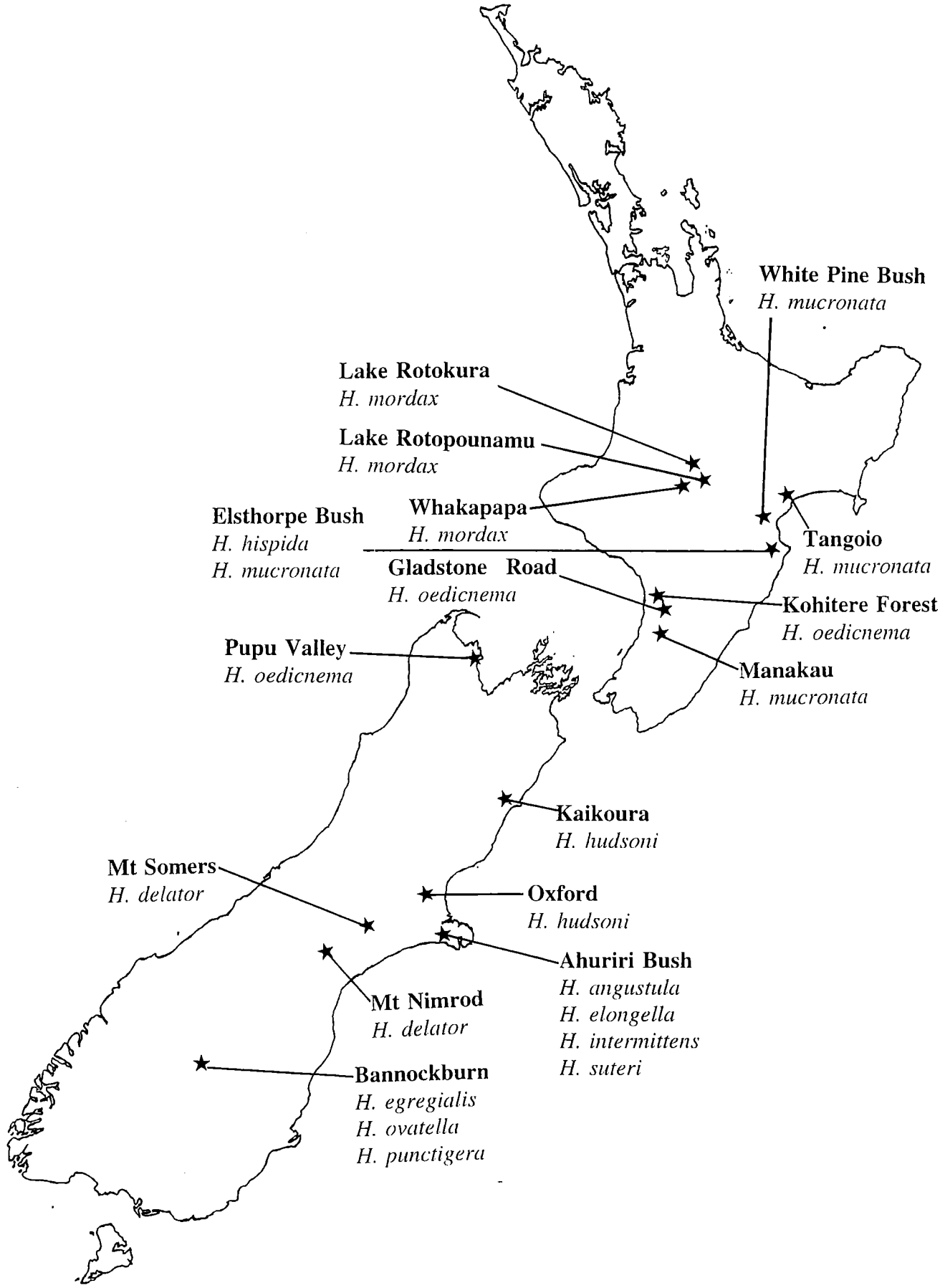
4.3.2 Sample preparation

Each individual was sexed and was identified using Butcher's (1984) key. Identities were confirmed by the specialists M.R. Butcher and J.I. Townsend. To avoid contamination with enzymes from their food, specimens were starved for 3 days in an incubator at a temperature of 15°C. After starvation, each specimen was placed in an Eppendorf tube and quick frozen in liquid nitrogen. The specimens were then kept in a deepfreeze at -80°C until used in the electrophoresis experiments.

4.3.3 Preparation of extracts for electrophoresis

After the specimens were taken from the deepfreeze, the intestinal tracts were removed from the specimens and put separately in a chilled block. Liquid nitrogen was then poured over the intestines and they were individually ground up with a glass tissue grinder in 30 µl of cold, newly prepared, extraction solution (0.01 M tris-hydroxymethyl aminomethane pH 7.00, 0.01 mM ethylene diaminetetra acetic acid, 0.1 % triton X, 0.1 % xylene, 0.1 % mercaptoethanol). The homogenate was centrifuged for 5 min at 10,000*g* at 4°C. The clear supernatant was transferred to an Eppendorf tube. The supernatant from each individual was divided into equal volumes of about 5 µl. The divided supernatant was stored in separate Eppendorf tubes. The advantage of separating the supernatant into small volumes is that enzyme denaturing by freezing and thawing the whole sample many times is avoided. The supernatant could be used immediately or quick frozen in liquid nitrogen and stored at -80°C in a deep freezer.

Figure 4.1 Collection sites of the 13 *Holcaspis* species


White Pine Bush
H. mucronata
Lake Rotokura
H. mordax
Lake Rotopounamu
H. mordax
Whakapapa
H. mordax
Elsthorpe Bush
H. hispida
H. mucronata
Tangoio
H. mucronata
Gladstone Road
H. oedicnema
Kohitere Forest
H. oedicnema
Pupu Valley
H. oedicnema
Manakau
H. mucronata
Kaikoura
H. hudsoni
Mt Somers
H. delator
Oxford
H. hudsoni
Ahuriri Bush
H. angustula
H. elongella
H. intermittens
H. suteri
Mt Nimrod
H. delator
Bannockburn
H. egregialis
H. ovatella
H. punctigera

4.3.4 Electrophoresis system

Electrophoresis was conducted using cellulose acetate. The cellulose acetate sheets (Helena Laboratories, Beaumont, Texas) were 60 x 76 mm (Titan III. Zip Zone, cat No. 3024). The Helena applicator kit, including two loading bases, with a row of eight sample wells, an applicator with eight capillary tips and a plate alignment base (Super Z base cat. No. 4086) was used.

The electrophoresis chamber, which was modified from a Helena chamber, consisted of two compartments with two electrodes in each compartment. A model 1000/500 Bio-Rad power supply was used because this power supply has two output terminals, allowing two gels to be run simultaneously.

4.3.4.1 Buffer systems and running conditions

Four buffer systems were used (see Table 4.1). Electrophoresis was carried out in refrigerator at 4 °C . The running time and electric current varied depending on the buffer used.

4.3.4.2 Electrophoresis procedure

Procedures for cellulose acetate electrophoresis were adapted from those recommended by Helena Laboratories, Beaumout, Texas. They were as follows:

1) Fifty millilitres of running buffer were poured into each of the outer compartments of the electrophoresis chamber. Two disposable paper wicks (filter paper Wat. No. 3, size 150 x 30 mm) were moistened with buffer, then draped over each support bridge.
2) The cellulose acetate plate was presoaked in the running buffer tank for 20 min until it was completely saturated.
3) A 5 µl of sample of supernatant was transferred to the well of a Zip Zone well plate with a micropipette. A maximum of eight samples can be placed in this plate. The cellulose acetate plates were then removed from the buffer tank,

blotted and aligned on the Super Z Aligning Base.

4) The Super Z Applicator was loaded by depressing the tips into the sample wells 3 or 4 times. About 0.5 µl of the supernatant were then transferred from the well plate to the cellulose acetate plate with the applicator.
5) The cellulose acetate plate was quickly placed, cellulose acetate side down, on the two wicks in electrophoresis chamber. The sample was at the cathode end. A cold metal plate was placed on top of the cellulose acetate plate. Ice cubes also were placed in the centre compartment to keep the chamber cool. The lid was fitted firmly. The electrophoresis chamber then was placed in a refrigerator at 4°C to keep it cool. The Bio-Rad power supply was set for the appropriate voltage and time before turning on the power supply.

4.3.5 Enzyme Staining

After electrophoresis, the cellulose acetate plate was removed from the chamber and placed in a specific substrate stain, staining procedures and recipes were modified from Shaw and Prasad (1970), Steiner and Joslyn (1979), Vallejos (1983), Richardson et al. (1986) and Pasteur *et al.* (1988). The plate was incubated in the staining solution at 37°C until the bands, which indicate enzyme activity, had been stained enough to be observed (about 15-20 min for most of the enzymes in this study). The plate was then removed from the reaction staining mixture, placed in destaining solution (water:methanol:acetic acid, 5:5:1) to stop the reaction, washed in water, and air dried.

For this study, an initial screening of enzyme systems from each species was carried out. The selected enzyme systems (Table 4.1) were limited to those enzymes that gave distinctive banding patterns in all species.

4.3.6 Data recording and analysis

To record the banding patterns, photographs were taken immediately after they appeared. Bands were also traced with tracing paper to record the patterns.

Table 4.1 The selected *Holcaspis* species enzymes with their Enzyme Commission (E.C.) classification numbers, buffer used for electrophoresis and the number of loci resolved for each enzyme studied.

Enzyme	E.C. number	Buffer	Number of loci
Acid phosphatase	3.1.3.2	Tris-citrate pH 7.0 (modified from Richardson *et al.* 1986)	2
Aldehyde dehydrogenase	1.2.1.5	Citrate-N-3 amino propyl morpholine pH 7.5 (modified from Easteal and Boussy 1987)	1
Aldehyde oxidase	1.2.3.1	Citrate-N-3 amino propyl morpholine pH 7.5	1
Adenylate kinase	2.7.4.3	Phosphate pH 7.0 (modified from Richardson *et al.* 1986)	1
Hexokinase	2.7.1.1	Citrate-N-3 amino propyl morpholine pH 7.5	2
Leucine aminopeptidase	3.4.11.1	Borate pH 8.0 (modified from Liebherr 1983)	2
Phosphoglucomutase	5.4.2.2	Phosphate pH 7.0	1
6-Phosphogluconate dehydrogenase	1.1.1.44	Phosphate pH 7.0	1
Glucose phosphate isomerase	5.3.1.9	Phosphate pH 7.0	1
Malic enzyme	1.1.1.40	Citrate-N-3 amino propyl morpholine pH 7.5	1

To express the position of a certain band, the mobility of bands was determined using the Relative Mobility Index (RMI) of Zurwerra *et al.* (1986). They suggested that some variation in the mobilities of band patterns could be reduced by using the Relative Mobility Index. Therefore, in order to minimize variation in comparing alleles from different gels in this study, the Relative Mobility Index was used as follows:

$$10[(M_i - M_{ref}) \div M_{ref}] + 100$$

Where M_i and M_{ref} are the distances (measured from the origin to top of the relevant band) of the sample and reference bands in millimetres. For this study, the bands from *Megadromus antarcticus* were used as the reference bands.

According to the relative mobility of alleles, different alleles at each locus were coded with an alphabetical designation. The most anodal allele was named as 'a', followed by 'b', 'c', etc., in order of electrophoretic relative mobility. In case of multiple loci, the loci were numbered sequentially from anode to cathode. Enzyme nomenclature is taken from the International Union of Biochemistry (Anon. 1978), and as suggested by Richardson *et al.* (1986). Table 4.1 presents the enzymes selected, their Enzyme Commission classification numbers, and the buffers used during electrophoresis. As the purpose of the work was an interspecific study, for every species, the data obtained from samples from different localities were lumped and the allelic frequencies found at every locus from each species were calculated.

The appropriate treatment of allozyme data is still being discussed, for both the phenetic and cladistic approaches (Buth 1984). Therefore, in this study, both cladistic and phenetic analyses were employed for comparative purposes.

4.3.6.1 Phenetic analysis

For phenetic analysis, allelic frequency data were converted into a measure of genetic distance among species. The genetic similarity (identity) between *Holcaspis* species was computed from allelic frequency data using Nei's similarity index (I) (Nei 1978). Nei's similarity index estimates the proportion of genes that are identical in

structure in two populations and ranges from 0 to 1. When I = 0, no alleles are in common, the two species are completely genetically different. When I = 1, the same alleles are in the same frequencies in both species, i.e., they are genetically identical.

Nei's (1972) index of genetic distance (D), which estimates the number of allelic substitutions per locus that have occurred in the separate evolution of two species, was also measured. The mean value of D can range from zero, which means there are no genetic differences between two species, to infinity. The D value is equal to the negative logarithm of I, (D = -Ln I). Thus a large value for I corresponds to a small value for D and *vice versa.*

Cluster analysis was performed on the genetic distance matrix, using the unweighted pair group method with arithmetic averages (UPGMA). This cluster analysis, performed with the SPSS computer package, generated a phenogram based on genetic distance values.

4.3.6.2 Cladistic analysis

The cladistic approach assesses relationships based on the possession of synapomorphic (shared, evolutionarily derived) characters, rather than on overall similarity. For this analysis, allelic frequency data were coded as character states. Both alleles and loci were treated as characters. The data were analyzed in two ways:

1) The independent allele model of Mickevich and Mitter (1981) was employed. The model uses a coding scheme in which each allelic entity is recognized as a separate character. Two character states, presence or absence, are possible. The allele was coded as 'present' if its frequency was 0.05 or greater, and 'absent' if its frequency was less than 0.05. The data matrix of character state 'presence/absence' was transformed into the binary numbers '1/0'.
2) Each locus, which includes a number of combinations of alleles, was treated as character state. The multistate coding of the locus, which was based on the entire allelic array, was applied and a chi square test was used to test whether the allele frequencies were significantly different between species. This coding was considered as a coarser grained matrix (Buth 1984).

In this analysis, phylogenetic relationships among *Holcaspis* species were inferred under the principle of maximum parsimony using the PAUP program (Swofford 1992) and *M. antarcticus* was used as an outgroup. The data matrices from both allele and locus character states were treated as unordered and the heuristic search option was employed to find the shortest phylogenetic tree.

4.4 Results and discussion

4.4.1 Selection of enzyme systems for allozyme study of *Holcaspis* species

Twenty enzyme systems were initially screened. Of these, six enzymes (aconitate hydratase, fumarate hydratase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and xanthine dehydrogenase) were rejected because the banding patterns showed poor resolution after staining. Both esterases and general protein gave good electrophoretic resolution but unreliable interpretation. This was because they produced too many bands. Therefore, to avoid the uncertainties associated with the interpretation of banding pattern, esterases and general protein were discarded. Two other enzyme systems (diaphorase and isocitrate dehydrogenase) could not be consistently scored in all specimens and were also rejected. The analysis was therefore limited to 10 enzyme systems that gave consistent interpretable banding patterns and also provided high electrophoretic resolution, as shown in Table 4.1. Thirteen presumptive loci, with a total 42 alleles were resolved.

4.4.2 Genetic variation within the genus *Holcaspis*

Table 4.2 records the mean number of alleles per locus (A) and the mean proportion of polymorphic loci per species (P), together with the observed heterozygosity (H). The mean proportion of polymorphic loci per species in *Holcaspis* species was 0.349 ± 0.131. This level of polymorphism corresponds well with Nevo's (1978) review of genetic polymorphism in insects. He reported that in insects (excluding *Drosophila*) the mean proportion of polymorphism was 0.329 ± 0.203. However, Liebherr (1986) reported the

Table 4.2 Genetic variability at 13 polymorphic loci in 13 species of *Holcaspis*

Species	Mean number of alleles per locus (A)	Mean polymorphisms per species (P)	Observed heterozygosity per individual (H)
H. angustula	1.230	0.153	0.037
H. delator	1.153	0.154	0.030
H. egregialis	1.384	0.385	0.159
H. elongella	1.307	0.307	0.111
H. hudsoni	1.538	0.538	0.158
H. intermittens	1.307	0.308	0.154
H. mordax	1.384	0.385	0.112
H. mucronata	1.461	0.462	0.131
H. oedicnema	1.384	0.385	0.207
H. ovatella	1.538	0.538	0.174
H. punctigera	1.230	0.231	0.059
H. suteri	1.538	0.538	0.153
H. hispida	1.230	0.231	0.091
Mean ± S.D.	1.378 ± 0.126	0.349 ± 0.131	0.119 ± 0.051

mean proportion of polymorphism in several species of *Agonum* (Carabidae) was 0.46-0.60.

As can be seen in Table 4.2, the mean observed heterozygosity in *Holcaspis* species was 0.119 ± 0.051. Nevo (1978) reported that the level of heterozygosity in insects averaged 0.074 ± 0.08. *Holcaspis delator* and *H. angustula* showed the lowest degree of polymorphism (0.154 and 0.153, respectively) and lowest average observed heterozygosity (0.030 and 0.037, respectively); *H. oedicnema* had the highest observed heterozygosity of 0.207. Liebherr (1986) reported the levels of heterozygosity in several *Agonum* species. These varied from *Agonum extensicolle* (Say) with an observed heterozygosity of 0.114-0.218 to *A. decorum* (Say) whose heterozygosity ranged from 0.218 to 0.278. Unlike the *Holcaspis* species, these two *Agonum* species are widespread flighted species, therefore

migration among populations and exchanging gene flow-variation can produce the high genetic variation. Avise and Selander (1972) reported heterozygosity of 0.218 in two populations of the carabid *Rhadine subterranea* (Van Dyke). Both *Agonum* and *Rhadine* belong to the tribe Platynini, a possible sister group to the Pterostichini. From this it can be seen that the average level of polymorphism and heterozygosity in *Holcaspis* species corresponded well with that reported from insects in general but was relatively low, except for *H. oedicnema* in comparison with other Carabidae where these parameters have been measured.

The low level of heterozygosity reported in this study may be partially due to the limited number of loci investigated and the relatively narrow range of sample sites. Almost all species examined came from single population samples. In the case of multiple samples, most sites were not far away from each other. Therefore these populations tended to be representative of specific localities. Moreover, *Holcaspis* species are flightless. Therefore they naturally would have relatively narrow geographic limits. Nevo (1978) stated that species that occurred in broad niches or were habitat generalist species, tended to show relatively high heterozygosity in contrast to the nearly homozygous patterns expected in narrow-niche or habitat specialist species. The results also showed that *H. oedicnema* has high heterozygosity of 0.207 in comparison with the other *Holcaspis* species. This species was represented by three populations, two of these were geographically close to each other in the south of North Island, near Levin. The other population came from the north of South Island, near Nelson. These two localities are isolated from one other, and the populations may have gradually become genetically differentiated. However, it is not necessarily a sufficient condition for species differentiation. Various genetic races can occur in some species (Ayala, 1975). Similar to its high heterozygosity, *H. oedicnema* showed the most morphological diversity among all *Holcaspis* species. This morphological diversity was also commented on by Butcher (1984). The allele frequencies of each species found at every locus are listed in Table 4.3 and the degree of genetic differentiation among the 13 *Holcaspis* species, as measured by Nei's indices of genetic similarity (I) and genetic distance (D), are presented in Table 4.4. The average genetic differentiation among all species of *Holcaspis* is 0.382 ± 0.142 and 1.055 ± 0.143 for I and D respectively. *Holcaspis mordax* and *H. punctigera* were

remotely related to the other 11 *Holcaspis* species with an average genetic distance of 1.294 ± 0.298 and 1.599 ± 0.527 respectively. The low level of genetic similarity and high level of genetic distance among the *Holcaspis* species is a reflection of the fact that any populations that belong to separate species have generally been shown to be genetically more different than congeneric populations (Ferguson 1988). Hsioa (1989) noted that the genetic distance, which measures genetic dissimilarity, is usually relatively large between species of Coleoptera. It may be inferred that there is much more genetic differentiation in Coleoptera above the species level than at the intraspecific level.

4.4.3 Phenetic analysis

The phenetic association of all 13 *Holcaspis* species by UPGMA clustering analysis of genetic distance data from Table 4.4 is presented in Figure 4.2. *Holcaspis mordax* and *H. punctigera* were clustered together and divergent from the rest of the group. Next to *H. punctigera* and *H. mordax*, were two sister groups. One was *H. oedicnema* and *H. delator* cluster together, followed by *H. hudsoni*, *H. mucronata* and *H. ovatella.* The other included the remainder of the *Holcaspis* species and was itself split into two groups. *Holcaspis angustula* clustered with the two species *H. egregialis* and *H. elongella.* Then *H. intermittens* and *H. hispida* were grouped together, followed by *H. suteri.* These results can be compared with the cladistic analysis.

4.4.4 Cladistic analysis

Cladograms using alleles as independent characters were constructed from the data matrix of allele character states (Table 4.5) using the maximum parsimony method. Two equally parsimonious trees, which were rooted using *M. antarcticus* as an outgroup, were obtained by the PAUP analysis as shown in Figure 4.3. When loci were coded as characters, a single most parsimonious tree was generated (Figure 4.4) from the data matrix shown in Table 4.6. The production of a single most parsimonious tree in this study, using loci as characters, would appeared to be the best fit cladogram to estimate the phylogenetic relationships of *Holcaspis* species. Moreover, the phylogenetic tree

Table 4.3 Allele frequencies of *Holcaspis* species at 13 presumptive loci in each species. Species sharing the same letter at a locus displayed alleles with the same electrophoretic mobility. (Species (1)=*Megadromus antarcticus*, (2)=*Holcaspis oedicnema*, (3)=*H. hudsoni*, (4)=*H. punctigera*, (5)=*H. egregialis*, (6)=*H. mordax*, (7)=*H. mucronata*, (8)=*H. ovatella*, (9)= *H. suteri*, (10)=*H. elongella*, (11)=*H. angustula*, (12)=*H. intermittens*, (13)=*H. hispida* and (14)=*H. delator*)

Species		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
n		10	32	13	8	10	18	18	22	14	8	5	20	8	10
Population		1	3	2	1	1	3	4	1	1	1	1	1	1	2
Enzyme															
AO	(a)	-	1.0	1.0	-	-	-	1.0	1.0	0.857	-	-	0.583	-	1.0
	(b)	-	-	-	-	0.90	-	-	-	0.143	1.0	1.0	0.417	1.0	-
	(c)	1.0	-	-	1.0	0.10	1.0	-	-	-	-	-	-	-	-
ALDH	(a)	-	1.0	1.0	-	-	-	0.833	1.0	0.928	-	0.143	0.667	-	1.0
	(b)	0.050	-	-	0.063	1.0	-	0.143	-	0.172	1.0	-	0.333	1.0	-
	(c)	0.950	-	-	0.937	-	1.0	-	-	-	-	0.857	-	-	-
ME	(a)	-	1.0	-	-	-	-	-	0.286	0.857	-	0.143	-	-	-
	(b)	-	-	-	-	-	-	-	-	0.143	1.0	0.857	1.0	1.0	0.80
	(c)	0.714	-	-	-	-	0.944	0.888	0.714	-	-	-	-	-	0.20
	(d)	0.186	-	1.0	1.0	1.0	0.056	0.111	-	-	-	-	-	-	-
PGI	(a)	0.928	0.231	-	0.750	-	0.888	-	1.0	1.0	1.0	1.0	1.0	1.0	-
	(b)	0.072	0.769	1.0	0.250	1.0	0.111	1.0	-	-	-	-	-	-	1.0
ACP1	(a)	-	0.30	-	-	-	-	-	-	1.0	-	-	1.0	1.0	-
	(b)	-	0.70	-	-	0.909	0.625	1.0	0.214	-	1.0	1.0	-	-	1.0
	(c)	1.0	-	1.0	1.0	0.091	0.375	-	0.786	-	-	-	-	-	-
ACP2	(a)	-	-	-	-	0.660	-	-	-	0.312	0.90	1.0	-	-	-
	(b)	-	1.0	0.75	-	0.340	1.0	-	0.125	0.688	0.10	-	-	-	-

Species		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
n		10	32	13	8	10	18	18	22	14	8	5	20	8	10
Population		1	3	2	1	1	3	4	1	1	1	1	1	1	2
Enzyme															
	(c)	-	-	0.25	-	-	-	1.0	-	-	-	-	1.0	1.0	-
	(d)	1.0	-	-	1.0	-	-	-	0.875	-	-	-	-	-	1.0
LAP1	(a)	-	-	-	-	-	-	-	0.071	-	-	-	-	-	-
	(b)	0.950	0.125	0.071	-	0.75	-	0.30	0.929	1.0	1.0	1.0	1.0	-	0.928
	(c)	0.050	0.875	0.929	1.0	0.25	1.0	0.70	-	-	-	-	-	1.0	0.072
LAP2	(a)	-	-	0.5	0.875	-	-	-	1.0	0.25	-	-	-	-	-
	(b)	-	-	0.5	0.125	-	1.0	0.857	-	0.75	0.875	-	-	0.625	-
	(c)	1.0	1.0	-	-	1.0	-	0.143	-	-	0.125	1.0	1.0	0.375	1.0
PGM	(a)	-	0.733	1.0	-	-	0.960	-	-	-	-	-	-	-	1.0
	(b)	0.733	0.267	-	0.960	-	0.040	-	0.791	-	-	-	0.785	-	-
	(c)	-	-	-	0.046	1.0	-	0.810	-	0.777	1.0	1.0	-	0.812	-
	(d)	0.267	-	-	-	-	-	0.200	0.209	0.223	-	-	0.225	0.188	-
PGD	(a)	-	-	0.90	-	1.0	0.75	1.0	-	-	0.80	-	-	-	-
	(b)	1.0	1.0	0.10	-	-	0.25	-	0.714	1.0	0.20	1.0	1.0	1.0	1.0
	(c)	-	-	-	1.0	-	-	-	0.286	-	-	-	-	-	-
HK1	(a)	0.330	1.0	0.890	1.0	-	0.777	-	-	-	-	1.0	-	-	-
	(b)	0.670	-	0.110	-	1.0	0.223	1.0	1.0	1.0	1.0	-	1.0	1.0	1.0
HK2	(a)	-	-	-	-	-	-	0.75	-	0.937	-	0.75	0.90	1.0	-
	(b)	0.880	0.593	-	-	0.750	-	-	-	-	0.916	0.25	-	-	1.0
	(c)	-	0.407	-	1.0	-	1.0	0.25	-	0.063	-	-	0.10	-	-
	(d)	0.120	-	1.0	-	0.250	-	-	1.0	-	0.084	-	-	-	-

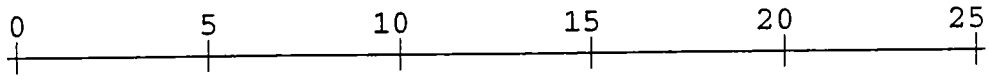
Species		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
n		10	32	13	8	10	18	18	22	14	8	5	20	8	10
Population		1	3	2	1	1	3	4	1	1	1	1	1	1	2
Enzyme															
AK	(a)	-	-	0.818	-	-	-	-	0.916	-	-	-	-	-	-
	(b)	-	0.509	-	-	-	-	-	-	1.0	-	-	-	0.928	-
	(c)	-	0.491	0.182	-	1.0	-	1.0	0.084	-	-	1.0	1.0	0.072	1.0
	(d)	1.0	-	-	1.0	-	1.0	-	-	-	1.0	-	-	-	-

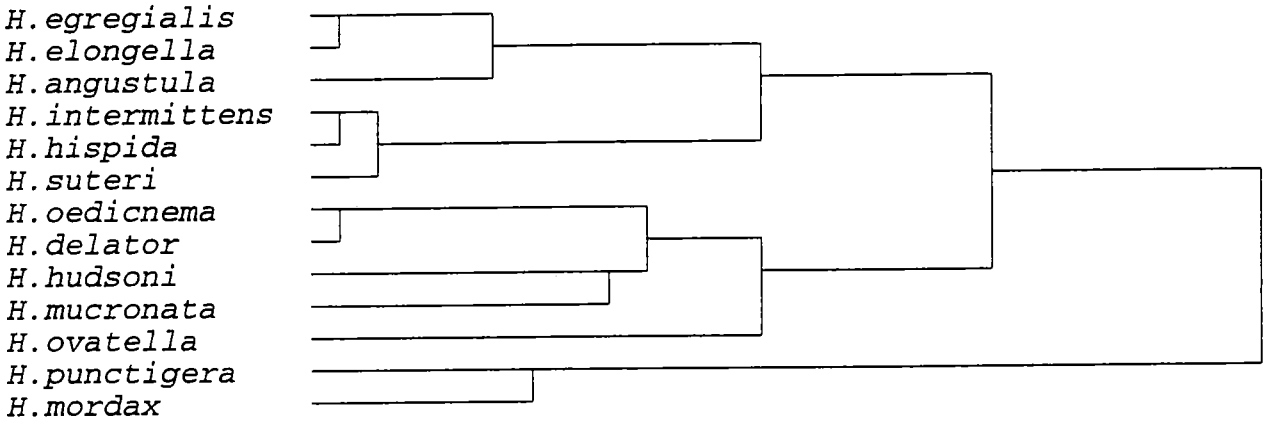
Table 4.4 Matrix comparison of Nei's genetic similarity coefficients (I) (below the diagonal) and genetic distance coefficients (D) (above the diagonal) among *Holcaspis* species.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *H. oedicnema*	-	0.578	1.320	0.970	0.850	0.800	1.032	0.752	1.604	0.823	1.035	0.983	0.418
2. *H. hudsoni*	0.561	-	1.090	1.030	0.693	0.699	0.816	1.203	1.683	2.145	1.937	1.903	0.951
3. *H. punctigera*	0.267	0.336	-	1.555	0.636	2.282	0.980	2.375	1.456	1.469	1.698	1.857	1.676
4. *H. egregialis*	0.379	0.400	0.211	-	1.487	0.553	1.579	1.241	0.409	0.610	1.007	0.983	0.631
5. *H. mordax*	0.427	0.500	0.529	0.226	-	0.954	1.456	1.350	0.944	0.975	1.715	1.328	1.532
6. *H. mucronata*	0.449	0.497	0.102	0.575	0.385	-	1.005	0.805	0.884	0.911	0.811	0.853	0.603
7. *H. ovatella*	0.356	0.442	0.375	0.206	0.233	0.366	-	0.605	1.234	1.145	0.757	1.431	0.693
8. *H. suteri*	0.471	0.300	0.093	0.289	0.259	0.447	0.546	-	0.761	0.827	0.497	0.441	1.052
9. *H. elongella*	0.201	0.186	0.233	0.664	0.389	0.413	0.291	0.467	-	0.608	0.913	0.605	0.906
10. *H. angustula*	0.443	0.181	0.230	0.543	0.377	0.402	0.318	0.437	0.544	-	0.634	0.855	0.789
11. *H. intermittens*	0.355	0.144	0.183	0.365	0.170	0.444	0.469	0.608	0.401	0.530	-	0.415	0.594
12. *H. hispida*	0.259	0.149	0.156	0.374	0.265	0.426	0.239	0.643	0.546	0.425	0.660	-	1.313
13. *H. delator*	0.658	0.386	0.187	0.532	0.216	0.547	0.500	0.408	0.404	0.454	0.552	0.269	-

Figure 4.2 Phenogram of genetic relationships of *Holcaspis* species derived from Nei's coefficient of genetic distance (D) clustered using the unweighted pair group method with arithmetic average (UPGMA).

Rescaled Dissimilarity Distance





generated by using loci as characters was somewhat similar to the topological tree using the phenetic analysis (Figures 4.2). This result supported the comment of Buth (1984) and Murphy (1993) that using loci rather than alleles as characters was better for estimating phylogenetic relationships. The results from the phenetic analysis and cladistic analysis, using both allele and loci as characters, showed highly congruent of grouping *H. egregialis*, *H. elongella,* and *H. angustula.* The results also showed that *H. punctigera* is the most distance species, which is supported by the highest average genetic distance (D= 1.598 ± 0.527) in comparison with the other *Holcaspis* species. In agreement with the phenetic analysis, the cladogram using loci as characters showed that *H. oedicnema* and *H. delator* were closely related species. Overall, both the cladogram using loci as characters and the phenogram using genetic distance data gave a more or less congruent pattern.

4.4.5 Systematics of *Holcaspis* species based on allozyme data compared with previous studies

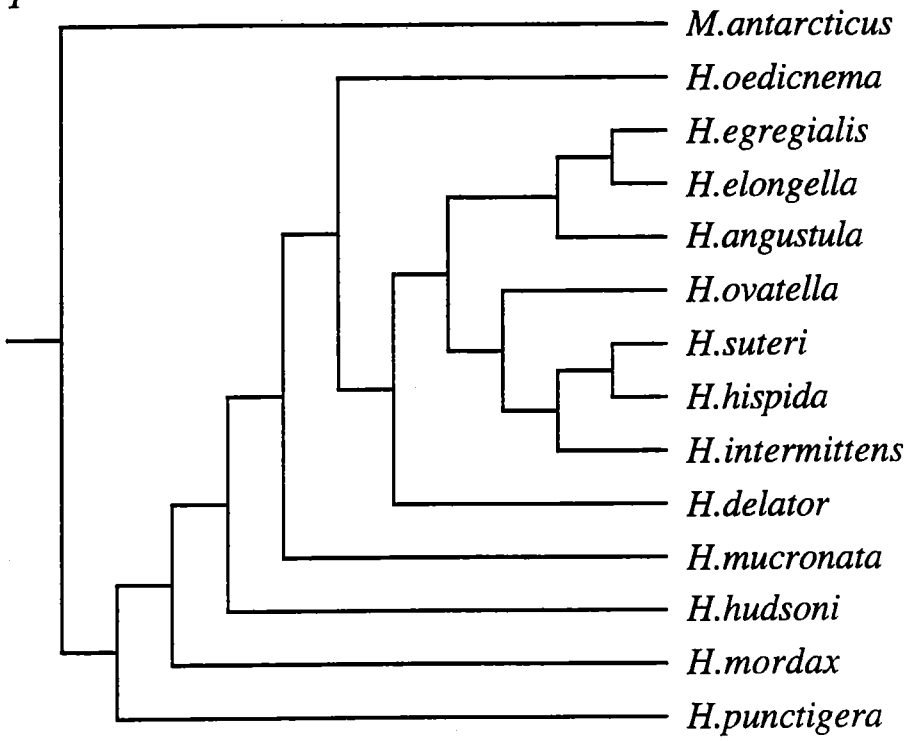
The results from the allozyme data showed some clades that strongly supported Butcher's (1984) conclusions based on morphology. Cladistic and phenetic analysis revealed two species, *H. mordax* and *H. punctigera*, that were allozymically the most divergent taxa and these at least clustered together in the phenogram similar to the result of Butcher's work. Both the phenogram and cladogram based on loci as characters showed congruence, grouping *H. oedicnema*, *H. delator* and *H. mucronata* together similar to Butcher (1984), who grouped these three species into the *oedicnema* group. In contrast to Butcher's work, this analysis discarded *H. hispida* from the *oedicnema* group. Corresponding to the conventional classification, the allozyme data also confirmed that *H. angustula*, *H. egregialis* and *H. elongella* were relatively closely related to each other. Unfortunately, other species of the *angustula* species group identified by Butcher (1984) were not available for study as live specimens.

Table 4.5 Presence/absence data matrix of *Holcaspis* species for the cladistic analysis coded by using 42 alleles as characters. (Allele frequencies that occurred at rates less than 0.05 are treated as absences.)

Species	Allele Character			
	1	2	3	4
	123456789012345678901234567890123456789012			
M. antarticus	00101100111100100010110010101010110101000 1
H. angustula	01010111001001010000100010010010101100001 0
H. delator	10010001100101000010110011000010010100001 0
H. egregialis	01101000010101011000110010010100010101001 0
H. elongella	01001001001001010000100010010100010101000 1
H. hispida	01001001001010000100010110011010011000011 0
H. hudsoni	10010000010101101100011101000110110001101 0
H. intermittens	10010001001010000100100010101010011010001 0
H. mordax	00100100010101101000010101000110110010000 1
H. mucronata	10011000110101000100110110011100011010001 0
H. oedicnema	10010010001101001000110011100010100110011 0
H. ovatella	10010010101001101011101000101011010001101 0
H. punctigera	00101100001100100010011100100001100010000 1
H. suteri	11011011001010011000101100011010011010010 0

Figure 4.3 Two cladograms of 13 *Holcaspis* species using 42 independent alleles as characters with *Megadromus antarcticus* as the outgroup.

1


M.antarcticus
H.oedicnema
H.egregialis
H.elongella
H.angustula
H.ovatella
H.suteri
H.hispida
H.intermittens
H.delator
H.mucronata
H.hudsoni
H.mordax
H.punctigera

2

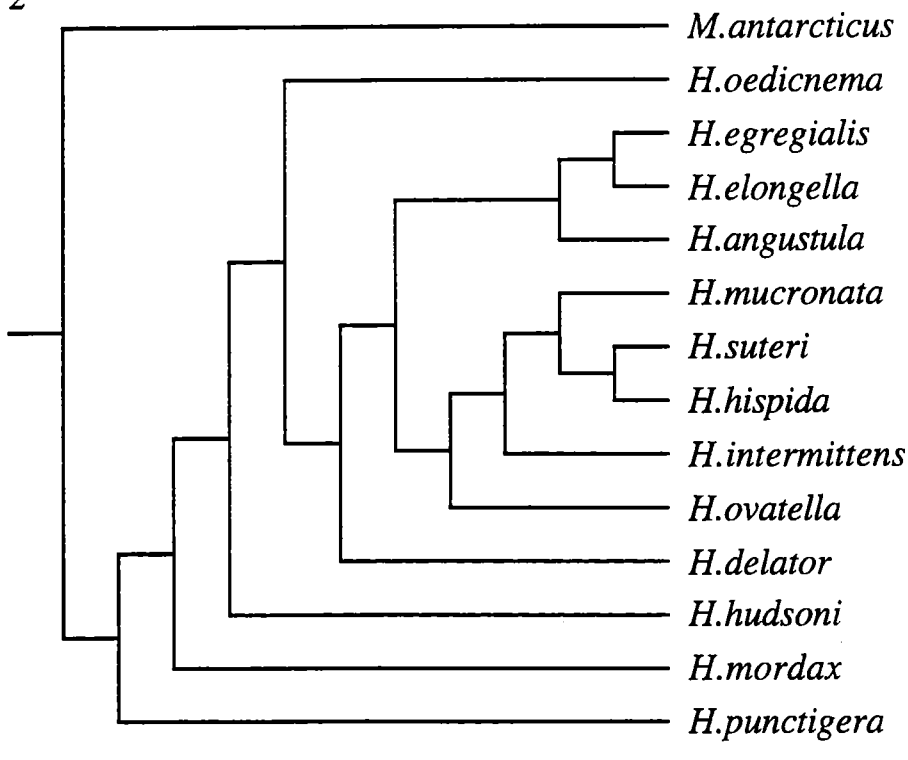
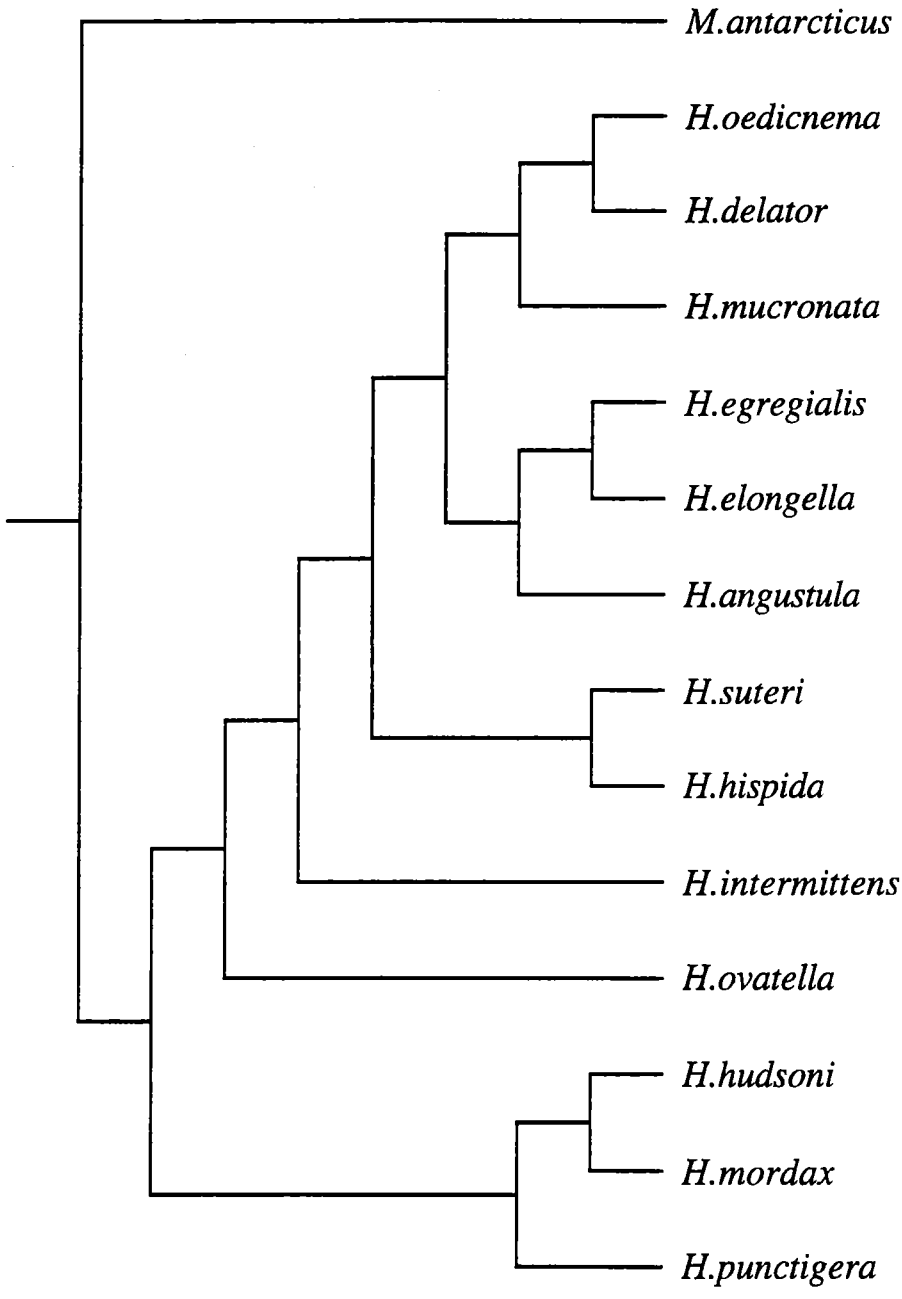

M.antarcticus
H.oedicnema
H.egregialis
H.elongella
H.angustula
H.mucronata
H.suteri
H.hispida
H.intermittens
H.ovatella
H.delator
H.hudsoni
H.mordax
H.punctigera

Table 4.6 Data matrix of *Holcaspis* species for the cladistic analysis coded by using 13 loci as characters.

Species	Locus Character 1 1 2 3 4 5 6 7 8 9 0 1 2 3
M. antarticus	2 2 2 0 1 3 0 2 1 1 1 1 4
H. angustula	1 2 1 0 0 0 0 2 2 1 0 0 3
H. delator	0 0 1 1 0 3 1 2 0 1 1 1 3
H. egregialis	1 1 3 1 0 0 0 2 2 0 1 1 3
H. elongella	1 1 1 0 0 0 0 2 2 0 1 1 4
H. hispida	1 1 1 0 2 2 1 1 2 1 1 0 2
H. hudsoni	0 0 3 1 1 1 1 0 0 0 0 3 0
H. intermittens	0 0 1 0 2 2 0 2 1 1 1 2 3
H. mordax	2 2 2 1 0 1 1 1 0 0 0 2 4
H. mucronata	0 0 2 1 0 2 1 1 2 0 1 0 3
H. oedicnema	0 0 0 1 0 1 1 2 0 1 0 1 2
H. ovatella	0 0 0 0 1 3 0 0 1 1 1 3 0
H. punctigera	2 2 3 0 1 3 1 0 1 2 0 2 4
H. suteri	0 0 0 0 2 1 0 1 2 1 1 0 2

Figure 4.4 A cladogram of 13 *Holcaspis* species using 13 loci as characters with *Megadromus antarcticus* as the outgroup.


M.antarcticus
H.oedicnema
H.delator
H.mucronata
H.egregialis
H.elongella
H.angustula
H.suteri
H.hispida
H.intermittens
H.ovatella
H.hudsoni
H.mordax
H.punctigera

4.5 Summary

The phylogenetic relationships of 13 species of the carabid beetle genus *Holcaspis* were investigated using the cellulose acetate electrophoresis technique. A phenogram and cladograms were constructed from a total of 42 variable enzyme products (alleles) of 13 gene loci from 10 enzyme systems. The phenogram was generated by UPGMA clustering using a genetic distance matrix. Cladograms were constructed using both independent allele and loci as characters. The cladogram from the locus data was highly congruent with the phenogram. The results showed that *H. punctigera* and *H. mordax* were more genetically divergent than the other *Holcaspis* species. Some of the clades generated by the two approaches were congruent with the previous classification of Butcher (1984) and the results showed strong congruence with the results from RAPD (Chapter 5). An attempt was also made to investigate the intraspecific allozyme variation. *Holcaspis oedicnema* revealed a marked degree of heterozygosity compared with the other species. However, the results for this species and all of the others remain inconclusive due to the relatively narrow range of sample sites available.

CHAPTER 5

Systematic relationships among *Holcaspis* species inferred from the RAPD-PCR method

5.1 Introduction

With the recent introduction of the polymerase chain reaction (PCR) and subsequent development of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), the opportunity has arisen to explore and address questions in many different areas of study, including insect systematics.

This chapter includes a brief discussion of the historical development of the PCR and RAPD-PCR, and also of the principles and the application of RAPD-PCR. However, the chapter focuses on an exploration of the application of RAPD-PCR to systematic studies of the carabid genus *Holcaspis*.

5.2 Literature review

5.2.1 History of the development of the polymerase chain reaction

The PCR is an *in vitro* method of amplification, or producing a large numbers of copies, of DNA fragments using enzymatic synthesis and a certain amount of oligonucleotide primers (Saiki *et al.* 1985; White *et al.* 1989; Eleles *et al.* 1992). The PCR technique was originally discovered by Saiki *et al.* (1985) and used as a diagnostic medical genetic test for sickle cell anaemia. The classical PCR technique of Saiki and his team was subsequently modified by Williams *et al.* (1990). They proposed a new PCR amplified DNA technique to detect DNA polymorphism which is the so called 'random amplified polymorphic DNA' (RAPD). They showed that RAPD based on the polymerase chain reaction technique (RAPD-PCR) was able to expose polymorphisms in the genomes of a wide variety of different species.

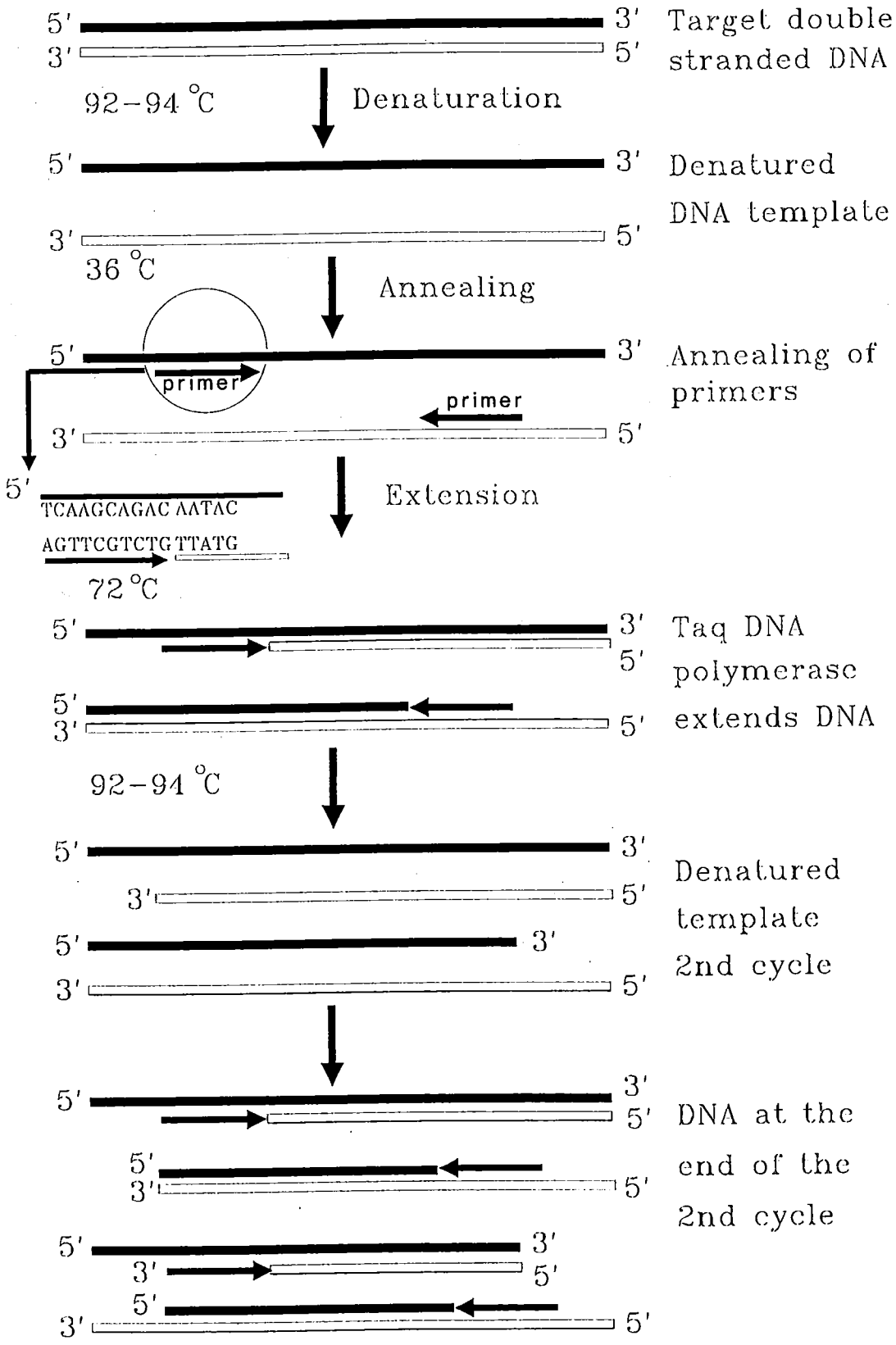
Unlike the classical PCR technique of Saiki *et al.* (1985), which uses a specific

primer (usually around 20-25 nucleotides long), to amplify genomic DNA in the reaction, the RAPD-PCR technique uses fragments of genomic DNA of various sizes that are randomly amplified using an oligonucleotide primer of single-stranded DNA, with 10 base pairs of arbitrary sequence and a thermostable enzymatic DNA polymerase (Williams *et al.* 1990).

5.2.2 The principle of the RAPD-PCR method

Apart from using low temperatures for the annealing step in the RAPD-PCR technique, basically, RAPD-PCR and classical PCR methods have the same principal processes involved in the reaction. The amplification reaction comprises three basic steps: DNA template denaturation, primer annealing, and new DNA stranded extension. The first step begins with a high temperature, approximately 92-94°C, for a specific length of time. The double stranded template DNA is then dissociated. Once the template DNA has been denatured, the temperature is dropped rapidly to around 36-37°C. This allows the oligonucleotide primer to anneal to its complementary sequence in the template DNA. The last step is extension. In this step, new DNA strands complementary to the single stranded template DNA are synthesised starting from the bound primers. After the annealing step, the temperature is raised to 72-74°C. At this temperature, the thermostable enzyme Taq DNA polymerase functions at maximum efficiency and assists extension of the complementary nucleotide sequence on the template DNA from the 3′ end of the primer sequence, which is annealed to the template DNA (Figure 5.1). These three steps are then repeated cyclically. The newly synthesised DNA strands are also available as new templates for further rounds of DNA synthesis in the following cycles of the reaction. The DNA is therefore amplified exponentially. Theoretically, the number of amplified DNA fragments that are generated by the RAPD-PCR method depends upon the probability that a given primer sequence will detect the presence of a complementary sequence in the target template DNA within a distance close enough to each other to be amplifiable (usually less than 2 kilobase pairs apart). It is also based on the size of the target genome (Waugh and Powell 1992). Typically, for RAPD-PCR, 45 cycles are performed. The size of the DNA fragments that can be amplified varies from 200 base

Figure 5.1 Schematic representation of the random amplified polymorphic DNA based on the polymerase chain reaction (RAPD-PCR). The three steps comprising one cycle: denaturation, annealing and extension, are illustrated.


5'
3'
3'
5'
Target double stranded DNA
92–94 °C
Denaturation
5'
3'
Denatured DNA template
3'
5'
36 °C
Annealing
5'
3'
primer
primer
Annealing of primers
3'
5'
5'
TCAAGCAGAC AATAC
AGTTCGTCTG TTATG
Extension
72 °C
5'
3'
5'
Taq DNA polymerase extends DNA
5'
3'
5'
92–94 °C
5'
3'
3'
5'
5'
3'
5'
Denatured template 2nd cycle
5'
3'
5'
3'
5'
DNA at the end of the 2nd cycle
5'
3'
3'
5'
5'
3'
5'

pairs up to 1.4 kilobases (Innis *et al.* 1990; Williams *et al.* 1990; Kambhampati *et al.* 1992). The various sizes of the amplification products can easily be resolved by standard electrophoresis and visualized by ultraviolet (UV) illumination in an ethidium bromide-stained agarose gel.

5.2.3 Application of the RAPD-PCR

Since the RAPD-PCR technique was developed, it has been widely used to address many problems in both plant and animal biology. The RAPD-PCR has recently been used for a wide range of genetics studies, such as genetic mapping, identification of genetic markers, population genetics and genetic relationships. Waugh and Powell (1992) pointed out that using RAPD-PCR provided a quick and useful system for monitoring the levels of diversity both between and within species. It is possible to construct the genetic profile or find genetic markers as well as study the genetic diversity of any organism. RAPD-PCR has been extensively used in plant breeding for crop improvement (Demeke *et al.* 1992; Vierling and Nguyen 1992). In addition, RAPD-PCR is being progressively used on some groups of insects to identify discrete genetic markers as well as being used in genetic mapping and to investigate the biogeography of different species (Black *et al.* 1992; Kambhampati *et al.* 1992). The RAPD-PCR technique has also been used to investigate evolutionary problems, including the determination of paternity and maternity (Scott *et al.* 1992). They demonstrated that in a population of beetles (*Nicrophorous tomentosus* Weber: Silphidae) the likelihood that the RAPD-PCR bands in the offspring are inherited from their parents is quite high. Similar work, done by Williams *et al.* (1990), also illustrated that the polymorphisms of DNA products from RAPD-PCR are inherited in a Mendelian manner in the progeny of a soybean cross.

The use of the RAPD-PCR technique for taxonomic purposes has been investigated recently. Demeke *et al.* (1992) investigated the potential taxonomic use of RAPD-PCR with *Brassica* spp. and discovered that the RAPD-PCR was able to solve the ambiguous relationships of the species. They suggested that RAPD-PCR appears to be useful for taxonomic studies at levels ranging from populations to species and maybe to genera.

In insects, the application of the RAPD-PCR technique has been limited to

identifying genetic markers in species and populations as well as investigating the biogeography of such species or populations (Black *et al.* 1992; Hunt and Page 1992; Kambhampati *et al.* 1992). Therefore, the purpose of this study was to evaluate RAPD-PCR as a novel, non-complicated and fast molecular technique for studying the genetic relationships, at the species level, of a model insect group such as the genus *Holcaspis*. In the study, data from the RAPD-PCR analysis are compared using both phenetic and cladistic methods.

5.3 Materials and methods

5.3.1 Beetle collection

The 86 individuals of 13 *Holcaspis* species used (see Table 5.1) in this study were field collected in various locations throughout the North and South Islands of New Zealand. Six specimens of *Megadromus antarcticus* were used as before as an outgroup for the purpose of the cladistic analysis. After collection, the beetles were starved and the gut systems were removed for use in allozyme work. The gutted remains were then stored at -80°C until they were used.

5.3.2 DNA extraction

Most DNA extraction protocols have the same basic steps: homogenization, lysis of all membranes, and the separation of DNA from cell debris and protein. In this study, the protocol used for extraction of genomic DNA from beetles was after Maniatis *et al.* (1982) and Henry *et al.* (1990) as follows:

1. Grind up an individual entire beetle (except intestine) under liquid nitrogen, then add homogenization buffer (25 mM ethylene diaminetetra acetic acid (EDTA), 0.5 % sodium dodecyl sulphate (SDS), 1 mg/ml proteinase K and 25 mM 2-mercaptoethanol in tris-ethylene diaminetetra acetic acid (TE) buffer), incubate 1 h at 50°C with occasional mixing.

Table 5.1 Sources of *Holcaspis* and *Megadromus antarctica* material used for DNA extraction

Taxon	Locality	Number of Specimens
H. angustula	Ahuriri Bush: Banks Peninsula, (South Island)	3
H. delator	Mt Somers, (South Island)	5
H. egregialis	Bannockburn: Central Otago (South Island)	2
H. elongella	Ahuriri Bush: Banks Peninsula	3
H. hispida	Elsthorpe Bush: Hawke's Bay (North Island)	8
H. hudsoni	Kaikoura: Marlborough (South Island)	8
	Oxford: North Canterbury (South Island)	2
H. intermittens	Ahuriri Bush: Banks Peninsula.	8
H. mordax	Whakapapa: Taupo	3
	Lake Rotokura	3
	Lake Rotopounamu (North Island)	3
H. mucronata	Elsthorpe Bush: Hawke's Bay	5
	White Pine Bush: north of Napier (North Island)	2
H. oedicnema	Gladstone Road: Levin	3
	Kohitere Forest: Levin (North Island)	4
	Pupu Valley, Golden Bay: Nelson (South Island)	6
H. ovatella	Bannockburn: Central Otago	7
H. punctigera	Bannockburn: Central Otago	4
H. suteri	Ahuriri Bush: Banks Peninsula	7
M. antarctica	Ahuriri Bush: Banks Peninsula	6

2. Microcentrifuge at low speed for 2 minutes. After that, transfer the supernatant to a new tube to remove the exoskeleton and discard the pellet.
3. Extract the supernatant twice with one volume of phenol and then once each with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and one volume of chloroform:isoamyl alcohol (24:1) respectively. Microcentrifuge each time at high speed for 2 min.
4. Pipette the aqueous phase into a new tube and add two and a half volumes of ice cold absolute ethanol alcohol to precipitate the DNA. Place at - 20°C for 1 h and then spin at high speed for 10 min.
5. Discard the supernatant and keep the DNA pellet. Wash the DNA pellet with 70 % ethanol and spin for 5 min.
6. Discard ethanol and dry the DNA pellet with a speed vacuum for 5 min.
7. Dissolve the dried DNA pellet in TE buffer. To remove residual RNA, DNA could be purified further by adding 10 µg/ml of RNase and incubating for 30 min at 37°C.
8. Quantify the amount of DNA by measuring the optical density at 260 nm. Store DNA samples at -20°C.

5.3.3 The RAPD Polymerase Chain Reaction

In order to achieve a satisfactory reaction and produce a usable banding pattern of randomly amplified polymorphic DNA, the essential parameters such as primer conditions, PCR programme and the concentration of the magnesium chloride solution in which the reaction takes place, need to be adjusted. The justification for the modifications of these parameters is discussed below.

5.3.3.1 Primer

Primers used for the random amplified polymorphic DNA are short single stranded 10 base oligonucleotides of random sequence.

5.3.3.1.1 *Primer Selection.* Twenty 10 base oligonucleotide primers with 50-70 % Guanine and Cytosine (G+C) contents were screened. Some of these primers were from kits G, H and F of Operon Technologies, Alameda, California. Other primers were synthesized by standard laboratory techniques by Oligos Etc. Inc., Wilsonville, Oregon, USA and a few primers used in this study were obtained from the Crop and Food Research Institute molecular laboratory, Lincoln, Canterbury. All 20 primers were first tested for amplification with all beetle DNA used. The primers that gave satisfactory results were then selected. The list of the 20 primers examined and the selected primers is given in Table 5.2.

5.3.3.1.2 *Primer Concentration.* In general, primer concentrations of 200 nM to 1000 nM have been used successfully in RAPD-PCR (Ausubel *et al.* 1993). In this study, primer concentrations of 200, 300, 500 and 1,000 nM were tested in the PCR and the concentration that gave the best amplification was selected for the following experiments.

5.3.3.2 Optimization of the cycling parameters

The PCR programme commonly used for RAPD analysis is as described in Figure 5.1. Ideally, 45 cycles of the three steps are used in a 5-6 h period (Williams *et al.* 1990). This process is very time consuming, may produce non-specific amplification bands and is inconvenient when a large number of samples is to be tested. The RAPD-PCR programme, therefore, was modified in order to minimise these disadvantages and suit the specific conditions for the amplification of genomic DNA from *Holcaspis* species. In this experiment, the primer code G-01 (AGT TCG TCT G) was used to amplify genomic DNA of *H. ovatella.* The optimization of conditions for the RAPD-PCR were as follows:

5.3.3.2.1 *Optimization of denaturing time.* The PCR programme was tested at different denaturing times at 94°C: 6, 12, 30 and 60 s. The total number of cycles was 35.

5.3.3.2.2 *Optimization of annealing time.* The PCR programme with 35 cycles was tested with the following annealing times at 36°C: 12, 30 and 60 s.

Table 5.2 The 20 primers examined for random amplified polymorphic DNA (RAPD) use. (*indicates primers selected for use)

Primer (code)	Sequence (5′-3′)	% G-C content	Sources of Primers
OPF-04	GGT GAT CAG G	60 %	OperonTechnologies
OPF-05	CCG AAT TCC C	60 %	Operon Technologies
OPF-06*	GGG AAT TCG G	60 %	Operon Technologies
OPF-08	GGG ATA TCG G	60 %	Operon Technologies
OPF-11	TTG GTA CCC C	60 %	Operon Technologies
OPF-17	AAC CCG GGA A	60 %	Operon Technologies
OPG-05	CTG AGA CGG A	60 %	Operon Technologies
OPG-07	GAA CCT GCG G	70 %	Operon Technologies
OPG-11	TGC CCG TCG T	70 %	Operon Technologies
OPG-12	CAG CTC ACG A	60 %	Operon Technologies
OPG-16	AGC GTC CTC C	70 %	Operon Technologies
OPG-17	ACG ACC GAC A	60 %	Operon Technologies
OPG-18*	GGC TCA TGT G	60 %	Operon Technologies
OPH-04*	GGA AGT CGC C	70 %	Operon Technologies
G-01*	AGT TCG TCT G	50 %	Oligos Etc. Inc.
J-09*	TCT GCC GTG A	60 %	Oligos Etc. Inc.
J-267/1*	AGG AGA TAC C	50 %	Oligos Etc. Inc.
J-267/2	ACA TAG ACG C	50 %	Oligos Etc. Inc.
J-267/4	GGA TGT CGA A	50 %	Oligos Etc. Inc
J-317/1	GAT AAC GCA C	50 %	Crop and Food Research, Lincoln

5.3.3.2.3 *Optimization of extension time.* Different extension times at 72°C of 30, 60 and 120 s were also tested with 35 programme cycles.

5.3.3.2.4 *Optimization of number of cycles.* PCR programmes comprising 35, 40 and 45 cycles were screened by using the optimum timing for each of the three steps in the

reaction process: denaturing, annealing and extension.

5.3.3.3 Optimization of magnesium chloride concentration

The magnesium chloride concentration affects primer annealing and DNA polymerase activity (Ausubel *et al.* 1993). Therefore it has to be readjusted for each primer used in order to get optimum amplification. Magnesium chloride concentrations ranging from 0 to 6 mM were used and selected primers were tested for optimum magnesium chloride concentration. The procedures for optimization of magnesium chloride concentration used in this experiment followed the standard protocol of magnesium chloride concentration optimization described by Ausubel *et al.* (1993).

5.3.4 DNA amplification conditions

In this experiment, all reactions were performed in 25 µl volume. In order to eliminate non specific amplification of DNA, the hot start technique was used. Hot start begins by heating the DNA template to a high temperature for a certain of time without adding the Taq DNA polymerase enzyme. Ideally, the high heat in the hot start cycle can aid in eradicating exotic DNA. After that, the temperature is then lowered to the extension temperature and the remaining reaction components are added.

The PCR components with 25 ng of each *Holcaspis* genomic DNA, optimised primers and magnesium chloride concentration and deionised water were assembled and 50 µl of light mineral oil was layered on the top of the reactants. The reaction tube was placed in a water thermalcycler (Autogene) and a hot start was performed by heating up to 94°C for 2 min. When the temperature went down to the extension temperature of 72°C, the remaining parts of the reaction solution were added through the mineral oil layer. Before being added through the oil layer, to avoid inaccuracies involved in pipetting very small quantities of solution, 1 unit of Taq enzyme (Boehringer) was made up with the Boehringer PCR buffer (10 mM tris-hydrochloric acid (tris- HCl) pH 8.3, 1.5 mM magnesium chloride, 50 mM potassium chloride and 0.01 % gelatin) and a solution of 0.1 mM each of 2´-Deoxycytidine 5´-tri-phosphate (dCTP), 2´-Deoxyguanosine 5´-triphosphate

(dGTP), 2′-Deoxyadenosine 5′-triphosphate (dATP) and 2′-Deoxythymidine 5′-triphosphate (dTTP) (Boehringer).

To detect possible contamination in the amplification reaction, a negative control, which contained all amplification reaction components except the beetle template DNA, was used every time amplification was carried out.

After the amplification was completed, the RAPD-PCR products were resolved by electrophoresis in a 1.4 % agarose gel containing 0.5 µg/ml ethidium bromide in tris-borate-EDTA buffer, visualized and photographed under ultraviolet (UV) light. The 100 base pairs DNA Ladder (Life Technologies) was used as a molecular weight marker.

5.3.5 Demonstration of the homologous bands of the RAPD-PCR product

In order to demonstrate the homologous nature of the RAPD-PCR amplification products, i.e., whether the bands that ran in the same position in the gel had a similar nucleotide sequence (and not just the same molecular weight but different sequence), Southern blot was performed.

After running the RAPD-PCR amplified products from each beetle species genomic DNA on 1.4 % agarose gel, the gel was photographed under ultraviolet light and the DNA fragments were transferred to a nylon membrane (GeneScreen Plus, Dupont) under alkaline conditions by the capillary transfer method, according to the manufacturer's instructions. A second aliquot of the RAPD-PCR product was run in a similar gel and the DNA bands of interest were eluted by running them into a DEAE-cellulose membrane. The method of fragment elution followed Ausubel *et al.* (1993). Eluted DNA was labelled with radioactive phosphorous (^{32}P) by the random primer labelling method, using the Amersham kit, according to the manufacturer's recommendations. These DNA fragments were used as radioactive probes for hybridization on the membrane containing the RAPD-PCR fragments from different beetle species. Probes were added to the membrane in 10 ml of hybridization solution containing 10 % of sodium dodecyl sulphate (SDS), 50 % of dextran sulphate and water, sealed in a plastic bag and incubated at 65°C overnight. Following hybridization, the membrane was removed from the hybridization solution in the bag. The unbound radioactive probe was removed from the membrane by washing

twice in 0.3 M sodium chloride-0.03 M sodium citrate at room temperature for 5 min with constant agitation, followed by two washes in 0.3 M sodium chloride-0.03 M sodium citrate and 1.0 % SDS at 65°C for 30 min with constant agitation. Finally, it was washed twice in 0.015 M sodium chloride-0.0015 M sodium citrate at room temperature for 30 min with constant agitation. The membrane was then exposed to an X-ray film for 1 h at -100°C, with an intensifying screen. After the membrane was removed, the film was developed.

In this experiment, the amplification banding patterns from the different species of *Holcaspis* that were used in Southern blot analysis were obtained using two primers: AGG AGA TAC C (J267/1) and TCT GCC GTG A (J-09). Testing for homology among the amplification products was performed on a total of five representative bands: two DNA fragments of gel applied with primer J267/1 from *H. elongella* and *H. suteri* and three DNA fragments of gel applied with primer G-09 from *H. ovatella* (indicated in Figure 5.5 and 5.6, respectively) were purified from agarose gels, labelled with ^{32}P, and used as probes in Southern blots on an array of RAPD-PCR products. The choice of selected DNA fragments was based on the fact that these bands were common to different species and that they were clear on the gel. The technique for testing the composition of bands is new and has been published (Hanboonsong *et al. 1992*).

5.3.6 Data recording and analysis

The mobility distance for each band of the amplified product and the molecular weight marker to the loading point on each agarose gel was measured in centimetres. The measurements were converted to base pair lengths using the computer programme of Schaffer and Sederoff (1981).

To determine the size of the bands from the RAPD-PCR amplification products with more accuracy, every RAPD-PCR product was run side by side with the 100 base pairs molecular weight marker. In cases where it was uncertain whether two bands from two different samples were of fragments the same size, the two samples were then combined and run together in the same lane on an agarose gel. If there were no separation of the bands, it was assumed that the two doubtful fragments had the same size. If there were

perceptible resolution of the bands, it demonstrated that the two fragments had different sizes.

The data were scored on presence or absence of the amplified fragments for all individuals. Two procedures utilized for analysis of the RAPD-PCR data are discussed below.

5.3.6.1 Cladistic method

The data matrix was generated from the individual fragment profiles of all species. Each individual profile was constructed using the criterion that if a given amplified fragment was present in an individual, it was assigned a '1' and when the fragment was absent, it was assigned a '0'.

Data were analyzed cladistically by considering the presence and absence of amplified fragments as binary characters. The data were converted into a matrix format and analysed using PAUP version 3.0 (Swofford 1992). Simple stepwise addition and tree bisection reconnection in the branch swapping option were selected for trial rearrangement to find the shortest tree. No assumptions of character weighting were made for this analysis; all the selected characters were treated as of equal weight. Because of the large number of taxa used, a heuristics search was employed to look for the most parsimonious trees. Rooting the trees and the direction of polarization of character states was determined by using an outgroup comparison (Watrous and Wheeler 1981). In this analysis, *Megadromus antarcticus*, as before, was used as the outgroup.

Assumptions on the direction of character changing in the RAPD-PCR analysis data were based on the Fitch method (Fitch 1971). This method searches for the most parsimonious evolutionary tree, which allows only the minimum of the number of evolutionary steps or changes of the characters in the evolutionary pathway. The Fitch model of character changing assumes that the rate of evolution of the characters may vary between lineages. Therefore, the characters were treated as unordered and so they can be transformed from any one state to any other state. The most parsimonious trees were selected by maximising the minimal number of character state transformations (steps) used. The strict consensus tree was used to combine all the most parsimonious

trees that resulted from the heuristic search.

5.3.6.2 Phenetic method

Besides the cladistic analysis, the RAPD-PCR data were also analyzed using both cluster and principal coordinate analysis (Gower 1966). For these analyses, all 86 individuals of 13 *Holcaspis* species were used. Each band in each individual of all beetle species was scored as '1' if present or '0' if absent. Pair-wise comparison based on both unique and shared fragments was used to generate two different similarity coefficients: simple-matching fragments and Jaccard's coefficient of similarity (Gower 1985). The fraction of simple-matching fragments between individuals was calculated by the equation:

$$F = 2\,N_{ab}/(N_a + N_b)$$

Where N_a and N_b are the number of fragments found in individuals 'a' and 'b' and N_{ab} is the number of fragments shared by individuals 'a' and 'b'.

Jaccard's coefficient of similarity was calculated from the following equation:

$$F = N_{ab}/N_t$$

Where N_{ab} is the number of fragments shared by individuals 'a' and 'b' and N_t is the number of unique fragments plus the number shared by individuals 'a' and 'b'.

The results of the coefficient of similarity from both the simple-matching fragments model and Jaccard's coefficient model were interpreted on the following basis. If the value of the coefficient of similarity was equal to '1', the two taxa had identical patterns of similarity, therefore, those two were close likenesses. If a coefficient of similarity value was close to '0', it indicated that the two taxa had completely different patterns. Consequently, those two taxa were distinct. Cluster analysis was performed using an unweighted pair group method with arithmetic averages (UPGMA) on the value of similarity coefficients between taxa. After clustering, phenograms were constructed. Using the pair-wise taxa similarity distance matrix, principal coordinates analysis was carried out to illustrate the distribution pattern of each species in multidimensional space. In the principal coordinate analysis, similar species are placed close together while the dissimilar species are further apart.

Both cluster analysis and principal coordinate analysis were performed using the statistical package Genstat version 5.0.

5.4 Results

5.4.1 Optimization of the RAPD-PCR conditions

5.4.1.1 DNA extraction

According to the DNA extraction protocol, high molecular weight DNA was isolated from *Holcaspis* species as shown in Figure 5.2. Unlike DNA from other eukaryotic cells, the beetle genomic DNA (*Holcaspis* species and *Megadromus antarcticus*) from the protocol showed a slightly brownish colour. The quantity of DNA isolated was 1.5-2.5 μg/μl, depending on the body size of beetles.

5.4.1.2 Primer selection for the RAPD-PCR of *Holcaspis* species

Six of the 20 oligonucleotide primers tested were selected. The selection was based on the criterion that chosen primers should generate informative banding patterns, with most amplified fragments shared within species and some amplified fragments shared between ingroup and outgroup species. In addition, the selected primers should consistently produce well amplified fragments that are sharp, clear and have a high intensity on the gel. Moreover, the amplified banding patterns should be well separated for ease of scoring. Faint bands that amplified irregularly were omitted from scoring.

These six selected primers, which contained 50-70 % G+C, yielded a total of 271 amplified fragments that met the above criteria. The number of scorable amplified fragments was 56, 41, 44, 48, 37 and 45 respectively from primers OPF-06, G-01, OPG-18, OPH-04, J-09 and J-267/1. The size of these amplified fragments ranged from 220 to 3018 base pairs (see Table 5.3).

Figure 5.2 Genomic DNA (lanes 1-4) from four individuals of *Holcaspis intermittens* resolved by electrophoresis in a 0.8 % agarose gel. Molecular weight markers (λ Hind III) were as indicated in lane 5 (scale shown in kilobase pairs, kb).

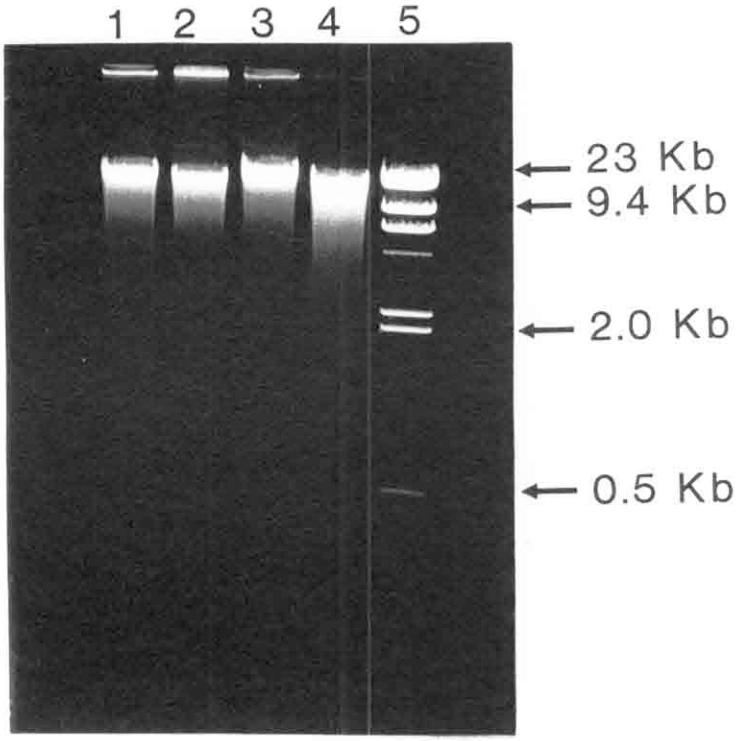

1
2
3
4
5
23 Kb
9.4 Kb
2.0 Kb
0.5 Kb

Table 5.3 The number of scorable amplified fragments and the range of fragment sizes from *Holcaspis* species from each of the six selected oligonucleotide primers.

Selected Primer	Sequence (5′-3′)	Range of Bands Size (bp)	Number of Scorable Fragments
OPF-06	GGG AAT TCG G	313-2620	56
G-01	AGT TCG TCT G	433-2636	41
OPG-18	GGC TCA TGT G	289-2178	44
OPH-04	GGA AGT CGC C	220-1540	48
J-09	TCT GCC GTG A	294-1693	37
J267/1	AGG AGA TAC C	388-3018	45

5.4.2 Description of RAPD-PCR conditions for use in amplification of genomic DNA of *Holcaspis* species

5.4.2.1 Primer and magnesium chloride concentation

Two essential parameters for conducting the RAPD-PCR process, primer and magnesium chloride concentration, were optimized in order to increase the efficiency of the RAPD-PCR reaction. It was found that the best-defined banding patterns were obtained using 300 nM of primer per 25 µl and 3 mM magnesium chloride for two of the primers (primers J-09 and G-01). For the rest of the primers (J-267/1, OPH-04, OPG-18 and OPF-06), 2 mM magnesium chloride gave a good amplification reaction. It was found that if there was less primer or a lower magnesium chloride concentration, there were fewer bands. In contrast, if more primer or magnesium chloride were used, a smear, resulting from non-specific amplification, would be visible on the gel. In addition, the amplification reaction was also unlikely to be successful if the amount of primer and

magnesium chloride concentration were not maximised.

5.4.2.2 The RAPD-PCR programme

Apart from the above two critical parameters, the three different PCR steps (denaturation, annealing and extension) for programming the RAPD were optimised. The results are presented in Figure 5.3

5.4.2.2.1 *Denaturation time* The results showed that denaturing times of 6 and 12 s for separation of the template DNA did not make any difference to the amplified products. When the denaturing time was increased to 30 or 60 s, the efficiency of amplification was decreased, resulting in loss of amplified fragments compared with a denaturing time of 6 or 12 s. This may be explained by the fact that a longer denaturation time will result in more Taq DNA polymerase inactivation. The life span of Taq DNA polymerase is reduced with increasing temperature (Yu and Pauls 1992). Therefore, longer incubation of Taq at 94°C may result in not enough enzyme activity in the later cycles. Hence, the shortest denaturing time, of 6 s, was chosen because it used less time, but was also able to generate informative and high resolution amplified products.

5.4.2.2.2 *Annealing time.* It was found that annealing times of 30 and 60 s gave the best results compared with 12 s, with no difference between 30 and 60 s.

5.4.2.2.3 *Extension time.* With the extension time, the results showed that there were no significant differences in amplified fragments between the three different extension times (30, 60 and 120 s). However, the longest extension time tested (120 s) resulted in higher background smear than the others.

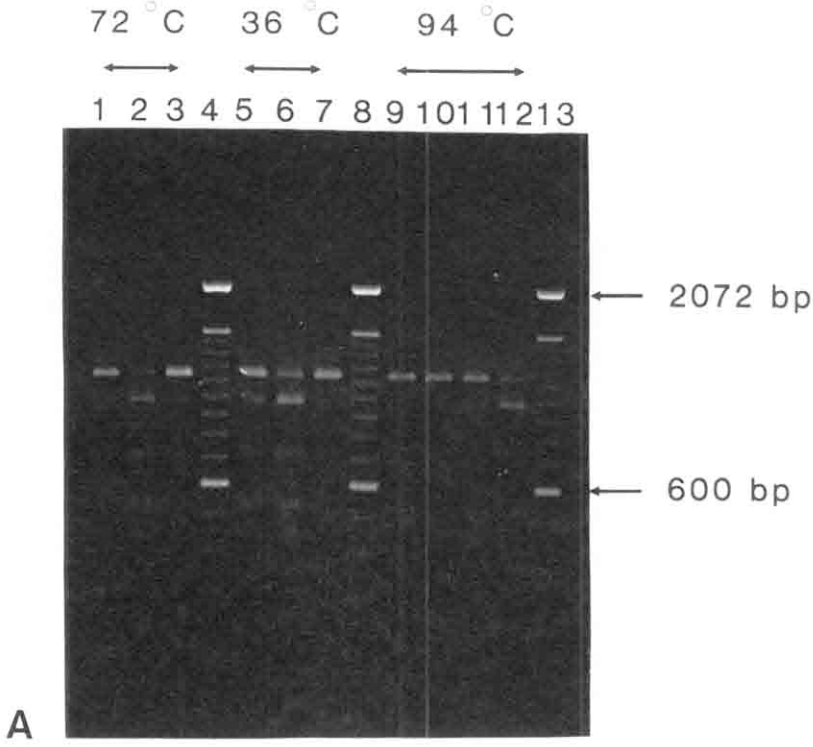
No large differences in banding pattern intensity were found using 35, 40 or 45 cycles in the PCR programme. Thus, 35 cycles of the PCR programme was chosen in order to cut the overall reaction time (Figure 5.3B).

Therefore, the optimal PCR programme conditions for analysis of *Holcaspis* species genomic DNA that gave the best RAPD-PCR amplified products were a:

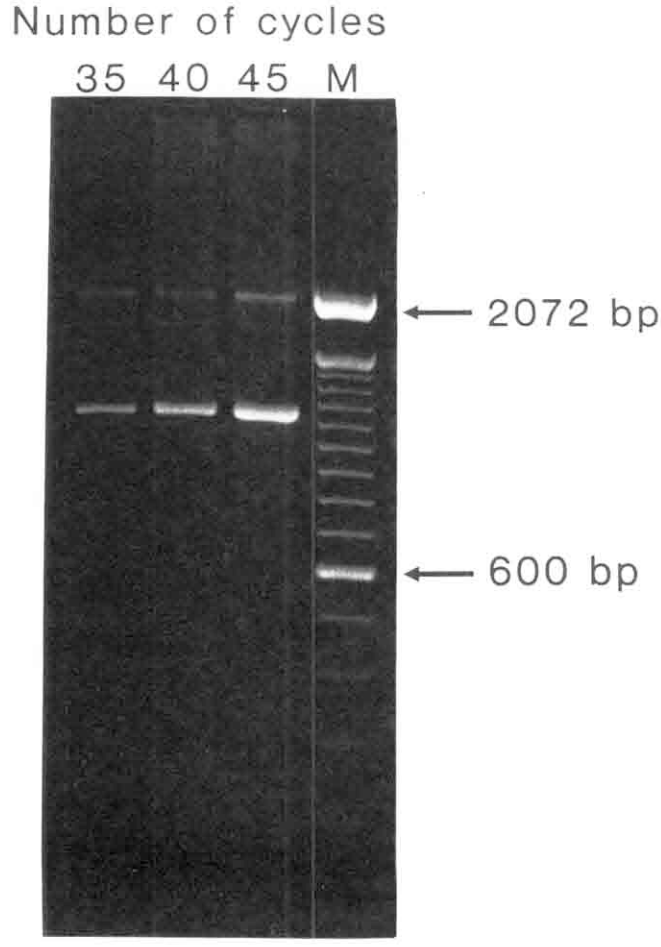
Figure 5.3 The results of optimization of the RAPD-PCR programme with *Holcaspis* species. Primer G-01 (AGTTCGTCTG) was used to amplify genomic DNA of *H. ovatella.* Amplification products were resolved by electrophoresis in a 1.4 % agarose gel and stained with ethidium bromide.

(A) The RAPD-PCR programme at 30 cycles of denaturing time at 94°C for 60, 30, 12 and 6 s (lanes 9, 10, 11 and 12), annealing time at 36°C for 60, 30 and 12 s (lanes 5, 6 and 7) and extension time at 72°C for 120, 60 and 30 s (lanes 1,2 and 3). Lane 13 was a 100 Base-Pair Ladder (molecular weight markers) whose scale is shown in base pairs (bP).

(B) The RAPD-PCR patterns were obtained from using PCR programme of 35, 40 and 45 cycles at: 94°C, 6 s; 36°C, 30 s; and 72°C, 30 s. 'M' indicates as molecular weight markers (100 Base-Pair Ladder) whose scale is shown in base pairs (bp).


72 °C
36 °C
94 °C
1 2 3 4 5 6 7 8 9 10 11 12 13
2072 bp
600 bp

A


Number of cycles
35 40 45 M
2072 bp
600 bp

B

6 s denaturation step at 94°C;

30 s annealing step at 36°C; and

30 s extension step at 72°C.

These three steps were repeated for 35 cycles. It took 2-3 h to complete all cycles (Figure 5.4).

5.4.3 Application of RAPD-PCR for systematics study

5.4.3.1 Southern blot technique

As illustrated in Figures 5.5 and 5.6, five representative bands, which were generated with two primers, were used as probes to hybridize to 25 co-migrating fragments. Of them, 22 fragments (equivalent 88 %) gave a positive signal on the Southern blot, showing that they were homologous to the probes. Thus, 88 % of co-migrating bands from RAPD-PCR result had similar sequences.

The homology among amplification products using Southern blot technique is shown in Table 5.4.

5.4.3.2 Genetic variation within and among *Holcaspis* species

At the interspecific level, a considerable degree of polymorphism was detected from RAPD-PCR using the six selected primers. Certain amplified bands appeared to be common to several species while others were present in some species and absent in others (Figures 5.7 and 5.8).

In contrast, at the intraspecific level, a much lower degree of polymorphism was detected. The genetic variation within *Holcaspis* species was recorded by counting the number of bands occurring in all individuals of each species and clarified by how many bands were monomorphic in all individuals examined. As can be seen in Table 5.5, there was some variation among individuals from each species with all six selected primers (Figure 5.7). The RAPD-PCR technique revealed that the percentage of amplified DNA regions that were monomorphic in all individuals of each species was essentially 53-76 %

Figure 5.4 Schematic representation of the optimization of the RAPD-PCR programme used for *Holcaspis* species in this study.

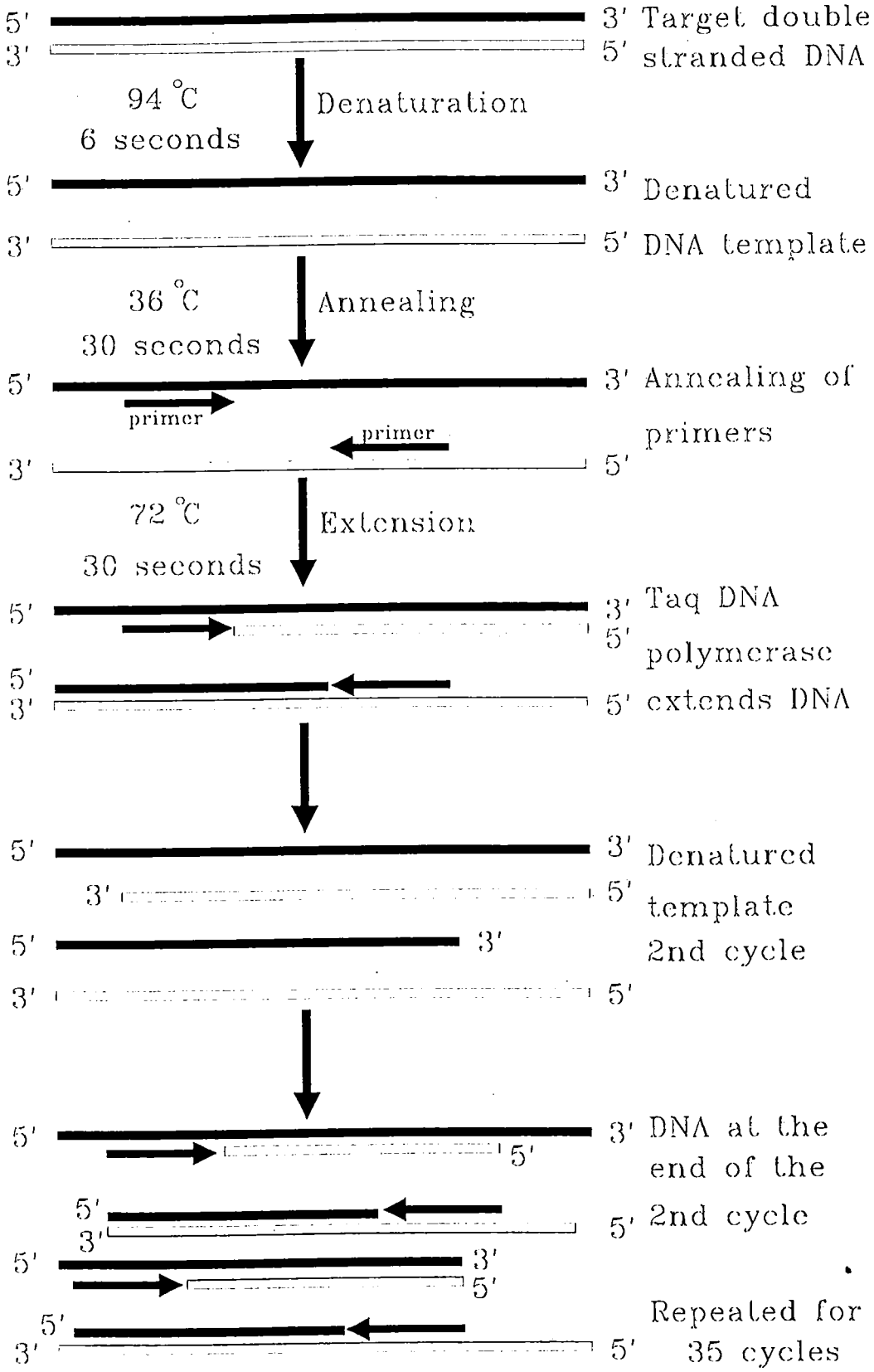
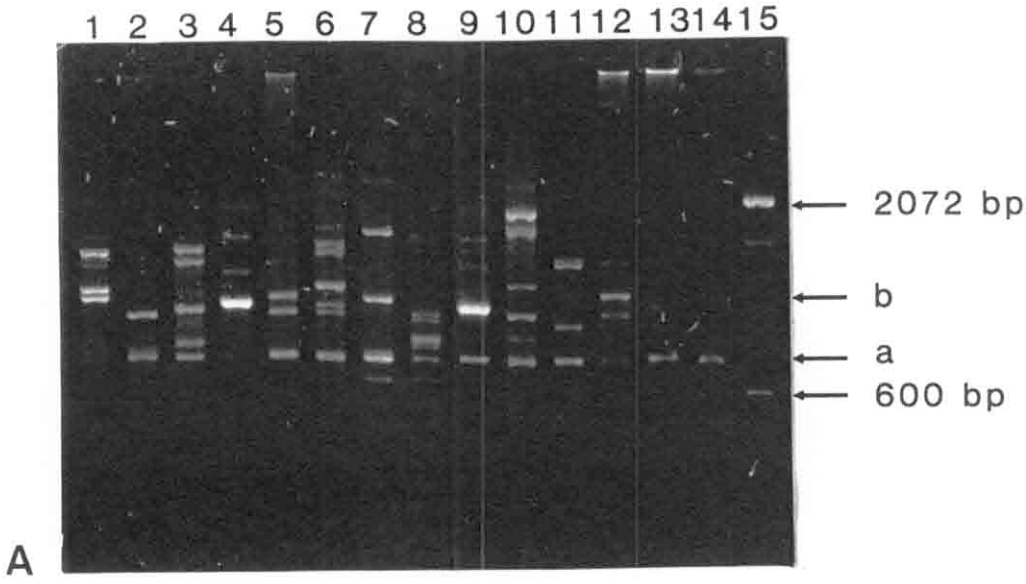

5'
3'
3' Target double
5' stranded DNA
94 °C
6 seconds
Denaturation
5'
3' Denatured
3'
5' DNA template
36 °C
30 seconds
Annealing
5'
3' Annealing of
primer
primer
primers
3'
5'
72 °C
30 seconds
Extension
5'
3' Taq DNA
5'
polymerase
5'
3'
5' extends DNA
5'
3' Denatured
3'
5'
template
5'
3'
2nd cycle
3'
5'
5'
3' DNA at the
5'
end of the
5'
5' 2nd cycle
3'
5'
3'
5'
5'
3'
Repeated for
5'
35 cycles

Figure 5.5 Southern blot test of homology with RAPD-PCR products. (Fragments amplified using primer AGG AGA TAC C. Amplification products were resolved by electrophoresis in a 1.4 % agarose gel and stained with ethidium bromide.)

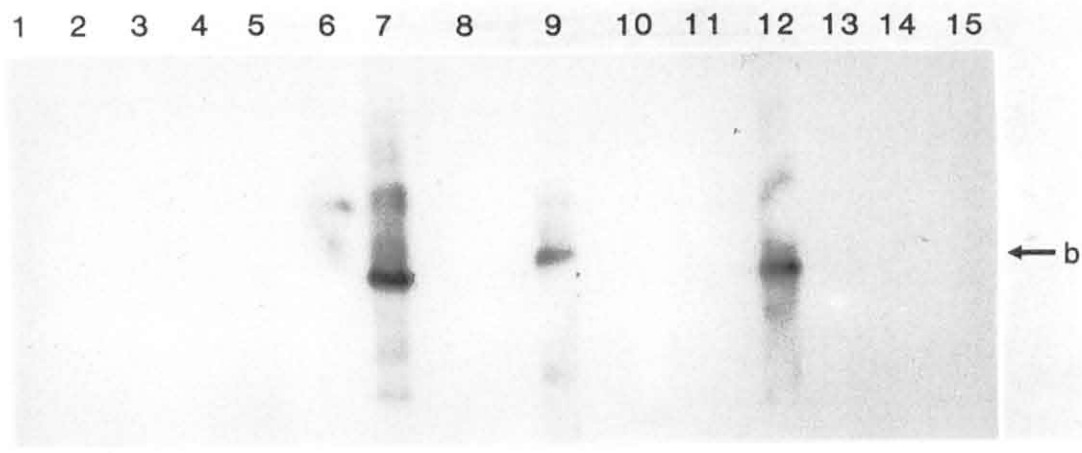
(A) Lanes 1-14: DNA from *Megadromus antarcticus*, *Holcaspis punctigera*, *H. mucronata*, *H. hudsoni*, *H. angustula*, *H. mordax*, *H. oedicnema*, *H. ovatella*, *H. egregialis*, *H. intermittens*, *H. hispida*, *H. suteri*, *H. elongella* and *H. delator* respectively. Lane 15 was a 100 Base-Pair Ladder (molecular weight marker) whose scale is shown in base pairs (bp). Arrows indicate two fragments, used as probes, that generated from the DNA of *H. elongella* and *H. suteri*, labelled as 'a' and 'b' respectively.

(B) Autoradiograph of Southern blot results of probing the array shown in Figure 5.5A with probes from DNA of *H. suteri* (as indicated by arrow).

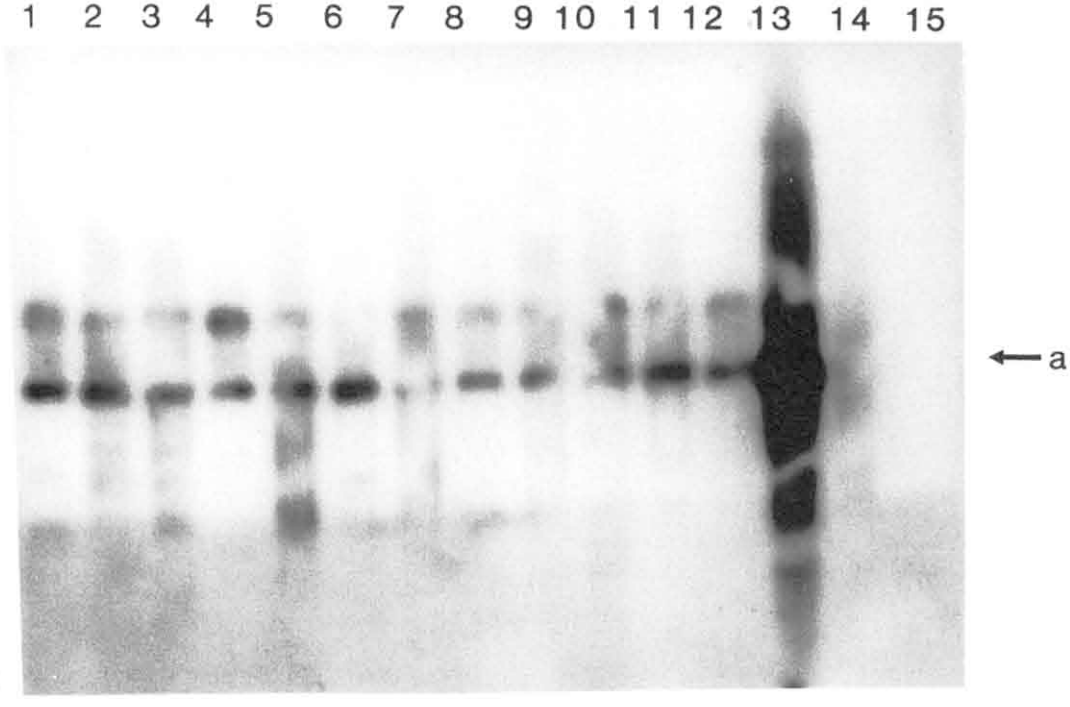
(C) Autoradiograph of Southern blot results of probing the array shown in Figure 5.5A with probes from DNA of *H. elongella* (as indicated by arrow).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
2072 bp
b
a
600 bp

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
b

B

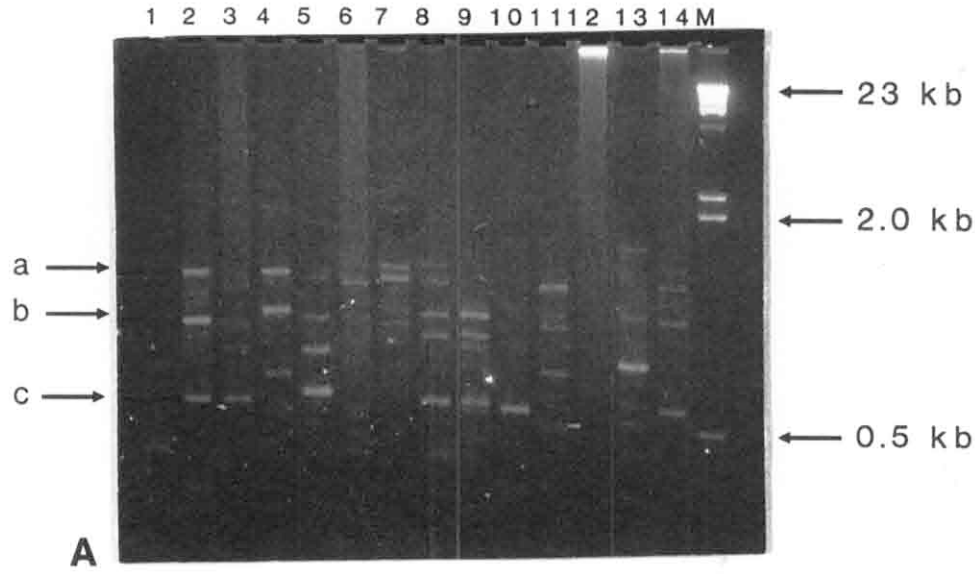

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
a

C

Figure 5.6 Southern blot test of homology with RAPD-PCR products. (Fragments amplified using primer TCT GCC GTG A. Amplification products were resolved by electrophoresis in a 1.4 % agarose gel and stained with ethidium bromide.)

(A) Lanes 1-14: DNA from *Holcaspis elongella*, *H. ovatella*, *H. egregialis*, *H. mucronata*, *H. hudsoni*, *M. antarcticus*, *H. mordax*, *H. suteri*, *H. angustula*, *H.intermittens*, *H.oedicnema*, *H. punctigera*, *H. delator* and *H. hispida* respectively. Lane 'M', molecular weight markers (λ Hind III) whose scale is shown in kilobase pairs, kb). Arrows indicate three fragments, used as probes, that generated from the DNA of *H. ovatella*.

(B) Autoradiograph of Southern blot results of probing the array shown in Figure 5.6A with probes from DNA of *H. ovatella* (as indicated by arrow 'c').


1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
23 kb
2.0 kb
0.5 kb
a
b
c
A

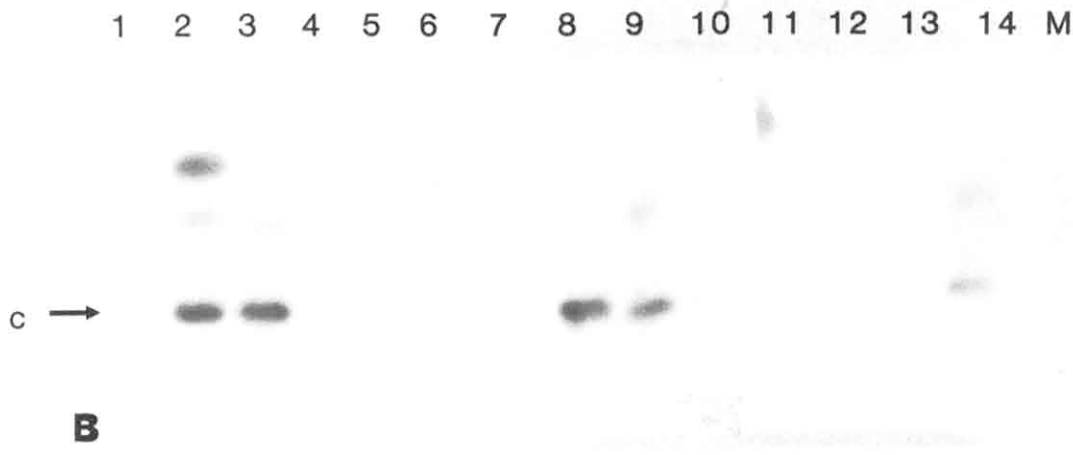

1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
c
B

Table 5.4 The number of co-migrating fragments and the number of bands recognised from the Southern blot technique from *Holcaspis* species and the percentage of homologous bands detected from Southern blot test.

Primer	DNA fragments used as probe	Number of co-migrating fragments	Number of fragments probed	Percentage of homologous bands
J267/1 (AGGAGATACC)	a (*H. elongella*)	14	13	92.85
J267/1 (AGGAGATACC)	b (*H. suteri*)	3	3	100
J-09 (TCTGCCGTGA)	a (*H. ovatella*)	2	1	50
J-09 (TCTGCCGTGA)	b (*H. ovatella*)	2	1	50
J-09 (TCTGCCGTGA)	c (*H. ovatella*)	4	4	100
Total	5	25	22	88

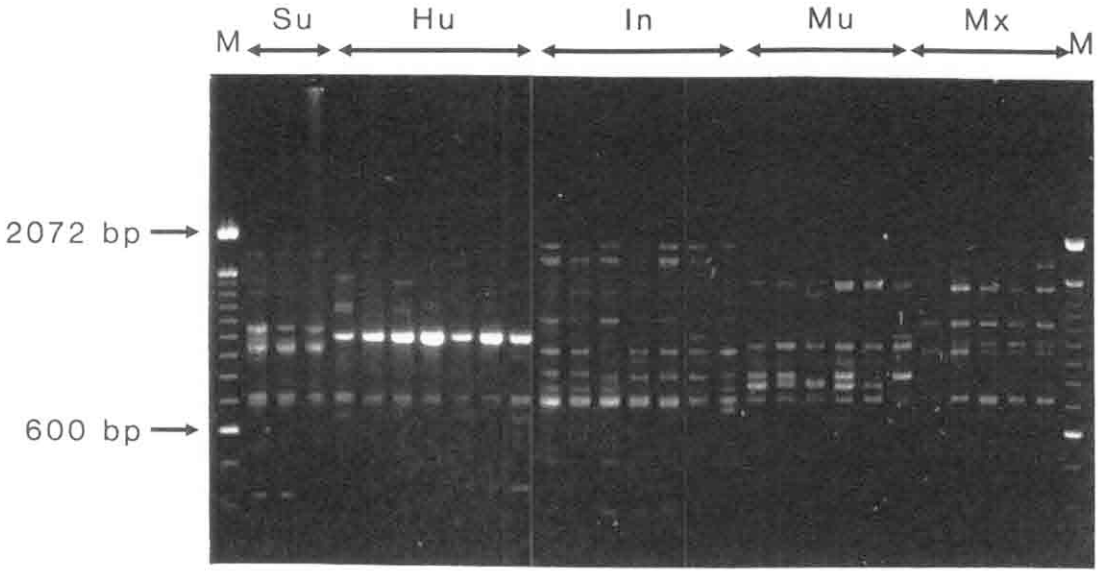
Table 5.5 The percentage of monomorphic fragments of amplified *Holcaspis* species and *Megadromus antarcticus* DNA from each of six primers.

Species	Percentage of Monomorphic Fragment from selected primers						
	OPF-06	G01	OPG-18	OPH-04	J09	J267/1	Mean ± S.D.
H. angustula	50	33	40	63	57	75	53.00 ± 15.35
H. delator	75	100	67	75	75	67	76.50 ± 12.16
H. egregialis	84	50	75	75	100	67	75.17 ± 16.70
H. elongella	72	60	50	57	67	60	61.00 ± 7.69
H. hispida	50	67	63	57	67	40	57.33 ± 10.71
H. hudsoni	63	40	50	80	67	67	61.17 ± 14.13
H. intermittens	40	33	67	100	80	75	65.83 ± 25.29
H. mordax	71	75	72	75	75	67	72.50 ± 3.21
H. mucronata	50	60	60	86	40	50	57.67 ± 15.77
H. oedicnema	11	50	18	17	25	14	22.50 ± 14.27
H. ovatella	75	67	50	80	75	83	71.67 ± 11.93
H. punctigera	45	67	75	67	50	50	59.00 ± 12.18
H. suteri	83	63	75	83	75	67	74.33 ± 8.16
M. antarcticus	60	50	67	67	57	72	62.18 ± 8.04

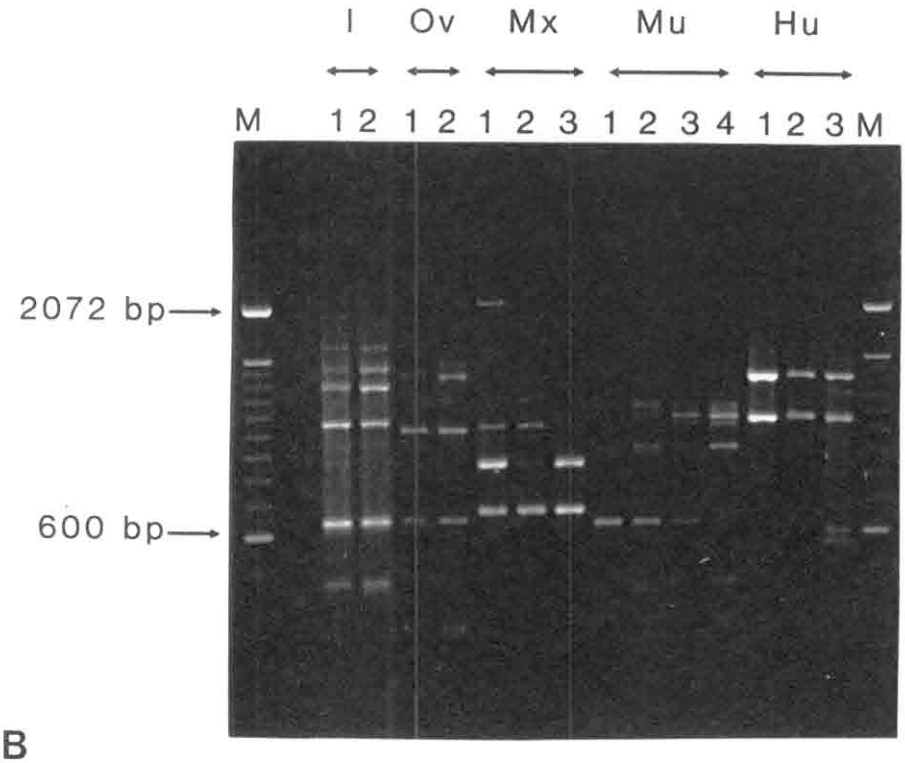
Figure 5.7 The inter-and intraspecific variation in amplified fragment patterns among the *Holcaspis* species. (Amplification products were resolved by electrophoresis in a 1.4 % agarose gel and stained with ethidium bromide.)

(A) Amplified fragments patterns using primer AGG AGA TAC C on DNA extracted from individuals of five species. Abbreviations Su, Hu, In, Mu and Mx refer to *H. suteri*, *H. hudsoni*, *H. intermittens*, *H. mucronata* and *H. mordax* respectively. Lane 'M's are molecular weight markers (100 Base-Pair Ladder) whose scale is shown in base pairs (bp).

(B) Amplified fragments patterns using primer TCT GCC GTG A on DNA extracted from individuals of five species. Abbreviations I, Ov, Mx, Mu, and Hu refer to *H. intermittens*, *H. ovatella*, *H. mordax*, *H. mucronata* and *H. hudsoni* respectively. Lanes 'M's are molecular weight markers (100 Base-Pair Ladder) whose scale is shown in base pairs (bp).


M
Su
Hu
In
Mu
Mx
M
2072 bp
600 bp

A

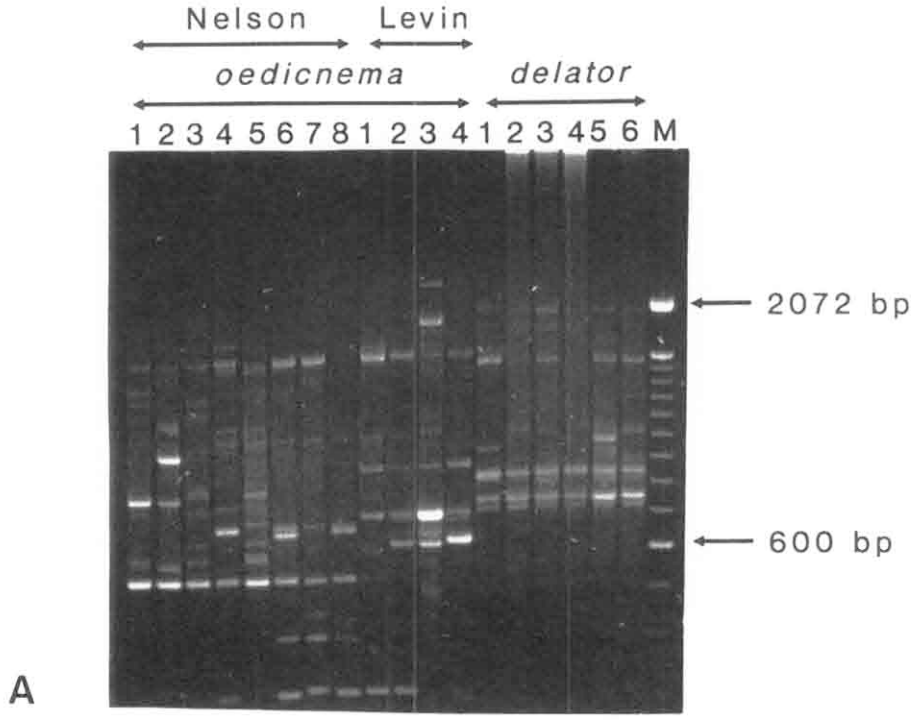

I
Ov
Mx
Mu
Hu
M 1 2 1 2 1 2 3 1 2 3 4 1 2 3 M
2072 bp
600 bp

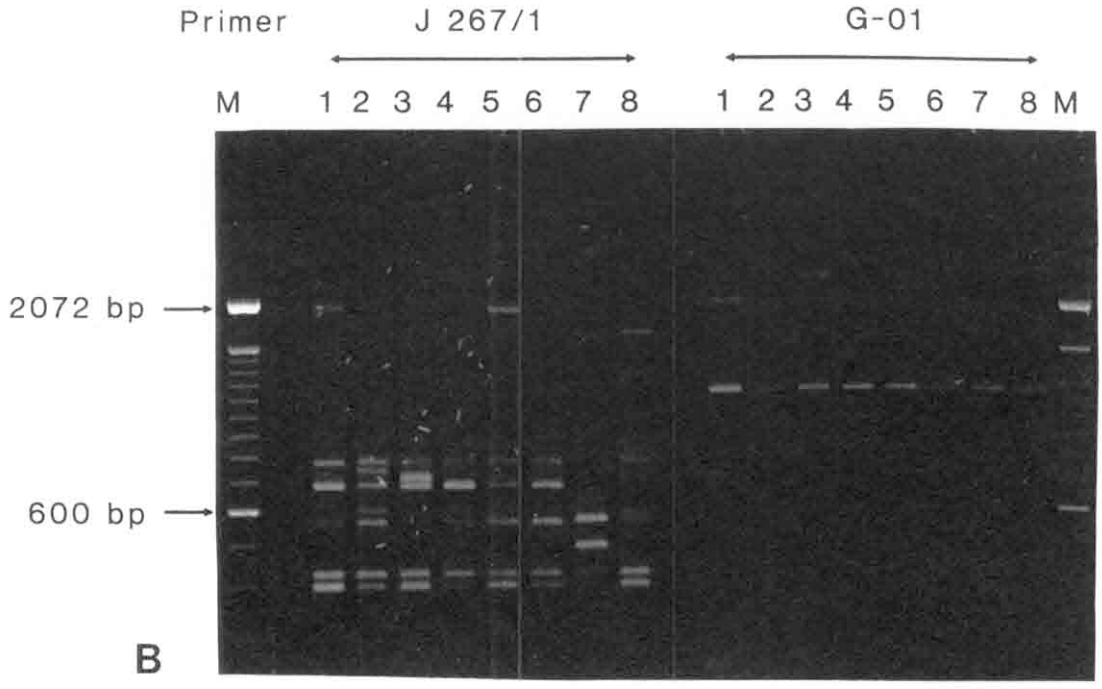
B

Figure 5.8 The intraspecific variation of amplified *Holcaspis* species fragment patterns resolved by electrophoresis in a 1.4 % agarose gel and stained with ethidium bromide.

(A) Representative results of the high variation of bands within *H. oedicnema* compared with *H. delator*, amplified patterns using primer GGG AAT TCG G. (*H. oedicnema* was collected from two strongly allopatric populations: Nelson and Levin). Lane 'M' is molecular weight markers (100 Base-Pair Ladder) whose scale is shown in base pairs (bp).

(B) Representative results of the variation within *H. hispida* as shown by two primers AGG AGA TAC C (J 267/1) and AGT TCG TCT G (G-01).


Nelson
Levin
oedicnema
delator
1 2 3 4 5 6 7 8 1 2 3 4 1 2 3 45 6 M
2072 bp
600 bp
A


Primer
J 267/1
G-01
M 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 M
2072 bp
600 bp
B

(Table 5.5). One species (*H. oedicnema*) contained only 22 % of monomorphic fragments and 77 % of polymorphic fragments in all individuals (as shown in Figure 5.8).

Both coefficients of similarity within species (simple-matching fragments: Table 5.6, and Jaccard's similarity coefficient: Table 5.7) are excessively high (as will be discussed later). The similarity within a species was 73-97 % or 70-96 % from simple-matching fragments and Jaccard's similarity respectively, except in *H. oedicnema*, which showed only 57 % or 52 % similarity.

5.4.4 Phenetic and cladistic analysis of the *Holcaspis* species

5.4.4.1 Phenetic analysis

The results of the phenetic analysis using either cluster analysis by an unweighted pair group method with arithmetic averages, or principal coordinate analysis, are shown in Figures 5.9 and 5.10. Both analyses from the RAPD-PCR data revealed a significant distinctness among the 13 *Holcaspis* species. The coefficient of similarity calculated from simple-matching fragments and Jaccard's coefficient was low as shown in Tables 5.6 and 5.7. The phenograms constructed from both simple matching fragments and Jaccard's coefficient were identical, except that the similarity coefficient obtained using Jaccard's approach was slightly smaller.

The similarity coefficient from simple-matching fragments between species (Table 5.6) ranged from about 27 % for the most closely related species (*H. mucronata* and *H. suteri*) to only 10% for those more distantly related (*H. mordax* and *H. punctigera*). In contrast, the coefficient of similarity within species was found to be extremely high. The similarity within a species was 73-97 %, except for in *H. oedicnema*, which showed only 57 % similarity.

The first three principal coordinates (PCO) of the similarity coefficients of 13 species accounted for 27 % of variation (Figure 5.10). The PCO depicts, more or less, the same pattern of species relationships as seen in the clustering analysis. However, the use of principal coordinates did not illustrate the correlation pattern among the species very well, since the variation was very low from those three dimensions.

Table 5.6 *Holcaspis* species pair-wise data matrix of simple-matching fragments similarity coefficients calculated within species (on the diagonal) and between species (below diagonal).

	Species												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *H. mordax*	0.725												
2 *H. mucronata*	0.100	0.909											
3 *H. hudsoni*	0.123	0.137	0.809										
4 *H. intermittens*	0.170	0.205	0.232	0.870									
5 *H. punctigera*	0.101	0.137	0.136	0.115	0.735								
6 *H. hispida*	0.094	0.155	0.125	0.183	0.051	0.783							
7 *H. suteri*	0.057	0.272	0.135	0.180	0.102	0.136	0.928						
8 *H. elongella*	0.147	0.159	0.202	0.199	0.122	0.192	0.188	0.875					
9 *H. egregialis*	0.170	0.146	0.111	0.199	0.098	0.140	0.119	0.265	0.958				
10 *H. angustula*	0.082	0.171	0.128	0.242	0.107	0.155	0.228	0.244	0.224	0.754			
11 *H. oedicnema*	0.101	0.186	0.141	0.225	0.118	0.151	0.186	0.191	0.147	0.136	0.574		
12 *H. ovatella*	0.118	0.161	0.174	0.142	0.065	0.144	0.217	0.141	0.129	0.215	0.107	0.939	
13 *H. delator*	0.060	0.140	0.130	0.162	0.095	0.103	0.154	0.091	0.067	0.097	0.173	0.142	0.968

Table 5.7 *Holcaspis* species pair-wise data matrix of Jaccard's similarity coefficient calculated within species (on the diagonal) and between species (below diagonal).

	Species												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *H. mordax*	0.715												
2 *H. mucronata*	0.053	0.836											
3 *H. hudsoni*	0.066	0.074	0.786										
4 *H. intermittens*	0.093	0.114	0.132	0.791									
5 *H. punctigera*	0.053	0.074	0.073	0.064	0.699								
6 *H. hispida*	0.050	0.087	0.067	0.101	0.026	0.728							
7 *H. suteri*	0.030	0.157	0.073	0.099	0.054	0.073	0.866						
8 *H. elongella*	0.080	0.087	0.113	0.110	0.065	0.106	0.104	0.779					
9 *H. egregialis*	0.093	0.079	0.059	0.110	0.052	0.075	0.063	0.153	0.937				
10 *H. angustula*	0.043	0.094	0.069	0.138	0.057	0.084	0.130	0.139	0.126	0.706			
11 *H. oedicnema*	0.054	0.103	0.076	0.127	0.063	0.082	0.103	0.106	0.081	0.073	0.522		
12 *H. ovatella*	0.063	0.088	0.096	0.076	0.034	0.078	0.121	0.076	0.069	0.121	0.057	0.886	
13 *H. delator*	0.031	0.075	0.069	0.088	0.050	0.055	0.083	0.048	0.034	0.051	0.096	0.076	0.957

Figure 5.9 The phenogram obtained from the random amplified polymorphic DNA data of *Holcaspis* species individuals using cluster analysis with unweighted pair group method with arithmetic averages (UPGMA).

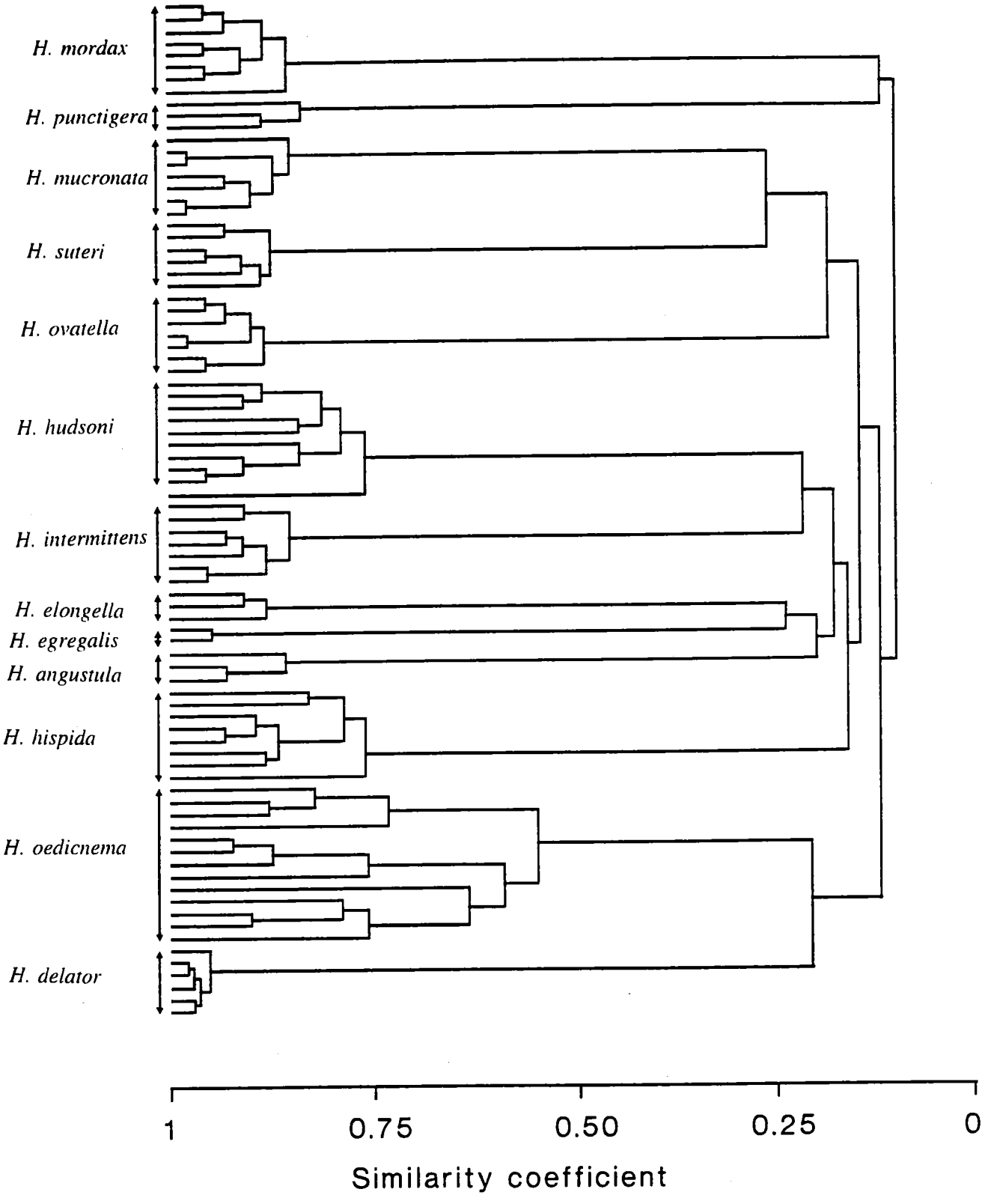
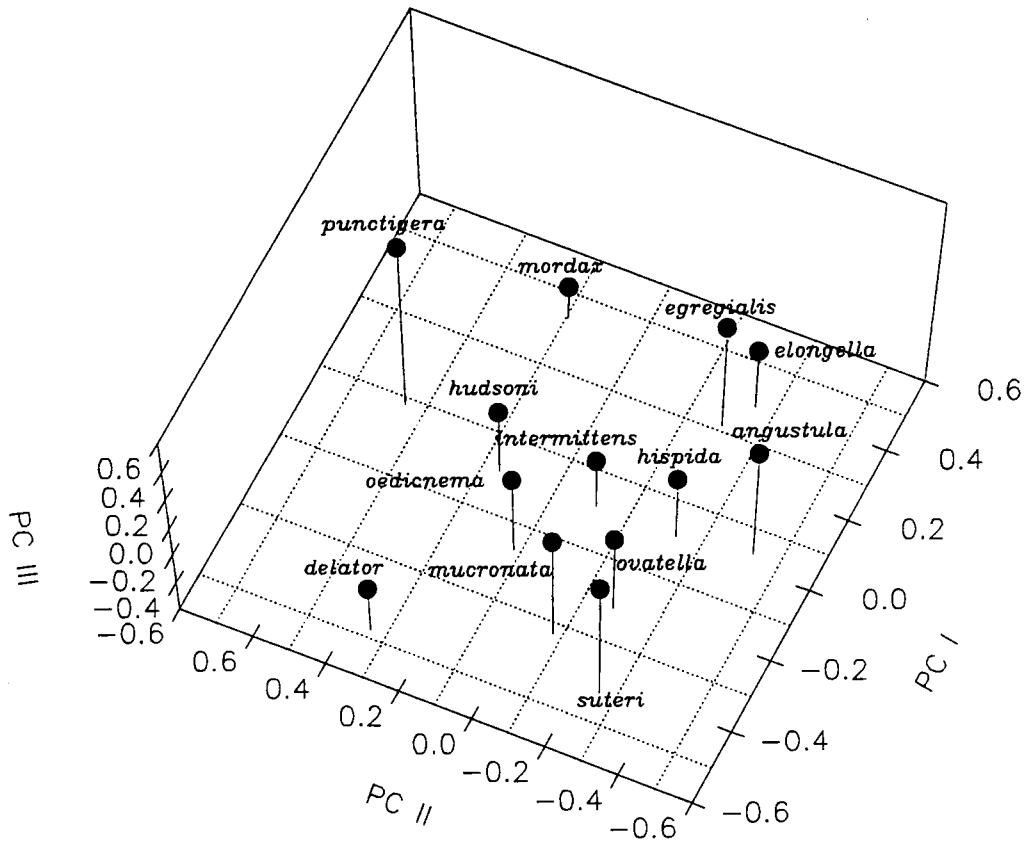

H. mordax
H. punctigera
H. mucronata
H. suteri
H. ovatella
H. hudsoni
H. intermittens
H. elongella
H. egregalis
H. angustula
H. hispida
H. oedicnema
H. delator
1
0.75
0.50
0.25
0
Similarity coefficient

Figure 5.10 Principal coordinate analysis of *Holcaspis* species, a three dimensional projection of the taxa on axes representing the first three dimensions. (The percentages of variation explained by each of the three dimension are: I = 10.17 % , II = 8.75 %, III = 8.13 %. The total variation explained by the model is 27.05 %.)


punctigera
mordax
egregialis
elongella
hudsoni
intermittens
angustula
hispida
oedicnema
delator
mucronata
ovatella
suteri
PC III
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
PC II
PC I

5.4.4.2 Cladistic analysis

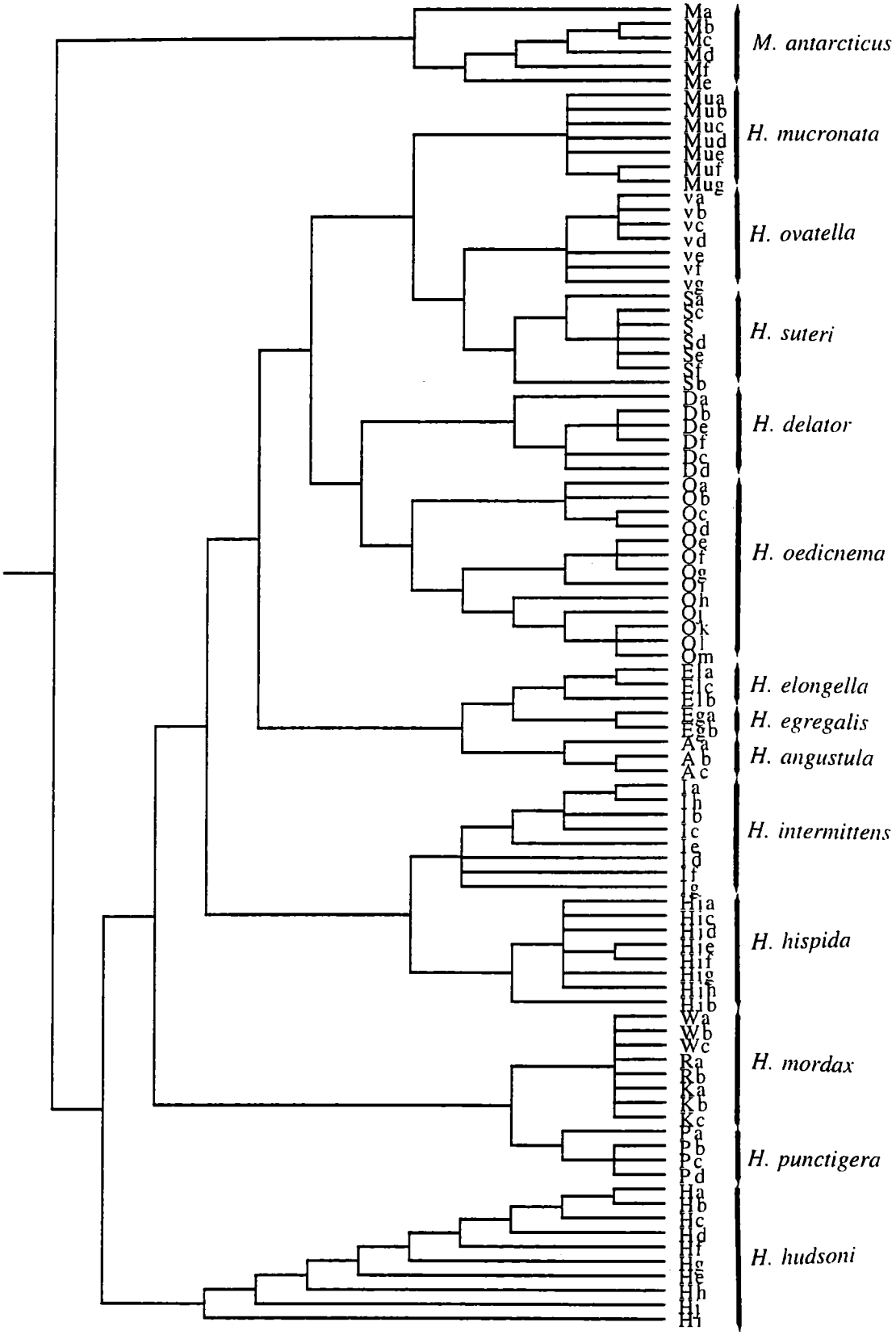
A total of 271 fragments from the six selected primers were treated as characters for cladistic analysis of 92 individuals from the 13 *Holcaspis* species and one outgroup species, *M. antarcticus*. The 265 fragments were phylogenetically informative (synapomorphic) characters. Only six characters were autoapomorphic and were excluded from the analysis. The Fitch parsimony analysis resulted in at least 300 equally parsimonious trees, requiring a total of 598 character changes with a consistency index (CI) of 0.443 (Kluge and Farris 1969). Calculation of the consistency index excluded uninformative characters (autoapomorphies). The strict consensus tree of individuals was constructed from the 300 parsimonious trees (Figure 5.11).

The cladogram of 13 *Holcaspis* species using the RAPD-PCR data is depicted in Figure 5.11. It shows *H. ovatella* and *H. suteri* grouped together and they are the sister group to *H. mucronata*. This group is then the sister group of *H. delator* and *H. oedicnema*. The sister group of *H. angustula* is a clade consisting of *H. elongella* and *H. egregialis*. *Holcaspis hispida* is clustered with *H. intermittens* and connected to the clade of *H. angustula*, *H. elongella* and *H. egregialis*. *Holcaspis mordax* and *H. punctigera* are together and connected to the clade of *H. intermittens* and *H. hispida*. The genome of *H. hudsoni* was found to be distinct from the genome of the other *Holcaspis* species. However, the separation of *H. hudsoni* from the other 12 *Holcaspis* species was slightly less supported by it only sharing a few synapomorphies.

5.5 Discussion

From this study, the use of the Southern blot technique was successful in confirming the similarity of DNA sequences between RAPD-PCR bands. However, the Southern blot technique also produced weak positive signals of additional bands that migrated to unexpected positions (as can be seen in Figures 5.5B and 5.5C). These bands were sometimes smaller or larger than the probe. However, their exact nature is unclear. They may correspond to: 1) the same DNA sequence amplified from an alternative primer binding site; 2) a sequence of DNA present at a different location but homologous (having

Figure 5.11 The strict consensus tree of all *Holcaspis* individuals produced from 300 most parsimonious trees using parsimony analysis.


M. antarcticus
H. mucronata
H. ovatella
H. suteri
H. delator
H. oedicnema
H. elongella
H. egregalis
H. angustula
H. intermittens
H. hispida
H. mordax
H. punctigera
H. hudsoni

a similar sequence) to the probe; 3) a single stranded DNA from the same sequence, which migrates to a different location in the agarose gel; or 4) heterologous DNA hybridizing to DNA from other bands that may have contaminated the probe during its purification. This contaminating DNA may produce the extra bands. Although the probe was checked after purification, giving a single band on an agarose gel, the quantity of DNA run in the gel was very low and it is possible that contaminating bands were not detected.

Despite these unexpected bands, the nature of which can be precisely determined only by sequencing, the Southern blots were informative. The washes, after hybridization, were done under stringent conditions. Under these conditions (wash temperature 65°C, low salt concentration), the probe will not bind to DNA that is not highly homologous. The melting temperature of the DNA hybrid is reduced by 1°C for every 1 % of sequence mismatch between the two strands (Bonner *et al.* 1973). Therefore the DNA to which the probe attaches is highly homologous and it is possible to conclude that in 88 % of the cases, co-migrating bands correspond to the same DNA.

An essential question when looking at characters being considered for use in phylogenetic studies is the homology of the characters. For phylogeny, a homologous character is an inheritable character. In this study, the amplified fragments (characters) from RAPD-PCR are direct portions of the genome, and therefore are highly heritable.

A possible weakness of this approach for phylogenetic studies is that the RAPD-PCR may amplify 'junk' (non-coding) DNA, such as DNA that does not contain genes that is thought to make up the majority of the total genomic DNA (Lewin 1990). This DNA is not expressed and therefore is under different evolutionary constraints. However, the primers selected had 50-70 % C+G content and are thus more likely to bind to expressed DNA, which has a high C+G content, than to non-expressed DNA.

The results of this study showed that the cladograms and phenogram produced from cladistic and phenetic analyses of *Holcaspis* RAPD-PCR data show relatively highly congruent relationships among *Holcaspis* species. The results, from both phenetic and cladistic analysis, confirmed the close relationship of *H. mordax* and *H. punctigera.* The similarity of these two species was suggested by Butcher (1984). Even though the two species are well separated geographically, they apparently share parts of the same gene

pool. The results also suggest that *H. punctigera* and *H. mordax* are more genetically divergent than the rest of *Holcaspis* species. This result agrees with the result from the allozyme data (Chapter 4). Also, similar to the allozyme data, *H. delator* and *H. oedicnema* were clustered together in both analyses. Butcher (1984) placed *H. delator* in the *oedicnema* species group, indicating a relatively close relationship. The grouping of the clade *H. elongella*, *H. egregialis* and *H. angustula* was congruent in both the phenetic and cladistic analyses and strongly congruent with the allozyme data results (Chapter 4).

This study has shown with the value of consistency index of 0.433 in that the tree derived from cladistic analysis gave considerable convergence (56 % homoplasy), as well as the low genetic similarity of each *Holcaspis* species from clustering analysis. The high degree of homoplasy may be explained by the large nucleotide diversities in the genomes of the *Holcaspis* species. Caccone and Powell (1990) pointed that there is a high rate of divergence in insect genomic DNA compared with other sorts of DNA. The same result was also shown in *Drosophila* where there was high population diversity in the genomic DNA (Caccone and Powell 1990).

At the intraspecific level, in both the cladograms and phenogram, the populations of *H. oedicnema* show a greater degree of heterogeneity within the species group, which suggests that there could be a species complex involved. Moreover, the study suggests that it is likely that the amount of genomic variation among *H. oedicnema* individuals was much greater than that detected in the other species. This coincided with a large number of polymorphisms or a low percentage of monomorphism of the genome (as shown in the Table 5.5). The result is supported by the high degree of variation in morphology of this species. Butcher (1984) noted that *H. oedicnema*, the only species distributed in both the North and South Islands, had extremely variable morphology. Characters such as body size, intensity and type of elytral sculpture, and number of setae on the pronotal margin all varied widely. Some distinctive populations can be recognised visually (Emberson, *pers. com.*). The extreme genetic variation within *H. oedicnema* also was supported by showing relatively high level of heterozygosity from allozyme data (Chapter 4) and it can be explained, as pointed out by Ayala (1975), that it occurs if there is little or no gene flow between populations of species that are geographically isolated. Consequently, the populations could gradually become genetically differentiated as a result of their

adaptation to different environments.

At this point, regarding the highly congruent result from both RAPD-PCR data and allozyme data, it can be concluded that the RAPD-PCR technique has been demonstrated to be a useful method for estimating the species relationships of *Holcaspis* and for detecting the genetic variation within species, in this case *H. oedicnema.*

In conclusion, apart from revealing the relationships among *Holcaspis* species, the results of this study also indicate that even though there is some variation among individuals within a species, some DNA fragments are conserved and are characteristic of a species and present in individuals of that species. Thus the unique banding pattern of all individuals can be used to construct a characteristic profile of such a species. This study has demonstrated that the RAPD-PCR technique has a high probability of correctly grouping together all individuals belonging to the same species. This phenomenon, of accurately grouping individuals, was similar to the work of Kambhampati *et al.* (1992). They found that the RAPD technique was sensitive in identifying an unknown species, which was correctly identified as belonging to the *Aedes albopictus* group.

The RAPD-PCR is very useful for work in which there is only a limited amount of tissue available. Thus it is particularly useful in insect taxonomy because it allows individual specimens to be characterized. Also, in species where no sequence information is available, other methods of genome analysis such as restriction fragmental length polymorphisms (RFLPs) or sequencing, may be too time consuming or require excessive tissue. In these cases, the RAPD-PCR may be the only realistic molecular method. Besides which it has the advantage of being a technically relatively simple and quick molecular technique.

5.6 Summary

The RAPD-PCR was applied to the genus *Holcaspis* in order to: 1) assess its value for systematics studies; 2) assess the degree of polymorphism within the genus; and 3) investigate whether this approach was suitable for studying the genetic relationships of *Holcaspis* species.

Optimal RAPD-PCR conditions were determined for primer concentration,

magnesium chloride concentration and the RAPD-PCR programme, for reproducible and formative of amplifying banding patterns of *Holcaspis* species.

A subset of *Holcaspis* species was evaluated for variability using a set of 20 random 10 base oligonucletiode primers. Six out of 20 tested primers gave formative scorable banding patterns and showed polymorphism between *Holcaspis* species.

The RAPD-PCR showed great promise for grouping individuals together in recognized species. Only *H. oedicnema* showed extreme intraspecific variation with 78 % polymorphic bands and approximately 57 % similarity coefficient.

A total of 271 band positions were scored on presence and absence (1/0) for all individuals studied. Pair-wise comparisons were used to generate simple-matching 's similarity coefficients using shared fragments and Jaccard's s imilarity coefficients using unique and shared fragments. These data were employed to construct phenograms using an unweighted pair group method with arithmetical averages. The two phenograms were identical. Principal coordinate analysis showed more or less the same similarity of species relationships as the phenograms. However, the pattern of species relationships was indistinct.

The cladistic method based on the principle of maximum parsimonious analysis was also applied for constructing phylogenetic relationships. The results showed that both the cladogram and phenogram derived from cladistic and phenetic methods were relatively highly congruent. The pattern of genetic relationships of *Holcaspis* species derived from RAPD-PCR data was relatively highly congruent with the results from allozyme data (Chapter 4). Therefore it can be suggested that the RAPD-PCR technique is a potentially useful technique to investigate phylogenetic relationships among taxa.

CHAPTER 6

The cladistics of *Holcaspis* species: congruence testing by taxonomic congruence and character congruence

6.1 Introduction

It is well known that when different character sets, from the same set of taxa are analyzed to estimate phylogeny, this often results in dissimilar, or similar but non-identical, trees (Farris 1971; Swofford 1991; Baum 1992; Vane-Wright *et al.* 1992). Conflicts in phylogenetic results from using different data sets have been found in many studies. For example, Vane-Wright *et al.* (1992) compared phylogenetic trees of milkweed butterflies (Nymphalidae: Danainae) derived from morphological data and chemical data which were based on the extraction of male volatile substances. They found incongruence between the morphological and chemical data. Shaffer *et al.* (1991) reported conflicting results in a study of the phylogenetic relationships of the salamander family Ambystomatidae, using allozyme data compared with the previous results obtained from morphological data.

Theoretically, phylogenies inferred from different character sets should be congruent with the true tree and therefore with each other (Penny and Hendy 1986; Swofford 1991). In practice, however, the ideal of perfect congruence is hard to achieve. Baum (1992) commented that different data sets may be reflecting the same phylogenetic scheme of those taxa but the characters used may be subjected to random error and, consequently, different samples of characters may, by chance, yield different results.

Until now, there has been considerable debate over whether different taxonomic results for the same set of taxa, from different character sets, can be minimized by using either consensus techniques or a combination technique (Miyamoto 1985; Hillis 1987; Faith 1988; Baum 1992). These approaches have been named '*taxonomic congruence*' and '*character congruence*' (or total evidence) methods, respectively (Kluge 1989; Shaffer *et al.* 1991; Vane-Wright *et al.* 1992). The taxonomic congruence method focuses on constructing consensus trees from the phylogenetic trees generated by different methods or

data sets and results in a compromise classification (Adam 1972; Mickevich 1978; Nelson 1979; Nelson and Platnick 1981; Schuh and Farris 1981; Rohlf 1982). The character congruence method combines all of the available character sets used into a single pooled data set for analysis (Kluge, 1989; Miyamoto, 1985).

The primary objective of this study was to elucidate the phylogenetic relationships of the genus *Holcaspis* using different, independently generated data sets. However, the phylogenetic trees derived from the three different character sets: morphological, allozyme and RAPD-PCR data (Chapters 3, 4 and 5, respectively), resulted in different inferred phylogenetic patterns.

Since the phenetic method, using morphometric analysis (Chapter 3) did not produce useful results to explain the relationships among *Holcaspis* species, this chapter will focus only on the cladistic analysis using maximum parsimony to investigate the phylogenetic relationships.

Thus, the purpose of this chapter is to estimate and assess the best-fit of the phylogenetic relationships in the genus *Holcaspis* from the three different data character sets: morphological, allozyme and RAPD-PCR data.

6.2 Materials and methods

In the analysis, the trees derived from the allozyme data were obtained from Chapter 4 (Figures 4.3 and 4.4). For the purpose of comparison, the other two character sets (morphological and RAPD-PCR) were reanalysed separately in order to have the same taxa as the trees derived from allozyme data.

The morphological data, comprising 26 morphological characters, were obtained from Chapter 3. Thirteen of the 30 *Holcaspis* species were used in the analysis.

The RAPD-PCR data were obtained from Chapter 5 . In order to keep the number of taxa manageable and for compatibility with the other two character data sets, the data from all individuals were lumped (Appendix 1). Each species was represented by all individual. The fragment profile of a species was generated using the criterion that the scorable fragment is characteristic of a species if it is shared by at least two individuals of that species. The data from both the morphological and RAPD methods were treated as

comprising unordered characters.

To derive the *Holcaspis* phylogenetic tree from three different character sets, both the character congruence method and the taxonomic congruence method (consensus technique) were used. All three data sets were combined in the character congruence method by: 1) adding the data sets of 26 morphological characters, 13 allozyme loci characters and 271 RAPD-PCR characters (Appendix 2); 2) adding the data sets of 26 morphological characters, 42 allozyme allele characters and 271 RAPD-PCR characters (Appendix 3). The resulting data sets were analyzed using the heuristic method with tree bisection reconnection in branch-swapping (100 random seedings) option of PAUP 3.0 (Swofford 1992). *Megadromus antarcticus* was used as the outgroup and all characters were treated as unordered.

For taxonomic congruence analysis, the shortest trees derived from each character set were combined by using the consensus tree option in PAUP 3.0 of Swofford (1992). There were two options used in this analysis: 1) trees derived from morphological data, allozyme locus data and RAPD-PCR data; and 2) trees derived from morphological data, allozyme allele data and RAPD-PCR data.

6.3 Results and discussion

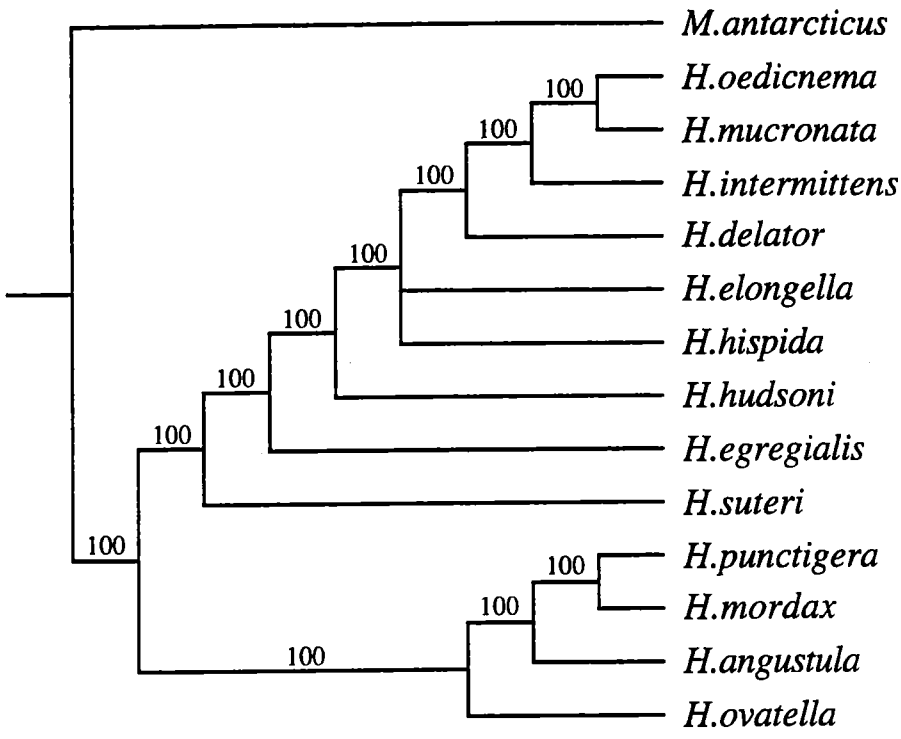
6.3.1 Phylogenetic trees based on each character set

Parsimony analysis of the 13 *Holcaspis* species using the 26 morphological characters (Chapter 3, Table 3.1) produced two shortest trees of length of 68 character changes, consistency index (CI) of 0.515 and retention index (RI) of 0.571. These trees were condensed as a majority rule consensus tree as shown in Figure 6.1.

From the allozyme analysis using the 42 alleles (electromorphs) and 13 loci obtained from Chapter 4 (Figures 4.3 and 4.4, respectively), two most parsimonious trees with a length of 125 character changes, CI of 0.336 and RI of 0.461 were derived by scoring each allele as a character. These two trees were condensed as a majority rule consensus tree as shown in Figure 6.3. A single, most parsimonious tree was obtained from the analysis by coding each locus as a character (Figure 4.4: length of 132 character changes,

Figure 6.1 Majority rule consensus tree of the two shortest trees derived from morphological data from *Holcaspis* species. (The percentage of two shortest trees that contain that component are indicated along each branch.)

Figure 6.2 Majority rule consensus tree of the two shortest trees derived from RAPD-PCR data of *Holcaspis* species. (The percentage of two shortest trees that contain that component are indicated along each branch.)


M.antarcticus
H.oedicnema
H.mucronata
H.intermittens
H.delator
H.elongella
H.hispida
H.hudsoni
H.egregialis
H.suteri
H.punctigera
H.mordax
H.angustula
H.ovatella
100

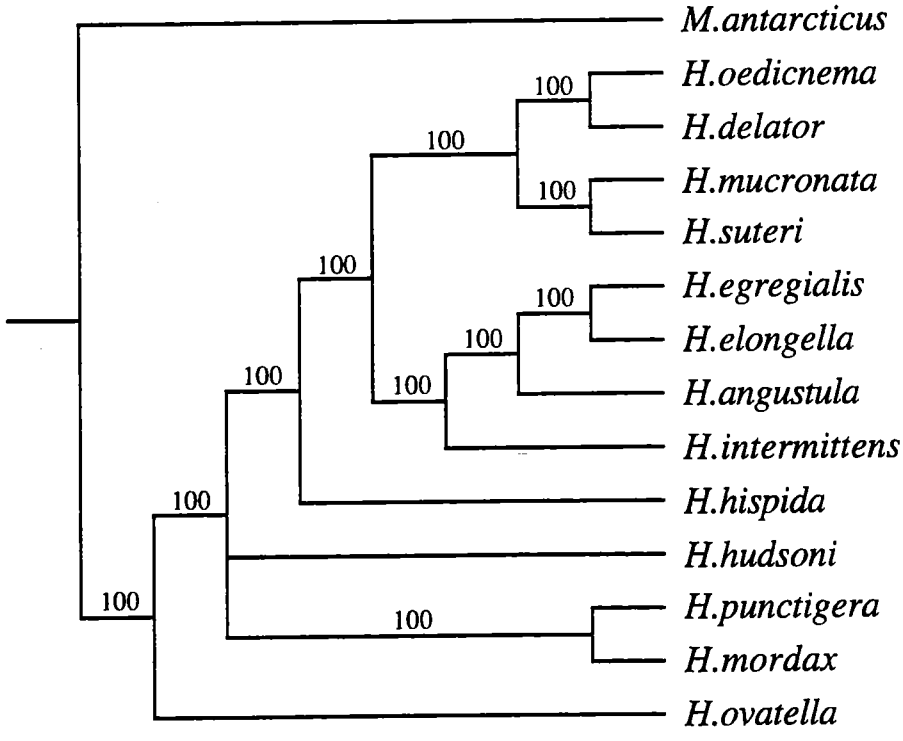
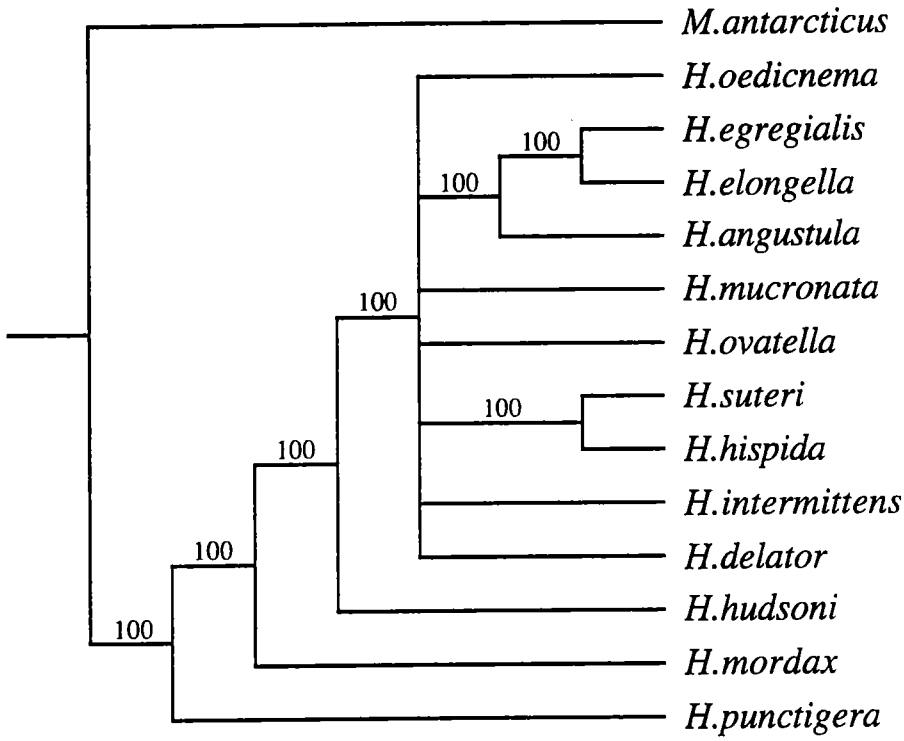

M.antarcticus
H.oedicnema
H.delator
H.mucronata
H.suteri
H.egregialis
H.elongella
H.angustula
H.intermittens
H.hispida
H.hudsoni
H.punctigera
H.mordax
H.ovatella
100

Figure 6.3 Majority rule consensus tree of *Holcaspis* species of the two shortest trees derived from allozyme data using independent allele as character. (The percentage of two shortest trees that contain that component are indicated along each branch.)


M.antarcticus
H.oedicnema
H.egregialis
H.elongella
H.angustula
H.mucronata
H.ovatella
H.suteri
H.hispida
H.intermittens
H.delator
H.hudsoni
H.mordax
H.punctigera
100
100
100
100
100
100
100

CI of 0.318 and RI of 0.416).

Parsimony analysis of the 271 characters of the RAPD-PCR from species pooled data (Appendix 2) also yielded two most parsimonious trees but of length of 394 character changes, CI of 0.660 and RI of 0.174. These two trees were condensed as a majority rule consensus tree (Figure 6.2).

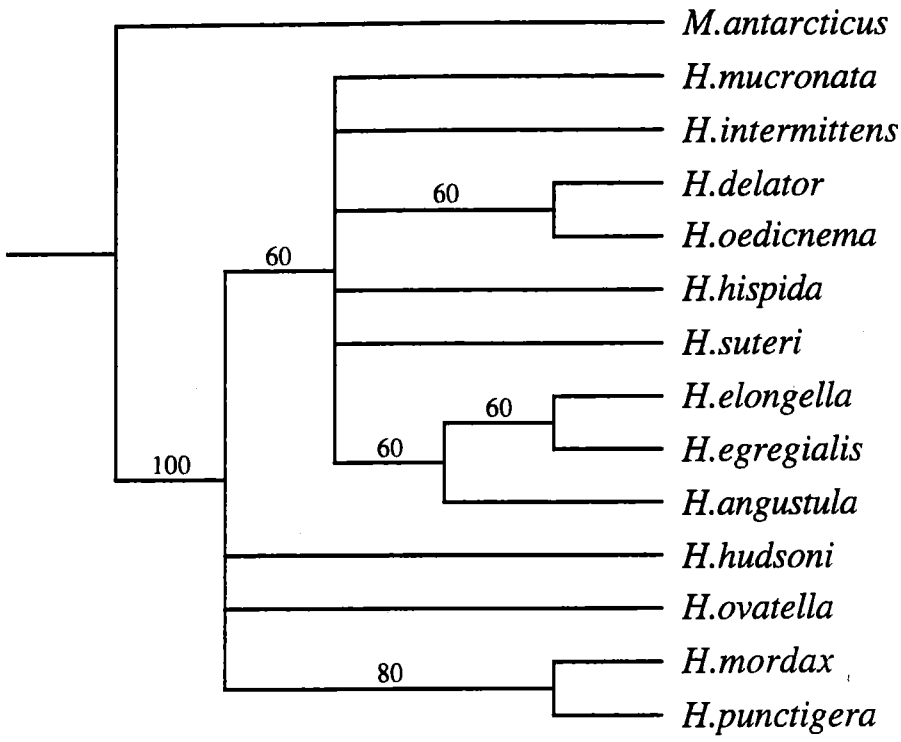
6.3.2 A comparison of the phylogeny of the genus *Holcaspis* using taxonomic congruence and character congruence methods

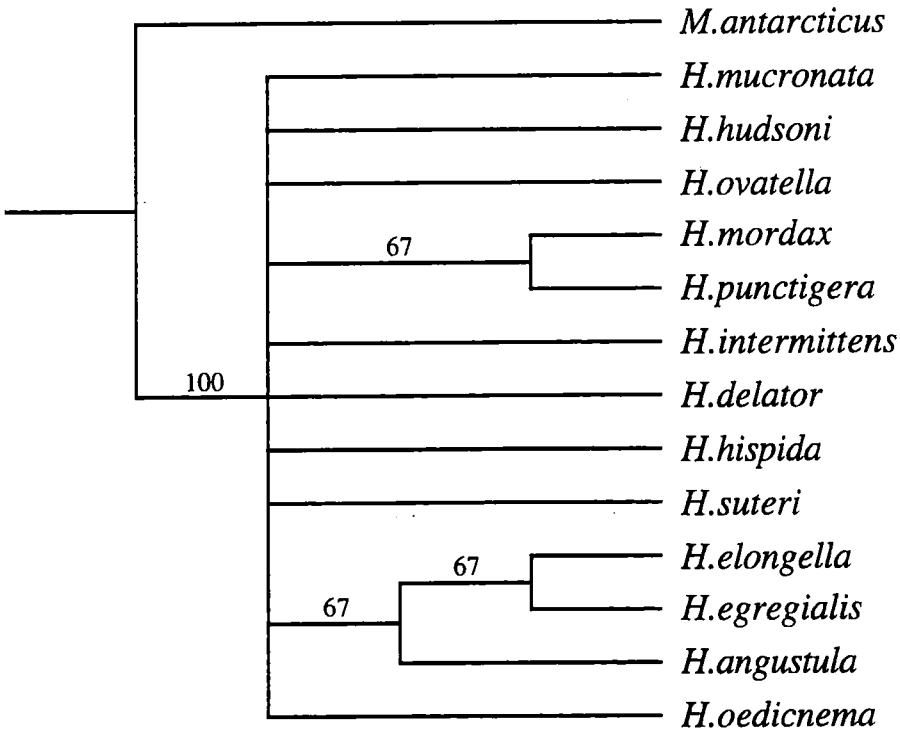
The results from the trees produced from allozyme data set (allele and locus) and RAPD-PCR data sets corresponded, with some groups in common (*oedicnema-delator*; *egregialis-elongella-angustula*). However, there was little congruence between the trees derived from morphological data and from the molecular data (allozyme data and RAPD-PCR data).

Following the taxonomic congruence, to maximize the information content for all trees derived from different character sets, shortest trees derived from morphological, allozyme (locus) and RAPD-PCR data sets (Appendix 2) and morphological, allozyme (allele) and RAPD-PCR data sets (Appendix 3) were summarized using majority-rule consensus as shown in Figures 6.4 and 6.5, respectively. It is suggested that the consensus index is an indicator providing a quantitative measure of the agreement among the trees. Consensus indices typically vary between 0 (implying no agreement among the rival classifications) and 1 (implying identity agreement possible) (Swofford 1991). In this study, the consensus tree derived from the morphological, allozyme (locus) and RAPD-PCR data sets produced consensus indices of: the component information index of Nelson (1979) of 0.455; Mickevich's (1978) consensus index of 0.250 and Rohlf's (1982) CI of 0.232. The consensus tree derived from the morphological, allozyme (allele) and RAPD-PCR data sets produced lower values of these consensus indices (component information index of Nelson (1979) of 0.333; Mickevich's (1978) consensus index of 0.119 and Rohlf's (1982) CI of 0.211). Therefore, it can be concluded that the consensus tree derived from the morphological, allozyme (locus) and RAPD-PCR data sets reflects a higher level of congruence than the consensus tree derived from the morphological,

Figure 6.4 Majority rule consensus tree of the five most parsimonious trees generated from morphological, allozyme (locus) and random amplified polymorphic DNA character data sets using the taxonomic congruence method. (The percentage of the five shortest trees that contain that component are shown along each branch.)

Figure 6.5 Majority rule consensus tree of the six most parsimonious trees generated from morphological, allozyme (independent allele) and random amplified polymorphic DNA, character data sets using the taxonomic congruence method. (The percentage of the six shortest trees that contain that component are shown along each branch.)


M.antarcticus
H.mucronata
H.intermittens
H.delator
H.oedicnema
H.hispida
H.suteri
H.elongella
H.egregialis
H.angustula
H.hudsoni
H.ovatella
H.mordax
H.punctigera
60
60
60
60
100
80


M.antarcticus
H.mucronata
H.hudsoni
H.ovatella
H.mordax
H.punctigera
H.intermittens
H.delator
H.hispida
H.suteri
H.elongella
H.egregialis
H.angustula
H.oedicnema
67
100
67
67

allozyme (allele) and RAPD-PCR data sets.

Estimates of *Holcaspis* species phylogeny using the character congruence method, from the combination of the morphological, allozyme (locus) and RAPD-PCR character sets and the combination of the morphological, allozyme (allele) and RAPD-PCR character sets, resulted in one and four maximum parsimonious trees of length of 463 and 618 character changes, CIs of 0.620 and 0.545, and rescaled consistency indices (RC) of 0.190 and 0.175, respectively (Figures 6.6 and 6.7).

Based on the assumption that a high index (CI and RC) value indicates high congruence among the character data sets used in phylogenetic hypotheses (Kluge and Farris 1969; Farris 1989), this study revealed that the trees derived from using morphological, allozyme (locus) and RAPD-PCR data sets showed greater congruence and inferred phylogenetic relationships than the trees derived from morphological, allozyme (allele) and RAPD-PCR data sets.

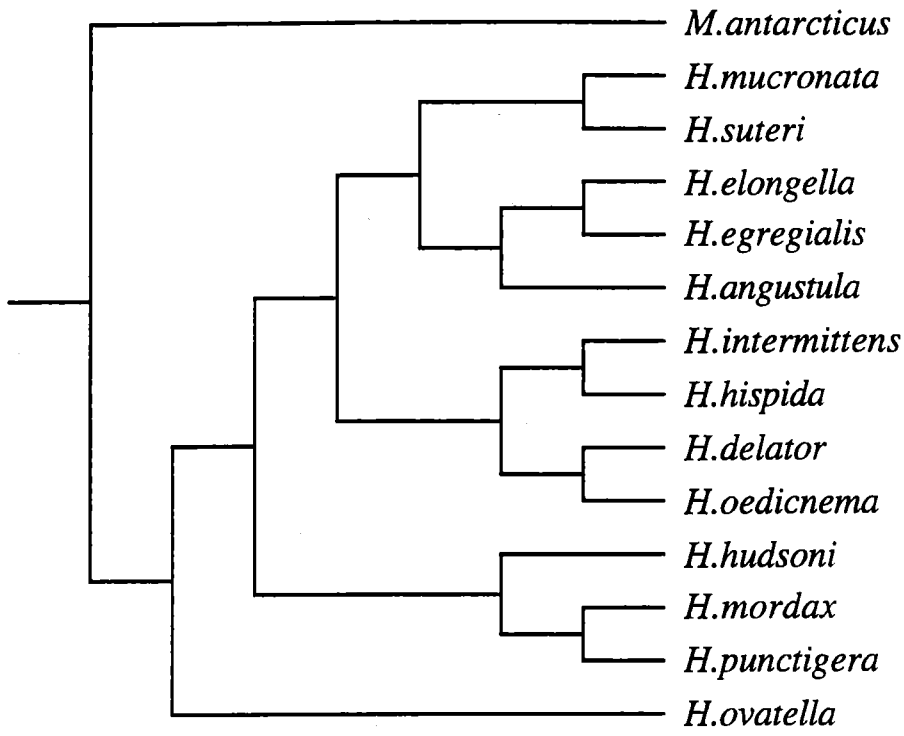
The results showed, with these two analyses (taxonomic congruence and character congruence), that trees derived from using locus data as a component of analysis, led to more resolved trees and better fit of phylogenetic relationships than using allele data. Murphy (1993) stated that the presence-absence coding of alleles, as characters, can result in the development of a less parsimonious solution than could be obtained by using loci as the characters.

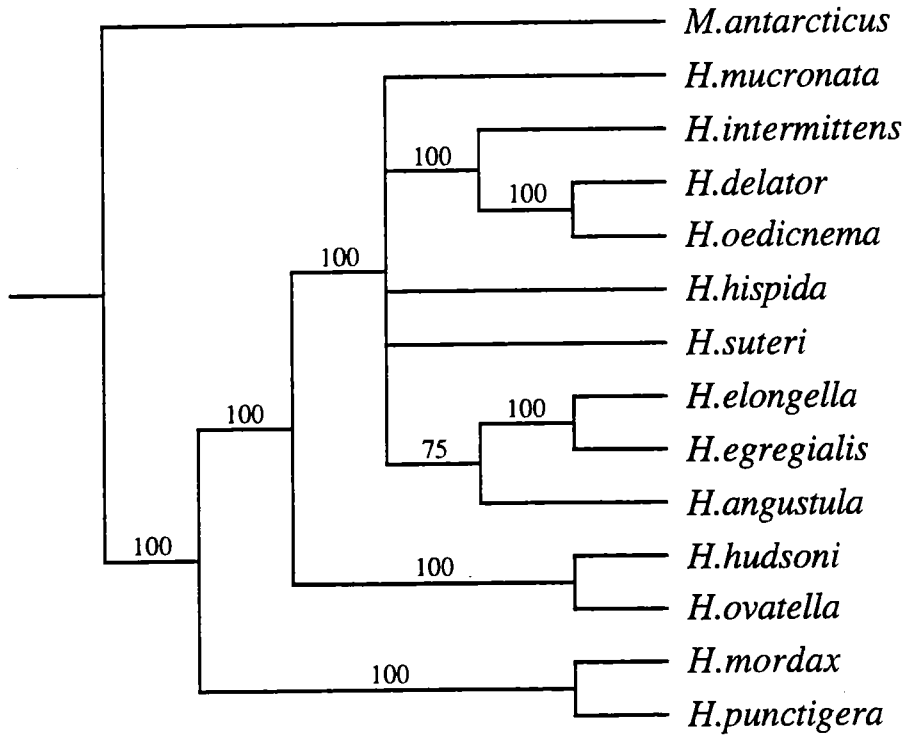
Comparison of the trees generated using taxonomic congruence and character congruence methods from morphological, allozyme (locus) and RAPD-PCR data revealed that the trees derived from the character congruence method, using a single pooled data set, gave more resolution and information, on *Holcaspis* phylogeny, than using the taxonomic congruence method (Figures 6.4 and 6.6). Similar results of higher resolution with character congruence than with taxonomic congruence trees were also obtained by Faith and Cranston (1991) and Vane-Wright *et al.* (1992).

The use of the taxonomic congruence method has been criticized on the basis that it only depicts the degree of agreement on congruence between classification, but adds nothing to the phylogeny. Moreover, this method provided a less parsimonious explanation than the combination of data sets for reanalysis, which has the advantage of greater information content and global parsimony (Miyamoto 1985; Hillis 1987; Kluge

Figure 6.6 A most parsimonious tree generated from morphological, allozyme (locus) and random amplified polymorphic DNA character data sets using character congruence method.

Figure 6.7 Majority rule consensus tree of the four most parsimonious trees generated from morphological, allozyme (independent allele) and random amplified polymorphic DNA character data sets using the character congruence method. (The percentage of the four shortest trees that contain that component are shown along each branch.)


M.antarcticus
H.mucronata
H.suteri
H.elongella
H.egregialis
H.angustula
H.intermittens
H.hispida
H.delator
H.oedicnema
H.hudsoni
H.mordax
H.punctigera
H.ovatella


M.antarcticus
H.mucronata
H.intermittens
H.delator
H.oedicnema
H.hispida
H.suteri
H.elongella
H.egregialis
H.angustula
H.hudsoni
H.ovatella
H.mordax
H.punctigera
100
100
100
100
100
75
100
100
100

1989). Baum (1992) stated that taxonomic congruence, or the consensus method, was of limited value for combining data sets, because the consensus method loses information, descriptive power and resolution.

6.3.3 The phylogeny of *Holcaspis* species

The tree constructed from a combination of morphological, allozyme (locus) and RAPD-PCR) characters using character congruence gives a best assessment of the phylogeny of *Holcaspis* species (Figure 6.6). The tree shows that *H. ovatella* is the most distinct species of the group, and that *H. punctigera* and *H. mordax* are closely related. These results support the previous groupings of Butcher (1984). *Holcaspis oedicnema*, *H. delator*, *H. intermittens* and *H. hispida* are grouped together, which also agrees with Butcher (1984). He grouped these species into the *oedicnema* group. However, the results do not support Butcher's grouping of species complexes. In comparison with Butcher's (1984) work, this study has rejected the grouping of *H. hudsoni* and *H. suteri* into the *hudsoni* species group. These results show that *H. mucronata* and *H. suteri* are more closely related species, while *H. hudsoni* is related to the *punctigera* group. However, the aims of this study were different from those of Butcher (1984). The results from this study were derived specially to determine phylogenetic relationships of the species, Butcher's work was based only on the grouping of morphological similarity among the species, but similarity can arised by descent and by convergent evolution. Therefore his groups, which imply species relationships, are not based on unambiguous evidence.

6.4 Summary

In this chapter, an estimation of phylogenetic relationships of *Holcaspis* species from morphological, allozyme, and random amplified polymorphic DNA based on polymerase reaction character data sets using character congruence revealed a single parsimonious tree with well resolved phylogenetic information (Figure 6.6). The trees generated using the allozyme (locus) data as one of the components in character congruence gave a better

character fit in phylogenetic hypotheses than those using independent alleles. The character congruence method of combining all character data sets produced a more informative result than the taxonomic congruence method.

CHAPTER 7

Concluding summary

7.1 Introduction

Ever since the New Zealand beetle genus *Holcaspis* was first described in 1886, understanding of the systematic relationships of the species has been limited, mainly, to species differentiation and identification. The relationships of the species to each other remained largely unknown. This study has explored different character data sets, techniques, and systematics methodologies in an attempt to understand and reveal *Holcaspis* species relationships. The outcomes of this study as well as the advantages and disadvantages of the different techniques and methodologies are discussed in this chapter.

7.2 Phylogeny of *Holcaspis* species derived from different methodologies

The task of systematics studies to assessing phylogenetic relationships is usually involved with two main themes: appropriate methodological models and informative character data sets (West and Faith 1990). Some systematics methodologies like phenetics and cladistics are well known. In phenetics, a commonly used procedure is the unweighted pair-group method with arithmetic mean (UPGMA), and a tree (phenogram) is constructed from a distance matrix. Unlike the phenetic method, the cladistic method produces trees (cladograms) from character states.

In general, the different methods, phenetics and cladistics, have been used to solve a range of diverse problems in systematics. However, the controversy between these two methods is still alive (Jensen 1983; Sokal 1983; Sneath 1988). The similar and dissimilar results from the same character data set produced by these two methods can be seen in many works (e.g., Buth 1984; Kessler and Avise 1984; Sourdis and Krimbas 1987; McIntyre 1988; Brasher *et al.* 1992). In this study, two data sets (allozyme data, Chapter 4; RAPD-PCR data, Chapter 5) were compared by both phenetic and cladistic methods.

The trees from each method for the allozyme data set were highly congruent (Figure 4.2 and Figures 4.3 and 4.4). The trees for the RAPD-PCR data set were also highly congruent (Figures 5.9 and 5.10)

These results are similar to those of Varvio-Aho *et al.* (1984). They presented the phylogenetic relationships of social wasps (Hymenoptera: Vespidae) from allozyme electrophoresis data using both phenetic and cladistic methods. They found that the trees derived from the two analytical methods were concordant. Another study, that of Brasher *et al.* (1992), using mitochondrial DNA data, found that trees from both phenetic and cladistic methods to investigate the phylogenetic relationships of lobsters (Decapoda: Palinuridae) were congruent. In addition, much evidence of congruency can be found in studies involving phenetic and cladistic methods using allozyme electrophoresis data or DNA data, e.g., Kessler and Avise (1984), McIntyre (1988), Randi *et al.* (1991), Barker *et al.* (1992).

According to Sneath (1988), the congruence of phylogenetic relationships derived from molecular data (allozyme and RAPD-PCR) in this study, using both phenetic and cladistic methods, can be explained. He claimed that if lineages have constant divergence and if the evolution rate is very consistent, then the likelihood is that the phenetic results will be very similar to those from cladistic methods. This phenomenon can often be seen in molecular character data. The reason that is usually given is that, compared with evolution at the phenotypic level, molecular evolution occurs with more consistency in evolutionary rate so that it is the basis for the so called molecular clock (Wilson *et al.* 1977; Kimura 1991). If lineages have a constant rate of divergence, then the phenetic method will be consistent with the cladistic method, as has been found by many authors, e.g., Colless (1970), Felsenstein (1978), Rohlf and Wooten (1988).

The relationships of *Holcaspis* species from morphological data, using both phenetic and cladistic methods were also investigated (Chapter 3). As was pointed out in that chapter, the morphometric analysis was not really adequate to reveal the species relationships in detail, when compared with the cladistic method, but it did reveal a relatively high probability of correctly classifying of the species. In addition, these two methods, using morphological data, gave incongruent species relationships patterns (Figures 3.8 and 3.11). This result is possibly because morphometric analysis does not set

any standard or model for selecting the characters to be studied; it has to involve a large number of characters, which are measured from all parts of the body. Consequently, the information in the data set often turns out to be highly biased in several ways. For example, 1) character measurements are often uneven by region as well as by orientation (e.g., the pronotum, Figure 3.5), 2) the character measurements do not have the same weight in all body areas used (e.g., only one character measurement (pleuron depth) observed laterally, and 3) morphometric analysis has been criticized for using only external character measurements, which cannot reflect the totality of the biological complexity of possible characters. The inconsistency of morphometrics and cladistics can also be explained on the basis that even though they used the same character type (morphological characters), they are disparate in the nature of the data sets. Unlike morphometrics, the cladistic method uses character states in *Holcaspis* that do not correlate with body size among species. Thus characters, such as the number of setae and the shape of structures (but not the size), are meaningful in the cladistic method but are totally different from morphometric characters.

At this point, it is possible to postulate that morphometrics is useful for generally grouping and identifying species. However, the question of whether evolutionary changes in morphology, reflecting phylogenetic relationships, are correlated with a tendency to change in size is still unknown. This question may be answered if the number of both individuals and characters used are more intensively investigated. Nevertheless, the likelihood that morphometrics can reflect genetically determined phylogenetic relationships is probably less than for cladistics.

It can be concluded that different patterns of species relationships using phenetic and cladistic methods will be seen from some character types such as morphological data. However, under some circumstances, such as the use of molecular data like allozymes and genomic DNA, the results of phenetic and cladistic analyses are likely to be consistent. Thus, phenetics and cladistics should not be treated as methods in contention, rather they can be used as interrelated methods to search for the pattern of relationships of any organisms.

McNeill (1983) commented that phenetics seeks to discern patterns of diversity; patterns that are the product of evolution. The pattern imposed by evolution is also what

cladistics seeks to discover. There are, however, differences in the way the pattern is discerned and in how it should be represented. Cladists are concerned only with the branching pattern of character state change, phenetics are more concerned with the overall extent and rate of change. However, there will often be a close concordance between the results of studies conducted of the same group whether phenetic or cladistic methods are used.

7.3 *Holcaspis* species phylogeny derived from different character sets

The choice of data sets is the other theme that is involved with systematics work. Two main character sets, morphological and molecular characters, have been used for many years. However, controversy over the usefulness of these different systematic data sets has appeared periodically.

Morphological characters are very convenient and can be obtained from the extensive collections of preserved specimens in museums. Most characters are easily observed by using ordinary, inexpensive equipment such as microscopes, compared with expensive molecular instruments. The unfavourable side of morphological characters is that they are sometimes subject to change in individuals due to environmental variation. In addition, as Cody (1973) discussed, character convergence often takes place in morphological characters and, indeed, may occur simultaneously with character divergence. This phenomenon was seen in Chapter 3 in that the cladograms derived from morphological characters showed a lower consistency index than those derived from molecular data. In other words, it is implied that there is a large amount of homoplasy among characters, which is caused by convergence, reversal or parallel evolutionary events in the lineage. This may be partly because of the nature of the *Holcaspis* morphological characters used. Some characters, like chaetotaxy (patterning of setae), can be useful in species identification, although they may vary within species. The inconsistency of a character like chaetotaxy may be due to it being subjected to environmental conditions. It is well known that external morphological characters are more vulnerable to environmental fluctuation than internal characters. Therefore, it would be useful to use more internal morphological characters, e.g., shape or ultrastructure of the alimentary tract, and

characters of the internal male and female reproductive organs. However, the limitation is that some of these characters can be only obtained by using living specimens.

Molecular data, like enzymes, have been well known for many years in helping to solve systematics problems, particularly for comparing races, subspecies and closely related species (Buth 1984; Menken and Ulenberg 1987; Daugherty *et al.* 1990). It has also been suggested that phylogenetic relationships, derived from these data, should give better estimates of the relationships than those from morphological data, since the molecular data are less influenced by changing environment. However, the limitation of these data is that they again require living specimens. In some cases, if the study deals with rare species or species that are hard to find, like some species of *Holcaspis*, then it may be impossible to obtain complete data sets, as was the case here. Unlike morphological characters, the banding patterns (characters) from enzymes are often difficult to interpret and sometimes the banding patterns appear ambiguous. In addition, the coding of enzyme data, whether to use locus or allele characters, is still being debated. It has been suggested here that coding loci as characters should give more accuracy than coding alleles as characters. As discussed in Chapter 6, the trees derived using locus characters are more congruent than those using independent alleles.

DNA molecular data have begun to be used extensively to investigate phylogenetic relationships at the nucleotide level. It has been suggested (e.g., Moritz and Hillis 1990; Swofford 1991) that molecular characters should give an even better assessment of phylogeny since these data are derived directly from the genome. Other merits of molecular data are that the data can be obtained from both living and, with some difficulty, dead specimens, especially since the recent development of techniques like the polymerase chain reaction. This latter technique provides significant advantages because it needs only very small tissue samples (as discussed in Chapter 5).

As the results have shown, the cladograms derived using allozyme and RAPD-PCR data sets are relatively highly congruent compared with the morphological data. In addition, the cladograms from both the allozyme and RAPD-PCR data showed less homoplasy. Therefore, it is suggested that the RAPD-PCR technique can be a promising novel technique to estimate the phylogenetic relationships. Moreover, the results showed that the RAPD-PCR technique is very useful in grouping and identifying species. It is

also able to identify relatively high variation in the genome within a species with allopatric populations like *H. oedicnema*. This evidence can be useful for monitoring speciation events and species diversity. In this particular species, it also suggests that *H. oedicnema* is polytypic and warrants further study to see if more than one species is involved.

This study has demonstrated that the RAPD-PCR technique has the potential to be a powerful new technique in systematics studies. It is very useful for species where no DNA sequencing information is available. Also, it is a technically simple, quick molecular technique and requires only a small amount of tissue. Therefore, it is useful for work that has only a limited amount of material. However, users of allozyme and RAPD-PCR data sets from molecular techniques generally should be concerned with the problem of homology since, at the molecular level, homology may be particularly difficult to discern, perhaps even more so than is the case with morphological characters. Therefore, great care should be taken in choosing the outgroup, or outgroups.

7.4 Future research

This study has provided some insights into the phylogenetic relationships and systematics of the genus *Holcaspis*. However, only some species have been intensively investigated for their relationships. Ideally, the relationships between all species in the genus should be investigated and these data can then be used to reflect the natural evolution of the genus. Museum specimens could be useful for assessing the phylogenetic relationships of the genus using molecular techniques such PCR. In addition, the phylogenetic information on *Holcaspis* could be transformed into a hierarchical classification. After determining the interrelationships among the species of genus, the end results of phylogenetics should provide information for a natural classification of *Holcaspis* species.

The morphological characters used in this study were derived only from adults. These data would be enhanced by the addition of data from other life history stages. Congruence of independently produced phylogenies from each life history stage would

greatly increase confidence in the species relationships and evolutionary pattern in *Holcaspis*, but details of larvae and other life stages are currently unknown.

The comparison of phylogenies derived by using RAPD-PCR and the other independent molecular character sets such as DNA sequencing, from different genes, would also make a very interesting study.

7.5 Conclusion

With regard to the objectives outlined in the Chapter 1, the following conclusions can be drawn about the systematics of the genus *Holcaspis*.

1. The phylogenetic relationships among the *Holcaspis* species derived from external morphological character data, using cladistic analysis were generally in accord with the previous species grouping of Butcher (1984). However, the cladogram derived from this analysis revealed a higher amount of homoplasy, suggesting character reversals or convergent evolution, in comparison with the molecular data. Therefore it can be concluded that some morphological characters of *Holcaspis* species have been rapidly changing independently over time and it would seem that they are more likely to have been influenced by environment.
2. Morphometric analysis can be useful for preliminary identification of individuals belonging to *Holcaspis* species, sex does not make any significant difference to identification. Morphometric analysis based on quantitative of morphological data could not give informative results about the relationships of *Holcaspis* species.
3. Selected enzyme systems provided a practical way to elucidate *Holcaspis* systematics. The phenogram and cladograms derived from allozyme data are relatively congruent. The results from the allozyme data substantially agree with the RAPD-PCR data results.
4. The RAPD-PCR technique can be reliably used for species identification. It also shows potential as a powerful new tool to study phylogenetic relationships among *Holcaspis* species and more widely. This study has demonstrated the way to handle the RAPD-PCR data in both quantitative assessment of genetic distance between species for the phenetic method and for the cladistic method. The results of both

methods are highly congruent and the relationships of *Holcaspis* species derived from the RAPD-PCR and allozyme are somewhat similar. Therefore, it is suggested the RAPD-PCR more accurately reconstructs the phylogenetic relationships of the genus *Holcaspis*. In addition, the study is a pioneer in demonstrating the use of the RAPD-PCR as a tool for phylogenetic studies (Chapter 5).

5. A pioneering method for testing for the homology of RAPD-PCR banding patterns using the Southern blotting has been developed (Chapter 5). From this study, Southern blotting confirms that the RAPD-PCR bands, which co-migrate, are likely to have the same base sequence. The homologosity of bands is 88 %.
6. This study has modified and developed the RAPD-PCR conditions, such as primers and magnesium chloride concentration, for the development of an efficient RAPD-PCR programme for *Holcaspis* species. With some adjustments, this may be a model to apply to other carabid beetles and, perhaps more widely, to other Coleoptera.
7. The phylogenetic relationships of *Holcaspis* species using the cladistic method based on a combination of three data sets (morphological, allozyme (locus) and RAPD-PCR) yielded a best estimate of the phylogeny of *Holcaspis* species (Figure 6.6). This study shows that *H. ovatella* is the most genetically distinct species of the group, and is followed by *H. punctigera* and *H. mordax*. The results revealed that both *H. punctigera* and *H. mordax* appear to be genetically very distinct species from the other species in the genus, which is not what one would have thought from Butcher (1984). However, these two species are widespread. *Holcaspis ovatella*, which appears to be the most genetically distinct species, has a quite limited geographical distribution, mainly in tussock grasslands in Central Otago. It is in a habitat that is vulnerable to development and degradation. Therefore, it is important that conservation measures be put in place for this species (*H. ovatella*) before it becomes a threatened species.
8. It was confirmed by both allozyme and RAPD-PCR data that *H. oedicnema* may be a complex species. *Holcaspis oedicnema* shows a relatively very high level of heterozygosity from the allozyme study and a high level of intraspecific variation from RAPD-PCR data. The high genetic divergence in *H. oedicnema* is easily

noticeable in both sets of molecular data, but was not so remarkable in morphological characters although the morphological variation in the species was commented on by Butcher (1984).

9. From these studies, *H. oedicnema* justifiably can be considered for conservation. Some of the more obvious morphological variants appear to be strongly geographically limited, which may make them vulnerable, and this study suggests that the *H. oedicnema* is probably a complex of species, which includes these distinct geographical forms.

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REFERENCES

Adams III, E.N. 1972. Consensus techniques and the comparison of phylogenetic trees. *Systematic Zoology* 21: 390-397.

Andersen, J. 1985. Ecomorphological adaptations of riparian Bembidiini species (Coleoptera: Carabidae). *Entomologia Generalis* 11: 41-46.

Anderson, W.W.; Berisford, C.W.; Kimmich, R.H. 1979. Genetic differences among five populations of the southern pine beetle. *Annals of the Entomological Society of America* 72: 323-327.

Anon. 1978. *Enzyme nomenclature.* Recommendations of the Nomenclature Committee of the International Union of Biochemistry on the Nomenclature and Classification of Enzyme. Academic Press, New York.

Ausubel, F.M.; Brent R.; Kingston R.E.; Moore D.D.; Seidman J.G.; Smith J.A.; Struhl K. 1993. *Current Protocols in Molecular Biology.* John Wiley & Sons, New York.

Avise, J.C. 1975. Systematic value of electrophoretic data. *Systematic Zoology* 23: 465-481.

Avise, J.C. 1983. Protein variation and phylogenetic reconstruction. Pp. 103-130 *in*: Oxford, G.S.; Rollinson, D. (eds). *Protein Polymorphism: Adaptive and Taxonomic Significance.* Academic Press, London.

Avise, J.C.; Selander, R.K. 1972. Evolutionary genetics of cave-dwelling fishes of the genus *Astyanax. Evolution* 26: 1-19.

Ayala, F.J. 1975. Genetic differentiation during the speciation process. *Evolution Biology* 8: 1-78.

Ayala, F.J. 1983. Enzymes as taxonomic characters. Pp. 3-26 *in*: Oxford, G.S.; Rollinson, D. (eds). *Protein Polymorphism: Adaptive and Taxonomic Significance.* Academic Press, London.

Barker, S.C.; Briscoe, D.A.; Close, R.L. 1992. Phylogeny inferred from allozymes in the *Heterodoxus octoseriatus* group of species (Phthiraptera: Booiidae). *Australian Journal of Zoology* 40: 411-422.

Bartlett, A.C. 1981. Isozyme polymorphisms in boll weevils and thurberia weevils from Arizona. *Annals of the Entomology Society of America* 74: 359-362.

Bates, H.W. 1874. On the geodaphagous Coleoptera of New Zealand. *Annals and*

Magazine of Natural History 4: 1-244.

Baum, B.R. 1992. Combining trees as a way of combining data sets for phylogenetic inference, and the desirability of combining gene trees. *Taxon* 41: 3-10.

Baverstock, P.R.; Cole, S.R.; Richardson, B.J.; Watts, C.H.S. 1979. Electrophoresis and cladistics. *Systematic Zoology* 28: 214-219.

Berlocher, S.H. 1984. Insect molecular systematics. *Annual Review of Entomology* 29: 403-433.

Black IV, W.C.; Duteau, N.M.; Puterka, G.J.; Nechols, J.M.; Pettorini, J.M. 1992. Use of the random amplified and polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids (Homoptera: Aphididae). *Bulletin of Entomological Research* 82: 151-159.

Bonde, N. 1977. Cladistic classification as applied to vertebrates. Pp. 741-804 *in*: Hecht, M.K.; Goody, P.C.; Hecht, B.M. (eds). *Major Patterns in Vertebrate Evolution.* Plenum Press, New York.

Bonner, T.I.; Brenner, D.J.; Neufeld, B.R.; Britten, R.J. 1973. Reduction in the rate of DNA reassociation by sequence divergence. *Journal of Molecular Biology* 81: 123.

Brasher, D.J.; Ovenden, J.R.; White, R.W.G. 1992. Mitochondrial DNA variation and phylogenetic relationships of *Jasus* spp. (Decapoda: Palinuridae). *Journal of the Zoological Society of London* 227: 1-16.

Brewer, G.J.; Sing, C.F. 1970. *An Introduction to Isozyme Techniques.* Academic Press, New York.

Britton, E.B. 1940. The Carabidae (Coleoptera) of New Zealand Part I, Pterostichini. *Transactions of the Royal Society of New Zealand* 69: 473-508.

Broun, T. 1880. *Manual of New Zealand Coleoptera* Part I. Wellington, New Zealand.

Broun, T. 1893. *Manual of the New Zealand Coleoptera* Parts V-VII. Wellington, New Zealand.

Brown, A.H.D. 1990. The role of isozyme studies in molecular systematics. Australian *Systematic Botany* 3: 39-46.

Brusca, R.C. 1990. *Invertebrates.* Sinauer Association Inc., USA.

Burne, J.C. 1987. A morphometric analysis of *Mordellistena* Costa in the Southwestern United States (Coleoptera: Mordellidae). *Pan-Pacific Entomologist* 63: 224-235.

Bush, G.L.; Hoy, M.A. 1984. Evolutionary process in insects. Pp. 248-278 *in*: Huffaker, C.B.; Rabb, R.L. (eds). *Ecological Entomology*. John Wiley & Sons, New York.

Butcher, M.R. 1984. A revision of genus *Holcaspis* (Coleoptera: Carabidae). *Journal of the Royal Society of New Zealand* 14: 47-99.

Butcher, M.R.; Emberson, R.M. 1981. Aspect of the biology of carabid beetles of Ahuriri Bush Scenic Reserve, Banks Peninsula. *Mauri Ora* 9: 59-70.

Buth, D.G. 1984. The application of electrophoretic data in systematic studies. *Annual Review of Ecology and Systematics* 15: 501-522.

Caccone, A.; Powell, J.R. 1990. Extreme rates and heterogeneity in insect DNA evolution. *Journal of Molecular Evolution* 30: 273-280.

Cain, A.J. and Harrison, G.A., 1960. Phyletic weighting. *Proceedings Zoological Society of the London* 135: 1-31

Camin, J.H.; Sokal, R.R. 1965. A method for deducing branching sequences in phylogeny. *Evolution* 19: 311-326.

Cann, R.L.; Stoneking, M.; Wilson, A.C. 1987. Mitochondrial DNA and human evolution. *Nature* 325: 31-36.

Chaudoir, M. 1865. Essai sur les féronies de Í Australie et de la Nouvelle-Zélande. *Bulletin de la Société Impériale des Naturalistes de Moscou* 38: 65-112.

Cody, L.M. 1973. Character convergence. *Annual Review of Ecology and Systematics* 4: 189-213.

Colless, D.H. 1970. The phenogram as an estimate of phylogeny. *Systematic Zoology* 19: 352-362.

Daugherty, C.H.; Cree, A.; Hay, J.M.; Thompson, M.B. 1990. Neglected taxonomy and continuing extinctions of tuatara (*Sphenodon*). *Nature* 347: 177-179.

Demeke, T.; Adams, R.P.; Chibbar, R. 1992. Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica*. *Theoretical and Applied Genetics* 84: 990-994.

Devonshire, A.L. 1989. The role of electrophoresis in the biochemical detection of insecticide resistance. Pp. 963-374 *in*: Loxdale, H.D.; Hollander, J.D. (eds). *Electrophoresis Studies on Agricultural Pests*. The Systematics Association Special Volume 39. Clarendon Press, Oxford.

Easteal, S.; Boussy, I.A. 1987. A sensitive and efficient isoenzyme technique for small arthropods and other invertebrates. *Bulletin of Entomological Research* 77: 407-415.

Eleles, R.A.; Warre, W.; Stamps, A. 1992. The PCR revolution. *European Journal of Cancer* 28: 289-293.

Eldredge, N.; Cracraft, J. 1980. *Phylogenetic Patterns and the Evolutionary Process: Method and Theory in Comparative Biology*. Columbia University Press, New York.

Faith, D.P. 1988. Consensus applications in the biological sciences. Pp. 325-332 *in*: Bock, H.H. (ed.). *Classification and Related Methods of Data Analysis*. Elsevier Science Publishers B.V., Amsterdam.

Faith, D.P.; Cranston, P.S. 1991. Could a cladogram this short have arisen by chance alone?: On permutation tests for cladistic structure. *Cladistics* 7: 1-28.

Farris, J.S. 1971. The hypothesis of nonspecificity and taxonomic congruence. *Annual Review of Ecology and Systematics* 2: 277-302.

Farris, J.S. 1972. Estimating phylogenetic trees from distance matrices. *American Naturalist* 106: 645-668.

Farris, J.S. 1977. On the phenetic approach to vertebrate classification. Pp. 823-850 *in*: Hecht, M.K.; Goody, P.C.; Hecht, B.M. (eds). *Major Patterns in Vertebrate Evolution*. Plenum Press, New York.

Farris, J.S. 1989. The retention index and homoplasy excess. *Systematic Zoology* 38: 406-407.

Felsenstein, J. 1978. The number of evolutionary trees. *Systematic Zoology* 27: 27-33.

Felsenstein, J. 1983. Parsimony in systematics: biological and statistical issues. *Annual Review of Ecology and Systematics* 14: 313-333.

Ferguson, A. 1980. *Biochemical Systematics and Evolution*. Blackie, London.

Ferguson, A. 1988. Isozyme studies and their interpretation. Pp. 184-201 *in*: Hawksworth, D.L. (ed.). *Prospects in Systematics*. The Systematics Association Special Volume 36. Clarendon Press, Oxford.

Fitch, W.M. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Systematic Zoology* 20: 406-416.

Fitch, W.M.; Mangoliash, E. 1967. Construction of phylogenetic tree. *Science* 155: 279-284.

Frelin, C.; Vuilleumier, F. 1979. Biochemical methods and reasoning in systematics. *Zeitschrift fuer Zoologische Systematik und Evolutionsforschung* 17: 1-10.

Gower, J.C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* 53: 325-338.

Gower, J.C. 1985. Measures of similarity, dissimilarity, and distance. Pp. 397-405 *in*: Kotz, S.; Johnson, N.L.; Read, C.B. (eds). *Encyclopedia of Statistical Science.* Volume V. Wiley, New York.

Groombridge, B. 1992. *Global Biodiversity: Status of the Earth's Living Resources.* Chapman & Hall, London.

Hanboonsong, Y.; Damak, S.; Emberson, R.M.; Scott, R.R. 1992. Molecular and biochemical systematics of carabid beetles of the genus *Holcaspis* Chaudoir. *Proceedings of the Ancient DNA, Human Origins, Molecular Systematics Conferences.* Wellington, New Zealand.

Harris, H.; Hopkinson, D.A. 1976. *Handbook of Enzyme Electrophoresis in Human Genetics.* Oxford American Elsevier Publishing Co., New York.

Hawksworth, D.L.; Bisby, F.A. 1988. Systematics: the keystone of biology. Pp. 3-30 *in*: Hawksworth, D.L. (ed.). *Prospects in Systematics.* The Systematics Association Special Volume 36. Clarendon Press, Oxford.

Hennig, W. 1966. *Phylogenetic Systematics.* University of Illinois Press, Urbana.

Henry, J.M.; Raina, A.K.; Ridgway, R.L. 1990. Isolation of high molecular-weight DNA from insects. *Analytical Biochemistry* 185: 147-150.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics* 18: 23-42.

Hsiao, T.H. 1989. Estimation of genetic variability amongst Coleoptera, p. 143-180 *in*: Loxdale, H.D.; Hollander, J.D. (eds). *Electrophoretic Studies on Agricultural Pests.* The Systematics Association Special Volume 39. Clarendon Press, Oxford.

Hunt, G.J.; Page, R.E. 1992. Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey-bee. *Theoretical and Applied Genetics 83:* 15-20.

Innis, M.A.; Gelfand, D.H.; Sninsky, J.J.; White, T.J. 1990. *PCR Protocols: A Guide to Methods and Applications.* Academic Press, New York.

Jardine, N.; Sibson, R. 1971. *Mathematical Taxonomy.* Wiley, London.

Jensen, R.J. 1983. A practical view of numerical taxonomy or should I be a pheneticist or a cladist? Pp. 53-71 *in*: Felsenstein, J. (ed.) *Numerical Taxonomy.* NATO series, New York.

Kambhampati, S.; Black IV, W.C.; Rai, K.S. 1992. RAPD of mosquito species and populations: techniques statistical analysis and application. *Journal of Medical Entomology* 29: 939-945.

Kambhampati, S.; Rai, K.S. 1991. Patterns of morphometric and allozyme variation in *Aedes albopictus. Entomologia Experimentalis et Applicata* 60: 193-201.

Kessler, L.G.; Avise, J.C. 1984. Systematic relationships among waterfowl (Anatidae) inferred from restriction endonuclease analysis of mitochondrial DNA. *Systematic Zoology* 33: 370-380.

Kimura, M. 1991. Some recent data supporting the neutral theory. Pp. 3-15 *in*: Kimura, M.; Takahata, N. (eds). *New Aspects of the Genetics of Molecular Evolution.* Japan Scientific Societies Press, Tokyo.

Kirk, V.M. 1971. Biological studies of a ground beetle *Pterostichus. Annals of the Entomological Society of America* 64: 540-544.

Kluge, A.G. 1983. Cladistics and the classification of the great apes. Pp. 151-177 *in*: Ciochan, R.L.; Corruccini, R.S.(eds). *New Interpretations of Ape and Human Ancestry.* Plenum, New York.

Kluge, A.G. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Systematic Zoology* 38: 7-25.

Kluge, A.G.; Farris, J.S. 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology* 18: 1-32.

Larochelle, A. 1979. A preliminary bibliography on life cycles and life histories of carabid beetles (Coleoptera). *Cordulia* 5: 72-75.

Lewin, B. 1990. *Genes IV.* Cambridge, Oxford University.

Liebherr, J.K. 1983. Genetic basis for polymorphism in the ground beetle, *Agonum decorum* (Say) (Coleoptera: Carabidae). *Annals of the Entomological Society of America* 76: 349-358.

Liebherr, J.K. 1986. *Cladistic Analysis of North American Platynini and Revision of the*

Agonum extensicolle Species Group (Coleoptera: Carabidae). University of California Press, Berkeley.

Lindroth, C.H. 1974. Coleoptera Carabidae. *Handbook of Identification of British Institute IV (2):* 1-148.

Luff, M.L. 1973. The annual activity pattern and life cycle of *Pterostichus madilus* (F.) (Col. Carabidae). *Entomologia Scandinaviea* 4: 259-273.

Luff, M.L. 1987. Biology of polyphagous ground beetles in agriculture. *Agriculture Zoology Review* 2: 237-278.

MacArthur, G. 1986. An electrophoretic contribution to the systematics of the genus *Wiseana* Viette (Lepidoptera: Hepialidae). M.Sc. thesis, Victoria University of Wellington.

Maddison, W.P.; Donoghue, M.J.; Maddison, D.R. 1984. Outgroup analysis and parsimony. *Systematics Zoology* 33: 83-103.

Maniatis, T.; Fritsch, E.F.; Sambrook, K.J. 1982. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mayr, E. 1969. *Principles of Systematic Zoology.* McGraw-Hill, New York.

Mayr, E. 1988. Recent historical developments. Pp. 31-43 *in*: Hawksworth, D.L. (ed.). *Prospects in Systematics.* The Systematics Association Special Volume 36. Clarendon Press, Oxford.

McIntyre, C.L. 1988. Variation at isozyme loci in Triticeae. *Plant Systematics and Evolution* 160: 123-142.

McNeill, J. 1983. The future of numerical methods in plant systematics: a personal prospect. Pp. 47-52 *in*: Felsenstein, J. (ed.) *Numerical Taxonomy.* NATO series, New York.

Menken, S.B.J.; Ulenberg, S.A. 1987. Biochemical characters in agricultural entomology. *Agricultural Zoology Reviews* 2: 305-360.

Michener, C.D.; Sakagami, S.F. 1990. Classification of the Apidae (Hymenoptera). *University of Kansas Science Bullentin* 54: 75-164.

Mickevich, M.F. 1978. Taxonomic congruence. *Systematic Zoology* 27: 143-158.

Mickevich, M.F.; Mitter, C. 1981. Treating polymorphic characters in systematics: a phylogenetic treatment of electrophoretic data. Pp. 45-58 *in*: Funk, V.A.; Brooks,

D.R. (eds). *Advances in Cladistics.* Proceedings of the First Meeting of the Willi Hennig Society. New York Botanical Garden, Bronx.

Miyamoto, M.M. 1985. Consensus cladograms and general classifications. *Cladistics* 1: 186-189.

Moore, B.P. 1965. Studies on Australian Carabidae (Coleoptera): 4. The Pterostichinae. *Transactions of the Royal Entomological Society of London* 117 (Pt.1): 1-32.

Moritz, C.; Hillis, D.M. 1990. Molecular systematics: context and controversies. Pp. 1-10 *in*: Hillis, D.M.; Moritz, C. (eds). *Molecular Systematics.* Sinauer Associates, Inc., Massachusetts.

Murphy, R.W. 1993. The phylogenetic analysis of allozyme data: invalidity of coding alleles by presence/absence and recommended procedures. *Biochemical Systematics and Ecology* 21: 25-38.

Murray, R.A.; Solomon, M.G.; Fitzgerald, J.D. 1989. The use of electrophoresis for determining patterns of predation in arthropods. Pp. 467-483 *in*: Loxdale, H.D.; Hollander, J.D. (eds). *Electrophoresis Studies on Agriculture Pests.* The Systematics Association Special Volume 39. Clarendon Press, Oxford.

Nei, M. 1972. Genetic distance between populations. *American Naturalist* 106: 283-292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.

Nelson, G.J. 1979. Cladistic analysis and synthesis: principles and definitions, with a historical note on Adanson's Familles des Plantes (1763-1764). *Systematic Zoology* 28: 1-21.

Nelson, G. 1985. Outgroups and ontogeny. *Cladistics* 1: 29-45.

Nelson, G.J.; Platnick, N. 1981. *Systematics and Biogeography: Cladistics and Vicariance.* Columbia University Press, New York.

Nevo, E. 1978. Genetic variation in natural populations: patterns and theory. *Theoretical Population Biology* 13: 121-177

Pape, T. 1992. Cladistic analyses of mosquito chromosome data in *Anopheles* subgenus *Cellia* (Diptera: Culicidae). *Mosquito Systematics* 24: 1-11.

Pasteur, N.; Pasteur, G.; Bonhomme, F.; Catalan, J.; Britton-Davidian, J. 1988. *Practical*

Isozyme Genetics. Ellis Horwood Limited Publishers, Chichester.

Patterson, C. 1987. *Molecules and Morphology in Evolution: Conflict or Compromise?*. Cambridge University Press, Cambridge.

Penny, D.; Hendy, M.D. 1986. Estimating the reliability of evolutionary trees. *Molecular Biology Evolution* 3: 403-417.

Powell, W.; Walton, M.P. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pests. Pp. 443-466 *in*: Loxdale, H.D.; Hollander, J.D. (eds). *Electrophoresis Studies on Agricultural Pests*. The Systematics Association Special Volume 39. Clarendon Press, Oxford.

Prager, E.M.; Wilson, A.C. 1978. Construction of phylogenetic trees for protein and nucleic acids: Empirical evaluation of alternative matrix methods. *Journal of Molecular Evolution* 11: 129-142.

Randi, E.; Fusco, G.; Lorensini R.; Crowe, T.M. 1991. Phylogenetic relationships and rates of allozyme evolution within the Phasianidae. *Biochemical Systematics and Ecology* 19: 213-221.

Richardson, B.J.; Baverstock, P.R.; Adams, M. 1986. *Allozyme Electrophoresis; A Handbook for Animal Systematics and Population Studies*. Academic Press, Sydney.

Ridley, M. 1985. *Evolution and Classification: the Reformation of Cladism*. Longman Group Limited, London.

Rodrigo, A.G.; Bergquist, P.R.; Bergquist, P.L.; Reeves, R.A. 1993. Are sponges animals? An investigation into the vagaries of phylogenetic inference. *In:* van Soest, R. (ed.). *Proceedings of the 4th International Porifera Congress*. (In press).

Rohlf, F.J. 1982. Consensus indices for comparing classifications. *Mathematical Biosciences* 59: 131-144.

Rohlf, F.J.; Wooten, M.C. 1988. Evaluation of the restricted maximum-likelihood method for estimating phylogenetic trees using simulated allele-frequency data. *Evolution* 42: 581-595.

Ross, H.H. 1974. *Biological Systematics*. Addison-Wesley, Reading, Massachusetts.

Saiki, R.K.; Scharf, S.; Faloona, F.; Mullis, K.B.; Horn, G.T.; Erlich H.A.; Arnheim A. 1985. Enzymatic amplification of B-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* 230: 1350-1354.

Schaffer, H.E.; Sederoff, R.R. 1981. Improved estimation of DNA fragment lengths from agarose gels. *Analytical Biochemistry* 115: 113-122.

Schuh, R.T.; Farris, J.S. 1981. Methods for investigating taxonomic congruence and their application to the Leptopodomorpha. *Systematic Zoology* 30: 331-351.

Scott M.P.; Haymes K.M.; Williams S.M. 1992. Parentage analysis using RAPD PCR. *Nucleic Acids Research* 20: 5493.

Scott-Ram, N.R. 1990. *Transformed Cladistics, Taxonomy and Evolution.* Cambridge University Press.

Shaffer, H.B.; Clark, J.M.; Kraus, F. 1991. When molecules and morphology clash: a phylogenetic analysis of the North American ambystomatid salamanders (Caudata: Ambystomatidae). *Systematic Zoology* 40: 284-303.

Sharp, D. 1886. On New Zealand Coleoptera, with descriptions of new genera and species. *Transactions of the Royal Dublin Society n.s.*, 3: 351-457.

Shaw, C.R.; Prasal, R. 1970. Starch gel electrophoresis a compilation of recipes. *Biochemical Genetics* 4: 297-320.

Simpson, G.G. 1961. *Principles of Animal Taxonomy.* Columbia University Press, New York.

Sluss, T.P.; Graham, H.M.; Sluss, E.S. 1982. Morphometric, allozyme, and hybridization comparisons of four *Lygus* species (Hemiptera: Miridae). *Annals of the Entomological Society of America* 75: 448-456.

Sneath, P.H.A. 1961. Recent developments in theoretical and quantitative taxonomy. *Systematic Zoology* 10: 118-197.

Sneath, P.H.A. 1988. The phenetic and cladistic approaches. Pp. 252-276 *in*: Hawksworth, D.L. (ed.). *Prospects in Systematics.* The Systematics Association Special Volume 36. Clarendon Press, Oxford.

Sneath, P.H.; Sokal, R.R. 1973. *Numerical Taxonomy.* Freeman, San Francisco.

Sober, E. 1983. Parsimony in systematics: philosophical issues. *Annual Review of Ecological Systematics* 14: 335-357.

Sokal, R.R. 1983. The phenetic-cladistics controversy: a personal view. Pp 38-41 *in*: Felsenstein, J. (ed.). *Numerical Taxonomy.* NATO series, New York.

Sokal, R.R.; Camin, J.H. 1965. The two taxonomies: areas of agreement and conflict.

Systematic Zoology 14: 176-195.

Sokal, R.R.; Sneath, P.H.A. 1963. *Principles of Numerical Taxonomy*. W.H. Freeman & Co., San Francisco.

Sourdis, J.; Krimbas, C. 1987. Accuracy of phylogenetic trees estimated from DNA sequence data. *Molecular Biology and Evolution* 4: 159-166.

Steiner, W.W.M.; Joslyn, D.J. 1979. Electrophoresis techniques for the genetic study of mosquitoes. *Mosquito News* 39: 35-54.

Steven, P.F. 1980. Evolutionary polarity of character states. *Annual Review of Ecological Systematics* 11: 333-358.

Swofford, D.L. 1991. When are phylogeny estimates from molecular and morphological data incongruent?. Pp. 295-333 *in*: Miyamoto, M.M. and Cracraft, J. (eds). *Phylogenetic Analysis of DNA Sequences*. Oxford University Press, New York.

Swofford, D.L. 1992. *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.0.* Illinois Natural History Survey, Champaign.

Swofford, D.L.; Berlocher, S.H. 1987. Inferring evolutionary trees from gene frequency data under the principle of maximum parsimony. *Systematic Zoology* 36: 293-325.

Thiele, H.U. 1977. *Carabid Beetles in Their Environments*. Springer-Verlag.

Thorpe, J.P. 1979. Enzyme variation and taxonomy: the estimation of sampling errors in measurements of interspecific genetic similarity. *Biological Journal Linnean Society* 11: 369-386.

Thorpe, J.P. 1983. Enzyme variation, genetic distance and evolutionary divergence in relation to levels of taxonomic separation. Pp. 131-152 *in*: Oxford, G.S.; Rollinson, D. (eds). *Protein Polymorphism: Adaptive and Taxonomic Significance*. Academic Press, London.

Urbani, C.B.; Bolton, B.; Ward, P.S. 1992. The internal phylogeny of ants (Hymenoptera: Formicidae). *Systematic Entomology* 17: 301-329.

Vallejos, C.E. 1983. Enzyme activity staining. Pp. 469-516 *in*: Tanksley, S.D.; Orton, T.J. (eds). *Isozymes in Plant Genetics and Breeding; Part A*. Elsevier, Amsterdam.

Vane-Wright, R.I.; Schultz, S.; Boppre, M. 1992. The cladistics of *Amauris* butterflies: congruence, consensus and total evidence. *Cladistics* 8: 125-138.

Varvio-Aho, S.L.; Pamilo, P.; Pekkarinen, A. 1984. Evolutionary genetics of social

wasps (Hymenoptera, Vespidae, *Vespula*). *Insectes Sociaux* 31: 375-386.

Vierling, R.A.; Nguyen, H.T. 1992. Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes. *Theoretical and Applied Genetics* 84: 835-838.

Wagner, W.H., Jr. 1980. Origin and philosophy of groundplan-divergence method of cladistics. *Systematic Botanical* 5: 173-93.

Watrous, L.E.; Wheeler, Q.D. 1981. The out-group comparison method of character analysis. *Systematic Zoology* 30: 1-11.

Watt, J.C., 1982. 1981 Presidential address: New Zealand beetles. *New Zealand Entomologist* 7: 213-221.

Waugh, R.; Powell W. 1992. Using RAPD markers for crop improvement. *Trends in Biotechnology* 10: 186-191.

West, J.G.; Faith, D.P. 1990. Data, methods and assumptions in phylogenetic inference. *Australian Systematic Botanical* 3: 9-20.

White, A. 1846. Insects. Pp. 4-5 *in*: Richardson, J.; Gray, J.E. (eds). *The Zoology of the Voyage of HMS Erebus and Terror During the Years 1893-1843*, Volume 2.

White, T.J.; Arnheim, N.; Erlich H. 1989. The polymerase chain reaction. *Trends in Genetics* 5: 185-189.

Williams, J.G.K.; Kubelik, A.R.; Livak, K.J.; Rafalski, J.A.; Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535.

Wilson, A.C.; Carlson, S.S.; White, T.J. 1977. Biochemical evolution. *A Review of Biochemical* 46: 573-639.

Yu, K.; Pauls, K.P. 1992. Optimization of the PCR program for RAPD analysis. *Nucleic Acids Research* 20: 2606.

Zurwerra, A.; Tomka, I.; Lampel, G. 1986. Morphological and enzyme electrophoresis studies on the relationships of the European *Epeorus* species (Ephemeroptera, Heptageniidae). *Systematic Entomology* 11: 255-366.

Appendix 1 Data matrix showing the presence/absence of 271 amplified DNA fragments from all *Holcaspis* individuals by the random amplified polymorphic DNA based on the polymerase chain reaction lumped

M. antarcticus	000000110101010100000000000000100001010000000000000000000101010000000100000010000000000000000000000001000100000000001010000000000000001000000
	0100000000000000010100000000000101100100000010000000000000000100000000000000001000000000100000000001000010100010010000000000000001000100
H. angustula	0000101000001000000100000000000000000100001000100010000000000100100000000000000000000001000000010000000000000001010000010000100000000000000
	0000000010000000010000000000001000000000000000010000000000000100000010000000101000000000000000000000000000000100000000010000000000000001
H. delator	0100000000000001001000101000000000100000000000010000010000000100000000010000000001100000011000011000000000000000010010000000000000000000
	0000000000100000100100010000000000000100000001000000000000000010010000000000000000000000000010000000000000000100010000000000001000000100
H. egregialis	0000000010000001010000000000000100000000000100000010000000100100000000000100001000100000000000000100000100010000000000000000100000000000
	0000000000000000100000000000100000000000000000000000000000100000001100000000010010000101000000000000000000101000100001000010000000100000
H. elongella	0010000001000000101000010000000000100000000000000000000000000000100000000000010000000100001000100000000000000000001000000110000100000000000
	0000000000000000010000000010010001000000000000000000000000000001000000010000000010010000000100001100000000101000000000100000000000000000000
H. hispida	0000010000000000000000010000100000001000000000000000000000000000100000001001000000000000100000001000010000100000000010000000000000000000000
	0000101000001010001000001000100000000010000000000000000100001011000000000000100011000000010000000000000000000000100000000000100010000000000
H. hudsoni	0010000000000101001100001000000010010000000000000000000000000000000000001000010000000000000000000000100010000100000010000010001000000001100000
	0000000001000000010000000000100000100000000100001000010010001100000010000000000000000000000000000000000000000000000000000000100000000001000010
H. intermittens	1101000000001010000010001000000000100010000100000100000000000100000000000001000000000000000000100001000000001001000010000000000001000001000
	0000000000000000000001001100000000001000000001010100100000000000100000110000000000100000000001000000000010000010000000000000000000010001
H. mordax	0000000010000001001000010010000001000000000000000100000000000000000000000001100001000000000000000000000001000000000000010000001000000000000
	0010010000000000001000000000100001000001001000000000100000000001010000010100000000000001010000000000000000000010000010000000001000001000
H. mucronata	0000001000000000000100000100000001000000010010000000000000001000000010110000000000010001000001000000000110000000010010001001000000000000
	0001000000000000010000100001001000100001000000000000000000001000000100000000010001001000000000000011000000010000000000100000010000000
H. oedicnema	0110001000101001000000000001000000100000000000001000000000000111010000000000100000000000100010101001001000010000000100001000000100000000
	0000000000100010010001001000000000000000010000000010000101000100000100001100000000010000001000000000000000000010010000000100000000100000
H. ovatella	0000000000000001010001000000000100000010001010000000010000100010100000001001000000010000000010010000000100100010100000000100000000000
	0000000000000100101000000100000000000000000000000001000100000100000000100000000000000001000000000100000000100000000000000100000000010
H. punctigera	0000001000000100100000001010010100001000000000000001000010000000000010000000000110000010100000000000000000000000000000000100000000010000000
	0000000100000000001010000000000000000000000000010000000000001000000010000000000000001000000000000000100000100000001000000000100000010100
H. suteri	0010000000000100100000000000000000100000010000010000000000000100000000000000100000100100001000100000000000100010100101000000000001000101010
	1001000000000000010000000110001100001000000000000000000000000100000000000001101000000000000000001110001001000100000000000000000000000010000

Appendix 2 Data matrix showing 26 morphological characters allozyme (13 loci) characters and 271 amplified DNA fragments of the random amplified polymorphic DNA based on the polymerase chain reaction characters for 13 *Holcaspis* species and *Megadromus antarcticus*.

M. antarcticus	2000000200000010001010002022201302111140000001101010101000000000000010000101000000000000000000001010100000001000000100000000000000000000000010001000000000010100
	0000000000000100000001000000000000000101000000000010110010000001000000000000000100000000000000010000000001000000000010000101000100100000000000000001000100
H. angustula	2001000300010101110000010012100002210030000101000010000010000000000000001000010001000100000000001001000000000000000000000010000000100000000000000101000010
	0001000000000000000000000010000000001000000000001000000000000000001000000000000100000010000000101000000000000000000000000000000010000000001000000000000001
H. delator	20000203110010101211201001001103120111301000000000000100100010100000000010000000000010000010000000100000000010000000001100000011000011000000000000000001001
	000000000000000000000000000001000001001000100000000000100000001000000000000000100100000000000000000000000000100000000000000001000100000000000100000000100
H. egregialis	2000020100010210030000100111310002201130000000010000001010000000000001000000000001000000100000001001000000000010000100010000000000001000001000100000000000
	0000001000000000000000000000000000010000000000010000000000000000000000000000000100000001100000000100100001010000000000000000101000100001000010000000100000
H. elongella	2020020311101210011001100111100002201140010000010000001010000100000000001000000000000000000000000000100000000000010000000100001000100000000000000000001000000
	1100001000000000000000000000000000001000000001001000100000000000000000000000000010000000100000000100100000010000110000000010100000000010000000000000000000
H. hispida	2000020301001210021110100111102211211020000010000000000000000100001000000100000000000000000000000001000000010010000000000010000000100001000010000000001000
	0000000000000000000000101000010100010000010001000000000100000000000000010000101100000000000010001100000001000000000000000000000010000000000100010000000000
H. hudsoni	2001020201000b11031020101100311110000300010000000001010001100001000000010010000000000000000000000000000000000010000100000000000000000000000100010000100000001000
	0010001000000011000000000000001000000010000000000100000100000001000010000100100011000000100000000000000000000000000000000000000000000000000100000000000100001
H. intermittens	2000020311001010021120101000102202111231101000000001010000010001000000001000100001000001000000000001000000000001000000000000000010000100000000100100001000
	0000000001000001000000000000000000001001100000000001000000001010100100000000000100000110000000000100000000010000000000010000010000000000000000000010001000
H. mordax	1100101100000200111011001022210111000240000000100000010010000100100000010000000000000010000000000000000000000000001100001000000000000000000000001000000000000
	0100000010000000000001001000000000001000000000100001000001001000000000100000000001010000101000000000000010100000000000000000000010000010000000001000001000
H. mucronata	2000020300001011021020101000210211201030000001000000000000100000100000001000000100100000000000000010000000101100000000000100010000010000000001100000000100
	1000100100000000000000010000000000001000010000100100010000100000000000000000000100000010000000001000100100000000000001100000001000000000001000000100000000
H. oedicnema	2010020311000111112111010100001011201012011000100010100100000000001000000100000000000010000000000001110100000000001000000000010001010100100100001000000010
	0001000000100000000000000000001000100100010010000000000000010000000010000101000100001000011000000000100000010000000000000000100100000001000000010000000
H. ovatella	2020000000100010031100013100001300111300000000000000000101000100000000010000001000101000000001000010001010000000100100000001000000001001000000100100010100
	0000000100000000000000000000100101000000100000000000000000000000100010000010000000100000000000000001000000000100000000010000000000000000100000000010
H. punctigera	1100000000010200111011101022301310120240000001000000100100000001010010100001000000000000010000100000000000100000000001100000101000000000000000000000000000
	0100000000010000000000000010000000001010000000000000000000000000100000000000100000000100000000000000100000000000000100000100000001000000000100000001001000
H. suteri	20000202000000100310000000000021012110200100000000001001000000000000000010000001000001000000000000010000000000000010000010010000100010000000000100010100101
	0000000000100010101010010000000000000100000001100011000010000000000000000000000000100000000000000110100000000000000000111000100100010000000000000000000010000

Appendix 3 Data matrix showing 26 morphological characters allozyme (42 alleles) characters and 271 amplified DNA fragments of the random amplified polymorphic DNA based on the polymerase chain reaction characters data for 13 *Holcaspis* species and *Megadromus antarcticus*.

M. antarcticus	20000002000000100010100020001011001111001000101100101010101101010001000000110101010100000000000001000010100000000000000000010101000000010000001 00000000000000000000010001000000000010100000000000000100000001000000000000010100000000000101100100000010000000000000001000000000000000000100 00000001000000000000100001010001001000000000000000001000100
H. angustula	2001000300010101110000010001010111001001010000100010010010101100001000001010000010100000100000000000000000100001000100010000000000100100000000000000 0000000100000000100000000000000010100000100001000000000000000000000000100000000010000000000010000000000000000100000000000001000000100000001010100 00000000000000000000000000100000000001000000000000001
H. delator	200002031100101012112010011001000110010100001011001100001001010000100100000000000001001000101000000000100000000000100000100000001000000000010000 00000110000001100001100000000000000000010010000000000000000000000000000000100000100100010000000000010000000100000000000000001001000000000000000 00000000010000000000000001000100000000000010000000100
H. egregialis	20000201000102100300001001011010000101010110001100100101000101010010000000001000000101000000000000100000000000100000010000000100100000000000010 00010001000000000000001000001000100000000000000001000000000000000000000000000000000100000000000100000000000000000000000000000000100000001100000000100 10000101000000000000000010100010000100001000000000100000
H. elongella	2020020311110121001100110010100100100100101000010001001010001010100010010000010000001010000100000000000100010000000000000000000000010000000000000 10000000100001000100000000000000000001000000110000100000000000000000000000000000001000000000100100010000000000000000000000000000000010000000100000000010 010000001000011000000000101000000000100000000000000000000
H. hispida	2000020301001210021110100101001001001010000100010110011010011000011000000100000000000000001000010000000100000000000000000000000000001000000001001000 000000000100000000100001000010000000001000000000000000000000000000001010000101000100000100010000000000100000000000000000000100001011000000000000100011 00000001000000000000000000000100000000000010001000000000000
H. hudsoni	200102020100061103102010111001000001010110110001110100011011000110100010000000000101001100001000000001001000000000000000000000000000000000000100001 00000000000000000000000100010000100000010000001000100000000110000000000000010000000100000000000100000100000000100001000010010001100000010000000000000 00000000000000000000000000000000000000010000000000001000010
H. intermittens	20000203110010100211201010100100010010100001001000101010100110100010110100000000010100000100010000000001000100001000001000000000001000000000000001 000000000000000010000100000000100100001000000000000100000100000000000000000000000010011000000000001000000000101010010000000000001000001100000000010 0000000001000000000000100000100000000000000000000010001001
H. mordax	11001011000002001110110010001001000101011010000101010001101100100001000000000100000010010000100100000010000000000000100000000000000000000000000000001 10000100000000000000000000000010000000000000001000000100000000000000100100000000000100000000010000100000100100000000010000000000101000001010000000 0000001010000000000000000000000100000100000000001000001000
H. mucronata	2000020300001011021020101010011000110101000100110110011100011010001000000001000000000000010000010000000100010000100100000000000000010000000010110 00000000001000100000100000000001100000000100100010010000000000000000100000000000000100001000010010001000010000000000000000000000001000000100000000010

0010010000000000000110000000100000000000100000010000000

H. oedicnema 2010020311000111121110101010010010001101001000110011100010100110011001100010001010010000000000100000010000000000000100000000000011101000000000
0100000000000100010101001001000010000000100001000000100000000000000000000010001001000100100000000000000001000000001000001010001000001000011000000000
0001000000100000000000000000001001000000010000000010000000

H. ovatella 2020000000100010031100013110010010101001101011101000101011010001101000000000000000000101000100000000010000001000101000000001000010001010000000001
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0000000100000000010000000001000000000000000001000000000010

H. punctigera 1100000000010200111011101000101100001100100010011100100001100010000100000010000001001000000010100101000010000000000000100001000000000000010000000
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0010000000000000000100000100000001000000000010000000101000

H. suteri 2000020200000001003100000000110110110010101001100010110001101001101001000010000000000100100000000000000001000000100000100000000000000010000000000000
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