Contemporary and Historical Influences on the Taxonomy and Distributions of Cherax Species in South Eastern Queensland, Australia

Author

Bentley, Andrew I

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CONTEMPORARY AND HISTORICAL INFLUENCES ON THE TAXONOMY AND DISTRIBUTIONS OF *CHERAX* SPECIES IN SOUTH EASTERN QUEENSLAND, AUSTRALIA

PhD Thesis

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Andrew I. Bentley B.Sc. (Hons)

School of Environment

Griffith University

Supervisors

Professor Jane Hughes (GU)

Dr. Daniel Schmidt (GU)

Griffith UNIVERSITY

SUMMARY

Freshwater crayfish are a highly diverse group of decapod crustaceans that are distributed across all but the Indian and Antarctic continents. Their broad distribution suggests a strong ability to disperse and adapt to a wide range of habitats and environmental niches. In particular, freshwater crayfish have been discovered in caves, burrows, streams, lakes and sometimes even terrestrial habitats. This dispersed distribution across a range of habitats is particularly evident for the genus *Cherax*, with at least 47 species identified from throughout Australia. With some species highly diverse and others widely distributed, it is unclear what effect geographic, behavioural and ecological isolation has had on the current biogeographic structure of Australian *Cherax*. By investigating variation across a nested series of scales, the primary aim of this study was to distinguish the historical and contemporary drivers that may have caused and maintained the high level of biodiversity observed in Australian and more specifically South-East Queensland (SEQ) *Cherax*.

Firstly, I estimated the current degree of biodiversity in eastern Australian *Cherax*. This was accomplished by using the phylogenetic diversity of twelve eastern Australian *Cherax* as an independent test on the current taxonomic/systematic classifications. Phylogenetic analysis of three mtDNA (COI, 16S, 12S) and four nuclear (ITS2, H3, 28S and GAPDH) genes identified a total of nineteen divergent lineages from twelve described species. These nineteen lineages were further separated into six species groups; *C. depressus*, *C. robustus*, *C. cuspidatus*, *C. dispar*, *C. destructor* and *C. quadricarinatus*. While phylogenetic analysis supported the taxonomic classification of a majority of the eastern Australian *Cherax* species, it did not support the distinction between *C. depressus* and *C. cairnsensis*. More specifically, although the distributions of the two species are separated by more than 1000km, phylogenetic analysis showed them as paraphyletic for every gene except COI. In contrast, a high level of biodiversity was observed within *C. cuspidatus* and *C. dispar*, with five highly divergent lineages identified within each. Of these ten lineages, four were previously un-identified; three lineages of *C. cuspidatus* (*C. cuspidatus* B, D & E) and one lineage of *C. dispar* (*C. dispar* E). The high intra-specific diversity observed for *C. cuspidatus* and *C. dispar* was particularly surprising with all but one lineage restricted to SEQ. This high diversity within single species' suggests that further taxonomic research is warranted on the two species. The contrasting level of geographic structure among *Cherax* species also suggests that biogeographic barriers may not have isolated and affected each species/lineage in the same way.

This variation in the effect of biogeographic barriers on Australian *Cherax* was investigated further with Bayesian biogeographic analyses. Using S-DIVA, this study estimated the historic biogeographic distributions and dispersal patterns of Australian *Cherax* based on their current geographic distribution. As *Cherax* are obligate freshwater species, it was hypothesised that similar biogeographic events and river catchment boundaries would affect each species alike. In contrast however, biogeographic analysis identified different biogeographic histories for each of the species groups. Historically, *Cherax* were estimated to have been distributed Australia wide, with separation into three ancestral bioregions (south-west, north and east of Australia) in the early-Miocene. Within eastern Australia, the Great Dividing Range (GDR) appeared to be the most significant barrier to *Cherax* dispersal, with only the *C. destructor* group estimated to have dispersed across the range into central Australia. Furthermore, the *C. destructor* group was estimated to have dispersed back across the range on two additional occasions, indicating a relatively strong 'terrestrial' dispersal ability compared to other *Cherax* species. Similarly, river catchment boundaries did not appear to restrict dispersal of the *C. depressus* species group, with the group distributed across eighteen coastal river catchments along a 1200km portion of the Queensland coastline. This relatively recent inter-catchment connectivity of *C. depressus* was unexpected for an obligate freshwater species, with the distribution instead resembling that of an amphidromous species. As the only *Cherax* species group distributed along central Queensland, the biogeographic history was unclear, however historical oceanic dispersal for *C. depressus* is unlikely with the species absent from all nearby coastal islands. Within SEQ specifically, river catchment boundaries did appear to limit dispersal of *Cherax* species similarly. This was particularly the case for *C. dispar* and *C. cuspidatus*, with intra-specific lineages typically differentiated among river catchments or biogeographic regions. Although the Mary River catchment was estimated to have been the origin for all eastern *Cherax*, a historical broad distribution as far south as the Logan-Albert River catchment during the late-Miocene was estimated. This SEQ wide distribution was only maintained for *C. dispar* and *C. robustus* however, with *C. cuspidatus* and *C. depressus* estimated to have reduced their distribution into the south and north respectively. This contrasting geographic structure of SEQ *Cherax* suggests that extrinsic biogeographic barriers and river catchment boundaries are not the only limiting factor on the current distributions of eastern Australian *Cherax*.

To investigate this further and identify what contemporary processes may be restricting the distribution and dispersal of SEQ *Cherax*, a phylogeographic approach was applied to two

sympatric SEQ species; *C. dispar* and *C. depressus*. As the two species differ in their ability to burrow, it was hypothesised that variations in the effect extrinsic biogeographic barriers have had on the current distributions and genetic variation of the species was likely to be caused by species-specific responses to the local environment and/or life history traits. Although both species were distributed across multiple SEQ river catchments, both showed a clear North/South divergence. While this North/South split generally corresponded with river catchment boundaries, current river boundaries did not appear to explain the divergences observed between three sympatric *C. dispar* lineages (A-C) in the Mary River catchment in the North. Instead, the sympatric distribution of the lineages suggests that divergence may be the result of allopatric divergence with relatively recent recolonisation, specifically from Tin Can Bay into the Mary River catchment. During this period, the two ends of Fraser Island may have also been colonised separately, with a phylogeographic break observed between south Fraser Island (and Tin Can Bay) and north Fraser Island (and Mary River catchment). In contrast, southern *C. dispar* lineages (D-E) showed no evidence of historical oceanic dispersal, with strong divergence observed between the mainland (*C. dispar* E) and islands of Moreton Bay (*C. dispar* D). While divergence between the two lineages pre-date both the last glacial maximum and the actual age of the islands, gene flow between the islands of Moreton Bay may also still be inhibited, with no haplotype sharing observed among the islands for *C. dispar* D. This strong differentiation and limited dispersal among river catchments was however not specific to *C. dispar* with AMOVA analyses identifying significant river catchment structure for *C. depressus* as well as all five *C. dispar* lineages. As *C. depressus* is relatively 'resistant' to drought conditions and able to remain stationary in ephemeral systems, it was hypothesised that *C. depressus* would have a relatively low dispersal ability compared to *C. dispar*. As expected, *C. depressus* showed low levels of dispersal in SEQ, with highly significant isolation by distance (IBD) analyses observed for both Euclidean and aquatic distances. This similar IBD effect for both creek and Euclidean distance may suggest that *C. depressus* is capable of dispersal 'terrestrially', particularly during high rainfall.

This IBD effect across multiple river catchments however, assumes that dispersal within a river catchment is equally likely to dispersal oceanically or 'terrestrially' across river catchment boundaries. To limit this inference and more accurately estimate the current level of dispersal for the two species, a population genetic approach was applied within a single sub-catchment (Tinana Ck) of the Mary River. More specifically, by examining molecular, morphological and environmental variation among populations, this study aimed to decipher the relative effect localised adaptation and geographic isolation has on dispersal and phenotypic variation in *C.*

dispar and *C. depressus*. As populations within a single sub-catchment are essentially connected, it was hypothesised that geographic distance would not have an effect on dispersal among *C. dispar* populations. In contrast however, both *C. dispar* lineages showed significant geographic structure among populations and isolation by creek distance effects, further supporting the suggestion of a very low dispersal capability for the species. Phenotypic variation in one *C. dispar* lineage (A) did not appear to be affected by geographic distance however, with significant correlations observed instead with environmental and genetic distances. This suggests that IBD and drift do not play a major role in the phenotypic variation of the lineage, with *C. dispar* A instead adapting morphologically to specific environmental conditions such as salinity and pH. Unlike *C. dispar*, *C. depressus* also showed a significant relationship between Euclidean and genetic distances among populations within the Mary River catchment. This suggests that although the ability to burrow may permit dispersal across ephemeral river systems for *C. depressus*, the species rarely disperses at all. While these differing dispersal abilities most likely restrict the distributions of each species differently, factors such as salinity, substrate type and competition were also estimated to have had a limiting effect. As *C. depressus* was only observed within the upper reaches of the mainland river systems and absent from all four coastal sand islands, high salinities may inhibit the survival of the species. The impact of salinity was not conclusive however, with *C. depressus* estimated to have dispersed across the entire Queensland coastline relatively recently. Instead an interaction between substrate type and inter-species competition was suggested to be the cause of the restricted *C. depressus* distribution. In particular, in habitats where *C. depressus* is unable to form a permanent burrow and seek refuge, *C. depressus* individuals may be outcompeted by the more mobile and aggressive *C. dispar* individuals. While a direct effect of competition was not observed, *C. dispar* individuals were significantly larger and less abundant in populations where both species occurred.

Although freshwater crayfish are distributed in a wide range of environmental niches worldwide, their ability to disperse is relatively limited. In particular, this study highlights the particularly high level of biodiversity and limited dispersal ability of SEQ *Cherax* species. As SEQ is currently undergoing major development, the threat of increased habitat destruction is of growing concern for the conservation of biodiversity. In particular, localised extinction within the region may have irreversible consequences on *Cherax* diversity, with five of the six resident species and all eleven resident lineages endemic to the region. This study highlights the need for conservation managers to consider both described species and molecular diversity when planning growth within SEQ.

DECLARATION

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

(Signed)_____

Andrew Bentley

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CHAPTER 1: GENERAL INTRODUCTION

1.1 SPECIATION AND GENETIC DISTINCTNESS

Understanding the cause of speciation or genetic distinctness is a fundamental biological concern. The understanding of how the continuous process of evolution can produce morphologically and/or genetically distinct groups known as species has fascinated many since Charles Darwin's 'On the Origin of Species' in 1859 (Darwin, 1859). Although still intensely disputed, Dobzhansky (1937), Muller (1942) and Mayr (1942) progressively modified the definition of species into the 'biological species concept' (BSC) (Mayr, 1942). The BSC states that 'species are groups of interbreeding populations that are reproductively isolated from other such groups'. The reproductive barriers separating these groups or different species are divided into two factors: 'pre-zygotic isolating factors' such as mate discrimination, geographic isolation and habitat preference or 'post-zygotic isolating factors' such as hybrid in-viability and sterility. The interaction between this reproductive isolation and the effects of selection and genetic drift, produce the morphological and genetic distinctiveness observed between both species and populations (Futuyma, 1989).

The process of speciation or genetic divergence can occur in populations distributed both allopatrically and sympatrically. Allopatric populations are those where gene flow between populations is restricted by physical barriers (Harrison, 2012; Mayr, 1942, 1963). The physical isolation of populations leads inevitably to evolutionary divergence through natural selection or genetic drift (Harrison, 2012; Mayr, 1942). Disjunct populations were originally thought to be the only cause of speciation. However a large number of studies have discovered that geographic isolation is not in fact necessary as speciation can occur between populations that are sympatric, even within a single interbreeding population (Abbott *et al.*, 2013; Dieckmann & Doebeli, 1999; Futuyma & Mayer, 1980; Tauber & Tauber, 1989). Allopatric divergence however is still recognised as a much more common and more likely speciation process (Futuyma, 1989; Harrison, 2012). Current patterns of reproductive isolation between populations can be divided into three general categories: geographic isolation, behavioural isolation and ecological isolation.

1.1.1 Geographic Isolation

Since Darwin in 1859, speciation due to geographic isolation has been studied intensively using a range of techniques, including morphological characteristics (Melville *et al.*, 2006; Relethford, 2004) and genetic techniques (Coyne, 1992; Coyne & Orr, 2004). By utilising these techniques, researchers are able to investigate patterns of speciation where geographical barriers have isolated populations for extensive periods of time. These barriers to gene flow lead to the genetic or morphological differentiation of populations over time by fixing alleles within each of the separated populations. The rate of this fixation is known to be negatively correlated with effective population size (Kimura, 1962). This rate of genetic drift between the populations can be hindered by movement of a small number of genes between populations per generation or by natural selection (Kimura, 1962). Natural selection can however only slow the rate of genetic drift if it is strongly selecting for identical alleles across the entire range of the taxon (Hartl & Clark, 1989; Slatkin, 1985). Patterns of speciation via geographic isolation can appear in both allopatrically and sympatrically distributed populations. Allopatrically distributed populations represent populations that are currently still isolated (Mayr, 1942) while sympatrically distributed populations may represent populations that have been intermittently affected by long term extrinsic barriers (e.g., Glaciations) but have subsequently expanded their ranges (Templeton *et al.*, 1987).

As a result of the almost continuous fluctuations in the global climate over the last 65 million years, the relative impact geographical barriers have had on species' distributions has similarly also fluctuated through time (Andrewartha & Birch, 1954; Parmesan, 2006). These historical changes in climate, predominantly in temperature, have intermittently expanded and contracted the distributions of a majority of species/populations (Houghton *et al.*, 2001; Jones *et al.*, 2001). When faced with ongoing climate change, populations typically respond in one of three ways (Gienapp *et al.*, 2008). First, the population may migrate or disperse elsewhere to a suitable habitat. Second, providing the climate remains within the species' tolerances, the population may persist by means of phenotypic plasticity (Parmesan, 2006). Or lastly, the population can adapt/evolve to the changed conditions through genetic mutation or changes in the gene frequency of already present mutations (Davis *et al.*, 2005). These major changes in the global climate also provide organisms with the unique opportunity to expand their distribution to regions that were previously unoccupied (Carey, 2009; Lambeck & Chappell, 2001). This is particularly the case for species that inhabit lowland coastal regions, with major changes in temperature periodically altering the global sea level and connecting landmasses that were previously isolated (Lambeck & Chappell, 2001). For freshwater organisms, these

periodic changes in the sea level can also provide the unique opportunity for species to disperse between river systems, with river connections sometimes possible at low sea level (Langford *et al.*, 1995; Schultz *et al.*, 2008).

1.1.2 Behavioural Isolation

Aside from being physically isolated, the dispersal ability/behaviour and breeding patterns of an organism can also limit gene flow between populations of any given species (Bubb *et al.*, 2006). If a species population in any given habitat prefers to inhabit the proximate population rather than disperse, an 'isolation by distance' and inbreeding effect may occur (Slatkin, 1985). When this occurs, a correlation may develop between the genetic differentiation of populations and their spatial separation (Johnston & Robson, 2009; Templeton *et al.*, 1987). Unlike the geographic isolation of populations, isolation by distance demonstrates a gradient of genetic differentiation with populations at the outer extremes of the total distribution more divergent than adjacent ones (Slatkin, 1985). The overall effect of this is expected to be lower for organisms with high dispersal ability/tendencies, as genes are more likely to be passed on to the outer extremities of their distribution. This contrasts to organisms with strong site fidelity (Hoffman *et al.*, 2006), with their genes remaining locally rather than passed on. As site fidelity behaviours are often sex specific, isolation by distance can also affect the genes of each sex differently (Greenwood, 1980; Ujvari *et al.*, 2008). This has been observed in mammals (Dobson, 1982; Greenwood, 1980), birds (Beck *et al.*, 2008; Greenwood, 1980) and invertebrates (Beirinckx *et al.*, 2006). The most common and effective methods of testing dispersal in organisms is either through direct tracking methods (Hestbeck *et al.*, 1991; Lebreton *et al.*, 2003) or investigating the amount of gene flow between populations (Hoffman *et al.*, 2006; Waser & Strobeck, 1998).

While behavioural isolation due to isolation by distance relies on geographic distance for divergence to occur, differing life histories or mating behaviours encourage speciation between sympatric populations (Bubb *et al.*, 2006). For reproduction and thus gene flow to be successful, individuals of a population must not only recognise the mating behaviour of one another but also breed at the same time and place (Palumbi, 2003). Because of this, any slight change to the timing, location or mating behaviour of a species initiates reproductive isolation and speciation within the population (Rundle & Nosil, 2005). Changes in the breeding 'timing' of a species can occur at a range of scales, with deviations to the breeding season the most obvious barrier to reproduction success (Palumbi, 2003; Reese, 1968).Predominantly, these

changes in breeding season are driven by external forces such as climate change (Beebee, 1995; Carey, 2009) and localised pressures like competition (Hancock & Bunn, 1997). Isolation can however also arise from slight changes to the daily behaviour of a population, such as converting to a more nocturnal lifestyle (Pianka, 1969). While a 'location' change sometimes leads to geographical isolation, variations in habitat preference and the location for producing offspring (Downey & Nice, 2013; Filchak *et al.*, 2000) can also encourage sympatric divergence. Unlike variations in the breeding 'time' and 'location' of a population, which primarily inhibit contact between individuals, variations in the mating behaviour or mate choice of a population restricts the identification of individuals as breeding partners . Some common characteristics that inhibit partner identification include variations in mating rituals (e.g., calls (Lachlan & Servedio, 2004; Price, 1998), dance (Salmon *et al.*, 1979; Tan *et al.*, 2008), colour patterns (Gray & McKinnon, 2007; Seehausen *et al.*, 1999) and size (Nagel & Schluter, 1998). Although sympatric speciation from these behavioural isolation mechanisms may be relatively common (Bolnick & Fitzpatrick, 2007), distinguishing it from other speciation processes is often difficult and problematic (Bird *et al.*, 2012; Bolnick & Fitzpatrick, 2007).

1.1.3 Ecological Isolation

The last category of reproductive isolation, ecological isolation, is associated with behavioural isolation and represents the possible environmental limitations on the dispersal and current distribution of a species. Ecological isolation encourages genetic divergence between populations that occupy different habitats as they become isolated from one another (Jones & Bergey, 2007; Pfenninger *et al.*, 2003). A species or population can be restricted to specific habitats either through competition, resource availability or an inability to survive in other conditions (Rundle & Nosil, 2005). The adaptation of a species to access a resource or survive in a habitat that other species cannot has been suggested to be the most probable cause of speciation in sympatrically distributed populations (Purvis & Hector, 2000), although, secondary contact between previously geographically isolated populations is also likely.

The most effective method of investigating the effect of environmental conditions on species distribution is by comparing both the abiotic and biotic conditions of the localised habitat with the species or lineages that are present (Rundle & Nosil, 2005). As abiotic and biotic conditions can vary drastically both locally and following a gradient, a comprehensive analysis is encouraged to include a range of habitat types at a range of locations (Abellán *et al.*, 2005). At a local scale, the depth or ephemerality of a stream can significantly influence the occurrence

of a freshwater organism as its survival can depend on the availability of refuge sites during times of drought. The availability of refuge sites during times of drought is not however always the most limiting factor, with refuge sites from predators also highly important, particularly for smaller freshwater organisms (Rundle & Nosil, 2005). At a large scale, a gradual change in abiotic and biotic conditions along an altitudinal gradient limits the distribution of freshwater organisms within a river system. This gradual change in environmental conditions significantly affects distributions of freshwater organisms as water conditions at different altitudes can vary significantly in temperature, salinity and flow (Hodkinson, 2005). The effect of elevation on species distribution has been observed in a wide range of freshwater taxa and is recognised as an important factor to include in any environmental analysis (Hodkinson, 2005).

Research into the effect of ecological isolation on a population has grown rapidly in the past ten years with the advent of landscape genetics (Manel *et al.*, 2003). By combining landscape ecology and population genetics, landscape genetics aims to provide insight into the influence of ecological processes or landscape ecology (Storfer *et al.*, 2006) on genetic variation and micro-evolutionary processes, such as gene flow, genetic drift and selection (Manel *et al.*, 2003). Although typically focused at the micro-evolutionary scale (within a species), landscape genetics can also be applied to determine current isolation processes that may lead to speciation in the future (Manel *et al.*, 2003). Unlike traditional population genetic studies, which were limited to spatial inference as a simple function of geographic distance, landscape genetics, through the inclusion of a matrix, allows the incorporation of both biological and ecological processes at the landscape level (Cushman *et al.*, 2013; Storfer *et al.*, 2006). Understanding how these landscape processes affect genetic connectivity provides insight into fundamental biological processes such as; metapopulation dynamics, speciation and ultimately the formation of species' distributions (Storfer *et al.*, 2006). More commonly, landscape genetics is utilised to identify specific barriers that reduce gene flow or genetic diversity, such as waterfalls, habitat variation and increased water flow (Holderegger & Wagner, 2008). This approach also provides the framework to predict the effects of anthropogenic barriers or management alternatives on genetic variation and population connectivity, and thus can subsequently assist in the identification of potential biological corridors for reserve/conservation design (Hale *et al.*, 2001; Holderegger & Wagner, 2008; Storfer *et al.*, 2006). Along with the identification of specific barriers to gene flow, landscape genetics can also be utilised to identify the influence of other landscape variables on genetic variation, including cover type (Keyghobadi *et al.*, 1999; Spear *et al.*, 2005), stream distance (Antolin *et al.*, 2006; Roach *et al.*, 2001), historic landscape configuration (Holzhauer *et al.*, 2006), water

flow rates (Michels *et al.*, 2001), ridge distances (Funk *et al.*, 2005; Pfenninger, 2002) and thermal cover (Scribner *et al.*, 2005), as well as the effect of landscape configuration on allelic fixation time (Ezard & Travis, 2006).

1.2 TOOLS IN EVOLUTIONARY STUDIES

1.2.1 Morphology

Morphological characteristics are still recognised as the most useful and common methods for differentiating between species (Hillis & Wiens, 2000). Although the use of genetics is fast replacing traditional taxonomic identification of species, its practicality in the field is still limited compared to the use of morphology. Morphological variation is believed to be a crucial component of the 'biological species concept' as populations are isolated from breeding either through physical incompatibility or inability to recognise one another as potential mates (Mayr, 1942). Studies of morphological variation in organisms generally follow one of two methods; traditional taxonomy or morphometrics.

Traditional taxonomy uses shared morphological characteristics to categorise organisms into their biological type or taxonomic rank (such as species or genus) (e.g., Riek, 1969). This method is very useful for naming and identifying species across the entire taxonomic spectrum with shared morphological characteristics assumed to portray shared evolutionary history (Mayr, 1942). The simplistic nature of traditional taxonomy also makes it more applicable to identify between species in the field via single morphological traits. Taxonomic identification between freshwater crayfish however has not been as easy or successful with large numbers of both sub-species and re-identifications made continuously (McCormack, 2013; Riek, 1951, 1969; Sokol, 1988). The difficulties faced when identifying between species of freshwater crayfish include irregular growth from ecdysis, convergent evolution of morphological traits (Munasinghe *et al.*, 2004a) and erratic morphological variation within a species (Riek, 1969; Sokol, 1988). These difficulties often lead to vague and 'averaged' taxonomic descriptions for species.

Morphometrics on the other hand has been highly successful in freshwater crayfish for identifying morphological variation among one or a few closely related species (Allegrucci *et al.*, 1992; Bertocchi *et al.*, 2008; Haddaway *et al.*, 2012). This is done by analysing variations in size and shape of a number of key morphological characteristics both within and between species (Bookstein, 1998; Dryden & Mardia, 1998) (e.g., Allegrucci *et al.*, 1992). This form of identifying morphological variation is especially useful in small scale studies where a number of small morphological dissimilarities may distinguish between species rather than one main morphological feature (Allegrucci *et al.*, 1992). Since the 1960's and 1970's when biometricians began using multivariate statistical tools to describe morphological shape via morphometrics, there have predominantly been two disciplines that have emerged; traditional morphometrics (Marcus, 1990; Reyment, 1991) or multivariate morphometrics (Blackith & Reyment, 1971) and geometric morphometrics (Dryden & Mardia, 1998; James Rohlf & Marcus, 1993). Traditional morphometrics consists of applying multivariate statistical analyses to sets of morphological variables such as linear distances, counts, ratios and angles. The covariation in these measurements are then quantified and the patterns of variation within and among samples are assessed using statistical analyses like Principle Component Analysis (PCA), factor analysis, Canonical Variate Analysis (CVA) and discriminant function analysis (Adams *et al.*, 2004). Two major issues that have been identified with traditional morphometrics are the difficulty to integrate curved edges into the analysis and the strong correlation between linear distances and size (Bookstein *et al.*, 1985). Some effort has been spent on developing methods to correct for size including size-free shape variables and patterns of shape variation (e.g., Jungers *et al.*, 1995; Sundberg, 1989).

Because of these issues, researchers have explored alternative methods of quantifying and analysing morphological shape and established geometric morphometrics. Referred to as 'morphometric synthesis' by Fred L Bookstein (1996a), geometric morphometrics combines multivariate statistics and methods to directly visualise the biological shape. When first theorised, this technique was limited predominantly to 'outline methods'. This method involved digitising points along an outline, fitting them to a mathematical function and comparing their coefficients using multivariate analyses (Rohlf, 1990). This approach however was quickly surpassed by 'landmark-based methods' due to its limitations and inconsistencies using different statistical methods (Adams *et al.*, 2004). Landmark-based methods begin with a collection of two- or three-dimensional coordinates of biological definable landmarks that are then optimised and rotated to remove any 'non-shape' variation. The remaining variation between the landmarks represents the variation in shape rather than length and can be

graphically represented for comparison using a variety of methods (Fred L Bookstein, 1996b; Rohlf & Slice, 1990; Slice, 1996). The current popularity of this method has also been facilitated by the growing availability of high quality images and image processing techniques (Cadrin & Friedland, 1999). A major limitation with the exclusive use of morphology or morphometrics however is in areas of hybridisation or hybrid zones. Within hybrid zones, interbreeding between species can result in a completely new phenotype (Allegrucci *et al.*, 1992; Jensen *et al.*, 2002). In areas where this may occur, combinations of both morphological and genetic techniques are likely to give more useful results.

1.2.2 Mitochondrial and Nuclear DNA

With the advent of molecular phylogenetics (Schuh, 2000) and phylogeography (Avise, 1998), genetic techniques have become an easy and quantitative way of exploring the historical effects of geographic isolation on different taxa. Combined with morphology, they provide accurate information on both inter and intra-species variation that can successfully aid in the identification and conservation of organisms (Palumbi & Cipriano, 1998). Since the discovery of the polymerase chain reaction, mitochondrial DNA has fast become the preferred marker for these studies to analyse genetic variation within or between species. The relatively clonal and maternal inheritance of the mitochondria along with its similarity across all eukaryotes provides great confidence in both the construction of gene trees and detection of population subdivisions (Moritz *et al.*, 1987). The ability of mtDNA to detect population subdivisions is primarily due to its haploid nature. As a maternally inherited haploid molecule, the effective population size of mtDNA is one quarter that of nuclear DNA (Birky-Jr *et al.*, 1989). This reduction in effective population size combined with a relatively high mutation rate leads to an increased effect of genetic drift (Birky-Jr *et al.*, 1989). It was however mtDNA's similarity across all eukaryotes that increased its popularity, as it allowed relatively cheap amplification using easily accessible 'universal' primers. In phylogenetics, mtDNA clonal inheritance allows the relatively accurate use of a molecular clock to estimate the time to the most recent ancestor (TMRCA) or 'coalescence time' (Crisci *et al.*, 2003; Page & Holmes, 1998). TMRCA is useful for assessing the likelihood of a number of competing hypotheses, such as vicariance and dispersal (Avise, 2004; Crisci *et al.*, 2003). Although extremely useful, there are a number of complications that limit the accuracy of molecular clocks, including DNA repair efficiency, different generation times between taxa, DNA replication intervals, mutation rates between regions of a genome and the reliance on fossil and biogeographic evidence for the separation times of taxa (Avise, 2000; Ho *et al.*, 2005). Heterogeneity among lineages can also be a major

source of error in molecular clocks, especially when the mutation rate used is not from the species of focus or one very similar (Arbogast *et al.*, 2002). Until recently, a common assumption in the use of molecular clocks was that the mutation rate of a gene is linear through time (Ho & Larson, 2006; Ho *et al.*, 2005). Ho *et al.* (2005) suggests however that due to purifying selection and saturation, the mutation rate of a gene may actually decrease over time from high short term mutation rates to slow stable rates. The main issue with the use of mtDNA in reconstructing the history of a species is it represents just a single marker and thus a single story (Chen & Jinzhong, 2009; Grechko, 2013). To gain a comprehensive understanding of the genetic variability of an organism, it is recommended to utilise both fast evolving mtDNA and slowly evolving nuclear DNA (nDNA) (Grechko, 2013). The slow nucleotide substitution rates of nDNA compared to mitochondrial DNA make them ideal for inter-species phylogenetic comparisons (Grajales *et al.*, 2007), particularly as they are less affected by saturation. Inter-species phylogenetic comparisons provide an initial understanding of the evolutionary relationships between taxa and are crucial as a comparison with morphological analysis (Austin *et al.*, 2003; Hillis & Wiens, 2000) and as a precursor for smaller scale geographic analysis (Bentley *et al.*, 2010; Bernatchez & Wilson, 1998). Phylogenetic comparisons combining both mtDNA and nDNA are commonly implemented for widely distributed species with plenty of taxonomic challenges like 'cryptic' species (Baker, Hughes*, et al.*, 2004; Bentley *et al.*, 2010) , sex biased dispersal (Prugnolle & de Meeus, 2002) and morphological homoplasy (Mueller *et al.*, 2004). Comparing the results from nDNA and mtDNA can identify mtDNA issues such as unrecognised hybridisation and introgression, ancestral polymorphism and nuclear paralogs of mtDNA (Chen & Jinzhong, 2009; Grechko, 2013; Lopez *et al.*, 1994; Sorenson & Quinn, 1998).

1.3 SPECIATION IN AUSTRALIAN CRAYFISH

The different techniques introduced above for identifying the processes of genetic divergence and gene flow have been successfully applied separately on freshwater crayfish, but very few studies have integrated genetic, morphological and ecological information for a comprehensive investigation into their historic and contemporary isolation. Traditionally, morphology was used to describe new crayfish species (Hobbs Jr., 1987; Riek, 1951, 1969) and interpret past evolutionary history (Riek, 1972). However its practicality for constructing phylogenies and estimating common ancestors in freshwater crayfish is questionable due to the convergence of morphological characters among species (Holdich, 2002). As different species can occupy similar habitats they often have similar adaptive morphological features

(e.g., burrowing species have reduced abdomens and larger, broader chelae) (Crandall & Buhay, 2008). Because of this, effective identification between species relies fundamentally on differences of either a specific morphological characteristic (e.g., pleopod in Genus *Orconectes*) or an accumulation of small morphological variants (Riek, 1951, 1969). This technique has been effective for distinguishing among a number of species of freshwater crayfish (Allegrucci *et al.*, 1992; Hobbs Jr., 1987) and is still the method used to identify species taxonomically (Coughran, 2005; Morgan, 1997; Riek, 1951).

The traditional method of using morphology to distinguish between species of freshwater crayfish is however fast being replaced by molecular approaches. The use of genetic information has identified a large number of areas of high biodiversity for freshwater crayfish, with the identification of new taxonomic species and cryptic species (Bentley *et al.*, 2010; Buhay *et al.*, 2007; Munasinghe *et al.*, 2004b). These areas of high biodiversity were investigated by Crandall and Buhay (2008) where they documented over 640 species of freshwater crayfish around the globe with two particular areas of high biodiversity and endemism (Southern Appalachian Mountains in south-eastern United States and south-eastern Australia). In 2000, Crandall *et al*. also used genetic distinctness to identify a monophyletic origin of freshwater crayfish, estimating a sister-group relationship between freshwater crayfish and clawed lobsters (Crandall *et al.*, 2000). As obligate freshwater organisms, freshwater crayfish are often highly endemic, showing patterns of strong geographic structure. In Australia, this has been observed by Whiting *et al.* (2000), who identified the north-west coast of Tasmania and south-east of the Australian continent as high conservation areas for freshwater crayfish and south-east Queensland and south-east Australia as areas of high endemism. In south-east Queensland, Bentley *et al.* (2010) discovered high geographic structuring both between and within lineages of the species *Cherax dispar*, with a total of four lineages discovered within the species. This within-species structure was also observed by Gouws *et al.* (2006) for *Cherax preissii* in the south-west of Western Australia with geographic structuring discovered between north and south populations. One of the strongest geographic structuring of freshwater crayfish in Australia however was described by Riek (1969), who observed geographic isolation between different species of *Euastacus*, with each species isolated to a different mountain top of eastern Australia.

This strong geographic structuring in freshwater crayfish is often attributed to the ecology and environmental requirements of the species. The inter-species geographic structuring described by Riek (1969) was thought to be caused by the environmental requirements of the species, as

this genus required cooler climates. Studies like this on the habitat requirements of freshwater crayfish are usually focused on one of three topics: the conservation of endangered species, understanding ways of stopping or limiting introduced species or understanding the environmental requirements of farmed crayfish. Although a number of freshwater crayfish species in Australia are endangered, most of the studies on the habitat requirements of freshwater crayfish are focused on conserving species in Europe (Benvenuto *et al.*, 2008; Scalici *et al.*, 2008; Smith *et al.*, 1996), Asia (Nakata *et al.*, 2003; Usio, 2007) and North America (Jones & Bergey, 2007). The general consensus from these studies however, is that most freshwater crayfish are highly specialised in terms of habitat requirements and often occupy specific microhabitats within a freshwater system. This was also suggested by Johnston and Robson (2009) in south-east Australia, where five sympatric species occupying the same landscape each had contrasting habitat requirements with a generalist species (*Cherax destructor*) and a highly specialised species (*Euastacus bispinosus*) and some in-between. Among these species, the key delimiting environmental factor that separated them was the percent of boulders and the abiotic/biotic environmental characteristics that come with it.

Although freshwater crayfish often tend to reside in specific habitats, the ability for them to disperse between habitats and/or populations is vitally important for their conservation. Some of the most comprehensive studies on small scale freshwater crayfish dispersal have been on the signal crayfish and rusty crayfish, two highly destructive invasive species in North America and Europe (Bubb *et al.*, 2004; Bubb *et al.*, 2006; Byron, 2001; Usio *et al.*, 2006; Wilson *et al.*, 2004). From these studies, it has been shown that the dispersal ability of freshwater crayfish is highly species specific, with generalist species, such as many invasive species, dispersing easily between habitats and populations (Bubb *et al.*, 2006). Generally however, dispersal of freshwater crayfish is relatively low, with dispersal limited to small scale movements within river catchment boundaries (Bentley *et al.*, 2010; Gouws *et al.*, 2006; Hughes & Hillyer, 2003).

Although there have been a large number of studies on freshwater crayfish around the world, very few have focused on the processes of speciation and the ecological requirements of Australian crayfish. Instead a number of studies on Australian freshwater crayfish have focused on the taxonomic identification of species or the current distribution of morphological and/or phylogenetic variation. In 2004, Ponniah and Hughes investigated the evolutionary history of the Queensland freshwater crayfish, identifying speciation via simultaneous vicariance for the Spiny Mountain Crayfish. The Queensland Spiny Mountain Crayfish however are relatively unique for Australian crayfish, with each species allopatrically distributed on separate

mountain tops. In contrast, Australian species are often distributed sympatrically across a wide range of environmental habitats and conditions. This is particularly the case for the genus *Cherax*, with distributions ranging from temperate rainforests to dry land ephemeral river systems. Further analysis combining the morphology, genetic and ecological information of Australian crayfish however may identify the evolutionary history of Australian crayfish and place a better understanding of the underlying processes that have driven freshwater crayfish speciation within Australia.

1.4 STUDY SPECIES

Of Australia's nine freshwater crayfish genera, *Cherax* is the most widespread and species-rich. Although the taxonomy of the genus is still incomplete, there are currently 45 species recognised throughout Australia (Crandall & Buhay, 2008; McCormack, 2013). The genus has three distinct centres of diversity in Australia; the southwest of Western Australia, the southeast of Queensland (SEQ) and the Cape York region in the north (Austin, 1996; Austin & Knott, 1996; Munasinghe *et al.*, 2004a; Munasinghe *et al.*, 2003). Within these regions *Cherax* occur in lakes, swamps, billabongs, farm dams, irrigation canals and bore drains, and also in slow, muddy rivers and creeks.

Of the three *Cherax* 'hotspots' of diversity found in Australia, SEQ alone holds approximately a fifth of the currently recognised Australian species (McCormack, 2013; Munasinghe *et al.*, 2004a). These species are distributed throughout the mainland coastal river catchments and the four adjacent sand islands, and most are endemic to SEQ (McCormack, 2013; Munasinghe *et al.*, 2004a; Riek, 1969; *Wildlife of Greater Brisbane*, 2007). These species include *Cherax robustus, Cherax dispar, Cherax depressus, Cherax punctatus, Cherax cuspidatus, Cherax urospinosus* and *Cherax sp. nov* (McCormack, 2013; Munasinghe *et al.*, 2004a). This number of described *Cherax* species from SEQ is also expected to double in the coming years with another five distinct morphological species already identified (McCormack pers comm). Although some of these species are common throughout Queensland, research on them has mainly been limited to either taxonomic or phylogenetic analysis, with only one phylogeographic study to date focused specifically on *C. dispar* (Bentley *et al.*, 2010). This project will develop on this previous research on *C. dispar* and compare its molecular variation with *C. depressus*. These two species were selected as they occur sympatrically throughout SEQ but are suggested to occupy contrasting habitat types (*Wildlife of Greater Brisbane*, 2007).

1.4.1 Cherax dispar (Slender Yabby)

Cherax dispar is relatively widely distributed throughout southeast Queensland, found as far north as Bundaberg and south as Logan (Figure 1.1). As a highly abundant species, it dominates the crayfish fauna in the freshwater systems of both North Stradbroke Island and Fraser Island, although it often shares the streams (particularly acidic ones) with other crustaceans such as *Macrobrachium tolmerum* and Caridina *indistincta* (*Wildlife of Greater Brisbane*, 2007). Due to its poor burrowing capabilities (Type 1 burrower (Horwitz & Richardson, 1986)), *C. dispar* tends to be restricted to perennial streams and coastal sand lakes rather than inland ephemeral streams (*Wildlife of Greater Brisbane*, 2007).

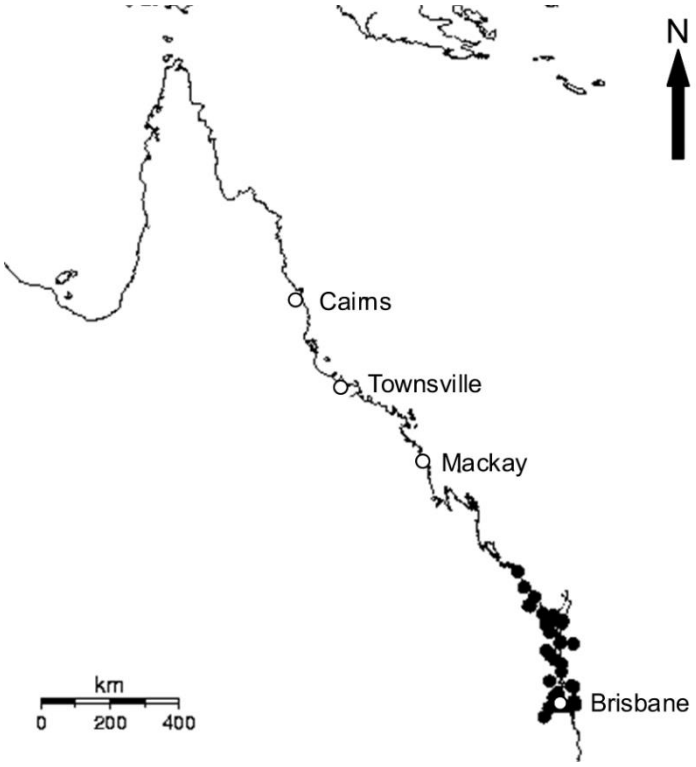


Figure 1.1: Estimated *Cherax dispar* distribution based on Queensland Museum holdings in 2007. Open and closed circles indicate major Queensland cities and *C. dispar* sample localities respectively.

C. dispar grows to about 75mm and bears a long and slender rostrum with two well-developed spines near the tip (Figure 1.2 & Figure 1.3). The carapace is greenish-grey to brown which blends well in the substrates of stream banks (Figure 1.2). Only the undersides of the claws are a deeper blue. Juvenile specimens of *C. dispar* have orange fingertips on their claws and are often misidentified as *C. depressus* (*Wildlife of Greater Brisbane*, 2007).

a)  b) 

Figure 1.2: Photographs of the a) dorsal view and b) rostrum of *Cherax dispar*. (Sourced from Bartholomai (1997) and self-taken respectively).

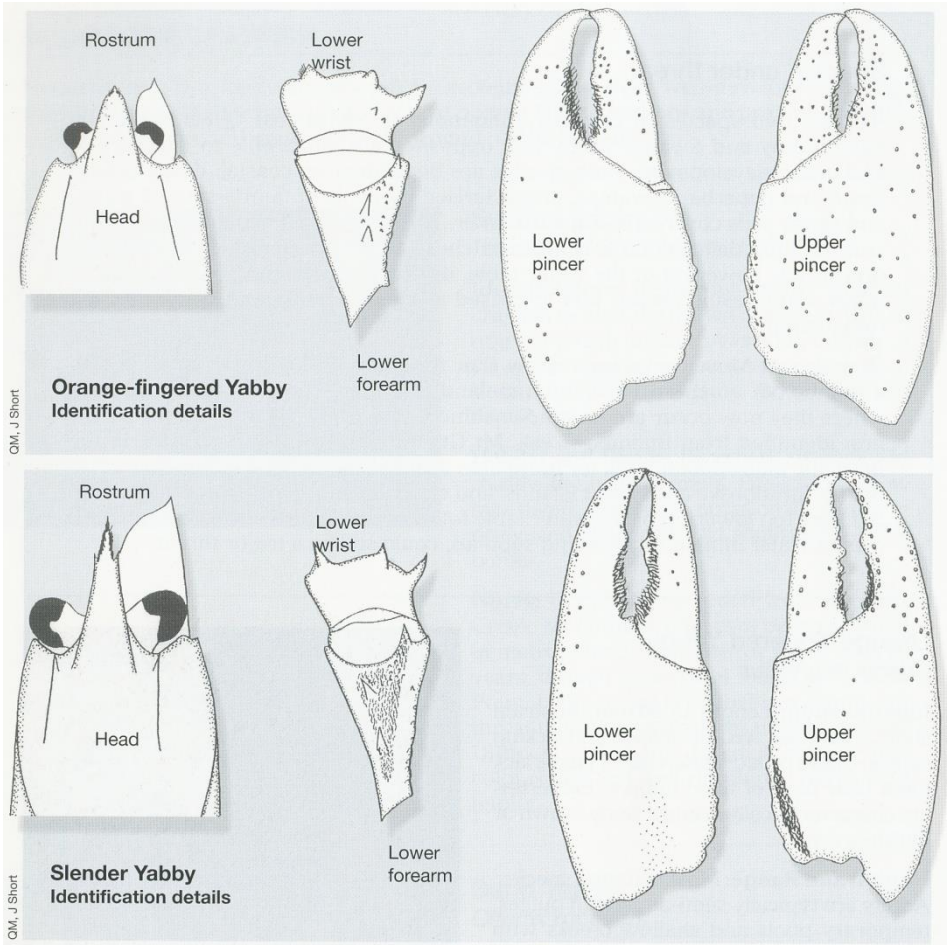


Figure 1.3: Identifiable morphological characteristics of *Cherax depressus* (Orange-fingered Yabby) and *Cherax dispar* (Slender Yabby). (Sourced from Museum (*Wildlife of Greater Brisbane*, 2007)).

Variation within the species *C. dispar* was first noticed by Riek (1951), when based on morphology alone. He erected an additional two subspecies to the nominal taxon. This variation was also further investigated genetically, where four potential cryptic species were identified; *C. dispar* A-D (Bentley *et al.*, 2010). Strong phylogeographic structure was discovered among the four cryptic species, with one isolated to the islands of Moreton Bay (*Cherax dispar* D), one distributed along the coast from the Brisbane River catchment to Noosa Heads (*Cherax dispar* C) and two distributed throughout the Mary River catchment and Fraser Island (*Cherax dispar* A & B) (Figure 1.4). Of these four clades, *Cherax dispar* A & C were the only clades discovered to occur sympatrically.

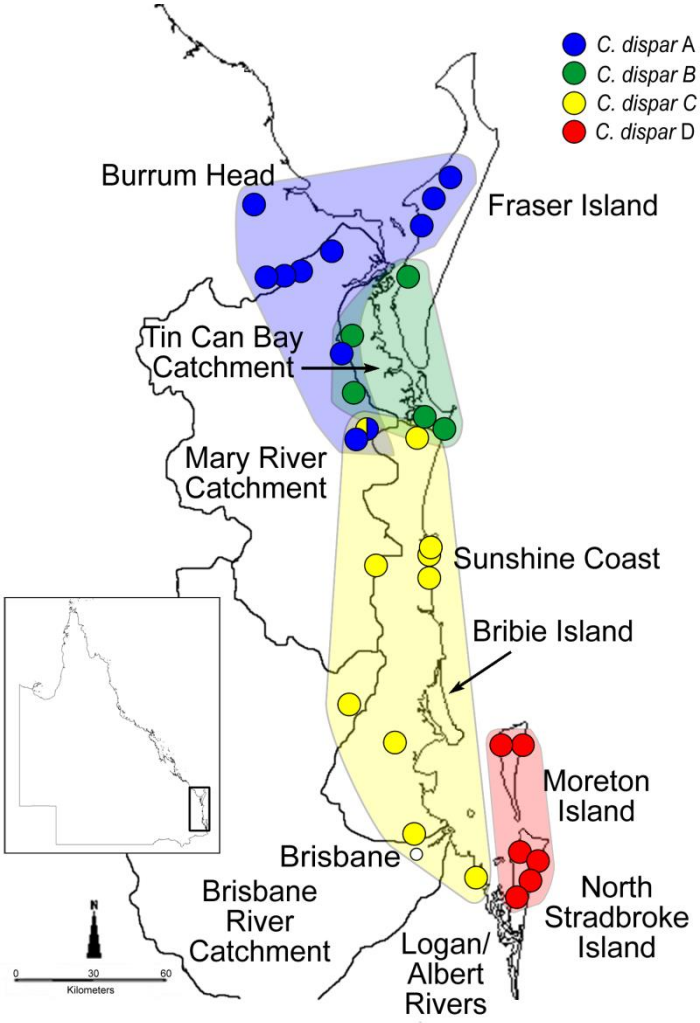


Figure 1.4: Distribution of four cryptic species of *Cherax dispar* within South East Queensland (Sourced from Bentley *et al.* (2010))

1.4.2 Cherax depressus (Orange-Fingered Yabby)

Cherax depressus is the most widely distributed *Cherax* species in Queensland, and is distributed along the entire coast of Queensland (Figure 1.5). Referred to as the *depressus* complex, the original distribution of *C. depressus* was suggested to be broken into an additional three species (Riek, 1969). The variants of the original taxon include *C. cairnsensis* and *C. waselli* (located in Cairns and Kuranda respectively, Nth Qld) and *C. gladstonensis* (located in Gladstone, central Qld) (Figure 1.5). *C. gladstonensis* has since been incorporated into *C. cairnsensis*, extending *C. cairnsensis*'s distribution across most of the Queensland coast (McCormack, 2013). The nominal taxon of *C. depressus* is restricted to the mainland of southern Queensland. Unlike *C. dispar*, *C. depressus* is a strong burrower (Type 2 burrower (Horwitz & Richardson, 1986)) and is capable of survival in semi-aquatic gullies, temporary pools and shallow creeks that have very limited to no flow (*Wildlife of Greater Brisbane*, 2007).

C. depressus is typically distinguishable from *C. dispar* by its short triangular rostrum with no spines (Figure 1.3 & Figure 1.6). It grows to approximately 90mm and bears claws that are generally broad with no hairs near the base or fixed fingers (Figure 1.3). The fingertips have the characteristic orange tips while their overall body colour is usually brown or bluish-green (Figure 1.6) (Riek, 1969; *Wildlife of Greater Brisbane*, 2007).

Although *C. depressus* is the most common species of crayfish in SEQ, very little is known concerning the ecological requirements of the species or any genetic or morphological variation within the species. As *C. depressus* is capable of burrowing down to the water table during drought periods, it would be predicted that the need to disperse for suitable habitat would be lower than that of *C. dispar*.

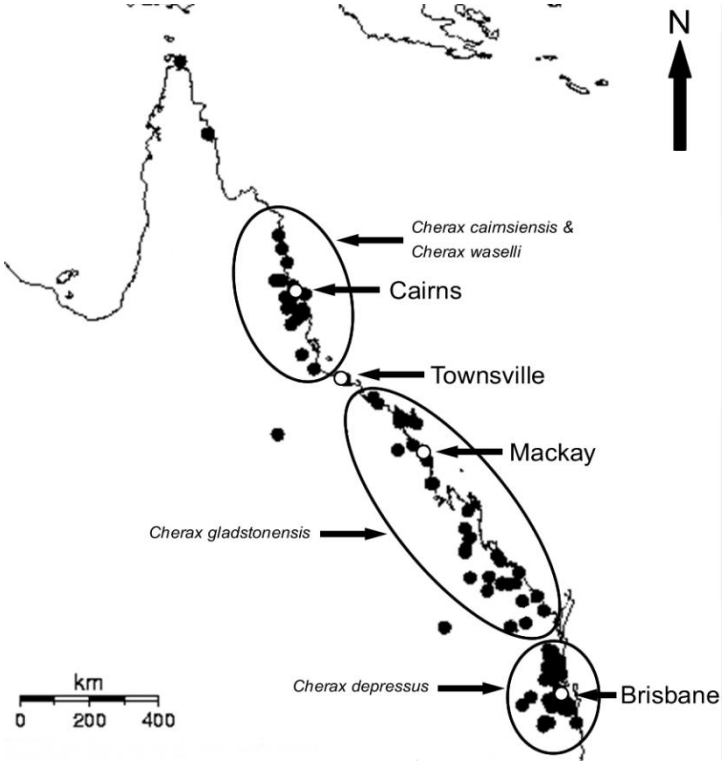


Figure 1.5: Estimated *Cherax depressus* distribution based on Queensland Museum holdings in 2007.Circled areas represent distributions of species within the *C. depressus* complex identified by Riek (1969).

a) b)

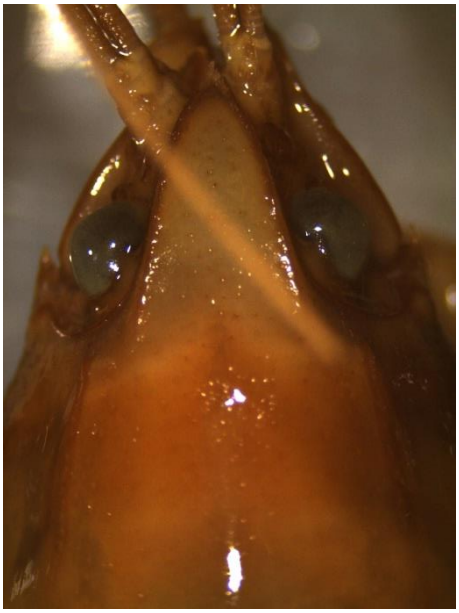


Figure 1.6: Photograph of a) dorsal view and b) rostrum of *Cherax depressus*. (Sourced from Bartholomai (1997) and self-taken respectively).

1.5 STUDY AIMS & HYPOTHESES

Although there have been a number of phylogeographic and phylogenetic studies conducted on freshwater crayfish in South East Queensland, none have comprehensively identified the historical and contemporary drivers that caused and maintained their high level of biodiversity. For each of the studies, there have been major limitations either in the number of or type of genes used, the number of individuals and a complete lack of any accompanying morphological information. Because of this, the studies could only speculate on the actual amount of diversity present and its causes.

The aim of this study therefore was to comprehensively identify the contemporary and historical causes of the current SEQ freshwater crayfish diversity, with specific focus on *C. dispar* and *C. depressus*. Using an integration of phylogenetics, morphometrics, phylogeography, population genetics and environmental information, I aim to identify whether geographic, behavioural or ecological isolation (or a combination of the three) is driving the current freshwater crayfish biodiversity in SEQ. As a biodiversity hotspot for a number of other freshwater organisms (Munasinghe *et al.*, 2004b; Unmack, 2001; Whiting *et al.*, 2000), SEQ provides an ideal location to determine whether the driving forces identified are specific to the species of focus or freshwater crayfish more generally.

Even though a number of studies have been conducted on the phylogeny and taxonomy of SEQ freshwater crayfish (McCormack, 2013; Munasinghe *et al.*, 2004b; Riek, 1951, 1969), there is still a large amount of confusion and inconsistency between them. In Chapter 3, I will identify the current phylogenetic relationships of SEQ *Cherax* and distinguish their validity as distinct taxonomic species. Currently most phylogenetic studies on SEQ freshwater crayfish use only a few individuals for each species (Munasinghe *et al.*, 2004b) or a limited number of genes (Bentley *et al.*, 2010; Munasinghe *et al.*, 2004b). These limitations have consistently been proven to produce misinterpretations or inconsistencies in results, as seen between Munasinghe *et al.* (2004b) and Bentley *et al.* (2010), with each study identifying support either for or against Riek (1951)'s separation of *C. dispar* into multiple subspecies. In this chapter I will analyse three mtDNA genes and four nDNA genes both separately and together to understand the extent of diversity that is consistent across all genes. From these phylogenies, I will also determine the evolutionary history of SEQ freshwater crayfish and estimate the potential number of taxonomic species within the region.

As freshwater crayfish in Australia are both widely distributed and highly diverse, they provide a unique opportunity for a comprehensive investigation into the biogeographic history of freshwater taxa in Australia. By comparing the current geographical relationships among the phylogenetic lineages identified in Chapter 3, Chapter 4 will estimate the historical biogeographic distributions and dispersal patterns of *Cherax* within Australia. With the continuous increase in aridity within Australia, the opportunities for obligate freshwater organisms to disperse throughout Australia have progressively become limited (Unmack, 2001). By comparing the biogeographic history of *Cherax* to other freshwater crayfish and fish, Chapter 4 aims to identify whether Australia's gradual change in climate has driven diversification within Australian freshwater fauna. A comparison between divergence estimates for *Cherax* species and other freshwater fauna will also identify the dispersal limitations and abilities among *Cherax* species and other freshwater taxa.

In Chapter 5, a phylogeographic approach will be applied to explore the recent history of *C. dispar* and *C. depressus*. While both the previous chapters investigated the historical causes of diversity in SEQ, Chapter 5 focuses specifically on the more recent drivers of divergence. In this chapter I aim to use the COI mtDNA phylogeographic pattern of *C. depressus* and each of the lineages of *C. dispar* to identify the geographic causes of the high freshwater crayfish diversity in SEQ and the overall effect life history has on *Cherax* dispersal. Comparisons between river catchment boundaries and physical distances along a river will be used to determine possible dispersal limitations for the species. As the two species have contrasting life histories, their dispersal limitations are expected to also differ.

Although phylogeographic patterns can explain the possible causes of the current diversity, they often lack the capability to explain the current distribution of a species, especially those that are sympatric. In the final data chapter, a finer scale approach will be used to identify the contemporary drivers of the current distribution of SEQ freshwater crayfish, specifically *C. dispar* and *C. depressus*. Using mtDNA variation, morphological characteristics and environmental conditions, Chapter 6 aims to identify if the current freshwater crayfish distribution is driven by environmental or behavioural factors. By comparing the contemporary patterns of connectivity and morphological plasticity among populations with their environmental conditions, I am to identify if environmental limitations are restricting the dispersal of freshwater crayfish.

Lastly the thesis finishes with a general discussion of the findings from each of the chapters to reinforce our knowledge of the contemporary and historic influences on the current SEQ freshwater crayfish distribution.

CHAPTER 2: GENERAL METHODS

2.1. SAMPLE COLLECTION

2.1.1 Large Scale

Crayfish specimens were sampled throughout every catchment of south-east Queensland covering the entire distribution of *Cherax dispar* and *Cherax depressus* (Riek, 1969; Queensland *Wildlife of Greater Brisbane*, pers comm). To cover distributions of both species, sampling was conducted in both permanent creeks and ephemeral waterholes/creeks (*Wildlife of Greater Brisbane*, 2007). Specimens were collected using a range of techniques including hand nets, traps (box and opera-house), electro-fishing, seine netting and by hand. Some specimens were also collected as part of other studies by the Department of Environment and Resource Management (DERM) and fellow staff at Griffith University (see Appendix 8.2 for specifics). Whole individuals were collected for small specimens while either a leg or claw was taken for larger individuals. When possible the third leg was taken as the leg is quicker than the claws to re-grow and causes the least disturbance to the mobility and feeding ability of a crayfish (McCormack, 1994). Some larger specimens were also collected whole for further morphological comparisons. All samples were either frozen in liquid nitrogen or preserved in 90-100% ethanol. A number of specimens from the entire geographic region of each species were also borrowed from the Queensland museum. These specimens were used to identify individuals taxonomically and to assign relevant species designations for other specimens.

The distribution of the two *Cherax* species of focus and all sampling in this study is spread across eight river catchments; Logan-Albert (LA), Brisbane (BR), Caboolture (CAB),Pine (PIN), Maroochy (MCHY), Mooloolah (MOO), Noosa (NR), Mary (MR) and Burrum (BUR), four coastal islands; North Stradbroke Island (NSI), Moreton Island (MI), Bribie Island (BI) and Fraser Island (FI) and a number of small creeks that flow directly into the Pacific Ocean; grouped into Glass House Mountains (GHM), Gold Coast (GC), Tin Can Bay (TCB) or Tingalpa (TIN) (Figure 2.1). Other *Cherax* species were also collected from the following river catchments; Waterpark Bay (WP), Shoalwater Bay (SWB) and the Murray-Darling Basin (MDB).

Figure 2.1: South East Queensland catchment boundaries.

2.1.2 Small Scale

2.1.2.1 Sampling Design

Fine scale sampling was conducted in the Tinana Ck sub-catchment of the Mary River. Four replicate sites were sampled at each of six different locations (Figure 2.2). Due to problems with site access or difficulty with capturing sufficient specimens, these six locations were later reduced to four for multiple sampling trips, with locations four and six removed. The sample sites were chosen to allow for the best analysis of both small scale and larger scale dispersal with sampling occurring in tributary creeks on either side of the main channel of Tinana Ck. Sampling at each replicate was repeated 2-3 times per season. Due to South East Queensland's highest rainfall and largest flood on record occurring in December 2010 and January 2011 (Meteorology, 2011) sampling had to be extended over a two year period with all summer samples collected at the end of 2011/beginning of 2012.

Sampling was conducted using hand-nets, seine nets and a combination of box traps and opera-house traps over a one hour period for each site. Samples were collected for further DNA analysis from ten specimens per site. As per large scale sampling, the third leg was taken from larger individuals with whole specimens only from smaller individuals and these were kept to a minimum. During the final two sampling trips, a number of whole adult specimens from each species at each site were collected for further morphological analysis. Photographs were taken of each of these individuals to record the colour before preservation. All individuals and legs were preserved in 90% ethanol.

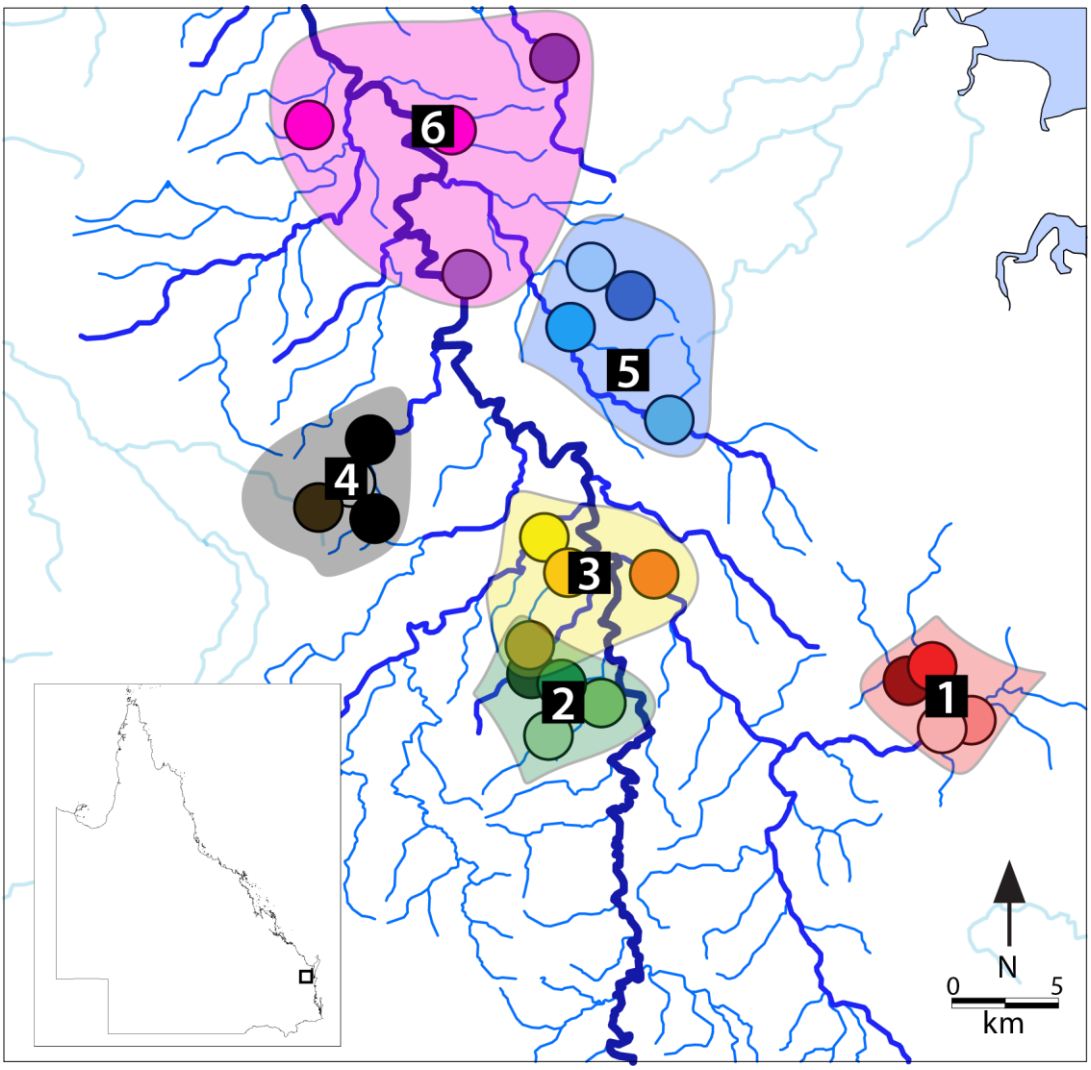


Figure 2.2: Small scale sampling sites within Tinana Ck in the Mary River catchment. Numbers represent sample areas.

2.1.2.2 Sample Measurements

All specimens collected were held in a large bucket containing water from the site of capture and recorded for the following twelve general measurements (nine of which are summarised in Table 2.1):

1. Species (*Cherax dispar*/*Cherax depressus*).

The distinguishing features between the two species are predominantly the rostrum, claw shape and carapace shape (Figure 1.3). Some smaller juvenile individuals were labelled as unknown due to difficulty in identifying these features.

2. Sex (Male/Female/Unknown).
3. Age (Juvenile, Sub-Adult, Adult).

The identification between these age classes was predominantly based on their length with less than 30mm a juvenile, between 30 and 50mm a sub-adult and more than 50mm a mature adult. These three size classes are indicative of individuals unable to reproduce (juveniles), individuals able to reproduce but have not attained adult characteristics (sub-adult) and mature breeding adults (adult) (McCormack, 1994, Bentley Pers. Comm.).

4. Length (From tip of rostrum to end of tail).
5. Rostrum Length (From tip to base).
6. Colour of the carapace.
7. Presence of orange tips on the chela (Yes/No).
8. Presence of eggs (whether in berry) (Yes/No).
9. Presence of *Temnocephalida* on the specimen (Yes/No) (Figure 2.3).

Temnocephalida are commensal turbellarian flatworms that inhabit the cavities of the mantle of *Cherax*. There are two species known to occupy the species of focus; *Temnocephalida christineae* and *Temnosewellia minor* (Cannon & Sewell, 2001). There are no known parasitic effects from *Temnocephalida* on the crayfish.

10. Presence of *Temnocephalida* eggs on the specimen (Yes/No) (Figure 2.3).
11. Area specimen was collected (Pool/Riffle/Run).
12. Whether the sample was possibly a recapture (Yes/No).

Due to the frequent sampling design in this study, estimation on whether a specimen has been recaptured is possible as sampling was more frequent than the regeneration time of a leg/claw (Brewis & Bowler, 1982).To limit the effect of resampling individuals, recaptured samples were excluded from non-temporal population analyses (AMOVA, Phylogeography). Although a loss of a leg/claw naturally is possible, it has been observed rarely and so should have little impact on this study.

Table 2.1: Number of *C. dispar* and *C. depressus* individuals caught for nine of the recorded general measurements.

Measurement		**Species**	
		C. dispar	*C. depressus*
Total Sample Size		1646	289
Sex	Male	661	105
	Female	651	90
	Unknown	334	94
Age	Juvenile	585	136
	Sub-Adult	744	117
	Adult	317	36
Rostrum Length	≤3mm	414	191
	4-6mm	848	86
	≥7mm	254	2
Orange-tip Chela	Present	948	260
	Absent	285	2
Eggs (In Berry)	Present	20	0
	Absent	1611	289
Temnocephalida	Present	77	4
	Absent	551	116
Temnocephalida eggs	Present	411	41
	Absent	1202	248
Area	Pool	1091	153
	Riffle	13	0
	Run	506	118
Recaptures	Yes	75	15
	No	1553	274

Figure 2.3: Photo of Temnocephalida on the underside of a *Cherax dispar*. Temnocephalida adults and eggs are circled in red and black respectively.

2.1.2.3 Environmental Characteristics

Biotic and abiotic environmental variables were recorded from three locations at each of the sample sites with one from the closest pool, riffle and run. These variables were measured during every sampling trip (where possible) to account for temporal variation. The following environmental variables were measured using either a Sonde (YSL) or portable DO, pH & Turbidity TPS meters (TPS, Brisbane), as indicated.

1. Water depth (at deepest accessible section)
2. Water velocity (m/s)
3. Water temperature (^{o}C) (Sonde, TPS)
4. pH (Sonde, TPS)
5. Dissolved oxygen (DO) (Sonde, TPS)
6. Percent dissolved oxygen (DO %) (Sonde)
7. Conductivity (µS/cm) (Sonde, TPS)
8. Specific conductivity (µS/cm) (Sonde)
9. Salinity (mS/cm) (TPS)
10. Turbidity (NTU) (TPS)

Turbidity was measured using a TPS meter when available. As a continual standard for the entire study, turbidity was also measured as the depth at which a white ruler could not be seen. Similar to the Secchi disk method (Preisendorfer, 1986), this measured the turbidity of the water from the surface. I recorded all measurements in the shade during the day to limit glare, daylight and sampler error.

11. % Substrate type (Bedrock, boulder, cobble, gravel, sand and silt)
12. Overhanging vegetation (scale 0-3)

Overhanging vegetation was recorded by the estimated percent of the creek that had branches from vegetation directly above. The scale was separated as follows:

0: 0%

1: 0-20%

2: 20-60%

3: 50-100%

13. Leaf litter (scale 0-3)

Leaf litter was scaled by the estimated percent of leaf litter covering a 20cm by 20cm square section of the creek. For both turbid and deep sections, a dip net was dragged over a 20cm section to estimate the amount of leaf litter. The scale was separated as follows:

0: 0%

1: 0-30%

2: 30-60%

3: 60-100%

14. Submerged tree roots (Scale 0-3)

The scale for submerged tree roots was estimated and scaled based on the percentage of the banks with which submerged tree roots were present. The scale was separated as follows:

0. 0%
1. 0-20%
2. 20-50%
3. 50-100%

15. Large woody debris (Present/absent)

Large woody debris was identified as the presence or absence of a branch or tree larger than 20cm long and 2cm diameter that is submerged in the water.

16. Small woody debris (Scale 0-3)

Small woody debris was identified as woody debris or twigs, smaller than those characterised as large woody debris. This variable was scaled by the percent covered in a one square meter section of the creek. Percentages were separated as follows:

0. 0%
1. 0-10%
2. 10-20%
3. 20-100%

17. Undercut banks (Present/absent)
18. Bank modifications (Scale 0-3)

Bank modifications were categorised into a scale as follows:

0. No modifications/natural condition
1. Some substrate used (e.g., gravel or mud)
2. A combination of a new substrate and cement was used
3. Bank has been concreted

19. Aquatic and riparian vegetation

The predominant species of vegetation discovered on the banks and in the channel were identified to the species level where possible.

2.2. MOLECULAR TECHNIQUES

2.2.1 DNA Extraction

A modification of the CTAB/phenol-chloroform DNA extraction protocol (Doyle & Doyle, 1987) was used to isolate total genomic DNA from a small portion (~1mm) of the abdomen, claw or leg of each individual. Each sample was placed in a 1.5mL Eppendorf tube containing 600 µL of 2 x CTAB extraction buffer (0.5M Tris HCL pH 9.0, 2M NaCl, 0.25M EDTA, 0.05M CTAB; Sigma, Sydney, Australia) and 5 µL of Proteinase K (20mg mL^{-1}). Each sample was homogenised with a plastic mortar, vortexed, then left to digest overnight at 55°C on a Thermoline dry block incubator. An extra 5 µL of Proteinase K was added to samples that had not completely digested by the next morning and left for another day.

Once digested, 600µL of chloroform-isoamyl (24:1) was added to each tube and centrifuged at 13500 rpm in an IEC Micromax centrifuge for 5 minutes. The supernatant from the upper phase which contains the DNA was removed and placed into a new 1.5 mL Eppendorf tube. A further 350µL of phenol and 350 µL of chloroform-isoamyl (24:1) was then added to each tube and allowed to mix on a Clements suspension mixer for 15 minutes. Once mixed each sample was centrifuged at room temperature for a further 5 minutes at 13500 rpm. The supernatant from the upper phase was removed and placed into a new 1.5mL Eppendorf tube. 600 µL of chloroform-isoamyl (24:1) was added to each tube and centrifuged for a further 5 minutes. The supernatant was again removed and incubated with 600 µL isopropanol at -20°C for 60 minutes to precipitate genomic DNA. Each tube was then centrifuged at 13500 rpm for 30 minutes, with the supernatant removed and the pellet washed in 1000 µL of 70% ethanol. Each tube was then centrifuged at 13500 rpm for a further 20 minutes and the supernatant removed. DNA pellets were then dried and resuspended in 50 µL ddH_2O.

2.2.2 DNA amplification

A total of seven mitochondrial and nuclear DNA fragments were amplified using polymerase chain reaction (PCR). The PCR amplifications were performed in 10 µL reaction volumes with concentrations as per

Table 2.2 and primers as per Table 2.3. PCR reactions were also vortexed and centrifuged for 10 seconds before the addition of 0.5 µL of template DNA. All PCR reactions were loaded into an Applied Biosystems GeneAmp PCR System 2700 and placed under the same PCR protocol or cycling conditions with differences only in annealing temperature (Table 2.3). The PCR protocol included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C denaturing for 30 seconds, the specific annealing temperature for 30 seconds and 72°C extension for 45 seconds. This was followed with 7 minutes at 72°C and a hold for an indefinite period at 4°C.

Table 2.2: Polymerase Chain Reaction (PCR) ingredient concentrations.

Ingredient	Stock Concentration	Reaction Concentration
10 x Polymerase Reaction Buffer (Fisher)	10U/µL	1U/µL
$MgCl_2$ (Fisher)	25mM	2.5mM
dNTP's (Bioline)	10000mM	250mM
Primer 1	10mM	0.4mM
Primer 2	10mM	0.4mM
Thermus aquaticus DNA (Taq) polymerase (Fisher)	1U/µL	0.02U/µL
ddH_2O	-	8.45µL

Table 2.3: PCR amplification primer details.

Gene	Primer Names	Annealing Temperature	Reference
COI	CRCOI-F & CRCOI-R	52	Cook *et al.* (2008)
	HCO & LCO	55	Folmer *et al.* (1994)
16S	16S-F-Car & 16S-R-Car	56	Von Rintelen *et al.* (2007)
12S	12SF & 12SR	56	Mokady *et al.* (1994)
ITS2	CAS5p8sFC & CAS28sB1d	54	Ji *et al.* (2003)
H3	H3F & H3R	55	Colgan *et al.* (1998)
28S	28SF & 28SR	55	Whiting *et al.* (1997)
GADPH	GADPHF & GADPHR	57	(Buhay *et al.*, 2007)

2.2.3 Sequencing

Completed Polymerase Chain Reactions (PCR) were run on 1% agarose gels (0.8g agarose, 0.5µL gel red/80mL) to check for the amplification success of the target gene fragment. 3 µL of PCR product was loaded into the agarose gel plates and submerged into 1 x TAE Buffer (4.84 g Tris, 1.142 mL glacial acetic acid and 2 mL 0.5 EDTA pH 8.0 in 1 L of H_2O). 100 Volts of current at 400 amps were run through each agarose gel for 25 minutes. Each gel was then visualised under a UVP trans-illuminator and compared against a DNA size marker (100bp DNA Ladder) to check for correct amplification.

Successful amplifications were purified using EXO SAP (Fermentas). Purifications were performed in 10.75 µL reaction volumes containing 9.5 µL of PCR product, 0.25 µL Exonuclease I and 1 µL Shrimp Alkaline Phosphate. The Exonuclease effectively removes residual single-stranded primers and any extraneous single-stranded DNA produced during the PCR and the Shrimp Alkaline Phosphate eradicates the remaining dNTP's from the PCR product. Samples were then loaded into an Applied Biosystems GeneAmp PCR System 2700 and incubated at 37°C for 35 minutes then heated at 80°C for 20 minutes. Each purified PCR product was then stored at 4°C.

The purified PCR product then underwent 10 µL sequencing reaction containing 2 µL of Terminator Mix 3.1 (Applied Biosystems), 2 µL of 5 x Terminator Mix Buffer (Applied Biosystems), 0.32 µL of the appropriate Primer, 0.5 µL of purified PCR product and 5.18 µL of ddH_2O. Samples were placed into an Applied Biosystems GeneAmp PCR System 2700 and run through the following protocol: an initial hold of 96°C for 1 minute, 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Samples were then kept at 4°C and out of direct light until sequencing clean-up procedure could be performed.

To clean-up the sequence reaction the entire contents of the sequencing reaction tubes were incubated at room temperature for 15 minutes in new 1.5 mL Eppendorf tubes with 10 µL ddH_2O, 5 µL 125mM EDTA and 60 µL 100% ethanol. The tubes were then centrifuged in an IEC Micromax centrifuge at 13500 rpm for 40 minutes. The supernatant was then quickly removed from each tube and the DNA pellet was rinsed in 200 µL of 70% ethanol before being centrifuged for a further 30 minutes. This step was then repeated with the supernatant once again removed and the DNA pellet rinsed in 200 µL of 70% ethanol prior to being centrifuged for another 30 minutes. The supernatant was removed from each tube and dried in a Thermoline dry block incubator at 50°C. The School of Biological and Biomedical Sciences, Griffith University then sequenced the DNA pellets on an automated sequencing machine (Applied Biosystems AB 3130xl).

2.2.4 PCR-RFLP

Due to the large sample sizes and high cost of sequencing, a number of individuals were identified genetically using a restriction enzyme. Many possible restriction enzymes were trialled, but the large phylogenetic range and variability of this study limited their effectiveness. Due to this limitation, the one-enzyme PCR-RFLP approach of Mirhendi *et al.* (2006) was applied. By using the restriction enzyme *Thermus* species (TseI) (NEB), this method identifies between the two pre-identified clades of *Cherax dispar* (A & B) (see Chapter 3) based on the 16S ribosomal gene. The enzyme TseI digests the 5' GCWGC 3' *C. dispar* A sequence fragment at the 383bp of the 16S gene while the *C. dispar* B fragment contains a mutation (5' GTAGC 3') restricting its digestion. Restriction enzyme digestions were performed in 10 µL reaction volumes containing 5 µL of PCR product, 0.01 µL of TseI restriction enzyme and 1ul of Buffer 3. Samples were then loaded into an Applied Biosystems GeneAmp PCR System 2700 and incubated at 65°C for two hours. Digested PCR reactions were then run on 1.5% agarose gels (1.2g agarose, 0.5µL gel red/80mL) to check for successful digestions. The agarose gels were submerged in 1 x TAE Buffer and run at 90 Volts of current at 400 amps for thirty minutes. Each gel was then visualised under a UVP trans-illuminator and compared against a DNA size marker (100bp DNA Ladder) and a positive control to check for successful digestion (Figure 2.4).

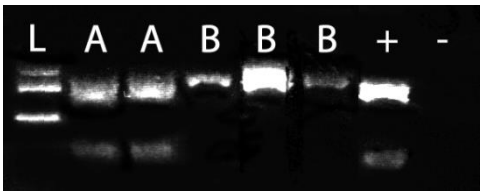


Figure 2.4: Agarose gel of successful restriction enzyme digestions. Symbols are L (100bp Ladder), A (*C. dispar* A), B (*C. dispar* B), + (Positive control) and - (Negative control).

2.2.5 Microsatellite

2.2.5.1 Design

A 50 µL DNA extraction of Cherax *dispar* was sent to the University of Otago High-Throughput DNA Sequencing Unit to be run on the Genome Sequencer FLX system, a 454 sequencing machine. From the run, a total of 23,265 sequence fragments were attained. These sequence fragments ranged from 66 to 810 base pairs in length with an average quality between 15 and 39.

Microsatellites or short sequence repeats (SSR) and their corresponding primers were identified from the sequence fragments using the software package MSAT Commander (Faircloth, 2008; Rozen & Skaletsky, 1999). MSAT Commander was run under default settings with the minimum number of microsatellite repeats for Mononuc, Dinuc, Trinuc, Tetranuc, Pentanuc, Hexanuc set as 10, 6, 4, 4, 4 and 4 respectively. Under these settings a total of 1035 microsatellite regions were discovered from 851 unique sequences. Of these 851 sequences, 168 contained flanking regions for primers that were 50bp or more long and forty seven of these had flanking regions completely SSR free.

Of the forty seven primer sets that were SSR free, twenty four were chosen for amplification based on the guidelines of Faircloth (2008) for successful amplification. These guidelines include an optimal temperature between 57-62^{o}C, a GC content above 50%, a 1-bp GC clamp, a maximum end-stability (ΔG) of 8 and a less than 5^{o}C optimal temperature difference between primers.

Preliminary tests for polymorphism and amplification success were conducted on the twenty four chosen primer sets using twenty four individuals; sixteen *Cherax dispar* and eight *Cherax depressus* from throughout South East Queensland. Of these, half amplified successfully and were polymorphic, with the other twelve either monomorphic, not amplifying effectively or displaying unreliable stuttering (Table 2.4). From these twelve polymorphic loci, a further ten were removed due to inconsistent amplification or high null allele frequency. Of the remaining two loci, one was polymorphic for *C. depressus* (CTO) and the other for both species (C9Y).

In view of the low success rate from the pyrosequencing microsatellite design, a further twenty four primer sets originally designed for another Queensland *Cherax*, *Cherax quadricarinatus* (Xie *et al.*, 2010) were trialled. Preliminary tests managed to amplify only four of the twenty four loci. Although two were polymorphic, all four had low amplification success (Table 2.5). As only two consistent microsatellite loci were identified, further analysis on microsatellite variation was omitted from this study.

Table 2.4: Microsatellite primers designed from pyrosequencing. Tail (5') numbers refer to tail ID in Table 2.2 of Real *et al.* (2009) and T_A is annealing temperature. Amplification success codes are; Poly (polymorphic), LA (low amplification), NA (no amplification), NUL (high # null alleles), Mono (monomorphic) and LV (low variation).

Locus	Primer Sequence (5'-3')	Repeat Motif	Tail (5')	T_A (°C)	Size Range (bp)	Amplification Success
CJF	F: TAGTGGTGGTAGTAGCAGTAATAG R: CCACTACTCTCTTCTTATTGTCTCTG	$(AGT)_5$	1	65	96-117	Poly, LA
CRL	F: ACCCCAGAAAAGGACTTGG R: TGTTGATGTGTGGAAGGAGG	$(ACC)_5$	1	56		NA
C14	F: GTGTGTTGTTCCGCTCTGC R: AGCAGCACTACTTCTTCTCC	$(AGG)_{11}$	1	56	181-184	Poly, LA
C9Y	F: CTGACACTAAAACACCTTCCCG R: GGACACACTGCCTTTCAACC	$(ATT)_7$	2	52	160-211	Poly
DIN	F: CACATCCTTCAGAGTACAGGC R: CAGCTGGTGTTGGCAGAG	$(ATT)_6$	2	56	137-203	LA
CN7	F: TGCTACTGTACTGGTCTCGTG R: GGGGCTCTTGGCATATACATC	$(AAT)_5$	2	52	168-174	LV, NUL
DCR	F: ACCTTTGACGAGTTTCGAGAG R: TGCTGATTTACAAACACGCTTC	$(ATT)_5$	3	56	152-209	Poly, NUL
DLT	F: ATTGTAATCCTTTTACCCTCTCTTC R: CAACATAGCCGGTGCAAGC	$(AACC)_5$	3	56	158-213	Poly, LA
CJL	F: GTACACAGTGACGCACACG R: ACGCACTGACTTTCTTGGG	$(AAGAT)_5$	3	60	167-211	Poly, LA
DN2	F: TCGAGTTTAACGGAATAGCGAG R: TTCAGCGTTCTATGACCTTTATG	$(ATT)_7$	4	56	173	Mono
CAK	F: GAAGAGACGGGGCCAAGAG R: CCAACATGGCTCACAGGC	$(ACAT)_4$	4	60	147-183	Poly, LA
B2W	F: GGCTCTACGGTGGGTTGTG R: GATGGGACATCGAACTTGGC	$(GGCT)_5$	4	56	189	Mono
CWW	F: ATGAAGATCGGTGAGGGGC R: CCTTGAGTGAGGTTTGCAGTC	$(ACTT)_7$	1	56	174	Mono
B5D	F: TGTCTCTGTTTTAGTTTCTCTGCTG R: AGGACCCCAATGGAAATAAGTC	$(AAGT)_5$	1	62		NA
CK9	F: TGACTTTGCATTTGGTGGGG R: TCAGGCAGGACTACAAAGGG	$(ACTT)_5$	1	56		NA
COQ	F: CCAAATCACCATCGATTACTCCC R: GGTGTTGCTAGGTTGGGTTAG	$(AACCT)_4$	2	62		NA
DP4	F: GTGATACCAGGGTGCCGTC R: CACAGGACCCCAATGGAAATAAG	$(AAGT)_4$	2	60	127-292	Poly, LA

Locus	Primer Sequence (5'-3')	Repeat Motif	Tail (5')	T_A (^oC)	Size Range (bp)	Amplification Success
C2X	F: AGCAAACTAACTTTAAGAACCGTG R: TTTGGTACCTACCCTGCCC	$(AAT)_{12}$	2	56		NA
CTS	F: GGGCTATTTCTGTATGGAATATAAAGG R: ACCTGGACTCCTTCCTTCTG	$(AAGG)_4$	3	56	155-450	Poly, LA
B46	F: GGTGATACCAGGGTGCCG R: ATCAGCAGCTCCACAGGAC	$(AAGT)_4$	3	56	159	Mono
CJ1	F: CTACCTCCTAGTGGAACGCC R: AGTGGCTTTCTTTTGTGCC	$(AAT)_9$	3	56	224-431	Poly, LA
DM3	F: CGGGCTATTTCTGTATGGAATATAAAGGAG R: AGGGCATAGATAACCAGAGAATAAG	$(AAGG)_4$	4	56	258-330	Poly, LA
CTO	F: GCCTCTGTTTGTTGTTGTGC R: ACACCGTCTCTCCTTTCGG	$(AGT)_8$	4	64	151-174	Poly
B8P	F: CTAGCACTCGCCTACCGAC R: CTGAAGAAGCCTGCTGTGC	$(ACCG)_4$	4	62	162-255	LA

Table 2.5: Microsatellite primers trialled from Xie *et al.* (2010). Abbreviations as per Table 2.4.

Locus	Primer Sequence (5'-3')	Repeat Motif	Tail (5')	T_A (^oC)	Size Range (bp)	Amplification Success
CQ16	F: GATAAATTTTCTTTGCGCGCTG R: GTCTTTCCTGAGGATTTGAT	$(AC)_{35}(CA)_5$	1	56		NA
CQ17	F: TCGCAGGCTGAGTTTCTATC R: TAGATGTAAATTTTGTGCAC	$(AC)_{28}$	1	56		NA
CQ18	F: ATTTCCTCCCTGGATGTTAC R: TATTCCACTTCCTGACGACT	$(AC)_{10}(AC)_{10}(AC)_6(AC)_6(AC)_{22}$	1	56		NA
CQ19	F: GATAAGCAGTGGCTACAAAA R: TAGTAGCGACCAGTGAAGAG	$(TG)_{16}$	1	56		NA
CQ20	F: CATAGCTGAAACTAGGCACG R: CCTAGTAGCAATCAGTGAAGAG	$(GT)_{25}(GT)_5(CT)_6$	2	52		NA
CQ21	F: CATTTGCCATTTTCCATACC R: GTAGCGACCAGTGAAGAGGC	$(TG)_{28}$	2	62		LA
CQ22	F: TAGTAACGACCAGTGAAGAGGC R: AATCAAGAGCCCCTCACCAG	$(CA)_{14}$	2	62		NA
CQ23	F: CGTCTGCGTGACTGACTCGT R: GCAGATAGAGGACCTAGTAGTGA	$(GT)_9(GT)_{10}(TG)_{21}$	3	65		NA
CQ24	F: GACCTCCAGAGTGAAGCGTT R: CAGAATCAACCCAAACCACG	$(TG)_{39}$	3	65		NA
CQ25	F: ATACAGCAGTTTCGGGTCAA R: GGTTTGATAAAGCTCATGGA	$(GT)_{11}(GT)_{12}$	3	60	243-399	Poly, LA
CQ26	F: GACCCCTGCAACCACAAATA R: TCTGCATTCTCTCAGCGAC	$(AC)_9(CA)_{21}(CA)_{12}(CA)_{63}(AC)_{18}$ $(AC)_{19}(AC)_{20}(AC)_{22}(GT)_5$	4	60		LA
CQ27	F: TAGTGGCGACCAGTGAAGAG R: AGGTTACCATTCATTCGTGT	$(AC)_{73}$	4	64		NA
CQ28	F: ATACTGTCGGAAGGAGAGGTGC R: TTCTACGACTACAAGGATGATGG	$(GTA)_{13}$	4	64	165-415	Poly, LA

2.2.5.2 Amplification

Microsatellite amplification costs were reduced by applying a multi-tailed approach to fluorescent labelling of primers as per Real *et al.* (2009). One of four unique 20-mer oligonucleotide tails designed by Real *et al.* (2009) were added to the 5' end of the forward primers to enable incorporation of a corresponding fluorescently labelled tagging primer in the PCR (Schuelke, 2000). The use of these four unique tails during amplification combines the economic benefits of universal M13 tailing while permitting multiplex amplification of up to four different fluorescent labels in a single reaction (Missiaggia & Grattapaglia, 2006; Real *et*

al., 2009). The four tagging primers used in this study were labelled with 6-FAM, VIC, NED and PET from the G5 fluorescent dye set (Applied Biosystems). Although multiplex amplification is the quickest and most economic method for microsatellite amplification, it was used minimally in this study. Multiplex combinations during the PCR process were trialled for a majority of the loci but not pursued due to poor PCR success. Instead each locus was amplified in separate PCR's and later combined into one solution. Also as a majority of the microsatellites used are of similar fragment lengths, post-PCR multiplex combinations were limited to just DCR & C9Y.

The PCR amplifications were performed in 7 µL reaction volumes. Each primer PCR contained 1.5mM $MgCl^2$, 1x reaction buffer (Fisher Bioreagents, Fisher Scientific Inc., VIC, Australia), 0.05 µM tailed forward primer, 0.2 µM reverse primer, 0.2 µM corresponding fluorescent tag, 0.2 µM dNTP's, 0.028 U *Thermus aquaticus* (Taq) (Fisher Bioreagents) and 20-60 ng/µl of template DNA. Each amplification was vortexed and centrifuged for 10 seconds before the addition of template DNA. All PCR reactions were loaded into an Applied Biosystems GeneAmp PCR System 2700 and placed under the same PCR protocol or cycling conditions with differences only in annealing temperature (Table 2.4 & Table 2.5). The PCR protocol used included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C denaturing for 30 seconds, the specific annealing temperature for 30 seconds and 72°C extension for 45 seconds. This was followed with 15 minutes at 72°C and a hold for an indefinite period at 4°C. Combined PCR products were then analysed on an ABI 3130 (Applied Biosystems) and scored on Genemapper v4.0 (Applied Biosystems)

2.3. MORPHOLOGICAL TECHNIQUES

Whole specimens collected during the small scale sampling mentioned above were measured for a number of morphological characteristics. These morphological characteristics included the length and width of a number of general body sections (rostrum, thorax, abdomen and tail), length and width of each of the chela segments, length of each segment of the walking legs and characteristics of a number of key features (spines, ridges etc.). These measurements were taken in millimetres to the nearest hundredth using a digital calliper under a dissection microscope. To remove any measuring bias, all measurements were taken prior to identifying the genetic signature of the individual. Digital photographs were also taken of the dorsal and ventral side of the Chelae and the dorsal side of the Cephalothorax for each individual using a Leica Microscope Camera. These photographs were later used for geometric analysis.

2.4. DATA ANALYSIS

2.4.1 MtDNA & Nuclear DNA

2.4.1.1 Phylogenetics

Within this study all sequences were originally aligned and edited using Sequencher 4.1.1 ("Sequencher® version 4.1.1 sequence analysis software," 2000) using default settings. Sequence gaps were not observed for the mitochondrial gene COI or the two nuclear genes; H3 and GADPH. Small sporadic indels were however observed in the other two mitochondrial genes 12S and 16S, with minimal effect on their sequence alignment. Sequences for these five genes were edited for ambiguities and trimmed to the same length using Sequencher 4.1.1. Sequences for the two nuclear genes ITS2 and 28S were aligned using the Muscle plugin (Edgar, 2004a, 2004b) within Jalview v2.8 (Waterhouse *et al.*, 2009) under default settings.

Phylogenetic analysis was conducted on all seven genes separately as well as the mitochondrial genes, nuclear genes and all genes combined. jModeltest version 2.1.1 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) was used to select the best-fit model of evolution for each of the datasets (Akaike Information Criterion, as recommended by Posada and Buckley (2004)). Three methods of phylogenetic analysis (tree building) were carried out for comparison; Bayesian, parsimony and maximum likelihood. These analyses were performed using Beast (A. J. Drummond & A. Rambaut, 2007; Drummond *et al.*, 2012), PAUP v4.0 (Swofford, 2002), and RAXML (Stamatakis *et al.*, 2008; Stamatakis *et al.*, 2005), the latter two utilised the online gateway CIPRES (Miller *et al.*, 2010).

A Bayesian molecular clock method was also used to estimate the time to most recent common ancestor for each of the species and any monophyletic lineages within. This was done by applying an estimated divergence rate during phylogenetic analysis in BEAST (A. J. Drummond & A. Rambaut, 2007; Drummond *et al.*, 2012). Due to the high variability and lack of research on freshwater crayfish nuclear genes, only mtDNA divergence rates were used. These estimates were compared between each gene separately as well as together.

2.4.1.2 Population Structure

A number of statistical methods were used to understand the sequence and population dynamics across all gene regions for all eastern Australian *Cherax* species. The level of sequence polymorphism for each species was calculated using the software package DNASP v5.10 (Librado & Rozas, 2009; Rozas, 2009). This calculation estimates the number of unique haplotypes (*Hn*), haplotype diversity (*Hd*), and current (θ_π) and historical (θ_w) genetic diversity for each species. A number of statistical methods were also used to understand the population structure of the COI mitochondrial gene for the two species of focus; *C. dispar* and *C. depressus* A series of Analyses of Molecular Variance (AMOVA's; Excoffier *et al.*, 1992) were also computed in Arlequin v3.5 (L. Excoffier & H. E. Lischer, 2010) to investigate the spatial distribution of genetic variation. The pairwise F_{ST} and Φ_{ST} estimates from the AMOVA calculations were also compared to geographic distances to estimate the effect of isolation by distance (Bohonak, 2002; Wright, 1943). NETWORK v4.6 (fluxus-engineering, 2013) was also used to visualise the relationships among haplotypes and their geographic location by constructing median joining parsimony networks (Bandelt *et al.*, 1999; Polzin & Daneshmand, 2003). Lastly, an Isolation By Distance (IBD) test was conducted using the Mantel test (Mantel, 1967) in Arlequin v3.5 (L. Excoffier & H. E. Lischer, 2010). A Mantel test computes a Pearson product-moment correlation coefficient between the genetic and geographic distance to estimate the effect distance has on the genetic diversity between populations (Legendre & Fortin, 2010), with significant correlations identified using a permutation procedure.

2.4.2 Morphometrics

2.4.3.1 Traditional Analysis

Due to the large number of traditional morphometric measurements that were recorded, both univariate and multivariate statistical analyses were conducted. Firstly a number of univariate Analyses of Variance (ANOVA's) were calculated on a number of basic body shape measurements of each crayfish, including the rostrum size, propodus length and width, orbit-carapace length (OCL) and areola length and width. To limit the effect of size on these tests, each measurement was standardised using both ratios and regression residuals, as recommended by Albrecht *et al.* (1993) and Corruccini (1987). All ANOVA's were calculated in R 3.0.2 (R Development Core Team, 2013), with pairwise t-tests estimated between lineages/species following significant *F* values. As an ANOVA is restricted to single variable at a time, they are extremely limited in their identification of the overall morphological differences between lineages/species. For a more comprehensive comparison between species/lineages, two multivariate approaches were applied; Principal Component Analysis (PCA) and

Discriminant Function Analysis (DFA). As the simpler of the multivariate analyses, the PCA uses orthogonal transformation to convert linearly uncorrelated variables into principal components. As this analysis method is highly sensitive to the relative scaling of the variables and the normalisation of the data, all measurements were first normally transformed within R 3.0.2. (R Development Core Team, 2013). Unlike PCA, DFA calculates the best discriminating components (discriminants) that explain the maximum level of variance between pre-defined groups. This difference allows the identification of specific continuous variables that distinguish between pre-defined groups (species/lineages) the most (Jolliffe, 2005). Both multivariate analyses were calculated in the software package R 3.0.2. (R Development Core Team, 2013).

2.4.3.2 Geometric Analysis

A major limitation of the traditional morphometric analysis methods is its reliance on comprehensive measurements to completely capture the overall size and shape of the organisms. To bypass this issue, a geometric analysis was also implemented, specifically on the chela and rostrum of each individual. These two morphological characteristics were chosen as they are often the characteristics of choice for identifying between species in freshwater crayfish (Allegrucci *et al.*, 1992; Riek, 1969). High definition photographs were taken with a Leica dissection microscope camera with the zoom adjusted depending on the size of the individual. Landmarks and semi-landmarks were defined using Fred L Bookstein (1996a)'s sliding semi-landmark point algorithm implemented for curved edges. To reduce computational demand, landmark definition and analysis was restricted to one side when possible, assuming bilateral duplication. The resulting configuration of landmarks was then superimposed by generalised Procrustes analysis (Marcus *et al.*, 1996; Rohlf & Slice, 1990). This procedure translates and rotates the landmark configurations to a common origin and scales them to unit centroid size. This centroid size was used as a proxy for rostrum/chela size and calculated for each individual as the square root of the sum of the squared deviations of landmarks from the centroid (Fred L. Bookstein, 1996). To evaluate the independence between this centroid size and the shape variables, a multivariate regression was computed in MorphoJ (Klingenberg, 2011). Once identified as independent, PCA and Canonical Variate Analyses (CVA) were implemented in MorphoJ (Klingenberg, 2011). These two methods differ as the CVA method identifies the shape features that best distinguish among known groups while a PCA requires no prior identification.

2.4.3 Environmental Characteristics

Similar to the traditional morphometric statistical analyses, the environmental characteristics were investigated using ANOVA, PCA and DFA methods. Combined these three methods provide a comprehensive analysis of any environmental factors that may separate sites/populations. To reduce bias towards certain duplicated variables, only one variable of each of conductivity (conductivity/specific conductivity), DO (DO/% DO) and turbidity (Secchi/NTU) were used. As freshwater crayfish are highly specialised in terms of habitat requirements, analysis was conducted on all three site sections (pool, riffle & run) separately as well as a combination of all three. By keeping the analyses separate, we can identify whether genetic or morphological variation within each of the *Cherax* species coincides with the overall environmental characteristics of each site or to the specific microhabitat conditions of a section.

CHAPTER 3: MOLECULAR PHYLOGENETICS AND TAXONOMIC CLASSIFICATION OF EASTERN AUSTRALIAN *CHERAX*

3.1 INTRODUCTION

Freshwater crayfish are a highly diverse group of decapod crustaceans that are distributed across all but the Indian and Antarctic continents (Crandall & Buhay, 2008). Five years ago there were 640 species worldwide with an average of 5-10 new species described every year (Crandall & Buhay, 2008). These species can be taxonomically separated into two superfamilies, the Northern Hemisphere Astacoidea and the Southern Hemisphere Parastacoidea. The southern Parastacoidea is composed of a single family, Parastacidae, consisting of 15 genera and over 170 species (Crandall & Buhay, 2008). Divergences within Parastacidae were estimated to pre-date the separation of the Gondwanan landmasses (Sanmartin & Ronquist, 2004) with poor terrestrial and oceanic dispersal and obligate freshwater requirements thought to be the primary cause (Toon *et al.*, 2010). Of the fifteen genera within Parastacidae, *Cherax* and *Euastacus* are the most diverse and widely distributed (Crandall & Buhay, 2008; McCormack, 2013). The two genera were estimated to have diverged from other Parastacids in the Early Cretaceous, with divergences within their groups not estimated to have occurred until much later in the Late Cretaceous to Paleogene periods (Toon *et al.*, 2010). The late divergence and broad distribution of the two genera suggests diversification may have initially been into new niches or climatically induced habitat changes (Toon *et al.*, 2010). Although a vicariant origin due to climatic changes can explain the distribution of *Euastacus* species across the mountaintops of Australia (Ponniah & Hughes, 2004), the evolutionary history of *Cherax* species is not so clear. Unlike *Euastacus*, *Cherax* are distributed throughout Australia (and some neighbouring islands) and are often sympatric with other *Cherax* species and/or genera (Munasinghe *et al.*, 2004a). This more complex distribution and evolutionary history of *Cherax* species has led to a number of taxonomic and evolutionary reviews of *Cherax* (Austin, 1996; Austin & Knott, 1996; Crandall *et al.*, 1999; Munasinghe *et al.*, 2004a, 2004b; Riek, 1969).

Within Australia, *Cherax* species can generally be grouped into three areas of high diversity; western, eastern and northern Australia (Munasinghe *et al.*, 2004a). The identification and suggested phylogenetic relationships between these areas of high biodiversity was first proposed by Riek (1969). Riek (1969) separated the genus *Cherax* into five groups based on morphology; destructor, punctatus, dispar, quinquecarinatus and a new genus Astaconephrops. These five groups did not correspond with the three

areas of high biodiversity with the punctatus and Astaconephrops groups encompassing more than one area. This implied that long-distance dispersal had played a major role in the speciation of *Cherax*, specifically for the punctatus group which encompassed species from both eastern and western Australia. Riek (1969) also suggested that the dispar group from eastern Australia is more closely related to species from Western Australia than other species from eastern Australia.

Riek (1969)'s separation of *Cherax* into five groups was later revised using molecular and morphological approaches into three monophyletic groups corresponding with the three high diversity areas (Austin, 1996; Austin & Knott, 1996; Munasinghe *et al.*, 2004a; Toon *et al.*, 2010). The western species was identified to have diverged first during the Miocene with a sister group relationship between the east and north species (Munasinghe *et al.*, 2004a). This Miocene divergence was also identified for freshwater fish (Unmack, 2001) and amphibians (Roberts & Maxson, 1985) and corresponds with the formation of the Nullarbor Plain in central Australia. The limited dispersal across Australia also suggests endemic speciation rather than long-distance dispersal as an explanation for the evolutionary history of *Cherax* (Munasinghe *et al.*, 2004a). These discrepancies between the morphology and genetics of *Cherax* also suggest that a number of the morphological characters may have evolved through parallel or convergent evolution and as such are poor tools for understanding the evolutionary history of *Cherax* (Austin & Knott, 1996; Munasinghe *et al.*, 2004a).

While this high variation and convergent evolution of morphological characters has placed scrutiny on the original taxonomic identifications of *Cherax* species (Munasinghe *et al.*, 2004a, 2004b), the molecular approaches used to review the taxonomy also contain their own inconsistencies and/or limitations. Most phylogenetic studies on *Cherax* either use only mitochondrial DNA (Crandall *et al.*, 1999; Munasinghe *et al.*, 2004a, 2004b), a limited number of individuals for each species (Munasinghe *et al.*, 2004a, 2004b) or a limited number of species (Bentley *et al.*, 2010; Crandall *et al.*, 1999; Toon *et al.*, 2010). Although mitochondrial DNA has been identified as a useful tool for phylogenetic comparisons, its sole use has been criticised, with conclusions to be made with caution (Grechko, 2013). This is particularly the case for phylogenetic studies on *Cherax*, with Nguyen, Murphy*, et al.* (2002) sequencing multiple copies of the same mitochondrial gene region and Bentley *et al.* (2010) identifying incongruence between mitochondrial and nuclear gene regions. Phylogenetic studies with small sample sizes also intrinsically have their own limitations, with genetic diversity both within and between species often missed. This was apparent for *Cherax dispar*, with evidence for and against Riek (1951)'s additional subspecies observed by Bentley *et al.* (2010) and Munasinghe *et al.* (2004b) respectively.

This chapter will examine the phylogenetic relationships of all eastern *Cherax* species to estimate the taxonomic identity and evolutionary relationships of freshwater crayfish in the region. As previous phylogenetic research on Australian *Cherax* has predominantly been focused only on mtDNA, their ability to confidently identify the evolutionary history and current taxonomic classification of *Cherax* has been limited. By identifying congruent evolutionary relationships between the phylogenies of seven gene regions (including four nuclear genes), this chapter aims to more comprehensively evaluate the current taxonomy of eastern *Cherax* and possibly identify new species within the region. As the species' sample sizes used in this chapter are also considerably larger than previous research, higher levels of intraspecific and interspecific genetic variation is also expected to be observed.

3.2 METHODS

3.2.1 Sample Area

Cherax specimens were collected as per the large scale sampling method mentioned in the previous chapter. This included specimens from as far north as Shoalwater Bay to as far south as the Gold Coast. As well as including the entire range of most SEQ *Cherax* species, this large sampling range also comprises a majority of the lineage breaks previously observed in other freshwater organisms (Hughes et al., 1999; Page & Hughes, 2007a; Page et al., 2004; Sharma & Hughes, 2009). A majority of these studies separate the sampling area into six geographic regions; Shoalwater Bay, Mary River & Fraser Island, Sunshine Coast, Brisbane & Gold Coast, Moreton Bay and the Murray-Darling Basin (Figure 3.1). Sequences of *Cherax* species located outside of SEQ were obtained for each gene from Genbank when available (Table 3.1). Due to the insufficient availability of comparable Genbank sequences for *C. austini*, *C. cartalacoolah*, *C. cid*, *C. leckii* and *C. waselli*, the species were omitted from this study. Similarly, *C. urospinosus* was also not included, with the species only known and now extinct from its type locality (Riek, 1969).

3.2.2 Gene Selection

3.2.2.1. Mitochondrial DNA

In this study, three areas of the mitochondrial DNA (mtDNA) were targeted for phylogenetic analysis; the 16S and 12S ribosomal DNA (16S rDNA, 12S rDNA) and cytochrome oxidase subunit I (COI). Although all three genes are in reality from a similarly inherited gene region, they each can be highly informative. The conserved nature of the 16S and 12S rDNA genes compared to that of the COI portion, make them ideal for phylogenetic and taxonomic studies. As the rDNA genes have a lower likelihood of saturation, they are more likely to establish relationships between distant taxa than the COI portion (Xia *et al.*, 2003). The combination of the two rDNA genes has commonly been used to identify phylogenetic relationships and

evolutionary history of freshwater crayfish in Australia (Munasinghe *et al.*, 2004a, 2004b; Shull *et al.*, 2005), with the 16S gene in particular popular for molecular clock approaches (Bentley *et al.*, 2010; Schultz *et al.*, 2007). The COI gene has also commonly been utilised in phylogenetic studies of *Cherax* (Bentley *et al.*, 2010), but its higher rate of divergence compared to the two rDNA genes make it the preferred gene for phylogeographic studies (Bentley *et al.*, 2010; Gouws *et al.*, 2006; Hughes & Hillyer, 2003). The popularity of the three genes in studies of *Cherax* phylogenetics provides easy comparisons with earlier studies by adding the available sequences from Genbank.

One of the potential problems that can arise from the direct sequencing of mitochondrial genes is the accidental sequencing of a nuclear homologue of the target fragment (Numts), a situation previously reported in other crustaceans (Williams & Knowlton, 2001) and *Cherax* (Nguyen, Murphy*, et al.*, 2002). Numts are the result of a gene transfer of a fragment of mtDNA to the nuclear genome (Bensasson *et al.*, 2001). Due to the loss of function from the transfer, and subsequent independent evolution, Numts are likely to display considerable divergence compared to the original protein coding mitochondrial gene. Therefore if not detected, interpretations from divergences between individuals sequenced for the Numts and the mitochondrial gene will be erroneous and misleading. The presence of Numts can typically be detected in sequences via a visual inspection of the sequence data for uncharacteristic amino acid changes, such as the presence of stop codons or an inappropriate sequence base frameshift change (Bensasson *et al.*, 2001). Numts can also commonly be identified by strongly contrasting relationships between individuals across multiple gene regions. To ensure the presence of Numts did not influence results in this study, both mtDNA and nuclear gene regions were sequenced and investigated for uncharacteristic amino acid changes.

Table 3.1: Sample size of current taxonomic eastern Australian *Cherax*. Genbank individuals included are indicated within parentheses with all references ($^{A-G}$ and $^{1-8}$) identified in Appendix 8.1.

Species	COI	16S	12S	28S	ITS2	GAPDH	H3
C. austini[1]	-	-	-	-	-	-	-
C. cairnsensis[2]	3	5 (3^{AB})	5 (2^{A})	4 (1^{B})	2	6	3
C. cartalacoolah[3]	-	-	-	-	-	-	-
C. cid[1]	-	-	-	-	-	-	-
C. cuspidatus[2]	43 (1^{E})	21 (3^{A})	8 (3^{A})	3 (1^{B})	10	8 (1^{B})	6
C. depressus[4]	163	12	10 (1^{A})	12	8	4	11
C. destructor albidus[5]	2 (2^{BF})	2 (2^{AC})	1 (1^{A})	1 (1^{B})	-	1 (1^{B})	-
C. destructor destructor[5]	9	13 (1^{A})	5 (3^{A})	2	3	1 (1^{B})	2
C. dispar[4]	467 (1^{B})	82 (1^{A})	34 (1^{A})	26 (1^{B})	45	19 (1^{B})	36
C. leckii[6]	-	-	-	-	-	-	-
C. parvus[3]	1 (1^{E})	1 (1^{A})	1 (1^{A})	1 (1^{B})	-	-	-
C. punctatus[5]	3	2 (1^{A})	4 (1^{A})	2	-	-	3
C. quadricarinatus[7]	-	1 (1^{D})	1 (1^{E})	3 (1^{E})	-	3 (2^{BG})	-
C. rhynchotus[4]	-	1 (1^{A})	1 (1^{A})	-	-	-	-
C. robustus[4]	25	7 (1^{A})	3	3	8	3 (1^{B})	3
C. rotundus[8]	-	1 (1^{A})	1 (1^{A})	-	-	1 (1^{B})	-
C. setosus[4]	-	1 (1^{A})	1 (1^{A})	-	-	1 (1^{B})	-
C. urospinosus[2]	-	-	-	-	-	-	-
C. wasselli[2]	-	-	-	-	-	-	-

3.2.2.2. Nuclear

Although a number of phylogenetic studies have been implemented on freshwater crayfish in Australia, very few have included both nuclear and mtDNA genes. Instead a majority have focused on phylogenetic interpretations from only mtDNA, assuming that the gene/s represent the evolutionary history of the entire genome for both sexes (Grechko, 2013). In *Cherax*, this has been shown to be misleading with multiple copies of the same mtDNA gene region sequenced (Numts) (Nguyen, Murphy*, et al.*, 2002). To limit the inherent issues of mtDNA only interpretations, four nuclear genes that have previously been used on freshwater crayfish in Australia will be also be used; ITS2, H3, 28S and GAPDH. These four genes will be analysed separately and combined with mtDNA to fully understand the evolutionary history of *Cherax* in South East Queensland.

The ribosomal nuclear gene 28S rDNA has been extensively used in phylogenetic studies of decapods around the world (Ahyong *et al.*, 2007; Ahyong & O'Meally, 2004; Mitsuhashi *et al.*, 2007; Porter *et al.*, 2005; Toon *et al.*, 2009). Its variable evolutionary rate makes it ideal for phylogenetic analyses at different taxonomic levels (Hillis & Dixon, 1991). This has been shown in practice for freshwater crayfish with the 28S gene region used at both the family (Toon *et al.*, 2010) and genus level (Shull *et al.*, 2005). In contrast, the ribosomal nuclear DNA in the internal transcribed spacer region (ITS2) between the 5.8S and 28S gene region has had limited use in freshwater crayfish studies. One reason for this may be the high amounts of intragenomic variation identified in the gene region for freshwater crayfish (Harris & Crandall, 2000). The practicalities for using ITS2 for interspecies phylogenetics however is still valid (Bentley *et al.*, 2010; Harris & Crandall, 2000), with Bentley *et al.* (2010) observing congruence between mtDNA and ITS2 at the species level. The two protein coding genes used in this study, Histone 3 (H3) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are commonly used in the systematics of arthropods (Porter *et al.*, 2005) and Decapods (Toon *et al.*, 2009). Their value however goes beyond the order level, with studies utilising the genes at the genus (Schultz *et al.*, 2009) and sub-genus levels (Buhay *et al.*, 2007).

3.2.3 Sequence alignment

Large subunit rDNA (LSU RNA) and Internal Transcribed Spacer (ITS) sequences are well known to vary considerably in length and house both highly conserved and very variable portions (including gaps) (Buckley *et al.*, 2000; Grajales *et al.*, 2007). This is particularly a problem during alignment, as there is no universally accepted method for LSU RNA sequence alignment (Chu *et al.*, 2006). Although it is the secondary structure of LSU RNA that makes alignment difficult, it may also help to explain the evolutionary history of the conserved and variable regions (Chen *et al.*, 2004; Grajales *et al.*, 2007). This is due to the differing functional roles and selective pressures placed on the various helix structures (Grajales *et al.*, 2007). These highly variable portions of LSU RNA have been identified as a particular problem in freshwater crayfish (Bentley *et al.*, 2010; Harris & Crandall, 2000; Toon *et al.*, 2009), specifically for the ITS (Bentley *et al.*, 2010; Harris & Crandall, 2000) and 28S (Toon *et al.*, 2009) genes as microsatellite repeat motifs are commonly found within them. To determine the effect these highly variable portions and gaps had on phylogenetic analyses, a combination of approaches was utilised. For the assessment of the effect of the highly variable microsatellite regions in the ITS2 and 28S gene regions, phylogenetic analyses were performed with and without their corresponding microsatellite regions. As these highly variable microsatellite regions may have a faster evolutionary rate and different model of evolution, their inclusion in phylogenetic analyses can skew or oversaturate the true phylogenetic relationships (Harris & Crandall, 2000).

Indels however, are widely recognised as valuable sources of information for phylogenetic inference (Simmons *et al.*, 2007; Simmons & Ochoterena, 2000; Wiens, 1998, 2003), with phylogenetic trees able to be inferred solely from them (Lloyd & Calder, 1991). Although indels can provide valuable information for phylogenetic analyses, there is still some debate on the most effective way to include them (Simmons *et al.*, 2007), if at all (Raymúndez *et al.*, 2002). The most accepted approaches implement multi-position gap characters, with variations predominantly between the inclusion of gap characters as either a separate alignment (Barriel, 1994; Baum *et al.*, 1994; Müller & Reisz, 2006; Simmons & Ochoterena, 2000) or as replacement characters (Bena *et al.*, 1998; Giribet & Wheeler, 1999). Although the coding of each nucleotide gap as a 5^{th}-state was identified as an effective method for phylogenetic analysis (Ogden & Rosenberg, 2007; Simmons *et al.*, 2007), its inherent issues of weighting multi-position gaps as a function of their length and treating overlapping non-homologous gaps as homologous can be problematic in highly variable regions (Simmons *et al.*, 2007). Two multi-position gap character approaches that were identified to perform well in highly variable regions are simple indel coding (SIC, Simmons and Ochoterena (2000)) and modified complex indel coding (MCIC, Muller (2006)). SIC is applied by scoring all gaps, regardless of length as separate presence/absence characters, with overlapping gaps scored as missing data (Simmons & Ochoterena, 2000). In addition to SIC, Simmons and Ochoterena (2000) also proposed and recommended complex indel coding (CIC) as a more appropriate approach as SIC does not incorporate all available information and can imply fewer steps than are biologically possible (Simmons & Ochoterena, 2000). CIC applies a separate symmetrical step matrix that allows for two steps between non-overlapping gaps in two sequences that are subsumed within a longer gap in a third (Simmons & Ochoterena, 2000). Due to criticisms from Graham *et al.* (2000), particularly on the asymmetry of the step matrix and its violations of triangle inequality, the CIC approach was later revised into MCIC (Muller, 2006). The MCIC approach integrates aspects of SIC and CIC and allows the violations of triangle inequality to be automatically corrected in PAUP* (Swofford, 2002). In this study, both SIC and MCIC methods were applied to indels within the ribosomal genes 12S, 16S, 28S and ITS2 using the software SeqState (Muller, 2005). MCIC's reliance on PAUP* to correct for triangle inequality restricted its application to parsimony phylogenetic analyses, with the SIC approach applied to both Bayesian and parsimony phylogenetic methods.

3.2.4 Phylogenetic analysis

Three methods of phylogenetic analysis were carried out in this study; maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetics (BP). These methods of phylogenetic inference can generally be separated into either nonparametric (e.g., MP) or parametric (e.g., BP & ML) methods based on their assumptions of the underlying evolutionary processes (Sanderson & Kim, 2000). There is still much debate on which is the most appropriate for reconstructing the 'true' phylogenetic tree (Gaucher & Miyamoto, 2005; Kolaczkowski & Thornton, 2004; Rindal & Brower, 2011). Nonparametric methods evaluate phylogenetic relationships based on a general metric with no assumption of a specific distribution (Sanderson & Kim, 2000). For example, the MP method assumes that changes (mutations) are relatively rare, and so the fewer the changes, the more likely the relationship or scenario. Although this simple approach performs well most of the time (Hillis *et al.*, 1994; Müller & Reisz, 2006), particularly using heterogeneous data (Kolaczkowski & Thornton, 2004), it can be strongly affected by 'long branch attraction' (Felsenstein, 1978; Gaucher & Miyamoto, 2005; Swofford *et al.*, 2001). 'Long branch attraction' is caused by an inherent bias in the estimation procedure where similarities due to convergent or parallel changes along long branches produce an artifactual phylogenetic grouping of taxa (Bergsten, 2005; Sanderson & Kim, 2000). This limitation in MP phylogenetics along with the gradual increases in computing power has shifted phylogenetic analyses towards the use of parametric methods (Felsenstein, 1973; Wiley & Lieberman, 2011). Parametric methods use likelihood based calculations to infer the most probable phylogenetic tree based on a specific chosen model. Although both BA & ML use these likelihood calculations, they are very different in their philosophical approaches to the question (Wiley & Lieberman, 2011). ML analysis uses a criterion-based approach where the preferred tree is the tree that has the highest probability of producing the observed data, given a specific model of evolution, tree topology and branch lengths between nodes (Felsenstein, 1973; Felsenstein, 1985). By using the model, ML calculates the probabilities of observing the data on a specified tree, one transformation series at a time. As the phylogenetic inferences from ML analyses depend on the chosen specific model of evolution, the accuracy of this inference does also (Sullivan & Joyce, 2005). Similar to MP analyses, ML analyses produce a point estimate of the best tree given the criterion provided. BA turns this principle on its head, and instead estimates the probability of the tree topology given the data and model. It does this by maximising the posterior probability (likelihood) of the tree by exploring probability space to find when the probability density is the highest (Wiley & Lieberman, 2011). Unlike MP and ML, BA results in a probability distribution of models that may contain one or more tree topologies (Wiley & Lieberman, 2011). This probability distribution provides the backdrop for further molecular clock approaches with error boundaries (A. J. Drummond & A. Rambaut, 2007). Due to the inherent strengths and weaknesses of each method, all three methods (MP, ML & BA) were applied to all seven genes separately as well as combined datasets of just

mitochondrial genes, nuclear genes and all genes together. The specific methods for each analysis are as follows.

The first phylogenetic method (Parsimony analysis) was conducted using the Parsimony Ratchet method of Nixon (1999) in PAUP* v4.0 (PAUPRat) (Swofford, 2002) on the CIPRES portal gateway (Miller *et al.*, 2010). Datasets with multiple genes were analysed as one concatenated dataset with their corresponding substitution models, calculated in jModeltest (Darriba *et al.*, 2012), included at the bottom of each input nexus file. Each analysis was run for 1000 iterations with 1000 of the best trees saved. This number of best trees saved was also set to automatically increase by 100 when reached. From these best trees, an extended majority rule consensus tree (Margush & McMorris, 1981) was calculated using the software plugin Consense (Felsenstein, 1993a) in Phylip (Felsenstein, 1989, 1993b) on the NCBS web server (Khadar, 2013). Parsimony trees with branch percentages were then created using Figtree (Rambaut, 2012).

The second phylogenetic method (Likelihood analysis) was also conducted on the CIPRES portal gateway (Miller *et al.*, 2010) using the fast bootstrapping method of RAXML (Stamatakis *et al.*, 2008; Stamatakis *et al.*, 2005). Similar to the parsimony method, the multi-gene datasets were concatenated into a single file. The substitution models however were set using the CIPRES portal. The number of bootstrap iterations used was automatically calculated by the RAXML procedure in CIPRES. Maximum Likelihood trees with their corresponding bootstrap values were then created using Figtree (Rambaut, 2012).

The last method (Bayesian analysis) was performed in Beast version 1.7.4 (A. J. Drummond & A. Rambaut, 2007; Drummond *et al.*, 2012). Input files for Beast were created in Beauti v1.7.4 (Drummond *et al.*, 2012) with each gene inserted separately and with site, clock and partition models unlinked. The substitution model and the corresponding priors for each gene were identified using jModeltest (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) and unlinked and implemented within Beauti. For datasets with multiple genes, analysis was performed using *BEAST (Heled & Drummond, 2010) with clock models unlinked and mtDNA trees linked. As all phylogenetic analyses were identifying interspecies relationships rather than intra species, a yule-birth rate tree prior was used as recommended by A. Drummond (pers. Comm.). All analyses were run for 10,000,000 iterations with parameters logged every 10,000 iterations. An appropriate burn-in was identified from Tracer v1.5 (Rambaut & Drummond, 2004) and implemented in TreeAnnotator v.1.7.4 (Drummond *et al.*, 2012) to calculate the Maximum clade credibility tree. Bayesian trees with posterior probabilities were then created in Figtree v1.4.0 (Rambaut, 2012).

3.2.5 Molecular divergence and clock calculations

To provide the basis for comparisons with earlier research on Australian *Cherax*, the molecular divergence and time since divergence for each of the species was estimated using a molecular clock approach. Although a molecular clock approach is a useful tool to estimate the timing of phylogenetic events, there are a number of potentially confounding factors that limit the accuracy of their estimates. These factors include (but are not limited to) variable metabolic rates between taxa, different generation times, DNA replication intervals, DNA repair efficiency, mutation rates between regions of a genome and the reliance of fossil and biogeographic evidence for the separation times of taxa (Graur & Martin, 2004; Ho *et al.*, 2005; Weir & Schluter, 2008). Despite these uncertainties, and with an adequate degree of caution, molecular clock estimates can place divergences in a temporal context and provide the framework to assess the likelihood of competing hypotheses, such as vicariance and dispersal (Avise, 2004; Waters & Craw, 2006).

Molecular clock estimates in this study were calculated by applying an estimated uncorrelated log-normal relaxed clock divergence rate during the Bayesian phylogenetic analysis in BEAST (A. J. Drummond & A. Rambaut, 2007; Drummond *et al.*, 2012). To estimate divergences between species, sequences were grouped into their corresponding species using the 'trait' feature in Beauti (Drummond *et al.*, 2012). Due to the high variability in substitution/divergence rates of nuclear sequences among species groups, divergence rates were only used for the three mitochondrial genes, CO1, 16S and 12S. For the CO1 gene, the closely related Caridean shrimp CO1 divergence rate of 1.4% per million years was used (Knowlton & Weigt, 1998; Morrison *et al.*, 2004; Page & Hughes, 2007a). For the 16S gene, a far slower rate of 0.65% per million years (Schubart *et al.*, 1998) from a Pleocyematan decapod was applied. Due to the low availability of divergence rates for the 12S gene, a more distantly related *Heliconius* Butterfly divergence rate of 2.3% per million years (Brower, 1994) was applied. To estimate the divergence rates of each of the nuclear genes, uniform priors were applied to nuclear mean ucld (mean divergence rate under the uncorrelated log-normal relaxed clock). Initial values for these priors were estimated based on an extrapolation from the exponential relationship between the mitochondrial variation and divergence rates. The nuclear divergence rate priors used were 0.3011, 0.0548, 0.1004, 1.7463 for GAPDH, H3, ITS and 28S respectively.

3.3 RESULTS

3.3.1 Genetic Diversity

3.3.1.1 Mitochondrial genes

A total of 716 *Cherax* individuals from throughout eastern Australia were sequenced for the mtDNA COI gene. These individuals consisted of 189 unique haplotypes from 145 locations, with genetic diversity relatively high (*Hd*: 0.98) but nucleotide diversity low (θ_π: 0.126) (Appendix 8.1). A 51 haplotype subset was used for all COI phylogenetic analyses which encompassed representative individuals for each major phylogenetic and geographic group in the dataset. These 51 representatives include five Genbank sequences (two from Queensland) and one *Euastacus* sequence. To limit the amount of missing information, all sequences used were cut to a 578bp fragment. Within these 578bp, a total of 358 mutations were identified across 221 sites (38%). Of these 358 mutations, only four were non-synonymous (1%). Three gaps were also identified within the dataset, all within individuals retrieved from Genbank. jModeltest selected the Three-Parameter Model v3 of substitution with unequal frequencies (TPM3uf) (Kimura, 1981), a proportion of invariable sites (0.586) and a Γ distribution of site-to-site variation (1.344).

Of the 716 *Cherax* individuals sequenced in the COI dataset, a subset of 138 was also sequenced for a 446bp fragment of the 16S gene. These 138 sequences include seventeen sequences from Genbank (11 from Qld) and a *Euastacus* sequence as an outgroup. The dataset showed a high level of genetic diversity (*Hd*: 0.97) consisting of 47 haplotypes which were all used for phylogenetic analyses (Appendix 8.1). Although the dataset showed a relatively low level of nucleotide diversity (θ_π: 0.078), 181 variable sites (40%) were observed within the sequence alignment, with a total of 237 mutations identified throughout. Unsurprisingly, due to the ribosomal nature of the 16S gene, 26 gap sites were also identified sporadically across the alignment. For both analyses when gap sites were included and omitted, jModeltest selected the more simple Hasegawa, Kishino and Yano Model (HKY) (Hasegawa *et al.*, 1985) of substitution for the 16S gene with a 0.306 and 0.303 Γ distribution of site-to-site variation and transition-transversion ratio of 4.3573 and 4.2664 when gaps were included and excluded respectively.

For the 12S gene, a subset of 60 individuals was sequenced for a 307bp fragment. From this relatively small gene fragment, a high level of genetic variation was observed (*Hd*: 0.99), with 41 unique haplotypes identified from the 52 individuals (Appendix 8.1). These 41 haplotypes included 17 from Genbank (10 from QLD) and a *Euastacus* individual. The low level of nucleotide diversity (θ_π: 0.082) was similar to the 12S gene region, with a total of 149 mutations observed across 105 variable sites (34%). 19 gap sites were also observed sporadically across the alignment. When the gap sites were included, the Tamura and Nei model

(TrN) (Tamura & Nei, 1993) of substitution was selected by jModeltest for the 12S gene region with a proportion of invariable sites (0.516) and a Γ distribution of site-to-site variation (0.737). This differed to analyses when the gap sites were omitted, with jModeltest selecting the Transitional Model (TIM1) (Posada, 2003) of substitution with a proportion of invariable sites (0.52) and a Γ distribution of site-to-site variation (0.737)

3.3.1.2 Nuclear genes

A total of 60 individuals were sequenced for an 841bp fragment of the 28S ribosomal nuclear gene, including six Genbank sequences and a *Euastacus* individual as an outgroup. From this alignment, only 19 unique haplotypes were identified (*Hd*: 0.67) and used for all phylogenetic analyses. Unlike the mtDNA genes, the nucleotide diversity was relatively low with 125 sites variable (15%) encompassing 133 mutations in total (θ_π: 0.005) (Appendix 8.1). Not included in these calculations was one ambiguous or heterozygous base in a single individual. A total of 271 gap sites were observed throughout the 28S alignment. Similar to Toon *et al.* (2009), a majority of the mutations and gaps occurred in a highly variable region between the 363 and 572bp's. When this highly variable region was included jModeltest selected the Three-Parameter Model v2 of substitution with unequal frequencies (TPM2uf) (Kimura, 1981), a proportion of invariable sites (0.553) and a Γ distribution of site-to-site variation (0.27). When the region was omitted, the Tamura & Nei (TrN) (Tamura & Nei, 1993) substitution model was selected with a Γ distribution of site-to-site variation (0.374)

Seventy-nine individuals were also sequenced for a 605bp fragment of the ITS2 gene region. From these individuals, a total of 38 haplotypes (*Hd*: 0.91) were identified and used in phylogenetic analyses. As the ITS2 gene region is both highly variable and rarely used in studies of Australian freshwater crayfish, there were no available Genbank sequences that could be included. Of the 605bp fragment used, 60 were variable (10%), incorporating a total of 71 mutations (θ_π: 0.027) (Appendix 8.1). On top of this variation, an extra 19 sites also contained ambiguous or heterozygous bases. A total of 128 gap sites were observed within the sequenced ITS2 gene region. Similar to the 28S gene, these mutations and gap sites were condensed to two highly variable regions between the 101 and 162bp's and the 415 and 605 bp's. When this highly variable region is included jModeltest selected the Three-Parameter Model v2 of substitution with unequal frequencies (TPM2uf) (Kimura, 1981) with a Γ distribution of site-to-site variation (0.213). When the region was omitted, jModeltest selected the simpler substitution model of Hasegawa, Kishino and Yano Model (HKY) (Hasegawa *et al.*, 1985) with a Γ distribution of site-to-site variation (0.163) and transition-transversion ratio of 2.0659.

Due to the poor PCR success of sequencing the GAPDH gene region, only 50 individuals were sequenced for a 648bp fragment. An extra nine sequences were obtained from Genbank to make a total of 24 haplotypes (*Hd*: 0.81). As no *Euastacus* individuals could be sequenced, two Genbank sequences of *Cherax quadricarinatus* were used as an outgroup. A total of 82 mutations were identified across 79 variable sites (12%) within the GAPDH gene region (θ_π: 0.011) (Appendix 8.1). An extra 10 sites within the alignment also included ambiguous or heterozygous bases. Unlike the two nuclear ribosomal gene regions, the protein coding GAPDH region did not contain any gaps. jModeltest selected the Transitional Model v2 of substitution with equal frequencies (TIM2ef) (Posada, 2003) and a proportion of invariable sites (0.623)

For the last and shortest gene fragment in this study, Histone 3 (H3), a total of 72 individuals were sequenced. As very few studies on *Cherax* in Queensland have utilised the H3 gene region, only one Genbank sequence could be included in this study. From the alignment of these individuals, 32 haplotypes (*Hd*: 0.37) were identified from 15 mutations across 14 variable sites (5%) (θ_π: 0.004) (Appendix 8.1). A majority of the phylogenetic information observed in the H3 gene alignment was from 27 sites containing ambiguous or heterozygous bases. Only two gap sites in close proximity were observed within the H3 gene region. jModeltest selected the Three-Parameter Model v3 of substitution (TPM3) (Kimura, 1981) with a proportion of invariable sites (0.807)

3.3.2 Tree Topologies

3.3.2.1 MtDNA

The thirteen species sequenced for mtDNA genes were all largely supported as separate taxonomic species within the *Cherax* genus. Along with these thirteen species, an additional five clades were also identified for both *C. dispar* and *C. cuspidatus* (Figure 3.1). The phylogenetic relationships between each of the species and clades were generally consistent across all three mtDNA genes with inconsistencies mainly limited to species with only one individual sourced from Genbank (*C. parvus*, *C. setosus*, *C. rotundus*, *C. rhynchotus* and *C. quadricarinatus*). Node support was highest delineating each species individually but generally dropped for intraspecific and deep nodes. This was especially the case in likelihood analyses with bootstrap values consistently lower than their parsimony and Bayesian posterior probability counterparts. For each of the mtDNA genes, species were categorised into one of six groups (if applicable); *C. dispar*, *C. destructor*, *C. depressus*, *C. robustus*, *C. cuspidatus* or *C. quadricarinatus* (Figure 3.1). Of these six groups, five were represented in the combined mtDNA phylogeny (Figure 3.3). Estimates of divergence dates between these groups varied dramatically among each of the mtDNA genes with the 16S and combined gene phylogenies representing a 'middle ground' between the upper and lower estimates of the COI and 12S genes respectively.

The identification of *C. dispar* as a separate taxonomic species was strongly supported, with monophyly observed for all mtDNA genes and all but one dataset within. *C. dispar* was only not monophyletic in the likelihood analysis of the 16S gene, where due to long-branch attraction or poor model selection the *C. rhynchotus* and *C. quadricarinatus* individuals were aligned within the *C. dispar* group. The divergence of *C. dispar* from other *Cherax* is estimated to have occurred during the Miocene (7.78mya) (Figure 3.3) with further divergences into five highly supported monophyletic clades (A-E). Although the evolutionary relationships between these clades vary across genes and analyses, a divergence into two group's; clades A-C and D-E, was consistently observed for all but one analysis. This split was highly supported by all analyses and genes (average nodal support of 86) except for the same 16S likelihood analysis mentioned previously. For both the COI and 16S mtDNA genes, clades B and C were more closely related to each other than to clade A. This is contrary to the 12S mtDNA gene, where clades A and B are more closely related (Figure 3.2). Divergence estimates between each of the clades were estimated to have occurred during the Pliocene (B-C; 2.58mya & A-BC; 3.47mya) (Figure 3.3). Similarly, divergence between the *C. dispar* clades D & E was also estimated to have occurred during the same period (3.27mya) (Figure 3.3). Unlike clades A, B & C, *C. dispar* D & E are distributed allopatrically, with one found only on coastal islands (D) and the other restricted to a small pocket of the mainland (E) (Figure 3.1).

Although *Cherax destructor* has the largest distribution of all *Cherax*, its genetic diversity is comparatively limited. Within the *C. destructor* group, three taxonomic species (*C. destructor* (*C. d. destructor* and *C. d. albidus*), *C. setosus* and *C. rotundus*) were strongly supported with monophyly observed for the 16S gene. A monophyletic relationship was not observed for the 12S gene however, with *C. destructor* and *C. setosus* paraphyletic (Figure 3.2). Similarly the separation of the *C. destructor* sub-species (*C. d. destructor* and *C. d. albidus*) was also supported for a majority of the 16S gene analyses (Figure 3.2) but not 12S gene analyses (Figure 3.2). Phylogenetic inconsistencies among the mtDNA genes were also observed between *C. destructor* and other *Cherax* species. For a majority of the analyses on the 16S gene, the *C. destructor* group was identified as a sister group to *C. dispar*, specifically for Bayesian methods and analyses with gaps coded. This relationship was however not commonly observed for the COI and 12S genes, with the *C. destructor* group predominantly identified as the most divergent SEQ *Cherax* species for the 12S (Figure 3.2) and COI gene (Figure 3.1). This early divergence from other SEQ *Cherax* was also observed for the combined mtDNA phylogeny (Figure 3.3). For the COI and 16S genes, the *C. destructor* group was estimated to have diverged from *C. dispar* during the Miocene (10-12mya). In contrast, phylogenies that show an early divergence from all other *Cherax* vary between 4.7mya for the 12S gene (Figure 3.2) to 18.23mya for the combined mtDNA analysis (Figure 3.3). Within the *C. destructor* group, *C. setosus* and *C. rotundus* were estimated to have diverged from *C. destructor* during the Miocene 6.7mya (Figure 3.2). Unsurprisingly,

divergence between the two sub-species (*C. d. destructor* and *C. d. albidus*) was estimated to have occurred far more recently (2.17-3.37mya (Figure 3.2)).

The *C. depressus* phylogenetic group identified in this study encompasses four burrowing species with wide chelae; *C. depressus*, *C. cairnsensis, C. punctatus* and *C. parvus*. The inclusion of *C. depressus* and *C. cairnsensis* together is not surprising with the species' originally identified as one when first taxonomically described (Riek, 1951). The monophyletic relationship of the two species was supported by strong nodal support (average 89) for all three mtDNA genes with only the likelihood analysis of the 16S gene not monophyletic. The separation of the two species is also highly supported by all three mtDNA genes for individuals sampled within this study (average nodal support 89). The 16S and 12S sequences from Genbank do not however support this separation (Figure 3.2). For the 16S gene, all analyses grouped the Genbank *C. depressus* sequence from the Brisbane River (AY191760) with the *C. cairnsensis* sequence from North Queensland (AY191761) (Figure 3.2). This incongruence was also observed for the 12S gene with the *C. depressus* individual from the Brisbane River (AY191731), identified as more closely related to *C. cairnsensis* than *C. depressus* (Figure 3.2). Even when these individuals are omitted, mtDNA molecular divergence estimates between the two species was low (2-8% (Table 3.3) with a Pliocene divergence 2.51mya estimated (Figure 3.3)). For the COI gene a north-south split was also identified within *C. depressus*, with sequences from the Brisbane River different to those from the Mary/Burrum Rivers and Tin Can Bay (Figure 3.1). The monophyletic grouping of *C. depressus* and *C. cairnsensis* with *C. punctatus* and *C. parvus* was also highly supported (average nodal support 70) for a majority of analyses. These species were only not monophyletic for parsimony analyses of the 12S gene. Within the group, a majority of analyses identified *C. parvus* as the most divergent species of the group with *C. punctatus* closely related to *C. depressus* and *C. cairnsensis*. Bayesian analysis of the COI gene differed from this placing *C. punctatus* and *C. parvus* as a separate monophyletic clade (Figure 3.1). The *C. depressus* group as a whole was estimated to have diverged from other eastern *Cherax* during the Miocene approximately 8.75mya (Figure 3.3).

In this study, *C. robustus* was highly supported as a highly diverged species with strong nodal support (average 100) for monophyly for all analyses of all genes. There was however some confusion as to what species was the closest relative, with each gene predominantly indicating different relationships. For all COI analyses and 16S analyses where gaps were coded, *C. robustus* was identified as monophyletic with the *C. cuspidatus* group. This relationship was also observed for both parametric based analyses of the three mtDNA genes combined (Figure 3.3). Parametric analyses of the 12S gene however, predominantly identified *C. robustus* as monophyletic with the *C. depressus* group. This also differed from a majority of the parsimony analyses of the 16S and 12S genes where *C. robustus* showed an early divergence from other *Cherax* species. Irrespective of these phylogenetic inconsistencies, divergence estimates for *C. robustus*

were relatively consistent with all but the 12S gene estimating that *C. robustus* diverged from other *Cherax* during the Miocene approximately 11-14mya (Figure 3.3). All three mtDNA genes also estimated that variation within the *C. robustus* clade diverged during the Pleistocene (0.68-1.38mya) (Figure 3.3).

Similar to *C. dispar*, five highly supported monophyletic clades (A-E) were identified within the *C. cuspidatus* group (Figure 3.1). These five clades formed a monophyletic *C. cuspidatus* group for all analyses of the mtDNA genes except for parsimony analysis of the 12S gene. This incongruence was evident, with nodal support values (average 57) far lower than their COI and 16S counterparts (average 98). The suggested evolutionary history of *C. cuspidatus* also differs among the genes with each gene identifying differing phylogenetic relationships. While the COI and combined mtDNA genes support a monophyletic relationship with *C. robustus*, the 16S gene suggests an earlier divergence from all SEQ *Cherax*. Irrespective of their phylogenies, the COI, 16S and combined analyses all estimate a Miocene (11-17mya) divergence from other *Cherax*. In contrast, the 12S gene estimates a more recent Pliocene (4.26mya) divergence from *C. dispar*, *C. depressus* and *C. robustus*. Among the five clades within *C. cuspidatus*, two highly divergent groups were identified; *C. cuspidatus* A & B and *C. cuspidatus* C, D & E. Although highly divergent, the distribution of these groups was not geographically separated with *C. cuspidatus* B & E both located within the Logan-Albert River (Figure 3.1). There were however some geographic boundaries for each of the clades, with *C. cuspidatus* A, B, C & D each restricted to NSW, the Logan-Albert River, the Pine River and the Maroochy Rivers respectively. *C. cuspidatus* E was the most widely distributed, encompassing the Logan-Albert River, Brisbane River, Tingalpa and Gold Coast (Figure 3.1). The molecular divergence between the two groups was approximately 7-14% for the three mtDNA genes (Table 3.3) with an estimated divergence during the Miocene (5.9-11.66mya) for the COI and 16S genes (Figure 3.1). The more recent Pliocene divergence among the clades (Figure 3.1) also showed highly variable molecular divergence estimates of 1-11% (Table 3.3).

The last species group, *C. quadricarinatus*, encompassed two species; *C. quadricarinatus* and *C. rhynchotus*. Sequences of both species were obtained from Genbank and thus analysis was limited to the 16S and 12S genes. For all but the likelihood analysis of the 16S gene, *the C. quadricarinatus* group was identified as the most divergent of the eastern *Cherax*. Support for this early divergence was particularly high for both genes with an average nodal support of 100 (Figure 3.2). Although both genes estimate a Miocene divergence from other Qld *Cherax*, the 16S gene estimates that the divergence occurred 13my earlier than that of the 12S gene (5mya).

3.3.2.1 Nuclear DNA Congruence

As expected from genes with slower mutation rates, the nuclear genes successfully identified interspecies taxonomic relationships but were inconsistent when determining intraspecific relationships. This was most evident for *C. dispar*, with the five highly divergent mtDNA lineages showing evidence of only two clear groups (A/B/C and D/E). Similar levels of intraspecific incongruence were also observed for *C. cuspidatus*, with the nuclear gene regions identifying differing relationships between the five *C. cuspidatus* mtDNA lineages (Figure 3.4 & 3.5). Similar to the mtDNA, the 28S gene region separated *C. cuspidatus* A, D & E with a major break separating *C. cuspidatus* A from the others (Figure 3.4). This similarity was not observed for other nuclear genes though, with *C. cuspidatus* A & E having the same haplotype for the GAPDH gene (Figure 3.5) and *C. cuspidatus* D & E combined for the H3 gene region (Figure 3.5).

Table 3.2: Classifications of the eastern Australian *Cherax* after Riek (1969), Austin (1996), Munasinghe *et al.* (2004b) and those supported by this study.

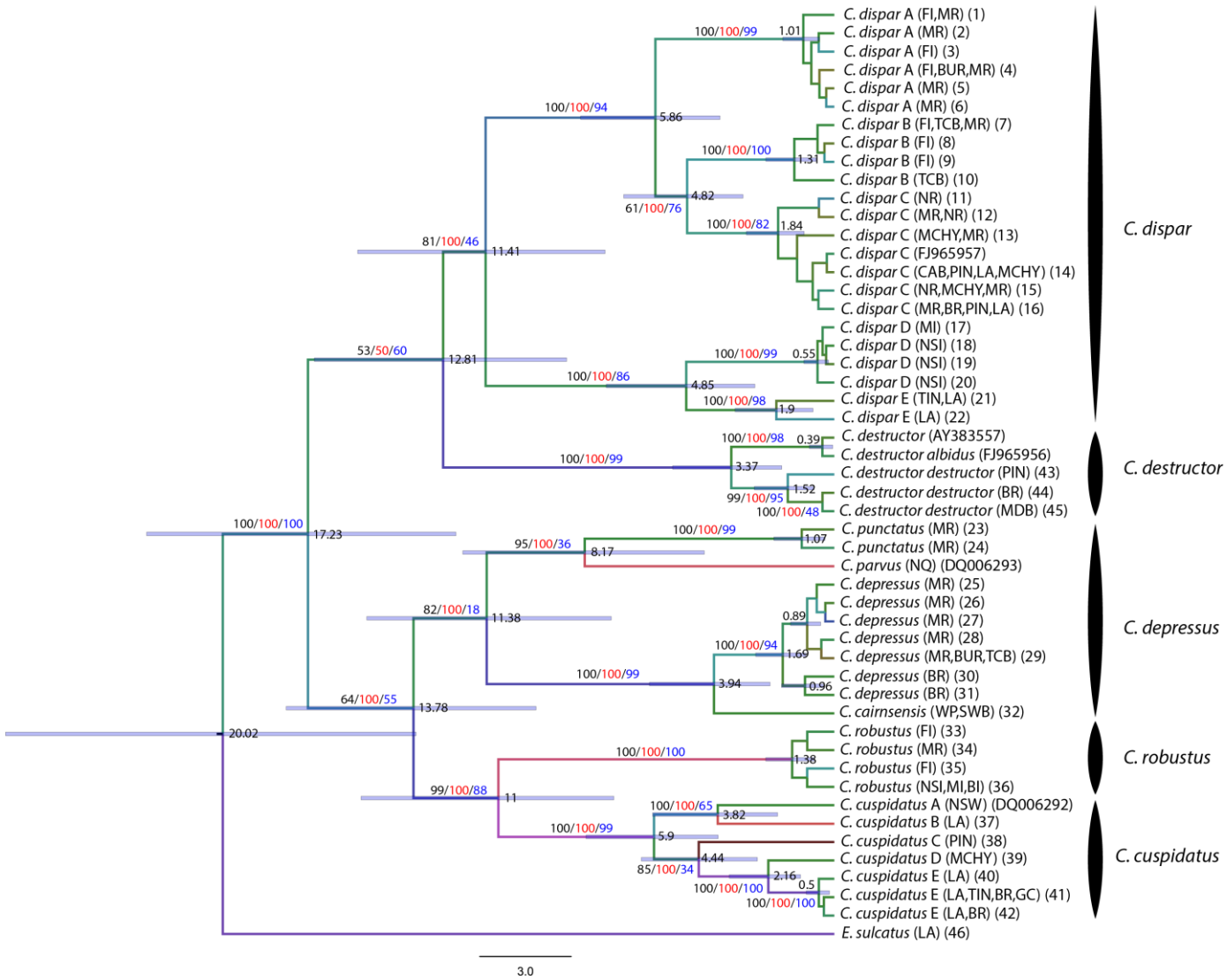
After Riek (1969)	After Austin (1996)	After Munasinghe *et al.* (2004b)	Present Study
C. albidus[5]	*C. destructor albidus*	*C. d. albidus*	*C. d. albidus*
C. austini[1]*	-	-	-
C. barretti[8]	-	-	-
C. bicarinatus[9]	*C. quadricarinatus*	*C. quadricarinatus*	*C. quadricarinatus*
C. cairnsensis[2]	*C. cairnsensis*	*C. cairnsensis*	*C. depressus subspecies*[#]
C. cartalacoolah[3]*	-	-	-
C. cid[1]*	-	-	-
C. cuspidatus[2]	*C. cuspidatus*	*C. cuspidatus*	*C. cuspidatus* A-B
C. davisi[8]	*C. destructor destructor*	*C. d. destructor*	*C. d. destructor*
C. depressus[4]	*C. depressus*	*C. depressus*	*C. depressus*
C. destructor[5]	*C. d. destructor*	*C. d. destructor*	*C. d. destructor*
C. dispar[4]	*C. dispar*	*C. dispar*	*C. dispar* B or C[#]
C. dispar crassus[4]	*C. dispar*	*C. dispar*	*C. dispar* B or C[#]
C. dispar elongatus[4]	*C. dispar* (North)[#]	*C. dispar*	*C. dispar* A[#]
C. dispar D[12]	-	-	*C. dispar* D-E[#]
C. esculus[2]	*C. d. destructor*	*C. d. destructor*	*C. d. destructor*
C. gladstonensis[2]	*C. cairnsensis*	*C. cairnsensis*	*C. depressus subspecies*[#]
C. leckii[6]*	-	-	-
C. neopunctatus[2]	*C. cuspidatus*	*C. cuspidatus*	*C. cuspidatus* A-B
C. nucifraga[11]*	-	-	-
C. parvus[3]*	-	*C. parvus*	*C. parvus*
C. punctatus[5]	*C. punctatus*	*C. punctatus*	*C. punctatus*
C. punctatus[5]*[+]	*C. cuspidatus*	*C. sp. nov.*	*C. cuspidatus* C-E
C. quadricarinatus[7]	*C. quadricarinatus*	*C. quadricarinatus*	*C. quadricarinatus*
C. rhynchotus[4]	*C. rhynchotus*	*C. rhynchotus*	*C. rhynchotus*
C. robustus[4]	*C. robustus*	*C. robustus*	*C. robustus*
C. rotundus[8]	-	*C. rotundus*	*C. rotundus*
C. rotundus setosus[4]	*C. destructor rotundus*	*C. setosus*	*C. setosus*
C. urospinosus[2]	-	-	-
C. wasselli[2]	*C. wasselli*	-	-

* Species described after Riek (1969). [1-12] indicate reference for original taxonomic description identified in Appendix 8.1. [+] Riek applied the *C. punctatus* name to a different taxon to that described by Clark (1936). [#] Classification only an estimate, with further analysis needed.

Table 3.3: Molecular divergence estimates between eastern *Cherax* species and lineages for the three mtDNA genes. Values in parentheses represent standard error for each estimate. * Molecular divergence estimates are among eastern *Cherax* and average among all *Cherax* and average among entire phylogeny respectively.

Species/lineage A	Species/lineage B	Gene		
		COI	16S	12S
C. dispar A	*C. dispar* B	11.31 (1.56)	3.25 (0.84)	1.98 (0.8)
C. dispar A	*C. dispar* C	11.30 (1.59)	2.98 (0.79)	3.37 (1.1)
C. dispar B	*C. dispar* C	10.25 (1.4)	2.62 (0.71)	2.01 (0.82)
C. dispar D	*C. dispar* E	9.67 (1.36)	4.18 (1.02)	2.77 (0.91)
C. dispar A, B & C	*C. dispar* D & E	19.67 (2.04)	5.79 (1.01)	5.41 (1.23)
C. cuspidatus A	*C. cuspidatus* B	11.02 (1.64)	9.69 (1.81)	
C. cuspidatus C	*C. cuspidatus* D	7.31 (1.25)	1.84 (0.63)	
C. cuspidatus C	*C. cuspidatus* E	8.49 (1.31)	2.35 (0.69)	
C. cuspidatus D	*C. cuspidatus* E	5.64 (0.99)	1.81 (0.61)	1.56 (0.73)
C. cuspidatus A & B	*C. cuspidatus* C, D & E	13.73 (1.51)	12.37 (1.86)	7.52 (1.93)
C. punctatus	*C. parvus*	18.32 (2.27)	8.57 (1.61)	10.55 (2.28)
C. depressus	*C. cairnsensis*	7.99 (1.24)	3.84 (0.7)	2.52 (0.64)
C. depressus (North)	*C. depressus* (South)	3.22 (0.67)		
C. punctatus & *C. parvus*	*C. depressus* & *C. cairnsensis*	19.16 (2.01)	7.34 (1.15)	8.08 (1.57)
C. destructor destructor	*C. destructor albidus*	6.80 (1.10)	2.41 (0.7)	4.43 (1.29)
C. setosus	*S. rotundus*		6.31 (1.34)	6.31 (1.76)
C. destructor	*C. setosus* & *C. rotundus*		6.95 (1.13)	6.26 (1.33)
C. quadricarinatus	*C. rhynchotus*		7.19 (1.45)	7.94 (1.94)
*C. robustus**	Rest	22-28, 29	12-14, 17, 18	9-12, 21, 24
C. depressus group*	Rest	22-25, 24	9-15, 16, 21	8-11, 18, 20
*C. dispar**	Rest	22-25, 26	9-15, 16, 20	7-11, 20, 23
C. cuspidatus group*	Rest	22-26, 25	14-15, 18, 23	7-9, 17, 22

a)


C. dispar A (FI,MR) (1)
C. dispar A (MR) (2)
C. dispar A (FI) (3)
C. dispar A (FI,BUR,MR) (4)
C. dispar A (MR) (5)
C. dispar A (MR) (6)
C. dispar B (FI,TCB,MR) (7)
C. dispar B (FI) (8)
C. dispar B (FI) (9)
C. dispar B (TCB) (10)
C. dispar C (NR) (11)
C. dispar C (MR,NR) (12)
C. dispar C (MCHY,MR) (13)
C. dispar C (FJ965957)
C. dispar C (CAB,PIN,LA,MCHY) (14)
C. dispar C (NR,MCHY,MR) (15)
C. dispar C (MR,BR,PIN,LA) (16)
C. dispar D (MI) (17)
C. dispar D (NSI) (18)
C. dispar D (NSI) (19)
C. dispar D (NSI) (20)
C. dispar E (TIN,LA) (21)
C. dispar E (LA) (22)
C. destructor (AY383557)
C. destructor albidus (FJ965956)
C. destructor destructor (PIN) (43)
C. destructor destructor (BR) (44)
C. destructor destructor (MDB) (45)
C. punctatus (MR) (23)
C. punctatus (MR) (24)
C. parvus (NQ) (DQ006293)
C. depressus (MR) (25)
C. depressus (MR) (26)
C. depressus (MR) (27)
C. depressus (MR) (28)
C. depressus (MR,BUR,TCB) (29)
C. depressus (BR) (30)
C. depressus (BR) (31)
C. cairnsensis (WP,SWB) (32)
C. robustus (FI) (33)
C. robustus (MR) (34)
C. robustus (FI) (35)
C. robustus (NSI,MI,BI) (36)
C. cuspidatus A (NSW) (DQ006292)
C. cuspidatus B (LA) (37)
C. cuspidatus C (PIN) (38)
C. cuspidatus D (MCHY) (39)
C. cuspidatus E (LA) (40)
C. cuspidatus E (LA,TIN,BR,GC) (41)
C. cuspidatus E (LA,BR) (42)
E. sulcatus (LA) (46)
C. dispar
C. destructor
C. depressus
C. robustus
C. cuspidatus
3.0

b)

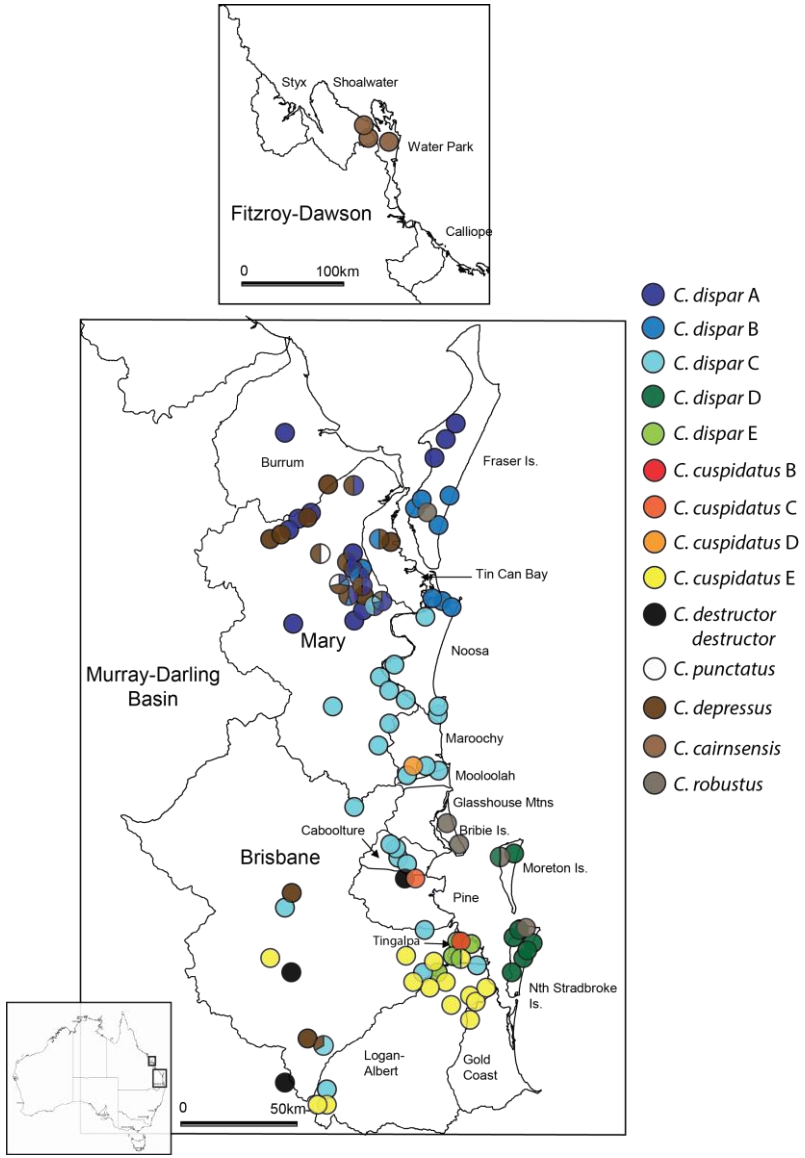

Styx
Shoalwater
Water Park
Fitzroy-Dawson
Calliope
0
100km
Burrum
Fraser Is.
Tin Can Bay
Mary
Noosa
Murray-Darling Basin
Maroochy
Mooloolah
Glasshouse Mtns
Caboolture
Bribie Is.
Brisbane
Moreton Is.
Pine
Tingalpa
Nth Stradbroke Is.
Logan-Albert
Gold Coast
0
50km
C. dispar A
C. dispar B
C. dispar C
C. dispar D
C. dispar E
C. cuspidatus B
C. cuspidatus C
C. cuspidatus D
C. cuspidatus E
C. destructor destructor
C. punctatus
C. depressus
C. cairnsensis
C. robustus

Figure 3.1: a) Bayesian analysis consensus phylogram of selected individuals from the COI dataset.b) Distribution of sample sites in South East Queensland for each of the species and lineages within. Bayesian posterior probabilities and MP and ML bootstrap values are displayed on each inter-species branch in black, red and blue respectively. Bold and underlined values indicate support values for complex gap coding and gap removal analysis that differ by more than twenty from the standard analysis. Divergence time estimates (MYA) are displayed on each node with node bars representing the 95% HPD. Branch colours indicate the mutation rate of that branch ranging from brown (slowest) to red (fastest). Each individual is labelled with its species, location and number or Genbank identifier. Location abbreviations are as per Chapter 2, with the addition of NQ (North Queensland), NSW (New South Wales), VIC (Victoria), NT (Northern Territory), CAL (Calliope River), QLD (Queensland), PR (Pioneer) and MDB (Murray-Darling Basin).

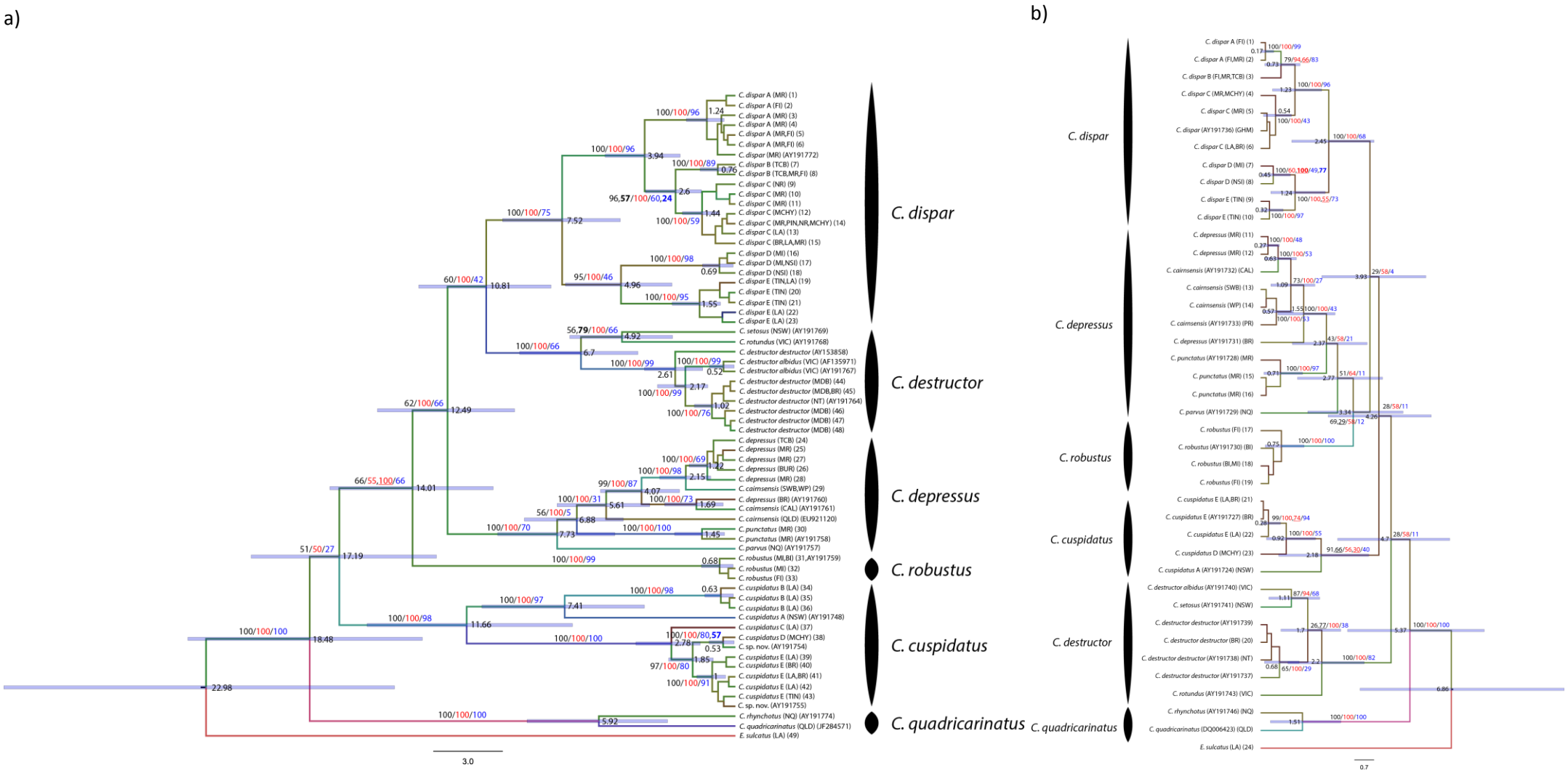


Figure 3.2: Bayesian analysis consensus phylogram of individuals from the a) 16S b) 12S dataset. Phylogram nodal support values, bars and location abbreviations as per Figure 3.1.

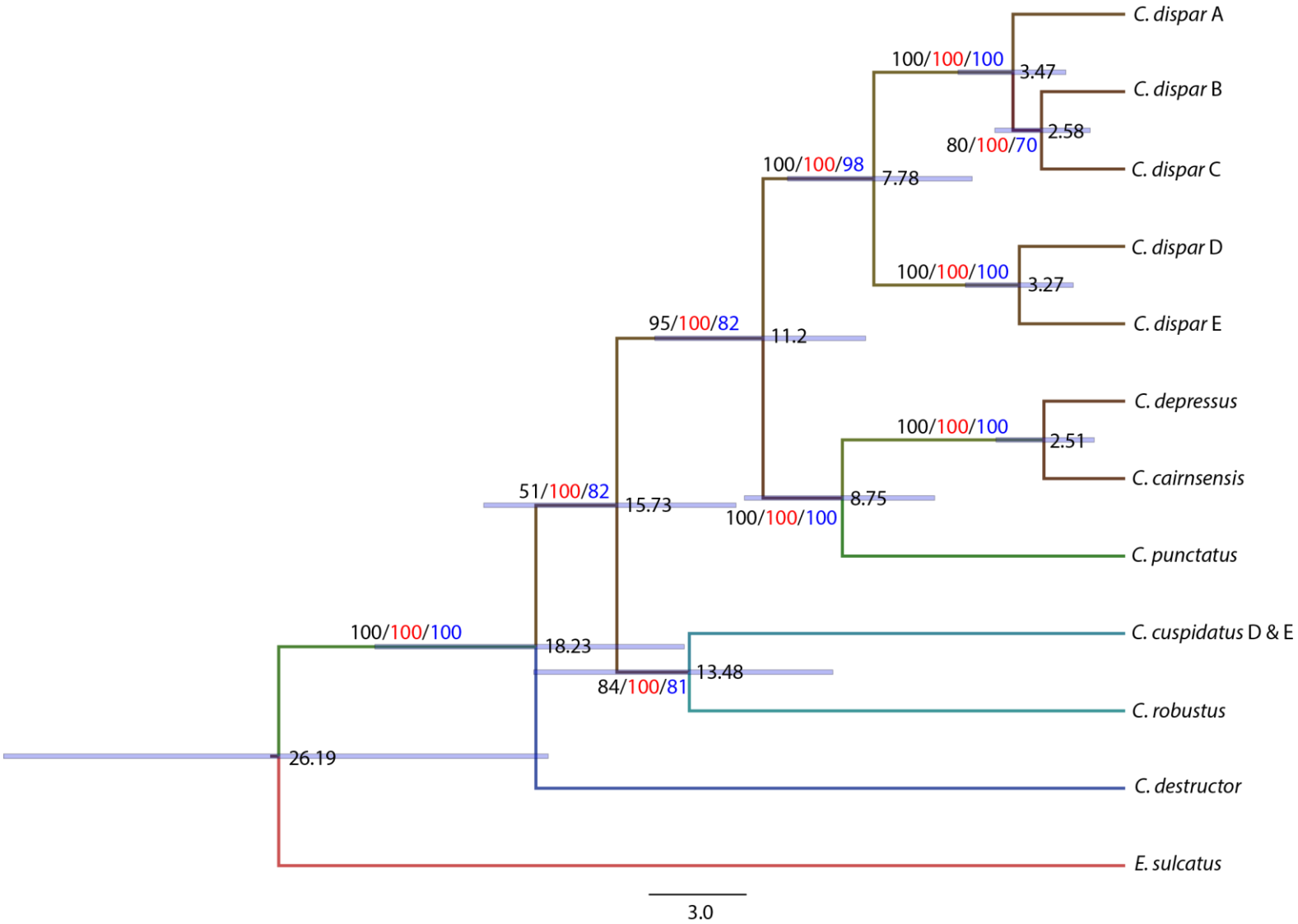


Figure 3.3: Bayesian *Beast consensus tree of selected individuals of the COI, 16S and 12S datasets. Phylogram nodal support values, bars and location abbreviations as per Figure 3.1.

Although the level of genetic diversity between the nuclear genes varies considerably, monophyletic support for the current taxonomic identifications of eastern *Cherax* species was strongly supported. The only two exceptions to this were in two separate genes for *C. destructor* and *C. dispar*. In the 28S gene region, *C. destructor destructor* was identified as paraphyletic with *C. destructor albidus* (Figure 3.4) and in the H3 gene region *C. dispar* was identified to be paraphyletic with *C. glaber* and *C. depressus* (Figure 3.5). Specifically, the *C. dispar* lineages A, B & C were identified to be closely related to *C. glaber* and the *C. dispar* lineages D & E paraphyletic with *C. depressus* (Figure 3.5). Along with taxonomic incongruences, some inconsistencies were also observed for the mtDNA species groups established previously. This was observed for the *C. destructor* species group with *C. setosus* and *C. rotundus* more closely related to *C. cuspidatus* than to *C. destructor destructor* and *C. destructor albidus* for the GAPDH gene region (Figure 3.5). A similar incongruence with the mtDNA species groups was also observed for the *C. depressus* species, with *C. punctatus* detached from *C. depressus* and *C. cairnsensis* for the H3 gene (Figure 3.5).

Strong congruence was also observed between the mtDNA and nuclear genes in distinguishing the phylogenetic relationships between *Cherax* species. For all mtDNA and nuclear genes, either *C. robustus* or *C. cuspidatus* were identified as the most divergent SEQ *Cherax* species with a sister species relationship often observed between them. Although not as consistent across genes, *C. dispar* was also identified to be closely related to *C. depressus* for all except the ITS2 and 16S genes. For both these genes, *C. dispar* was identified as closely related to the *C. destructor* group and *C. depressus* closely related to *C. cuspidatus* and *C. destructor* for the ITS2 and 16S genes respectively. Of all *Cherax* species in SEQ, *C. destructor* was the most inconsistent across the genes. Predominantly for most of the genes, *C. destructor* was identified as closely related to either the *C. dispar* or *C. depressus* species groups. In the COI, 12S and H3 genes however, *C. destructor* was estimated to have a diverged from other SEQ *Cherax* far earlier, sometimes at the same time as *C. cuspidatus* or *C. robustus*. Congruence between mtDNA and nuclear DNA was also observed for the North Queensland species group *C. quadricarinatus* with GAPDH also showing an early divergence from other eastern *Cherax* (Figure 3.5).

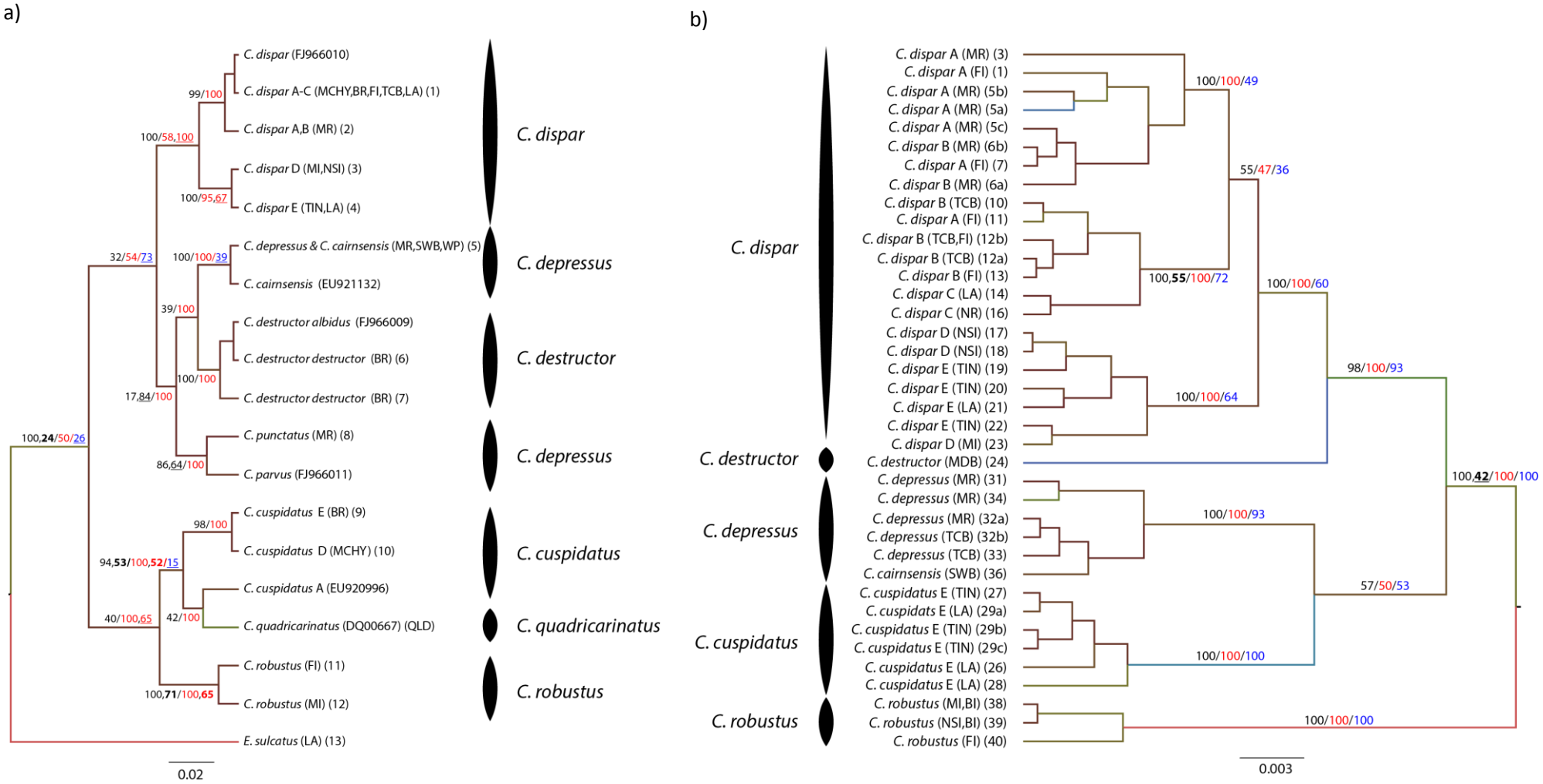


Figure 3.4: Bayesian analysis consensus phylogram of individuals from the a) 28S and b) ITS2 dataset. Phylogram nodal support values and location abbreviations as per Figure 3.1.

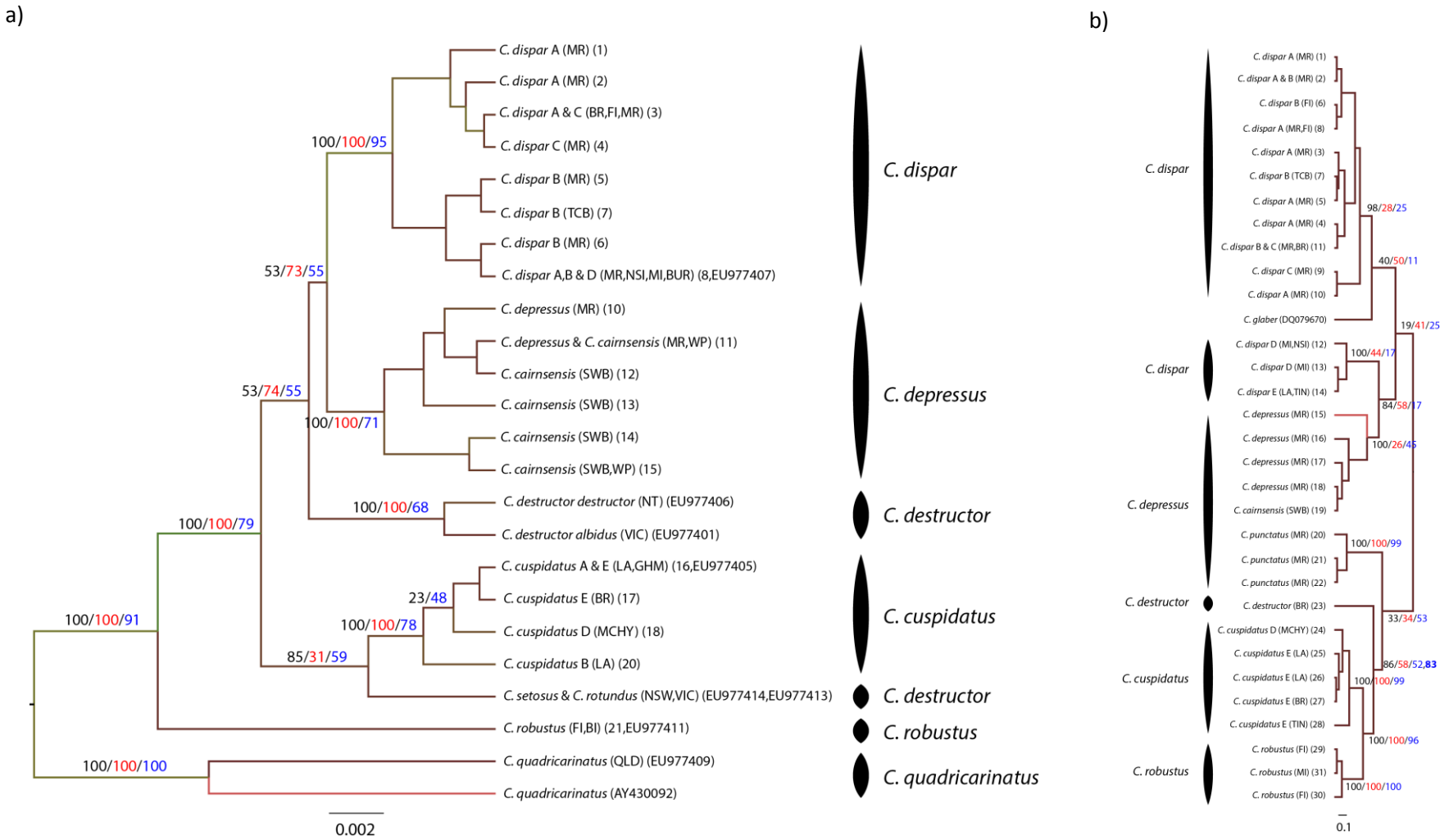


Figure 3.5: Bayesian analysis consensus phylogram of individuals from the a) GAPDH and b) H3 dataset. Phylogram nodal support values and location abbreviations as per Figure 3.1.

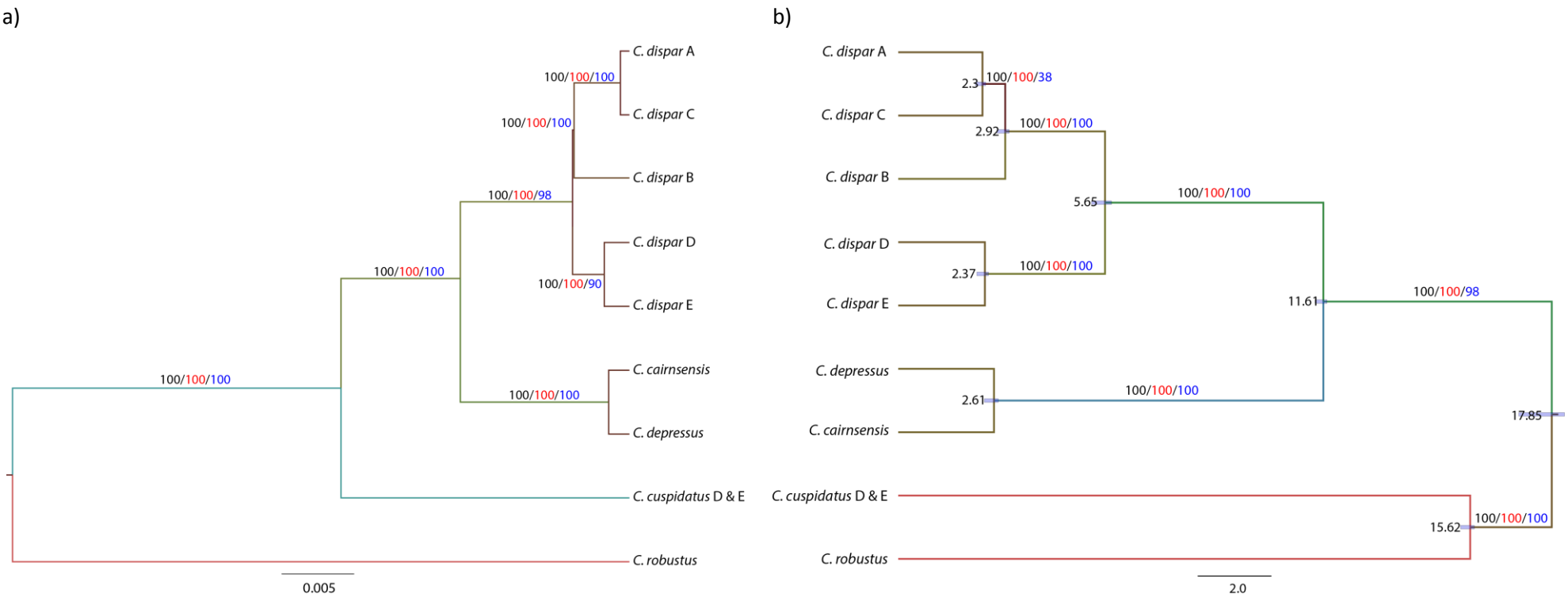


Figure 3.6: Bayesian *Beast consensus tree of selected individuals of the a) nDNA b) both nDNA & mtDNA datasets. Phylogram nodal support values and bars as per Figure 3.1.

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3.4 DISCUSSION

3.4.1 Phylogenetic and Taxonomic Species

The use of a molecular lineage based approach to identify and validate taxonomic species has been implemented for a variety of organisms (Hebert *et al.*, 2003; Lee, 2004; Wiens & Penkrot, 2002), including freshwater crayfish (Grandjean *et al.*, 2000; Munasinghe *et al.*, 2004b). Using this approach, the three mtDNA and four nuclear genes in this study strongly support the classification of at least fifteen *Cherax* species throughout eastern Australia. The fifteen species currently identified are the result of continuous amendments from molecular (Austin, 1996; Munasinghe *et al.*, 2004b) and morphological (Austin, 1996; Riek, 1969) information. Of these fifteen species, the two species, *C. wasselli* and *C. urospinosus* could not be obtained by either field sampling or from Genbank. However, the remaining thirteen species consistently formed monophyletic groups with high nodal support for a majority of the mtDNA and nuclear phylogenetic analyses. Along with these thirteen taxonomically identified taxa, an additional three highly divergent possible new species were identified, one within *C. dispar* (*C. dispar* D/E) and two from within *C. cuspidatus* (*C. cuspidatus* B & D/E). Of these three possible species, only *C. cuspidatus* B had not previously been identified. Previous analysis on *C. dispar* identified *C. dispar* D as a highly divergent lineage separate from the nominal taxon (Bentley *et al.*, 2010), while *C. cuspidatus* D/E was previously recognised by Munasinghe *et al.* (2004b) and labelled *Cherax sp. nov*. pending taxonomic identification. Divergence estimates between each of these taxa (including the three new taxa) were at a similar level to other Decapods (Daniels *et al.*, 2002; Palumbi & Benzie, 1991) and freshwater crayfish (Munasinghe *et al.*, 2004b; Munasinghe *et al.*, 2003; Toon *et al.*, 2009). Typical mtDNA interspecies divergence estimates for freshwater Decapods range from 4-11%, 8-10% and 15% for the 16S (Grandjean *et al.*, 2000; Munasinghe *et al.*, 2004b; Munasinghe *et al.*, 2003; Schultz *et al.*, 2007), 12S ((Munasinghe *et al.*, 2004b; Munasinghe *et al.*, 2003; Palumbi & Benzie, 1991) and COI (Munasinghe *et al.*, 2003) genes respectively.

Although many studies have solely utilised mtDNA divergences to classify taxonomic species (Hebert *et al.*, 2003; Lee, 2004; Munasinghe *et al.*, 2004b), there are a number of inherent issues with this approach. These can include discrepancies between gene trees and organismal trees, mtDNA introgression, multiple copies of the mitochondria and variable mutation rates between organisms and genes (Grechko, 2013). For these reasons, taxonomic classifications and interpretations of eastern Australian *Cherax* in this study are made by utilising concordances between seven genes and morphological analyses from prior studies (Austin,

1996; Austin & Knott, 1996; Riek, 1951, 1969; Sokol, 1988). Using this information, support for or against the current taxonomic classification of each eastern Australian *Cherax* species is discussed below according to six mtDNA species groups.

3.4.1.1 Cherax dispar

The analysis of *C. dispar* in this study strongly supports the classification of *C. dispar* as at least one distinct taxonomic species. All seven mtDNA and nuclear genes supported a monophyletic group for the species, with high divergences both within the species and from other eastern *Cherax*. Within the species, one new highly divergent monophyletic lineage (*C. dispar* E) was identified in addition to the four divergent lineages (A-D) that were previously observed (Bentley *et al.*, 2010),. This high variation within *C. dispar* is consistent with Riek (1951), who initially divided the species into three subspecies; *C. dispar*, *C. dispar crassus* and *C. dispar elongatus*. The geographic distribution of the three subspecies however, does not coincide with the separate mtDNA lineages within this study. Based on each subspecies' distribution (Riek, 1951), *C. dispar* B or C could coincide with the nominal species *C. dispar* or *C. dispar crassus*, while the distribution of *C. dispar elongatus* matches that of *C. dispar* A. The nominal species *C. dispar* did however show a similar distribution to the *C. dispar* C lineage (Riek, 1951). Although the subspecies' suggested by Riek (1951) do not necessarily match the mtDNA lineages in this study, the high divergences between the three lineages (10-11%, 3%, 2-3% for COI, 16S & 12S respectively) are higher than other typical taxonomically identified subspecies, *C. destructor destructor* and *C. destructor albidus*. Similar to the *C. destructor* subspecies, *C. dispar* A, B & C were also observed to be paraphyletic for all four nuclear genes. Even though the *C. dispar* lineages A, B and C were not supported by both mtDNA and nuclear genes, a taxonomic distinction may still be warranted, with Austin (1996) also identifying a split between the northern (*C. dispar* A or B) and southern (*C. dispar* C) mainland *C. dispar* samples for both morphology and nuclear allozyme information.

Along with the three *C. dispar* lineages A, B & C, two other highly divergent lineages were also observed as a sister clade within *C. dispar* (D & E). Previous research based on mtDNA and the ITS gene, identified the clade *C. dispar* D as a separate highly divergent cryptic species of *C. dispar* (Bentley *et al.*, 2010). This high level of divergence for the mtDNA genes (20%, 6%, 5% for COI, 16S and 12S respectively) and congruence with three of the four nuclear genes support the classification of a new taxonomic species. Variance between the two clades at this level is similar or higher than divergences between taxonomic species of other freshwater crayfish (Grandjean *et al.*, 2000; Schultz *et al.*, 2007) and *Cherax* (Munasinghe *et al.*, 2004b;

Munasinghe *et al.*, 2003). As well as being highly divergent monophyletic lineages, *C. dispar* D & E also have highly restricted geographic distributions, with *C. dispar* E restricted to the lower reaches of the Logan River and Tingalpa Creek and *C. dispar* D found only on three coastal sand islands (Figure 3.1). These restricted distributions for *C. dispar* D & E may also explain the limited research on the lineages, with previous studies limiting their sampling within these regions (Austin, 1996; Riek, 1951, 1969). Although *C. dispar* D & E differ at a similar level to *C. dispar* A, B & C (10%, 4% and 3% for COI, 16S and 12S respectively), the lack of morphological research on the lineages limit the ability to classify them taxonomically. With further morphological research on the five lineages, specifically D & E, distinct taxonomic classification may be warranted.

3.4.1.2 Cherax depressus group

In this study, the *C. depressus* species group consists of four described species that span the entire Queensland coastline; *C. depressus*, *C. cairnsensis*, *C. punctatus* and *C. parvus*. The recognition of these four species from the ten originally identified as the *C. punctatus* group (Riek, 1969) was first suggested by Munasinghe *et al.* (2004b) based on mtDNA analysis. Although some nuclear genes in this study suggest further separation of *C. punctatus* from this group, mtDNA strongly supports the monophyletic classification of each of the four species. With broad chelae also a shared feature among the group, three of the species (*C. punctatus*, *C. depressus* and *C. cairnsensis*) are capable of burrowing deep into the water table during drought conditions (Riek, 1951, 1969; Short & Davie, 1993). This 'terrestrial' characteristic of *C. punctatus*, along with its original taxonomic identification (Clark, 1941), was disputed by Riek (1969). Further analysis by Austin (1996) and Munasinghe *et al.* (2004b) however, identified that Riek (1969)'s findings were based on individuals from a different species and *C. punctatus* was in fact a distinct terrestrial species. This result was also supported in this study, with both mtDNA and nuclear DNA identifying *C. punctatus* as a distinct monophyletic group. This combination of high mtDNA divergence (18-20%, 7-9% and 7-10% for COI, 16S and 12S respectively), nuclear gene monophyly and morphological distinction (Austin, 1996; Clark, 1941) strongly affirms the taxonomic identification of *C. punctatus* as a species. This level of evidence for taxonomic distinction was not available for the relatively poorly researched *C. parvus*. The limited level of research on *C. parvus* may be due to the comparatively new taxonomic identification of the species (Short & Davie, 1993) and its highly restricted distribution in North Queensland. Phylogenetic analysis of a single sequence for each of the mtDNA genes did however support the separation of *C. parvus* from other similar *Cherax* species, with divergence estimates at a similar level to other interspecies comparisons (18-

22%, 7-9% and 9-10% for COI, 16S and 12S respectively). This distinction was especially evident for both ribosomal mtDNA genes, with the divergence of *C. parvus* estimated to be earlier than all other species within the *C. depressus* species group.

Although a number of studies have continuously reviewed the taxonomy of *C. depressus* and *C. cairnsensis* (Austin, 1996; Munasinghe *et al.*, 2004b; Riek, 1969), no consensus has been reached on how best to classify them. When first taxonomically identified, *C. depressus* was thought to be distributed throughout Queensland, with morphological variants acknowledged along the Queensland coast (Riek, 1951). These morphological variants were later separated into four distinct species (*C. depressus*, *C. cairnsensis*, *C. gladstonensis* and *C. wasselli*) by Riek (1969). The taxonomic distinction of *C. gladstonensis* as a separate species was not supported by Austin (1996), who, using allozyme and morphological data, did not observe abrupt changes between the *C. cairnsensis* and *C. gladstonensis* distribution boundaries. Austin (1996) did however support the classification of *C. wasselli* as a distinct species, identifying it as part of a completely separate group. Austin (1996) also identified a slight break between two SEQ sites and the rest of Queensland, defining individuals north and south of Brisbane as *C. cairnsensis* and *C. depressus* respectively. This geographical break between *C. depressus* and *C. cairnsensis* was also observed by Munasinghe *et al.* (2004b) with mtDNA, who identified samples south of Brisbane as *C. depressus* and north of Gladstone as *C. cairnsensis*. This clear differentiation between the two was not evident in this study, with the two paraphyletic for every gene except COI. The low divergence estimates of 8% observed between the two species for the COI gene was also similar to other subspecies divergences in *Cherax* (7%). This lack of consistency between mtDNA, nuclear DNA and morphological data (Austin, 1996; Riek, 1951, 1969) in identifying between the two species, suggests a reclassification for the two species may be required.

3.4.1.3 Cherax robustus

As the most differentiated eastern *Cherax* species in this study, the classification of *C. robustus* as a distinct taxonomic species is strongly supported. For all seven mtDNA and nuclear genes, *C. robustus* formed a highly diverged monophyletic clade. Nucleotide divergences between *C. robustus* and other eastern *Cherax* range from 22-28%, 11-14% and 9-12% for the COI, 16S and 12S genes respectively. Similar to *C. parvus*, the limited amount of morphological research on the species may be due to its highly restricted distribution in sandy, coastal acidic swamps. As a majority of these areas are now developed, *C. robustus* is predominantly restricted to the four coastal sand islands off the South East Queensland coast, with the species presumed to be

extremely rare or extinct on the mainland. Although no morphological studies have been conducted on *C. robustus* recently, the strong congruence between mtDNA and nuclear DNA support the original taxonomic classification by Riek (1951).

3.4.1.4 Cherax cuspidatus group

The delineation of *C. cuspidatus* as a distinct species group was highly supported in this study, with six of the seven genes showing a monophyletic relationship for the group. Second only to *C. robustus*, the *C. cuspidatus* group was also highly differentiated from other eastern *Cherax* species with mtDNA divergences of 22-26%, 14-15% and 7-9% for the COI, 16S and 12S genes respectively. Similar to *C. dispar*, phylogenetic analysis of the mtDNA genes also identified five highly divergent monophyletic lineages distributed across South East Queensland and Northern NSW. Classification of each of these lineages into separate species was not supported in this study, with low divergences (6-16%, 2-13% and 2-8% for COI, 16S and 12S respectively) and nuclear paraphyly observed between some lineages. A clear delineation into at least two clades (AB-CDE) was supported, with mtDNA divergence estimates (14%, 12%, 8% for the COI, 16S and 12S respectively) higher than other interspecies comparisons in this study. This split was also identified by earlier molecular (Munasinghe *et al.*, 2004b) and morphological (Austin, 1996) studies, with Munasinghe *et al.* (2004b) classifying the northern SEQ clade as a new species (*C. sp. nov.*). This classification differs to Riek (1969)'s original classification of northern SEQ as *C. punctatus* and southern (NSW) as either *C. rotundus*, *C. cuspidatus* or *C. neopunctatus*. These three southern species were later revised by Austin (1996) and Munasinghe *et al.* (2004b), who placed *C. rotundus* within the *C. destructor* complex and synonymised *C. cuspidatus* and *C. neopunctatus*. Although Munasinghe *et al.* (2004b) identified the McPherson Range as a clear biogeographic barrier between the south *C. cuspidatus* and north *C. sp. nov* species, it was not observed in this study. In particular, the *C. cuspidatus* B lineage from the Logan-Albert River in SEQ was identified as more closely related to *C. cuspidatus* A from Northern NSW than other SEQ *C. cuspidatus* individuals.

Within this study, the northern *C. sp. nov* species that was recognised by Munasinghe *et al.* (2004b) was identified as a part of the *C. cuspidatus* D lineage within the *C. cuspidatus* C, D & E clade. Although *C. cuspidatus* D does not share the same haplotype as either of the other two lineages in this clade, mtDNA divergence estimates from *C. cuspidatus* E were relatively low (COI; 5.64%, 16S; 1.81% and 12S; 1.56%). Whilst *C. cuspidatus* D & E are very similar, *C. cuspidatus* C was more divergent within the clade, with divergences around 7-8% and 2% for the COI and 16S genes respectively. Due to poor sampling and sequencing success, *C.*

cuspidatus C was only sequenced for these two genes. With these low mtDNA divergences and no apparent morphological differences (Austin, 1996), there is no evidence to separate the three lineages taxonomically. The nominal taxon, *C. cuspidatus*, as classified by Austin (1996) and Munasinghe *et al.* (2004b), was identified in this study as *C. cuspidatus* A. Contrary to previous studies (Austin, 1996; Munasinghe *et al.*, 2004b), *C. cuspidatus* A was identified as similar to SEQ samples of the lineage *C. cuspidatus* B, rather than the unidentified species *C. sp. nov*. (*C. cuspidatus* D). In contrast to *C. cuspidatus* C-E, the distinction between *C. cuspidatus* A & B was highly supported in this study, with mtDNA divergence estimates (COI; 11% and 16S; 10%) similar to other species of freshwater crayfish (Grandjean *et al.*, 2000). Further delineation into distinct taxonomic species however is dependent on further analyses using morphological information.

3.4.1.5 Cherax destructor group

With the largest distribution of all *Cherax* species groups, the *C. destructor* group has been heavily researched both taxonomically and genetically. Since *C. destructor* was first recognized by Clark (1936), a number of revisions have been made on the distribution of the nominal species and the identification of its closest relatives (Austin, 1996; Hughes & Hillyer, 2003; Munasinghe *et al.*, 2004b; Nguyen & Austin, 2005). Riek (1969) proposed a *C. destructor* species group containing four species; *C. destructor*, *C. albidus*, *C. esculus and C. davisi*. This species group was later drastically reduced, with the synonymy of *C. esculus* and *C. davisi* with the nominal taxon *C. destructor* (Austin, 1996; Campbell *et al.*, 1994; Sokol, 1988). The taxonomic delineation of the former two species, *C. destructor* and *C. albidus*, has also been under scrutiny, with the two taxa identified as separate species (Riek, 1969; Sokol, 1988), subspecies (*C. destructor destructor* and *C. destructor albidus*) (Austin, 1996; Hughes & Hillyer, 2003; Munasinghe *et al.*, 2004b; Nguyen & Austin, 2005) or a single species (Austin *et al.*, 2003). This difficulty to distinguish between the two species was also evident in this study with monophyletic separation observed for the COI, 12S and GAPDH genes and paraphyly for the 16S and 28S genes. This paraphyly for both mtDNA and nuclear genes along with a low level of mtDNA divergence between the two species (6.8%, 2.41% and 4.43% for the COI, 16S and 12S genes respectively) does not support the two species classification by Sokol (1988), with molecular and morphological information instead supporting delineation into at most sub-species (Austin, 1996; Nguyen & Austin, 2005).

In addition to the nominal taxon subspecies, Austin *et al.* (2003) also suggested the inclusion of *C. setosus* and *C. rotundus* in the *C. destructor* species group. Similar to the previous *C. destructor* subspecies, the taxonomic classification of these two species has also been under continuous revisions with *C. setosus* classified as a single taxon with *C. rotundus* (Riek, 1969), a subspecies of *C. rotundus* (Riek, 1951), a subspecies of *C. destructor* (Austin, 1996) and a separate species (Austin *et al.*, 2003; Munasinghe *et al.*, 2004b). The latter classification as separate species was highly supported in this study with the two species showing interspecific divergence levels for both the 16S (6.31%) and 12S (6.31%) genes. Surprisingly, although the distributions of *C. rotundus* and *C. setosus* are separated by *C. destructor*, a monophyletic sister group relationship to the two *C. destructor* subspecies was still observed.

3.4.1.6 Cherax quadricarinatus group

Although both species of the *C. quadricarinatus* species group (*C. quadricarinatus* and *C. rhynchotus*) are distributed within Queensland, both are significantly different from other Qld *Cherax* (15-17% and 16-17% average divergence for 16S and 12S genes respectively). This high variance from other Qld *Cherax* is not surprising, as Austin (1996) and (Munasinghe *et al.*, 2004a) identified the two species as part of the 'Northern' *Cherax* biodiversity hotspot. The separation of these two biodiversity hotspots was also supported using morphological and allozyme analyses (Austin, 1995; Austin, 1996), with a clear break along the Qld coast near Cooktown (Munasinghe *et al.*, 2004b).

3.4.2 Phylogenetic Congruence

Although the delineation and taxonomic classification of each species and their corresponding species groups was well supported, the phylogenetic relationship and history among them was not. There was low nodal support and continuous inconsistencies when estimating the phylogenetic relationships between the species groups. These inconsistencies were observed across each of the genes, analysis methods and coding methods. For the three multi-gene analyses three monophyletic groups were observed within eastern Australian *Cherax*; the *C. dispar* and the *C. depressus* group, the *C. robustus* and *C. cuspidatus* group and the *C. destructor* group. Combined analysis of mtDNA identified the *C. destructor* group as the most divergent eastern *Cherax* species group, with the group estimated to have diverged from other eastern *Cherax* during the early Miocene approximately 18mya. This initial divergence estimate of eastern *Cherax* was significantly later than the 50mya estimate by Toon *et al.* (2010) and slightly earlier than Munasinghe *et al.* (2004a) estimate of 12-18mya.

Although *C. dispar* and the *C. destructor* group were identified as sister clades for the COI, 16S and ITS2 genes, there was strong nodal support for a close affinity with the *C. depressus* group for all three analyses of combined datasets. This inconsistency across single gene analyses was similar to previous molecular research with studies both supporting (Munasinghe *et al.*, 2004b; Toon *et al.*, 2010) and challenging (Austin, 1996; Bentley *et al.*, 2010; Munasinghe *et al.*, 2004a) the grouping with *C. depressus*. Similarly, previous morphological research also challenged this grouping, identifying *C. cuspidatus* (Riek, 1969) and northern Australia *Cherax* (Austin, 1996) as *C. dispar*'s closest morphological group. Riek (1969) also went on to advocate that the *C. dispar* and *C. cuspidatus* group is more similar to western Australian *Cherax* than to other eastern Australian *Cherax*. Of the five eastern *Cherax* groups, the *C. destructor* group was the most inconsistently classified, with almost every analysis identifying a different phylogenetic relationship for the group. The highest nodal support was observed for an early divergence from all eastern *Cherax*. This separate classification of the group is consistent with Riek (1969), who suggested the group separated from other eastern *Cherax* relatively early. As the only eastern *Cherax* species group not to occur naturally in SEQ, an early divergence of the *C. destructor* group is not surprising, with the group estimated to have diverged from other eastern *Cherax* approximately 18mya. There is however very limited support for this early divergence with both molecular (Austin, 1996; Bentley *et al.*, 2010; Munasinghe *et al.*, 2004a, 2004b) and morphological studies (Austin, 1996) also showing contradictory results.

3.5 CONCLUSION

Results observed in this study demonstrate that even after a number of previous taxonomic revisions (Austin, 1996; Munasinghe *et al.*, 2004b; Riek, 1969), the most appropriate taxonomy for eastern *Cherax* may still not be complete. Using a combination of mtDNA, nDNA and morphological information, previous studies estimated a total of fifteen species and one sub-species distributed throughout central and eastern Australia. By comparing the mtDNA and nDNA phylogenies in this study with previous morphological research, this study increased the number of taxonomic species to sixteen with at least an additional six lineages requiring further investigation. Included in this estimate are two new species, one from within the *C. cuspidatus* species and one from within *C. dispar*. Although morphological evidence delineating the new species from their nominal taxon was limited, genetic divergences between them were at a similar level to those observed between other freshwater crayfish species (Grandjean *et al.*, 2000; Ponniah & Hughes, 2004) and genera (Schultz, 2009). This study also suggests a possible reclassification of *C. cairnsensis* and *C. depressus*. With very little

morphological support (Austin, 1996) and paraphyly observed for all nDNA, the two species exhibited support for a possible subspecies classification. Although a high level of molecular support was observed for each of the fourteen taxonomic classifications in this study, the availability of supporting morphological research was limited. This was especially the case for new species and sub-species classifications, indicating scope for future comprehensive morphological research on taxa in the area.

CHAPTER 4: BIOGEOGRAPHIC HISTORY OF A HIGHLY DIVERSE AUSTRALIAN FRESHWATER CRAYFISH GENUS (*CHERAX*)

4.1. INTRODUCTION

With a monophyletic origin and global distribution, the freshwater crayfish are estimated to have separated from their saline relatives, the clawed lobsters (Nephropoidea), within Pangaea during the Triassic period approximately 185-225mya (Crandall *et al.*, 2000). The later separation of Pangaea into northern (Laurasia) and southern (Gondwana) landmasses also corresponds with the current taxonomic separation of freshwater crayfish into two superfamilies; Astacoidea and Parastacoidea. The origin of these two superfamilies is suggested to have occurred within the two current centres of biodiversity; in the south-eastern Appalachian Mountains for the northern Astacoidea, and in southeast Australia for the southern Parastacoidea (Crandall & Buhay, 2008). Although Parastacoidea is distributed throughout a majority of the Gondwanan landmasses, the biogeographic history of the superfamily does not necessarily mirror that of other widely distributed taxa (Sanmartin & Ronquist, 2004; Toon *et al.*, 2010). While a majority of other taxa follow a southern Gondwanan pattern (Sanmartin & Ronquist, 2004), southern freshwater crayfish observe an East (Madagascar, India, Australia, Antarctica & New Zealand) to West (South America, Africa) diversification with divergences pre-dating continental separation (Toon *et al.*, 2010). This was evident with early divergence estimates identifying that freshwater crayfish did not use the Kerguelen Plateau between Madagascar/India and Antarctica/Australia/New Zealand 120-80mya (Krause *et al.*, 1997) or the Drake Passage connecting South America and Antarctica 31mya (Lawver & Gahagan, 2003; Lawver *et al.*, 1992). This early diversification pre-dating continental separation was also evident in Australia, with *Ombrastacoides* & *Spinastacoides* from Tasmania (Australia; Figure 4.1) more closely related to New Zealand and Madagascar genera than other Australian genera (Crandall *et al.*, 1999; Toon *et al.*, 2010).

Riek (1972) originally split Parastacids based on morphology into three groups; *Engaeus* (*Engaeus*, *Tenuibranchiurus*, *Parastacus* & *Engaewa*), *Cherax* (*Cherax*, *Paranephrops*, *Parastacoides* (now *Ombrastacoides* and *Spinastacoides*), *Geocherax*, *Gramastacus* & *Samastacus*) and *Euastacus* (*Euastacus*, *Euastacoides*, *Astacopsis* & *Astacoides*). Separation between these three groups was predominantly based on the male genitalia shape and the plane of chela movement, with *Engaeus* (vertical plane) divergent from *Euastacus* & *Cherax* (horizontal plane). This split was later revised using molecular information by Toon *et al.* (2010), who, excluding *Ombrastacoides* & *Spinastacoides*, identified monophyletic divergence between each of the continents and islands. Toon *et al.* (2010) also identified a close relationship between the *Cherax* and *Engaeus* groups, with *Cherax* species identified as a divergent monophyletic group, sister to other genera within the *Cherax* and *Engaeus* groups. Although Riek (1972) originally grouped *Cherax* with *Geocherax* and *Gramastacus*, he did suggest a more ancient divergence for the genus, separating them with *Paranephrops* and *Parastacoides* from New Zealand and Tasmania respectively. Unlike the *Euastacus* group, which is restricted to eastern Australia, both the *Cherax* and *Engaeus* groups are distributed across Australia, with species/genera in both Western Australia and Eastern Australia. This similar distribution of the two closely related groups may indicate a historic Australia wide distribution, with separation between the two groups occurring via niche evolution. This process of divergence was first mentioned by Riek (1972) who suggested their differing chelae plane may indicate contrasting burrowing styles; with the *Engaeus* group predominantly being burrowers.

Even though the *Engaeus* group is thought to be far more proficient at burrowing down into the water table (Riek, 1972), it is the *Cherax* group that currently inhabits the dry ephemeral streams throughout Australia, populating the most diverse range of habitats and having the widest distribution. With a distribution spanning most of Australia and the Torres Strait (Figure 4.1), the *Cherax* distribution can be separated into three regions based on biodiversity; southwest Australia, north Australia and eastern Australia (Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009). The high biodiversity in each of these areas suggests endemic speciation within each area as the probable mechanism for the current high diversity. The phylogenetic history among each of these three groups is however still unresolved with Munasinghe *et al.* (2004a), Toon *et al.* (2010) and Schultz *et al.* (2009) identifying the southwest, north and east groups as the most divergent respectively. For all three studies, the separation between the east and southwest groups was estimated to have occurred at minimum during the Miocene, 16-14 Mya (Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009; Toon *et al.*, 2010). This Miocene

divergence coincides with the formation of the Nullarbor Plain (Figure 4.1) and mirrors divergence estimates between genera within the *Engaeus* group (Schultz *et al.*, 2009; Toon *et al.*, 2010) and species of freshwater fish (Unmack, 2001). The *Cherax* and *Engaeus* groups east of the Nullarbor Plain have contrasting distributions, with the *Engaeus* group restricted to the south east of Australia (including Tasmania) and *Cherax* distributed along the northern coast, the Torres Strait, within inland Australia and along the entire eastern coast (excluding Tasmania) (Figure 4.2) (Munasinghe *et al.*, 2004b; Schultz *et al.*, 2009).

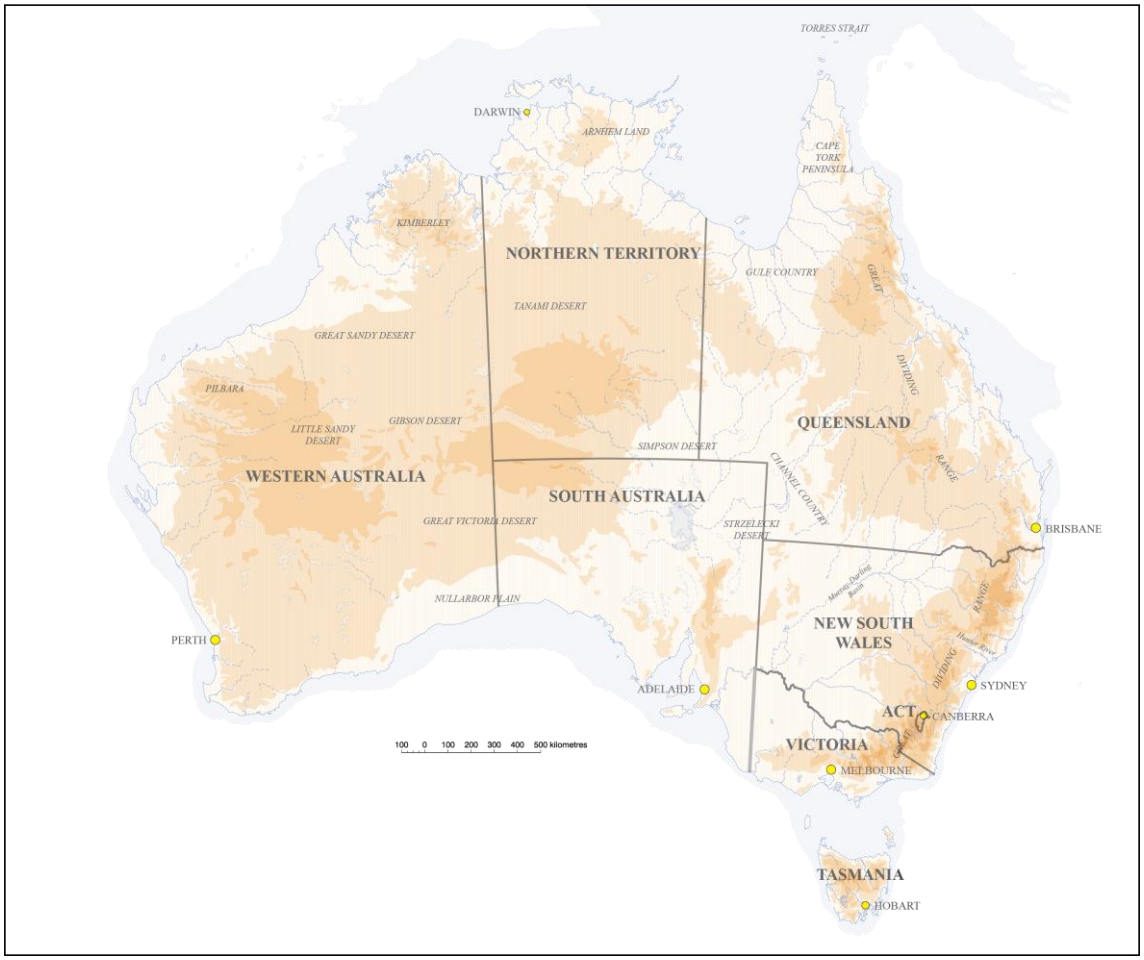

Figure 4.1: Topographic map of Australia and its arid regions.

Within the eastern *Cherax* group, the Great Dividing Range (GDR) and northern river catchment boundaries (Figure 4.1) represented a significant barrier to dispersal, separating the northern *Cherax* group and restricting a majority of the previously identified eastern species groups (Figure 4.2). Of all the species groups, only *C. destructor* was observed on both sides of the GDR, with *C. destructor* and *C. setosus* on the west and east side of the range respectively. Although the eastern *Cherax* group is distributed along most of the east coast and inland Australia, 64% inhabit South-East Queensland (SEQ) (east of the GDR) (Figure 4.2), with 78% of

these endemic to the region. High species richness in SEQ was also observed for freshwater fish (Unmack, 2001), but a majority of the fish species in the area were also observed west of the GDR. Munasinghe *et al.* (2004b) also suggested that the high *Cherax* species richness and endemism within SEQ indicates it as the most probable origin for the eastern *Cherax* group. Munasinghe *et al.* (2004b) went on to identify two major dispersal events across the GDR; the first from east (SEQ) to west (Murray-Darling Basin) (Figure 4.1) to establish *C. destructor* and the second from west (MDB) to east (Hunter River) (Figure 4.1) to establish *C. setosus*.

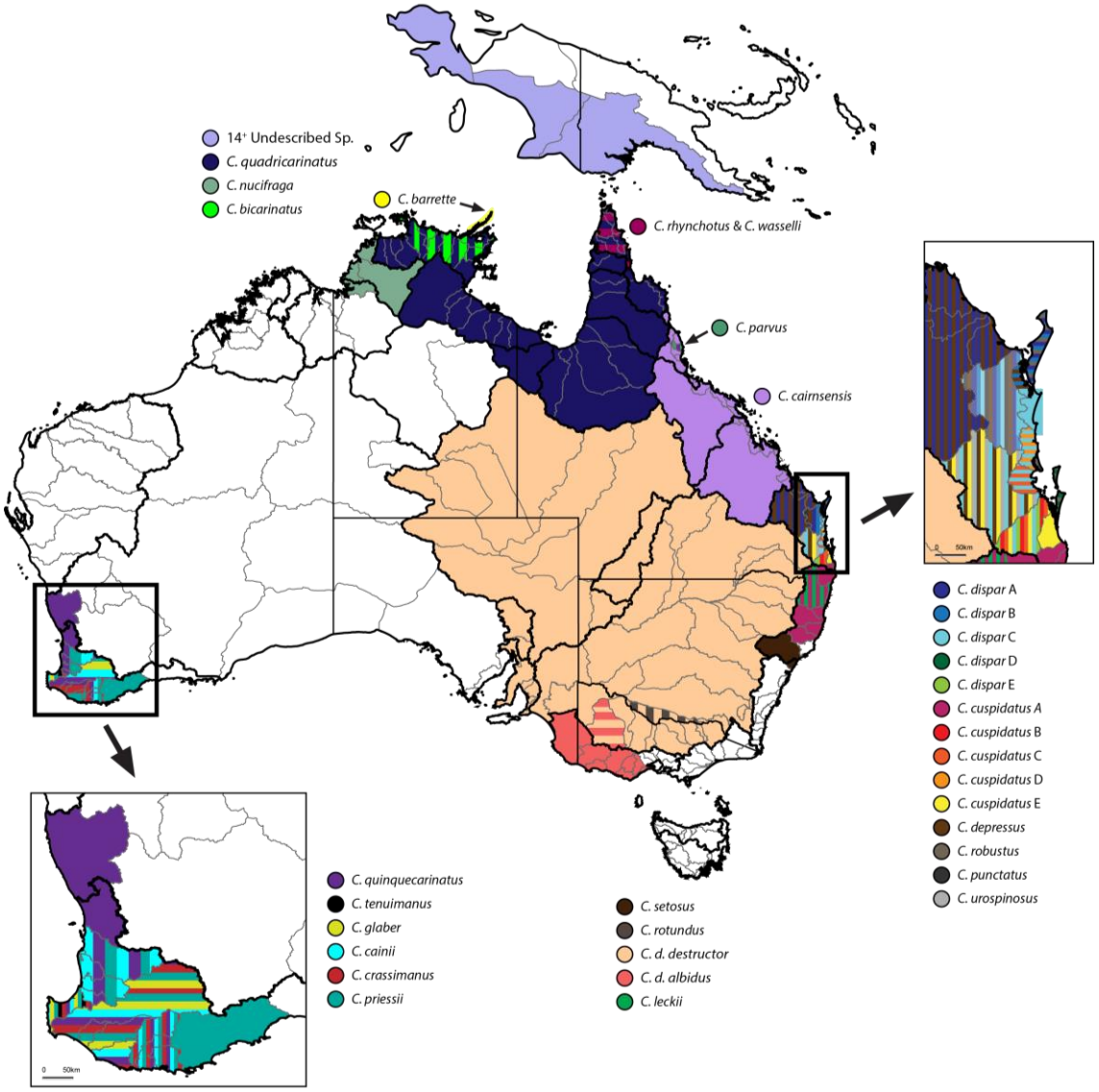

Figure 4.2: Estimated distribution boundaries of current Australian *Cherax* species. Striped distributions indicate river catchments inhabited by multiple species.

As freshwater crayfish in Australia are both widely distributed and highly diverse, they represent a unique opportunity for a comprehensive view into the biogeographic history of freshwater organisms in Australia. This ideal situation has stimulated a large number of biogeographic studies on freshwater crayfish in Australia within the last decade with specific focus on the widely distributed *Euastacus*, *Engaeus* and *Cherax* genera (Bentley *et al.*, 2010; Munasinghe *et al.*, 2004a; Nguyen *et al.*, 2004; Ponniah & Hughes, 2004; Ponniah & Hughes, 2006; Shull *et al.*, 2005; Toon *et al.*, 2010). While a majority of the biogeographic analyses on *Euastacus* and *Engaeus* utilise both mitochondrial and nuclear DNA (Ponniah & Hughes, 2006; Schultz *et al.*, 2009), comprehensive biogeographic analyses focused specifically on *Cherax* are limited to only mtDNA (Munasinghe *et al.*, 2004a). The sole use of mtDNA has consistently been observed to misrepresent the true biogeographic history of a species (Grechko, 2013). This is especially the case for *Cherax,* with the mtDNA study of Munasinghe *et al.* (2004a) identifying a differing relationship between the three biogeographic regions than Toon *et al.* (2010) and Schultz *et al.* (2009), who also utilised nuclear DNA. Both Schultz *et al.* (2009) and Toon *et al.* (2010) however, included only a limited number of *Cherax* species from Australia.

This chapter will investigate the biogeographic history of *Cherax* in Australia by examining the current phylogenetic and geographic relationships among a majority of the Australian *Cherax* taxa. As all Australian *Cherax* are obligate freshwater species, I hypothesise that similar biogeographic events and river catchment boundaries will affect each *Cherax* species similarly. Additionally, variations between the biogeographic history of *Cherax* and other freshwater crayfish and fish will also identify the driving forces of diversification within Australian freshwater fauna and help identify the dispersal abilities or limitations among the genus.

4.2. METHODS

4.2.1 Sequence Collection

Cherax individuals were collected as per the large scale sampling method identified in Chapter 2. The same individuals sequenced for a combination of the seven genes (COI, 16S, 12S, 28S, GAPDH, H3 and ITS2) in Chapter 3 were also used in this study. In addition to these sequences, 317 sequences were attained from previous research on *Cherax* via Genbank (Table 4.1). The inclusion of these extra *Cherax* sequences allowed a more comprehensive analysis of Australian *Cherax*, with a majority of the Australian species sequenced for each gene. Due to the low availability of individuals and research on *Cherax* species from northern Australia and the Torres Strait, only sequences of *C. quadricarinatus* and *C. rhynchotus* could be included in this study. With the exclusion of *C. urospinosus* from SEQ, sequences of all *Cherax* species in southwest Australia and eastern Australia were included in the analysis.

Table 4.1: Previous molecular research on Australian *Cherax* and the corresponding sequences that was included in this study.

Source	COI	16S	12S	28S	GAPDH	H3	Total
Munasinghe *et al.* (2004a)		28 (AY191748-74)	27 (AY191720-46)				55
Munasinghe *et al.* (2003)	14 (AF493618-31)	10 (AF492801-10)	14 (AF492771-84)				38
Shull *et al.* (2005)	3 (DQ006292-4)	3 (DQ006550-5)	3 (DQ006421-3)				9
Toon *et al.* (2010)	5 (FJ965956-9, EU921142)	1 (EU921120)	1 (EU921113)	6 (FJ966009-12, EU921132-9)			13
Unpublished	7 (AF510181-7)	7 (AY153856, AY211980, AY153855-60)	1 (AY211981)				15
Nguyen *et al.* (2004)		31 (AY150034-7, AF500591-617)					31
Crandall *et al.* (1999)		9 (AF135970-8)					9
Schultz *et al.* (2007)		2 (EF493070-80)					2
Nguyen, Meewan*, et al.* (2002)		7 (AF395852-8)					7
Lawler and Crandall (1998)		3 (AF0442446-8)					3
Schultz *et al.* (2009)		2 (EU977342-3)			14 (EU977401-15)		16
Liu *et al.* (2011)		1 (JF284571)				2 (JF284590-9)	3
Baker *et al.* (2008)		16 (EU244878-93)					16
Porter *et al.* (2005)				1 (DQ079783)		1 (DQ079670)	2
Toon *et al.* (2009)				1 (EU920996)		1 (EU921048)	2
Gouws *et al.* (2010)	76 (HM641052-128)						76
Gouws *et al.* (2006)	17 (EF118807-23)						17
Adam D. Miller *et al.* (2004)	1 (NC011243)	1 (NC011243)	1 (NC011243)				3

4.2.2 Phylogenetic Construction

Due to the large dataset used in this chapter, phylogenetic construction was only performed using *BEAST (Heled & Drummond, 2010) within Beast, version 1.7.4 (A. Drummond & A. Rambaut, 2007; Drummond *et al.*, 2012). This method of phylogenetic construction combined the sequences from all seven genes for each species to construct a 'species' tree. This 'simplified' phylogeny integrated both nuclear and mtDNA as well as intraspecific variation within each species to construct a comprehensive tree that portrayed only inter-specific variation. The use of a species tree method is the most appropriate for biogeographic analysis of Australian *Cherax*, as the method provides a much better estimation of the current species topology. By taking advantage of the multispecies coalescent model, the species tree method treats loci as independent replicates of speciation, providing a better model for understanding historic speciation in the genus. For each of the seven gene regions, sequences were aligned using the Muscle algorithm (Edgar, 2004a, 2004b) with default settings within the software package Jalview (Waterhouse *et al.*, 2009). Each aligned gene region was implemented separately into Beauti v1.7.4 (Drummond *et al.*, 2012) to create the input files for later use within Beast. As a number of species were sequenced only for the mtDNA genes, analyses were performed on both the three mtDNA genes separately and a combined dataset of all seven genes. This comparison between a more representative phylogeny and an extrapolated phylogeny offers a comprehensive analysis of the evolutionary history of the genus. For both these analyses, the site and clock model for each gene region unlinked and mtDNA partition trees linked. This permitted the specific substitution model and corresponding prior estimates that were identified using jModeltest (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) to be implemented for each gene region. To estimate the divergence times between each of the *Cherax* taxa, the molecular rate of the three mtDNA genes were also included as per Chapter 3. Also similar to Chapter 3, the molecular rates for the four nuclear genes were estimated using an uncorrelated log-normal relaxed clock with an initial uniform prior rate implemented from previous analyses. As *Beast performs an interspecies comparison, a Yule-Species Tree model was used for phylogenetic tree construction, as recommended by A. Drummond (pers. comm.). All analyses were run for 10,000,000 iterations with parameters logged every 10,000 iterations. An appropriate burn-in was subsequently identified using Tracer v1.5 (Rambaut & Drummond, 2004) and implemented within the software package TreeAnnotator v.1.7.4 (Drummond *et al.*, 2012) to calculate the Maximum clade credibility tree. Bayesian trees with node ages were then created in Figtree v.1.4.0 (Rambaut, 2012).

4.2.3 Historical Biogeographic Analysis

To estimate the historic distribution and possible causes of speciation within Australian *Cherax*, a historic biogeographical analysis was implemented. Traditionally historical biogeographic analyses examine the current and historic distribution patterns of taxa to identify the processes that have shaped their distributions over time. These biogeographic processes can primarily be separated into one of six categories/events; extinctions (partial and complete), range expansions, vicariance and dispersal (partial and complete) (Futuyma, 1998). Speciation by vicariance is predominantly identified as allopatric speciation driven by the emergence of a barrier, whereas speciation by dispersal is caused by the colonisation of an organism across a pre-existing barrier (Platnick & Nelson, 1978). More recently, with the advent of molecular datasets and improved analytical methods in phylogenetics, historic biogeographic analyses have focused on utilising complex phylogenetic information to statistically classify speciation processes throughout a phylogeny. Biogeographic methods that utilise this phylogenetic information generally follow one of four basic models; diffusion, island, Hierarchical vicariance (HVM) or reticulate models (Ronquist & Sanmartín, 2011). While all four models can effectively estimate the biological processes that have produced the current species distribution, they each perform best under certain circumstances.

The diffusion model, unlike the other three models, simulates dispersal across a continuous landscape with movements typically following a random walk pattern (Brownian motion) (Lemmon & Lemmon, 2008). As the diffusion model focuses on organism movements within a continuous landscape, it's applicability is best for population level interpretations with accuracy typically limited by the sampling density both spatially and temporally (Knowles, 2009; Ronquist & Sanmartín, 2011). In contrast, the focus of the island model is on the dispersal of organisms between discrete defined areas. As suggested in the name, the typical application for this is to interpret dispersal events among islands. The model however, is similarly applicable to any landscape where discrete populations are separated by a barrier limiting dispersal (e.g., river catchments, mountain ranges and fragmented habitats). To estimate the dispersal of organisms, the island model treats distribution as an unordered character and finds the optimal relationship with the minimum number of character changes (Fitch, 1970). Although this implies the cost of moving between discrete areas is the same, a cost matrix can be implemented to accurately represent the cost of dispersal, carrying capacity and level of biotic exchange between areas (Bremer, 1995; Ronquist, 1994, 1995; Sanmartín *et al.*, 2008). As one of the most popular models in phylogenetic biogeography, the hierarchical vicariance model (HVM) describes the fragmentation of a contiguous ancient area by

successive fragmentation and vicariance. Each barrier to dispersal separates an ancestral area into two, with allopatric speciation or vicariance occurring across the break. Due to a number of cost optimisation limitations in the model, HVM can only consider four of the six biogeographic events mentioned previously, with complete extinction (organism completely disappears from an area) and complete dispersal (organism switches from on area to another) overlooked (Ronquist, 2003). Another major limitation to HVM is the assumption that ancestors were restricted to a single area, as widespread extant taxa are incompatible within HVM.

The last of the four models, reticulate models, identify the speciation events that have affected the evolution of a group and its current biogeographic distribution by analysing individual nodes using a weighted cost matrix (Page, 1994; Ree *et al.*, 2005; Ree & Smith, 2008; Ronquist, 1997; Ronquist & Nylin, 1990). By analysing each individual node they can estimate alternative cycles of dispersal (range expansion across a dispersal barrier) and vicariance (as in the HVM) through history. Unlike the HVM and island models, which assume that each lineage occupies a single area at any one point in time, reticulate models also allow the inclusion of widespread lineages. Currently the two most popular reticulate models are dispersal-extinction-cladogenesis (DEC) (Ree *et al.*, 2005) and dispersal-vicariance analysis (DIVA) (Ronquist, 1997). Both methods utilise a range expansion and contraction model but differ in how they analyse ancestral range across multiple areas (Ronquist & Sanmartín, 2011). The DEC analysis requires that between-area-vicariance events separate a single area from the remainder of the ancestral range (alloperipatric speciation) while DIVA analysis permits classical vicariance events in which each daughter occupies more than one area. Although DEC analyses are arguably the most realistic models for estimating historical biogeography, they are still in their infancy and poorly understood. Due to the high complexity of the DEC analyses the statistical power of the analyses is also a major concern. DIVA however, is a powerful alternative to DEC analyses (Ronquist, 1997). The method reconstructs the ancestral distribution of a phylogeny by optimising a three-dimensional cost matrix, in which colonisations and extinctions 'cost' more than vicariance and within-area speciation events (Lamm & Redelings, 2009; Ronquist, 1997). Unlike other model-based methods (Ree *et al.*, 2005; Ree & Smith, 2008; Sanmartín *et al.*, 2008), DIVA requires limited prior information and provides rapid results. This simplicity has made DIVA analyses popular when it is not feasible or desirable to take evidence from other organisms or geological history into account (Barber & Bellwood, 2005; Prieto-MÁRquez, 2010) or when the geological history does not conform to the HVM (Mansion *et al.*, 2008; Oberprieler, 2005; Sanmartín, 2003).

For the analysis and estimation of the historical biogeography of Australian *Cherax* in this study, the Bayesian DIVA model was implemented. This model was selected over the more complex DEC approach, as the evolution and biogeographic history of Australian *Cherax* is currently poorly understood. Although estimates of dispersal and vicariance events could be inferred from other Australian crayfish groups, these estimates would be considerably affected by the contrasting habitat preference and dispersal ability among Australian crayfish species (Johnston & Robson, 2009; Schultz *et al.*, 2009). Also as the history of the Australian river systems do not resemble the typical conditions for the island and HVM models, they were not investigated. All DIVA analyses in this study were conducted within the software package S-DIVA (Yu *et al.*, 2010) with default settings. This package was selected over the original DIVA analysis as it integrates the phylogenetic uncertainty of the species tree into its inference of biogeographic history. This is particularly important as phylogenetic uncertainty was detected for Australian *Cherax* (see previous chapters). For all analyses, the distribution of each of the species was specified based on thirteen discrete bio-regions (Figure 4.3). These bio-regions were selected based on thirty-one known freshwater biogeographic regions previously identified by Unmack (2001), with multiple bordering biogeographic regions inhabited by a single species grouped together (i.e. Central Australia, Northern Queensland and Central Queensland) . Due to the high species diversity and complexity in the South-East Queensland biogeographic region, analysis was performed with the region as a single group and separated into five additional regions. These additional SEQ biogeographic regions were identified based known biogeographic regions for a number of freshwater crayfish (Austin, 1996; Bentley *et al.*, 2010; Munasinghe *et al.*, 2004b; Riek, 1969). The current distribution of each of the species in this study was obtained from the crayfish project (McCormack, 2013), previous research (Austin, 1996; Austin & Knott, 1996; Munasinghe *et al.*, 2004a, 2004b; Riek, 1969) and sampling from this study. In this study, the estimated geographic distribution of each node was also constrained to a maximum of four localities (one more than the three major areas of high biodiversity). As this constraint can greatly affect the inferences from DIVA analyses, separate analyses were also conducted with an eight locality restriction and no restriction. Results from these analyses are not included however as they showed minimal difference from the default four locality constraint.

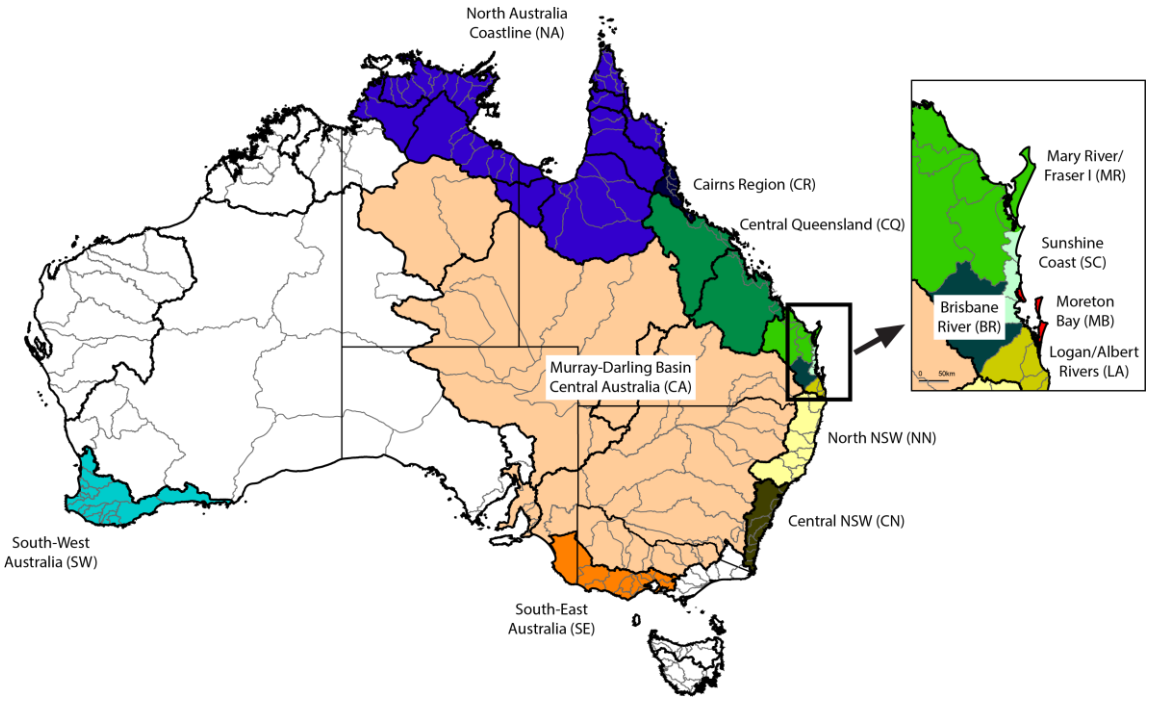

Figure 4.3: Australian biogeographic regions for *Cherax*.

4.3. RESULTS

4.3.1 Genetic Diversity

Of the 2010 sequences obtained in this study, 548 unique haplotypes were identified and included across the seven genes. Of these, a majority were for one of the three mtDNA genes (75%), with another 83% of these evenly distributed between the COI and 16S genes (Table 4.2). Possibly due to the high frequency of the three mtDNA genes, the mtDNA genes also showed considerably higher variation than their nuclear gene counterparts (Table 4.2). This was especially the case for the ribosomal genes with the 16S and 12S genes 42% and 34% variation compared to only 3% and 10% for the 28S and ITS genes respectively. Although the COI gene showed a relatively high level of variation (26%), only 1% of the base changes were identified as non-synonymous. This may indicate a high mutation rate and chance of back mutations with a low rate of fixed mutations. Surprisingly, a large number of gap/ambiguous sites were also observed for the three mtDNA genes. This was especially the case for the COI gene, with the 171 observed gap/ambiguous sites at a similar level to the 28S and ITS genes, which are both known to contain highly variable regions. These gap/ambiguous sites within the COI gene were however predominantly observed within sequences obtained from previous studies and as such could not be re-sequenced for accuracy. Similar to the previous chapter, a

large number of gap sites were observed in the highly variable portions of the ITS and 28S genes.

Table 4.2: Observed molecular diversity and chosen model of substitution for the seven genes used in this study

Gene	# Haplotypes	# bp	# Variable Sites	# Mutations	# Gap or Ambiguous Sites	Total Ambiguous Bases	Model of Substitution
COI	171	578	153 (26%)	257bp	171bp	140bp	GTR+I+G
16S	173	451	189 (42%)	264bp	92bp	10bp	TPM2uf+I+G
12S	69	311	105 (34%)	153bp	34bp	0bp	TrN+G
28S	24	894	29 (3%)	36bp	204bp	1bp	TrN+G
GAPDH	34	648	36 (6%)	38bp	10bp	16bp	TIM2ef+I
H3	39	296	70 (24%)	83bp	41bp	85bp	K80+G
ITS	38	605	60 (10%)	71bp	144bp	32bp	TPM2uf+G

4.3.2 Tree Topologies

For both the mtDNA and combined gene analyses, three highly supported (nodal bootstrap support 100) clades were observed that corresponded closely with geographic regions; south-west Australia, northern Australia and eastern/central Australia (Figure 4.3). Of these clades, the eastern/central Australia clade was identified as the most divergent, with a sister relationship observed between the south-western and northern Australia clades. Although strong nodal support was observed for the divergence of the eastern/central Australia clade (100), the support separating the remaining two clades was less conclusive (86 & 93) (Figure 4.4 & 4.6). Within the south-western Australia clade, two major species groups were identified for both phylogenetic analyses; *C. crassimanus*, *C. glaber* and *C. preissii* & *C. quinquecarinatus*, *C. tenuimanus* and *C. cainii*. Although the separation of these two groups does not clearly coincide with any geographic pattern, it was phylogenetically well supported with nodal support of 100 for both analyses.

Within the eastern Australia clade, strong nodal support (99-100) was observed for each of the species groups identified within the previous chapter. Support was considerably lower however when identifying the phylogenetic relationships among each of these species groups. This was most evident when comparing the two analyses, with contrary relationships and patterns observed. For the mtDNA analysis, a hierarchical like phylogenetic pattern was identified, with successive divergence of each species group (Figure 4.4). This analysis identified the *C. destructor* species group as the most divergent clade within the eastern Australia group followed sequentially by *C. depressus*, *C. robustus*, *C. cuspidatus* and *C. dispar*. This pattern was not well supported however as the divergence of the *C. depressus* and *C. robustus* species groups observed nodal support of only 47 and 41 respectively. Contrary to the mtDNA analysis, the combined gene analysis identified more of a mid-point phylogenetic pattern within the eastern Australia clade (Figure 4.6). While this pattern generally observed higher nodal support, the relationship among the species groups contrasts to those observed in the mtDNA analysis. Across both analyses, the only consistent relationship was the close affinity between the *C. cuspidatus* species group and *C. robustus*. While this relationship was not as simple in the mtDNA phylogeny, the combined gene analysis showed strong support for their close affinity (72). The most prominent difference between the two analyses however is the exchange of this group (*C. cuspidatus* and *C. robustus*) with the *C. destructor* group. For the mtDNA analysis, *C. cuspidatus* and *C. robustus* have a close affinity to *C. dispar* with *C. destructor* separate to all other eastern *Cherax*. In contrast the combined analysis identified a close affinity between *C. destructor* and *C. dispar*, with *C. cuspidatus* and *C. robustus* identified as the most divergent of the eastern *Cherax*. The close affinity between *C. destructor* and *C. dispar* was poorly supported though with the relationship observing the lowest support of the entire phylogeny (62).

4.3.3 Historical Biogeographic Distributions

Biogeographic analysis of the two phylogenies identified an Australia wide distribution as the most probable origin for *Cherax*, with taxa distributed across all three major biogeographic regions (south-west Australia, northern Australia and eastern Australia) (Figure 4.5 & 4.7). While this three locality distribution of the basal node was clear for the combined analysis (100%), a total of 30 possible distribution combinations were identified for the mtDNA analysis, with the three locality distribution the most probable (41%) (Figure 4.4). The divergence of the eastern *Cherax* from the other two regions was estimated to have occurred approximately 16mya, with the northern and south-western groups subsequently diverging approximately 13-12mya. Due to the variation in phylogenies between the mtDNA and combined analyses, the estimated biogeographic history for eastern *Cherax* was highly inconsistent between the analyses.

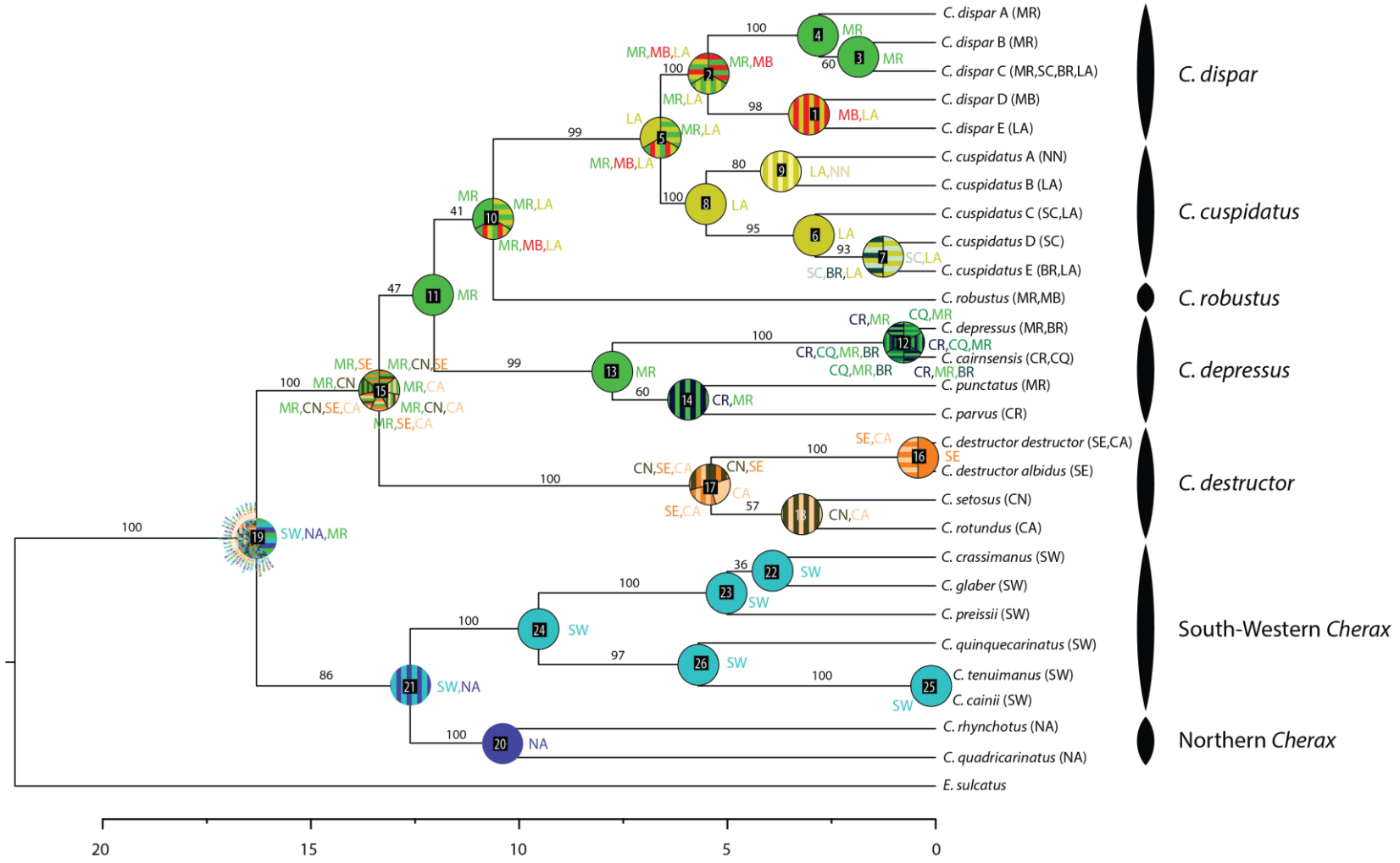

Figure 4.4: Bayesian *Beast consensus tree of all mtDNA genes with estimated node biogeographic distributions. Pie proportions represent the probability of the specific distribution with locality abbreviations as per Figure 4.3. Legend indicates million years before present.

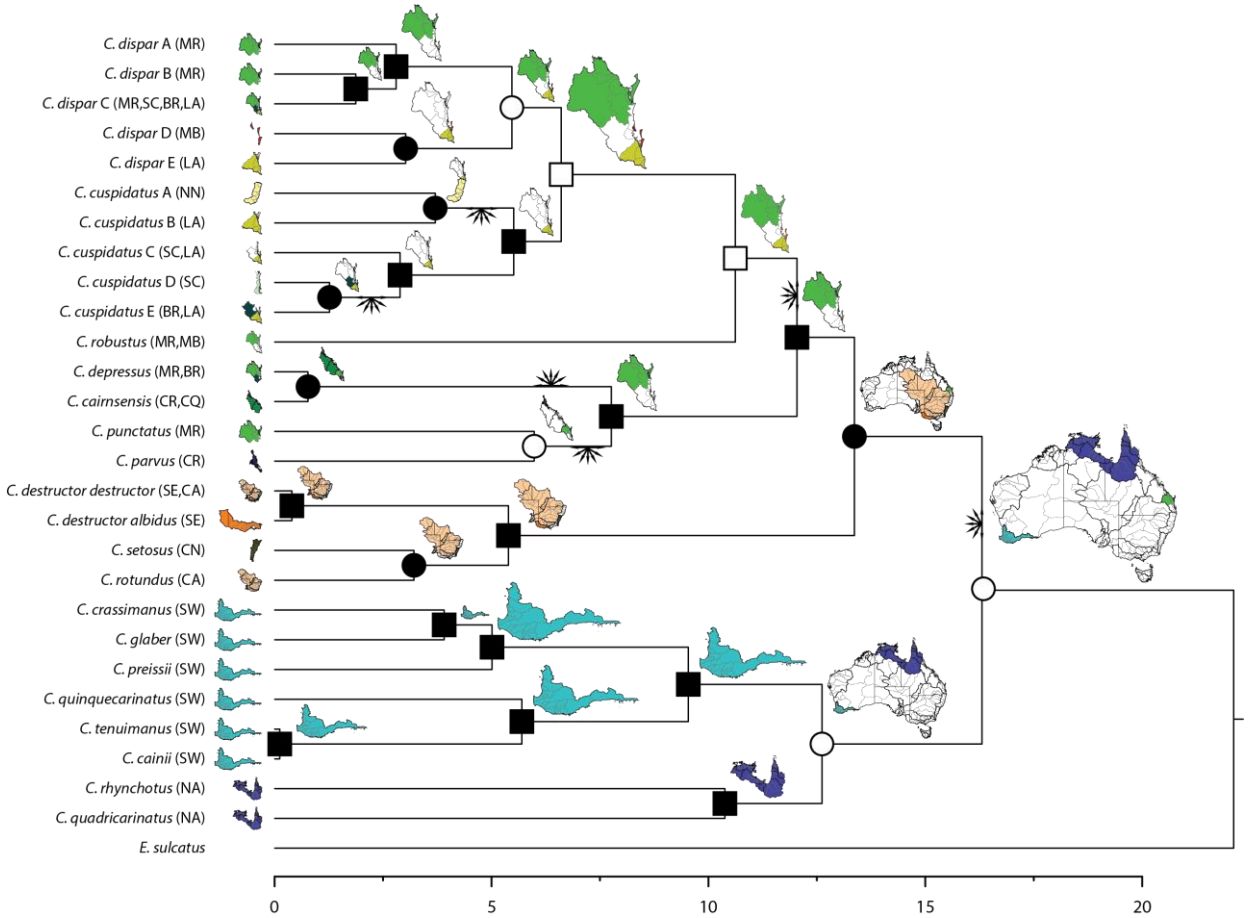

Figure 4.5: Summary of the optimal ancestral distribution reconstruction of Australian *Cherax* based on three mtDNA genes. At each node, the optimal distribution is indicated with its catchment shape (not to scale) and colour as per Figure 4.3. Symbols: ●: vicariance event, ■: duplication (sympatric speciation) event, ✳: dispersal event. Hollow nodes indicate possible extinction events, inferred because the subsequent vicariance event takes place between areas that are not geographically adjacent.

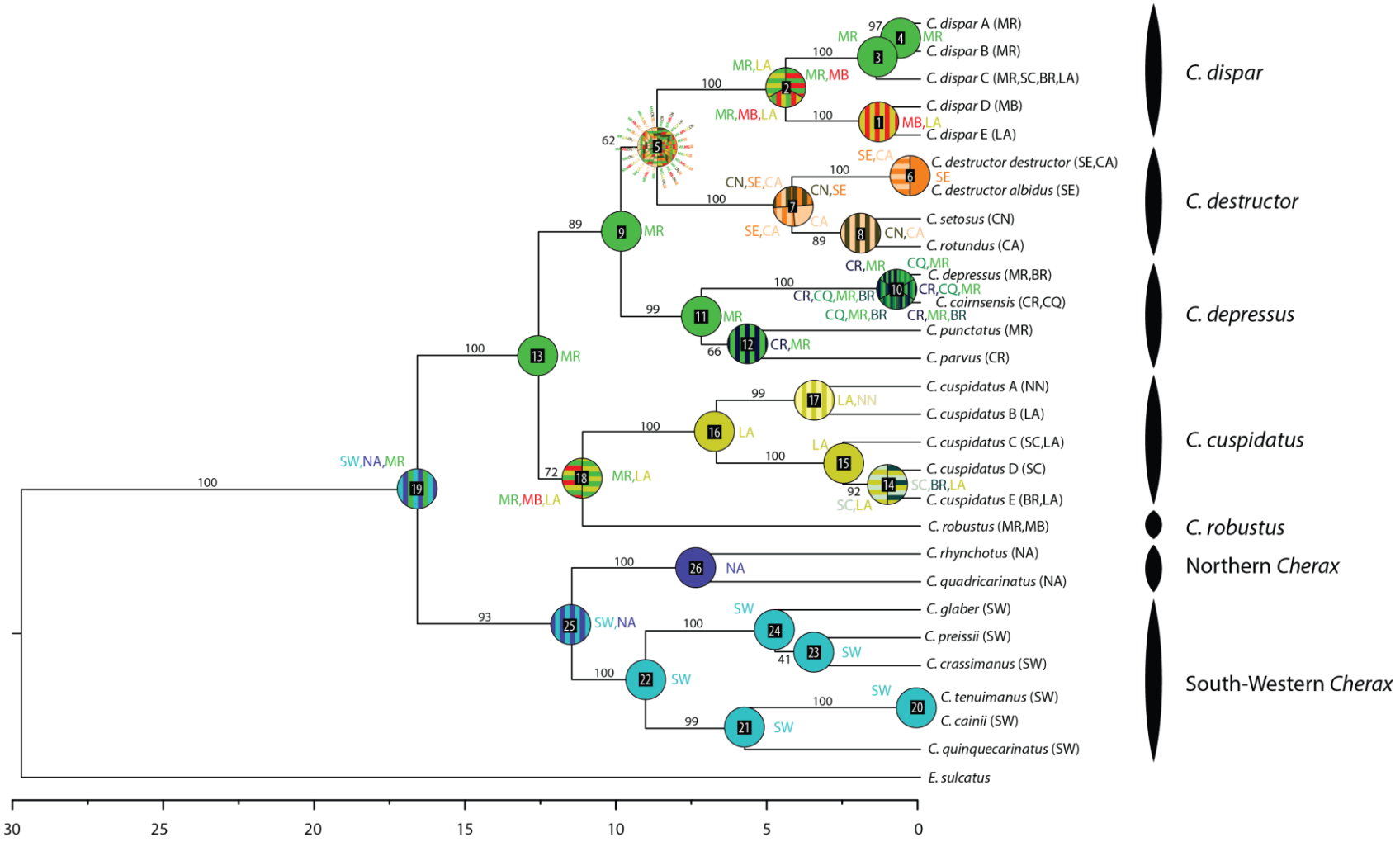

Figure 4.6: Bayesian *Beast consensus tree of all genes with estimated node biogeographic distributions. Pie proportions represent the probability of the specific distribution with locality abbreviations as per Figure 4.3. Legend indicates million years before present.

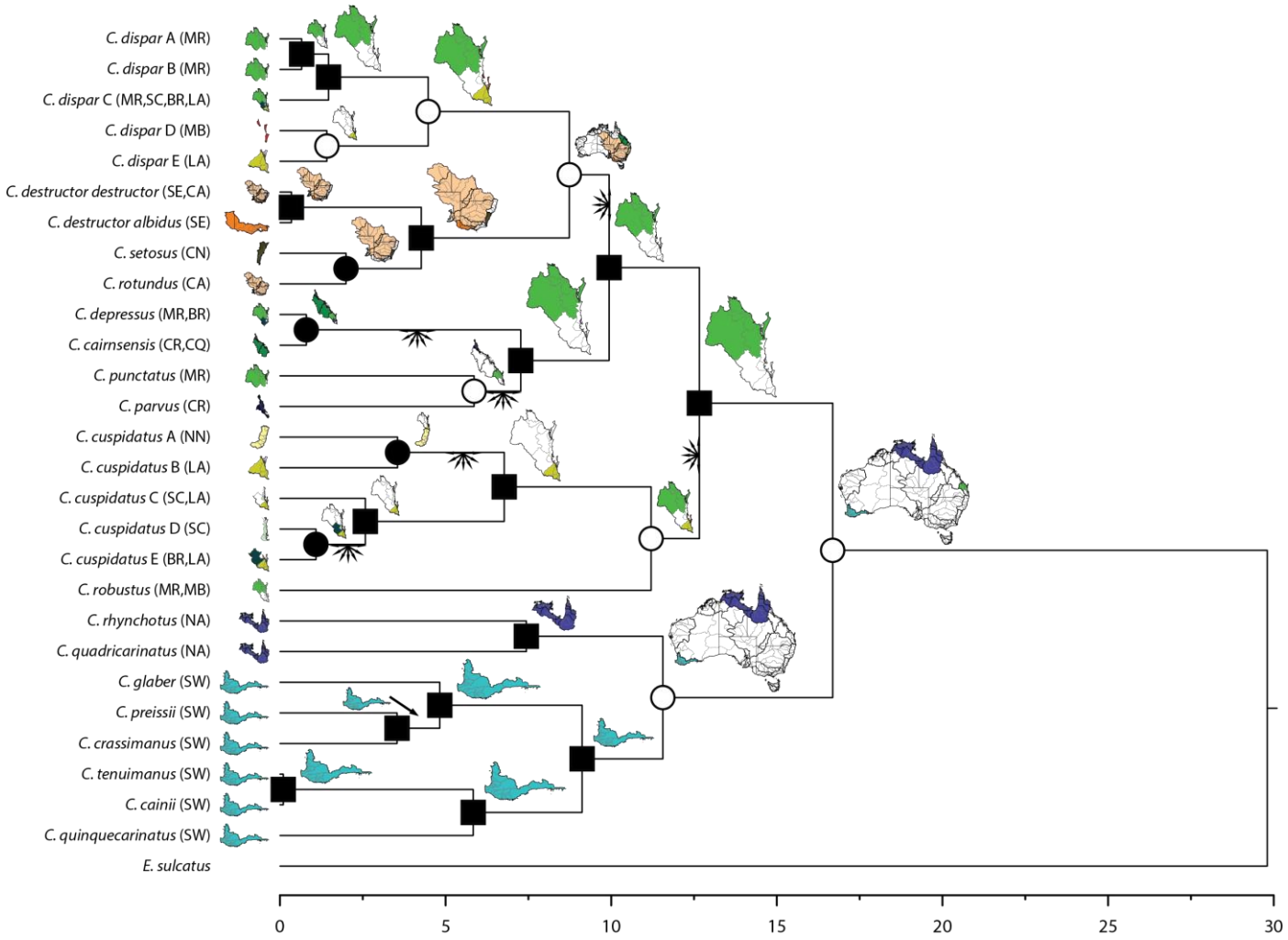

Figure 4.7: Summary of the optimal ancestral distribution reconstruction of Australian *Cherax* based on all seven genes. At each node, the optimal distribution is indicated with its catchment shape (not to scale) and colour as per Figure 4.3. All symbols are as per Figure 4.5.

For both analyses, the dominant biogeographic force influencing the historic distribution of eastern *Cherax* was dispersal or range expansion rather than vicariance. This was evident with a majority of the more basal nodes estimated to either include or only occur within the MR catchment (Figure 4.5). Prior to the speciation into each of the main species groups, the main difference between the two analyses was their estimated timing of dispersal across the Great Dividing Range (GDR). For the mtDNA analysis, this dispersal event from the MR into the Murray-Darling Basin (CA) was estimated to have occurred prior to 13mya (Figure 4.5) with subsequent vicariance either side of the GDR. The combined gene analysis however estimated the event far later (8mya) when *C. destructor* diverged from *C. dispar* (Figure 4.7). Excluding this dispersal event, all other movements between the species groups were limited to along the eastern coastline, predominantly in a southern direction into Moreton Bay (MB) and the Logan-Albert Rivers (LA). A connection between the MR and LA rivers was estimated to have occurred approximately 12-10mya, but with each analysis estimating contrasting biogeographic patterns post-divergence. While both analyses estimated the two river systems were still connected approximately 5mya, the mtDNA analysis estimated the connection was never lost (Figure 4.5).

When focused on each species group within eastern *Cherax*, both analyses estimated the same historical distributions and subsequent movements between regions. For all species groups except *C. destructor*, the origin of the group was estimated in either the MR or LA catchments or a combination of the two. For the *C. dispar* group, the group was estimated to have originated across both catchments, with a simple vicariance between the catchments observed. In contrast, within the *C. depressus* species group, a long distance range expansion from the MR catchment was estimated approximately 7mya. This range expansion was estimated to have reached approximately 1200km north to Cairns (CR) and south to the Brisbane River (BR). From this initial range expansion, two separate speciation events via vicariance were estimated along the Queensland coast, one separating *C. depressus* (MR) from *C. cairnsensis* (CQ) and another separating *C. punctatus* (MR) from *C. parvus* (CR). Although six possible distributions were estimated prior to the vicariance of *C. depressus* and *C. cairnsensis*, the most probable distribution was along the entire Queensland coast (CR, MR, CQ, BR), with the MR the most likely and BR least. Similar to the *C. depressus* species group, the *C. cuspidatus* group was also estimated to have undergone two separate speciation events while distributed within the LA (and partly Northern NSW (NN)). While subsequent vicariance approximately 6mya between NN and LA was the most probable cause of divergence between *C. cuspidatus* A & B, the biogeographic history of the remaining three lineages was not as clear.

The most probable biogeographic pattern for the three lineages was estimated to be via range expansion from LA into the Sunshine Coast (SC) region. This estimate was tentative at best however, with only a few individuals having ever been sampled for *C. cuspidatus* C & D, providing only limited information on their current true distribution. The last species group, *C. destructor*, was the only eastern species group not to speciate within South-East Queensland (SEQ). Within the group, two separate dispersal events across the GDR were estimated, one from Central Australia (CA) to South-East Australia (SE) and another from CA to Central NSW (CN). Because of the independent and recent (1mya) nature of these two events, the estimated shared distribution among the four *C. destructor* species was uncertain. Of the three localities estimated, CA and SE were the two most probable origins for *C. destructor*, although they were not estimated together exclusively.

4.4. DISCUSSION

4.4.1 Biogeographic History of Cherax in Australia

Phylogenetic analysis of Australian *Cherax*, estimated an Oligocene diversification from *Euastacus* approximately 30-25mya. This estimate, although consistent across both analyses, is significantly more recent than those estimated in previous studies, with an Eocene, Early-Cretaceous and Early-Jurassic divergence estimated by Schultz *et al.* (2009), Toon *et al.* (2010) and Bracken-Grissom *et al.* (2014) respectively. These discrepancies between previous studies coincide with the molecular clock approach used, with fossil calibrated divergence estimates (Bracken-Grissom *et al.*, 2014; Toon *et al.*, 2010) considerably pre-dating those based on mutation rates alone (Schultz *et al.*, 2009). Biogeographic analysis in this study more specifically also identified three areas of ancestral distribution for *Cherax,* in the south-west, north and east of Australia. While a specific river catchment was not identified for the origin of the south-west and northern populations, the Mary River and Fraser Island catchment (MR) was identified as the most probable origin for all eastern *Cherax*. This SEQ origin for eastern freshwater taxa was also identified by Unmack (2001), who identified SEQ as the shared distribution for a number of eastern freshwater fish. Due to the inconsistencies between the divergence estimates of a number of studies on *Cherax*, contrary theories have been proposed on the dispersal mechanisms that connected/maintained this wide distribution throughout Australia. The possibly more robust Jurassic/Cretaceous fossil calibrated estimates from Bracken-Grissom *et al.* (2014) and Toon *et al.* (2010) coincide with a warm and swampy Australian climate where a number of large rivers, lakes and swamps were spread across

Australia (Frakes, 1997; Grant-Mackie *et al.*, 2000). These large rivers may have facilitated the dispersal of freshwater crayfish throughout central Australia with subsequent vicariant divergence occurring during the mid-Cretaceous when Australia was inundated by large shallow seas (Frakes *et al.*, 1987). This inundation of Australia may also have driven vicariant divergence between the *Cherax* and *Engaeus* groups with post-inundation recolonisation and admixture possible. Although the more recent Eocene dispersal estimate from Schultz *et al.* (2009) was during a far cooler period, it also corresponds with a period of high saturation when swamp taxa were becoming increasingly prominent throughout inland Australia (Macphail *et al.*, 1994; Martin, 2006). During this period, the *Cherax* and *Engaeus* groups are estimated to have inhabited different niches, promoting morphological (Riek, 1969, 1972) and molecular (Horwitz & Adams, 2000; Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009) divergence between the two.

In the early-Miocene, 17mya, biogeographic analysis suggested a divergence between the eastern MR population of *Cherax* and the northern and south-western populations (Figure 4.6). This biogeographic divergence coincided with increased aridity within central Australia and the formation of the Nullarbor Plain, a major barrier to dispersal for Australian *Cherax* (Benbow, 1990; Martin, 2006). This restricted dispersal across central Australia has also been observed in a number of taxa, including birds (Toon *et al.*, 2003), fish (Unmack, 2001), freshwater crayfish (Horwitz & Adams, 2000; Munasinghe *et al.*, 2004a; Riek, 1969; Schultz *et al.*, 2009) and plants (Hopper, 1979; Hopper & Gioia, 2004), with the last freshwater migration across the region estimated to have occurred by the late-Miocene (Benbow, 1990; Unmack, 2001). Although the early-Miocene divergence estimate in this study coincides with the formation of the Nullarbor Plain and estimates from a number of other freshwater taxa studies (Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009; Unmack, 2001), it is significantly later than Toon *et al.* (2010)'s Cretaceous estimate using a fossil calibrated molecular clock. The Miocene divergence estimated from this study does however concur with other mutation rate calibrated phylogenetic studies on *Cherax* (Austin & Knott, 1996; Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009) and the divergence between the freshwater crayfish genera *Engaeus* (South-East Australia) and *Engaewa* (South-West Australia) (Schultz *et al.*, 2009). During this same period, gene flow was also estimated to have ceased between the northern and south-western *Cherax* populations (Figure 4.6), coinciding with the spread of the Great Sandy Desert throughout central Australia and the coastal regions of Western Australia. Due to the low number of northern *Cherax* samples, the identification of the origin and possible dispersal route between the northern and south-western populations is however still unknown. This

very poor northern *Cherax* sample size may also be the cause for disparities observed among *Cherax* phylogenetic studies. While Schultz *et al.* (2009) similarly observed an initial divergence of the eastern lineage, Toon *et al.* (2010) and Munasinghe *et al.* (2004a) instead suggested that the northern or western lineages initially diverged from the other *Cherax*.

Contrary to the estimated biogeographic distribution of the most recent *Cherax* ancestor, the biogeographic distribution of the eastern lineage was inconsistent across the two analyses. For the combined analysis, the eastern lineage of *Cherax* was estimated to have remained within South-East Queensland (SEQ) until 7.5mya. This differs to the mtDNA analysis which estimated a dispersal event approximately 12.5mya across the Great Dividing Range (GDR) into Central Australia (CA) and along the eastern coastline from South-East Australia (SE) to the MR catchment. Although both analyses estimated this dispersal event across the GDR, the combined gene analysis estimated the occurrence far later (7.5mya). Interestingly, this later estimate was also observed by previous *Cherax* studies based only on mtDNA genes (Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009). This inconsistency between the results of this study and previous research using only mtDNA indicates possible incomplete lineage sorting for the mtDNA genes, with the more recent dispersal across the GDR the more probable estimate. When this dispersal across the GDR is overlooked, a majority of the more basal nodes were estimated to have remained within the MR, with dispersal only occurring into the Moreton Bay (MB) and Logan-Albert (LA) catchments approximately 10mya. The combined gene analysis estimates that this range expansion event occurred on two separate occasions, once when the *C. robustus* and *C. cuspidatus* species groups speciated and another when *C. dispar* diverged from *C. destructor* (Figure 4.7). The mtDNA analysis however estimated a single range expansion event, with the *C. dispar*, *C. cuspidatus* and *C. robustus* common ancestors remaining distributed throughout the catchments until relatively recently (Figure 4.4). This Late-Miocene connection between the LA and MR catchments was also observed for the forest-restricted frogs, *Litoria pearsoniana* (McGuigan *et al.*, 1998), suggesting the climate may have been both cooler and wetter during this period.

4.4.2 Divergence of Eastern Cherax Species Groups

4.4.2.1 Cherax dispar complex

Biogeographic analysis of the *C. dispar* species group identified three equally likely origin combinations for the group. These combinations include the Mary River catchment (MR) and one or both of the Moreton Bay (MB) and Logan-Albert catchments (LA). Although both analyses specifically suggested these three catchments as the original distribution for the group, the *C. dispar* species group would have also had to disperse through either the Brisbane (BR) and/or Sunshine Coast (SC) regions to reach either MB or LA (Figure 4.7). Through vicariance, the original three catchment distribution subsequently diverged into north/south geographical regions, with the MR (*C. dispar* A, B & C) lineages separating from the LA and MB (*C. dispar* D & E) lineages both geographically and phylogenetically. This north/south separation within SEQ was also observed for a number of freshwater (Hughes *et al.*, 1999; Murphy & Austin, 2004; Page & Hughes, 2007a; Wong *et al.*, 2004; Woolschot *et al.*, 1999) and terrestrial taxa (McGuigan *et al.*, 1998). More specifically, the Miocene separation between these two regions coincides with a warm change in SEQ conditions compared to the Murray Basin, with palynofloras in SEQ predominately Araucariaceae (Dettmann & Clifford, 2003; Macphail *et al.*, 1994). This warming of SEQ in the Miocene was also estimated to have affected the dispersal of freshwater shrimp (Page & Hughes, 2007a), fish (Page *et al.*, 2004) and frogs (McGuigan *et al.*, 1998).

As both lineages of the southern group of *C. dispar* are currently endemic to separate regions in SEQ and distributed either side of MB, speciation was estimated to have occurred allopatrically with increasing sea level in the Late-Pliocene. This Late-Pliocene divergence however pre-dates both the last glacial maximum (30,000 years ago) (Lambeck & Chappell, 2001) and the actual age of the Moreton Bay islands (North Stradbroke Island: 150, 000 years ago (Pickett *et al.*, 1985; Tejan-Kella *et al.*, 1990)), suggesting the formation of the islands may not be the cause of speciation between the lineages. Instead, speciation may have occurred within the SEQ mainland prior to the formation of the islands with subsequent dispersal of a single lineage during low sea level. This pattern was also observed at a much smaller geographical scale by Page and Hughes (2007a), who identified a Miocene/Pliocene divergence between cryptic species on the east and west of a single island (North Stradbroke Island) within MB.

As all three northern lineages of *C. dispar* currently still reside within the MR, speciation could have occurred allopatrically (with subsequent re-colonisation) or sympatrically by inhabiting differing ecological niches or micro habitats (Johnston & Robson, 2009; Pfenninger *et al.*, 2003; Wellborn & Cothran, 2004). Small scale separation of freshwater crayfish has been observed by Johnston and Robson (2009), who identified specific habitat preferences for five sympatric freshwater crayfish species. As the *C. dispar* B lineage is predominately distributed close to the coastline in TCB, the two lineages (*C. dispar* A & B) may have also once been separated allopatrically, with the TCB coastal system only recently connecting to the MR catchment. This separation between TCB and the MR was also observed for freshwater fish (Page *et al.*, 2004) shrimp (Page & Hughes, 2007a; Sharma & Hughes, 2009) and crayfish (Bentley *et al.*, 2010). Of the northern *C. dispar* lineages, *C. dispar* C observed the most surprising biogeographic pattern, with a major range expansion down the entire SEQ coastline approximately 2mya. As the lineage still resides within the MR, three possible explanations could explain the current distribution. The first possibility suggests sympatric speciation within the MR with a recent dispersal down the SEQ coastline. The other two possible explanations estimate a more ancient dispersal from the MR with either subsequent recent re-colonisation back into the MR or continued gene flow between the catchments. This SEQ wide distribution is common in a number of freshwater taxa (Baker, Sheldon*, et al.*, 2004; Chenoweth & Hughes, 2003; Murphy & Austin, 2004; Page & Hughes, 2007a, 2007b; Page *et al.*, 2004; Sharma, 2006; Unmack, 2001), with some studies estimating a historic river paleo-drainage parallel to the SEQ coastline as the probable connection between river catchments (Hughes *et al.*, 1999; Page & Hughes, 2007a). A majority of these studies however, observe a significant divergence between the MR and southern river catchments with mainly diadromous fish observing similarities between regions (Cook *et al.*, 2012). As *C. dispar* is an obligate freshwater species, a likeness to diadromous fish is unexpected. Since *C. dispar* C was only ever observed at one location within the MR catchment, a more likely explanation is recent colonisation into the MR via avian (Charalambidou *et al.*, 2005; Proctor, 1964) or anthropogenic sources (Reynolds & Souty-Grosset, 2012; Stefani *et al.*, 2011). To determine the likely cause of this biogeographic pattern, a further fine-scale study would be needed.

4.4.2.2 Cherax robustus and C. cuspidatus complex

Since the first *Cherax* dispersal event south out of the MR 12mya, *C. robustus* has sustained its divergence from other eastern *Cherax* species. Although *C. robustus* has a wide distribution throughout SEQ (MR, MB and SC (Queensland Museum pers. comm.), it showed minimal intraspecific and high interspecific diversity. This deep divergence with low intraspecific diversity may either be due to its rarity (possible bottleneck) (*Wildlife of Greater Brisbane*, 2007)or recent dispersal after prolonged divergence. As a species that inhabits low-land coastal swamps (*Wildlife of Greater Brisbane*, 2007), *C. robustus* is thought to be able to tolerate low levels of salinity (*Wildlife of Greater Brisbane*, 2007), signifying that either possibility is conceivable.

Approximately 5my after the dispersal and divergence of *C. robustus* from the MR, *C. cuspidatus* was also estimated to have diverged from other eastern *Cherax* within LA. Although the two analyses estimated divergence from different species groups (mtDNA; *C. dispar*, combined; *C. robustus*), they both estimated a MR, LA and MB distribution for their common ancestor. Within the *C. cuspidatus* complex, two lineages were estimated to have dispersed along the eastern coast in opposing directions. A similar biogeographic history to *C. dispar* C was observed for one lineage (*C. cuspidatus* C, D & E) with high connectivity estimated in the north across the BR, SC and LA catchments. The uncertainty observed when estimating the historic biogeography of this northern lineage may be a result of poor sampling, with only a few individuals ever caught for *C. cuspidatus* C & D. This low sample size would have a large effect on the historic biogeographic estimates, as the total distribution for the clades are essentially unknown. The high divergence and overlapping distributions observed among the three northern lineages suggests possible intermittent vicariant speciation and recolonisation as a result of changing sea levels (Horwitz, 1988; Miller *et al.*, 2005). During these intermittent sea level changes 4mya, the other lineage of *C. cuspidatus* (*C. cuspidatus* A & B) was also estimated to have dispersed south across the McPherson Range into Northern NSW (NN), with subsequent vicariant speciation. This biogeographic break has been observed in a number of other freshwater (Munasinghe *et al.*, 2004b; Page & Hughes, 2007a; Unmack, 2001) and terrestrial taxa (D. Chapple *et al.*, 2011; D. G. Chapple *et al.*, 2011; James & Moritz, 2000; Keogh *et al.*, 2003), and represents a strong barrier for dispersal for all non-diadromous aquatic taxa. The McPherson Range is a particularly strong biogeographic barrier as it contains both a steep ocean floor (Veevers *et al.*, 1991) and high coastal mountain ranges (Australia, 2013). Although the last known volcanism and major drainage change in SEQ occurred 22.6-27.2mya (Johnson *et al.*, 1989), connectivity across the McPherson range was estimated to

have occurred prior to the Pliocene for frogs (James & Moritz, 2000; McGuigan *et al.*, 1998), snakes (Keogh *et al.*, 2003) and skinks (D. Chapple *et al.*, 2011; D. G. Chapple *et al.*, 2011). While the McPherson range may present a major barrier for freshwater taxa, dispersal across the range during this period may have been common for less aquatically restricted taxa, such as plants (Burke *et al.*, 2013) and Sugar-gliders (Malekian *et al.*, 2010).

4.4.2.3 Cherax depressus complex

Both biogeographic analyses of the *C. depressus* species group identified the MR as the most probably origin for the entire complex. From within the MR, approximately 7.5mya, *C. depressus* was estimated to have dispersed as far north as Cairns (CR), with a shared distribution between the two localities estimated for the *C. punctatus* and *C. parvus* most recent common ancestor (Figure 4.7). As the two regions are approximately 1200km apart, vicariance approximately 6mya was the most probable cause of speciation between the highly divergent species. This strong biogeographic break between the CR and MR regions coincides with a number of other freshwater (McGlashan & Hughes, 2002; Unmack, 2001; Wong *et al.*, 2004) and terrestrial (Brown *et al.*, 2006; D. Chapple *et al.*, 2011; D. G. Chapple *et al.*, 2011; James & Moritz, 2000) taxa and may correspond with the Burdekin Gap, a dry corridor that dates back to before the Pliocene (Joseph & Moritz, 1994; Joseph *et al.*, 1993). The strong influence of the Burdekin Gap as a biogeographical barrier to dispersal is not predominantly limited to freshwater taxa however with large divergences observed in birds (Joseph & Moritz, 1994; Joseph *et al.*, 1993; Nicholls & Austin, 2005), mammals (Brown *et al.*, 2006; Pope *et al.*, 2001) and skinks (D. Chapple *et al.*, 2011; D. G. Chapple *et al.*, 2011). Surprisingly, unlike a number of these taxa, no remnant intra-specific variation was observed along the Queensland coast, with a disjunct distribution observed between MR and CR. This lack of intra-specific diversity among the two species may be the result of either poor sampling or mass extinction of related taxa across Queensland. Although the biogeographic history of the two species is unclear in this study, more comprehensive sampling along the entire Queensland coast would identify the most probable biogeographic history. A similar split distribution to *C. punctatus* and *C. parvus* was however also observed within a single clade of closely related Melanotaeniid rainbow fish, with *Melanotaenia eachamensis* and *M. duboulayi*, located in NQ and SEQ respectively (McGuigan *et al.*, 2000).

Although the *C. depressus* and *C. cairnsensis* phylogenetic lineage is also distributed along the Queensland coastline, the biogeographic history for the group was not as conclusive. Contrary to the *C. punctatus* and *C. parvus* divergence, *C. cairnsensis* and *C. depressus* were estimated to have diverged during the Pleistocene only a million years ago. Prior to this divergence, biogeographic analysis identified six evenly possible distribution combinations across four localities. Of the four localities, the three most commonly estimated were the CR, Central Queensland (CQ) and MR, with a distribution south into the Brisbane River (BR) less likely. Although biogeographic analysis identified this distribution approximately a million years ago, the actual Queensland wide dispersal event may have occurred during the same period as the *C. punctatus* and *C. parvus* lineage 6mya. If this was the case, gene flow throughout Queensland would have been maintained for approximately 5mya. Although continued gene flow across the entire Queensland coastline for 5my seems improbable for obligate freshwater crayfish, high connectivity across the region has been observed in a number of freshwater fish (McGuigan *et al.*, 2000; Unmack, 2001) and crustaceans (Murphy & Austin, 2004). If however the dispersal of the lineage north out of the MR was a separate event, it most probably occurred approximately 1.5mya, coinciding with a glacial maximum (Lambeck & Chappell, 2001). Surprisingly, the observed subsequent allopatric speciation and biogeographic break between *C. cairnsensis* in NQ and CQ and *C. depressus* in MR and BR is analogous to amphidromous crustaceans (Cook *et al.*, 2012) and saltwater taxa (Chenoweth *et al.*, 2002; Haig *et al.*, 2010) rather than obligate freshwater species (Unmack, 2001; Wong *et al.*, 2004). For saltwater taxa, this break coincides with the enlargement of the Queensland continental shelf, which has predominantly had an effect during the tumultuous sea-level changes of the Pleistocene (Haig *et al.*, 2010).The most prominent Queensland biogeographic break for both freshwater and terrestrial taxa is rather the Burdekin Gap in NQ (Brown *et al.*, 2006; Pusey *et al.*, 1998; Unmack, 2001), a break not observed for *C. cairnsensis*. However as no *C. cairnsensis* samples north of the Burdekin Gap were included in this study, the overall intraspecific effect from the gap for *C. cairnsensis* is unclear.

4.4.2.4 Cherax destructor complex

In addition to the largest distribution of all *Cherax* species, the *Cherax destructor* complex also showed the most variable and indefinite biogeographic estimates for its divergence from other *Cherax*. Unlike all other eastern *Cherax*, the *C. destructor* complex is also the only *Cherax* species that occurs west of the GDR. Since its divergence from either all other *Cherax* (mtDNA analysis) or the *C. dispar* complex (combined analysis), biogeographic analysis identified four

almost equally possible distributions for the group across three separate localities (Central Australia (CA), Central NSW (CN) and South-East Australia (SE)). Of the four possibilities, a shared distribution across all three localities was estimated as the most probable origin for the group, with CA the most common. This CA origin for the *C. destructor* complex indicates a major east to west dispersal event from the MR catchment across the GDR approximately 13-8mya. This connection between SEQ and the MDB was also observed for a number of freshwater fish (McGlashan & Hughes, 2001a; McGuigan *et al.*, 2000; Musyl & Keenan, 1996; Unmack, 2001), turtles (Baggiano, 2012) and frogs (McGuigan *et al.*, 1998), with species distributed on both sides of the range. Unlike these freshwater taxa, the GDR in SEQ remained a prominent biogeographic barrier for *Cherax*, with subsequent Miocene divergence for taxa either side of the range. Divergence estimates for freshwater fish, turtles and frogs however suggest that dispersal across the range continued until relatively recently (Baggiano, 2012; McGlashan & Hughes, 2001a; McGuigan *et al.*, 1998). Although *Cherax* are also freshwater taxa, this contrary biogeographic pattern may indicate a lowland/warmer climate preference for *C. destructor* compared to other freshwater taxa, with drainage connection possibly via upland streams (McGuigan *et al.*, 1998). Surprisingly, since the dispersal of *C. destructor* across the GDR, both divergent lineages were estimated to have remained sympatric within the Murray-Darling Basin in CA. Speciation may still have occurred via geographic vicariance for *C. rotundus* however, with the species currently isolated to the Barmah Forest of the Murray River (Munasinghe *et al.*, 2004a, 2004b).

Since its divergence and dispersal from SEQ, two subsequent major dispersal events have also been estimated for the *C. destructor* complex. The first of which was estimated to have occurred from west to east across the GDR in the Hunter River region during the Pliocene, 2.5 Mya. This dispersal between the Murray-Darling Basin (CA) and CN via the north-east of the Hunter River is recognised as a dispersal corridor between the catchments for freshwater crayfish (Austin *et al.*, 2003; Munasinghe *et al.*, 2004a, 2004b; Schultz *et al.*, 2009; Schultz *et al.*, 2007), fish (Jerry, 2005; Jerry, 2008; Jerry & Woodland, 1997; Adam D Miller *et al.*, 2004; Thacker *et al.*, 2007; Unmack, 2001) and shrimp (McClusky, 2007). Freshwater connectivity across this region of the GDR is estimated to have occurred by either river capture (Haworth & Ollier, 1992; Ollier & Pain, 1994) or recent/ongoing dispersal (Unmack, 2001) in areas where elevation is relatively low (McGlashan & Hughes, 2001a; Thacker *et al.*, 2007). Similar to the SEQ region of the GDR, freshwater fish dispersal across this region of the GDR was estimated to have continued until relatively recently, compared to the Pliocene divergence estimates of *Cherax* (Munasinghe *et al.*, 2004a, 2004b) and *Gramastacus* (Schultz *et al.*, 2009). Through

vicariance, this 2.5my biogeographic barrier to dispersal for freshwater crayfish promoted speciation with *C. setosus* and *C. rotundus* distributed on the east and west side of the GDR respectively.

The second major dispersal event by *C. destructor* was estimated to have occurred from north to south in South-East Australia (SE). Dispersal within this region was estimated to have occurred from the Wimmera River region of CA across the Grampians into SE (Schultz *et al.*, 2009; Schultz *et al.*, 2007) approximately one million years ago. This recent connection between CA and SE was also observed for other freshwater crayfish (Crandall *et al.*, 1999; Munasinghe *et al.*, 2004a, 2004b; Nguyen *et al.*, 2004; Schultz *et al.*, 2009) and fish (Adam D Miller *et al.*, 2004). Although gene flow between the two regions is estimated to have ceased for *C. destructor*, it is thought that there has not been adequate time for speciation to occur (Munasinghe *et al.*, 2004a, 2004b). Speciation may however be in progress with the sub-species *C. destructor albidus* currently isolated to the SE region and deviating from the CA *C. destructor*.

4.4.3 Cherax Dispersal Mechanisms

The widespread distribution of freshwater species throughout geographically isolated river systems implies historical connectivity between the regions. The degree of connectivity between these regions is generally determined by the life history of the taxa and geographical history of the region (Hughes *et al.*, 2009). For obligate freshwater taxa, dispersal between river systems is typically rare and explained either via historical river connections (Unmack, 2001) or by third party translocation (bird or human) (Charalambidou *et al.*, 2005; Gittenberger *et al.*, 2006; Proctor, 1964). Although dispersal across rivers can occur during times of low sea level and/or high volcanism, the low frequency of these periods produce patterns of high inter-specific diversity between each river catchment (Unmack, 2001; Van Der Beek *et al.*, 1999). Characteristically, obligate freshwater species therefore tend to have small isolated distributions (Hughes *et al.*, 2009; Unmack, 2001). In contrast, freshwater species with varying forms of diadromy (migration between freshwater and estuarine/marine habitats) have the capability of among-river dispersal over a range of spatial scales (Chubb *et al.*, 1998; McDowall, 2004; Page *et al.*, 2005; Waters *et al.*, 2001). For example, catadromy (migration of freshwater species to saltwater for reproduction) often produces patterns of isolation by distance among coastal rivers (Stephen F. Chenoweth *et al.*, 1998; Jerry & Baverstock, 1998). Freshwater taxa with a diadromous life history stage generally exhibit widespread distributions

with less genetic diversity (Chubb *et al.*, 1998; Cook *et al.*, 2006; Page, von Rintelen*, et al.*, 2007a). Additionally, variations of these typical patterns are frequently observed when historic transitions between diadromous and obligate freshwater life histories occur (Cook *et al.*, 2006; Goto & Arai, 2003; Lee & Bell, 1999; Page, von Rintelen*, et al.*, 2007a; Taylor *et al.*, 1996; Taylor & McPhail, 1999; Waters & Wallis, 2001). For some freshwater species, such as frogs, crabs and crayfish, terrestrial dispersal across river catchment boundaries is also possible during humid conditions (Daniels *et al.*, 2006; Fitzpatrick *et al.*, 2009; Hughes & Hillyer, 2003; O'Brien, 2007). Although this may not be a common occurrence for freshwater crayfish, it can potentially explain gene flow between adjacent river catchments when historical river connection is improbable (Hughes & Hillyer, 2003).

Biogeographic analysis in this study identified a wide range of life history traits among Australian *Cherax* species. As obligate freshwater taxa, each *Cherax* was expected to inhabit relatively small distributions with high genetic drift among river catchments. This high endemicity and interspecific diversity was predominantly observed among south-western Australian and SEQ *Cherax*, with most species in these regions highly endemic. Within south-west Australia, all six species were endemic to a small isolated coastal region of Western Australia (Austin & Knott, 1996). This isolated geographic distribution indicates a long term inability for the species to disperse out of the region via either long distance oceanic dispersal (Unmack, 2001) or among adjacent river catchments (Hughes *et al.*, 2009). Interestingly, all six species within this region also showed sympatric distributions, overlapping across the same river catchment (Austin & Knott, 1996). Although this sympatric distribution among south-western *Cherax* can also be indicative of a diadromous species, connectivity among the river catchments was instead suggested to have occurred during low sea level when rivers were connected (Gouws *et al.*, 2006; Unmack, 2001). Through intermittent allopatric speciation and recolonisation this fluctuating connectivity during glacial maxima may also have driven the observed high interspecific diversity and sympatric distributions among the species. Gouws *et al.* (2006) also suggested the high vagility and desiccation tolerance of *Cherax preissii* indicated terrestrial connectivity during humid conditions as the most probable explanation for contemporary dispersal throughout the region. This terrestrial connectivity among river catchments may also explain the similarly wide distributions of *C. crassimanus* and *C. quinquecarinatus*. In contrast, the remaining three species (*C. glaber*, *C. tenuimanus* and *C. cainii*) are restricted to the same three adjacent rivers (Austin & Knott, 1996; Austin & Ryan, 2002), a biogeographic pattern characteristic of obligate freshwater species with limited dispersal ability both terrestrially and oceanically (Avise, 2000; Hughes *et al.*, 2009).

Unlike south-west Australia, *Cherax* from Eastern Australia had a wide range of biogeographic patterns and life history traits. Although *C. cuspidatus* and *C. dispar* are sympatric for a large portion of their distributions, they both appear to be restricted by contrary biogeographic breaks. In areas where the two species are sympatric (Brisbane River, Logan-Albert Rivers and Sunshine Coast) dispersal was estimated to have occurred via river connectivity during low sea levels (Page & Hughes, 2007a; Page *et al.*, 2004). This contemporary connectivity among the three catchments was also observed for other obligate freshwater taxa such as Oxleyan Pigmy Perch (Hughes *et al.*, 1999) and shrimp (Chenoweth & Hughes, 2003; Woolschot *et al.*, 1999), suggesting dispersal among the catchments may have been relatively easy for freshwater species. Outside of this sympatric zone, *C. dispar* was also estimated to have dispersed across the Mary River/Brisbane River break and onto all four coastal sand islands, whereas *C. cuspidatus* was restricted to mainland Australia, with dispersal only across the McPherson Range. Although both species are sympatric, this contrasting biogeographic history may indicate contrary life histories for the two species. As *C. dispar* is distributed on all four coastal sand islands and along the coastal regions of the mainland, the species may tolerate varying levels of salinity. This salinity tolerant life history for *C. dispar* may suggest that dispersal across the Mary River/Brisbane River break required some form of oceanic dispersal. This required salinity tolerance was further evident as *Caridina* (Page & Hughes, 2007b) and *Paratya* (Cook *et al.*, 2006) shrimp, two genera with estuary inhabiting ancestors, also showed connectivity across the break (Cook *et al.*, 2006; Page & Hughes, 2007a, 2007b). Although no estuary inhabiting *Cherax* relatives are known, *C. dispar* has been observed in shallow creeks within 100m of the coastline (Bentley Pers. Comm.). This biogeographic history across the break was also observed for *C. robustus*, another coastal *Cherax* distributed on all four sand islands. Although a comparison across the break is not possible when no *C. robustus* individuals were observed on the mainland south of the break, Queensland Museum records indicate the species was once distributed down the entire SEQ coastline. This euryhaline life history may however be limited for both *C. dispar* and *C. robustus*, with the two species only distributed as far north as the Mary River and south as the McPherson Range (Bentley *et al.*, 2010; Garvie).

In contrast to *C. dispar* and *C. robustus*, dispersal across the Mary River/Brisbane River break was not observed for *C. cuspidatus*. Instead dispersal was observed south across the McPherson Range, indicating *C. cuspidatus* may exhibit a contrasting life history. As the McPherson Range coincides with a steep ocean sea floor and is consistently humid, dispersal south across the range may be limited oceanically with dispersal terrestrially more probable

(James & Moritz, 2000; Keogh *et al.*, 2003). Although dispersal across the range has ceased for the past four million years, a similar level of divergence across the region was also observed for terrestrial taxa such as frogs (James & Moritz, 2000) and skinks (D. Chapple *et al.*, 2011; D. G. Chapple *et al.*, 2011). This similar biogeographic history to terrestrial species suggests *C. cuspidatus* may be capable of terrestrial dispersal and tolerate desiccation more than saline conditions. This is further evident with *C. cuspidatus* also absent from all four coastal sand islands and limited by the Mary River/Brisbane River biogeographic break. Similar levels of terrestrial dispersal have also been observed for other freshwater crayfish (O'Brien, 2007; Short, 2000), including *Cherax destructor* (Campbell *et al.*, 1994; Hughes & Hillyer, 2003; Nguyen *et al.*, 2004). Unlike *C. cuspidatus* however, the strong dispersal ability of *C. destructor* has also facilitated its movement throughout central Australia and across the GDR on multiple occasions (Hughes & Hillyer, 2003; Schultz *et al.*, 2009). While it is unclear whether the dispersal across the GDR was by historic river capture or terrestrial dispersal (Haworth & Ollier, 1992; Ollier & Pain, 1994; Unmack, 2001), it is acknowledged that *C. destructor* has dispersed terrestrially throughout central Australia (Hughes & Hillyer, 2003; Nguyen *et al.*, 2004; O'Brien, 2007). As the central Australia River systems are dominated by naturally unpredictable ephemeral rivers, during times of drought *C. destructor* is capable of both burrowing into the water table and traversing overland between waterholes (O'Brien, 2007). This adaptability to the harsh conditions of central Australia and strong dispersal ability has facilitated the broad distribution for the species, with *C. destructor* currently the most broadly distributed Australian freshwater crayfish (Munasinghe *et al.*, 2004a). Although *C. destructor* is distributed throughout eastern Australia, the species exhibits very low intra-specific diversity, a characteristic of species with high dispersal ability.

Similar to *C. destructor*, the *C. depressus* complex also showed relatively low levels of diversity across most of the Queensland coastline. Unlike *C. destructor* however, dispersal throughout the Queensland coast required movement across eighteen coastal river catchments (Figure 4.3). While water-borne dispersal across this region can be accomplished with fewer dispersal events, dispersal most probably required an oceanic stage (Chenoweth *et al.*, 2002; Cook *et al.*, 2012). This is especially evident with the biogeographic history of *C. depressus* and *C. cairnsensis* resembling that of amphidromous crustaceans (Cook *et al.*, 2012) and saltwater taxa (Chenoweth *et al.*, 2002; Haig *et al.*, 2010). This biogeographic history however is contrary to both freshwater and terrestrial taxa in the region, which predominantly observe a divergence within the Burdekin River (Brown *et al.*, 2006; Pusey *et al.*, 1998; Unmack, 2001), a break not observed for *C. cairnsensis*. Although most amphidromous crustaceans utilise water

flow and ocean currents to disperse juveniles along the coastline (Cook *et al.*, 2012; Ford & Kinzie III, 1994; Luton *et al.*, 2005), this may not necessarily be the case for freshwater crayfish (Toon *et al.*, 2010). Instead *C. depressus* and *C. cairnsensis* may exhibit a tolerance of elevated salinities, with dispersal along the coastline transpiring during periods of high rainfall when surface water is mostly freshwater. Most surprisingly however, although *C. cairnsensis* is estimated to have dispersed oceanically, no individuals have been observed on any of the Queensland coastal islands (McCormack, 2013). This may however be a remnant from poor sampling of Queensland islands or local extinctions (Barry & Campbell, 1977). Due also to poor sampling, inferences on the biogeographic and life history of Northern *Cherax* species is significantly limited in this study. Although *C. quadricarinatus* observed a similar wide coastal distribution to the *C. depressus* complex, a majority of its coastal connectivity can be attributed to freshwater dispersal via the formation of the freshwater Lake Carpentaria (S. F. Chenoweth *et al.*, 1998; Gopurenko & Hughes, 2002) during the Pleistocene (Torgersen *et al.*, 1983; Torgersen *et al.*, 1985). As many more *Cherax* species also inhabit Northern Australia and Papua New Guinea, further research may also identify a number of amphidromous, terrestrial and obligate freshwater species across the region.

CHAPTER 5: PHYLOGEOGRAPHIC STRUCTURE OF TWO *CHERAX* SPECIES WITH CONTRASTING RESPONSES DURING DROUGHT CONDITIONS

5.1 INTRODUCTION

Defined as the total variation of living organisms that exist on our planet, biological diversity or biodiversity describes the variation observed within species (genetic diversity), between species (species diversity) and within ecosystems (habitat diversity). High levels of biodiversity within a group of organisms can occur due to a number of causes, including geographic and ecological isolation (Purvis & Hector, 2000). The effect geographic fragmentation has on a species is determined by the structure of the landscape it inhabits and the species' ability to disperse (Avise, 2000). The strong structure of freshwater landscapes is particularly limiting for freshwater taxa, with mountain ranges (Calsbeek *et al.*, 2003), dry land (Unmack, 2001) and oceans (Benstead *et al.*, 2003) predominantly constraining freshwater taxa within river basins (catchments) and major drainage divisions (watersheds) (Bohonak & Jenkins, 2003; Page & Hughes, 2014; Poff *et al.*, 1997). This limited dispersal between river catchments is suggested to be a major factor for the high population structure often observed in freshwater invertebrates (Bohonak & Jenkins, 2003; Hurwood & Hughes, 2001) and fish (Unmack, 2001). Strong structuring of the freshwater landscape, together with long-term climate change, are examples of large-scale "extrinsic" factors (Havel & Shurin, 2004; McMillen-Jackson & Bert, 2003). In theory, these large-scale "extrinsic" factors should severely constrain the dispersal of all freshwater taxa equally, promoting similar distributions and structuring among species. This is not always the case, with some taxa widely distributed while others have narrow ranges. These small-scale variations in geographic structure among freshwater taxa are most likely explained by "intrinsic" factors, such as species-specific responses to local environments and different life history traits (Havel & Shurin, 2004; McMillen-Jackson & Bert, 2003; Poff *et al.*, 1997). The interaction between these intrinsic and extrinsic factors will primarily determine the current distribution, intra-specific genetic structure and community structure of individual species (Havel & Shurin, 2004; Poff *et al.*, 1997).

A comprehensive understanding of the intrinsic and extrinsic factors that have shaped the current and historic distribution of taxa (Havel & Shurin, 2004; McMillen-Jackson & Bert, 2003) can greatly assist the design of conservation practices for both regions and taxa. Studies focused on a single species however are unable to effectively decipher between the effects of each factor, with comparisons between the geographic structure of different taxa (comparative phylogeography; (Avise, 2000)) a more comprehensive approach. If geographic patterns are concordant between taxa, extrinsic factors are most likely dominant, with intrinsic factors more likely when patterns are significantly different between taxa (Avise, 2000). Comparative phylogeography is particularly effective when comparisons are made across closely related taxa (Bohonak, 1999), with comparisons within/between species and within/between intraspecific phylogroups also highly effective. The clear geographic boundaries and hierarchical/nested geographic structure of freshwater habitats (Bohonak & Jenkins, 2003; Ward, 1998) provides a great framework for the comparison of geographic structure in freshwater taxa.

As one of Australia's freshwater biodiversity hotspots (Crandall & Buhay, 2008; Unmack, 2001), South East Queensland provides an excellent location to compare the geographic structure of a wide range of freshwater taxa and identify the overall influence that intrinsic and extrinsic factors have had on the biogeographic history of freshwater taxa in the region. The diverse range of freshwater habitats in SEQ along with the intermittent connectivity of four (mostly) sand islands along the coastline, has been suggested as the most probable cause of the high SEQ freshwater biodiversity (Munasinghe *et al.*, 2004b; Unmack, 2001; Whiting *et al.*, 2000). While previous research has recognised the saline conditions surrounding the coastal islands of Moreton Bay as a significant barrier to dispersal for the Oxleyan Pygmy Perch (Hughes *et al.*, 1999), Ornate Rainbow Fish (Page, Bentley*, et al.*, 2007; Sharma & Hughes, 2011), freshwater shrimp (Page & Hughes, 2007a) and freshwater crayfish (Bentley *et al.*, 2010), salinity was not recognised as a significant barrier between Fraser Island and the adjacent mainland for the Pygmy Perch and Rainbow fish (Hughes *et al.*, 1999; Page, Bentley*, et al.*, 2007). In addition to oceanic isolation, Page and Hughes (2014) also identified high levels of latitudinal diversity within SEQ, with a significant North/South biogeographic break observed for sixteen obligate freshwater species. For nine of these species, a break was identified between the northern Mary River catchment and the southern Brisbane River catchment (Page & Hughes, 2014). As a single divergence between these two regions is most likely (Page et al 2014), variations in the presence of the break may indicate differing dispersal abilities or life histories (Page & Hughes, 2007b; Sharma & Hughes, 2011).

Similar to other SEQ freshwater taxa, freshwater crayfish in SEQ are highly diverse, with over fifteen species across three genera (*Cherax*, *Euastacus* and *Tenuibranchiurus*) (McCormack, 2013). Within *Cherax* alone, SEQ holds approximately a fifth of the currently recognised Australian species (McCormack, 2013; Munasinghe *et al.*, 2004b), with all except one endemic to the region (McCormack, 2013). While geographic isolation has been recognised as the dominant influence on freshwater crayfish biodiversity in SEQ, the overall impact of each geographic barrier is highly dependent on the life history of each species (Schultz *et al.*, 2007). Within *Euastacus*, a genus restricted to cool upland streams, phylogeographic isolation between species was estimated to have resulted from the large geographic distances between upland mountain tops in Australia (Ponniah & Hughes, 2006). In contrast, previous analysis on *C. dispar*, a species that inhabits permanent lowland streams, identified river catchment boundaries and saline conditions as the dominant barriers to dispersal (Bentley *et al.*, 2010). Similar river catchment based boundaries were also observed in other Australian freshwater crayfish (Schultz, 2009) and more specifically other *Cherax* species (Austin & Knott, 1996; Gouws *et al.*, 2006). As all *Cherax* tend to inhabit lowland river systems, differing phylogeographic histories are thought to be predominantly influenced by 'intrinsic' factors such as physiological tolerances and life history characteristics (McMillen-Jackson & Bert, 2003). One such prominent life history trait is the ability to burrow during drought conditions. The overall influence of this trait is most noticeable between *C. destructor* and *C. dispar*, with the latter restricted solely to permanent streams and unable to survive in ephemeral regions (Bartholomai, 1997). In contrast, *C. destructor* has been able to disperse throughout inland Australia and across multiple river catchment boundaries (Hughes & Hillyer, 2003).

Although all freshwater crayfish seek refuge of some form and most are capable of burrow construction (Horwitz & Richardson, 1986; Riek, 1969), their ability and tendency to form burrows vary considerably among both genera and species (Hobbs Jr, 1981; Horwitz & Richardson, 1986). This is especially the case within Australia, with some species of *Engaeus* essentially terrestrial; able to burrow and survive completely separate from the water-table (Horwitz & Richardson, 1986). While species with this 'terrestrial' characteristic would be expected to be widely dispersed, they are instead often restricted to the cooler humid conditions of upland mountain tops (Horwitz & Adams, 2000; Richardson & Swain, 1980). Most freshwater crayfish species however, rely on some form of connection to the water-table, either through surface flow or groundwater (Horwitz & Richardson, 1986). This dependence on freshwater, restricts dispersal to within contemporary riverine structures (Murphy & Austin, 2004), with exceptions often implying ancient riverine connections or drainage re-arrangements (McGlashan & Hughes, 2001b; Page & Hughes, 2014; Schultz *et al.*, 2008).

Variances in the burrowing capabilities of freshwater crayfish may however also influence the ability and tendency for species to disperse across river catchments. In particular, strong burrowing crayfish are known to be able to disperse 'terrestrially' across river catchment boundaries during humid or high rainfall conditions (Hughes & Hillyer, 2003; O'Brien, 2007). Although strong burrowing crayfish may be capable of 'terrestrial' dispersal, they instead tend to remain 'stationary' during drought conditions; searching for burrow spaces, occupying existing burrows or constructing new burrows (Acosta & Perry, 2001). In contrast, species that are unable to vertically burrow rely on a high dispersal ability to inhabit ephemeral river systems, specifically to be able to seek refuge during or recolonise after dry periods (Acosta & Perry, 2001; Loftus *et al.*, 1992). With a high tendency to disperse, obligate freshwater species also often utilise low sea level river drainage connections for dispersal between river catchments (Bentley *et al.*, 2010; Hughes *et al.*, 1999; Page & Hughes, 2007a; Schultz *et al.*, 2008). Few studies however have specifically compared between closely related sympatric species with differing burrowing capabilities to comprehensively determine what effect burrowing capability may have on population structure.

In this chapter a phylogeographic approach will be applied to explore the recent biogeographic history of *C. depressus* and each of the *C. dispar* lineages identified in Bentley *et al.* (2010) and Chapter 3. By building on previous results and comparing genetic variation in COI mtDNA among each of the *C. dispar* lineages with other freshwater taxa, I aim to identify any intrinsic or extrinsic factors that may inhibit the dispersal of the species across river drainages. By investigating the effect terrestrial and aquatic distance has on the genetic variation of each lineages/species, I also aim to determine which dispersal pathway lineages of *C. dispar* historically used to disperse. As an obligate freshwater species, I hypothesise that current river catchment boundaries represent a stronger barrier to dispersal for *C. dispar* lineages, particularly compared to the more 'terrestrial' species, *C. depressus*. More specifically, I hypothesise that a stronger relationship between genetic variation and terrestrial distance will be identified for *C. depressus*, a species capable of burrowing during drought conditions, compared to *C. dispar*, a species that cannot.

5.2 METHODS

Molecular techniques, data alignment, network creation and molecular diversity methods used here are as outlined in Chapter 2. More specific methods for population analyses are outlined below.

5.2.1 Population Structure

A series of Analyses of MOlecular VAriance (AMOVA's;Excoffier *et al.*, 1992) were computed in Arlequin 3.5.1.2 (L. Excoffier & H. E. L. Lischer, 2010) to investigate the spatial distribution of genetic variation for *C. depressus* & *C. dispar* using the CO1 gene. Analysis of molecular variance was used to test the null hypothesis of no association between genetic structure and geographic structure. The analysis compares genetic divergence at three hierarchical levels, which were analysed for the large sampling design and separately for *C. depressus* and each *C. dispar* clade. The three hierarchical levels for all species and clades were: among catchments (F_{CT}), among sites within catchments (F_{SC}) and among all sites (F_{ST}). Both *F*-statistics (genetic structure based on haplotype frequency only) and Φ-statistics (genetic structure based on haplotype frequency and genetic divergence) were estimated. The river catchments included were Brisbane River, Burrum River, Caboolture-Pine Rivers, Fraser Island, Logan-Albert Rivers, Maroochy River, Mary River, Moreton Island, Noosa River, North Stradbroke Island and Tin Can Bay, with each coastal island counted as a single river catchment. Sites with low sample sizes were combined with nearby sites (<10km) within the same river catchment. If a nearby site was not available, the site was omitted from population analysis but presented in the network construction. To maintain clarity in the map presentations, sites within two kilometres were pooled and coloured alike. A breakdown of the sites included in population analysis can be found in Appendix 8.2.

5.2.2 Isolation by Distance

A number of Mantel's tests (Mantel, 1967) were also performed on *C. depressus* and each *C. dispar* clade to test for Isolation by Distance (IBD). A Mantel test computes a Pearson product-moment correlation coefficient to test for a significant correlation between the genetic and geographical distance within each species (Legendre, 2000). Each Mantel test was implemented in Arlequin 3.5.1.2 (L. Excoffier & H. E. L. Lischer, 2010) with 10,000 permutations. Analyses were performed using both river distance (oceanic dispersal) and straight line (Euclidean) distances between pairs of sites. This allowed testing of the distance on both within-channel/oceanic dispersal and overland terrestrial dispersal among catchments. Slatkin's linearised distance ($D=F_{ST}/(1-F_{ST})$ (Slatkin, 1995) was used as the genetic distance between sites. This modified F_{ST} is more appropriate for IBD analyses

within one-dimensional habitats (Rousset, 1997, 2000), such as streams and rivers. Due to the low sample size and genetic diversity of *C. dispar* E, it was omitted from all population analyses.

5.3 RESULTS

Preliminary phylogenetic analysis on SEQ *Cherax* in Chapter 3 identified six taxonomically recognised species that inhabit SEQ (*C. dispar*, *C. punctatus*, *C. depressus*, *C. cuspidatus*, *C. robustus* and *C. destructor*). Due to the low sample sizes obtained for a majority of these species, the population analysis in this chapter focused only on *C. dispar* and *C. depressus*. Within these two species, an additional five phylogenetic groups were discovered within *C. dispar*; with *C. dispar* A, B & C differentiated from *C. dispar* D & E.

5.3.1 Geographical distributions

The five highly divergent *C. dispar* clades identified in Chapter 3 were strongly structured both genetically and geographically. The three sister clades *C. dispar* A, B & C were all restricted to the 'North' of SEQ, with *C. dispar* A & B confined to Fraser Island and the Mary River catchment (including Tin Can Bay) and *C. dispar* C limited to the Sunshine Coast, a small part of the Mary River catchment and the Brisbane River catchment (Figure 5.1). Although *C. dispar* C had a far more southerly distribution than *C. dispar* A & B, all three clades were observed within the Tinana Creek region of the Mary River catchment. This region however is located at the outer edge of each of the clades' respective distributions. In contrast to the three 'Northern' clades, the two sister lineages *C. dispar* D & E were entirely geographically differentiated from one another. They were distributed adjacent to one another with *C. dispar* D only observed on two of the coastal sand islands (North Stradbroke Island and Moreton Island) and *C. dispar* E distributed in the adjacent mainland coastal regions (Tingalpa Ck and the Logan-Albert Rivers) (Figure 5.1).

Although *C. dispar* A & B were distributed within the same river catchments (Mary River (including Tin Can Bay) and Fraser Island), strong spatial structuring was still observed between them. The most notable split was between the North and South of Fraser Island and between the Tin Can Bay coastal region and the Mary River catchment. Of the two clades, *C. dispar* B appeared to have the most restricted distribution, confined to the coastline (Tin Can Bay), the southern end of Fraser Island and the coastal edges of the Mary River catchment. In contrast, *C. dispar* A was distributed throughout the northern end of the Mary River catchment and Fraser Island. *C. dispar* A was however not

observed within the coastal catchment of Tin Can Bay. Of the five *C. dispar* lineages, *C. dispar* C had the widest distribution, with populations observed down the entire SEQ coastline (Figure 5.1). While *C. dispar* A & B were generally restricted to the more northerly end of the Mary River catchment, *C. dispar* C was observed in the upper reaches of the catchment in the south. This region of the Mary River catchment is located substantially inland from the mouth of the river and adjacent to the coastal Sunshine Coast catchments (Figure 5.1).

Unlike *C. dispar*, *C. depressus* was only observed on the mainland of SEQ, with no populations observed on any of the four coastal sand islands (Figure 5.1). Within the mainland SEQ populations, individuals of *C. depressus* were also only discovered within the Mary River (including Tin Can Bay) and Brisbane River catchments. Although the sampling effort in this study varied throughout SEQ, the *C. depressus* population sizes appeared to be larger in the Mary River than the Brisbane River. Unlike *C. dispar*, *C. depressus* was rarely discovered in any of the SEQ coastal streams with only a few samples caught around Tin Can Bay. This was even more apparent south of the Mary River catchment, with *C. depressus* only observed in the upper reaches of the Brisbane River. Although *C. depressus* did not appear to co-occur with other *Cherax* species in the coastal streams, the species was often discovered co-inhabiting with *C. dispar* and *C. punctatus* in the Mary River catchment.

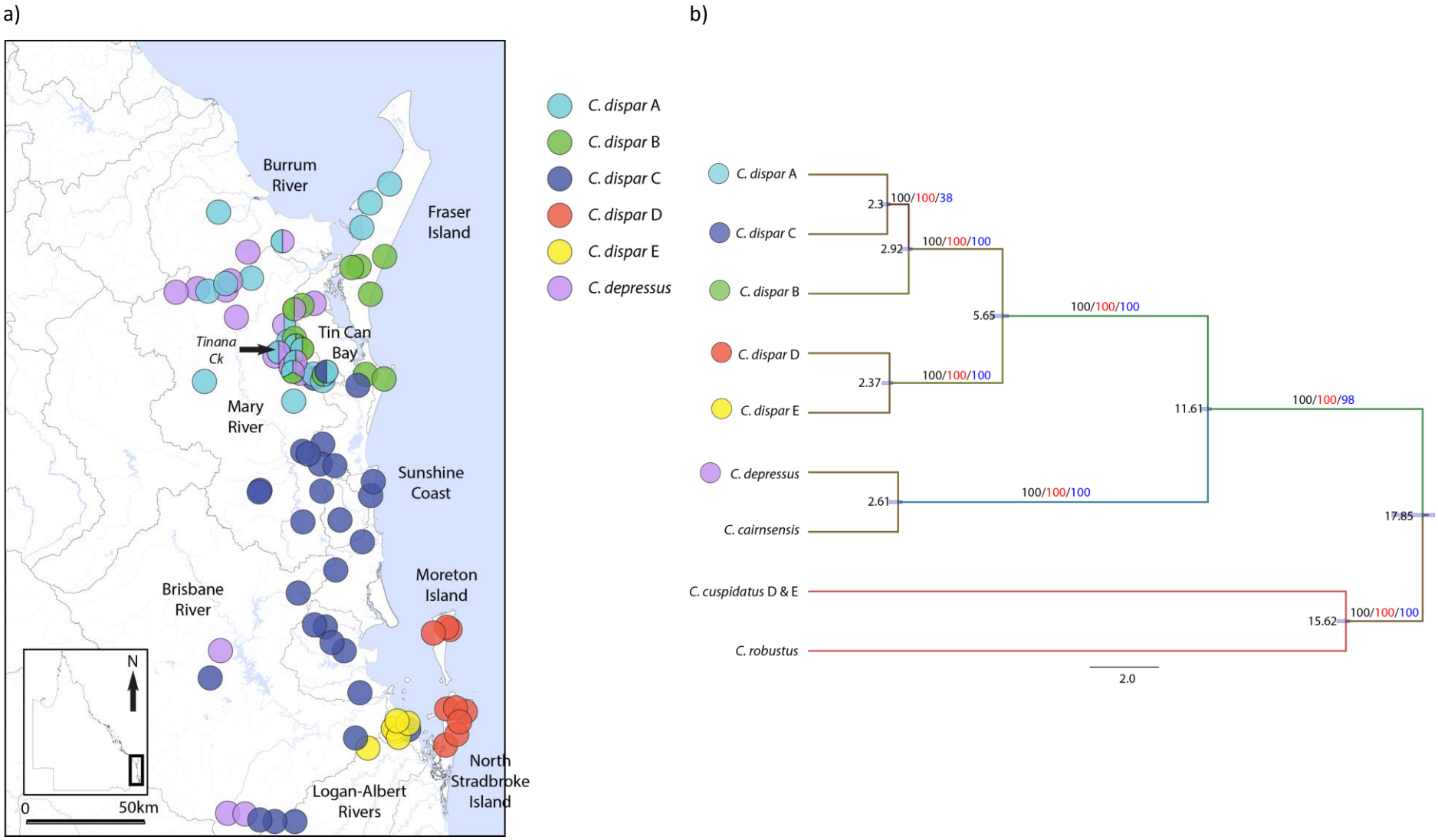

Figure 5.1: a) Distribution of *C. depressus* and *C. dispar* individuals in South-East Queensland. b) Bayesian *Beast consensus tree of selected individuals based on both nDNA and mtDNA datasets (Sourced from Figure 3.6). Phylogram values and bars as per Figure 3.1.

5.3.2 C. dispar A population structure

A total of 159 *C. dispar* A individuals across 29 localities were sequenced for a 625bp fragment of the COI mtDNA gene. From these 159 sequences, a relatively high level of genetic diversity was observed (*Hd*: 0.877) with 38 unique haplotypes identified (Figure 5.3). While genetic diversity appeared relatively equal across the river catchments, nucleotide diversity was significantly higher within Fraser Island (θ_{π}: 0.0115) (Appendix 8.2). Due to the low sample size/variation observed in some localities, the 29 sampled localities were later pooled into 22 and 17 sites for population analysis and network presentation respectively (Table 8.4).

C. dispar A displayed significant differentiation among sites within river catchments (F_{SC}) and among all sites (F_{ST}) for both *F*-Statistic and ϕ-Statistic values (Table 5.1). River catchments however, did not account for a significant amount of the variation unless genetic divergence was included (ϕ_{CT}=0.47, *p*=0.004) (Table 5.1). This conflicting result between F_{CT} and ϕ_{CT} was presumably because closely related haplotypes tended to occur in the same river catchment.

As expected from coalescent theory (Posada & Crandall, 2001), the interior haplotypes of the *C. dispar* A network (8, 11, 18) were the most abundant and widely distributed, with star like patterns around each interior haplotype (Figure 5.3). Strong geographic structuring was also observed within the network with all three interior haplotypes and their closest derived haplotypes distributed strictly within the Tinana Ck sub-catchment of Mary River (Figure 5.3). Individuals from Fraser Island, northern Mary River and the Burrum River were instead isolated to the tips of the network. Interestingly, all three Fraser Island populations were highly divergent from one another, with haplotypes from each population located at separate tips of the network (Figure 5.3). Similarly, although haplotypes 6, 37 and 38 were sampled from the same site, they were located at opposing ends of the network.

Due to the high number of highly divergent haplotypes that were geographically proximate, there was no significant correlation between the direct geographic distance and genetic distance (*r*=0.106, *p*=0.181) (Figure 5.2). Instead, there was a significant correlation or isolation by distance (IBD) when aquatic distances between sites were used (*r*=0.536, *p*<0.001) (Figure 5.2). This contrasting result between aquatic and geographic IBD analyses, suggests dispersal of *C. dispar* A individuals occurred through creeks and across the ocean, rather than terrestrially across river catchment boundaries.

Table 5.1: *C. dispar* A; Analysis of molecular variance between river catchments. F_{CT}, F_{SC} and F_{ST} sources represent among river catchments, among sites within river catchments and within sites respectively. Fixation values are calculated using F_{ST} & ϕ_{ST} values. **p*<0.05 ***p*<0.01 ****p*<0.001.

Source	% of Total Variation	*F*-Statistic	*p*-Value	Source	% of Total Variation	ϕ-Statistic	*p*-Value
F_{CT}	3.81	0.038	0.329	ϕ_{CT}	47.33	0.473	0.004**
F_{SC}	49.60	0.516	0.000***	ϕ_{SC}	38.13	0.724	0.000***
F_{ST}	46.59	0.534	0.000***	ϕ_{ST}	14.55	0.855	0.000***

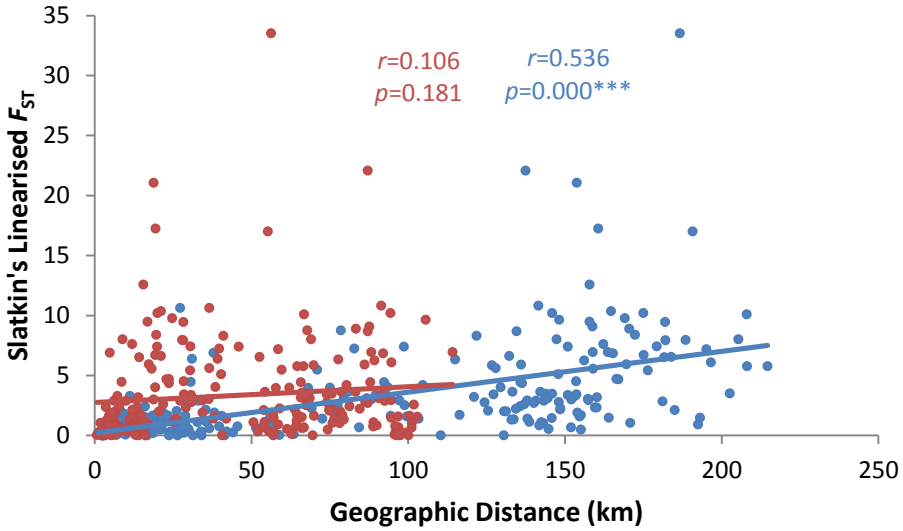

Figure 5.2: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* A populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene. Correlation (*r*) and significance for Mantel's are presented.

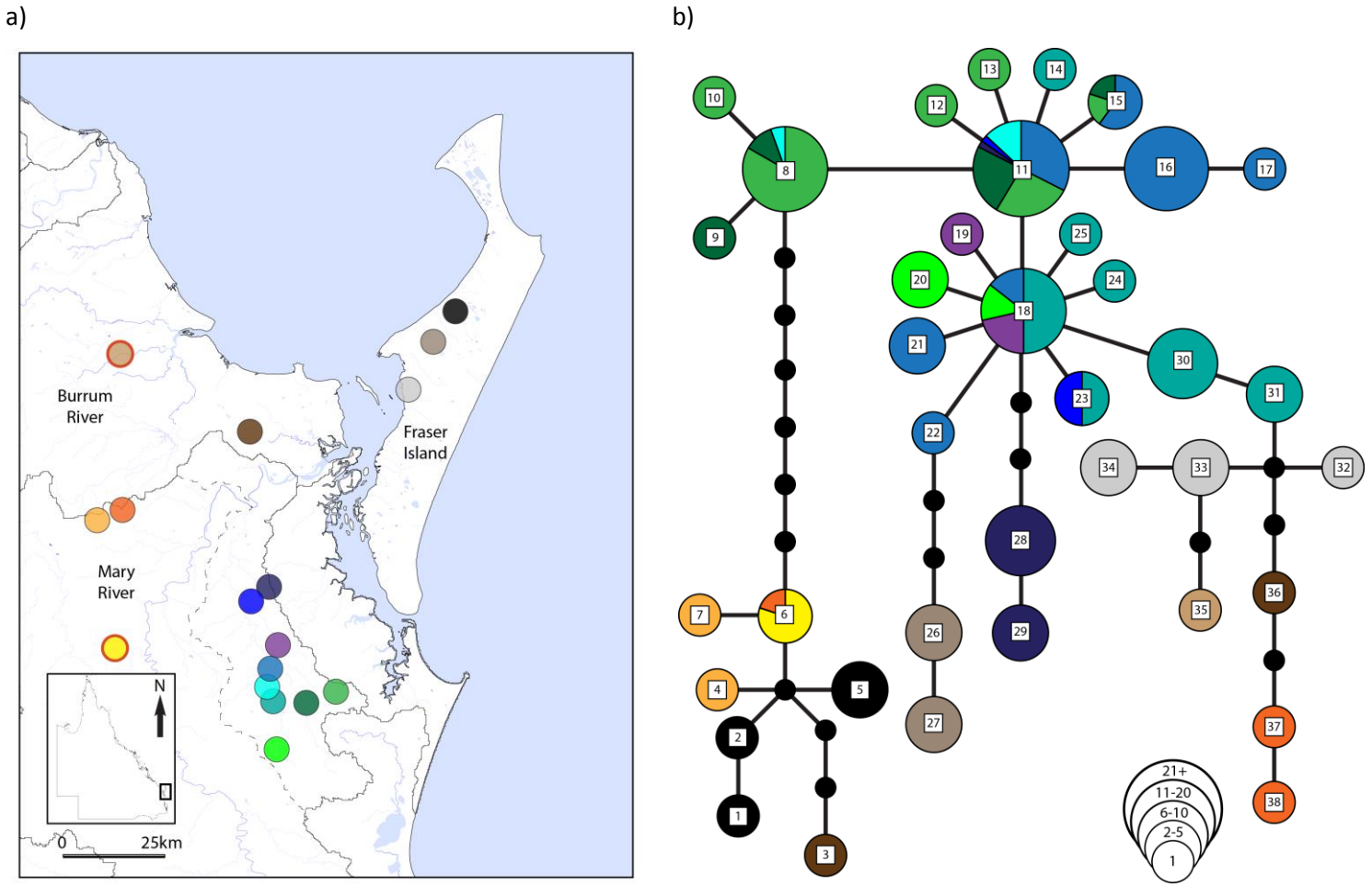

Figure 5.3: a) Distribution of *C. dispar* A individuals within SEQ.b) Genealogy network for *C. dispar* A. Dashed line in map represents border of the Tinana Ck sub-catchment. Each line in the network represents a single mutational change. Small black dots represent undetected haplotypes. Circle size of each haplotype is proportional to overall frequency of the haplotype with colours and numbers corresponding to their sample location and haplotype number respectively. Red outlined sites were not included in population analyses.

5.3.3 C. dispar B population structure

From 116 *C. dispar* B individuals across 18 populations, a total of 32 unique haplotypes were observed from a 638bp COI fragment (Figure 5.5). Unlike *C. dispar* A, the *C. dispar* B genetic and nucleotide diversity was not similar among the river catchments, with the Mary River catchment (*Hd*: 0.283) less diverse than both Fraser Island (*Hd*: 0.943) and Tin Can Bay (*Hd*: 0.708) (Appendix 8.2). Of the 18 sites sampled, only 11 were used for both population analyses and network presentation (Table 8.5). Similar to *C. dispar* A, individuals from the Tinana Ck sub-catchment (except haplotypes 17 & 18) were highly differentiated from other populations (Figure 5.5). This geographic structuring was also observed within Fraser Island, with haplotypes split into two groups corresponding with the east and west of the island (Figure 5.5). Haplotype 29 was the only haplotype that did not correspond with this break, with the haplotype represented by a single individual more than 8bp divergent from all other haplotypes. Two groups of haplotypes were also observed in the Tin Can Bay region, with one group occurring across both populations (haplotypes 15-16 & 19-23) and the other only observed within one site and highly divergent from all other haplotypes (haplotypes 30-32) (Figure 5.5).

This strong geographic structuring was also supported at all levels of molecular variance for both *F*-Statistic and ϕ-Statistic values (Table 5.2). As expected for an obligate freshwater species, a larger proportion of the variation was between the three water catchments (38% and 30%), than between sites within the water catchments (18% and 23%). This strong geographical structuring was also observed for both Mantel tests, with a significant IBD effect observed for both direct (r=0.316, p=0.032) and aquatic (r=0.485, p=0.009) geographic distances (Figure 5.4: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* B populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene.). As expected, the more significant correlation was observed when aquatic geographic distances were used. This catchment based differentiation between *C. dispar* B populations coincides with the results of AMOVA analyses, suggesting that both terrestrial landscape and water catchment boundaries are significant barriers to dispersal.

5.3.4 C. dispar C population structure

Although *C. dispar* C showed the largest distribution of the *C. dispar* lineages, individuals were only obtained from 32 localities. From these 32 populations, a relatively high level of diversity was observed (θ_π: 0.016), with 27 unique haplotypes (*Hd*: 0.924) observed from only 84 individuals

sequenced for a 640bp COI fragment (Appendix 8.2). As a number of the populations were geographically similar or contained limited genetic variation, only 15 sites were used for population analyses and 19 for network presentation (Figure 5.7). Although some geographic differentiation among river catchments was observed from the network, catchment boundaries did not impact all individuals equally (Figure 5.7). This was specifically observed within the Mary River and Noosa River catchments, with the closely related haplotypes 22-27 all distributed within this region, while other individuals from the Mary River catchment were distributed throughout the network (Figure 5.7). Some geographic structuring was observed within the Sunshine Coast however, with haplotypes 14-18 all located across neighbouring river catchments.

Table 5.2: *C. dispar* B; Analysis of molecular variance between river catchments.

Source	% of Total Variation	*F*-Statistic	*p*-Value	Source	% of Total Variation	ϕ-Statistic	*p*-Value
F_{CT}	37.51	0.375	0.006**	ϕ_{CT}	30.23	0.302	0.005**
F_{SC}	17.84	0.285	0.000***	ϕ_{SC}	22.56	0.323	0.000***
F_{ST}	44.65	0.554	0.000***	ϕ_{ST}	47.21	0.528	0.000***

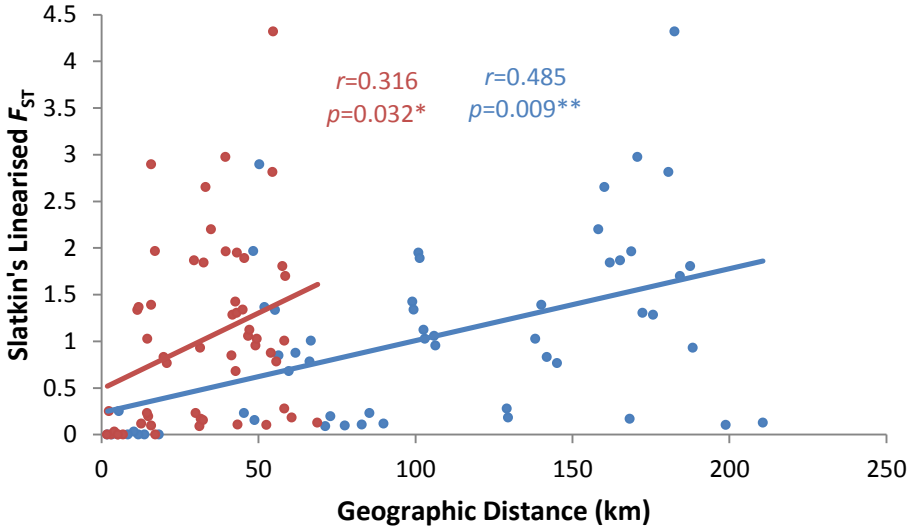

Figure 5.4: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* B populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene. Correlation (*r*) and significance for Mantel's are presented.

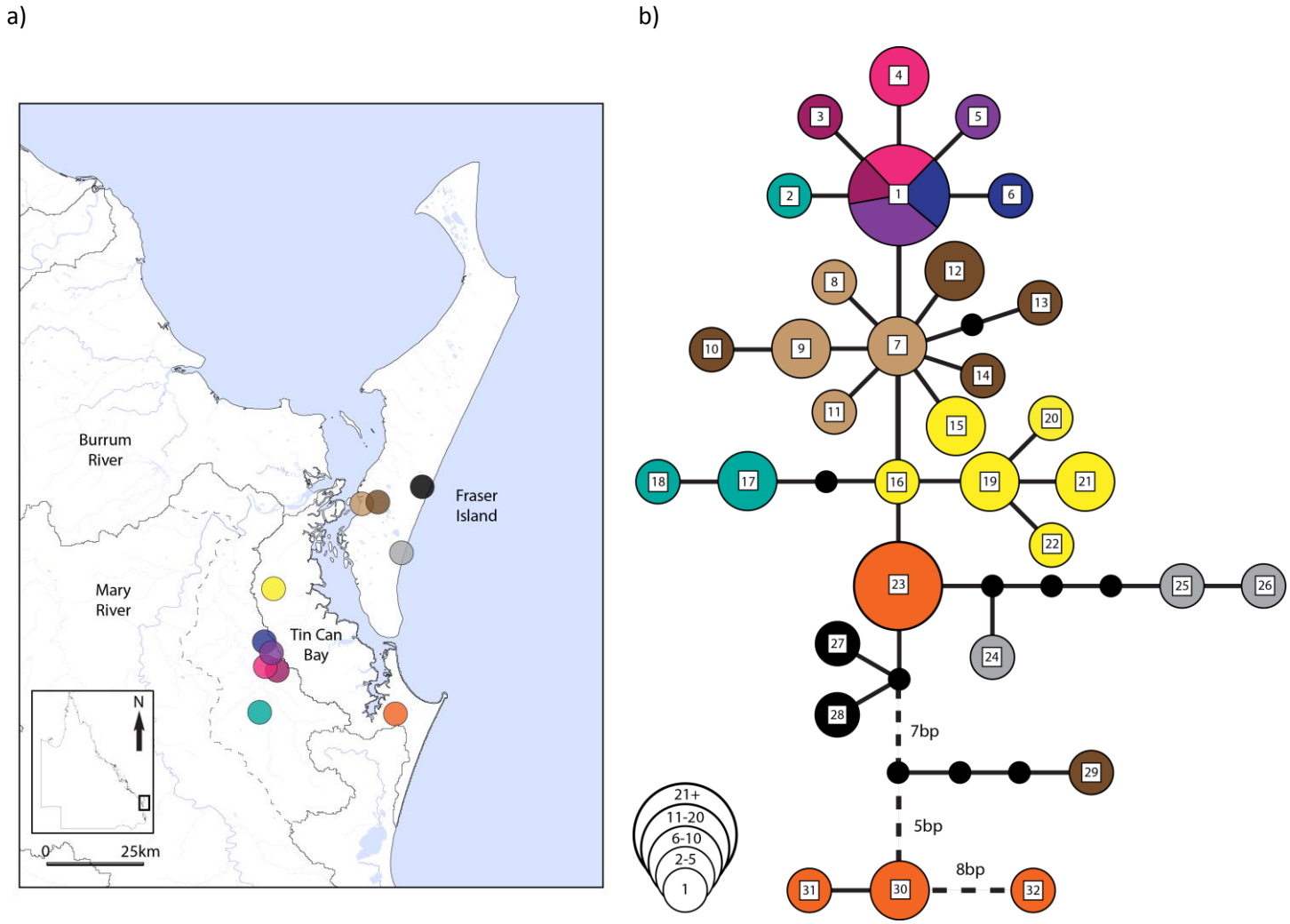

Figure 5.5: a) Distribution of *C. dispar* B individuals within SEQ. Dashed line represents border of the Tinana Ck sub-catchment b) Genealogy network for *C. dispar* B.

Due to this patchiness in geographic structuring, the AMOVA results only detected significant catchment differentiation when genetic distance was not included (*F*-Statistics) (Table 5.3). All levels of the analysis were highly significant (p<0.001), although the majority of the variation was within sites (48%) rather than among catchments (19%). When genetic distance was included, significant geographic differentiation was observed only among sites within catchments (ϕ_{SC}=0.734, p<0.001) and among all sites (ϕ_{ST}=0.759, p<0.001) (Table 5.3). In contrast to *C. dispar* A & B, a significant IBD effect was observed for direct geographic distance (Figure 5.6) and not for aquatic distance (r=0.336, p=0.007). This geographic isolation, accompanied with inconsistent catchment differentiation, suggests that dispersal across river catchments may have occurred terrestrially.

Table 5.3: *C. dispar* C; Analysis of molecular variance between river catchments.

Source	% of Total Variation	*F*-Statistic	*p*-Value	Source	% of Total Variation	ϕ-Statistic	*p*-Value
F_{CT}	19.34	0.193	0.001***	ϕ_{CT}	9.38	0.094	0.256
F_{SC}	32.50	0.403	0.000***	ϕ_{SC}	66.51	0.734	0.000***
F_{ST}	48.16	0.518	0.000***	ϕ_{ST}	24.11	0.759	0.000***

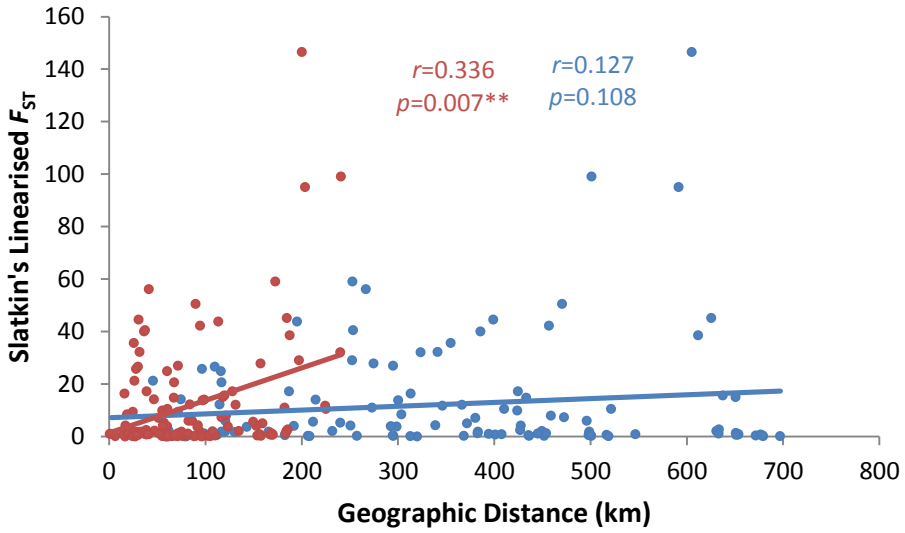

Figure 5.6: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* C populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene. Correlation (r) and significance for Mantel's are presented.

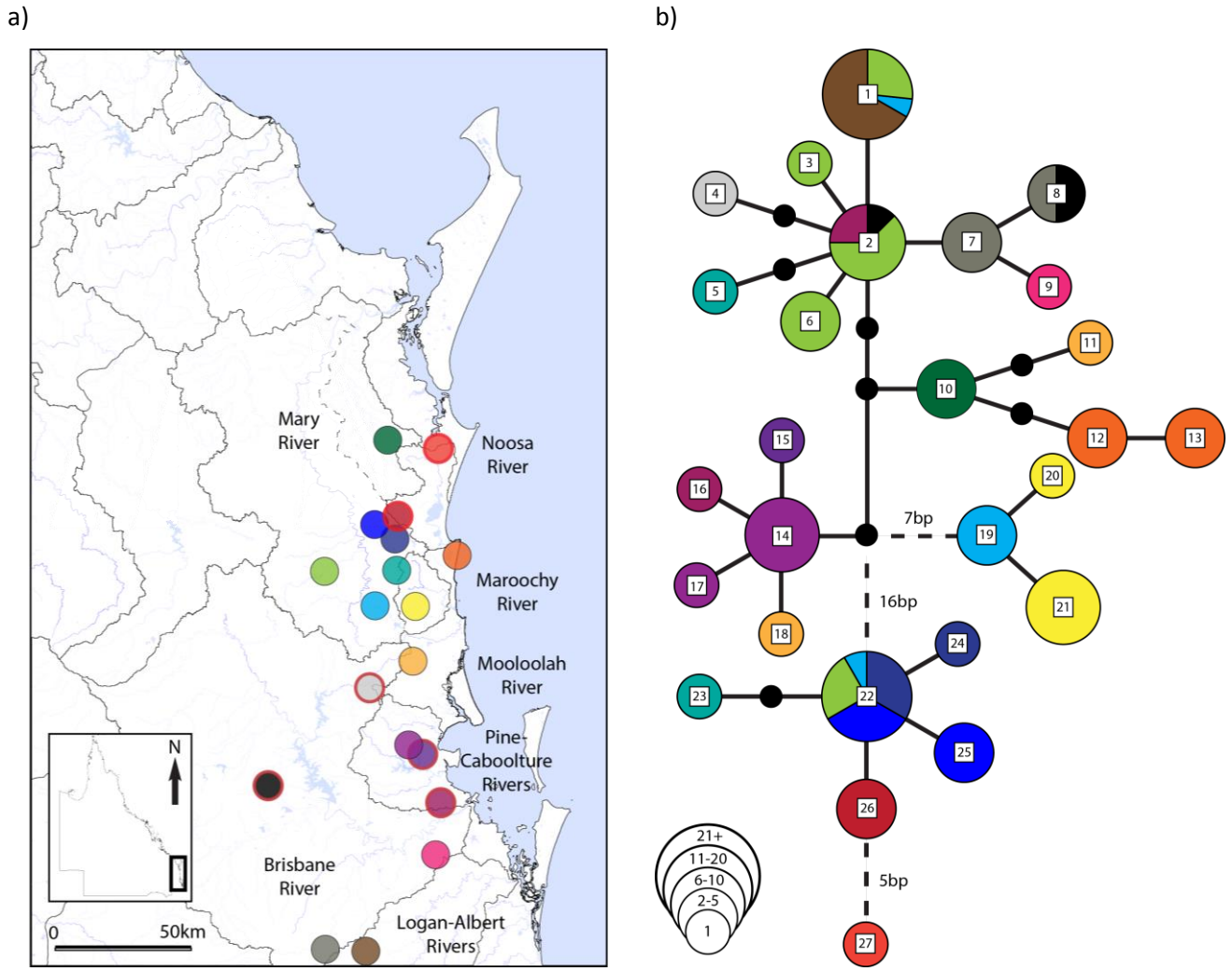

Figure 5.7: a) Distribution of *C. dispar* C individuals within SEQ. b) Genealogy network for *C. dispar* C. Dashed line in map represents border of the Tinana Ck sub-catchment

5.3.5 C. dispar D and E population structure

Of the five *C. dispar* lineages, *C. dispar* D & E were the most geographically restricted. This very isolated distribution for the two lineages limited sampling to only 75 individuals from 14 populations for *C. dispar* D and 12 individuals from 6 populations for *C. dispar* E. Sequencing of an 841bp COI fragment of *C. dispar* D individuals identified 26 haplotypes spread throughout North Stradbroke Island and Moreton Island (Figure 5.9). Although a number of individuals were sequenced from Moreton Island (*N*: 22), only four unique haplotypes were identified (*Hd*: 0.260), far less than those from Nth Stradbroke Island (*Hn*: 23 & *Hd*: 0.93) (Appendix 8.2). As a large proportion of the sites sampled for *C. dispar* D were within close proximity on the western coast of North Stradbroke Island, the 14 populations were amalgamated into eight distinct populations for both population analyses and network presentation (Figure 5.9). This was also the case for *C. dispar* E populations, with two of the six sites merged into proximate sites within the same river catchment (Figure 5.9). As sequencing of a 701bp COI fragment of *C. dispar* E individuals identified eight haplotypes from only twelve individuals, population analysis could not confidently be conducted on this lineage.

Although the *C. dispar* E lineage was not analysed statistically, a clear geographic pattern was observed. There was no haplotype sharing either between the river catchments (Logan-Albert Rivers and Tingalpa Ck) or between sites (Figure 5.9). The 21bp genetic divergence between the two river catchments also far exceeded the geographic divergence in any other *C. dispar* lineage. However, some geographic admixture was observed within the Tingalpa Ck catchment, with haplotypes 2 & 6 both from the same population but on opposite sides of the network (Figure 5.9). Similar to *C. dispar* E, *C. dispar* D was strongly differentiated both between and within the two islands, with a clear split observed between the east and west of North Stradbroke Island. Contrary to expectation, the populations on either side of North Stradbroke Island were as divergent from each other as they were from populations on Moreton Island (Figure 5.9).

The strong geographic structuring observed for *C. dispar* D was supported at all levels of molecular variance using both *F*-statistic and ϕ-statistic values (Table 5.4). As expected, a much higher percentage of the variation was explained between the islands (28% and 45%) than between sites within the islands (20% and 35%). The geographic structure among sites within the islands however was more significant ($p<0.001$) (Table 5.4). Similar to *C. dispar* C, which inhabits mainland SEQ, *C. dispar* D individuals were also estimated to have dispersed terrestrially or directly ($r=0.509$, $p=0.007$), rather than aquatically via the ocean ($r=-0.071$, $p=0.616$) (Figure 5.8).

Table 5.4: *C. dispar* D; Analysis of molecular variance between river catchments.

Source	% of Total Variation	*F*-Statistic	*p*-Value	Source	% of Total Variation	ϕ-Statistic	*p*-Value
F_{CT}	28.41	0.284	0.016*	ϕ_{CT}	44.74	0.447	0.011*
F_{SC}	20.10	0.281	0.000***	ϕ_{SC}	35.04	0.634	0.000***
F_{ST}	51.49	0.485	0.000***	ϕ_{ST}	20.23	0.798	0.000***

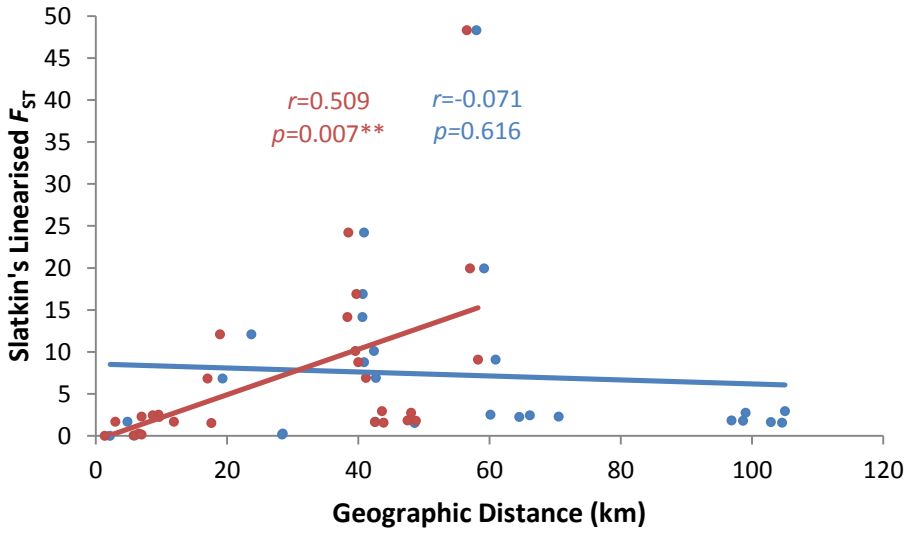

Figure 5.8: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* D populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene. Correlation (*r*) and significance for Mantel's are presented.

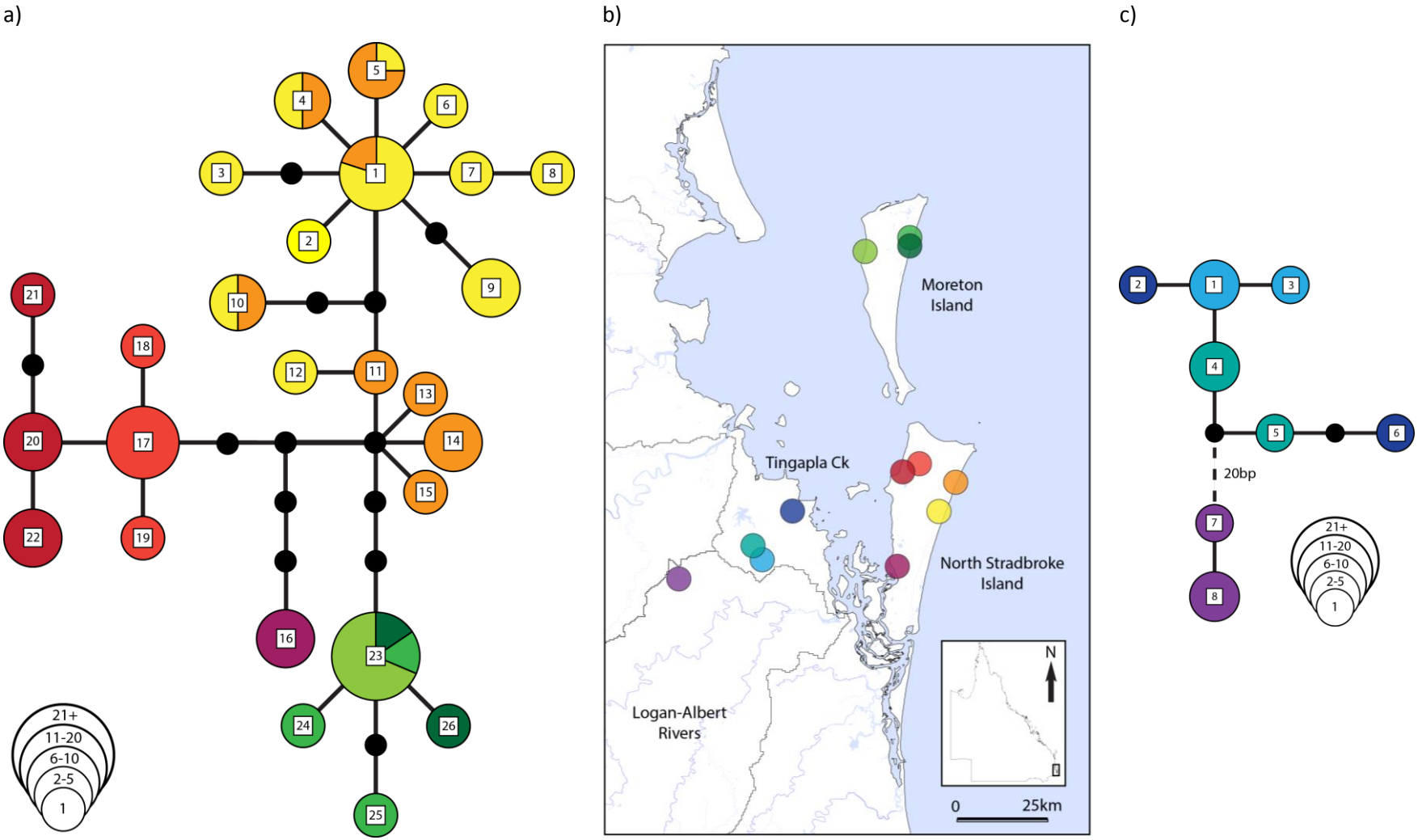

Figure 5.9: a) Genealogy network of *C. dispar* D. b) Distribution of *C. dispar* D and *C. dispar* E individuals within SEQ. c) Genealogy network for *C. dispar* E.

5.3.6 C. depressus population structure

A total of 28 haplotypes were identified from a 602bp COI fragment of 169 *C. depressus* individuals (Figure 5.11). Even though only eight of the 169 individuals were sampled from within the Brisbane River catchment, four unique haplotypes were identified, with the nucleotide diversity (θ_π: 0.011) within the catchment much higher than the other three catchments (Appendix 8.2). Although the 169 *C. depressus* individuals were originally sampled from 26 localities, they were later combined into 16 geographically distinct populations for both population analysis and network presentation (Figure 5.11). The *C. depressus* network identified a clear north-south geographic break between populations in the Mary River/Tin Can Bay region and populations in the Brisbane River (Figure 5.11). Geographic differentiation was also observed within the northern populations of *C. depressus*, with haplotypes in the Tinana Ck sub-catchment (except 23 & 24) separate from other northern populations (Northern Mary River, Tin Can Bay & Burrum River). Although *C. depressus* was only found at two sites in the south, the distinction was clear with 10bp distance between them and the northern sites.

Based on ϕ-statistics, this differentiation among geographic regions was significantly supported for all levels of molecular variance (Table 5.5). Significant differentiation among catchments was however not observed for *F*-statistics (F_{CT}=0.038, p=0.329), with differentiation instead among populations within river catchments (F_{SC}=0.516, p<0.001) (Table 5.5). This poor statistical distinction between river catchments was most likely influenced by a single population within the Burrum River, as it shared haplotypes with a number of individuals from the Mary River and Tin Can Bay catchments (haplotypes 11 & 19). When genetic distance was included, genetic variation was predominantly explained between river catchments (47%). This geographic structuring was also supported by the Mantel tests, with a significant correlation observed between genetic distance and both direct (r=0.439, p=0.012) and aquatic (r=0.397, p=0.012) geographic distances (Figure 5.10). This significant IBD effect from both geographic distances suggests the dispersal ability of *C. depressus* may be limited, particularly compared to the five *C. dispar* lineages.

Table 5.5: *C. depressus*; Analysis of molecular variance between river catchments.

Source	% of Total Variation	*F*-Statistic	*p*-Value	Source	% of Total Variation	ϕ-Statistic	*p*-Value
F_{CT}	3.81	0.038	0.329	ϕ_{CT}	47.33	0.473	0.004**
F_{SC}	49.60	0.516	0.000***	ϕ_{SC}	38.13	0.724	0.000***
F_{ST}	46.59	0.534	0.000***	ϕ_{ST}	14.55	0.855	0.000***

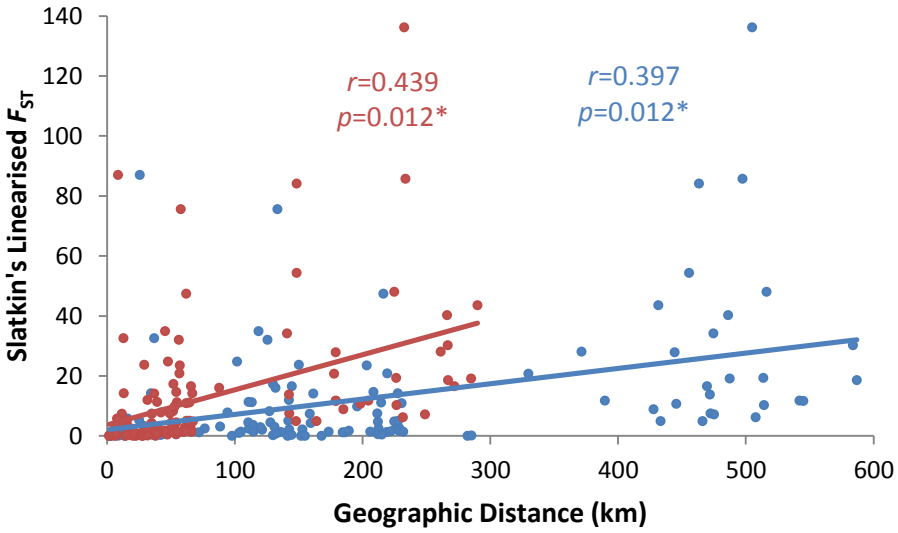

Figure 5.10: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. depressus* populations against their corresponding direct (Red) and creek/ocean (Blue) geographic distance for the COI mtDNA gene. Correlation (*r*) and significance for Mantel's are presented.

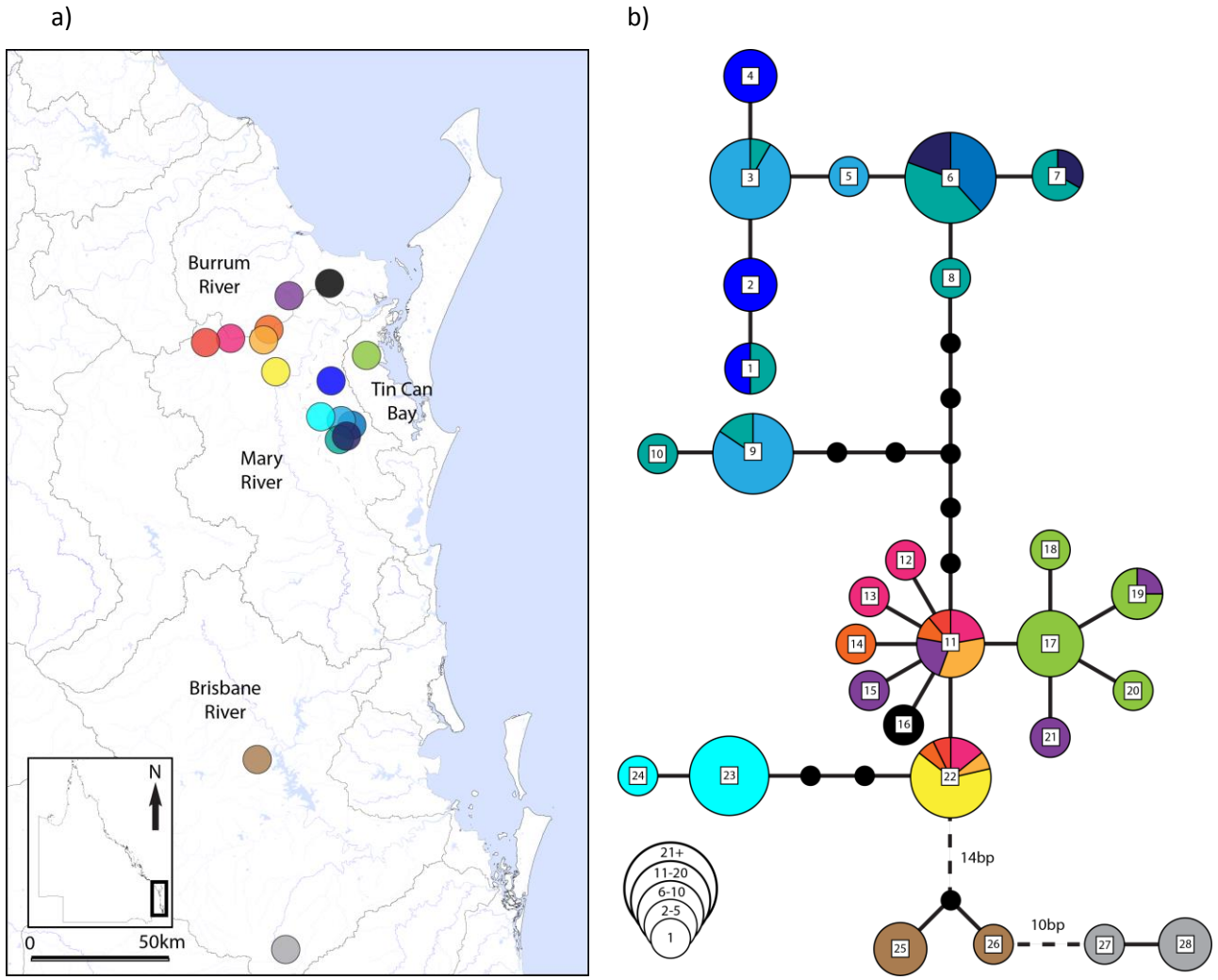

Figure 5.11: a) Distribution of *C. depressus* individuals within SEQ. b) Genealogy network for *C. depressus.*

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5.4 DISCUSSION

5.4.1 Distribution of two SEQ freshwater crayfish

Similar to many other freshwater fish (Hughes *et al.*, 1999; Sharma & Hughes, 2011; Unmack, 2001) and crayfish (*Wildlife of Greater Brisbane*, 2007), both *C. dispar* and *C. depressus* have a wide SEQ distribution spread across multiple river catchments (Figure 5.1). Across this wide distribution, a clear North/South geographic divergence was observed for both species. While river catchment boundaries are thought to have been the driving force for this North/South split in freshwater taxa (Hughes *et al.*, 1999; Murphy & Austin, 2004; Page & Hughes, 2007b; Sharma & Hughes, 2011), a similar break has also been observed for terrestrial species (McGuigan *et al.*, 1998), which are unlikely to be influenced directly by catchment boundaries. Similarly, the divergence between the northern lineages of *C. dispar* (A-C) does not necessarily correspond with river catchment boundaries, as all three lineages are instead sympatric within the Mary River catchment (Figure 5.1). More specifically, all the lineages inhabit the upper reaches of the Tinana Ck sub-catchment at the outer edges of each of their distributions (Figure 5.1). This sympatric distribution may be the result of allopatric divergence with subsequent re-colonisation or sympatric divergence by inhabiting differing ecological niches or microhabitats (Pfenninger *et al.*, 2003; Wellborn & Cothran, 2004). As *C. dispar* B is predominantly distributed near the coastline in TCB and south Fraser Island, previous analysis estimated the two lineages (*C. dispar* A & B) may have once inhabited separate river systems, with recent colonisation of *C. dispar* B into the Mary River catchment (Bentley *et al.*, 2010). Due to the low number of sites where the three lineages were sympatric, it was also suggested that divergence between the three lineages may have been maintained as a result of differing ecological requirements (Bentley *et al.*, 2010). Further sampling in this study however has identified many more sites where *C. dispar* A & B are sympatric, suggesting instead that other factors may currently be maintaining the divergence.

Of the northern *C. dispar* lineages, *C. dispar* C had the largest geographic distribution, with a distribution covering the majority of the SEQ coastline (Figure 5.1). This distribution down the Sunshine Coast coastline has also been observed in a number of freshwater taxa (Baker, Sheldon*, et al.*, 2004; Chenoweth & Hughes, 2003; Murphy & Austin, 2004; Page & Hughes, 2007a, 2007b; Page *et al.*, 2004; Sharma, 2006; Unmack, 2001), with a historic river paleo-drainage parallel to the SEQ coastline estimated as the probable connection between the river catchments (Hughes *et al.*, 1999; Page & Hughes, 2007a). Most studies however, have reported a significant break between the Mary

River and the Sunshine Coast (Page & Hughes, 2007a; Sharma & Hughes, 2011), with mostly only diadromous fish being genetically similar in the two regions (Cook *et al.*, 2012). As *C. dispar* C was only found in one site within the Tinana Ck sub-catchment of the Mary River, dispersal into this region was most likely through recent colonisation possibly via an external force (Reynolds & Souty-Grosset, 2012; Stefani *et al.*, 2011). Unlike the northern lineages of *C. dispar*, *C. dispar* D & E had the most geographically restricted distributions, with *C. dispar* D endemic to two of the coastal islands of Moreton Bay, and *C. dispar* E only in the nearby mainland catchments (Figure 5.1). While the oceanic conditions surrounding Fraser Island did not appear to have influenced the distribution of the northern *C. dispar* lineages, the saline conditions of Moreton Bay appear to have represented a significant barrier to dispersal for *C. dispar* D & E. The rise of sea level is not likely to be the driving factor for divergence between the two lineages however, with their divergence pre-dating (Chapter 3) both the last glacial maximum and the actual age of the islands (Tejan-Kella *et al.*, 1990). While the saline conditions of Moreton Bay appear to have restricted the dispersal of *C. dispar* E across to the nearby coastal islands, dispersal appears to have also been limited inland (Figure 5.1). Although this may be due to a preference for the specific habitat offered by coastal streams, the lineage may also have been outcompeted by the *C. dispar* C lineage, which also inhabits the inland region of the Logan-Albert Rivers (Figure 5.1).

While *C. dispar* was distributed within almost all river catchments in SEQ, *C. depressus* appeared to have a much more restricted distribution, with notable absences from all four coastal sand islands and the Sunshine Coast Region (Figure 5.1). As the creeks within these regions are all predominantly permanent coastal streams, the absence of *C. depressus* from these regions may be due to a number of factors including habitat preference, dispersal opportunity and competition. Since *C. depressus* is a burrowing freshwater crayfish that prefers to inhabit stagnant ephemeral streams (*Wildlife of Greater Brisbane*, 2007), the increased water flow, sand dominated substrate and salinity of coastal streams may have inhibited survival within these regions (Horwitz & Richardson, 1986; Nakata *et al.*, 2003). In particular, both increases in water flow and sandy substrate would hinder burrow construction for the species (Horwitz & Richardson, 1986; Nakata *et al.*, 2003) and subsequently leave it exposed to predation, competition and desiccation .(Berrill & Chenoweth, 1982). The predominant barrier to dispersal for *C. depressus* however is more likely to be a low tolerance to saline conditions. As dispersal down the SEQ coastline to the coastal islands was estimated to have occurred through historical river paleo-drainages (Hughes *et al.*, 1999), *C. depressus*' low salinity tolerance may have inhibited its survival in coastal regions and subsequently restricted its opportunity to disperse. Instead dispersal south from the Mary River into the Brisbane River

catchment may have occurred 'terrestrially', where the two catchments share a boundary in their upper reaches (Figure 5.11). Dispersal across this region is more likely as it avoids all Sunshine coast river catchments and coastal regions.

5.4.2 Extrinsic drivers on phylogeographic structure

As an obligate freshwater species, the dispersal of *C. dispar* is expected to be strongly restricted to freshwater 'corridors', and ultimately river catchment boundaries (Burridge *et al.*, 2008; Hughes *et al.*, 2009). Dispersal across river catchments is therefore expected to be rare, with dispersal predominantly possible via river connections at low sea level (Horwitz, 1988; Page & Hughes, 2007a), 'terrestrially' via river capture (Burridge *et al.*, 2006; Hurwood & Hughes, 2001), temporary connections from floods (Yam & Dudgeon, 2005) or even the intervention of a third party such as humans (Havel & Shurin, 2004) or birds (Charalambidou *et al.*, 2005; Hebert *et al.*, 2003). This strong differentiation and limited dispersal among river catchments was observed for all five *C. dispar* lineages, with analyses of molecular variance identifying significant structure among river catchments for at least one genetic statistic (F_{CT}/ϕ_{CT}). Of the five *C. dispar* lineages*, C. dispar* B showed the strongest geographic structure at all levels of AMOVA analysis (Table 5.2). This significant geographic structure was also supported with significant IBD analyses for both freshwater and direct geographic distances (Figure 5.4), suggesting that dispersal of the lineage is extremely limited. *C. dispar* B's relatively small geographic distribution is also akin to a species with limited dispersal ability, as dispersal across large distances is uncommon. A phylogeographic break was observed between south Fraser Island and the adjacent mainland (Tin Can Bay), and north Fraser Island and the Mary River catchment, suggesting that the two ends of Fraser Island may have been colonised separately (Figure 5.5). The close geographic proximity between north Fraser Island and the mouth of the Mary River advocates it as the most probable corridor for colonisation between the two regions (Page *et al.*, 2012). Page *et al.* (2012) went on to suggest that colonisation of south Fraser Island may have instead occurred via a historical river connection from the south. A similar recent connectivity between Fraser Island and Tin Can Bay was also found for the freshwater fish *Rhadinocentrus ornatus* (Sharma & Hughes, 2011).This clear colonisation history of Fraser Island was not observed for *C. dispar* A, with some Fraser Island haplotypes more closely related to haplotypes found at the most distant mainland sample sites than the closest (Figure 5.3). A clear differentiation between each of the Fraser Island populations was observed however, with each population genetically distinct at opposing sides of the genetic network (Figure 5.3). As well as indicating a lack of dispersal among Fraser Island sites, this patchy phylogeographic structure also suggests

colonisation of each population may have occurred separately. This pattern of intermittent historical dispersal between Fraser Island and the adjacent mainland is typical of a species that commonly disperses during fluctuating sea levels (Hewitt, 2001). The significant IBD for freshwater distance observed for *C. dispar* A may also indicate that dispersal to Fraser Island occurred through the Mary River channel, rather than directly (Figure 5.2). This Mary river connection coincides with the geographic location of the Fraser Island populations, with all three sites located opposite the mouth of the Mary River (Figure 5.3).

Although *C. dispar* D did not show a significant difference among catchment phylogeographic structure, a clear geographic differentiation was observed both between Moreton Island and North Stradbroke Island and within North Stradbroke Island (east and west) (Figure 5.9). This east/west split on North Stradbroke Island was also observed for a number of other freshwater taxa, including fish (*Hypseleotris compressa* (Page, Bentley*, et al.*, 2007), *Nannoperca oxleyana* (Page, Bentley*, et al.*, 2007) & *Rhadinocentrus ornatus* (Page, Bentley*, et al.*, 2007; Sharma & Hughes, 2011)) and shrimp (*Caridina indistincta* (Page & Hughes, 2007a). Unlike previous analysis, which observed less genetic distance between the two sides of North Stradbroke Island than between it and Moreton Island (Bentley *et al.*, 2010), a similar level of genetic distance was observed between all three regions (Figure 5.9). This similar genetic differentiation instead suggests that colonisation of both islands may have occurred simultaneously, with subsequent divergences occurring *in situ*. Page and Hughes (2007a) suggested dispersal to both islands may have occurred during low sea level, when rivers which now flow into Moreton Bay would have continued past the stranded dune fields. This similarity between the mainland and Moreton Bay was also supported by the close affinity both geographically and genetically between *C. dispar* D & E, with a historical distribution across the entire region likely. While the saline conditions would have restricted dispersal between the islands, dispersal across North Stradbroke Island was most likely restricted by the ancient dunes separating the two sides of the island (Page *et al.*, 2012; Ward, 1978). Similar to *C. dispar* D, *C. dispar* E showed high levels of divergence between river catchments, suggesting a relatively poor dispersal ability (Figure 5.9). This strong geographic structuring across the relatively small geographic distribution of *C. dispar* E was most surprising, with historical dispersal between the two catchments expected during fluctuating sea levels (Page & Hughes, 2007a). A high divergence between the two catchments may instead suggest that Tingalpa Ck may have historically run north towards the Brisbane River, particularly during times of low sea level (Page & Hughes, 2007a) .

Considering the two species, only *C. dispar* C and *C. depressus* were observed in both the Mary River and Brisbane River catchments (Figure 5.1). While a number of other freshwater taxa also show similar geographic distributions, a majority of the species show a significant phylogeographic break between these two catchments (Murphy & Austin, 2004; Page, Bentley*, et al.*, 2007; Page & Hughes, 2014; Woolschot *et al.*, 1999). While there was high genetic divergence between the two regions in *C. depressus* (Figure 5.11), the two catchments shared haplotypes for *C. dispar* C (Figure 5.7). As *C. dispar* C was also distributed throughout the Sunshine Coast, the extrinsic factors within the region most likely affected the two species differently (Havel & Shurin, 2004; McMillen-Jackson & Bert, 2003). Interestingly, both species did still show some geographic structuring within the region, with significant among catchment structure and a correlation between direct geographic distance and genetic distance (Table 5.3 &Table 5.5). This direct distance IBD correlation was most surprising for *C. dispar* C, as it indicated the lineage may have dispersed along the Sunshine Coast coastline 'terrestrially'. This surprising result may however be due to an initial 'terrestrial' dispersal event from the Mary River into the Sunshine Coast with subsequent aquatic/oceanic dispersal via a historical river paleo-drainage parallel to the coastline. The lack of *C. depressus* individuals along the Sunshine Coast suggests that dispersal for the species more than likely occurred inland directly between the Mary River and Brisbane River.

5.4.3 Life history or evolutionary history?

As *C. dispar* does not tend to burrow, its distribution is considerably restricted to permanent creeks, with localised extinction occurring during times of drought (*Wildlife of Greater Brisbane*, 2007). As a species that requires re-colonisation to inhabit ephemeral systems, particularly post-drought *C. dispar* was expected to have a high dispersal ability (Cook *et al.*, 2007). In contrast, *C. depressus* is relatively resistant to drought conditions, with the ability to burrow down into the water table and remain 'stationary' in ephemeral systems (Schultz *et al.*, 2007). Because of this resistant characteristic, it was expected that *C. depressus* would have a low tendency to disperse throughout the ephemeral streams of SEQ. This lower dispersal for *C. depressus* was supported in this study, with highly significant results for all levels of AMOVA analysis (Table 5.5) and both IBD tests (Figure 5.10). The similar IBD effect for both creek distance and direct geographic distance, may also suggest that *C. depressus* is capable of dispersal 'terrestrially' and able to survive in dry conditions (Bubb *et al.*, 2006). Similar cross river catchment dispersal has also been observed in *C. destructor*, another burrowing freshwater *Cherax* (Hughes & Hillyer, 2003). More specifically, *C. destructor* was estimated to have dispersed between the Murray-Darling Basin and the Lake Eyre Basin (Hughes &

Hillyer, 2003) and across the Great Dividing Range on multiple occasions (Munasinghe *et al.*, 2004a). For *C. depressus*, this 'terrestrial' dispersal may have occurred directly from the Mary River catchment into the Brisbane River catchment. In contrast, dispersal of *C. dispar* appears to have occurred predominantly along the coastline, possibly via historical river drainages during times of low sea level. This was particularly the case for *C. dispar* C, with individuals observed in almost every coastal SEQ river catchment.

Interestingly, while *C. dispar* appears to be distributed throughout each of the river catchments, *C. depressus* is predominantly observed within the upper reaches of each catchment (Figure 5.1). This is particularly the case within the Brisbane River catchment. This highly restricted distribution in the Brisbane River catchment may be due to competition pressures, with *C. cuspidatus*, another burrowing *Cherax*, also inhabiting the catchment. Although *C. depressus* still inhabits the same sites as *C. dispar*, their contrasting life histories and burrowing capabilities may limit their interactions or resource competition (Johnston & Robson, 2009). The similar distributions between *C. depressus* and *C. dispar* A & B across the Mary River, Burrum River and Tin Can Bay, also suggests that both species may have dispersed throughout the region at the same time, with the same extrinsic forces influencing their dispersal patterns (McMillen-Jackson & Bert, 2003).

CHAPTER 6: POPULATION CONNECTIVITY WITHIN A HETEROGENEOUS SUBCATCHMENT OF MARY RIVER

6.1 INTRODUCTION

The reconstruction of the evolutionary history of an organism requires a comprehensive understanding of both the biogeographic history of the organism and the contemporary micro-evolutionary processes (Funk, 1998; Schluter, 2000, 2001). While biogeography is an effective approach to investigate an organisms' historical distribution at a large scale, it is ecological variables that tend to explain its contemporary distribution as a result of selective pressures (Rundle & Nosil, 2005). In general, the current distribution of a species is restricted to locations where physicochemical conditions are appropriate, resources are available and competitors or predators do not preclude them (Barbaresi *et al.*, 2007; Rundle & Nosil, 2005). These restrictions on the distribution of a species often maintain genetic divergence between populations that occupy different habitats within a single connected system (Jones & Bergey, 2007; Pfenninger *et al.*, 2003). As a result, situations where closely related species occur sympatrically are of particular interest to evolutionary biologists, with ecological variables suggested to be one of the most probable factors maintaining diversity within a freshwater system (Schluter, 2001).

Throughout a species' distribution, landscapes can differ in both physical condition (habitat complexity, substratum type, salinity, temperature etc.) and ecology (competitive interactions, predation pressure and feeding behaviour) (Jones *et al.*, 2007; Rundell & Price, 2009). As a result landscape heterogeneity between habitats or populations can impede dispersal and subsequently gene flow between populations (Holderegger & Wagner, 2008). Through divergent selection and environmental pressures, this landscape heterogeneity may also result in notable phenotypic variation among populations (Schluter, 2009; Taylor & McPhail, 1999). While phenotypic variation within populations may reflect the underlying genetic structure of the species, geographically separated populations may also differ morphologically due to founder effects, migration patterns, mating systems or local adaptation (Palumbi, 2003; Schluter, 2009). This phenotypic plasticity and ability to adapt to the environment inhabited during ontogeny is a key factor for a species' potential

to colonise, survive and reproduce (Davidson *et al.*, 2011; Gotthard & Nylin, 1995). In particular, differing environmental conditions can strongly affect the behavioural, physiological and morphological characteristics of the species (Hollander *et al.*, 2006; Idaszkin *et al.*, 2013; Pigliucci, 1996). Therefore, combined analysis of molecular connectivity, morphological variation and environmental conditions is crucial for a fundamental understanding of the processes affecting a species' contemporary distribution (Rundle & Nosil, 2005; Triponez *et al.*, 2013). This is particularly the case for freshwater organisms, with species highly susceptible to variations in their habitat (Bloom *et al.*, 2013; Dudgeon *et al.*, 2006).

Significant relationships between the connectivity of freshwater organisms and their environment have been observed in a number of freshwater organisms. In particular, water flow (Kerby *et al.*, 2005; Nakagawa, 2013), salinity (Matthews, 1998; Nielsen *et al.*, 2003), substrate type (Johnston & Robson, 2009; Pusey *et al.*, 1993; Usio, 2007) and temperature (Leathwick *et al.*, 2005; Mantyka-Pringle *et al.*, 2014; Ponniah & Hughes, 2006) are common characteristics that restrict distributions of freshwater species. Although sometimes restricted by substrate type, freshwater crayfish are known for their high adaptability to changing water conditions (Crandall & Buhay, 2008; Haddaway *et al.*, 2012) and phenotypic plasticity in varying environments (Austin & Knott, 1996; Campbell *et al.*, 1994; Haddaway *et al.*, 2012). This strong adaptability and phenotypic plasticity of freshwater crayfish is of particular interest to studies on introduced and aquaculture species, with interest specifically in the environmental tolerance limits (Capinha *et al.*, 2013; Kendall & Schwartz, 1964; Mills & Geddes, 1980) and optimal growth conditions for the organisms (De Bock & López Greco, 2010; Geddes & Smallridge, 1993). To distinguish between the effects of the environment and genetic inheritance on morphology, studies predominantly control for either the genetic composition (Dimmock *et al.*, 2004; Swain *et al.*, 1991) or a specific environmental variable (Matsuzaki *et al.*, 2012; Zanotto & Wheatly, 1993) within a controlled setting. Although studies of freshwater crayfish within a natural setting are common, they often focus on either sympatric highly diverged species or geographically isolated lineages/morphotypes that do not compete for resources (Johnston & Robson, 2009; Jones *et al.*, 2007). Interpretations from this approach are therefore limited, as they lack information on potential gene flow and environmental connectivity in combination (Barton & Hewitt, 1989).

The freshwater crayfish of the Mary River catchment provide a unique opportunity to investigate the effect of environment on connectivity and morphology. Although relatively small compared to other Australian river catchments, the Mary River is inhabited by four sympatric, highly divergent *Cherax*

species; *C. dispar*, *C. depressus*, *C. punctatus* and *C. robustus*. Within just the Tinana Creek sub-catchment of the Mary River, the previous chapter further identified three divergent, geographically structured lineages of *C. dispar*, two of which are common within the region. Although high diversity and strong geographic structure was observed, there is potential for dispersal and gene flow in the region, with populations closely interconnected hydrologically. As a typical Australian ephemeral system, the Tinana Creek sub-catchment is also environmentally heterogeneous with variations in salinity, turbidity, water velocity and habitat type across a comparatively small area. It is this environmental heterogeneity that is believed to maintain sympatry among the four *Cherax* species, with contrasting environmental preference and life histories limiting interactions and resource competition (Johnston & Robson, 2009). This was specifically the case for *C. depressus* and *C. dispar*, two species with contrasting life histories that share a majority of their distributions but typically inhabit different microhabitats (Bartholomai, 1997). It is however unclear what contemporary mechanisms currently maintain divergence among *C. dispar* lineages. In previous research on the *C. dispar*, we hypothesised that divergence between two lineages of *C. dispar* occurred from geographic isolation and subsequent dispersal, with the region's environmental heterogeneity maintaining contemporary separation (Bentley *et al.*, 2010). This hypothesis was speculative though, with no environmental information included (Bentley *et al.*, 2010).

In this chapter a finer scale approach will be used to identify the micro-evolutionary processes currently restricting the distribution of the two sympatric *C. dispar* lineages identified in earlier chapters. By examining molecular, morphological and environmental variation among populations of *C. dispar*, this chapter aims to determine if phenotypic variation is driven by localised adaptation or isolation. In general the aim was to address the following;

1. Does phenotypic variation among *C. dispar* populations reflect the level of gene flow among them?
2. Does morphological variation among populations of *C. dispar* coincide with local environmental conditions more than genetic variation (i.e., phenotypic plasticity)?
3. Is genetic connectivity among *C. dispar* populations restricted by specific environmental conditions?
4. Do these ecological limitations coincide with that of *C. depressus*, a species capable of burrowing in drought conditions?

6.2 METHODS

Cherax individuals were collected from the Tinana Ck sub-catchment of the Mary River as per the small scale sampling design outlined in Chapter 2 (Figure 6.1). Molecular techniques, data alignment, and general population analyses were performed as per Chapter 2. More specific methods for population analyses are outlined below. To comprehensively understand the connectivity of all *C. dispar* individuals in the region, genetic and morphological analyses were performed on each *C. dispar* lineage separately as well as combined. The relationship between the genetic, morphological and environmental variation of each site was also analysed to distinguish between geographical and ecological isolation.

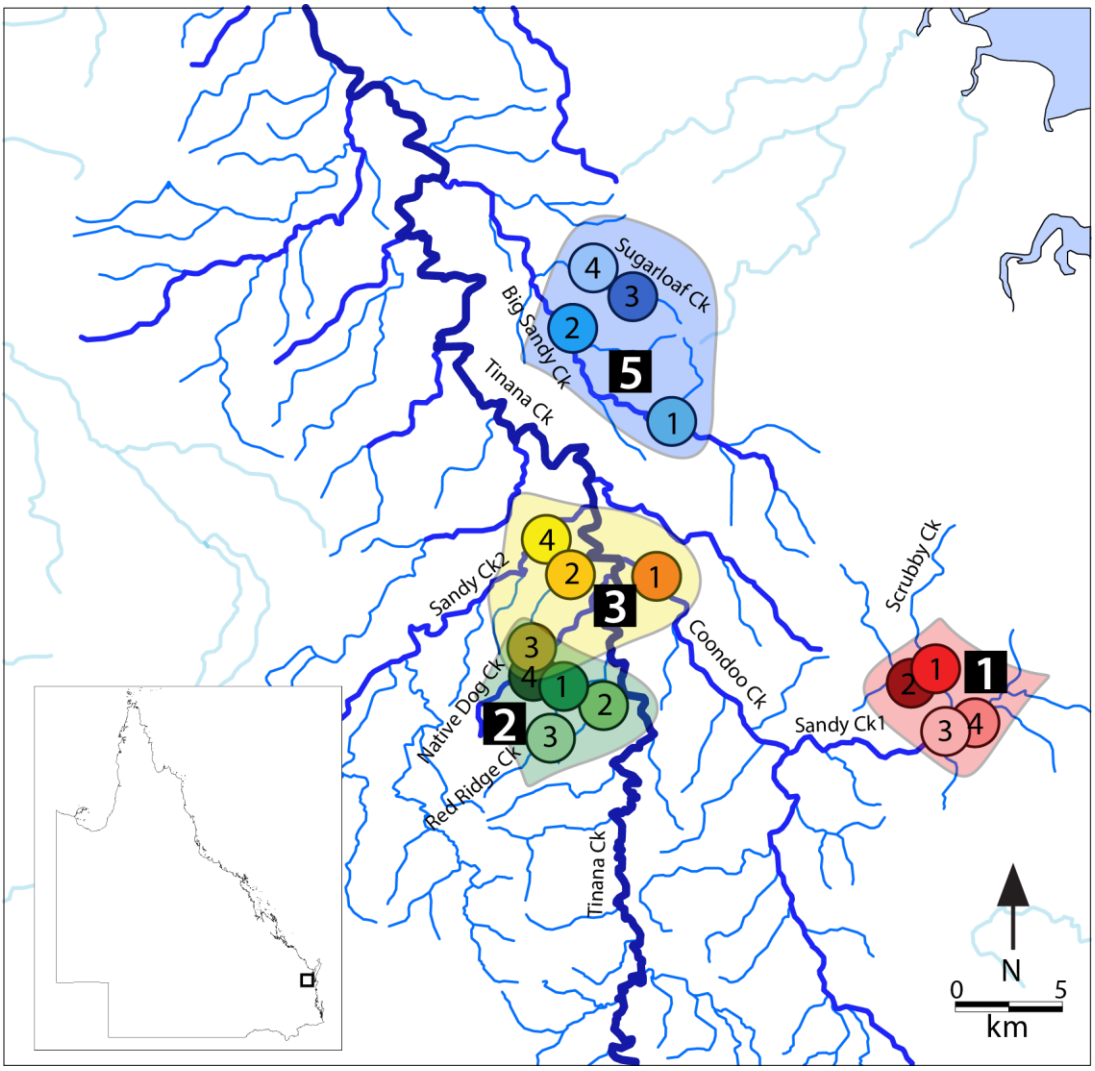


Figure 6.1: Sampling design within the Tinana Ck sub-catchment of the Mary River. Numbers in boxes and circles represent sample areas and sites respectively.

6.2.1 Morphological Measurements

During the small scale sampling of this study, a total of 119 adult *Cherax* individuals were collected whole from sixteen different sites for morphological analysis. Of these 119 *Cherax* individuals, ten were identified as *Cherax depressus* (5 male/5 female) and 109 as *Cherax dispar* (73 male/46 female). Using laboratory techniques described in Chapter 2, the 109 *C. dispar* individuals were later separated into 74 *C. dispar* A individuals and 35 *C. dispar* B individuals. A total of fifty one morphological characteristics were measured to the nearest hundredth millimetre for all *Cherax* individuals under a dissection microscope using a digital calliper. These fifty one measurements were total length (TL), orbital carapace length (OCL), areola length (ARL), areola width (ARW), thorax width (TW), thorax length (TAL), carapace length (CL), carapace depth (CD), cephalon width (CW), rostrum length (RL), rostrum width (RW), rostrum acumen length (RAL), marginal spine length (MSL), abdomen width (AbW), suborbital spine length (SSL), antennal scale length (ASL), antennal scale width (ASW), # spines on left thorax (LTS), # spines on right thorax (RTS), outer ramus length (ORL), outer ramus width (ORW), inner ramus length (IRL), inner ramus width (IRW), telson length (TEL), telson width (TEW), 1st cheliped segment length (1CL), 1st cheliped segment width (1CW), 2nd cheliped segment length (2CL), 2nd cheliped segment width (2CW), # spines on left 2nd cheliped segment (L2CS), # spines on right 2nd cheliped segment (R2CS), 2nd cheliped spine length (2CSL), 3rd cheliped segment length (3CL), 3rd cheliped segment width (3CW), propodus length (PL), propodus width (PW), propodus depth (PD), palm length (PAL), dactyl length (DL), 2nd leg length (2LL), 2nd leg propodus length (2LPL), 2nd leg propodus width (2LPW), 2nd leg dactyl length (2LDL), 3rd leg length (3LL), 3rd leg propodus length (3LPL), 3rd leg propodus width (3LPW), 3rd leg dactyl length (3LDL), 4th leg length (4LL), 4th leg propodus length (4LPL), 5th leg length (5LL) and 5th leg propodus length (5LPL) (Figure 6.2). To remove measurement bias, all measurements were taken prior to the identification of the species, lineage and sex of the individual.

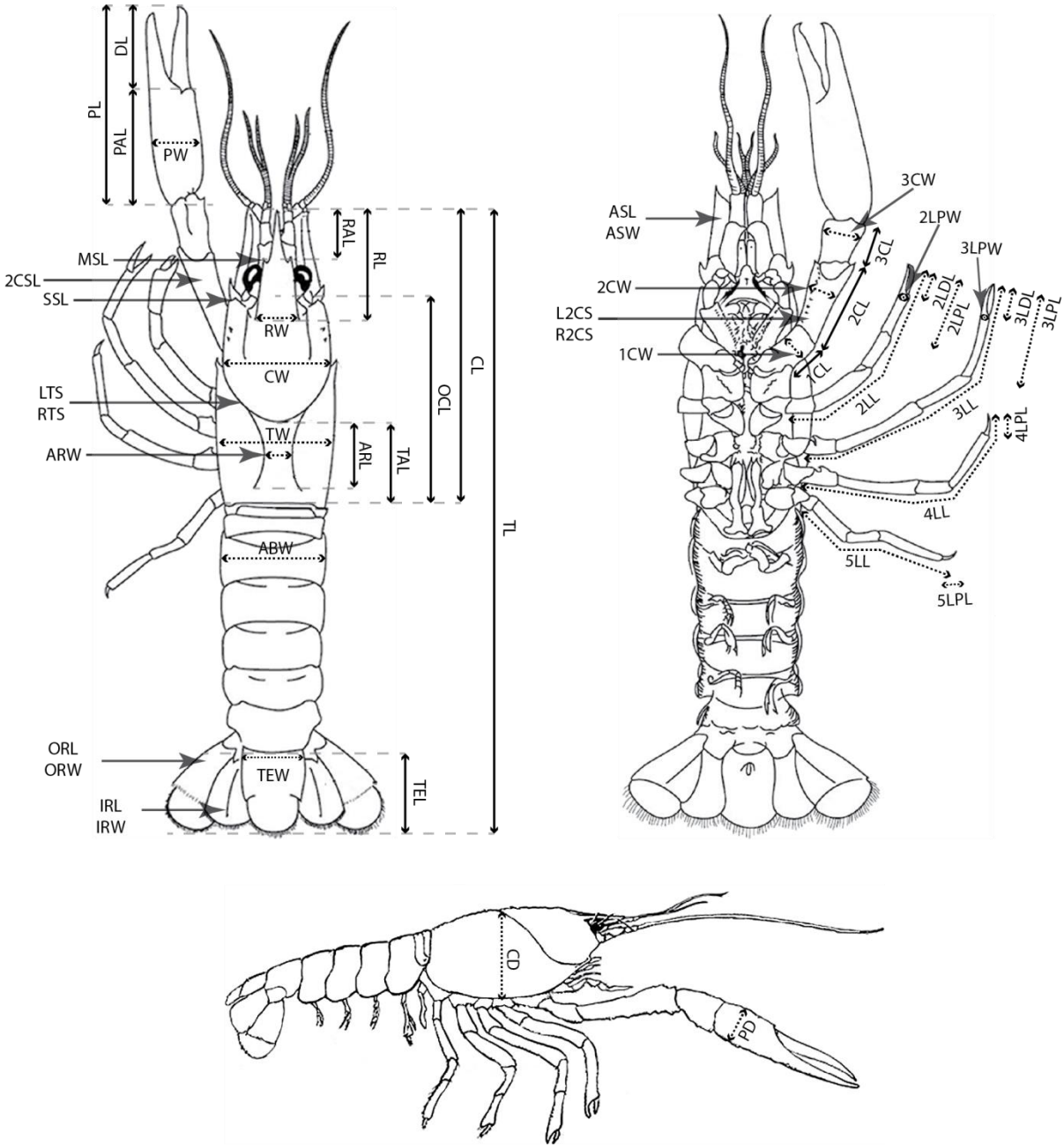


Figure 6.2: Morphological measurements taken for each *Cherax* individual. Outline drawings modified from Loughman and Simon (2011) and Hobbs (1989).

Digital photographs were also taken of the dorsal and ventral side of the chelae and the dorsal side of the cephalothorax for each individual using a Leica Microscope Camera. Digital photographs were loaded in ImageJ 1.46r (Rasband, 2012) with landmarks placed using the plugin Point Picker. A total of nine landmarks were used for the dorsal side of the chelae and seven for the ventral side (Figure 6.3). For the cephalothorax, fifteen landmarks were used, with a majority placed around the rostrum of the individual (Figure 6.3).

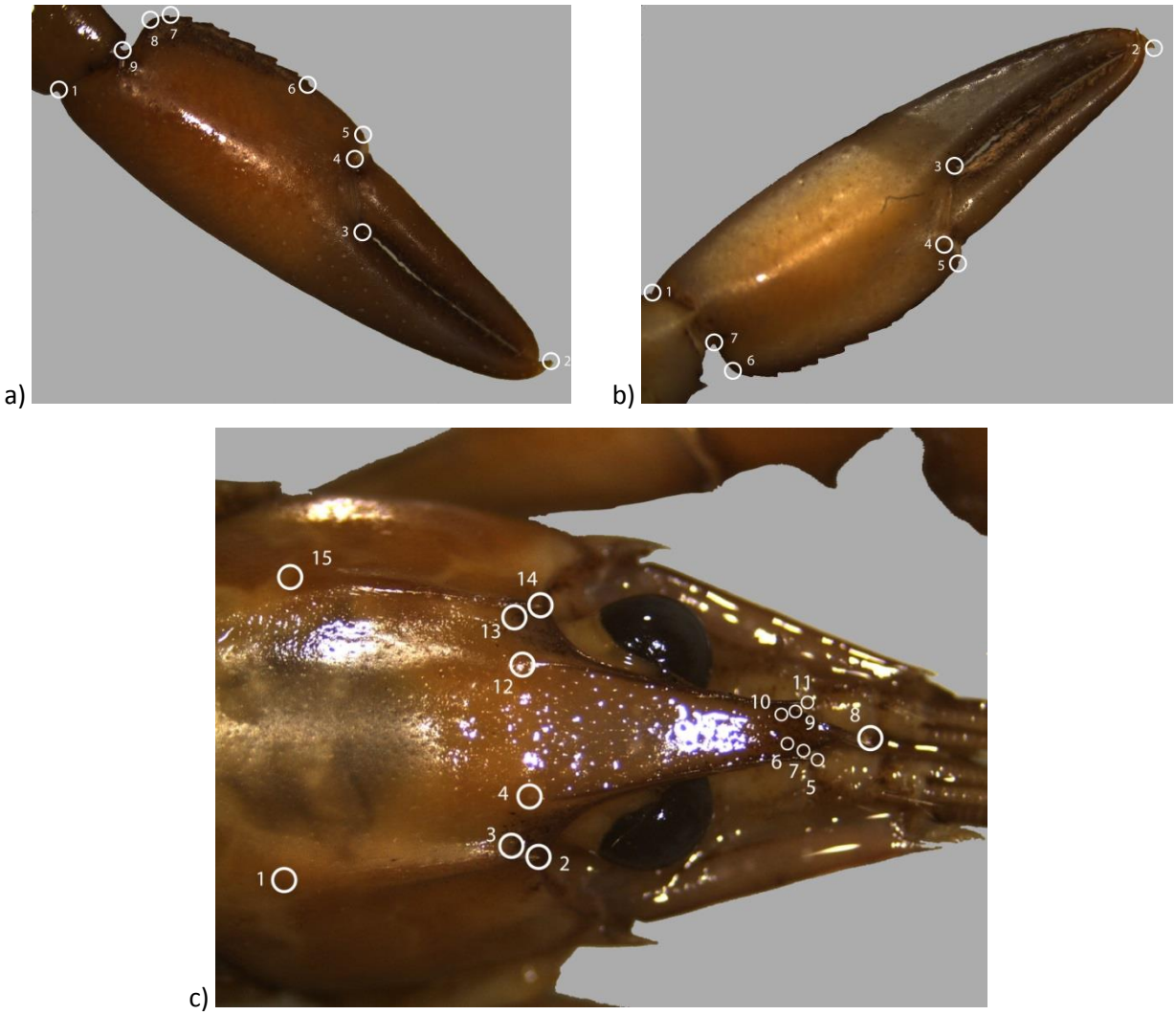


Figure 6.3: Distribution of landmarks on the a) chelae dorsal view, b) chelae ventral view and c) cephalothorax used to describe morphological variation in *Cherax* individuals.

6.2.2 Analysis

6.2.2.1 Population Structure

To investigate the spatial distribution of genetic variation in *C. dispar* and *C. depressus*, a number of AMOVA's were computed in Arlequin 3.5.1.2 (L. Excoffier & H. E. L. Lischer, 2010). As the two species are hypothesised to have contrasting dispersal behaviours, specifically within ephemeral river systems, two geographic population groupings were used. The first grouping (Areas 1-5) investigates geographic distance as a potential barrier to dispersal, with populations grouped into four regions based on their direct geographic proximity (Figure 6.1). The second grouping examines aquatic distance as a barrier, with populations grouped by their respective creeks (Figure 6.1). Due to the difficulty of sampling deep flowing creeks, no specimens were collected from the Tinana Ck main

channel. For the two analyses three hierarchical analyses were computed with the highest hierarchical level as among regions and among creeks respectively. *F*-statistics were calculated for each level of the analysis. To specifically view the relationship among all population pairs, a multidimensional scaling plot (MDS) was also constructed for each lineage/species in R 3.0.2 (R Development Core Team, 2013) using Slatkin's linearised F_{ST} between sites.

6.2.2.2 Isolation by Distance

To further understand the contemporary dispersal ability of *C. dispar* and *C. depressus* and compliment any geographic structure identified previously, a number of Mantel tests (Mantel, 1967) were performed. By investigating the relationship between geographic and genetic distance, a Mantel test identifies if distance is a significant barrier to gene flow for a species. As this chapter is restricted to a relatively small scale sampling design within a single river catchment, geographic distances used in this chapter do not imply either oceanic dispersal or dispersal across river catchment boundaries. Instead, as the populations are estimated to be currently 'connected', the geographic distances provide a more realistic measurement of the current distance needed to disperse between populations. Similar to the previous chapter, all Mantel tests were carried out in Arlequin 3.5.1.2 (L. Excoffier & H. E. L. Lischer, 2010) with 10,000 permutations using both river distance (aquatic dispersal) and Euclidean distances (terrestrial dispersal) between pairs of sites. For within lineage/species comparisons, Slatkins linearised distance ($D=F_{ST}/(1-F_{ST})$ (Slatkin, 1995) was used as the genetic distance between sites.

6.2.2.3 Environmental Analysis

While the combination of genetic and geographic information is an extremely powerful tool for the investigation of population structure in a species, it can be limited in its ability to determine the fundamental processes that maintain divergence within or between populations (Rundle & Nosil, 2005). One of the most effective methods to investigate these processes is by examining the relationship between the abiotic and biotic conditions of habitats, with variation in the species, lineages or populations present (Rundle & Nosil, 2005). This combination of environmental information and population genetics can provide insight into the influence of ecological processes on genetic variation and micro-evolutionary processes, such as gene flow, genetic drift and selection (Manel *et al.*, 2003). Species or populations can be isolated to specific habitats through competition, resource availability or an inability to survive in other conditions (Rundle & Nosil, 2005). To characterise the environmental characteristics of each site, five biotic and eight abiotic

measurements were taken at each section (pool/riffle/run) of each site; overhead branches, small wooded debris, large wooded debris, submerged tree roots, leaf litter, pH, dissolved oxygen, conductivity, turbidity, depth, temperature, velocity and salinity. A detailed description of the methods used can be found within Chapter 2.

To investigate environmental variation in the Tinana Ck region, both univariate and multivariate analyses were conducted on the biotic and abiotic characteristics separately and a combination of the two. As some freshwater crayfish are highly specialised in terms of habitat requirements (Bartholomai, 1997; McCormack, 2013), all univariate analyses were also analysed for an interaction with microhabitat (pool/riffle/run). To investigate the habitat preference of each species/lineage of *C. dispar* and *C. depressus* and allow comparison with previous genetic analyses, analysis was performed with sites grouped by the species present, the area and the creek (Figure 6.1). Sites inhabited only by *C. depressus* were excluded from analyses among areas and creeks as the environmental conditions varied significantly from other sites. In addition, to explore the effect inter-species competition has on each species, analysis was conducted on individual size and abundance across sympatric and non-sympatric sites. Analysis was performed on each variable separately with a number of ANalyses Of Variance (ANOVA). A number of pairwise t-tests comparing between groups were also performed for all significant *F* Values.

As ANOVA's can only test a single variable at a time they are extremely limited in their investigation of the overall environmental differences between sites/populations (Gauch, 1982). To achieve a more comprehensive comparison between sites, two multivariate approaches were applied; Principal Component Analysis (PCA) and Discriminant Analysis (DA). PCA uses orthogonal transformation to convert linearly uncorrelated variables into principal components. This transformation highlights the similarities and differences between each of the sites by summarising the maximum variance in each principal component. As the simplest of the eigenvector-based multivariate analyses, the PCA requires no prior information before analysis. In contrast, the DA calculates the best discriminating components (discriminants) that explain the maximum level of variance between pre-defined groups. This difference allows the identification of specific continuous variables that distinguish between the pre-defined groups (Jolliffe, 2005). All ANOVA, PCA and DA analyses were run within R 3.0.2 (R Development Core Team, 2013) with results represented using graphs created in Microsoft Excel 2007 (Microsoft, 2007).

6.2.2.4 Morphological Analysis

MORPHOMETRIC ANALYSIS

Morphological variation was investigated using both univariate (ANOVA) and multivariate (PCA, DA) methods. Prior to analysis, each length measurement was standardised using the allometric equation $Y = aX^b$ to remove the effect of variation in Total Length (X) on all measurements (Y) (Tzeng, 2004). More specifically, all length measurements were standardised according to the equation $M_S = M_0 (L_X/L_0)^b$, where M_S is the standardised measurement, M_0 is the length of the measured character, L_X is the arithmetic mean of the total length (TL) for all *Cherax* and L_0 is the standard length of each specimen. Parameter b was evaluated as the slope of regression of $\log_{10}M_0$ on $\log_{10}L_0$ using all *Cherax* in each group (Ferrito *et al.*, 2007; Lleonart *et al.*, 2000). Univariate analyses were performed on the basic body shape measurements of each crayfish, specifically the rostrum size (RL), propodus length (PL) and width (PW), orbital carapace length (OCL) and areola length (AL) and width (AW). All univariate and multivariate analyses were performed in R 3.0.2.(R Development Core Team, 2013), with pairwise *t*-tests performed for all significant *F* values. To test the plasticity of certain morphological characters to their local environmental conditions, analysis was conducted both between each species/lineage and between their geographic regions/creeks. A comparison between the two analyses provides insight into the morphological traits that distinguish each species/lineage and that vary according to the local habitat. Due to the large morphological difference between *C. dispar* and *C. depressus* and the restricted distribution of *C. dispar* B, comparisons between geographic regions and creeks were only performed for *C. dispar* A individuals.

GEOMETRIC ANALYSIS

A major limitation of traditional morphometric analysis is its reliance on a comprehensive number of measurements to achieve an accurate representation of an organism size and shape (Bookstein, 1982; Corti *et al.*, 1988; Strauss & Bookstein, 1982). Landmark-based geometric analysis instead is a relatively simple morphometric method that identifies differences among groups by comparing overall shape rather than lengths (Adams *et al.*, 2004). The overall shape of an individual is identified by optimising and rotating a number of two or three dimensional biological co-ordinates (landmarks) to remove all 'non-shape' variation. This method imposes no restriction on the direction of variation or localisation of shape changes. As the size and shape of a freshwater crayfish thorax and chelae are commonly used to identify between species, analyses in this study were restricted to the cephalothorax and chelae (ventral/dorsal) of each individual. Landmarks and semi-landmarks were defined using (Fred L Bookstein, 1996a)'s sliding semi-landmark point algorithm implemented using the Point Picker plugin in ImageJ 1.46r (Rasband, 2012). Landmarks were positioned twice for each

photograph so that human variation due to landmark placement may be removed. The configuration of landmarks was then optimised and superimposed using the generalised Procrustes method (Marcus *et al.*, 1996; Rohlf & Slice, 1990) in MorphoJ 1.05b (Klingenberg, 2011). This method translates and rotates the landmark configurations to a common origin and scales them to unit centroid size (Márquez *et al.*, 2012). Analyses were only conducted between species/lineages. Comparisons between the groups were tested using PCA and Canonical Variate Analyses (CVA) in MorphoJ 1.05b (Klingenberg, 2011). Unlike PCA, the CVA method identifies the shape features that best distinguish the pre-defined groups. Significant variation in morphological shapes was represented using an outline diagram of the region in MorphoJ 1.05b (Klingenberg, 2011).

6.2.2.5 Correlation Analysis

To distinguish the relationships among ecology, morphology and gene flow in *C. dispar* and *C. depressus* individuals, a number of Mantel and Partial Mantel tests (Mantel, 1967) were performed. Unlike the standard Mantel test, a Partial Mantel test allows for a comparison among three or more variables. This is achieved by examining the relationship between two variables while controlling for variation in the others. In this study, Mantel tests were performed to test for significant correlations between the genetic, morphological and environmental distances among *C. dispar* and *C. depressus* populations, with Partial Mantel tests used to control for the effect of spatial distance. By taking into account the effect of spatial distance, analyses can distinguish between 'causally' related or spatially structured relationships between distances. This is particularly important as neighbouring populations are expected to be relatively similar genetically, morphologically and environmentally. Similar to previous IBD analyses, both creek (aquatic) and direct (Euclidean) distances were controlled for. By investigating the relationship between each of the genetic, morphological and environmental variables, a Mantel test can identify the contemporary micro-evolutionary processes affecting *C. dispar* lineages and *C. depressus*. To distinguish if these contemporary evolutionary processes are congruent within and between *C. dispar* lineages, analyses were also performed both separately for each *C. dispar* lineage and combined. From these Mantel tests, a significant correlation between genetic and environmental distance suggests that *Cherax* dispersal is ecologically restricted by specific habitat preferences (Lowe *et al.*, 2012). If however, morphological variation is predominantly due to drift and isolation, a significant correlation between genetic and morphological distances would be expected (Merilä & Crnokrak, 2001). In contrast, if phenotypic variation is instead due to adaptation to the local environment, a correlation between environmental and morphological distances would be expected (Ramstad *et al.*, 2010). All Mantel

tests were carried out in Arlequin 3.5.1.2 (L. Excoffier & H. E. L. Lischer, 2010) with 10,000 permutations. Slatkins linearised distance ($D=F_{ST}/(1-F_{ST})$ (Slatkin, 1995) was used as the genetic distance between sites while Mahalanobis distances from previous DA analyses used for morphological and environmental distances. For analyses when the *C. dispar* lineages were combined, the conventional F_{ST} genetic distance measurement (Wright, 1943) was used. Analysis of *C. depressus* populations was also limited to comparisons of only genetic and environmental distance due to the small number of individuals for which there was morphological data.

6.3 RESULTS

6.3.1 Genetic Diversity

6.3.1.1 Cherax dispar A

A total of 107 *C. dispar* A individuals across 13 sites were sequenced for a 625bp fragment of the COI mtDNA gene (Figure 6.4). *C. dispar* A individuals were predominantly observed in Areas 1 and 3, with only three individuals sampled from within Area 5 (Appendix 8.3). Although a similar number of haplotypes were observed in Areas 1, 2 and 3 (*Hn*=7, 8 and 7 respectively), genetic (*Hd*: 0.805) and nucleotide (θ_{π}: 0.002) was highest within Area 2. Significant F_{ST} values ($p<0.05$) were observed for the majority of the *C. dispar* A pairwise population comparisons, with 47 out of 78 comparisons (60%) significant (Table 8.11). The MDS plot shows the *C. dispar* A populations generally clustered into their corresponding areas, with only Area 2 and 5 genetically indistinct (Figure 6.5). The highest degree of intra-area diversity was observed within Area 3, with A3S4 and A3S2 both genetically and geographically separated from the remaining two populations (Figure 6.4). The close affinity between the geographic and genetic distances for *C. dispar* A populations was supported with Mantels' test, with a significant correlation between genetic distance (Slatkins' linearised F_{ST}) and creek distances ($p=0.048$) (Figure 6.6). This geographic structure among *C. dispar* A populations was also supported by Analyses of Molecular Variance (AMOVA), with a significant level of genetic variation explained by both groupings (by area and creek) (Table 6.1). Contrary to the Mantel Test, the division of populations by their geographic proximity (by Area) explained a slightly higher amount of variation (35.19%; $p<0.001$) than among creek localities (30.09%; $p<0.01$).

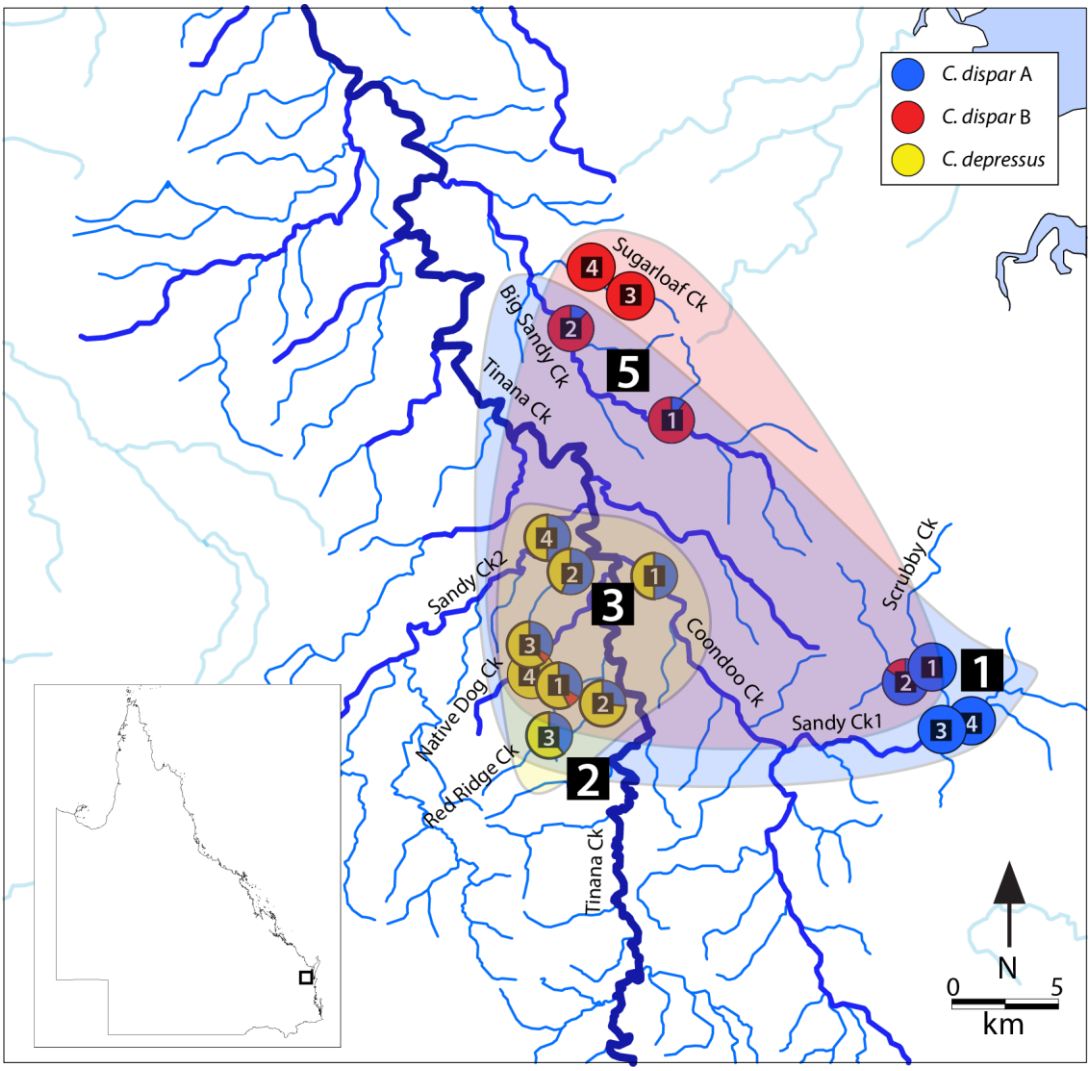


Figure 6.4: Distribution of *C. dispar* and *C. depressus* individuals within the Tinana Ck sub-catchment of Mary River. Pie charts represent percentage of each species sampled.

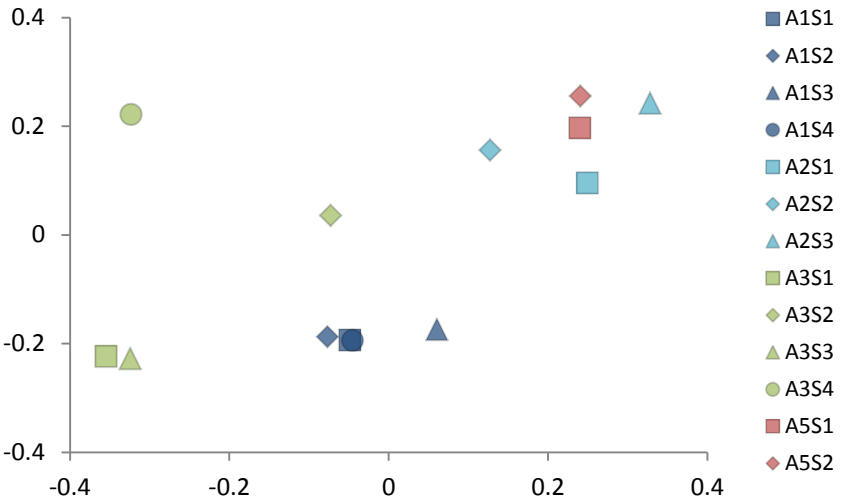


Figure 6.5: Multidimensional Scaling Plot (MDS) of mtDNA variation using F_{ST} as a measure of differentiation for 13 populations of *C. dispar* A. Population colours are assigned according to sampling region.

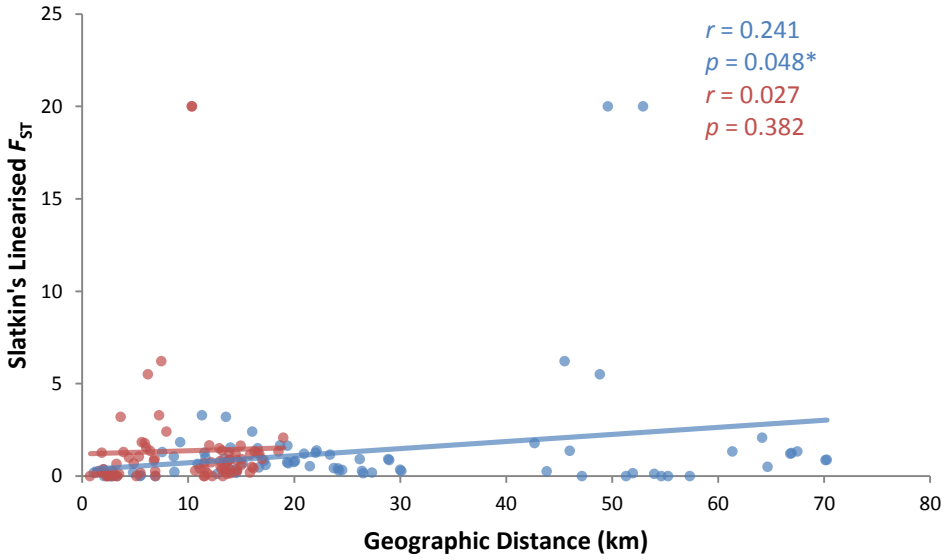


Figure 6.6: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* A populations against their corresponding Euclidean (Red) and creek (Blue) geographic distance. Line of best fit, correlation (*r*) and significance for Mantel's are presented.

Table 6.1: Analysis of molecular variance between Tinana Ck regions and creeks for *C. dispar* and *C. depressus.* Significant *F* values indicated as *p<0.05 **p<0.01 ***p<0.001.

Source	*C. dispar* A		*C. dispar* B		*C. depressus*	
	Area	*Creek*	*Area*	*Creek*	*Area*	*Creek*
Among regions (F_{CT})	0.352***	0.301**	0.944*	0.798*	0.051	0.83**
% Variation	35.19	30.09	94.36	79.82	5.13	82.99
Among populations within regions (F_{SC})	0.154***	0.157**	0.028	0.021	0.815***	0.092
% Variation	9.95	10.97	0.16	0.43	77.28	1.56
Within populations (F_{ST})	0.451***	0.411***	0.945***	0.803***	0.824***	0.846***
% Variation	54.86	58.93	5.48	19.75	17.59	15.44

6.3.1.2 Cherax dispar B

For lineage B of *C. dispar*, a total of 59 individuals across seven sites were sequenced for a 638bp fragment of the COI mtDNA gene (Figure 6.4). A majority of the *C. dispar* B individuals sampled in this study were from Area 5 with only four individuals caught outside of this region (Appendix 8.3). While each of these four individuals outside of Area 5 were genetically distinct, individuals sampled within Area 5 exhibited very low genetic (*Hd*: 0.174) and nucleotide (θ_π: 0.000) diversity (Appendix 8.3). Significant F_{ST} values were observed for only one third (7/21) of the pairwise population comparisons (Table 8.12). This low level of divergence among *C. dispar* B populations was also observed for MDS analysis of F_{ST} with all populations within Area 5 indistinct (Figure 6.7). Similar to *C. dispar* A, the population from Area 3 was also the most differentiated (Figure 6.7). A strong relationship between genetic and geographic distance was also observed for Mantels' tests, with significant correlations observed between genetic distance (Slatkins' linearised F_{ST}) and both Euclidean (p=0.01) and creek (p=0.012) distances (Figure 6.8). Similar to *C. dispar* A, strong geographic structure among *C. dispar* B populations was also supported by AMOVAs, with a significant level of genetic variation explained by both groupings (by area and creek) (Table 6.1).

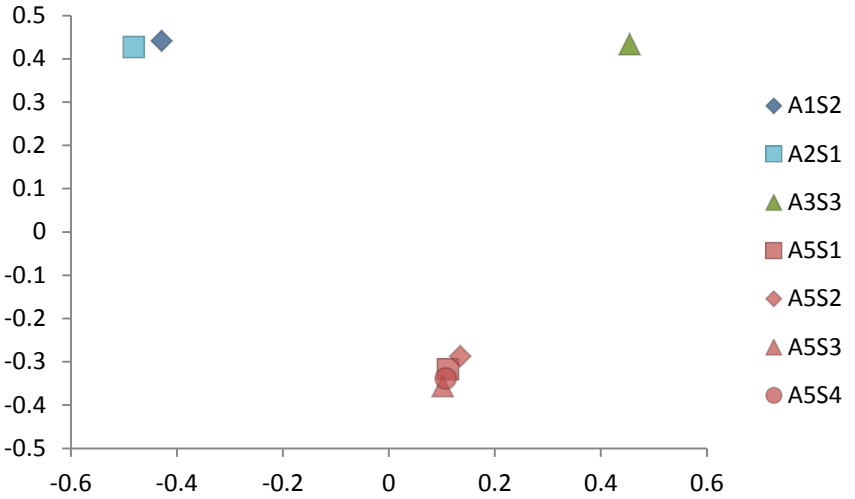


Figure 6.7: Multidimensional Scaling Plot (MDS) of mtDNA variation using F_{ST} as a measure of differentiation for 7 populations of *C. dispar* B. Population colours are assigned according to sampling region.

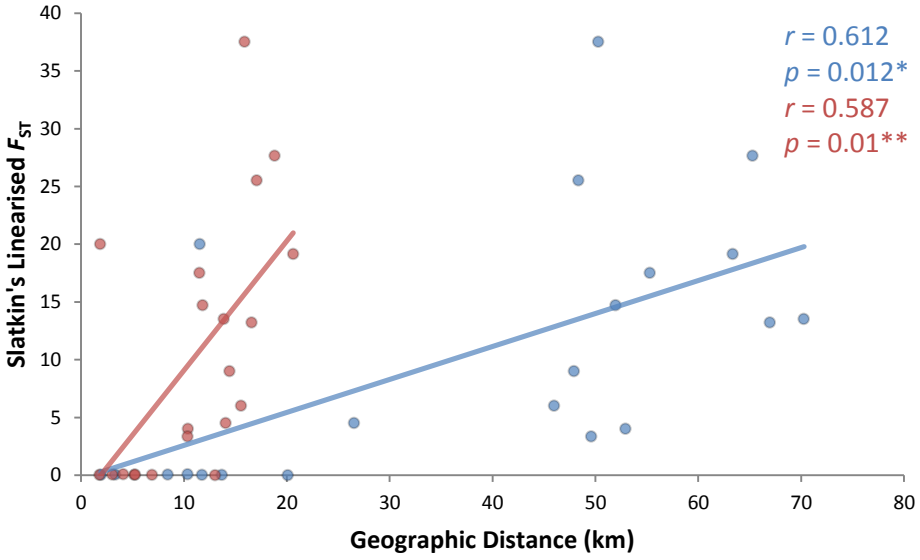


Figure 6.8: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. dispar* B populations against their corresponding Euclidean (Red) and creek (Blue) geographic distance. Line of best fit, correlation (*r*) and significance for Mantel's are presented.

6.3.1.3 Cherax depressus

A 620bp fragment of the COI mtDNA gene was sequenced from 97 *C. depressus* individuals across eight sites. Unlike *C. dispar*, *C. depressus* was not observed within Areas 1 or 5. Although a similar number of individuals were sampled from Areas 2 & 3 (49 & 48 respectively), Area 3 exhibited a far higher level of genetic (*Hd*: 0.691) and nucleotide (θ_π: 0.005) diversity, despite the fact that it contained only half the number of haplotypes (*Hn*: 4) (Appendix 8.3). Pairwise F_{ST} population comparisons within Areas 2 and 3 were significant for half of the comparisons (14/28) (Table 8.13). Similar to *C. dispar* A, *C. depressus* populations from Area 3 showed the highest level of intra-area variation with populations distributed throughout the MDS plot (Figure 6.9). A similar pattern to *C. dispar* A was also observed for the A3S1 population, with it distinct from all other Area 2 and 3 populations. Unlike both *C. dispar* lineages however, the genetic variation among the populations of *C. depressus* did not correlate with aquatic distance, with a significant Mantels result only observed for Euclidean distances (p=0.0283) (Figure 6.10).Although no correlation was observed between the genetic and aquatic distances among populations, a significant structure was observed among creeks for AMOVA analysis (p=0.007) (Table 6.1). Also, although a significant correlation between genetic and Euclidean distance was observed, no significant structure was identified among Areas (p=0.368) (Table 6.1).

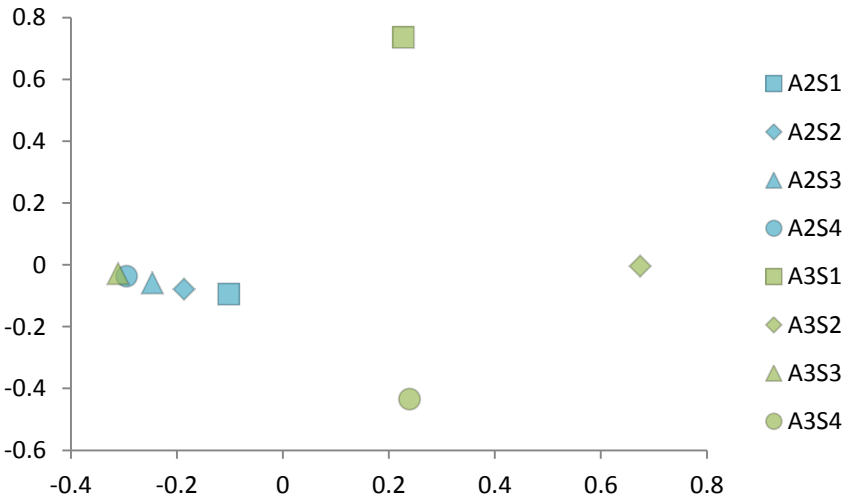


Figure 6.9: Multidimensional Scaling Plot (MDS) of mtDNA variation using F_{ST} as a measure of differentiation for 8 populations of *C. depressus*. Population colours are assigned according to sampling region.

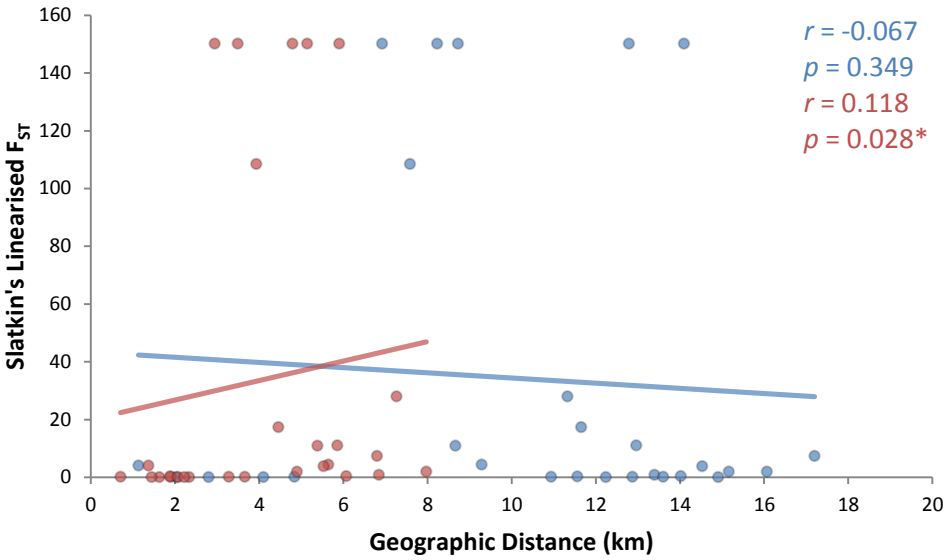


Figure 6.10: Scatterplot of Slatkin's linearised F_{ST} values between pairs of *C. depressus* populations against their corresponding Euclidean (Red) and creek (Blue) geographic distance. Line of best fit, correlation (r) and significance for Mantel's are presented.

6.3.2 Environmental Diversity

6.3.2.1 Univariate Analysis

Although all sixteen sites sampled within this study are relatively close and hydrologically connected, there was substantial variation for many of the environmental characteristics (Table 6.2). ANalyses Of VAriance (ANOVA) identified significant differences between both creeks and areas for pH, dissolved oxygen (DO), conductivity, depth, velocity and salinity (Table 6.2). For analyses of depth and velocity, a significant interaction with microhabitat was observed. In particular, pools within each Area and creek were identified as both deeper and slower than runs and riffles. For the analysis of conductivity and salinity, Red Ridge Ck and Area 2 exhibited significantly higher readings than all other creeks/Areas (Table 8.15). Along with the conductivity and salinity, the pH of Red Ridge Ck was also significantly higher than all creeks except the nearby Sandy Ck 2 and Native Dog Ck. When sites were grouped by the species present, significant differences were identified for pH, small wooded debris (SWD), submerged tree roots (STR), leaf litter (LL), conductivity, velocity, salinity and turbidity (Table 6.2), with sites inhabited by both species (*C. dispar* and *C. depressus*) significantly different from single species sites for all except STR, velocity and turbidity (Table 8.15). When sites inhabited by *C. depressus* only were removed, significant differences were observed for pH, DO and

conductivity, with only DO specifically different between sites inhabited by the two *C. dispar* lineages (Table 8.15).

Table 6.2: Analysis of variance of eleven environmental variables for four treatment groups. Significant *p* values are indicated as *p<0.05 **p<0.01 ***p<0.001. Underlined values indicate a significant interaction with microhabitat.

	Area	Creek	Species	*C. dispar* Lineages
pH	0.0001***	0.0001***	0.0001***	0.049*
Overhead Branches	0.336	0.42	0.271	0.537
Small Wooded Debris	0.377	0.086	0.048*	0.553
Submerged Tree Roots	0.737	0.756	0.029*	0.821
Leaf Litter	0.795	0.197	0.039*	0.169
Dissolved Oxygen	0.068	0.03*	0.789	0.05*
Conductivity	0.0001***	0.0001***	0.0001***	0.012*
Depth	0.0001***	0.0001***	0.088	0.858
Velocity	0.0001***	0.001***	0.02*	0.84
Salinity	0.0001***	0.013*	0.032*	0.191
Turbidity	0.501	0.059	0.0001***	0.218

6.3.2.2 Cluster Analysis

Both Discriminant Function Analysis (DA) and Principal Component Analysis (PCA) determined sites inhabited by *C. depressus* alone as the most divergent in terms of environmental variables measured (Figure 6.11). DA analysis identified turbidity as the factor contributing most to this divergence (1.00), whereas PCA also indicated velocity and salinity as significant contributors (Figure 6.11). Both analyses identified minimal differences between sites inhabited by *C. dispar* only and sites with both *C. dispar* and *C. depressus*. Both analyses identified a higher salinity and conductivity for sites inhabited by both species and higher velocity for *C. dispar* only sites (Figure 6.11). Neither of the cluster analyses could differentiate between the two *C. dispar* lineages clearly, with only DA analysis identifying a slight difference (Figure 6.11).

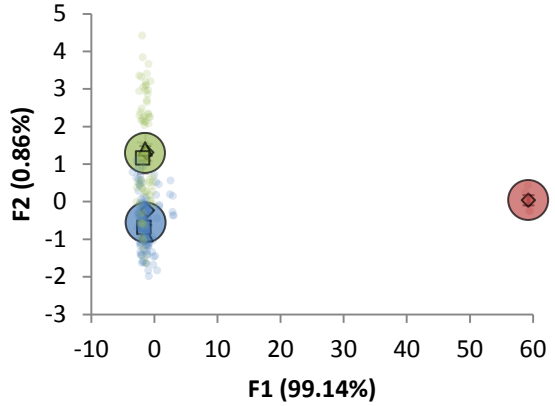
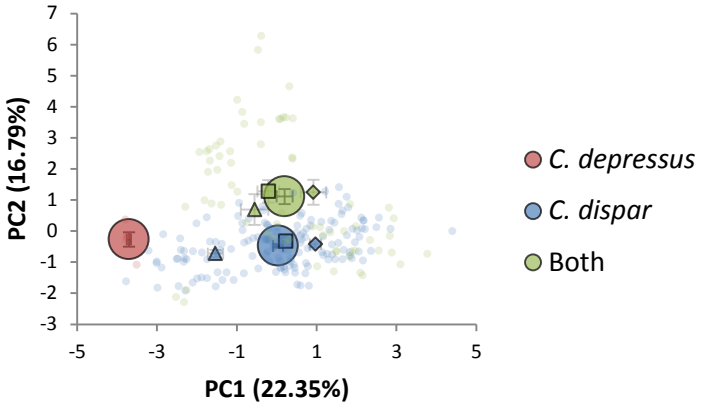
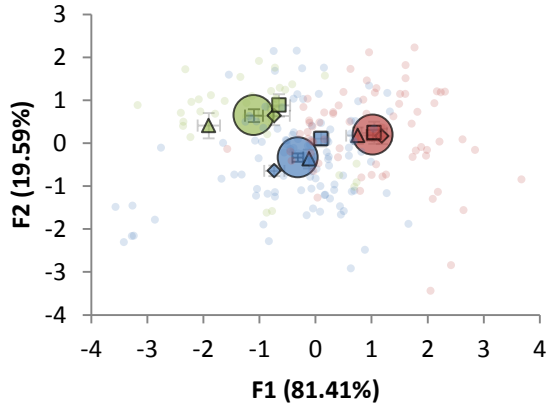
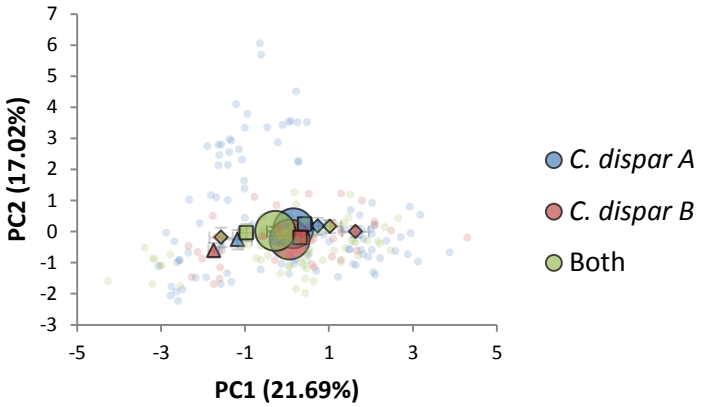
	Discriminant Function Analysis	Var	F1	F2	Principal Component Analysis	Var	PC1	PC2
Species		Turb	**1.00**	0.04		SWD	**0.81**	0.16
		pH	**0.09**	**0.44**		STR	**0.68**	*0.05*
		Temp	**0.05**	0.03		LL	**0.68**	0.12
		Sal	0.02	**0.83**		Depth	0.61	0.07
		DO	0.00	*0.03*		OB	0.49	0.10
		OB	-0.08	0.06		pH	-0.07	**0.52**
		Cond	-0.08	**0.68**		DO	-0.11	0.45
		Vel	-0.11	*-0.18*		Cond	-0.11	**0.81**
		Depth	-0.12	*-0.02*		Temp	-0.13	0.36
		LL	*-0.21*	0.35		Sal	*-0.29*	**0.81**
		SWD	*-0.23*	0.19		Turb	*-0.33*	*-0.02*
		STR	*-0.30*	0.07		Vel	*-0.49*	*-0.24*
C. dispar lineages		Sal	**0.41**	-0.04		SWD	**0.79**	0.13
		Cond	**0.36**	*-0.25*		LL	**0.69**	0.13
		SWD	**0.30**	-0.08		STR	**0.63**	*0.01*
		STR	0.19	**0.14**		Depth	0.61	*0.03*
		OB	0.07	**0.31**		OB	0.46	0.04
		Temp	0.01	-0.04		Turb	0.14	0.19
		Vel	-0.02	-0.06		pH	0.02	**0.55**
		Depth	0.06	0.05		Temp	-0.10	0.35
		DO	-0.13	**0.64**		DO	-0.12	0.43
		pH	*-0.26*	*-0.22*		Cond	*-0.15*	**0.80**
		LL	*-0.34*	-0.15		Sal	*-0.28*	**0.81**
		Turb	*-0.45*	*-0.38*		Vel	*-0.62*	*-0.27*

Figure 6.11: Discriminant Function Analysis and Principal Component Analysis of environmental variation among sites containing different compositions of species and lineages. Corresponding tables represent Factor Scores for each analysis. Values in **bold** and *italic* indicate the top and bottom three Factor Scores for each eigenvector/axis respectively. Large circles represent each group centroid with smaller square, diamond and triangle shapes signifying the centroid for the pools, riffles and runs respectively. Standard error for each point is shown using error bars. The variation explained by each eigenvector is indicated on its corresponding axis.

6.3.3 Morphological Diversity

6.3.3.1 Observational Analysis

Across the entire sampling period, a total of 1944 freshwater crayfish were sampled from eight separate trips to the region. From these sampling trips, on average a higher number of *C. dispar* individuals were caught each sampling effort than *C. depressus* (Figure 6.12). In sites where the two species were sympatric, significantly less *C. dispar* individuals were caught (Figure 6.12). Analysis of variance also identified a significant effect on individual size from species co-occurrence with both species significantly larger in sites where they were sympatric (Figure 6.12).

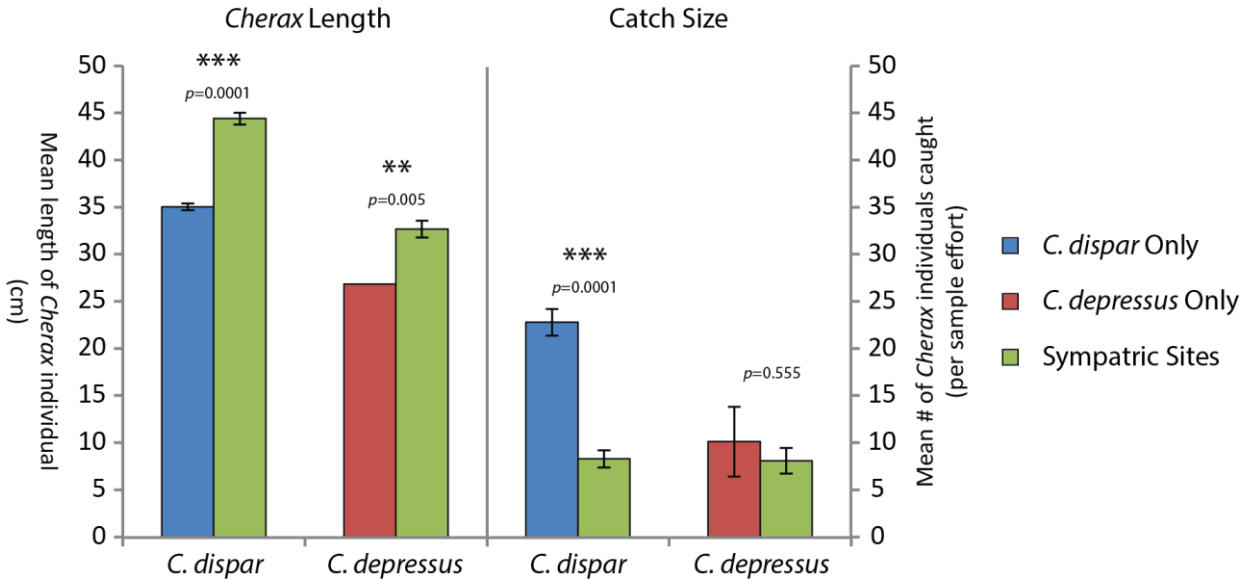


Figure 6.12: Average catch size and individual size of *C. dispar* and *C. depressus* individuals across sympatric and non-sympatric sites. Standard error and ANOVA results are represented above each comparison with significant *p* values indicated as *$p<0.05$ **$p<0.01$ ***$p<0.001$.

6.3.3.2 Morphometric Analysis

Prior to the analysis of all fifty one morphological measurements combined, fourteen basic body shape measurements were analysed using a series of ANOVA's. There were significant differences between male and female individuals for twelve morphological measurements, with only carapace length (CL), areola width (ARW) and rostrum length (RL) not significant. Eleven morphological measurements were also identified as significantly different between the species (*C. depressus* and *C. dispar* A & B); orbital carapace length (OCL), areola length (ARL), ARW, carapace depth (CD), carapace width (CW), thorax width (TW), thorax length (TAL), RL, propodus

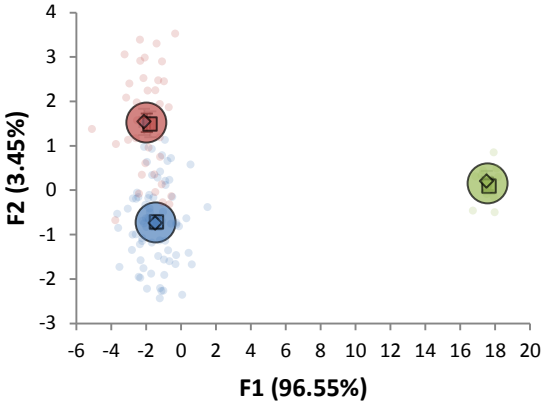
width (PW), propodus depth (PD) and dactyl length (DL). For all significant measurements, *C. depressus* individuals were significantly different to the two *C. dispar* lineages. ANOVA analysis on the OCL and CD also identified a significant effect from the sex of the individual, with male and female *C. depressus* individuals showing significantly larger OCL and CD lengths respectively (Table 8.16). Further analysis specifically on *C. dispar* showed significant differences between the two lineages for TL, ARL, CD, TAL, RL and OCL (Table 8.16), with *C. dispar* B exhibiting significantly larger TL, CD and RL measurements and *C. dispar* A larger ARL and TAL. A significant difference between the four sample areas was also observed for three of the fourteen morphological measurements; TW, TAL and DL. The TW of individuals was significantly wider for Area 2 and thinner for Area 1. Area 3 was distinguishable by a smaller DL and Areas 2 & 3 possessed longer TAL than individuals from Area 1 & 5. Analyses of variance identified the morphological measurement ARL as significantly different among creeks, in addition to the previous three measurements (TW, TAL and DL). On average, individuals from Native Dog Ck had longer areolas (ARL), individuals from Red Ridge Ck were wider (TW) and the thoraxes were longer (TAL) in Native Dog Ck, Sandy Ck 2 and Red Ridge Ck (Table 8.16).

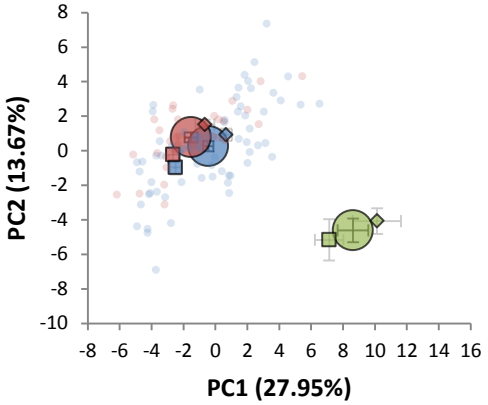
Both cluster analysis methods effectively distinguished between the two species; *C. depressus* and *C. dispar* (Figure 6.13). The contributing factors for this distinction differed between analyses however, with DA analysis identifying TAL, 2LPW, ARL and 3LPW, and PCA analysis 3CW, 2CW, PD and PW. PCA analysis failed to differentiate between the two *C. dispar* lineages both with *C. depressus* individuals included and excluded (Figure 6.13). PCA analysis did differentiate between the male and female *C. dispar* individuals with females longer (TL, RAL, ASL, RL) and males having larger claws (2CW, PL, PW, PD). Unlike PCA analysis, DA analyses successfully distinguished between the two *C. dispar* lineages for both analyses. When *C. depressus* individuals were included, *C. dispar* A exhibited larger TAL, CL, PW and TW and *C. dispar* B longer RW, IRL, 4LPL and TL (Figure 6.13). When *C. depressus* individuals were excluded, both the *C. dispar* lineages and their sexes were distinct. Similar to PCA analysis the two sexes differed along the x axis (F1) with females longer (TL, IRL, ORL, RL) and the claws of males larger (3CL, PL, 1CW, PD). The two *C. dispar* lineages instead differed along the y axis (F2) with *C. dispar* A having larger bodies (TAL, CL, OCL and IRW) and *C. dispar* B longer rostrum and head measurements (ASL, RAL, MSL, RL).

PCA analysis on the morphological variation across the four areas did not clearly identify distinct differences among the areas, with only individuals from Area 3 appearing to be slightly differentiated (Figure 8.2). In contrast, DA analysis effectively isolated the three areas, with each area highly divergent (Figure 8.2). The separation of individuals from Area 3 was generally due to

their larger body sizes, with individuals having a longer and wider thorax (ARL, TAL, TW). Similar to the variation across the three areas, PCA analysis also had difficulty identifying morphological variation among the creeks (Figure 8.2). DA analysis however, grouped Native Dog Ck and Sandy Ck 2 together based on their larger body sizes (ARL, TW, TAL) (Figure 8.2). The most unique creek identified from DA analysis was Red Ridge Ck, where individuals had longer walking legs (4LL, 3LL) and chelae (DL).

Species

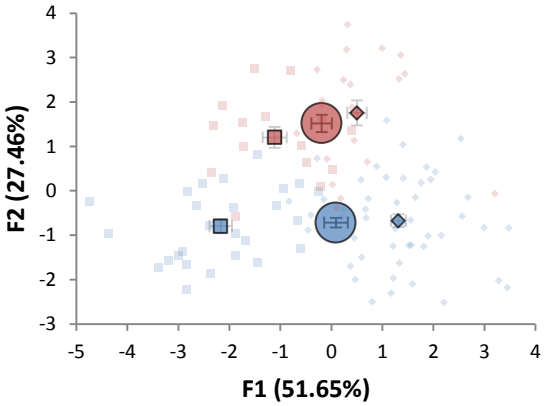


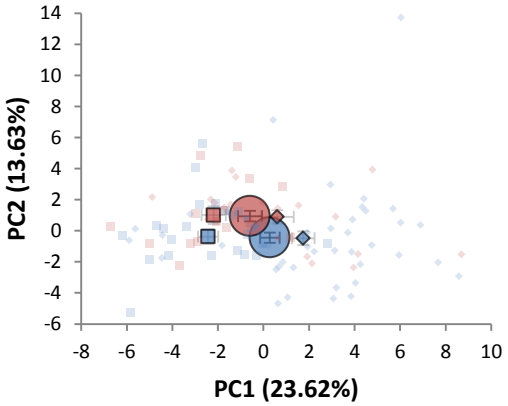


Discriminant Function Analysis	Var	F1	F2
	TAL	**0.75**	*-0.21*
	2LPW	**0.72**	0.14
	ARL	**0.70**	-0.10
	LTS	*-0.75*	-0.05
	RAL	*-0.76*	0.25
	RTS	*-0.76*	-0.04
	RW	0.032	**0.39**
	IRL	-0.16	**0.30**
	4LPL	-0.09	**0.26**
	PW	0.44	*-0.15*
	CL	0.165	*-0.18*

Principal Component Analysis	Var	PC1	PC2
	3CW	**0.92**	0.08
	2CW	**0.92**	0.16
	PD	**0.89**	0.20
	RAL	*-0.70*	0.44
	RL	*-0.73*	0.49
	ASL	*-0.75*	0.55
	2CL	0.34	**0.77**
	2CSL	-0.32	**0.63**
	PAL	0.51	**0.61**
	CD	0.23	*-0.31*
	ORL	0.22	*-0.31*
	ARL	0.71	*-0.38*

C. dispar lineages

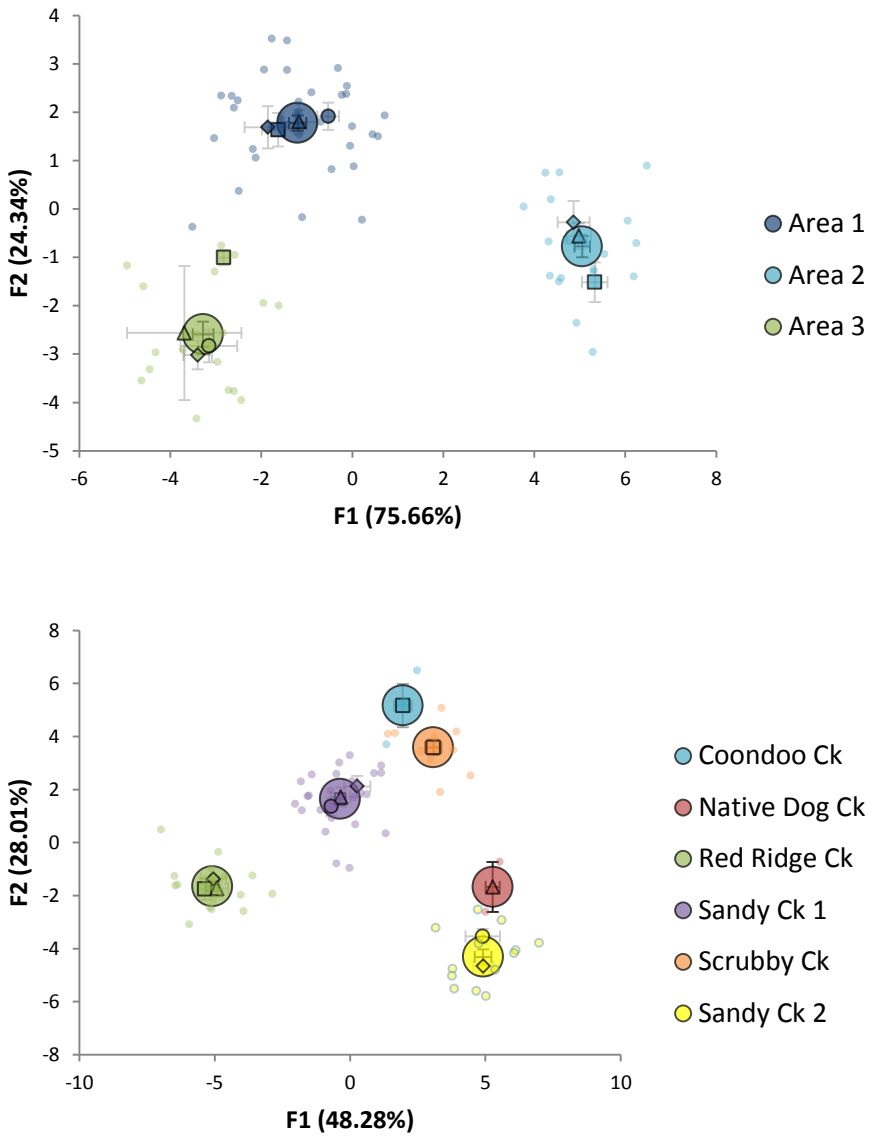




Discriminant Function Analysis	Var	F1	F2
	3CL	**0.64**	-0.05
	PL	**0.61**	-0.04
	1CW	**0.56**	0.02
	ORL	*-0.25*	0.21
	TL	*-0.42*	0.21
	IRL	*-0.45*	0.28
	ASL	-0.16	**0.48**
	RAL	-0.13	**0.43**
	MSL	-0.10	**0.37**
	OCL	0.27	*-0.19*
	CL	0.13	*-0.20*
	TAL	0.35	*-0.35*

Principal Component Analysis	Var	PC1	PC2
	2CW	**0.93**	*-0.20*
	PL	**0.92**	-0.15
	PW	**0.92**	*-0.26*
	PD	0.91	*-0.25*
	RL	-0.32	**0.64**
	ASL	*-0.33*	**0.72**
	RAL	*-0.34*	0.46
	TL	*-0.62*	0.52
	3LL	0.19	**0.63**

Figure 6.13: Discriminant Function Analysis and Principal Component Analysis of morphological variation among sites containing different compositions of species and lineages. Corresponding tables represent Factor Scores for each analysis. Values in **bold** and *italic* indicate the top and bottom three Factor Scores for each eigenvector/axis respectively. Large circles represent each group centroid with smaller square and diamond shapes signifying the centroid for the females and males respectively. Standard error for each point is shown using error bars. The variation explained by each eigenvector is indicated on its' corresponding axis.



Var	F1	F2
DL	**0.361**	0.271
TW	**0.306**	*-0.338*
4LL	**0.293**	**0.454**
3LDL	*-0.075*	0.283
IRL	*-0.112*	0.264
LTS	*-0.255*	0.085
4LPL	0.151	**0.483**
2CSL	0.052	**0.397**
ARL	0.181	*-0.261*
TAL	0.147	*-0.468*

Var	F1	F2
LTS	**0.280**	0.118
IRL	**0.087**	0.321
2LPW	**0.029**	-0.121
4LL	*-0.382*	**0.459**
3LL	*-0.395*	0.388
DL	*-0.459*	0.135
4LPL	-0.19	**0.437**
ASL	-0.248	**0.427**
ARL	-0.057	*-0.208*
TW	-0.167	*-0.432*
TAL	-0.008	*-0.473*

Figure 6.14: Discriminant Function Analysis of *C. dispar* A morphological variation among sites within different Areas and Creeks. Corresponding tables represent Factor Scores for each analysis. Values in **bold** and *italic* indicate the top and bottom three Factor Scores for each eigenvector/axis respectively. Large circles represent each group centroid with smaller square, diamond, triangle and circle shapes signifying the centroid for the four sites respectively. Standard error for each point is shown using error bars. The variation explained by each eigenvector is indicated on its' corresponding axis.

6.3.3.3 Geometric Analysis

Similar to morphometric analysis, geometric analysis of shape variation identified a significant difference between the claws and rostrum of *C. dispar* and *C. depressus* (Figure 6.15). In general, both the claw and body shape of *C. depressus* individuals were significantly broader and the rostrum of *C. dispar* significantly longer (Figure 6.15). Geometric differences between the two *C. dispar* lineages were not as obvious however, with a significant distinction between the lineages observed only for the rostrum (Figure 6.15). Geometric analysis of the shape variation in the rostrum showed that *C. dispar* B has a significantly longer rostrum than *C. dispar* A.

6.3.4: Local Variation in C. dispar and C. depressus

A total of thirty Mantel and Partial Mantel's tests were performed on the environmental, morphological and genetic variation between *C. dispar* and *C. depressus* population pairs (Table 6.3). From the thirty tests, the strongest correlation was observed between the genetic and morphological variation of *C. dispar* A with a significant result observed when variation in geographic distance was included and excluded (p<0.006) (Table 6.3). A significant correlation for *C. dispar* A populations was also observed between environmental and morphological variation, but only when variation in aquatic distance was controlled for (p=0.033). In contrast to *C. dispar* A, *C. dispar* B populations showed a negative correlation between genetic and morphological variation (Figure 6.16) with a significant relationship instead observed between genetic and environmental variation; when variation in geographic distance was removed (p<0.02). Similar results were also observed when the two lineages were combined with a significant relationship observed between genetic variation and both environmental (p=0.003) and morphological (p=0.001) variation when creek distance was controlled for. Unlike *C. dispar* B, no correlation between genetic and environmental variation was observed for *C. depressus*.

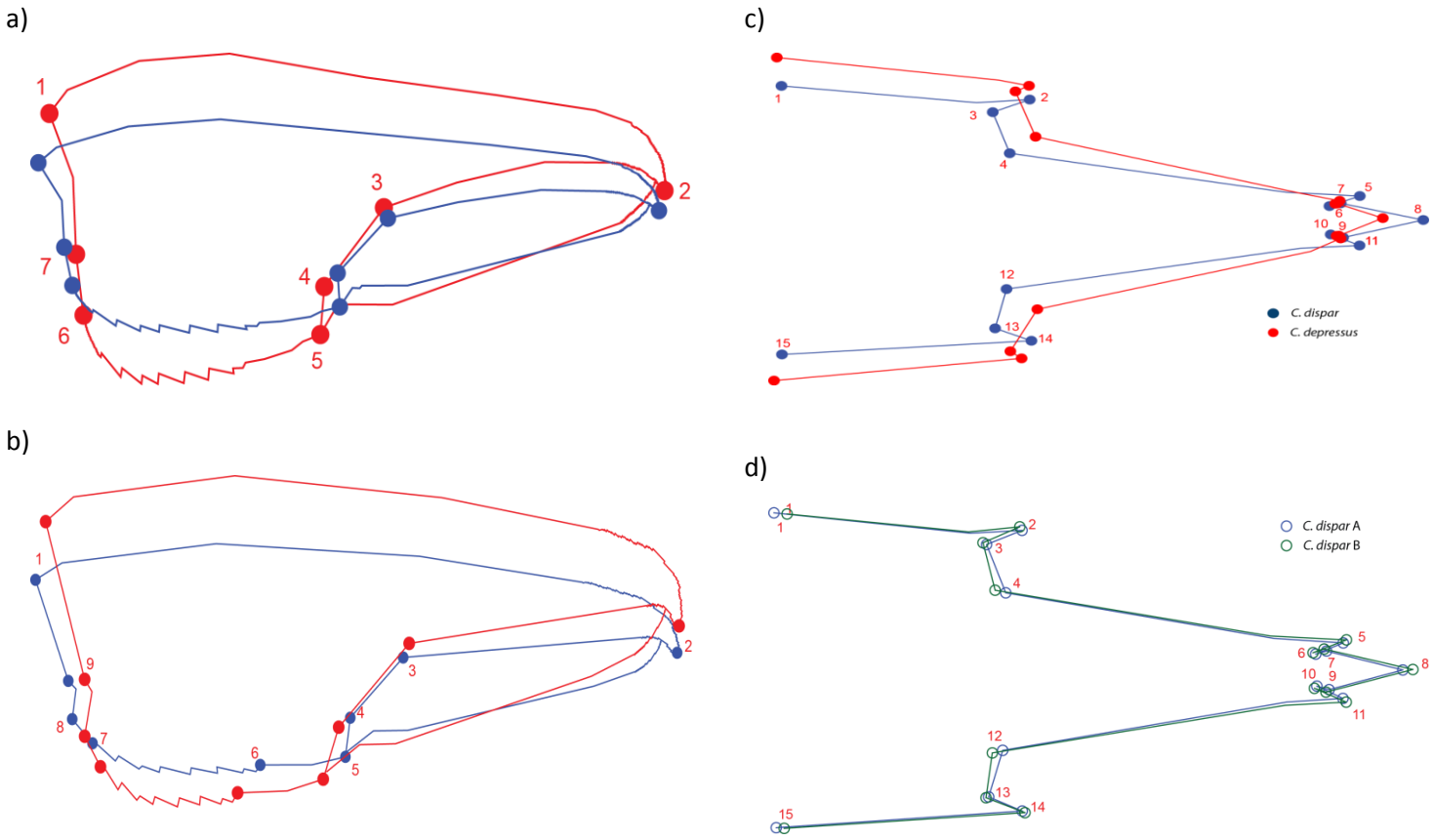


Figure 6.15: Warped outline comparison of *C. dispar* and *C. depressus* a) claw (ventral) b) claw (dorsal) and c) rostrum. d) Warped outline comparison of *C. dispar* lineages rostrum. Dots and circles represent the landmark points selected for geometric analysis. Warped outlines illustrate changes in the average CV score for each species/lineage.

Table 6.3: Mantel and Partial Mantel test results for comparisons between genetic (Slatkins F_{ST} and F_{ST}), morphological (Morph) and environmental (Env) distance among *C. dispar* lineages and *C. depressus* populations. Results from Partial Mantel tests are separated according to the geographic distance controlled for (Creek or Euclidean). Significant values indicated as *p<0.05 **p<0.01 ***p<0.001.

Species	Comparison	*Mantel Test*		*Partial Mantel Tests*			
				Creek Distance		Euclidean Distance	
		p	*r*	*p*	*r*	*p*	*r*
C. dispar A	Slatkins F_{ST}-Env	0.451	-0.048	0.502	0.018	0.493	-0.046
	Slatkins F_{ST}-Morph	0.001***	0.419	0.006**	0.375	0.001***	0.42
	Env-Morph	0.084	0.34	0.033*	0.415	0.096	0.344
C. dispar B	Slatkins F_{ST}-Env	0.111	0.505	0.004**	0.601	0.014*	0.579
	Slatkins F_{ST}-Morph	0.372	-0.004	0.327	-0.022	0.635	0.011
	Env-Morph	0.208	0.568	0.201	0.566	0.211	0.476
C. dispar combined	F_{ST}-Env	0.353	0.04	0.003**	0.402	0.15	0.114
	F_{ST}-Morph	0.358	-0.054	0.001***	0.409	0.173	0.11
	Env-Morph	0.083	0.318	0.098	0.279	0.085	0.315
C. depressus	Slatkins F_{ST}-Env	0.487	-0.009	0.494	0.001	0.478	0.016

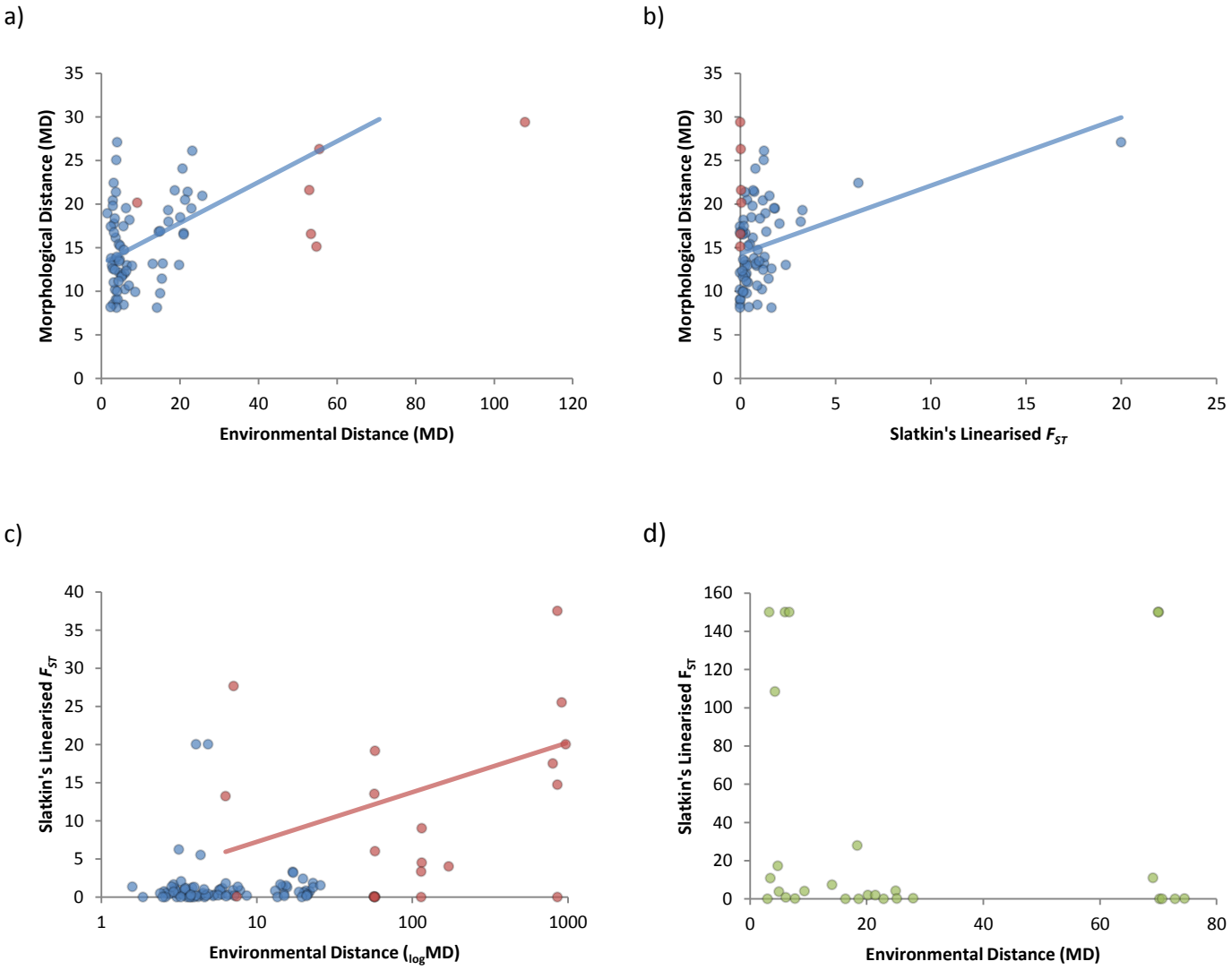


Figure 6.16: Scatterplot comparison of distance between pairs of *C. dispar* populations for a) Morphological and environmental Mahalanobis distances, b) Slatkin's Linearised F_{ST} and morphological Mahalanobis distance, c) Slatkin's linearised F_{ST} and environmental Mahalanobis distance and d) Slatkin's linearised F_{ST} and environmental Mahalanobis distances between pairs of *C. depressus* populations. Line of best fit is presented for each significant relationship. Blue, red and green points represent *C. dispar* A, *C. dispar* B and *C. depressus* populations respectively.

6.4 DISCUSSION

6.4.1 Gene Flow and Genetic Structure

Considering the relatively small geographic range of the present study (30km), *C. dispar* demonstrated comparatively restricted contemporary gene flow among populations, with significant structure observed among the pre-defined areas and creek localities for both lineages. Although freshwater crayfish are not likely to be constrained entirely to river networks, with terrestrial dispersal possible (Lodge *et al.*, 2000), most studies on freshwater crayfish identify unique structure among river catchments rather than within (Bentley *et al.*, 2010; Fetzner & Crandall, 2003; Hughes & Hillyer, 2003; Smith & Smith, 2009). *C. dispar* showed significant geographic structure among populations within a single river catchment (Mary River), further supporting the suggestion of a very low dispersal capability for the species. Restricted dispersal ability for *C. dispar* was also supported by analyses of IBD, with significant correlations between genetic and aquatic distance observed for both lineages. While *C. dispar* B also showed a significant correlation with geographic (Euclidean) distance measurements, the difference between the two distance measurements for the lineage was minimal, indicating river channels as a more appropriate avenue for contemporary dispersal (Fetzner & Crandall, 2003). This strong geographic structure in *C. dispar* was most prevalent in populations within Area 5, with populations from the region unique for both lineages.

Interestingly, although Area 3 populations are geographically proximate, a number were particularly divergent from other populations in the region. The identity of these populations differed depending on the lineage of focus, with A3S4 divergent for *C. dispar* A and A3S3 for *C. dispar* B. This patchy structure in Area 3 was also observed for *C. depressus*, but with all four populations divergent. Geographically, no clear pattern was observed for these four populations, with two near the main channel (A3S1 and A3S2), one nearby to other populations (A2S1) and the last on the outer edge of the region (A3S3). In contrast to both *C. dispar* lineages, a significant IBD correlation for *C. depressus* was only observed with Euclidean geographic distance (Figure 6.10). *C. dispar* and *C. depressus* have been hypothesised to have contrasting life histories and dispersal abilities (Bentley *et al.*, 2010; Short, 2000). This contrasting life history may explain the different patterns of geographic structure between the two species with dispersal across-land more common in *C. depressus*.

6.4.2 Micro-evolutionary drivers of phenotypic variation

Morphological characteristics are still recognised as the most useful and common method for differentiating between species (Hillis & Wiens, 2000). While this method is very useful for naming and identifying species across the entire taxonomic spectrum, it fundamentally relies on the assumption that shared morphological characteristics portray shared evolutionary history (Mayr, 1942). This however is not always the case for freshwater crayfish, with a large number of freshwater crayfish species continuously re-classified (McCormack, 2013; Riek, 1951, 1969, 1972; Sokol, 1988), predominantly post-molecular analysis (Austin, 1996; Austin & Knott, 1996; Austin *et al.*, 2003; Larson *et al.*, 2012; Munasinghe *et al.*, 2004b; Nguyen & Austin, 2005; Shull *et al.*, 2005). The major difficulties faced when distinguishing between species of freshwater crayfish include irregular growth from ecdysis, convergent evolution of morphological traits (Munasinghe *et al.*, 2004a) and phenotypic plasticity in variable environmental conditions (Austin & Knott, 1996; Riek, 1969; Sokol, 1988). These issues were not of concern when identifying between *C. dispar* and *C. depressus* with both morphometric and geometric analysis clearly distinguishing between them. In general, *C. depressus* individuals had a significantly larger and wider body with a shorter rostrum. These differing morphological traits coincide with those identified by Riek (1969). Along with a larger body, geometric analysis also identified a significantly wider chela on *C. depressus*, a trait that is characteristic of freshwater crayfish with a burrowing life history (Schultz *et al.*, 2009).

Although not as clear as the distinction between *C. dispar* and *C. depressus*, both morphometric and geometric analysis identified a significant difference in morphology between *C. dispar* A and *C. dispar* B. In general DA analysis identified a larger rostrum on *C. dispar* B and a larger body on *C. dispar* A. Unlike the other analyses, PCA analysis could not distinguish between the two lineages, with a majority of the variation instead between sexes. Not surprisingly, the chela size was identified as significantly larger for *C. dispar* males than females. As male freshwater crayfish use their chelae for aggressive territorial fights (McCormack, 1994), predator protection (Stein, 1976) and reproductive activities (Snedden, 1990), the size of their chelae is likely to be under selective pressure (Snedden, 1990; Stein, 1976). Surprisingly, Stein (1976) identified large chelae in male freshwater crayfish as essential only for reproductive activities, with chela size reduced in non-mating males. In contrast, the telson length was identified as significantly larger for *C. dispar* females. Similar to the chela size, this morphological characteristic may also be under strong selection with females with a longer telson possibly able to hold more fertilised eggs during the reproductive process (McCormack, 1994). Although the two lineages were identified as morphologically distinct, a correlation between morphological and genetic distances was not

observed in analyses when the two *C. dispar* lineages were combined (Table 6.3). A correlation was however observed when variation in aquatic geographic distance was removed, suggesting that phenotypic variation is fundamentally driven by genetic distance (gene flow) and not geographic proximity (Merilä & Crnokrak, 2001). This insignificant correlation with geographic variation included was surprising, with freshwater crayfish expected to be genetically and phenotypically adaptive to their local environment. In particular, phenotypic variation often coincides with an environmental cline, such as in birds (Lehtonen *et al.*, 2009), mammals (Storz, 2002) and fish (Bradbury *et al.*, 2006).

Even though the two *C. dispar* lineages share a relatively recent evolutionary history (Chapter 3), the current micro-evolutionary processes affecting phenotypic variation appear to differ between them. Similar to the combined *C. dispar* analysis, *C. dispar* A showed a significant correlation between morphological and genetic distances. This correlation was observed when variation in geographic distance was both included and excluded, suggesting that IBD and drift do not play a major role in the phenotypic variation of the lineage. While phenotypic variation in *C. dispar* was attributed solely to genetic variation, a correlation was also observed between phenotypic and environmental variation for *C. dispar* A when aquatic distance was accounted for. Such a pattern might be expected when the morphology of a species adapts to specific environmental conditions such as temperature, salinity, water flow, or other factors that influence local optimal morphology (Mayr 1963, James 1970 (See Storz)). Two environmental characteristics that appeared to influence phenotypic variation in *C. dispar* A were salinity and pH. While the relative effect of salinity and pH on freshwater crayfish has been frequently studied (Kendall & Schwartz, 1964; Malley, 1980; Zanotto & Wheatly, 1993), most research focused on the survival limits for introduced species rather its effect on morphology (Mills & Geddes, 1980). Studies on aquaculture freshwater crayfish species however identified a significant reduction in Calcium absorption and overall size/growth of crayfish in low salinity and acidic environments (Kendall et al 1964, Wheatly & Gannon 1995, Zanotto et al 1993). A similar effect was also observed in this study with individuals from the saline and high pH Red Ridge Ck, Native Dog Ck and Sandy Ck 2 exhibiting significantly longer and wider bodies than individuals in other creeks. While this suggests that salinity and pH had an effect on the morphology of Tinana Ck freshwater crayfish, it cannot be ignored that other natural contributing factors that were not measured may have also influenced their morphology (Edwards *et al.*, 2009; Jones *et al.*, 2007).

One such natural ecological process that is not always accurately represented within a controlled setting is inter-species competition (Capelli & Munjai, 1982; Gherardi & Daniels, 2004). While it can be difficult to completely gauge the effect competition has on a species within its natural environment, studies have identified a significant influence on species distributions (Capelli & Munjai, 1982; Hill & Lodge, 1999; Pigot & Tobias, 2013), survival (Hanshew & Garcia, 2012; Hill & Lodge, 1999) and morphology (Haddaway *et al.*, 2012; Naspleda *et al.*, 2012). While there did not appear to be any significant effect from *C. depressus* on the distribution of *C. dispar*, a significant effect was observed on the morphology and abundance of *C. dispar* (Figure 6.12). This was particularly evident with *C. dispar* individuals significantly longer and wider but less abundant in regions where the two species co-inhabit (Figure 6.12). This result is not surprising with extreme levels of physical competition common between two sympatric species that may share resources (Capelli & Munjai, 1982; Gherardi & Daniels, 2004). While we cannot rule out other contributing factors for this reduction in the size and abundance of *C. dispar* individuals, further laboratory research on the behavioural reaction of *C. dispar* to the presence of other freshwater crayfish could test this hypothesis.

6.4.3 Ecological barriers in a heterogeneous system

Through variation in life history traits, freshwater crayfish distributions are often ecologically restricted from specific habitats or ecological conditions (Barbaresi *et al.*, 2007; Usio, 2007). Ecology-related variables that limit freshwater crayfish distributions include abiotic conditions (Kutka *et al.*, 1996; Usio & Townsend, 2000), refuge availability/substratum type (Barbaresi *et al.*, 2007; Benvenuto *et al.*, 2008; Johnston & Robson, 2009; Kutka *et al.*, 1996; Rabeni, 1985; Usio & Townsend, 2000), altitude (Growns & Marsden, 1998) and habitat type (Jones *et al.*, 2007). For sympatric species, differences in life history traits may allow two species to co-occur, with each species able to make use of specific micro-habitats and resources (Benvenuto *et al.*, 2008; Clavero *et al.*, 2009). This was the case for *C. dispar* and *C. depressus* within the Tinana Ck region of Mary River. As a 'burrowing' species, *C. depressus* is capable of surviving in ephemeral river systems, where water flow can cease completely resulting in very high turbidity and drought. Although *C. depressus* is able to survive in these conditions, the species is also commonly observed and capable of surviving in less turbid, flowing creeks. While *C. depressus* appears to be able to inhabit a wide range of habitats, there appeared to be a restriction on the species' distribution, with *C. depressus* missing from Areas 1 and 5. Unlike Areas 2 and 3, these two regions are low in turbidity and high in velocity. While velocity did not appear to be a primary limitation on the dispersal of *C. depressus*, an ecological consequence of high velocities, such as reduced leaf litter, heavier substratum type or

differing macrophyte diversity may have inhibited their survival (Nakagawa, 2013). A similar impact from high velocities and depth was also identified by Nakata *et al.* (2003) who noticed a significant effect on the burrow choice in the Japanese crayfish. As only a relatively limited number of ecological variables were measured in this study, it cannot be dismissed that the *C. depressus* distribution may instead be limited by an environmental variable not measured, such as substratum type (Johnston & Robson, 2009). Interestingly, although *C. dispar* has a limited capability to burrow, it appeared to show a higher adaptability to the aquatic conditions in the region, with only one shallow (5cm), stagnant and highly turbid site uninhabited by the species (A2S4).

Similar to analysis between the two species, a clear ecological distinction between the *C. dispar* lineages was also supported in this study, with a significant correlation between genetic and ecological variation observed when variation in aquatic geographic distance was removed. This significant correlation only when geographic variation was controlled for suggests that gene flow in *C. dispar* individuals is fundamentally driven by ecological variation, not geographic proximity. This result is particularly surprising with a strong geographic structure observed for both *C. dispar* lineages. Instead this result may have arisen from a relatively recent re-colonisation of *C. dispar* B into the region, with each species predominantly inhabiting different regions and habitats (*C. dispar* A; Area 1, *C. dispar* B; Area 5). Studies on the artificial introduction of exotic species provide ideal examples to compare the effect of recent re-colonisation events, with repercussions ranging from complete extinction (Capelli & Munjai, 1982; Hill & Lodge, 1999; Hughes *et al.*, 2003), alteration of life history (Fawcett *et al.*, 2010; Hanshew & Garcia, 2012), hybridisation (Lawrence *et al.*, 2000; Lodge *et al.*, 2012) and co-inhabitancy (Jackson *et al.*, 2014; Jones & Bergey, 2007). This competitive interaction is possible, with *C. dispar* A almost completely absent from Area 5, the region closest to the remainder of *C. dispar* B's distribution (Chapter 5). The relatively slow encroachment of *C. dispar* B throughout Tinana Ck may however be due to the poor dispersal ability or ecological limitations identified for the lineage. Further research using fast evolving microsatellite loci may shed more light on the issue and identify the contemporary dispersal patterns of the two lineages.

CHAPTER 7: GENERAL DISCUSSION

7.1 EASTERN CHERAX PHYLOGENY AND ITS IMPLICATIONS ON CURRENT TAXONOMY

Since the advent of the taxonomic system, morphological variation has been the preferred tool to identify new species (Hillis & Wiens, 2000) and interpret their past evolutionary history (Riek, 1972). While still crucial for taxonomic classification, the applicability of morphological variation to construct freshwater crayfish phylogenies is problematic. As the morphological differentiation among freshwater crayfish species relies heavily on either specific morphological characteristics or an accumulation of small morphological variants (Riek, 1951, 1969), interpretations are strongly influenced by convergent evolution (Holdich, 2002). This is particularly apparent across crayfish with similar life history traits; for example burrowing species typically exhibit reduced abdomens and larger, broader chelae (Crandall & Buhay, 2008). Molecular techniques however, are fast becoming a favoured method to locate and identify potential new taxonomic species (Doyle, 1992). A molecular approach to taxonomic identification still however has a number of inherent issues, including the choice of an 'appropriate' species level of molecular divergence (Blaxter, 2004) and applicability in identification of species in the field. A molecular approach instead is more appropriate as a supporting or pre-cursor tool to the taxonomic process, using genetic information as an independent test on the current taxonomic/systematic classifications (Dayrat, 2005). It is this combination of morphological and molecular methods that were used in this study to investigate the current Eastern *Cherax* taxonomy.

Currently a total of eighteen taxonomic *Cherax* species and one sub-species have been identified along the Australian Eastern coast (McCormack, 2013). Of these eighteen species, six could not be obtained or analysed and so were not investigated further. While a majority of the remaining twelve species were supported by phylogenetic analysis, *C. depressus* and *C. cairnsensis* were not. Although taxonomically delineated, phylogenetic analysis did not support their taxonomic distinction at the species level, with very low mtDNA divergence and paraphyly observed for all nuclear genes. This weak taxonomic distinction was further supported, with two closely related species on a nearby coastal island more morphologically and genetically distinct than *C. depressus* and *C. cairnsensis*, two species distributed more than 1000km apart (Coughran *et al.*, 2012). This result suggests that Riek

(1951) original taxonomic identification of a single species across the entire Queensland coast may be more appropriate, with genetic divergence currently occurring due to geographic separation. Unlike the *C. depressus* group, a large number of previous studies have focused on the morphological, phylogenetic and taxonomic status of the *C. destructor* complex (Austin, 1996; Munasinghe *et al.*, 2004b; Nguyen *et al.*, 2005; Riek, 1969). While the lack of morphological analysis in this study limits taxonomic interpretations, the phylogenetic results support the taxonomic delineation by Austin *et al.* (2003) of *C. setosus* and *C. rotundus*. Phylogenetic results do not however support the separation *of C. destructor* into two separate taxonomic species (Riek, 1969), instead observing low divergence and sympatry (Austin, 1996; Hughes & Hillyer, 2003; Munasinghe *et al.*, 2004b; Nguyen & Austin, 2005).

In contrast to the *C. depressus* group, phylogenetic analysis suggested that *C. dispar* and *C. cuspidatus* should be divided into a number of new species, with five highly divergent lineages identified within each. Previous research on *C. cuspidatus* by Austin (1996) and Munasinghe *et al.* (2004b) suggested the identification of a new species (*C. sp. nov*) within SEQ, with the nominal taxon distributed within Northern NSW. Although a similar genetic differentiation was observed in this study (Chapter 3), results did not coincide with the geographic boundaries previously identified. Instead *C. cuspidatus* A (nominal taxon) was genetically similar to *C. cuspidatus* B (SEQ lineage) but divergent from three other SEQ lineages (*C. cuspidatus* A-C). While this does support the suggestion by Munasinghe *et al.* (2004b) of the potential for a new taxonomic species, it does not support a clear geographic separation between them. As the specific genetic lineage of individuals analysed by Austin (1996) for morphology is unclear, a delineation into distinct taxonomic species from this study would be speculative at best. It is clear however that as a single highly diverse species, *C. cuspidatus* warrants further research.

Since its initial identification over sixty years ago (Riek, 1951), the single species classification of the *C. dispar* complex has been under scrutiny (Austin, 1996; Bentley *et al.*, 2010; Munasinghe *et al.*, 2004b; Riek, 1951). In particular, Riek (1951) suggested an additional two sub-species (*C. dispar elongatus* and *C. dispar crassus*) distributed in Fraser Island and Caboolture respectively. Similarly, a north/south split was also supported by Austin (1996) and earlier research (Bentley *et al.*, 2010), with a distinction between the Mary and Brisbane River for molecular, electrophoretic and morphometric analysis. While phylogenetic results support the distinction of a lineage from Fraser Island (Bentley *et al.*, 2010), the addition of new individuals did not support a distinction between individuals from Caboolture and the Brisbane River (nominal taxon). Instead, the two genetically

distinct lineages identified in the 'north' within the Mary River and Fraser Island region (Bentley 2010), were further supported morphologically (Chapter 6). The lack of diversity in the north observed by Riek (1951) and Austin (1996) also did not contradict these morphological and phylogenetic results, with sampling in both studies restricted to regions inhabited by only a single lineage. Although morphological analysis in this study was limited geographically, the clear distinction observed between the two northern lineages (*C. dispar* A & B) suggests that a more comprehensive and broader taxonomic study would more than likely identify the lineages taxonomically.

Interestingly, although Riek (1951) and Austin (1996) both suggested a north/south split within *C. dispar*, previous phylogenetic analysis on the species instead observed the highest level of genetic divergence between the islands of Moreton Bay and the mainland (Bentley *et al.*, 2010). Unlike previous analysis, this strong divergence was supported by both mtDNA and nuclear phylogenies, with mtDNA divergences at a similar level to most other species (Grandjean *et al.*, 2000; Schultz *et al.*, 2007). In addition, phylogenetic analysis also identified a new divergent monophyletic lineage (*C. dispar* E) from the mainland of Moreton Bay (Tingalpa). The restricted distribution for *C. dispar* D & E may explain the limited research on the lineages, with most previous studies not sampling within the region (Austin, 1996; Riek, 1951, 1969). Although Riek (1951) did sample Moreton Bay individuals, he did not differentiate them from the nominal taxon. He did however note that Moreton Island samples exhibited a different morphology with a blunt sternal keel and longer thinner body. While phylogenetic analysis supports a distinction for Moreton Bay individuals, further morphological analysis is needed before a taxonomic classification can be made.

7.2 HISTORICAL CONNECTIVITY OF AUSTRALIAN CHERAX

Since its separation from Antarctica, the Australian landscape has undergone a substantial climatic shift towards a more arid and temporal climate (Martin, 2006; Steffen *et al.*, 2009). This shift has had a particular effect on the survival and dispersal of Australia wide freshwater species such as *Cherax*, with distinct bio-regions identified in the South-West, North and East of Australia. While the historical timeline for the separation of these regions is uncertain (Schultz *et al.*, 2009; Toon *et al.*, 2010), it is clear that dispersal among the regions has been restricted for at least 25 million years. This historically restricted dispersal across central Australia is also evident in a wide range of taxa including birds (Toon *et al.*, 2003), fish (Unmack, 2001), freshwater crayfish (Horwitz & Adams, 2000; Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009) and plants (Hopper & Gioia, 2004). For a number of

these species, the Nullarbor Plain appeared to be the most significant barrier for dispersal, with species from South-West Australia both highly diverse and endemic (Munasinghe *et al.*, 2004a; Schultz *et al.*, 2009). In addition to the limited dispersal across central Australia, biogeographic and phylogeographic analysis of *Cherax* also identified a highly restricted dispersal ability for the genus (Chapter 5). This was specifically the case in SEQ species, with a majority of the species' restricted to a single river catchment or biogeographic region. Historical dispersal events across river catchments for *Cherax* species may therefore indicate a relatively major biogeographic event or change in conditions.

With a third of the Australian *Cherax* species currently distributed within SEQ, it was not surprising that SEQ (Mary River specifically) was identified as a freshwater crayfish biodiversity hotspot (Whiting *et al.*, 2000) and the most likely origin for the group. Although eastern Australian *Cherax* are distributed along almost the entire eastern coastline, dispersal appeared significantly inhibited by the GDR. Of the eastern Australian species groups, only the *C. destructor* complex exhibited a relatively strong 'terrestrial' dispersal ability, with at least three major inter-basin dispersal events across the GDR estimated. The central Australian origin that was identified for the *C. destructor* group suggests a major east to west dispersal event across the GDR from SEQ approximately 8mya. Although this connection was also observed for a number of freshwater fish (McGlashan & Hughes, 2001a; McGuigan *et al.*, 2000; Musyl & Keenan, 1996; Unmack, 2001), turtles (Baggiano, 2012) and frogs (McGuigan *et al.*, 1998), dispersal across the range appeared inhibited far earlier for *Cherax* (Miocene). From central Australia, the *C. destructor* ancestor was also estimated to have dispersed across the GDR from West to East in the Hunter River region (Jerry, 2008; Thacker *et al.*, 2007) and North to South in the Wimmera River region (Schultz *et al.*, 2007). Although these dispersal events suggest a more 'terrestrial' dispersal path, these connections were also observed in other freshwater crayfish (Austin *et al.*, 2003; Crandall *et al.*, 1999; Munasinghe *et al.*, 2004a, 2004b; Schultz *et al.*, 2007), fish (Jerry, 2008; Adam D Miller *et al.*, 2004; Thacker *et al.*, 2007; Unmack, 2001) and shrimp (McClusky, 2007). This suggests that dispersal across these regions is most likely due to a major biogeographic event such as river rearrangement (Haworth & Ollier, 1992; Ollier & Pain, 1994) rather than 'terrestrial' dispersal.

Similar to the *C. destructor* complex, the *C. depressus* complex also exhibited a relatively large geographic range (1200km), with a distribution across most of the Queensland coastline. Unlike *C. destructor*, inter-catchment connectivity was estimated to have been maintained until relatively recently with two sister groups within the complex both showing a NQ (*C. cairnsensis* and *C. parvus*) to SEQ (*C. depressus* and *C. punctatus*) break. This multi-species connectivity across the entire Queensland coastline was surprising, as dispersal through the region crosses eighteen coastal river catchments. While water-borne dispersal across the region can be accomplished with fewer dispersal events, the biogeographic pattern of the group resembles that of amphidromous crustaceans (Cook *et al.*, 2012) and saltwater taxa (Chenoweth *et al.*, 2002; Haig *et al.*, 2010), suggesting an oceanic dispersal method. Even though amphidromous crustaceans can utilise water flow and ocean currents to disperse juveniles along the coastline (Cook *et al.*, 2012; Ford & Kinzie III, 1994; Luton *et al.*, 2005), this is unlikely the case for *C. depressus*. More specifically, both phylogeographic and population analysis of *C. depressus* identified a preference for low salinity conditions, with the species only distributed in the upper reaches of SEQ and absent from high salinity populations in the Mary River catchment (Chapter 6). Similarly, if *C. depressus* was able to utilise oceanic currents for juvenile dispersal, populations would be expected on a number of the coastal islands. In contrast, no individuals from the entire *C. depressus* complex were observed on any coastal island. Although it is unclear how the current biogeographic pattern has arisen, further sampling along the Queensland coastline should shed more light on the species' history.

Within SEQ specifically, river catchments appeared to be the most limiting factor for *Cherax* dispersal. This was particularly the case for *C. dispar* and *C. cuspidatus*, which each exhibiting strong genetic differentiation among river catchments. Although the Mary River catchment is estimated to have been the origin for all eastern *Cherax*, a historical broad distribution as far south as the Logan-Albert River catchment approximately 10mya was estimated prior to the divergence of most SEQ species. This Late-Miocene connection between SEQ river catchments was also observed in forest-restricted frogs (McGuigan *et al.*, 1998), suggesting the climate may have been both cooler and wetter during this period. Of all the SEQ species, only *C. dispar* and *C. robustus* (islands only) maintained a SEQ wide distribution with *C. cuspidatus* and *C. depressus* estimated to have reduced their distribution to only the Logan-Albert and Mary River catchments respectively. This high connectivity among SEQ river catchments for *C. dispar* was only observed for lineage C, with individuals observed along the entire SEQ coastline. Similar SEQ wide distributions have been observed in some other freshwater taxa (Chenoweth & Hughes, 2003; Murphy & Austin, 2004; Page, von Rintelen*, et al.*, 2007b; Unmack, 2001), with some studies suggesting a historic river paleo-

drainage parallel to the coastline (Hughes *et al.*, 1999; Page & Hughes, 2007a). Unlike *C. dispar*, the precise biogeographic history of the *C. cuspidatus* complex was difficult to estimate, with sampling and distribution estimates relatively poor. In particular, it was unclear what mechanisms have driven the high sympatry observed within the Logan-Albert River Catchment for the five *C. cuspidatus* lineages. A more comprehensive sampling effort however may provide a better understanding of their history, and possibly identify the pathway at which the species crossed the McPherson Range into Northern NSW.

7.3 DOES DISPERSAL ABILITY REFLECT LIFE HISTORY IN TWO SYMPATRIC *CHERAX*

As identified previously, the strong structure of freshwater landscapes are particularly limiting on the dispersal of freshwater taxa, with mountain ranges (Calsbeek *et al.*, 2003) and oceans (Benstead *et al.*, 2003) predominantly constraining freshwater taxa within river basins (catchments) and major drainage divisions (watersheds) (Bohonak & Jenkins, 2003; Poff *et al.*, 1997). Variations in the effect of these extrinsic barriers on dispersal across multiple species are therefore suggested to be caused by 'intrinsic' factors such as species-specific responses to local environments and different life history traits (Havel & Shurin, 2004; McMillen-Jackson & Bert, 2003; Poff *et al.*, 1997). One such prominent life history trait that may affect dispersal of freshwater crayfish is the ability to burrow down into the water table (Schultz, 2009). Although the ability to burrow does not appear to be homologous (Crandall & Buhay, 2008), its frequency across freshwater crayfish genera implies an inherent importance for the survival of the species (Crandall & Buhay, 2008; Schultz *et al.*, 2009). It is unclear however, what effect this trait has on the ability for a species to disperse. Two species that provide an ideal opportunity to explore this effect are *C. dispar* and *C. depressus*. As both species are estimated to have originated within the Mary River catchment but possess contrasting burrowing capabilities. Therefore variations in their biogeographic and phylogeographic structures may be due to their burrowing capabilities.

Even though biogeographic and phylogeographic results similarly identified river catchment boundaries as significant barriers to dispersal for both species, the SEQ distributions of the species differed significantly. This was particularly evident in SEQ's coastal regions with *C. depressus* only observed within the upper reaches of the mainland river systems and absent from all four coastal sand islands. Surprisingly, although both species were distributed across multiple river catchments,

phylogeographic analysis identified a relatively low dispersal ability and significant isolation by distance effect for each species. While this implies both species are capable of dispersal across river catchment boundaries, the geographic pathway taken may differ between them. In particular, the significant relationship between Euclidean and genetic distance identified for *C. depressus* in the Mary River catchment suggests that the ability to burrow may permit more frequent over-land dispersal than would be expected for an obligate freshwater species (Schultz, 2009). This capability to disperse 'terrestrially' has been documented in other freshwater crayfish (Lodge *et al.*, 2000; Schultz, 2009), including *Cherax* (Gouws *et al.*, 2006; Nguyen *et al.*, 2004) and *Paranephrops* (Apte *et al.*, 2007). More specifically, the ability to burrow and disperse terrestrially is suggested to have facilitated the dispersal of *C. destructor* throughout central Australia and across the GDR on a number of occasions (Munasinghe *et al.*, 2004a; Nguyen *et al.*, 2004).

While contrasting dispersal abilities can significantly alter the distributions of species, it does not appear to be the only cause for the disjunct distributions in this study. As *C. depressus* is absent from SEQ's coastal regions and islands, salinity may also be a contributing factor. The negative effect salinity has on the distribution and survival of freshwater taxa has been extensively studied both globally (Kelly *et al.*, 2006; Kendall & Schwartz, 1964) and within Australia (Mills & Geddes, 1980; Nielsen *et al.*, 2003; Williams & Williams, 1991). In particular, salinity is suggested to have an adverse effect on larval survival (Nielsen *et al.*, 2003) and the moulting process (Wheatly & Gannon, 1995) of freshwater crayfish. Macro-crustaceans however are estimated to be one of the most tolerable freshwater taxa to changes in salinity (Nielsen *et al.*, 2003). This inconclusive effect from salinity was also observed for *C. depressus* with the species distributed 1200km and eighteen coastal river catchments away from its' closest relative (*C. cairnsensis*). This biogeographic pattern is similar to amphidromous and saltwater taxa (Cook *et al.*, 2012; Haig *et al.*, 2010), suggesting that the species may have dispersed along the Queensland coastline relatively recently. Neither species however has been observed on any of the nearby coastal islands, indicating another factor is most likely restricting the *C. depressus* distribution. For a burrowing species, the principal substrate type may also be an inhibiting factor on the distribution of the species (Schultz, 2009). As all four SEQ coastal islands and regions are predominantly sand, this may hinder the ability of *C. depressus* to form burrows (Horwitz & Richardson, 1986; March & Robson, 2006). This effect of sandy substrate was also observed on the SEQ mainland with *C. depressus* absent from highly sandy regions within the Mary River catchment. This inability to seek refuge may expose *C. depressus* to high flow or high predatory conditions that are not ideal for the species (Horwitz & Richardson, 1986). Similarly,

March and Robson (2006) identified a higher density of *Engaeus* and *Geocherax* crayfish burrows in regions where the soil was least compacted.

Although substrate type appears to have a significant impact on the distribution of *C. depressus*, it is most likely not the only contributing factor. This is particularly evident as *C. depressus* is capable of surviving in artificially set up conditions with either sand or no substrate (Bentley pers. Obs.). Instead, the disjunct distribution of *C. depressus* is mostly likely due to an interaction of a number of factors. One additional factor that was not investigated comprehensively in this study is the presence of *C. dispar*. As *C. dispar* is a highly mobile and aggressive species (Wilson *et al.*, 2007), it may outcompete *C. depressus* in habitats where *C. depressus* is unable to burrow and seek refuge. While a direct effect of their co-inhabitancy was not observed, a significant effect on the size and abundance of *C. dispar* was, with *C. dispar* individuals larger and less abundant when both species co-inhabited. Capelli and Munjai (1982) identified a similar interaction between inter-species competition and substrate type with the level of shelter displacement among *Orconectes* species dependent on the available substrate. As neither species seem to co-inhabit sandy habitats, it is difficult to completely gauge the effect competition has on their distribution and survival. Further laboratory research using a similar approach to Capelli and Munjai (1982) for *C. depressus* may provide greater insight into this hypothesis.

7.4 CONCLUSION

Although freshwater crayfish are distributed worldwide and have managed to adapt to a wide range of environmental niches, their ability to disperse appears relatively limited. This restricted dispersal and strong geographic structuring of freshwater crayfish has been observed across all Australian genera (Ponniah & Hughes, 2004; Schultz *et al.*, 2009; Toon *et al.*, 2010) and suggests a high level of localised adaptation. With the freshwater ecosystem now recognised as one of the most endangered ecosystems in the world (Dudgeon *et al.*, 2006), this restricted ability to disperse to new habitats is of concern for the future of Australian freshwater crayfish. Threats to Australian freshwater crayfish can be grouped into five categories; overexploitation (Benstead *et al.*, 2003), water pollution (Aparicio *et al.*, 2000), flow modification (Kingsford, 2000; McIvor *et al.*, 2000), destruction or degradation of habitat (Aparicio *et al.*, 2000) and invasion by exotic species (Elvira, 1998). As SEQ is an area undergoing major development, the threat of increased habitat destruction and demand for freshwater by human populations is of growing concern for the survival of SEQ freshwater crayfish. In particular, localised extinction within the region may have irreversible consequences, with five of

the six resident crayfish species recognised as endemic to the region (McCormack, 2013). With more than one-third of the world's crayfish species in decline or threatened with extinction (Ricciardi & Rasmussen, 1999), the conservation of SEQ's extremely bio-diverse and localised crayfish fauna is vital for the future. The conservation and management of the freshwater crayfish in SEQ requires comprehensive identification of each of the species, along with an assessment of their population dynamics and ecological requirements (Benstead *et al.*, 2003). Through the conservation of each species and intraspecific genetic variation, freshwater crayfish populations can remain viable into the future. While this study did not comprehensively identify all the taxonomic and biological aspects of SEQ freshwater crayfish, it did provide an important step in the right direction.

APPENDICES

8.1 PHYLOGENETIC ANALYSIS

Table 8.1: Taxonomic classifiers of eastern Australia *Cherax*.

#	Reference	Species
1	Coughran *et al.* (2012)	*C. austini* & *C. cid*
2	Riek (1969)	*C. cairnsensis, C. cuspidatus, C. urospinosus* & *C. wasselli*
3	Short and Davie (1993)	*C. cartalacoolah* & *C. parvus*
4	Riek (1951)	*C. depressus, C. dispar, C. rhynchotus, C. robustus* & *C. setosus*
5	Clark (1936)	*C. destructor albidus, C. destructor destructor* & *C. punctatus*
6	Coughran (2005)	*C. leckii*
7	von Martens (1868)	*C. quadricarinatus*
8	Clark (1941)	*C. rotundus*
9	Gray (1845)	*C. bicarinatus*
10	Munasinghe *et al.* (2004b)	*C. sp. nov.*
11	Short (1991)	*C. nucifraga*
12	Bentley *et al.* (2010)	*C. dispar* D

Table 8.2: Reference and Genbank accession number for individuals used in phylogenetic analyses.

#	Reference	Gene	Accession #
A	Munasinghe *et al.* (2004b)	16S	AY191748, 54-55, 57-61, 64, 67-69, 72, 74
		12S	AY191724, 27-33, 37-41, 43, 46
B	Toon *et al.* (2010)	COI	FJ965956-7
		16S	EU921120
		28S	FJ966009-11, EU921132
		GAPDH	EU977401, 05-07, 09, 11, 13-14
C	Crandall *et al.* (1999)	16S	AF135971
D	Liu *et al.* (2011)	16S	JF284571
E	Shull *et al.* (2005)	COI	DQ006292-3
		12S	DQ006423
		28S	DQ006677
F	Adam D. Miller *et al.* (2004)	COI	AY383557
G	Schultz *et al.* (2009)	GAPDH	AY430092

Table 8.3: Sample size (*N*), unique haplotype number (*Hn*), haplotype diversity (*Hd*) and current (θ_π) and historical (θ_w) genetic diversity of all eastern Australian *Cherax* for each gene analysed.

Species	**Gene**																			
	COI			16S			12S			28S			GAPDH			H3			ITS		
	N	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)
C. cairnsensis	4	3 (0.833)	0.167 (0.014)	4	3 (0.833)	0.042 (0.039)	5	5 (1)	0.019 (0.019)	4	1 (0)	0 (0)	9	5 (0.861)	0.003 (0.003)	1	1 (-)	-	2	1 (0)	0 (0)
C. cuspidatus A	1	1 (-)	- (-)	1	1 (-)	-	6	6 (1)	0.017 (0.018)	1	1 (-)	-	1	1 (-)	-	-	-	-	-	-	-
C. cuspidatus B	1	1 (-)	-	8	3 (0.679)	0.003 (0.002)	-	-	-	-	-	-	1	1 (-)	-	-	-	-	1	1 (-)	-
C. cuspidatus C	3	2 (0.667)	0.002 (0.002)	2	1 (0)	0 (0)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. cuspidatus D	1	1 (-)	-	2	2 (1)	0.005 (0.005)	2	2 (1)	0.01 (0.01)	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-
C. cuspidatus E	37	12 (0.877)	0.006 (0.006)	8	6 (0.893)	0.005 (0.006)	4	3 (0.833)	0.007 (0.008)	1	1 (-)	-	5	1 (0)	0 (0)	4	2 (0.5)	0.003 (0.004)	8	2 (0.25)	0.001 (0.002)
C. depressus	167	27 (0.852)	0.011 (0.02)	17	6 (0.831)	0.009 (0.014)	3	3 (1)	0.025 (0.025)	12	1 (0)	0 (0)	4	2 (0.5)	0.001 (0.001)	12	1 (0)	0 (0)	9	8 (0.972)	0.005 (0.005)
C. d. albidus	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-	-	-	-	-	-	-
C. d. destructor	10	7 (0.911)	0.021 (0.028)	15	6 (0.571)	0.007 (0.011)	4	4 (1)	0.01 (0.011)	2	2 (1)	0.001 (0.001)	1	1 (-)	-	1	1 (-)	-	2	1 (0)	0 (0)
C. dispar A	167	37 (0.883)	0.006 (0.012)	23	7 (0.743)	0.003 (0.004)	3	3 (1)	0.009 (0.009)	11	1 (0)	0 (0)	8	1 (0)	0 (0)	11	1 (0)	0 (0)	17	5 (0.625)	0.003 (0.006)
C. dispar B	123	32 (0.748)	0.006 (0.015)	15	2 (0.248)	0.002 (0.002)	1	1 (-)	-	4	1 (0)	0 (0)	5	1 (0)	0 (0)	9	1 (0)	0 (0)	12	4 (0.773)	0.003 (0.003)

Species	Gene																			
	COI			16S			12S			28S			GAPDH			H3			ITS		
	N	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)	*N*	*Hn* (*Hd*)	θ_π (θ_w)
C. dispar C	85	28 (0.925)	0.018 (0.024)	23	7 (0.783)	0.005 (0.006)	5	5 (1)	0.007 (0.008)	7	1 (0)	0 (0)	4	2 (0.5)	0.002 (0.002)	1	1 (-)	-	7	3 (0.524)	0.008 (0.011)
C. dispar D	75	26 (0.9)	0.0051 (0.011)	18	3 (0.307)	0.001 (0.001)	2	2 (1)	0.01 (0.01)	4	1 (0)	0 (0)	2	1 (0)	0 (0)	4	1 (0)	0 (0)	5	3 (0.8)	0.002 (0.002)
C. dispar E	16	7 (0.85)	0.014 (0.013)	7	5 (0.905)	0.007 (0.007)	2	2 (1)	0.007 (0.007)	4	1 (0)	0 (0)	-	-	-	2	1 (0)	0 (0)	5	3 (0.7)	0.003 (0.003)
C. parvus	1	1 (-)	-	1	1 (-)	-	2	1 (0)	0 (0)	1	1 (-)	-	-	-	-	-	-	-	-	-	-
C. punctatus	3	3 (1)	0.015 (0.015)	2	2 (1)	0.016 (0.016)	3	3 (1)	0.011 (0.011)	2	1 (0)	0 (0)	-	-	-	3	2 (0.667)	0.002 (0.002)	-	-	-
C. quadricarinatus	-	-	-	1	1 (-)	-	1	1 (-)	-	1	1 (-)	-	2	2 (1)	0.088 (0.088)	-	-	-	-	-	-
C. rhynchotus	-	-	-	1	1 (-)	-	1	1 (-)	-	-	-	-	-	-	-	-	-	-	-	-	-
C. robustus	25	9 (0.787)	0.008 (0.013)	8	3 (0.464)	0.002 (0.002)	4	4 (1)	0.01 (0.011)	3	2 (0.667)	0.002 (0.002)	3	1 (0)	0 (0)	3	1 (0)	0 (0)	8	2 (0.25)	0.003 (0.005)
C. rotundus	-	-	-	2	1 (0)	0 (0)	1	1 (-)	-	-	-	-	1	1 (-)	-	-	-	-	-	-	-
C. setosus	-	-	-	2	2 (1)	0.06 (0.06)	2	2 (1)	0.007 (0.007)	-	-	-	1	1 (-)	-	-	-	-	-	-	-
All	716	189 (0.976)	0.126 (0.059)	138	47 (0.968)	0.078 (0.07)	60	41 (0.998)	0.082 (0.075)	60	19 (0.672)	0.005 (0.007)	50	24 (0.81)	0.011 (0.028)	73	32 (0.371)	0.004 (0.005)	79	38 (0.905)	0.027 (0.025)

8.2 PHYLOGEOGRAPHIC ANALYSIS

Table 8.4: Study sample sites, haplotype distribution and analysis groupings for *C. dispar* A individuals.

Sample site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Mary	Scrubby Ck	A1S1	A. Bentley	-25.965306	152.894497	11	8, 11	A1S2, A1S3, A1S4	-
River	Sandy Ck 1	A1S2	A. Bentley	-25.96935	152.888231	11	8-9, 11-12	A1S1, A1S3, A1S4	-
	Sandy Ck 2	A1S3	A. Bentley	-25.994544	152.886922	3	8, 13	A1S1, A1S2, A1S4	-
	Sandy Ck 3	A1S4	A. Bentley	-25.982858	152.907464	5	8, 11	A1S1, A1S2, A1S3	-
	Red Ridge Ck 1	A2S1	A. Bentley	-25.97057	152.757825	7	14, 24, 30-31	A2S2, A2S3	-
	Red Ridge Ck 2	A2S2	A. Bentley	-25.975331	152.77322	4	15, 18, 23, 25	A2S1, A2S3	-
	Red Ridge Ck 3	A2S3	A. Bentley	-25.987767	152.757792	11	18, 30	A2S1, A2S2	-
	Native Dog Ck 1	A2S4	A. Bentley	-25.968608	152.751083	1	8	A3S3	A3S3
	Coondoo Ck 1	A3S1	A. Bentley	-25.929325	152.79098	11	11, 18	A3S2, A3S4	-
	Sandy Ck 4	A3S2	A. Bentley	-25.926375	152.761767	13	11, 15-17, 21-22	A3S1, A3S4	-
	Native Dog Ck 2	A3S3	A. Bentley	-25.955681	152.74868	6	11	A2S4	A2S4
	Sandy Ck 5	A3S4	A. Bentley	-25.915744	152.754652	11	11, 15-16, 18	A3S1, A3S2	-
	Rocky Ck	A4S3	A. Bentley	-25.887144	152.680619	1	19	A5S1, A5S2	A5S1, A5S2
	Big Sandy Ck 1	A5S1	A. Bentley	-25.873428	152.798958	1	18	A4S3, A5S2	A4S3, A5S2
	Big Sandy Ck 2	A5S2	A. Bentley	-25.864172	152.770286	2	18	A4S3, A5S1	A4S3, A5S1

Sample site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
	Susan R	HBSR	T. Page	-25.394783	152.708317	2	3, 36	-	-
	Myrtle Ck	MC86	A. Bentley	-25.5689	152.423517	2	6, 38	MMAC	MMAC
	Wide Bay Ck	MWW	T. Page	-26.00992	152.38104	4	6	-	Omitted
	Mariana Ck	MMAC	A. Bentley	-25.541581	152.540758	1	37	MC86	MC86
	Coondoo Ck 2	MCC	T. Page	-25.99275	152.840633	15	8, 10-11, 15	-	-
	Charley Hart Ck	MCHC	A. Bentley	-25.591986	152.370608	2	4	-	-
	Logging Ck	MLC2	T. Page	-25.740817	152.755833	11	11, 28-29	-	-
	Stockyard Ck	RC	A. Bentley	-25.766683	152.7166	1	11	MTC	MTC
	Tinana Ck	MTC	T. Page	-25.819933	152.72225	1	23	RC	RC
	Ross Ck	TCRC	A. Bentley	-26.096233	152.7556	4	18, 20	-	-
Burrum River	Isis R	BIR	T. Page	-25.226767	152.420633	1	35	-	Omitted
Fraser	Bogimbah Ck	FBU	DERM	-25.304783	153.05645	7	32-34	-	-
Island	Bowaraddy Ck	FIBO	DERM	-25.133083	153.165033	6	1-2, 5	-	-
	Coongul Ck	FICO	DERM	-25.197083	153.11065	4	26-27	-	-

Table 8.5: Study sample sites, haplotype distribution and analysis groupings for *C. dispar* B individuals.

Sample Site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Mary	Sandy Ck 1	A1S2	A. Bentley	-25.96935	152.88823	1	17	A2S1, A3S3	A2S1, A3S3
River	Red Ridge Ck 1	A2S1	A. Bentley	-25.97057	152.75783	2	17-18	A1S2, A3S3	A1S2, A3S3
	Native Dog Ck 2	A3S3	A. Bentley	-25.95568	152.74868	1	2	A1S2, A2S1	A1S2, A2S1
	Big Sandy Ck 1	A5S1	A. Bentley	-25.87343	152.79896	9	1, 3	-	-
	Big Sandy Ck 2	A5S2	A. Bentley	-25.86417	152.77029	14	1, 4	-	-
	Sugarloaf Ck 1	A5S3	A. Bentley	-25.82869	152.78264	19	1, 5	-	-
	Sugarloaf Ck 2	A5S4	A. Bentley	-25.8165	152.77044	13	1, 6	-	-
Fraser	Alligator Ck	FIAL	G. McGregor	-25.4901	152.99607	9	7-9, 11	-	-
Island	Gerowweea Ck	FIGE	T. Page	-25.59893	153.08442	2	25-26	FIGO	FIGO
	Govi Ck	FIGO	T. Page	-25.59928	153.09283	1	24	FIGE	FIGE
	Lake Wabby	FILW	T. Page	-25.45	153.13333	2	27-28	-	-
	Rocky Ck	FIRC	T. Page	-25.47272	153.00962	6	10, 12-14, 29	-	-
Tin Can	Big Tuan Ck 1	TCBT	T. Page	-25.68528	152.78765	9	15-16, 19-22	TCBT2, TCBT4	TCBT2, TCBT4
Bay	Big Tuan Ck 2	TCBT2	T. Page	-25.6955	152.76425	1	15	TCBT, TCBT4	TCBT, TCBT4
	Big Tuan Ck 4	TCBT4	T. Page	-25.6875	152.78305	1	19	TCBT, TCBT2	TCBT, TCBT2
	Freshwater Lake	TCFR	T. Page	-25.99747	153.14187	10	23	TCPC, TCSE	TCPC, TCSE
	Poona Ck	TCPC	T. Page	-25.964300	153.111750	6	23	TCFR, TCSE	TCFR, TCSE
	Searys Ck	TCSE	A. Bentley	-25.97355	153.07232	7	23, 30-32	TCPC, TCFR	TCPC, TCFR

Table 8.6: Study sample sites, haplotype distribution and analysis groupings for *C. dispar* C individuals.

Sample Site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Brisbane	Reynolds Ck	BRG	SGM & AJP	-28.0106	152.5552	1	8	BRG3	BRG3
River	Reynolds Ck 3	BRG3	T. Page	-28.000694	152.569916	3	2	BRG	BRG
	Running Ck	BRE	DJH & TG	-27.3406	152.3736	1	2	-	Omitted
	Stony Ck	QdiWo	J. Short	-26.954839	152.777875	1	8	-	Omitted
Caboolture-	Burpengary Ck	NSB	A. Bentley	-27.177458	152.919813	3	14, 17	PCR, PCR2	PCR, PCR2
Pine	Caboolture R	PCR	DJH & TG	-27.0957	152.8387	3	14	NSB, PCR2	NSB, PCR2
Rivers	Caboolture R 2	PCR2	DJH & TG	-27.109	152.8867	1	14	NSB, PCR	NSB, PCR
	Kedron Brook	QdiKe	R. McKay	-27.408347	153.032291	1	9		Omitted
	Oxley Ck	BOC	T. Page	-27.6109	153.0231	1	2	QdiBr	QdiBr
	Lagoon Ck	PDM	A. Bentley	-27.211297	152.97405	1	15		Omitted
	Hilliards Ck	QdiBr	S. Cook	-27.563211	153.256977	1	16	BOC	BOC
Logan-	Teviot Brook	TDS	D. Sternberg	-28.156194	152.571833	9	1	LTT	LTT
Albert Rivers	Teviot Brook 2	LTT	T. Page	-28.162138	152.558305	1	1	TDS	TDS
Maroochy	Bluegum Ck	NBG	SGM & TG	-26.8535	152.9844	1	18	AMS	AMS
River	Sippy Ck	AMS	T. Page	-26.733	153.04607	1	11	NBG	NBG
	Whalleys Ck	MPW	T. Page	-26.63217	152.95044	1	20	APC	APC
	Coes Ck	APC	T. Page	-26.64109	152.93649	9	21	MPW	MPW

Sample Site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Mary	Chinaman Ck	MCRC	A. Bentley	-26.498997	152.863256	2	5, 23	-	-
River	Obi Obi Ck	OOC	D. Sternberg	-26.634028	152.783722	6	1-2, 19, 22	-	-
	Cooroora Ck	MSC	T. Page	-26.36552	152.85924	4	22, 24	MLM	MLM
	Lake McDonald	MLM	T. Page	-26.38531	152.92937	1	22	MSC	MSC
	Six Mile Ck	6MN	D. Sternberg	-26.329694	152.809194	5	22, 25	6MN2	6MN2
	Six Mile Ck 2	6MN2	D. Sternberg	-26.318514	152.7836	1	22	6MN	6MN
	Scrubby Ck	A1S1	A. Bentley	-25.965306	152.894497	2	10	-	-
	Yabba Ck	MYY	T. Page	-26.49133	152.59296	9	1-3, 6	-	-
	Yabba Ck 2	DTW	D. Sternberg	-26.498278	152.591639	6	1, 22	-	-
Noosa	Teewah Ck	NTE	T. Page	-26.0242	153.025267	1	27	-	Omitted
River	Kin Kin Ck	NKK	T. Page	-26.28522	152.8727	2	26	-	Omitted
	Castaways Ck	NCC	A. Bentley	-26.438967	153.104633	4	12-13	NMC	NMC
	Marcus Ck	NMC	A. Bentley	-26.4506	153.102	2	13	NCC	NCC

Table 8.7: Study sample sites, haplotype distribution and analysis groupings for *C. dispar* D individuals.

Sample Site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Moreton	Cravens Ck	MICR2	T. Page	-27.113167	153.37	14	23-24	-	-
Island	Honeyeater Lake	MIH	T. Page	-27.09625	153.433467	3	23, 25	MIBL	MIBL
	Blue Lagoon	MIBL	T. Page	-27.092967	153.440883	1	23	MIH	MIH
	Unnamed Ck	MIU	G. McGregor	-27.107111	153.440194	4	23, 26	-	-
North	Blue Lake	SBLO	A. Bentley	-27.534517	153.488633	14	1- 10, 12	SIBLA, SHL	SIBLA, SHL
Stradbroke	Blue Lake 2	SIBLA	T. Page	-27.522	153.497633	2	1	SBLO, SHL	SBLO, SHL
Island	Hering Lagoon	SHL	T. Page	-27.575767	153.469637	2	1	SBLO, SIBLA	SBLO, SIBLA
	Key-Hole Lagoons	SKHL	A. Bentley	-27.4864	153.511783	12	1, 4, 6, 10, 11, 13-15	-	-
	Aranarawai Ck	SHA	A. Bentley	-27.453617	153.451267	10	17-19	-	-
	Campebah Ck	SMS	G. McGregor	-27.468717	153.4258	8	20-22	-	-
	Little Canalpin Ck	SLC	T. Page	-27.62255	153.41915	3	16	-	-

Table 8.8: Study sample sites, haplotype distribution and analysis groupings for *C. dispar* E individuals.

Sample Site				Co-ordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network grouping	Population Grouping
Logan-Albert Rivers	Hilliards Ck	LHA	SGM & AJP	-27.5312	153.25	1	6	LCC	LCC
	Coolnwynpin Ck	LCC	SGM & AJP	-27.5255	153.2033	1	2	LHA	LHA
	Scrubby Ck	LSC	SGM & AJP	-27.6409	153.0701	3	7-8	-	-
	Tingalpa Ck	LTS	SGM & AJP	-27.5979	153.1862	1	4	LTS2	LTS2
	Tingalpa Ck 2	LTS2	SGM & AJP	-27.5762	153.1821	3	4-5	LTS	LTS
	Tingalpa Ck 3	LTMC	SGM & AJP	-27.6113	153.2043	3	1, 3	-	-

Table 8.9: Study sample sites, haplotype distribution and analysis groupings for *C. depressus* individuals.

Sample Site				Coordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network Grouping	Population Grouping
Mary	Red Ridge Ck	A2S1	A. Bentley	25.97057	152.757825	12	6-8, 10	A2S2, A2S3	-
River	Red Ridge Ck	A2S2	A. Bentley	-25.9753306	152.77322	11	6, 9	A2S1, A2S3	MTC2
	Red Ridge Ck	A2S3	A. Bentley	-25.987767	152.757792	17	1, 3, 6	A2S1, A2S2	-
	Native Dog Ck	A2S4	A. Bentley	-25.968608	152.751083	9	6	A3S3, A3S1	A3S3, A3S1
	Tinana Ck	MTC2	T. Page	-25.976883	152.78765	1	7	-	A2S2
	Coondoo Ck	A3S1	A. Bentley	-25.929325	152.79098	11	9	A2S4, A3S3	A2S4, A3S3
	Sandy Ck 2	A3S2	A. Bentley	-25.926375	152.761767	10	3	A3S4	A3S4
	Native Dog Ck	A3S3	A. Bentley	-25.9556806	152.74868	11	6	A2S4, A3S1	A2S4, A3S1
	Sandy Ck 2	A3S4	A. Bentley	-25.915744	152.754652	2	3, 5	A3S2	A3S2
	Spudo Gully	BGF	A. Bentley	-25.901997	152.670692	6	23-24	A4S3, A4S4	A4S3, A4S4
	Rocky Ck	A4S3	A. Bentley	-25.887144	152.680619	4	23	BGF, A4S4	BGF, A4S4
	Spudo Gully	A4S4	A. Bentley	-25.89832	152.682953	3	23	BGF, A4S3	BGF, A4S3
	Susan River	HBSR	T. Page	-25.394783	152.708317	1	16	-	Omitted
	Eel Ck	MCLC	A. Bentley	-25.618525	152.230608	2	11, 22	-	-
	18 Mile Ck	MEMC	A. Bentley	-25.554975	152.495339	3	11, 14, 22	-	-
	Myrtle Ck	MMC	T. Page	-25.607283	152.455017	4	11, 22	MLC	MLC
	Lary Ck	MLC	T. Page	-25.73525	152.5024	9	22	MMC	MMC
	Middle Ck	MMIC	A. Bentley	-25.609364	152.301153	6	11-13, 22	-	-

Sample Site				Coordinates		Sample Size		Analysis Grouping	
Catchment	Creek	Code	Sampler	Latitude	Longitude	Count	Haplotypes	Network Grouping	Population Grouping
	Stockyard Ck	RC	A. Bentley	-25.766683	152.7166	8	1-2, 4	-	-
Brisbane	Esk Ck	BEE	T. Page	-27.2255	152.42812	3	25-26	-	-
River	Reynolds Ck	BRG2	T. Page	-28.011556	152.55652	4	27-28	BWN	BWN
	Warill Ck	BWN	T. Page	-27.988247	152.478256	1	28	BRG2	BRG2
Burrum River	Longbridge Ck	BLC	T. Page	-25.436033	152.55095	4	11, 15, 19, 21	-	-
Tin Can	Little Tuan Ck	TCLT	A. Bentley	-25.664369	152.845972	5	18-20	TCBT, TCBT2	TCBT, TCBT2
Bay	Big Tuan Ck	TCBT	T. Page	-25.685283	152.78765	3	17	TCLT, TCBT2	TCLT, TCBT2
	Big Tuan Ck	TCBT2	T. Page	-25.6955	152.76425	6	17	TCLT, TCBT	TCLT, TCBT

Table 8.10: Sample size (*N*), unique haplotype number (*Hn*), haplotype diversity (*Hd*) and current (θ_π) and historical (θ_w) genetic diversity of *C. dispar* lineages and *C. depressus* for the COI mitochondrial gene.

Population	Diversity Statistic				
	N	*Hn*	*Hd*	θ_π	θ_w
C. dispar A	165	38	0.877	0.006	0.012
Burrum R	1	1	-	-	-
Fraser I	17	8	0.897	0.012	0.009
Mary R	147	29	0.846	0.004	0.009
C. dispar B	116	32	0.787	0.006	0.015
Fraser I	21	14	0.943	0.007	0.011
Mary R	59	8	0.283	0.001	0.003
Tin Can Bay	36	10	0.708	0.009	0.011
C. dispar C	84	27	0.924	0.016	0.014
Brisbane R	7	4	0.810	0.003	0.003
Caboolture R	7	2	0.286	0.000	0.001
Logan-Albert R	11	2	0.182	0.002	0.003
Maroochy R	12	4	0.455	0.006	0.008
Mary R	36	11	0.840	0.017	0.012
Noosa R	9	4	0.806	0.017	0.014
Pine R	2	2	1	0.011	0.011
C. dispar D	75	27	0.903	0.005	0.009
Moreton I	22	4	0.260	0.000	0.001
Nth Stradbroke I	53	23	0.930	0.005	0.008
C. dispar E	12	8	0.924	0.014	0.012
Logan-Albert R	5	4	0.900	0.021	0.017
Tingalpa Ck	7	4	0.810	0.002	0.002
C. depressus	169	28	0.857	0.011	0.012
Brisbane R	8	4	0.750	0.011	0.008
Burrum R	4	3	0.833	0.002	0.003
Mary R	144	19	0.808	0.008	0.007
Tin Can Bay	13	4	0.603	0.001	0.002

8.3: POPULATION CONNECTIVITY

Table 8.11: *C. dispar* A pairwise F_{ST} from COI mtDNA data in the Mary River catchment. Below diagonal: F_{ST} (θ); Bold value: significant F_{ST} (θ); Above diagonal: *P*-value; Underline value: significant at α= 0.05. Site codes see Table 8.4.

	A1S1	A1S2	A1S3	A1S4	A2S1	A2S2	A2S3	A3S1	A3S2	A3S3	A3S4	A5S1	A5S2
A1S1		0.550	0.568	0.991	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.991	0.045
A1S2	0.000		0.450	0.991	0.000	0.000	0.000	0.000	0.000	0.198	0.000	0.991	0.018
A1S3	0.000	0.021		0.505	0.009	0.099	0.000	0.009	0.036	0.009	0.009	0.991	0.108
A1S4	0.000	0.000	0.000		0.018	0.009	0.000	0.036	0.036	0.108	0.009	0.991	0.135
A2S1	**0.428**	**0.446**	**0.270**	**0.373**		0.045	0.009	0.000	0.000	0.000	0.000	0.991	0.162
A2S2	**0.423**	**0.404**	**0.276**	**0.316**	**0.217**		0.018	0.000	0.099	0.000	0.000	0.991	0.991
A2S3	**0.556**	**0.579**	**0.599**	**0.622**	**0.152**	**0.260**		0.000	0.000	0.000	0.000	0.991	0.459
A3S1	**0.294**	**0.218**	**0.624**	**0.344**	**0.646**	**0.513**	**0.766**		0.009	0.991	0.000	0.991	0.027
A3S2	**0.251**	**0.217**	**0.245**	**0.152**	**0.405**	0.140	**0.451**	**0.174**		0.099	0.018	0.991	0.234
A3S3	0.212	0.133	**0.547**	0.294	**0.557**	**0.397**	**0.761**	0.000	0.104		0.000	0.991	0.009
A3S4	**0.478**	**0.462**	**0.535**	**0.475**	**0.606**	**0.481**	**0.706**	**0.563**	**0.142**	**0.501**		0.991	0.063
A5S1	0.462	0.467	0.333	0.571	0.000	0.000	0.000	0.846	0.000	1.000	0.576		0.991
A5S2	**0.546**	**0.554**	0.571	0.674	0.124	0.000	0.108	**0.861**	0.206	**1.000**	0.640	0.000	

Table 8.12: *C. dispar* B pairwise F_{ST} from COI mtDNA data in the Mary River catchment. Below diagonal: F_{ST} (θ); Bold value: significant F_{ST} (θ); Above diagonal: *P*-value; Underline value: significant at α= 0.05. Site codes see Table 8.5.

	A1S2	A2S1	A3S3	A5S1	A5S2	A5S3	A5S4
A1S2		0.991	0.991	0.063	0.045	0.027	0.117
A2S1	0.000		0.991	0.036	0.000	0.009	0.000
A3S3	0.818	1.000		0.153	0.036	0.117	0.207
A5S1	0.931	**0.946**	0.800		0.342	0.505	0.667
A5S2	**0.930**	**0.936**	**0.769**	0.039		0.153	0.351
A5S3	**0.965**	**0.974**	0.900	0.025	0.068		0.712
A5S4	0.950	**0.962**	0.857	0.007	0.048	0.005	

Table 8.13: *C. depressus* pairwise F_{ST} from COI mtDNA data in the Mary River catchment. Below diagonal: F_{ST} (θ); Bold value: significant F_{ST} (θ); Above diagonal: *P*-value; Underline value: significant at α= 0.05. Site codes see Table 8.8.

	A2S1	A2S2	A2S3	A2S4	A3S1	A3S2	A3S3	A3S4
A2S1		0.477	0.063	0.288	0.000	0.000	0.027	0.081
A2S2	0.000		0.640	0.991	0.000	0.000	0.477	0.108
A2S3	0.102	0.000		0.505	0.000	0.000	0.486	0.081
A2S4	0.068	0.000	0.000		0.000	0.000	0.991	0.099
A3S1	**0.809**	**0.915**	**0.965**	**1.000**		0.000	0.000	0.036
A3S2	**0.650**	**0.790**	**0.879**	**1.000**	**1.000**		0.000	0.306
A3S3	**0.120**	0.023	0.017	0.000	**1.000**	**1.000**		0.000
A3S4	0.217	0.405	0.648	0.916	**0.991**	0.799	**0.945**	

Table 8.14: Sample size (*N*), unique haplotype number (*Hn*), haplotype diversity (*Hd*) and current (θ_π) and historical (θ_w) genetic diversity of *C. depressus* and two *C. dispar* lineages for the COI mitochondrial gene.

Population	Diversity Statistic				
	N	*Hn*	*Hd*	θ_π	θ_w
C. dispar A	107	18	0.807	0.002	0.005
Area 1	37	7	0.638	0.001	0.003
Area 2	22	8	0.805	0.002	0.004
Area 3	45	7	0.649	0.001	0.001
Area 5	3	1	0	0	0
Coondoo Ck	14	2	0.143	0	0.001
Native Dog Ck	6	1	0	0	0
Red Ridge Ck	22	8	0.805	0.002	0.004
Sandy Ck 1	21	5	0.652	0.001	0.002
Sandy Ck 2	25	7	0.733	0.002	0.002
Scrubby Ck	16	4	0.650	0.001	0.002
Stockyard Ck	3	1	0	0	0
C. dispar B	59	8	0.283	0.001	0.003
Area 1	1	1	-	-	-
Area 2	2	2	1	0.002	0.002
Area 3	1	1	-	-	-
Area 5	55	5	0.174	0	0.001
Big Sandy Ck	23	3	0.245	0	0.001
Native Dog Ck	1	1	-	-	-
Red Ridge Ck	2	2	1	0.002	0.002
Sandy Ck 1	1	1	-	-	-
Sugarloaf Ck	32	3	0.123	0	0.001
C. depressus	97	8	0.615	0.004	0.003
Area 2	49	7	0.301	0.002	0.004
Area 3	48	4	0.691	0.006	0.003
Coondoo Ck	15	1	0	0	0
Native Dog Ck	23	1	0	0	0
Red Ridge Ck	40	7	0.362	0.002	0.004
Sandy Ck 2	19	2	0.105	0	0

Table 8.15: ANOVA analysis and pairwise *t*-test comparisons of eleven environmental variables in the Mary River catchment, with sites grouped by species/lineage present, the area and the creek. Bold value: significant at α= 0.05; Underline value: significant interaction effect from micro-habitat; Letters (A-C): significantly different groups from pairwise *t*-test comparisons.

Variable	ANOVA				Pairwise *t*-test Comparisons									
	Treatment Group				Area				Species			*C. dispar* lineages		
	Area	Creek	Species	*C. dispar* lineages	1	2	3	5	*C. dispar*	*C. depressus*	Both	A	B	Both
pH	**0.001**	**0.001**	**0.001**	**0.049**	B	A	A	B	B	AB	A	A	AB	B
Overhead Branches	0.336	0.420	0.271	0.537	-	-	-	-	-	-	-	-	-	-
Small Wooded Debris	0.377	0.086	**0.048**	0.553	-	-	-	-	AB	B	A	-	-	-
Submerged Tree Roots	0.737	0.756	**0.029**	0.821	-	-	-	-	A	B	A	-	-	-
Leaf Litter	0.795	0.197	**0.039**	0.169	-	-	-	-	AB	B	A	-	-	-
Dissolved Oxygen	0.068	**0.030**	0.789	**0.050**	-	-	-	-	-	-	-	B	A	AB
Conductivity	**0.001**	**0.001**	**0.001**	**0.012**	B	A	B	B	B	B	A	AB	B	A
Depth	**0.001**	**0.001**	0.088	0.858	C	C	A	B	-	-	-	-	-	-
Velocity	**0.001**	**0.001**	**0.020**	0.840	A	B	B	B	A	B	B	-	-	-
Salinity	**0.001**	**0.013**	**0.032**	0.191	B	A	B	B	B	AB	A	-	-	-
Turbidity	0.501	0.059	**0.001**	0.218	-	-	-	-	B	A	B	-	-	-

Table 8.16:ANOVA analysis and pairwise *t*-test comparisons of fifteen morphological measurements for five treatment groups; species, lineage, area, creek and sex. *p*-values are shown with; Bold value: significant at α= 0.05; Underline value: significant interaction effect from sex of individual at α= 0.05; Letters (A-C): significantly different groups from pairwise *t*-test comparisons. Morphology measurement abbreviations see Figure 6.2. Additional abbreviations are; COO (Coondoo Ck), BSC (Big Sandy Ck), SUG (Sugarloaf Ck), NAD (Native Dog Ck), SCR (Scrubby Ck), SAN1 (Sandy Ck 1), SAN2 (Sandy Ck 2), RRC (Red Ridge Ck), *C. dis* (*Cherax dispar*) and *C. dep* (*Cherax depressus*).

Variable	ANOVA					Pairwise *t*-test Comparisons													
	Treatment Group					Area				Species			Creek						
	Area	Creek	Species	*C. dis* Lineages	Sex	1	2	3	5	*C. dis* A	*C. dis* B	*C. dep*	COO	BSC	SUG	NAD	SCR	SAN1	SAN2	RRC
TL	0.076	**0.015**	**0.028**	0.065	**0.001**	-	-	-	-	B	A	B	A	B	-	AB	AB	AB	B	B
OCL	0.758	0.451	**0.007**	0.370	**0.015**	-	-	-	-	B	B	A	-	-	-	-	-	-	-	-
ARL	0.207	**0.028**	**0.001**	0.345	**0.001**	-	-	-	-	B	B	A	AB	B	-	A	AB	B	AB	AB
ARW	0.523	0.800	**0.001**	0.095	0.121	-	-	-	-	B	A	C	-	-	-	-	-	-	-	-
CL	0.368	0.477	0.119	0.322	0.827	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD	0.262	0.519	**0.002**	**0.014**	**0.002**	-	-	-	-	B	B	A	-	-	-	-	-	-	-	-
CW	0.148	0.325	**0.001**	0.316	**0.013**	-	-	-	-	B	B	A	-	-	-	-	-	-	-	-
TW	**0.011**	**0.014**	**0.001**	0.313	**0.021**	B	A	AB	AB	B	B	A	C	ABC	-	ABC	BC	C	AB	A
TAL	**0.004**	**0.004**	**0.001**	**0.015**	**0.003**	B	A	A	B	B	C	A	B	B	-	A	B	B	A	A
RL	0.291	0.316	**0.001**	**0.004**	0.097	-	-	-	-	B	A	C	-	-	-	-	-	-	-	-
PL	0.342	0.162	0.930	0.716	**0.001**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PW	0.677	0.649	**0.001**	0.221	**0.001**	-	-	-	-	B	B	A	-	-	-	-	-	-	-	-
PD	0.416	0.409	**0.001**	0.460	**0.001**	-	-	-	-	B	B	A	-	-	-	-	-	-	-	-
PAL	0.847	0.504	0.290	0.664	**0.001**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL	**0.001**	**0.001**	**0.003**	0.638	**0.001**	A	A	A	A	B	B	A	AB	A	-	B	AB	A	AB	A

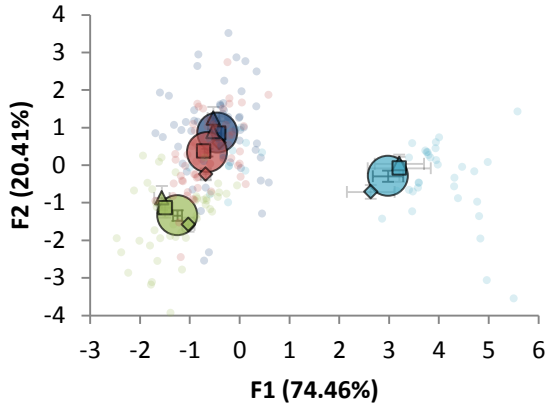
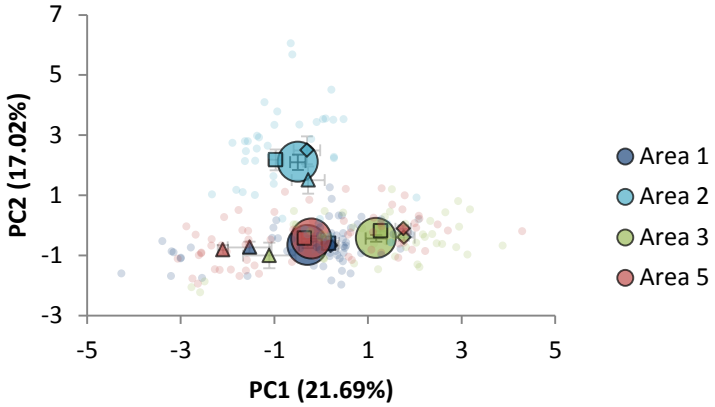
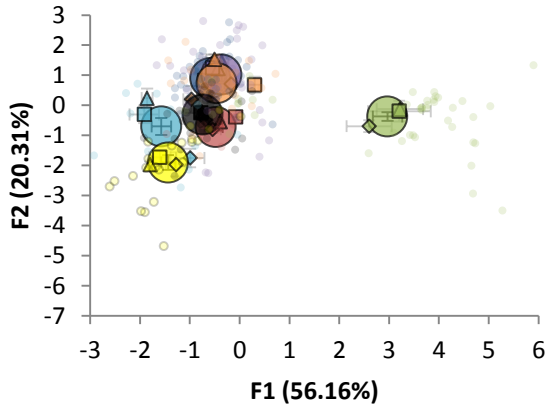
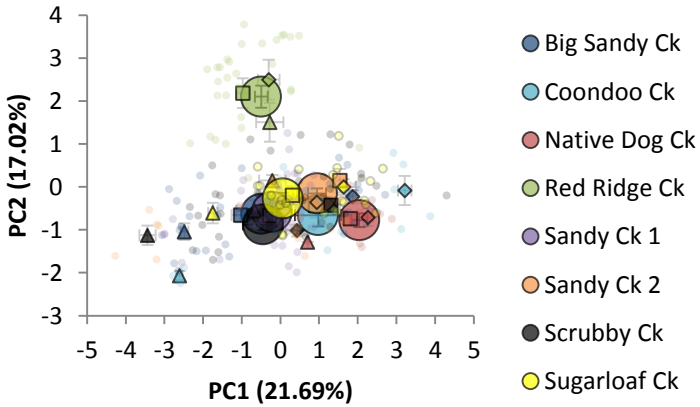
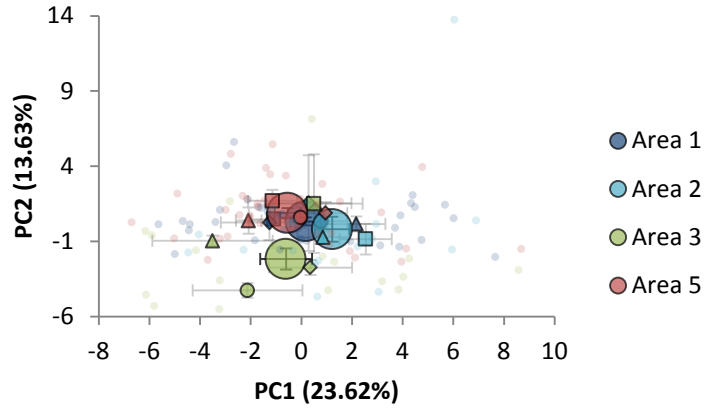
	Discriminant Function Analysis	Var	F1	F2	Principal Component Analysis	Var	PC1	PC2
Area		Salinity	**0.95**	-0.02		SWD	**0.79**	0.13
		Cond	**0.73**	-0.29		LL	**0.69**	0.13
		pH	**0.34**	*-0.47*		STR	**0.63**	*0.01*
		DO	0.27	**0.27**		Depth	0.61	*0.03*
		Temp	0.14	**0.17**		OB	0.46	0.04
		Turbidity	0.14	-0.26		Turbidity	0.14	0.19
		LL	0.00	*-0.50*		pH	0.02	**0.55**
		SWD	-0.03	-0.34		Temp	-0.10	0.35
		STR	-0.08	-0.01		DO	-0.12	0.43
		Velocity	*-0.09*	**0.29**		Cond	*-0.15*	**0.80**
		OB	*-0.17*	*-0.44*		Salinity	*-0.28*	**0.81**
		Depth	*-0.28*	-0.37		Velocity	*-0.62*	*-0.27*
Creek		Salinity	**0.94**	-0.02		SWD	**0.79**	0.13
		Cond	**0.71**	-0.29		LL	**0.69**	0.13
		pH	**0.33**	*-0.52*		STR	**0.63**	*0.01*
		DO	0.24	**0.07**		Depth	0.61	*0.03*
		Temp	0.14	**0.15**		OB	0.46	0.04
		Turbidity	0.12	*-0.38*		Turbidity	0.14	0.19
		LL	0.01	*-0.52*		pH	0.02	**0.55**
		SWD	-0.02	-0.24		Temp	-0.10	0.35
		STR	-0.05	0.06		DO	-0.12	0.43
		Velocity	*-0.08*	**0.27**		Cond	*-0.15*	**0.80**
		OB	*-0.16*	-0.35		Salinity	*-0.28*	**0.81**
		Depth	*-0.31*	-0.25		Velocity	*-0.62*	*-0.27*

Figure 8.1: Discriminant Function Analysis and Principal Component Analysis of environmental variation among Areas and Creeks. Corresponding tables represent Factor Scores for each analysis. Values in **bold** and *italic* indicate the top and bottom three Factor Scores for each eigenvector/axis respectively. Large circles represent each group centroid with smaller square, diamond and triangle shapes signifying the centroid for the pools, riffles and runs respectively. Standard error for each point is shown using error bars. The variation explained by each eigenvector is indicated on its' corresponding axis. Environmental variable abbreviations see Chapter 6.

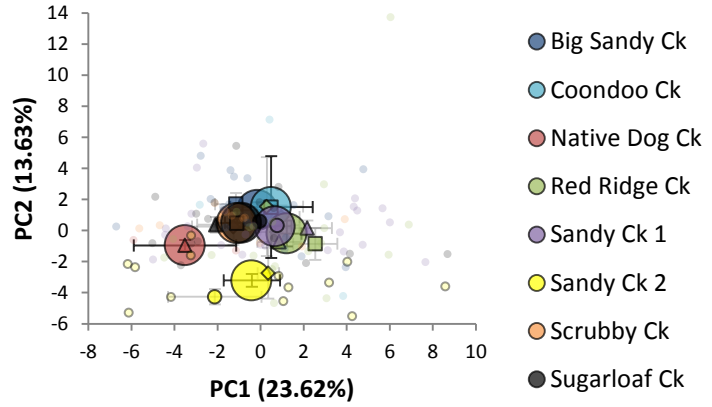
Principal Component Analysis

Area



Var	PC1	PC2
2CW	**0.93**	*-0.20*
PL	**0.92**	-0.15
PW	**0.92**	*-0.26*
PD	**0.91**	*-0.25*
RL	*-0.32*	**0.64**
ASL	*-0.33*	**0.72**
RAL	*-0.34*	0.46
TL	*-0.62*	0.52
3LL	0.19	**0.63**
4LL	0.12	**0.62**
3CW	0.90	*-0.20*

Creek



Var	PC1	PC2
2CW	**0.93**	*-0.20*
PL	**0.92**	-0.15
PW	**0.92**	*-0.26*
PD	**0.91**	*-0.25*
RL	*-0.32*	**0.64**
ASL	*-0.33*	**0.72**
RAL	*-0.34*	0.46
TL	*-0.62*	0.52
3LL	0.19	**0.63**
4LL	0.12	**0.62**
3CW	0.90	*-0.20*

Figure 8.2: Principal Component Analysis of morphological variation among Areas and Creeks. Corresponding table represents Factor Scores for each analysis. Values in bold and italic indicate the top and bottom three Factor Scores for each eigenvector/axis respectively. Large circles represent each group centroid with smaller square, diamond, triangle and circle shapes signifying the centroid for the four sites respectively. Standard error for each point is shown using error bars. The variation explained by each eigenvector is indicated on its' corresponding axis. Morphology measurement abbreviations see Figure 6.2.

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