THESIS

Investigations into the taxonomy, phylogeography and history of native (*Parartemia*) and exotic (*Artemia*) brine shrimps in Australia

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Thesis declaration

I Md Aminul Islam verify that in submitting this thesis; the thesis is my own account of the research conducted by me, except where other sources are fully acknowledged in the appropriate format, the extent to which the work of others has been used is documented by a percent allocation of work and signed by myself and my Principal Supervisor, the thesis contains as its main content work which has not been previously submitted for a degree at any university, the university supplied plagiarism software has been used to ensure the work is of the appropriate standard to send for examination, any editing and proof-reading by professional editors comply with the standards set out on the Graduate Research School website, and that all necessary ethics and safety approvals were obtained, including their relevant approval or permit numbers, as appropriate.

Candidate

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Statement of contributions to thesis

In accordance with the Murdoch University Graduate Degrees Regulations, it is acknowledged that this thesis represents the work of the Candidate with contributions from their supervisors and, where indicated, collaborators. The Candidate is the majority contributor to this thesis with no less than 75% of the total work attributed to their efforts.

Candidate **Principal supervisor**

Statement of thesis structure

This thesis comprises seven chapters:

Chapter 1: General Introduction

Chapter 2: Comprehensive Literature Review

Chapters 3 to 5: Data Chapters (manuscripts)

Chapter 6: General Discussion

Chapter 7: General Conclusions

Each data chapter (3-5) comprises an abstract, introduction, materials and methods, results, discussion and conclusion, forming cohesive standalone manuscripts. These manuscripts adhere to the author guidelines of the respective journals, to which each manuscript is published, submitted or planned for submission. Preceding each manuscript, a statement of author attribution outlines the contributions of each author.

It is important to note that there may be some overlap among chapters (1-5), especially in the General Introduction (Chapter 1) and the introduction sections of other chapters (2-5). However, this intentional overlap serves to provide contextual meaning for the content within each chapter. The thesis concludes with a unified reference list encompassing the cited literature across all chapters.

General Abstract

Australia has numerous salt lakes. These lakes have a rich, endemic invertebrate fauna, which face a range of threats. *Parartemia* is one of the most speciose and salt-tolerant taxa found in these lakes. Unlike *Artemia*, a well-studied brine shrimp with an almost global range, *Parartemia* is less well known and only found in Australia. This PhD study investigated the taxonomy, evolutionary history and phylogeography of *Parartemia*. I conducted molecular-based phylogenetic and species delimitation analyses and compared the results to morphological information about species and species groups. The molecular and morphological data were mainly congruent, although some morphospecies showed large amounts of genetic divergence. I found two new morphospecies, three cryptic species and one synonymy. A time-calibrated *16S* phylogeny indicated that speciation and deeper divergence in *Parartemia* occurred between about 40 and 10 million years ago, which broadly coincides with a general increase in the aridity of the Australian climate but predates estimates of the timing of such in some other salt lake taxa. I conducted phylogeographic analyses of *cytochrome c oxidase 1* (*COI*) sequence variation in the widespread species *P. cylindrifera* and *P. longicaudata*. Each of these species comprised a series of divergent lineages, mainly with restricted spatial distributions. Populations of these species were typically isolated in single salt lakes. I also investigated the distributions, identity and phylogeography of *Artemia* in natural salt lakes in Australia. My genetic and distributional data show that lineages of diploid parthenogenetic *Artemia* and *A. franciscana* are currently spreading in natural lakes in Western Australia. Further research is needed to determine whether *Artemia* will negatively impact on *Parartemia* and other endemic species. Overall, my results have provided information that will be useful for planning for the management and future studies of native and exotic brine shrimps in Australia.

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Chapter 1

Chapter 1. General Introduction

"Conservation is the protection of wildlife from irreversible harm" (Hambler, 2004). Over time, the term 'wildlife' has gradually been replaced by the broader term 'biodiversity' (Hambler, 2004; Swingland, 2001). Biodiversity encompasses the range of living organisms, their genetic variations and the communities and ecosystems they form, as well as the evolutionary and ecological processes that support their functioning (Noss & Cooperrider, 1994). Why should we prioritise biodiversity conservation? In part because humans rely on biodiversity for a range of essential products and services (Hambler, 2004; Lynch *et al.*, 2023). Furthermore, biodiverse ecosystems are more resilient to disturbance and more likely to sustain the ecological and evolutionary processes that generate new biodiversity (Chapin *et al.*, 2000; Linders *et al.*, 2019). Nevertheless, only ~ 1.2 - 1.9 million of an estimated 5 - 15 million species have been described (Costello *et al.*, 2013; Jackson *et al.*, 2022; Mora *et al.*, 2011; Stork, 1997). Furthermore, taxonomic effort has not been evenly spread across taxa or ecosystems (Di Marco *et al.*, 2017; Donaldson *et al.*, 2016; Troudet *et al.*, 2017).

To conserve biodiversity, it is essential to first document it (Bolam *et al.*, 2019; Buxton *et al.*, 2021; Cook *et al.*, 2010; Pino-Del-Carpio *et al.*, 2014). Thus, taxonomy and systematics play an integral role in biodiversity research and conservation planning (Khuroo *et al.*, 2007). However, identifying meaningful taxonomic units for conservation is problematic (Zachos, 2018). This stems in part from the ongoing debate about what constitutes a species (Frankham *et al.*, 2012; Zachos, 2016, 2018), from disagreements about which biological entities (e.g., populations, species or ecosystems) and attributes (genetic diversity, ecological function or evolutionary distinctiveness) should be prioritised (DeWoody *et al.*, 2021; Milot *et al.*, 2020; Nielsen *et al.*, 2023; Radinger *et al.*, 2023), and from complications arising from evolutionary processes such as hybridisation, introgression and asexuality (Karbstein *et al.*, 2024). Nevertheless, in practice, species are the predominant units in biodiversity research and conservation planning. For example, species names are often needed in legislation relating to environmental protection and biodiversity conservation (Mace, 2004) and species lists (e.g., vulnerable, endangered and critically endangered) are widely used in conservation planning (Mace, 2004; Mallet, 2001; Rodrigues *et al.*, 2006). Traditionally, species identifications were based on morphological features, sometimes in combination with ecological and/or karyotype data (Hillis, 1987; Jackson, 1971; Van Valen, 1976). Over the past 25 years, molecular data have played an increasingly prominent role in species delimitation (Antil *et al.*, 2023; DeSalle

& Goldstein, 2019; Hubert & Hanner, 2015) and are often used to test species hypotheses based on morphology (e.g., see Lobo *et al.*, 2017; Palandačić *et al.*, 2017; Song *et al.*, 2018). However, proposals for molecular data to replace rather than complement traditional data sources (Hebert *et al.*, 2003; Hebert & Gregory, 2005) have faced justified criticism (see Buhay, 2009; Karabanov *et al.*, 2023; Meier *et al.*, 2006; Song *et al.*, 2008; Will *et al.*, 2005). Instead, integrative taxonomy, which combines multiple lines of evidence, usually including both DNA and morphological evidence, is the preferred approach because it generates robust species hypotheses (Dayrat, 2005; Lawrie *et al.*, 2023; Padial *et al.*, 2010; Rahman, 2024; Schlick-Steiner *et al.*, 2010; Sheth & Thaker, 2017).

Tracking and managing genetic variation within species are important for the development of effective conservation strategies (Hoban *et al.*, 2021; Pauls *et al.*, 2013). Monitoring genetic diversity can identify populations with reduced adaptive and evolutionary potential (Hoban *et al.*, 2020) or those that are susceptible to inbreeding depression (Keller & Waller, 2002) or pathogens (Gibson, 2022). These results can be used to help develop conservation priorities and/or mitigation strategies (Frankham, 2010; Hendry *et al.*, 2010; Jensen *et al.*, 2019; Pauls *et al.*, 2013). Phylogeographic studies are used to identify major evolutionary lineages within a species, document the distributions of these lineages, and elucidate the contemporary and historical processes that shape these distributions (Avise, 2000, 2009; Emerson & Hewitt, 2005). In some cases, such evolutionary significant units may be more appropriate units of analysis in conservation than species (Hutama *et al.*, 2017; Moritz, 2002; Ryder, 1986; Willi *et al.*, 2022). Connectivity, the exchange of individuals between the assemblages of species in different locations (Cowen & Sponaugle, 2009), plays a crucial role in shaping a species' ecology and evolution. It influences population growth, resilience to environmental disturbances, and rates of divergence and adaptation (Gardner *et al.*, 2015 and references therein). An understanding of connectivity and patterns of gene flow is needed to establish the spatial scales that are relevant for the management of species, for example, by elucidating the boundaries of demographically-independent or genetically homogeneous units within a species (Pember *et al.*, 2020; Sexton *et al.*, 2024).

Invertebrates are a significant component of global biodiversity, representing approximately 98 % of all animal species (Mather, 2023). They play essential roles in ecosystem regulation and functioning, and their ecological importance is widely recognised (Malmqvist, 2002; Prather *et al.*, 2013; Saccò *et al.*, 2021; Wallace & Webster, 1996). The volume of research work on invertebrates is now 60 % higher than it was two to three decades ago (Di Marco *et*

al., 2017). Nevertheless, invertebrates tend to be underrepresented in global conservation efforts, particularly lesser-known ones (Barua *et al.*, 2012; Caldwell *et al.*, 2024). Although small in area, small standing waterbodies contain a significant fraction of global invertebrate biodiversity (De Meester *et al.*, 2005; Dudgeon *et al.*, 2006; Saccò *et al.*, 2021). This includes a diverse range of crustaceans, such as branchiopods, copepods and ostracods (see Brendonck *et al.*, 2008; Forro *et al.*, 2008; Lawrie *et al.*, 2021; Martens *et al.*, 2008), that are especially common in temporary and/or saline waterbodies where there is a general absence of fish predators (De Deckker, 1983). These crustaceans play a variety of ecological roles and are key to the functioning of the associated communities (e.g., Brendonck *et al.*, 2022; Saccò *et al.*, 2021).

The conservation of salt lake invertebrates and ecosystems is one area that requires more attention (Lawrie *et al.*, 2021; Saccò *et al.*, 2021; Williams, 2002). Salt lakes are unique ecosystems that can be either temporary or permanent bodies of non-marine water with salinities > 3 g/L and have no recent connection to marine water (Bayly, 1967; Bayly & Williams, 1966). These ecosystems typically contain a unique salt-adapted fauna, dominated by invertebrates (Jellison *et al.*, 2008; Lawrie *et al.*, 2021; Williams, 1998). Charles Darwin even acknowledged their distinctiveness, stating, "we have a little world within itself, adapted to these little inland seas of brine" (Darwin, 1839). Typically found in arid and semi-arid regions worldwide, where evaporation exceeds precipitation, salt lakes have a global volume estimated at 85,000 km^3, rivalling that of freshwater bodies estimated at 105,000 km^3 (Shiklomanov, 1990; Williams, 2002). Despite their ubiquity and abundance, until recently salt lakes have typically received less study than freshwater ecosystems partly due to their remote locations and misconceptions about their importance (De Deckker, 1983; Last, 2002; Saccò *et al.*, 2021; Williams, 1981; Williams, 2002; Zadereev *et al.*, 2020). However, in addition to their unique invertebrates, they are an important aquatic habitat for wildlife species in arid and semi-arid regions, particularly waterbirds which may feed on the invertebrates (Pedler *et al.*, 2018; Williams, 2002; Zadereev *et al.*, 2020). Salt lakes also have aesthetic and cultural significance, economic and recreational benefits (Saccò *et al.*, 2021; Williams, 2002). Furthermore, these lakes provide excellent opportunities for testing ecological and evolutionary theories (De Meester *et al.*, 2005; Jellison *et al.*, 2008; Lawrie *et al.*, 2024; Saccò *et al.*, 2021) and accordingly previous studies have provided insights into adaptation, speciation and evolutionary dynamics in salt lake invertebrates (e.g., see Finston, 2002; Lawrie, 2023; Rahman, 2024; Whitehead, 2005; Williams & Mellor, 1991).

Salt lake ecosystems face numerous threats from human activities, such as surface inflow diversions, groundwater pumping, mining, secondary salinisation, biological disturbances, pollution and anthropogenically induced global climate changes (Heydari & Jabbari, 2012; Jellison *et al.*, 2008; Saccò *et al.*, 2021). These threats are geographically widespread and often lead to irreversible effects (Jellison *et al.*, 2008; Timms, 2005; Williams, 2002). For example, in central Mexico, excessive groundwater pumping for irrigation has resulted in the disappearance of most temporary and shallow permanent salt lakes (Jellison *et al.*, 2008). Due to their sensitivity to minor alterations in hydrological budgets, salt lakes are being affected by climate change, primarily through changes in precipitation and more rapid evaporation due to higher temperatures, which prolong the drying phases of temporary lakes and increase the salinity of permanent ones (IPCC, 2001; Jellison *et al.*, 2008; Williams, 2002).

Australia is renowned for its extensive salt lakes (De Deckker, 1983; Saccò *et al.*, 2021; Timms, 2005). With approximately 70 % of Australia having an arid and semi-arid climate, it is unsurprising that over 80 % of wetlands are saline, covering a vast area exceeding 100,000 km^2 (Anon 1911 in Timms, 2005). Most of the salt lakes are concentrated in Western Australia and South Australia, with some in Victoria (including maar lakes) and a few in other states (De Deckker, 1983; Timms, 2005). The hydrology of Australian salt lakes is diverse, some are permanent, but most undergo either seasonal filling during summer or winter rainfall, or episodic filling years or even decades apart, typically only after unseasonal heavy rainfall linked to cyclonic events (Lawrie *et al.*, 2021; Timms, 2005). Most Australian salt lakes are alkaline and dominated by sodium and chloride ions, closely resembling the ionic composition of seawater. However, some are naturally acidic due to the influence of acidic groundwater (Bayly & Williams, 1966; Bowen & Benison, 2009; Timms, 2009b). Outside of Australia, acidic salt lakes are very rare and linked to volcanic activity (Bowen & Benison, 2009; Moors *et al.*, 2023). The diverse characteristics of Australian salt lakes have played a crucial role in shaping the current biotic compositions of these ecosystems (see Lawrie *et al.*, 2021 and references therein).

Early studies on Australian salt lakes primarily focused on describing their distributions, general physiography and geochemical properties, and resulted in a comprehensive understanding of their physical and chemical characteristics (information summarised in Bowler, 1981; Mernagh *et al.*, 2016). The fundamental details of the biota of Australian salt lakes were established by the 1980s due to the seminal work of Geddes, De Deckker, Timms and Williams (e.g., De Deckker, 1983; Geddes, 1981; Geddes, 1983; Timms, 1983; Timms,

1987; Williams, 1984). These details include recognition of the presence of a diverse range of endemic invertebrates, especially among crustaceans. Recent estimates suggested that at least one family, two subfamilies, 11 genera and 74 species of crustaceans and molluscs are endemic to Australian salt lakes (Lawrie *et al.*, 2021). Since the 1980s, there have been scattered studies on the invertebrates of Australian salt lakes, including on their taxonomy (e.g., Halse & McRae, 2004; Lawrie *et al.*, 2023; Martens *et al.*, 2012; Timms, 2010; Timms & Hudson, 2009), community composition in Western Australia (e.g., ARL, 2004, 2006, 2009), conservation status (e.g., Timms, 2005; Timms *et al.*, 2009) and ecology (e.g., Lawrie *et al.*, 2024; Rahman *et al.*, 2024), as well as several reviews (e.g., Lawrie *et al.*, 2021; Rahman *et al.*, 2023; Timms, 2020). Nevertheless, there are still major gaps in knowledge regarding the fauna of Australian salt lakes, including for the endemic brine shrimp *Parartemia*.

Brine shrimps are often regarded as extremophiles due to their occurrence in hypersaline waters (Remigio *et al.*, 2001; Rogers, 2024; Timms, 2014). There are two types of brine shrimps, *Parartemia* and *Artemia* (Timms, 2014). *Parartemia*, which prior to this PhD study had 18 described morphospecies (Timms, 2012b), is endemic to Australia and is one of the most speciose genera in Australian salt lakes (Lawrie *et al.*, 2021; Timms, 2012b). All *Parartemia* species are bisexual and typically occur in shallow temporary salt lakes (Geddes, 1981; Timms, 2012b, 2014). While most species inhabit alkaline water, a few also occur in acidic lakes (Timms, 2014). Some *Parartemia* species display broad geographical distributions, while others are restricted to specific regions or only known from single sites (Timms, 2012b; Timms *et al.*, 2009). *Artemia* occurs in diverse inland saline habitats, such as temporary and permanent salt lakes, solar saltworks, salt ponds and coastal lagoons, worldwide except for Antarctica (Rogers, 2024; Van Stappen, 2002). It includes both bisexual lineages, with nine currently recognised species (Asem *et al.*, 2023), and polyphyletic unisexual lineages that exhibit varying ploidy levels (Asem *et al.*, 2016; Asem *et al.*, 2024a; Baxevanis *et al.*, 2006). Australia does not have native species of *Artemia*, however, bisexual and unisexual populations of this taxon now occur here (ARL, 2009; Geddes, 1979; McMaster *et al.*, 2007; Pinder *et al.*, 2002; Ruebhart *et al.*, 2008; Williams & Geddes, 1991).

The threats faced by Australian salt lakes mirror those faced by salt lakes globally (see above), but their impact is especially notable given the country's abundance of salt lakes and widespread agricultural and mining activities (Timms, 2005). The impacts are concerning given the high levels of unique biodiversity contained in these lakes. Climate change is intensifying aridity in Australia, particularly in the southwest region, where declining winter

rainfall is causing waterbodies to hold water for increasingly shorter periods (Atkinson *et al.*, 2021). Secondary salinisation is another major threat and is especially problematic across large areas of southwestern Australia (Pinder *et al.*, 2009). It is driven by human activities, like irrigation and replacing deep-rooted plants with shallow-rooted crops, which cause saline ground water to rise and results in salt accumulating in the soil and surface waters (see Timms, 2005 and references therein). It can convert temporary salt lakes to permanent ones, posing significant risks to *Parartemia* due to their preference for temporary salt lakes (Timms, 2005; Timms *et al.*, 2009). It has been predicted that secondary salinisation will cause the extinction of a third of the invertebrate species in wetlands in Western Australia's Wheatbelt region by the end of this century (Halse *et al.*, 2003; Timms, 2005). Some *Parartemia* populations in this region may have already gone extinct, as several studies have reported being unable to find *Parartemia* at sites where it was previously found (Pinder *et al.*, 2009; Timms *et al.*, 2009). Additionally, available evidence indicates that unisexual *Artemia* (ARL, 2004, 2006; McMaster *et al.*, 2007), and possibly also *A. franciscana* (ARL, 2009), are spreading in natural salt lakes in Australia. In general, invasive species/lineages of *Artemia* are known for their remarkable adaptability in diverse habitats (see Ruebhart *et al.*, 2008) and can take advantage of habitat alterations and disturbances (McMaster *et al.*, 2007). Whether exotic populations of *Artemia* pose a threat to *Parartemia* and/or other endemic invertebrates in Australian salt lakes is not clear.

The purpose of my PhD research is to fill some of the knowledge gaps regarding the biology of the endemic brine shrimp *Parartemia* and exotic *Artemia* in Australian salt lakes. The specific goals are to: (1) use published and unpublished information to review the biology of native *Parartemia* and exotic *Artemia* in Australia (Chapter 2); (2) use molecular data to assess species boundaries and relationships in *Parartemia* (Chapter 3); (3) investigate the evolutionary history and phylogeography of *Parartemia* (Chapter 4); and (4) explore the distribution, identity and phylogeography of *Artemia* in Australian natural salt lakes (Chapter 5). The results contribute to our understanding of the unique biodiversity of Australian salt lakes and will facilitate the development of informed conservation plans for *Parartemia*.

Chapter 2

Chapter 2. A review on brine shrimps in Australia – the endemic *Parartemia* and exotic *Artemia*

Chapter linking statement

Chapter 1 (General Introduction) provided a broad overview of the topics covered in this thesis. This chapter provides a thorough literature review of aspects of the biology of *Parartemia* and *Artemia* brine shrimps in Australia. It includes a detailed assessment of the distributions of *Parartemia* and *Artemia* species in Australia.

2.1. Introduction

Salt lakes, which are found on all continents including Antarctica (Saccò *et al.*, 2021), can be either temporary or permanent and are characterised by salinities greater than 3 g/L, although their salinity is typically much higher (Bayly & Williams, 1966; Williams, 1964). These lakes are predominantly found in dry regions where evaporation exceeds precipitation and can be much more common than freshwater bodies in these regions (Williams, 2002). The ecological and economic significance of salt lakes is sometimes overlooked in favour of freshwater systems, resulting in significant gaps in our knowledge of these systems (Lawrie *et al.*, 2021; Saccò *et al.*, 2021; Williams, 2002).

In Australia, salt lakes are particularly numerous, accounting for over 80 % of the country's lakes and wetlands (De Deckker, 1983; Timms, 2005). Most Australian salt lakes are shallow, temporary and alkaline, dominated by NaCl (Bayly & Williams, 1966; Timms, 2005; Williams, 1998). However, there are also some naturally acidic salt lakes with pH levels as low as 3 (Timms, 2012b). Australian salt lakes harbour a rich and highly endemic fauna, particularly crustaceans (De Deckker, 1983; Lawrie *et al.*, 2021). One of the most notable is the endemic brine shrimp *Parartemia* (Lawrie *et al.*, 2021; Remigio *et al.*, 2001; Timms, 2014).

All brine shrimp belong to two monogeneric families, the Artemiidae, which comprises the genus *Artemia*, and the Parartemiidae, which comprises the genus *Parartemia*. These two families make up the suborder Artemiina in the order Anostraca (Timms, 2014; Weekers *et al.*, 2002). Both *Artemia* and *Parartemia* are found in coastal and inland enclosed saline waters (Timms *et al.*, 2009; Van Stappen, 2002). There are native species of *Artemia* on all continents except Antarctica and Australia, however, some *Artemia* species have spread to regions outside of their native range, including Australia (Asem *et al.*, 2018; Ruebhart *et al.*, 2008). Overall, *Artemia* can be found in a range of places, including coastal, inland and high-altitude salt lakes and commercial saltworks (Asem *et al.*, 2024c; Bowen *et al.*, 1988; Eimanifar *et al.*, 2014; Muñoz & Pacios, 2010; Van Stappen, 2002). In contrast, the lesser-known *Parartemia* is found only in Australian salt lakes (Islam *et al.*, 2024; Remigio *et al.*, 2001; Timms, 2014).

The bisexual species *A. franciscana* is native to the Americas but has spread across most of the globe and is now found in Asia, Europe, Africa and Australia (see Asem *et al.*, 2018; Horváth *et al.*, 2018; Muñoz *et al.*, 2014; Ruebhart *et al.*, 2008; Thirunavukkarasu *et al.*, 2024). Human activities have directly contributed to the spread of this brine shrimp in association with its use in the aquaculture and saltworks industries. Its use in aquaculture as a dietary item gained

prominence in the 1930s and greatly expanded with a boom in fish and shrimp farming that started in the 1960s (Dhont & Sorgeloos, 2002; Sorgeloos, 1980). *Artemia franciscana* has mainly spread via introductions to saltworks where it is used to control phytoplankton blooms and accelerate evaporation by promoting red-pigmented bacteria (Jones *et al.*, 1981; Ruebhart *et al.*, 2008; Sorgeloos & Tackaert, 1991; Van Stappen *et al.*, 2020; Van Stappen *et al.*, 2007). *Artemia franciscana* can outcompete some other *Artemia* species and therefore poses a significant threat to native *Artemia* populations in areas where it has spread (Eimanifar *et al.*, 2014; Muñoz *et al.*, 2014; Naceur *et al.*, 2010; Ruebhart *et al.*, 2008; Sainz-Escudero *et al.*, 2022; Scalone & Rabet, 2013).

The first record of *A. franciscana* in Australia refers to a deliberate introduction of this species into the Port Alma saltworks (Inkerman Creek) in Queensland in the 1960s (Clark & Bowen, 1976; Ruebhart *et al.*, 2008). Unisexual (parthenogenetic) *Artemia* also occur in Australia, but whether they arrived in via bird-mediated dispersal or human-facilitated introductions has been debated (see McMaster *et al.*, 2007). Regardless, they now occur in various coastal and inland salt lakes in Western Australia (ARL, 2004, 2006; McMaster *et al.*, 2007; Timms, 2014). *Artemia franciscana* occurs in some Australian saltworks (Asem *et al.*, 2018; Ruebhart *et al.*, 2008) and probably also in some natural salt lakes that were once used for salt extraction (Timms, 2014). An unpublished technical report has mentioned the presence of this species in three natural lakes in Western Australia (ARL, 2009).

Australian salt lakes face threats from global climate change and other anthropogenic activities (Timms, 2005). Human activities such as agriculture and mining can result in significant disturbances in salt lakes, and secondary salinisation is a major problem in some areas (e.g., Halse *et al.*, 2003; McMaster *et al.*, 2007; Pinder *et al.*, 2009; Timms *et al.*, 2009). While most species of *Parartemia* can tolerate high salinity, they are typically found in pristine episodic or seasonal lakes and may be vulnerable to secondary salinisation and other disturbances (Pinder *et al.*, 2009; Timms, 2005; Timms *et al.*, 2009). Unisexual *Artemia* and especially *A. franciscana*, by virtue of their greater dispersal powers and adaptability, could represent a threat to *Parartemia* (Ruebhart *et al.*, 2008). Up-to-date information on the biology of *Parartemia* and these *Artemia* biotypes is needed to effectively conserve the former and manage the spread of the latter in Australia.

Geddes (1981) first reviewed the biology of both *Artemia* and *Parartemia*, focusing on their distribution within Australia and comparative physiology. An updated version of this work appeared as a chapter in the book '*Artemia* Biology' co-authored by Williams and Geddes

(1991). Over 30 years later, Timms (2014) reviewed halophilic anostracans in Australia, which provided a brief overview of the distribution and history of *Artemia* in Australia and more details on the biology of *Parartemia*. Both *Artemia* and *Parartemia* have also been briefly discussed in a recent review of Australian halophilic invertebrates (Lawrie *et al.*, 2021). However, a recent comprehensive review updating Geddes' pioneering work has not been attempted. This review is intended to fill this gap.

This chapter provides an overview of aspects of the biology of *Parartemia* and *Artemia*, focusing on those species that occur in Australia. This review is mainly based on published and unpublished literature that is independent of my PhD research. Relevant information on the taxonomy and distribution of both *Parartemia* and *Artemia* in Australia from my PhD research (see Chapters 3 and 5) has, however, been included to ensure that the taxonomic and distributional information is up to date (see 'Methods' section for more details).

2.2 Methods

Published articles and reports on the general biology of *Parartemia* and *Artemia* were located through searches on Google Scholar and Scopus databases using various keywords, such as 'brine shrimp', 'brine shrimp Australia', '*Artemia*', '*Parartemia*', 'salt lakes Australia', 'saline lakes Australia', 'brine shrimp biology', 'brine shrimp ecology', 'Anostraca', 'Artemiina', 'inland crustaceans Australia' and 'inland aquatic invertebrates Australia'. Searches were made up to October 2024 and yielded a final selection of 168 articles, including 138 journal articles (mostly peer-reviewed articles), 20 book sections, one thesis and nine other document types (i.e., online database, conference proceedings, electronic book section, magazine article and government document).

Some information on the distributions of *Parartemia* and *Artemia* in Australia were obtained from the above searches. Additional information was obtained from a partially unpublished dataset from the Department of Biodiversity, Conservation and Attractions (DBCA), which included information, dating from August 1994 to December 2023, on the locations and physicochemical properties of sites containing *Artemia* and *Parartemia* in Western Australia. Access to these data can be obtained via DBCA with reasonable request. Additionally, data from three unpublished reports submitted to the DBCA (ARL, 2004, 2006, 2009) were included. Another unpublished raw dataset, used to create *Parartemia* distribution maps in Timms *et al.* (2009), was generously made available by Brian V. Timms. I also included site details for some specimens that the Western Australian Museum, the Tasmanian Museum and Art Gallery and Stantec Australia Pty Ltd contributed to this PhD research (see Chapter 3 for details).

Finally, site and water quality data obtained during field sampling between September 2017 and May 2023 for this PhD research and related projects are also included and are available in supplementary Tables S2.1 and S2.2.

Water quality parameters temperature, salinity and dissolved oxygen were presented in units of degrees Celsius (°C), grams per liter (g/L) and percentage (%), respectively. For salinity measurements recorded in total dissolved solids (TDS), which might include various dissolved organic matter as well as salt (Williams & Sherwood, 1994), a correction factor of 0.91 (Bayly & Williams, 1966) was applied to convert TDS to salinity (g/L). In instances where conductivity data (mS cm^{-1}) were available instead of salinity, conversion to salinity (g/L) was done using the formula of Williams (1986). Although this formula is intended for converting

conductivity with the range of 5 - 100 mS cm^{-1}, some records above 100 mS cm^{-1} were converted when it was not possible to obtain salinity data in another way.

2.3 Taxonomy

The first written record of a brine shrimp, where it was referred as an 'aquatic dog', dates to the 10th century AD (see Asem & Eimanifar, 2016). In 1755, Schlösser referred to brine shrimp from a saltworks near Lymington in England as an 'unknown insect' (see Kuenen & Baas-Becking, 1938). Initially identified as *Cancer salinus* Linnaeus, 1758, the brine shrimp was later reclassified as *Artemia salina* by Leach (1819). In 1903, Sayce introduced the genus *Parartemia* when describing specimens of *Parartemia zietziana* Sayce, 1903 from a lake near Lake Alexandrina in South Australia. Prior to 2002, *Parartemia* was placed in the Parartemiinae in the fairy shrimp family Branchipodidae under the order Anostraca. However, Weekers *et al.* (2002) used genetic and morphological evidence (notably the last abdominal segment and telson are fused in brine shrimp but free in fairy shrimp) to propose a new dedicated suborder, Artemiina, comprising the families Artemiidae and Parartemiidae, a classification which has been widely adopted (Castellucci *et al.*, 2022; Islam *et al.*, 2024; Rogers, 2013, 2024; Timms, 2014).

The taxonomy of *Artemia* has been widely debated and has undergone major changes over time (see Asem *et al.*, 2024b; Asem *et al.*, 2023; Gajardo & Beardmore, 2012; Rogers, 2013; Sainz-Escudero *et al.*, 2021). Sainz-Escudero *et al.* (2021) have summarised the history of this taxonomy (also see references therein), which they divided into three phases. The early phase mainly used morphological features to define species, but later investigations revealed that many of these features were plastic and often only applicable to specific populations. The second phase emphasised data on the reproductive mode and reproductive isolation, and occasionally also on cytogenetics or protein profiles. According to Sainz-Escudero *et al.* (2021), the third phase and current phase relies mainly on molecular data. Sainz-Escudero *et al.* (2021) proposed the presence of five independent taxonomic units within *Artemia*, corresponding to *A. persimilis*, *A. salina*, *A. urmiana*, *A. sinica* and *A. monica*. They argued that *A. tibetiana* should be synonymised with *A. urmiana* and followed Muñoz *et al.*'s (2013) recommendation that *A. franciscana* should be synonymised with *A. monica.* However, their findings are based mainly on DNA sequence data and have been subject to several corrections and updates (e.g., Asem *et al.*, 2024a; Asem *et al.*, 2024b; Asem *et al.*, 2023). For example, Asem *et al.* (2024b) used egg morphology and ecological information to make a case that *A.*

franciscana and *A. monica* are valid separate species. Asem *et al.* (2023) used molecular and morphological data to identify two new bisexual *Artemia* species (*A. amati* and *A. sorgeloosi*) from Asia and to suggest that *A. tibetiana* and *A. urmiana* are valid species. The most recent information, which is based on multiple lines of evidence, indicates that the number of bisexual *Artemia* species is nine (Table 2.1), plus a variety of parthenogenetic lineages (see below). The main morphological features used to discriminate the bisexual species are the structures of the frontal knob, gonopod, brood pouch and cercopods (Asem *et al.*, 2023; Mura & Brecciaroli, 2004; Mura & Gajardo, 2011). A taxonomic key that uses morphological features to identify all currently recognised bisexual species of *Artemia* except *A. monica* is available in Asem *et al.* (2023).

Parthenogenetic *Artemia* have polyphyletic origins and exhibit various ploidies, ranging from diploid to pentaploid (Abatzopoulos *et al.*, 1986; Asem *et al.*, 2016; Baxevanis *et al.*, 2006; Maniatsi *et al.*, 2011; Rode *et al.*, 2022; Triantaphyllidis *et al.*, 1998). Therefore, the taxonomic status of parthenogenetic *Artemia* is not straightforward. In 1906, Artom first used a term 'Artemia partenogenetica' to describe a parthenogenetic variety, but it was not used as a binomial name (Artom, 1906). In 1974, Barigozzi suggested a modified term 'Artemia parthenogenetica' to encompass all parthenogenetic forms (Barigozzi, 1974). But the binomen *Artemia parthenogenetica* was first used by Bowen and Sterling (1978). Later, many scientists used this name for taxonomic convenience, but this practice conflicts with the biological species concept and some other species concepts because parthenogens with different ploidy levels and polyphyletic origins cannot be assigned to a single species name (Abatzopoulos *et al.*, 2002b; Asem *et al.*, 2024a; Baxevanis *et al.*, 2006). The study of Sainz-Escudero *et al.* (2021) synonymised *A. parthenogenetica* with the bisexual species *A. urmiana*. However, this synonymisation is not valid. In a subsequent paper, Sainz-Escudero *et al.* (2022) emphasised the polyphyletic origins of parthenogenetic *Artemia* and recognised parthenogenetic populations of both *A. urmiana* and *A. sinica*. Furthermore, Asem *et al.* (2024a) have pointed out, there are some important morphological and molecular differences between *A. urmiana* and parthenogenetic *Artemia*. This review therefore follows the suggestion of Asem *et al.* (2024a) and refers to parthenogenetic lineages of *Artemia* according to their ploidy level, e.g., diploid parthenogenetic *Artemia*, however, it uses the term unisexual/s when the identity of the lineage is unknown.

Parartemia, with at least 21 species (all bisexual), has far higher species richness than *Artemia* (Table 2.1). Many of the *Parartemia* species have been uncovered within the past 25 years

(Islam *et al.*, 2024; Timms, 2010; Timms & Hudson, 2009), a trend also observed for some other invertebrate taxa from Australian salt lakes, e.g., giant ostracods (Halse & McRae, 2004; Rahman *et al.*, 2023) and gastropods (Lawrie *et al.*, 2023). Most *Parartemia* species show characteristic morphological differences (Islam *et al.*, 2024; Timms, 2012b). The main morphological features for identifying P*arartemia* species are the structure of the second antenna basal antennomeres in males and the last few thoracic segments in females (Timms, 2012b).

Table 2.1: List of bisexual *Artemia* and *Parartemia* species.

Taxonomic Hierarchy		
Kingdom	Animalia Linnaeus, 1758	
Phylum	Arthropoda von Siebold, 1848	
Subphylum	Crustacea Brünnich, 1772	
Class	Branchiopoda Latreille, 1817	
Order	Anostraca Sars, 1867	
Suborder	Artemiina Weekers et al., 2002	
Family	Artemiidae Grochowski, 1896	Parartemiidae Daday, 1910
Genus	*Artemia* Leach, 1819	*Parartemia* Sayce, 1903
Species	*Artemia monica* Verrill, 1869	*Parartemia acidiphila* Timms & Hudson, 2009
	Artemia persimilis Piccinelli and Prosdocimi, 1968	*Parartemia auriciforma* Timms & Hudson, 2009
	Artemia salina (Linnaeus, 1758)	*Parartemia bicorna* Timms, 2010
	Artemia sinica Cai, 1989	*Parartemia contracta* Linder, 1941
	Artemia urmiana Gunther, 1900	*Parartemia cylindrifera* Linder, 1941
	Artemia franciscana Kellogg, 1906	*Parartemia extracta* Linder, 1941
	Artemia tibetiana Abatzopoulos *et al*.,1998	*Parartemia informis* Linder, 1941
	Artemia amati Asem *et al*., 2023	*Parartemia laticaudata* Timms, 2010
	Artemia sorgeloosi Asem *et al*., 2023	*Parartemia longicaudata* Linder, 1941[1]
		Parartemia minuta Geddes, 1973
		Parartemia mouritzi Timms, 2010
		Parartemia purpurea (a)[2]
		Parartemia purpurea (b)[2]
		Parartemia purpurea (c)[2]
		Parartemia serventyi Linder, 1941
		Parartemia triquetra Timms & Hudson, 2009

	Parartemia veronicae Timms, 2010
	Parartemia yarleensis Timms & Hudson, 2009
	Parartemia zietziana Sayce, 1903
	Parartemia sp. ‘y’ (yet to be formally described)[3]
	Parartemia sp. ‘z’ (yet to be formally described)[3]

[1]synonym: *Parartemia boomeranga*; [2]cryptic species within *Parartemia purpurea*; [3]new *Parartemia* species (details in Islam *et al.*, 2024).

2.4 Morphology

Artemia and *Parartemia* are easily differentiated by their size and morphology. *Artemia* adult males measure about 8 - 10 mm and adult females measure about 10 - 12 mm (Asem *et al.*, 2023; Criel & Macrae, 2002a). In contrast, adult males in *Parartemia* show a wider size range, from 10.5 to 26.7 mm (Table 2.2) and the females are usually smaller than males. Both genera possess an elongated segmented body (Fig. 2.1), comprising head, thorax and abdominal segments.

Table 2.2: Data on the mean male size and water quality parameters for different *Parartemia* species based on field records. Data sources include published literature (Timms, 2012b; Timms *et al.*, 2009), field trips conducted for this PhD research (details in Table S2.2) and records from the Department of Biodiversity, Conservation and Attractions.

Species	Mean male size (mm)	Temperature (°C)	Salinity (g/L)	Dissolved Oxygen (%)	pH
Parartemia acidiphila	12.1	21.2-24.3	35-210	73.8-111.3	3.0-7.4
Parartemia auriciforma	11.5	-	-	-	-
Parartemia bicorna	21.6	-	22-105	-	7.5-8.8
Parartemia contracta	20.1	16.4-31.7	31.3-240	71.9-103.6	3.5-6.9
Parartemia cylindrifera	22.3	12.47-23.07	3-140	75.5-151.5	6.6-9.8
Parartemia extracta	17.0	14.5-30.17	18.6-100	88.2-167.2	7.6-9.1
Parartemia informis	26.7	10.0-30.2	21.0-263.9	52.6-124.2	6.5-9.6
Parartemia laticaudata	17.5	-	8-141	-	8.2
Parartemia longicaudata	25.4	9.4-25.5	8.5-291.2	60.9-127.9	5.3-9.1
Parartemia minuta	14.2	-	2-255	-	-
Parartemia mouritzi	10.5	16.3	20-95	93.1	4.1-7.0
Parartemia purpurea (a)	18.8	16.21-19.19	92.9-149	87.6-118	7.8-8.4
Parartemia purpurea (b)	21.7	28.31	100.2	106.4	7.9
Parartemia purpurea (c)	20.8	14.17-28.5	56.4-120.8	68-152.8	7.9-8.9
Parartemia serventyi	21.2	17.1-28.3	15-262	89.1-118.6	4.0-8.5
Parartemia triquetra	19.5	-	-	-	-
Parartemia veronicae	13.6	-	74-225	-	-
Parartemia yarleensis	18.0	-	-	-	-
Parartemia zietziana	19.3	15.97	22-353	106.6	7.5-10.0
Parartemia sp. ‘y’	20.1	20.05-24.5	46.8-65.3	83.5-105.7	8.2
Parartemia sp. ‘z’	22.9	24.7	120.9	105.9	8

The head region of male brine shrimps is distinctive, particularly the structure of the second pair of antennae, which consists of basal and distal antennomeres (Fig. 2.2). In *Artemia*, the distal antennomere is flattened and widest at the midpoint, tapering sharply to the apex. In contrast, the distal antennomere of *Parartemia* is tubular and medially curved (some with medial tumidity), tapering towards the apex, and terminating with a sharp point (Timms, 2012b). As for the basal antennomere, *Artemia* displays a distinct frontal knob on its medial margin (see Fig. 2.2), a feature of taxonomic importance (see Asem *et al.*, 2023). In contrast, the basal antennomeres of *Parartemia* are proximally fused and bear various outgrowths. These outgrowths hold significant taxonomic value for distinguishing *Parartemia* species (see Islam *et al.*, 2024; Timms, 2010, 2012b; Timms & Hudson, 2009). Females of *Parartemia* have a recurved spine on the labrum, a feature that distinguishes them from all other anostracan taxa (see Fig. 2.1).

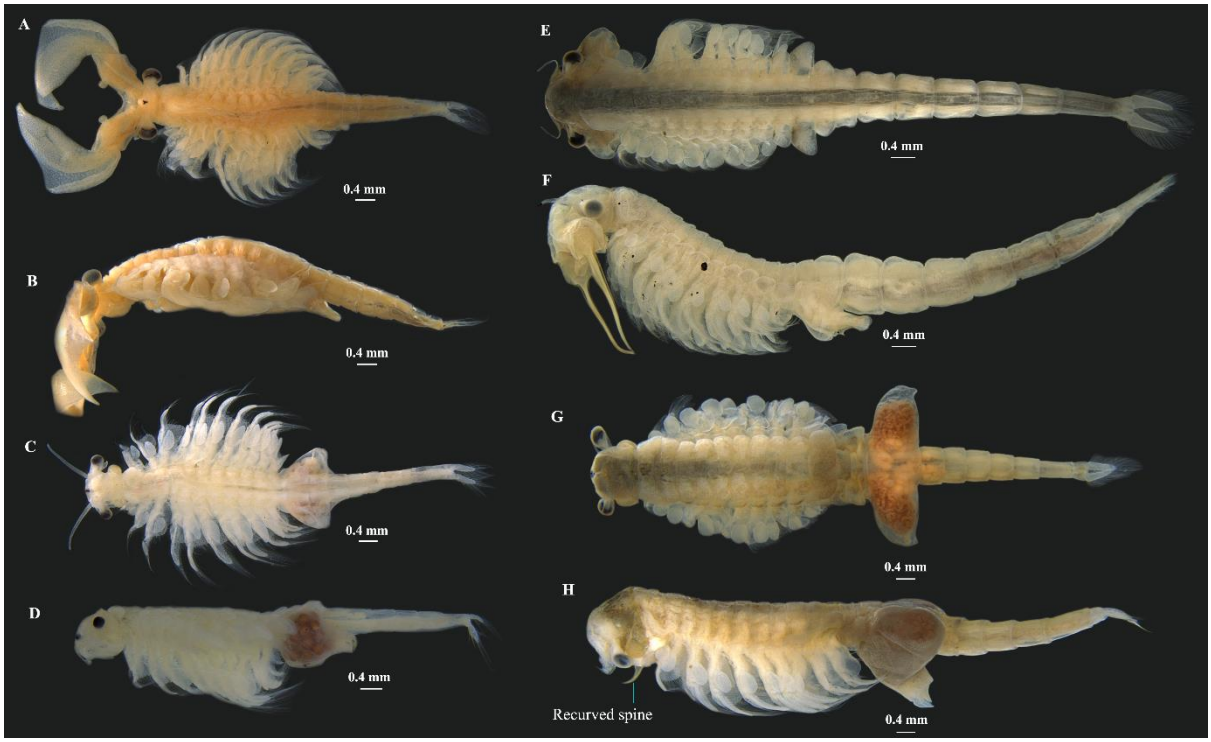

Fig. 2.1: Comparison of gross morphology of *Artemia* and *Parartemia*. The left column shows (A) dorsal view and (B) lateral view of male *Artemia* as well as (C) dorsal view and (D) lateral view of female *Artemia*. The right column shows (E) dorsal view (*Parartemia* sp. 'z') and (F) lateral view (*Parartemia informis*) of male *Parartemia* as well as (G) dorsal view (*Parartemia* sp. 'z') and (H) lateral view (*Parartemia informis*) of female *Parartemia*.

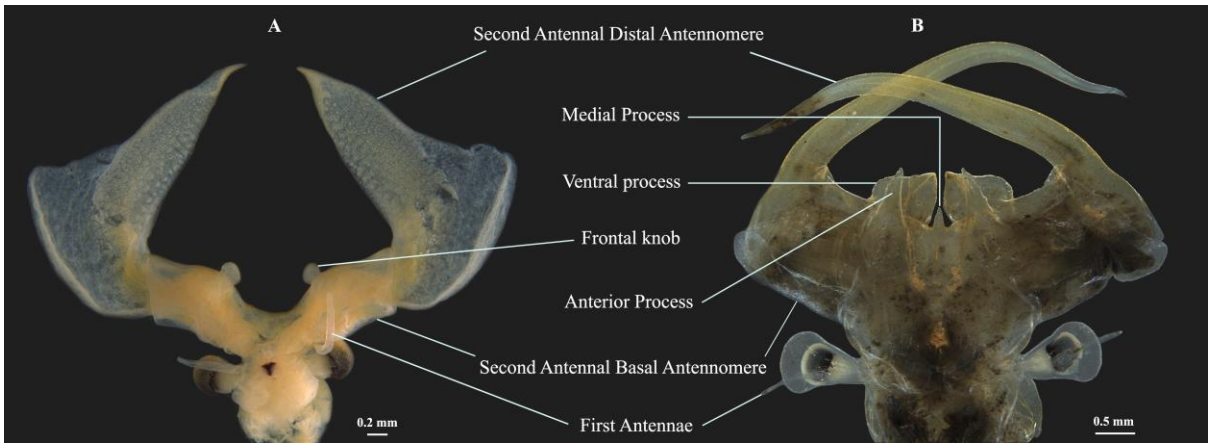

Fig. 2.2: Male heads of (A) *Artemia* (*A. franciscana*) collected from an unnamed lake near Dalwallinu (Marchagee 12 in Table S2.1) and (B) *Parartemia* (*P. bicorna*) from Lake Carey (specimens provided by the Stantec Australia Pty Ltd.).

The thoracic region in brine shrimp comprises 13 segments (I-XIII), with the first 11 (I–XI) each typically bearing a pair of flattened, leaf-like appendages called thoracopods. The thoracic segments I–XI in males of both *Parartemia* and *Artemia* generally have little taxonomic value. However, two exceptions are *P. serventyi* and *Parartemia* sp. 'z', where the eleventh thoracic segment exhibits extended lateral lobes, providing a distinctive trait that aids in easily separating these two species from other morphologically similar species (Fig. 2.3). The morphology of the thoracic segments VII-XI in females of *Parartemia* has some taxonomic importance. Species-specific modifications on these thoracic segments in *Parartemia* females allow the appropriate males' second pair of antennae to fit perfectly during copulation (see Geddes, 1973; Rogers, 2002; Timms, 2012b).

The last two segments (XII-XIII) of the thoracic region in brine shrimp are partially fused genital segments. Males have ventral gonopods, while females possess a brood pouch. Although the gonopod morphology of *Artemia* can be taxonomically informative (see Asem *et al.*, 2023; Brendonck & Belk, 1997; Mura & Brecciaroli, 2004; Triantaphyllidis *et al.*, 1997), further study is needed to determine if this is the case for *Parartemia* (but see Brendonck & Belk, 1997; Geddes, 1973). Female *Artemia* and *Parartemia* can usually be distinguished by the presence of two posteriorly directed ventral anteromedial hooks on the brood pouch in the former (Fig. 2.4; also see Rogers, 2002).

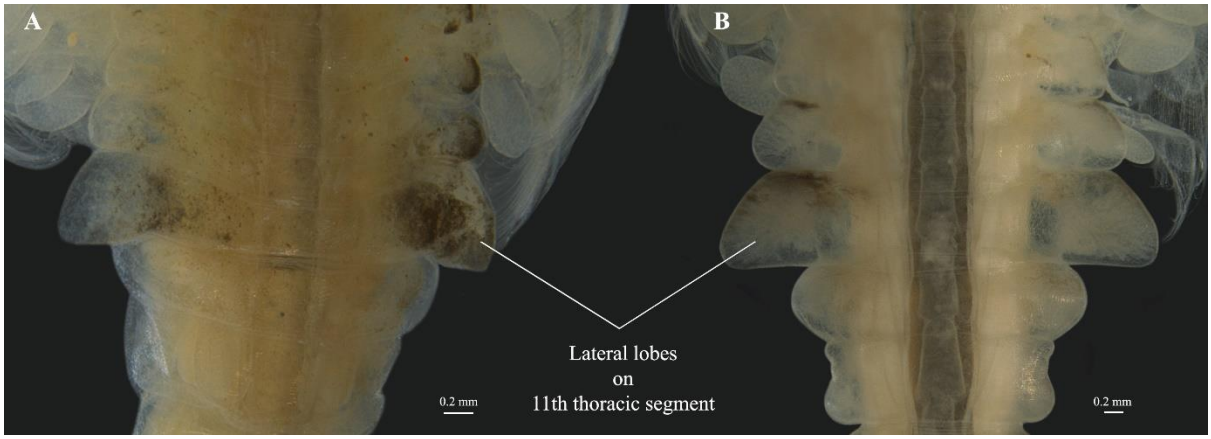

Fig. 2.3: The extended lateral lobes on the eleventh thoracic segment of (A) male *Parartemia serventyi* collected from an unnamed lake in Esperance area (Esperance 14 in Table S2.2) and (B) male *Parartemia* sp. 'z' collected from another unnamed lake in Esperance area (Esperance 23 in Table S2.2).

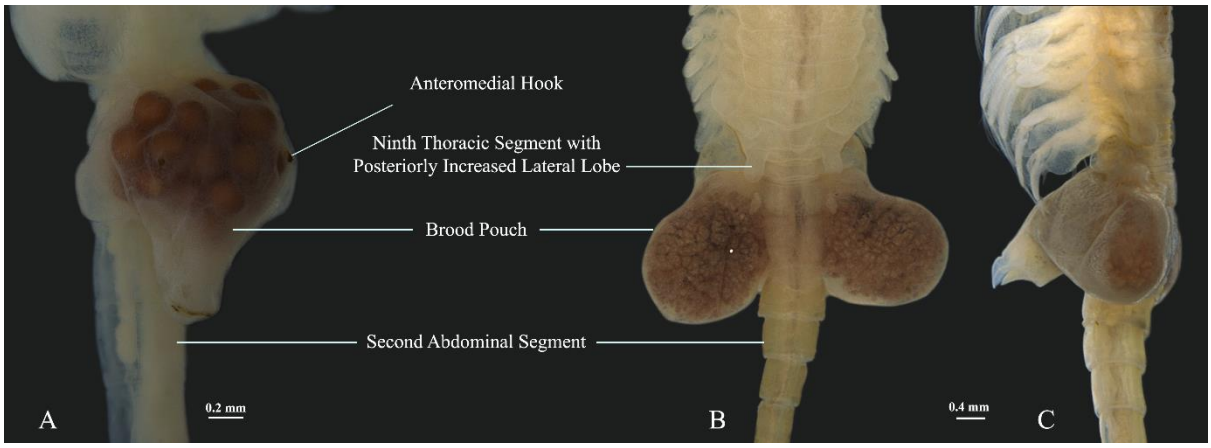

Fig. 2.4: Brood pouch of (A) *Artemia* (diploid parthenogenetic *Artemia*) collected from the Pink Lake on Rottnest Island (Rottnest Pink Lake in Table S2.1) and (B and C) *Parartemia* (*Parartemia informis*) collected from an unnamed lake near Marne (Wongan Hills 2, Lake 6 in Table S2.2).

The abdominal region of brine shrimps comprises six relatively uniform segments without appendages, although some species may have abdominal hairs (Timms, 2012b). Differentiating the last abdominal segment from the telson is challenging, and they are viewed as fused segments (Criel & Macrae, 2002a; Rogers, 2024; Timms, 2010; Weekers *et al.*, 2002). The telson bears a pair of cercopods, which carry varying numbers of setae that may have taxonomic value (see Asem *et al.*, 2023; Mura *et al.*, 2006). In certain instances, the morphology of the abdominal segments of *Parartemia* varies among species and can be taxonomically informative. For example, the first abdominal segment is widest in *P. laticaudata* but not in

other species, whereas the last abdominal segment is longest only in *P. longicaudata* (see Timms, 2010, 2012b).

2.5 Ecology: Salinity and Temperature

While most branchiopods occur in fresh- or brackish water (Brendonck *et al.*, 2008), both *Artemia* and *Parartemia* are halophilic (Rogers, 2024; Timms, 2012a).

Artemia generally occur in saline waters where NaCl is the major salt but are also found in other salt waters, e.g., some lakes in Nebraska, USA (potassium-rich), Mono Lake in California, USA and Qixiang Lake in Tibetan Plateau, China (carbonate-rich) and Chaplin Lake in Saskatchewan, Canada (sulphate-rich) (Asem *et al.*, 2024b; Asem *et al.*, 2024c; Van Stappen, 1996).

Laboratory tests have confirmed that *A. franciscana* (then called *A. salina*, but eggs were sourced from the USA) can survive in seawater concentrations as low as 10%, extending up to saturated brine (Croghan, 1958c). In natural habitats, active individuals of this species have been recorded in salinities exceeding 300 g/L (Lenz & Browne, 1991). During our field trips, active individuals of bisexual *Artemia* (confirmed as *A. franciscana*; see Chapter 5) were collected from a salinity range of 35.7-193.5 g/L and those of unisexual *Artemia* (confirmed as diploid parthenogenetic *Artemia*; see Chapter 5) from 71.9-186 g/L (see Table S2.1 for detailed salinity information and other water quality parameters). However, these field records do not take into account other physical and chemical parameters or the interactions between parameters. Mitchell and Geddes (1977) examined brine shrimps in the St Kilda saltworks (Dry Creek near Adelaide), South Australia. At that time, the site was known to harbour both unisexual *Artemia* and *Parartemia* (*P. zietziana*). According to Mitchell and Geddes (1977), *Artemia* at this site were documented within a salinity range of 186 to 330 g/L, while *P. zietziana* occurred in a slightly lower range of 112 to 258 g/L. However, diploid parthenogenetic *Artemia* occur in Lake Hayward, a coastal lake in Western Australia characterised by stratification with an epilimnion salinity ranging from 65 to 110 g/L (Savage & Knott, 1998a; also see Chapter 5). Thus, the absence of unisexual *Artemia* at salinity levels below 186 g/L in the St Kilda saltworks might be linked to the presence of *P. zietziana*, although the site is now solely occupied by *A. franciscana* (Asem *et al.*, 2018; Timms, 2014).

Initially, it was thought that *Parartemia* could only tolerate moderate salinity (Kuenen, 1938), but the upper limit for most species is now known to be much higher (see Table 2.2). Geddes (1976) reported that individuals of *P. zietziana* remain active for a long period until the lake water moves toward salt saturation. Based on field records, the salinity range for *P. zietziana* is 22 - 353 g/L (De Deckker & Geddes, 1980; Timms *et al.*, 2009), which is the broadest reported, with the highest upper limit, for any *Parartemia* species (see Table 2.2). However, *P. zietziana* has been monitored in multiple lakes throughout the year (De Deckker & Geddes, 1980), so we may have more accurate data on its upper salinity tolerance compared to other species. No salinity information is available for three *Parartemia* species: *P. auriciforma*, *P. triquetra* and *P. yarleensis*. Most *Parartemia* species occur in alkaline salt lakes but a few also occur in acidic waters (see pH data in Table 2.2).

Temperature also plays a crucial role in the survival and abundance of brine shrimps. *Artemia* has not been reported in frost-prone or tundra climatic areas, where extremely cold weather restricts its existence (Van Stappen, 2002). However, unisexual *Artemia* along with *A. tibetiana* have been recorded from the Lagkor Co Lake in Tibet (Maccari *et al.*, 2013a), where the air temperature ranges from approximately -26 to 24 °C and the average water salinity is about 60 g/L (Abatzopoulos *et al.*, 1998). *Artemia* is abundant in this lake during summer (Van Stappen, 2002). A recent study confirmed the presence of at least three bisexual *Artemia* species - the exotic *A. franciscana* and the native *A. tibetiana* and *A. sorgeloosi* - along with diploid parthenogenetic *Artemia* from the Tibetan Plateau (see Asem *et al.*, 2024c).

In Australia, the adults of diploid parthenogenetic *Artemia* experience a significant reduction in numbers, or even complete die-offs, during late summer in Lake Hayward where water temperatures in the epilimnion range from 10 - 30 °C (Savage & Knott, 1998a). A new generation appears when temperature falls, and recruitment of the adults takes place during late winter. We collected diploid parthenogenetic *Artemia* between 1st October 2022 and 30th April 2023 from lakes with water temperature ranging from 15.7 - 28.2 °C (Table S2.1).

Active individuals of *A. franciscana* in the Great Salt Lake, where this species is native, start to appear at ~10 °C (in April), can endure temperatures up to ~27 °C during the summer and disappear when temperatures are ~11 °C (in November) (Stephens & Gillespie, 1976). There is no information about the impacts of temperature on this species in Australian salt lakes, however, we found this species in temperatures ranging from 21.4 - 30 °C in Western Australian salt lakes (Table S2.1).

For *Parartemia*, temperature exerts a significant impact on the salinity tolerance of *P. zietziana* (Geddes, 1975a, 1981; Marchant & Williams, 1977d). In laboratory tests, *P. zietziana* exhibited a broad salinity tolerance, spanning from 4.8 to 301 g/L at 10 °C. However, this tolerance range decreased to 12.8-119 g/L when the temperature was increased to 30 °C (Geddes, 1981). This association between temperature and salinity tolerance probably also exists for other *Parartemia* species, but further experimental investigations are needed to elucidate the details. It is important to consider this association when interpreting field data on the salinity distributions of *Parartemia* species, which will have been recorded over a range of temperatures.

2.6 Physiology: Osmoregulation and Respiration

The invertebrates of hypersaline lakes, like *Artemia* and *Parartemia*, have to cope with high and variable salt levels. Croghan studied osmoregulation in *A. franciscana* (then called *A. salina*, but eggs obtained from the USA) and found that the osmotic pressure of the haemolymph was largely independent of external salinity, rising only slightly with increased salinity and becoming markedly hypo-osmotic in highly saline water (Croghan, 1958b). Also, the ratios of various ions in the haemolymph remain relatively constant, differing considerably from those in the external water, with sodium and chloride acting as the primary ions regulating haemolymph osmotic pressure. In another study, Croghan (1958a) suggested that the gut fluid of *A. franciscana* is hypo-osmotic to external water but hyper-osmotic to the haemolymph, implying that NaCl moves from the gut fluid into the haemolymph. Croghan (1958a) also indicated that excess NaCl in the haemolymph can be excreted through gills. The hypo-osmoregulatory capacity of *A. franciscana* has also been observed in *A. salina* (see Sellami *et al.*, 2020) and is likely characteristic of all *Artemia* species.

Geddes studied osmoregulation in *P. zietziana* (Geddes, 1975b; Geddes, 1975c), which exhibits hyper-osmotic regulation in mildly saline water and robust hypo-osmotic regulation in high salinity water. The ionic compositions of the haemolymph and gut fluid of *P. zietziana* differ, yet they are isosmotic at all salinities. In high salinity conditions, gut fluid is dominated by magnesium ions, whereas the haemolymph is dominated by sodium and chloride ions. The gut fluid consistently receives NaCl-rich water through both oral and anal openings, and the sodium and chloride ions are then transported to the haemolymph. Any water loss from the haemolymph through exosmosis is similarly replenished through the gut fluid. Excess salt in the haemolymph is excreted through the gills. Geddes's findings are therefore broadly

comparable to Croghan's description of osmoregulation in *Artemia*, possibly reflecting their common evolutionary origins, although the gut fluid and haemolymph are isosmotic in *Parartemia* but not in *Artemia* (Geddes, 1975c).

Generally, higher salinity reduces the availability of dissolved oxygen in a salt lake (Mitchell & Geddes, 1977). However, both *Artemia* and *Parartemia* can survive in waters with very low oxygen concentrations. For example, unisexual *Artemia* (reported as *A. salina*, but based on the timing and study site, should be unisexual *Artemia*) has a critical oxygen limit as low as 0.9 mg/l (Geddes, 1981; Mitchell & Geddes, 1977), whereas the critical oxygen limit for *P. zietziana* is as low as 1.8 mg/l (Marchant & Williams, 1977a). Additionally, both *Artemia* and *Parartemia* demonstrate a comparable respiration rate over these threshold limits. For example, *A. franciscana* (reported as *A. salina* but eggs sourced from California) exhibits a respiration rate of 0.9 - 4.0 mgO_2 per hour per individual at a salinity of 140 g/L, temperature of 25 °C, and individual bodyweight of 0.05 - 0.4 mg (Gilchrist, 1956, 1958), while *P. zietziana* displays a similar respiration rate of 0.9 - 4.6 mgO_2 per hour per individual under the same conditions (Marchant & Williams, 1977a).

Artemia has long been recognised for its capacity to produce haemoglobin (Gilchrist, 1954; Lochhead & Lochhead, 1941), which enables individuals to survive under extremely low oxygen concentrations. *Artemia* typically increase haemoglobin production once the salinity reaches a certain level. For instance, Gilchrist (1954) carried out investigations on *A. franciscana* in salinity ranging from 112 - 280 g/L and noted that haemoglobin was only detected when the salinity exceeded 125 g/L. Mitchell and Geddes (1977) studied unisexual *Artemia* and, in conjunction with the findings of Gilchrist (1954), concluded that the haemoglobin of *Artemia* becomes functional when the oxygen concentration falls below approximately 2.0 mg/l. *Parartemia zietziana* synthesises haemoglobin of a similar molecular weight as of *Artemia*, but the amount is minute (only around 0.01 % in the haemolymph), and it is not functionally involved in oxygen transportation and does not appear to be a response to low oxygen concentrations (Manwell, 1978). According to Manwell (1978), the haemoglobin in *P. zietziana* may be involved with heme (iron transport) rather than oxygen transportation but further study is required to confirm this (Coleman *et al.*, 2001). It is interesting that, despite the above observations, at least some species of *Parartemia* can still tolerate very high salinity and therefore very low levels of dissolved oxygen. There is a need to better understand how *Parartemia* species meet their oxygen requirements in high salinity water.

The haemoglobin of *Artemia* is a dimeric structure consisting of two multi-domain polymers that function freely in the haemolymph without excretory loss due to their large sizes (Coleman *et al.*, 1998; Manning *et al.*, 1990; Matthews *et al.*, 1998). Each polymer, named T and C, comprises nine globin domains, resulting in a quaternary structure consisting of eighteen domains, with each polymer being encoded by a single gene (Matthews *et al.*, 1998). The T and C globin genes have evolved from a single nine-domain gene through duplication, which is believed to have originated from an ancient single-domain globin gene through multiplications (Manning *et al.*, 1990; Matthews *et al.*, 1998). In contrast, the haemoglobin of *Parartemia* is encoded by a single nine-domain globin gene called P, which is estimated to have originated from a nine-domain globin gene in the common ancestor of *Artemia* and *Parartemia* at least 85 million years ago (Mya) (Coleman *et al.*, 1998). In the *Artemia* lineage, this ancestral gene is believed to have diverged into T and C globin genes approximately 60 Mya (Matthews *et al.*, 1998). The T globin gene in particular has undergone considerable modification in *Artemia* and may allow the production of large amounts of haemoglobin mentioned above (Coleman *et al.*, 2001). This paragraph does not refer to species names for either *Artemia* or *Parartemia* because these names were not given in the cited papers. However, based on information about the source of specimens, the species are probably *A. franciscana* and *P. zietziana*.

2.7 Food and Feeding Habits

Both *Artemia* and *Parartemia* are filter feeders (Mura, 1995). *Artemia* primarily feed on planktonic algae (Marden *et al.*, 2020; Van Stappen, 2002), but also consume bacteria and organic particles (Marden *et al.*, 2020; Savage & Knott, 1998b; Van Stappen, 1996). In a study conducted by Reeve (1963a) using *A. franciscana* (eggs sourced from the Great Salt Lake in the USA), filtration and ingestion rates were largely influenced by the age of the brine shrimp and the concentration of food in the water. Another study by Reeve (1963b) observed that *A. franciscana* feeds on particles regardless of their nutritional value. However, the filtration process in *Artemia* exhibits strong size selectivity (Dobbeleir *et al.*, 1980; Fernández, 2001). Fernández (2001) proposed that *A. franciscana* has a strict food particle size ranging from 6.8 to 27.5 µm, with an optimum size of approximately 16.0 µm.

For *Parartemia*, feeding has only been studied in *P. zietziana*, the primary food source is organic particles from sediments (Marchant & Williams, 1977b). Marchant and Williams (1977c) demonstrated that the organic content of the faeces of *P. zietziana* is notably higher

than that of the sediment. They concluded that *P. zietziana* exhibits selective feeding behaviour, targeting energy-rich organic particles during feeding, which is different from the nonselective feeding suggested for *Artemia*. Timms (2012b) made a general comment that *Parartemia* feed on nonliving organic particles as well as benthic diatoms but did not provide any supporting details. Further research is necessary to gain a more comprehensive understanding of feeding behaviour in *Parartemia* and whether this behaviour varies among species.

The natural ecosystems of which *Artemia* is a part typically exhibit a simple trophic structure, seemingly devoid of food competition, which allows *Artemia* to dominate (Van Stappen, 1996). However, the abundance of *Artemia* can be influenced by food availability, as seen in studies showing that, in the Great Salt Lake, the abundance of *A. franciscana* is positively correlated with that of the phytoplankton (Stephens & Gillespie, 1976; Wurtsbaugh & Gliwicz, 2001). In the case of *Parartemia*, Marchant and Williams (1977d) revealed that *P. zietziana* in a salt lake in Victoria experienced continuous but variable mortality despite favourable salinity and temperature conditions, and the absence of significant predators. The authors suggested that the most plausible cause of this mortality was food shortage, either due to a direct scarcity of food or inability to assimilate enough food. Specific nutritional requirements have not been studied for any *Parartemia* species.

2.8 Reproduction and Life History

One of the main differences between *Artemia* and *Parartemia* is that the former has both bisexual (sexually reproducing) and parthenogenetic lineages, whereas all *Parartemia* species are bisexual and presumably sexually reproducing. The parthenogenetic *Artemia* lineages (ranging from diploid to pentaploid) have evolved from Asian bisexual *Artemia* lineages (Asem *et al.*, 2024a and references therein). Diploid parthenogenetic *Artemia* reproduce via automixis whereas the polyploids use apomixis (Innes & Dufresne, 2020; Muñoz *et al.*, 2010).

Females of *Artemia* and *Parartemia* can reproduce through both ovoviviparity, where active nauplii are directly released, or oviparity, with the production of resting eggs (Criel & Macrae, 2002b; Geddes, 1976; Marchant & Williams, 1977d; Van Stappen, 1996). The resting eggs are often referred to as ‘cysts’, but the correct term is ‘eggs’ (see Asem *et al.*, 2024b). For *Artemia*, ovoviviparous versus oviparous reproduction is influenced by ecological factors such as temperature, salinity, dissolved oxygen, light intensity and food availability (Asil *et al.*, 2013; D'Agostino & Provasoli, 1968; Nambu *et al.*, 2004; Sorgeloos, 1975; Versichele & Sorgeloos, 1980; Yang & Sun, 2023). The reproductive mode of *A. franciscana* in the Alviso Salt Ponds

and the Great Salt Lake is usually influenced by water temperature, with the production of resting eggs initiated when temperature drops (Carpelan, 1957; Stephens & Gillespie, 1976). According to Stephens and Gillespie (1976), *A. franciscana* in the Great Salt Lake survives summer temperatures up to 27 °C, but individuals produce resting eggs before dying off during winter, when temperatures generally range between 1 to 9 °C. In a multi-factor laboratory experiment, conducted at temperatures between 10 and 30 °C and salinities ranging from 15 to 180 g/L, food availability had a major impact on the mode of reproduction in *A. franciscana,* with females tending to shift from oviparity to ovoviviparity as food increased (Belovsky *et al.*, 2024). In Lake Hayward, Western Australia, where epilimnion water temperatures range from 10 to 30°C and salinity levels vary between 65 to 110 g/L, diploid parthenogenetic *Artemia* produces resting eggs during the summer season (Savage & Knott, 1998a). Based on the above, *Artemia* demonstrates an oviparous mode of reproduction under unfavourable conditions, such as low or high temperatures and low food availability (see also Marden *et al.*, 2020).

The factors influencing the reproductive mode of *Parartemia* species are not yet fully understood. Geddes (1981) indicated that, based on field observations, *P. zietziana* produces active nauplii under stable salinity conditions and switches to producing resting eggs at high salinity and low dissolved oxygen (Geddes, 1976; Marchant & Williams, 1977d). In a highly episodic salt lake in Western Australia (Lake Yindarlgooda), *P. veronicae* produced resting eggs at higher salinity levels before dying off and emerged from resting eggs after sufficient rainfall (see Campagna, 2007). According to Campagna (2007), during prolonged hydroperiods, individuals of this species may produce active nauplii.

In general, *Artemia* is capable of producing up to 300 eggs or active nauplii per clutch every four days (Van Stappen, 1996). Browne and Wanigasekera (2000) examined the reproductive characteristics of four bisexual *Artemia* species and unisexual *Artemia* under different salinity and temperature conditions in the laboratory. They found that the reproductive output of the different species varied significantly with these factors. Optimal conditions for *A. franciscana* were 24 °C and 120 g/L salinity, where it produced 74.6 ± 34.9 (mean ± SD) offspring (eggs or active nauplii) per clutch, 5.4 ± 3.8 clutches per female and a total of 470.5 ± 438.5 offspring per female. In comparison, the unisexual *Artemia* exhibited the highest reproductive output, with 57.3 ± 11.7 offspring per clutch, 10.8 ± 2.1 clutches per female and a total of 603 ± 102.2 offspring per female, under the optimal conditions same as for *A. franciscana*. In natural habitats, females of *A. franciscana* in the Great Salt Lake reportedly produce 30 - 50 offspring

per clutch (Wirick, 1972 in MacDonald & Browne, 1989). Unisexual *Artemia* in Salin de Giraud, France, produced 10 - 100 offspring per clutch (MacDonald & Browne, 1989). Regarding *Parartemia*, Geddes (1976) reported that oviparous *P. zietziana* females produce an average of 24 - 205 offspring per clutch, but no information is available on the total number of clutches produced per female for this or any other *Parartemia* species. *Parartemia* eggs sink and persist on lake beds until hatching, in contrast to the floating eggs of most *Artemia* species (Asem *et al.*, 2024b; Geddes, 1981; Zhou *et al.*, 2022).

Shepard and Hill (2001) described egg morphology for some anostracan species, including. *A. franciscana* and *A. monica*. They found that those of the former had a smooth external surface whereas those of the latter had a wrinkled surface with circular discoidal projections. Thus these two species can be distinguished via egg morphology (see also Asem *et al.*, 2024b). Asem and Sun (2014) investigated the surface morphology of eggs in several unisexual *Artemia* populations and found both inter- and intrapopulation variations. Consequently, these authors cautioned against relying on egg surface morphology for distinguishing among unisexual *Artemia* lineages. Timms *et al.* (2004) described the egg morphology of some *Parartemia* species and reported that they were smooth but with some inpocketing. Campagna (2007) examined egg morphology in *P. veronicae* and *P. laticaudata* and found notable distinctions between these two species (see Table 2.3). However, information about egg morphology in other *Parartemia* species, as well as about whether this morphology is subject to environmental modification, is needed to properly understand the taxonomic value of eggs.

Table 2.3: Egg morphology of *Parartemia* species, information sourced from Timms *et al.* (2004) and Campagna (2007). No information on egg morphology is available for species not listed here.

Species	Egg Shape	Egg diameter	Alveolar layer	Tertiary layer
P. contracta	Spherical, smooth external surface with some inpockets	250-275 µm	Uniform and subequal rounded small vesicles with short struts	Thin
P. cylindrifera	Spherical, smooth external surface with several inpockets	204-225 µm	Uniform and mostly solid	Thin
P. informis	Spherical	162-176 µm	Immature sample	-
P. laticaudata	Spherical and a large inpocketing at one side, hairy dark brown external surface with large pores	260-320 µm	Two lamellate sublayers with unequal vesicles	-
P. minuta	Spherical, smooth external surface with several inpockets	180-232 µm	Uniform and solid	Thin
P. veronicae	Spherical and a large inpocketing at one side, smooth light brown external surface with visible pores	285-322 µm	Uniform with subequal rounded small vesicles	Thin
P. zietziana	Spherical to hemispherical, smooth external surface with some inpockets at one side	180-208 µm	Uniform and solid	Thin

The resting eggs of both *Artemia* and *Parartemia* are widely recognised for their remarkable resilience against various adverse environmental conditions. Many studies have demonstrated the capacity of *Artemia* eggs to withstand extreme temperatures, severe desiccation, repeated hydration, prolonged anoxic conditions and intense UV radiation (Lenormand *et al.*, 2018 and references therein). The primary defensive features of the resting eggs include the structure of the egg shell and the presence of stress-response proteins (e.g., artemin, p26 and hsp70), alcohol-soluble carbohydrates and trehalose (Clegg, 2005; Clegg & Campagna, 2006; Clegg & Trotman, 2002; Hibshman *et al.*, 2020). These proteins and trehalose are maintained at high levels in the resting eggs until emergence (Clegg, 2005). Notably, the presence of artemin and p26, once thought to be exclusive to *Artemia* eggs, have also been identified in the eggs of *P. laticaudata* and *P. veronicae* (Clegg & Campagna, 2006). Although our understanding of the molecular mechanisms underlying desiccation resistance and stress tolerance of *Artemia* eggs is relatively advanced (see Hibshman *et al.*, 2020; Marden *et al.*, 2020), it is much more limited for *Parartemia*, particularly regarding the role of different molecular chaperones and the specific biochemical pathways contributing to their stress resilience.

Environmental factors influencing the hatching of *Artemia* resting eggs have been well-documented (Asil *et al.*, 2012; Belovsky *et al.*, 2024; Clegg, 1964; Dana & Lenz, 1986; Dey *et al.*, 2023; Marden *et al.*, 2020; Sorgeloos, 1980; Triantaphyllidis *et al.*, 1995; Vanhaecke *et al.*, 1981; Vanhaecke & Sorgeloos, 1989), although much of the information is derived from just *A. franciscana*. An early study by Clegg (1964) reported that higher salinity, approximately 100 g/L (Geddes, 1981), hindered the hatching of resting eggs of this species obtained from a commercial company in Hayward, California, USA. In its natural habitat, the Great Salt Lake, *A. franciscana* exhibits seasonal hatching patterns, with early evidence of hatching emerging in April as spring temperatures rise (Marden *et al.*, 2020). Optimal environmental conditions required for hatching *Artemia* eggs can vary between species, conspecific populations and different unisexual strains, with temperature and salinity playing significant roles (Dey *et al.*, 2023; Geddes, 1981; Vanhaecke & Sorgeloos, 1989). For *Parartemia*, Geddes (1976) found that the eggs of *P. zietziana* can hatch at salinities of 50 to 200 g/L. In laboratory tests, Campagna (2007) showed that a salinity below 55 g/L (80 mS cm^{-1}) is more suitable for hatching *P. veronicae* eggs. However, the full extent of species-specific salinity requirements and the potential importance of other environmental influences on the hatching of *Parartemia* eggs is largely unknown.

In general, *Artemia* develop rapidly and can transition from nauplius to adult within eight days (Van Stappen, 1996). The adults can then survive for several months in suitable environmental conditions (Van Stappen, 1996). An anecdotal report suggests that *Artemia*, when kept as pets, may live up to a year, with some claims indicating lifespans of up to five years (White, 2022). *Artemia franciscana,* in particular, has the ability to complete up to eight generations within a single year in permanent salt ponds (Carpelan, 1957). However, the number of generations per year can vary depending on environmental conditions and/or species. For example, diploid parthenogenetic *Artemia* in Lake Hayward (a permanent lake), Western Australia, completes just a single generation per year (Savage & Knott, 1998a).

The relatively well-studied *P. zietziana* from eastern Australia has been reported to have one or two generations per year (Geddes, 1976) or possibly two or three generations (Marchant & Williams, 1977d). Another species, *P. minuta*, also from eastern Australia, is presumed to complete one generation per year (Timms, 2007, 2009a), although the actual number may vary depending on the length of suitable hydroperiods (Timms, 2014), which is likely applicable for all or most *Parartemia* species, given that they typically occupy temporary habitats filled by seasonal or unpredictable rainfall. Further study is needed to document the life-history characteristics and population dynamics of *Parartemia* species.

2.9 Distribution of Brine Shrimp in Australia

Artemia have been recorded from Queensland, Victoria, South Australia and Western Australia (see Fig. 2.5). Bisexual *Artemia* has been reported from 17 sites in Australia, comprising six saltworks (located in Port Alma and Bowen in Queensland, St Kilda and Mulgundawa in South Australia and Port Hedland and Dampier in Western Australia; see Chapter 5), three lakes formally used for salt extraction (an unnamed lake near Port Augusta in South Australia and Hutt Lagoon and Lake Koorkoodine in Western Australia; see Timms, 2014) and eight natural salt lakes in Western Australia that have not been used for salt extraction (see Chapter 5). The bisexual *Artemia* in Australia are usually referred to as *A. franciscana,* sometimes on the basis of the original source of the eggs (e.g., see Ruebhart *et al.*, 2008) but sometimes no reason is given (e.g., ARL, 2009). This identification has been confirmed using electrophoretic data for the *Artemia* in the saltworks at Port Alma (Queensland) (Ruebhart *et al.*, 2008 and references therein) and mitochondrial DNA data for the *Artemia* in saltworks at St Kilda (Dry Creek near Adelaide) and Mulgundawa (near Lake Alexandrina) in South Australia and Port Hedland and

Dampier in Western Australia (Asem *et al.*, 2018). The findings of this PhD research suggest that bisexual *Artemia* in natural salt lakes are also *A. franciscana* (see Chapter 5).

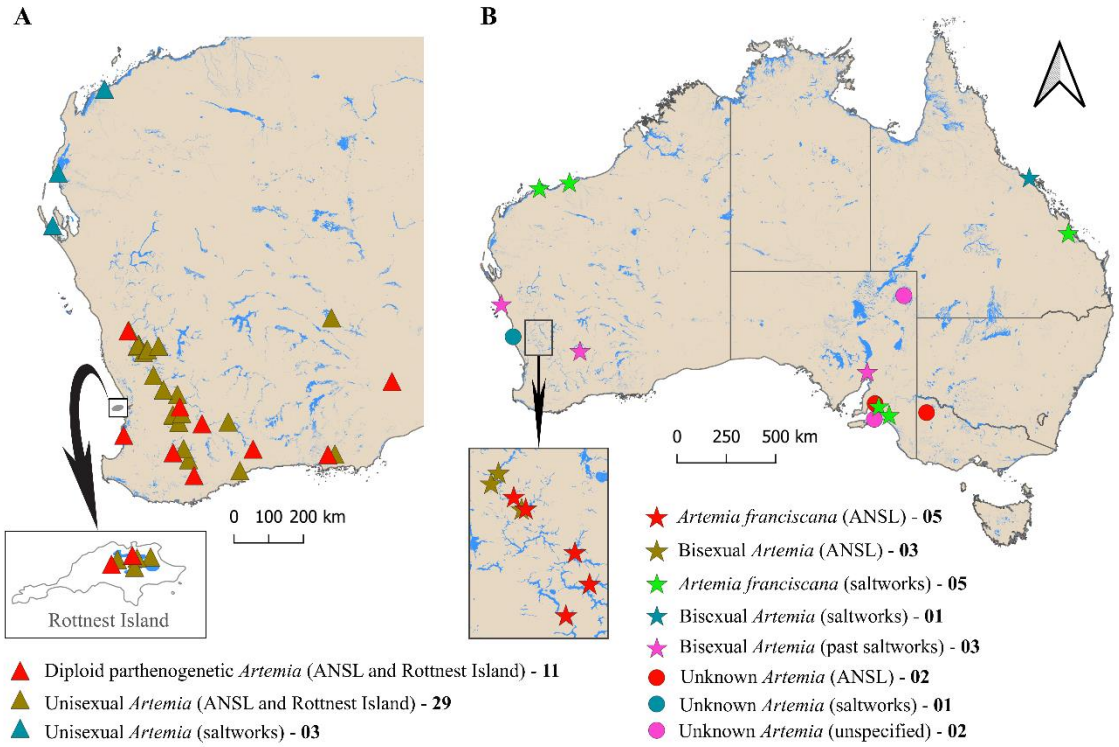

Fig. 2.5: Approximate locations of currently known *Artemia* populations in Australia: (A) unisexual populations and (B) bisexual and unknown (sex ratio not specified) populations. ANSL indicates Australian natural salt lakes that have not been used/modified for salt extraction. Number of records per category is in bold. Species name is provided only if identity has been confirmed via molecular data (see Chapter 5). This diagram is also presented in Chapter 5.

Unisexual *Artemia* are currently known from at least 43 sites, all of which are in Western Australia. These include saltworks in Shark Bay, Lake McLeod and Onslow (McMaster *et al.*, 2007; Timms, 2014), and 40 natural salt lakes on Rottnest Island and the mainland (Fig. 2.5). This PhD research has identified the unisexual *Artemia* in the natural salt lakes as a type of diploid parthenogenetic *Artemia* (see Chapter 5).

Parartemia occur in all Australian states and are known from at least 435 sites (Fig. 2.6 - Fig. 2.9). These sites collectively harbor at least 21 *Parartemia* species, with 16 species occurring in Western Australia, 13 of which are endemic to this state (Table 2.4). South Australia is home to seven species, three of which are exclusive to this state. Only one or two species occur in

Victoria, Tasmania, New South Wales, Queensland and the Northern Territory and none of these jurisdictions has any endemic species (see Table 2.4). A total of five species are found in multiple states, with *P. minuta* occurring in four states (South Australia, Victoria, New South Wales and Queensland). Some species are widespread within a state, such as *P. longicaudata*, *P. serventyi* and *P. informis* in Western Australia (see Fig. 2.6 – Fig. 2.8).

The distributions of the 21 *Parartemia* species across Australian drainage divisions are summarised in Table 2.4 (Fig. 2.10 and Fig. 2.11 to see the locations of drainage divisions). The South Western Plateau (SWP) drainage division harbors at least one population of all *Parartemia* species, except *P. extracta*, with eight species only known from this division. Within the SWP division, most *Parartemia* populations are located at the western edge in Western Australia and in the southeastern corner in South Australia (see Fig. 2.10 and Fig. 2.11). However, three species, *P. auriciforma*, *P. triquetra* and *P. yarleensis*, have populations in intermediate locations, which cover the extremely arid Great Victorian Desert and Nullarbor Plain. Despite the arid conditions, the former two species are restricted to this region (see Fig. 2.7 and Fig. 2.8). The South West Coast (SWC) drainage division in Western Australia also contains a high diversity of species. Ten species occur there, including *P. extracta*, which is only known from this division (see Table 2.4).

Parartemia populations are predominantly situated in the southern half of Australia in areas with an average annual rainfall of about 200 to 600 mm (Fig. 2.10) and average annual temperatures of about 15 to 21 °C (Fig. 2.11). Numerous salt lakes also occur in these areas (De Deckker, 1983; Timms, 2005). However, more surveys in arid regions in central Australia are likely to reveal additional populations and species (Timms, 2010).

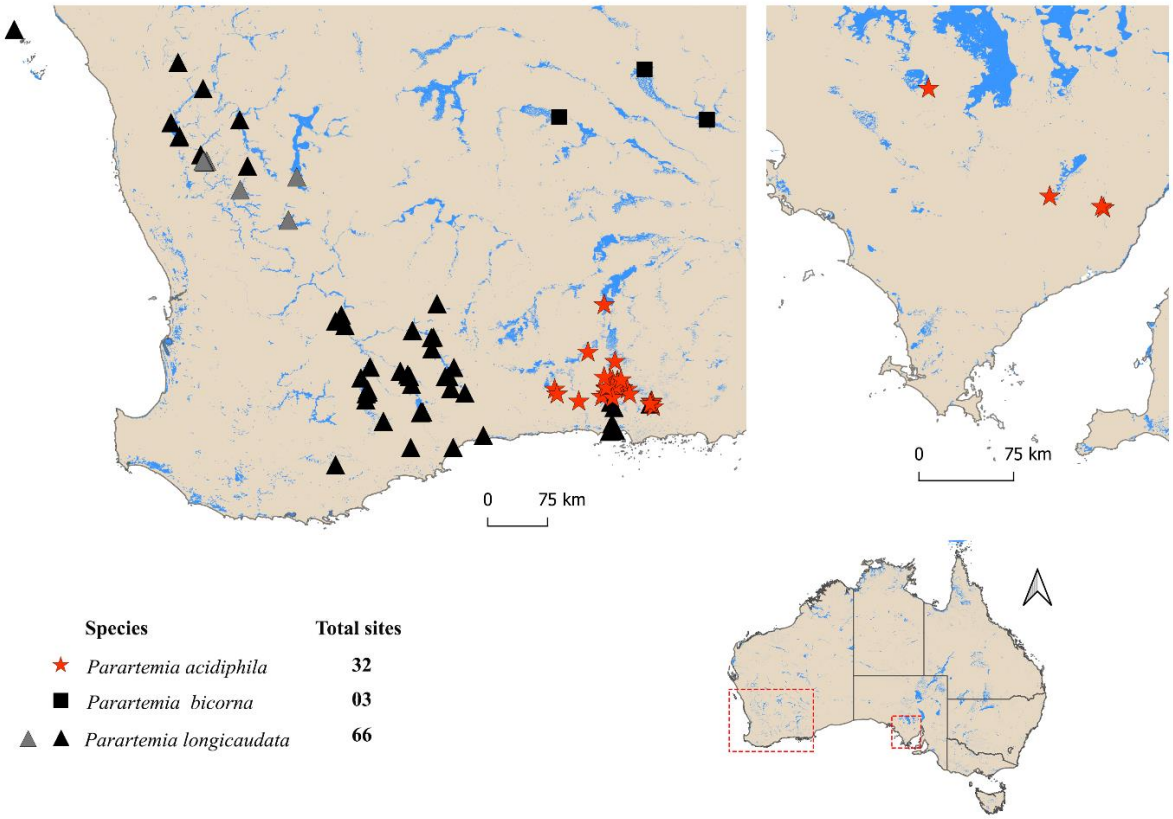

Fig. 2.6: Approximate locations of currently known sites for three *Parartemia* species. Grey triangles indicate the approximate location of the *P. boomeranga* morphotype, which has been synonymised with *P. longicaudata* (black triangles) in Islam *et al.* (2024). Maps have been created using QGIS 3.32 (www.qgis.org). Data for surface hydrology in blue on the maps are sourced from the national surface water database of Geoscience Australia (www.ga.gov.au).

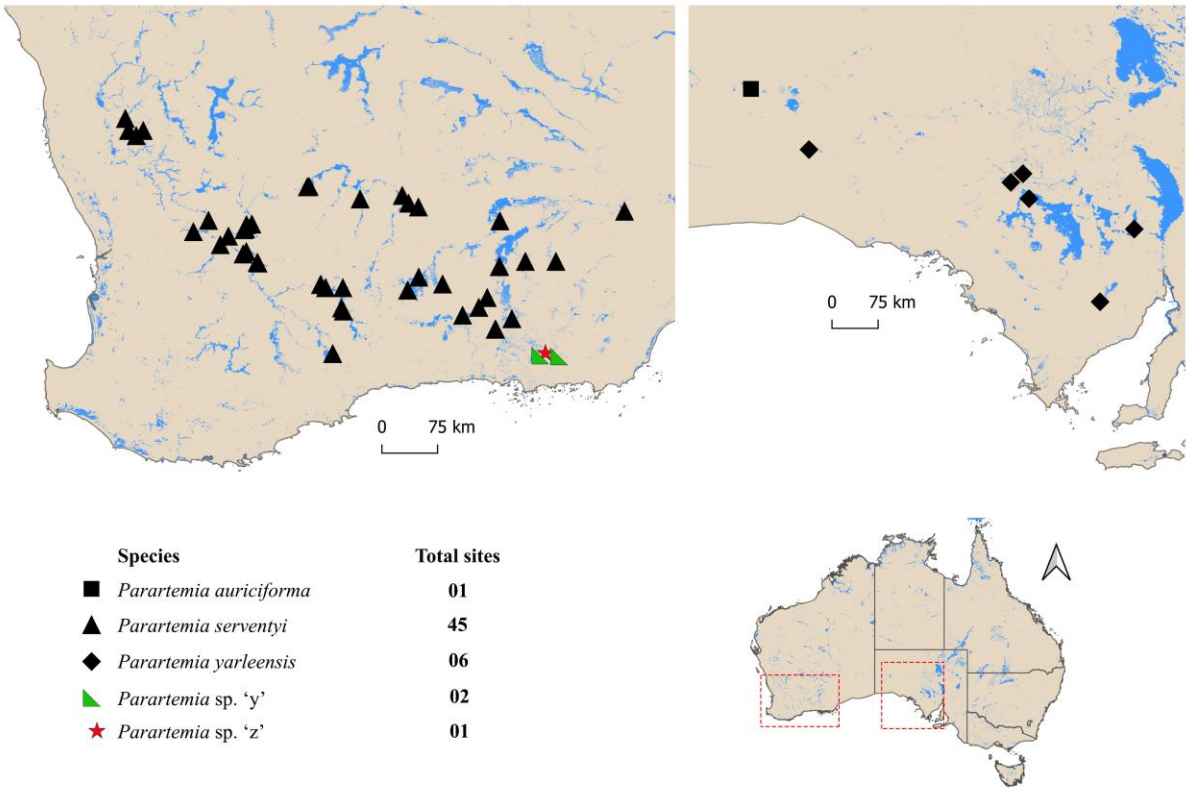

Fig. 2.7: Approximate locations of currently known sites for five *Parartemia* species. Maps have been created using QGIS 3.32 (www.qgis.org). Data for surface hydrology in blue on the maps are sourced from the national surface water database of Geoscience Australia (www.ga.gov.au).

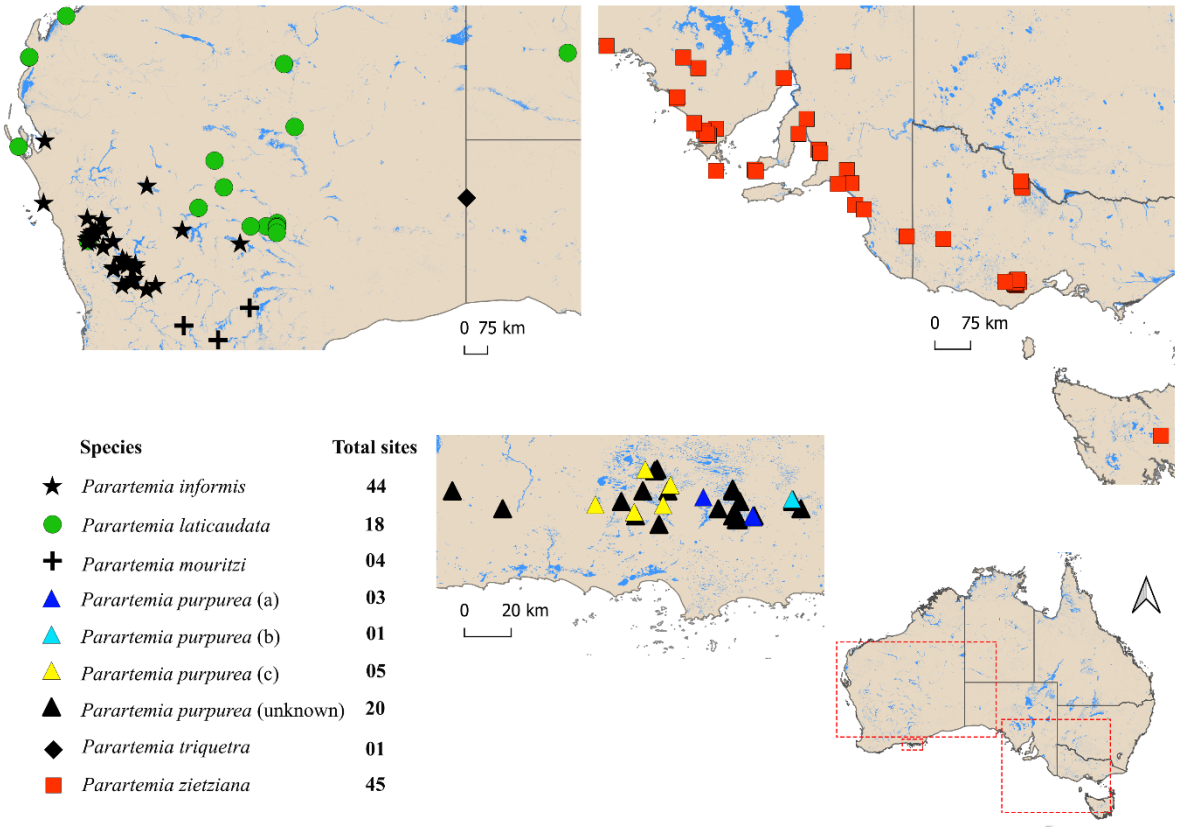

Fig. 2.8: Approximate locations of currently known sites for six *Parartemia* morphospecies species, plus three cryptic species within *P. purpurea* (a-c) confirmed by molecular data (Islam *et al.*, 2024). Black triangles indicate sites with the *P. purpurea* morphotype but for which there are no molecular data. Maps have been created using QGIS 3.32 (www.qgis.org). Data for surface hydrology in blue on the maps are sourced from the national surface water database of Geoscience Australia (www.ga.gov.au).

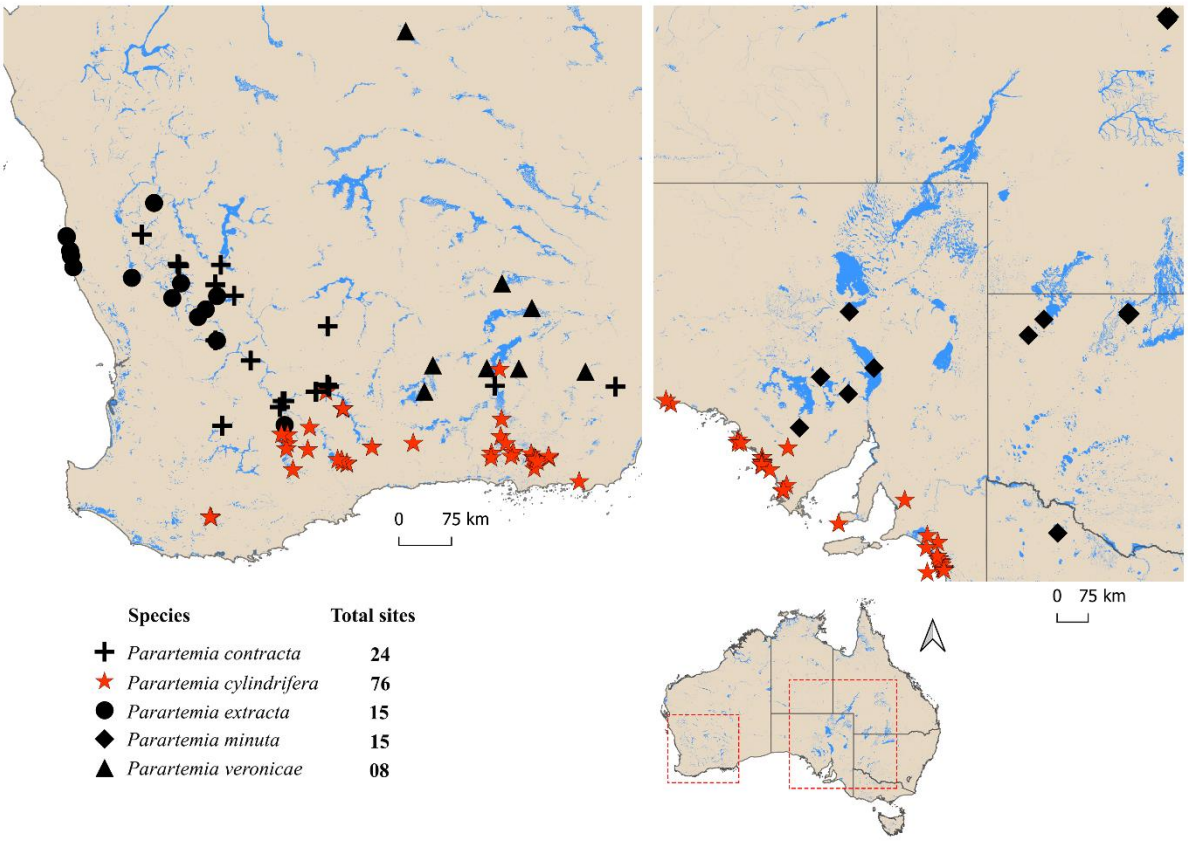

Fig. 2.9: Approximate locations of currently known sites for five *Pararartemia* species. Maps have been created using QGIS 3.32 (www.qgis.org). Data for surface hydrology in blue on the maps are sourced from the national surface water database of Geoscience Australia (www.ga.gov.au).

Although multiple *Pararartemia* species have overlapping geographic distributions, it is rare to find more than one species in the same water body (Timms, 2014; Timms *et al.*, 2009). When different *Pararartemia* species occur in closely located habitats, the habitats are often separated by land barriers or narrow sand bars (Timms *et al.*, 2009). Instances where two species appear to coexist usually result from one of them being washed into a habitat from adjacent locations (Timms, 2012b). This lack of coexistence might be attributed to the Monopolization Hypothesis (De Meester *et al.*, 2002), resulting in the exclusion of new arrivals by congeneric or conspecific occupants (see Chapter 4). Such habitat monopolisation is expected to minimise gene flow among populations and lead to speciation in anostracans like *Pararartemia* (Rogers, 2015).

Table 2.4: Distribution of *Parartemia* species across Australian states and territories and twelve drainage divisions. Key to states and territories – WA = Western Australia, SA = South Australia, VIC = Victoria, NSW = New South Wales, QLD = Queensland, TAS = Tasmania and NT = Northern Territory. Key to Australian drainage divisions – SWC = South West Coast, SWP = South Western Plateau, SAG = South Australian Gulf, SEC = South East Coast, TAS = Tasmania, MDB = Murray-Darling Basin, NEC = North East Coast, LEB = Lake Eyre Basin, CC = Carpentaria Coast, TTS = Tanami-Timor Sea coast, NWP = North Western Plateau and PG = Pilbara-Gascoyne. Data on Australian drainage divisions are sourced from the Bureau of Meteorology (www.bom.gov.au) and are depicted in Fig. 2.10 and Fig. 2.11.

Species	WA	SA	VIC	NSW	QLD	TAS	NT
Parartemia acidiphila	SWC, SWP	SWP, SAG					
Parartemia auriciforma		SWP					
Parartemia bicorna	SWP						
Parartemia contracta	SWC, SWP						
Parartemia cylindrifera	SWC, SWP	SWP, SAG, MDB					
Parartemia extracta	SWC						
Parartemia informis	PG, SWC, SWP						
Parartemia laticaudata	PG, SWC, SWP, NWP						TTS
Parartemia longicaudata	PG, SWC, SWP						
Parartemia minuta		SWP, SAG, LEB	MDB	LEB, MDB	LEB		
Parartemia mouritzi	SWC, SWP						
Parartemia purpurea (a)	SWP						
Parartemia purpurea (b)	SWP						
Parartemia purpurea (c)	SWC, SWP						
Parartemia serventyi	SWC, SWP						
Parartemia triquetra		SWP					
Parartemia veronicae	SWP						
Parartemia yarleensis		SWP, SAG					

Parartemia zietziana		SWP, SAG, LEB, MDB, SEC	MDB, SEC			TAS	
Parartemia sp. ‘y’	SWP						
Parartemia sp. ‘z’	SWP						
Total	**16**	**07**	**02**	**01**	**01**	**01**	**01**

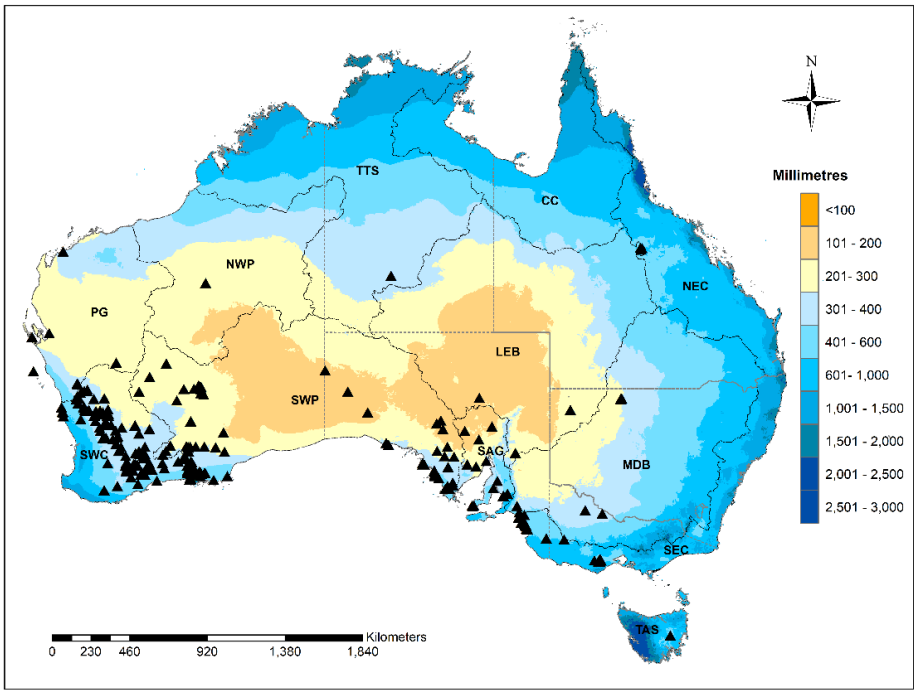

Fig. 2.10: Distribution of *Parartemia* on a map showing average annual rainfall (standard 30-year records between 1961-1990 from the Bureau of Meteorology, www.bom.gov.au). To enhance visibility, certain *Parartemia* sites in proximity were omitted. The map also shows the locations of drainage divisions, denoted by bold abbreviated names, with the corresponding full names provided in Table 2.4.

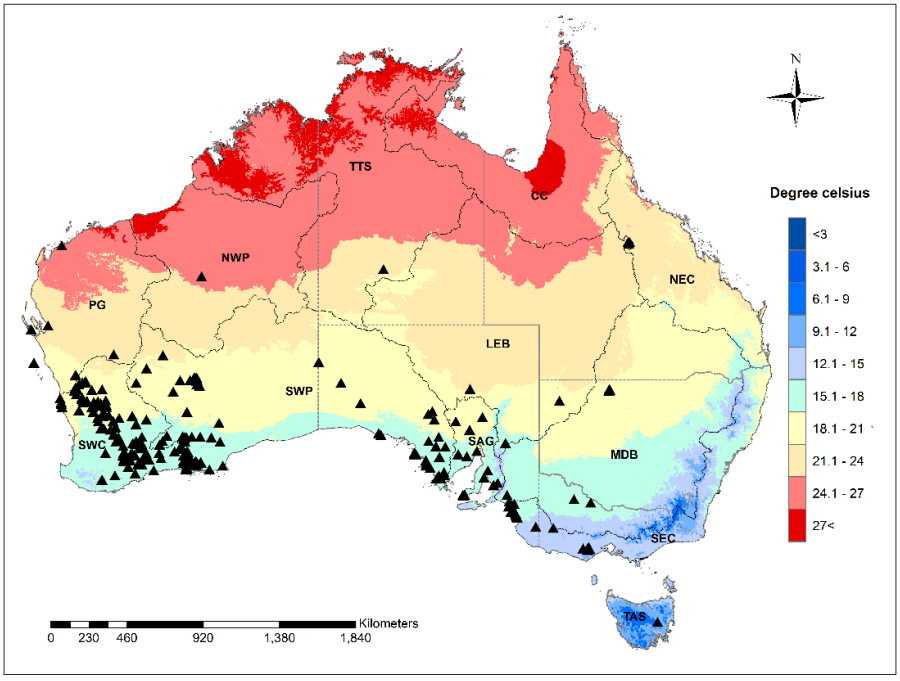

Fig. 2.11: Distribution of *Parartemia* on a map showing average annual temperature (standard 30-year records between 1961-1990 from the Bureau of Meteorology, www.bom.gov.au). To enhance visibility, certain *Parartemia* sites in proximity were omitted. The map also shows the

locations of drainage divisions, denoted by bold abbreviated names, with the corresponding full names provided in Table 2.4.

2.10 Egg Banks and Dispersal

As noted above, both *Artemia* and *Parartemia* produce resting eggs. These eggs accumulate in habitats, forming egg banks in the sediments or along the shoreline from which active populations are re-established when more favourable conditions return (Campagna, 2007; Rogers, 2015). These eggs are essential for buffering populations against local extinction during dry or other unfavourable environmental conditions (De Meester *et al.*, 2002; Rogers, 2014a, 2015; Schwentner & Richter, 2015). Interestingly, in some anostracan and other branchiopod species, it has been shown that only a fraction of eggs hatch during a single wet period (Pinceel *et al.*, 2021; Pinceel *et al.*, 2017; Rogers, 2014a; Schwentner & Richter, 2015). Hatching is delayed in the remaining eggs until one or more subsequent hydroperiods. This staggered hatching is regarded as bet hedging strategy that minimising the chances of a catastrophic loss of individuals in a poor year (Pinceel *et al.*, 2017; Rogers, 2014a, 2015). It means that the active individuals present in a population at one time are from a mix of generations, which may increase population inbreeding and facilitate local adaptation (see Rogers, 2014a, 2015). Such staggered hatching has been observed in *P. veronicae* (Campagna, 2007) and likely occurs in other *Parartemia* species as well.

Both *Artemia* and *Parartemia* rely solely on passive dispersal of resting eggs (Rogers, 2015). Human activities have played an important role in the dispersal of some *Artemia* species, most notably *A. franciscana*. This species is used in saltworks, aquaculture and the aquarium trade and as a result has been deliberately transported by humans across many different regions, including into Australia, possibly from North America (see Introduction). Unintentional human-mediated dispersal through motor vehicles and footwear has also been reported (Waterkeyn *et al.*, 2010). Non-human dispersal vectors are more difficult to pin down, but birds and wind are likely to be important (Amat *et al.*, 2005; Camara, 2001; Green *et al.*, 2023; McMaster *et al.*, 2007; Muñoz *et al.*, 2014; Van Stappen, 1996). Birds are capable of transporting individuals over large distances and may travel directly from one habitat to another, increasing the chances of dispersal (see Green *et al.*, 2023; Rogers, 2015). Eggs of *A. franciscana* and unisexual *Artemia* have been shown to remain viable after passage through alimentary canal of birds such as Dunlin (*Calidris alpina*) and Redshank (*Tringa tetanus*) (Green *et al.*, 2005; Sánchez *et al.*, 2012; Sánchez *et al.*, 2007), and eggs can also attach to

their feathers and legs (Green *et al.*, 2023; Sánchez *et al.*, 2012; Van Stappen, 1996). For some anostracan species, the hatchability of eggs increases if they have passed through the alimentary canal of birds, implying that the eggs are adapted for dispersal via predator ingestion (Rogers, 2014a).

Modes of dispersal in *Parartemia* are not well known. Unlike *Artemia*, deliberate human transportation is unlikely. Also, unlike the floating eggs of most *Artemia* species, so far as is known those of *Parartemia* sink and remain in bottom sediments (Geddes, 1981), which will reduce the chances of wind- and flood-mediated dispersal (Timms *et al.*, 2009). Birds are said to be important dispersal vectors of *Parartemia* (Campagna, 2007; Remigio *et al.*, 2001), although sinking might reduce the chances of eggs being accidentally ingested, or adhering to the external surfaces of birds (McMaster *et al.*, 2007). A range of wading birds feed in Australian salt lakes (Kingsford *et al.*, 2010; Pedler *et al.*, 2018; Timms *et al.*, 2009; Williams *et al.*, 1998) and a few species, such as Banded Stilt, are known to feed on *Parartemia* (Pedler *et al.*, 2018). In fact, *Parartemia* is probably an important component of the diet of many migratory birds that visit these lakes during the northern hemisphere winter. Kangaroos are common in arid and semi-arid areas where salt lakes are common and may also carry eggs in their fur (Timms & Halse, 2020).

Population genetic data are typically used to assess patterns of dispersal and gene flow in anostracans and other crustaceans from lentic environments (Asem *et al.*, 2024c; Finston, 2002; Sainz-Escudero *et al.*, 2023). Some species show a complex relationship between dispersal and gene flow, which is sometimes called the dispersal-gene flow paradox (see De Meester *et al.*, 2002; Rogers, 2014a, 2015; Schwentner & Richter, 2015). The basis of this paradox is that, although some species are widely distributed and capable of rapidly colonising new habitats, i.e., are good dispersers, they show high levels of genetic differentiation even over fine spatial scales, i.e., experience negligible gene flow. The paradox is usually explained via the Monopolization Hypothesis of De Meester *et al.* (2002), where gene flow into occupied habitat patches is limited by the residents' higher fitness and monopolisation of resources. Despite its invasive capacity, high levels of population genetic differentiation have been observed in *A. franciscana* both within its natural range (Frisch *et al.*, 2021) and in areas that it has invaded (Subramani *et al.*, 2021). Some of this differentiation is linked to the hydrochemistry of the environment, implying that adaptation may play a role in limiting gene flow in this species (Frisch *et al.*, 2021).

2.11 Conservation Status of Australian Endemic *Parartemia*

The only *Parartemia* species currently listed on the IUCN is *P. contracta*, which is categorised as 'Vulnerable' based on an assessment in 1996 (IWCSG, 1996). However, this assessment appears to have been based on incomplete data as the species is now recorded from 24 sites, and there is no evidence that it has disappeared from any known habitat. Timms *et al.* (2009) proposed that its status should be changed, releasing it from the IUCN 'Vulnerable' status.

Timms *et al.* (2009) recommended evaluating *P. bicorna* and *P. mouritzi* for potential listing as Priority 1 species by the DBCA in Western Australia. This informal listing is for species that may be threatened species but do not meet the criteria for listing under Western Australia's Biodiversity Conservation Act because of survey or data deficiencies. Priority 1 species are 'poorly-known species' - known from few locations or absent on conservation lands (CCWAFF, 2023). *Parartemia bicorna* is known from three sites and *P. mouritzi* from four sites in Western Australia (see distributional information). Additionally, *P. purpurea* (b) and *Parartemia* sp. 'z' are each known from a single site, while *Parartemia* sp. 'y' is known from two sites, *P. purpurea* (a) from three sites and *P. purpurea* (c) from five sites. All these species warrant evaluation for inclusion in the Priority 1 category. The evaluation should include an investigation into the exact identity of the *P. purpurea* morphotype that has been recorded from another 20 sites but not yet assessed using molecular data (see Fig. 2.8). In South Australia, two species, namely *P. auriciforma* and *P. triquetra*, are each known from only a single site. Timms *et al.* (2009) has suggested that these species are afforded some protection because these sites are in remote locations.

Timms *et al.* (2009) proposed that *P. boomeranga* and *P. extracta* should be considered for 'Vulnerable' status on the IUCN Red List (www.iucnredlist.org). Timms *et al.* suggested that *P. extracta* should be listed because its known distribution range is shrinking. However, the updated distributional information presented in this chapter does not support the view that the distribution of *P. extracta* is shrinking. Timms *et al.* suggested that *P. boomeranga* should be listed because it is either extremely rare or possibly extinct. However, this morphotype was collected from five lakes during this PhD research. Furthermore, molecular data suggest that this morphotype should be synonymised with *P. longicaudata*, which is more common and widespread (Islam *et al.*, 2024).

The implications of the recent spread of *Artemia* in natural salt lakes in Western Australia, especially *A. franciscana*, for *Parartemia* and other native species needs urgent investigation.

To prevent new *Artemia* introductions, Ruebhart *et al.* (2008) recommended actions such as conducting comprehensive risk assessments before introducing *A. franciscana* into saltworks, prohibiting the keeping of pet *Artemia* and implementing strict regulations for using *A. franciscana* in aquaculture. Lake degradation may facilitate the spread of *Artemia* for several reasons. For example, increased salinity, from secondary salinisation or decreasing rainfall, can favour halophiles like *Artemia*. Reduced hydroperiods may also favour *Artemia*, which are effective dispersers with rapid reproductive rates (Van Stappen, 1996). Additionally, increased eutrophication due to excessive fertiliser runoff from agricultural fields may benefit species like *Artemia* that feed on algae in the water column. Also, in some areas, secondary salinisation can convert temporary salt lakes into semi-permanent or permanent ones, which may increase the opportunity for *Artemia* to colonise these lakes (see Timms, 2005). Thus, mitigating the degradation of Australian salt lakes should help to reduce the spread of *Artemia* in these lakes.

Even without *Artemia*, salt lake degradation poses threats to *Parartemia* species (see Introduction). Timms (2005) has outlined management strategies for reversing degradation, which are not reiterated here. Considering the multitude of potential threats to native *Parartemia*, establishing a laboratory egg bank aimed at preserving the genetic diversity within and between *Parartemia* species may be a worthwhile conservation initiative.

2.12 Conclusion

Brine shrimps are so named because they primarily inhabit salt lakes, mainly hypersaline ones. While *Artemia* has low species richness, an almost global distribution and is relatively well studied, *Parartemia* is endemic to Australia, has much higher species richness but is poorly studied. In fact, our understanding of many aspects of the biology of *Parartemia* derives from only *P. zietziana*, leaving many gaps. However, taxonomic and distributional knowledge of *Parartemia* is relatively well established, especially when the results of this PhD research are considered. Western Australia is an important location for brine shrimps in Australia. At least 16 *Parartemia* species are found in this state, with 13 only occurring here and both bisexual and unisexual *Artemia* have invaded natural salt lakes in this state. Further research is needed to evaluate the impact of this invasion on the salt lake ecosystems.

2.13. Supplementary Tables and Figures

Table S2.1: Field data for collections of *Artemia franciscana* and diploid parthenogenetic *Artemia* made during this PhD research. Data include site location, sampling date and temperature, salinity, dissolved oxygen and pH at the time of collection (when available). All locations are in Western Australia.

Location	**Site Name**	**Date of sampling**	**Latitude**	**Longitude**	**Taxa**	**Temp (°C)**	**Salinity (g/L)**	**DO (%)**	**pH**
Gunyidi	Marchagee 11	12/10/2022	-30.115814	116.286304	*Artemia franciscana*	21.39	93.11	-	7.77
Dalwallinu	Marchagee 12	12/10/2022	-30.198687	116.370877		23.22	91.33	-	7.85
Marne	Wongan Hills 11	16/10/2022	-30.506496	116.717221		28.81	193.5	-	7.02
Lake Ninan	Lake Ninan 2	16/10/2022 and 28/01/2023	-30.949596	116.653710		22.64	35.74	-	7.9
Kondut	Kondut 1	28/01/2023	-30.727821	116.820120		29.97	164.76	-	7.73
Anderson Lake	Anderson Lake	1/10/2022	-34.182192	117.965194	Diploid parthenogenetic *Artemia*	-	-	-	-
Wittenoom Hills	Esperance 42	2/10/2022	-33.50895	122.28685		19.36	137.04	67.5	6.87
Ravensthorpe	Newdagate 10	3/10/2022	-33.331509	119.869434		19.44	71.87	88.3	8.06

Badjaling	Quairading Pink Lake	6/11/2022	-31.973552	117.505184		22.49	140.62	-	7.37
Womarden	Three Springs 11	23/11/2022	-29.515824	115.832682		28.15	185.99	-	7.27
Rottnest Island	Lake Baghdad	26/11/2022	-31.995912	115.525028		22.64	118.38	-	8.32
Rottnest Island	Rottnest Pink Lake	26/11/2022	-32.001567	115.511901		22.49	106.4	-	7.97
Lime Lake	Norring Lake	30/04/2023	-33.449242	117.285425		15.66	132.52	-	7.99
Kondinin	Kondinin Lake	30/04/2023	-32.512940	118.220610		19.87	112.98	-	8.44

Table S2.2: Field data for collections of different *Parartemia* species made during this PhD research. Data include site location, sampling date and temperature, salinity, dissolved oxygen and pH at the time of collection (when available). Locations are in Western Australia except a few in South Australia as indicated by 'SA' after the location name.

Location	**Site Name**	**Date of sampling**	**Latitude**	**Longitude**	**Taxa**	**Temp (°C)**	**Salinity (g/L)**	**DO (%)**	**pH**
Grass Patch	Esperance 16	12/09/2020	-33.136987	121.965285	*Parartemia acidiphila*	24.02	98.68	83.6	4.31
Mount Ney	Esperance 24	13/09/2020	-33.438657	122.392712		21.64	104.08	75.5	3.5
Mount Ney	Esperance 32	04/09/2019 and 23/09/2021	-33.508967	122.410974		21.22	71.85	111.3	6.2
Neridup	Esperance 34	23/09/2021	-33.471019	122.382336		21.9	108.06	98	5.05
Neridup	Esperance 35	23/09/2021	-33.467023	122.367464		24.34	95.29	73.8	4.39
Jilakin	Kondinin 5	26/08/2020	-32.581316	118.431073	*Parartemia contracta*	18.92	77.76	71.9	6.27
Jilakin	Jilakin 1	19/08/2020	-32.676675	118.355247		16.39	72.03	103.6	5.65
Hyden	Hyden 4	26/08/2020	-32.355594	119.134036		27.98	103.45	73.9	4.05
Badgerin Rock	Cowcowing 3	05/03/2020	-30.735187	117.337013		31.69	31.32	84.6	5.96
Hyden	Hyden-5	17/08/2021	-32.437035	118.922445		16.89	41.89	93.6	6.94
Neridup	Esperance 8	13/09/2020	-33.4980	122.4012		20.64	84.38	114.8	7.87

Wittenoom Hills	Esperance 20	12/09/2020	-33.393300	122.046633		22.16	90.5	84.5	6.62
Scaddan	Esperance 21	12/09/2020	-33.455407	122.016637		23.07	28.06	145.1	9.76
Holt Rock	L. Varley 3	19/08/2020	-32.708471	119.359619		20	12.57	142.7	8.33
Tenterden	Frankland 1	27/08/2020	-34.416769	117.252365		13.28	16.01	102.8	7.68
Mount Madden	Ravensthorpe 1	16/08/2020	-33.315098	119.814935		15.23	54.37	84.2	8.76
Holt Rock	L. Varley 2 (= Var-1)	19/08/2020	-32.704707	119.358251	*Parartemia cylindrifera*	21.4	48.49	151.5	7.41
Grass Patch	Esperance 17	12/09/2020	-33.252057	121.931928		21.28	39.97	86.8	8.3
Pingrup	Pingrup	14/10/2017	-33.670854	118.564158		29.6	94.15	136.4	8.72
Neridup	Esperance 33	23/09/2021	-33.508491	122.409129		22.17	97.35	75.5	7.73
Neridup	Esperance 30	18/08/2021	-33.543448	122.432428		18.69	17.22	104.6	8.24
Elliston, SA	Elliston	03/08/2022	-33.632156	134.872246		12.47	42.17	105.8	8.4
Sheringa, SA	Lake Tungketta	01/08/2022	-33.762754	135.098527		13.8	50.29	112.3	8.34

Leeman	Green Head 2	30/07/2019 and 04/08/2021	-29.987320	114.986895	*Parartemia extracta*	23.3	35.78	167.2	9.1
Leeman	Green Head 1	31/07/2019	-29.974886	114.980817		22.01	25.41	136.1	8.73
Dowerin	Dowerin-1	04/08/2021	-31.253627	117.060872		19.66	26.89	140.7	8.24
Lake Ninan	Lake Ninan-1	04/08/2021	-30.953402	116.654574		18.28	25.33	146.3	7.93
Jurien Bay	Jurien Bay 4	15/09/2021	-30.206705	115.038112		14.52	18.6	88.2	8.91
Booralaming	Cow-2	05/03/2020	-30.922094	117.363814		30.17	35.89	107	8.75
North Tammin	Wyola-2	31/10/2021	-31.626042	117.358562		22.81	85.3	107.9	7.98
North Tammin	Wyola-3	31/10/2021	-31.626133	117.360922		23.47	68.5	98.6	7.63
Dudawa	Morawa 2	08/08/2019	-29.405722	115.888194	*Parartemia informis*	13.79	21.02	101.4	7.94
Booralaming	Cow-2	05/03/2020	-30.922094	117.363814		30.17	35.89	107	8.75
Merkanooka	Morawa 3	08/08/2019 and 15/09/21	-29.299638	115.913111		15.91	42.52	108.5	8.29
Womarden	Morawa 1	08/08/2019	-29.448866	115.879115		13.55	46.13	113.2	7.9
Morawa	Morawa 4	08/08/2019	-29.184522	116.086731		17.42	104.16	100.4	6.51

Marne	Wongan Hills 2, Lake 6	25/08/2018 and Sept. 2017	-30.510341	116.709957		-	-	-	-
Booralaming	Cowcowing 6	04/08/2021	-30.934874	117.388860		-	-	-	-
Yallabatharra	Hut-1	04/09/2020	-28.207383	114.287526		26.93	49.63	101.9	8.54
Kalannie	Maxine's Pond	22/08/2020	-30.367059	117.190395		17.22	23.46	119.3	6.91
Mongers Lake	Monger's Lake-2	15/08/2021	-29.542408	116.709323		16.51	106.99	112.7	8.49
Morawa	Koolanooka	15/08/2021	-29.26874	116.032221		20.41	49.46	108.7	8.4
Kalannie	Kalannie 2	15/08/2021	-30.281587	117.072370		18.16	42.49	91.2	7.8
Morawa	Morawa Bridge	15/08/2021	-29.246981	116.011079		18.79	77.45	93.2	8.28
Womarden	Three Springs 7	15/09/2021	-29.577527	115.821453		24.9	90.64	81.2	8.16
Womarden	Three Springs 2	15/09/2021	-29.575023	115.822197		28.03	26	89.3	9.16
Rothsay	Monger's Lake-3	15/09/2021	-29.543749	116.699672		27.94	53.07	71.9	9.59
Latham	Latham 1	15/09/2021	-29.736231	116.354658		23.77	49.54	79.7	8.77
Latham	Latham 3	15/09/2021	-29.736814	116.359168		20.44	34.76	71.9	9.52
Latham	Latham 4	15/09/2021	-29.737895	116.358878		16.96	70.81	124.2	7.71
Lyndon	Coral Bay-1	13/04/2022	-23.127919	113.786264		-	-	-	-

Womarden	Three Springs-11	23/11/2022	-29.515824	115.832682	*Parartemia laticaudata*	28.15	185.99	-	7.27
Camel Lake	Stirling 1	20/08/2020 and 05/08/2021	-34.306644	118.027642	*Parartemia longicaudata*	16.32	8.49	94.8	8.37
Pingrup	Cairolcup-1	19/08/2020	-33.707084	118.687531		11.07	111.01	96.2	6.35
Lake King	Lake King-2	16/08/2020	-33.090770	119.540744		16.25	73.1	89.2	8.53
Hyden	Hyden 6	17/08/2021	-32.454396	119.091053		17.41	55.13	84.5	7.57
Pink Lake	Pink Lake-1	18/08/2021	-33.838279	121.833288		13.09	89.1	83.8	8.56
Scaddan	Esperance 28	18/08/2021	-33.514871	121.869683		9.4	162.27	60.9	7.18
North Island	Abrolhos-1	24/08/2021	-28.297237	113.595343		-	-	-	-
Mount Madden	Lake King 3/Rav-5	17/08/2021	-33.313708	119.811999		19.76	42.83	105.3	7.67
Carnamah	Three Springs 5	15/09/2021	-29.783126	115.871530		21.35	120.02	92.2	8.11
Pingrup	Leke Magenta 1	22/09/2021	-33.577858	119.229112		21.64	79.24	97.4	8.14
Pingrup	Lake Magenta 2	22/09/2021	-33.576338	119.228724		19.29	83.56	95.3	8.01
Pingrup	Lake Magenta 3	22/09/2021	-33.575465	119.206876		21.97	34.55	127.9	9.06

Pingrup	Lake Magenta 4	22/09/2021	-33.585244	119.199468		16.67	41.21	82.6	8.28
South Newdegate	Lake Magenta 7	24/09/2021	-33.196573	119.075532		15.03	79.8	97.5	8.06
South Lake Grace	Lake Grace 3	24/09/2021	-33.303721	118.477823		19.63	28.01	116.9	5.28
North Lake Grace	Lake Grace 2	24/09/2021	-32.955887	118.505980		17.14	32.46	79.9	8.03
Lake Grace	Lake Grace 1	24/09/2021 and 21/09/2022	-33.107453	118.377491		25.35	147.34	114.2	7.79
Kurrenkutten	Bendering Rd-1	31/10/2021 and 21/09/2022	-32.380481	118.157547		25.53	59.46	96.8	7.79
Marne	Near WH-2	11/08/2020	-30.511147	116.711458		-	-	-	-
Lake Moore	Lake Moore	15/08/2021	-30.333737	117.492973		23.68	100.08	79.1	8.09
Gunyidi	Marchagee 3	16/09/2021	-30.119139	116.222031		16.12	112.54	62.2	7.99
Gunyidi	Marchagee 4	16/09/2021	-30.119420	116.213778		19.19	85.16	83	8.19

Gunyidi	Marchagee 5	16/09/2021 and 12/10/2022	-30.117236	116.201455		19.43	68.46	79.7	8.34
Hyden	Hyden 9	17/08/2021	-32.462249	119.174969	*Parartemia mouritzi*	16.27	26.25	93.1	4.13
Neridup	Esperance 7	15/08/2020	-33.539844	122.430503	*Parartemia purpurea* (a)	19.19	92.9	87.6	7.8
Wittenoom Hills	Esperance 29	18/08/2021	-33.446943	122.197356		16.21	120.51	93.1	8.04
Neridup	Esperance 31	23/09/2021	-33.531295	122.426558		19.14	149	118	8.35
Beaumont	Esperance 9	13/09/2020	-33.455955	122.608653	*Parartemia purpurea* (b)	28.31	100.24	106.4	7.91
Grass Patch	Esperance 1	12/09/2020	-33.318431	121.927802	*Parartemia purpurea* (c)	25.57	61.36	101.5	7.88
Gibson	Esperance 4	14/08/2020	-33.516317	121.876323		14.17	56.42	83	8.85
Scaddan	Esperance 19	12/09/2020	-33.390527	122.044960		23.71	120.83	152.8	8.18
Scaddan	Esperance 3	12/09/2020	-33.481497	121.696884		19.69	59.23	108.2	8.18
Esperance	Esperance 36	14/08/2020	-33.482778	122.010556		28.5	137	68	7.91
Varley	Lake Varley 4	19/08/2020	-32.765616	119.398004		20.86	102.2	98.7	5.98

Ardath	Corrijin-1	Sept. 2018	-32.08701	118.144322		-	-	-	-
Bruce Rock	Yerding-1	Sept. 2018	-31.92715	117.979964		-	-	-	-
Hyden	Hyden 3	26/08/2020	-32.415574	119.085077		23.02	29.64	96.1	5.99
Salmon Gums	Esperance 14	14/08/2020	-33.081987	121.685399		21.06	84.38	102.5	8.49
Varley	Lake Varley 5	19/08/2020	-32.810349	119.424893		22.23	105.1	96.9	4.62
Hyden	Hyden 7	17/08/2021	-32.462961	119.160903		17.1	31.03	105.7	4.9
South Doodlakine	Baandee Lake	05/09/2021	-31.600911	117.946518	*Parartemia serventyi*	19.76	121.28	118.6	5.25
Hines Hill	Pontifex Rd-1	05/09/2021	-31.582883	117.966390		23.36	71.96	95.5	4.78
Hines Hill	Pontifex Rd-2	05/09/2021	-31.587377	117.967899		24.85	63	95	4.42
Hines Hill	Hines Hill-1	05/09/2021	-31.517009	118.062735		23.84	73.02	92.7	4.53
Magenta	Lake Magenta 5	24/09/2021	-33.442913	119.266142		12.54	81.58		4.33
Mount Caroline	Mount Stirling	31/10/2021	-31.823409	117.592579		28.27	97.61	89.1	7.14
Hyden	Hyden-2	26/08/2020	-32.415375	119.0866		-	-	-	-
Lake Hamilton, SA	Lake Hamilton	01/08/2022	-34.022875	135.281496	*Parartemia zietziana*	15.97	120.43	106.6	8.11

Beaumont	Esperance 25	13/09/2020	-33.486080	122.636330		24.54	46.81	83.5	8.21
Wittenoom Hills	Esperance 22	13/09/2020 and 18/08/2021	-33.473564	122.355036	*Parartemia* sp. ‘y’	20.05	65.27	105.7	8.2
Wittenoom Hills	Esperance 23	13/09/2020 and 18/08/2021	-33.473025	122.353382	*Parartemia* sp. ‘z’	24.67	120.91	105.9	7.99

Chapter 3

Chapter 3. A molecular assessment of species boundaries and relationships in the Australian brine shrimp *Parartemia* (Anostraca: Parartemiidae)

The following chapter was drafted in accordance with the guidelines of the journal ***Invertebrate Systematics*** and has now been published (https://doi.org/10.1071/IS24044).

The following authors contributed to this manuscript as outlined below.

Authorship order	Contribution (%)	Concept development	Data collection	Data analysis	Drafting manuscript	Revision of manuscript
Md Aminul Islam	79	X	X	X	X	X
Jennifer Chaplin	12	X	X			X
Angus Lawrie	3		X			X
Mahabubur Rahman	3					X
Adrian Pinder	3					X

Contribution indicates the total involvement each author has had in this project. Placing an 'X' in the remaining boxes indicates which aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

Candidate **Principal Supervisor**

Chapter Linking Statement

The review (Chapter 2) provided some important insights into the taxonomy of *Parartemia*, drawing from the content presented in this first data chapter. This chapter (Chapter 3) focuses on using molecular data to explore species relationships within the genus *Parartemia* and to check the validity of morphologically described species. A confirmed list of *Parartemia* species is essential for developing targeted conservation strategies and advancing further research.

3.0 Abstract

Australian salt lakes contain a diverse range of endemic invertebrates. The brine shrimp *Parartemia* is amongst the most speciose and salt-tolerant of these invertebrates. The morphotaxonomy of *Parartemia* is well established but there has only been limited molecular assessment of the phylogenetic relationships and boundaries of the morphospecies. We used multiple genetic markers (nuclear *28S* and mitochondrial *16S* and *COI*) and tree-building methods (Bayesian inference and maximum likelihood) to investigate the phylogeny of *Parartemia*. We also used species delimitation methods to test the validity of morphological species designations. The data set included all but two of the 18 described *Parartemia* morphospecies, collected from a total of 93 sites from across southern Australia plus some sequences from GenBank. The results identified large amounts of molecular divergence (e.g., *COI p*-values of up to 25.23 %), some groups of closely related species (which also usually shared some morphological similarities) and some distinctive species, although the relationships among divergent lineages were generally not well resolved. The most conservative set of results from the species delimitation analyses suggests that the morphotaxonomy is largely accurate, although many morphospecies comprised divergent genetic lineages separated by *COI p*-values of up to 17.02 %. Two putative new morphospecies, three cryptic species and one synonymy were identified. Our findings improve the knowledge of *Parartemia* taxonomy and will facilitate the development of future studies and conservation of this taxon.

3.1 Introduction

Most biological disciplines depend on adequate taxonomy, particularly accurate species determination (Bortolus, 2008; Jackson *et al.*, 2022), which ideally should be supported by multiple lines of evidence (Padial *et al.*, 2010). Nevertheless, only approximately 1.2 to 1.9 million of an estimated 5 to 15 million species have been described (Costello *et al.*, 2013; Jackson *et al.*, 2022; Mora *et al.*, 2011; Stork, 1997). Furthermore, taxonomic effort has not been evenly spread across taxa or ecosystems (Di Marco *et al.*, 2017; Donaldson *et al.*, 2016; Troudet *et al.*, 2017). The biodiversity of inland saline waters is one area that requires better documentation (Lawrie *et al.*, 2021; Saccò *et al.*, 2021).

Salt lakes are exceedingly common in Australia, where they are usually ephemeral and show variable and often very high salinities (Bayly & Williams, 1966; De Deckker, 1983; Lawrie *et al.*, 2021). Some invertebrate taxa have undergone substantial diversification in these environments or their precursors (De Deckker, 1983; Lawrie *et al.*, 2023; Lawrie *et al.*, 2021; Rahman *et al.*, 2023; Remigio *et al.*, 2001). The endemic brine shrimp *Parartemia*, the only genus in the Parartemiidae (Weekers *et al.*, 2002), is probably the best example of this. This taxon and its closest known relative *Artemia* are often regarded as extremophiles due to their occurrence in hypersaline lakes (Timms, 2014). Divergence between these two lineages is ancient, dating back to around the breakup of Gondwana (Anufriieva & Shadrin, 2013; Coleman *et al.*, 1998). Native *Artemia* species occur on all continents except Australia and Antarctica (Muñoz & Pacios, 2010; Ruebhart *et al.*, 2008), however, *Parartemia* is endemic to Australia. Although much more widespread, *Artemia* with nine sexually reproducing species plus a range of parthenogens (Asem *et al.*, 2024a; Asem *et al.*, 2023; Rogers, 2013) appears to have far fewer species than *Parartemia*, which has 18 described morphospecies (Timms, 2014).

The first described *Parartemia* species was *P. zietziana* by Sayce (1903), which occurs in South Australia, Victoria and Tasmania (Timms *et al.*, 2009). Prior to this, all *Parartemia* specimens were probably misidentified as *Artemia* (Timms, 2014). *Parartemia zietziana* was considered to be the sole representative of the genus until Linder (1941) described six species from Western Australia: *P. contracta*, *P. cylindrifera*, *P. extracta*, *P. informis*, *P. longicaudata* and *P. serventyi*. Another species, *P. minuta*, was described by Geddes (1973). More recently, Timms and Hudson (2009) added four morphospecies (*P. acidiphila*, *P. auriciforma*, *P. triquetra* and *P. yarleensis*) from South Australia and Timms (2010) added another six (*P. bicorna*, *P. boomeranga*, *P. laticaudata*, *P. mouritzi*, *P. purpurea* and *P. veronicae*) from

Western Australia, giving a total of 18 described morphospecies. A detailed identification guide for *Parartemia* morphospecies has been provided by Timms (2012b). Three male morphological characters – the ventral process, medial process, and the space between the second antenna basal antennomeres – are important in species diagnoses (Timms, 2012b). Although *Parartemia* morphotaxonomy is well established, it has been subject to only limited molecular testing (see below). More intensive sampling is also likely to reveal more species (Timms, 2010).

Remigio *et al.* (2001) conducted the only previous study on the molecular phylogeny of *Parartemia*. The results were mainly based on 341 bp of the mitochondrial *16S* rRNA gene and a small number of specimens per species (mostly just one) from eight known species and two putative new species (corresponding to their samples POP4 and POP5). The results suggested that *Parartemia* morphospecies are genetically distinct, with high levels of genetic divergence between species. The study identified four main clades (labelled A to D), two of which contained the haplotypes of multiple species whereas the other two contained haplotypes of single distinctive species. Their molecular data supported some previous hypotheses of species relationships based on limited morphological traits but not others. *Parartemia minuta* was the most genetically distinctive species in their dataset. A comprehensive analysis, with a more complete set of species and additional genetic loci, is required to fully understand *Parartemia* systematics.

Salt lake environments in Australia (and elsewhere) are deteriorating due to the effects of global climate change (e.g., mainly increasing aridity) and other anthropogenic effects (Jellison *et al.*, 2008; Timms, 2005; Williams, 2002). This has led to general concerns about the fate of the invertebrate inhabitants of these lakes (Lawrie *et al.*, 2021; Pinder *et al.*, 2009; Timms, 2005; Timms *et al.*, 2009). Some species of *Parartemia* have been singled out as vulnerable to extinction, although in some cases this appears to be based on incomplete data. For example, *P. contracta* currently has 'Vulnerable' status on the IUCN Red List based on a 1996 assessment, but since that assessment more populations have been discovered and the listing may be unnecessary (Timms *et al.*, 2009). On the other hand, Timms *et al.* (2009) suggested that *P. boomeranga* (formerly *Parartemia* sp. 'c') and *P. extracta* should be assessed for inclusion in the IUCN Red List as 'Vulnerable' because distributional records indicate that the ranges of these species are contracting. In a separate study, Timms (2012b) suggested that *P. boomeranga* was extremely rare or possibly even extinct. To manage their conservation, a

thorough understanding of the phylogeny, taxonomy and distributions of *Parartemia* species is needed (see Rogers & Aguilar, 2020).

We used multiple molecular markers to investigate the phylogenetic relationships among almost all described *Parartemia* morphospecies, typically with multiple representatives from multiple populations of each morphospecies. We also used single mitochondrial DNA loci (*16S* and *COI*) and species delimitation methods to assess the validity of 16 described *Parartemia* morphospecies that were included in this study as well as of two putative new morphospecies found in our collections. Finally, we compared our results to those of Remigio *et al.* (2001) to test the validity of their findings regarding the phylogeny of *Parartemia*, which were based on a more limited data set.

3.2 Materials and Methods

3.2.1 Specimen collection and preservation

We collected *Parartemia* specimens from 84 salt lakes in Australia (mainly Western Australia) between September 2017 and August 2022 (Fig. 3.1 and supplementary Table S3.1). Specimens were collected using a dip net and then euthanised by freezing and preserved in 100% ethanol. Specimens from a site in Tasmania (provided by the Tasmanian Museum and Art Gallery) and eight sites in Western Australia (from the Department of Biodiversity, Conservation and Attractions, the Western Australian Museum and the Stantec Australia Pty Ltd) were also used (details in Table S3.1).

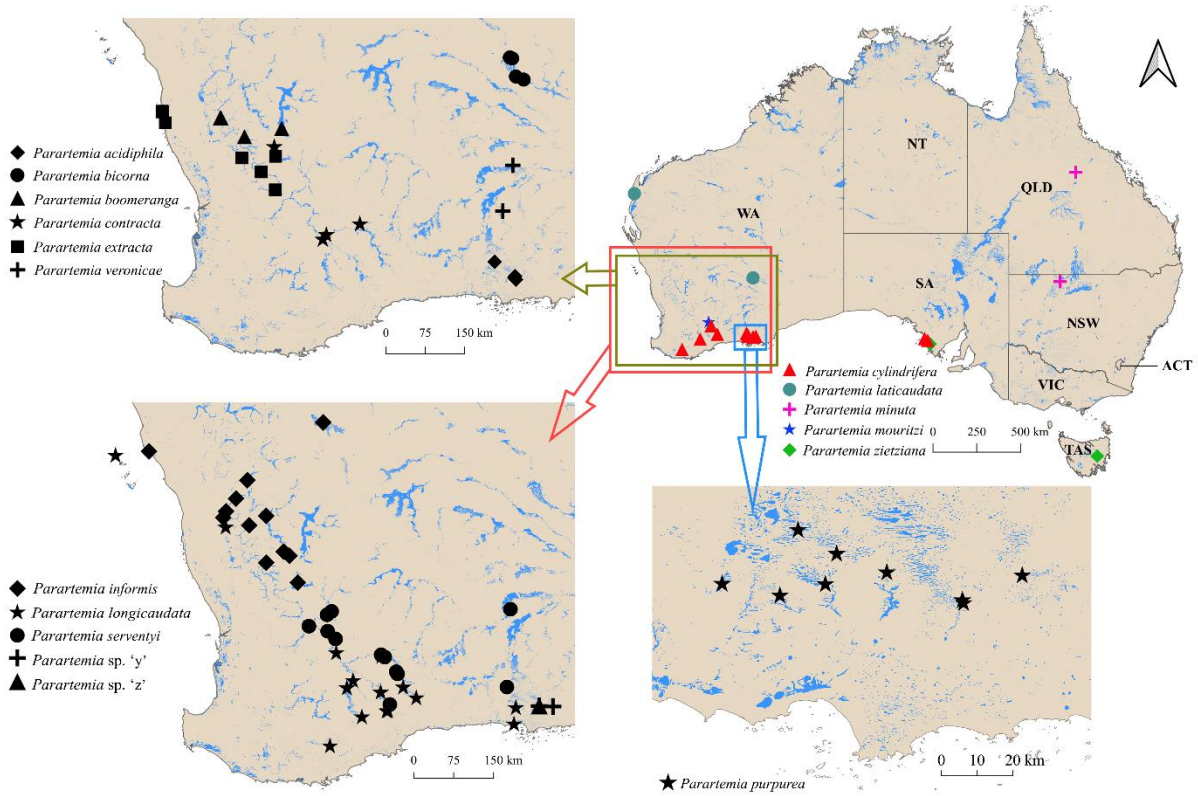

Fig. 3.1: Approximate locations of *Parartemia* collection sites. Sites in close proximity may not always be distinguishable in the figure (see detailed information in supplementary Table S3.1). The hydrological information, shown in blue, is sourced from the national surface water database of Geoscience Australia (www.ga.gov.au). The full Australian map includes the state/territory boundaries, WA: Western Australia; SA: South Australia; NT: Northern Territory; QLD: Queensland; NSW: New South Wales; ACT: Australian Capital Territory; VIC: Victoria; and TAS: Tasmania. No specimens of P. minuta were collected in this study but

GenBank sequences for this species from Lake Buchanan in QLD and an unspecified site in the Paroo area in NSW were included (details in Table S3.1).

3.2.2 Identification of morphospecies

All *Parartemia* specimens were identified to morphospecies using the morphological criteria of Timms (2012b). All known morphospecies were represented in the samples except for *P. auriciforma*, *P. yarleensis* and *P. minuta*. *Parartemia auriciforma* is only known from one site in central Australia, *P. yarleensis* is only known from inland South Australia and *P. minuta* occurs in inland areas in South Australia, Victoria, New South Wales and Queensland (Timms *et al.*, 2009). Our attempts to obtain either fresh or ethanol-preserved specimens of these three species were unsuccessful. However, for *P. minuta*, we included *28S* and *16S* sequences from GenBank (see below) and morphological data from Timms (2012b). We collected two groups of specimens whose morphology did not match those of any described species (see results) - herein these groups are referred to as *Parartemia* sp. 'y' and *Parartemia* sp. 'z'.

3.2.3 DNA extractions, PCR amplification and sequencing

Genomic DNA was extracted from thoracic segments V through VII or thoracopods III through VII using a Masterpure™ Complete DNA and RNA Purification Kit (Epicentre®) following the manufacturer's instructions. Taxonomically significant characters (head, genital segments and abdomen) were left intact for later cross-checking with molecular findings if required. Negative controls, i.e., assays with reagents but no added tissue/DNA, were included in every extraction to check for contamination.

One nuclear genetic region (*28S*) and two mitochondrial genetic regions (*16S* and *COI*) were used. Details of the PCR primers and amplicon lengths are given in Table 3.1. Except for the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), all primers were designed specifically for *Parartemia* because it was not possible to amplify the target region from all species and/or all populations of a species with preexisting primers (see Tables 3.1, S3.2 and S3.3).

Table 3.1: Primers used for amplifying *28S* (~ 873 bp), *16S* (~ 494 bp) and *COI* (658 bp) genetic regions from *Parartemia* species in this study. All primers were designed in this study, except for the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Details of primers used for each species are in Tables S3.2 and S3.3.

Name	Forward Primers	Name	Reverse Primers
28S			
28S11	ACAAGTACCGCGAGGGAAAGT	28S32	CGCCAGTTCTGCTTACCAAAA
28S71	TGGTAAACTCCATCTAAGGCTAA		
16S			
16SarPara	CGCCTGTTTAACAAAAACATAGC	16SbrPara	TGAACTCAGATCACGTAGGG
COI			
LCO1490	GGTCAACAAATCATAAAGATATTGG	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA
LCOPara	CAATCACAAAGATATTGGAACCC	HCOPara	ACTTCAGGGTGACCAAAAAATCAG
COI101	GCACCTATTATCGGCCACTTT	COI102	TGGTGGGCTCAGACAACAAA
Facid-1	TCTACGAACCATAGGGACATTG	Rboom-2	TTCTGGGTGACCAAAAAACCAG
Fboom-2	ACTCTACAAACCATAAGGACATTG	Rinfo-1	CCTCTGGATGGCCGAAAAATC
Fext-2	ATTCTACGAATCACAAGGATATTGG	Rlong-1	CTTCTGGGTGACCAAAAAACCA
Finfo-1	TATGCAACGCTGACTATATTCTAC		
Finfo-3	TATGCAACGCTGGCTGTACTC		
Flong-1	ACTCTACAAATCATAAGGACATCG		
Flong-2	ACTCTACAAATCATAAGGACATTGG		
Fser-1	ACTCTACAAACCATAAGGACATCG		

PCR reaction volumes were 25 µL containing 5 µL GoTaq® Reaction Buffer (Promega), 0.5 µL dNTPs (10 mM per nucleotide), 0.25 µL each of forward and reverse primers (10 µM), 2 µL $MgCl_2$ (25 mM), 0.35 µL bovine serum albumin (10 µg/µL), 0.125 µL GoTaq® G2 Hot Start Taq Polymerase, 1 µL DNA and adjusted to the final volume using PCR grade water. PCR reactions for all three markers were (i) 95 °C initial denaturation temperature for 5 min; (ii) 40 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 45 sec; and (iii) a final extension at 72 °C for 7 min. PCR products were purified using Exo-SAP purification (Dugan *et al.*, 2002) and sequenced in the forward and reverse directions in an automatic ABI 3700 sequencer (Applied Biosystems®) by Macrogen Inc. (South Korea).

3.2.4 Sequence data

The sequencing chromatograms were visualised in Chromas v2.6.5 (Technelysium Pty Ltd., Australia). Forward and reverse sequences were compared, and any ambiguities were corrected. The consensus *16S* sequences were aligned using MAFFT online version (see http://mafft.cbrc.jp/alignment/server/) with the G-INS-i strategy, while the consensus *COI* sequences were aligned using MUSCLE (ver. 5, see http://www.drive5.com/muscle/; Edgar, 2004) in MEGA X (ver. 10.2.6, see https://www.megasoftware.net; Kumar *et al.*, 2018). The *28S* sequences were aligned as described for *16S* except that we used the Q-INS-i strategy and made manual adjustments by eye in regions containing indels. We found no evidence that nuclear copies of *COI* had inadvertently been included in our *Parartemia* dataset as, for example, there were few amino acid substitutions, and no indels or stop codons, in the translated sequences (see Raupach & Radulovici, 2015). Haplotypes in the *28S*, *16S* and *COI* datasets were identified using DnaSP (ver. 6.12.03, see http://www.ub.edu/dnasp/; Rozas *et al.*, 2017). All new haplotypes have been deposited in GenBank with accession numbers listed in Table S3.1. This study also used GenBank sequences for *Parartemia* (including one *28S* and two *16S* haplotypes of *P. minuta*) and a range of outgroup taxa (see Tables S3.1 and S3.4). A multigene (*28S*, *16S* and *COI*) concatenated dataset was assembled in MEGA X (Kumar *et al.*, 2018), using individuals for which data from at least two of the three targeted genetic regions were available.

3.2.5 Phylogenetic analysis

Phylogenetic analyses were conducted using Bayesian inference (BI) and maximum likelihood (ML) frameworks on the concatenated dataset and on single locus *28S*, *16S* and *COI* datasets.

The former is widely recognised as the most reliable approach for phylogenetic analysis (Wiens & Moen, 2008). The concatenated dataset was used to investigate species relationships within *Parartemia*. The *16S* and *COI* markers were also used to investigate the validity of *Parartemia* morphospecies, as they are effective in identifying crustacean species (Costa *et al.*, 2007; Raupach & Radulovici, 2015; Remigio *et al.*, 2001).

The best nucleotide substitution models (GTR+I+G for *28S* and TrN+G+I for *16S* and *COI*) were selected using jModelTest (ver. 2.1.9, see https://github.com/ddarriba/jmodeltest2; Darriba *et al.*, 2012) based on the Bayesian information criterion (BIC). Maximum likelihood molecular clock tests conducted in MEGA X (Kumar *et al.*, 2018) revealed that the partitioned concatenated dataset and the single locus datasets did not adhere to the assumptions of a strict clock. BI analysis for each dataset was separately conducted in BEAST v1.10.4 (Suchard *et al.*, 2018), employing the identified substitution models (see above) with the uncorrelated relaxed clock and coalescent constant population size as tree priors. The analysis was run for 50 million generations, and the estimated sample size (ESS) was checked by looking at the log-output file in Tracer (ver. 1.7.2, see https://github.com/beast-dev/tracer/releases/tag/v1.7.2; Rambaut *et al.*, 2018). A burn-in of 25% of the initial trees was discarded, and the final tree was produced in TreeAnnotator (a BEAST-distributed program) and visualised in FigTree (ver. 1.4.4, A. Rambaut, see http://tree.bio.ed.ac.uk/software/figtree/). The analysis was also performed for each dataset using the uncorrelated relaxed clock and Yule process as tree priors, but the ESS was low (< 200), even after increasing the MCMC chain length to 100 million generations, and thus the results were discarded.

The ML phylogenetic analysis was performed separately for the concatenated, *28S*, *16S* and *COI* datasets on the IQ-TREE web server (see http://iqtree.cibiv.univie.ac.at; Trifinopoulos *et al.*, 2016) using 5000 ultrafast bootstrap replicates and the above-mentioned substitution models.

3.2.6 Validity of *Parartemia* morphospecies and pairwise distances

Three species delimitation analyses were employed to assess the validity of *Parartemia* morphospecies using the *16S* and *COI* datasets (without outgroups). Firstly, the Assemble Species by Automatic Partitioning (ASAP), which relies on pairwise genetic distances (Puillandre *et al.*, 2021), was conducted on the ASAP website (see https://bioinfo.mnhn.fr/abi/public/asap) using the Kimura (K80) substitution model (Kimura, 1980) and default settings. Of the ten best ASAP partition schemes included in the results, the

one with the lowest ASAP score and another one with the smallest number of partitions were chosen. Secondly, the maximum likelihood implementation of the Multi-rate Poisson tree processes (mPTP) (Kapli *et al.*, 2017) was performed on the mPTP webserver (see https://mptp.h-its.org) with default settings using a BI phylogenetic tree generated by BEAST (Suchard *et al.*, 2018) based on the same parameters as outlined in the "Phylogenetic analysis" section (see above). Lastly, the General Mixed Yule Coalescent (GMYC) analysis (Fujisawa & Barraclough, 2013) was performed in R (ver. 4.3.3, R Foundation for Statistical Computing, Vienna, Austria, see https://www.r-project.org/) using the same BI phylogenetic tree as used for mPTP (see Michonneau, 2016 for further details).

MEGA X (Kumar *et al.*, 2018) was used to compute uncorrected *p*-distances and Kimura two-parameter (K2P) (Kimura, 1980) distances between *Parartemia* haplotypes in the *28S*, *16S* and *COI* datasets.

3.3 Results

3.3.1 General information

After aligning and trimming, a total of 28 *28S* (674 bp excluding gaps), 100 *16S* (474 bp excluding gaps) and 161 *COI* (658 bp) haplotypes of *Parartemia* were identified in sequences from 50, 113 and 232 individuals, respectively.

Substantial genetic divergence was present in the *16S* (e.g., *p*-distance of up to 20.34 %) and especially the *COI* (e.g., *p*-distance of up to 25.23 %) genetic regions in *Parartemia*. As expected, divergence in the *28S* region was more limited (e.g., *p*-distance of up to 2.8 %), with few differences between most species (Table S3.5). Accordingly, the relationships among *Parartemia* species were poorly resolved in *28S* phylogeny (Fig. S3.1), although the *28S* data did provide strong support for the monophyly of *Parartemia* (see below).

3.3.2 Monophyly of *Parartemia* and species relationships

All *Parartemia* haplotypes formed a well-supported monophyletic group in both BI and ML concatenated phylogenetic trees (BPP = 1 and 97% bootstrap value; Fig. 3.2 and Fig. S3.2), as well as in the BI and ML *28S* (Fig. S3.1) and *16S* trees (see Fig. 3.3 and Fig. S3.3) and the ML *COI* tree (see Fig. S3.4). In the BI *COI* tree, all *Parartemia* haplotypes formed a monophyletic group, but the node support was low (see Fig. 3.4).

Although some aspects of the relationships among *Parartemia* species were not well resolved, particularly for deeper divergences, some species groups and divergent species were evident. The haplotypes of five species (*P. bicorna*, *P. contracta*, *P. informis*, *P. laticaudata* and *P. veronicae*) were invariably grouped together in a single usually well-supported clade in the BI and ML concatenated, *16S* and *COI* trees (species group A in Fig. 3.2-3.4). A sixth species, *P. triquetra*, for which there were no *28S* or *COI* data, was also included in this group in the *16S* trees (Fig. 3.3 and Fig. S3.3). Similarly, *Parartemia* sp. 'z' and the *P. boomeranga* and *P. longicaudata* morphotypes always formed a single group (species group B in Fig. 3.2-3.4). *Parartemia cylindrifera* and *Parartemia* sp. 'y' also consistently grouped together, forming a well-supported clade in the BI trees; this clade was present but less strongly supported in the ML trees (species group C in Fig. 3.2-3.4). *Parartemia zietziana* and the different lines of *P. purpurea* (see below) also usually grouped together but the clade was not always well supported (species group D in Fig. 3.2-3.4). The concatenated and *16S* BI trees also indicated that, although *P. minuta* and *P. mouritzi* showed considerable divergence from each other, they

formed a base group in the phylogeny (species group E in Fig. 3.2 and Fig. 3.3; no *COI* data are available for the former species). Three species (*P. serventyi*, *P. extracta* and *P. acidiphila*) were each distinctive (see Fig. 3.2-3.4).

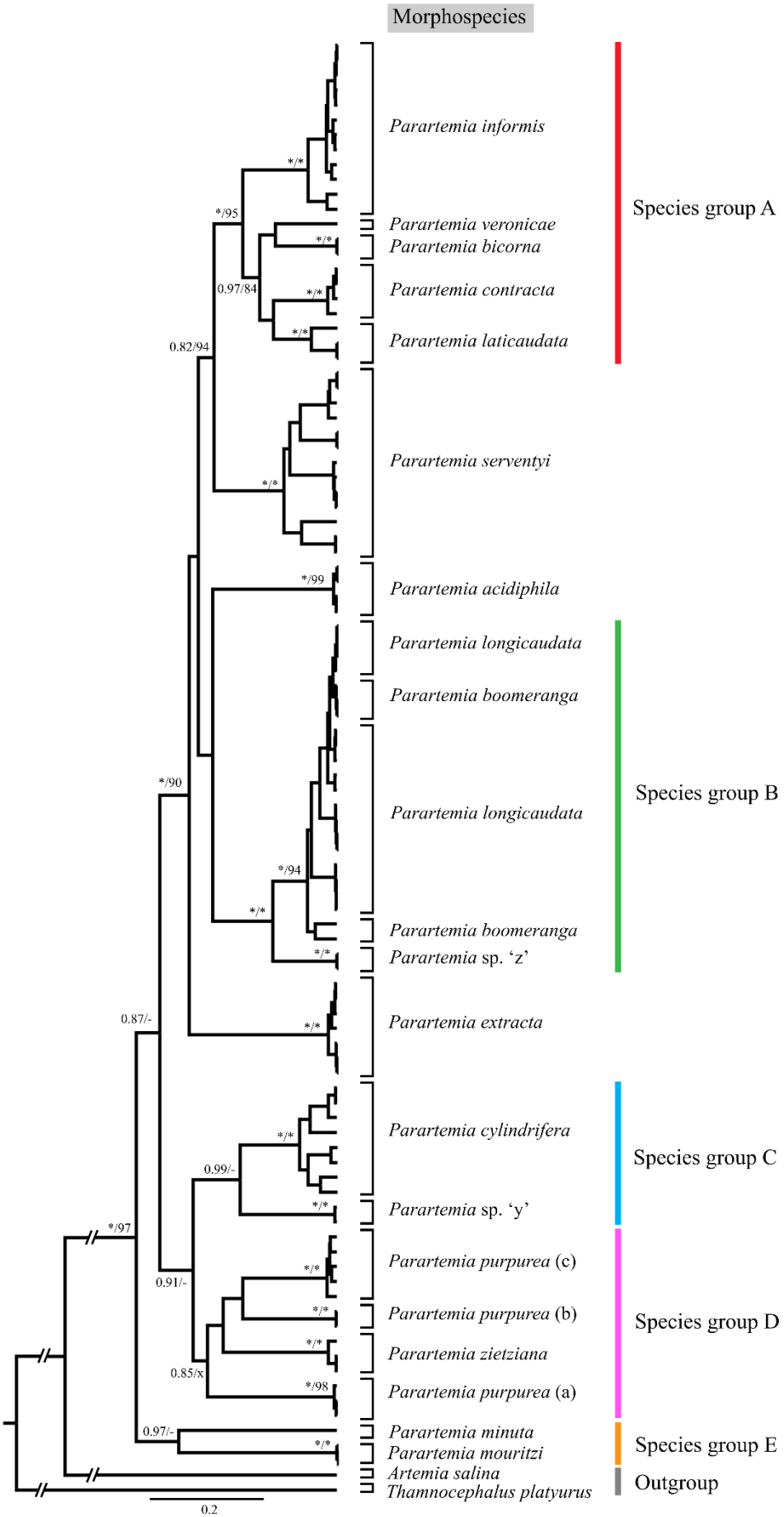
Morphospecies
Pararartemia informis
Pararartemia veronicae
Pararartemia bicorna
Pararartemia contracta
Pararartemia laticaudata
Pararartemia serventyi
Pararartemia acidiphila
Pararartemia longicaudata
Pararartemia boomeranga
Pararartemia longicaudata
Pararartemia boomeranga
Pararartemia sp. 'z'
Pararartemia extracta
Pararartemia cylindrifera
Pararartemia sp. 'y'
Pararartemia purpurea (c)
Pararartemia purpurea (b)
Pararartemia zietziana
Pararartemia purpurea (a)
Pararartemia minuta
Pararartemia mouritzi
Artemia salina
Thamnocephalus platyurus
Species group A
Species group B
Species group C
Species group D
Species group E
Outgroup
/
*/95
0.97/84
0.82/94
*/99
*/90
*/94
0.87/-
0.99/-
*/97
0.91/-
0.85/x
*/98
0.97/-
0.2

Fig. 3.2: Bayesian inference (BI) phylogenetic tree for the *Parartemia* concatenated dataset (*COI*, *16S* and *28S*). Maximum likelihood (ML) phylogenetic tree is available in supplementary Fig. S3.2. Bayesian Posterior Probability (BPP, when ≥ 0.80) and bootstrap values from the ML tree (when ≥ 80%) are indicated at nodes (BPP/bootstrap). For nodes where one value was above the threshold and the other was below, the latter is indicated by hyphen '-'. BPP values of 1 and bootstraps of 100% are indicated by asterisks '*'. Node with bootstrap value 'x' indicates that the node-level species composition is not supported by the ML phylogenetic tree. Species names are given based on *Parartemia* morphotaxonomy (morphospecies). Coloured bars to the right of the tree indicate species groups identified within *Parartemia* in this study, named alphabetically (A-E).

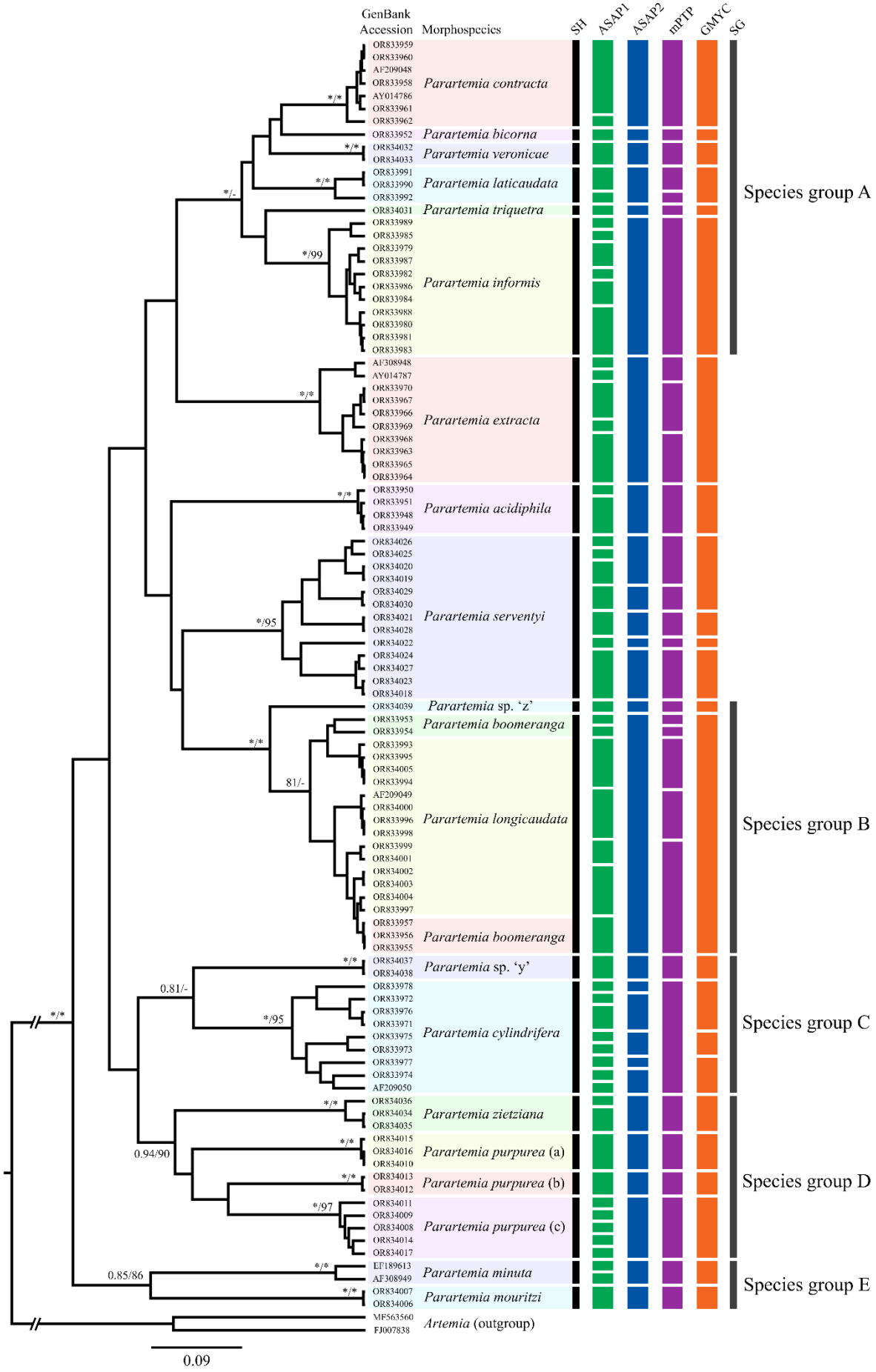
GenBank Accession
Morphospecies
SH
ASAP1
ASAP2
mPTP
GMYC
SG
OR833959
OR833960
AF209048
OR833958
AY014786
OR833961
OR833962
Pararartemia contracta
OR833952
Pararartemia bicorna
OR834032
OR834033
Pararartemia veronicae
OR833991
OR833990
OR833992
Pararartemia laticaudata
OR834031
Pararartemia triquetra
OR833989
OR833985
OR833979
OR833987
OR833982
OR833986
OR833984
OR833988
OR833980
OR833981
OR833983
Pararartemia informis
AF308948
AY014787
OR833970
OR833967
OR833966
OR833969
OR833968
OR833963
OR833965
OR833964
Pararartemia extracta
OR833950
OR833951
OR833948
OR833949
Pararartemia acidiphila
OR834026
OR834025
OR834020
OR834019
OR834029
OR834030
OR834021
OR834028
OR834022
OR834024
OR834027
OR834023
OR834018
Pararartemia serventyi
OR834039
Pararartemia sp. 'z'
OR833953
OR833954
Pararartemia boomeranga
OR833993
OR833995
OR834005
OR833994
AF209049
OR834000
OR833996
OR833998
OR833999
OR834001
OR834002
OR834003
OR834004
OR833997
Pararartemia longicaudata
OR833957
OR833956
OR833955
Pararartemia boomeranga
OR834037
OR834038
Pararartemia sp. 'y'
OR833978
OR833972
OR833976
OR833971
OR833975
OR833973
OR833977
OR833974
AF209050
Pararartemia cylindrifera
OR834036
OR834034
OR834035
Pararartemia zietziana
OR834015
OR834016
OR834010
Pararartemia purpurea (a)
OR834013
OR834012
Pararartemia purpurea (b)
OR834011
OR834009
OR834008
OR834014
OR834017
Pararartemia purpurea (c)
EF189613
AF308949
Pararartemia minuta
OR834007
OR834006
Pararartemia mouritzi
MF563560
FJ007838
Artemia (outgroup)
/
*/-
*/99
/
/
*/95
/
81/-
/
0.81/-
*/95
/
0.94/90
/
*/97
0.85/86
/
/
Species group A
Species group B
Species group C
Species group D
Species group E
0.09

Fig. 3.3: Bayesian inference (BI) phylogenetic tree of *Parartemia 16S* haplotypes. Maximum likelihood (ML) phylogenetic tree is available in Fig. S3.3. Bayesian Posterior Probability (BPP, when ≥ 0.80) and bootstrap values from the ML tree (when ≥ 80%) are indicated at nodes (BPP/bootstrap). For nodes where one value was above the threshold and the other was below, the latter is indicated by hyphen '-'. BPP values of 1 and bootstraps of 100% are indicated by asterisks '*'. Each haplotype is denoted by its GenBank accession number followed by its morphospecies name. The green, blue, magenta and orange vertical rectangles indicate recovered species partitions based on Assemble Species by Automatic Partitioning (ASAP) lowest score scheme (ASAP1), ASAP least partition scheme (ASAP2), Multi-rate Poisson Tree Processes (mPTP) and Generalized Mixed Yule Coalescent (GMYC) analyses, respectively. Species hypotheses (SH) determined by this study are indicated by black bars under the SH heading. Grey-coloured bars to the right of the coloured rectangles indicate species groups (SG) identified within *Parartemia* in this study, named alphabetically (A-E).

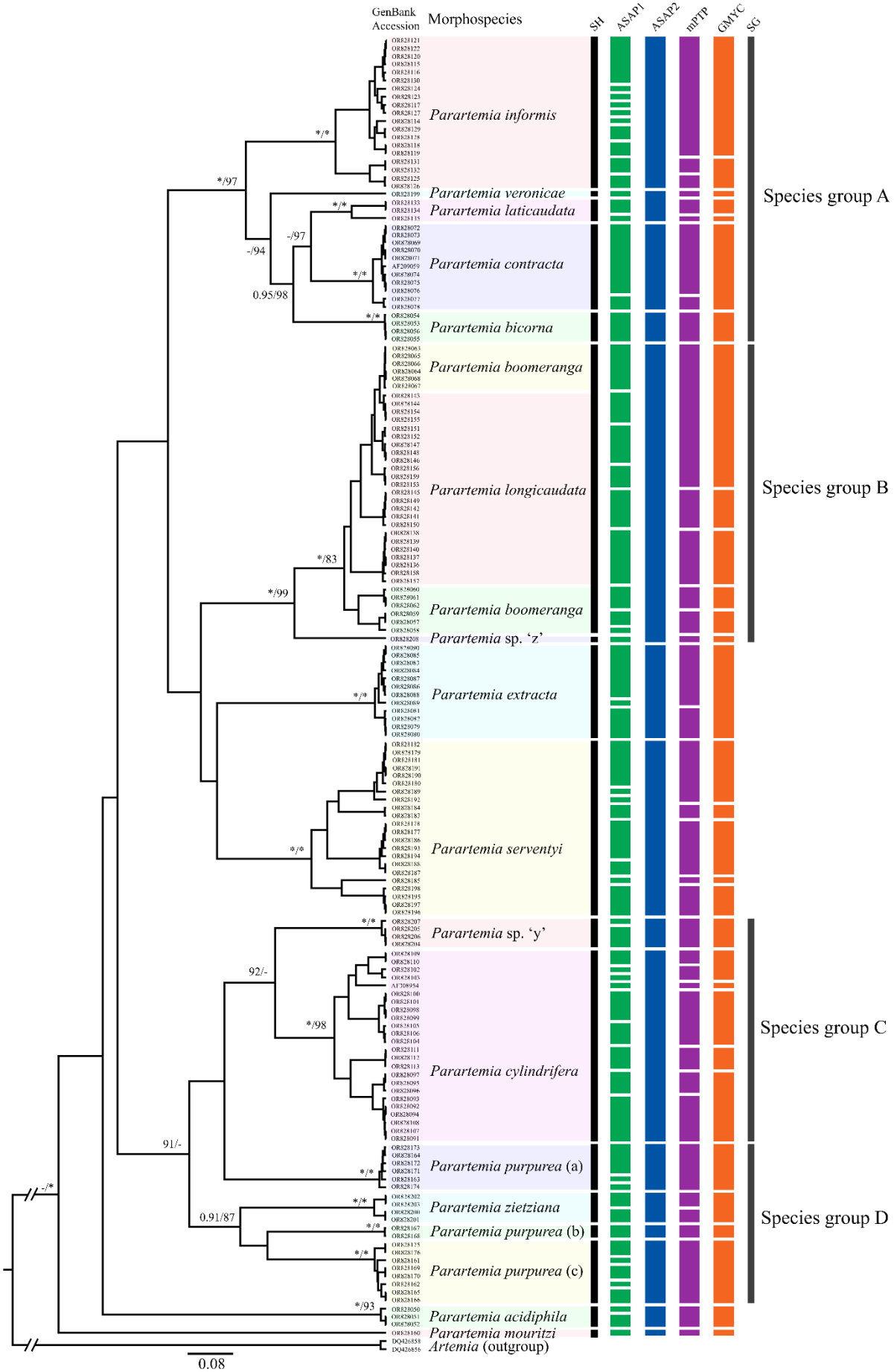
GenBank Accession
Morphospecies
SH
ASAP1
ASAP2
mPTP
GMYC
SG
Pararartemia informis
Pararartemia veronicae
Pararartemia laticaudata
Pararartemia contracta
Pararartemia bicorna
Pararartemia boomeranga
Pararartemia longicaudata
Pararartemia boomeranga
Pararartemia sp. 'z'
Pararartemia extracta
Pararartemia serventyi
Pararartemia sp. 'y'
Pararartemia cylindrifera
Pararartemia purpurea (a)
Pararartemia zietziana
Pararartemia purpurea (b)
Pararartemia purpurea (c)
Pararartemia acidiphila
Pararartemia mouritzi
Artemia (outgroup)
Species group A
Species group B
Species group C
Species group D
/
*/97
-/97
-/94
0.95/98
*/83
*/99
92/-
*/98
91/-
0.91/87
*/93
0.08

Fig. 3.4: Bayesian inference (BI) phylogenetic tree of *Parartemia COI* haplotypes. Maximum likelihood (ML) phylogenetic tree is available in Fig. S3.4. Bayesian Posterior Probability (BPP, when ≥ 0.80) and bootstrap values from the ML tree (when ≥ 80 %) are indicated at nodes (BPP/bootstrap). For nodes where one value was above the threshold and the other was below, the latter is indicated by a hyphen '-'. BPP values of 1 and bootstraps of 100 % are indicated by asterisks '*'. Each haplotype is denoted by its GenBank accession number followed by its morphospecies name. The green, blue, magenta and orange vertical rectangles indicate recovered species partitions based on Assemble Species by Automatic Partitioning (ASAP) lowest score scheme (ASAP1), ASAP least partition scheme (ASAP2), Multi-rate Poisson Tree Processes (mPTP) and Generalized Mixed Yule Coalescent (GMYC) analyses, respectively. Species hypotheses (SH) determined by this study are indicated by black bars under the SH heading. Grey-coloured bars to the right of the coloured rectangles indicate species groups (SG) identified within *Parartemia* in this study, named alphabetically (A-E; Species group E is not shown due to the absence of *COI* data for *P. minuta*).

The morphology of the medial process (MP) and/or medial space (MS) in the heads of males in species that occurred in the same species groups (A to E) in the molecular phylogenies were usually similar (Table 3.2; Fig. 3.5). For example, the 6 species in group A all had an undivided medial process (Fig. 3.5). Apart from *P. yarleensis*, which was not included in the molecular phylogeny, *P. serventyi* was the only other species to show this feature (Table 3.2) and, although the *P. serventyi* haplotype clade was distinctive, it was closest to species group A clade in the concatenated trees (Fig. 3.2 and Fig. S3.2). Another example is group C, which was formed by *P. cylindrifera* and *Parartemia* sp. 'y', the only *Parartemia* species that have a small bifid medial process (Table 3.2). *Parartemia minuta* and *P. mouritzi* in species group E had different MS structures but are the only known *Parartemia* species possessing basolateral spines (see Fig. 3.5).

Table 3.2: Structural features of the medial process (MP) and medial space (MS) in the male head in *Parartemia* species. Photographs of these morphological features are in Fig. 3.5.

Morphospecies	Description
Parartemia acidiphila Timms and Hudson, 2009	MP present (large with small bifid apex)
Parartemia cylindrifera Linder, 1941 *Parartemia* sp. 'y' (this study)	MP present (small bifid structure)
Parartemia bicorna Timms, 2010 *Parartemia contracta* Linder, 1941 *Parartemia informis* Linder, 1941 *Parartemia laticaudata* Timms, 2010 *Parartemia serventyi* Linder, 1941 *Parartemia triquetra* Timms and Hudson, 2009 *Parartemia veronicae* Timms, 2010 *Parartemia yarleensis* Timms and Hudson, 2009*	MP present (small, medium or large; no bifid apex)
Parartemia auriciforma Timms and Hudson, 2009* *Parartemia boomeranga* Timms, 2010 *Parartemia longicaudata* Linder, 1941 *Parartemia* sp. 'z' (this study)	MP absent; MS broad (flat, concave or convex)
Parartemia extracta Linder, 1941	MP absent; MS round
Parartemia minuta Geddes, 1973*	MP absent; MS open V-shaped
Parartemia mouritzi Timms, 2010	MP absent; MS flat with a small V-shaped central notch
Parartemia purpurea Timms, 2010 *Parartemia zietziana* Sayce, 1903	MP absent; MS either absent or an almost closed diamond-shaped

* The morphological features of these species were determined from photographs in Timms (2012b).

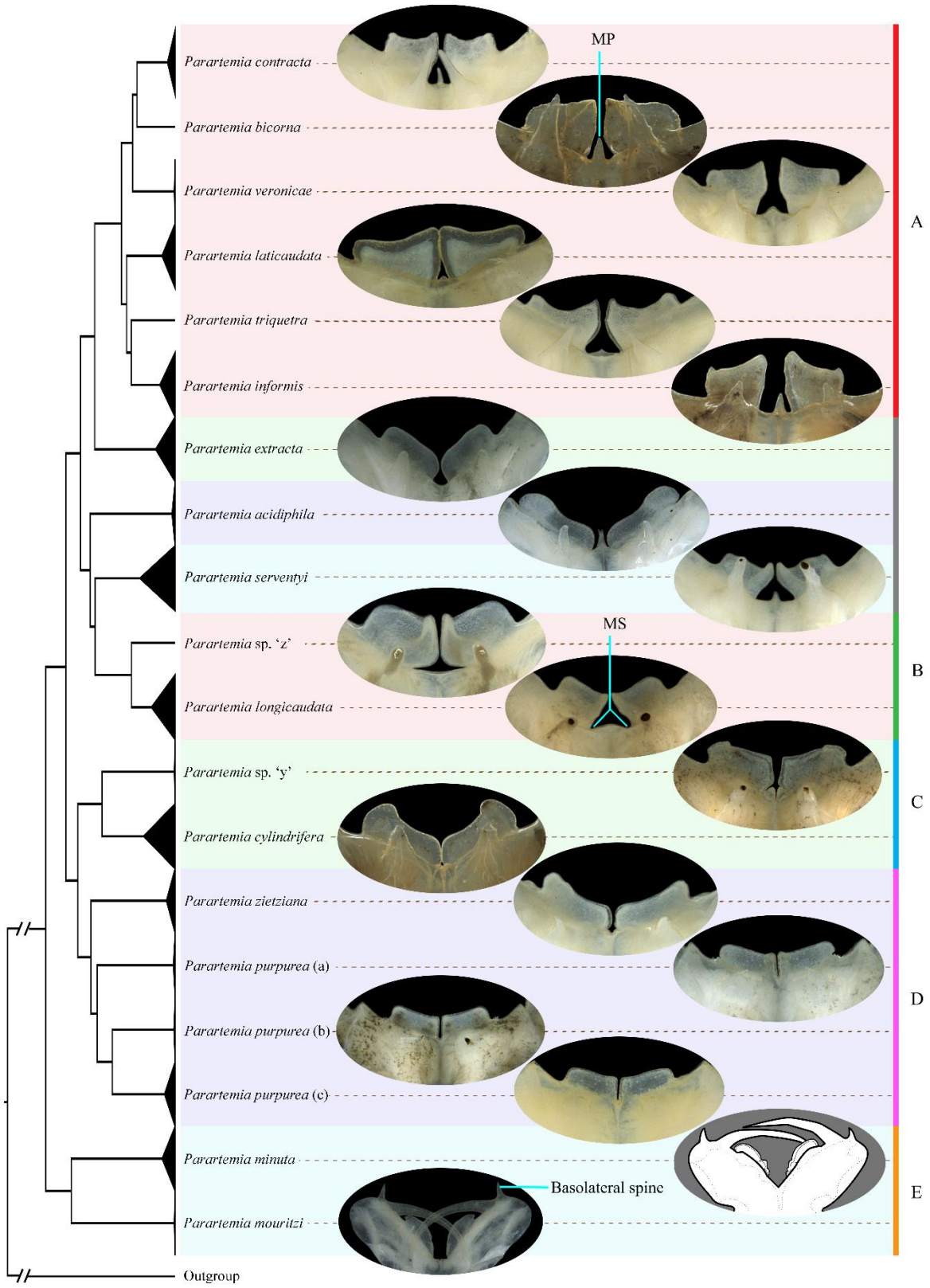

Fig. 3.5: Photographs showing the morphology of the medial process (MP) and/or medial space (MS) in the heads of male Parartemia from 19 species confirmed in this study. The male head of P. minuta has been redrawn from Timms (2014). Coloured bars indicate five species groups (A to E) in Parartemia identified in the molecular phylogenies. The grey-coloured bar indicates

the three molecularly distinctive species. The tree topology is derived from the collapsed 16S phylogenetic tree (Fig. 3.3).

3.3.3 Species delimitation

The three species delimitation methods identified different numbers of partitions among the *Parartemia* morphospecies. For the *16S* dataset, the ASAP scheme with the lowest score (ASAP1; threshold distance 0.01) gave 56 partitions and the ASAP least partition scheme (ASAP2; threshold distance 0.06) and the mPTP and GMYC methods yielded fairly similar results of 27, 30 and 24 partitions, respectively (Fig. 3.3 and Table 3.3). For the *COI* dataset, the GMYC (31) and mPTP (38) methods and especially ASAP1 (62; threshold distance 0.01) gave more partitions than ASAP2 (15; threshold distance 0.16) (Fig. 3.4 and Table 3.3). As is described below, the ASAP2 scheme showed the closest match with the *Parartemia* morphospecies. The other methods indicate the presence of one or more partitions within most morphospecies (Table 3.3). Our species hypotheses mainly conform with the ASAP2 scheme (see Table 3.3 and below).

For both the *16S* and *COI* genetic regions, the maximum amount of intraspecific genetic distance varied among species and tended to be larger in species that were sampled from a greater number of sites (see Tables 3.4 and S3.1). For any one species, the maximum intraspecific genetic distance was always less than its minimum genetic distance from another species, but overall, there was a limited amount of overlap between minimum interspecific and maximum intraspecific distances for both the *COI* and *16S* datasets (Tables 3.4, S3.6 and S3.7).

Eight of the 18 *Parartemia* morphospecies (16 described and two newly identified undescribed species) were each represented by a single well-supported clade (or a distinct haplotype when the species was represented by a single haplotype) that exactly corresponded to a single ASAP2 partition in both *16S* and *COI* datasets (Fig. 3.3 and Fig. 3.4; also see Table 3.3). Another two species, *P. minuta* and *P. triquetra* (represented by two and a single haplotype, respectively), each corresponded to a single ASAP2 partition in the *16S* dataset but were not included in the *COI* dataset (Table 3.3). For this group, genetic distances were highest in *P. extracta*, which was collected from seven sites plus two *16S* sequences from GenBank and had maximum *p*-distances of 7.53 % for *16S* and 3.65 % for *COI.* They were also high in *P. informis*, which was collected from 12 sites and had maximum *p*-distances of 6.28 % for *16S* and 12.16 % for *COI* (Tables 3.4 and S3.1). The geographic distributions of the divergent *16S* lineages within each of these species did not overlap (Fig. S3.5 and Fig. S3.6). This species group also included

the putative new morphospecies *Parartemia* sp. 'y', which was collected from two sites and represented by two *16S* and four *COI* haplotypes (Table 3.3), with maximum *p*-distances of 0.21 % and 1.37 % respectively (Table 3.4). The minimum *p*-distance between the haplotypes of *Parartemia* sp. 'y' and those of any other species (*P. cylindrifera*) was 12.16 % for *16S* and 15.65 % for *COI* (Table S3.6).

The remaining eight morphospecies fell into four categories: (i) morphospecies that formed single ASAP2 partitions in the *COI* but not the *16S* dataset, (ii) morphospecies that formed single ASAP2 partitions in the *16S* but not the *COI* dataset, (iii) multiple morphospecies that were combined into a single ASAP2 partition in both the *16S* and *COI* datasets, and (iv) a single morphospecies that was spilt into multiple ASAP2 partitions in both the *16S* and *COI* datasets.

Category (i) comprised *P. cylindrifera* and *P. serventyi*, each of which formed multiple (5) partitions in *16S* ASAP2 analysis (Table 3.3), despite each being represented by a single well-supported clade in the phylogenetic trees (see Fig. 3.2-3.4) and only a single partition in the *COI* ASAP2 analysis. These two species, which were sampled from respectively 12 (plus one *COI* and one *16S* sequence from GenBank) and 13 sites (Table S3.1), had the highest *16S* and *COI* genetic distances of all sampled species (Table 3.4). Within each species, the distributions of the divergent *16S* lineages did not overlap, although those of lineages C and E in *P. serventyi* bordered each other (see Fig. S3.7 and Fig. S3.8).

Category (ii) included *P. laticaudata* and *P. veronicae*, which were combined into a single partition in the *COI* ASAP2 analysis (Table 3.3). This was surprising because these two species did not form a monophyletic group in any of the phylogenetic analyses (see Fig. 3.2-3.4) and were partitioned via the more conserved *16S* region. Although the minimum genetic distances between the haplotypes of these two species, e.g., *p*-distances of 9.24 % for *16S* and 14.44 % for *COI*, were amongst the lowest found in the *Parartemia* species (see Table S3.6), that between the *16S* haplotypes of *P. verconicae* and *P. informis* was only 8.39 % (Table S3.6). Category (ii) also included the putative new species *Parartemia* sp. 'z', which was combined into a single partition with *P. boomeranga* and *P. longicaudata* in the *COI* analysis; it also grouped with these species in the ML *16S* tree (Fig. S3.3). *Parartemia* sp. 'z' was represented by single *16S* and *COI* haplotypes. The minimum *p*-distances between these haplotypes and those of the *P. longicaudata-boomeranga* morphotypes, at 9.39 % for *16S* and 13.98 % for *COI*, were low for interspecific comparisons (Table S3.6). Nevertheless, the haplotypes of *Parartemia* sp. 'z' were separated from those of *P. longicaudata-boomeranga* in most of the

phylogenetic analyses (see Fig. 3.2-3.4) and formed their own partition in all species delimitation analyses except *COI* ASAP2 (Table 3.3).

Table 3.3: Number of populations (p; excluding sequences obtained from GenBank with uncertain site details) and individuals (n) assayed, number of haplotypes detected (h) and results of species delimitation analyses (ASAP1: Assemble Species by Automatic Partitioning lowest score scheme, ASAP2: Assemble Species by Automatic Partitioning least partition scheme, mPTP: Multi-rate Poisson Tree Processes and GMYC: Generalized Mixed Yule Coalescent method) for *COI* and *16S* datasets for *Parartemia* morphospecies. The number of species represented by each morphospecies, based on the results of this study, is given in the final column.

Morphospecies	***16S***							***COI***							**No. of species**
	p	n	h	ASAP 1	ASAP 2	mPTP	GMYC	p	n	h	ASAP 1	ASAP 2	mPTP	GMYC	
Parartemia acidiphila	4	4	4	2	1	1	1	4	8	3	2	1	1	1	1
Parartemia bicorna	1	4	1	1	1	1	1	1	8	4	1	1	1	1	1
Parartemia contracta	4	7	7	2	1	1	1	4	13	11	2	1	2	1	1
Parartemia cylindrifera	8	9	9	8	5	1	3	12	29	24	9	1	7	5	1
Parartemia sp. ‘y’	2	4	2	1	1	1	1	2	6	4	2	1	1	1	1*
Parartemia extracta	7	10	10	5	1	3	1	7	17	12	3	1	2	1	1
Parartemia informis	12	13	11	6	1	1	1	12	29	19	10	1	3	2	1
Parartemia boomeranga	5	5	5	7	1	5	1	5	13	12	9	1	6	5	1+
Parartemia longicaudata	15	18	14					15	36	24					
Parartemia sp. ‘z’	1	2	1	1	1	1	1	1	3	1	1		1	1	1*
Parartemia minuta	-	2	2	2	1	1	1	-	-	-	-	-	-	-	1
Parartemia mouritzi	1	2	2	1	1	1	1	1	3	1	1	1	1	1	1
Parartemia purpurea	9	10	10	7	3	3	3	9	18	16	9	3	3	3	3#
Parartemia serventyi	13	14	13	7	5	5	4	13	32	22	8	1	5	5	1
Parartemia triquetra	1	1	1	1	1	1	1	-	-	-	-	-	-	-	1
Parartemia laticaudata	2	3	3	2	1	2	1	2	10	3	2	1	2	2	1

Parartemia veronicae	1	2	2	1	1	1	1	1	1	1	1		1	1	1
Parartemia zietziana	2	3	3	2	1	1	1	2	6	4	2	1	2	1	1
Total	**88**	**113**	**100**	**56**	**27**	**30**	**24**	**91**	**232**	**161**	**62**	**15**	**38**	**31**	**19**

* new species; + conspecific morphotypes; and # cryptic species.

Table 3.4: Summary of species-level pairwise genetic distances (%) for 100 *16S* and 161 *COI* haplotypes in *Parartemia* species confirmed in this study. N: number of haplotypes per species; M. Intra.: maximum intraspecific distance; and Inter.: range in interspecific distance. Further details are in supplementary Tables S3.6 and S3.7.

Species	***16S* rRNA**					***COI* mtDNA**				
	N	K2P-distance		*p*-distance		N	K2P-distance		*p*-distance	
		M. Intra.	Inter.	M. Intra.	Inter.		M. Intra.	Inter.	M. Intra.	Inter.
P. acidiphila	4	1.49	13.97-23.06	1.47	12.58-19.54	3	1.86	20.19-27.70	1.82	17.33-22.49
P. bicorna	1	-	10.69-18.70	-	9.85-16.35	4	0.46	18.20-27.10	0.46	15.81-22.04
P. contracta	7	3.02	10.38-21.21	2.93	9.62-18.03	11	4.42	16.86-29.47	4.26	14.74-23.71
P. cylindrifera	9	10.73	12.62-23.03	9.83	11.51-19.50	24	14.08	17.88-29.30	12.46	15.65-23.71
P. extracta	10	8.08	13.30-21.19	7.53	12.13-18.24	12	3.79	20.95-28.51	3.65	17.93-22.95
P. informis	11	6.67	8.97-22.75	6.28	8.39-19.29	19	13.75	17.64-28.51	12.16	15.35-22.95
P. laticaudata	3	4.56	9.95-20.52	4.40	9.24-17.65	3	11.66	16.43-29.98	10.49	14.44-24.01
P. longicaudata-P. boomeranga	19	8.06	10.14-23.44	7.52	9.39-19.71	36	13.12	16.06-28.05	11.70	13.98-22.80
P. minuta	2	4.58	15.88-21.85	4.39	14.23-18.62	-	-	-	-	-
P. mouritzi	2	0.21	16.38-23.40	0.21	14.68-19.75	1	-	21.95-27.92	-	18.84-22.80
P. purpurea (a)	3	0.63	14.76-24.59	0.63	13.21-20.34	6	2.81	20.43-29.47	2.74	17.63-23.71
P. purpurea (b)	2	0.42	15.26-23.06	0.42	13.60-19.50	2	0.46	20.99-30.38	0.46	17.93-24.32
P. purpurea (c)	5	4.34	12.85-22.62	4.18	11.74-19.08	8	3.93	20.67-32.02	3.80	17.78-25.23
P. serventyi	13	10.49	11.36-24.59	9.60	10.46-20.34	22	20.21	18.25-32.02	17.02	15.96-25.23
P. triquetra	1	-	9.85-21.85	-	9.21-18.62	-	-	-	-	-
P. veronicae	2	0.21	8.97-20.52	0.21	8.39-17.61	1	-	16.43-26.48	-	14.44-21.73
P. zietziana	3	3.47	13.48-22.27	3.35	12.16-18.87	4	4.28	20.45-27.20	4.10	17.63-22.34
Parartemia sp. ‘y’	2	0.21	13.56-19.80	0.21	12.16-17.19	4	1.38	17.88-27.70	1.37	15.65-22.49

Parartemia sp. ‘z’	1	-	10.14-21.12	-	9.39-18.20	1	-	16.06-27.42	-	13.98-22.34

Category (iii) comprised *P. boomeranga* and *P. longicaudata*, which in combination corresponded to a single ASAP2 partition in both the *16S* (a single partition was also suggested by the *16S* GMYC analysis; Fig. 3.3 and Table 3.3) and (along with *Parartemia* sp. 'z') in the *COI* datasets (see above; also see Fig. 3.4 and Table 3.3). Together, these species formed a single clade in the phylogenetic trees (which also included *Parartemia* sp. 'z' in the ML *16S* tree; Fig. S3.3), but neither morphotype formed an exclusive monophyletic group within this broader clade (see Fig. 3.2-3.4). Similarly, although the other species delimitation methods identified multiple partitions within the broader *P. boomeranga-P. longicaudata* clade, none of these partitions exactly corresponded to haplotypes representing one or the other morphotype (Fig. 3.3 and Fig. 3.4). The amount of divergence (*p*-distance) within the *P. longicaudata-boomeranga* complex ranged up to 7.52 % for *16S* and 11.7 % for *COI*, which was less than that in some other species (Table 3.4). The *P. longicaudata* morphotype was common and widely distributed whereas we only found the *P. boomeranga* morphotype at five sites in the northern Wheatbelt within 125 km of each other (Table S3.1 and Fig. S3.9). The *P. boomeranga-P. longicaudata* complex included a widespread lineage (E) whose distribution overlapped or bordered that of the other four lineages (A to D) whose distributions did not overlap (see Fig. S3.9).

Parartemia purpurea was the only morphospecies in category (iv). Representatives of this taxon fell into three well-supported clades (a, b and c) in the phylogenetic trees (see Fig. 3.2-3.4). Some details of the relationship among these clades varied between datasets and tree-building methods (Fig. 3.2-3.4). Regardless, each of the three clades corresponded to separate ASAP2 partitions (also for the mPTP and GMYC methods) in both the *16S* and *COI* datasets (Fig. 3.3 and Fig. 3.4) and differed from each other by *p*-distances of at least 13.6 % and 18.24 % for the *16S* and *COI* data, respectively (see details in Table S3.6), which are greater than the maximum intraspecific distance recorded in any other morphospecies (Table 3.4). On this basis, we propose that '*P. purpurea*' comprises three cryptic species that herein are called *P. purpurea* (a), *P. purpurea* (b) and *P. purpurea* (c) (see Fig. 3.2-3.4). The morphology of some specimens of each species were checked but no characteristic differences were found. All three species were found within 85 km of each other in the Esperance hinterland, but their geographical distributions did not overlap (see Fig. 3.6).

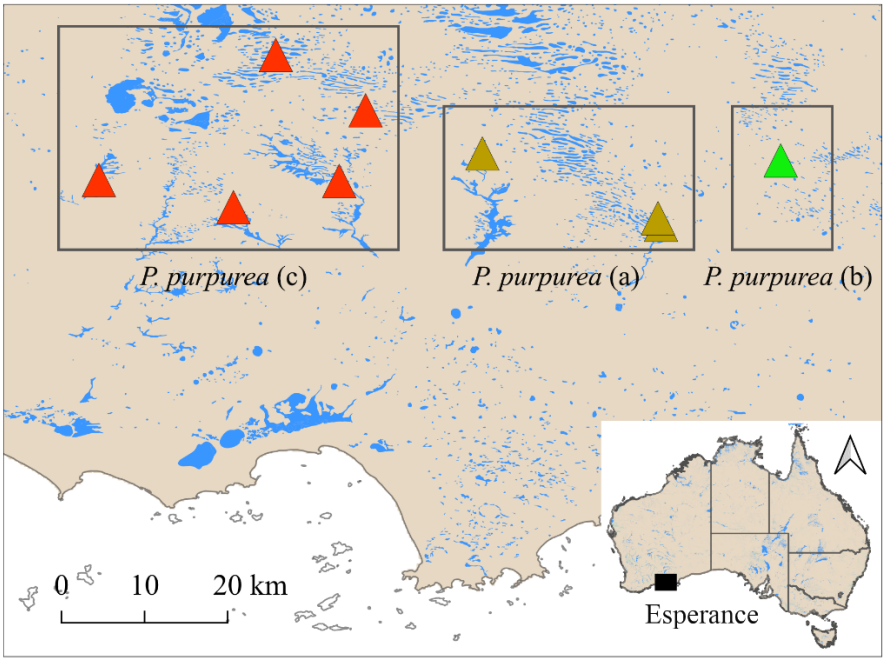

Fig. 3.6: Distribution of three cryptic species of *P. purpurea*. Site details are in supplementary Table S3.1

3.3.4 Morphology of new species

The overall morphology of the putative new species *Parartemia* sp. 'y' was like that of *P. cylindrifera* (with which it also had the most molecular similarities). However, the ventral processes in the males of *Parartemia* sp. 'y' featured nearly rectangular distolateral outgrowths and obtuse distomesial corners, whereas those of other species including *P. cylindrifera* had round distolateral corners protruding ventrally and round distomesial corners (Fig. 3.7).

The overall morphology of *Parartemia* sp. 'z' was similar to the *P. boomeranga* and *P. longicaudata* morphotypes (with which it also had the most molecular similarities) but the thickened medial edges of the ventral processes in the males were strongly concave (boomerang-shape) contrasting with their weakly concave (boomerang-shape) or cylindrical shape in *P. boomeranga-P. longicaudata* (Fig. 3.7). *Parartemia* sp. 'z' was also easily discernible from *P. boomeranga-P. longicaudata* by the substantially larger lateral lobes on the eleventh thoracic segment in males, which were more than double the size of lateral bulges on the first genital segment (Fig. 3.7).

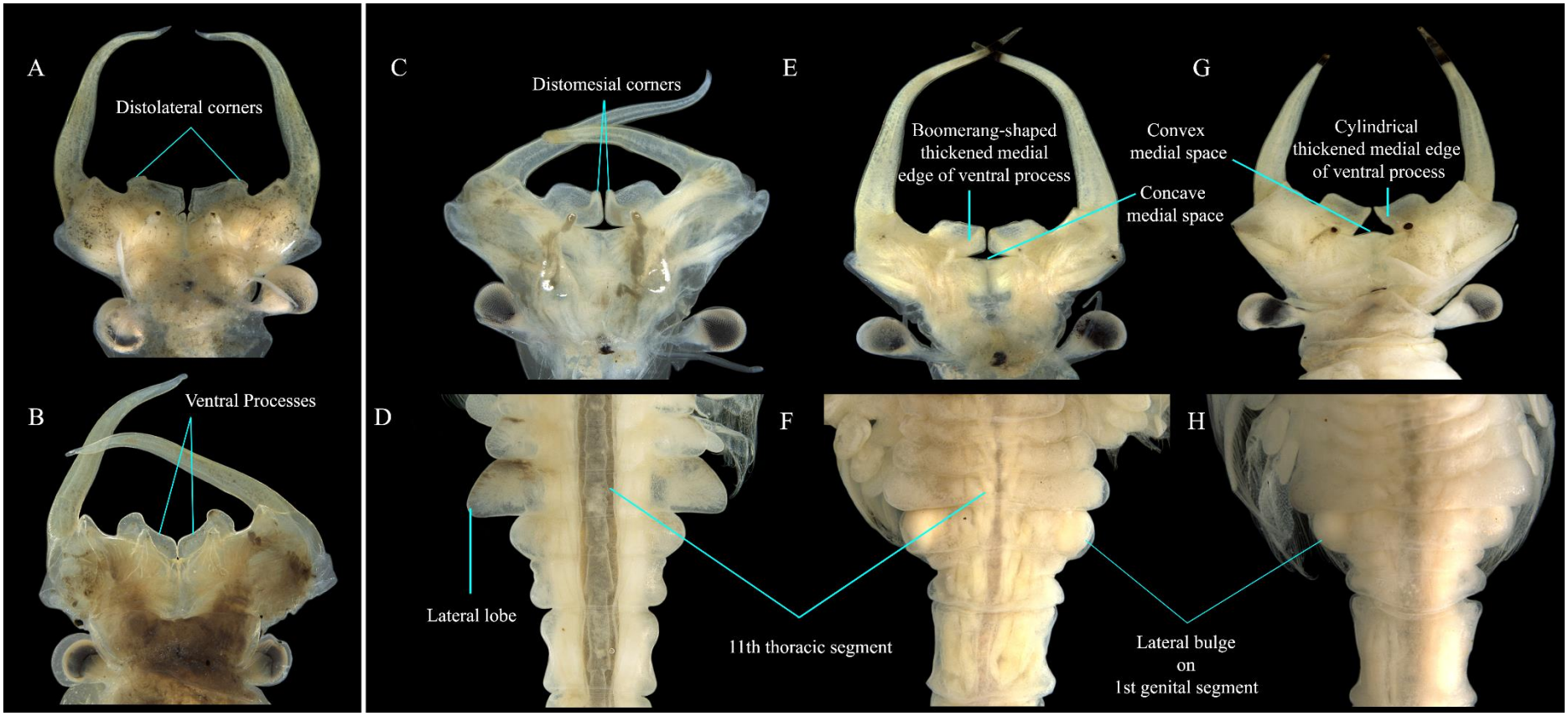

Fig. 3.7: Photographs of putative new species of *Parartemia* showing distinctive morphological features relative to morphologically similar species. Comparison of male head of *Parartemia* sp. 'y' (A) with that of *P. cylindrifera* (B). Comparison of male head and posterior thoracic and anterior abdominal segments of *Parartemia* sp. 'z' (C and D) with those of the *P. boomeranga* (E and F) and *P. longicaudata* (G and H) morphotypes. Morphological features mentioned in the text are indicated.

3.3.5 *Parartemia* species POP4 and POP5

To determine the species identity of POP4 and POP5 from Remigio *et al.* (2001), we trimmed our *16S* sequences and compared them to two short *16S* sequences (335 bp and 337 bp) from POP4 and POP5 on GenBank (data not presented). The sequence for POP4 (accession number AY014794) was identical to the corresponding region in one of three *P. laticaudata* haplotypes. The sequence for POP5 (accession number AY014795) was similar (minimum and maximum *p*-distances of 2.2 and 4.3 %, respectively) to the corresponding region in one of five haplotypes found in *P. purpurea* (c), but highly divergent from the next closest taxa based on *16S*, which were *P. purpurea* (b) and *P. purpurea* (a) (minimum *p*-distances of 16.1 % and 17.1 %, respectively). These findings indicate that POP4 corresponds to *P. laticaudata* and POP5 to *P. purpurea* (c).

3.4 Discussion

This study provides the first molecular phylogeny of *Parartemia* based on an almost complete suite of species and broad geographic sampling. The findings generally support suggestions that this taxon is monophyletic, includes large amounts of molecular divergence, generally exhibits congruent patterns of genetic and morphological divergence and contains a large number of species (see Lawrie *et al.*, 2021; Remigio *et al.*, 2001; Timms, 2014). It also provides evidence of two new morphospecies and of cryptic speciation.

3.4.1 Molecular divergence

The amount of molecular divergence found in *Parartemia* was large, as was also noted by Remigio *et al.* (2001). Using *COI p*-distances as an example, the maximum divergence in *Parartemia* (25.23 %) was higher than that reported for a range of other branchiopod genera, such as *Triops* (15.6 %; Meusel & Schwentner, 2017), *Limnadopsis* (18.6 %; Schwentner *et al.*, 2011), *Eocyzicus* (19.0 %; Schwentner *et al.*, 2014), *Ozestheria* (21.2 %; Schwentner *et al.*, 2015) and *Artemia* (21.8 %; Muñoz *et al.*, 2008). It was also greater than that detected in other invertebrate genera that have a long history in Australian salt lakes or their precursors, for example, *Coxiella* gastropods (18.9 %; Lawrie *et al.*, 2023) or *Australocypris* giant ostracods (18.9 %; Rahman, 2024). The extreme conditions in salt lakes may accelerate the rate of molecular evolution in halophilic cladocerans and anostracans, like *Parartemia* (Hebert *et al.*, 2002). Such rate heterogeneity would help to explain why divergence levels in *Parartemia* are high relative to branchiopods from fresh or low salinity water but not necessarily in comparison with *Artemia* or other genera of halophilic crustaceans. It may also be that some *Parartemia* lineages are particularly old and/or that habitat specialisation has driven divergence among some lineages (see below).

3.4.2 Phylogenetic relationships

Some aspects of the phylogeny of *Parartemia*, particularly deeper divergences, were not fully resolved in this study. This may be because the selected genetic markers were not suitable for higher-level taxonomic resolution in *Parartemia* and/or because these deeper divergences may be associated with rapid cladogenesis (e.g., see Kang *et al.*, 2008; Pinceel *et al.*, 2013b; Whitfield & Kjer, 2008). However, some groups of related species and some distinctive species were evident. The fact that species thought to be closely related based on the molecular data usually also showed similarities in the structures of their medial processes or medial space in

the males adds evidence that these groups are real. Like Remigio *et al.* (2001), we found that *P. minuta* (which occurs in central and eastern Australia) was usually positioned at the base of the phylogeny and, in our study, usually grouped with *P. mouritzi* (which occurs in Western Australia and was not sampled by Remigio *et al.*, 2001). These are the only two *Parartemia* species known to possess basolateral spines (see Timms, 2012b), providing further evidence that they are sister species. Like Remigio *et al.* (2001), we found that *P. extracta* was a distinctive species. Remigio *et al.* (2001) found two new species of *Parartemia*, which they called POP4 and POP5 and which we have identified as *P. laticaudata* and *P. purpurea* (c), respectively. The species groups that Remigio *et al.* (2001) called clades A and B were not apparent in our samples. For example, that study placed *P. longicaudata* (which included their POP1, POP3 and POP6 samples), *P. cylindrifera*, *P. purpurea* (c) (then POP5) and *P. zietziana* in clade A whereas our study usually placed *P. purpurea* (c) and *P. zietziana* in the same clade but *P. longicaudata* in a different clade and *P. cylindrifera* in yet another one. The discrepancies between the results of this study and those of Remigio *et al.* (2001) can be attributed to the results of the latter study being based on only a short *16S* fragment and only a small subset of species (see Introduction). Increased taxonomic representation has been shown to alter perceptions of species relationships in a range of taxa, including *Branchinella* fairy shrimps in Australia (Pinceel *et al.*, 2013b).

On the basis that new anostracans species are expected to arise from widespread species in peripheral habitats, Rogers (2015) predicted that anostracan genera will comprise a series of small clades, each containing a basal widespread species and a derived species with a narrow distribution at the periphery of the widespread one (see also Rogers & Aguilar, 2020). Our phylogenies provide several examples of widespread and narrowly distributed sister species including (with the widespread species listed first) - *P. cylindrifera* and *Parartemia* sp. 'y', *P. longicaudata-boomeranga* and *Parartemia* sp. 'z', *P. informis* and *P. triquerta* and *P. minuta* and *P. mouritzi* (see Timms *et al.*, 2009 and below for distributional information). However, other aspects of the relationships between these sister species do not fit with the above prediction. For example, relative to their widespread counterparts, the narrow range species were usually ancestral in the phylogenies and/or had either overlapping (e.g., *P. cylindrifera* and *Parartemia* sp. 'y') or disjunct distributions (e.g., *P. informis* and *P. triquerta).* Intraspecific divergence within some widespread *Parartemia* species may more closely conform with the above prediction and will be examined in detail in a future study on phylogeographic patterns in *Parartemia*.

3.4.3 Species delimitation

The most recent previous estimate of the number of *Parartemia* morphospecies was 18 (Timms, 2010). Our study suggests that *Parartemia* consists of at least 21 species. These species comprise: (i) 13 described morphospecies that have been confirmed using the molecular data in this study; (ii) two putative new morphospecies; (iii) three cryptic species of *P. purpurea*; (iv) a species encompassing the *P. longicaudata* and *P. boomeranga* morphotypes; and (v) two described morphospecies that were not included in this study. These data suggest that *Parartemia* is the most speciose genus of halophilic invertebrates found in Australia, notwithstanding that the taxonomy of some of the other invertebrate groups is rudimentary (see Lawrie *et al.*, 2021). Based on the currently available data, the next most diverse genus is *Coxiella* (a gastropod) with at least 15 species, although recent molecular evidence indicates that these species are spread over several unrecognised genera (four clades in Lawrie *et al.*, 2023). *Australocypris* with 10 species is the most species-rich genus in Mytilocypridinae giant ostracods (Rahman, 2024). *Parartemia* is much more species-rich than *Artemia*, which only has nine recognised species even though *Artemia* is essentially globally distributed (Asem *et al.*, 2023; Rogers, 2013). Compared to *Artemia*, speciation in *Parartemia* is likely facilitated by their heavy, sinking resting eggs, which tend to retard dispersal (McMaster *et al.*, 2007; Timms *et al.*, 2009). Australia also has a diverse range of *Branchinella* fairy shrimps in fresh or low-salinity water (Pinceel *et al.*, 2013b; Rogers & Timms, 2014). This diversity and that in *Parartemia* is probably linked to a long history of aridity and persistent ephemeral water bodies in the Australian landscape (Rogers & Timms, 2014). Rogers (2015) and references therein have argued that species diversity in anostracans will be enhanced in an older landscape like Australia where more occupied habitats will favour speciation by habitat specialisation over colonisation of vacant habitats. This fits with the high levels of ecological specialisation apparent among *Parartemia* species, e.g., with regard to pH and/or substrate geochemistry (see Timms, 2012b; Timms *et al.*, 2009).

Our species hypotheses for *Parartemia* are based on the most conservative results from the species delimitation analyses (i.e., the ASAP scheme with the least number of partitions), which tended to match morphological species boundaries. This fits with the suggestion that morphological diagnosis of anostracan species is generally straightforward (Rogers & Aguilar, 2020), although this is not always the case (e.g., see Ketmaier *et al.*, 2008; Pinceel *et al.*, 2013b; Rogers & Aguilar, 2020). Many *Parartemia* morphospecies comprised multiple divergent lineages that were partitioned in some of the species delimitation analyses. Other than for the

three lineages of *P. purpurea* (discussed below), we have interpreted the divergent lineages within morphospecies as examples of intraspecific variation rather than as cryptic species for the following reasons. (1) The presence of morphologically similar but genetically divergent lineages may be linked to an accelerated rate of molecular evolution in *Parartemia* (see above). (2) With the exception of *P. longicaudata*, the geographic distributions of the divergent lineages of morphospecies were essentially nonoverlapping. Also, conspecific divergent lineages never co-occurred in the same water body (see Fig. S3.5-S3.9). (3) Species delimitation methods are sensitive to the population structure of a species and sometimes delimit genetically divergent populations within a species (Gaytán *et al.*, 2020; Luo *et al.*, 2018). (4) Defining species based on molecular data alone is challenging (Fišer *et al.*, 2018; Jörger & Schrödl, 2013). (5) We wanted to avoid the pitfalls of taxonomic inflation (see Padial & De la Riva, 2006).

We found two putative new morphospecies of *Parartemia* - *Parartemia* sp. 'y' and *Parartemia* sp. 'z' - in our samples. These taxa exhibited both distinctive morphological and genetic characteristics relative to other described species. The evidence is stronger for *Parartemia* sp. 'y', which was found at two sites, than for *Parartemia* sp. 'z', which was only found at a single site. The results of this study provide the first evidence of cryptic species in *Parartemia*, namely *P. purpurea* (a), *P. purpurea* (b) and *P. purpurea* (c), although cryptic species have been reported in a range of anostracans (e.g., Pinceel *et al.*, 2013b; Rogers, 2014b) and other branchiopods (e.g., Meusel & Schwentner, 2017; Schwentner *et al.*, 2013). The three *P. purpurea* lineages were consistently separated from each other in the species delimitation analyses and did not always form a single well-supported clade in the phylogenetic trees. No morphological differences were detected among individuals from these lineages, including in relation to characters important in species diagnosis in *Parartemia* (see Introduction), although it is possible that further scrutiny could reveal some subtle differences. Ecological and/or physiological specialisation may have been crucial in driving cryptic speciation in *P. purpurea* (e.g., Rogers, 2014b).

The two new *Parartemia* morphospecies as well as the three cryptic species of *P. purpurea* were found in only the Esperance hinterland region of Western Australia. This brings the total number of *Parartemia* species found in this region to nine (out of 21), five of which have only been found in this region (this study and Timms *et al.*, 2009). In general, the region is well known for the diverse range of invertebrates found in its salt lakes (see Timms, 2009b). *Coxiella* gastropods (Lawrie *et al.*, 2023) and *Australocypris* ostracods (Rahman *et al.*, 2023)

also have high species richness and multiple endemic species in this region. The region hosts a large number and variety of salt lakes (see Timms, 2009b for details), which are probably important factors driving divergence and diversity. It also seems likely that this region has served as a refugium in evolutionary time, limiting extinctions, possibly because of favourable hydrological and/or climatic conditions (e.g., see Davis *et al.*, 2013; Jansson, 2003).

Our results suggest that the *P. boomeranga* and *P. longicaudata* morphotypes comprise a single monophyletic lineage. These results are based on a comprehensive sampling of these morphotypes. *Parartemia boomeranga* was sampled across its documented range, including three lakes near the type locality (unnamed lake east of Gunyidi, -30.12, 116.24; see Timms, 2010). *Parartemia longicaudata* was also sampled across its entire known geographical range, from Esperance to the Houtman Abrolhos Islands, including the neotype locality (Pink Lake in Esperance; see Fig. S3.9 and Timms, 2010). Morphological differences between *P. boomeranga* and *P. longicaudata* are minor and confined to the thickened medial edges of the ventral processes in males (boomerang-shaped in the former and cylindrical in the latter) and the shape of the medial space (concave in the former and convex in the latter) (see Fig. 3.7 and Timms, 2010). Regardless, the *P. boomeranga* and *P. longicaudata* morphotypes did not form reciprocally monophyletic groups in the molecular phylogeny, so each morphotype must have evolved more than once or may be environmentally induced. Following the guidelines set by the International Code of Zoological Nomenclature (ITZN, 1999), *P. longicaudata* being named first has nomenclatural priority over *P. boomeranga*.

3.4.4 Conservation implications

Timms *et al.* (2009) provided a detailed assessment of the conservation status of *Parartemia* species. This assessment can be updated using the improved taxonomic and associated distributional information on *Parartemia* generated by this study.

Timms *et al.* (2009) proposed that *P. boomeranga* (then *Parartemia* sp. 'c') should be assessed as potentially vulnerable. Timms (2012b) later advised that this species was extremely rare and at risk of extinction. However, we encountered this species at five sites within the reported range of this species, including Lake Moore (in the northern wheatbelt region in Western Australia) from which this taxon has previously been recorded (Timms *et al.*, 2009) and three sites that are near the type locality (see above). Furthermore, the molecular results suggest that the *P. boomeranga* morphotype is not a valid species but is synonymous with the common and

widely distributed *P. longicaudata*, which is not regarded as threatened (see Timms *et al.*, 2009).

The two new species and the three cryptic species of *P. purpurea* discovered in this study all have narrow geographic distributions. Except for *P. purpurea* (c), these species are known from between only one and three sites in the Esperance hinterland region. All these species have sites in the Kau Rock Nature Reserve and/or the nearby Beaumont Nature Reserve and are therefore offered some protection but are nonetheless somewhat vulnerable given their rarity and restricted distributions. *Parartemia purpurea* (c) is known from 5 or 6 sites (depending on whether we resampled Remigio *et al.*'s (2001) POP5 collection site, the exact location of which was not reported) but all are in the Esperance hinterland and none are in nature reserves. The above distributional information does not consider 20 other sites in the Esperance hinterland that were not sampled in this study but are reported to contain the *P. purpurea* morphotype (see Rogers & Timms, 2014; Timms, 2010; Timms *et al.*, 2009). Regardless, as an area of evolutionary importance with high species diversity (see above and Timms *et al.*, 2009), the Esperance hinterland should be a priority site for *Parartemia* conservation (see Davis *et al.*, 2013; Moritz, 2002).

3.4.5 Limitations and future work

Future phylogenetic studies on *Parartemia* should aim to include *P. auriciforma* and *P. yarleensis*, which were missing from this study, as well as more samples from salt lakes in remote areas, which have typically been poorly studied and may harbour a high proportion of undiscovered species (Timms, 2010). It is also important to formally describe and name the new species discovered in this study so they can be taken into account in conservation planning and legislation (e.g., see Mace, 2004; Padial & De la Riva, 2006). A better understanding of the evolutionary significance of the divergent lineages within morphospecies is also needed. This could be facilitated via assessments of phylogeographic structures of these species (e.g., see Seidel *et al.*, 2009).

The phylogenetic results of this study are based on a total of three different genetic markers, which were not sufficient to resolve the relationships among some lineages, particularly those reflecting deeper divergences. Future studies should include more markers/loci to improve the resolution of phylogenetic relationships in *Parartemia*, although there is no simple answer as to the minimum number of markers/loci needed to produce a robust phylogeny or whether markers should be selected at random or systematically (Gatesy *et al.*, 2007).

3.5 Conclusion

Our study indicates that *Parartemia* comprises at least 21 species, including two putative new morphospecies and three cryptic species. The molecular data revealed five groups of related species that were also largely supported by morphological data. The molecular data were also mainly consistent with morphospecies designations, although many morphospecies contained large amounts of divergence. Overall, the results highlight the importance of using both molecular and morphological data for providing robust species hypotheses in *Parartemia.* The improved taxonomic information for *Parartemia* will support the development of future studies and conservation assessments of this taxon.

3.6 Supplementary Tables and Figures

Table S3.1: *Parartemia* specimens used for sequence data in this study with GenBank accession numbers. Specimens that we collected have been lodged with the Western Australian Museum (WAM). The remains of specimens that were used for DNA extractions have been given individual registration numbers (WAM Reg. No. Individual), other specimens collected from the same site have been given sample registration numbers (WAM Reg. No. Sample).

SL	Morphospecies Name	Site ID (WAM Reg. No. Sample)	Latitude and Longitude	Individual ID (WAM Reg. No. Individual)	GenBank Accession Number		
					COI	16S	28S
1	*P. acidiphila*	Esperance 16 (C84797)	-33.136987, 121.965285	Esp-16.1 (C84868)	OR828050	OR833948	×
				Esp-16.3 (C84869)	OR828050	×	×
2		Esperance 24 (C84798)	-33.438657, 122.392712	Esp-24.1 (C84870)	OR828050	OR833949	OR834040
				Esp-24.2 (C84871)	OR828050	×	×
3		Esperance 32 (C84799)	-33.508967, 122.410974	Esp-32.1 (C84872)	OR828051	OR833950	OR834040
				Esp-32.2 (C84873)	OR828051	×	×
4		Esperance 34 (C84800)	-33.471019, 122.382336	Esp-34.1 (C84874)	OR828052	OR833951	×
				Esp-34.2 (C84875)	OR828050	×	×
1	*P. bicorna*	Lake Carey[4]	-29.311261, 122.573451	LN9634.2	OR828053	OR833952	OR834041
				LN9634.3	OR828054	×	×
				LN9634.4	OR828053	×	×
			-28.845632, 122.283433	LN31213.1	OR828053	OR833952	OR834041
				LN31213.3	OR828053	×	×
				LN31213.6	OR828055	×	×
			-28.866558, 122.331809	LN10215.1	OR828056	OR833952	×
				LN10215.5	OR828053	×	×
			-29.246474, 122.411221	LN30046.1	×	OR833952	×

1	*P. boomeranga*	Near Wongan Hills-2 (C84841)	-30.511172, 116.711515	nWH-2.1 (C84876)	OR828057	×	×
				nWH-2.2 (C84877)	OR828058	OR833953	OR834042
				nWH-2.3 (C84878)	OR828059	×	×
2		Lake Moore (C84842)	-30.333737, 117.492973	Moo-1.1 (C84879)	OR828060	×	×
				Moo-1.2 (C84880)	OR828061	OR833954	OR834042
				Moo-1.3 (C84881)	OR828062	×	×
3		Marchagee 3 (C84843)	-30.119139, 116.222031	Mar-3.1 (C84882)	OR828063	×	×
				Mar-3.2 (C84883)	OR828064	OR833955	OR834042
4		Marchagee 4	-30.119420, 116.213778	Mar-4.1 (C84884)	OR828065	×	×
				Mar-4.2 (C84885)	OR828066	OR833956	OR834051
5		Marchagee 5 (C84844)	-30.117236, 116.201455	Mar-5.1 (C84886)	OR828067	OR833957	×
				Mar-5.2 (C84887)	OR828068	×	×
				Mar-5.3 (C84888)	OR828064	×	×
				Mar-5.5 (C84889)	×	×	OR834051
1	*P. contracta*	Kondinin 5 (C84801)	-32.581316, 118.431073	Kondi-5.1 (C84890)	×	OR833958	×
				Kondi-5.2 (C84891)	OR828069	OR833959	OR834043
				Kondi-5.3 (C84892)	OR828070	×	×
				Kondi-5.4 (C84893)	OR828071	×	×
2		Jilakin 1 (C84802)	-32.676675, 118.355247	Jila-1.3 (C84894)	OR828072	×	×
				Jila-1.4 (C84895)	OR828073	×	×
				Jila-1.5 (C84896)	OR828072	OR833960	×
3		Hyden 4 (C84803)	-32.355594, 119.134036	Hy-4.3 (C84897)	OR828074	×	×
				Hy-4.4 (C84898)	OR828075	×	×
				Hy-4.5 (C84899)	OR828076	OR833961	×
4		Cowcowing 3 (C84804)	-30.735187, 117.337013	Cow-3.1 (C84900)	OR828077	×	×
				Cow-3.2 (C84901)	OR828078	OR833962	OR834043
				Cow-3.3 (C84902)	OR828078	×	×

5		AF209048*	-	-	×	AF209048	×
6		AY014786*	-	-	×	AY014786	×
7		AF209059*	-	-	AF209059	×	×
1	*P. extracta*	Green Head 1 (C84815)	-29.974886, 114.980817	Green-1.2 (C84903)	OR828079	OR833963	×
				Green-1.3 (C84904)	OR828079	×	×
				Green-1.4 (C84905)	OR828079	×	×
				Green-1.5 (C84906)	OR828080	×	×
2		Green Head 2 (C84814)	-29.987320, 114.986895	Green-2.2 (C84907)	OR828081	OR833964	OR834044
				Green-2.3 (C84908)	OR828082	×	×
				Mix-1.1 (C84909)	×	OR833965	×
				Mix-1.2 (C84910)	OR828082	×	×
3		Dowerin 1 (C84816)	-31.253627, 117.060872	Dow-1.1 (C84911)	OR828083	×	×
				Dow-1.3 (C84912)	OR828084	OR833966	OR834045
				Dow-1.4 (C84913)	OR828085	×	×
4		Lake Ninan 1	-30.953402, 116.654574	Nin-1.1 (C84914)	OR828086	OR833967	×
				Nin-1.3 (C84915)	OR828087	×	×
				Nin-1.4 (C84916)	OR828088	×	×
5		Jurien Bay 4 (C84817)	-30.206705, 115.038112	Juri-4.1 (C84917)	OR828080	×	×
				Juri-4.2 (C84918)	OR828080	OR833968	×
6		Cowcowing 2	-30.922094, 117.363814	Mix-1.8 (C84919)	OR828089	OR833969	×
7		Wyola 2 (C84818)	-31.626042, 117.358562	Wy-2.1 (C84920)	OR828090	OR833970	OR834045
8		AF308948*	-	-	×	AF308948	×
9		AY014787*	-	-	×	AY014787	×
1	*P. cylindrifera*	Esperance 17	-33.252057, 121.931928	Esp-17.1 (C84921)	OR828091	×	×

		(C84811)		Sty-2.1 (C84922)	OR828107	OR833976	×
				Sty-2.2 (C84923)	OR828107	×	×
				Sty-2.3 (C84924)	OR828108	×	×
2		Esperance 20 (C84805)	-33.393300, 122.046633	Esp-20.1 (C84925)	OR828092	×	×
3		Esperance 21 (C84806)	-33.455407, 122.016637	Esp-21.1 (C84926)	OR828093	OR833971	OR834046
				Esp-21.3 (C84927)	OR828094	×	×
4		Esperance 30 (C84813)	-33.543448, 122.432428	Esp-30.1 (C84928)	OR828095	×	×
				Esp-30.2 (C84929)	OR828095	×	×
5		Esperance 33	-33.508491, 122.409129	Esp-33.1 (C84930)	OR828096	×	×
				Esp-33.2 (C84931)	OR828097	OR833972	×
6		Lake Varley 2 (C84810)	-32.704707, 119.358251	Var-2.1 (C84932)	OR828098	×	×
				Var-2.2 (C84933)	OR828099	×	×
7		Lake Varley 3 (C84807)	-32.708471, 119.359619	Var-3.1 (C84934)	OR828100	×	×
				Var-3.2 (C84935)	OR828100	OR833973	×
				Var-3.3 (C84936)	OR828101	×	×
8		Frankland 1 (C84808)	-34.416769, 117.252365	Frank-1.1 (C84937)	OR828102	×	×
				Frank-1.2 (C84938)	OR828103	OR833974	OR834047
9		Ravensthorpe 1 (C84809)	-33.315098, 119.814935	Rav-1.1 (C84939)	OR828104	×	×
				Rav-1.2 (C84940)	OR828105	OR833975	OR834046
				Rav-1.3 (C84941)	OR828106	×	×
10		Pingrup (C84812)	-33.670854, 118.564158	Pin-1.1 (C84942)	OR828109	OR833977	OR834048
				Pin-1.3 (C84943)	OR828110	×	×
11		Elliston	-33.632156, 134.872246	Elli-1.1 (C84944)	OR828111	OR833978	×
				Elli-1.2 (C84945)	OR828111	×	×
12		Lake Tungketta	-33.762754, 135.098527	Tung-1.1 (C84946)	OR828112	×	×
				Tung-1.2 (C84947)	OR828113	×	×

				Tung-1.3 (C84948)	OR828113	×	×
13		AF209050*	-	-	×	AF209050	×
		AF308954*	-	-	AF308954	×	×
1	*P. informis*	Cowcowing 2	-30.922094, 117.363814	Mix-1.7 (C84949)	OR828114	OR833979	×
2		Morawa 1 (C84819)	-29.448866, 115.879115	Mor-1.1 (C84950)	OR828115	×	×
				Mor-1.2 (C84951)	OR828115	OR833980	OR834049
3		Morawa 4 (C84820)	-29.184522, 116.086731	Mor-4.1 (C84952)	OR828116	OR833981	×
				Mor-4.2 (C84953)	OR828115	×	×
4		Wongan Hills 2, Lake 6 (C84821)	-30.510341, 116.709957	WH.L-6.1 (C84954)	OR828117	OR833982	×
				WH.L-6.3 (C84955)	OR828118	×	×
				WH.L-6.6 (C84956)	OR828119	×	×
5		Hut Lagoon 1 (C84822)	-28.207383, 114.287526	Hut-1.1 (C84957)	OR828120	OR833983	×
				Hut-1.2 (C84958)	OR828121	×	×
				Hut-1.3 (C84959)	OR828122	×	×
6		Maxine's Pond (C84823)	-30.367059, 117.190395	Max-1.5 (C84960)	OR828123	OR833984	×
				Max-1.6 (C84961)	OR828124	×	×
7		Lake Monger's 2 (C84824)	-29.542408, 116.709323	Mong-1.1 (C84962)	OR828125	OR833985	OR834049
				Mong-1.3 (C84963)	OR828126	×	×
8		Kalannie 2 (C84825)	-30.281587, 117.072370	Deca-1.1 (C84964)	OR828127	OR833986	OR834050
9		Three Springs 7 (C84826)	-29.577527, 115.821453	TS-7.1 (C84965)	OR828115	OR833980	×
10		Latham 4 (C84827)	-29.737895, 116.358878	Lath-4.1 (C84966)	OR828128	OR833987	×
				Lath-4.3 (C84967)	OR828129	×	×
11		Burra Lake[4]	-28.808827, 116.313526	LN30648.1	OR828130	OR833988	×
			-28.804976, 116.321268	LN31270.2	OR828115	OR833980	×
				LN31270.3	OR828115	×	×

				LN30133.1	OR828115	×	×
			-28.808827, 116.313526	LN30126.2	OR828115	×	×
				LN30126.3	OR828115	×	×
12		Lake Austin[4]	-27.609441, 117.889275	LN3108.1	OR828131	OR833989	×
				LN3108.2	OR828132	×	×
				LN3108.3	OR828131	×	×
			-27.510674, 117.810955	LN4793.1	OR828132	×	×
1	*P. laticaudata*	Lake Carey[4]	-29.235278, 122.408054	LN30050.1	OR828133	×	×
				LN30050.2	OR828134	OR833990	×
				LN30050.3	OR828133	×	×
				LN30050.4	OR828133	×	×
				LN9202.1	OR828133	×	×
				LN9202.2	OR828133	OR833991	×
				LN9202.5	OR828133	×	×
2		Coral Bay (C84828)	-23.127919, 113.786264	Bay-1.1 (C84968)	OR828135	OR833992	×
				Bay-1.2 (C84969)	OR828135	×	×
				Bay-1.3 (C84970)	OR828135	×	×
1	*P. longicaudata*	Camel Lake	-34.306644, 118.027642	Mix-1.4 (C84971)	OR828136	OR833993	OR834051
				Mix-1.5 (C84972)	OR828136	×	×
				Mix-1.6 (C84973)	OR828137	×	×
2		Cairolcup 1 (C84829)	-33.707084, 118.687531	Cup-1.1 (C84974)	OR828138	×	×
				Cup-1.2 (C84975)	OR828139	OR833994	×
				Cup-1.4 (C84976)	OR828140	OR833995	OR834051
3		Lake King-2 (C84830)	-33.090770, 119.540744	King-2.1 (C84977)	OR828141	OR833996	OR834052
				King-2.2 (C84978)	OR828142	×	×
4		Hyden 6 (C84831)	-32.454396, 119.091053	Hy-6.1 (C84979)	OR828143	OR833997	×
				Hy-6.2 (C84980)	OR828144	×	×

5		Pink Lake (C84832)	-33.838279, 121.833288	Pink-1.1 (C84981)	OR828145	OR833996	×
				Pink-1.2 (C84982)	OR828145	×	×
				Pink-1.3 (C84983)	OR828145	OR833996	×
6		Esperance 28 (C84833)	-33.514871, 121.869683	Esp-28.1 (C84984)	OR828145	OR833998	OR834053
				Esp-28.2 (C84985)	OR828145	×	×
7		Abrolhos Island (C84834)	-28.295996, 113.594432	Abro-1.1 (C84986)	OR828146	×	×
				Abro-1.2 (C84987)	OR828147	OR833999	×
				Abro-1.3 (C84988)	OR828148	×	×
8		Ravensthorpe 5 (C84835)	-33.313882, 119.812912	Rav-5.1 (C84989)	OR828149	×	×
				Rav-5.2 (C84990)	OR828149	×	×
				King-3.1 (C84991)	OR828150	OR834000	OR834052
9		Three Springs 5 (C84836)	-29.783126, 115.871530	TS-5.1 (C84992)	OR828151	×	×
				TS-5.2 (C84993)	OR828152	OR834001	OR834054
10		Lake Magenta 1 (C84837)	-33.577858, 119.229112	Mag-1.1 (C84994)	OR828153	OR834002	OR834051
				Mag-1.2 (C84995)	OR828153	×	×
11		Lake Magenta 4	-33.585244, 119.199468	Mag-4.1 (C84996)	OR828153	OR834003	OR834055
				Mag-4.2 (C84997)	OR828153	×	×
12		Lake Magenta 7	-33.196573, 119.075532	Mag-7.1 (C84998)	OR828154	OR834004	×
				Mag-7.2 (C84999)	OR828155	×	×
13		Lake Grace 2 (C84838)	-32.955887, 118.505980	Grace-2.1 (C85000)	OR828154	OR834004	OR834052
				Grace-2.2 (C85001)	OR828156	×	×
14		Lake Grace 1 (C84839)	-33.107453, 118.377491	Grace-1.1 (C85002)	OR828157	OR834005	OR834051
				Grace-1.2 (C85003)	OR828158	×	×
15		Bendering Road 1 (C84840)	-32.380481, 118.157547	Ben-1.1 (C85004)	OR828155	OR834004	×
				Ben-1.2 (C85005)	OR828154	×	×
				Ben-1.3 (C85006)	OR828159	×	×
16		AF209049*	-	-	×	AF209049	×

1	*P. minuta*	EF189613*	-	-	×	EF189613	×
		EF189656*	-		×	×	EF189656
2		AF308949*	-	-	×	AF308949	×
1	*P. mouritzi*	Hyden 9 (C84845)	-32.462249, 119.174969	Hy-9.1 (C85007)	OR828160	OR834006	OR834056
				Hy-9.2 (C85008)	OR828160	OR834007	×
				Hy-9.3 (C85009)	OR828160	×	×
1	*P. purpurea*	Esperance 1	-33.318431, 121.927802	Esp-1.1 (C85010)	OR828161	OR834008	OR834057
2		Esperance 4	-33.516317, 121.876323	Esp-4.1 (C85011)	OR828162	OR834009	OR834057
3		Esperance 7 (C84846)	-33.539844, 122.430503	Esp-7.1 (C85012)	OR828163	×	×
				Esp-7.3 (C85013)	OR828164	OR834010	OR834058
4		Esperance 19 (C84850)	-33.390527, 122.044960	Esp-19.1 (C85014)	OR828165	OR834011	OR834059
				Esp-19.2 (C85015)	OR828166	×	×
5		Esperance 9 (C84849)	-33.455955, 122.608653	Esp-9.3 (C85016)	OR828167	OR834012	OR834060
				Esp-9.4 (C85017)	OR828168	OR834013	×
				Esp-9.5 (C85018)	OR828168	×	×
6		Esperance 3 (C84851)	-33.481497, 121.696884	Esp-3.2 (C85019)	OR828169	OR834014	OR834057
				Esp-3.4 (C85020)	OR828170	×	×
7		Esperance 29 (C84847)	-33.446943, 122.197356	Esp-29.1 (C85021)	OR828171	×	×
				Esp-29.3 (C85022)	OR828172	OR834015	OR834061
8		Esperance 31 (C84848)	-33.531295, 122.426558	Esp-31.1 (C85023)	OR828173	OR834016	OR834057
				Esp-31.2 (C85024)	OR828173	×	×
				Esp-31.4 (C85025)	OR828174	×	×
9		Esperance 36 (C84852)	-33.482778, 122.010556	Esp-36.1 (C85026)	OR828175	OR834017	OR834062
				Esp-36.3 (C85027)	OR828176	×	×
1	*P. serventyi*	Lake Varley 4 (C84853)	-32.765616, 119.398004	Var-4.1 (C85028)	OR828177	OR834018	×
				Var-4.2 (C85029)	OR828178	×	×
				Var-4.3 (C85030)	OR828177	×	×

2		Corrigin 1 (C84854)	-32.08701, 118.144322	Com-1.1 (C85031)	OR828179	OR834019	×
				Com-1.2 (C85032)	OR828180	×	×
				Com-1.3 (C85033)	OR828181	×	×
3		Yerding 1 (C84855)	-31.92715, 117.979964	Yerd-1.1 (C85034)	OR828181	OR834020	×
				Yerd-1.2 (C85035)	OR828181	×	×
				Yerd-1.3 (C85036)	OR828182	×	×
4		Hyden 3 (C84856)	-32.415574, 119.085077	Hy-3.1 (C85037)	OR828183	OR834021	×
				Hy-3.2 (C85038)	OR828183	×	×
				Hy-3.3 (C85039)	OR828184	×	×
5		Esperance Pond A (C84857)	-33.081987, 121.685399	Pond-A.1 (C85040)	OR828185	OR834022	×
				Pond-A.2 (C85041)	OR828185	×	×
				Pond-A.3 (C85042)	OR828185	×	×
6		Lake Varley 5 (C84858)	-32.810349, 119.424893	Var-5.1 (C85043)	OR828186	OR834023	OR834063
				Var-5.2 (C85044)	OR828186	×	×
				Var-5.3 (C85045)	OR828186	×	×
7		Hyden 7 (C84859)	-32.462961, 119.160903	Hy-7.1 (C85046)	OR828187	×	×
				Hy-7.2 (C85047)	OR828188	OR834024	×
8		Pontifex Road 2 (C84860)	-31.587377, 117.967899	Ponti-2.3 (C85048)	OR828189	OR834025	OR834064
				Ponti-2.4 (C85049)	OR828190	×	×
9		Hines Hill 1 (C84861)	-31.517009, 118.062735	Hines-1.3 (C85050)	OR828191	×	×
				Hines-1.4 (C85051)	OR828192	OR834026	OR834064
10		Lake Magenta 5 (C84862)	-33.442913, 119.266142	Mag-5.1 (C85052)	OR828193	×	×
				Mag-5.2 (C85053)	OR828194	OR834027	OR834065
11		Mount Stirling (C84863)	-31.823409, 117.592579	MS-1.2 (C85054)	OR828179	OR834019	×
12		Hyden 2 (C84864)	-32.415375, 119.0866	Hy-2.1 (C85055)	OR828183	OR834028	×

13		Lake Lefroy[4]	-31.472584, 121.758688	LN31044.2	OR828195	OR834029	×
				LN31044.3	OR828196	×	×
			-31.469333, 121.755552	AQ16001.1	OR828197	×	×
				AQ16001.2	OR828198	OR834030	×
1	*P. triquetra*	C77314[3]	-28.019722, 129.026944	C77314.1	×	OR834031	×
1	*P. veronicae*	C77310 and C77311[3]	-32.069444, 122.137222	C77310.1	×	OR834032	×
				C77311.1	×	OR834033	×
2		ADS029[2]	-31.110000, 122.344722	ADS29.1	OR828199	×	OR834066
1	*P. zietziana*	G5291[1]	-42.1468, 147.4283	G5291.1	OR828200	OR834034	OR834067
				G5291.2	OR828201	OR834035	×
				TAS-1.3	OR828200	×	×
2		Lake Hamilton	-34.022875, 135.281496	Hami-1.1 (C85056)	OR828202	OR834036	×
				Hami-1.2 (C85057)	OR828203	×	×
				Hami-1.3 (C85058)	OR828202	×	×
1	*Parartemia* sp. ‘y’	Esperance 22 (C84866)	-33.473564, 122.355036	Esp-22.1 (C85059)	OR828204	OR834037	OR834068
				Esp-22.2 (C85060)	OR828205	×	×
				Esp-22.3 (C85061)	OR828206	×	×
2		Esperance 25 (C84865)	-33.486080, 122.636330	Esp-25.1 (C85062)	×	OR834038	×
				Esp-25.2 (C85063)	OR828207	OR834038	×
				Esp-25.3 (C85064)	OR828207	OR834038	×
				Esp-25.4 (C85065)	OR828207	×	×
1	*Parartemia* sp. ‘z’	Esperance 23 (C84867)	-33.473025, 122.353382	Esp-23.1 (C85066)	OR828208	OR834039	OR834069
				Esp-23.3 (C85067)	OR828208	×	×
				Esp-23.5 (C85068)	OR828208	OR834039	×

*Sequences obtained from GenBank. Specimens provided by [1]the Tasmanian Museum and Art Gallery (TMAG), [2]the Department of Biodiversity, Conservation and Attractions (DBCA), [3]the Western Australian Museum (WAM), and [4]Stantec Australia Pty Ltd.

Table S3.2: Species-specific primers used for amplifying the *28S* genetic region in *Parartemia*. Primer sequences are in Table 3.1.

Morphospecies	**Forward primer**	**Reverse primer**
Parartemia acidiphila	28S71	28S32
Parartemia bicorna	28S71	28S32
Parartemia boomeranga	28S11	28S32
Parartemia contracta	28S71	28S32
Parartemia extracta	28S11	28S32
Parartemia cylindrifera	28S11	28S32
Parartemia informis	28S11	28S32
Parartemia longicaudata	28S11	28S32
Parartemia mouritzi	28S71	28S32
Parartemia purpurea	28S71	28S32
Parartemia serventyi	28S71	28S32
Parartemia veronicae	28S11	28S32
Parartemia zietziana	28S71	28S32
Parartemia sp. ‘y’	28S71	28S32
Parartemia sp. ‘z’	28S11	28S32

Table S3.3: Species-specific primers used for amplifying the *COI* genetic region in *Parartemia*. Primer sequences are in Table 3.1.

Morphospecies	**Forward primer**	**Reverse primer**
Parartemia acidiphila	COI101	HCO2198
	Facid-1	HCO2198
Parartemia bicorna	LCO1490	HCO2198
Parartemia boomeranga	COI101	COI102
	Fboom-2	Rboom-2
	Flong-2	Rlong-1
Parartemia contracta	LCO1490	HCO2198
Parartemia extracta	LCO1490	HCO2198
	LCOPara	HCO2198
	LCOPara	COI102
	COI101	HCO2198
	COI101	HCOPara
	Fext-2	Rinfo-1
Parartemia cylindrifera	LCO1490	HCO2198
Parartemia informis	LCO1490	HCO2198
	LCOPara	HCOPara
	LCOPara	COI102
	Fext-2	Rinfo-2
	Finfo-1	Rinfo-1
Parartemia laticaudata	LCO1490	HCO2198
Parartemia longicaudata	COI101	COI102
	COI101	HCOPara
	Flong-1	Rlong-1
	Flong-2	Rlong-1
Parartemia mouritzi	Finfo-1	HCO2198
Parartemia purpurea	LCO1490	HCO2198
	Finfo-3	HCO2198
	Flong-2	Rlong-1
	Fser-1	HCO2198
Parartemia serventyi	LCO1490	HCO2198
	COI101	HCO2198
	Finfo-3	HCO2198
	Fser-1	HCO2198
Parartemia zietziana	Finfo-1	HCO2198
Parartemia sp. ‘y’	LCO1490	HCO2198
Parartemia sp. ‘z’	COI101	COI102

Table S3.4: Details of outgroup data used for *28S*, *16S*, *COI* and concatenated phylogenetic analyses.

Taxa	Genetic region	GenBank accession number
For *28S* dataset		
Artemia salina	*28S*	AF169697
Artemia salina	*28S*	X90461
Artemia sp.	*28S*	AY210805
Thamnocephalus platyurus	*28S*	AF209046
Branchinella occidentalis	*28S*	AY744895
Eubranchipus sp.	*28S*	AF209044
Artemiopsis stefanssoni	*28S*	AF209045
Lepidurus arcticus	*28S*	AF209047
Triops australiensis	*28S*	EF189662
Lynceus biformis	*28S*	EF189653
Eulimnadia texana	*28S*	AY851444
Leptestheria kawachiensis	*28S*	EF189649
Cyclestheria hislopi	*28S*	AF532878
Moina affinis	*28S*	AF532882
Chydorus sphaericus	*28S*	AF532891
Acantholeberis curvirostris	*28S*	AF532890
Ilyocryptus sp.	*28S*	AF532892
Ceriodaphnia sp.	*28S*	AF532889
Daphnia magna	*28S*	EU370436
Cypridopsis uenoi	*28S*	AB674997
Cypretta seurati	*28S*	AB675000
For *16S* dataset		
A. franciscana	*16S*	MF563560
Artemia salina	*16S*	FJ007838
For *COI* dataset		
Artemia salina	*COI*	DQ426856
Artemia salina	*COI*	DQ426858
For concatenated dataset		
Artemia salina	*28S*, *16S* and *COI*	AF169697, FJ007838, MT495441
Thamnocephalus platyurus	*28S*, *16S* and *COI*	AF209046, AF209057, AF209066

Table S3.5: Minimum and maximum pairwise *28S* genetic distances (*p*-distance, %) matrix for *Parartemia* species, confirmed in this study. 1: *P. acidiphila*, 2: *P. bicorna*, 3: *P. contracta*, 4: *P. cylindrifera*, 5: *P. extracta*, 6: *P. informis*, 7: *P. longicaudata*/*boomeranga*, 8: *P. minuta*, 9: *P. mouritzi*, 10: *P. purpurea* (a), 11: *P. purpurea* (b), 12: *P. purpurea* (c), 13: *P. serventyi*, 14: *P. veronicae*, 15: *P. zietziana*, 16: *Parartemia* sp. 'y', and 17: *Parartemia* sp. 'z'.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
2	0.59															
3	0.15	0.73														
4	1.17-1.32	1.47-1.61	1.03-1.17													
5	0.59-0.73	1.17-1.32	0.73-0.88	1.17-1.61												
6	0.59-0.73	0.88-1.03	0.73-0.88	1.47-1.91	0.59-0.88											
7	0.59-0.88	1.17-1.32	0.73-1.03	1.47-1.91	0.59-1.03	0.29-0.73										
8	1.76	1.76	1.62	1.17-1.47	1.76-1.91	1.91-2.06	2.06-2.20									
9	2.21	2.06	2.21	1.62-1.77	2.51	2.65-2.80	2.51-2.80	1.91								
10	0.59-0.73	0.88-1.03	0.73-0.88	0.44-1.02	1.03-1.32	1.03-1.32	0.88-1.32	1.61-1.76	1.62-1.91							
11	1.32	1.62	1.47	0.88-1.17	1.47-1.62	1.62-1.76	1.62-1.76	2.05	2.21	0.59-1.03						
12	0.59-0.88	0.88-1.17	0.73-1.03	0.59-0.88	1.17-1.32	1.17-1.32	1.17-1.32	1.47-1.76	1.62-1.91	0.00-0.59	0.73					
13	0.00-0.15	0.59-0.73	0.15-0.29	1.17-1.47	0.59-0.88	0.59-0.88	0.59-1.03	1.76-1.91	2.21-2.36	0.59-0.88	1.32-1.47	0.59-1.03				
14	0.29	0.29	0.44	1.17-1.32	0.88-1.03	0.88-1.03	0.88-1.03	1.76	2.21	0.59-0.73	1.32	0.59-0.88	0.29-0.44			
15	0.88	1.17	1.03	0.44-0.73	1.03-1.17	1.17-1.32	1.17-1.32	1.61	1.77	0.15-0.59	0.44	0.29	0.88-1.03	0.88		
16	0.88	1.17	1.03	0.59-0.73	1.17-1.32	1.47-1.61	1.47-1.61	1.47	1.77	0.59-0.73	1.17	0.59-0.88	0.73-1.03	0.88	0.73	
17	0.29	0.88	0.44	1.17-1.47	0.29-0.44	0.29-0.44	0.29-0.59	1.76	2.51	0.73-0.88	1.32	0.88	0.29-0.44	0.59	0.88	1.17

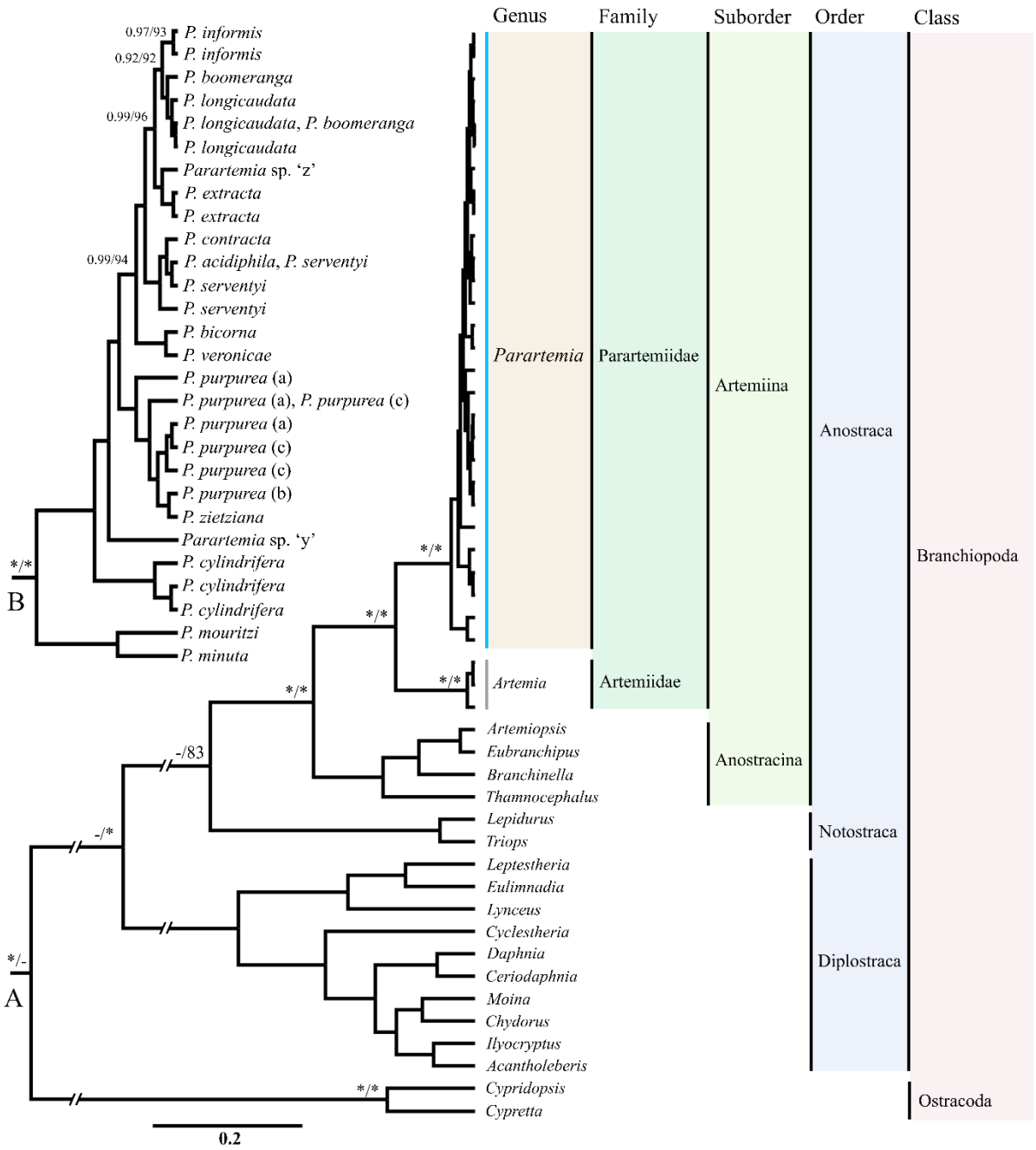

Fig. S3.1: Bayesian inference (BI) phylogenetic tree of *Parartemia 28S* haplotypes. A) whole tree and B) close up of branch containing *Parartemia* haplotypes. Bayesian Posterior Probability (BPP, when ≥ 0.80) are indicated at nodes, as are the bootstrap values from maximum likelihood (ML) phylogenetic analysis (when ≥ 80%) (BPP/bootstrap). The ML tree is not presented. For nodes where one value was above the threshold and the other was below, the latter is indicated by hyphen '-'. BPP values of 1 and bootstraps of 100% are indicated by asterisks '*'. GenBank accession numbers of *Parartemia* haplotypes are in supplementary Table S3.1 and of other haplotypes in Table S3.4.

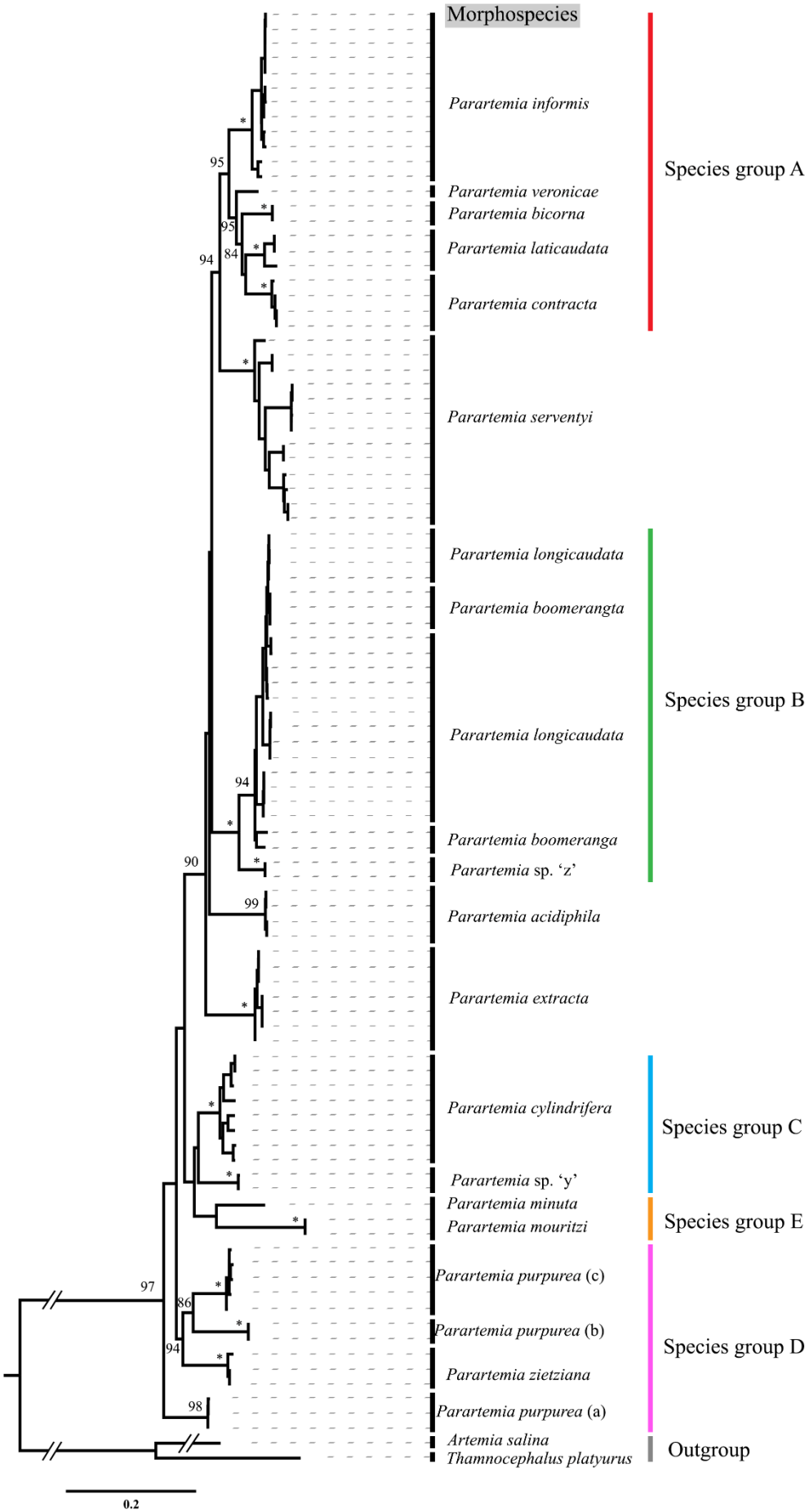
Morphospecies
Parartemia informis
Parartemia veronicae
Parartemia bicorna
Parartemia laticaudata
Parartemia contracta
Parartemia serventyi
Parartemia longicaudata
Parartemia boomerangta
Parartemia longicaudata
Parartemia boomeranga
Parartemia sp. 'z'
Parartemia acidiphila
Parartemia extracta
Parartemia cylindrifera
Parartemia sp. 'y'
Parartemia minuta
Parartemia mouritzi
Parartemia purpurea (c)
Parartemia purpurea (b)
Parartemia zietziana
Parartemia purpurea (a)
Artemia salina
Thamnocephalus platyurus
Species group A
Species group B
Species group C
Species group E
Species group D
Outgroup
95
95
84
94
94
90
99
97
86
94
98
0.2

Fig. S3.2: Maximum likelihood (ML) phylogenetic tree of *Parartemia* based on the concatenated dataset (*COI*, *16S* and *28S*). Bootstrap values ≥ 80% are indicated at nodes. Nodes with 100% bootstrap support are indicated by asterisks '*'. Taxa are identified by their morphospecies names. Coloured bars to the right of the figure indicate species groups identified within *Parartemia* in this study (see text).

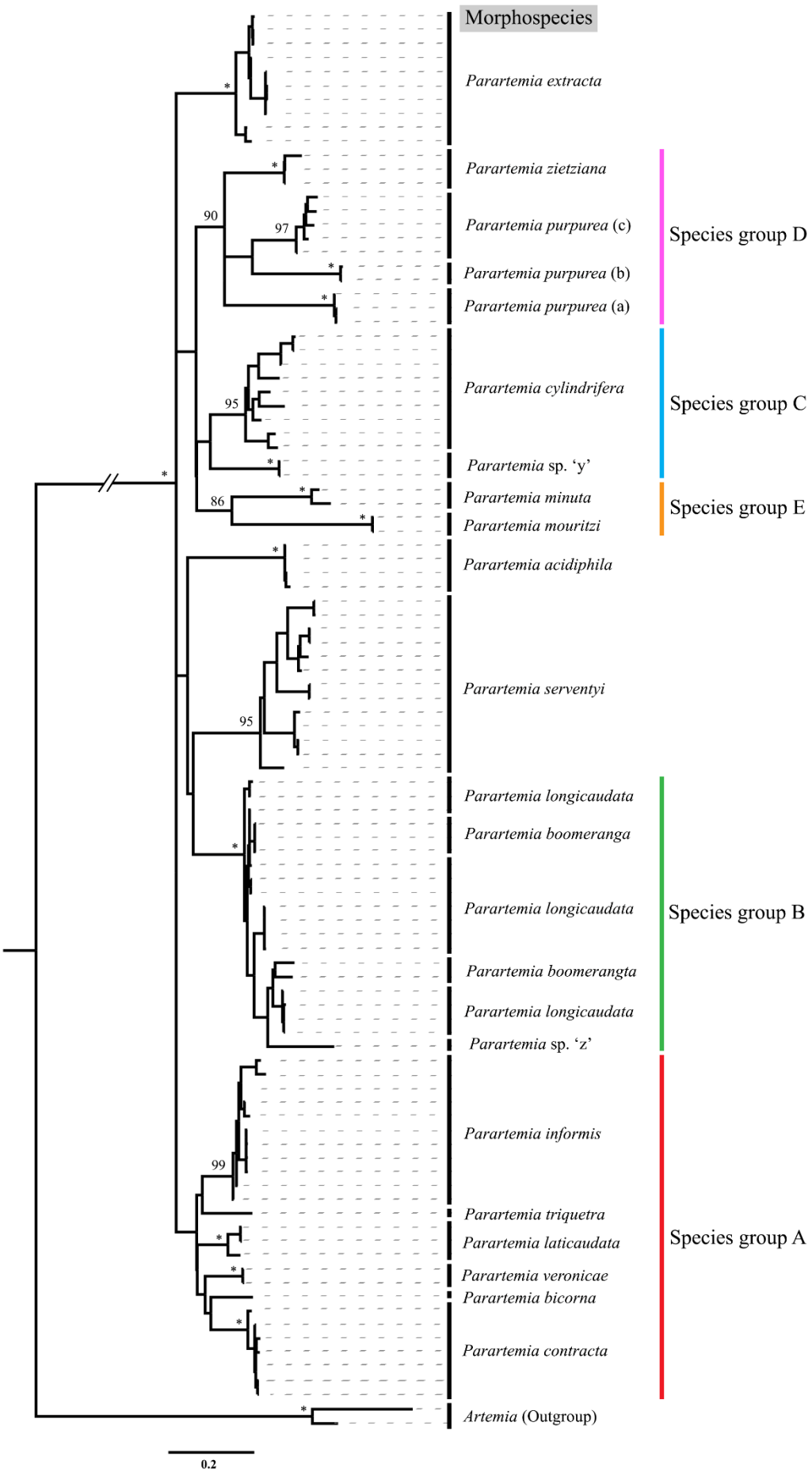
Morphospecies
Parartemia extracta
Parartemia zietziana
Parartemia purpurea (c)
Parartemia purpurea (b)
Parartemia purpurea (a)
Parartemia cylindrifera
Parartemia sp. 'y'
Parartemia minuta
Parartemia mouritzi
Parartemia acidiphila
Parartemia serventyi
Parartemia longicaudata
Parartemia boomeranga
Parartemia longicaudata
Parartemia boomerangta
Parartemia longicaudata
Parartemia sp. 'z'
Parartemia informis
Parartemia triquetra
Parartemia laticaudata
Parartemia veronicae
Parartemia bicorna
Parartemia contracta
Artemia (Outgroup)
Species group D
Species group C
Species group E
Species group B
Species group A
90
97
95
86
95
99
0.2

Fig. S3.3: Maximum likelihood (ML) phylogenetic tree of *Parartemia 16S* haplotypes. Bootstrap values ≥ 80% are indicated at nodes. Nodes with 100% bootstrap support are indicated by asterisks '*'. Taxa are identified by their morphospecies names. Coloured bars to the right of the figure indicate species groups identified within *Parartemia* in this study (see text).

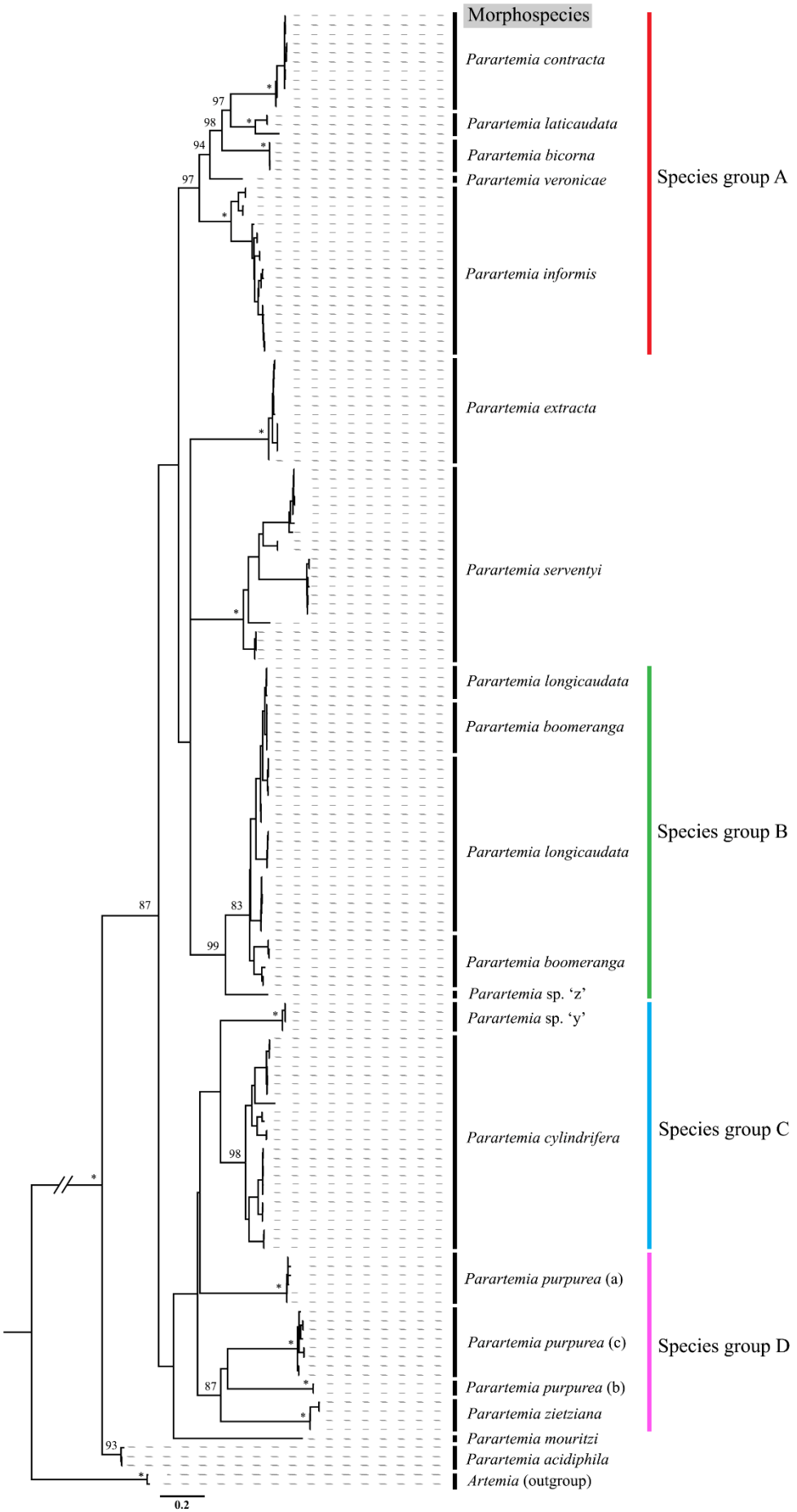
Morphospecies
Parartemia contracta
Parartemia laticaudata
Parartemia bicorna
Parartemia veronicae
Parartemia informis
Parartemia extracta
Parartemia serventyi
Parartemia longicaudata
Parartemia boomeranga
Parartemia longicaudata
Parartemia boomeranga
Parartemia sp. 'z'
Parartemia sp. 'y'
Parartemia cylindrifera
Parartemia purpurea (a)
Parartemia purpurea (c)
Parartemia purpurea (b)
Parartemia zietziana
Parartemia mouritzi
Parartemia acidiphila
Artemia (outgroup)
Species group A
Species group B
Species group C
Species group D
97
98
94
97
87
83
99
98
87
93
0.2

Fig. S3.4: Maximum likelihood (ML) phylogenetic tree of *Parartemia COI* haplotypes. Bootstrap values ≥ 80% are indicated at nodes. Nodes with 100% bootstrap support are indicated by asterisks '*'. Taxa are identified by their morphospecies names. Coloured bars to the right of the figure indicate species groups identified within *Parartemia* in this study (see text). Species group E is not shown due to the absence of *COI* data for *P. minuta*.

Table S3.6: Minimum and maximum pairwise genetic distances (*p*-distance, %) matrix between 19 species of *Parartemia* (confirmed in this study) based on *16S* (below the diagonal) and *COI* (above the diagonal) markers. 1: *P. acidiphila*, 2: *P. bicorna*, 3: *P. contracta*, 4: *P. cylindrifera*, 5: *P. extracta*, 6: *P. informis*, 7: *P. laticaudata*, 8: *P. longicaudata*/*boomeranga*, 9: *P. minuta*, 10: *P. mouritzi*, 11: *P. purpurea* (a), 12: *P. purpurea* (b), 13: *P. purpurea* (c), 14: *P. serventyi*, 15: *P. triquetra*, 16: *P. veronicae*, 17: *P. zietziana*, 18: *Parartemia* sp. 'y', and 19: *Parartemia* sp. 'z'.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1		19.8-20.4	20.1-21.1	18.5-21.1	19.2-20.4	18.8-20.8	20.7-22.0	17.3-19.5	-	19.9-20.4	20.2-21.3	19.8-20.1	19.3-20.2	18.2-21.3	-	18.5-18.8	19.5-21.9	21.1-22.5	18.4-18.7
2	14.1-14.9		16.6-17.8	19.0-21.6	18.5-19.8	16.4-18.7	16.4-17.6	19.6-22.0	-	21.9-22.0	19.6-20.1	19.5-19.8	20.1-21.6	17.2-20.7	-	15.8-16.0	19.9-20.8	20.5-21.4	18.1-18.2
3	13.6-14.9	10.0-11.3		19.0-22.0	18.4-21.0	15.8-18.8	14.7-16.1	18.2-21.4	-	21.0-22.5	21.6-23.7	19.5-20.4	21.1-22.6	17.6-22.3	-	15.7-16.3	19.3-21.0	20.4-21.4	21.6-22.3
4	14.3-17.8	13.4-15.9	15.1-18.0		18.4-22.0	17.2-21.7	18.4-21.9	17.6-22.3	-	18.8-21.1	17.9-21.6	17.9-20.5	18.1-21.1	18.2-23.7	-	17.3-20.7	17.6-20.7	15.7-17.9	19.2-20.7
5	15.1-17.8	13.8-15.1	12.8-15.1	13.0-18.0		17.9-23.0	18.2-19.9	18.2-21.0	-	21.3-22.2	20.1-21.7	21.0-22.0	19.0-20.8	18.1-21.0	-	18.7-19.6	19.9-21.7	19.8-20.8	19.6-20.8
6	13.8-15.7	10.0-11.1	10.3-13.0	11.5-17.2	12.1-14.9		15.8-19.9	16.0-20.7	-	20.7-22.8	18.7-21.1	18.7-22.2	19.8-22.2	16.0-21.6	-	15.4-17.8	19.2-21.9	18.4-21.1	17.8-19.3
7	15.1-17.0	9.9-10.3	11.1-12.0	14.0-16.5	13.0-14.0	9.4-11.1		17.0-19.8	-	21.3-21.4	21.1-23.4	19.3-20.2	20.8-24.0	18.1-22.0	-	14.4-15.8	19.2-19.6	20.8-22.0	17.9-18.5
8	13.8-17.0	13.8-16.1	12.1-15.9	12.3-18.0	13.6-16.7	10.7-15.5	12.3-14.6		-	21.1-22.3	19.0-21.3	18.2-21.3	20.5-22.8	17.3-22.6	-	16.9-19.6	18.4-21.6	19.0-21.6	14.0-16.3
9	16.8-17.2	15.5-16.3	16.3-17.0	14.3-16.7	15.7-18.0	15.1-17.2	15.7-17.2	14.2-17.8		-	-	-	-	-	-	-	-	-	-
10	18.7-19.5	15.1	17.2-18.0	15.7-19.5	17.2-18.2	14.7-16.4	15.1-15.7	17.8-19.3	16.6-16.8		19.5-19.9	21.4-21.7	19.0-20.2	19.5-21.3	-	19.9	20.4-20.5	19.0-19.5	21.0
11	18.7-19.5	16.1-16.4	16.8-18.0	13.2-17.2	15.5-17.6	17.0-19.3	17.0-17.7	16.8-19.7	15.9-16.8	19.3-19.8		20.5-21.6	19.3-21.4	19.9-22.5	-	20.5-21.0	19.6-21.3	17.6-19.2	20.5-21.1
12	17.6-18.5	15.3	16.1-17.0	14.6-16.7	16.3-17.6	15.9-18.2	14.9-16.3	15.5-17.6	15.1-16.5	19.1-19.5	14.3-14.7		18.2-19.8	21.3-24.3	-	18.8-19.0	18.7-19.3	20.2-20.8	18.2-18.7
13	15.9-17.4	13.6-14.3	13.8-16.4	12.4-15.1	13.8-16.8	11.7-14.7	12.4-15.7	14.0-17.6	15.5-17.6	16.4-17.4	14.9-16.1	13.6-15.1		21.3-25.2	-	20.2-21.7	17.8-19.5	20.1-21.3	21.0-21.7
14	14.5-18.7	13.2-15.5	10.5-15.1	14.9-18.0	14.6-18.0	11.1-16.5	11.1-16.3	12.8-17.0	15.5-18.0	16.1-19.5	17.4-20.3	14.9-17.2	14.9-19.1		-	17.6-20.5	18.4-22.3	18.1-22.0	19.0-21.9
15	14.7-15.3	11.3	9.6-10.5	14.3-17.4	12.8-14.4	9.2-10.7	10.3-11.1	11.7-14.0	17.6-18.6	15.9	18.0	16.7-17.2	14.1-14.9	13.0-15.9		-	-	-	-
16	12.6-13.2	9.9-10.1	9.9-11.7	13.4-17.6	12.4-14.7	8.4-9.9	9.2-10.3	10.7-13.4	16.1-16.4	14.9-15.1	16.6-17.0	16.1-16.4	13.2-14.5	12.4-15.7	12.0-12.2		20.2-21.0	21.1-21.6	19.5

17	13.7-14.5	13.6-14.6	13.2-14.6	12.8-17.2	14.4-16.4	14.0-15.1	13.8-14.7	14.2-16.4	15.3-15.9	15.7-16.4	14.3-15.1	14.2-16.1	12.2-14.3	15.3-18.9	13.0-13.4	13.7-14.3		19.5-20.4	19.5-19.9
18	15.9-16.6	14.2-14.4	15.5-16.3	12.2-14.4	14.2-15.7	13.4-14.9	14.3-15.7	14.4-15.9	14.9-15.3	17.0-17.2	16.4-16.8	15.3-15.9	14.5-15.7	14.7-17.0	15.3-15.5	14.9-15.3	15.1-15.7		20.1-20.4
19	16.8-17.6	14.2	13.0-14.6	13.6-17.4	14.2-15.1	13.8-15.7	15.3-15.9	9.4-11.5	16.3-17.6	17.8-18.0	17.6-17.8	18.2	14.9-16.4	14.7-16.3	14.9	14.1-14.3	15.1-16.1	15.3-15.5	

Table S3.7: Minimum and maximum pairwise genetic distances (K2P distance, %) matrix between 19 species of *Parartemia* (confirmed in this study) based on *16S* (below the diagonal) and *COI* (above the diagonal) markers. 1: *P. acidiphila*, 2: *P. bicorna*, 3: *P. contracta*, 4: *P. cylindrifera*, 5: *P. extracta*, 6: *P. informis*, 7: *P. laticaudata*, 8: *P. longicaudata*/*boomeranga*, 9: *P. minuta*, 10: *P. mouritzi*, 11: *P. purpurea* (a), 12: *P. purpurea* (b), 13: *P. purpurea* (c), 14: *P. serventyi*, 15: *P. triquetra*, 16: *P. veronicae*, 17: *P. zietziana*, 18: *Parartemia* sp. 'y', and 19: *Parartemia* sp. 'z'.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1		23.6-24.5	24.0-25.7	21.7-25.6	22.7-24.5	22.4-25.2	25.0-27.1	20.2-23.2	-	23.5-24.2	24.2-25.8	23.3-23.8	22.7-24.1	21.4-25.7	-	21.9-22.3	22.8-26.5	25.6-27.7	21.6-22.1
2	15.8-17.0		19.4-21.1	22.3-26.1	21.8-23.6	19.0-22.2	19.2-20.9	23.3-27.1	-	26.6-26.8	23.3-24.0	23.0-23.4	23.8-26.1	19.7-24.7	-	18.2-18.4	23.5-24.9	24.4-25.8	21.0-21.3
3	15.3-17.0	11.0-12.5		22.3-26.9	21.6-25.4	18.1-22.5	16.9-18.8	21.2-26.1	-	25.1-27.4	26.1-29.5	22.8-24.2	25.3-27.6	20.6-27.6	-	18.1-19.0	22.6-25.1	24.2-25.8	26.2-27.4
4	16.0-20.7	15.0-18.2	17.5-21.2		21.4-26.8	19.9-26.7	21.5-26.5	20.4-27.6	-	21.9-25.3	21.0-26.6	21.0-24.7	21.2-25.8	21.2-29.3	-	20.0-24.8	20.5-24.8	17.9-21.0	22.5-24.8
5	17.2-20.9	15.5-17.1	14.2-17.2	14.5-21.1		20.9-28.5	21.4-23.8	21.5-25.5	-	25.7-27.1	23.9-26.4	25.3-27.0	22.3-25.0	21.2-25.5	-	22.1-23.5	23.7-26.4	23.6-25.2	23.7-25.6
6	15.5-17.9	10.9-12.1	11.2-14.5	12.6-19.9	13.3-16.7		18.1-24.0	18.4-25.2	-	24.6-27.9	21.9-25.7	21.9-27.1	23.4-27.1	18.3-26.4	-	17.6-21.1	22.4-26.5	21.4-25.6	20.8-23.0
7	17.1-19.7	10.7-11.2	12.2-13.3	15.7-19.0	14.4-15.7	10.1-12.1		19.7-23.6	-	25.6-25.7	25.4-28.9	22.7-24.0	25.0-30.0	21.3-27.0	-	16.4-18.2	22.5-23.2	24.9-26.8	21.0-21.9
8	15.4-19.8	15.5-18.5	13.4-18.3	13.7-21.0	15.2-19.3	11.5-17.6	13.6-16.5		-	25.3-27.2	22.1-25.7	21.2-25.7	24.4-27.9	20.0-28.1	-	19.4-23.3	21.4-26.1	22.4-26.1	16.1-19.2
9	19.2-19.9	17.6-18.7	18.7-19.6	16.0-19.3	17.9-21.1	17.0-19.8	17.8-19.8	15.9-20.8		-	-	-	-	-	-	-	-	-	-
10	21.8-23.1	17.0	19.7-20.9	17.8-23.0	19.8-21.2	16.4-18.6	17.0-17.8	20.6-22.7	19.0-19.3		22.9-23.5	25.8-26.2	22.3-24.0	22.9-25.6	-	23.6	24.0-24.2	22.3-22.9	25.3
11	21.8-23.1	18.4-18.7	19.3-21.1	14.8-20.1	17.6-20.4	19.5-22.8	19.6-20.5	19.3-23.4	18.2-19.4	22.8-23.4		24.6-26.2	22.8-25.9	23.5-27.4	-	24.6-25.3	23.1-25.6	20.4-22.7	24.4-25.3
12	20.3-21.4	17.4	18.4-19.5	16.5-19.3	18.6-20.3	18.1-21.2	16.8-18.7	17.5-20.4	17.0-19.0	22.4-23.1	16.0-16.5		21.4-23.6	25.6-30.4	-	22.1-22.4	21.9-22.8	24.1-25.0	21.2-21.9
13	18.2-20.3	15.3-16.1	15.5-18.9	13.6-17.1	15.5-19.3	12.8-16.6	13.7-18.0	15.7-20.5	17.6-20.5	18.6-20.1	16.8-18.5	15.3-17.1		25.5-32.0	-	24.1-26.5	20.7-23.1	24.0-25.7	25.2-26.3
14	16.2-21.9	14.8-17.8	11.4-17.2	16.9-21.1	16.4-21.0	12.0-19.1	12.1-18.8	14.1-19.6	17.6-21.1	18.4-23.0	20.3-24.6	16.8-19.9	16.8-22.6		-	20.7-24.9	21.4-27.2	21.1-26.8	22.5-27.0
15	16.6-17.4	12.3	10.4-11.4	16.0-20.2	14.1-16.2	9.9-11.6	11.1-12.1	12.8-15.7	20.4-21.8	18.0	20.9	19.1-19.7	15.7-16.7	14.4-18.2		-	-	-	-
16	14.0-14.8	10.7-11.0	10.8-13.2	14.9-20.5	13.7-16.6	9.0-10.7	10.0-11.2	11.6-15.1	18.4-18.7	16.7-17.0	19.0-19.6	18.4-18.7	14.7-16.4	13.7-18.0	13.3-13.5		24.0-25.2	25.5-26.2	23.3

17	15.2-16.3	15.2-16.5	14.7-16.6	14.2-20.1	16.2-18.8	15.7-17.1	15.4-16.5	15.9-18.8	17.3-18.2	17.8-18.7	16.1-17.2	16.0-18.6	13.5-16.3	17.4-22.3	14.4-14.9	15.3-16.1		22.9-24.2	22.9-23.5
18	18.2-19.0	16.1-16.4	17.8-19.0	13.6-16.4	15.9-17.9	14.9-16.8	16.0-18.0	16.3-18.3	16.8-17.4	19.5-19.8	18.8-19.4	17.3-18.2	16.3-18.0	16.7-19.7	17.3-17.6	16.9-17.5	17.1-18.1		23.9-24.4
19	19.2-20.4	16.1	14.4-16.6	15.1-20.2	15.9-17.0	15.4-17.8	17.5-18.3	10.1-12.7	18.6-20.4	20.6-20.9	20.5-20.8	21.1	16.7-18.7	16.7-18.7	16.7	15.8-16.1	17.0-18.5	17.4-17.7	

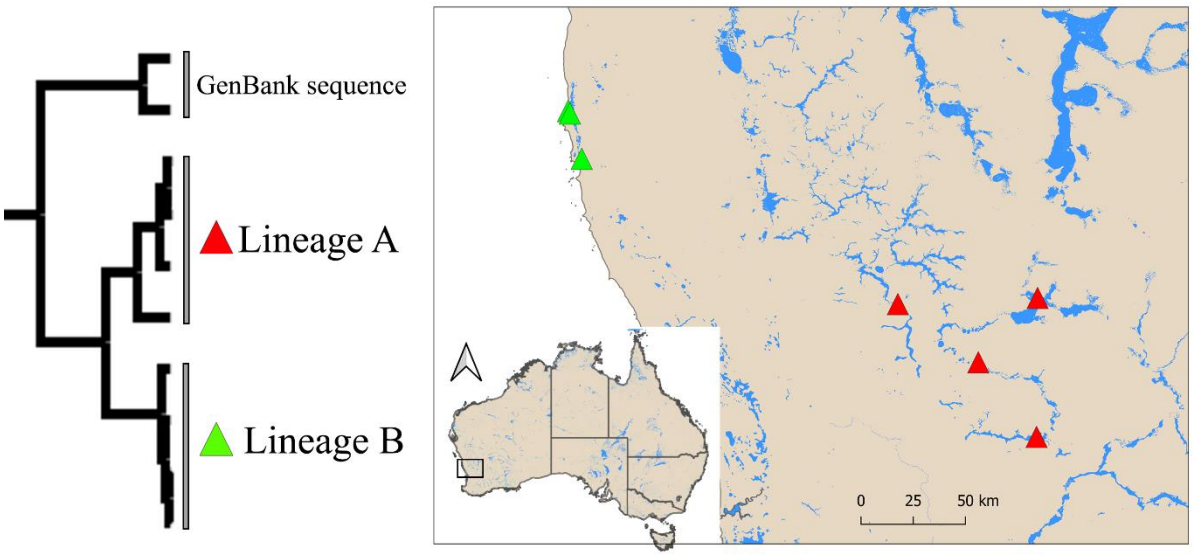

Fig. S3.5: Distributions of major *16S* genetic lineages in *P. extracta*. An excerpt from the *16S* BI tree (Fig. 3.3) has been included to identify the lineages. See supplementary Table S3.1 for site details.

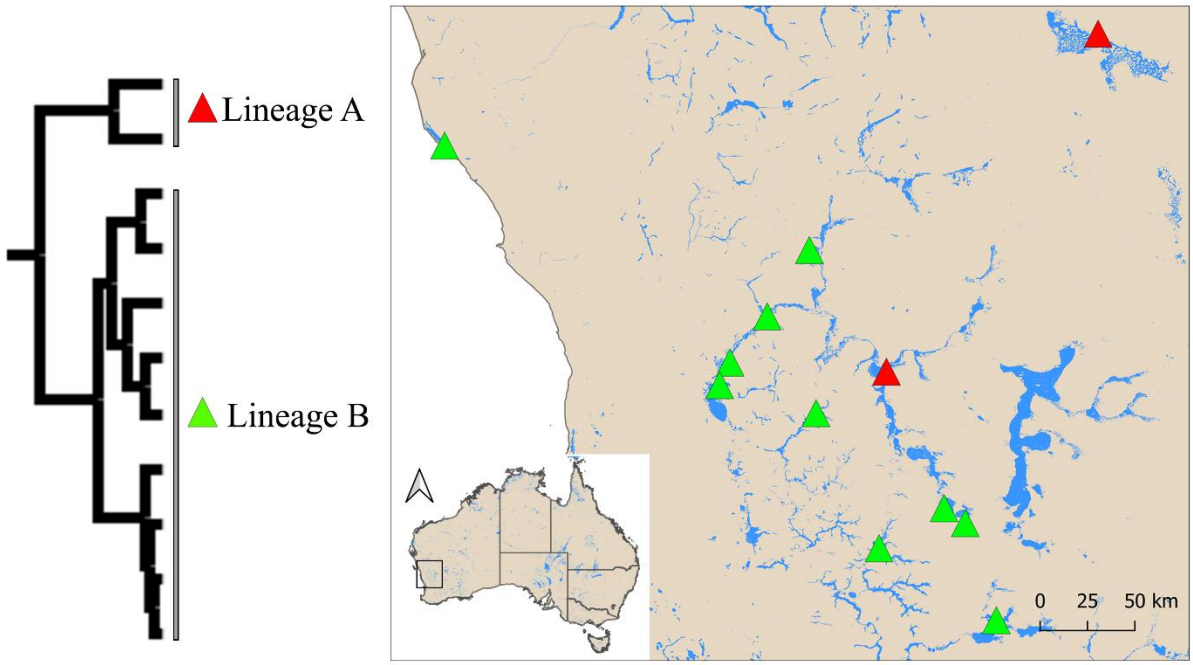

Fig. S3.6: Distributions of major *16S* genetic lineages in *P. informis*. An excerpt from the *16S* BI tree (Fig. 3.3) has been included to identify the lineages. See supplementary Table S3.1 for site details.

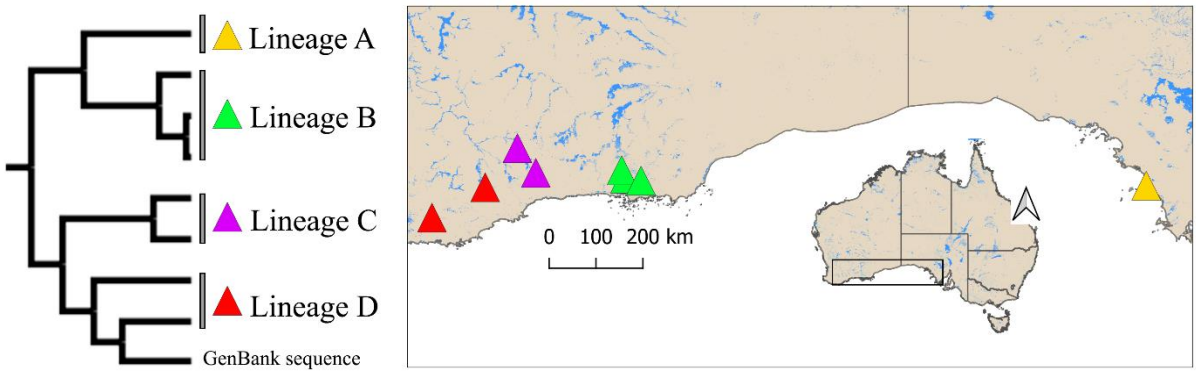

Fig. S3.7: Distributions of major *16S* genetic lineages in *P. cylindrifera*. An excerpt from the *16S* BI tree (Fig. 3.3) has been included to identify the lineages. See supplementary Table S3.1 for site details.

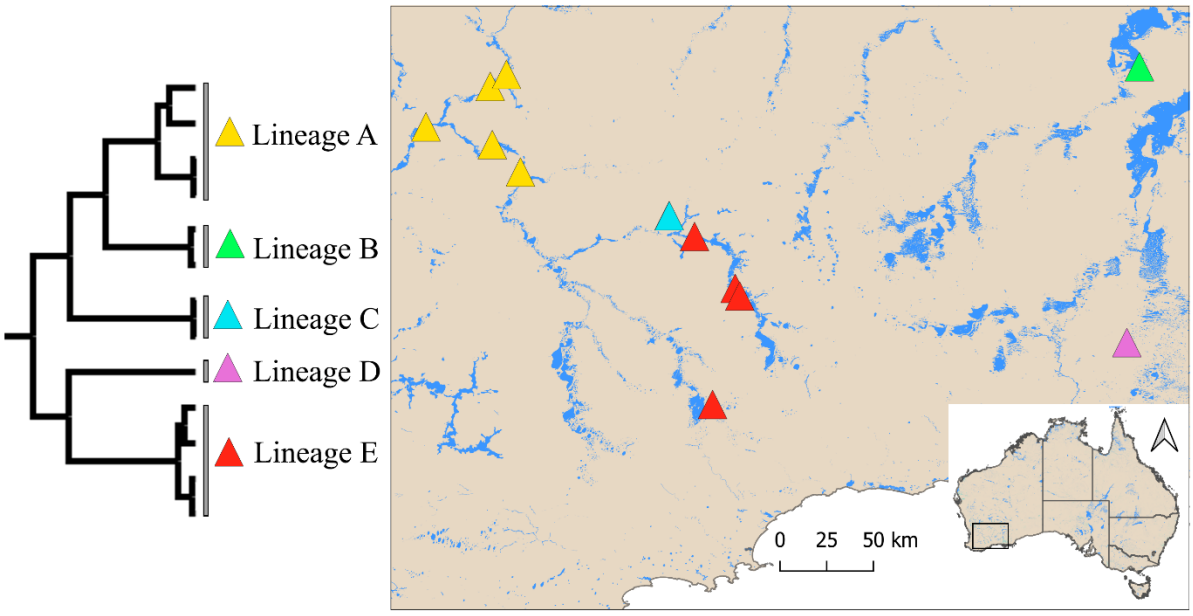

Fig. S3.8: Distributions of major *16S* genetic lineages in *P. serventyi*. An excerpt from the *16S* BI tree (Fig. 3.3) has been included to identify the lineages. See supplementary Table S3.1 for site details.

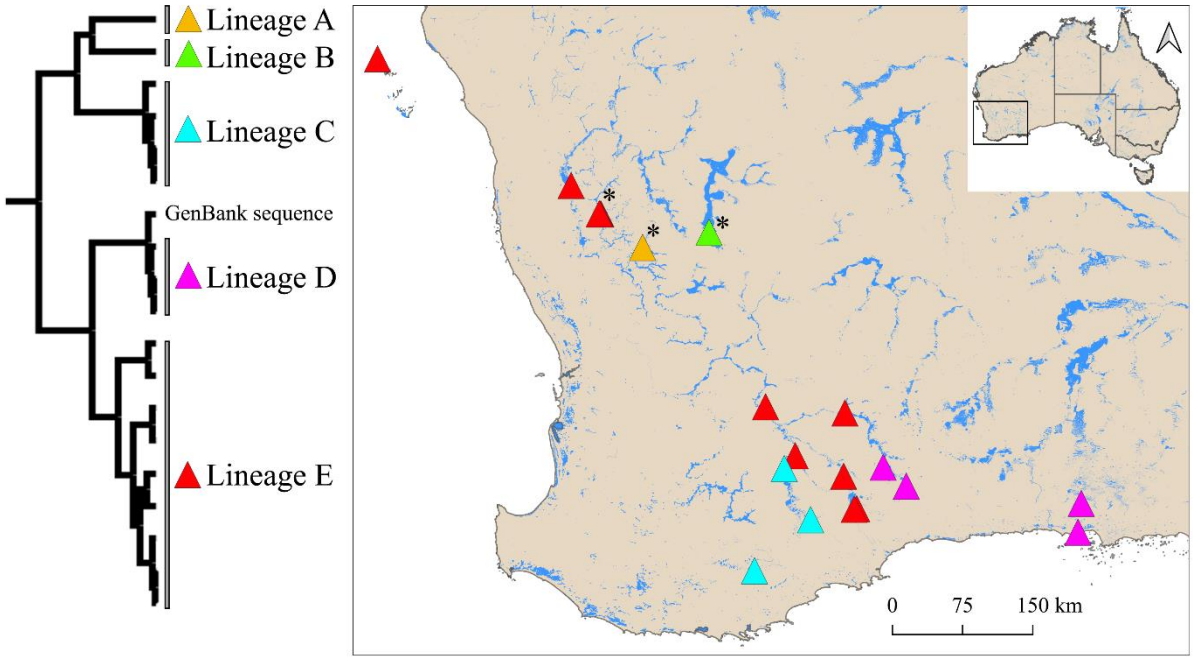

Fig. S3.9: Distributions of major *16S* genetic lineages in *P. longicaudata*. An excerpt from the *16S* BI tree (Fig. 3.3) has been included to identify the lineages. Sites marked with asterisks (*) indicate the *P. boomeranga* morphotypes. See supplementary Table S3.1 for site details.

Chapter 4

Chapter 4: Evolutionary history and phylogeography of *Parartemia*: a speciose brine shrimp from Australian salt lakes

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Chapter Linking Statement

Chapter 3 examined the taxonomy and phylogeny of *Parartemia*. Molecular data confirmed the validity of most morphospecies but also revealed the presence of two putative new species, three cryptic species and one synonymy. Many morphospecies were found to contain large amounts of molecular divergence. Building upon this foundation, Chapter 4 investigates the evolutionary history of *Parartemia* and the phylogeography of two widespread *Parartemia* species.

4.0. Abstract

Salt lakes are widespread in Australia and host many endemic invertebrates. This includes the diverse brine shrimp genus *Parartemia*. However, information about the mechanisms underlying the evolution of this unique and diverse fauna is mostly speculative. This study used molecular (mitochondrial *16S* marker) data to construct a time-calibrated phylogeny that encompassed nearly all known species of the genus, with the results suggesting that deep divergences and speciation in *Parartemia* occurred between around 40 and 10 million years ago (Mya), which is earlier than what has been reported for some other invertebrates from arid and semi-arid areas of Australia. The study also used mitochondrial *cytochrome c oxidase I* (*COI*) to investigate the phylogeography of two common and widely distributed species: *P. cylindrifera* and *P. longicaudata*. Their populations were typically localised to individual salt lakes, with no or little gene flow between them, possibly because established conspecific residents can impede gene flow into a habitat. These two species also contained highly divergent lineages. Long distance dispersal into vacant habitats towards the edge of the species' range appears to be an important driver of this divergence. Conservation efforts should focus on protecting a representative range of existing *Parartemia* populations as well as salt lake ecosystems in general to safeguard the ecological and evolutionary processes that generate diversification and adaptation in *Parartemia*.

4.1 Introduction

Conserving biodiversity is one of the most important tasks in modern biology (Hambler & Canney, 2013; Rands *et al.*, 2010). Biodiversity is a product of evolution but our views on the conservation of biodiversity and ecosystem function are often based around a 'static' view of biological systems (Santamaría & Mendez, 2012). In addition to documenting biodiversity, it is important to consider the evolutionary processes that generate, maintain and erode this diversity when developing conservation plans and trying to understand how contemporary ecosystems function (Avise, 2009; Avise *et al.*, 2016; Brooks *et al.*, 1992; Dobzhansky, 1973; Hendry *et al.*, 2010).

Around 65 million years ago (Mya), during the early Cenozoic era, Australia had a warm, humid climate and extensive rainforests (Fujioka & Chappell, 2010; Martin, 2006). However, the paleoclimate has overall become cooler and drier since at least the time Australia separated from Antarctica (during the late Eocene to early Oligocene, around 40 to 30 Mya) and then drifted northward (McGowran *et al.*, 2000; Owen *et al.*, 2017). A rapid transition to an even drier and cooler climate started during the mid-Miocene, around 15 Mya, marked by the cessation of consistent water flows in much of the ancient drainage systems in Western Australia (Byrne *et al.*, 2008; Martin, 2006; McCurry *et al.*, 2022). In Australia, the overall trend towards a drier climate has continued to the present day (Martin, 2006; Quilty, 1994), although it was briefly interrupted by a wetter period in the early Pliocene (Sniderman *et al.*, 2016). The glacial and interglacial cycles of the late Pliocene-Pleistocene commenced around 3 Mya (Pinceel *et al.*, 2013a). Traditionally, these glacial and interglacial periods were believed to be associated with arid and humid conditions respectively (Byrne *et al.*, 2008; Fujioka & Chappell, 2010; Martin, 2006), however, recent evidence suggests that the reverse association applied in southern Australia (Weij *et al.*, 2024).

Environmental shifts, often associated with biotic extinctions, can also help to create new or vacant habitats that may be populated by new lineages, potentially leading to diversification and/or adaptation (Byrne *et al.*, 2008; Crowley & North, 1988; Gillespie, 2004; Pinceel *et al.*, 2013a). The distribution and genetic structures of numerous taxa, spanning both aquatic and terrestrial environments, were significantly influenced by climatic transitions during the mid- to late-Miocene and Pliocene, characterised by deep divergences and major speciation events (Byrne *et al.*, 2008). Pleistocene climatic fluctuations drove diversifications in many species, often resulting in fragmented populations (Byrne, 2008; Byrne *et al.*, 2008; Whitehead, 2005).

The extent of interspecific and intraspecific diversification varies across taxa, depending on their adaptive capabilities (Rix *et al.*, 2015).

The aridification of the Australian landscape over geological time scales has given rise to numerous salt lakes (salinity > 3 g/L, Bayly & Williams, 1966), constituting over 80 % of the region's wetlands by area (Timms, 2005). Many of these salt lakes fill irregularly and may go several years or even decades without water, and can remain highly saline for extended periods during wet periods (De Deckker, 1983; Timms, 2005). These challenging environmental conditions, coupled with Australia's historical isolation, have fostered the emergence of a unique and diverse endemic invertebrate fauna (De Deckker, 1983; Lawrie *et al.*, 2021; Remigio *et al.*, 2001). Despite their distinctiveness and historical importance, the evolutionary history and phylogeographic patterns of the invertebrates within these salt-lake ecosystems remain largely unknown. Limited studies, such as those on the terrestrial tiger beetle genus *Pseudotetracha* (López-López *et al.*, 2016), the copepod *Calamoecia clitellata* (Whitehead, 2005), the snail *Coxiella* (Lawrie, 2023) and the ostracod *Australocypris* (Rahman, 2024) are consistent with the view that the aridification of the Australian climate had a huge impact on ecological and evolutionary processes in these unique ecosystems.

The brine shrimp *Parartemia* is one of the most noteworthy inhabitants of Australian salt lakes. It comprises at least 21 species that are found only in Australia and which tend to favour the extreme environment of hypersaline lakes (Islam *et al.*, 2024; Timms, 2014). The closest known relative of *Parartemia* is *Artemia* - their distant ancestors probably lived in Panthalassa lagoons in the Tethys Sea around 400 - 300 Mya (Anufriieva & Shadrin, 2013). These two lineages likely diverged around the time when east Gondwana (including Australia) separated from Africa about 140 - 120 Mya (Reeves & De Wit, 2000; Smith *et al.*, 2004; Upchurch, 2008; Wilford & Brown, 1994). The history of *Parartemia* in Australia and factors contributing to its diversification and distribution are little known.

Inland waterbodies in Australia are under stress, where many of them including salt lakes are undergoing rapid changes because of global climate change and local anthropogenic activities (Halse *et al.*, 2003; Timms, 2005). This study was partly prompted by reports of the disappearance of several *Parartemia* populations in Western Australia's Wheatbelt region, as well as concerns about the long-term viability of some species (see Pinder *et al.*, 2009; Timms, 2012b; Timms *et al.*, 2009). Furthermore, evidence-based management strategies for *Parartemia* are needed, not only for the sustainability of this distinctive taxon but also for that of the many bird species that rely on *Parartemia* and other salt lake invertebrates as a food

source to varying degrees (Kingsford *et al.*, 2010; Pedler *et al.*, 2018; Timms *et al.*, 2009; Williams *et al.*, 1998). Some of these avian species have breeding cycles closely linked to the presence of water in salt lakes. For example, Banded Stilts are predicted to breed only once or twice during their long lifespan when previously dry salt lakes are inundated with rainwater triggering a mass emergence of *Parartemia* (Pedler *et al.*, 2018).

To improve our understanding of the processes that have shaped the evolution and ecology of *Parartemia* species, this study used DNA data to (i) investigate the evolutionary history of *Parartemia*, exploring potential links between the diversification in this unique brine shrimp and past environmental changes; and (ii) examine the phylogeography of two widely distributed *Parartemia* species, *P. cylindrifera* and *P. longicaudata*, exploring the processes that underpin intraspecific diversity and divergence in these species.

4.2 Materials and Methods

4.2.1 Genetic data for time-calibrated phylogeny

The mitochondrial *16S* marker was used to investigate the evolutionary history of *Parartemia*. The *16S* marker was chosen for two reasons. Firstly, *16S* sequence data are available for almost all *Parartemia* species. Our data set encompassed 19 out of a total of 21; only *P. auriciforma* and *P. yarleensis* were not included. Secondly, the interspecific divergence in the mitochondrial *COI* region, another potential marker, was found to be relatively high (Islam *et al.*, 2024), making it difficult to elucidate evolutionary relationships among distantly related species in particular. A similar observation was reported by Pinceel *et al.* (2013a) for the Australian fairy shrimp (*Branchinella*). The *16S* sequences used in this study were generated mostly by Islam *et al.* (2024), comprising sequences from 105 *Parartemia* individuals, plus the inclusion of sequences from eight individuals generated by Remigio and Hebert (2000), Remigio *et al.* (2001) and Richter *et al.* (2007). GenBank accession numbers for the *16S* dataset are AF209048-AF209050, AF308948, AF308949, AY014786, AY014787, EF189613 and OR833948-OR834039. Six *Artemia* sequences were included as an outgroup in the time-calibrated phylogeny and were obtained from GenBank: *A. persimilis* (FJ007808 and FJ007810), *A. salina* (FJ007838 and FJ007839) and *A. franciscana* (JN572922 and MF563560).

The validity of 19 *Parartemia* species included in the time-calibrated phylogeny (see above) was confirmed by Islam *et al.* (2024), which highlighted some differences from previous taxonomic information. These differences involved combining the *P. boomeranga* with *P. longicaudata* morphotypes into a single species, recognising three cryptic species within *P. purpurea* (a, b and c) and two new morphospecies (*Parartemia* sp. ‘y’ and *Parartemia* sp. ‘z’).

4.2.2 Genetic data for phylogeography

A fragment of *COI* gene was used to investigate the phylogeography of *P. cylindrifera* and *P. longicaudata*. This gene region was chosen because it exhibits more variation within species than the *16S* region and has been successfully used to investigate the phylogeography of *Artemia* species (see Eimanifar *et al.*, 2015; Eimanifar & Wink, 2013; Muñoz *et al.*, 2008; Sainz-Escudero *et al.*, 2022). Specimens of *P. cylindrifera* and *P. longicaudata* were collected from 10 and 18 salt lakes, respectively, between October 2017 and October 2022 (Table 4.1). All *P. longicaudata* sampling sites were in Western Australia (this species only occurs in this

state; see Timms *et al.*, 2009). Most *P. cylindrifera* sampling sites were also in Western Australia but two were in South Australia (see Table 4.1). Previously, *COI* sequences were generated for up to four individuals from each of these sites by Islam *et al.* (2024) (GenBank accession numbers in supplementary Table S4.1), but here we substantially expanded the number of sequences by including more individuals, i.e., 197 individuals of *P. cylindrifera* and 295 individuals of *P. longicaudata*. The total *P. cylindrifera* and *P. longicaudata* datasets contained *COI* sequences from 220 and 340 individuals, respectively (Table 4.1). The translated *COI* sequences did not contain any indels or stop codons or a large number of amino acid substitutions, i.e., we found no evidence of nuclear-mitochondrial DNA pseudogenes among these sequences (see Raupach & Radulovici, 2015). The new *COI* sequences have been deposited in GenBank under the accession numbers OR984215-OR984411 and OR984435-OR984729.

All *COI* sequences were generated following the procedures detailed in Islam *et al.* (2024) for DNA extraction and PCR amplification. The primers used for PCR amplification were LCO1490 and HCO2198 (Folmer *et al.*, 1994) for *P. cylindrifera* and Flong-2 and Rlong-1 (Islam *et al.*, 2024) for *P. longicaudata*. All PCR products were sent to Macrogen Inc. in South Korea (https://dna.macrogen.com) for Exo-SAP purification (Dugan *et al.*, 2002) and subsequent forward and reverse sequencing in an automatic ABI 3700 sequencer (Applied Biosystems®). The resulting sequencing chromatograms were visualised using Chromas v2.6.5 (Technelysium Pty Ltd., Australia). Forward and reverse sequences were generated for each individual; consensus sequences were generated in MEGA X (Kumar *et al.*, 2018).

4.2.3 Sequence processing

Sequences were aligned using the G-INS-i iterative refinement method in MAFFT (http://mafft.cbrc.jp/alignment/server/) for *16S* and MUSCLE (Edgar, 2004) in MEGA X (Kumar *et al.*, 2018) for *COI*. Haplotypes for both genetic regions were identified using DnaSP v6 (Rozas *et al.*, 2017). jModelTest v2.1.9 (Darriba *et al.*, 2012) was used to determine the best nucleotide substitution model based on the Bayesian information criterion (BIC) for the *16S* dataset (TrN+I+G) and for the *COI* datasets of *P. cylindrifera* (K80+G) and *P. longicaudata* (TN93+G).

Table 4.1: Information about sampling localities and sites and sample sizes for *COI* sequences for *Parartemia cylindrifera* and *P. longicaudata*. One location was in South Australia (marked SA) and the remainder were in Western Australia. Numbers in the column 'Previous' refer to individuals sequenced as part of a related study by Islam *et al.* (2024) (further details are in supplementary Table S4.1). Asterisks (*) in the column 'Site Code' indicate sites with the *P. boomeranga* morphotype, which has been synonymised with *P. longicaudata* (see Islam *et al.*, 2024).

	Locality	Latitude	Longitude	Site Code	Number of individuals sequenced		
					Previous	New	Total
P. cylindrifera							
1	Esperance Area	-33.25205	121.93192	Esp17	4	36	40
2		-33.3933	122.04663	Esp20	1	19	20
3		-33.4554	122.01663	Esp21	2	18	20
4		-33.54344	122.43242	Esp30	2	18	20
5	Southeast of Frankland	-34.41676	117.25236	Fra1	2	18	20
6	South of Pingrup	-33.67085	118.56415	Pin1	2	18	20
7	Northwest of Ravensthorpe	-33.31509	119.81493	Rav1	3	17	20
8	Lake Varley Nature Reserve	-32.7047	119.35825	Var2	2	18	20
9	Eyre Peninsula (SA)	-33.63215	134.87224	Elli1	2	18	20
10		-33.76275	135.09852	Tun1	3	17	20
P. longicaudata							
1	Houtman Abrolhos Islands	-28.29611	113.59432	Abr1	3	17	20
2	Northwest of Kondinin	-32.38048	118.15754	Ben1	3	17	20
3	Camel Lake Nature Reserve	-34.30664	118.02764	Cam1	3	17	20
4	Cairlocup Nature Reserve	-33.70708	118.68753	Cup1	3	17	20
5	Esperance Area	-33.51487	121.86968	Esp28	2	18	20

6		-33.83827	121.83328	Pink1	3	17	20
7	Lake Grace	-33.10745	118.37749	Gra1	2	18	20
8	East of Hyden	-32.45439	119.09105	Hy6	2	18	20
9	Near Lake King	-33.09077	119.54074	Kin2	2	18	20
10	Near Lake Magenta	-33.57785	119.22911	Mag1	2	18	20
11		-33.58524	119.19946	Mag4	2	10	12
12		-33.19657	119.07553	Mag7	2	7	9
13	Beside Gunyidi-Wubin Rd	-30.11942	116.21377	Mar4*	2	17	19
14		-30.11723	116.20145	Mar5*	3	17	20
15	Lake Moore	-30.33373	117.49297	Moo1*	3	17	20
16	Beside Northam-Pithara Rd	-30.51112	116.71148	nWH2*	3	17	20
17	Northwest of Ravensthorpe	-33.3137	119.81199	Rav5	3	17	20
18	South of Three Springs	-29.78312	115.87153	TS5	2	18	20

4.2.4 Time-calibrated phylogeny

The test for substitution saturation (Xia & Lemey, 2009; Xia *et al.*, 2003) of the *16S* dataset was conducted using DAMBE v7 (Xia, 2018). The molecular clock for the phylogeny was calibrated using the estimated separation time of east Gondwana (including Australia) from Africa around 140 - 120 Mya (Reeves & De Wit, 2000; Smith *et al.*, 2004; Upchurch, 2008; Wilford & Brown, 1994) as the time when *Artemia* and *Parartemia* last shared a common ancestor. The reasons for using this calibration are set out in the discussion.

A maximum likelihood molecular clock test was conducted in MEGA X (Kumar *et al.*, 2018), and revealed that the *16S* dataset did not conform to the assumptions of a strict clock. Divergence times were therefore estimated using a relaxed molecular clock with the coalescent constant population model, run for 50 million generations under the previously outlined evolution model in BEAST 2.6.7 (Bouckaert *et al.*, 2019). The parameter distributions estimated by BEAST were analysed in Tracer v1.7.2 (Rambaut *et al.*, 2018), ensuring that estimated sample size (ESS) values exceeded 200. The final maximum clade credibility tree was generated using the BEAST distributed program TreeAnnotator (with a 25% burn-in and 0.80 posterior probability limit) and visualised using FigTree v1.4.4 (Rambaut, 2018).

4.2.5 Phylogeographic analyses

The *P. cylindrifera* and *P. longicaudata COI* datasets were analysed separately. Population-level genetic diversity indices, i.e., number of polymorphic sites, number of parsimony informative sites, number of haplotypes, haplotype diversity, nucleotide diversity, average number of nucleotide difference, Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997), were calculated in DnaSP v6 (Rozas *et al.*, 2017).

Two separate maximum likelihood trees for *P. cylindrifera* and *P. longicaudata* were constructed in MEGA X (Kumar *et al.*, 2018) using 1000 bootstrap replications and the models of evolution outlined above. Additionally, median joining haplotype network (Bandelt *et al.*, 1999) was constructed for each species in PopART (Leigh & Bryant, 2015). To investigate whether groupings of closely related conspecific sites exhibited any specific distributional patterns aligning with Australian river catchments (www.bom.gov.au), separate distribution maps were generated for both species using QGIS 3.32 (www.qgis.org).

Exact tests for population differentiation (Raymond & Rousset, 1995), specifically for those sites with shared haplotypes, were performed in Arlequin v3.5 (Excoffier & Lischer, 2010).

The analysis involved a Markov chain with 100,000 steps and a burn-in of 10,000 steps, with a significance level set at 0.05.

4.3 Results

4.3.1 Evolutionary history of *Parartemia*

The time-calibrated phylogeny was constructed using 99 *16S* haplotypes (430 bp excluding gaps) from 113 *Parartemia* individuals representing 19 species. The substitution saturation index (*Iss* = 0.27) was significantly lower than the critical value (*Iss.c* = 0.71), indicating that this dataset contains useful phylogenetic information.

The time-calibrated phylogenetic tree suggests that divergence amongst extant *Parartemia* species dates to circa 38.6 Mya, when the *P. minuta* and *P. mouritzi* lineage separated from the remaining species, although the confidence limits around the time estimate are very large (58.5 to 20.9 Mya; Fig. 4.1). The tree also suggests that the lineages representing each species were established by around 10 Mya (see Fig. 4.1) and some species (typically ones sampled from a range of sites) have been accumulating divergence for millions of years (Fig. 4.1).

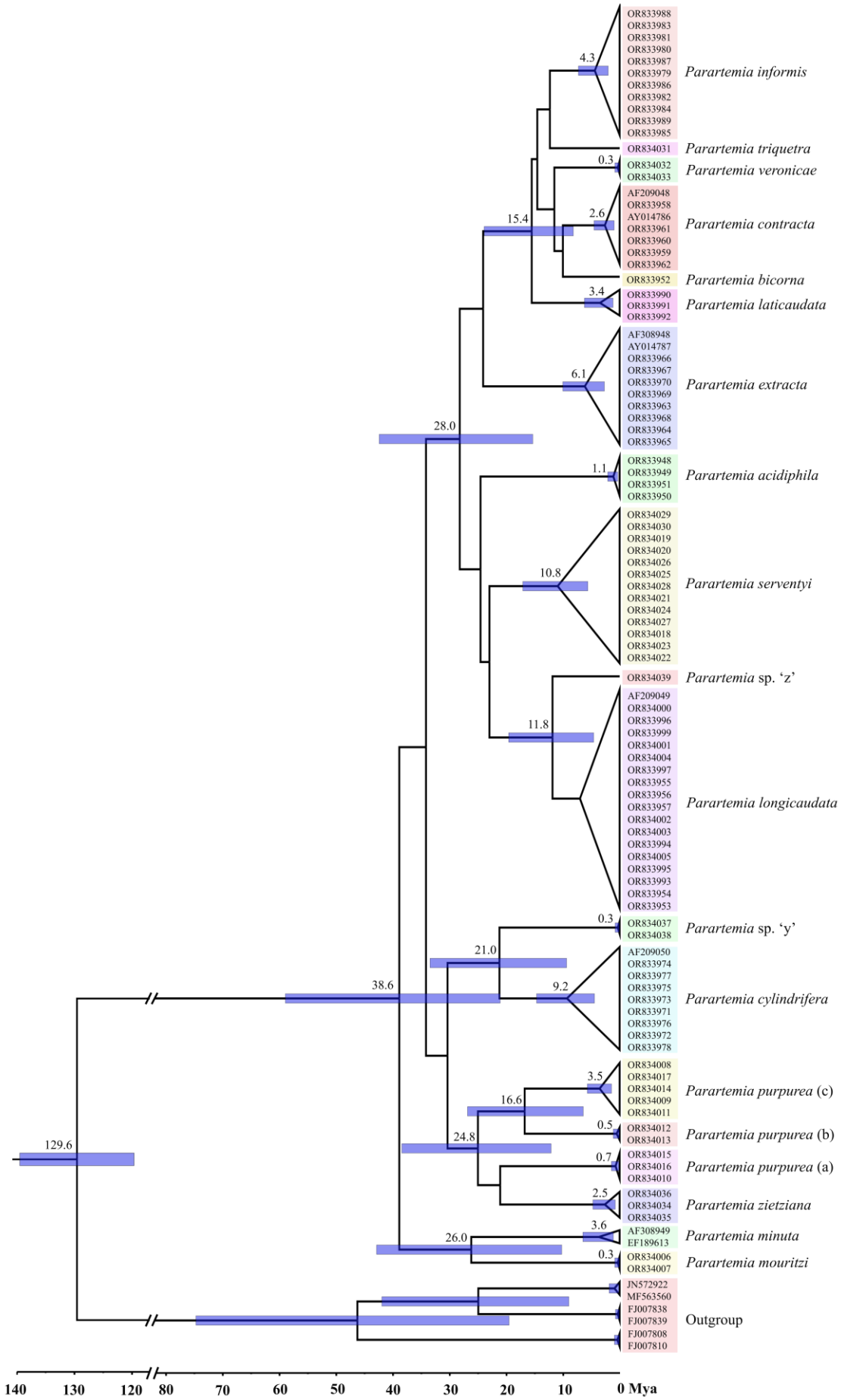
OR833988
OR833983
OR833981
OR833980
OR833987
OR833979
OR833986
OR833982
OR833984
OR833989
OR833985
Parartemia informis
OR834031
Parartemia triquetra
OR834032
OR834033
Parartemia veronicae
AF209048
OR833958
AY014786
OR833961
OR833960
OR833959
OR833962
Parartemia contracta
OR833952
Parartemia bicorna
OR833990
OR833991
OR833992
Parartemia laticaudata
AF308948
AY014787
OR833966
OR833967
OR833970
OR833969
OR833963
OR833968
OR833964
OR833965
Parartemia extracta
OR833948
OR833949
OR833951
OR833950
Parartemia acidiphila
OR834029
OR834030
OR834019
OR834020
OR834026
OR834025
OR834028
OR834021
OR834024
OR834027
OR834018
OR834023
OR834022
Parartemia serventyi
OR834039
Parartemia sp. 'z'
AF209049
OR834000
OR833996
OR833999
OR834001
OR834004
OR833997
OR833955
OR833956
OR833957
OR834002
OR834003
OR833994
OR834005
OR833995
OR833993
OR833954
OR833953
Parartemia longicaudata
OR834037
OR834038
Parartemia sp. 'y'
AF209050
OR833974
OR833977
OR833975
OR833973
OR833971
OR833976
OR833972
OR833978
Parartemia cylindrifera
OR834008
OR834017
OR834014
OR834009
OR834011
Parartemia purpurea (c)
OR834012
OR834013
Parartemia purpurea (b)
OR834015
OR834016
OR834010
Parartemia purpurea (a)
OR834036
OR834034
OR834035
Parartemia zietziana
AF308949
EF189613
Parartemia minuta
OR834006
OR834007
Parartemia mouritzi
JN572922
MF563560
FJ007838
FJ007839
FJ007808
FJ007810
Outgroup
4.3
0.3
2.6
15.4
3.4
6.1
28.0
1.1
10.8
11.8
0.3
21.0
38.6
9.2
3.5
16.6
0.5
129.6
24.8
0.7
2.5
3.6
26.0
0.3
140
130
120
80
70
60
50
40
30
20
10
0 Mya

Fig. 4.1: Time-calibrated Bayesian maximum clade credibility tree of *Parartemia 16S* haplotypes. Nodes with a posterior probability ≥ 0.80 are annotated. Node values indicate divergence times in million years ago (Mya), and node bars represent the 95 % confidence intervals of the time estimates. Triangles are used to join conspecific haplotypes, with triangle heights indicating the time since the haplotypes diverged and triangle widths indicating the number of haplotypes in the analysis. Tip labels give GenBank accession numbers.

4.3.2 Phylogeography, genetic diversity and demographic history

4.3.2.1 *Parartemia cylindrifera*

A total of 64 *COI* (658 bp) haplotypes (PC01-PC64) were identified in 220 individuals of *P. cylindrifera* from 10 sites (see Table S4.2), with four to ten haplotypes per site (Table 4.2).

The amount of *COI* variation in *P. cylindrifera* was large and partly linked to geography (see Fig. 4.2). Most sites contained their own unique and distinctive set of closely related haplotypes. However, the sampling sites (Elli1 and Tun1) on the Eyre Peninsula in South Australia shared a distinctive group of related haplotypes. Similarly, three sites (Esp17, Esp20 and Esp21) around Esperance in Western Australia shared another distinctive group of related haplotypes (Fig. 4.2).

Three haplotypes (PC56-PC58) were shared between the two Eyre Peninsula sites, which were separated by 25 km, and one haplotype (PC30) was shared among three sites from Esperance, which were separated from each other by 7.4 to 24 km (see Fig. 4.2). No haplotypes were shared between these three sites and a fourth site near Esperance (Esp30), which was located a minimum of 39.5 km from these other sites and contained its own distinctive group of haplotypes (Fig. 4.2).

The phylogenetic tree shows two main groups of *COI* lineages in *P. cylindrifera*. The first group combines two lineages from the Esperance region with one from the Eyre Peninsula (Fig. 4.2). Although the number of mutational steps between the lineages from these two locations was large, it is nevertheless interesting that the Esperance lineages were more closely aligned with the one from the Eyre Peninsula (separated by about 1,153 km) than to those from other sites in Western Australia, which were located much closer (e.g., Rav1 is only 197 km from Esp17). The second group comprised lineages from the four Western Australian sites located west of Esperance (Fra1, Pin1, Rav1 and Var2) (see Fig. 4.2). Each of these sites was spatially

isolated (the minimum distance to the next sampling site was 80.2 km) and contained a distinctive lineage/group of haplotypes. The relationships among the lineages from these sites were not clearly linked to geographic location or river catchment (Fig. 4.2).

Exact tests were used to test for differences in haplotype composition between those *P. cylindrifera* sites that shared haplotypes. The results (*p*-values) revealed significant differences in haplotype composition between the two sites from the Eyre Peninsula (Elli1 and Tun1; $p = 0.01$), as well as between the three sites from the Esperance region (Esp17, Esp20 and Esp21; $p < 0.01$) (see Table 4.3). However, we found no evidence of a significant difference in the haplotype compositions of two samples of *P. cylindrifera* collected from Esp17 in August 2019 (Esp17a) and September 2020 (Esp17b) ($p = 0.42$; Table 4.3).

Haplotype diversity in *P. cylindrifera* at the different sites was moderate to high, ranging from 0.60 (Elli1) to 0.88 (Tun1) but nucleotide diversity was typically low (maximum of 0.006) (Table 4.2), reflecting the fact that each site contained only closely related haplotypes (see above).

The results of the neutrality tests did not provide any clear evidence of departures from expectations for drift-mutation equilibrium for most *P. cylindrifera* sites (Table 4.2). However, the values of both Fu's FS and Tajima's D were negative for five sites, indicating an excess of rare haplotypes and rare mutation, respectively, although only the former values were significantly different from zero for three sites (Table 4.2; also see Table S4.2).

Table 4.2: *COI* diversity indices for populations of *Parartemia cylindrifera* and *P. longicaudata*, including site codes (details are in Table 4.1), number of individuals sequenced (N), number of polymorphic sites (V), number of parsimony informative sites (P), number of haplotypes (H), haplotype diversity (Hd) (standard deviation in parentheses), nucleotide diversity (Phi) (standard deviation in parentheses), average number of nucleotide difference (K) and values of Tajima's D and Fu's Fs (asterisks denote statistical significance; $p < 0.05$).

Site code	N	V	P	H	Hd	Phi	K	Tajima's D	Fu's Fs
P. cylindrifera									
Esp17	40	16	12	10	0.79 (0.054)	0.005 (0.0005)	3.12	-0.55	-0.63
Esp20	20	6	2	7	0.64 (0.118)	0.001 (0.0004)	0.93	-1.44	-3.84*
Esp21	20	7	7	6	0.81 (0.052)	0.004 (0.0005)	2.94	1.61	0.85
Esp30	20	21	12	7	0.64 (0.118)	0.005 (0.002)	3.46	-1.58	0.45
Elli1	20	5	3	4	0.60 (0.077)	0.002 (0.0003)	1.64	0.49	1.21
Fra1	20	11	11	4	0.66 (0.092)	0.006 (0.0014)	3.63	0.61	3.99
Pin1	20	11	2	5	0.74 (0.058)	0.003 (0.001)	1.76	-1.53	0.35
Rav1	20	8	5	7	0.84 (0.056)	0.003 (0.0004)	1.93	-0.49	-1.32
Tun1	20	8	4	9	0.88 (0.043)	0.003 (0.0004)	1.88	-0.89	-3.58*
Var2	20	9	2	10	0.80 (0.088)	0.002 (0.0004)	1.24	-1.76	-7.29*
P. longicaudata									
Abr1	20	8	0	7	0.52 (0.135)	0.001 (0.0004)	0.80	-2.17*	-4.43*
Ben1	20	20	18	5	0.56 (0.114)	0.011 (0.0028)	7.35	1.16	6.09*
Cam1	20	10	3	7	0.52 (0.135)	0.002 (0.0008)	1.27	-1.92*	-2.68*
Cup1	20	12	9	7	0.82 (0.058)	0.005 (0.0008)	3.11	-0.29	0.12
Esp28	20	-	-	1	-	-	-	-	-
Gra1	20	13	9	10	0.90 (0.043)	0.005 (0.0005)	3.37	-0.29	-2.29
Hy6	20	10	10	7	0.77 (0.081)	0.006 (0.0005)	3.90	1.34	0.82
Kin2	20	9	4	9	0.82 (0.073)	0.002 (0.0005)	1.63	-1.23	-4.23*
Mag1	20	19	17	4	0.36 (0.131)	0.005 (0.0028)	3.42	-1.37	3.73
Mag4	12	25	18	6	0.68 (0.148)	0.011 (0.0039)	7.09	-0.64	2.01
Mag7	9	22	18	4	0.58 (0.183)	0.012 (0.0044)	8.11	0.01	4.15
Mar4	19	30	10	10	0.74 (0.111)	0.006 (0.0028)	4.09	-2.07*	-1.75
Mar5	20	19	0	9	0.65 (0.122)	0.003 (0.0011)	1.90	-2.44*	-3.54*
Moo1	20	11	3	9	0.80 (0.073)	0.004 (0.0004)	2.38	-0.83	-2.61*
nWH2	20	23	20	8	0.86 (0.049)	0.010 (0.0021)	6.50	0.01	1.67
Pink1	20	3	0	3	0.20 (0.115)	0.0005 (0.0003)	0.30	-1.72	-1.14
Rav5	20	8	6	4	0.43 (0.126)	0.003 (0.001)	1.81	-0.66	1.50
TS5	20	5	5	4	0.73 (0.067)	0.003 (0.0004)	1.83	0.92	1.53

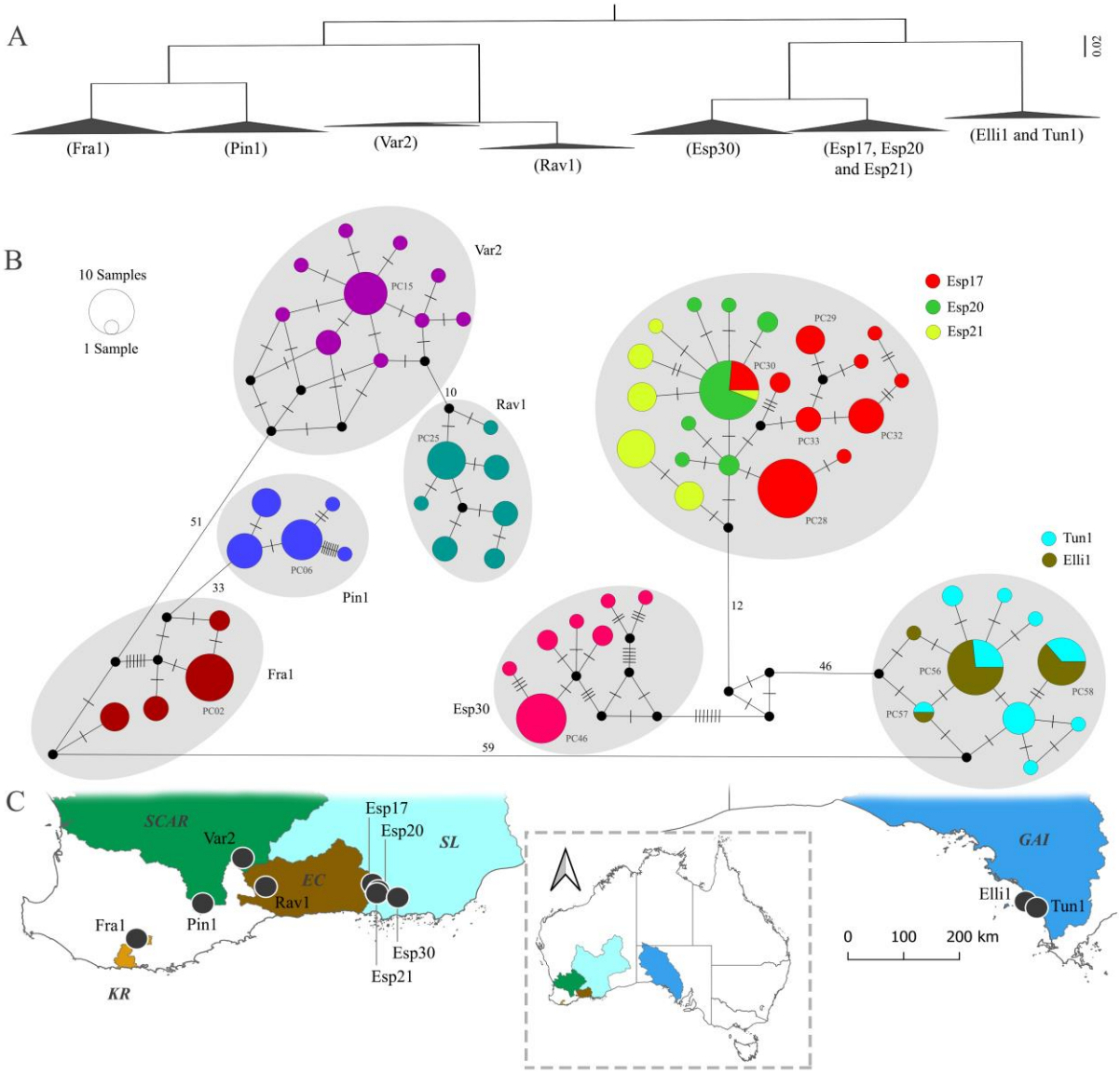

Fig. 4.2: Maximum likelihood phylogenetic tree (top; A) and haplotype network (middle; B) for *COI* haplotypes of *Parartemia cylindrifera*, with a site distribution map at the bottom (C). Site codes are shown; site details are in Table 4.1. Labels for some common and/or shared haplotypes are shown in the haplotype network (B); further details are in supplementary Table S4.2. Hatch marks and black dots in the haplotype network (B) indicate mutational steps between haplotypes and missing haplotypes, respectively. Mutational steps between haplotypes greater than ten are indicated by numbers. The coloured areas on the map (C) highlight relevant Australian river catchments (www.bom.gov.au): Kent River (KR), Swan Coast–Avon River (SCAR), Esperance Coast (EC), Salt Lake (SL) and Gairdner (GAI).

Table 4.3: Results of exact tests (*p*-values) for differences in haplotype composition among *Parartemia cylindrifera* sites with at least one shared haplotype. Site details are available in Table 4.1. Two samples were collected from Esp17: one in August 2019 (Esp17a) and another in September 2020 (Esp17b). 'N/A' indicates that site pairs do not share haplotypes.

Site code	Esp17 (a)	Esp17 (b)	Esp20	Esp21	Elli1	Tun1
Esp17 (a)	-					
Esp17 (b)	0.42	-				
Esp20	0.00	0.00	-			
Esp21	0.00	0.00	0.00	-		
Elli1	N/A	N/A	N/A	N/A	-	
Tun1	N/A	N/A	N/A	N/A	0.01	-

4.3.2.2 *Parartemia longicaudata*

A total of 102 *COI* (658 bp) haplotypes (PL01-PL102) were identified in 340 individuals of *P. longicaudata* from 18 sites (details in Table S4.3). The number of haplotypes per site varied from one to ten (Table 4.2).

Parartemia longicaudata contained six *COI* lineages, one of which occupied a central position in the network (see Fig. 4.3). The other (peripheral) lineages were typically very divergent from each other and this central lineage (see Fig. 4.3). For example, the lineage in Moo1 (Lake Moore) was at least 45 mutational steps from the lineage at the nWH2 site (near Wongan Hills), which in turn was at least another 46 steps from any other/the central lineage (see Fig. 4.3). However, a lineage comprising haplotypes from TS5 and Abr1 was only ~13 steps from the central lineage, implying a more recent separation (see Fig. 4.3).

The distribution of *COI* variation in *P. longicaudata* was only partly linked to geography. Haplotypes from the same site were always in the same lineage (see Fig. 4.3). Each peripheral lineage typically included haplotypes from sites that were in the same general area or river catchment but did not always include haplotypes from every site in that area or catchment (Fig. 4.3). The haplotypes from any remaining sites occurred in the central lineage (Fig. 4.3). For example, one lineage comprised haplotypes from four sites (Kin2, Esp28, Pink1 and Rav5) in and around the Esperance area but haplotypes from some other 'nearby' sites, e.g., Mag1, Mag4 and Mag 7, were in the central lineage (see Fig. 4.3). The Kin2 and Mag7 sites are only 45 km

apart whereas the Kin2 and Rav5 sites are a minimum of 192 km from the Esp28 and Pink1 sites.

There was no sharing of haplotypes among sites represented in peripheral lineages of *P. longicaudata*, except for Pink1 and Esp28, which are located 36 km apart and shared one haplotype (Fig. 4.3). This shared haplotype was the only one present in Esp28. Haplotype sharing was more extensive among sites represented in the central lineage, although it was still limited. Three (PL14, PL16 and PL18) haplotypes were shared between Mar4 and Mar5, which are separated by only 820 metres (Fig. 4.3). The Mar4 site also shared one haplotype (PL45) with three sites (Ben1, Mag1 and Mag4), and another haplotype (PL23) with four sites (Ben1, Mag4, Mag7 and Hy6). The Mar4 site was a minimum of 312 km apart from these other sites. Another haplotype (PL35) was shared between Ben1 and Mag7, which are 124 km apart.

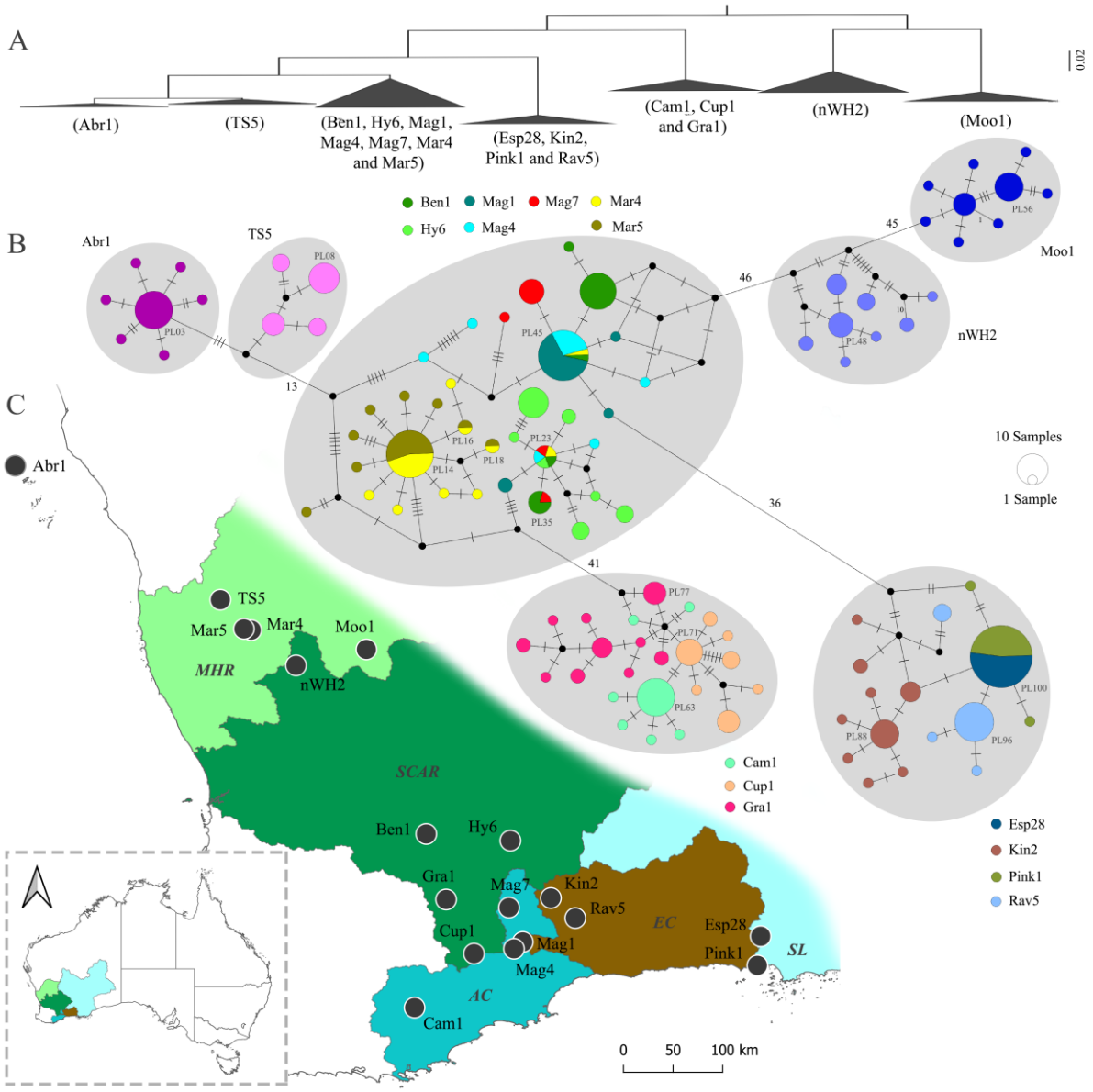

Fig. 4.3: Maximum likelihood phylogenetic tree (top; A) and haplotype network (middle; B) for *COI* haplotypes of *Parartemia longicaudata* with a site distribution map at the bottom (C). Site codes are shown; site details are in Table 4.1. Labels for some common and/or shared haplotypes are shown in the haplotype network (B); further details are in supplementary Table S4.3. Hatch marks and black dots in the haplotype network (B) indicate mutational steps between haplotypes and missing haplotypes, respectively. Mutational steps between haplotypes greater than ten are indicated by numbers. The coloured areas on the map (C) highlight relevant Australian river catchments (www.bom.gov.au): Moore-Hill Rivers (MHR), Swan Coast-Avon River (SCAR), Albany Coast (AC), Esperance Coast (EC) and Salt Lake (SL).

The results of exact tests for differences in haplotype composition between *P. longicaudata* sites with shared haplotypes revealed significant differences between most such sites, but not for three pairs that were in proximity (see Table 4.4; Fig. 4.3). These were Mar4 and Mar5 (p = 0.93; 820 metres apart), Mag1 and Mag4 (p = 0.10; 2.7 km apart) and Esp28 and Pink1 (p = 0.49; 36 km apart).

Table 4.4: Results of exact tests (p-values) for differences in haplotype composition among *Parartemia longicaudata* sites with at least one shared haplotype. Site details are available in Table 4.1. 'N/A' indicates that site pairs do not share haplotypes.

Site code	Ben1	Hy6	Mag1	Mag4	Mag7	Mar4	Mar5	Esp28	Pink1
Ben1	-								
Hy6	0	-							
Mag1	0	N/A	-						
Mag4	0	0	0.10	-					
Mag7	0	0	N/A	0	-				
Mar4	0	0	0	0	0	-			
Mar5	N/A	N/A	N/A	N/A	N/A	0.93	-		
Esp28	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-	
Pink1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.49	-

Haplotype diversity in most sites was moderate to high, ranging from 0.52 (Abr1 and Cam1) to 0.90 (Gra1), but was low (< 0.50) at three sites (Mag1, Pink1 and Rav5) (see Table 4.2). Nucleotide diversity was usually low (≤ 0.006), but higher (ranging from 0.010 to 0.012) at four sites (Ben1, Mag4, Mag7 and nWH2) (Table 4.2).

The results of the neutrality tests provided clear evidence of departures from expectations for drift-mutation equilibrium for seven of the 17 *P. longicaudata* sites (Table 4.2). These included Ben1, which was the only site with positive values of both Fu's Fs and Tajima's D with at least one value (Fs) that was significantly different from zero (see Table 4.2). The remaining six sites had negative values for both Fu's Fs and Tajima's D (Table 4.2), with either one (Kin2, Mar4 and Moo1) or both values (Abr1, Cam1 and Mar5) significantly different from zero (Table 4.2).

4.4 Discussion

The findings of this study improve our understanding of the evolution of *Parartemia*, one of the most speciose genera in the rich, endemic invertebrate fauna of Australian salt lakes. The time-calibrated *16S* phylogeny suggests that deeper divergences and speciation in *Parartemia* occurred between about 40 and 10 Mya (late Eocene to late middle Miocene). Phylogeographic analysis of *COI* variation in two common and widespread species indicates that these species (especially *P. cylindrifera*) are subdivided into a series of divergent genetic lineages with very restricted spatial distributions.

4.4.1 Evolutionary history of *Parartemia*

Based on the results of the time-calibrated phylogeny, major diversification and speciation in *Parartemia* occurred between about 40 and 10 Mya, coinciding with Australia's transition to a more arid climate (see Introduction). However, we did not find evidence of an acceleration in divergence in *Parartemia* in the mid-Miocene, a period marked by a rapid increase in aridification (details in Introduction). During the Pleistocene, intraspecific divergences in *Parartemia* increased but there is no evidence that new species evolved, which is a common pattern in taxa in arid and semi-arid zones in Australia (see Byrne *et al.*, 2008 and references therein). Assuming that the time estimates are accurate, major divergence and speciation occurred earlier in *Parartemia* than it did in *Australocypris* ostracods (Rahman, 2024) and *Coxiella* gastropods (Lawrie, 2023) from Australian salt lakes, and also earlier than in *Pseudotetracha* tiger beetles (López-López *et al.*, 2016) from dry salt lake beds. It may have occurred around the same time in *Branchinella* fairy shrimps from fresh and low-salinity waterbodies in Australia depending on which time estimate is used (see Pinceel *et al.*, 2013a). Thus, diversification in *Parartemia* appears to be old and is probably tied to a long history of aridity, abundant salt lakes and the persistence of ephemeral water bodies in the Australian landscape (see Islam *et al.*, 2024; Remigio *et al.*, 2001; Rogers & Timms, 2014; Timms, 2005).

It is challenging to establish definitive links between diversification patterns and past climatic or other events. The above estimates of divergence time are derived from a molecular clock that was calibrated using the estimated time of the separation of east Gondwana from Africa, leading to the divergence of *Artemia* and *Parartemia* from their most recent common ancestor (see Anufriieva & Shadrin, 2013; Reeves & De Wit, 2000; Smith *et al.*, 2004; Upchurch, 2008; Wilford & Brown, 1994). There are limitations associated with using such an old geological event for calibrating our molecular clock (see Guindon, 2020; Heads, 2005) but fossil

calibration was not possible due to an absence of *Parartemia* fossils. Sainz-Escudero *et al.* (2021) calibrated their phylogeny for *Artemia* using a putative fossil of *A. salina* but their results include what appear to be some unrealisically recent divergence estimates. For example, they suggest that *A. persimilis* separated from other congeners around 15 Mya when biogeographic evidence (the separation of South America from Africa) indicates that this split probably occurred around 80 to 90 Mya (see Baxevanis *et al.*, 2006). We decided against using a 'universal' rate of *16S* evolution because molecular divergence in *Parartemia* may be unusually high (Hebert *et al.*, 2002). Pinceel *et al.* (2013a) used two mutation rates for timing events in the evolution of fairy shrimp *Branchinella* - 0.00205 mutations per site per million years (based on 13 vertebrates and invertebrates; Lynch & Jarrell, 1993) and 0.0048 mutations per site per million years (based on crabs; Sturmbauer *et al.*, 1996). If we had used these rates, our estimates of divergence times in *Parartemia* would have been much older.

4.4.2 Phylogeography of *Parartemia*

The haplotype compositions of the assemblages of *P. cylindrifera* in different salt lakes were very distinctive, indicating that the populations of this species are confined to individual salt lakes. Haplotype sharing in this species was limited to populations in proximity (separated by up to 25 km) and regardless even these populations had different haplotype compositions. This genetic distinctiveness indicates that the amount of gene flow among *P. cylindrifera* populations is negligible (see Mallet, 2001). The above conclusions also apply to *P. longicaudata*, except that this species had some nearby populations (separated by a maximum of 36 km) with similar haplotype compositions and so appears to be able to sustain some gene flow over short distances. *Parartemia longicaudata* also shared haplotypes over larger distances (up to 478 km). This difference could be because, compared to *P. cylindrifera*, the genotypes of *P. longicaudata* are more generalists with broader ecological tolerances and therefore more likely to persist in a broader range of sites. Certainly, field records show that the salinity range of *P. longicaudata* (31 - 240 g/L) is much broader than that of *P. cylindrifera* (3 - 140 g/L) and most other *Parartemia* species (Timms, 2012b).

A population of *P. longicaudata* occurs in a salt lake on North Island in the Houtman Abrolhos group (Abr1), which is located some 60 km from mainland Western Australia. These are low lying islands that, depending on the island, were entirely or partly submerged during a high sea level stand approximately 6,000 years ago (see Collins *et al.*, 2006; Collins *et al.*, 2004). It is likely that the salt lake on North Island formed and therefore has been colonised by *P.*

longicaudata within the past 6,000 years. This is supported by the finding that the *P. longicaudata* haplotypes on North Island were a minimum of only three mutational steps away from those from the nearest sampled population on the mainland (TS5). It suggests that this and possibly other *Parartemia* species may be effective at colonising newly created habitats (see below).

Like *P. cylindrifera* and *P. longicaudata*, many species of passively dispersed crustaceans from lentic environments show high levels of genetic differentiation even over small spatial scales, despite being widely distributed and apparently having a high dispersal potential (e.g., see Asem *et al.*, 2024c; De Meester *et al.*, 2002; Lopes da Cunha *et al.*, 2021; Muñoz *et al.*, 2008; Pinceel *et al.*, 2013a; Rodríguez-Flores *et al.*, 2020). This apparent paradox may be explained via the Monopolization Hypothesis of De Meester *et al.* (2002), which suggests that genetic differentiation arises in these species due to a combination of founder effects and limited ongoing gene flow. According to the hypothesis, individuals are effective colonisers of vacant habitats but may be prevented from colonising occupied habitat by resident conspecifics, which have a numerical advantage (see De Meester *et al.*, 2002; Emami-Khoyi *et al.*, 2023; Rogers, 2015; Schwentner & Richter, 2015) and also a fitness advantage if they have adapted to local conditions (see De Meester *et al.*, 2002; Kawecki & Ebert, 2004; Rogers, 2015; Schwentner & Richter, 2015).

A range of passively dispersed lentic crustaceans (Okamura & Freeland, 2002; Rogers, 2014a; Schwentner & Richter, 2015), including *P. veronicae* (Campagna, 2007) and probably other *Parartemia* species as well, display asynchronous egg hatching. Thus, for these species only a fraction of the fertilised eggs in an egg bank will hatch in any one hydroperiod and those that do hatch in the same period will comprise a mix of generations (Rogers, 2014a; Schwentner & Richter, 2015). Rogers (2014a, 2015) argued that this admixture of generations will increase inbreeding levels and thereby facilitate the rapid evolution of local adaptation. Another possibility is that an egg bank will contain an admixture of genotypes that differ in their capacity to hatch and develop under a particular set of environmental conditions, such that the individuals/genotypes that are active in a waterbody at any one time would have high fitness in the prevailing conditions (see Okamura & Freeland, 2002; Rogers, 2015). Both scenarios depend, however, on the presence of adaptively significant variation (see Kawecki & Ebert, 2004), which may not initially be present if a population is founded by one or a few related individuals. Experimental and temporal genetic studies are needed to determine how well *Parartemia* populations conform to the Monopolization Hypothesis and associated predictions.

Rogers (2015) predicted that new anostracan species evolve in habitats at the periphery of the existing range of species. According to this argument, a small number of individuals establish a population in a vacant habitat at the edge or outside of the existing range of the species. The population rapidly expands and adapts to local environmental conditions, thereby monopolising the habitat and impeding gene flow from other sites (see above), sometimes culminating in ecological speciation. The phylogeographic structure of *P. cylindifera* is largely consistent with this prediction. This species consists of several highly divergent mitochondrial (*COI*) lineages with narrow geographical distribution, but with no close association between lineage distribution and geography or catchment area. This lack of association suggests that divergence has followed long distance dispersal into a new area rather than vicariance (see Boileau & Hebert, 1991). Most lineages comprised a small group of closely related haplotypes that were confined to a single habitat and may derive from a single or several closely related founders followed by *in situ* mutation (see below). Lineages may regularly go extinct, especially those in more astatic habitats (see Okamura & Freeland, 2002; Rogers, 2015), but any lineage that persists for a long time and/or that undergoes ecological diversification could conceivably evolve into a new species.

One or more haplotypes were shared between *P. cylindrifera* habitats ≤ 25 km apart indicating that there is a higher chance of dispersal among nearby populations compared to distant ones. This is probably because there is a greater chance of eggs being transported among close sites via the localised movements of birds and other dispersal vectors (Brendonck & Riddoch, 1999; Havel & Shurin, 2004; Hessen *et al.*, 2019). Also, if the environmental conditions at nearby sites are similar, then any dispersal between these sites is more likely to be realised. Alternatively, the rate of dispersal/gene flow between nearby populations may be sufficient to limit the evolution of local adaptation (see Rogers, 2015), thus removing one potential barrier to realised dispersal.

Parartemia cylindrifera is one of only two *Parartemia* species that occurs in both south-western and south-eastern Australia (Timms *et al.*, 2009). The amount of mitochondrial divergence in this species between the Eyre Peninsula in South Australia and Western Australia was very large. The giant ostracod *Australocypris insularis*, another common and widespread species in salt lakes (Rahman *et al.*, 2023), also shows deep divergence (plus some more recent connections) between these two regions (Rahman, 2024). These two regions are separated by the vast arid Nullarbor Plain (see Webb & James, 2023 for a physical description of the Nullarbor Plain), which is a major climatic biogeographic barrier in southern Australia (e.g.,

see Crisp & Cook, 2007; Rix *et al.*, 2015). However, it is difficult to attribute east-west divergence in *P. cylindrifera* to vicariance associated with the formation of or climate change on the Nullarbor Plain because this species shows comparable and sometimes even greater divergence over much smaller spatial scales in Western Australia. According to the time-calibrated phylogeny, the Eyre Peninsular lineage of *P. cylindrifera* last separated from a Western Australian lineage about 5.5 Mya, although the confidence limits of this estimate were broad. Around this time, the climate of the Nullarbor Plain may have been slightly wetter than it is today (Webb & James, 2023), which may have assisted *P. cylindrifera* to disperse across this barrier and colonise the Eyre Peninsula. Documentation of genetic variation in this species at sites further east of the Eyre Peninsula is needed to fully understand the relationship between populations of this species on either side of the Nullarbor Plain.

The phylogeographic structure of *P. longicaudata* was complex. It showed evidence of divergence in peripheral habitats in the form of several mitochondrial (*COI*) lineages that appear to have been derived from a central lineage and occupy sites at the periphery of the range of this species. The lineage in Lake Moore, a huge (~120 km long and ~10 - 20 km wide) salt playa in Western Australia (see Beard, 2000), was particularly distinctive and may have been isolated in this waterbody for a very long time. Such a large and old habitat may have afforded resident lineages some protection against extinction. Williams (1984) suggested that regularly rather than episodically filled lakes are the centres of evolution for the Australian salt lake fauna. The presence of a highly divergent *Parartemia* lineage in Lake Moore, which only intermittently holds water, does not support this suggestion. Nor does it support the suggestion that genetic differentiation is likely to be reduced in large habitats due to repeated colonisation (see De Meester *et al.*, 2002), but it is consistent with Rogers' (2015) prediction that peripheral isolation is a major source of divergence in anostracans.

Haplotype sharing was more common (i.e., occurred in half of the sites) and widespread (i.e., occurred over a larger distance, up to 478 km) in *P. longicaudata* compared to *P. cylindrifera* and mostly involved haplotypes in the central lineage of this species. It may be that there is a relatively high density of suitable habitats/conspecific populations within the distribution of the central lineage, making dispersal more likely. Furthermore, Rogers (2015) suggested that in areas where suitable habitat is concentrated, the amount of dispersal may be sufficient to overcome any advantage that residents have over immigrants (see above), making realised dispersal more likely. Regardless, although the amount of gene flow in *P. longicaudata* is very

limited, it does appear to greater than that in *P. cylindrifera*, at least over short and moderate distances.

4.4.3 Genetic diversity and demographic history

Populations of *P. cylindrifera* had moderate to high levels of haplotype diversity coupled with low nucleotide diversity, as did most populations of *P. longicaudata*. This is a common pattern in populations of anostracan and other lentic crustaceans (e.g., see Asem *et al.*, 2024c; Eimanifar *et al.*, 2015; Ketmaier *et al.*, 2012; Maturana *et al.*, 2020; Muñoz *et al.*, 2008; Rahman, 2024; Scheihing *et al.*, 2011; Young *et al.*, 2013). It suggests that the haplotypes in a population have recently diversified from a single or group of closely related haplotypes, possibly in association with a demographic expansion following a bottleneck or a founder event. For both *Parartemia* species, the typically limited differences among the haplotypes at the same site was in stark contrast to the large gaps that were often present between these haplotypes and those at other sites. This could be because of an ongoing turnover of haplotypes within sites, e.g., due to repeated bottlenecks, the existence of 'intermediate' haplotypes at other sites that were not sampled and/or the loss of populations/habitats that had once contained these intermediate haplotypes (e.g., Ketmaier *et al.*, 2012). Diversifying selection could have also played a role.

The results of the neutrality tests were mixed. Values of Tajima's D and/or Fu's Fs were both negative, which is consistent with expectations for demographic expansion (see Excoffier & Lischer, 2011 and references therein), for about half of the populations in both species, although at least one value was significantly different from zero for only three populations in *P. cylindrifera* and six in *P. longicaudata*. One of these was the *P. longicaudata* on the Houtman Abrolhos Islands, which has been founded within the past 6,000 years (see above). Values Tajima's D and/or Fu's Fs were both positive for a few populations in both species, possibly indicating a demographic contraction, although only one positive value (for Ben1 in *P. longicaudata*) was significantly different from zero.

Egg banks will promote the persistence of genetic diversity in *Parartemia* populations by enabling individuals to survive during unfavourable environmental conditions (e.g., see Bilton *et al.*, 2001; Rother *et al.*, 2010). The risk of massive losses of individuals/genetic diversity during a poor season will be greatly reduced if only a small proportion of eggs hatch during any one hydroperiod (see Okamura & Freeland, 2002; Rogers, 2014a; Rogers, 2015; Schwentner & Richter, 2015), as has been recorded for *P. veronicae* (Campagna, 2007).

Schwentner and Richter (2015) have suggested that chance events associated with individuals hatching from a genetically diverse egg bank, coupled with genetic drift, could artificially inflate genetic differentiation among populations. However, we assessed temporal variation in the haplotype composition of one *P. cylindrifera* population over a ~12 month period and found no evidence of any significant difference. Thus, based on this limited testing, the haplotype compositions of *Parartemia* populations are stable in the short term and the genetic differences that we observed among *Parartemia* sites predominantly reflect spatial rather than temporal differences.

4.4.4 Conservation implications

The conservation of *Parartemia* populations is important not only for their intrinsic value but also because they are an important food source for a range of waterbirds (see Introduction). Most *P. cylindrifera* and *P. longicaudata* populations contained moderate to high levels of *COI* diversity, although nucleotide diversity was usually low and the link between the *COI* diversity and the evolutionary potential of the populations is not clear (e.g., see Milot *et al.*, 2020; Teixeira & Huber, 2021). Developing effective conservation strategies for these *Parartemia* species is challenging because they contain multiple divergent lineages and sublineages that tend to have localised distributions. Also, populations are typically confined to individual waterbodies. To be effective, conservation efforts must therefore be implemented across a range of spatial scales. Protecting a representative range of existing *Parartemia* populations is important, but safeguarding the processes that promote diversification and adaptation in *Parartemia* is also essential. This could be achieved by conserving salt lake ecosystems in general, including 'unoccupied habitat', which appear to serve as foci for genetic divergence in *Parartemia* (see above).

4.4.5 Limitations and future directions

As discussed above, the interpretation of the time-calibrated phylogeny is limited by uncertainty around the rate of molecular evolution of the *16S* marker in *Parartemia*. The results of this study are also limited by the fact that a single marker was used for the time-calibrated phylogeny (*16S*) and for phylogeographic analyses (*COI*). Given their lack of recombination, these mitochondrial DNA markers are well suited to addressing phylogenetic and phylogeographic questions (Castro *et al.*, 1998; DeSalle *et al.*, 2017) and have consistently provided valuable insights into such questions for a range of taxa for over 20 years (e.g., Bowen *et al.*, 2014; Kerr *et al.*, 2005; Kim & Hwang, 2023; Lawrie, 2023; López-López *et al.*, 2016;

Pinceel *et al.*, 2013a; Rahman, 2024; Schön *et al.*, 2015). However, discrepancies between patterns of variation in mitochondrial and nuclear loci are also well known (Dool *et al.*, 2016; Toews & Brelsford, 2012; Tóth *et al.*, 2017) and data from other independent (nuclear) loci are needed to complement the information on the phylogeny and phylogeography of *Parartemia* species provided in this study. Genomic data in particular have the potential to provide high resolution insights into the evolutionary history and phylogeography of species (e.g., Hughes *et al.*, 2018; McCartney-Melstad *et al.*, 2018; Sainz-Escudero *et al.*, 2023; Stange *et al.*, 2018), although they also have some limitations in this respect (see Carstens *et al.*, 2012; Fuentes-Pardo & Ruzzante, 2017; Leaché *et al.*, 2015; Leaché & Oaks, 2017).

4.5 Conclusion

Diversification in *Parartemia* appears to have occurred earlier than in some other salt lake taxa after the Australian paleoclimate had started to aridify but prior to a rapid increase in aridification in the mid-Miocene. The populations of two widely distributed *Parartemia* species were typically localised to individual salt lakes, with no or little gene flow between them. Established conspecific residents may impede immigration into occupied habitat. Both species also contain highly divergent lineages. Long distance dispersal into vacant habitats towards the edge of the species' range appears to be an important driver of this divergence.

4.6 Supplementary Tables and Figures

Table S4.1: *COI* sequence information of *Parartemia cylindrifera* and *P. longicaudata* obtained from Islam *et al.* (2024), along with GenBank accession numbers. The number in parentheses '()' is the number of individuals representing the GenBank sequence when that number is more than one. Population site codes are given; site details are in Table 4.1.

	Site code	Number of individuals sequenced	GenBank accession number
P. cylindrifera			
1	Esp17	4	OR828091, OR828107 (2) and OR828108
2	Esp20	1	OR828092
3	Esp21	2	OR828093 and OR828094
4	Esp30	2	OR828095 (2)
5	Elli1	2	OR828111 (2)
6	Fra1	2	OR828102 and OR828103
7	Pin1	2	OR828109 and OR828110
8	Rav1	3	OR828104, OR828105 and OR828106
9	Tun1	3	OR828112 and OR828113 (2)
10	Var2	2	OR828098 and OR828099
P. longicaudata			
1	Abr1	3	OR828146, OR828147 and OR828148
2	Ben1	3	OR828154, OR828155 and OR828159
3	Cam1	3	OR828136 (2) and OR828137
4	Cup1	3	OR828138, OR828139 and OR828140
5	Esp28	2	OR828145 (2)
6	Gra1	2	OR828157 and OR828158
7	Hy6	2	OR828143 and OR828144
8	Kin2	2	OR828141 and OR828142
9	Mag1	2	OR828153 (2)
10	Mag4	2	OR828153 (2)
11	Mag7	2	OR828154 and OR828155
12	Mar4	2	OR828065 and OR828066
13	Mar5	3	OR828064, OR828067 and OR828068
14	Moo1	3	OR828060, OR828061 and OR828062
15	nWH2	3	OR828057, OR828058 and OR828059
16	Pink1	3	OR828145 (3)
17	Rav5	3	OR828149 (2) and OR828150
18	TS5	2	OR828151 and OR828152

Table S4.2: List of *COI* haplotypes of *Parartemia cylindrifera* and information on the number of individuals sequenced per haplotype (n) and their distribution across populations. Population site codes are given; site details are in Table 4.1.

Hap.	n	Site code									
		Fra1	Pin1	Var2	Rav1	Esp17	Esp20	Esp21	Esp30	Elli1	Tun1
PC01	4	4									
PC02	11	11									
PC03	3	3									
PC04	2	2									
PC05	6		6								
PC06	8		8								
PC07	4		4								
PC08	1		1								
PC09	1		1								
PC10	1			1							
PC11	1			1							
PC12	1			1							
PC13	3			3							
PC14	1			1							
PC15	9			9							
PC16	1			1							
PC17	1			1							
PC18	1			1							
PC19	1			1							
PC20	3				3						
PC21	1				1						
PC22	2				2						
PC23	1				1						
PC24	3				3						
PC25	7				7						
PC26	3				3						

PC27	1					1					
PC28	17					17					
PC29	4					4					
PC30	17					4	12	1			
PC31	1					1					
PC32	6					6					
PC33	3					3					
PC34	1					1					
PC35	2						2				
PC36	1						1				
PC37	1						1				
PC38	1						1				
PC39	1						1				
PC40	2						2				
PC41	7							7			
PC42	4							4			
PC43	4							4			
PC44	3							3			
PC45	1							1			
PC46	12								12		
PC47	1								1		
PC48	2								2		
PC49	1								1		
PC50	2								2		
PC51	1								1		
PC52	1								1		
PC53	2					2					
PC54	1					1					
PC55	1									1	
PC56	15									11	4
PC57	2									1	1

PC58	11									7	4
PC59	1										1
PC60	5										5
PC61	1										1
PC62	1										1
PC63	1										1
PC64	2										2
Total	**220**	**20**	**20**	**20**	**20**	**40**	**20**	**20**	**20**	**20**	**20**

Table S4.3: List of *COI* haplotypes of *Parartemia longicaudata* and information on number of individuals sequenced per haplotype (n) and their distribution across populations. Population site codes are given; site details are in Table 4.1.

Hap.	n	Site code																	
		Abr1	TS5	Mar5	Mar4	Ben1	Hy6	Mag7	Mag1	Mag4	nWH2	Moo1	Cam1	Cup1	Gra1	Kin2	Rav5	Pink1	Esp28
PL01	1	1																	
PL02	1	1																	
PL03	14	14																	
PL04	1	1																	
PL05	1	1																	
PL06	1	1																	
PL07	1	1																	
PL08	9		9																
PL09	3		3																
PL10	5		5																
PL11	3		3																
PL12	1			1															
PL13	1			1															
PL14	22			12	10														
PL15	1			1															
PL16	2			1	1														
PL17	1			1															
PL18	2			1	1														
PL19	1			1															
PL20	1			1															
PL21	1				1														
PL22	1				1														
PL23	5				1	1	1	1		1									
PL24	1				1														
PL25	1				1														
PL26	1				1														
PL27	1					1													
PL28	13					13													
PL29	9						9												

PL30	2						2												
PL31	3						3												
PL32	1						1												
PL33	1						1												
PL34	3						3												
PL35	5					4		1											
PL36	6							6											
PL37	1							1											
PL38	2								2										
PL39	1								1										
PL40	1								1										
PL41	1									1									
PL42	1									1									
PL43	1									1									
PL44	1									1									
PL45	25				1	1			16	7									
PL46	2										2								
PL47	2										2								
PL48	6										6								
PL49	4										4								
PL50	3										3								
PL51	1										1								
PL52	1										1								
PL53	1										1								
PL54	5											5							
PL55	1											1							
PL56	8											8							
PL57	1											1							
PL58	1											1							
PL59	1											1							
PL60	1											1							
PL61	1											1							
PL62	1											1							
PL63	14												14						

PL64	1												1					
PL65	1												1					
PL66	1												1					
PL67	1												1					
PL68	1												1					
PL69	1												1					
PL70	1													1				
PL71	7													7				
PL72	5													5				
PL73	3													3				
PL74	2													2				
PL75	1													1				
PL76	1													1				
PL77	5														5			
PL78	2														2			
PL79	4														4			
PL80	2														2			
PL81	2														2			
PL82	1														1			
PL83	1														1			
PL84	1														1			
PL85	1														1			
PL86	1														1			
PL87	2															2		
PL88	8															8		
PL89	4															4		
PL90	1															1		
PL91	1															1		
PL92	1															1		
PL93	1															1		
PL94	1															1		
PL95	1															1		
PL96	15																15	
PL97	3																3	

PL98	1																1		
PL99	1																1		
PL100	38																	18	20
PL101	1																	1	
PL102	1																	1	
Total	**340**	**20**	**20**	**20**	**19**	**20**	**20**	**9**	**20**	**12**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**

Chapter 5

Chapter 5. Invasion of salt lakes: the brine shrimp *Artemia* in Australia

The following chapter has been drafted in accordance with the journal ***Hydrobiologia***, and the manuscript is currently under review.

The following authors contributed to this manuscript as outlined below.

Authorship order	Contribution (%)	Concept development	Data collection	Data analysis	Drafting manuscript	Revision of manuscript
Md Aminul Islam	79	X	X	X	X	X
Jennifer Chaplin	12	X	X			X
Angus Lawrie	3		X			X
Mahabubur Rahman	3					X
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Contribution indicates the total involvement each author has had in this project. Placing an 'X' in the remaining boxes indicates which aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

Candidate **Principal Supervisor**

Chapter Linking Statement

The previous data chapters (Chapter 3 and Chapter 4) enhanced our understanding of Australian brine shrimp *Parartemia*, covering species relationships, validity of morphospecies, evolutionary history and phylogeography. The current chapter (Chapter 5) presents up-to-date information on the distribution of exotic *Artemia* in Australia and investigates the identity and phylogeography of the species in Australian natural salt lakes. These results provide important background information for use by future studies to assess the risk that *Artemia* represents to *Parartemia* and other native fauna in these lakes.

5.0 Abstract

This study examines the distribution, identity and phylogeography of exotic *Artemia* in Australian natural salt lakes (ANSL), focusing on Western Australia. We used a *cytochrome c oxidase subunit 1* marker to establish that the bisexual *Artemia* in ANSL are *A. franciscana*, represented by a haplotype that has also been found in some Australian saltworks and many other countries. Similarly, the unisexual *Artemia* in these lakes belong to a diploid parthenogenetic lineage that is very common and widespread outside of Australia. We used a combination of published and new and preexisting unpublished data to provide an up-to-date account of *Artemia* distribution in Australia. We provide records of parthenogenetic *Artemia* co-occurring with the endemic brine shrimp *Parartemia* and of *A. franciscana* displacing parthenogenetic *Artemia* in an ANSL. Genetic and distributional information support the view that both *Artemia* biotypes have recently been spreading among ANSL. Data from the *internal transcribed spacer 1* gene region indicate that parthenogenetic *Artemia* have invaded coastal and inland lakes in Western Australia via Rottnest Island, which appears to have been colonised by multiple clones from Asia. This study establishes baseline data needed to assess the impacts of *Artemia* invasions on the rich endemic fauna of ANSL, especially *Parartemia*.

5.1 Introduction

Invasive species are a threat to biodiversity (Elton, 2020; Gherardi, 2007; Mooney & Hobbs, 2000; Sala *et al.*, 2000; Simberloff, 2010) and their economic and ecological consequences are a major global concern (Crystal-Ornelas & Lockwood, 2020; Cuthbert *et al.*, 2021; Zenni *et al.*, 2021). The term invasive species is variously defined (Boonman-Berson *et al.*, 2014; Pereyra, 2016), but herein we follow the definition of Simberloff (2010) that "an invasive species is one that arrives (often with human assistance) in a habitat it had not previously occupied, then establishes a population and spreads autonomously". The invasion process typically involves the introduction and establishment of an invasive species, followed by the spread of that species and its ecological impact on native species and communities (see Allendorf & Lundquist, 2003; Renault *et al.*, 2022). It has been suggested that lake ecosystems are particularly vulnerable to the effects of invasive species (see Havel *et al.*, 2015; Reynolds & Aldridge, 2021; Sala *et al.*, 2000) because for example, these effects may rapidly impact the entire ecosystem (Reynolds & Aldridge, 2021).

Salt-lake ecosystems are exceedingly abundant in Australia, where it is estimated that more than 80 % of lakes and wetlands are saline (De Deckker, 1983; Timms, 2005). Australian salt lakes contain an unusually diverse endemic fauna but they are vulnerable to a range of threats (Lawrie *et al.*, 2021; Timms, 2005). Some of these threats, for example, those associated with agriculture and mining (Halse *et al.*, 2003; Timms, 2005) or global climate change (IPCC, 2001; Jellison *et al.*, 2008; Kirono *et al.*, 2012; Pittock, 2003; Williams, 2002), are well documented. However, potential threats from invasive species, most notably the exotic brine shrimp *Artemia*, have received much less attention (but see McMaster *et al.*, 2007; Ruebhart *et al.*, 2008; Timms, 2014).

Brine shrimp (suborder Artemiina) are divided into two monogeneric families, the Parartemiidae and the Artemiidae (Timms, 2014; Weekers *et al.*, 2002). The former comprises 21 bisexual species of *Parartemia*, which are endemic to Australia (Islam *et al.*, 2024; Timms, 2012b, 2014). The latter comprises *Artemia*, which is essentially globally distributed and includes both bisexual and unisexual lineages (Asem *et al.*, 2016; Asem *et al.*, 2023; Browne & Bowen, 1991; Van Stappen, 2002). Recent data (see Asem *et al.*, 2024b; Asem *et al.*, 2023) suggest that there are nine bisexual species, as follows: *A. salina* (Linnaeus 1758), *A. sinica* Cai 1989, *A. urmiana* Günther 1899, *A. amati* Asem et al. 2023, *A. tibetiana* Abatzopoulos et al. 1998, *A. sorgeloosi* Asem et al. 2023, *A. franciscana* Kellogg 1906, *A. monica* Verrill 1869

and *A. persimilis* Piccinelli and Prosdocimi 1968. Unisexual *Artemia* includes a range of diploid and polyploid lineages that are now known to be polyphyletic and so no longer classified together as the species *A. parthenogenetica* (Abatzopoulos *et al.*, 2002a; Asem *et al.*, 2016; Asem *et al.*, 2024a; Maccari *et al.*, 2013a; Maniatsi *et al.*, 2011; Muñoz *et al.*, 2010), although that binomen is still sometimes used (e.g., Wang *et al.*, 2024). We have followed the suggestion of Asem *et al.* (2024a) and refer to parthenogenetic lineages of *Artemia* according to their ploidy level, e.g., diploid parthenogenetic *Artemia*, however, we use the term unisexual/s when the identity of the lineage is unknown.

Both unisexual *Artemia* lineages and *A. franciscana* have invaded areas outside of their native range. Unisexual lineages, which originated in Asia (Asem *et al.*, 2016; Muñoz *et al.*, 2010), have spread to the Mediterranean region, greater Africa and also Australia (Baxevanis *et al.*, 2014; McMaster *et al.*, 2007; Muñoz & Pacios, 2010; Sainz-Escudero *et al.*, 2022). *Artemia franciscana*, which is native to the Americas, has invaded locations across the globe, including Africa, Asia, Europe, New Zealand as well as Australia (see Horváth *et al.*, 2018; Muñoz *et al.*, 2014; Ruebhart *et al.*, 2008; Thirunavukkarasu *et al.*, 2024). Its spread between continents is primarily attributed to human activities associated with saltworks, aquaculture and the aquarium trade (Amat *et al.*, 2005; Eimanifar *et al.*, 2014; Horváth *et al.*, 2018; Muñoz *et al.*, 2014; Ruebhart *et al.*, 2008; Thirunavukkarasu *et al.*, 2024).

Artemia franciscana has a high reproductive capacity (Amat *et al.*, 2005; Ruebhart *et al.*, 2008) and broad ecological tolerances (Browne & Wanigasekera, 2000), which can provide it with a competitive advantage over its congeners. When *A. franciscana* invades the habitats of other *Artemia* species, the native populations typically disappear within a few years (Abatzopoulos *et al.*, 2006; Amat *et al.*, 2005; Ruebhart *et al.*, 2008; Thirunavukkarasu *et al.*, 2024; Valsala *et al.*, 2015; Vikas *et al.*, 2012). Laboratory trials have confirmed the competitive superiority of *A. franciscana* over some other *Artemia* species, with the overall ranking being *A. franciscana* > unisexual *Artemia* > *A. salina* (Amat *et al.*, 2005; Browne & Halanych, 1989; Ruebhart *et al.*, 2008). This is borne out in the field where unisexual *Artemia* has invaded habitats of native *A. salina* in the Mediterranean and Africa (Baxevanis *et al.*, 2014; Sainz-Escudero *et al.*, 2022; Van Stappen, 2002) and *A. franciscana* is now replacing both of these *Artemia* in some areas (Amat *et al.*, 2005; Baxevanis *et al.*, 2014; Muñoz *et al.*, 2014). The effects of *Artemia* invasions on native fauna other than congeners are unknown.

Records of *Artemia* in Australia date back to 1855 but questions have been raised about whether some or all of the early reports relate to misidentified specimens of native brine (*Parartemia*)

and/or fairy (*Branchinella*) shrimps (see Geddes, 1979; McMaster *et al.*, 2007; Timms, 2014). Confirmed records of bisexual *Artemia* in Australia date back to a report of the deliberate introduction of *A. franciscana* into the Inkerman Creek saltworks in Port Alma, Queensland in the 1960s from a packet of commercial eggs (Clark & Bowen, 1976; Ruebhart *et al.*, 2008). Bisexual *Artemia* are now known from a total of six coastal saltworks in Queensland, South Australia and Western Australia and in three lakes where salt extraction used to occur - an unnamed lake near Port Augusta in South Australia and Hutt Lagoon and Lake Koorkoordine in Western Australia (McMaster *et al.*, 2007; Pinder *et al.*, 2002; Ruebhart *et al.*, 2008; Timms, 2014). Molecular data (*cytochrome c oxidase subunit 1*, *COI*) have identified *A. franciscana* as the species present in the St Kilda and Mulgundawa saltworks in South Australia and in those at Port Hedland and Dampier in Western Australia (Asem *et al.*, 2018). Timms (2014) reported that bisexual *Artemia* is not known to occur in any Australian natural salt lake that has not been used/modified for salt extraction. However, we found an unpublished report that mentions the presence of *A. franciscana* in three such lakes in the northern Wheatbelt region of Western Australia (ARL, 2009), although the report does not mention how this species was identified.

Unisexual *Artemia* is known from a range of sites in Western Australia, comprising saltworks (at Shark Bay, Lake McLeod and Onslow; Timms, 2014), coastal lakes on Rottnest Island (some of which were once subject to salt extraction; Jamet, 2021; Lennon, 2017) and lakes on the Swan Coastal Plain and further inland in the Wheatbelt region (McMaster *et al.*, 2007), as well as remote Lake Boonderoo in the Esperance-Goldfields region (Timms, 2014). They were once present in the St Kilda saltworks (dry creek near Adelaide) in South Australia but have been replaced by *A. franciscana* (Asem *et al.*, 2018; Timms, 2014). Timms (2014) indicated that unisexual *Artemia* occurred in another saltworks in South Australia, near Lake Alexandria, although the exact location was not specified. Asem *et al.* (2018) reported the presence of *A. franciscana* in a saltworks near Lake Alexandria, referred to as Mulgundawa. If this is the site that Timms was referring to, then the unisexual *Artemia* in this saltworks has also been replaced by *A. franciscana*.

In their study of the origins and phylogeography of diploid parthenogenetic *Artemia*, Muñoz *et al.* (2010) included two partial (531 bp) *COI* sequences, obtained from GenBank (AY953368 and AY953369), of unisexual *Artemia* from an unspecified location/s in Australia. They found that these sequences belonged to a diploid parthenogenetic lineage of *Artemia* that was closely related to an undescribed bisexual species from Kazakhstan (= *A. amati* in Asem *et al.*, 2023). McMaster *et al.* (2007) also reported that the unisexual *Artemia* in salt lakes in Western

Australia were a type of diploid parthenogen but they did not mention how they determined this. Using allozyme data, McMaster *et al.* (2007) found that unisexual *Artemia* on Rottnest Island contained relatively high clonal diversity, possibly reflecting multiple introductions, compared to salt lakes on the Western Australia mainland. These authors suggested that a coastal lake on the mainland (Lake Hayward) was the source of emigrants to three salt lakes further inland in the Wheatbelt region on the basis that the clonal compositions of these four lakes were very similar. DNA sequence data, which typically contain a stronger phylogenetic signal than allozyme data (Avise, 2012), are needed to test these hypotheses about the spread of unisexual *Artemia* in Western Australia.

The main published sources of information on the distribution of unisexual and bisexual *Artemia* in Australia, i.e., McMaster *et al.* (2007) and Ruebhart *et al.* (2008), respectively, are now over 15 years old. If these taxa are spreading in natural systems, then it is likely that published information underestimates their current distributions. The first aim of our study was therefore to use published, unpublished and newly acquired data to provide an up-to-date account of the distribution of bisexual and unisexual *Artemia* in Australia, focusing on natural salt lakes (ANSL). The second aim was to use mitochondrial DNA (*COI*) sequence data to identify the types of bisexual and unisexual *Artemia* that are present in the ANSL. A third aim was to use *COI* and *internal transcribed spacer 1* (*ITS-1*) to investigate the phylogeography of unisexual and bisexual *Artemia* in ANSL. For the unisexuals, we used the results to test the hypotheses that multiple clones of unisexual *Artemia* have invaded lakes on Rottnest Island and that Lake Hayward is the source of unisexual populations in salt lakes in inland Western Australia.

5.2 Materials and Methods

5.2.1 *Artemia* distribution and collections

To produce up-to-date information on the distribution of unisexual and bisexual *Artemia* in Australia, we combined data from our field collections (see below) with information from published sources, three unpublished reports (ARL, 2004, 2006, 2009) to the Department of Biodiversity, Conservation and Attractions (DBCA), records maintained by DBCA (dating from October 1997 to November 2022) and publicly available data in Atlas of Living Australia (www.ala.org.au). We only included records that included specific date and location details. Older records that were not included in a review by Geddes (1979) were excluded because they could not be confirmed. The list of sources is given in supplementary Table S5.1. To find published and publicly available sources, various keywords were used to search in Google Scholar and Scopus databases. Keywords included 'brine shrimp Australia', '*Artemia* Australia', 'Salt Lakes Australia', 'Salt Lakes Threats', 'Anostraca Australia', 'Artemiina Australia', 'Crustacea Australia', 'Aquatic Invertebrates Australia', 'Aquatic Invasive Species Australia'.

We collected unisexual *Artemia* (only females detected) from two lakes on Rottnest Island (BAG01 and ROT11) and seven ANSL (AND01, ESP42, KL11, NEW10, NOR01, QUA01 and TS11) and bisexual *Artemia* (both males and females detected) from a total of five ANSL (KON01, MAR11, MAR12, NIN02 and WH11). All sites are in Western Australia. These collections were made between October 2022 and April 2023 using a dip net (site details in Table 5.1). Despite sampling over 200 ANSL in Western Australia between 2017 and 2023, these are the only sites where we found *Artemia*.

After collection, specimens were transported to a laboratory at Murdoch University, washed in freshwater to remove salt, euthanised by freezing and finally preserved in 100 % ethanol for analysis. Ethanol-preserved samples of unisexual *Artemia* from another two ANSL (BOO01 and HAY01) were obtained from the DBCA (Table 5.1).

Table 5.1: Details of *Artemia* collected from Australian salt lakes and used in the genetic analyses. DBCA: Department of Biodiversity, Conservation and Attractions, Western Australia. Location coordinates and additional details are available in supplementary Table S5.1.

Sl.	Location	Lake Name	Site ID	Source
		Unisexual *Artemia*		
01	Lake Toolbrunup	Anderson Lake *	AND01	This study
02	Rottnest Island	Lake Baghdad	BAG01	
03	Zanthus	Lake Boonderoo	BOO01	DBCA
04	Wittenoom Hills	Unnamed lake beside Mount Ney Rd *	ESP42	This study
05	Preston Beach	Lake Hayward	HAY01	DBCA
06	Kondinin	Lake Kondinin *	KL11	This study
07	Ravensthorpe	Unnamed lake beside Beatty Rd *	NEW10	
08	Lime Lake	Lake Norring	NOR01	
09	Badjaling	Quairading Pink Lake	QUA01	
10	Rottnest Island	Rottnest Pink Lake	ROT11	
11	Womarden	Unnamed lake beside Perenjori-Three Springs Rd	TS11	
		Bisexual *Artemia*		
01	Kondut	Unnamed lake beside Kondut S E Rd *	KON01	This study
02	Gunyidi	Unnamed lake beside Gunyidi-Wubin Rd *	MAR11	
03	Dalwallinu	Unnamed lake beside Miling N Rd *	MAR12	
04	Lake Ninan	Lake Ninan	NIN02	
05	Marne	Unnamed lake beside Damboring E Rd *	WH11	

* Newly identified *Artemia* sites.

5.2.2 DNA extraction, PCR and sequencing

Genomic DNA was extracted from thoracic segments of mature *Artemia* or the whole body of immature specimens (when only a few mature specimens were collected from a site) using the MasterPure™ Complete DNA and RNA Purification Kit according to the manufacturer's instructions. A DNA negative was included in every set of extractions to detect potential contamination.

Two genetic regions were amplified. (i) A 658 bp region of *COI* via the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) for unisexual *Artemia* and via the primers 1/2COI_Fol-F and 1/2COI_Fol-R (Muñoz *et al.*, 2008) for the bisexuals. (ii) A 1177 bp

amplicon comprising the nuclear *ITS-1* region along with parts of the adjacent *18S* rRNA and *5.8S* rRNA regions via the primers 18d and R58 (Baxevanis *et al.*, 2006; Hillis & Dixon, 1991) for both unisexual and bisexual *Artemia*. PCR reactions were performed in a final volume of 25 µL, containing 2.5 µL of Thermo Scientific 10X DreamTaq Buffer (supplemented with 20 mM $MgCl_2$), 1.25 µL of dNTPs (10 mM per nucleotide), 0.5 µL of each primer (10 µM), 0.35 µL of bovine serum albumin (10 µg/µL), 0.125 µL of DreamTaq DNA Polymerase, 0.5 µL of template DNA, and PCR grade water to adjust the final volume. The PCR reaction conditions were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 45 sec; and a final extension at 72 °C for 7 min.

ExoSAP-IT purification was used to remove unincorporated primers and dNTPs from the PCR products (Dugan *et al.*, 2002). Purified PCR products were sequenced in both forward and reverse directions using an ABI 3700 automated sequencer (Applied Biosystems) at Macrogen, Inc. in South Korea.

5.2.3 Sequence processing and alignment

Sequencing chromatograms were examined using Chromas v2.6.5 (Technelysium Pty. Ltd., Queensland, Australia). Heterozygous sites in the *ITS-1* region were identified by visually looking at the sequencing chromatograms and labelled using the codes of the International Union of Pure and Applied Chemistry (IUPAC) (Cornish-Bowden, 1985). The final consensus sequences were confirmed using MEGA X software (Kumar *et al.*, 2018). We found no evidence that nuclear copies of *COI* had inadvertently been included in our *Artemia* dataset as, for example, there were minor amino acid substitutions, and no indels or stop codons, in the translated sequences (see Raupach & Radulovici, 2015). The alignment of the *COI* sequences was performed in MEGA X using MUSCLE (Edgar, 2004), while the *ITS-1* sequences were aligned using the MAFFT online platform with the Q-INS-i strategy (http://mafft.cbrc.jp/alignment/server/).

5.2.4 Identity of unisexual and bisexual *Artemia*

Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of *COI* variation were used to determine the species identity of unisexual and bisexual *Artemia* in Australia. The dataset comprised 111 and 50 *COI* sequences for, respectively, unisexual and bisexual *Artemia* from our collections, plus 520 *COI* sequences for a range of *Artemia* obtained from GenBank

(details in Table S5.2). The *COI* region was selected for this analysis because of its ability to elucidate relationships among bisexual and unisexual lineages of *Artemia* (Asem *et al.*, 2016; Maniatsi *et al.*, 2011). DnaSP v6 (Rozas *et al.*, 2017) was used to identify the different haplotypes in this dataset. Three sequences of *Parartemia*, the closest known relative of *Artemia*, were obtained from GenBank (AF308954, AF209059 and AF209060) and used as an outgroup in these analyses.

The BI phylogenetic analysis was conducted in BEAST v1.10.4 (Suchard *et al.*, 2018). The best substitution model, i.e., HKY+G, was selected using the Bayesian Information Criterion (BIC) in jModelTest v.2.1.9 (Darriba *et al.*, 2012). The molecular clock test in MEGA X (Kumar *et al.*, 2018) indicated that the model without a molecular clock assumption provided the best fit for the dataset. The uncorrelated lognormal relaxed clock model with coalescent constant size tree prior were assigned in the BEAST program BEAUti, and the analysis was run for 50 million generations. The log-output file generated by the main BEAST program was evaluated in Tracer v1.7.2 (Rambaut *et al.*, 2018) to check the Effective Sample Size (ESS) and ensure that the ESS values are greater than 200. A 25% burn-in was applied to discard potentially unreliable trees. The final tree was generated in TreeAnnotator (another BEAST program) and visualised using FigTree v1.4.4 (Rambaut, 2018).

The ML phylogenetic analysis was conducted on the IQ-TREE web server (see http://iqtree.cibiv.univie.ac.at; Trifinopoulos *et al.*, 2016) using 5000 ultrafast bootstrap replicates, employing the same substitution model that was used for the BI analysis.

5.2.5 Phylogeography of unisexual *Artemia*

To investigate the phylogeography of unisexual *Artemia* in Australian lakes, our *COI* sequences from 111 individuals from 11 sites (see Tables 5.1 and S5.3) and *ITS-1* sequences from 98 individuals from ten sites (see Tables 5.1 and S5.4; it was not possible to produce *ITS-1* data for individuals from BOO01 due to repeated PCR amplification failures) were combined with, respectively, *COI* sequences from 267 individuals (generated by Maccari *et al.*, 2013a; Muñoz *et al.*, 2010) and *ITS-1* sequences from 57 individuals (generated by Asem *et al.*, 2016; Baxevanis *et al.*, 2006; Maccari *et al.*, 2013a) from sites in Europe, Africa and Asia. These pre-existing sequences were obtained from GenBank and selected because detailed site information was available. Further details with GenBank accession numbers are in Table S5.3 for *COI* and Table S5.4 for *ITS-1*.

DnaSP v6 (Rozas *et al.*, 2017) was used to estimate genetic diversity indices (i.e., number of polymorphic sites, number of parsimony informative sites, number of haplotypes, haplotype diversity and nucleotide diversity) for the *COI* dataset. For the *ITS-1* dataset, unique sequences were treated as different clones. The Median Joining algorithm (Bandelt *et al.*, 1999) in PopART v1.7 (Leigh & Bryant, 2015) was used to create a *COI* haplotype network and *ITS-1* clone network, as well as to develop the *COI* haplotype and *ITS-1* clone distribution map.

5.2.6 Phylogeography of *bisexual Artemia* in ANSL

The phylogenetic analysis indicated that bisexual *Artemia* in ANSL are *A. franciscana*. *COI* and *ITS-1* sequence data were used to investigate the phylogeography of this species in these lakes. The *COI* dataset comprised newly generated sequences for 50 *A. franciscana* from five ANSL (see Tables 5.1 and S5.5) as well as all GenBank sequences for this species from Australian saltworks, i.e., the 67 individuals from the St Kilda, Mulgundawa, Port Hedland and Dampier saltworks (generated by Asem *et al.*, 2018). We also included *COI* data from 127 individuals from the Great Salt Lakes and San Francisco Bay site in the USA from GenBank (generated by Eimanifar *et al.*, 2015; Muñoz *et al.*, 2013) to provide context for the Australian data. See Table S5.5 for additional details, including GenBank accession numbers.

The *ITS-1* dataset comprised newly generated sequences from 50 *A. franciscana* individuals from five ANSL (see Tables 5.1 and S5.6). The *ITS-1* sequences included both homozygous and heterozygous sites. Consequently, prior to analysis, the initial 50 unphased *ITS-1* sequences were transformed into 100 reconstructed sequences using the PHASE algorithm (Stephens & Donnelly, 2003; Stephens *et al.*, 2001) in DnaSP v6 (Rozas *et al.*, 2017). Further details with GenBank accession numbers are in Table S5.6.

Genetic diversity indices for the *COI* and *ITS-1* datasets were estimated using DnaSP v6 (Rozas *et al.*, 2017). The Median Joining algorithm (Bandelt *et al.*, 1999) in PopART v1.7 (Leigh & Bryant, 2015) was used to create haplotype networks and develop haplotype distribution maps for both datasets.

5.3 Results

5.3.1 Distribution of *Artemia* in Australia

Unisexual *Artemia* has been recorded from 43 sites in Australia, comprising three saltworks, six lakes on Rottnest Island, and 34 ANSL on the Australian mainland (Fig. 5.1, Table S5.1). Bisexual *Artemia* has been recorded from 17 sites across Australia (Fig. 5.1, Table S5.1), made up of six saltworks, three natural salt lakes previously used for salt extraction (past saltworks), and another eight ANSL. *Artemia* has also been documented from five other sites in Australia (two ANSL, one saltworks and two unspecified sites) but the records did not specify whether they were bisexual or unisexual (Fig. 5.1). These records include cases of both unisexual and bisexual *Artemia* co-occurring in the same ANSL, and of unisexual and/or bisexual *Artemia* co-occurring with *Parartemia* species in six ANSL (see Tables 5.2, S5.1). All ANSL sites (not associated with salt extraction) with unisexual and/or bisexual *Artemia* are in Western Australia.

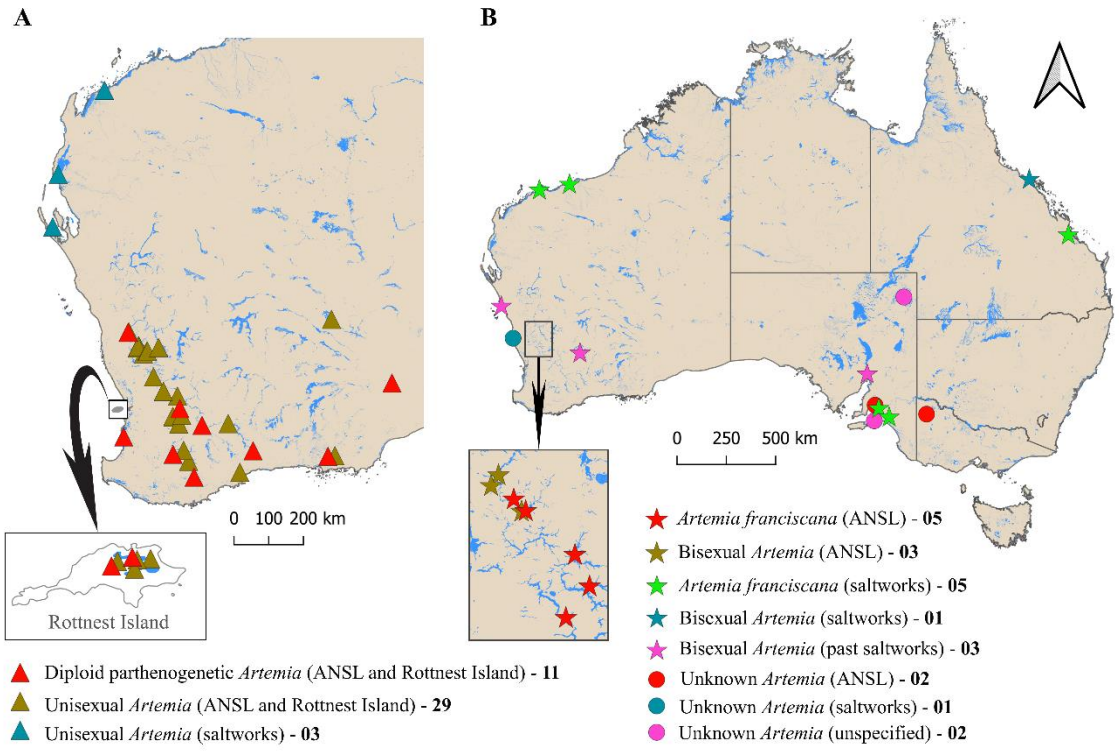

Fig. 5.1: Approximate locations of known *Artemia* populations in Australia: (A) unisexual populations and (B) bisexual and unknown (sex ratio not specified) populations. ANSL indicates Australian natural salt lakes that have not been used/modified for salt extraction. Number of records per category is in bold. Species name is provided only if identity has been

confirmed via molecular data. Multiple sites in proximity may not be always clearly distinguishable (refer to supplementary Table S5.1 for detailed information on sites). Maps have been created using QGIS 3.32 (https://www.qgis.org). Data for surface hydrology in blue on the maps are sourced from the national surface water database of Geoscience Australia (www.ga.gov.au).

Table 5.2: Australian natural salt lakes from which (i) both unisexual and bisexual *Artemia* and/or (ii) both *Artemia* and *Parartemia* have been observed. The 'Site' column indicates either the site ID used in this study (see Table 5.1) or the site code used in the source. Location coordinates are available in supplementary Table S5.1.

Site	Taxa	Observation date	Source
AND01	Diploid parthenogenetic *Artemia*	October 2022	This study
	Parartemia longicaudata	September 1998	DBCA
NIN02	*Artemia franciscana*	October 2022 and January 2023	This study
	Unisexual *Artemia*	September 1999 and no date in McMaster *et al.* (2007)	DBCA and McMaster *et al.* (2007)
TS11	Diploid parthenogenetic *Artemia*	November 2022	This study
	Parartemia laticaudata		
W001	Unisexual *Artemia*	September 2008	ARL (2009)
	Bisexual *Artemia*	August 2008	
	Parartemia serventyi	August 2004	ARL (2004)
	Parartemia contracta	August and September 2008	ARL (2009)
W002	Unisexual *Artemia*	November 2003 and October 2008	ARL (2004) and ARL (2009)
	Parartemia contracta	August 2004 and August 2005	ARL (2004) and ARL (2006)
W004	Bisexual *Artemia*	August 2008	ARL (2009)
	Parartemia serventyi	August 2004	ARL (2004)
	Parartemia longicaudata	August 2008	ARL (2009)
W012	Unisexual *Artemia*	August and October 2008	ARL (2009)
	Bisexual *Artemia*	September 2008	
W018	Unisexual *Artemia*	November 2003	ARL (2004)
	Parartemia serventyi	August 2004	

5.3.2 Identity of unisexual and bisexual *Artemia* in Australia

The *COI* sequences used to build the BI and ML phylogenetic trees were between 446 and 658 bp long. The entire dataset included 95 haplotypes from a range of locations including four Australian saltworks. However, only three *COI* haplotypes (APD02, APD20 and APD21) were found in 111 individuals of unisexual *Artemia* from two sites on Rottnest Island and nine mainland sites and another haplotype (HAP01) in 50 bisexual *Artemia* from five ANSL (Fig. 5.2 and Fig. S5.1). Haplotypes APD02, APD20 and APD21 grouped with those from a lineage of diploid parthenogenetic *Artemia* that was closely related to a clade containing *A. amati* and *A. tibetiana* (Fig. 5.2). Haplotypes AU-1 and AU-2 (see Fig. 5.2), the two partial (531 bp) *COI* sequences from unisexual *Artemia* from an unspecified location/s in Australia (used in Muñoz *et al.*, 2010), occurred in the same group. Haplotype HAP01 clustered with those of *A. franciscana* (Fig. 5.2) and has also been found in populations of this species from two saltworks in Australia and Great Salt Lake and the San Francisco Bay site in the USA (discussed below).

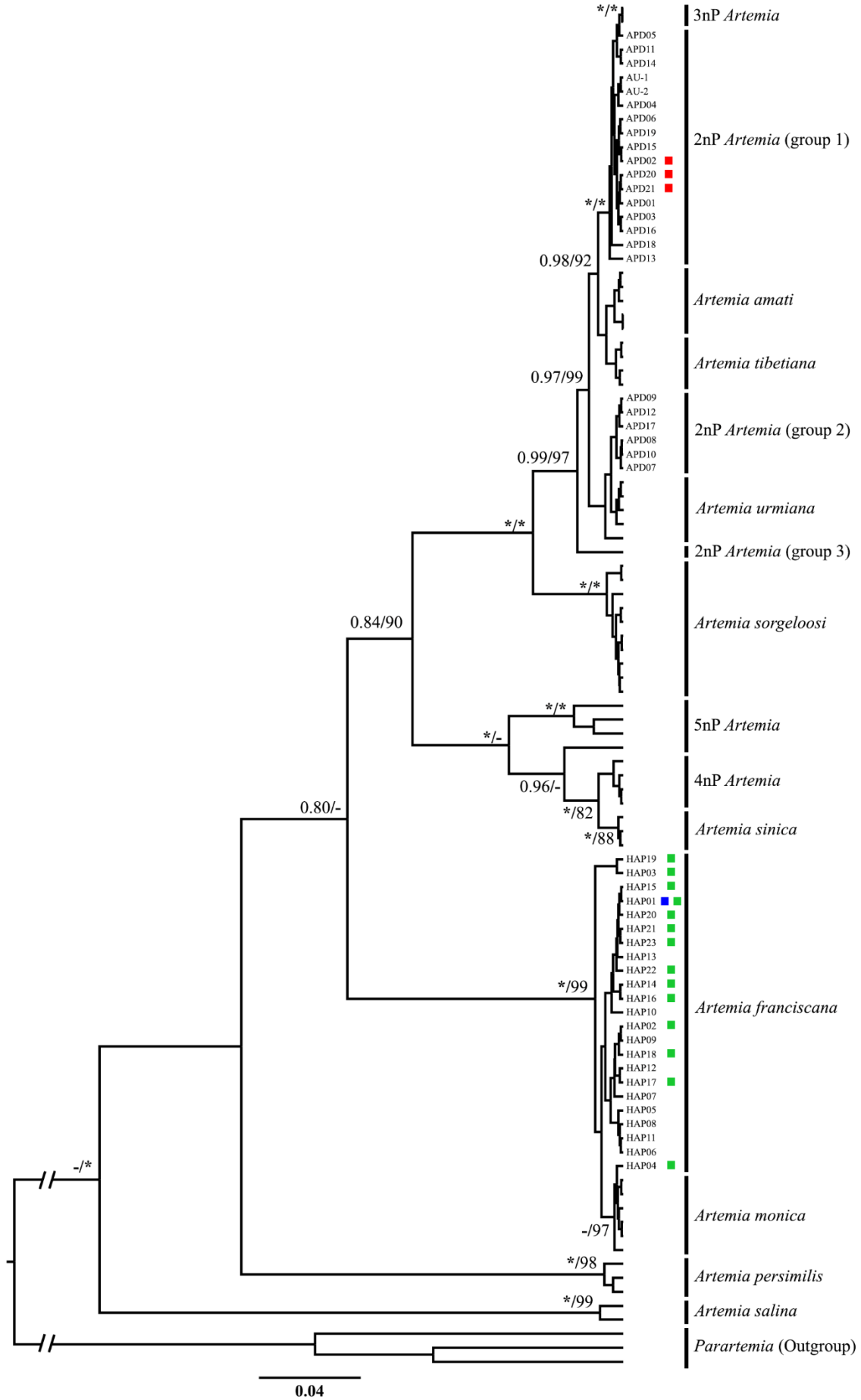
3nP *Artemia*
APD05
APD11
APD14
AU-1
AU-2
APD04
APD06
APD19
APD15
APD02
APD20
APD21
APD01
APD03
APD16
APD18
APD13
2nP *Artemia* (group 1)
Artemia amati
Artemia tibetiana
APD09
APD12
APD17
APD08
APD10
APD07
2nP *Artemia* (group 2)
Artemia urmiana
2nP *Artemia* (group 3)
Artemia sorgeloosi
5nP *Artemia*
4nP *Artemia*
Artemia sinica
HAP19
HAP03
HAP15
HAP01
HAP20
HAP21
HAP23
HAP13
HAP22
HAP14
HAP16
HAP10
HAP02
HAP09
HAP18
HAP12
HAP17
HAP07
HAP05
HAP08
HAP11
HAP06
HAP04
Artemia franciscana
Artemia monica
Artemia persimilis
Artemia salina
Parartemia (Outgroup)
0.98/92
0.97/99
0.99/97
0.84/90
0.80/-
0.96/-
*/82
*/88
*/99
*/98
-/97
-/*
0.04

Fig. 5.2: Bayesian inference (BI) and Maximum Likelihood (ML) phylogenetic analyses of *Artemia* based on the mitochondrial *COI* region (446 - 658 bp). The ML tree is available in Supplementary Fig. S5.1. Bayesian Posterior Probability (BPP, when ≥ 0.80) and bootstrap values from the ML tree (when ≥ 80%) are shown at the nodes as BPP/bootstrap. For nodes where one value was above the threshold and the other was below, the latter is indicated by a hyphen (-). Nodes with a BPP value of 1 and a bootstrap value of 100% are marked with an asterisk (*). APD01 - APD21 are labels for haplotypes (614 bp) of diploid parthenogenetic *Artemia* from Muñoz *et al.* (2010), Maccari *et al.* (2013a) and this study. AU-1 and AU-2 denote two shorter sequences (531 bp) of diploid parthenogenetic *Artemia* from an unspecified location/s in Australia from GenBank (AY953368 and AY953369, see Supplementary Table S5.2). HAP01 - HAP23 are labels for haplotypes (446 bp) of *A. franciscana* from Asem *et al.* (2018), Muñoz *et al.* (2013); Eimanifar *et al.* (2015) and this study. Red and blue symbols indicate unisexual and bisexual *Artemia* haplotypes from Australian salt lakes, respectively. Green symbols indicate *A. franciscana* haplotypes found in four Australian coastal saltworks (from Asem *et al.*, 2018). 2nP, 3nP, 4nP, and 5nP denote diploid, triploid, tetraploid, and pentaploid parthenogens, respectively. Lineage 2nP *Artemia* (group 3) is a putative third diploid parthenogenetic *Artemia* lineage represented by rare males from Kujalnik (rmKUJ), identified by Maccari *et al.* (2013b).

5.3.3 Phylogeography of unisexual *Artemia* in Australian lakes

After aligning and trimming, the *COI* sequences used to investigate the phylogeography of diploid parthenogenetic *Artemia* on Rottnest Island and in ANSL on the mainland were 614 bp in length. Three different *COI* haplotypes (APD02, APD20 and APD21) were found in 111 individuals from 11 Australian sites (see above and Fig. 5.3 and Fig. 5.4). The total dataset also contained another 18 *COI* haplotypes from sixteen other countries.

COI diversity was low in this taxon in the Australian lakes. Only three of eleven sites had more than one haplotype and haplotype diversity was only low to moderate in these three sites, ranging from 0.100 ± 0.088 in BAG01 to 0.467 ± 0.132 in NEW10 (see Table 5.3). Nucleotide diversity was also low ranging from 0.00016 ± 0.00014 in BAG01 to 0.00076 ± 0.00021 in NEW10 (Table 5.3).

The APD02 haplotype was found in ten of eleven Australian sites. It was the only haplotype found at seven sites (including ROT11 on Rottnest Island and Lake Hayward) and the most common haplotype at one of the remaining sites (BAG01 on Rottnest Island) (see Fig. 5.3).

This haplotype is also very common and widespread outside of Australia (see Fig. 5.3 and Fig. 5.4). APD20 and APD21 were unique to the Australian samples and, given that they each differ from APD02 by only 1 bp, are probably recent mutational derivatives of APD02 (see Fig. 5.4). APD20 was only found on Rottnest Island. APD21 was found in three (NEW10, NOR01 and QUA01) of the ANSL in the Wheatbelt region of Western Australia (see Fig. 5.3), suggesting that this haplotype evolved in and is spreading among lakes in this region (see below for more details).

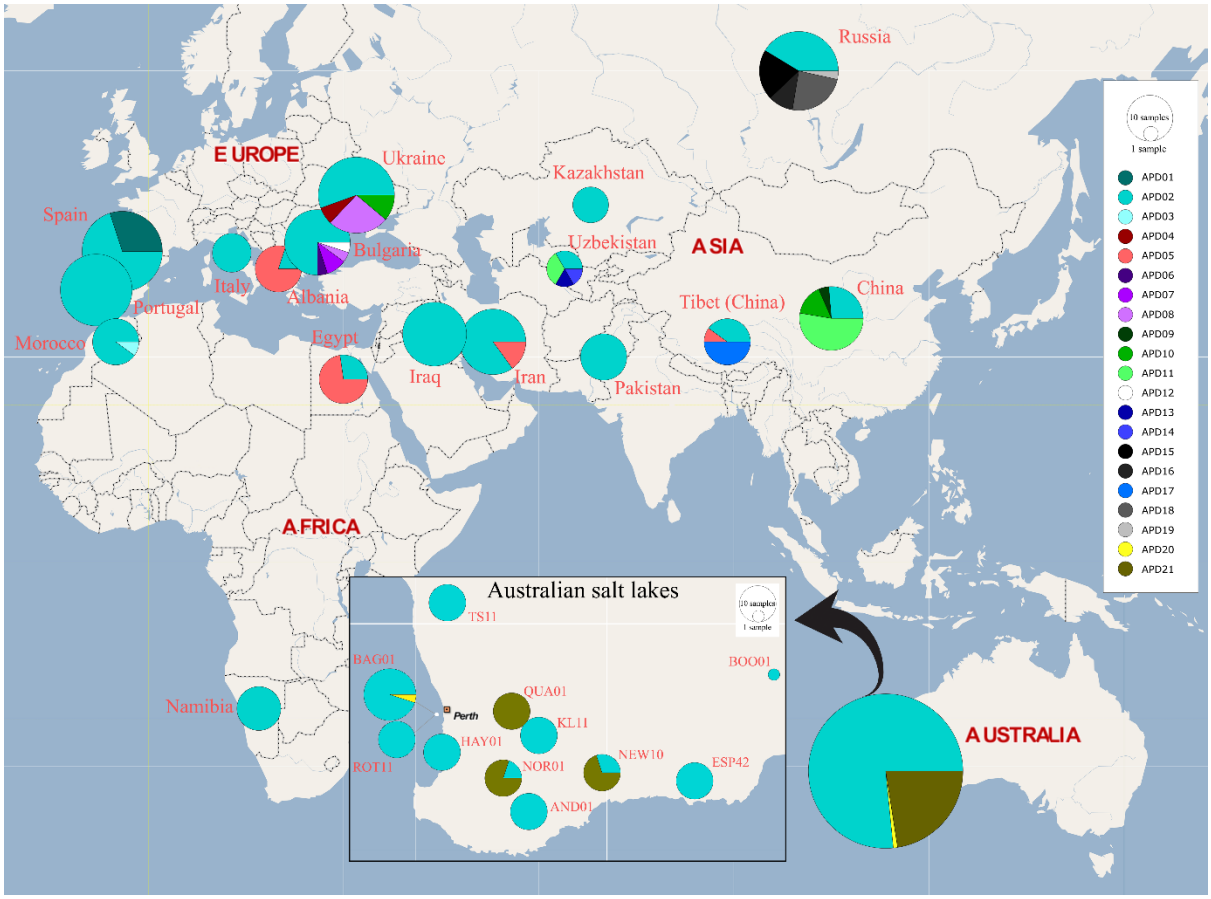

Fig. 5.3: Map showing the distribution and abundance of 21 *COI* (614 bp) haplotypes of diploid parthenogenetic *Artemia* from eleven Australian salt lakes and sixteen other countries. Details of Australian sites are in Table 5.1.

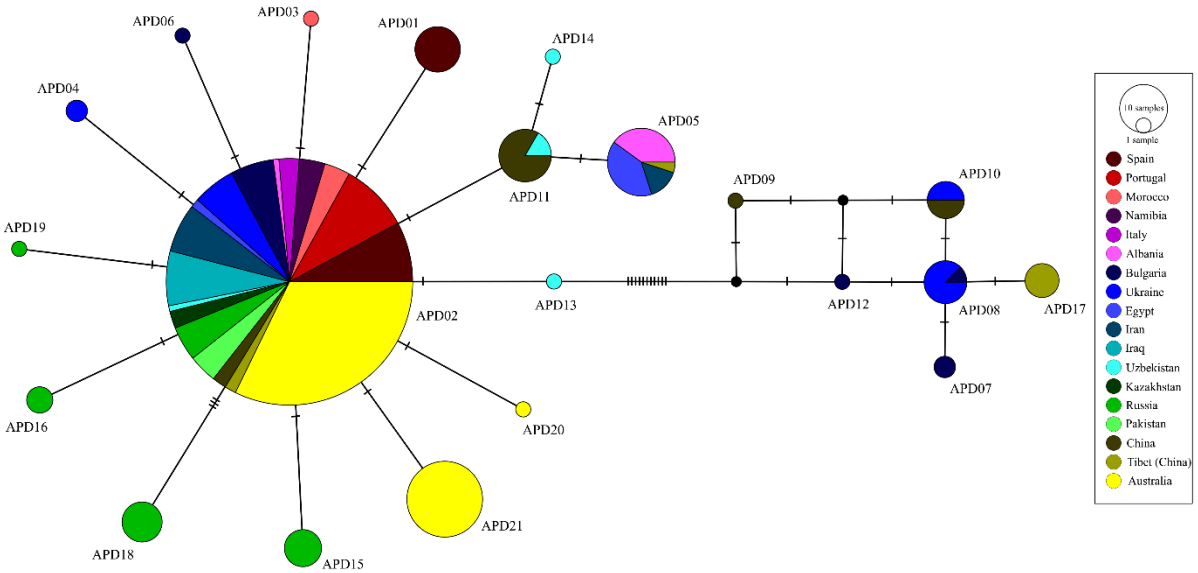

Fig. 5.4: Haplotype network for 21 *COI* (614 bp) haplotypes of diploid parthenogenetic *Artemia* from eleven Australian salt lakes and sixteen other countries. Hatch marks indicate the number of mutational steps between haplotypes and black dots indicate missing haplotypes. Details of Australian sites are in Table 5.1.

Table 5.3: *COI* diversity in diploid parthenogenetic *Artemia* from eleven Australian salt lakes and sixteen other countries. N: number of sequences; V: number of polymorphic sites; P: number of parsimony informative sites; H: number of haplotypes; Hd: haplotype diversity; and Phi: nucleotide diversity. Numbers in parentheses after each country denote the contributing sites for each country, applicable when multiple sites are involved. Further details are in Table S5.3.

Site	N	V	P	H	Hd	Phi
Australian sites						
AND01	10	0	0	1	0	0
BAG01	20	1	0	2	0.100 ± 0.088	0.00016 ± 0.00014
BOO01	1	0	0	1	0	0
ESP42	10	0	0	1	0	0
HAY01	10	0	0	1	0	0
KL11	10	0	0	1	0	0
NEW10	10	1	1	2	0.467 ± 0.132	0.00076 ± 0.00021
NOR01	10	1	1	2	0.356 ± 0.159	0.00058 ± 0.00026
QUA01	10	0	0	1	0	0
ROT11	10	0	0	1	0	0
TS11	10	0	0	1	0	0
Country-based						
Australia (11)	111	2	1	3	0.366 ± 0.045	0.00060 ± 0.00008
Spain (3)	30	1	1	2	0.434 ± 0.070	0.00071 ± 0.00011
Portugal (2)	24	0	0	1	0	0
Morocco	10	1	0	2	0.200 ± 0.154	0.00033 ± 0.00025
Namibia	9	0	0	1	0	0
Italy	7	0	0	1	0	0
Albania	10	2	2	2	0.356 ± 0.159	0.00116 ± 0.00052
Bulgaria	20	16	15	5	0.442 ± 0.133	0.00804 ± 0.00259
Ukraine (3)	27	16	16	4	0.630 ± 0.076	0.01161 ± 0.00129
Egypt (2)	11	2	2	2	0.436 ± 0.133	0.00142 ± 0.00043
Iran	20	2	2	2	0.268 ± 0.113	0.00087 ± 0.00037
Iraq	19	0	0	1	0	0
Uzbekistan	6	3	1	4	0.867 ± 0.129	0.00206 ± 0.00052
Kazakhstan	6	0	0	1	0	0
Russia (3)	29	6	5	5	0.741 ± 0.049	0.00283 ± 0.00038
Pakistan	10	0	0	1	0	0
China (2)	19	16	16	4	0.661 ± 0.084	0.00920 ± 0.00269
Tibet (China)	10	17	15	3	0.644 ± 0.101	0.01422 ± 0.00190

The *ITS-1* dataset for diploid parthenogenetic *Artemia* consisted of 991 bp sequences from *155* individuals from ten Australian lakes and 12 other countries (details in Table S5.4). A total of 17 variable sites, including 5 heterozygous positions, and 16 clones (pGen-1 to pGen-16) were present (Table 5.4). Six clones (pGen-9 and pGen-12 to pGen-16) were found in 98 individuals from the Australian lakes (Fig. 5.5, Fig. 5.6). Five of these clones were exclusive to these lakes, but pGen-9 has also been found in China, Iran, Russia and Uzbekistan but not in Europe or Africa (see Fig. 5.5, Fig. 5.6), suggesting its potential origin in Asia. Clone pGen-9 was found in BAG01 on Rottnest Island (see Fig. 5.5) but not in any ANSL on the mainland. However, clone pGen-14, which differs from pGen-9 by 1 bp and is probably a mutational derivative of pGen-9, was found in a lake in Esperance (ESP42; see Fig. 5.5, Fig. 5.6). Three other clones (pGen-12, pGen-13 and pGen-16) also occur in Rottnest Island (Fig. 5.5). These clones differ from each other by 1 bp and pGen-12 and pGen-16 are probably mutational derivatives of pGen-13 (see Fig. 5.6). Clone pGen-13 shows only 1 bp difference and is probably derived from pGen-3, which occurs in China and Namibia but not in Australia (Fig. 5.5, Fig. 5.6). The presence of both pGen-9 and pGen-13 on Rottnest Island supports the hypothesis that multiple clones of diploid parthenogenetic *Artemia* have invaded this location.

Clone pGen-16 was only found on Rottnest Island, however, pGen-12 and/or pGen-13 were also found in all inland ANSL except QUA01 (see Fig. 5.5). Given this and that Lake Hayward only contained pGen-13, it appears that Rottnest Island is the main original source of diploid parthenogenetic *Artemia* in the inland lakes, not Lake Hayward. The immediate origin of clone pGen-15, which occurs in three inland ANSL (NEW10, NOR01 and QUA01), is uncertain. It is likely a mutational derivative of one of two missing clones (see Fig. 5.6). One of these missing clones differs from pGen-9 (which was found in Australia) by 1 bp and from each of pGen-7 and pGen-8 (which have only been found in Egypt) by 1 bp. The other missing clone differs from pGen-3 (which occurs in China and Namibia) by 1 bp. Thus, the data do not discriminate whether pGen-15 arose at a location within or outside of Australia. However, except for two individuals from NEW10 with APD02, every individual with the pGen-15 genotype also had the APD21 *COI* haplotype. This implies that the APD21 - pGen-15 combination may have arisen in the NEW10 population and then spread to NOR01 and QUA01.

Table 5.4: Details of *ITS-1* clones of diploid parthenogenetic *Artemia* encountered in the dataset. The aligned sequence was 991 bp and had 17 variable, including five heterozygous, sites. N: number of sequences; A: adenine; C: cytosine; G: guanine; T: thymine; K: G-T; M: A-C; W: A-T; and Y: C-T. Dots represent matching sites

Clones	**N**	**Genotype**																
		17 bp	35 bp	66 bp	214 bp	238 bp	261 bp	372 bp	422 bp	517 bp	519 bp	540 bp	541 bp	567 bp	665 bp	684 bp	710 bp	813 bp
pGen-1	1	G	T	G	C	C	A	C	T	T	C	G	C	C	A	T	C	C
pGen-2	1	.	.	.	A	.	.	.	.	.	.	T	A	.	.	.	.	.
pGen-3	9	.	.	.	A	A	C	.	C	.	.	T	A	.	.	A	.	.
pGen-4	2	.	.	A	A	A	.	.	C	.	.	T	A	.	.	.	.	.
pGen-5	8	.	.	.	A	A	.	.	C	.	A	T	A	.	.	.	.	.
pGen-6	3	.	.	.	A	A	.	.	C	.	M	T	A	.	.	.	.	.
pGen-7	1	.	.	.	A	A	.	.	C	.	.	T	A	.	G	W	.	.
pGen-8	1	.	.	.	A	A	.	T	C	.	.	T	A	.	.	W	.	.
pGen-9	29	.	.	.	A	A	.	.	C	.	.	T	A	.	.	.	.	.
pGen-10	2	.	.	.	A	A	.	.	C	.	A	T	A	.	.	.	Y	.
pGen-11	1	.	G	.	A	A	C	.	C	.	.	T	A	A	.	A	.	.
pGen-12	38	.	.	.	A	A	C	.	C	K	.	T	A	.	.	A	.	T
pGen-13	21	.	.	.	A	A	C	.	C	.	.	T	A	.	.	A	.	T
pGen-14	9	A	.	.	A	A	.	.	C	.	.	T	A	.	.	.	.	.
pGen-15	27	.	.	.	A	A	M	.	C	.	.	T	A	.	.	W	.	.
pGen-16	2	.	.	.	A	A	C	.	C	G	.	T	A	.	.	A	.	T

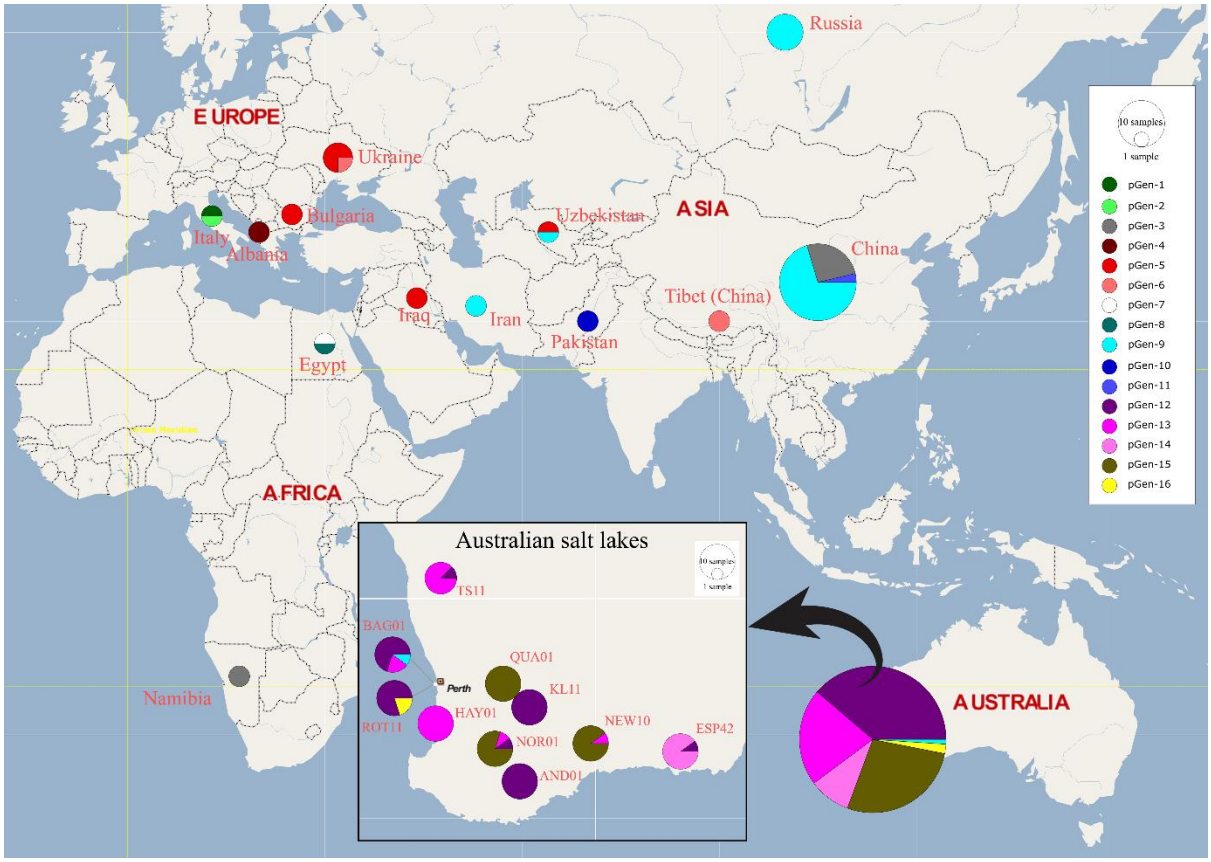

Fig. 5.5: Map showing the distribution and abundance of 16 *ITS-1* (991 bp) clones of diploid parthenogenetic *Artemia* from ten Australian salt lakes and twelve other countries. Details of Australian sites are in Table 5.1.

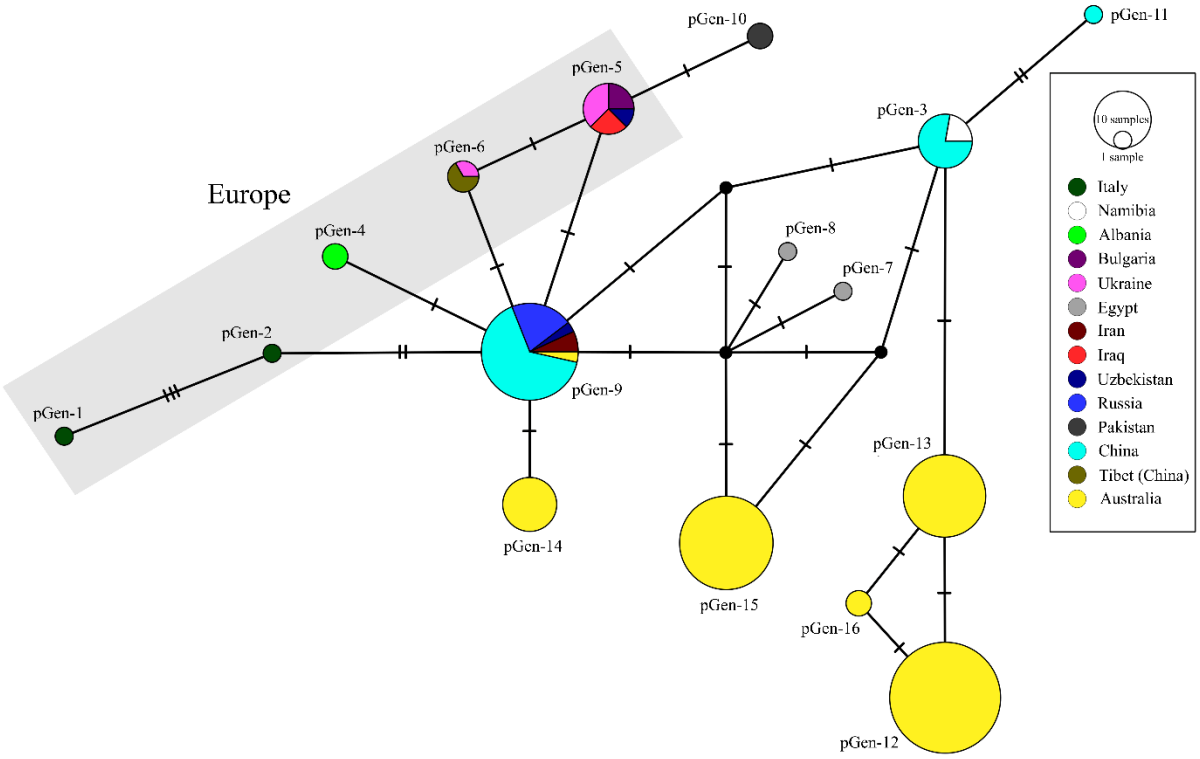

Fig. 5.6: Clone network for 16 *ITS-1* (991 bp) clones of diploid parthenogenetic *Artemia* from ten Australian salt lakes and twelve other countries. Hatch marks indicate the number of mutational steps between clones and black dots indicate missing clones. Details of Australian sites are in Table 5.1.

5.3.4 Phylogeography of *A. franciscana* in Australian lakes

After aligning and trimming, the *COI* sequences used to investigate the phylogeography of *A. franciscana* in the ANSL were 446 bp in length. The *COI* dataset had a total of 23 haplotypes (HAP01 - HAP23). Only HAP01 was found in 50 individuals from five ANSL, compared to 14 haplotypes from 67 individuals in the four Australian saltworks and 13 from 127 individuals from San Francisco Bay site and the Great Salt Lake (Fig. 5.7, Fig. 5.8; also see Table S5.5). HAP01 also occurred at the Port Hedland saltworks in Western Australia and the St Kilda saltworks in South Australia, as well as both USA sites (Great Salt Lake and San Francisco Bay) (Fig. 5.7, Fig. 5.8). It was the most common haplotype at the Port Hedland, St Kilda and San Francisco Bay sites (Fig. 5.7). The levels of *COI* diversity in *A. franciscana* in a single lake were less than those in any one Australian saltworks, with exception of Dampier which only contained HAP03 (Fig. 5.7; Table S5.7). These levels were also less than those in the Great Salt Lake and San Francisco Bay sites (Fig. 5.7; Table S5.7).

Some of the haplotypes in the Australian saltworks were rare, unique to one site and only one mutational step from a common haplotype and could be *in situ* mutational derivatives. For example, HAP14 - HAP16 at St Kilda and HAP20 - HAP23 at Port Hedland are probably mutational derivatives of HAP01 whereas HAP17 and HAP18 at Mulgundawa may be derived from HAP02 (see Fig. 5.8).

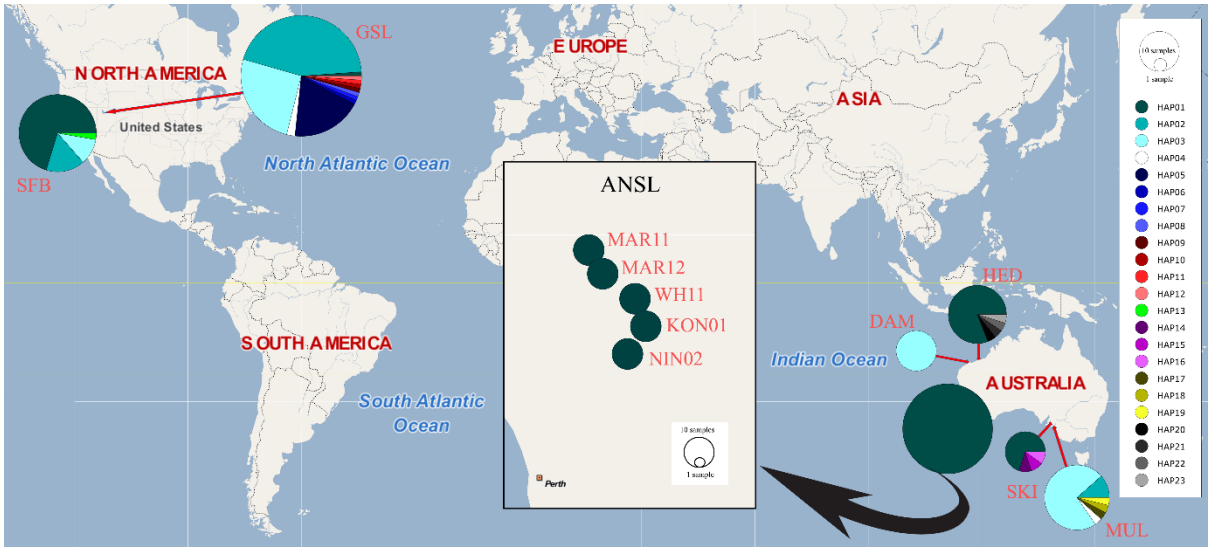

Fig. 5.7: Map showing the distribution and abundance of 23 *COI* (446 bp) haplotypes of *Artemia franciscana* from five Australian natural salt lakes (ANSL) and four Australian saltworks: St Kilda (SKI), Mulgundawa (MUL), Port Hedland (HED) and Dampier (DAM) and from the Great Salt Lake (GSL) and San Francisco Bay (SFB) sites in the USA. Site details of ANSL are in Table 5.1.

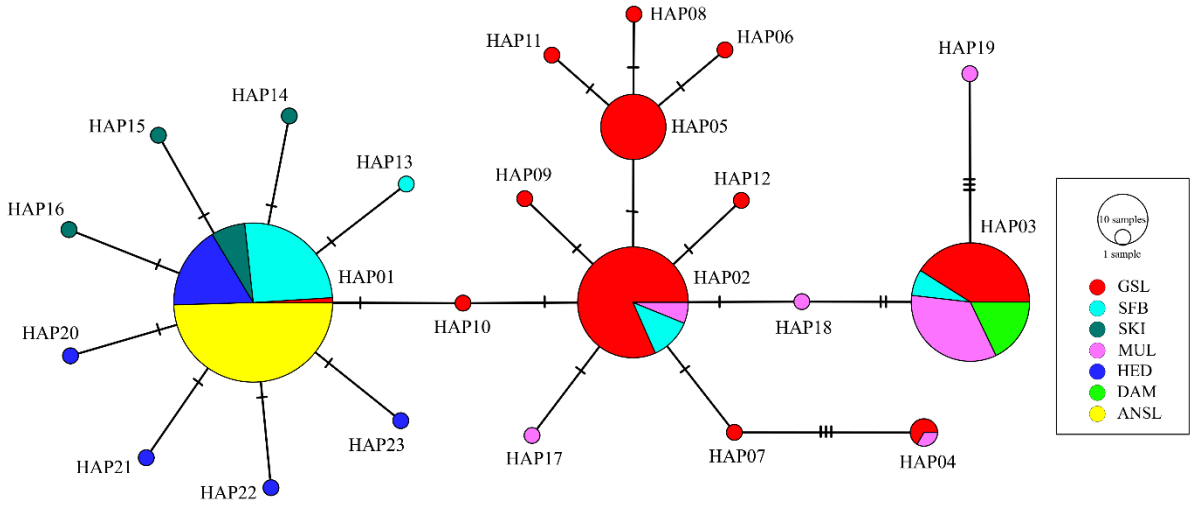

Fig. 5.8: Haplotype network for 23 *COI* (446 bp) haplotypes of *Artemia franciscana* from five Australian natural salt lakes (ANSL) and four Australian saltworks: St Kilda (SKI), Mulgundawa (MUL), Port Hedland (HED) and Dampier (DAM) and from the Great Salt Lake

(GSL) and San Francisco Bay (SFB) sites in the USA. Hatch marks indicate the number of mutational steps between haplotypes. Site details of ANSL are in Table 5.1.

A total of six *ITS-1* (1177 bp) haplotypes (bITS-1.1 to bITS-1.6) were identified in 100 reconstructed sequences from 50 *A. franciscana* from the five ANSL (details in Table S5.6). The number of haplotypes ranged from six in MAR12 near Dalwallinu, one of the most northern sites in the sampling area, to three in Lake Ninan (NIN02), the most southerly sampling site for this species (see Fig. 5.9). The sample from MAR12 also had the highest haplotype (0.789 ± 0.057) and nucleotide (0.00106 ± 0.00017) diversity, whereas a near-by site (MAR11) had four haplotypes but the lowest level of haplotype diversity (0.553 ± 0.111) and of nucleotide diversity (0.00060 ± 0.00016) (Table 5.5). The most abundant haplotype was bITS-1.1 (Fig. 5.9). This haplotype plus two others (bITS-1.2 and bITS-1.5) were found at all five ANSL (see Fig. 5.9). All *ITS-1* haplotypes were within four mutational steps of each other (Fig. 5.9).

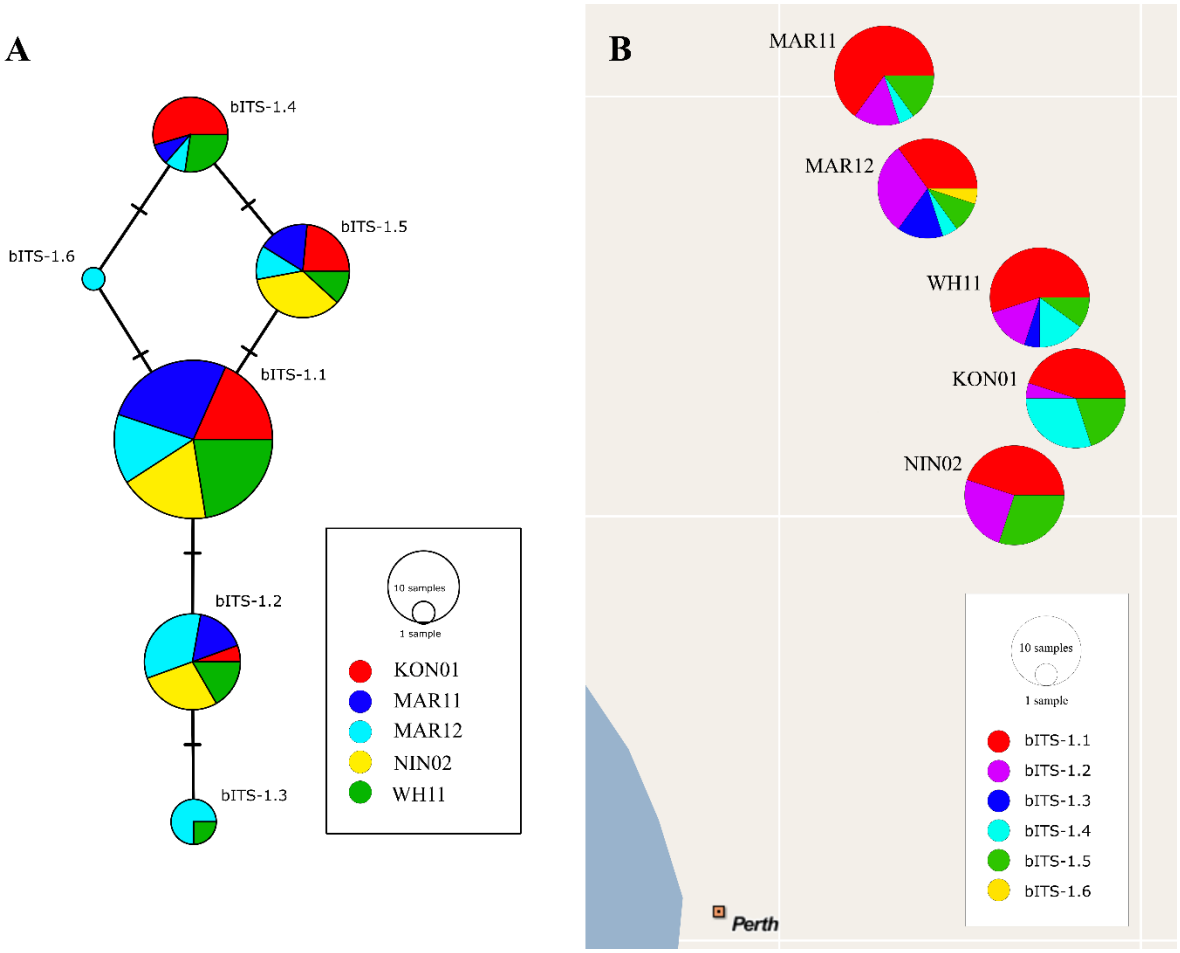

Fig. 5.9: *ITS-1* (1177 bp) haplotype information for *Artemia franciscana* from five natural salt lakes (ANSL) in Western Australia. (A) Haplotype network, with hatch marks indicating the number of mutational steps between haplotypes, and (B) the distribution and relative abundance of haplotypes. Site details of ANSL are in Table 5.1.

Table 5.5: *ITS-1* diversity in *Artemia franciscana* from five Australian natural salt lakes (ANSL). N: number of unphased sequences; n: number of reconstructed sequences; V: number of polymorphic sites; P: number of parsimony informative sites; H: number of haplotypes; Hd: haplotype diversity; and Phi: nucleotide diversity. Site details of ANSL are in Table 5.1.

Site	N	n*	V	P	H	Hd	Phi
KON01	10	20	3	2	4	0.679 ± 0.074	0.00091 ± 0.00013
MAR11	10	20	3	2	4	0.553 ± 0.111	0.00060 ± 0.00016
MAR12	10	20	4	4	6	0.789 ± 0.057	0.00106 ± 0.00017
NIN02	10	20	2	2	3	0.679 ± 0.052	0.00071 ± 0.00009
WH11	10	20	4	3	5	0.674 ± 0.098	0.00093 ± 0.00020

* Reconstructed sequences were obtained from the unphased *ITS-1* (1177 bp) sequences using the PHASE algorithm (Stephens & Donnelly, 2003; Stephens *et al.*, 2001) in DnaSP v6 (Rozas *et al.*, 2017).

5.4 Discussion

We used *COI* data to confirm that the unisexual *Artemia* in ANSL belong to a common and widespread lineage of diploid parthenogenetic *Artemia* (lineage group B in Maccari *et al.*, 2013a). We also show that the bisexuals are *A. franciscana* and exhibit a common and widespread *COI* haplotype (e.g., haplotype Af10 in Muñoz *et al.*, 2013; Haf04 in Muñoz *et al.*, 2014). Despite extensive sampling of the invertebrates in ANSL in the past 20 years (e.g., Islam *et al.*, 2024; Lawrie *et al.*, 2023; Pinder *et al.*, 2005; Rogers & Timms, 2014; Timms, 2009b, 2018), unisexual/diploid parthenogenetic *Artemia* have been recorded from a total of only 34 ANSL on the Australian mainland, and bisexual/*A. franciscana* is known from only eight ANSL that have not been used for salt extraction, plus three that have (Timms, 2014). Nevertheless, our distributional and genetic data indicate that both *Artemia* biotypes have recently been spreading among ANSL in Western Australia.

5.4.1 Species identity

Maccari *et al.* (2013a) identified three mitochondrial (*COI*) lineages of diploid parthenogenetic *Artemia*. (1) Lineage B, which was monophyletic and closely related to an undescribed species from Kazakhstan and some populations of *A. tibetiana*; (2) Lineage A, which was polyphyletic and closely related to *A. urmiana;* and (3) a putative third lineage represented by two rare males from Kujalnik. We now know that the undescribed species from Kazakhstan is *A. amati* (Asem *et al.*, 2023) and that Maccari et al.'s *A. tibetiana* that were not closely related to lineage B is *A. sorgeloosi* (Asem *et al.*, 2023). Although some aspects of our *COI* phylogeny do not exactly match that of Maccari *et al.* (2013a), our *COI* data, which were derived from a total of 111 individuals from Rottnest Island and a range of mainland sites in Western Australia, indicate that unisexual *Artemia* in ANSL in Australia (see group 1 in Fig. 5.2) belong to lineage B of Maccari *et al.* (2013a), as do haplotypes AU-1 and AU-2, from an unspecific location/s in Australia (see Fig. 5.2 and Muñoz *et al.*, 2010). Sainz-Escudero *et al.* (2022) reported the presence of parthenogenetic populations of *A. urminana* in Australia, although the source of their data is not clear. The apparent discrepancy between these results *versus* ours seems to be that Sainz-Escudero *et al.* (2022) have largely followed the classification suggested by Sainz-Escudero *et al.* (2021), i.e., have used a different taxonomy.

To date, diploid parthenogenetic *Artemia* and *A. franciscana* are the only *Artemia* known to occur in Australia (e.g., Muñoz *et al.*, 2010; Timms, 2014; and this study). Based on eDNA data, Campbell *et al.* (2023) suggested that *A. tibetiana*, as well as unisexual *Artemia* (which

they referred to as *A. parthenogenetica*), occurs in five lakes (Lake Baghdad, Lake Vincent, Garden Lake, Herschel Lake and Serpentine Lake) on Rottnest Island. However, they only collected unisexual *Artemia* in net surveys from these sites. We assayed a total of 30 individuals from Lake Baghdad and another lake on Rottnest Island (ROT11, see Table 5.1) but only found evidence of diploid parthenogenetic *Artemia*. Similarly, McMaster *et al.* (2007) only reported the presence of '*A. parthenogenetica*' from Rottnest Island (they did not mention any specific lake). Since eDNA sequence reads do not always provide reliable species-level identifications (Klymus *et al.*, 2017) and no specimens have been observed, we suggest that it is unlikely that *A. tibetiana* occurs on Rottnest Island.

5.4.2 Phylogeography of diploid parthenogenetic *Artemia* in ANSL

Almost all (77 %) individuals of diploid parthenogenetic *Artemia* in salt lakes on Rottnest Island and the mainland had the APD02 *COI* haplotype. This haplotype also occurs in Asia, Europe and Africa (Fig. 5.3; see also Maccari *et al.*, 2013a; Muñoz *et al.*, 2010). The two other *COI* haplotypes (APD20 and APD21) are known only from ANSL and appear to be recent mutational derivatives of APD02. In fact, the evidence suggests that APD21 - pGen-15 clone arose in the NEW10 population and has spread to some other sites in the Wheatbelt region (see results). The AU-1 and AU-2 haplotypes differed from APD02 by one and three mutational steps, respectively, but the significance of AU-1 and AU-2 is not clear as their source location/s is unknown (see Introduction).

The *ITS-1* data support the proposal of McMaster *et al.* (2007) that multiple clones of diploid parthenogenetic *Artemia* have invaded the lakes on Rottnest Island. The introduction of parthenogenetic *Artemia* to Australia has been the subject of three main hypotheses. (1) Intentional human-mediated introduction from European salterns (McMaster *et al.*, 2007). (2) Unintentional human-mediated introduction during the importation of *A. franciscana* eggs that inadvertently included some from unisexual *Artemia* (see Campos-Ramos *et al.*, 2003; Endebu *et al.*, 2013; Ruebhart *et al.*, 2008). (3) Bird-mediated dispersal from Asia (McMaster *et al.*, 2007). At least 20 species of migratory shorebirds visit Rottnest Island using the East Asian - Australasian flyway (Mather, 2020). Option 2 seems unlikely because confirmed records of parthenogenetic *Artemia* on Rottnest Island date back to 1959 (see Geddes, 1979; McMaster *et al.*, 2007), which is prior to the first records of *A. franciscana* being imported into Australia (see Introduction). McMaster *et al.* (2007) argue in favour of the third hypothesis, suggesting that parthenogenetic *Artemia* first invaded Australia via the lakes on Rottnest Island before

spreading to other coastal sites and then to inland sites. Their evidence included that fewer migratory birds visit inland areas of Western Australia compared to Rottnest Island and other coastal areas. Given that our *ITS-1* data suggest that the lineage of diploid parthenogenetic *Artemia* in Australia likely arrived from Asia, our findings support the third hypothesis. However, like McMaster *et al.* (2007), we cannot rule out the possibility that parthenogenetic *Artemia* were deliberately introduced into the Rottnest Island salt lakes from Europe, especially since salt was harvested from these lakes from the 1830s to 1952 (Jamet, 2021).

The *ITS-1* data do not support the suggestion by McMaster *et al.* (2007) that Lake Hayward, which is located on the Swan Coastal Plain, is the source of unisexual *Artemia* further inland in the Wheatbelt region in Western Australia. Instead, these data suggest that Rottnest Island is the main source of these *Artemia* in Lake Hayward and lakes in the Wheatbelt and other inland areas.

5.4.3 Phylogeography of *A. franciscana* in ANSL

The amount of *COI* diversity in populations of *A. franciscana* in ANSL was low, with only one haplotype detected in 50 individuals from five sites. In contrast, up to six *COI* haplotypes, sometimes including putative *in situ* mutational derivatives, were present in populations of this species in Australian saltworks (see Asem *et al.*, 2018). Similarly, some populations of diploid parthenogenetic *Artemia* in the ANSL appear to contain *in situ* mutational derivatives (see above). The presence of a single *COI* haplotype of *A. franciscana* in the ANSL supports the idea that this species has only recently invaded these sites. This fits with other observations such as that the only previous record of this species in ANSL, other than from those from which salt has been harvested (see Timms, 2014), was in the same general area (northern Wheatbelt region in Western Australia) in 2009 (ARL, 2009) and that so far this species is only known from a total of eight such ANSL. This is despite extensive sampling of salt lakes in Western Australia having taken place before and after 2009 (e.g., see ARL, 2004; Islam *et al.*, 2024; Lawrie *et al.*, 2023; Pinder *et al.*, 2005; Rogers & Timms, 2014; Timms, 2009b). Certainly, *A. franciscana* has only recently invaded Lake Ninan, as DBCA records from 1999 and McMaster *et al.* (2007) only mentioned parthenogenetic *Artemia* at this site.

The nuclear *ITS-1* diversity in *A. franciscana* in the ANSL was slightly higher compared to mitochondrial *COI* diversity, which was also the case for diploid parthenogenetic *Artemia*. This could be due to the effects of reduced effective population size, decreased mutation rate and/or purifying selection on the mitochondrial marker (see Ellegren, 2009). The number of *ITS-1*

haplotypes in *A. franciscana* populations in the ANSL tended to decline from north to south. Assuming that *ITS-1* variation is selectively neutral, this might indicate that *A. franciscana* has experienced repeated founder effects as it is spreading in a predominantly southwards direction in ANSL in the sampling area (e.g., see Boileau & Hebert, 1991; De Meester *et al.*, 2002).

The *COI* haplotype (HAP01) representing *A. franciscana* in the ANSL also occurs in the Port Headland saltworks in Western Australia as well as in the St Kilda saltworks in South Australia (Asem *et al.*, 2018). This species has most likely invaded the ANSL via the Port Headland or another saltworks with this haplotype, although there is no definitive evidence of this. Haplotype HAP01 is also known from a broad range of sites in the Americas (where it is native; haplotype Af10 in Muñoz *et al.*, 2013) as well as Asia (H6 in Eimanifar *et al.*, 2014) and Europe (Haf04 in Muñoz *et al.*, 2014). Muñoz *et al.* (2014) found Haf04 in 14 out of 16 Mediterranean sites they sampled, attesting to its remarkable ability to invade new habitats.

5.4.4 Distribution of *Artemia* in ANSL

Unisexual *Artemia* (which are presumably all diploid parthenogenetic *Artemia*) are now known from a total of 34 ANSL in mainland Western Australia. We did not find any definitive reports of unisexual *Artemia* from ANSL in other regions of Australia, although some faunal surveys have been conducted in these regions (e.g., Rogers & Timms, 2014; Timms, 2007; Timms, 2009c, 2018). We have also conducted limited sampling in western Victoria and on the Eyre Peninsula between 2019 and 2024 but have not encountered any *Artemia* in ANSL in these regions. Our sampling revealed four previously unreported ANSL sites with unisexual *Artemia* (see Table 5.1), all of which were within the known distribution of this taxon in ANSL, which ranges from about Three Springs (29°31' S 115°50' E) in the north (Timms, 2014) to Lake Carey (~ 29°05' S 122°22' E) in the east (Campagna, 2007) and remote Lake Boonderoo (31°10' S 124°21' E) on the Nullabor Plain in the southeast (Timms, 2014).

Prior to this study, bisexual *Artemia/Artemia franciscana* was reported to occur in three ANSL from which salt had previously been extracted (Timms, 2014). This species may have been deliberately introduced to these sites by humans. It was also reported in three ANSL that have not been used for salt extraction (ARL, 2009) and may have naturally dispersed to these sites. These ANSL were located within 32 km of each other in the northern Wheatbelt region in WA. We found *A. franciscana* in another five ANSL sites with no history of salt extractions in the same general area but up to 88 km further south (in Lake Ninan) than those previously reported. Since bisexual *Artemia* has been reported from Lake Koorkoordine (31°10' S 119°19' E), a

natural lake at the eastern edge of the Wheatbelt region from which salt was once collected (Timms, 2014), it would also be useful to sample ANSL in this area, although to date we have not observed any *Artemia* at sites in and around Baandee Lake (31°36' S 117°57' E) about 100 km to the west. Similarly, ANSL around any saltworks containing *A. franciscana* should be regularly monitored for the presence of this species.

We have confirmed an unpublished report in ARL (2009) that unisexual and bisexual *Artemia* sometimes co-occur in the same ANSL (see Table 5.2). Moreover, we found evidence of *A. franciscana* displacing unisexual *Artemia* in Lake Ninan, as we only observed the former species in this lake on 16th October 2022 and 28th January 2023, whereas unisexual *Artemia* had previously been present (see above for details). This fits with reports of *A. franciscana* outcompeting unisexual *Artemia* in other locations (e.g., see Amat *et al.*, 2005; Thirunavukkarasu *et al.*, 2024; Vikas *et al.*, 2012). Despite suggestions that the presence of *Parartemia* may inhibit *Artemia* from colonising an ANSL (McMaster *et al.*, 2007), bisexual and/or unisexual *Artemia* have been found at some *Parartemia* sites (see Table 5.2). We found active individuals of both diploid parthenogenetic *Artemia* and *P. laticaudata* in a natural lake (TS11) in the northern Wheatbelt on 23rd November 2022, however, the ability of these two taxa to co-exist in the longer term is not clear. In October 2022, we observed unisexual *Artemia* but not *Parartemia* in Anderson Lake in the eastern Wheatbelt, although *P. longicaudata* had been collected from this lake in September 1998 (see Table 5.2).

Many migratory and nomadic birds feed in the ANSL and move over large distances in both coastal and inland areas (Blakers *et al.*, 1984; Geering *et al.*, 2007; Mather, 2020). McMaster *et al.* (2007) proposed that these birds play an important role in spreading unisexual *Artemia* among ANSL. This is likely also the case for *A. franciscana* (e.g., see Frisch *et al.*, 2021; Muñoz *et al.*, 2013). Wind can also disperse *Artemia* eggs (Graham & Wirth, 2008). Since the eggs of *A. franciscana* and parthenogenetic *Artemia* float whereas those of *Parartemia* sink to the lake bottom (Geddes, 1981; Williams & Geddes, 1991), the former may be more likely to be caught in the wind (Parekh *et al.*, 2014) or ingested by or attached to a bird then the latter (McMaster *et al.*, 2007).

5.4.5 Threats to endemic fauna and future directions

The impact of *Artemia* on the native fauna of ANSL is not known, however, the potential for impacts on biodiversity seems high as these lakes contain an unusually diverse endemic fauna, including native brine shrimp (Islam *et al.*, 2024; Lawrie *et al.*, 2021; Rahman *et al.*, 2023;

Ruebhart *et al.*, 2008). It has been suggested that unisexual *Artemia* mainly occurs in degraded or secondary salinised lakes (McMaster *et al.*, 2007; Timms, 2005, 2014). Even if this is confirmed, it is still potentially problematic as the quality of ANSL is typically declining due to a general increase in the aridity of the climate in southern Australia and secondary salinisation (Timms, 2005).

Future studies on this topic should prioritise the following:

1. Impact of *Artemia* on native fauna, especially *Parartemia*. Detailed temporal sampling and experimental studies are needed to confirm whether the presence of *Artemia* will negatively impact *Parartemia* and other invertebrates in ANSL and, if so, how and under what circumstances.

2. Ongoing monitoring of *Artemia* in ANSL. Further sampling of ANSL in Western Australia, where unisexual and bisexual *Artemia* lineages have become established, is needed to gain more information on the rate and pattern of spread of these lineages. It is also important to monitor ANSL outside of Western Australia, especially those in the vicinity of saltworks, to check for the presence of *Artemia*.

3. Population genomic studies of *Artemia* in Australia. Population genomic data offer the potential to reconstruct invasion routes, document demographic changes and measure pre-and post-adaptation for *A. franciscana* and parthenogenetic *Artemia* in ANSL (e.g., see Chen *et al.*, 2020; Coleman & Bowen, 2022; North *et al.*, 2021; Sainz-Escudero *et al.*, 2023).

5.5 Conclusion

This study provides up-to-date information on the distribution of unisexual and bisexual *Artemia* in Australia. It identifies unisexual and bisexual *Artemia* in ANSL as, respectively, a type of diploid parthenogenetic *Artemia* and *A. franciscana*. The results support the hypothesis that there have been multiple introductions of unisexual *Artemia* on Rottnest Island, probably from Asia. The results also suggest that Rottnest Island is the direct source of unisexual *Artemia* in ANSL in inland areas of Western Australia contrary to previous suggestions that they colonised these inland areas via a coastal lake (Lake Hayward) on the mainland. The spread of *A. franciscana* among ANSL appears to be very recent and broadly occurring in a north-to-south direction in Western Australia. The spread of *Artemia* in ANSL is ongoing and future studies are needed to assess the potential impact of *Artemia* on the brine shrimp *Parartemia* and other endemic fauna.

5.6 Supplementary Tables and Figures

Table S5.1: Details of known sites for *Artemia* in Australia, including site name and location and source of information. Species identity is provided only in cases for which the identity has been confirmed by genetic data. ANSL: Australian Natural Salt Lakes; DBCA: Department of Biodiversity, Conservation and Attractions; ALA: Atlas of Living Australia (www.ala.org.au); and BDBSA: Biological Databases of South Australia. The 'Site' column indicates the site ID used in this study (see Table 5.1), lake name, DBCA site code, location or the site code used in the source.

	Site	Species (Habitat)	Latitude	Longitude	Source
Bisexual *Artemia*					
1	KON01	*A. franciscana* (ANSL)	-30.7278	116.8201	This study
2	MAR11		-30.1158	116.2863	
3	MAR12		-30.1986	116.3708	
4	NIN02 [A]		-30.9495	116.6537	
5	WH11		-30.5064	116.7172	
6	W001 [A, B]	(ANSL)	-29.9466	116.1790	ARL (2009)
7	W004 [B]		-30.2015	116.3416	
8	W012 [A]		-30.0217	116.1270	
9	Port Alma (Rockhampton)	*A. franciscana* (saltworks)	-23.5948	150.7397	Bowen *et al.* (1978); Abreu-Grobois and Beardmore (1982); Ruebhart *et al.* (2008); Timms (2014); Asem *et al.* (2018)
10	St Kilda [C, D]		-34.7359	138.5389	
11	Mulgundawa [C]		-35.2944	139.2104	
12	Port Hedland [C]		-20.3353	118.6392	
13	Dampier		-20.7053	116.7005	
14	Bowen	(saltworks)	-20.0198	148.2257	Ruebhart *et al.* (2008)
15	Port Augusta	(past saltworks)	*	*	Timms (2014)
16	Hutt Lagoon		-28.1688	114.2511	
17	Lake Koorkoordine		-31.1776	119.3155	

Unisexual *Artemia*					
1	AND01 [B]	Diploid parthenogenetic *Artemia* (Rottnest Island and ANSL)	-34.1826	117.9636	This study
2	ESP42		-33.5089	122.2868	
3	KL11		-32.5129	118.2206	
4	NEW10		-33.3315	119.8694	
5	ROT11		-32.0015	115.5119	
6	BOO01		-31.1483	124.3551	This study; DBCA; ALA; McMaster *et al.* (2007); Timms (2014)
7	HAY01		-32.8849	115.6920	
8	NOR01		-33.4492	117.2854	
9	QUA01		-31.9735	117.5051	
10	BAG01		-31.9959	115.5250	
11	TS11 [B]		-29.5158	115.8326	
12	W001 [A, B]	(Rottnest Island and ANSL)	-29.9466	116.1790	ARL (2004), ARL (2006) and ARL (2009)
13	W002 [B]		-29.9473	116.1728	
14	W006		-30.2049	116.3308	
15	W011		-30.0223	116.1235	
16	W012 [A]		-30.0217	116.1270	
17	W016		-29.9744	116.1537	
18	W018 [B]		-30.1252	116.4458	
19	W019		-30.1259	116.4406	
20	W070		-30.1305	116.4616	
21	ABP016		-32.2448	117.2821	DBCA
22	ABP023		-32.4569	119.0658	
23	ABP028		-32.2234	117.2797	
24	ABP042		-32.4664	117.4700	
25	ABP056		-31.4209	116.9733	
26	ABP157		-33.6743	117.7874	
27	SPM021 (Lake Dumbleyung)		-33.3194	117.6247	
28	SPS052		-32.1872	117.5655	
29	SPS097		-31.5713	117.4280	
30	NIN02 (Lake Ninan) [A]		-30.9495	116.6537	

31	Garden Lake		-31.9969	115.5365	DBCA; McMaster *et al.* (2007); Campagna (2007); Timms (2009b); Timms (2014); Campbell *et al.* (2023); Rogers and Timms (2014)
32	Herschel Lake		-31.9975	115.5280	
33	Lake Vincent		-31.9986	115.5157	
34	Serpentine Lake		-32.0034	115.5264	
35	Fitzgerald River		*	*	
36	Goomalling		*	*	
37	Howick		-33.4908	122.5111	
38	Lake Carey		*	*	
39	East of Three Springs		-29.5015	115.8579	
40	East of Wubin		-30.0076	116.8178	
41	Shark Bay (Useless Loop)	(saltworks)	-26.1159	113.4002	McMaster *et al.* (2007); Timms (2014)
42	Lake McLeod		-24.4182	113.5743	
43	Onslow		-21.7171	115.0710	
Unknown *Artemia* (sex ratio not specified)					
1	Linga	(ANSL)	-35.0833	141.6166	Australian Museum
2	Adelaide International Bird Sanctuary		-34.5026	138.3037	BDBSA
3	ADS027	(saltworks)	-30.2077	115.0102	DBCA
4	Cooper's Creek	(unknown)	*	*	Williams and Geddes (1991)
5	Port Adelaide		*	*	

* Exact location uncertain; approximate location is illustrated in Fig. 5.1.

[A] ANSL where unisexual and bisexual *Artemia* have been found (details in Table 5.2).

[B] ANSL where *Artemia* and *Parartemia* have been found (details in Table 5.2).

[C] Unisexual *Artemia* once reported from these saltworks (McMaster *et al.*, 2007; Mitchell & Geddes, 1977; probably Timms, 2014) but is no longer present.

[D] *Parartemia* once reported from this saltworks (Mitchell & Geddes, 1977) but is no longer present.

Table S5.2: Details of *COI* sequences for different *Artemia* species used in the phylogenetic analyses.

Taxa	# seq	GenBank accession number	References
A. franciscana	244	Sequences in Table S5.5	-
A. monica	6	KF663037-42	(Muñoz *et al.*, 2013)
A. persimilis	3	DQ119647, HM998992, and EF615593	Hou *et al.* (2006), Wang *et al.* (2008), and Maniatsi *et al.* (2011)
A. salina	6	KF691509-14	Eimanifar *et al.* (2014)
A. sinica	5	KF691298-99 and KF691300-02	Eimanifar *et al.* (2014)
A. urmiana	5	JX512748-52	Eimanifar and Wink (2013)
A. tibetiana	4	KF707855, KF707923, KF707927 and KF707928	Maccari *et al.* (2013a)
A. amati	5	GU591385-87, MZ189884 and MZ189888	Muñoz *et al.* (2010) and Asem *et al.* (2023)
A. sorgeloosi	11	KF691215-18, KF691245-49, MZ189919 and MZ189920	Eimanifar *et al.* (2014) and Asem *et al.* (2023)
Diploid parthenogenetic *Artemia*	382	Sequences in Table S5.3 and AY953368-69 and KC193664-65	(Maccari *et al.*, 2013b)
Triploid parthenogenetic *Artemia*	2	HM998997 and HM998999	Maniatsi *et al.* (2011)
Tetraploid parthenogenetic *Artemia*	4	KU183954-57	Asem *et al.* (2016)
Pentaploid parthenogenetic *Artemia*	4	KU183968-71	Asem *et al.* (2016)

Table S5.3: Details of *COI* sequences of diploid parthenogenetic *Artemia* used to investigate the phylogeography of this taxon in Australian salt lakes. N = the number of individuals used in the analysis. When a single haplotype is represented by multiple individuals, the number of individuals is given in parentheses '()'. Similarly, when a single GenBank accession number is represented by multiple individuals, the number of individuals is given in double parentheses '(())'. If different accession numbers represent a single haplotype, the last two digits of the accession numbers are enclosed in square brackets '[]'. Further details for the Australian sites are in Table 5.1 and for other sites are in the reference cited in the table.

Site	Country	N	Haplotypes	GenBank Accession	References
AND01	Australia	10	APD02 (10)	OR8259[27-36]	This study
BAG01		20	APD02 (19)	OR8259[37-38, 40-56]	
			APD20 (1)	OR825939	
BOO01		1	APD02 (01)	OR825957	
ESP42		10	APD02 (10)	OR8259[58-67]	
HAY01		10	APD02 (10)	OR8259[68-77]	
KL11		10	APD02 (10)	OR8259[78-87]	
NEW10		10	APD02 (3)	OR8259[88, 92, 97]	
			APD21 (7)	OR8259[89-91, 93-96]	
NOR01		10	APD02 (2)	OR8260[06-07]	
			APD21 (8)	OR8259[98-99], OR8260[00-05]	
QUA01		10	APD21 (10)	OR8260[08-17]	
ROT11		10	APD02 (10)	OR8260[18-27]	
TS11		10	APD02 (10)	OR8260[28-37]	
ODI	Spain	13	APD01 (9)	DQ426824 ((9))	Muñoz *et al.* (2010)
			APD02 (4)	DQ426825 ((4))	
BOS		10	APD02 (10)	DQ426825 ((10))	
GAT		7	APD02 (7)	DQ426825 ((7))	
SEN	Portugal	12	APD02 (12)	DQ426825 ((12))	
RIO		12	APD02 (12)	DQ426825 ((12))	
LAR	Morocco	10	APD02 (9)	DQ426825 ((9))	
			APD03 (1)	DQ426826	
NAM	Namibia	9	APD02 (9)	DQ426825 ((9))	
MAR	Italy	7	APD02 (7)	DQ426825 ((7))	
ALB	Albania	10	APD02(2)	KF7077[94, 98]	Maccari *et al.* (2013a)
			APD05(8)	KF7077[90-93, 95-97, 99]	
ATA	Bulgaria	20	APD02(15)	DQ426825 ((5)), KF707720-25, KF7078[00, 2-4]	Muñoz *et al.* (2010) and Maccari *et al.* (2013a)
			APD06(1)	GU591382	
			APD07(2)	GU591383, KF707726	
			APD08(1)	GU591384	
			APD12(1)	KF707801	
KUJ	Ukraine	2	APD04 (2)	GU591380 ((2))	Muñoz *et al.* (2010)

OYB		10	APD08(7)	KF7078[10-11, 13-14, 16-18]	Maccari *et al.* (2013a)
			APD10(3)	KF7078[12, 15, 19]	
KOY		15	APD02(15)	KF7077[00-09], KF7078[05-09]	
WAD	Egypt	6	APD05 (6)	GU591381 ((6))	Muñoz *et al.* (2010)
EGY		5	APD02(3)	KF7077[86-87, 89]	Maccari *et al.* (2013a)
			APD05(2)	KF7077[85, 88]	
URM	Iran	20	APD02(17)	KF7077[10-19, 67-69, 71-74]	
			APD05(3)	KF7077[65-66, 70]	
IRA	Iraq	19	APD02(19)	KF7077[27-45]	
ARA	Uzbekistan	6	APD02(2)	KF7078[20, 24]	
			APD11(2)	KF7078[23, 25]	
			APD13(1)	KF707821	
			APD14(1)	KF707822	
BJU	Kazakhstan	6	APD02(6)	DQ426825 ((6))	Muñoz *et al.* (2010)
MAL	Russia	10	APD02(3)	KF7078[27, 32-33]	Maccari *et al.* (2013a)
			APD15(5)	KF7078[26, 28, 31, 34-35]	
			APD16(2)	KF7078[29-30]	
MOI		10	APD02(2)	KF7078[67, 73]	
			APD18(7)	KF7078[65-66, 68-70, 72, 74]	
			APD19(1)	KF707871	
BOL		9	APD02(7)	KF7078[36-40, 42, 44]	
			APD15(1)	KF707841	
			APD16(1)	KF707843	
PAK	Pakistan	10	APD02(10)	KF7077[75-84]	
AIB	China	9	APD02(5)	KF7077[47-48, 50, 53-54]	
			APD09(1)	KF707746	
			APD10(3)	KF7077[49, 51-52]	
GAH		10	APD11(10)	KF7077[55-64]	
LAG	Tibet (China)	10	APD02(4)	KF7078[45-46, 52-53]	
			APD05(1)	KF707850	
			APD17(5)	KF7078[47-49, 51, 54]	

Table S5.4: Details of nuclear *ITS-1* clones of diploid parthenogenetic *Artemia* used to investigate the phylogeography of this taxon in Australian salt lakes. N = the number of individuals used in the analysis. When a single clone is represented by multiple individuals, the number of individuals is given in parentheses '()'. If different accession numbers represent a single clone, the last two digits of the accession numbers are enclosed in square brackets '[]'. Further details for the Australian sites are in Table 5.1 and for other sites are in the reference cited in the table.

Site	Country	N	Clones	GenBank Accession	References
AND01	Australia	10	pGen-12 (10)	OR8277[77-86]	This study
BAG01		10	pGen-9	OR827788	
			pGen-12 (7)	OR8277[89-94, 96]	
			pGen-13 (2)	OR8277[87, 95]	
ESP42		10	pGen-12	OR827802	
			pGen-14 (9)	OR8277[97-99], OR8278[00-01, 03-06]	
HAY01		10	pGen-13 (10)	OR8278[07-16]	
KL11		10	pGen-12 (10)	OR8278[17-26]	
NEW10		10	pGen-15 (9)	OR8278[27-35]	
			pGen-13	OR827836	
NOR01		10	pGen-15 (8)	OR8278[37-44]	
			pGen-13	OR827845	
			pGen-12	OR827846	
QUA01		10	pGen-15 (10)	OR8278[47-56]	
ROT11		10	pGen-12 (8)	OR8278[57-64]	
			pGen-16 (2)	OR8278[65-66]	
TS11		8	pGen-12 (1)	OR827869	
			pGen-13 (7)	OR8278[67-68, 70-74]	
TCL	Italy	2	pGen-1	DQ201279	Baxevanis *et al.* (2006)
			pGen-2	DQ201278	
NAM	Namibia	2	pGen-3 (2)	DQ2012[81-82]	
ALB	Albania	2	pGen-4 (2)	KF7362[74-75]	Maccari *et al.* (2013a)
ATA	Bulgaria	2	pGen-5 (2)	KF7362[58-59]	
OYB	Ukraine	2	pGen-5 (2)	KF7362[76-77]	
KOY		2	pGen-5	KF736257	
			pGen-6	KF736255	
EGY	Egypt	2	pGen-7	KF736266	
			pGen-8	KF736269	
URM	Iran	2	pGen-9 (2)	KF7362[53-54]	
IRA	Iraq	2	pGen-5 (2)	KF7362[64-65]	
ARA	Uzbekistan	2	pGen-5	KF736279	
			pGen-9	KF736278	
MAL	Russia	2	pGen-9 (2)	KF7362[80-81]	
MOI		2	pGen-9 (2)	KF7362[84-85]	

BOL		2	pGen-9 (2)	KF7362[82-83]	
PAK	Pakistan	2	pGen-10 (2)	KF7362[70, 73]	
AIB	China	7	pGen-9 (7)	KF7362[60-61], KU1838[15-19]	Asem *et al.* (2016) and Maccari *et al.* (2013a)
GAH		7	pGen-9 (7)	KF7362[62-63], KU1838[25-29]	
BAR		5	pGen-9 (5)	KU1838[00-04]	
AQQ		7	pGen-3 (7)	KU1838[30-36]	
		1	pGen-11	KU183837	
LAG	Tibet (China)	2	pGen-6 (2)	KF7362[86, 89]	Maccari *et al.* (2013a)

Table S5.5: Details of *COI* sequences of *Artemia franciscana* used to investigate the phylogeography of this taxon in Australian natural salt lakes (ANSL). N = the number of individuals used in the analysis. When a single haplotype is represented by multiple individuals, the number of individuals is given in parentheses '()'. Similarly, when a single GenBank accession number is represented by multiple individuals, the number of individuals is given in double parentheses '(())'. If different accession numbers represent a single haplotype, the last two digits of the accession numbers are enclosed in square brackets '[]'. Further details for the ANSL are in Table 5.1 and for other sites are in the reference cited in the table.

Site	Country (Habitat)	N	Haplotype	GenBank Accession	References
KON01	Australia (ANSL)	10	HAP01 (10)	OR8258[76-85]	This study
MAR11		10	HAP01 (10)	OR8258[86-95]	
MAR12		10	HAP01 (10)	OR8258[96-99], OR8259[00-05]	
NIN02		10	HAP01 (10)	OR8259[06-15]	
WH11		10	HAP01 (10)	OR8259[16-25]	
SKI	Australia (saltworks)	10	HAP01 (7)	MK6133[32-33, 35-39]	Asem *et al.* (2018)
			HAP14	MK613334	
			HAP15	MK613331	
			HAP16	MK613330	
MUL		26	HAP02 (3)	MK6133[00, 02, 04]	
			HAP03 (19)	MK6132[84-94, 96, 98-99], MK6133[03, 05–07, 08]	
			HAP04	MK613283	
			HAP17	MK613301	
			HAP18	MK613297	
			HAP19	MK613295	
HED		21	HAP01 (17)	MK6133[09-12, 14-16, 18-21, 23-25, 27-29]	
			HAP20	MK613326	
			HAP21	MK613322	
			HAP22	MK613317	
			HAP23	MK613313	
DAM		10	HAP03 (10)	MK6132[73-82]	
GSL	USA	90	HAP01	KF662960	

	(saltworks)		HAP02 (40)	KF662968 ((2)), KJ8634[30, 32-35, 37, 40-42, 44-49, 51-53, 56-59, 61, 64-65, 68-70, 72-73, 75-78, 80, 83, 88, 90]	Muñoz *et al.* (2013); Eimanifar *et al.* (2015)
			HAP03 (23)	KF662970 ((21)) and KF662976 ((2))	
			HAP04 (2)	KF662971 ((2))	
			HAP05 (17)	KF662977, KJ8634[31, 36, 38-39, 43, 55, 60, 66-67, 71, 74, 79, 81-82, 87, 89]	
			HAP06	KJ863450	
			HAP07	KJ863454	
			HAP08	KJ863462	
			HAP09	KJ863463	
			HAP10	KJ863484	
			HAP11	KJ863485	
			HAP12	KJ863486	
SFB		37	HAP01 (26)	KF662960 ((26))	Muñoz *et al.* (2013)
			HAP02 (6)	KF662968 ((6))	
			HAP03 (4)	KF662970 ((4))	
			HAP13	KF662975	

Table S5.6: Details of the *ITS-1* sequences found in *Artemia franciscana* from Australian natural salt lakes (ANSL). Site details of ANSL are in Table 5.1.

Site	Unphased sequences	Reconstructed sequences*	Haplotypes	GenBank Accession
KON01	10	20	bITS-1.1 (9)	OR827767-76
			bITS-1.2 (1)	
			bITS-1.4 (6)	
			bITS-1.5 (4)	
MAR11	10	20	bITS-1.1 (13)	OR827757-66
			bITS-1.2 (3)	
			bITS-1.4 (1)	
			bITS-1.5 (3)	
MAR12	10	20	bITS-1.1 (7)	OR827747-56
			bITS-1.2 (6)	
			bITS-1.3 (3)	
			bITS-1.4 (1)	
			bITS-1.5(2)	
			bITS-1.6 (1)	
NIN02	10	20	bITS-1.1 (9)	OR827737-46
			bITS-1.2 (5)	
			bITS-1.5 (6)	
WH11	10	20	bITS-1.1 (11)	OR827727-36
			bITS-1.2 (3)	
			bITS-1.3 (1)	
			bITS-1.4 (3)	
			bITS-1.5 (2)	

* Reconstructed sequences were obtained from the unphased *ITS-1* (1177 bp) sequences using the PHASE algorithm (Stephens & Donnelly, 2003; Stephens *et al.*, 2001) in DnaSP v6 (Rozas *et al.*, 2017).

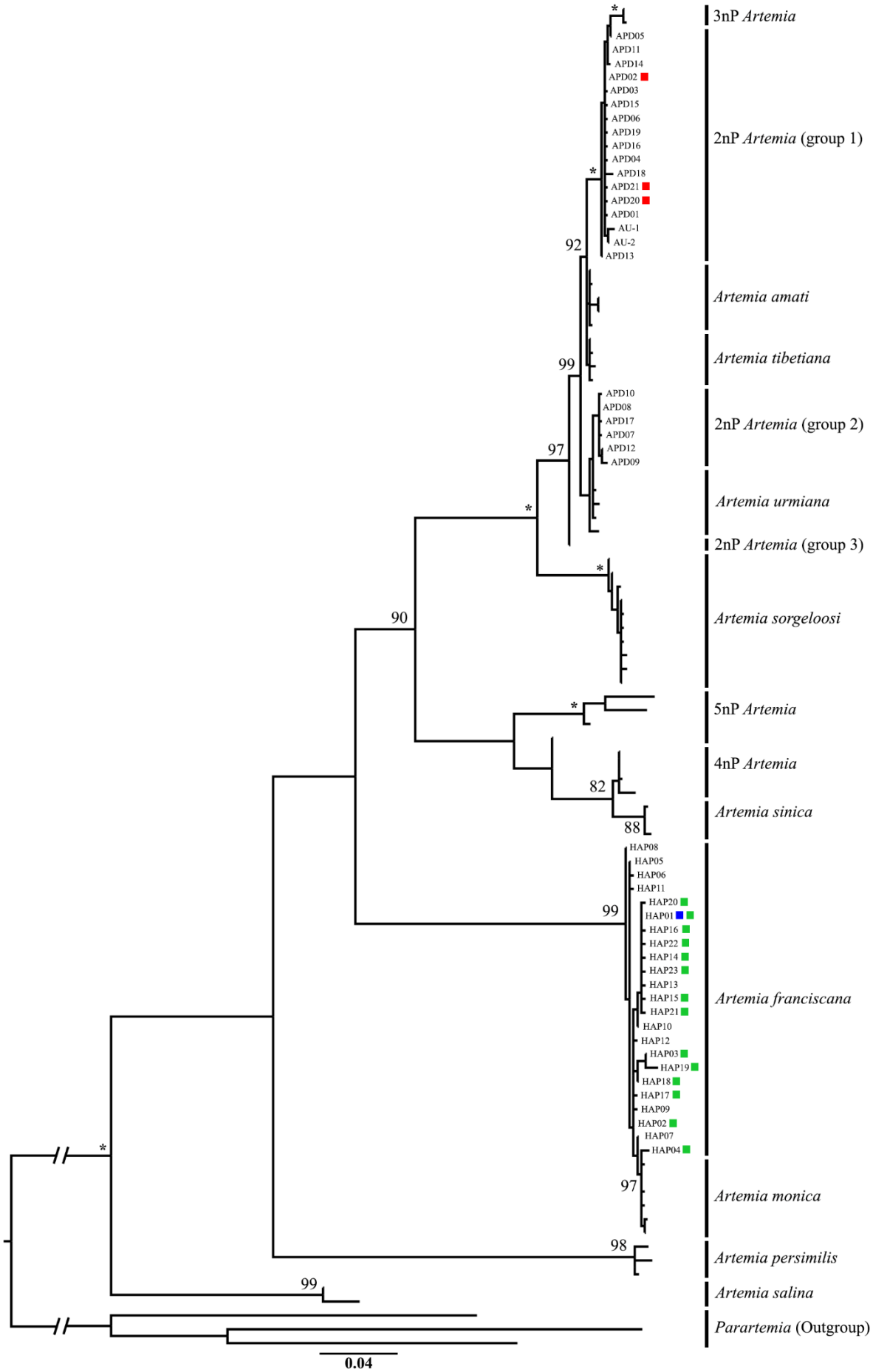
3nP Artemia
APD05
APD11
APD14
APD02
APD03
APD15
APD06
APD19
APD16
APD04
APD18
APD21
APD20
APD01
AU-1
AU-2
APD13
2nP Artemia (group 1)
Artemia amati
Artemia tibetiana
APD10
APD08
APD17
APD07
APD12
APD09
2nP Artemia (group 2)
Artemia urmiana
2nP Artemia (group 3)
Artemia sorgeloosi
5nP Artemia
4nP Artemia
Artemia sinica
HAP08
HAP05
HAP06
HAP11
HAP20
HAP01
HAP16
HAP22
HAP14
HAP23
HAP13
HAP15
HAP21
HAP10
HAP12
HAP03
HAP19
HAP18
HAP17
HAP09
HAP02
HAP07
HAP04
Artemia franciscana
Artemia monica
Artemia persimilis
Artemia salina
Parartemia (Outgroup)
92
99
97
90
82
88
99
97
98
99
0.04

Fig. S5.1: Maximum Likelihood (ML) phylogenetic analysis of *Artemia* based on the mitochondrial *COI* region (446 - 658 bp). Bootstrap values ≥ 80% are indicated at nodes. Nodes with 100% bootstrap support are indicated by asterisks '*'. APD01 - APD21 are labels for haplotypes (614 bp) of diploid parthenogenetic *Artemia* from Muñoz *et al.* (2010), Maccari *et al.* (2013a) and this study. AU-1 and AU-2 denote two shorter sequences (531 bp) of diploid parthenogenetic *Artemia* from an unspecified location/s in Australia from GenBank (AY953368 and AY953369, see Table S5.2). HAP01 - HAP23 are labels for haplotypes (446 bp) of *A. franciscana* from Asem *et al.* (2018), Muñoz *et al.* (2013); Eimanifar *et al.* (2015) and this study. Red and blue symbols indicate unisexual and bisexual *Artemia* haplotypes from Australian salt lakes, respectively. Green symbols indicate *A. franciscana* haplotypes found in four Australian coastal saltworks (from Asem *et al.*, 2018). 2nP, 3nP, 4nP, and 5nP denote diploid, triploid, tetraploid, and pentaploid parthenogens, respectively. Lineage 2nP *Artemia* (group 3) is a putative third diploid parthenogenetic *Artemia* lineage represented by rare males from Kujalnik (rmKUJ), identified by Maccari *et al.* (2013b).

Table S5.7: *COI* diversity in *Artemia franciscana* from five Australian natural salt lakes (ANSL), four Australian saltworks and the Great Salt Lake (GSL) and San Francisco Bay (SFB) in the USA. N: number of sequences; V: number of polymorphic sites; P: number of parsimony informative sites; H: number of haplotypes; Hd: haplotype diversity; and Phi: nucleotide diversity. ANSL site details are in Table 5.1.

Site	N	V	P	H	Hd	Phi
ANSL						
KON01	10	0	0	1	0	0
MAR11	10	0	0	1	0	0
MAR12	10	0	0	1	0	0
NIN02	10	0	0	1	0	0
WH11	10	0	0	1	0	0
Australian saltworks						
SKI	10	3	0	4	0.533 ± 0.180	0.00135 ± 0.00053
MUL	26	10	3	6	0.465 ± 0.116	0.00350 ± 0.00098
HED	21	4	0	5	0.352 ± 0.131	0.00086 ± 0.00035
DAM	10	0	0	1	0	0
USA sites						
GSL	90	13	8	12	0.708 ± 0.031	0.00415 ± 0.00035
SFB	37	5	4	4	0.480 ± 0.087	0.00255 ± 0.00052

Chapter 6

Chapter 6. General Discussion

6.1 Thesis Overview

The invertebrate fauna of Australian salt lakes is rich with high levels of endemism (De Deckker, 1983; Lawrie *et al.*, 2021; Rahman *et al.*, 2023). *Parartemia* brine shrimps are an important component of this fauna (Timms, 2014). *Parartemia* is a highly divergent lineage - its closest known relative is *Artemia* from which it separated approximately 140 - 120 million years ago during the breakup of Gondwana (Reeves & De Wit, 2000; Smith *et al.*, 2004; Upchurch, 2008; Wilford & Brown, 1994). It is one of the most species-rich genera in Australian salt lakes (see Islam *et al.*, 2024; Lawrie *et al.*, 2021). This PhD research investigated the taxonomy, evolutionary history and phylogeography (of selected species) of *Parartemia* to gain insights into the diversity patterns present in this ancient lineage and some of the processes that may have contributed to these patterns. The findings are needed to develop evidence-based conservation plans for *Parartemia* in view of the deteriorating quality of salt lake environments in Australia (see Pinder *et al.*, 2009; Timms, 2005; Williams, 2002). The findings are also useful for addressing hypotheses about the evolutionary history and biogeography of anostracans (e.g., see Rogers, 2015) and salt lake invertebrates (e.g., see Williams, 1984, 1985, 1998). *Artemia* brine shrimps also occur in Australian salt lakes but appear to be recent arrivals (Ruebhart *et al.*, 2008). This PhD research also investigated the distribution, identity and phylogeography of *Artemia* in Australian natural salt lakes to collect baseline data needed to assess the risk that *Artemia* biotypes may present to the biodiversity of these lakes.

6.2 Literature Review and Distributional Information

Chapter 2 provided an overview of aspects of the biology of *Parartemia* and *Artemia* in Australia. It identified important similarities and dissimilarities between these two taxa, including that *Parartemia* exhibits a remarkable level of diversity, comprising 21 known species, contrasting with the widely distributed *Artemia*, which consists of nine bisexual species, plus some parthenogenetic lineages (Asem *et al.*, 2024a; Asem *et al.*, 2024b; Asem *et al.*, 2023). It also identified some important knowledge gaps for both genera. For example, it noted that most of the available information on the general biology of *Parartemia* is derived from just *P. zietziana*. A key component of this chapter is the inclusion of an up-to-date assessment of the distribution of *Parartemia* species, which was based on published and

unpublished data, including from my field records comprising *Parartemia* samples from a total of 113 sites in Western Australia (mainly), South Australia and Tasmania. The updated distributional data support previous conclusions that species diversity in *Parartemia* is high in Western Australia (see Remigio *et al.*, 2001; Timms *et al.*, 2009), with at least 16 out of 21 species occurring in this state and 13 of these not found anywhere else. The exact reason for the high species diversity in Western Australia is not known but the pattern is repeated in some other salt invertebrates (see Lawrie *et al.*, 2021), including mytilocypridine ostracods (Rahman *et al.*, 2023) and *Coxiella* gastropods (Lawrie *et al.*, 2023), and is probably linked to the abundance and variety of salt lakes in this state (see Timms, 2009b; Timms *et al.*, 2009). The updated distributional information for *Parartemia* is crucial for conservation planning and assessment, which requires a sound knowledge of species' distributions (Villero *et al.*, 2017). For example, it has been suggested that special protection be applied to *P. extracta* because its range is shrinking (see Timms *et al.*, 2009) but my data show that the range of this species is not shrinking.

6.3 Phylogeny and Taxonomy of *Parartemia*

Effective biodiversity and conservation research depends on accurate and reliable taxonomy (Mace, 2004; Mallet, 2001; Rodrigues *et al.*, 2006). In the past 25 years, considerable progress has been made towards improving the taxonomy of some key groups of invertebrates from Australian salt lakes, including *Parartemia* (Timms, 2010; Timms & Hudson, 2009), ostracods (Halse, 2002; Halse & McRae, 2004; Rahman, 2024), *Coxiella* (Lawrie *et al.*, 2023) and *Triops* (Meusel & Schwentner, 2017; Murugan *et al.*, 2009). Overall, the results of these studies have revealed many new species and sometimes even new genera (e.g., Lawrie *et al.*, 2023). Chapter 3 provided the first molecular phylogeny and species delimitation analyses for *Parartemia* based on an almost complete suite of species and broad geographic sampling. Patterns of genetic and morphological variation were largely congruent, and the molecular data confirmed the validity of most described morphospecies, however, two new morphospecies and three cryptic species were identified. This study therefore adds to the growing record of invertebrate biodiversity of Australian salt lakes. The results support previous suggestions that the amount of molecular divergence in *Parartemia* species is typically very large (Remigio *et al.*, 2001) and identified some groups of closely related species (which also usually shared some morphological similarities), plus some distinctive species, although the relationships among divergent lineages were generally not well resolved. Overall, the data indicate that *Parartemia* with a total of 21 known species, including three cryptic species as well as the morphospecies

P. auriciforma and *P. yarleensis* that were not included in this study, is the most specious genus in Australian salt lakes, where it has undergone a remarkable radiation (Remigio *et al.*, 2001). The revised *Parartemia* species list, and improved distributional information, will aid management authorities in devising targeted conservation strategies for this taxon.

6.4 Evolutionary History and Phylogeography of *Parartemia*

Biodiversity is dynamic and it is important to consider the evolutionary processes that generate, maintain and erode this diversity when developing conservation plans and investigating how contemporary ecosystems function (Avise, 2009; Avise *et al.*, 2016; Brooks *et al.*, 1992; Dobzhansky, 1973; Hendry *et al.*, 2010). Chapter 4 used a time-calibrated *16S* phylogeny to explore the evolutionary history of *Parartemia* and *COI* data to investigate the phylogeography of two widely distributed species, *P. cylindrifera* and *P. longicaudata*. This was done to elucidate some of the ecological and evolutionary processes underlying the divergence and diversity in *Parartemia* that was documented in Chapter 3. The findings suggest that deep divergence and speciation in *Parartemia* occurred roughly between 40 to 10 million years ago (late Eocene to late middle Miocene), coinciding with increases in the aridity of the Australian climate after Australia separated from Antarctica (Kear *et al.*, 2016; McGowran *et al.*, 2000; Owen *et al.*, 2017). Deep divergence and speciation appear to have occurred earlier in *Parartemia* than in some other salt lake invertebrates (e.g., Lawrie, 2023; Rahman, 2024). The chapter concluded that diversification in *Parartemia* is old and probably tied to a long history of aridity, abundant salt lakes and the endurance of ephemeral water bodies in the Australian landscape (see Islam *et al.*, 2024; Remigio *et al.*, 2001; Rogers & Timms, 2014).

The *COI* data suggest that populations of *P. cylindrifera* and *P. longicaudata* are typically confined to individual salt lakes, although the latter species may sustain some gene flow over small spatial scales. Like a range of other lentic crustaceans that rely on passive dispersal (e.g., see Asem *et al.*, 2024c; De Meester *et al.*, 2002; Lopes da Cunha *et al.*, 2021; Muñoz *et al.*, 2008; Pinceel *et al.*, 2013a; Rodríguez-Flores *et al.*, 2020), these two species are widespread and appear to have good dispersal abilities, but their gene flow is extremely limited. This is most likely explained via the Monopolization Hypothesis (De Meester *et al.*, 2002), which predicts that gene flow among established populations is impeded because conspecific residents have a numerical (see also Emami-Khoyi *et al.*, 2023; Rogers, 2015) and an adaptive advantage over immigrants (De Meester *et al.*, 2002; Rogers, 2015; Schwentner & Richter, 2015). Relatively high levels of inbreeding, linked to a mixing of generations via asynchronous

hatching of egg clutches, may facilitate the rapid evolution of local adaptation in resident populations (see Rogers, 2014a; Rogers, 2015). *Parartemia cylindrifera* and *P. longicaudata* include highly divergent mitochondrial lineages, most of which had very localised distributions. The evidence suggests that large amounts of divergence tended to follow dispersal into new areas, which is consistent with Rogers (2015) prediction that new anostracan species evolve in habitats at the periphery of the existing range of species. In addition to protecting a representative range of existing *Parartemia* populations, Chapter 4 highlighted the importance of conserving salt lake ecosystems in general, including 'unoccupied habitat', which appear to serve as foci for genetic divergence in *Parartemia*. As Williams (2002) stated "the value of many salt lakes (particularly episodically-filled ones in arid regions) may lie more in their role as part of a mosaic within a wide landscape than as an individual lake".

6.5 Distribution, Identity and Phylogeography of *Artemia* in Australian Salt Lakes

In general, invasive species can cause a significant reduction in biodiversity in the areas that they invade (Linders *et al.*, 2019; North *et al.*, 2021). It is currently unknown whether the presence of *Artemia* represents a threat to the biodiversity of Australian salt lakes, however, in general, invasive species have a broad range of detrimental effects on native species and their communities (e.g., see Renault *et al.*, 2022). There is a need to generate information that will support a meaningful assessment of the risk that *Artemia* poses to the native species and communities of Australian natural salt lakes. Accordingly, Chapter 5 of this PhD research investigated the distribution, identity and phylogeography of *Artemia* in Australian natural salt lakes. It used *COI* data to show that the unisexual *Artemia* from salt lakes on Rottnest Island and the Western Australian mainland are a type of diploid parthenogenetic *Artemia* that is widely distributed outside of Australia. These data also show that the bisexual *Artemia* in natural salt lakes in Western Australia belong to a common and widespread mitochondrial lineage of *A. franciscana*. The chapter collated information from published and unpublished sources, including my own field records, to provide an up-to-date account of the distribution of unisexual and bisexual *Artemia* in Australia. The results show that both diploid parthenogenetic *Artemia* and *A. franciscana* occur in natural salt lakes that have not been used for salt extraction in Western Australia, with the implication that *Artemia* has autonomously dispersed into these habitats and has the potential to continue to spread unaided. Prior to this research, the only previous mention of *A. franciscana* in such lakes was in an unpublished technical report (ARL, 2009) that has largely gone unnoticed (e.g., Lawrie *et al.*, 2021; Timms, 2014). Furthermore, the distributional and genetic data presented in this thesis indicate that *A.*

franciscana has only recently invaded natural salt lakes in the northern wheatbelt region of Western Australia and is expanding its current range, predominantly in a southerly direction. It has apparently displaced unisexual *Artemia* in Lake Ninan within the past ~20 years. Genetic data were used to test McMaster *et al.* (2007) hypotheses about the invasion route of diploid parthenogenetic *Artemia* in Western Australia. These data supported their hypothesis that multiple clones of this lineage, possibly from Asia, colonised Rottnest Island but do not support a second hypothesis that these *Artemia* invaded lakes in inland areas via Lake Hayward, a coastal lake on the Western Australian mainland. Instead, my data suggest that they spread directly from Rottnest Island to inland areas. Two findings are notable from a conservation perspective. (1) Confirmation that a lineage of *A. franciscana*, which has invaded a range of regions outside of Australia (e.g., see Eimanifar *et al.*, 2014; Muñoz *et al.*, 2014), has recently colonised and is spreading in natural salt lakes in Western Australia. (2) Contrary to previous suggestions (e.g., McMaster *et al.*, 2007), diploid parthenogenetic *Artemia* have invaded some *Parartemia* habitats. These habitats provide an ideal starting point for investigating the potential impacts of *Artemia* invasions on *Parartemia* populations.

6.6 Limitations and Future Research

Although this PhD research has significantly advanced our understanding of *Parartemia* and *Artemia* in Australia, there are some limitations/unanswered questions that need to be addressed in future research.

Outstanding taxonomic work includes the need for formal descriptions of the two new morphospecies, *Parartemia* sp. 'y' and *Parartemia* sp. 'z', so these species can be considered in conservation planning and legislation (e.g., see Mace, 2004; Padial & De la Riva, 2006). Future taxonomic studies should aim to include the *P. auriciforma* and *P. yarleensis* morphospecies, which are known from one and six sites, respectively, in remote areas of South Australia (Timms *et al.*, 2009) but were not included in this study. Finally, future taxonomic sampling should focus on remote and less-explored areas of Australia, where there is an increased chance of discovering new species (Timms, 2010).

The results on the evolutionary history and the phylogeography of *Parartemia* presented in this thesis were based on single mitochondrial markers. Consequently, it is important to use nuclear markers to provide an independent test of the main findings (e.g., see Tóth *et al.*, 2017). The phylogeography of additional *Parartemia* species should be studied to assess the generality of the results obtained for *P. cylindrifera* and *P. longicaudata*. Population genomic data could be

used to provide a high-resolution assessment of patterns of gene flow and the demographic history of populations (e.g., see McCartney-Melstad *et al.*, 2018; Sainz-Escudero *et al.*, 2023). Manipulative experiments are needed to test whether restricted gene flow in *P. cylindrifera* and *P. longicaudata* conforms with the specific predictions of the Monopolization Hypothesis (see De Meester *et al.*, 2002; Rogers, 2015).

There is an urgent need to assess the impact of *Artemia* on the native fauna of Australian salt lakes, especially *Parartemia*. This requires both experimental studies and comprehensive temporal sampling at sites where both *Parartemia* and *Artemia* co-occur. Further monitoring of natural salt lakes in Western Australia is needed to better understand the dynamics of how both diploid parthenogenetic *Artemia* and *A. franciscana* are spreading in this region. It would also be prudent to monitor salt lakes outside of Western Australia, especially those near saltworks containing *Artemia,* as early detection of and rapid response to the presence of *Artemia* may be the best way to stop an invasion occurring in other locations (e.g., see Reaser *et al.*, 2020). Lastly, population genomic data could be used to infer migration rates among *Artemia* populations and to improve our understanding of the evolutionary processes, particularly pre- and post-invasion adaptive changes, underpinning the spread of *Artemia* in natural salt lakes in Australia (see North *et al.*, 2021).

Chapter 7

Chapter 7. General Conclusions

My PhD research has significantly advanced our knowledge of *Parartemia*, an old lineage of brine shrimp that has undergone a remarkable radiation in Australian salt lakes. It used molecular data to provide an independent test of the morphotaxonomy of *Parartemia*, which was already well established. Although largely confirming the existing morphotaxonomy, two new morphospecies, three cryptic species and one synonymy were discovered. Updated information on the distribution of *Parartemia* species is also presented. Combined with the improved taxonomic understanding, this will support future studies on this group and aid in the development of targeted conservation plans.

This research used a time-calibrated phylogeny and phylogeographic data to investigate the ecological and evolutionary processes that have shaped diversification and divergence in *Parartemia*. The results suggest that diversification in *Parartemia* is old and linked to a long history of aridity and abundant salt lakes and ephemeral water bodies in the Australian landscape. Phylogeographic analyses revealed that the widespread species *P. cylindrifera* and *P. longicaudata* included large amounts of genetic divergence and diversity, much of which has a highly localised distribution. This distribution makes the development of effective conservation plans challenging. The potential importance of 'unoccupied habitat' as foci for genetic divergence in *Parartemia* was recognised, as was the need for conservation plans to include protections for the evolutionary processes that generate, maintain and erode this diversity in *Parartemia*.

Although it was already known that *Artemia* was present in some natural salt lakes in Australia, my PhD research has clarified some important details regarding the distribution, identity and phylogeography of the *Artemia* biotypes that are present. These new details, particularly confirmation that the highly invasive *A. franciscana* is spreading in salt lakes in Western Australia and that diploid parthenogenetic *Artemia* have invaded some *Parartemia* habitats, highlight the need for an urgent assessment of the risk that these *Artemia* pose to *Parartemia* and other native fauna in these salt lakes.

An experimental approach is needed to test some of the ideas about the mechanisms underlying restricted gene flow in *Parartemia* species and the evolution and role of local adaptation in this process. This type of approach is also needed to assess the competitive ability of *Artemia* relative to *Parartemia*. Population genomic data can be used to provide a high-resolution

picture of gene flow patterns and of the evolutionary and demographic histories of both *Parartemia* and *Artemia* populations in natural salt lakes in Australia.

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