Dispersal, population genetics and taxonomy of selected aquatic macroinvertebrates in ephemeral river systems

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Contents

Thesis Abstract

Ephemeral rivers make up a large portion of the world's river systems and yet in the past they receive little attention compared to perennial rivers. In addition to this, interest into the dispersal of aquatic macroinvertebrates until recently has also received little attention. Recent work has uncovered that aquatic macroinvertebrate dispersal may be more limited than previously thought. I investigated the dispersal and population structure of aquatic macroinvertebrates across two ephemeral catchments in South Australia using both genetic techniques as well as direct measures of dispersal.

Previous studies on *Paratya australiensis* have shown that it is not a single species, but rather a species complex made up of multiple linages, some of which have been shown to be reproductively isolated. We analysed the CO1 region of the mitochondrial DNA and compared that to sequence from previous studies (chapter 2). Both lineages 4 and 8 were found which are also found in the head waters of the Murray River and we suggest they used this as a dispersal pathway from eastern Australia.

In order to assess population structure within and between the study catchments we developed microsatellite primer pairs for the shrimp (Chapter 3). The primer pairs developed in chapter 3 were used to look at the population structure of the shrimp across the Broughton and Wakefield Catchments of South Australia (chapter 4). The results showed distinct variation between catchments and additional structuring within the Broughton Catchment. We suggested that even though there were no definitive barriers along the water course that it was

impossible for the shrimp to pass, that repeated times in isolation have led to population differentiation.

One of the challenges to the study macroinvertebrates has always been identification. We looked at two morphologically very similar damselflies, *Ischnura heterosticta* and *Austroagrion watsoni* to investigate the benefit of genetic techniques in species identification (chapter 5). The mitochondrial sequence showed the rate of incorrect morphological identification at approximately 50%. This highlights both the need for accurate identification as well as the power of genetic techniques for identifying morphologically similar species.

One of the most successful methods for direct dispersal measurement is the light trap. We designed and trialled a new type of light trap for catching emerging caddisflies (chapter 6). The light traps were used in a short study to assess their ability to detect dispersal direction of Caddisfly from a permanent water pool within an ephemeral river using a concentric ring design (chapter 7). The results showed that the majority of individuals dispersed along the river channel and that there was a trend to disperse downstream.

The Ecology of the ephemeral rivers is often different to perennial river theory. This difference is the subject of an increasing research effort and this thesis contributes significant new information to the discussion. Through the development of new methods and the use of genetic techniques this thesis has documented previously unknown patterns and suggests areas for future work.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma at any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text

Douglas Green

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Overview of thesis content

Chapters two through seven of this thesis present original research in the format of scientific papers. For this reason each chapter has a separate reference list. Tables and figures are presented after the references of each chapter. Chapters three and six are already published while chapters three, four, five and seven are yet to be submitted for publication.

All chapters have been written by me. My supervisors, Duncan Mackay and Molly Whalen, are co-authors due to their significant input, advice and guidance. Laurence Clarke is also co-author of chapter three owing to his significant input and advice with the project. Michael Gardner is also a co-author on chapter five due to his input and advice through the development of analysis of the project.

Chapter three:

Green, D.J., Clarke, L.J., Mackay, D.A., and Whalen, M.A. (2011) Microsatellite markers for the freshwater shrimp *Paratya australiensis* (Atyidae). *Conservation Genetics Resources* 3, 295-296

Chapter six:

Green, D.J., Mackay, D.A., and Whalen, M.A. (2012) Next generation light traps: The use of LED light technology, *Australian Entomology*, 39, 189-194

Chapter 1

Thesis Introduction

Ephemeral rivers are a large part of the aquatic world (Tooth, 2000) comprising about 60% of the river length in the United States of America (Nadeau and Rains, 2007) and being found across areas of Africa, Asia, Australia, New Zealand, Europe and the Americas (Larned *et al.*, 2010). Despite this, studies of ephemeral rivers have been less common than studies of perennial rivers in the past, with interest in these systems increasing markedly in the last decade or two (Larned *et al.*, 2010). The predominance of studies of perennial streams has led to ideas from perennial streams being applied to ephemeral systems (Bilton *et al.*, 2001). However, not all rivers are the same and the degree of connectivity is thought to be not only a key driver of the physical river environment (Tockner *et al.*, 1999) but also a key driver of the ecology of the river (Larned *et al.*, 2010). One of the key aspects of the ecology of ephemeral rivers is the movement of fauna into and out of the river channel and their mechanisms for coping with the periodic loss or reduction of aquatic habitat.

Dispersal in freshwater ecosystems has been subject to many myths in the past and these have led to reduced effort in understanding the dynamics of macroinvertebrate dispersal (Bohonak and Jenkins, 2003). The notion that freshwater invertebrates disperse frequently and over large geographic distances is something that is deeply ingrained, stemming back to Darwin's 'Duck's feet hypothesis' in *On the origin of Species* (Darwin, 1859). In their review of dispersal of freshwater invertebrates Bohonak and Jenkins (2003) found that even though most species of freshwater invertebrates are thought to have the potential

to disperse widely, the empirical evidence suggests that this ability may often not be fully realised. Instead they found that most freshwater invertebrates tend to have low dispersal rates and that generalisations are misguided as even within families there is a great deal of variation in dispersal capability (e.g. Wilcock *et al.*, 2007). The dispersal ability of organisms will affect their population dynamics, resilience, colonisation ability and population genetics (De Meutter *et al.*, 2007). For these reasons it is important to understand how different organisms disperse and the ways in which different dispersal modes affect population dynamics.

Dispersal studies have generally been conducted using either genetic techniques to infer dispersal ability, or by actively tracking individuals in controlled dispersal experiments. Theoretical studies have shown that the degree of connectivity between populations is a major influence on the overall genetic diversity with genetic drift being more prevalent in those populations with less connectivity (Slatkin, 1977, Lowe and Allendorf, 2010). In freshwater systems this pattern has been shown in some species (e.g. some bivalves, Machordom *et al.*, 2003) but not in others (e.g. shrimp species, Carini *et al.*, 2006). This difference in population genetic structure has been thought to be due to different dispersal mechanisms (Carini *et al.*, 2006, Wilcock *et al.*, 2007). In the past genetic studies have focused on one or two species, however, multi-species work is needed in order to explore how different species disperse in a similar environment (De Meester and Declerck, 2005).

Although tracking of individuals is often difficult with insects, particularly aquatic macroinvertebrates (Humphries, 2002), several studies have examined the dispersal abilities of various aquatic macroinvertebrates. A general trend that has

been found is that individuals of many species tend to actively disperse upstream to counteract drift downstream (Elliot, 2003). Drift refers to the movement of individuals down-stream as a result of water flow (Brittain and Eikeland, 1988). Different methods have been trialled to investigate drift including laboratory simulations, cage studies in the field and mark-recapture studies however; little has been done to validate particular methods (Elliot, 2003). Another aspect of macroinvertebrate dispersal is the role of winged adult life stages, with overland dispersal thought to be a key factor in dispersal of winged macroinvertebrates (Miller *et al.*, 2002). Previous studies have revealed correlations between observed dispersal and genetic differentiation (Miller *et al.*, 2002), however most of these studies have been conducted at relatively small spatial scales, observing dispersal at distances of up to 100 metres and dispersal over greater distances is poorly studied. However, the combination of genetic techniques and observational data appears to be a powerful tool in assessing and examining dispersal abilities.

When studying dispersal in aquatic macroinvertebrates, it is important to note that there is a range of dispersal abilities. These varying abilities are thought to affect the amount of gene flow between populations. Carini *et al.* (2006) examined the genetic diversity of two obligate freshwater species, a freshwater snail *Notopala sublineata* (Gastropoda: Viviparidae) and a free-swimming crustacean *Macrobrachium australiense* (Decapoda: Palaemonidae) comparing main channel pools and satellite pools. They found that there was little differentiation between *M. australiense* populations in the pools indicating that they are able to move between the pools during flow events with ease. However, *N. sublineata* did show some genetic differences that were hypothesised to be due to its lesser dispersal ability.

Wilcock *et al.* (2001) studied genetic differentiation in *Plectrocnemia conspersa*, a Caddisfly from Europe. They showed, using microsatellite markers, that even though dispersal events over tens of kilometres are frequent, over longer distances founder effects were evident. This would suggest that while short distance dispersals are common, larger scale dispersal events are rare. It also suggests that combined small scale dispersal events are not sufficient to overcome the longer distance founder effects. Likewise, Wilcock *et al.* (2007) looked at a core region and sites centred on this core region at a radius of 15, 40 and 100 km. They found that *P. conspersa* had weak genetic differentiation across large distances and that barriers, such as geologic formations or large urban areas, affected gene flow. Conversely they found that *Plectrocnemia flavomaculatus* exhibited strong genetic differentiation and suggested that this was because *P. flavomaculatus* has more limited dispersal. They concluded that *P. conspersa* was a stronger disperser and was able to overcome most obstacles but was constrained by large tracts of landscape with few larval sites.

Colonisation of new habitats is directly related to the dispersal ability of the species. It has been shown that in some systems there is a predictable succession of colonising invertebrates and that the order of succession correlates with dispersal ability (Barnes, 1983). Colonisation will also be affected by location and connectivity with other populations. Where there is a direct link between new habitat and source a different set of factors play a role (e.g. direct dispersal to the new habitat by larval macroinvertebrates as well as winged dispersal by adults (De Meutter *et al.*, 2006)) compared to that of a disconnected system (Barnes, 1983).

The aquatic organisms that inhabit systems that experience disconnections of aquatic habitat (ephemeral systems) show many differences to those adapted to perennial systems. These include differences in life history strategies (e.g. diapause, Denlinger, 2008) and increased tolerance to decreasing water quality (Boulton and Lake, 1992). It is now established that the use of refuge habitat is a vital part of the survival of many of these species. However, how the use of these refuges affects to dispersal behaviour and population structure is still poorly understood, particularly in ephemeral waterways.

Ephemeral waterways are common across much of central and southern Australia. The seasonal wetting and drying of ephemeral rivers means the obligate aquatic flora and fauna are frequently threatened by a loss of habitat. Refugia are areas where special environmental circumstances enable a species or a community of species to survive after extinction in surrounding areas (Caballero and Toro, 2002). In Australia's temperate climate the role of refugia in aquatic systems differs slightly from the traditional view. The remnant habitat, in the form of permanent pools, is formed naturally by the process of drying of ephemeral streams and rivers instead of through climate shift or human interferences (McManhon and Finlayson, 2003). The presence of permanent pools as refugia in the riverscape is vital for the persistence of obligate aquatic species (Carini *et al.*, 2006, Sheldon *et al.*, 2010). Despite the importance of permanent pools, studies of the use and importance of refugia and their effect on the biota that use them have only become prevalent in the last decade or so, driven mainly by studies of desert rivers (Box *et al.*, 2008).

Many of the permanent pools within ephemeral rivers are maintained by inflows of groundwater (Brunke and Gonser, 1997). The inflow of groundwater into the

pools is dependent on the levels of groundwater available and across much of Australia ground water resources are under threat from increased groundwater usage and decreasing water quality (Brunke and Gonser, 1997). It is important for land managers to understand how the loss of refugia will affect populations of aquatic organisms, and their ecology and genetic diversity.

Many of the smaller ephemeral rivers of southern Australia's agricultural region have received little attention. The majority of the studies for this thesis were conducted in the Clare Valley region of South Australia, located approximately 120 kilometres north of Adelaide. The region is used quite extensively for agriculture and is particularly known for wine production. Recently the area was made a prescribed area, meaning that all water use in the area is subject to regulation in order to reduce the effects of over-exploiting the groundwater (Favier *et al.*, 2004). The two river systems that originate in the Clare Valley are the north-flowing Broughton Catchment and the south-flowing Wakefield Catchment. Both have several sub-catchments that provide an excellent opportunity to observe the dispersal of organisms across various boundaries. The rivers themselves are highly dependent on groundwater and the seasonal rising of the water table (Favier *et al.*, 2000, Favier *et al.*, 2004). The deeper pools are maintained year-round by groundwater and in winter with the increased rainfall in the area the water table rises to connect the pools with a base flow, averaging about 600 ml per year (Favier *et al.*, 2004).

Thesis aims and content

The aims of this thesis are broken down into three main sections that reflect different aspects of the ecology, genetics and taxonomy of several invertebrates

that occupy an ephemeral river system. The first section was to examine the population genetics of the obligate aquatic macroinvertebrate *Paratya australiensis* in an ephemeral river system. Specifically the aims were to;

Identify the mitochondrial lineages of *Paratya australiensis* present in the Broughton and Wakefield Catchments; compare the lineages present with those identified in previous studies and to interpret these findings with regard to possible dispersal pathways **(Chapter 2).**

Develop microsatellite primers for *Paratya australiensis* **(Chapter 3).**

Investigate the population genetic structure of *Paratya australiensis* within and between two ephemeral catchments **(Chapter 4).**

The second objective was to investigate the usefulness of genetic techniques in identifying morphologically similar larval Odonata. Specifically the aim was to;

Verify the identification of larval Odonata based on morphology using genetic techniques **(Chapter 5).**

The third objective was to examine dispersal in adult Trichoptera using light traps. Specifically, the aims were to;

Design and construct light traps using LED technology and readily available materials **(Chapter 6).**

Establish a method for the quantification of dispersal direction of emerging adult Trichoptera **(Chapter 7).**

The data chapters of this thesis have been formatted for publication in the scientific literature. Chapters 3 and 6 have been published. Chapter 3 was

published in *Conservation Genetic Resources* and is referred to in this thesis as Green *et al.* (2011). Chapter 6 was published in *Australian Entomology* and referred to in this thesis as Green *et al.* (2012). Chapters 2, 4, 5 and 7 are not yet published.

References

Barnes, L. E. 1983. The colonization of ball-clay ponds by macroinvertebrates and macrophytes. *Freshwater Biology,* 13**,** 561-578.

Bilton, D. T., Freeland, J. R. & Okamura, B. 2001. Dispersal in freshwater invertebrates. *Annual review of Ecology and Systematics,* 31**,** 159-181.

Bohonak, A. J. & Jenkins, G. 2003. Ecological and evolutionary significance of dispersal by freshwater invertebrates. *Ecology Letters,* 6**,** 783-796.

Boulton, A. J. & Lake, S. P. 1992. The ecology of two intermittent streams in Victoria, Australia. II. Comparisons of faunal composition between habitats, rivers and years. *Freshwater Biology,* 27**,** 99-121.

Box, J. B., Duguid, A., Read, R. E., Kimber, R. G., Knapton, A., Davis, J. & Bowland, A. E. 2008. Central Australian waterbodies: The importance of permanence in a desert landscape. *Journal of Arid Environments,* 72**,** 1395-1413.

Brittain, J. E. & Eikeland, T. J. 1988. Invertebrate drift - A review. *Hydrobiologia,* 166**,** 77-93.

Brunke, M. & Gonser, T. 1997. The ecological significance of exchange processes between rivers and groundwater. *Freshwater Biology,* 37**,** 1-33.

Caballero, A. & Toro, M. A. 2002. Analysis of genetic diversity for the management of conserved subdivided populations. *Conservation Genetics,* 3**,** 289-299.

Carini, G., Hughes, J. M. & Bunn, S. E. 2006. The role of waterholes as 'refugia' in sustaining genetic diversity and variation of two freshwater species in dryland river systems (Western Queensland, Australia). *Freshwater Biology,* 51**,** 1434-1446.

Darwin, C. 1859. *On the origin of species,* London, John Murray.

De Meester, L. & Declerck, S. 2005. The study of biodiversity in freshwater habitats: societal relevance and suggestions for priorities in science policy. *Hydrobiologia,* 542**,** 1-9

De Meutter, F. V., De Meester, L. & Stoks, R. 2007. Metacommunity structure of pond macroinvertebrates: Effects of dispersal mode and generation time. *Ecology,* 88**,** 1687-1695.

De Meutter, F. V., Stoks, R. & De Meester, L. 2006. Lotic dispersal of lentic macroinvertebrates. *Ecography,* 29**,** 223-230.

Denlinger, D. L. 2008. Why study diapause? *Entomological Research,* 38**,** 1-9.

Elliot, J. M. 2003. A comparative study if the dispersal of 10 species of stream invertebrate. *Freshwater Biology,* 48**,** 1652-1668.

Favier, D., Rixon, S. & Scholz, G. 2000. A River Management Plan for the Wakefield Catchment. Adelaide: Environmental Protection Agency.

Favier, D., Scholz, G., VanLaarhoven, J. & Bradley, J. 2004. A River Management Plan for the Broughton Catchment. Adelaide: The Department of Water, Land, Biodiversity and Conservation.

Humphries, S. 2002. Dispersal in drisft-prone macroinvertebrates: a case for density-independence. *Freshwater Biology,* 47**,** 921-929.

Larned, S. T., Datry, T., Arscott, D. B. & Tockner, K. 2010. Emerging concepts in tempory-river ecology. *Freshwater Biology,* 55**,** 717-738.

Lowe, W. H. & Allendorf, F. W. 2010. What can genetics tell us about population connectivity? *Molecular Ecology,* 19**,** 3038-3051.

Machordom, A., Araujo, R., Erpenbeck, D. & Ramos, M. A. 2003. Phylogeography and conservation genetics of endangered European Margaritiferidae (Bivalvia: Unionoidea). *Biological Journal of the Linnean Society,* 73**,** 235-252.

McManhon, T. A. & Finlayson, B. L. 2003. Droughts and anti-droughts: the low flow hydrology of Australian rivers. *Freshwater Biology,* 48**,** 1147-1160.

Miller, M. P., Bilinn, D. W. & Keim, P. 2002. Correlations between observed dispersal capabilities and patterns of genetics differentiation in populations of four aquatic insect species from the Arazona White Mountains, U.S.A. *Freshwater Biology,* 47**,** 1660-1673.

Nadeau, T. L. & Rains, M. C. 2007. Hydrological connectivity of headwater streams and downstream waters: how science can inform policy. *Hydrological Processes,* 43**,** 118-133.

Sheldon, F., Bunn, S. E., Hughes, J. M., Arthington, A. H., Balcombe, S. R. & Felows, C. S. 2010. Ecological roles and threats to aquatic refugia in arid landscapes: dryland river waterholes. *Marine and Freshwater Research,* 61**,** 885-595.

Slatkin, M. 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. *Theoretical Population Biology,* 12**,** 253-262.

Tockner, K., Pennetzdorfer, D., Reiner, N., Schiemer, F. & Ward, J. V. 1999. Hydrological connectivity, and the exchange of organic matter and nutrients in a dynamic river-floodplain system (Danybe, Austria). *Freshwater Biology,* 41**,** 521-535.

Tooth, S. 2000. Process, form and change im dryland rivers: a review of current research. *Earth-Science Reviews,* 51**,** 67-107.

Wilcock, H. R., Bruford, M. W., Nichols, R. A. & Hildrew, A. G. 2007. Landscape, habitat characteristics and the genetic population structure of two caddisflies. *Freshwater Biology,* 52**,** 1907-1929.

Wilcock, H. R., Hildrew, A. G. & Nichols, R. A. 2001. Genetic differentiation of a European caddisfly: past and present gene flow among fragmented larval habitats. *Molecular Ecology,* 10**,** 1821-1834.

Chapter 2

Mitochondrial sequence reveals provides further insights into the biogeography of *Paratya australiensis* from South Australia

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Key Words: *Paratya,* Mitochondrial, Ephemeral, Cryptic species, Dispersal

Abstract

The use of mitochondrial sequence information for the detection and estimation of population structure is becoming increasingly prevalent in ecology. The ability to examine inter and intraspecies relationships allows for the estimation of population structure and the detection of possible dispersal pathways. *Paratya australiensis* Kemp (Decapoda: Atyidae) is a common obligate aquatic macroinvertebrate found across much of Australia. Previous studies have examined dispersal pathways into and through eastern Australia, but the spatial extent of previous studies has not ranged into South Australia. We use mitochondrial Cytochrome c oxidase subunit 1 (CO1) sequence data to examine the population structure of *P. australiensis* in two adjacent catchments in the mid-north of South Australia. We found two distinct mitochondrial clusters associated with two previously identified widespread lineages but no spatial divergence. We propose two alternative dispersal pathways with the combination of the two being most likely.

Introduction

The use of genetic techniques to assess population structure provides insights into the dispersal, times of isolation and degree of connections between populations. This expanding field has increasingly been applied to the aquatic fauna of Australia as reviewed by Hughes *et al.* (2009). Genetic techniques in aquatic ecology have been used in many ways, including examining dispersal pathways (e.g. Page *et al.*, 2005, Hughes *et al.*, 2013), population structure (e.g. Faulks *et al.*, 2010), phylogeny (e.g. Futahashi, 2011) and species identification, particularly of stygofauna (Cook *et al.*, 2012).

Aquatic decapods are increasingly well-studied across Australia given their presence in almost every waterway and their obligate aquatic nature. Several studies have used mitochondrial DNA (mtDNA) to examine the phylogeography of *Cherax destructor*, uncovering several different mtDNA clades across Australia separating out across the major water basins of Australia, the Lake Eyre Basin and the Murray Darling Basin (Austin *et al.*, 2003, Hughes and Hillyer, 2003). These results suggest that the two basins have been colonised early and have had limited connection since. This pattern is also seen in the freshwater prawn, *Macrobrachum australiense* (Murphy and Austin, 2004). Both *C. destructor* and *M. australiense* have demographic populations on each side of the Great Dividing Range belonging to the same mitochondrial clade indicating that for each species there has been more recent connections between these populations than between those in the Eyre and Murray Darling Basins. Both of these patterns were also observed in the freshwater shrimp *Paratya australiensis* (Cook *et al*., 2006). Similar patterns have been identified within the genus *Caridina* (Atyidae) with distinct divergence between groups found based on physical boundaries (Page *et al.*, 2007). *Caridina* is thought to differ from other aquatic decapods as the genetic data suggest that there were multiple entries onto the Australian continent (Page *et al.*, 2007).

The genus *Paratya* has previously been studied across a wide range of spatial scales. The spatial distribution of the genus covers much of the southern Pacific from Japan through Norfolk and Lord Howe Islands to Australia and New Zealand. Page et al. (2005) examined the biogeographic relationships between the species of *Paratya* across this geographic range in order to examine colonisation pathways. Mitochondrial DNA analysis uncovered no obvious point of origin. The analysis also showed there are two deeply divergent clades that separate the northern populations of Japan, Korea and Siberia and the southern Pacific populations (Page *et al.*, 2005). For those populations of the south Pacific region, analysis of sequence data suggests that these populations have dispersed from a source population, currently unknown, into the region rather than originating from a Gondwanan species that diverged following the breakup of Gondwana (Page *et al.*, 2005). The mode of dispersal is unclear with several hypotheses considered from Darwin's Duck Feet hypothesis (Darwin, 1859), through to dispersal through saline waters through either freshwater plumes or by individuals with an increased tolerance to salinity (Page *et al.*, 2005). Populations of semi-salinity tolerant freshwater fish have shown evidence of post Gondwanan dispersal through the south pacific subsequent radiation around landmasses (McDowall, 2002). Other evidence suggests that dispersal of freshwater biota is the result of a synergy of dispersal facilitators allowing freshwater paths to open up across the oceans (Measey *et al.*, 2007).

The populations of *P. australiensis* across eastern Australia have been shown to have several divergent lineages. Hurwood et al. (2003) used a combination of allozyme and mtDNA sequence data to examine population genetic structure in the Conondale Range, Queensland. The results showed different mitochondrial clades with many populations with fixed allelic differences at loci. Baker et al. (2004) used mtDNA to measure spatial genetic structure in three catchments used as the water supply for Sydney, New South Wales, Australia. They proposed restricted dispersal and gene flow overlaid with structuring based on the topography of the catchment in upland catchments. This work

expanded by Cook et al. (2006) who added further mitochondrial data and additional sites across eastern Australia. Cook et al. (2006) identified nine divergent lineages of *P. australiensis* across the study area with varying levels of isolation and geographical extent. Cook et al. (2006) suggest that the existing lineage structure observed is the result of multiple amphidromy-freshwater life history transitions with additional evidence of secondary contact between lineages through inland-coastal movement.

The average pairwise model-corrected genetic distance of sampled populations of *P. australiensis* across eastern Australia ranges from 0.028 to 0.081 and indicates that *P. australiensis* may be a complex of cryptic species (Cook *et al.*, 2006). Further evidence for the presence of cryptic species in *P. australiensis* comes from a translocation study of Hughes *et al.* (2003). The authors reported the local extinction of the native lineage due to the low viability of crosses after the mixing two of the most common lineages present based on Cook et al. (2006), lineage 4 and lineage 6 (Hughes *et al.*, 2003). Subsequently, Cook et al. (2007) investigated this further using a combination of allozyme and mitochondrial sequence data examining two of the most common lineages present based on Cook *et al.* (2006), lineage 4 and lineage 8. The results indicated that the two lineages were reproductively isolated.

Knowledge of the population structure of *P. australiensis* is largely limited to studies conducted in mainly eastern Australia and to a lesser extent the Lake Eyre Basin. However *P. australiensis* is reportedly an Australia-wide species (Williams, 1977) and it is still unclear how the populations in other river systems of Australia are related to the lineages identified by Cook *et al.* (2006). As part of a larger study, individuals of *P. australiensis* were sampled from two adjacent catchments in South Australia and their population structure was examined using a fragment of the mitochondrial cytochrome *c* oxidase sub unit 1 (CO1) gene. The aim of the present study was to ascertain how the populations from the river systems in the mid-north of South Australia were related to the populations already sampled by Cook *et al.* (2006) and to investigate possible methods of

colonisation. It was hypothesised that given the close proximity of the two study catchments, they would likely share the same mitochondrial lineages. It was also hypothesised that the lineages identified in this study would differ from those previously identified given that this is a geographically separate drainage basin from the other areas studied by Hurwood *et al.* (2003) and Cook *et al.* (2006).

Methods

Study Sites

The sample sites were located across two catchments in the mid-north region of South Australia. The Broughton and Wakefield Catchments both have ephemeral headwater originating from the Clare Valley, with more permanent reaches downstream. Headwaters in both of the catchments contain permanent pools sustained by groundwater (Favier *et al.*, 2000, Favier *et al.*, 2004). Shrimp were sampled from twelve sites across both catchments using a pond net (four in the Wakefield and eight in the Broughton, Figure 1). Approximately 40 individuals were samples at each site. Individuals were placed in 100% ethanol on ice then later stored at 4˚C. More sites were sought in the headwaters of the Hill River and the Wakefield River to reduce the gap between sampling locations. However, no permanent water was found that contained populations of *P. australiensis* in this region.

Mitochondrial Sequencing

Mitochondrial CO1 sequence data were analysed in order to determine the extent of haplotype diversity, and to determine if any of the different lineages identified by Cook *et al.* (2006) were present. Up to 32 individuals from each sampling site were randomly selected from the samples collected and sequenced. PCRs were performed in a 25 μl reaction using 2 μl of a 1/20 dilution of template DNA, 0.1 μl of Amplitaq Gold (Life Technologies, Carlsbad, California), 1 μl of 25 mM $MgCl_2$, 2.5 μl of 10X PCR Gold

Buffer, 0.5 µl of 10mM dATP, 0.5 µl of 10mM dCTP, 0.5 µl of 10mM dGTP, 0.5 µl of 10mM dTTP, 1µl of 0.5µM Forward Primer, 1µl of 0.5µM Reverse Primer and 15.4µl of molecular grade H_2O. The primers used were ParaCOI-L and ParaCOI-H (Cook *et al.*, 2006). PCR conditions were a two-step program of 8 minutes at 94°C followed by 15 cycles of 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 50 seconds, followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 50 seconds with a final extension step of 4 minutes at 72°C. Sequencing was performed using the forward primer by AGRF (Adelaide, Australia). Sequence data was compared to existing sequence data on Genbank (Benson *et al.*, 2011) to ensure the correct target organism.

The CO1 sequence from *Paratya howensis* (Genbank accession: AY622605) was sourced from Genbank and added to the sequence data collected and aligned as an outgroup, as suggested by Cook et al. (2006). A representative sequence from each of the lineages identified by Cook et al. (2006) was added to the alignment for comparative purposes (Genbank accession numbers in Figure 2). Sequences were aligned using Mega (Version 5.05, Tamura *et al.*, 2011).

Phylogenetic Reconstruction

The CO1 data were analysed to estimate a phylogeny using BEAST (Version 1.7.3.0, Drummond *et al.*, 2012) with 50 million MCMC, with a burn-in of 20 million MCMC based on the Trace. The data were not partitioned based on codon position or other parameters. Results of the MCMC was sampled every 1000 iterations. The HKY substitution model was used for base frequency estimation and the Bayesian Skyline plots were used to model diversification. The phylogeny was built as a maximum clade credibility tree using TreeAnnotator with target node heights maintained (Lemey *et al.*, 2009, Drummond *et al.*, 2012).

Haplotype Network Reconstruction

Network analysis was undertaken using TCS (Clement *et al.*, 2000) to examine how the haplotypes were related to each other and the degree of differentiation between them. The connection limit, the difference at which the program separates networks, was set to 95% which corresponded to approximately 8bp. In order to compare the frequencies of the major lineages identified between the two catchments, a Chi-Squared test of heterogeneity was performed.

Results

Sequence Results

A total of approximately 480 individuals of *P. australiensis* were collected from the study populations, of which 387 were sequenced. This resulted in 323 mitochondrial sequences that were analysed from the sample populations, ranging from 17 to 32 individuals per site (average 27). These sequences were combined with additional sequence data from Cook et al. (2006) and the outgroup to give a total of 338 sequences and a complete (no missing base pairs) alignment length of 418 base pairs. Of the 418 sites, 63 sites were parsimony informative with 48 of them representing singletons. There were a total of 242 zero-fold degenerate sites, 48 two-fold degenerative sites and 64 four-fold degenerative sites.

Phylogenetic Analysis

The phylogeny estimated with BEAST indicated that there were two distinct clades present (posterior probability 1.0) (Figure 2). The first clade identified in the phylogeny included sequence data from all six variations of lineage 4 from Cook et al. (2006) as well as approximately 18% of the individuals from the current study. The sequences from this study which were identified as being similar with Lineage 4B from the Cook et al. (2006) study (Linage 4B embedded within Cluster 1, Figure 2) were also the same

sequences that were identified as being part of the network associated with lineage 4 (Figure 3). These sequences as well as the representative sequence for lineage 4B are shown collapsed in Figure 2 for ease of viewing. The second clade included representative sequences for Lineage 1, 2, 3, 5, 6, 7, 8 and 9 from Cook et al. (2006) as well as approximately 82% of the individuals from the present study. The individuals from the present study within this clade were most genetically similar with lineage 8 from Cook et al. (2006). These individuals are the same individuals that were identified in the haplotype network in Figure 4 and are shown collapsed in Figure 2 (Cluster 2), again, for ease of viewing.

Haplotype Analysis

There were a total of 38 unique haplotypes that were included in the dataset, these included the outgroup and the 14 sequences from Cook et al. (2006) leaving 23 haplotypes from the study area (Table 1). Haplotypes 1, 2 and 30 comprised Cluster 1, while haplotypes 3, 5, 6, 7, 8, 21 through 34 and 36-37 comprised cluster 2. The most common haplotype was haplotype 3 with 232 individuals, followed by haplotype 1 with 39 individuals. There were 18 haplotypes that were represented by only one individual. Eleven of these singletons were from the Wakefield Catchment and seven were from the Broughton Catchment, with five from one site (Broughton 1, the most downstream site in the catchment, Figure 1). All haplotypes represented by more than one individual were represented in both catchments.

The TCS network analysis identified two groups of haplotypes at 95% connection (8 steps), the total number of substitutions between the two networks was 30 base pair changes. The smaller of the groups included haplotypes 1, 2 and 30 from this study with a total of 59 individuals (Figure 3). This network also included Lineage 4B and Lineage 4C from Cook et al. (2006) with Lineage 4B being considered the most similar (separated by two base pair substitutions). The most frequently sampled of the two clusters

contained 264 individuals from this study across 20 unique haplotypes (Figure 4). Haplotype 3 contained the majority of individuals, with haplotype 23 and haplotype 5 being the only other haplotypes in the network with more than one representative (twelve and three times respectively). Included in this group was Lineage 8 from Cook et al. (2006) separated from the majority of individuals by 1.44% (6 base pairs). The number of base pairs between the two clusters was 18. All of the other sequences from Cook et al. (2006) were separated from these groups by more than 8bp changes, as was the outgroup *P. howensis*.

Chi-Square Analysis

The split between the two major clades in the phylogeny did not strongly reflect the geographic separation of the Broughton and Wakefield catchments (Figure 4). Individuals from Cluster 2 (Figure 2) were distributed across both catchments, whereas individuals belonging to Cluster 1 (Figure 2) were more predominate in the Broughton Catchment and not found at all in two sites, one on the Hill River in the Broughton Catchment and the most downstream sites on the Wakefield River. Cluster 1 (Figure 2) was predominantly from the Broughton Catchment, with only four of the 57 individuals being from the Wakefield Catchment (χ=12.59, df = 1, P<0.01). Within each catchment, there was no obvious spatial structuring of the distribution of the two major lineages.

Discussion

The results of this study show that the two study catchments share mitochondrial lineages, supporting the first hypothesis. While both catchments have the same lineages present there is some variation between them in the frequency of the different lineages present. It is also noted that the two clusters present are associated with lineages identified previously by Cook et al. (2006) and further investigated by Cook et al. (2007). Lineages 4 and 8 are both widespread lineages distributed across much of eastern Australia and notably in the upper Murray River catchment (Goulburn River and Ovens

River in the Murray River Catchments and Horton River in the Darling Catchment). Given the dispersal abilities of *P. australiensis*, it is likely that both of the lineages are present along the length of the Murray River. The dispersal pathway from the Murray River to the Mid North of South Australia is less clear with several possible explanations. Overland dispersal of the shrimp is not entirely out of question with evidence from other species of Decapoda suggesting that overland dispersal occurs, although it is not common (Hurwood and Hughes, 2001). Overland dispersal from the Murray Basin to the two study catchments would require multiple cross-catchment migrations, including a crossing of the Mt. Lofty Ranges in South Australia. Given the findings of previous studies of apparent connections across the Great Dividing Range of eastern Australia (Hughes *et al.*, 1996, Hurwood and Hughes, 2001), the possibility of such dispersal events cannot be ruled out. It should be noted however, that the terrestrial environment is less conducive to overland dispersal. Southern Australia is generally dryer than the sub-tropical study area of Hurwood and Hughes (2001).

Another possible dispersal pathway is via the sea from catchment to catchment along the coast of South Australia. Several Victorian rivers (Hopkins, Barwon and the Werribee Rivers) flowing south to Bass Strait contain only Lineage 8 (Cook *et al.*, 2006). There is evidence that *P. australiensis* has some ability to tolerate saline waters (Walsh and Mitchell, 1995) that might allow for costal marine dispersal events between catchments, although not all lineages of *P. australiensis* display tolerance of saline waters (Page *et al.*, 2005). It may be that both dispersal theories are possible. Lineage 4 is common through the Darling River while it is unobserved in southern Victoria (Cook *et al.*, 2006). Conversely, Lineage 8 is common along the coast of southern Victoria and, though it is present in the Darling and Murray Rivers, it is not a common lineage (Cook *et al.*, 2006). It is possible that Lineage 4 dispersed along the Murray River while Lineage 8 dispersed along the coastline, as suggested by Cook et al. (2006).

There is a difference in the frequencies of the two observed lineages in the two catchments. Of particular note were the two sites that showed only individuals associated with lineage 8, of which there was one per catchment. The other pattern was both the Hill River sites showed an excess of these individuals. It has been demonstrated that it is possible for one lineage to outcompete another (Hughes *et al.*, 2003). If this is what we are observing here, then it would suggest that the introduction of one of the clusters is more recent with the effects still becoming apparent or still moving towards a stable strategy (regarding community assemblage). At this stage the reason for this difference in unclear. The number of individuals sampled in this study is considerably higher per site than previous studies, which allows for a more in-depth analysis of the frequencies of the different haplotypes and it allows for greater confidence that rare lineages and haplotypes are represented. Lineage 4 and 8 have been found together in multiple catchments across Australia at varying frequencies (Cook *et al.*, 2007). The Cook et al. (2007) study identified variations of the frequencies of lineage 4 and 8 over the course of two years and suggest that the assemblage can vary in a stochastic fashion. Cook et al. (2007) concluded both of the lineages have similar responses to steep stream gradients suggesting that both lineages struggle with barriers to dispersal but the different lineages showed different responses to lowland rivers with lineage 8 showing increased isolation among lowland streams indicating that lowland streams may act as a barrier to dispersal for Lineage 8 and not for Lineage 4. The reason for the difference in the two catchments observed in this study is not clear and further work is needed to identify if this is due to a fitness difference associated with one of the lineages, abiotic factors (water chemistry, temperature, salinity) or simply part of the stochastic variation postulated by Cook et al. (2007).

The increased number of singleton haplotypes in the Wakefield Catchment over the Broughton and the high number observed at the Broughton 1 site represents an unresolved issue in the data. Previous studies on *P. australiensis* have not investigated

the presence of singletons. Singletons, apart from one (Hap 30), were all found in the more abundant group, lineage 8. This may be due to recent expansion into the area or some dispersal from outside areas.

This study introduces more information to the growing body of knowledge of *P. australiensis.* The study catchments have extended the sampled area across more of the range of *P. australiensis* into previously un-sampled drainage basins separated by over 500 kilometres and the Mt. Lofty Ranges. The individuals sampled in this study cluster together with previously identified lineages which have been documented as being reproductively isolated, even though they occur together across much of the their range (Cook *et al.,* 2007). It was hypothesised, given the large geographic distance between these populations and those previously studied combined with the different drainage basin, that there would be different lineages present. Further work is needed to validate the hypothesis that the Murray River is the main dispersal path for the populations in the present study. In addition, more sampling locations across more of its distribution, including sampling of the populations of Tasmania and additional sampling from southern Australia, are required to gain a more complete picture of *P. australiensis* in Australia. There have been previous attempts to split the complex into different species based on morphology (Riek, 1953), however, the taxonomic splits between the different species and sub-species were subsequently removed (Williams, 1977). Recently there have been documented differences in morphology between some lineages (Hancock *et al.,* 1998), however, these have been not been investigated in the context of genetic differences identified in the last decade by several studies.

If the hypothesis that the shrimp present in the catchments studied dispersed down the River Murray and then up the coastline of South Australia, then it could be expected that the genetic diversity of *P. australiensis* in the River Murray is relatively low when compared to the multitude of lineages present on the eastern coast of Australia (Cook *et al.,* 2006). Other Decapods have shown similar wide ranging clades through the Murray

River. *Cherax destructor* has been shown to have multiple sympatric clades in the Murray River, some of which range over 1700kms (Nguyen *et al.*, 2004). Future work may want to focus on smaller scale differences of population structure between the lineages to provide insights into contemporary dispersal through the Murray River system and possibly some of the catchments of the southern Mt. Lofty Ranges in South Australia.

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References

Austin, C. M., Nguyen, T. T. T., Meewan, M. M. & Jerry, D. R. 2003. The taxonomy and phylogeny of the *Cherax destructor* complex (Decapoda: Parastacidae) examined using mitochondrial 16S sequences. *Australian Journal of Zoology,* 51**,** 99-110.

Baker, A. M., Hughes, J. M., Dean, J. C. & Bunn, S. E. 2004. Mitochondrial DNA reveals Phylogenetic structuring and cryptic diversity in Australian freshwater macroinvertebrate assemblages. *Marine and Freshwater Research,* 55**,** 629-640.

Benson, D. A., Karsch-Mizachi, I., Lipman, D. J., Ostell, J. & Sayers, E. W. 2011. GenBank. *Nucleic Acids Research,* 39**,** D32-D37.

Clement, M., Posada, D. & Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology,* 9**,** 1657-1660.

Cook, B. D., Abrams, K. M., Marshall, J. C., Perna, C. N., Choy, S., Guzic, M. T. & Cooper, S. J. B. 2012. Species diversity and genetic differentiation of stygofauna (Syncarida : Bathynellacea) across an alluvial aquifer in north-eastern Australia. *Australian Journal of Zoology,* 60**,** 152-158.

Cook, B. D., Baker, A. M., Page, T. J., Grant, S. C., Fawcett, J. H., Hurwood, D. A. & Hughes, J. M. 2006. Biogeographic history of an Australian freshwater shrimp, *paratya australiensis*: the role of history transition in phylogeographic diversification. *Molecular Ecology,* 15**,** 1083-1093.

Cook, B. D., Bunn, S. E. & Hughes, J. M. 2007. A comparative analysis of population structuring and genetic diversity in sympatric lineages of freshwater shrimp (Atyidae: *Paratya*): concerted or independent responces to hydrographic factors? *Freshwater Biology,* 52**,** 2156-2171.

Darwin, C. 1859. *On the origin of species,* London, John Murray.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. 2012. Bayesiam phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution***,** doi:10.1093/molbev/mss075.

Faulks, L. K., Gilligan, D. M. & Beheregaray, L. B. 2010. Islands of water is a sea of dry land: hydrological regime predicts genetic diverisy and dispersal in the widespread fish from Australia's arid zone, the golden perch (*Macquaria ambigua*). *Molecular Ecology,* 19**,** 4723-4737.

Favier, D., Rixon, S. & Scholz, G. 2000. A River Management Plan for the Wakefield Catchment. Adelaide: Environmental Protection Agency.

Favier, D., Scholz, G., VanLaarhoven, J. & Bradley, J. 2004. A River Management Plan for the Broughton Catchment. Adelaide: The Department of Water, Land, Biodiversity and Conservation.

Futahashi, R. 2011. A revisional study of Japanese Dragonflies based on DNA analysis. *Tombo,* 53**,** 67-74.

Hancock, M., Hughes, J. M. & Bunn, S. E. 1998. Influence of genetic and environmental factors on egg and clutch sizes among populations of *Paratya australiensis* Kemp (Decapoda: Atyidae) in upland rainforest streams, south-east Queensland. *Oecologia,* 115**,** 483-491.

Hughes, J. M., Bunn, S. E., Choy, S. & Pearson, R. G. 1996. Genetic differentiation among populations of Caridina zebra (decapoda: Atyidae) in tropical rainforest streams, northern Australia. *Freshwater Biology,* 36**,** 289-296.

Hughes, J. M., Goudkamp, K., Hurwood, D. A., Hancock, M. & Bunn, S. E. 2003. Translocation causes extinction of a local population of the freshwater shrimp *Paratya australiensis*. *Conservation Biology,* 17**,** 1007-1012.

Hughes, J. M. & Hillyer, M. J. 2003. Patterns of connectivity among populations of *Cherax destructor* (Decapoda: Parastacidae) in western Queensland, Australia. *Marine and Freshwater Research,* 54**,** 587-596.

Hughes, J. M., Huey, J. A. & Schmidt, D. J. 2013. Is realised connectivity among populations of aquatic fauna predictable from potential connectivity? *Freshwater Biology,* 58**,** 951-966.

Hughes, J. M., Schmidt, D. J. & Finn, D. S. 2009. Genes in streams: Using DNA to understand the movement of freshwater fauna and their riverine habitat. *BioScience,* 59**,** 573-583.

Hurwood, D. A. & Hughes, J. M. 2001. Nested Clade analysis of the freshwater shrimp, *Caridina zebra,* (Decapoda: Atyidae), from north-eastern Australia. *Molecular Ecology,* 10**,** 113-125.

Hurwood, D. A., Hughes, J. M., Bunn, S. E. & Cleary, C. 2003. Population structure in the freshwater shrimp (*Paratya australiensis*) infered from allozymes and mitochondrial DNA. *Heredity,* 90**,** 64-70.

Lemey, P., Rambaut, A., Drummond, A. J. & Suchard, M. A. 2009. Bayesian phylogeography finds its roots. *PLOS Computational Biology,* 5**,** e1000520.

McDowall, R. M. 2002. Accumulating evidence for a dispersal biogeography of southern cool temperate freshwater fishes. *Journal of Biogeography,* 29**,** 207-219.

Measey, G. J., Vences, M., Drewes, R. C., Chiari, Y., Melo, M. & Bourles, B. 2007. Freshwater paths across the ocean: molecular phylogeny of the frog *Ptychadena newtoni* gives insights into amphibian colonization of oceanic islands. *Journal of Biogeography,* 34**,** 7-20.

Murphy, N. P. & Austin, C. M. 2004. Phylogeography of the widespread Australian freshwater prawn *Macrobrachium australiense* (Decapoda: Palaemonidae). *Journal of Biogeography,* 31**,** 1065-1072.

Nguyen, T. T. T., Austin, A. D., Meewan, M. M., Schultz, M. B. & Jerry, D. R. 2004. Phylogeography of the freshwater crayfish *Cherax destructor* Clark (Parastacidae) in inland Australia: historical fragmentation and recent range expansion. *Biological Journal of the Linnean Society,* 83**,** 539-550.

Page, T. J., Baker, A. M., Cook, B. D. & Hughes, J. M. 2005. Historical transoceanic dispersal of a freshwater shrimp: the colonization of the South Pacific by the genus *Paratya* (Atyidae). *Journal of Biogeography,* 32**,** 581-593.

Page, T. J., von Rintelen, K. & Hughes, J. M. 2007. An island in the stream: Australia's place in the cosmopolitan world of the Indo-West Pacific freshwater shrimp (Decapoda: Atyidae: *Caridina*). *Molecular Phylogenetics and Evolution,* 43**,** 645-659.

Riek, E. F. 1953. The Australian freshwater prawns of the family Atyidae. *Records of the Australian Museum,* 23**,** 111-121.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution,* 28**,** 2731-2739.

Walsh, C. J. & Mitchell, B. D. 1995. The freshwater shrimp *Paratya australiensis* (Kemp, 1917) (Decapoda: Atyidae) in estuaries of south-western Victoria, Australia. *Marine and Freshwater Research,* 46**,** 959-965.

Williams, W. D. 1977. Some Aspects of the Ecology of *Paratya australiensis* (Crustacea : Decapoda : Atyidae). *Australian Journal of Marine and Freshwater Research,* 28**,** 403-415.

Table 1: Haplotype information for the P. australiensis individuals sequenced from the Broughton and Wakefield Catchments. Geographic coordinates for the sample sites are available from the Authors on request.

		Broughton Catchment								Wakefield Catchment			
	Site	**Broughton 1**	**Broughton 2**	**Broughton 3**	**Broughton 4**	**Hill 1**	**Hill 2**	**Freshwater 2**	**Freshwater 2**	**Wakefield 1**	**Wakefield 2**	**Wakefield 3**	**Wakefield 4**
Cluster 1	Hap1	7	10	7	3			3	6	1	1	1	
	Hap2	4	2	2	6			2	2	1			
	Hap30	1											
Cluster 2	Hap3	13	18	20	20	30	29	24	19	24	15	8	12
	Hap5	1			1					1			
	Hap6						1						
	Hap7								1				
	Hap21									1			
	Hap22									1			
	Hap23	2								2		6	2
	Hap24	1											
	Hap25									1			
	Hap26												1
	Hap27												1
	Hap28												1
	Hap29											1	
	Hap31											1	
	Hap32										1		
	Hap33										1		
	Hap34										1		
	Hap35	1											
	Hap36	1											
	Hap37	1											

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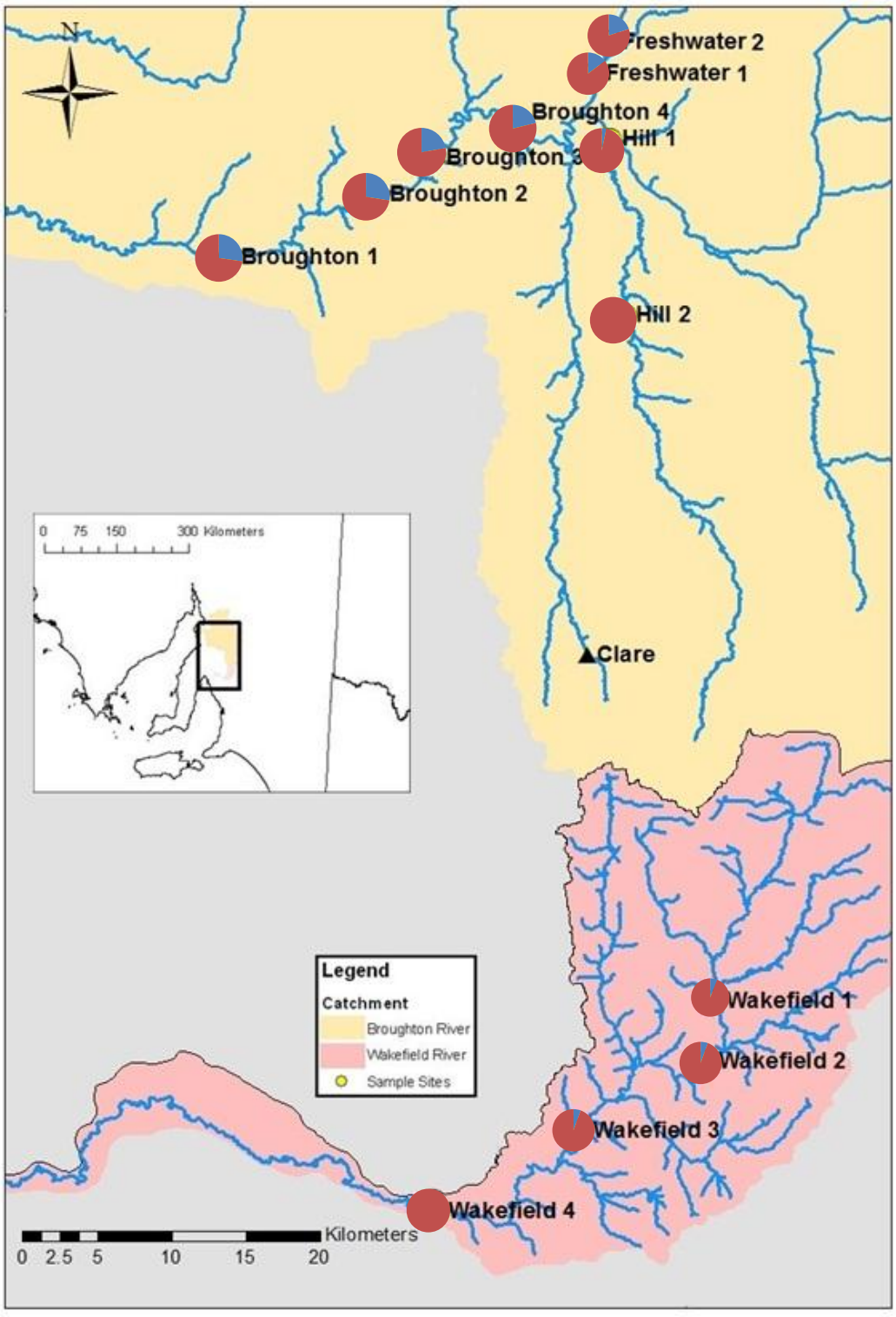

Figure 1: Map of the study sites across the Broughton and Wakefield Catchments in South Australia. Pie charts indicate the relative frequencies of 'Cluster 1' (blue) and 'Cluster 2' (red).

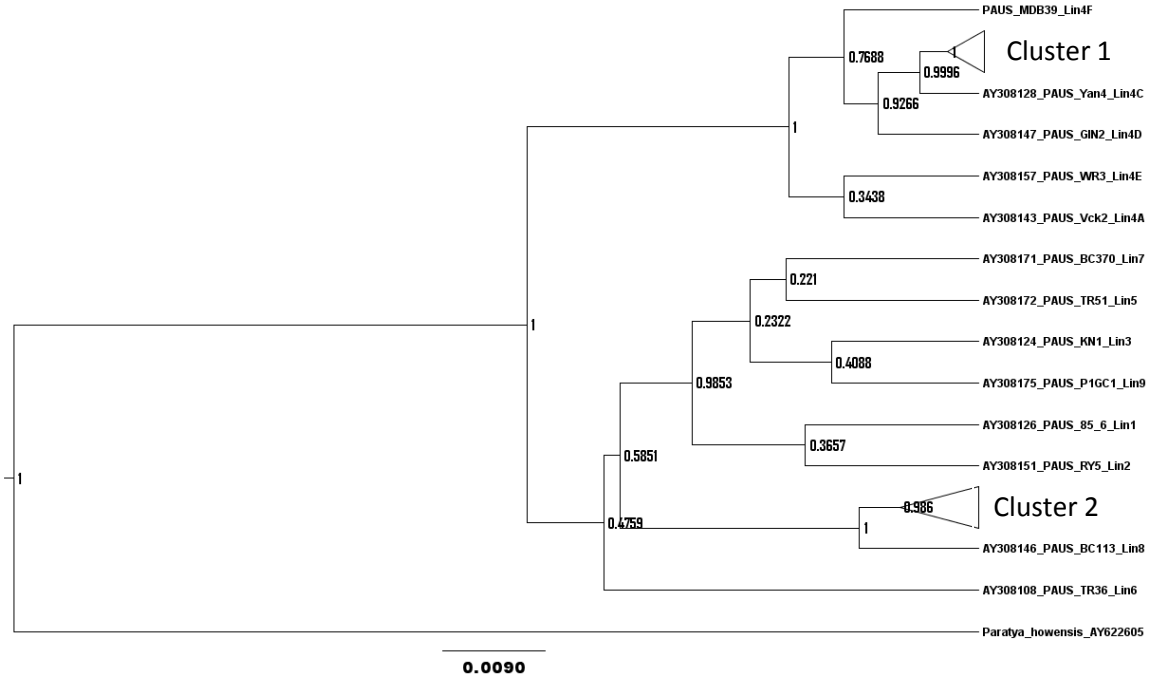

Figure 2: Maximum clade credibility tree produced using TreeAnnotator (Version 1.7.3.0) from the BEAST output of the CO1 sequence data. The posterior probabilities are shown for each node and the divergence distance scale is below the figure. The two main clusters of sequence from the present study are collapsed to show their position relative to other species. Genbank Sequence data are identified by their accession number.

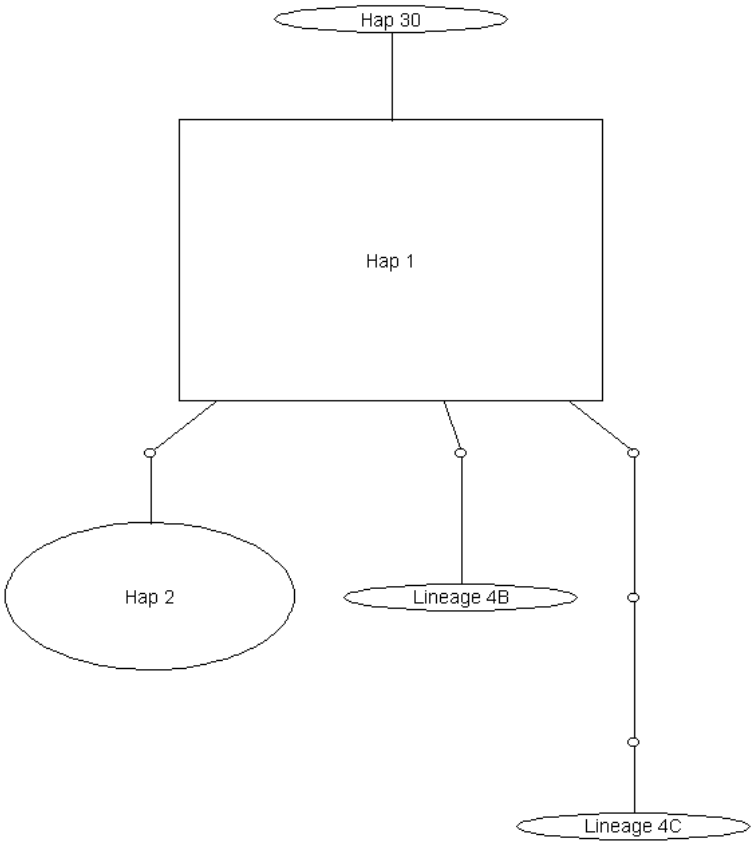

Figure 3: Network diagram generated using TCS of the first cluster of haplotypes (Cluster 1) identified in the network analysis. Each node represents a base pair change. The haplotypes 'Lineage 4B' and 'Lineage 4C' are representative sequence from Cook et al. (2006). There were 39 individuals identified with 'Hap 1', 19 individuals with 'Hap 2', while 'Hap 30' was only identified once.

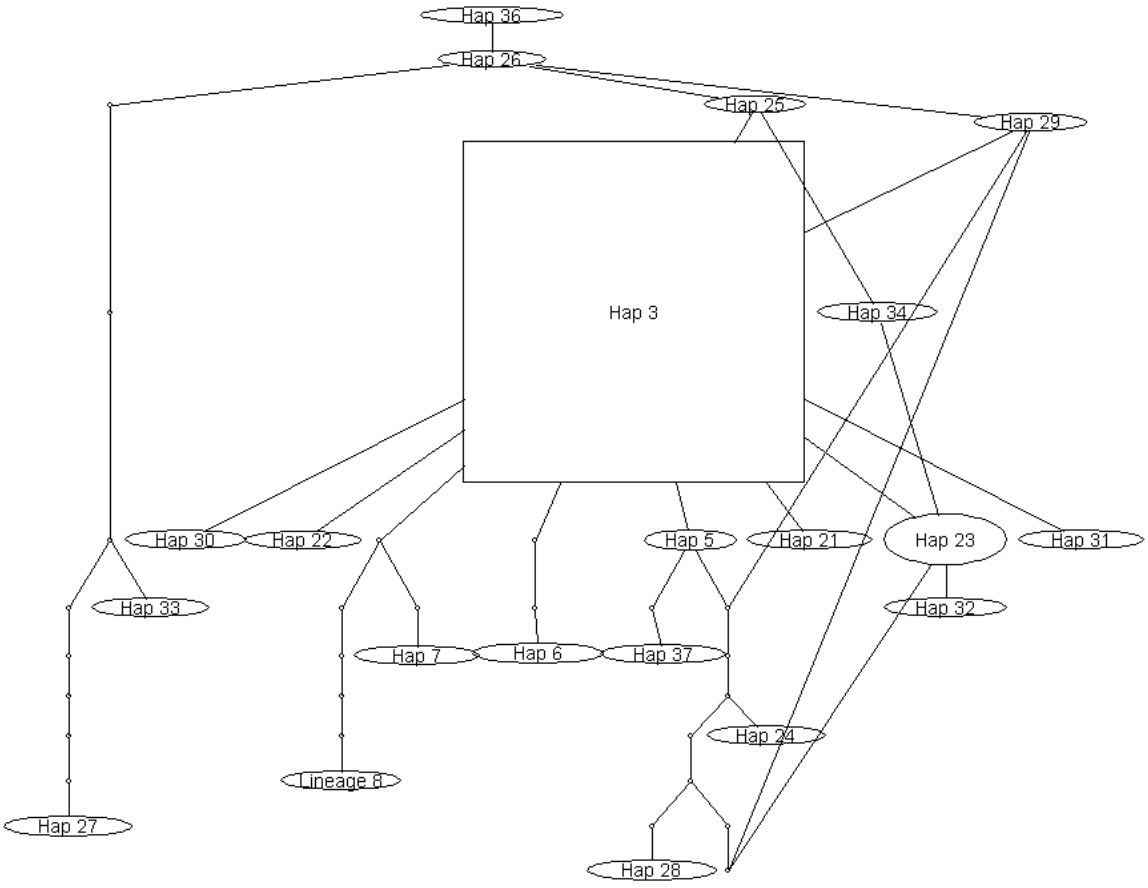

Figure 4: Network diagram generated using TCS of the second cluster of haplotypes (Cluster 2) identified in the network analysis. Each node represents a base pair change. The haplotype 'Lineage 8' is representative sequence from Cook et al. (2006). 'Hap 3' was identified 232 times, 'Hap 23' was identified 12 times, 'Hap 5' was identified 3 times while the rest of the haplotypes were singletons.

Chapter 3

Microsatellite Markers for the Freshwater Shrimp *Paratya australiensis* (Atyidae)

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Keywords: *Paratya,* freshwater shrimp, South Australia, Macroinvertebrate, Microsatellites, 454 sequencing

Abstract

Paratya australiensis is a common freshwater shrimp found in most catchments in south-eastern Australia. Microsatellite loci were isolated from a partial genomic library created using 454 sequencing. Of the 25 tested, 13 were found to be polymorphic, however, for pooling purposes only 12 were used for genotyping. The number of alleles per locus varied from 2 to 14 in a population from Wakefield River, South Australia, and the mean (range) observed and expected heterozygosity were 0.512 (0.136-0.909) and 0.590 (0.165-0.788) respectively across all alleles. These microsatellites will be used to study the genetic structure of populations in two catchments in South Australia.

Introduction

The distribution of the widespread freshwater shrimp *Paratya australiensis* Kemp covers much of southern and eastern Australia. *Paratya australiensis* was described by Kemp (1917) to encompass Australian material of the genus *Paratya* including material from the Australian territory of Norfolk Island, which he considered to represent a distinct subspecies from the typical form on mainland Australia. Reik (1953) subsequently described a new subspecies of *P. australiensis* and two additional species of *Paratya* from Australia. However, Williams (1977) recognised only one Australia-wide species, *P. australiensis*. More recently, genetic studies have shown that *P. australiensis* may be a species complex (Hughes *et al.*, 1995, Baker *et al.*, 2004, Cook *et al.*, 2006, Cook *et al.*, 2007). Previous mitochondrial and allozyme studies on this species indicated a high level of structuring resulting from limited dispersal (Hughes et al., 1995, Hurwood *et al.*, 2003). This was further supported by the findings of Cook (2006), who uncovered evidence of nine separate lineages throughout eastern Australia. We developed microsatellites for *P. australiensis* to explore dispersal potential and examine genetic structure at a finer scale.

Methods

Individuals were collected from the Broughton and Wakefield catchments in South Australia and preserved on ice in 100% ethanol. DNA was extracted from tail muscle tissue using a modified Gentra method (Gentra Systems Inc. Minneapolis, US). The cell lysis step and the DNA precipitation step were both extended to 24 hours and all centrifuge times were doubled to ensure both maximum yield and clean product.

DNA from four individuals was pooled, then split across one eighth of a plate and sequenced on a Roche GS-FLX system (Roche, Penzburg, Germany) at the Australian Genome Research Facility (AGRF, Brisbane, Australia). This provided 121252 sequences between 150 and 450 bp long. These sequences were analysed for the presence of dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide microsatellites over 8 repeats long using MSATCOMMANDER (version 0.8.2, Faircloth, 2008). We identified 1086 potential microsatellite loci and designed primers for these using the Primer3 feature in MSATCOMMANDER (Primer 3.1.1.1). We used the program MicroFamily (version 1.2, Meglecz, 2007) to identify microsatellites present more than once in the data set, and removed these loci as well as those containing microsatellite-type repeats within the primer sequence, yielding 324 possible primer combinations (see Appendix 1).Twenty-five loci were selected based on repeat number and type. Primers were labelled with both forward and reverse MRT tags for genotyping (see Hayden *et al.*, 2007, Hayden *et al.*, 2008).

Results and Discussion

Optimum primer concentrations were determined using three individuals. PCRs were performed in a 12 μl reaction containing approximately 10 ng of genomic DNA, 75 nM of dye-labelled tagF, 75 nM of unlabelled tagR and a concentration of locus-specific primers between 10-60 nM. The PCR cycling conditions used were as described in Hayden et al. (2008). Genotyping was performed by capillary electrophoresis at AGRF (Adelaide, Australia). Of the 25 loci, 16 consistently amplified a product of the expected size at one or several locus-specific primer concentrations. We found 13 of these loci to be polymorphic after

genotyping eight individuals from several sites across the Broughton and Wakefield catchments However, locus Pa_16 was homozygous in all eight individuals, and may be sex-linked or from mitochondrial DNA. The remaining 12 loci were separated into two pools for capillary electrophoresis (Table 1) and used to genotype 40 individuals from the Wakefield River, South Australia. Observed and expected heterozygosity were calculated using GenAlEx (Version 6.2, Peakall and Smouse, 2006). Hardy-Weinberg equilibrium and linkage disequilibrium between loci was estimated using GenePop (4.1.10) on the web (Raymond and Rousset, 1995). Null allele frequencies were estimated using MICRO-CHECKER (Brookfield 1 estimator, van Oosterhout *et al.*, 2004). The number of alleles per locus ranged from two to 14 with an average of 7.16. Mean (range) observed and expected heterozygosity was 0.512 (0.136-0.909) and 0.590 (0.165-0.788) respectively across all loci. We found no evidence of linkage disequilibrium between loci; however, Pa_07, Pa_10 and Pa_14 showed significant deviations from Hardy-Weinberg equilibrium, all exhibiting a heterozygote deficit. The reason behind this is unclear at this point, however, we suspect populations may be experiencing inbreeding owing to the current drought reducing available habitat. Locus Pa_03 also showed a heterozygote deficit, however, we also found evidence for null alleles at this locus (estimated frequency = 0.1386). These microsatellites are currently being used to examine the genetic structure of *Paratya australiensis* in two catchments in the Clare Valley, South Australia.

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References

Baker, A. M., Hurwood, D. A., Krogh, M. & Hughes, J. M. 2004. Mitochondiral DNA signatures of restricted gene flow within divergent lineages of an atyid shrimp (*Paratya australiensis*). *Heredity,* 93**,** 196-207.

Cook, B. D., Baker, A. M., Page, T. J., Grant, S. C., Fawcett, J. H., Hurwood, D. A. & Hughes, J. M. 2006. Biogeographic history of an Australian freshwater shrimp, *paratya australiensis*: the role of history transition in phylogeographic diversification. *Molecular Ecology,* 15**,** 1083-1093.

Cook, B. D., Bunn, S. E. & Hughes, J. M. 2007. A comparative analysis of population structuring and genetic diversity in sympatric lineages of freshwater shrimp (Atyidae: *Paratya)*: concerted or independent responses to hydrological factors. *Freshwater Biology,* 52**,** 2156-2171.

Faircloth, B. C. 2008. MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources,* 8**,** 92-94.

Hayden, M. J., Nguyen, T. M., Waterman, A. & Chalmers, K. J. 2008. Multiplex-ready PCR: A new method for multiplexed SSR and SNP genotyping. *BMC Genomics,* 9**,** 80.

Hayden, M. J., Nguyen, T. M., Waterman, A., McMichael, G. L. & Chalmers, K. J. 2007. Application of multiplax-ready PCR for fluroescence-based SSR genotyping in barley and wheat. *Molecular Breeding,* 21**,** 271-281.

Hughes, J. M., Bunn, S. E., Kingston, D. M. & Hurwood, D. A. 1995. Genetic differentiation and dispersal among populations of *Paratya australiensis*

(Atyidae) in rainforest streams in southeast Queensland, Australia. *Journal of the North American Benthological Society,* 16**,** 158-173.

Hurwood, D. A., Hughes, J. M., Bunn, S. E. & Cleary, C. 2003. Population structure in the freshwater shrimp (*Paratya australiensis)* inferred from allozymes and mitochindrial DNA. *Heredity,* 90**,** 64-70.

Kemp, S. 1917. XVII. Notes on Crustacea Decapoda in the Indian Museum. XI. Atyidae of the genus *Paratya* (= *Xiphocaridina*). *Reconds of the Indian Museum,* 13**,** 293-302.

Meglecz, E. 2007. MicroFamily: A computer program for detecting flanking region similarities among different microsatellite loci. *Molecular Ecology Notes,* 7**,** 18-20.

Peakall, R. & Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 228-295.

Raymond, M. & Rousset, F. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity,* 86**,** 248-249.

Riek, E. F. 1953. The Australian prawns of the family Atyidae. *Records of the Austrlian Museum,* 23**,** 111-121.

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shiply, P. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes,* 4**,** 535-538.

Williams, W. D. 1977. Some Aspects of the Ecology of *Paratya australiensis* (Crustacea : Decapoda : Atyidae). *Australian Journal of Marine and Freshwater Research*, 28**,** 403-415.

Table 2: Microsatellite primer sequences and product information for *Paratya australiensis* including dye used for MRT pooling.

	Locus	Repeat motif	Forward Primer	Reverse Primer	Primer conc (nM)	Dye	Size Range (bp)	N_a	H_o	H_e	GenBank Accession number
			Sequence	Sequence							
Pool 1	Pa_03	(AC)^20	ACGACGTTGTAAAAGAGACGGCAAACCAAGAGC	CATTAAGTTCCCATTACCGTGACAAGTTCTGGGAAAG	20	FAM	196-206	3	0.333	0.562	HQ010424
	Pa_06	(AC)^13	ACGACGTTGTAAAACCTCTCAATGTCTTCTTTGTTGC	CATTAAGTTCCCATTACCCTGGCCATTTTCTCAAGC	10	NED	213-217	2	0.182	0.165	HQ010425
	Pa_07	(ATT)^33	ACGACGTTGTAAAAGCCTGTCCTACAGATCCTCG	CATTAAGTTCCCATTAACTGTCCAGCTTGTCTCCTC	20	VIC	225-324	14	0.409	0.788	HQ010426
	Pa_10	(AAT)^24	ACGACGTTGTAAAATGTTTTATACCTGAAGTGCGG	CATTAAGTTCCCATTAAAAGGATCTGCCTAAATCATCAC	20	PET	277-338	5	0.136	0.462	HQ010427
	Pa_14	(ACT)^22	ACGACGTTGTAAAACGCCAAGTTATGATAGGGTCAG	CATTAAGTTCCCATTATCATCAAAGTAGTACAGTTAAGGAGC	20	FAM	244-277	3	0.182	0.468	HQ010430
	Pa_18	(ATT)^20	ACGACGTTGTAAAAAGAACTCCTTCAGCCCCAC	CATTAAGTTCCCATTAACAAACGGAACCCTACCCC	40	FAM	297-336	6	0.318	0.439	HQ010433
	Pa_21	(ATGT)^21	ACGACGTTGTAAAATGACACTTGTATAAAGGGAGCATC	CATTAAGTTCCCATTAACTACGAGACGGGAAAGCG	20	PET	224-260	10	0.864	0.852	HQ010434
Pool 2	Pa_12	(ATT)^23	ACGACGTTGTAAAAACATGCCCTATGGAAGGAGAC	CATTAAGTTCCCATTAACCATAAATCCACTCACATACTGAC	20	NED	252-286	9	0.909	0.753	HQ010428
	Pa_13	(ATT)^22	ACGACGTTGTAAAAACTTCACCGCGTCCATTTG	CATTAAGTTCCCATTATGCCATCCTACTGAAGATTTGG	20	VIC	240-290	14	0.818	0.771	HQ010429
	Pa_15	(AAT)^21	ACGACGTTGTAAAAGCTTTCGAATCAAGGGCTCC	CATTAAGTTCCCATTACCACGCTGGAGTGATTATGAG	20	FAM	295-423	8	0.773	0.737	HQ010431
	Pa_17	(ATT)^20	ACGACGTTGTAAAAATAACCTGTATGTATCTGCAACG	CATTAAGTTCCCATTACTCTTCGTCTTTCTTGGCTG	20	FAM	253-284	5	0.5	0.474	HQ010432
	Pa_24	(ATCT)^17	ACGACGTTGTAAAAAGTTGACGGCATTGATATGTTCC	CATTAAGTTCCCATTAACAGAACACAGACACACAAACC	10	PET	229-265	7	0.545	0.584	HQ010435

*bp: Base pairs, N_a: Number of alleles, H_o: Observed heterozygosity, H_e: Expected heterozygosity

Appendix 1: Sequence information for all 324 primers identified for microsatellite loci in *Paratya australiensis*

Name	454 Sequence	Repeat	Left Primer		Right Primer		Product length (bp)
			Sequence	Melting Temp (°C)	Sequence	Melting Temp (°C)	
Pa_01	FU1G3FE02Q059K	(AT)^33	TGGAACCCTTGTTTGTGAGAG	58.835	ACCGGTTACGGTTACGTTAG	58.111	225
Pa_02	FU1G3FE02RCXX2	(AG)^25	TGCTACCGCTCACAGGATG	60.232	CGTCGTCTACTCTCGTCTACTC	59.954	239
Pa_03	FU1G3FE02SF68U	(AC)^20	GAGACGGCAAACCAAGAGC	59.865	CCGTGACAAGTTCTGGGAAAG	59.877	180
Pa_04	FU1G3FE02PYTW7	(AG)^19	GGGTGAATGGGGAGTAATTGAG	59.216	TCGGTAGTAGATCAAAAGAAACCG	59.358	211
Pa_05	FU1G3FE02R3ZF4	(AT)^16	CTGGTAGTGAACAGCGAGC	58.988	GTTTACGGTACGTTACCCCG	59.11	227
Pa_06	FU1G3FE01BZ30G	(AC)^13	CCTCTCAATGTCTTCTTTGTTGC	58.963	CCCTGGCCATTTTCTCAAGC	60.223	187
Pa_07	FU1G3FE01ESWIY	(ATT)^33	GCCTGTCCTACAGATCCTCG	60.081	ACTGTCCAGCTTGTCTCCTC	59.791	208
Pa_08	FU1G3FE02SVXNG	(ATT)^26	TGTGATGGAAAGGGGTTTGAC	59.177	ACCCTACGAAACCAAACGAC	58.877	219
Pa_09	FU1G3FE02PO4QF	(ATT)^25	TGCGGTATTTGGGTAATGGAG	58.839	AGATCACCCTACGAACTCTAGC	59.551	242
Pa_10	FU1G3FE01AHRBO	(AAT)^24	TGTTTTATACCTGAAGTGCGG	57.016	AAAGGATCTGCCTAAATCATCAC	57.412	289
Pa_11	FU1G3FE01D0DIZ	(AAT)^24	AAGCCCTAGAGCCAACACC	60.081	AGGACCATGAATTCGTTCTTTCG	60.076	249
Pa_12	FU1G3FE02RMF8L	(ATT)^23	ACATGCCCTATGGAAGGAGAC	60.006	ACCATAAATCCACTCACATACTGAC	59.429	246
Pa_13	FU1G3FE01BLF2B	(ATT)^22	ACTTCACCGCGTCCATTTG	59.494	TGCCATCCTACTGAAGATTTGG	58.883	225
Pa_14	FU1G3FE02RBOPT	(ACT)^22	CGCCAAGTTATGATAGGGTCAG	59.364	TCATCAAAGTAGTACAGTTAAGGAGC	59.456	245
Pa_15	FU1G3FE01CT29P	(AAT)^21	GCTTTCGAATCAAGGGCTCC	60.011	CCACGCTGGAGTGATTATGAG	59.271	271
Pa_16	FU1G3FE02Q5CQC	(ATT)^21	TGTGCATGCTCTCCTCTCG	60.232	AAAATAGCCCGCCATGCAG	59.633	237
Pa_17	FU1G3FE01AVHC2	(ATT)^20	ATAACCTGTATGTATCTGCAACG	57.265	CTCTTCGTCTTTCTTGGCTG	57.206	246
Pa_18	FU1G3FE01ENJHI	(ATT)^20	AGAACTCCTTCAGCCCCAC	59.697	ACAAACGGAACCCTACCCC	60.004	289
Pa_19	FU1G3FE02THMAS	(ATGT)^25	TCTGTTTACACACACACCTTTG	57.8	AAACACGACACGCACTGAC	59.723	243
Pa_20	FU1G3FE02RPEMG	(AGGT)^22	CTTGACCAATGCCTAGACCC	58.714	ACATGAATGTTATCGACACGG	57.186	237
Pa_21	FU1G3FE02SL11L	(ATGT)^21	TGACACTTGTATAAAGGGAGCATC	59.346	ACTACGAGACGGGAAAGCG	60.231	227
Pa_22	FU1G3FE02TO7OK	(ATGT)^19	GATGCAGTGCTGGCTTCG	59.935	GTCCGTACGTCCATCCGTG	60.956	196
Pa_23	FU1G3FE01BTM2A	(ATCT)^17	TGTCTGTGTCTTGCTCCTACC	60.146	ACAAGGAAAGGTTTCAACCCAC	60.009	242
Pa_24	FU1G3FE02Q9Z9J	(ATCT)^17	AGTTGACGGCATTGATATGTTCC	60.137	ACAGAACACAGACACACAAACC	60.012	232
Pa_25	FU1G3FE02QVL7X	(CTGT)^17	CGTTACCGCTTGCAGGATG	60.013	AGACAAATTATCAGACTCTCTAACAGC	59.8	218
Pa_26	FU1G3FE01BXV6J	(GT)^10	ACTGGCTCTCTGAAATGCG	58.594	ACACACACACGCACACAC	59.551	99
Pa_27	FU1G3FE02TWXZX	(AAT)^9	GTATTATAGGAAGAGGGAAAGCGTG	59.842	GCTTGGCTGCTATTTCTTGTG	58.873	100
Pa_28	FU1G3FE02QXNIQ	(GT)^8	CAAGCCTGAGCTTCATTTTCTG	58.917	ACACAAACAACTATTTCACTTACGC	59.562	109
Pa_29	FU1G3FE01DGFI2	(GCT)^9	ACACATCAGCATACATCACAAAC	58.458	TGAAGGTGAACGAAGCTGG	58.44	127

Pa_30	FU1G3FE01DCLAS	(ACAT)^16	GGATCATGTGTAATCATAAGGGTTG	58.565	TCGTATCGTATCGTATCGTATCG	58.205	131
Pa_31	FU1G3FE01DDQZF	(ATT)^19	TCTGTGGTTGTGGCCCTG	60.244	GGTGCAAATTCCATCTACCAGC	60.661	133
Pa_32	FU1G3FE02S6QNV	(AAT)^13	TGTGTAGAGTCAAACGCATTC	57.324	TGGGCTGCTTACAAATGTTCC	59.872	135
Pa_33	FU1G3FE02SN1HR	(ATCT)^16	AGTAATGGTCATCCTCGACAAC	58.637	TCATAGCAATACCATCTTCGAGTG	59.234	136
Pa_34	FU1G3FE01C4K4D	(ATT)^18	TTTCTCGCTGCAAGGAGTTG	59.517	ATCTACTATTTTACTCCCGAGCC	58.047	149
Pa_35	FU1G3FE01D4TZL	(ACAG)^15	TTGGTAGACAGGCAGACAAAC	58.914	ACTTTACTATCTCTGGTTACAGGC	58.552	149
Pa_36	FU1G3FE02PLCD8	(ACTG)^11	CCCTGAAAACCATGTGTATAACCC	60.375	TCCTCGACAATGCGGGAC	60.164	149
Pa_37	FU1G3FE02QB2AV	(AGG)^19	GCAAGTATGACCAAGTGGAGC	60.012	CCGTCCTGCTACCTCGC	60.252	149
Pa_38	FU1G3FE02QSNUQ	(AT)^10	ATGTGGGGCTTCCCTCTTG	60.08	GATCCGACTTACGGACAAAACC	60.079	149
Pa_39	FU1G3FE02RXHTD	(CT)^8	AGCGCTGAATGACCCTCTG	60.532	AATACGTAGTTTCTCAGCTTCCC	59.009	149
Pa_40	FU1G3FE02TFX4V	(GT)^8	TCTTCGTGAATGGTTTAAGGTGAG	59.773	TTCTTTAGTGCAAGCAAATCATCC	59.054	149
Pa_41	FU1G3FE01BIVEA	(CT)^9	CACCGACCTAGTCATGGGC	60.605	AGAGTTATTGGATGAAGAGGATTCTG	59.278	150
Pa_42	FU1G3FE02PKE45	(AC)^11	GGAAGATACAATGCGGGAGG	58.868	TCTGGTTACAGGCTGAGTG	57.127	150
Pa_43	FU1G3FE01C1T4X	(AT)^32	GCTACCGGATGAGCCTTGG	60.977	ACTCGTCTCTCTACTCTCTCTC	58.111	152
Pa_44	FU1G3FE01CBWVG	(CTT)^8	CTCACGGAATGCAAACCCG	60.231	CCCAAATCAAACTATGCAGCG	58.943	152
Pa_45	FU1G3FE02PXAEA	(AT)^10	TGGCAAGTACTCTCTCCCG	59.176	AGAGTGATGAGTGGGTGGAAG	59.868	152
Pa_46	FU1G3FE02RK9FL	(AC)^8	TCATGAATCTTTCCCATTCATCC	57.398	TCCATGCCTCTTCTATTCAAAACC	59.829	152
Pa_47	FU1G3FE02S4EO0	(ATT)^17	TGGTACGTTTTCTTCACATACTCG	59.838	TCCGCTGTTGTTTAAAGGGG	59.147	152
Pa_48	FU1G3FE02TZ1SF	(ATT)^8	TTGATGGGCCTCATAGTGTC	57.755	TGTCAGTGTTGGTTACAGGAG	58.292	152
Pa_49	FU1G3FE01DIHMQ	(CT)^8	CTGTCTTCGGCTCGTGTTG	59.58	AGCGTAGAGGAGGACACCC	61.145	153
Pa_50	FU1G3FE02SKEWX	(GT)^19	TGATAGTTTTCAAATGAGAGGACG	57.795	ACTTGAGAAACAACTAACACGC	58.216	153
Pa_51	FU1G3FE02Q06UX	(CT)^8	TGATTTGCGTAATGACAGCC	57.539	GCTTCATCGTCCTTGTGAGAC	59.747	154
Pa_52	FU1G3FE02S7G8C	(ATC)^8	CCAATGTACAACAACATTCATCATC	58.015	ACTATAGCACCAGCAGACG	57.394	154
Pa_53	FU1G3FE01B86AH	(AC)^8	TGACGGTACCTGGGAGTTG	59.4	GTGTGCGCGTGTGTATGTG	60.871	155
Pa_54	FU1G3FE02PX3D7	(GT)^8	ATGGGTGAGTGTGGTTCCC	60.003	AGGTGAATTTTGCACCGAC	57.176	155
Pa_55	FU1G3FE02QZVDX	(ATT)^11	TCGGTGTGCCTGGATATGG	59.93	TGACCCGGTTAAGATTCCCC	59.859	155
Pa_56	FU1G3FE01C81MV	(GT)^9	TTGCAGAGGGGCGTAAGAG	60.157	CCATGGACAACCAACAGCC	59.783	156
Pa_57	FU1G3FE02SFER1	(ATT)^16	AGAATGGGCGTAGATGCCG	60.679	CGGAGGGGATCAATTTGTTGG	60.01	156
Pa_58	FU1G3FE01AK1J5	(GT)^11	ACATTCCTGCAAACAGAGATTGG	60.263	GGTTACAGGACACGCAAAAC	58.34	157
Pa_59	FU1G3FE01BAWRL	(AAT)^10	TGCTTTATATGCCGATTGTGGG	59.88	TGCATCTGAGTGATTGTATTAAGC	58.155	157
Pa_60	FU1G3FE01ETV33	(AC)^12	ACTAACACTGAATTCCCTCGC	58.783	TGATAATTGTGATCGAGTGTTGAG	57.867	158
Pa_61	FU1G3FE02Q5H98	(GT)^13	GGTCATCGCAACATGAAGTG	58.201	TAATGACCTGGGCGGTGTG	60.459	159
Pa_62	FU1G3FE02RUEPZ	(AT)^8	GAGCTGAGCAGTTTACTTCGAG	59.824	AACCGTGCTTGAATGTGTG	57.495	159
Pa_63	FU1G3FE01BO17C	(ATT)^17	ACGTATGTATTGTAATTATCGTCCC	57.654	GACTGGGTTTGCTCCTTGG	59.106	160
Pa_64	FU1G3FE01BP68V	(ACT)^10	TTCCCCGGAGACACAACAG	60.006	AGTAGTAGTACGCAAACAGAAGTAG	58.925	160

Pa_65	FU1G3FE02R7N4D	(AT)^8	AGAAATACACAGAGGATAAAACCCC	59.362	TGCGCAGAGAATAGGATCG	57.864	160
Pa_66	FU1G3FE01AQJSI	(GT)^8	CACTTAAACTCATATTTCGATTGGC	57.857	GCCGGAGAAAGAAAACACCC	60.152	161
Pa_67	FU1G3FE01ELL0I	(CT)^13	TGGCAACGACGTCACAAAG	59.721	GTACCGCCGACTACGAGAG	60.086	161
Pa_68	FU1G3FE01EI21O	(AAT)^9	GGTCAGAGGTTATTGTGACTCC	58.9	GGGGTTATCAAGGTGTGGTG	58.926	162
Pa_69	FU1G3FE02PKIF4	(AG)^8	AGAGGAAAGCGAATTGCGTC	59.662	CCTCTCTCTCTCCTTATTCTATCTCC	59.673	162
Pa_70	FU1G3FE02S2YPH	(AT)^8	AGCCTGAAAGAAGAAGGTGC	58.856	ACAAGCTGGACTTGTTGGTG	59.364	162
Pa_71	FU1G3FE02SZKZ2	(AT)^11	GGCACCATAGATGGACTTGC	59.44	AGGTTTGTAGTTCCCGATTTAAG	57.3	162
Pa_72	FU1G3FE02R3LSN	(AC)^12	CGGCCCCATTCAATCACAC	59.935	TGAGCATTGACATAAAAGGGAGAG	59.587	163
Pa_73	FU1G3FE02S5NOP	(AAG)^8	GGTGAATAGAATGCTGTACTGGC	60.2	AGGTAGGTAGACCACACAATGG	59.941	163
Pa_74	FU1G3FE02TOPCD	(ATCT)^13	TAGCTCCTGCCAAGTCAGC	60.157	ACTATAAGTTCTTGCCTCTCTGTC	58.558	163
Pa_75	FU1G3FE01D1UYQ	(AGAT)^16	TGGAATAACTGAGCTGCAAGTG	59.686	TGTGACATGTGAGGACTTTTG	57.143	164
Pa_76	FU1G3FE01AJEBF	(AG)^22	TCAAGCTGCAACAACTGCC	60.009	TTACCGTCCTTCCGGTTCC	59.781	165
Pa_77	FU1G3FE01EAHFF	(CT)^8	GGAAGGAGGATGCTAGCCC	60.005	TTGGAAGCAGTTTTGGAGAAG	57.4	165
Pa_78	FU1G3FE02PQ3VK	(AG)^8	ACACAATGGGACGTACAGAG	58.008	TCTTCTGCCTGCCTATCCC	59.236	165
Pa_79	FU1G3FE02TDO9Q	(ACC)^8	TGTTAGCCCCGAAAATGGG	58.485	CCATTCCCACTGCTACTTCC	58.714	165
Pa_80	FU1G3FE02TWVQI	(ACT)^8	ACATCTGTGACTACAACTTCCAC	59.134	ACTCAATGCTTGAATCTATGCC	57.584	165
Pa_81	FU1G3FE02Q42XK	(GT)^8	GTGTGGGTGGGTTTTGGTG	59.932	AGCTTGAAACTCAACTGTTTGC	58.98	166
Pa_82	FU1G3FE02QBOKN	(CT)^11	TTTCCTTTGCAGTCAGTTAGAC	57.332	CCGTTCAAGACTACTCTCTGTTG	59.523	166
Pa_83	FU1G3FE01BSOK8	(AG)^15	GGAAGAAAACTAGACTTAGGGTGTG	59.84	ACAAACCGTACAACAGGCG	59.423	167
Pa_84	FU1G3FE02TN224	(AAT)^23	GTTTTGAAACGTTGTATGTTTGGTG	59.288	TCCCTTACACGGAGTATTTCTTTAG	58.906	167
Pa_85	FU1G3FE01BC2RG	(AC)^10	TGACATAACTTTGTTCCGGC	57.096	TCCAAGCCCGTGACCTTAC	60.082	168
Pa_86	FU1G3FE01CP4I0	(ATT)^20	TTGTACGTGCCGATTGTGG	59.202	ACAACCTCAAGTAGTTAATCACG	57.332	168
Pa_87	FU1G3FE01ELG14	(AAT)^22	GGTGCCCATTTGCTGCTAC	60.232	AGAAACACATACAAAGCCACAAG	58.576	168
Pa_88	FU1G3FE02RNTYI	(AG)^9	GACATCTGTTGCGTTTCAATGG	59.507	ATCATCTCTCCGGTGCCTC	59.323	168
Pa_89	FU1G3FE01BWJUC	(ATCT)^12	ATTACGTGGTCGTTTTGACG	57.515	GGATGGATGGATGGATGGATG	58.757	169
Pa_90	FU1G3FE01EKAVK	(ATT)^21	GGGGTGCAACAGGTATTGATTG	60.598	TAAGCCGACAAAGCAAGGC	59.49	169
Pa_91	FU1G3FE02PFB4C	(AAT)^8	TGCTTGGTTCATAGTCATGTGC	60.012	AGGACCGTCTGTATGTCCG	59.259	169
Pa_92	FU1G3FE02R1DCJ	(CT)^10	GACCCTGTTTCTTTGGCCTC	59.508	ATGAGCTAAGCGCCTACGG	60.38	169
Pa_93	FU1G3FE02R6YQ1	(CTGT)^8	TGATTGGGTAGATTTGAAGTGTC	57.295	TCCTATGACATTCCCCAGGC	59.637	169
Pa_94	FU1G3FE02RASM0	(AG)^12	GGGGACTCTCTGTATCGGC	59.708	GTCATTGTTTGTTCCAGTATGAATCG	60.018	169
Pa_95	FU1G3FE02TN07U	(CTT)^20	TGTCCCATTCAGTAATGACAATC	57.357	GAAGGGAAGGGAGGTAAGGG	59.563	169
Pa_96	FU1G3FE01DTS55	(AAT)^16	ACGACCAGACCACAATGCC	61.055	CGTTAGTGTTTGTTGCCTTAAGATAG	59.526	170
Pa_97	FU1G3FE02QLBZY	(AAT)^14	ACTCTCTTCAGCTCCATACG	57.218	TGTGCTCATGGGATTTATGTCAG	59.57	170
Pa_98	FU1G3FE02RC6Q0	(ATT)^18	GTTTGGTTTCTGGGGTCCG	59.411	GTTGCATAACCCCGATGGC	60.01	170
Pa_99	FU1G3FE02S0K58	(AAAT)^8	TGTGAGTGAAGATGACTAACCAG	58.628	CTCCATCGGTGGGTACAGG	59.932	171

Pa_100	FU1G3FE01CJG9W	(ACAT)^12	TGAAGATCAACTTTCTGCCTTG	57.655	TGCTGTATCTGAAAGATTGCAC	57.803	172
Pa_101	FU1G3FE01DRPG5	(AAC)^8	CTGTTGCAGCCTTATCTGCC	60.011	GGGATGTTGATAGTATTCCTGATGG	59.604	172
Pa_102	FU1G3FE02QCMSO	(AC)^9	GCTTTTCGCTTGTGGATGC	58.997	TTGTCTGTGTGCGTGTGTG	59.647	172
Pa_103	FU1G3FE01BYSO6	(AAT)^8	AGAGGCAGAGACTTCACGG	59.481	CCACAATTGCTAATGCTGCTG	59.209	173
Pa_104	FU1G3FE01EM37F	(CT)^9	CGAGTAGGCAAGGGCAAAG	59.27	CAACTGGAATTGCCACGGG	60.158	173
Pa_105	FU1G3FE02TMMHL	(ATT)^12	TGTTGTTGTTTTGTTGTTGCAG	58.024	GCTAACTCCGAATTTATTGGTGAATG	59.852	173
Pa_106	FU1G3FE02Q1NBW	(AAT)^16	CTCAGTCGATCGTCAATACTTCC	59.465	TGACACGAATTTTGTAGGTGTATG	58.285	174
Pa_107	FU1G3FE02RRVPB	(GCT)^11	GGTATCACTGCTTGTATGGACG	59.628	TGCAGCAGAACAGAACAGC	59.417	174
Pa_108	FU1G3FE01CWYR4	(CT)^8	CACCGACCTAGTCATGGGC	60.605	TCAATAGTGGCGTTGTTGC	57.277	176
Pa_109	FU1G3FE01DZ4YL	(ACAT)^8	TCAATGGCTGGCTGTTTGG	59.402	GTGACTACTGCTGCTCTTGC	59.664	176
Pa_110	FU1G3FE02S39F8	(ATGT)^15	AGAGCAAAATCATGTCCCGTG	59.671	GGATGTGTTTTAATGGTTGCGG	59.5	176
Pa_111	FU1G3FE01B4OIV	(ATT)^17	AGCCATGACAACCCTTTCC	58.399	GGCCTAGCTGCCCTGTG	60.504	177
Pa_112	FU1G3FE01B7QDB	(ATGT)^20	ATGTGCGGACGGAAGGATG	60.902	CACATGCACTTACATTGTGCTG	59.505	177
Pa_113	FU1G3FE01BOOGF	(AGT)^11	ATTGGGTCGGTGGTTCGAG	60.457	GACTAGACTTGCTGCTGCTAC	59.209	177
Pa_114	FU1G3FE01D29GD	(ATGT)^20	GTATGAGCGTGCGATGTGAG	59.878	ACCCACCCACCTACCTACC	60.703	177
Pa_115	FU1G3FE02PL34K	(AAT)^12	TGGTTACCGTCCGCTTGAC	60.75	ATACGCAAGACTGCGGTTG	59.278	177
Pa_116	FU1G3FE02TTSIO	(AT)^10	AAGCCTCTAAAGCAACGCC	59.189	TGGCAGTGGTAGACAGTATG	57.409	177
Pa_117	FU1G3FE01A463M	(AC)^8	AATGCCCGTTCTGAAGCTC	58.887	CGTTGATTTTGGTGTTTAGTGGTC	59.781	179
Pa_118	FU1G3FE01D2US3	(AG)^12	TGAAAGGCAGTTTTCTCCATC	57.191	GGGGACAGTTTCATTAGTCTACC	59.003	179
Pa_119	FU1G3FE01DARPU	(ATGT)^15	CACTACAAGGCAGCTGGTC	58.896	ACCTACTTACGTGCAGTACC	57.438	179
Pa_120	FU1G3FE01DBA47	(ATCT)^8	AGCCAACTCTGAAACTTCACC	59.186	GGTGGCGATGAAAGTGATACG	60.148	179
Pa_121	FU1G3FE01BCKI6	(ATT)^13	TCCTGCTGCTCAGACATCG	60.232	TCCTCAGTGATAGCCATAGGTC	59.276	180
Pa_122	FU1G3FE02TSSYE	(ATGT)^11	AGGGTGAATGGGAGTAATTGAG	58.268	TCCTCAAGGTAAACCCGGC	60.081	180
Pa_123	FU1G3FE01AKR0R	(AC)^9	GCATCATCGGCGTTAAGGTG	60.43	AACGAGGAAAGTGTATTGTGTG	57.68	181
Pa_124	FU1G3FE01DW3HA	(CTT)^12	CCCCTTTAGCTTGCCTGTTC	59.579	AGCATTACACTTGCCTTCAGC	59.943	182
Pa_125	FU1G3FE01EPMR6	(CT)^8	GAGGCTCTTGATTAGTCCCTG	58.294	GGCCAAGTCATCACGCTAC	59.351	182
Pa_126	FU1G3FE02SUXL1	(ATT)^9	GCATGAAAGCAATAACTCACTTCG	59.959	GGCTGATGAGGAATGCTGTG	59.727	184
Pa_127	FU1G3FE01DVINU	(AGG)^13	CAAGCAGACAAGACAGGCG	59.867	TGGGTTGTACCTTGGGTGG	59.926	185
Pa_128	FU1G3FE02RIM3P	(AAT)^15	TGTGCTCGTAGTTCCCAGTC	60.152	ACCTCTGTGGTTTGGTTTGG	58.99	185
Pa_129	FU1G3FE02PGC1R	(AAT)^9	TGGAGCTAACTCATCAGGCG	60.295	TCTAGCCTTGGTGTATTGTCG	58.236	186
Pa_130	FU1G3FE01DNFV7	(GGAT)^9	TTGGCATCCATGTCCCTCC	60.157	ACTCGAGAGTAAACGCTGTTG	59.079	187
Pa_131	FU1G3FE02SIPNI	(AG)^18	TGCCAACCACGAAATGCAC	60.379	CTCCTACTATCTTTCCTTACTTCTTTC	57.748	187
Pa_132	FU1G3FE02TL8IF	(ACT)^15	GCAACTACAACCACCACCAC	60.082	GGCCAATGAACTGCGTCTG	60.231	187
Pa_133	FU1G3FE01A9K80	(ATT)^9	CTTTATGATGTATTCTAGAGAGAGTGC	58.145	ACTCTGAGGCTTTCCTGCG	60.457	188
Pa_134	FU1G3FE01B1NQQ	(ATT)^12	TCTCTTGATGTTATGAGAGCCTTTTC	59.958	GCCCTACGAAACCAAACGAC	60.222	188

Pa_135	FU1G3FE01CJS4D	(AT)^10	CGTACGTTACTGGACGGTTTG	60.017	ACACTCGCATTCTACTGTTCAC	59.434	188
Pa_136	FU1G3FE02RAIRL	(AC)^9	AAAGCATGTACACCCACGC	59.49	TCACTGAAACAGCACCACAC	59.371	188
Pa_137	FU1G3FE02SAZQD	(AAT)^14	ATATGCCGATTGTGGGTTTG	57.21	TCGGTTGGGAAAGGGGATG	60.08	189
Pa_138	FU1G3FE02SCFJ5	(AG)^15	AGGTTCCTTTTGATCTACTACCG	58.431	CGTCGTCGTCGTCTCTCTC	60.372	189
Pa_139	FU1G3FE01BNMRS	(CTT)^16	ATTCACGGCGGTCATTTCC	59.266	TCGATGATGAGGAGCAAAACAG	59.493	190
Pa_140	FU1G3FE01EXEOI	(ACAT)^12	TGTTGTTTGTTCAATTCGTGCC	59.566	TCTGTATCGCCATATTTGCTTG	57.676	190
Pa_141	FU1G3FE02SD8V1	(AAT)^10	CTACCATTGCTGCCACTACC	59.084	ACTGCTTGCTTAGAACAACTG	57.5	191
Pa_142	FU1G3FE02QS6J1	(AGG)^15	AAGAGGTGTGAGGGAGTGC	59.701	GCCTCTTCCCCTTCCCATTC	60.878	192
Pa_143	FU1G3FE02R0919	(AC)^9	TCTGTCATGAAGCCTGAGC	57.901	CACTCGTTCTTTTGCATTCAGAG	59.285	192
Pa_144	FU1G3FE01CAOFX	(GT)^14	TGATATGTGTCTGTGTATTGTGGC	59.893	ACCTACCTCCTACCTACCTACC	59.733	193
Pa_145	FU1G3FE02SRP88	(ACCT)^8	TTAGCATCGACTGTCACCC	57.611	GGGTGGGAAGGTAGGTAGG	58.845	193
Pa_146	FU1G3FE01A297J	(AAT)^13	TTGATCCCTCATCGACCCC	59.238	TAGTCTCGTGCAGCCCTTG	60.158	194
Pa_147	FU1G3FE01EFCZO	(ATT)^14	TGCCGTTTGTAGGTTCTTGAG	59.265	TGGGGCAATAAGGTTTCGC	59.179	194
Pa_148	FU1G3FE02PH9ON	(ATGT)^9	TCGATGGAGAGGACAGTGG	58.869	ATACATACACCACGCACGC	59.06	194
Pa_149	FU1G3FE02Q1DHL	(AC)^9	ACTAACTTGCTCAAGAAGACGAC	59.457	AGGCCCTTCAAAACCGAG	57.929	195
Pa_150	FU1G3FE01CJKTU	(ACAT)^13	CAAGTGCCTCCCGTTTGAG	59.493	AGCTTATTTGTACTCGCGTATCG	59.773	196
Pa_151	FU1G3FE01D4GRQ	(AAT)^8	TGTGTGCATGGGTAAATATGTTATG	58.736	TGGCTGGGTGAATTCTTGC	59.099	196
Pa_152	FU1G3FE02SYFMJ	(GT)^8	GCTAAAATTCCAGGATTGTCATATAGC	59.534	ACAAGGTGGTAGAATGGAGC	57.974	196
Pa_153	FU1G3FE02SZJXP	(ATT)^8	TCTCAATTATTTCCTCTTCGTCTTC	57.701	ACGACGTGATCAAAGGAGG	57.927	196
Pa_154	FU1G3FE01D1AP4	(CT)^8	ACCTGTGGCTTTTGTACTCAC	59.188	TGTTGTTGATGCTGTCGTTG	57.983	197
Pa_155	FU1G3FE01DLX8C	(GT)^10	AGATAAACGAGTGAATGCTCTGC	59.89	GTTGCTGCTGCTGATGCTG	60.887	197
Pa_156	FU1G3FE02SEY5X	(AG)^37	TGTGCCTTGGACAGACCTC	60.006	CGTAACCGAACGTACCGAC	59.088	197
Pa_157	FU1G3FE02TVD1W	(GAT)^9	ACGTGATAAAGGAATGTTAAGAGG	57.539	TTCTATCACTATCACCAGCATAATC	57.094	197
Pa_158	FU1G3FE01BCKSB	(AAT)^13	AGACTTTATAGGCATTGCTTGG	57.238	TGGCTTCATCACCACCATC	58.182	198
Pa_159	FU1G3FE02RO53P	(ATCT)^9	TTGATTTAAGCCAACCGTAGTAG	57.384	TGACTTTCCACAAGCAAGCTC	59.807	198
Pa_160	FU1G3FE02SHF13	(ATC)^9	TGACGTAACCCCAAACCCTC	60.368	GTTGCGTTAGGGTCCGAAG	59.28	198
Pa_161	FU1G3FE02QECB1	(AAT)^12	CAACTGGAACATCTGCAGGG	59.582	AGACAAACGGATGAATGTGTG	57.574	200
Pa_162	FU1G3FE02RBSAK	(ATT)^11	GTTAGGCGGGTAGGGATGG	60.006	AGACAACACTTAACACTAGCAAC	57.772	200
Pa_163	FU1G3FE02RM305	(CT)^8	TTTTCGCTGCGTTGAGGAC	59.793	TGATGTCGTTGTTGGTCGC	59.501	200
Pa_164	FU1G3FE02RV4R1	(ATT)^21	TGAGAACAAAATGTACGAGCG	57.682	CATACGCCAACACGCGG	59.934	200
Pa_165	FU1G3FE02SQ4X6	(AAT)^8	AATTACATGCCAAGCAGAGAC	57.343	CTACCTGCCCAAGGGAGTG	60.157	200
Pa_166	FU1G3FE01AVRC0	(AAT)^16	GCTCTTTCAATATAGGACAAATCTGC	59.409	AGATGAGAAACAAGCTCGGTC	58.788	201
Pa_167	FU1G3FE01ET9UU	(AG)^10	ACAGATTAGCAGCACGAAGG	58.733	ATTGGTCCCAGTGCTTGGC	61.373	201
Pa_168	FU1G3FE02QZT12	(AG)^35	TGAATGGTGGCGAGTTTGC	59.787	CGTCTACGTCGTCGTCTC	57.78	201
Pa_169	FU1G3FE02TT8ZM	(AGT)^9	TCTCACTTGGCAAATTCAGCTC	59.946	TGCTGCTACCACTACTACTAC	57.06	201

Pa_170	FU1G3FE01CGN8I	(ATT)^10	AGTGAGTGACTGTGTACGTG	57.966	TGCTTAGTAAACGGACCAAAC	57.229	202
Pa_171	FU1G3FE02RI86J	(AAT)^16	TGCTCTTAATGTGGAAACTGC	57.558	TCTATACAAGTATTCCTCTACGGG	57.389	202
Pa_172	FU1G3FE02RNY1H	(CT)^8	GTTCTTGTGCATGCCCCTC	59.86	AGACAGAGAAGGCGGAACC	59.78	202
Pa_173	FU1G3FE01BOUOG	(GT)^9	CCTCATGGCAATATCCTGCTC	59.326	TGGCAATTTGTAATTCAGGAAGC	58.946	203
Pa_174	FU1G3FE01CV06G	(AAT)^17	GTGACTGGTGAGCGGATAG	58.087	TTGGTCCCGACTTCTGTGG	60.006	203
Pa_175	FU1G3FE02TADU2	(ATT)^8	ACACATGACTGACTCTCGC	57.936	GTGTAATGATTCGTGTCTTGCC	58.74	203
Pa_176	FU1G3FE01C9CAN	(AAT)^11	ACCACTACTACTGCTGCCAC	60.152	AGACACATGAATGTTGAGAACCG	60.013	204
Pa_177	FU1G3FE02PZT2O	(ATTT)^10	AGCAATTTTCACGTAGCTGGG	59.943	GCAATTTTGGAACTGATGCGG	59.748	204
Pa_178	FU1G3FE02Q4OPE	(AC)^8	ACTTGAAATGTGACCTCCAAC	57.123	AGACTAAGGTGTAAATACCCTGC	58.421	204
Pa_179	FU1G3FE02SL3BI	(CTT)^9	CTTGGCTCCGCAAACTACAG	59.942	ATCAGGGACAGGGAAAGCG	60.157	205
Pa_180	FU1G3FE02QU8HQ	(AAT)^8	TGCAAAATTAGCGTAGGTGG	57.167	TCCTTACTACACAAAGATATTGCG	57.618	206
Pa_181	FU1G3FE02TX0DT	(ATT)^18	GCAATACGCATTCAGTAGTACCC	60.261	CAGACATCACAACTAAACTAAAGTG	57.164	206
Pa_182	FU1G3FE01B4EOD	(ACT)^8	CAGCAACATCACCACCACC	59.786	AGGAAAGGACAATAAAGCTAAAGAG	57.847	208
Pa_183	FU1G3FE02P7PDI	(AAT)^10	GCGTGGGCACACTAACAAC	60.449	GGCTAGGTCAACATGGTCG	58.675	208
Pa_184	FU1G3FE02RK64W	(AT)^8	CATCGTCTCCCCAGTGACC	60.232	CCCCGAAGCAGAGGGAAC	60.48	208
Pa_185	FU1G3FE02SLDXJ	(AC)^12	CATTCTCAAGCCTGCGCTC	60.012	ACGAAGTCGAATTTGAAGAAGGG	60.014	208
Pa_186	FU1G3FE02SXLUN	(AGAT)^9	TCGAGTGCAAAGAAGGGTTTG	59.808	TGGAATCTCTGTCCGTCCG	59.559	208
Pa_187	FU1G3FE02PLXIH	(GGCT)^8	TGGTCCTTCGATTATGAGAAATGC	59.953	TGGGTAGCGTAGTATTGGGG	59.352	209
Pa_188	FU1G3FE02SL2WU	(CTT)^12	CGCTCAGTAAACAGGCGAG	59.362	CAGCTGTGGAGGAGAGCC	60.164	209
Pa_189	FU1G3FE01C6HZQ	(AGAT)^10	GTGGGAATGGGGTACGAGG	60.233	TCTGTACTGTCTGCCTGTCTG	59.873	210
Pa_190	FU1G3FE02Q0PAU	(AGG)^8	CCATCAGTCCCGGGTTTAG	57.971	ACTGTCCATCCTTCTCAGTCC	59.59	210
Pa_191	FU1G3FE02R8MEJ	(AAT)^15	TGGGCTAAACCTGAACCCC	60.002	ACTCAACTTCTACCGACCCAG	59.872	210
Pa_192	FU1G3FE01EL9ZD	(AGAT)^8	GAGGTCCCCGTTTTGTGTG	59.416	GTCTATCGGTCGGTCGGTC	60.085	212
Pa_193	FU1G3FE01DOY9I	(AAT)^13	AGTACGAATAGTCGAGAAATGTTG	57.644	ACTGGCATTAGCCATCATACTG	59.021	213
Pa_194	FU1G3FE02TRG9K	(GT)^11	TCCTCGGGATGTTCAGGTTG	60.151	GTGGTGCTACTGATGACGC	59.356	214
Pa_195	FU1G3FE02TTOUP	(GAT)^18	TACAAGTGCGTTTCGTGGC	59.794	CTCCAACTCCTCCTCGTCC	59.557	214
Pa_196	FU1G3FE01DQDTF	(ATT)^15	GAGTCAAGTCCCCGAAAGC	58.898	TGAAATATCCTCAGTTCCAAAGCG	60.133	215
Pa_197	FU1G3FE02P1UBZ	(ACC)^8	AGCTCGTTATGGTGCTTCTTC	59.131	GAGGTTGGAGACCACACTTATG	59.162	215
Pa_198	FU1G3FE02S1KY6	(AAT)^19	CATGAACGTCAAGTCGTGGG	59.944	CCAGTTGTCCCTACATTTAGCC	59.486	215
Pa_199	FU1G3FE01C3011	(AGAT)^14	CTCAGGAATGTGCAGCGAG	59.352	TACTCCGGCTTGAATTGGC	58.581	216
Pa_200	FU1G3FE01EOH84	(CTGT)^10	TCCGCATCCAAGTGTTTGTG	59.798	TCAACTTCGTCGATATTTACGGC	59.955	216
Pa_201	FU1G3FE02PTNUA	(GT)^12	TCGTTTACCCTCTGCTGGC	60.457	GCAAGGCTCTGTTCCTTCTG	59.585	216
Pa_202	FU1G3FE02R9RBX	(ATGT)^12	AAGCTATTCAGTTTGGGCTTC	57.257	TGATAAGGGCTGTGGTAGAC	57.097	216
Pa_203	FU1G3FE02RVUYF	(ATT)^17	ACCCATACTGCAACGCAATC	59.655	GACTAGCCAGCCGTGTAGG	60.306	216
Pa_204	FU1G3FE02SUHM4	(ATT)^10	TGGGCGTTCTACCCATTCG	60.531	CAGCAACAACCAACAACAACG	60.082	216

Pa_205	FU1G3FE01BAJWF	(ATGT)^8	TCTTTCTTCTTCTCCATCAATGTC	57.469	ACGTTAACACCGTAAACTGC	57.429	217
Pa_206	FU1G3FE01BGQLR	(AAT)^8	TCCTACGCCTCTTCTCTGC	59.257	TGCAGTGCTGGTTTTGGAG	59.333	217
Pa_207	FU1G3FE02PPSC5	(AAT)^13	TGCAGAAACTCGATTCTTTCG	57.676	TGTCTTCGGAATATGCAGTACC	58.706	217
Pa_208	FU1G3FE01BEM2W	(AG)^8	CACGGAGAGGAATCCAGGG	59.931	TGCGGTAATTGCCCCTTTG	59.481	218
Pa_209	FU1G3FE02ROXLI	(AAT)^20	ACGTAAACGCCCGTCCTG	60.781	AAAGGGGTTCTAAGTTTGTGTC	57.233	218
Pa_210	FU1G3FE01A3WND	(ATT)^11	AGGGGAACAAACACTGAATTTAAC	58.617	TGTGGCACTAAAGGAAACGC	59.799	219
Pa_211	FU1G3FE01ALXIU	(AT)^9	GTCATTTTGACCTGGCTGTTTG	59.369	TGTTAATGCTGTTGAAGCAGAG	58.001	219
Pa_212	FU1G3FE01BG12X	(AAT)^10	ACTCGGCTCTAGCATTGGG	59.931	TTTGCTATGCCTGCACTGG	59.181	219
Pa_213	FU1G3FE02SMK6P	(ACAG)^14	TCTTAGCTACAAACAAGAGATTGC	58.036	TCCAGATGGTAATGTGTTCACG	59.165	219
Pa_214	FU1G3FE02RZF36	(GAT)^8	CCTTTGCCGCTTCATCAGG	59.936	CGCTACTGGATAATGGATGACG	59.502	220
Pa_215	FU1G3FE01C2NXL	(ATT)^8	GGCACCATAGATGGACTTGC	59.44	TGTTGGGGAAACTTCAGCC	58.635	223
Pa_216	FU1G3FE01EAS3A	(ACAT)^12	TCAAATGGAGTGCCCGTGG	61.061	AGCGCATGTCACGACTTTG	59.867	223
Pa_217	FU1G3FE02RGF1Q	(AT)^9	TGCACCTGAAACTACCGCC	61.053	AGTCGAGATGATGAGGGCTAC	59.529	223
Pa_218	FU1G3FE02TXR9R	(ATGT)^16	ACCCCTCTGTGTCTCATTTTG	58.622	TACCTCCGTCCGTGCATTC	60.232	223
Pa_219	FU1G3FE01B3Z4L	(AG)^8	CTGGGGACGTCACAAAAGC	59.79	GACTTCTGCGGTTCTTCGC	59.942	224
Pa_220	FU1G3FE01C2P26	(ACT)^11	CTCCGACTCAGCGTGTCTC	60.594	CGTCATAGTAGAAGTAGTAGAAGTAGC	59.063	224
Pa_221	FU1G3FE02SSOM4	(AT)^8	TTTCTTAGGGGACGGCCAG	59.776	GCTTTCTGTGCAACGAGGC	60.811	224
Pa_222	FU1G3FE02SVBID	(AAT)^12	TTGTAATATGAGCCAGCAAGAAG	57.688	GGGATTGTATGCATTGTGCAAG	59.303	224
Pa_223	FU1G3FE01DHAYG	(AT)^9	GGATTGATTTTGGGGTACGGG	59.735	CCCCGGTTCCAACCCTC	60.002	225
Pa_224	FU1G3FE02P0UXB	(ATT)^15	TGTGTCAGTGTGGTGTATGTATG	59.2	AGAACGAAGCAGCCACATC	58.896	225
Pa_225	FU1G3FE02TK5T8	(AT)^8	ACCGCTTTATTTACCTTGCG	57.544	TGTTTCTTTTGGCCACCCC	59.241	225
Pa_226	FU1G3FE02QET78	(AGT)^18	GACTGAGCCCTGGAACCTC	60.157	ACCGGTTCTCGCAGATG	57.288	226
Pa_227	FU1G3FE02RA42D	(ACAT)^8	TCAGTCCGTTCTTCCGTTC	57.859	GGCAGCGTTTACACCAAGC	60.813	226
Pa_228	FU1G3FE02SHDC8	(ATT)^12	TCGAAGGCGTTTGAGTTTCAG	59.88	AGGCTAGTAATCAAGTTAATGGGC	59.402	227
Pa_229	FU1G3FE02SOQPP	(CT)^9	GAGACTCAAGCACTGGCTC	58.603	CTGTTCGTGTGTGACTGCG	60.159	228
Pa_230	FU1G3FE02SVZG3	(ATT)^9	GGTTACCCTGACCATAGGGC	60.296	ACGAAAATAGTAAGGAATGGTGTAG	57.693	228
Pa_231	FU1G3FE02QMKLR	(ACAG)^9	TCCTACCAAACATCCTTCCATC	58.273	ACTAGTCGGCAACCCTGTC	59.781	229
Pa_232	FU1G3FE01AMPE2	(GAT)^9	AGAGCACATCCTTCCGGTC	59.856	TGCGTCACATAACCAACTTCC	59.605	230
Pa_233	FU1G3FE02Q8CQ6	(ATT)^14	AAAGGCTCACTTACGTTTTCC	57.495	GGTTTGTCATGTTTCATGGCTC	59.174	230
Pa_234	FU1G3FE02R0GPA	(AT)^8	TTTGCCGACACATCCACAC	59.416	ACATAGGTCTGCCCTGCAC	60.157	230
Pa_235	FU1G3FE02TE4Q9	(CCG)^10	GGCGCAGATTTCTGTAGTCG	59.807	ACCACAGTGCTGGAGTCG	60.007	230
Pa_236	FU1G3FE01CBVC9	(AAAT)^8	TTATTCCTCTGTGCGCGTG	58.984	TGTCGGGTTTTGTAAATGGATG	57.985	231
Pa_237	FU1G3FE02Q2SXI	(ATT)^9	GGAGACACTCCAAGACATGC	59.017	GGTCATTGTGGTACAACCCG	59.586	232
Pa_238	FU1G3FE02RRF2X	(AAT)^10	ATTGCGAATCAACAAAACACAG	57.447	AAAATTCCTATCCAAGCTCTACG	57.18	232
Pa_239	FU1G3FE02SK152	(ATT)^12	TCCTCTGTGGTTGTGACCC	59.623	TCCCAAAGCAATGGTACGTC	58.938	232

Pa_240	FU1G3FE02TXAGC	(AT)^9	AACGCTCATGGATCTCGGG	60.307	AGGGATCTCGTTCGTAAGTCG	60.08	232
Pa_241	FU1G3FE01DAR9I	(CT)^8	TGTATTCTCGGCTGATGGC	58.06	CCAACATTCAGTTAACCGTACAAG	59.07	234
Pa_242	FU1G3FE02Q0UOO	(AAT)^8	TTCTTTATATGCCGATTGTGGG	57.317	TTGCTTGATGATCTTACTCTACTG	57.057	234
Pa_243	FU1G3FE02Q8LI5	(GCT)^8	TTTTGGACACTGAAGCCCC	58.635	TCTAGGTAGCCAATACCCGC	59.429	234
Pa_244	FU1G3FE02R8U2N	(AAT)^18	TGGGTTAAAAGAAAGAAAAGCTGG	58.861	GGCGTCTATTCCTATGTTAGTGG	59.204	234
Pa_245	FU1G3FE01BF7TL	(AT)^11	ATACCGACCAAGGCTGGAG	59.549	AGCTACAACATGCAACGCC	59.863	235
Pa_246	FU1G3FE01C0J0Y	(AAT)^18	CAGCAGTTTATCCAGCCTATCG	59.691	ACTGACCGAAACGTAAAACCAC	60.142	235
Pa_247	FU1G3FE01CB5PL	(ATT)^9	GGTGATTTGAGAAAATATGCAGGTG	59.728	GTATAGGGGTTAGTATGACTTATCTTG	57.412	235
Pa_248	FU1G3FE01DLP2Q	(AAT)^11	TGGAGAAATCATTGGTTGTGTATC	58.014	TGATACCAAAGTTGGGGTGC	58.847	235
Pa_249	FU1G3FE01EC6F4	(ACAT)^10	ACCTGTCTGCCTGTCTGTC	59.704	AGATGAAGCTCAGGTGCCG	60.532	235
Pa_250	FU1G3FE02Q5VB8	(AG)^8	TCCCTCTCCCCTCTCAACC	60.39	ATGGAAACGTCAGCCCATC	58.577	235
Pa_251	FU1G3FE02SHA23	(ATT)^15	ACTTGCGTATTTGTGTGCG	57.978	ACCTTTCCTTAAGAGACGGAAAAC	59.711	235
Pa_252	FU1G3FE02TPYLB	(AC)^8	GCAATGCTATGTCCCGTTAATAC	58.721	GCACACTCAACACCCTTCC	59.414	235
Pa_253	FU1G3FE01DKWW1	(ACG)^9	GGCACAACGCCAGTAGATG	59.645	CGGCGTCCCTCTTGGATAC	60.675	236
Pa_254	FU1G3FE01C6HD6	(AT)^13	TGAACTTTGGTCATGTGAACTG	57.859	TCCTCGTGTCGTCGTTCG	60.165	237
Pa_255	FU1G3FE01CHV14	(ATT)^9	TGTACACCGCCAACTTTCC	58.737	AGAGGAAATGGAAGAAGCAAGAAG	59.769	237
Pa_256	FU1G3FE02PJ0UD	(AAT)^8	GACGCCTGTTCATGTGAGTG	59.944	GAGTGTACCAACGGCTTTCC	59.59	237
Pa_257	FU1G3FE02RX1GU	(ATT)^8	CTCGTCAAAGTTAGAGGTGGAC	59.176	AGACCAAATCCGTTGCTGG	58.804	237
Pa_258	FU1G3FE02SDOAJ	(ATGT)^9	AGATGTATGCATGTATGTAGGTATG	57.152	TCACACCCTAAGTTTAGTTTTCGTC	60.015	237
Pa_259	FU1G3FE02SK2JG	(ATT)^8	CCCACATCAATAATAGTAAAATCACGC	59.965	ATTGCTACTCCCACGCTTC	58.286	237
Pa_260	FU1G3FE01ALPJ7	(ACT)^8	GGTACTTGAACCACGGACAC	59.238	AATTTGCACGCTTGCTGCG	61.745	238
Pa_261	FU1G3FE02R8RZE	(ACTC)^11	AGCGAGTTGTGTATTTGTGAG	57.317	ACCGAATTATGAAGGCTTTGC	57.97	238
Pa_262	FU1G3FE02RMLSS	(AAT)^12	AGATACGTGCGAGAGAGCC	59.714	TTTTGCGCCCGTTCTCAAG	60.085	239
Pa_263	FU1G3FE01A1B9S	(ATT)^18	TTTTGGAGTATCGTTAGCTGTG	57.222	GACGTCAGTCGAGACCCAAG	60.849	240
Pa_264	FU1G3FE01CL1LO	(AAAT)^9	AATTGTGCGTCCAATGCTC	57.64	TCAAGTACCTCTCTGTTGACG	57.835	240
Pa_265	FU1G3FE02THELH	(ATGT)^10	CCCCTCTCCCGTAACCTTC	59.55	ACAGTGCAACATTTTGTATATAGCG	59.275	241
Pa_266	FU1G3FE02TRUII	(AG)^9	ACCTCAGCCAGTCATTAAGTC	58.146	ATGGCTCTGAACCCAGAAG	57.48	241
Pa_267	FU1G3FE01ATFD8	(AC)^9	CAAGCACAAAGGACCTGGC	60.083	TCCCTTCGTTTCCTTGCCC	60.688	242
Pa_268	FU1G3FE01EFR3B	(ATTT)^11	GGTACGATTGCCTACGGTG	58.765	TCCTGCCTCCTCCACAAAG	59.698	242
Pa_269	FU1G3FE01EGHHN	(GT)^9	TGCTCAGCCACTAGTTCCG	60.158	ACAAGACAGAGGAGCCACG	60.083	242
Pa_270	FU1G3FE02PR87K	(AAT)^14	TGGCAAAATGTGCAACAAAATG	58.463	AGTGCGCAGTTAAAGTCGAAG	59.948	242
Pa_271	FU1G3FE02QQUO1	(AAC)^11	CGTGTAAGTGCGGGGTTTC	59.867	GTCGTCGTTGTCGTCGTTG	60.229	242
Pa_272	FU1G3FE01ELQ0J	(ATT)^17	CTTTCAAGTTTGTTTAAGCTTGGTG	58.765	AAGATCCCCGAAAGCTCCC	59.851	243
Pa_273	FU1G3FE02PP2UD	(AT)^8	TTGCATCCAGTGGTCTCCC	60.081	ATAGATTGGGCGTAGGGGC	59.699	243
Pa_274	FU1G3FE02Q3OOA	(AT)^10	GCGTGATGAATGTAATGTTGAGG	59.158	TGGGACCAGAGGGTCATTC	59.076	243

Pa_275	FU1G3FE02P4NI9	(AG)^9	GTTAGCCCAAACGCAGACG	60.231	ACCCAACTTCCTTACCGACC	60.079	244
Pa_276	FU1G3FE02QBPHZ	(AAT)^14	TCTGATGAGAGCTGACTAGAAGTG	60.133	TCCAGACAAAGAAGATTTCCCC	58.742	244
Pa_277	FU1G3FE01ENNQK	(ATT)^13	GGCGAGAGTCCCGAGAAAG	60.599	TTCCTTCATGTTGCTGTTGAG	57.485	245
Pa_278	FU1G3FE02P06IW	(AC)^9	ACTCTACGGACAGAACACGG	59.869	TGACTAGCAAGGAGTTGCAC	58.582	245
Pa_279	FU1G3FE01CL28J	(CT)^9	ACCAAACGCAAGTTTACGC	58.19	CCTTGGGTACAGCGCAC	58.701	246
Pa_280	FU1G3FE01EPS5N	(AAT)^13	ACAGTGTGAAACAAAGTCTCCTG	59.825	CGTTCTTTCCCGTCACATGG	59.943	246
Pa_281	FU1G3FE02P32HQ	(AC)^8	CAATGCCTGGTTACGGCAG	59.937	AGAGGGTGAAGACTTGGGG	58.995	246
Pa_282	FU1G3FE02R0UIC	(ATT)^8	AGAGTCTGGGTGAAGGAAGG	59.124	AGGAACGAAACGAAGACAAGAG	59.37	246
Pa_283	FU1G3FE02SOPVG	(AGT)^16	TGCAGTGGATAAAGTGTTGGC	59.874	TTCCTCCGCCTTCTTTATTTTG	57.715	246
Pa_284	FU1G3FE02TULJK	(AC)^10	AGCCTAAGCTTCGCAAGTG	58.896	CTTGGAGTTGCTTTGTTGCC	58.886	246
Pa_285	FU1G3FE01DRIFR	(AAT)^8	ACGAATCAAGTCTCTGAAAGCTC	59.518	AGCATATCGCACATGGGTTG	59.44	247
Pa_286	FU1G3FE02QLDY2	(ATT)^13	ACGGTGTGTGTTTCGTTTCC	60.012	AGGCCTATGGTTATGCCCG	60.004	247
Pa_287	FU1G3FE01CSOXO	(ATT)^13	TACCAGACGCCACCGATTG	60.529	ACAGACATGCACATACCACAC	59.33	248
Pa_288	FU1G3FE01DNQ8F	(ATT)^8	AGCGACTGAGTATTTATTGGAAGAG	59.494	TGTTCTTGAAGGCTTGAGTTTCC	60.137	248
Pa_289	FU1G3FE02TSVGS	(AT)^8	GTACCAGGTGGGTTCAGGG	60.081	TCTGGAGAGCTTGCCTGTC	59.779	248
Pa_290	FU1G3FE01A6B3Q	(ACT)^12	TGCTTACCTCACCCACTTG	57.727	GACGGTGACAAAATAGAGTGC	58.014	249
Pa_291	FU1G3FE01AT9DX	(AAT)^13	CAGTTGGTTTGGTAGAGAAAAGC	58.898	TGTGACGTGGGTATTATTTTCG	57.559	249
Pa_292	FU1G3FE01CAZDW	(AGT)^9	CCGATGAACTGCGTCTGTG	59.652	TCATTGCCATCAGTTATGAGAGG	59.062	249
Pa_293	FU1G3FE02PZT3E	(AAT)^15	GCACTGCCATTTCCCAGAC	59.86	TGAGACATAGATGTAGTTAAGGAGG	57.955	249
Pa_294	FU1G3FE02QC1KQ	(AAT)^9	GGGACTTACGTGGTACTTGG	58.374	TCGGAGGAATTATACTGGGTTAG	57.717	249
Pa_295	FU1G3FE02SITYR	(ATT)^15	CCGCACATCCTGTTCTTCAG	59.661	CGAACTAGTACAGAACTCTTGAGG	59.125	249
Pa_296	FU1G3FE02SUBAH	(CT)^8	TCATGTATTTTCGTCAATTCACCC	58.575	AAGTAGAGAGTGAAGAGGAAGTAG	57.451	249
Pa_297	FU1G3FE01EPS8W	(AT)^10	TGATCAACAGCACCAACGC	59.79	GGTGTGACATGTATTTGTAATTAGGG	59.069	255
Pa_298	FU1G3FE02TCUF1	(ATT)^49	TCTTGTTTTCTTTGCAGGTCAG	58.121	CTAACCCCAACCCCAACCC	60.695	261
Pa_299	FU1G3FE02SVHP3	(AGC)^10	AGTAGTAGTAGCAGCAGTAGTAGAG	59.257	AGCTAATACGTGACTATGACTCG	58.408	262
Pa_300	FU1G3FE01CWTI4	(AAT)^15	GGCTGAGCTTAATCGGTGG	59.046	ATGAAACCTATAATGTACCTTCCTG	57.183	272
Pa_301	FU1G3FE02QL9ZH	(ATT)^20	GCGCGCACGTGTGTATTG	61.273	GAATGGTTGACGTATCAATTATCAG	57.164	272
Pa_302	FU1G3FE02QDZTB	(ATGT)^12	ATGCATAAATCGTCTCACTTCC	57.342	TACGAGCGTGAATGTGTGC	59.286	283
Pa_303	FU1G3FE01C7FSS	(ACAT)^8	TCGCAGCTTCCTGTGTAGG	60.158	TGTCAGAATAGCAAGTAAGGGTG	58.939	284
Pa_304	FU1G3FE01B5TR2	(AAT)^8	AGTACTCTGGTCAGCAACG	57.547	TGTGTGAAACCAAAACTCATCTTC	58.875	286
Pa_305	FU1G3FE02RKU1L	(ATTT)^11	CTCATATTTCCAGGGTCCCATC	58.747	CTGAAAACAAGGAAAAGAAAACAGC	58.993	287
Pa_306	FU1G3FE02P75U1	(ATTT)^10	CAAGCATTTTAGCCATGAACTCC	59.332	ACCTGACTATCTTTTAGGTACTTTG	57.083	290
Pa_307	FU1G3FE01D2YC7	(AAAT)^10	GTGTTTTATGCTTTTGGGAGTGAATAG	60.23	AGGCCCTGATGAGCACTAAC	60.224	291
Pa_308	FU1G3FE01BO83U	(GT)^9	TCACATTGTCTGGGGTGGC	60.689	AGTAGTTGTGGTAGTGGTTATCG	58.444	292
Pa_309	FU1G3FE02QZIH4	(ATT)^14	TGCTTTCTAGTTGCTCTTTGC	57.845	GCGATAACGAGGCTTGCTG	60.086	295

Pa_310	FU1G3FE02SPBK9	(ATGT)^11	ACTCTGGTCCATGTTTGCAG	58.857	AAAGAGTAGACCGAAATGAAAGG	57.309	297
Pa_311	FU1G3FE02RQMBA	(ATT)^11	CGTTAGGGTCTTGCCGTTC	59.28	CCTTGAATGAATTCCTAATTTGCTCC	59.792	298
Pa_312	FU1G3FE02SDHM3	(ATT)^15	TGCAGTTGATGAGTTATTGTACC	57.631	ACGAGAAAGGAAAACATGATCTGG	60.073	298
Pa_313	FU1G3FE02SSALI	(AAT)^19	CAGTCAAATCATTCAAGAAAACAGC	58.654	GGTAGGGAAGTTGCTCATTATAGG	59.223	298
Pa_314	FU1G3FE01BO77O	(ATT)^12	CGAACGATTGAGACTTGGTCAG	60.079	CAAAGTACAGCTAAGAAACAAAACC	58.078	299
Pa_315	FU1G3FE01CVCTN	(AAT)^15	GGCTGAGCTTAATCGGTGG	59.046	GTGGCCTTTGGTCAGACAG	59.113	299
Pa_316	FU1G3FE02RAB6K	(AAT)^11	GTGAGTAAGTGAAGCTGAGAAG	57.241	CTGCTGTATTCATGTGTTATAGTAGTG	58.795	299
Pa_317	FU1G3FE02TD3G5	(AC)^8	AGGATTTGAAGTAGAGAGGGAC	57.403	GTTGTGTCTGTCTGTGTGTG	57.64	299
Pa_318	FU1G3FE01D5JNQ	(GT)^9	TTCTTATACATCAAGATGAGTCGAG	57.069	AGAAATTAACGCTATCATCGGC	57.757	313
Pa_319	FU1G3FE01A38B6	(AAAT)^9	ACCTGACATCTTTTGGGTACTTTG	59.951	AGCTATGAGCTAGTGATATGAATCAAG	59.529	320
Pa_320	FU1G3FE01CHYY8	(AAT)^15	AGCTGGGAAGAAATTGCCAC	59.502	GCTAAGCTGTATTGCTCTGG	57.042	344
Pa_321	FU1G3FE02R6J3R	(ATT)^13	GGAATCCTATTGATTATGTGGGACG	60.13	ACTACAAATGCTGCTATGGATG	57.32	352
Pa_322	FU1G3FE02Q96Q4	(ATT)^14	CAGTCCCCTTACGTACCCC	59.552	AAGGGACAGACAGACTTGG	57.021	373
Pa_323	FU1G3FE02QCHH9	(AAT)^20	TGCGAGTCTATATCCGTTAGC	57.921	TTCCCGGTCGGTCGTAAAG	60.158	375
Pa_324	FU1G3FE02Q0VCF	(AAT)^14	ACCAGTAATGTATTTATCTCACGAC	57.53	TTCCGGTGGAGAGGAAACG	60.083	381

Chapter 4

Temporary accommodation – the influence of ephemeral pools on the population genetic structure of a freshwater shrimp, *Paratya australiensis* (Atyidae), across two catchments in South Australia

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Abstract

Ephemeral waterways are characteristic of many Australian landscapes and will, given several current climate change predictions, become more common. How their ephemeral nature affects the ecology of such waterways, and in particular, the population genetics of aquatic organisms, is not well understood. *Paratya australiensis* is an obligate freshwater macroinvertebrate which has been the subject of several population genetic studies. In this study, microsatellites were used to investigate population structure across two catchments in South Australia. Three hundred and seventy-seven individuals were genotyped at nine microsatellite loci across twelve sites in two adjacent catchments. The microsatellite results indicated clear genetic differences between the shrimps from the two catchments. There was, however, little genetic differentiation among sites within the Wakefield catchment compared to that among sites within the Broughton Catchment. The observed structuring was consistent with the 'Death Valley' model of river population structure. We propose that the genetic structure observed is not the result of a single period of isolation but the result of repeated short-term isolation events caused by the ephemeral nature of the catchments. We suggest that while a single short-term isolation event may not be sufficient to result in significant population structuring, the compounded effects of many of these events is sufficient to create population structuring within and between catchments.

Introduction

Ephemeral waterways make up a large proportion of the world's aquatic habitats particularly in locations with dry climates such as parts of Africa, Australia, the Mediterranean, North and South America (Tooth, 2000). Larned et al. (2010) presented three conceptual models for the biodiversity of temporary rivers. The first model suggests that the hydrological connectivity controls the meta-community and meta-ecosystem dynamics and that these communities may become longitudinally nested with populations, and within communities being in a constant state of flux. The second model relates temporary rivers to path-dynamic theory and predicts the fluctuation of large-scale biodiversity due to the variation in water levels and the alternation between aquatic and terrestrial habitat with peak biodiversity occurring between fully terrestrial and fully inundated states. The third conceptual model focuses on the biochemical aspects of ephemeral rivers and explores how wetting and drying of organic matter moving longitudinally through the river accelerates biochemical processes. The first conceptual model is predicted to also apply to the population level as well, with periods of connectivity and isolation driving population genetic structure on a spatial scale, both within and between catchments.

Southern Australia's temperate climate results in many streams remaining dry for much of the year, with flow only resuming when either the groundwater table is elevated enough to sustain flow or when there is sufficient surface runoff (Boulton and Lake, 2008). During periods of no flow, much of the fauna of these intermittent streams must rely on either dispersing to more permanent water sources, or persisting in a dormant, desiccation-resistant stage or residing in deeper permanent pools (James *et al.*, 2008, Sheldon *et al.*, 2010). For obligately

aquatic fauna with homolimnic lifecycles, permanent deep pools act as refugia over the dry months. During times of flood, these ephemeral systems experience an exponential growth in productivity and biodiversity owing to increased habitat, lack of competition and increased resources (Sheldon *et al.*, 2010). This leads to a 'boom and bust' ecology which is dictated by the variation in flows through the river system (Kingsford *et al.*, 1999, Balcombe and Arthington, 2009). The use of permanent water as refugia has been examined in several contexts including studies of refugia, colonisation from refugia and the ability of fauna to deal with deteriorating water conditions (Caruso, 2002). However, there has been little study of how the genetic structure of aquatic organisms may be affected by ephemeral flow regimes (Faulks *et al.*, 2010), with the exception of the Lake Eyre Basin fish populations which have been studied in several different contexts including gene flow and landscape processes (Huey *et al.*, 2008, Huey *et al.*, 2011). Currently, much conservation management of streams is based upon ideas and models derived from connected stream systems (Larned *et al.*, 2010) and additional data on the population genetic structure of organisms from ephemeral streams are likely to promote a more effective management of such systems. This is becoming increasingly important as aquatic ecosystems are coming under increased threat from a multitude of sources. Of particular concern with regard to aquatic ecosystems is increasing aridity through global climate change and an increasing demand for irrigation and drinking water, trends that may drive once permanent water courses into a more ephemeral condition (Brooks, 2009).

Interest in ephemeral waterways has increased over the last decade. This increase has been due to several factors, primarily the increasing body of knowledge indicating that they are more complex than previously thought and the need to

understand existing ephemeral systems as well as to understand the impacts of global drying trends on perennial rivers. For example, modelling of various different scenarios in the ephemeral systems of north-east Spain has suggested that those river systems will become more ephemeral, resulting in a loss of biodiversity (Otero *et al.*, 2011). Research undertaken in North America has confirmed the importance of ephemeral waterways for breeding amphibians, fish and microcrustaceans (Brooks, 2009). Kadye and Chakona (2012) demonstrated high levels of variability in the fish community in intermittent streams in Zimbabwe and identified the importance of permanent pools within the stream network for the persistence of these communities. Santos and Stevenson (2011) identified that similar pools in non-perennial streams are able to support diverse communities and contribute to overall river biodiversity. The increasing body of work on these ephemeral systems is revealing their importance for aquatic communities on a global scale.

The dispersal of macroinvertebrate populations has been subject to many myths in the past (Bohonak and Jenkins, 2003). Early theories such as Darwin's 'Duck Feet Hypothesis', predicted frequent overland dispersal facilitated by vectors such as waterbirds, however a growing body of work suggests that this is not a major vector (Bohonak and Jenkins, 2003). Several recent population genetic studies have found distinct population structuring within populations of macroinvertebrates across varying degrees of spatial separation (e.g. Wilcock et al., 2007, Hughes et al., 1996), suggesting limited levels of dispersal and gene flow.

The population genetics of aquatic organisms in freshwater systems may reflect both the life history of the organisms and the physical structure of the watercourse

that they inhabit (Hughes *et al.*, 2009). Short and Caterino (2009) investigated the influence of habitat on the genetic structure of three species of water beetle and found that life history played a larger role in determining genetic structure than habitat type. This idea was supported by Miller et al. (2002) in their study of four aquatic macroinvertebrate species. However, Marten et al. (2006) reviewed data for 150 aquatic species and found that there was a difference in population structure between species found in lentic (more permanent) and lotic (more ephemeral) water sources, with lotic species displaying greater population differentiation. Short and Caterino (2009) developed this observation into a general hypothesis that organisms living in ephemeral waterways will have more developed dispersal abilities than those found in more permanent water courses as a way of ensuring persistence by dispersing away from drying waterways to more permanent water. However, when considering the effect of the ephemeral nature of waterways on obligately aquatic organisms, there is still a knowledge gap, particularly with regard to population genetic structure.

Faulks et al. (2010) addressed this gap with their study on the effect of hydrological regime on genetic diversity of the golden perch. They found that the genetic structure and diversity are significantly influenced by the hydrological regime, though there were higher rates of dispersal than predicted given the isolating nature of desert rivers, suggesting that dispersal may be more common than initially hypothesised. This work was supported by Huey et al. (2011) who identified higher than expected gene flow between waterholes in a desert river system. This work demonstrated strong metapopulation dynamics are present in ephemeral rivers in the Lake Eyre Basin, Australia. Carini et al. (2006) explored how pools outside a main river channel affected the population structure of

Macrobrachium australiense and found higher levels of connectivity between the river and the out-of-channel pools than expected. However, how these results may relate to in-stream population structure within a main river channel is still unclear. Although the roles of adult flight (e.g. Miller *et al.*, 2002) and of downstream drift (e.g. Anholt, 1995) in influencing population structure of aquatic invertebrates have been examined, the effect of the shifting nature of ephemeral waterways, from flowing waters to isolated pools through to hyporhic conditions, on population structure remains relatively unstudied (Larned *et al.*, 2010, Sheldon *et al.*, 2010).

Four models have been proposed to describe the population genetic structuring of aquatic macroinvertebrates. Meffe and Vrijenhork (1988) hypothesized two different models for obligate aquatic fauna. The 'Death Valley' model is most often applied to spring populations. Populations in this model are isolated in areas of suitable habitat with no connection to one another. These populations are genetically distinct as a result of selection pressures and genetic drift, however, they show no structuring that can be explained by geographic location. The 'stream hierarchy' model proposed by Meffe and Vrijenhork (1988) predicts that populations will show a hierarchical population structure that reflects the drainage basin. Those populations in the head waters of one sub-catchment will be highly distinct from those in another, with the genetic distance becoming less towards the catchment outlet. However, not all freshwater macroinvertebrates are obligately aquatic. The third model is the 'headwater' model. This model applies to organisms that specialize in the headwaters of catchments and that have at least limited terrestrial dispersal ability (Hughes *et al.*, 2009). In this model populations from different catchments that are geographically close will be more genetically

similar than those from the same catchment but separated by larger geographic distances, both terrestrially and in-stream. The reality for most freshwater fauna is that population structure most likely represents a combination of these models, wherein the dendritic nature of streams underlies the species' life history, giving rise to the fourth model, the 'dendritic network model' (Hughes *et al.*, 2009). In this model the dendritic nature of streams will cause some structuring in the upper order streams, however, overland dispersal may link geographically close populations. "Isolation by distance" is a model that is not specifically related to aquatic macroinvertebrates but it should also be considered when considering larger, more capable overland dispersers. Populations in this model will show genetic differentiation based on geographic distance and dispersal methodology (overland or in stream), irrespective of catchment structure (Slatkin, 1977, Hughes *et al.*, 2009).

Paratya australiensis Kemp (Decapoda: Atyidae) is an obligately aquatic species and unfacilitated terrestrial dispersal by this species is highly unlikely (Hughes *et al.*, 1995). In order to examine how the ephemeral nature of waterways will affect the population structure of *P. australiensis*, we examined the population genetic structure of this organism using microsatellite markers across the Broughton and Wakefield catchments in South Australia. Mitochondrial and allozyme studies have indicated that there is a high level of genetic structuring among populations of this species across much of Australia as a result of limited dispersal (Hughes *et al.*, 1995, Hurwood *et al.*, 2003). Cook et al. (2006) indicated that there may be as many as nine reproductively isolated clades across eastern and south-eastern Australia and Cook et al. (2007) found evidence that two of these lineages are reproductively isolated. There has been, however, limited study of the within

catchment population structure of *P. australiensis* or of the genetics of populations across southern Australia. Baker et al. (2004) used mitochondrial DNA to examine within and between catchment differences of three of the lineages and found widespread lineages but proposed high levels of structuring in the headwaters due to the mountainous region. Similarly, Hurwood et al. (2003) found high levels of structuring based on allozyme and mitochondrial markers..

As an obligate aquatic species common across much of southern and eastern Australia (Williams, 1977), *P. australiensis* may well represent a useful model organism for understanding how organisms respond to the variability of flows in ephemeral streams and provide some insights into the role of dispersal in these systems.

Adding the information about population structure into the ever growing body of knowledge on ephemeral systems will allow conservation managers to better understand these systems. There is already limited knowledge on how drying trends may affect these systems in regards to macroinvertebrates and fish community structure (Santos and Stevenson, 2011, Kayde and Chakona, 2012) and phylogenetic knowledge (Cook *et al.*, 2006, Otero *et al.*, 2011). With the addition of localised genetic work, managers working in any ephemeral system will be better equipped to make decisions on issues of changes to flow regimes, restoration areas and translocations. We hypothesised that the two study catchments would form separate clusters and that there would be distinct structuring within each catchment following the stream hierarchy model (Meffe and Vrijenhork, 1988). Moreover, we hypothesized that shrimps in locations isolated by a lack of flow during dry conditions would show greater levels of

genetic differentiation than those in locations which experience more regular connecting flows.

Methods

Study Species

The freshwater shrimp, *Paratya australiensis*, is a relatively well-studied freshwater macroinvertebrate. Initially described as a single species covering much of southern and eastern Australia by Kemp (1918), Reik (1953) later described a new subspecies of *P. australiensis* and two additional species of *Paratya* from Australia. However, Williams (1977) recognized the original Australia-wide *P. australiensis*. More recent work has uncovered evidence that *P. australiensis* may be a species complex (Cook *et al.*, 2006).

Study Sites

The two catchments selected for study were the Broughton and the Wakefield Catchments in the mid-north region of South Australia (Figure 1). Both of these catchments have ephemeral headwaters flowing into more permanent rivers downstream and have headwaters originating in the hills around the Clare Valley, a prominent wine growing region of South Australia (Favier *et al.*, 2000, Favier *et al.*, 2004). *P. australiensis* occurs throughout both of these catchments and during dry months is restricted to the permanent pools that persist owing to groundwater influx (E. Bestland, *pers. comm.)*. Thus, such pools serve as refugia during the no-flow periods. The Wakefield Catchment covers 690 square kilometres and is a relatively simple catchment with no tributaries containing permanent pools of sufficient size and volume to sustain shrimp populations other than the main

channel of the Wakefield River itself (Favier *et al.*, 2000). The Broughton Catchment is much larger, covering a total of 5671 square kilometres, and has several major tributaries including Yakilo Creek, Hutt River, Hill River, Freshwater Creek, Bundaleer Creek, Rocky River and Crystal Brook (Favier *et al.*, 2004). A total of 12 sites were selected across the two catchments, four along the Wakefield River and eight in the Broughton Catchment with four along the Broughton River, two on the Hill River and two on Freshwater Creek (Figure 1). All sites were selected based on the presence of permanent water during the summer months, either in the form of a permanent pool or groundwater-based perennial flow. The four sites on the Wakefield River consisted of reaches of flowing surface water between 500 metres and 1000 metres long that were seasonally isolated from each other by dry sections. The sites located in the Broughton Catchment were mostly isolated pools with the exception of the two upper Broughton River sites (UpperBro 1 & UpperBro2) which were isolated reaches of flowing water 500 metres and 900 metres long, respectively.

Genetic Techniques

Individuals were collected from each site and immediately stored in 100% ethanol on ice. DNA was extracted from a portion of tail muscle using the Gentra method (Gentra Systems Inc. Minneapolis, US).

Twelve microsatellite primers were designed based on Roche 454 sequence data and PCR conditions and genotyping was carried out as described in Green et al. (2011).

Scoring errors and the frequencies of null alleles (Brookfield 1 estimator) were estimated using MICRO-CHECKER (version 2.2.3, van Oosterhout *et al.*, 2004).

The number of alleles, allelic frequencies and observed and expected heterozygosity per site and per locus were estimated using GenAlEx (version 6.3, Peakall and Smouse, 2006). Given the high number of alleles per locus, Jost's *D* was calculated as a measure of genetic distance between populations using SMOGD (Jost, 2008, Crawford, 2010). F_{ST} values, for comparison to Jost's *D*, and tests for linkage disequilibrium, were calculated using GENEPOP on the web (version 1.2, Raymond and Rousset, 1995). Testing for selection was conducted by examining F_{ST} vs. H_E outliers using LOSITAN at 99% confidence (Beaumont and Nichols, 1996, Antao *et al.*, 2008). Inbreeding coefficients were estimated using FSTAT (500 permutations, Goudet, 1995). Isolation by distance effects were examined using Mantel Tests based on Jost's D and in-stream distances in GenAlEx (version 6.3). Effective population size was estimated using ONeSAMP (Tallmon *et al.*, 2008). An AMOVA was performed to assess the difference between and within catchments and sample sites using GenAlEx (version 6.3).

Population structure was estimated using STRUCTURE (Pritchard *et al.*, 2000). Default settings were maintained with a burn in of 200,000 cycles and 1,000,000 MCMC repetitions. Evanno et al.'s (2005) method of identifying the number of clusters via the deltaK (ΔK) value was used in STRUCTURE HARVESTER (Earl, 2010). This was compared to clusters produced by PCA plots of Jost's D in GenAlEx (version 6.3).

The cluster analysis and Structure analysis clusters were analysed using a hierarchal AMOVA in GenAlEx (version 6.3) to provide a third line of evidence of population structure.

Additional work has characterised the mitochondrial COI sequences of the study specimens (Green et al. unpub. data) In order to examine the association between the mitochondrial lineages identified and the results of the microsatellite analysis, we conducted assignment tests using both STRUCTURE and Geneclass (Piry *et al.*, 2004). In addition, the microsatellite data were split based on mitochondrial lineage, and the microsatellite analyses were repeated and the results compared to those of the full microsatellite dataset to assess consistency.

Results

Microsatellite Results

The locus Pa_03 was removed from the microsatellite analysis as the amount of missing data from genotyping proved excessive (34% of individuals, not correlated to mitochondrial lineage) and was attributed to imperfect primer design. This left 11 other polymorphic loci, with the number of alleles per locus varying from 9 to 39 (mean 17.8). There was no evidence for mis-scoring or allele drop out at any loci, however, MICRO-CHECKER identified both Pa_06 and Pa_14 as having null alleles (0.166 and 0.167 respectively, $p<0.05$) and therefore these loci were excluded from all further analysis. Mean (range) observed and expected heterozygosity across all sites was 0.560 (0.480 – 0.690) and 0.680 (0.640 – 0.820) respectively (Table 1). The effective population size across the whole sample range was 313.63 (Lower 95% CI = 150.13, Upper 95% CI = 813.69). When split across the two catchments, the effective population size in the Broughton Catchment was 668.81 (Lower 95% CI = 326.32, Upper 95% CI = 2190.64) while in the Wakefield Catchment it was 99.71 (Lower 95% CI = 64.61,

Upper 95% CI = 244.84). Table 2 contains the genetic summary statistics across the nine study loci.

Deviations from Hardy-Weinberg were found in 53 of the 108 tests conducted with most showing heterozygote deficit. However, no locus or population showed consistent deviations across all tests. In order to increase the number of individuals per group, samples were pooled according to PCA analysis of Jost's D values (Figure 4). Again there were significant deviations from Hardy-Weinberg equilibrium in 33 of the 45 tests. These tests were duplicated for the two lineages identified across the study area, and are presented later.

The mean number of alleles per locus was significantly lower in the Wakefield Catchment than in the Broughton Catchment (6.50 and 10.00 respectively, t = 4.76, df = 4.60, $p< 0.01$). Mean allelic richness per locus was also lower in the Wakefield Catchment (6.19) than in the Broughton Catchment (7.33), however, this difference was not significant. Observed and expected heterozygosity were lower in the Wakefield Catchment than in the Broughton Catchment. F_{IS} values indicated significant levels of inbreeding within all populations except Wakefield 3 ($p< 0.00046$, adjusted p-value, Table 1).

STRUCTURE Results

The results from the STRUCTURE and STRUCTURE Harvester analysis across both catchments indicated a maximum ΔK value at K=2, corresponding to the two separate catchments (Figure 3). This difference was also identified in the AMOVA analysis (F =0.091, df = 1 & 10, $p<0.005$). STRUCTURE was run again on each catchment separately in order to test for any underlying within-catchment structuring. The Wakefield Catchments showed no significant peaks in the ΔK

values, suggesting a lack of significant population structuring within this catchment. However, there was a large peak at K=3 for the Broughton Catchment, suggesting some sub-structuring within this catchment. Again, this difference was also identified in the AMOVA analysis ($F= 0.035$, $df = 10 \& 365$, $p<0.005$). Further analysis of the STRUCTURE plots indicated that the three sub-populations in the Broughton catchment corresponding to the downstream Broughton River sites (Broughton 1 & Broughton 2), the upstream Freshwater creek site (Freshwater 2) and the Hill River sites (Hill 1 & Hill 2) (Refer Figure 1). This sub-structuring was not as distinct as the division between the Broughton and the Wakefield Catchments with some individuals assigned to different clusters. Visual analysis of the STRUCTURE bar plots (Figure 4) also suggested that the two upstream Broughton River sites (Broughton 3 & Broughton 4) and the downstream Freshwater Creek (Freshwater 1) site are a mix of the three identified genetic clusters.

The clusters identified in the STRUCTURE output are similar to those produced via the PCA plot of the Jost's *D* measures of genetic distance. These show the Wakefield sites distinct from those of the Broughton Catchment (Figures 3, 4 & 5). In the PCA plot the upper Broughton River sites (Broughton 3 & Broughton 4) and the lower Freshwater Creek site (Freshwater 1) cluster together as well, but this cluster is situated in the middle of the other three Broughton Catchment clusters, possibly indicating that it is a mix of all the clusters as suggested by the STRUCTURE output.

The hierarchical AMOVA showed that the majority of the variation observed in the data was within the populations (82%, Table 3). There was a significant difference found in all tests. That the majority of the variation was found within

the clusters limits the conclusions that can be drawn from the analysis. The permuted pairwise PhiST values for the within cluster analysis showed significant differences between all clusters ($p<0.02$, Table 4). Again, the within population variation is limiting the effectiveness of the analysis.

Mantel Test Results

A Mantel test provided evidence of an 'isolation by distance' effect across all study sites ($r^2 = 0.6643$, $p<0.05$). Further Mantel Tests were conducted to explore the observed structuring in the Broughton Catchment. There was no observable pattern using either in-stream distance or geographic distance ($p>0.05$). Spatial autocorrelation tests showed a similar pattern with a significant effect being observed across all study sites; however, there was no observable effect across the individual catchments.

Assignment Testing Results

Given the multiple lineages identified in the mitochondrial results (Green et al., unpublished data), additional testing was undertaken to confirm the importance of catchment effects within lineages. Microsatellite data were split based on the two major mitochondrial lineages identified ('4' and '8') and the microsatellite analysis repeated on those individuals from lineage 8 (there were insufficient individuals from lineage 4 for analysis). All of the methods used on the full dataset were repeated based on only lineage 8 individuals for comparison to those from the full dataset. STRUCTURE analysis showed the same patterns in both the full microsatellite data set and the lineage 8 dataset. The divergence between catchments was very distinct with the same sub-populations being identified in the Broughton Catchment (Figure 5). The PCA analysis again showed these same

patterns (Figure 6). The sites from the Wakefield Catchment clustered together more tightly in the ‘lineage 8’ results; however, all of the patterns observed in the complete dataset were mirrored in the ‘lineage 8’ dataset. Based on these finding, we concluded that the observed differences in the microsatellite data between the catchments did not simply arise from differences between separate mitochondrial lineages but is more likely based on more contemporary genetic processes.

Given that individuals from lineages 4 and 8 have previously been demonstrated to be reproductively isolated in sympatry in the Upper Murray Basin in Victoria by Cook et al. (2007), further testing was undertaken. Assignment testing was undertaken based on catchment, mitochondrial clade, sample site and grouping based on the microsatellite analysis. GeneClass assignment testing assigned 96.5% of individuals correctly based on catchment, the highest result from all tests, 32% were correctly identified based on sample location, the lowest result, 76% were correctly assigned on microsatellite group and 67.8% were correctly assigned based on mitochondrial lineage. In order to verify results assignment testing was undertaken in STRUCTURE. STRUCTURE correctly assigned approximately 96% of individuals from the study area based on catchment. Only 57% of individuals were correctly assigned to their correct lineage using the same method. This suggests that the catchment, rather than lineage, is primarily responsible for the observed population structure.

Discussion

The microsatellite results revealed genetic differentiation between the two catchments and within the Broughton Catchment. The STRUCTURE analysis, as well as the PCA plots of both the full data set and the lineage 8 data set, indicated that there are distinct sub-populations that correspond to the two catchments sampled in this study. This suggests that there is no overland dispersal between these two catchments as also indicated from mitochondrial and allozyme results from Hughes et al. (1995) and Cook et al. (2006) for *P. australiensis* in other regions. There was also no population structuring within the Wakefield River shown in the STRUCTURE analysis. Given that the sites along the Wakefield River are less ephemeral and are connected more frequently by flows (Favier *et al.*, 2000) than those in the Broughton catchment, this result is not unexpected (see Carini *et al.*, 2006). In contrast, Hughes et al. (1995) found larger genetic differences between populations on opposite ends of a single catchment than across catchments on a similar spatial scale. This difference most likely results from the structural difference between the catchments, with the Wakefield Catchment being relatively flat with sampling sites ranging from 100 to 290 m above sea level across about 20.7 kilometres (35.8 in-stream kilometres), compared to mountain range sites (see Hughes et al. 1995).

Our analyses also indicated that there was further structuring in the Broughton Catchment. The structuring indicated that the two Hill River sites (Hill 1 & 2) clustered together, the two downstream Broughton River (Broughton 1 & 2) sites clustered together, the upstream Freshwater Creek (Freshwater 2) site formed a separate cluster, and the two upstream Broughton River sites (Broughton 3 & 4) and the downstream Freshwater Creek site (Freshwater 1) clustered together,

comprising an admixture of the former three separate clusters (Figure 1). The isolation of the upstream Freshwater Creek site was expected given that this site would only be connected by surface flows on average once every six years (Favier *et al.*, 2004). There is also a large stepped dam constructed in 1904 separating the upper and lower Freshwater Creek sites. While the stepped design of the dam was designed to allow for easier upstream movements of fauna, it requires large volume flows to breach the dam. While there is evidence that *P. australiensis* actively moves upstream to counter downstream larval drift (Hancock and Hughes, 1999), the rates and outcomes of this behaviour are not currently known, and this population could possibly be isolated for longer periods of time than suggested by the flows. In addition, the average precipitation in the catchment only exceeds the average potential evapotranspiration for about 2-2.5 months of the year, meaning that there is only a short window of opportunity for dispersal when there is sufficient rain to connect the isolated pools (Favier *et al.*, 2004). Thus, we hypothesise that the observed population differentiation between the upper and lower sites on Freshwater Creek is most likely a compounded effect from repeated long time spans in isolation from the rest of the catchment.

The clustering of the Hill River sites is most likely caused by the same compounded effect. Despite the larger geographic distance between the two Hill River sites, there is only a 36 meter altitude difference between the two sites across approximately 18 kilometres of stream, meaning even a slight rise in the groundwater table will result in flows along the entire reach. Given the extremely low slope, the Hill River will vary on a yearly basis from isolated pools to a single long stretch of water, with very little flow. The groundwater-driven surface flow stops 250 meters downstream of the lower Hill River site (Hill 1) and reappears

near the junction of Freshwater Creek and the Broughton River, approximately 5 kilometres downstream. This break in flow is present on a more permanent basis with only substantial flows able to produce and maintain a surface flow through this section. In addition, there are three large drops (between 2-4 meters) in the river channel through this section forming a barrier to upstream dispersal. These barriers to dispersal both up and down the river isolate the Hill River from the rest of the Broughton Catchment for extended periods of time. The data suggest that these periods without dispersal have resulted in genetic differentiation between the shrimps in the Hill River and those in the rest of the catchment.

The two lower Broughton River sites formed a separate genetic cluster which was unexpected given that there are connections between these and the upstream sites in most years. However, this link has not been formed in the past 6-8 years owing to the drought conditions prevalent in the years preceding this study. While we would not expect to see divergence over this single time-frame, it is possible that the compounded effects of many periods of separation could lead to this divergence. Even though these sites were at the end of the catchment, there is no evidence of an overall isolation by distance effect within this catchment as indicated by the Mantel tests. The populations in the lower Broughton River sites appear to be distinct from the others within the catchment, suggesting insufficient connection to counteract genetic drift.

While there is evidence that shrimp will actively disperse upstream to counter the downstream drift due to current (Hancock and Hughes, 1999), the differentiation observed among the Broughton Catchment populations suggests that there is insufficient time during which these pools are connected to allow for upstream dispersal. Likewise, given the general lack of flow through the catchment, flow

does not appear to be sufficient to facilitate drift downstream. While there are connections periodically, the observed genetic differentiation may represent the compounded result of several periods without connection. The recent drought (2001-2009, van Dijk *et al.*, 2013) will have enhanced this as the time without flow would have been longer than traditionally experienced in this system, however, it is unlikely that the structuring seen is the result of one single time period without connection. Given the large effective population sizes observed in these systems, it is unlikely that drift would be evident over the estimated eight generations that would have occurred during the drought period.

The presence of the two main mitochondrial lineages in the study area (Green et al., unpublished) may call into question the interpretation of the microsatellite analysis, namely that the differentiation of microsatellite genotypes reflects the hydrological structuring of the catchments. However, the results of the comparison between the microsatellite results of lineage 8 and the full microsatellite dataset, as well as the assignment testing, suggest that the presence of the mitochondrial lineages does not invalidate these conclusions. There are individuals from each mitochondrial lineage present in the clusters identified in the STRUCTURE analysis. The comparatively rapid divergence of microsatellite markers compared to mitochondrial DNA can lead to incomplete lineage sorting and this is not uncommon in animals as discussed by Funk and Omland (2003) and expanded on by McKay and Zink (2010). Funk and Omland (2003) also make note that homoplasies in markers shared between study organisms may also make identifying factors causing divergence difficult to interpret given current methods.

The structuring of the Broughton Catchment in this study raises an interesting insight into the population structure models proposed for freshwater systems.

While there is evidence that there is a mixing of individuals at the confluence of the three river systems, at the extremes, both upstream and downstream, there is evidence of population differentiation. This structuring is similar to the 'Death Valley' model. This model is most commonly applied to isolated pools such as mound spring systems where sites can drift in different genetic directions with no influence of other sites. This appears to conflict with the higher than expected levels of dispersal found in other ephemeral systems. For example, Faulks et al. (2010) and Huey et al. (2011) identified that fish had higher than expected levels of dispersal though catchments. They both hypothesized that there was a mixing of individuals during higher flows that triggered breeding giving rise to a metapopulation structure within the Lake Eyre Basin rather than an assemblage of single isolated populations. Carini et al. (2006) also identified higher level of connectivity between main river channel pools and side pools in aquatic prawns. Again, they hypothesized that the prawns took advantage of the periods of connection and actively dispersed between pools. While *P. australiensis* has been shown to actively disperse in responce to flow (Hancock and Hughes, 1999), the data suggest that the barriers to dispersal, either distance between permanent pools or instream barriers are not overcome with suffcient frequency to allow for genetic mixing. It could be argued that intermittent streams may pose the need for an additional model which combines aspects of the Death Valley model and the stream hierarchy model.

In studies examining freshwater systems in Australia there has been little effort invested in examining how the ephemeral nature of many of southern Australia's waterways will affect the population structure of a species. This ephemeral nature will become particularly important for obligately aquatic fauna. It was

hypothesized that there would be distinct structuring between the two catchments studied as well as some sub-structuring in both catchments following the stream hierarchy model put forward by Meffe and Vrijenhork (1988). The microsatellite data indicated that there was distinct population structuring between the two catchments and within the Broughton Catchment. The structuring in the Broughton Catchment appears to be based around barriers to dispersal, dispersal ability and the ephemeral nature of the system rather than location in the stream network. If the population structure followed the stream hierarchy model we would expect to see the Hill River and Freshwater Creek as the distinct populations with evidence of mixing of the two populations in the upper Broughton River sites and total mixture of these two groups by the lower Broughton River sites. The structuring observed is more akin to the 'Death Valley' model, normally applied to spring populations (Meffe and Vrijenhork, 1988). This suggests that in these waterways the barriers to dispersal are key to defining population clusters, even though these barriers are temporary, being broken with occasional surface flow. It also suggests that the temporary isolation caused by the ephemeral nature of the waterways causes a compounding effect leading to greater population structuring than expected.

The implications of this work need to be taken into account when considering the management of populations in ephemeral waterways. The microsatellites reveal significant population structuring at a catchment scale and sub-structuring within the Broughton Catchment. Previous studies have identified that translocated lineages competitively exclude endemic lineages of *P. australiensis* (Fawcett *et al.*, 2010) while the data presented here shows that even on relatively small spatial scales there can be divergences within the population structure. These should

factor into the risk assessment of management activities such as translocations of breeding stock for ecosystem restoration.

While this work has focused on a single species from a small geographic location, the results have more widespread implications. While there is likely no model that can be applied to all ephemeral river systems, the results presented here illustrate the need to treat these systems differently and that given the current drying trends we can expect to see changes in population structure as rivers become more ephemeral.

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References

Anholt, B. R. 1995. Density dependence resolved the stream drift paradox. *Ecology,* 76**,** 2235-2239.

Antao, T., Lopes, A., J., L. R., Beja-Pereira, A. & Luikark, G. 2008. LOSITAN: A workbench to detect molecular adaptation based on a Fst-outlier method. *BMC Bioinformatics,* 9**,** 323.

Baker, A. M., Hughes, J. M., Dean, J. C. & Bunn, S. E. 2004. Mitochondrial DNA reveals Phylogenetic structuring and cryptic diversity in Australian freshwater macroinvertebrate assemblages. *Marine and Freshwater Research,* 55**,** 629-640.

Balcombe, S. R. & Arthington, A. H. 2009. Temporal changes in fish abundance in response to hydrological variability in a dryland floodplain river. *Marine and Freshwater Research,* 60**,** 146-159.

Beaumont, M. A. & Nichols, R. A. 1996. Evaluating loci for the use in the genetics analysis of population structure. *Proceedings of the Royal Society of London,* 263**,** 1619-1626.

Bohonak, A. J. & Jenkins, G. 2003. Ecological and evolutionary significance of dispersal by freshwater invertebrates. *Ecology Letters,* 6**,** 783-796.

Boulton, A. J. & Lake, S. P. 2008. Effects of drought on stream insects and its ecological consequences. *In:* LANCASTER, J. & BRIERS, R. A. (eds.) *Aquatic Insects: Challenges to Populations.* Oxfordshire: CABI International.

Brooks, R. T. 2009. Potential impacts of global climate change on the hydrology and ecology of ephemeral freshwater systems of the forests of the northeastern United States. *Climate Change,* 95**,** 469-483.

Carini, G., Hughes, J. M. & Bunn, S. E. 2006. The role of waterholes as 'refugia' in sustaining genetic diversity and variation of two freshwater species in dryland river systems (Western Queensland, Australia). *Freshwater Biology,* 51**,** 1434-1446.

Caruso, B. S. 2002. Temporal and spatial patterns of extreme low flows and effects on stream ecosystems in Otago, New Zealand *Journal of Hydrology,* 257**,** 115-133.

Cook, B. D., Baker, A. M., Page, T. J., Grant, S. C., Fawcett, J. H., Hurwood, D. A. & Hughes, J. M. 2006. Biogeographic history of an Australian freshwater shrimp, *paratya australiensis*: the role of history transition in phylogeographic diversification. *Molecular Ecology,* 15**,** 1083-1093.

Cook, B. D., Bunn, S. E. & Hughes, J. M. 2007. A comparative analysis of population structuring and genetic diversity in sympatric lineages of freshwater shrimp (Atyidae: *Paratya*): concerted or independent responces to hydrographic factors? *Freshwater Biology,* 52**,** 2156-2171.

Crawford, N. G. 2010. SMOGD: software for the measurement of genetic diversity. *Molecular Ecology Resources,* 10**,** 556-557.

Earl, D. 2010. *Structure Harvester v0.56.4* [Online]. Available: http://taylor0.biology.ucla.edu/struct_harvest/ [Accessed 06/08/2010.

Evanno, G., Regnuat, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* 14**,** 2611-2620.

Faulks, L. K., Gilligan, D. M. & Beheregaray, L. B. 2010. Islands of water is a sea of dry land: hydrological regime predicts genetic diverisy and dispersal in the widespread fish from Australia's arid zone, the golden perch (*Macquaria ambigua*). *Molecular Ecology,* 19**,** 4723-4737.

Favier, D., Rixon, S. & Scholz, G. 2000. A River Management Plan for the Wakefield Catchment. Adelaide: Environmental Protection Agency.

Favier, D., Scholz, G., VanLaarhoven, J. & Bradley, J. 2004. A River Management Plan for the Broughton Catchment. Adelaide: The Department of Water, Land, Biodiversity and Conservation.

Fawcett, J. H., Hurwood, D. A. & Hughes, J. M. 2010. Consequences of a translocation between two divergent lineages of the *Paratya australiensis* (Decapoda:Atyidae) complex: reproductive success and relative fitness. *Journal of the North American Benthological Society,* 29**,** 1170-1180.

Funk, D. J. & Omland, K. E. 2003. Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evoloution, and Systematics,* 34**,** 397-423.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity,* 86**,** 485-486.

Green, D. J., Clarke, L. J., Mackay, D. A. & Whalen, M. A. 2011. Microsatellite markers for the freshwater shrimp *Paratya australiensis* (Atyidae). *Conservation Genetics Resources,* 3**,** 295-296.

Hancock, M. A. & Hughes, J. M. 1999. Direct measures of instream movement in freshwater shrimp using a genetic marker. *Hydrobiologia,* 416**,** 23-32.

Huey, J. A., Baker, A. M. & Hughes, J. M. 2008. The effect of landscape processes upon gene flow and genetic diversity in an Australian freshwater fish, *Neosilurus hyrtlii*. *Freshwater Biology,* 53**,** 1393-1408.

Huey, J. A., Schmidt, D. J., Balcombe, S. R. M., J. C. & Hughes, J. M. 2011. High gene flow and metapopulation dynamics detected for three species in a dryland river system. *Freshwater Biology,* 56**,** 2378-2390.

Hughes, J. M., Bunn, S. E., Kingston, D. M. & Hurwood, D. A. 1995. Genetic differentiation and dispersal among populations of *Paratya australiensis* (Atyidae) in rainforest streams in southeast Queensland, Australia. *Journal of the North American Benthological Society,* 14**,** 158-173.

Hughes, J. M., Schmidt, D. J. & Finn, D. S. 2009. Genes in streams: Using DNA to understand the movement of freshwater fauna and their riverine habitat. *BioScience,* 59**,** 573-583.

Hurwood, D. A., Hughes, J. M., Bunn, S. E. & Cleary, C. 2003. Population structure in the freshwater shrimp (*Paratya australiensis*) infered from allozymes and mitochondrial DNA. *Heredity,* 90**,** 64-70.

James, A. B. W., Dewson, Z. S. & Death, R. G. 2008. Do stream macroinvertebrates use instream refugia in response to severe short-term flow reduction in New Zealand streams? *Freshwater Biology,* 53**,** 1316-1334.

Jost, L. 2008. Gst and its relatives do not measure differentiation. *Molecular Ecology,* 17**,** 4015-4026.

Kayde, W. T. & Chakona, A. 2012. Spatial and temporal variation of fish assemblages in two intermittent streams in north-western Zimbabwe. *African Journal of Ecology,* 50**,** 428-438.

Kemp, S. 1918. XVII. Notes on Crustacea Decapoda in the Indian Museum. XI. Atyidae of the genus *Paratya* (= *Xiphocaridina*). *Reconds of the Indian Museum,* 13**,** 293-302.

Kingsford, R. T., Curtin, A. L. & Porter, J. 1999. Water flows on Cooper Creek in arid Australia determine 'boom' and 'bust' periods for waterbirds. *Biological Conservation,* 88**,** 231-248.

Larned, S. T., Datry, T., Arscott, D. B. & Tockner, K. 2010. Emerging concepts in tempory-river ecology. *Freshwater Biology,* 55**,** 717-738.

Marten, A., Brandle, M. & Brandle, R. 2006. Habitat type predicts genetic population differentiation in freshwater invertebrates. *Molecular Ecology,* 15**,** 2643-2651.

McKay, B. D. & Zink, R. M. 2010. The causes of mitochondrial DNA gene tree paraphyly in birds. *Molecular Phylogenetics and Evolution,* 54**,** 647-650.

Meffe, G. K. & Vrijenhork, R. K. 1988. Conservation genetics in the managemnt of desert fishes. *Conservation Biology,* 2**,** 157-169.

Miller, M. P., Bilinn, D. W. & Keim, P. 2002. Correlations between observed dispersal capabilities and patterns of genetics differentiation in populations of four aquatic insect species from the Arazona White Mountains, U.S.A. *Freshwater Biology,* 47**,** 1660-1673.

Otero, I., Boada, M., Babia, A., Pla, P., Veyreda, J., Sabate, S., Gracia, C. & Penuelas, J. 2011. Loss of water avaliability and stream biodiversity under land abandonment and climate change in a Mediterranean catchment (Olzinelles, NE Spain). *Land Use Policy,* 28**,** 207-218.

Peakall, R. & Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Populatuion genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 228-295.

Piry, S., Alapetite, J., Cornuet, D., Paetkau, L., Baudoin, L. & Estoup, A. 2004. GENECLASS2: A Software for Genetic Assignment and First-Generation Migrant Detection. *Journal of Heredity,* 95**,** 536-539.

Pritchard, J. K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics,* 155**,** 945-959.

Raymond, M. & Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity,* 86**,** 248-249.

Santos, A. N. & Stevenson, R. D. 2011. Comparison of macroinvertebrate diversity and community structure among perennial and non-perennial headwater streams. *Northeastern Naturalist,* 18**,** 7-26.

Sheldon, F., Bunn, S. E., Hughes, J. M., Arthington, A. H., Balcombe, S. R. & Felows, C. S. 2010. Ecological roles and threats to aquatic refugia in arid landscapes: dryland river waterholes. *Marine and Freshwater Research,* 61**,** 885-595.

Short, A. E. Z. & Caterino, M. S. 2009. On the validity of habitat as a predictor of genetic structure in aquatic systems: a comparative study using California water beetles. *Molecular Ecology,* 18**,** 403-414.

Slatkin, M. 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. *Theoretical Population Biology,* 74**,** 5463-5467.

Tallmon, D. A., Koyuk, A., Luikart, G. & Beaumont, M. A. 2008. ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. *Molecular Ecology Resources,* 8**,** 299-301.

Tooth, S. 2000. Process, form and change im dryland rivers: a review of current research. *Earth-Science Reviews,* 51**,** 67-107.

van Dijk, A. I. J. M., Beck, H. E., Crosbie, R. S., de Jeu, R. A. M., Liu, Y. Y., Podger, G. M., Timbal, B. & Viney, N. R. 2013. The Mellennium Drought in southeastern Australia (2001-2009): Natrual and human causes and implications for water resources, ecosystems, economy and society. *Water Resources Research,* 49**,** 1040-1057.

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shiply, P. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes,* 4**,** 535-538.

Williams, W. D. 1977. Some Aspects of the Ecology of *Paratya australiensis* (Crustacea : Decapoda : Atyidae). *Australian Journal of Marine and Freshwater Research,* 28**,** 403-415.

Table 3: Genetic diversity across catchments and sampled populations. N – number of individuals sampled in population, N_A – number of alleles per locus, A_R – allelic richness, H_O – observed heterozygosity, H_E – expected Heterozygosity, F_{IS} – inbreeding coefficient.

Catchment	Population	*Flowing*	*N*	N_A	A_R	H_O	H_E	F_{IS}
Broughton	Broughton 1	Yes	34	10.11	7.66	0.67	0.77	0.19**
	Broughton 2	Yes	29	10.44	7.96	0.61	0.77	0.11**
	Broughton 3	No	36	10.67	6.63	0.66	0.81	0.18**
	Broughton 4	No	40	10.22	5.81	0.63	0.82	0.15**
	Freshwater 1	No	40	10.22	6.03	0.69	0.79	0.10**
	Freshwater 2	No	31	10.67	7.89	0.62	0.71	0.14*
	Hill 1	No	32	7.78	8.49	0.58	0.71	0.22**
	Hill 2	No	38	9.89	8.19	0.58	0.72	0.21**
	Mean ± S.D.		35.00 ± 3.91	10.11 ± 0.88	7.33 ± 0.96	0.63 ± 0.04	0.76 ± 0.04	0.16 ± 0.04
Wakefield	Wakefield 1	Yes	19	7.56	4.56	0.48	0.70	0.20**
	Wakefield 2	Yes	20	7.33	5.89	0.64	0.72	0.05**
	Wakefield 3	Yes	20	4.67	5.61	0.60	0.64	0.23
	Wakefield 4	Yes	38	6.44	8.70	0.54	0.67	0.30**
	Mean ± S.D.		24.25 ± 7.95	6.50 ± 1.14	6.19 ± 1.53	0.56 ± 0.06	0.68 ± 0.03	0.19 ± 0.09

* $P<0.01$, ** $P<0.001$

Table 4: Genetic diversity across the investigated loci. N – number of individuals sampled in population, N_A – number of alleles per locus, A_R – allelic richness, H_O – observed heterozygosity, H_E – expected Heterozygosity, F_{IS} – inbreeding coefficient.

Locus	N		Na		Ho		He		Fis	
	All	Lin 8	All	Lin 8	All	Lin 8	All	Lin 8	All	Lin 8
Pa-07	23.583	16.33	13.42	10.92	0.59	0.58	0.84	0.81	0.30	0.29
Pa_10	23.583	16.33	5.83	4.83	0.31	0.32	0.58	0.57	0.47	0.44
Pa_12	23.583	16.33	8.33	7.67	0.75	0.77	0.79	0.78	0.06	0.01
Pa_13	23.583	16.33	9.58	8.83	0.76	0.76	0.83	0.82	0.09	0.07
Pa_15	23.583	16.33	9.42	8.08	0.76	0.74	0.82	0.80	0.08	0.08
Pa_17	23.583	16.33	6.17	5.92	0.65	0.63	0.68	0.68	0.04	0.07
Pa_18	23.583	16.33	6.08	5.25	0.35	0.39	0.52	0.54	0.33	0.28
Pa_21	23.583	16.33	7.75	7.17	0.80	0.79	0.79	0.78	-0.02	-0.01
Pa_24	23.583	16.33	5.83	5.33	0.61	0.57	0.72	0.69	0.15	0.18

Table 3: Summary hierarchical AMOVA table based on clusters identified by the PCA plots and STRUCTURE analysis. SS – Sum of Squares, MS – Mean Sum of Squares

	df	SS	MS	Est. Var.	%
Among Catchments	1	166.047	166.047	1.267	13%
Among Clusters	3	100.321	33.440	0.484	5%
Within Clusters	278	2266.569	8.153	8.153	82%
Total	282	2532.936		9.904	100%

Table 4: PhiST values generated through the Hierarchical AMOVA illustrating the differences between the clusters observed. PhiPT values are below the diagonal and the probability based on 99 permutations is shown above the diagonal.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Cluster 1	0.000	0.010	0.010	0.010	0.010
Cluster 2	0.090	0.000	0.010	0.010	0.010
Cluster 3	0.079	0.094	0.000	0.010	0.010
Cluster 4	0.018	0.079	0.038	0.000	0.010
Cluster 5	0.185	0.190	0.164	0.175	0.000

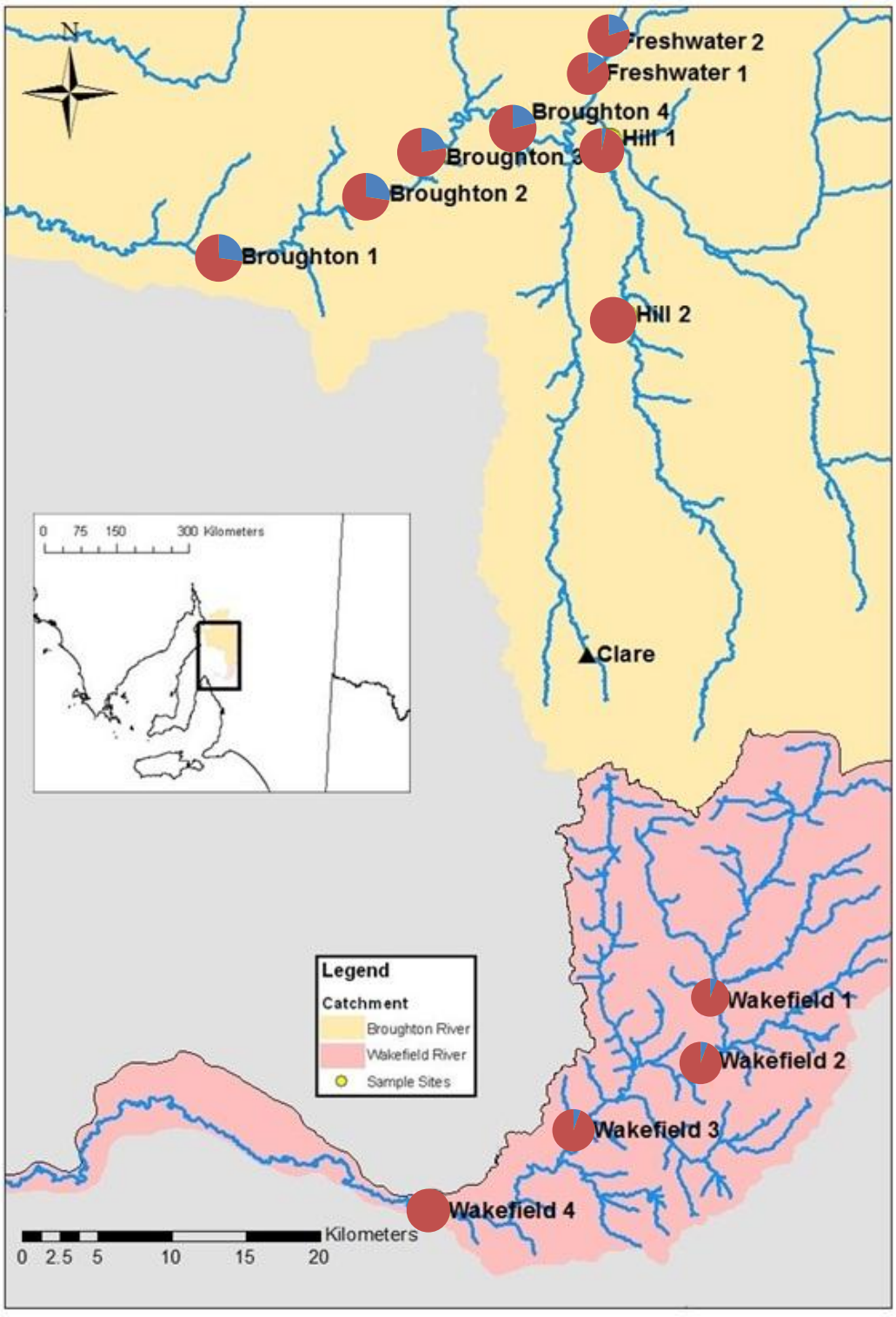

Figure 1: Map of the study sites across the Broughton and Wakefield Catchments in South Australia. Pie charts indicate the relative frequencies of 'Cluster 1' (blue) and 'Cluster 2' (red).

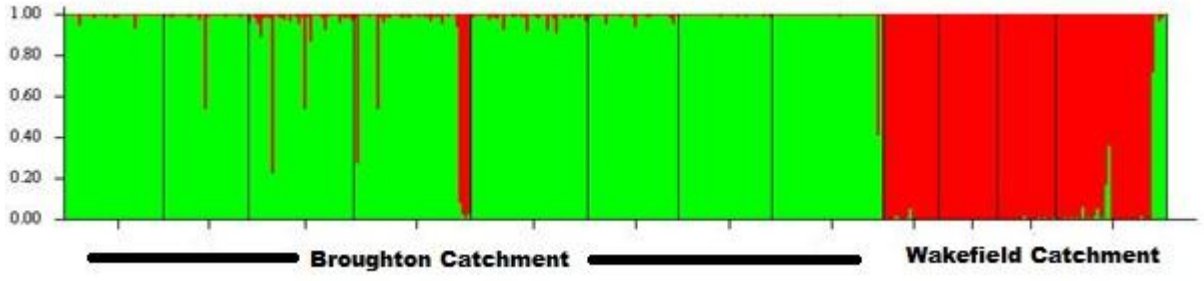

Figure 2: STRUCTURE output for all microsatellite data across both catchments for K=2.

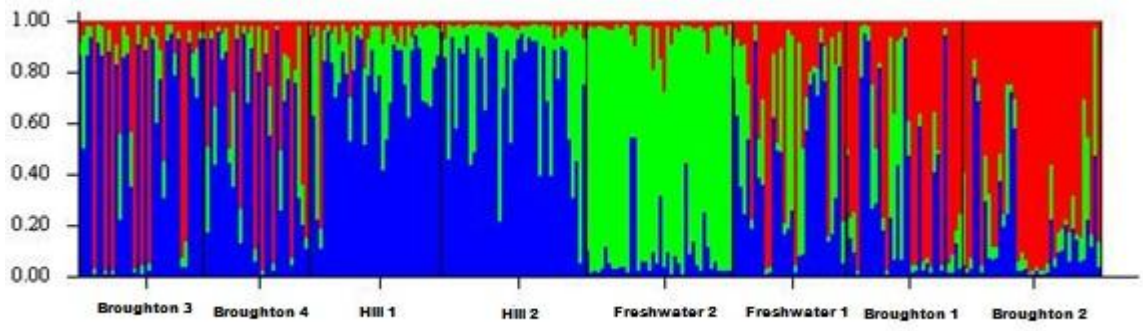

Figure 3: STRUCTURE output of all the microsatellite data for the Broughton Catchment for K=3.

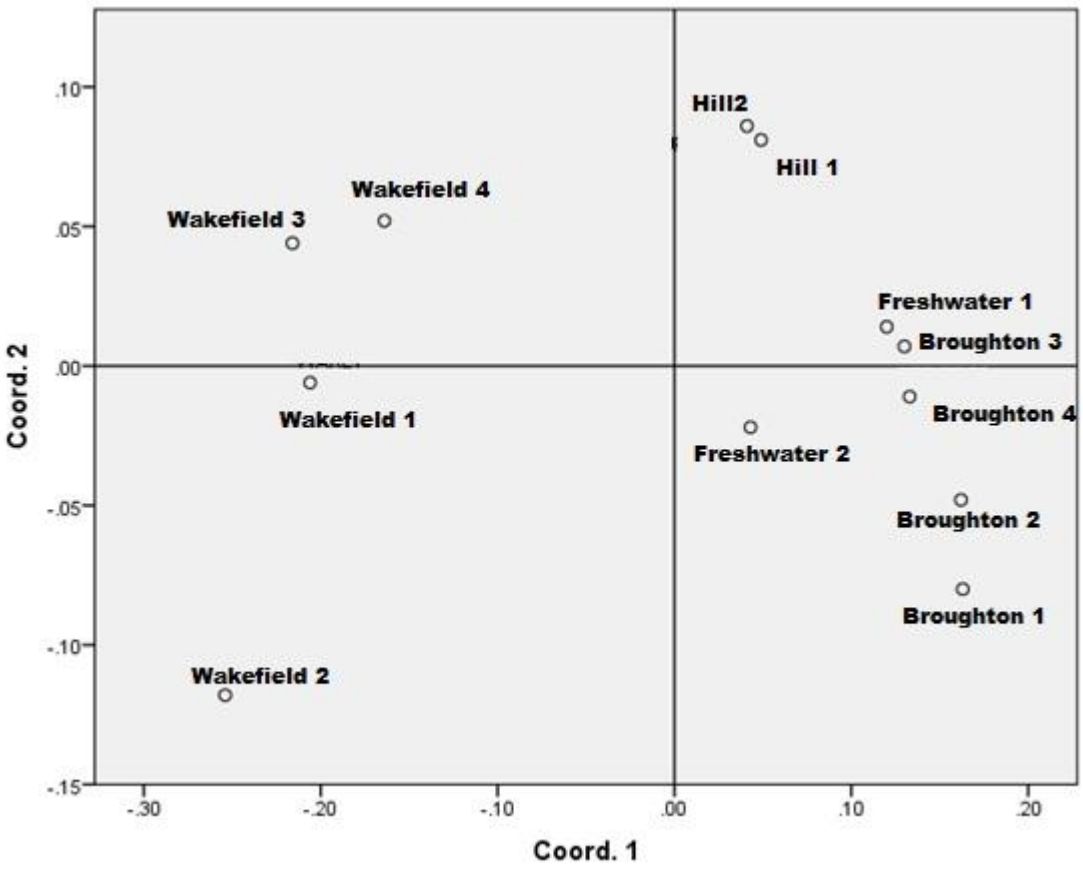

Figure 4: PCA plot of the Jost's *D* results for all microsatellite data across the Broughton and Wakefield Catchments.

Figure 5: STRUCTURE output for the microsatellite data from those individuals identified as being from Lineage 8 across both catchments for K=2.

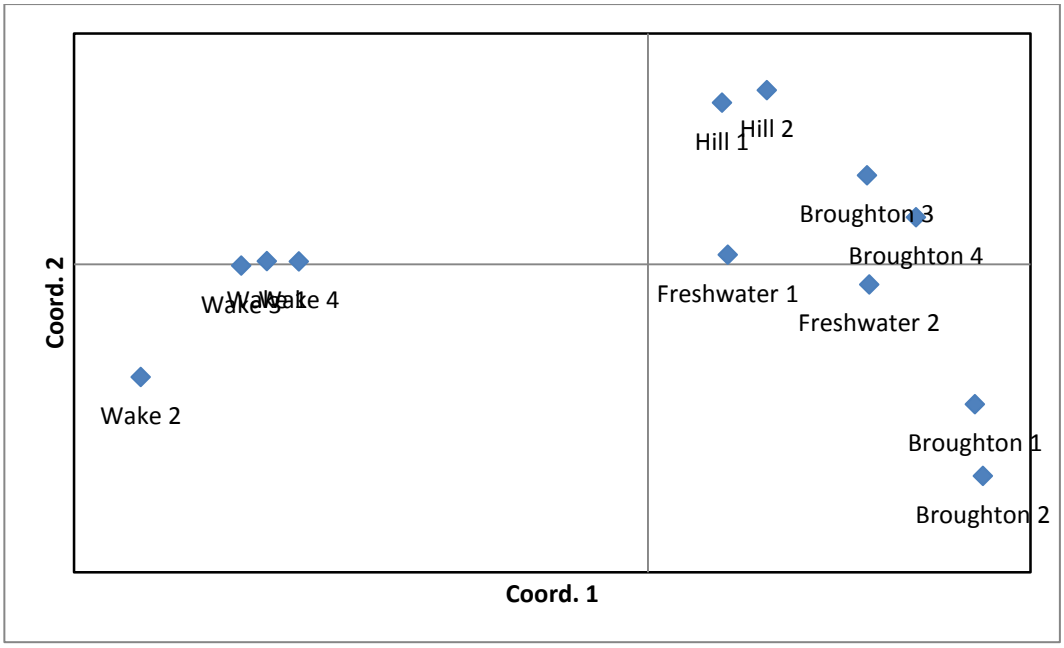

Figure 6: PCA plot of the Jost's *D* results for those individuals identified as being from lineage 8 across the Broughton and Wakefield Catchments.

Chapter 5

Accurate identification of larval damselflies: genetic clues to subtle morphology

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Abstract

The correct identification of study specimens is paramount to the integrity of ecological studies. Aquatic macroinvertebrate identification can be difficult as diagnostic characteristics are often subtle and subject to variation within species. Incorrect identifications of aquatic macroinvertebrates have implications for their use as bioindicators of environmental parameters such as water quality or community health. We used the mitochondrial cytochrome oxidase I gene that has been widely used to 'barcode' metazoan taxa to examine two species of damselfly from the Coenagrionidae family. Initial morphological identification was difficult due to subtle differences between species. The COI sequence data indicated that approximately 50% of the individuals were identified incorrectly based solely on morphology. Our study highlights the fact that analysis based only on morphological identifications may seriously reduce the utility of a taxonomic study and demonstrates the importance of DNA-based identification for ecological studies.

Introduction

The correct identification of study specimens is paramount to most biological studies. At a fundamental level, species identification is the backbone of most ecological studies with most findings being tied to a specific species or group of species. However, taxonomy is often ignored in ecological studies as highlighted by Bortolus (2008). Of the 80 papers reviewed by Bortolus (2008), 62.5% made no reference to taxonomic literature, expert knowledge or any form of support for the identification of the species studied. Furthermore, only 2.5% of the included articles reported placing material in reference collections to allow others to verify identifications (Bortolus, 2008). This trend is concerning as accurate identifications in macroinvertebrate studies are particularly difficult due to often subtle diagnostic morphological features. Macroinvertebrate species level identification is made even more complex with early instars often not displaying diagnostic characters and with increasing numbers of cryptic species being uncovered through genetic techniques (Baker *et al.*, 2004, Bickford *et al.*, 2006). For these reasons many macroinvertebrate sampling protocols for assessments of habitat quality only identify individuals to higher taxonomic levels (Bailey *et al.*, 2001).

The use of genetic markers to identify species is becoming increasingly common with the proliferation of genetic sequencing techniques and technologies (Hebert *et al.*, 2003, Herbert *et al.*, 2003, Rach *et al.*, 2008). While the use of this technology to delineate species previously unstudied species without the aid of any morphological study has met with some resistance (e.g. Will and Rubinoff, 2003, Moritz and Cicero, 2004, Ebach and Holdrege, 2005, Will *et al.*, 2005, Kvist, 2013), its utility in identifying species, both in the sense of recognising

new species and in the sense of species identification, is now widely acknowledged (Hogg and Hebert, 2004, Young *et al.*, 2013). These techniques allow researchers to quickly and accurately identify study species or provide support for identifications based on morphological characters, even in previously collected museum collections (Zuccon *et al.*, 2012). This ability to use genetic techniques to separate recognised species becomes increasingly important or useful when examining species in groups with relatively low levels of morphological variation (Rach *et al.*, 2008, Porco *et al.*, 2012, Zhou *et al.*, 2012).

Studies in freshwater biology often use species as surrogates or bioindicators of environmental parameters such as water quality or community health (Resh and Unzicker, 1975, Chessman and McEvoy, 1998). In addition, there are several rapid biodiversity assessment protocols such as AusRivAS (Smith *et al.*, 1999) and RIVPACS (Davy-Bowker *et al.*, 2006) that rely on fast, accurate identification of specimens to draw conclusions on river health relative to optimal conditions. The reference condition approach builds upon a baseline of community composition that represents a natural and preferably undisturbed community state. The assessment process then compares the compositional dissimilarity of surveyed sites (observed) with an environmentally similar reference condition (expected). The ratio of observed species composition to expected species composition (O:E) indicates the discrepancy between the surveyed community and the reference condition and this is then linked to the level of human disturbance (Smith *et al.*, 1999).

While rapid assessment protocols such as AusRivAS (Smith *et al.*, 1999) often identify taxa only to family level, they still rely on accurate identification of individuals to at least the family level. These methods have been widely adopted

across much of Europe, Canada and Australia for baseline biological surveys and monitoring programs (Resh *et al.*, 2006). One of the limitations with such assessment protocols is the effect of species that are sensitive to stress, in particular organic pollutants which often appear to be less widespread (Clark and Murphy, 2006).. Given that often samples are only identified to family level, such rare taxa can often be lumped with other, more common representatives of the same family (Marshall *et al.*, 2006).

A further potential limitation of rapid assessment protocols that has received little attention is the effect of incorrect identification of samples and the effect this may have on metrics and the conclusions drawn from them. Haase et al. (2006) identified considerable errors arising from the morphological misidentification of some macroinvertebrates in their audit of the STAR project (see Furse *et al.*, 2006). These errors were detectable in most of the calculated metrics and their study identified a real need for quality control of identification procedures. These misidentification errors were identified again in a separate study of macroinvertebrate sorting and identification by Haase et al. (2010). Other studies have suggested that biological survey data should have additional metadata attached indicating how the identification was undertaken, and by whom, in order to indicate the confidence placed in the identification (Hebert *et al.*, 2003, Stribling *et al.*, 2003, Deiner *et al.*, 2013). For example, the barcode of life project (Stockle and Herbert, 2008) that aims to use a fragment of mitochondrial DNA to identify species, much like a barcode, has protocols around the identification of species that entail the collection of additional metadata including notes on species identification (Weigt *et al.*, 2012).

Several barriers to correct identification may exist. Misidentification of difficult to identify species or genera due to lack of training or experience can be countered by increased training and quality control (Nerbonne and Vondracek, 2003, Haase *et al.*, 2006, Haase *et al.*, 2010). However, not all misidentification can be traced back to inadequate training. There are other sources of error introduced through more fundamental issues with the identification of biological organisms. In particular, with aquatic macroinvertebrates it can be difficult to identify taxa using existing taxonomic keys based on morphological characters due to the presence of cryptic species (Stoks *et al.*, 2005, Rach *et al.*, 2008). Also, there may be species complexes which are not well-understood and in need of further study. Cryptic variation within recognised species has been identified for nearly 300 years across many different taxa (Bickford *et al.*, 2006). With the increasing use of genetic techniques in the study of biological systems, particularly DNA sequencing, the rate at which these species are being identified is rapidly increasing (Pfenninger and Schwenk, 2007), although there is still much debate over the use of genetic information alone for delimiting species, with no agreed level of genetic divergence marking a universal split between species (Rach *et al.*, 2008). Instead, intraspecific genetic divergence needs to be compared to that between the putative taxa ((i.e. the 'Barcode Gap'; e.g. Puillandre *et al.*, 2012).

The Odonata (Insecta) are one of the most obvious and well-known groups of aquatic insects and are an important part of most aquatic ecosystems (Cummins, 1973). Due to their common nature, Odonata members are often represented in aquatic surveys (Hawking and Theischinger, 2004). There have been several cryptic lineages identified within the Odonata and this group of aquatic insects

highlight the problems of relying solely on morphological assessments for species identification. For instance, in their examination of DNA barcoding in Odonata, Rach et al. (2008) found several groups where genetic divergence was not mirrored morphologically. Stoks et al. (2005), using mitochondrial sequence information, also identified several cryptic lines of damselflies within the genus *Enallagma* across the Holarctic. They concluded that a combination of adult mate selection and variation at common traits that drive larval ecological performance have resulted in parallel evolution. Hogg and Hebert (2004) examined the effectiveness of DNA barcodes in identifying species of Collembola from the Canadian Arctic. They found that they were able to identify all species correctly, with within species divergences being consistently less than 1% and between species divergences being over 8%. They conclude that DNA barcoding is useful as a complimentary identification method to morphology-based identification.

In addition to the issues associated with cryptic species, the larvae of many species of Odonata are only identifiable in their final instar (Theischinger, 2009). One way that this issue has been dealt with in biological surveys is by the grouping or lumping of small instars together into a single category, however this results in a loss of information similar to that lost through identification to higher levels only (Marshall *et al.*, 2006).

Damselflies of the family Coenagrionidae are among the most widely distributed Odonata (Askew, 2004, Theischinger and Hawking, 2006). In Australia they are represented by 13 genera, two of which are endemic (Theischinger and Hawking, 2006). The identification of the larvae of these genera has proven difficult in the past with diagnostic characteristics being subjective and overlapping between species and genera (Hawking and Theischinger, 2004). Identification of adult

specimens is somewhat easier as the inclusion of characters associated with the genitalia and with markings across the head and tails provides additional diagnostic characters (Theischinger, 2009). However, the larval stages are more easily captured and represented in surveys.

Ischnura heterosticta (Burmeister) is a widespread and common damselfly from the family Coenagrionidae that has been used in several studies in the past. Warfe and Barmuta (2004) used it as a model predator in their examination of the effects of habitat complexity on predation success. Kefford et al. (2006) detailed the growth response of larvae to saline waters. Due to its Australia-wide presence, it is also common in ecological surveys and studies of Odonata (e.g. Hawking and New, 2002). Given its prominent role in such applications, it would be useful to be able to rapidly and reliably identify larvae of this species using simple morphological criteria that has been verified by comparisons to genetic data. In order to assess potential barriers to correct identification of this taxon, and as part of a larger study of the population genetics of several aquatic macroinvertebrates, we compared the groupings of putative larvae of *I. heterosticta* derived from mitochondrial (COI) sequence data with identifications based on morphological criteria using a current taxonomic key (Theischinger, 2009). Mitochondrial sequences were also compared to those of several adult specimens and to the existing COI sequence data collection on Genbank submitted by Futahashi (2011).

Methods

Field sites and sampling

Individuals were sampled from four sites across two catchments centred around the Clare Valley, South Australia. Sites IW1 and IW4 were from the Wakefield Catchments (33.919S, 138.817E and 34.167 S, 138.522 E respectively). Sites IB1 and IB7 were from the Broughton Catchment (33.529 S, 138.523 E and 33.464 S, 138.625E, respectively). The waterways through this region are ephemeral and recede to a series of permanent pools for most of the year (Favier *et al.*, 2000, Favier *et al.*, 2004). Larvae were sampled from sites across both catchments using a pond net. Adults were collected from two sites (one per catchment) using butterfly nets. Individuals were placed in 100% ethanol on ice then later stored at 4˚C. Individuals were putatively identified in the laboratory using Theischinger (2009) as *Ischnura heterosticta*. Sixty-three larvae and eight adults were selected for analysis.

Genetic Techniques

DNA was extracted from a section of tail using a modified Gentra Extraction Protocol. PCRs were performed in a 25 μl reaction using 2 μl of a 1/10 dilution of template DNA, 0.1 μl of Amplitaq Gold (Life Technologies, Carlsbad, California), 1 μl of 25mM $MgCl_2$, 2.5 μl of 10X PCR Gold Buffer, 0.5 μl of 10mM dATP, 0.5 μl of 10mM dCTP, 0.5 μl of 10mM dGTP, 0.5 μl of 10mM dTTP, 1 μl of 0.5μM Forward Primer, 1 μl of 0.5μM Reverse Primer and 15.4 μl of molecular grade water. Primers used were LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') designed by Folmer et al. (1994)

in order to target the CO1 region of the mitochondrial DNA. PCR conditions were 95°C for 10 minutes, 34 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 50 seconds with a final extension step of 72°C for 6 minutes. Sequencing of the CO1 region of the mitochondrial DNA was performed using the forward primer by AGRF (Adelaide, Australia). Sequences were aligned using Mega (Version 5.05, Tamura *et al.*, 2011). Additional sequence was gathered from Genbank from Futahashi (2011). The alignment was trimmed to remove all gaps in the data, there was no missing data with any of the sequences.

Phylogenetic Reconstruction

A phylogeny was then constructed to examine relationships between individuals using BEAST (Drummond *et al.*, 2012). The data were not partitioned based on codon position or other parameters. Results of the MCMC was sampled every 1000 iterations. Given the variable base frequencies observed in the data, the HKY substitution model was used on estimated base frequencies and the Bayesian Skyline process was used as the model of diversification. The HKY method is a robust method of substitution that makes few assumptions about the data and/or populations. It allows for variable base frequencies like we had in this data and has only one rate of transition and transversion. The phylogeny was built as a maximum clade credibility tree using TreeAnnotator (Version 1.7.3.0) with target node heights maintained (Lemey *et al.*, 2009, Drummond *et al.*, 2012).

Automatic Barcode Gap Detection

Automatic barcode gap detection was used to estimate hypothetical species based on the barcode gap, comparing intra vs. interspecies divergence at COI (Puillandre *et al.*, 2012). The automatic barcode gap detection analysis uses

ranked pairwise differences between the barcode sequence. By observing the slope of the ranked pairwise differences the program is able to detect significant changes in the pairwise differences between groups of species. It uses the 'gaps' to infer candidate species (Puillandre *et al.*, 2012). Puillandre et al. (2012) also suggest that the candidate species information be used in conjunction with other data to determine species.

Default parameters were used (Pmin = 0.001, Pmax = 0.1, 10 steps, X = 15 and 20 Nb bins). The pairwise distance matrix was created in Mega (Tamura *et al.*, 2011) using the Jukes-cantor model with uniform rates among sites and no variance estimation. These hypothetical species were then compared to the species used in the construction of the phylogeny to examine any splits between the sequenced individuals.

Results

Sampling Results

Specimens were examined in the field using a hand lens to identify the family to which they belonged. Field sampling resulted in 30-40 late or final instar Coenagrionidae larvae being collected at each of the four study sites. Once the larvae were identified to species level using Theischinger (2009), 20 individuals per site were selected haphazardly for sequencing. All eight adult Zygoptera that were captured across the two sampling sites were identified to species level and sequenced.

Larval Morphological Identification

All larvae were identified using Theischinger (2009) as being *Ischnura heterosticta*, however, difficulties in the identification process were acknowledged, particularly with immature larvae. For this reason only final or near final instars were used for this study.

All individuals were easily identified to the family Coenagrionidae using Theischinger (2009), but the identification to species of larval stages of taxa within Coenagrionidae became more difficult, particularly when assessing character states involving the pattern of the premental setae (step 8, page 146, Theischinger, 2009) and the shape and length of the prementum (Step 11 & 12, Pages 146 & 147, Theischinger, 2009). The leads for step 8 are 'premental setae in a straight line' or 'premental setae in a curved row'. This orientation of the premental setae is an important character for separating larvae of several different genera and, of importance to this study, in separating *Ischnura* and *Austroagrion*. All individuals identified appeared to have setae arranged in a straight line. This character was based on the position of the innermost setae in the specimens examined. Furthermore, the position of those small setae on the side of the labium are significant as they only tend to extend below the setae in the middle if they are in a "straight-line" setae (A. Bush, Pers. Comm.). The number of premental setae were also variable, with some individuals having five pairs of setae while others had six, both being included at this point in the identification process. Adding to the difficulty of identification, it was common for individuals to be missing setae, presumably from damage during prey capture.

With respect to the shape and length of the prementum used in step 11, there was a range of sizes observed in individuals at the final instar stage. The leads for this step are ‘prementum c. 1.5mm long; dorsal branch of labial palps basally narrower than ventral branch’ or ‘prementum 2.3-2.5mm; dorsal branch of labial palps basally about as wide as ventral branch’. The values for the length of the prementums of the final instars ranged from 1.8 to 2.4mm, allowing for easy identification of some individuals, while others were distinguished based on the labial palps which showed very limited differentiation. This issue was continued through step 12 which compares the length:width ratio of the prementum, ‘prementum stout, length:greatest width ratio c. 1.15; six to seven palpal setae’ or ‘prementum slimmer, length:greatest width ratio c. 1.125; generally five palpal setae’, with the differences between the diagnostic characteristics becoming increasingly subtle. Diagnostic characters were based on differences of around 0.16mm. Given that the overall size of final instars varied by over 7mm, this character was difficult to assess consistently. Geographical range was also included at this point in the key as a distinguishing character in the key as there which assumes that there is no spatial overlap in distribution.

Adult Morphological Identification

Identification of adults was considerably easier than that of the larvae. Wing venation and colour patterns were easily identifiable and were not affected by overall size of the individual. Of the eight adult individuals sampled in the study area and identified, one was *I. heterosticta,* five were *Austroagrion watsoni* and two were *Austrolestes annulosus,* which was not identified at all in the larval study. Identification of the adults was achieved quickly using Theischinger (2009), with the only issue being the geographical range of *A. watsoni,* which the

key suggested was not found in this region of southern Australia. The only other member of this family reported to occur in the study region is *Austroagrion cyane.* However, the distinctive colouration on section 8 of the tail suggested that these individuals were *A. watsoni* (Theischinger, 2009). Current knowledge of the distribution of most Australian Odonata is currently incomplete (Hawking and Theischinger, 2004).

Phylogeny Reconstruction

A total of 63 larvae and 8 adult damselflies were sequenced. Once the additional sequence data from Futahashi (2011) from Genbank were added, there was a total of 108 sequences of 433 base pairs. The phylogeny derived from the mtDNA data indicated that there were two distinct clades in the sequence data collected (posterior probability 0.921) (Figure 1). One of the clades grouped together with the *Ischnura heterosticta* sequence data from Futahashi (2011) (Figure 2). Further support for this identification was provided by the adult sequence data. The second cluster was identified as *Austroagrion watsoni* based on the morphological identification of the adult specimens (Figure 3). *Austroagrion* was not examined by Futahashi (2011) and there were no sequences for this genus on Genbank.

The clade containing *A. watsoni* (Clade A in Fig. 1) diverged from *Ischnura, Pacificagrion, Enallagma* and *Amophostigma* gathered from Genbank, (Futahashi, 2011) data by 13%. Clade A formed a sister group to a single individual, IB1 18. The other clade contained multiple genera. The genus *Ischnura* (among others) separated from *Aciagrion migratum* with strong support (posterior probability 1.0) with 11% sequence divergence. The splits between the remaining individuals within this clade were not well supported (posterior

probability >0.9) with the exception of some of the terminal nodes between the individuals from the current study identified as *I. heterosticta* (forming Clade B in Fig. 1) and *I. rufostigma* (posterior probability 1.0), and between *I. heterosticta* and *I. senegalensis* (posterior probability 1.0). Within the two clusters of individuals from the present study there was limited differentiation except at terminal nodes between single individuals (see expanded clades in Figures 2 and 3). Within Clade A there was strong support separating IB1.26 and IB1.30 (posterior probability 0.92, Figure 2). Both of these individuals are from the same sample location. There was also a strong support for the difference between *A. watsoni* (Adult 8) and IB1.25 and IB7.02 (posterior probability 0.99, Figure 2). All of these individuals were sampled within the Broughton Catchment. Within Clade B there was only one strongly supported node separating IW1.26 and IB1.05 (posterior probability 0.98, Figure 3). These individuals were from different catchments but there was no additional evidence supporting a difference between catchments that may have been expected due to the strong site fidelity observed in other species of Damselfly (Geenen *et al.*, 2000).

There was a single representative from *Ischnura aurora* within the dataset as well, based on the sequence data from Futahashi (2011). The placement of individuals in the phylogeny also indicated some discrepancies between the genetic data and the current classifications of some taxa, in particular, the placement of *Pacificagrion* within the genus *Ischnura*.

Automatic Barcode Gap Discovery

The first and second partition of the data showed 44 groups at a prior maximal distance of 0.00100 and 0.00167, respectively. Partition three through five

showed 25 groups up to a prior maximal distance of 0.00774. Partitions 6 through 9 found 24 groups up to a prior maximal distance of 0.05995. The tenth partition found one group at 0.1000 (Figure 4). The 24 hypothetical species identified in the last 3 partitions represent the 24 species identified in the phylogeny (Figure 1). The extra group for partitions three through five was individual IB1.18. This individual is also identified as divergent from *A. watsoni* in the phylogeny, although the posterior probability is low (<0.9, Figure 2). The groups represented by partitions one and two begin to split the individuals of *A. watsoni* and *I. heterosticta.* These splits are matched in the phylogeny but not supported by the posterior probability values (<0.9).

Discussion

Identification of Coenagrionid larvae such as *I. heterosticta* using only morphological traits can be time-consuming and difficult, owing to the small and often subtle morphological differences that separate taxa. These difficulties are particularly challenging for aquatic biologists engaged in studies such as rapid biodiversity assessments that involve the rapid processing of large numbers of larval specimens. We have uncovered problems with current taxonomy of the Coenagrionidae by using barcoding sequences. Our study highlights that analysis based on current taxonomic designations could seriously influence interpretations of other ecological patterns.

The different species identified in this study are all widespread, common and sympatric (Theischinger and Hawking, 2006, Theischinger, 2009). Hawking and Theischinger (2004) comment that a detailed taxonomic study and subsequent key for the larvae of these species is lacking and that this has been an impediment to

aquatic surveys. The development of the larval key for Odonata by Theischinger (2009) removes much of this impediment, however, its usefulness for some families is limited. The distinction between the two species *I. heterosticta* and *A. watsoni* in the key of Theischinger (2009) centres around the position of setae in the prementum (step 8). The resolution of this character can be difficult in many individuals as the difference between the curved line of setae and the straight line of setae is minimal and often determined by the position of a single seta at the end of the row. The considerable variation among individuals and the frequent occurrence of missing setae in collected specimens also make this character state difficult to reliably assess.

The subsequent step (step 11) in the key involving the size and shape of the prementum, as well as sample location, is also difficult to use as distinguishing features because the size of the prementum varies considerably based on the size of the individual and there is also substantial variation among individuals of the study species. Furthermore, knowledge of the distribution of many species of Odonata is often anecdotal or incomplete (Hawking and Theischinger, 2004).

The difficulty of creating a key based on larval morphological criteria for such subtly differentiated organisms, such as some of the larvae of this family of damselfly, is considerable. The incorporation of genetic data in taxonomic assessments of such organisms is likely to be rewarding. With the increased use of genetic barcoding (Rach *et al.*, 2008) and other genetic marker systems for identification, as well as the availability of genetic data in online services such as Genbank, it has become much easier to characterize and compare individuals genetically (Benson *et al.*, 2011). Though there is some hesitation to use this technology for the classification of new species due to differing views about the

relative importance of reproductive isolation, genetic differences or morphology in separating species (Will and Rubinoff, 2003), its usefulness in cases such as the present study seems clear (Rach *et al.*, 2008). The decreasing cost associated with genetic sequencing and the emergence of third party sequencing services should also lead to an increase in the number of researchers utilising this option for identification of study species to verify morphological identifications.

The reasons behind the lack of differentiation in larval morphology among several species of this family are unclear. The marked difference in the larval and adult life forms in Odonates presumably gives rise to a complex combination of selective pressures. The physical characteristics of the larval stage may suggest convergent selective pressures resulting in similar camouflage colourations and functional feeding appendages. In the winged adult stage, sexual selection may become a more prominent driver with males of different species developing distinctive bright colours. The effects of these different pressures are still poorly understood, however it is apparent that larval morphology has either changed very little since speciation or has converged across several genera, though there are no detailed studies of this.

The drivers of differentiation between species in morphology are complex with multiple drivers often acting simultaneously (Orr and Smith, 1998, Nosil *et al.*, 2009). Cryptic species may arise where multiple selective pressures result in convergent evolution or where insufficient time has passed for morphological differences to become evident among diverging lineages. Studies examining the morphology of Echinoderm larvae have identified a convergent simplification of larval forms to better cope with shared marine environmental stressors

(McEdward and Janies, 1997). Similar strategies have been seen in other marine invertebrate larvae, even across phyla (Belmonte *et al.*, 1997).

Damselflies are one of the most common aquatic macroinvertebrates across the world. As such they often play a major role in many rapid assessment protocols and environmental monitoring programs. While some assessment protocols only identify individuals to family level, hence avoiding difficulties with identification of species (e.g. Chessman, 1995, Chessman, 2003), other studies examining the biodiversity and species diversity of sites are likely to face difficulties with the specific identification of damselfly larvae.

The consequences of such misidentifications are unclear. Some suggest that they may be widespread and that insufficient attention to taxonomic identifications in ecological studies can have far reaching effects on our understanding of ecosystem structuring and functioning (Hewlett, 2000, Bortolus, 2008, Monk *et al.*, 2012). Alternatively, the argument has be made that those species that are similar enough to be particularly subject to misidentification are likely to be similar in biology and ecology, and therefore errors in identification among such species are of little consequence. Our view is that correct identifications are an integral part of the characterisation of levels of biodiversity such as those found in surveys of the status of the 'health' of aquatic ecosystems.

While this study has centred around one specific issue of identification, it does highlight the role that genetic techniques can play in species identification. The increased use of genetic barcoding in species identification has been accompanied by several debates concerning the roles of traditional morphology-based taxonomy and the genetic delineation of species (Will and Rubinoff, 2003, Rach

et al., 2008, Ratnasingham and Hebert, 2013). While the issues associated with this argument are not the basis of this work, the correct identification of organisms appears to be increasingly based on genetic markers (Porco *et al.*, 2012). The proliferation of genetic information in services such as Genbank (Benson *et al.*, 2011) has allowed for easy and rapid comparison of genetic information. While we do not suggest the routine sequencing of all study specimens, as the cost alone may be prohibitive, sequence data from a subsample of specimens should be considered in situations where species identifications are difficult. There is also the opportunity for clusters or groupings that are identified from genetic markers to suggest or highlight the presence of previously unrecognized morphological traits useful for taxonomic purposes.

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References

Askew, R. R. 2004. *The Dragonflies of Europe*, Colchester, UK, B H & A Harley Ltd.

Bailey, R. C., Norris, R. H. & Reynoldson, T. B. 2001. Taxonomic resolution of benthic macroinvertebrate communities in bioassessments. *Journal of the North American Benthological Society,* 20**,** 280-286.

Baker, A. M., Hughes, J. M., Dean, J. C. & Bunn, S. E. 2004. Mitochondrial DNA reveals Phylogenetic structuring and cryptic diversity in Australian freshwater macroinvertebrate assemblages. *Marine and Freshwater Research,* 55**,** 629-640.

Belmonte, G., Miglietta, A., Rubino, F. & Boero, F. 1997. Morphological convergence of restingstages of planktonic organisms: a review. *Hydrobiologica,* 355**,** 159-165.

Benson, D. A., Karsch-Mizachi, I., Lipman, D. J., Ostell, J. & Sayers, E. W. 2011. GenBank. *Nucleic Acids Research,* 39**,** D32-D37.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K. & Das, I. 2006. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution,* 22**,** 148-155.

Bortolus, A. 2008. Error cascades in the biological sciences: the unwanted consequences of using bad taxonomy in ecology. *AMBIO: A Journal of the Human Environment,* 37**,** 114-118.

Chessman, B. C. 1995. Rapid assessment of rivers using macroinvertebrates: A procedure based on habitat-specific sampling, family level identification and a biotic index. *Australian Journal of Ecology,* 20**,** 122-129.

Chessman, B. C. 2003. New sensitivity grades for Australian river macroinvertebrates *Marine and Freshwater Research,* 54**,** 95-103.

Chessman, B. C. & McEvoy, P. K. 1998. Towards diagnostic biotic indices for river macroinvertebrates. *Hydrobiologia,* 364**,** 169-182.

Clark, R. T. & Murphy, J. F. 2006. Effect of locally rare taxa on the precision and sensitivity of RIVPACS bioassessment of freshwaters. *Freshwater Biology,* 51**,** 1924-1940.

Cummins, K. W. 1973. Trophic relations of aquatic insects. *Annual Review of Entomology,* 18**,** 183-206.

Davy-Bowker, J., Clarke, R. T., Johnson, R. K., Kokes, J., Murphy, J. F. & Zahradkova, S. 2006. A comparison of the European Water Framework Directive physical typology and RIVPACS-type models as alternative methods of establishing reference conditions for benthic macroinvertebrates. *Hydrobiologica,* 566**,** 91-105.

Deiner, K., Knapp, R. A., Boiano, D. M. & May, B. 2013. Increased accuracy of species lists developed for alpine lakes using morphology and cytochrome oxidase I for identification of specimens. *Molecular Ecology Resources,* Early View**,** DOI: 10.1111/1755-0998.12130.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. 2012. Bayesiam phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution***,** doi:10.1093/molbev/mss075.

Ebach, M. C. & Holdrege, C. 2005. DNA barcoding is no substitute for taxonomy. *Nature*, 697**,** 697.

Favier, D., Rixon, S. & Scholz, G. 2000. A River Management Plan for the Wakefield Catchment. Adelaide: Environmental Protection Agency.

Favier, D., Scholz, G., VanLaarhoven, J. & Bradley, J. 2004. A River Management Plan for the Broughton Catchment. Adelaide: The Department of Water, Land, Biodiversity and Conservation.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhork, R. K. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3**,** 294-299.

Furse, M., Hering, D., Moog, O., Verdonschot, P., Johnson, R., Brabec, K., Gritzalis, K., Buffagni, A., Pinto, P., Friberg, N., Murray-Bligh, J., Kokes, J., Alber, R., Usseglio-Polatera, P., Haase, P., Sweeting, R., Bis, B., Szoszkiewicz, K., Soszka, H., Springe, G., Sporka, F. & Krno, I. j. 2006. The STAR project: context, objectives and approaches. *Hydrobiologia*, 566**,** 3-29.

Futahashi, R. 2011. A revisional study of Japanese Dragonflies based on DNA analysis. *Tombo*, 53**,** 67-74.

Geenen, S., Jordaens, K., De Block, M., Stoks, R. & De Bruyn, L. 2000. Genetic differentiation and dispersal among populations of the damselfly *Lestes viridis* (Odonata). *Journal of the North American Benthological Society,* 19**,** 321-328.

Haase, P., Murray-Bligh, J., Lohse, S., Pauls, S., Sundermann, A., Gunn, R. & Clarke, R. 2006. Assessing the impacts of errors in sorting and identifying macroinvertebrate samples. *Hydrobiologica,* 566**,** 505-521.

Haase, P., Pauls, S., Schinderhutte, K. & Sundermann, A. 2010. First audit of macroinvertebrate samples from an EU water framework directive monitoring program: human error greatly lowers precision of assessment results. *Journal of the North American Benthological Society,* 29**,** 1279-1291.

Hawking, J. & New, T. R. 2002. Interpreting dragonfly diversity to aid in the conservation assessment: lessons from the Odonata assemblage at Middle Creek, north-eastern Victoria, Australia. *Journal of Insect Conservation,* 6**,** 171-178.

Hawking, J. & Theischinger, G. 2004. Critical speies of Odonata in Australia. *Internation Journal of Odonatology,* 7**,** 113-132.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. W. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society Biological Sciences,* 270**,** 313-321.

Herbert, P. D. N., Ratnasingham, S. & de Waard, J. R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species *Proceedings of the Royal Society Biological Sciences,* 270**,** S96-S99.

Hewlett, R. 2000. Implication of taxonomic resolutiuon and sample habitat for stream species - level identifications. *Journal of the North American Benthological Society,* 20.

Hogg, I. D. & Hebert, P. D. N. 2004. Biological identification of springtails (Hexapoda: Collembola) from the Canadian Arctic using mitochondrial DNA barcodes. *Canadian Journal Of Zoology,* 82**,** 749-754.

Kefford, B. J., Zalizniak, L. & Nugegoda, D. 2006. Growth of the damselfly *Ischnura hetersticta* is better in saline water than freshwater. *Environmental Polution,* 141**,** 409-419.

Kvist, S. 2013. Barcoding in the dark?: A critical view of the sufficiency of zoological DNA barcoding databases and a plea for broader integration of taxonomic knowledge. *Molecular Phylogenetics and Evolution,* 69**,** 39-45.

Lemey, P., Rambaut, A., Drummond, A. J. & Suchard, M. A. 2009. Bayesian phylogeography finds its roots. *PLOS Computational Biology,* 5**,** e1000520.

Marshall, J. C., Steward, A. L. & Harch, B. D. 2006. Taxanomic resolution and quantification of freshwater macroinvertebrate samples from and Australia dryland river: the benefits and costs of using species abundance data. *Hydrobiologica,* 572**,** 171-194.

McEdward, L. R. & Janies, D. A. 1997. Relationships among development, ecology, and morphology in the evolution of Echinoderm larvae and life cycles. *Biological Journal of the Linnean Society,* 60**,** 381-400.

Monk, W. A., Wood, P. J., Hannah, M., Extence, C. A., Chadd, R. P. & Dunbar, M. J. 2012. How does macroinvertebrate taxonomic resolution influence ecohydrological relationships in riverine ecosystems. *Ecohydrology,* 5**,** 36-45.

Moritz, C. & Cicero, C. 2004. DNA barcoding: promise and pitfalls. *PLOS Biology,* 2004**,** e354.

Nerbonne, J. F. & Vondracek, B. 2003. Volunteer macroinvertebrate monitoring: assessing training needs through examining error and bias in untrained volunteers. *Journal of the North American Benthological Society,* 22**,** 152-163.

Nosil, P., Harmon, L. J. & Seehausen, O. 2009. Ecological explanations for (incomplete) speciation. *Trends in Ecology and Evolution,* 24**,** 145-156.

Orr, M. R. & Smith, T. B. 1998. Ecology and speciation. *Trends in Ecology and Evolution,* 13**,** 502-506.

Pfenninger, M. & Schwenk, K. 2007. Cryptic animal species are homogeneously distrubuted among taxa and biogeographical regions. *Evolutionary Biology,* 7**,** 121-127.

Porco, D., Bedos, A., Greenslade, P., Janion, C., Skarzynski, D., Stevens, M. I., van Vuuren, J. & Deharveng, L. 2012. Challenging species delimination in Collembola: cryptic diversity amond common springtails unveiled by DNA barcoding. *Invertebrate Systematics,* 26**,** 470-477.

Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. 2012. ABGD, automatic barcode gap discovery for primary species delimitation. *Molecular Ecology,* 21**,** 1864-1877.

Rach, J., DeSalle, R., Sarkar, I. N., Schierwater, B. & Hadrys, H. 2008. Character-based DNA barcoding allows discrimination of genera, species and populations in Odonata. *Journal of the Royal Society Biological Sciences,* 275**,** 237-247.

Ratnasingham, S. & Hebert, P. D. N. 2013. A DNA based registry for all animal species: The barcode index number (BIN) system. *PLos ONE,* 8**,** e66213, doi:10.1371/journal.pone.0066213.

Resh, V. H., Norris, R. H. & Barbour, M. T. 2006. Design and implementation of rapid assessment approaches for water resource monitoring using benthic macroinvertebrates. *Australian Journal of Ecology,* 20**,** 108-121.

Resh, V. H. & Unzicker, J. D. 1975. Water quality monitoring and aquatic organisms: the importance of species identification. *Journal (Water Pollution Control Federation),* 47**,** 9-19.

Smith, M. J., Kay, W. R., Edward, D. H. D., Papas, P. J., Richardsons, K. S. J., Simpson, J. C., Pinder, A. M., Cale, D. J., Horwitz, P. H. J., Davis, J. A., Yung, F. H., Norris, R. & Halse, S. A. 1999. AusRivAS: Using macroinvertebrates to assess ecological condition of rivers in Western Australia. *Freshwater Biology,* 41**,** 269-282.

Stockle, M. Y. & Herbert, P. D. N. 2008. Barcode of life. *Scientific American,* 299**,** 82-88.

Stoks, R., Nystrom, J. L., May, M. L. & McPeek, M. A. 2005. Parallel evolution in ecological and reproductive traits to produce cryptic damselfly species across the Holarctic. *Evolution,* 59**,** 1976-1788.

Stribling, J. B., Moulton II, S. R. & Lester, G. T. 2003. Determining the quality of taxanomic data. *Journal of the North American Benthological Society,* 22**,** 621-631.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution,* 28**,** 2731-2739.

Theischinger, G. 2009. Identification Guide to the Australian Odonata. Department of Environment, Climate Change and Water NSW.

Theischinger, G. & Hawking, J. 2006. *The complete field guide to Dragonflies of Australia,* Collingwood, Australia, CSIRO Publishing.

Warfe, D. M. & Barmuta, L. A. 2004. Habitat structural complexity mediates the foraging success of multiple predator species. *Oecologia,* 141**,** 171-178.

Weigt, L. A., Driskell, A. C., Ormos, A., Meyer, C. & Collins, A. 2012. Introductions to animal DNA barcoding protocols. *Methods in Molecular Ecology,* 858**,** 11-16.

Will, K. W., Mishler, B. D. & Wheeler, Q. D. 2005. The perils of DNA barcoding and the need for intergrative taxonomy. *Systematic Biology,* 54**,** 844-851.

Will, K. W. & Rubinoff, D. 2003. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics,* 20**,** 47-55.

Young, M. K., McKelvey, K. S., Pilgrim, K. L. & Schwartz, M. K. 2013. DNA barcoding at riverscape scales: assessing biodiversity among fishes of the genus Cottus (Teleostei) in northern Rocky Mountain streams. *Molecular Ecology Resources,* 13**,** 583-595.

Zhou, Z. J., Li, R. L., Huang, D. W. & Shi, F. M. 2012. Molecular identification supports most traditional morphological species of *Ruspolia* (Orthoptera: Conocephalinae). *Invertebrate Systematics,* 26**,** 451-456.

Zuccon, D., Brisset, J., Corbari, L., Puillandre, N., Utge, J. & Samadi, S. 2012. An optimised protocol for barcoding museum collections of decapod crustaceans: a case study for a 10-40 year-old collection. *Invertebrate Systematics,* 26**,** 592-600.

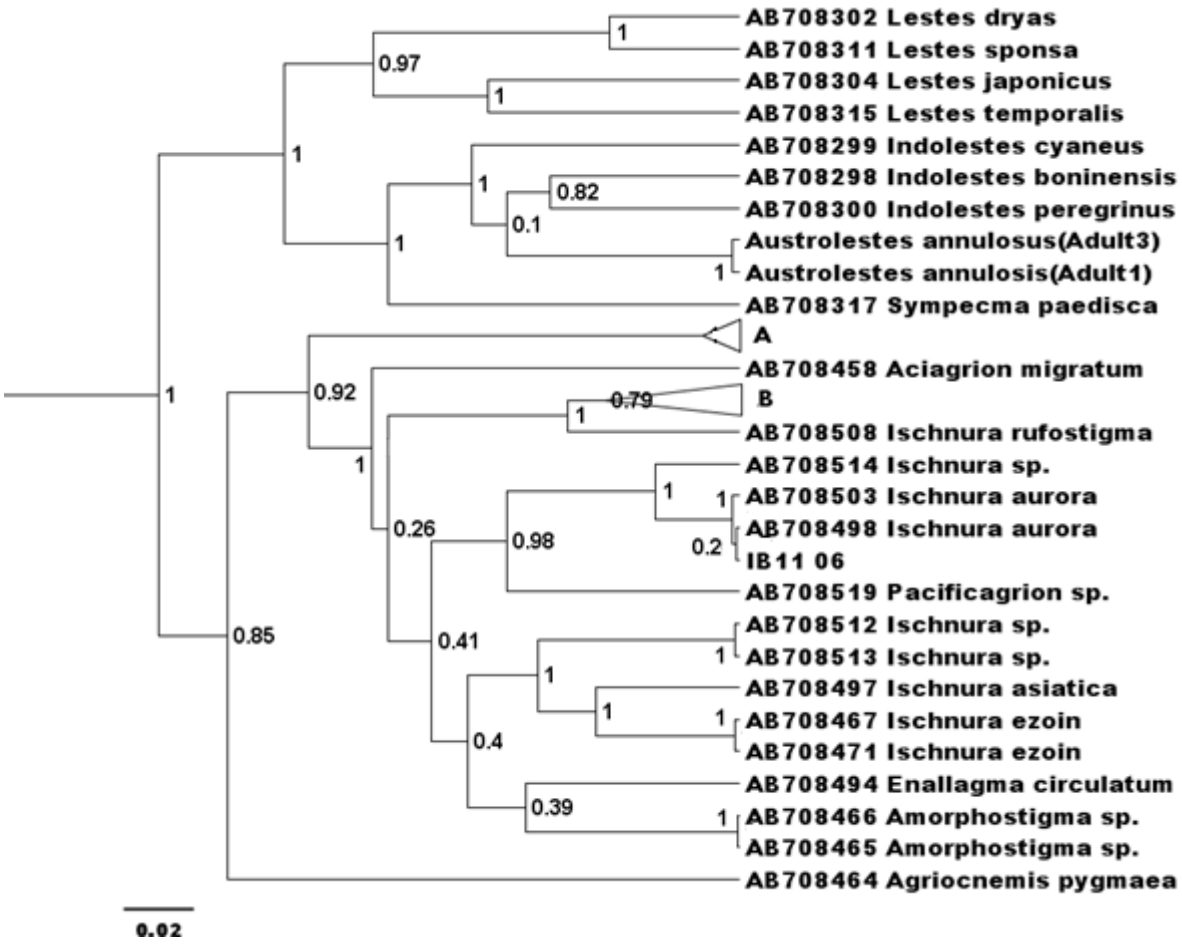

Figure 1: Maximum clade credibility tree produced using TreeAnnotator (Version 1.7.3.0) from the BEAST output of the CO1 sequence data. The posterior probabilities are shown for each node and the divergence distance scale is below the figure. The two main cluster of sequence from the present study are collapsed to show their position relative to other species. Clade A (expanded in Figure 2) contains those individuals identified as *A. watsoni* including the representative sequence from Futahashi (2011). Clade B (expanded in Figure 3) contains those individuals identified as *I. heterosticta* including a representative sequence the Genbank sequence from Futahashi (2011). Genbank Sequence data are identified by their accession number; larval individuals are labelled with a five digit code while the adult sequence data is labelled as such.

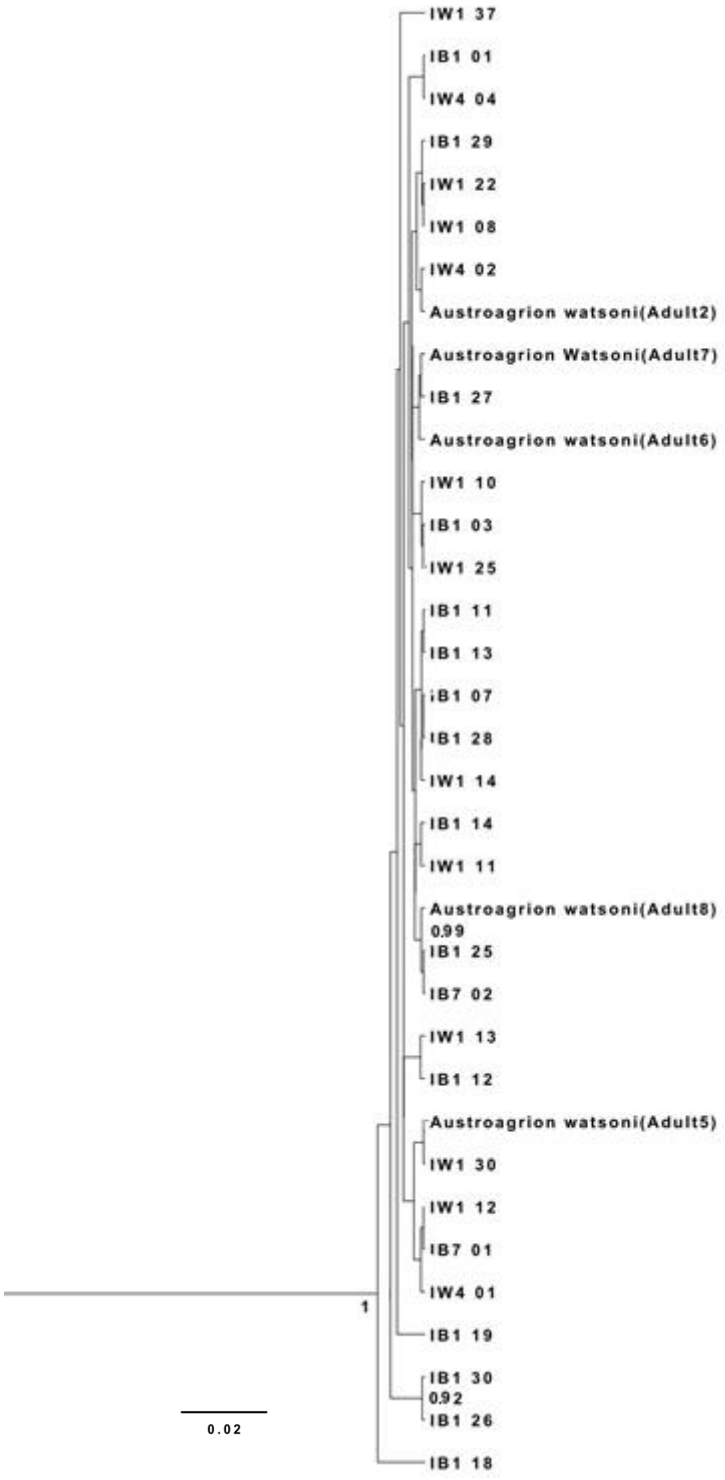

Figure 2: Expanded view of the maximum clade credibility tree produced using Tree Annotator from the BEAST output of the CO1 sequence data of Clade A from Figure 1. The posterior probabilities greater than 0.90 are shown and the divergence distance scale shown. The adult sequence data is identified by species name. Larval individuals are labelled with a five character code. The first digit is I, the second character is the source catchment (B = Broughton, W = Wakefield), the third character represents the capture site, the last two digits are the individual number.

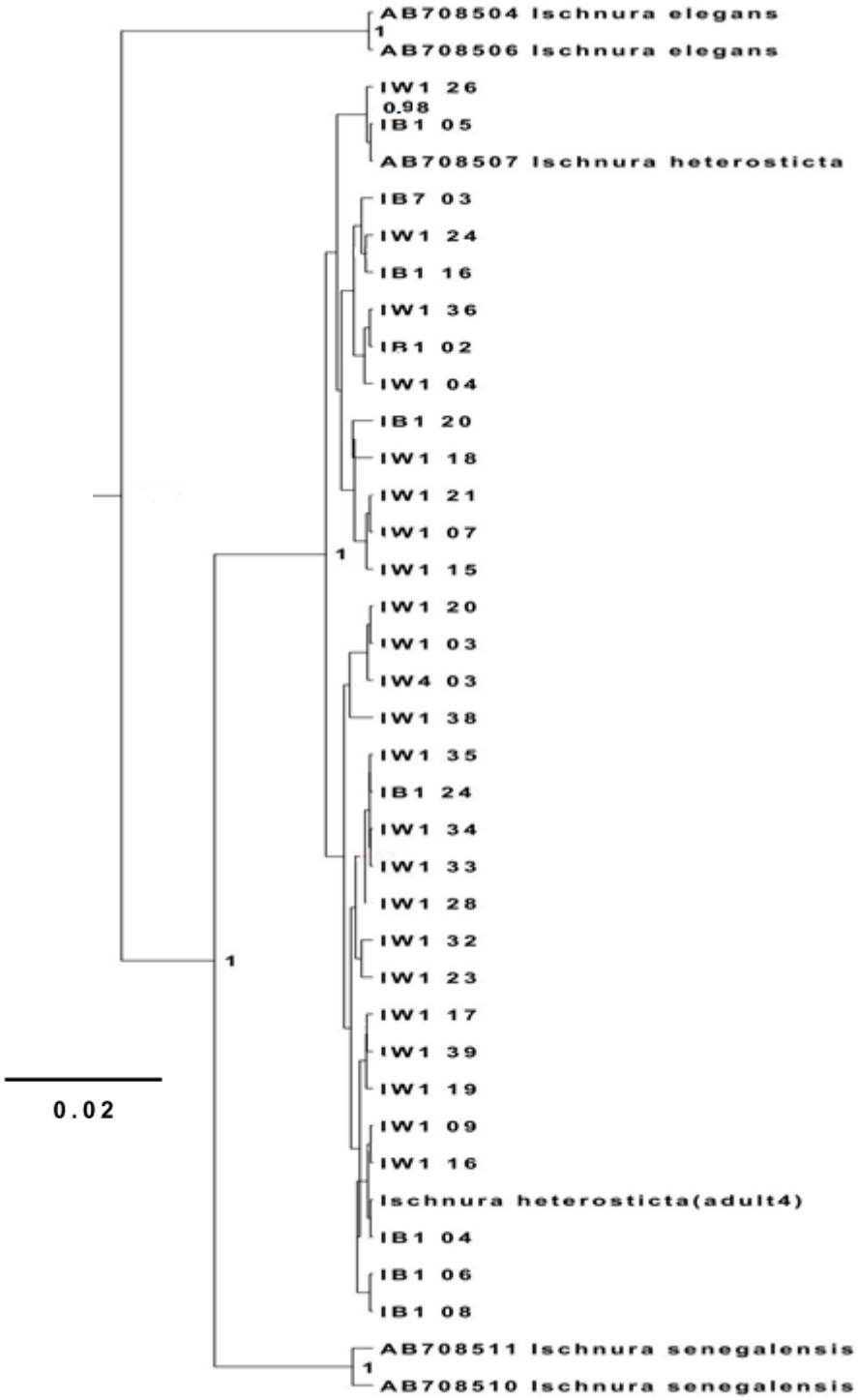

Figure 3: Expanded view of the maximum clade credibility tree produced using Tree Annotator from the BEAST output of the CO1 sequence data of Clade B from Figure 1. The posterior probabilities greater than 0.90 are shown and the divergence distance scale shown. The adult sequence data is identified as such. Larval individuals are labelled with a five character code. The first digit is I, the second character is the source catchment (B = Broughton, W = Wakefield), the third character represents the capture site, the last two digits are the individual number.

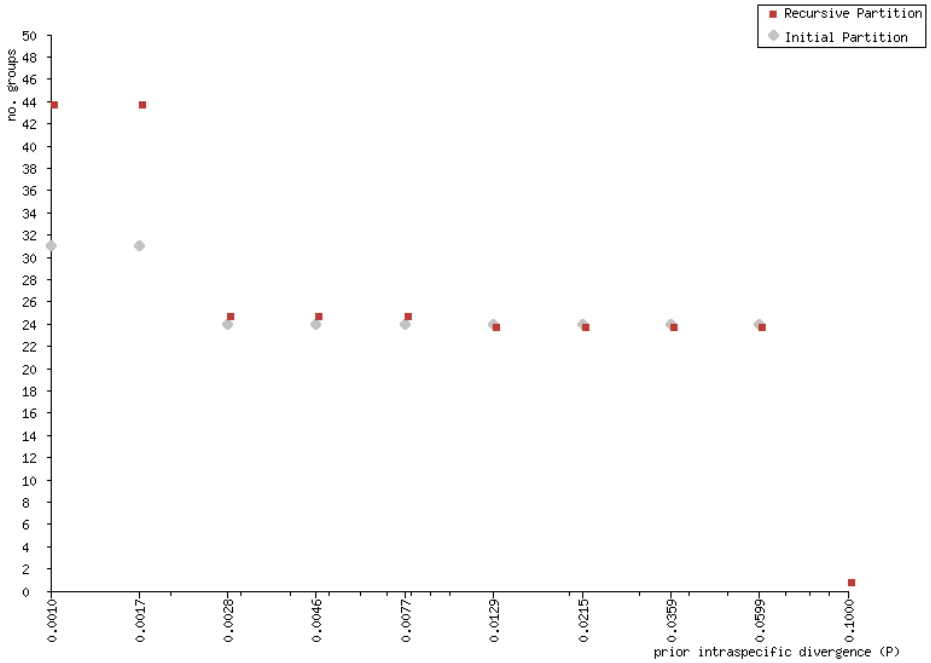

Figure 4: Automatic barcode gap detection (Puillandre *et al.*, 2012) partition results based on a pairwise distance matrix generated using the Jukes-cantor model with uniform rates among sites and no variance estimation showing the number of groups detected at varying levels of intraspecific divergence. The number of groups represents the number of hypothesised species contained within the sequence dataset.

Chapter 6

Next generation insect light traps: The use of LED light technology in sampling emerging aquatic macroinvertebrates

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Abstract

Light traps have long been a popular choice for baseline surveys of winged invertebrates from mosquitos to moths and there have been many variations on light trap designs over the years. While their use in urban environments is facilitated by the availability of close power sources, field use has always been limited by the requirement of power to run traditional lights. Traditional fluorescent tubes often do not run for more than 12 hours from a traditional 12 volt power source such as a car battery. We trialled LED lights as a replacement for traditional fluorescent bulbs for catching emerging aquatic macroinvertebrates. Initial trials with white LEDs were disappointing, with the catch amounting to chance contact with the trap. However, when ultraviolet LEDs were used, there was no significant difference to the traditional fluorescent trap of the same design. While the fluorescent trap used most or all of the available battery power, the LED lights had used less than 10% of the available power. We suggest that LEDs can be used to replace the more power demanding traditional lights for use in light traps.

Introduction

Light traps have been used for insect trapping for over 100 years. In that time there have been many variations in design with some being extremely complex, involving both lights and fans (Venter *et al.*, 2009), while others have remained simple (Scanlon and Petit, 2008). The source of light has also varied, beginning with flames and moving on to incandescent bulbs, and in more recent times, fluorescent tubes. Most current traps employ either an incandescent bulb or actinic fluorescent tube as the light source, as the spectrum of light emitted from these bulbs is effective for attracting insects (Sambaraju and Phillips, 2008). However, the power used by these light sources has always been an issue. Typically, small bulbs of around 6-9 watts are used which require either a fixed power source or a large power supply to power the light for an entire night. A common power source used is a 12 volt battery which will power such lights for approximately 6-8 hours, depending on the amp-hours of the battery. Given that the flight period of different insects varies from dusk until dawn, this means that standard light sources may fail and not attract a portion of the available insect population (Williams, 1935, Scalercio *et al.*, 2009).

Over the last decade light-emitting diodes (LEDs) have become increasingly popular as a replacement for standard incandescent bulbs or fluorescent bulbs as they are cheaper, run cooler, are more resistant to damage and use considerably less power. LEDs are also a much more focused light source with a narrow spectrum of light (generally 5 nanometres) and either a narrow beam (generally 25 degrees) or wide beam (Moreno and Sun, 2008). This allows for specific lighting characteristics to be selected and tailored for a specific purpose. Previous work has indicated that the use of LEDs increased capture rates of sand flies by

50% (Cohnstaedt *et al.*, 2008); however, the effectiveness of LEDs in attracting other types of insects has been little investigated. The purpose of this study was to examine whether LEDs could be used as a substitute for an actinic fluorescent bulb in a conventional light trap, and to examine the effect of this substitution on capture rates of emerging aquatic macroinvertebrates.

Method

For this study three different lights were trialled. All light sources used were attached to a "heath" style trap that employs three transparent upright vanes radiating out from a central point and light source. The vanes sit over a vertical funnel leading into a chamber where the insects are trapped until collection. In order to keep the trap stable under windy conditions the vanes were anchored to a stake. All lights were attached to an 18 amp-hour 12 volt battery (5-in-1 Power station/Jump starter (MB-3594), PowerTech). The first light source trialled was a commercially available 8 watt actinic fluorescent bulb (E700, Australian Entomological Supplies Pty. Ltd, Australia). The second was two banks of four white LEDs (6500nm, 3000 millicandela), and the third was two banks of nine 2000 millicandela 'UV/black light (395nm)' LEDs (Figure 1).

These traps were trialled in the Sturt River Gorge, South Australia on the 5th through 8th of December 2011. Given the documented variation in catch due to weather conditions (Williams, 1940, Yela and Holyoak, 1997) and moonlight (Bowden and Church, 1973, Yela and Holyoak, 1997) these details were recorded. Two of the actinic fluorescent light type and two of the UV LED light trap were trialled over four consecutive nights. The traps were placed alongside pools separated by a minimum of 50 meters and at least one riffle section (Figure

2). No other trap was visible from the trap location. The LED light traps were always directed towards the water, facing the steep side of the river valley. Traps were set at 8pm and were collected at 7am.

Individuals were identified to Order using the CSIRO online invertebrate key (CSIRO, 2011). In order to rule out any effect of sampling date on the results a one-way ANOVA was used. Differences between the samples collected by the different styles of trap were analysed using a series of independent samples t-tests for total number of individuals sampled per trap, total orders sampled per trap and the number of each order sampled per trap, treating the nightly catches as replicates. All statistical analysis was performed in IBM SPSS Statistics (Version 19).

Results and Discussion

The weather conditions varied little over the sampling period. There was light cloud cover ranging from 10-20% on each of the sampling nights. The moon phase was day 11 through 15. The wind direction and speed varied from night to night, however, due to the location of the trapping site, a well vegetated river gorge, the effect of wind was likely minimal. There was no significant effect of sampling date on the invertebrates caught shown by the independent samples t-tests conducted for the total number of individuals caught, as well as on each individual order (all results $p>0.05$).

The White LED light traps were relatively ineffective, with the insect catch apparently amounting to no more than incidental collision with the clear vanes (total 7 individuals) and were discarded after the first two nights. Therefore, we focused on comparing the UV LED traps and the actinic fluorescent trap. The

results indicated that there was little difference between the catch from either trap type. The most commonly caught insects were Trichoptera, followed by Coleoptera (Figure 4). When looking at the total insect abundance, there were on average slightly fewer individuals caught in the UV LED traps, however, this difference was not significant (Figure 3, $t=0.490$, $df=13.982$, $p=0.631$). Independent samples t-tests were also done on individual orders to see if there was an order specific difference in the sample. There was a trend towards more Lepidoptera and Diptera in the actinic light traps, however, this was found to be not significant using an independent samples t-test for the four replicates ($p>0.05$). It is possible that these results are related to the 360 degree spread of light from the actinic bulb rather than the 120 degree spread of light from the UV LED traps. In addition, the light from the UV LEDs was directed largely over the water body, rather than towards the vegetation. Given that all orders trapped in this study appear to be attracted to both light sources we hypothesise that, given a full 360 degree spread of light (achieved by adding more LEDs or modifying the arrangement of the LEDs), the results may have been more similar.

Power consumption was measured using the inbuilt voltmeter on the jump starter battery packs and analysed using an independent samples t-test. The power consumption significantly differed between the two trap types as expected ($t=32.16$, $df= 8.84$, $p<0.00$, $n=4$). While running off 18 amp-hour batteries the LED light traps used, on average, less than 10 per cent of the available power while the actinic fluoro used on average 92.5 percent of the available power, with some trials using 100 percent. This may have led to discrepancies among catches as it was unclear when the battery power was exhausted for some of the fluorescent light traps.

Given the results of this study, we propose that UV LEDs may often be used in place of traditional light sources in insect light traps. LEDs can be easily retrofitted to any existing light trap and are inexpensive to buy. They are also more durable, longer lasting, more power efficient and easier to repair. The LED light traps used in this study were constructed from commonly available materials for less than $60AUD each. LEDs also commonly run on 12 volts DC, which reduces the risk of electric shock to the operator as fluorescent tubes may require high voltages to start and inverters to run. This study found no significant differences in the abundance or composition of the insects caught by LED-based and fluorescent tube based light traps, even when the LEDs only illuminated 120 degrees while using less than an eighth of the power of the fluorescent lights. While we believe that UV LED light traps are a good replacement for actinic light traps, largely because of their lower power consumption and more robust design, we believe considerably more work is required to assess the relative attractiveness of LED and traditional light sources to specific insect orders.

References

Bowden, J. & Church, B. M. 1973. The influence of moonlight on catches of insects in light-traps in Africa. Part II. The effect of moon phase on light-trap catches. *Bulletin of Entomological Research,* 63**,** 129-142.

Cohnstaedt, L., Gillen, J. I. & Munstermann, L. E. 2008. Light-emitting diode technology improves insect trapping. *Journal of the American Mosquito Control Association,* 24**,** 331-334.

CSIRO. 2011. *Key to the Invertebrates* [Online]. CSIRO. Available: http://www.ento.csiro.au/education/key/couplet_01.html [Accessed 6 December, 2011 2011].

Moreno, I. & Sun, C. 2008. Modeling the radiation pattern of LEDs. *Optics Express,* 16**,** 1808-1819.

Sambaraju, K. R. & Phillips, T. W. 2008. Responses of adult *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) to light and combinations of attractants and light. *Journal of Insect Behaviour,* 21**,** 422-239.

Scalercio, S., Infusino, M. & Woiwod, I. P. 2009. Optimising the sampling window for moth indicator communities. *Journal of Insect Conservation,* 13**,** 583-591.

Scanlon, A. T. & Petit, S. 2008. Biomass and biodiversity of nocternal aerial insects in an Adelaide City park and implications for bats. *Urban Ecosystems,* 11**,** 91-106.

Venter, G. J., Labuschagne, K., Hermanides, K. G., Boikanyo, S. N. B., Majatladi, D. M. & Morey, L. 2009. Comparison of the efficiency of five suction light traps under field conditions in South Africa for the collection of *Culicoides* species. *Veterinary parasitology,* 166**,** 299-307.

Williams, C. B. 1935. The times of activity of certain nocternal insects, chiefly Lepidoptera, as indicated by a light trap. *Transactions of the Royal Society of London,* 83**,** 523-556.

Williams, C. B. 1940. An analysis of four years capture of insects in a light trap. Part1I.I The effect of weather conditions on insect activity; and the estimation and forecasting of changes in the insect population. *Transactions of the Royal Society of London,* 90**,** 227-306.

Yela, J. L. & Holyoak, M. 1997. Effects of moonlight and meteorological factors on light and bait trap catches of noctuid moths (Lepidoptera: Noctuidae). *Entomological Society of America,* 26**,** 1283-1290.

Figure 1: Constructed Light trap showing banks of LEDs and general setup of upright clear vanes positioned over a funnel.

Figure 2: Sampling sites used for trialling the light traps in the Sturt River Gorge, South Australia. Site a: 35° 2'58.49"S, 138°36'25.96"E. Site b: 35° 2'57.18"S, 138°36'27.73"E. Site c: 35° 2'58.49"S, 138°36'30.52"E. Site d: 35° 3'0.69"S, 138°36'32.77"E.

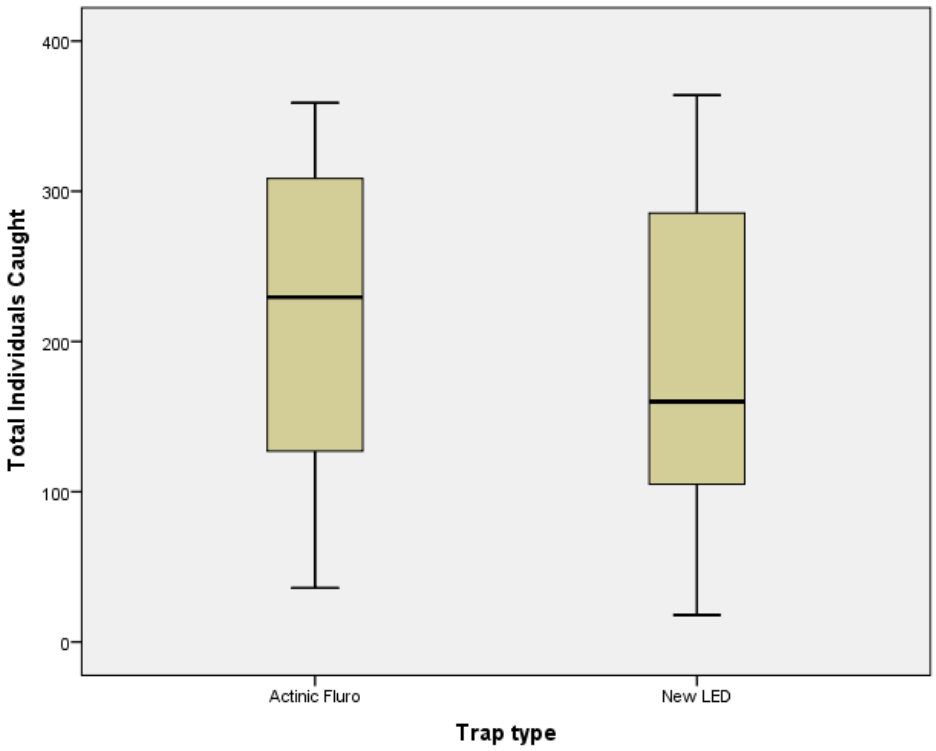

Figure 3: Box plot of total individuals caught in the different styles of trap per night generated using IBM SPSS Statistics Version 19 (8 replicates). Bars represent minimum and maximum number of individuals caught per night, the middle bar represents the median.

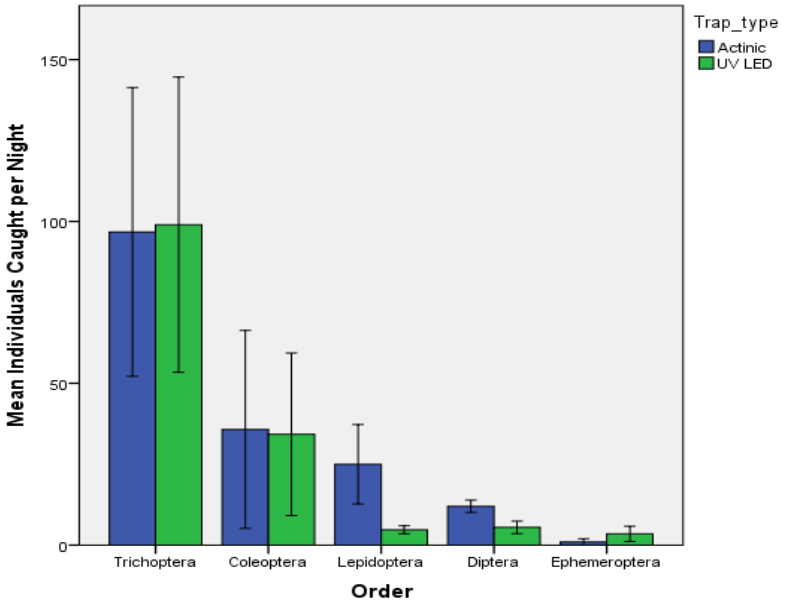

Figure 4: Mean and error bar plot (+/- 1 standard error, 8 replicates) of the five most abundant orders caught in both UV LED and Actinic light traps (generated using IBM SPSS Statistics Version 19).

Chapter 7

Detecting Dispersal Direction of Trichoptera in ephemeral aquatic systems using concentric rings of light traps

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Green *et al.* (unpublished)

Abstract

The ability to quantify the extent and direction of dispersal of emerging aquatic macroinvertebrates is a vital link in understanding the dynamics of their populations. Different theories suggest different patterns in dispersal and these patterns affect the growth and survival of populations. Some theories suggest that emerging adults will disperse upstream in order to counteract downstream larval drift while other studies suggest that dispersal is less directed. Ephemeral waters are an additional complication when considering dispersal in aquatic systems, as dispersing organisms must contend with the constant wetting and drying of habitat and the variable availability of refugia. In this study we examine the use of light traps arranged in a concentric ring around permanent waterholes to quantify the amount and direction of dispersal in emerging adult Trichoptera. The results also suggested a tendency to disperse downstream rather than upstream or across paddocks. Quantifying distance travelled would call for a larger design with greater distances between traps.

Introduction

Dispersal in aquatic macroinvertebrates has received increasing attention over the last decade. There have been several studies examining active dispersal directly (e.g. Elliot, 2003) and an increasing number of genetic studies (e.g. Miller *et al.*, 2002). These studies are beginning to build an understanding of how these organisms disperse. Riverine systems are a special case in ecology as they represent a distinctly fragmented landscape subject to many anthropogenic inputs and threats. Müller (1982) discussed the 'colonisation cycle' of aquatic macroinvertebrates in which it is suggested that adults will actively disperse upstream in order to counteract downstream larval drift. Other work has suggested that the downstream drift of larval invertebrates represents an excess in population density and that sufficient larvae will remain upstream for populations to persist (Walters, 1961, Walters, 1965). Under this model a random direction of flight may be sufficient to maintain upstream populations (Walters, 1961, Walters, 1965). More recent work using stable isotope analysis suggested a new movement upstream of dispersing adult stoneflies (Macneale *et al*., 2005). While there is evidence to support both these hypotheses, they are both based on perennial systems. The question remains how does the ephemeral nature of many of southern Australia's waterways affect dispersal behaviour?

Ephemeral systems are different to traditional river systems in that they do not flow for much of the year (Boulton, 2003). This seasonal wetting and drying of water systems has resulted in a 'boom and bust' ecology (Sheldon *et al.*, 2010). During the dry months, the river system retreats into a series of more permanent waterholes which sustain the flora and fauna until flow is resumed through

increased surface run-off or increased groundwater input (Boulton, 2003). This ephemeral nature has been shown to affect community composition (Sheldon *et al.*, 2002), trophic dynamics (Spencer *et al.*, 1999) and population genetics of both aquatic macroinvertebrate and fish (Bohonak and Roderick, 2001). However, little effort has been expended in examining how it may affect the direct dispersal from one patch of habitat to another. During this period of no flow, there is likely to be an increased risk of dispersing from a natal water body as there may be no indication of the distance to the next water body. The documented use of waterways as travel guides for dispersal (e.g. Petersen *et al.*, 1999) will aid the dispersing adults in finding appropriate habitat, however, the overall chances of finding suitable habitat are lessened in periods of reduced or no flow, reducing proportionally with increasing distances between pools.

Trichoptera are common aquatic macroinvertebrates with species inhabiting almost all aquatic environments. In Australia there are over 25 families of Trichoptera representing over 500 species (Gooderham and Tsyrlin, 2002). Given their common nature and documented range of sensitivity to pollutants, they have become a key indicator species of water quality (Bonada *et al.*, 2004). As they are a group of interest, their dispersal behaviour has also been examined. Collier and Smith (1998) examined the dispersal of adult Trichoptera in forested perennial streams in New Zealand and identified that there was a trend for dispersing individuals to follow the river channel, supporting previous studies (e.g. Sode and Wilberg-Larsen, 1993). However, other studies have shown large dispersal distances over cropland, up to several kilometres (Kovats *et al.*, 1996). It has been suggested that the vegetation density surrounding the watercourse may play a role in confining individuals to the river channel (Collier and Smith, 1998).

The purpose of this study was to test an approach for quantifying the direction and magnitude of dispersal of emerging Trichoptera from permanent pools in an ephemeral river system. In addition, we initially hypothesised that dispersing individuals would be more likely to remain within the river channel rather than disperse across land to increase the likelihood of finding suitable habitat (Collier and Smith, 1998). Additionally, it was hypothesised that the majority of dispersing individuals would disperse upstream in line with 'colonisation cycle' (Müller, 1982).

Methods

Study Sites

Insect trapping was undertaken in summer, January 2012. Two catchments were selected for study, the Broughton and the Wakefield Catchments, in the mid-north region of South Australia (Favier *et al.*, 2000, Favier *et al.*, 2004). Isolated pools were selected from relatively straight sections of river in the more ephemeral headwaters. Pools were termed 'isolated' when there were no other pools (including farm dams) within at least 500 metres in any direction. All pools selected were known to house Trichoptera larvae (D. Green, unpublished data). A total of six pools were selected for sampling, one from the Hill River in the Broughton Catchment and five from the Wakefield River in the Wakefield Catchment.

Field work

Light trapping was done using LED light traps as described in Green *et al*. (2012). Six sites were selected based on their isolation from other water bodies,

established initially by analysing area maps and later confirmed on site. The six sites were visited on six consecutive nights and traps were put out at each site for one night. It was assumed that given the isolation of the pools, that all individuals captured in traps around a pool originated from the study pool and did not disperse in from other areas. Nine traps were used per site. One trap was located at the edge of the pool. The others were arranged in two concentric rings centred on the pool at 15 metres and 30 metres distance from the water's edge with the light source directed towards the pool (Figure 1). The traps were set at 6pm, two hours before sunset, and collected at 9am the following morning, two hours after sunrise. Our expectation was that the emerging Trichoptera would disperse in their chosen direction, encounter one of the light traps and be collected for processing. The concentric ring layout of traps was designed to detect the direction of dispersal, and the relative number of individuals dispersing in each given direction. Samples were placed in a sealed container to be sorted later in the lab. Trichoptera were identified using Neboiss (1992).

Statistical Analysis

Descriptive statistics were calculated in SPSS (Version 20.0.0.1). A random block ANOVA using trap location as a fixed factor and site as a random factor was used in order to examine the effect of trap location on Trichoptera catch. The following planned comparisons among trap catches were conducted: in-stream versus cross-stream, upstream versus downstream, inner ring versus outer ring and downstream outer versus other in-stream sites. These comparisons were undertaken for both the total number of Trichoptera found and for numbers of each species of Trichoptera found.

Results

A total of 348 Trichoptera was captured across the sampling period (Table 1). Of these, 218 were captured in the river channel, while 80 were caught in the traps outside the river channel. Of those remaining in the river channel, 39 Trichoptera were collected in the upstream close trap, 34 in the upstream far trap, 43 in the downstream close trap and 102 in the downstream far trap. There were two species of Trichoptera caught. The first species, *Ecnomus cygnitus*, had 105 individuals and is a small, relatively common Trichoptera often associated with permanent pools and slow moving waterways (Neboiss, 1992). The second species, *Triplectides australis*, is a larger species of Trichoptera also associated with slower flowing waters (Neboiss, 1992) and was represented by 243 individuals.

Weather conditions were conducive to successful insect trapping (Collier *et al.*, 1997). There was limited cloud cover except during the first night when high cloud was accompanied with relatively high winds. Daily temperatures ranged from 30 through 37 degrees Celsius while minimum night time temperatures ranged from 17 to 20 degrees Celsius. The moon was near full and waning during the sample period. Given the high wind and cloud levels on the first night, the catch was severely reduced (one individual) and this night was excluded from further study leaving five sample nights.

There was no significant difference between the numbers of Trichoptera caught in the closer traps and the far traps ($p>0.05$, Table 1). There was, however, a significant difference between the average number of Trichoptera caught in the traps in the river channel and those caught in the cross-stream traps (F=15.270,

df=1, $p<0.002$). Of the individuals remaining in the river channel, it was found that significantly more were dispersing downstream (F=8.356, df1, $p<0.01$). Further examination identified significantly more individuals on average caught in the further downstream traps than the other in-stream traps (F=17.820, df=1, $p<0.001$).

When the two species were analysed separately, the pattern was very similar (Table 1). The data for *E. cygnitus* showed that there was no significant difference between the mean numbers found in the close traps and the far traps ($p>0.05$), however, they were significantly more likely to remain in the river channel rather than disperse across land (F=14.644, df=1, $p<0.002$). Of those remaining in the river channel, again they were more likely to disperse downstream (F=8.073, df=1, $p<0.01$) with more individuals caught in the further downstream trap over other in-stream traps (F=17.140, df=1, P<0.001).

T. australis showed the same patterns. There was no significant difference in the number of individuals trapped by the close traps as opposed to the far traps ($p>0.05$). The same significant trends were identified in the cross-stream versus downstream and upstream versus downstream comparisons. There were significantly more individuals caught in the in-stream traps than in the across paddock traps (F=12.443, df=1, $p<0.003$). When the individuals that remained in the river channel were examined, it was found that significantly more individuals were trapped downstream rather than upstream, (F=6.6787, df=1, $p<0.05$) and significantly more individuals were trapped in the further downstream trap than the other in-stream traps (F=14.503, df=1, $p<0.002$).

Discussion

The results of this study indicate that adult *E. cygnitus* and *T. australis* will disperse downstream more often than upstream and are more likely to remain in the river channel than disperse across paddocks. Dispersing across land rather than following the river channel is a risk for the dispersing individual as there is less chance that the individual will find suitable habitat. The chances of finding suitable habitat are even further reduced when considering the environment where this study has been undertaken. The Clare Valley and surrounding area are relatively dry over the summer months with very little standing water available for Trichoptera. The standing water available outside of the river channel is mostly bore-water-fed artificial water bodies, including farm dams and livestock water troughs.

In perennial systems it has been demonstrated that the direction of dispersal in some species of Trichoptera is upstream biased (Bagge, 1995), however, dispersal by caddisflies has not previously been examined in ephemeral systems. The significant trend of dispersal downstream observed in this study suggests that there is an advantage to dispersing downstream rather than upstream. It is suggested that dispersing downstream will lead to a higher likelihood of locating suitable habitat for colonisation as the frequency of pools increases downstream (Favier *et al.*, 2000, Favier *et al.*, 2004). This suggests that the emerging caddisflies have a mechanism for detecting the direction of flow or detecting slope, given that they are emerging from an isolated pool within the river channel.

Other species of Trichoptera have been found to disperse across land, up to several kilometres from water sources (Jackson and Resh, 1989, Kovats *et al.*,

1996). While the long-term fate of the cross-paddock dispersers is currently unknown, this dispersal behaviour may allow for colonisation of new habitat on rare occasions.

The observation that the two study species of Trichoptera tend to remain in the river channel rather than dispersing across paddocks is consistent with other studies (Sode and Wilberg-Larsen, 1993, Collier and Smith, 1998). When this result is examined in the context of dispersal success, it is logical given that the chances of locating a suitable habitat to disperse to is increased by following the river channel rather than dispersing across land. There were still a number of individuals dispersing upstream as well. Of the in-stream trap sites, the lowest recorded numbers (16%) were from the upstream far site, followed by the upstream close site (18%). This indicates that there are still a number of individuals dispersing upstream allowing for colonisation of upstream pools. While this may be seen as a more risky behaviour, it is not as risky as dispersing across land given that all study sites had pools located upstream, albeit over 500 metres away. Unfortunately, there has been little work undertaken looking at the rate of successful dispersal of Trichoptera. Most of the work looking at dispersal success has centred around the dispersal of invasive species (Hayes and Barry, 2008). Recolonisation work can provide some insights into the dispersal success of different species with suggestions most winged invertebrates will recolonise restored river systems if they are within 5 kilometres of source population (Sundermann *et al.*, 2011). This would suggest that dispersing Trichoptera in the current study rivers may be able to locate suitable habitat by following the river channel.

The numbers of Trichoptera sampled by the traps showed a significant trend for dispersal in a downstream direction. The distance of dispersal was not as readily observable with the study design given the relatively small distances between the inner and outer rings of traps compared to the overall distances over which some species of Trichoptera are able to disperse (Kovats *et al.*, 1996). However, the concentric ring design used in this study allowed for the identification of dispersal directionality from the source pools and this design may be suitable for detecting the dispersal direction of other emerging Trichoptera. Further development of this method should also aim to include some either chemical or genetic techniques to compliment the observed dispersal similar to the techniques used by (Macneale *et al.*, 2005)

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References

Bagge, P. 1995. Emergence and upstream flight of lotic mayflies and caddisflies (Ephemeroptera and Trichoptera) in a lake outlet, central Finland. *Entomologica Fennica,* 6**,** 91-97.

Bohonak, A. J. & Roderick, G. K. 2001. Dispersal of invertebrates among temporary ponds: are genetic estimates accurate? *Israel Journal of Zoology,* 47**,** 367-386.

Bonada, N., Zamora-Munoz, C., Rieradevall, M. & Prat, N. 2004. Ecological profiles of caddisfly larvae in Mediterranean streams: implications for bioassessment methods. *Environmental Polution,* 132**,** 509-521.

Boulton, A. J. 2003. Parallels and contrasts in the effects of drought on stream macroinvertebrate assemblages. *Freshwater Biology,* 48**,** 1173-1185.

Collier, K. J. & Smith, B. J. 1998. Dispersal of adult caddisflies (*Trichoptera)* into forests alongside three New Zealand streams. *Hydrobiologia,* 361**,** 53-65.

Collier, K. J., Smith, B. J. & Baillie, B. R. 1997. Summer light-trap catches of adult Trichoptera in hill-country catchments of contrasting land use, Waikato, New Zealand. *New Zealand Journal of Marine and Freshwater Research,* 31**,** 623-634.

Elliot, J. M. 2003. A comparative study if the dispersal of 10 species of stream invertebrate. *Freshwater Biology,* 48**,** 1652-1668.

Favier, D., Rixon, S. & Scholz, G. 2000. A River Management Plan for the Wakefield Catchment. Adelaide: Environmental Protection Agency.

Favier, D., Scholz, G., VanLaarhoven, J. & Bradley, J. 2004. A River Management Plan for the Broughton Catchment. Adelaide: The Department of Water, Land, Biodiversity and Conservation.

Gooderham, J. & Tsyrlin, E. 2002. *The Waterbug Book: A guide to the freshwater macroinvertebrates of temperate Australia,* Collingwood, CSIRO Publishing.

Green, D. J., Mackay, D. A. & Whalen, M. A. 2012. Next generation insect light traps: the use of LED light technology in sampling emerging aquatic macroinvertebrates. *Australian Entomologist,* 39**,** 189-194.

Hayes, K. R. & Barry, S. C. 2008. Are there any consistant predictors of invasion success? *Biological Invasions,* 10**,** 483-506.

Jackson, J. K. & Resh, V. H. 1989. Distrubution and abundance of adult aquatic insects in the forest adjacent to a northern Californian stream. *Environmental Entomology,* 18**,** 278-283.

Kovats, Z. E., Cibrowski, J. J. & Corkum, L. D. 1996. Inland dispersal of adult aquatic insects. *Freshwater Biology,* 36**,** 265-276.

Macneale, K. H., Peckarsky, B. L. & Likens, G. E. 2005. Stable isotopes identify dispersal patterns of stonefly populations living along stream corridors. *Freshwater Biology,* 50**,** 1117-1130.

Miller, M. P., Bilinn, D. W. & Keim, P. 2002. Correlations between observed dispersal capabilities and patterns of genetics differentiation in populations of four aquatic insect species from the Arazona White Mountains, U.S.A. *Freshwater Biology,* 47**,** 1660-1673.

Müller, K. 1982. The colonization cycle of freshwater insects. *Oecologia,* 52**,** 202-242.

Neboiss, A. 1992. *Illustrated keys to the families and genera of Australian Trichoptera. I. Adults,* No. 9, Australian Society for Limnology Special Publication.

Petersen, I., Winterbottom, J. H., Orton, S., Friberg, N., Hildrew, A. G., Spiers, D. C. & Gurney, W. S. C. 1999. Emergence and lateral dispersal of adult Plecoptera and Trichoptera from Broadstone Stream, U.K. *Freshwater Biology,* 42**,** 401-416.

Sheldon, F., Boulton, A. J. & Puckridge, J. T. 2002. Conservation value of variable connectivity: aquatic invertebrate assemblages of channel and floodplain habitats of a central Australian arid-zone river, Cooper Creek. *Biological Conservation,* 103**,** 13-31.

Sheldon, F., Bunn, S. E., Hughes, J. M., Arthington, A. H., Balcombe, S. R. & Felows, C. S. 2010. Ecological roles and threats to aquatic refugia in arid landscapes: dryland river waterholes. *Marine and Freshwater Research,* 61**,** 885-595.

Sode, A. & Wilberg-Larsen, P. 1993. Dispersal of adult Trichoptera at a Danish forest brook. *Freshwater Biology,* 30**,** 439-446.

Spencer, M., Blaustein, L., Schwartz, S. S. & Cohen, J. E. 1999. Species richness and the proportion of predatory animal species in temporary freshwater pools: relationships with habitat size and permanence. *Ecology Letters,* 2**,** 157-166.

Sundermann, A., Stoll, S. & Haase, P. 2011. River restoration success depends on the species pool of the immediate surroundings. *Ecological Applications,* 21**,** 1962-1971.

Walters, T. F. 1961. Standing crop and the drift of stream bottom organisms. *Ecology,* 42**,** 532-537.

Walters, T. F. 1965. Interpretation of invertebrate drift in streams. *Ecology,* 46**,** 327-334.

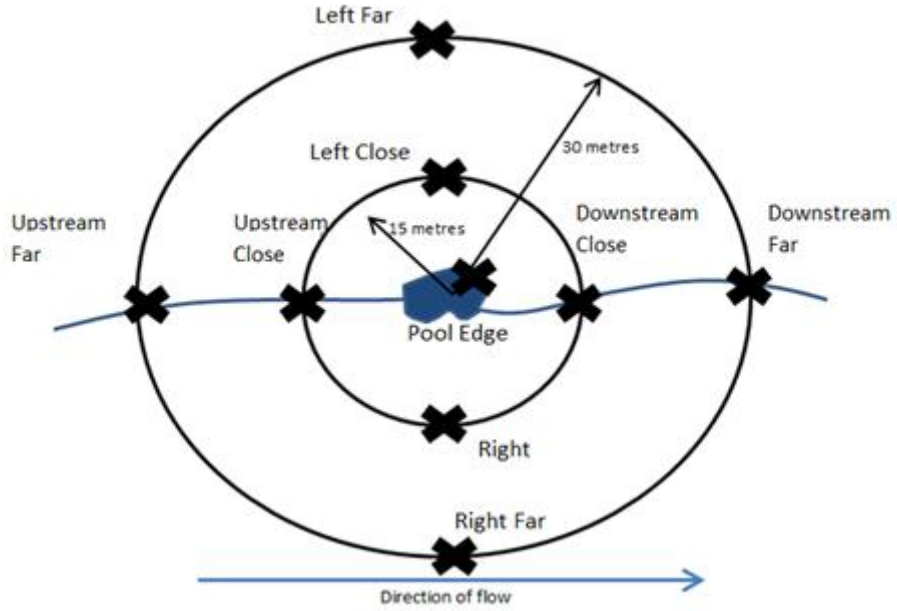

Figure 2: The layout of the light traps around the isolated study pools. The inner, 'near' ring was set at 15 metres from the water's edge and the outer or 'far' ring was set at 30 metres from the water's edge.

Table 5: Summarised statistics of the number of all Trichoptera caught across all five sampling nights.

	All Trichoptera					*Ecnomus cygnitus*					*Triplectides australis*				
Trap Location	**N**	**Mean**	**Standard Deviation**	**Min**	**Max**	**N**	**Mean**	**Standard Deviation**	**Min**	**Max**	**N**	**Mean**	**Standard Deviation**	**Min**	**Max**
Pool Edge	50	10	9.8	0	28	35	3	2.6	0	7	15	7	7.3	0	21
Upstream Close	39	7.8	5.9	2	18	30	1.8	2.2	0	6	9	6	3.8	2	12
Upstream Far	34	6.8	6.8	1	20	20	2.8	1.3	1	5	14	4	5.6	0	15
Downstream Close	43	8.6	10.6	0	27	29	2.8	3.9	0	10	14	5.8	6.7	0	17
Downstream Far	102	20.4	8.6	7	31	70	6.4	2.6	4	10	32	14	6.3	3	21
Left Close	14	2.8	2.8	0	7	9	1	1.1	0	3	5	1.8	1.8	0	4
Left Far	17	3.4	3.8	0	8	11	1.2	1.2	0	3	6	2.2	2.7	0	6
Right Far	22	4.4	3.6	0	10	15	1.4	0.8	0	2	7	3	3.0	0	8
Right Close	27	5.4	4.4	0	12	24	0.6	0.5	0	1	3	4.8	4.1	0	11

Chapter 8

General Discussion

This thesis utilised several techniques to investigate the genetics, population structure, taxonomy and dispersal of selected aquatic macroinvertebrates from across the Broughton and Wakefield Catchments of the Mid North of South Australia. Genetic studies of populations of *P. australiensis* showed that while the mitochondrial lineages present in the study catchments are also found across south-eastern Australia, microsatellite data shows fine-scale population differences across a relatively small spatial scale.

In a different application of the same genetic techniques, mitochondrial sequence data were used to identify two different species of larval Odonata which are morphologically very similar.

The final two research chapters demonstrated the development and use of light traps to detect dispersal directionality of emerging Trichoptera from isolated pools. In this final section, I summarise and discuss the key findings of this thesis and suggest areas for further research.

The population genetic structure of P. australiensis

The research presented in chapters two, three and four centre on the population genetics of *P. australiensis.* The work conducted by Page *et al.* (2005), Hurwood *et al.* (2003) Cook *et al.* (2006) and Cook *et al.* (2007) has shown that *P. australiensis* is a species complex with nine lineages identified across eastern Australia, with some of those lineages being reproductively isolated. The findings from Chapter 2 use mitochondrial sequence data to show how *P. australiensis*

from the two catchments studied here are related to those found across eastern Australia. The two lineages in the present study are both lineages that were previously identified by Cook *et al.* (2006). Both of these lineages are widespread across much of eastern Australia and most importantly, they are present in the headwaters of the Murray River. The distribution of these lineages provides possible insight into the dispersal pathway from the eastern side of Australia to the study catchments. Page *et al.* (2005) suggest that *P. australiensis* entered Australia through one or more connections from the north and dispersed down the eastern coast. Cook *et al.* (2006) suggest that dispersal links across the Great Dividing Range may occur more often than previously thought given the lineages present on both sides of the range. Once *P. australiensis* entered the Murray-Darling Basin it is likely they would have dispersed its length leaving only a relatively short migration to the rivers of the western Mt. Lofty Ranges including the Broughton and Wakefield Catchments studied here.

Mitochondrial DNA gives an insight into historic dispersal, connections and isolations, while microsatellite markers can reflect changes on a more contemporary time scale (Bohonak and Jenkins, 2003). Chapter four examined the population genetic structure among and within the study catchments with the use of microsatellite markers. The microsatellite results showed not only distinct differences between the two study catchments but structuring within the Broughton Catchment as well. This structuring across a relatively small spatial scale did not closely resemble that expected from any of the hierarchical models suggested by Hughes *et al.* (2009) or that demonstrated in other studies of obligate aquatic fauna in permanent pools (Huey *et al.*, 2008). The differences observed in the Broughton Catchment appear to be related to barriers to dispersal

along an ephemeral river, such as man-made weirs, cascades or dry sections of riverbed, with the ephemeral nature of the river system appearing to play a large role in population structure. Differences over small spatial scales have been observed before in aquatic macroinvertebrates (Carini *et al.*, 2006) and I suggest that the compounding effect of multiple periods in isolation has caused the population structuring observed in the Broughton Catchment, while the divergence between the Wakefield and the Broughton Catchments is related to the inability of *P. australiensis* to cross the catchment boundary on a regular basis. In perennial rivers, such repeated periods of isolation are less likely, however, barriers to dispersal also occur (e.g. dams, waterfalls) and some studies have indicated how anthropogenic impacts such as dams may be beginning to drive increasing differentiation in migratory species in modified aquatic systems (e.g. Bull Trout, Neraas and Spruell, 2001). The degree of such divergence will be affected by various factors such as effective population size, generation time, time in isolation (both collective and in any once instance), among others, and would likely not be limited to macroinvertebrates, as isolation of fish populations has been identified across modified rivers (e.g. Neraas and Spruell, 2001).

The lineages present in the study catchments have previously been shown to be reproductively isolated in the headwaters of the Murray River Catchment using a combination of mtDNA and allozyme data (Cook *et al.*, 2007). In the present study, the microsatellite data suggested that the two lineages present were not reproductively isolated in the Broughton and Wakefield Catchments and that the two lineages were interbreeding. The difference in reproductive isolation identified by Cook *et al*. (2007) and the findings of chapter 4 suggests some unidentified differences between lineages 4 and 8 in the headwaters of the Murray

River and lineages 4 and 8 found in the mid-north region of South Australia. The incomplete lineage sorting indicated by the comparison of the microsatellite and mitochondrial sequence data in chapters 2 and 4 has been observed before in other organisms and is thought to result from the differing divergence times of mitochondrial DNA and microsatellite sequences (Funk and Omland, 2003, McKay and Zink, 2010).

Previous work on the population genetics of *P. australiensis* has provided an understanding of possible colonisation pathways and subsequent population divergence across much of eastern Australia (Page *et al.*, 2005, Cook *et al.*, 2006). The mitochondrial sequence data examined in chapter 2 has extended the spatial coverage of the collective mitochondrial dataset. Lineage 8 is common across much of eastern Australia and is the only lineage identified in several south flowing rivers in Victoria (Cook *et al.*, 2006). Westerly dispersal from these rivers around the coastline and between catchments following the life history variation hypothesis of Cook *et al.* (2006) is one possible dispersal pathway into the South Australian catchments. The dispersal of lineage 4 into South Australia is more likely to have followed the River Murray given that the majority of lineage 4 populations are in the Darling River Catchment (Cook *et al.*, 2006). It is possible that lineage 8 could have followed this pathway as well given its presences in the Murray Catchment (Cook *et al.*, 2007).

The species complex of *P. australiensis* covers much of Australia (Williams, 1977). Despite the additional data provided by this study from the western edge of the range of this species, there remains a considerable gap between the study sites from eastern Australia and the mid-north of South Australia. Sequence information from additional sites along the length of the River Murray, including

its major tributaries, as well as from additional sites from the western Mt. Lofty Ranges, would improve our understanding of the links between the eastern populations and those studied in the Broughton and Wakefield Catchments. The addition of sequence data from these additional sites should also clarify the dispersal pathway from the River Murray to the Western Mt. Lofty Ranges and beyond. The collection of additional sequence data should extend into other areas of *P. australiensis* range as well such as Western Australia and Tasmania (Williams, 1977). Additional information on the dispersal of *P. australiensis* could also shed light on previous dispersal pathways for other obligate aquatic macroinvertebrates across the continent given the similar patterns observed between other decapods and *Paratya* in previous studies (Murphy and Austin, 2004, Nguyen *et al.*, 2004).

Chapter 4 demonstrated the ability of microsatellite markers for *P. australiensis* to detect divergence across sites separated by distances less than 20kms but by the presence of a barrier to dispersal The same methodology could be applied to any aquatic macroinvertebrate to investigate population genetic structure on the same or larger scale depending on the perceived dispersal ability of the study organism. The population divergence identified within the two study catchments supports the results of other studies examining the population genetic structure of macroinvertebrates (e.g. Miller *et al.*, 2002) suggesting that the realised dispersal of macroinvertebrates is often less than the hypothesised dispersal. The permanent pools of the Broughton and Wakefield Catchments used for these studies contain a diverse community of macroinvertebrates and limited fish populations (Green *et al.*, 2010). Studying a range of species with varying dispersal abilities across the same study area would provide insight into the influence of dispersal ability on

population genetic structure over the same spatial scale (Bohonak and Jenkins, 2003) and I propose these two catchments provide a suitable area to conduct further studies into the effect of ephemeral waterways of population genetic structure based on the results of the microsatellite study of *P. australiensis*. Conducting the studies at the same sites on the same catchments would allow a detailed comparison of dispersal without confounding factors of different study catchments influencing the results.

The role of genetics in identification of species

Chapter 5 used genetic techniques to identify two different species of damselfly that are morphologically similar at the larval stage. The rate of errors in identification of specimens in many ecological studies is often unknown but existing estimates are far higher than should be acceptable (Bortolus, 2008). The initial misidentification on morphological grounds of a large proportion of the larval study specimens in chapter 5 highlights the possible benefits of the use of genetic techniques in ecological studies as suggested in the literature (e.g. Hebert *et al.*, 2003, Zhou *et al.*, 2012). The species used in this paper are morphologically very similar at the larval life history stage, with the morphological diagnostic features being subtle and difficult to distinguish.

The results of Chapter 5 clearly indicate the potential utility of genetic markers in the identification of specimens in the survey of aquatic biodiversity where larval forms of macroinvertebrates are common and often difficult to identify, particularly with early instars. The approximately 50% error in identification based on morphology highlighted the problems with relying on a single method of morphological identification, particularly when diagnostic characters are difficult

to identify. The availability of a database containing genetic information for a large number of aquatic organisms allowed for the use of additional sequence information in order to add greater confidence to the genetic identification of individuals (Benson *et al.*, 2011). In addition, although not studied here, genetic identification can allow for the identification of instars at any stage (Hawking and Theischinger, 2004). The automatic barcode gap detection analysis also provides additional information not previously available through morphological identifications (Puillandre *et al.*, 2012). Though not identified in this study, the identification of new or cryptic species for further investigation or genetic evidence for the merging of two species can be suggested by the automatic barcode gap detection analysis.

The genetic techniques we utilised in our studies are becoming cheaper, faster and more accessible to researchers, making the use of these techniques more feasible and common. For rapid assessments of macroinvertebrate biodiversity, such as AusRivAS (Smith *et al.*, 1999), the routine use of genetic identification of specimens may not be feasible as the time and cost associated, though limited, are still not 'rapid' and these studies require identification to species level (Haase *et al.*, 2006). The invertebrate COI mtDNA primer pairs developed by Folmer et al. (1994) were used in both Chapter 5 and, though modified slightly by Cook *et al.* (2006), Chapter 2. The use of these primers allowed for both the increased matches from genetic database searches and a reduced development period for the genetic protocol. The results of this study demonstrate that where species level identification is required, the use of genetic techniques to verify identifications should be considered for early instar specimens and those species where identification is difficult.

Active measures of dispersal from permanent pools

The use of genetic techniques can allow for inference of dispersal across different time scales (Pritchard *et al.*, 2000, Bohonak and Roderick, 2001). However, in order to quantify contemporary dispersal, use of direct measures will always be needed (Kovats *et al.*, 1996, Bilton *et al.*, 2001, Bohonak and Jenkins, 2003). Ideally research would have been undertaken to measure the dispersal of *P. australiensis* and relate this back to the findings of chapters 2 and 4. Unfortunately, the study was undertaken during the end of a drought period and the river systems were not flowing. Lab measures of active dispersal in flow chambers were considered, similar to Hancock and Bunn (1999), however, these could not be resourced.

The results of chapter 7 supported the notion that dispersing adult Trichoptera will tend to follow river channels rather than overland dispersal (Sode and Wilberg-Larsen, 1993, Collier and Smith, 1998). This is not to suggest that caddisflies are not capable of overland dispersal, as previous studies have found caddisflies dispersing across catchment boundaries (Jackson and Resh, 1989, Kovats *et al.*, 1996). The study presented in chapter 7 was designed to assess the utility of the light traps we developed in chapter 6 to detect the direction of dispersal. The findings of this preliminary study give an indication that the preferred direction of dispersal of emerging Trichoptera is downstream. This finding is different to that predicted by several common theories of dispersal in winged macroinvertebrates which suggest that most dispersing adults will disperse upstream to counteract downstream larval drift (Petersen *et al.*, 1999, Bilton *et al.*, 2001, Bohonak and Jenkins, 2003). In order to validate this finding

more work needs to be done as the sample dataset is limited in both time and space.

The ‘colonisation cycle’ theory (Müller, 1982) relates the dispersal of macroinvertebrates to the direction of flow. In ephemeral systems there may not be flow during the lifecycle of the species. In addition, in a perennial system it is likely that there will be water upstream. In ephemeral systems, there is a higher likelihood of finding water downstream (Larned *et al.*, 2010) which may explain the dispersal behaviour observed in this limited study. The ability to detect dispersal direction in a larger scale study (both in time and space) will provide more evidence of how these organisms disperse in ephemeral systems.

Overview and Future Research Directions

The exploration of dispersal of macroinvertebrates and the discussion of ephemeral rivers are both topics that have, until recently, been poorly represented in the literature (Bilton *et al.*, 2001, Larned *et al.*, 2010). This thesis expands the existing body of knowledge surrounding both of these fields. Previous studies have examined dispersal though river systems of eastern Australia (e.g. Murphy and Austin, 2004, Nguyen *et al.*, 2004, Page *et al.*, 2005, Cook *et al.*, 2006). Chapters 2 and 4 have extended this work for *P. australiensis* into South Australia and into ephemeral rivers. Theories presented in Cook *et al.* (2006) are used to help interpret the presence of previously identified lineages over 600 kilometres from the current most westerly identified population. This spatial expansion of the mitochondrial dataset for *P. australiensis* also allows for additional inferences to be made based on the relationship between dispersal patterns of *P. australiensis*

and other decapods such as *Cherax destructor* (Nguyen et al., 2004) and *Macrobrachum australiense* (Murphy and Austin, 2004).

The structuring observed in the microsatellite data for *P. australiensis* is evidence for the ephemeral nature of the study catchments influencing the population genetic structure. There is limited evidence in the literature of this effect. Investigations into the effect of population isolation have shown divergence based on limited dispersal ability between isolated pools in aquatic snails (Carini *et al.*, 2006) and studies of migratory fish have demonstrated barriers to dispersal in perennial waterways will affect population structure (Neraas and Spruell, 2001). On a larger scale a similar pattern is evident when examining the desert dwelling fish of the Lake Eyre Basin (Huey *et al.*, 2008). Strong between catchment variation and mild within catchment structuring was shown following the stream hierarchy model (Huey *et al.*, 2008).

The studies described in this thesis highlight the role and importance of genetic techniques for studies of aquatic invertebrates. Whether their use is limited to species identification, to species delineation or used to examine population structure between pools separated by as little as a few kilometres, or as many as hundreds of kilometres, it is clear that genetic techniques are a vital tool for ecologists. The rate of advancement of genetic techniques and technology that was evident even through the timeframe of the presented work, serves to make genetics more accessible to more researchers, though, it will never replace the need for field surveys and on ground studies.

References

Benson, D. A., Karsch-Mizachi, I., Lipman, D. J., Ostell, J. & Sayers, E. W. 2011. GenBank. *Nucleic Acids Research,* 39**,** D32-D37.

Bilton, D. T., Freeland, J. R. & Okamura, B. 2001. Dispersal in freshwater invertebrates. *Annual review of Ecology and Systematics,* 31**,** 159-181.

Bohonak, A. J. & Jenkins, G. 2003. Ecological and evolutionary significance of dispersal by freshwater invertebrates. *Ecology Letters,* 6**,** 783-796.

Bohonak, A. J. & Roderick, G. K. 2001. Dispersal of invertebrates among temporary ponds: are genetic estimates accurate? *Israel Journal of Zoology,* 47**,** 367-386.

Bortolus, A. 2008. Error cascades in the biological sciences: the unwanted consequences of using bad taxonomy in ecology. *AMBIO: A Journal of the Human Environment,* 37**,** 114-118.

Carini, G., Hughes, J. M. & Bunn, S. E. 2006. The role of waterholes as 'refugia' in sustaining genetic diversity and variation of two freshwater species in dryland river systems (Western Queensland, Australia). *Freshwater Biology,* 51**,** 1434-1446.

Collier, K. J. & Smith, B. J. 1998. Dispersal of adult caddisflies (*Trichoptera)* into forests alongside three New Zealand streams. *Hydrobiologia,* 361**,** 53-65.

Cook, B. D., Baker, A. M., Page, T. J., Grant, S. C., Fawcett, J. H., Hurwood, D. A. & Hughes, J. M. 2006. Biogeographic history of an Australian freshwater shrimp, *paratya*

australiensis: the role of history transition in phylogeographic diversification. *Molecular Ecology,* 15**,** 1083-1093.

Cook, B. D., Bunn, S. E. & Hughes, J. M. 2007. A comparative analysis of population structuring and genetic diversity in sympatric lineages of freshwater shrimp (Atyidae: *Paratya*): concerted or independent responces to hydrographic factors? *Freshwater Biology,* 52**,** 2156-2171.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhork, R. K. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology,* 3**,** 294-299.

Funk, D. J. & Omland, K. E. 2003. Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evoloution, and Systematics,* 34**,** 397-423.

Green, D. J., Mackay, D. A. & Whalen, M. A. 2010. Macroinvertebrate sampling methodology in deep insream pools: a comparison of methods. *Transactions of the Royal Society of South Australia,* 134**,** 143-145.

Haase, P., Murray-Bligh, J., Lohse, S., Pauls, S., Sundermann, A., Gunn, R. & Clarke, R. 2006. Assessing the impacts of errors in sorting and identifying macroinvertebrate samples. *Hydrobiologica,* 566**,** 505-521.

Hancock, M. A. & Bunn, S. E. 1999. Swimming response to water current in Paratya australiensis Kemp, 1917 (Decapoda, Atyidae) under laboratory conditions. *Crustaceana,* 72**,** 313-323.

Hawking, J. & Theischinger, G. 2004. Critical speies of Odonata in Australia. *Internation Journal of Odonatology,* 7**,** 113-132.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. W. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society Biological Sciences,* 270**,** 313-321.

Huey, J. A., Baker, A. M. & Hughes, J. M. 2008. The effect of landscape processes upon gene flow and genetic diversity in an Australian freshwater fish, *Neosilurus hyrtlii*. *Freshwater Biology,* 53**,** 1393-1408.

Hughes, J. M., Schmidt, D. J. & Finn, D. S. 2009. Genes in streams: Using DNA to understand the movement of freshwater fauna and their riverine habitat. *BioScience,* 59**,** 573-583.

Hurwood, D. A., Hughes, J. M., Bunn, S. E. & Cleary, C. 2003. Population structure in the freshwater shrimp (*Paratya australiensis*) infered from allozymes and mitochondrial DNA. *Heredity,* 90**,** 64-70.

Jackson, J. K. & Resh, V. H. 1989. Distrubution and abundance of adult aquatic insects in the forest adjacent to a northern Californian stream. *Environmental Entomology,* 18**,** 278-283.

Kovats, Z. E., Cibrowski, J. J. & Corkum, L. D. 1996. Inland dispersal of adult aquatic insects. *Freshwater Biology,* 36**,** 265-276.

Larned, S. T., Datry, T., Arscott, D. B. & Tockner, K. 2010. Emerging concepts in tempory-river ecology. *Freshwater Biology,* 55**,** 717-738.

McKay, B. D. & Zink, R. M. 2010. The causes of mitochondrial DNA gene tree paraphyly in birds. *Molecular Phylogenetics and Evolution,* 54**,** 647-650.

Miller, M. P., Bilinn, D. W. & Keim, P. 2002. Correlations between observed dispersal capabilities and patterns of genetics differentiation in populations of four aquatic insect species from the Arazona White Mountains, U.S.A. *Freshwater Biology,* 47**,** 1660-1673.

Müller, K. 1982. The colonization cycle of freshwater insects. *Oecologia,* 52**,** 202-242.

Murphy, N. P. & Austin, C. M. 2004. Phylogeography of the widespread Australian freshwater prawn *Macrobrachium australiense* (Decapoda: Palaemonidae). *Journal of Biogeography,* 31**,** 1065-1072.

Neraas, L. P. & Spruell, P. 2001. Fragmentation of riverine systems: the genetic effects of dams on bull trout (Salvelinus confluentus) in the Clark Fork River system. *Molecular Ecology,* 10**,** 1153-1164.

Nguyen, T. T. T., Austin, A. D., Meewan, M. M., Schultz, M. B. & Jerry, D. R. 2004. Phylogeography of the freshwater crayfish *Cherax destructor* Clark (Parastacidae) in inland Australia: historical fragmentation and recent range expansion. *Biological Journal of the Linnean Society,* 83**,** 539-550.

Page, T. J., Baker, A. M., Cook, B. D. & Hughes, J. M. 2005. Historical transoceanic dispersal of a freshwater shrimp: the colonization of the South Pacific by the genus *Paratya* (Atyidae). *Journal of Biogeography,* 32**,** 581-593.

Petersen, I., Winterbottom, J. H., Orton, S., Friberg, N., Hildrew, A. G., Spiers, D. C. & Gurney, W. S. C. 1999. Emergence and lateral dispersal of adult Plecoptera and Trichoptera from Broadstone Stream, U.K. *Freshwater Biology,* 42**,** 401-416.

Pritchard, J. K., Sterphens, M. & Donnelly, P. 2000. Inference of population structure using multilicus genotype data. *Genetics,* 155**,** 945-959.

Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. 2012. ABGD, automatic barcode gap discovery for primary species delimitation. *Molecular Ecology,* 21**,** 1864-1877.

Smith, M. J., Kay, W. R., Edward, D. H. D., Papas, P. J., Richardsons, K. S. J., Simpson, J. C., Pinder, A. M., Cale, D. J., Horwitz, P. H. J., Davis, J. A., Yung, F. H., Norris, R. & Halse, S. A. 1999. AusRivAS: Using macroinvertebrates to assess ecological condition of rivers in Western Australia. *Freshwater Biology,* 41**,** 269-282.

Sode, A. & Wilberg-Larsen, P. 1993. Dispersal of adult Trichoptera at a Danish forest brook. *Freshwater Biology,* 30**,** 439-446.

Williams, W. D. 1977. Some Aspects of the Ecology of *Paratya australiensis* (Crustacea : Decapoda : Atyidae). *Australian Journal of Marine and Freshwater Research,* 28**,** 403-415.

Zhou, Z. J., Li, R. L., Huang, D. W. & Shi, F. M. 2012. Molecular identification supports most traditional morphological species of *Ruspolia* (Orthoptera: Conocephalinae). *Invertebrate Systematics,* 26**,** 451-456.