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PRIMARY RESEARCH PAPER

Genetic characterization of Argentinean *Artemia* species with different fatty acid profiles

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Abstract The Anostracan genus *Artemia* is composed by several sibling species reproductively isolated, but identical or very similar in outward appearance. The genus shows also an underlying striking variability from the biochemical point of view, regarding especially the fatty acid profile of the cysts and nauplii. In Argentina, *Artemia* is represented by two bisexual species: *A. franciscana* and *A. persimilis*. Former studies have shown that *A. franciscana* is present in northern of 36° and that *A. persimilis* is constrained southwards of 37° S. In general, there is good agreement between morphological and cytogenetic comparisons of Argentinean populations with respect to species discrimination. However, new Argentinean *Artemia* populations are being analyzed morphologically and it becomes necessary to further investigate if the genetic adscription of these populations is congruent with the results obtained from the current morphological analyses. Restriction fragment length polymorphism (RFLP) analysis of a fragment of the 16S rRNA mitochondrial gene was used to investigate the genetic diversity and population structure of 10 new *Artemia* populations from Argentina. The mitochondrial DNA (mtDNA) results showed a similar pattern to that of previous cytogenetic and morphological analyses with the two Argentinean species appearing as highly divergent. The presence of *A. persimilis* in southern Argentina and the southernmost Chilean population was confirmed unveiling a novel picture of species distribution in the country. *A. franciscana* showed a unique haplotype. Populations of *A. persimilis* appeared highly structured, although their clustering did not follow a clear geographic pattern. The different Argentinean *Artemia* populations analyzed were characterized by high variability in their fatty acids, showing both marine- and freshwater-type profiles. For the first time, the investigation of the relatedness between the fatty acid composition in *Artemia* and genetic markers was attempted. The study aimed at the putative association of molecular markers with marine versus freshwater-type populations. A lack of correlation between RFLP patterns at mtDNA and the fatty acid (FA) profiles was found in the *A. persimilis* populations which was discussed from the point of view of two main genetic hypotheses and/or phenotypic plasticity.

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Introduction

The brine shrimp *Artemia* (Branchiopoda, Anostraca) is widely distributed in salterns and hypersaline water bodies all over the world, except Antarctica (Triantaphyllidis et al., 1998; Van Stappen, 2002). In the New World, the genus is represented by two bisexual species; *A. franciscana*, present in North, Central and South America, and *A. persimilis*, which to date has been found in some hypersaline biotopes in Argentina (Amat et al., 1994, 2004; Cohen et al., 1999) and in southern Chile (Gajardo et al., 1999, 2004).

Several studies have dealt with the characterization of Argentinean *Artemia* populations, although a definite picture for this part of the world is still far from complete. Amat et al. (1994) presented a preliminary account of the distribution of the genus *Artemia* in Argentina. Later, Cohen et al. (1999) provided evidence through morphological analyses and cross-breeding tests that Argentinean populations from Salinas Grandes de Hidalgo, Laguna Callaqueo, Laguna Colorada Chica and Salinas Chicas belong to *A. persimilis*. Moreover, there is evidence that brine shrimp populations from Mar Chiquita and Las Tunas belong cytogenetically to *A. franciscana* (Papeschi et al., 2000). This was further corroborated through morphometric and reproductive studies by Amat et al. (2004). These authors suggested the likely exclusive presence of *A. persimilis* southwards of 37° S and the possible existence of an occasional hybridization between *A. franciscana* and *A. persimilis* populations in Las Tunas lagoon. Generally, the differentiation between *A. franciscana* and *A. persimilis* as gauged by allozyme and cytogenetic studies (Abreu-Grobois, 1987; Badaracco et al., 1987; Baratelli & Barigozzi, 1990; Colihueque & Gajardo, 1996; Gajardo et al., 1998; Rodríguez Gil et al., 1998) is congruent with the findings obtained by multivariate discriminant analysis of morphometric characters (Hontoria & Amat, 1992; Cohen et al., 1999).

New Argentinean *Artemia* populations are being analyzed morphologically and through cross-breeding tests, and results are corroborating the presence of *A. persimilis* in southern Argentina. It is therefore critical to investigate if the conclusions drawn from the previous studies match with results obtained from molecular DNA analyses. This will allow a more detailed evaluation of patterns of morphological and genetic differentiation with the aim of species identification and subsequent determination of distribution areas in Argentina.

Together with the progressive aquaculture development worldwide, *Artemia* has become the most frequently used organism as live food for culturing fish and crustacean larvae (Lavens & Sorgeloos, 2000). The nutritional value of nauplii seems to be determined by their content of $n-3$ highly unsaturated fatty acids (Watanabe et al., 1980; Léger et al., 1986; Navarro, 1990). This led Watanabe et al. (1980) to classify different *Artemia* populations according to their fatty acid (FA) profile and suitability for feeding marine or freshwater organisms, in two broad groups (phenotypes). The so-called marine-types are characterized by a high percentage of eicosapentaenoic acid (EPA, 20:5$n-3$) and a low percentage of linolenic acid (LNA, 18:3$n-3$). The freshwater-types show low levels of EPA and high levels of LNA. Traditionally, although with a lack of sound scientific evidence, marine-type profiles have been associated to the marine-type nature of primary productivity in coastal ecosystems (Vos et al., 1984). However, marine-type profiles have also been reported in inland biotopes (Navarro et al., 1993, Van Stappen et al., 2003). In order to complete the picture, recently, in the most complete survey carried out on *A. persimilis*, Ruiz et al. (2007) have reported for the first time freshwater-type FA profiles in coastal Argentinean ecosystems. Hypotheses for the factors that could determine these and other patterns were provided in that study, including the existence of an underlying genetic component.

The aim of the present work was two-fold. First, to present genetic data on *A. persimilis* and *A. franciscana* populations from Argentina and one (southernmost) *A. persimilis* population from Chile. Second, to infer the genetic structure of *A. persimilis* populations in order to discern, whether, RFLP markers correlate with the patterns of biochemical (fatty acid) data (marine vs. freshwater-type). The investigation was based on the 16S rRNA gene of the mitochondrial (mt) DNA and the method of RFLP genotyping that has proven reliable for fast and easy identification of species and populations of *Artemia* (Baxevanis & Abatzopoulos, 2004; Gajardo et al.,

2004; Baxevanis et al., 2005, 2006) and for studies of genetic population structure (Palumbi, 1996).

Materials and methods

Study locations and sample collection

Between 1992 and 2003 Argentina was explored from north to south in search of hypersaline biotopes hosting brine shrimp populations. In addition, Los Cisnes (CIS) lagoon from southern Chile was sampled and incorporated in the present study. The population from Los Cisnes lagoon is the southernmost *Artemia* population ever sampled.

The biotopes and populations included in the study are summarized in Table 1. In total, 12 populations were investigated including reference samples of *A. franciscana* (San Francisco Bay, SFB) and *A. persimilis* (Buenos Aires, BAI) (Gajardo et al., 2004). Note that all combinations of coastal/inland and marine/freshwater FA profiles in *A. persimilis* were available for the study.

Stock culture conditions

Cysts were hatched in sea water at 28°C under continuous aeration and illumination (hatching percentage > 80%). After 24 h, newly hatched nauplii were transferred to 4 l plastic containers.

The animals were fed ad libitum (density: 5–8 × 10^5 cells ml^{-1}) with a mixture (1:1, v/v) of two algal species, *Dunaliella salina* and *Tetraselmis suecica*, under mild aeration at a 12D:12L photoperiod. The salinity was maintained at 70‰ and the temperature at 24 ± 1°C. The medium was monitored for food and renewed every two days. With the onset of sexual maturity (survival > 90%), evidenced by the ovisac development in females and antenna in males, adult specimens (similar ratio of females and males) were collected from the mass cultures and preserved in absolute ethanol (Merck).

Mitochondrial DNA analysis

Total DNA was extracted from 18–20 individuals from each population using the Chelex protocol described in Estoup et al. (1996).

Part of the 16S rRNA gene was amplified using the universal primers L_{2510} and H_{3080} (Palumbi, 1996). PCR reactions were prepared in a final volume of 20 µl and were composed of 0.5 pmoles of each primer, 0.125 mM of each dNTP, 10 mM Tris–HCl (pH 8.4), 25 mM KCl, 1.5 mM $MgCl_2$ and 0.025 U

Table 1 List of the *Artemia* populations studied, with their types of habitats, their respective FA (fatty acid) profiles (from Ruiz et al., 2007) and precise localities in Argentina and Chile

Locality	Abbreviation	Species	Type of habitat/ FA profile[a]	Coordinates	IATS[b]/ARC[c] code No
Hidalgo (Argentina)	HID	*A. persimilis*	I/F	37°08′ S, 63°31′ W	5/–
Algarrobo (Argentina)	ALG	*A. persimilis*	I/M	40°22′ S, 63°13′ W	4/–
Palos Blancos (Argentina)	PBL	*A. persimilis*	I/M	39°28′ S, 62°45′ W	9/–
El Ingles (Argentina)	ING	*A. persimilis*	C/F	40°43′ S, 62°27′ W	3/–
Caleta Olivia (Argentina)	COL	*A. persimilis*	C/F	46°27′ S, 67°31′ W	7/–
San Julian (Argentina)	SJU	*A. persimlis*	C/M	49°17′ S, 67°46′ W	11/–
Las Tunas (Argentina)	TUN	*A. franciscana*	I/M	33°45′ S, 62°19′ W	14/–
Mar Chiquita (Argentina)	CHI	*A. franciscana*	I/M	30°39′ S, 62°36′ W	16/–
Santiago del Estero	EST	*A. franciscana*	I/F	27°21′ S, 64°13′ W	12/–
Los Cisnes (Chile)	CIS	*A. persimlis*	C/?	53°15′ S, 70°10′ W	15/–
Buenos Aires (Argentina)[d]	BAI	*A. persimilis*	C/?	34°30′ S, 58°20′ W	–/1321
San Francisco Bay (USA)[d]	SFB	*A. franciscana*	C/?	37°41′, 122°14′	–/1258

[a] I: Inland, C: coastal; F: freshwater, M: marine

[b] Instituto de Acuicultura de Torre de la Sal, Castellón, Spain

[c] *Artemia* Reference Center, Ghent, Belgium

[d] Reference samples (Gajardo et al., 2004)

of *Taq* DNA polymerase (Invitrogen, Carzlsband, California). The amplification profile consisted of an initial denaturation at 95°C for 4 min, 32 cycles of 94°C for 50 s, 48°C for 50 s, 72°C for 1 min and a final extension step of 72°C for 5 min. PCR products (5 µl) were digested with seven restriction endonucleases, electrophoretically separated in 1.5% agarose gels, stained with ethidium bromide, visualized and photographed under UV light. The restriction enzymes used were: *Not*I, *Alu*I, *Dpn*II, *Dde*I, *Hae*III, *Rsa*I and *Msp*I (New England BioLabs®, Beverly, MA). For molecular weight size standard, a 100 bp ladder (New England BioLabs®, Beverly, MA) was used.

Data analysis

Distinct endonuclease restriction patterns were identified by a specific letter. Each specimen was assigned a multi-letter code (restriction profiles for each enzyme were given letter designations in order of discovery A, B, etc.) that described its composite mtDNA genotype. Samples from San Francisco Bay, USA (SFB; *A. franciscana*) and Buenos Aires, Argentina (BAI; *A. persimilis*) were used as reference material (Gajardo et al., 2004).

Haplotype and nucleotide diversity values within populations as well as nucleotide divergence estimates between haplotypes and populations (Nei & Tajima, 1981; Nei, 1987; Nei & Miller, 1990) were computed using REAP 4.0 software (McElroy et al., 1992). The input data were site differences among haplotypes, inference of which was aided by the published sequence of the whole mtDNA of *A. franciscana* (Valverde et al., 1994).

The extent of geographical structuring of genetic variation was assessed by quantifying the inter-group component of total variance by F statistics using the analysis of molecular variance model (AMOVA, Excoffier et al., 1992) implemented in ARLEQUIN 3.01 (Excoffier et al., 2005). Molecular variance components and F statistics were calculated using the pairwise difference method. Significance of each F_{ST} value was evaluated by a permutation test (1,000 iterations).

Heterogeneity in haplotype frequency distributions was tested for *A. persimilis* populations with the Monte Carlo permutation procedure (Roff & Bentzen, 1989) implemented in the REAP 4.0 package.

Correlation of pairwise nucleotide divergence estimates with geographical distances for all *A. persimilis* populations was evaluated with the Mantel permutation procedure (Mantel, 1967) available in ARLEQUIN 3.01 (Excoffier et al., 2005). Trees based on distance methods were constructed using the UPGMA algorithm of the Neighbour module in PHYLIP 3.57c package (Felsenstein, 2004).

Biochemical analysis (fatty acid composition)

Fatty acid profiles of six selected *A. persimilis* populations available from Ruiz et al. (2007) were included in the present study and were classified as marine (M) or freshwater (F) (Table 1). In order to examine the relationship of genetic divergence with PUFA profiles some tests were performed. Biochemical divergence among the six *A. persimilis* populations was estimated as pairwise Euclidean distances and displayed in a UPGMA dendrogram (SPSS, 12.0). Firstly, Euclidean distances were calculated with ten fatty acids (16:0, 16:1$n-9$, 16:1$n-7$, 18:0, 18:1$n-9$, 18:1$n-7$, 18:2$n-6$, 18:3$n-3$, 20:4$n-6$, 20:5$n-3$) and the ratio 16:0/16:1 (Table 2). Secondly, only the best diagnostic fatty acids (i.e. 18:3$n-3$ and the ratio 16:0/16:1) of the freshwater- and marine-type character were included to estimate the biochemical divergence between populations (Table 3). Additionally, a matrix pairwise F_{ST} values (Table 4) for the six *A. persimilis* populations was calculated. Correlation between biochemical and genetic distances (nucleotide divergence and F_{ST}) was tested by applying a Mantel test.

Table 2 Rescaled distance matrix of the fatty acid percentages (16:0, 16:1$n-9$, 16:1$n-7$, 18:0, 18:1$n-9$, 18:1$n-7$, 18:2$n-6$, 18:3$n-3$, 20:4$n-6$, 20:5$n-3$) and the ratio 16:0/16:1 from the cysts of Argentinean populations of *Artemia* (Ruiz et al., 2007)

	HID	ALG	PBL	ING	COL	SJU
HID	0.000					
ALG	1.000	0.000				
PBL	0.939	0.235	0.000			
ING	0.000	0.965	0.897	0.000		
COL	0.363	0.718	0.762	0.344	0.000	
SJU	0.862	0.151	0.344	0.838	0.567	0.000

Table 3 Rescaled distance matrix based on the 18:3*n*−3 fatty acid percentage and the ratio 16:0/16:1 from the cysts of Argentinean populations of *Artemia* (Ruiz et al., 2007)

	HID	ALG	PBL	ING	COL	SJU
HID	0.000					
ALG	0.981	0.000				
PBL	0.764	0.188	0.000			
ING	0.000	1.000	0.783	0.000		
COL	0.275	0.675	0.459	0.295	0.000	
SJU	0.931	0.020	0.140	0.951	0.626	0.000

Table 4 Population pairwise F_{ST} estimates

	HID	ALG	PBL	ING	COL	SJU
HID	0.000					
ALG	1.000	0.000				
PBL	0.699	0.095	0.000			
ING	1.000	0.000	0.095	0.000		
COL	0.859	0.158	0.111	0.158	0.000	
SJU	1.000	1.000	0.832	1.000	0.903	0.000

Results

A total of 236 individuals of both species were scored with the seven restriction enzymes. The size of the PCR-amplified mtDNA fragment was 535 bp.

Four restriction enzymes (*Not*I, *Alu*I, *Dde*I and *Hae*III) produced species-specific patterns. The restriction enzyme *Rsa*I detected polymorphism for *A. franciscana* whereas *Dpn*II, *Dde*I and *Msp*I detected polymorphism for *A. persimilis*.

In total, seven composite mtDNA genotypes were found (Table 5, Fig. 1). The maximum number of haplotypes (five, Pers1 to Pers5) was observed within *A. persimilis* while only two were detected in *A. franciscana* (Fran1, Fran2; Fig. 1). The most common haplotype was Pers3, observed in four populations of *A. persimilis*. The PBL population showed the highest number of haplotypes (three), followed by COL and CIS with two haplotypes each. *Artemia franciscana* samples showed a unique haplotype for the Argentinean populations, which differed from the one found in the control population SFB at one restriction site for the enzyme *Rsa*I. The remaining populations (HID, ALG, ING, SJU, BAI, TUN, CHI, EST and SFB) showed no haplotype diversity. Nucleotide diversity was highest in the CIS population (Table 5).

Estimates of nucleotide divergence among the seven haplotypes are shown in Table 6. The associated standard errors for each comparison are also reported. The highest degree of sequence divergence is between haplotypes Fran1 and Pers1

Table 5 Composite genotypes, haplotype diversity ($h \pm$ SE), % nucleotide diversity (π) and samples sizes (n) across populations. Composite genotypes are denoted with capital letters, each one corresponding to the restriction pattern obtained by a restriction enzyme in the following order: *Not*I, *Alu*I, *Dpn*II, *Dde*I, *Hae*III, *Rsa*I, *Msp*I

Haplotype		HID	ALG	PBL	ING	COL	SJU	TUN	CHI	EST	CIS	BAI	SFB
Fran1	AAAAAAA												1.00
Fran2	AAAAABA							1.00	1.00	1.00			
Pers1	BBBBBBB						1.00					1.00	
Pers2	BBBBBBA	1.00		0.10							0.72		
Pers3	BBABBBA		1.00	0.75	1.00	0.80							
Pers4	BBABBBB					0.20					0.28		
Pers5	BBACBBA			0.15									
n		20	20	20	20	20	20	20	20	18	18	20	20
h		–	–	0.426	–	0.337	–	–	–	–	0.425	–	–
SE				0.122		0.110					0.099		
π		–	–	0.010	–	0.005	–	–	–	–	0.012	–	–
Mean h[a]	0.149												
SE[a]	0.041												
Mean π[a]	0.0023												
SE[a]	0.0000016												

[a] *A. persimilis* populations

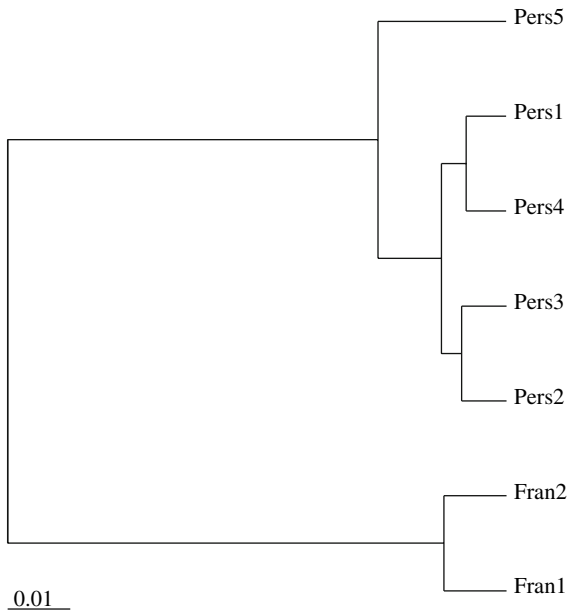

Fig. 1 UPGMA dendrogram of the seven mtDNA composite genotypes identified in the *Artemia* populations studied

(0.188 ± 0.076). The Fran1 haplotype is unique to SFB whereas Pers1 is present in both BAI and SJU populations. Average nucleotide divergence among Pers and between Fran haplotypes were 0.027 ± 0.024 and 0.020 ± 0.021, respectively, while the average nucleotide divergence obtained between the two groups of haplotypes (Pers/Fran) was 0.160 ± 0.066.

The divergence values were used to cluster haplotypes using the UPGMA method. The obtained cladogram is shown in Fig. 1. Two well-differentiated groups are evident, represented by the *A. persimilis* and *A. franciscana* haplotypes. Pers5 (present in PBL) appears as the basal haplotype of the *A. persimilis* clade.

Pairwise comparisons of mtDNA nucleotide sequence divergence (d_A) for the populations studied, corrected for inter-population diversity (d_{XY}), are shown in Table 7. The average nucleotide diversity and nucleotide divergence among all the populations studied were 0.085 ± 0.0000835 and 0.083 ± 0.000085, respectively. The nucleotide divergence estimates between *A. franciscana* and *A. persimilis* populations ranged from 13.8 to 18.8%, whereas, the average divergence among *A. franciscana* and *A. persimilis* populations were 1% and 1.2%, respectively.

The pairwise nucleotide divergence values were used as an input matrix for the generation of a dendrogram for the twelve populations studied (Fig. 2). Again, two groups are evident. The *A. persimilis* populations (first group) are well separated from the *A. franciscana* populations (second group). Within the former, the populations harbouring the haplotype Pers3 in high frequency (PBL, COL, ING and ALG) form a distinct clade that branches off the clade of BAI, SJU, CIS and HID. Within this clade, two other subclades appear, one that clusters populations in which the haplotype Pers1 is the most common (BAI, SJU), and the other clustering the populations in which the most frequent haplotype is Pers2 (CIS, HID).

All global F_{ST} tests of population differentiation showed significant differences in haplotype frequencies among all populations ($F_{ST} = 0.97$, $P < 0.05$). Most of the observed variation (91.41%) was due to differences between the two identified species, whereas only 7.23% was due to differences among populations within species. Only 1.36% of the total variation accounted for polymorphism within populations. The F_{ST} value for the *A. persimilis* group was

Table 6 Estimates of nucleotide divergence between haplotypes (below diagonal). Standard errors are shown above the diagonal

Haplotype		Fran1	Fran2	Pers1	Pers2	Pers3	Pers4	Pers5
Fran1	AAAAAAA		0.021	0.076	0.072	0.068	0.072	0.055
Fran2	AAAAABA	0.020		0.072	0.068	0.062	0.068	0.051
Pers1	BBBBBBB	0.188	0.175		0.016	0.025	0.016	0.036
Pers2	BBBBBBA	0.175	0.160	0.013		0.018	0.025	0.031
Pers3	BBABBBA	0.160	0.145	0.028	0.014		0.018	0.025
Pers4	BBABBBB	0.175	0.160	0.013	0.028	0.014		0.031
Pers5	BBACBBA	0.137	0.124	0.054	0.041	0.028	0.041	

Table 7 Mean nucleotide diversity (d_{XY}) (above diagonal) and nucleotide divergence (d_A) (below diagonal) between the *Artemia* populations studied

Population	HID	ALG	PBL	ING	COL	SJU	TUN	CHI	EST	CIS	BAI	SFB
HID		0.014	0.017	0.014	0.017	0.013	0.160	0.160	0.160	0.008	0.013	0.175
ALG	0.014		0.006	0.000	0.003	0.028	0.145	0.145	0.145	0.014	0.028	0.160
PBL	0.012	0.001		0.006	0.008	0.030	0.143	0.143	0.143	0.018	0.030	0.158
ING	0.014	0.000	0.001		0.003	0.028	0.145	0.145	0.145	0.014	0.028	0.160
COL	0.015	0.000	0.001	0.000		0.025	0.148	0.148	0.148	0.016	0.025	0.163
SJU	0.013	0.028	0.025	0.028	0.023		0.175	0.175	0.175	0.013	0.000	0.188
TUN	0.160	0.145	0.138	0.145	0.146	0.175		0.000	0.000	0.160	0.175	0.020
CHI	0.160	0.145	0.138	0.145	0.146	0.175	0.000		0.000	0.160	0.175	0.020
EST	0.160	0.145	0.138	0.145	0.146	0.175	0.000	0.000		0.160	0.175	0.200
CIS	0.002	0.008	0.007	0.008	0.007	0.007	0.154	0.154	0.154		0.013	0.175
BAI	0.013	0.028	0.025	0.028	0.023	0.000	0.175	0.175	0.175	0.007		0.188
SFB	0.175	0.160	0.153	0.160	0.161	0.188	0.020	0.020	0.020	0.169	0.188	

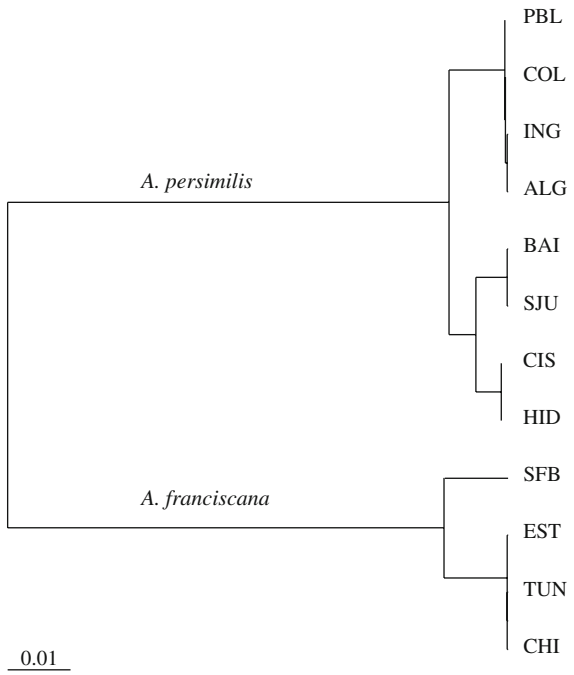

Fig. 2 UPGMA dendrogram clustering all *Artemia* populations according to pairwise sequence divergence (restriction sites data)

very high ($F_{ST} = 0.81$, $P < 0.05$) with only 19.08% of the variation occurring within populations.

Significant heterogeneity in haplotype frequencies among *A. persimilis* populations was found by the Monte Carlo approach of Roff & Bentzen (1989)

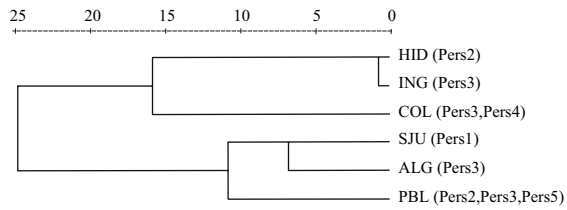

Fig. 3 UPGMA phenogram based on biochemical divergence between *A. persimilis* populations (Euclidean distance between 10 fatty acids and the ratio 16:0/16:1). All populations are labelled with their respective mtDNA haplotype

($\chi^2 = 338.23$, $P < 0.01$). The Mantel test for correlation of pairwise nucleotide divergence with geographical distance between all pairs of examined *A. persimilis* populations was not significant ($P = 0.47$).

Mantel tests showed no significant correlations among population divergences in FA profiles (Fig. 3), calculated as fatty acid content matrix (Table 2) or only considering the ratio 16:0/16:1 and 18:3$n-3$ (Table 3), and mtDNA nucleotide divergence ($P = 0.98$ and $P = 0.77$, respectively). Mantel tests also revealed a lack of association ($P = 0.94$ and $P = 0.70$) between divergence in FA profiles and *A. persimilis* pairwise F_{ST} estimates. Therefore, patterns of genetic diversity found in *A. persimilis* populations and differences between samples grouped by marine/freshwater profile were not linked.

Discussion

The publication of the complete mtDNA sequence of *A. franciscana* (Valverde et al., 1994) has triggered the use of several DNA-based techniques (AFLP, RAPD and RFLP) (Badaracco et al., 1995; Triantaphyllidis et al., 1997; Sun et al., 1999; Gajardo et al., 2004; Baxevanis et al., 2005, 2006) as molecular tools for the characterization of populations and the evaluation of evolutionary relevant problems in the brine shrimp.

The present study provides molecular evidence, based on 16S mtDNA gene, for the presence of the two New World species *A. franciscana* and *A. persimilis* in Argentina and in the southernmost Chile (Los Cisnes, Tierra de Fuego). This finding, together with a number of other investigations (Amat et al., 1994; Gajardo et al., 1995, 1998; Rodriguez-Gil et al., 1998; Cohen et al., 1999; Zuñiga et al., 1999; Papeschi et al., 2000; Gajardo et al., 2004) on genetic variability, morphometric characterization and cross-breeding tests, offers new information into the genetic diversity and evolutionary relationships of *A. franciscana* and *A. persimilis*.

Of the total set of 10 populations analyzed here, 7 of them were grouped with the BAI *A. persimilis* reference sample (Fig. 2), and three with SFB (*A. franciscana*). The ascription of some Argentinean populations to both species had already been reported on the basis of morphometric (Amat et al., 1994, 2004; Cohen et al., 1999) and cytogenetic evidence (Papeschi et al., 2000), demonstrating the high correlation of these tools in species identification. The CIS population from Chile is the southernmost *Artemia* population ever sampled. It has been identified as *A. persimilis*, confirming once again the presence of this species in southern Chile (Gajardo et al., 2004).

Haplotype and nucleotide diversity was observed only in *A. persimilis* populations (PBL, COL and CIS). No haplotype diversity within *A. franciscana* populations was found. In part, this is in accordance with the results of Gajardo et al. (2004) on Chilean populations, since, these authors found higher mean haplotype and nucleotide diversity values for *A. persimilis* than for *A. franciscana* populations by studying RFLP markers. Compared with the results obtained by Gajardo et al. (2004) (mean $h = 0.279 \pm 0.019$; mean $\pi = 0.0025$), the genetic diversity measures for the *A. persimilis* populations studied here are apparently different (mean $h = 0.149 \pm 0.041$; mean $\pi = 0.0023$), presumably as a result of increased taxon sampling for *A. persimilis* in the current study. This is a useful finding considering that *A. persimilis* diverged earlier from the common ancestor of the genus than *A. franciscana* as mentioned by the studies of Gajardo et al. (2001, 2002) and Baxevanis et al. (2006).

Based on the number of samples analyzed in the current work, a rough picture of the distribution patterns of *A. persimilis* and *A. franciscana* in Argentina is obtained. It is likely that *A. franciscana* has dispersed in the different Argentinean ecosystems via inoculation or waterfowl, as demonstrated in the Mediterranean basin by Amat et al. (2005) and Green et al. (2005). However, more studies are needed for a thorough examination of patterns of genetic structure and distribution of *A. franciscana* in Argentinean sites.

The presence of a species boundary at 37°–38° S between *A. franciscana* (to the north) and *A. persimilis* (to the south), provides some indications for the particular fitness of each species in relation to the temperature regimes of their respective habitats. Recent results (Medina et al., 2007) demonstrate the superior status of several fitness-related traits measured in *A. franciscana* at higher temperatures. This might shed more light to patterns of expansion of the two New World species in Argentina.

The population from TUN deserves special attention because of the unusual characteristics previously reported by some authors. Papeschi et al. (2000) ascribed this population cytogenetically to *A. persimilis*. Also, Amat et al. (2004) using life-history traits and cross-breeding tests raised the possibility for the presence of hybrids within this population, in accordance with Papeschi et al. (2000). This situation could be the same to that previously reported by Gajardo et al. (2004) with the population from Pichilemu salterns (Chile), indicating the possible presence of sites harbouring hybrid populations. Our molecular analysis has shown that the mtDNA haplotypes found in the TUN sample belong to *A. franciscana*. Previous cytogenetic (Papeschi et al., 2000) and morphometric (Amat et al., 2004) results for CHI and recent ones (unpublished data) for EST have illustrated that both populations belong to *A. franciscana*, which is in complete agreement with

the results obtained in this study using genetic markers. However, mtDNA markers alone are not adequate for detecting hybrids because of their maternal inheritance, and therefore nuclear markers would be needed to demonstrate the presence of hybrids in a population.

Tests for population differentiation gave significant differences ($P < 0.05$) between the two species (% of variation within = 1.36). Heterogeneity in haplotype frequencies among *A. persimilis* populations was confirmed by the Monte Carlo approach. Moreover, an extremely high value of F_{ST} (0.81) was found. The absence of significance in Mantel tests showed that the genetic differences among populations cannot be explained through isolation by distance. In Fig. 2, the UPGMA dendrogram shows that the populations CIS and HID cluster together despite being geographically very distant (~1,850 km). The same situation occurs with populations BAI and SJU which are similarly apart. This result points to an isolation of *A. persimilis* populations and differentiation due to genetic drift in the presence of limited gene flow, or to the possibility of extreme founder-effects shaping up population structure. In addition, the prospected Argentinean biotopes vary substantially in chemical conditions (Dragó & Quirós, 1996; Amat et al., 2004; Ruiz et al., 2007) and this ecological diversity may have an additional effect on the genetic structure and variability of the respective *Artemia* populations.

Related with the second aim of the present work, although brine shrimp are naturally low in PUFA content (Navarro, 1990; Van Stappen, 1996), different strains have shown variability in the proportions of some fatty acids such as EPA and LNA (Tizol-Correa et al., 2006; Ruiz et al., 2007). Demonstrating a genetic basis for these differences could result in the identification of markers for tagging the marine and freshwater-types in *Artemia*. Two genetic hypotheses could be advanced to explain the divergence in fatty acid profiles. First, biochemical divergence could be the result of genetic drift. Since drift would affect the whole genome, including cytoplasmic genomes, a drift-based scenario could give rise to similar patterns of divergence at genomic regions that are not functionally related with the lipid metabolism, including mtDNA, and biochemical loci. Alternatively, it can be considered that certain fatty acid profiles provide some adaptive advantage, with genetic divergence being limited to the genes responsible for such profiles. In this scenario, natural selection would cause divergence at PUFA metabolism-related genes, but not necessarily at the mitochondrial genome.

We have examined the correlation between genetic variability at the mtDNA and variability in the fatty acid profile in populations of *A. persimilis,* as this was the only species that showed haplotype and nucleotide diversity in our study, and we found no association between RFLP patterns at mtDNA and fatty acid profiles. This result supports the second model presented above, suggesting that genetic differentiation underlying the biochemical profiles, if present, is limited to the genes responsible for fatty acid metabolism, perhaps on an adaptive basis. However, two other alternatives should be taken into account. First, it is possible that the investigated markers have not been able to detect genetic divergence between freshwater- and marine-type profiles due to the variance among loci in the effect of drift and differences in evolutionary rates among genomic regions. Other mtDNA regions with higher evolutionary rates, or other nuclear markers, could show a parallel pattern of divergence between the genetic and the biochemical levels.

A second possibility is that the pattern of biochemical divergence is caused only or mainly by differences in the environment between the two groups of populations. It has been suggested that the fatty acid profile of *Artemia* and other zooplankton reflects the fatty acid profile of their food (Léger et al. 1986; Torrentera & Tacon, 1989; Navarro & Amat, 1992) and some authors (Vos et al., 1984; Léger et al., 1986; Lavens et al., 1989) have proposed that the variation in PUFA content among cyst batches might be due to variations in the composition of the food for the parental *Artemia* populations in the natural habitat. Consequently, phenotypic plasticity may explain why striking biochemical differences within *A. persimilis* populations from different habitat types were not correlated with divergence in the assayed genetic markers.

Additional studies with other markers, particularly with those derived from or located near the genes involved in fatty acid metabolism, such as genes coding for desaturases, would be extremely useful to get further insights into the possible genetic basis of PUFA profiles in *Artemia*. These studies will have to

wait until the major fatty acid desaturase genes in *Artemia* are isolated. Furthermore, classical genetic experiments designed to partition genetic and environmental components of the variability in PUFA profiles remain to be done. Concluding, our mtDNA analysis of restriction polymorphisms has provided novel and complementary data on the distribution of *A. persimilis* and *A. franciscana* in a region of South America. In particular, the current work has contributed significantly to a more informative description of *A. persimilis*, a primitive species largely undersampled in previous studies, and *A. franciscana*, a lineage with an extraordinary potential for range expansion. Although a number of unknown populations exist and higher resolution assays are still needed, our results are of great value for further research in a possibly unique region of the world where two bisexual *Artemia* species come into contact. Moreover, with the increasing variety of *Artemia* strains reaching the aquaculture market, it becomes very interesting to further investigate and to map alternative markers and other genomic regions, besides those analyzed here, potentially associated to *Artemia* fatty acid patterns.

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