A study on *Tenuibranchiurus* and the evolution of the burrowing clade of Australian freshwater crayfish

PhD Thesis

Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

February 2015

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ABSTRACT

The overall aim of this thesis was to investigate the morphological and molecular diversity within *Tenuibranchiurus*, and to utilise these data to further the understanding of the evolution of this freshwater crayfish and the other genera of the Australian burrowing clade from both a phylogenetic and biogeographic perspective. The genus *Tenuibranchiurus* occurs within coastal eastern Australia and currently represents the largest gap in knowledge within this clade of crayfish. It is also the only monotypic parastacid genus, containing the single species *T. glypticus*. Additionally, it has morphological, phylogenetic, and geographical attributes that are not exhibited by other members of the burrowing clade, or by other freshwater fauna found throughout its distributional range. Examination and clarification of these unique features has the potential to elucidate evolutionary processes determining the distribution and genetic structure of the genus, the burrowing clade, and the freshwater fauna of coastal eastern Australia.

Examination and analysis of the morphological and molecular characteristics of *Tenuibranchiurus* identified the presence of two distinct genera, with the populations from Queensland representing *Tenuibranchiurus* and those from New South Wales representing a proposed new genus, *Gen. nov.*. The analyses also supported the recognition of six species within *Tenuibranchiurus* (including the previously recognised *T. glypticus*), and two within *Gen. nov.*. All species within both genera exhibited highly restricted and/or highly disjunct distributions. The molecular data suggest that species within both genera are highly structured spatially; this is likely the result of cyclical population retractions and expansions, and shifting distributions in response to changes in sea level and increased aridity altering available and accessible habitat.

The present day distribution of *Tenuibranchiurus* and *Gen. nov.* species has been strongly influenced by historical climate events. Dispersal most likely occurred during periods of lowered sea level which would have exposed swampy coastal plains, creating a dispersal pathway for populations to use as they followed a moisture gradient as the sea receded. Genetic divergence, and subsequent speciation, likely occurred through vicariance as a result of population isolation, habitat heterogeneity, and/or subsequent sea level oscillations. These processes were estimated to have occurred over ~50 million years, commencing with the

splitting of an ancestral genus into *Tenuibranchiurus* and *Gen. nov.*, and subsequent divergence resulting in the eight species present today.

With the molecular diversity of *Tenuibranchiurus* and *Gen. nov.* clarified, the phylogenetic relationships between all of the burrowing clade genera were re-examined. However, the relationship between the three oldest genera (*Engaewa*, *Engaeus sensu stricto*, and *E. lyelli*) could not be resolved, probably as a result of almost simultaneous divergence. These genera were estimated to have diverged during the Cretaceous, followed by *Gramastacus* (also during the Cretaceous), *Geocharax* (Palaeocene), and *Tenuibranchiurus* and *Gen. nov.* (Eocene). Investigation of the biogeographic history of the burrowing crayfish suggests that the genera (and species within them) display highly concordant distributional patterns, appearing to be influenced largely by climate change and fluctuating sea level rather than by drainage architecture, which is the most commonly accepted freshwater paradigm. Therefore, the burrowing clade crayfish, and potentially other parastacid genera, can be viewed as ecologically removed from other freshwater taxa, requiring detailed historical climate and geological data in combination with dispersal models in order to reconstruct their biogeographic histories.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed) _____

Kathryn Dawkins

ACKNOWLEDGEMENTS

To my principle supervisor Professor Jane Hughes, thank you for undertaking this project with me and giving me the freedom to mould and re-mould it how I chose over the many years. Thank you also for your support and advice. To my associate supervisors Professor Clyde Wild and Dr. James Furse, thank you for your proof-reading and helpful suggestions.

Special thanks are given to Andrew Bentley and Quinton Burnham for providing additional sample material for my genetic analyses, and to Stephen Keable for helping me gain access to the Australian Museum collections. This research was funded by the Griffith School of Environment, and made possible with an Australian Postgraduate Award. Additional funding was supplied by the Australian Rivers Institute and Griffith Graduate Research School for conference attendance to present my work. All specimens from this study were collected under permits WITK08599510, WISP08599610, and TWB/01/2011 issued by the Department of Environment and Resource Management.

I would also like to thank the plethora of people who have given me assistance whenever I ran into one of the many pitfalls that seem to surround molecular analyses on crayfish. Specifically, to Dan Schmidt, Bob (aka Andrew Bentley), Jesse Breinholt, Alicia Toon, Kathryn Real, and Jemma Somerville for answering countless emails on genetic techniques and lab questions, and allowing me to hassle you any time for explanations. A huge thank you also is warranted for Michael Arthur who never complained when I would turn up unannounced at his office door with a stack of statistical analyses to discuss.

This project would never have been possible without my field lackeys; you were both my helpers and my sanity-keepers, so thank you to James Furse, Seanan Wild, Amanda Dawson, Diana Virkki, and Shane Howard for the many hours (and prescription sunglasses, Seanan) you sacrificed for me.

I am eternally grateful for the support I received from my friends during this journey. To DD and Bones, you were my coffee support team and my lunch buddies, providing invaluable meaningless chit-chat to break up the hard slog that is a PhD. You were also (and still are) close friends and helped me through a lot. Manana and Seaman, you followed in our footsteps and decided to join a team of crazy people doing their PhD's – for reasons I still do

not understand. You were also there as close friends and staunch supporters, and I cannot thank you enough for being there through this entire journey. You will always be my Joben and favourite woo-girl. I am also grateful to Jason Coughran for being my office buddy and friend when I moved to Perth for the last year and a half of my PhD. I always enjoyed talking about how time might not even be real, to then look up and realise we had just wasted an hour of it.

Last, but not least, are my family. Mum and Dad, you supported me throughout my degree; from undergraduate, to Honours, through to a PhD that seemed like it would never end. Although the times I had to camp out at your house were because I had a lot of hard lab work ahead of me, the little things like packed lunches and treaties made it so much easier for me. Thank you also to Anne and Rohan who did their best to understand what I was doing and why I was doing it – I know that you will encourage Kate and Sarah to follow the path of science, no matter what you may say now. To my other set of parents, Kay and Geoff, thank you for putting up with both Quinton and I as we both struggled through the last steps of our PhD journey. It could not have been easy dealing with the daily mood-swings that are part and parcel of that, so I am grateful to you for sticking with us. Three of the most important supporters in this journey were in fact my puppies; Molly, Nikki, and Rowdy. You provided sympathetic ears when I needed a chat, and endless hours of fun and laughter – something not to be dismissed lightly during such a long journey.

Finally, and my biggest thank you yet, is for my Q. We came into each other's lives at perhaps the most difficult points – I don't know any other couple that were crazy enough to try and finish two PhD's at the same time, and I suspect there is a reason for that. You have been my biggest supporter, taking on the tasks of keeping me happy, sane, fed, and relaxed as if it were easy. You survived it through the many emotional ups and downs and the tears that came with both of these. No one will ever truly understand how important you were to this whole process, even perhaps me, but I thank you from the bottom of my heart for being there every step of the way for these last three years. I am forever in debt to you, but I know I have forever to repay you.

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1.0 General Introduction

1.1 BIOGEOGRAPHY

Biogeography endeavours to describe the processes and mechanisms that have led to the distributions of biota through the discovery of spatial distribution patterns, and by extension explain their evolution (Santos and Amorim 2007). This is achieved by combining information about geographic patterns of variation, both within and between species, with historical, demographic, and/or environmental events. For instance, physiological and behavioural adaptations can result from both abiotic and biotic factors (such as intra- and inter-specific interactions and environmental interactions), and influence species' distributions (Spellerberg and Sawyer 1999). Added to this are historical forces occurring over large geological timescales, such as sea level change, changing climate, and plate tectonics (Spellerberg and Sawyer 1999). Given that such processes are most apparent at large geographic scales and their influence on species' distributions are likewise evident over broad areas, much biogeographical work has used continental- or landmass-scale areas as analytical units (Kozak *et al.* 2006; Pyron and Burbrink 2010).

Although there is a general lack of biogeographical studies looking at small scales, due primarily to the difficulty in defining boundaries within changing landmasses, an exception to this is the biogeography of freshwater species. Freshwater taxa have featured prominently in such studies, as the nature of rivers and lakes somewhat replicates island-like characteristics (Hughes *et al.* 2009). River basins are bound by both land and sea, therefore the movement of obligate freshwater species tends to be higher within basins than between them (Page and Hughes 2014). Thus, these discrete geographic boundaries provide ideal parameters and demonstrable patterns for use in biogeographic studies, both at large and small scales.

Within the freshwater biota, the biogeographic and speciation patterns of invertebrate taxa have been somewhat overlooked, despite their propensity to retain strong genetic signatures of their phylogeographic history (Koizumi *et al.* 2012). Many invertebrate species that display low dispersal abilities survive in small, isolated populations, which acts to preserve the continuity of their phylogeographic signal (Daniels *et al.* 2002; Price *et al.* 2010), thus making them ideal candidates for biogeographic analysis. One impediment to this is a general lack of comprehensive phylogenetic studies on this group of organisms. As such, detailed information on patterns of genetic diversity, or even the recognition of distinct

genetic entities (e.g. species), is required for such groups before meaningful biogeographic patterns can be discerned (Pfenninger and Schwenk 2007; Cook *et al.* 2008a; Cumberlidge and Daniels 2014).

1.2 SPECIATION IN FRESHWATER SYSTEMS

Speciation is the process by which one ancestral species diverges to form two or more descendant species, and occurs as a result of some form of isolation (e.g. genetic, morphological, geographic), causing the cessation of dispersal and thus gene flow (Templeton 1981; Wiens 2004b; de Queiroz 2007). When populations cease exchanging genes differentiation occurs, with populations beginning to accrue genetic differences at neutral loci through mutations, which results in changes in allele frequencies as genetic drift takes place (Chenoweth and Hughes 2003). Additionally, the process of natural selection will act on geographic locales differently, leading to either an increase or decrease in genetic variation between populations (Lenormand 2002). These four main evolutionary processes (i.e. gene flow, genetic drift, mutation, natural selection) determine genetic variability and, therefore, are drivers of the speciation process.

Population connectivity (and thus the ability to exchange genes) within freshwater systems is subject to different environmental conditions than that of terrestrial and marine systems. For most freshwater species, pathways of connectivity are arranged in a dendritic fashion (i.e. drainage networks) rather than (theoretically, at least) in any direction across a plane (Hughes *et al.* 2013). Added to this is the influence of life history (and associated dispersal ability) of the species in question (Ockinger *et al.* 2010), as the connectivity of species may be governed by factors such as flight capacity (e.g. aquatic insects), diadromous migrations, or obligate freshwater requirements. By observing the genetic relatedness of populations the degree of connectivity between them can be inferred, with the genetic structure of freshwater organisms following one of five models of connectivity; stream hierarchy model, death valley model, isolation by distance, panmixia, or headwater model (see Hughes *et al.* 2013). While these models can describe relatively recent patterns of gene flow, they do not extend to inferring the historical processes driving the degree of connectivity.

Like any other organisms, freshwater taxa are subject to stochastic events that can shape their genetic diversity; however, there are other large scale processes that, while they may be rare occurrences, are highly influential for freshwater species. Such events include changes in sea level, drainage rearrangements, river capture, and freshwater pulses into the ocean (Unmack 2001). As sea level changes over time, this can greatly affect the connectivity and dispersal ability of freshwater species. For instance, if sea level lowers, the width of the exposed continental shelf increases, potentially allowing separate drainages to coalesce; alternatively, if sea level rises, previously connected drainages may be sundered and begin to flow independently. Drainage rearrangements and river capture work in much the same way, whereby tectonic activity may cause drainage pathways to either coalesce or diverge, resulting in either new connections or population isolation. Finally, pulses of freshwater into the ocean (for example, due to large scale flooding events) have the potential to allow previously isolated drainages to become connected by providing a dispersal pathway through the temporary removal of the marine barrier.

Each of these processes has the potential to impact the genetic diversity of populations. This may be through the provision of dispersal pathways retaining gene flow between populations (or reconnecting populations), or it may be in the form of population isolation causing a cessation of gene flow. It is this last outcome that has the potential to lead to sufficient genetic divergence between entities to give rise to new species.

1.3 DEFINING A SPECIES

The publication of Darwin's *On the Origin of Species* saw the start of an intense debate regarding what a 'species' is, and how speciation occurs; and yet there is still no general consensus on either subject. There are numerous definitions of what should be considered a 'species' (see Table 1.1). Some species concepts have become less favoured as new information has emerged and flaws are found, while others remain but are disputed. Mayr (1963) outlined several species concepts including the typological species concept (coined by Charles Darwin), non-dimensional species concept, and interbreeding-population concept. Added to that list is his own biological species concept (Mayr 1942), which defines species as 'groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups'; a definition that has since been widely used throughout the literature. However, one of the major downfalls of this concept is being able to recognise

speciation when organisms are not co-occurring and therefore one does not know if they can potentially interbreed – a requirement for this species concept.

The lack of one clearly accepted definition of a 'species' creates obvious limitations as what one person regards as a species may not be regarded as being so by another person, which is often further exacerbated by differences of opinion between fields of study. For the purposes of this study, the General Lineage Concept (GLC; de Queiroz 1998) will be applied. The GLC defines a species as a metapopulation lineage evolving separately from other lineages. The advantage of using this particular species concept is that it unites the various other concepts by allowing any evidence of lineage separation (and thus any property emphasised by the alternative concepts) to be used as evidence for species delimitation (de Queiroz 2007). Not only does this allow multiple lines of evidence to be used, but it also allows the evolutionary processes that have caused divergence between lineages to be examined.

Table 1.1. Summary of the major species concepts modified from de Queiroz (2007).

Species Concepts	Brief Definition	Reference(s)
Biological	Interbreeding (natural reproduction resulting in viable and fertile offspring)	Wright (1940); Mayr (1942); Dobzhansky (1950)
Isolation	Intrinsic reproductive isolation (absence of interbreeding between heterospecific organisms based on intrinsic properties, as opposed to extrinsic [geographic] barriers	Mayr (1942); Dobzhansky (1970)
Recognition	Shared specific mate recognition or fertilization system (mechanisms by which conspecific organisms, or their gametes, recognize one another for mating and fertilization)	Paterson (1985); Masters *et al.* (1987); Lambert and Spencer (1995)
Cohesion	Phenotypic cohesion (genetic or demographic exchangeability)	Templeton (1989, 1998)
Ecological	Same niche or adaptive zone (all components of the environment with which conspecific organisms interact)	Van Valen (1976); Andersson (1990)
Evolutionary	Unique evolutionary role, tendencies, and historical fate Diagnosability (qualitative, fixed difference)	Simpson (1951); Wiley (1978); Mayden (1997); Grismer (1999, 2001)
Genotypic cluster	Form a genotypic cluster (deficits of genetic intermediates; e.g. heterozygotes)	Mallet (1995)
Phenetic	Form a phenetic cluster (quantitative difference)	Michener (1970); Sokal and Crovello (1970); Sneath and Sokal (1973)
Cladistics	Species are unbranched segments or lineages in an organismal phylogeny. Ancestor becomes extinct when lineage splits	Hennig (1966); Ridley (1989); Meier and Willmann (2000)
Monophyletic	Monophyly (consisting of an ancestor and all of its descendants; commonly inferred from possession of shared derived character states)	Rosen (1979); Donoghue (1985); Mishler (1985)
Genealogical	Exclusive coalescence of alleles (all alleles of a given gene are descended from a common ancestral allele not shared with those of other species)	Baum and Shaw (1995); see also Avise and Ball (1990)
Diagnosable	Diagnosability (qualitative, fixed difference)	Nelson and Platnick (1981); Cracraft (1983); Nixon and Wheeler (1990)
Lineage	Species are independent lineages	de Queiroz (1998)

1.4 IDENTIFYING A SPECIES

Taxonomic groups can be defined using several different approaches. One such method, and the method adopted by this study, is an integrative taxonomic approach, whereby several lines of evidence are used to delineate taxonomic groups. Two evidentiary pathways that will be employed here (and are commonly used in other studies) are (1) the use of morphology, where shared morphological features are used to indicate shared ancestry and thereby group organisms that are most similar, whether that may be at a species, genus, family level or so on, and (2) the use of molecular techniques to identify genetically distinct units, again at differing taxonomic levels.

Using a taxonomic approach that examines several lines of evidence is important as there can often be discordance among gene trees, between gene trees and species trees, and between molecular and non-molecular (i.e. morphological) characters (e.g. Shaw 2002; Avise 2004; Hall and Katz 2011; Tilley *et al.* 2013). Genealogical discordance, especially between mitochondrial and nuclear trees, can occur for a number of reasons with one of the most prominent being the differing times it takes for these gene regions to coalesce. Alleles at nuclear genes complete the coalescent process much more slowly than those at mitochondrial loci due to the tendency of mitochondrial DNA (mtDNA) to accumulate nucleotide substitutions several times faster than nuclear DNA (nuDNA) (i.e. effective population size (Ne) of mtDNA is one quarter of that of nuDNA; Avise 2000). Species and gene tree conflicts may arise when the sorting of gene lineages at speciation (i.e. in the ancestral gene pool) is incomplete, as random fixation reached in descendant taxa can produce topological discordance between the two trees (Avise 2000).

Finally, morphological incongruence with molecular data may occur as a result of the plasticity of some organisms. This can occur in two ways, either through multiple morphological forms representing the same genotype (i.e. morphological plasticity), or one morphological form masking the presence of multiple species (i.e. cryptic species). Within crustaceans, there are numerous examples of this phenomenon. For example, Murphy and Austin (2003, pg 174) examined both the morphology and mtDNA of species of Australian shrimp and found that the morphological features that are used for taxonomic differentiation between three genera are “phylogenetically unreliable and plastic”. Silva *et al.* (2010, pg 443) found that in a British crab species “genetic and phenotypic characters are in some way independent” as the low levels of genetic diversity did not reflect the high morphometric

variability. Breinholt *et al.* (2012) found that previously defined relationships based on morphology are inconsistent with phylogenetic relationships within the crayfish subgenus *Cambarus* Girard, and extended this conclusion to suggest that convergent evolution has influenced morphological characters within both the studied subgenus and other invertebrates. These examples provide support for the use of integrative taxonomy, an aim that this study advocates by using both genetics and morphology to reduce any bias and possible confusion within the taxonomy of the study organism.

1.5 FRESHWATER CRAYFISH

There are two recognised superfamilies of freshwater crayfish; Astacoidea De Haan 1841 and Parastacoidea Huxley 1879 (Hobbs 1974). Within the first are the families Astacidae and Cambaridae, and within the second the Parastacidae. The astacids and cambarids are found in the Northern Hemisphere exclusively, with the parastacids found only in the Southern Hemisphere (Crandall *et al.* 2000b). The three families can be distinguished from one another on the basis of differences in morphology, embryonic development, post-hatching behaviour, and secondary sexual characteristics (especially in males) (Riek 1969; Holdich and Reeve 1988).

The distribution of the astacids extends from North America to Europe, north- and east-Asia, and Japan, while the cambarids occur in North America and east-Asia (Hobbs 1974; Holdich 1993). Parastacids are found in the Southern Hemisphere (Holdich 1993), including Australia, New Zealand, South America (Riek 1969; Mills *et al.* 1994), Madagascar and New Guinea (Holthius 1986). It is interesting to note that freshwater crayfish do not occur naturally on the African continent, Indian sub-continent, Central America, most of Asia, or Antarctica (Holdich 1993).

Despite the almost global distribution of freshwater crayfish, North America and Australia host by far the highest diversity, with over 450 and 170 species occurring in these regions, respectively (Crandall *et al.* 2000b; Crandall and Buhay 2008). The presence of two such centres of diversity has previously led some to debate the origin of freshwater crayfish (i.e. whether there have been multiple transitions to freshwater from a marine ancestor(s)); however, molecular analyses support a monophyletic origin of freshwater crayfish occurring during the Triassic period in the super-continent Pangaea (Crandall *et al.* 2000b; Crandall and Buhay 2008). Fossil evidence and phylogenetic relationships provide support for this theory

and additionally support the two superfamilies forming due to the splitting of Pangaea into Laurasia and Gondwana (Crandall *et al.* 2000b; Crandall and Buhay 2008).

In contrast to some Northern Hemisphere freshwater crayfish genera, the parastacid genera represent monophyletic groups (Crandall *et al.* 2000b), suggesting that they are much older in origin (Crandall and Buhay 2008). The centre of diversity for the Parastacidae occurs in south-east Australia, with all but 11 of the described species occurring east of central Australia (Whiting *et al.* 2000). Crayfish are distributed over a large portion of the continent of Australia (Figure 1.1), and occupy a wide range of habitat types including coastal, alpine, and arid habitats (Riek 1959). The Parastacidae is comprised of 15 genera, nine of which are endemic to Australia (Riek 1969, 1972; Hansen and Richardson 2006), while the genus *Cherax* Erichson is also found in New Guinea and surrounding islands as well as Australia (Clark 1936; Holthius 1986).

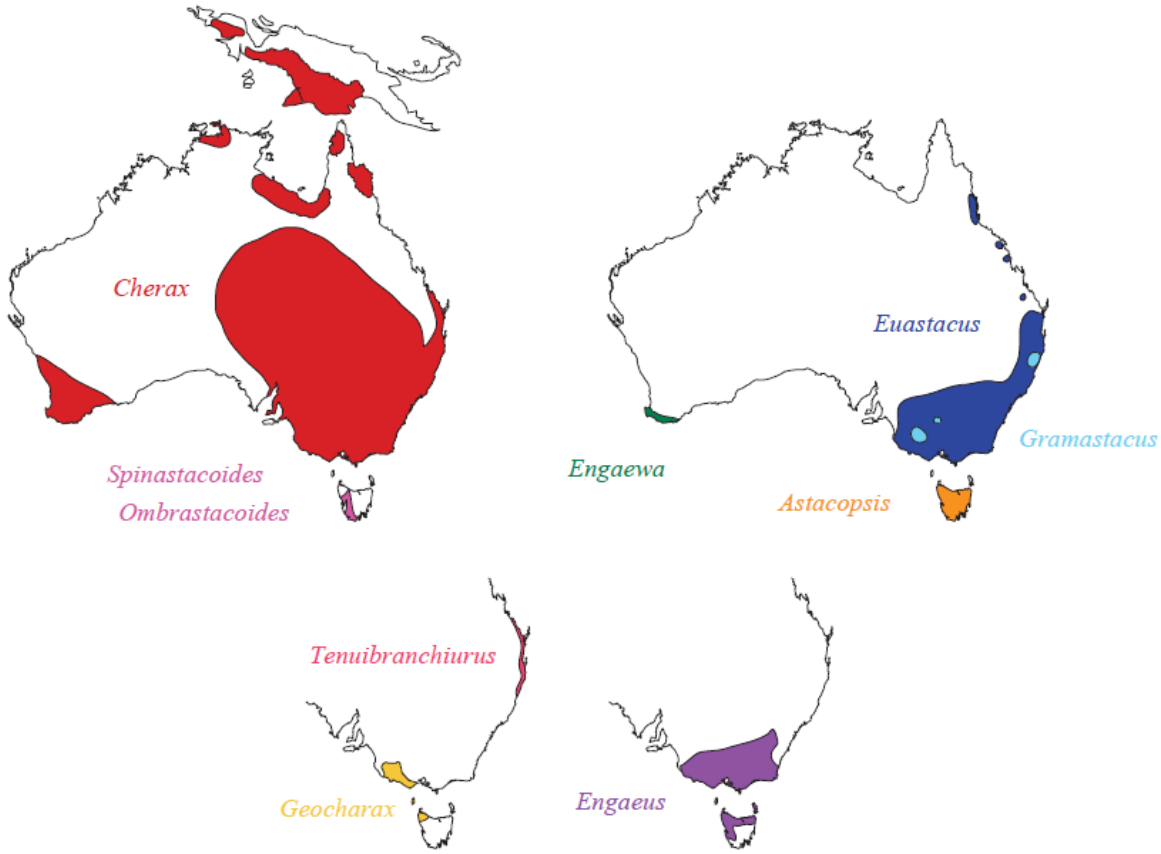


Figure 1.1. Distribution of the Australian and New Guinea freshwater crayfish. Figure adapted from Toon *et al.* (2010).

1.6 BURROWING CLADE OF AUSTRALIAN CRAYFISH

Although all freshwater crayfish have the ability to burrow to some degree, a phylogenetically related group of crayfish within the Australian crayfish has been termed the 'burrowing clade' (*sensu* Burnham 2014). This clade currently includes five described genera (*Tenuibranchiurus* Riek, *Gramastacus* Riek, *Geocharax* Clark, *Engaeus* Erichson, and *Engaewa* Riek) and one yet to be described genus; a lineage containing the species *Engaeus lyelli* Clark, which may in fact represent two species (*sensu* Schultz *et al.* 2009). As this new genus is not yet described, it shall be referred to as *E. lyelli* throughout the remainder of the document and be considered as a distinct genus, while the remainder of the genus *Engaeus* shall be referred to as *Engaeus sensu stricto*. The unusual characteristics of this group of crayfish have been highlighted in several studies as they display distinctive morphological features. Such features are the result of adaptations to their often extreme burrowing habit and include a reduction in the size and width of the abdomen, oblique/vertically orientated chela, and a vaulted carapace (Hobbs 1969, 1975; Holdich 2002). As such, they were classified by Riek (1972) as belonging to two groups of crayfish; the moderate burrowers (*Gramastacus*, *Geocharax*) and the strong burrowers (*Engaeus*, *Engaewa*, *Tenuibranchiurus*). However, this was revised by Horwitz (1988b, 1990) who placed them into a single group upon the discovery of the presence of an abdominal anterolateral flap which is unique among all freshwater crayfish and thought to be the result of common ancestry between these genera. This grouping has since been supported by several molecular studies (i.e. Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014), which have found this clade to be monophyletic to the exclusion of all other genera.

Although they form a close phylogenetic group, the distributions of genera within this clade is unusual (Figure 1.2), with *Engaewa* located in the far south-west of Australia, *Engaeus*, *Geocharax*, *E. lyelli*, and one described and one undescribed species of *Gramastacus* located in the south-east of Australia (including Tasmania), and finally *Tenuibranchiurus* and one species of *Gramastacus* on the central-eastern coast of Australia. These highly disjunct distributions raise questions related to the historical biogeographic processes that have influenced such a diverse and wide-ranging group of crayfish. In addition to their apparent geographically disparate distributions, they have also been highlighted as representing a phylogenetically confusing group, with no studies to date able to resolve the phylogenetic relationships between the genera, and there have been highly conflicting estimates of the age

of the various genera (e.g. Crandall *et al.* 1999; Schultz *et al.* 2007; Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014).

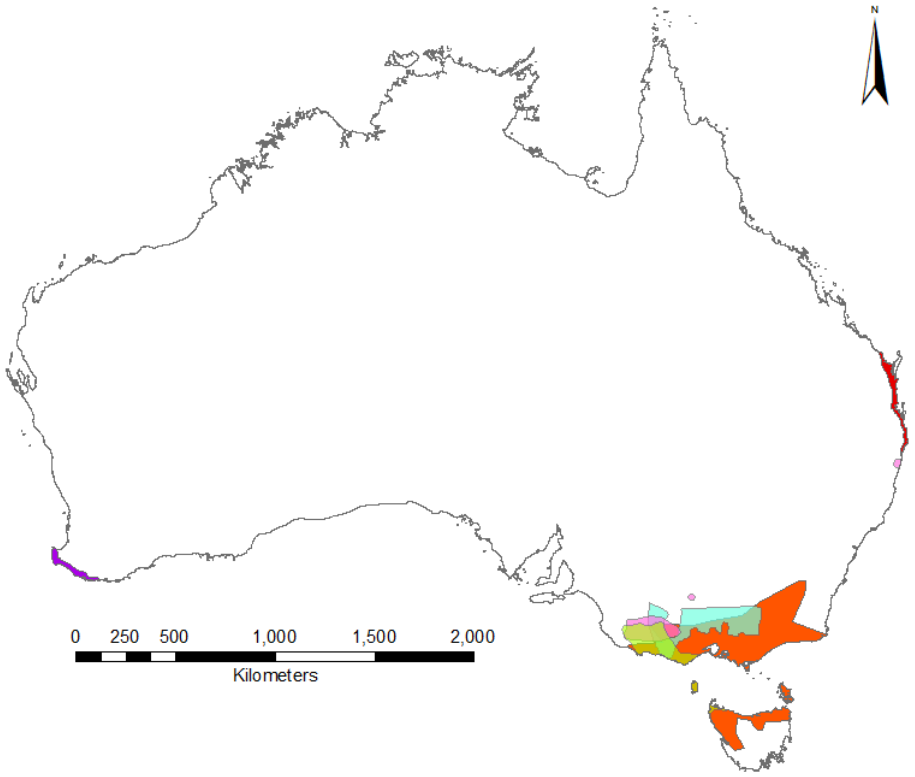


Figure 1.2. Distribution of the burrowing clade genera. Purple=*Engaewa*, orange=*Engaeus sensu stricto*, blue=*E. lyelli*, green=*Geocharax*, pink=*Gramastacus*, red=*Tenuibranchiurus*.

The most thoroughly studied burrowing clade genus is *Engaeus*, with investigations including: a full taxonomic revision that included detailed morphological, demographic, and ecological notes (Horwitz 1990); an examination of the secondary sexual characteristics of females from this genus (Horwitz 1988b); a biogeographic study of two species from the genus (Horwitz 1988a); an electrophoretic evaluation of species from this genus (Horwitz *et al.* 1990); multiple molecular studies focussing primarily on *Engaeus* and examining their phylogeny and the biogeography of some species (Schultz *et al.* 2007; Schultz *et al.* 2008; Schultz *et al.* 2009), and; many other related publications (e.g. Suter 1977; Suter and Richardson 1977; Horwitz *et al.* 1985a; Horwitz *et al.* 1985b; March and Robson 2006).

Although the subject of few publications, *Engaewa* represents a relatively thoroughly examined genus, as it served as a model organism in the study of Burnham (2014), and prior to that was the focus of a study by Horwitz and Adams (2000). Thus, there is a substantial amount of information available on the biology, ecology, morphology, biogeography, and

molecular diversity of this genus. *Geocharax* and *Gramastacus* have also been studied across multiple disciplines, forming part of a broad biogeographic discussion, taxonomic revisions, and molecular, life history, and habitat use studies (e.g. Zeidler and Adams 1990; March and Robson 2006; Schultz *et al.* 2007; Johnston and Robson 2009; Schultz *et al.* 2009; Johnston *et al.* 2010; McCormack 2014). The other member of the burrowing clade genera, *Tenuibranchiurus*, is the least studied of all, with only two small studies commenting on their biology and ecology (Harding and Williamson 2003, 2004) and two studies on their genetic diversity (Horwitz 1995; Dawkins *et al.* 2010). It is this last genus that forms the major focus of this study.

1.7 GENUS *TENUIBRANCHIURUS*

Parastacid genera are generally highly speciose, with new species and genetically diverse groups still being regularly described (e.g. Coughran 2005; Hansen and Richardson 2006; Coughran *et al.* 2012; Furse *et al.* 2013). The most notable exception to this is the genus *Tenuibranchiurus*, which contains the smallest parastacid species (Figure 1.3). Although it has previously been highlighted as containing genetically diverse groups (see Horwitz 1995; Dawkins *et al.* 2010), this genus contains only the single described species *Tenuibranchiurus glypticus* Riek (1951), endemic to the central-eastern coast of Australia, and represents the only monotypic parastacid genus[1]. This species currently has no official conservation status assigned to it, although it has been listed under the IUCN Red List as Endangered (Coughran *et al.* 2008).

Figure 1.3. Photograph of the lateral view of a *Tenuibranchiurus* specimen (~25 mm total length).

[1] Although the genus *E. lyelli* is a potential exception to this, the genus is as yet undescribed and has been suggested to contain two distinct species (see Schultz *et al.* 2009).

Because so few studies have been undertaken on *Tenuibranchiurus*, very little is known about its basic biology or ecology. When initially describing the genus, Riek (1951) stated that they prefer wallum swamps and, since then, two small studies have suggested that members of this genus prefer vegetated and tannin-stained ephemeral waters (Harding and Williamson 2003), and are more likely to be active nocturnally than diurnally (Harding and Williamson 2004). The only other available information pertinent to this genus relates to its burrowing habits. Riek (1951) stated that *Tenuibranchiurus* specimens dig burrows up to three feet in length and Horwitz and Richardson (1986) classified them (along with the other members of the burrowing clade with the exception of *Gramastacus*) as constructing Type 2 burrows (i.e. burrows connected to the water table). However, in an investigation by P. Horwitz (unpublished data), it was noted that the burrow structure and the strength of the association of *Tenuibranchiurus* specimens and their burrows was difficult to ascertain, due both to habitat characteristics (e.g. water depth, detritus on substrate) and the activities of other freshwater organisms. For instance, many other small invertebrate species burrow into the substrate, making it difficult to tell what organism constructed burrows found. Additionally, *Cherax* species often live in the same habitats as *Tenuibranchiurus* and can be found in the same burrow system, making it unclear as to which species is responsible for its construction.

Despite so little being known about the genus *Tenuibranchiurus*, it is an interesting biogeographic model. Except for *Tenuibranchiurus* and one species of *Gramastacus* (*Gramastacus lacus* McCormack), the remainder of the burrowing clade is distributed along the southern coast of mainland Australia and Tasmania (Figure 1.2). The distribution of *Tenuibranchiurus*, as well as their atypical body form relative to the other burrowing crayfish (e.g. lack of constriction at 1st abdominal segment) and their possession of both specialised and primitive attributes, led Riek (the author of their original description) to hypothesise in a later paper that *Tenuibranchiurus* may represent the descendant of a different marine ancestor to that which gave rise to the other Australian crayfish (Riek 1959). Although this theory was later discounted by the author when he suggested a single freshwater invasion (see Riek 1972), it highlights the unique morphological attributes and geographical distribution of this genus of crayfish. As with all members of the burrowing clade, *Tenuibranchiurus* is only moderately constrained by freshwater availability as it can persist in temporary or marginal habitat; thus making it a useful organism to test the applicability of freshwater speciation models (e.g. the models of Hughes *et al.* (2013) referred to in section 1.2) to atypical freshwater species.

In relation to many parastacids, and particularly to the other members of the burrowing clade, the distribution of *T. glypticus* is relatively large, with several species of both *Cherax* and *Euastacus* Clark occurring within the same area. Additionally, its distribution spans biogeographically significant boundaries that have been shown to influence the genetic diversity of many other freshwater species. The current distribution of *Tenuibranchiurus* extends along the coastal regions of south-east Queensland (Qld) and northern New South Wales (NSW). A significant biogeographic break has been identified between these two regions, with this break representing either the northern- or southern-most distributional limit of many freshwater taxa, or representing a region of genetic divergence (e.g. James and Moritz 2000; Munasinghe *et al.* 2004b; Page *et al.* 2004; Cook *et al.* 2006; Thacker *et al.* 2007; Bentley *et al.* 2010; Unmack and Dowling 2010; Page and Hughes 2014). Additionally, particularly within the south-east Qld region, drainage basin boundaries have been found to significantly influence the genetic structure of freshwater species (see Page and Hughes 2014). Yet, currently the species *T. glypticus* appears to span all of these potential biogeographic boundaries.

1.8 OBJECTIVES AND THESIS OUTLINE

The unique morphological, phylogenetic, and geographical attributes described in the preceding section lead to questions surrounding the genus *Tenuibranchiurus*, for instance: (1) is the current taxonomy of a single species within *Tenuibranchiurus* accurate?; (2) can patterns of diversity within other freshwater taxa with similar distributions to *Tenuibranchiurus* give insights into the distribution of this genus (and *vice versa*)?; (3) as this genus represents a peripheral isolate of the burrowing clade's distribution, is their current phylogenetic placement accurate (i.e. do they represent an earlier/later branch in the burrowing clade's phylogeny)?; and, finally (4) what biogeographic processes have led to the disjunct distribution of *Tenuibranchiurus* from the rest of the burrowing clade (e.g. climate change, sea level fluctuations, geological events). These questions will form the basis of this study, as well as informing on processes that have occurred throughout the entire burrowing clade.

With these questions in mind, the overall aim of this thesis is:

> "To clarify the morphological and molecular diversity present within *Tenuibranchiurus* as it represents the largest gap in knowledge within the burrowing clade, and to use this information to further the understanding of the evolution of all burrowing clade genera, both from a phylogenetic and a biogeographic perspective."

The generally speciose nature of Australian crayfish genera, in combination with the phylogenetic, morphological, and biogeographic inferences surrounding *Tenuibranchiurus* has introduced an interesting premise; either the genus *Tenuibranchiurus* represents an anomaly within the parastacids (i.e. it is the only monotypic genus in an otherwise speciose group), or the current taxonomy does not accurately reflect the true diversity present within this genus. In order to provide clarity to the evolution of the burrowing clade, these propositions need to be resolved. Chapter 3 seeks to resolve the taxonomy of this genus using morphological data as part of an integrative taxonomic approach. The original taxonomic description of *Tenuibranchiurus* by Riek (1951) is currently the only morphological work that has been undertaken on this genus. As this description was based on specimens from only two localities within Qld, morphological characterisation of newly discovered populations (extending both north and south of the type locality) is yet to be undertaken.

As part of the integrative taxonomic approach, molecular data across populations will also be analysed in Chapter 4, with the aim of clarifying the genetic diversity identified by previous studies (i.e. Horwitz 1995; Dawkins *et al.* 2010). Phylogenetic analyses and species delimitation methods across multiple genes (two mitochondrial and three nuclear genes) will be undertaken, which will allow a comprehensive analysis of the molecular diversity within *Tenuibranchiurus*. Combining the morphological and molecular results will provide an accurate framework (i.e. taxonomy that reflects evolutionary relationships) on which to further explore all of the burrowing clade genera.

Once this has been determined, the biogeography of *Tenuibranchiurus* can be explored in detail. In Chapter 5, both the contemporary and historical biogeography of this genus will be investigated. In order to do this, first the phylogeographic structure of the genus will be examined to infer the degree of contemporary population connectivity, with the timing of divergence events within the genus explored and subsequently correlated with the occurrence of historical geological events. The historical biogeography will include determining the ancestral distributions of each of the genetic units within *Tenuibranchiurus*, with divergence estimates calculated using all of the available sequence data (i.e. two mitochondrial and three nuclear genes).

With the molecular diversity and biogeography of *Tenuibranchiurus* explored in detail, understanding the evolution of the burrowing clade becomes a feasible goal. However, although each genus in the clade is generally well understood individually, when considered collectively, there is still a lack of agreement between studies as to their phylogenetic relationships and age. As *Tenuibranchiurus* represents the largest deficiency in the molecular data prior to this study, it is hypothesised in Chapter 6 that, with this rectified, a more accurate phylogeny of the burrowing clade can be inferred, and previously proposed (and widely varying) hypotheses can be further tested. With the burrowing clade phylogeny and age clarified, the biogeographic history of the entire clade will be explored in detail in Chapter 7. In order to do this, divergence estimates will be correlated with geological data and estimates of ancestral ranges for each of the genera. A biogeographic description will be provided at both a generic and specific level, allowing the first complete biogeographic model of the evolution of this group of organisms to be produced.

2.0 General Methods

2.1 SPECIMEN COLLECTION

2.1.1 Selection and Details of Sampling Locations

In order to obtain a representative sample from the entire distribution of *Tenuibranchiurus*, sampling locations were located throughout coastal Qld and NSW (Appendix One). In order to sample likely habitat throughout the entire potential distribution of *Tenuibranchiurus*, sampling localities were either identified from vegetation maps (specifically looking for the distribution of *Melaleuca*, as this has been highlighted as suitable *Tenuibranchiurus* habitat previously: Riek 1951; Dawkins *et al.* 2010) using Geographic Information Systems (GIS) software (ArcView 3.2 GIS), through previous collection records (see Riek 1951; Crandall *et al.* 1999; Harding and Williamson 2003; Bentley 2007; Schultz *et al.* 2007; Schultz *et al.* 2009; Dawkins *et al.* 2010), or opportunistically (i.e. locations identified in person as potentially suitable habitat that were not shown by GIS datum). Table 2.1 and Figure 2.1 show sampling localities from which *Tenuibranchiurus* have been collected both in this study and those previously reported in the literature. All locations where *Tenuibranchiurus* have currently been found are as close as 1 km and no further than ~25 km from the coast; further supporting the previously assumed coastal distribution of this genus. Several localities identified from the literature were visited but sampling efforts failed to uncover any *Tenuibranchiurus*. Both Eumundi (Eu) and Mooloolaba (Moo) appear to have been developed for housing or infrastructure and populations there have likely been extirpated, and Kinkuna National Park (KNP) and Tuan State Forest North (TSFN) were too dry to sample when visited due to a lack of rain throughout the region, although crayfish may still be present. Bribie Island 1&2 (BRB1 and BRB2), were not visited as suitable habitat in close proximity to these sites was sampled instead (i.e. Bribie Island (BRB)).

Although specimens were collected from the Type Locality (TL) in Caloundra, Qld, during this study, they were damaged and could not be used for morphological examination. Therefore, in addition to specimens collected in this study, specimens from the Australian Museum lodged by Riek for the original description of *Tenuibranchiurus* were also accessed. These type specimens were used for morphological analyses, and the specimens collected in this study were used for molecular analyses. All specimens from this study were collected under permits WITK08599510, WISP08599610, and TWB/01/2011 issued by the Department of Environment and Resource Management.

Table 2.1. Sampling localities and GPS co-ordinates where *Tenuibranchiurus* have been collected (refer also to Figure 2.1). TL=Type locality, *=This study, [1]=Bentley 2007, [2]=Crandall *et al*. 1999, [3]=Dawkins *et al*. 2010, [4]=Schultz *et al*. 2007, [5]=Schultz *et al*. 2009, [6]=P. Horwitz unpublished data.

State	Sampling Location ID	General Locality	Latitude °S	Longitude °E
Qld	KNP[4]	Kinkuna National Park	25.060339	152.397875
	HB[1*]	Hervey Bay	25.394783	152.708317
	MAR[3*]	Maryborough	25.482139	152.759083
	TSFN[1]	Tuan State Forest (North)	25.695500	152.764250
	TSFS[*] A	Tuan State Forest (South)	25.926375	152.761767
	C	Tuan State Forest (South)	25.955681	152.748683
	E	Tuan State Forest (South)	25.962658	152.790983
	F	Tuan State Forest (South)	25.975608	152.773217
	G	Tuan State Forest (South)	25.982858	152.907464
	H	Tuan State Forest (South)	25.994544	152.886922
	TEW[5*]	Tewantin State Forest	26.377000	152.979444
	LW[*]	Lake Weyba	26.441142	153.056111
	Eu[4]	Eumundi	26.466667	152.933889
	Moo[4]	Mooloolaba	26.700000	153.083333
	BER[*]	Beerburrum Scientific Area	26.858928	153.002642
	Q2[6]	Beerburrum Scientific Area	n/a	n/a
	TL1[5]	Bells Creek, Caloundra (TL)	26.855306	153.091861
	TL2[5*]	Bells Creek, Caloundra (TL)	26.863667	153.096278
	TL3[2*]	Bells Creek, Caloundra	26.798422	153.048689
	Q1[6]	Caloundra	n/a	n/a
	Q3[6]	Caboolture	n/a	n/a
	BRB1[5]	Bribie Island, Bongaree	27.045722	153.175556
	BRB2[1]	Bribie Island	27.045867	153.175600
	BRB[*]	Bribie Island	27.050169	153.167250
	GC1[3*]	Molendinar, Gold Coast	27.967583	153.374361
	GC2[3*]	Southport, Gold Coast	27.966000	153.384028
NSW	LH[3*]	Lennox Head	28.748178	153.592519
	BNP1[3*]	Broadwater National Park	29.055819	153.425011
	BNP2[3*]	Broadwater National Park	29.077700	153.399550
	N1[6]	Tabbimoble	n/a	n/a
	LakeH[3*]	Lake Hiawatha	29.826397	153.277569

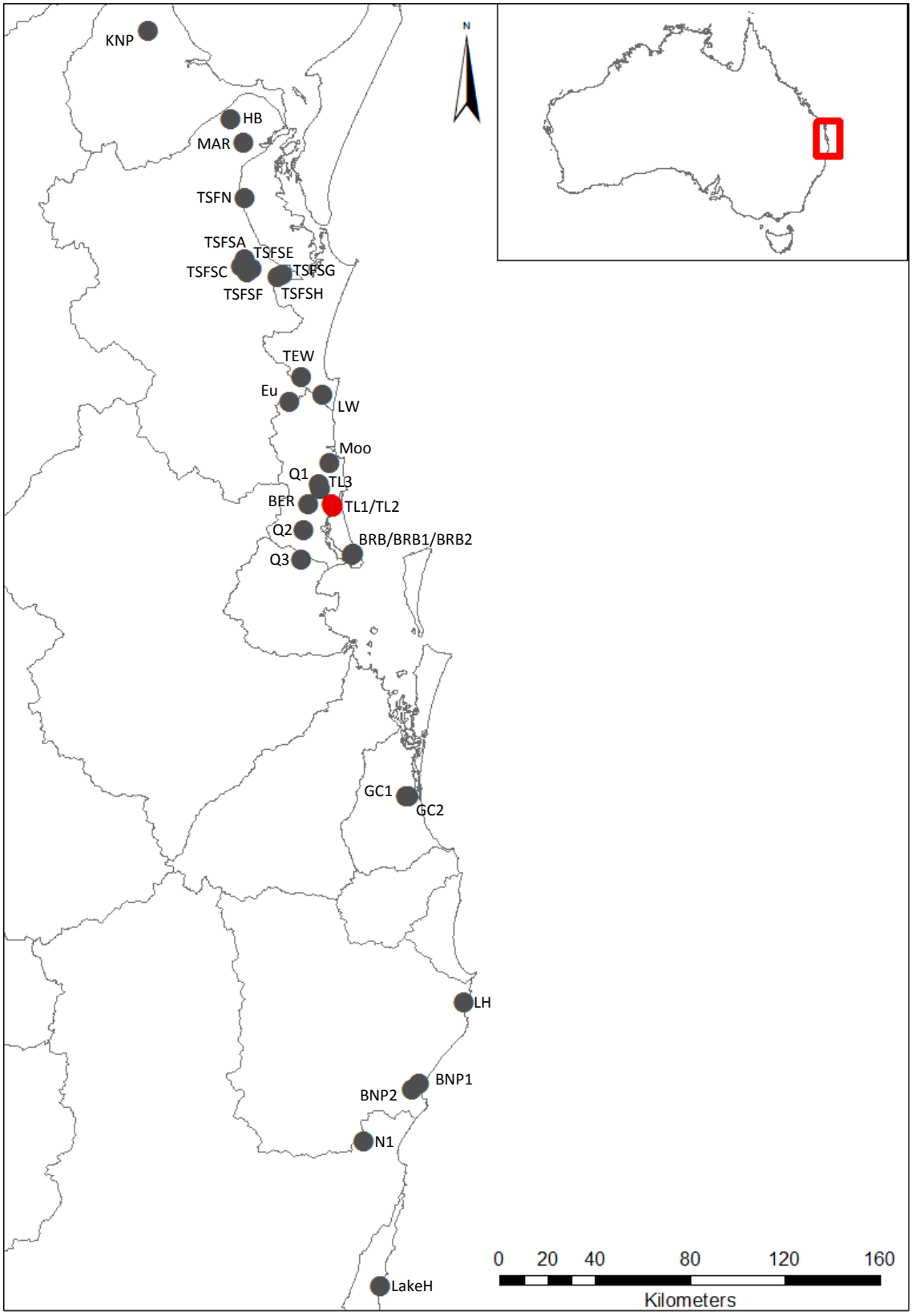


Figure 2.1. Locations within Queensland and New South Wales in Australia where *Tenuibranchiurus* were collected during this study and have been reported within the literature. Red circles denote the Type Locality. Refer to Table 2.1 for location details, and Appendix One for additional locations sampled. For sources of GIS data see section 2.3.

2.1.2 Sampling Protocol

Crayfish were collected using a fine-meshed sweep net (1 mm aperture) to sweep through both the vegetation within the water body, as well as the leaf litter and detritus accumulated on the bottom of pools. Initially a bait-pump was also used to pump out burrows and around submerged logs (see Dawkins *et al.* 2010), but this method was discontinued due to poor success rates. The majority of sampling was conducted from February to June (2008-2011) to take advantage of the seasonal rains during this time.

Where whole specimens were required for later morphological analysis, they were stored separately on ice in the field, frozen at -20°C on return to the laboratory, and preserved separately in 70% ethanol. However, at the majority of sampling locations, a single chela was removed from each specimen and stored separately in 70% ethanol for later genetic analysis, with the specimen then released otherwise unharmed. This was done to reduce any impact of removing several specimens from a population at once. Where possible, a minimum of seven genetic samples and five morphological samples were collected from each site, as this has been considered in a number of studies to be sufficient to provide a robust genetic analysis to identify significant genetic divergence (such as evolutionarily significant units: Austin *et al.* 2003; Ponniah and Hughes 2004; Schultz *et al.* 2007) and should also be sufficient to detect morphological variability.

2.1.3 Specimen Notes

Tenuibranchiurus catch numbers were variable between sampling locations and also temporally variable when locations were revisited. *Tenuibranchiurus* were found within burrow systems, but were predominantly collected in the accumulated rotting detritus or around the base of vegetation clumps or tree trunks. The habitats in which *Tenuibranchiurus* were found varied from those conditions previously described, and are outlined in Appendix Two. Often specimens from the genus *Cherax* were found in sympatry with *Tenuibranchiurus*; however, the extent of the association between the two genera is unclear. Sizes of specimens varied and ranged between approximately 5-14 mm occipital carapace length (OCL; Morgan 1997). The presence of young-of-year specimens was noted (i.e. specimens <5 mm OCL) but they were not retained for further analysis. Data were also collected on the reproductive biology of specimens. Although only a few gravid females were found during the study, these data were recorded and are discussed in Appendix Three,

as they provide previously undescribed insights into this genus which may prove useful for future studies.

2.2 LABORATORY METHODS

2.2.1 DNA Extraction

A variation of the cetyltrimethyl ammonium bromide (CTAB)/phenol-chloroform extraction protocol (Doyle and Doyle 1987) was used to extract DNA. A small amount of tissue was placed in a 1.5 mL Eppendorf tube with 700 µL 2×CTAB buffer (1M Tris HCl pH 8.0, 4 M NaCl, 0.5 M EDTA, 0.1 M CTAB) and homogenised using a plastic mortar and pestle. Five µL of Pro K (20 mg mL^{-1}) was added and the sample vortexed and incubated at 65ºC overnight on a Thermoline dry block incubator. After incubation, the following process was undertaken in order to remove proteins and lipids from the extractions; 300 µL Phenol (25:1) and 300 µL chloroform-isoamyl (24:1) were added to each tube, mixed on a Clements suspension mixer for 15 min, then centrifuged at 13500 rpm for 3 min in an IEC Micromax centrifuge. The supernatant was siphoned into a new labelled tube with the remainder discarded, 600 µL chloroform-isoamyl (24:1) was added, tubes were mixed for 5 min, then centrifuged for 3 min. The supernatant was again siphoned into a new labelled tube with the remainder discarded, 600 µL of cold isopropanol was added to each tube, and the solution was mixed to precipitate the DNA. The samples were then left at -70ºC for 30 min, defrosted and centrifuged for 15 min to allow a pellet of DNA to form. The supernatant was removed, leaving the DNA pellet, which was then rinsed in 1 mL 70% ethanol and centrifuged for 2 min. The ethanol was then removed and the tubes were placed on a heatblock at 50ºC until the samples were dry. The dried DNA was resuspended in 100 µL ddH_2O and stored at 4ºC until further analysis took place.

2.2.2 Mitochondrial and Nuclear DNA

Although a variety of DNA gene regions can be used to investigate genetic diversity, the selection of a molecular marker is based largely on the scale of the relationship being assessed (e.g. population versus species level). In crayfish studies in particular, the two most commonly used gene regions are the mitochondrial COI gene and 16S (Schubart 2009). These have been used widely for inferring crayfish phylogenetic and phylogeographic relationships in Australia (e.g. Crandall *et al.* 1999; Shull *et al.* 2005; Hansen and Richardson 2006; Ponniah and Hughes 2006; Schultz *et al.* 2007), New Zealand (e.g. Apte *et al.* 2007), Europe (e.g. Grandjean and Souty-Grosset 2000; Grandjean *et al.* 2002), and both North and

South America (e.g. Crandall *et al.* 2000a; Taylor and Hardman 2002; Barriga-Sosa *et al.* 2010; Dillman *et al.* 2010; Breinholt *et al.* 2012). Although these two markers are by far the most widely used in crayfish studies, additional markers employed include GAPDH (Buhay *et al.* 2007; Schultz *et al.* 2009), H3 (Buhay *et al.* 2007), 12S rRNA (Munasinghe *et al.* 2003; Shull *et al.* 2005; Buhay *et al.* 2007), 28S rRNA (Shull *et al.* 2005; Breinholt *et al.* 2012), and the Internal Transcribed Spacer region 2 (ITS2) (Bentley *et al.* 2010).

For this study, two mitochondrial gene regions (cytochrome oxidase subunit 1 (COI) and 16S ribosomal RNA (16S)) and three nuclear gene regions (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone-3 (H3), arginine kinase (AK)) were amplified (see Table 2.2 for primer list). Additional genes were trialled (i.e. 12S, 18S, 28S, and EF2), and while all were successfully amplified, they either showed no variation or could not be amplified consistently.

The COI gene region has a relatively high mutation rate in comparison to 16S, making it useful for inferring the current genetic structure between populations, whereas the more conservative 16S gene was used to examine deeper phylogenetic relationships. The nuclear gene region GAPDH has been successfully used in other studies on parastacid crayfish (Schultz *et al.* 2009), as well as northern hemisphere crayfish (Buhay *et al.* 2007). These studies found this nuclear gene region to be useful for distinguishing between closely related species as well as examining deeper phylogenetic relationships. The gene H3 has also been used in crayfish studies (e.g. Toon *et al.* 2009) and exhibits moderate levels of divergence useful for examining relationships within a genus, although it should be noted that the genus tested in the study of Toon and collaborators (i.e. *Euastacus*) is much older than most and this gene may therefore not be appropriate for recently diverged species (Toon *et al.* 2009). Finally, the AK gene was originally developed for northern hemisphere crayfish (J.W. Breinholt unpublished data) and as yet no data are available on its divergence levels between species or genera; however, it was included in the analysis as preliminary results showed genetic variation.

Table 2.2. Forward and reverse primers used for molecular analyses.

Gene region	Primers (5'⟶3')	Reference	Fragment length
COI	CRCOI-F: CWACMAAYCATAAAGAYATTGG CRCOI-R: GCRGANGTRAARTARGCTCG	Cook *et al.* (2008b)	644bp
16S	16S-ar: CGCCTGTTTATCAAAAACAT 16S-br: CCGGTCTGAACTCAGATCACGT	Palumbi *et al.* (1991)	449bp
GAPDH	G3PCq157-F: TGACCCCTTCATTGCTCTTGACTA G3PCq981-R: ATTACACGGGTAGAATAGCCAAACTC	Schultz *et al.* (2009)	563bp
H3	H3-AF: ATGGCTCGTACCAAGCAGACVGC H3-AR: ATATCCTTRGGCATRATRGTGAC	Colgan *et al.* (1998)	264bp
AK	AKcray-F: CTACCCCTTCAACCCCTGCCTT AKcray-R: CGCCCTCTGCTTCGGTGTGCTC	J.W. Breinholt unpublished data	538bp

2.2.2.1 Amplification

The PCR reactions for COI contained the following components; 2.0 µL DNA extract, 0.4 µL forward and reverse primer (10mM), 0.32 µL dNTP (10mM), 0.7 µL $MgCl_2$ (50 mM), 1.25 µL buffer (10×), 0.35 µL Astral Red *Taq* (1U/µL), and 7.08 µL ddH_2O. The 16S reactions contained; 2.0 µL DNA extract, 0.4 µL forward and reverse primer (10mM), 0.3 µL dNTP (10mM), 0.63 µL $MgCl_2$ (50 mM), 2.50 µL buffer (5×), 0.35 µL Bioline Mango *Taq* (5U/µL), and 6.19 µL ddH_2O. The GAPDH, H3, and AK reactions used; 1.5 µL DNA extract, 0.4 µL forward and reverse primer (10mM), 0.2 µL dNTP (10mM), 0.6 µL $MgCl_2$ (50 mM), 1.0 µL buffer (10×), 0.3 µL Astral Red *Taq* (1U/µL), and 5.6 µL ddH_2O. For all analyses, one positive and one negative control were included, with PCR reactions performed on a GeneAmp PCR System 2700 (Applied Biosystems, www.appliedbiosystems.com).

The PCR cycling conditions for each gene were as follows: (a) COI – 94ºC for 5 min; 40 cycles of: 30 sec at 94ºC, 1 min at 55ºC, 30 sec at 72ºC; then 7 min at 72ºC with the reaction stored at 4ºC, (b) 16S – 94ºC for 10 min; 40 cycles of: 45 sec at 94ºC, 45 sec at 52ºC, 1.5 min at 72ºC; then 7 min at 72ºC with the reaction stored at 4ºC, (c) GAPDH and AK – 94ºC for 3 min; 40 cycles of: 30 sec at 94ºC, 30 sec at 57ºC, 30 sec at 72ºC; then 5 min at 72ºC with the reaction stored at 4ºC, and (d) H3 – 94ºC for 3 min; 45 cycles of: 30 sec at 94ºC, 30 sec at 50ºC, 1 min at 72ºC; then 5 min at 72ºC with the reaction stored at 4ºC.

2.2.2.2 DNA sequencing

An enzymatic purification procedure was undertaken using EXO SAP (Fermentas, www.fermentas.com) on successfully amplified samples and contained the following: 5.0 µL amplified PCR product, 0.25 µL Exonuclease I, and 1.0 µL Shrimp Alkaline Phosphate. This solution was incubated at 37ºC for 35 min, heated at 80ºC for 20 min, held at 15ºC, then stored at 4ºC until sequenced. Sequencing reactions contained; 1.0 µL purified PCR product,

1.0 µL of the forward primer (CRCOI-F/16S-ar/GAPDH-F/H3-AF/AKcray-F), 5.0 µL Terminator Mix (Applied Biosystems, www.appliedbiosystems.com), 2.0 µL Terminator Mix Buffer (5×) (Applied Biosystems, www.appliedbiosystems.com), and 5.5 µL ddH_2O. Reactions were performed under the following conditions: initial hold of 96ºC for 1 min; 30 cycles of: 96ºC for 10 sec, 50ºC for 5 sec, 60ºC for 4 min; then a final hold at 4ºC. Samples were then cleaned and sequenced on an automated sequencing machine (Applied Biosystems 3130, www.appliedbiosystems.com) at the Griffith University DNA Sequencing Facility. Sequences were edited using Sequencher 4.9 (GeneCodes 2009) and aligned using the MUSCLE addition in MEGA5 (Edgar 2004). Alignments were then checked and edited by hand if necessary.

2.2.3 Microsatellite Library Development

Two microsatellite libraries were constructed using the methods outlined in Appendix Four. However, although a range of PCR and cycling conditions were trialled, no primers consistently amplified across all samples and this approach was eventually discarded. Therefore, all analyses were confined to DNA sequence data. All developed microsatellite primers are included in Appendix Four for use in any future studies.

2.2.4 Sequencing

A total of 127 *Tenuibranchiurus* specimens were sequenced for the COI gene fragment, 59 for 16S, 93 for GAPDH, 57 for H3, and 46 for AK. Additional specimens were also sequenced for inclusion as outgroups from the genera *Gramastacus*, *Geocharax*, *Engaeus*, *Engaewa*, and *Cherax*. Where these outgroup sequences could not be obtained (i.e. due to non-amplification), alternative sequences were retrieved from GenBank (details in Appendix Five).

2.3 GIS MAPPING

Data were obtained from Australian Government Geoscience Australia (www.ga.gov.au) (GEODATA 9 Second Digital Elevation Model (DEM-9S)) and Bureau of Meteorology (www.bom.gov.au) (Geofabric Surface Cartography). These data sources were used to produce a series of maps throughout this thesis; thus any figures that do not have a source provided in the figure title were created by the author using these data in ESRI ArcGIS 10.2.

3.0 Morphological Analysis of *Tenuibranchiurus*

3.1 INTRODUCTION

3.1.1 The Role of Morphology and Morphometrics in Taxonomy

Morphological examination is considered the 'traditional' form of taxonomy, whereby shared morphological features are used to classify an organism into a species, genus, family, and so forth. However, morphological characters are complex, non-neutral markers that can arise or be lost through a variety of processes, which could potentially lead to an under- or over-estimation of species diversity (Lefébure *et al.* 2006; Burnham and Dawkins 2013). With this in mind, one may question why the need for morphological taxonomy still exists, given that reconstructing phylogenies using molecular data is becoming increasingly popular.

Wiens (2004a) and Jenner (2004) each provide insightful reviews of how morphology is still necessary and relevant, both at the present and into the future. Examples given within their arguments include: (1) examination and comparison of fossil data with modern organisms, which is useful not only for examining the relationship of fossils to extant taxa but also for providing fossil calibration for determining the time of divergence events – a process that is only possible if the correct inferences are made about how fossils are aligned to living taxa, (2) the use of fossils to elucidate the process of character evolution, (3) increasing the accuracy of phylogenetic inferences made using other data forms (e.g. molecular), (4) examination and subsequent placement within a phylogeny for taxa that are rare, existing only as preserved specimens, and may never be collected again, and (5) the morphological description of all living species on Earth is not close to being achieved, but the description of species using other techniques is even further away.

Given the historical role of morphological taxonomy, the probable requirement of this technique well into the future, and the role morphology can play in an integrative taxonomic approach, the importance of morphological taxonomy is evident. However, the act of morphological character examination itself has inherent problems (e.g. Swiderski *et al.* 1998). One concern is that any character state is subject to the interpretation of the observer. For instance, while one observer with specimens displaying the full range of character states (e.g. absent, small, medium, large) may be able to distinguish between them, another observer with only a subset of states visible may not definitively be able to assign a specimen to a character state (e.g. is the character small or medium). This problem can potentially be

overcome by a thorough and in-depth explanation by the describer, in the form of both a written description as well as detailed drawings of each state of the character in question. In addition to this, taxonomic studies have progressively started to incorporate morphometric statistics to reduce potential subjectivity in conclusions drawn through morphology alone (Mutanen and Pretorius 2007). Morphometric statistics analyse metric characters whereby a certain feature, for example the chela length, is measured and recorded. Both morphological and morphometric methods have been used for freshwater crayfish and have been utilised for diagnostic purposes (i.e. species identification and/or description) (e.g. Sokol 1988; Austin and Knott 1996; Bunn *et al.* 2008; Mathews *et al.* 2008).

Despite a number of recognised issues in using morphology to understand evolutionary relationships, this should not preclude the utilisation of morphological information in conjunction with other data sources to perform taxonomic revisions. Very few species descriptions are performed without the description of morphological characters, and the relevance of morphology in the future has been established. Thus, this chapter will utilise morphological information as a line of evidence in an integrative taxonomic approach.

3.1.2 Genus *Tenuibranchiurus*

Tenuibranchiurus is considered to be a monotypic lineage within a monophyletic clade containing the other Australian burrowing crayfish, (*Gramastacus*, *Geocharax*, *Engaewa*, *Engaeus sensu stricto*, and *E. lyelli* (*sensu* Schultz *et al.* 2009)) (Horwitz 1988b). This relationship (proposed by Horwitz (1988b)) was based primarily on morphological and behavioural adaptations to their burrowing habit, and has subsequently been supported by various molecular studies (e.g. Crandall *et al.* 1999; Schultz *et al.* 2007; Schultz *et al.* 2009; Toon *et al.* 2010). Although there is a large amount of morphological diversity within each of the six burrowing genera, a number of morphological similarities have been shown between them (see Horwitz 1990). As certain characters have been found that blur the distinction between some of these genera, Horwitz (1990) suggested that the overlap and variability of morphological characters between the burrowing crayfish could indicate that the current generic distinctions between them may not be the best representation of true evolutionary relationships. Indeed, the splitting of *Engaeus* into *Engaeus sensu stricto* and *E. lyelli* by Schultz *et al.* (2009) based on molecular data supports this.

The only previous morphological work that has been published on *Tenuibranchiurus* is the original description by Riek in 1951 (see Appendix Six). Riek described the genus as having a Qld distribution and separated it from the other Qld crayfish genera (i.e. *Euastacus* and *Cherax*) on the basis of the nature of the podobranchs, abdominal somite, and telson, and on the orientation of chelae and the branchial formula (see Riek 1951 for complete key). Although the morphological description for *Tenuibranchiurus* is fairly thorough for the characters examined, the number of characters examined is relatively small and morphological variability is not covered in any great detail. As the original description by Riek was based on specimens from only two localities (with the second locality consisting of only two specimens), there is almost certainly as yet unaccounted-for morphological diversity present within this genus.

Since its original description, the distribution of *Tenuibranchiurus* has been extended to coastal northern NSW (Dawkins *et al.* 2010). In addition to this, based on the results of a preliminary genetic study, Dawkins *et al.* (2010) suggested that the genus *Tenuibranchiurus* may, in fact, contain two (or more) species. The two genetically divergent groups most likely to represent species were identified as those populations from Qld, and the new populations from NSW. This highlights the need for a morphological revision to be undertaken on this genus of crayfish; an issue that this chapter seeks to address.

3.2 CHAPTER AIM

The purpose of this chapter is to examine the morphological variation within the genus *Tenuibranchiurus* and use these data to test the currently accepted taxonomy (i.e. a single species within this genus) through multiple morphological analyses.

3.3 METHODS

Wherever possible, the same specimens were included in both the morphological and morphometric analyses and all locations from which whole specimens were collected were included in the analyses (i.e. excluding locations where only chelae were collected); these sampling locations spanned the entire geographic extent of the genus. To examine the degree of morphological variation within and between sampling locations, each location was treated as a 'population' (and shall be referred to as such) and therefore a separate operational taxonomic unit.

Initial examination of the morphology of these crayfish identified taxonomic characters that were not consistent with the original description of *Tenuibranchiurus*. Additionally, Qld and NSW specimens were found to be substantially different from each other, to the extent that they warrant separate analyses. As a result of this finding, four specimens of *Gramastacus lacus* were also included for morphometric and morphological examination. This approach was taken for a number of reasons; (1) specimens from the genus *Gramastacus* represent the most geographically proximate group of similar crayfish (i.e. they are found just to the south of the NSW populations and are considered to be phylogenetically closely-related), (2) the degree of morphological variation within the Qld/NSW populations could span the morphological separation of *Tenuibranchiurus* and *Gramastacus*, thereby resulting in a bridge between the taxa, and (3) if the morphology of the *Gramastacus* specimens does not span the differences between Qld and NSW, it may align with one of the groups and identify the need for a re-examination of the current taxonomy of this taxon also.

3.3.1 Morphometric Measurements

Morphometric measurements were made on specimens from seven Qld and three NSW sampling locations (see Table 3.1 for number of specimens measured). All measurements were made with digital callipers to a precision of 0.01 mm except where specified. Damaged body parts were excluded from analyses. Measurements were divided into three categories; chela, cephalothorax, and rostrum, with all measurements shown in Figure 3.1. In addition to this, the OCL was also measured. This measurement was taken from the posterior orbital margin to the centre of the posterior carapace margin. Appendix Seven shows a full list of the measurements for all populations.

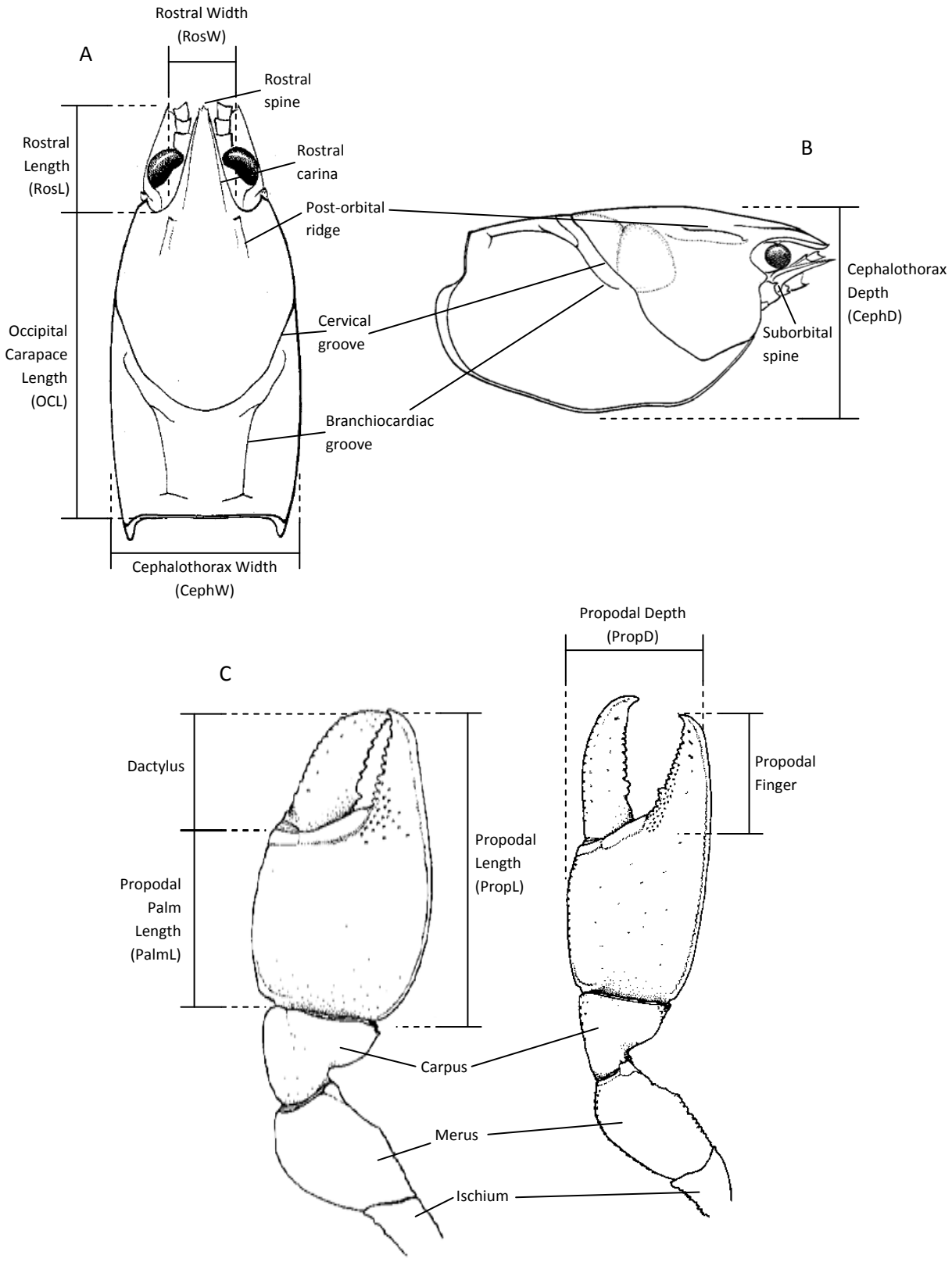


Figure 3.1. Stylised drawings of a freshwater crayfish of the dorsal (A) and lateral (B) view of the cephalothorax showing the features from which morphological characters and morphometric measurements were scored. Representations of the lateral view of a large dimorphic (left) and isomorphic (right) chelae are shown in (C) with the features from which morphological characters and morphometric measurements were scored. Figures A and B adapted from Riek (1972) and C from Horwitz and Adams (2000).

For the chela measurements, specimens were identified as being either heterochelous or homochelous (this study represents the first time heterochelosity has been reported in *Tenuibranchiurus*). The definition of each given by Horwitz and Adams (2000, pg 657) will be adopted in this study, where heterochelous is when "two chelae differ in their size, proportions, setation, tuberculation and the form of the cutting edges to give a 'large dimorphic chela' and a 'small dimorphic chela'" and homochelous is when "two chelae do not differ markedly in their proportions even though their sizes may be different, to give two equally proportioned (but not necessarily equally sized) or 'isomorphic' chelae" (see Figure 3.1c). In this study, the terms isomorphic, small dimorphic, and large dimorphic will be used.

For the chela morphometrics, three measurements were made; propodal depth (PropD), propodal length (PropL), and propodal palm length (PalmL). The PropD was measured at the deepest part of the chela (when viewed laterally), the PropL was measured from the tip of the chela to where it joins the carpus, and the PalmL was measured from the articulation of the dactylus to where the chela joins the carpus. The dactyl length was also measured initially, but discarded as the curve of the dactylus makes it unreliable to measure. Measurements were made for both the left and right chelae and recorded separately. Where regenerate chelae were present, these were not measured as they have been shown to display different relative dimensions (due to growth patterns) to fully formed chelae (Sokol 1988; Austin and Knott 1996).

Cephalothorax measurements included the cephalon width (CephW) and cephalon depth (CephD). The CephW was measured either side of where the cervical groove runs across the top of the carapace, and CephD was measured from in front of the first walking leg to the top of the carapace.

Rostral measurements included the rostral length (RosL) and rostral width (RosW), and were measured using a graticule in a binocular microscope as the digital callipers were too large to make accurate measurements due to the small size of the rostrum. The RosL was measured from the tip of the rostrum to the imaginary line running between the posterior orbital margin, and the RosW was measured from where the rostral edge joins the orbital margin. Because of the small sizes involved, the ratio between length and width was recorded rather than raw measurements.

Table 3.1. Number of specimens from which morphometric measurements were recorded for each sampling location from Queensland and New South Wales for the chela, cephalothorax, and rostrum. Unless damaged or regenerate, both chelae were measured for each specimen. Cephalothorax and rostral measurements were not made on specimens with damage to those features.

Group	Pop[n]	Number of specimens measured		
		Chela	Cephalothorax	Rostrum
Qld	HB	8	8	8
	MAR	5	6	6
	TL	6	6	6
	BER	10	10	10
	BRB	4	6	5
	TEW	10	10	10
	GC2	5	5	5
NSW	LH	9	10	11
	BNP1	5	5	5
	LakeH	8	9	9

3.3.2 Morphology

The original description for *Tenuibranchiurus* did not provide many characters that could potentially be useful diagnostic characters. However, full morphological reviews for closely related genera (e.g. *Engaeus*, *Engaewa*, *Gramastacus*, *Geocharax*) have been undertaken and these were used as a general guide as to what features may be important for this genus (see Erichson 1846; Clark 1936; Riek 1967, 1972; Horwitz 1990; Zeidler and Adams 1990; Horwitz and Adams 2000 for descriptions of these genera).

A total of 31 characters were examined initially (see Appendix Eight), with these condensed to 19 informative characters (excluding those unique to either Qld or NSW). Once the final character summaries were completed for all populations, character states were determined. A combination of discrete and continuous characters were used, and between two and five character states were defined for each character. For example, some characters could be described as either present or absent, others as a gradient (e.g. small, medium, large), and others could have two or more different expressions (e.g. granulate, punctate). Where size classes were used as character states, relative sizes of features were adapted from Morgan (1997) which provides graphical representations of many spination patterns for the genus *Euastacus*, thereby avoiding subjectivity between specimen examinations. Table 3.2 shows the final list of characters and character states used, and the relevant morphological structures are shown in Figure 3.1.

Table 3.2. Final list of characters and their associated character states used for all morphological analyses for characters present in Queensland and New South Wales.

Character Code	Character	Character State				
		A	B	C	D	E
2	Rostral carinae length	Extending to posterior margin of orbit and reaching to approximately ½ of the way along rostrum	Extending to posterior margin of orbit and reaching to approximately $^{2}/_{3}$ of the way along rostrum	Extending to posterior margin of orbit and reaching to tip of rostrum but not fusing with tip		
3	Rostral carinae definition	Very poorly to poorly developed, inconspicuously raised	Moderately developed, moderately raised	Well developed, conspicuously raised		
4	Rostral spine	Absent	Very small	Small	Medium	
5	Post-orbital ridges	Very poorly developed	Poorly developed	Moderately developed		
9	Cephalothorax punctation (*Dorsal*; anterior to cervical groove)	Sparse	Moderate			
10	Cephalothorax punctation (*Dorsal*; posterior to cervical groove)	None to very sparse	Sparse	Moderate		
11	Cephalothorax granulation (*Lateral*)	Moderate	Dense			
13	Branchiocardiac groove	Transverse grooves present just before posterior margin of the cephalothorax	No transverse grooves present just before posterior margin of the cephalothorax			
17	Basipodite spine	Absent	Very small	Medium	Large	Very large
19	Suborbital spine	Absent	Very small	Small	Medium	Large
24	Propodal palm granulation (*Ventral*)	Small, no carinae	Large, no carinae	Margin smooth and carinate		
25	Propodal palm granulation (*Dorsal*)	Small	Moderate	Large	Irregular row of small tubercles along margin	Irregular row of large tubercles along margin
26	Propodal palm granulation/punctation (*Lateral*)	Small granulation	Large granulation	Very sparsely punctate		
31	Propodal finger granulation/punctation (*Lateral*)	Smooth, sparse granulation ventrolaterally	Smooth halfway with dense granulation posteriorly	Very sparsely punctate		
34	Dactylus granulation (*Dorsal*)	Small	Moderate	Large	Margin with 2 rows of tubercles	
38	Ventromeral spine	Absent	Medium	Large	Very large	
42	Meral margin tubercles (*Dorsal*)	One row	Two rows			
43	Meral spine (*Dorsal*)	Absent	Present			
46	Ischium (Dorsal margin)	Smooth	One row of small tubercles	One row of large tubercles	Two rows of small tubercles	

3.3.3 Statistical Analyses

3.3.3.1 Morphometric analyses

All morphometric statistical analyses were performed in the statistical packages SPSS version 20 (SPSS 2011) and Primer6 (Primer-E Ltd. 2007). All analyses performed on an individual data set (those outlined within the next three paragraphs) were performed using SPSS, and all combined analyses used Primer, except for discriminant function analyses which also utilised SPSS.

Because allometric scaling is known to occur for some characters in some species of crayfish (Sokol 1988), differing rates of development due to the age of a specimen were tested for within populations. Sexual dimorphism has also been shown to exist in some crayfish genera (e.g. Horwitz 1990; Grandjean and Souty-Grosset 2000; Streissl and Hödl 2002; Sint *et al.* 2007), and therefore sex was also tested separately for allometric scaling. Raw measurements (i.e. PropL, PalmL, PropD, CephW, and CephD) were initially regressed against OCL for each population separately. After the linearity of each population's relationship was assessed for each variable, the data for each population were grouped into a single data set. Sex was then separated, and each variable (with populations grouped) regressed against OCL.

Because all measurements were found to be isometric with respect to OCL (i.e. growth did not lead to a change in proportion), log-transformation of the data (as has been used in other crayfish studies; e.g. Sokol 1988; Austin and Knott 1996) was not necessary. All of the data displayed strong linear relationships without transformation when regressed against OCL, and examination of the residuals showed that there was no increase in the residual with an increase in the dependent variable. Therefore, in order to eliminate the bias caused by size differences of measured specimens, all measurements were normalised for size by dividing them by the corresponding OCL, as has been performed in other studies (e.g. Chambers *et al.* 1979; Sint *et al.* 2007).

With the potential influence of allometric scaling discounted, each variable was then tested to see if there was a difference between sex (i.e. although neither sex scales allometrically, they may grow at different rates). Using a General Linear Model (GLM) with sex as a covariate, the standardised chela and cephalon measurements (i.e. standardised for specimen size) for each population were tested. If a significant effect of sex was found, a post-hoc analysis

using the Estimated Marginal Means (EMM) with a Bonferroni correction was performed to show in which populations the differences occurred. Once the effect of sex was tested for, differences between populations were assessed using a GLM and, where significant differences were found between populations, a Tukey post-hoc analysis was performed to show where the differences occurred. The GLM tests were performed on all three data sets (i.e. chela, cephalothorax, and rostrum).

Because individual variables may not always show a clear pattern of separation between groups, all of the morphometric data were combined and analysed together. Principle components analyses (PCA) were performed to summarise patterns in the data. PCA summarises relationships among variables by transforming potentially correlated variables into principle components (i.e. uncorrelated variables) (Jackson 1991). This reduces the complexity of the data, with each successive principal component accounting for as much of the variability within the data as possible (i.e. the first accounts for the majority, the second as much of the remaining variability as possible, and so on) (Jackson 1991). As PCA is used for continuous numerical data, it is ideally suited for use on morphometric data.

The combined data were also tested using a discriminant function analysis (DFA). Rather than creating a summary of the patterns seen in the data, this tests the efficacy of the data for assigning each individual accurately to the correct population (as designated *a priori*). This is achieved by taking into account the within-group correlation between characters and therefore maximising the separation between groups relative to the within-group variance (Thorpe 1976; Austin and Knott 1996). Although this method introduces an *a priori* bias into the analysis, it was only used as a single line of evidence in a multi-facetted approach. The independence of each variable was tested using a tolerance test. Those variables that failed were excluded from the analysis as they represent a near-linear combination of other variables within the analysis.

3.3.3.2 Morphological analyses

The morphological data were analysed using the statistical package Primer6 (Primer-E Ltd. 2007). Each specimen was entered as a 'sample', and each character state was used as a 'variable'. The data were partitioned into two separate data sets (Qld and NSW), and each data set only included those variables present within each of these groups as, although each morphological character was assessed for all specimens, some individual character states only occurred within Qld populations and some only within NSW populations. A total of 48 variables for 51 Qld specimens and 33 variables for 25 NSW specimens were included in the analyses.

In order to visualise the relationship between populations based on morphological characters, non-metric multidimensional scaling (MDS) plots with 1000 restarts were created for each data set (i.e. Qld and NSW). The MDS represents the similarities or differences between sampling units. This test creates a distance matrix between each possible pair of sampling units and uses this to create a graphical representation of dissimilarities in as few dimensions (i.e. axes) as possible. MDS is suited to discontinuous data, and is therefore most appropriate for categorised morphological data (Edkins *et al.* 2007). The combined morphology data were also tested using a DFA, with a tolerance test conducted on the variables.

Population aggregation analysis (PAA) is one of the few non-tree based methods for diagnosing species boundaries using morphological data. Other methods (e.g. Wiens and Penkrot methods (Wiens and Penkrot 2002) and Templeton's Test for Cohesion (Templeton 2001)) use morphological data but are tree-based and therefore require some form of knowledge about the evolution of populations/characters (Sites and Marshall 2003). PAA was developed by Davis and Nixon (1992) and is a formal codification of traditional taxonomic methods using diagnostic character differences; in this case, morphology. Profiles are developed for each population that summarise the character states present. Populations are 'lumped' into species in an iterative process, whereby populations with identical profiles or no fixed character differences are combined. Where there is at least one fixed character difference, populations or groups are separated into species. PAA has some drawbacks, namely the requirement for diagnostic characters to be present at 100% frequency in a population. The requirement of fixed character states raises two issues; (1) under-sampling of characters may lead to an underestimation of the number of species present as fixed states

may not be observed, and (2) under-sampling of specimens may lead to an overestimation of species as traits that are in fact polymorphic may only be observed in one (seemingly fixed) state (Wiens and Servedio 2000). Character fixation in PAA is difficult to support statistically, due to the large sample size or number of assessed characters required to give statistical confidence. Wiens and Sevedio (2000) developed criteria that are less restrictive (e.g. 95% confidence that all character states have been sampled) to allow statistical support. However, due to the limited number of specimens that could be collected, coupled with the general paucity of characters present for these crayfish, this method could not be employed. Therefore, the results from the PAA analyses were treated as one line of evidence in a multi-facetted approach, rather than prescriptive species diagnoses.

3.3.3.3 Combined morphometric and morphology analyses

Multidimensional scaling plots with 1000 restarts were created for both the Qld and NSW data sets. The combined morphology data were also tested using a DFA, with a tolerance test conducted on the variables.

3.4 RESULTS

3.4.1 Comparison to *Gramastacus lacus*

The original 51 identified morphological characters were examined across four *Gramastacus lacus* specimens in addition to the *Tenuibranchiurus* specimens. Of these characters, 26 were found to be informative and were separated into four categories; (i) shared between Qld and NSW to the exclusion of *G. lacus*, (ii) shared between *G. lacus* and NSW to the exclusion of Qld, (iii) shared between *G. lacus* and Qld to the exclusion of NSW, and (iv) not shared between any of the groups (Table 3.3). It should be noted that although some groups may share the same character (e.g. granulate), they do not necessarily express that character to the same degree (e.g. density of granulation). Characters that were shared between all three groups are not shown.

While Qld and NSW populations were observed to be substantially different from each other morphologically, when characters were analysed using MDS (Figure 3.2) they did have a number of characters that were shared between them (Table 3.3). The shape of the antennal squame, development of the post-orbital ridge, presence of granulation on both the dorsal meral and lateral carpal surfaces, presence of punctation on the mesial dactylus surface, presence of meral setae, and presence of a terminal meral spine are all shared traits that *G. lacus* did not have. The presence of punctations on some surfaces of the propodus and carpus, and granulation of the ventral meral surface were traits shared between NSW and *G. lacus* to the exclusion of Qld. The presence of branchiostegal and ventromeral spines, granulation of the propodal palm, and absence of carinae on the propodus were shared between Qld and *G. lacus* to the exclusion of NSW. Finally, the rostral carinae length, and the degree of granulation/punctation on the ventral and mesial surfaces of the propodal finger, lateral dactyl surface, and ventral surface of the propodal palm are all characters that were different between the three groups.

Five characters were identified that varied among the groups but had some overlap between them, which (along with the characters identified in the comparison between all groups) could potentially be useful for detection of clinal variation. These characters are included at the bottom of Table 3.3 and include the degree of definition of the rostral carinae, granulation/punctation of the mesial propodal palm surface, presence/absence of the dorsal meral spine, granulation/punctation/tuberculation of the dorsal dactyl surface, and possessing isomorphic and/or dimorphic chela.

As well as being morphologically differentiated, comparison of a variety of morphometric measurements also demonstrated clear separation between groups (based on isomorphic specimens; Figures 3.3, 3.4, and 3.5). The standard error plots showed *G. lacus* as the most highly differentiated, with substantial differences in the propodal and palm length, all chela ratios, and the cephalothorax ratio. Although there was considerable overlap in the Qld and NSW specimens, a difference could be seen in the overall chela shape; the propodus depth/propodus length, and propodus depth/palm length. In the PC plots (Figure 3.5), the first axis accounted for 76.1% of the total variation and was correlated most highly with CephD/CephW, PropL/OCL, PalmL/OCL, and RosL/RosW. The second axis accounted for 12.6% of the variance and was most highly correlated with RosL/RosW, and the size-standardised PropL, PalmL, and PropD.

The morphometric measurements (Figures 3.3, 3.4, and 3.5) clearly supported Qld and NSW being distinct from *G. lacus*. Additionally, the morphological results showed characters separating each group, supporting the division of each of the three groups into distinct taxonomic units (Figure 3.2). Therefore, separate analysis of Qld and NSW specimens was required and, as such, the results for the remainder of this chapter will be presented separately for these two groups.

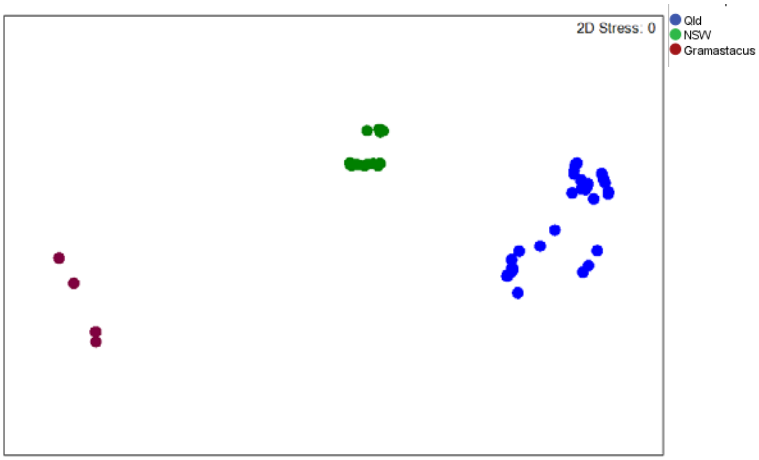


Figure 3.2. Multi-dimensional scaling plot of the relationship found between Queensland and New South Wales groups of *Tenuibranchiurus glypticus* and *Gramastacus lacus*. Analysis performed on 21 morphological variables.

Table 3.3. Morphological character differentiation between Queensland and New South Wales specimens of *Tenuibranchiurus glypticus*, and *Gramastacus lacus*. Refer to Table 2.1 for sampling locality codes.

	Character	Character expression		
		Qld	NSW	*Gramastacus lacus*
Qld and NSW versus *Gramastacus lacus*	Antennal squame	Distal		Mid-length
	Post-orbital ridge development	Poor to moderately		Very well developed
	Dactylus (mesial)	Smooth		Punctate
	Ventral meral inner edge	Does not terminate in spine		Terminates in spine
	Dorsal meral surface	Granulate		Smooth
	Carpus lateral surface	Granulate		Punctate
	Ventral meral surface	Setose		No setae
NSW and *Gramastacus lacus* versus Qld	Propodal palm (lateral)	Granulate	Punctate	
	Propodal finger (lateral)	Granulate	Punctate	
	Ventral meral surface	Smooth	Granulate	
	Carpus dorsal surface	Granulate	Punctate	
Qld and *Gramastacus lacus* versus NSW	Branchiostegal spine(s)	Present	Absent	Present
	Propodal palm (dorsal)	Granulate	One or two rows of tubercles	Granulate
	Ventromeral spine	Present	Absent	Present
	Propodal palm (ventral)	No carinae	Carinate	No carinae
	Propodal finger (ventral)	No carinae	Carinate	No carinae
Qld versus NSW versus *Gramastacus lacus*	Rostral carinae length	Does not reach tip of rostrum	Reaches tip of rostrum, does not fuse with tip	Reaches tip of rostrum, fuses with tip
	Propodal finger (ventral)	Granulate	Smooth	Punctate
	Propodal finger (mesial)	Granulate posteriorly and smooth anteriorly	Smooth, or ventromesially granulate and dorsomesially smooth	Punctate
	Propodal palm (ventral)	Granulate	Smooth	Punctate
	Dactylus (lateral)	Smooth	Very small punctation	Large punctation
Variable characters displaying some overlap	Rostral carinae definition	Poorly to moderately (TEW sometimes well developed)	Well developed	Very well developed
	Propodal palm (mesial)	Granulate	LH – Granulate LakeH,BNP1 – Punctate	Punctate
	Dorsal meral spine	TL,TEW,GC2 – Absent HB,MAR,BRB,BER – Present	Always absent	Always present
	Dactylus (dorsal)	Granulate	LakeH – Granulate LH,BNP1 – Two rows of tubercles	Punctate
	Chela type	Isomorphic	Isomorphic or dimorphic	Isomorphic

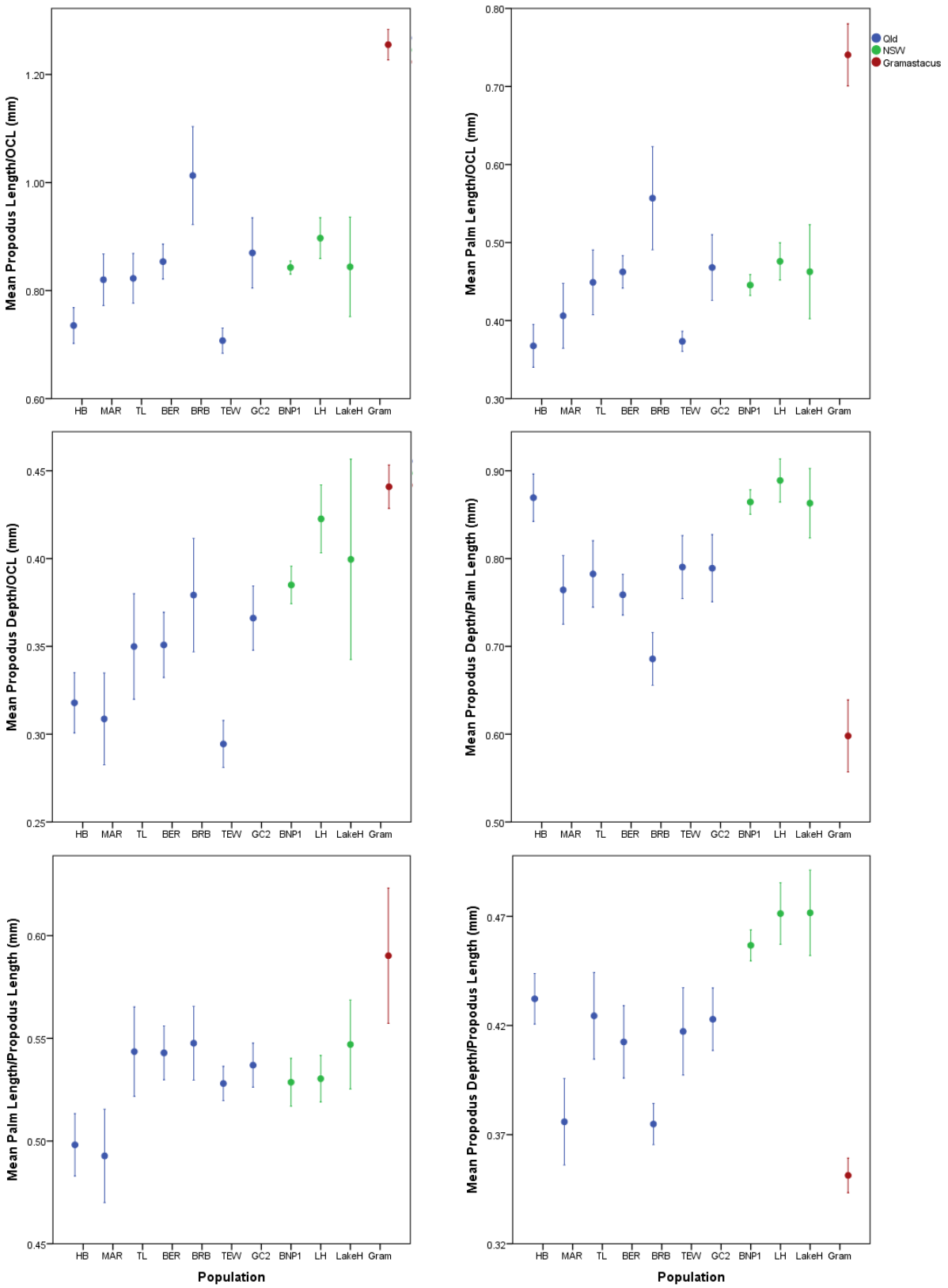


Figure 3.3. Comparison of chela morphometric measurements for isomorphic specimens of Queensland and New South Wales groups of *Tenuibranchiurus glypticus* and *Gramastacus lacus*. Refer to Table 2.1 for sampling locality codes.

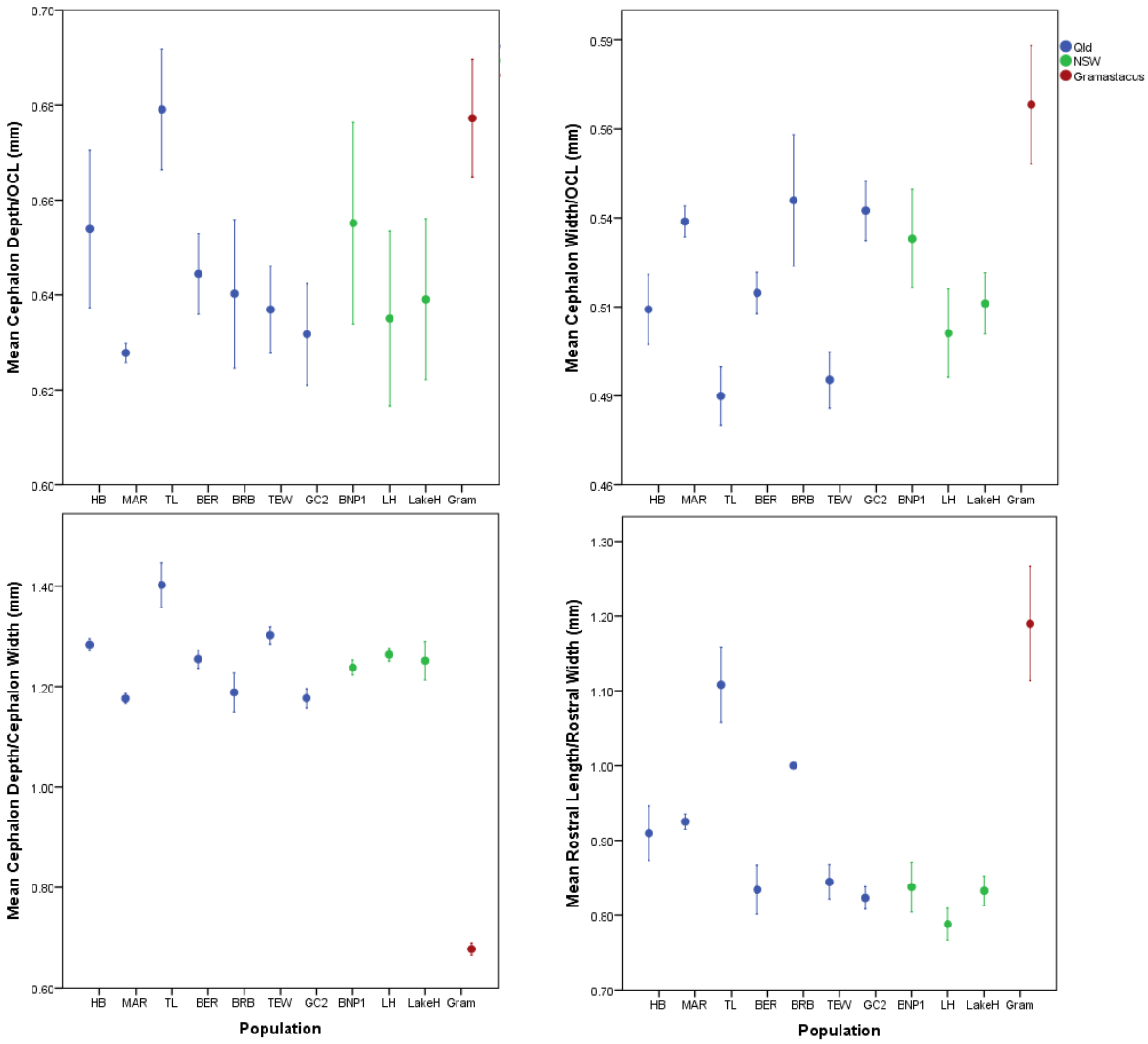


Figure 3.4. Comparison of cephalothorax and rostral morphometric measurements isomorphic specimens of Queensland and New South Wales groups of *Tenuibranchiurus glypticus* and *Gramastacus lacus*. Refer to Table 2.1 for sampling locality codes.

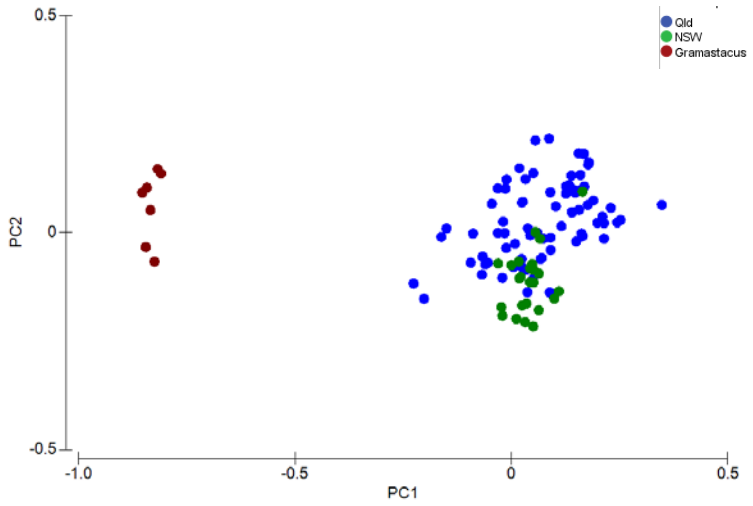


Figure 3.5. Plots of the principal component scores of the first two axes based on morphometric variables from Queensland and New South Wales groups of *Tenuibranchiurus glypticus* and *Gramastacus lacus* specimens. Analysis performed on 10 morphometric variables.

3.4.2 Queensland Morphometric Analyses

Morphometric analyses were performed on the following variables: PropL, PalmL, PropD, CephD, CephW, RosL/RosW. Although significant differences were found between populations for each of these variables (when analysing size-standardised variables or chela ratios), no consistent patterns were found that grouped particular populations. Therefore, the results of these single variable analyses are presented and discussed in Appendix Nine.

3.4.2.1 Combined morphometric analysis for Queensland populations

The structure of the first five principal components (using chela, cephalon, and rostral data) is shown in Appendix Ten, Table A10.1. The first axis accounted for 51.1% of the total variation and was correlated most highly with PropL/OCL, PalmL/OCL, PropD/OCL, PropD/PalmL, and CephD/CephW. The second axis accounted for 27.0% of the variance and was most highly correlated with CephD/CephW, CephD/OCL, and RosL/RosW. Combining the first two components clearly separated TL from all other populations, with BRB also generally distinct though with two specimens showing a small overlap with other populations (Figure 3.6). Exclusion of these populations from the PCA did not improve the separation of the remaining specimens.

Specimens from TL had a distinctive rostrum; the only population in which it was longer than it was broad; and a much higher cephalothorax ratio (i.e. cephalon depth was greater than other populations in comparison to the width). The main factors attributable to the difference seen at BRB were PropL/OCL, PropD/OCL, PalmL/OCL, and PalmL/PropL, with specimens showing distinctive long, slender chelae.

The DFA assigned 83.5% of specimens to the correct population (Table 3.4). TL was the only population for which specimens were always correctly assigned, while the highest discrepancy occurred when identifying BRB specimens as 40% were allocated to MAR. Discrimination was based mainly on the standardised chela measurements and chela ratios, rather than the cephalon and rostrum (Appendix Ten, Table A10.2). The first three discriminant functions accounted for 88.8% of the total variation, with the individual scores and population centroids shown in Figure 3.7. Again, both graphs showed TL completely separate from all other populations, with the relationships between other populations less clear.

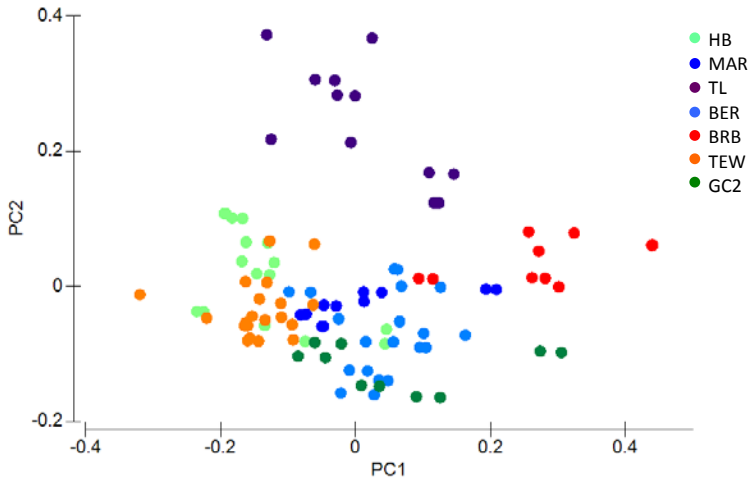


Figure 3.6. Plots of the principal component scores of the first two axes based on morphometric variables from Queensland populations of *Tenuibranchiurus*. Analysis performed on 10 morphometric variables for 51 specimens from seven populations.

Table 3.4. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for seven Queensland populations of *Tenuibranchiurus* based on all morphometric data collected.

		Predicted Group Membership							
	Pop[n]	HB	MAR	TL	BER	BRB	TEW	GC2	Total
Count (%)	HB	11 (78.6)	-	-	2 (14.3)	-	1 (7.1)	-	14 (100.0)
	MAR	-	7 (70.0)	-	-	2 (20.0)	-	1 (10.0)	10 (100.0)
	TL	-	-	10 (100.0)	-	-	-		10 (100.0)
	BER	-	1 (6.7)	-	13 (86.7)	-	-	1 (6.7)	15 (100.0)
	BRB	-	2 (40.0)	-	-	3 (60.0)	-	-	5 (100.0)
	TEW	1 (6.7)	-	-	-	-	14 (93.3)	-	15 (100.0)
	GC2	-	-	-	2 (20.0)	-	-	8 (80.0)	10 (100.0)
83.5% of original grouped cases correctly classified									

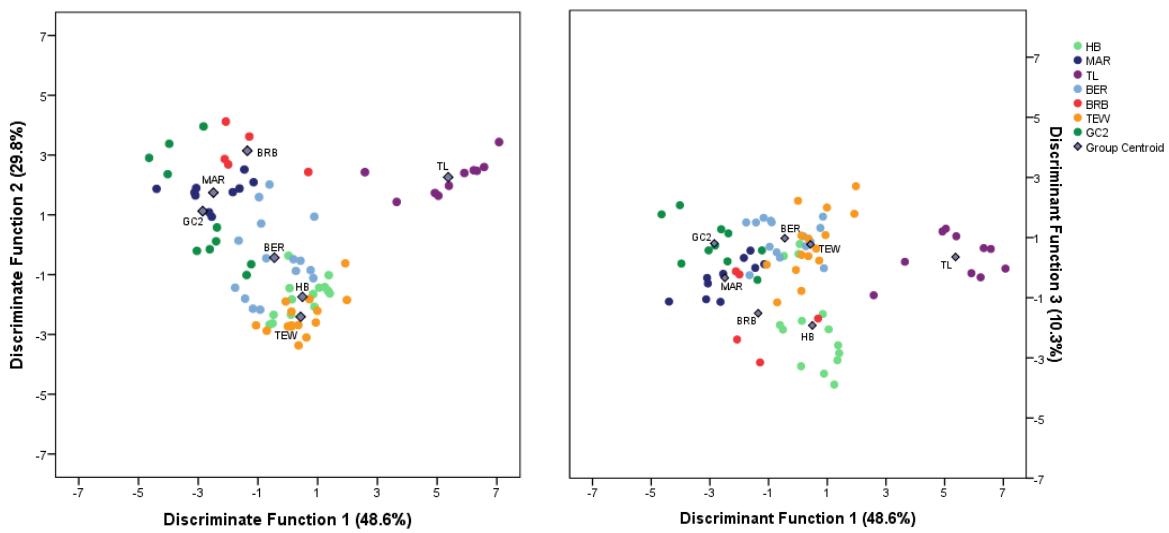


Figure 3.7. Individual discriminant scores and group centroids on combinations of the first three discriminant functions for seven Queensland populations of *Tenuibranchiurus* based on the combined morphometric data. The three axes account for 48.6%, 29.8%, and 10.3% of the total variation, respectively.

3.4.3 Queensland Morphological Analyses

No morphological characters were found to be sexually dimorphic, so all features apply equally to males and females. No intersexed specimens (i.e. where male and female gonopores are present) were found.

The MDS plot showed distinct differences between Qld populations, with no overlap of specimens between populations and all populations clearly distinct from each other (Figure 3.8), although MAR and BER were similar. The DFA analysis assigned 98% of specimens to the correct population (Table 3.5). GC2 was the only population where all specimens were not correctly classified, with one specimen assigned to TL. Rostral carina definition, suborbital spine size, and ventromeral spine size were most strongly correlated with the first function, rostral spine size and post-orbital ridge definition with the second function, and rostral carinae length with the third function (Appendix Ten, Table A10.3). The first three discriminant functions accounted for 91.3% of the total variation, with the individual scores and population centroids shown in Figure 3.9. Graphing the first two discriminant scores separated TEW, GC2, MAR, and TL from the other populations, with the separation less clear when the second and third scores were plotted.

The PAA found fixed differences for four out of the seven populations (GC2, TEW, HB, and BRB), with TL, BER, and MAR grouped (Table 3.6; see Appendix Thirteen for full list of character states). The diagnostic characters for GC2, TEW, HB, and BRB were as follows: GC2 = ischium dorsal margin smooth, moderate sized granulation on the dactyl dorsal margin; TEW = moderately dense granulation over cephalothorax, large sized granulation on the propodal palm lateral surface; HB = small sized granulation on the dactyl dorsal margin; and BRB = ischium dorsal margin with two rows of tubercles, propodal finger lateral surface smooth until halfway with dense granulation posteriorly.

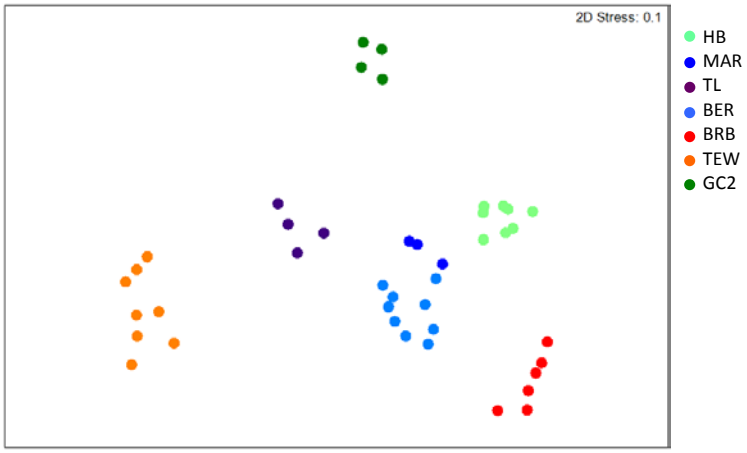


Figure 3.8. Multi-dimensional scaling plot of the relationship found between Queensland populations of *Tenuibranchiurus*. Analysis performed on 48 character states across 19 morphological characters for 51 specimens from seven populations.

Table 3.5. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for seven Queensland populations of *Tenuibranchiurus* based on 48 character states across 19 morphological characters.

		Predicted Group Membership							
	Pop[n]	HB	MAR	TL	BER	BRB	TEW	GC2	Total
Count (%)	HB	8 (100.0)	-	-	-	-	-	-	8 (100.0)
	MAR	-	6 (100.0)	-	-	-	-	-	6 (100.0)
	TL	-	-	6 (100.0)	-	-	-	-	6 (100.0)
	BER	-	-	-	10 (100.0)	-	-	-	10 (100.0)
	BRB	-	-	-	-	6 (100.0)	-	-	6 (100.0)
	TEW	-	-	-	-	-	10 (100.0)	-	10 (100.0)
	GC2	-	-	1 (20.0)	-	-	-	4 (80.0)	5 (100.0)
98.0% of original grouped cases correctly classified									

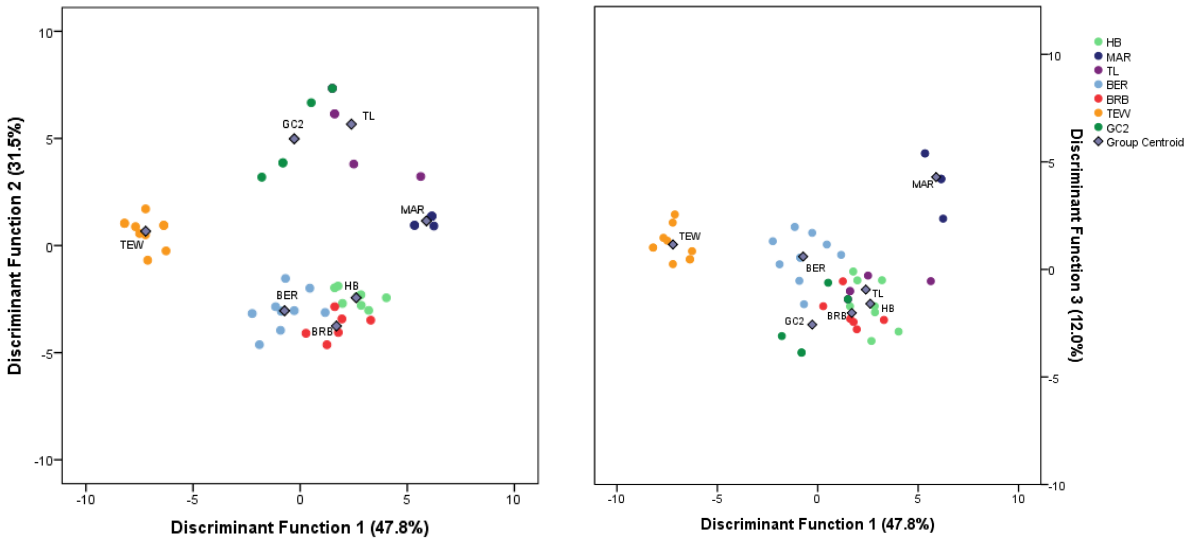


Figure 3.9. Individual discriminant scores and group centroids on combinations of the first three discriminant functions for seven Queensland populations of *Tenuibranchiurus* based on the combined morphology data. The three axes account for 47.8%, 31.5%, and 12.0% of the total variation, respectively.

Table 3.6. Fixed differences determined using population aggregation analysis performed on 48 character states across 19 morphological characters from seven Queensland populations of *Tenuibranchiurus*. States highlighted in dark grey represent fixed differences. Dotted lines indicate potential species groupings.

	Characters and associated character states				
	46	11	26	31	34
GC2	A	B	A	A	B
TEW	B	A	B	A	C
HB	B	B	A	A	A
BRB	D	B	A	B	C
TL	B	B	A	A	C
BER	B	B	A	A	C
MAR	B	B	A	A	C

3.4.4 Queensland Combined Morphometric and Morphological Analyses

The MDS plot showed distinct differences between Qld populations, with no overlap between populations (Figure 3.10), although MAR and BER were similar. The DFA analysis assigned 100% of specimens to the correct population (Table 3.7). Rostral carina length, cephalothorax dorsal anterior punctation, palm length, propodal depth, propodal depth/propodal length, and palm length/propodal length were most strongly correlated with the first function. Rostral carina definition, ventromeral spine size, propodal length, and propodal depth/palm length were strongly correlated with the second function, and post-orbital ridge definition with the third function (Appendix Ten, Table A10.4). The first three discriminant functions accounted for 89.7% of the total variation, with the individual scores and population centroids shown in Figure 3.11. Graphing the first two discriminant scores separated all populations except for TEW and GC2, with the separation between these two populations much clearer when the third scores were included.

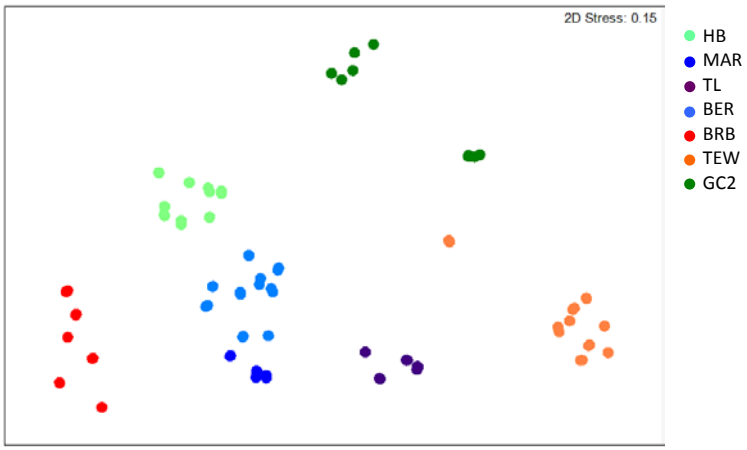


Figure 3.10. Multi-dimensional scaling plot of the relationship found between Queensland *Tenuibranchiurus* populations. Analysis performed on 19 morphological characters and 10 morphometric measurements for 51 specimens from seven populations.

Table 3.7. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for seven Queensland populations of *Tenuibranchiurus* based on 19 morphological characters and 10 morphometric measurements.

		Predicted Group Membership							
	Pop[n]	HB	MAR	TL	BER	BRB	TEW	GC2	Total
Count (%)	HB	14 (100.0)	-	-	-	-	-	-	14 (100.0)
	MAR	-	10 (100.0)	-	-	-	-	-	10 (100.0)
	TL	-	-	10 (100.0)	-	-	-	-	10 (100.0)
	BER	-	-	-	15 (100.0)	-	-	-	15 (100.0)
	BRB	-	-	-	-	5 (100.0)	-	-	5 (100.0)
	TEW	-	-	-	-	-	15 (100.0)	-	15 (100.0)
	GC2	-	-	-	-	-	-	10 (100.0)	10 (100.0)
100.0% of original grouped cases correctly classified									

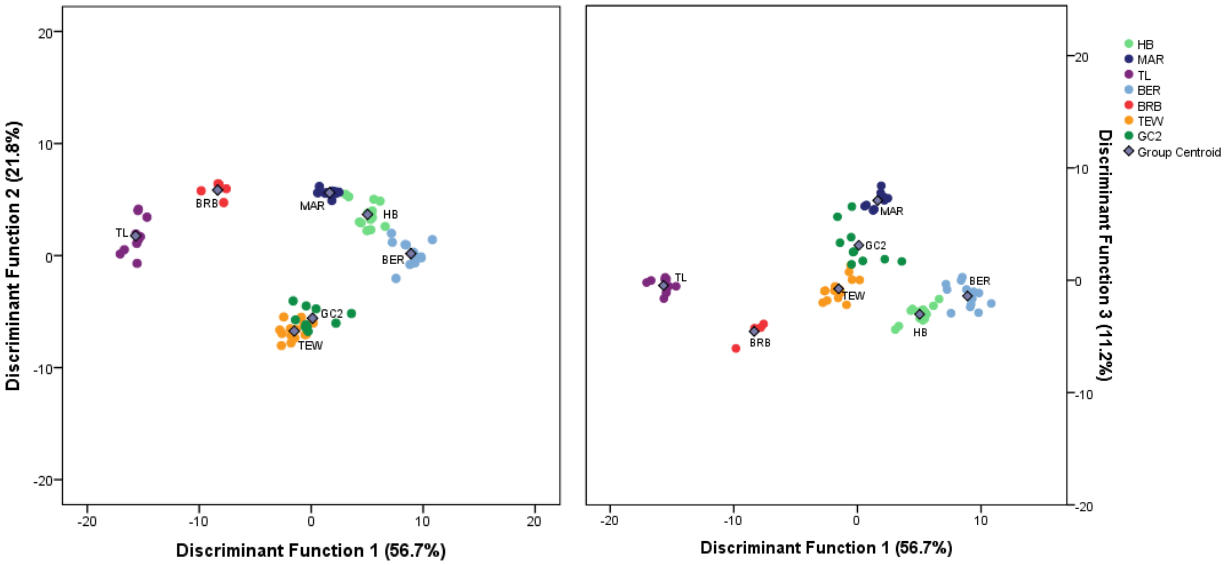


Figure 3.11. Individual discriminant scores and group centroids on combinations of the first three discriminant functions for seven Queensland populations of *Tenuibranchiurus* based on the combined morphology and morphometric data. The three axes account for 56.7%, 21.8%, and 11.2% of the total variation, respectively.

3.4.5 Summary for Queensland

Significant differences were found between populations for each of the morphometric variables examined. Specimens from TL were the most distinguishable, showing distinctive cephalothorax and rostral measurements. They displayed a relatively deep and narrow cephalothorax, and are the only specimens to have a rostrum longer than it is broad. Other populations were also differentiated for certain features. For BRB, the most distinctive characteristic was the shape of the chelae, with specimens exhibiting a long, slender claw, and it is also the only population to have a rostrum of equal length and width. HB, TEW, and MAR also showed some distinctive characteristics; however, these were usually shared with at least one other population and are therefore not useful as diagnostic tools.

The chela variables (both standardised measurements and ratios) were shown by the DFA not to be strong predictors for population assignment. Combining all of the morphometric measurements allowed only one population (TL) to be positively identified (members assigned correctly 100% of the time by DFA and clearly separated by PCA), with TEW being the only other population to score relatively highly in the DFA (93.3%). All other populations scored relatively low using DFA and were not separated by either the discriminant function plots or PCA.

Analyses of the morphological characters showed clearer results than the morphometrics. With the exception of GC2 in the DFA analysis, both the MDS and DFA successfully separated all populations on the basis of morphology. In addition to this, GC, TEW, HB, and BRB all had fixed differences to the exclusion of all other populations that could be used for diagnostic purposes; however, TL, BER, and MAR formed a single morphological group based on the PAA criteria. Although morphological differences between populations are clearer and more readily identifiable than through the use of morphometrics, the overall pattern is still complex.

Combining both morphometric and morphological data improved the strength of separation between populations, and also correctly identified all GC2 specimens using the DFA where the use of only morphological data did not. When the two data sources were combined, all populations could be differentiated from one another.

3.4.6 New South Wales Morphometric Analyses

As for the Qld analyses, the single variable NSW morphometric analyses are presented in Appendix Eleven, as no consistent patterns were found that grouped particular populations.

3.4.6.1 Combined morphometric analysis for New South Wales populations

Because populations from NSW displayed both isomorphic and dimorphic chelae, each chela type had to be tested separately for the combined analysis. The structure of the first five principal components is shown in Appendix Twelve, Table A12.1. For isomorphic specimens, the first axis accounted for 54.2% of the total variation, and was correlated most highly with the standardised chela measurements. The second axis accounted for a further 17.6% of the variance and was most highly correlated with the cephalothorax and rostral measurements. For dimorphic specimens, the first axis accounted for 81.6% of the total variation, and again was correlated most highly with the standardised chela measurements. The second axis accounted for a further 8.5% of the variance and was most highly correlated with the chela, cephalothorax, and rostral ratio measurements. For both isomorphic and dimorphic specimens, combining the first two components did not clearly separate any populations, although there was slight separation suggested for dimorphic specimens (Figure 3.12).

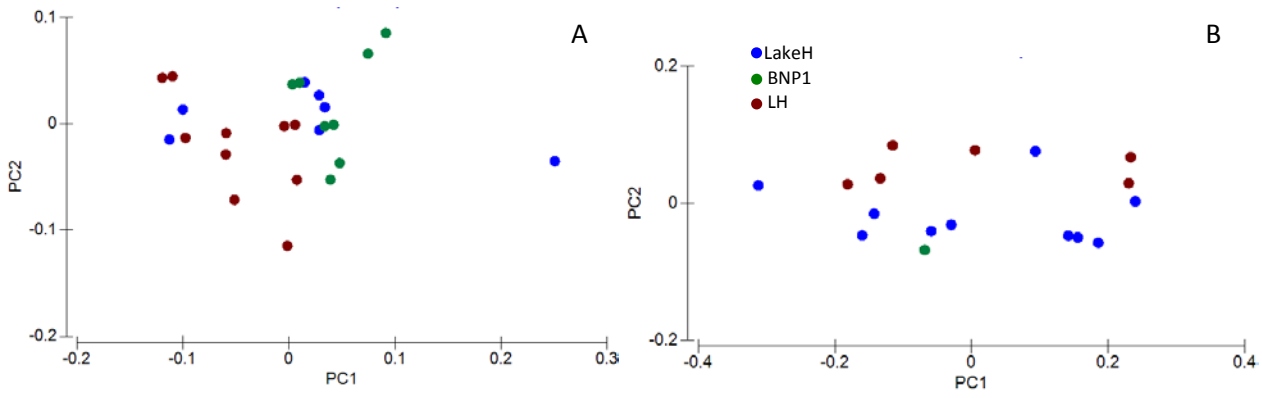


Figure 3.12. Plots of the principal component scores of the first two axes based on morphometric variables from New South Wales populations of *Tenuibranchiurus*. Analysis performed on all morphometric variables, with specimens having either isomorphic (A) or dimorphic (B) chelae separated. A total of 24 specimens from three populations were examined.

The DFA assigned 66.7% and 93.8% of specimens to the correct population for isomorphic and dimorphic specimens, respectively (Table 3.8). For isomorphic specimens, no population was completely identified correctly, with specimens from both LakeH and BNP1 assigned to alternative populations. Correct assignment of dimorphic specimens was much higher, with 100% for both LH and BNP1 (although BNP1 was represented by only one specimen), and 90% for LakeH. Only two discriminant functions were calculated, with the first accounting for 61.7% and 79.8% of the total variation for isomorphic and dimorphic specimens, respectively (Appendix Twelve, Table A12.2). The individual scores and population centroids are shown in Figure 3.13.

Table 3.8. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for three New South Wales populations of *Tenuibranchiurus* based on all morphometric data collected, with isomorphic and dimorphic specimens separated.

		Predicted Group Membership								
		Isomorphic				Dimorphic				
	Pop[n]	LakeH	BNP1	LH	Total	Pop[n]	LakeH	BNP1	LH	Total
Count (%)	LakeH	3 (60.0)	1 (20.0)	1 (20.0)	5 (100.0)	LakeH	9 (90.0)	-	1 (10.0)	10 (100.0)
	BNP1	1 (12.5)	6 (75.0)	1 (12.5)	8 (100.0)	BNP1	-	1 (100.0)	-	1 (100.0)
	LH	3 (37.5)	-	5 (62.5)	8 (100.0)	LH	-	-	5 (100.0)	5 (100.0)
	66.7% of original grouped cases correctly classified					93.8% of original grouped cases correctly classified				

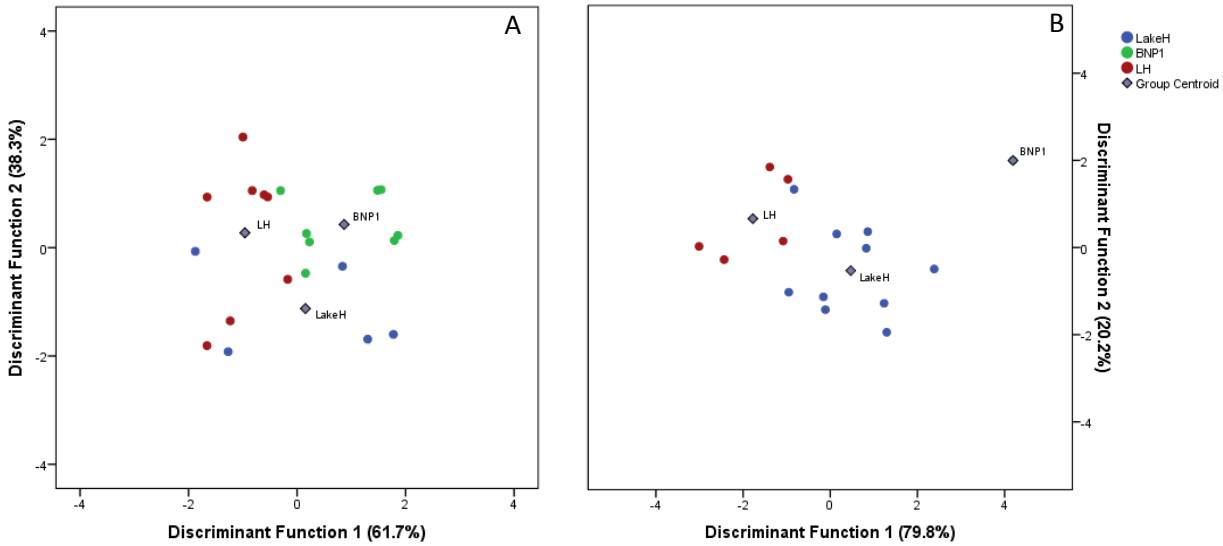


Figure 3.13. Individual discriminant scores and group centroids on combinations of the first two discriminant functions for three New South Wales populations of *Tenuibranchiurus* based on the combined morphometric data. Specimens were separated based on having either (A) isomorphic or (B) dimorphic chelae.

3.4.7 New South Wales Morphological Analyses

No morphological characters were found to be sexually dimorphic, so all features apply equally to males and females. No intersexed specimens were found. Morphological characters did not vary with chela type (i.e. ISO, SD, LD) except for the size of granulation on the dorsal dactyl surface for specimens from LakeH. In this instance, the ISO and LD exhibited state C (large) where the SD showed state B (moderate). As morphological analyses were undertaken on all chela types together, LakeH was simply recorded as having two expressions for that character.

The MDS analysis of morphological characters clearly separated all NSW populations (Figure 3.14) and the DFA analysis assigned 100% of specimens to the correct population (Table 3.9). Post-orbital ridge development, branchiocardiac groove, and dactylus dorsal granulation were most strongly correlated with the first function, and suborbital spine with the second function (Appendix Twelve, Table A12.3). The first discriminant function accounted for 86.2% of the total variation, with the individual scores and population centroids shown in Figure 3.15. Graphing the first two discriminant scores clearly separated all populations.

The PAA found fixed differences between all three populations (Table 3.10; see Appendix Thirteen for full list of character states). The diagnostic characters for each of the populations were as follows: LH = rostral spine very small or medium, propodal palm dorsal surface with small tubercles; BNP1 = cephalothorax with sparse punctation anterior to cervical groove, cephalothorax with sparse punctation posterior to cervical groove; and LakeH = ischium dorsal margin with one row of large tubercles, moderate to large sized granulation on the dactyl dorsal margin.

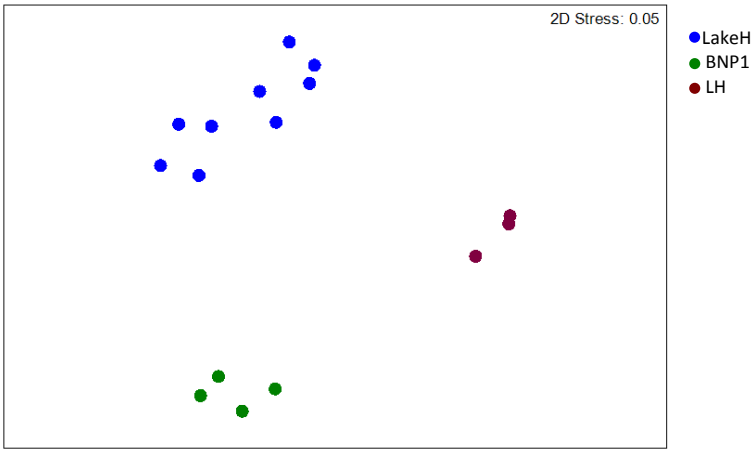


Figure 3.14. Multi-dimensional scaling plot of the relationship found between New South Wales populations of *Tenuibranchiurus*. Analysis performed on 33 character states across 19 morphological characters for 25 specimens from three populations.

Table 3.9. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for three New South Wales populations of *Tenuibranchiurus* based on all morphological data collected.

		Predicted Group Membership			
	Pop[n]	LakeH	BNP1	LH	Total
Count (%)	LakeH	9 (100.0)	-	-	9 (100.0)
	BNP1	-	5 (100.0)	-	5 (100.0)
	LH	-	-	11 (100.0)	11 (100.0)
100% of original grouped cases correctly classified					

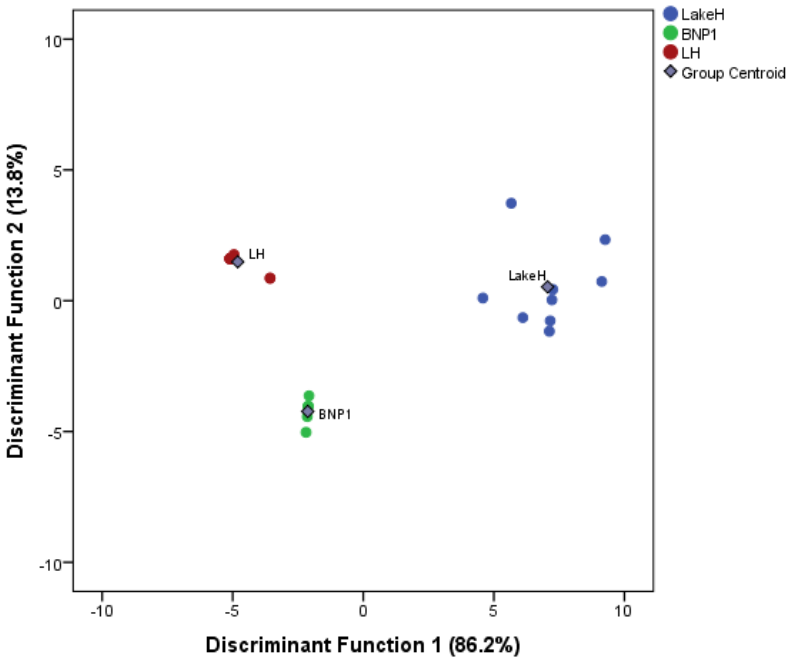


Figure 3.15. Individual discriminant scores and group centroids on the first two discriminant functions for three New South Wales populations of *Tenuibranchiurus* based on the combined morphology data. The axes account for 86.2% and 13.8% of the total variation, respectively.

Table 3.10. Fixed differences determined using population aggregation analysis performed on 48 character states across 19 morphological characters from three New South Wales populations of *Tenuibranchiurus*. States highlighted in dark grey represent fixed differences. Dotted lines indicate potential species groupings.

	Characters and associated character states					
	4	46	9	10	25	34
LH	BD	B	B	A	D	D
BNP1	A	B	A	B	E	D
LakeH	A	C	B	A	E	BC

3.4.8 New South Wales Combined Morphometric and Morphological Analyses

The MDS plot showed all populations (for both isomorphic and dimorphic specimens) to be separate (Figure 3.16). The DFA analysis assigned 100% of specimens (both isomorphic and dimorphic) to the correct population (Table 3.11). For isomorphic specimens, post-orbital ridge development, basipodite and suborbital spine size, and CephD/CephW were most strongly correlated with the first function, and PalmL/OCL with the second function (Appendix Twelve, Table A12.4). For dimorphic specimens, post-orbital ridge development, PropL/OCL, and PalmL/PropL were all strongly correlated with the first function and PropD/OCL with the second (Appendix Twelve, Table A12.4). The first discriminant function accounted for 99.2% and 97.3% of the total variation for isomorphic and dimorphic specimens, respectively, with the individual scores and population centroids shown in Figure 3.17.

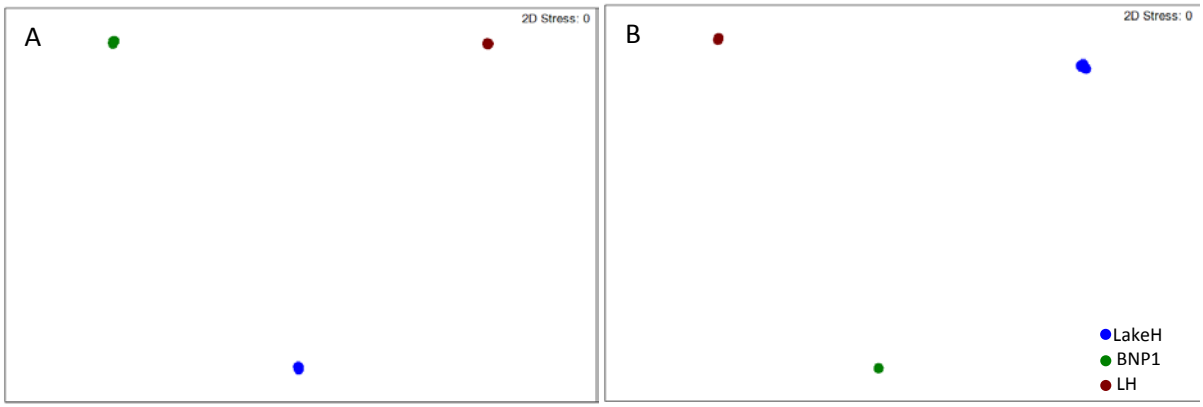


Figure 3.16. Multi-dimensional scaling plot of the relationship found between New South Wales populations of *Tenuibranchiurus* with specimens possessing isomorphic (A) or dimorphic (B) chelae separated. Analysis was performed on 19 morphological characters and 10 morphometric measurements for 25 specimens from three populations (Note: the specimens from each population are strongly clustered and appear as a single point on the graphs).

Table 3.11. Classification table of the number of specimens correctly classified by discriminant function analysis into known groups for three New South Wales populations of *Tenuibranchiurus* based on 19 morphological characters and 10 morphometric measurements, with specimens possessing isomorphic or dimorphic chelae separated.

		Predicted Group Membership								
		Isomorphic				Dimorphic				
	Pop[n]	LakeH	BNP1	LH	Total	Pop[n]	LakeH	BNP1	LH	Total
Count (%)	LakeH	5 (100.0)	-	-	5 (100.0)	LakeH	10 (100.0)	-	-	10 (100.0)
	BNP1	-	8 (100.0)	-	8 (100.0)	BNP1	-	1 (100.0)	-	1 (100.0)
	LH	-	-	8 (100.0)	8 (100.0)	LH	-	-	5 (100.0)	5 (100.0)
	100.0% of original grouped cases correctly classified					100.0% of original grouped cases correctly classified				

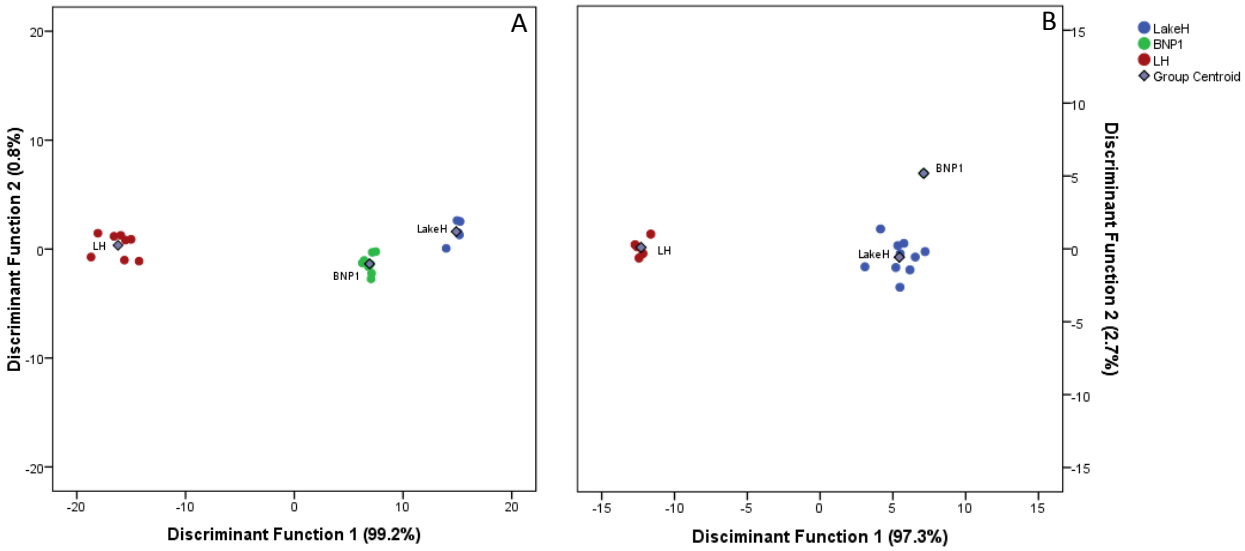


Figure 3.17. Individual discriminant scores and group centroids on combinations of the first two discriminant functions for three New South Wales populations of *Tenuibranchiurus* based on the combined morphology and morphometric data. Specimens were separated by having either (A) isomorphic or (B) dimorphic chelae.

3.4.9 Summary for New South Wales

Significant differences were found between populations for some of the morphometric variables examined. Specimens from LH were most distinguishable from the other NSW populations, showing distinctive chela, cephalothorax, and rostral measurements (though not always significantly different to all other populations). Because not all measurements were available for each population (i.e. some chela measurements for BNP1), it is impossible to make definitive statements about how each population may differ from another for those features. However, in general, LH specimens have shallower small dimorphic chelae with a shorter palm, and also show a shorter palm in their large dimorphic chelae. They also have a short and broad rostrum, and a quite narrow and shallow cephalothorax. Specimens from LakeH and BNP1 are very similar in their measurements, with the only significant difference found in the width of the cephalothorax, where BNP1 is much broader than LakeH.

Combining all of the morphometric measurements allowed dimorphic specimens from LH to be positively identified using the DFA (members assigned correctly 100% of the time); however, separation of the populations was not as clear using PCA. Although LH specimens were mostly separate from the other populations when visualised on the PC plots, there was a slight overlap with specimens from LakeH, which can also be seen in the DFA where one specimen from LakeH was incorrectly assigned to LH. No populations could be clearly separated from each other when examining isomorphic specimens only.

Clear morphological differences existed between the three NSW populations, with the MDS, DFA, and PAA identifying each population as a distinct entity. Combining the morphometric and morphological data also showed that after classifying a specimen as isomorphic or dimorphic, it can be accurately assigned to one of the three populations, as there are clear differences between all of them.

3.5 DISCUSSION

The morphological differences found between specimens examined in this study, in addition to previously identified genetic divergence (see Dawkins *et al.* 2010), provides additional support for the supposition of Horwitz (1990); namely, that the generic level relationships within the burrowing clade should be re-evaluated. Although morphological discrepancies exist between the original *Tenuibranchiurus* description and all populations examined, the morphological differences between Qld and NSW specimens are so vast that their inclusion within the same taxonomic group requires reconsideration.

Although the morphology of the two groups is significantly different, the occurrence of morphological plasticity within freshwater crayfish (e.g. Austin and Knott 1996; Grandjean and Souty-Grosset 2000; Breinholt *et al.* 2012) needs to be addressed as a potential cause of variation. Incorporating *Gramastacus* specimens into this analysis allowed an objective examination of the morphological patterns, as they represent both a phylogenetically and geographically proximate member of the burrowing clade, and are therefore the most likely taxon to highlight any discrepancies that may exist in the current taxonomy through the overlap of morphological characters with Qld and/or NSW specimens.

Given its geographic location, if clinal variation within a single morphologically variable group (i.e. where Qld, NSW, and *Gramastacus* represent a single taxon) could explain the diversity seen, intuitively NSW would represent the intermediary group and, therefore, display an intermediate phenotype. Not only did very few of the morphological character states examined correspond with such a gradient, the NSW specimens did not simply display an intermediate expression of all characters, rather a variety of distinct diagnostic characters. Furthermore, comparison with the morphological characters of the other described burrowing genera (i.e. *Geocharax*, *Engaewa*, *Engaeus*) showed both morphological similarities between many of the genera for some characters (a result that is not surprising given that they belong to the same functional group), and extreme morphological diversity for others. Therefore, the possibility of a morphologically variable taxon that includes all three groups is rejected.

A second possibility, whereby the morphology of *Gramastacus* aligns with either Qld or NSW, was explored and similarly rejected. *Gramastacus* did not align closely with either group, as it displayed some morphological characters shared with Qld to the exclusion of NSW, and others that were shared with NSW to the exclusion of the Qld, and also many characters that were not shared with either group. Additionally, differences in the morphometric measurements were most evident between *Gramastacus* specimens and both Qld and NSW. The failure of *Gramastacus* to align closely with either Qld or NSW morphologically suggests they belong to a separate taxonomic group from both the Qld and NSW specimens (and therefore does not provide a morphological bridge between the taxa, or align with either group).

Although *Gramastacus* was the most morphometrically differentiated, differences in the overall chela form (for isomorphic specimens) were still evident between Qld and NSW. In general, the Qld specimens displayed a longer and more slender chela shape than the NSW specimens. There were also vast differences in their overall morphology, with almost as many differences between Qld and NSW as between these two groups and *Gramastacus*. One pronounced difference was the occurrence of dimorphic chelae within the NSW populations only. Although this is not an uncommon occurrence in burrowing crayfish, it is interesting to note that none of the Qld populations showed this trait. As suggested by Horwitz (1990), this may be a result of the degree to which each of these groups burrow (although the specific reasons were not expounded upon by Horwitz), as it has been suggested that species which occupy burrows connected to permanent waters (i.e. Type 1a and 1b *sensu* Horwitz and Richardson 1986) are less likely to show heterochelosity (i.e. chelae dimorphism).

These two groups of crayfish have previously been highlighted as being genetically divergent by Dawkins *et al.* (2010), with the authors suggesting this may represent a species-level difference. However, the identification of numerous invariable and diagnostic characters between Qld and NSW specimens (equivalent to the comparison between each of these groups and *Gramastacus*) suggests that a generic distinction between these two groups may

be warranted. While the morphological data presented here may be sufficient for the erection of a new genus[2], support for this will be examined using molecular analyses in Chapter 4.

3.5.1 Queensland Populations

When looking at the ratio of chela dimensions to OCL the only population to show a unique relationship was BRB, where all specimens were of a roughly similar overall size yet displayed widely varying chela measurements. This relationship was not seen in any other population, where all chela measurements increased proportionally with an increase in OCL. The relationship seen within BRB could be explained by a variety of processes that are potentially unique to this island population. For example, these processes could include (1) intraspecific competition driving individuals to direct energy towards chela growth for increased dominance in interactions (e.g. resource acquisition or reproductive success through mate attraction), (2) interspecific competition with other aquatic fauna endemic to BRB (e.g. fish, other crayfish species) requiring energy to be directed to chela growth to increase their chance of survival, or (3) the chela of specimens examined are regenerate and, therefore, do not display the same dimensions as fully developed chela yet exhibit the correct morphological character states of fully formed chela (and were not recognised as being regenerate). The latter explanation is the least likely scenario, as other specimens from this population were identified as having regenerate chelae through their deviation from correct morphology as well as incorrect chela dimensions (and were therefore not included). A situation where some regenerate chelae show accurate morphological characters while others do not is highly improbable. Although both (1) and (2) are possible scenarios, the presence of a single female from this population with a larger OCL than the males that displayed smaller chela ratios perhaps lends additional support to (1); whereby males may be more likely to expend more energy on chela growth for reproductive dominance. However, the small number of specimens available from both sexes prohibits explicit conclusions; rather, the collection of multiple specimens covering a wide range of OCL would help clarify a potentially unique adaptation not seen in other populations (see Appendix Three for additional discussion on the unique nature of this population).

[2] For example, the erection of *Gramastacus* as a genus by Riek (1972) was based primarily on sexual morphologies and to a lesser extent the cephalothoracic grooves, and the key differentiating feature used in the erection of *Ombrastacoides* and *Spinastacoides* by Hansen and Richardson (2006) was the presence of terminal uropod spines (although this division was also supported by molecular data).

Analysis of chela morphometrics highlighted differences between sex for some populations (MAR, BRB, and TL). The results from MAR are potentially attributable to a single large male measured from this population possibly skewing the data and creating a significant difference, where this may not be a true indication of sex separation. Additionally, the other male specimen measured from this population was of a similar size and displayed similar chela ratios to the females examined. The small sample size and the overlap between some male and female specimens prevent any strong conclusions regarding sex separation to be made. For the BRB population, only a single female was measured that was larger than the male specimens, yet displayed smaller chela ratios. As data from only one female was available, no conclusions that this may represent sexual dimorphism can be drawn. Finally, specimens from TL show a more complex relationship, where female specimens tended to show smaller chela ratios than males; however, the smaller male specimen had similar ratios to the largest female, but both had larger ratios than the other smaller female specimens, and smaller ratios than the largest male. Again, the small sample size and some overlap between sex means that no definitive statement regarding sexual dimorphism can be made. Although no strong conclusions can be drawn from these data alone, the potential for sexual dimorphism within these populations should be investigated further.

Although there were general trends evident for both the morphometric and morphological data, the overall relationship between populations is somewhat blurred. In light of the patterns of morphological variation seen in other crayfish species (i.e. Sokol 1988; Austin and Knott 1996; Hansen *et al.* 2001), two potential scenarios are shown by the data; (1) the genus *Tenuibranchiurus* is comprised of a single species that has low levels of morphometric variation but is morphologically plastic across its range, or (2) the genus *Tenuibranchiurus* is represented by multiple species that are morphometrically conservative but morphologically diagnosable to some extent. Both scenarios are equally plausible, and the current data are insufficient to determine which is correct. As stated by Austin and Knott (1996), due to the potential for morphological plasticity (or conservatism) in crayfish species, the conventional approach to taxonomy (i.e. morphology based) may not be appropriate for many species groups; rather, molecular approaches may be necessary to elucidate species boundaries, with these data then used to determine the significance of observed morphological variation. This is particularly relevant when small sample sizes, like those available for this study, are all that is available.

3.5.2 New South Wales Populations

The morphology of NSW specimens was somewhat less complex, with no differences in sex or irregular growth patterns evident. Additionally, although the morphometric data were not always conclusive, clear morphological differences were found between all three populations examined. Although the differences between the NSW populations were more pronounced than in Qld, the same problem is still apparent with respect to species delineation. Whether there is a single morphologically variable species, or multiple species with identifiable diagnostic characters, is still unclear. In light of the fact that differentiation is less complex, the presence of multiple species could arguably be a more plausible hypothesis. However, a precautionary approach shall be adopted and pre-emptive conclusions avoided, whereby the morphological results found here shall be interpreted in relation to the molecular data presented in the following chapter.

4.0 Molecular Analysis of *Tenuibranchiurus*

4.1 INTRODUCTION

4.1.1 The Role of Molecular Data in Taxonomy

There has been an increasing awareness of issues related to morphological taxonomy (Burnham and Dawkins 2013), which can either under- or over-estimate the diversity within a genus. One cause of underestimation of diversity is morphological conservatism where multiple species (cryptic species) have been classified into a single species because they cannot be morphologically differentiated (Daniels *et al.* 2003; Bickford *et al.* 2006; Pfenninger and Schwenk 2007). This can occur as speciation does not always result in morphological diversification (i.e. through stabilising selection); a process known as morphologically static cladogenesis (Bickford *et al.* 2006). Causes of this morphological stasis include adaptation to behavioural, physiological, reproductive, or ecological conditions, where these adaptations may not be expected to result in morphological changes (Bickford *et al.* 2006). Cryptic species are frequently found unintentionally through other (usually genetic) studies, and it is as yet unknown how often cryptic species may arise, the evolutionary timescale at which they may occur, or the environments (if any) in which they are more likely to be found (Pfenninger and Schwenk 2007). Freshwater systems have been found to harbor many morphologically cryptic species, including macro-invertebrates and fish assemblages (e.g. Baker *et al.* 2003; Daniels *et al.* 2003; Baker *et al.* 2004; Cook *et al.* 2006; Cook *et al.* 2008a) and many instances of cryptic diversity have been reported in freshwater crayfish (both sympatric and allopatric species) (e.g. Hansen *et al.* 2001; Apte *et al.* 2007; Schultz *et al.* 2007; Mathews *et al.* 2008; Bentley *et al.* 2010; Dawkins *et al.* 2010; Sinclair *et al.* 2011).

In response to changes in biotic and/or abiotic conditions, species may display morphological plasticity, rather than conservatism. This plasticity, known as phenotypic plasticity, is where a single genotype produces different morphological phenotypes in response to a change in conditions (Pfennig *et al.* 2010). This expression can vary where conditions alter throughout the range of a species, as a single phenotype is unlikely to confer high fitness in all circumstances (Via *et al.* 1995), potentially leading to an overestimation of species diversity if all morphological forms are deemed to represent unique species. Morphological plasticity has been demonstrated in freshwater crayfish, with the most notable example in Australia being the genus *Cherax*. This genus has been found to exhibit multiple phenotypic forms

within a single species, presumably in response to varying freshwater habitats. For example, Austin and Knott (1996) showed a direct correlation between habitat variation and morphological variation within three species of *Cherax* (*Cherax crassimanus* Riek, *Cherax quinquecarinatus* Gray, *Cherax preissii* Erichson) across a wide range of freshwater habitats, ranging from semi-permanent swamps to deeper permanent rivers. The problems associated with the occurrence of both morphological plasticity and conservatism can potentially be overcome through the use of molecular data, where relatedness can be determined without potentially confounding morphologies.

Although the utility of DNA sequences is vast, its place in taxonomy raises challenges that must be circumvented as there is the potential for it to confound signatures of population level and species level histories (Edwards 2008). This can occur when gene trees constructed from a single locus differ from the true genealogical history of a species (Sunnucks 2000; Hey and Machado 2003), although this problem that can potentially be overcome by estimating gene trees from multiple unlinked loci. Using multiple loci from different areas of the genome (e.g. mtDNA and nuDNA) can account for the different patterns of evolution expressed by each; whereby alleles at nuclear genes complete the coalescent process much more slowly than those at mitochondrial loci due to the tendency of mitochondrial genes to accumulate nucleotide substitutions several times faster due to their lower N_e, and thereby becoming diagnostic of taxa more rapidly (Sunnucks 2000).

Once a species tree has been inferred, additional testing is often undertaken to provide support for the proposed species' groups. A range of statistical analyses are available for testing species boundaries and, as there is currently no universally accepted way to define species, there are also a range of critiques on these methods (e.g. Sneath and Sokal 1973; Brower 1999; Wiens and Servedio 2000; Tautz *et al.* 2002; Wiens and Penkrot 2002; Lipscomb *et al.* 2003; Seberg *et al.* 2003; Sites and Marshall 2003; Tautz *et al.* 2003; Blaxter 2004; Ebach and Holdrege 2005; Will *et al.* 2005; Yang and Rannala 2010). Under the GLC (the species concept of this study, as outlined in Chapter 1) any evidence of lineage separation can be evidence for the existence of different species (de Queiroz 2007); as such, the identification of numerous corroborating lines of evidence (through the use of multiple tests) will be seen as lending support to any species boundaries that are defined. Therefore, although no single test is currently universally accepted, the apparent need to choose a

particular method is circumvented by using a selection of techniques, as multiple lines of evidence under the GLC is seen as increasing the rigour of species delimitation. This is the approach taken in this thesis, and the results will be considered to represent the best estimate of species boundaries until such time as new data become available.

4.1.2 Genus *Tenuibranchiurus*

As outlined in the previous chapter, there is very little information available on the genus *Tenuibranchiurus*. There is currently only one molecular study that has been undertaken specifically on the genus (see Dawkins *et al.* 2010), while other publications included *Tenuibranchiurus* within a greater phylogenetic reconstruction (e.g. Crandall *et al.* 1999; Crandall *et al.* 2000a; Rode and Babcock 2003; Schultz *et al.* 2007; Schultz *et al.* 2009; Toon *et al.* 2010). The study of Dawkins *et al.* (2010) identified the presence of two genetically divergent groups, both of which showed genetic variability within them. The two most divergent groups aligned with populations from Qld and NSW and were suggested to potentially represent species that diverged as a result of long-term historical geographic isolation (Dawkins *et al.* 2010), although no further testing was undertaken to confirm this. It has also been suggested by Horwitz (1995), on the basis of electrophoretic and geographic differences, that there is unrecognised genetic diversity within the genus. This chapter seeks to expand on the results of these studies and quantify the genetic diversity present within *Tenuibranchiurus*.

4.2 CHAPTER AIM

The purpose of this chapter is to examine the molecular variation within the genus *Tenuibranchiurus* and use these data to test the currently accepted taxonomy (i.e. a single species within this genus) through multiple species delimitation approaches.

4.3 METHODS

4.3.1 Phylogenetic Analyses

The nuclear sequence files with ambiguity codes (i.e. GAPDH, H3, AK) were run separately through DnaSP v. 5 (Librado and Rozas 2009) in order to phase each sequence. Where there was low confidence in a sequence reconstruction, the specimen was removed. The phased nuclear sequences were used to infer phylogenetic trees for each of the genes.

All coding genes (COI, GAPDH, H3, AK) were tested for saturation to check that the phylogenetic signal was not overwhelmed by substitutions using the program Dambe v. 5.3.21 (Xia 2013). All sequences for each gene were also checked for stop codons. The data for each of the five genes were run separately through jModeltest v. 0.0.1 (Posada 2008) to determine the best-fit model of evolution. Although some phylogenetic programs do not allow model selection, certain parameters from these models can be implemented to enable these programs to apply the closest available model.

Individual gene trees were then inferred for each of the five genes using both a Maximum Likelihood (ML) and Bayesian approach, with outgroups included. ML trees were constructed using RAxML v. 7.4.4 (Stamatakis 2006; Stamatakis *et al.* 2008) through the CIPRES Science Gateway v. 3.3 (Miller *et al.* 2010) (GTR+CAT model of evolution, automatically halt bootstrapping), and Bayesian trees were constructed using MrBayes v. 3.2.0 (Ronquist *et al.* 2012) (two replicate Markov chain Monte Carlo (MCMC) analyses, four chains in each analysis (one cold, three heated); all standard deviations of the partition frequencies (SD) were <0.01, effective sample size (ESS) values >100, and PSRF+ ≈1.000; see Table 4.1 for further settings used for each gene). Trees were visualised using the program Figtree v. 1.4.0 (Rambaut 2012). Branches on trees were considered highly supported if bootstrap values (BS) were >70% and Bayesian posterior probabilities (Pp) were >0.95. Weak support was inferred by BS of 50-70% and Pp of 0.90-0.95.

Combined gene trees were also inferred using both ML and Bayesian analyses. Specimens were included in the data set if they were sequenced for at least four of the five genes. Again, RAxML was used for the ML tree and MrBayes for the Bayesian tree. Within the ML analysis, each gene was entered as a separate DNA-partition, the GTR+CAT model used, and bootstrapping automatically halted. For the Bayesian analysis, each gene was entered as a separate partition, the same parameters as the individual gene analyses were entered for each

gene, the statefreq, ravmat, shape, and pinvar all unlinked, the ratepr set as variable, and the analysis set to stop when the SD<0.0099 (all ESS>100, PSRF+ ≈1.000, and the final Ngen was 1,715,000). The same analysis was performed at least twice to verify topological convergence and homogeneity of posterior clade probabilities between runs. The first 25% of samples were discarded as burnin, with the resulting trees visualised using the program Figtree.

Although the individual gene trees did not all show the same relationships, both they (with the exception of AK) and the combined gene trees did show a very prominent separation between Qld and NSW populations. In light of this, genetic distances between Qld and NSW, distances between these two groups and the outgroups, and distances between the outgroups, were calculated using both COI and 16S data to compare the degree of separation. These distances were calculated in MEGA5 (Tamura *et al.* 2011) using the net between group mean distances with 1000 bootstrap replicates (gamma distribution with shape parameter = 1, Maximum Composite Likelihood (MCL) model, positions containing gaps and missing data were eliminated). Because of the high level of separation between Qld and NSW (see section 4.4.1) all further analyses were conducted on these two groups separately.

Table 4.1. Settings used for MrBayes Bayesian tree analysis. Nst indicates model complexity (determined by jModeltest), Rates indicates +I+G for model selected (determined by jModeltest), Ngen is the number of generations the analysis was run for, and Temp determines the ease at which the chains could swap.

Gene	Nst	Rates	Ploidy	Ngen	Temp
COI	6	Invgamma	Haploid	2,500,000	0.1
16S	2	Gamma	Haploid	1,500,000	0.1
GAPDH	6	Invgamma	Diploid	7,320,000	0.2
H3	6	Gamma	Diploid	2,500,000	0.1
AK	6	Gamma	Diploid	2,000,000	0.1

4.3.2 Species Delimitation

Throughout the remainder of the chapter, the term 'lineage' will be used to refer to the genetic groups being tested for species-status to avoid confusion in terminologies. Once an assessment has been made as to the status of these lineages in section 4.5.2, they will then be referred to as 'species'. In order to test lineages for species-status, a form of iterative taxonomy was used (following the discussions of O'Meara 2010; Yeates *et al.* 2010; Niemiller *et al.* 2012). First, an initial lineage hypothesis (H_A) was formed based on the best representation of a species tree (i.e. a combined gene tree). This was then tested using alternative methods, with lineage boundaries defined to form a series of alternative hypotheses ($H_{B1,2,3}$ etc.). If both H_A and H_B concur, then this will represent the accepted lineage boundaries (and therefore species boundaries); however, if they do not agree, then alternative explanations will be sought for the cause of discordance (i.e. a biological or evolutionary explanation), and the lineage/species boundaries refined accordingly. Only the mitochondrial data were used in testing the alternate hypotheses, as the nuclear gene sample sizes were limited and individually were not very informative; for instance, most of the nuclear gene trees contained numerous polytomies and thus could not be used to identify genetically divergent groups. However, the nuclear data did prove useful for improving the resolution of phylogenetic relationships when used in combination with the mitochondrial data, and were therefore used in order to obtain the initial lineage hypothesis.

4.3.2.1 Combined gene trees

Lineages that could potentially represent distinct species were identified using a comparison of branch lengths within and between the groupings evident in the combined gene trees (both ML and Bayesian). These groupings were used to form the initial hypothesis (i.e. H_A), with these lineage boundaries tested using the methods outlined below.

4.3.3 Testing of Lineages

4.3.3.1 Haplotype networks

Following the suggestion of Hart *et al.* (2006), the connection (or lack thereof) of networks can be considered to represent the extent of lineage sorting, and therefore the presence of distinct lineages. Where multiple networks exist, this may indicate the presence of multiple species that have been separated for enough time that lineage sorting has rendered them distinct (based on a connection limit of 95% in parsimony networks) (Hart *et al.* 2006). Although this method has generally been used for marine organisms (Hart *et al.* 2006 and

references therein), similar concepts have become widely used for population genetic analyses generally (e.g. Templeton 2001; Avise 2004) on the premise that haplotype networks represent the reticulate structure of gene flow within species, as opposed to the hierarchical structure between higher taxon levels. Using this adapted method, haplotype networks were created for the COI and 16S data sets using the program TCS v. 1.21 (Clements *et al.* 2000). Initial analyses used a cut-off value of 95% between networks as recommended by Hart *et al.* (2006); however, this was reduced to 90% to allow further connections to be examined and a more relaxed lineage hypothesis to be suggested.

4.3.3.2 Genetic distances

The genetic distances between the hypothesised lineages and between specimens for both COI and 16S were calculated and graphed to determine whether a barcoding gap existed. As the intent of this test was to provide support for, or refutation of, the initial lineage hypothesis, lineages were pre-defined and genetic distances categorised as representing either intra- or inter-lineage distances. For the purposes of this study, a barcoding gap was defined as a clear separation (or 'gap') between the highest intra-lineage and lowest inter-lineage genetic distances measured between the suggested lineages. Although a standard threshold has been suggested by Hebert *et al.* (2004) for recognising distinct species (10× average intraspecific difference), this approach was not followed as it has been shown that there are vastly different rates of divergence for both different taxa and different genetic markers (Avise 2009). Rather, a recognisable distinction between the inter- and intra-lineage distances was considered potential evidence for distinct species. Analyses were undertaken for Qld and NSW specimens separately.

Relative divergences between genetic groups were calculated in MEGA5. To determine inter-lineage divergence, the number of base substitutions per site was estimated from the net average between groups of sequences and the diversity between specimens was determined by calculating the number of base substitutions per site between each sequence, both using a MCL model with 1000 replicates. The rate variation among sites was modelled with a gamma distribution with a shape parameter of 1, with positions containing gaps and missing data eliminated. This was performed for both COI and 16S, with all unique haplotypes included.

4.3.3.3 Genetic measures

An analysis of molecular variance (AMOVA) was used to calculate variation within and among clusters of sequences, as implemented in Arlequin v. 3.1 (Excoffier *et al.* 2005). Sequences were initially clustered into those defined by the lineage hypothesis. To determine whether the initial lineage hypothesis was supported, additional splits evident within the combined gene tree that were deemed to plausibly represent lineages were also tested, as well as groups based on the geographic division of populations (i.e. sampling location).

The AMOVA calculates three statistics; Φ_{ST}, Φ_{SC}, and Φ_{CT}, all of which are based on both the haplotype frequency and genetic divergence. Φ_{ST} measures variation among all populations, and Φ_{SC} measures among populations within groups. Φ_{CT} estimates variation among groups. It has been suggested that an F_{CT} value >0.95 can represent evidence for accurate species groupings (i.e. >95% of the genetic variation can be attributed to differences among groups) (Monaghan *et al.* 2005). Using the Φ_{CT} estimate as a surrogate for F_{CT} (as this estimate includes genetic divergence as well as haplotype frequency), this can provide an approach to delineate taxa based on population genetic analyses by interpreting the AMOVA results used to calculate intra- versus inter-cluster variation in a way analogous to F-statistics (Wright 1978). The criterion to determine the appropriate number of lineages using this method is where an increase in the number of suggested lineages does not appreciably increase the Φ_{CT} estimate for those lineages.

4.3.3.4 K/θ method

The initial lineage hypothesis was also tested using the K/θ method (Birky *et al.* 2005; Birky and Barraclough 2009; Birky *et al.* 2010). Although this method was originally developed for asexual organisms and termed the 4X rule, it has been further developed and shown to be effective for the mtDNA region of sexual organisms (Birky 2013). This method provides a simple way of defining species groups based on specimens/populations that form clusters (i.e. clades) that are separated by genetic gaps too deep to be ascribed to random genetic drift within a species and, therefore, must be due to diversifying selection or long-term physical isolation (Apte *et al.* 2007).

Using the groups from the chosen lineage hypothesis, sister clades were identified from the combined gene trees and statistical support for these was tested. Sequence divergences were estimated within (d) and between each sister clade using uncorrected p-distances calculated in MEGA5. Nucleotide diversity (π) was then calculated using $\pi=dn/(n-1)$, where n is the number of samples per clade. Theta (θ) was then estimated as $\theta=2Ne\mu$ (where Ne is the effective populations size and μ is mutation rate per base pair per generation) by calculating $\pi/(1-4\pi/3)$ within each clade. If d=0 (as it did for one clade in this study), then π can alternatively be calculated as $2/Ln(n-1)$, where L is the length of the sequence. K was then calculated for each sister-clade comparison (using MEGA5) as the uncorrected net between group mean distance, with this divided by the highest θ in the comparison (as this is the more conservative approach) to provide K/θ. Where sister clades were poorly defined in the tree, K was estimated between all potential sister clades in the polytomy, with the clade of the lowest K considered to be the sister clade. Finally, if the K/θ value was greater than four, then the sister clades were accepted as different lineages.

4.4 RESULTS

A total of 127 *Tenuibranchiurus* samples (either whole specimens or chelae) were collected across 20 field locations (Table 4.2). Specimens collected by the author that had previously been sequenced for the COI and 16S genes (see Dawkins *et al.* 2010) were included in the data set (*n*=51), with additional analyses undertaken on nuclear markers.

Table 4.2. Number of *Tenuibranchiurus* specimens sequenced for each gene fragment from each of the sampled locations as well as outgroup sequences included (see Appendix Five for outgroup sequence details).

State	Location ID	Number of specimens analysed				
		COI	16S	GAPDH	H3	AK
Qld	KNP	-	1	-	-	-
	HB	1	4	-	4	4
	MAR	10	4	9	5	3
	TSFN	2	2	-	-	-
	TSFS A	4	1	4	1	1
	C	14	3	12	4	4
	E	4	2	4	2	2
	F	3	1	3	1	1
	G	4	-	4	-	-
	H	1	-	1	-	-
	TEW	7	3	5	3	4
	LW	7	4	7	5	4
	Eu	-	1	-	-	-
	Moo	-	1	-	-	-
	BER	7	2	5	2	2
	TL1	-	1	1	-	-
	TL2	-	2	1	1	1
	TL3	1	2	-	-	-
	BRB1	-	-	1	-	-
	BRB2	4	-	-	-	-
	BRB	6	6	-	6	6
	GC1	8	3	5	5	3
	GC2	7	3	6	4	3
NSW	LH	13	5	10	4	3
	BNP1	13	4	9	4	2
	BNP2	2	1	2	1	-
	LakeH	9	3	4	5	3
Total		127	59	93	57	46
Gramastacus spp.		6	6	4	6	4
Geocharax spp.		3	2	3	1	1
Engaeus spp.		2	2	1	3	1
Engaewa spp.		3	3	3	3	2
Cherax spp.		1	1	1	1	-
Total including outgroups		142	73	105	71	54

4.4.1 Phylogenetic Analyses

Within *Tenuibranchiurus* COI sequences, 225 of 644 bases were variable with 35, 14 and 176 first, second, and third codon position changes, respectively. A total of 48 transitions and 17 transversions were observed across all nucleotide sites, with 190 of these sites parsimony informative. Within the 16S sequences, 98 of 468 bases were variable. A total of 23 transitions and nine transversions were observed across all nucleotide sites, of which 85 sites were parsimony informative. Within GAPDH 38 of 563 bases were variable and 35 parsimony informative, within H3 38 of 264 bases were variable and 11 parsimony informative, and within AK 18 of 538 were variable and 18 parsimony informative.

The haplotype reconstructions are summarised in Table 4.3, where numbers of homozygous and heterozygous specimens sequenced for the three nuclear genes are shown. AK was the only nuclear gene where there was only a single heterozygous site. No coding genes were found to have experienced substitution saturation, and no stop codons were found in any genes. The models selected by jModeltest for each gene were as follows: COI=TrN+I+G model; 16S=HKY+G model; GAPDH=TIM2ef+I+G model; H3=TIM3ef+G model; and AK=TrN+G model.

4.4.1.1 Tree topologies

Each of the five genes had very different levels of polymorphism, reflected in their differing degrees of phylogenetic resolution (Figures 4.1 through 4.8). In the nuclear gene trees, each specimen was represented by two sequences (to account for the phased nuclear data). For all nuclear trees, the two sequences for each specimen were recovered within the same nesting within the tree.

Both the ML and Bayesian COI trees showed poor support for the relationship between genera, with BS values and Bayesian Pp low (Figures 4.1 and 4.2); however, the grouping of specimens from each genus (and the distinct Qld and NSW groups) were consistent between trees. The arrangement of the terminals for the NSW group was highly supported in both trees, with the LH population forming a sister-group to BNP1&2 and LakeH (BS 98%, Pp 1). The NSW groups were placed outside *Geocharax* and *Gramastacus* outgroups, although this arrangement was poorly supported. The relationships between the Qld groups were much less clear. Both trees show divergence between some specimens from both TSFS and BER. For the remaining populations the same relationship was found for both trees, with TEW and

LW forming a sister-relationship with the HB and GC populations (BS 84%, Pp 1). The sister-grouping of HB and GC1&2 was only supported in the ML tree (BS 78%, Pp 0.72).

The 16S trees also showed poor support for generic level relationships, although there was clear separation between the Qld and NSW groups (Figure 4.3). Again, although there was poor support for the branching patterns between genera, the groupings were consistent between trees. As with COI, the grouping of the NSW populations was highly supported in both ML and Bayesian trees, with LH forming a sister-group with all other NSW populations (BS 85%, Pp 0.99). There were no supported relationships between the Qld groups. Also, the NSW groups were again placed outside of the outgroups, with the monophyly of this clade to the exclusion of the rest highly supported (BS 85%, Pp 0.99).

Table 4.3. Number of heterozygous and homozygous specimens for each of the three nuclear genes for each sampling location. Dashes indicate where no specimens were sequenced for the gene.

State	Location ID	Homozygous			Heterozygous		
		GAPDH	H3	AK	GAPDH	H3	AK
Qld	KNP	-	-	-	-	-	-
	HB	-	4	2	-	0	2
	MAR	0	0	2	9	5	1
	TSFN	-	-	-	-	-	-
	TSFS A	1	1	1	3	0	0
	C	4	4	3	8	0	1
	E	1	1	1	3	1	1
	F	3	1	1	0	0	0
	G	2	-	-	2	-	-
	H	0	-	-	1	-	-
	TEW	3	2	2	2	1	2
	LW	5	0	0	2	5	4
	Eu	-	-	-	-	-	-
	Moo	-	-	-	-	-	-
	BER	5	2	0	0	0	2
	TL1	1	0	0	0	0	0
	TL2	1	1	1	0	0	0
	TL3	-	-	-	-	-	-
	BRB1	1	-	-	0	-	-
	BRB2	-	-	-	-	-	-
	BRB	-	6	6	-	0	0
	GC1	2	0	2	3	5	1
	GC2	6	0	3	0	4	0
NSW	LH	8	0	3	2	4	0
	BNP1	7	2	0	2	2	2
	BNP2	1	0	-	1	1	-
	LakeH	3	6	3	1	0	0

The GAPDH trees showed the same grouping of specimens contained within each genus, but poor support for the relationships among the genera (Figure 4.4). The sister-relationship between Qld and NSW groups was highly supported by both trees (BS 96%, Pp 1) and both trees supported the monophyly of the Qld (BS 100%, Pp 1) and NSW (BS 92%, Pp 1) groups. No relationships within either Qld or NSW groups were supported in either tree. Support for relationships between genera for the H3 gene was not consistent between trees (Figure 4.5). A sister-relationship between Qld/NSW and *Geocharax*/*Gramastacus* was supported in the ML tree (BS 72%), and the monophyly of the NSW populations was highly supported across both trees (BS 89%, Pp 0.99). The monophyly of all genera (including the separate Qld and NSW groups) was supported by the AK data (Figure 4.6). Only the ML tree supported the sister-relationship between the Qld and NSW groups (BS 78%, Pp 0.50). The sister-group of LH and all other NSW populations was supported in both trees (BS 98%, Pp 1).

The individual gene trees showed different arrangements between groups as well as some poorly supported branching patterns (especially in the nuDNA). However, both the Bayesian and ML combined gene trees showed the same arrangement between all groups and genera (Figures 4.7 and 4.8). The sister-relationship between Qld and NSW populations was highly supported (BS 97%, Pp 1), as well as the monophyly of both the Qld and NSW groups (BS 100%, Pp 1 for both). The grouping of LH and the BNP and LakeH complex was highly supported (BS 100%, Pp 1), with the suggestion of some geographic structuring occurring within the complex.

Both combined trees suggested the presence of multiple groups within Qld, although they were not all supported. Six clades were evident within the Qld populations, with the monophyly of all but two highly supported (as these were represented by single specimens). The first clade included MAR specimens and some TSFS specimens (BS 90%, Pp 1), and the second was the remaining TSFS specimens as well as BRB, TL, and some BER specimens (BS 96%, Pp 1). The two clades for which monophyly could not be established were represented by the remaining BER specimens for the first, and HB for the second. The final two clades consisted of TEW and LW specimens (BS 100%, Pp 1) and GC specimens (BS 100%, Pp 1). There was also some geographic structuring evident within each of the clades.

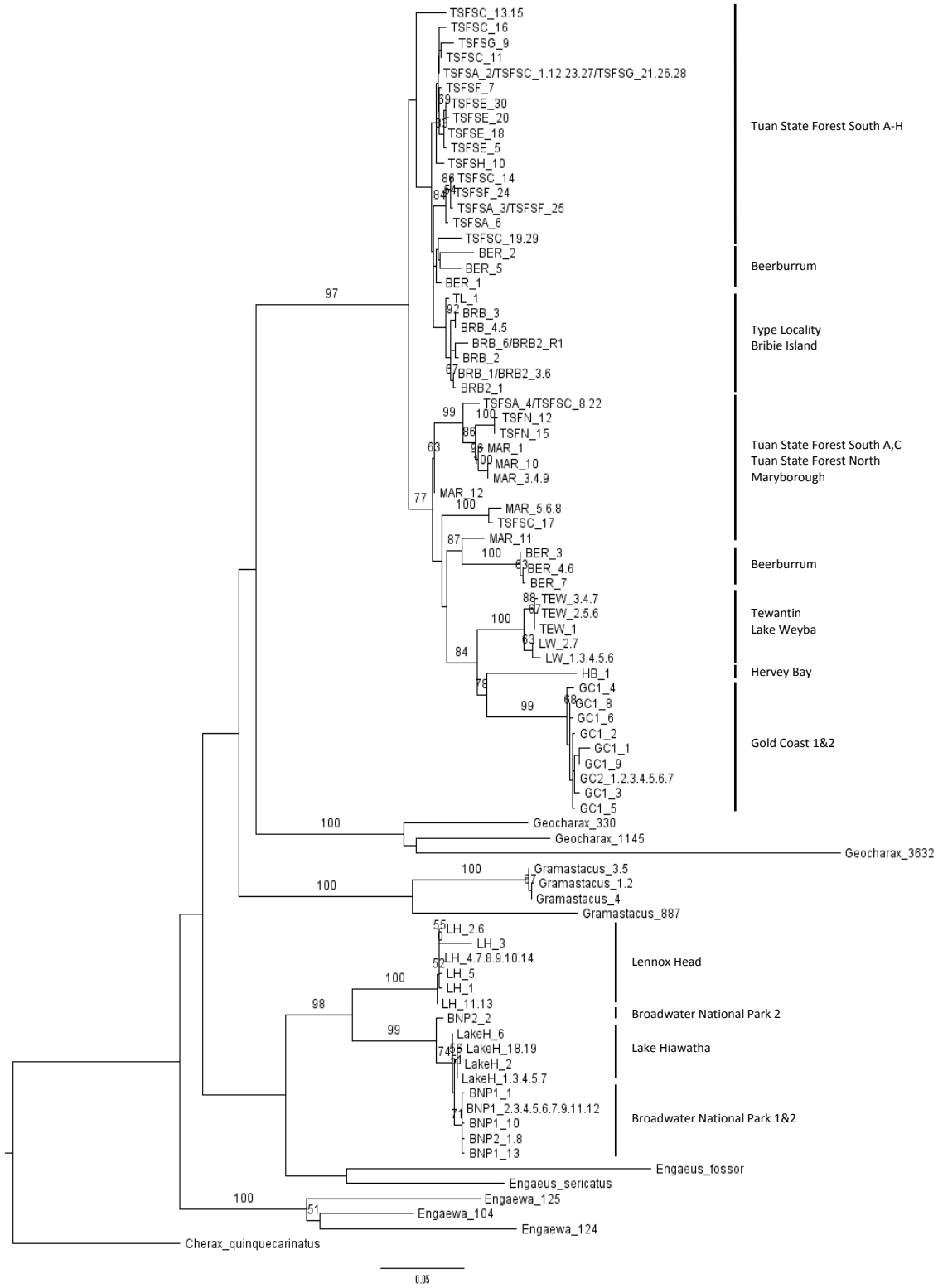


Figure 4.1. Maximum Likelihood inference gene tree based on the COI data set. Numbers on branches represent bootstrap values, with only values >50% shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

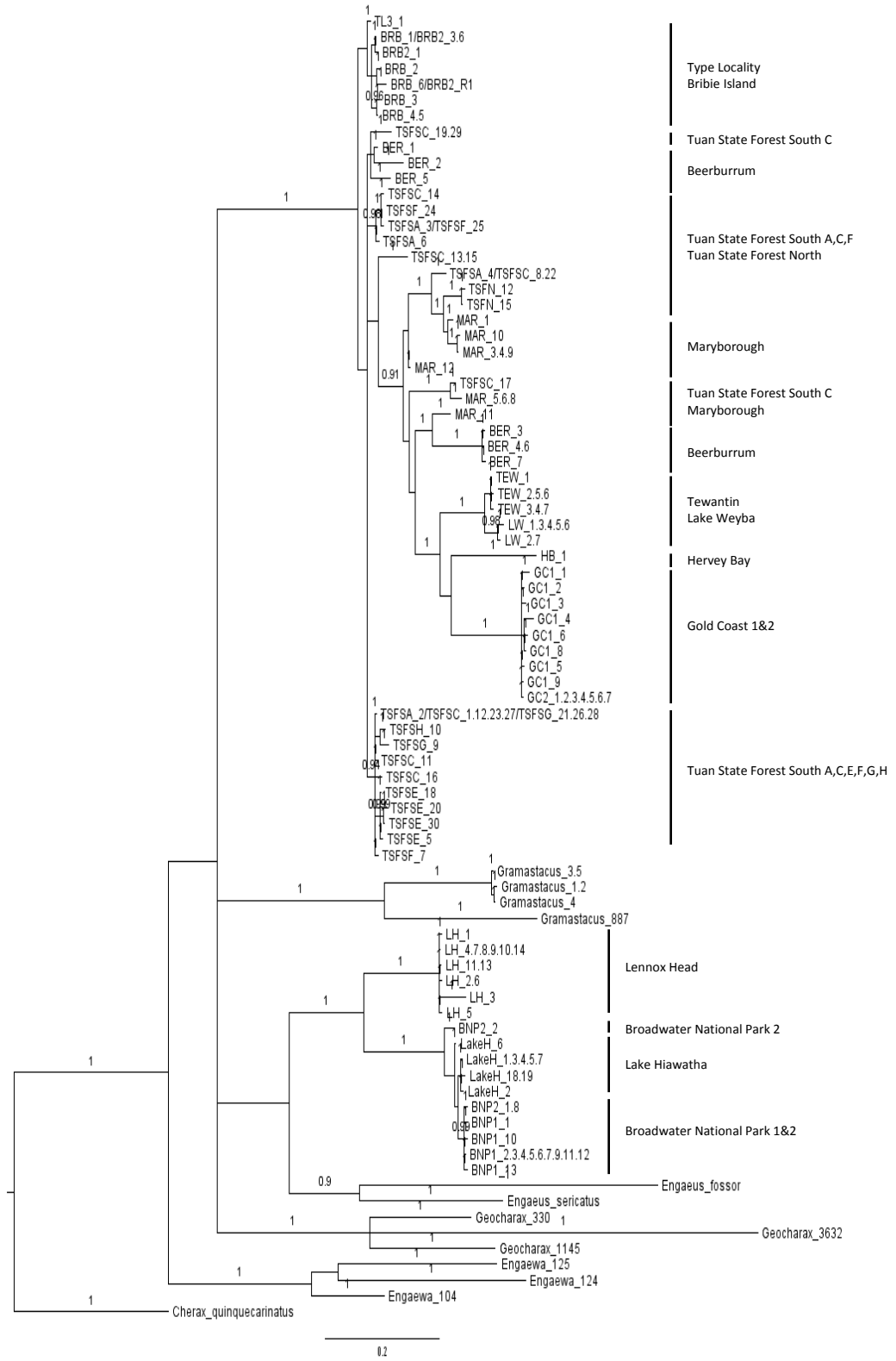


Figure 4.2. Bayesian inference gene tree based on the COI data set. Numbers on branches represent posterior probabilities, with only values >90 Pp shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

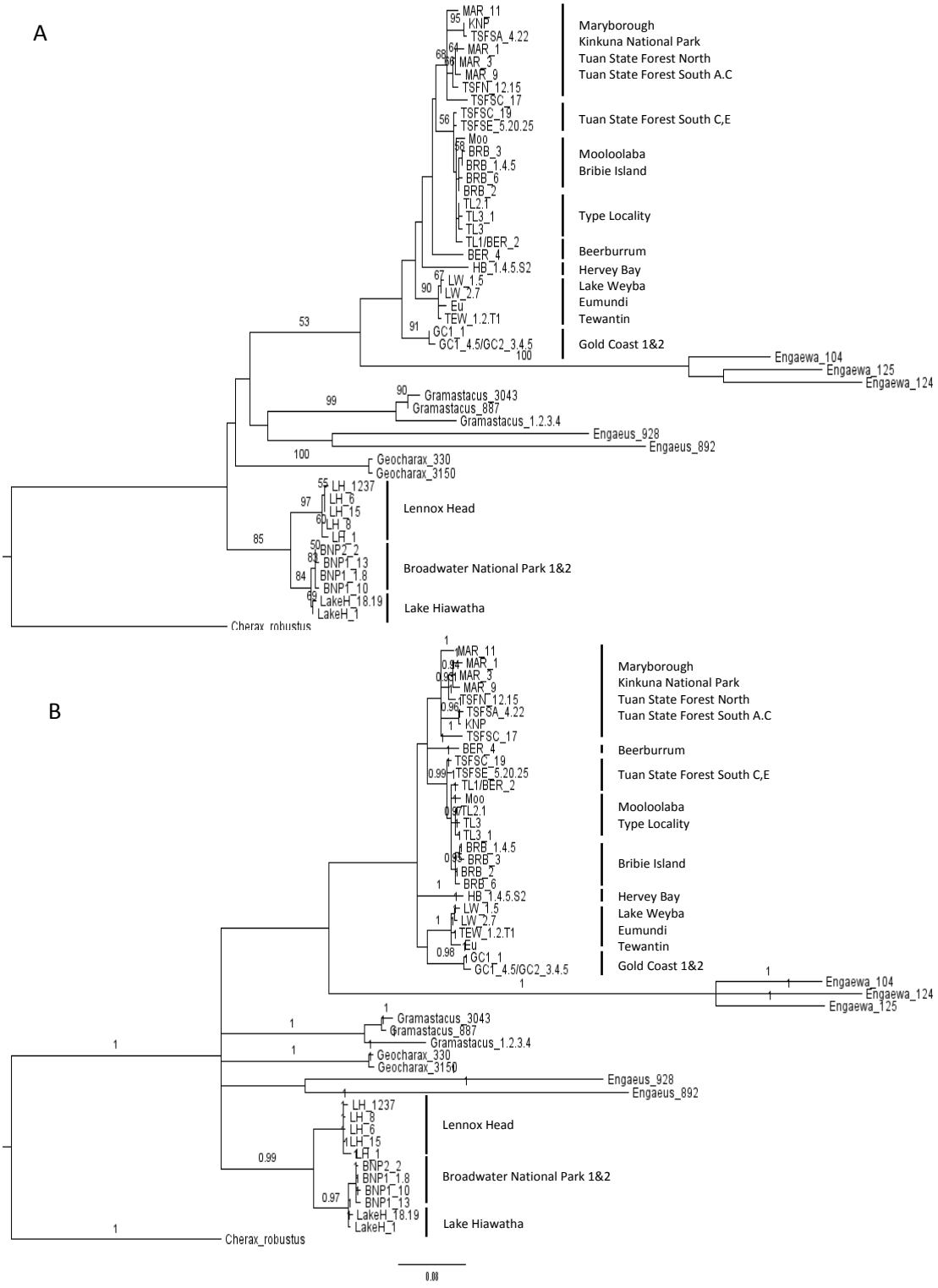


Figure 4.3. (A) Maximum Likelihood inference gene tree based on the 16S data set. Numbers on branches represent bootstrap values, with values only >50% shown. (B) Bayesian inference gene tree based on the 16S data set. Numbers on branches represent posterior probabilities, with values >90 Pp only shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

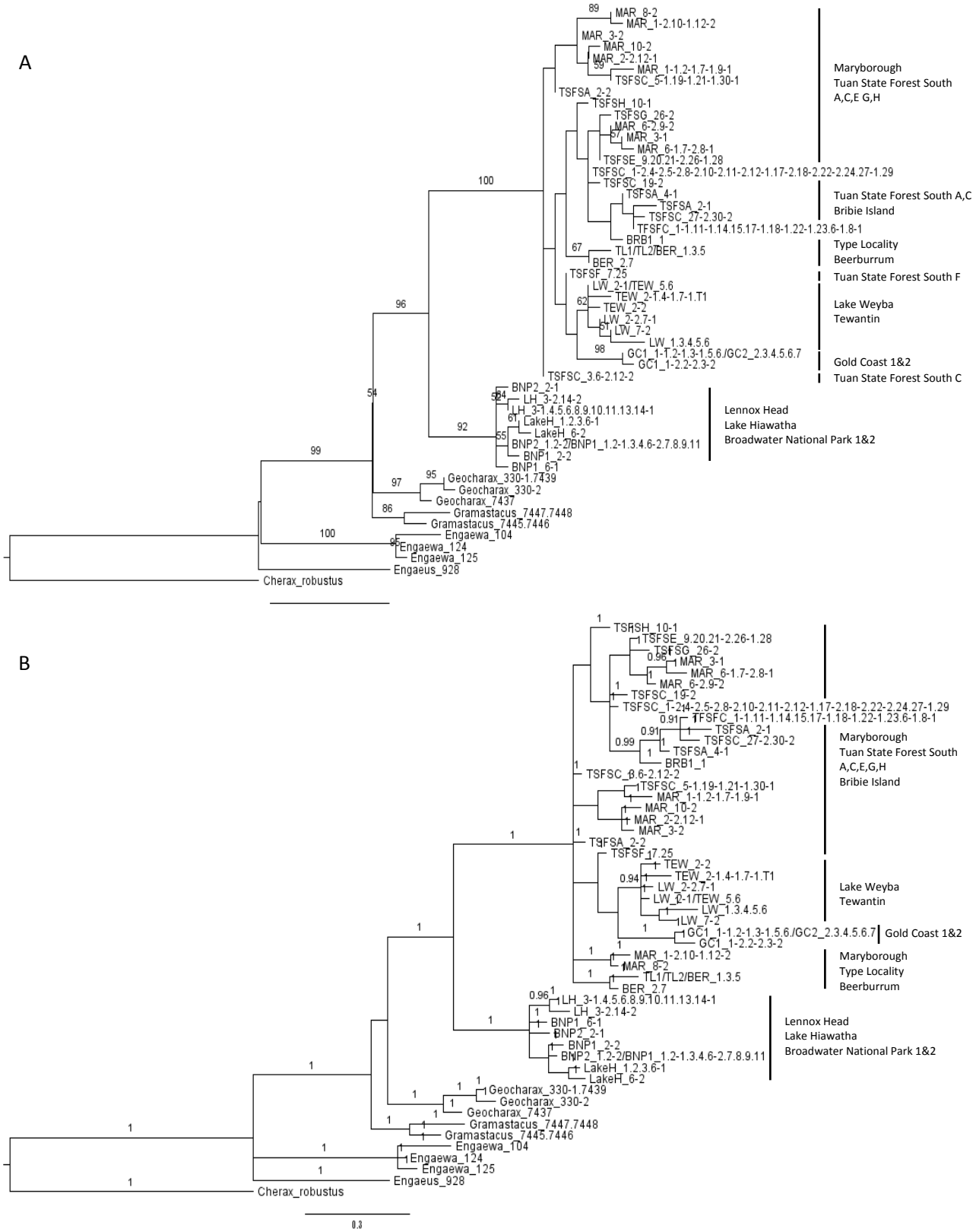


Figure 4.4. (A) Maximum Likelihood inference gene tree based on the GAPDH data set. Numbers on branches represent bootstrap values, with values only >50% shown. (B) Bayesian inference gene tree based on the GAPDH data set. Numbers on branches represent posterior probabilities, with values >90 Pp only shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

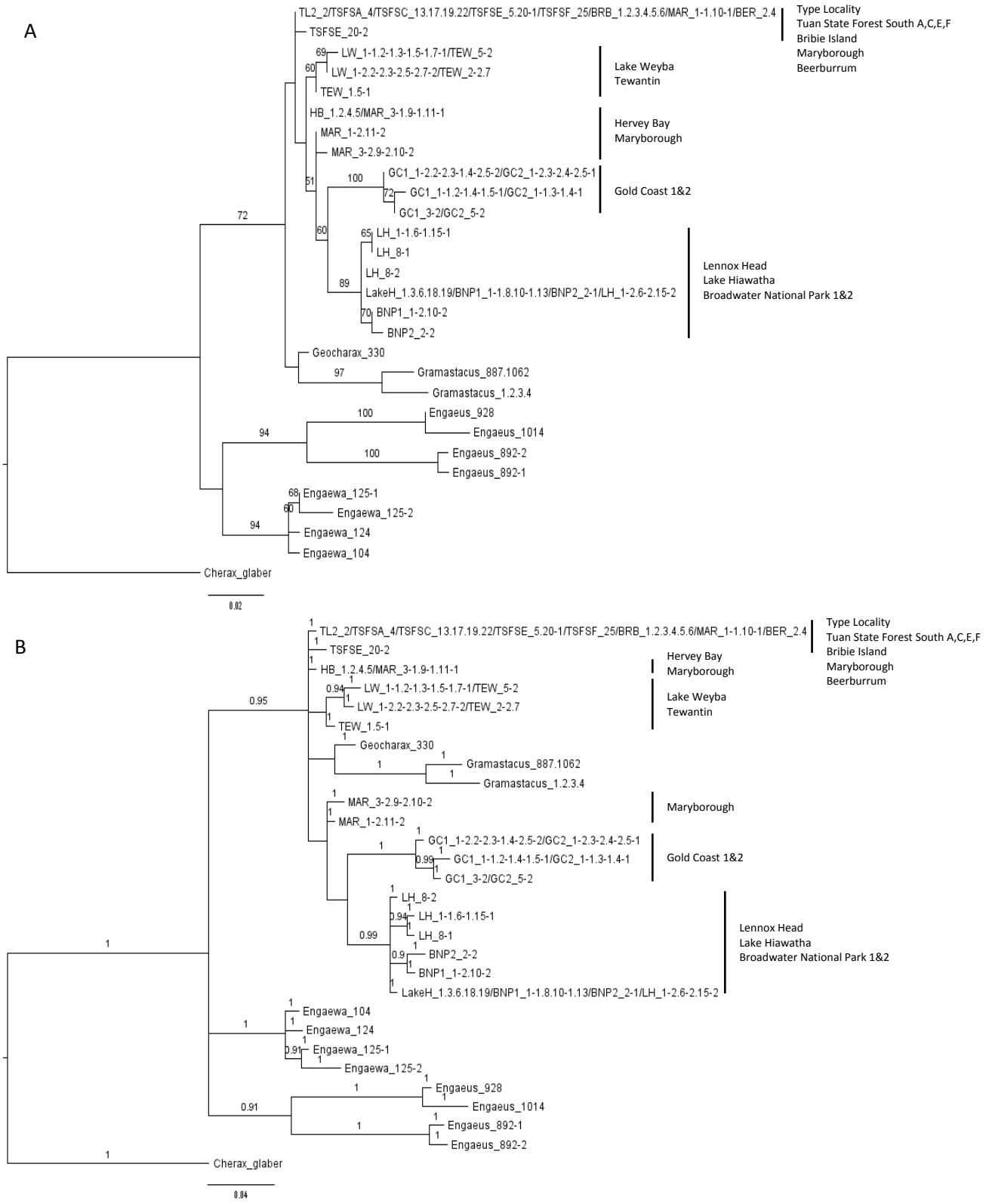


Figure 4.5. (A) Maximum Likelihood inference gene tree based on the H3 data set. Numbers on branches represent bootstrap values, with values only >50% shown. (B) Bayesian inference gene tree based on the H3 data set. Numbers on branches represent posterior probabilities, with values >90 Pp only shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

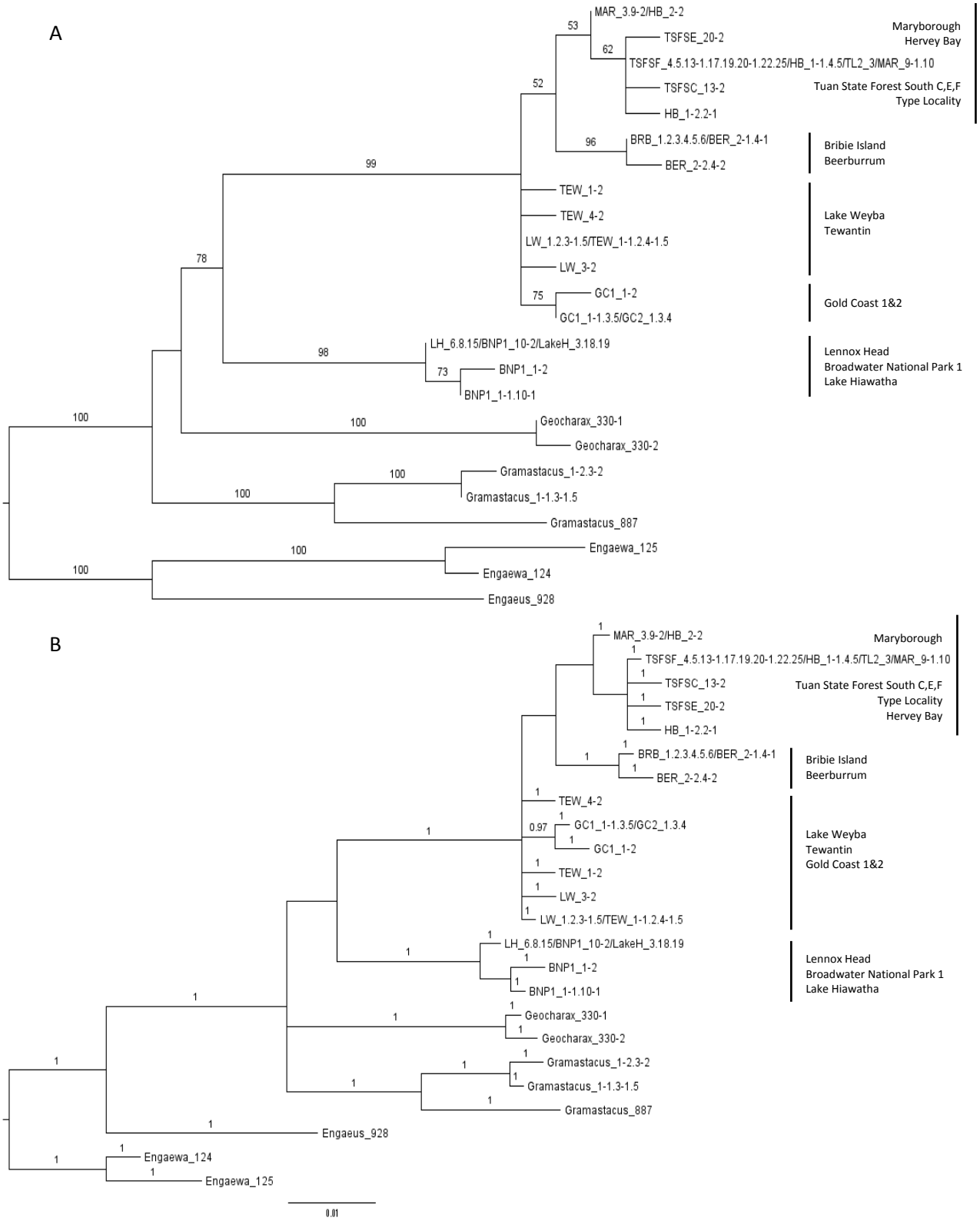


Figure 4.6. (A) Maximum Likelihood inference gene tree based on the AK data set. Numbers on branches represent bootstrap values, with values only >50% shown. (B) Bayesian inference gene tree based on the AK data set. Numbers on branches represent posterior probabilities, with values >90 Pp only shown. Sampling locations are delineated by an underscore. Multiple specimens from the same location are separated by a period, multiple locations with the same haplotype are separated by a slash.

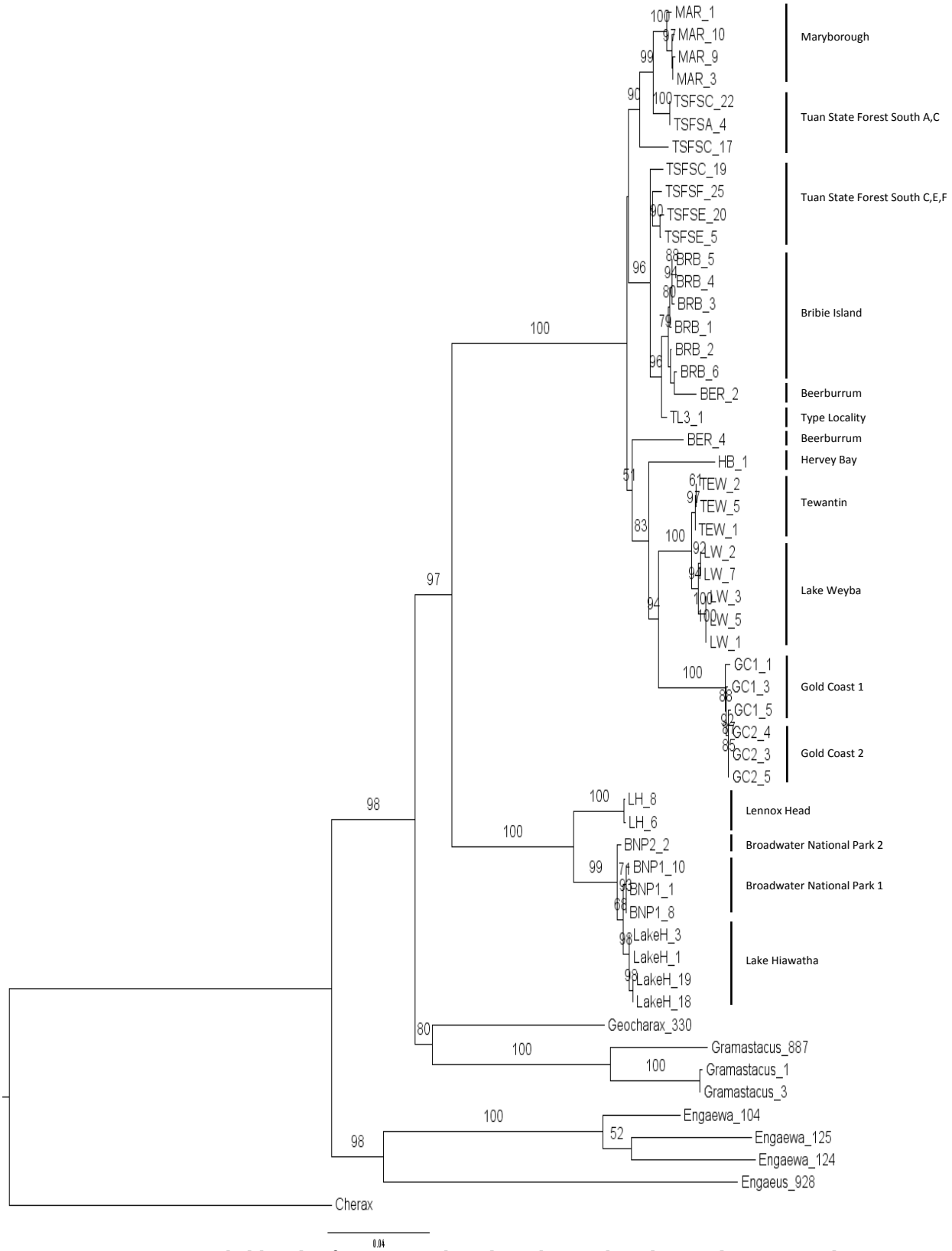


Figure 4.7. Maximum Likelihood inference tree based on the combined gene data set. Only unique haplotypes are shown. Numbers on branches represent bootstrap values, with values only >50% shown. Sampling locations are delineated by an underscore.

Figure 4.8. Bayesian inference gene tree based on the combined gene data set. Numbers on branches represent posterior probabilities, with only values >90 Pp shown. Sampling locations are delineated by an underscore.

4.4.1.2 Genetic distances between genera

The genetic distances calculated between the Qld and NSW groups using COI and 16S were 16.0% and 12.7%, respectively (Tables 4.4 and 4.5). These distances were as large as, or in some cases larger, than the distances calculated between these two groups and the other genera (with the exception of distances calculated between *Cherax* and other genera, as would be expected). Furthermore, some distances between pairs of the other genera were smaller than those between the Qld and NSW groups for both COI and 16S (e.g. *Geocharax* versus *Engaeus* = 13.7% and 6.7%, *Gramastacus* versus *Engaeus* = 11.7% and 8.1%; Tables 4.4 and 4.5).

Table 4.4. Estimates of net evolutionary divergence between groups of COI sequences with a MCL model. Standard error estimates are shown above the diagonal.

	Qld	NSW	*Geocharax*	*Gramastacus*	*Engaeus*	*Engaewa*	*Cherax*
Qld	-	0.030	0.035	0.050	0.028	0.036	0.052
NSW	0.160	-	0.037	0.054	0.042	0.034	0.051
Geocharax	0.156	0.164	-	0.062	0.033	0.044	0.059
Gramastacus	0.185	0.206	0.203	-	0.034	0.052	0.070
Engaeus	0.109	0.086	0.137	0.117	-	0.023	0.047
Engaewa	0.164	0.154	0.160	0.169	0.103	-	0.050
Cherax	0.256	0.256	0.261	0.294	0.195	0.228	-

Table 4.5. Estimates of net evolutionary divergence between groups of 16S sequences with a MCL model. Standard error estimates are shown above the diagonal.

	Qld	NSW	*Geocharax*	*Gramastacus*	*Engaeus*	*Engaewa*	*Cherax*
Qld	-	0.022	0.023	0.029	0.026	0.033	0.044
NSW	0.127	-	0.020	0.022	0.023	0.036	0.044
Geocharax	0.140	0.113	-	0.024	0.023	0.039	0.048
Gramastacus	0.161	0.117	0.129	-	0.025	0.047	0.046
Engaeus	0.101	0.072	0.067	0.081	-	0.036	0.046
Engaewa	0.175	0.191	0.212	0.244	0.138	-	0.074
Cherax	0.240	0.240	0.257	0.242	0.189	0.347	-

4.4.2 Species Delimitation

4.4.2.1 Combined gene tree

As outlined in section 4.4.1.1, there were several groupings evident within both Qld and NSW (Figures 4.7 and 4.8). There were multiple highly supported monophyletic groups evident within Qld, forming six proposed lineages. Working from the top section of the tree downwards in Figure 4.9, Lineage 1 consisted of the MAR population and specimens from TSFSA&C (TSFSA;4/TSFSC;17,22), and Lineage 2 was formed from the TL population, BRB, a single specimen from BER (specimen 2), and specimens from TSFSC,E,F (TSFSC;19/TSFSE;5,20/TSFSF;25). Lineage 3 was represented on the tree by a single specimen from BER (specimen 4), and a single HB specimen represented Lineage 4, while Lineage 5 was represented by two populations (TEW and LW), and Lineage 6 consisted of specimens from the two Gold Coast populations (GC1&2). The two monophyletic clades evident within the NSW populations were strongly supported by both combined gene trees, and formed Lineage 7 (LH) and Lineage 8 (LakeH, BNP1&2) (Figure 4.9). Although there was some structuring evident within Lineage 8, the branching patterns were very shallow and were therefore not explored as potential distinct lineages.

The lineages as defined below represent the best estimate of potential species boundaries to be further tested. The summary for the lineage hypothesis (H_A) to be tested is as below.

Lineage 1: Maryborough, Tuan State Forest South

Lineage 2: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum

Lineage 3: Beerburrum

Lineage 4: Hervey Bay

Lineage 5: Tewantin, Lake Weyba

Lineage 6: Gold Coast

Lineage 7: Lennox Head

Lineage 8: Lake Hiawatha, Broadwater National Park

As the combined gene tree was inferred only using specimens that were successfully sequenced for four out of the five genes, not all sampling locations were represented on the tree (i.e. TSFN, KNP, Moo, Eu). Therefore, specimens representing these locations were not included in the original lineage hypothesis, but were assigned through the hypothesis testing.

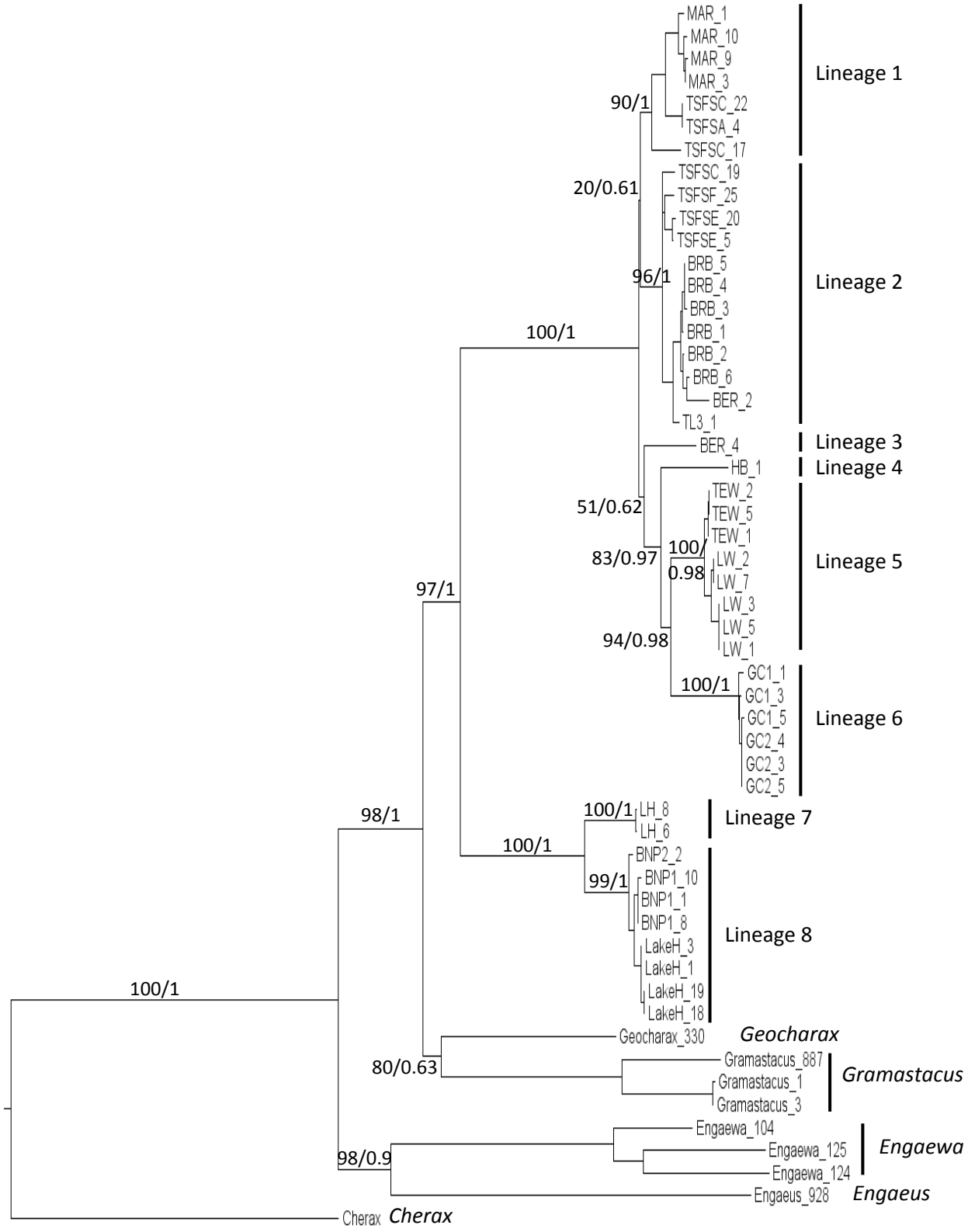


Figure 4.9. Phylogram showing the initial lineage hypothesis (H_A) to be tested for Queensland and New South Wales. Bootstrap values are shown followed by posterior probabilities for the major nodes.

4.4.2.2 Haplotype networks

From the 127 *Tenuibranchiurus* COI sequences, a total of 81 haplotypes were identified, with no haplotypes shared between sampling locations. The COI data were divided into six Qld networks (with four singletons) and two NSW networks (Figures 4.10 and 4.11). The arrangement of the Qld networks indicates that complex genetic patterns exist within and between populations.

From the 16S data, 36 haplotypes were identified from 57 sequences, where two of the haplotypes were shared between populations (one haplotype between GC1 and GC2, one haplotype between TL and BER). The 16S data were divided into four Qld networks (with two singletons) and two NSW networks (Figures 4.12 and 4.13). The 16S data resolved the relationship between two of the COI networks (haplotypes from MAR, TSFN, and TSFS).

The relationship between the NSW specimens was much clearer than for the Qld specimens, as they were separated into two groups in both the COI and 16S networks; those from LH (with one singleton in the COI network), and those from BNP1&2 and LakeH.

The COI and 16S networks showed strong congruence for the placement of haplotypes into distinct groups, with 16S helping to resolve some of the more complex patterns seen in the Qld populations. Based on these haplotype arrangements, the most conservative definition of lineages (i.e. H_{B1}) (including additional populations not defined by H_A (i.e. TSFN, KNP, Moo, Eu)) is outlined below.

Lineage 1: Maryborough, Tuan State Forest North and South, Kinkuna National Park

Lineage 2: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum, Mooloolaba

Lineage 3: Beerburrum

Lineage 4: Hervey Bay

Lineage 5: Tewantin, Lake Weyba, Eumundi

Lineage 6: Gold Coast

Lineage 7: Lennox Head

Lineage 8: Lake Hiawatha, Broadwater National Park

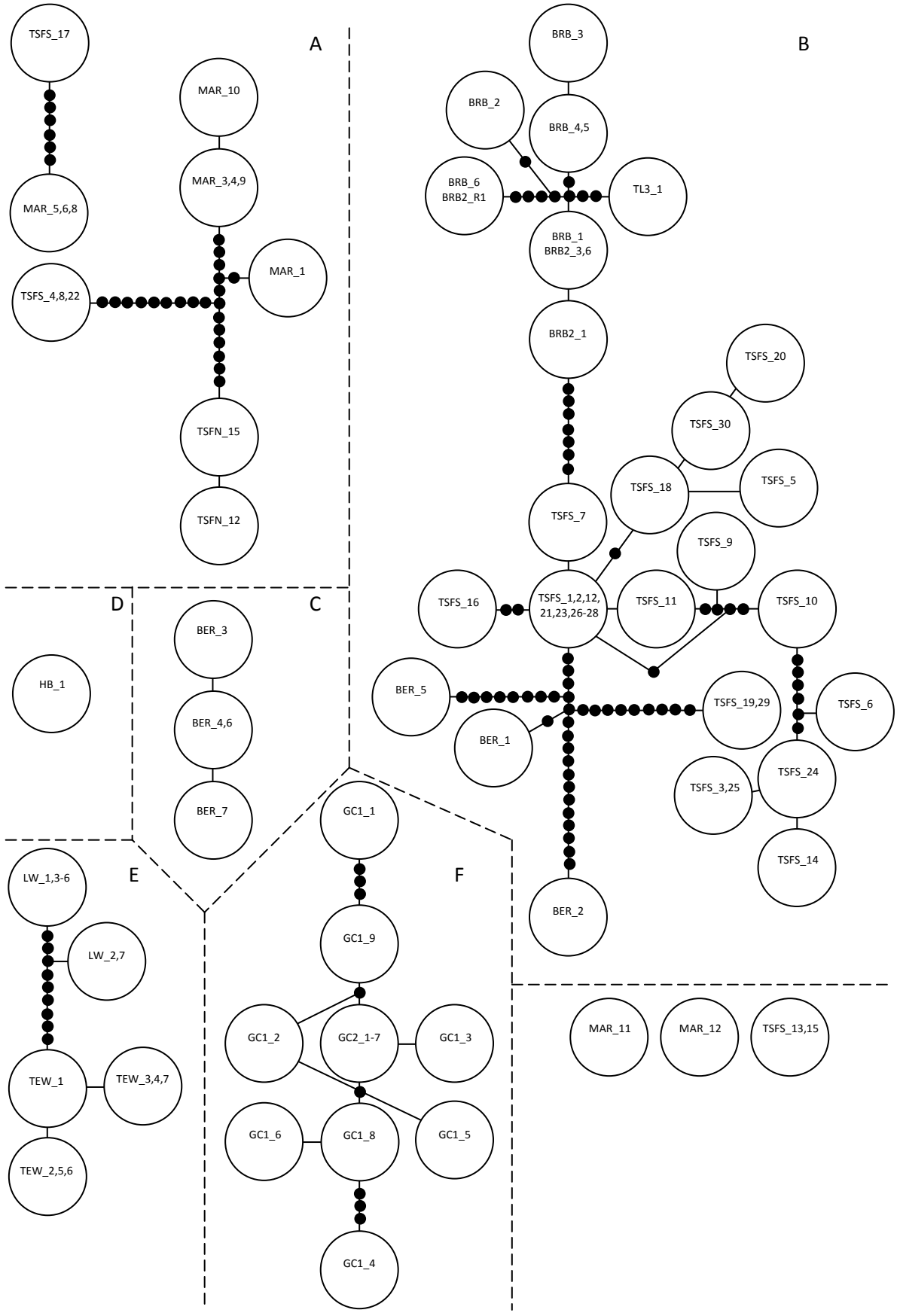


Figure 4.10. COI haplotype networks for Queensland specimens using a 90% connection limit corresponding to H_{B1} Lineages 1 through 6 (A through F, respectively). Singletons that were not assigned to a lineage are shown at the bottom right.

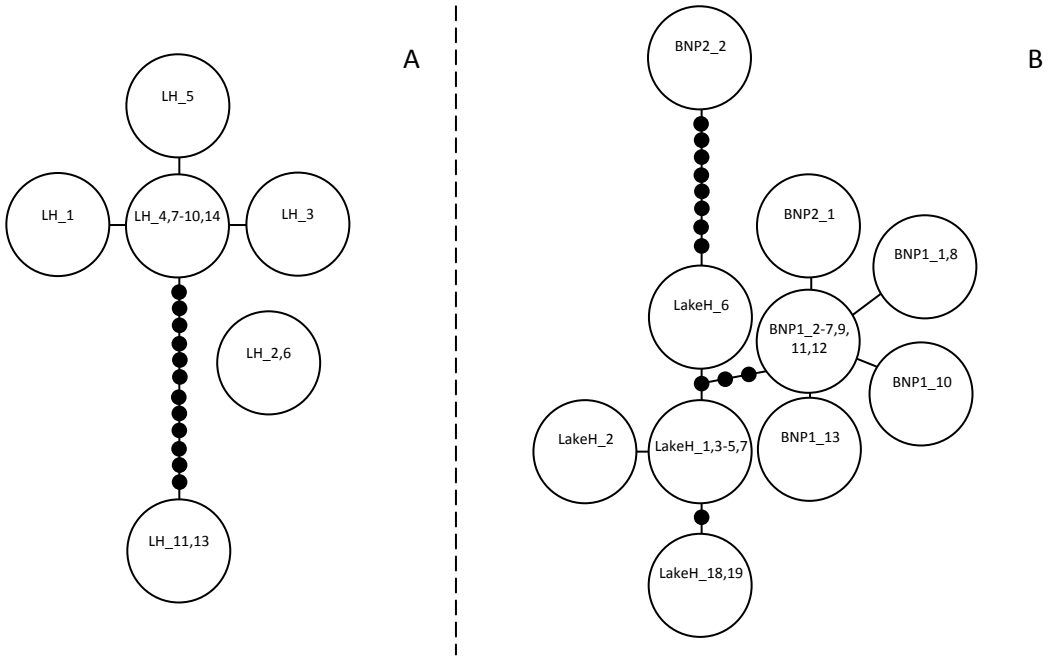


Figure 4.11. COI haplotype networks for New South Wales specimens using a 90% connection limit corresponding to H_{B1} Lineages 7 and 8 (A and B, respectively).

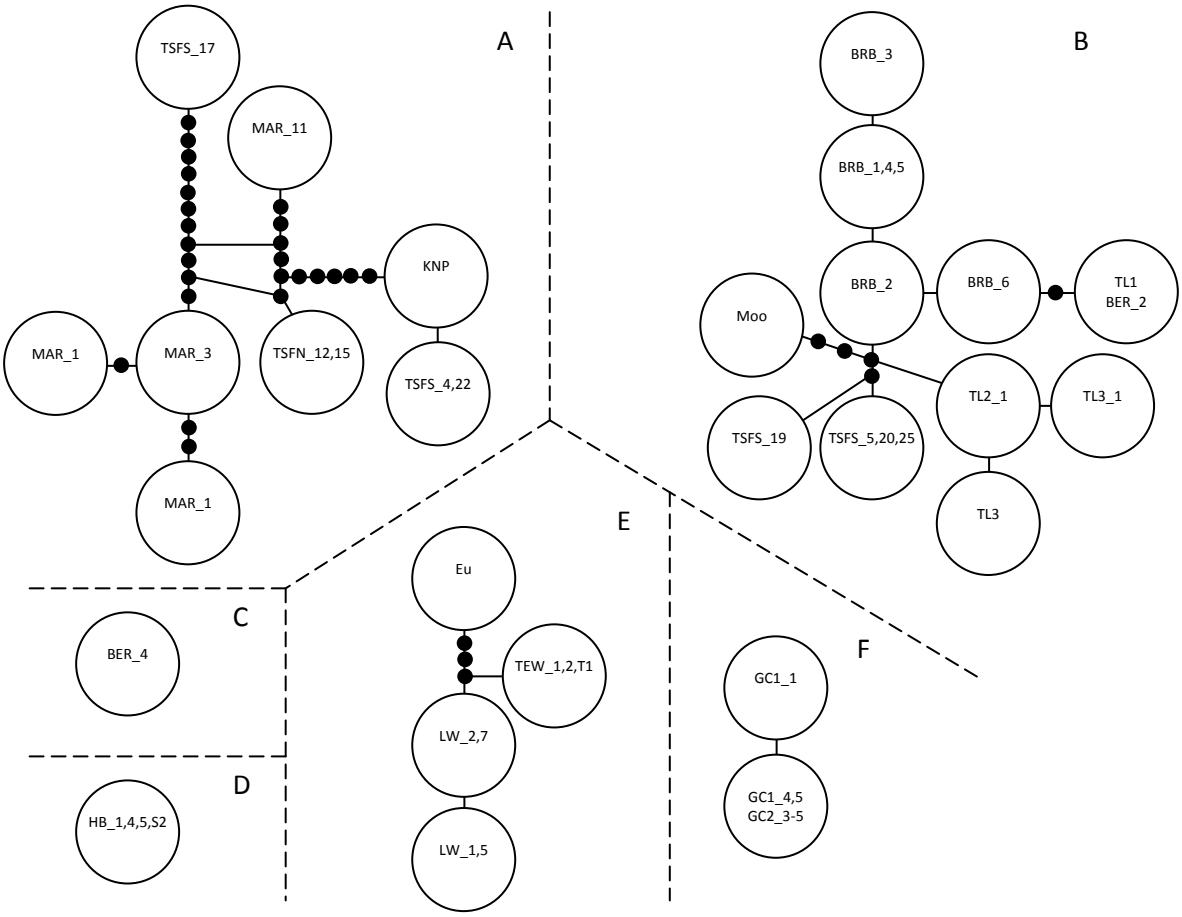


Figure 4.12. 16S haplotype networks for Queensland specimens using a 90% connection limit corresponding to H_{B1} Lineages 1 through 6 (A through F, respectively).

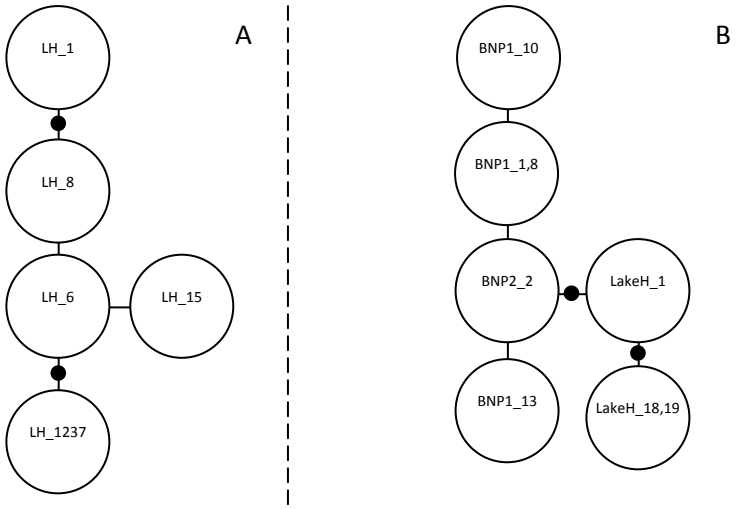


Figure 4.13. 16S haplotype networks for New South Wales specimens using a 90% connection limit corresponding to H_{B1} Lineages 7 and 8 (A and B, respectively).

4.4.2.3 Genetic distances

The COI data show some overlap of the intra- and inter-lineage estimates within Qld, resulting in no usable barcoding gap for lineage separation (Figure 4.14a). Where the overlap occurs, the low inter-lineage estimates are attributable to the Lineage 1 vs. Lineage 2 comparison, and the high intra-lineage estimates are seen between specimens within Lineage 1. However, it should be noted that many estimates between these two lineages fall in the higher range of the inter-lineage estimates as well as the low range.

The 16S data for Qld populations show a clearer relationship between lineages (Figure 4.14c). Although there is a very small overlap between the intra- and inter-lineage distances (occurring between two specimens from Lineage 1), this represents an overlap of less than 0.01%. If the existence of this overlap is disregarded temporarily, it can be seen that there is a small gap occurring at 2.8-3.0%. However, despite there not being a distinguishable gap due to the overlap, identification of the majority of lineages through the comparison of intra- and inter-lineage distances was clear and distinguishable.

When the estimates within and between Lineage 1 and 2 specimens were removed from both the COI and 16S data (with the comparison between these two lineages and all other lineages remaining), a clear barcoding gap was seen in both data sets (Figure 4.14,b&d). In COI the gap occurred between 1.7-4.7%, and at 0.9-3.5% for 16S. This shows that all other Qld groups (i.e. Lineage 3 through 6) represent clear lineages based on the barcoding approach using both COI and 16S data.

For NSW populations, there was a clear barcoding gap between the two lineages (i.e. Lineage 7 and 8), occurring between 1.5-6.6% for the COI data and 0.7-3.0% for the 16S data (Figure 4.15).

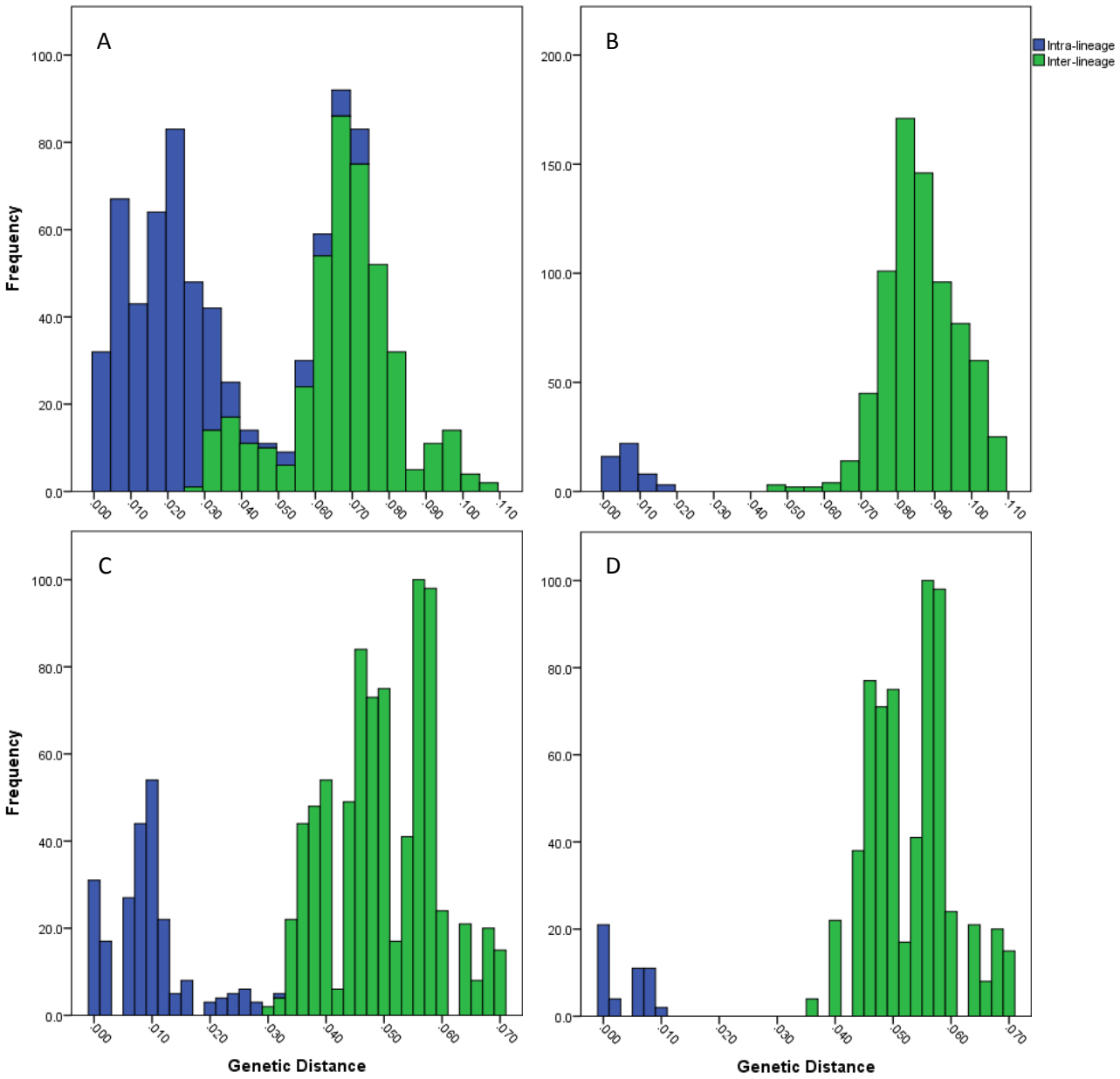


Figure 4.14. Intra- and inter-lineage genetic distance estimates for Queensland lineages showing (A) COI estimates for all lineages, (B) COI estimates without comparisons between Lineage 1 and 2, (C) 16S estimates for all lineages, and (D) 16S estimates without comparisons between Lineage 1 and 2.

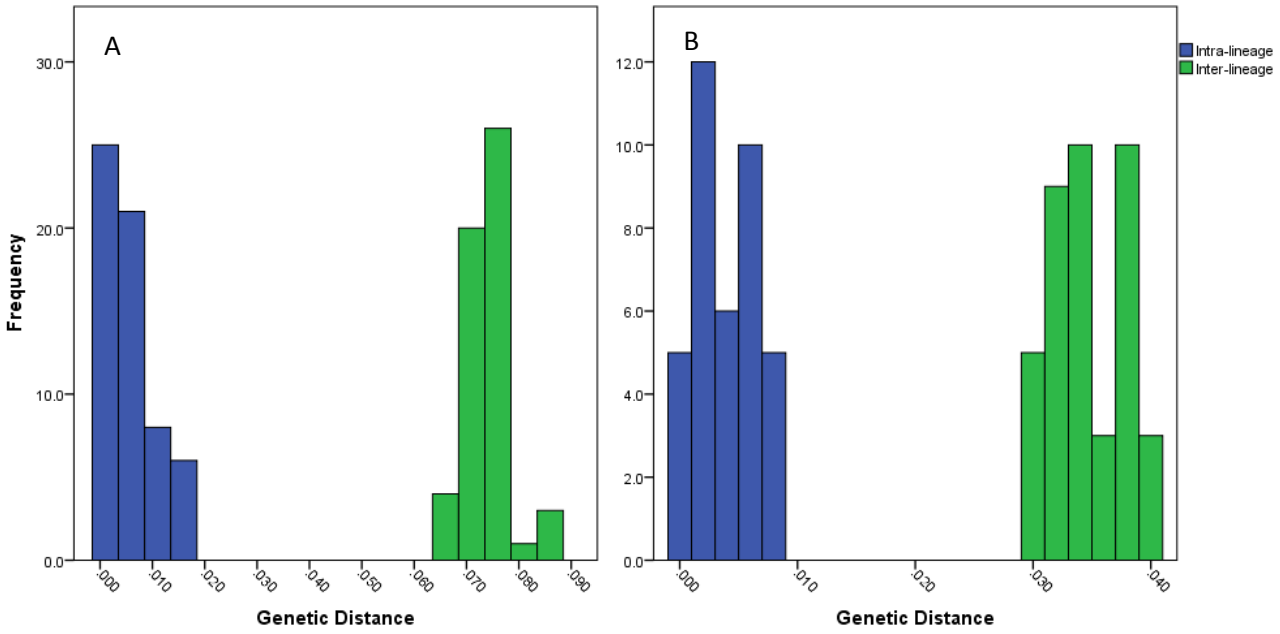


Figure 4.15. Intra- and inter-lineage genetic distance estimates for New South Wales lineages showing (A) COI and (B) 16S estimates for all lineages.

Although there is some ambiguity in the Qld COI data as to the separation of Lineage 1 and 2, the 16S data show support for the original lineage hypothesis. Because of the deeper phylogenetic inferences provided by 16S in addition to the fact that there were many genetic distances within and between Lineage 1 and 2 falling within the expected distributions, the original lineage hypothesis for Qld populations is considered supported by this analysis. The two NSW lineages are clearly separate based on both the COI and 16S data and are therefore also supported. Therefore, the lineages supported by this analysis (i.e. H_{B2}) are as follows:

Lineage 1: Maryborough, Tuan State Forest North and South, Kinkuna National Park

Lineage 2: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum, Mooloolaba

Lineage 3: Beerburrum

Lineage 4: Hervey Bay

Lineage 5: Tewantin, Lake Weyba, Eumundi

Lineage 6: Gold Coast

Lineage 7: Lennox Head

Lineage 8: Lake Hiawatha, Broadwater National Park

4.4.2.4 Genetic measures

A total of eight lineage arrangements, including the initial lineage hypothesis, were deemed plausible based on the combined gene tree and sampling locations and were tested using an AMOVA (Table 4.6). The process of assigning the potential lineages is outlined in Table 4.7, where a hierarchical approach was taken to split the tree into major genetic groups, minor genetic groups, and geographic localities. As there was no logical reason for combining the NSW lineages for the AMOVA analysis based on either the phylogenetic or geographic information, the NSW populations were considered to consist of the LH lineage and the LakeH/BNP lineage. Further testing was considered appropriate for the Qld lineages to verify the initial hypothesis of six lineages.

Figure 4.16 shows an increase in the Φ_{CT} estimate, with a plateau reached at six lineages for both the COI and 16S estimates. These six Qld lineages represent the most parsimonious arrangement of the specimens into lineages, with H_{B3} defined as follows:

Lineage 1: Maryborough, Tuan State Forest North and South

Lineage 2: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum

Lineage 3: Beerburrum

Lineage 4: Hervey Bay

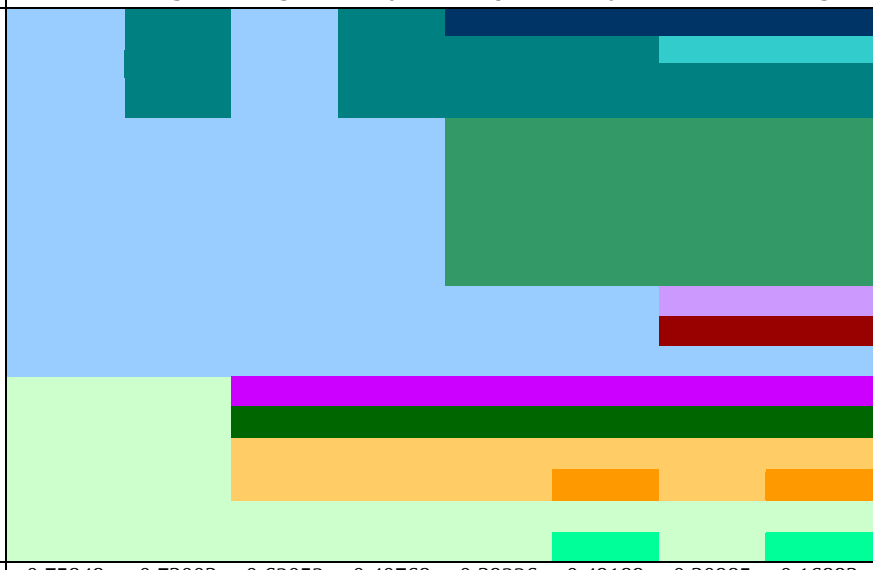
Lineage 5: Tewantin, Lake Weyba

Lineage 6: Gold Coast

Lineage 7: Lennox Head

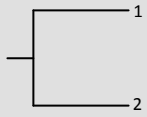
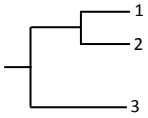
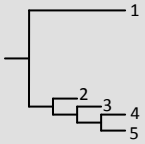
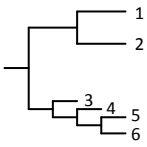
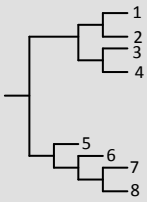
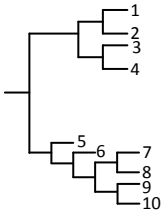
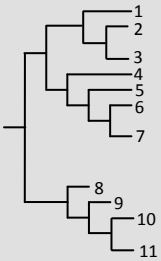
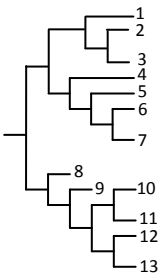
Lineage 8: Lake Hiawatha, Broadwater National Park

Table 4.6. Summary of possible lineages based on the combined gene tree and their associated Φ-statistics for COI and 16S. See Table 4.7 for explanation of how potential lineages were determined. Where specimens from the same sampling location are split into two or more groups, details are included below the table for clarification.

Location ID	Number of potential lineages							
	2	3	5	6	8	10	11	13
MAR								
TSFN								
TSFSA[a]								
C[b]								
A								
C								
E								
F								
G								
H								
TL								
BRB								
BER[c]								
BER[d]								
HB								
LW								
TEW								
GC1								
GC2								
COI – Φ_{SC}	0.75848	0.73003	0.62052	0.40768	0.39226	0.49189	0.30985	0.16883
COI – Φ_{ST}	0.83245	0.82362	0.84592	0.82845	0.81145	0.80721	0.80969	0.80564
COI – Φ_{CT}	0.30627	0.34669	0.59395	0.71038	0.68975	0.62057	0.72424	0.83371
16S – Φ_{SC}	0.87218	0.84538	0.77989	0.53957	0.47467	0.56598	0.24716	0.43330
16S – Φ_{ST}	0.91463	0.91177	0.92051	0.91225	0.90574	0.90342	0.90123	0.89906
16S - Φ_{CT}	0.33209	0.42938	0.63887	0.80942	0.82056	0.77748	0.86880	0.92958

a=TSFSA;4
b=TSFSC;8,17,22
c=BER;1,2,5
d=BER;3,4,6,7

Table 4.7. Process of assigning genetic groups/populations to potential lineages within Queensland.

Number of potential lineages	Explanation	Graphical representation	Populations included
2	Major division within Qld		1 = MAR, TSFN, TSFS, TL, BRB, BER 2 = HB, BER, TEW, LW, GC
3	Major division within top portion of tree		1 = MAR, TSFN, TSFS 2 = TSFS, TL, BRB, BER 3 = HB, BER, TEW, LW, GC
5	Major divisions within bottom portion of tree		1 = MAR, TSFN, TSFS, TL, BRB, BER 2 = BER 3 = HB 4 = TEW, LW 5 = GC
6	Major divisions within both top and bottom portion of tree		1 = MAR, TSFN, TSFS 2 = TSFS, TL, BRB, BER 3 = BER 4 = HB 5 = TEW, LW 6 = GC
8	Minor divisions within both top and bottom portion of tree		1 = MAR 2 = TSFN, TSFS 3 = TSFS 4 = TL, BRB, BER 5 = BER 6 = HB 7 = TEW, LW 8 = GC
10	All geographic localities separated within bottom portion of tree		1 = MAR 2 = TSFN, TSFS 3 = TSFS 4 = TL, BRB, BER 5 = BER 6 = HB 7 = TEW 8 = LW 9 = GC1 10 = GC2
11	All geographic localities separated within top portion of tree		1 = MAR 2 = TSFN 3 = TSFS 4 = TSFS 5 = TL 6 = BRB 7 = BER 8 = BER 9 = HB 10 = TEW, LW 11 = GC
13	All geographic localities separated within entire tree		1 = MAR 2 = TSFN 3 = TSFS 4 = TSFS 5 = TL 6 = BRB 7 = BER 8 = BER 9 = HB 10 = TEW 11 = LW 12 = GC1 13= GC2

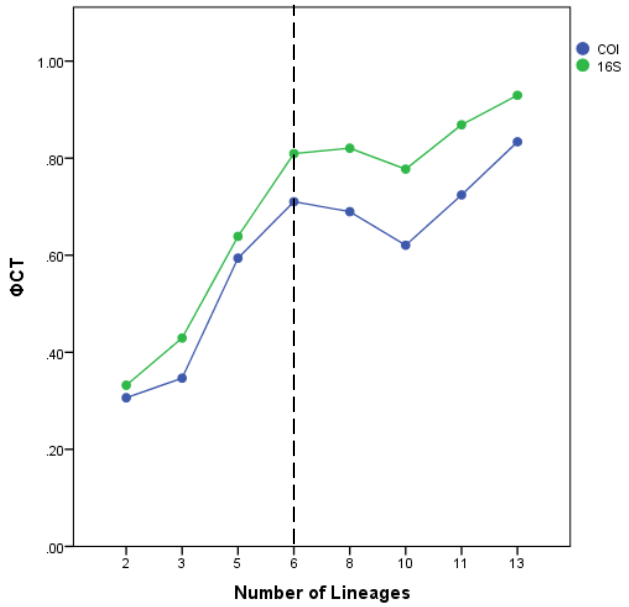


Figure 4.16. Φ_{CT} values for potential lineages for both COI and 16S for Queensland specimens. The dotted line indicates the most likely delimitation at six lineages.

4.4.2.5 K/θ method

The sister clades within Qld and NSW were tested using the K/θ method for a delimitation of eight lineages using both COI and 16S data (Table 4.8). In some instances, sister clades that were defined by the lowest K-distance (as they were ambiguous based on the combined gene tree) differed between the COI and 16S data sets. In these cases, only the relevant K/θ comparison for the applicable gene was calculated. For both COI and 16S data, all lineages were supported with the exception of the split between Lineage 1 and 2 (both genes), and Lineage 1 and 3 (16S).

Considering this, the lineages supported by this analysis and form H_{B4} are as follows:

Lineage 1/2: Maryborough, Tuan State Forest North and South, Bribie Island, Type Locality, Beerburrum

Lineage 3: Beerburrum

Lineage 4: Hervey Bay

Lineage 5: Tewantin, Lake Weyba

Lineage 6: Gold Coast

Lineage 7: Lennox Head

Lineage 8: Lake Hiawatha, Broadwater National Park

Table 4.8. K/θ values for both COI and 16S for comparisons between sister clades within Queensland and New South Wales. Where specimens from the same sampling location are split into two or more lineages, details are included below the table for clarification. Dashes are used where sister clades differ between COI and 16S.

Sister Clade 1	Sister Clade 2	K/θ	
		COI	16S
Lineage 1	Lineage 2	0.78	1.41
Lineage 2	Lineage 1	0.78	1.41
Lineage 3	Lineage 1	-	1.67
	Lineage 5	6.99	-
Lineage 4	Lineage 5	7.18	-
	Lineage 6	-	32.84
Lineage 5	Lineage 6	6.71	-
	Lineage 2	-	4.92
Lineage 6	Lineage 5	6.71	8.24
Lineage 7	Lineage 8	16.03	6.48

Lineage 1 = MAR&TSFN&TSFSA (specimen 4) &TSFSC (specimens 8,17,22)
Lineage 2 = TSFSA-H (specimens 1-3,5-7,9-12,14,16,18-21,23-30)&BRB&TL&BER (specimens 1,2,5)
Lineage 3 = BER (specimens 3,4,6,7)

4.5 DISCUSSION

4.5.1 Phylogenetic Relationships

Based on a preliminary data set, Dawkins *et al.* (2010) highlighted the presence of two genetically divergent groups within *Tenuibranchiurus* and from this suggested the potential presence of two distinct species within the genus. The phylogenetic reconstruction in this chapter supports the presence of these two divergent groups; however, the larger data set used, as well as additional nuclear genes analysed, suggests that the recognition of the two groups should be at a generic rather than specific level. Inclusion of the most closely related genera (i.e. *Gramastacus*, *Geocharax*, *Engaeus*, and *Engaewa*) in the analyses shows that the genetically divergent entities represented by the Qld and NSW groups each form monophyletic clades to the exclusion of all other genera. While the splitting of a monophyletic grouping into two genera is arguably arbitrary, the degree of divergence suggests it is warranted.

Although it is difficult to define what degree of separation is necessary between genera at a molecular level (Rach *et al.* 2008), within the parastacids it has previously been suggested that generic status should be attributed to a group of species where there is a *substantial degree of genetic difference combined with morphological distinction* (Hansen and Richardson 2006). The morphological distinction between Qld and NSW populations has been clearly established in Chapter 3, and based on the genetic distances presented in this chapter, there is strong support for a generic division. For instance, the genetic distance between Qld and NSW is larger than that seen between *Engaeus* and both *Geocharax* and *Gramastacus* for both gene fragments, and between *Engaewa* and both *Geocharax* and *Engaeus* for COI. Other genera also show smaller genetic distances when compared to either Qld or NSW than these two groups do with each other. Regardless of which genera were genetically closer to each other, the distance between Qld and NSW is at least as large as those between existing genera, thereby supporting their separation into two distinct genera.

As the currently described genus (i.e. *Tenuibranchiurus*) represents those specimens collected from within Qld (based on the location from which the type specimens were collected), specimens collected from NSW will belong to a new genus. Until a formal description is completed, and throughout the remainder of this thesis, the new genus will be referred to as *Gen. nov.*, and consists of the following populations; Lennox Head, Broadwater National Park 1 & 2, and Lake Hiawatha.

The individual gene trees had differing levels of support at some of the major nodes, and there was discordance between some genes for the arrangement of both *Tenuibranchiurus* and *Gen. nov*. specimens. Although not always statistically supported, most specimens grouped in a similar fashion across the mitochondrial gene trees, with the exception of the most northerly *Tenuibranchiurus* populations (i.e. Maryborough, Tuan State Forest North and South, Type Locality, Bribie Island, and Beerburrum). The nuclear gene trees displayed a significant number of polytomies, making the relationship between most specimens difficult to interpret. Discordance between gene trees has been widely recognised, particularly in studies of recent and rapidly radiating species (Buckley *et al.* 2006). The resulting topological differences can be attributed to the stochastic nature of the coalescent process, for example lineage sorting, gene duplication, and introgression (Maddison 1997; Buckley *et al.* 2006). Due to the randomness of the coalescent, the same population history can be reflected in different genealogical arrangements at different loci, a process that is most likely the cause of the discordance between the genes analysed in this study. However, some general patterns could still be seen within the nuclear data, with the Gold Coast specimens and Tewantin/Lake Weyba specimens consistently grouped together across all nuclear gene trees, and the Lennox Head specimens and Broadwater National Park/Lake Hiawatha specimens also consistently grouped together.

The combined gene trees were highly supported at major nodes and were highly concordant, with the same specimens consistently grouped for both analyses (ML and Bayesian). There were no differences in the topology of the two trees, with the only notable disparity being the difference in the support value at the (Beerburrum (Hervey Bay/Tewantin/Lake Weyba/Gold Coast)) node, which was not supported in the Bayesian tree and was weakly supported in the ML tree.

4.5.2 Species Delimitation

The separation of specimens into Lineages 3 through 8 was highly supported by all species delimitation methods used. Support for the distinction between Lineages 1 and 2 was dependent upon the method and gene used. It has been found that recently diverged species are harder to distinguish than older species using the barcoding approach, with problems most likely attributable to incomplete lineage sorting resulting in the lack of a barcoding gap (van Velzen *et al.* 2012 and references therein). Additionally, when using the K/θ method, the high levels of genetic diversity found within each lineage (rather than low levels between them) may have resulted in these two lineages not being supported. Alternatively, as has been found by other studies, retained ancestral variation between two recently diverged clades may mask their current genetic isolation using the K/θ method as divergence will follow a continuum and therefore no single percentage will work in every case (Druzhinina *et al.* 2012). Although this method has proven useful for other studies of sexual organisms (e.g. Marrone *et al.* 2010; Leasi *et al.* 2013; Reniers *et al.* 2013), the results presented here suggest that it may not be suitable for delineating between some species where intraspecific diversity is high. In light of this, and considering the support shown by the other species delimitation methods (i.e. haplotype networks and AMOVA), Lineage 1 and 2 are accepted as independently evolving lineages and, therefore, species.

The final species designation using the COI and 16S haplotype networks, genetic distances, and genetic measures (formulated as $H_{B1,2,3}$) agreed with the initial hypothesis (H_A). Throughout the remainder of this thesis the species will be referred to as outlined below and will include the nominated populations. The already described species *Tenuibranchiurus glypticus* (i.e. those populations grouped with the Type Locality) will retain this species name. It should be noted that in two sampling locations multiple distinct species exist (i.e. Tuan State Forest South and Beerburrum); these cases of sympatry will be discussed in Chapter 8.

> *Tenuibranchiurus* sp. nov. 1: Maryborough, Tuan State Forest North and South, Kinkuna National Park
>
> *Tenuibranchiurus glypticus*: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum, Mooloolaba
>
> *Tenuibranchiurus* sp. nov. 2: Beerburrum

Tenuibranchiurus sp. nov. 3: Hervey Bay

Tenuibranchiurus sp. nov. 4: Tewantin, Lake Weyba, Eumundi

Tenuibranchiurus sp. nov. 5: Gold Coast

Gen. nov. sp. nov. 1: Lennox Head

Gen. nov. sp. nov. 2: Lake Hiawatha, Broadwater National Park

5.0 Biogeography of *Tenuibranchiurus* and *Gen. nov.*

5.1 INTRODUCTION

Phylogeography allows the spatial arrangement of genetic lineages and species to be visualised in order to identify how the genetic heritage of conspecific individuals is linked (Avise 2009). The two principal hypotheses employed to explain how the current distributions of genetic lineages evolved are vicariance and dispersal (Parenti and Humphries 2004; Ponniah and Hughes 2004). Drawing correlations between the timing and location of historical geological events, genetic divergence, and geographic distribution of diversity allows the processes responsible for contemporary distribution patterns of taxa to be evaluated.

Freshwater species in particular may show strong genetic structuring throughout the landscape, allowing the observed distribution of diversity to be examined using contemporary geography and/or palaeo-structure (Schultz *et al.* 2008). The biogeographic restraints that affect freshwater organisms (e.g. restriction to freshwater habitat and dispersal being dependant on the degree of freshwater connectivity; Unmack 2001) have resulted in discernable genetic patterns even at very small scales (e.g. within a single stream: Hughes *et al.* 2009; Page and Hughes 2014). Although these patterns may be strongly correlated with contemporary riverine structure, frequent discrepancies have meant that historical riverine connections and rearrangements have often been implied as the underlying cause of genetic diversity (e.g. McGlashan and Hughes 2000, 2001; Nguyen *et al.* 2004; Waters *et al.* 2007; Jerry 2008).

Phylogeographic studies on Australian freshwater crayfish have primarily been focussed on the three most widespread genera (i.e. *Euastacus*, *Engaeus*, and *Cherax*; Munasinghe *et al.* 2004a; Nguyen *et al.* 2004; Ponniah and Hughes 2004; Shull *et al.* 2005; Ponniah and Hughes 2006; Schultz *et al.* 2008; Bentley *et al.* 2010; Toon *et al.* 2010), or have investigated more ancient connections between genera (e.g. Schultz *et al.* 2009; Toon *et al.* 2010). However, investigating the phylogeographic relationships within and between *Tenuibranchiurus* and *Gen. nov.* represents an opportunity to test previous assumptions and models related to the biogeography of freshwater fauna throughout central-eastern Australia as well as the historical biogeography of the burrowing clade and Australian freshwater crayfish in general.

5.2 CHAPTER AIM

As *Gen. nov.* is a newly proposed genus from this study (see Chapter 4), no current information exists on the separation of this genus from *Tenuibranchiurus* nor the diversification within this genus, and the only available estimates of divergence times relating to *Tenuibranchiurus* are either at the generic-level (i.e. between *Tenuibranchiurus* and *Geocharax*: Schultz *et al.* 2009; Toon *et al.* 2010) or based on limited within-genus data (Dawkins *et al.* 2010). Additionally, all previous genetic analyses on *Tenuibranchiurus* have been limited to mtDNA or a very small number of sequences, potentially obscuring or misrepresenting the true biogeographic history of this genus. No biogeographic information specific to either genus exists, nor have they been analysed as part of a comparative biogeographic approach for the central-eastern coast. This chapter will therefore seek to understand the historical and contemporary forces that define the degree and distribution of diversity within these taxa by (1) investigating whether the contemporary phylogeographic structure of species within both genera highlights recent connectivity across the landscape, (2) determining the timing of divergence between species and genera, and (3) comparing estimates of divergence times and corresponding geological events in order to reconstruct ancient distributions and the factors that shaped them.

5.3 METHODS

5.3.1 Genetic Diversity

Genetic distances were calculated within each putative species of *Tenuibranchiurus* and *Gen. nov.* using both the COI and 16S data from Chapter 4, as these represent the most complete data available. Distances were calculated in MEGA5 using the within group mean distance with 1000 bootstrap replicates (gamma distribution with shape parameter = 1, MCL model, positions containing gaps and missing data were eliminated).

Basic summary statistics were also calculated to explore the distribution of diversity within each of the *Tenuibranchiurus* and *Gen. nov.* species. The program DnaSP was used to calculate the haplotype diversity and nucleotide diversity for both the COI and 16S data.

The current genetic and geographic structure of *Tenuibranchiurus* and *Gen. nov.* was then examined via haplotype networks. Following the protocol in Chapter 4 (section 4.3.3.1), both COI and 16S networks were created using the program TCS v. 1.21 with a cut-off value of 90%. The putative ancestral haplotype was determined by the program through calculating the outgroup weights based on haplotype frequency and connectivity. These networks were then plotted in a geographic framework using ArcGIS v. 10.2, allowing the presence of shared and unique haplotypes to be visualised in order to explore the genetic connectivity of populations within species.

5.3.2 Neutrality Tests

Two tests of neutrality were carried out (Tajima's D and Fu's Fs) for each gene fragment using Arlequin 3.5 (Excoffier *et al.* 2005). These tests are used to detect any recent demographic changes or non-neutral evolution within populations. For example, a positive value for Tajima's D indicates a recent bottleneck, a negative D indicates selection or a recent population expansion has occurred, and a negative value for Fu's Fs suggests that there has likely been a recent demographic expansion (Tajima 1989; Fu and Li 1993). All but two results[3] were non-significant and therefore these tests are not discussed further.

[3] Tajima's D was significant (p=0.008) for *Gen*. nov. sp. nov. 1 (value=-2.01) for the COI gene data, and Fu's Fs was significant (p=0.015) for *T. glypticus* (value=-3.75) for the 16S gene data.

5.3.3 Ancestral Range Reconstruction and Timing of Divergence

Ancestral distributions of *Tenuibranchiurus* and *Gen. nov.* were reconstructed using the program RASP (Reconstruct Ancestral State in Phylogenies) v. 2.1 (Yu *et al.* 2010, 2013) by Bayesian binary MCMC (BBM) analysis. This package allows geographic distributions to be inferred for ancestral nodes, as well as inferring the occurrence of 'events' including dispersal, vicariance, and extinction (Ronquist 2004). Three sets of results are produced by the analysis; results of run one, results of run two, and results of the combined runs. The outputs from these results include the probabilities of alternative ancestral ranges at each node, and the associated dispersal, vicariance, and extinction costs. The results of the combined runs were used for all further analyses.

In order to run the RASP analysis, a species phylogeny of *Tenuibranchiurus* and *Gen. nov.* was first constructed in the program *BEAST v. 1.8.0 (Heled and Drummond 2010) following the species designations specified in Chapter 4. All sequences for all genes (i.e. COI, 16S, GAPDH, H3, and AK) were utilised, with blank sequences inserted where a sequence for any particular gene was not available for a species. Before analysis, each gene was tested separately for the assumption of a molecular clock using a likelihood ratio test (LRT) implemented in PAUP* (Swofford 2003). The LRT failed to reject the hypothesis of a molecular clock for 16S and AK (p=0.342 and 0.317, respectively), but rejected it for COI, GAPDH, and H3 (p<0.05). Therefore, an uncorrelated lognormal relaxed clock was used to account for non-clocklike evolution. Additionally, models of evolution were specified for each gene. Although the optimal models were calculated in Chapter 4 (section 4.4.1), as only three models are available in *BEAST the best-fit models were designated as follows: COI=TN93+I+G, 16S=HKY+G, GAPDH=TN93+I+G, H3=TN93+G, and AK=TN93+G.

Uninformative uniform priors (0-100) were used as the ucld.mean for the nuclear genes (GAPDH, H3, AK), and an informed normal prior for the ucld.mean for COI and 16S (mean=0.0085, stdev=7.653×10^{-4}; mean=0.003575, stdev=4.719×10^{-4}, respectively). These informed priors were determined using reported substitution rates for similar organisms, and represent the range of different values stated in the literature for COI (1.4-2.0%) (e.g. Schubart *et al.* 1998; Wares and Cunningham 2001; Morrison *et al.* 2004; Page and Hughes 2007a) and 16S (0.53-0.9%) (e.g. Sturmbauer *et al.* 1996; Schubart *et al.* 1998; Stillman and Reeb 2001; Schultz *et al.* 2009). Trees representing the mtDNA genes were linked and a

yule-birth rate tree prior used for all genes. All ucld.stdev were adjusted to represent a plausible distribution (exponential, initial value=2, mean=0.5), the ploidy type specified, and the COI and 16S trees were linked.

Two runs were performed using random starting trees, each with a total of 25 million generations sampling every 1000 generations. An 'empty alignment' was also run (i.e. without nucleotide data, using only the set priors), to examine the influence of the assigned priors on the parameters. The runs were checked for convergence, the ESS values (>200 was considered appropriate), and the burnin determined using the program Tracer v. 1.5 (Rambaut and Drummond 2007). The post-burnin trees were combined using LogCombiner v. 1.8.0 (Drummond and Rambaut 2007). After the burnin was removed, a total of 45,000 trees remained in the species tree. The post-burnin trees were then annotated using TreeAnnotator v. 1.8.0 (Drummond and Rambaut 2007) to provide a 'condensed' tree for RASP. Using the species phylogeny created by *BEAST, divergence times were estimated for the entire tree based on the evolution rates entered for COI and 16S and estimated for the nuclear genes.

For the BBM analysis, the 45,000 trees produced by *BEAST were loaded, as well as the condensed tree, and the following parameters set; 5 million cycles, 30 chains, sampling every 100 cycles, a maximum of 7 areas, null root distribution, and F81+G model. The distributions for each of the tips were assigned *a priori* from a total of seven geographical regions; (A) Burrum River, (B) Mary River, (C) Noosa River, (D) Maroochy River, (E) South Coast, (F) Richmond River, and (G) Bellinger River. These areas represent the present day catchments from which *Tenuibranchiurus* and *Gen. nov.* are currently known.

5.4 RESULTS

5.4.1 Species Distributions and Genetic Diversity

The distribution of the genus *Tenuibranchiurus* is restricted to south-east Qld and spans a distance of ~350 km along a narrow coastal strip. Using the species groups determined in Chapter 4, the distributions of the six *Tenuibranchiurus* species are shown in Figure 5.1. The range of the single described species (*T. glypticus*) was previously restricted to the two localities referred to in its original description by Riek (1951); namely Caloundra and Mt Gravatt[4]. The distribution of this species has been extended, now reaching from Tuan State Forest to Bribie Island (although it is highly disjunct), and the species occurs in sympatry with two other species at the extremities of its mainland range (i.e. *T.* sp. nov. 1 in the north and *T.* sp. nov. 2 in the south; Figure 5.1). The distribution of *T.* sp. nov. 1 extends from Tuan State Forest to Kinkuna National Park (the northern-most limit of this genus), which is the second-largest range within *Tenuibranchiurus* (after *T. glypticus*). Although the range of *T.* sp. nov. 1 is smaller that that of *T. glypticus*, the COI genetic diversity within this species is almost 2.5 times larger than that of *T. glypticus*, and substantially larger than all other species (Table 5.1). Both the COI and 16S sequences produced a high percentage of unique haplotypes within both *T.* sp. nov. 1 and *T. glypticus* and a large number of mutational steps were evident between haplotypes within each of these species (Table 5.1).

The remaining four *Tenuibranchiurus* species have restricted distributions, with *T.* sp. nov. 2 and 3 currently only represented by single populations, *T.* sp. nov. 4 known from three populations (one of which (Eumundi) may have become extinct (see Chapter 2, section 2.1.1)), and *T.* sp. nov. 5 found in two nearby populations (Figure 5.1). Admittedly, the sample size of *T.* sp. nov. 2 and 3 were markedly smaller; however, all four of these species show very low levels of intraspecific diversity for both genes and fairly similar haplotype and nucleotide diversities; although *T.* sp. nov. 5 displayed very few connected haplotypes for the 16S gene (Table 5.1).

[4] Although all subsequent studies nominally designated collected specimens as *T. glypticus*, this was due to the presumed monotypy of the genus and are therefore not included in the previous distributional extent outlined here.

The distribution of the newly proposed genus *Gen. nov.* is limited to NSW and currently extends from Lennox Head to Lake Hiawatha; a distance of ~125 km (Figure 5.1). The distribution of *Gen. nov.* sp. nov. 1 is restricted to the northern-most population at Lennox Head, and the disjunct distribution of *Gen. nov.* sp. nov. 2 extends from Broadwater National Park to Lake Hiawatha, with these two regions separated by ~85 km. Although the distribution of *Gen. nov.* sp. nov. 2 is seemingly divided and much larger than *Gen. nov.* sp. nov. 1, both species have comparable levels of haplotypic and nucleotide diversities (relatively high and low, respectively), and both exhibit very low intraspecific diversity (Table 5.1).

All species from both genera are considered to have a coastal distribution, with the most 'inland' population (Tuan State Forest; an area of sympatry between *T.* sp. nov. 1 and *T. glypticus*) occurring 25-30 km from the coast. The most 'coastal' populations were found in *T. glypticus* (Bribie Island), and *Gen. nov.* sp. nov. 1 and 2 (Lennox Head and Lake Hiawatha, respectively), with all populations within these species occurring as close as ~1 km from the coastline.

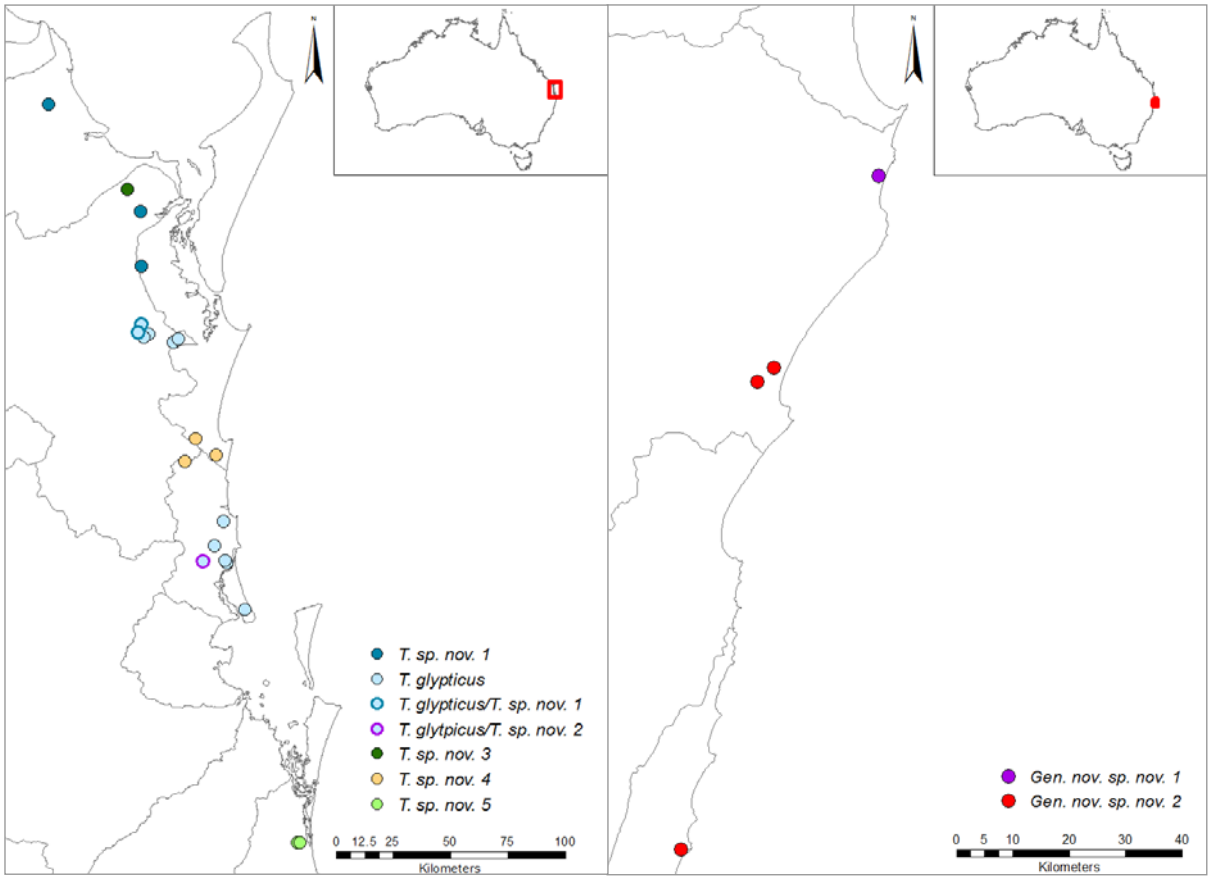


Figure 5.1. Species distributions for *Tenuibranchiurus* (left) and *Gen. nov.* (right) and the catchment boundaries of the regions. For sources of GIS data see section 2.3.

Table 5.1. Sequence divergence estimates within *Tenuibranchiurus* and *Gen. nov.* species, and haplotype and nucleotide diversity measures using COI and 16S data. Dashes are used where values could not be calculated due to sample size or haplotype composition.

		# sequences		Sequence divergence		Haplotype diversity		Nucleotide diversity	
		COI	16S	COI	16S	COI	16S	COI	16S
Tenuibranchiurus	sp. nov. 1	16	10	0.045	0.019	0.925	0.956	0.0375	0.0188
	glypticus	38	17	0.019	0.013	0.999	0.941	0.0164	0.0073
	sp. nov. 2	4	1	0.002	-	0.833	-	0.0016	-
	sp. nov. 3	1	4	-	0.000	-	0.000	-	0.000
	sp. nov. 4	14	8	0.010	0.005	0.813	0.821	0.0097	0.0052
	sp. nov. 5	15	6	0.007	0.002	0.800	0.333	0.0046	0.0015
Gen. nov.	sp. nov. 1	13	5	0.003	0.004	0.782	0.900	0.0045	0.0041
	sp. nov. 2	24	8	0.004	0.004	0.826	0.857	0.0056	0.0045

To examine the current genetic and geographic structure of *Tenuibranchiurus* and *Gen. nov.*, COI and 16S haplotype networks were visualised and placed within a geographic context (Figures 5.2 through 5.6). For some species, the pattern of haplotype distributions did not relate to geographic proximity, nor were haplotype relationships always consistent across the two gene regions.

The COI and 16S haplotypes for *T.* sp. nov. 1 followed a north-south pattern, with the ancestral haplotype for each gene occurring in the most northerly population (MAR for COI, KNP for 16S; Figure 5.2). For the 16S network, the maximum number of haplotypes within a single population was four (MAR) and the only shared haplotype was between TSFSA and TSFSC. There were a large number of missing haplotypes within the 16S network, with almost all populations separated by multiple mutational steps (Figure 5.2). The single exception to this was the close relationship between the ancestral haplotype in KNP (the most northerly population) and the haplotype shared across TSFSA and TSFSC (the most southerly populations). Phylogeographic structuring within the COI networks was strong. Again, MAR displayed the highest number of haplotypes within a single population, and a single shared haplotype was seen between TSFSA and TSFSC (Figure 5.2). There were a large number of mutational steps between all populations, with a single haplotype from both MAR and TSFSC forming a separate network.

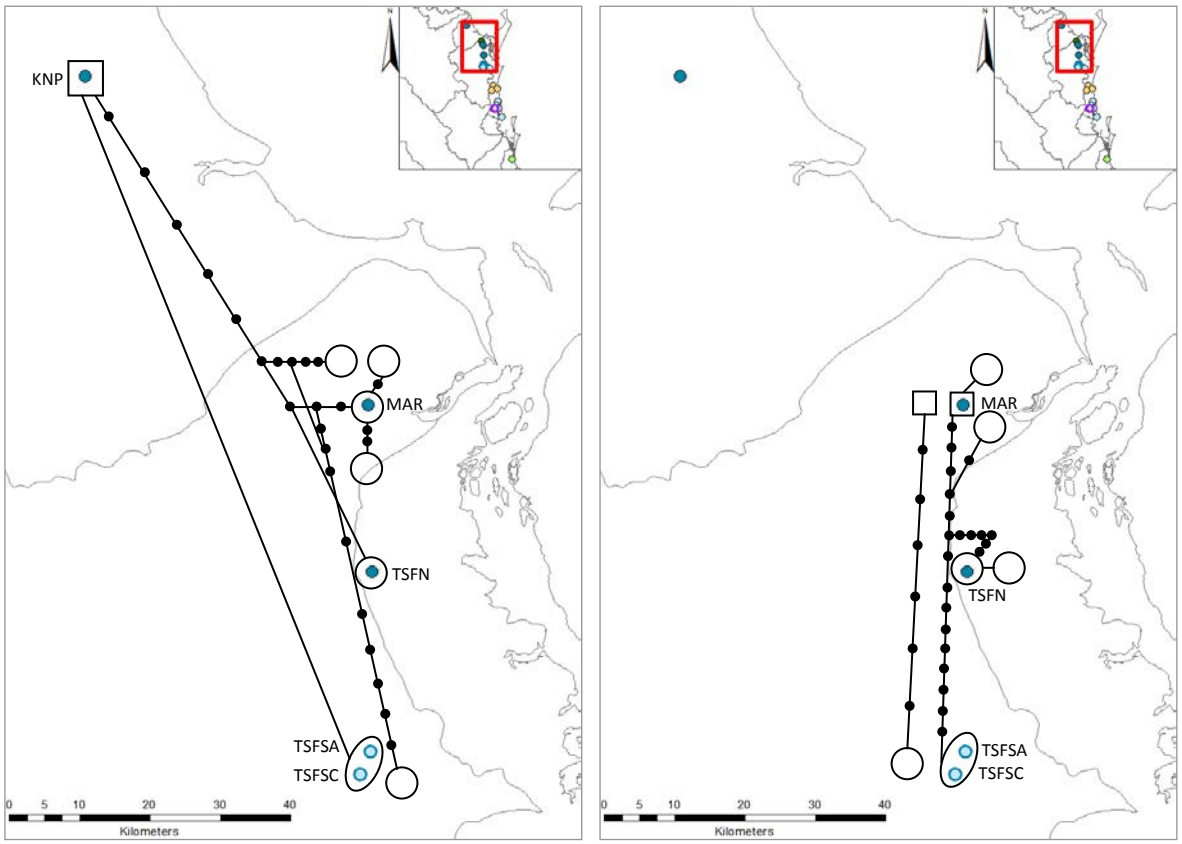


Figure 5.2. Haplotype networks for *T.* sp. nov. 1 using 16S (left) and COI (right) data. Catchment boundaries are shown in grey. Haplotypes are placed around the sampling locations (coloured dots) they were found at or, where there are multiple haplotypes per location, they are placed as close as possible to the corresponding sampling location. Ancestral haplotypes are denoted by a square. For sources of GIS data see section 2.3.

The ancestral COI and 16S haplotypes for *T. glypticus* were not geographically consistent. In the COI network it was located in the most northerly populations (TSFS), but in the 16S network it was in the most southerly population (BRB) (Figure 5.3). Within the 16S network there were two shared haplotypes; one between TL1 and BER, and the other between TSFSE and TSFSF. The maximum number of haplotypes within a single population was four (BRB), and most populations were separated by only a small number of mutational steps (Figure 5.3). The COI haplotypes were arranged in a roughly geographically concordant pattern, although relationships between haplotypes were complex, especially in the northern-most populations. The ancestral haplotype was shared across three populations (TSFSA, TSFSC, TSFSG), with an additional shared haplotype between TSFSA and TSFSF (Figure 5.3). Again, BRB displayed the highest number of haplotypes for a single population, and all populations were separated by a large number of mutational steps. There were also a large number of missing haplotypes within the BER, BRB, TSFSC, and TSFSF populations.

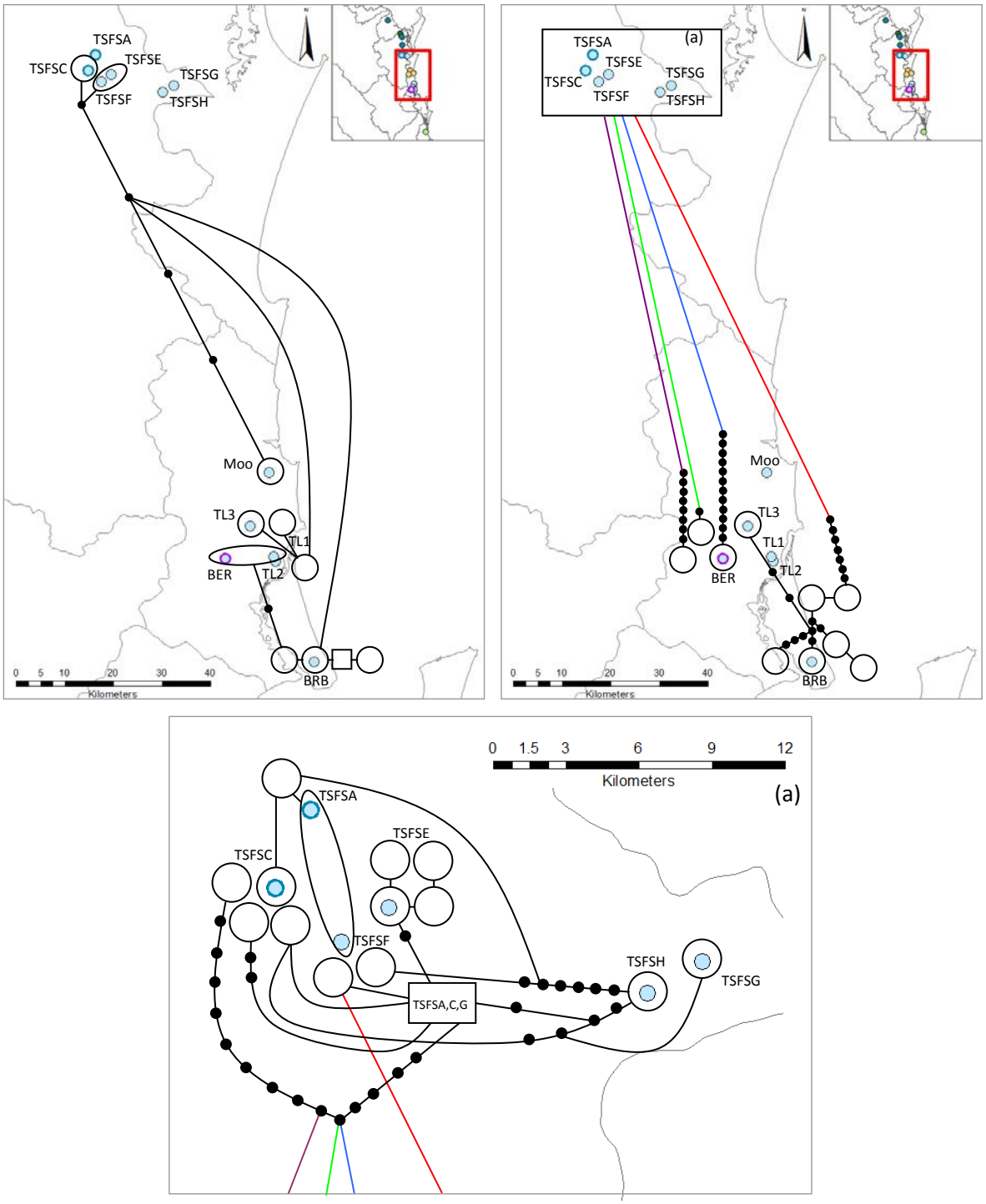


Figure 5.3. Haplotype networks for *T. glypticus* using 16S (left) and COI (right) data. The rectangle in the right figure denoted by an (a) is expanded below it and displays the COI haplotype network for the most northerly populations, with coloured lines identifying the connections between the enlarged image and the full distribution map. The ancestral COI haplotype (rectangle) shown in (a) is not placed proximate to its geographic location as it spans multiple populations. Catchment boundaries are shown in grey. Haplotypes are placed around the sampling locations (coloured dots) they were found at or, where there are multiple haplotypes per location, they are placed as close as possible to the corresponding sampling location. The ancestral 16S haplotype is denoted by a square. For sources of GIS data see section 2.3.

Tenuibranchiurus sp. nov. 4 showed simple COI and 16S networks (Figure 5.4). The ancestral haplotypes were located in TEW in the COI network, and LW in the 16S network. Haplotypes from TEW and LW clustered most closely with other haplotypes from the same population in the COI network. The 16S network was simple, with LW the only population to show multiple haplotypes.

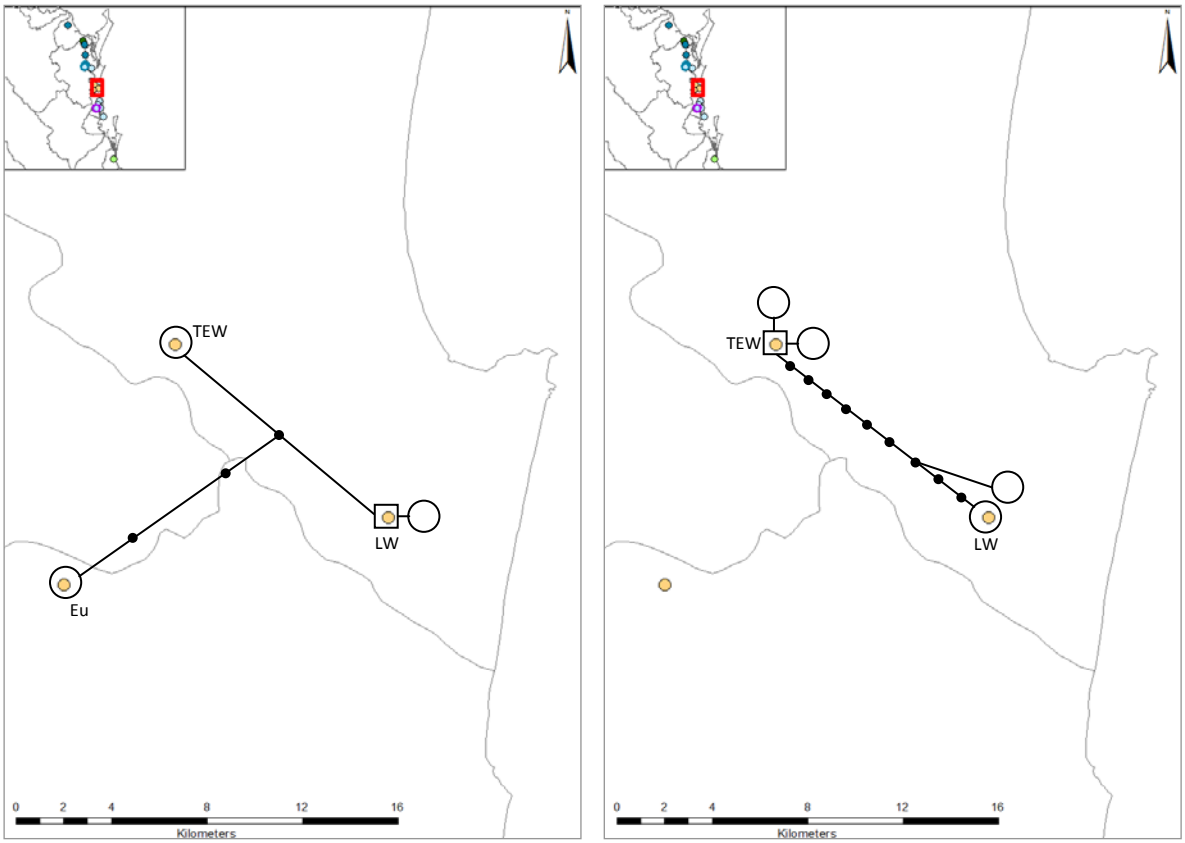


Figure 5.4. Haplotype networks for *T.* sp. nov. 4 using 16S (left) and COI (right) data. Catchment boundaries are shown in grey. Haplotypes are placed around the sampling locations (coloured dots) they were found at or, where there are multiple haplotypes per location, they are placed as close as possible to the corresponding sampling location. Ancestral haplotypes are denoted by a square. For sources of GIS data see section 2.3.

The pattern shown by *T.* sp. nov. 5 haplotypes were also straightforward, with two 16S haplotypes across two populations and the ancestral haplotype shared between them (Figure 5.5c), and many closely related COI haplotypes with all GC2 occurring as a single central ancestral haplotype (Figure 5.5c).

Both *T.* sp. nov. 2 and 3 only occur in single populations, and therefore have no geographic pattern to explore. *Tenuibranchiurus* sp. nov. 2 was represented by a single specimen in the 16S data, and four specimens consisting of three connected haplotypes in the COI data (Figure 5.5a). *Tenuibranchiurus* sp. nov. 3 displayed only one haplotype in both data sets, with the 16S haplotype recovered from four specimens and the COI from one specimen (Figure 5.5b).

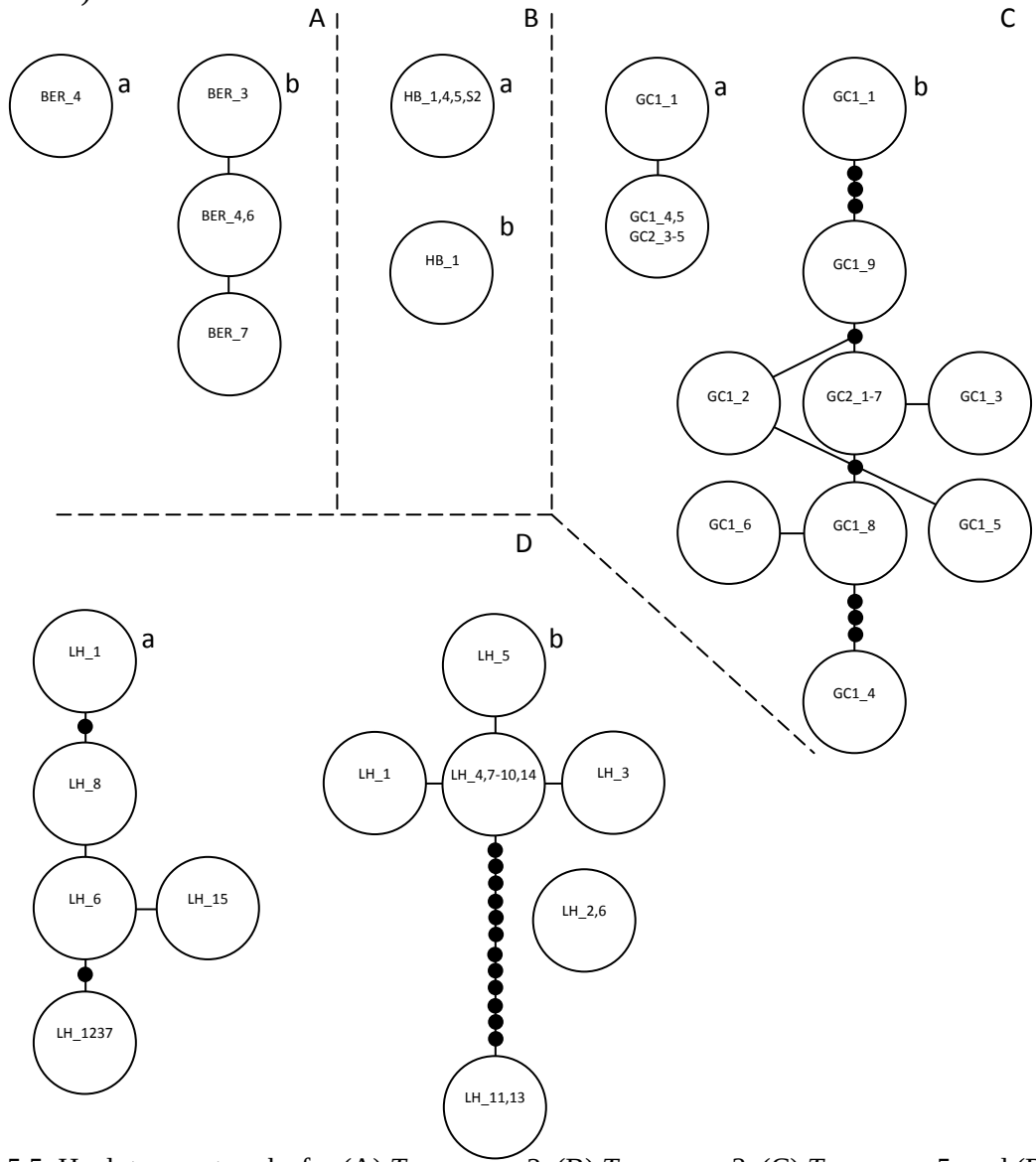


Figure 5.5. Haplotype networks for (A) *T.* sp. nov. 2, (B) *T.* sp. nov. 3, (C) *T.* sp. nov. 5, and (D) *Gen. nov.* sp. nov. 1. 16S networks are denoted by an (a) and COI by a (b).

The *Gen. nov.* sp. nov. 1 specimens displayed a simple network for COI, with 12 steps to the outermost haplotype and one singleton. The 16S network was also simple, consisting of five closely related haplotypes (Figure 5.5d). The *Gen. nov.* sp. nov. 2 16S network was straightforward, with haplotypes arranged geographically and all closely related. BNP2 was the only population with a single haplotype. In the COI network, all populations displayed multiple haplotypes, with the largest number of mutational steps within a population between the two BNP2 haplotypes (Figure 5.6). The ancestral 16S haplotype was located at BNP2 and at BNP1 for COI, both of which are the most northerly populations (Figure 5.6).

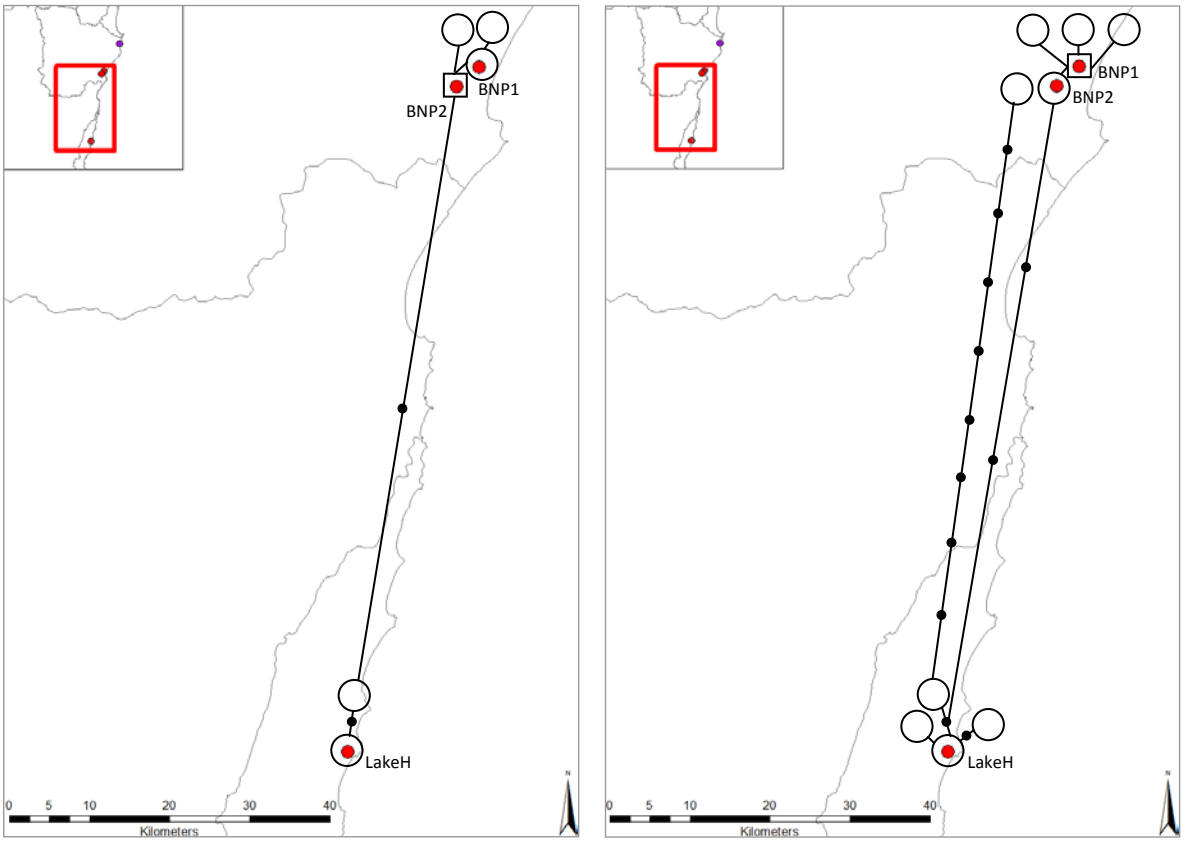


Figure 5.6. Haplotype networks for *Gen. nov.* sp. nov. 2 using 16S (left) and COI (right) data. Catchment boundaries are shown in grey. Haplotypes are placed around the sampling locations (coloured dots) they were found at or, where there are multiple haplotypes per location, they are clustered around the sampling location. Ancestral haplotypes are denoted by a square. For sources of GIS data see section 2.3.

5.4.2 Historical Biogeography

The reconstruction of ancestral distributions using BBM suggested that both dispersal and vicariance have acted to shape the current distribution of species within the genus *Tenuibranchiurus* and *Gen. nov.* (12 dispersal, three vicariant, and zero extinction events – see Figure 5.7 for a graphical representation of the following discussion, and the associated map). The RASP analysis suggested the ancestor of these genera (node 1) most likely occurred in the Richmond River catchment (F; 61.23% probability), and from there dispersed into the Mary River (B) during the Miocene, giving rise to the ancestral *Tenuibranchiurus* (node 3). A vicariant event also occurred at node 1 that gave rise to the ancestral *Gen. nov.* that remained in the Richmond River (node 2; 82.28% probability). From the Richmond River, the ancestral *Gen. nov.* then dispersed into the Bellinger River (G) during the Late Miocene, giving rise to *Gen. nov.* sp. nov 1 and 2.

The ancestral *Tenuibranchiurus* (node 3) most likely occurred in the Mary River (B; 39.29% probability), with this ancestor dispersing into the Noosa River (C) during the Late Miocene, as well as undergoing a vicariant event to give rise to the ancestor of *T.* sp. nov. 3 (node 5). At node 5, no events were suggested as *T.* sp. nov. 3 remained in the Mary River (B; 80.84% probability) and the ancestral range at node 6 was also most likely in this catchment (45.68% probability). The ancestor at node 6 then dispersed across the Mary River (B), Burrum River (A), Noosa River (C), and Maroochy River (D) catchments during the Pliocene, giving rise to *T.* sp. nov. 1 and the ancestral species at node 7. The ancestral distribution at node 7 most likely occurred across the Mary River and Maroochy River catchments (BD; 67.48% probability), with *T. glypticus* remaining in this range and *T.* sp. nov. 2 retreating into the Maroochy River catchment during the Pleistocene.

The ancestral distribution of the remaining two species (node 4; *T.* sp. nov. 4 and 5) was ambiguous, with two almost equally probable options; the Maroochy River (D) or Noosa River (C) (27.82% and 27.46% probability, respectively). Using the most favoured range at D, the descendants dispersed into the South Coast (E) and Noosa/Maroochy River (CD) catchments during the Miocene, giving rise to the descendant species.

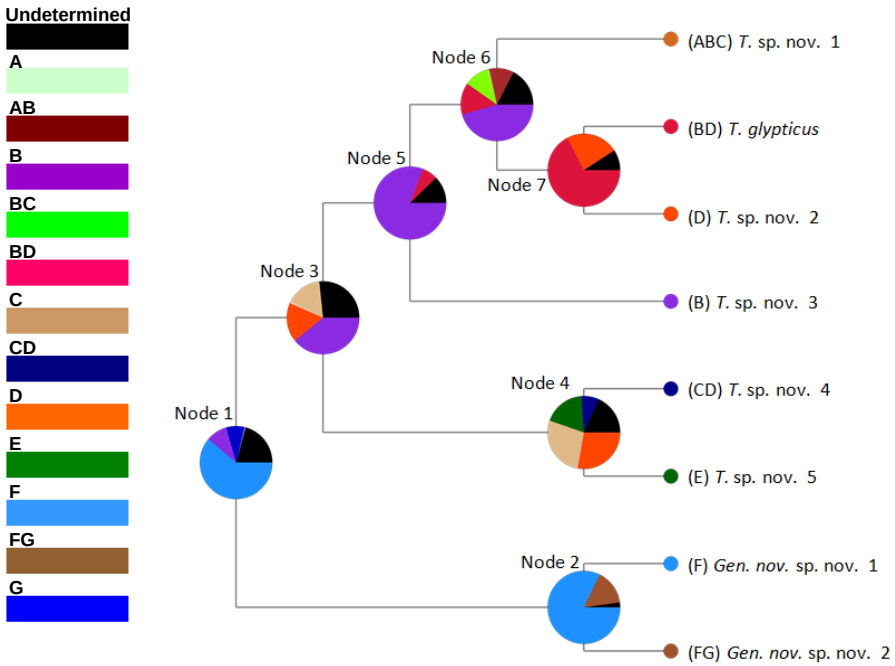


Table 5.2. Estimated age of nodes in Figure 5.7 and associated 95% confidence intervals. All values are given in millions of years ago.

Node	Mean node age	Upper bound	Lower bound
1	43.8	62.5	28.3
2	6.4	10.7	2.5
3	10.9	15.3	7.4
4	8.5	12.7	4.8
5	6.6	10.9	2.5
6	2.9	4.6	1.6
7	1.2	2.3	0.3

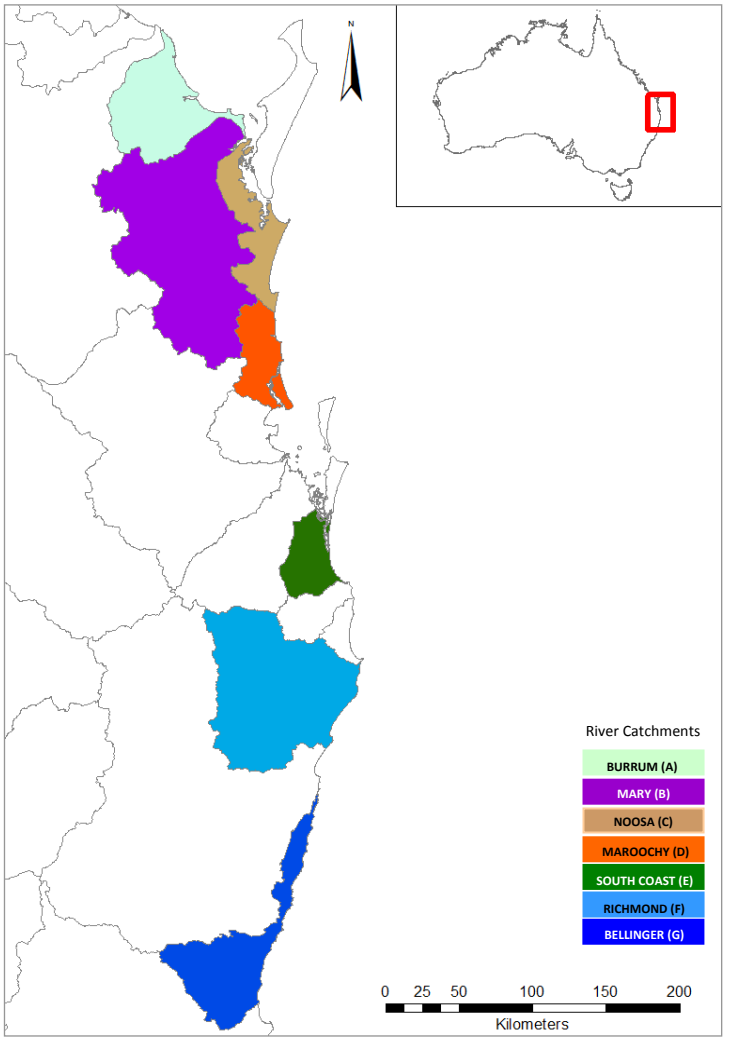


Figure 5.7. Estimated node ancestral distributions for *Tenuibranchiurus* and *Gen. nov.*. Pie proportions and colour indicate the probability of the associated distribution shown in map. Phylogeny transformed to show topology, branch lengths not indicative of time or mutation rates. Catchments are as follows: A=Burrum River, B=Mary River, C=Noosa River, D=Maroochy River, E=South Coast, F=Richmond River, G=Bellinger River. For sources of GIS data see section 2.3.

5.5 DISCUSSION

The Australian freshwater crayfish fauna is ancient in origin (Crandall and Buhay 2008; Toon *et al.* 2010), and has its centre of biodiversity in the south-east corner (SEC) of the continent (Crandall and Buhay 2008). Four of the seven burrowing clade genera (including *E. lyelli*), and an additional three other parastacid genera, all occur within the SEC, making this a highly diverse region and likely the origin of the burrowing clade (Crandall and Buhay 2008; Schultz *et al.* 2009). It is likely that the ancestor of *Tenuibranchiurus*/*Gen. nov.* occurred within the SEC region and subsequent dispersal events and/or range expansions northwards resulted in the present distribution of these two genera. The following discussion will not consider how these genera came to be distributed along the central eastern coast as this will be explored in Chapter 7; rather, this discussion will focus on the processes that have occurred since then. Throughout this discussion, when referring to the processes leading to diversification within each of the genera, the lineages leading to each node shown in Figure 5.7 will be referred to by an N followed by the node number. For example, the lineage that existed prior to the divergence at node 5 will be referred to as 'ancestral N5'. The discussion of the diversification of lineages will follow a temporal sequence. The timescale being discussed here reaches back into the Eocene and, therefore, the inferences provided should be viewed as a best estimation of the events that occurred based on the available ecological, biological, molecular, and historical climate data.

Following a northward dispersal pattern of the burrowing clade genera out of their centre of origin, it was found that the ancestral *Tenuibranchiurus*/*Gen. nov.* most likely originated in the Richmond River catchment in NSW, where a subsequent vicariance event resulted in the formation of the two genera. This divergence event was estimated to have occurred during the Middle Eocene[5] (~43.8 million years ago (mya)), but may have been as late as the Early Oligocene (~28.3 mya). Prior to this, during the Palaeocene (65.5-55.8 mya), conditions in Australia were warm and humid with many swamps and rainforest as the dominant vegetation type (Martin 2006). Throughout the Early Eocene (55.8-49.0 mya), conditions remained humid and temperatures increased, with this epoch becoming the warmest during the Cenozoic era (Martin 2006). The humid climate during both the Palaeocene and the Early

[5] Dates estimated in the following chapter using data from all of the burrowing clade genera suggest this date may be slightly earlier (50 mya; Early Eocene); however, they differ by only six million years and the confidence intervals of the two estimates are largely overlapping.

Eocene, in combination with the abundance of swampy habitat, would have provided favourable conditions for the ancestral *Tenuibranchiurus*/*Gen. nov.* to have expanded its range northwards from northern NSW into southern Qld. It appears that this expansion was followed by a vicariance event that resulted in two genera.

Studies undertaken on taxa that show divergences across the Qld-NSW border region have generally focused on within species-level divergences that occurred much more recently than the estimates of this study (e.g. James and Moritz 2000; Unmack 2001; Page and Hughes 2007a; Chapple *et al.* 2011a; Chapple *et al.* 2011b). The largest barrier within this region, and one that is frequently cited as a cause of genetic divergence (e.g. see previous references and Keogh *et al.* 2003; Munasinghe *et al.* 2004b), is the McPherson Range (a remnant of the Tweed Volcano), which is found along the border between Qld and NSW. The volcano is estimated to have erupted between 22-20 mya (McDougall and Wilkinson 1967; Webb and McDougall 1967; Wellman and McDougall 1974), during the Early Miocene, thus placing its formation after the divergence of *Tenuibranchiurus* and *Gen. nov.*. It is, therefore, unlikely to have caused the rift between the two genera, although it likely reinforced their continued separation. Discounting this volcano as the cause of the initial separation means there is no obvious geological barrier that would have caused vicariance between *Tenuibranchiurus* and *Gen. nov.*. However, during the middle-to-late Eocene (49.0-34.0 mya), the climate of Australia was starting to cool, with conditions becoming sub-tropical to temperate rather than tropical, though still with rainforest as the dominant vegetation (Christophel and Greenwood 1989; Martin 2006). It is possible that the decreasing temperatures throughout the end of the Eocene, shifts in coastal vegetation types, and climatic oscillations (Quilty 1984; Martin 2006) could have (individually or collectively) been responsible for the vicariance between the genera.

The next event to occur was during the Late Miocene in the Mary River catchment (~10.9 mya). This catchment is at the northern end of the current distribution of *Tenuibranchiurus*, meaning that the ancestral *Tenuibranchiurus* must have dispersed into this region at some point during the previous 30 million years. The cooling trend seen at the end of the Eocene continued into the Early Oligocene (34.0-28.5 mya), with this period being cool and very wet within south-eastern Australia and swamps becoming prominent (Martin 2006). Sea level fluctuated widely and there was a major drop in sea level during the Middle Oligocene (Martin 2006), which has been estimated to have been as much as 200-250 meters

lower than present (Quilty 1984). A fall of this magnitude would have exposed the entire continental shelf across northern NSW and southern Qld, and the low-lying terrain combined with the wet climate characteristic of this epoch would have created swampy habitat favourable for dispersal (e.g. Horwitz 1988a) (Figure 5.8a).

Although the sea level subsequently rose at the end of the Oligocene (Quilty 1984; Martin 2006), wet conditions and an increase in humidity continued into the Early Miocene (23.0-16.4 mya), potentially further facilitating the dispersal of this genus into the northerly drainages. Numerous fossilised wetland bird species indicate conditions would have been ideally suited to this (Martin 2006). As all subsequent events within *Tenuibranchiurus* were estimated to originate within the northern region, this indicates that the range of the ancestral *Tenuibranchiurus* either shifted northwards (with subsequent southerly extinctions) or contracted during this time. As the analysis did not suggest any extinction events, range contraction throughout the Middle Miocene (16.4-11.2 mya) is likely the cause of the northern ancestral distribution (Figure 5.8b).

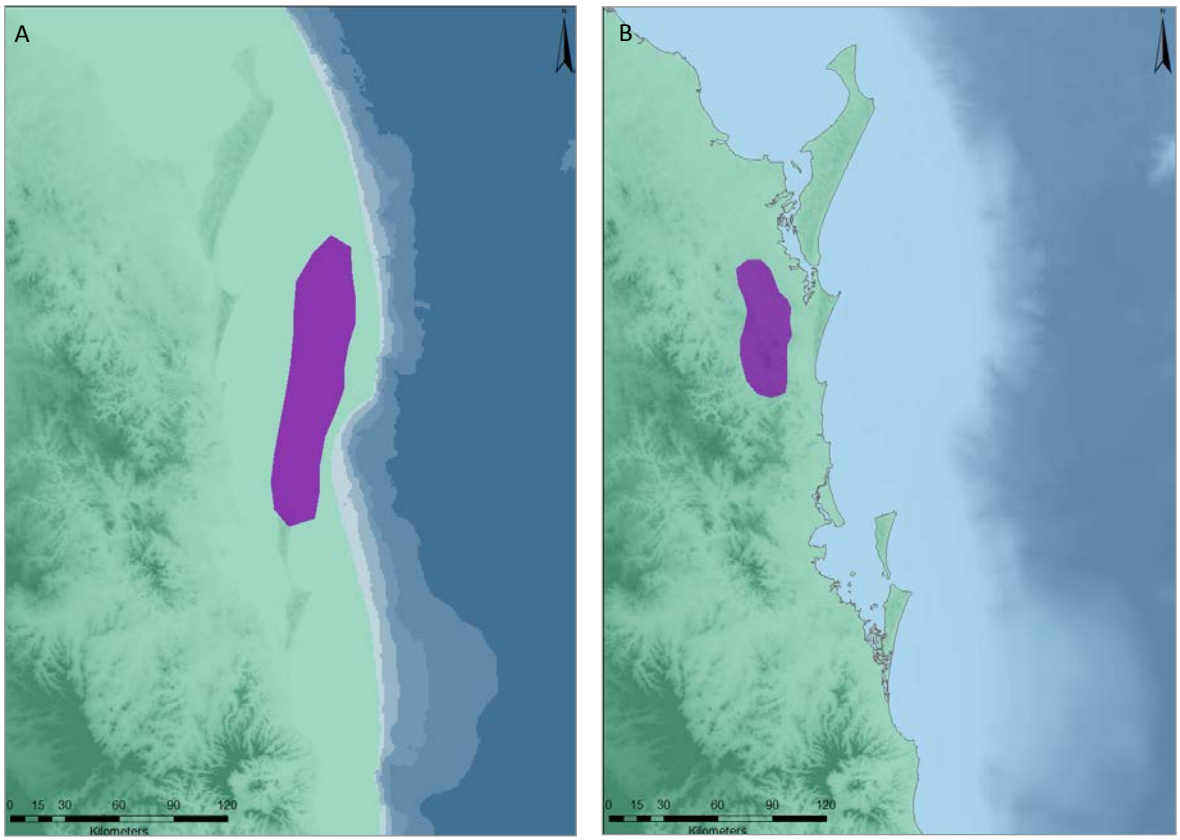


Figure 5.8. Hypothetical distribution of the ancestral *Tenuibranchiurus* (purple) during (A) Middle Oligocene where the sea level was -200 meters below present, and (B) Middle Miocene. For sources of GIS data see section 2.3. Note: the islands did not form until the late Pleistocene and should not be considered as part of the coastline.

During this epoch, conditions began to cool once again, the sea level fell and aridity started to become a feature (Quilty 1984; Martin 2006). Drying of the climate continued during the Late Miocene (11.2-5.3 mya), which corresponded with the ancestral *Tenuibranchiurus* dispersing into the Maroochy River catchment. This was most likely in response to the lowered sea level causing populations to shift their distributions in a coastal direction in an attempt to maintain access to sufficient moisture (either as an above-ground source or an accessible water-table), as has been suggested for other members of the burrowing clade (Horwitz 1988a; Schultz *et al.* 2008). As populations moved towards the coast, a vicariance event estimated by the analysis suggests that the species fractured into two groups (likely in a response to the drying conditions) with one persisting in the Maroochy River catchment (ancestral N4) and the other remaining in the Mary River catchment in areas where they could persist due to favourable local conditions (i.e. in microrefugia), giving rise to the ancestral N5 (Figure 5.9a).

The ancestral species that followed the receding coastal zone into the Maroochy River catchment (ancestral N4) underwent an additional two dispersal events during the Late Miocene (~8.5 mya), both southwards into the South Coast catchment and northwards to span both the Maroochy River and Noosa River catchments. Although the Late Miocene was a period of drying climate, the low-lying coastal regions would still have facilitated dispersal of this crayfish, due largely to its ability to burrow during unfavourable conditions, meaning it relies only on moist soil conditions rather than access to freshwater channels. As such, the lowered sea level during the Late Miocene would have exposed at least part of the continental shelf, thus providing a flat and likely swampy expanse of coastal habitat which would have allowed dispersal southwards along this coastal corridor (Figure 5.9b). Continued drying and an increase in sea level (reaching approximately the level seen today; Haq *et al.* 1987) are likely the cause of vicariance within the ancestral species, which gave rise to the descendant species, namely *T.* sp. nov. 4 and 5 (Figure 5.9c).

For the ancestral species that remained in the Mary River catchment (ancestral N5), a divergence event occurred some two million years later (~6.6 mya) that resulted in two species which both remained in this catchment. This divergence resulted in *T.* sp. nov. 3 and the ancestral N6. No events were estimated by the analysis for node 5, but it is likely that speciation occurred as a result of the isolation of populations. Those populations that remained in the Mary River catchments would have been in a region of high habitat

heterogeneity. This would have resulted in the occurrence of disjunct populations reliant on areas of sufficient moisture (unlike those in coastal habitats which would be more widely distributed), thus promoting divergence and the formation of two species in different areas within the Mary River catchment (Figure 5.9d). Genetic discontinuities have also been found within this region for other freshwater species including freshwater crayfish, shrimp, and fish, making this scenario highly plausible (e.g. Page *et al.* 2004; Page and Hughes 2007b; Thacker *et al.* 2007; Bentley 2014; Page and Hughes 2014).

During this period of diversification within *Tenuibranchiurus*, the ancestral *Gen. nov.* remained in the Richmond River catchment before subsequently expanding its range into the Bellinger River catchment. This occurred over an extended period of time; potentially 35 million years. In a similar situation to that described for the dispersal of *Tenuibranchiurus*, *Gen. nov.* may have expanded its range southwards during the low sea level stand of the Middle Oligocene, when the continental shelf was exposed and swampy conditions favourable for dispersal persisted (e.g. Horwitz 1988a) (Figure 5.10a). This is further supported by the presence of a major tributary bisecting the distribution of this genus; the Richmond River tributary. It follows that the range expansion of the ancestral *Gen. nov.* into both catchments occurred during a period of lowered sea level and exposed coastal plains, as the geographical boundaries defining these catchments would have been greatly lessened in such a situation (i.e. the coastal plain is much flatter and rivers tend to become more diffuse, shallower and more slowly flowing). An almost synchronous divergence date is estimated for the rise of the two *Gen. nov.* species and the divergence at the ancestral N5 (~6.4 mya), and it is likely that similar conditions were responsible. Although the ancestral *Gen. nov.* maintained its distribution across the two catchments throughout the increased sea level of the Late Oligocene and the wet and humid conditions of the Early Miocene, conditions started to become increasingly arid during the mid-to-late Miocene. This drying environment would likely have caused contractions within the distribution of this crayfish, resulting in a cessation of gene flow between the two resultant species; *Gen. nov.* sp. nov. 1 and 2 (Figure 5.10b).

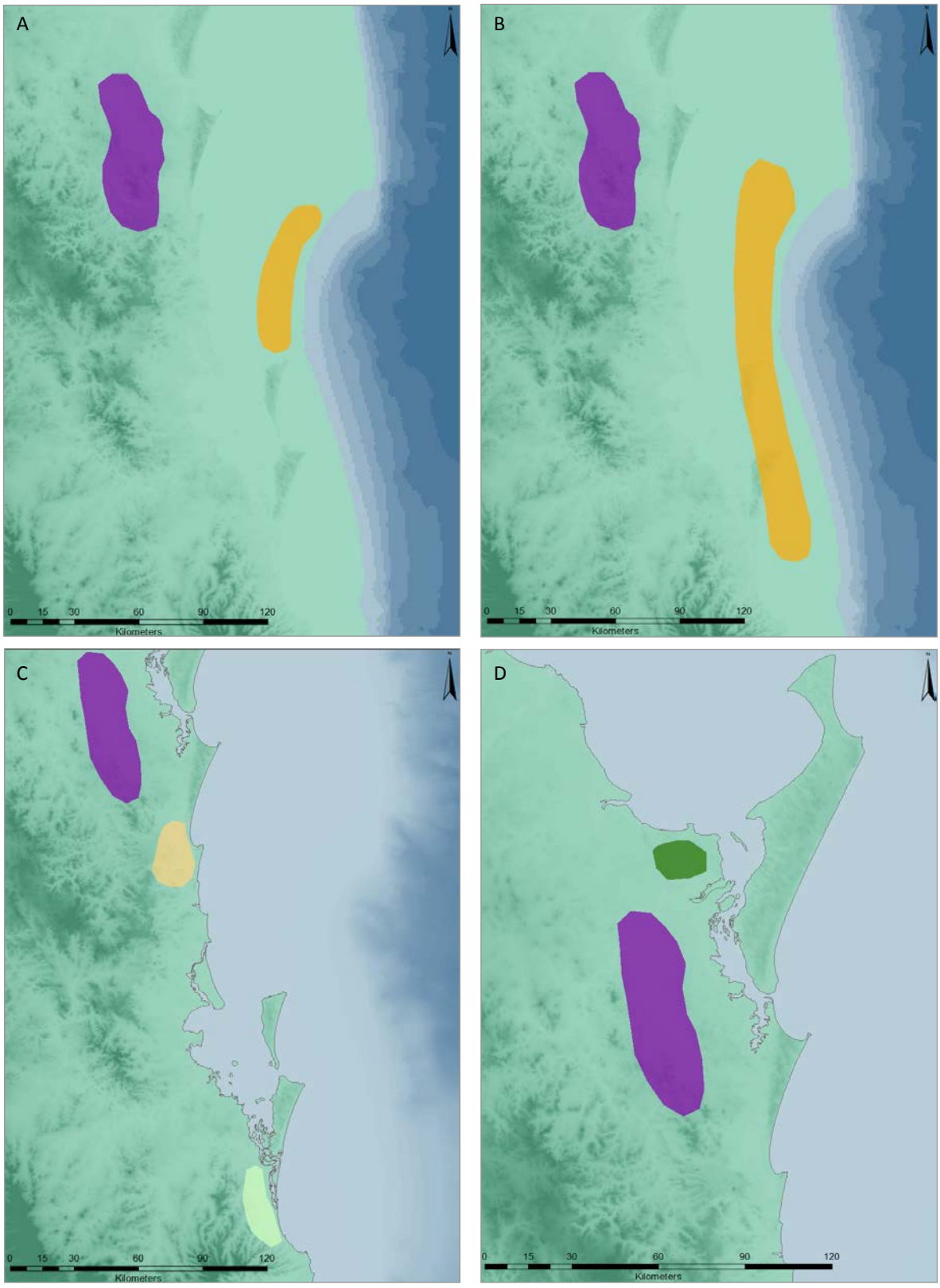


Figure 5.9. Hypothetical distribution of (A) the ancestral N5 (purple) and ancestral N4 (orange), with ancestral N4 dispersing north and south in (B). In (C) the ancestral N4 diverges to produce *T.* sp. nov. 4 (light orange) and *T.* sp. nov. 5 (light green). (D) The ancestral N5 diverges to give rise to *T.* sp. nov. 3 (dark green) and ancestral N6 (purple). All events occurred during the Late Miocene, with sea level in A and B shown at -100 meters below present. For sources of GIS data see section 2.3. Note: the islands did not form until the late Pleistocene and should not be considered as part of the coastline.

Shortly after this (during the Late Miocene through to the Late Pliocene (3.6-2.4 mya)), the ancestral N6 is estimated to have expanded its distribution across the Mary River catchment to also inhabit the Burrum, Noosa, and Maroochy River catchments. It has been suggested that there was a brief wetter period during the Early Pliocene in eastern Australia (5.3-3.6 mya), as evidenced by a resurgence of moisture dependent vegetation (Martin 2006). It is possible that it was during this period that the ancestral N6 utilised this brief period of wetter climate to extend its range into and across the four catchments (Figure 5.11a). Following this, there was a period of sea level rise during the Late Pliocene (Quilty 1984) and the climate returned to drier conditions (Martin 2006). It is likely that this drying climate caused the subsequent isolation of populations through range contractions and gene flow ceased (~2.9 mya), giving rise to *T.* sp. nov. 1 (distributed across the Burrum, Mary, and Noosa River catchments) and the ancestral N7 (distributed across the Mary and Maroochy River catchments). Although both species occurred in the Mary River catchment, the current distribution of *T.* sp. nov. 1 suggests it occupied a more northerly range within this catchment and ancestral N7 a more southerly range (Figure 5.11b).

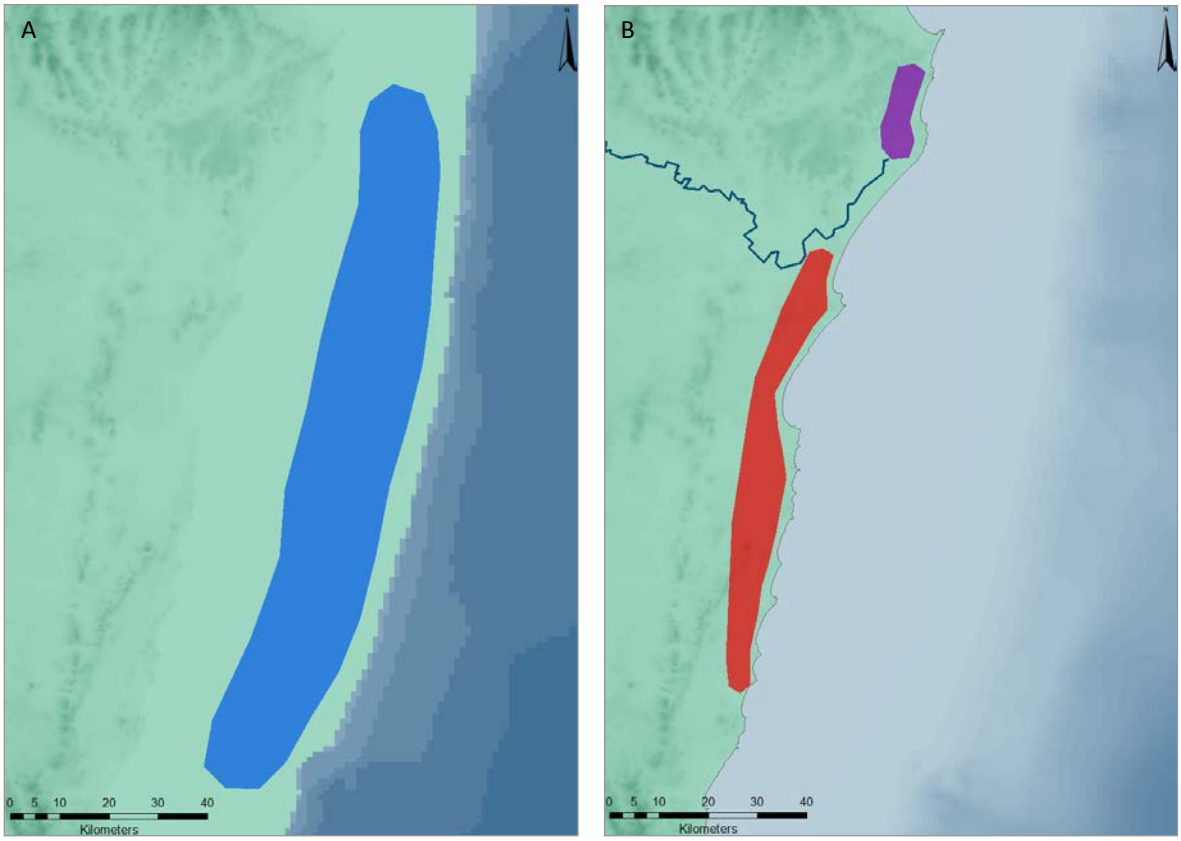


Figure 5.10. Hypothetical distribution of (A) the ancestral *Gen. nov.* (blue) during the Middle Oligocene where the sea level was -200 meters below present, with this diverging in (B) to produce *Gen. nov.* sp. nov 1 (purple) and *Gen. nov.* sp. nov. 2 (red) during the Late Miocene. The Richmond River is also shown in B. For sources of GIS data see section 2.3.

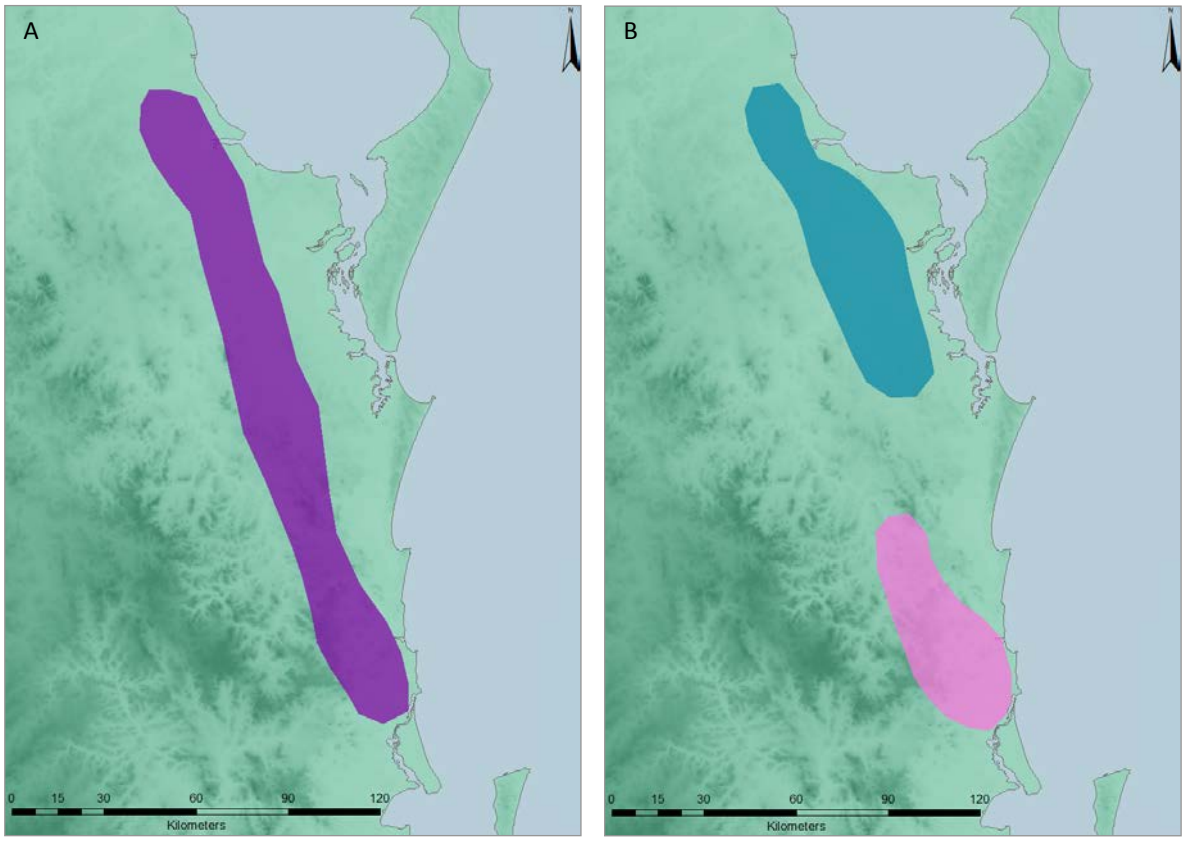


Figure 5.11. Hypothetical distribution of (A) the ancestral N6 (purple) during the Early Pliocene, with this diverging in (B) to give rise to *T.* sp. nov. 1 (blue) and ancestral N7 (pink) during the Late Pliocene. For sources of GIS data see section 2.3. Note: the islands did not form until the late Pleistocene and should not be considered as part of the coastline.

By the end of the Pliocene, the climate was very similar to that of the modern-day, although it was still wetter than present (Martin 2006). The following epoch, the Pleistocene (2.4-0.011 mya), was characterised by repeated glacial and interglacial cycles of cold and dry, and warm and wet conditions, respectively. Sea level fluctuated during this time, with the highstands (i.e. interglacial periods) no more than 6 meters above present sea level (Ward 1985; Murray-Wallace 2002), and the lowstands (i.e. glacial periods) up to 40-50 meters below present sea level (Byrne *et al.* 2008). It was throughout the Late Pliocene/Early Pleistocene that the ancestral N7 was distributed across the Mary and Maroochy River catchments, before diverging during the Early Pleistocene (~1.2 mya) to give rise to *T. glypticus* and *T.* sp. nov. 2. In the same process that was described by Horwitz (1988a) and Burnham (2014) for other crayfish from the burrowing clade, it is likely that the ancestral N7 followed the shifting coastline during periods of sea level fall, leaving behind isolated populations (Figure 5.12a). As sea level rose again, the coastal populations shifted in response and subsequently came into contact with those populations that had remained *in situ* (Figure

5.12b). As the sea level fluctuated repeatedly during the Pleistocene this would have occurred a number of times and, as hypothesised by Horwitz (1988a), if sufficient divergence had occurred then two species could have resulted; a coastally adapted species (i.e. *T. glypticus*) and an inland resident species (i.e. *T.* sp. nov. 2). It is worth noting that this is the only example within *Tenuibranchiurus/Gen. nov.* entirely consistent with the model proposed by Horwitz (1998a). However, a similar process (i.e. contraction and expansion of populations in coastal habitats in response to sea-level fluctuations) seems to have resulted in divergence parallel to the coastline in the remaining species, rather than at right angles to it.

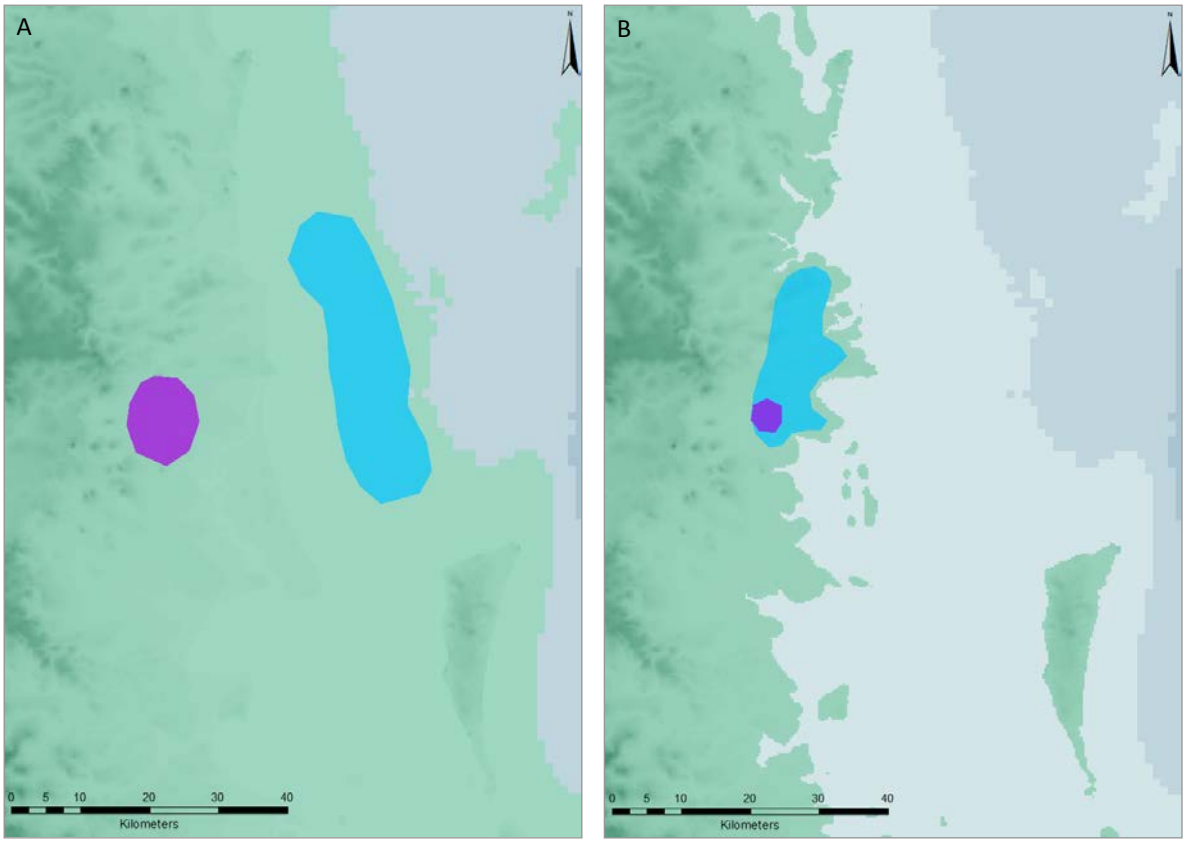


Figure 5.12. Hypothetical distribution of *T. glypticus* (blue) and *T.* sp. nov. 2 (purple) during fluctuating sea level of the Late Pliocene and Early Pleistocene. Low sea level in (A) is shown at -50 meters below present, and high sea level in (B) is at +6 meters above present. For sources of GIS data see section 2.3. Note: the islands did not form until the late Pleistocene and should not be considered as part of the coastline.

The fluctuating climate throughout the Pleistocene drove sea level change and, with an increasing trend towards aridity, strongly influenced the current distribution of species within both genera. Although the overall trend during this epoch was a decrease in precipitation, up until approximately 120 thousand years ago (kya) it was still wetter than today (Martin 2006). At approximately 120 kya, global sea level was ~6 m above present, but falling to ~70 m below present level by 50 kya (Rohling *et al.* 1998). At 20 kya the sea level was at its lowest during the Pleistocene, reaching between 120-150 m below the present level (Ferland *et al.* 1995; Rohling *et al.* 1998; Murray-Wallace *et al.* 2005; de Bruyn and Mather 2007). This period also represents the last glacial period, which was characterised by particularly arid and harsh conditions (Martin 2006). Although globally sea level has risen since then, it has been estimated that as recently as 8 kya the sea level off eastern Australia was as much as 120 m below present, which was the last period of lowered sea level (Hughes *et al.* 1999; Page *et al.* 2006; Martin *et al.* 2014). Australia then experienced a rapid sea level rise, reaching approximately 1.5 m above present at 7.8 kya (Martin *et al.* 2014), to then gradually fall to reach the sea level seen today between 6-2 kya (Pickett *et al.* 1985; Martin *et al.* 2014). Specific to eastern Australia, during the Pleistocene and Holocene, sea level was higher than present on only three occasions and never more that +3 m in Qld and +5 m in NSW (Ward 1985; Murray-Wallace and Belperio 1991). This fluctuating eustatic rise and fall (sometimes on a rapid scale) has likely strongly contributed to the distributions and genetic diversity of *Tenuibranchiurus* and *Gen. nov.*.

Considering the fluctuations in sea level along the eastern Australian coast, an interesting aspect of the distribution of *Tenuibranchiurus* is that of all the islands in SEQ (Bribie, North Stradbroke, Moreton, and Fraser) only a single species occurs on a single island; *T. glypticus* on Bribie Island. Bribie Island represents the closest island to the mainland, hugging the coastline and separated by the relatively narrow Pumicestone Passage. The majority of the island is only ~5 meters above sea level (a.s.l.), with the highest point measured at ~12 meters a.s.l.; however, it is also very flat, creating poor surface drainage and thus large parts of the island are swampy or prone to inundation (Isaacs and Walker 1983), providing an ideal habitat for *Tenuibranchiurus*. The island is of Pleistocene origin (Jones *et al.* 1978), coinciding with the time estimated for the dispersal of the ancestor to *T. glypticus* across the Mary and Maroochy River catchments (the latter of which includes Bribie Island). As the Pumicestone Passage is shallow and narrow, dispersal across this area at periods of low sea level is highly likely, as evidenced by the presence of *T. glypticus*.

In light of the current distributions of other *Tenuibranchiurus* species, the question as to why they are not present on the other islands is raised. The distribution of the ancestral *T. glypticus* is thought to be within the southern region of the Mary and Maroochy River catchments with subsequent northwards expansion. Although Fraser Island was formed at approximately 700 kya (Longmore 1997), well after the inception of *T. glypticus*, it would still remain plausible that it could have been later colonised during the northward expansion. However, although Fraser Island lies to the north, the contemporary distribution of *T. glypticus* could suggest that a more inland dispersal route was used (rather than coastal), thus not bringing the species into contact with the island. Alternatively, it is also plausible that at some point *Tenuibranchiurus* did inhabit this coastal island but has since become extinct there. The isolated nature of island populations can prohibit metapopulation processes (at least over short-to-medium timescales), so that stochastic or biological processes could be responsible for there no longer being any *Tenuibranchiurus* populations on the other islands (Hanski 1998).

A similar situation to above could be proposed for *T.* sp. nov. 1; however, perhaps a more plausible explanation is related to the age of this species. *T.* sp. nov. 1 speciated approximately 2.9 mya, when it diverged from the ancestor to *T. glypticus*. It is hypothesised here that the ancestral distribution of *T.* sp. nov. 1 was similar to that seen today. Therefore, it is possible that between the period of speciation to the inception of Fraser Island, populations of *T.* sp. nov. 1 had become highly constrained by landscape characteristics and unable to disperse into this new area. This scenario is also the most likely reason that *T.* sp. nov. 3 is not found on Fraser Island, as it originated even earlier at ~6.6 mya, and for why *T.* sp. nov. 5 does not occur on North Stradbroke Island[6]. North Stradbroke has been estimated to be ~150 kya old, forming well after the origin of *T.* sp. nov. 5 (~8.5 mya) and likely not colonised due to habitat specificity and dispersal ability.

[6] Although *Tenuibranchiurus* (now recognised as *T.* sp. nov. 5) has previously been suggested to occur on this island (through anecdotal reports), extensive sampling by this study and other freshwater studies (e.g. Marshall *et al.* 2011; Page *et al.* 2012) have failed to locate any populations.

Contemporary phylogeographic structure is fairly congruent across all species from both genera. High haplotype diversity is evident for most species from both genera, and nucleotide diversity is relatively low, indicating that haplotypes are generally common within a species and vary little from each other. However, few haplotypes are shared between sampling locations, indicating that although haplotypes are closely related within a species they are usually unique to a particular population. This haplotype pattern is indicative of a spatially highly structured species, which would be expected in populations that have been isolated for significant periods of time, in species that have had many populations lost, and also where the number of individuals within populations has, at times, been low, which would result in low effective population sizes and the loss of rare alleles (Avise 2000). This is further evidenced by the high number of missing haplotypes within all multi-population species. While this would perhaps be expected in species with large and disjunct distributions (i.e. *T. glypticus*, *T.* sp. nov. 1, *Gen. nov.* sp. nov. 2), it was also evident in *T.* sp. nov. 4, which is comprised of three neighbouring populations. These haplotype patterns, in combination with both the disjunct distributions of some species and restricted distributions of the remaining species, are likely the result of cyclical population retractions and shifting distributions in response to changes in sea level and increased aridity altering available and accessible habitat. Additionally, altered habitat connectivity due to human land use has likely impacted the more disjunct species, potentially removing populations through extirpations and contributing to the geographic patterns seen today. It is evident that there is little to no contemporary connectivity between populations within species of either genus, making the occurrence of migration between populations highly unlikely.

6.0 Phylogeny and Divergence of the Burrowing Clade

6.1 INTRODUCTION

Although the monophyly of the Parastacidae has been strongly supported by a number of studies, the relationships between some of the parastacid genera remain unresolved. Of particular interest to this study is the phylogenetic relationships between the genera of the burrowing clade, which have been presented in a number of studies (e.g. Crandall *et al.* 1999; Sinclair *et al.* 2004; Schultz *et al.* 2007; Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014), usually as part of a larger phylogenetic investigation. Of those studies that have focussed specifically on this group of burrowing crayfish, most have employed multi-gene analyses with at least one nuclear gene. However, sample sizes utilised in those studies have generally been small and the data incomplete or disproportionate across genera. Additionally, in previous phylogenetic reconstructions very few sequences have been available for the genus *Tenuibranchiurus* and none for the proposed *Gen. nov.*. Therefore, a revision of the phylogeny of this burrowing clade is required, both in terms of resolving relationships between genera and for the purpose of obtaining accurate estimates of when they diverged from each other.

6.1.1 Phylogenetic Inferences

Previous phylogenetic reconstructions have not been able to resolve the position of some of the burrowing genera (i.e. Crandall *et al.* 1999; Schultz *et al.* 2007; Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014) (Figure 6.1). The most recent study of Burnham (2014) using the same gene regions as this study (i.e. 16S, GAPDH; Figure 6.1a) could not resolve the relationship between *Engaewa* and *Engaeus sensu stricto* unless *E. lyelli* was removed from the phylogeny, where a sister-relationship was then weakly supported. The study of Toon *et al.* (2010) used different gene regions than this study has utilised but also could not resolve the placement of *E. lyelli*, and additionally did not support a sister-relationship between *Tenuibranchiurus* and *Geocharax* which has previously been suggested (Figure 6.1b). Schultz *et al.* (2009) was again not able to resolve the relationship between *Engaewa*/*Engaeus sensu stricto*/*E. lyelli* (using both 16S and GAPDH), although they did provide support for the relationship between *Tenuibranchiurus* and *Geocharax* (Figure 6.1c). Schultz *et al.* (2007) is the only study to give support to the placement of both *Engaeus sensu stricto* and *E. lyelli*; however, this study was based on a single gene (16S) and did not include the genus *Engaewa* and is therefore of limited usefulness (Figure 6.1d). Finally, the study of

Crandall *et al.* (1999) provided support for the placement of all genera except *Engaewa* and the sister-relationship between *Tenuibranchiurus* and *Geocharax*; however, the tree provided was un-rooted and one of the *Engaewa* sequences was erroneous, most likely representing a North American crayfish (see Burnham 2014), and therefore the results of Crandall *et al.* (1999) should be interpreted with considerable caution (Figure 6.1e). Only two of these previously outlined studies could determine which node was the ancestral/basal node, but the results were conflicting, likely due to the different genera included in the analyses (*Engaewa* in Toon *et al.* (2010), *E. lyelli* in Schultz *et al.* (2007)).

6.1.2 Estimating Timing of Divergence

Previous estimates of the dates of phylogenetic splitting between and within genera of the burrowing clade vary substantially. The most relevant and comparable studies are those of Toon *et al.* (2010) and Schultz *et al.* (2009), as they use representatives from all of the burrowing genera and utilise similar gene regions. Although both of these studies used a Bayesian approach to determine divergence dates, the study of Toon *et al.* (2010) utilised a multi-gene approach and employed the program MULTIDIVTIME with fossil data used to calibrate various nodes within their phylogeny (which contained all Parastacidae genera), whereas the single gene analysis of Schultz *et al.* (2009) employed the program BEAST and calibrated the analysis using published mutation rates for the 16S gene fragment in crabs, with the analysis restricted to the burrowing clade genera.

The dates estimated by Toon *et al.* (2010) suggest that the genera are ancient in origin (originating during the Cretaceous), whereas Schultz *et al.* (2009) provided significantly more recent estimates (originating during the Eocene). Although only a subset of the burrowing clade was examined, the analyses of Burnham (2014) somewhat support the dates suggested by Toon *et al.* (2010), as the divergence of *Engaewa* was estimated to have occurred ~122 mya compared to the estimates of ~124 mya by Toon *et al.* (2010) versus ~40 mya by Schultz *et al.* (2009). Additionally, fossilised parastacid burrows considered to most closely resemble those of *Engaeus* have been dated to ~116-106 mya (Martin *et al.* 2008), confirming that the earlier estimates of Burnham (2014) and Toon *et al.* (2010) are plausible, as strongly burrowing crayfish occurred during this same period.

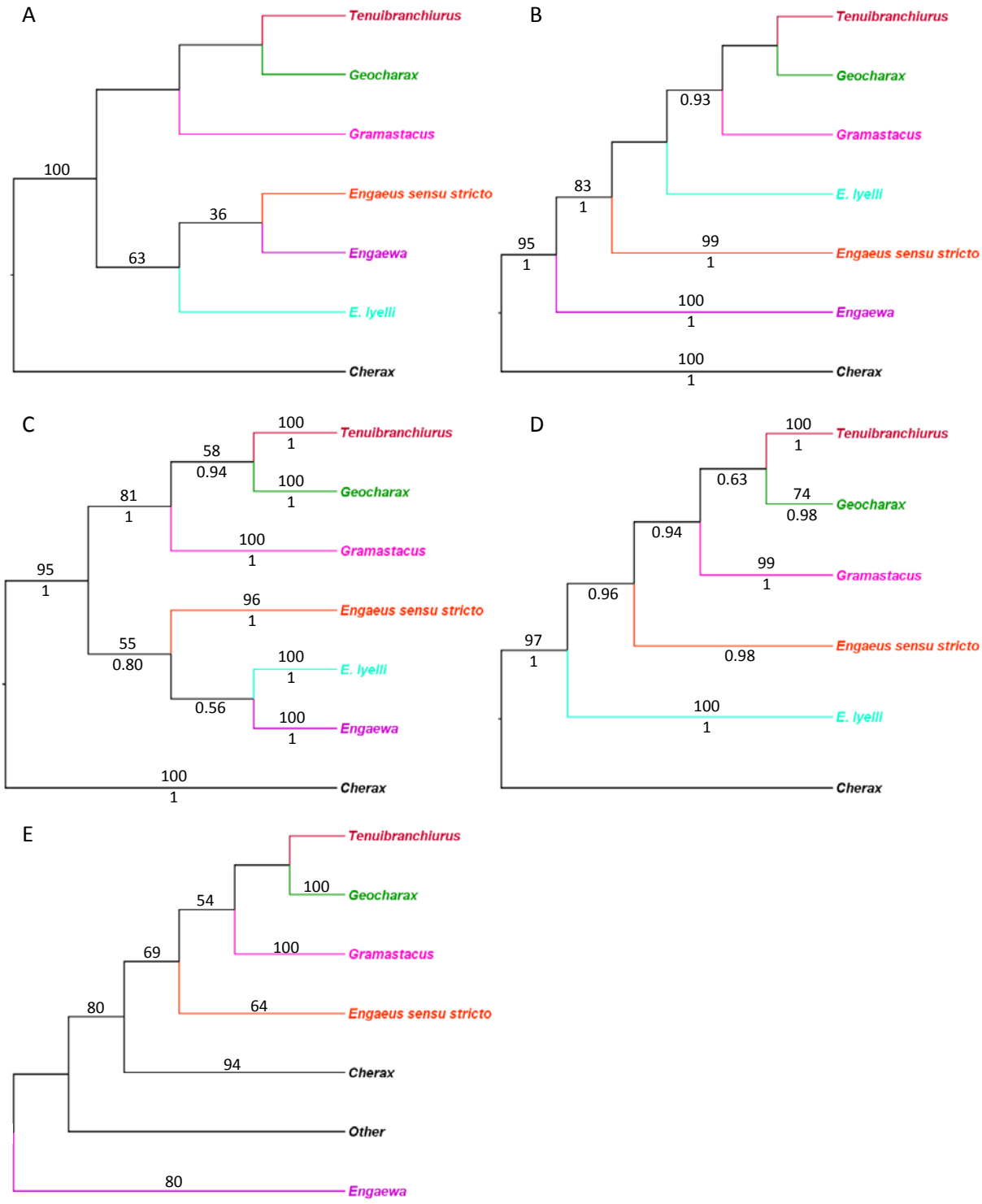


Figure 6.1. Alternative phylogenetic hypotheses of the relationship between the genera of the burrowing clade from (A) Burnham (2014), (B) Toon *et al.* (2010), (C) Schultz *et al.* (2009), (D) Schultz *et al.* (2007), and (E) Crandall *et al.* (1999). All phylogenies are transformed to show topology, with branch lengths not indicative of time or mutation rates. Support values are shown where they were given in the studies, with bootstrap values above and posterior probabilities below branches.

6.2 CHAPTER AIM

While acknowledging the limited scope of some of the previous studies on the burrowing clade genera, it is somewhat surprising that the phylogenetic positioning of a number of genera are still not resolved; particularly in light of the fact that the majority of discrepancies occur between *Engaewa*, *Engaeus sensu stricto*, and *E. lyelli*, of which the former two are probably the most extensively studied and thoroughly sampled within the clade. Additionally, studies investigating the timing of divergence within the parastacids that have included the burrowing clade have presented conflicting results, with estimates of some of the most ancient divergences within the clade up to ~80 million years apart (see Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014). Therefore, the purpose of this chapter is to utilise all available molecular data (obtained from both this and previous studies), in order to clarify the phylogeny and timing of divergence of the burrowing clade genera. This will allow the phylogenetic positioning of *Tenuibranchiurus* and *Gen. nov.* to be confirmed, and thus their relationship to the other burrowing clade genera, both in terms of phylogeny and age.

6.3 METHODS

6.3.1 Burrowing Clade Phylogenetic Reconstruction

A full phylogeny of the burrowing clade genera was reconstructed using the combined 16S and GAPDH sequences from this study and from GenBank, as these genes represent the most complete data available. The final alignment included representative taxa from all of the burrowing genera where both 16S and GAPDH sequences were available, and with sequences from *Cherax robustus* Riek also included as an outgroup taxon. For this analysis, a ML tree was constructed using RAxML as described in Chapter 4 (section 4.3.1).

6.3.2 Species Tree Construction

A species tree using all available 16S and GAPDH sequences for all of the burrowing clade genera was created using *BEAST. The sequences representing *Tenuibranchiurus* and *Gen. nov.* were those analysed in Chapter 4. For the remainder of the burrowing genera (i.e. *Gramastacus*, *Geocharax*, *Engaeus sensu stricto*, *E. lyelli* and *Engaewa*), all available sequences for the gene regions were retrieved from GenBank and Burnham (2014) in addition to those sequenced in this study. All sequences were collectively aligned using MAFFT v. 7.058 (Katoh and Standley 2013). Sequences that were problematic for the alignment (e.g. causing ambiguous gaps in the alignment, many nucleotide sites designated as N, etc.) were removed from the analysis as their accuracy could not be verified without chromatograms. In the final alignment, all currently known species of *Gramastacus* (two spp.), *Geocharax* (four spp.), and *Engaewa* (seven spp.) were represented, as well as 30 of the 34 *Engaeus sensu stricto* species and specimens of *E. lyelli*. Sequences from *C. robustus* were also included in the alignment as an outgroup taxon. All species were represented in the 16S alignment, but blank sequences needed to be inserted into the GAPDH alignment for species where sequence data were not available. As species were represented by multiple sequences across genes, sequences were grouped into their corresponding species using the 'trait' feature.

An uncorrelated lognormal relaxed clock was used as an alternative to a strict clock model as it allows the analysis to account for non-clocklike evolution and rate heterogeneity among branches. An uninformative uniform prior (0-100) was used as the ucld.mean for GAPDH as no substitution rate is available for this gene, and an informed normal prior for the ucld.mean for 16S was used (mean=0.003575, stdev=4.719×10^{-4}), following Chapter 5 (section 5.3.3). All ucld.stdev were adjusted to represent a plausible distribution (exponential, initial value=2,

mean=0.5) and the ploidy type specified. The evolutionary models were recalculated using jModeltest for each gene using the full data set and, selecting from the models available in *BEAST, were both assigned as GTR (+G for 16S, +I+G for GAPDH).

Two runs were performed using random starting trees, each with a total of 50 million generations sampled every 1000 generations, and an empty alignment was also run. The runs were checked for convergence, the ESS values examined, and the burnin determined using the program Tracer. The post-burnin trees were combined and resampled at a frequency of 2000 due to their large size, by using LogCombiner, then annotated using TreeAnnotator and visualised using Figtree. Using the species phylogeny created by *BEAST, divergence times were estimated for the entire tree based on the evolutionary rates entered for 16S and estimated for GAPDH.

6.4 RESULTS

6.4.1 Phylogeny of the Burrowing Clade Genera

The genera of the burrowing clade formed a monophyletic grouping, with the split between these genera and the *Cherax* species highly supported (Figure 6.2; BS 100%). All genera within the burrowing clade also formed highly supported monophyletic clades (BS ≥98%). The relationships between the four most recently diverged genera (i.e. *Tenuibranchiurus, Gen. nov.*, *Geocharax*, and *Gramastacus*) were highly supported (BS ≥94%); however the relationships between the remaining three genera (*Engaewa*, *Engaeus sensu stricto*, *E. lyelli*) were unclear. A sister-relationship between *Engaewa* and *Engaeus sensu stricto* was suggested, though not supported (BS 44%), and the node leading to *E. lyelli* was placed basal to this, although this again was not well supported (BS 27%).

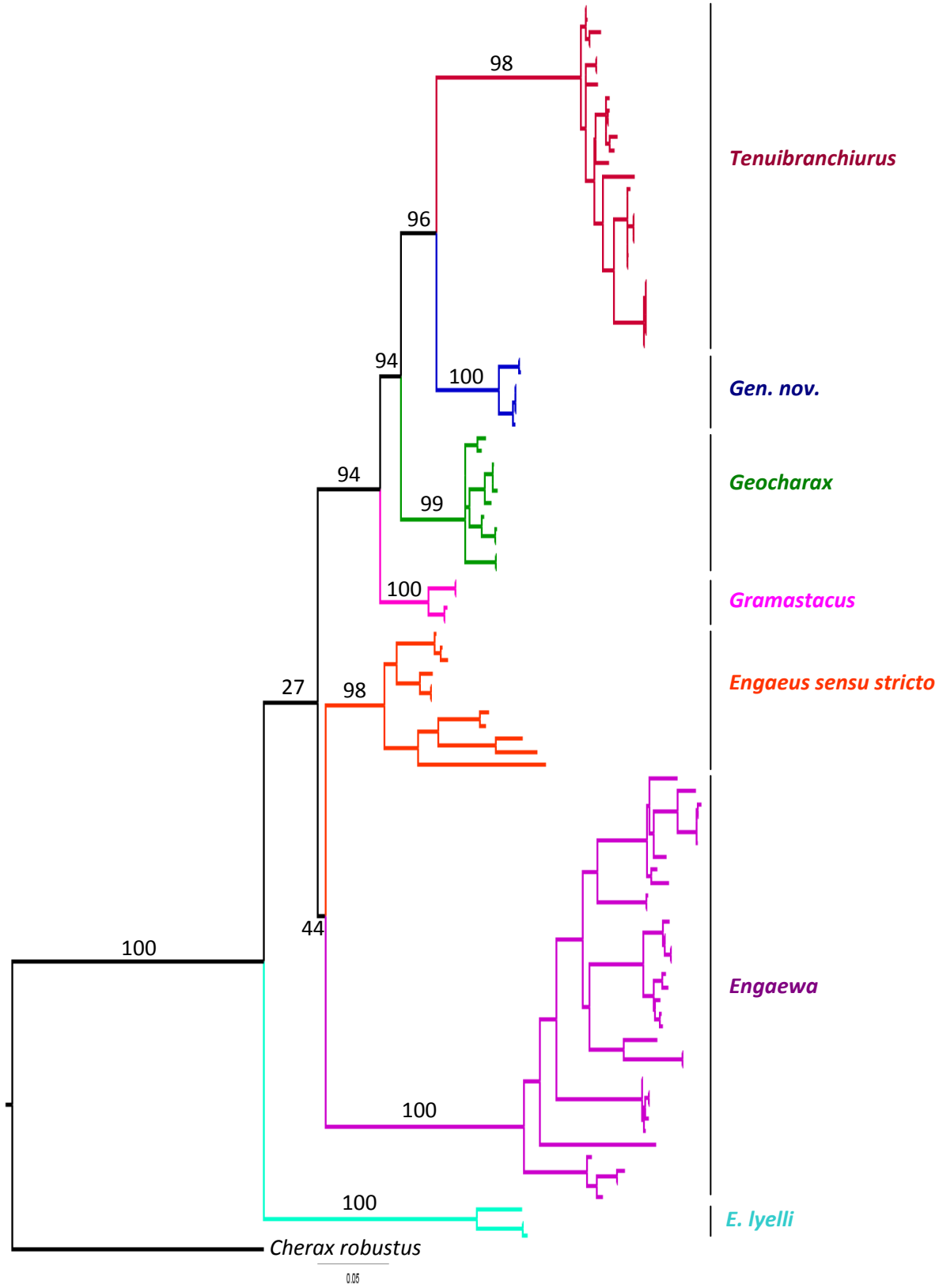


Figure 6.2. Combined 16S and GAPDH maximum likelihood tree showing the relationships between genera of the burrowing clade. Bootstrap support values for major branches are given, and species within each genus are grouped by colour. *E. lyelli* is considered to be a distinct genus (after Schultz *et al*. 2009).

6.4.2 Timing of Divergence of the Burrowing Clade Genera

Based on the *BEAST phylogenetic reconstruction, all genera were considered monophyletic with high nodal support (Pp ≥0.94; Figure 6.3), except for *E. lyelli* as this was represented by only a single species. All sister-relationships were supported except between *Tenuibranchiurus*/*Gen. nov.* (Pp 0.64) and *E. lyelli*/*Engaeus sensu stricto*/*Engaewa* (Pp 0.41). However, the sister-relationship between *Tenuibranchiurus* and *Gen. nov.* was supported in previous multi-gene phylogenies throughout this study (see section 6.4.1 above (BS 96%) and Chapter 4, Figures 4.7 and 4.8 (BS 97%, Pp 1)). The phylogeny presented in section 6.4.1 could also not resolve the relationship between *E. lyelli*/*Engaeus sensu stricto*/*Engaewa*.

Based on the *BEAST divergence times, the most recent common ancestor to the burrowing clade, and also the direct ancestor of *Engaewa*, originated ~131 mya (node 2; Figure 6.3, Table 6.1). Radiation of the remaining burrowing genera commenced shortly after, with the ancestral *E. lyelli*, *Engaeus sensu stricto*, and *Gramastacus* established during the Cretaceous (nodes 3-5; Figure 6.3). Despite the early origin of *Engaewa*, diversification among the extant lineages did not occur until the Palaeocene, continuing through to the Oligocene (Figure 6.3). *Engaeus sensu stricto* followed a similar pattern, though with additional recent diversification during the Miocene and Pliocene. Although established during the Cretaceous, the diversification of *Gramastacus* is comparable to *Geocharax* (Palaeocene origin), *Gen. nov.* and *Tenuibranchiurus* (Eocene origin), as most radiations within these genera occurred during the Miocene/Pliocene period (Figure 6.3, Table 6.1).

Table 6.1. Comparison of divergence time estimates within the burrowing clade (BC) and their associated 95% confidence intervals (reported as ranges where available) across multiple studies.

	Node	This study[a] (16S & GAPDH)		Toon *et al.* 2010[b] (16S, COI, 18S, 28S)		Schultz *et al.* 2009[c] (16S)	
		Age (mya)	Range (mya)	Age (mya)	Range (mya)	Age (mya)	Range (mya)
1	*Cherax* vs. BC	152	224-98	134	-	45	-
2	*Engaewa*	131	185-88	124	150-97	40	-
3	*E. lyelli*	116	163-80	100	-	37	55-20
4	*Engaeus sensu stricto*	95	137-64	110	-	32	48-17
5	*Gramastacus*	73	104-47	90	-	25	39-13
6	*Geocharax**	58	83-38	75	-	20	32-10
7	*Tenuibranchiurus* vs. *Gen. nov.*	50	72-31				

*As *Gen. nov.* is a newly proposed genus from this study, previous studies have estimated the split between *Geocharax* and *Tenuibranchiurus* rather than *Tenuibranchiurus* and *Gen. nov.*. As such, these cells are combined in the summaries of other studies.
Dates estimated using the program: a. *BEAST, b. MULTIDIVTIME, c. BEAST

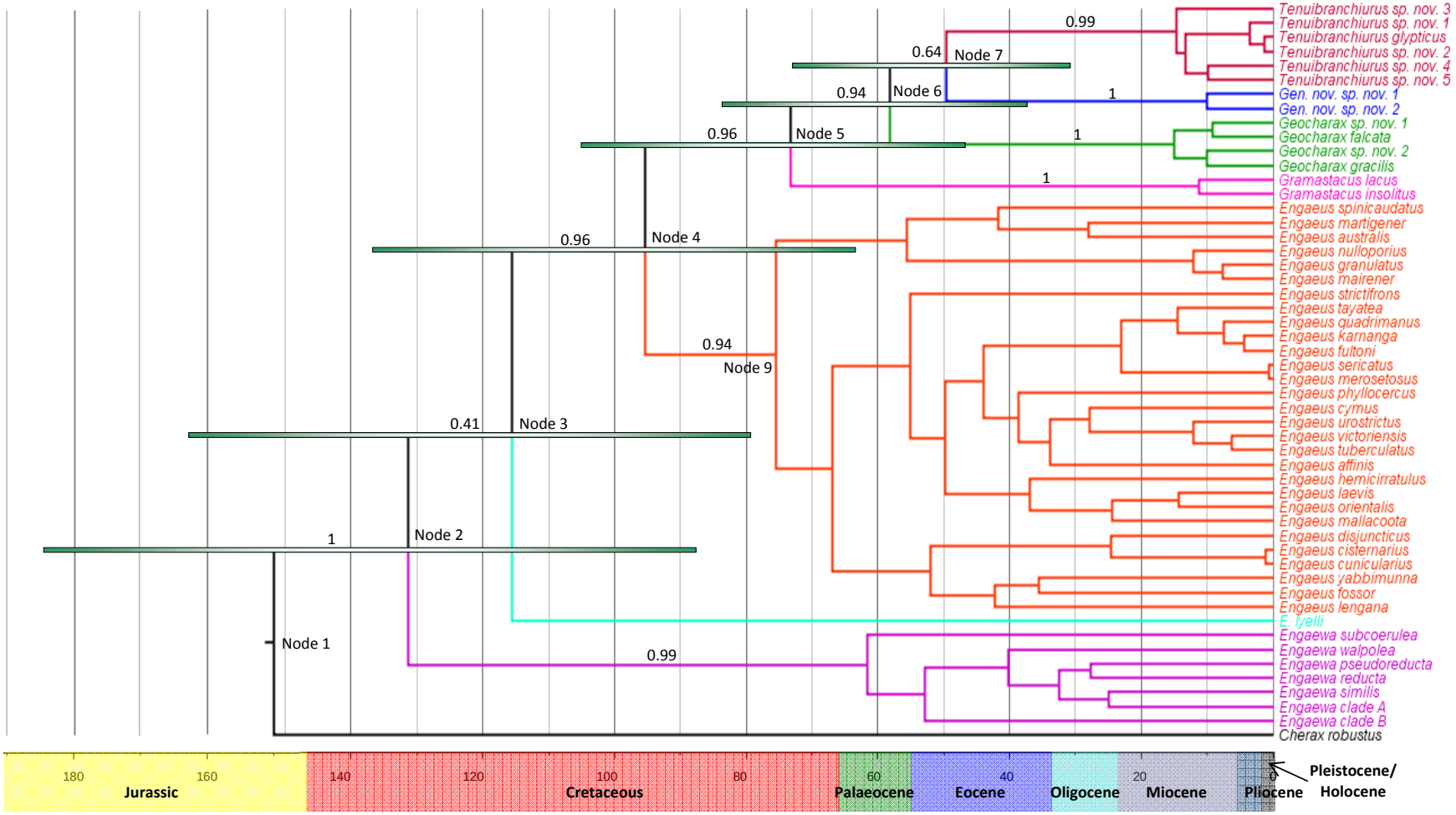


Figure 6.3. Divergence estimates calculated by *BEAST for all burrowing clade genera using 16S and GAPDH genes. The 95% highest posterior densities (HPD) are shown using node bars for the divergence events between genera. Divergence times are shown as millions of years ago with geological epochs/periods indicated. Bayesian posterior probabilities are only shown for major branches.

6.5 DISCUSSION

6.5.1 Phylogeny of the Burrowing Clade Genera

The monophyly of the burrowing clade has previously been supported by morphological and molecular data (Horwitz 1988b; Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014). This study further confirms this, with the presence of the burrowing clade recovered in 100% of the iterations. Furthermore, the monophyly of each genus is supported (BS ≥98%), including the newly proposed genus *Gen. nov.*.

This study found strong support for the placement and relationship between all of the most recently derived genera (i.e. *Gramastacus*, *Geocharax*, *Gen. nov.*, and *Tenuibranchiurus*), but could not resolve the relationship between *Engaewa*/*Engaeus sensu stricto*/*E. lyelli*. Although the node leading to *E. lyelli* was suggested to be basal to the rest of the burrowing clade, this arrangement was not statistically supported. If the unsupported branches were to be collapsed, a polytomy with four branches would result; one from which the most recently derived genera would branch, and the remaining three representing *Engaewa*, *Engaeus sensu stricto*, and *E. lyelli*.

This study cannot resolve all phylogenetic relationships within the burrowing clade and further highlights the uncertainty of the relationship between *Engaewa*, *Engaeus sensu stricto*, and *E. lyelli*. That an unclear association between these genera is repeatedly found suggests that perhaps a near-simultaneous divergence happened between them, thus obscuring the true relationship even when a comprehensive data set is utilised. This study does, however, clarify the association between *Tenuibranchiurus* and *Geocharax*. Although a supported sister-relationship was found between these two genera by Schultz *et al.* (2007) and Schultz *et al.* (2009), the remaining studies did not support this. The recognition of the new genus *Gen. nov.* clarifies the phylogeny of this part of the burrowing clade, with *Tenuibranchiurus* and *Gen. nov.* representing sister-taxa and the youngest genera, with *Geocharax* placed as the next most closely related genus.

6.5.2 Timing of Divergence of the Burrowing Clade Genera

The results of this study were formulated using the program *BEAST, which is a modified version of BEAST used by Schultz *et al.* (2009)[7]. Although the estimates of Toon *et al.* (2010) were based on both mitochondrial and nuclear gene regions (as in this study), the nuclear gene regions differed to those employed here, the divergence estimates were calculated using a different Bayesian approach, and Toon *et al.* (2010) used fossils to calibrate the phylogeny, rather than mutation rates. In contrast, the analysis of Schultz *et al.* (2009) was based on a similar Bayesian approach to this study; however, only a single gene was analysed and therefore only a single mutation rate was used to calibrate the tree.

The dates estimated by this study and by Toon *et al.* (2010) suggest that the genera are ancient in origin, whereas Schultz *et al.* (2009) provided significantly more recent estimates. Although the estimates of Toon *et al.* (2010) are slightly different to those from this study, they fall well within the range estimated here, whereas those of Schultz *et al.* (2009) are outside even the youngest estimated bounds of this study. The majority of mean node ages between genera were estimated in this study to have occurred during the Cretaceous period except for *Geocharax* (Palaeocene), *Tenuibranchiurus* (Eocene), and *Gen. nov.* (Eocene). This is similar to the results of Toon *et al.* (2010) where all genera were estimated to have diverged during the Cretaceous, but in contrast to the estimates of Schultz *et al.* (2009) which fall between the Eocene and Miocene. Additional support for a more ancient burrowing clade origin is also given by Burnham (2014) and Martin *et al.* (2008).

Using the most comprehensive data set of burrowing clade genera and species to date, in combination with a multi-gene analysis using one of the most advanced dating software packages, has allowed the timing of divergence within the burrowing clade to be clarified. The estimates align with those of Toon *et al.* (2010) and Burnham (2014), suggesting that the most recent common ancestor to the burrowing clade occurred during the Cretaceous. The three oldest genera (*Engaewa*, *E. lyelli*, and *Engaeus sensu stricto*) also originated during the Cretaceous followed closely by *Gramastacus*, with the three youngest genera (*Geocharax*, *Tenuibranchiurus*, and *Gen. nov.*) originating during the Palaeocene and Eocene.

[7] In *BEAST, the roots of individual gene trees are estimated (in this case, five gene trees) using estimated mutation rates, with the trees then combined using the multispecies coalescent to estimate the species tree root, rather than through concatenation as used in BEAST (Heled and Drummond 2010).

6.6 SUMMARY

Although not all phylogenetic relationships within the burrowing clade could be resolved with statistical support, it would appear that there was near-simultaneous divergence of the oldest three genera (*Engaewa*, *E. lyelli*, *Engaeus sensu stricto*) during the Cretaceous, followed by subsequent divergence events of *Gramastacus*, *Geocharax*, *Gen. nov.*, and *Tenuibranchiurus*. Only limited data have previously been available for *Tenuibranchiurus* and until this study *Gen. nov.* was unknown and, therefore, the inclusion of the most complete data available for these two genera, in addition to the inclusion of all available genetic data for species within the burrowing clade, makes this study the most comprehensive to date. Thus, with the clarification of the phylogeny and timing of divergence of the burrowing clade genera, accurate biogeographic inferences surrounding this clade can now be made.

7.0 Biogeography of the Burrowing Clade

7.1 INTRODUCTION

The freshwater crayfish are an old and ecologically diverse group, estimated to have diverged from their marine ancestor (the clawed lobsters; Nephropoidea) approximately 280 mya (Porter *et al.* 2005). They dispersed throughout the freshwater systems of the super-continent Pangaea during the remainder of the Permian and into the Triassic period, after which they were subsequently divided into the two freshwater crayfish superfamilies, coinciding with the splitting of Pangaea into Laurasia and Gondwana (forming Astacoidea and Parastacoidea, respectively) approximately 185 mya (Crandall *et al.* 2000b; Crandall and Buhay 2008). The presence of fossilised crayfish and crayfish burrows in Permian and Triassic deposits (Hasiotis and Mitchell 1993; Bedatou *et al.* 2008; Martin *et al.* 2008), and morphological and phylogenetic estimates supporting their monophyletic origin (Ortmann 1902; Hobbs 1974; Crandall *et al.* 2000b; Porter *et al.* 2005) confirms this ancient origin.

The Southern Hemisphere superfamily Parastacoidea is comprised of a single family, Parastacidae, which consists of 15 extant genera. Divergences within the parastacids are estimated to have occurred prior to Gondwana fragmenting into the continents seen today (Sanmartin and Ronquist 2004); a supposition supported by genetic analyses which suggest that the monophyletic grouping of the Parastacidae indicate they are much older in origin than the Northern Hemisphere crayfish (Crandall *et al.* 2000b; Crandall and Buhay 2008). The sequence of events during the fragmentation of Gondwana into the continents seen today commenced ~165 mya and are generally agreed upon (see Storey 1995; Sanmartin and Ronquist 2004; Upchurch 2008). This sequence started with the rifting of India/Madagascar, Antarctica/Australasia, and Africa/South America, in a process which was completed approximately 140 mya. These landmasses then continued to separate into today's continents, with Africa and South America separating by ~110-95 mya, India and Madagascar by ~88-84 mya, and lastly Antarctica and Australasia by ~35 mya (Toon *et al.* 2010).

Of the 15 described extant parastacid genera, ten occur on the Australian continent, of which nine are endemic (i.e. *Astacopsis* Huxley, *Engaeus*, *Engaewa*, *Euastacus*, *Geocharax*, *Gramastacus*, *Ombrastacoides* Hansen and Richardson, *Spinastacoides* Hansen and Richardson, and *Tenuibranchiurus*), as *Cherax* are distributed widely over both Australia and New Guinea. In addition to these genera is the newly discovered genus from this study (*Gen. nov.*) and the yet to be described genus *E. lyelli* of Schultz *et al.* (2009). The Parastacidae have their centre of diversity in the SEC of Australia, with all but 11 described species occurring east of central Australia (Whiting *et al.* 2000). Additionally, fossil records of an extinct species of crayfish (*Palaeoechinastacus australianus* Martin) have been documented from south-eastern Australia and dated to ~106-116 mya (Martin *et al.* 2008), indicating that this region has long been occupied by the parastacids. Studies on extant genera suggest that the high species richness within the parastacid genera is the result of a combination of vicariance, dispersal, and isolation events (e.g. Horwitz 1988a; Munasinghe *et al.* 2004a; Nguyen *et al.* 2004; Ponniah and Hughes 2004; Shull *et al.* 2005; Schultz *et al.* 2009; Toon *et al.* 2010).

7.2 CHAPTER AIM

Although the biogeography of the burrowing clade genera has been explored in part by other studies (see Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014), the estimates of divergence times within the clade have been reported to have occurred at vastly different times and thus are inconsistent. In addition to being based on conflicting data, these studies focus on either a single genus (i.e. Burnham 2014), form only a broad and brief discussion on the biogeographic processes across the burrowing clade (i.e. Schultz *et al.* 2009), or are a general discussion on Parastacidae as a whole (i.e. Toon *et al.* 2010). Therefore, in light of the clarification provided by the results of Chapter 6, the aim of this chapter is to use these data (as they represent arguably the most accurate and comprehensive burrowing clade phylogeny and dating estimates available), in combination with geological data from this time period obtained from the literature, to produce the first complete biogeographic model of the evolution of this group of burrowing crayfish, both at a generic and specific level.

7.3 METHODS

7.3.1 Ancestral Range Reconstruction

Ancestral distributions of each of the burrowing clade genera were reconstructed using the program RASP v. 2.1 (Yu *et al.* 2010, 2013) by BBM analysis. As in Chapter 5 (section 5.3.3), the geographic distributions for ancestral nodes and dispersal, vicariance, and extinction events were estimated (Ronquist 2004) from the combined runs.

For the BBM analysis, the 25,000 trees produced by *BEAST (see Chapter 6, section 6.3.2) were loaded, as well as the condensed tree and the following parameters set; 1 million cycles, 30 chains, sampling every 100 cycles, a maximum of 7 areas, null root distribution, and F81+G model. The distributions for each of the tips were assigned *a priori* from a total of seven geographical regions adapted from Unmack (2001); (A) south-east Qld (SEQ), (B) north-eastern NSW (NEN), (C) south-eastern Australia (SEA), (D) Tasmania, (E) King Island, (F) Flinders and Cape Barren Islands, and (G) Western Australia (WA). These areas represent the present day regions through which the burrowing clade genera are distributed (Figure 7.1).

7.4 RESULTS

7.4.1 Historical Biogeography of the Burrowing Clade Genera

Based on the divergence times estimated in Chapter 6 and the BBM analysis, the following inferences were made about the biogeography of the burrowing clade. The BBM analysis suggested the ancestor to the burrowing clade most likely occurred in SEA (C; 47.07% probability). From there, the ancestral burrowing clade genus dispersed into WA (G; 99.07% probability), where a vicariance event resulted in divergence, giving rise to the ancestral *Engaewa* (node 8) during the Cretaceous (Figure 7.3). Diversification within this ancient genus occurred during the Palaeocene through to the Oligocene (Figure 7.2). The genus that remained in SEA (node 3: C; 97.46% probability) subsequently diverged to become *E. lyelli* and the ancestor at node 4, both of which were distributed within SEA (Figure 7.3). One dispersal event was estimated at node 4, with this resulting in the ancestor at node 5 (within SEA: C; 93.46% probability) and the ancestral *Engaeus sensu stricto* (node 9) distributed across both SEA and Tasmania (CD; 36.91% probability). *Engaeus sensu stricto* followed a similar speciation pattern to *Engaewa*, as it began during the Cretaceous but underwent additional recent diversification during the Miocene and Pliocene (Figure 7.2).

Although no events were estimated, the ancestor at node 5 diverged to give rise to the ancestral *Gramastacus* (node 10) and the ancestor at node 6, both within SEA (C; 84.58% and 90.88% probability, respectively) (Figure 7.3). The event estimated at node 6 suggested that this genus expanded its range from SEA (C) to span across to NEN (B), where a vicariance event resulted in the ancestral *Geocharax* (node 11) located within SEA (C; 96.45% probability) and the ancestor at node 7 within NEN (B; 35.95% probability) (Figure 7.3). This ancestral genus would eventually give rise to the ancestral *Gen. nov.* and *Tenuibranchiurus* (nodes 12 and 13, respectively) during the Eocene.

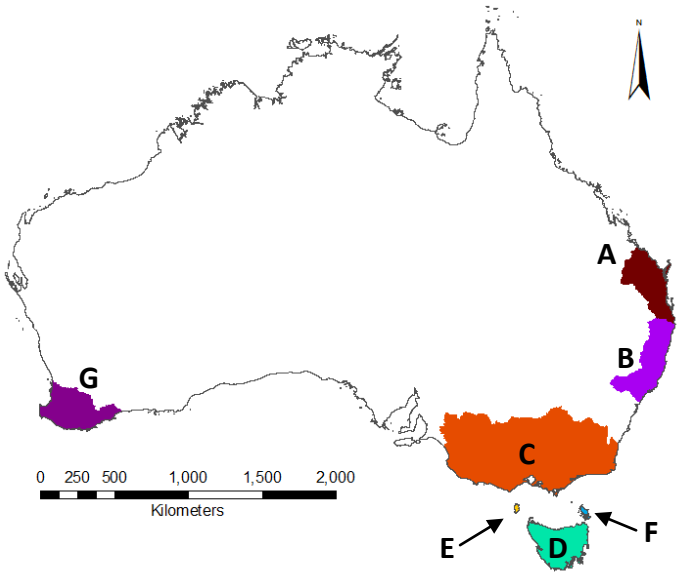


Figure 7.1. Biogeographic regions for determining the ancestral distributions of the burrowing clade genera. Regions are as follows: A=south-east Queensland, B=north-eastern New South Wales, C=south-eastern Australia, D=Tasmania, E=King Island, F=Flinders and Cape Barren Islands, G=Western Australia. For sources of GIS data see section 2.3.

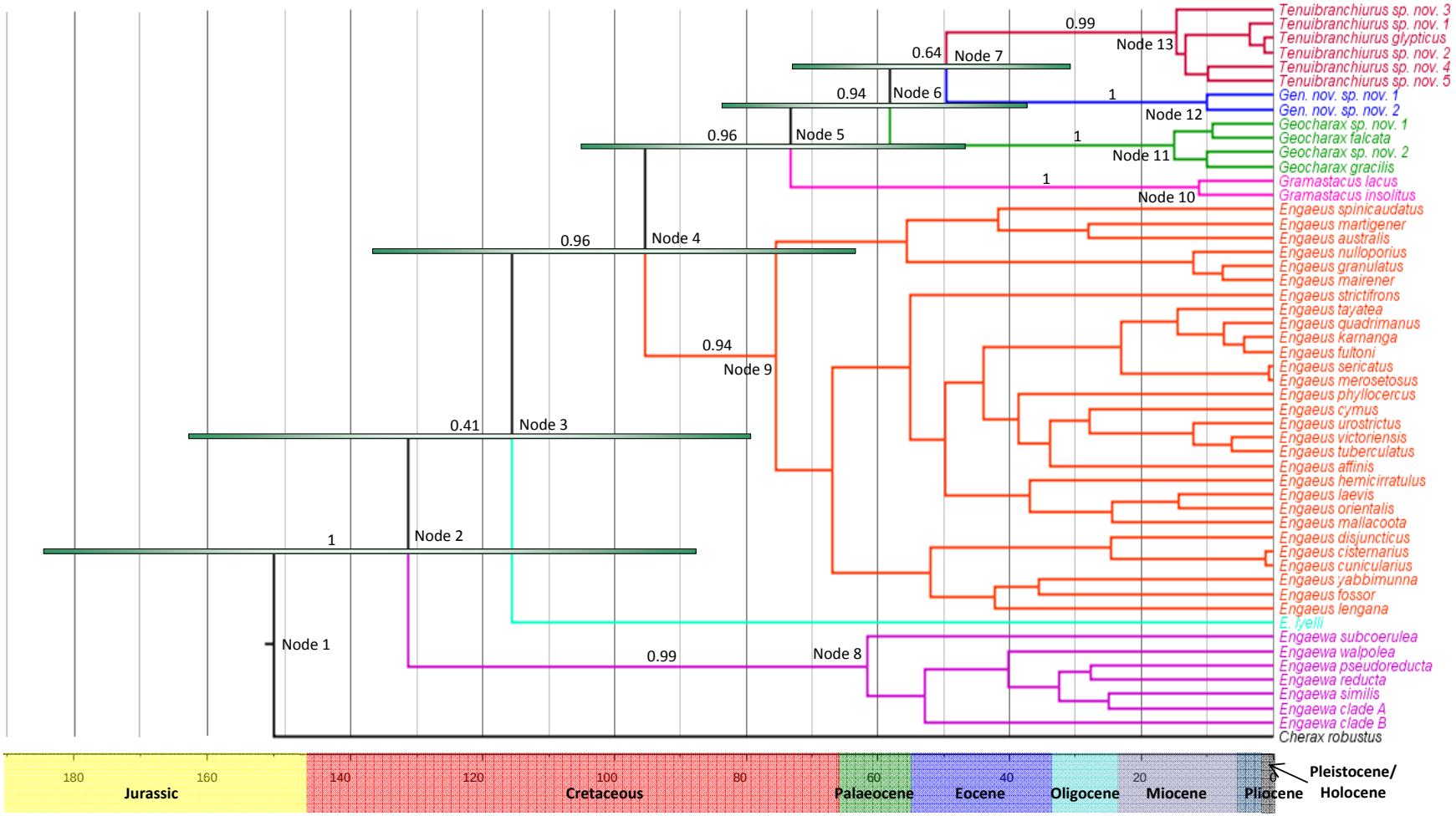


Figure 7.2. Divergence estimates calculated by *BEAST for all burrowing clade genera using 16S and GAPDH genes. The 95% highest posterior densities (HPD) are shown using node bars for the divergence events between genera. Divergence times are shown as millions of years ago with geological epochs/periods indicated. Bayesian posterior probabilities are only shown for major branches. Node numbers correspond with those in Figure 7.3. Figure modified from Figure 6.3.

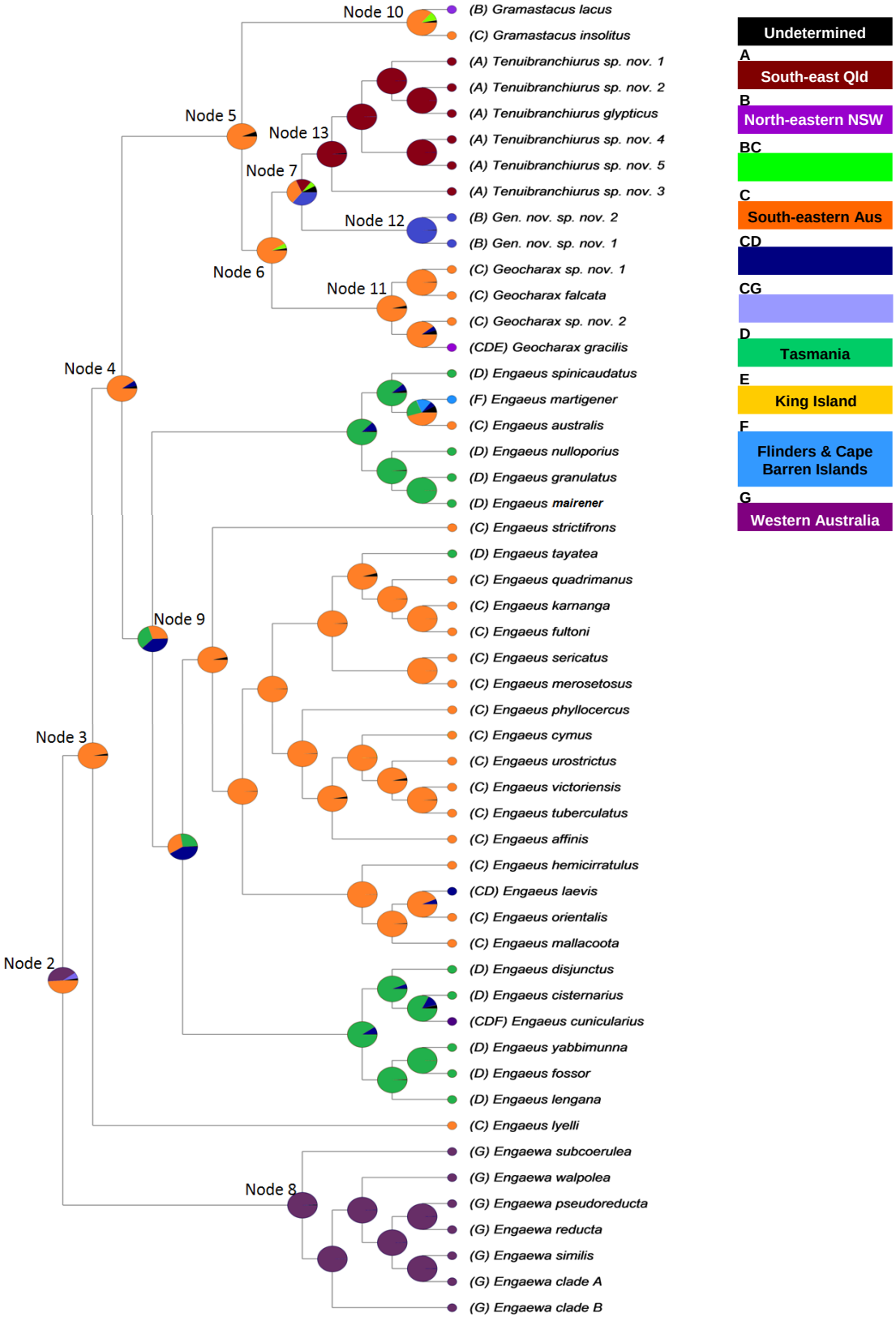


Figure 7.3. Estimated node ancestral distributions for the burrowing clade genera. Pie proportions and colour indicate the probability of the associated distribution shown in Figure 7.1. Phylogeny transformed to show topology, branch lengths not indicative of time or mutation rates. Node numbers correspond with those in Figure 7.2.

7.5 DISCUSSION

7.5.1 Historical Biogeography of the Burrowing Clade Genera

Before the proposed biogeographic explanation of the evolution of the burrowing clade is discussed, it should be noted that the timescale over which these processes occurred is vast and, therefore, the inferences provided herein are a best estimate of the events that occurred based on ecological, biological, molecular, and historical climate data. The processes driving diversification of the burrowing clade genera have only been briefly discussed previously by Schultz *et al.* (2009). The biogeographic discussion of their study is general in nature, with few correlations drawn between divergence events and specific geographic events. Although all genera are mentioned, the majority of the discussion is formed around the genus *Engaeus sensu stricto* and the species within. Additionally, the estimates of timing of divergences of Schultz *et al.* (2009) do not agree with the results of this study, which is now the most comprehensive analysis of divergence dating for this group of crayfish. Therefore, the biogeographic inferences drawn in the study of Schultz *et al.* (2009) do not align with the epochs discussed here, making the combined discussion of this and Chapter 5 the most complete discussion of the biogeographic history of all of the genera of the burrowing clade[8]. Following the process of Chapter 5, throughout this discussion the ancestral lineages shown in Figure 7.3 will be referred to as an N followed by the corresponding node number.

Origin of the Burrowing Clade

Freshwater crayfish diverged from their marine ancestor in the Permian (~280 mya) during the existence of the super-continent Pangaea (Porter *et al.* 2005). When Pangaea split into Laurasia and Gondwana ~185 mya, the two superfamilies of crayfish were formed on each landmass (Astacoidea and Parastacoidea, respectively). Coinciding with this division, the climate of the area that now forms Australia entered an arid phase (Fawcett *et al.* 1994). At the beginning of the Middle Jurassic (~176 mya), the climate of the region reverted to humid conditions and high precipitation, with milder winters ensuing (Fawcett *et al.* 1994). These conditions continued until the Late Jurassic (~145 mya), during which Gondwana began to separate into three landmasses (India/Madagascar, Antarctica/Australasia, Africa/South America), in a process that spanned between 165-140 mya.

[8] As the biogeographic history of *Tenuibranchiurus* and *Gen*. nov. has been described previously in Chapter 5, it will not be presented again in this chapter.

During the Late Jurassic (~152 mya), the ancestor of the burrowing clade diverged from the ancestral *Cherax*. It is likely that the splitting of Gondwana, significant changes in climate at the early-mid Jurassic boundary, and fluctuating sea level throughout this entire period (see Haq *et al.* 1987) all contributed to the division of the two groups. The fracturing of Gondwana would have created additional coastlines and potentially increased features along the coastal margin, and an increasingly wet climate would likely have created new habitat both in type and extent, promoting dispersal of these freshwater crayfish. Additionally, fluctuating sea level and changing coastlines would have created barriers at various points in time. Increased dispersal opportunities as well as intermittent barriers could have promoted speciation and, through continued cessation of gene flow, this could quite plausibly have resulted in two distinct groups (i.e. *Cherax* and the burrowing clade). It is likely that ecological separation would have played a part in their formation, with a more burrowing-form arising either in response to periods of dry conditions or as a result of expansion into novel habitats (i.e. increased coastal margins).

Diversification of the Burrowing Clade

When Antarctica/Australasia separated from the other southern continents ~140 mya, the western margin of Australia was inundated (Quilty 1984). There was also a major marine transgression over much of Australia (~155-120 mya) (Frakes *et al.* 1987), resulting in the formation of the Eromanga Sea. This transgression formed large 'islands' over present-day Australia, with the south-west of Australia almost completely sundered from south-east Australia and Antarctica (Veevers 2004). It was during this time that the ancestral *Engaewa* diverged from the remainder of the burrowing clade (~131 mya). It is likely that the ancestor to the burrowing clade was widely distributed across the wet coastal margins of southern Australia, with the subsequent separation of the western portion of Australia (where *Engaewa* is now restricted to) and its extended isolation resulting in the divergence of this ancient genus (Figure 7.4). This has previously been suggested by Burnham (2014), who reported that the origin of *Engaewa* lies during the Cretaceous (~122 mya), potentially as a result of its isolation during the substantial marine division of Australia.

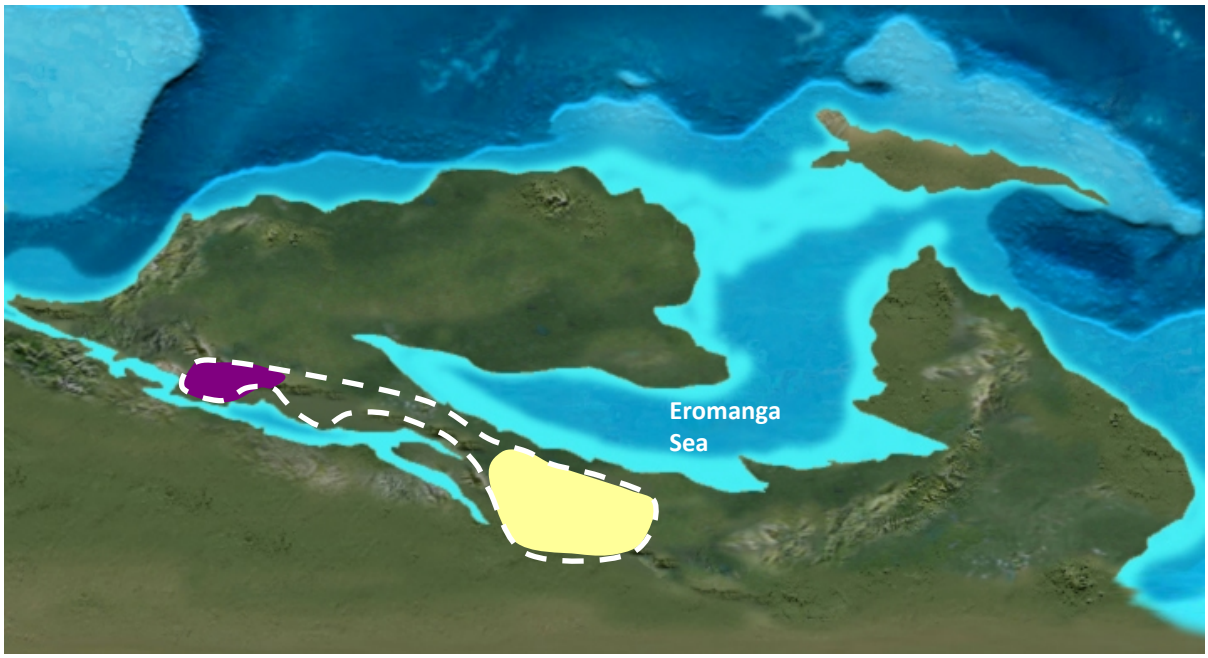


Figure 7.4. Hypothetical distribution of the ancestral burrowing clade genus (dashed line) and the subsequent divergence of *Engaewa* (purple) from ancestral N3 (yellow) during the Early Cretaceous (~122 mya). Figure modified from Blakey (2008).

During this same period, the ancestral *E. lyelli* also diverged from the rest of the burrowing clade (~116 mya). The Early Cretaceous was a period of cool and wet conditions (Quilty 1994), with the coldest climates in the southeast of Australia (Frakes 1997). As *E. lyelli* is monotypic (or only contains a small number of species; see Schultz *et al.* 2009) and has a restricted distribution, this suggests that it derived from either a single ancestral species with a restricted range, or the distribution of its ancestor was repeatedly reduced and genetic diversity lost. Although there is no current geological feature isolating *E. lyelli*, it is possible that both extensive volcanism along the southern Victorian basins (Quilty 1994) and fluctuating sea level led to the formation of barriers, contributing to the isolation of populations and leading to eventual cessation of gene flow between the ancestral *E. lyelli* and the ancestral N4 (Figure 7.5).

Figure 7.5. Hypothetical distribution of ancestral N3 (dashed line) and the subsequent divergence of *E. lyelli* (cyan) from ancestral N4 (yellow) during the Early Cretaceous (~116 mya). Triangle symbols denote hypothetical volcanic area. Figure modified from Blakey (2008).

Following this, uplift of Australia (especially in the east) forced the regression of the ocean at ~99 mya, with south-western drainage across southern Australia forming an ocean between Australia and Antarctica (Twidale 1994; Veevers 2006), but with an overland connection between these continents remaining through the eastern margin (coinciding with present-day Tasmania) (Veevers 2006). Although precipitation was still prevalent (Hill 2004), humidity during the Late Cretaceous decreased in comparison to the Early Cretaceous, inland drainage was poor, and swamps in the south-east were much less prevalent than previously, being restricted to the Gippsland and Otway Basins (south of present day Victorian coastline) (Quilty 1984). These factors would have resulted in reduced habitat connectivity and availability, likely resulting in the contraction of ranges into suitable coastal habitat for the ancestral N4. Not only would this have resulted in the isolation of populations, and thus genetic divergence (to eventually give rise to *Engaeus sensu stricto* and ancestral N5 ~95 mya), but such divergence would likely have been further magnified by the changing climate through the occurrence of ecological separation (Figure 7.6). For instance, it is possible that during such a period of reduced wetness the strong burrowing habit of the proto-*Engaeus* (as suggested by fossilised burrows; Martin *et al.* 2008) could potentially have been reinforced, whereas those populations that were able to remain in highly suitable and moist habitats would have retained a less-specialised form (i.e. ancestral N5), thus resulting in ecological separation and subsequent speciation.

Figure 7.6. Hypothetical distribution of ancestral N4 (dashed line) and the subsequent divergence of *Engaeus sensu stricto* (orange) from ancestral N5 (yellow) during the Late Cretaceous (~95 mya). Figure modified from Blakey (2008).

Although some 20 million years later (~73 mya), the divergence of ancestral N5 to give rise to *Gramastacus* and ancestral N6 also took place during the Late Cretaceous. There are two plausible (and not mutually exclusive) causes for the divergence event. The reduced wetness of the Late Cretaceous could have further isolated populations in a similar scenario to that of *Engaeus sensu stricto* discussed above. Additionally, the Eastern Highlands (a mountain range running along the eastern and south-eastern coast of Australia) originated ~90 mya (Unmack 2001), potentially dividing the distribution of the ancestral N5 and resulting in *Gramastacus* and ancestral N6 due to prolonged separation. It has been suggested that the present distribution of *Gramastacus* may have been the result of dispersal through the Murray and Wimmera Rivers (Schultz *et al.* 2009), and it is possible that the headwaters of these tributaries are also the historical refugia for this genus. As the climate continued to dry and the Eastern Highlands formed, some of the ancestral N5 populations may have retreated into the more permanent headwaters further inland, leaving the more coastally distributed populations behind, and thus giving rise to *Gramastacus* (inland) and the ancestral N6 (coastal) (Figure 7.7). *Gramastacus* is one of the lesser burrowing crayfish from this clade, which lends further support to the supposition that this genus originated in a habitat of at least semi-permanent surface water.

Figure 7.7. Hypothetical distribution of ancestral N5 (dashed line) and the subsequent divergence of *Gramastacus* (pink) from ancestral N6 (yellow) during the Late Cretaceous (~73 mya). Figure modified from Blakey (2008).

The beginning of the Palaeocene marked a period of warming, reaching its peak in the Early Eocene (Byrne *et al.* 2011; Sluijs *et al.* 2011). In SEA, the climate was cool temperate during the Early Palaeocene, with precipitation and runoff considerable, and humidity high (Quilty 1994; Martin 2006). Coastal vegetation was predominantly swampy and all the major river basins of today were established (Veevers 1991). The Early Eocene remained humid, with temperatures increasing to become the warmest during the Cenozoic era, and the climate of SEA became sub-tropical (Martin 2006). The conditions throughout these two epochs would have promoted the dispersal of crayfish, with one of two possible dispersal routes used; either a coastal route, or an inland route. Although the burrowing clade was restricted to SEA during this period (with the exception of *Engaewa*) and was found primarily within the coastal regions, it is likely that the dispersal route followed to colonise the eastern coast was via inland drainage systems. The Eastern Highlands run along the eastern and southern coastal margins and, as the continental shelf along the southern part of the eastern coast is narrow (Harris *et al.* 2005), lowered sea level would only produce a thin strip of marginal swampy habitat. The absence of any crayfish from the burrowing clade being found between the most easterly *Engaeus sensu stricto* and *Gramastacus lacus* in central coastal NSW today further suggests that the coastal route was not used for this dispersal event.

Support for an inland dispersal route being a viable option for freshwater organisms is shown by numerous studies that have recognised the Murray-Darling province as being a historical dispersal corridor (see Hurwood and Hughes 1998; Unmack 2001; Austin *et al.* 2003; Munasinghe *et al.* 2004a; Nguyen *et al.* 2004; Thacker *et al.* 2007). Additionally, further support for an inland dispersal route lies in the present distribution of the easterly *Tenuibranchiurus* and *Gen. nov.* (although the formation of these genera from the ancestral N7 did not happen until later). The wet conditions of the Palaeocene and the significant runoff from the established basins indicate that both the headwaters of these systems, and the surrounding areas, were likely very wet, providing ideal conditions for the ancestral N6 to disperse across the headwaters during periods of connection. As the ancestral N6 dispersed across these headwaters, invariably populations would have remained in the moist conditions in the coastal regions, and gene flow would eventually cease. Those that remained *in situ* would have diverged to become *Geocharax*, and those that dispersed into newly available habitat would become the ancestral N7 (~58 mya) (Figure 7.8).

Figure 7.8. Hypothetical distribution of ancestral N6 (dashed line) and the subsequent divergence of *Geocharax* (green) from ancestral N7 (yellow) during the Palaeocene (~58 mya). Figure modified from Blakey (2008).

It is likely that the ancestral N7 continued to disperse across headwaters until reaching the swampy systems of the east coast. However, in order to colonise these coastal areas, it must have crossed the Eastern Highlands. Many freshwater fish species are found both east and west of this formation (Unmack 2001), suggesting that this has been accomplished multiple times by various freshwater taxa (Unmack 2001 and references therein). There are several areas of lower relief in the Eastern Highlands at the boundary of the Murray-Darling and Eastern provinces, including the headwaters of the Clarence and Hunter Rivers in NSW (Unmack 2001). Following the results in the Chapter 5, the ancestral distribution of *Tenuibranchiurus*/*Gen. nov.* (i.e. ancestral N7) was in the Richmond River catchment which is just to the north of the Clarence River catchment. This supports the supposition that an inland dispersal pathway through the Murray-Darling basin was used by ancestral N7 during the wet and humid climate of the Palaeocene/Early Eocene, by crossing the Eastern Highlands through the topographically subdued headwaters of the Clarence River and following this to the coast, in order to establish its distribution within the coastal regions of the Richmond River catchment. Subsequent vicariance as a result of temperatures beginning to cool, shifts in coastal vegetation types, and climatic oscillations (Quilty 1984; Martin 2006) would have given rise to *Tenuibranchiurus* and *Gen. nov.* ~50 mya (as outlined in Chapter 5).

7.6 SUMMARY

It can be seen that the majority of divergence events can be correlated strongly with changes in climate. Most of these events can be characterised by fine scale movements in response to changing climate and fluctuating sea level, in a similar fashion to the model proposed by Horwitz (1988a) (and adopted by Burnham 2014 and Schultz *et al.* 2009) of coastal populations diverging from those left *in situ* as they follow a receding coastline. Ecological specialisation and the use of refugia (discussed in detail in Burnham (2014)) may also have been important factors for promoting divergence between the burrowing clade genera. While it was generally fine scale factors that were useful in describing most of the events affecting the burrowing clade, there were also exceptions to this. The inception of *Engaewa* was likely the result of major geological changes (i.e. substantial marine transgression across Australia), and colonisation of the eastern coast and the subsequent origin of the ancestor to *Tenuibranchiurus* and *Gen. nov.* likely resulted from dispersal events across a relatively large distance within the southern portion of Australia.

8.0 General Discussion

8.1 Morphological and Phylogenetic Implications for Taxonomy

At the outset of this study, a single genus (*Tenuibranchiurus*) was to be examined for morphological variability, as it represented a single anomaly in the Australian parastacids (i.e. the only monotypic genus). Despite previous studies suggesting there was variability within this genus (e.g. Horwitz 1995; Dawkins *et al.* 2010), the degree of morphological variability detected in this study was unexpected. Crayfish from two regions in particular (i.e. Qld and NSW) differ substantially in their morphologies, and a range of analyses confirmed the distinctiveness of these two regions from the closely related and geographically proximate *Gramastacus lacus* as well as from each other. These data provide strong support for the recognition of the crayfish of each of these regions as distinct genera. Molecular analyses further confirmed this generic level distinction, with both individual and combined gene trees providing support for the separation of the two groups from each other, as well as from the other burrowing clade genera; thus dividing the formerly single genus into *Tenuibranchiurus* (Qld populations) and *Gen. nov.* (NSW populations).

That such extreme morphological variation could exist without previous detection highlights how little work has been undertaken on these disjunct burrowing clade crayfish and that any additional studies on this group have the potential to unearth novel insights. This is further supported as additional analyses suggest there are interesting morphological and morphometric relationships between populations within each genus. Comparison of morphometric variables between *Tenuibranchiurus* populations suggests that, although some general trends are evident, variation is minimal. This is also the case for comparisons between *Gen. nov.* populations. In contrast, analysis of morphological characters show the presence of distinct clusters within each genus, which are more clearly defined when the morphometric data are added. Although fixed morphological differences (and thus diagnostic characters) exist between all of the analysed *Gen. nov.* populations (i.e. Lennox Head; Broadwater National Park 1; Lake Hiawatha), they are not present between all *Tenuibranchiurus* populations analysed, with only the following distinctions supported: Gold Coast; Tewantin; Hervey Bay; Bribie Island; and Type Locality/Beerburrum/Maryborough. Although fixed differences form the basis of morphological taxonomy and, as such, each of these morphological groups could feasibly be suggested to represent a distinct species, because of the complex relationships found, and the tendency for morphological plasticity or

conservatism to exist within freshwater crayfish, no suggestion of distinct species has been made at this stage. Rather, two potential scenarios are suggested; (1) each genus is comprised of a single species that has low levels of morphometric variation but is morphologically plastic across its range, or (2) each genus is represented by multiple species that are morphometrically conservative but morphologically diagnosable (to some extent). As such, inclusion of molecular data provides necessary additional evidence with which to analyse these alternative scenarios.

The final outcome of the species delimitation through molecular analyses supports the presence of two species within the newly proposed genus *Gen. nov.*, namely; Lennox Head (*Gen. nov.* sp. nov. 1) and Broadwater National Park 1&2 and Lake Hiawatha (*Gen. nov.* sp. nov. 2). Although the morphological analysis suggests that there are fixed differences between *Gen. nov.* sp. nov. 2 populations, this is not supported by the molecular data. This would suggest that Scenario 2 above is applicable to this genus, with the additional caveat that *Gen. nov.* sp. nov. 2 represents a morphologically variable species across its range.

Within *Tenuibranchiurus*, the molecular analyses support the presence of six species, one of which is the previously described *T. glypticus*. Of these species, three were identified as having diagnostic characters in the morphological analyses, namely; *T.* sp. nov. 3 (Hervey Bay), *T.* sp. nov. 4 (Tewantin, Lake Weyba, Eumundi), and *T.* sp. nov. 5 (Gold Coast), although it should be noted that not all *T.* sp. nov. 4 populations have been morphologically examined. Although the Bribie Island population shows morphologically diagnosable characters to the exclusion of all other populations, in the molecular analyses it is placed in *T. glypticus* along with the Tuan State Forest South, Type Locality, Beerburrum, and Mooloolaba populations. Populations of *T.* sp. nov. 1 (Maryborough, Tuan State Forest North and South, Kinkuna National Park) have overlapping characters with populations analysed from both *T. glypticus* and *T.* sp. nov. 2. Therefore, morphological characterisation of *Tenuibranchiurus* species is difficult, with *T.* sp. nov. 3, 4, and 5 representing morphometrically conservative but morphologically diagnosable species, whereas *T.* sp. nov. 1, 2, and *T. glypticus* represent species that have low levels of morphometric variation but are morphologically variable.

Comparison of the morphological and molecular data highlights the need for integrative taxonomic techniques to be employed, particularly for organisms that display such complex phenotypic and genotypic relationships; a supposition that has been supported in other studies of freshwater crustaceans (e.g. Hansen *et al.* 2001; Page *et al.* 2005; Mathews *et al.* 2008). Prior to this study, all crayfish analysed were presumed to form part of the single monotypic genus *Tenuibranchiurus* (Dawkins *et al.* 2010), however, this study has identified not only multiple species within this genus, but also recognised an entirely new genus with multiple species of its own. With the molecular and morphological diversity of *Tenuibranchiurus* and *Gen. nov.* characterised, it was possible to examine the evolution and biogeography of the two genera within an accurate framework, thus providing novel insights into a previously understudied group of crayfish.

8.2 FUTURE CONSERVATION CONSIDERATIONS

Currently, the most prevalent concern regarding the long term persistence of *Tenuibranchiurus* and *Gen. nov.* (and likely many of the burrowing clade genera) is the alteration of habitat, primarily through anthropogenic impacts (e.g. Taylor *et al.* 2007; Coughran and Furse 2012). Although historical biogeographic events have strongly influenced the current distribution of species within these genera, extensive geographic isolation as a result of contemporary habitat fragmentation throughout their ranges will undoubtedly affect their future survival. The contemporary phylogeographic structure between *Tenuibranchiurus* and *Gen. nov.* populations shows very little connectivity between populations within species; indicating that populations are highly constrained by landscape characteristics and migration is unlikely (Avise 2000, 2009). As such, if population extirpations were to occur it is unlikely that these areas would be recolonised by neighbouring populations. Additionally, few haplotypes were shared between sampling locations, making each population a unique (and therefore irreplaceable) reservoir for genetic material (Moritz 1994). Therefore, future conservation action will be needed to conserve as much of the genetic variation of *Tenuibranchiurus* and *Gen. nov.* species, and may be warranted at population level, species level, or both.

As well as conservation concern due to human-mediated impacts, there are also conservation issues resulting from distributional overlaps between *Tenuibranchiurus* species. Such overlaps may cause inter-specific competition with the potential to lead to population extinctions (Liebherr and Hajek 1990; Westman and Savolainen 2001). Three cases of sympatry are currently known within *Tenuibranchiurus*, with one case occurring between *T. glypticus* and *T.* sp. nov. 2, and two between *T. glypticus* and *T.* sp. nov. 1. Although sympatry has been described within several parastacid genera (e.g. Suter and Richardson 1977; Horwitz *et al.* 1985a; Honan and Mitchell 1995; Austin and Knott 1996; Hansen *et al.* 2001; Hansen and Richardson 2002; Sinclair *et al.* 2011), it has only been described to occur within two genera of the burrowing clade; *Engaeus sensu stricto* (Suter and Richardson 1977; Richardson and Horwitz 1987; Horwitz *et al.* 1990; Schultz *et al.* 2009) and *Engaewa* (Burnham 2014), with the latter example suggested to be an 'artificial' situation. It has been suggested that sympatry between freshwater crayfish species is rarely complete (Horwitz 1986) and, as such, the extent of previously suggested cases of sympatry are unclear. For instance, Horwitz (1990) and Suter and Richardson (1977) both suggested that sympatry between *Engaeus* species will always occur with some form of separation (e.g. longitudinal or transverse separation), and this has similarly been suggested for other parastacid genera (Hansen and Richardson 2002).

As no detailed information on the behaviour, ecology, or reproductive morphology of the *Tenuibranchiurus* species were collected, the type and degree of sympatry between them are also unclear. If it is assumed that *Tenuibranchiurus* act in a similar way to other burrowing clade genera, then it is likely that some form of separation exists between sympatric species. At all locations of sympatry, both species were collected at the same time from the same waterbody. This suggests that behavioural separation (e.g. being active at different times of the day) between the species is likely not occurring and, as the same sampling method was used, perhaps microhabitat separation may also be excluded. While an interesting study in itself, the extent of sympatry requires further investigation for conservation purposes as well, as the continued persistence of both species in a single area may not represent a lasting situation. As *Tenuibranchiurus* species appear to represent very similar organisms, a competitive advantage is likely to occur in one species, resulting in the decline and possible extirpation of the congener from that area. In the case of sympatry between *T. glypticus* and *T.* sp. nov. 2, if the outcompeted species is the latter, the removal of this species from the area would also result in the extinction of this species.

8.3 BIOGEOGRAPHY OF *TENUIBRANCHIURUS* AND *GEN. NOV.*

8.3.1 Distributional Patterns

One of the basic tenets of biogeographic studies is to seek congruence in distributional patterns between taxa to draw inferences on the relationship between the landscape and the taxa within it (Page and Hughes 2014). Congruence between taxa can highlight processes such as vicariance, environmental change, and dispersal, and where shared discontinuities among taxa (e.g. species boundaries, genetic groupings) exist, the presence of biogeographic barriers can be inferred (Riddle *et al.* 2008). As numerous factors can affect the response of an organism to an event, congruence between multiple taxa would not always be expected; for instance, vicariance events may not affect all taxa equally, with differing ecological tolerances potentially altering the type or degree of response (Crisp *et al.* 1995). Despite this, testing for both spatial and temporal congruence across co-distributed taxa is an important part of the biogeographic approach.

In relation to the biogeography of Australian fauna and flora, the break up of the landmass of Gondwana into the present-day Southern Hemisphere continents was a significant process in the evolution of the taxa of these regions (Toon *et al.* 2010). Australia separated from all other landmasses over a period of 130 million years (165-35 mya), with the last separation being between Australia and Antarctica (Christophel and Greenwood 1989; Martin 2006). Throughout this time, the eastern margin of Australia (the current distribution of *Tenuibranchiurus* and *Gen. nov.*) was subject to fluctuating sea level (both higher and lower than present-day), volcanic activity from the late Mesozoic through the Quaternary, and a climate that progressively changed (Quilty 1984; Christophel and Greenwood 1989; Unmack 2001; Martin 2006; Byrne *et al.* 2008). Historically, movement by freshwater taxa throughout this region would have been challenging. It has been suggested that few drainages would have coalesced even with lowered sea level (Unmack 2001), as the continental shelf throughout the area is narrow compared to the rest of the continent (Murray-Wallace 2002; Harris *et al.* 2005). These factors mean this region of Australia represents an interesting study area for biogeographers, and many studies have been conducted on its freshwater biota in an attempt to explain and reconstruct the biogeographic history (e.g. Unmack 2001; Shull *et al.* 2005; Cook *et al.* 2006).

The present day distribution of *Tenuibranchiurus* and *Gen. nov.* species can be interpreted through the influence of historical events. It is likely that most dispersal events occurred during periods of lowered sea level, due to the exposure of coastal plains creating dispersal pathways and/or populations moving in response to following the moisture gradient as it receded eastwards. Genetic divergence between populations would have occurred as they became separated by heterogeneous habitat resulting in relatively widespread and coastally-distributed groups and highly-restricted inland-distributed groups. These divergences would have been exacerbated by vicariance resulting from drying conditions, shrinking (and dividing) suitable habitat patches, and/or later sea level rise further dividing populations. These repeated divergence events (along with any extinctions) would eventually have resulted in the six *Tenuibranchiurus* and two *Gen. nov.* species present today. It will be argued in section 8.4.2 that these same processes underlie a biogeographic model for all burrowing clade genera, which will be outlined in detail.

The six species identified within *Tenuibranchiurus* are restricted to the coastal areas of SEQ. When the ranges of *Tenuibranchiurus* species are compared to other similarly restricted freshwater fauna, a lack of spatial concordance between them is evident. The distributions of the vast majority of freshwater taxa present in SEQ extend well beyond this region, with only three being completely restricted to coastal SEQ (*Cherax dispar, Cherax robustus, Caridina sp. A*) (Chenoweth and Hughes 2003; Page and Hughes 2007a, 2007b; Bentley 2014), and an additional eight restricted to coastal SEQ and NEN (*Cherax cuspidatus*, *Caridina sp. C*, *Crinia tinnula*, *Melanotaenia duboulayi*, *Nannoperca oxleyana*, *Retropinna SEQ*, *Retropinna CEQ*, *Rhadinocentrus ornatus*) (Hughes *et al.* 1999; Chenoweth and Hughes 2003; Page *et al.* 2004; Renwick 2006; Hammer *et al.* 2007; Page and Hughes 2007a, 2007b; Unmack *et al.* 2013; Bentley 2014; Page and Hughes 2014). These taxa demonstrate distributions of genetic lineages that generally are not concordant with *Tenuibranchiurus* species, with the distribution of most species encompassing the entire range of all species within this genus. The only area that shows some form of concordance is where the distributions of *T.* sp. nov. 1 and *T. glypticus* overlap (i.e. in the northern Mary River catchment). In this area, the distributions of *Cherax dispar* and *C. robustus*, *Retropinna SEQ* and *Retropinna CEQ*, as well as lineages within *C. dispar* (i.e. A, B, and C) show some overlap, indicating that this region may be an important area for species turnover. A number of other taxa have distributions that are limited by either the northern or southern limit of SEQ, suggesting that although SEQ (generally) may not itself possess a unique freshwater fauna, nor have clear

patterns within it, its boundaries may indicate regions where there is a high turnover of species.

The distribution of species within *Tenuibranchiurus* is interesting, as there is perhaps no other coastal freshwater taxon in the region that contains both highly restricted species as well as comparatively widespread species, yet has all of its species wholly contained within this relatively small and discrete area. The lack of concordance between distributional breaks (when compared to other freshwater-dependent species) suggests that intrinsic factors likely dictate the distribution of *Tenuibranchiurus* species; these same factors appear to characterise all of the burrowing clade genera. Such factors result in relatively small ranges for species within burrowing clade genera (based on calculations provided by Burnham 2014; IUCN 2014), as well as the propensity for the distributional boundaries of species not to be restricted by drainage basins (based on distributions outlined in Horwitz 1990; Schultz *et al.* 2007; Burnham 2014; McCormack 2014). Although much of the previous work on the biogeographic and phylogeographic patterns of freshwater taxa has identified drainage basins as a key unit for influencing the distributions of freshwater fauna (Wishart and Davies 2003), it would appear that *Tenuibranchiurus* and *Gen. nov.* (along with the other burrowing clade genera) operate outside of this paradigm.

It is not entirely surprising that *Tenuibranchiurus* and *Gen. nov.* species do not confirm to a drainage basin driven model of distribution (as previously noted), as some other Australian freshwater crayfish have also previously been identified as not conforming to typical freshwater patterns. For instance, the genus *Euastacus*, which is distributed throughout much of eastern Australia, does not fit within a drainage basin driven paradigm. As many of the species within this genera are confined to isolated mountain tops separated from each other by topographically and climatically inhospitable environments (Ponniah and Hughes 2004, 2006), their distributional and diversity patterns are driven by geography rather than drainage architecture. Although no specific biogeographic studies have been undertaken, it has also been suggested that the species boundaries within *Parastacoides* Clark (subsequently split into the genera *Ombrastacoides* and *Spinastacoides* by Hansen and Richardson (2006)) are not associated with physical or environmental barriers (Hansen and Richardson 2002), such as those of drainage boundaries. Additionally, the dispersal of *Parastacoides* species was found to generally occur via terrestrial dispersal rather than through streams (Hansen and Richardson 2002), thus removing them from the characteristics of these systems.

Based on these examples and the pattern of diversity present within the study taxa, it can be argued that the burrowing clade genera (along with some other parastacid genera) and the species within them do not fit within many of the current management practices for freshwater taxa, and it is argued that these burrowing crayfish taxa should be considered as a distinct category of freshwater fauna requiring a more complex model to understand the distribution of diversity within the group.

8.4 PHYLOGENY AND HISTORICAL BIOGEOGRAPHY OF THE BURROWING CLADE

8.4.1 Phylogeny and Timing of Divergence

Although the monophyly of the burrowing clade has been supported in previous studies (Horwitz 1988b; Schultz *et al.* 2009; Toon *et al.* 2010; Burnham 2014), the phylogenetic relationship between genera of the clade has never been fully resolved. As this study utilised all available sequence data for one mitochondrial (16S) and one nuclear (GAPDH) gene, in addition to new sequence data for *Tenuibranchiurus* and *Gen. nov.*, it represents the most comprehensive phylogenetic study of the burrowing clade to date. However, the phylogenetic relationship was not statistically resolved for all of the genera (specifically between *Engaewa*, *Engaeus sensu stricto,* and *E. lyelli*), which supports the conclusions of previous studies; namely, that this relationship cannot be resolved with currently available data and/or methods. Using such a comprehensive data set and still not being able to provide a statistically supported branching pattern indicates that it is likely the three oldest genera diverged almost simultaneously, creating a polytomy whereby the order of branching is unable to be resolved (Humphries and Winker 2010). This is further supported by the *BEAST analysis, where all of these genera were estimated to have originated during the same period (the Cretaceous), although this analysis did suggest that *Engaeus sensu stricto* was perhaps the last of the three to diverge. Subsequent divergence events between the remainder of the burrowing clade genera were phylogenetically supported, with *Gramastacus* the next to diverge (during the Cretaceous) followed by *Geocharax* (Palaeocene) and finally the divergence of *Tenuibranchiurus* and *Gen. nov.* (Eocene).

The estimates of divergence times between the burrowing clade genera prior to this study were conflicting, with Schultz *et al.* (2009) suggesting all events occurred during the Eocene/Miocene and Toon *et al.* (2010) reporting dates during the Cretaceous. Neither of these studies included the newly proposed genus *Gen. nov.* and, as such, their most recent divergence event was calculated between *Tenuibranchiurus* and *Geocharax*. With the inclusion of this newly proposed genus as well as representatives from all but four burrowing clade species (from *Engaeus sensu stricto*) this study was able to clarify the evolution of this group of crayfish and therefore provide an accurate framework on which to build biogeographic inferences.

8.4.2 Biogeographic Model

The phylogeographic assessment paradigms often applied to freshwater taxa (i.e. stream hierarchy model, death valley model, isolation by distance, panmixia, or headwater model as outlined by Hughes *et al.* 2013) appear to only tell part of the biogeographic story of the burrowing crayfish. Based on the distribution of genetic variation within *T. glypticus*, Hughes *et al.* (2013) suggested that they conform to the death valley model (i.e. individuals are unable to migrate and therefore there is no inter-population gene exchange (as first defined by Meffe and Vrijenhoek (1988))), as did Burnham (2014) for *Engaewa* spp.. While this may explain the present distribution of haplotype diversity (specifically each population representing a unique genetic entity evolving in isolation), in order to truly understand these taxa a biogeographic model that can explain the distribution of genetic diversity within these crayfish (both inter- and intra- species, and inter- and intra- generic) in a historical context needs to be formulated. Such a model has been described, in part, by Horwitz (1988a) as outlined below. However, it should be noted that his model was restricted to describing the dispersal and speciation of a few *Engaeus* species in southern Australia, rather than across genera.

1. The lowland forms are adhered to the microclimate of freshwater habitats in the coastal region, following the coast as the sea levels rise and fall.
2. When the sea levels fall, populations can be left behind by the lowland forms, and these 'highland isolates' may develop into distinct species provided that they undergo sufficient divergence between the time of isolation and the time when contact between the lowland and highland forms can be re-established.

3. If sufficient divergence occurs, and if the highland species and the lowland species come back into contact, then longitudinal zonation along a creek system may develop with one species in the upper reaches, the other species in the lower reaches, and perhaps some overlap in the middle.

The model of Horwitz (1988a) was further expanded by Burnham (2014) who included the concept of historical climate refugia as regions where isolates persist, and also included the possibility of both coastal and inland (i.e. across headwater) dispersal depending on climatic conditions, in order to explain the contemporary and historical distribution of *Engaewa* species; however, again, this was limited to a single genus in a single area. Although the models of Horwitz (1988a) and Burnham (2014) were formulated to describe the biogeography of species within a genus that are confined to a relatively small area, the strong ecological and biological similarities of crayfish within the burrowing clade genera (i.e. the burrowing habit, low mobility, coastal distributions etc.) suggest the core tenets of these models may be applicable more widely. By using the premise of Horwitz (1988a) and Burnham (2014), but expanding the concept to include large scale climate changes incorporating sea level fluctuations, shifting habitat zones, and geological events on a continental scale, and including robust divergence estimates for all taxa within the group this study provides a possible explanation of how the individual taxa formed (outlined in Chapter 7).

8.4.3 Historical Biogeography

As stated previously, the data of this study suggest that these crayfish are truly ancient in origin. Throughout the last ~150 million years members of this group have dispersed out of a southern/south-eastern centre of origin, and as they have done so they have diversified to form the seven genera recognised in this study. All bar two of the divergence events between genera were likely the result of relatively small-scale distributional shifts in response to changing climate (specifically related to rainfall patterns) and the influence of environmental heterogeneity on population persistence. Of all the burrowing clade genera, the origin of *Engaewa* was perhaps the most extreme event to occur, with the ancestor to the burrowing clade that likely once occurred across a large portion of the southern margin reduced to two isolates; one in the far south-west (the ancestral *Engaewa*) and one in the south-east. This likely occurred in response to a major geological change; the marine transgression across large portions of Australia. The event resulting in the origin of the ancestor to

Tenuibranchiurus and *Gen. nov.* would have been another exception to the general pattern. This event most likely resulted from long distance dispersal across a temporary corridor through the interior of SEA.

Speciation within each of the burrowing clade genera can also be explained by similar patterns throughout, as could be expected due to the similar dispersal and ecological characteristics displayed across the genera. Changing climate and associated fluctuating sea level, were likely the main driving forces behind speciation. Periods of low sea level could force most populations to follow the receding coast, while leaving others behind *in situ*, which would eventually result in genetic divergence (and thus speciation) between them. Almost all speciation events within these crayfish can be correlated with relatively small distributional shifts in response to climate and/or sea level with one exception; speciation within *Gramastacus*. Of all the burrowing clade genera, this is the only one to display a vastly disjunct species distribution, with *Gramastacus insolitus* Riek occurring in the south-west of Victoria, an undescribed species ~250 km to the north-east of this, and *G. lacus* in the north-east of NSW. This distribution is best explained in a similar way to the dispersal of the ancestor to *Tenuibranchiurus* and *Gen. nov.*, whereby a long distance dispersal event through the interior of SEA occurred during times of favourable climate (Schultz *et al.* 2009), thus resulting in the three disjunct species. Support for this interpretation comes from this area having been recognised as a historical dispersal corridor for other freshwater taxa (Unmack 2001; Austin *et al.* 2003; Munasinghe *et al.* 2004a; Nguyen *et al.* 2004; Thacker *et al.* 2007; Jerry 2008).

8.5 CONCLUSION

The overall aim of this thesis was:

> "To clarify the morphological and molecular diversity present within *Tenuibranchiurus* as it represents the largest gap in knowledge within the burrowing clade, and to use this information to further the understanding of the evolution of all burrowing clade genera, both from a phylogenetic and a biogeographic perspective."

Prior to this study, *Tenuibranchiurus* was considered to be the only monotypic parastacid genus, but containing undefined genetic groups. It has now been divided into two separate genera (*Tenuibranchiurus* and *Gen. nov.*), each with multiple species, that await taxonomic description. The distributional patterns of *Tenuibranchiurus* and *Gen. nov.* species align closely with patterns common to all burrowing clade genera, rather than being dictated by historical freshwater processes that occurred throughout their distributions. It is important for future studies to recognise that these taxa appear to operate outside of commonly accepted freshwater paradigms, and are somewhat ecologically removed from other freshwater taxa, appearing to largely be influenced by climate change and fluctuating sea level rather than drainage architecture. Furthermore, it is important to examine any new populations of burrowing crayfish found from both a morphological and molecular perspective as this study (and previous studies) on the burrowing clade have highlighted the potential for any new area surveyed to host a novel group of crayfish (either as a new genetic variant within a previously identified species or an entirely new species). Using the novel information obtained in this study, detailed phylogenetic relationships and biogeographic histories were reconstructed for all of the burrowing clade genera, as well as inferences formed on the speciation processes that have occurred within each genus. This group of crayfish represents an ancient and diverse group, originating in the Cretaceous and diversifying through repeated dispersal and vicariance events in response to climate change, sea level fluctuations, and major geologic events. As well as representing an interesting group of fauna, the burrowing clade crayfish also represent a unique perspective for informing on biogeographic processes in the areas they inhabit.

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APPENDIX ONE

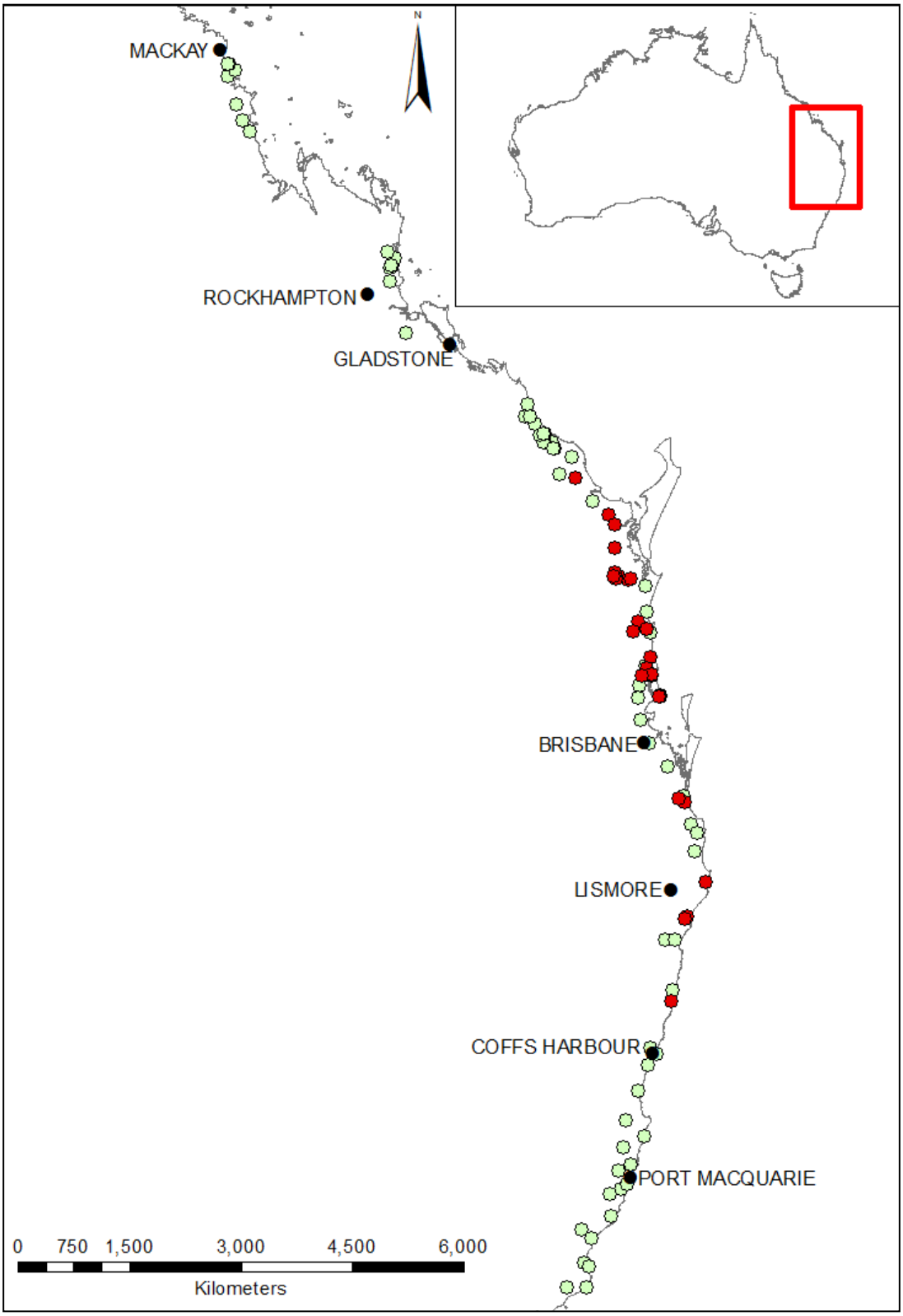


Figure A1.1. Locations that were visited to sample for *Tenuibranchiurus.* Green circles indicate where no *Tenuibranchiurus* were found, red circles indicate where they were successfully collected, black circles are major populated places. For sources of GIS data see section 2.3.

APPENDIX TWO

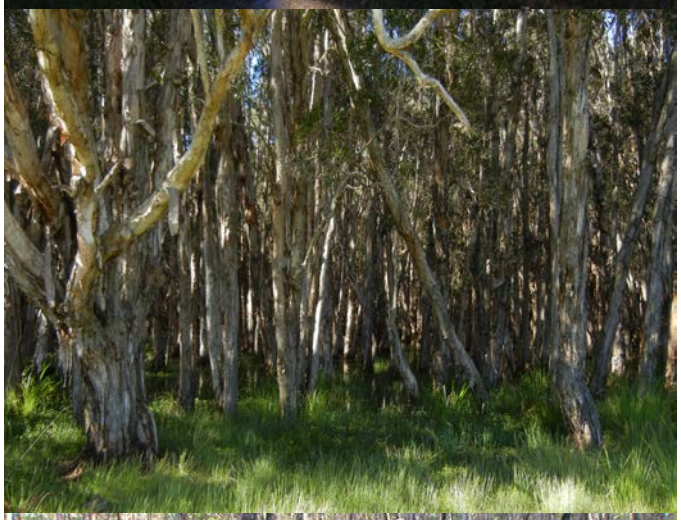
Previous habitat characterisation of *Tenuibranchiurus* was limited to *Melaleuca* swampland (Riek 1951; Dawkins *et al.* 2010). However, due to the opportunistic sampling protocol of this study, alternative habitats have now been highlighted as suitable for this genus (and *Gen. nov.* as recognised in this thesis). Of the 20 collection localities in this study, 14 had alternative habitat structure to *Melaleuca* swamp; Hervey Bay, Tuan State Forest South (A-H), Tewantin State Forest, Lake Weyba, Beerburrum, Type Locality 3, Bribie Island, Broadwater National Park , and Lake Hiawatha. The different habitat types where specimens were found are outlined in Table A2.1. The remainder of sites where specimens were collected during this study were typical *Melaleuca* swamps.

Although many of the collection locations in this study were within protected areas such as national parks, state forests, and scientific areas, there were also a number in disturbed regions such as pine plantations and residential areas. These findings suggest that sampling for these crayfish (and possibly other members of the burrowing clade) should not be restricted to a narrow range of habitats based on previous site descriptions, which may have important ramifications for any development proposals. Furthermore, additional investigations are warranted to determine the resilience of these crayfish to disturbance in order to assess whether populations living in disturbed areas are viable in the long term.

Table A2.1. Collection locations with alternative habitat types to what was previously considered to be characteristic of *Tenuibranchiurus*.

Collection Location	Habitat Description
Hervey Bay	Flooded tyre tracks adjacent to *Melaleuca* swamp (also collected from swamp)
Tuan State Forest South (A-H)	A variety of habitat types, though none were swamps: creeks for C and H, backwash pools from creeks for the remainder, and *Melaleuca* was not present at A and E
Tewantin State Forest	Flooded *Melaleuca* forest (i.e. not swampland)
Lake Weyba	Water tank on private property
Beerburrum	A deep waterhole as well as the adjacent flooded heathland with small patches of *Melaleuca* present but not dominant
Type Locality 3	Flooded tyre tracks at an elevated portion of road that was not adjacent to *Melaleuca* swamp
Bribie Island	Flooded depression within heathland where no *Melaleuca* was present
Broadwater National Park 2	Deep stream channel with slow flowing water and *Melaleuca* present on stream banks
Lake Hiawatha	Natural drainage channel (dissected by a dirt road) within heathland with no *Melaleuca* present

Hervey Bay (HB): Flooded tyre track adjacent to *Melaleuca* swamp. The adjacent swamp was also sampled, with *Tenuibranchiurus* also collected from there.



Maryborough (MAR): Large *Melaleuca* swamp with accumulated leaf litter, sedges and decomposing logs present in water. *Tenuibranchiurus* were mostly caught around the bases of sedges.

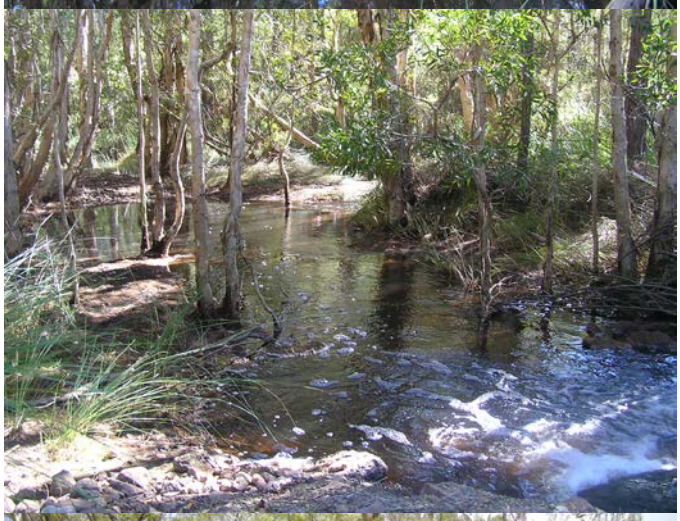
Tuan State Forest North (TSFSN): Muddy pool with clay soil, and sedges as the dominant vegetation. *Tenuibranchiurus* were not collected from this site during this study as water was not present at time of sampling.

Tuan State Forest South A (TSFSA): An isolated pool with red clay/loam sediment and very turbid red water. *Melaleuca* were not the dominant vegetation type.

Tuan State Forest South C (TSFSC): Leaf litter accumulated on the bottom of the creek that was very shallow, muddy and stagnant. *Melaleuca* were present at this site.

Tuan State Forest South E (TSFSE): Backflow pool next to the main channel. There were large amounts of leaf litter and the pool dries when there has been little rain. The site is dominated by sedge grass and large gums.

Tuan State Forest South G (TSFSG): Backflow pool adjacent to a small creek that runs almost all year round. There was little leaf litter and some sandy sediment.



Tuan State Forest South H (TSFSH): Small creek that runs almost all year round. There was a large amount of *Melaleuca* and some sedge grasses.

Beerburrum (BER): *Tenuibranchiurus* were collected at a deep waterhole (shown), as well as in the adjacent flooded heathland. Small patches of *Melaleuca* were present but the not dominant vegetation component.

Lennox Head (LH): *Melaleuca* swamp with accumulated leaf litter, sedge plants, and decomposing logs. The swamp was mostly shallow, and individuals of *Gen*. nov. were mostly collected from bases of sedges.

Broadwater National Park 1 (BNP1): *Melaleuca* swamp with accumulated leaf litter, sedge plants, and decomposing logs. The depth of the swamp varied. *Gen*. nov. specimens were most successfully caught from the shallower sections and around the bases of sedges and logs.

Lake Hiawatha (LakeH): A natural drainage channel was dissected by a dirt road within heathland. There were no *Melaleuca* present.

APPENDIX THREE

Mirroring the difficulties in defining a specific habitat type for these crayfish, the reproductive pattern for these crayfish is difficult to explain. All collections of gravid females previous to this study have occurred in April (i.e. two from Maryborough, Qld (Dawkins 2008; Dawkins *et al.* 2010), one near Caloundra, Qld (P. Horwitz unpublished data), and four near Tabbimoble, NSW (P. Horwitz unpublished data)) (Table A3.1). However, on 7 September 2011, two gravid females were collected from Bribie Island (i.e. Spring). Although the collection information is limited, based on all previous studies it would appear that *Tenuibranchiurus* and *Gen. nov.* are summer brooders, carrying their eggs over the Summer/Autumn period and releasing their young in late Autumn/Winter. However, the collection of gravid *T. glypticus* females in September does not support this pattern, and may suggest the occurrence of reproductive plasticity and/or response to environmental variation. Further investigation into these possibilities is warranted, as it may be that their reproductive strategy is flexible (possibly even including more than one brood per year) or it may be that the Bribie Island population is unique.

The possibility that the Bribie Island population possesses a unique reproductive strategy may have support based on the morphological revision undertaken in this study. The Bribie Island individuals were found to have unusual chelae growth, where individuals of a similar size had widely differing chela measurements (section 3.5.1); a trait not displayed by any other population. Although this could possibly be a result of intraspecific competition, this occurrence, coupled with the anomalous reproductive strategy, could hint at the possibility of incipient speciation. Being the only island locality for *Tenuibranchiurus*, Bribie Island is likely to have been subject to differing environmental conditions than other mainland populations (e.g. increased effects from rising/lowering sea levels, more extreme climate and/or habitat change, altered rainfall patterns and groundwater hydrology). This could potentially have caused population demographics to change in response (e.g. increases/decreases in population size, altered breeding cycle), thereby being a catalyst for incipient speciation. The phylogenies presented in Chapter 4 show Bribie Island as a distinct cluster, however they are clearly still currently part of *T. glypticus* and therefore have not undergone complete speciation. The process of incipient speciation has been suggested for some other crustacean species occurring as peripheral, isolated, or island populations (e.g. Wilson and Hessler 1987; Austin and Knott 1996; Hayd and Anger 2013), and the current

isolation of Bribie Island from the mainland populations may lead to the complete speciation of this population in the future. However, more information is required on all populations in order to draw strong conclusions about the reproductive patterns of this genus as a whole.

Table A3.1. Collection localities sampled during this study and by *P. Horwitz (unpublished data) as well as the time of year when *Tenuibranchiurus* were found. The presence of adult *Tenuibranchiurus* or *Gen. nov.* are indicated by darkened cells, young-of-year are indicated with a Y, and gravid females with a G.

State	Location ID	January	February	March	April	May	June	July	August	September	October	November	December
Qld	HB												
	MAR				Y,G								
	TSFS A												
	C												
	E												
	F												
	G												
	H												
	TEW					Y							
	LW												
	BER					Y							
	Beerburrum*				Y								
	TL2					Y							
	TL3												
	Caloundra*				Y,G								
	BRB									G			
	GC1	Y											
	GC2												
NSW	LH					Y							
	BNP1					Y	Y						
	BNP2												
	Tabbimoble*				Y,G								
	Lake H					Y							

APPENDIX FOUR

Extracted DNA from two individuals were separately digested in the following reaction: 10.0 µL DNA, 1.0 µL RNase, 1.0 µL *DpnII*, 1.5 µL *DpnII* Buffer (10×), and 1.5 µL ddH_2O. The reaction was left at 37ºC overnight, with an additional 1.0 µL *DpnII* subsequently added and left for another 2 hours. Linkers were then ligated to the DNA fragments in the following reaction: 2.0 µL digested DNA, 2.0 µL SauLinker, 1.5 µL T4 Ligase, 1.5 µL T4 Ligase buffer (1×), 0.5 µL ATP, and 7.5 µL ddH_2O (see Table A4.1 for primers). This was then incubated at room temperature for 2 hours and then 16ºC overnight.

A pre-enrichment PCR was then run on the resulting solution as follows: 2.0 µL ligated DNA, 3.0 µL SauLA (10 µM), 4.0 µL dNTP (10 mM), 3.0 µL $MgCl_2$ (50 mM), 10.0 µL buffer (5×), 0.3 µL Bioline Mango *Taq* (5U/µL), and 27.7 µL ddH_2O. The PCR cycling conditions were as follows: 94ºC for 5 min; 35 cycles of: 30 sec at 58ºC, 40 sec at 72ºC, 30 sec at 95ºC; then 4 min at 72ºC with the reaction stored at 4ºC. The PCR product was then run out on a 2% agarose gel, with a 400-800 bp size selection made. This was then gel extracted using the Qiagen gel extraction kit and eluted in 20 µL ddH_2O.

The gel extracted DNA for the two individuals was then mixed and separated into two equal quantities for further analysis. Two reactions containing the following were then run: 15.0 µL 2×Hyb solution (60 µL 20×SSC, 1.0 µL 10% SDS, 39 µL ddH_2O), 6.0 µL ligated PCR product, 6.0 µL probe mix (dimer/trimer mix for reaction 1, tetramer mix for reaction 2), and 3.0 µL ddH_2O. The PCR cycling conditions for each reaction were as follows: 94ºC for 5 min; 5 sec at 70ºC, with the temperature then dropped by 2ºC every 5 secs until 50ºC and held for 10 mins; then the temperature dropped by 2ºC every 5 secs until 40ºC; then quickly ramped down and stored at 15ºC.

Table A4.1. Universal primers used for microsatellite pre- and post-enrichment, and amplification.

Primer ID	Sequence
SauLA	GCGGTACCCGGGAAGCTTGG
SauLB	GATCCCAAGCTTCCCGGGTACCGC
M13-F	GTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGAC

The resulting DNA was then purified using Streptavidin MagneSphere® Paramagnetic Particles. The magnetic beads were rinsed three times in 600 µL 0.5×SSC and resuspended in 534.6 µL 6×SSC and 5.4 µL 10% SDS, then split into two aliquots. Into each of these aliquots 30 µL of the above DNA/probe mix was added (i.e. the dimer/trimer mix into one, the tetramer mix into the second). These were incubated at room temperature for 20 mins while mixed on a Clements suspension mixer. The beads were then 'captured' (magnets were used to draw the beads out of suspension) and the supernatant was removed and discarded. The beads were then mixed gently with 297 µL 2×SSC and 3 µL 10% SDS, captured again, the supernatant discarded, with this step repeated once. The cleaned beads were then mixed gently with 147 µL 2×SSC, 150 µL ddH_2O, and 3 µL 10% SDS, captured, the supernatant discarded, and this step repeated once. The beads were then mixed gently with 147 µL 2×SSC, 150 µL ddH_2O, and 3 µL 10%SDS, incubated for 15 mins at 45ºC, captured, and the supernatant discarded. The beads were then mixed gently with 147 µL 2×SSC, 150 µL ddH_2O, and 3 µL 10%SDS, incubated at 50ºC, captured, and the supernatant discarded. The captured beads were then eluted in 100 µL 0.1M NaOH at 80ºC for 10 mins. The beads were again captured, but this time the supernatant was removed and kept, with the beads discarded. The solution was neutralised with 100 µL TE (pH 7.5). The neutralised solution was then mixed by pipette with 22 µL NaOAc/EDTA solution, with 444 µL 95% ethanol then added and mixed by inverting the tube several times. This was placed on ice for at least 15 mins then centrifuged for 10 mins at 13500 rpm. The supernatant was discarded, 500 µL 70% ethanol added, and the solution centrifuged for 1 min. All of the supernatant was then carefully pipetted off, with the sample air dried until no trace of ethanol was left. The pellet that remained was resuspended in 25 µL 0.1×TE and left to hydrate for at least 20 mins.

A post-enrichment PCR was then performed on the hydrated solution and used the following; 2.0 µL ligated DNA, 3.0 µL SauLA (10 µM), 4.0 µL dNTP (10 mM), 3.0 µL $MgCl_2$ (50 mM), 10.0 µL buffer (5×), 0.3 µL Bioline Mango *Taq* (5U/µL), and 27.7 µL ddH_2O. The PCR cycling conditions were as follows: 94ºC for 5 min; 35 cycles of: 30 sec at 58ºC, 40 sec at 72ºC, 30 sec at 95ºC; then 4 min at 72ºC with the reaction stored at 4ºC. The PCR product was then run out on a 2% agarose gel, with a 400-800 bp size selection made. This was then gel extracted using the Qiagen gel extraction kit and eluted in 20 µL ddH_2O.

The previous steps (from paragraph three of this section) were repeated, with the resulting DNA prepared for cloning by using the following; 2.5 µL post-enrichment PCR product, 1.0 µL dilute salt solution (50mM), 0.5 µL Invitrogen pCR2.1-TOPO vector, and 2.0 µL ddH_2O. This was then mixed and incubated at room temperature for 10 mins, then placed onto ice in preparation for cloning. Cloning consisted of the following reaction; 2 µL of the above solution, 25 µL *Escherichia coli*, and 30 µL ddH_2O. These reactions were transferred to 0.1 cm cuvettes that had been cooled on ice, 250 µL of SOC medium added, placed into a pulser set to 1.85 kV and zapped, then placed into a shaker for 2 hrs at 37ºC at 200 rpm to allow the cells to grow.

Cells were then plated out by adding 50 µL of cloned cells and 50 µL SOC medium onto three agar-ampicillin plates (for both dimer/trimer and tetramer reactions) and incubated at 37ºC overnight. Colonies were then picked and placed in 10 µL ddH_2O for immediate analysis.

Microsatellite Amplification and Screening

Microsatellite colonies were amplified in the following reaction; 2.0 µL DNA, 0.4 µL forward and reverse primer (10mM) (M13-F and M13-R), 0.2 µL dNTP (10mM), 0.6 µL $MgCl_2$ (50 mM), 1.0 µL buffer (10×), 0.3 µL Astral Red *Taq* (1U/µL), and 5.1 µL ddH_2O. The PCR cycling conditions were as follows: 94ºC for 4 min; 35 cycles of: 30 sec at 94ºC, 40 sec at 53ºC, 1 min at 72ºC; then 4 min at 72ºC with the reaction stored at 4ºC. Colonies that PCR'd successfully were then cleaned and sequenced, then checked and edited using Sequencher 4.9. Primers were designed from those sequences that showed microsatellite repeat regions using the program BLAST. Fluorescent tags (FAM, VIC, NED, or PET) were added to either the forward or reverse primer sequence.

The first group of primers designed (Ten_01 through Ten_15; Table A4.2) were tested using the method described below. However, amplification success was low and therefore the entire Microsatellite Library Development section described above needed to be repeated up until this step, in order to design a second group of primers (4G, 6F, 6H, 7A, 7C, 7F, 8G, 12C; Table A4.2). Again, amplification success was low and again the Development section was repeated and a third group of primer designed (A11, C5, F1, F3, 12D, 8F; Table A4.2)

The PCR reactions for the initial testing of the first batch of primers (i.e. Ten01 through Ten15) contained the following components; 0.8 µL DNA extract, 0.02 µL tagged primer (10mM), 0.08 µL non-tagged primer (10mM), 0.08 µL fluorescent tag, 1.5 µL *bozine serum albumin* (BSA), 0.2 µL dNTP (10mM), 0.7 µL $MgCl_2$ (50 mM), 1.0 µL buffer (10×), 0.27 µL Fisher White *Taq* (5U/µL), and 5.35 µL ddH_2O. This reaction was modified for further testing of the first batch of primers as well as for the initial testing of the second batch (i.e. 4G, 6F, 6H, 7A, 7C, 7F, 8G, 12C), and consisted of the following; 0.8 µL DNA extract, 0.01 µL tagged primer (10mM), 0.4 µL non-tagged primer (10mM), 0.3 µL fluorescent tag, 1.5 µL BSA, 0.2 µL dNTP (10mM), 0.4 µL $MgCl_2$ (50 mM), 1.0 µL buffer (10×), 0.3 µL Astral Red *Taq* (1U/µL), and 5.0 µL ddH_2O. All reactions were performed on a GeneAmp PCR System 2700 (Applied Biosystems, www.appliedbiosystems.com).

The amplification temperatures were varied in order to find the optimal temperature for each primer, but the general cycling conditions were as follows: 94ºC for 5 min; 35 cycles of: 30 sec at 94ºC, 30 sec at 50ºC through 65ºC, 40 sec at 72ºC; then 40 min at 72ºC with the reaction stored at 4ºC. Although a range of cycling conditions were trialled, no primers consistently amplified across all samples and were thus discarded.

Table A4.2. Microsatellite primers developed.

Group	Primer ID	Forward and Reverse (5` → 3`)	Tag
1	Ten_01	TGCTCCTGCAGCCCGGTCCT GGCGTGGAATGTGTTCCGACCG	FAM
	Ten_02	TGTTGTGGGTCGCAACCTGGGG CGGCGTGGGTGACTGTGTTCCG	VIC
	Ten_03	AGCTCAAGCGTGGTCTAACTCACCCA TCCGACCGCTCTAACCCACTGACC	NED
	Ten_04	CCAGCTCCTACGTGGCCTGGGTC TGTGGGGTCGCAACCTGGGG	PET
	Ten_05	CGACTGTTGTGGGTCGCATCCTGG GCTTCTGGAGGGAAGGTGCCTCGT	FAM
	Ten_06	TGCCGTCACGTCTCCCGTCA TGGGAGACTGTGTTCCGACCGCT	FAM
	Ten_07	TTGGAAGAGGCAGTGGTCGAGGCAG TGTGGTCATGTCCCTCCTTTCTGCTCA	VIC
	Ten_08	ACCCGCTGGAGAGACTGTGGGA AGCCTGGTCGCGAACCTCTGAACC	NED
	Ten_09	ACGTGTGCCTACCCGGGCGT CCGACCGCTCTAACCCACTGAGC	PET
	Ten_10	TGGATGAGGCAGAGGTCGAGGCA GTGTGCTCCTAGGTGCTGCCGGT	FAM
	Ten_11	GCTCGATTCCTACTCGTTCAGCGCC ACCGCCCTTCACATGAGCAAACAC	FAM
	Ten_12	GGTGTTCCAGACACTACCTGCTTGTCT ACCACAATACACCAGGACTGACGGTCG	VIC
	Ten_13	GGGGCCAGGAGCTGTGTCTCG CCGACCGCTCTAACCCACTGAGC	NED
	Ten_14	CGCAAACAACACTGTATCCCGGCCT GACGTCCTCCACTCACACATTCCCA	PET
	Ten_15	ACCATGAGTGACGCAGCTTCCAGG GGCACCAGGTTGAATAAGCGTGGACC	FAM
2	4G	TGCATAATGTCAACTGGACAGCA GCTGCAAGGCGATTAAGTTG	VIC
	6F	CGGCACACAGAGCTGGGACA GCTGCAAGGCGATTAAGTTG	VIC
	6H	TATTTGACACCAGACATACAAAG GCTGCAAGGCGATTAAGTTG	VIC
	7A	CTCTTCGCTATTACGCCAGC CTCTGTCCCGCTGTTCTGCG	FAM
	7C	AAGGTGGTCAGTGAAAACA GTGGTGGCCAGCTGGTG	NED
	7F	AAGGTGGTCAGTGAAAACA TAGAGTCGACCTGCAGGCAT	PET
	8G	CGGCCGCCAGTGTGATGGAT GTGGTGGCCAGCTGGTG	NED
	12C	TGTGTGTGTTCTGTGTCGGTGG GTGGTGGCCAGCTGGTG	NED
3	A11	GGCGGGGCCAGGAGCTGTGTC GCGGTCGGAACACAGTCACCC	FAM
	C5	GGCCCATGCTGACTCATCCTC GAGTGGTGTAGGTGTCGTGTACA	NED
	F1	GGCGGGGCCAGGAGCTGTGTC GACCAGGTGGCAGGAACAACTACC	FAM
	F3	CGACAAGAGCCTCAGTCATCCC GTGTGAGTGGAGGACGTCAG	FAM
	12D	ACCCCGGCACACAGAGCTGGG CCCAAGCTTCCCGGGTACAGCAAGGGC	VIC
	8F	GAGGGACTGCGGTACCCGGGA GGTGTGTCCCAGCCTGC	PET

APPENDIX FIVE

Additional sequences obtained from GenBank with their associated Accession Number, specimen ID used in this study for molecular analyses, and the gene fragment analysed. * is used to indicate the Accession Number corresponds to the COI gene region, and ** to indicate the H3 gene region.

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Cherax glaber	Cherax_glaber	-	-	DQ079670**	✓		
Cherax quinquecarinatus	Cherax_quinquecarinatus	-	-	HM641111*	✓		
Cherax robustus	Cherax_robustus	EU977343	EU977412	-	✓	✓	✓
Engaeus affinis	CCE1.2	EU977344	-	-			✓
	CCE1.3	EU977345	-	-			✓
	CCE1.4	EU977346	-	-			✓
	MV1	EU977347	-	-			✓
Engaeus australis	MV6	EU977348	-	-			✓
Engaeus cisternarius	MV7	EF493110	-	-			✓
	MV9	EU977349	-	-			✓
	Q75	EU977350	-	-			✓
Engaeus cunicularius	Engaeus_cunicularius	AF135980	-	-			✓
Engaeus cymus	A29	EU977352	EU977417	-		✓	✓
	K4	AY223709	EU977418	-		✓	✓
Engaeus disjuncticus	Engaeus_disjuncticus	EF493102	-	-			✓
	MV51	EU977353	-	-			✓
Engaeus fossor	Engaeus_fossor	-	-	EU921144*	✓		
	Engaeus_fossor	AF135979	-	-			✓
	KC619	EU921121	-	-			✓
	MV53	EF493103	-	-			✓
Engaeus fultoni	AIR2.1	EF493042	EU977419	-		✓	✓
	ELZ1.1	EU977355	-	-			✓
	GEL1.1	EU977358	EU977420	-		✓	✓
	MV56	EU977356	-	-			✓
	PDS1.1	EU977354	-	-			✓
	STV1.1	EU977357	-	-			✓
Engaeus granulatus	MV98	EU977359	-	-			✓
Engaeus hemicirratulus	MV58	EF493104	-	-			✓
Engaeus karnanga	MV10	EU977360	EU977421	-		✓	✓
	MV59	EF493105	-	-			✓
Engaeus laevis	LEL1.1	EF493088	-	-			✓
	MV63	EF493106	-	-			✓
Engaeus lengana	MV224	EU977362	-	-			✓
	MV66	EU977363	-	-			✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Engaeus lengana	MV67	EU977361	-	-			✓
Engaeus lyelli	DUN1.1	EU977365	EU977422	-		✓	✓
	ENF1.2	EF493073	EU977423	-		✓	✓
	Engaeus_lyelli	AY223711	-	-			✓
	KC1180	FJ965950	-	-			✓
	MOY2.1	EU977366	-	-			✓
	MOY3.1	EU977367	-	-			✓
	MOY5.1	EU977368	-	-			✓
	MOY5.4	EU977369	-	-			✓
	MV71	EF493107	-	-			✓
	MV72	EF493108	-	-			✓
	NRN1.1	EU977370	EU977424	-		✓	✓
	NRN2.1	EF493121	-	-			✓
	RED2.1	EU977371	-	-			✓
	STA1.1	EU977372	-	-			✓
Engaeus mairener	MV78	EF493109	-	-			✓
Engaeus mallacoota	MV107	EF493096	-	-			✓
Engaeus martigener	MV80	EF493111	-	-			✓
Engaeus merosetosus	Engaeus_merosetosus	AY223712	-	-			✓
	WPC2.1	EF493153	-	-			✓
Engaeus nulloporius	MV84	EF493112	-	-			✓
Engaeus orientalis	MV85	EF493113	-	-			✓
Engaeus phyllocercus	A27	EF493041	EU977425	-		✓	✓
Engaeus quadrimanus	MOR1.2	EU977373	-	-			✓
	MOR1.3	EU977374	-	-			✓
	MOR2.2	EU977375	-	-			✓
	MV187	EU977377	-	-			✓
	WAN1.1	EU977376	-	-			✓
Engaeus sericatus	AIR3.1	EU313346	EU977426	-		✓	✓
	CUR2.1	EU313368	EU977427	-		✓	✓
	Engaeus_sericatus	-	-	FJ965960*	✓		
	Engaeus_sericatus	AF135981	-	-			✓
	PAN1.3	EU313402	EU977428	-		✓	✓
	PEN1.4	EF493125	-	-			✓
	WAL1.1	EU313411	EU977429	-			✓
Engaeus spinicaudatus	MV198	EU977379	-	-			✓
	MV89	EF493114	-	-			✓
Engaeus strictifrons	CRP1.1	EU977381	-	-			✓
	DTS1.1	EU977382	-	-			✓
	FIT1.3	EU977383	-	-			✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Engaeus strictifrons	KRK2.1	EU977384	-	-			✓
	MCP1.1	EU977385	-	-			✓
	MYA2.7	EU977386	-	-			✓
	TWH1.1	EF493149	-	-			✓
	WOO1.1	EU977387	-	-			✓
Engaeus tayatea	MV92	EU977388	-	-			✓
Engaeus tuberculatus	MCS1.2	EU977389	EU977430	-		✓	✓
	MON1.4	EU977390	-	-			✓
Engaeus urostrictus	HAR1.1	EU977391	EU977431	-		✓	✓
	MV94	EF493115	-	-			✓
Engaeus victoriensis	YRG1.1	EU977393	-	-			✓
Engaeus yabbimumma	MV116	EF493101	-	-			✓
Engaewa clade A	7	-				✓	✓
	8	JQ613144	-	-			✓
	9	JQ613151	-	-			✓
	10	JQ613107	-	-			✓
	11	JQ613113	-	-		✓	✓
Engaewa clade B	33	-	-	-			✓
	42	-	-	-			✓
	45	-	-	-			✓
	73	-	-	-			✓
	78	-	-	-			✓
	79	-	-	-			✓
	99	-	-	-		✓	✓
	107	-	-	-			✓
Engaewa pseudoreducta	104	JQ613110	-	-	✓	✓	✓
	105	JQ613111	-	-		✓	✓
	125	JQ613118	-	-	✓	✓	✓
Engaewa reducta	2	JQ613129	-	-			✓
	3	JQ613132	-	-			✓
	4	JQ613136	-	-		✓	✓
	15	JQ613128	-	-			✓
	24	JQ613130	-	-			✓
	26	JQ613131	-	-			✓
	80	JQ613145	-	-		✓	✓
	85	JQ613148	-	-			✓
	86	-	-	-		✓	✓
	89	JQ613150	-	-			✓
	90	JQ613152	-	-			✓
	92	JQ613153	-	-			✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Engaewa reducta	102	JQ613109	-	-		✓	✓
	124	JQ613117	-	-	✓	✓	✓
	133	JQ613123	-	-		✓	✓
	139	-	-	-		✓	✓
	141	JQ613125	-	-		✓	✓
	145	JQ613127	-	-		✓	✓
Engaewa similis	13	JQ613122	-	-			✓
	14	JQ613124	-	-			✓
	30	JQ613133	-	-		✓	✓
	32	JQ613134	-	-			✓
	34	JQ613135	-	-		✓	✓
	50	JQ613137	-	-		✓	✓
	52	JQ613138	-	-		✓	✓
	58	JQ613139	-	-		✓	✓
	59	JQ613140	-	-			✓
	60	JQ613141	-	-			✓
	61	JQ613142	-	-			✓
	81	JQ613146	-	-			✓
	82	JQ613147	-	-		✓	✓
	101	JQ613108	-	-			✓
	109	JQ613112	-	-		✓	✓
	118	JQ613122	-	-			✓
	119	JQ613114	-	-			✓
	122	JQ613115	-	-			✓
	123	JQ613116	-	-			✓
	126	JQ613119	-	-		✓	✓
	127	JQ613120	-	-			✓
	128	JQ613121	-	-			✓
	142	JQ613126	-	-		✓	✓
Engaewa subcoerulea	35	-	-	-		✓	✓
	39	-	-	-			✓
	51	-	-	-		✓	✓
	56	-	-	-		✓	✓
	116	-	-	-			✓
	135	-	-	-			✓
	146	-	-	-		✓	✓
	149	-	-	-			✓
Engaewa walpolea	46	-	-	-			✓
	47	-	-	-			✓
	62	-	-	-		✓	✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Engaewa walpolea	63	-	-	-			✓
	64	-	-	-			✓
	69	-	-	-			✓
	70	-	-	-			✓
	71	-	-	-			✓
	72	-	-	-			✓
	74	-	-	-		✓	✓
	75	-	-	-			✓
	76	-	-	-		✓	✓
	77	-	-	-			✓
	94	-	-	-			✓
	95	-	-	-			✓
	96	-	-	-		✓	✓
	114	-	-	-			✓
	138	-	-	-		✓	✓
Geocharax falcata	3632	-	-	AF493632*	✓		
	7437/SWC1.1	EF493144	EU977437	-	✓	✓	✓
	FWC1.1	EF493076	EU977435	-		✓	✓
	FWC1.2	EF493077	-	-			✓
	FYN1.1	EF493078	-	-			✓
	MWC1.1	EF493116	-	-			✓
	RED1.1	EF493134	EU977436	-		✓	✓
	RED1.2	EF493135	-	-			✓
	SWC1.2	EF493145	-	-			✓
	TRC1.1	EF493147	-	-			✓
	TRC1.3	EF493148	-	-			✓
Geocharax gracilis	1145	-	-	EU921145*	✓		
	3150	EF493150	-	-	✓		
	7439/MSQ1.3	EF493095	EU977439	-	✓	✓	✓
	BOG1.1	EF493046	-	-			✓
	BRY1.2	EF493052	EU977438	-		✓	✓
	CLS1.2	EF493054	-	-			✓
	CUR2.2	EF493058	-	-			✓
	CUR4.2	EF493059	-	-			✓
	EMU1.2	EF493072	-	-			✓
	KCR1.2	EF493084	-	-			✓
	MAR1.1	EF493090	-	-			✓
	MCK1.1	EF493091	-	-			✓
	MV110	EF493097	-	-			✓
	MV111	EF493098	-	-			✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Geocharax gracilis	MV113	EF493099	-	-			✓
	MV114	EF493100	-	-			✓
	PET1.1	EF493129	-	-			✓
	PIY1.2	EF493130	-	-			✓
	SLC1.1	EF493140	-	-			✓
	SPC1.4	EF493141	-	-			✓
	TMC1.1	EF493146	EU977440	-		✓	✓
	WAL1.3	EF493150	-	-			✓
	WAL1.5	EF493151	-	-			✓
	YAL1.3	EF493154	-	-			✓
Geocharax sp. nov. 1	BIG1.1	EF493044	-	-			✓
	BRA1.1	EF493048	-	-			✓
	BRN1.1	EF493050	-	-			✓
	BRN1.2	EF493051	-	-			✓
	BRN1.H113	EF493049	-	-			✓
	DER1.1	EF493061	-	-			✓
	DWY1.1	EF493064	-	-			✓
	DWY2.1	EF493065	-	-			✓
	EEL1.1	EF493068	EU977441	-		✓	✓
	GRR1.1	EF493081	-	-			✓
	KNB1.1	EF493085	EU977442	-		✓	✓
	LYN1.1	EF493089	-	-			✓
	PCP1.1	EF493122	-	-			✓
	ROC1.1	EF493137	-	-			✓
Geocharax sp. nov. 2	BNX1.4	EF493045	-	-			✓
	BOO1.1	EF493047	-	-			✓
	CAR1.10	EF493053	-	-			✓
	COX1.6	EF493056	-	-			✓
	CRP3.1	EF493057	-	-			✓
	CWF1.H118	EF493060	-	-			✓
	DNM1.2	EF493062	-	-			✓
	DUT1.H39	EF493063	-	-			✓
	ELL1.4	EF493071	-	-			✓
	FIT1.4	EF493074	-	-			✓
	FIT1.5	EF493075	-	-			✓
	GOW1.1	EF493079	-	-			✓
	HWK1.1	EF493082	-	-			✓
	JNS1.1	EF493083	-	-			✓
	KNC1.1	EF493086	EU977443	-		✓	✓
	KON1.1	EF493087	-	-			✓

Species	Sample ID	GenBank Accession Number			Chapter 4	Chapter 6	
		16S	GAPDH	Other	Gene Trees	Full Phylogeny	Species Tree
Geocharax sp. nov. 2	MOY1.10	EF493092	-	-			✓
	MOY3.6	EF493093	-	-			✓
	MYA2.1	EF493117	-	-			✓
	PEN2.1	EF493126	-	-			✓
	PEN3.9	EF493128	-	-			✓
	SER1.1	EF493138	-	-			✓
	SER2.3	EF493139	EU977444	-		✓	✓
	SRY1.1	EF493142	-	-			✓
	STF1.1	EF493143	-	-			✓
	WHC1.4	EF493152	-	-			✓
Gramastacus insolitus	1062	-	-	EU921062**	✓		
	3043	EF493043	-	-	✓		
	74453/3BX1.1	EF493040	EU977445	-	✓	✓	✓
	7446/DWY3.1	EF493066	EU977446	-	✓	✓	✓
	BCK1.1	EF493043	-	-			✓
	COX1.1	EF493055	-	-			✓
	DWY3.2	EF493067	-	-			✓
	EEL1.8	EF493069	-	-			✓
	MOY5.5	EF493094	-	-			✓
	PCP1.20	EF493123	-	-			✓
	PDR1.2	EF493124	-	-			✓
	PEN3.1	EF493127	-	-			✓
	RED3.1	EF493136	-	-			✓
Gramastacus lacus	7447/MYL1.1	EF493118	EU977447	-	✓	✓	✓
	7448/MYL1.3	EF493120	EU977448	-	✓	✓	✓
	MYL1.2	EF493119	-	-			✓
Tenuibranchiurus sp.	BRB1_1/BRB1.1	-	EU977452	-	✓	✓	
	Eu	EF493132	-	-	✓	✓	✓
	KNP	EF493131	-	-	✓	✓	✓
	Moo	EF493133	-	-	✓	✓	✓
	TEW_T1/TEW1.1	EU977400	EU977453	-	✓	✓	✓
	TL1/BEL1.2	EU977397	EU977450	-	✓	✓	✓
	TL2/BEL2.2	EU977398	EU977451	-	✓	✓	✓
	TL3	AF135998	-	-	✓	✓	✓

APPENDIX SIX

Genus *Tenuibranchiurus*

Cephalothorax shorter than abdomen; carapace higher than broad; cervical groove deeply impressed, rounded; branchiocardiac grooves prominent; areola wide; rostral carinae reduced or almost absent; sternal keel narrow, posterior pair of lateral processes large and flattened; male genital aperture on an arcuate medial projection of the coxopodite of the fifth periopods. The aperture is on the ventral extremity of the projection. Abdomen slightly wider than cephalothorax, smooth, pleural margins of somites rounded; first abdominal somite with pleural portions very much reduced; telson without transverse suture, entirely calcareous; branchial formula typically 18 + ep.; stem of podobranch not produced into a wing-like expansion; pleurobranchs reduced, typically to one situated on the last thoracic somite.

This genus is separated chiefly on the branchial arrangement. The gill-structure approaches most closely to that of *Parastacoides* Clark in the reduction in size of the posterior arthrobranchs and in the number of pleurobranchs, but the trend has proceeded further in *Parastacoides* which has no pleurobranchs. In most specimens of *Tenuibranchiurus glypticus* there is only the somewhat reduced posterior pleurobranch but in some females there are three, quite pronounced pleurobranchs on the last three thoracic somites.

Tenuibranchiurus glypticus

Diagnosis.— Small; eyes reduced; cephalothorax higher than broad; abdomen longer than cephalothorax; great chelae 80 to 85 per cent. of the body length; propodus and dactylus lying in a vertical plane.

Description of Adult.— Carapace finely punctate, branchiostegites finely tuberculate; carapace much shorter than abdomen, much higher than broad, two and one-half times as long as broad; cervical groove deeply impressed, very oblique laterally; branchiocardiac grooves strongly marked, not meeting the cervical groove dorsal but being carried anterolaterally just below it for some distance, posteriorly ending in small, irregular, transverse grooves just before the posterior border of the cephalothorax; areola wide, only a little more than twice as long as broad, sides almost parallel posteriorly; rostrum broad, reaching only to the base of the third segment of the antennular peduncle, one and one-half times as long as broad, carinae poorly developed and in some cases partly obsolete; post-orbital ridges very much reduced; eyes relatively small, slightly greater in diameter than one-

half the width of the rostrum at its base; antennule with the inner and outer flagella of equal size; antenna extending to the third segment of the abdomen, scaphocerite very broad anteriorly, ending in a short, sharp spine reaching to the middle of the second segment of the antenna and extending just beyond the rostrum; interantennal spine triangular, sharply pointed; exopodite of the third maxilliped long and flagellate; sternal keel narrow, moderately sharp, first two pairs of lateral processes rudimentary, third pair small, posterior pair large and broad, slightly flattened, processes between the fifth periopods small, lateral processes of the sternal keel without conspicuous openings; abdomen slightly wider than cephalothorax; telson rounded, one and one-half times as long as broad, a blunt spine on each lateral margin towards the posterior border; uropod rounded, slightly longer than telson, each ramus with a longitudinal, median carina ending in a small spine towards the posterior margin, outer rami each with a transverse suture along which there are a number of very fine spines; telson and uropods bordered with numerous long setae; lobes at base of uropods rounded; pleural portions of abdominal somites each with a few, long, thin setae; great chelae long and stout, 80 to 85 per cent. of the body length, held so that the dactylus lies vertically above the propodus and not medially to it; propodus two and one-half times as long as broad (lateral view), viewed dorsally at least four times as long as broad, upper margin feebly tuberculate with several irregular rows of tubercles, lower margin smooth. The tubercles extend over the whole of the dorsal and lateral surfaces of the propodus decreasing in size towards the ventral margin. Dactylus one-third as long as propodus, upper margin very feebly tuberculate; cutting edges of propodus and dactylus each with one or two well-developed tubercles; upper margins of carpus and merus feebly tuberculate; podobranchs without lateral, wing-like expansions, anterior arthrobranchs much larger than the posterior ones. Typically there is only a single pleurobranch, situated on the last thoracic somite, but occasionally in the female there are three, more strongly developed pleurobranchs on the last three thoracic somites. In the female the great chelae are slightly smaller and the abdomen very slightly wider than in the male.

Colour.— Greyish-brown tending to bluish-grey on the great chelae.

APPENDIX SEVEN

Table A7.1. Morphometric measurements for *Tenuibranchiurus* specimens. MC=missing chela; MP=missing portion of chela; R=regenerate; D=damaged.

	Specimen ID	Sex	Chela type	Occipital carapace length (mm)	Propodus length (mm)		Palm length (mm)		Propodus depth (mm)		Cephalothorax width (mm)	Cephalothorax depth (mm)	Rostral length/rostral width (mm)
					RHS or LD	LHS or SD	RHS or LD	LHS or SD	RHS or LD	LHS or SD			
Hervey Bay	HB_1	female	Isomorph	6.54	4.55	4.46	2.18	2.18	1.86	1.87	3.23	4.17	1.00
	HB_2	male	Isomorph	6.57	4.73	R	2.21	R	1.95	R	3.25	4.19	0.96
	HB_3	male	Isomorph	8.49	7.34	6.52	4.04	3.38	3.13	2.64	4.18	5.32	0.81
	HB_4	male	Isomorph	6.04	4.64	4.31	2.25	2.21	2.03	2.01	3.09	4.02	0.92
	HB_5	male	Isomorph	6.04	4.49	4.39	2.10	2.16	1.87	1.80	3.01	3.81	0.92
	HB_6	female	Isomorph	7.32	5.00	5.07	2.49	2.39	2.23	2.17	3.81	4.94	1.00
	HB_7	female	Isomorph	10.54	9.11	R	5.10	R	4.20	R	5.38	6.65	0.85
	HB_8	female	Isomorph	7.11	4.93	4.83	2.41	2.39	2.24	2.22	3.93	5.17	0.82
Maryborough	MAR_1	female	Isomorph	9.10	7.27	7.18	3.44	3.52	3.00	3.02	4.98	5.71	0.90
	MAR_2	male	Isomorph	11.36	10.98	10.67	5.93	5.90	4.21	4.10	5.96	7.12	0.95
	MAR_3	male	Isomorph	13.57	R	MC	R	MC	R	MC	7.17	8.46	0.93
	MAR_5	female	Isomorph	9.12	6.71	6.85	3.33	2.96	2.60	2.13	4.90	5.72	0.91
	MAR_7	male	Isomorph	9.39	7.63	7.81	3.80	3.99	2.79	2.92	5.00	5.96	0.94
	MAR_8	female	Isomorph	9.31	7.34	7.33	3.43	3.42	2.54	2.74	4.97	5.84	0.92
Type Locality	P11971	female	Isomorph	8.63	7.60	7.38	4.06	4.34	3.08	2.85	4.37	5.46	1.11
	P11972-1	female	Isomorph	8.40	R	6.39	R	3.24	R	2.77	3.93	5.81	1.03
	P11972-2	female	Isomorph	7.85	R	5.66	R	2.84	R	2.32	3.67	5.38	1.21
	P11972-3	male	Isomorph	7.12	6.64	6.58	3.87	3.87	3.00	3.00	3.47	4.91	0.97
	P11972-4	male	Isomorph	6.75	5.49	5.45	2.93	3.07	2.51	2.53	3.29	4.60	1.15
	P11972-5	female	Isomorph	6.63	5.06	5.09	2.57	2.69	1.97	1.99	3.27	4.60	1.17
Beerburrum	BER_8	female	Isomorph	9.76	8.93	MP	4.70	4.33	3.80	3.22	5.01	6.56	0.79
	BER_9	female	Isomorph	8.75	7.50	R	4.03	R	2.87	R	4.45	5.56	0.76
	BER_10	female	Isomorph	9.22	7.92	7.63	4.07	3.96	2.90	2.80	4.50	5.84	0.75
	BER_11	female	Isomorph	8.07	7.26	7.46	4.15	4.41	3.15	3.01	4.09	5.20	0.83
	BER_12	male	Isomorph	7.15	5.16	5.41	2.74	2.89	2.00	2.20	3.72	4.81	0.88
	BER_13	male	Isomorph	7.97	7.27	6.81	3.94	3.60	2.98	2.61	4.21	5.08	0.94

	Specimen ID	Sex	Chela type	Occipital carapace length (mm)	Propodus length (mm)		Palm length (mm)		Propodus depth (mm)		Cephalothorax width (mm)	Cephalothorax depth (mm)	Rostral length/rostral width (mm)
					RHS or LD	LHS or SD	RHS or LD	LHS or SD	RHS or LD	LHS or SD			
Beerburrum	BER_14	male	Isomorph	7.09	6.25	R	3.27	R	2.65	R	3.67	4.64	0.94
	BER_15	female	Isomorph	7.29	MC	5.68	MC	3.18	MC	2.60	3.92	4.73	0.88
	BER_16	male	Isomorph	8.89	8.20	7.79	4.22	4.55	3.43	3.49	4.54	5.39	0.85
	BER_17	female	Isomorph	10.17	8.31	R	4.71	R	3.93	R	5.16	6.50	0.73
Bribie Island	BRB_7	male	Isomorph	8.15	R	8.68	R	4.92	R	3.39	4.12	5.26	1.00
	BRB_8	male	Isomorph	8.36	9.59	9.67	5.56	5.42	3.63	3.49	4.42	5.53	1.00
	BRB_9	female	Isomorph	8.54	7.45	7.31	3.89	3.80	2.84	2.83	4.50	5.07	1.00
	BRB_10	female	Isomorph	9.37	R	R	R	R	R	R	4.97	6.06	1.00
	BRB_11	male	Isomorph	8.23	8.24	8.16	4.36	4.53	3.00	2.95	4.48	5.12	D
	BRB_12	female	Isomorph	9.94	R	R	R	R	R	R	6.00	6.68	1.00
Tewantin	TEW_8	male	Isomorph	6.12	R	4.43	R	2.46	R	1.99	2.79	3.84	0.82
	TEW_9	female	Isomorph	5.67	4.28	MC	2.31	MC	1.76	MC	2.76	3.69	0.80
	TEW_10	male	Isomorph	6.55	5.02	R	2.64	R	2.10	R	3.26	4.28	0.79
	TEW_11	female	Isomorph	6.79	R	4.91	R	2.54	R	1.57	3.31	4.29	0.96
	TEW_12	female	Isomorph	6.38	4.68	4.69	2.39	2.54	1.89	1.82	3.04	3.84	0.88
	TEW_13	female	Isomorph	6.48	R	4.42	R	2.35	R	1.93	3.28	4.21	0.90
	TEW_14	male	Isomorph	6.58	4.60	4.85	2.40	2.48	1.85	1.89	3.08	4.02	0.83
	TEW_15	female	Isomorph	6.09	4.52	4.09	2.39	2.09	2.10	1.85	3.12	4.01	0.83
	TEW_16	female	Isomorph	6.48	4.31	4.39	2.41	2.28	1.83	1.79	3.27	4.03	0.83
	TEW_17	male	Isomorph	5.49	3.90	R	1.98	R	1.59	R	2.74	3.64	0.80
Gold Coast	GC2_6	male	Isomorph	7.99	6.99	7.34	3.87	3.86	2.94	2.95	4.38	4.95	0.80
	GC2_7	female	Isomorph	8.43	6.98	7.03	3.66	3.89	2.93	2.99	4.43	5.16	0.80
	GC2_8	male	Isomorph	5.77	4.55	4.43	2.38	2.29	1.94	2.02	3.15	3.79	0.82
	GC2_9	male	Isomorph	6.87	5.53	5.42	2.98	2.83	2.40	2.45	3.56	4.31	0.84
	GC2_10	male	Isomorph	7.84	8.17	8.23	4.63	4.48	3.09	3.41	4.29	5.04	0.86
Lake Hiawatha	LakeH_2	male	Isomorph	8.54	5.70	R	2.98	R	2.54	R	4.37	5.32	0.86
	LakeH _4	male	Dimorph	10.25	10.14	8.17	5.82	4.27	5.40	3.70	5.41	6.35	0.85
	LakeH _5	male	Dimorph	8.34	7.86	6.25	4.50	3.11	3.87	2.66	4.19	5.25	0.84
	LakeH _7	female	Dimorph	10.05	9.48	8.07	5.62	4.40	5.31	3.63	5.40	6.59	0.78

	Specimen ID	Sex	Chela type	Occipital carapace length (mm)	Propodus length (mm)		Palm length (mm)		Propodus depth (mm)		Cephalothorax width (mm)	Cephalothorax depth (mm)	Rostral length/rostral width (mm)
					RHS or LD	LHS or SD	RHS or LD	LHS or SD	RHS or LD	LHS or SD			
Lake Hiawatha	LakeH _8	male	Dimorph	7.70	6.95	5.81	3.95	3.21	3.55	2.71	3.86	4.91	0.80
	LakeH _9	male	Isomorph	8.83	R	R	R	R	R	R	4.60	5.95	0.76
	LakeH _10	male	Dimorph	11.66	12.74	9.86	7.49	4.95	7.07	4.60	5.97	7.63	0.73
	LakeH _11	male	Isomorph	11.50	10.45	10.65	6.00	5.67	5.18	5.25	5.66	7.40	0.80
	LakeH _12	male	Isomorph	7.11	6.11	6.10	3.26	3.49	2.84	2.80	3.69	4.38	0.73
Broadwater National Park	BNP1_1	female	Dimorph	7.93	7.36	R	4.31	R	3.74	R	4.25	5.31	0.86
	BNP1_2	female	Isomorph	9.37	7.81	7.66	3.93	4.03	3.55	3.42	5.00	6.33	0.91
	BNP1_8	male	Isomorph	7.77	6.78	6.67	3.64	3.69	3.16	3.18	4.33	5.35	0.83
	BNP1_14	male	Isomorph	6.06	5.13	5.02	2.61	2.72	2.30	2.29	3.10	3.75	0.73
	BNP1_15	male	Isomorph	6.65	5.62	5.58	2.97	2.96	2.54	2.53	3.42	4.24	0.71
Lennox Head	LH_2	female	Dimorph	9.16	9.02	7.08	4.84	3.32	4.78	2.99	4.69	5.85	0.87
	LH_4	female	Isomorph	10.72	R	9.11	R	4.60	R	4.15	5.59	7.14	0.75
	LH_7	female	Isomorph	7.49	6.56	6.39	3.44	3.51	2.95	3.00	3.83	4.84	0.80
	LH_11	female	-	9.30	9.13	8.28	4.82	4.04	4.18	3.68	D	D	0.87
	LH_13	female	-	7.68	6.77	R	3.22	R	3.35	R	3.73	4.74	0.92
	LH_14	male	Isomorph	7.24	6.61	6.77	3.49	3.55	3.11	3.27	3.76	4.72	0.91
	LH_15	male	-	6.78	6.25	5.28	3.45	2.67	3.04	2.27	3.27	4.24	0.83
	LH_16	male	Dimorph	8.60	8.33	R	4.48	R	4.36	R	4.16	5.41	0.80
	LH_17	male	Isomorph	9.05	R	7.47	R	3.98	R	3.79	4.31	5.31	0.80
	LH_18	female	Dimorph	9.85	10.09	7.60	5.70	3.57	5.23	3.17	4.89	6.22	0.82
	LH_19	male	Isomorph	10.30	10.00	9.82	5.23	5.46	4.50	4.76	4.99	6.43	0.78

APPENDIX EIGHT

Table A8.1. List of characters and their associated character states examined across all specimens. * indicates characters used in final statistical analyses.

Character Code	Character	Character State				
		A	B	C	D	E
1	Rostral length (relative to the base of the 3rd antennal segment)	Base to middle of second	Base of second to base of third	Middle of second to base of third		
2*	Rostral carinae length	Extending to posterior margin of orbit and reaching to approximately ½ of the way along rostrum	Extending to posterior margin of orbit and reaching to approximately $^{2}/_{3}$ of the way along rostrum	Extending to posterior margin of orbit and reaching to tip of rostrum but not fusing with tip		
3*	Rostral carinae definition	Very poorly to poorly developed, inconspicuously raised	Moderately developed, moderately raised	Well developed, conspicuously raised		
4*	Rostral spine	Absent	Very small	Small	Medium	
5*	Post-orbital ridges	Very poorly developed	Poorly developed	Moderately developed		
9*	Cephalothorax punctation (*Dorsal*; anterior to cervical groove)	Sparse	Moderate			
10*	Cephalothorax punctation (*Dorsal*; posterior to cervical groove)	None to very sparse	Sparse	Moderate		
11*	Cephalothorax granulation (*Lateral*)	Moderate	Dense			
13*	Branchiocardiac groove	Transverse grooves present just before posterior margin of the cephalothorax	No transverse grooves present just before posterior margin of the cephalothorax			
17*	Basipodite spine	Absent	Very small	Medium	Large	Very large
18	Branchiostegal spine	Absent	Present			
19*	Suborbital spine	Absent	Very small	Small	Medium	Large
23	Chela shape	Isomorphic	Dimorphic			
24*	Propodal palm granulation (*Ventral*)	Small, no carinae	Large, no carinae	Margin smooth and carinate		
25*	Propodal palm granulation (*Dorsal*)	Small	Moderate	Large	Irregular row of small tubercles along margin	Irregular row of large tubercles along margin
26*	Propodal palm granulation/punctation (*Lateral*)	Small granulation	Large granulation	Very sparsely punctate		

Character Code	Character	Character State				
		A	B	C	D	E
27	Propodal palm granulation/punctation (*Mesial*)	Very small granulations	Moderately dense and small granulations	Dense and large granulations	Very small granulations anteriorly and ventrally, very slightly and sparsely punctate posteriorly	Moderately dense and moderate granulations dorsally, decreasing in density ventrally
29	Propodal finger (*Ventral*)	No carina	Carinate			
31*	Propodal finger granulation/punctation (*Lateral*)	Smooth, sparse granulation ventrolaterally	Smooth halfway with dense granulation posteriorly	Very sparsely punctate		
32	Propodal finger granulation (*Mesial*)	Smooth	Very small ventromesially, dorsomesially smooth	Moderate posteriorly, smooth anteriorly for the first ¾		
34*	Dactylus granulation (*Dorsal*)	Small	Moderate	Large	Margin with 2 rows of tubercles	
35	Dactylus punctation (*Lateral*)	Smooth	Very sparse			
37	Dorsomeral spine	Absent	Present			
38*	Ventromeral spine	Absent	Medium	Large	Very large	
39	Ventral meral inner edge tubercles	Large	Very large			
40	Ventral meral outer edge tubercles	Small	Small, decreasing in size towards anterior, with one or two small tubercles inside outer row			
41	Ventral meral surface	Smooth, dense setae	Granulate, moderate setae			
42*	Meral margin tubercles (*Dorsal*)	One row	Two rows			
43*	Meral spine (*Dorsal*)	Absent	Present			
46*	Ischium (Dorsal margin)	Smooth	One row of small tubercles	One row of large tubercles	Two rows of small tubercles	
49	Carpal dorsal surface	Granulate	Punctate			

Table A8.2. List of all characters analysed across all specimens. Ticks indicate character state displayed. Refer to Table A8.1 for character details.

	Character	1			2			3			4				5			9		10			11		13		17					18		19					23		24			25					26		
	Specimen ID	a	b	c	a	b	c	a	b	c	a	b	c	d	a	b	c	a	b	a	b	c	a	b	a	b	a	b	c	d	e	a	b	a	b	c	d	e	a	b	a	b	c	a	b	c	d	e	a	b	c
Hervey Bay	HB_1		✓		✓			✓				✓			✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	HB_2		✓		✓			✓				✓			✓			✓		✓			✓			✓				✓		✓					✓		✓			✓					✓		
	HB_3		✓		✓			✓				✓			✓			✓		✓			✓		✓				✓			✓					✓		✓			✓					✓		
	HB_4		✓		✓			✓					✓		✓			✓		✓			✓		✓				✓			✓					✓		✓			✓					✓		
	HB_5		✓		✓			✓					✓		✓			✓		✓			✓			✓	✓					✓					✓		✓			✓					✓		
	HB_6		✓		✓			✓					✓		✓			✓		✓			✓		✓				✓			✓					✓		✓			✓					✓		
	HB_7		✓		✓			✓					✓		✓			✓		✓			✓			✓				✓		✓					✓		✓			✓					✓		
	HB_8		✓		✓			✓			✓				✓			✓		✓			✓			✓			✓			✓					✓		✓			✓					✓		
Maryborough	MAR_1	✓				✓		✓				✓			✓			✓		✓			✓		✓				✓			✓					✓		✓				✓				✓		
	MAR_2	✓				✓		✓					✓		✓			✓		✓			✓		✓				✓			✓					✓		✓				✓				✓		
	MAR_3	✓				✓		✓				✓			✓			✓		✓			✓		✓				✓			✓					✓		✓				✓				✓		
	MAR_5	✓				✓		✓					✓		✓			✓		✓			✓		✓				✓			✓					✓		✓				✓				✓		
	MAR_7	✓				✓		✓					✓		✓			✓		✓			✓		✓					✓		✓					✓		✓				✓				✓		
	MAR_8	✓				✓		✓					✓		✓			✓		✓			✓		✓				✓			✓					✓		✓				✓				✓		
Type Locality	P11971		✓		✓				✓		✓				✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	P11972-1		✓		✓			✓			✓				✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	P11972-2		✓		✓			✓				✓			✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	P11972-3		✓		✓			✓				✓			✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	P11972-4		✓		✓			✓			✓				✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	P11972-5		✓		✓			✓				✓			✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
Beerburrum	BER_8		✓		✓				✓			✓			✓			✓			✓		✓		✓					✓		✓					✓		✓				✓				✓		
	BER_9		✓		✓			✓				✓			✓			✓			✓		✓			✓				✓		✓					✓		✓				✓				✓		
	BER_10		✓		✓				✓			✓			✓			✓			✓		✓		✓					✓		✓					✓		✓				✓				✓		
	BER_11		✓		✓				✓				✓		✓			✓			✓		✓			✓			✓			✓					✓		✓				✓				✓		
	BER_12		✓		✓				✓			✓			✓			✓			✓		✓		✓					✓		✓					✓		✓				✓				✓		
	BER_13		✓			✓			✓			✓			✓			✓			✓		✓		✓					✓		✓					✓		✓				✓				✓		
	BER_14		✓			✓			✓			✓			✓			✓			✓		✓		✓				✓			✓					✓		✓				✓				✓		
	BER_15		✓		✓			✓				✓			✓			✓			✓		✓			✓				✓		✓					✓		✓				✓				✓		
	BER_16		✓		✓			✓				✓			✓			✓			✓		✓			✓				✓		✓					✓		✓				✓				✓		
	BER_17		✓		✓			✓				✓			✓			✓			✓		✓			✓			✓				✓				✓		✓				✓				✓		

	Character	1			2			3			4				5			9		10			11		13		17					18		19					23		24			25					26		
	Specimen ID	a	b	c	a	b	c	a	b	c	a	b	c	d	a	b	c	a	b	a	b	c	a	b	a	b	a	b	c	d	e	a	b	a	b	c	d	e	a	b	a	b	c	a	b	c	d	e	a	b	c
Bribie Island	BRB_7		✓		✓			✓			✓				✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	BRB_8		✓			✓			✓				✓		✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	BRB_9		✓		✓			✓					✓		✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	BRB_10		✓			✓			✓		✓				✓			✓		✓			✓		✓			✓				✓					✓		✓			✓					✓		
	BRB_11		✓					✓							✓			✓		✓			✓		✓					✓			✓				✓		✓			✓					✓		
	BRB_12		✓		✓				✓				✓		✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
Tewantin	TEW_8		✓			✓			✓		✓				✓			✓		✓			✓			✓				✓		✓			✓				✓			✓			✓				✓		
	TEW_9		✓		✓			✓			✓				✓			✓		✓			✓		✓					✓		✓			✓				✓			✓			✓				✓		
	TEW_10		✓			✓			✓			✓			✓			✓		✓			✓			✓				✓		✓			✓				✓			✓			✓				✓		
	TEW_11		✓		✓			✓			✓				✓			✓		✓			✓			✓					✓		✓				✓			✓			✓				✓		
	TEW_12		✓			✓				✓	✓				✓			✓		✓			✓			✓				✓		✓				✓			✓			✓				✓			✓		
	TEW_13		✓			✓				✓	✓				✓			✓		✓			✓			✓		✓				✓				✓			✓			✓				✓			✓		
	TEW_14		✓		✓			✓			✓				✓			✓		✓			✓			✓					✓		✓				✓			✓			✓				✓			✓		
	TEW_15		✓		✓			✓			✓				✓			✓		✓			✓			✓				✓		✓			✓				✓			✓				✓			✓		
	TEW_16		✓		✓			✓			✓				✓			✓		✓			✓			✓				✓		✓			✓				✓			✓				✓			✓		
	TEW_17		✓		✓			✓			✓				✓			✓		✓			✓			✓					✓		✓				✓			✓			✓				✓			✓		
Gold Coast	GC2_6		✓		✓			✓			✓				✓				✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	GC2_7		✓		✓			✓			✓					✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	GC2_8		✓		✓			✓			✓				✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	GC2_9		✓		✓			✓			✓					✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
	GC2_10		✓		✓			✓			✓				✓			✓		✓			✓		✓					✓		✓					✓		✓			✓					✓		
Lake Hiawatha	LakeH_2	✓				✓			✓		✓				✓			✓		✓			✓		✓		✓					✓		✓					✓		✓			✓					✓		
	LakeH _4	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓				✓					✓			✓		✓			✓					✓		
	LakeH _5	✓				✓			✓		✓				✓			✓		✓			✓			✓	✓					✓		✓					✓		✓			✓					✓		
	LakeH _7	✓				✓			✓		✓				✓			✓		✓			✓		✓		✓					✓		✓					✓		✓			✓					✓		
	LakeH _8	✓				✓			✓		✓					✓		✓		✓			✓			✓		✓				✓			✓				✓		✓			✓					✓		
	LakeH _9	✓				✓			✓		✓					✓		✓		✓			✓			✓		✓				✓		✓					✓			✓			✓					✓		
	LakeH _10	✓				✓			✓		✓				✓			✓		✓			✓			✓		✓				✓			✓				✓		✓			✓					✓		
	LakeH _11	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓				✓			✓				✓			✓			✓					✓		
	LakeH _12	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓				✓			✓				✓			✓			✓					✓		

	Character	1			2			3			4				5			9		10			11		13		17					18		19					23		24			25					26		
	Specimen ID	a	b	c	a	b	c	a	b	c	a	b	c	d	a	b	c	a	b	a	b	c	a	b	a	b	a	b	c	d	e	a	b	a	b	c	d	e	a	b	a	b	c	a	b	c	d	e	a	b	c
Broadwater	BNP1_1	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓					✓			✓					✓		✓				✓				✓		
	BNP1_2	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓					✓			✓					✓		✓				✓				✓		
	BNP1_8	✓				✓			✓		✓				✓			✓		✓			✓		✓			✓					✓				✓				✓		✓				✓				✓		
	BNP1_14	✓				✓			✓		✓					✓		✓		✓			✓		✓			✓					✓			✓					✓		✓				✓				✓		
	BNP1_15	✓				✓			✓		✓				✓			✓		✓			✓		✓				✓				✓			✓					✓		✓				✓				✓		
Lennox Head	LH_2	✓				✓			✓				✓			✓			✓	✓			✓		✓		✓					✓		✓						✓		✓			✓					✓		
	LH_4	✓				✓			✓				✓			✓			✓	✓			✓		✓		✓					✓		✓					✓			✓			✓					✓		
	LH_7	✓				✓			✓				✓			✓			✓	✓			✓		✓			✓				✓		✓					✓			✓			✓					✓		
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APPENDIX NINE

A9.1 Chela morphometrics for Queensland populations

The chela analyses were divided into two parts. Part One investigates whether there were population-specific differences in the relative sizes of chelae (i.e. standardised for overall size using OCL), whereas Part Two explored whether there is a difference in the ratio of one chela measurement to another between populations. For Part One, the size-standardised PropD, PropL, and PalmL were compared between populations, whereas these chela measurements were compared to each other in Part Two. For all analyses, the potential effect of both size-dependent development and sex was tested for (see Chapter 3 section 3.3.3.1 for more detail).

Part One

The raw measurements for PropL, PalmL, and PropD were graphed against OCL for each population separately to check for a linear relationship. Initial tests showed an abnormal relationship within BRB, where an increase in all chela measurements was not due to an increase in OCL (i.e. all specimens had roughly the same OCL but very variable chela measurements; discussed in Chapter 3 section 3.5.1). This was the only population to show an irregular relationship and therefore it was removed, with the remainder of the populations graphed together, with sex separated (Figure A9.1). For all chela measurements, there was a strong linear relationship for both males and females in relation to OCL, indicating that there is not a point at which the chela dimensions change from isometric to allometric scaling. It should be noted, however, that there was a trend for males to have larger chela dimensions than females for all comparisons.

The effect of sex on all size-standardised variables was tested using a GLM. There was a significant difference found between sex ($p<0.001$). Post-hoc analyses showed a significant difference between sex for PropL/OCL for BRB ($p<0.001$) and MAR ($p=0.011$), PalmL/OCL for BRB ($p<0.001$), MAR ($p=0.002$), and TL ($p=0.019$), and PropD/OCL for BRB ($p=0.017$), MAR ($p=0.041$), and TL ($p<0.001$) (Figure A9.2). Although the differences found may be indicative of sexual dimorphism in these three populations, it was decided that sex would not be separated in further analyses. This approach was taken for two reasons; (1) as sample sizes are already relatively small for each population and splitting them by sex would decrease them substantially to the point where some sex-specific populations would be represented by only a single specimen, thereby decreasing the power of any statistical

analysis performed and increasing the potential for false or misleading conclusions, and (2) as it was the more conservative approach to take, where any variation in the characters was increased, therefore decreasing the likelihood of separating populations into groups/species where it was not warranted. This reasoning is discussed in further detail in Chapter 3 section 3.5.1.

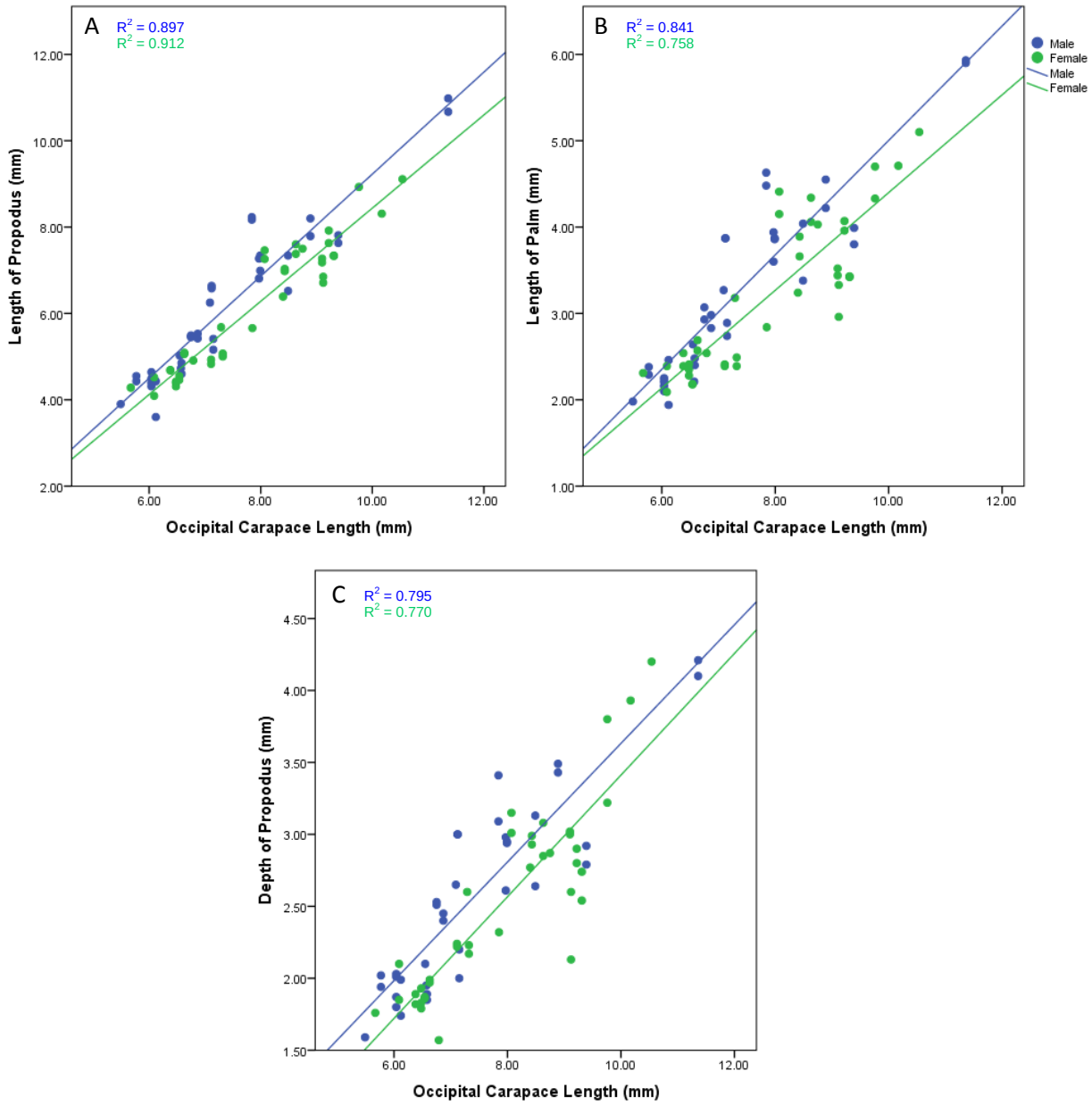

Figure A9.1. Regression of chela dimensions relative to occipital carapace length across Queensland populations (excluding BRB) with males and females separated. Occipital carapace length (mm) plotted against (A) propodal length (mm), (B) palm length (mm), and (C) propodal depth (mm). 75 chelae were measured from 44 *Tenuibranchiurus* specimens across six populations.

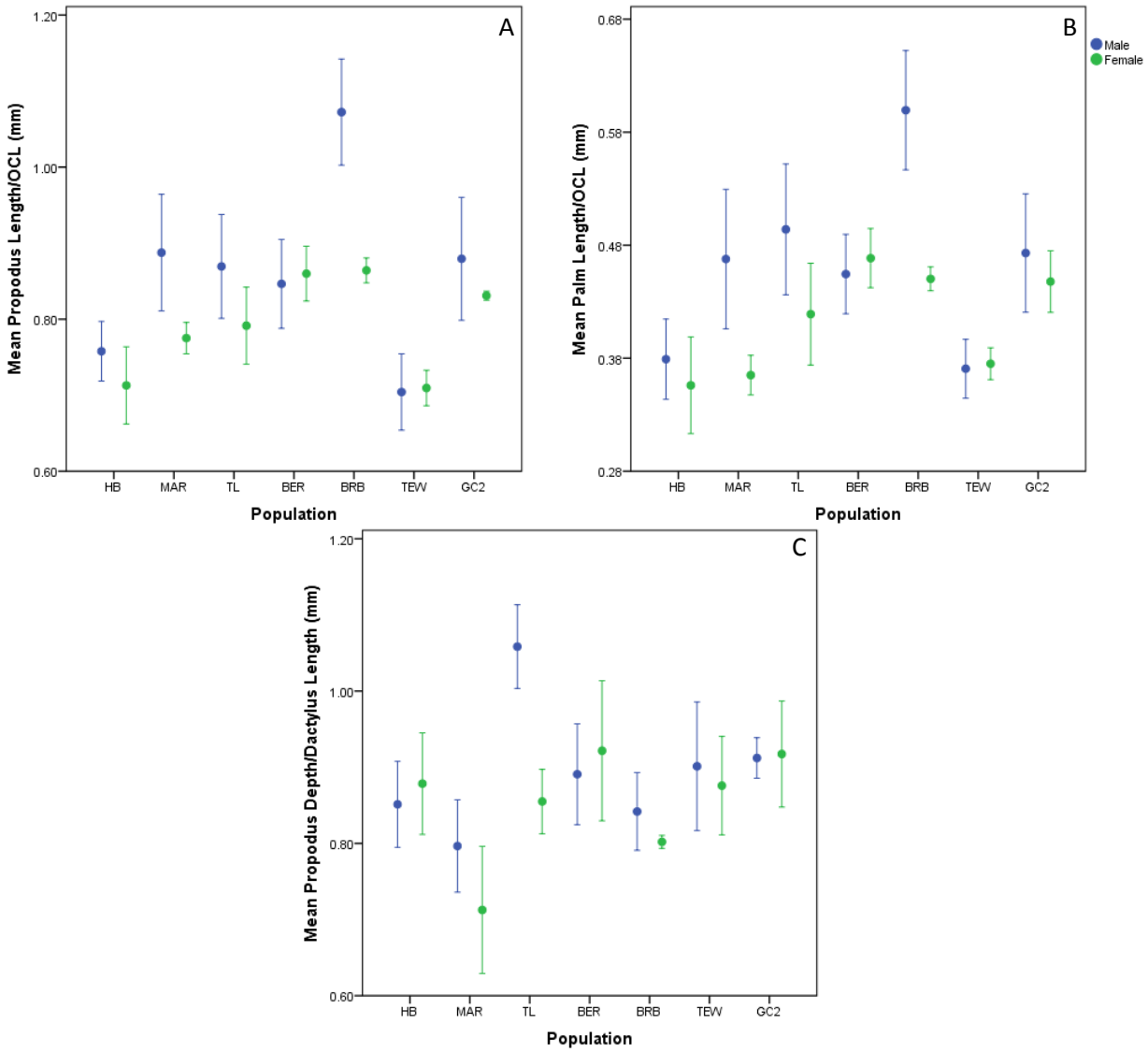

Figure A9.2. Observed differences in sex found within some Queensland populations. Standard error plot (±2 S.E.) showing the mean (A) propodal length to occipital carapace length (mm) showing a significant difference between sex at MAR and BRB, (B) palm length to occipital carapace length (mm) showing a significant difference between sex at MAR, BRB, and TL, and (C) propodal depth to occipital carapace length (mm) showing a significant difference between sex at MAR, BRB, and TL. 82 chelae were measured from 48 *Tenuibranchiurus* specimens across seven populations.

The GLM found a significance difference between populations (p<0.001) for all variables, with the post-hoc results graphed below (Figure A9.3). BRB had a significantly different PropL/OCL and PalmL/OCL ratio from all other populations (p<0.05), whereas HB and TEW also tended to have much smaller PropL/OCL and PalmL/OCL ratios than the other populations, though not always significantly different. These trends did not hold as strongly for the comparison of PropD/OCL. Although BRB was still the highest, the ratio was not significantly different to all other populations. HB and TEW were again among the smallest ratios; however, MAR was also quite small and statistically not significantly different to HB and TEW.

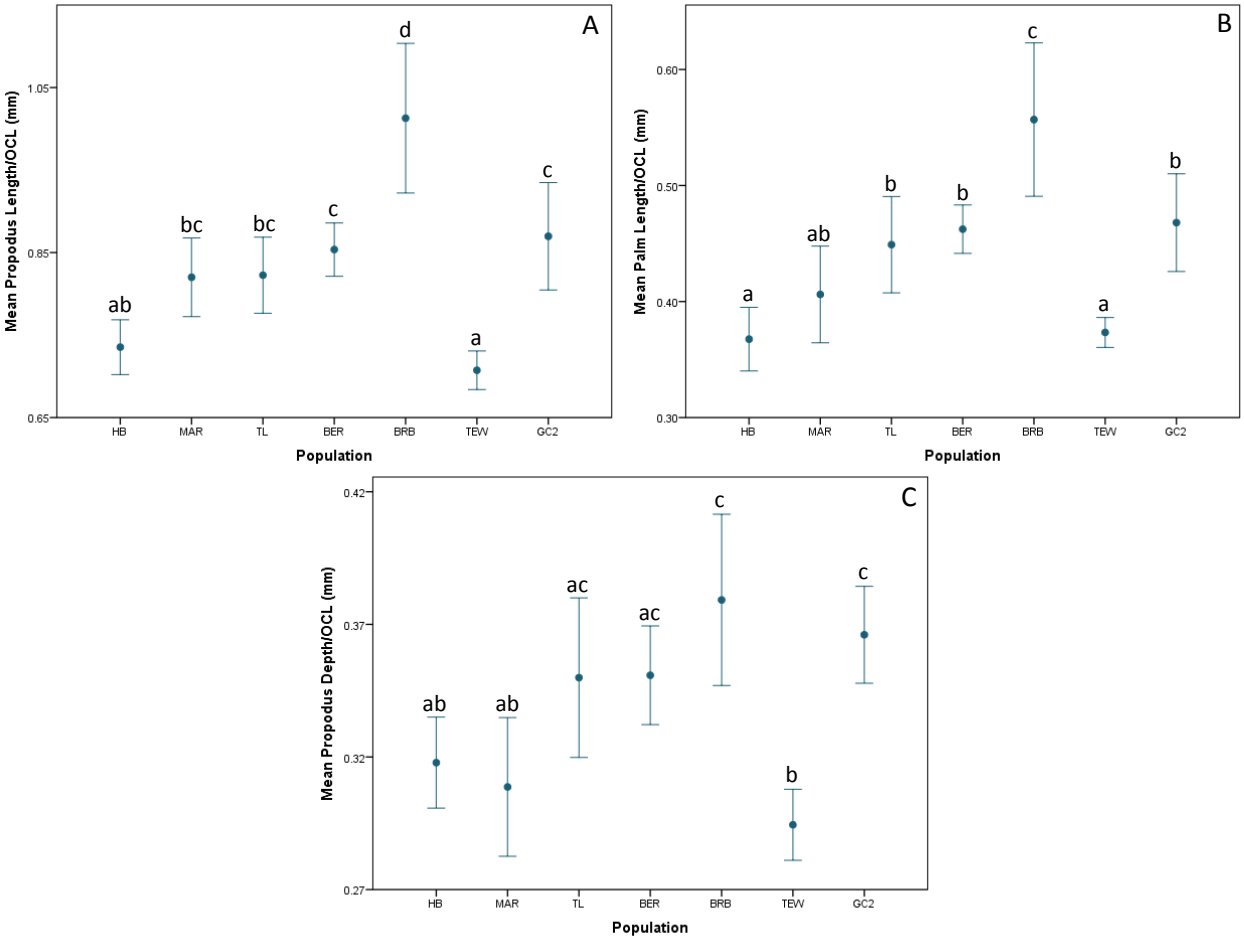

Figure A9.3. Observed differences in chela dimensions relative to occipital carapace length found between Queensland populations. Standard error plot (±2 S.E.) showing the mean (A) propodal length to occipital carapace length (mm), (B) palm length to occipital carapace length (mm), and (C) propodal depth to occipital carapace length (mm). Significant differences are indicated by lowercase letters. 82 chelae were measured from 48 *Tenuibranchiurus* specimens across seven populations.

Part Two

To test for the effect of sex, a GLM was performed on all chela comparisons (i.e. PropD/PalmL, PropD/PropL, and PalmL/PropL). There was a significant difference found between sex for PalmL/PropL (p=0.006), with the post-hoc showing a significant difference in populations MAR (p=0.001) and TL (p=0.014). As for the Part One analyses, the more conservative approach was taken, with sex analysed together.

There was a significant difference between populations (p<0.001) for all chela comparisons. A Tukey post-hoc analysis indicated where the differences occurred and are graphed (Figure A9.4). The results showed that HB had a significantly higher PropD/PalmL ratio than all other populations (p<0.05) and BRB was a lot lower than most other populations, though not always statistically significant. Both HB and MAR showed a significantly lower PalmL/PropL ratio than all other populations (p<0.05), and both MAR and BRB had a significantly lower PropD/PropL ratio than all other populations (p<0.05), except BER.

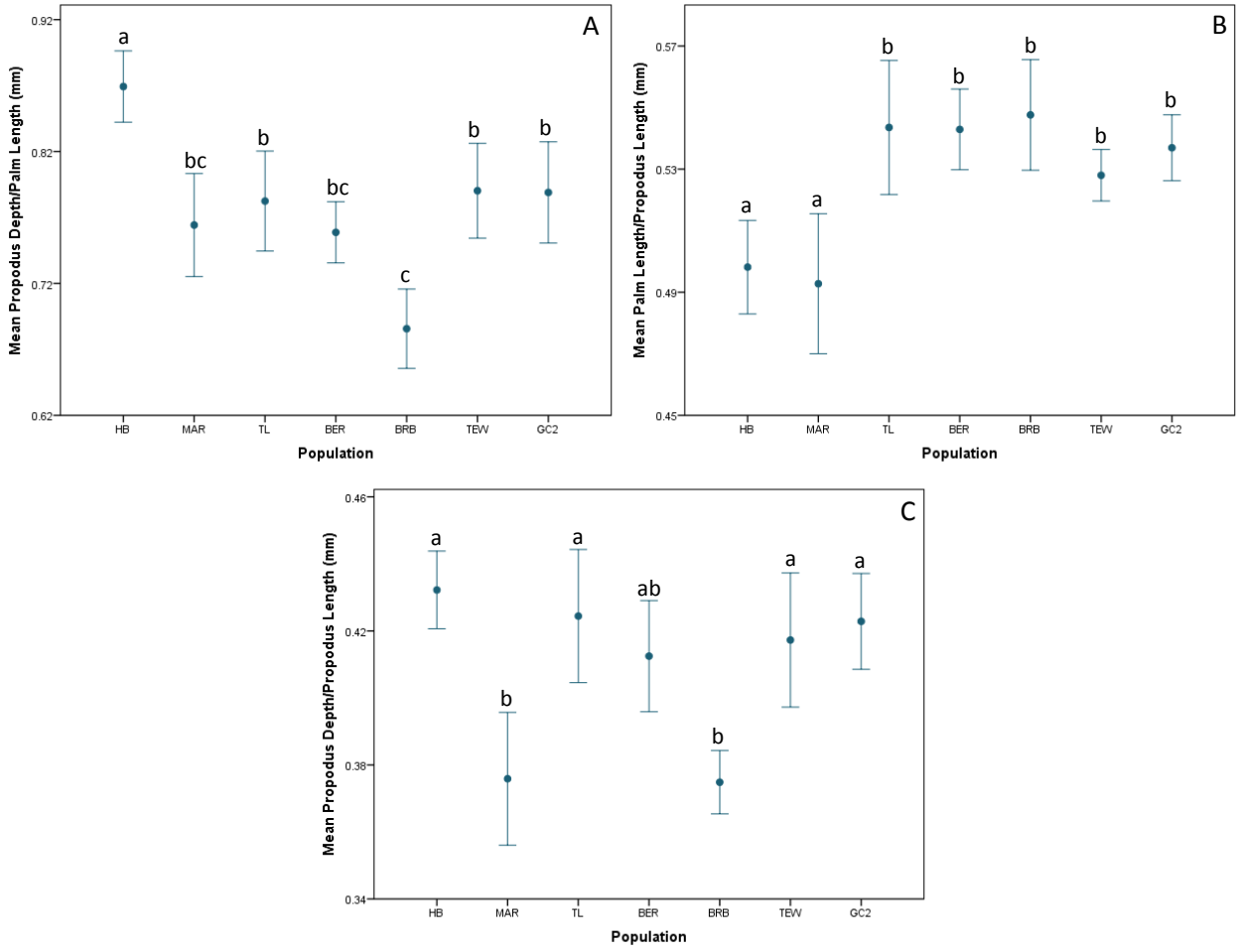

Figure A9.4. Observed differences between chela dimensions found between Queensland populations. Standard error plot (±2 S.E.) showing the mean (A) propodal depth to palm length (mm), (B) palm length to propodal length (mm), and (C) propodal depth to propodal length (mm). Significant differences are indicated by lowercase letters. 82 chelae were measured from 48 *Tenuibranchiurus* specimens across seven populations.

A9.2 Cephalothorax morphometrics for Queensland populations

The raw measurements for CephD and CephW were graphed against OCL to check for a linear relationship, with sex graphed separately (Figure A9.5). A strong linear relationship was found for both males and females for both CephD and CephW in relation to OCL, indicating no change in growth rate with age or sex. There was no significant difference (p>0.05) between sex at any population for either variable, and therefore they were not separated in further analyses.

A significant difference was found between populations (p<0.001), with the results of the post-hoc analysis graphed (Figure A9.6). Both TL and TEW had a significantly smaller CephW/OCL than all other populations (p<0.05), and MAR, BRB, and GC2 significantly larger (p<0.05). TL also had significant difference in CephD/OCL to all other populations (p<0.01) except HB. When comparing CephD to CephW, TL again had a significantly higher ratio than all other populations (p<0.001), with MAR, BRB, and GC2 significantly lower than all other populations (p<0.05).

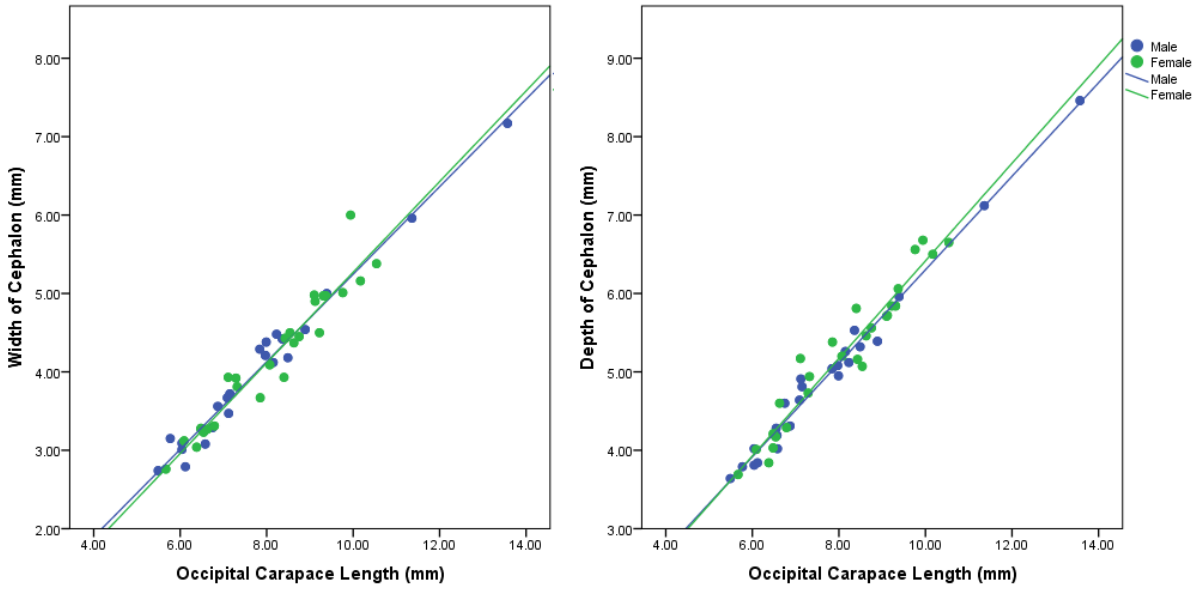

Figure A9.5. Regression of cephalothorax dimensions relative to occipital carapace length across all Queensland populations. Occipital carapace length (mm) plotted against (A) cephalothorax depth (mm), and (B) cephalothorax width (mm). 51 *Tenuibranchiurus* specimens across seven populations were measured.

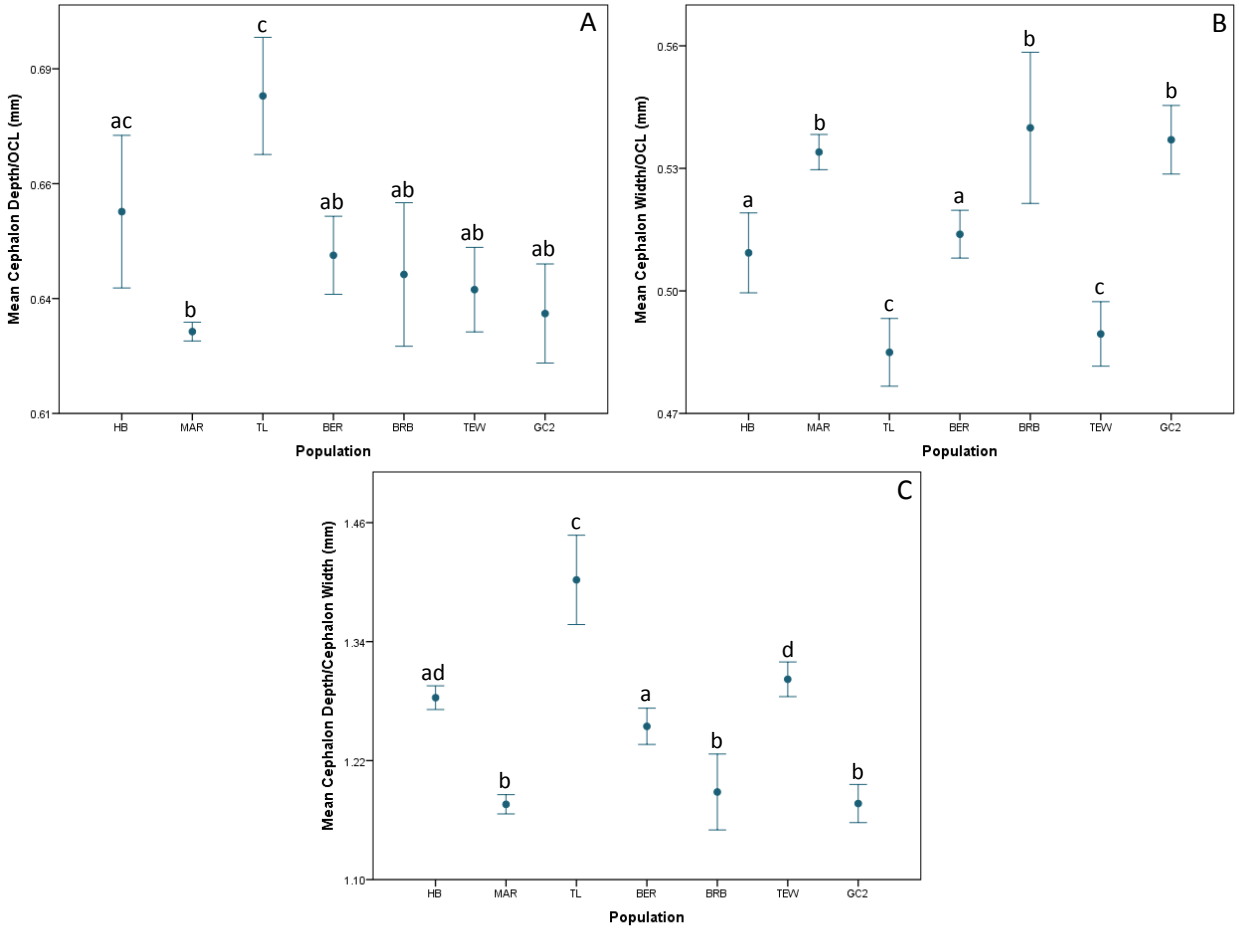

Figure A9.6. Observed differences in cephalothorax dimensions relative to occipital carapace length and each other within Queensland populations. Standard error plot (±2 S.E.) showing the mean (A) cephalothorax depth to occipital carapace length (mm), (B) cephalothorax width to occipital carapace length (mm), and (C) cephalothorax depth to width (mm). Significant differences are indicated by lowercase letters. 51 *Tenuibranchiurus* specimens across seven populations were measured.

A9.3 Rostral morphometrics for Queensland populations

Because the rostral measurements were recorded as a ratio, they did not need to be compared against OCL to check for the effect of size-related development. The effect of sex was tested and there was no significant difference found between sex across populations ($p>0.05$).

A significant difference was found between populations ($p<0.001$), with the results of the post-hoc analysis graphed (Figure A9.7). TL had a significantly higher rostral ratio than all other populations ($p<0.05$), with BRB the next highest and also significantly different to all other populations ($p<0.05$). HB and MAR were not significantly different from each other ($p>0.05$), and BER, TEW, and GC2 also formed a statistically similar group with the lowest ratio of all populations.

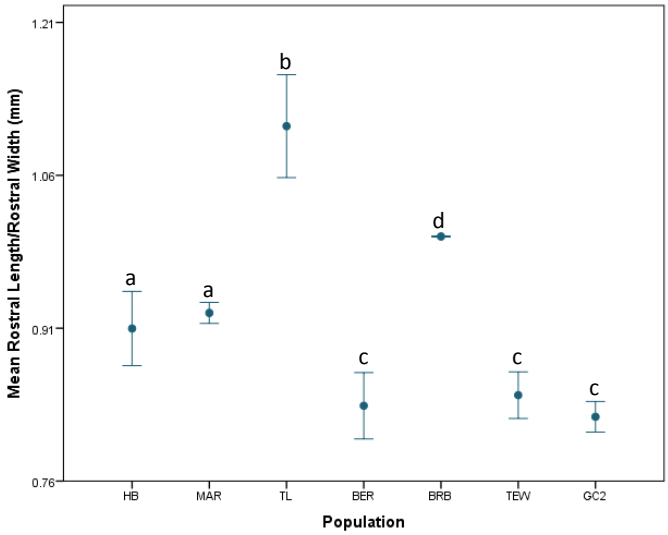

Figure A9.7. Observed differences in rostral ratios within Queensland populations. Standard error plot (±2 S.E.) showing the mean rostral width to rostral length (mm). Significant differences are indicated by lowercase letters. 50 *Tenuibranchiurus* specimens across seven populations were measured.

APPENDIX TEN

Table A10.1. Factor loadings for morphometric variables from a principal component analysis of seven Queensland populations. PTV = percentage of total variance accounted for by the component, CP = cumulative percentage of total variance accounted for.

Variable	PC1	PC2	PC3	PC4	PC5
PropL/OCL	0.714	-0.027	0.157	-0.150	0.419
PalmL/OCL	0.475	-0.008	0.259	-0.049	-0.237
PropD/OCL	0.205	-0.006	0.255	-0.334	-0.162
PropD/PalmL	-0.343	0.022	0.136	-0.765	0.165
PropD/PalmL	-0.096	0.005	0.238	-0.342	-0.392
PalmL/PropL	0.111	-0.004	0.215	0.070	-0.612
CephD/CephW	-0.226	0.449	0.717	0.306	0.219
CephW/OCL	0.077	-0.083	-0.158	-0.182	0.141
CephD/OCL	-0.021	0.114	0.162	-0.091	0.317
RosL/RosW	0.162	0.882	-0.396	-0.147	-0.134
PTV	51.1	27.0	11.8	7.0	1.8
CP	51.1	78.2	90.0	97.0	98.8

Table A10.2. Standardised canonical discriminant function coefficients for used morphometric variables from discriminant analysis of seven Queensland populations.

Variable	Function					
	1	2	3	4	5	6
PropL/OCL	0.226	1.625	6.870	-3.775	-3.429	-5.764
PalmL/OCL	-6.156	-1.604	-12.829	-5.176	6.817	9.597
PropD/OCL	4.776	0.286	2.446	8.770	-2.587	-1.633
PropD/PalmL	3.016	-3.615	-1.977	5.176	-4.085	1.791
PropD/PropL	-5.920	3.239	-0.196	-10.466	5.276	-0.117
PalmL/PropL	4.787	-1.794	4.420	5.138	-5.285	-2.053
CephD/CephW	-1.604	8.422	5.558	4.276	6.459	6.879
CephW/OCL	-2.293	7.799	4.785	4.018	5.666	6.063
CephD/OCL	2.563	-8.656	-5.371	-4.121	-6.978	-7.443
RosW/RosL	0.691	0.624	-0.322	-0.075	0.153	-0.037
% explained variance	48.6	29.8	10.3	5.7	3.9	1.6
Cumulative %	48.6	78.5	88.8	94.5	98.4	100.0
Canonical correlation	0.928	0.891	0.755	0.652	0.578	0.410

Table A10.3. Standardised canonical discriminant function coefficients for used morphological variables from discriminant analysis of seven Queensland populations.

Variable	Code	Function					
		1	2	3	4	5	6
Rostral carinae length (halfway)	2a	0.243	-0.006	0.878	0.516	1.603	-0.544
Rostral carinae length (two-thirds)	2b	0.580	-0.192	1.269	1.186	1.100	0.094
Rostral carinae definition (very poor to poor)	3a	1.616	0.085	-0.138	0.383	0.702	0.620
Rostral carinae definition (moderate)	3b	0.974	-0.099	0.043	-0.404	0.296	0.872
Rostral spine (absent)	4a	-0.140	0.572	-0.472	0.113	0.125	0.429
Rostral spine (very small)	4b	-0.171	0.341	-0.564	-0.623	0.153	-0.157
Rostral spine (small)	4c	-0.225	-0.119	0.332	-0.289	0.074	0.367
Post-orbital ridge development (poor)	5b	-0.381	-0.574	-0.410	0.219	0.365	0.294
Branchiocardiac groove (transverse grooves)	13a	0.404	0.276	-0.316	-0.220	-0.093	-0.137
Basipodite spine (medium)	17c	0.108	0.274	-0.083	-0.168	0.534	-0.187
Basipodite spine (large)	17d	0.314	0.451	-0.233	-0.221	0.519	0.188
Suborbital spine (small)	19c	-0.807	-0.367	0.829	0.377	0.081	-0.927
Suborbital spine (medium)	19d	-0.361	-0.258	1.046	0.269	0.593	-0.134
Ventromeral spine (large)	38c	0.700	-0.509	0.080	-0.136	0.231	-0.069
Dorsal meral margin granulation (one row)	42a	-0.095	0.408	0.301	-0.364	0.583	0.147
% explained variance		47.8	31.5	12.0	3.9	3.6	1.2

Table A10.4. Standardised canonical discriminant function coefficients used for included morphological and morphometric variables from discriminant analysis of seven Queensland populations.

Variable	Code	Function					
		1	2	3	4	5	6
Propodus Length/OCL (mm)	-	1.814	4.373	0.780	0.398	0.786	-5.438
Palm Length/OCL (mm)	-	-5.131	-1.119	-2.516	-5.544	-2.295	4.066
Propodus Depth/OCL (mm)	-	2.112	-3.818	1.363	4.578	1.971	2.509
Propodus Depth/Palm Length (mm)	-	1.424	-2.448	0.202	-1.508	3.752	5.706
Propodus Depth/Propodus Length (mm)	-	-2.670	5.119	-1.509	-1.088	-4.804	-7.017
Palm Length/Propodus Length (mm)	-	2.620	-1.713	1.039	1.146	3.099	1.958
Cephalon Width/OCL (mm)	-	-0.043	-0.026	0.605	-0.153	0.398	-0.370
Rostral Width/Rostral Length (mm)	-	-0.477	0.651	-0.430	0.569	-0.052	-0.016
Rostral carinae length (halfway)	2a	3.348	0.135	1.763	0.968	-0.527	-0.079
Rostral carinae length (two-thirds)	2b	2.890	0.262	1.865	0.203	-0.817	-0.207
Rostral carinae definition (very poor to poor)	3a	-1.368	1.274	-0.103	-0.556	0.096	-0.032
Rostral carinae definition (moderate)	3b	-0.579	0.627	-0.320	0.481	-0.422	-0.482
Rostral spine (absent)	4a	-0.912	-0.654	-0.003	0.059	0.390	0.279
Rostral spine (very small)	4b	-0.518	-0.406	-0.352	0.697	0.211	0.147
Rostral spine (small)	4c	-0.020	0.052	-0.067	0.412	-0.359	-0.282
Post-orbital ridge development (poor)	5b	1.028	-0.337	-1.451	-0.347	0.368	-0.018
Cephalothorax dorsal anterior punctation (sparse)	9a	-2.583	0.164	-0.388	-0.680	-0.229	-0.187
Branchiocardiac groove (transverse grooves)	13a	-0.418	-0.117	-0.075	0.178	0.302	0.088
Basipodite spine (medium)	17c	-0.317	-0.166	-0.008	0.334	0.092	0.274
Basipodite spine (large)	17d	-0.734	-0.016	-0.132	0.607	0.022	0.320
Suborbital spine (small)	19c	0.992	-0.069	0.456	-0.480	-0.248	0.373
Suborbital spine (medium)	19d	0.745	0.081	0.525	-0.406	-0.394	0.100
Ventromeral spine (large)	38c	-0.143	1.051	-0.309	-0.101	-0.244	0.119
Dorsal meral margin granulation (one row)	42a	-0.037	-0.293	0.321	0.274	-0.071	0.182
% explained variance		56.7	21.8	11.2	4.6	3.2	2.4

APPENDIX ELEVEN

A11.1 Chela morphometrics for New South Wales populations

The chela analyses for NSW were slightly different to Qld, in so far as there were three different chela types involved in the analyses; isomorph (ISO), small dimorph (SD), and large dimorph (LD). First, the data were checked to make sure that these three chela types could be accurately identified by differences in chela shape. The most informative chela comparison was found to be PropD/Dactylus Length (DactL). This showed that ISO ranged from 0.91-1.09, the SD from 0.79-1.04, and LD from 1.13-1.38. Therefore, an individual identified as having dimorphic chelae will always have a SD chela with a PropD/DactL ratio <1.04 and the LD >1.13, making the presence of dimorphism identifiable. Table A11.1 shows other comparisons that can be used to elucidate the presence of dimorphism, with Figure A11.1 displaying the proportion of isomorphic and dimorphic individuals within each population.

Table A11.1. Morphometric comparisons showing the presence of isomorphic and dimorphic chelae.

Chela type	PropD/DactL	PropL/OCL	PalmL/OCL	PropD/OCL
ISO	0.91-1.09	0.67-0.97	0.35-0.53	0.30-0.46
SD	0.79-1.04	0.75-0.85	0.36-0.44	0.32-0.39
LD	1.13-1.38	0.90-1.09	0.51-0.64	0.46-0.61
All dimorphs will have:	SD≤1.04, LD≥1.13	SD≤0.85, LD≥0.9	SD≤0.44, LD≥0.51	SD≤0.39, LD≥0.46

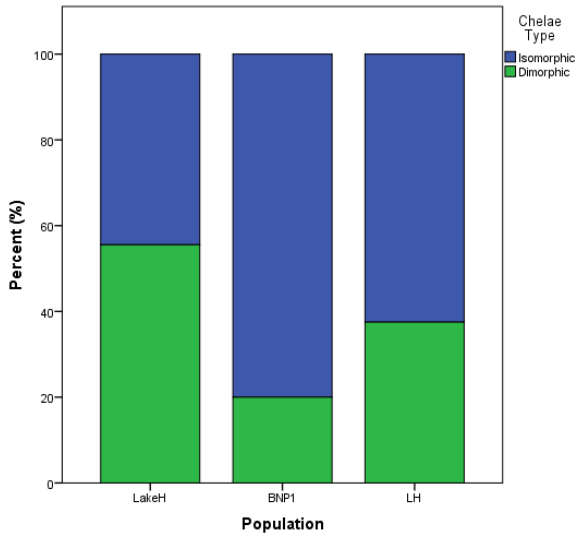

Figure A11.1. The proportion of isomorphic and dimorphic individuals within each New South Wales population. LakeH *n*=9, BNP1 *n*=5, and LH *n*=11.

To determine if the three chela types could be analysed together, or whether the data set would have to be partitioned, a GLM analysis was run on all chela variables, which showed a significant difference between chela types ($p<0.05$). Therefore ISO, SD, and LD were analysed separately for all further analyses. The pattern of differences are best represented in Figure A11.2 which demonstrates the typical pattern of large dimorphs having higher chela values (standardised against OCL) and small dimorphs having lower values with isomorphs possessing intermediate values for all comparisons.

As for the Qld populations, the chela analyses were divided into two parts; however, all analyses were performed separately for each chela type (i.e. ISO, SD, LD). Part One investigates whether there were population-specific differences in the relative sizes of chela measurements (i.e. standardised for overall size using OCL) within each chela type, whereas Part Two explores whether there was a difference in the ratio of one chela measurement to another between populations for each chela type. For Part One, the size-standardised PropD, PropL, and PalmL were compared between populations, whereas these chela measurements were compared to each other in Part Two. For all analyses, the potential effect of both size-dependent development and sex was tested for.

Part One

The raw measurements for PropL, PalmL, and PropD were graphed against OCL to check for a linear relationship. Initial tests showed a linear relationship for all populations. Sex was then graphed separately to detect any presence of sexual dimorphism. For all chela measurements, there was a strong linear relationship for both males and females in relation to OCL, indicating that there is not a point at which the chela dimensions change from isometric to allometric scaling (Figure A11.3). It should be noted, however, that the relationship between males and females was not consistent across chela types, most likely due to small sample sizes. The effect of sex was then tested using a GLM and found to be non-significant for all chela variables and for all chela types ($p>0.05$). Therefore, sex was not separated for further analyses.

There was no significant difference between populations for any variables for either ISO or LD ($p>0.05$), but there was a significant difference in SD for PalmL/OCL between LakeH and LH ($p=0.038$). By looking at the relationship between PalmL and OCL for SD, LakeH

had a significantly higher increase in the palm length with OCL growth than LH (Figure A11.4). There were no differences found with BNP1 as there was no SD chela measurements recorded for this population.

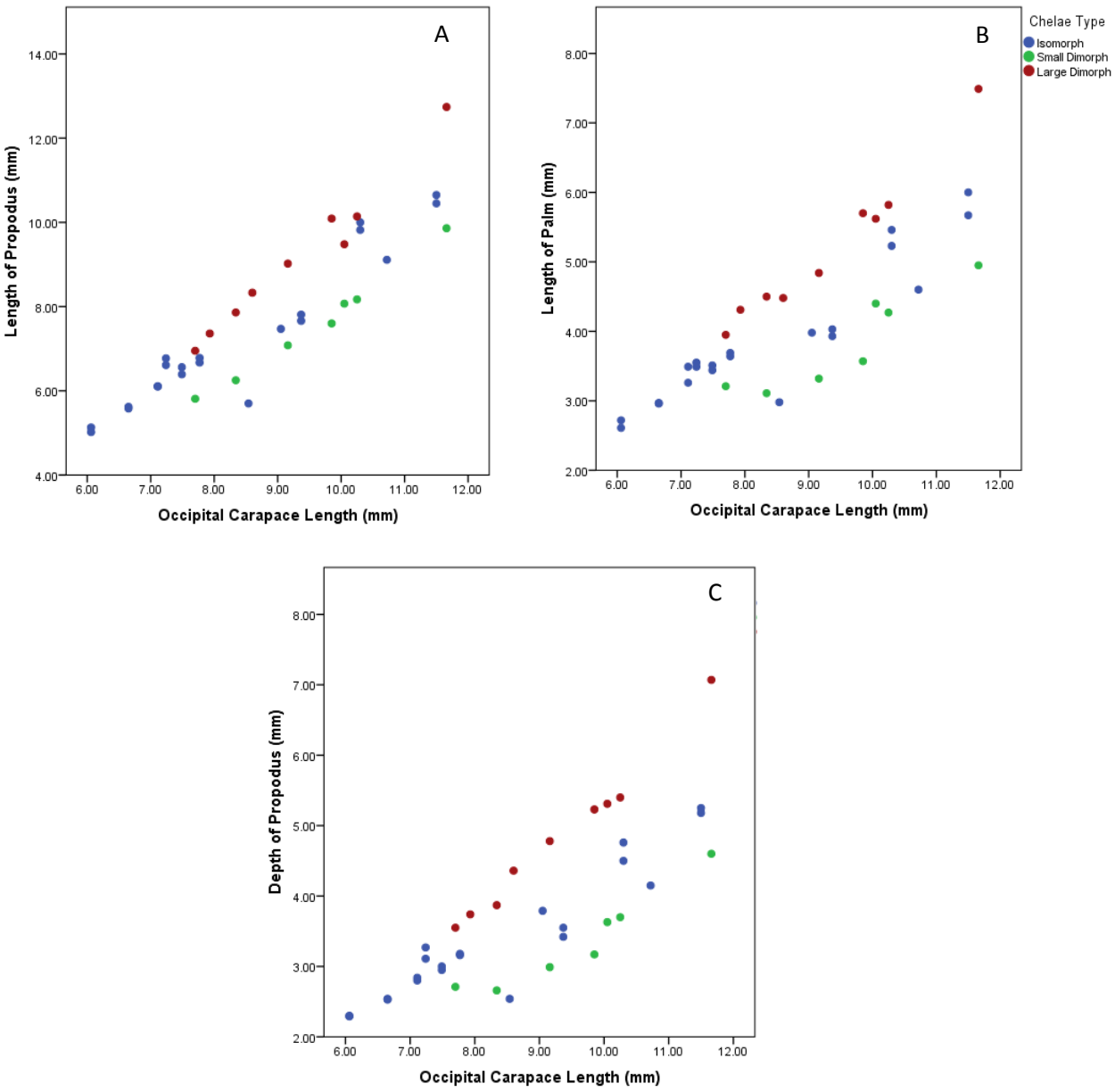

Figure A11.2. Regression of chela dimensions relative to occipital carapace length across all New South Wales populations separated into chela type. Occipital carapace length (mm) plotted against (A) propodal length (mm), (B) palm length (mm), and (C) propodal depth (mm). 37 chelae were measured from 22 *Tenuibranchiurus* specimens divided into isomorphic and small and large dimorphic chelae.

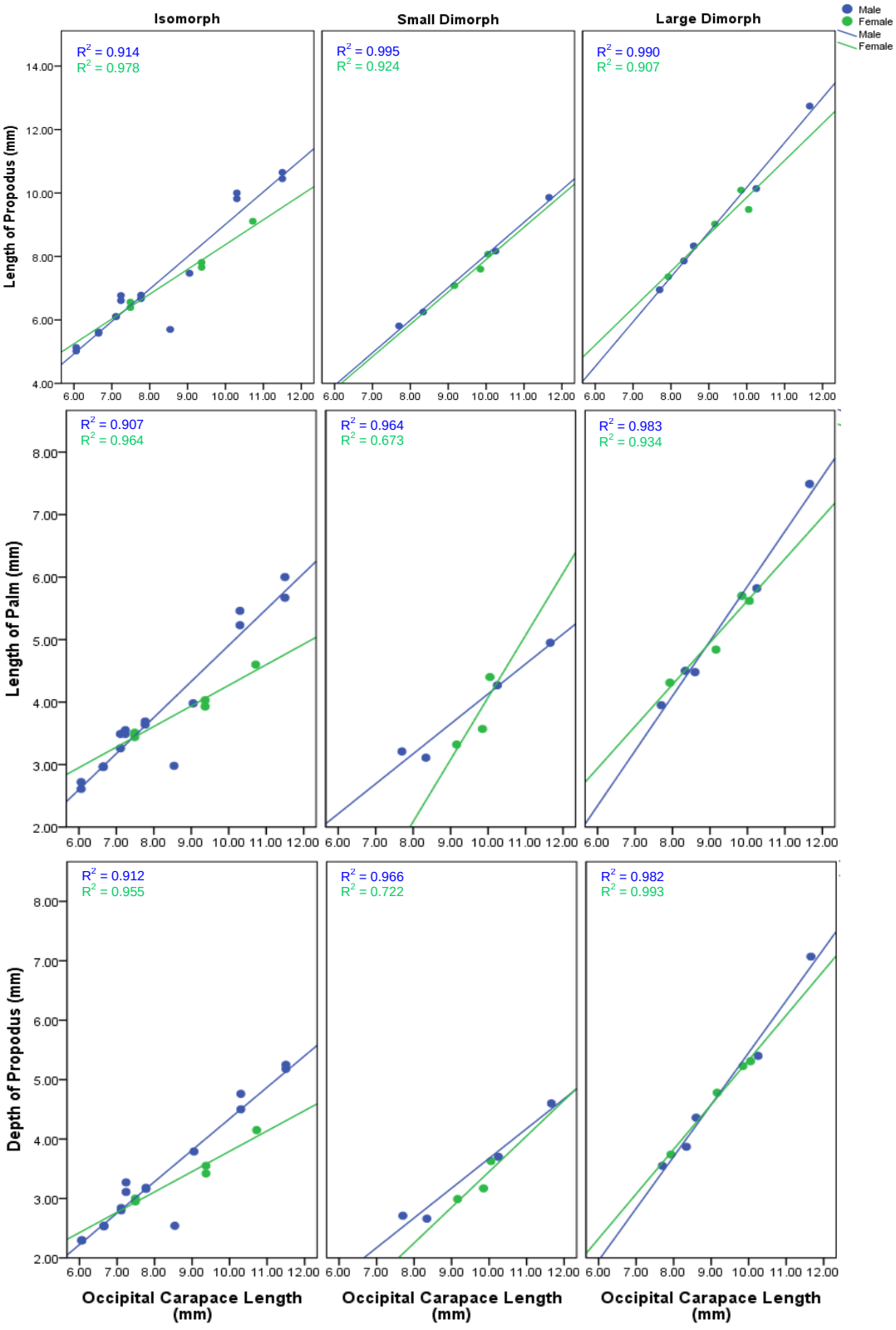

Figure A11.3. Regression of chela dimensions relative to occipital carapace length across all New South Wales populations. Propodal length, palm length, and propodal depth (mm) plotted against occipital carapace length (mm), with sex separated. 37 chelae were measured from 22 *Tenuibranchiurus* specimens divided into isomorphic and small and large dimorphic chelae across three populations.

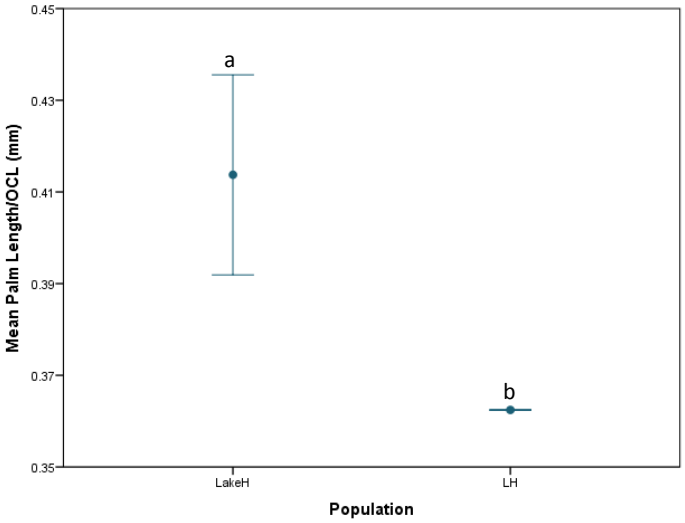

Figure A11.4. Observed differences in palm length relative to occipital carapace length for small dimorphic chelae within New South Wales populations. Standard error plot (±2 S.E.) showing the mean palm length to occipital carapace length (mm) with a significant difference between LakeH and LH. Significant differences are indicated by lowercase letters. 7 chelae were measured, *n*=5 and *n*=2 for LakeH and LH, respectively.

Part Two

As stated in Part One, the relationships between OCL and the raw chela measurements were found to be linear, and so the possible influence of size-determined development was discounted. There were no significant differences between sex for any chela type or variable (p>0.05), and so sex was not separated for further analyses.

There was no significant difference between populations for ISO (p>0.05), a significant difference in SD for PropD/PropL (p=0.049) and both SD and LD for PalmL/PropL (p=0.032 and 0.025, respectively). A post-hoc analysis showed that both the SD and LD differences were between LakeH and LH (Figure A11.5).

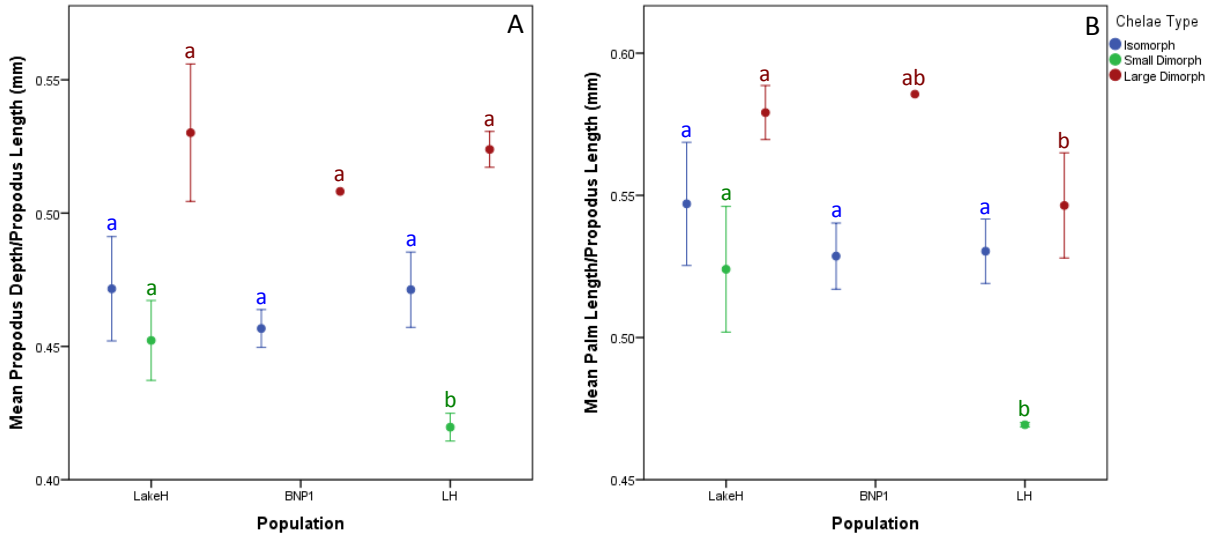

Figure A11.5. Observed differences in chela dimensions relative to each other within New South Wales populations. Standard error plot (±2 S.E.) showing the mean (A) propodal depth to propodus length (mm) showing a significant difference in both SD and LD between LakeH and LH, and (B) palm length to propodal length (mm) showing a significant difference in SD between LakeH and LH. Error bars are not shown when calculations yielded infinite results. Significant differences are indicated by lowercase letters. 46 chelae were measured from 22 *Tenuibranchiurus* specimens divided into isomorphic and small and large dimorphic chelae across three populations.

A11.2 Cephalothorax morphometrics for New South Wales populations

The raw measurements for CephD and CephW were graphed against OCL to check for a linear relationship, with sex graphed separately (Figure A11.6). A strong linear relationship was found for both males and females for both CephD and CephW in relation to OCL, indicating no change in growth rate with age or sexual dimorphism. It was also determined that there was no difference in measurements between individuals with different chela types (i.e. isomorphs and dimorphs). A GLM also found there was no significant difference (p>0.05) between sex at any population for either variable, and therefore they were not separated in further analyses.

There was a significant difference between all populations for CephW/OCL (p<0.05), and between BNP1 and LH for CephD/OCL (p=0.005). There was also a very slight significant difference found between both LakeH and BNP1 with LH for CephD/CephW (p=0.05) (Figure A11.7).

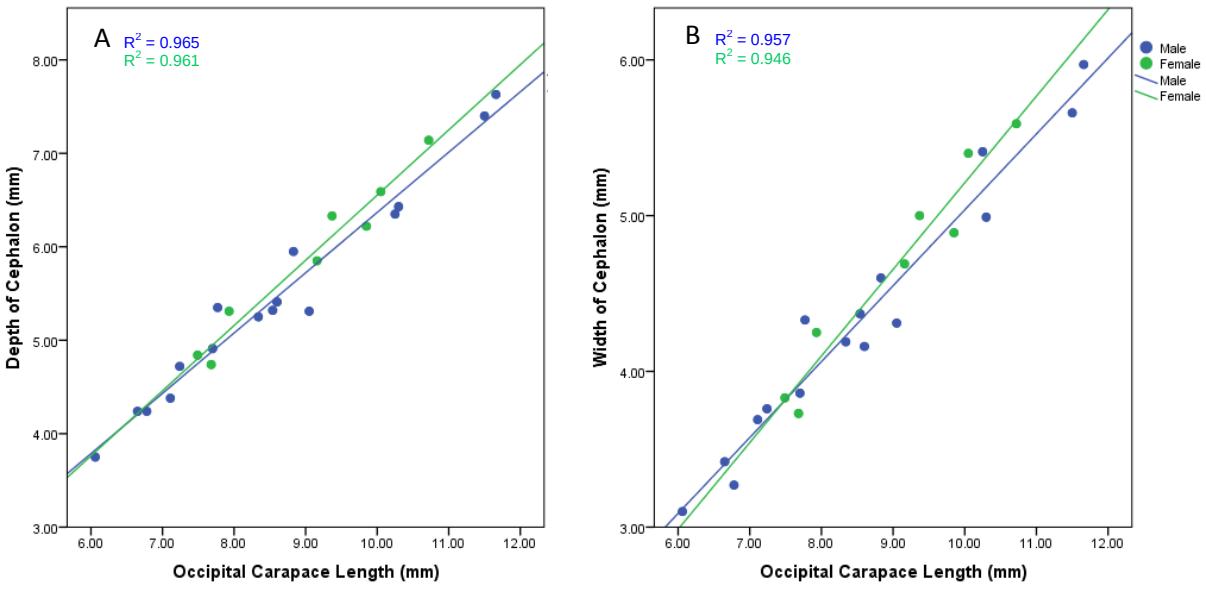

Figure A11.6. Regression of cephalothorax dimensions relative to occipital carapace length across all New South Wales populations. Cephalothorax depth and width (mm) plotted against occipital carapace length (mm) with sex separated. 24 *Tenuibranchiurus* specimens were measured across three populations.

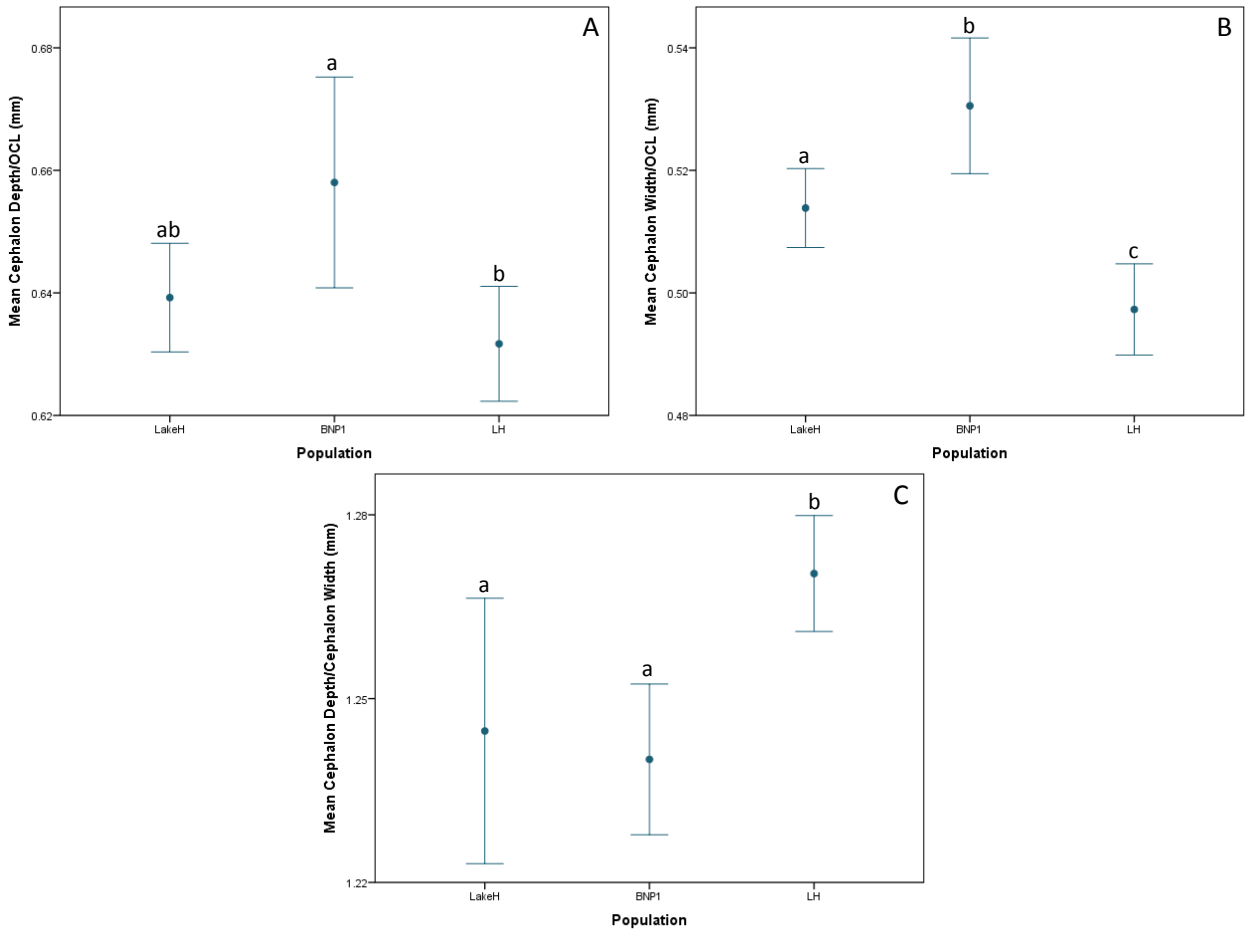

Figure A11.7. Observed differences in cephalothorax dimensions relative to occipital carapace length and each other within New South Wales populations. Standard error plot (±2 S.E.) showing the mean (A) cephalothorax depth to occipital carapace length (mm) showing a significant difference between BNP1 and LH, (B) cephalothorax width to occipital carapace length (mm) showing a significant difference between all populations, and (C) cephalothorax depth to width (mm) showing a slight significant difference between both LakeH and BNP1 with LH. Significant differences are indicated by lowercase letters. 24 *Tenuibranchiurus* specimens were measured across three populations.

A11.3 Rostral morphometrics for New South Wales populations

There was no difference in measurements between individuals with different chela types (i.e. isomorphs and dimorphs), nor between sex (p>0.05) across populations. There was a significant difference between BNP1 and LH for RosL/RosW (p=0.025), and an almost significant difference between LakeH and LH (p=0.054) (Figure A11.8).

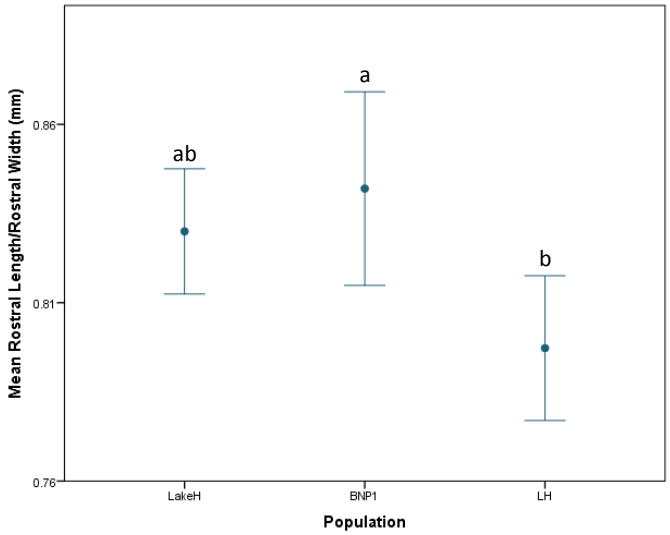

Figure A11.8. Observed differences in rostral ratios within New South Wales populations. Standard error plot (±2 S.E.) showing the mean rostrum width to rostrum length (mm) showing a significant difference between BNP1 and LH. Significant differences are indicated by lowercase letters. 25 *Tenuibranchiurus* specimens were measured across three populations.

APPENDIX TWELVE

Table A12.1. Factor loadings for morphometric variables from a principal component analysis of three New South Wales populations with isomorphic and dimorphic specimens separated. PTV = percentage of total variance accounted for by the component, CP = cumulative percentage of total variance accounted for.

	Isomorphic					Dimorphic				
Variable	PC1	PC2	PC3	PC4	PC5	PC1	PC2	PC3	PC4	PC5
PropL/OCL	-0.698	0.181	0.125	-0.001	0.375	-0.602	0.168	0.009	0.013	0.494
PalmL/OCL	-0.412	0.192	0.283	-0.110	-0.148	-0.490	-0.278	-0.115	-0.129	0.086
PropD/OCL	-0.427	0.007	-0.013	0.083	0.027	-0.525	0.047	0.078	0.069	-0.159
PropD/PalmL	-0.142	-0.323	-0.503	0.385	0.365	-0.158	0.543	0.368	0.381	-0.382
PropD/PropL	-0.109	-0.101	-0.062	0.043	-0.200	-0.247	-0.079	0.078	0.016	-0.513
PalmL/PropL	-0.053	0.115	0.255	-0.143	-0.385	-0.183	-0.427	-0.119	-0.216	-0.288
CephD/CephW	-0.231	0.254	-0.579	0.159	-0.593	-0.016	0.422	0.046	-0.828	-0.149
CephW/OCL	0.100	0.194	-0.133	-0.473	0.336	-0.010	-0.252	-0.158	0.244	-0.232
CephD/OCL	0.024	0.373	-0.469	-0.513	0.113	-0.016	-0.093	-0.129	-0.142	-0.383
RosL/RosW	0.251	0.749	0.097	0.546	0.195	0.049	-0.399	0.884	-0.146	0.097
PTV	54.2	17.6	12.2	6.8	5.3	81.6	8.5	4.1	3.5	1.6
CP	54.2	71.8	83.9	90.7	96.0	81.6	90.1	94.2	97.7	99.2

Table A12.2. Standardised canonical discriminant function coefficients for used morphometric variables from discriminant analysis of three New South Wales populations with isomorphic and dimorphic specimens separated.

Variable	Isomorphic		Dimorphic	
	Function		Function	
	1	2	1	2
PropL/OCL	-0.706	2.544	0.102	9.406
PalmL/OCL	0.532	-1.744	4.969	-3.012
PropD/OCL	-0.111	-0.809	-6.094	-8.764
PropD/PropL	-	-	1.141	2.672
CephD/CephW	-0.218	0.184	0.951	1.353
CephW/OCL	0.560	0.525	1.335	1.232
RosW/RosL	0.473	-0.291	0.446	0.791
% explained variance	61.7	38.3	79.8	20.2
Cumulative %	61.7	100.0	79.8	100.0
Canonical correlation	0.655	0.564	0.856	0.640

Table A12.3. Standardised canonical discriminant function coefficients for included morphological variables from discriminant analysis of three New South Wales populations

Variable	Code	Function	
		1	2
Rostral spine (very small)	4b	0.030	0.032
Post-orbital ridge development (very poor)	5a	1.588	-1.188
Post-orbital ridge development (poor)	5b	-0.012	-0.143
Branchiocardiac groove (transverse grooves)	13a	-1.447	-0.201
Basipodite spine (absent)	17a	-0.608	0.467
Basipodite spine (very small)	17b	0.015	0.184
Suborbital spine (absent)	19a	-0.448	1.746
Suborbital spine (very small)	19b	0.030	0.370
Dactylus dorsal granulation (moderate)	34b	1.412	0.673
% explained variance		86.2	13.8

Table A12.4. Standardised canonical discriminant function coefficients for used morphological and morphometric variables from discriminant analysis of three New South Wales populations.

Variable	Code	Isomorphic Function		Dimorphic Function	
		1	2	1	2
PropL/OCL	-	3.507	0.350	-6.547	1.314
PalmL/OCL	-	1.281	1.428	-0.365	0.527
PropD/OCL	-	-1.674	-0.579	2.605	-3.620
PalmL/PropL	-	-	-	4.243	1.665
CephD/CephW	-	-5.010	-0.272	-0.288	-0.044
CephW/OCL	-	0.195	-0.786	-	-
RosW/RosL	-	-3.367	-0.277	-	-
Post-orbital ridge development (very poor)	5a	9.176	1.204	3.834	-0.735
Post-orbital ridge development (poor)	5b	7.055	0.577	2.807	1.104
Branchiocardiac groove (transverse grooves)	13a	-	-	-2.067	1.028
Basipodite spine (absent)	17a	3.045	0.740	-1.789	0.340
Basipodite spine (very small)	17b	6.334	0.093	-	-
Suborbital spine (very small)	19b	5.099	-0.014	-	-
% explained variance		99.2	0.8	97.3	2.7
Cumulative %		99.2	100.0	97.3	100.0
Canonical correlation		0.998	0.783	0.994	0.836

APPENDIX THIRTEEN

Full list of morphological characters and character states across Queensland and New South Wales populations used in the population aggregation analyses. Refer to Appendix Eight for explanation of character codes.

	PAA Qld - Characters and associated character states																		
	2	3	4	5	13	17	19	38	42	43	46	9	10	11	24	25	26	31	34
GC2	A	A	A	BC	AB	D	E	B	A	A	A	B	C	B	B	A	A	A	B
TEW	AB	BC	AB	B	AB	CDE	CD	D	A	A	B	A	B	A	B	C	B	A	C
HB	A	A	BD	B	AB	CDE	CDE	C	AB	B	B	B	C	B	A	A	A	A	A
BRB	AB	AB	BD	B	AB	CDE	DE	C	B	B	D	A	C	B	A	B	A	B	C
TL	A	AB	AB	A	A	D	E	CD	A	A	B	A	B	B	A	C	A	A	C
BER	AB	AB	BCD	B	AB	CDE	DE	C	AB	B	B	B	C	B	A	C	A	A	C
MAR	B	A	CD	A	A	DE	DE	C	A	B	B	B	C	B	A	C	A	A	C

	PAA NSW - Characters and associated character states																		
	2	3	4	5	13	17	19	38	42	43	46	9	10	11	24	25	26	31	34
LH	C	C	BD	C	A	AB	A	A	A	A	B	B	A	B	C	D	C	C	D
BNP1	C	C	A	BC	A	BC	BC	A	A	A	C	B	A	B	C	E	C	C	D
LakeH	C	C	A	BC	AC	ABC	AB	A	A	A	C	B	A	B	C	E	C	C	BC