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Genetic identification of South African *Artemia* species: invasion, replacement and co-occurrence

ATHANASIOS D. BAXEVANIS[1], STEFANIA MANIATSI[1], DIMITRIOS KOUROUPIS[1*], KONSTANTINOS MARATHIOTIS[1*], ILIAS KAPPAS[1], HORST KAISER[2] AND THEODORE J. ABATZOPOULOS[1]

[1]Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece, [2]Department of Ichthyology and Fisheries Science, PO Box 94, Rhodes University, 6140, South Africa
*These two authors made an equal contribution to the work.

Detailed molecular data of Artemia *(Crustacea, Anostraca) from South Africa are scarce. Here, we provide for the first time genetic data on the species status and phylogeography of* Artemia *from this region after an extensive sampling expedition. Our 16S rRNA RFLP analysis of 27* Artemia *populations, including seven from South African sites, provides evidence for the presence of a mixed bisexual and parthenogenetic component in this area. Phylogenetic and network analyses reveal that three (out of seven)* Artemia *populations from South Africa belong to the invasive* A. franciscana*, two of them belong to* A. salina *while the last two contain both parthenogenetic and bisexual (*A. salina*) individuals. From a total of 18 haplotypes identified, seven were recorded from South Africa. To our knowledge, this is the first confirmed report of* A. franciscana *in this region. Its invasiveness is reaffirmed by evidence for the complete replacement of the native* A. salina *population from Velddrif Saltworks. However, in other cases, parthenogens seem to modify the capacity of* A. franciscana *to replace natives. Results on the genetic identification of South African* Artemia *provide insights into the dynamics of invasion and co-occurrence and highlight effects on species interactions and on biodiversity in inland aquatic invertebrates.*

Keywords: brine shrimp, endemicity, biogeography, Africa, species interactions, *Artemia*

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INTRODUCTION

Artemia is a branchiopod crustacean commonly known as the brine shrimp. Currently, six well-characterized bisexuals (*Artemia salina* Leach 1819, *Artemia urmiana* Günther 1899, *Artemia franciscana* Kellogg 1906, *Artemia persimilis* Piccinelli & Prosdocimi 1968, *Artemia sinica* Cai 1989, and *Artemia tibetiana* Abatzopoulos, Zhang & Sorgeloos 1998) and a large number of obligate parthenogenetic strains are widely recognized (Triantaphyllidis *et al.*, 1997; Abatzopoulos *et al.*, 2002a, b; Gajardo *et al.*, 2002, 2004; Baxevanis & Abatzopoulos, 2004; Baxevanis *et al.*, 2006; Kappas *et al.*, 2009; Maniatsi *et al.*, 2009a, 2011). All bisexual species are diploid, while asexual populations may be diploid, polyploid or a mixture of different ploidies (Abatzopoulos *et al.*, 1986, 2002a; Maniatsi *et al.*, 2011).

Artemia is typically found in saline and hypersaline coastal or inland habitats worldwide with the exception of Antarctica. These communities are considerably diverse in terms of their physical, chemical and biotic conditions (Triantaphyllidis *et al.*, 1998; Van Stappen, 2002). Like other inland water organisms, *Artemia* shows a patchy distribution in continental 'islands', with limited capacity for active dispersal (Bilton *et al.*, 2001). From a biogeographical perspective, a sharp divide can be outlined demarcating the native distributions of *A. persimilis* and *A. franciscana* in the 'New World' from those of the other bisexuals and parthenogens in the 'Old World'. However, since the early 1980s, *A. franciscana* populations have also been recorded in Europe (Portugal, Spain, France and Italy) (Thiéry & Robert, 1992; Amat *et al.*, 2005; Green *et al.*, 2005; Mura *et al.*, 2006), in Asia (China, Iran, Japan and India) (Mito & Uesugi, 2004; Abatzopoulos *et al.*, 2006; Van Stappen *et al.*, 2007; Hajirostamloo & Pourrabbi, 2011; Vikas *et al.*, 2012), in Australia and New Zealand (Vanhaecke *et al.*, 1987; Ruebhart *et al.*, 2008) and in Africa (Morocco, Madagascar, Kenya, Tunisia) (Triantaphyllidis *et al.*, 1998; Amat *et al.*, 2005; Kaiser *et al.*, 2006; Kappas *et al.*, 2009; Ben Naceur *et al.*, 2010). The spread and the establishment of *A. franciscana* populations worldwide are mainly attributed to anthropogenic introductions (i.e. intentional and non-intentional inoculations in saltworks, use in aquaculture as live feed or pet trade activities) and secondarily to passive dispersal of *Artemia* cysts by waterfowl and/or wind (Figuerola & Green, 2002; Van Stappen, 2002). During the last decade, a number of studies (e.g. Amat *et al.*, 2005; Mura *et al.*, 2006; Van Stappen *et al.*, 2007; Vikas *et al.*, 2012) have provided conclusive evidence for the capacity of *A. franciscana* to successfully invade biotopes that were previously occupied by autochthonous *Artemia* species. The collapse of biogeographical barriers that had kept biotas effectively isolated for millions of years has been dramatic

Corresponding author:
T.J. Abatzopoulos
Email: abatzop@bio.auth.gr

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since the Age of Exploration (early 15th Century to the middle of the 16th Century—Mooney & Cleland, 2001). In fact, the effects of human-assisted invasions may reasonably be several orders of magnitude greater compared with those of prehistoric invasions (Ricciardi, 2007). This is particularly true for biotic interchanges of diapausing invertebrates to the point that such events may alter significantly the native distributions of taxa, at least in a regional scale. In this respect, *Artemia* constitutes an invaluable model organism for the study of biological invasions and their subsequent effects on the evolutionary trajectory of endemic species.

Studies on biodiversity and distribution of *Artemia* have led to the suggestion of several historical biogeographical scenarios (Baxevanis *et al.*, 2006; Maniatsi *et al.*, 2011). The key element for these efforts was the genetic analysis of a great number of *Artemia* populations with several molecular markers (i.e. 16S mtDNA RFLP analysis, AFLPs, COI and ITS sequencing and microsatellites) (Abatzopoulos *et al.*, 2002a and references therein; Bossier *et al.*, 2004; Gajardo *et al.*, 2004; Baxevanis *et al.*, 2005, 2006; Kappas *et al.*, 2009; Maniatsi *et al.*, 2009a, b, 2011; Muñoz *et al.*, 2010). Species delineation based on genetic data and measurement of genetic diversity is fundamental to assessing biodiversity. However, for several parts of the world there is still a paucity of this kind of data. For example, no genetic data are available for the species status of the numerous *Artemia* populations in Siberia (Mura & Nagorskaya, 2005; Van Stappen *et al.*, 2009).

A similar pattern is observed in Africa, especially in its southern part. The most thorough review on *Artemia* distribution is provided by Kaiser *et al.* (2006) but it is mainly confined to the southern part of the African continent. Additionally, there are quite a few studies concerning exclusively or in part North African countries (e.g. Hontoria & Amat, 1992; Baxevanis *et al.*, 2004; El-Bermawi *et al.*, 2004; El-Magsodi *et al.*, 2005; Amarouayache *et al.*, 2009; El-Gamal, 2010; Ben Naceur *et al.*, 2012) while there is only one study focusing on two populations from southern Africa (Triantaphyllidis *et al.*, 1996). All studies referenced above provide information about demographic, reproductive, lifespan and hatching characteristics, survival, maturation and growth rates, morphometry and biometry, but only the study by El-Gamal (2010) includes genetic data. South African sites are included only occasionally as part of broader research efforts (e.g. Baxevanis *et al.*, 2006; Kappas *et al.*, 2009; Maniatsi *et al.*, 2011).

From the above it is evident that the genetic identification of *Artemia* populations from South Africa using molecular techniques is necessary in order to fill in some of the gaps in our knowledge about the genetic make-up of *Artemia* in the African continent and to provide useful data for assessing potential biodiversity and phylogeographic patterns within the genus. Over the years 16S rRNA RFLP analysis has proved to be a reliable and cost-effective technique for the genetic screening of *Artemia* populations (Baxevanis & Abatzopoulos, 2004; Gajardo *et al.*, 2004; Baxevanis *et al.*, 2005; Mura *et al.*, 2005; Baxevanis *et al.*, 2006; Mura *et al.*, 2006). As a result, the establishment of a large databank of 16S RFLP patterns from several *Artemia* populations has made genetic screening a relatively straightforward task. Thus, the present study provides novel molecular data regarding the species status of seven South African *Artemia* populations, their genetic structuring and phylogeographic pattern.

MATERIALS AND METHODS

Details of the *Artemia* populations used in this study as well as their sites are given in Table 1. We compiled a dataset of 27 *Artemia* populations including seven populations from South Africa (for details, see Kaiser *et al.*, 2006) genetically analysed for the first time, six populations from North Africa, South Europe and South Asia that are well-characterized with other markers (see Maniatsi *et al.*, 2011), and 14 reference populations for all bisexuals and parthenogens (previously analysed by Baxevanis *et al.* (2005) and Baxevanis (2006)). DNA isolation and subsequent mtDNA RFLP analysis were performed only for the seven *Artemia* populations from South Africa, since RFLP data were available for the rest of the populations (Baxevanis *et al.*, 2005, 2006).

MtDNA RFLP analysis

DNA was extracted from 403 encysted embryos (209 extractions were used finally, 29–30 cysts per population) following the protocol of Maniatsi *et al.* (2011). Part of the 16S rRNA gene was amplified using the universal primers L2510 and H3080 (Palumbi, 1996). Polymerase chain reactions (PCRs) were performed in a total volume of 75 μl containing 6 μl of DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 0.15 mM of each dNTP, 45 pmoles of each primer and 4 U of Taq-DNA polymerase (Invitrogen, Carlsbad, California, USA). Amplifications were performed on a PTC-100® Peltier thermal cycler (MJ Research) with the following temperature profiles and conditions: initial denaturation at 95°C for 5 min, 33 cycles consisting of 94°C for 50 s, 50°C for 50 s, 72°C for 1 min and a final extension step at 72°C for 5 min. PCR products (4–7 μl) were digested with eight restriction endonucleases, separated electrophoretically in 1.5% agarose gels, stained with ethidium bromide, visualized and photographed under UV light. The following restriction enzymes were used: *Alu*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I and *Taq*^{a}I (New England BioLabs, Beverly, Massachusetts, USA). For molecular weight size standard a 100 bp ladder (New England BioLabs, Beverly, Massachusetts, USA) was used.

Data analysis

Single endonuclease restriction patterns were denoted by a specific letter. Thus, each individual was characterized by a multi-letter code describing its composite mtDNA genotype. The raw data were fragment profiles, but analysis was carried out based on site differences since these could be accounted for by the gain or loss of particular restriction sites (REs). For site inference, the published sequence of the complete *Artemia franciscana* mtDNA was used (GI: 5835051, Valverde *et al.*, 1994). Haplotype diversity values (*h*) within populations were computed using Arlequin 2.0 (Schneider *et al.*, 2000). The same software was also used to test for population differentiation in African populations based on haplotype frequencies (using 10,000 randomizations) and to evaluate the degree of population genetic structure (F_{ST}) through analyses of molecular variance (Excoffier *et al.*, 1992). AMOVA was performed for African *Artemia salina* and African parthenogenetic populations, separately. Haplotype divergence values were computed using the statistical package REAP 4.0 (McElroy *et al.*, 1991) and a UPGMA tree was drawn with the MEGA4 software (Tamura *et al.*,

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Table 1. List of *Artemia* populations used in this study.

Locality	Coordinates	Abbreviation	Species status	Reference
Coega Cerebos, South Africa	33°46′S 25°40′E	KOE	Present study	Kaiser *et al.*, 2006
Kleinzee Yacht Club, South Africa	29°40′S 17°04′E	KYZ	Present study	Kaiser *et al.*, 2006
Klipfontein Saltworks, South Africa	32°57′S 18°13′E	KFS	Present study	Kaiser *et al.*, 2006
Missionvale Salt, South Africa	33°52′S 25°32′E	MIV	Present study	Kaiser *et al.*, 2006
Saldanha Steel, South Africa	33°00′S 18°02′E	SAD	Present study	Kaiser *et al.*, 2006
Swartkops Marina, South Africa	33°51′S 25°34′E	SMS	Present study	Kaiser *et al.*, 2006
Velddrif Saltworks, South Africa	32°43′S 18°12′E	VSW	Present study	Kaiser *et al.*, 2006
Qarun, Egypt	29°30′N 30°30′E	QAR	Parthenogenetic	Maniatsi *et al.*, 2011
Borg el, Arab, Egypt	31°05′N 30°03′E	BOR	Parthenogenetic	Maniatsi *et al.*, 2011
Port Said, Egypt	31°09′N 32°22′E	PSA	Parthenogenetic	Baxevanis, 2006
Tanggu, China	39°00′N 117°40′E	TAG	Parthenogenetic	Maniatsi *et al.*, 2011
Puttalam, Sri Lanka	08°02′N 79°50′E	PUT	Parthenogenetic	Maniatsi *et al.*, 2011
Citros, Greece	40°22′N 22°34′E	CIT	Parthenogenetic	Maniatsi *et al.*, 2011
Mesi, Greece	40°59′N 25°13′E	MES	Parthenogenetic	Maniatsi *et al.*, 2011
M. Embolon, Greece	40°38′ N 22°58′E	MEM	Parthenogenetic	Baxevanis *et al.*, 2006
Jiangsu, China	34°40′N 119°30′E	JIA	Parthenogenetic	Baxevanis *et al.*, 2006
Swakopmund, Namibia	22°40′S 14°34′E	NAM	Parthenogenetic	Baxevanis *et al.*, 2006
Ankiembe, Madagascar	12°19′S 49°17′E	MAD	Parthenogenetic	Baxevanis *et al.*, 2006
Urmia Lake, Iran	37°20′N 45°40′E	URM	*A. urmiana*	Baxevanis *et al.*, 2005
Lagkor Co, Tibet	32°03′N 84°13′E	TIB	*A. tibetiana*	Baxevanis *et al.*, 2005
Yuncheng, China	35°00′N 111°00′E	SIN	*A. sinica*	Baxevanis *et al.*, 2005
Sant' Antioco, Italy	39°02′N 08°30′E	SAN	*A. salina*	Baxevanis *et al.*, 2006
Larnaca, Cyprus	34°54′N 33°37′E;	LAR	*A. salina*	Baxevanis *et al.*, 2006
Sfax, Tunisia	35°45′N 10°43′E	SAL	*A. salina*	Baxevanis *et al.*, 2005
Wadi el Natrun, Egypt	30°10′N 30°27′E	WAN	*A. salina*	Baxevanis *et al.*, 2006
San Francisco Bay, USA	37°28′N 122°30′W	SFB	*A. franciscana*	Baxevanis *et al.*, 2005
Buenos Aires, Argentina	34°30′S 58°20′W	PER	*A. persimilis*	Baxevanis *et al.*, 2005

2007). A phylogenetic network was constructed with Network 4.6.0.0 (www.fluxus-engineering.com) using the median-joining (MJ) algorithm (Bandelt *et al.*, 1999).

RESULTS

The size of the PCR-amplified mtDNA fragment was ~535 bp. Routine screening of all populations revealed a total of 25 cutting sites corresponding to 107 nucleotides. The fragment patterns generated by each enzyme are shown in Table 2. In total, 18 composite genotypes were identified and assigned to different haplotypes (Table 3). The maximum number of observed haplotypes within population samples was four (SMS population) (Table 4). Ten out of 18 haplotypes were private.

Haplotype frequencies across all populations together with mean haplotypic diversity estimates are given in Table 4. Only one haplotype was present in four out of seven South African *Artemia* populations (KFS, VSW, KYZ and SAD), while for the rest, haplotypic diversity ranged from 0.022 ±0.02 (SMS) to 0.093 ±0.57 (KOE). Seven out of 18 haplotypes identified in this study were recorded only in *Artemia* populations from South Africa (H12, H13, H14, H15, H16, H17 and H18). The SMS and KOE populations shared common haplotypes with LAR and WAN (*A. salina*), while KOE and MIV populations possessed haplotypes (H7 and H5, respectively) which appeared in the parthenogenetic populations. The most frequent haplotype and the one with the greatest geographical distribution was H2 (identified in nine out of 27 populations studied), while H17 was the rarest one, present in only two MIV individuals.

In order to visualize the relationships among haplotypes, a UPGMA dendrogram resulting from the distance matrix between haplotypes was computed (Figure 1). Three clusters were revealed, each one corresponding to the major phylogroups of *Artemia*: the first cluster contained haplotypes from *Artemia salina*, the second one included haplotypes identified in *Artemia franciscana* while the third cluster contained haplotypes present in parthenogenetic populations and Asian bisexuals (*Artemia sinica*, *Artemia urmiana* and *Artemia tibetiana*). *Artemia persimilis* haplotype (H11) was basal to the rest. According to the UPGMA dendrogram, the presence of *A. franciscana*, *A. salina* and parthenogenetic *Artemia* was verified. More specifically, SMS and KYZ populations contained haplotypes belonging to *A. salina*, while VSW, KFS and SAD individuals possessed haplotypes grouped with the one present in reference material of *A. franciscana*. Finally, MIV and KOE populations showed two components: one parthenogenetic (haplotypes H5 and H7) and one bisexual (*A. salina*). The most common haplotype (H2) was not present in South Africa.

The MJ phylogenetic network (Figure 2) of haplotypes reflected the topology of the UPGMA tree. The first cluster (Cluster I) contained *A. franciscana* haplotypes, the second (Cluster II) comprised *A. salina* haplotypes while the third one consisted of Asian bisexual species and parthenogenetic populations (Cluster III). *Artemia persimilis* haplotype (H11) was placed as outgroup. The constructed network verified that three out of seven *Artemia* populations from South Africa belong to the invasive *A. franciscana* (i.e. KFS, VSW and SAD), two of them belong to *A. salina* (i.e. SMS and KYZ) while the last two (i.e. KOE and MIV) presented both parthenogenetic and bisexual (*A. salina*) components. Figure 2

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Table 2. Fragment size estimates (base pairs) of restriction patterns observed in the 16S rRNA region among the *Artemia* populations analysed.

***Alu*I**	**A**	**B**	**C**	**D**	**E**
340	–				
285			–		
260		–			
220				–	–
195	–		–	–	–
155		–			
120		–			–
65*				–	
55*			–	–	

***Dde*I**	**A**	**B**	**C**
385		–	
360	–		–
150	–	–	
100			–
50*			–
25*	–		–

***Dpn*II**	**A**	**B**	**C**	**D**
230	–	–	–	–
180	–		–	
125	–	–		
115			–	–
110		–		–
70*		–		–
10*			–	–

***Hae*III**	**A**	**B**	**C**	**D**
470	–			
390			–	–
235		–		
235		–		
145				–
80*			–	
65*	–	–	–	

***Msp*I**	**A**	**B**
535	–	
295		–
240		–

***Not*I**	**A**	**B**
535	–	
300		–
235		–

***Rsa*I**	**A**	**B**
535		–
330	–	
205	–	

***Taqa*I**	**A**	**B**	**C**	**D**	**E**	**F**
420				–		
320			–		–	
260	–					–
250		–				
170		–				
120	–				–	
100			–			–
55*	–	–	–	–	–	–
40*	–					–
40*	–	–	–	–		–
20*					–	–
20*	–	–	–	–	–	–

*, these fragments could not be visualized because of their small size.

Table 3. Composite genotypes assigned to haplotypes. The enzymes used were in the following order: *Alu*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I and *Taq*^{a}I.

Haplotype	Composite genotype
H1	DAAAAABC
H2	DACABABC
H3	DACABABF
H4	DAAABABC
H5	CCAAAABD
H6	CCAABABD
H7	CAAAAABD
H8	AAAAAAAA
H9	AACCAABE
H10	AADCAABE
H11	BBBBBBBB
H12	AACAAAAA
H13	AACDBAAE
H14	AAACAABE
H15	CCCAAABE
H16	EACCAABE
H17	ABAAAAAA
H18	ACACAABE

shows the REs patterns that were responsible for the grouping. *Artemia persimilis* presented different patterns in seven out of eight REs while the three clusters differed mainly due to the patterns produced by *Taq*^{a}I and *Alu*I.

In order to determine whether the observed genetic diversity within *A. salina* was dictated by geographical separation, populations that possessed haplotypes belonging to *A. salina* were placed in two groups; the first group included populations from North Africa (i.e. SAL, SAN, LAR and WAN) and the second one comprised populations from South Africa (i.e. SMS, KOE, MIV and KYZ). It should be noted that for mixed populations (i.e. KOE and MIV), only the bisexual component was included in the analysis. According to the AMOVA results the F_{ST} value was 94.5%. However, 99.11% of the variation was attributed to differences among populations within groups, while the variance between groups was negligible. These results are indicative of a limited geographical subdivision in *A. salina* mtDNA (based on 16S RFLP analysis).

DISCUSSION

Updating *Artemia* biogeography is an arduous and continuous endeavour (the first organized research effort to compile a detailed list of *Artemia* sites throughout the world was in 1915 by Abonyi (see Van Stappen, 2002)). The current trend in *Artemia* biogeography is to provide as detailed information as possible, including data on genetic components, instead of a mere exposition of coordinates, local names and an indication regarding mode of reproduction (bisexual or parthenogenetic). In the present study, we have tried to resolve ambiguities regarding the species status of seven *Artemia* populations from South Africa by employing RFLP analysis and to identify possible biogeographical patterns that have shaped the distribution of *Artemia* in South Africa.

Invertebrates are widely distributed in South Africa. According to Hamer (2010), there are ~60,000 invertebrate species in South Africa, 3000 of which are crustaceans (Griffiths, 1999). However, very limited information exists on *Artemia* biogeography (e.g. Amat *et al.*, 1995; Kaiser *et al.*, 2006) in this region. Our results provide genetic evidence for the presence of two bisexual *Artemia* species, namely *A. salina* and *A. franciscana*, and a number of parthenogenetic strains in South Africa. According to the phylogenetic and network analyses, 11 haplotypes were detected; three of them were grouped with the reference *A. franciscana* material (namely H8, H12 and H17), six were grouped with *A. salina* (i.e. H9, H13, H14, H15, H16 and H18) and two haplotypes (i.e. H5 and H7) clustered with the group of bisexual Asian species and parthenogenetic strains. To our knowledge, this is the first confirmed report of *A. franciscana* in South Africa, although its presence has been implied by Kaiser *et al.* (2006). Based on a survey of 127 *Artemia* records in the African continent, it was estimated that from 41 populations with known reproductive mode about 12% could be hypothetically assigned to *A. franciscana* (Kaiser *et al.*, 2006). The authors also stressed the need for species verification using molecular techniques and the current work comes to fill this critical gap on *Artemia* biogeography.

One striking observation is the complete absence of the most common and widespread haplotype H2 (found in nine out of 27 populations) from South Africa. However, there are cases like the Egyptian sites BOR, PSA, QAR where H2 is present while it is absent from WAN (all these sites are in very close proximity, see Tables 1 and 4). Also, the neighbouring Chinese sites JIA and SIN are fixed for different haplotypes (H6 and H5, respectively). Therefore, signs of narrow endemicity as well as widespread occurrence of clones, as reported previously in Maniatsi *et al.* (2011), are also appearing here. Given this pattern, the absence of H2 from South Africa could be the result of repeated bottlenecks in North African sites coupled with unfavourable conditions in South Africa which restrain its distribution.

The spatial and temporal presence of *A. franciscana* in South Africa coincides with the significant growth of aquaculture activities in the region from the beginning of the 21st century (http://www.fao.org/fishery/countrysector/FI-CP_ZA/3/en; accessed 30 January 2014). It should be noticed that *A. franciscana* has been recorded in other African countries, such as Kenya (Kaiser *et al.*, 2006), Madagascar (Triantaphyllidis *et al.*, 1998) and Tunisia (Ben Naceur *et al.*, 2010). The current findings expand the *A. franciscana* distribution to the southern part of the African continent.

Of particular interest is the *Artemia* population from Velddrif Saltworks (VSW); this population had been previously identified as *A. salina* (Amat *et al.*, 1995). The results of the present study indicate that since then, the native *A. salina* population has been replaced by the invasive *A. franciscana*. Similar cases have been recorded in numerous localities, mainly in the western Mediterranean, providing evidence for the great capacity for expansion of *A. franciscana* (Amat *et al.*, 2005; Mura *et al.*, 2011). Moreover, laboratory experiments have shown that *A. franciscana* is able to outcompete *A. salina* and parthenogenetic strains within two or three generations (Abatzopoulos *et al.*, 2002a). However, there are documented cases of habitats where populations of *A. franciscana* and parthenogens co-exist. For example, Mura *et al.* (2006), based on a multidisciplinary analysis, revealed the presence of *A. franciscana* among native parthenogens in Margherita di Savoia (Italy) while Amat *et al.* (2005) recorded their co-existence in Mar Chica (Morocco) and Aigues Mortes

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Table 4. Haplotype frequencies, sample size (N) for each population and mean haplotypic diversity (*h*) (standard error, SE) of the studied *Artemia* populations. For abbreviations and details, see Tables 1 and 3.

	PER	LAR	WAN	SAL	SAN	SFB	SIN	URM	TIB	TAG	NAM	MAD	PUT	CIT	MES	JIA	BOR	MEM	PSA	QAR	SMS	KFS	KOE	VSW	KYZ	MIV	SAD
H1																	0.5	0.766									
H2								1.00	1.00	0.33	1.00	1.00	0.64				0.13		0.6	1.00							
H3										0.67																	
H4													0.36														
H5							1.00										0.37	0.233								0.93	
H6														1.00		1.00											
H7															1.00								0.3				
H8						1.00													0.4			1.00					
H9		1.00	1.00																		0.82		0.7				
H10				1.00	1.00																						
H11	1.00																										
H12																								1.00			
H13																									1.00		
H14																					0.10						
H15																					0.04						
H16																					0.04						
H17																											1.00
H18																										0.07	
N	30	27	26	30	28	30	30	30	30	27	30	29	28	27	28	30	28	30	25	27	29	30	30	30	30	30	30
h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.066	0.0	0.0	0.017	0.0	0.0	0.0	0.068	0.04	0.143	0.0	0.022	0.0	0.093	0.0	0.0	0.023	0.0
SE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.044	0.0	0.0	0.017	0.0	0.0	0.0	0.044	0.03	0.082	0.0	0.02	0.0	0.057	0.0	0.0	0.021	0.0

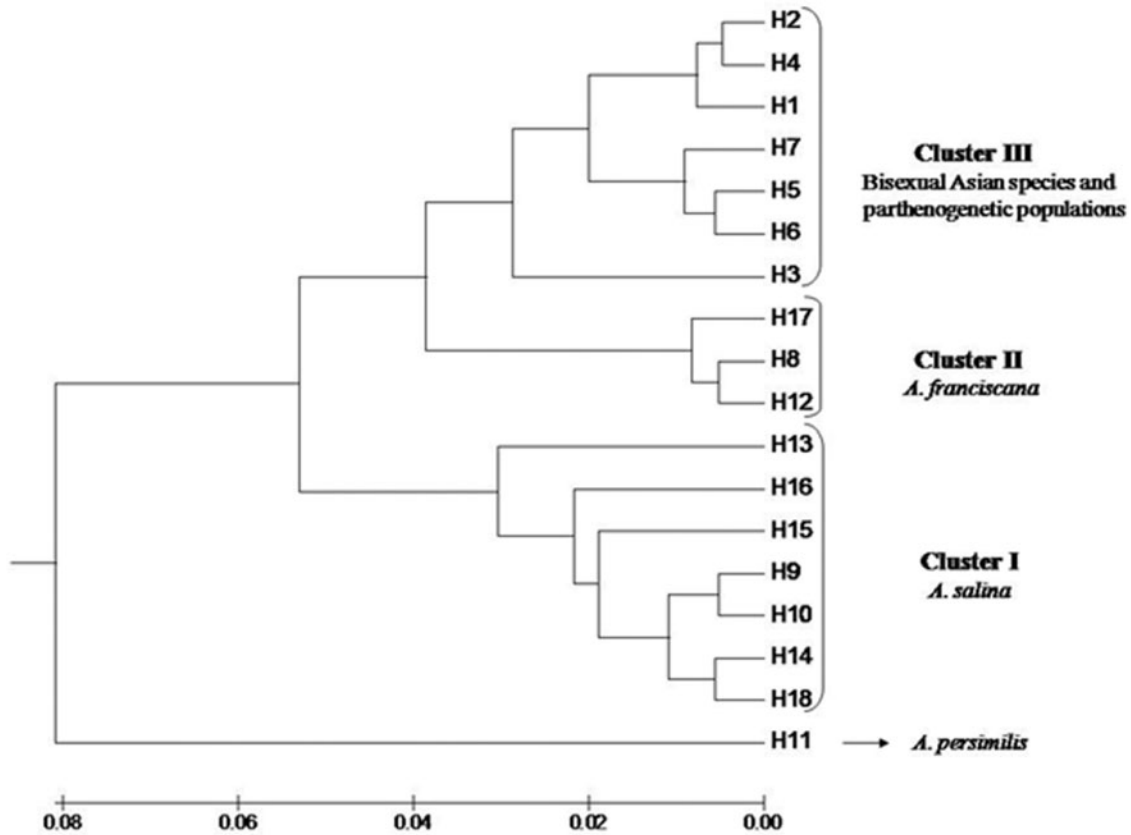

Fig. 1. UPGMA dendrogram clustering the 18 *Artemia* haplotypes recorded in this study. For more details, see Tables 1 and 3.

(France). In South Africa we found no populations harbouring both *A. franciscana* and parthenogenetic haplotypes. This fact in conjunction with the reported cases above probably indicates that although *A. franciscana* is an advantaged invader, the particular outcome in each case may be quite specific and dependent on certain conditions.

In contrast, co-existence of *A. salina* and parthenogenetic strains has been documented in two of the examined populations (KOE and MIV, see Figure 1). This observation reinforces the idea that parthenogenetic strains may share a habitat with bisexual species (apart from *A. franciscana*). For example, there are confirmed cases of co-existence of parthenogens with *A. salina* in Spain and in Italy (Amat *et al*., 1995, 2005), with *Artemia sinica* in North and Central China (Van Stappen, 2002), and with *Artemia urmiana* in Iran (Abatzopoulos *et al*., 2006) and Crimea (Abatzopoulos *et al*., 2009; Shadrin & Anufrijeva, 2012).

The finding that co-occurrence and replacement have affected the biogeography of *Artemia* in South Africa provides the tools to better understand the expansion of a non-indigenous species, such as *A. franciscana*. Given the frequency of *A. franciscana* introductions throughout the world, relevant field data should provide valuable comparisons on the competitive potential of *A. franciscana* with different relatives (i.e. *A. salina*, parthenogens). For example, it would be of interest to determine whether bisexual competitors suffer more severely compared with cases where one competitor is asexual. In general, the full spatial and temporal range of the events linked to invasion is not well defined, since only a fraction of sites have been investigated during a few restricted time intervals (Gertzen & Leung, 2011). To aggravate the problem, current rates of migration and dispersal of organisms are significantly higher compared with historical fluctuations in biota (Mooney & Cleland, 2001), thus opening the scene for novel species interactions. The monitoring of *Artemia* populations (especially in Africa) should be continuous and as extensive as possible in order to assess the rate of change in their composition. In general, this is a specially pressing issue in biodiversity studies, as several populations may go extinct even before they have been surveyed. The rate of change of saline biotopes is particularly rapid due both to the effects of desertification and human activities. A typical example is the wide-scale biodiversity loss that has taken place in the Aral Sea Basin within a very short period of time (Micklin, 2007).

Phylogenetic and network analyses showed that seven out of the 18 haplotypes found were present solely in the South African populations (i.e. H12–H18). The *A. salina* haplotypes from South Africa exhibit distinct phylogenetic histories when compared to those originating from the eastern and/or western Mediterranean (although they clustered together within the *A. salina* group). Furthermore, the AMOVA results showed that most of the genetic variation is distributed over a regional scale (among *A. salina* populations), while the genetic variation between the two geographical groups (*A. salina* populations originated from North and South Africa) is negligible. The existence of unique (but low in frequency) haplotypes in *A. salina* populations from South Africa (see Table 4) and the absence of genetic structure between *A. salina* from North and South Africa (AMOVA results) indicated that local adaptation mechanisms and a constant gene flow between South and North African populations have

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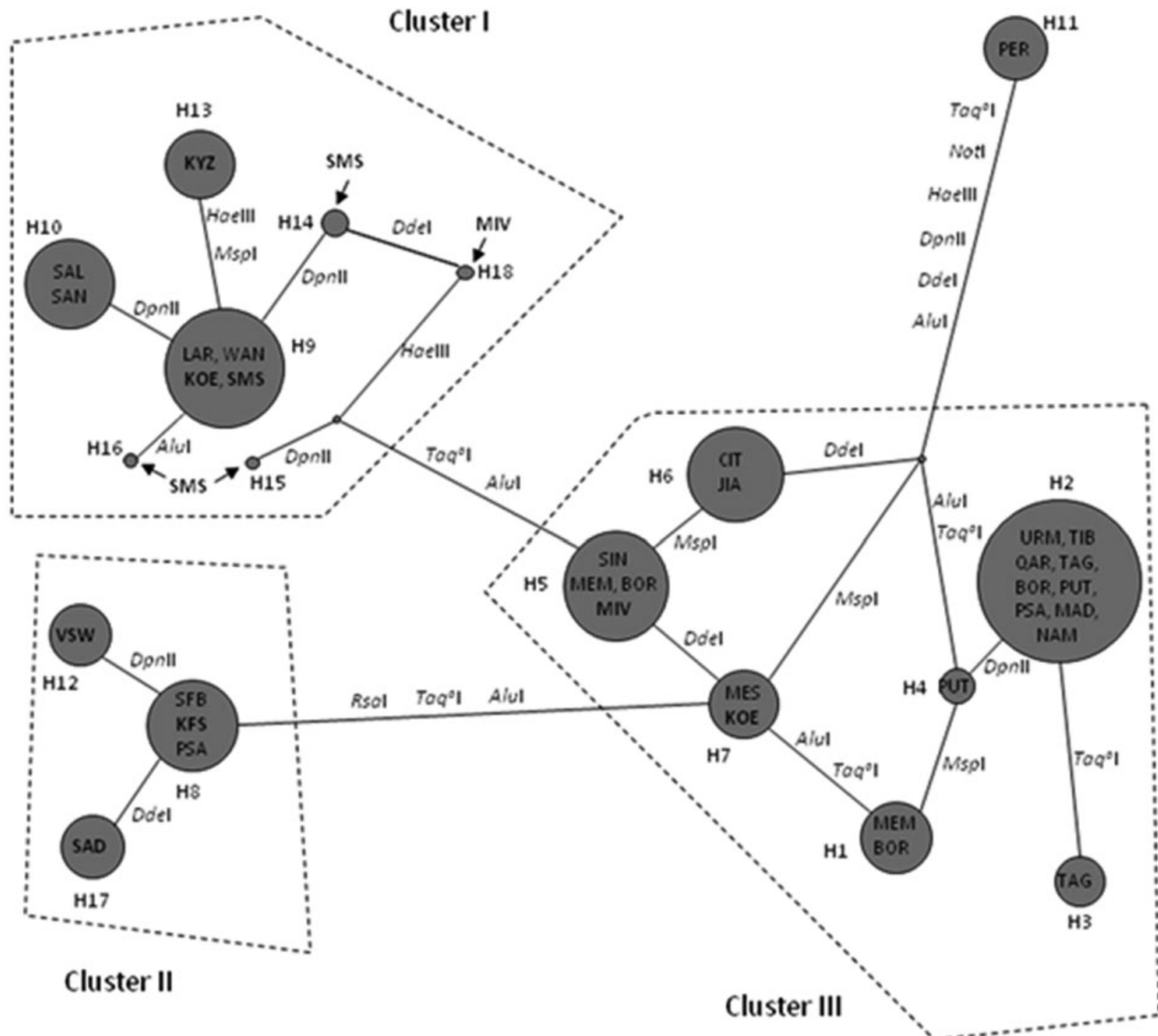

Fig. 2. Median-joining phylogenetic network based on haplotypes recorded in this study. Enzymes responsible for composite genotype changes are shown along lines connecting the different haplotypes. Populations sharing a specific haplotype are also indicated. For more details, see Tables 1 and 3.

shaped the genetic structure of *A. salina* in South Africa. A possible mechanism for the homogenization of geographically distinct gene pools is through passive dispersal of *Artemia* cysts by wind and/or migratory waterfowl (Green & Figuerola, 2005; Vanschoenwinkel *et al.*, 2008a, b). *Artemia* cysts may 'travel' for more than 1000 km in the digestive tract of water birds (Green & Figuerola, 2005). Furthermore, two of the major shorebird migratory flyways (East Atlantic and West Asia/West Africa flyways) overlap in the southern Africa region (Hickman *et al.*, 2001); the first one covers the western Mediterranean Basin and the western African coasts while the second crosses areas of western Asia, eastern Mediterranean and eastern African coasts.

The vast majority of cysts used in aquaculture industry originate from a limited number of sources (Dhont & Sorgeloos, 2002). On this basis, *A. franciscana* haplotypes from different parts of the 'Old World' are expected to show complete identity or minimum site changes compared with those of the same species from the USA. However, that was not the case with the *A. franciscana* haplotypes recorded in South Africa. Moreover, two different haplotypes were identified in South African *A. franciscana* (Figures 1 and 2). The existence of distinct haplotypes implies either that the invasion happened well in the past or the existence of a relic *A. franciscana* population. Fast genetic and reproductive differentiation of *A. franciscana* in a new environment has been previously documented (Kappas *et al.*, 2004; Mura *et al.*, 2006). In particular, evidence for significant differentiation of inoculated *A. franciscana* (San Francisco Bay stock) was apparent as early as within a year after inoculation in Vietnamese saltworks (Kappas *et al.*, 2004). Therefore, a probable scenario for explaining the presence of distinct haplotypes within South African *A. franciscana* populations is the rapid adaptation and establishment of new populations following the release (accidental or not) of USA cysts. This possibility is reinforced by the presence of both native (SFB) and distinct *A. franciscana* haplotypes in South Africa, yet its downside concerns the complete absence of published records or even anecdotal reports on *A. franciscana* inoculations in this region. The other possibility, of a relic *A. franciscana* lineage, can be safely excluded on the basis of low divergence of the distinct *A. franciscana* haplotypes (H12 and H17).

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The genetic results of the current study provide firm evidence for the existence of a bisexual (*A. franciscana* and *A. salina*) and a parthenogenetic component in South African *Artemia* populations. Comparisons with other published data reveal that populations of the invasive species *A. franciscana* have been established in South Africa, while the previous occurrence of *A. salina* has been substantially restricted. Parthenogenetic *Artemia* strains seem to impede the capacity of *A. franciscana* to replace native populations, as documented in other cases (Pinto *et al*., 2013). The combination of invasion, co-occurrence and replacement appears to have shaped the *Artemia* distribution in South Africa. The monitoring of the South African *Artemia* populations using molecular markers (as well as other *Artemia* populations from the African continent) is strongly recommended in order to gain a better understanding of the biogeography of the genus both from an ecological and a historical perspective.

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Correspondence should be addressed to:
T.J. Abatzopoulos
Department of Genetics Development and Molecular Biology
School of Biology, Aristotle University of Thessaloniki
54124 Thessaloniki, Greece
email: abatzop@bio.auth.gr

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