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Phylogeography and population genetic structure of an exotic invasive brine shrimp, *Artemia* Leach, 1819 (Crustacea : Anostraca), in Australia

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Abstract. Native American *Artemia franciscana* has become an introduced species in the Old World due to the rapid development of the aquaculture industry in Eurasia. The recent colonisation of *A. franciscana* in Mediterranean regions and Asia has been well documented, but Australia is a continent where the dispersal of this species is not well understood. In the present study, we sequenced the cytochrome oxidase subunit I (*COI*) and examined the phylogenetic relationships, haplotype network and population genetic structure of *Artemia* from four geographical localities in Australia and two American native localities. Our results confirmed the colonisation of Australia in all four localities by *A. franciscana*. First, we document the occurrence of *Artemia* in Mulgundawa and St Kilda localities in Australia. The Dampier population is a monomorphic population, but there is high genetic variation and a degree of demographic expansion observed in other introduced *A. franciscana* populations in Australia. This observation suggests an interaction between environmental conditions and adaptive potentials of *A. franciscana*. Our findings imply that populations from St Kilda and Port Hedland might have originated from a San Francisco Bay source, while the two other locations resulted from admixture between Great Salt Lake and San Francisco Bay sources, perhaps resulting from secondary introduction events.

Additional keywords: Australian *Artemia*, biodiversity, introduced species, mtDNA-*COI*.

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Introduction

The brine shrimp *Artemia* is a primitive microcrustacean inhabiting many hypersaline habitats worldwide such as inland salt lakes, coastal saltworks, salt ponds and lagoons (Van Stappen 2002). *Artemia* can withstand extreme environmental conditions such as high salinity (7.0 – 340 g/L) and ionic compositions in the natural environment due to its unique osmoregulation mechanism (Post and Youssef 1977; Bowen *et al*. 1985; Lenz 1987; Browne *et al*. 1988; Liu and Zheng 1990).

The genus *Artemia* consists of seven bisexual species and numerous parthenogenetic populations with different ploidy levels (Asem *et al*. 2010; Asem *et al*. 2016). Three bisexual species occur naturally in the New World: *Artemia monica* Verrill, 1869 (Mono Lake, USA), *Artemia franciscana* Kellogg, 1906 (North America, Central America and South America) and *Artemia persimilis* Piccinelli & Prosdocimi, 1968 (Argentina and Chile). The other four bisexual species are native to the Old World, namely *Artemia salina* (Linnaeus, 1758) (Mediterranean basin), *Artemia urmiana* Gunther, 1899 (Lake Urmia, Iran, and the Crimean salt lakes, Russia), *Artemia sinica* Cai, 1989 (China and Mongolia) and *Artemia tibetiana* Abatzopoulos, Zhang & Sorgeloos 1998 (Qinghai–Tibetan Plateau, China). Previous studies have documented that the Tibetan populations were placed in two different groups in the phylogenetic trees of the mitochondrial *COI* marker, while all of them represented a single clade when analysed with the nuclear marker *ITS1* (Maccari *et al*. 2013; Eimanifar *et al*. 2014). This contradiction could be attributed to a hybridisation event that occurred between two ancestors. Two new species were recently described from Mongolia (Asia), namely *Artemia frameshifta* and *Artemia murae* (Naganawa and Mura, 2017), although the taxonomic status of these taxa requires confirmation. The biosystematics of these two new species were determined by single individual differentiation from the cytochrome oxidase subunit I (*COI*) sequence combined with morphological parameters, whereas morphometric study and population genetic analysis have

not previously been examined. Additionally, the existence of males has not been investigated in *A. frameshifta* so that the reproductive mode of that population is still doubtful. Parthenogenetic populations are obligate clones containing di-, tri-, tetra-, penta- and heteroploids, and inhabit the Old World and Oceania (Sun *et al*. 1999; Abatzopoulos *et al.* 2003).

Artemia has been broadly used as live food in the fishery and aquaculture industry, especially in the coastal areas of eastern Asia (Van Stappen 2008). It is also used to improve the quality of sodium chloride production in solar salt-fields by aiding in the control of phytoplankton blooms and increasing the density of red-pigmented bacteria to accelerate evaporation (Jones *et al*. 1981; Ruebhart *et al*. 2008). *Artemia* is a model organism in many biological fields, including phylogeography and population genetics (Kappas *et al*. 2011), molecular and cellular biology (Li *et al*. 2017), bioassay toxicity (Rajabi *et al*. 2015) and bioencapsulation (Vázquez-Silva *et al*. 2017).

Since 1950, cysts of *A. franciscana* have been exported overseas from the USA for applications in fishery markets. Genetic studies have documented that these exports originated primarily from two major natural sources in the USA, namely the Great Salt Lake, Utah (GSL), and San Francisco Bay, California (SFB) (Van Stappen 2008; Muñoz 2009; Eimanifar *et al*. 2014). Phylogeographic analysis revealed that the expansion of *A. franciscana* to non-native regions has resulted in rapid colonisation of numerous regions across Eurasia (Amat *et al*. 2005; Mura *et al*. 2006; Van Stappen 2008; Muñoz 2009; Ben Naceur *et al*. 2010; Scalone and Rabet 2013; Eimanifar *et al*. 2014; Horvath *et al*. 2018). Phylogenetic analysis of *Artemia* has not previously been conducted in Australia because of difficulties in obtaining adequate samples.

Previous studies have suggested the introduction of *A. franciscana* into Australia (Clark and Bowen 1976; Geddes 1979, 1981; Abreu-Grobois and Beardmore 1982; Geddes and Williams 1987; Vanhaecke *et al*. 1987; Pinder *et al*. 2002; McMaster *et al*. 2007) but there was no evidence using genetic barcoding to support the regional colonisation of *A. franciscana* in Australia. The aim of the present study was to perform a phylogenetic analysis of *Artemia* populations from Dampier, Mulgundawa, Port Hedland and St Kilda in Australia to confirm the taxonomical status of *Artemia* in these localities. Here we sequenced the mitochondrial *COI* gene and determined the genetic diversity, population genetic structure and the genetic source of *Artemia* populations as compared with two American native populations of *A. franciscana* from GSL and SFB.

Materials and methods

Origin of samples and sample analysis

In total, 67 individuals of bisexual *Artemia* were collected from four geographical sites in Australia in summer 2011 (Fig. 1). The sampling sites, with their abbreviations, geographical coordinates, IPMB code numbers and number of individuals analysed, are summarised in Table 1.

Total DNA was separately extracted from part of the antenna of male and female shrimps (1 : 1) following the Chelex® 100 Resin method (Bio-Rad Laboratories, USA). The samples were crushed, incubated for 2.5–3 h at 60°C (tubes were vortexed every 30 min) and then a final 10 min at 80°C. Then the tubes were centrifuged at 10 000 rpm for 1 min and the supernatant phase was directly used in the PCR reaction (Montero-Pau *et al*. 2008; Eimanifar and Wink 2013; Asem *et al*. 2016). All extracted DNA was stored at –80°C for further genetic analyses.

A fragment of the mitochondrial cytochrome oxidase subunit I (*COI*) was amplified. PCR was performed in a final reaction volume of 50 µL in a thermocycler (Biometra, Tgradient, Germany) with Taq DNA polymerase (Bioron, GmbH, Germany) according to conditions published previously (Eimanifar and Wink 2013). The *COI* partial fragment (~588 bp) was amplified using the metazoan invertebrates' universal primers L*COI*490/HC02198 (Folmer *et al*. 1994). PCR amplification was carried out under the following conditions: a cycle of 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 60 s at 45°C, and 60 s at 72°C, with a final step of 5 min at 72°C. Before sequencing, PCR products were purified using standard procedures (Eimanifar and Wink 2013).

Sequence alignment and phylogenetic analyses

Sequences were aligned using MEGA 7.0.26 with default parameters (Kumar *et al.* 2016). A lack of pseudogenes enabled utilisation of the protein-coding sequence; additionally, multiple mutations or deletion(s) and duplication(s) were not observed.

To estimate the phylogenetic relationship among samples collected from Australia and other species, *COI* reference sequences of bisexual species and parthenogenetic populations including di-, tri-, tetra- and pentaploidy were downloaded from GenBank (Table 2). The phylogenetic tree was generated using Bayesian Inference (BI) (Huelsenbeck and Ronquist 2001), as implemented in MrBayes 3.2.2 on XSEDE (Miller *et al*. 2010). For BI the best-fitting nucleotide substitution model was calculated based on MrModelltest 2.2 (Nylander 2004) and HKY +G was chosen as the best-fit model. Additionally, for posterior probabilities, the values <0.94 and ≥0.95 were considered to be low and high, respectively (Alfaro *et al*. 2003).

To find the origin and genealogical relationships among haplotypes of Australian samples and *A. franciscana* (more information in Results), a median network was performed using the median-joining algorithm in Network 5.0.0.3 (Bandelt *et al*. 1999). The sequences of *A. franciscana* were chosen from two natural habitats in the USA: GSL and SFB (Table S1, Supplementary Material).

For each population, the number of polymorphic sites (S), total number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), haplotype ratio (Hr), nucleotide diversity (Pi) and average number of nucleotide differences (k) were calculated using DnaSP 5.10 (Librado and Rozas 2009). Expected heterozygosity, F_{ST} (an overall population differentiation index), mismatch distribution, Harpending's Raggedness index (Hri) and sum of squared deviations (SSD) were computed in Arlequin 3.5 (Excoffier and Lischer 2010).

Results

All *COI* sequences of *A. franciscana* from Australia had 18 variable sites, of which four sites were parsimony informative and 14 sites were singletons. The total *COI* sequences of native American *A. franciscana* displayed 12 variable sites, of which five sites were parsimony informative and seven sites were singletons.

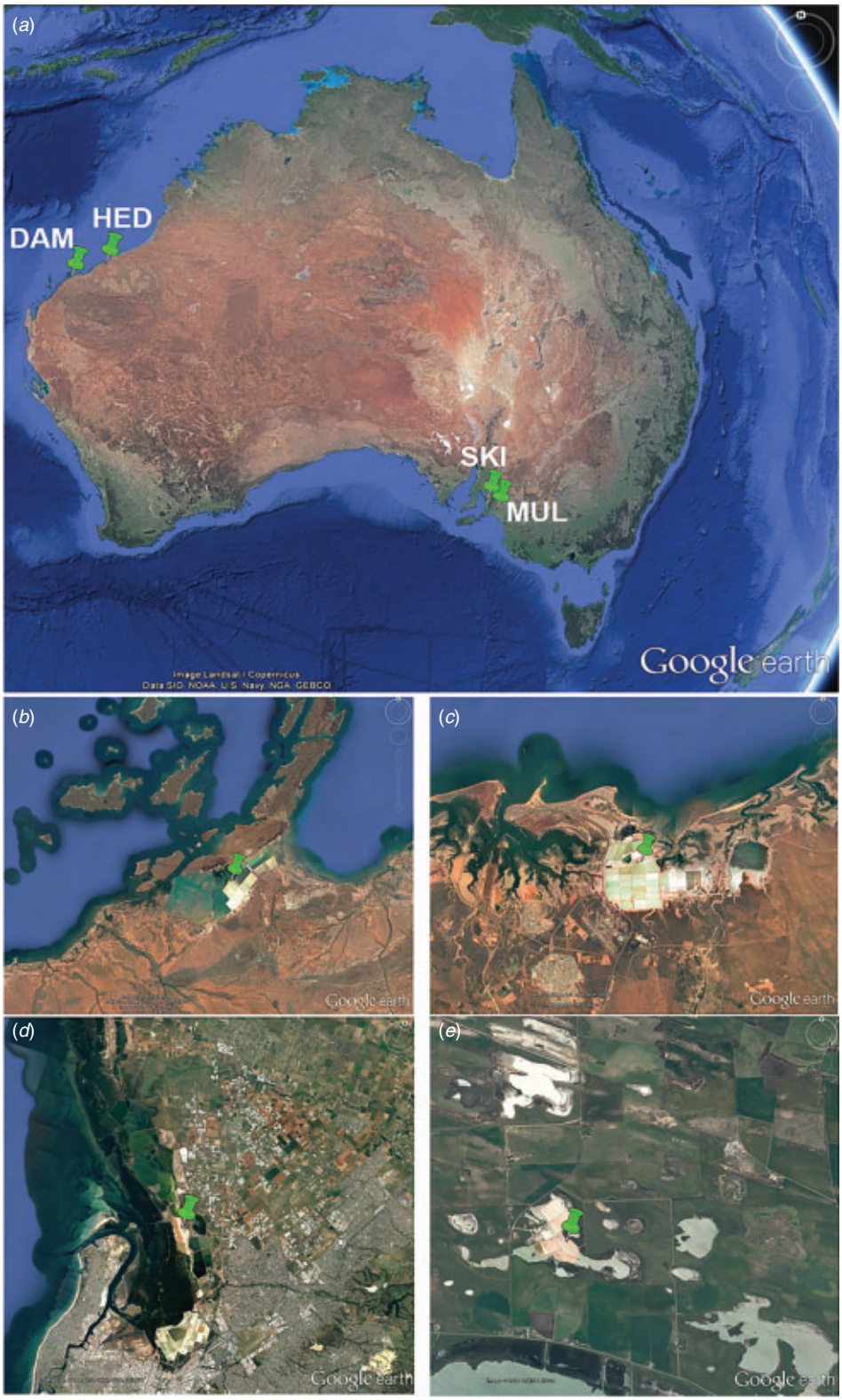

Fig. 1. (*a*) Map of *Artemia* sampling sites in Australia, (*b*) DAM: Dampier, (*c*) HED: Port Hedland, (*d*) SKI: St Kilda, (*e*) MUL: Mulgundawa. Map data © Google 2018.

Table 1. Origin of *Artemia* samples used for this study
IPMB, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany; WA, Western Australia; SA, South Australia

Site	Abbreviation	Geographic coordinates	IPMB voucher no.	No. of individuals	Reference
Dampier, WA	DAM	20°42′19.38″S, 116°42′2.09″E	66843	10	Ruebhart *et al*. (2008); McMaster *et al*. (2007)
Mulgundawa, SA	MUL	35°17′39.84″S, 139°12′37.76″E	66844	26	This study
Port Hedland, WA	HED	20°20′26.57″S, 118°39′20.98″E	77593	21	Ruebhart *et al*. (2008)
St Kilda, SA	SKI	34°44′09.49″S, 138°32′20.34″E	66849	10	This study

Table 2. Species information and GenBank accession numbers
Pop., population

Species/population	Abbreviation	No. of individuals	Accession nos	Reference
A. urmiana	URM	4	JX512748–751	Eimanifar and Wink (2013)
A. sinica	SIN	4	KF691298–301	Eimanifar *et al*. (2014)
A. tibetiana	TIB	4	KF691215–218	Eimanifar *et al*. (2014)
A. salina	SAL	4	KF691512–515	Eimanifar *et al*. (2014)
A. persimilis	PER	4	DQ119647	Hou *et al*. (2006)
			HM998992	Maniatsi *et al*. (2011)
			EF615594	Wang *et al*. (2008)
			EF615593	Wang *et al*. (2008)
A. franciscana	FRA	4	KJ863440–443	Eimanifar *et al*. (2014)
Diploid Pop.	DI	4	KU183949–952	Asem *et al*. (2016)
Triploid Pop.	TRE	3	HM998997–999	Maniatsi *et al*. (2011)
Tetraploid Pop.	TETR	4	KU183954–957	Asem *et al*. (2016)
Pentaploid Pop.	PEN	4	KU183968–971	Asem *et al*. (2016)
Unidentified[A]	DAM	10	MK613273–282	This study
	MUL[B]	26	MK613283–308	This study
	HED	21	MK613309–329	This study
	SKI[B]	10	MK613330–339	This study

[A]Australian samples
[B]New record site.

The phylogenetic tree revealed that all examined *Artemia* individuals from Australia clustered in the clade of *A. franciscana* (Fig. 2). The haplotype distribution network analysis of the *A. franciscana* complex was performed to determine the population structure of individuals, but a geographically unique haplotype could not be distinguished (Fig. 3). Most sequences belonged to H1 (31.3%) and H2 (28.5%). The haplotype frequency of *A. franciscana* from SFB for H1, H2 and H3 haplotypes were 70.3%, 16.2% and 10.8%, respectively. *A. franciscana* from GSL grouped in the H2 and H4 haplotypes with frequencies of 62.3% and 26.2%, respectively. Two localities from Australia had the greatest H1 haplotype frequency: HED (80.96%: 17 individuals out of 21) and SKI (70%: 7 individuals out of 10). There were seven haplotypes around H1 with a frequency of 1.0 (Fig. 3). All *A. franciscana* sequences from the DAM locality were recovered in H3. The MUL locality consisted of 73.1% H3 and 11.5% H2 haplotype frequencies (Fig. 3, Tables S2, S3 in the Supplementary Material).

The population differentiation test (F_{ST}) among the examined populations suggested that there is no significant differentiation between DAM and MUL (4.8%), SKI and HED (1.3%) and SKI and SFB localities (7.8%), respectively. A significant population differentiation was observed between the GSL and SFB localities (60.9%). The highest variation was revealed between DAM and SKI (90.9%) and DAM and HED (91.9%), and the lowest was observed between HED and SFB (10.3%), respectively (Table 3).

All estimated genetic indices for the examined localities are summarised in Table 4. The lowest genetic variation was observed in the DAM location, which had only a single haplotype. Two localities, SKI and MUL, exhibited the highest genetic variation. The highest-ranking levels of Hd (0.533 ± 0.180) and Hr (0.4) were found in SKI, whereas the MUL locality had the highest values for Pi (0.00349 ± 0.0023), k (1.554) and H_{exp} (0.0013 ± 0.016) (Table 4). Between the native American populations, the highest values for Hd (0.550 ± 0.058) and Hr (0.15) were found in GSL, whereas the other genetic indices were highest in the SFB locality. In total, among all American and Australian populations, the highest values of Pi (0.00349 ± 0.0023), expected heterozygosity (0.0013 ± 0.016) and k (1.554) were recorded in MUL. The highest values for Hr (0.4) and Hd (0.550 ± 0.058) were observed in SKI and GSL, respectively (Table 4).

We calculated mismatch distributions for pairwise differences from exotic and native populations of *A. franciscana*. These revealed that the SKI, HED and GSL localities had a unimodal pattern, whereas MUL and SFB localities showed a pattern likely to be multimodal. Additionally, the indices of SSD and Hri for all examined localities were non-significant, except

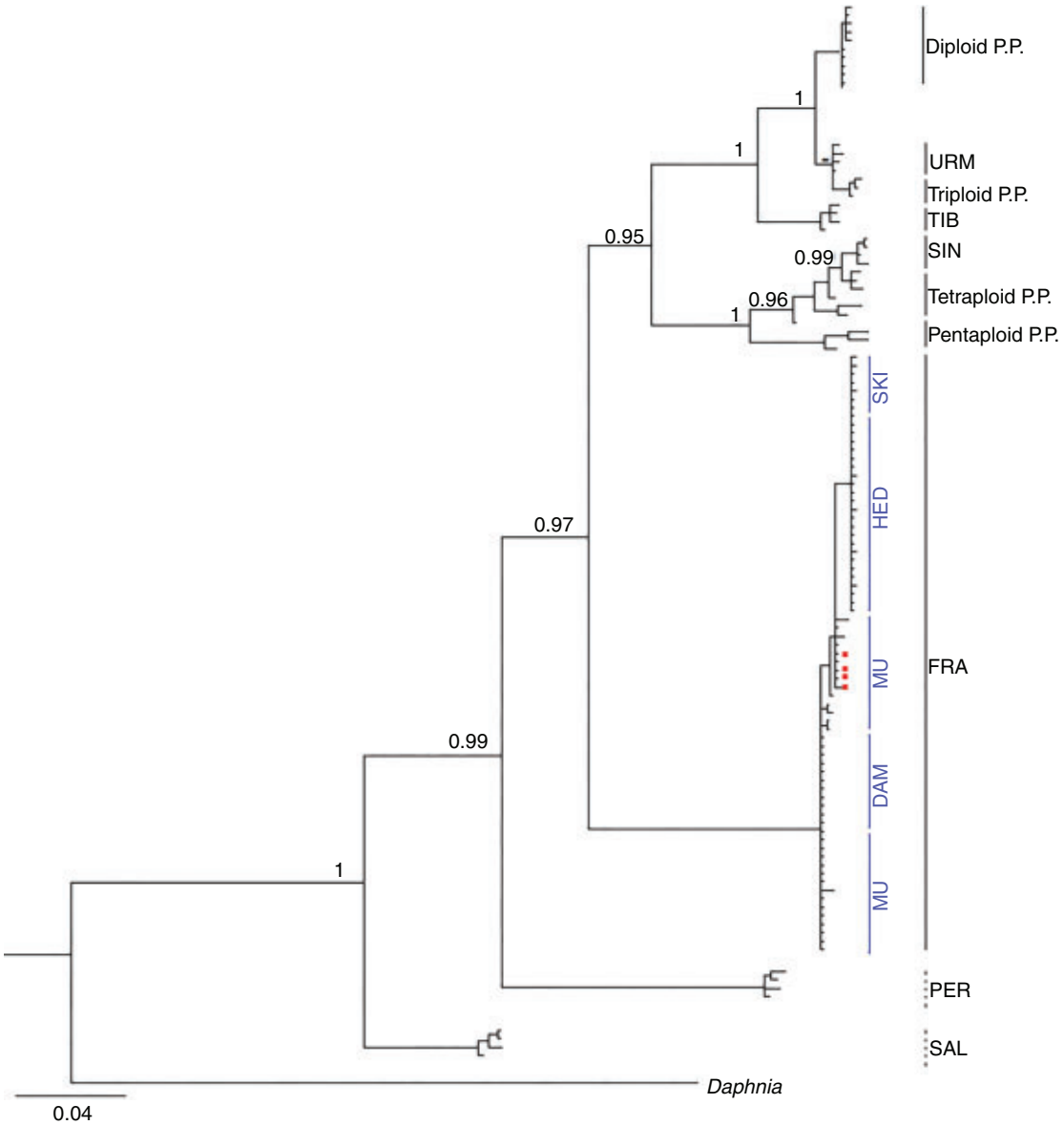

Fig. 2. *COI* phylogeny of *Artemia* based on a Bayesian inference approach. The numbers behind major nodes denote posterior probabilities. *Daphnia tenebrosa* (HQ972028) was used as an outgroup. Red dots show the position of reference sequences of *A. franciscana*. P.P., parthenogenetic population; URM, *Artemia urmiana*; TIB, *Artemia tibetiana*; SIN, *Artemia sinica*; FRA, *Artemia franciscana*; PER, *Artemia persimilis*; SAL, *Artemia salina*; DAM, Dampier; MUL, Mulgundawa; HED, Port Hedland; SKI, St Kilda.

for MUL, where we observed a significant SSD value ($P < 0.001$) (Fig. 4).

Discussion

The occurrence of American *A. franciscana* in Australia had been suggested for three geographical regions (11 localities) in Australia, including western (seven sites), southern (one site) and Queensland (three sites) (Ruebhart *et al*. 2008). Our findings confirmed the colonisation of Australia with *A. franciscana*. Previously, parthenogenetic populations have been reported from two localities in Australia – Port Hedland and Dry Creek (Van Stappen 2002) – but our findings could not support the existence of parthenogenetic populations in these localities. In the present study, we have documented the first scientific record of *Artemia* in Mulgundawa and St Kilda localities in southern Australia, which are clearly colonised by *A. franciscana*. Brine shrimps from GSL and SFB have often been introduced to other regions of the world for aquaculture production of *Artemia* cysts and biomass (Sorgeloos *et al*. 2001; Amat *et al*. 2005; Eimanifar *et al*. 2014; Muñoz *et al*. 2014; Saji *et al*. 2019) therefore these populations were included in this analysis.

Mitochondrial markers reflect the maternal evolutionary pathway and are important for understanding the passageways

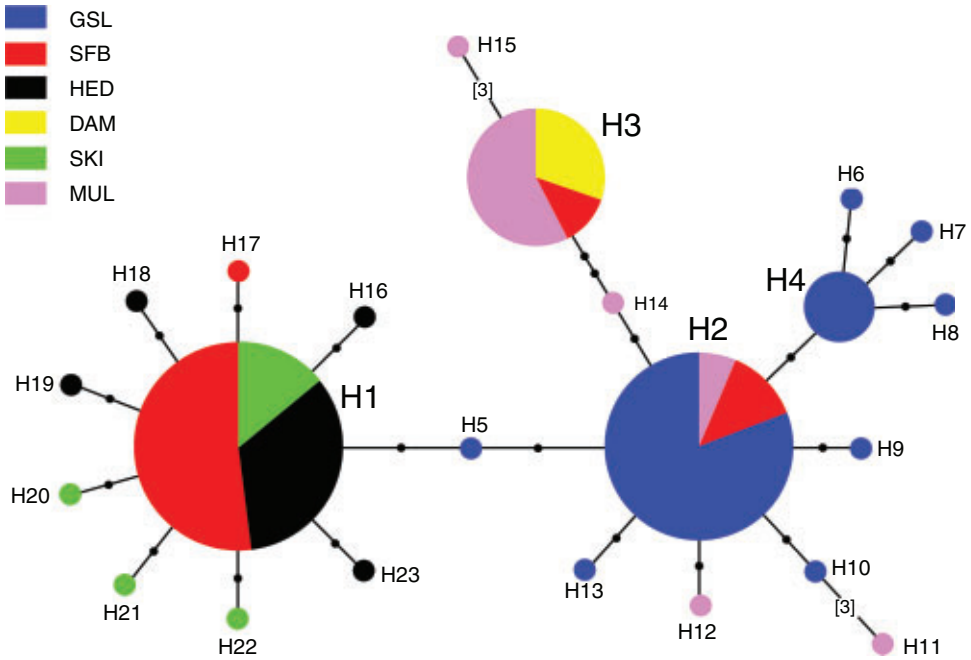

Fig. 3. The relationship of *COI* haplotype distribution among *Artemia franciscana* individuals from Great Salt Lake (GSL), San Francisco Bay (SFB) and Australian populations (MUL, Mulgundawa; SKI, St Kilda; HED, Port Hedland; DAM, Dampier).

Table 3. Pairwise population matrix of F_{ST} values from *COI* loci
Results are shown as percentages. ns, non-significant; *, $P < 0.05$; **, $P < 0.001$

Site	DAM	MUL	ADE	HED	GSL
MUL	4.8^{ns}				
SKI	90.9**	63.1**			
HED	91.9**	68.6**	1.3^{ns}		
GSL	84.1**	68.1**	75.7**	77.5**	
SFB	69.7**	52.2**	7.8^{ns}	10.3**	60.9**

and elucidating the source of invasive species in non-indigenous habitats (Ashton *et al*. 2008; Ficetola *et al*. 2008; Mabuchi *et al*. 2008; Gaubert *et al*. 2009). The genetic structure of mitochondrial *COI* in the Mediterranean *Artemia* populations have clearly documented an invasion of *A. franciscana* from the GSL and SFB (Muñoz *et al*. 2014; Horvath *et al*. 2018). Phylogeographical analysis of Asian populations has documented colonisation by *A. franciscana* from multiple origins in both America and Europe (Eimanifar *et al*. 2014). Haplotype distribution revealed *A. franciscana* in Al Wathba Wetland Reserve (United Arab Republic; Abu Dhabi), likely originating from the GSL (Saji *et al*. 2019). Our results strongly support the idea that *A. franciscana* found in the HED and SKI

Table 4. Population genetic indices for Australian and native American *A. franciscana* based on *COI* loci
N, no. of sequences; S, no. of polymorphic (segregating) sites; Eta, total no. of mutations; h, no. of haplotypes; Hd, haplotype (gene) diversity; Hr, no. of haplotypes/no. of sequences; Pi, nucleotide diversity; k, average no. of nucleotide differences; Exp. Het., expected heterozygosity

Genetic indices	DAM	MUL	SKI	HED	GSL	SFB
N	10	26	10	21	61	37
S	0	10	3	4	8	5
Eta	0	10	3	4	8	5
h	1	6	4	5	9	4
Hd	0	0.465	0.533	0.352	0.550	0.480
(s.d.)		(0.116)	(0.180)	(0.131)	(0.058)	(0.087)
Hr	0.1	0.23	0.4	0.24	0.15	0.11
Pi	0	0.00349	0.00135	0.00085	0.0015	0.0025
(s.d.)		(0.0023)	(0.00131)	(0.0009)	(0.0012)	(0.0018)
k	0	1.554	0.600	0.381	0.666	1.135
Exp. Het.	0	0.0034	0.0013	0.0008	0.0014	0.0025
(s.d.)		(0.029)	(0.016)	(0.008)	(0.021)	(0.026)

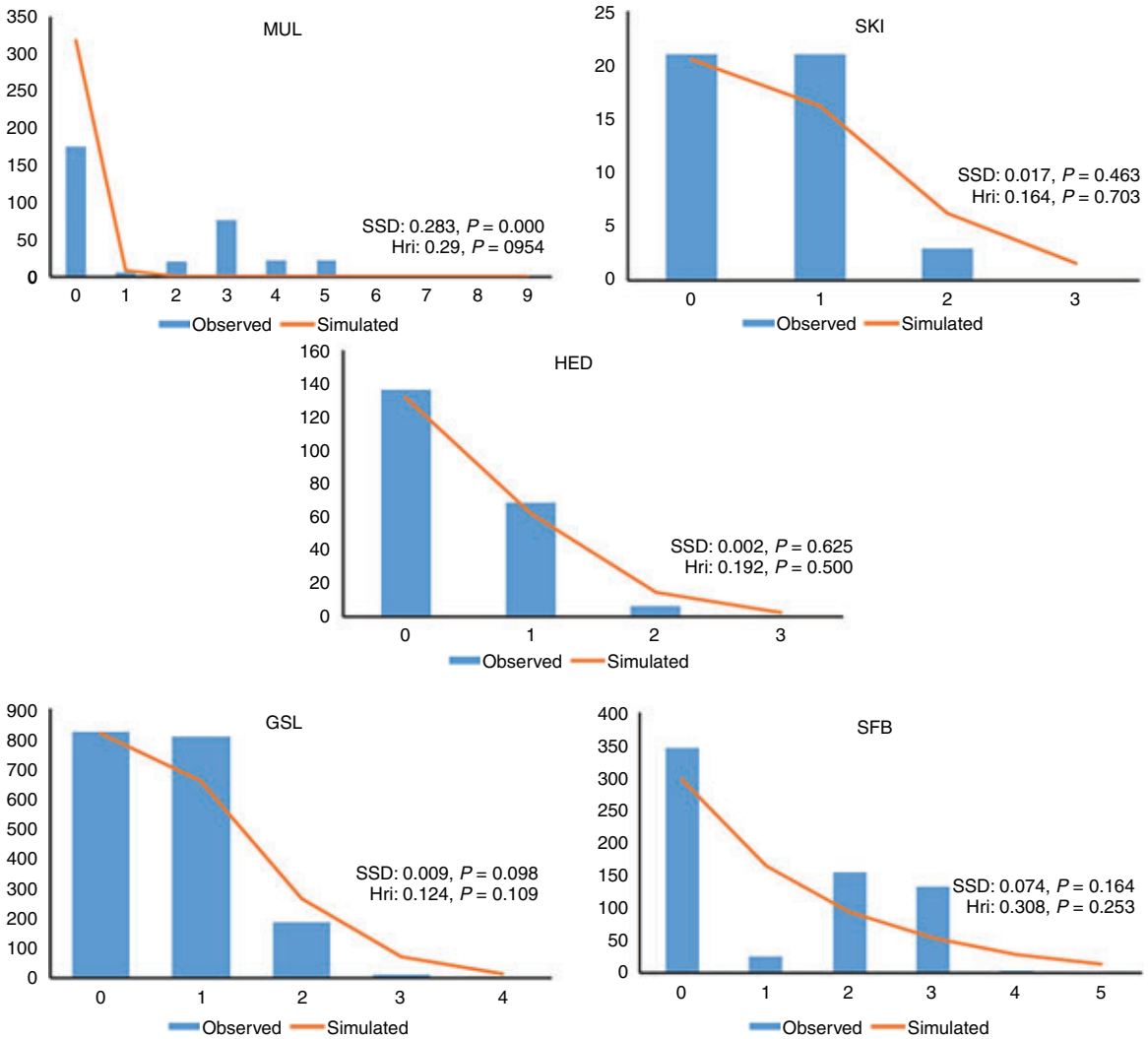

Fig. 4. Observed mismatch distributions and their curve fit to simulated models of demographic expansion. MUL, Mulgundawa; SKI, St Kilda; HED, Port Hedland; DAM, Dampier; GSL, Great Salt Lake; SFB, San Francisco Bay.

localities is derived from commercialised SFB populations in the USA, based on similar haplotypes (Fig. 3). These two groups also possessed the lowest values for the population differentiation index (F_{ST}) between SFB and HED/SKI (Table 3).

Interestingly, we found a single haplotype (H3) connected to the main haplotype H2 from GSL, consisting of SFB (12.12%), DAM (30.03%) and MUL (57.58%) localities. All sequences of DAM and 73.06% of MUL individuals belonged to this haplogroup, while it held only 10.82% of total SFB sequences. Generally, the observed haplotype pattern of DAM could suggest an origin in the SFB, while MUL possessed an intermediate structure between GSL and SFB (Fig. 3, Tables S2, S3, Supplementary Material). In contrast, the high values of F_{ST} among GSL/SFB and DAM/MUL, and in particular between SFB and DAM (69.7%) are considerable and cannot corroborate the suggested network distribution (Table 3). Ordinarily, introduction from the multiple sources (both GSL and SFB) could explain this observation. As an alternative hypothesis, the origin of MUL may be secondary introduction from other sources, in particular eastern Asia, where *A. franciscana* cysts from the Mekong Delta (Vietnam) and Bohai Bay (China) are easily obtainable in aquaculture markets (Van Stappen *et al.* 2007; Muñoz *et al.* 2014; Le *et al.* 2018).

The F_{ST} value was strongly significant between the two American populations, GSL and SFB (60.9%) (see Table 3). This result is similar to previous calculations (59.3%) performed by Muñoz *et al.* (2014). The pattern of haplotype frequencies (Fig. 3), as well as the value of F_{ST}, strongly suggest that there is a high degree of genetic separation between the GSL and SFB populations.

Typically, the invasive populations have lower genetic variation in their non-native locations compared with the original population (Golani *et al.* 2007). Reduction of haplotype variation and low intraspecific genetic differentiation has also been

observed for introduced *A. franciscana* in Vinh Chau (Vietnam) as compared with its source population from SFB, likely due to founder effects (Kappas *et al.* 2009). In contrast, Eimanifar *et al.* (2014) showed that the genetic diversity of invasive Asian *A. franciscana* is higher than in GSL and native Asian species. Similar results have been recorded in some invasive Mediterranean populations (Hontoria *et al.* 2012; Muñoz *et al.* 2014). Our results indicate that DAM is a static population with no genetic variation, which may be the result of a founder effect and population bottleneck during the process of colonisation. In general, MUL had the highest genetic diversity among the populations examined in this work. This finding might be due to multiple introductions by human-mediated dispersal events or secondary introductions, although, higher genetic diversity can be the result of adaptive pressure and/or physiological plasticity of the exotic population in a non-native region (Dlugosch and Parker 2008; Ruebhart *et al.* 2008; Vikas *et al.* 2012; Muñoz *et al.* 2014; Eimanifar *et al.* 2014). We propose that environmental conditions in new habitats could also have exerted selective pressure during development of the invasive population.

Two locations from Australia (SKI and HED) and GSL showed a unimodal structure of mismatch distribution, as well as a low and non-significant value of SSD and Hri, which indicate a recent demographic expansion. These findings suggest high adaptive potential and physiological plasticity of exotic Australian populations in the new habitats. The demographic history of MUL and SFB presented a complex structure indicated by multimodal patterns of mismatch distribution due to demographic equilibrium. A significant value of SSD ($P < 0.001$) in MUL confirmed these results, but a non-significant value of SSD in SFB and a non-significant value of Hri in both populations highlights the existence of demographic expansion. The results of this study indicate that the native American and Australian *A. franciscana* populations have undergone some degree of demographic expansion, with the exception of the DAM population.

In conclusion, our results verify the previous observations of the colonisation of Australia by invasive *A. franciscana* populations. Yet these populations harbour higher levels of genetic variation than the native America population, in contrast to other previously studied taxa (see Golani *et al.* 2007). *A. franciscana* possesses a faster filter-feeding rate and higher reproductive rate than the native species (Amat *et al.* 2007; Sanchez *et al.* 2016). It is also immune to the reduced feeding rate caused by cestode parasites, contrary to the native populations (Sanchez *et al.* 2016). These characteristics provide a high adaptive potential for *A. franciscana* in new non-indigenous regions, ultimately resulting in replacement of native species. Although there are many more populations of *A. franciscana* in America, the findings confirmed that Great Salt Lake, Utah, and/or San Francisco Bay, California, should be the most likely source for all the current invasion populations outside America. The utilisation of *Artemia* in aquaculture production without regard to potential environmental hazards threatens the biodiversity of *Artemia* worldwide.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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