Recent evolutionary history of the Australian freshwater turtles *Chelodina expansa* and *Chelodina longicollis.*

by

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Kate Hodges with *Chelodina (Macrochelodina) expansa* from upper River Murray.

Photo by David Thorpe, Border Mail.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Kate Meredith Hodges

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Publications associated with this thesis

Chapter 2 – Phylogeography of Chelodina expansa

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Chapter 3– Phylogeography of Chelodina longicollis

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The above publications are reproduced in this thesis without change apart for formatting.

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Summary

Of all the global biomes, freshwater ecosystems are the most degraded and freshwater taxa suffer the highest extinction rates on record. Given the degradation of river basins in Australia specifically, there is a pressing need to characterise Australian freshwater biodiversity and the evolutionary processes that maintain it. In this thesis I examine Australian freshwater biodiversity by integrating across multiple disciplines of biogeography, phylogeography, ecology, and phylogenetics. I address questions in each of these disciplines using two Australian freshwater turtles in the genus *Chelodina*. The patterns, diversity, and evolutionary processes I uncover scale to the broader freshwater community and can facilitate effective conservation and better understanding of Australian freshwater ecosystems.

Chapter 2 presents a mitochondrial phylogeographic investigation of *C. expansa*. I uncovered two divergent haplogroups representing discrete regional populations each with independent evolutionary trajectories. One haplogroup is found in the inland Murray-Darling Basin, and on the east coast south of the Conondale Range and on southern Fraser Island. The other is found only on the east coast north of the Conondale Range and on northern Fraser Island. Few studies have explicitly examined relationships among inland and coastal bioregions and I show that with extensive sampling, complex and cryptic patterns can emerge that are concordant across a range of other freshwater taxa. This chapter improves understanding of how taxa respond to regional scale biogeographic boundaries, and highlights new and important phylogeographic breaks and centres of diversity within regions. I conclude with a call to uncover and recognise cryptic microbiogeographic regions for more directed freshwater conservation and biodiversity management.

In Chapter 3 I investigate the phylogeographic structure of *C. longicollis*. I predicted this species would have highly connected populations and would show insensitivity to biogeographic barriers owing to its strong capacity for overland dispersal and specific adaptions to terrestriality. Contrary to expectations, and in a pattern similar to low vagility freshwater vertebrates, *C. longicollis* revealed two ancient mitochondrial haplogroups with clear geographic partitioning either side of the Great Dividing Range. This pattern is overlayed with signatures of recent gene flow over the longstanding biogeographic barrier, likely facilitated by late Pleistocene and ongoing anthropogenic landscape change. I discuss how evolutionary and biogeographic processes can dominate at different times in freshwater species to create complex patterns of population divergence and connectivity. I demonstrate that even in a highly vagile freshwater species, the divergent effects of landscape history and hydrological boundaries often overwhelm the homogenising effects of life history.

Chapter 4 examines the nature and extent of mitochondrial gene tree – species tree discordance within *Chelodina*. I was drawn to investigate this issue as phylogeographic exploration of *C. expansa* and *C. longicollis* revealed three instances of mitochondrial haplotype exchange between the two species. A multilocus phylogenetic approach of the broader species tree revealed extensive mitonuclear discordance and high levels of mitochondrial paraphyly. I found that the mitochondrial genome of *C. expansa* is completely replaced with that of either *C. longicollis* or *C. canni*. This chapter adds to a small but growing set of case studies demonstrating complete mitochondrial replacement. It is the second only reported case of dual mitochondrial genome capture and the first reported case in a reptile. The *C. longicollis* mitochondrial genome has also been partially replaced with that of *C. canni*. Estimates of common ancestry for mitochondrial and nuclear lineages, plus coalescent simulations of gene flow suggest these patterns are not a

result of deep coalescence but rather multiple and ancient asymmetric introgressive events within and between subgenera. In Chapter 5 I use palaeodistribution and ecological niche modelling to explore the biogeographic, ecological, and climatic arena that led to ancient introgression. I support inferences made earlier in favour of neutral demographic disparity driving introgression from the common into the rare species during Plio/Pleistocene glacial aridity.

This thesis significantly improves our knowledge of southeast Australian freshwater biogeography. The pervasive yet equivocal influence of the Great Dividing Range on the evolutionary history of freshwater species is emphasised. Life history characteristics such as strong dispersal capacity and adaptations for terrestriality are shown to be unreliable predictors of population connectivity, and phylogeographic concordance among species illustrates broad-scale biogeographic processes that reach beyond taxonomy, life history, and ecology.

This thesis also reframes our understanding of the relationships and historic interactions among freshwater turtles in *Chelodina*. In synthesising evidence from multiple disciples I show that phylogeographic patterns have been shaped by complex evolutionary and ecological interactions between each species, and with *C. canni*. Molecular dating of *C. longicollis* and *C. expansa* haplogroups indicate that signals of expansion and diversification track recent post-hybridisation events and demographic histories unique to each species. Deeper mitochondrial genetic structure however reflects repeated and temporally separated episodes of mitochondrial genome capture during Plio/Pleistocene glacial aridity. The patterns I uncover of ancient, repeated, asymmetric introgression among Australian freshwater turtles is unique and of great biogeographic, evolutionary, and ecological interest. I conclude this thesis by highlighting future directions to better

understand the biogeography of freshwater systems, and the cause and extent of

introgression in *Chelodina*.

Chapter 1

General Introduction

Of all the global biomes, freshwater ecosystems are the world's most degraded and threatened (Kingsford & Biggs, 2012; Keith *et al*., 2013). Intense agriculture, water extraction, pollution, river regulation, inversion of natural flow regimes, climate change, and invasive species imperil freshwater fauna with the highest extinction rates on record (Richter *et al*, 1997; Ricciardi & Rasmussen, 1999; Rahel, 2002; Abell, 2002; Poff *et al*., 2012). Urgent calls for a comprehensive and systematic approach to the conservation of Australian freshwater biodiversity (Kingsford & Nevill, 2005) have been met by a multitude of increasingly sophisticated methods. Systematic conservation planning, conservation decision theory, process-based models, and CARE principles (Margules & Pressy, 2000; Linke *et al*., 2011; Nel *et al*., 2011; Turak *et al*., 2011) all have strong theoretical foundations and all claim to consider each level of biological organisation. Rarely do they deliver, however for freshwater biodiversity at the genetic level.

Knowledge of the spatial and temporal patterns of genetic diversity and the evolutionary processes that give rise to them is crucial for an integrated approach to biodiversity conservation. Recognition of underlying genetic diversity is the first step towards its conservation and attention directed at the basis of biodiversity delivers a framework for assessment at higher levels. Given the global threat to freshwater ecosystems, and the degradation of semi-arid river basins in Australia specifically (MDBA, 2010; LeBlanc *et al*., 2012; MDBA Sustainable Rivers Audit, 2012), there is a pressing need to characterise genetic diversity of Australian freshwater taxa to understand the evolutionary and ecological context in which such diversity arose and to understand the processes that maintain it.

In this thesis, I use molecular phylogenetic data to investigate the recent evolutionary history and phylogeographic structure of two species of Australian freshwater turtle, the eastern long-necked turtle *Chelodina (Chelodina) longicollis* (Shaw, 1794) and the broad shelled turtle, *Chelodina (Macrochelodina) expansa* (Gray, 1857). These species are freshwater obligates and widely distributed throughout inland and coastal eastern Australia. They occur in a variety of endangered ecological communities and ecosystems, and with a range of endangered fish species. Environmental changes of the freshwater landscapes inhabited by *C. expansa* and *C. longicollis* are well characterised, the range of freshwater habitats they occupy is broad, and an understanding of their life history traits is well established. Each of the above factors combine to make Australian freshwater turtles an ideal yet underutilised model group for comparative phylogeographic inquiry in aid of freshwater biodiversity conservation.

In this opening chapter, I begin with an introduction to phylogeography and its utility in the conservation and bioregionalisation of freshwater ecosystems. Then I introduce the study species and provide an overview of relationships among *Chelodina* subgenera. Because phylogeographic patterns are inherently related to biogeographic history, I also provide an account of landscape evolution and biogeography of eastern Australia and the Murray-Darling Basin. Finally, I provide an introduction to gene tree–species tree discordance to give context to Chapter 4 where I describe unexpected asymmetric mitochondrial introgression. I conclude this general introduction with the thesis aims and objectives addressed by the substantive data chapters.

Phylogeography and the bioregionalisation of freshwater systems

Phylogeography is a success story within biology and is heralded as the discipline that bridged phylogenetics and population genetics (Avise *et al*., 1987). As a sub-discipline of

biogeography, phylogeography investigates the geographical distribution of genealogical lineages and clades, and implicitly recognises that the products and processes of evolution should be regarded within a spatio-temporal framework. As such, it is also the discipline that allows us to "see genes in space and time" (Hewitt, 2001). Phylogeography includes in its scope, dispersal, vicariance, and extinction on the geographic distribution of lineages, and thus has the capacity to test traditional biogeographic paradigms. Furthermore, when disparate taxa show concordant phylogeographic patterns, the biogeographic history of entire landscapes can be inferred (Avise, 2009). Where co-distributed taxa each show different patterns of genetic structure, the comparative approach illuminates species-specific processes such as habitat specificity, dispersal ability and differential resistance to barriers.

Phylogeographic analysis in the freshwater realm is uncovering a vast number of previously unrecognised evolutionary lineages and astonishingly high levels of genetic diversity across many freshwater taxa (Adams *et al*., 2013; Hammer *et al*., 2014; Huey *et al*., 2014). The expectation that freshwater taxa tend to have lineages defined by catchments (Hughes *et al*., 2009) is often countered by species-specific patterns that show unexpected gene flow across significant catchment boundaries which are commonly believed to prevent biological dispersal (*e.g*. Slechtova *et al.*, 2004; Craw *et al.*, 2008). Comparative phylogeography can highlight hydrological and species-specific attributes of freshwater taxa that can inform conservation at the landscape scale. Hydrological attributes include the detection of cryptic drainage connectivity, community level responses to catchment boundaries, unexpected instances of population isolation, and the locations of fine scale genetic diversity which is typical of freshwater systems. Comparative phylogeography can explicitly test the assumption of catchment constraint and quantify

relationships between and within catchments, thus illuminating the influence of hydrology on biotic distribution. Species-specific attributes revealed though comparative phylogeographic investigation include desiccation and salinity resistance, the capacity for overland dispersal, and the extent to which hydrology and vagility interact to influence population processes.

Phylogeography is celebrated for its interdisciplinary research agenda. Since its inception the field has advanced knowledge on human evolution and the interactions of our ancestors with other members of the genus (Li *et al*., 2008; Oppenheimer, 2012). It has revealed the genetic legacy of Quaternary glacial cycles (Hewitt, 2000), and the origin and pace of modern viral pandemics (Zehender *et al*., 2012). Phylogeography has also contributed to conservation biology and biodiversity management though characterisation of phylogenetic diversity and endemism, and Evolutionarily Significant Units (Moritz, 1994; Faith *et al*., 2004; Bryne, 2007; Rosauer *et al*., 2009). Recent emphasis on hypothesis testing has incorporated coalescent theory and species distribution modelling and has added much needed rigor and ecological realism to the field (Carstens *et al*., 2005; Carstens & Richards, 2007; Knowles, 2009; Chan *et al*., 2011; Marske *et al*., 2013; Alvarado-Serrano & Knowles, 2014). Parallels between the disciples of comparative phylogeography and historical biogeography have also long been recognised (Riddle, 1996; Zink, 1996; Arbogast & Kenagy, 2001; Diniz-Filho *et al*., 2008). Despite this, the power of phylogeography to lend an important evolutionary perspective to its parent discipline of biogeography is yet to be fully realised. This is perhaps most apparent in the subject of biogeographic regionalisation (bioregionalisation) which so far rarely integrates phylogeographic thinking.

Chapter 1 – General introduction

Bioregionalisation aims to systematically delineate nested geospatial regions of biotic distinctness across the landscape within which macroecological patterns and processes are maintained. This aim is somewhat provocative as the extent to which bioregions are artificial constructs or are indeed natural and discoverable entities is difficult to determine (Mackey *et al.*, 2008). At best, biogeographical regions are imperfect summaries of biological and ecological diversity as not all taxa respond to biogeographical barriers in the same way. Nonetheless, bioregionalisation provides a useful tool for simplifying and outlining complex patterns of biodiversity and has been applied across terrestrial and marine environments at a global scale (see Olson *et al.*, 2001; Spalding *et al.*, 2007). Bioregionalisation has outcomes for both theoretical and conservation oriented studies. It provides a theoretical framework to investigate and test patterns of biotic diversity and change, and also lays foundations for systematic biodiversity conservation and landscape scale conservation planning.

Various methods have been used to define bioregions. Contemporary studies integrate broad scale ecosystem drivers (climate, elevation, terrain parameters), ecosystem responses (primary productivity, vegetation structure), and taxonomic composition (species richness and endemism). This achieves the want to represent compositionalist and functionalist perspectives, and succeeds in global bioregionalisation that recognises macroevolutionary history. At the continental scale, bioregionalisation methods refine taxonomic information to include species level phylogenies and the spatial distribution of individual taxa. This method has proven fruitful in Australia as regions of high endemism and biodiversity hotspots are well described for the terrestrial (Cracraft, 1991; Thackway & Cresswell, 1995; Crisp *et al.*, 1999; Ladiges *et al.*, 2011) and marine (Butler *et al.*, 2001; Lyne & Hayes, 2005; Waters *et al.*, 2010; Woolley *et al.*, 2013) environment. The

imperilled Australian freshwater realm however has received only limited attention (see

Unmack, 2001; Abell *et al*., 2008; www.feow.org) (Figure 1.1). The above methods for

bioregionalisation have much to offer biodiversity conservation at global and continental

scales. They are too broad however to translate to the regional scale as they fail to

recognise genetic diversity at the population level.

A phylogeographic approach is well suited to regional scale bioregionalisation.

Phylogeography can identify spatially explicit units of biodiversity with a cohesive

evolutionary past, provide an understanding of the relationships between units, and detect

shared patterns of isolation and connectivity. Comparative phylogeography can also test

and refine broader scale bioregions already defined. Such an approach is urgently needed

to aid conservation of the Australian freshwater environment, and Unmack's (2001)

Australian bioregions for freshwater fishes present a range of biogeographic hypotheses

that are ideal to test using comparative phylogeographic analysis.

Freshwater turtles present an excellent model group for comparative

phylogeographic study and freshwater bioregionalisation. Turtles are intimately tied to the

freshwater environment, however life history traits such as a strong proclivity in some

species for terrestrial dispersal and an ability to occupy a range of freshwater habitats place

turtles as an intermediate between freshwater fish and low-vagility terrestrial mammals

(Walker & Avise, 1998). This presents the opportunity to uncover cryptic hydrological

connectivity between freshwater bioregions. Further, the long generation time of turtles

enables the retention of genetic signatures of past biogeographic events and demographic

perturbations for longer than taxa with shorter generations. For these reasons early
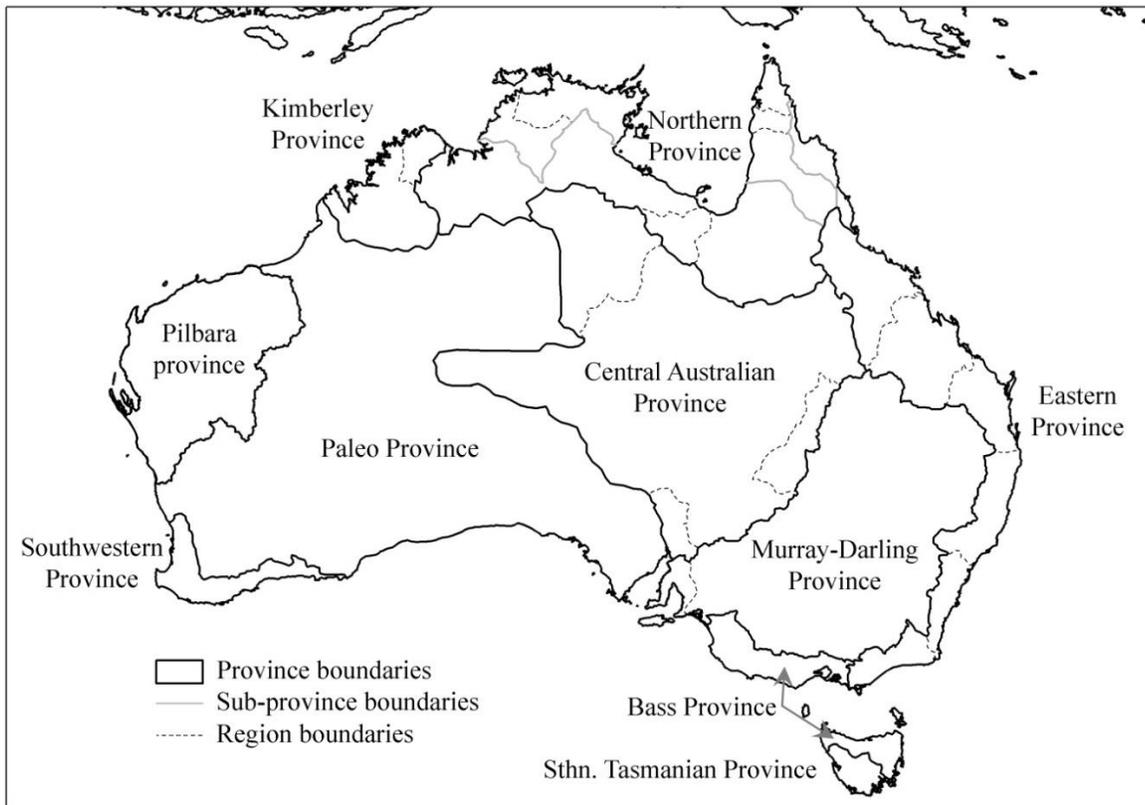
Figure 1.1 Australian freshwater biogeographic provinces for fish proposed by Unmack (2001). To date, this is the only attempt at freshwater bioregionalisation in Australia.

phylogeographic studies employed freshwater turtles as a model system to illustrate biogeographic patterns (*e.g*. Walker & Avise, 1998).

The utility of the group has continued in the northern hemisphere with many phylogeographic studies revealing high levels of genetic diversity, cryptic lineages, and important insights into the spatial distribution of freshwater biodiversity at regional and local scales (Weisrock & Janzen, 2000; Starkey *et al*., 2003; Stephens & Wiens, 2003; Spinks & Shaffer, 2005; Fritz *et al*., 2009; Spinks & Shaffer 2009). Despite demonstrated utility as a sensitive model system, Australian freshwater turtle species are very poorly represented in phylogeographic and biogeographic studies. Australian freshwater biogeographic work routinely relies on fish and macroinvertebrate model systems. Only very recently have turtles been used to infer evolutionary forces driving Australian freshwater biodiversity (Georges *et al*., 2013; Todd *et al*., 2013, 2014).

The Australian long-necked turtles: *Chelodina*

The Australian freshwater turtle fauna is dominated by the family Chelidae Gray, 1825 (suborder: Pleurodira) which contains aquatic or semi-aquatic freshwater species also found in New Guinea, the islands of Timor and Roti, and South America. The family is not known as fossils outside of this distribution and is consequently considered of Gondwanan origin. *Chelodina* Fitzinger, 1826 is one of seven genera endemic to the Australian region, and arose in the mid-Eocene approximately 47 Mya (million years ago) (Near *et al*., 2005). *Chelodina* represents a clearly defined monophyletic group and is characterised by exceptionally long necks that have evolved independently of South American long-necked genera (Georges *et al*., 1998). The 11 *Chelodina* species fall into three long-recognised clades first proposed (but not named) by Goode (1967) and later confirmed using serological (Burbridge, 1974), morphological (Thomson *et al*., 1997) and allozyme data

(Georges & Adams, 1992; Georges *et al*., 2002). These subgeneric groups were finally assigned subgenera rank by Georges & Thomson (2010) – *Chelodina*, *Macrochelodina* Wells & Wellington, 1985 and *Macrodiremys* McCord & Ouni, 2007. Taxonomy in this thesis follows that of Georges & Thomson (2010), Kennett *et al*. (2014), and the recommendations of Kaiser *et al*. (2013).

The subgenus *Chelodina* comprises *C. longicollis*, *C. canni*, and *C. steindachneri* of Australia; *C. novaeguineae*, *C. reimanni* and *C. pritchardi* of New Guinea; and *C. mccordi* of Timor and Roti Islands. This subgenus is characterised by a narrow head, a relatively short and thin neck compared to the other species, and a broad plastron (Georges and Thomson, 2010). The subgenus *Macrochelodina* is characterised by a broad head, narrow plastron, and a robust and long neck (hence the name "snake-necked turtles") evolved for a strike and suck method of piscivory (Georges *et al*., 2002 and authors therein; Georges & Thomson, 2005). Subgenus *Macrochelodina* comprises *C. expansa*, *C. oblonga* (formerly *rugosa*) and *C. burrungandjii* of Australia; and *C. parkeri* of New Guinea. The subgenus *Macrodiremys,* is monotypic with *C. colliei* found only in Western Australia.

The currently accepted phylogeny for the three sub-genera (Figure 1.2) was established by Georges et al. (2002) through consolidation of genetic and morphological data. They paired subgenera *Chelodina* and *Macrodiremys* as sister clades, to the exclusion of subgenus *Macrochelodina*. This disagreed with earlier work that placed *C. colliei* with the species now assigned to subgenus *Macrochelodina* (Goode, 1967; Legler, 1981). Although support for the *C. colliei – Macrochelodina* clade remains controversial, it is supported by morphology (Thomson et al., 1997), mitochondrial sequence data (Seddon et al., 1997) and allozymes (Georges & Adams 1992; Georges et al., 2002).
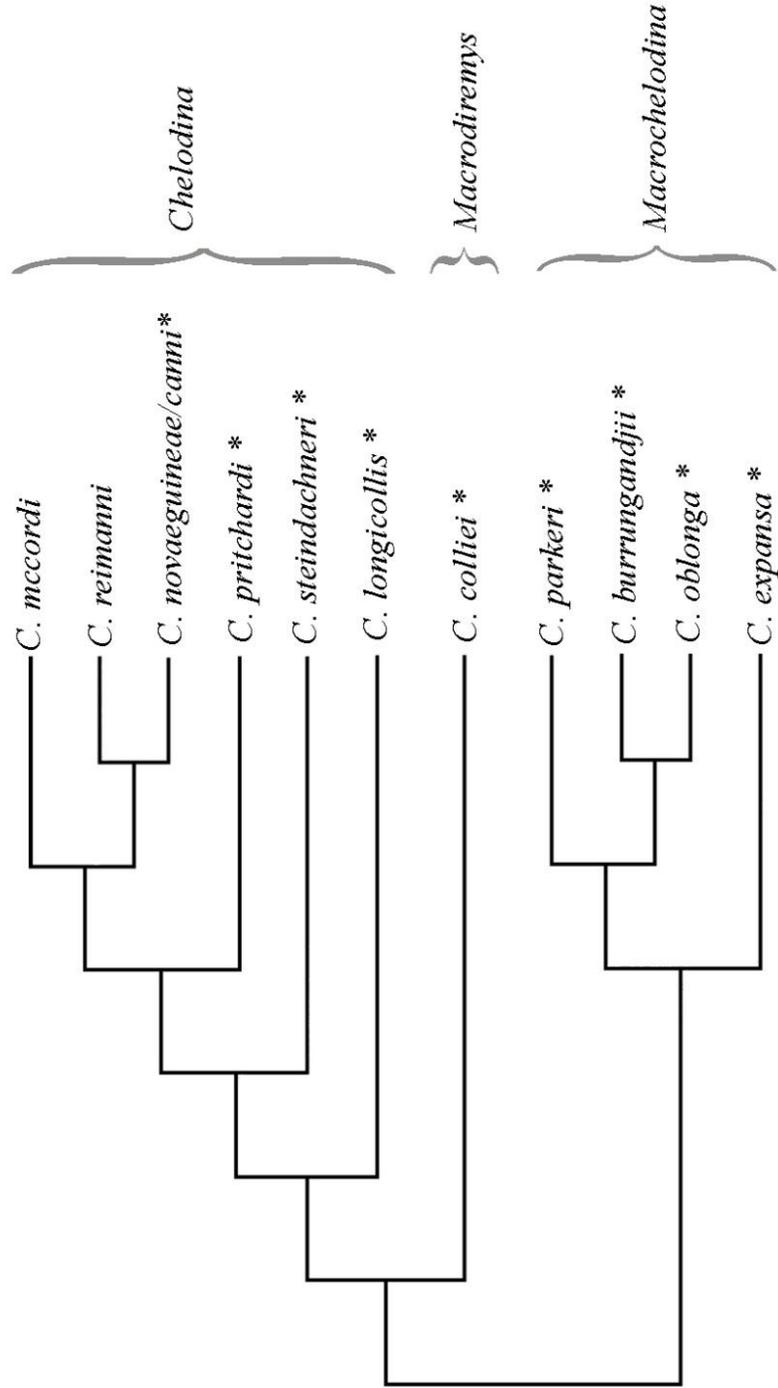
Figure 1.2 Phylogeny for the *Chelodina* with subgeneric groups *Chelodina*, *Macrodiremys*, and *Macrochelodina* based on allozymes and morphological data (modified from Georges *et al.*, 2002). In Georges *et al.* (2002) *C. novaeguineae* was indistinct from *C. canni* and so here both are placed on the same branch. * indicates species studied in this thesis

Natural hybridisation within and between species in the subgenera *Chelodina* and *Macrochelodina* is not uncommon. *Chelodina longicollis* and *C. canni* hybridise to yield viable offspring on the boundaries of their distributions in the Styx catchment in coastal Queensland (Georges *et al*., 2002). *Chelodina oblonga* and *C. burrungandjii* hybridise where their ranges overlap in Arnhem Land of northern Australia (Georges *et al*., 2002; Alacs, 2008). In the latter instance, backcrossing of hybrids into the paternal species (*C. burrungandjii*) has led to widespread introgression of the *C. oblonga* mitochondrial haplotype (Alacs, 2008). Between subgenera, natural and morphologically distinctive hybrids have been documented between *C. canni* and *C. oblonga* where the two species are sympatric in northern Australia. Second generation hybrids were not known to occur until an allozyme study identified a single morphologically indistinguishable individual as a backcrossed form of *C. canni* (Georges *et al*., 2002; Alacs, 2008). Hybridisation between distantly related subgenera and evidence of successful backcrossing raises the possibility of further, as yet undetected introgression occurring throughout the group.

Study species

This thesis focusses on *C. longicollis* and *C. expansa* and the recent evolutionary history of these species with *C. canni*. Typical of many freshwater turtles, all species display life history strategies of delayed maturity and high adult survivorship (Shine & Iverson, 1995).

Chelodina longicollis is ubiquitous and widely distributed throughout inland and coastal south eastern Australia (Figure 1.3). The species inhabits a broad range of freshwater environments and occurs in greatest abundance in shallow ephemeral wetlands and disconnected water bodies with an abundance of slow-moving invertebrate prey (Chessman, 1984a, 1988; Georges *et al*., 1986). An exceptional dispersal capacity at the landscape scale and a strong propensity to utilise terrestrial environments (Roe & Georges,
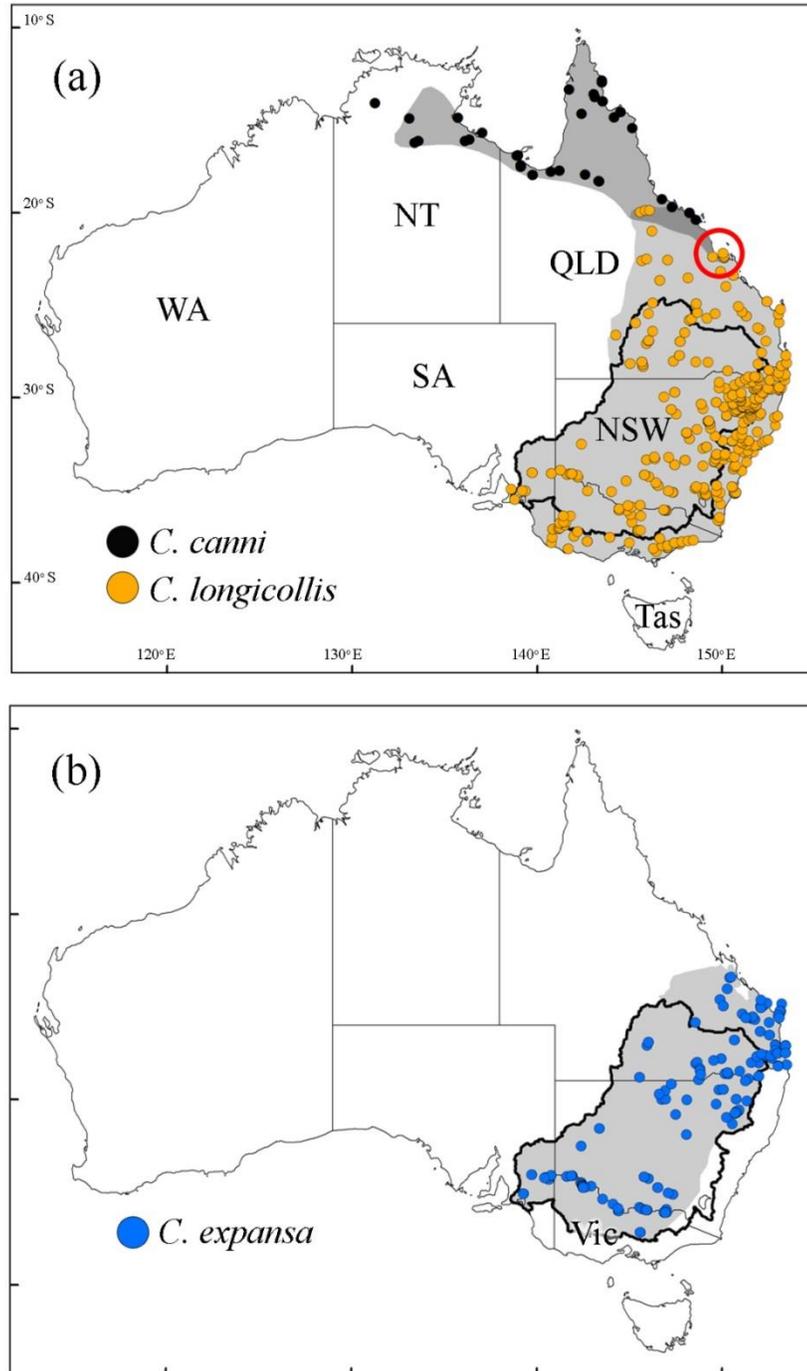
Figure 1.3 Distribution of (a) *C. longicollis* **and** *C. canni*, **and (b) distribution of** *C. expansa*. **Circles represent all collection localities and museum records (http://iae.canberra.edu.au/cgi-bin/locations.cgi). Note the region of overlap between** *C. canni* **and** *C. longicollis* **in coastal Queensland. Location of Styx catchment is circled in red. Thick black line indicates the periphery of the Murray-Darling Basin. Abbreviations: WA, Western Australia; NT, Northern Territory; SA, South Australia; QLD, Queensland; NSW, New South Wales; Vic, Victoria; Tas, Tasmania.**

2007; Roe & Georges, 2008b) allow the species to exploit highly productive ephemeral

systems which are often several kilometres from permanent rivers. When disturbed on

land, individuals wholly conceal the head and neck within the shell (Figure 1.4) and release

a pungent fluid from inguinal and axillary scent glads, presumably to repel terrestrial

predation.

      Chelodina longicollis displays some degree of cold tolerance (Chessman, 1988)

and can maintain populations at montane sites in the high elevation southeast Australian

uplands (793 m a.s.l) where snowfall and severe frosts are common during winter.

Maximum adult body sizes (measured by straight line carapace length) vary geographically

and with habitat productivity, and there is some degree of sexual size dimorphism with

females growing to larger sizes than males. Maximum female size ranges between 216

mm-282 mm, and maximum male size between 188 mm-249 mm (Kennett *et al*., 2009).

Mating occurs in the Austral spring (September) followed by nesting in late spring and

early summer (October – January). Incubation time is between 110-150 days, and

hatchlings emerge in autumn (March-April). Males reach maturity at 7-8 years and

females at 10-12 years (Chessman, 1978).

      Chelodina expansa is broadly sympatric with, but morphologically and ecologically

distinct from *C. longicollis*. *Chelodina expansa* is widely distributed throughout the inland

Murray-Darling Basin and on the southeast Queensland coast, and offshore populations

occur on Fraser Island, Moreton Island and Stradbroke Island. The species is listed as

Threatened in parts of its range in the Murray-Darling Basin (DSE, 2010), and Near

Threatened under IUCN red listing (Bower & Hodges,2014). *Chelodina expansa* occurs at

low densities and although traditionally considered a riverine species (Cann, 1998), recent

studies demonstrate that they are more frequently represented in permanent lakes and

Figure 1.4 Dorsal and ventral view of a live female *C. longicollis*. Note how the individual is completely concealed in the shell. Photo by Arthur Georges in Kennett *et al.* (2009).

billabongs connected to the main river channel (Hamann *et al.*, 2008; De Lathouder *et al.*,

2009; Bower *et al.*, 2012; pers obs).

The species is carnivorous and predominately feeds on fast moving prey items such

as decapod crustaceans and fish which it captures via ambush or foraging (Chessman,

1983; Meathrel *et al.*, 2002). The species is Australia's largest chelid (Figure 1.5) though

there is marked sexual dimorphism where males are smaller and mature at a smaller size

class than females (Spencer, 2002). Maximum adult female size can reach 500 mm with

an additional neck length between 65 and 75% of the overall carapace length (Cann, 1998).

Size does not appear to vary geographically, except that individuals from Fraser Island are

markedly smaller and darker compared to mainland populations (Cann 1998; pers obs.)

(Figure 1.6). Mating occurs in the Austral late summer and early autumn (February –

March) followed by nesting through autumn and early winter (April – June) (Legler,

1985). This tropical nesting pattern is unusual in Australian freshwater turtles at high

latitudes as most temperate species nest predominantly in the spring and summer. The

reproductive biology of *C. expansa* sets them apart from other members of the subgenus

and there has been substantial research on the physiological aspects of embryonic

development (Booth 1998a, 1998b, 2000, 2002a, 2002b). Developing embryos exhibit

secondary diapause which enables them to overwinter in the nest, resulting in a long

incubation period occasionally up to 522 days (Goode & Russell, 1968). Males reach

maturity at 9-11 years, and females at 14-15 years (Spencer, 2002).

Figure 1.5 Dorsal and ventral view of an adult female *Chelodina expansa*. Note the narrow plastron and the inability of the animal to fully conceal its neck and limbs inside the shell. Photo by Deborah Bower in Bower & Hodges (2014).

Figure 1.6 Sub-adult male *Chelodina expansa*, Fraser Island, Queensland Australia. Note the dark skin coloration. Photo by Kate Hodges.

Chelodina canni (Figure 1.7) was described by McCord and Thomson (2002) from the *C. novaeguineae* species complex. *Chelodina canni* is readily distinguished from C. *longicollis* by a wide, robust head. Adults are distinguished by a wide, rounded carapace with a moderately deep midvertebral trough (McCord & Thomson, 2002). The distribution of *C. canni* encompasses Cape York Peninsula and the Gulf of Carpentaria, with a southern boundary extending from coastal north east Queensland where it shares a narrow zone of sympatry with *C. longicollis*, to the Daly Waters region of the Northern Territory (Figure 1.3). An offshore population occurs on Maria Island in the Gulf of Carpentaria. The species inhabits semi-permanent and seasonally ephemeral water bodies. In its western distribution *C. canni* aestivates in muddy waterholes that dry annually, though in other parts of its range it is similar to *C. longicollis* and will migrate overland to more permanent water during the dry season (Kennett *et al*., 1992). Further similarity to *C. longicollis* is demonstrated by the production of a distinctive pungent odour when handled (Cann, 1998; McCord & Thomson, 2002). Reproductive biology of *C. canni* is not well characterised beyond observations by Kennett *et al*. (1992): nesting is thought to occur in the tropical dry season (April – June), followed by an incubation period of 120 days, and hatching in the late dry to early wet season (September – November). Hybrids between *C. canni* and *C. longicollis* are distinguished by a blend of characters from both species and a deformity of the intergular scute and the underlying bone (McCord & Thomson, 2002).

Figure 1.7 *Chelodina canni.* **Photo by Erika Alacs.**

Environmental evolution and biogeography of eastern Australia

Australia is a geologically stable continent with the last major orogenic event being the uplift of the Great Dividing Range approximately 90 Mya (Wellman, 1979). This uplift occurred as the continent rifted from Antarctica and moved though 20° of latitude to its current location (Hill, 2004). Except for incidents of river capture in the late Cretaceous (Haworth & Ollier, 1992), and some volcanism throughout the Miocene (Johnson, 1989), the catchment boundaries of the east coast and the inland Murray-Darling Basin have changed little since this time. Humid environments dominated south eastern Australia from the Oligocene, with gradual drying throughout the Miocene and Pliocene and significantly dry episodes in the late Miocene from 10-7 Mya (Bowman & Yates, 2006) and in the Late Pliocene (Kershaw *et al*., 1994; Martin, 1998; Hill, 2004). An inland sea covered the south-western corner of the Murray-Darling Basin during the late Miocene, and retreated in the Pliocene approximately 2 Mya (Page *et al*., 1996; Williams, 2011). Following this retreat and the uplift of the Pinnaroo Block, a large freshwater lake, Bungunnia, formed in the region and persisted until about 700,000 years ago (Page *et al*., 1996). The draining of Lake Bungunnia in the early Pleistocene was coincident with major climatic oscillations across the continent with the onset of glacial cycles, but it was not until about 350,000 years ago, when glacial cycles intensified, that major climatic and landscape changes affected Australia's biota.

In the mid to late Pleistocene, glacial periods exceeded past aridity thresholds and resulted in widespread dune building, expansion of open steppe grasslands, depression of eucalypt forests to protected isolates, and extension of coastal plateaus (Hill, 2004). Paradoxically, at glacial maxima even though precipitation was greatly reduced, denuded slopes and plains plus low evaporation rates combined to enhance catchment efficiency. In

the upper catchments of the south-eastern Murray-Darling Basin, this resulted in seasonally large and powerful rivers dominated by glacial spring runoff, and in the retention of surface water in foothill lakes (Page *et al.*, 1996; Bowler *et al.*, 2005; Kemp & Spooner, 2007). Despite this fluvial activity, glacial periods were times of severe environmental stress for Australia's biota and for freshwater taxa specifically. Intense cold and extensive salinisation owing to a raised water table (Bowler *et al.*, 2005) saw severe range contraction and enforced allopatry of many freshwater dependent species. Interglacials saw a return to warm, humid and wet conditions with recharged lacustrine systems, reconnection of forest and woodland habits, and marine flooding of low-lying coastal plains (Bowler *et al.*, 2005).

The biogeographic history of eastern Australia and the inland Murray-Darling Basin is strongly dictated by the Great Dividing Range. This mountain belt is the only major range in Australia and is the dominant topographic feature on the east of the continent. It presents a substantial biogeographic barrier to Australian taxa throughout most of its length, separating inland and coastal drainages and protecting the coastal periphery from inland aridity (Figure 1.8).

Compared to mountain ranges of other continents, the Great Dividing Range has a somewhat subdued character. Ollier (1982) described it as a "cartographic myth" and Jones (2006) as a "scarcely distinguishable landform". Indeed, remarkably low relief country characterises the Great Dividing Range at the headwaters of the Fitzroy and Burnett drainages at the northern boundary of the Murray-Darling Basin (1, in Figure 1.8), and at the headwaters of the Hopkins and Glenelg drainages where the range commences in the south (2, in Figure 1.8). The southern region however represents the edge of the uplifted plateau, and includes a major escarpment up to 1,000 m. a.s.l on its eastern periphery. Several well defined geocols (broad regions of low elevation) characterise this section of

the Great Dividing Range and link the Murray-Darling Basin and coastal bioregions. All

occur among otherwise high elevation landscapes and are found at the headwaters of

Clarence, Hunter and Hawkesbury coastal drainages (Haworth & Ollier, 1992) (Figure 1.8).

Of particular interest to this thesis is the Hunter geocol which extends into the east coast

Hunter Valley and presents a biogeographic barrier to mesic adapted biota in the form of a

dry open lowland (Moussalli *et al*., 2005; Chapple *et al*., 2011; Rix & Harvey, 2012) also

known as the Cassilis Gap. A further biogeographic barrier on the east coast is presented

by the McPherson Range, though this is in the form of a humid upland forest (James &

Moritz, 2000; Schauble & Moritz, 2001; Keogh, 2003; Crisp *et al*., 2004; Colgan *et al*.,

2009; Lucky, 2011).

No biogeographic barriers have been described within the Murray-Darling Basin.

This semi-arid inland basin is characterised by very low elevational gradients, wandering

lowland river channels, and large distributary floodplains. Climate is characterised by

drought/flood cycles that commonly span decades. The current hydrological regime is

among the most variable in the world (Puckridge *et al*., 1998) and the region has been

broadly described as a "sea of dry land" (Faulks, 2010).

Australian ecosystems have been shaped by increasing aridification since the

Miocene, and freshwater environments in particular have experienced significant changes

over short geological time scales. Landscape evolution and climate oscillations in eastern

Australia likely had a strong influence on the distributions, divergence patterns, and

demography of freshwater taxa. This biogeographic history provides a backdrop to

contemporary genetic structure in freshwater turtles and a crucial context within which

patterns of freshwater biodiversity can be explored.
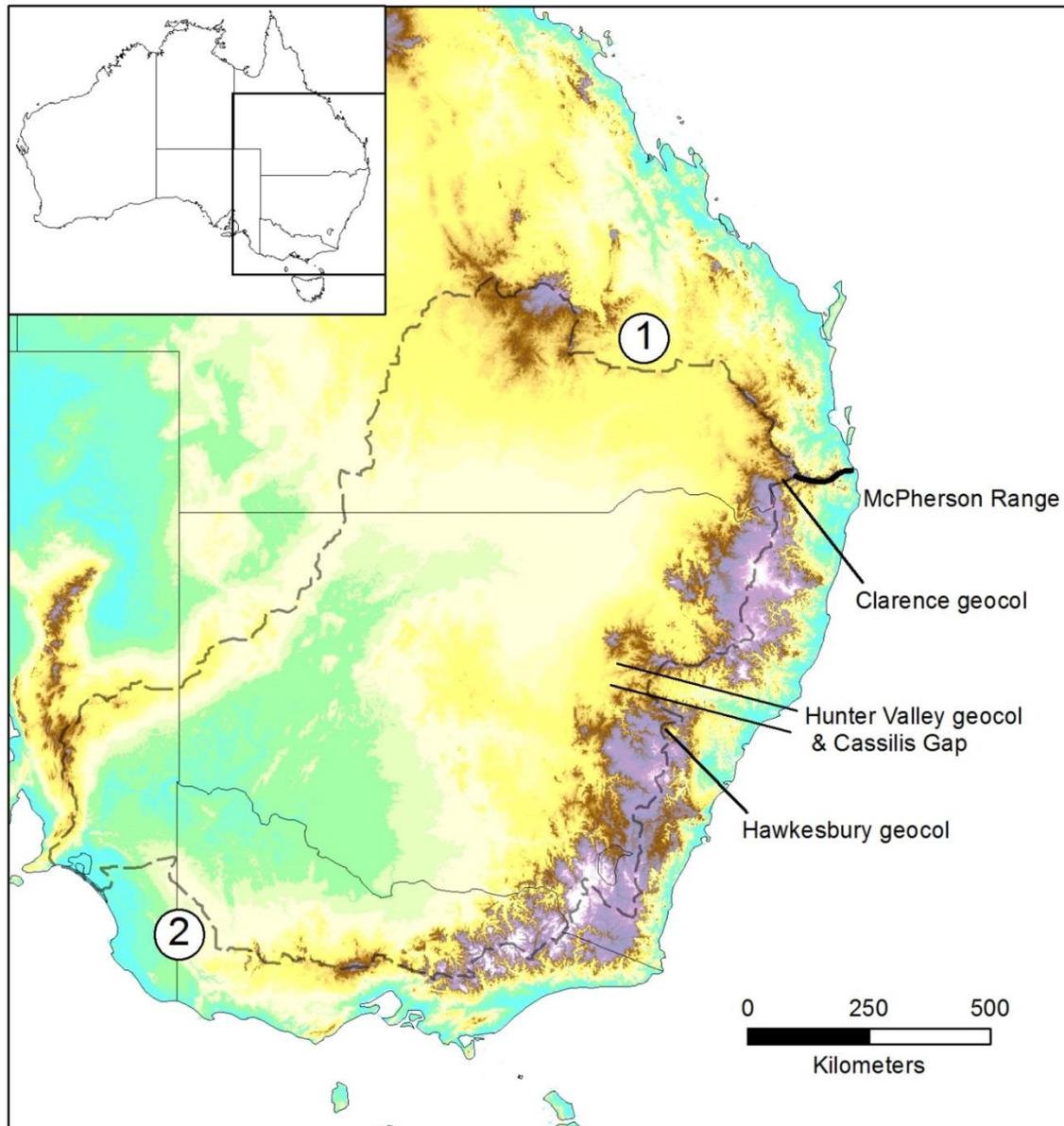
Figure 1.8 Eastern Australia showing the high elevation Great Dividing Range (brown to purple), the lowland interior Murray-Darling Basin (yellow to green) delineated by the grey dashed line, and the low lying coastal plains (blue). Labels (1) and (2) indicate low relief sites where the Great Dividing Range commences in the north and south. Geocols in the Clarence, Hunter, and Hawkesbury drainages and the upland McPherson range are also indicated.

An inescapable biological reality: gene tree–species tree discordance

Genes and species are different entities and genealogical histories can vary from locus to locus despite sharing the same population and speciation history. A result of this variation is gene tree–species tree discordance, species level polyphly, and erroneous evolutionary inference. Variation among gene histories in a species can result from natural selection, which may only affect some loci; molecular events such as horizontal gene transfer, gene duplication, and recombination that typically affect only small segments of the genome; stochastic properties of populations such as size, which randomly determines if lineages are retained or lost; and historical or contemporary introgression in which alleles from one species penetrate the gene pool of another though hybridisation and backcrossing of hybrids. These processes shape idiosyncratic gene histories and can ultimately lead to branch length differences and topological discord among gene trees and with the underlying species tree. Given this inherent gene tree heterogeneity and the biological reality of a single true species tree, molecular phylogenetic research must acknowledge gene tree–species tree discordance as an "inescapable biological reality" (Knowles, 2009; Knowles & Kubatko, 2010) and gene trees as only a "local optimum" (Edwards, 2009) in pursuit of the true species tree.

Much incongruence between gene trees and the species tree arises through variability among gene trees in time to most recent common ancestor, also known as coalescent stochasticity (Funk & Omland, 2003; Hudson & Turelli, 2003; Joseph *et al*., 2006; Eytan & Hellbery, 2010). Coalescent stochasticity is a product of stochastic lineage sorting whereby gene lineages within a species are randomly retained or lost in successive generations. Retained lineages over time come to represent deeper portions of the gene tree and reflect ancient polymorphisms. The longer a gene lineage persists through the

evolution of a species, so too does its chance of failing to coalesce with conspecific lineages increase, owing to their stochastic loss. Coalescence is then more likely to occur with a similarly ancient lineage from a different but closely related species, resulting in gene tree–species tree discordance for that locus. This manifestation of coalescent stochasticity is known as incomplete lineage sorting or deep coalesce because coalescing lineages have not 'sorted' by species, and because coalescence is 'deep' in the gene tree reflecting ancient polymorphism and a time prior to speciation. Coalescent stochasticity has gained much attention in phylogenetics. An extensive analytical framework and methods have developed for phylogenetic reconstruction that takes into account discrepancies among individual gene trees resulting from incomplete lineage sorting (Ronquist & Huelsenbeck, 2003; Maddison & Knowles, 2006; Carstens & Knowles, 2007; Liu & Pearl, 2007; Wakeley, 2008; Degnan & Rosenberg, 2009; Edwards, 2009; Knowles, 2009; Kubatko *et al*., 2009; Liu *et al*., 2009; Heled & Drummond, 2010).

The genealogical pattern of incompletely sorted lineages also characterises early and intermediate stages of population divergence. As diverging populations progresses though neotypy, allotypy, and allophyly to reciprocal monophyly (Omland *et al*., 2006) genealogical discordance is widespread and species affiliation of individuals is unclear. This outcome is temporary however and is importantly different to gene tree–species tree discordance as a result of deep coalescence. In the case of deep coalescence, speciation is considerably progressed such that most gene lineages are concordant and accurately reflect species affiliation, while the lineages discordant with the species tree reflect the persistence of ancient polymorphism.

Gene tree–species tree discordance via deep coalescence is primarily driven by effective population size and phylogenetic branch length (Zink & Barrowclough, 2008;

Degnan & Rosenberg, 2009). Deep coalescence will be more likely when effective population sizes are large, thus increasing the chance of ancient lineages persisting in the population; and when phylogenetic branch lengths between species are short, thus allowing gene lineages to extend deeper into the gene tree. As a result, gene tree–species tree discordance via deep coalescence is more likely to characterise rapid species diversifications and species with large effective population sizes.

A classic and well described example of lineage sorting as a function of effective population size is demonstrated by the common discordance between nuclear and mitochondrial genealogies (mitonuclear discordance) within a species (Palumbi *et al*., 2001; Ting *et al*., 2008; Knowles, 2009; Leaché, 2010; Fijarczyk *et al*., 2011; Toews & Brelsford, 2012). The mitochondrial genome is haploid and uni-parentally inherited in most animals and is thus characterised by a genetic effective population size approximately one-quarter that of the nuclear genome which is diploid and bi-parentally inherited. A smaller genetic effective population drives mitochondrial gene lineages to complete the lineage sorting process and resolution to reciprocal monophyly four times more rapidly (assuming a 1:1 sex ratio and similar reproductive success for males and females) than nuclear gene lineages. Owing to the larger effective population size of the nuclear genome, ancient nuclear gene lineages are more frequent and may persist in the population for longer compared to those in the mitochondrial genome. These ancient lineages coalescence deep in the gene tree and thus genealogical patterns in the nuclear genome are more likely to lag behind those recovered from mitochondrial lineages. An inverse relationship between effective population size and the speed with which reciprocal monophyly is achieved predicts that nuclear lineages will recover gene tree–species tree discordance more often than mitochondrial lineages which are more likely to recover reciprocal

monophyly and gene tree–species tree congruence. Coalescent stochasticity plays a dominant role impelling phylogenetic discordance among gene trees, between genomes, and among these and the underlying species tree. It does not however account for all cases of gene tree–species tree incongruence.

An additional process contributing to phylogenetic discordance between genomes and with the underlying species tree is introgressive hybridisation. Initially this involves interspecific hybridisation and the exchange of alleles between species. Subsequent backcrossing of hybrid offspring with the parental species allows lineages of one species to introgress into the gene pool of the other. Mitochondrial introgression in particular has a considerable phylogenetic effect because all mitochondrial genes are inherited together owing to a lack of recombination in this genome. Introgression of the maternal species' mitochondrial genome against a nuclear background of the paternal species creates extreme intraspecific mitonuclear discordance because the mitochondrial lineages will always reflect their interspecific origin. Superficially, introgressive hybridisation is difficult to distinguish from incomplete lineage sorting as the two processes leave similar genetic signatures of polyphyly (McGuire *et al*., 2007; Ting *et al*., 2008; Degnan & Rosenberg, 2009; McKay & Zink 2010). In the case of introgression however, lineage divergence must post-date the speciation event and shared lineages will show some degree of localised geographic structure. This is unlike incomplete lineage sorting where shared lineages are likely to be randomly distributed among loci and geographically in the population (Funk & Omland, 2003; McGuire *et al*., 2007; Toews & Brelsford, 2012).

Hybrids between closely related species are not as rare as previously considered in animals (Sequeira *et al*., 2011) and mitonuclear discordance in distantly related non-sister species is being increasingly documented and attributed to introgressive hybridisation

events rather than to deep coalescence (Zha *et al*., 2008, Kronforst, 2008; Kubatko, 2009; Toon *et al*., 2012; Toews & Brelsford, 2012). Cases of hybridisation among freshwater turtles in particular are also growing (Georges *et al*., 2002; Spinks & Shaffer, 2007, 2009; Stuart & Parham, 2007; Alacs, 2008; Freedberg & Myers, 2012) and are especially noteworthy owing to deep divergence (~5 Myr) and morphological differentiation of the species involved (Freedberg & Myers, 2012). Introgressive hybridisation effectively sets lineage divergence time (as measured from current genetic diversity) between species back to zero, and if hybridisation is between sister species it will have the effect of shortening or eliminating the branch separating the pair but will otherwise not change species tree topology. If introgression occurs between non-sister taxa however, species tree topology will be paraphyletic, making hybridisation between distantly related taxa easier to distinguish from incomplete lineage sorting.

The inevitability of gene tree–species tree discordance should drive molecular phylogeneticists to actively uncover it. Gene tree–species tree discordance is not a hindrance to evolutionary inquiry, indeed the idiosyncratic genealogical realisations of different loci can be as informative as the species tree itself (Linnen, 2010).   Incongruence can highlight important evolutionary and genetic processes, and can inform the timing of significant species level events such as rapid radiations, population demographic change, and hybridisation. Studies that exclude the ability to detect gene tree–species tree discordance at the outset by not sampling multiple independent loci or multiple geographically dispersed individuals (*i.e.* potentially different lineages) risk inferring species monophyly by chance (Rosenberg, 2007), and making uninformed evolutionary inferences on the true branching history of speciation.

Thesis aims and objectives

The purpose of this thesis is to investigate the mitochondrial phylogeographic structure of the freshwater turtles *C. expansa* and *C. longicollis*. In doing so, I aim to advance knowledge on the biogeographic arena and evolutionary processes that shape east Australian freshwater systems and apply this knowledge to freshwater bioregionalisation.

An unexpected outcome of the phylogeographic investigation was the discovery of shared mitochondrial haplotypes between the two turtle species and extensive mitonuclear discordance. This directed a second aim to understand the nature and extent of discordance, and investigate the historical biogeographic processes behind potential mitochondrial introgression.

Specific objectives of this thesis are stated in the form of the following questions:

1. Does mitochondrial phylogeographic structure for *C. expansa* and other co-distributed freshwater taxa support the freshwater bioregions previously proposed for eastern Australia? Do east Australian freshwater bioregions need refining on the basis of this data? (Chapter 2).

2. What influence does a species-specific propensity for terrestriality have on the phylogeographic structure of *C. longicollis*? How does this information affect our understanding of east Australian freshwater bioregions? (Chapter 3).

3. What evolutionary processes have led to haplotype sharing between *C. expansa* and *C. longicollis*? Is there evidence of mitochondrial introgressive hybridisation and if

so how and when did it occur? (Chapter 4).

4. What past biogeographic conditions lead to the mitochondrial introgression described for *C. expansa* and *C. longicollis*? Was introgression driven by local mitochondrial adaption? Where did introgression occur? (Chapter 5).

In addressing the above questions I integrate across multiple disciplines including phylogeography, biogeography, molecular phylogenetics, and ecological niche modelling. This approach takes into account the broad domain of demographic, evolutionary, ecological, climatic, and landscape variables that have influenced the results I report. Multiple lines of evidence are essential to provide robust hypothesis generation and testing, and I believe a multidisciplinary approach contributes to improved understanding and management of contemporary evolutionary relationships and patterns of diversity.

The results of this thesis are presented in a series of data chapters each written as a stand-alone manuscript for publication. The results obtained on the phylogeographic structure of *C. expansa* (Chapter 2) and *C. longicollis* (Chapter 3) are combined and built on in Chapter 4 to direct investigations on mitochondrial haplotype sharing and the possibility of ancient mitochondrial introgressive hybridisation. Similarly, the results of Chapter 4 shape the questions and research agenda in Chapter 5 where I use ecological niche modelling to explore the climatic and ecological conditions that may have facilitated ancient introgression. Chapter 6 is my final synopsis where I show where I have delivered new knowledge and highlight research questions for future study.

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Chapter 2

Phylogeography of the Australian freshwater turtle *Chelodina expansa* reveals complex relationships among inland and coastal bioregions.

Abstract

We examined range-wide mitochondrial phylogeographic structure in the riverine freshwater turtle *Chelodina expansa* to determine if this species exhibits deep genetic divergence between coastal and inland hydrological provinces as seen in co-distributed freshwater taxa. We sequenced two mitochondrial loci, genealogical relationships were assessed using a network approach, and relationships among biogeographic regions were tested using analyses of molecular variance. Population history was evaluated using neutrality tests, indices of demographic expansion, and mismatch analyses. Twenty one haplotypes were recovered across two mitochondrial haplogroups separated by *ca* 4% nucleotide divergence. The haplogroups have discrete geographic boundaries but only partially support a hypothesis of deep divergence between coastal and inland bioregions. The first haplogroup comprises populations from the inland Murray-Darling Basin and from coastal catchments south of the Mary River in southeast Queensland. The second haplogroup comprises populations from coastal catchments north of the Mary River. Cryptic phylogeographic barriers separating adjacent coastal populations are congruent with those demonstrated for other freshwater taxa and may result from the combined influences of the Conondale Range and alluvial deposits at the mouth of the Mary River. Our study demonstrates that freshwater taxa commonly display genetic differentiation within a biogeographic region where no boundaries have been recognised, highlighting the need to uncover cryptic microbiogeographic regions to aid conservation of freshwater biota.

Introduction

Traditional biogeographic regions reflect hierarchical levels of biological, physical and ecological distinctness (Mackey *et al*., 2008) however they are at best imperfect summaries of biological and ecological diversity as not all taxa respond to biogeographic barriers in

the same way. Biogeographic regionalisation is well established in Australia for the terrestrial environment (Cracraft, 1991; IBRA, 2012) whereas identification of freshwater biogeographic regions has received very limited attention. Unmack (2001) detailed freshwater biogeographic provinces for fishes at the continental scale, and created a range of hypotheses to test using phylogenetic analysis. A freshwater bioregionalisation is urgently needed for the development of a comprehensive and representative system for the conservation of freshwater biodiversity. Comparative phylogeographic datasets of freshwater taxa have huge potential to contribute to the bioregionalisation of Australian freshwater systems as they capture the evolutionary processes that generate underlying patterns of biodiversity, and illuminate fine scale intraspecific diversity typical of freshwater systems. Freshwater taxa are excellent candidates for comparative phylogeographic inquiry as population structure is often complex owing to the dendritic and hierarchical nature of the freshwater habitat, and disparate freshwater species are likely to be equally affected by spatial and temporal hydrological connectivity. Further, the influences of climate and landscape are magnified in freshwater systems as these two factors dictate flow regime, meaning freshwater taxa can be more sensitive indicators of biogeographic patterns that may be undetectable using other ecological groups.

A growing number of studies have investigated phylogeographic patterns in Australian freshwater taxa that span two major freshwater biogeographic regions identified by Unmack (2001): the Murray-Darling Basin and the coastal Eastern Province (Figure 2.1). The Murray-Darling Basin is a large semi-arid river basin in eastern Australia characterised by very low elevational gradients. The Eastern Province includes the deeply dissected plains on the perimeter of the east Australian coast, and has been separated from the Murray-Darling Basin since the uplift of the Great Dividing Range approximately 90 Mya (Wellman, 1979). Deep phylogenetic divergence typify freshwater taxa that span

these provinces (Table 2.1; Figure 2.2) and allopatric speciation of Murray cod

(*Maccullochella peelii)*, Eastern freshwater cod (*Maccullochella ikei)*, and Mary River cod

(*Maccullochella mariensis*) is also attributed to the biogeographic distinctness of the

Murray-Darling Basin and the Eastern Province (Rowland, 1993).

Timing of phylogenetic divergence post-dates the uplift of the Great Dividing

Range, and Mio-Pliocene range expansion arising from temporary hydrological

connectivity between the Eastern Province and the inland Murray-Darling Basin is

generally used to explain the presence of divergent lineages in each biogeographic region.

Phylogenetic divergence has been described also at smaller spatial scales within the Eastern

Province (Table 2.1). For example, freshwater fishes and invertebrates in southeast

Queensland (SE Queensland), a region within the Eastern Province, show phylogeographic

breaks between the Brisbane and Mary catchments, between the Pine and Mary catchments,

and between north and south Fraser Island. Collectively, these comparative

phylogeographic studies demonstrate that freshwater taxa show concordance in their

recognition of freshwater biogeographic boundaries, and that they display significant

genetic differentiation at small geographic scales where no boundaries have been

recognised. Knowledge of which species are impeded by freshwater biogeographic

boundaries, and documentation of cryptic barriers to gene flow are important steps towards

conservation of imperilled freshwater ecosystems.
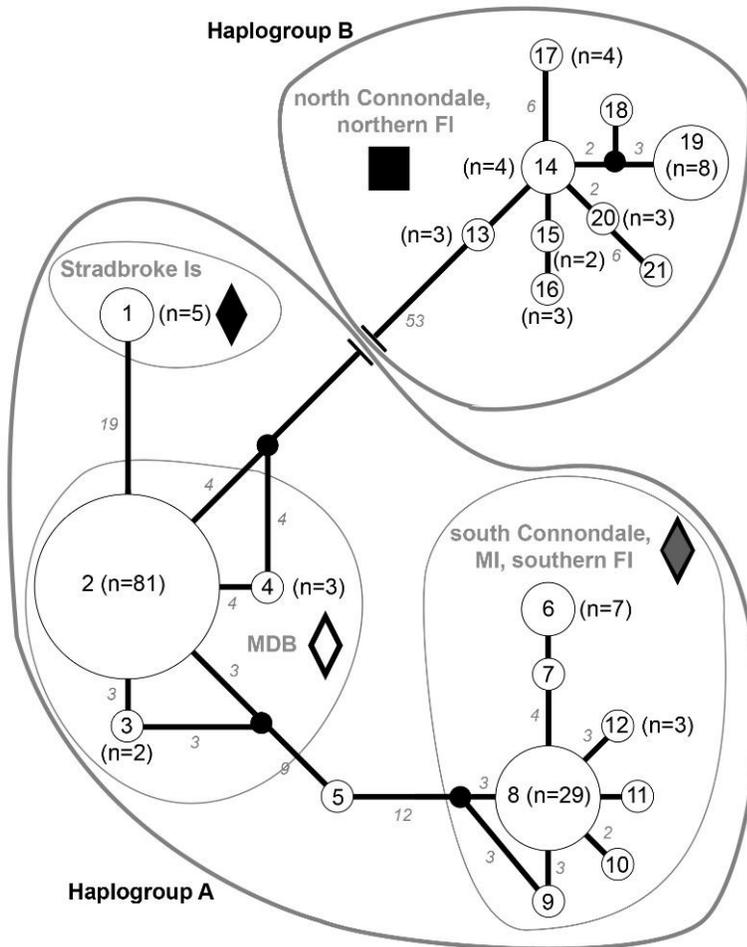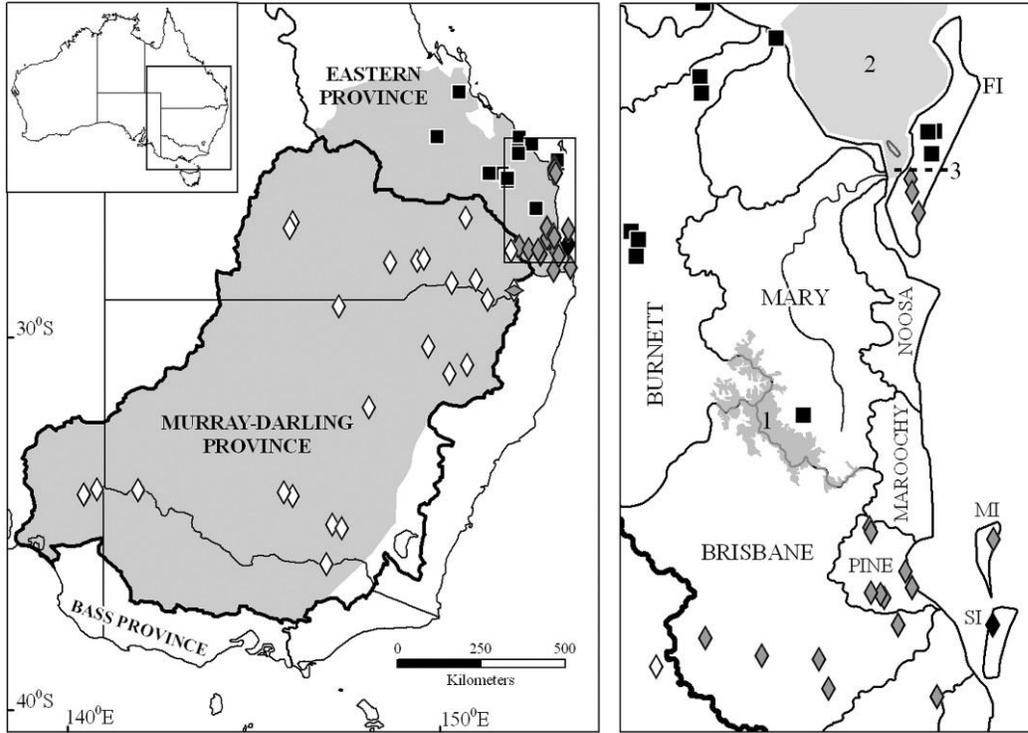
Figure 2.1 (previous page) Map of *C. expansa* collection localities and median-joining haplotype network. Diamonds indicate Haplogroup A and subgroups are delineated in the network: open diamonds represent Murray-Darling Basin haplotypes, grey diamonds represent South Conondale + MI + FI haplotypes, and the black diamond represents the Stradbroke Island (SI) haplotype. Black squares indicate haplogroup B which includes North Conondale + FI haplotypes. Shading in the left panel shows *C. expansa* distribution, light black lines are State boundaries, and heavy black lines delineate freshwater biogeographic provinces of Unmack (2001). Haplotype 5 is presented as a grey diamond in a horizontal alignment. Right panel illustrates the SE Queensland region of the Eastern Province and thin black lines delineate catchments and the Mary River. Shaded regions indicate the Conondale Range (1) and the Mary River palaeo coastal plain (2). Dashed line (3) highlights the Mary River ridge delineating north and south Fraser Island. Number of mutational steps (>1) in the haplotype network are indicated in grey, circle area is proportional to the number of individuals sharing a haplotype, haplotype number is given inside the circle, and the number of individuals (>1) is indicted in parentheses. Abbreviations: FI, Fraser Island; MI, Moreton Island; SI, Stradbroke Island.

Table 2.1 Summary of genetic divergences and diversity in a range of freshwater taxa sympatric with *C. expansa*. Species denoted with an asterisk (*) are compared to *C. expansa* in Figure 2.2.

Species	Divergence between MDB and EP	Divergence between Brisbane and Mary catchments	Divergence between Mary and Burnett catchments	Divergence between Mary and Pine catchments	Diversity within Burnett catchment	Diversity within Fitzroy-Dawson catchment.	Divergence between north and south Fraser Island	Reference
Broad-shelled turtle (*Chelodina expansa*)	●	●	●	●	●	●	●	This study
White-throated snapping turtle (*Elseya albagula*)	●	●	●	●	●	●	●	Todd *et al*., 2013
Australian freshwater prawn (*Macrobrachium australiense*)	●	●	●	●	●	●	●	Murphy & Austin, 2004*; Page & Hughes, 2014.
Australian smelt (*Retropinna semoni*)	●	●	●	●	●	●	●	Hammer *et al.,* 2007; Page & Hughes, 2014.
Hardyhead (*Craterocephalus stercusmuscarum fulvus*)	●	●	●	●	●	●	●	Unmack & Dowling, 2010*
Southern purple spotted gudgeon (*Mogurnda adspersa*)	●	●	●	●	●	●	●	Faulks *et al*., 2008*
Freshwater catfish (*Tandanus tandanus*)	●	●	●	●	●	●	●	Musyl & Keenan, 1996; Jerry, 2008; Page & Hughes, 2014.
Golden perch (*Macquaria ambigua*)	●	●	●	●	●	●	●	Musyl & Keenan, 1992; Faulks *et al.*, 2010*
Midgley's carp gudgeon (*Hypseleotris* sp.)		●	●	●	●	●	●	Thacker *et al*., 2007*; Page & Hughes, 2014.
Flathead gudgeons (*Philypnodon macrostomus* and *P. grandiceps*)		●	●	●	●	●	●	Thacker *et al*., 2008; Page & Hughes, 2014.
Freshwater crayfish (*Cherax dispar*)		●					●	Bentley *et al*., 2010

							Reference
Lungfish (*Neoceratodus forsteri*)	●	●	●	●	●	●	Frentiu *et al.*, 2001
Pacific blue-eye (*Pseudomugil signifer*)			●	●	●	●	Wong *et al*., 2004*; Page *et al*., 2012; Page & Hughes, 2014.
Ornate rainbowfish (*Rhadinocentrus ornatus*)			●	●	●	●	Page *et al.*, 2004
Honey blue-eye (*Pseudomugil mellis*)						●	Page *et al*., 2012
Freshwater shrimp (*Caridina indistincta*)	●		●			●	Page *et al*., 2012; Page & Hughes, 2014.
Crimson-spotted rainbowfish (*Melanotaenia duboulayi*)						●	Page *et al*., 2012
Carp gudgeon (*Hypseleotris galii*)						●	Page *et al*., 2012
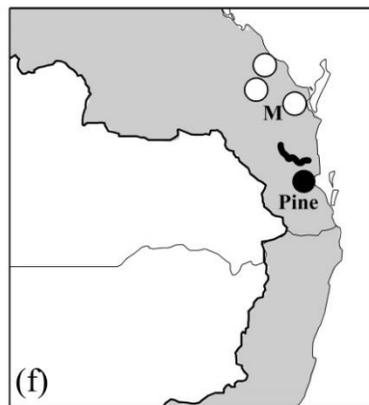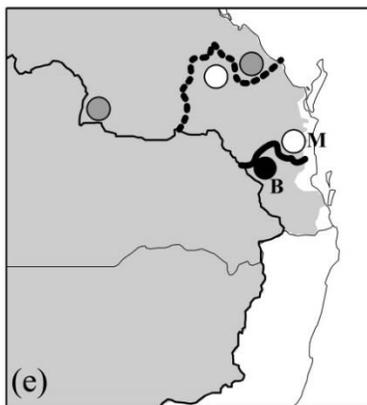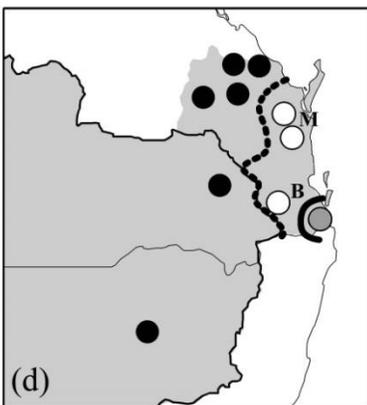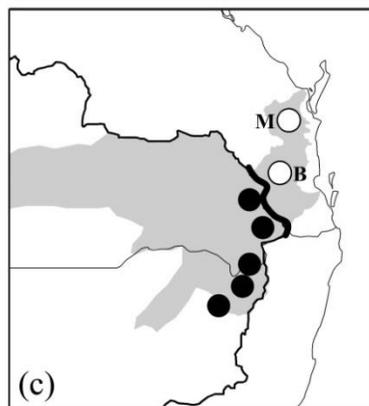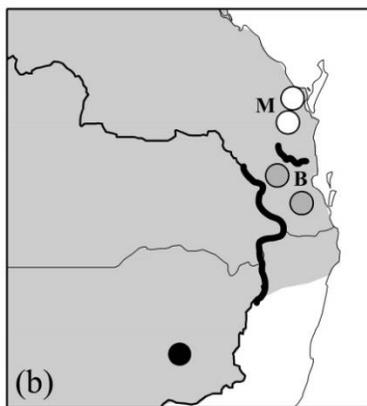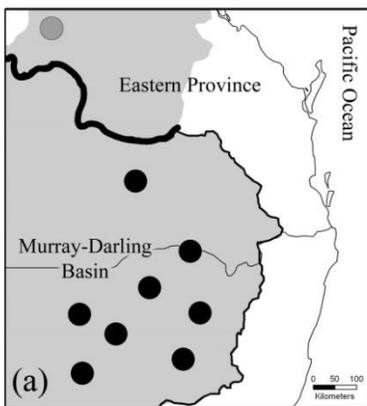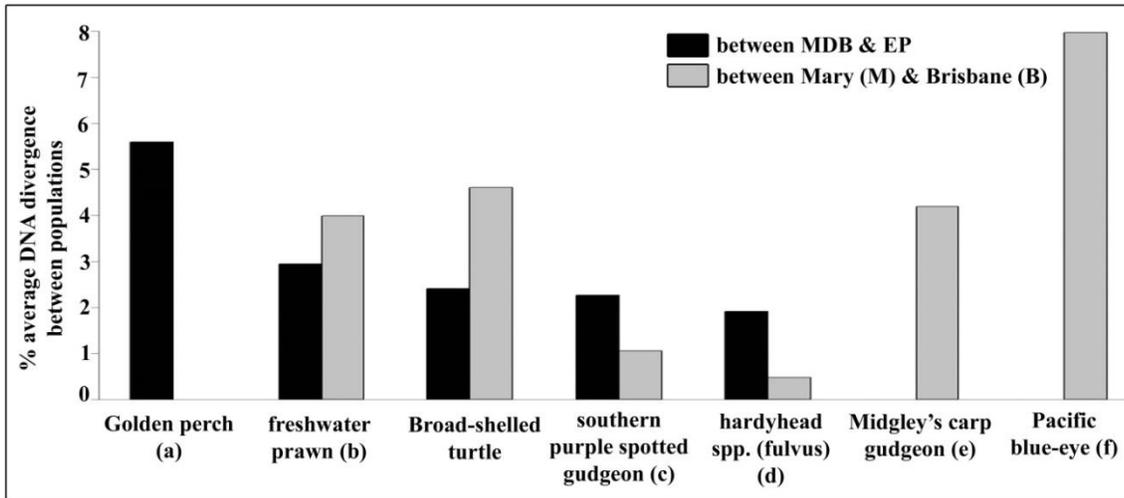
Figure 2.2 (previous page) Comparison of nucleotide divergences (*D*xy) and maps indicating the location of putative phylogeographical breaks for (a) Golden perch; (b) freshwater prawn; (c) southern purple spotted gudgeon; (d) hardyhead spp. (*fulvus*); (e) Midgleys carp gudgeon; and (f) Pacific blue-eye. Black bars indicate divergence between Murray-Darling Basin and Eastern Province populations, and grey bars indicate divergence between populations in the Brisbane and Mary catchments. Shaded regions in maps indicate species distribution, dots indicate sampling location and are coloured according to phylogenetic group, thick black lines represent phylogeographical breaks based on primary phylogenetic divergence, and dotted lines represent phylogeographical breaks based on secondary phylogenetic divergence. M, Mary catchment; B, Brisbane catchment. Note that sequences representing the grey dot (Nerang catchment) in map (d) were not included in Murray-Darling Basin/Eastern Province *D*xy calculations. Note also that sequence divergence for (f) Pacific blue-eye is only between the Mary and Pine catchments.

We investigate the mitochondrial phylogeographic structure of a wide-ranging freshwater turtle, *Chelodina expansa* Gray 1857 (Chelonia: Chelidae). Turtles present a unique and useful model for comparative phylogeography as they are tied intimately to the freshwater environment, although their differential capacity for terrestrial dispersal can highlight species-specific influences. *Chelodina expansa* occurs at low densities in riverine environments and connected lakes and billabongs in the inland Murray-Darling Basin and coastal SE Queensland (Bower *et al.*, 2012). Offshore populations occur on Fraser, Moreton and Stradbroke Islands. The range of *C. expansa* encompasses two major biogeographic regions defined by Unmack (2001) and mirrors that of other freshwater taxa that have been phylogeographically analysed at this continental scale and also at finer regional scales in SE Queensland. Unmack's (2001) biogeographic regions, and the results of previous studies create specific and testable hypotheses that we use as a basis for this study. We use mitochondrial nucleotide sequence variation and extensive sampling of *C. expansa* to test the following: (1) that Murray-Darling Basin and Eastern Province populations are highly differentiated; (2) that a phylogeographic break exists within SE Queensland populations; and (3) that a phylogeographic break exists between north and south Fraser Island. Our study demonstrates that, while freshwater taxa recognise regional scale biogeographic boundaries, they also display genetic differentiation where no boundaries have been recognised, highlighting the need to uncover cryptic microbiogeographic regions to aid conservation of freshwater biota.

Materials and Methods

Sampling

Tissue samples were obtained from 164 *Chelodina expansa* from across the geographic range of the species. Sixty three samples were collected between 2006 – 2009 using

snorkel-traps (modified crab traps), seine nets, or by hand capture with mask and fins. Skin tissues were obtained from the vestigial webbing on the hind foot and immediately placed in 95% ethanol. The remaining 101 samples (skin, muscle and blood) were sourced from the University of Canberra Wildlife Tissue Collection. Specimen details and collection localities are provided in Appendix 2A. Taxonomy follows that of Georges and Thomson (2010).

DNA extraction, fragment amplification and sequencing

Total genomic DNA was isolated using a standard salt extraction protocol (Sambrook & Russell, 2001) and quality was confirmed by gel electrophoresis. We targeted a 630 bp fragment of the mitochondrial *ND4* gene using primers ND4 (Arevalo *et al*., 1994) and ND4intR (Fielder *et al*., 2012) and a 470 bp fragment of the mitochondrial *control region* including part of *tRNA^{Proline}* (hereafter referred to as *control region*, *CR*) using primers TCR500 (Engstrom *et al*., 2002) and PS15841 (Engstrom *et al*., 2004). These fast evolving mitochondrial genes were targeted as studies at similar taxonomic levels in other turtle groups revealed they uncover informative intraspecific variation (Spinks & Schaffer, 2005). Annotation was based on *C. oblonga* (formerly *rugosa*) (Genbank # HQ172157; Wang *et al*., 2011).

Separate PCR amplifications for each fragment were conducted in 25 µl reactions containing 50-100 ng DNA, 1xPCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 pmol each of primer, 10 mg/ml BSA (New England Biolabs), 0.8 M Betaine, and 1 unit DNA polymerase (Bioline BioTaq Red). PCR temperature cycling for both fragments was 94°C for 120s, 30 cycles of 94°C for 30s, 51°C for 30s, 72°C for 45s, and a final elongation step at 72°C for 300s; PCR was performed using an Eppendorf Mastercycler 5333 (version 2.30). Amplicons were purified with polyethylene glycol (Sambrook & Russell, 2001) and

sequenced in both directions by Macrogen (Seoul, South Korea) using an ABI 3730XL

DNA automated sequencer. Sequences were edited, assembled, and consensus sequences

determined using Geneious Pro 5.3.4 (BioMatters Inc.). Sequences were aligned using

CLUSTALX 1.81 (Thompson *et al.*, 1997). The final alignment was 1,042 concatenated bp

comprising 595 bp *ND4*, 68 bp *tRNA^{Proline}* and 379 bp *CR* (GenBank accession numbers

KJ469917-KJ469958 for haplotypes. Data available from the Dryad Digital Repository:

doi:10.5061/dryad.5vd3q).

Mitochondrial authenticity

Our study relies entirely on mtDNA and thus incorporates the risk of inadvertently

amplifying nuclear-mitochondrial pseudogenes and making incorrect evolutionary

inferences. For this reason, we used four methods to investigate the mitochondrial origin

of the sequences examined: (1) Chromatograms were checked for instances of double

peaks and ambiguous bases which would indicate nuclear paralogue polymorphism – none

were found; (2) Coding regions were translated into amino acid sequence using the

standard vertebrate mitochondrial genetic code, and checked for premature stop codons

indicative of non-functional nuclear paralogues - none were found; (3) A partition

homogeneity test (PAUP* v.4.0b10; Swofford, 2002) did not detect incongruent signal

between *ND4* and *CR* sequences (p=0.11), indicating the two mitochondrial regions were

tracking the same evolutionary history; (4) Application of a mitochondrial enrichment and

serial dilution procedure. For the serial dilution, two samples of *C. expansa* (AA32871,

AA5283 – see [Appendix 2A](#)) were re-extracted and enriched for mtDNA using a Wako

mtDNA Extractor Kit (Wako Chemicals, USA). Two serial dilution procedures

(Donnellan *et al*., 1999) were performed: one on the pair of mtDNA enriched samples; and

one on six other *C. expansa* standard gDNA samples. DNA in each dilution was amplified

for the mitochondrial *12s* RNA fragment and the single copy nuclear intron *R35* (primers

provided in [Appendix 2B](#)). The *12s* fragment was chosen as it has been shown not to have

nuclear paralogues in the Chelodina (Seddon *et al*., 1997). *R35* was used as it is known as

a single copy and robust nuclear marker (Fujita *et al*., 2004). Mitochondrial fragments

amplified in serial dilutions beyond those at which nuclear DNA amplified in all samples.

These end-point dilutions were used as template to amplify *ND4* and *CR* fragments, and

resultant sequences were compared with those obtained directly from untreated and

undiluted template. In no cases did sequences obtained after serial dilution differ from

untreated template. Collectively, the above four results are consistent with the

amplification of genuine mitochondrial sequences.

Haplotypic relationships

To determine intraspecific relationships among haplotypes, a median-joining haplotype

network was constructed on concatenated *ND4* and *CR* sequences using Network 4.610

(Fluxus Technology Ltd) with epsilon value 0 and maximum parsimony optimisation. The

number of mutational steps between haplotypes were obtained using the 'statistics' option.

BEAST 2.0.2 (Bouckaert *et al*., 2013) was used to estimate timing of molecular

divergence between major haplogroups. Data were partitioned by domain (3 partitions:

ND4, *tRNA^{Proline}*, and *control region*), substitution models and clock parameters were

unlinked, and the best model of nucleotide substitution for each partition was determined

automatically using the add-on RB BEAST. We tested each domain for clocklike

evolution using likelihood ratio tests analyses in PAUP*. The null hypothesis of clocklike

evolution across all lineages could not be rejected for *ND4* or *tRNA^{Proline}*, but was rejected

for the *control region* ($P<0.05$). Accordingly, we estimated the *ND4* and *tRNA^{Proline}* under

a strict clock, and *control region* under a relaxed uncorrelated lognormal molecular clock.

To calibrate the molecular clocks we followed the approach of Rabosky *et al*. (2007) and used the mean of reptilian mtDNA divergence rates of 0.47% - 1.32% per million years (Zamudio & Green, 1997) scaled per lineage per million years and modelled under a normal distribution. This mean mitochondrial divergence rate of 0.895% per million years is consistent with a rate estimated from fossil chelid turtles by Georges *et al*. (2013) (0.86% per million years, but see discussion regarding Bluff Downs vs Redbank Plains fossils). Furthermore, when this rate is independently applied to a larger *Chelodina* and *Elseya* dataset (data not shown), the timing of the split between the two genera (37 Mya, 95% HPD: 29 – 46 Mya) is similar to that obtained by Near *et al*. (2005) (46.74 ± 5.49 Mya) using nuclear markers *R35*, *Rag-1* and mitochondrial *cytochrome b*. Corroborative rate and date comparisons provide good evidence that our chosen mitochondrial divergence rate is appropriate. MCMC chains were run for 70 million generations with sampling every 7,000 steps yielding a total of 10,000 trees. Convergence and burn-in were assessed in TRACER v1.5 (Rambaut & Drummond, 2007), and chronophylogenies were visualised in FIGTREE 1.3.1 (Rambaut, 2009). We used TREEANNOTATOR 2.0.2 to calculate maximum clade credibility for each tree, and applied a burnin of 1000.

Phylogeographic analyses

We assessed phylogeographic patterns of mitochondrial haplotypes using two approaches: (1) analyses of molecular variance (AMOVA) to test the significance of haplotype variation within and between predefined continental scale biogeographic regions, and finer scale geographic regions in SE Queensland; and (2) spatial analyses of molecular variance (SAMOVA) to identify maximally differentiated populations without using biogeographic regions defined *a priori*. AMOVA using 1,000 bootstrap replicates were calculated in ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Regions include the Murray-Darling Basin

and the SE Queensland region of the Eastern Province excluding offshore islands. Within

SE Queensland, two secondary regions were identified based on separation by the

Conondale Range which divides the Mary and Brisbane catchments and is broadly

consistent with phylogeographic breaks reported in co-distributed freshwater fishes and

invertebrates. These two areas are named North Conondale and South Conondale. The

North Conondale region includes the Mary, Burnett, Baffle, and Fitzroy-Dawson

catchments. The South Conondale region includes the Brisbane, Pine, Logan-Albert, and

South Coast catchments. The following hierarchical partitions were used for the AMOVA:

(1) among all catchments; (2) among all catchments in the Murray-Darling Basin; (3)

among all catchments in SE Queensland; (4) between the Murray-Darling Basin and SE

Queensland; (5) between North Conondale and South Conondale; (6) among all catchments

in North Conondale; and (7) among all catchments in South Conondale. SAMOVA using

10,000 iterations were calculated in SAMOVA 1.0 (Dupanloup *et al*., 2002) across 61

discrete *C. expansa* populations. We explored values of K (number of population groups)

from 2 to 12 each with 200 simulated annealing processes (conditions). We selected a

value of K which corresponded to the point where among group genetic variation (FCT)

reached a plateau.

Molecular diversity indices including the number of segregating sites (S),

haplotype diversity (hd), nucleotide diversity (π), average number of nucleotide differences

(k), and an index of nucleotide divergence (D_{xy}: the average number of nucleotide

substitutions per site between haplogroups) were calculated for each major haplogroup in

DNASP 5.10.01 (Librado & Rozas, 2009). Nucleotide divergences (D_{xy}) were also

calculated from GenBank deposited sequences for a range of sympatric freshwater taxa to

provide context for *C. expansa* divergences described in this study (taxa identified in Table

2.1). Tajima's (1989) D statistic was calculated to test if haplogroups conformed to or

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departed from models of neutral evolution owing to population expansion. Fs (Fu, 1997) and R2 (Ramos-Onsin and Rozas, 2002) were used to test for demographic stability. All tests were performed in DNASP with 10,000 simulations via the coalescent to obtain 95% confidence intervals. To test further for conformation to a model of sudden demographic expansion, mismatch analyses using 2,000 bootstrap replicates were performed, and the raggedness statistic (Rg of Harpending, 1994) was calculated in ARLEQUIN. Time since expansion was calculated using the Tau values (τ) provided by the mismatch analysis and the equation $t = \tau 2\mu$ (Rogers & Harpending, 1992; Schneider & Excoffier, 1999), where μ is the mutation rate per generation per gene. To calculate μ we applied the same divergence rate for mtDNA as per our BEAST analysis. The rate per site per year was converted to a rate per generation (G) with generation time 23 years, using the equation $G = a + (s/(1-s))$ where a is the age of maturity and s is the expected adult survival rate. We used $a = 11.5$ as the average of the earliest age of maturity for *C. expansa* males (9 years) and females (14 years) (Spencer, 2002) and adult annual survival rate of $s = 0.921$ (Spencer & Thompson, 2005).

Results

Phylogeographic relationships

Twenty one haplotypes were recovered from concatenated *ND4* and *CR* mitochondrial sequences from 164 *C. expansa* (haplotype frequencies for each catchment are provided in Appendix 2C). Haplotypes clustered into two major haplogroups and had allopatric distributions (Figure 2.1). The two major haplogroups, hereafter referred to as Haplogroup A and Haplogroup B were differentiated by 53 mutational steps. Haplogroup A comprised three distinct sub-groups with geographic distributions that corresponded exclusively to (1) Stradbroke Island, (2) the Murray-Darling Basin, and (3) the South Conondale region

including Moreton Island and Southern Fraser Island (hereafter referred to as the South Conondale + MI + FI region). Haplotype 5 was an exception to this result as it was found in the Murray-Darling Basin, while positioned intermediately in the network between the Murray-Darling Basin haplotypes and South Conondale + MI + FI haplotypes. The Murray-Darling Basin had very low diversity with only four haplotypes (2-5), and a single widespread haplotype (Haplotype 2) accounting for 92% of all sequences recovered. The South Conondale + MI + FI region was moderately diverse with seven haplotypes (6-12), and was characterised by a star-like structure with the most common haplotype (Haplotype 8) being widespread among catchments in this region. Stradbroke Island was monomorphic (Haplotype 1) and clustered with the Murray-Darling Basin population, although separated by 19 mutational steps. Haplogroup B had a geographic distribution exclusive to the North Conondale region and northern Fraser Island (hereafter referred to as the North Conondale + FI region). This region was highly diverse with nine haplotypes (13-21). These displayed elements of a star-like structure, though a large number of mutational steps (up to 6) separated some terminal haplotypes. Excepting Haplotype 5, and the haplotypes from Fraser Island that occurred in both major haplogroups, divisions in the network correlate to distinct and non-overlapping geographic regions.

Uncorrected nucleotide divergence (D_{xy} = average number of nucleotide substitutions per site between populations) between haplogroups A and B was 4.29%, corresponding to 34 fixed differences (nucleotide sites where all the sequences in the first haplogroup differ from all the sequences in the second haplogroup). Within haplogroup A, there are 11 fixed differences between the Murray-Darling Basin group and the South Conondale + MI + FI group, corresponding to a divergence of 1.19%. The number of fixed differences and average nucleotide divergences between Stradbroke Island Haplotype 1 and the Murray-Darling Basin group were 9 and 0.87% respectively. Haplotypes from north

(Haplogroup B) and south (Haplogroup A) of Fraser Island exhibited deep nucleotide divergence of 4.54%, and 47 fixed differences. Dating analysis determined that divergence between Haplogroup A and B occurred approximately 4.2 Mya (2.8 – 5.7 Mya 95% HPD) in the early Pliocene. Within Haplogroup A divergences occurred in the early Pleistocene with the Murray-Darling Basin haplotypes and Stradbroke Island haplotype diverging approximately 1.4 Mya (0.7 – 2.1 Mya 95% HPD) followed by the Murray-Darling Basin haplotypes and the South Conondale + MI + FI haplotypes diverging approximately 1.1 Mya (0.6 – 1.7 Mya 95% HPD).

Nucleotide divergence between the Murray-Darling Basin haplotypes and all haplotypes from the Eastern Province was 2.41%. Twice this divergence was found between the two geographically proximate populations on the coast (South Conondale and North Conondale) within the Eastern Province. These populations each belonged to different haplogroups and were distinguished by 40 fixed differences and deep nucleotide divergence of 4.61%. This pattern is mirrored in the freshwater prawn (*Macrobrachium australiense*) where nucleotide divergence between the Murray-Darling Basin and Eastern Province haplotypes is 2.94%, and nucleotide divergence between the Brisbane and Mary drainages (equivalent to South Conondale and North Conondale respectively) is greater at 3.99% (Figure 2.2).

The inverse pattern is found in a subspecies of flyspeckled hardyhead (*Craterocephalus stercusmuscarum fulvus*) and southern purple spotted gudgeon (*Mogurnda adspersa*) where divergence between the Murray-Darling Basin and the Eastern Province haplotypes is 1.91% and 2.27% respectively, and divergence between the Brisbane and Mary drainages is smaller at 0.48% and 1.05% respectively. For further context, golden perch (*Macquaria ambigua*) exhibits 5.59% nucleotide divergence between Murray-Darling Basin and Eastern Province (Fitzroy drainage) haplotypes. Nucleotide

divergence between haplotypes of Midgley's carp gudgeon (Thacker *et al*., 2007) in the Brisbane and Mary drainages is roughly equivalent to *C. expansa* and the freshwater prawn at 4.19%, and Pacific blue-eye (*Pseudomugil signifer*) exhibits very deep divergence of 7.98% between these two proximate coastal bioregions.

Phylogeographic subdivision

Our AMOVA indicated significant genetic structure among all catchments encompassing the range of *C. expansa* ($\theta_{ST} = 0.601$, P<0.001). Among-catchments AMOVA within the Murray-Darling Basin region revealed no differentiation ($\theta_{ST} = 0.082$, P=0.118). There was significant high differentiation between the Murray-Darling Basin and SE Queensland regions ($\theta_{CT} = 0.528$, P<0.001), and among all catchments in SE Queensland ($\theta_{ST} = 0.426$, P<0.001). The primary source of variation in SE Queensland was attributed to significant differentiation between South Conondale and North Conondale haplotypes ($\theta_{CT} = 0.253$, P<0.05), and among North Conondale catchments ($\theta_{ST} = 0.468$, P<0.001). No substructure was observed among South Conondale catchments ($\theta_{ST} = 0.033$, P=0.262). AMOVA could not be reliably calculated for Fraser Island owing to low sample sizes. SAMOVA distinguished the same broad geographic groups we had recognised *a priori* based on previous biogeographic and phylogeographic studies. The best partitioning of genetic diversity was obtained with two (FCT = 0.878) to seven (FCT = 0.979) population groups. Past this point the value of K plateaued considerably. When K = 2, SAMOVA recovered the deep mitochondrial phylogeographic split between the combined Murray-Darling Basin and South Conondale haplotypes, and the North Conondale haplotypes. At K= 3 Murray-Darling Basin haplotypes and the South Conondale haplotypes were separated, with the Stradbroke haplotypes and the intermediate Haplotype 5 grouped with the Murray-Darling Basin. At K= 4 the Stradbroke haplotypes were distinguished from the Murray-Darling

Basin, and Haplotype 5 remained clustered with the Murray-Darling Basin up to K=9. For values of K from 5 to 7 the North Conondale group became increasingly differentiated: first with recognition of a group containing all individuals from northern Fraser Island and the Mary catchment (Haplotype 19), and followed by a group representing individuals from the Fitzroy-Dawson drainage (Haplotype 17). Finally, individuals in the Burnett catchment were separated into two groups each representing a population on the lowland coastal plateau, and a population occurring higher in tributaries of the Burnett.

Tests for demographic expansion

Haplotype diversity in the Murray-Darling Basin was very low (Hd = 0.112), consistent with no differentiation in AMOVA and SAMOVA. Haplotype diversity for South Conondale + MI + FI was moderate (Hd = 0.524), driven by diversity on the islands rather than in the South Conondale region alone. Haplogroup B exhibited the highest diversity (Hd = 0.877), and Stradbroke Island had the lowest (monomorphic). Estimates of Tajima D and Fs were significantly negative (Table 2.2) for Murray-Darling Basin haplotypes (D = -1.13, $p<0.02$; Fs = -2.15, $p< 0.05$). Negative values of Tajima D indicate a departure from neutrality and suggest population growth, or a selective sweep. Negative values of Fs indicate an excess of low frequency younger variants which is expected under a model of population expansion. The same indices were not significant for South Conondale + MI + FI haplotypes (D = -0.99; Fs = -2.72) and North Conondale + FI haplotypes (D = 0.29; Fs = -0.4), signalling selective neutrality and conformation to a model of population size stability in these Eastern Province groups. R2 statistics are not significant for any network group examined, though the tendency towards small positive values for Murray-Darling Basin haplotypes (R2 = 0.029) indicate an excess of low frequency variants consistent with a signal of population expansion.

Mismatch distributions (Figure 2.3) and Harpending's raggedness statistics (Rg) contradicted inferences of population size stability (D, Fs, R2) for the South Conondale + MI + FI group and the North Conondale + FI group. Each group fits the expected mismatch distribution under a model of sudden demographic expansion, and this inference was especially strong for the South Conondale + MI + FI group owing to a unimodal distribution similar to that of the Murray-Darling Basin group. Observed mismatch means for Murray-Darling Basin, South Conondale + MI + FI, and North Conondale + FI haplotypes were 0.114, 0.862, and 3.32 respectively. Non-significant Rg values meant that the null hypothesis of population expansion could not be rejected in any case. The shape of the North Conondale + FI distribution was multimodal and right shifted, reflecting its high mismatch mean. Notwithstanding discrepancy of the mismatch results with other indices, an approximate estimate of time since expansion was calculated for each major network group (excluding Stradbroke Island on account of monomorphism) .

Table 2.2 Molecular diversity indices: number of sequences (n), number of haplotypes (h), number of segregating sites (S), haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide differences (k). Tests for population stability: Tajima's D (1989), Fu's Fs (1997), Ramos-Onsin and Rozas's R2 (2002), and Harpending's (1994) raggedness index (Rg). Time since expansion (Kya) is based on Tau (τ) values provided by mismatch analyses, brackets indicate upper and lower confidence interval of Tau at alpha = 0.1. Details for Stradbroke Island are not given owing to monomorphism, and Haplotype 5 is excluded owing to its intermediate position in the network. * _p_<0.05; ** _p_<0.02.

Haplotype group	n	h	S	Hd ±SD	π	k	D (95% CI)	Fs (95% CI)	R2 (95% CI)	Rg	τ	time since expansion
MDB	86	3	2	0.112 ± 0.046	0.00011	0.114	- 1.13** (-1.04 - 1.74)	9.3095	0.029 (0.023 - 0.244)	0.615	3	14 (2.5-14)
South Conondale + MI + FI	43	7	6	0.524 ± 0.083	0.00015	0.861	-0.99 (-1.55 - 1.98)	-2.72 (-3.17 - 4.08)	0.074 (0.050 - 0.232)	0.101	1.97	9 (0 - 18)
North Conondale + FI	29	9	12	0.877 ± 0.034	0.00319	3.325	0.29 (-1.73 - 1.86)	-0.4 (-4.36 - 5.52)	0.136 (0.032 - 0.155)	0.049	4.54	21 (9.6-29)
all	164	21	64	0.723 ± 0.033	0.01681	17.479	1.49 (-1.56 - 1.90)	12.62 (-13.92 - 14.00)	0.136 (0.044 - 0.140)	0.118**	-	-
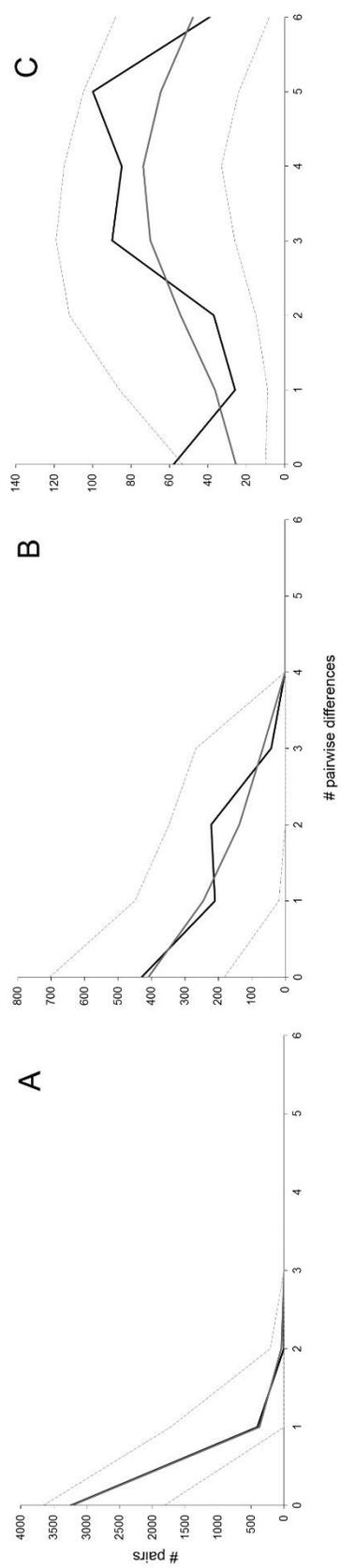
Figure 2.3 Mismatch distributions under the sudden expansion model for (A) the Murray-Darling Basin population, (B) the South Conondale + MI + FI population, and (C) the North Conondale + FI population. Black lines indicate the observed distribution, grey lines indicate the expected distribution under the sudden expansion model, and dashed grey lines indicate the upper and lower confidence interval based on alpha = 0.1.

The Murray-Darling Basin group expanded approximately 14 Kya, and the South

Conondale + MI + FI and North Conondale + FI groups expanded approximately 9 Kya and

21 Kya respectively.

Discussion

Our principle aim is to describe the phylogeographic structure of *C. expansa* to assess

relationships among freshwater bioregions. The high variability and rapid coalescence time

of mitochondrial DNA has particular utility for this aim, and we recognise that the

matrilineal history we present is only indicative of the demographic history of *C. expansa*

more broadly. We do not make specific inferences regarding *C. expansa* population

genetics; rather we highlight mitochondrial connections and disjunctions across the

freshwater landscape, and the presence and origins of independently evolving haplogroups.

Relationships between the Murray-Darling Basin and Eastern Province

We hypothesised that variation between *C. expansa* in the Murray-Darling Basin and

Eastern Province would follow a pattern similar to sympatric freshwater taxa and be highly

differentiated owing to the Great Dividing Range which delimits these two bioregions.

Indeed, mitochondrial phylogeographic relationships, divergence indices, and analyses of

molecular variance all clearly differentiate mitochondrial sequences from the Murray-

Darling Basin and Eastern Province. The distinction between freshwater fauna in these

bioregions has long been recognised, however our study illustrates that the mountain range

separating them is equivocal in its influence.

　　　Chelodina expansa haplotypes in the Murray-Darling Basin are more closely related

to those from the coastal South Conondale region in the Eastern Province than the two

Eastern Province regions are related to each other. A similar pattern is found in the

freshwater prawn (*Macrobrachium australiense*) where divergence between the two Eastern Province regions is greater than divergence between the Eastern Province and the Murray-Darling Basin. Overall, we can confirm the broad mitochondrial phylogeographic distinction between populations of *C. expansa* in the Murray-Darling Basin and Eastern Province, however the details of this distinction are more complex than previously thought.

Origins and expansion of the Murray-Darling Basin population

Divergence of *C. expansa* Murray-Darling Basin and South Conondale + MI + FI haplotypes occurred approximately 1.1 Mya in the early Pleistocene, and we propose the present Murray-Darling Basin haplotypes were established at this time by colonisers from the coast. It is unknown if *C. expansa* inhabited the Murray-Darling Basin prior to 1.1 Mya as signatures of earlier invasions may have been lost. Colonisation of the Murray-Darling Basin from ancestral populations in the Eastern Province has been demonstrated in other freshwater associated taxa (sedge frog, James & Moritz, 2000; gudgeon, Faulks *et al.*, 2008; catfish, Jerry, 2008) and invasion of the Murray-Darling Basin by coastal *C. expansa* is suggested to explain why populations in the Murray-Darling Basin have retained a tropical reproductive biology despite their range extending into the temperate zone as far south as the Lower Murray drainage (Legler & Georges, 1993).

Freshwater turtles are not restricted to the aquatic environment, and although *C. expansa* is not considered highly vagile on land (Cann, 1998), it does move considerable distances to nest, and could presumably cross low relief drainage divides, especially during the wetter early Pleistocene interglacials. A high escarpment on the Great Dividing Range separates the South Conondale population from the Murray-Darling Basin and offers no opportunity for dispersal. We suggest the present South Conondale distribution of *C. expansa* is a northern remnant of a southerly-distributed extinct population that colonised

the Murray-Darling Basin in the Pleistocene. Low relief drainage divides such as geocols

(regions of broad, low relief in otherwise topologically complex landscapes) at the

headwaters of the Clarence and Hunter Rivers in the Eastern Province have assisted gene

flow across the Great Dividing Range in multiple species of fishes, either by drainage

rearrangement or direct dispersal (Unmack, 2001; McGlashan and Hughes, 2001; Thacker

et al., 2007; Faulks *et al.*, 2008), and could have facilitated ancient migration from the

Eastern Province to the Murray-Darling Basin in *C. expansa*. This hypothesis is supported

by Haplotype 5 which is found in the eastern Murray-Darling Basin yet is positioned

between the Murray-Darling Basin and the South Conondale + MI + FI network groups.

The single individual representing this haplotype may represent a low frequency ancient

polymorphism retained from the early Pleistocene when the Murray-Darling Basin was

colonised from now-extinct populations on the coast.

    After the last glacial maximum, mismatch distribution suggests the *C. expansa*

Murray-Darling Basin haplogroup rapidly expanded *in situ* approximately 14 Kya. This

may have been facilitated by a range of hydrological and climatic factors, for example,

climate warming coincident with the final stages of alpine deglaciation (complete by

15,800 years ago, Barrows *et al.*, 2001; Gingele *et al.*, 2007), and a fluvial pulse in southern

Australia after deglaciation (13,500 to 11,500 years ago) (Gingele *et al.*, 2007). Extensive

lateral channel migration (Page & Nanson, 1996) may have further facilitated expansion

and assisted in rapid and widespread dispersal of the few haplotypes present in the Murray-

Darling Basin today. Recent population expansion in the Murray-Darling Basin has been

suggested for a range of other freshwater species such as fishes and crustaceans (Austin *et

al.*, 2003; Nguyen *et al.*, 2004; Hughes & Hillier, 2006; Hammer *et al.*, 2007; Faulks *et al.*,

2008). In these taxa, expansion from multiple Pleistocene refugia is inferred from

significant genetic structure. Thus in the Murray-Darling Basin, there is no evidence that

the most recent expansion of *C. expansa* 14Kya originated from multiple refugia as very low haplotype diversity seen in this species is more consistent with a genetic bottleneck caused by survival in a single refugium.

Stability or expansion of the South Conondale population?

The South Conondale + MI + FI group yields signals of both mitochondrial population stability and demographic expansion. Neither AMOVA nor SAMOVA detect any intra-group divergence indicative of *in situ* diversification, and the landscape history of the region is amenable to an inferred population expansion approximately 9 Kya. Low sea levels and a dry phase on the coast in the early Holocene exposed the continental shelf in the region until 6 Kya (Donders *et al.*, 2006), promoting high hydrological connectivity and the opportunity for demographic expansion. Such palaeoecological and climatic conditions may have been typical in the region up to 6 Kya, and low genetic diversity and a lack of phylogeographic subdivision in the South Conondale + MI + FI group may have characterised populations prior to 9 Kya. Alternatively, high hydrological connectivity around 9 Kya may have acted to erase prior signals of phylogeographic subdivision if they had occurred, and removed genetic diversity through introgressive replacement. Both scenarios are consistent with the data and we cannot speculate on the mitochondrial phylogeographic structure that may have existed in the region prior to 9 Kya.

Diversification of North Conondale populations

Evidence of long-term demographic stability and mitochondrial diversification is strongest for the North Conondale + FI group. Significant molecular differentiation (AMOVA and SAMOVA) among and within catchments in the North Conondale region suggest the presence of population isolates in this region. Similar genetic substructure has been

described in this region in other freshwater taxa (Table 2.1). Subdivision is possible owing to the deeply dissected mountainous landscape of the North Conondale region which presents a mosaic of freshwater isolates. Population stability and differentiation is not immediately apparent in the mismatch analysis which appears inconsistent with this result. The shape of the mismatch distribution however does not characterise a recent and rapid expansion. The mismatch distribution is right shifted, providing a signal of ancient demographic expansion approximately 21 Kya, possibly at the height of the last glacial cycle. Further, the multimodal and ragged distribution suggests either: (1) the existence of population subdivision in the region with stable population sizes (Harpending *et al*., 1998), or (2) a series of temporally separated expansion episodes resulting from intermittent connectivity among population isolates (Rogers & Harpending, 1992; Ray *et al*., 2003; Bos *et al.*, 2008). Significant molecular differentiation (SAMOVA) among *C. expansa* in the North Conondale region suggests the presence of population isolates in this region, and genetic substructure has also been described in other freshwater taxa between the Mary and Burnett catchments, and within the Fitzroy-Dawson catchment (Table 2.1). In support of the second mismatch interpretation; we propose that mitochondrial sequences of *C. expansa* populations diversified in landscape isolates of the North Conondale region, and that intermittent gene flow and demographic growth occurred during glacial periods with exposure of the broad continental shelf and associated coalescence of lower reaches of the Mary and Burnett, and Baffle and Fitzroy-Dawson rivers (Unmack, 2001; Thacker *et al*., 2008).

Phylogeographic barriers in Southeastern Queensland

Phylogeographic breaks of varying extent and age have been documented in freshwater fishes and invertebrates in SE Queensland between the Brisbane and Mary catchments and

the Pine and Mary catchments (Page & Hughes, 2014; Table 2.1). The Conondale Range

delineates the Brisbane and Mary catchments, and based on the above studies we used this

obvious geographic feature to define *C. expansa* mainland populations *a priori*. *Chelodina*

expansa exhibited deep nucleotide divergence between North Conondale and South

Conondale regions, and SAMOVA partitioning indicated that the largest genetic break in *C.*

expansa occurred between these two populations, thus confirming the biogeographic

significance of the Brisbane/Mary drainage divide. This divide may also influence the

distribution of subspecies in another freshwater turtle, *Emydura macquarii*. The range of *E.*

m. macquarii is similar to *C. expansa* haplogroup A and extends throughout the Murray-

Darling Basin into the Eastern Province south from the Brisbane catchment. *Emydura*

macquarii krefftii broadly follows the range of *C. expansa* haplogroup B in the Eastern

Province north from the Mary catchment. The subspecies are distinguished by the presence

(*E. m. krefftii*) or absence (*E. m. macquarii*) of a prominent yellow post-ocular stripe

(Georges & Thomson, 2010) and contact of morphological forms may be restricted by the

Brisbane/Mary drainage divide. Deep haplotypic divergence in *C. expansa*,

phylogeographic breaks in freshwater fishes and invertebrates, plus the presence of

different subspecies of *Emydura macquarii* across the Brisbane/Mary drainage divide

highlight the Conondale Range as an important phylogeographic barrier in freshwater taxa.

The influence of the Conondale Range does not extend all the way to the coast

however. For example, *C. expansa* haplotypes in the South Conondale region are also

found on the southern extent of FI. Similar patterns are observed in Oxleyan pygmy perch

(*Nannoperca oxleyana*) (Hughes *et al.*, 1999), ornate rainbowfish (*Rhadinocentrus ornatus*)

(Page *et al.*, 2004), and freshwater crayfish (*Cherax dispar*) (Bentley *et al.*, 2010). These

species display close relationships among populations in the South Conondale region and

those in the low lying Noosa and Maroochy catchments, despite these latter populations

occurring in the North Conondale region. These complex relationships suggest an additional cryptic phylogeographic barrier, and Hughes *et al.* (1999) propose that a ridge at the mouth of the Mary River may influence the genetic structure of freshwater taxa in the region. High energy erosional processes in the upper Mary catchment contributed to large amounts of sediment and alluvial deposits on the Mary River coastal plain (Bridges *et al.*, 1990; Feng & Vasconcelos, 2007) when this region was exposed during low sea levels in the Plio/Pleistocene (Grimes, 1992). We propose that a depositional zone existed near the mouth of the present-day Mary River where the ancient channel abruptly turned northward (Grimes, 1992), and that this depositional zone is synonymous with the ridge proposed by Hughes *et al.* (1999). We hypothesise that the Mary River ridge divides freshwater populations directly north and south of the present-day river mouth.

Chelodina expansa haplotypes on Fraser Island are distributed across two major haplogroups with non-overlapping distribution of the south (haplogroup A) and north (haplogroup B) mitochondrial sequences. Nucleotide divergence is approximately 4.5% which is considered 'deep' (>4%, Page *et al.,* 2012), and represents the second largest mitochondrial nucleotide divergence reported for the island, only surpassed by the freshwater yabby *Cherax dispar* (8.4%). The mitochondrial phylogeographic break described in *C. expansa* is concordant with a range of freshwater fishes and invertebrates (Page *et al.,* 2012) and aligns with the present-day mouth of the Mary River and its associated Plio/Pleistocene depositional ridge. The times of divergence in *C. expansa* and other taxa pre-date the age of the island itself (Page *et al.,* 2012) and imply that the Mary River ridge influenced the distribution of freshwater taxa before the Fraser Island landmass was established in the late Pleistocene (Tejan-Kella *et al.*, 1990). The most recent colonisation of *C. expansa* on Fraser Island may have been as simple as the process outlined Bentley *et al.* (2010) whereby southern Fraser Island was colonised by populations

expanding from the south (*i.e. ex situ* refugia in the South Conondale region), and northern

Fraser Island was colonised by populations expanding from the north (*i.e. ex situ* refugia in

the Mary and Burnett drainages). However, indices of long term demographic stability for

the haplotypes of the populations of *C. expansa* from northern Fraser Island suggest an

alternate process. Instead of establishing via expansion from the north, northern Fraser

Island populations may have persisted in much the same location as they are today, while

late Pleistocene wind action and dune building (Petherick *et al.*, 2008) caused Fraser Island

to form around them.

Systematic and management implications

The divergent mitochondrial haplogroups that we uncovered are evidence of discrete

regional populations of *C. expansa* each with independent evolutionary trajectories. Cann

(1998) noted that population east and west of the Great Dividing Range show

morphological variation and that populations from Fraser Island are morphologically

distinctive. We do not regard taxonomic recognition of these groups necessary as we

demonstrate that morphological variation is not indicative of evolutionary divergence.

Strongly concordant phylogeographic patterns across a range of freshwater taxa

differentiate populations in the Murray-Darling Basin and Eastern Province, and within SE

Queensland and Fraser Island. This concordance illustrates broad-scale biogeographic

processes that reach beyond taxonomy, life history, and ecology to shape similar patterns of

colonisation, expansion, and diversification. Shared phylogeographic breaks across

disparate taxa suggest that the regions we identified may harbour evolutionary significant

units (Moritz, 1994) that could serve as new biogeographic and 'microbiogeographic'

provinces for freshwater taxa. More data from highly polymorphic genetic markers may be

used to test these conclusions and could help refine management units to direct bioregional

conservation strategies. Significant genetic diversity at the finest of spatial scales is likely to be widespread in freshwater taxa and we propose that a comparative phylogeographic approach combined with high resolution sampling across the distribution of species is essential to understanding the biogeography and conservation of imperilled freshwater systems.

Acknowledgements

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Supporting information

Archived data

Data deposited at Dryad (Hodges, Donnellan & Georges, 2014).

Appendix 2A – Specimens examined

Data for *Chelodina expansa* are given by major catchment (in bold), local drainage, number

of specimens collected (in square parentheses), latitude and longitude and specimen

number(s) (Wildlife Tissue Collection, University of Canberra, UC<Aus> in GenBank). *

denotes samples used in the mitochondrial enrichment and serial dilution procedure.

<u>Southeast Queensland sub-province:</u> **Baffle catchment:** Littabella creek [1] (24.6358 S

152.106 E) J83694; **Brisbane catchment:** Bremmer Creek [1] (27.6493 S 152.635 E)

AA33027, Brisbane [5] (27.4913 S 152.997 E) AA33194-98, Laidley Creek [1] (27.6298 S

152.375 E) AA32892, Lockyer Creek [1] (27.5493 S 152.115 E) AA41603, Warrill Creek

[1] (27.7809 S 152.68 E) AA32963; **Burnett catchment:** Barambah Creek [3] (25.7208 S

151.81 E) AA2331-32 / AA2366, [1] (25.7974 S 151.8 E) AA2367, Burnett River [1]

(25.593 S 151.315 E) AA10145, [3] (25.685 S 151.777 E) AA2360-61 / AA2363, [1]

(24.974 S 152.091 E) AA00268, [4] (25.051 S 152.099 E) AA276 / AA4642 / AA4648 /

AA4654, [1] (25.614 S 151.592 E) AA295, [1] (25.583 S 151.661 E) AA296, Burrum

River [1] (24.796 S 152.442 E) AA00246; **Fitzroy-Dawson catchment:** Dawson River [3]

(24.603 S 149.913 E) AA32871*-73, Rockhampton [1] (23.4 S 150.5 E) ABTC76454;

Fraser Island: Dilli Village lagoon [2] (25.599 S 153.092 E) AA5283* / AA5285, Lake

Birrabeen [1] (25.506 S 153.062 E) AA33696, Lake Coomboo [1] (25.223 S 153.168 E)

AA33863, Lake Garawongera [1] (25.326 S 153.153 E) AA33737, Lake McKenzie [1]

(25.446 S 153.056 E) 1172, South Boomerang Lake [1] (25.226 S 153.135 E) AA33997;

Logan-Albert catchment: Albert River [1] (28.198 S 153.040 E) AA41626, [4] (27.818 S

153.175 E) 323 / 328 / 337 / 343; **Mary catchment:** Mary River [5] (26.525 S 152.564 E)

AA4502 / AA4565 / AA4569-71; **Moreton Island:** Honeyeater Lake [2] (27.095 S

153.435 E) AA4300 / AA4292; **Pine catchment:** North Pine River [1] (27.043 S 152.869

E) AA33551, [1] (27.063 S 152.872 E) J83695, [1] (27.245 S 153.03 E) AA33066,

Sandgate Lagoons [15] (27.318 S 153.063 E) AA941-55, South Pine River [1] (27.366 S

152.933 E) 31, [1] (27.35 S 152.916 E) 3030, [1] (27.343 S 152.872 E) AA46429; **South

coast catchment:** Currumbin Creek [2] (28.132 S 153.488 E) AA5296-97; **Stradbroke

Island:** Brown Lake [5] (27.490 S 153.432 E) AA46516-18 / AA46520-21.

Murray-Darling Basin province: Border Rivers catchment: Dumaresq River [3]

(28.988 S 151.277 E) AA20598 / AA20620-21, Macintyre River [11] (28.548 S 150.301

E) AA32782-88 / AA32790 / AA32796-98, [1] (28.465 S 150.958 E) AA33103, Severn

River [2] (28.733 S 151.983 E) 365 / 371; **Condamine catchment:** Bokhara River [1]

(29.164 S 147.279 E) AA33875, Condamine River [6] (26.8 S 150.678 E) AA32804-07 /

AA32820-21, [7] (27.68 S 151.89 E) AA32956-62, Condamine-Culgoa River [1] (27.991

S 148.659 E) AA33099; **Darling catchment:** Darling River [6] (34.112 S 141.917 E)

AA33112 / AA33152-54 / AA33182-83; **Macquarie catchment:** Macquarie River [1]

(31.888 S 148.092 E) AA32170; **Moonie catchment:** Moonie River [1] (27.894 S

149.559 E) AA46409, [7] (27.956 S 149.382 E) AA46411-17; **Murray catchment:**

Murray River [6] (34.084 S 140.781 E) AA18731 / AA20454 / AA20468-69 / AA20495-

96, [1] (34.218 S 140.453 E) AA20528, [6] (34.113 S 141.891 E) AA33107-11 /

AA33115, [2] (34.23 S 140.44 E) ABTC51969 / CE_003, [6] (36.092 S 146.948 E)

AA20675-79 / AA20691, [3] (36.092 S 146.948 E) 625-267; **Murrumbidgee catchment:**

Murrumbidgee River [3] (35.123 S 147.351 E) AA10784 / AA10787 / AA10789, [1]

(34.272 S 146.032 E) AA32108, [2] (34.173 S 145.801 E) AA32147 / AA32165, [1]

(35.032 S 147.101 E) MB007; **Namoi catchment:** Namoi River [2] (30.75 S 150.716 E)

757 / 759, [2] (30.972 S 150.254 E) AA32419-20, [1] (30.242 S 149.683 E) AA32426;

Warrego catchment: Warrego River [3] (26.906 S 146.033 E) AA13179 / AA13204 /

AA13239, [1] (27.074 S 145.957 E) AA13303.

Voucher numbers are for the Wildlife Tissue Collection at the University of Canberra

(http://iae.canberra.edu.au/cgi-bin/locations.cgi). Where available, photo vouchers

available on request.

Appendix 2B – 12s and R35 primers used for mitochondrial serial dilution test

End point serial dilutions were amplified for the mitochondrial *12s* RNA fragment and the

single copy nuclear intron *R35*.

12s primers:

H1478 (5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'), Kocher *et al*., 1989.

L1091 (5'- AGCTTCAAACTGGGATTAGATACCCCACTAT-3'), Kocher *et al*., 1989.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca, and

A. C. 1989. Wilson. Dynamics of mitochondrial DNA evolution in animals: Amplification

and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*

of the United States of America 86: 16.

<u>*R35* primers</u>:

R35_int_F (5'-CCTNTCAGCTYCTTTCCAT-3'), this study.

R35Ex1 (5'-GCAGAAAACTGAATGTCTCAAAGG-3'), Fujita *et al*., 2004.

Fujita, M. K., Engstrom, T. N., Starkey, D. E., and Shaffer, B. H. (2004) Turtle phylogeny: Insights from a novel nuclear intron. *Molecular Phylogenetics and Evolution* **31**: 1031–1040.

Appendix 2C – Haplotype frequencies for each catchment

Mitochondrial haplotypes and haplotype frequencies recovered in each catchment. n refers to number of individuals, n haps refers to the number of haplotypes in each catchment. Catchments are grouped by regions described in the text.

Region	Catchment	n	n haps	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21
Murray-Darling Basin n=87 n haps=4	Murray	24	1		1																			
	Murrumbidgee	7	2		0.71	0.29																		
	Macquarie	1	1		1																			
	Namoi	5	1		1																			
	Border Rivers	17	2		0.94			0.06																
	Moonie	8	1		1																			
	Condamine	15	2		0.80		0.20																	
	Darling	6	1		1																			
	Warrego	4	1		1																			
South Conondale n=37 n haps=5	South Coast	2	1								1													
	Logan-Albert	5	2								0.80		0.20											
	Pine	21	2						0.33		0.67													
	Brisbane	9	3							0.11	0.78	0.11												
North Conondale n=26 n haps=9	Mary	5	1																			1		
	Burnett	16	7													0.19	0.25	0.06	0.19		0.06		0.19	0.06
	Baffle	1	1															1						
	Fitzroy-Dawson	4	1																	1				
	Stradbroke Island	5	1	1																				
	Moreton Island	2	2								0.50			0.50										
	Fraser Island	7	3								0.14						0.43					0.43		

Chapter 3

Significant genetic structure despite high vagility revealed through mitochondrial phylogeography of an Australian freshwater turtle, *Chelodina longicollis*.

Abstract

Restriction to the freshwater environment plays a dominant role in the population genetic structure of freshwater fauna. In taxa with adaptations for terrestriality however, the restrictions on dispersal imposed by drainage divides may be overcome. We investigate the mitochondrial phylogeographic structure of the eastern long-necked turtle *Chelodina longicollis*, a widespread Australian freshwater obligate with strong overland dispersal capacity and specific adaptations to terrestriality. We predict such characteristics make this freshwater species a strong candidate to test how life-history traits can drive gene flow and inter-basin connectivity, overriding the constraining effects imposed by hydrological boundaries. Contrary to expectations, and similar to low vagility freshwater vertebrates, we found two ancient mitochondrial haplogroups with clear east/west geographic partitioning either side of the Great Dividing Range. Each haplogroup is characterised by complex genetic structure, demographically stable sub-populations, and signals of isolation by distance. This pattern is overlayed with signatures of recent gene flow, likely facilitated by late Pleistocene and ongoing anthropogenic landscape change. We demonstrate that the divergent effects of landscape history can overwhelm the homogenising effects of life history traits that connect populations, even in a highly vagile species.

Introduction

Freshwater organisms are collectively limited by barriers such as marine and terrestrial habitats, and this restriction plays a dominant role in their genetic structure at the broad level of whole river drainage basins. Relationships among freshwater populations also often reflect the dendritic structure of streams, and the nested hierarchy of tributaries and rivers within catchments. This stream hierarchy predicts that freshwater populations will have high connectivity and low genetic structure in populations within, but not among

catchments, for a particular river basin (Hughes *et al*., 2009). For obligate freshwater

fauna, drainage divides present barriers to population connectivity (Banarescu, 1990) and

many studies have implicated their influence in shaping the evolution of freshwater faunal

lineages. Inability to disperse across drainage divides creates isolated populations that over

time provide opportunity for differentiation, divergence, and ultimately allopatric

speciation. For example, studies in Australia highlight the eastern uplands of the Great

Dividing Range that separate inland and coastal bioregions as a driver of allopatric

speciation in freshwater cod (*Maccullochella* spp., Rowland, 1993; Nock *et al*., 2010), and

phylogenetic divergence in multiple species of fish (Faulks *et al*, 2008, 2010; Hammer *et*

al., 2007; Unmack & Dowling, 2010; Unmack, 2001), freshwater crustaceans (Murphy &

Austin, 2004), and a low vagility turtle (Hodges *et al*., 2014). In such cases genetic

divergence is a function of isolation by limited hydrological connectivity, rather than

isolation by distance *per se*.

There are exceptions to population connectivity being driven by contemporary

drainage divides and the dendritic and hierarchical nature of freshwater systems. Changes

in stream organisation in recent geological history have facilitated enduring or intermittent

inter-basin connectivity through drainage reversals (Burridge *et al*., 2007; Unmack *et al*.,

2012), exposure of the continental shelf (Ruzzante *et al*., 2011) and flooding at low relief

drainage divides (Masci *et al*., 2008). Furthermore, species with tolerance of saline

conditions can move between drainage basins via a coastal marine corridor or infrequent

freshwater plumes that connect neighbouring catchments (Jerry & Cairns, 1998). Dispersal

capacity including flight ability, desiccation resistance, temperature tolerance, and

propensity for overland migration can also determine if freshwater taxa overcome drainage

divides (*e.g*. Slechtova *et al.*, 2004; Craw *et al.*, 2008). Each of the above abiotic

(geological history) and biotic (life history) processes can leave genetic signatures in

populations that contradict assumptions of population divergence based on traditional

biogeography and hydrological architecture. Here we use mitochondrial phylogeography

of an obligate freshwater turtle with strong terrestrial dispersal capacity to test the extent to

which its vagile life history traits mitigate the otherwise dominant influence of drainage

divides.

The Eastern Long-necked Turtle (*Chelodina longicollis*) is one of Australia's most

widespread and ubiquitous species of chelid. It is continuously distributed throughout four

major freshwater biogeographic regions (Unmack, 2001): the Bass Province, the Eastern

Province, the Murray-Darling Basin (MDB), and the Cooper and Bulloo drainages in the

Central Australian Province (Figure 3.1). The MDB and the Central Australian Province

are large inland semi-arid river basins characterised by low elevational gradients and

braided distributary channels. These inland regions are separated from the coastal Bass

Province and Eastern Province by the Great Dividing Range which formed in the

Cretaceous approximately 90 Mya (Wellman, 1979). Compared to mountain ranges of

other continents, the Great Dividing Range has a subdued character with low to moderate

elevational gradients throughout much of its length. It is particularly subdued at the

drainage boundary between the MDB the Bass Province and at the boundary between the

MDB the Fitzroy-Dawson and Burnett drainages. Exchange of freshwater taxa across the

Great Dividing Range has been long recognised (Musyl & Keenan, 1992, 1996; McGlashan

& Hughes, 2001; Unmack, 2001; Murphy & Austin, 2004; Cook *et al*., 2006; Hammer *et*

al., 2007; Thacker *et al.*, 2007; Jerry, 2008; Faulks *et al.*, 2008, 2010; Unmack & Dowling,

2010; Hodges *et al.*, 2014) and we expect *C. longicollis* to easily transverse regions with

low to moderate elevational gradients.
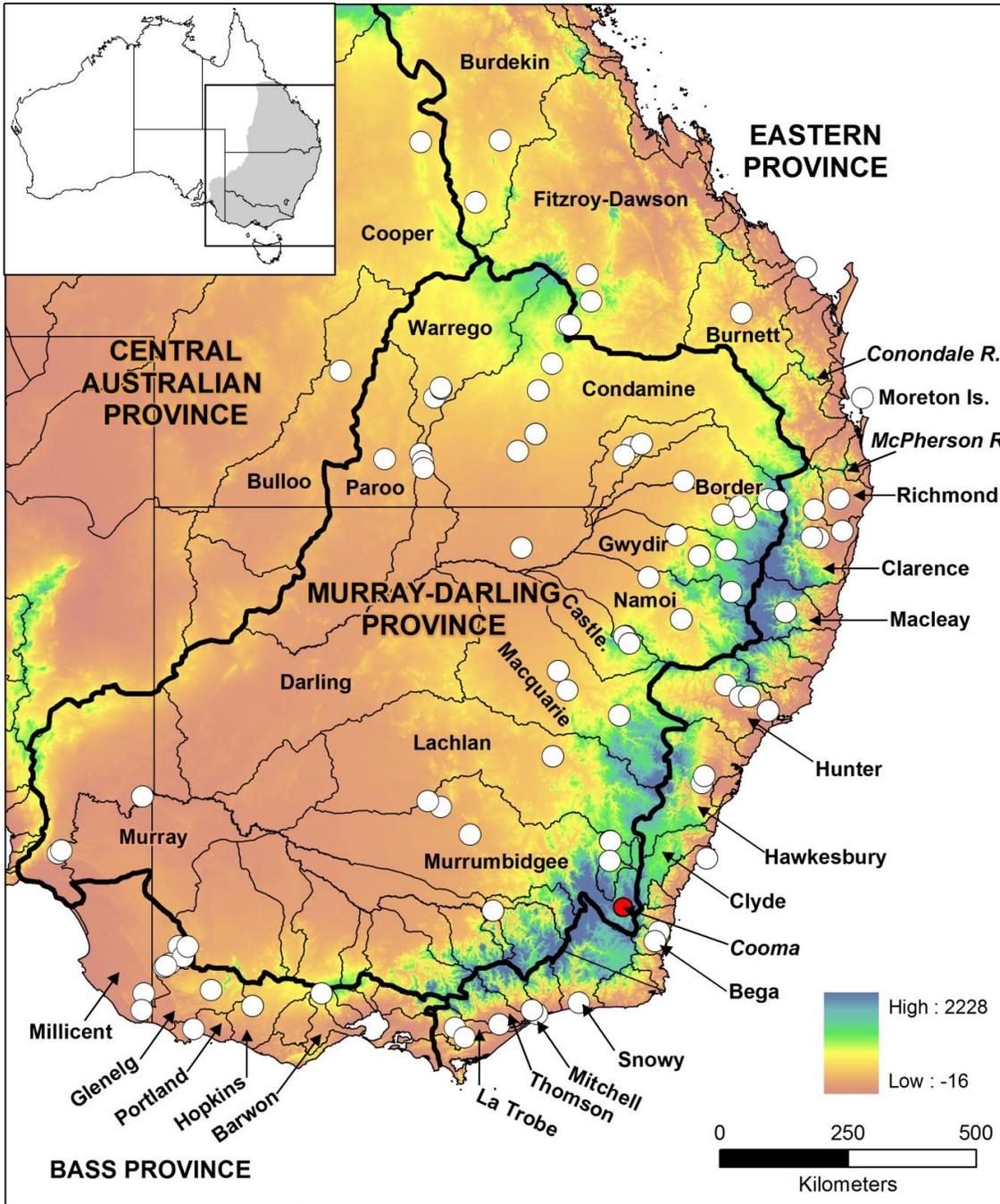
Figure 3.1 **(previous page) Distribution of** *C. longicollis* **(shaded region in inset) and collection localities (white circles). Thick black lines delineate major freshwater biogeographic regions as per Unmack (2001), thin black lines delineate drainages, red circles indicate major cities and localities referred to in the text. Underlying colour indicates elevation in meters. The Great Dividing Range occurs at the interface of the inland Murray-Darling Basin and eastern seaboard drainages. Note the central sections of the Great Dividing Range are high elevation and the north and south are subdued.**

Chelodina longicollis occupies a broad suite of freshwater habitats throughout its range. The species occurs in greatest abundance in shallow ephemeral wetlands and disconnected water bodies, including artificial environments such as farm dams and irrigation channels, with an abundance of slow-moving invertebrate prey (Chessman, 1984a; 1988). It is active in water temperatures as low as 12°C (Kennett *et al*., 2009) and displays the greatest cold tolerance of any Australian turtle with nesting populations at montane sites such as in the vicinity of Cooma, NSW. The species belongs to the family Chelidae and, unlike the Testudinidae, uses a 'gape and suck' method of predation (Parmenter, 1976) which renders it and all members of the family obligate freshwater species. Despite this *C. longicollis* is a strong disperser at the landscape scale with a high propensity to utilise terrestrial environments (Roe & Georges, 2007, 2008).

Terrestrial forays are reported in 91% of males and 75% of females in some populations, with individual movements up to 1,470 m over the course of a year (Roe & Georges, 2007). Average time spent in the terrestrial environment before returning to a wetland is approximately 2 months, though terrestriality of up to 16 months is possible (Roe & Georges, 2008). This propensity for terrestrial migration enables *C. longicollis* to exploit highly productive disconnected ephemeral systems (Kennett & Georges, 1990), and to find permanent water to escape periodic drought conditions (Roe & Georges, 2007). To cope with extended periods of terrestriality *C. longicollis* has evolved specific water conserving adaptations (Roe *et al*., 2008). It is able to draw its head, neck, limbs and tail tightly within the shell to both reduce exposure to predation and minimize evaporative water loss (Chessman, 1984b). To limit desiccation the species has the capacity to store and reabsorb water from the cloacal bladder, adjust uric acid excretions, limit cutaneous

water loss, and drink pooled water from terrestrial leaf litter (Rogers, 1966; Chessman, 1984b; Roe, 2008).

Chelodina longicollis is the most vagile of Australian chelids. The related and broadly sympatric broad-shelled turtle (*C. expansa*) however has no specific adaptions for terrestriality and is restricted to permanent water bodies connected to main river channels (Bower & Hodges, 2014). As a consequence, mitochondrial phylogeographic structure in *C. expansa* is dictated by long-standing drainage divides. Mitochondrial nucleotide divergence between the MDB and Eastern Province bioregions in this species is 2.41%, and deeper divergence of 4.61% is found between the Mary and Brisbane drainages in the Eastern Province itself (Hodges *et al*., 2014). Eastern Province drainage divides similarly dictate deep phylogeographic structure in the Australian snapping turtle (*Elseya albagula*) and Krefft's river turtle (*Emydura macquarii krefftii*) (Todd *et al*., 2013, 2014). Compared to *C. longicollis*, *C. expansa* and *Em. m. krefftii* have poor dispersal capacity, and *Els. albagula* can be considered sedentary (Todd *et al*., 2013). Given that phylogeographic structure is closely tied to species vagility, especially in freshwater taxa, we expect genetic patterns in *C. longicollis* to be quite unlike that described for other Australian chelids. Rather, we expect phylogeographic structure in *C. longicollis* to be comparable to that of the common snapping turtle (*Chelydra serpentina*) from eastern and central North America.

Chelodina longicollis is broadly similar to *Chelydra serpentina* in that they both have an extensive range, a degree of cold tolerance, and strong terrestrial dispersal ability (Obbard & Brooks, 1981a,b; Costanzo *et al*., 1995). *Chelydra serpentina* exhibits almost no mitochondrial sequence variation across its distribution in the southeastern United States, suggesting its dispersal capacity and cold tolerance either allowed it to resist population subdivision during Pleistocene glacial periods (Walker & Avise, 1998), or

facilitated its rapid expansion during interglacials from a single population. We expected to find a similar pattern of limited mitochondrial genetic diversity in *C. longicollis*, and any diversity that we do find we expect to be broadly dispersed throughout the range of the species and not limited by drainage divides and bioregional boundaries. We use mitochondrial nucleotide sequences to test the following: (1) that there is no genetic subdivision between the MDB, Eastern Province, Bass Province and Central Australian Province; and (2) that genetic structure is dominated by signals of panmixia. Our study builds on the phylogeographic dataset for sympatric freshwater taxa in eastern Australia (Hughes *et al*., 2013; Hodges *et al*., 2014) to test the extent of influence freshwater bioregions and hydrological connectivity have on genetic structure. *Chelodina longicollis* represents the maximum dispersal capacity of an obligate Australian freshwater vertebrate and can potentially highlight the upper limit beyond which freshwater bioregions have no effect on phylogeographic partitioning.

Materials and Methods

Sampling

We obtained tissue samples from 274 *Chelodina longicollis* from 94 localities across 33 drainages throughout the geographic range of the species (Figure 3.1). Skin samples were obtained from the webbing of the clawless digit on the hind foot and immediately placed in 95% ethanol for transport and storage. Sample details and collection localities are provided in Appendix 3A. Taxonomy follows that of Georges and Thomson (2010).

The mitochondrial regions examined and the procedures for DNA extraction, PCR amplification and PCR product purification follow those for *C. expansa* (Hodges *et al.,* 2014) and thus the two studies are directly comparable. Briefly, we targeted a 630 bp fragment of the mitochondrial *ND4* gene, and a 470 bp fragment of mitochondrial *control*

region including *tRNA^{Pro}* (hereafter collectively referred to as *control region – CR*). Sequencing was performed in both directions using an ABI 3730XL DNA automated sequencer by Macrogen (Seoul, South Korea) and sequences were edited, assembled, and consensus sequences determined using Geneious Pro 5.3.4 (BioMatters Inc.). Sequences were aligned using ClustalX 1.81 (Thompson *et al.*, 1997) to yield final edited alignments of 1,042 bp, comprising 595 bp of *ND4*, 68 bp of *tRNA^{Pro}* and 379 bp of *control region* (GenBank accession numbers for *ND4* haplotypes KM581393-KM581420; GenBank accession numbers for *CR* haplotypes KM581421-KM581448). Four methods, described in greater detail in Hodges *et al.* (2014), were used to confirm the genuine mitochondrial origin of the sequences and minimise the chance of undetected inclusion of nuclear paralogues in our analyses. Appendix 3A details the two samples used in the present study for the mitochondrial enrichment procedure.

Population genetic structure

A median-joining haplotype network was constructed on concatenated *ND4* and *CR* sequences using NETWORK v4.610 (Fluxus Technology Ltd) with $\varepsilon = 0$ and maximum parsimony post processing. Molecular divergence indices were estimated in DNASP v5 10.01 (Librado & Rozas, 2009) using the average number of nucleotide substitutions per site between groups (D_{xy}) with 1,000 bootstrap replicates. A molecular dating analysis was implemented using the Bayesian approach in BEAST v1.6.1 (Drummond *et al.*, 2007) to estimate time to most recent common ancestor for major genetic groups. Models of evolution were specified in MODELTEST 3.7 (Posada & Crandall, 1998): *tRNA^{Pro}* K80, *control region* TrN+G, *ND4* HKY+G. Domains were tested for clocklike evolution in PAUP* (Swofford, 2002) and *tRNA^{Pro}* and *ND4* were estimated under a strict molecular clock model and *control region* was estimated under a relaxed uncorrelated lognormal

clock. We applied a mitochondrial divergence rate of 0.895% per Myr (Zamudio &

Greene, 1997; Rabosky *et al.*, 2007) scaled per lineage per Myr and modelled under a

normal distribution. This divergence rate is consistent with a rate estimated from fossil

chelid turtles by Georges *et al*. (2013) (0.86% per million years) and has been applied

successfully elsewhere (Hodges *et al*., 2014; Todd *et al*., 2014). MCMC chains were run

for 40 million generations with sampling every 1,000 steps yielding a total of 40,000 trees.

Convergence was checked and parameters assessed using TRACER1.5 (Rambaut &

Drummond, 2007).

Correlation between geographic and genetic distance (isolation by distances) was

assessed using Mantel tests implemented in GENALEX version 6.41 (Peakall & Smouse,

2006) and significance tests were carried out using 9,999 permutations. We also performed

analyses of molecular variance (AMOVA) from haplotype frequencies using 1,000

bootstrap replicates in ARLEQUIN v3.5.1.2 (Excoffier & Lischer, 2010) to investigate

partitioning of genetic variation based on the four major freshwater biogeographic regions

defined *a priori*. We first combined all individuals by drainage and performed AMOVAs

with three levels: within drainages, among drainages within regions, and among regions.

Four analyses were performed: (1) among all four freshwater biogeographic regions; (2)

the MDB versus Eastern Province; (3) the MDB versus Bass Province; and (4) the Bass

Province versus Eastern Province. Because of insufficient samples from the Central

Australian Province, we excluded this region from paired analyses of variation.

Historical population demography

Number of segregating sites (S), haplotype diversity (hd), nucleotide diversity (π), and

average number of nucleotide differences (k) were calculated in DNASP. Tajima's (1989)

D statistic, *Fs* (Fu, 1997), and R2 (Ramos-Onsin & Rozas, 2002) were calculated to test if

populations were conforming to models of neutral evolution and demographic stability, or were departing from these states owing to population expansion. All tests were performed in DNASP and significance was estimated using the distribution of random samples generated by 10,000 coalescent simulations.

Results

Haplotypic relationships

Twenty eight mitochondrial haplotypes were recovered from concatenated *ND4* and *CR* mitochondrial sequences from 274 *C. longicollis* representing the geographic range of the species. Haplotypes fall into two major haplogroups, A and B (Figure 3.2), separated by 44 mutational steps, and D_{xy} sequence divergence of 4.38%.

Bayesian dating analysis estimated the most recent common ancestor of the two haplogroups occurred 6.53 Mya (95% highest posterior density (HPD) =4.89–8.26 Mya) in the late Miocene, and the TMRCA for each haplogroup in the early Pleistocene, Haplogroup A: 1.29 Mya (95% HPD=0.71–1.95 Mya); Haplogroup B: 1.5 Mya (95% HPD=0.89–2.17 Mya). Haplogroups A and B comprise 13 and 15 haplotypes respectively. Divergence within each haplogroup is low with an average of 1.8 substitutions separating haplotypes in Haplogroup A, and 3.3 separating haplotypes in Haplogroup B. Despite low divergence, the haplotype network reveals a complex genetic structure with little evidence of star-like patterns (*i.e.* many recently evolved haplotypes), common haplotypes not always located centrally, and a large number of mutational steps separating terminal haplotypes in Haplogroup B.
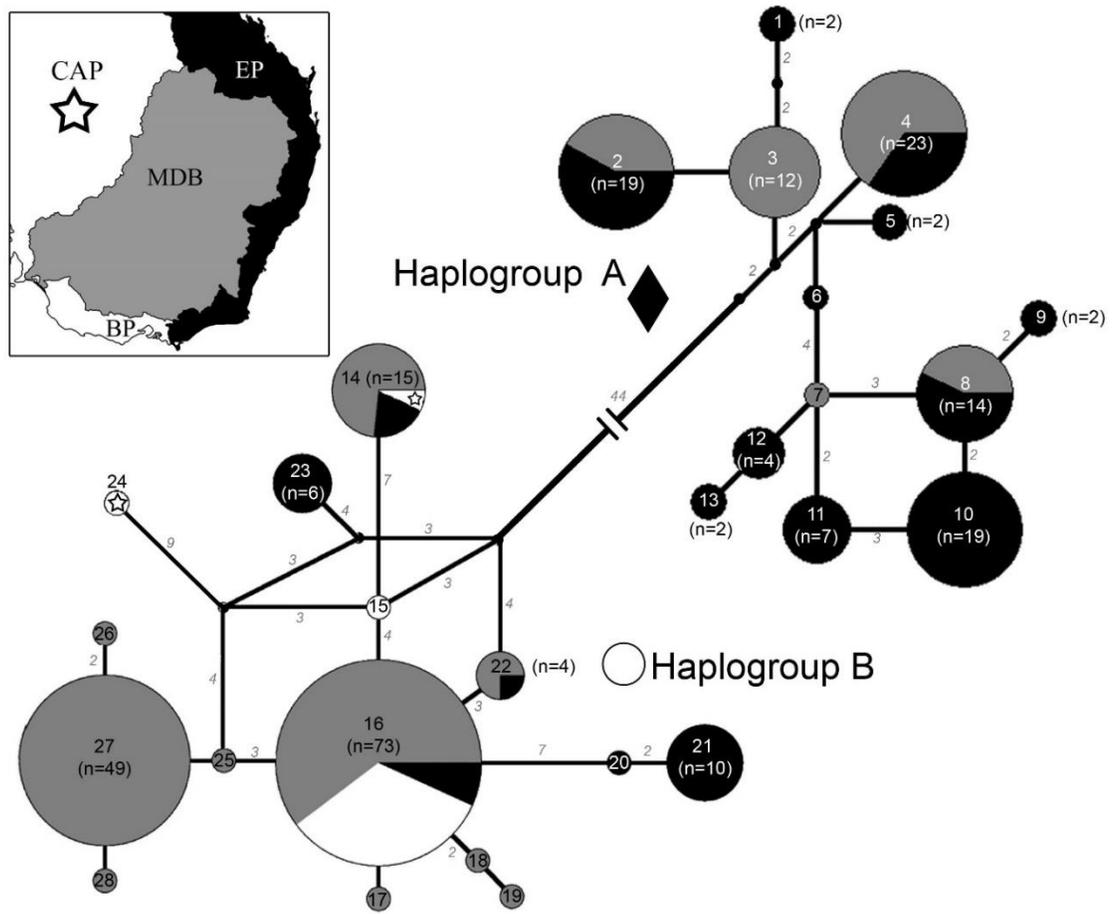
Figure 3.2 Median-joining haplotype network of the 28 *C. longicollis* haplotypes. Number of mutational steps (>1) in the haplotype network are indicated in grey, circle area is proportional to the number of individuals sharing a haplotype, haplotype number is given inside the circle, number of individuals (>1) is indicted in parentheses. Haplotypes are coloured by representation of individuals from each of the four major freshwater biogeographic regions: grey indicates the Murray-Darling Basin (MDB), white indicates the Bass Province (BP), black indicates the Eastern Province (EP), star indicates the Central Australian Province (CAP). Black diamond (Haplogroup A) and white circle (Haplogroup B) are consistent with symbology presented in Figure 3.3.

Phylogeographic relationships

The two major haplogroups do not strictly correspond to freshwater biogeographic regions

defined *a priori* however there is clear geographic structure in their distributions (Figure

3.3). Haplogroup A tends to have an easterly distribution associated with the eastern

uplands of the MDB and coastal Eastern Province drainages from Moreton Island in the

north to the southern boundary of the Eastern Province. Haplogroup B tends to have a

westerly distribution associated with the entire MDB, the Bass Province, and the north-

western drainages of the Eastern Province. Haplogroups A and B both occur in the Eastern

Province but their ranges do not overlap nor do they occupy the same drainages. In the

MDB however the two haplogroups are sympatric in the eastern uplands of the Border

Rivers, Namoi, and Castlereagh drainages. The drainage with the highest haplotype

diversity in the Eastern Province is the Hunter (n=16 individuals and 5 haplotypes, Hd =

0.76) and the highest haplotype diversity in the MDB is in the Namoi (n=9 individuals and

4 haplotypes, Hd = 0.75). Haplotype sharing among bioregions is moderate with six out of

the 28 haplotypes (21.4%) found in more than one major freshwater bioregion. Overall,

there are seven broad locations where haplotype sharing occurs between freshwater

bioregions (Figure 3.3). Haplotype frequencies for each drainage division are available in

Appendix 3B.

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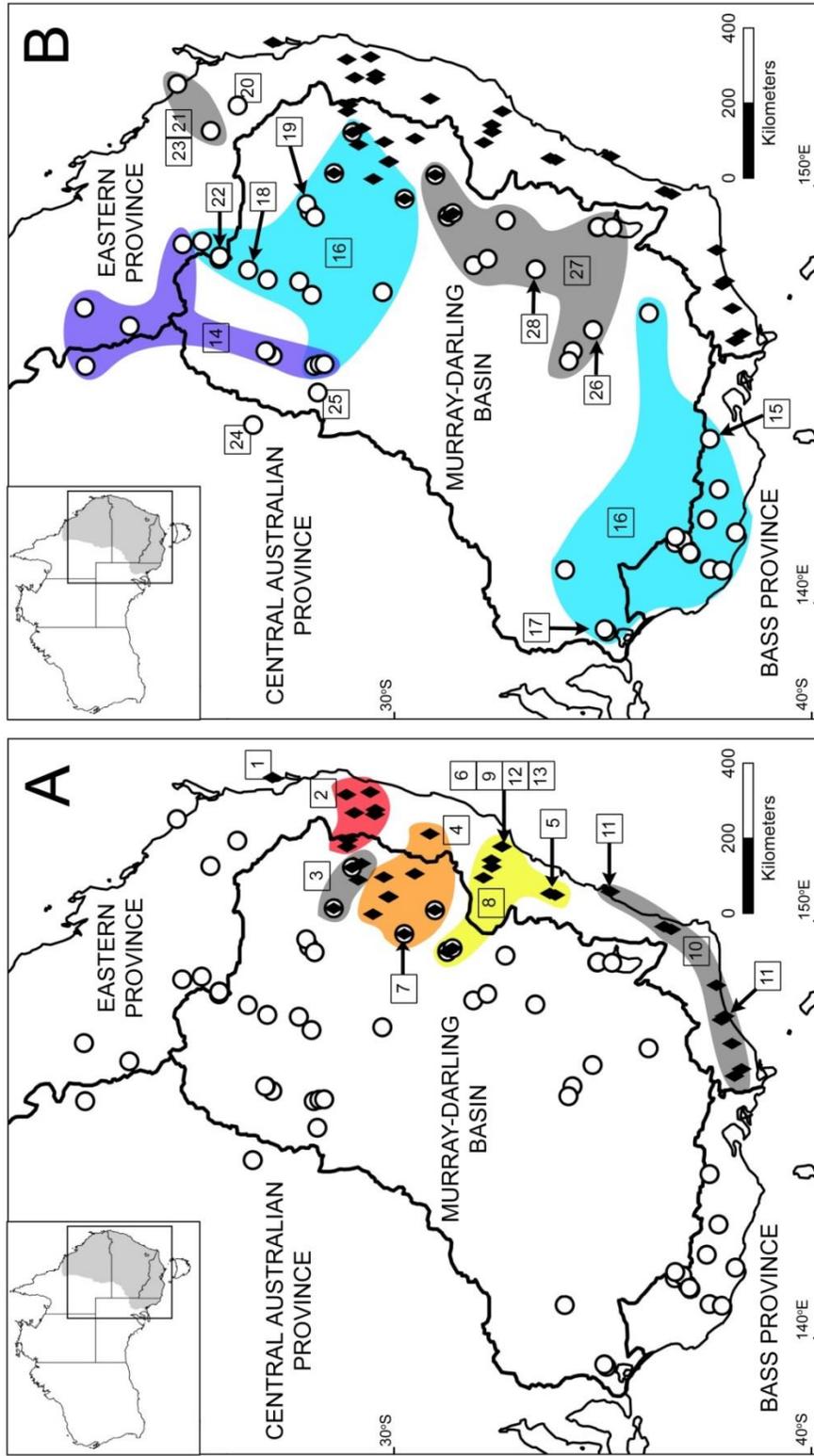
Figure 3.3 Phylogeographic structure of the two major haplogroups in *C. longicollis*. Each panel indicates the location of individuals in Haplogroup A (black diamonds) and Haplogroup B (white circles). Note that Haplogroups overlap at some sites. Panel A and B highlight the distribution and number of Haplogroup A and B haplotypes respectively. Coloured groups encompass all individuals with the same haplotype, and grey shaded groups indicate haplotypic distributions that do not cross freshwater province boundaries. Numbers presented in boxes indicate haplotype number, black lines indicate boundaries of the four major freshwater biogeographic regions.

Analysis of molecular variance

Analysis of molecular variance (Table 3.1) among the four freshwater biogeographic regions apportioned 12.48% of total genetic variation among regions (P<0.05), 52.4% among drainages within regions (P <0.001), and 35.12% within drainages (P <0.001). Genetic differentiation was significant between the MDB and the Eastern Province with only 6.54% apportioned between regions (P<0.05), 56.06% among drainages within regions (P <0.001), and 37.4% within drainages (P <0.001). Between the MDB and the Bass Province 20.04% of variation was shared between regions (P<0.05), 48.13% among drainages within regions (P<0.001), and 31.83% within drainages (P <0.001). Finally, between the Eastern Province and the Bass Province 29.88% of variation was shared between regions (P<0.001), driven primarily by the widespread Haplotype 16 and a lack of diversity in the Bass Province. 41.18% was apportioned among drainages within regions (P <0.001), and 28.94% within drainages (P <0.001).

Isolation by distance

Results from Mantel tests on each haplogroup infer significant positive correlation between genetic and geographic distance. Haplogroup A yields a strong signal of isolation by distance (Rxy = 0.565, P<0.001), and a moderate signal characterises Haplogroup B (Rxy = 0.23, P<0.001). We also tested for isolation by distance across the entire range of *C. longicollis* (94 collection localities) and again a significant but weaker signal of isolation by distance was found (Rxy = 0.118, P<0.001).

Table 3.1 Hierarchical analysis of molecular variance (AMOVA) results for *Chelodina longicollis* mitochondrial haplotype frequency data. SS, sum of squares; MDB, Murray-Darling Basin; EP, Eastern Province; BP, Bass Province

Source of variation	d.f	SS	Variance components	Variation %	Fixation index	P-value
Among all biogeographic regions						
among regions	3	15.01	0.06	12.48	$F_{CT} = 0.125$	0.003
among drainages within regions	30	64.35	0.25	52.40	$F_{SC} = 0.599$	0.000
within drainages	240	39.99	0.17	35.12	$F_{ST} = 0.649$	0.000
total	273	119.35	0.47			
Between the MDB and EP						
between regions	1	7.13	0.03	6.54	$F_{CT} = 0.065$	0.034
among drainages within regions	25	63.56	0.27	56.06	$F_{SC} = 0.600$	0.000
within drainages	220	39.32	0.18	37.40	$F_{ST} = 0.626$	0.000
total	246	110.01	0.48			
Between the MDB and BP						
between regions	1	6.29	0.10	20.04	$F_{CT} = 0.200$	0.020
among drainages within regions	15	37.91	0.23	48.13	$F_{SC} = 0.602$	0.000
within drainages	163	24.61	0.15	31.83	$F_{ST} = 0.682$	0.000
total	179	68.8	0.47			
Between the BP and EP						
between regions	1	8.84	0.17	29.88	$F_{CT} = 0.299$	0.001
among drainages within regions	18	26.24	0.24	41.18	$F_{SC} = 0.587$	0.000
within drainages	97	16.05	0.17	28.94	$F_{ST} = 0.710$	0.000
total	116	51.13	0.57			

Haplogroup demographic analyses

Haplotype diversity (Table 3.2) is higher in Haplogroup A (Hd = 0.86) than in Haplogroup B (Hd = 0.71). Estimates of Tajima D and Fs are not significant for either haplogroup (A: D = 0.92, P = 0.85; Fs = 0.72, P = 0.66) (B: D = -0.72, P = 0.23; Fs = -1.04, P = 0.41) supporting the null hypothesis that the gene fragments associated with each lineage are selectively neutral and conform to a model of population size stability. The R2 statistic is not significant for either haplogroup further supporting demographic stability.

Discussion

Turtles such as *C. longicollis* are intermediate in life history traits, such as dispersal capacity and an ability to occupy a range of freshwater habitats, when compared with freshwater fish and low-vagility terrestrial mammals (Walker & Avise, 1998). In contrast to expectations for other vertebrate freshwater obligates, in *C. longicollis* we predicted highly connected populations and insensitivity to traditional freshwater biogeographic boundaries. Instead we found two divergent mitochondrial haplogroups with east/west geographic partitioning, genetic structure within each haplogroup, signals of historic demographic stability, and isolation by distance. These patterns are overlayed with signatures of recent population connectivity and haplotype sharing among bioregions.

Table 3.2 Molecular diversity indices: number of sequences (n), number of haplotypes (h), number of segregating sites (S), haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide differences (k). Tests for population stability: Tajima's *D* (1989), Fu's *Fs* (1997), Ramos-Onsin and Rozas's R2 (2002).

Haplogroup	n	h	S	Hd ±SD	π	k	*D* (95% CI)	*Fs* (95% CI)	R2 (95% CI)
A	108	13	16	0.864 ± 0.013	0.00389	4.06	0.92 (-1.62 - 1.95)	0.72 (-7.19 - 7.87)	0.13 (0.04 - 0.16)
B	166	15	23	0.71 ± 0.025	0.00289	3.00	-0.72 (-1.60 - 1.98)	-1.04 (-6.88 - 7.48)	0.06 (0.03 - 0.15)

East/west divergence

Chelodina longicollis mitochondrial haplogroups diverged approximately 6.53 Mya in the late Miocene. The maintenance of this ancient signature in contemporary populations seems at odds with the species dispersal capacity and its potential to transverse low to moderate elevation regions of the Great Dividing Range. The processes that lead to late Miocene mitochondrial divergence are uncertain as these signatures have been replaced with diversity acquired since the early Pleistocene. We cannot speculate on the Mio/Pliocene distributions of ancestral Haplogroups A and B; however the antiquity of each group suggests long-term demographic decoupling of mitochondrial lineages. We suggest the barrier presented by the ancient and topographically complex Great Dividing Range drove independent evolution of the two mitochondrial lineages and has also maintained separate distributions of the two contemporary haplogroups at least since the early Pleistocene. A range of sympatric freshwater taxa including fish, crustaceans, and a turtle also display intraspecific phylogeographic structure in varying extent and age as a result of the Great Dividing Range (Rowland, 1993; Unmack, 2001; Murphy & Austin, 2004; Hammer *et al*., 2007; Faulks *et al*., 2008, 2010; Unmack & Dowling, 2010; Hodges *et al*., 2014). Despite its often subdued character, this landscape feature is an important driver of evolutionary diversity in freshwater taxa, regardless of life history.

We acknowledge that phylogeographic breaks can arise without long-term barriers to gene flow (Irwin, 2002) and that mitochondrial haplotypic relationships do not necessarily reflect the organismal history of a species. However breaks without barriers are more likely to occur in low vagility species. Also, there is evidence for an association between *C. longicollis* east/west mitochondrial divergence and morphological traits. Cann (1998) recognised two morphological forms within *C. longicollis*: eastern distributed

specimens collected in the Eastern Province have long ovoid to oblong shaped carapaces (Cann, 1998; Goode, 1967), whereas the carapaces from western distributed specimens in the Bass Province and the MDB are wider and 'more squat' (Cann, 1998). Cann suggested these two morphological forms may highlight distinct *C. longicollis* populations, and our mitochondrial genetic data support this claim, though in the absence of nuclear gene data we do not recognise the different haplogroups as requiring taxonomic recognition. Future work could investigate if individuals from different haplogroups correspond to Cann's putative morphotypes, focussing especially on the site of distributional overlap.

The persistent influence of the Great Dividing Range is visible today in *C. longicollis*. Higher elevation montane environments, such as those at the interface of the Murrumbidgee and Snowy drainages, appear to inhibit connectivity in southeast Australia between populations in the MDB and Eastern Province. Mitochondrial gene flow at these locations appears absent even with the widespread contemporary presence of farm dams which *C. longicollis* regularly inhabit. Limited cold tolerance may be acting to constrain *C. longicollis* dispersal in this region. Although *C. longicollis* is active at low temperatures (Kennett *et al*, 2009) and nests at montane sites on the southeast tablelands (Cooma, 793m above sea level; pers. obs), these attributes appear insufficient to allow gene flow over the Great Dividing Range in this region.

Unexpected diversity

Contrary to expectations of panmixia, signals of isolation by distance and significant mitochondrial genetic diversity characterise each haplogroup. Isolation by distance reflects equilibrium between gene flow and genetic drift and is established over long time periods with stable populations and limited barriers to dispersal. Neutrality indices also support demographic stability and historically subdivided populations within each haplogroup. We

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propose these patterns result from population contraction and persistence in the MDB and Eastern Province during recent Pleistocene glacial oscillations.

Haplogroup A diversity

High mitochondrial genetic diversity and signals of population subdivision and demographic stability in Haplogroup A suggest the eastern population of *C. longicollis* has long persisted in the Eastern Province. Further, highly localised haplotypes point to a pattern of range contractions during Pleistocene aridity and population persistence in multiple freshwater isolates. The complex topography of the Eastern Province could have harboured multiple refugia during glacial cycles. Freshwater taxa including shrimp (*Paratya australiensis*; Cook *et al*., 2006), hardyhead (*Craterocephalus marjoriae*; Unmack & Dowling, 2010), smelt (*Retropinna semoni*; Hammer *et al*., 2007), and flathead gudgeon (*Philypnodon macrostomus*; Thacker *et al*., 2008) show similarly localised haplotypes and isolation by distance. The Hunter drainage in particular likely played an important role in harbouring and promoting diversity during Pleistocene population contraction. This drainage has the highest haplotype diversity in *C. longicollis*, and also harbours a divergent lineage of the freshwater catfish *Tandanus tandanus* (Jerry, 2008).

Haplogroup B diversity

Haplogroup B has a strong association with the MDB and we expected this region above all others to show very limited genetic structure. While haplotype 16 has an enormous distribution extending over 1,500km, signals of isolation by distance and moderate mitochondrial genetic diversity dominate. The two most common haplotypes are separated by a large number of mutational steps from the B group haplotypes (20, 21 & 23) endemic to the Eastern Province. We propose that throughout the LGM, both the MDB and the

Eastern Province independently harboured Haplogroup B haplotypes that originated from earlier diversification. Pleistocene refugia in the MDB have been suggested on the basis of localised divergent haplotypes and significant genetic structure in freshwater fish and crustaceans (Austin *et al.*, 2003; Nguyen *et al.*, 2004; Hughes & Hillier, 2006; Hammer *et al.*, 2007; Faulks *et al.*, 2008). The upland regions of the Border Rivers, Gwydir, and Namoi drainages in particular are strong candidates for Pleistocene refugia in *C. longicollis*. These headwaters contain ancestral haplotypes of the southern purple spotted gudgeon (*Mogurnda adspersa*; Faulks *et al.*, 2008), and the highly restricted and endangered Western Sawshelled turtle *Myuchelys bellii* (Fielder *et al.*, 2012). Relictual turtle populations are the product of range contraction from a formally widespread distribution (Fielder *et al.*, 2012), and highlight the headwaters of the Border Rivers, Gwydir, and Namoi drainages as suitable refuge sites for freshwater fauna in the present day, and possibly during the LGM.

Highly localised and divergent haplotypes in the north and northwest Eastern Province suggests this region also harboured population isolates during the LGM. The Fitzroy-Dawson and the Burnett drainages both present a mosaic of freshwater isolates where haplotypes could have persisted through hostile Pleistocene conditions. A close relationship between the Burnett drainage and the northern MDB characterises carp gudgeon (*Hypseleotris klunzingeri* and *H. galii*; Thacker *et al.*, 2007); lineages of dwarf flathead gudgeon (*Philypnodon macrostomus*; Thacker *et al.*, 2008); and lineages of hardyhead (*Craterocephalus stercusmuscarum fulvus*; Unmack & Dowling, 2010), and expose this area as a potentially important source of diversification before expansion into the MDB.

Phylogeographic break in the Eastern Province

A mitochondrial phylogeographic break occurs in *C. longicollis* between the Richmond and Burnett drainages. Potential drivers of this break include the McPherson Range and the Conondale Range. The McPherson Range forms the high elevation (~1,500 m. above sea level) northern boundary of the Richmond drainage and acted as a significant barrier to gene flow in some species throughout the Miocene and Pliocene (McGuigan *et al*., 1998; Keogh *et al*., 2003; Chapple *et al*., 2011; Smissen *et al*., 2013). The Conondale Range delineates the Mary and Brisbane drainages and is an influential biogeographic barrier in many freshwater species including a turtle (Page & Hughes, 2014; Hodges *et al*., 2014). Future sampling is required in *C. longicollis* to determine the exact location of the phylogeographic break, and if haplogroups overlap or have hard boundaries in this region.

Phylogeographic break between the Bass Province and Eastern Province

We did not observe any gene flow between the Eastern Province and the Bass Province. These two freshwater bioregions showed the strongest signal of differentiation despite bordering each other in southern Australia and a continuous distribution of *C. longicollis* throughout. We predicted *C. longicollis* would be insensitive to this freshwater bioregional boundary as the region is characterised by open low-lands, a habitat type over which gene flow should readily occur. Further, high population connectivity in *C. longicollis* is expected to have dominated this region during the last glacial cycle owing to the presence of the freshwater Lake Bass on the Pleistocene land-bridge between southeast Australia and the island of Tasmania (Blom & Alsop, 1988). Unmack *et al*. (2012) suggest divergence between the Bass Province and Eastern Province in fish populations may have been maintained during Pleistocene aridity by limited floodplain connectivity surrounding Lake Bass and potentially high salinities in the lake itself. High regional aridity during the LGM

coupled with severe localised salinisation in southeast Australia (Bowler *et al*., 2005) may

have also limited the distribution of *C. longicollis* Haplogroups A and B, and population

connectivity over this region may have not yet recovered from earlier contractions.

Sampling of geographically intermediate populations of *C. longicollis* in this region is

necessary to ascertain the exact location and extent of this phylogeographic break.

Haplotype sharing demonstrates contemporary connectivity between bioregions

Chelodina longicollis populations are characterised by seven instances of haplotype sharing

between major freshwater biogeographic regions. The geographic extent of haplotype

sharing differs markedly between haplogroups with limited distributions in Haplogroup A,

and vast distributions in Haplogroup B. Shared haplotypes can be interpreted variously as

evidence of contemporary gene flow, convergence, or the retention of ancestral haplotypes

in disconnected populations. We recognise haplotype sharing as an indicator of very recent

and potentially ongoing gene flow as all instances are characterised by geographic

proximity and contemporary environmental conditions that promote connectivity.

Four cases of mitochondrial haplotype sharing between freshwater bioregions occur

across lowland drainage divides. These characterise Haplogroup B and occur in the

northwest and southwest MDB. Population connectivity in *C. longicollis* is expected at

these locations owing to indistinguishable drainage divides and broad low plains that

ensure hydrological connection during wet periods. Affinity between bioregions bordering

the northwest MDB is demonstrated in many other freshwater taxa. Connectivity between

the Condamine and Fitzroy-Dawson drainages is evident in golden perch (*Macquaria*

ambigua; Musyl & Kennan, 1992; Faulks *et al*., 2010) and in populations of Midgley's carp

gudgeon (*Hypseleotris* sp.; Thacker *et al*., 2007). Faunal connections among the Burdekin,

Warrego, and Cooper Creek drainages characterise eight species of freshwater fish

(Unmack, 2001; Thacker *et al*., 2007). Population connectivity in the southwest between

the MDB and the Bass Province is demonstrated in sister lineages of the river blackfish

(*Gadopsis marmoratus*; Miller *et al*., 2004), Australian smelt (*Retropinna semoni*; Hammer

et al., 2007), subspecies of the freshwater crayfish (*Cherax destructor*; Nguyen *et al*.,

2004), and in populations of flathead gudgeon (*Philypnodon grandiceps*; Thacker *et al*.,

2008).

     Three incidences of haplotype sharing occur across complex and relatively high

elevation landscapes (average elevation approximately 850 m. above sea level). These

characterise Haplogroup A and occur at the eastern boundary of the MDB and the Eastern

Province. We propose haplotype sharing represents very recent population expansion from

the east to the west. This directionality is inferred as the shared haplotypes all belong to

Haplogroup A which has a strong affiliation with the Eastern Province and signatures of

expansion from *in situ* refugia.

     An ephemeral upland wetland complex encompassing the Clarence and Macleay

drainages in the Eastern Province and the Border Rivers and Gwydir drainages in the MDB

(Bell *et al*., 2008) may facilitate sharing of haplotypes 2 and 4 over the Great Dividing

Range. These wetlands formed during late Pleistocene glacial cycles (Haworth *et al*., 1999)

and may explain the limited extent of these haplotypes in the MDB as *C. longicollis*

populations in the Eastern Province were only afforded the opportunity to expand eastward

over the Great Dividing Range very recently. Sharing of haplotype 8 between the Eastern

Province and the upland MDB is likely assisted by the Cassilis Gap at the headwaters of the

Hunter drainage. The Cassilis Gap is a broad open valley and a well-known biogeographic

barrier to upland forest adapted fauna (Moussalli *et al*., 2005; Colgan *et al*., 2009; Chapple

et al., 2011; Rix & Harvey, 2012). The same landscape features that inhibit north/south

dispersal in terrestrial species assist east/west dispersal in *C. longicollis* and a range of

freshwater fish (Unmack, 2001; Jerry, 2008). A similar pattern characterises the Burdekin Gap in the Eastern Province. There, an arid corridor contributes to vicariance in terrestrial faunal lineages yet freshwater turtle species are relatively insensitive to the north/south "gap" (Todd *et al*., 2014).

Recent mitochondrial gene flow in *C. longicollis* from the Eastern Province into the MDB may be also assisted by permanent water provided by farm dams. The upper reaches of drainages in the Eastern Province became major agricultural areas after European settlement in the 1800s, and saw the proliferation of privately owned dams. *Chelodina longicollis* is abundant in these artificial permanent water bodies and it is possible these new habitats assist contemporary populations to extend from the Eastern Province into the upland MDB. Furthermore the recency of this habitat availability is consistent with the limited geographic extent of Haplogroup A in the MDB.

Conclusion

The longstanding biogeographic impediment of the Great Dividing Range, plus Pleistocene climate change has significant influence on the recent evolutionary history of Australian freshwater taxa, and a far greater impact on *C. longicollis* than predicted. In contrast to expectations of insensitivity to barriers, we find east/west phylogeographic partitioning dating to the Miocene, and caused by the Great Dividing Range which on global standards is of relatively low elevation. In contrast to predictions of panmixia we instead find signals of isolation by distance and diversity within each haplogroup shaped by diversification within, and limited connectivity among multiple Pleistocene refugia.

Mitochondrial phylogeography of *C. longicollis* demonstrates that different evolutionary processes dominate at different times to create complex patterns of divergence and connectivity. Landscape history has driven ancient patterns of mitochondrial

divergence and diversity, and overwhelmed life history traits that could connect populations. Contemporary processes however have re-instated the influence of life history with some populations dominated by dispersal and gene flow, leading to sympatry of haplogroups. As such, the eastern and western distributions of *C. longicollis* may be moving from divergence towards homogenisation as the convergent effects of gene flow between bioregions has a greater impact than the divergent effects of genetic drift between them.

Acknowledgements

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Supporting information

Appendix 3A – Specimens examined

Data for *Chelodina longicollis* are given by freshwater biogeographic region, drainage (in bold), local river or region, number of specimens collected (in square parentheses), latitude and longitude and specimen number(s) (Wildlife Tissue Collection, University of Canberra, UC<Aus> in Genbank). Drainage names follow those recommended by the Bureau of Meteorology (1997). * denotes samples used in the mitochondrial enrichment and serial dilution procedure.

Central Australian Province: Bulloo River: Bulloo River [1] (26.6107 S 144.2680 E) AA18058. **Cooper Creek:** Thompson River [1] (22.5933 S 145.6779 E) 98ClonDunn-2.

Murray-Darling Basin: Border rivers: Severn River [12] (28.8667 S 151.7833 E) 3061-66, (29.1380 S 150.9842 E) AA20775-78, (28.8978 S 151.9447 E) QM J84640, J85191;

Beardy Creek [6] (29.2107 S 151.3789 E) AA20575-80; Dumaresq River [2] (28.9889 S

151.2777 E) AA20597,619; Macintyre River [2] (28.5484 S 150.3014 E) AA32770,89.

Castlereagh River: Coonabarabran region [7] (31.3996 S 149.3432 E) AA32178-84;

Castlereagh River [8] (31.2675 S 149.2810 E) AA32193-95, (31.2719 S 149.2558 E)

AA32401-05. **Condamine-Culgoa rivers:** Wallam Creek [4] (26.9561 S 147.7439 E)

737-40; Narran River [4] (29.7167 S 147.4502 E) AA32086-89; Box Creek [5] (25.8033 S

148.2396 E) AA32262-64,66,68; Maranoa River [1] (26.4891 S 147.9815 E) AA33076;

Mungallala Creek [1] (28.0201 S 147.3810 E) AA33085; Neabul Creek [5] (27.7169 S

147.6995 E) AA33086-90. **Gwydir River:** Roumala Creek [5] (30.4956 S 151.1329 E)

188-92; Hall's Creek [4] (29.8595 S 150.5812 E) AA20742, (29.8631 S 150.5709 E)

AA32438-40; Gwydir River [1] (29.4941 S 150.1648 E) AA32431; Myall Creek [1]

(29.7548 S 151.0483 E) AA32435. **Lachlan River:** Lake Forbes [5] (33.3820 S 148.0010

E) AA10928-30,42-43; **Macquarie River:** Fine Gold Dam [6] (32.6651 S 149.1678 E)

AA11833-38; Macquarie River [7] (31.8887 S 148.0924 E) AA32167,68*,69, (32.2256 S

148.2478 E) AA32173-74, (31.8887 S 148.0924 E) AA32175,77. **Moonie River:** Moonie

River [7] (28.0909 S 149.2470 E) 733, (27.8948 S 149.5599 E) AA46402-05,07,08.

Murray-Riverina: Murray River [2] (36.0929 S 146.9484 E) 174, 620, AA20685-89.

Lower Murray: Murray River [6] (34.0860 S 140.7896 E) AA20514-17, (35.0333 S

139.3702 E) AA32107, (35.0867 S 139.3066 E) AA37272. **Murrumbidgee River:** Yass

River [5] (34.8646 S 149.0093 E) AA20629,32-34,37; Murrumbidgee River [4] (34.2729 S

146.0322 E) AA32127, (34.7546 S 146.5447 E) MB016-17, 22; Barren Box Swamp [8]

(34.1735 S 145.8017 E) AA32146,AA33212,14-19; Molonglo River [4] (35.2190 S

149.0010 E) AA42303-06; **Namoi River:** Gunnedah region [4] (30.9742 S 150.2588 E)

AA32414,15*,16, (30.9742 S 150.2588 E) AA32422; Namoi River [5] (30.9722 S

150.2542 E) AA32418,(30.2425 S 149.6839 E) AA32424-25, 27-28. **Paroo River:** Eulo

Creek [1] (28.1606 S 145.0448 E) AA18024. **Warrego River:** Warrego River [10]

(28.0684 S 145.6787 E) AA10161-64,AA10229-31, (28.3202 S 145.7274 E) AA13049-

50, (28.2051 S 145.7144 E) AA13057; Sanford Park Lagoon [6] (26.9234 S 146.0372 E)

AA13149-54; Sanford Park Waterhole [1] (26.9066 S 146.0333 E) AA13168; Quilberry

Waterhole [1] (27.0804 S 145.9231 E) AA13355.

Eastern Province: **Burdekin River:** Alpha Creek [1] (23.6520 S 146.6380 E) 00ChlBurd;

Mistake Creek [1] (22.5637 S 147.0728 E) AA19284. **Burnett River:** Cable House Creek

[6] (24.7966 S 152.4419 E) AA00240,47-49,AA00903,5; Munduberra region [10]

(25.5933 S 151.3153 E) AA11789,91,AA11821-28; Barambah Creek [1] (26.2380 S

151.9264 E) AA33554. **Fitzroy River (Qld):** Carnarvon Creek [1] (24.9239 S 148.6004

E) AA19310; Hutton Creek [4] (25.8143 S 148.3079 E) AA32033-35, (25.8103 S 148.299

E) AA32036, (25.8143 S 148.3079 E) AA32300; Dawson River [1] (25.3920 S 148.668

E) AA32283. **Moreton Island:** Blue Lagoon [2] (27.0874 S 153.4379 E) AA4270,87.

Bega River: Bega region [2] (36.6317 S 149.7954 E) 765,766; Bega River [1] (36.4621 S

149.8643 E) AA33490. **Clarence River:** Clarence River [7] (29.0513 S 152.5898 E)

193,195,196, (29.5490 S 152.6647 E) AA18685, (29.5376 S 152.5503 E) AA18706-08;

Broadwater Creek [3] (29.4277 S 153.0847 E) AA18589-91. **Clyde River-Jervis Bay:**

Blacks Waterhole [5] (35.1740 S 150.7003 E) BL001-005. **Hawkesbury River:** Nepean

River [3] (33.8570 S 150.6180 E) 997, (33.7278 S 150.6557 E) 741/744. **Hunter River:**

Hunter River [2] (32.1333 S 151.0333 E) 194,200; Lake St Claire [2] (32.3332 S

151.2911 E) AA18392-93; Williams River [6] (32.5820 S 151.7839 E) AA18398-03;

Lostock Dam [6] (32.3322 S 151.4543 E) AA18461-66. **Macleay River:** Georges Creek

[8] (30.8523 S 152.0871 E) 3050-55,57,58. **Richmond River:** Richmond River [1]

(28.8579 S 153.0257 E) AA18538. **Snowy River:** Orbost lagoon [1] (37.7139 S 148.4563

E) AA33489. **Thomson River:** Stradford wetlands [4] (38.0944 S 147.0577 E)

AA33462,69,71,72. **Latrobe River:** Traralgon Creek [2] (38.3192 S 146.4468 E)

AA33457, (38.1516 S 146.2830 E) AA33459.

Bass Province: Barwon River: Leigh River [3] (37.5526 S 143.9353 E) AA33453-55.

Hopkins River: Lake Bolac region [10] (37.7666 S 142.722 E) AA33440-46,48-50.

Millicent Coast: Gymbowen region [1] (36.7288 S 141.5861 E) AA33430; Goroke region

[1] (36.7185 S 141.4333 E) AA33431; Karnak region [1] (36.9009 S 141.5022 E)

AA33432; Edenhope region [4] (37.0288 S 141.2717 E) AA33433-34, (37.0901 S

141.1773 E) AA33435, (37.0662 S 141.2029 E) AA33436; Hamilton region [2] (37.4883

S 141.9952 E) AA43387-88; Penola region [1] (37.5477 S 140.8164 E) ABTC51959;

Blue Lake [1] (37.8444 S 140.7776 E) ABTC51976. **Mitchell River (Vic):** Paynesville

region [2] (37.9123 S 147.7194 E) AA33473-74; MacLeod's Morass [9] (37.8423 S

147.6303 E) AA33476-84. **Portland Coast:** Fitzroy River (Vic) [1] (38.1768 S 141.6861

E) AA33437.

Voucher numbers are for the Wildlife Tissue Collection at the University of Canberra

(http://iae.canberra.edu.au/cgi-bin/locations.cgi); photo vouchers available on request.

Appendix 3B – Haplotype frequencies for each drainage

freshwater biogeographic region	drainage	n	n haps	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Murray-Darling Basin	Murray	13	2																12	1											
	Murrumbidgee	21	2																										1	20	
	Lachlan	5	2																											4	1
	Macquarie	13	1																											13	
	Castlereagh	15	2								6																			9	
	Namoi	9	4				4			1									1											3	
	Gwydir	11	1				11																								
	Border Rivers	22	3		8	12													2												
	Moonie	7	2																6		1										
	Condamine	20	3																16	1					3						
	Warrego	18	2														11		7												
	Paroo	1	1																									1			
Central Australian Province	Bulloo	1	1																								1				
	Cooper	1	1														1														
Eastern Province	Richmond	1	1		1																										
	Clarence	10	1		10																										
	Macleay	8	1				8																								
	Hunter	16	5						1		7	2			4	2															
	Hawkesbury	3	2					2			1																				
	Clyde	5	2										1	4																	
	Bega	3	1										3																		
	Snowy	1	1										1																		

freshwater biogeographic region	drainage	n	n haps	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Eastern Province	Mitchell	11	2										8	3																	
	Thomson	4	1										4																		
	La Trobe	2	1										2																		
	Burnett	17	3																				1	10		6					
	Fitzroy-Dawson	7	3														1		5						1						
	Burdekin	2	1														2														
	Moreton Island	2	1	2																											
Bass Province	Barwon	3	2															1	2												
	Hopkins	10	1																10												
	Portland	1	1																1												
	Glenelg	2	1																2												
	Millicent	9	1																9												

Chapter 4

Rampant asymmetric introgression between subgenera of Australian freshwater turtles (*Chelodina*) illuminates dual mitochondrial genome capture.

Abstract

I investigate the extent of mitochondrial introgression in the phylogenetic history of south-eastern Australian freshwater turtles of the genus *Chelodina*. A multilocus phylogenetic approach with extensive geographic sampling of the common long-neck turtle *C. longicollis* and the broad-shelled turtle *C. expansa* revealed extensive mitonuclear discordance and high levels of mitochondrial paraphyly. Half of the *C. longicollis* haplotypes recovered are derived from *C. canni*. *Chelodina expansa* is entirely dissociated from its subgenus (*Macrochelodina*) and is instead characterised by mitochondrial lineages derived either from *C. longicollis* (57% of haplotypes) or *C. canni* (43% of haplotypes). This demonstrates the second reported incidence of dual mitochondrial genome capture, and the first for a reptile. Estimates of common ancestry for mitochondrial and nuclear lineages, plus coalescent simulations of gene flow suggest these patterns are not a result of deep coalescence. Rather, mitonuclear discordance is a result of multiple and ancient Plio/Pleistocene introgressive events within and between subgenera. I uncover strong signals of unidirectional gene flow from *C. longicollis* to *C. expansa* and attribute this to the neutral effect of demographic disparity driving introgression from the common into the rare species.

Introduction

Discordance between a species tree and its embedded gene trees is an "inescapable biological reality" (Knowles, 2009; Knowles & Kubatko, 2010). This has necessitated a shift in thinking from the straightforward use of gene trees to directly infer species phylogeny, to the application of mixed mutational models and the coalescent framework to accommodate inherent variability among gene lineages (Ronquist & Huelsenbeck, 2003; Wakeley, 2008). This variability can highlight important evolutionary processes that blur

species delimitation such as gene duplication, natural selection, incomplete lineage sorting, and hybridisation. Far from being a hindrance to evolutionary inquiry, species tree and gene tree discordance improves our understanding of the evolutionary process (Spinks & Shaffer, 2009; Linnen 2010).

Simply observing gene tree and species tree discordance does not inform the source of that discordance. Incomplete lineage sorting and hybridisation can generate similar phylogenetic patterns of paraphyly despite each occurring on opposite sides of a speciation event. Coalescent-based methods can go some way to differentiate between the two processes however distinction is not always possible particularly in the case of recently diverged species with large population sizes (Sang & Zhong, 2000; Funk & Omland, 2003; Linnen & Farrell, 2007; Belfiore *et al.*, 2008; McKay & Zink, 2010). Reports of mitonuclear discordance in distantly related non-sister species highlight that hybridisation between distant taxa is not as rare as previously considered (Georges *et al*., 2002; Zha *et al*., 2008, Kronforst, 2008; Kubatko, 2009; Toon *et al*., 2012). Given the possibility of natural hybridisation between even distantly related species, multilocus phylogenetic studies need to extend beyond immediate sister taxa, and should include multiple and geographically dispersed individuals to avoid monophyly being observed because of inadequate sampling (McKay & Zink, 2010).

Here I use a multilocus dataset with wide taxonomic and geographic sampling to explore phylogenetic relationships in the Australasian freshwater turtle genus *Chelodina*. The genus comprises obligate freshwater long-neck and snake-neck turtles and is divided into three subgenera – *Chelodina* which comprises *C. longicollis*, *C. canni, C. pritchardi*, *C. novaeguineae, C. mccordi, C. steindachneri*; *Macrochelodina* which comprises *C. expansa*, *C. oblonga* (formerly *rugosa,* see Kennett *et al*., 2014), *C. parkeri*, *C. burrungandjii*; and *Macrodiremys* which comprises solely *C. colliei* (Georges & Thomson,

2010). I was drawn to explore possible discordance between the species tree and embedded gene trees in this group as natural hybrids are common and have been documented within and between subgenera. *Chelodina longicollis* and *C. canni* hybridise on the boundaries of their distributions in coastal Queensland (Georges *et al*., 2002), and *C. oblonga* and *C. burrungandjii* hybridise where their ranges overlap in Arnhem Land of northern Australia (Georges *et al*., 2002). Between subgenera, natural hybrids have been documented between *C. canni* and *C. oblonga* where the two species are sympatric (Georges *et al*., 2002). These examples and additional studies of cyrptodiran turtles (Stuart & Parham, 2004; Spinks & Shaffer, 2007, 2009; Freedberg & Myers, 2012) demonstrate hybridisation and introgression among close and distantly related turtles may be reasonably widespread in sympatric or parapatric species.

The potential for introgressive hybridisation poses particular challenges for inferring species in single gene studies, and for inferring an absence of introgression when wide geographic sampling from regions of ancient or recent sympatry is not included. Early molecular phylogenetic work on *Chelodina* suffered these challenges and provided only fragmentary insight into the evolutionary history of the genus. Where nuclear and mitochondrial markers were used, the potential to uncover introgressive hybridization was impeded by poor taxonomic and geographic representation of samples (Georges *et al*., 1998). Where multiple taxa and the full geographic extent of species were represented, studies only explored relationships using nuclear markers (allozymes) and not mitochondrial markers (Georges & Adams, 1992; Georges *et al*., 2002). Given the seemingly high propensity for hybridisation within *Chelodina* I see a need for targeted exploration of potential mitonuclear discordance to evaluate the extent and frequency of this phenomenon.

I take a multilocus approach to evaluate phylogenetic relationships within the *Chelodina* and to explore the evolutionary history of *C. expansa* and *C. longicollis* in particular. Aside from belonging to different subgenera, *C. expansa* and *C. longicollis* also differ in morphology, life history, behaviour, and ecology (Cann, 1998). Despite this, they are a good candidate species pair for testing hybridization owing to their extensive sympatric range in Australia's inland Murray-Darling Basin, and southeast Queensland coast (Figure 4.1). It must be noted that the data regarding mitochondrial variation have been obtained as part of a larger phylogeographic study which investigated *C. expansa* and *C. longicollis* separately (Hodges *et al*., 2014; Hodges *et al*., 2015). Questions regarding introgression and how mitochondrial sequence variation in *C. expansa* relates to that found in *C. longicollis* remain unexplored. The present study fills these gaps and interprets mitochondrial sequence variation in the context of the broader species tree.

Specifically I investigate the following propositions: (1) that mitochondrial and nuclear gene tree topologies track the same evolutionary history; (2) that *C. expansa* and *C. longicollis* are reciprocally monophyletic for the species tree; and (3) that there is no evidence of gene flow between *C. expansa* and *C. longicollis*. I use the phylogeny of the genus *Chelodina* proposed in Georges *et al*. (2002) as a hypothesis against which I compare findings, and I interpret results with reference to the demographic and biogeographic arena that likely shaped sympatric populations throughout their history.
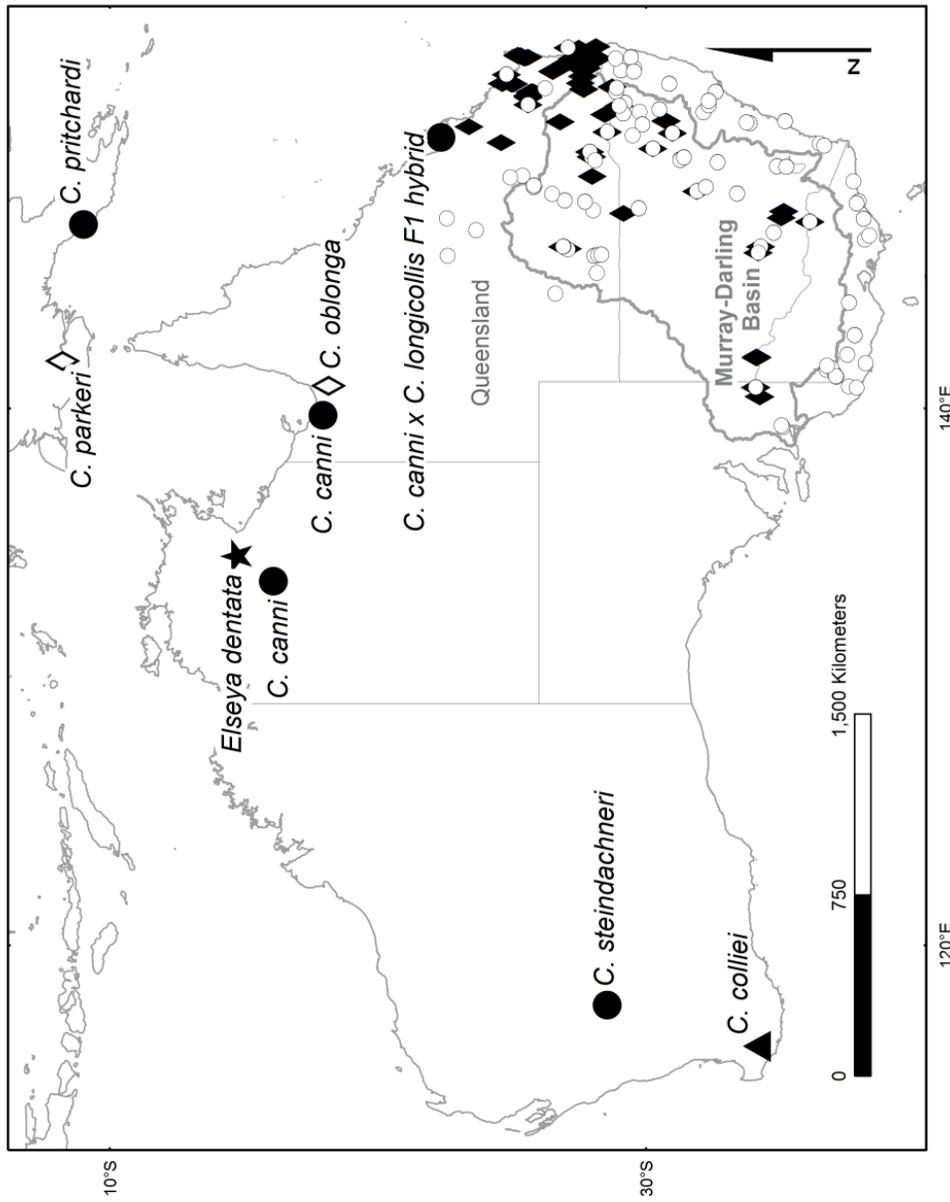
Figure 4.1 Australia and southern New Guinea with all collection localities of *C. longicollis* (open circles) and *C. expansa*   (black diamonds), and collection locations of out-group taxa and the *C. canni x C. longicollis* hybrid. The thin grey lines indicate Australian state borders, and the thick grey line delineates the Murray-Darling Basin.

Materials and Methods

Taxon sampling

For the mitochondrial dataset, I used comparable *ND4* and *control region* sequences from previous mitochondrial phylogeographic studies to yield 21 *C. expansa* haplotypes from 164 individuals (Hodges *et al*., 2014), and 28 *C. longicollis* haplotypes from 274 individuals (Hodges *et al*., 2015). For the nuclear dataset I sequenced three nuclear genes for 6 specimens each of *C. expansa* and *C. longicollis* representing major mitochondrial haplogroups. Representatives of the three subgenera were also included in each dataset: *Chelodina* was represented by *C. canni* (two specimens in the mitochondrial dataset, and one specimen in the nuclear dataset) and one specimen each of *C. pritchardi*, *C. steindachneri* and an F$_1$ *C. canni* x *longicollis* hybrid (adult male). *Macrochelodina* was represented by one specimen each of *C. oblonga* and *C. parkeri*. *Macrodiremys* was represented by its nominate *C. colliei*. The final mitochondrial dataset comprised 58 specimens, and the nuclear dataset comprised 20 specimens (specimen list provided in Appendix 4A). All phylogenetic trees were rooted with *Elseya dentata*. Tissue collection for *C. expansa* and *C. longicollis* follow Hodges *et al*. (2014). Tissues for other members of the *Chelodina* and for *E. dentata* were sourced from skin, muscle and blood samples held at the University of Canberra Wildlife Tissue Collection (GenBank UC <Aus>).

DNA sequencing

DNA was isolated using a standard salt extraction protocol (Sambrook & Russell, 2001). For the mitochondrial dataset I targeted a 630 bp fragment of the mitochondrial *ND4* gene, and a 470 bp fragment of the mitochondrial *control region* including part of *tRNA^{Proline}* (hereafter collectively referred to as *control region*, *CR*). Procedures for mitochondrial

fragment amplification, primers, and sequencing were the same for all taxa and are described in Hodges *et al*., (2014). Tests for mitochondrial authenticity for *C. expansa* and *C. longicollis* are also described in Hodges *et al*. (2014) and are not revisited here except to say that the results of all four tests were consistent with the amplification of genuine mitochondrial sequences. The final alignment for the mitochondrial dataset comprised 595 bp *ND4*, 69 bp *tRNA^{Pro}*, and 391 bp *control region.*

For the nuclear dataset I targeted a 380 bp fragment of the nuclear proto-oncogene *C-mos* as previous work on pleurodiran (Georges *et al*., 1998) and cyrptodiran turtles (Le *et al*., 2006; Le & McCord, 2008) revealed strong interspecific diversity at this locus. A 465 bp fragment from an intron in the *R35* neural transmitter gene, and a 475 bp fragment from an intron in the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene also were targeted as they revealed different levels of diversity in other turtle systematic studies (*R35*: Vargas-Ramirez *et al*., 2010; Engstrom, 2004; *Gapdh*: Spinks & Shaffer, 2005). Primer details and PCR amplification, sequencing and editing details are given in supporting material ([Appendix 4B](#), [4C](#)). Sequences were aligned using ClustalX 1.81 (Thompson *et al*., 1997) to yield final alignments of 357 bp *C-mos*, 453 bp *R35*, and 411 bp *Gapdh*.

Phylogenetic analyses

Four phylogenetic analysis methods were used to allow complete exploration of the dataset and comparison of topology among analyses. Methods included maximum parsimony (MP), maximum likelihood (ML), mixed model Bayesian analyses, and Bayesian coalescent analyses. Prior to phylogenetic analysis I examined substitutional saturation of each coding gene using DAMBE v 5.2.15 (Xia & Lemey, 2009) and the best model of evolution for each locus using MODELTEST 3.7 (Posada & Crandall, 1998). I found no

evidence of saturation in coding loci (P<0.001 in all cases). Mean within and among

lineage divergence were calculated using p-distances in MEGA5 (Tamura *et al*., 2011).

Maximum parsimony and ML analysis were performed on each dataset using

PAUP* v.4.0b10 (Swofford, 2002). Partition homogeneity tests could not reject the null

hypothesis of homogeneity among loci in each dataset (mtDNA: P=0.11; nDNA: P= 0.06-

0.82) and loci within each dataset were concatenated for each analysis. Maximum

parsimony used a heuristic search method, TBR branch swapping, and assumed equally

weighted and unordered character changes. Gaps were coded as a fifth state and branch

support was estimated using 10,000 non-parametric pseudo-replicates. I consider bootstrap

values in excess of 70 to be indicative of support for the associated node, and bootstrap

values in excess of 90 to be strong support. ML analyses used a heuristic search method

with substitution estimates and gamma parameters estimated using AIC criteria in

MODELTEST 3.7. The concatenated mitochondrial dataset fit the TVM+G model, and the

concatenated nuclear dataset fit the K81uf+G model. Support for ML clades was

calculated using 100 pseudo-replicates for the mitochondrial dataset and 1,000 pseudo-

replicates for the nuclear dataset. Both datasets used stepwise addition, 10 random-

sequence addition replicates, and TBR branch swapping. The mitochondrial dataset

produced 570 equally parsimonious trees (length=710), and a tree likelihood score of -

4507.17. The nuclear dataset produced 36 equally parsimonious trees (length =155), and a

tree likelihood score of -2388.46.

Mixed model Bayesian analyses were implemented in MRBAYES v3.1 (Ronquist &

Huelsenbeck, 2003) for each dataset to overcome restrictions of model homogeneity across

all lineages. Each dataset was partitioned by gene region and by codon position for coding

sequence (5 partitions each) as harmonic mean Bayes factors (Kass & Raftery, 1995)

estimated in the program TRACER v1.5 (Rambaut & Drummond, 2007) preferred this

regime compared to less complex arrangements (partitioned by locus, and partitioned).

The following models of evolution were used: *C-mos* positions 1 and 2, JC; *C-mos* position

3, K2P; *R35* and *Gapdh*, HKY; *ND4* positions 1 and 3, GTR; *ND4* position 2, HKY+I;

tRNA^Pro, K80; and *control region*, TrN+G. The parallel processor version of MRBAYES

v3.1 was used with 6 replicate simultaneous runs of Metropolis-coupled Markov chain

Monte Carlo (MCMCMC) sampling to ensure overall tree-space was well sampled and to

avoid becoming trapped in local optima. Analyses for both datasets were conducted over

10 million generations, with 8 incrementally heated chains, 2 swaps per generation, and

sampling every 1,000 generations. Model parameters and rate multipliers we unlinked

across all classes using the "prset ratepr = variable" command to accommodate rate

variation among partitions. Post burnin parameters and trees from all 6 independent runs

were combined to create consensus phylograms. Clade credibilities were taken from the

majority rule consensus tree and clades with support above 95% were considered very

robust. Diagnostics to confirm chain stationarity and convergence included standard

deviations of split frequencies (Huelsenbeck *et al*., 2001), potential scale reduction factors

(Barley, 2010), and visual inspection of stationarity of log-likelihood values and effective

sample sizes using TRACER v1.5. The 'cumulative' and 'compare' diagnostic functions in

AWTY (Wilgenbusch *et al*., 2004) were used to determine 10% burnin for both datasets.

Standard deviations of split frequencies were 0.0037 for the nuclear dataset and 0.0035 for

the mitochondrial dataset and potential scale reduction factors were 1.000 for both. ESS

values were always above 3,000 for the mitochondrial dataset, and above 6,000 for the

nuclear dataset.

To estimate the overall species tree in a coalescent framework, a Bayesian

coalescent approach was implemented in BEST v2.3 (Edwards *et al*., 2007; Liu & Pearl,

2007; Liu *et al*., 2008). This method avoids concatenation required in likelihood and other

Bayesian methods, and uses importance sampling to infer the species tree after estimating each gene tree individually. Only the nuclear dataset was investigated in this analysis as earlier methods demonstrated mitonuclear discordance and the possibility of mitochondrial introgression which would violate assumptions of the analysis. Importantly for the nuclear dataset, BEST can take incomplete lineage sorting into account and thus provide a more robust inference of the species tree. Species assignment was determined *a priori*, and the F_1 *C. canni* x *C. longicollis* hybrid was designated as an individual species. BEST was conducted using 2 simultaneous MCMCMC runs for 45 million generations using chain heating and model parameters as per the MRBAYES analysis. Standard diagnostics were used to determine chain stationarity and convergence, and analysis in TRACER v1.5 indicated the first 4.5 million steps (10%) were sufficient burnin.

Tests of mitochondrial monophyly

Evidence of hybridisation between *Chelodina* species suggests introgressive processes could affect the mitochondrial dataset. Mitochondrial introgression leads to nonmonophyly of species in the mitochondrial gene tree, and this possibility was tested in *C. expansa* and *C. longicollis* using Bayesian constraint analyses on the mitochondrial dataset in MRBAYES. In four separate analyses I constrained to monophyly the *C. expansa* subgeneric group, the *C. longicollis* subgeneric group, all *C. expansa*, and all *C. longicollis*. The same chain heating, model parameters, and chain convergence diagnostics were used as per the MRBAYES analysis. Posterior probabilities of constrained versus unconstrained trees were compared using harmonic mean Bayes factors.

Divergence dating

BEAST 2.0.2 (Bouckaert *et al*., 2013) was used to estimate timing of molecular divergence within each dataset. Data were partitioned by locus (3 partitions each), substitution models and clock parameters were unlinked, and the best model of nucleotide substitution for each partition was determined automatically using the add-on RB BEAST. Each locus was tested for clocklike evolution (PAUP* v.4.0b10) and likelihood ratio tests were used to assess significant differences between likelihood scores of trees with a free rates model and those with a global molecular clock enforced. The null hypothesis of clocklike evolution could not be rejected for *ND4*, *tRNA^Pro^*, and *R35* and I estimated these loci under a strict molecular clock. The null hypothesis was rejected for *CR*, *Gapdh* and *C-mos* (all P<0.05), and these loci were estimated under a relaxed uncorrelated lognormal molecular clock.

For each dataset internal calibration was based on known divergence time between *Elseya* and *Chelodina* generic groups. Earliest diagnostic *Elseya* and *Chelodina* fossils in Eastern Australia are dated to 45 Mya in the mid-Eocene (de Broin & Molnar, 2001). In molecular phylogenetic studies using nuclear markers *R35*, and *Rag-1* and mitochondrial *cytochrome b*, *Elseya*/*Chelodina* divergence is estimated at 46.74 ± 5.49 Mya (Near *et al*., 2005). These corroborate dates provide extremely robust information for internal calibration. I followed Near *et al*., (2005) and characterised the basal node representing *Elseya dentata*/*Chelodina* using a normally distributed prior with a mean of 46.74 and standard deviation of 2.2 to reflect the 5.49 error in the 95% highest posterior density (HPD).

For the mitochondrial dataset, I applied a divergence rate of 0.895% per Myr (Zamundio & Greene, 1997; Rabosky *et al.*, 2007) scaled per lineage per Myr and modelled under a normal distribution (mean, 0.004475; standard deviation, 3.0E-4). This

divergence rate is consistent with a rate estimated from fossil chelid turtles by Georges *et al*. (2013) (0.86% per million years) and has been applied successfully elsewhere (Hodges *et al*., 2014; Todd *et al*., 2014; Hodges *et al*., 2015). A Yule branching process appropriate to interspecific data was used, and starting trees were estimated using the UPGMA method. MCMC chains were run for 40 million generations with sampling every 1,000 steps yielding a total of 40,000 trees. Convergence and burn-in were assessed in TRACER using methods previously described, and chronophylogenies were visualised in FIGTREE v1.3.1 (Rambaut, 2009). TREEANNOTATOR v1.6.1 was used to calculate maximum clade credibility for each tree, and apply a burnin of 6,000 for both datasets. Mitochondrial and nuclear dataset parameters were also run with an empty alignment to examine the influence of the priors on posterior distributions. In analyses without data the posterior distributions were highly similar to the original priors, and in analyses with full sequence alignments the posterior distributions were orders of magnitude different to the original priors indicating informative data (Sanders & Lee, 2007).

Tests for gene flow

To investigate the possibility of post divergence gene flow between *C. longicollis* and *C. expansa*, isolation with migration analysis was implemented in the program IM (12.17.09; Hey, 2010). This coalescent analysis examined four loci (concatenated mitochondrial sequences + three nuclear loci) and only samples with the full complement of three mitochondrial and three nuclear loci were used, resulting in 6 *C. longicollis* and 5 *C. expansa* samples. To ensure IM assumptions of selective neutrality and absence of within-loci recombination were met, coalescent simulations in DNAsp 5.1 (Librado & Rozas, 2009) were used to calculate Tajima's D (Tajima, 1989) and to implement the four-gamete test (Hudson, 1984). No significant signals of selection were recovered for each locus

(P>0.1), and the minimum number of recombinant events was always zero for nuclear loci, indicating the data did not violate key assumptions of the IM program (Hey & Nielson, 2004). A single index of mean generation time for *C. expansa* and *C. longicollis* was calculated using the equation $G = \alpha + (s/(1-s))$ where α is the average age of maturity and *s* is the average adult survival rate (Sæther *et al.*, 2005). I used a combined average age of maturity $\alpha = 10$ years (Spencer, 2002; Kennett *et al.*, 2009), and a combined average adult survival rate $s = 0.843$ (Spencer & Thompson, 2005; Parmenter, 1985; Roe & Georges, 2008) to provide an estimate of $G = 15$. The same mitochondrial mutation rate was applied as per the divergence dating analysis (0.895% sequence divergence per million years), scaled per year, per locus according to the IM documentation resulting in a rate of 4.654 x 10^{-6}. Inheritance scalars were 0.25 for the concatenated mitochondrial dataset, and 1 for each nuclear locus to account for differences in the effective population size of each genome. I applied the HKY model of evolution to the mitochondrial dataset, to *Gapdh*, and *R35*; and the infinite sites model to *C-mos*. Population size change was incorporated into the IM model (–j 9 option) as lineages of *C. expansa* displayed signals of recent demographic expansion (Hodges *et al.*, 2014).

Preparatory analyses were run using wide priors to investigate mixing and convergence requirements. The final analysis used the following priors: population size (q) of 4 for *C. longicollis* and 6 for *C. expansa*, population splitting time (t) of 4, migration rate prior (m1) describing gene flow from *C. longicollis* to *C. expansa* (backwards in time, in the coalescent direction) of 35, and a migration rate prior (m2) describing gene flow from *C. expansa* to *C. longicollis* (backwards in time, in the coalescent direction) of 200. Three independent replicates were run with different random starting seeds using 20 parallel Markov chains with geometric heating parameters –g1 0.9 and –g2 0.85. Analyses were run for 10 million steps with the initial 2 million discarded as burnin. Each analysis was

characterised by high Markov chain update and swapping rates, indicating adequate mixing in each run. Convergence of Markov chains on the same optima was confirmed with high ESS values (>110), low autocorrelations, and similar parameter distributions in each individual run. Parameter estimates were taken from the peak of posterior distributions (Neilson & Wakeley, 2001) and confidence intervals were taken from the 90% HPD. The geometric mean of mutation rate scalars estimated in each run was used to convert parameters to biologically meaningful values using formulas provided in the IM documentation. An analysis run without data (-j0 option) determined the priors were not driving the signals recovered in the final analysis.

Results

Alignment data

The concatenated mitochondrial dataset displayed 290 variable sites of which 167 are parsimony informative. *Chelodina longicollis* and *C. expansa* shared mitochondrial haplotypes on three occasions. A 2 bp diagnostic indel was present in the mitochondrial control region at position 672-673 in the concatenated dataset. Samples from mitochondrial clade A (samples 7-12) exhibited a CA at this position, and samples from clade B (samples 1-6, *C. canni*, and *C. canni* x *C. longicollis* hybrid) except for *C. pritchardi* exhibited a 2bp gap. This indel appears to be diagnostic of mitochondrial clade assignment in *C. expansa* and *C. longicolli*s. The concatenated nuclear dataset (excluding *E. dentata*) is conserved with 53 variable sites of which 30 are parsimony informative. Sequencing at the *Gapdh* locus failed for three samples: *C. steindachneri*, *C. parkeri*, and a sample representing *C. expansa* haplotype 01 (AA46516). These samples were excluded from analyses of the nuclear dataset except the divergence dating analysis where the *Gapdh* locus was treated as missing data for these samples.

Phylogenetic results

Nuclear dataset

Nuclear topologies always recovered reciprocal monophyly of the subgenera *Chelodina* (clade 1) and *Macrochelodina* (clade 2) (Figure 4.2), and monophyly of *C. longicollis* and *C. expansa* within each. Uncorrected ingroup p-distances for the nuclear dataset ranged from 0.1% to 2.4% (Table 4.1). Average p-distance within *Chelodina* subgenus (clade 1) is 0.4% (0.1% - 0.9%) and within *Macrochelodina* subgenus (clade 2) is 0.9% (0.1% - 2.1%).

Average p-distance within *C. longicollis* is 0.1% (0.1% - 0.2%) and within *C. expansa* was 0.4% (0.1% - 0.7%). Nuclear relationships among the three subgenera were counter to previous hypotheses. Georges *et al*. (2002) placed *Macrodiremys* as a sister subgenera to *Chelodina*, with the *Macrochelodina* placed outside this pair. I found an alternate structure with *Macrodiremys* positioned outside a *Chelodina*, *Macrochelodina* subgeneric pair. This relationship was recovered in likelihood and all Bayesian analyses (MRBAYES, BEAST, & BEST) though with poor to moderate support for the *Chelodina*/*Macrochelodina* pair (likelihood bootstrap: 51; MRBAYES posterior probability: 83; BEAST posterior probability 50; BEST posterior probability: 71). Parsimony indicated poor support for all arrangements among the subgenera and the bootstrap 50% majority-rule consensus tree yielded a trichotomy.

Mitochondrial dataset

Phylogenetic analyses on the mitochondrial dataset yielded very similar topologies. Comparison with the nuclear dataset demonstrates some phylogenetic concordance (Figure 4.2): A *C. oblonga* and *C. parkeri* sister pair represents the subgenus *Macrochelodina*; a clade containing *C. pritchardi*, *C. canni* and the F_1 *C. canni* x *C. longicollis* hybrid

represents part of the subgenus *Chelodina*, with *C. longicollis* in clade A (following

haplogroup designation in Hodges *et al*., 2014 and in Hodges *et al*., 2015) representing the

remainder. This congruence between the mitochondrial and nuclear topologies is the

exception rather than the rule and serves more generally to demonstrate extensive

mitochondrial paraphyly across *Chelodina* and *Macrochelodina* subgenera. Of the 21

haplotypes observed among the 164 *C. expansa* individuals, not one is grouped with con-

subgeners *C. oblonga* and *C. parkeri*. Instead *C. expansa* occurs with the paraphyletic *C.*

longicollis across two internal mitochondrial clades A and B within the subgenus

Chelodina.

Clade A reflects the nuclear tree position of *C. longicollis* and contains 46% of *C.*

longicollis haplotypes (haplotypes 1-13) and 57% of *C. expansa* haplotypes (haplotypes 1-

12), with *C. longicollis* haplotype 3 and *C. expansa* haplotype 1 identical. Clade B is sister

to *C. canni* and contains the F$_1$ *C. canni* x *C. longicollis* hybrid, the remaining 54% of *C.*

longicollis haplotypes (haplotypes 14-28), and the remaining 43% of *C. expansa* haplotypes

(haplotypes 13-21). *Chelodina longicollis* haplotype 16 is identical to *C. expansa*

haplotype 14, and *C. longicollis* haplotype 22 is identical to *C. expansa* haplotype 13.

Table 4.1 Summary of uncorrected p-distances among non-identical *Chelodina sp.* sequences for the concatenated nuclear dataset. Clades designation follows Figure 4.2

nDNA	mean	SD	range
all samples	1.1%	0.7%	0.1% - 2.4%
within			
clade 1	0.4%	0.3%	0.1% - 0.9%
clade 2	0.9%	0.7%	0.1% - 2.1%
Chelodina		*same as clade 1*	
Macrochelodina		*same as clade 2*	
C. longicollis	0.1%	0.0%	0.1% - 0.2%
C. expansa	0.4%	0.2%	0.1% - 0.7%
between			
1 - 2	1.6%	0.3%	0.11% - 2.4%
C. longicollis - C. expansa	1.6%	0.1%	0.14% - 1.9%

Figure 4.2 (previous page) Mitochondrial (left) and nuclear (right) phylograms inferred through maximum likelihood analysis. The subgenus *Chelodina* is presented in orange, the subgenus *Macrochelodina* in blue, out-group is not shown. Numbers indicate node support for parsimony bootstrap replicates/maximum likelihood bootstrap replicates/mixed model Bayesian posterior probabilities (Mr Bayes). Branch support is only given where a single value exceeds 90, except for the position of *C. colliei* in the nuclear dataset. A single 100 indicates that the node received 100% bootstrap and posterior probability for all analyses. -- indicates that branch support is not applicable owing to differences in topology between analyses. Grey lines linking phylograms highlight congruent phylogenetic placement between datasets. Black diamond, black circle, and open circle indicate the three instances of haplotype sharing between *C. longicollis* and *C. expansa*. Scale bar applies to both phylograms and indicates substitutions per site.

Bayesian constraint analyses tested conformations to mitochondrial monophyly of the *Chelodina*, the *Macrochelodina*, and of *C. longicollis*, and *C. expansa* in four separate analyses. In all cases posterior probability distributions of constrained trees had significantly lower log likelihood scores than unconstrained topologies. Furthermore, Bayes factors supported unconstrained trees over all constraints to monophyly and decisively reject all confirmations to monophyly tested. This provides strong support for the mitochondrial paraphyly observed, demonstrates that the mitochondrial genome is tracking an evolutionary history different to that of the species tree.

Uncorrected mean p-distance between clade A and B is high at 4.4% (3.9% - 5%) which also reflects the mean p-distance between *C. longicollis* and *C. expansa* (Table 4.2). Mean within clade p-distance is low in clade A (0.8%, 0.1% - 1.5%) and clade B (0.5%, 0.1% - 1.2%). Paraphyly of *C. longicollis* and *C. expansa* and their close mitochondrial relationship is reflected by a large average within species p-distance of 2.5% in each case, and almost identical p-distance range (*C. longicollis*: 0.1% - 4.9%; *C.* expansa: 0.1% - 4.8%). Average sequence p-distance between the *C. oblonga*, *C. parkeri* pair and members of the subgenus *Chelodina* (excluding *C. steindachneri*) is 14.26% which reflects the true mitochondrial distance between *Macrochelodina* and *Chelodina* were it not for *C. expansa* paraphyly. The maximum uncorrected p-distance in the mitochondrial dataset is 15.4% which is driven exclusively by *C. expansa* paraphyly and characterises the upper range of sequence divergence within the *Macrochelodina*. The sister pair of clade B and *C. canni* are separated by a low mean p-distance (1.85%, 1.57% - 2.15%), and within clade B both *C. longicollis* and *C. expansa* are equally close to the F_1 *C. canni* x *C. longicollis* hybrid (mean p-distance *C. longicollis* 0.49%, 0.2% - 0.98%; *C. expansa* 0.45%, 0.09% - 0.59%).

The placement of *Macrodiremys colliei* in the mitochondrial dataset is inconclusive and incongruent with the nuclear data. This monotypic species lies between *C.*

steindachneri and the remainder of *Chelodina*, however support for a *Macrodiremys*,

Chelodina node varies across analyses (parsimony bootstrap: 59; likelihood bootstrap: 50;

MRBAYES posterior probability: 93; BEAST posterior probability 75).

Estimates of TMRCA

The three subgenera share a MRCA broadly in the early to mid-Miocene with nuclear and

mitochondrial estimates at 14.3 Mya and 22.52 Mya respectively (Table 4.3). Subgenera

Chelodina and *Macrochelodina* share a MRCA later in the mid Miocene at 12.58 Mya

estimated from nuclear DNA. This date could not be estimated in the mitochondrial dataset

owing to species tree incongruence. Speciation within the *Chelodina* and *Macrochelodina*

occurred concurrently throughout the late Miocene. *Chelodina oblonga* and *C. parkeri*

represent the true placement of the *Macrochelodina* in the mitochondrial dataset, and both

datasets suggest a MRCA of this subgeneric group in the late Miocene (7.10 Mya, nDNA:

10.27 Mya, mtDNA). The placement of *C. canni* and *C. pritchardi* is also concordant

between datasets and TMRCA for this pair is in the early to middle Pliocene at 5.22 Mya

for mitochondrial and 3.19 Mya for nuclear DNA. Based on nuclear DNA, a Pliocene

TMRCA characterises *C. expansa* (4.86 Mya) and *C. longicollis* (2.99 Mya). The

mitochondrial MRCA for *C. expansa* is the same as the MRCA for the whole *Chelodina*

generic group (22.52 Mya) and reflects *Macrochelodina* paraphyly.

Table 4.2 Summary of uncorrected p-distances among non-identical *Chelodina sp.* sequences for the concatenated mitochondrial dataset. Clades designation follows Figure 4.2.

mtDNA	mean	SD	range
all samples	4.0%	3.7%	0.1% - 15.4%
within			
clade A	0.8%	0.4%	0.1% - 1.5%
clade B	0.5%	0.2%	0.1% - 1.2%
Chelodina	3.1%	2.6%	0.1% - 10.8%
Macrochelodina	4.5%	4.7%	0.1% - 15.4%
C. longicollis	2.5%	2.0%	0.1% - 4.9%
C. expansa	2.5%	1.9%	0.1% - 4.8%
between			
A - B	4.4%	0.2%	3.9% - 5.0%
C. longicollis - C. expansa	4.4%	0.2%	3.9% - 5.0%
hybrid - *C. longicollis* **clade B**	0.49%	0.20%	0.2% - 0.98%
hybrid - *C. expansa* **clade B**	0.45%	0.09%	0.29% - 0.59%

Table 4.3 Time to most recent common genetic ancestor inferred in BEAST analyses of mitochondrial and nuclear datasets. Molecular dates not provided (n/a) are erroneous owing to mitochondrial paraphyly. * *Macrodiremys* does not affect this mitochondrial estimate as this taxon is placed within the *Chelodina* . ** *C. steindachneri* is excluded from the *Chelodina* subgeneric group in the mitochondrial estimate as inclusion is not supported in the mitochondrial phylogeny. *** This estimate for mitochondrial DNA is based on the TMRCA for all *C. longicollis* in clade A only as this is the putative true *C. longicollis* clade.**

TMRCA of taxa within the genus *Chelodina*	dataset	molecular date (Mya)	95% HPD (Mya)	Neogene period
*Chelodina, Macrochelodina, Macrodiremys**	mtDNA	22.52	(18.36 - 26.81)	early to mid Miocene
	nDNA	14.3	(8.08 - 21.22)	
Chelodina & Macrochelodina	mtDNA	n/a	n/a	mid Miocene
	nDNA	12.58	(6.92 - 18.32)	
Macrochelodina	mtDNA	n/a	n/a	late Miocene
	nDNA	9.58	(4.99 - 14.59)	
C. oblonga & C. parkeri	mtDNA	10.27	(7.44 - 13.29)	late Miocene
	nDNA	7.10	(3.06 - 11.41)	
*Chelodina***	mtDNA	6.53	(4.89 - 8.26)	late Miocene
	nDNA	8.52	(3.77 - 13.42)	
C. canni & C pritchardi	mtDNA	5.22	(3.71 - 6.79)	early to mid Pliocene
	nDNA	3.19	(0.85 - 6.10)	
C. expansa	mtDNA	n/a	n/a	early Pliocene
	nDNA	4.86	(1.94 - 8.36)	
*C. longicollis****	mtDNA	2.21	(1.35 - 3.08)	mid to late Pliocene
	nDNA	2.99	(0.79 - 5.76)	
C. canni & hybrid	mtDNA	2.24	(1.41 - 3.16)	late Pliocene
	nDNA	1.96	(0.22- 4.12)	
hybrid, *C. longicollis*, & *C. expansa*	mtDNA	1.50	(0.89 - 2.17)	early Pleistocene
	nDNA	n/a	n/a	

Mitochondrial clade A reflects the species tree position of *C. longicollis* and both datasets estimate a *C. longicollis* TMRCA in the late Pliocene at 2.21 Mya for mitochondrial and 2.99 Mya for nuclear DNA. A similar late Pliocene common ancestor is estimated in both datasets for *C. canni* and the F_1 *C. canni* x *longicollis* hybrid (mtDNA: 2.24 Mya; nDNA: 1.96 Mya). The mitochondrial estimate of 2.24 Mya also describes the MRCA of *C. canni* and all clade B taxa. Common ancestry for clade B specifically is 1.5 Mya (0.89 – 2.17 Mya 95% HPD) in the early Pleistocene, and this date also corresponds to common ancestry of both *C. longicollis* and *C. expansa* with the F_1 *C. canni* x *C. longicollis* hybrid.

Coalescent isolation & migration analysis

Coalescent analysis using IM suggest the data broadly fit an island model where species divergence is ancient, and gene flow between species is steady (Hey & Nielson, 2004). The posterior distribution for population split time (t) between *C. longicollis* and *C. expansa* does not include zero and has a peak at 1.18 Mya in the early Pleistocene (Figure 4.3B). This date is not meaningful however as I cannot reject very ancient population split times. An essentially infinite posterior on split time is consistent with ancient divergence between *C. longicollis* and *C. expansa* estimated in BEAST on the nuclear dataset (13.27 Mya). Effective population size for the ancestor (qA) of *C. longicollis* and *C. expansa* cannot be reliably estimated, except to say that is larger than both descendant species. Effective population size of *C. longicollis* (q1) has a clear peak at 100,608 individuals and *C. expansa* (q2) has a much smaller effective population of 29,093 individuals (Figure 4.3A).

Gene flow between *C. longicollis* and *C. expansa* is unidirectional (Figure 4.3C) with strong signals of gene flow from *C. longicollis* to *C. expansa* (m2, forwards in time in

the conventional direction). No gene flow from *C. expansa* to *C. longicollis* (m1, forwards in time) is inferred as the lower 90% HPD for this parameter includes the lowest bin indicating zero gene flow in this direction. Gene flow forwards in time from *C. longicollis* to *C. expansa* peaks at 8.48 x 10^{-5} migrants per generation per gene copy. This figure is not meaningful in itself however as the curve for is high and flat, indicating that larger estimates of gene flow from *C. longicollis* to *C. expansa* are equally probable.

Discussion

All mitochondrial sequences in *C. expansa* are derived either from *C. longicollis* or *C. canni*, providing the second example of dual mitochondrial capture (see Liu *et al*., 2011), and the first example for a reptile. Furthermore, approximately half of all *C. longicollis* mitochondrial sequences are derived from *C. canni* demonstrating partial mitochondrial replacement in this species. Our results refute technical errors such as incorrect taxonomy, species misidentification, unidentified paralogues, and incorrect phylogenetic inference. Wide taxon sampling and consistent results across analyses of both datasets indicate that well-supported mitonuclear discordance is an accurate representation of gene tree histories within the *Chelodina* genus.
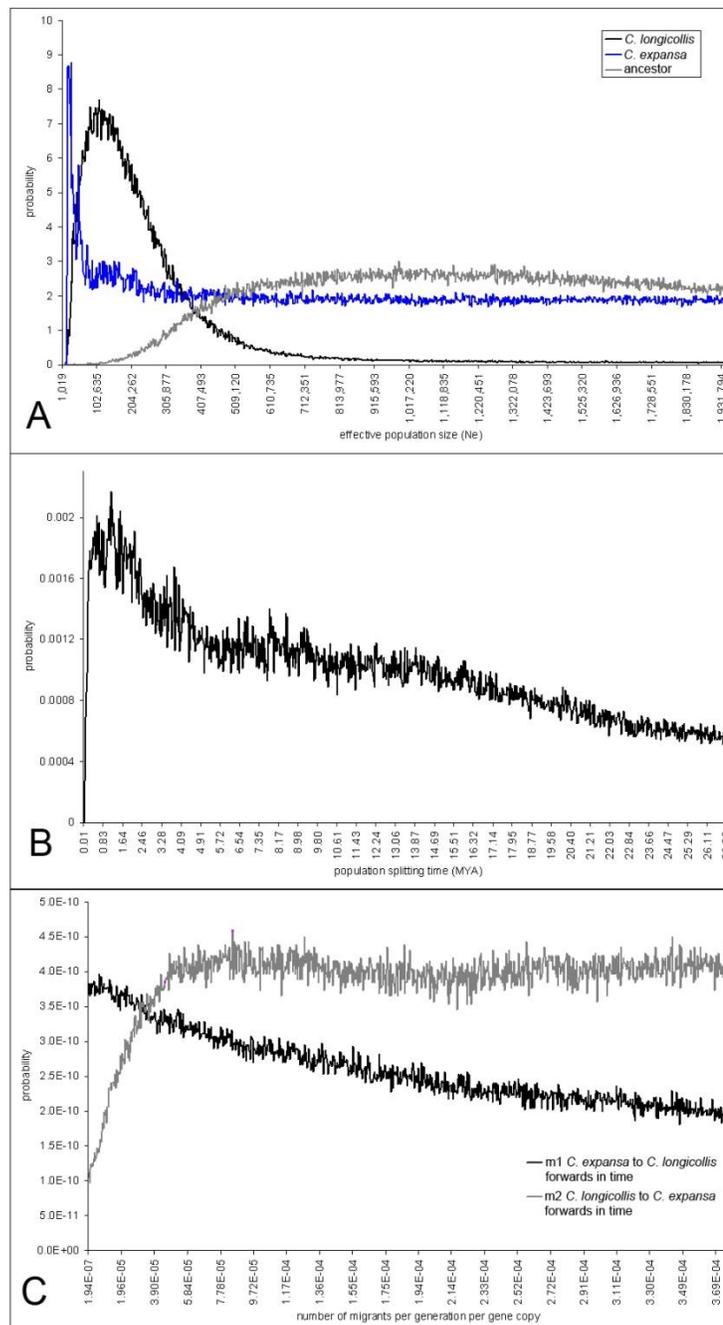
Figure 4.3 Posterior probability distributions of parameters estimated from coalescent analyses of mitochondrial and nuclear sequences data in IM. (A) Effective population sizes of *C. longicollis* (black line), *C. expansa* (blue line), and their inferred ancestor (grey line) in numbers of individuals. (B) Divergence time in millions of years between *C. longicollis* and *C. expansa*. (C) Gene flow (number of migrants per generation per gene copy) estimates between *C. longicollis* and *C. expansa* forwards in time, not in the coalescent direction.

No support for deep coalescence

Incomplete lineage sorting (ILS) of mitochondrial lineages is occasionally invoked to explain mitonuclear discord, especially in closely related species (Funk & Ormland, 2003; Joseph *et al*., 2009; Rheindt *et al*., 2009). Recently diverged species and ancient rapid radiations are inclined to incompletely sorted lineages as short branch lengths (few generations) allow persistence of ancestral polymorphisms through a common ancestor to the daughter taxa (Sang & Zhong, 2000). Rapid radiation makes ILS a good candidate to explain apparent capture of *C. canni* mitochondrial DNA by *C. longicollis*. Nuclear and mitochondrial data suggest the common ancestor of true *C. longicollis* (clade A) and *C. canni* diverged in the late Miocene (mtDNA: 6.35 Mya; nDNA: 8.35 Mya) followed closely by divergence of *C. canni* and *C. pritchardi* in the early to mid Pliocene (mtDNA: 5.09 Mya; nDNA: 3.19 Mya). An ancient rapid radiation is also supported by short internodes and poor resolution of relationships in the *Chelodina* subgeneric group in the Bayesian coalescent analysis (BEST). Deep coalescence requires that an ancestral mitochondrial lineage was lost from *C. pritchardi* and yet maintained in *C. canni* and *C. longicollis* for at least 6 million years post divergence. These conditions are falsified by a late Pliocene (2.24 Mya) mitochondrial MRCA of *C. canni* and *C. longicollis* clade B haplotypes, reflecting timing of gene capture by *C. longicollis* rather than deep coalescence of mitochondrial lineages. Furthermore, the expectation that mitochondrial lineages should sort to monophyly before nuclear ones owing to the smaller effective population size and faster mutation rate of mitochondrial DNA (Palumbi *et al*., 2001; Funk & Ormland, 2003; Ting *et al*., 2008) is not met. This is demonstrated by reciprocal monophyly of *C. longicollis* and *C. canni* in the nuclear dataset. Thus ILS is not consistent with the data and a more likely explanation for the sister relationship between *C. longicollis* clade B

haplotypes and *C. canni* is hybridisation in the late Pliocene. This assertion is strengthened by monophyly of clade B *C. longicollis* haplotypes and a known F$_1$ *C. canni* x *C. longicollis* hybrid.

If deep coalescence of mitochondrial lineages is an unlikely explanation for *C. longicollis* paraphyly, it is an even more improbable explanation for the unusual mitochondrial polyphyly recovered in *C. expansa*. First, *C. expansa* is not closely related to either *C. longicollis* or *C. canni* and nuclear DNA consistently recovers *C. expansa* monophyly. Second, deep coalescence of mitochondrial lineages could not have produced *C. expansa* polyphyly as the youngest nodes where polyphyly is present (Clade A: 2.21 Mya; Clade B: 1.5 Mya) are much later (younger) than an expected Miocene divergence between *C. expansa* and *C. longicollis* and *C. canni*. Finally and most crucially, shared haplotypes between *C. expansa* and *C. longicollis* are hard to explain as anything but post divergence gene flow.

Dual mitochondrial capture by *C. expansa*

Partial mitochondrial lineage replacement through introgression by a closely related species is reasonably common (Funk & Ormland, 2003; Petit & Excoffier, 2009; Toews & Brelsford, 2012) and has produced a close relationship between *C. longicollis* and *C. canni* in this study. Less common is complete replacement of the mitochondrial genome across the entire range of one species with that of another (Spinks & Shaffer, 2009; Unmack *et al.*, 2011; Zieliński *et al.*, 2013). Extinction of *C. expansa* mitochondrial DNA identified in this study adds to a small set of examples of complete mitochondrial genome replacement (Liu *et al.*, 2011; Zieliński *et al.*, 2013) but with two unprecedented differences: (1) two taxa contribute to contemporary *C. expansa* mitochondrial DNA, and (2) one of these is a backcrossed hybrid (Figure 4.4).
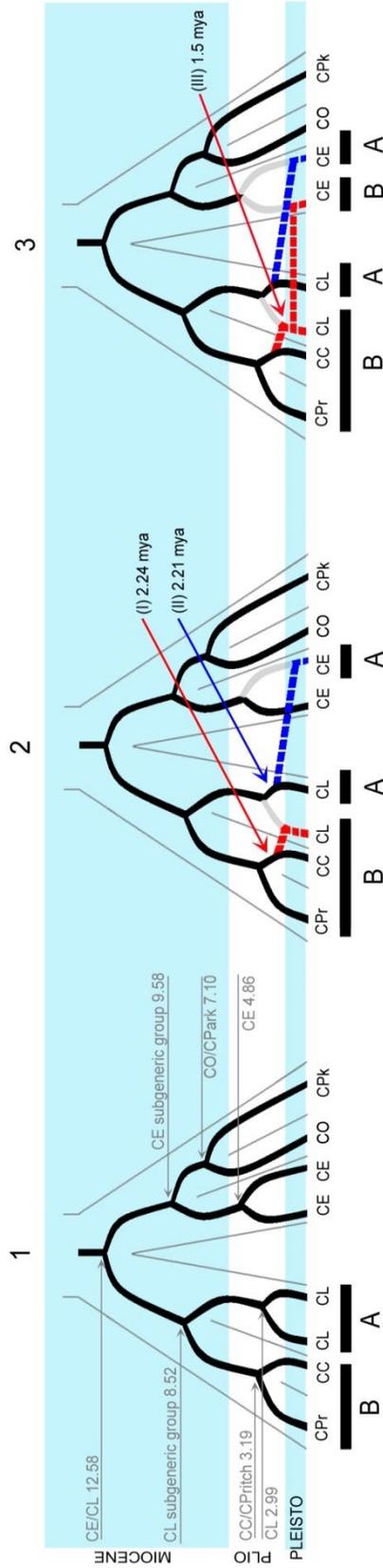
Figure 4.4 Stylised mitochondrial gene tree (thick black lines) within the Chelodina species tree (thin grey lines). Text in grey refers to divergence times (Mya) based on nuclear genes, text in black refers to divergence times based on mitochondrial genes. Bars labelled A and B correspond to the 2 major mitochondrial clades identified in this study. Panel 1 illustrates mitonuclear gene tree concordance. Panel 2 illustrates the first and second episode of mitochondrial introgression (I) between *C. canni* and *C. longicollis* (red dashed line) and (II) between *C. longicollis* and *C. expansa* (blue dashed line) respectively. Panel 3 illustrates the third episode of mitochondrial introgression (III) whereby *C. canni* and *C. expansa* hybridise "by proxy" via introgressed *C. longicollis*. (CPr) *Chelodina pritchardi*, (CC) *Chelodina canni*, (CL) *Chelodina longicollis*, (CE) *Chelodina expansa*, (CO) *Chelodina oblonga*, (CPk) *Chelodina parkeri*.

The placement of *C. expansa* in clade A is simply explained by hybridisation with true *C. longicollis* and capture of this species' mitochondrial DNA through introgression. Evolutionary mechanisms that place *C. expansa* in clade B are less easy to interpret and I rely on a very close mitochondrial relationship between *C. expansa* and the F_1 *C. canni* x *C. longicollis* hybrid to support a hypothesis of double-introgression and associated mitochondrial replacement. I posit that *C. canni* and *C. longicollis* hybridised in the first instance and introgressed to ensure *C. canni* mitochondrial lineages partially replaced those of *C. longicollis*. Next, *C. longicollis* individuals possessing *C. canni* mitochondrial DNA hybridised with sympatric *C. expansa*. Backcrossing effected double-introgression of *C. canni*, effectively causing *C. canni* and *C. expansa* to hybridize "by proxy" (Marshall *et al*., 2011). Importantly, the *C. canni* mitochondrial DNA characterising *C. expansa* in clade B is more closely related to the F_1 *C. canni* x *C. longicollis* hybrid than to *C. canni* itself, indicating that the alternate scenario of direct *C. canni* hybridisation with *C. expansa* in the first instance is not supported.

Capture of *C. longicollis* mitochondrial DNA by one population of *C. expansa* (clade A), followed by capture of *C. canni* mitochondrial DNA (clade B) by another population of *C. expansa* is extraordinary especially given the ancient divergence of *Macrochelodina* and *Chelodina* approximately 12.58 Mya. This scenario establishes the first case of dual mitochondrial capture by a reptile, and surpasses divergence times involved in dual mitochondrial capture reported in Chinese hares (*Lepus* spp.) where interspecific genetic variation was low and species diverged only 3 Mya (Liu *et al*., 2011). This research in the *Chelodina* also warns against studies that rely only on mitochondrial DNA, have poor taxonomic sampling, or limited geographic coverage of samples.

Introgression from common into rare species

Mitochondrial introgressive hybridisation within and between *Chelodina* subgenera is a recurring feature in the evolutionary history of the genus, yet not all sympatric taxa are affected. I find no evidence of introgression from *C. expansa* into *C. longicollis*, and no evidence of *C. canni* paraphyly through capture of *C. expansa* or *C. longicollis* (though this is based on two specimens only). This pattern is consistent with unidirectional mitochondrial introgression and is supported in this study by evidence of gene flow only from *C. longicollis* to *C. expansa*.

Neutral processes such as demographic disparity between sympatric species can drive extensive asymmetric introgression, and complete mitochondrial replacement does not necessarily require the action of selective forces. Where hybridisation is possible, the neutral model predicts that a species with a small effective population size will be massively introgressed by the mitochondrial DNA of the more common species (Currat *et al*., 2008; Excoffier *et al*., 2009). Genetic drift ensures genes flow from the larger towards the smaller population at a greater rate than the reverse, thus asymmetric introgression can be particularly prevalent during biological invasion as an expanding species is rare and is likely to experience more genomic dilution (Petit & Excoffier, 2009; Excoffier *et al*., 2009).

I tentatively support neutral demographic processes over selective forces driving asymmetric mitochondrial introgression for three reasons. (1) The strong directionality of hybridisation I observe in freshwater turtles is consistent with data on contemporary effective population sizes (*C. canni* > *C. longicollis* > *C. expansa*) and the natural rarity of *C. expansa* in particular (Bower & Hodges, 2014). (2) Non-significant Tajima's D characterises mitochondrial clade B of both *C. expansa* and *C. longicollis* (Hodges *et al*.,

2014; Hodges *et al*., 2015). Clade B is associated with *C. canni*, and if a mixed cytonuclear combination involving *C. canni* mitochondrial DNA were positively selected, I would expect evidence of selection in this demographic index (Tajima, 1989). Non-significant Tajima's D indicates long term population stability and diversification, not selection. (3) Significantly negative Tajima's D characterises mitochondrial clade A in *C. expansa* (Hodges *et al*., 2014), hinting at a selective advantage conferred by the true *C. longicollis* mitochondrial genome. However this result can also be interpreted as recent and rapid demographic expansion, and mismatch indices corroborate this interpretation (Hodges *et al*., 2014). Some studies claim complete mitochondrial replacement though drift alone is unlikely (Irwin, 2009). Although I cannot completely rule out selective forces, I suggest that asymmetric introgression though neutral demographic processes is the most parsimonious explanation for the data at this time.

Did Plio/Pleistocene aridity elicit introgression?

Time to most recent common ancestor for each mitochondrial clade occurs in the late Pliocene and early Pleistocene. This coincides with the onset of Plio/Pleistocene aridity across much of Australia that saw many species contract their ranges to more mesic refugia (Kershaw *et al*., 1994; Hill, 2004; Martin, 2006; Petherick *et al*., 2008). These historical climatic conditions may have effected introgression thorugh isolation in sympatric refugia, and through magnifying effective population size disparity. Demographic disparity could have been amplified during isolation in refugia, or simply owing to specific-species responses to aridity where some species suffered large range contractions and become rare, while others persisted and were more common (McPeek & Gavrilets, 2006; Linnen & Farrell, 2007). Future work is needed using ecological niche modelling to examine the range dynamics of freshwater turtles during Plio/Pleistocene aridity, and clarify regions of

ancient sympatry where introgression may have occurred. Tests for ecological niche identity between mitochondrial clades of *C. expansa* and *C. longicollis* can also examine the hypothesis of neutral demographic processes creating introgression as opposed to selective advantage.

Revisiting the *Chelodina* species tree

Aside from extensive mitonuclear discordance driven by *C. longicollis* and *C. expansa,* this study also highlights inconsistencies in relationships among the three *Chelodina* subgenera. The mitochondrial dataset places the monotypic *Macrodiremys* within the *Chelodina* however this interpretation is dictated by the placement of *C. steindachneri* and it is unclear if this is a true reflection of the mitochondrial species tree, evidence of deep coalescence or introgression, or an artefact of poor phylogenetic resolution. A short internode supports *Macrodiremys* in the mitochondrial gene tree and poor phylogenetic resolution is demonstrated by weak branch support in parsimony and likelihood bootstrap analyses (MP: 59; ML: 50). Bayesian analyses yielded stronger support for this short internode (Mr. Bayes: 93: BEAST: 75) however this could be attributable to unpredictable Bayesian posterior probability values when attempting to resolve a hard or near-hard polytomy (Lewis *et al*., 2005; Leaché & McGuire, 2006).

The placement of *Macrodiremys* in the nuclear dataset is inconsistent with both the mitochondrial gene tree, and the proposed species tree. Georges *et al*. (2002) present *Macrodiremys* as sister subgenera to the *Chelodina.* This study however supports *Macrodiremys* outside a *Macrochelodina*, *Chelodina* pair, albeit with weak support. These inconsistent relationships among subgenera are indicative of an ongoing controversy in the placement of *Macrodiremys* more generally (Georges & Adams, 1992; Seddon *et al*., 1997; Georges *et al*., 2002). Resolution requires more nuclear loci and flexible probability

priors on internodes allowing the exploration of rapid diversification and the potential for a true hard polytomy (*e.g.* Rannala & Yang, 2013).

Conclusions

This study significantly reframes our understanding of relationships and historic interactions among freshwater turtles in the *Chelodina* genus. A small amount of interspecific gene flow can have far reaching evolutionary consequences; introgressive hybridisation can occur between subgenera that have been separated for over 12 million years, and allopatric populations of a single species can each be characterised by a different interspecific mitochondrial genome. The results of this study are consistent with other work on freshwater turtles that suggest the degree of introgressive hybridisation within this family could be quite high (Stuart & Parham, 2007; Freedberg & Myers, 2012). Within the Chelidae, introgressive hybridisation is almost certainly much higher than currently described, and the frequency of hybridisation in nature more broadly is likely considerably underestimated. Signatures of introgression are only exposed though broad taxonomic and biogeographic sampling and I emphasise the call for future systematic and phylogeographic work to sample extensively across and within species (McKay & Zink, 2010; Marshall *et al*, 2011). Without comprehensive sampling of multiple gene trees and individuals, monophyly may be observed by chance, and the complexity of evolutionary interactions among species significantly underestimated.

Acknowledgements

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Supporting information

Appendix 4A – *Specimens examined*

Outgroup taxa are presented with species name, local drainage or region, state/province, country, latitude and longitude (in parentheses), and specimen number(s) (Wildlife Tissue Collection, University of Canberra, UC<Aus> in Genbank). Specimens of *C. expansa* and *C. longicollis* are taxa are presented by haplotype number (as per Hodges *et al*., 2014; Hodges *et al*., 2015) with local drainage or region, Australian state, latitude and longitude (in parentheses), and specimen number(s) (Wildlife Tissue Collection, University of Canberra, UC<Aus> in Genbank). * denotes the 6 specimens each of *C. longicollis* and *C.*

expansa used in the nuclear dataset. Abbreviations for Australian states are: ACT

(Australian Capital Territory), NT (Northern Territory), QLD (Queensland), SA (South

Australia), Vic. (Victoria), and WA (Western Australia).

Outgroups:

Chelodina canni (used in both nuclear and mitochondrial datasets), Nicholson River, QLD,

Australia (17.961 S 139.752 E), AA20218; *Chelodina canni* (used in mtDNA dataset

only), Roper River, NT, Australia (16.1177 S 133.5723 E), 657; *Chelodina pritchardi*,

Kerema-Moresby, Central Province, Papua New Guinea (9.031 S 146.868 E), AA21711;

Chelodina steindachneri, Yarra Yarra Lakes, WA (28.558 S 117.779 E), ATP501; *C.*

canni x *longicollis* hybrid, Shoalwater Creek, QLD, Australia (22.354 S 150.106 E),

AA00912; *Chelodina oblonga*, Flinders River, QLD, Australia (18.160 S 140.855 E),

AA20447; *Chelodina parkeri*, Fly River, Western Province, Papua New Guinea (8.245 S

141.767 E), AA42953; *Chelodina colliei*, Wilgarup River, WA (34.110 S 116.238 E),

ATP401; *Elseya dentata*, Roper River, NT, Australia (14.713 S 134.504 E), AA32205.

*Chelodina expansa***:**

Haplotype 01: Stradbroke Island, QLD (27.49 S 153.432 E), **AA46516***-18, 20-21.

Haplotype 02: Border Rivers, NSW (28.733 S 151.983 E), 365; Border Rivers, NSW

(28.989 S 151.278 E), AA20598, 620-621; Border Rivers, QLD (28.465 S 150.959 E),

AA33103;

Border Rivers, QLD (28.548 S 150.301 E), AA32782-88, 90, 96-98; Condamine River,

NSW (29.164 S 147.279 E), AA33875; Condamine River, QLD (26.8 S 150.679 E),

AA32805, 07, 21; Condamine River, QLD (27.68 S 151.89 E), AA32956-62; Condamine

River, QLD (27.991 S 148.659 E), AA33099; Darling River, NSW (34.112 S 141.917 E),

AA33112, 52-54, 82-83; Macquarie River, NSW (31.889 S 148.092 E), AA32170; Moonie

River, QLD (27.895 S 149.56 E), AA46409; Moonie River, QLD (27.957 S 149.383 E),

AA46411-17;

Murray River, NSW (34.113 S 141.891 E), AA33107-11, 15; Murray River, QLD

(34.218 S 140.454 E), AA20528; Murray River, SA (34.084 S 140.782 E), **AA18731***,

AA20454, 68-69, 95-96; Murray River, SA (34.23 S 140.44 E), ABTC51969, CE_003;

Murray River, NSW (36.093 S 146.948 E), 625-27, AA20675-79, 91; Murrumbidgee

River, NSW (34.174 S 145.802 E), AA32147, 65; Murrumbidgee River, NSW (34.273 S

146.032 E), AA32108;

Murrumbidgee River, NSW (35.032 S 147.101 E), MB007; Murrumbidgee River, NSW

(35.124 S 147.352 E), AA10787; Namoi River, NSW (30.243 S 149.684 E), AA32426;

Namoi River, NSW (30.75 S 150.717 E), 757, 759; Namoi River, NSW (30.972 S

150.254 E), AA32419-20; Warrego River, QLD (26.907 S 146.033 E), AA13179, 204,

239;

Warrego River, QLD (27.075 S 145.958 E), AA13303.

Haplotype 03: Murrumbidgee River, NSW (35.124 S 147.352 E), AA10784, 89.

Haplotype 04: Condamine River, QLD (26.8 S 150.679 E), AA32804, 06, 20.

Haplotype 05: Border Rivers, NSW (28.733 S 151.983 E), 371.

Haplotype 06: Pine Rivers, QLD (27.318 S 153.064 E), AA00942, 44, 48, 51-54.

Haplotype 07: Brisbane River, QLD (27.649 S 152.635 E), AA33027.

Haplotype 08: Brisbane River, QLD (27.491 S 152.997 E), AA33195-98; Brisbane River, QLD (27.549 S 152.116 E), AA41603; Brisbane River, QLD (27.63 S 152.375 E), AA32892; Brisbane River, QLD (27.781 S 152.68 E), AA32963; Fraser Island, QLD (25.446 S 153.057 E), 1172; Logan-Albert Rivers, QLD (27.818 S 153.175 E), 323, 28, 43; Logan-Albert Rivers, QLD (28.198 S 153.04 E), AA41626; Moreton Island, QLD (27.096 S 153.436 E), AA4300; Pine Rivers, QLD (27.043 S 152.869 E), AA33551; Pine Rivers, QLD (27.063 S 152.873 E), J83695; Pine Rivers, QLD (27.246 S 153.031 E), AA33066; Pine Rivers, QLD (27.318 S 153.064 E), AA00941, 43, 45-47, 49-50, 55; Pine Rivers, QLD (27.344 S 152.873 E), AA46429; Pine Rivers, QLD (27.35 S 152.917 E), 3030; Pine Rivers, QLD (27.367 S 152.933 E), 31P; South Coast catchment, QLD (28.133 S 153.488 E), AA5296-97.

Haplotype 09: Brisbane River, QLD (27.491 S 152.997 E), AA33194.

Haplotype 10: Logan-Albert Rivers, QLD (27.818 S 153.175 E), 337.

Haplotype 11: Moreton Island, QLD (27.096 S 153.436 E), AA4292.

Haplotype 12: Fraser Island, QLD (25.507 S 153.063 E), AA33696; Fraser Island, QLD (25.599 S 153.093 E), **AA5283***, 85.

Haplotype 13: Burnett River, QLD (25.615 S 151.592 E), **AA00295***, 96; Burnett River, QLD (25.797 S 151.8 E), AA2367.

Haplotype 14: Burnett River, QLD (25.051 S 152.099 E), **AA4642***; Burnett River, QLD (25.685 S 151.778 E), AA2360, 63; Burnett River, QLD (25.721 S 151.81 E), AA2331.

Haplotype 15: Baffle creek, QLD (24.636 S 152.106 E), J83694; Burnett River, QLD (25.051 S 152.099 E), AA00276.

Haplotype 16: Burnett River, QLD (24.797 S 152.442 E), AA00246; Burnett River, QLD (24.974 S 152.091 E), AA00268; Burnett River, QLD (25.051 S 152.099 E), AA4648.

Haplotype 17: Fitzroy-Dawson River, QLD (23.4 S 150.5 E), ABTC76454; Fitzroy-Dawson River, QLD (24.603 S 149.913 E), **AA32871***-73.

Haplotype 18: Burnett River, QLD (25.051 S 152.099 E), AA4654.

Haplotype 19: Fraser Island, QLD (25.223 S 153.168 E), AA33863; Fraser Island, QLD (25.227 S 153.136 E), AA33997; Fraser Island, QLD (25.327 S 153.154 E), AA33737; Mary River, QLD (26.526 S 152.565 E), AA4502, 65, 69-71.

Haplotype 20: Burnett River, QLD (25.685 S 151.778 E), AA2361; Burnett River, QLD (25.721 S 151.81 E), AA2332, 66.

Haplotype 21: Burnett River, QLD (25.593 S 151.315 E), AA10145.

*Chelodina longicollis***:**

Haplotype 01: Moreton Island, QLD (27.087 S 153.438 E), AA4270, 87.

198

Haplotype 02: Border Rivers, NSW (28.867 S 151.783 E), 3061-66; Border Rivers, QLD (28.898 S 151.945 E), J84640, J85191; Clarence River, NSW (29.051 S 152.59 E), 193R_4, 195R_4, 196R_4; Clarence River, NSW (29.428 S 153.085 E), AA18589-91; Clarence River, NSW (29.538 S 152.55 E), AA18706-08; Clarence River, NSW (29.549 S 152.665 E), AA18685; Richmond River, NSW (28.858 S 153.026 E), AA18538.

Haplotype 03: Border Rivers, NSW (28.989 S 151.278 E), AA20619; Border Rivers, NSW (29.138 S 150.984 E), AA20775-78; Border Rivers, NSW (29.211 S 151.379 E), **AA205**75, **76***-80; Border Rivers, QLD (28.548 S 150.301 E), AA32789.

Haplotype 04: Gwydir River, NSW (29.494 S 150.165 E), AA32431; Gwydir River, NSW (29.755 S 151.048 E), AA32435; Gwydir River, NSW (29.86 S 150.581 E), AA20742; Gwydir River, NSW (29.863 S 150.571 E), AA32438-40; Gwydir River, NSW (30.496 S 151.133 E), 188R_4, 189R_3, 190R_4, 191R_3, 192R_2; Macleay River, NSW (30.852 S 152.087 E), 3050-55, 57-58; Namoi River, NSW (30.243 S 149.684 E), AA32424-25; Namoi River, NSW (30.972 S 150.254 E), AA32418; Namoi River, NSW (30.974 S 150.259 E), **AA32415***.

Haplotype 05: Hawkesbury River, NSW (33.728 S 150.656 E), 744; Hawkesbury River, NSW (33.857 S 150.618 E), 997.

Haplotype 06: Hunter River, NSW (32.582 S 151.784 E), AA18398.

Haplotype 07: Namoi River, NSW (30.243 S 149.684 E), AA32427.

Haplotype 08: Castlereagh River, NSW (31.272 S 149.256 E), AA32401, 04; Castlereagh River, NSW (31.4 S 149.343 E), AA32179, 81-83; Hawkesbury River, NSW (33.728 S 150.656 E), 741; Hunter River, NSW (32.133 S 151.033 E), 194R_4, 200R_3; Hunter River, NSW (32.333 S 151.291 E), AA18392, 93; Hunter River, NSW (32.582 S 151.784 E), AA18399, 401, 403.

Haplotype 09: Hunter River, NSW (32.582 S 151.784 E), AA18400, 02.

Haplotype 10: Bega River, NSW (36.462 S 149.864 E), AA33490; Bega River, NSW (36.632 S 149.795 E), 765-66; Clyde River, NSW (35.174 S 150.7 E), BL-003; La Trobe River, Vic. (38.152 S 146.283 E), AA33459; La Trobe River, Vic. (38.319 S 146.447 E), AA33457; Mitchell River, Vic. (37.842 S 147.63 E), AA33476, 80-84; Mitchell River, Vic. (37.912 S 147.719 E), AA33473, 74; Snowy River, Vic. (37.714 S 148.456 E), AA33489; Thomson River, Vic. (38.094 S 147.058 E), **AA334**62, **69***, 71-72.

Haplotype 11: Clyde River, NSW (35.174 S 150.7 E), BL-001-2, 4-5; Mitchell River, Vic. (37.842 S 147.63 E), AA33477-79.

Haplotype 12: Hunter River, NSW (32.332 S 151.454 E), AA18461-66.

Haplotype 13: Hunter River, NSW (32.332 S 151.454 E), AA18464, 66;

Haplotype 14: Burdekin River, QLD (22.564 S 147.073 E), AA19284; Burdekin River, QLD (23.652 S 146.638 E), 00ChlBurd; Eyre catchment, QLD (22.593 S 145.678 E), 98ClonDunn-2; Fitzroy-Dawson River, QLD (24.924 S 148.6 E), AA19310; Warrego

River, QLD (26.907 S 146.033 E), AA13168; Warrego River, QLD (26.923 S 146.037 E),

AA13149-54; Warrego River, QLD (27.08 S 145.923 E), AA13355; Warrego River, QLD

(28.205 S 145.714 E), AA13057; Warrego River, QLD (28.32 S 145.727 E), AA13049-

50.

Haplotype 15: Barwon River, Vic. (37.556 S 143.935 E), AA33455.

Haplotype 16: Barwon River, Vic. (37.553 S 143.935 E), **AA33453***-55; Border Rivers,

NSW (28.989 S 151.278 E), AA20597; Border Rivers, QLD (28.548 S 150.301 E),

AA32770; Condamine River, NSW (29.717 S 147.45 E), AA32086-89; Condamine River,

QLD (25.803 S 148.24 E), AA32262, 68; Condamine River, QLD (26.956 S 147.744 E),

737-40; Condamine River, QLD (27.717 S 147.7 E), AA33086-90; Condamine River,

QLD (28.02 S 147.381 E), AA33085; Fitzroy-Dawson River, QLD (25.392 S 148.668 E),

AA32283; Fitzroy-Dawson River, QLD (25.81 S 148.299 E), AA32036; Fitzroy-Dawson

River, QLD (25.814 S 148.308 E), AA32033-35; Hopkins River, Vic. (37.767 S 142.722

E), AA33440-46, 48-50; Millicent catchment, SA (37.548 S 140.816 E), ABTC51959;

Millicent catchment, SA (37.844 S 140.778 E), ABTC51976; Millicent catchment, Vic.

(36.719 S 141.433 E), AA33431; Millicent catchment, Vic. (36.729 S 141.586 E),

AA33430; Millicent catchment, Vic. (36.901 S 141.502 E), AA33432; Millicent

catchment, Vic. (37.029 S 141.272 E), AA33433, 34; Millicent catchment, Vic. (37.066 S

141.203 E), AA33436; Millicent catchment, Vic. (37.09 S 141.177 E), AA33435;

Millicent catchment, Vic. (37.488 S 141.995 E), AA43387-88; Moonie River, QLD

(27.895 S 149.56 E), AA46402-05; Moonie River, QLD (27.957 S 149.383 E), AA46407-

08; Murray River, SA (34.086 S 140.79 E), AA20514-17; Murray River, SA (35.087 S

139.307 E), AA37272; Murray River, NSW (36.093 S 146.948 E), 174R_4, 620,

AA20685-89; Namoi River, NSW (30.243 S 149.684 E), AA32428; Portland River, Vic.

(38.177 S 141.686 E), AA33437; Warrego River, QLD (28.068 S 145.679 E), AA10161-

64, AA10229-31.

Haplotype 17: Murray River, SA (35.033 S 139.37 E), AA32107.

Haplotype 18: Condamine River, QLD (26.489 S 147.981 E), AA33076.

Haplotype 19: Moonie River, QLD (28.091 S 149.247 E), 733.

Haplotype 20: Burnett River, QLD (26.238 S 151.926 E), AA33554.

Haplotype 21: Burnett River, QLD (24.797 S 152.442 E), AA00903; Burnett River, QLD

(25.593 S 151.315 E), AA11789, 91, AA11821-26, 28.

Haplotype 22: Condamine River, QLD (25.803 S 148.24 E), **AA322**63-**64***, 66; Fitzroy-

Dawson River, QLD (25.814 S 148.308 E), AA32300.

Haplotype 23: Burnett River, QLD (24.797 S 152.442 E), AA00240, 47-49, AA00905;

Burnett River, QLD (25.593 S 151.315 E), AA11827.

Haplotype 24: Bulloo River, QLD (26.611 S 144.268 E), AA18058.

Haplotype 25: Paroo River, QLD (28.161 S 145.045 E), AA18024.

Haplotype 26: Murrumbidgee River, NSW (34.755 S 146.545 E), MB016.

Haplotype 27: Castlereagh River, NSW (31.268 S 149.281 E), AA32193-95; Castlereagh River, NSW (31.272 S 149.256 E), AA32402-3, 05; Castlereagh River, NSW (31.4 S 149.343 E), AA32178, 80, 84; Lachlan River, NSW (33.382 S 148.001 E), AA10928-30, 42; Macquarie River, NSW (31.889 S 148.092 E), **AA321**67, **68***, 69, 75, 77; Macquarie River, NSW (32.226 S 148.248 E), AA32173, 74; Macquarie River, NSW (32.665 S 149.168 E), AA11833-38; Murrumbidgee River, ACT (35.219 S 149.001 E), AA42303-06; Murrumbidgee River, NSW (34.174 S 145.802 E), AA32146, AA33212, 14-19; Murrumbidgee River, NSW (34.273 S 146.032 E), AA32127; Murrumbidgee River, NSW (34.755 S 146.545 E), MB017, 22; Murrumbidgee River, NSW (34.865 S 149.009 E), AA20629, 32; Murrumbidgee River, NSW (34.875 S 149.013 E), AA20633-34, 37; Namoi River, NSW (30.974 S 150.259 E), AA32414, 16, 22.

Haplotype 28: Lachlan River, NSW (33.382 S 148.001 E), AA10943.

Appendix 4B – Nuclear primer details

Locus	Primer Name	Primer sequence (5' – 3')	Source
C-mos	G136	TCCAATCTTGCACACACCC	1
	G137	AAGCAGGTGAAGAAATGCAG	1
Gapdh	H950	CATCAAGTCCACAACACGGTTGCTGTA	2
	L890	ACCTTTAATGCGGGTGCTGGCATTGC	2
R35	R35_int_F	CCTNTCAGCTYCTTTCCAT	3
	R35Ex1	GCAGAAAACTGAATGTCTCAAAGG	3

1 - Georges, A., Birrell, J., Saint, K., McCord, W. & Donnellan, S.C. 1998. A phylogeny for side necked turtles (Chelonia: Pleurodira) based on mitochondrial and nuclear gene sequence variation. *Biological Journal of the Linnean Society* **67**: 213–246.

2 - Friesen, V. L., Congdon, B. C., Walsh, H. E., Birt, T. P. 1997. Intron variation in marbled murrelets detected using analyses of single-stranded conformational polymorphisms. *Molecular Ecology* **6**: 1047–1058.

3 - Fujita, M. K., Engstrom, T. N., Starkey, D. E., and Shaffer, B. H. 2004. Turtle phylogeny: Insights from a novel nuclear intron. *Molecular Phylogenetics and Evolution* **31**: 1031–1040.

Appendix 4C – Nuclear loci PCR procedures

Separate PCR amplifications for each nuclear locus were conducted in 25 µl reactions containing 50-100 ng gDNA. *C-mos* was amplified using 10xPCR buffer, 3 mM MgCl$_2$, 0.1 mM of each dNTP, 0.8 pmol of each primer (Table xx), 1 µg BSA (New England Biolabs), 0.6 M Betaine, and 0.04 unit DNA polymerase (Bioline BioTaq Red). PCR cycling for *C-mos* was performed under the following conditions: 94°C for 180s, 35 cycles of 94°C for 45s, 54°C for 45s, 72°C for 60s, and a final elongation step at 72°C for 300s. *R35* was amplified using 10xPCR buffer, 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.25 pmol of each primer (Table xx), 1 µg BSA (New England Biolabs), 0.8 M Betaine, and 0.04 unit DNA polymerase (Bioline BioTaq Red). PCR cycling for *R35* was performed under the following conditions: 94°C for 300s, 35 cycles of 94°C for 30s, 55°C for 90s, 72°C for 120s, and a final elongation step at 72°C for 600s. *Gapdh* was amplified using 10 x PCR buffer, 1.25 mM MgCl$_2$, 0.1 mM of each dNTP, 0.25 pmol of each primer (Table xx), 1 µg BSA (New England Biolabs), 0.8 M betaine, and 0.04 unit DNA polymerase

(Bioline BioTaq Red). PCR cycling for *Gapdh* was performed under the following

conditions: 94℃ for 120s, 30 cycles of 94℃ for 30s, 55℃ for 30s, 72℃ for 45s, and a

final elongation step at 72℃ for 300s. Amplicons were purified with polyethylene glycol

(PEG) (Sambrook and Russell, 2001) and sequenced in both directions using an ABI

3730XL DNA automated sequencer (Macrogen; Seoul, South Korea). Sequences were

edited, assembled, and consensus sequences determined using Geneious Pro 5.3.4

(BioMatters Inc.).

Chapter 5

Ecological niche modelling demonstrates drivers and locations of ancient asymmetric mitochondrial introgression among Australian freshwater turtles.

Abstract

Ecological niche modelling is applied to test hypotheses on the drivers and locations of ancient asymmetric mitochondrial introgression among freshwater turtles in eastern Australia. Three freshwater turtle species, *Chelodina expansa*, *C. longicollis*, and *C. canni*, have an intriguing history of mitochondrial introgression, with three introgressive events occurring during Plio/Pleistocene glacial aridity. *Chelodina expansa* and C. *longicollis* are each characterised by the same two highly divergent mitochondrial haplogroups. Distributions of these within each species are either allopatric or parapatric, drawing the question of whether introgression was driven by adaption of each haplogroup to local conditions. Tests of ecological niche identity could not reject niche convergence of haplogroups within each species, discrediting local adaption and the possibility that selection facilitated introgression. Instead, a neutral introgressive processes involving population size disparity is supported. Palaeodistribution models for each species highlight three key locations of ancient sympatry that likely accommodated Plio/Pleistocene introgression and fixation of locally acquired lineages. All are on the Australian east coat and include regions surrounding the Hunter catchment, Fraser Island, and the Styx catchment. Phylogeographic data independently confirms the importance of these locations in the recent evolutionary history of freshwater turtles, with evidence of modern hybrid-zones, ancient diversification, and long-term population persistence. The results of this study emphasise the important role ecological niche modelling can play in exposing the underlying processes that drive biogeographic patterns of introgression, and in testing hypotheses generated though other lines of inquiry.

Introduction

Asymmetrically introgressed mitochondrial DNA often provides the sole remaining signature of ancient hybridisation (Weisrock *et al*., 2005) and is increasingly being documented across a wide range of taxa. Introgression frequently has discernible biogeographic patterns and these can give insight into the history and dynamics of past species interactions (McGuire *et al*., 2007; Wielstra & Arntsen, 2012, 2014; Zielinski *et al*., 2013). Interpretation of the biogeographic pattern of introgression requires some statement as to process, however the drivers behind such patterns are often underexplored and unknown (Toews & Brelsford, 2012). Although introgression is inherently a genetic phenomenon, the sole use of genetic information to infer biogeographic process is contentious and deficient as generic statistical tests using phylogeographic data provide little biological insight (Alvarado-Serrano & Knowles, 2014).

Biogeographic processes are influenced by environmental variation over space and time. By integrating spatially explicit environmental data with phylogeographic data, a more robust estimation of biogeographic process is possible. Ecological niche modelling (ENM) enables this integration by estimating present-day and palaeo distributions of species, populations, and lineages. Comparing current and past species distributions allows insights into historical biogeography such as range dynamics, ancient habitat configurations, and the location and extent of past species interactions. For example, ecological niche models can identify regions of environmental stability that might have served as refugia during glacial maxima where species could, in principle, persist overtime. If the locations of these stable regions are similar across closely related species, sites can be identified that potentially supported introgressive hybridisation and fixation of locally acquired mitochondrial lineages. These insights can inform about the biogeographic,

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ecological, and evolutionary processes that lead to and drive the spatial patterns of asymmetric mitochondrial introgression. Ecological niche modelling provides an independent data source to generate hypotheses on biogeographic processes, or to test hypotheses generated from other sources such as phylogeographic studies (Waltari & Hickerson, 2013; Alvarado-Serrano & Knowles, 2014). In this study I use ENMs to test hypotheses on the drivers and locations of ancient introgressive hybridisation among freshwater turtles.

Study system: *Chelodina* freshwater turtles

Three freshwater turtle species, the broad-shelled turtle *Chelodina expansa*, the eastern long-necked turtle *C. longicollis*, and the northern long-necked turtle *C. canni*, have an intriguing history of asymmetric mitochondrial introgression.   Three temporally separated episodes of introgression have occurred from the late Pliocene to the early Pleistocene (Table 5.1): (1) from *C. canni* to *C. longicollis* an estimated 2.24 million years ago (Mya), (2) from *C. longicollis* to *C. expansa* 2.21 Mya, and (3) from *C. canni* to *C. expansa* (by proxy though backcrossed *C. longicollis* individuals possessing the *C. canni* mitochondrial genome) 1.5 Mya. Two highly divergent mitochondrial haplogroups, A and B, characterise *C. expansa* and *C. longicollis*. The *C. expansa* mitochondrial genome has been completely replaced with that of either *C. longicollis* (haplogroup A, 82% of *C. expansa* individuals) or *C. canni* (haplogroup B, 18% of *C. expansa* individuals). The *C. longicollis* mitochondrial genome has been partially replaced with that of *C. canni* (haplogroup B, 60% of *C. longicollis* individuals), with the remaining *C. longicollis* individuals (40%) characterised by the 'true' mitochondrial genome for this species (haplogroup A) (see Chapter 4 for details).

Table 5.1 Three episodes of mitochondrial introgression were identified in Chapter 4 and are outlined below. Approximate timing of introgressive events is based on time to the most recent common ancestor of the populations involved. Dates were generated in a mitochondrial molecular dating analysis using a Bayesian approach. 95% highest posterior density (HPD) represents the 95% probability distribution and can be considered similar to a confidence interval.

species involved	direction of mtDNA introgression	timing of introgression Mya (95% HPD)	mtDNA haplogroup
C. canni x *C. longicollis*	Unidirectional: *C. canni* into *C. longicollis*	2.24 (1.41 – 3.16) late Pliocene	B
C. longicollis x *C. expansa*	Unidirectional: *C. longicollis* into *C. expansa*	2.21 (1.35 – 3.08) late Pliocene	A
C. longicollis x *C. expansa*	Unidirectional: *C. canni* into *C. expansa (*via *C. longicollis*)	1.5 (0.89 – 2.17) early Pleistocene	B

The haplogroups have a complex geographic arrangement across species (Figure 5.1). Haplogroup A and B are allopatric in *C. expansa* and parapatric in *C. longicollis*. Haplogroup A encompasses a "southern" *C. expansa* population with haplotypes associated with the Murray-Darling Basin and the South Conondale region, and an "eastern" *C. longicollis* population with haplotypes associated with the southeast coast and a small region of the Murray-Darling Basin. Haplogroup B encompasses a "northern" *C. expansa* population associated the North Conondale region, and a "western" *C. longicollis* population associated with the Murray-Darling Basin, southwest coast and northeast coast. Adding to this complexity, haplotypes that are shared between species are not sympatric or geographically proximate.

In Chapter 4, I hypothesised that neutral processes such as effective population size disparity drove extensive asymmetric mitochondrial introgression among the three species of freshwater turtle. Where hybridisation is possible, this neutral model predicts that a rare species with a necessarily small effective population size will become massively introgressed into by mitochondrial lineages of the more common species (Currat *et al*., 2008; Excoffier *et al*., 2009). Given that introgression occurred during arid and cold conditions of the Plio/Pleistocene, I further hypothesised that these conditions drove introgression. This may have occurred though isolation of freshwater turtle species in shared refugia, and though amplified demographic disparity where some species and not others experienced range contraction and poor habitat suitability.
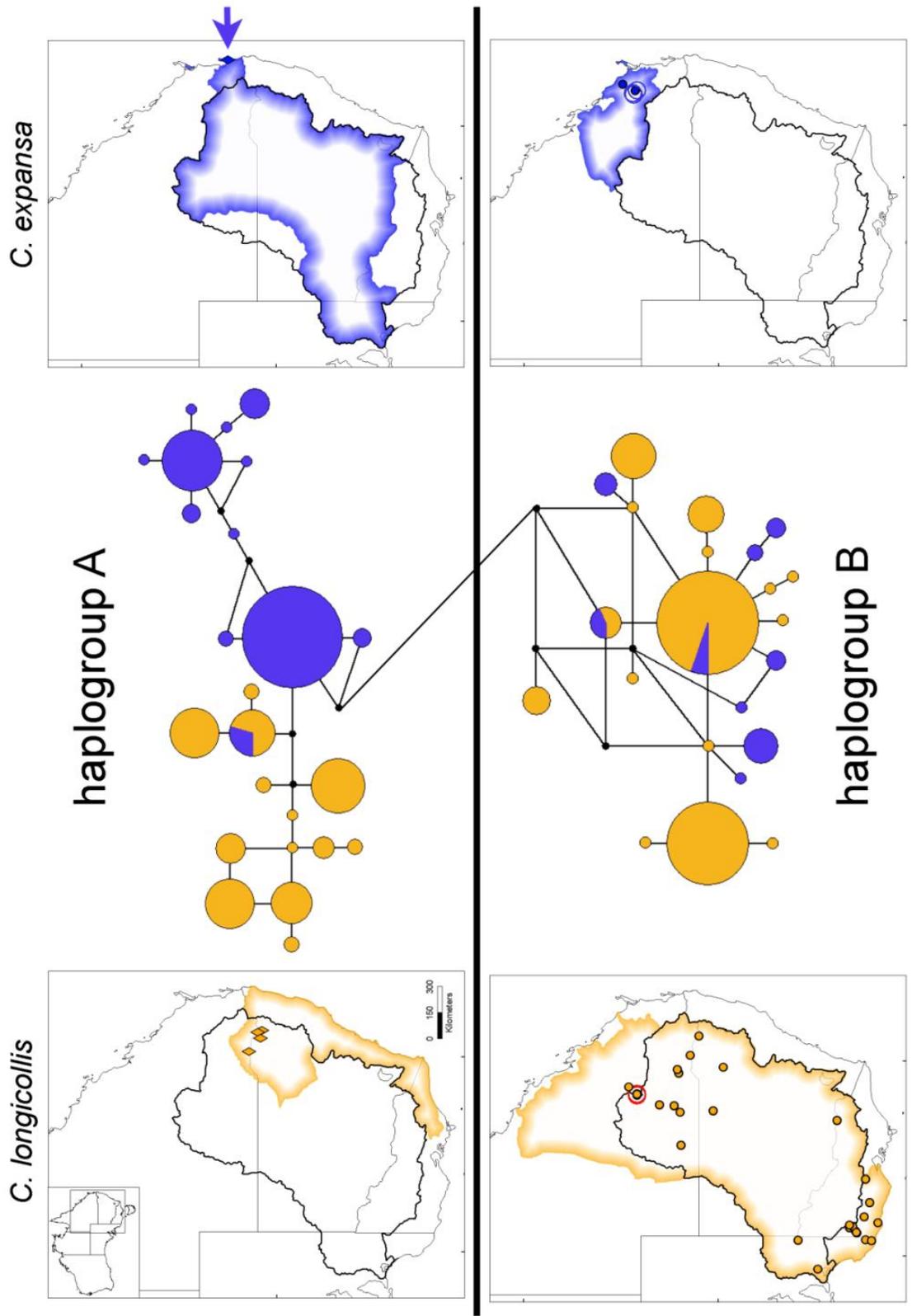
Figure 5.1 (previous page) Geographic distribution of haplogroups A and B in *C. longicollis* (orange) and *C. expansa* (blue), and a median-joining haplotype network of the 46 haplotypes identified in total. Thick back line on distribution maps indicates the boundary of the Murray-Darling Basin, and symbols indicate locations of individuals with shared haplotypes. In haplogroup A, orange and blue diamonds represent locations of the same haplotype in each species. In haplogroup B, closed orange and blue circles, and open red and blue circles each represent locations of the same haplotypes in each species. Circle area in the haplotype network is proportional to the number of individuals characterised by a haplotype. Mutational steps are not provided but see Hodges *et al*. (2014) for a detailed breakdown of the 21 *C. expansa* haplotypes, and Hodges *et al*. (2015) for a detailed breakdown of the 28 *C. longicollis* haplotypes.

Testing for introgression driven by neutral demographic processes is challenging (Currat *et al*., 2008). Here I use ecological niche modelling to investigate niche similarity between mitochondrial haplogroups as a proxy test of the null hypothesis. If introgression was driven by neutral demographic effects and not adaptive processes, I expect that the mitochondrial haplogroups within each species will display niche conservatism. The opposite case, niche divergence, might indicate local adaption of mitochondrial haplogroups and asymmetric introgression owing to selective advantage. To examine if Plio/Pleistocene aridity drove introgression, I use ecological niche modelling to assess past range dynamics, habitat suitability, and sympatry. I test the following three hypotheses: (1) that mitochondrial haplogroups A and B respectively within *C. expansa* and *C. longicollis* demonstrate niche conservatism, thus supporting neutral mitochondrial introgression over selective advantage; (2) that the ranges of *C. canni* and *C. longicollis* overlap when modelled under arid conditions, thus permitting introgression; and (3) that the range of *C. expansa* contracts to a minimum of two habitat isolates that are both sympatric with *C. longicollis* when modelled under arid conditions, thus permitting dual mitochondrial capture by *C. expansa*.

Materials and Methods

Occurrence and environmental data

I used tissue records, museum records, and verified sighting data from the Institute for Applied Ecology Wildlife Tissue Collection (http://piku.org.au/cgi-bin/locations.cgi) for *C. expansa*, *C. longicollis*, *C. canni*, and F$_1$ *C. canni + longicollis* hybrids to estimate the full known distribution of each species. The dataset included locations for 744 *C. expansa*, 3,022 *C. longicollis*, 94 *C. canni*, and 93 F$_1$ *C. canni + longicollis* hybrids. Taxonomy follows that of Georges and Thomson (2010). To examine possible niche differentiation

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associated with geographically discrete haplogroups within *C. expansa* and *C. longicollis*, I used location data for 134 individuals of *C. expansa* haplogroup A, 29 individuals of *C. expansa* haplogroup B, 108 individuals of *C. longicollis* haplogroup A, and 166 individuals of *C. longicollis* haplogroup B (specimens as per Hodges *et al*., 2014, Hodges *et al*., 2015). Mitochondrial haplogroups within each species are considerably diverged and sequenced individuals can be unambiguously assigned to either haplogroup A or B.

To construct ENMs, nineteen spatially explicit bioclimatic variables representing contemporary and palaeo (LGM, 21 Kya) terrestrial data were downloaded from WorldClim databases (contemporary, http://www.worldclim.org/ Hijmans *et al*., 2005; LGM http://www.worldclim.org/past). I used two LGM model simulations: (1) from the Community Climate System Model v3 (CCSM, Collins *et al*., 2006), created through the Palaeoclimate Modelling Intercomparison Project (http://pmip2.lsce.ipsl.fr/); and (2) from the Model for Interdisciplinary Research on Climate v3.2 (MIROC, Hasumi & Emori, 2004). The climate data for the LGM is the best available approximating the cool and arid conditions that also typified the late Pliocene and early Pleistocene periods when hybridisation occurred. It is assumed that habitat configurations modelled under LGM conditions are reasonably equivalent to those experienced during the late Pliocene and early Pleistocene. The nineteen bioclimatic variables describe variation in temperature, precipitation, and seasonality and can be used to infer a taxon's climatic niche which may also be considered an estimate of the Grinnellian or fundamental niche (Soberón, 2007). Other factors influencing distribution of taxa such as vegetation, food resources, predators, and competitors can be used to infer a taxon's realised or Eltonian niche; however these are not considered in the present study as they are difficult to measure at broad geographic scales.

Ecological niche modelling

For each target taxonomic unit I generated ENMs using MAXENT (maximum entropy modelling) v3.3.3 (Phillips & Dudik, 2008). MAXENT v3.3.3 uses a machine learning algorithm enabling spatial distribution modelling of presence-only data (Phillips *et al*., 2006). Known occurrence locations are used to determine a range of environmental variables that best predict a taxon's current geographical distribution. The machine-learning algorithm computes a probability of geographical distribution over mapped grid cells as a function of the cell's environmental variables, which can reflect contemporary or past climatic conditions. The final model presents a taxon's potential current geographical distribution, and the projected (palaeo) geographical distribution under past climate. An important limitation of this method is the assumption that bioclimatic limits applying to species today will have also applied to the same extent in the past and that there has been no evolution of environmental niche.

To address the first hypothesis of niche conservatism between haplogroups within *C. expansa* and within *C. longicollis*, present-day ENMs were developed separately for haplogroups A and B of both species using MAXENT v3.3.3 and all bioclimatic variables (see next section for tests of ecological similarity between haplogroups). To address the second and third hypotheses of range contraction and overlap among *C. expansa*, *C. longicollis*, and *C. canni*, I developed present-day ENMs based on terrestrial climate layers. These ENMs were then hindcasted to LGM conditions using MIROC and CCSM simulations. Three separate ENMs were developed incorporating: (1) all *C. expansa* occurrence records, (2) combined occurrence records of *C. longicollis* and F_1 *C. canni* + *longicollis* hybrids (hereafter referred to as the *C. longicollis* group) (n=3,115), and (3) combined occurrence records of *C. canni* and F_1 *C. canni* + *longicollis* hybrids (hereafter

referred to as the *C. canni* group) (n=187). The hybrid occurrence records were pooled separately with *C. canni* and *C. longicollis* as there were too few discrete hybrid locations to create robust ENMs for this taxon.

To obtain ENMs that are realistic and able to be transferred to another time period, the physiological limitations study taxa must be adequately represented, and the effects of multicollinearity among data layers reduced (Zielinski *et al*., 2013; Radosavljevic & Anderson, 2014; Weilstra & Artnzen, 2014). Ecological niche models for *C. expansa*, the *C. longicollis* group, and the *C. canni* group were initially developed using all nineteen bioclimatic variables. For each species, multicollinearity among variables was assessed using the correlation function in the program ENMTOOLS v1.4.3 (Warran *et al*., 2010). A final subset of bioclimatic variables were obtained for each species by selectively pruning highly correlated variables (Pearson's correlation > 0.7) and preferentially retaining those that were expected to influence freshwater turtle habitat availability and nesting outcomes. Retained variables included those that that influence the presence of water bodies (*i.e*. seasonal variation in evaporation and precipitation), hot and cold extremes of the year (*i.e*. temperature seasonality, maximums and minimums), drought/dry season incidence (*i.e*. precipitation of the driest quarter), and frost incidence (*i.e*. precipitation of the coldest quarter). The final model for *C. expansa* used eight variables, the *C. longicollis* group used a different eight, and the *C. canni* group used five. A full description of bioclimatic variables used to develop ENMs for each species is provided at Appendix 5A - Bioclimatic variables used for ENMs.

All ENMs were calculated in MAXENT v3.3.3 using a random 25% of occurrence points set aside as test data, the maximum number of iterations (500), and with duplicate samples removed. I used a convergence threshold of 0.00001, regularization multiplier of 1, and maximum number of background points of 10,000. All runs were created with auto

features, response curves, jack-knife tests, and logistic output format. Model performance was evaluated using area under the curve (AUC) values where AUC < 0.5 indicate models with no predictive ability (predictions no better than random) and AUC values near 1.0 indicate models with perfect predictions (Pearce & Ferrier, 2000).

Tests of ecological niche similarity and range overlap

To test if ENMs generated for each mitochondrial haplogroup within species were identical (niche conservatism) or different (niche divergence), ecological exchangeability was assessed in ENMTOOLS v1.4.3 using niche overlap, identity test and background test. Niche overlap and identity tests were performed among present-day distributions generated for *C. expansa*, the *C. longicollis* group, and the *C. canni* group; and range overlap tests were performed among palaeodistributions for each species. All niche overlap, identity, background, and range overlap tests were quantified using Schoener's D as calculated in ENMTOOLS v1.4.3. Rödder and Engler (2011) demonstrated this similarity index outperforms other metrics such Warren's *I* (Warren *et al*., 2008) and relative ranks (*RR*; Warren & Seifert, 2011).

Niche overlap tests (Warren *et al*., 2008) measure empirical pair-wise niche similarity based on predictions of habitat suitability. Niche overlap values range from 0 (niche divergence) to 1 (niche conservatism with identical ecological envelopes). The niche identity test examines if a niche overlap value for a taxon pair is more or less similar than expected by chance. The niche identity test pools georeferenced data points for a pair of taxa and randomises data point identity to create two new population samples of the same size as the original group (Warren *et al*., 2010). MAXENT v3.3.3 is used to generate an ecological niche model for each of the two new populations, and these are compared in ENMTOOLS v1.4.3 using niche similarity indices. This process is repeated to generate a

null distribution of niche similarity scores. The empirically observed niche overlap value is then compared to the identity test null distribution to assess if niche overlap is significantly different (one-tailed test) from expected. The hypothesis of niche identity (conservatism) between taxa is rejected when the empirical niche overlap value is lower (closer to 0) than the values expected from the null distribution. The niche identity test is very strict and niche conservatism is expected only when taxa inhabit the same set of environmental conditions and have the same suite of environmental conditions available to them (Warren *et al*., 2010). Availability of the same environmental conditions is unlikely to characterise allopatric taxa however and in these instances the background test is required.

I used the background test to infer niche differentiation or conservatism between allopatric haplogroups in *C. expansa*, and parapatric haplogroups in *C. longicollis*. The background test considers regional differences in the habitat (environmental conditions) available to each taxon and asks if ecological niche models are more or less similar than expected based on the differences of the environmental background in which each taxon resides. Null distributions are created by comparing an ENM of one taxon to an ENM generated from random points within the geographic range of the other taxon. I performed the background test in both directions for *C. expansa* haplogroups and *C. longicollis* haplogroups (*i.e*. the ENM for *C. expansa* haplogroup A was tested against the geographic background characteristic of *C. expansa* haplogroup B, and vice versa)   Geographic background for each haplogroup was defined by the minimum number of drainages that encompassed occurrence localities and follows the distribution of each haplogroup presented in Figure 5.1. Similar to the identity test, the empirically observed niche overlap value was then compared to the background test null distribution.   Niche conservatism is supported if the niche overlap value is above (closer to 1) the 95% confidence interval of the null distribution, and niche divergence is supported if the niche overlap value is below

(closer to 0) the 95% confidence interval of the null distribution (two-tailed test). Where the null hypothesis cannot be rejected, the niche similarity (or divergence) between taxa is no different than expected based on the regional differences in habitat available to each taxon (Warren *et al*., 2008, 2010). If the background test was significant in one direction but not in the other, I follow the conservative approach of Blair *et al*. (2013) and considered this support for the null hypothesis. All null distributions were developed using 100 pseudo-replicates. Significance at the 0.01 level was determined if Schoener's D falls outside the null distribution, and at the 0.05 level if Schoener's D falls within the highest or lowest 5% of the null distribution.

Finally, range overlap tests were used to quantify sympatry among present-day and palaeo distributions generated for *C. expansa*, the *C. longicollis* group, and the *C. canni* group. The range overlap test requires a threshold value at which either taxon is to be considered "present". For each range overlap test, I applied a threshold value by averaging each taxon pair's minimum training presence logistic provided in MAXENT v3.3.3. Threshold values using this method are as follows: *C. expansa* vs. the *C. longicollis* group = 0.0185; the *C. longicollis* group vs. the *C. canni* group = 0.069; and the *C. canni* group vs. *C. expansa* = 0.0785.

Results

All ecological niche models had mean test AUC values > 0.8 indicating a good ability to discriminate between presence and absence locations and good ability to predict observed distributions (Table 5.2). The ecological niche of *C. expansa* is best predicted by precipitation of the coldest quarter (BIO19) with a contribution of 62.7% to the model. This variable was highly correlated with precipitation of the driest month (BIO14, r=0.842) and precipitation of the driest quarter (BIO17, r=0.864). The ecological niche of the *C.*

longicollis group is best predicted by precipitation of the driest quarter (BIO17) with a

contribution of 55.1% to the model. This variable was highly correlated with maximum

temperature of the warmest month (BIO05, r=0.835) and precipitation of the driest month

(BIO14, r=0.954). Finally, the ecological niche of the *C. canni* group is best predicted by

temperature seasonality (BIO04) with a contribution of 72.4% to the model. This variable

is associated with hot and cold extremes of the year and was highly correlated with seven

other variables: minimum temperature of the coldest month (BIO06, r=0.826), temperature

annual range (BIO07, r=0.879), mean temperature of the coldest quarter (BIO11, r=0.744),

annual precipitation (BIO12, r=0.773), precipitation of the wettest month (BIO13, r=0.875),

precipitation of the wettest quarter (BIO16, r=0.884), and precipitation of the warmest

quarter (BIO18, r=0.759).

Table 5.2 Predictive ability of MAXENT models generated using present-day climatic conditions. n total = the total number of presence localities provided to the model, n model = the number of presence localities used to build the model (*i.e.* duplicates removed), SD = standard deviation.

	n total	n model	Test AUC	SD
C. expansa haplogroup A	134	46	0.960	0.012
C. expansa haplogroup B	29	16	0.846	0.004
C. longicollis haplogroup A	108	38	0.950	0.015
C. longicollis haplogroup B	166	60	0.824	0.032
C. expansa	744	153	0.937	0.013
*C. longicolli*s group	3,115	671	0.900	0.009
C. canni group	187	39	0.931	0.017

Haplogroup niche differentiation

Initial tests of niche overlap and identity between allopatric haplogroups in *C. expansa* and parapatric haplogroups in *C. longicollis*, indicate that haplogroups within species occupy niches that are significantly more different than expected. Background tests however give qualification to these initial results and provide a different interpretation that takes into account habitat available to each haplogroup. Ecological niche models for *C. expansa* haplogroups A and B differ significantly from the expected niche identity null distribution and appear to be highly divergent (D=0.33, p<0.01) (Figure 5.2a), the background tests however indicate that *stronger divergence* is expected. Mitochondrial haplogroup niches in *C. expansa* are more *similar* (niche conservatism) (p<0.01) than expected given the differences in environment and habitat experienced by each haplogroup (Figure 5.2b). The opposite case of niche divergence would be indicated if the observed niche overlap value (D) was to the left of both null distributions in Figure 5.2b.

In *C. longicollis*, ENMs for haplogroups A and B appear to be highly divergent (D=0.362, p<0.01) (Figure 5.3a). Nevertheless, the background test against the geographic range of *C. longicollis* haplogroup A (Figure 5.3b grey bars) could not reject the null hypothesis, meaning that the amount of niche divergence between mitochondrial haplogroups could be explained by differences in available environment and habitat. In the background test against the geographic range of haplogroup B (Figure 5.3b white bars), the null hypothesis was rejected (p<0.01) and the empirically observed niche overlap was closer to 1, providing evidence for greater niche conservatism than expected given differences in the habitat available to each haplogroup. Because background tests for *C. longicollis* are significant in one direction but not the other, I support the null hypothesis that haplogroup niche divergence is no more (or less) than expected

Present-day ecological niche models

Given the result of niche conservatism between *C. expansa* haplogroups, and no support for niche divergence between *C. longicollis* haplogroups, I combined occurrence data of each haplogroup within each species to create present-day and palaeo distribution models. Present-day distribution models for each species largely agree with their realised distribution.

The present-day *C. expansa* ENM (Figure 5.4a) predicts continuous habitat suitability throughout the lowlands of the Murray-Darling Basin, eastward into the Hunter catchment, and north in coastal south eastern Queensland. Very high suitability is predicted for Fraser Island, the lowland regions of the Border Rivers and the Condamine River in the northern Murray-Darling Basin, and the River Murray and Murrumbidgee River floodplains in the southern Murray-Darling Basin. Very low habitat suitably is broadly predicted throughout the far western Murray-Darling Basin and moderate to high elevation regions of the Great Dividing Range.

The *C. longicollis* group ENM (Figure 5.5a) predicts broad habitat suitability throughout the eastern lowlands and uplands of the Murray-Darling Basin but low suitability in the west, similar to *C. expansa*. Three discrete areas of high habitat suitability are predicted in the Murray-Darling Basin corresponding to the mid-Murray region, the upland tributaries of the Macquarie River, and the upland tributaries of the Namoi and Gwydir Rivers. A continuous band of suitable habitat extends throughout the southeastern coastal plain from western Victoria northward to near the Styx River catchment in Queensland. An area of unsuitable habitat in southeast Australia corresponds with the high elevation alpine region of the Great Dividing Range.
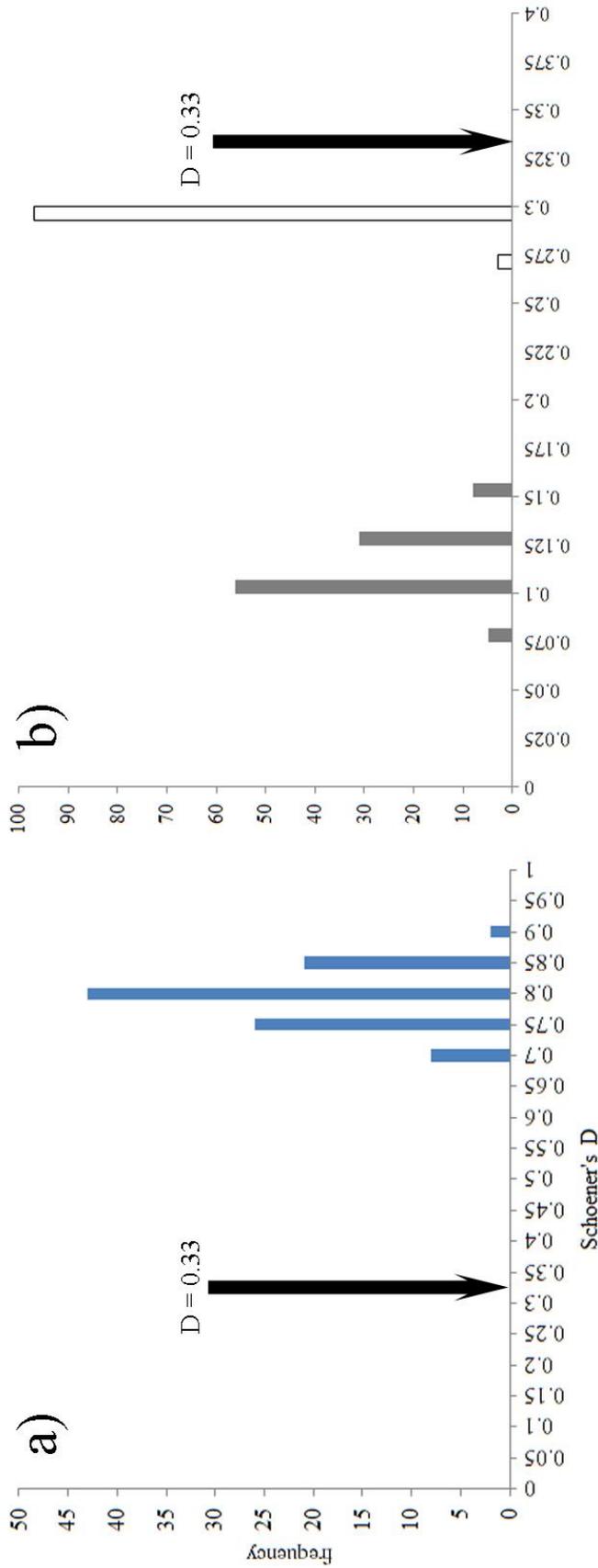
Figure 5.2 (a) *Chelodina expansa* **mitochondrial haplogroup niche identity null distribution, and (b) background test null distributions. The black arrows illustrate where the empirically observed niche overlap value falls compared to the null distribution of pseudo-replicated values. In the right panel, grey bars represent values for random samples within the geographic range of *C. expansa* haplogroup A, and white bars represent values for random samples within the geographic range of *C. expansa* haplogroup B.**
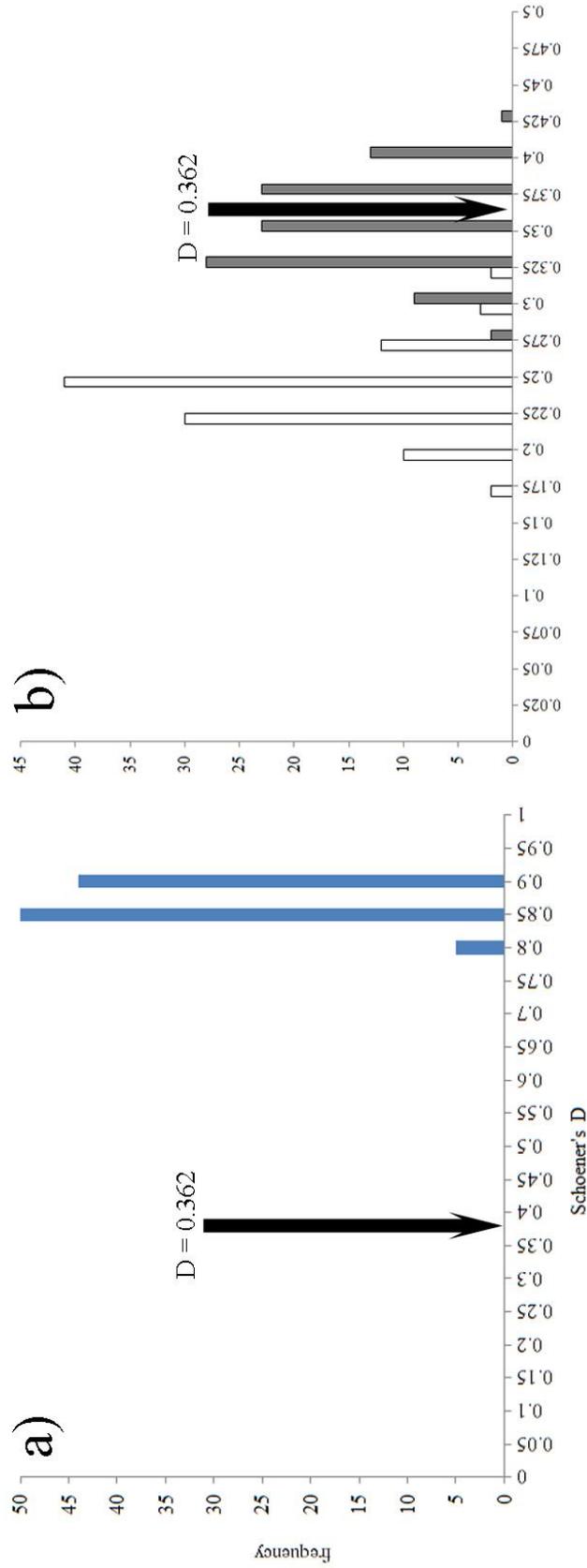
Figure 5.3 **(a)** *Chelodina longicollis* **mitochondrial haplogroup niche identity null distribution, and (b) background test null distributions. The black arrows illustrate where the empirically observed niche overlap value falls compared to the null distribution of pseudo-replicated values. In the right panel, grey bars represent values for random samples within the geographic range of *C. longicollis* haplogroup A, and white bars represent values for random samples within the geographic range of *C. longicollis* haplogroup B.**

The ENM for the *C. canni* group (Figure 5.6a) predicts two disjunct distributions separated by a low suitability region centred on the Black Mountain corridor. One region of suitable habitat is predicted across a broad area of northern Australia's monsoonal tropics encompassing the Top End and Cape York Peninsula. The other is a narrow band of habitat fringing the east coast from the Black Mountain corridor in the north, to Fraser Island in the south. In northern Australia, the model predicts very high habitat suitability associated with the southwest coastline of the Gulf of Carpentaria. On the east coast, high habitat suitability corresponds to the Daintree River and Endeavour River catchments, the Ross River catchment near Townsville, the Proserpine catchment, and the Waterpark Creek catchment north of Rockhampton. Areas of unsuitable habitat for the *C. canni* group correspond to the rise of the sandstone escarpment in the Top End, and the interface with the Great Dividing Range in the east.

Niche similarity between species is in accordance with predicted present-day distributional overlap (Figure 5.7). Tests of ecological niche similarity indicate that *C. expansa* and the *C. longicollis* group occupy the same niche (D=0.688), while the niche of the *C. canni* group is significantly different to both the *C. longicollis* group (D=0.091, p<0.01) and C. *expansa* (D=0.088, p<0.01).
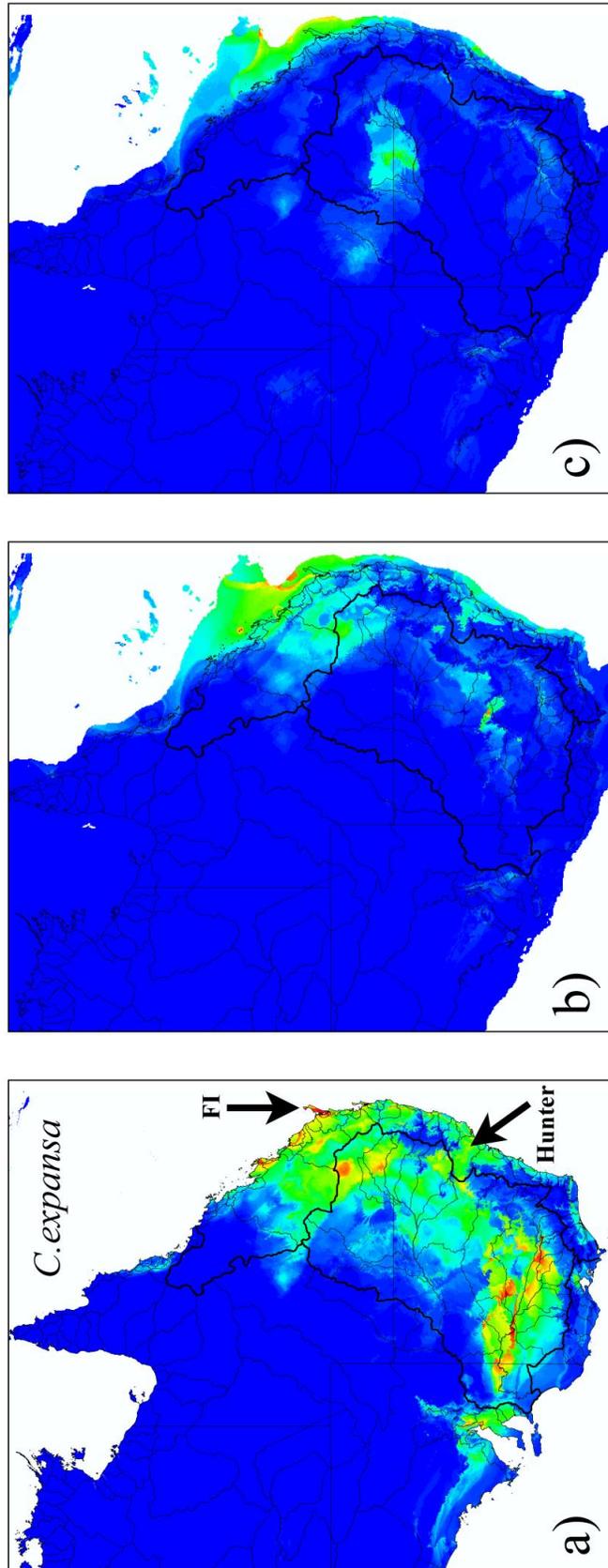
Figure 5.4 ENMs generated for *C. expansa* under modern climatic conditions (a) and paleoclimate using LGM MIROC climate variables (b) and LGM CCSM climate variables (c). Colours represent predicted probability of presence from low (blue) to high (red) suitability. Thin black lines represent catchment boundaries, and the thick black line outlines the boundary of the Murray-Darling Basin. Some sites mentioned in the text are provided. FI: Fraser Island.
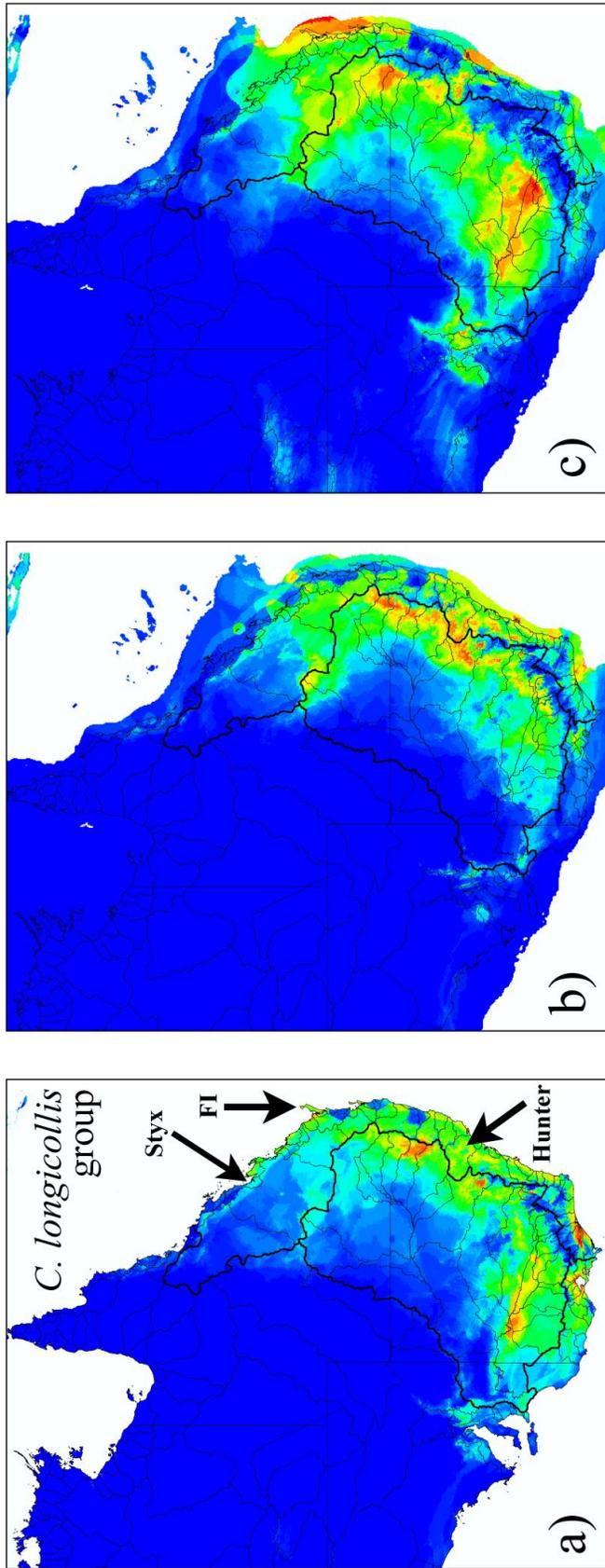
Figure 5.5 ENMs generated for the *C. longicollis* group under modern climatic conditions (a) and paleoclimate using LGM MIROC climate variables (b) and LGM CCSM climate variables (c). Colours represent predicted probability of presence from low (blue) to high (red) suitability. Thin black lines represent catchment boundaries, and the thick black line outlines the boundary of the Murray-Darling Basin. Some sites mentioned in the text are provided. FI: Fraser Island.

Palaeo ecological niche models

Palaeodistributions of *C. expansa* indicate a substantial reduction of suitable habitat during glacial aridity. Both MIROC (Figure 5.4b) and CCSM Figure 5.4c) simulations show strong distributional contraction to the northeast, centred on the broad continental shelf north of, and including present-day Fraser Island. A small region of low to moderate habitat suitability around the Hunter catchment coastal plain in the southeast is also evident in both simulations. Range overlap between *C. expansa* ENMs simulated under MIROC and CCSM is moderate at 0.689.

For the *C. longicollis* group, both MIROC and CCSM palaeodistributions (Figure 5.5b, c) indicate maintenance of the present-day widespread distribution during glacial aridity, with the majority of the Murray-Darling Basin and the east coast remaining ecologically suitable. Range overlap between the *C. longicollis* group ENMs simulated under MIROC and CCSM is very high at 0.994. Small differences between the two LGM simulations occur at the local scale however with different regions of high predicted habitat suitability.

The MIROC simulation predicts very high habitat suitability associated with the eastern uplands of the Murray-Darling Basin encompassing the upper tributaries of the Macquarie, Namoi, Gwydir, and Border Rivers. This band of high suitability extends onto the coast and incorporates a small distribution in the Hunter catchment. The CCSM simulation predicts high habitat suitability in the Murray-Darling Basin focussed on the mid-Murray and mid-Murrumbidgee catchments in the south, and the mid-Border Rivers catchment in the north. On the coast, CCSM agrees with MIROC in predicting moderate to high suitability in the Hunter catchment, however CCSM also predicts a band of high habitat suitability on the exposed continental shelf near present-day Fraser Island.
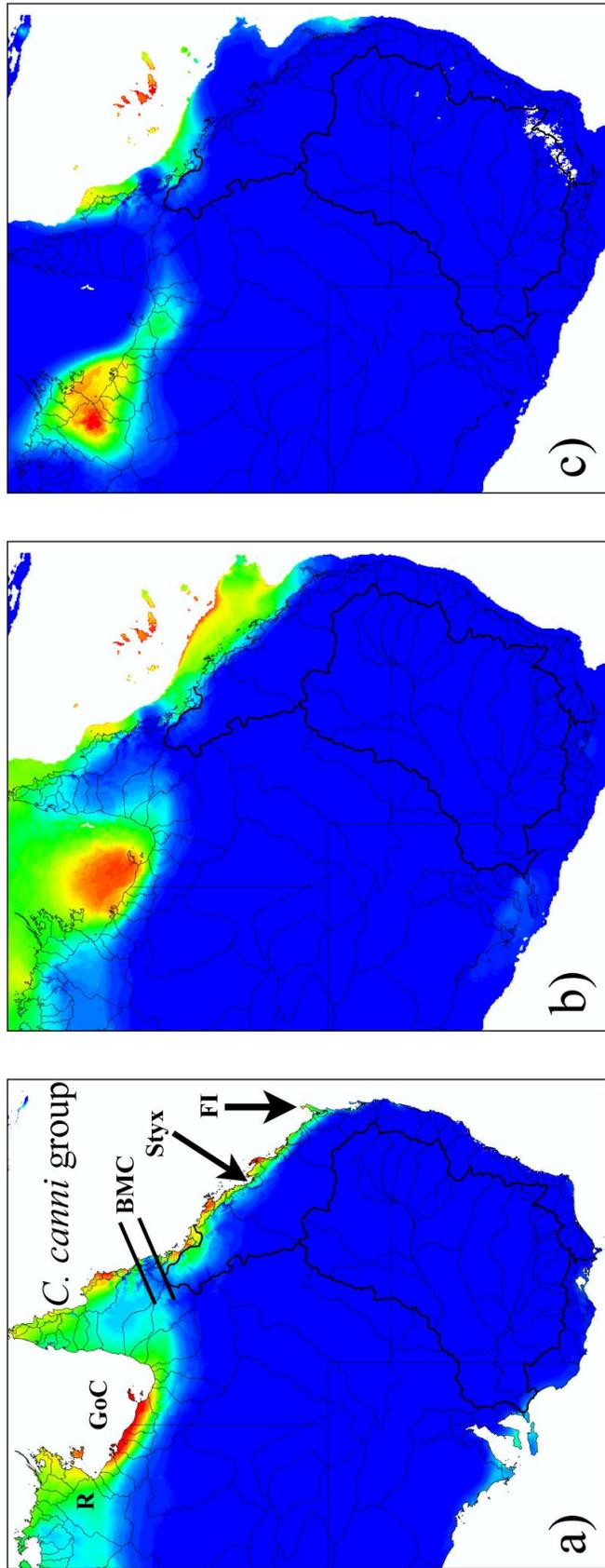
Figure 5.6 ENMs generated for the *C. canni* group under modern climatic conditions (a) and paleoclimate using LGM MIROC climate variables (b) and LGM CCSM climate variables (c). Colours represent predicted probability of presence from low (blue) to high (red) suitability. Thin black lines represent catchment boundaries, and the thick black line outlines the boundary of the Murray-Darling Basin. Some sites mentioned in the text are provided. **R: Roper River catchment; GoC: Gulf of Carpentaria, BMC: Black Mountain Corridor, FI: Fraser Island.**
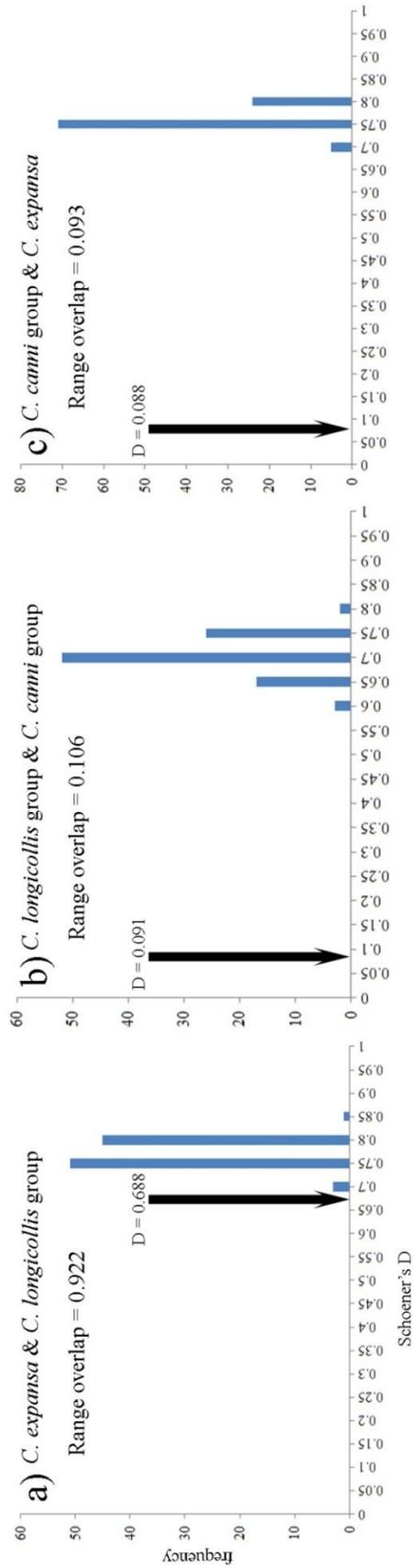
Figure 5.7 Niche identity null distributions between present-day ENMs of *C. expansa* and the *C. longicollis* group (a), the *C. longicollis* group and the *C. canni* group (b), and the *C. canni* group and *C. expansa* (c). Black arrows illustrate where the empirically observed niche overlap value falls compared to the null distribution of pseudo-replicated values. Range overlap value is based on the overlap of predicted distributions given in ENMs.

Palaeodistributions for the *C. canni* group indicate large and disjunct distributions encompassing the Top End and the east coast continental shelf. Range overlap between ENMs for the *C. canni* group simulated under MIROC and CCSM is moderate at 0.715. The MIROC simulation (Figure 5.6b) predicts a region of high habitat suitability in Lake Carpentaria (present-day Gulf of Carpentaria), and another encompassing the broad east coast continental shelf from the present-day Black Mountain Corridor in the north to present-day Fraser Island in the south. The CCSM simulation (Figure 5.6c) predicts a Top End distribution centred on the Roper River catchment in eastern Arnhem Land, and two disjunct northeast coast distributions each with low to moderate habitat suitability. Similar to the present-day ENM for the *C. canni* group, these two northeast CCSM distributions appear to be separated by the Black Mountain Corridor. There is also a small region of low habitat suitability centred on Fraser Island.

Range overlap during Plio/Pleistocene aridity

There is large overlap of *C. expansa* and the *C. longicollis* group palaeodistribution (Table 5.3). Overlap is centred on the broad east coast continental shelf around present-day Fraser Island. Palaeodistributions for the *C. canni* group have small range of overlap with *C. expansa* and the *C. longicollis* group, and in both cases this is also located on the east coast continental shelf, slightly north of present-day Fraser Island.

Discussion

No evidence of niche differentiation between haplogroups

Species that are widely distributed across heterogeneous landscapes are often characterised by locally adapted populations with distinct environmental tolerances and ecological niches (Cheviron & Brumfield, 2009; Fournier-Level *et al*., 2011; Banta *et al*., 2012). This

emphasises the role of geographical isolation in lineage divergence and ecological

speciation (Rundle & Nosil, 2005). The current distribution of mitochondrial haplogroups

A and B in *C. expansa* and *C. longicollis* are broad, and sharply defined as either allopatric

or parapatric, drawing the question of whether they each originated though local adaptation

and are kept separate by ecological niche divergence.

The mitochondrial haplogroup pairs in *C. expansa* and *C. longicollis* each use

statistically different niche space, as demonstrated by the rejection of niche identity.

However, when the environment and habitat available to each haplogroup is taken into

account, neither pair display significant niche divergence. Indeed, mitochondrial

haplogroups A and B in *C. expansa* exhibited greater niche *conservatism* than expected by

chance even though they each occur in regions with different environmental and habitat

characteristics. Some caution must be exercised when applying ENMs to study niche

differentiation because niche partitioning and local adaption may occur on a microhabitat

scale unable to be captured with the bioclimatic variables applied in this analysis

(Alvarado-Serrano & Knowles, 2014).

Introgression via neutral or adaptive processes?

The role of the mitochondrial genome in oxidative phosphorylation and basic metabolic

functioning make it highly visible to selection and inclined to local adaptation (Cheviron &

Brumfield, 2009). Positive selection for favourable metabolic properties can drive

mitochondrial introgression beyond initial hybrid zones and lead to replacement sweeps

across the entire distribution of a species, especially in organisms that occur across a

variety of temperature regimes (Rheindt & Edwards, 2011). The mitochondrial genome in

poikilotherms such as freshwater turtles is particularly susceptible to adaptive introgression

given that poikilotherm mitochondria experience external temperatures

Table 5.3 Pairwise range overlap based on palaeodistributions modelled under MIROC and CCSM datasets.

species pairs	MIROC range overlap	CCSM range overlap
C. expansa vs *C. longicollis* group	0.944	0.898
C. longicollis group vs. *C. canni* group	0.120	0.117
C. canni group vs. *C. expansa*	0.280	0.137

 (Ballard & Whitlock, 2004). Invoking a series of selective sweeps to explain introgression in freshwater turtles is seductive. It is difficult to explain complete replacement of one species' mitochondrial DNA, mitochondrial introgression with considerably less nuclear introgression, repeated waves of mitochondrial introgression, and fixation of mitochondrial DNA of one species across more than half the range of another, without invoking adaptive processes (Ballard & Whitlock, 2004; McGuire *et al*., 2007; Irwin *et al*., 2009; Toews & Brelsford, 2012). Such patterns strongly characterise the current study, however introgression via neutral processes cannot be rejected on available evidence.

It is possible that signals of past niche differentiation and local adaption have been erased over time, or that local adaptation cannot be captured using the available bioclimatic variables. In each case, we would expect a biogeographic pattern where the distribution of different haplogroups is discrete and non-overlapping, consistent with adaptation of each haplogroup to a different environment. This is not the case in the present study. Mitochondrial haplogroup A (in *C. expansa)* and haplogroup B (in *C. longicollis*) are sympatric throughout the vast and thermally diverse inland Murray-Darling Basin, and are each also distributed throughout the coastal margin in both species. Selective advantage of one mitochondrial genome over another cannot be accepted given this sympatry.

To concede an adaptive scenario, one must accept that the mitochondrion of *C. longicollis* is better adapted to conditions in the Murray-Darling Basin but only when against a nuclear background of *C. expansa* and not *C. longicollis* itself. Simultaneously, one must accept that the mitochondrion of *C. canni*, which occurs in less extreme thermal environments, is better adapted to the conditions in the Murray-Darling Basin only when against nuclear background of *C. longicollis* and not *C. expansa*. Collectively, a lack of support for niche differentiation between haplogroups and sympatry of different

mitochondrial haplogroups is difficult to reconcile with any adaptive scenario. The null

hypothesis of neutral introgression remains the most parsimonious explanation at this stage.

C. longicollis captured *C. canni* near the Styx catchment

Palaeodistributions for *C. canni* demonstrate that this species was not present throughout

the Murray-Darling Basin during glacial aridity. This result excludes the possibility that *C.*

canni had a historical and extensive inland distribution and hybridised in situ with resident

C. longicollis. Instead, palaeodistributions demonstrate *C. canni* and *C. longicollis*

persisted together during glacial aridity on the coast in the Styx River catchment. This

modelled sympatry occurs at the southern distributional extent of *C. canni* and the northern

distributional extent of *C. longicollis* in the same location observed today where the species

hybridise to produce viable offspring (Georges et al., 2002). If the LGM hindcast ENMs

are a reasonable approximation of freshwater turtle distributions during the arid late

Pliocene, this region is a good candidate site for the first episode of asymmetric

mitochondrial introgression 2.24 Mya.

In this episode the local population of *C. longicollis* captured the mitochondrial

genome of *C. canni*, effecting mitochondrial paraphyly of *C. longicollis* and

characterisation in haplogroup B. The Styx River catchment and surrounding area has

clearly played an important role in the evolutionary history of *C. longicollis*, possibly since

the late Pliocene. Further work is required to determine the modern frequency of hybrids in

this area and illuminate any landscape, demographic, or selective forces that might drive

local introgression of the *C. canni* mitochondrial genome.

After introgression and fixation of the *C. canni* mitochondrial haplogroup, the local

C. longicollis appears to have tracked warming conditions to expand into and diversify

within the Murray-Darling Basin. This must have occurred prior to the early Pleistocene as

Hodges *et al.* (2015) report time to the most recent common ancestor of *C. longicollis* haplogroup B at 1.5 Mya. It is unknown if expansion of introgressed *C. longicollis* into the Murray-Darling Basin replaced or founded the first populations of the species in the region.

Palaeodistributions demonstrate persistence of *C. longicollis* in the Murray-Darling Basin during LGM aridity, and such persistence may have also characterised populations prior to 1.5 Mya. Sound conclusions of original establishment of *C. longicollis* in the Murray-Darling Basin are beyond the scope of this study and would benefit from fossil evidence and a distribution model based on climatic conditions particular to the early Pleistocene.

C. expansa **introgression**

Palaeodistributions for *C. expansa* indicate a substantial decrease in distribution, and low to moderate habitat suitability during glacial aridity. Changes in population and range size are correlated (Excoffier *et al.*, 2009; Arenas *et al.*, 2012). Here I associate a decrease in range with reduced effective population size, and low habitat suitability with low ancestral population density. Palaeodistributions for *C. expansa* are consistent in their support for two disjunct regions of habitat suitability. One encompasses a small site in the Hunter catchment coastal plain, and the other occurs to the north across a broad region on the continental shelf around Fraser Island. These regions have high habitat suitability in *C. longicollis* palaeodistributions and highlight two distinct refugia where different mitochondrial lineages likely introgressed into *C. expansa* in the arid late Pliocene and early Pleistocene.

***C. expansa* captured *C. longicollis* near the Hunter catchment**

Hindcasted habitat suitability in the Hunter catchment coastal plain is moderate for *C. expansa* and very high for *C. longicollis*, translating to population size disparity where *C. expansa* was rare and *C. longicollis* more common. Following this, I suggest that late Pliocene sympatry and population size disparity in this region allowed *C. expansa* to capture the 'true' *C. longicollis* mitochondrial genome 1.29 Mya, thus positioning *C. expansa* in haplogroup A. *Chelodina expansa* is not currently distributed in the Hunter catchment and no *C. expansa* fossils are known from the area despite this region supporting high modelled habitat suitability. The Hunter catchment is a well-known biogeographic barrier to terrestrial forest-adapted fauna (Moussalli *et al*., 2005; Chapple *et al*., 2011; Rix & Harvey, 2012), and has recently been highlighted as a region of stability for freshwater fauna, having the highest haplotype diversity in *C. longicollis* consistent with long-term population persistence (Hodges *et al*., 2015). Palaeodistributions presented here for *C. expansa* and *C. longicollis* reinforce the significance of this region for freshwater turtles, and support the possibility raised in Hodges *et al*. (2014) that *C. expansa* had an ancient southerly coastal distribution.

Expansion of *C. expansa* out of the Hunter catchment, perhaps during Pleistocene glacial minima, may have established populations on the coast to the north and in the Murray-Darling Basin to the west, each fixed for the *C. longicollis* mitochondrial genome. The South Conondale *C. expansa* population represents the northern extent of this past expansion event, and is today the sole remnant of a hypothesised historical coastal distribution. It is worth noting that the *C. expansa* population in the Murray-Darling Basin has a signature of recent and rapid expansion approximately 14 Kya (Hodges *et al*., 2014). Hodges *et al*. (2014) suggest this expansion occurred *in situ*, however palaeodistributions

for *C. expansa* presented here demonstrate a lack of suitable refuge habitat in the Murray-Darling Basin. Habitat suitability in the Hunter catchment raises an alternative possibility that this region maintained a population of *C. expansa* until very recently, and possibly acted as an *ex situ* source for the recent Murray-Darling Basin expansion.

C. expansa captured *C. canni* near Fraser Island

Sympatry of *C. expansa* and *C. longicollis* populations on the continental shelf around Fraser Island likely facilitated the third and final episode of introgression 1.5 Mya. In this event, *C. expansa* hybridised with the local introgressed population of *C. longicollis* to capture the mitochondrial genome of *C. canni*. Phylogeographic studies of *C. expansa* and *C. longicollis* both demonstrate a history of intraspecific isolation and diversification in the coastal region around Fraser Island, specifically in the Mary, Burnett, and Fitzroy-Dawson drainages, and on Fraser Island itself (Hodges *et al*., 2014; Hodges *et al*., 2015). These locations have moderate to high hindcasted habitat suitability in both species and are strong candidate sites for harbouring refugia and facilitating introgression. Alternatively, population expansion onto the newly exposed continental shelf around Fraser Island may have enabled introgression especially if *C. expansa* was rare and *C. longicollis* more common, thus exposing *C. expansa* to greater genomic dilution. Hindcasted habitat suitability on the continental shelf around Fraser Island is moderate for *C. expansa* and very high (CCSM) for *C. longicollis*, supporting population size disparity required for the above scenario.

Biogeography of shared haplotypes

The biogeographic arrangement of shared haplotypes between *C. expansa* and *C. longicollis* presents some challenges for interpretation. Does haplotype sharing evidence

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the location of ongoing gene flow, or is it simply retained ancestral polymorphisms from past introgression? Ongoing gene flow is an unlikely explanation as shared haplotypes are not sympatric and are not shared between proximate regions. If gene flow between species was ongoing one would expect clustering of shared haplotypes at the site of introgression, or at least nearby within the same catchment. Further argument against ongoing gene flow is a lack of evidence supporting contemporary introgression between *C. expansa* and *C. longicollis* at nuclear DNA. Examination of more sensitive nuclear markers such as microsatellites or SNPs would however strengthen this assertion. An alternate explanation for haplotype sharing involves retention (by *C. expansa*) of the 'donor' species' (being *C. longicollis*) polymorphisms that were captured during Plio/Pleistocene introgression. Shared haplotypes occupying an internal (ancestral) position in the haplotype network (Figure 5.1) supports this conclusion. With increasing time since last gene flow and with stochastic lineage sorting erasing polymorphisms that date to the time of introgression, haplotypes from a donor species that persist in the introgressed species are more likely to be phylogenetically basal (Funk & Omland, 2003). The lack of geographic clustering of shared haplotypes can be attributed to shifting species distributions after introgression and dissipation of shared variation close to the original hybrid zone.

Conclusions

Ecological niche modelling confirms that ancient arid and cold conditions likely led to population sympatry among freshwater turtles in eastern Australia. Palaeodistribution models highlight locations where asymmetric mitochondrial introgression and fixation of locally acquired lineages likely occurred. These hindcasts strengthen assertions that coastal regions around the Hunter catchment, Fraser Island, and the Styx catchment hosted important interactions between species, with significant evolutionary consequences. Future

studies on other freshwater taxa could explore whether similar refugia are identified and if these coincide with expected genetic signatures of persistence and diversification. The data at hand do not support niche divergence or local adaption of mitochondrial haplogroups within a species. Although this result warrants further investigation, it highlights the important role ecological niche modelling can play in testing mechanisms supposedly driving introgression, and in understanding the role of ecology in diversification.

The results of the present study reveal the influence of landscape-scale processes in shaping the biogeographic pattern of asymmetric mitochondrial introgression. Biogeographic signatures of introgression become progressively diluted with increasing time since last gene flow. The pattern of shared haplotypes in this study and the distribution of mitochondrial lineages more broadly typify a scenario where introgression was ancient, but not so ancient that ancestral polymorphisms have been lost and all haplotypes have become derived. This study demonstrates how ecological niche modelling can expose the previously unrecognised spatio-temporal element of introgression, and provide key insights into historical biogeography. Biogeographic patterns of asymmetric mitochondrial introgression are increasingly being described. There is an exciting future for ecological niche modelling to integrate further with phylogeography and shift the focus from simply documenting biogeographic patterns towards exploring the underlying biogeographic process behind introgression.

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Supporting information

Appendix 5A - Bioclimatic variables used for ENMs

C. expansa **n = 8**

BIO4 = Temperature Seasonality; BIO6 = Min Temperature of Coldest Month; BIO8 = Mean Temperature of Wettest Quarter; BIO9 = Mean Temperature of Driest Quarter; BIO11 = Mean Temperature of Coldest Quarter; BIO16 = Precipitation of Wettest Quarter; BIO18 = Precipitation of Warmest Quarter; BIO19 = Precipitation of Coldest Quarter.

C. longicollis **group n = 8**

BIO4 = Temperature Seasonality; BIO6 = Min Temperature of Coldest Month; BIO9 = Mean Temperature of Driest Quarter; BIO10 = Mean Temperature of Warmest Quarter; BIO15 = Precipitation Seasonality (Coefficient of Variation); BIO16 = Precipitation of Wettest Quarter; BIO17 = Precipitation of Driest Quarter; BIO18 = Precipitation of Warmest Quarter.

C. canni **group n = 5**

BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp)); BIO4 = Temperature Seasonality; BIO9 = Mean Temperature of Driest Quarter; BIO10 = Mean Temperature of Warmest Quarter; BIO17 = Precipitation of Driest Quarter.

Chapter 6

Synopsis

This thesis was originally conceived as a study in phylogeography, yet the results provide new and extraordinary insights into the phylogenetic relationships and recent evolutionary history of the *Chelodina* more broadly. The sampling scheme I employed facilitated detection of two highly divergent haplogroups with distinct geographic boundaries, extensive mitochondrial introgression, complete mitochondrial replacement in *C. expansa*, and partial mitochondrial replacement in *C. longicollis*. Broad sampling of individuals across different environments also facilitated examination of haplogroup ecological niche and tests of niche differentiation. Previous phylogenic studies of *Chelodina* excluded at the outset the possibility of discovering the above results and methods by not sampling multiple geographically dispersed populations and their associated lineages.

Future molecular genetic studies in *Chelodina* must be mindful that species tree inferences are sensitive to geographic sampling. Relationships based on mitochondrial DNA especially will change depending on which specimens are used. For *C. expansa*, specimens from the Murray-Darling Basin and South Conondale region will represent *C. longicollis*. Specimens from the North Conondale region will represent *C. canni*. Similarly for *C. longicollis*, specimens from the Murray-Darling Basin, south east Queensland, and western Victoria will represent *C. canni*. The risk of incorrect evolutionary inference and inaccurate species assignments is likely more prevalent in studies on freshwater turtles than currently recognised. Wide geographic distributions of many species and preponderance for introgression and reproductive compatibility make it especially important for studies on this group to employ a multilocus phylogenetic approach and extensive geographic sampling from regions of ancient or recent sympatry. Current studies on freshwater turtles

complex Great Dividing Range a result of *C. longicollis* utilising Pleistocene upland wetlands? Could this same pattern result from the permanent water provided by contemporary farm dams? If agricultural development is facilitating anthropogenic gene-flow between divergent genetic lineages and across historically strong bioregional barriers, what are the management implications for freshwater bioregions and for their utility more generally? Further work is needed to assess if major freshwater biogeographic barriers such as the Great Dividing Range may be only of historical importance in some species, especially in the face of contemporary anthropogenic change in the freshwater landscape.

Synthesising the results of Chapter 2 and Chapter 3 highlights the contrasting phylogeographic patterns between *C. expansa* and *C. longicollis*. While both species share a similar temporal and spatial pattern of east/west lineage divergence, genetic structure within each westerly distributed lineage in the Murray-Darling Basin is vastly different between species. *Chelodina expansa* is characterised by a recently evolved haplotype network and recent and rapid population expansion, likely from a single ex-situ refugium. *Chelodina longicollis* is characterised by signals of isolation by distance reflecting time enough to establish equilibrium between gene flow and genetic drift. I attribute these contrasting patterns to idiosyncratic species-specific responses to Pleistocene climatic cycling. I propose that Pleistocene climatic oscillations in Australia afforded species the opportunity to shift their distributions according to species-specific requirements and capabilities. During glacial aridity, small differences in life histories between species would have been magnified and resulted in idiosyncratic, irregular and localised extinctions and range contractions. For *C. expansa* and *C. longicollis* in particular, dissimilarity in life history traits such as cold tolerance and the ability to abandon drying habitat in search of permanent water likely made the difference between persistence and local extinction in the Murray-Darling Basin.

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The second purpose of this thesis was to investigate haplotype sharing and extensive mitonuclear discordance in *C. expansa* and *C. longicollis*, and the biogeographic conditions that led to the patterns described. I demonstrated that haplotype sharing between species, and extensive mitonuclear discordance, is a result of multiple episodes of introgressive hybridisation within and between *Chelodina* subgenera during the Plio/Pleistocene (Chapter 4). I established that the original *C. expansa* mitochondrial lineage is extinct owing to "dual" mitochondrial capture of both *C. longicollis* and *C. canni*. This is the first reported incidence of dual mitochondrial capture in a reptile and only the second time dual mitochondrial capture has been reported more generally (*c.f.* Chinese hares *Lepus* spp. Liu *et al*., 2011). Further, capture of the *C. canni* lineage represents a case of double introgression whereby *C. expansa* hybridised with an introgressed *C. longicollis* population carrying *C. canni* mitochondrial DNA.

Mitochondrial introgression requires reproductive compatibility post species divergence and a lack of, or weak prezygotic and postzygotic isolating barriers. Reproductive compatibility and introgression between ancient lineages has precedence among freshwater turtles. Freedberg & Myers (2012) describe introgression between species of *Graptemys* that diverged approximately 5 Mya. The Plio/Pleistocene introgression I describe in Chapter 4 between *Chelodina* subgenera of early Miocene origin is extraordinary and represents the greatest reported divergence between hybridising freshwater turtle species. This new knowledge draws questions regarding reproductive compatibility. Has reproductive compatibility persisted for a substantial period in the history of *Chelodina*? Or have *C. longicollis*, *C. canni*, and *C. expansa* each lost their reproductive isolating mechanisms? Reproductive isolation is believed to increase with divergence between taxa and sympatric species pairs are considered to have stronger pre

and postzygotic isolating barriers than those in allopatry (Coyne & Orr, 1989, 1997, 2004). This study however may demonstrate that deep divergence and sympatry are not necessarily correlated with reproductive isolation. Reproductive isolation may indeed be a prolonged process among turtle species and reproductive compatibility may persist for substantial periods in a phylogeny.

I applied a demographic lens to the question of how mitochondrial capture and introgressive hybridisation occurred (Chapter 4). I argued that the action of selective forces is not necessarily required, and that the neutral process of demographic disparity between sympatric species likely drove introgression. This neutral process posits that a species with a small effective population size will be massively introgressed by the mitochondrial DNA of the more common species. Accordingly, introgression from resident (more common) into invading (less common) species is the rule and population expansions provide the most common opportunity (Currat *et al*., 2008). More specifically, interglacial expansions and post-glacial secondary contact is often invoked as the primary driver of asymmetric introgression (Taberlet *et al*., 1998; Hewitt, 2004; Babik *et al*., 2005; Morgan *et al*., 2010; Rheindt & Edwards, 2011). I used palaeo-ecological niche modelling to investigate if these biogeographic conditions are consistent with the timing of introgression in *Chelodina*, and to test the hypothesis that introgression was neutral and not selective (Chapter 5).

Different to other studies, I found that asymmetric introgression occurred during glacial aridity and was likely driven by contraction to glacial isolates rather that expansion from them (Chapter 5). Instead of exposing secondary contact zones between recently joined biotas, I highlighted the locations of ancient glacial isolates where sympatric "primary contact" (Toews & Brelsford, 2012) afforded the opportunity for introgression. Specifically, I highlighted the Hunter catchment, Fraser Island, and the Styx catchment as key locations of introgression and fixation of locally acquired lineages during

Plio/Pleistocene aridity. I found no evidence supporting selection of one mitochondrial haplotype over another (Chapter 5). Haplogroups within a species did not display local mitochondrial adaptation and were not kept separate by ecological niche divergence. These new results lend further support to the hypothesis raised in Chapter 4 that asymmetric mitochondrial introgression was likely driven by neutral demographic processes.

Future directions

A number of important research directions have emerged from my thesis that can guide future study to better understand the biogeography of freshwater systems, and the extent and cause of mitochondrial introgression in *Chelodina*.

Future investigations on the concordance of phylogeographic patterns among freshwater taxa are warranted. Taxa such as water rats (*Hydromys*), water dragons (*Physignathus*), and water skinks (*Eulamprus*) are good candidates to compare with freshwater fish and turtles. Sites of significance include regions of high diversity and potential for endemism such as Fraser Island, and the Fitzroy-Dawson, Burnett, Mary and Hunter catchments. My research also highlights areas where further resolution of genetic structure would be useful for conservation and management. Questions remain about the extent and influence of the Conondale Range, and of the phylogeographic break on Fraser Island. Does the influence of these features extend to *C. longicollis*? Do these features delineate evolutionary significant units in freshwater taxa? Both these regions have a high potential to harbour freshwater genetic diversity and endemism at very small spatial scales. Future work should undertake fine scale sampling and assemble data from highly polymorphic genetic markers to answer these questions, and to help define management units to direct regional conservation strategies.

Future research on mitochondrial introgression in *Chelodina* should focus on assessing more nuclear loci and testing for mitochondrial selection. Next-Generation Sequencing methods are ideal for generating nuclear datasets and testing for gene flow, and signals of ancient gene flow can now be investigated in non-model organisms as NGS technologies converge on longer read lengths. If future research uncovers nuclear introgression between freshwater turtle species, the legacy of introgressed nuclear elements should also be explored. Questions regarding the spatial extent of nuclear gene penetration, introgressed nuclear DNA conferring selective advantage, and the possibility that fixated introgressed nuclear DNA could lead to the formation of novel hybrid lineages should drive the research agenda. These questions could take advantage of new techniques in comparative transcriptomics which can detect outlier loci under selection. Equally important, future research should also examine nuclear regions that show no signs of introgression but elevated levels of divergence to gain insights into genomic regions responsible for reproductive isolation and maintaining species identity.

More extensive tests for selection are required. Prezygotic and postzygotic selective forces, undetected in this study, could favour the frequency or viability of one hybrid class over another and impel introgression. Assortative mating for example, disruption of co-adapted gene complexes, and Haldane's rule could each act independently or operate alongside demographic disparity to drive asymmetric introgression. Future studies investigating the existence and extent of premating and postmating barriers in *Chelodina* are required to address the influence of prezygotic and postzygotic selective forces. I was unable to reject the null scenario of neutral asymmetric mitochondrial introgression via demographic disparity. Future work should test this hypothesis more rigorously especially since intra and interspecific variation in protein coding regions of the mitochondrial

genome are increasingly being attributed to selection (Ballard & Rand 2005; Ballard & Melvin 2010; Meiklejohn *et al*., 2007; Scott *et al*., 2011).

Research should be directed towards exploring the relative fitness of mitochondrial genomes in different environments and in different freshwater turtle populations. Investigations should focus on whether a mixed mitonuclear phenotype confers a selective advantage compared to a locally typical genomic profile, and also if introgressed mitochondria differ phenotypically from those of the type species. Finally, mitochondrial respiration and thermal adaption in particular should also be studied to determine their effect on whole animal fitness, and if this differs between introgressed and non-introgressed populations.

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