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Characterization of polymorphic microsatellite markers in the brine shrimp *Artemia* (Branchiopoda, Anostraca)

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Abstract

The brine shrimp *Artemia* is a complex genus containing sexual species and parthenogenetic lineages. *Artemia franciscana* is native to America and its cysts (diapausing eggs) are used worldwide as a food source in aquaculture. As a consequence, this anostracan has become an invasive species in many hypersaline aquatic ecosystems of other continents. Parthenogenetic *Artemia* lineages occur only in the Old World. Ten and five microsatellite markers were developed to characterize two populations for *A. franciscana* and two populations for diploid parthenogenetic *Artemia*, respectively. For *A. franciscana* the number of alleles ranged from 11 to 58 per locus, while for parthenogens the number of alleles ranged from three to 10. The levels of heterozygosity in *A. franciscana* and in parthenogens ranged from 0.115 to 0.976 and from 0.000 to 0.971, respectively. These microsatellite loci showed a high population assignment power, which will be useful for future studies of population genetics and invasive processes in *Artemia*.

Keywords: *Artemia franciscana*, *Artemia parthenogenetica*, hypersaline ecosystem invasions, population assignment analysis, population genetics

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The brine shrimp *Artemia* is a zooplankter inhabiting hypersaline waterbodies around the world. *Artemia franciscana* is native in North, Central and South America (down to latitude 37°S), while parthenogenetic *Artemia* are exclusive to the Old World. Diapausing eggs (cysts) of the American brine shrimp, harvested from natural populations, are a major food source in aquaculture and exported across the world. The two main commercial sources of cysts are San Francisco Bay (SFB) in California and Great Salt Lake (GSL) in Utah. *Artemia franciscana* is now present as an exotic and invasive species in several Mediterranean countries, Iran, Russia, Vietnam, New Zealand, Australia and China.

To date, the population genetic, phylogeographical and evolutionary studies on *Artemia* have been based on allozyme, random amplified polymorphic DNAs, restriction fragment length polymorphisms and mitochondrial or nuclear DNA sequences (Abatzopoulos *et al*. 2002; Muñoz *et al*. 2008). No microsatellite DNA markers have previously been isolated in the genus *Artemia*. Here we describe the isolation of 10 polymorphic microsatellite loci for *A. franciscana* and five for the diploid parthenogenetic *Artemia (*hereafter *Artemia parthenogenetica*), using the magnetic bead-based enrichment method described previously by Jones *et al*. (2002) and the Glenn *et al*. (2000) method as is detailed in Galarza *et al*. (2006), respectively.

For the Jones *et al*. (2002) method, high molecular genomic DNA of eight adult specimens of *A. franciscana* was isolated using a cetyltrimethyl ammonium bromide protocol (Muñoz *et al*. 2008). Approximately 100 μg of DNA was partially digested with a cocktail of seven restriction enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments in the size range of 300–750 bp were ligated to adapter primers (20 bp), which contained a *Hin*dIII site at the 5′ end. Four enriched parallel libraries were prepared to be subjected to magnetic bead capture using Biotin-CA_{15}, Biotin-GA_{15}, Biotin-AAC_{12} and Biotin-ATG_{12} in a protocol

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provided by the manufacturer (CPG, Inc.). Captured molecules with repetitive sequences were amplified and restricted with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of the pUC19 plasmid and introduced into *Escherichia coli* strain DH5α by electroporation (ElectroMax, Invitrogen).

A total of 45 different microsatellite-containing clones were identified in *A. franciscana*, while 27 were identified in *A. parthenogenetica*. Only 31 and 19 polymerase chain reaction (PCR) primer pairs, respectively, could be designed using Primer 3 (Rozen & Skaletsky 2000) and OLIGO version 6.4 (Molecular Biology Insight, Inc.) software. Seventeen and eight of the primer pairs, respectively, yielded products of varying size, within the clonal expected size range, from 10 different brine shrimp samples and were selected for fluorescent labelling.

PCR amplifications were carried out in 20-μL total volume containing 2 μL of 10× PCR buffer (Bioline), 1.5–3.0 mM of $MgCl_2$ (Bioline), 0.25–0.60 μM of each primer (see Table 1 for details), 250 μM of each dNTPs, 0.5 μL of 20 mg/mL bovine serum albumin (BSA) (Roche Diagnostics), and 0.5 U *Taq* DNA polymerase (Bioline). Reaction conditions were as follows: an initial denaturation step at 95 °C for 5 min, 16–21 cycles consisting of 60 s at 95 °C, 60 s at 60 °C decreasing 1 °C per cycle, and 60 s at 72 °C. Then, 24 additional cycles were performed consisting of a step of 30 s at 95 °C, 30 s at 40 °C, and 30 s at 72 °C, and a final supplementary extension step of 15 min at 72 °C.

Ten out of the 17 polymorphic markers were screened in a set of 88 individual cysts from two US populations [GSL (n = 42), and SFB (n = 46)] in *A. franciscana*. Five out of the eight loci for *A. parthenogenetica* were screened in a set of 50 individual cysts from two Spanish populations [Cabo de Gata, GAT (n = 15), and La Mata, MAT (n = 35)]. DNA was isolated from individual cysts using the Montero-Pau *et al*. protocol (2008). Individuals were genotyped by assessing allele size on an ABI 3130xl Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma), and NED, PET and VIC (Applied Biosystems) together with LIZ 500 size standard (Applied Biosystems). Due to some screened loci showing peaks with one base of difference, we added a palindromic sequence (5′-GTGTCTT-3′) at the 5′ end of their unlabelled reverse primer (see Table 1) in order to minimize the generation of stutter peaks (Brownstein *et al*. 1996). Allele scoring was carried out in two independent laboratories using GeneMapper version 3.7 software (Applied Biosystems). Sequences of loci were deposited in GenBank (EU888832–EU888846).

Observed and expected heterozygosities, deviations from Hardy–Weinberg equilibrium (HWE) expectations and linkage disequilibrium were calculated using Arlequin version 2.000 (Schneider *et al*. 2000). Sequential Bonferroni corrections for multiple tests were applied using a global P value of 0.05 (Rice 1989). The program Micro-Checker version 2.2.3 (van Oosterhout *et al*. 2004) was used to test for null alleles, large allele dropout and scoring errors due to stutter peaks.

The number of alleles detected at each locus ranged from 11 to 58 for *A. franciscana*, and from three to 10 for *A. parthenogenetica*, identifying frequent single-base alleles. Observed heterozygosity ranged from 0.115 to 0.976 for *A. franciscana* and from 0.000 to 0.971 for *A. parthenogenetica* showing in some loci excess of heterozygosity probably due to its reproduction mode (see Table 1). Six and four loci showed significant departures from HWE expectations after Bonferroni corrections in *A. franciscana* from SFB and GSL, respectively. Only two loci showed departures from HWE in both populations (Af_B139TAIL and Af_B105TAIL). One and two loci showed significant departures from HWE in *A. parthenogenetica* from GAT and MAT, respectively. Analyses performed with Micro-Checker indicated homozygote excess at loci with HWE departures was due to the presence of null alleles. Although two independent laboratories assigned identical genotypes, Micro-Checker identified scoring error in locus Af_A108 at *A. franciscana* from SFB population due to shortage of heterozygote genotypes in alleles of one repeat unit difference. For *A. franciscana,* there was significant linkage disequilibrium for two loci in SFB (Af_B9, Af_A136), two loci in GSL (Af_B117TAIL, Af_B139TAIL), and one locus in both populations (Af_B105TAIL) (see Table 1 for details), while no significant linkage disequilibrium was found in the parthenogens. Additionally, no evidence for large allele dropout was observed.

Finally, population assignment analysis (PAA) and principal coordinates analysis (PCA) (see Fig. S1) were assessed with GENALEX version 6 (Peakall & Smouse 2006) using the 10 loci described in Table 1, showing a high discriminatory power between populations, and 100% of assignment capacity in the *A. franciscana* specimens analysed. The same assignment capacity was observed for *A. parthenogenetica* populations (figure not shown). Although some loci need to be used with care given the presence of null alleles, these new microsatellite markers will provide useful tools to assess genetic variation and genetic structure in the populations of these two *Artemia*, and should be useful to identify the source of introduction events in the Mediterranean Basin and elsewhere. Thus, these results could assist in future conservation plans.

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Table 1 Characterization of polymorphic microsatellite marker isolates for *Artemia* showing name of locus with fluorescent labels used in primer sequences.

Locus and GB Acc. No.	Primer sequences (5′–3′)	Repeat sequence	T_a	$MgCl_2$ (mM)	N_A	Size range (bp)	San Francisco Bay population					Great Salt Lake population				
							H_O	H_E	P	*FNA*	Linked loci	H_O	H_E	P	*FNA*	Linked loci
Artemia franciscana																
Af_A108	F: FAM-AGTGGCACAACATCCTTTTG	$(CA)_{13}$	60–40	3.0	12	150–177	0.357	0.804	< 0.001*	0.268		0.704	0.724	0.186		
EU888840	R: AACCACGCATGTGTTTTCAT															
Af_B117TAIL	F: VIC-CGTACCTTCAAGAGCTTTAGTC	$(TC)_{25}$	60–40	3.0	25	154–207	0.800	0.796	0.785			0.341	0.917	< 0.001*	0.306	B105TAIL/
EU888834	R: GTGTCTTGCTGATATTGTTTGCTTGTTC															B139TAIL
Af_B10	F: VIC-CTTTGCCTGGATCTCTAACAAA	$(CT)_{18}$	60–40	3.0	11	212–236	0.357	0.355	0.224			0.822	0.819	0.635		
EU888838	R: AGGAGGGTAAAGGGTAAAAAGG															
Af_A104TAIL	F: PET-ATGCACACACGTTCACACTC	$(CA)_{16}$	60–40	3.0	19	97–144	0.115	0.685	< 0.001*	0.384		0.683	0.879	0.019		
EU888841	R: GTGTCTTGGCCTATTGTAGCAAAGTGG															
Af_B139TAIL	F: PET-GAAGGAAAGGAAGAAGGATTAG	$(GA)_{30}$	60–40	2.0	58	238–390	0.167	0.900	< 0.001*	0.395		0.786	0.984	< 0.001*	0.096	B109/
EU888833	R: GTGTCTTAGAGCAAAGCCAAACTCAT															B117TAIL/B9
Af_B9	F: VIC-ACCTCCAAACACACAAAGTTG	$(GA)_{23}$	60–40	2.0	24	236–281	0.579	0.926	< 0.001*	0.182	B105TAIL	0.850	0.905	0.170		
EU888832	R: CCCGTTTCTCTCTCTCTCTATG										/A136					
Af_B109	F: PET-CACTCGCTAGGTTCAGAGTAAC	$(GA)_{14}$	60–40	2.0	39	194–288	0.976	0.949	0.967			0.953	0.973	0.518		
EU888836	R: ACCCTTATCTGGTGGTAAAGTC	$AA(GA)_5$														
Af_B105TAIL	F: NED-GGCAGATCAGTTTGACAGGAC	$(GA)_{16}$	60–40	3.0	31	266–314	0.536	0.950	< 0.001*	0.210	A136/B9	0.179	0.913	< 0.001*	0.394	B117TAIL/
EU888837	R: GTGTCTTAGATTACGCCAACGGTTGTAG	$GG(GA)_{13}$														A104TAIL
Af_B11	F: PET-GGGGAATTAAGTGGATTG	$(CT)_{17}$	60–40	3.0	37	286–338	0.909	0.935	0.906			0.868	0.945	0.133		
EU888835	R: TCCCTAGTTCAACATACCAC															
Af_A136	F: PET-TCTGGAAACCCTGATTAGACG	$(TG)_{10}$	60–40	3.0	19	215–317	0.189	0.814	< 0.001*	0.363	B105TAIL/B9	0.738	0.790	0.513		
EU888839	R: CGTCACTCGACACACAAACAT															
Artemia parthenogenetica																
Apdq01TAIL	F: FAM-ATTGGCCAGCTCTTCTGTTG	$(TG)_{19}$	60–45	1.5	3	175–183	Mon.	Mon.	—			0.000	0.056	0.014		
EU888844	R: GTGTCTTGCATCCGATTTTGTTCCTGT															
Apdq02TAIL	F: VIC-ACGCTGACTTTTGGTTGATC	$(ACT)_{20}$	60–45	2.0	4	229–250	0.200	0.494	0.004*	0.239		Mon.	Mon.	—		
EU888845	R: GTGTCTTCCTGGAAGGGTGTAAAGAGT															
Apdq03TAIL	F: NED-CACGAAAACAAGCCGTGATAG	$(GA)_{11}$	60–45	2.0	5	212–238	0.933	0.651	0.122			0.057	0.057	1.000		
EU888842	R: GTGTCTTTCTCTCTCTGCCTTTCTGTCC															
Apdq04TAIL	F: VIC-GGACATTTTCGTTTCCAGTG	$(AG)_{21}$	60–45	1.5	4	308–318	0.400	0.515	0.020			0.000	0.111	< 0.001*	0.210	
EU888843	R: GTGTCTTTCTGCAGCGTTGGACTATTG															
Apdq05TAIL	F: PET-CAGAGTAAATCGACCATGTG	$(AG)_{35}$	60–45	2.0	10	85–181	0.800	0.660	0.700			0.971	0.668	< 0.001*	–0.319	
EU888846	R: GTGTCTTAGAGCAAATCTCTCCTCTCC															

Notes: GB Acc. no., GenBank Accession no.; T_a, annealing temperature (see text for details); N_A, number of alleles for each locus; H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; *P, P* value of exact test using Markov chains with a confidence interval of 95%. *FNA,* frequency of null alleles based on the Oosterhout method (Micro-Checker software); Mon., monomorphic locus. Asterisks indicate significant departure from Hardy–Weinberg equilibrium after Bonferroni correction.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Plot of principal coordinates analysis (PCA) performed with GENALEX software using the 10 polymorphic microsatellite loci described for *Artemia franciscana* in Table 1. Black squares and white triangles indicate individuals from the San Francisco Bay and Great Salt Lake populations, respectively.

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Figure 1