Chapter 1
General Introduction

1.1 Population genetic structure and genetic models

The genetic structure of natural populations is dependent on the interaction between the biology of species, their environment and evolutionary processes: natural selection, mutation, gene flow and genetic drift (Slatkin, 1987).

Dispersal and gene flow, which refer respectively to the movement of individuals and gametes among populations, are closely associated and important in dictating the structure of populations. Generally, species with efficient mechanisms of dispersal, inhabiting continuous environments, have high rates of gene flow between subpopulations, which leads populations to genetic homogeneity on a large geographical scale. Species with low dispersal abilities often exhibit low levels of gene flow between subpopulations. In the absence of selection, limited gene flow will lead to genetic differentiation among subpopulations due to genetic drift (Slatkin, 1981). The rate at which subpopulations differentiate is largely a function of the effective population size because as it becomes smaller, the loss of variation due to random drift is faster (Crow and Aoki, 1984). When gene flow between subpopulations is absent, differential selection pressure can lead to genetic differentiation, which under biological species concepts may result in allopatric speciation (Templeton, 1989).

Many species are composed of a large number of local populations, among which gene flow is variable in space and time. The spatial arrangement of these subpopulations alone can profoundly influence the degree of genetic differentiation displayed in species (Slatkin, 1987). Several population genetic models have been developed for estimating patterns of genetic exchange among subpopulations, under the assumption that selection does not affect alleles or haplotypes. For example, in a continuously distributed species, genetic differences between subpopulations should increase with the distance separating them, when the effective dispersal distance is the only barrier to random mating. Wright (1943) first outlined this process, naming it “isolation by distance”. A pattern of spatial differentiation that arises in populations when dispersal is likely to occur only among adjacent discrete demes is described more accurately by the “stepping stone” models

(Kimura and Weiss, 1964). Populations in linear stepping stone models (one-dimensionally distributed species) have only two connections from which to receive migrants, whereas populations in two-dimensional stepping stone models have at least four, ignoring edge effects (Kimura and Weiss, 1964). Because migrants come mainly from neighbouring populations in the linear case, they are more likely to be closely related and thus less likely to introduce new genes into the population (Crow and Aoki, 1984). The "island model" (Wright, 1943) describes a pattern of spatial differentiation similar to the two-dimensional stepping stone models. Wright assumed that a population is subdivided into many subpopulations ("islands") of equal effective population size, all of which exchange genes with equal probability.

1.1.1 *F*-statistics as a measure of gene flow

Wright (1931) showed that there is a non linear relationship between the amount of gene flow and the degree of genetic differentiation among subpopulations, as measured by *F*-statistics. F_{ST}, based on allele frequency distributions, is the proportion of variance in allele frequencies that is among subpopulations (Wright, 1951; Nei, 1987). The degree of among subpopulation differentiation at neutral marker loci as estimated by F_{ST} indicates the expected degree of population differentiation as a result of the combined effect of genetic drift and gene flow (Wright, 1951; Rogers, 1986; Lande, 1992). For example, the use of F_{ST} as an indirect measure of gene flow is suggested by Wright's island model. The island populations can be treated as replicates that are characterised by two parameters: effective population size (N_e) and the migration rate (m). The strength of genetic drift is proportional to $1/N_e$, while the strength of gene flow is proportional to m. Wright proposed that at equilibrium:

$$F_{ST} = 1 / (4N_em + 1)$$

F_{ST} quickly approaches 0, as Nem (the effective number of migrants) increases. Theoretical values of F_{ST} range from zero to one, with zero indicating panmixia and one indicating complete demographic independence among populations.

1.1.2 Effective population size

In an ideal population every individual is considered equal in reproductive capacity, there is no overlap in the individuals from one generation to the next, mating occurs at random and the population remains at a constant size over time (Wright, 1969). There is

a variety of ways in which real populations can depart from ideal populations. For example, unequal sex ratio, changes in effective population size, differential reproductive success and overlapping generations. These deviations cause departures from predictions based on ideal populations (Hartl and Clark, 1989).

One of the most dramatic ways in which changes in effective population size influence molecular evolution occurs when a population experiences a rapid and severe decrease in the number of individuals ("population bottleneck"), perhaps because of natural disaster or disease. Bottlenecks greatly reduce the amount of genetic variation in populations because only a small number of alleles will survive the drop in size (Nei *et al.*, 1975; Barret and Richardson, 1985).

Effective population size will also change if a small group of individuals become isolated from the main population (i.e. colonisation of habitats) or in the case of re-colonisation of habitats after extinction of local populations. Because these isolated individuals will only carry a small proportion of the genetic variation from the population of origin, genetic drift can largely alter their genetic structure ("founder effect"). It is likely that the founding population will, by chance, have lost some alleles present in the population of origin (analogous to a bottleneck), or that alleles at low frequencies in the population of origin will be overrepresented in the new population (Hartl and Clark, 1989). Thus, a combination of the founder effect and genetic drift will enhance genetic differentiation among local populations (Whitlock and McCauley, 1990).

Habitat fragmentation is predicted to have considerable long-term effects on genetic and demographic viability of populations, due to the combined effects of reduced effective population size and increased isolation (Frankel and Soulé, 1981). Generally, overall levels of genetic diversity are expected to be lower in fragmented than in continuously distributed populations, because genetic drift has an increased effect when local effective population size and/or migration rate are reduced. Therefore, the genetic structure of a fragmented population leads to isolated and genetically discrete demes with, generally, low levels of genetic variability and high levels of genetic differentiation (Wright, 1931).

1.2 Gene genealogies and the coalescent process

Traditionally, allele frequencies and their geographical distribution have been used to quantify population subdivision and estimate the amount of gene flow between subpopulations (Wright, 1931, 1943). However, these approaches do not allow the investigation of how these alleles are related to each other. Furthermore, very different evolutionary processes may generate similar distributions of allele frequencies, which are then hard to distinguish through simple 'summary statistics', such as F_{ST} (e.g. Larson, 1984). Genetic surveys using restriction site or mitochondrial DNA sequences provide information on the evolutionary relationships of the genetic variants or haplotypes. Genetic variation (obtained from mtDNA sequence data) is often presented as 'gene trees', which describe the phylogenetic relationship of the sequences sampled from a population (Page and Holmes, 1998). The coalescent theory predicts the structure of gene trees for populations with different demographic histories, that is how genealogies are affected by changes in population size and structure (Felsenstein, 1971; Griffiths, 1980; Tavaré, 1984; Hudson, 1990, 1998). The coalescence theory could be explained as follows. For two gene sequences sampled in a population subjected to genetic drift alone, it is possible to trace their lineage backwards in time to when they shared a common ancestral allele. This is the point at which the two gene lineages 'coalesce' and the time at which this occurs is the 'coalescent time'. Continuing backwards in time, the number of lineages is reduced by one at each coalescence, creating nodes on the gene tree. Eventually, the gene tree will arrive at the single ancestral allele from which all the alleles have descended. This is called 'the most recent common ancestor' (Hudson, 1998).

1.3 Phylogeography

Phylogeography is a discipline that investigates underlying processes governing the geographic distributions of genealogical lineages (Avise *et al.*, 1987; Avise, 1994). The qualitative approach to phylogeography devised by Avise *et al.* (1987) explored the relationship between lineage divergence and the extent of geographical partitioning among haplotypes. Biological inferences were derived by overlaying haplotype trees or phylogenies on to a geographical distribution of sample locations. Avise (2000)

proposed five phylogeographic categories to define phylogeographic patterns that continually arise in phylogeographic analysis.

Category I occurs where lineages are separated by large mutational distances, confined to separate areas of a species` range (allopatric lineages). Such large phylogeographic discontinuities often arise from long-term extrinsic barriers to gene flow.
Category II occurs where lineages are deeply separated, but sympatric. This pattern could arise in species with large evolutionary effective population size and high gene flow, where some anciently separated lineages might by chance have been retained, whereas many intermediate genotypes have been lost over time by gradual lineage sorting. Category II patterns may also arise as a consequence of secondary admixture between allopatrically evolved populations.
Category III occurs where allopatric lineages are separated by shallow mutational distances. The implication is that contemporary gene flow has been low enough to promote genetic divergence among populations relatively recently isolated, through lineage sorting and random drift.
Category IV describes broadly sympatric lineages with recent evolutionary connections. This pattern is expected in populations with high levels of gene flow, when the effective population size is small and they have not been sundered by long term biogeographic barriers (i.e. panmixia).
Category V describes lineages with varied distribution but with shallow evolutionary separation. It is intermediate between Categories III and IV, and involves common lineages that are widespread plus closely related lineages confined to one or a few local populations. It occurs when gene flow between populations is limited.

Intraspecific phylogeography investigates the genetic structure of populations, allowing discrimination between the relative importance of historical and contemporary processes, by reconstructing the main sequence of events that have generated the current genetic structure. The use of statistical phylogeographic analysis, such as nested clade analysis (Templeton 1993, 1994, 1998; Crandall and Templeton, 1993), allows the discrimination among various biological explanations that may have determined an observed phylogeographic pattern.

1.3.1 Phylogeographic statistics: nested clade analysis

Nested clade analysis and other statistical procedures that use a coalescence approach to investigate genetic variation (Hudson *et al.*, 1992; Slatkin, 1989; Slatkin and Maddison 1989; Templeton, 1993; Templeton *et al.*, 1995; Templeton and Georgiadis, 1996) have an enhanced power over *F*-statistics, because they allow greater precision of gene flow estimation, and can separate population structure (recurrent forces, such as gene flow) from historical events (fragmentation and range expansion). For example, when a species has been fragmented into two or more subpopulations that experience no gene flow at all between them, if they had a relatively recently shared ancestry, the population could still display some genetic similarity. Yet, their shared ancestry can create F_{ST} values less than one, erroneously implying gene flow. Alternatively, when a species has recently expanded its range over a large area from some smaller subpopulations within the ancestral range, there would be much genetic similarity over the recent colonised area, leading to an overestimate of gene flow (Larson, 1984). The use of *F*-statistics or an algorithm that assumes that all geographical associations are due to gene flow (e.g. Slatkin and Maddison, 1989) can therefore yield an estimator of *Nm* that is biologically misleading (Templeton, 1998).

Nested clade analysis (Templeton, 1993, 1994, 1998; Crandall and Templeton, 1993) overlays an estimated gene tree upon geography in a rigorous statistical framework, designed to measure the strength of any geography/phylogeny association and to suggest the evolutionary processes responsible. Probably the main limitation of NCA occurs as a result of a false inference or biological misidentification (Templeton, 2004). False inferences could arise from the evolutionary stochasticity of the coalescent process itself, from the haplotype tree being skewed or otherwise altered by natural selection or from inadequacies in NCA and/or its inference keys (Templeton, 2004). NCA inferences can be validated by known 'a priori' information about historical events, such as range expansion or fragmentation. When no strong prior expectations are available, as in the case of specific patterns of gene flow, NCA can be validated with simulations. Various patterns of gene flow could be simulated and then tested with NCA (Templeton, 2004). Another approach to validate NCA is through a 'cross-validation' method, using data from multiple mtDNA regions (Templeton, 2002). NCA procedures are presented in the Chapter 2.

1.3.2 Comparative phylogeography

Phylogeography has become a powerful approach for elucidating contemporary geographical patterns of evolutionary subdivision within species and species complexes. A relatively recent extension of this approach is the comparison of phylogeographic patterns of multiple co-distributed taxonomic groups, or 'comparative phylogeography' (Avise, 1994). Comparative phylogeographic studies (Avise, 2000; Riddle *et al.*, 2000; Sullivan *et al.*, 2000) have revealed pervasive and previously unrecognised biogeographic patterns, which suggest that vicariance has played a more important role in the historical development of modern biotic assemblages than current taxonomy would indicate. In both historical biogeography and comparative phylogeography, the most parsimonious explanation for multiple co-distributed taxonomic groups to exhibit common spatial patterns of evolutionary subdivision is that they have been subjected to the same environmental history (Avise, 2000).

Comparative phylogeography complements analytical methods of historical biogeography, including cladistic approaches aimed at reconstructing the history of the place. In addition, because the phylogenies used are typically based on molecular data, it is possible to test hypotheses within the likelihood frameworks and to estimate important biogeographic and population parameters, such as the timing of population separation and speciation events, and the estimation of changes in effective population size over time. All of this information can be evaluated within the context of independent data that are obtained from the geological, climatological, palaeontological and palynological records (Arbogast and Kenagy, 2001).

1.4 Molecular markers

1.4.1 Allozymes

Genetic studies of diploid organisms using allozyme electrophoresis produce data that are normally based on co-dominant alleles at autosomal loci. Allozyme electrophoresis is used to provide genetic markers, in the form of single gene allelic variation, to be used in determining the population structure of a species (Richardson *et al.*, 1986). Moreover, when allozymes display genetic variation, they are good indicators of gene

flow. F_{ST} is an indirect measure of gene flow, estimating the proportion of genetic variation partitioned among populations (Wright, 1951; Nei, 1987).

A disadvantage is that selection may affect allozyme loci (Sokal *et al*., 1989). Local environmental variation may drive natural selection to operate differently among local populations affecting allozyme variation. Alternatively, balancing selection may lead to homogeneity of allele frequencies among subpopulations (Reeb and Avise, 1990). Nevertheless, valuable information about populations can be obtained by analysing multiple loci. If one of the loci is particularly affected by selection, it can usually be identified. Another disadvantage of allozymes is that they can not be used for phylogeographic purposes because the historical relationships of alleles at a locus remain unknown (Slatkin and Maddison, 1989).A final limitation of allozymes is that the total amount of detectable variation may be limited.

1.4.2 Mitochondrial DNA

Mitochondrial DNA has a fourfold smaller effective population size than nuclear genes, due to its uniparental maternal inheritance and a non-recombining haploid nature. The smaller effective population size and high mutation rate, that provides a large number of alleles, amplify the effects of genetic drift resulting in an elevated rate of differentiation between isolated subpopulations compared to nuclear genes (Birky *et al.* 1989). These attributes make a gender specific marker such as mitochondrial DNA a powerful locus for detecting patterns of demographic structure in natural systems (Wollenberg and Avise, 1998). It may be particularly useful in those systems characterised by large effective population sizes or where populations have recently increased it (i.e. due to range expansions), where maximum discriminatory power is essential. Mitochondrial DNA has also been largely used to investigate intraspecific phylogeographic patterns (Avise, 2000). Assuming that all organisms share a common ancestor with genetic divergence over time (Huelsenbeck and Rannala, 1997), the reconstruction of genealogical lineages for a population provides information regarding the history of that population.

1.4.3 Microsatellites

Microsatellites are repeat motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes. In the last few years they have become very popular molecular markers used with applications in many different fields (i.e. forensic DNA studies, population genetics and conservation/management of biological resources). Microsatellites are usually highly polymorphic, non-coding co-dominant DNA markers that are less likely to be affected by selection than allozymes, because they occur in non-coding regions of the genome. Thus, they tend to show high levels of variation within populations and can be useful for examining genetic differentiation on a fine scale.

The major drawback of microsatellites is that they need to be isolated *de novo* from most species that are being examined for the first time. This is due to the fact that microsatellites are usually found in non-coding regions where the nucleotide substitution rate is higher than in coding regions. Consequently, the strategy of designing 'universal primers' matching conserved sequences, which is very effective for mtDNA (Kocher *et al.*, 1989), is more problematic for microsatellites. Another problem with microsatellite usage can occur during their amplification by polymerase chain reaction (PCR). 'Null alleles' occur when one or more alleles fail to amplify during PCR, 'stuttering' when slight changes occur in the allele size during PCR and 'large allele dropout' when large alleles do not amplify as efficiently as small alleles. Also, because of this high mutation rate, 'homoplasy' of alleles is highly possible. Microsatellite alleles generally refer to DNA fragments of different size (electromorphs). Electromorphs can be identical in state (i.e. have identical size), but may not necessarily be identical by descent due to convergent mutation(s) (Estoup *et al.*, 2002).

1.5 Genetic structure of freshwater populations

Freshwater invertebrates occur in habitats that represent discrete sites surrounded by inhospitable terrestrial landscapes. Some aquatic insects possess a flying stage in their life cycle that allows them to disperse across drainage boundaries (Hughes *et al.*, 1998; Miller *et al.*, 2002), but in general, movements of obligate freshwater species are very much limited by the physical nature and arrangement of the riverine system (McGlashan *et al.*, 2001; Hurwood and Hughes, 2001). Thus, populations of obligate freshwater

fauna are expected to show a high degree of genetic structuring, as physical barriers are likely to impede gene flow (Gyllensten, 1985; Ward *et al.*, 1994). Meffe and Vrijenhoek (1988) proposed a model to describe the distribution of obligate freshwater species, where patterns of genetic variation among populations at drift/migration equilibrium should conform to the hierarchical nature of the river systems that they inhabit. 'The hierarchical model of gene flow' (Meffe and Vrijenhoek, 1988) predicts that the level of genetic differentiation between subpopulations from within river drainages will be significantly less than between isolated river systems. Also, genetic differentiation will be higher between subcatchments than between streams within subcatchments. Within drainages, where in-stream barriers are not significant, a pattern of isolation by stream distance may be expected. Genetic studies of some species conform to these expectations (e.g. the Pacific Blue-eye *Pseudomugil signifera*, McGalashan and Hughes, 2002; the freshwater shrimp *Caridina* sp., Woolschot *et al.*, 1999) while many others do not (e.g. some freshwater fish species: Hurwood and Hughes, 1998; McGalashan and Hughes, 2000, and crustaceans Hurwood and Hughes, 2001). These unexpected patterns of genetic differentiation have been attributed to a number of factors, such as relatively recent drainage rearrangements (Hurwood and Hughes, 1998) and terrestrial dispersal (Hurwood and Hughes, 2001).

1.5.1 Phylogeography of freshwater species

Freshwater drainages are spatially disconnected and obligate aquatic populations are expected to be genetically structured accordingly. Contemporary gene flow between river catchments is likely to be extremely limited. Thus, given significant evolutionary time with limited or absent gene flow, each river system should attain reciprocal monophyly, reflecting a Category I phylogeographic pattern that is consistent with the hierarchical model of gene flow.

Indeed, the distribution of a species may be dependent largely on historical events. Landscape and river courses continually change over geological time, and adjacent drainages may be connected repeatedly by headwaters streams, outlet merges (e.g. Bermingham and Avise, 1986; McGuigan *et al.*, 2000) or drainage and river course rearrangements (Hurwood and Hughes 1998; McGalashan and Hughes, 2000). For example, over shorter temporal scales, extensive floods may connect waterways, by inundating low divides, whereas droughts can isolate them (Hughes and Hillyer, 2003).

The relative importance of these processes can leave distinct phylogenetic 'signatures' useful in reconciling species' biology with population connectivity and earth history (McGalashan and Hughes, 2000).

1.6 Focus of this study

1.6.1 Western Queensland river systems and its aquatic fauna

Rivers in arid and semi-arid areas of Western Queensland, Australia, have flow regimes with episodic extensive floods and many small or no flow phases, associated with wet and dry seasons respectively. Usually rainfall occurs during the north Australian Monsoon (November to March) leading to intense but erratic rainfalls that bring floods to the rivers in most years. The hydrology of these dryland rivers is also strongly influenced by the irregular atmospheric circulation phenomena, the El Niño-Southern Oscillation (ENSO), responsible for prolonged dry weather and La Niña, associated with major flooding episodes (roughly, once in 20 years) (Allan 1985; Allan 1988; Kotwicki & Isdale 1991; Kotwiki & Allan 1998). Driven by this highly unpredictable rain regime, dryland rivers in Western Queensland typically experience episodic floods, where they are highly connected by a dominant system of anastomosing channels and extremely low or no flow periods (Puckridge *et al*., 1998, 2000). During low or no flow periods, water persists only in relatively wide and deep sections of the river channels, which are called 'waterholes', which serve as refugia for aquatic species during protracted intervals between floods (Knighton & Nanson 1994; Walker *et al.*, 1995; Sheldon *et al.*, 2002). These waterholes undergo continuous water loss by evaporation between episodic floods (Hamilton *et al.*, in review). Evaporation rates are largely variable between waterholes, some may experience larger loss of water than others and may, even rarely, dry out completely (Puckridge *et al*., 1998, 2000), reducing population sizes or ensuring the extinction of local populations of freshwater species without a resistant stage in their life cycle, respectively. In such discontinuous riverine habitat, passive and active movement of freshwater obligate invertebrates may be achieved in currents only during wet seasons, when water is flowing in rivers and the nearby floodplains.

1.6.2 Palaeoclimate

During the Pleistocene, periods of high rainfall alternated with periods of very dry weather, associated with interglacial and glacial phases respectively (Kershaw and Nanson, 1993; Alley, 1998). The period of maximum precipitation and the greatest fluvial activity was indicated as between about 110 000 and 80 000 years ago, when greatly expanded lakes dominated much of the Australian continent (Kershaw and Nanson, 1993; Alley, 1998; Croke *et al.*, 1999). These climate oscillations changed the hydrological flood regimes in Western Queensland significantly, potentially modifying levels of connectivity between populations of aquatic species. Eventually, the modern ephemeral regime of annual floods became established in the Lake Eyre Basin after about 3000-4000 yr BP (Gillespie *et al.*, 1991; Magee *et al.*, 1995).

1.6.3 Freshwater invertebrates inhabiting Western Queensland rivers

Until recently, the geographical extent of colonisation events of aquatic fauna associated with the episodic floods was thought to be extensive, due to the intensity of the currents and the low topographic relief of the region (Gibling *et al*, 1998). However, recent studies using molecular markers have provided novel insight into patterns of dispersal of freshwater invertebrates in Western Queensland. Contemporary gene flow appeared to be absent across drainage boundaries and very limited between catchments for three species with apparently good dispersal capabilities: *Cherax destructor* (Decapoda: Parastacidae) (Hughes and Hillyer, 2003), *Velesunio* sp. (Hughes *et al*, in review), and *Macrobrachium australiense* (Decapoda: Palaemonidae) (Cook *et al.*, 2002). It was suggested that despite the low topographic relief of the area, the occasional floods might not provide enough movements of individuals to ensure complete panmixia of populations within this region.

The species targeted in this study are two endemic freshwater invertebrates inhabiting permanent and ephemeral waterholes of Western Queensland: *Notopala sublineata* (Gastropoda: Viviparidae) and *M. australiense* (Decapoda: Palaemonidae). *N. sublineata* has been listed as endangered in the 2003 *IUCN* Red List of Threatened Species, since it is now considered extinct in some areas (Sheldon and Walker, 1993). The limited vagility and the absence of a larval stage (the species is viviparous) suggest

that *N. sublineata* should have low dispersal capabilities. *M. australiense* is the most common and abundant macro-invertebrate species in these water systems (Lee and Fielder, 1983; Sheldon and Walker, 1998). It appears to have good dispersal capabilities, due to a planktonic larval phase in its life cycle and its good swimming abilities (Williams, 1980).

1.6.4 Specific hypotheses of this study

Western Queensland river systems at the present time provide a very fragmented and variable habitat for freshwater biota. Rivers consist of a series of disconnected pools, where populations of freshwater fauna are isolated for long periods of time. Aquatic species are most likely to migrate between neighbouring pools during floods when ephemeral channels connect them. Also, it has been accepted that palaeoclimatological changes of the Quaternary have created significant changes in the hydrology and connectivity between river systems in Western Queensland. Yet, the impact of these contemporary and historical processes on freshwater species inhabiting this area is still unclear. The current distribution of genetic variation in populations is shaped by both historical and contemporary processes. The temporal information provided by mtDNA analyses can assist in determining the extent to which the population structure in Western Queensland results from recurrent processes, such as ongoing gene flow, versus historical processes, such as range expansion.

In this study, I will test the following hypotheses:

1. It was hypothesised that freshwater invertebrates, will display high levels of genetic structure among populations, because physical barriers represented by terrestrial inhospitable habitat, are likely to impede gene flow between populations inhabiting isolated pools. This hypothesis was tested for *N. sublineata* and for *M. australiense*. The results were discussed in chapter 3 and 4 respectively.

The choice of two species with dramatically different dispersal abilities will allow an assessment of the extent to which gene flow is determined by specific dispersal abilities or environmental factors (such as pattern of river flows). For example, if only *M. australiense* displayed high level of gene flow between populations, this would suggest that the network of ephemeral channels and floodplains allowed movements of species

with good dispersal abilities, implying that specific dispersal abilities play a primary role in maintaining levels of gene flow between populations. On the contrary, if both the species displayed limited gene flow between populations, this would suggest that habitat fragmentation impeded movements of any aquatic species, implying that specific dispersal abilities play a secondary role in maintaining levels of gene flow between populations. Results of this comparative approach will be discussed in chapter 6.

2. It is widely accepted that there have been significant changes in patterns of connectivity between rivers in Western Queensland during the Pleistocene (Kershaw and Nanson, 1993; Alley, 1998; Croke *et al.*, 1999). It was hypothesised that levels of gene flow between populations of aquatic species were higher during the Pleistocene (likely movements of individuals across catchment boundaries) and that they have been isolated relatively recently. Thus, in order to ascertain the relative contribution of historical versus contemporary processes that determine the actual genetic structure of populations within the region, the phylogeographic patterns of two species in the region (*N. sublineata* and *M. australiense*) were investigated in this study. The results were discussed in chapter 3 and 4 respectively.

Historical geomorphological events are likely to have had a major influence on the distribution of genetic variation, and phylogeographic patterns of co-distributed freshwater species should reflect these changes. If freshwater species in Western Queensland have had a shared biogeographic history, they will exhibit analogous spatial patterns of evolutionary subdivision. A common set of historical events that had geographically affected a group of ancestrally co-distributed organisms in a similar way should leave a phylogeographic signature in these populations. Phylogeographic patterns of the two species targeted in this study and three species inhabiting the same region (*Cherax destructor*, studied by Hughes and Hillyer, 2003 and *Velesunio* species A and C by Hughes *et al.*, in review) were compared and discussed in chapter 6. As historical changes in connectivity between these river systems are likely to have had a similar effect on populations, it would be expected that all invertebrate species will be affected in the same fashion.

3. The effects of the highly unpredictable flow regime on the genetic variability of aquatic populations were tested by formulating two hypotheses. First, if populations of obligate freshwater organisms inhabiting less persistent waterholes are more likely to

experience periodic bottlenecks than those inhabiting more persistent ones, they would be expected to have lower levels of genetic diversity. Second, if populations inhabiting less persistent waterholes periodically undergo local extinction with subsequent recolonisation, there should be higher levels of genetic differentiation among them, due to the founder effects, than among those populations inhabiting more persistent waterholes (Whitlock and McCauley, 1990). These hypotheses were tested for *N. sublineata* and for *M. australiense*. The results were discussed in chapter 5.

Chapter 2
General Methods

This chapter provides an overview of the techniques and analyses adopted for this study. Where analyses were restricted to specific questions, these are described in the relevant chapter.

2.1 Study species

For the present study, two endemic species of Australian obligate freshwater invertebrates were selected: *Notopala sublineata* (Gastropoda: Viviparidae) and *Macrobrachium australiense* (Decapoda: Palaemonidae) for the following reasons: a) they tend to share the same habitat, waterholes in dry and semi-dry river systems of Western Queensland b) they have apparently different dispersal capabilities, due to their dissimilar life history c) where they occur, they are usually abundant d) *N. sublineata* populations are rapidly declining in some areas, whereas *M. australiense* is still widespread and abundant throughout the region.

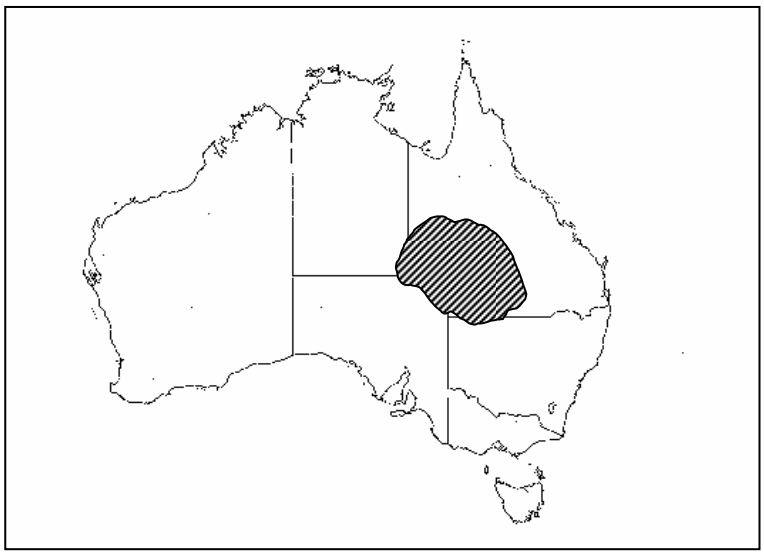
2.1.1 *Notopala sublineata*

There are approximately 4 species of *Notopala* (Sheldon & Walker 1993) found in Australia, mostly located in the northern part of the continent. *N. sublineata* (Gastropoda: Viviparidae) (Figure 2.1) is an endemic Australian freshwater snail that has been listed as endangered in the 2003 *IUCN* Red List of Threatened Species. The species was widely distributed in river systems of central Australia but now it has declined rapidly over the last few decades, apparently as a result of weir building and other activities associated with river flow management (Figure 2.2) (Sheldon and Walker, 1997). *N. sublineata* now seems to be virtually extinct throughout the Murray-Darling natural waterways, although there have been reports of some populations surviving in irrigation pipelines (Sheldon and Walker, 1993). However, the species is still common within the Lake Eyre basin of Western Queensland. It inhabits permanent and ephemeral waterholes, where it is found along the banks, attached to logs and rocks or crawling in the mud, feeding on bacterial biofilms. *N. sublineata* is typical of the family, with a conical-turbinate shell, a brown to greenish-brown periderm, often with

darker spiral bands and a corneous concentrically ringed operculum. The snails are dioecious and viviparous with internal fertilisation. The embryos are retained in a modified oviduct, arranged in a developmental sequence with small eggs at the rear and miniature adults at the front (Stoddart, 1982). *N. sublineata* is able to avoid dehydration by tightly sealing the aperture with the operculum (Sheldon, unpublished). However, it is unlikely to be able to survive prolonged drying out. The limited vagility, the benthic behaviour and the absence of a larval stage suggest that *N. sublineata* possesses very limited dispersal abilities.

Figure 2.1 *N. sublineata* shells.

Figure 2.2 Approximate geographic distribution of *N. sublineata* in Australia, derived from various sources (Sheldon and Walker, 1993, 1997; Ponder, 1997).

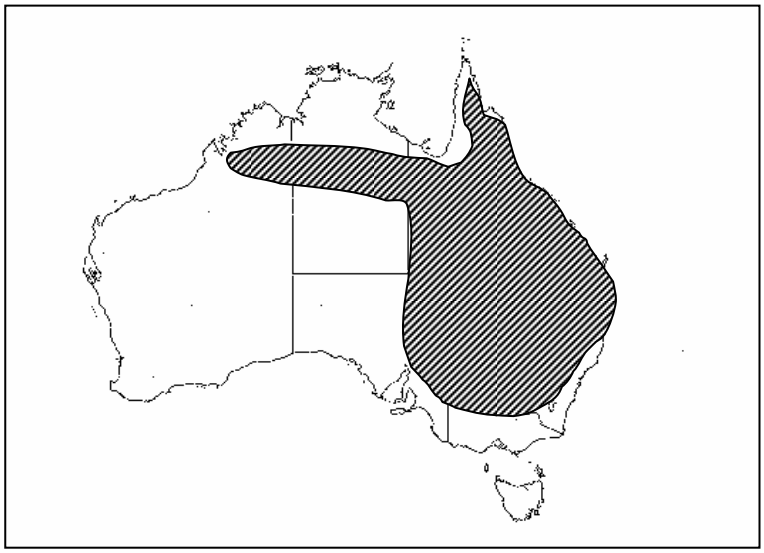


2.1.2 *Macrobrachium australiense*

The freshwater prawn *Macrobrachium australiense* (Decapoda: Palaemonidae) (Holthuis, 1950) (Figure 2.3) is the most common and abundant endemic macro-invertebrate species throughout northern (except coastal areas of North Territory), central and eastern Australia (Figure 2.4) (Short, in press). The species occurs in both lotic (i.e. flowing) and lentic (i.e. non-flowing) freshwater environments, where they seek refuge under submerged logs, tree roots and rocks along river banks (Lee & Fielder 1983, 1984). In particular, it occurs in open water, pond and lake habitats of arid and semi arid rivers throughout the study area (Lee and Fielder, 1983; Sheldon and Walker, 1998). *M. australiense* has a head-thorax with irregular grey blotches and lengthwise dark bands on palm and wrist of claws (Short, in press). The species completes both its adult and juvenile life history stages in freshwater habitats and it produces few large eggs that remain attached to the swimmerets of the female. *M. australiense* has a non-feeding larva with three stages completed in six days (Fiedler 1970; Lee & Fiedler 1982). This freshwater prawn appears to have good dispersal capabilities, due to a planktonic larval phase in its life cycle and its good swimming abilities, but it is not able to survive drying out (Williams, 1980).

Figure 2.3 *M. australiense* (Holthuis, 1950), (Photo courtesy of W. Hadwen).

Figure 2.4 Geographic distribution of *Macrobrachium australiense* in Australia (Short, in press)



2.2 Study area

Arid and semiarid river systems in Western Queensland, Australia (Figures 2.5a and b) are characterized by an unpredictable and highly variable nature of their climatic and hydrological regimes. They have a flow regime with episodic very large floods and many small or no flow periods (Kotwicki and Allan, 1998). During no flow periods, rivers consist of a series of isolated permanent and ephemeral waterholes, where obligate freshwater species are confined, and much of the channel is dry. One of the most distinctive features of this system is the preponderance of permanent and ephemeral "waterholes" which represent relatively wide and deep sections of the channels, ranging in length from 100m to over 20 Km (Knighton & Nanson 1994). Permanent waterholes (Figure 2.6a) are very large pools that never dry out completely, while ephemeral waterholes (Figure 2.6b) experience recurrent significant losses of water and eventually they completely dry out (Pickup 1991).

Sampling sites were located in the Lake Eyre basin and the northern regions of the Murray-Darling basin (Figure 2.7). The Lake Eyre basin is an endorheic system and it is characterised by temporary streams and rivers (Knighton and Nanson, 1994). The Western part of the study area included the four major river systems on the north-eastern side of the Lake Eyre basin: the Bulloo, the Cooper, the Diamantina and the

Georgina catchments. All these catchments terminate at Lake Eyre except for the Bullo which terminates in a series of wetlands in northern New South Wales. The Murray-Darling basin is exorheic, drawing water from two major systems: the Murray and the Darling rivers, ultimately draining to the sea through river systems along the South Australian coast. The catchments selected within the eastern part of the study area are all tributaries of the Murray-Darling basin and they were: Balonne-Condamine, Border Rivers, Warrego and Paroo.

Figure 2.5 Western Queensland river systems landscape overview during dry (a) and wet (b) periods.

a)

b)

Figure 26 More persistent (a) and less persistent (b) waterholes during the dry season in Western Queensland. Water level is reduced in the permanent waterhole, whereas the ephemeral is almost completely dry.

a)

b)

2.3 Sampling design and methods

2.3.1 Sampling design

For this study, a hierarchical sampling design was constructed by selecting the two major basins of Western Queensland: Lake Eyre and Murray-Darling and then four catchments within each basin (Figures 2.7, 2.8 and Tables 2.1, 2.2). Within each catchment, a number of sites were chosen, depending on the size of the catchment, accessibility of the area and species abundance. Eventually, within sites between one and four waterholes were sampled (Figure 2.8). Where possible, a balanced design

within and between catchments was adopted, to test the hypotheses of contemporary gene flow versus historical processes and of high levels of genetic structure in populations within the region. However, there were some geographic gaps in the sampling because in certain parts of the study area, no specimens of *N. sublineata* were encountered (notably all the Murray-Darling catchments), presumably due to the extinction of this species in those areas. To define genetic structure of populations on a fine scale (10Km, within sites) and to test the hypothesis that permanent waterholes serve as "refugia" for genetic diversity, *N. sublineata* and *M. australiense* were sampled from one permanent and generally between one and two ephemeral waterholes in each designated site. To assign the ephemeral or permanent nature to waterholes at each site, I referred to the local authorities, such as the Department of Natural Resources, the local National Parks authorities, and farmers within the study area.

Figure 2.7 Map showing major catchments and sampling sites in Western Queensland. For waterhole groupings within sites, refer to Table 2.1 and Table 2.2.

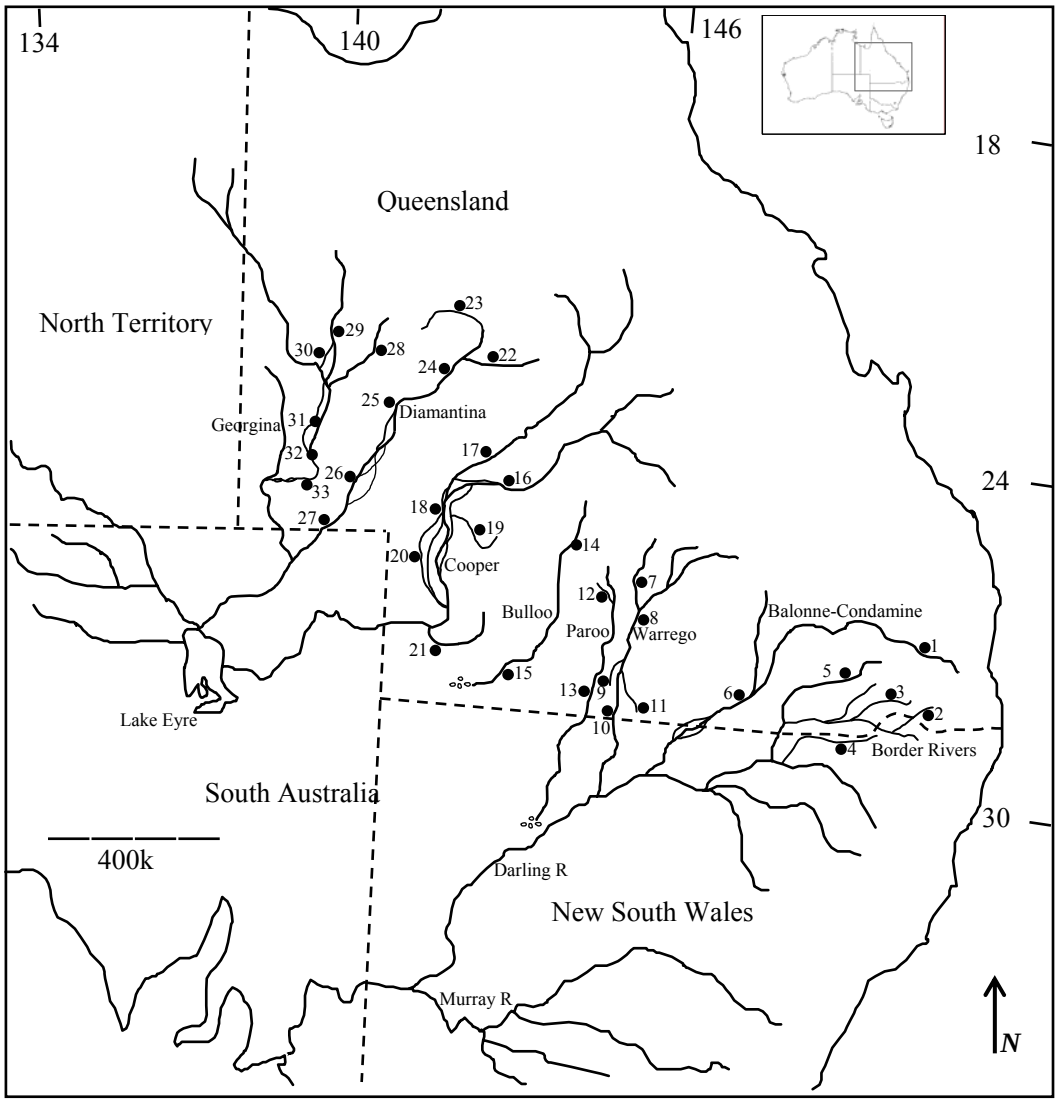


Figure 2.8 Sampling design indicates the two major basins: Lake Eyre and Murray-Darling and then four catchments within each one. Number of sites and waterholes within them varies between catchments *.

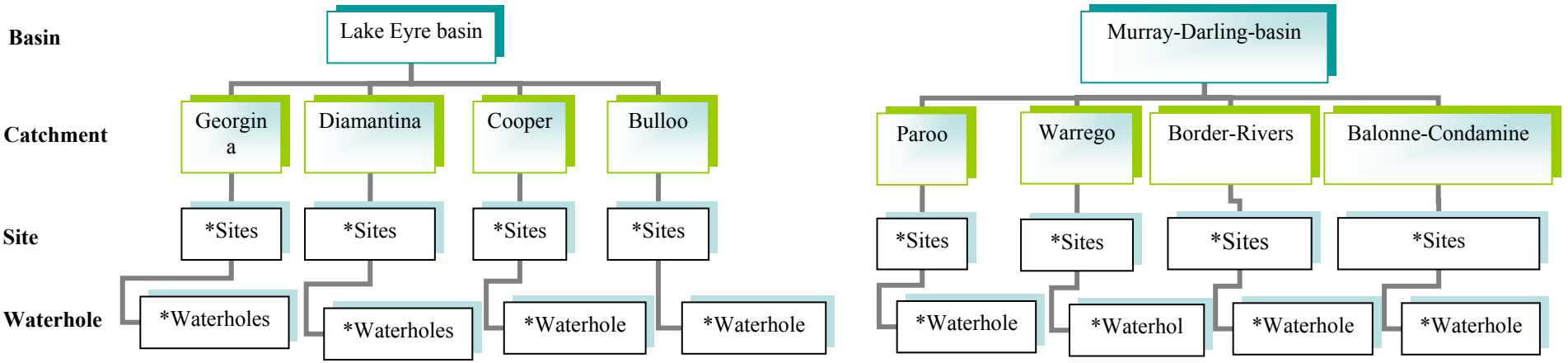


Table 2.1 Waterhole groupings within sampling sites and catchments in the Lake Eyre basin for *N. sublineata* and *M. australiense*. For geographic location, refer to Figure 2.3, where site numbers are displayed. Waterhole numbers are displayed when there is more than one waterhole within a site. * indicates the permanent waterhole at each site.

Catchment	Site No.	Waterhole No.	Site	Waterhole	*N. sublineata*	*M. australiense*
Bulloo	14	-	Bulloo R. at Quilpie	Quilpie	√	-
Bulloo	15	-	Bulloo R. at Thargomindah	Thargomindah	√	√
Cooper	16	-	Cooper Ck. at Retreat	Retreat	√	√
Cooper	17	a	Cooper Ck. at Noondah	Top*	√	√
Cooper	17	b	Cooper Ck. at Noondah	Pelican	√	√
Cooper	17	c	Cooper Ck. at Noondah	Waterloo	√	√
Cooper	18	a	Cooper Ck. at Windorah	Murken*	√	√
Cooper	18	b	Cooper Ck. at Windorah	Currareva	√	-
Cooper	18	c	Cooper Ck. at Windorah	Shedh	√	√
Cooper	18	d	Cooper Ck. at Windorah	Mayfield	-	√
Cooper	19	a	Kyabra Ck. at Springfield	Homestead*	√	√
Cooper	19	b	Kyabra Ck. at Springfield	Warranee	√	√
Cooper	19	c	Kyabra Ck. at Springfield	One Mile	√	√
Cooper	20	a	Cooper Ck. at Tanbar	Tanbar*	√	√
Cooper	20	b	Cooper Ck. at Tanbar	Yalungah	√	√
Cooper	20	c	Cooper Ck. at Tanbar	Yappi	-	√
Cooper	21	-	Wilson R. at Noccundra	Noccundra	√	√
Diamantina	22	-	Wockingham Ck. at Winton	Wockingham	√	√
Diamantina	23	-	Diamantina R. at Winton	Combo	√	√
Diamantina	24	a	Diamantina R. at Verdun Valley	Turkey*	-	√
Diamantina	24	b	Diamantina R. at Verdun Valley	Fish	√	√
Diamantina	25	a	Diamantina R. at Diamantina Lakes	Stock*	√	√
Diamantina	25	b	Diamantina R. at Diamantina Lakes	Middle	√	√
Diamantina	26	a	Diamantina R. at Monkira	Makara*	-	√
Diamantina	26	b	Diamantina R. at Monkira	Niggah	-	√
Diamantina	27	-	Diamantina R. at Birdsville	Birdsville	-	√
Georgina	28	-	Hamilton R. at	Bulla Bulla	√	√
Georgina	29	-	Georgina R. at Boulia	Boulia	-	√
Georgina	30	-	Bourke R.	Boomerang		√
Georgina	31	-	Georgina R. at Marion Downs	Four Miles	√	√
Georgina	32	-	King Ck. At Cluny	King	√	-
Georgina	33	-	Eyre Ck. at Lake Koolivo	Cuttaburra	√	-

Table 2.2 Waterhole groupings within sampling sites and catchments in the Murray-Darling basin for *M. australiense* (no live specimens of *N. sublineata* were found). For geographic location, refer to Figure 2.3, where site numbers are displayed. Waterhole numbers are displayed when there is more than one waterhole within a site. * indicates the permanent waterhole at each site.

Catchment	Site No.	Waterhole No.	Site	Waterhole	*M. australiense*
Balonne-Condomine	1	-	Condomine R. at Brigalow	Brigalow	√
Border Rivers	2	-	Whalan Ck. at Salt Bush	Salt Bush	√
Border Rivers	3	-	Weir R. at Kanowna	Kanowna	√
Border Rivers	4	-	Gnungarah Ck. at Gnungarah	Gnungarah	√
Border Rivers	5	-	Commoron Ck. at Iron Bark	Iron Bark	√
Balonne-Condomine	6	-	Balonne R. at St George	St George	√
Warrego	7	-	Ward R. at Charleville	Charleville	√
Warrego	8	a	Warrego R. at Quilberry	Quilberry*	√
Warrego	8	b	Warrego R. at Quilberry	SP	√
Warrego	8	c	Warrego R. at Quilberry	SP lagoon	√
Warrego	9	a	Warrego R. at Glencoe	Glencoe*	√
Warrego	9	b	Warrego R. at Glencoe	Wogganorrah	√
Warrego	9	c	Warrego R. at Glencoe	Key	√
Warrego	10	a	Warrego R. at Thurulgoona	Thurulgoona*	√
Warrego	10	b	Warrego R. at Thurulgoona	Noorama	√
Warrego	10	c	Warrego R. at Thurulgoona	Disco	√
Warrego	11	a	Cuttaburra Ck. at Tinnenburra	Binya*	√
Warrego	11	b	Cuttaburra Ck. at Tinnenburra	Red	√
Warrego	11	c	Cuttaburra Ck. at Tinnenburra	Mirage	√
Warrego	11	d	Cuttaburra Ck. at Tinnenburra	Tinnenburra	√
Paroo	12	-	Paroo R. at Cheepie	Cheepie	√
Paroo	13	-	Paroo R. at Eulo	Eulo	√

2.3.2 Field sampling and tissue storage

M. australiense was caught using a hand-hauled seine net (1mm mesh size), and *N. sublineata* was gathered along the entire perimeter of each waterhole and not from a single location. The snails tend to live in aggregates under logs or rocks; therefore by collecting from a large area, the possibility of having a limited number of families was reduced. The specimens were then separated into sealed and labelled plastic bags and placed in a liquid nitrogen container for transport back to the laboratory. The samples

were then transferred into a -80˚C freezer, until required for further analysis. The study species were sampled over the course of three field trips during dry seasons, between June 2001 and June 2002 (no major flood events were recorded within the study area during these two years): in the first field trip, the specimens were collected from the Bulloo and Cooper catchments, in the second from the Murray-Darling catchments and in the third one from Diamantina and Georgina catchments. Between 2001 and 2002 there were no major floods, therefore waterholes were not likely to have been reconnected between sampling trips.

2.4 Molecular techniques

2.4.1 *Notopala sublineata*

2.4.1.1 Allozyme electrophoresis

The whole digestive gland of each snail was removed by cracking the top of the shell. The digestive gland was chosen because it contains much less mucus than other parts of the body snails'. It appears that the use of tissues with a high percentage of mucus in the genetic analysis produces very unclear bands. The enzymes were extracted by homogenising a small piece of tissue (0.03mg) in 75µl of grinding buffer (pH 9.0) containing 0.2M Tris, 0.0001 EDTA Free acid, 0.1M NH_4Cl, 0.11M Glucose, 20 ml of 0.022M NaN_3 per litre. The supernatant was gathered by centrifuging for 20 minutes in a Multifuge 35-R Heraesus (Kendro Lab products) refrigerated centrifuge maintained at 4˚C. Seventeen enzyme systems were screened to detect polymorphic loci using cellulose acetate electrophoresis (Titan III, Helena Laboratories, Beaumount, TX, USA) and staining systems similar to Richardson *et al.* (1986) (Table 2.3). Initially, all enzymes were run for 45 minutes at 200V (TG buffer system: Tris-glycine pH8.5; 25mM Tris and 171mM glycine, Richardson *et al.* 1986) as well as at 150V (TC buffer system: Tris-citrate pH7.0; 75mM Tris and 25mM citric acid, Richardson *et al.* 1986) to assess whether systems were expressed reliably and were polymorphic. The eighteen systems screened were sufficient to find at least seven polymorphic loci. Table 2.4 shows loci found to be reliably polymorphic, such that resolved bands were clear and consistent, and expression followed the quaternary structure normally associated with the enzymatic system (Richardson *et al*., 1986). Where possible, 50/60 individuals per waterholes were analysed. At each locus, I numbered the alleles according to their

electrophoretic mobility relative to the most common allele in all the samples. A control sample from a different site was always included, on each plate, to eliminate the possibility of misreading the bands. All enzymes coded for only one locus.

Table 2.3 Allozyme loci screened in this study for *N. sublineata*.

Enzyme system	**Locus abbreviation**	**Enzyme number**	**Quaternary structure**
Glucosephosphate isomerase	*PGI*	5.3.1.9	dimer
Phosphoglucomutase	PGM	2.7.5.1	monomer
Aspartate aminotransferase	*AAT*	2.6.1.1	dimer
β-Esterase	*β-EST*	3.1.1.1	monomer
Peptidase-leucine-tyrosine	*PEPA*	3.4.11	dimer
Peptidase-leucine-glycine-glycine	*PEPB*	3.4.11	monomer
Peptidace-lysine-leucine	*PEPC*	3.4.11	dimer
Alcohol dehydrogenase	*ADH*	1.1.1.1	dimer
Hexokinase	*HK*	2.7.1.1	monomer
Lactate dehydrogenase	*LDH*	1.1.1.27	tetramer
Mannose-phosphate isomerase	*MPI*	5.3.1.8	monomer
6-Phosphogluconate dehydrogenase	*PGD*	1.1.1.44	dimer
Malate dehydrogenase	*MDH*	1.1.1.37	dimer
Malic enzyme	*ME*	1.1.1.40	tetramer
Adenylate kinase	*ADK*	2.7.4.3	monomer
Isocitrate dehydrogenase	*IDH*	1.1.1.42	dimer
Fructose 1-6 dehydrogenase	*F1,6PDH*	3.1.3.11	tetramer

Table 2.4 Polymorphic loci detected for *N. sublineata* and optimum running conditions. Number of alleles per locus is presented in appendices 3.1, 3.2 and 3.3.

Locus	**Number of Applications**	**Position on the plate**	**Running time (minutes)**	**Voltage (V)**	**buffer system**
ADK	4	4	90	200	TG
β-EST	6	4	90	200	TG
PGI	2	4	90	200	TG
PEPA	6	4	45	150	TC
PGM	8	4	75	150	TC
PGD	8	4	75	150	TC
F1,6PDH	4	4	75	150	TC

2.4.1.2 Mitochondrial DNA

2.4.1.2.1 DNA extraction

Total genomic DNA was extracted from the foot tissue using a modification of the CTAB / phenol-chloroform procedure of Doyle & Doyle (1987). Approximately 20mg of tissue were combined and ground with 700µl of extraction buffer (1 M Tris-HCl pH 8.0, 4 M NaCl, 0.5 M EDTA, 10g CTAB, hexadecyltrimethylammoniumbromide; Sigma # H5882) and 5µl of proteinase K (20mg/mL). The samples were incubated overnight at 65°C. In sequential steps, chloroform-isoamyl alcohol (24:1), phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) were added at equal volumes to the supernatant in order to remove proteins and lipids. After each step the samples were mixed slowly on a rotator for 10 min, spun (5 min at 17350 g); then the supernatant was piepetted off and added to a new 1.5 Eppendorf tube. DNA was precipitated at -80°C with 600µl of cold isopropanol, then centrifuged at 17350 g for 20 min and washed with 1000µl of 70% ethanol. The pellets were vacuum dried for 30 min and resuspended in 100µl ddH_2O and stored at 4 °C.

2.4.1.2.2 Polymerase Chain Reaction (PCR)

The fragment of mtDNA assayed for *N. sublineata* was a partial sequence of the protein coding cytochrome oxidase subunit I (CO-I). This fragment was selected for the study as it has generally proven to be useful at the intraspecific level due to its variability and to its relatively high evolutionary rate in invertebrates (Palumbi and Benzie, 1991; Brower, 1994; Folmer *et al.*, 1994). The oligoprimers LCO-1490, 5`> GGT CAA CAA ATC ATA AAG ATA TAT TGG<3`, and HCO-2198, 5`> TAA ACT TCA GGG TGA CCA AAA AAA TCA 3`< (Folmer *et al.*, 1994) were used to amplify a fragment of approximately 710bp in length. mtDNA CO-I region was amplified in 25µl reaction volumes containing 1µl of template DNA (around 0.1 µg), 1µl of each primer (10mM), 15 nmol each of dATP, dGTP, dCTP and dTTP (Biotech International Limited), 1.25µl $MgCl_2$, 2.5µl of 10x reaction buffer (100mM tris-HCl pH 8.3 and 500mM KCl) (Biotech Internaitonal Limited) and 1U of *Thermus aquaticus* DNA polymerase (Biotech International Limited). PCR was performed on the Geneamp PCR System 9700 (PE Applied Byosystems), with an initial denaturation at 94 °C for 5 minutes;

followed by 35 cycles of: denaturation at 94 °C for 30 seconds, annealing temperature at 50°C for 30 seconds and 72 °C for 45 seconds; and a final extension step at 72 °C for 7 minutes that terminated each reaction. Each PCR reaction was visualised and its length verified using agarose gel electrophoresis. Two microlitres of PCR product were mixed with 2µl of loading dye (Bromophenol Blue), loaded into a 1.6% agarose gel (Bio-Rad ultrapure DNA grade agarose) alongside a marker of known size and concentration (Lambda DNA/eco 91 I by MBI Fermentas). The gel was electrophoresed for 20 minutes at 100V in TAE running buffer with 100mg/l ethidium bromide for staining. After the run, PCR products were visualised and photographed under a UV light source.

2.4.1.2.3 Direct Sequencing

PCR products were purified using a Quiaquick agarose gel purification kit (Quiagen Pty Ltd, Victoria, Australia). 25µl of PCR product was loaded on to a 1.6% agarose gel and run for 30 minutes at 100V. The visualised bands were cut from the gel, under a UV light source, using a sterile scalpel and unincorporated nucleotides and primers were removed by purifying the gel with Quiaquick columns according to the manufacturer's protocol. The concentration of the resulting purified DNA was determined by electrophoresing 2µl of product alongside a marker of known size and concentration (Lambda DNA/eco 91 I by MBI Fermentas). Sequencing reactions were performed by adding 10-20ng of purified DNA to 3.2 pmoles of primer, 2µl sequencing Big Dye Terminator (Perkin Elmer), 2µl 5xBuffer (Applied Biosystems) and adjusting to 10µl with ddH_2O. Each individual was directly sequenced on an automated 377 Applied Biosystems sequencer using LCOI 1490. A few samples were sequenced from each waterhole in both directions, to check sequence accuracy. Thermocycling conditions were 25 cycles of 95˚C for 30 seconds, 50˚C for 15 seconds and 60˚C for 4 minutes. After the sequencing reaction, DNA was precipitated with 76% ethanol, cleaned with 70% ethanol and the pellets vacuum dried. The sequences were aligned using the SEQUENCHER^{TM} package (1995, Gene Codes Corporation, Inc.).

2.4.1.3 Microsatellites

Two microsatellite loci (Table 2.5) were successfully isolated, after randomly screening the genome of *Notopala sublineata* for simple sequence-repeat regions (Rassmann *et al.*, 1991), using the protocol outlined by Gopurenko *et al.* (2002). Approximately 10µg

of genomic DNA extracted from the foot tissue of a single snail, using a modification of the CTAB procedure of Doyle & Doyle (1987), were digested for 3 hours with restriction enzyme *Sau*3A1. After separation on a 1.5% agarose gel, DNA fragments in the size range of 200-800 base pair were excised, purified and ligated to an equal volume of plasmid vector pUC18 (Amersham-Pharmacia). The plasmid had previously been digested with *Bam*HI and dephosphorylated to create overhanging ends to match those resulting from the *Sau*3A1 digest. Recombinant plasmids were electrophorated into competent *Escherichia coli* cells (strain JM109, Promega) and incubated for one hour at 37˚C. Cells were spread onto agar plates containing LB-Ampicillin and incubated overnight at 37˚C to promote selective growth of transformed colonies. A total of 2200 recombinant colonies were picked up from plates and incubated overnight in a grid formation on new LB-Ampicillin agar plates and later stored at 4˚C. Recombinant colonies were blotted from the plates on to filter membranes (Hybond-N, Amersham). DNA from this transfer was cross-linked with the membrane, denaturated and probed with oligonucleotides [$(ACC)_8$, $(AAC)_8$, $(AAG)_8$, $(AGC)_8$, $(ACG)_8$, $(ACT)_8$, $(CA)_{15}$, $(AG)_{12}$] that had been end-labelled with [γ^{32}P]dATP (Perkin Elmer). Cross linked single-stranded DNA was hybridised with the probes overnight, before exposed on to X-ray film at -80˚C for 12 hours. Autoradiographs revealed forty positive clones that hybridised with probed repeats. Colonies containing repeats were thus identified and picked from the stored agar plates and cultured overnight at 37˚C. Plasmid DNA was extracted from cultures by alkaline-lysis miniprep and sequenced using Big Dye Terminator (Perkin Elmer), and universal plasmid primers (M13 F & R, Amersham Pharmacia Biotech). Sequences were determined by electrophoresis on an automated 377 Applied Biosystems sequencer. Thirty of the clones contained recognisable microsatellites arrays. Only eight of the thirty candidate microsatellites had a sufficient flanking region for primer design. Primers were designed to maximise annealing temperature and minimise flanking regions. Polymerase chain reaction amplification of targeted microsatellites was successful for seven of the primer pairs (Table 2.5). PCR reactions contained 50-100ng template DNA, 0.25U *Taq* DNA polymerase (GIBCO BRL), 0.25mM dNTPs, 2.5$MgCl_2$, 0.5μM each primer (one primer end-labelled with fluorescent HEX, refer to Table 2.5), in 1x proprietary reaction buffer (50mM KCl, 20mM Tris-HCl pH 8.4, GIBCO BRL) up to 12.5μL total reaction volume. PCR reactions were cycled with the following temperature profile: 94˚C denaturing for 1 min, followed by five cycles of 94˚C for 28 seconds, annealing temperature less 4˚C (Table 2.5) for 28 seconds and 72˚C for 40 seconds; 32 cycles of 94˚C for 28 seconds

annealing temperature for 28 seconds, 72˚C for 40 seconds followed by a final 72˚C extension of 7 minutes. Denaturated PCR products and TAMRA (Genescan-350) size markers (Applied Biosystems) were electrophoresed through 5% denaturing acrylamide gels using a GelScan 2000 rig (Corbett Research) and analysed for product size using ONE-DSCAN (Scanalytics) software. Microsatellite analysis of *N. sublineata* indicated that only two loci were polymorphic (N3.3 and N3.1, refer to Table 2.5).

Table 2.5 Two microsatellite loci isolated from *N. sublineata*, forward and reverse primer sequences, observed repeat motif, size and annealing temperature.

Name of primer	Sequences	Microsatellites repeats	Alleles size (bps)	Annealing temperature
N3.3 Forward N3.3 Reverse	HEX 5'-TAA CAG TGC GTG TAT TCT AGG 3' 5' – AAA TAT TAT GTG TAT TTG TGC 3'	$(CT)_5TATT(CA)_4…(CA)_6$	132	50˚C 51˚C
N3.1 Forward N3.1 Reverse	HEX 5'- TCC TAA CTC TTC ATT GCT TTC 3' 5' – AAA AGA AAG ATC CGA TAC AAG 3'	$(TC)_{11}$	107	48˚C 57˚C

2.4.2 *Macrobrachium australiense*

2.4.2.1 Mitochondrial DNA extraction

Mitochondrial DNA was extracted from caudal muscle tissue of *M. australiense* using the alkaline lysis protocol outlined in Tamura and Aotsuka (1988). Approximately 50mg of tissue was manually homogenised in a 1.5ml Eppendorf tube containing 1ml of chilled homogenising buffer (0.25M sucrose, 10mM EDTA, 30mM Tris-HCl adjusted to pH 7.5), using a microhomogeniser to ensure that most of the cells were broken. The homogenate was then centrifuged at 1 000g for 1 minute in order to pellet the nuclei and cellular debris. The supernatant was then transferred into a fresh 1.5ml tube and centrifuged at 12 000g for 10 minutes to pellet the mitochondria. The supernatant was discarded and the pellets were resuspended in 100µl of 10mM Tris-EDTA buffer (pH 8.0), containing 0.10mM EDTA and 15mM NaCl. To this solution, an additional 200µl of freshly prepared 0.18M NaOH containing 1% sodium dodecyl sulphate (SDS) was added, in order to break down the membrane of the mitochondria. After briefly vortexing, the solution was left on ice for 5 minutes and then 150µl of ice chilled potassium acetate (3M potassium and 5M acetate) was added, to bring the pH to neutrality. The solution was vortexed again and then returned to ice for a further 5

minutes. The solution was centrifuged at 12 000g for 5 minutes and transferred to a fresh tube, along with an equal volume of phenol-chloroform (one part buffer-saturated phenol and one part chloroform: isoamyl alcohol, 24:1). After centrifuging at 12 000g for 2 minutes, the aqueous phase was transferred to a fresh tube along with two volumes of 100% ethanol, in order to precipitate mitochondrial DNA. The resulting pellets were washed in 70% ethanol and then vacuum dried. Once dry, the pellets were resuspended in 50µl of ddH_2O and stored at 4˚C.

2.4.2.2 Polymerase Chain Reaction (PCR)

As for *N. sublineata*, the fragment of mtDNA assayed for *M. australiense* was a partial sequence of the protein coding cytochrome oxidase subunit I (CO-I). A 710 base pair fragment of mitochondrial DNA cytochrome *c* oxidase subunit I (COI) was amplified using polymerase chain reaction (PCR) with primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Reactions contained 0.5µl of 10mM dNTP's, 1.25µl of 50mM $MgCl_2$, 2.5 of 10 X polymerase reaction buffer, 0.1µl of *Taq* polymerase (Biotech International Limited), 1µl of each primer (10µM), 4µl of mtDNA template and adjusted to a final volume of 25µl with ddH_2O. PCR was performed on the Geneamp PCR System 9700 (PE Applied Byosystem); DNA was initially denatured at 94 °C for 5 minutes followed by 35 cycles of: denaturation at 94 °C for 30 seconds; annealing temperature at 55°C for 30 seconds and at 72 °C for 45 seconds and a final extension step at 72 °C for 7 minutes terminated each reaction. The success of each reaction was verified as described above for *N. sublineata.*

2.4.2.3 Direct Sequencing

PCR products for *M. australiense* were purified, sequenced and cleaned as described above for *N. sublineata.* Also for this species, each individual was directly sequenced using LCOI 1490 and a few samples from each waterhole were sequenced in both directions to check sequence accuracy. As for *N. sublineata* the sequences were aligned using the SEQUENCHER^{TM} package (1995, Gene Codes Corporation, Inc.).

2.5 Data analyses

2.5.1 Allozymes and microsatellites

The Biosys-1 package, version 1-7 (Swofford & Selander, 1989) was used to estimate allele frequencies and the genetic variability in terms of mean number of alleles per locus and the gene diversity (direct-count heterozygosities and unbiased estimates of expected heterozygosities under Hardy-Weinberg expectations) per population and locus. Deviation from Hardy-Weinberg equilibrium was investigated using exact tests at individual loci, all loci and across loci and populations, and the overall significance of multiple tests was evaluated by a combined probability test found in GENEPOP version 3.1 (Raymond & Rousset, 1995) for allozymes and in MICROCHECKER version 2.2.0 for microsatellites. This method tests whether the proportion of genotypes is as expected from a random combination of alleles. Mean number of alleles per locus and the gene diversity (direct-count heterozygosities and unbiased estimates of expected heterozygosities under Hardy-Weinberg expectations) for each population and locus were calculated using the Biosys-1 package, version 1-7 (Swofford & Selander, 1989).

To quantify the levels of population subdivision, I used the hierarchical partitioning of molecular variance according to Excoffier *et al.* (1992), using the software Arlequin version 2.0 (Schneider et al., 2000). Analysis of molecular variance was performed in order to test the null hypothesis that genetic structure was not associated with drainage structure. In freshwater systems the boundaries of the catchments inherently provide the grouping structure. Therefore, based on the expected population structure, population subdivision was examined with hierarchical partitioning among and within catchments. Variance components were used to calculate *F*-statistics that are analogues of the θ of Weir and Cockerham (1984), which is an unbiased estimate of F_{ST}. For this study the three *F*-statistics produced by this analysis are:

F_{ST}: the proportion of genetic variation between all sampled populations (i.e. waterholes)

F_{SC}: the proportion of genetic variation between populations within groups (i.e. catchments)

F_{CT}: the proportion of genetic variation between groups within the total sample

Significance for these statistics was tested using a non-parametric permutation procedure (Excoffier et al., 1992), implemented in Arlequin, incorporating 10 000 permutations. The resulting p values are the probability of getting values for the *F*-statistics by chance. A significant F_{ST} indicates that populations sampled do not represent a panmictic population and that there is restricted gene flow between at least two sites. A significant F_{SC} implies that there are differences between sites within a river system. A significant F_{CT} would support the proposed population structure in that there is significant genetic differentiation between catchments. A non-significant F_{CT} suggests that the proposed population structure delineated by the current catchment lines is artificial (Excoffier et al., 1992). To conform to the hierarchical model of gene flow proposed by Meffe and Vrijenhoek for freshwater populations, where levels of genetic differentiation between populations from within a river catchment will be significantly less than that between isolated river catchments, F_{CT} should be significantly greater than F_{SC}. If this is not the case, there is an implication that contemporary gene flow within catchments, or the lack of gene flow between catchments, is not the dominant force influencing the observed genetic structure. Under this scenario, significant barriers exist within catchments, gene flow between catchments is occurring, or alternatively, historical events have impacted the current population structure.

2.5.2 Mitochondrial DNA

Since COI is a mitochondrial coding gene, the assumption of neutrality at this gene was tested using Tajima`s D statistic (1989) in the program Arlequin version 2.0 (Schneider *et al*., 2000). The amount of genetic variation per nucleotide θ can be estimated using either the number of segregating sites S, or the average number of pairwise differences between the sequences in the samples π. Under neutral evolution, estimates based on S and π give the same value of θ. This lead Tajima (1989) to propose that the difference D, between the estimates of θ given by S and π could test the neutrality of the mutations (D=0 under neutrality).

Nucleotide diversity, haplotype diversity, ratio of transition, transversion and number of polymorphic sites were calculated for all waterholes also using Arlequin version 2.0.

2.5.2.1 Population structure and phylogeography

The spatial distribution of genetic variation was investigated using analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) and nested clade analysis (NCA; Templeton *et al.*, 1995). AMOVA incorporates genetic divergence among extant haplotypes and it is very useful to test hypotheses of catchment associations but it does not provide any information about historical processes or coalescence. Nested clade analysis achieves this by reconstructing the main sequence of events that have generated the current genetic structure. Haplotype networks are utilised in the evolutionary analysis of the spatial distribution of genetic variation and NCA allows the discrimination among various biological explanations for any detected geographical association (Templeton *et al.*, 1995 and Templeton, 1998).

AMOVA was performed in Arlequin, both Φ-statistics (based on haplotype frequencies and molecular pairwise differences) and *F*-statistics (haplotype frequencies only) were estimated to examine population subdivision as specified for allozyme analyses. The relationship between genetic distance and geographic distance was tested for significance using the Mantel's (1967) test implemented in Arlequin.

Nested clade analysis was used to test the null hypothesis of no association between clades and geographic location. Nested Clade analysis as described by Templeton *et al.* (1995) can determine whether the observed genetic pattern is the result of historical evolutionary processes (e.g. range expansion and fragmentation) or alternatively, due to restricted contemporary gene flow between isolated sites. In nested clade analysis, a haplotype tree is used to define a nested series of branches (clades) that are utilised in the evolutionary analysis of the spatial distribution of genetic variation (Templeton, 1998). A haplotype network displaying evolutionary relationships among sampled haplotypes was constructed using the program TCS version 1.13 (Clement et al., 2000), which incorporates the cladogram estimation algorithm described by Templeton et al., 1992. This program provides the 95% parsimoniously plausible branch connections between haplotypes. The nested clade information (location: interior vs. tip), sample size for each haplotype and geographical location of each clade are then entered into the software package GeoDis (Posada et al., 2000) which calculates two parameters: "clade dispersion" and "clade displacement", and tests them for significance at the 5% level using a permutation technique (see Posada et al., 2000). Geographical rather than stream

distances were utilised for the nested clade analysis in the present study, because dispersal may occur during flood times when populations are likely to be connected by a complex network of channels. Clade dispersion (Dc) is the average distance of all individuals in clade X from the geographical centre of that clade and the clade displacement (Dn) is the average distance of individuals in clade X from the geographical centre of the clade in the next highest nesting level. Where significant Dc and/or Dn values are detected, a set of criteria (outlined in the "inference key"; Templeton, 1998) is used to discriminate between the effects of contemporary (e.g. gene flow) and historical processes (e.g. allopatric fragmentation and range expansion) (Templeton 1998). GeoDis version 2.0 (with 10 000 resampling events; Posada et al., 2000) was used to test for significant associations between haplotype and geography. Where significance was detected, the updated version (2001) of the inference key by Templeton (1998) was employed to determine the likely cause of the association (downloaded from the Crandall home page: http://www.inbio.byu.edu/faculty/kac/Crandall_lab). The three main biological factors identified as causing a significant spatial/temporal association of haplotype variation summarised from Templeton (1993, 1998); Castelloe and Templeton (1994); Templeton *et al.* (1995) and Posada *et al.* (2000) are:

Haplotype patterns under restricted gene flow. When a mutation first occurs, the resulting new haplotype is found only in its area of origin. Each passing generation increases the chance of the persistent haplotype spreading to new locations. The ancestral haplotype is older than its mutational offshoots and therefore should have a wider geographical distribution. In addition, since the ancestral haplotype is expected to be most frequent near its site of geographical origin, most mutational derivatives of the ancestral haplotype will also occur near the ancestral site. Hence, clade distances should increase with time under the model of restricted gene flow. This also means that tip clades will have a smaller geographical range than clades in which they are nested and the geographical centres of all clades nested together should be close.

Haplotype patterns due to past fragmentation. Fragmentation events occur when an ancestral population had become subdivided into two or more subpopulations with no gene flow among them. After a fragmentation event, as the generations go by, the accumulation of independent mutations will cause the isolates to become genetically differentiated. Under population fragmentation expectations, both tip and interior clades should have significantly small clade distances and also a significant restriction of clade distances at a high clade level. This pattern should either not be recurrent in the

cladogram, or if it affects more than one topological section of the cladogram due to ancestral polymorphism, these different topological subsets should be associated with geographically congruent subpopulations.

Haplotype patterns due to range expansion. Range expansion results in recently evolved haplotypes becoming geographically widespread, i.e. large clade distance. Thus, the distinction between tip and interior clades that is evident in restricted gene flow, breaks down or may be reversed. Some haplotypes, remote from the source of range expansion events, will cause tip clades to display large nested clade distances because the interior haplotypes from which they mutated will also be found in the ancestral range.

2.5.2.2 Demographic history: mismatch distribution

To investigate the history of populations, I analysed the distribution of nucleotide pairwise differences between individuals, or mismatch distributions (Rogers and Harpending, 1992; Harpending *et al.*, 1993). Analysis of the mismatch distribution provides an insight into the historical demography of a population. It can discriminate between a population that has remained stable over time and one that has undergone a sudden population expansion from an initial smaller founding population (Roger and Harpending, 1992). Theoretically, a population at equilibrium that has remained stable over time should produce a declining geometric curve (Roger and Harpending, 1992). However, empirical data and computer simulations have shown that a stable population is more likely to produce a multi-modal distribution as the rise of new mutations is offset by the loss of variation due to random genetic drift, resulting in random lineage sorting (Roger and Harpending, 1992; Harpending, 1994). In contrast, the pairwise differences in a population that has expanded in the past will fit a Poisson distribution due to the rate of the accumulation of new mutations being greater than the loss of variation through drift.

In this study, populations from different catchments were considered as one large population for *N. sublineata* and two (Lake Eyre and Murray-Darling basins) for *M. australiense*. Contemporary levels of gene flow between populations are expected to be low, but to have been higher in the past. The relationship between gene flow and geographic distance was tested for significance using the Mantel's (1967) test implemented in Arlequin.

The expected mismatch distribution was generated using the software DnaSP version 4.0 (Rozas *et al.*, 2004). Testing whether the observed distribution of pairwise differences deviated significantly from that expected under the sudden population expansion model was accomplished by estimating the raggedness (r) (Harpending, 1994), which determines wether a distribution is more ragged than expected under the expansion model, using Arlequin version 2.0 (Schneider *et al*., 2000). The least-squares approach of Schneider and Excoffier (1999) was also used, in Arlequin. This method uses the sum of square deviance (SSD) between observed and expected distributions and assumes that the expected distribution is the true one. To calculate the significance of the statistics, both methods use a coalescent simulation test (Hudson, 1990), implemented in Arlequin.

The time and magnitude of an inferred population expansion was determined by calculating the expected mean pairwise differences (θ), and units of mutational time (τ). $\tau = 2ut$ (u = the mutation rate over the fragment assayed; t = time in generations); $\theta_0 = 2N_0u$ (N_0 = population size before expansion); and $\theta_1 = 2N_1u$ (N_1 = population size after expansion). These parameters were calculated in DnaSP.

The COI molecular clock rate is unknown for both *N. sublineata* and *M. australiense*. A mutation rate of 8.36×10^{-6} was applied for *N. sublineata*, based on the molecular clock calibrated for *Hydrobia* (Gastropoda). The substitution rate for this species is based on the split of the *Hydrobia*/*Peringia*-lineage and the lineage of a recently discovered endemic hydrobiine taxon that evolved on a Mediterranean island 5.33 Millions years ago (Wilke, unpublished).The relevant molecular clock rate was 1.83% population divergence per million years. Therefore, the mutation rate estimated for the COI 457bp of *N. sublineata* was estimated at 8.36×10^{-6} substitutions/locus/year (the generation time is one year, Williams, 1980). For *M. australiense*, a mutation rate of 11×10^{-6} was applied, based on the molecular clock calibrated for *Caridina* (Decapoda) by Shank *et al.* (1999). The substitution rate is based on the separation of Pacific and Caribbean caridean taxa, due to the rise of the Panamanian Isthmus (Knowlton *et al.*, 1993) and relevant molecular clock rate was estimated at 2.2 % population divergence per million years. Therefore, the mutation rate estimated for the COI 505bp of *M. australiense* was estimated at 1×10^{-5} substitutions/locus/year (the generation time is one year, Williams, 1980).

2.5.2.3 Divergence times between populations: MDIV

For each species, estimates of scaled divergence times between populations were made (T, using $T = t/N_{ef}$, where t = divergence time in generations, N_{ef} = effective female population size), migration rate (M, where $M = N_{ef}\, m$) and the parameter theta (θ, using $\theta = 2N_{ef}\, u$, where N_{ef} = effective female population size, u = mutation rate per sequence per generation which is the product of μ, mutation rate of the entire sequence, and m_T, the number of nucleotides in the sequence) among pairs of catchments, under a coalescent Bayesian framework, as implemented in the program MDIV (Nielsen, 2002). MDIV is a program that simultaneously calculates these demographic parameters using a Markov chain Monte Carlo approach. The program was run under the HKY finite sites model (which does not assume that only one mutation occurs at each site but takes into account the possibility of multiple hits, differences in the nucleotide frequencies and the presence of transition/transversion bias; Nielsen and Wakeley, 2001) with a markov chain length of 5×10^6 cycles and a 10% burn-in using a uniform (0, 100) prior assumed for kappa, the parameter relating to transition/transversion bias. For each catchment comparison, multiple chains of different length were run, to ensure parameter convergence, as suggested by Nielsen (2002). MDIV estimated values for theta ($\theta = 2N_{ef}u$), migration rate ($M = 2N_{ef}m$), time of population divergence ($T = t/N_{ef}$) and expected time to the most common ancestor ($TMRCA = tu$) (Nielsen and Wakeley, 2001). Likelihood values for θ, M and T were calculated and the value with the highest posterior probability accepted as the best estimate. For *M. australiense*, T and $TMRCA$ were calculated using the mutation rate estimated at 1×10^{-5} substitutions/locus/year. For *N. sublineata*, T and $TMRCA$ were calculated using the mutation rate estimated at 8.36×10^{-6} substitutions/locus/year (as for in mismatch analysis).

2.5.2.4 Comparative analysis

The main idea of historical biogeography is that co-distributed species are likely to have shared a common history (Rosen, 1978; Wiley, 1988). A comparison of a number of species with similar biogeographic distribution provides more robust conclusions regarding inferred historical events. Therefore, species that display similar phylogeographic patterns may support the idea that they have shared a common history and they may reveal aspects of the nature of the historical events.

A qualitative approach was taken in the comparison of phylogeographic patterns of the two species targeted in this study and three freshwater invertebrates inhabiting the same region. The first species is the freshwater crayfish, *Cherax destructor* (Hughes and Hillyer, 2003), the second and third one are the freshwater mussels, *Velesunio* species A and C (Hughes *et al.*, in review) (details for these species are discussed in chapter 6). Comparisons were made to investigate the extent to which the actual population structure of populations in Western Queensland is due to contemporary versus historical processes. Similarities in the degree of conformity to contemporary processes were investigated for each species, through results of AMOVA's, whereas historical processes were investigated by comparing inferences of NCA's and divergence times between catchments, inferred by MDIV's. Comparisons of the demographic history for *N. sublineata* and *M. australiense* were accomplished by investigating results of their mismatch distribution.

Chapter 3
Population genetic structure and phylogeography of *Notopala sublineata*

3.1 Introduction

This chapter focuses on the study of genetic structure of the endangered Australian freshwater snail, *Notopala sublineata* (Gastropoda: Viviparidae). As discussed in chapter 2, populations of *N. sublineata* have declined rapidly over the last decade, and they may now be only found in the Lake Eyre basin (Sheldon and Walker, 1993) (Figure 3.1). Between flood events, freshwater species are isolated within waterholes and the terrestrial sections between them represent insurmountable barriers to dispersal. Thus, passive and active movement of freshwater invertebrates is probably achieved only during wet seasons, when water is flowing in rivers and the nearby floodplains.

N. sublineata is expected to have limited dispersal capabilities, since it is benthic and viviparous. Species with low dispersal abilities are often found to exhibit high levels of genetic differentiation among subpopulations and low levels of genetic diversity within them. This is because genetic drift within fragmented subpopulations tends to reduce genetic diversity and gene flow is insufficient to erase (mitigate) its effects, leading to substantial local genetic differentiation (Slatkin, 1987). Habitat fragmentation may also have significant effects on the genetic structure of subdivided populations because physical barriers are likely to impede gene flow.

In this chapter I tested the following hypotheses for *N. sublineata* as presented in chapter one (hypotheses one and two). A combined approach with both allozyme and mitochondrial markers was used for this species.

1. It was hypothesised that *N. sublineata* inhabiting Western Queensland rivers, will display high levels of genetic structure in populations throughout its distribution (i.e. among waterholes within a catchment and among catchments), because physical barriers represented by terrestrial inhospitable habitat, are likely to impede gene flow between populations inhabiting isolated pools.

2. It was hypothesised that climate oscillations of the Pleistocene may be directly correlated with historically higher levels of connectivity between rivers that might have facilitated aquatic dispersal across catchment boundaries. Thus it was expected to find some evidence for historical gene flow between populations of *N. sublineata* across catchment boundaries.

To test the relative effects of historical and contemporary processes on the population structure of this species, a phylogeographic (coalescent) approach was adopted.

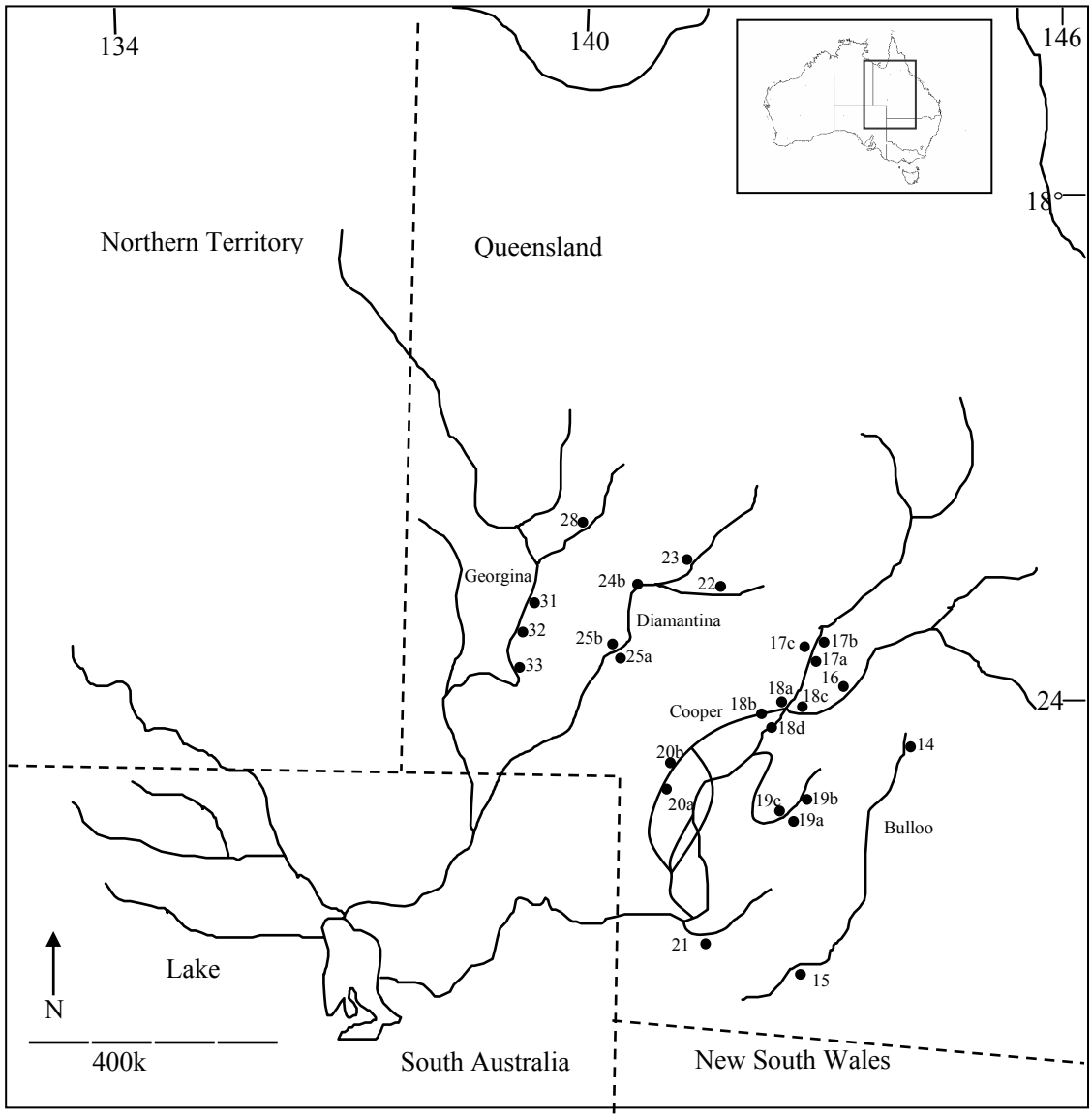
3.2 Specific methods

Figure 3.1 shows the waterholes that were sampled for *N. sublineata* throughout the four major catchments on the north-eastern side of the Lake Eyre Basin: Bullo, Cooper, Diamantina and Georgina. In total, twenty-four waterholes were sampled from sixteen sites. Sample sizes for mitochondrial DNA are given in Tables 3.3 and 3.4; for allozymes in Appendices 3.1, 3.2 and 3.3.

Allozyme procedures and general data analyses are described in detail in the general methods section. *F*-statistics were estimated to examine population subdivision. The following hierarchical levels were investigated: a) within and among the four major catchments (Bulloo, Cooper, Diamantina and Georgina) and b) within and between two groups, formed by adjacent catchments: Diamantina-Georgina and Cooper-Bulloo.

DNA extraction, PCR, sequencing procedures and general data analyses, are explained in the general methods section (chapter 2). Both Φ-statistics (based on haplotype frequencies and molecular pairwise differences) and *F*-statistics (haplotype frequencies only) were estimated to examine population subdivision using the same hierarchical partitioning as in allozyme analyses.

Figure 3.1 Map of the northeastern section of the Lake Eyre basin, illustrating the four major catchments: Bullo, Cooper, Diamantina and Georgina (refer to Table 2.1). A total of 24 waterholes, indicated by circles in the map, were sampled from these catchments. Bulloo catchment: 14.Quilpie; 15.Thargomondah; Cooper catchment: 18a.Murken; 18b.Currareva; 18c.Shed; 17c.Waterloo; 17a.Top; 17c.Pelican; 16.Retreat; 20a.Tanbar; 20a.Yalungah; 19a.Homestead; 19b.One Mile; 19b.Warranee; 21.Noccundra; Diamantina catchment: 22.Wockingham; 23.Combo; 24b.Fish; 25a.Stock route; 25b.Middle and Georgina catchment: 28.Bulla Bulla; 31.Four Mile; 32.King and 33.Cuttaburra.



3.3 Results

3.3.1 Allozyme Analysis

Sample sizes, allele frequencies and observed heterozygosity estimates for each waterhole and locus are displayed in Appendices I, II and III. Allele frequencies at each locus are illustrated as pie diagrams in Figures 3.2a, b, c, d, e, f and g. As expected, levels of variation within populations were very low, with the common allele at frequencies above 0.85 (Appendices 3.1, 3.2 and 3.3). In addition, at all loci, the same allele was most common in all the waterholes except for *βEst* at the Bulla Bulla waterhole (Georgina catchment), where the most common allele was the most rare at all the other sites. A few loci (*Pgi*, *Adk*, *F1,6pdh* and *βEst*) that were found to be polymorphic in the original screening were observed to be monomorphic in some waterholes within the study area. In particular, *Pgi* was not variable in any of the waterhole populations of the Diamantina and Georgina catchments. Multiple probability tests performed across all populations revealed no significant deviation from HWE proportions.

Figure 3.2a Pie charts representing allele frequencies at the *Adk* locus for *N. sublineata* in 20 waterholes

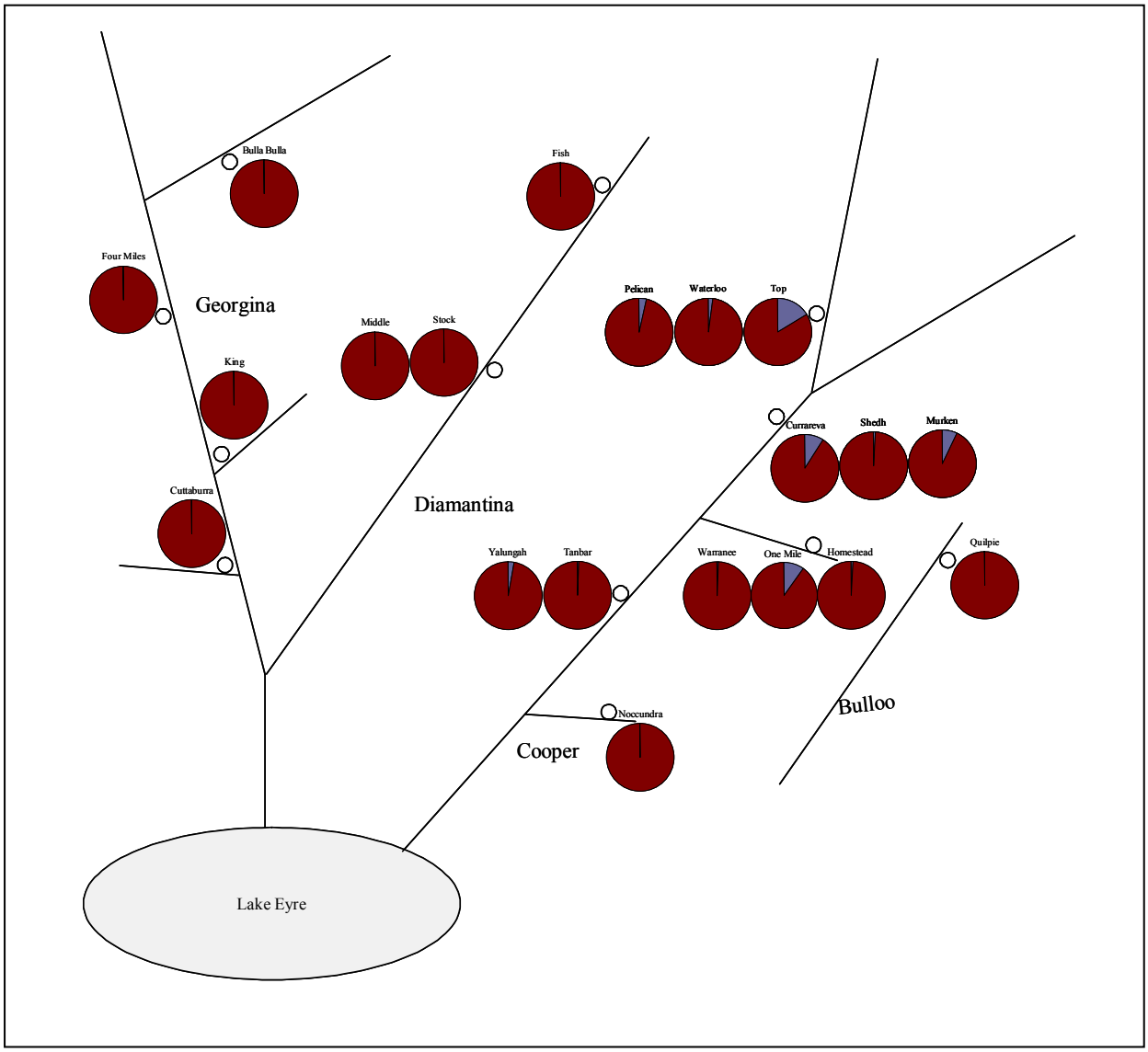


Figure 3.2b Pie charts representing allele frequencies at the *βEst* locus for *N. sublineata* in 20 waterholes

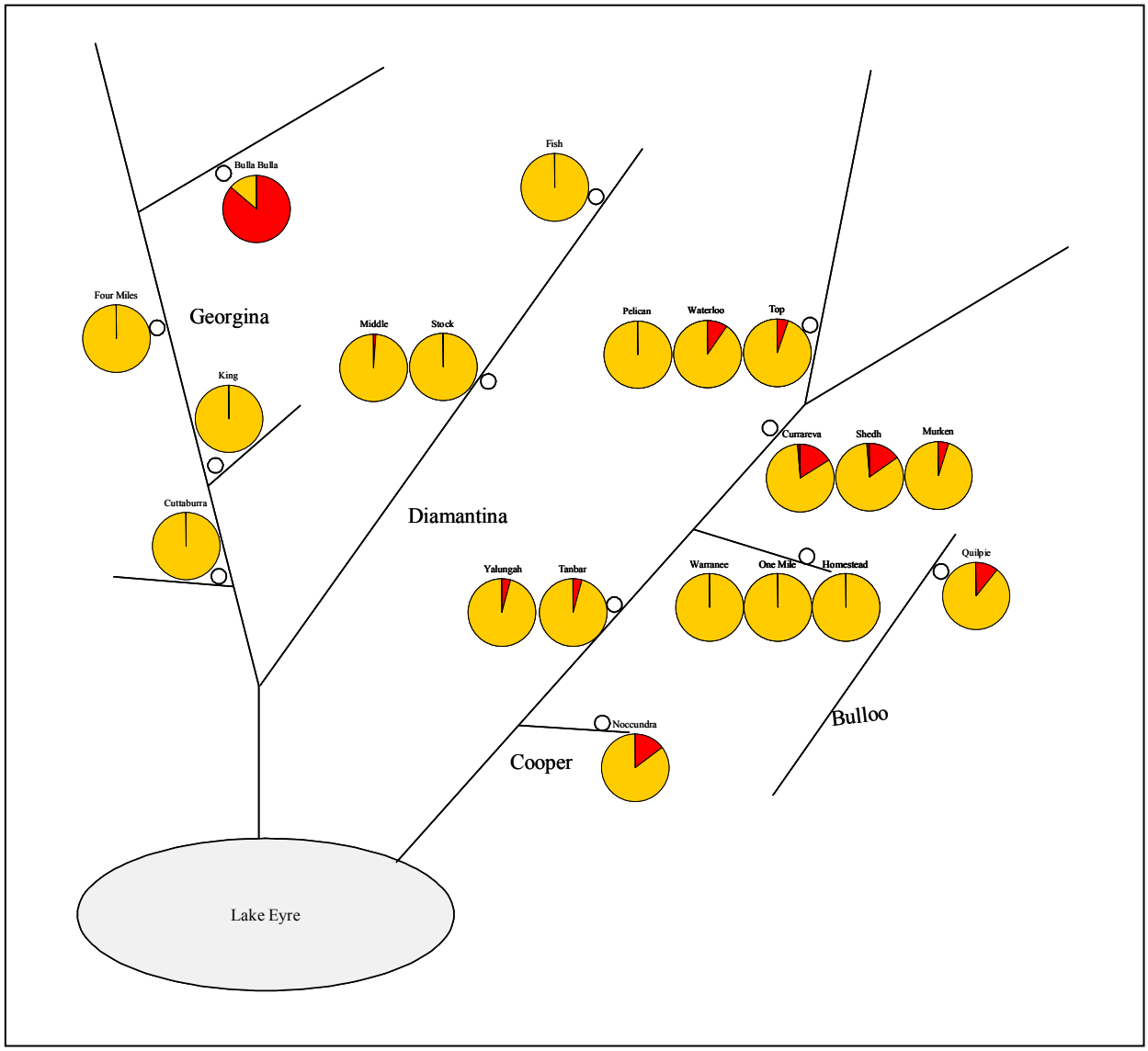


Figure 3.2c Pie charts representing allele frequencies at the *F1,6pdh* locus for *N. sublineata* in 20 waterholes

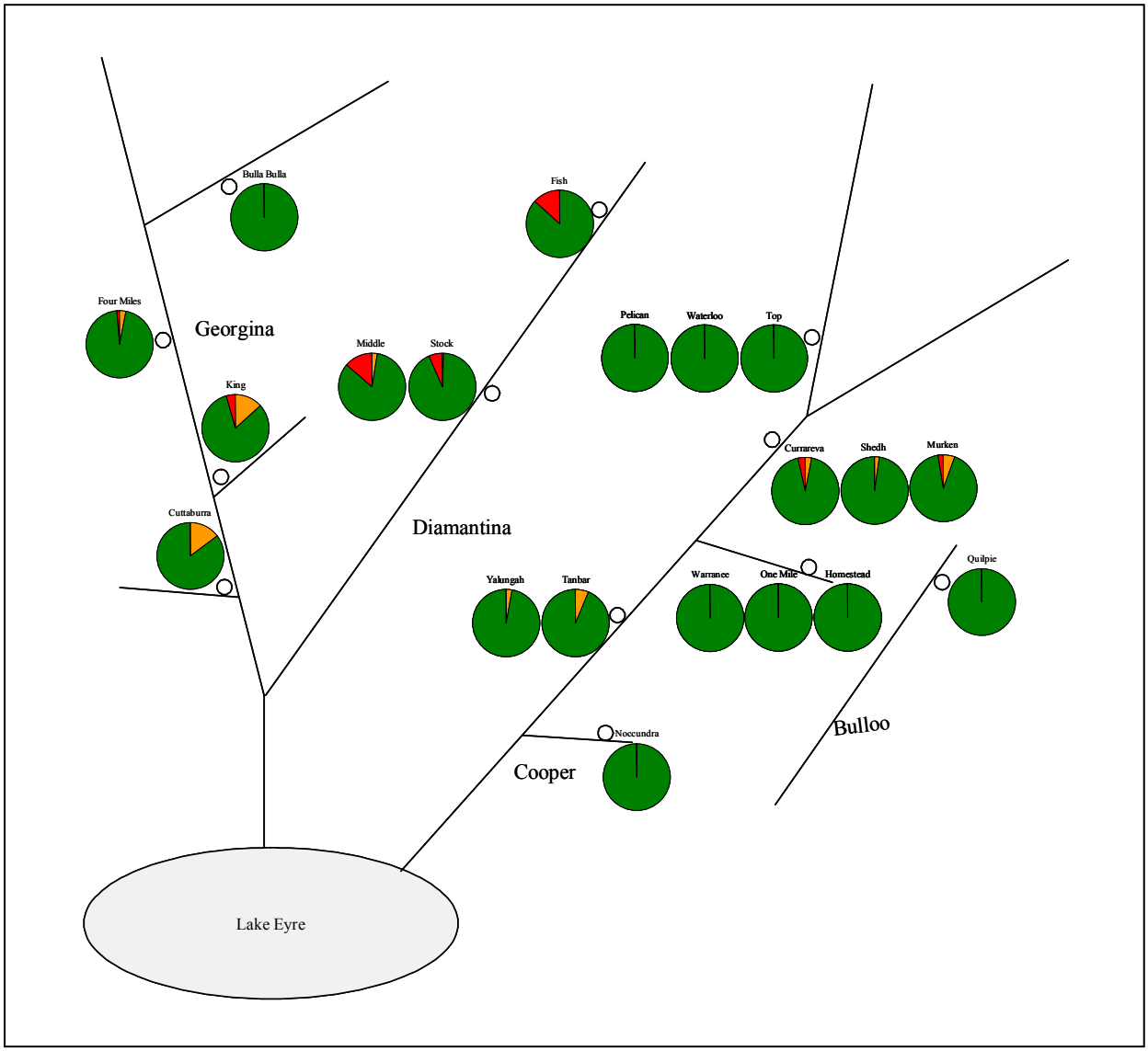


Figure 3.2d Pie charts representing allele frequencies at the *Pep a* locus for *N. sublineata* in 20 waterholes

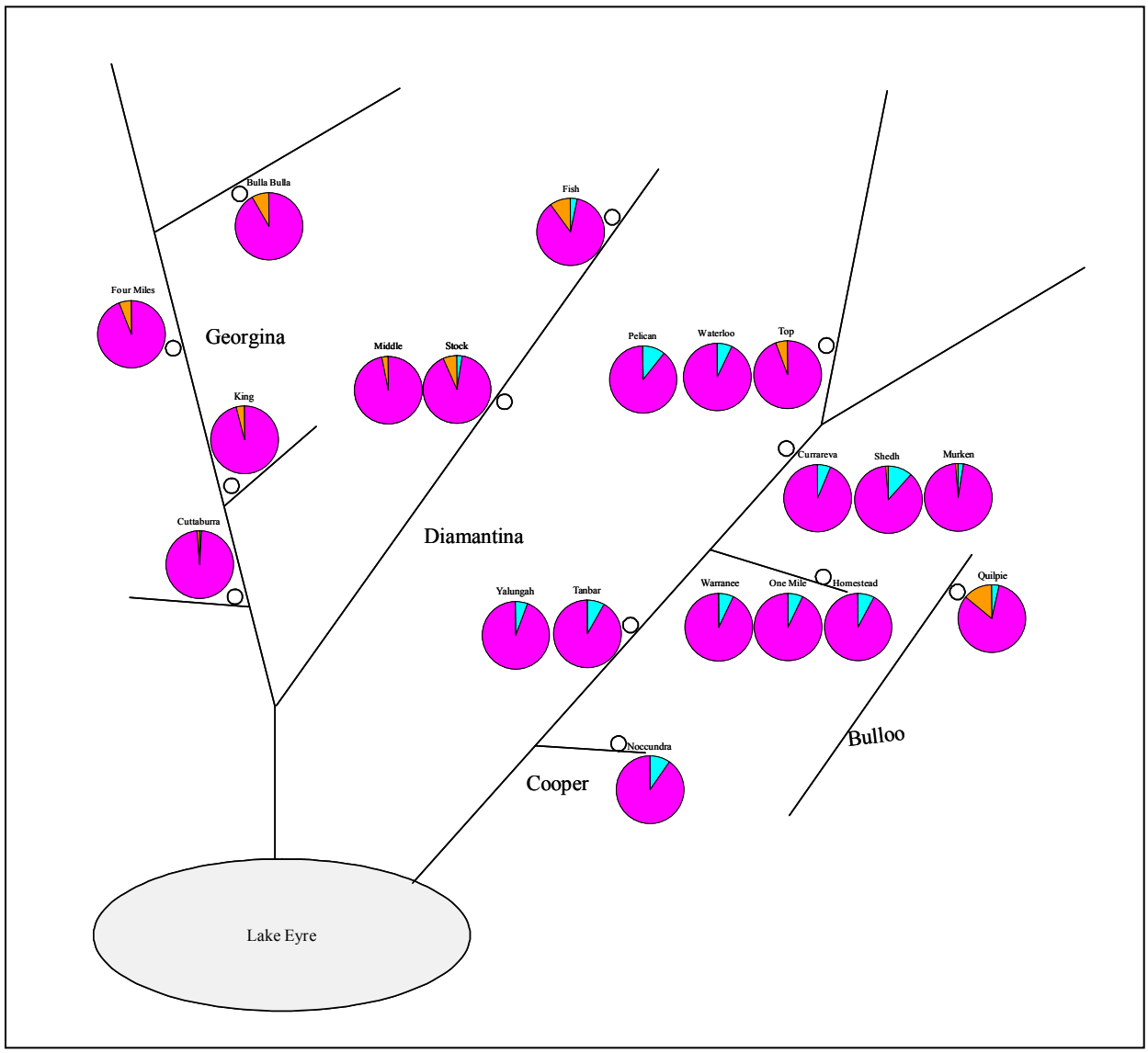


Figure 3.2e Pie charts representing allele frequencies at the *Pgd* locus for *N. sublineata* in 20 waterholes

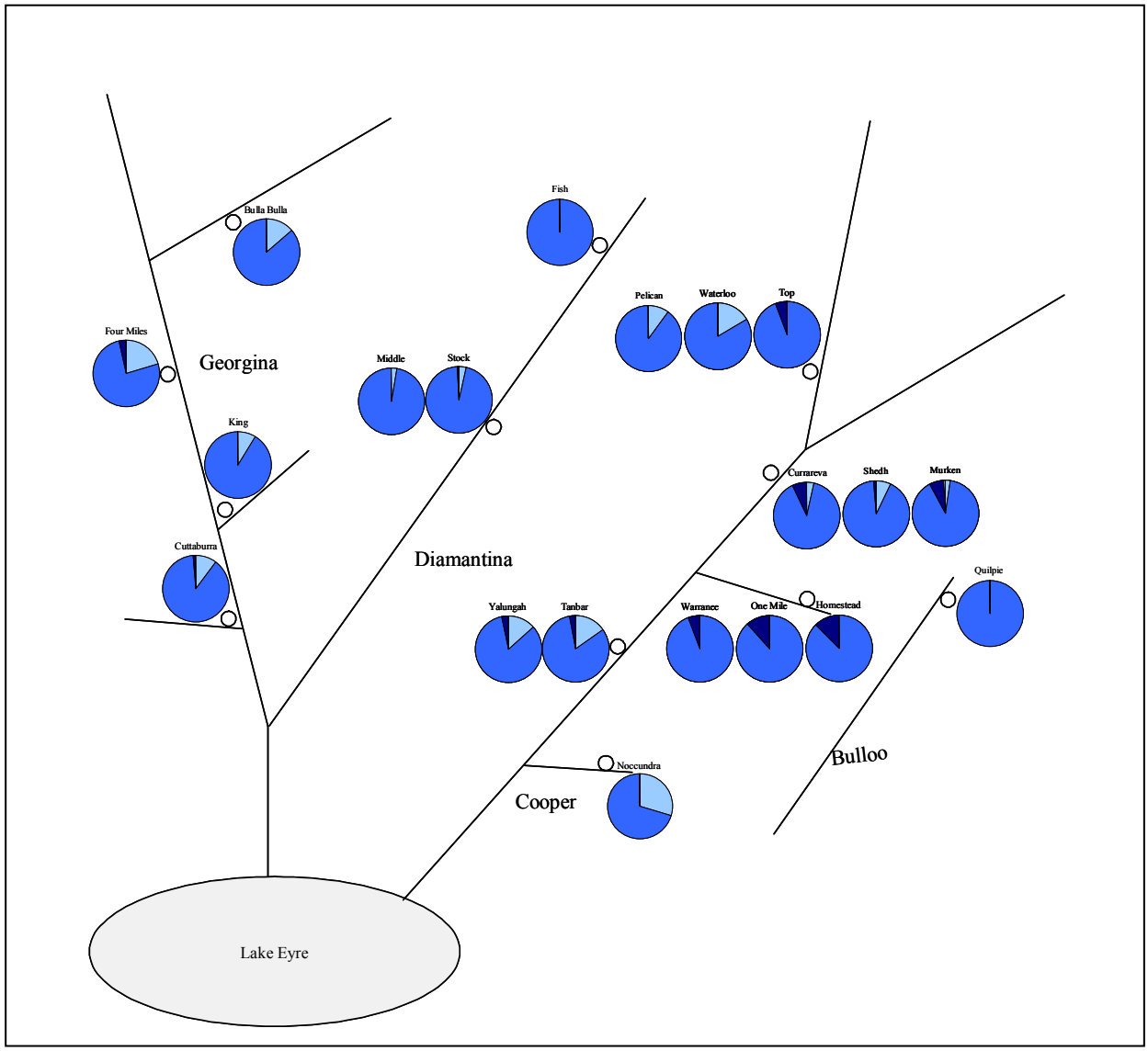


Figure 3.2f Pie charts representing allele frequencies at the *Pgi* locus for *N. sublineata* in 20 waterholes

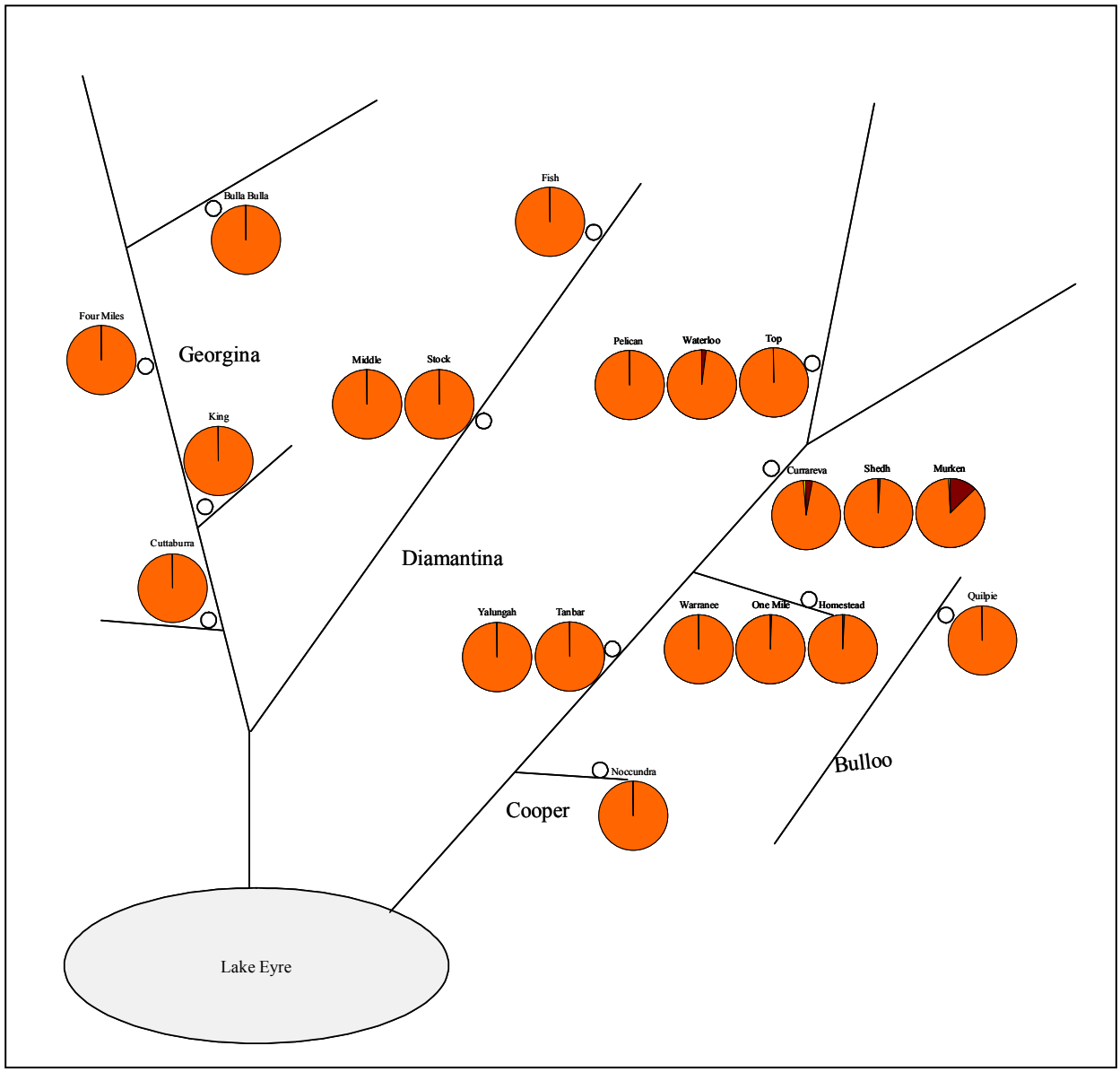
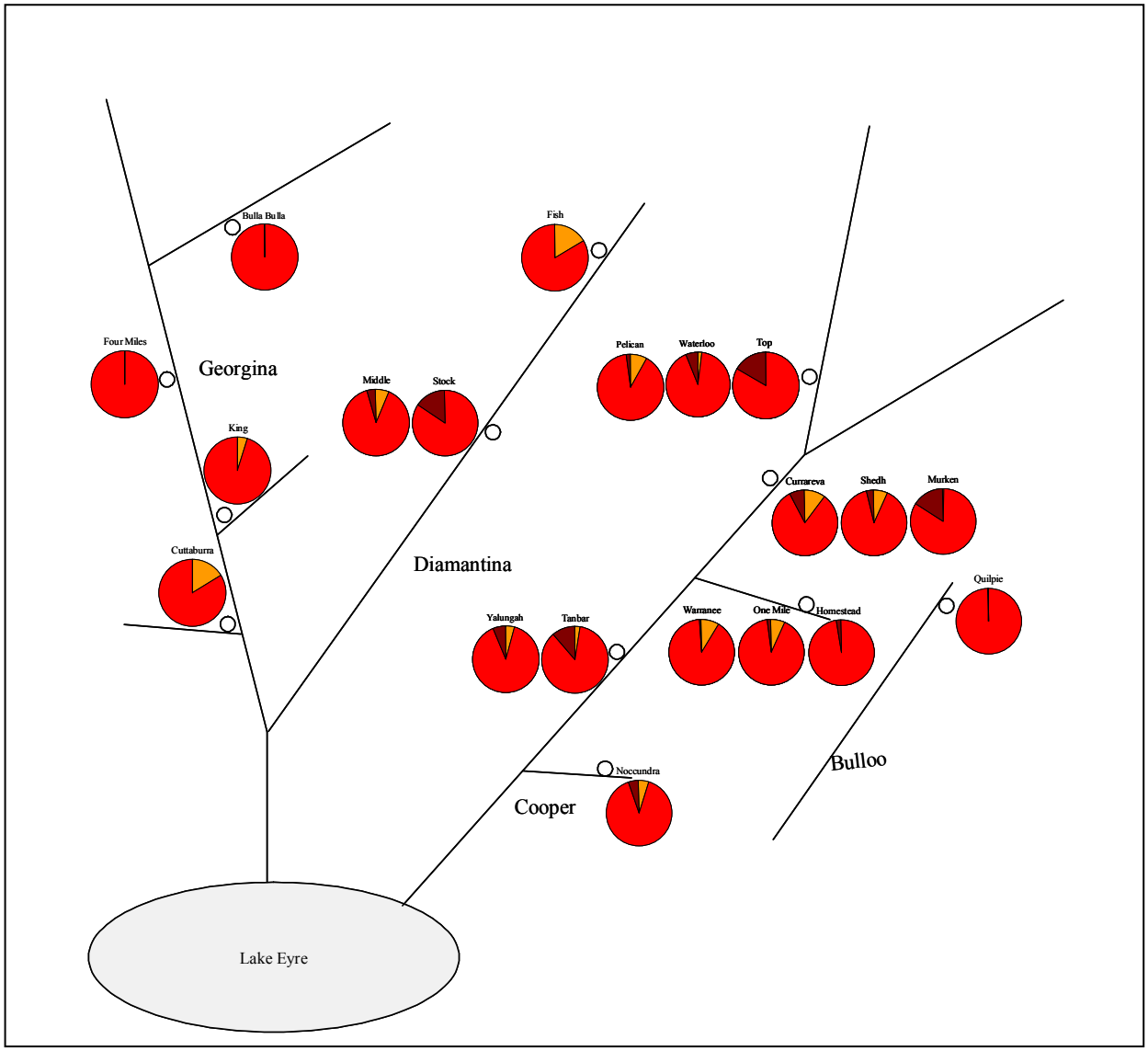


Figure 3.2g Pie charts representing allele frequencies at the *Pgm* locus for *N. sublineata* in 20 waterholes



3.3.1.1 Analysis of molecular variance

As was hypothesised, *N. sublineata* displayed high levels of genetic structure among populations throughout its distribution (Table 3.1 and Table 3.2). There was significant differentiation among waterholes within catchments (F_{SC}: 0.0713) and among waterholes within the two groups formed by adjacent catchments: Cooper-Bulloo and Diamantina-Georgina (F_{SC}: 0.0731). However, AMOVA indicated no significant hierarchical structuring among catchments or between groups of adjacent catchments. Accordingly, F_{SC} values were generally observed to be greater than F_{CT} values, indicating that there tended to be more differentiation among waterholes within catchments than between catchments overall.

It would be expected that the closer the populations are, the greater is the potential for genetic exchange and hence a lower level of genetic differentiation. Thus, a positive correlation between geographic distance and genetic distance would support this expectation and suggest a signature of isolation by distance across the region. However, the Mantel's test indicated that there was no relationship between geographic distance and genetic divergence (r = 0.18 *P* = 0.1) which is concordant with the lack of population structure by catchment implied by the preceding AMOVA analyses.

Table 3.1 Results of AMOVA for populations of *N. sublineata* showing *F*-Statistics for allozymes. The levels of partitioning investigated, included: among catchments (Cooper, Bulloo, Diamantina and Georgina), among waterholes within catchments and among waterholes.

Level of partitioning	*F*-Statistics	P
Among Catchments [Cooper][Bulloo][Diamantina][Georgina]	F_{CT}: 0.007	>0.099
Among waterholes within catchments	F_{SC}: 0.071	<0.001
Within waterholes	F_{ST}: 0.078	<0.001

Table 3.2 Results of AMOVA for populations of *N. sublineata* showing *F*-Statistics for allozymes. The levels of partitioning investigated, included: among the groups (Cooper-Bulloo and Diamantina-Georgina)

Levels of partitioning	*F*-Statistics	P
Among groups [Cooper/Bulloo][Diamantina/Georgina]	F_{CT}: 0.004	>0.66
Among waterholes within groups	F_{SC}: 0.073	<0.001
Within waterholes	F_{ST}: 0.078	<0.001

3.3.2 mtDNA Analysis

No significant deviation from neutrality was detected in the mitochondrial data (D=-1.3, P>0.09; Tajima, 1989). Using direct sequencing, sequence information for 256 *Notopala sublineata* were obtained from 4 catchments in the study area. 457 base pairs

of unambiguous sequence were generated, describing 40 polymorphic sites (8.75%), resulting in 55 haplotypes (Table 3.3, 3.4, 3.5 and Figure 3.3). The transition (Ts) to tranversion (Tv) substitution ratio was 4:1. Unlike the allozymes, mitochondrial haplotype (gene) diversity estimates were high and variable (ranging from 0 to 1.00, mean 0.9098 +/- 0.0124), reflecting the high number of haplotypes encountered at most sites.

Table 3.3 Haplotype distributions in each waterhole within the Bulloo (B) and Cooper (C) catchments. Sample size (n) is indicated for each waterhole. For waterhole name and geographic location, refer to Figure 3.1

Catchment	B	B	C	C	C	C	C	C	C	C	C	C	C	C	C
Waterhole	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
n	17	3	11	8	10	8	8	11	7	15	13	16	11	12	9
Haplotypes															
H1					1	6	2								
H2		1		3											
H3				1		2		9							
H4							1								
H5								2							
H6							1								
H7							1								
H8				1											
H9					6										
H10									3						
H11									1						
H12					1										
H13				1											
H14	14								1						
H15	2														
H16										2					
H17											1				
H18											1				
H19											2				
H20			1	1											
H21			6												
H25			1												
H26			2												
H27					1										
H29															9
H30										4	8				
H31										1					
H32	1		1	1	1				1	8	1	16	11	11	
H33														1	
H34									1						
H37		1													
H38		1													
H40							1								
H41							2								

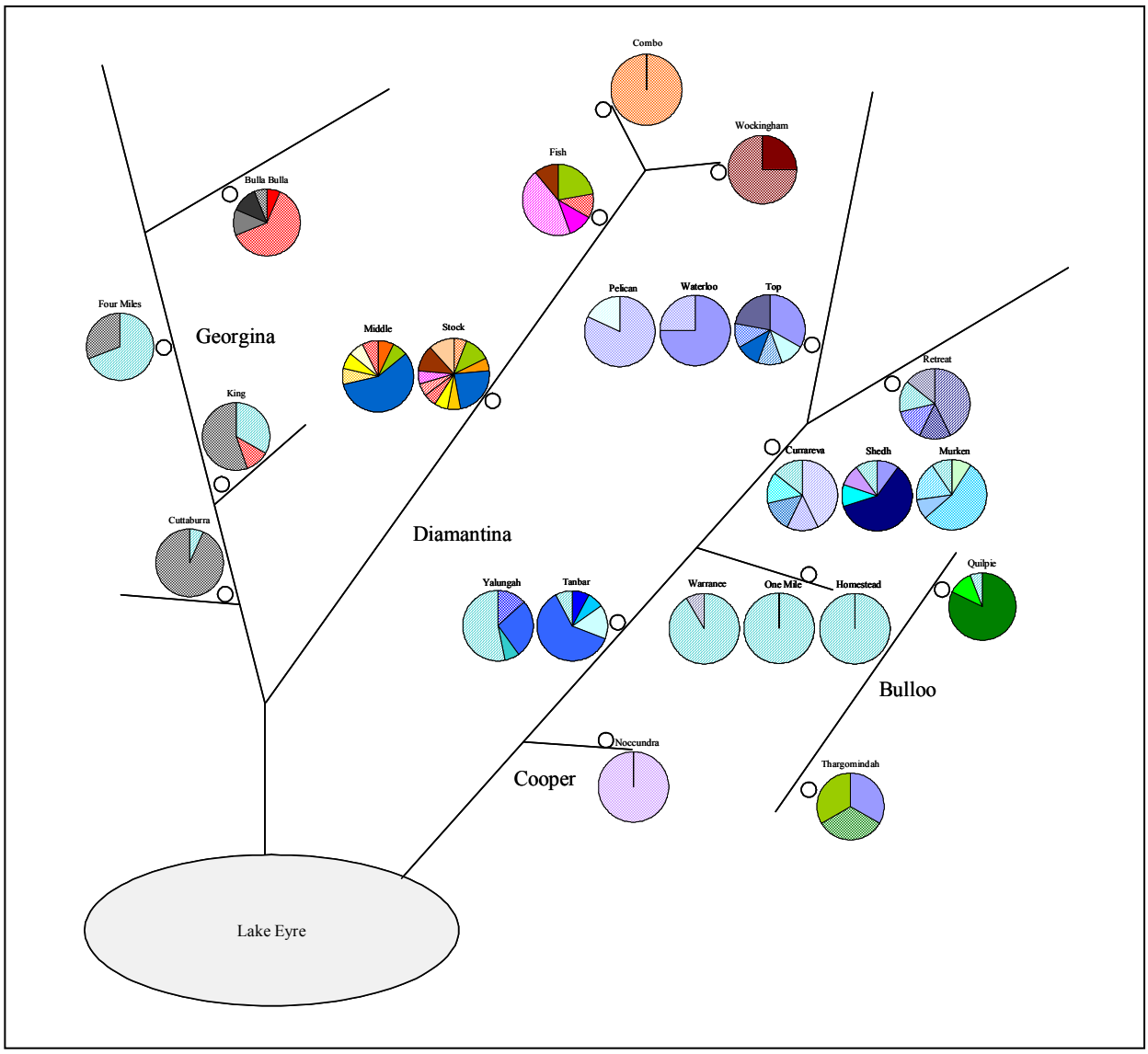
Table 3.4 Haplotype distributions in each waterhole within the Diamantina (D) and Georgina (G) catchments. Sample size (n) is indicated for each waterhole. For waterhole name and geographic location, refer to Figure 3.1

Catchment	D	D	D	D	D	G	G	G	G
Waterhole	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
n	2	4	9	17	13	16	13	9	15
Haplotypes									
H22		1							
H23		3							
H32							9	3	1
H35				1					
H36	2								
H38			2	2	1				
H39				1					
H42				4	8				
H43				1					
H44					1				
H45				1	1				
H46					1				
H47						1			
H48			1	1	1	10		1	
H49				1					
H50						2			
H51						2			
H52						1	4	5	14
H53			1						
H54			4						
H55				1					
H56			1	2					
H57				2					

Table 3.5 Variable sites for 55 haplotypes of the control region from 256 *Notopala sublineata* specimens.

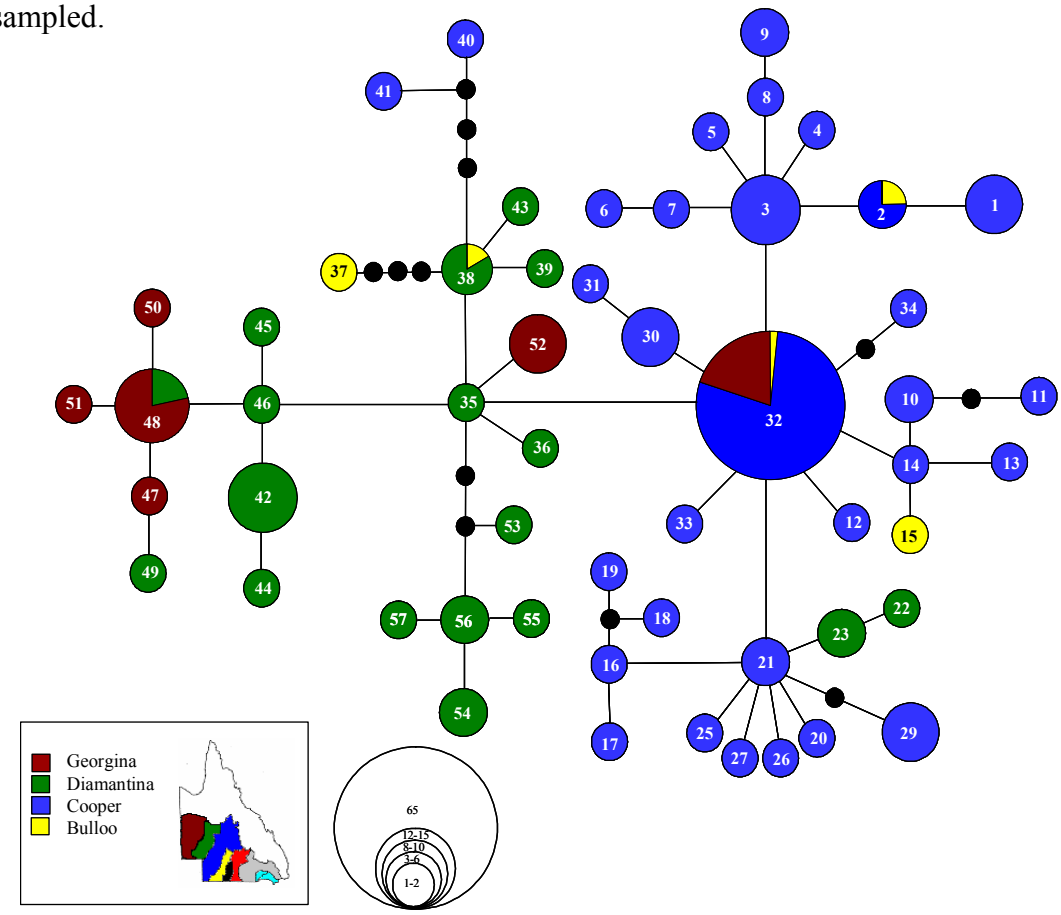
```
                                Position
[         11111 1111111222 2222222222 2233333333 3333444444 4]
[    1266602345 6678899112 5555667788 9901234445 5677000145 5]
[    3514791381 3951769179 0139891836 2873210132 8136569821 4]

H-1  AAGGAATTAA ATTTGGGTAG GTAAATATTT TGTCAGTGGT ACTACAAATT G
H-10 ........G. .......... .......... ...T..C..C .T........ .
H-11 ....G...G. .......... .........G ...T..C..C .T........ .
H-12 ........G. .......... .......... ...T..C... .T........ .
H-13 ...A....G. .......... .......... ...T..C..C .......... .
H-14 ........G. .......... .......... ...T..C..C .......... .
H-15 ........G. ........G. .......... ...T..C..C .......... .
H-16 .G......G. .......... .......... ..CT..C... .......... .
H-17 .G.....CG. .......... .......... ..CT..C... .......... .
H-18 .G......G. ..C....... ..G....... ..CT..C... .......... .
H-19 .G......G. .......... ..G....... ..CT..C... .T........ .
H-2  .......... .......... .......... ......C... .......... .
H-20 ........G. .......... .......... ..CT..C... .T........ .
H-21 ........G. .......... .......... ..CT..C... .......... .
H-22 ....G...G. ...C...... .......... ..CT..C... .........C .
H-23 ....G...G. .......... .......... ..CT..C... .......... .
H-25 ........G. .......... ...G...... ..CT..C... .......... .
H-26 ........G. ....A..... .......... ..CT..C... .......... .
H-27 ........G. .......... .......... ..CT..C... ........A. .
H-28 ........G. .......... ......G..C ..CT..C... .......... .
H-29 ........G. .........C .........C ..CT..C... .......... .
H-3  ........G. .......... .......... ......C... .......... .
H-30 ........G. G......... .......... ...T..C... .......... .
H-31 ........G. G......... .......... ...T.TC... .......... .
H-32 ........G. .......... .......... ...T..C... .......... .
H-33 ........G. .C........ .......... ...T..C... .......... .
H-34 ........G. .......... .......C.. ...T..C... ......G... .
H-35 ........G. .......... .......... ...TC.C... ..C....... .
H-36 G.....A.G. .......... .......... ...TC..... ..C....... .
H-37 ........G. ....AA.... ........C. ...TC.CA.. ..C....... A
H-38 ........G. .....A.... .......... ...TC.C... ..C....... .
H-39 ....G...G. .....A.... .......... ...TC.C... ..C....... .
H-4  ........G. .......... .......... .....AC... .......... .
H-40 ........G. ..C..A.... ....G..... C..TC.C..C ..C....... .
H-41 ........G. ..C..A.... ...GG..... ...TC.C..C ..C....... .
H-42 ........G. .......... ...G...... ...TC.C... ..C....G.. .
H-43 ........G. .....A.... .......... ..CTC.C... ..C....... .
H-44 ........G. .......... ...C...... ...TC.C... ..C....G.. .
H-45 ..A.....G. .......... .......... ...TC.C... ..C....G.. .
H-46 ........G. .......... .......... ...TC.C... ..C....G.. .
H-47 ...A....G. .......... .......... ..CTC.C... ..C....G.. .
H-48 ...A....G. .......... .......... ...TC.C... ..C....G.. .
H-49 ........G. .......... .......... ..CTC.C... ..C....G.. .
H-5  ...A....G. .......... .......... ......C... .......... .
H-50 ...A....G. ..C....... .......... ...TC.C... ..C....G.. .
H-51 ...A....G. .......... .......... ...TC.C... ..C..G.G.. .
H-52 ........GG .......... .......... ...TC.C... ..C....... .
H-53 ........G. ......A... .......... ...TCTC... .TC....... .
H-54 ....G...G. ......A... .......... ...TCTC..C ..C....... .
H-55 ....G...G. ......A... .......... ..GTCTC... ..C....... .
H-56 ....G...G. ......A... .......... ...TCTC... ..C....... .
H-57 ....G...G. ......A... .......... ...TCTC... ..CG...... .
H-58 ....GG..GG .......C.. AC.G.C.... .ACTCAC.A. TTC....... .
H-6  ........G. .......... .......... ..C...C... .G..T..... .
H-7  ........G. .......... .......... ......C... .G........ .
H-8  ........G. .......... .......... ......C... .T........ .
H-9  ........G. .......... .......... ......C... .T........ A
```

Figure 3.3 Pie charts representing mitochondrial DNA haplotype frequencies in each waterhole for *N. sublineata* (refer to Table 3.3 and 3.4).



The genealogy network showing catchment distribution for each haplotype (Figure 3.4) indicated a very strong geographic distribution pattern. Of the 55 haplotypes, 31 were restricted to Cooper-Bulloo and 21 to Diamantina-Georgina adjacent catchments (Table 3.3 and 3.4). Only two haplotypes occurred in more than one non-adjacent catchment (haplotype 32 in the Bulloo, Cooper and Georgina and haplotype 38 in the Diamantina and Bulloo). Very few haplotypes were shared between adjacent catchments (haplotype 48 between Georgina and Diamantina and haplotypes 2 and 14 between Cooper and Bulloo). A general trend was also noted for populations belonging to the same catchment to share only a few haplotypes, and have a relatively high number of unique haplotypes. In addition, the haplotypes shared were internal within the network. Coalescent theory predicts that the haplotypes or clades on the tips of the network are highly likely to be younger than the interior haplotypes or clades to which the tips are connected (Castelloe and Templeton, 1994), suggesting that the sharing of haplotypes detected between catchments is not due to contemporary gene flow.

Figure 3.4 Genealogy network showing distribution by catchment for each haplotype and depicting the number of base pair difference between them for *N. sublineata*. Circle size of each haplotype is proportional to overall frequency in the sample. Solid lines represent a single mutation and smallest black circles represent haplotypes that were not sampled.



3.3.2.1 Analysis of molecular variance

The hierarchical analysis of molecular variance, based on haplotype frequencies and molecular divergence (Φ-statistics) as well as haplotype frequencies only (*F*-statistics), did not differ significantly from the findings of the allozyme analysis, in that there was strong genetic structure in the study area (Table 3.6 and 3.7). It revealed significant subdivision between waterholes in the total sample (Φ_{ST}: 0.625; F_{ST}: 0.463 P<0.001), among waterholes within catchments (Φ_{SC}: 0.477; F_{ST}: 0.410 P<0.001) and among the four catchments (Φ_{CT}: 0.284 P<0.001; F_{CT}: 0.089 P<0.01). AMOVA also indicated high levels of genetic differentiation between the two groups, (Diamantina/Georgina and Cooper/Bulloo) when Φ-statistics (Φ_{CT}: 0.326 P<0.001) were used, but not when haplotype frequencies alone were used. As previously observed for allozyme data, Φ_{SC} estimates were generally higher than Φ_{CT} values, suggesting that the genetic variation was higher between waterholes within catchments than between catchments overall.

As for allozyme data, the Mantel's test indicated that there was no relationship between geographic distance and genetic divergence (r = 0.1 *P* = 0.12) which is concordant with the lack of population structure by catchment showed by the preceding AMOVA analyses.

Table 3.6 Results of AMOVA for populations of *Notopala sublineata* showing *F*-Statistics and Φ-statistics for mtDNA. The levels of partitioning investigated, included: among catchments (Cooper, Bulloo, Diamantina and Georgina), among waterholes within catchment and among waterholes. * P<0.001 ** P<0.01

Levels of partitioning	*F*-Statistics	Φ-Statistics
Among Catchments [Cooper] [Diamantina] [Georgina] [Bulloo]	F_{CT}: 0.089**	Φ_{CT}: 0.284*
Among Waterholes Within Catchments	F_{SC}: 0.411*	Φ_{SC}:0.477*
Among all waterholes	F_{ST}: 0.463*	Φ_{ST}:0.625*

Table 3.7 Results of AMOVA for populations of *Notopala sublineata* displaying showing *F*-Statistics and Φ-statistics for mtDNA. The levels of partitioning investigated, included: among the groups (Cooper-Bulloo and Diamantina-Georgina) * P<0.001.

Levels of partitioning	*F*-Statistics	Φ-Statistics
Among Groups (adjacent catchments) [Cooper-Bulloo] [Diamantina-Georgina]	F_{CT}: 0.039	Φ_{CT}: 0.326*
Among Waterholes Within Groups	F_{SC}: 0.436*	Φ_{SC}:0.486*
Among all waterholes	F_{ST}: 0.458*	Φ_{ST}:0.625*

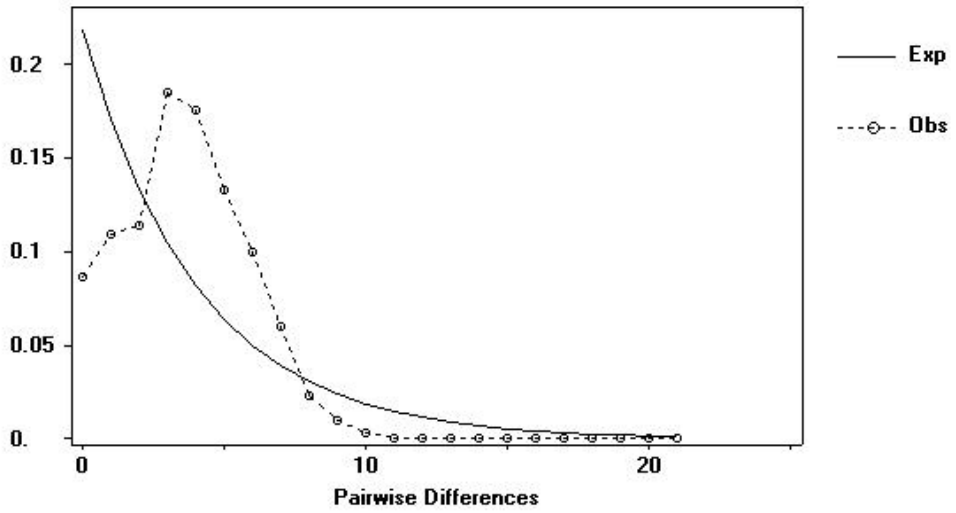
3.3.2.2 Population demographic history: mismatch distribution

There was a maximum of three base pair differences between any two adjacent nodes in the haplotype network (Figure 3.4). The short internodal distances are consistent with a star phylogeny that is indicative of rapid population expansion (Avise *et al.*, 1984). As a population expands, the effects of random genetic drift are reduced, thereby increasing the probability of retaining new mutations. The hypothesis of a sudden expansion is supported by the mismatch distribution (Figure 3.5) with no significant difference between the observed and expected distribution under the expansion model (SSD = 0.0035, P = 0.8; r = 0.014, P = 0.93).

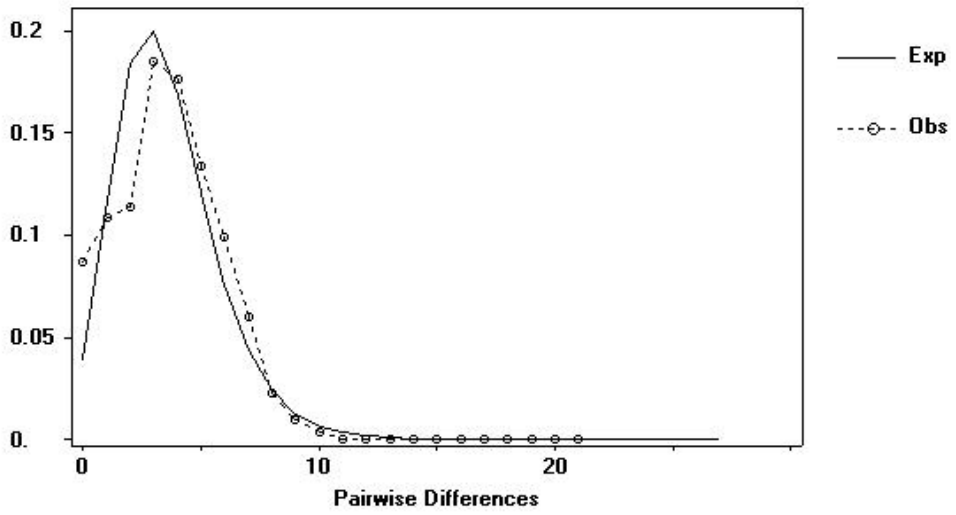
The calculated θ parameters (θ_0 = 1.04; θ_1 = 1000) suggest that *N. sublineata* population underwent a rapid range expansion in Western Queensland. Based on the mutation rate estimated at 8.36 x 10^{-6} substitutions/locus/year for the COI 457bp of *N. sublineata* and the calculated units of mutational time (τ = 2.55), the expansion event was estimated to have occurred around 150 000 years ago.

Figure 3.5 Distribution of pairwise nucleotide differences for *N. sublineata*. The dashed line is the observed distribution of the population in figures a) and b), the continuous line is the expected geometric distribution indicative of a) a stable population and b) a population that has undergone an expansion (expected Poisson distribution)

a)



b)



3.3.2.3 Nested clade analysis

The nested haplotype cladogram comprises 25 one-step clades, 11 two-step clades, 4 three-step clades, 2 four-step clades and the total cladogram (Figure 3.6). The null hypothesis of no association between genetic variability and geographical distribution was rejected for several clades at each clade level (Table 3.8). Two main events were detected by NCA within this species: a recurrent pattern of restricted gene flow and a

series of historical range expansions. The inferences from the relatively young one-step, one of the two-step, some of three-step and one of the oldest four-step clade levels revealed a pattern of restricted gene flow with isolation by distance. The inference key (Templeton, 1998) suggested that contiguous range expansion between the two adjacent catchments, Cooper and Bulloo, was the most likely process to explain the pattern of geographic variation in the two-step clades 2-10, 2-11. Contiguous range expansion was also proposed to explain the distribution of clade 4-2. Inferences from clades 2-8 and 3-4 suggested long distance colonisation between Cooper and Diamantina and among the four catchments respectively.

Figure 3.6 Nested Clade diagram with 95% plausible set of haplotype connections with clade nesting for *Notopala sublineata*. Clades are nested according to rules outlined in Templeton *et al.* (1995). Each line in the network represents one mutational change. Small black circles represent the inferred non-detected interior haplotypes. The number inside each circle identifies the detected haplotypes, as in Table 6 and Appendix 4. Rounded rectangles indicate the haplotypes grouped together into the step clades. The level of the nested clade is given by: 1-x for 1-step clades, 2-x for 2-step clades, 3-x for 3-step clades and 4-x for 4-step clades where x is the number identifying individual clades.

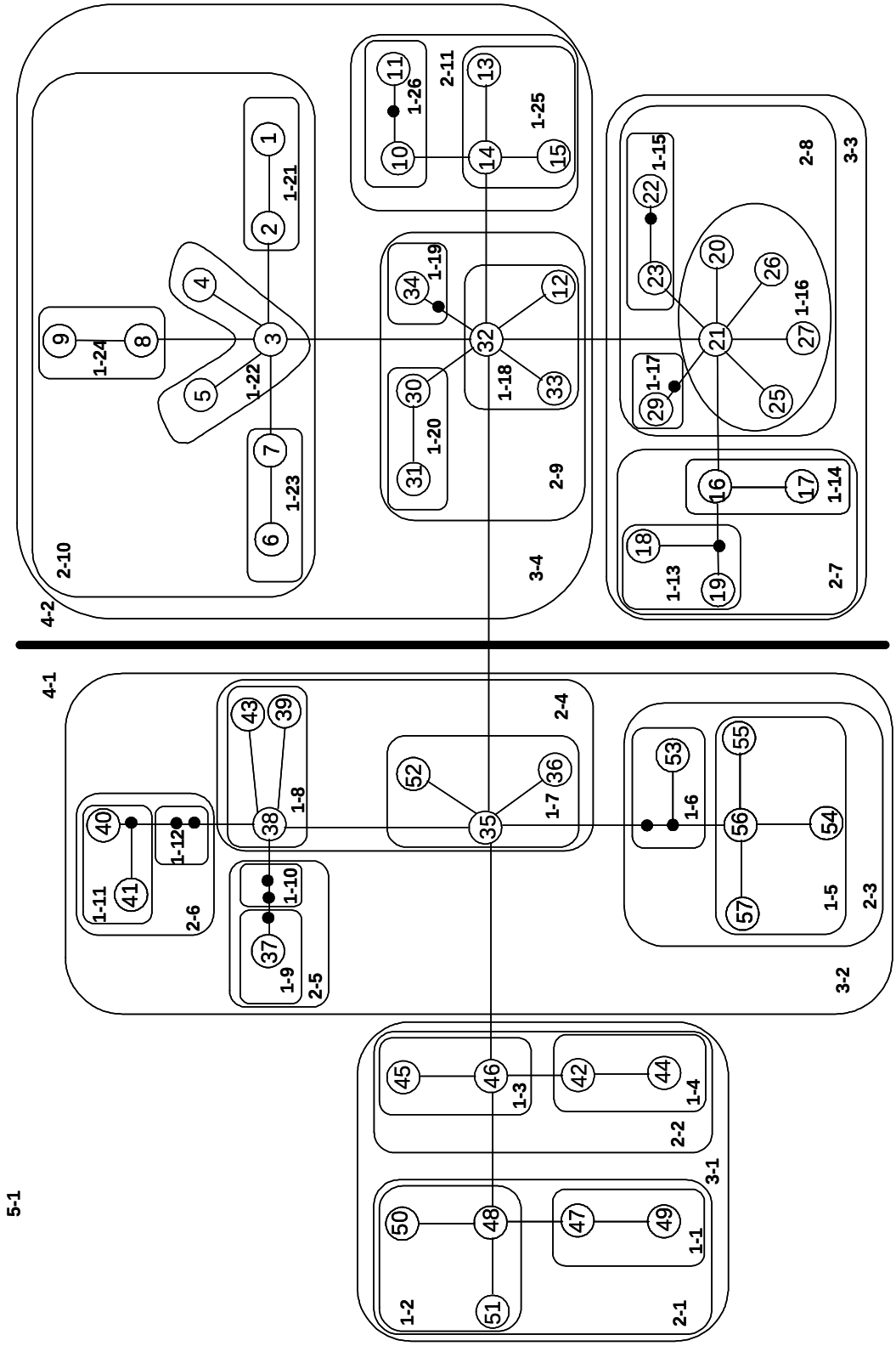
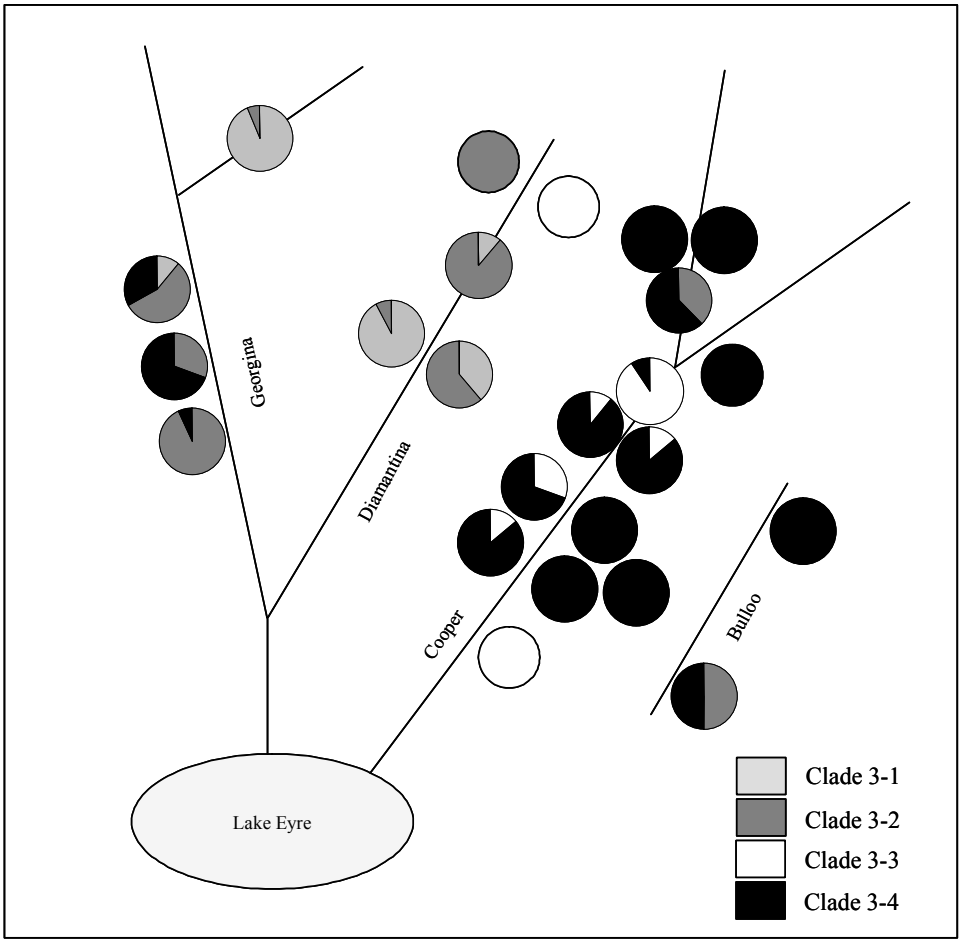


Table 3.8 Results of the NCA of the geographical distance for CO-I haplotypes. The nested design is given in Figure 3.6, as are the haplotype and clade designations. A superscript "s" indicates that the distance is significantly small at the 5% level and a superscript "L" indicates that it is significantly large. "Inference Key ⇒ Conclusion" refers to the key in Templeton (1998), numbers indicate steps followed in the inference key to obtain the conclusion. RGF/IBD is restricted gene flow with isolation by distance, RGF/LDD is restricted gene flow but with long distance dispersal and RE is range expansion. D_c, D_n and I-T represent the geographical dispersion of a clade, the distance between all individuals with haplotype x from those with haplotype y, and the average distance between tip and interior clades, respectively

Nesting	Location	No.	D_c	D_n	χ^2 - P	Inference Key⇒ Conclusion
	One-step clades					
1-7	Interior	35	0	77.33	0	1→2→3→4⇒RGF/IBD
	Tip	36	0	**233.55**^{L}		
	Tip	52	**82.80**^{S}	146.58		
		I-T	-79.49	-79.72		
1-21	Tip	1	**23.17**^{s}	111.73	0.002	1→2→3→4⇒RGF/IBD
	Interior	2	136.50	166.02		
		I-T	113.32	54.29		
	Two-step clades					
2-4	Interior	1-7	173.27	184.88	0	1→2⇒Inconclusive
	Interior	1-8	278.20	269.94		
		I-T	-	-		
2-8	Tip	1-15	0	**310.37**^{L}	0	1→2→11⇒LDC RE
	Interior	1-16	**11.47**^{S}	**24.19**^{S}		
	Tip	1-17	**0**^{S}	**286.43**^{L}		
		I-T	11.47	**-269.60**^{S}		
2-9	Interior	1-18	**120.35**^{L}	**120.42**^{L}	0	1→2→3→4⇒RGF/IBD
	Tip	1-19	0	123.33		
	Tip	1-20	**118.09**^{L}	**72.78**^{L}		
		I-T	**-**	**-**		
2-10	Tip	1-21	132.99	127.11	0	1→2→11⇒Contiguous RE
	Interior	1-22	**23.72**^{S}	76.67		
	Tip	1-23	0	80.13		
	Tip	1-24	**13.76**^{S}	**71.17**^{S}		
		I-T	**-59.24**^{S}	-28.37		
2-11	Interior	1-25	**122.47**^{S}	**176.03**^{S}	0.002	1→2→11⇒Contiguous RE
	Tip	1-26	0	**189.15**^{L}		
		I-T	122.47	**-13.11**^{S}		
	Three-step clades					
3-1	Tip	2-1	**50.34**^{S}	78.48	0	1→2→3→4⇒RGF/IBD
	Interior	2-2	**41.34**^{S}	70.76		
		I-T	-9.00	-7.71		
3-2	Tip	2-3	**46.13**^{S}	**130.89**^{S}	0	1→2→3→4→5→6⇒Inconclusive
	Interior	2-4	203.89	217.25		
	Tip	2-5	0	**487.45**^{L}		
	Tip	2-6	**0**^{S}	179.57		
		I-T	**170.05**^{L}	52.85		
3-3	Tip	2-7	**2.24**^{S}	91.77	0	1→2→3→4⇒RGF/IBD
	Interior	2-8	199.17	197.70		
		I-T	**196.93**^{L}	105.92		
3-4	Interior	2-9	**102.30**^{S}	**94.58**^{S}	0	1→2→3→4→5→6→7⇒RGF/LDD
	Tip	2-10	**98.83**^{S}	153.51		
	Tip	2-11	**180.49**^{L}	**206.79**^{L}		
		I-T	-29.93	**-81.47**^{S}		
	Four-step clades					
4-1	Tip	3-1	**74.48**^{S}	**89.83**^{S}	0	1→2→3→4⇒RGF/IBD
	Interior	3-2	**216.22**^{L}	**211.32**^{L}		
		I-T	**141.74**^{L}	**121.49**^{L}		
4-2	Tip	3-3	**184.63**^{L}	**182.60**^{L}	0	1→2→11⇒Contiguous RE
	Interior	3-4	**127.83**^{S}	**128.51**^{S}		
		I-T	**-56.80**^{S}	**-54.09**^{S}		
Total	Tip	4-1	**173.55**^{S}	**250.30**^{L}	0	1→2⇒Inconclusive
	Tip	4-2	**140.92**^{S}	**168.92**^{S}		

Figure 3.7 A stylized map showing the distribution of clades, identified in Figure 3.6 at the 3-step clades level, across the sampling area for *N. sublineata*.



3.3.2.4 Population divergence times: MDIV

According to the coalescent-based analysis, time since populations of *N. sublineata* have diverged (*T*) in Western Queensland was relatively recent (140 000/300 000 years ago) (Table 5). Populations inhabiting adjacent catchments (Georgina-Diamantina and Cooper-Bulloo) have diverged roughly 140 000 years ago. Populations from the Diamantina catchment have diverged about 250/300 000 years ago from populations of the group of catchments Cooper-Bulloo. Populations from the Georgina catchment have diverged more recently (approximately 150/170 000 years ago) than populations of the Diamantina catchment from populations of the Cooper-Bulloo catchment group, despite being geographically more distant. This pattern may be the result of more recent independent colonisation events involving populations from the Georgina and the Cooper, Bulloo catchments.

Table 3.9 Pairwise estimates of $\theta = 2N_{ef}u$, migration rates ($M = N_{ef}m$), time since divergence (T) and time to the most common ancestor ($TMRCA$) based on analysis of mtDNA COI using the MDIV program. Values show those with the highest likelihood scores for θ and M. Estimates for T and $TMRCA$ are calculated in years before present (YBP).

	Bulloo	Cooper	Diamantina
Cooper	θ= 1.95 *M*= 0.16 *T*= 140 000 YBP *TMRCA*= 280 000 YBP	–	
Diamantina	θ= 1.61 *M*= 0.06 *T*= 250 000 YBP *TMRCA*= 430 000 YBP	θ= 2.84 *M*= 0.07 *T*= 330 000 YBP *TMRCA*= 370 000 YBP	–
Georgina	θ= 0.49 *M*= 0.08 *T*= 170 000 YBP *TMRCA*= 600 000 YBP	θ= 0.49 *M*= 0.01 *T*= 150 000 YBP *TMRCA*= 600 000 YBP	θ= 2.69 *M*= 0.15 *T*= 140 000 YBP *TMRCA*= 280 000 YBP

3.4 Discussion

3.4.1 Genetic population structure in *Notopala sublineata*

Species with inefficient mechanisms of dispersal inhabiting a discontinuous habitat, such as *Notopala sublineata* in Western Queensland, are likely to be subdivided into many arrays of localised populations. Limited exchange of individuals between these localised populations was expected to lead to genetic differentiation among them. The data presented in this chapter strongly support the hypothesis that *N. sublineata* displays high levels of genetic structure among populations throughout its distribution in Western Queensland. This is evident from the AMOVA's, which showed that the populations of *N. sublineata* are significantly subdivided at all spatial scales examined (i.e. among and within catchments). Also, a high number of unique haplotypes was detected in most of the waterholes and some sites contained unique clades (Wilson, Cooper catchment and Wockingham, Diamantina catchment; Figure 1), suggesting that a geographic barrier has isolated these site for long enough to allow the local populations to evolve a unique set of haplotypes.

N. sublineata seems to be subdivided into many arrays of localised populations with limited exchange of individuals between them. This genetic pattern conforms to what is known of the biology-behaviour of the species and the contemporary structure of its riverine environment. Within the riverine systems of Western Queensland, freshwater snails are strictly confined in waterholes and the surrounding patches generated by erratic flow patterns represent insurmountable barriers to such aquatic dispersal, contributing to the isolation of neighbouring populations. During major floods, water flows in rivers and may allow movements of snails between waterholes. However, despite the potential for aquatic dispersal during floods, it appears that the sedentary behaviour, intrinsic to the snails, induces them to tend to remain in the waterhole of origin. This may suggest that movements of individuals are limited within catchments and absent across catchment boundaries.

The stream hierarchy model proposes that populations of aquatic species from different catchments should be genetically more differentiated than populations within the same catchment (Meffe and Vrijenhoek, 1988). This model has been developed for conventional stream systems and it is possible that aquatic species inhabiting endorheic river systems with a highly variable flood regime, such as the Lake Eyre basin, will not be consistent with the model. Nevertheless, it was predicted that populations in different catchments would have been more differentiated than within, because levels of connectivity are likely to be higher within than between catchments, anyway. Contrary to this prediction, *N. sublineata* populations displayed higher values of genetic variation between waterholes within catchments than between catchments overall. To explain this result, it was proposed that gene flow and genetic drift have not yet reached equilibrium on a large geographical scale (among catchments). A non-equilibrium explanation is that the pattern is the result of historical gene flow. When a panmictic population expands its range and occupies new habitats that then becomes subdivided, a number of generations must pass for genetic differences to accumulate among newly established demes. The speed of approach to equilibrium between gene flow and genetic drift is dependent upon both the effective population size and the probability of migration per generation (Crow and Aoki, 1984). If there has not been sufficient time since colonisation or if populations are extremely large, populations will appear similar genetically while being isolated demographically. It can be demonstrated that it would take a significant period of time for *N. sublineata* to reach equilibrium over the entire Western Queensland catchments, whereas the process will occur between neighbouring

waterhole populations much faster (refer to Crow and Aoki, 1984). An explanation for the fact that the differentiation among catchments was marginally significant for mitochondrial DNA, but non-significant for allozymes, is the smaller effective population size of mitochondrial than nuclear genes (Birky *et al.*, 1989). The smaller N_e will lead mitochondrial genes to approach equilibrium faster than nuclear genes (Crow and Aoki, 1984).

3.4.2 Evidence for some historical gene flow between populations

The data presented in this chapter strongly support the hypothesis that climate oscillations of the Pleistocene may be directly correlated with historically higher levels of connectivity throughout Western Queensland, facilitating episodic dispersal of aquatic species across catchment boundaries.

The gene tree lineages of *N. sublineata* can be defined by a combination of the Category III and V of the five categories to define phylogeographic patterns proposed by Avise (2000). In Category III most or all haplotypes are closely related, yet are localised geographically. The implication is that contemporary gene flow has been low enough in relation to population size to have permitted lineage sorting and random drift to promote genetic divergence among populations, which were in historical contact recently. Category V involves common lineages that are widespread and closely related lineages that are 'private' (each confined to one of few nearby locations). This phylogeographic outcome implies low or modest contemporary gene flow between populations that are connected tightly in history. Both these phylogeographic patterns describe case scenarios where contemporary levels of gene flow are low between populations, yet were somehow historically connected. In gene tree lineages of the freshwater snails, all the haplotypes are closely related, separated by no more than three step changes, yet populations have a relatively high number of 'private' haplotypes, thus localised geographically. Some sites even contained unique clades (Wilson, Cooper catchment and Wockingham, Diamantina catchment; Figure 3.1), suggesting that a geographic barrier might have isolated these sites for long enough to allow the local populations to evolve a unique set of haplotypes. One haplotype occurred in all catchments except for the Diamantina and another one was shared by two distant catchments (Diamantina and Bulloo). Very few haplotypes were shared between adjacent catchments. In addition, the haplotypes shared were internal within the network. Coalescent theory predicts that the

haplotypes or clades on the tips of the network are highly likely to be younger that the interior haplotypes or clades to which the tips are connected (Castelloe and Templeton, 1994), suggesting that the sharing of haplotypes detected between catchments is not due to contemporary gene flow.

N. sublineata populations clustered into two main biogeographical groups corresponding with the adjacent catchments: Cooper-Bulloo and Diamantina-Georgina. Populations of *N. sublineata* from the Cooper-Bulloo have been separated relatively recently from the geographically distant Georgina catchment group approximately 150/170 000 years ago, and 250/330 000 years ago from the Diamantina catchment. The time since adjacent catchments have been separated was estimated around 140 000 years ago.

It appears that *N. sublineata* underwent a sudden population expansion around 150 000 years ago, according to the mismatch distribution analysis. Contemporary dispersal of the freshwater snails is very limited and when it occurs is likely to be among neighbouring waterholes. Therefore, there has only been sufficient time since the last recolonisation event to alter haplotype frequencies over small geographic distances, among waterhole populations within catchments. At the large spatial scale, among catchments, the genetic signal of recolonisation still persisted in the data. In concordance with these findings, there seems to be no relationship between genetic distance and geographic distance. The non-significant correlation (r) between genetic distance and geographic distance from the Mantel's test provides further evidence to suggest that populations of *N. sublineata* have not yet reached equilibrium.

Inferences from NCA supported the pattern of past range expansions of *N. sublineata*. A pattern of range expansion can arise if some older (interior) haplotypes are left in the ancestral area, while younger (tip) haplotypes originating from the expanding population can be geographically widespread and/or distant from the ancestral area of origin (Templeton *et al.*, 1995). The oldest range expansion is contiguous and involves clades occurring in the Cooper, the Bulloo and the Georgina. Later in time there is evidence for a second range expansion, in fact clades 2-10 and 2-11 occur in the Cooper and Bulloo and 2-9 is shared among the Cooper, Bulloo and the distant Georgina representing evidence for some long distance colonisation. Figure 3.6 shows the distribution of clades at the 3-step level, indicating some sharing of clades among

distant catchments. Two more recent contiguous range expansions are observed between the Cooper and the Bulloo and some haplotypes shared within clade 2-8 between the Cooper and the top of the Diamantina suggest a long-distance range expansion.

Palaeogeological data provided evidence that perhaps in the past *N. sublineata* moved across catchment boundaries, generating an expansion of its population. During the Pleistocene, periods of high increased rainfall associated with interglacial phases changed the hydrology in Western Queensland significantly, modifying levels of connectivity between rivers (Kershaw and Nanson, 1993; Alley, 1998). It was suggested that very intense rainfall provided enough water to bring the rivers to flood across catchment boundaries and to reduce geographic distance between the headwaters of catchments. Palaeogeological records indicate the greatest fluvial activity and a period of maximum precipitation (Kershaw and Nanson, 1993) approximately between 110 000 and 80 000 ago, when greatly expanded lakes dominated much of the Australian continent (Kershaw and Nanson, 1993; Alley, 1998; Croke *et al.*, 1999). It was proposed that extensive floods and temporary internal lakes resulted in high levels of connectivity between waterholes during interglacial phases, promoting some movement of freshwater snails within and among catchments, resulting in a series of historical population range expansions. The late Pleistocene signalled the gradual stabilisation of the climate towards the contemporary quasi-periodic arid hydrological regime of Western Queensland (Alley, 1998). Eventually, the modern ephemeral regime of annual floods became established in the Lake Eyre Basin about 3000-4000 years ago (Gillespie *et al.*, 1991; Magee *et al.*, 1995).

In conclusion, the data presented in this chapter indicated that some episodic dispersal of *N. sublineata* across catchment boundaries was possible during the Pleistocene, due to different patterns of river connectivity, but that contemporary movements of individuals are extremely limited both between and within catchments. At the present time, Western Queensland represents a mosaic of aquatic landscapes. As a consequence the species is subdivided into many arrays of isolated local populations with very limited gene flow between them.

Chapter 4

Population structure of *Macrobrachium australiense*: the role of contemporary and historical processes

4.1 Introduction

Dispersal is the outward spreading of organisms from their point of origin and it can affect the evolution and population genetics of a species, only if successful colonisation is followed by breeding of subsequent generations. Dispersal is essential for the maintenance of gene flow and genetic diversity, and also for colonisation and re-colonisation of habitats. Generally, species with low dispersal capabilities exhibit a tendency towards genetic fragmentation and species with efficient mechanisms of dispersal have limited genetic structuring of their subpopulations (Slatkin, 1987).

Movements of continuously aquatic freshwater species are very much limited by the physical nature and arrangement of the riverine system, because the terrestrial landscapes represent insurmountable barriers (McGlashan *et al.*, 2001; Hurwood and Hughes, 2001). Thus, often species with apparently good dispersal abilities present surprisingly high levels of population subdivision (Hughes *et al.*, in review and Hughes and Hillyer, 2003).

This chapter focuses on the study of genetic structure of the freshwater prawn, *Macrobrachium australiense* (Decapoda: Palaemonidae). This species appears to have good dispersal capabilities, as suggested by its wide distribution in Australian rivers and its life history features as described by Williams (1980). As previously discussed in this study, passive transport by water currents and/or active swimming of freshwater invertebrates in Western Queensland rivers may be achieved only during wet seasons when water is flowing in rivers and the nearby floodplains.

A previous study, conducted on this species by Cook *et al.* (2002), showed significant population structure between catchments but found very low genetic differentiation between populations within catchments. They used mostly allozyme analysis, although

a fragment of the cytochrome oxidase I gene was sequenced for a small number of individuals. It was suggested that terrestrial watersheds represent an insurmountable barrier to dispersal between catchments, generating genetic differentiation between populations from isolated catchments. On the contrary, genetic homogeneity in populations of *M. australiense* within catchments suggested that levels of connectivity and dispersal were high. Nevertheless, Hughes *et al.* (in review) demonstrated that, using mtDNA markers, species with apparently efficient mechanisms of dispersal could display low levels of gene flow between populations within catchments in the same geographic region. Due to the haploid nature and the maternal mode of inheritance, mtDNA has a fourfold smaller effective population size than nuclear markers, increasing its sensitivity to any limited gene flow between populations (Birky *et al*., 1989). Therefore, it would be more likely to detect genetic structure of the species using mtDNA than allozymes. However, even if the population structure of mtDNA variation supports the allozyme study of Cook *et al.* (2002), there is still the question as to what processes led to the observed structure. The traditional methods for estimating rates of gene flow between subpopulations rely on *F*-statistics calculated from allelic frequency variation (e.g. Slatkin, 1981). However, the retention of ancestral alleles in subdivided populations can lead to an F_{ST} value less that one, implying some levels of contemporary gene flow even when dispersal is non-existent (Templeton, 1998). The problem can be overcome by employing temporal information available for molecular data.

The main aim of this chapter was to determine the extent to which the population structure of *M. australiense* in Western Queensland results from recurrent versus historical processes (Templeton *et al.*, 1995) by testing two hypotheses. Firstly it was hypothesised that *M. australiense* will display high levels of genetic structure among populations throughout its distribution (i.e. among waterholes within a catchment and among catchments). Although the species has potential for long distance dispersal, due to its good dispersal capabilities, the terrestrial inhospitable habitat and the erratic flow patterns of the rivers are likely to impede gene flow between populations inhabiting isolated pools. Thus, it is expected that the good dispersal abilities of the species will play a secondary role in determining level of gene flow between populations.

The second hypothesis was that climate oscillations of the Pleistocene may be directly correlated with historical higher levels of connectivity between rivers that might have

facilitated aquatic dispersal across catchment boundaries. Thus it was expected to find some evidences for historical gene flow between populations of *M. australiense.*

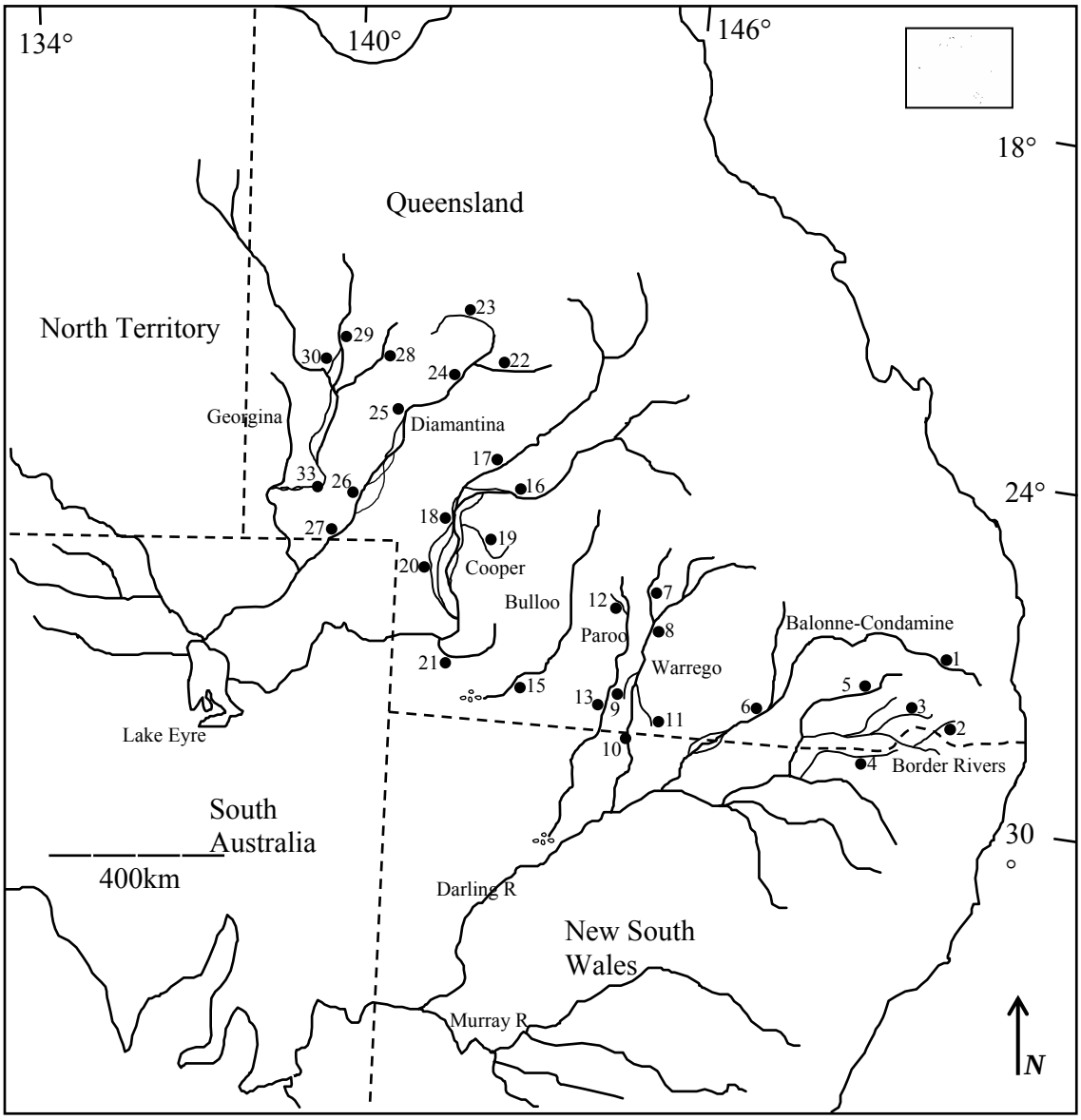
4.2 Specific methods

Sampling sites are localised within the Lake Eyre basin and the northern regions of the Murray-Darling basin (Figure 4.1). *M. australiense* was sampled from waterholes in the four major catchments (Bulloo, Cooper, Diamantina and Georgina) and tributaries within the Lake Eyre basin as well as in the four major catchments (Balonne-Condamine, Border Rivers, Warrego and Paroo) and tributaries within the upper Murray-Darling basin (Figures 4.1 and 2.8). Further details are presented in chapter 2 (general methods)

4.2.1 mtDNA Analysis

DNA extraction, PCR, sequencing procedures and general data analyses, are explained in the general methods section (chapter 2). Φ-Statistics (based on haplotype frequencies and molecular divergence) and *F*-Statistics (haplotype frequencies only) were used to assess population subdivision. Hierarchical partitioning was calculated between and within the two drainages: Lake Eyre basin and Murray-Darling basin. In addition, separate hierarchical AMOVA's were calculated within and among catchments for both the Lake Eyre and Murray-Darling basins.

Figure 4.1 Study area and sampling sites for *M. australiense.* Site names and catchment groupings are: Georgina catchment: Hamilton River at Bulla Bulla waterhole (28), Georgina River at Boulia (29), Burke River at Boomerang waterhole (30), Eyre creek at Cuttaburra crossing (33); Diamantina catchment: Wockingham creek at Winton (22), Diamantina River at Combo waterhole (23), Diamantina River at Verdun Valley (24), Diamantina River at Diamantina Lakes (25), Diamantina River at Monkira (26), Diamantina River at Birdsville (27); Cooper catchment: Thompson River at Noondah (17), Barcoo River at Retreat (16), Kyabra creek at Springfield (19), Cooper creek at Windorah (18), Cooper creek at Tanbar (20), Wilson River at Noccundra (21); Bulloo catchment: Bulloo River at Thargomindah (15); Paroo catchment: Paroo River at Cheepie (12), Paroo River at Eulo (13); Warrego catchment: Ward River at Charleville (7), Warrego River at Quilberry (8), Warrego River at Glencoe (9), Warrego River at Thurulgoona (10), Cuttaburra creek at Tinnenburra (11); Balonne-Condomine catchment: Condomine River at Brigalow (1), Balonne River at St George (6); Border Rivers catchment: Whalan creek at Salt Bush (2), Weir River at Kanowna (3) Gnungarah creek at Gnungarah (4), Commoron creek at Iron Bark (5).



4.3 Results

The mitochondrial data did not deviate significantly from predictions of neutrality (Tajima`s D: -1.02079 *P*= 0.14; Tajima, 1989). Sequence data for 402 specimens of *M. australiense* collected from eight catchments were acquired, using direct sequencing. Based on a 505 base pair fragment of COI of unambiguous sequence, 98 unique haplotypes were identified (Tables 4.1, 4.2 and 4.3) of which 63% represented single individuals. Of the 505 base pairs sequenced, 76 (15%) sites were polymorphic. The transition (T_s) to transversion (T_v) substitution ratio was 5.3:1. Haplotype gene diversity values (mean: 0.923+/- 0.0075) were high and variable; reflecting the large number of haplotypes detected at most sites. Haplotype frequencies are presented as pie charts in Figures 4.2 and 4.3.

It was evident that the haplotype network was divided into two distinct groups corresponding to the Lake Eyre and Murray-Darling basins. The Murray-Darling clade was more closely related to the Diamantina-Georgina clade than to the Cooper clade (Figure 4.4, Tables 4.1 and 4.2). No haplotypes were shared between the two basins.

Within the Lake Eyre basin two further groups could be distinguished. The first group contained mostly samples collected in the Diamantina, the Georgina and the Bulloo catchments (Figure 4.1), but a single haplotype (H-2) was also found in the Cooper catchment. The network indicated that haplotype 2 was at the centre of this group (Figure 4.4 and Table 4.1). The second group included haplotypes from throughout the Cooper catchment. Haplotype 37 was the centre of this group and was detected in all sites with the exception of the Wilson River at Noccundra (Figure 4.4 and Table 4.1). The Wilson River population (site number 21 in Figure 4.1 and Table 4.1) represented a unique subclade (haplotypes: 38, 39, 40 and 42).

The group represented by the Murray-Darling haplotypes was complex and there was no clear grouping of samples according to catchments. In fact, there was extensive haplotype sharing between waterholes among adjacent and non-adjacent catchments. Haplotypes 90 and 76 were internal to the network and widespread across the Murray-Darling basin. Haplotype 76 occurred in the Warrego, Border Rivers and Balonne-Condamine catchments while haplotype 90 was widespread across all the catchments within the Murray-Darling basin (Figure 4.4 and Table 4.2) except for the population of

Glencoe in the Warrego (site number 9 in Figure 4.1 and Table 4.2). In fact, samples from this population contained a unique subclade (59, 65, 66, 67, 68, 69, 70, 71 and 72).

Table 4.1 Distribution of haplotypes detected in *M. australiense* within the Lake Eyre basin. For site designation, refer to Figure 1. Catchments: Bulloo (B), Cooper (C), Diamantina (D) and Georgina (G).

Catchments	B	C	C	C	C	C	C	D	D	D	D	D	D	G	G	G	G
sites	15	18	19	20	17	16	21	25	27	26	24	23	22	28	33	30	29
Haplotypes																	
H1														1			
H2	2	2						9	2	7	12	5	6	8	7	9	10
H3															1		
H4											1		1				
H5															1	1	
H6											1						
H7													1				
H8										1							
H9														1			
H10															1		
H11								2		1	1	1					
H12								1		1							
H13											1						
H14											1						
H15	1																
H16	4																
H17	1																
H18								1	2	3	3	1	1				
H19									2	2			1				
H20										1							
H21								1									
H22								1		1		1					
H23								2		2							
H24	1																
H25									1			1	1				
H26								2	1	1		1					
H27		2															
H28			1														
H29			1	5													
H30					2												
H31			1														
H32		2		2													
H33				2													
H34				1													
H35						1											
H36		1															
H37		11	13	9	14	5											
H38							3										
H39							1										
H40							1										
H41					1												
H42							1										
H43		2	1														
H45					2												
H46					2												
H47			2	1													
H48			2														
H49		1															
H50						1											
H51								1									

Table 4.2 Distribution of haplotypes detected in *M. australiense* within the Murray-Darling basin. For site designation, refer to Figure 1. Catchments: Warrego (W), Paroo (P), Balonne-Condomine (BC) and Border Rivers (BR).

Catchments	W	W	W	W	W	P	P	BC	BC	BR	BR	BR	BR
Sites	8	11	9	10	7	12	13	1	6	2	3	4	5
Haplotypes													
H52							1						
H53												1	2
H54								1					
H55											1		1
H56											1		
H57											1		
H58				2									
H59			1										
H60										2	1	1	1
H61				1									
H62	1												
H63										1			
H64									1				
H65			2										
H66			12										
H67			1										
H68			1										
H69			1										
H70			3										
H71			1										
H72			1										
H73					1								
H74		1		2		1	5						
H75										1			
H76	2			1				5	5	3		3	3
H77											1		
H78									1		1	1	
H79												1	
H80								1					
H81				1									
H82	1			2				1					
H83		1											
H84		4											
H85											1	1	
H86	1												
H87	1	1		3	1				1				
H88						1	2						
H89										1			
H90	7	8		8	8		4		1	2	3	3	2
H91							2						
H92												1	
H93													1
H94										1			
H95												1	
H96													1
H97										1	1	1	
H98							1						

Table 4.3 76 variable sites for 98 haplotypes of COI fragment from 402 *M. australiense* specimens

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[                111111111 1111122222 2222222233 3333333333 3333333444 4444444444 444445]
[     1122235566 7111233555 6788913344 6667779900 0112334466 6788999000 0123444456 788990]
[     5814544737 8147928069 5213263736 1470391706 9281692503 6526039235 8763145702 426254]
 H-1  ACGTCTCAAT CTCCCTTATA CCCAAGTAGT ATCAGAACCG CCTAATCCCG AGCCTAGCCA ACAAACTTAA ATGACC
 H-3  .......... .......... .......... .........A .......... .......... ..C....... ......
 H-4  .......... .......... .......... .......... .......... ...T...... ..C....... ......
 H-5  .......... T......... .......... .......... .......... .......... ..C....... ......
 H-6  .......... T.T....... .......... .......... .......... .......... ..C....... ......
 H-7  .......... ..T....... .......... .......... .......... .......... ..C....... ......
 H-8  .......... .......... ....G..... .......... .......... .......... ..C....... ......
 H-2  .......... .......... .......... .......... .......... .......... ..C....... ......
 H-9  .......... .......... .......... .......... .A........ .......... ..C....... ......
 H-10 .......... .......C.. .......... .......... .......... .......... ..C....... ......
 H-11 .......... .......... .......... .......... .......... ..T....... ..C....... ......
 H-12 .......... .......... .......... .......... .......... .......... ..C...G... ......
 H-13 ....T..... .........G .......... .......... .......... .......... ..C...G... ......
 H-14 .......... .........G .......... .......... .......... .......... ..C....... ......
 H-15 .......... .C........ T......... .......... .......... .......... ..C....... ......
 H-16 .......... .......... T......... .......... .......... .......... ..C....... ......
 H-17 .......... .......... T......... .......... .......... ........T. ..C....... ......
 H-18 .......... .......... .......... ....A..... .......... ........T. ..C....... ......
 H-19 .......... .......... .......... .......... .......... ........T. ..C....... ......
 H-20 .......... .......... .......... .......... .......T.. ........T. ..C....... ......
 H-21 .......... .......... .......... .......... .......... ....C...T. ..C....... ......
 H-22 .......... .......... .....A.... .......... .......... ........T. ..C....... ......
 H-23 .......... .....C.... .......... .......... .......... ........T. ..C....... ......
 H-24 .......... .C........ ........A. .......... .......... .......... ..C....... ......
 H-25 .......... .......... ........A. .......... .......... .......... ..C....... ......
 H-26 .......... .......... .......... .......... ..C....... .......... ..C....... ......
 H-27 .T........ .......... .......... .......... ..C....... .......... ..C.....G. ......
 H-28 .T........ .......... .......... .......... ...G...... .....G.... ..C....... G.....
 H-29 .T........ .......... .......... .......... ...G...... .....G.... ..C....... ......
 H-30 .T....A... .......... .......... T......... ...G...... .....G.... ..C....... ......
 H-31 .T........ .......... .......... .......... ...G...... .......... ..C....... ......
 H-32 .T........ .......... .......... .......... ...G...... ......A... ..C....... ......
 H-33 .T........ .......... .......... .......... .......... .....G.... ..C....... ......
 H-34 .......... .......... .......... .......... .......... .....G.... ..C....... ......
 H-35 .T........ .......... .......... ....A..... .......... .......... ..C....... ......
 H-36 .T..T..... .......... .......... .......... .......... .......... ..C....... ......
 H-37 .T........ .......... .......... .......... .......... .......... ..C....... ......
 H-38 .T........ .......... .......... .......... .......... .......... ..C....... .G....
 H-39 .T........ .......... .......... .......... .......... ........G ..C....... .G....
 H-40 .T........ .......... .......... .......... ..C....... .......... ..C....... .G....
 H-41 .T........ .......... .......... .......... .......... ........G ..C....... ......
 H-42 .T........ .......... .......... .....G.... .......... .......... ..C....... .G....
 H-43 .T........ .......... .......... .....G.... .......... .......... ..C....... ......
 H-44 .TA....... .......... .......... .....G.... .......... .......... ..C....... ......
 H-45 .T........ .......... .......... .......... .......... .......... ..C....... G.....
 H-46 .T........ ......C... .......... .......... .......... .......... ..C....... ......
 H-47 .T........ .......... .......... .......... .......... .......... ..C....... ....T.
 H-48 .T........ .......... .......... .C........ .......... .......... ..C....... ......
 H-49 .T........ .......... .T........ .......... .......... .......... ..C....... ......
 H-50 .T........ ...T...... .......... ......G... .......... .......... ..C....... ......
 H-51 .......... .......... .......... .......... .......... ......A... ..C....... ......
 H-52 .......... .......... .......... .......... .......T.A ......A... ..C....... ..A...
 H-53 ........G. .......... ...G....CC ...GA..... ..C....... ......AT.. ..C....C.. ..A...
 H-54 ...C....G. .......... ...G....CC ....A..... .....CT.. ......AT.. ..C....C.. ..A...
 H-55 ...C...... .......... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-56 G..C...... .......... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-57 ...C...... .......G.. ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A..T
 H-58 ...C....G. .......G.. ...G....CC ....A..... ..C....... ......AT.. ..CC...C.. ..A..T
 H-59 ...C....G. .......G.. ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-60 ...C....G. .......G.. ...G....CC ....A..... .TC....... ......AT.. ..C....C.. ..A...
 H-61 ...C...... .......G.. ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-62 ...C....G. .......... ...G..A.CC G...A..... ..C....... ......AT.. ..C....C.. ..A...
 H-63 .T.C....G. .......... ...G....CC G...A..... ..C....... ......AT.. ..C....C.. ..A...
 H-64 ...C....G. .......... ...G.A..CC ....A..... ..C....T.. ......AT.. ..CC...C.. ..A...
 H-65 ...C....G. .......... ...G....CC ....A..... ..C....T.. ......AT.. ..CC...C.. ..A...
 H-66 ...C....G. .......... ...G....CC ....A..... ..C....T.. ....C.AT.. ..CC...C.. ..A...
 H-67 ...C....G. ......C... ..TG....CC ....A..... ..C....... ....G.AT.. .TCC...C.G ..A...
 H-68 ...C....G. .......... ...G....CC ....A..... ..C....... ....A.AT.. ..CC...C.. ..A...
 H-69 ...C....G. .......... ...G....CC ....A..... ..C....... ......AT.. ..CC...C.. ..A...
 H-70 ...C....G. .......... ...G....TC ....A..... ..C.G..... ......AT.. ..CC...C.. ..A...
 H-71 ...CTC..G. .......... ...G....CC ....A..... T.C....... ......AT.. ..CC...C.. ..A...
 H-72 ...CTC..G. .......... ...G....CC ....A..... T.C....... ......AT.. ..C....C.. ..A...
 H-73 ...CTC..G. .......... ...G....CC ....A..... T.C....... ......AT.. ..C....C.. ..AG..
 H-74 ...C....G. .......... T..G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-75 ...C....G. .......... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-76 ...C....G. ......C... ...G....CC ....A..... ..C....... .A....AT.. ..C....C.. ..A...
 H-77 ...C....G. ......C... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-78 ...C....G. .......... ..TG....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-79 ...C....G. ....T..... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-80 ...C....G. .......... ...G....CC ....A..... ..C....... G.....AT.. ..C....C.. ..A...
 H-81 ...C....G. .......... ...G....CC ....A..... ..C....... ....A.AT.. ..C....C.. ..A...
 H-82 ...C....G. .......... ...G....CC ....A..... ..CT...... ....A.AT.. ..C....C.. ..A...
 H-83 ...C....G. .......... ...G....CC ....A...A. ..C....... ....G.AT.. ..C....C.. ..A...
 H-84 ...C....G. .C........ ...G....CC ....A..... ..C....... ....G.AT.. ..C....C.. ..A...
 H-85 ...C....G. .......... ...G....CC ....A..... ..C....... ....G.AT.. ..C....C.. ..A...
 H-86 ...C..T.G. .......... ...G....CC ....A..... ..C....T.. ....G.AT.. ..C....C.. ..A...
 H-87 ...C....G. .......... ...G....CC ....A..... ..C....T.. ......AT.. ..C....C.. ..A...
 H-88 ...C....G. .......... ...G....CC ....A..... ..C.C..T.. ......AT.. ..C....C.. ..A...
 H-89 ...C....GC .......... ...G....CC ....A..... ..C....T.. ......AT.. ..C.G..C.. ..A...
 H-90 ...C....G. .......... ...G....CC ....A..T.. ..C....T.. ......AT.. ..C....C.. ..A...
 H-91 ...C.....C .......... ...G....CC ....A..... ..C....... ......AT.. ..C....C.. ..A...
 H-92 ...C....G. .......... ...G...GCC ....A..... ..C....... ....G.AT.. ..C....C.. ..A...
 H-93 ...C....G. .......... ...G....CC ....A..... ..C....T.. .....GAT.. ..C..A.C.. ..A...
 H-94 ...C....G. .C........ ...G....CC ....A..... ..C....T.. ......AT.. ..C....C.. ..A...
 H-95 ...C....G. .......... ...G....CC ..T.A..... ..C....T.. ......AT.. ..C....C.. ..A...
 H-96 ...C....G. .......... ...G....CC ....A..... ..C....TT. ......AT.. ..C....C.. ..A...
 H-97 ...C...GG. .......... ...G....CC ....A..... ..C....T.. ......AT.. ..C....C.. ..A...
 H-98 ...C....G. .......... ...G....CC ....A..... ..C....T.. ......AT.. G.C....C.. ..A...
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Figure 4.2 Pie charts representing mitochondrial DNA haplotype frequencies in each waterhole within the Lake Eyre basin for *M. australiense* (refer to Figure 4.1 and Tables 4.1 and 4.2).

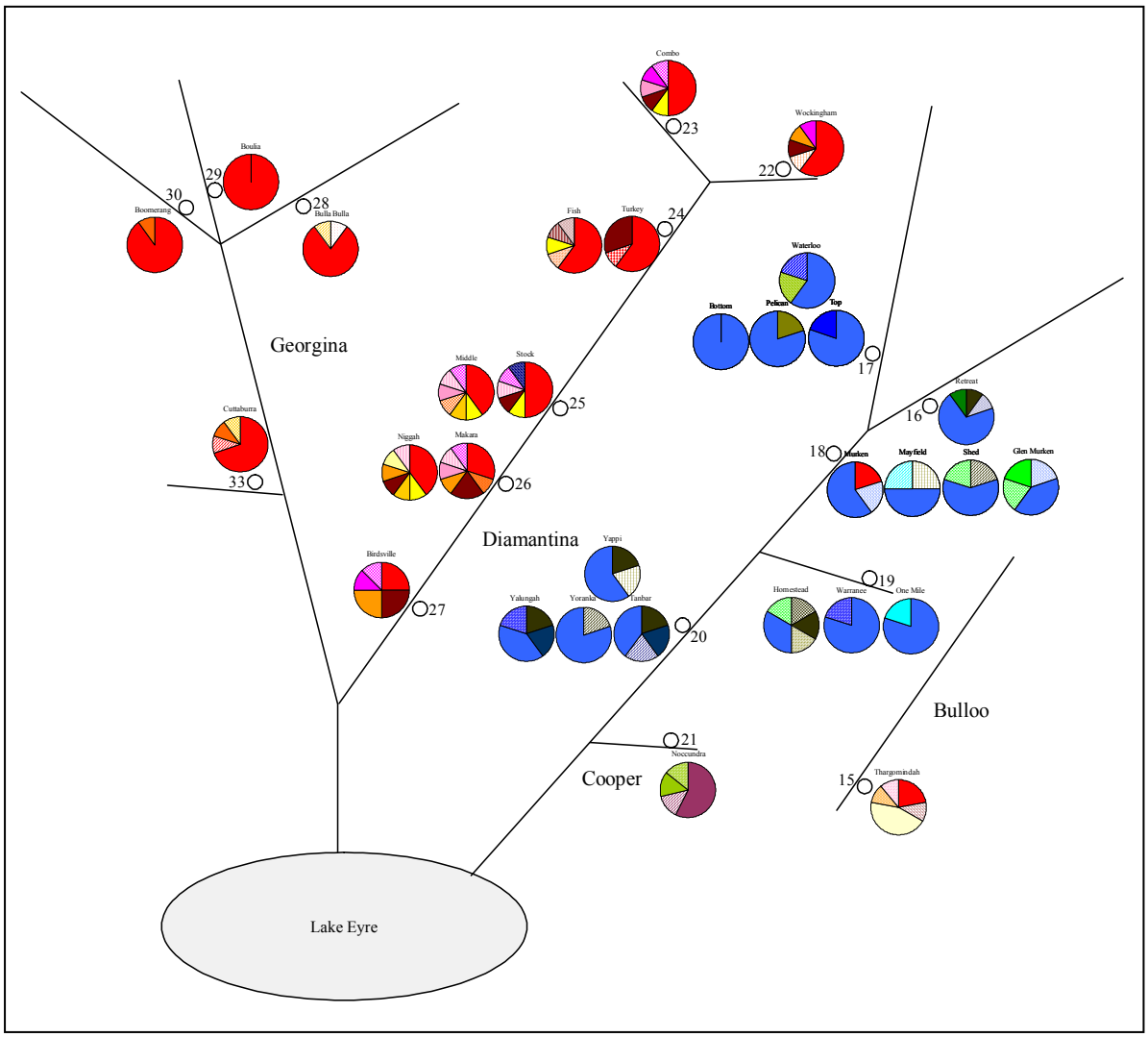


Figure 4.3 Pie charts representing mitochondrial DNA haplotype frequencies in each waterhole within the Murray-Darling basin for *M. australiense* (refer to Figure 4.1 and Tables 4.1 and 4.2).

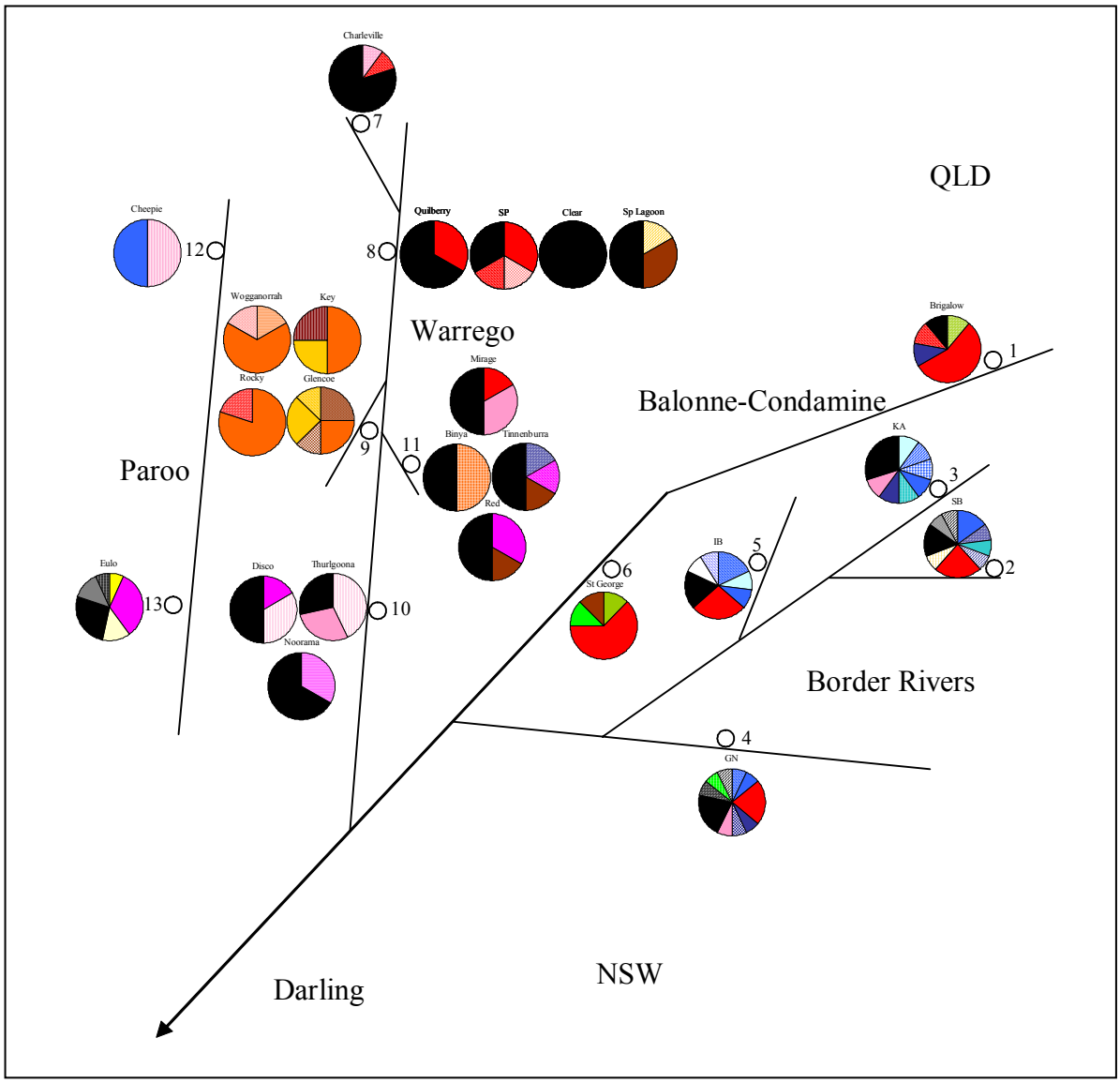
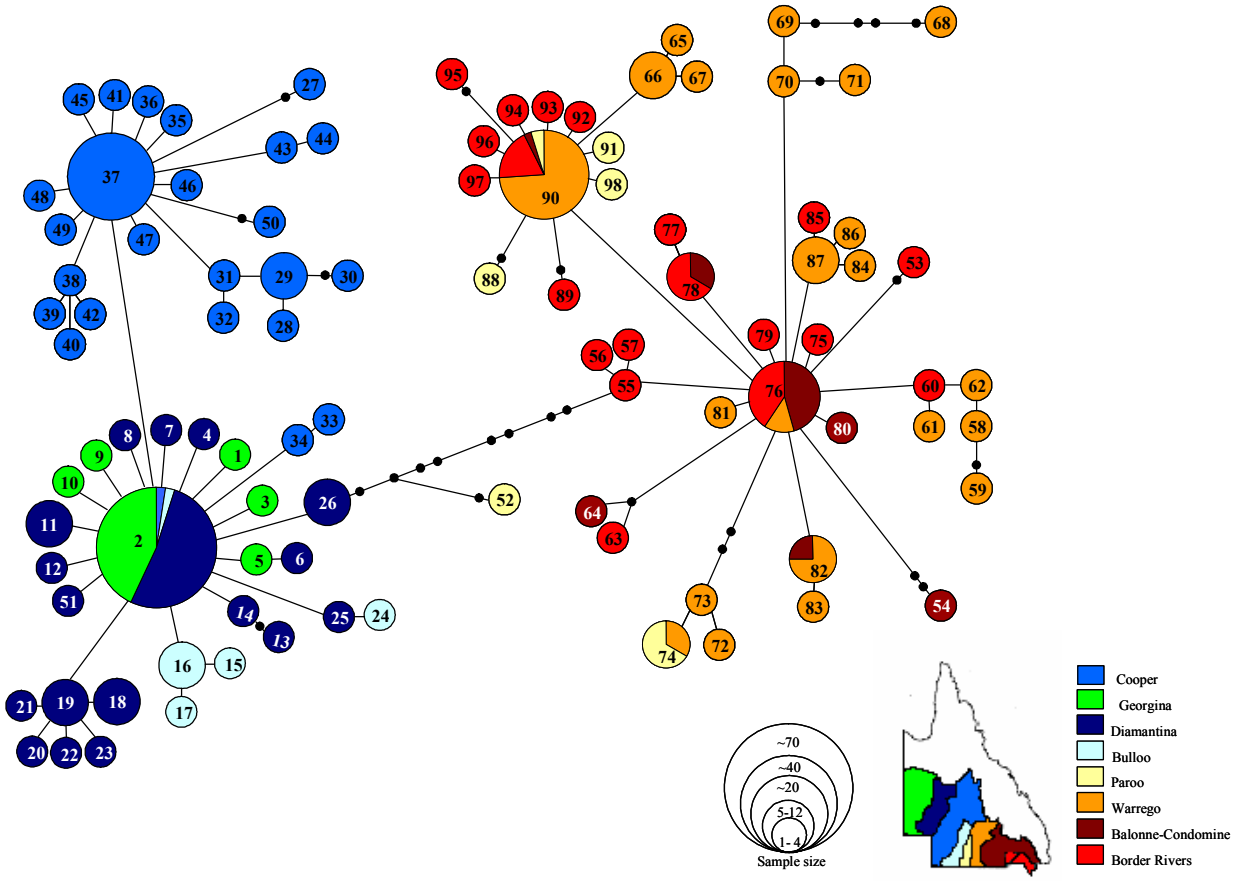


Figure 4.4 Haplotype and genealogy network, indicating catchment distribution of each haplotype (1-98). Circle size for each haplotype is proportional to overall frequency in our sample. Note that proportions of colours corresponding to catchment, within each pie chart, are representative of relative number of that haplotype from each catchment. Haplotypes not detected in the sample are indicated by a small solid black circle.



4.3.1 Analysis of molecular variance

Analysis of molecular variance of a total of 402 *M. australiense* samples detected significant levels of genetic variation at all the spatial scales examined. There were highly significant levels of genetic differentiation of populations between and within the major drainages and among all catchments (Table 4.4). Within the Lake Eyre basin, significant levels of genetic differentiation of populations between the two groups formed by adjacent catchments: Cooper-Bulloo and Diamantina-Georgina were detected. Also, significant values were observed among and within the four catchments (Table 4.5). Within the Murray-Darling basin estimates of genetic variation were significant among populations within catchments, but not significant among the four catchments (Φ_{CT}: 0.04805 P= 0.16+/- 0.0043) (Table 4.6a). Moreover, within the

Murray-Darling basin, the estimate of Φ_{SC} was higher than Φ_{CT}, suggesting that genetic variation is higher between populations within catchments than between catchments overall (Table 4.6a). However, this result seems to be largely due to the fact that the population from the Glencoe site in the Warrego (number 9 in Figure 4.1) did not share any haplotypes with other populations from the same catchment (Table 4.3). Indeed when the Glencoe site was removed from the analyses, both Φ_{SC} and F_{SC} were not significant and lower than Φ_{CT} and F_{CT}, respectively (Table 4.6b).

Table 4.4 Results of the analysis of molecular variance for populations of *M. australiense*, displaying *F*-Statistics and Φ-Statistics. The levels of partitioning investigated, included: between the two drainages: the Lake Eyre Basin and the Murray-Darling and also: among all the eight catchments * P<0.001.

Level of partitioning	***F*-Statistics**	**Φ-Statistics**
Among drainages [Lake Eyre Basin] [Murray-Darling Basin]	F_{CT}: 0.139*	Φ_{CT}: 0.843*
Among all catchments [Cooper] [Diamantina] [Georgina] [Bulloo] [Paroo] [Balonne-Condomine] [Warrego] [Border Rivers]	F_{CT}: 0.240*	Φ_{CT}: 0.806*

Table 4.5 Results of the analysis of molecular variance for populations of *Macrobrachium australiense*, displaying the *F*-statistics and Φ-statistics. The levels of partitioning investigated, included: between the groups corresponding to adjacent catchments (Bulloo-Cooper and Diamantina-Georgina) and among and within the four catchments of the Lake Eyre Basin *P<0.001.

Level of partitioning	***F*-statistics**	**Φ-Statistics**
Among groups within Lake Eyre Basin [Cooper-Bulloo] [Diamantina-Georgina]	F_{CT}: 0.288*	Φ_{CT}: 0.387*
Among catchments within Lake Eyre Basin [Cooper] [Diamantina] [Georgina] [Bulloo]	F_{CT}: 0.295*	Φ_{CT}: 0.411*
Among populations within catchments	F_{SC}: 0.032*	Φ_{SC}: 0.064*

Table 4.6 Results of the analysis of molecular variance for populations of *Macrobrachium australiense*, displaying the percentage variation and associated Φ-statistics and the *F*-statistics, a) all sites b) with Glencoe site excluded. The levels of partitioning investigated, included: among and within the four catchments of the Murray-Darling Basin. *P<0.001

a)

Level of partitioning	*F*-Statistics	Φ-Statistics
Among Catchments within Murray-Darling Basin [Paroo] [Balonne-Condomine] [Warrego] [Border Rivers]	F_{CT}: 0.042	Φ_{CT}: 0.048
Among populations within catchments	F_{SC}: 0.155*	Φ_{SC}: 0.121*

b)

Level of partitioning	*F*-Statistics	Φ-Statistics
Among Catchments within Murray-Darling Basin [Paroo] [Balonne-Condomine] [Warrego] [Border Rivers]	F_{CT}: 0.127*	Φ_{CT}: 0.098*
Among populations within catchments	F_{SC}: 0.026	Φ_{SC}: 0

The results of the Mantel test indicated that there is a positive relationship between genetic distance and geographic distance within the Lake Eyre basin (r = 0.54 *P*<0.001), which is concordant with the significant population structure by catchments implied by preceding AMOVA analysis. The Mantel's test clearly indicated that there was no relationship between geographic distance and genetic divergence within the Murray-Darling basin (r = 0.04 *P* = 0.3) which is concordant with the lack of population structure by catchment implied by the AMOVA when all sites where included in the analysis. However, when Glencoe site was removed, the Mantel's test detected a positive relationship between genetic distance and geographic distance (r = 0.3 *P* =0.038). These tests suggest that there is a signature of isolation by geographic distance within both basins in Western Queensland.

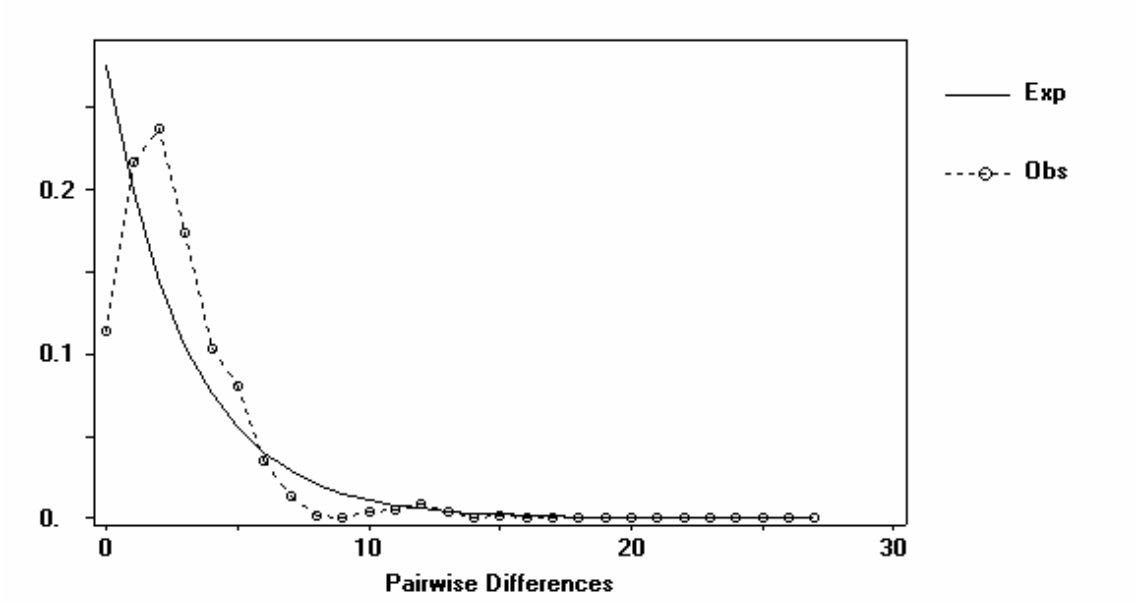
4.3.2 Population demographic history: mismatch distribution

There was a maximum of one and four base pair differences between any two adjacent nodes in the haplotype network within the Lake Eyre and the Murray-Darling basin, respectively (Figure 4.4). Eight base pair difference separated the two basins. The short internodal distances are consistent with a star phylogeny that is indicative of rapid population expansion (Avise *et al.*, 1984). The hypothesis of a sudden expansion within each basin is also supported by the mismatch distribution (Figures 4.5 and 4.6) with no significant difference between the observed and expected distribution under the expansion model (Murray-Darling basin: SSD = 0.002, P = 0.63; r = 0.023, P = 0.79; Lake Eyre basin: SSD = 0.0005, P = 0.41; r = 0.04 P = 0.35).

The calculated θ parameters indicate that the *M. australiense* population underwent a rapid range expansion within the Murray-Darling basin ($\theta_0 = 0$; $\theta_1 = 11$) and within the Lake Eyre basin ($\theta_0 = 0$; $\theta_1 = 3268$). Based on the mutation rate estimated at 6×10^{-6} substitutions/locus/year for the COI 505bp of *M. australiense* and the calculated units of mutational time (Murray-Darling $\tau = 1.13$; Lake Eyre $\tau = 1.8$), the expansion event was estimated to have occurred in the Murray-Darling around 90 000 years ago and in the Lake Eyre basin around 56 000 years ago.

Figure 4.5 Distribution of pairwise nucleotide differences for *M. australiense* within the Murray-Darling basin. The faint line is the observed distribution of the population in figures a) and b), the continuous line is the expected geometric distribution indicative of a) a stable population and b) a population that has undergone an expansion (expected Poisson distribution)

a)



b)

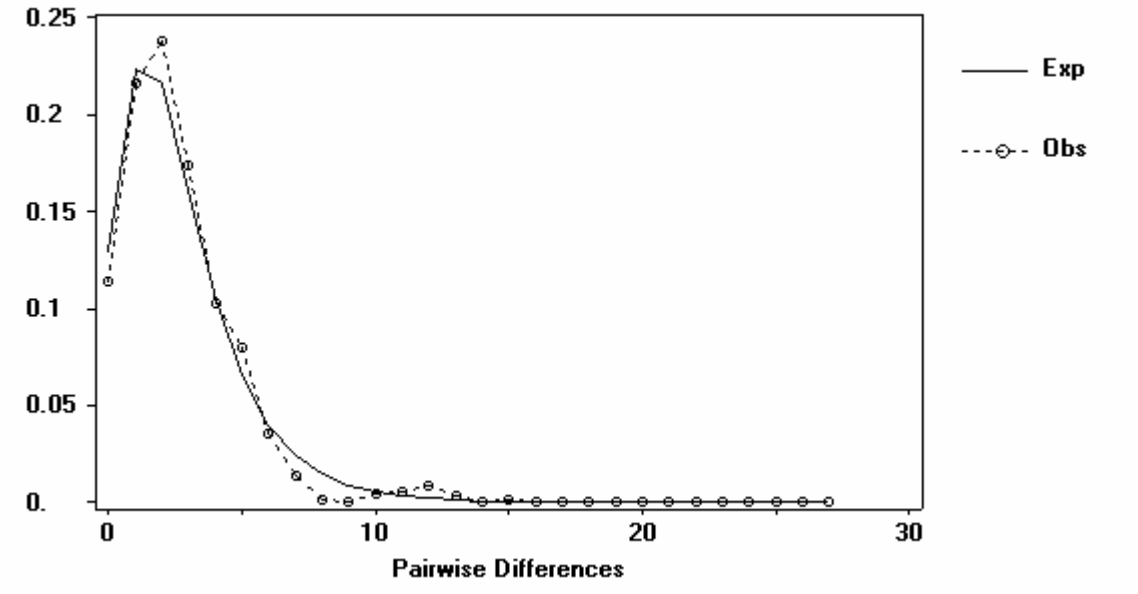
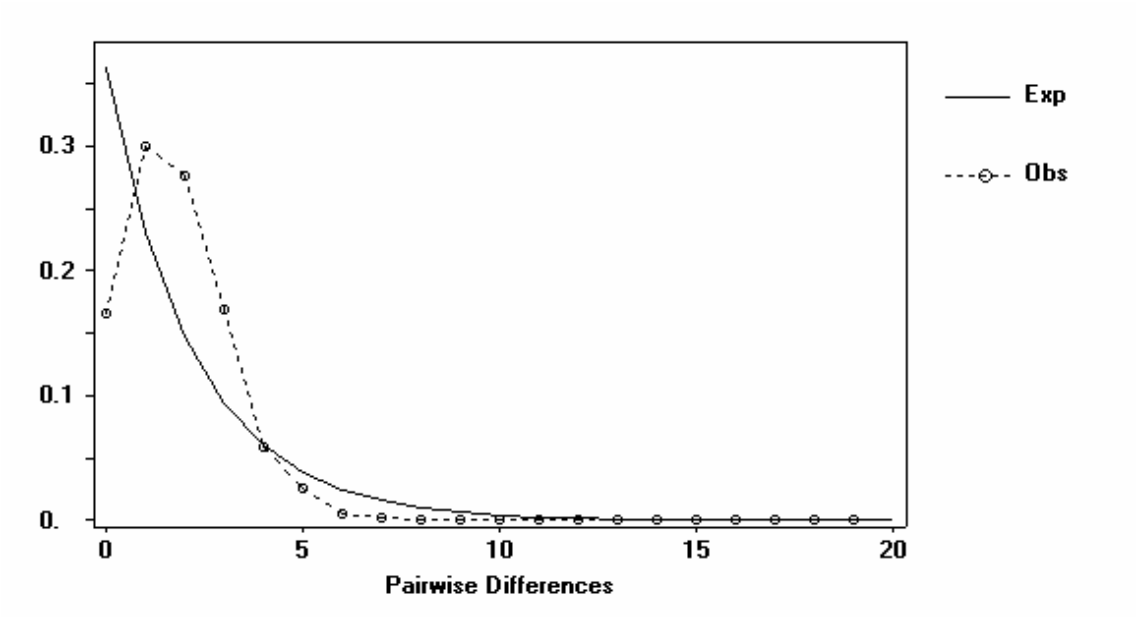
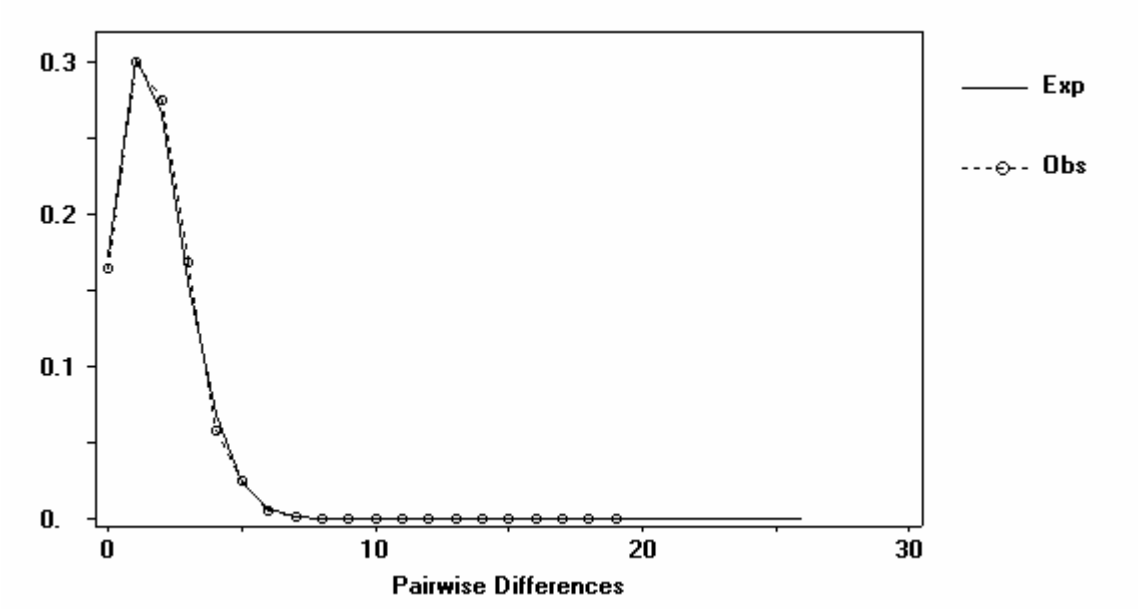


Figure 4.6 Distribution of pairwise nucleotide differences for *M. australiense* within the Lake Eyre basin. The faint line is the observed distribution of the population in figures a) and b), the continuous line is the expected geometric distribution indicative of a) a stable population and b) a population that has undergone an expansion (expected Poisson distribution)

a)



b)



4.3.3 Nested clade analysis

The 98 haplotypes were partitioned into forty-two 1-step clades, eleven 2-step clades, four 3-step clades and two 4-step clades (Figures 4.7 and 4.8). The haplotype cladogram revealed two divergent clades: 4-1 and 4-2 (Figure 4.8). Haplotypes within clade 4-1 (3-2 and 3-1) belonged to the Lake Eyre basin, whereas haplotypes within clade 4-2 (3-3 and 3-4) belonged to the Murray-Darling basin (Figure 4.8 and tables 4.1, 4.2). The level of genetic divergence between these clades was approximately 1.6% (uncorrected p-distance).

Figure 4.7 1-step clade nesting for the hypothesised network with 95% plausible set of haplotype connections. Clade step delineation is shown next to each nesting box. A solid branch between haplotype 1- 98, is representative of a single base pair change in the mitochondrial sequence.

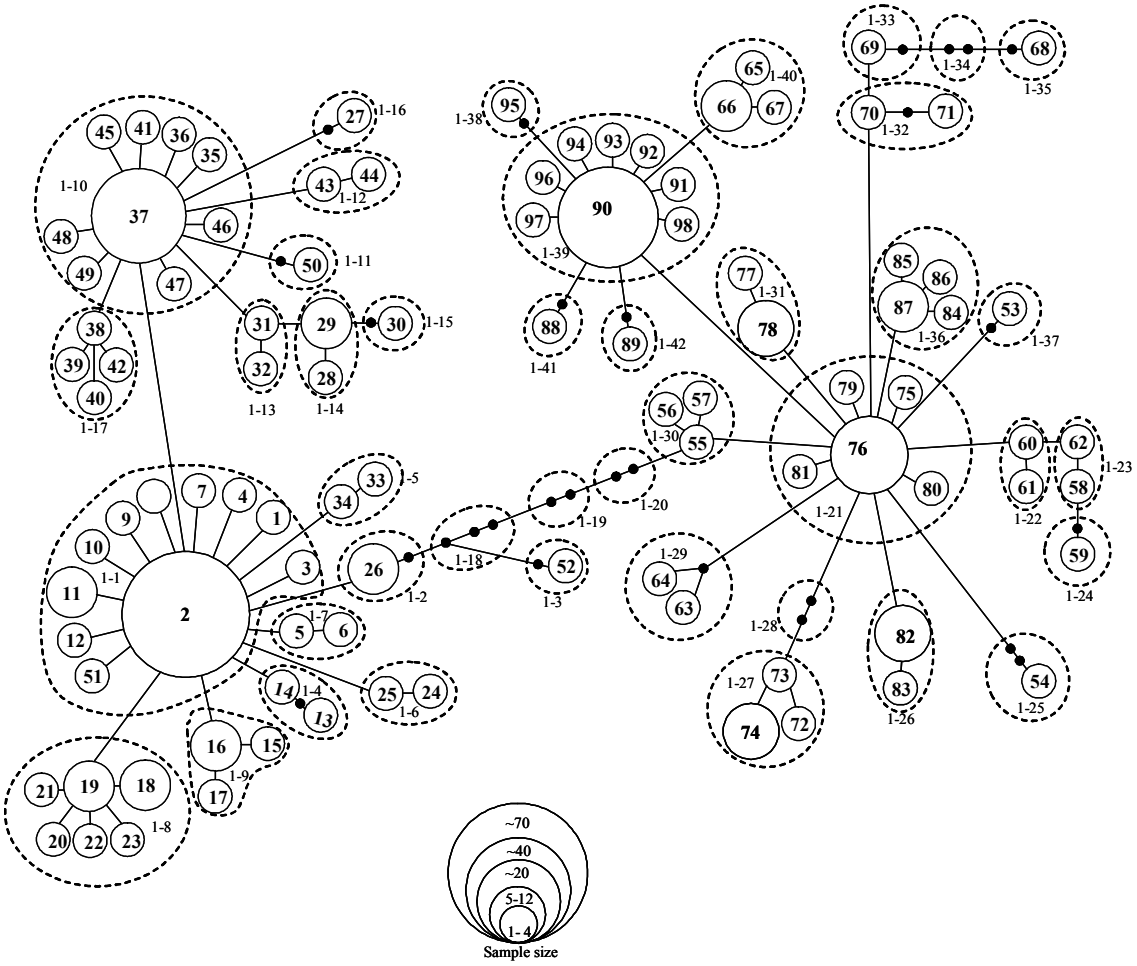
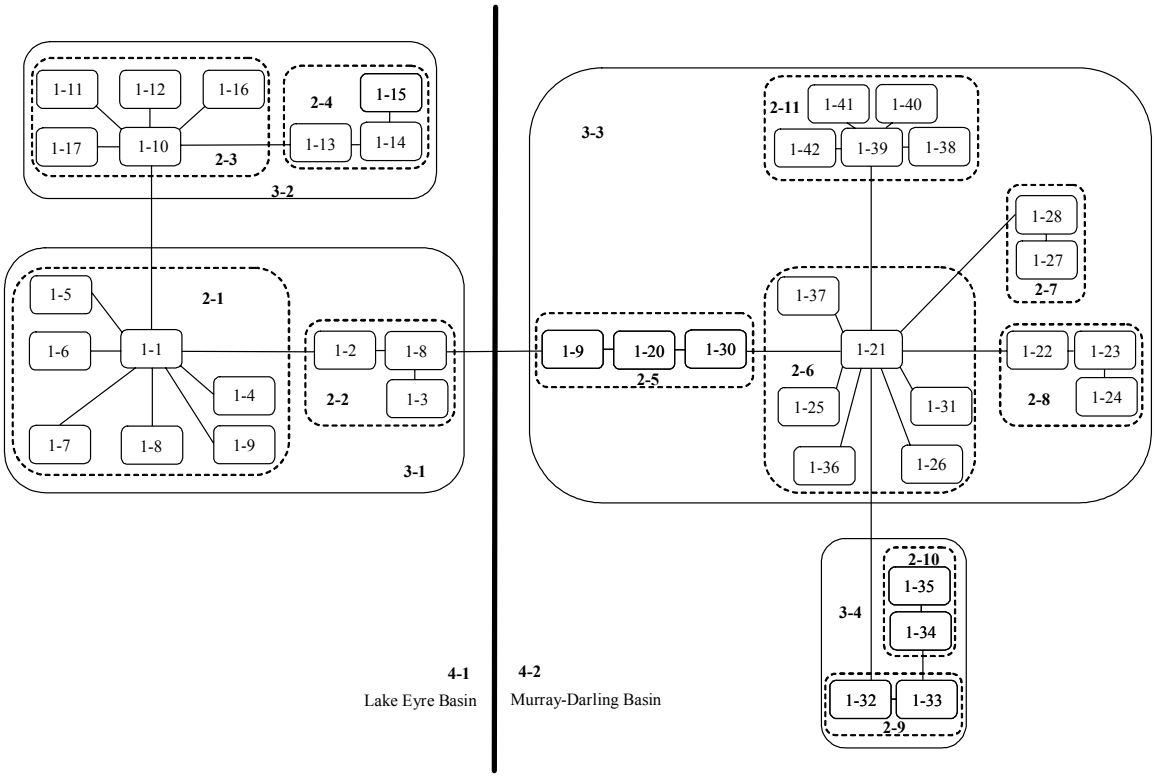


Figure 4.8 2-, 3- and 4-step clade nesting for the hypothesised network with 95% plausible set of haplotype connections. Clade step delineation is shown next to each nesting box. A solid branch between haplotype 1- 98, is representative of a single base pair change in the mitochondrial sequence.



Contingency analyses indicated no significant associations between nested clades and geography (P>0.05) for any 1-step clades, except 1-36 (Table 4.7). The Inference Key (Templeton, 1998) indicated a pattern of geographic variation in clade 2-11 (from the Murray-Darling basin), consistent with restricted gene flow with isolation by distance. For clades 2-1 and 2-3 (from the Diamantina/Georgina and Cooper respectively) range expansion with long distance colonisation was suggested. Contiguous range expansion was indicated for clade 3-3 (from the Murray-Darling). Range expansion with long distance colonisation was suggested for clade 4-1 (from the Lake Eyre basin) and restricted gene flow with isolation by distance was indicated for clade 4-2 (from the Murray-Darling). The outcome for the total cladogram was inconclusive (Figure 4.8 and Table 4.7).

Table 4.7 Nested clade analysis showing clade (D_C), nested (D_N) and interior-tip clade (I-T) distances. Only clades with significant permutational chi-square probabilities for geographic structure have been included in the table. Significantly small or large values for 'D_C' , 'D_N' and 'I-T' are in bold and indicated by 'S' and 'L', respectively. 'Inference Key ⇒ Conclusion' refers to update version of Inference Key (Templeton, 1998), whereby numbers indicate the steps and conclusions are abbreviated as follow: 'RE LDC' range expansion, long distance colonisation; 'RGF IBD' restricted gene flow with isolation by distance; 'C RE' contiguous range expansion.

Nesting	Location	No.	D_c	D_n	χ^2-P	Inference Key ⇒ Conclusion
	One-step	**Clades**				
1-36	Tip	84	**0**^{S}	**74.13**^{S}	0.026	1→2→3→5→6
	Tip	85	53.21	**275.21**^{L}		⇒ Inconclusive
	Tip	86	0	152.31		
	Interior	87	105.02	124.44		
		I-T	89.81	-18.30		
	Two-step	**Clades**				
2-1	Interior	1-1	**149.80**^{S}	**166.69**^{S}	0	1→2→11→12→13
	Tip	1-4	0	154.90		⇒ RE LDC
	Tip	1-5	**0**^{S}	209.88		(Diamantina/Georgina)
	Tip	1-6	**296.84**^{L}	**294.58**^{L}		
	Tip	1-7	143.81	166.68		
	Tip	1-8	170.35	172.20		
	Tip	1-9	**0**^{S}	**0**^{L}		
		I-T	13.11	**-64.86**^{S}		
2-3	Interior	1-10	**76.33**^{S}	**81.06**^{S}	0	1→2→11→12→13
	Tip	1-11	0	71.76		⇒ RE LDC
	Tip	1-12	**21.67**^{S}	**30.32**^{S}		(Cooper)
	Tip	1-16	44.60	63.77		
	Tip	1-17	**0**^{S}	**237.86**^{L}		
		I-T	62.80	**-53.38**^{S}		
2-6	Interior	1-21	148.59	152.54	0.02	1→11→17
	Tip	1-25	0	23.55		⇒ Inconclusive
	Tip	1-37	78.54	191.52		
	Tip	1-31	114.05	164.93		
	Tip	1-26	146.67	186.52		
	Tip	1-36	138.94	**239.64**^{L}		
		I-T	24.30	**-51.52**^{S}		
2-11	Interior	1-39	**201.30**^{L}	196.32	0.001	1→2→3→4
	Tip	1-40	**0**^{S}	**104.44**^{S}		⇒ RGF IBD
	Tip	1-41	57.24	216.20		(Murray-Darling)
	Tip	1-42	0	239.45		
	Tip	1-38	0	251.72		
		I-T	**192.71**^{L}	**61.00**^{L}		
	Three-step	**Clades**				
3-3	Interior	2-5	**40.20**^{S}	239.25	0.001	1→2→11→12
	Interior	2-6	**172.69**^{S}	210.63		⇒ C RE
	Tip	2-7	**99.19**^{S}	239.07		(Murray-Darling)
	Tip	2-8	182.73	206.74		
	Tip	2-11	**189.54**^{S}	208.56		
		I-T	-15.36	0.8484		
	Four-step	**Clades**				
4-1	Tip	3-2	**100.15**^{S}	**186.37**^{S}	0	1→2→11→12→13
	Interior	3-1	**194.29**^{S}	**223.92**^{L}		⇒ RE LDC
		I-T	**94.14**^{L}	**37.55**^{L}		(Lake Eyre)
4-2	Interior	3-3	212.95	212.73	0.003	1→2→3→4
	Tip	3-4	**0**^{S}	**157.95**^{S}		⇒ RGF IBD
		I-T	**212.95**^{L}	**54.78**^{L}		(Murray-Darling)
	Total	**Cladogram**				
	Tip	4-1	**211.09**^{S}	**338.42**^{S}	0	1→ Inconclusive
	Tip	4-2	**211.62**^{S}	**396.16**^{L}		

4.3.4 Population divergence times: MDIV

One population of approximately 10-15 sequences was randomly chosen from each catchment and I estimated values for θ, *M*, *T*, and *TMRCA* for 28 sets of pairwise comparisons. Estimates based on time since the populations of the two major basins have diverged (*T*) ranged between roughly 500 000 and 1 000 000 years ago. It was estimated that within the Lake Eyre basin, populations in different catchments became separated between 50 000 and 200 000 years ago, whereas within the Murray-Darling appeared to have been separated more recently, between 20 000 and 90 000 years ago (Table 5).

Estimates of gene flow based on migration rate (*M*) between populations from the two major basins and between those within the Lake Eyre basin were very low. Estimates of *M* between populations from the Murray-Darling were low to moderate ranging from a value of 1.24 (between the Border Rivers and the Paroo catchments) to 23.25 (between the Warrego and the Balonne-Condamine catchments) (Table 4.8).

Table 4.8. Pairwise of female effective population sizes ($\theta = N_{ef}u$), migration rates ($M = N_{ef}m$), time since divergence (T) and time to the most common ancestor ($TMRCA$) based on analysis of mtDNA COI using the MDIV program. Values with the highest likelihood scores for θ and M. Estimates for T and $TMRCA$ are calculated in years before present ($_{YBP}$).

	Balonne-Condamine	Border Rivers	Warrego	Paroo	Bulloo	Cooper	Diamantina
Border Rivers	θ= 3.2 *M*= 12.55 *T*= 20000 *TMRCA*=270000$_{YBP}$	–					
Warrego	θ= 2.3 *M*= 23.25 *T*= 40000 *TMRCA*= 788000 $_{YBP}$	θ= 3.8 *M*= 19.4 *T*= 35000 *TMRCA*= 520000 $_{YBP}$	–				
Paroo	θ= 2.22 *M*= 2.02 *T*= 20000 *TMRCA*= 765000 $_{YBP}$	θ= 5.03 *M*= 1.24 *T*= 90000 *TMRCA*= 436600 $_{YBP}$	θ= 3.57 *M*= 2.9 *T*= 50000 *TMRCA*= 588000 $_{YBP}$	–			
Bulloo	θ= 1.83 *M*= 0.01 *T*= 750000 *TMRCA*= 997000 $_{YBP}$	θ= 4.18 *M*= 0.01 *T*= 700000 *TMRCA*= 901600 $_{YBP}$	θ= 2.2 *M*= 0.1 *T*= 750000 *TMRCA*= 828300 $_{YBP}$	θ= 3.54 *M*= 0.1 *T*= 500000 *TMRCA*= 670000 $_{YBP}$	–		
Cooper	θ= 2.77 *M*= 0.01 *T*= 800000 *TMRCA*= 867000 $_{YBP}$	θ= 4.73 *M*= 0.02 *T*= 800000 *TMRCA*= 721000 $_{YBP}$	θ= 2.69 *M*= 0.12 *T*= 750000 *TMRCA*= 753000 $_{YBP}$	θ= 3.85 *M*= 0.1 *T*= 500000 *TMRCA*= 633000 $_{YBP}$	θ= 2.59 *M*= 0.2 *T*= 200000 *TMRCA*= 293000 $_{YBP}$	–	
Diamantina	θ= 2.34 *M*= 0.01 *T*= 750000 *TMRCA*= 903000 $_{YBP}$	θ= 4.61 *M*= 0.03 *T*= 800000 *TMRCA*= 863000 $_{YBP}$	θ= 2.62 *M*= 0.1 *T*= 800000 *TMRCA*= 833000 $_{YBP}$	θ= 3.46 *M*= 0.1 *T*= 500000 *TMRCA*= 633000 $_{YBP}$	θ= 2.28 *M*= 0.3 *T*= 200000 *TMRCA*= 273300 $_{YBP}$	θ= 3 *M*= 0.2 *T*= 260000 *TMRCA*= 270000 $_{YBP}$	–
Georgina	θ= 1.64 *M*= 0.09 *T*= 1000000 *TMRCA*= 1110000 $_{YBP}$	θ= 3.69 *M*= 0.01 *T*= 850000 *TMRCA*= 943000 $_{YBP}$	θ= 2.1 *M*= 0.09 *T*= 1000000 *TMRCA*= 1090000 $_{YBP}$	θ= 3.13 *M*= 0.1 *T*= 750000 *TMRCA*= 778000 $_{YBP}$	θ= 1.62 *M*= 0.3 *T*= 100000 *TMRCA*= 330000 $_{YBP}$	θ= 2.33 *M*= 0.2 *T*= 221000 *TMRCA*= 303000 $_{YBP}$	θ= 2.42 *M*= 0.4 *T*= 50400 *TMRCA*= 270000 $_{YBP}$

4.4 Discussion

4.4.1 Genetic population structure in *Macrobrachium australiense*

Until recently, the geographical extent of colonisation events of aquatic fauna associated with the quasi-periodic flow regime in Western Queensland was thought to be extensive due to the intense floods and the low topographic relief of the region (Gibling 1998). However, recent studies using molecular markers have provided novel insight into patterns of dispersal and connectivity of freshwater invertebrates within this region. In particular, a study of allozyme differentiation among populations of *M. australiense* in Western Queensland indicated that this species conformed to the hierarchical model of gene flow, that is population subdivision was significant between but not within catchments (Cook *et al*., 2002). The observed structure in the allozyme study was interpreted as the result of absent dispersal between catchments, due to the insurmountable barriers represented by terrestrial habitat, and high dispersal within catchments, facilitated through episodic flooding.

The analyses in the current study, using partial sequence from the mitochondrial CO-I gene, confirm these results to the extent that there was more genetic differentiation between than within catchments within the Lake Eyre and the Murray-Darling basins. The high level of genetic structure detected in the mitochondrial data suggested not only extremely limited dispersal between catchments, as in Cook *et al.* (2002), but also among waterholes within catchments. The apparent discrepancy between nuclear and mitochondrial markers is probably explained by the fact that the effects of genetic drift are amplified in the mitochondrial genome due to its smaller effective population size. Mitochondrial DNA is more sensitive to limited gene flow than nuclear markers resulting in an elevated rate of differentiation between isolated populations. Therefore, due to the increased sensitivity of mitochondrial genes to any limited gene flow between populations, the species displayed high levels of genetic variation in populations throughout its distribution (i.e. between and also within catchments). The possibility of gender-biased gene flow was excluded because there are no records in the literature of different behaviour for male and female prawns.

The significant population structure by catchments within basins implied by the analyses of molecular variance and the signature of isolation by distance provide evidence that populations of *M. australiense* are at equilibrium between gene flow and genetic drift. When a panmictic population expands its range and occupies new habitats that then become subdivided, a number of generations must pass for genetic differences to accumulate among newly established demes. An approximation for the time required for a haploid population to go half way to equilibrium can be expressed as: $t_{½} = (\ln 2)/(2m + 1/N_e)$, where m is probability of migration per generation, N_e the effective population size and $t_{½}$ is the time in generations (Crow and Aoki, 1984). It is possible that migration rates in *M. australiense* have been high enough for the population to have reached equilibrium between gene flow and genetic drift.

Habitat fragmentation may reduce exchange of individuals between adjacent populations, leading to an increase of differentiation through random drift. The Western Queensland region is a fragmented habitat for aquatic species. During dry periods, water becomes restricted to waterholes and rivers turn into dryland, resulting in a formidable system of barriers for freshwater fauna. It is only during occasional large floods that water fills up the channel systems and provides the opportunity for aquatic dispersal to neighbouring waterholes (Knighton and Nanson, 1997). It was suggested that the main consequence of this mosaic of aquatic landscape in Western Queensland is the subdivision of *M. australiense* into many arrays of localised populations with very limited gene flow at all spatial scales (i.e. between and within catchments). Although the Georgina, the Diamantina and the Cooper catchments are connected via Lake Eyre during episodic flood events, there seems to be very little gene flow between them. Lake Eyre probably does not serve as a connection between these catchments because for much of the time it is a vast dry salt pan and flood waters that fill it become highly saline (during the flood of the 1950 the salinity of Lake was recorded at 39 ‰; Bayly and Williams, 1973). This elevated salinity is likely to act as a barrier to dispersal for *M. australiense* because the species can not tolerate salinity greater than 25 ‰ (Denne, 1968).

The likely interpretation of the contemporary genetic subdivision of *M. australiense* is that: a) despite the potential for long distance dispersal of the species, the episodic floods do not ensure a good network of connectivity among waterholes and/or b) during

floods the freshwater prawns do not maximise their potential for dispersal and tend to remain in the waterhole of origin. Freshwater organisms can accumulate during floods into refugial areas of low hydraulic disturbance, where they can avoid harsh physical conditions, thus, avoiding dispersal (Winterbottom *et al.*, 1997).

4.4.2 Evidence for some historical gene flow between populations

The data presented in this chapter support the hypothesis that climate oscillations of the Pleistocene may be directly correlated with historically higher levels of connectivity between rivers throughout Western Queensland. It was suggested that extensive floods and temporary internal lakes, associated with the interglacial phases of the Pleistocene, promoted movement of freshwater prawns across catchment boundaries, resulting in population range expansions within basins. No evidence of historical gene flow was detected between the Lake Eyre and Murray-darling basins, resulting in the high genetic divergence between the corresponding populations.

Apart from the phylogeographic break between Lake Eyre and Murray-Darling basins, there was a maximum of four base pair differences between any two adjacent nodes in the haplotype network (Figure 4.4). Similarly to *Notopala sublineata* haplotype network, *M. australiense* can be described as intermediate between category III and V, where all the haplotypes are closely related, separated by no more than four step changes, yet populations have a relatively high number of 'private' haplotypes, thus localised geographically. It was suggested that this phylogeographic pattern described case scenarios where contemporary levels of gene flow are low between populations, yet were somehow historically connected.

Isolation and subsequent genetic divergence can occur in populations of obligate freshwater species inhabiting separate drainages because the terrestrial habitat can represent an insurmountable barrier, precluding dispersal and connectivity among drainages. Populations of *M. australiense* clustered into two main clades, corresponding geographically to the two major drainage basins: the Lake Eyre and the Murray-Darling, which appeared to have diverged between 500 000 and 1 000 000 years ago. This major division between these drainages was also detected by Cook *et al.* (2002) based on allozyme data. It was estimated that populations of *M. australiense* in different

catchments of the Lake Eyre basin became separated much earlier than populations of the Murray-Darling basin (Table 4.8). In this study it was suggested that the later separation of population within the Murray-Darling was due to a combined effect of geographic distance and hydrology patterns. The channels of the Murray-Darling are organised in a dendritic structure whereby smaller tributary systems feed into increasingly fewer but longer channels (Williams 1980; Young, 1999). These channels are less ephemeral than those in the Lake Eyre basin, therefore increasing the potential for connectivity between them.

It appeared that *M. australiense* has undergone sudden population expansions around 56 000 and 90 000 years ago within the Murray-Darling and the Lake Eyre respectively, according to the mismatch distribution analysis. The time of the population range expansion events roughly correspond to the period of the greatest fluvial activity and a period of maximum precipitation, when greatly expanded lakes dominated much of the Australian continent (approximately between 110 000 and 80 000 years ago), as indicated by palaeogeological records indicate (Kershaw and Nanson, 1993; Alley, 1998; Croke *et al.*, 1999). The extensive floods and temporary internal lakes apparently resulted in high levels of connectivity between waterholes, promoting movements of freshwater prawns within and among catchments, possibly resulting in the historical population range expansions.

The data presented in this study, also suggested that *M. australiense* colonised the Bulloo and the Cooper from the Georgina/Diamantina, possibly during the Pleistocene. The structure of the haplotype network indicated that the Cooper clade was derived from the Georgina/Diamantina clade, which is internal within the network and connected to the Murray-Darling group. Similarly all the haplotypes of the Bulloo cluster with the Georgina/Diamantina clade.

In conclusion, this study indicated that the population of *M. australiense* is highly subdivided (i.e. between and within catchments), suggesting that the dispersal abilities of the species play a secondary role in determining levels of gene flow between subpopulations. Also, there is evidence that levels of gene flow between and within catchments might have been higher in the past than at the present time. It was suggested

that possibly episodic dispersal of *M. australiense* across catchment boundaries was possible during the Pleistocene.

Chapter 5
Effects of persistence time of waterholes on genetic diversity in two freshwater species in Western Queensland

5.1 Introduction

As previously discussed in this study, arid and semiarid river systems in Western Queensland are characterized by the unpredictable and highly variable nature of their hydrological regimes as a result of the episodic nature of rain events in the region. These dryland rivers typically experience episodic floods and extremely low or no flow periods (Puckridge *et al*., 1998, 2000). During low or no flow periods, water persists only in relatively wide and deep sections of the river channels, which are called 'waterholes' (Knighton & Nanson 1994). These isolated waterholes serve as refugia for aquatic species during protracted intervals between floods (Walker *et al.*, 1995; Sheldon *et al.*, 2002). These waterholes undergo continuous water loss by evaporation between episodic floods (Hamilton *et al.*, in review). Evaporation rates are highly variable between waterholes, in part due to their different sizes, the effective fetch for wind action, the height and width of riparian vegetation, and the degree of channel incision below the levees, which affect wind-induced turbulence at the water surface, exposure of the water surface to solar heating, and convective air circulation above the water surface (Brutsaert, 1982). Thus, some waterholes may experience larger loss of water than others and may, even if rarely, dry out completely (i.e. Yalungah waterhole, in October 2002, Hamilton *et al.*, in review), reducing population sizes or ensuring the extinction of local populations of freshwater species without a resistant stage in their life cycle, respectively.

Colonisation and recolonisation of the waterholes by aquatic fauna are most likely to happen during flood events, when water is flowing in the channels. Patterns of dispersal of freshwater species, associated with these flood events, are still largely unexplored. Chapters 3 and 4 strongly suggest that levels of dispersal are equally low on a large scale (i.e. between catchments and between sites within catchments) for aquatic species

with both good and limited dispersal abilities (Hughes and Hillyer, 2003, Hughes *et al.*, in review; Carini and Hughes, in review; Carini and Hughes in press). In this chapter, patterns of dispersal for both the study, species have been investigated on a much smaller scale than in chapter 3 and 4. In this part of the study I try to determine if movements of freshwater species are limited also between waterholes within sites. If so, it is possible that aquatic species find refuge in waterholes when river channels are dry.

In this chapter the effects of habitat fragmentation and the periodical local extinction/recolonisation regime, driven by the highly unpredictable flow regime, on the genetic variability of aquatic populations inhabiting Western Queensland were investigated. I tested two hypotheses. First, if populations of obligate freshwater organisms inhabiting less persistent waterholes are more likely to experience periodic bottlenecks than those inhabiting more persistent ones, they would be expected to have lower levels of genetic diversity. Small populations will tend to lose genetic variation by drift more rapidly than larger populations (Wright, 1931; Nei *et al.*, 1975; Gilpin and Soulé, 1986; Lande, 1995). Second, if populations inhabiting less persistent waterholes periodically undergo local extinction with subsequent recolonisation, there should be higher levels of genetic differentiation among them, due to the founder effects, than among those populations inhabiting more persistent waterholes (Whitlock and McCauley, 1990).

These hypotheses were tested for both the species targeted in this study: *N. sublineata* and *M. australiense* throughout waterholes in Western Queensland.
Allozymes, microsatellites and mitochondrial markers were used to compare levels of genetic diversity and patterns of genetic differentiation in *N. sublineata*, whereas only mitochondrial DNA sequences were used for *M. australiense*, because Cook *et al.* (2002) already indicated in their study that allozyme markers had limited variation within catchments. Due to the haploid nature and the maternal mode of inheritance, mtDNA has a fourfold smaller effective population size than nuclear markers, increasing its sensitivity to any limited gene flow between populations (Birky *et al*., 1989). Therefore, any effects of reductions in effective population are more easily detected using mtDNA markers than allozymes.

5.2 Specific Materials and methods

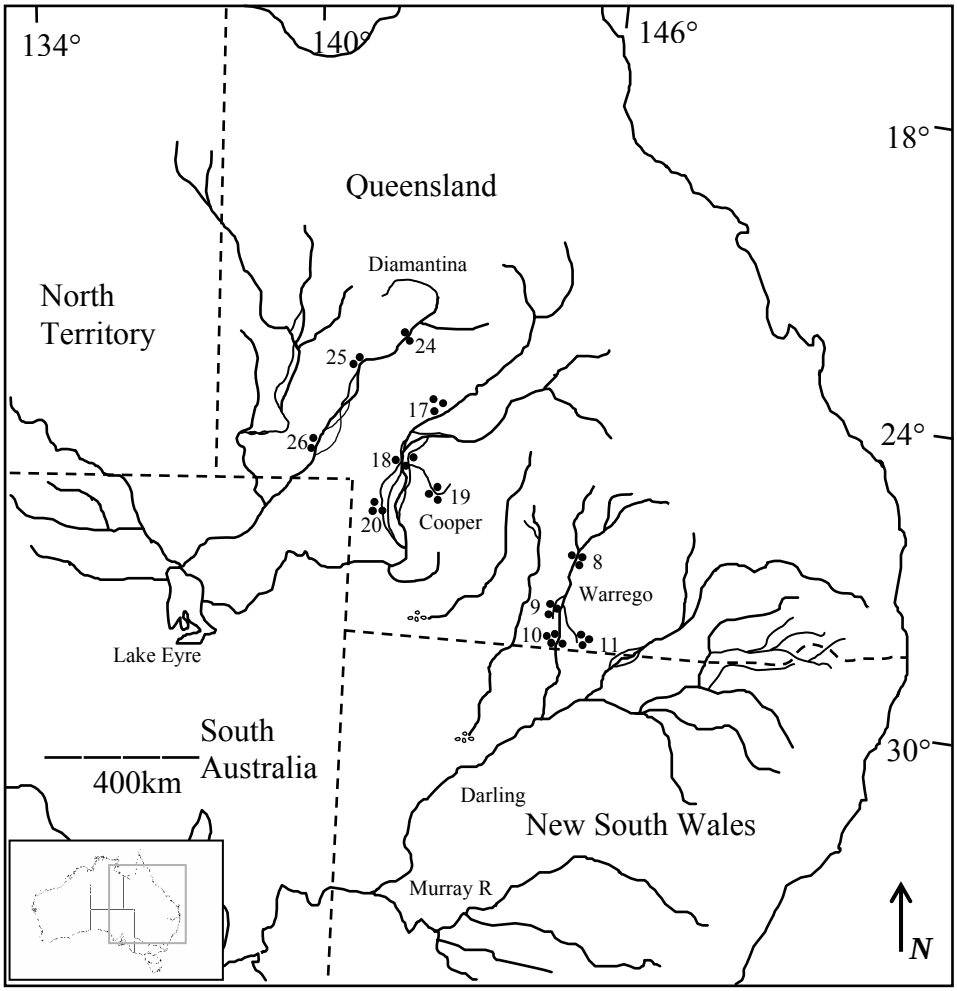
Sampling sites were within the Lake Eyre basin and the northern regions of the Murray-Darling basin (Figure 5.1). In this study, large river pools that rarely dry out were called 'more persistent waterholes', whereas river pools that experience recurrent significant losses of water and potentially could dry out completely, were called 'less persistent waterholes'. To assign the less persistent or more persistent nature to waterholes at each site, I referred to the local authorities, such as the Department of Natural Resources, from the local National Parks authorities, and farmers within the study area. Moreover, in a recent study, fractional water loss by evaporation in waterholes was estimated from the increase in conservative ion (Na^+ and Cl^-) concentration and independently from evaporative fractionation of oxygen and hydrogen isotopes in water. Persistence time of waterholes (defined as the time to evaporate to 10% of bankfull volume) was estimated, thus it was possible to discern between more persistent and less persistent river pools. These data were only available for waterholes within the Cooper catchment (Hamilton *et al.*, in review) (Table 5.1).

N. sublineata and *M. australiense* were sampled from one more persistent and a number of less persistent waterholes in each designated site, throughout Western Queensland major catchments (Figure 5.1 and Table 5.1). *N. sublineata* was sampled from five sites (four within the Cooper and one within the Diamantina catchments), represented by twelve waterholes: five more persistent and seven less persistent. For microsatellite analysis, only three sites were used, all within the Cooper catchment (Table 5.1) because I had to drop two sites. I could not include the Noonbah site, because the more persistent waterhole (Top) had not a suitable sample size for microsatellite analysis, and the Diamantina site due to difficulties in laboratory procedures. *M. australiense* was sampled from ten sites (three within the Warrego, four within the Cooper and three within the Diamantina catchments), represented by thirty-one waterholes: eleven more persistent and twenty less persistent (Table 5.1). In this section of the study, it was not possible to use samples from all the sites used in the previous chapters (3 and 4) for the analyses, because in order to test the above hypotheses it was necessary to have a minimum two waterholes at each site (one more persistent and one less persistent).

Table 5.1 Waterholes and their sample sizes. More persistent waterholes within sites are indicated by a star. M-D: Murray-Darling and LE: Lake Eyre basin.

					M. australiense	***N. sublineata***	***N. sublineata***	***N. sublineata***
	Catchment	**Site**	**Waterhole**	**Persistence in time (months)**	**mtDNA sample size**	**mtDNA sample size**	**Allozymes sample size**	**Microsatellites sample size**
M-D	Warrego	Quilberry 8	Quilberry*	-	7	-	-	-
M-D	Warrego	Quilberry 8	SP	-	7	-	-	-
M-D	Warrego	Quilberry 8	SP lagoon	-	7	-	-	-
M-D	Warrego	Glencoe 9	Glencoe*	-	8	-	-	-
M-D	Warrego	Glencoe 9	Wogganorah	-	7	-	-	-
M-D	Warrego	Glencoe 9	Key	-	7	-	-	-
M-D	Warrego	Thurulgoona 10	Thurulgoona*	-	8	-	-	-
M-D	Warrego	Thurulgoona 10	Noorama	-	6	-	-	-
M-D	Warrego	Thurulgoona 10	Disco	-	6	-	-	-
M-D	Warrego	Binya 11	Binya*	-	7	-	-	-
M-D	Warrego	Binya 11	Red	-	6	-	-	-
M-D	Warrego	Binya 11	Mirage	-	6	-	-	-
M-D	Warrego	Binya 11	Tinnenburra	-	6	-	-	-
LE	Cooper	Noonbah 17	Top*	22.8	7	8	~ 10	-
LE	Cooper	Noonbah 17	Pelican	14.8	7	8	~ 54	-
LE	Cooper	Noonbah 17	Waterloo	16.5	7	11	~ 66	-
LE	Cooper	Windorah 18	Murken*	17.5	7	11	~ 56	60
LE	Cooper	Windorah 18	Mayfield	13.6	7	-	-	-
LE	Cooper	Windorah 18	Shedh	10.6	7	10	~ 42	43
LE	Cooper	Springfield 19	Homestead*	-	7	16	~ 44	59
LE	Cooper	Springfield 19	Warranee	12.8	7	11	~ 54	-
LE	Cooper	Springfield 19	One Mile	8.6	7	12	~ 63	59
LE	Cooper	Tanbar 20	Tanbar*	19.1	7	13	~ 54	56
LE	Cooper	Tanbar 20	Yappi	7.5	7	-	-	-
LE	Cooper	Tanbar 20	Yalungah	6	7	15	~ 33	31
LE	Diamantina	Verdun Valley 24	Turkey*	-	10	-	-	-
LE	Diamantina	Verdun Valley 24	Fish	-	10	-	-	-
LE	Diamantina	Diamantina Lakes 25	Stock*	-	10	17	~ 55	-
LE	Diamantina	Diamantina Lakes 25	Middle	-	10	13	~ 37	-

Figure 5.1 Study area and sampling sites for *M. australiense* and *N. sublineata.* Site names are: 8. Quilberry: **Quilberry**, SP and SP lagoon; 9. Glencoe: **Glencoe**, Woggannorah and Key; 10. Thurulgoona**: Thurulgoona**, Disco and Noorama; 11. Binya**: Binya**, Red, Mirage and Tinnenburra; 17. Noonbah: **Top**, Pelican and Waterloo; 18. Windorah: **Murken**, Mayfield and Shedh; 19. Springfield: **Homestead** Warranee and One Mile; 20. Tanbar: **Tanbar**, Yappi and Yalungah; 24. Verdun Valley: **Turkey** and Fish; 25. Diamantina Lakes: **Stock** and Middle; 26. Makara: **Monkira** and Niggah (more persistent waterholes are indicated in bold characters within each grouping).



Allozyme, mitochondrial DNA and microsatellite procedures and data analysis adopted in this section have been outlined in chapter 2, general methods. Although only two microsatellite loci were developed, they have been incorporated in this chapter. The results were analysed in conjunction with allozymes and mitochondrial DNA.

Mitochondrial DNA procedures and data analysis adopted in this section have been outlined in chapter 2, general methods.

5.2.1 Comparison of genetic diversity between more persistent and less persistent waterholes

To test the hypothesis that populations of obligate freshwater organisms inhabiting less persistent waterholes have lower levels of genetic diversity than those inhabiting more persistent waterholes at a site, estimates of expected heterozygosity were compared (from allozyme) for *N. sublineata* as well as haplotype and nucleotide diversity measures (from mitochondrial data) for both species between less persistent and more persistent waterholes, using the Wilcoxon signed-rank test features in the package SPSS version 11.0. Where there were data for more than one less persistent waterhole at a site, I used the mean value. In addition, a non parametric correlation test was performed (one-tailed-test) in the package statview version 5.12 for Macintosh to investigate if there was a positive relationship between genetic diversity estimates and persistence of waterholes within the Cooper catchment.

5.2.2 Genetic structure among waterholes

For both species, pairwise Φ_{ST} (for mtDNA), F_{ST} (for allozymes, microsatellites and mtDNA) were estimated between populations of more persistent and less persistent waterholes at each site (refer to Figure 5.1 and Table 5.1), using the package Arlequin version 2.0 (Schneider *et al.*, 2000). To test the hypothesis that populations inhabiting less persistent waterholes were more genetically differentiated than populations inhabiting more persistent waterholes, Φ_{ST} and F_{ST} were calculated between all less persistent and all more persistent waterholes.

5.3 Results

5.3.1 *N. sublineata* and *M. australiense*

Allele frequency estimates for seven polymorphic allozyme loci are displayed in Appendices I, II and III. Allele frequency estimates for two polymorphic microsatellite loci are displayed in Appendix IV.

In this chapter, sequence information for 145 *N. sublineata* from 12 waterholes was used for the analyses. No significant deviations from neutrality were detected in the

mitochondrial data (D= -1.17, *P*>0.12). 457 base pairs of unambiguous sequence were generated, describing 29 polymorphic sites (6.34%), resulting in 36 haplotypes. Sequence information for 232 *M. australiense* from 29 waterholes was used for the analyses. No significant deviations from neutrality were detected in the mitochondrial data (D= -0.28, *P*=0.42; Tajima, 1989). 505 base pairs of unambiguous sequence were generated, describing 52 polymorphic sites (10.3%), resulting in 54 haplotypes.

5.3.2 Comparison of genetic diversity between more persistent and less persistent waterholes

Overall mitochondrial haplotype and nucleotide diversity estimates were high for both *N. sublineata* (0.89+/-0.02 and 0.013+/-0.007, respectively) and *M. australiense* (0.92+/-0.01 and 0.016+/-0.008, respectively) (Figure 5.2, 5.3). For populations of *N. sublineata*, allozyme data generally showed low levels of genetic variability, in terms of the mean number of alleles per locus and expected heterozygosity, whereas high levels were recorded for microsatellites (Figures 5.4 and 5.5).

There were no significant differences in genetic diversity between more persistent and less persistent waterholes for either of the species (Table 5.2). In fact, contrary to expectations, in many cases genetic diversity appeared to be equal or higher in less persistent than in more persistent waterholes (Figures 5.2, 5.3, 5.4 and 5.5).

No correlation was detected between genetic diversity of *M. australiense* and water persistence time in waterholes within the Cooper catchment (haplotype diversity: Rho = 0.152, Z = -0.008 *P*>0.05; nucleotide diversity Rho = -0.02, Z = 0.043 *P*>0.05). On the contrary, a strong correlation was detected between genetic diversity of *N. sublineata* and water persistence time in waterholes within the Cooper catchment at allozyme loci (expected heterozygosity: Rho = 0.72 Z = 2.03 *P*<0.05) and mitochondrial sequences ((haplotype diversity: Rho = 0.628, Z = 1.8 *P*<0.05; nucleotide diversity Rho = 0.728, Z = 2.06 *P*<0.05). Only the allozyme mean number of alleles per locus did not show a significant relationship with persistence time (Rho = 0.2 Z = 0.57 *P*>0.05).

Figure 5.2 Estimates of haplotype and nuclear diversity for *M. australiense*. Black columns indicate the more persistent waterhole in each grouping within each site. For site number and grouping details, refer to Figure 5.1. *P*>0.05 calculated with Wilcoxon signed-rank test (Table 5.2).

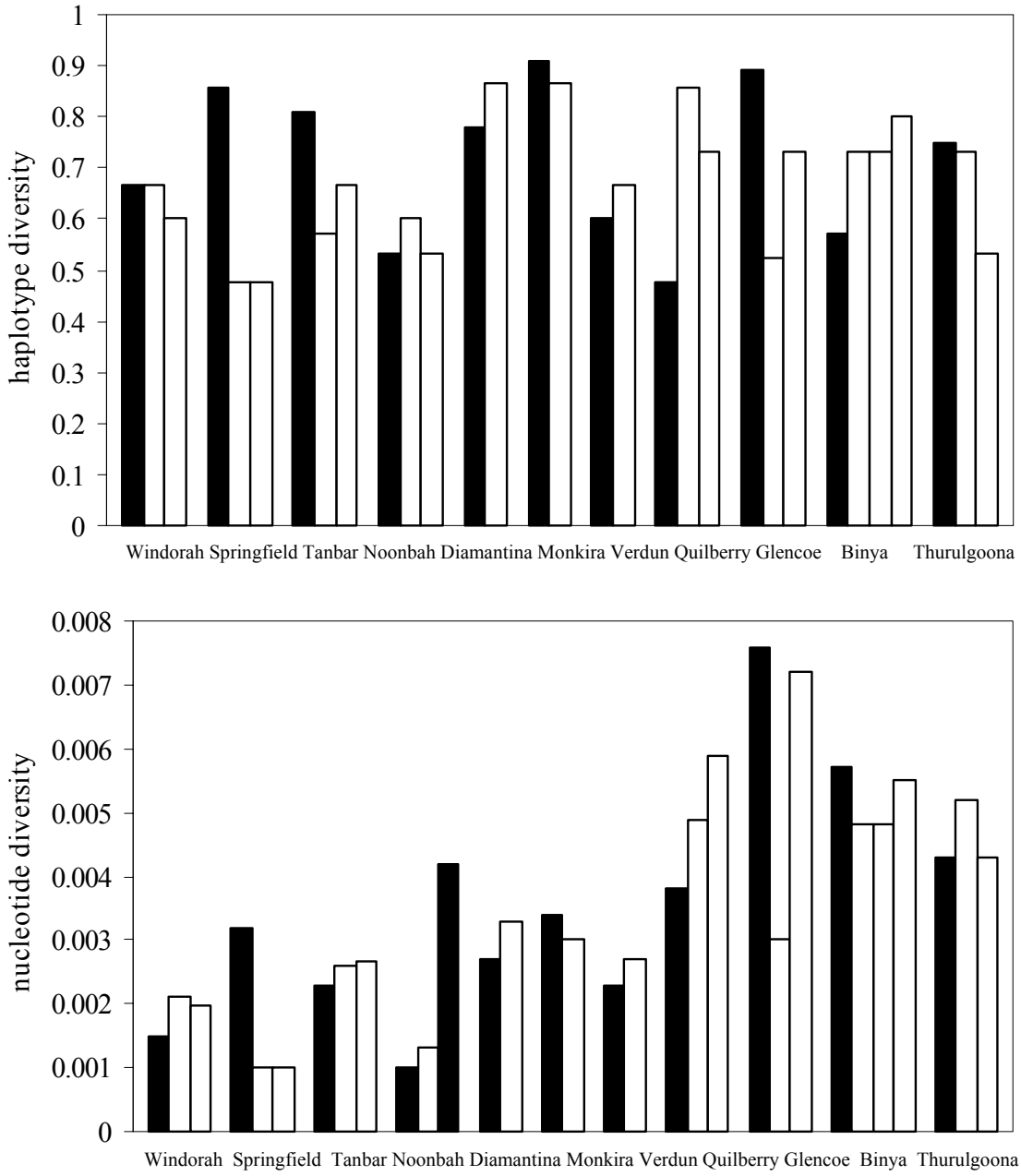


Figure 5.3 Estimates of haplotype and nuclear diversity for *N. sublineata*. Black columns indicate the more persistent waterhole in each grouping within each site. For site number and grouping details, refer to Figure 5.1. $P>0.05$ calculated with Wilcoxon signed-rank test (Table 5.2). Estimates from Homestead and One Mile waterholes (Springfield site) had zero values.

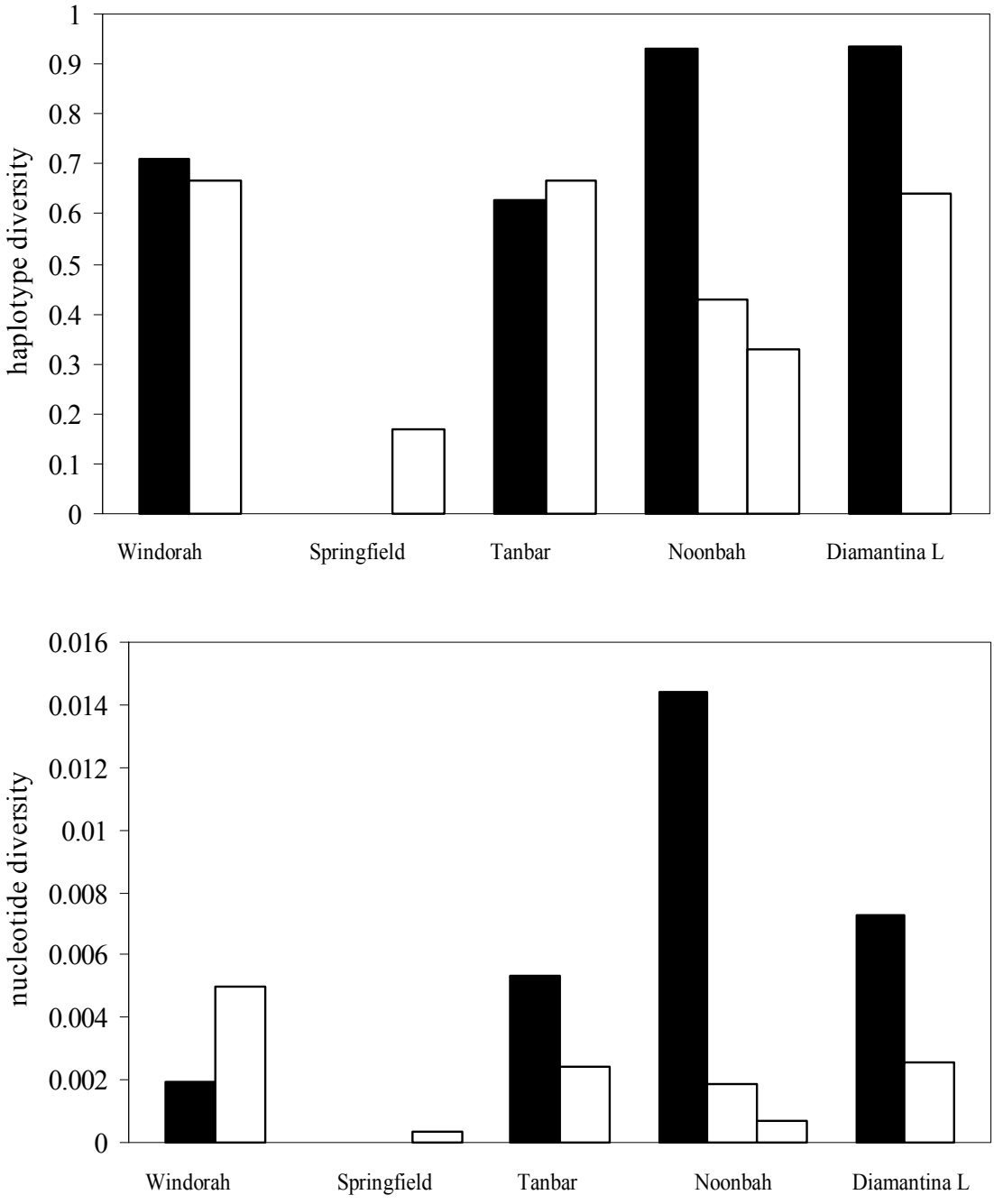


Figure 5.4 Estimates of mean number of alleles per locus and expected heterozygosity for *N. sublineata* from allozymes data. Black columns indicate the more persistent waterhole in each grouping within each site. For site number and grouping details, refer to Figure 5.1. $P>0.05$ calculated with Wilcoxon signed-rank test (Table 5.2).

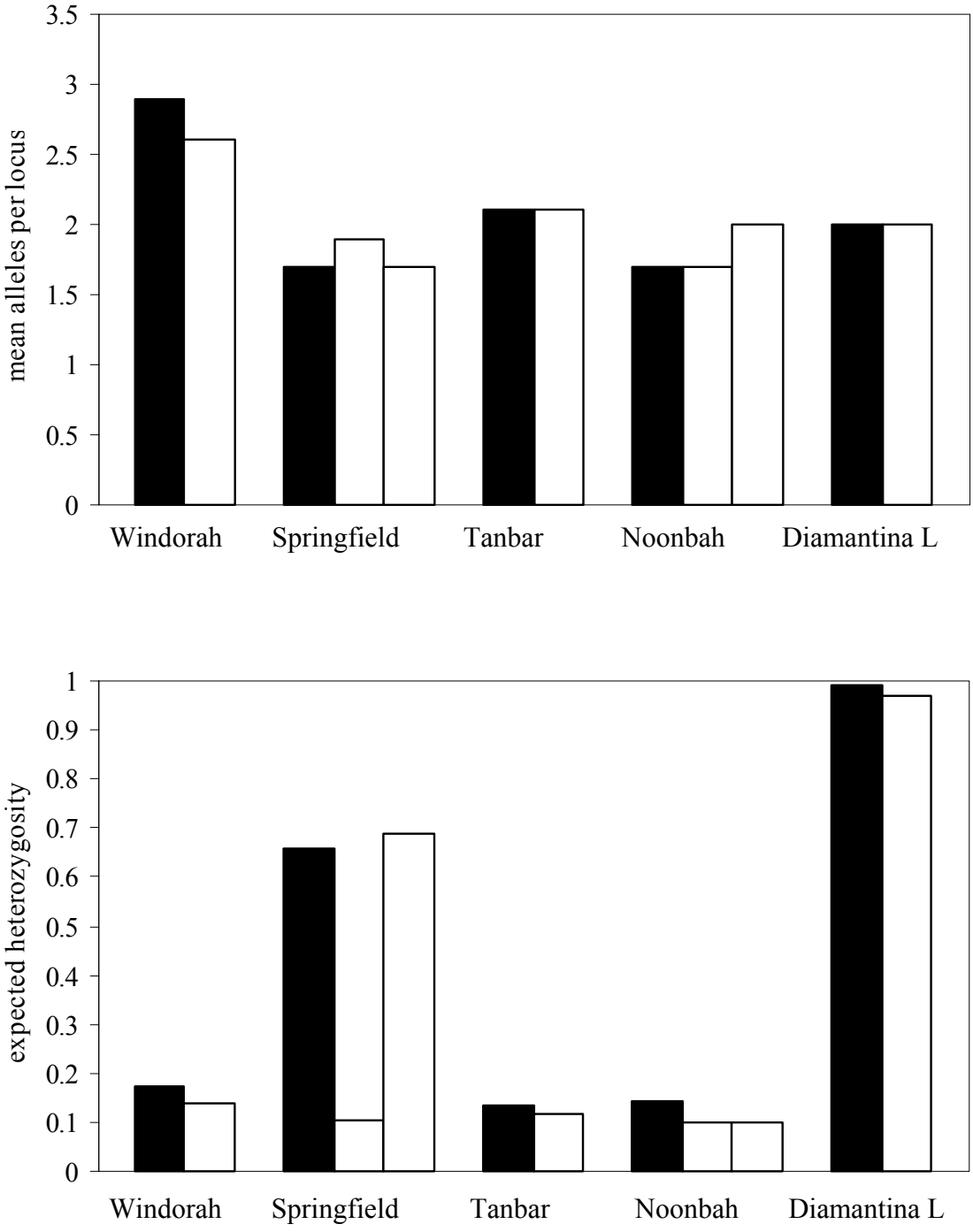


Figure 5.5 Estimates of mean number of alleles per locus and expected heterozygosity for *N. sublineata* from microsatellite data, at the locus N3.1 and N3.3. Black columns indicate the more persistent waterhole in each grouping within each site. For site number and grouping details, refer to Figure 5.1. $P>0.05$ calculated with Wilcoxon signed-rank test (Table 5.2).

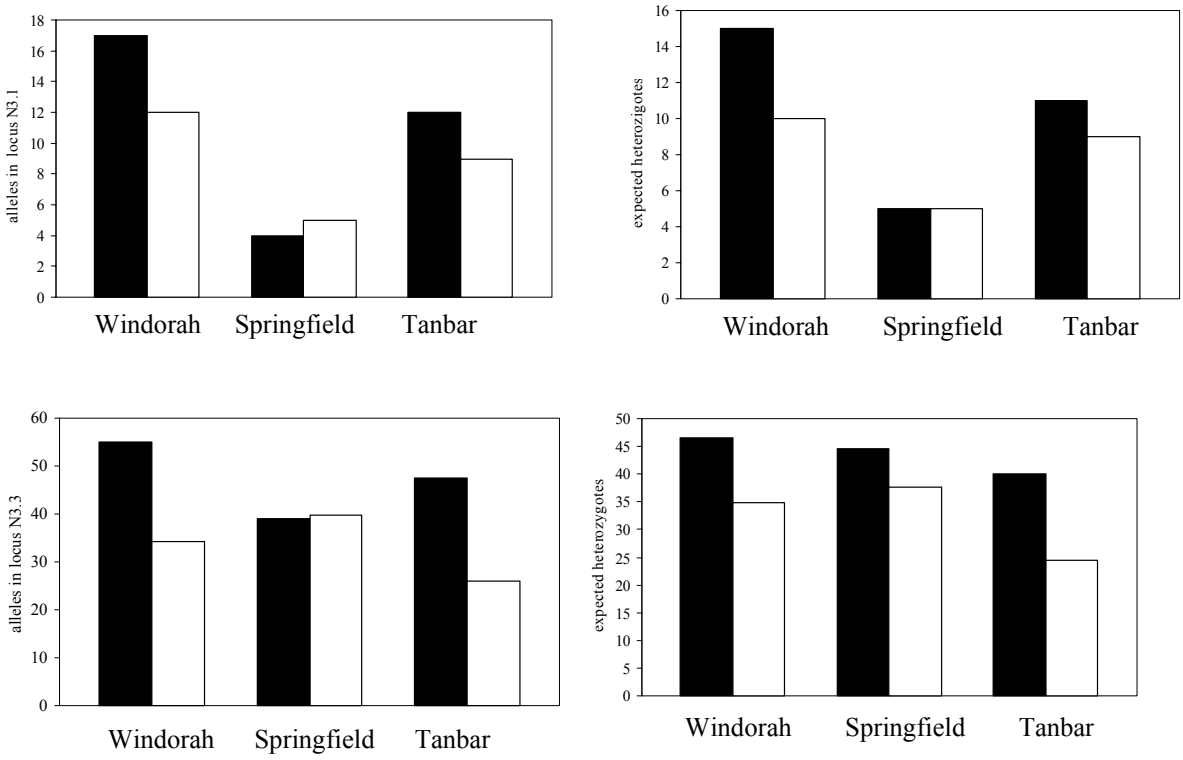


Table 5.2 Significance (*P*) of the Wilcoxon Signed Ranks test statistic, which compare genetic diversity estimates between more persistent and less persistent waterholes for a) *M. australiense* and b) *N. sublineata*.

		mtDNA		Allozymes		Microsatellites	
	Catchments	Nucleotide diversity	haplotype diversity	H_e	Mean allele per locus	allele per locus	H_e
	Cooper	0.593	0.285	-	-	-	-
a) *N. sublineata*	Diamantina	0.655	0.18	-	-	-	-
	C-D	-	-	0.5	0.785	0.61	0.45
	Cooper	0.465	0.655	-	-	-	-
b) *M. australiense*	Diamantina	0.285	0.3	-	-	-	-
	Warrego	0.715	0.72				

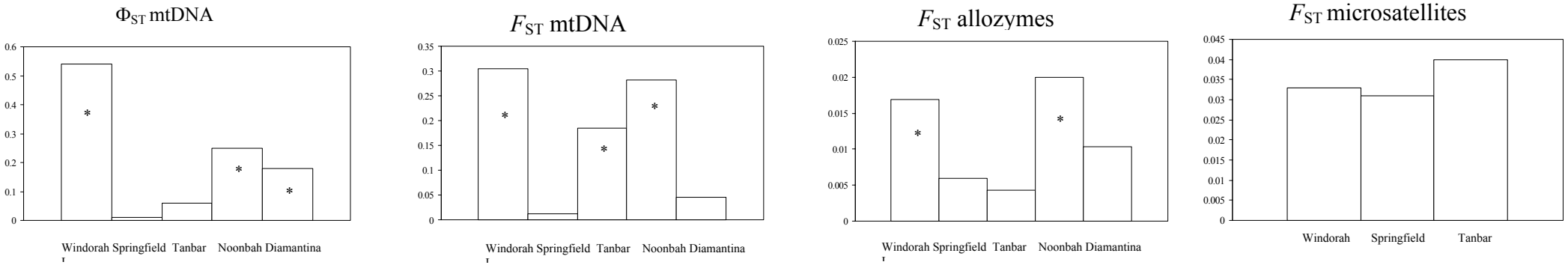
5.3.3 Genetic structure among waterholes

Estimates of pairwise Φ-statistics and *F*-statistics for mtDNA and *F*-statistics for allozymes between populations inhabiting more persistent and less persistent waterholes within sites (refer to Figure 5.1 and Table 5.1), varied considerably among sites for *N. sublineata* (Figure 5.6). On the basis of pairwise Φ-statistics for mtDNA, populations were significantly differentiated in three out of the six sites: Windorah, Noonbah and Diamantina Lakes (Figure 5.6); on the basis of pairwise *F*-statistics for mtDNA, in three out of the six sites: Windorah, Noonbah and Tanbar and on the basis of pairwise *F*-statistics for allozymes, in two out of the six sites: Windorah and Noonbah (Figure 5.6). In contrast, on the basis of pairwise *F*-statistics for microsatellites none of the populations of *N. sublineata* were significant within sites (Windorah, Tanbar and Springfield) (Figure 5.6). None of the populations of *M. australiense* were significantly differentiated at a site based on either Φ-statistics or *F*-statistics (Figure 5.6).

The hypothesis that populations inhabiting less persistent waterholes were genetically more differentiated (higher estimates) than populations inhabiting more persistent waterholes was tested by comparing Φ_{ST}, F_{ST}, allozyme F_{ST} and microsatellite F_{ST} estimated between all less persistent and all more persistent waterholes belonging to the same basin for both *N. sublineata* and *M. australiense* (Figure 5.7). Contrary to expectations, Φ_{ST}, F_{ST} for mtDNA and allozyme F_{ST} estimates were all highly significant ($P<0.001$) and also indicated that there was generally less differentiation among populations in less persistent than among populations in more persistent waterholes (Figure 5.7). Microsatellite F_{ST} estimates were low and not significant, probably due to the fact that there were only three close geographically sites (within the central region of the Cooper catchment).

Figure 5.6 a) *N. sublineata,* estimates of pairwise Φ-statistics and *F*-statistics for mtDNA data, *F*-statistics for allozymes and *F*-statistics for microsatellites between populations of more persistent and less persistent waterholes at each site. b) *M. australiense* estimates of pairwise Φ-statistics and *F*-statistics. *P<0.05

a) *N. sublineata*



b) *M. australiense*

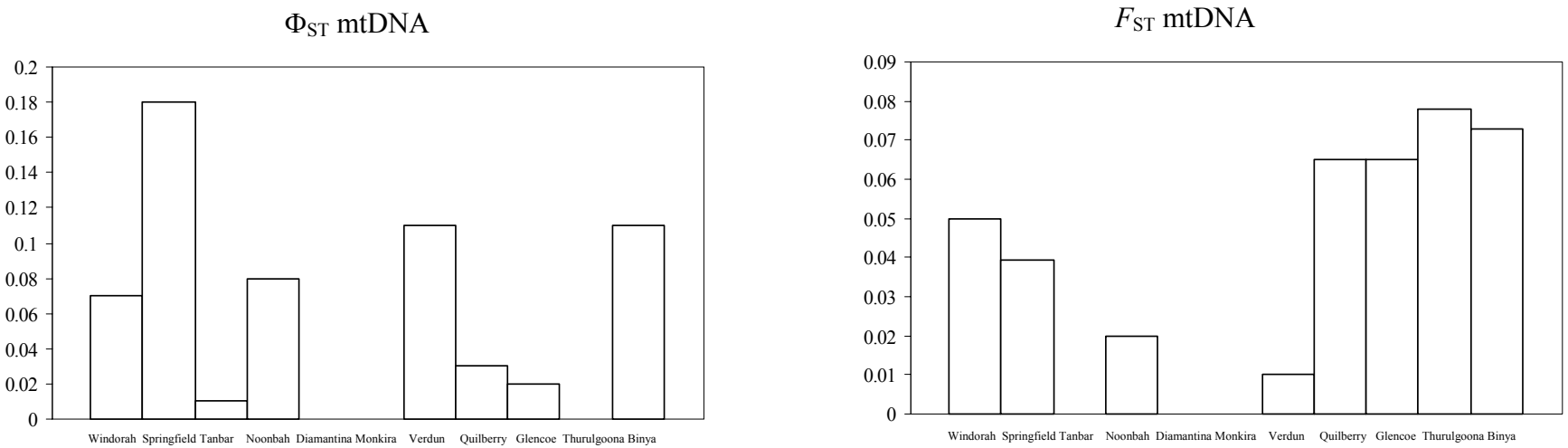
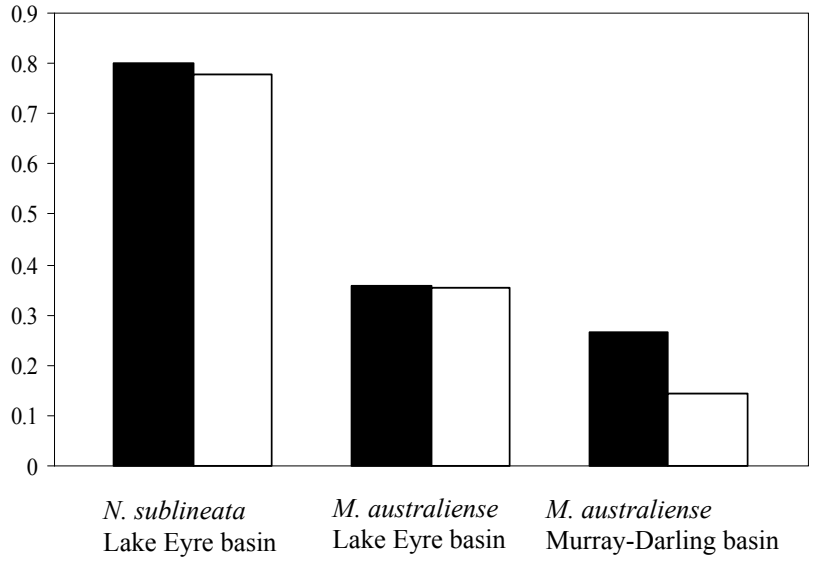
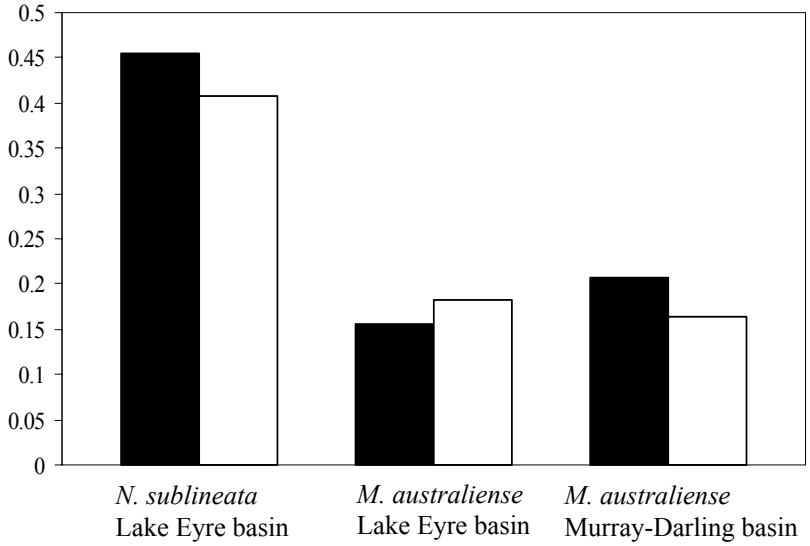


Figure 5.7 Estimates of a) Φ_{ST} for mtDNA, b) F_{ST} for mtDNA and c) F_{ST} for allozymes among all more persistent and all less persistent waterholes in *N. sublineata* and *M. australiense.*

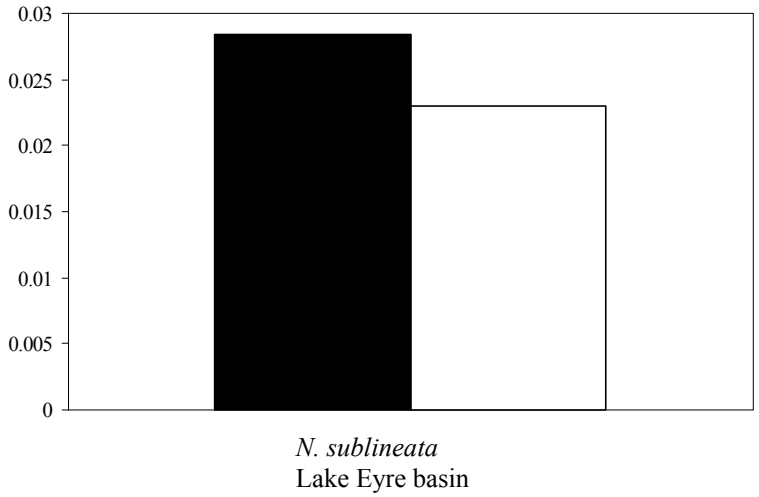
a) Φ_{ST}



b) F_{ST}



c) F_{ST}



5.4 Discussion

Generally, overall levels of genetic diversity are expected to be lower in fragmented than in continuously distributed populations, because genetic drift has an increased effect when local effective population size and/or migration rate are reduced (Wright, 1931). Genetic studies of some species conform to these expectations (e.g. some species of freshwater mussels, Machordom *et al.*, 2003) while many others do not, presenting overall high levels of genetic variability, as for the terrestrial snail, *Helix aspersa* (Arnaud *et al.*, 2003) and the freshwater mussel *Discus macclintocki* (Ross, 1999). In both these studies, it was suggested that unexpectedly high levels of genetic diversity were due to the particular population structure of the species (metapopulations). For example, terrestrial snails tend to be structured into numerous demes, with low migration rate between them (metapopulation structure). As stressed by Peacock and Ray (2001), the loss of genetic variability is not a general scheme in metapopulations with high turnover.

In Western Queensland, due to the unpredictable nature of their habitat, populations of obligate freshwater species inhabiting less persistent waterholes are likely to go through bottleneck events more often than populations inhabiting more persistent ones, and have their effective population size reduced each time. Thus, I expected lower levels of genetic diversity in populations inhabiting less persistent waterholes than in those in more persistent waterholes. Contrary to these predictions, the observed levels of genetic diversity in both *N. sublineata* and *M. australiense* were not low, particularly for mitochondrial DNA, which showed high nucleotide and haplotype diversity in both more persistent and less persistent waterholes. According to the Wilcoxon test, there was no tendency for genetic diversity to be lower in less persistent than in more persistent waterholes. Thus, genetic diversity of populations inhabiting less persistent waterholes does not seem to have been reduced by frequent bottleneck events. On the contrary, the non parametric correlation test on waterholes in the Cooper catchment detected a positive correlation between water persistence time and genetic diversity in *N. sublineata* but not in *M. australiense.* To explain this apparent discrepancy, I suggest that there was a small correlation between persistence time of waterholes and genetic diversity, but perhaps the Wilcoxon test was not sensitive enough to reveal it, probably due to the general high estimates of genetic diversity. Because no data on persistence time were available for the Homestead waterhole (more persistent waterhole at

Springfield site, Figure 5.1), it was not included in the non parametric correlation test. The absence of this site may have affected the analysis for *N. sublineata*, since its genetic diversity is generally lower than the less persistent waterholes at this site (Figures 5.3 and 5.4). Also, I suggest that no correlation between persistence time of waterholes and genetic diversity was detected in *M. australiense* by either of the tests because of its large population size (Lee and Fielder, 1983; Sheldon and Walker, 1998). Thus, any changes in genetic diversity were less detectable than in *N. sublineata*.

Population turnover can have a profound effect on the genetic differentiation of local populations (Slatkin, 1977). In the presence of frequent extinctions and recolonisations, a combination of the founder effect and genetic drift will enhance genetic differentiation among local populations (Whitlock and McCauley, 1990). It was hypothesised that this would have been the exact scenario for populations of *N. sublineata* and *M. australiense* inhabiting more persistent and less persistent waterholes. During severe droughts, water in less persistent waterholes may be extremely reduced or even dry out completely, resulting in large reductions or the extinction of obligate freshwater invertebrate populations. At the following flood event, they would be recolonised, most likely, by a small number of individuals from the nearest more persistent waterholes, assuming that apparently long distance colonisation events are very rare within this region (Chapters 3 and 4). If the less persistent waterholes are colonised by a small number of individuals, it was predicted that each of them would contain a different subset of genotypes available from the more persistent waterholes. Therefore, higher genetic differentiation among populations inhabiting less persistent were expected than among more persistent waterholes. However, I found highly significant genetic differentiation among populations from both less persistent and more persistent waterholes. This indicates that not only populations from less persistent but also those from more persistent waterholes were very dissimilar genetically. There was no difference in levels of genetic differentiation between populations of either *N. sublineata* or *M. australiense* inhabiting less persistent and more persistent waterholes. If anything, the reverse trend seemed to occur: there was slightly more variation among populations from more persistent than from less persistent waterholes. Therefore, there was no evidence that founder effects were greater in less persistent than in more persistent waterholes.

Levels of genetic differentiation among populations of *N. sublineata* within the same site were quite variable. At some sites, populations from different waterholes were very

similar, while at others there were significant differences. Populations inhabiting waterholes at the Windorah and at the Noonbah sites, both within the Cooper catchment, were genetically differentiated for both allozyme and mitochondrial markers. This suggests that at some sites, some less persistent waterholes may have been more isolated from their neighbouring more persistent waterhole than others. Perhaps these sites are less likely to be connected even during intense flood events than others. Information from a local resident (Angus Emmot of Noonbah station) indicates that Top is connected to Pelican and Waterloo waterholes only by high river flow events (*In*: Hamilton *et al.*, in review). At most sites however, it appears that some flow pulses are sufficient to connect populations and keep allele frequencies similar. Whatever the mechanisms are that isolate some populations of *N. sublineata*, they do not seem to have the same effect on *M. australiense*, possibly because of its better dispersal abilities.

In chapter 3 and 4 populations of *N. sublineata* and *M. australiense* were described as highly genetically structured on a large spatial scale. It was therefore suggested that movements of aquatic species were limited between catchments and between sites within catchments. Yet, the data presented in this chapter strongly support the possibility that some movements of freshwater species occur on a very small scale, as indicated by the non-significant levels of genetic differentiation between waterholes within the sites.

These results imply that freshwater organisms tend to utilise waterholes as refugia during dry periods but that during flood events can be washed out into neighbouring waterholes (within the same site). The possibility of freshwater invertebrates being transported for long distances it appears to be very low. This is because firstly major flood events that connect catchments are rare and second because the survivorship of individuals washed out into floodplains and open water has been suggested to be very low. The temperature of the water in the floodplains is very high and also the chance to be predated by other animals is high, since it is harder to find hiding places.

The acceptance of aquatic movements on a very small scale would explain the results of this chapter. Populations retain high variation as long as individual populations remain viable (Gilpin, 1991; Whitlock and McCauley, 1990). Populations of freshwater invertebrates and their genes moving across waterholes within sites might assist in

maintaining high genetic diversity. Also, multiple independent colonisation events, from several neighbouring more persistent waterholes, may reintroduce a large proportion of the total genetic variation, reducing the founder effect in less persistent waterholes.

These exchanges of individuals between waterholes within sites during floods apparently help to maintain overall high levels of genetic diversity as well as low levels of genetic differentiation within sites, in populations of *N. sublineata* and *M. australiense* inhabiting both less persistent and more persistent waterholes.

Chapter 6
Comparative phylogeography

6.1 Introduction

The current distribution of genetic variation in populations is shaped by both historical and contemporary processes (Templeton *et al.*, 1995). Comparative analyses assist in discriminating between the contrasting effects of contemporary dispersal versus vicariant events. In highly structured populations, dispersal is a stochastic event and it is not always expected to result in similar patterns for co-distributed species, whereas a common set of historical vicariant events is expected to geographically structure a group of sympatric species in a similar way (Avise, 2000). To date, several studies have found evidence of phylogeographic congruence among co-distributed taxa. For example, frequent climate change during the late Pleistocene has led to the concept that many high latitude plant and animal communities in Europe and North America are of a very recent origin, having been established by northward range expansion from southern refugia following the end of the last glacial maximum, 14-20kyr ago (Williams *et al.*, 1998; Hewitt 1996, 1999, Pielou, 1991). Evidence from fossil, pollen and other physical and biological residues strongly support the hypothesis that many species have undergone the same dramatic latitudinal shift in response to climate changes (Whitlock and Barlein, 1997, Williams *et al.*, 1998). In the case of organisms with poor palaeontologic records, comparative phylogeography has been a powerful tool to infer changes in the geographical distribution of the species (Hewitt 1996, 1999). The observed patterns of phylogeographic congruence suggest that the taxa examined have a long-standing geographical association with one another and have attained a common pattern of geographical subdivision as a result of being subject to the same environmental history. Thus, it may be expected that the ability to investigate the processes underlying the current distribution of genetic variation in freshwater populations in Western Queensland river systems is considerably increased through the comparative study of several species. This is particularly important when differentiating between historical and contemporary processes.

In Western Queensland, contemporary dispersal between river systems is expected to be very unlikely at all spatial scales (i.e. between and within catchments), due to the inhospitable nature of the dividing watersheds. Thus, it was hypothesised that all species (with good or poor dispersal abilities) should display high levels of population structure in this region. It has also been hypothesised that hydrological changes of the Pleistocene might have had a major influence on the distribution of genetic variation, and phylogeographic patterns of co-distributed freshwater species should exhibit analogous spatial patterns of evolutionary subdivision. Ongoing extensive flood events could change patterns of connectivity between rivers, thereby providing dispersal pathways for freshwater species otherwise isolated. The receding floodwaters would then leave populations effectively isolated in their respective river systems. Rare long-distance dispersal in strongly structured populations would generate a pattern of high levels of differentiation between populations, but with some sharing of haplotypes or clades among distant river systems. This particular scenario might be expected also as a consequence of historical isolating events, such as changes in hydrological flood regimes and levels of connectivity between rivers, where all co-distributed species should be affected equally. The temporal information provided by mtDNA analyses can assist in determining the extent to which the population structure of Western Queensland results from recurrent processes, such as ongoing gene flow, versus historical processes, such as range expansion (Avise, 2000).

There are several methods for making direct comparisons between the phylogenetic structures of independent taxa. Phylogenies of many species may result in regionally monophyletic clades due to biogeographic barriers separating them (Avise, 1989). In this case, it would be appropriate to construct a general area cladogram based on congruence among the area cladograms derived from phylogenetic analyses of the multiple co-distributed taxa (Sullivan *et al.*, 2000). Congruity of phylogeographic structure among independent taxa to infer common histories can then be quantitatively tested through a number of methods (e.g. Page, 1994). Gene-tree lineages for both the study species are separated by shallow mutational distances, with little regional monophyly. Thus, a qualitative approach to the analyses was considered more suitable.

6.2 Concordance in phylogeographic patterns

Phylogeographic patterns of the two species targeted in this study can be compared with three freshwater invertebrates inhabiting the same region. One of the species is the freshwater crayfish, *Cherax destructor*. Population structure and phylogeographic patterns of this species have been investigated by Hughes and Hillyer in a recent study (2003). *C. destructor* has a similar geographic distribution to *M. australiense* because, unlike *N. sublineata*, it still inhabits river systems of the Murray-Darling basin. Unlike *M. australiense*, the species lacks a planktonic larval stage (Lake and Sokol, 1986) and is strongly territorial, although it is thought to be capable of some terrestrial dispersal (Riek, 1959). The second and third species are the freshwater mussels, *Velesunio* species A and C. Phylogenetic and phylogeographic patterns of this species have been studied recently by Hughes *et al.* (Baker *et al.*, 2003). Species A inhabits waterholes within the Lake Eyre basin (Georgina, Diamantina and Cooper catchments), whereas species C appears to be widespread in the Murray-Darling basin, the Cooper and the Bulloo catchments. *Velesunio* species are thought to be very long-lived (Walker *et al.*, 2001) and are largely sedentary. Their only real opportunity for dispersal occurs in the larval stage (Pennack, 1978), which attaches to gills or fins of fishes.

In this chapter, comparisons of the phylogeographic patterns of the above described species have been made depending on their data availability.

6.2.1 Comparing phylogeographic patterns across freshwater species in Western Queensland

In Western Queensland, a large phylogenetic break was detected between the Lake Eyre and Murray-Darling basins by Cook *et al.* (2000), Hughes and Hillyer (2003) and Hughes *et al.* (in review) in populations of *M. australiense*, *C. destructor* and a freshwater mussel, *Velesunio* C, respectively. In this study, the same phylogeographic break was detected for one of the target species, *M. australiense*. No data are available for *N. sublineata*, since this species is apparently extinct from the Murray-Darling channels. In all cases, no haplotypes were shared between populations belonging to the two basins. Thus, it was suggested that gene flow has not occurred recently between the two basins for quite sometime, resulting in the high genetic divergence between the corresponding populations. Based on data for *Velesunio* species (Hughes *et al.*, in review) and *M. australiense* (Carini and Hughes, in review), it was estimated that

populations from the Lake Eyre and the Murray-Darling have become separated around 500 000 and 1 000 000 years ago.

Haplotype networks of *N. sublineata*, *M. australiense*, *C. destructor*, *Velesunio* A and C, displayed most haplotypes as closely related, yet localised geographically, generally by catchments (Figures 6.1, 6.2, 6.3, 6.4 and 6.5) and few common haplotypes that are widespread across catchment boundaries but no haplotypes are shared between the Lake Eyre and the Murray-Darling basins. Interestingly, the number of internal haplotypes shared across distant catchments was higher within the Murray-Darling than the Lake Eyre basin.

Figure 6.1 Genealogy network showing catchment's distribution for each haplotype and depicting the number of base pair difference between them for *N. sublineata*, circle size of each haplotype is proportional to overall frequency in the sample.

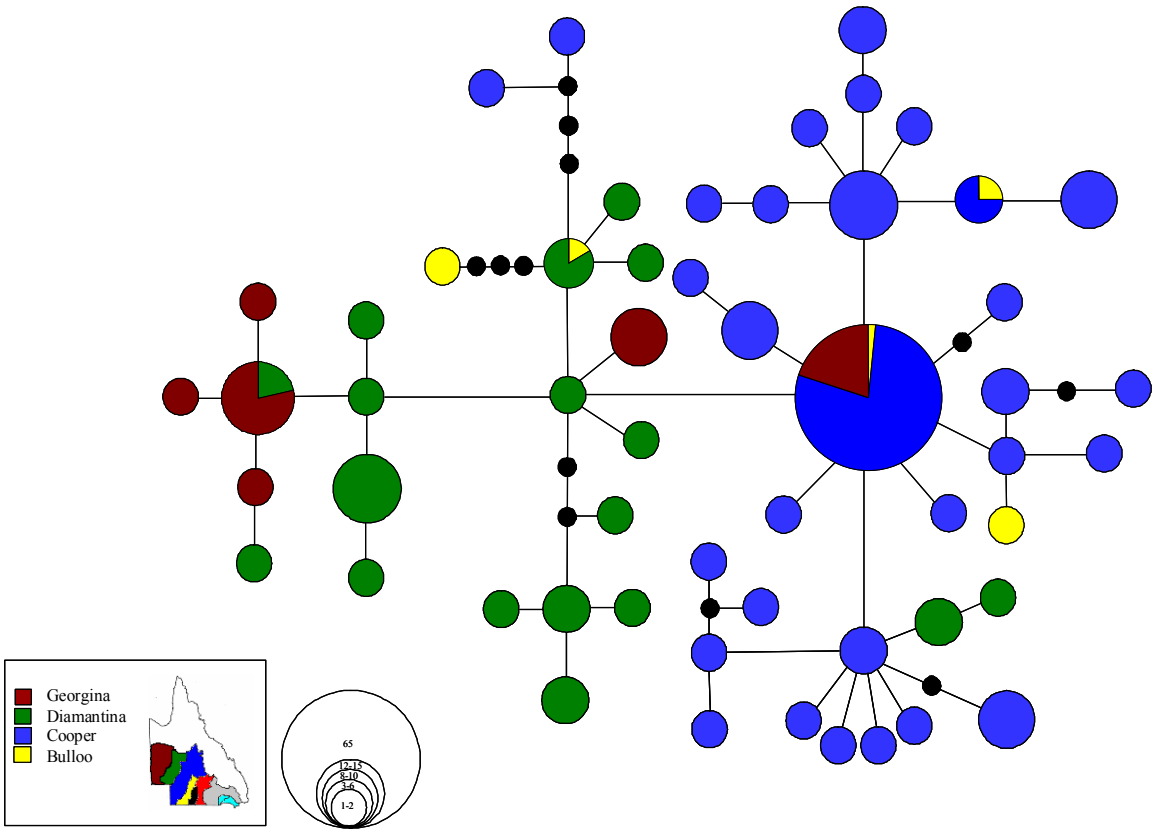


Figure 6.2 Genealogy network showing catchment's distribution for each haplotype and depicting the number of base pair difference between them for *Velesunio* species A (Hughes *et al*., in review).

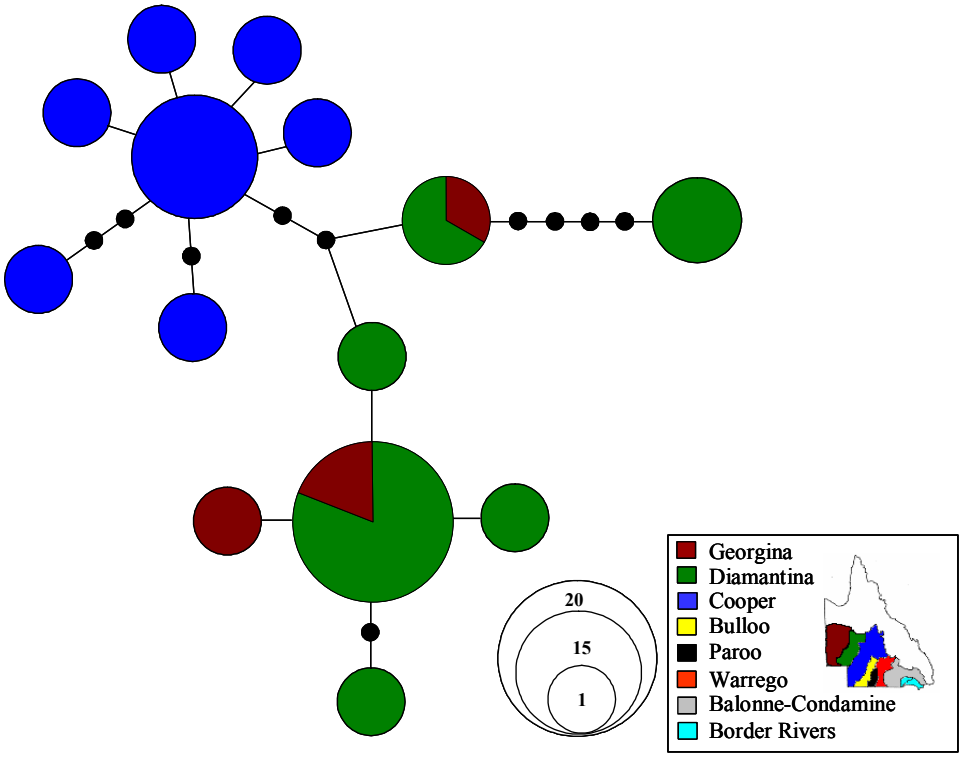


Figure 6.3 Genealogy network showing catchment's distribution for each haplotype and depicting the number of base pair difference between them for *C. destructor* (Hughes and Hillyer, 2003).

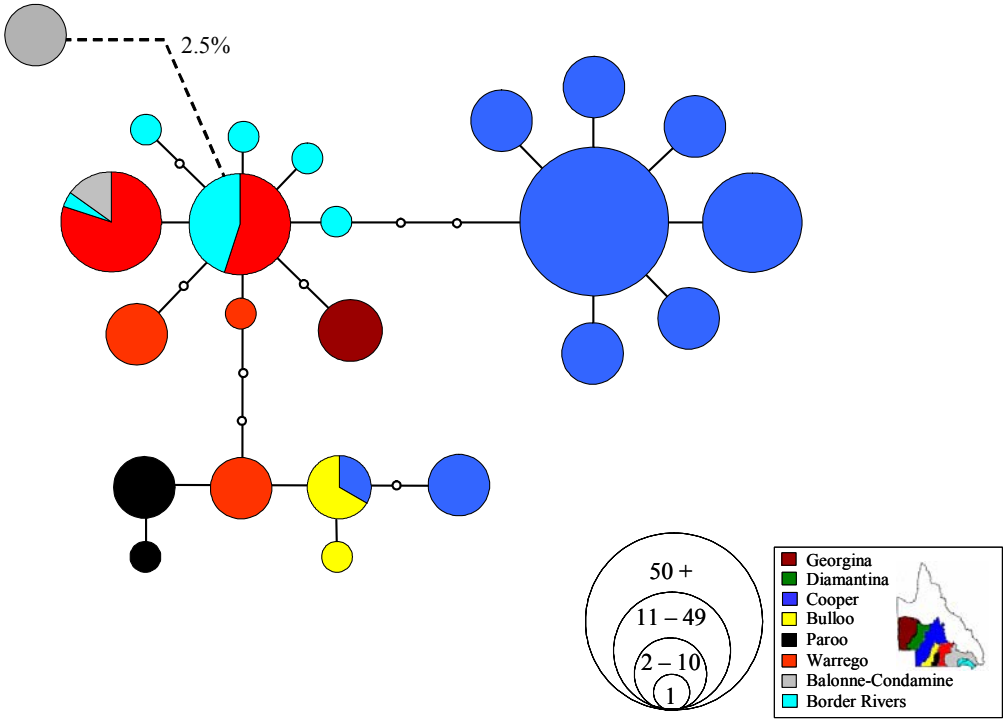


Figure 6.4 Genealogy network showing catchment's distribution for each haplotype and depicting the number of base pair difference between them for *M. australiense.*

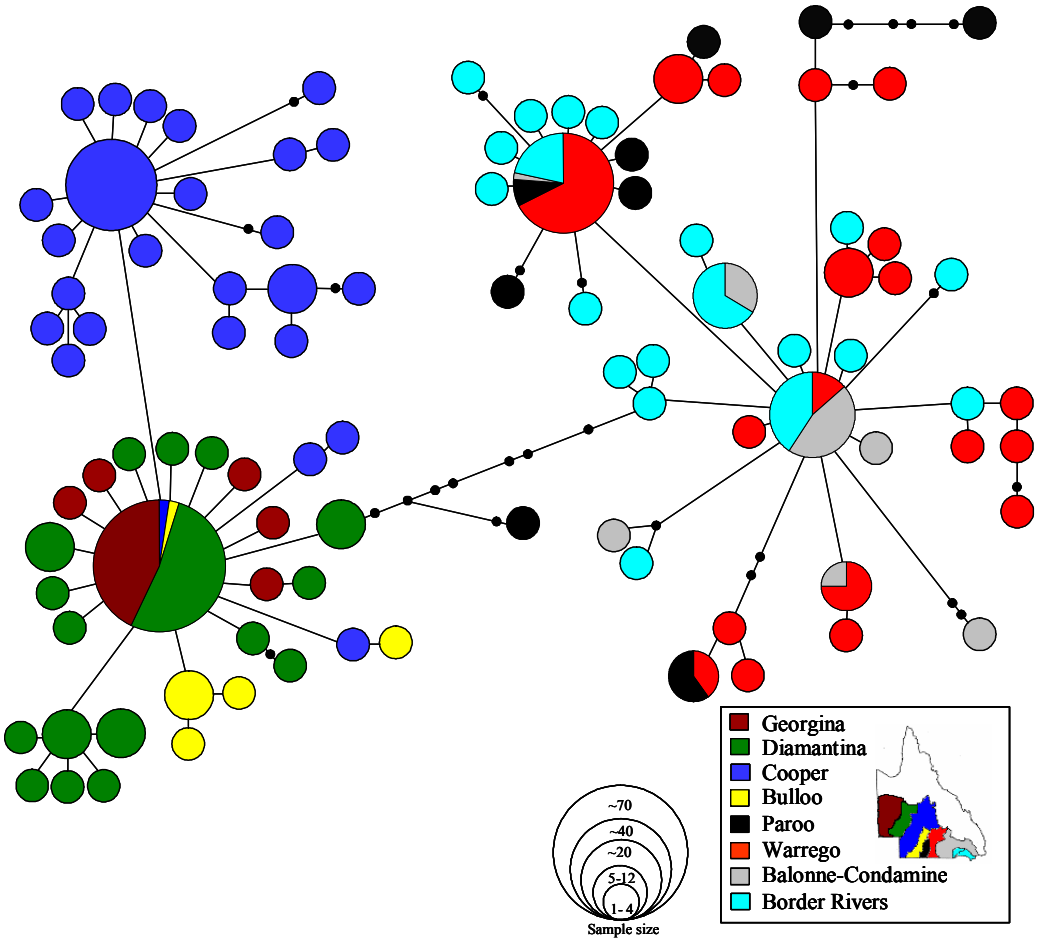
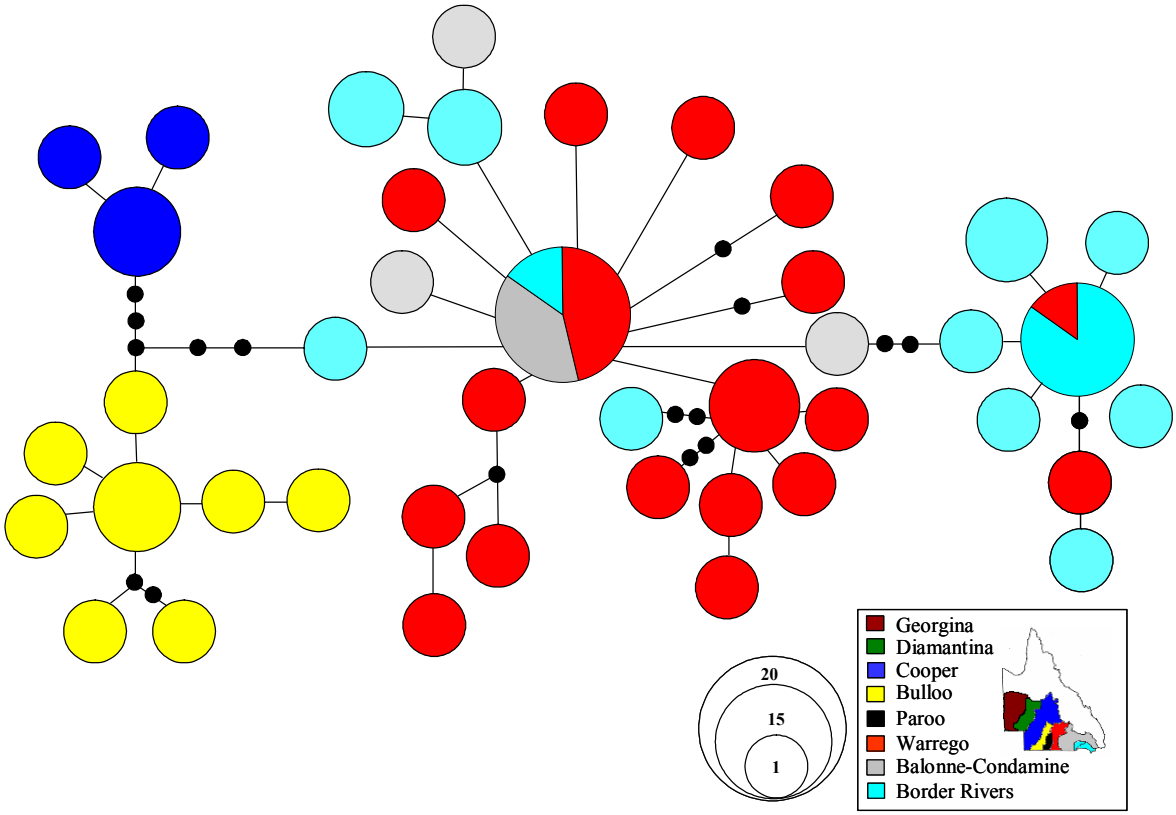


Figure 6.5 Genealogy network showing catchment's distribution for each haplotype and depicting the number of base pair difference between them for *Velesunio* species C (Hughes *et al*., in review).



6.2.2 Comparing AMOVA's across freshwater species in Western Queensland: evidence for a common pattern of contemporary restricted gene flow

AMOVA's have been used to compare population structure across Western Queensland among *N. sublineata*, *M. australiense* and *Cherax destructor* (Hughes and Hillyer, 2003) (Tables 6.1 and 6.2). The most evident similarity between the three species is the high genetic structure of the corresponding populations at all geographical scales. There are some differences in the respective distributions of genetic variation. Patterns of connectivity among populations of obligate freshwater species are expected to be related to the hierarchical nature of the river systems that they inhabit (Meffe and Vrijenhoek, 1988). However, this model has been developed for conventional stream systems and it is unlikely that aquatic species inhabiting quasi-periodic river systems with a highly variable flood regime of Western Queensland will be consistent with these expectations. Nevertheless, it was expected that populations in different catchments would have been more differentiated than within, because levels of connectivity are likely to be higher within than between catchments, anyway. Genetic variation of *M.*

australiense reflects this expectation within the Lake Eyre basin, but not within the Murray-Darling, where genetic variation is higher between populations within catchments (Φ_{SC}) than between catchments overall (Φ_{CT}) (Table 6.2). However, this result seems to be largely due to the fact that the population from the Glencoe site in the Warrego (number 9 in Figure 4.1) did not share any haplotypes with other populations from the same catchment (Table 4.2). Indeed when the Glencoe site was excluded from the analysis, genetic variation was higher between than within catchments. Patterns of genetic variation in *N. sublineata* and *C. destructor* show a different scenario. Neither of the two the species conform to the expectations, because the degree of genetic differentiation between populations within catchments is much larger than the amount of variation between catchments overall (Tables 6.1 and 6.2). One possible explanation to account for the observed pattern is that the species have had insufficient time to attain equilibrium between gene flow and genetic drift in Western Queensland (refer to chapter 3 and Hughes and Hillyer, 2003). If sufficient time has not passed since colonisation or if populations are extremely large, populations will appear similar genetically while being isolated demographically (Crow and Aoki, 1984). It was suggested that during the Pleistocene, particularly wet seasons generated extensive floods that might have resulted in population range expansions of freshwater fauna in Western Queensland. It will take a significant period of time for *C. destructor* and *N. sublineata* populations to reach equilibrium over the entire river system in the region, whereas the process will occur between neighbouring populations much faster (Crow and Aoki, 1984). Unlike *N. sublineata* (viviparous and adult benthic behaviour) and *C. destructor* (no planktonic larvae, territorial behaviour and unlikely terrestrial dispersal over large distances in such inhospitable environment), *M. australiense* have very good dispersal abilities (planktonic larvae and strong swimming adults) that might have increased the probability of migration per generation and shortened the time to reach equilibrium over large distances. Other evidence that support the hypothesis of higher levels of dispersal in *M. australiense* than *N. sublineata* and *C. destructor* is that this species shows the lowest value of genetic variation within catchments, once Glencoe site is removed from the analysis (Table 6.2).

Table 6.1 Results of the AMOVA analyses for *N. sublineata* and *M. australiense*, within the Lake Eyre basin.

Level of partitioning	**Species**	**Φ-statistics**	**P**
Among Catchments (Lake Eyre) [Cooper] [Diamantina] [Georgina] [Bulloo]	*N. sublineata*	Φ_{CT}: 0.284	<0.001
	M. australiense	Φ_{CT}: 0.411	<0.001
Among Waterholes Within Catchments	*N. sublineata*	Φ_{SC}:0.477	<0.001
	M. australiense	Φ_{SC}: 0.064	<0.001
Among all waterholes	*N. sublineata*	Φ_{ST}:0.625	<0.001
	M. australiense	Φ_{ST}: 0.449	<0.001

Table 6.2 Results of the AMOVA analyses for *C. destructor* (Hughes and Hillyer, 2003) and *M. australiense*, within the Murray-Darling basin. † Results of the AMOVA for *M. australiense* after Glencoe site was removed from analyses.

Level of partitioning	**Species**	**Φ-statistics**	**P**
Among Catchments (Murray-Darling) [Paroo] [Balonne-Condomine] [Warrego] [Border Rivers]	*C. destructor*	Φ_{CT}: 0.532	<0.01
	M. australiense	Φ_{CT}: 0.048	>0.05
		Φ_{CT}: 0.098†	<0.01
Among Waterholes Within Catchments	*C. destructor*	Φ_{SC}: 0.819	<0.001
	M. australiense	Φ_{SC}: 0.121	<0.001
		Φ_{SC}: 0.00†	>0.05
Among all waterholes	*C. destructor*	Φ_{ST}: 0.915	<0.001
	M. australiense	Φ_{ST}: 0.163	<0.001

A pattern of restricted gene flow between populations is also suggested by the haplotype distributions, i.e. limited sharing between catchments (and only internal haplotypes), the fact that unique clades were contained by some sites and a generally high number of haplotypes indicate that gene flow has not been sufficient to homogenise the respective subpopulations. For example, the Wilson site contained a unique clade for both the two study species (Tables 3.3 and 4.1) and for *Velesunio* lineage D (Hughes *et al.*, in

review), indicating that a geographic barrier has isolated this site for long enough to allow the local populations of these freshwater invertebrates to evolve a unique set of haplotypes, despite their different dispersal capabilities.

The data presented in this study strongly display high levels of genetic structure in freshwater populations throughout their distribution in Western Queensland. These results suggest that gene flow of freshwater invertebrates is extremely limited between and within catchments. A pattern of restricted gene flow is generally largely the result of the combined effect of specific biological traits of a species and the characteristics of the environment that they inhabit. The two species targeted in this study as well as others (Hughes and Hillyer 2003; and Hughes *et al.*; in review) share the same habitat but they have very different potential dispersal abilities. Nevertheless, all of these species displayed a general pattern of restricted gene flow within the region, suggesting that dispersal abilities are not the dominant force influencing the genetic structure of these populations in Western Queensland river systems. These freshwater invertebrates seem to be divided into many arrays of localised populations and despite the potential for movement of individuals at flood times, very little occur.
The interpretation of this genetic subdivision implies that
a) Despite the potential for long distance dispersal of some aquatic species, the episodic floods do not ensure a good network of connectivity among waterholes as suggested.
And/or
b) Dispersal capabilities of some species (such as *M. australiense*) have been overestimated.
And/or
c) Freshwater organisms tend to utilise waterholes as refugia not only during dry periods but also during floods. Freshwater invertebrates many tend to remain in the waterhole of origin during floods, accumulating into refugial areas of low hydraulic disturbance, thus avoiding dispersal. Examples of such behaviour exist in the literature, even if in a different environment (Winterbottom *et al.*, 1997).

6.2.3 Evidence for historical gene flow between populations

Inferences from the comparative analyses show large concordance across *N. sublineata* (refer to chapter 3), *M. australiense* (refer to chapter 4), and *C. destructor* (Hughes and Hillyer, 2003). These patterns support the idea that, while dispersal does not currently

occur between catchments, physical arrangements of connectivity have changed over time. This prediction was not supported in *Velesunio* species A, which inhabits the Cooper, Diamantina and Georgina. Not only there were no haplotypes shared between distant catchments but also most of the clades that showed significant geographic relationships suggested restricted gene flow and isolation by distance. In *Velesunio* species C the only evidence of range expansions is within catchment, not between them (Hughes *et al.*, in review). Further studies on fishes inhabiting these catchments should help to resolve this issue, since freshwater mussels rely on them to disperse across floodplains.

In haplotype networks of *N. sublineata*, *M. australiense* and *C. destructor*, all the haplotypes are generally closely related within the Lake Eyre and the Murray-Darling basins, yet populations have a relatively high number of 'private' haplotypes, thus localised geographically (Figures 6.1, 6.2 and 6.3). In addition, the haplotypes shared were internal within the networks. Coalescent theory predicts that the haplotypes or clades on the tips of the network are highly likely to be younger than the interior haplotypes or clades to which the tips are connected (Castelloe and Templeton, 1994), suggesting that the sharing of haplotypes detected between catchments is not a result of contemporary gene flow, but is more likely due to the retention of ancestral haplotypes in different populations.

The short internodal distances displayed by the haplotype networks of *N. sublineata*, *M. australiense* and *C. destructor* are consistent with a star phylogeny that may be indicative of rapid population expansion (Avise *et al.*, 1984). Inferences of the nested clade analysis indicated a series of range expansions for these three species both between adjacent catchments (i.e. Cooper-Diamantina for *N. sublineata* and *M. australiense*; Cooper-Bulloo for *N. sublineata*; Cooper-Bulloo-Paroo for *C. destructor*) and between distant catchments (i.e. Cooper-Bulloo-Georgina for *N. sublineata*; Cooper-Bulloo-Georgina-Diamantina as well as within the Murray-Darling basin for *M. australiense*; Georgina-Murray-Darling for *C. destructor*). It was suggested in chapters 3 and 4 of this study and by Hughes and Hillyer (2003) that these range expansion events might have been associated with higher levels of connectivity between catchments during the interglacial phases of the Pleistocene, which might have allowed dispersal of aquatic species across catchment boundaries. Inter-catchment transfer of aquatic species might have been facilitated by a series of flooding events and the

presence of vast lake formations across central Australia during periods of high rainfall (Alley, 1998; Croke *et al.*, 1999). In addition, the headwaters of catchments might have been geographically closer than the present time. Thus, it might have been possible even for small flood events to connect catchments and for aquatic species with some terrestrial abilities, such as *C. destructor* (Hughes and Hillyer, 2003), to move across catchments boundaries.

6.3 Conservation implications

It was indicated by Moritz (1994) that a prerequisite for managing biodiversity is the identification of populations with independent evolutionary histories. The different molecular tools that may be applied to endangered populations or species could provide limits for the units to manage, preserving the total diversity of the taxon. Both shallow and deep genealogical separation within species can be informative in a conservation context. Conspecific populations delimited by shallow phylogenetic gaps could be connected genealogically through ongoing gene flow or relatively recent gene flow (Avise, 1992). A management unit for conservation (MU) was defined as a population that exchanges few migrants with others and that has been geographically isolated long enough to be genetically distinct and independent: 'autonomous' (Avise, 1989; Daugherty *et al.*, 1990; Moritz, 1994). One or a set of conspecific populations with a distinct, long-term evolutionary history clearly separated from others by deep phylogenetic gaps (deep separated lineages) is defined as 'evolutionarily significant unit' (ESU) (Moritz, 1995, Ryder, 1986; Avise, 1992). As such, ESUs are the primary sources of historical genetic diversity within a species (Moritz, 1995). Thus, their identification and interpretation should be taken in consideration for conservation plans of species (Avise, 1992).

It also has been suggested that comparative analyses of phylogeographies for co-distributed species can not only provide insights into the biogeographical history of a region (Avise, 1992; Riddle, 1996), but also extend the use of intraspecific phylogeography to identify evolutionarily significant assemblages of species (Avise, 1992; Moritz, 1995). It has been proposed that comparative analyses of phylogeographies provides a way to investigate geographical patterns of the genetic component of biodiversity as one element of assessing priorities for conservation planning (Avise, 1992; Moritz, 1995).

The outcomes of this research are particularly important because the Western Queensland rivers have just begun to suffer the impact of activities especially associated with river flow management. One of the species targeted in this study, *N. sublineata*, has declined rapidly over the last decade and it is now endangered. This species seems to be virtually extinct in natural waterways throughout the Murray-Darling basin, although, there have been reports of some populations surviving in irrigation pipelines (Sheldon and Walker, 1993). From the comparison of the phylogeographic patterns of a number of co-distributed species in Western Queensland, it appears that populations from the Murray-Darling and the Lake-Eyre constitute two deeply separated groups. *M. australiense* and *Velesunio* C populations from the Lake Eyre and the Murray-Darling basins represent two 'evolutionarily significant units' as outlined by Moritz (1995). *N. sublineata* and *C. destructor* and *Velesunio* A populations constitute a single ESU, throughout their distribution. Within each basin, the species seem to be subdivided into many arrays of isolated populations with absent (between catchments) or low gene flow (within catchments). It was suggested that these populations have been separated long enough to develop private lineages and high levels of genetic variation, but not long enough to produce deep separations among lineages. The implications of restricted gene flow and population fragmentation reported here for *N. sublineata*, *M. australiense*, *C. destructor* (Hughes and Hillyer, 2003) and *Velesunio* sp (Hughes *et al.*, in review) may affect the long-term survival of these species. It was suggested that in the case of local extinction events the chances of recolonisation would be very low, despite the good dispersal abilities of some species. An example that could support this prediction is the very recent and fast decline of *N. sublineata* from the eastern catchments of Western Queensland (such as the Warrego and Paroo). Because of the poor dispersal abilities of the species and the heterogeneity of the habitat, the snails might have not been able to recolonise those areas. The data presented in this study may suggest that in general for all the species examined, populations within the Lake Eyre basin may be considered as two management unit for conservation (Diamantina-Georgina and Cooper-Bullo catchments) whereas populations within the Murray-Darling may be considered as only one.

6.4 Conclusion

At the present time, Western Queensland represents a mosaic of aquatic landscapes. As a consequence, species are subdivided into many arrays of isolated local populations with very limited gene flow between them. In this study of two freshwater invertebrates in Western Queensland river systems, the degree of genetic differentiation between populations is indicative of restricted gene flow between and among catchments, despite the potential dispersal capabilities of the species. However, low levels of genetic differentiation among waterholes within sites, in populations of *N. sublineata* and *M. australiense* indicate that within most sites (very fine scale) populations inhabiting waterholes are exchanging individuals and genes during floods.

As historical changes in connectivity between river systems in Western Queensland are likely to have had a similar effect on populations, it would be expected that all invertebrate species will be affected in the same fashion. The fact that aquatic species with different biological-ecological traits displayed a signature of historical movements across catchment boundaries in Western Queensland, sometime in the past, raises the possibility of a shared history. Similarities between *N. sublineta*, *M. australiense*, *Velesunio* species (Hughes *et al.*, in review) and *C. destructor* (Hughes and Hillyer, 2003) suggest that common historical events, such as interglacial periods during the Pleistocene causing extensive floods along with wide lake formations modifying patterns of connectivity among catchments might have played a major role in determining the population structure of these freshwater invertebrates.

In conclusion, this study indicated that episodic dispersal of *N. sublineata* and *M. australiense* across catchment boundaries was possible during the Pleistocene, due to different patterns of river connectivity, but that contemporary movements of individuals are extremely limited both between and within catchments.

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Appendices

Appendix I Sample size, allele frequencies and observed heterozygosity for *Notopala sublineata* in the Cooper catchment. Each name corresponds to a waterhole, description of waterhole codes is presented in Figure 3.1.

Locus		Currareva	Murken	One Mile	Tanbar	Yalungah	Homestead
Adk							
	n	43	55	63	54	33	44
	1	0.093	0.073	0.103	0.009	0.03	0.011
	2	0.907	0.927	0.897	0.991	0.97	0.989
βEst							
	n	46	57	63	55	33	44
	1	0.174	0.053	0	0.045	0.045	0
	2	0.826	0.947	1	0.955	0.955	1
F1,6pdh							
	n	47	60	64	53	33	44
	1	0.032	0.058	0	0.066	0.03	0
	2	0.936	0.917	1	0.934	0.97	1
	3	0.032	0.025	0	0	0	0
PepA							
	n	46	51	60	53	33	44
	1	0.065	0.029	0.075	0.085	0.061	0.08
	2	0.935	0.961	0.925	0.915	0.939	0.92
	3	0	0.01	0	0	0	0
Pgd							
	n	43	57	64	55	33	44
	1	0.035	0.026	0	0.155	0.136	0
	2	0.895	0.895	0.883	0.818	0.833	0.875
	3	0.07	0.07	0.117	0.027	0.03	0.125
	4	0	0.009	0	0	0	0
Pgi							
	n	46	57	63	55	33	44
	1	0.033	0.132	0.008	0	0	0.011
	2	0.957	0.86	0.992	1	1	0.989
	3	0.011	0.009	0	0	0	0
Pgm							
	n	47	56	64	52	33	44
	1	0.106	0.009	0.07	0.029	0.045	0
	2	0.819	0.83	0.914	0.856	0.894	0.977
	3	0.074	0.161	0.016	0.115	0.061	0.023
	Ho	0.191	0.189	0.102	0.136	0.126	0.052
	S.E.	0.036	0.036	0.035	0.042	0.043	0.028

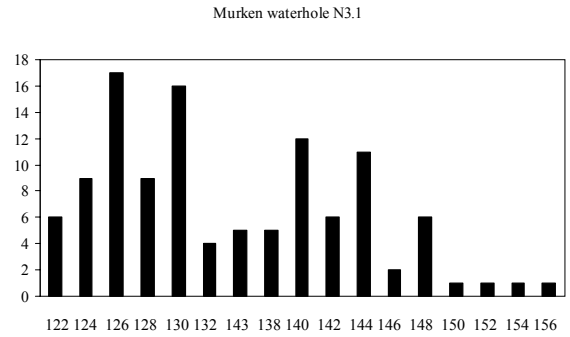
Appendix II Sample size, allele frequencies and observed heterozygosity for *Notopala sublineata* in the Cooper catchment. Each name corresponds to a waterhole, description of waterhole codes is presented in Figure 3.1.

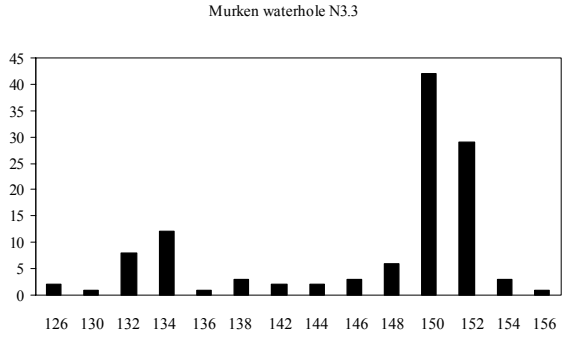
Locus		Waterloo	Pelican	Noccundra	Warranee	Shedh	Top
Adk							
	n	65	54	10	54	42	9
	1	0.023	0.037	0	0.009	0.012	0.167
	2	0.977	0.963	1	0.991	0.988	0.833
βEst							
	n	65	54	10	54	42	9
	1	0.1	0	0.15	0	0.167	0.056
	2	0.9	1	0.85	1	0.833	0.944
F1,6pdh							
	n	66	54	10	54	42	9
	1	0	0	0	0	0.024	0
	2	1		1	1	0.976	1
	3	0	0	0	0	0	0
Pep a							
	n	66	54	10	54	42	9
	1	0.076	0.111	0.1	0.074	0.119	0
	2	0.924	0.889	0.9	0.926	0.869	0.944
	3	0	0	0	0	0.012	0.056
Pgd							
	n	66	54	10	54	42	9
	1	0.167	0.102	0.3	0	0.071	0
	2	0.833	0.898	0.7	0.944	0.917	0.944
	3	0	0	0	0.056	0.012	0.056
Pgi							
	n	66	54	10	54	42	9
	1	0.023	0	0	0	0.012	0
	2	0.977	1	1	1	0.988	1
Pgm							
	n	66	54	10	54	42	9
	1	0.023	0.083	0.05	0.093	0.071	0
	2	0.917	0.898	0.9	0.898	0.893	0.833
	3	0.061	0.019	0.05	0.009	0.036	0.167
	Ho	0.1	0.101	0.129	0.069	0.153	0.143
	S.E.	0.029	0.040	0.047	0.032	0.047	0.053

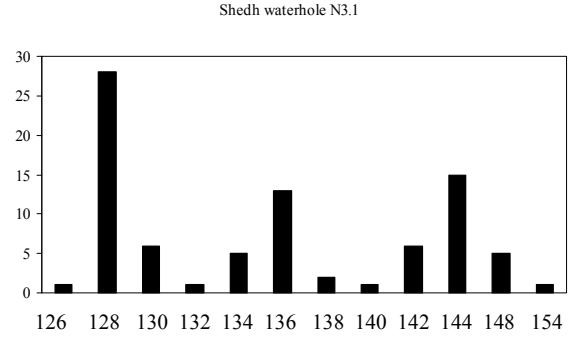
Appendix III Sample size, allele frequencies and observed heterozygosity for *Notopala sublineata* in the Bulloo, Diamantina and Georgina catchments. Each name corresponds to a waterhole, description of waterhole codes is presented in Figure 3.1.

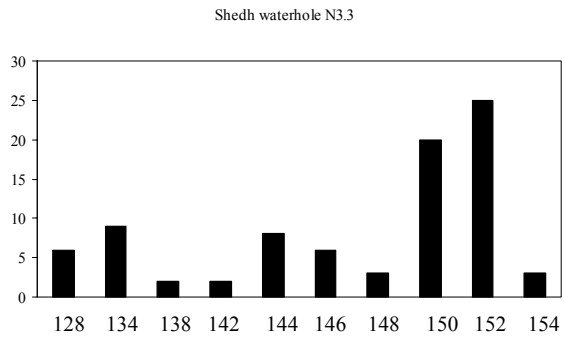
Locus		Middle	Stock	Bulla Bulla	King	Fish	Four Miles	Cuttaburra	Quilpie
Adk									
	n	55	37	18	50	15	44	43	14
	1	0	0	0	0	0	0	0	0
	2	1	1	1	1	1	1	1	1
βEst									
	n	55	37	18	50	15	44	43	14
	1	0	0.014	0.861	0	0	0	0	0.107
	2	1	0.986	0.139	1	1	1	1	0.893
F1,6ph									
	n	55	37	18	44	15	44	43	14
	1	0.009	0.027	0	0.136	0	0.034	0.151	0
	2	0.927	0.838	1	0.818	0.867	0.955	0.849	1
	3	0.064	0.135	0	0.045	0.133	0.011	0	0
PepA									
	n	55	37	18	50	15	44	43	14
	1	0.027	0	0	0	0.033	0	0.012	0.036
	2	0.909	0.973	0.917	0.96	0.867	0.943	0.977	0.821
	2	0.064	0.027	0.083	0.04	0.1	0.057	0.012	0.143
Pgd									
	n	55	37	18	50	15	44	43	14
	1	0.036	0.027	0.139	0.09	0	0.205	0.105	0
	2	0.955	0.973	0.861	0.91	1	0.761	0.884	1
	3	0.009	0	0	0	0	0.034	0.012	0
Pgi									
	n	55	37	18	50	15	44	43	14
	1	0	0	0	0	0	0	0	0
	2	1	1	1	1	1	1	1	1
	3	0	0	0	0	0	0	0	0
Pgm									
	n	55	37	18	50	15	44	43	14
	1	0	0.068	0	0.05	0.167	0	0.163	0
	2	0.845	0.892	1	0.95	0.833	1	0.837	1
	3	0.155	0.041	0	0	0	0	0	0
	Ho	0.099	0.097	0.087	0.097	0.124	0.091	0.123	0.082
	S.E.	0.0430	0.047	0.043	0.045	0.059	0.060	0.054	0.055

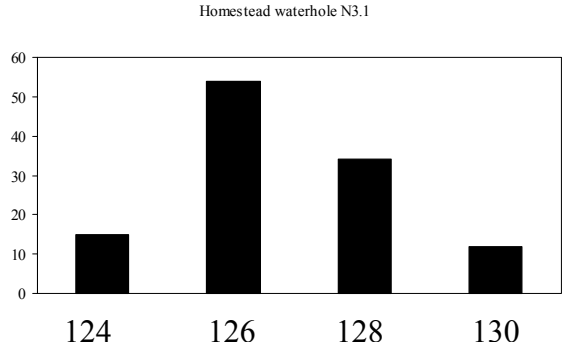
Appendix IV Allele frequencies for microsatellite data at the loci N3.1 and N3.3 for *N. sublineata* in six waterholes (Figure 3.1).

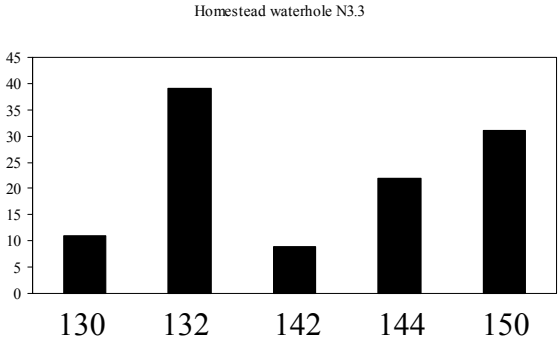


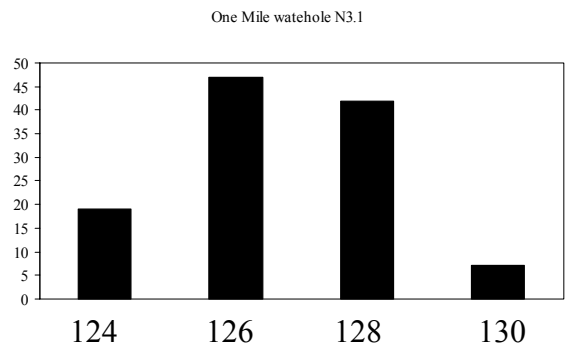


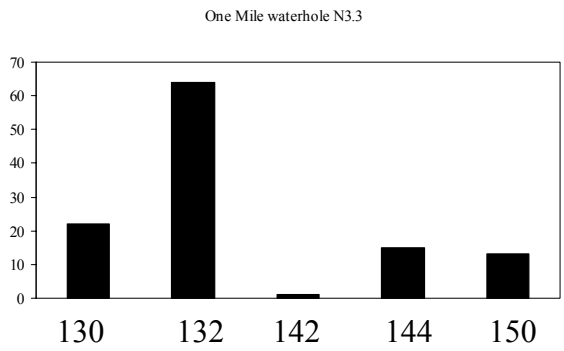




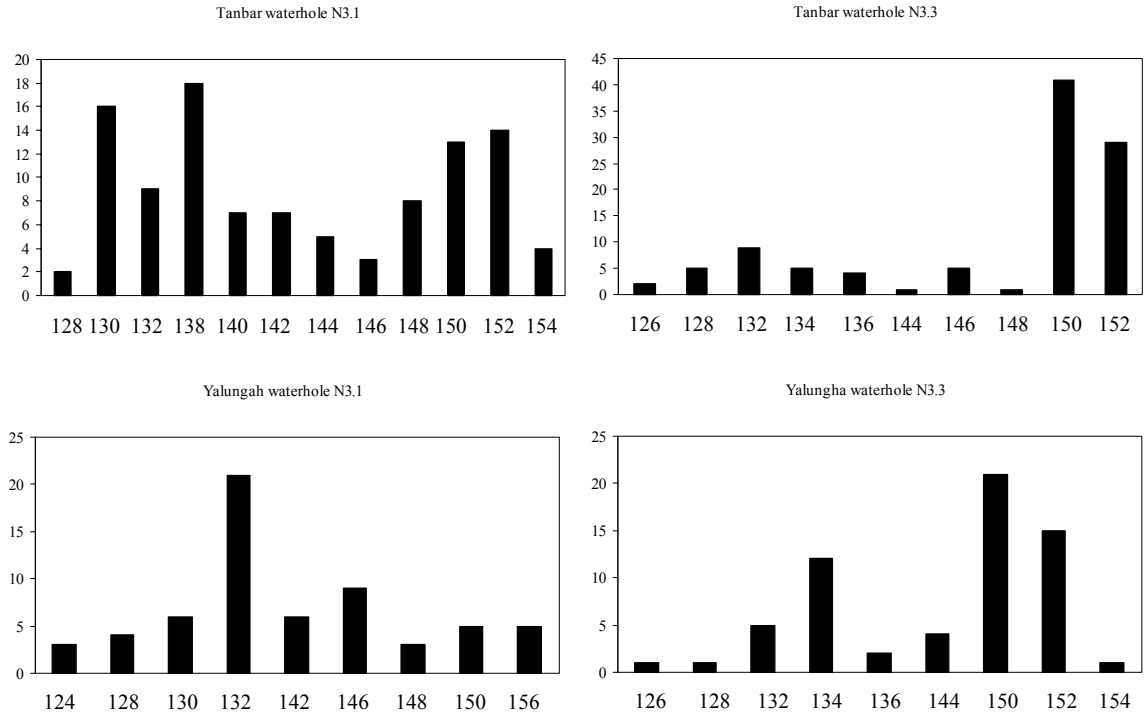








Appendix IV continued.



Papers arising from this study

Carini G, Hughes JM (in press). Population structure of *Macrobrachium australiense* (Decapoda: Palaemonidae) in Western Queensland, Australia: the role of contemporary and historical processes. *Heredity*.

Carini G, Hughes JM (in review). Population genetic structure and phylogeography of an endangered freshwater snail, *Notopala sublineata* (Gastropoda: Viviparidae) in Western Queensland, Australia.

Carini G, Hughes JM and Bunn SE (in review). Effects of persistence time of waterholes on genetic diversity in two freshwater species in Western Queensland, Australia.