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Fine-scale population genetic structure in *Artemia urmiana* (Günther, 1890) based on mtDNA sequences and ISSR genomic fingerprinting

Amin Eimanifar · Michael Wink

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Abstract We investigated the genetic variability and population structure of the halophilic zooplankter *Artemia urmiana* from 15 different geographical locations of Lake Urmia using nucleotide sequences of *COI* (mtDNA *cytochrome c oxidase subunit I*) and genomic fingerprinting by ISSR-PCR (inter-simple sequence repeats). According to sequence data, *A. urmiana* exhibits a high level of haplotype diversity with a low level of nucleotide diversity. The haplotype spanning network recognized 36 closely related unique haplotypes. ISSR profiles confirmed a substantial amount of genomic diversity with a low level of population structure. No apparent genetic structure was recognized in Lake Urmia but rather a random geographic distribution of genotypes indicating a high degree of panmixia. The population genetic data indicate the possibility of an individual's relationship, implying that differentiation of individuals is not affected by ecological factors. Therefore, the *A. urmiana* population from Lake Urmia should be considered as a single management unit for conservation.

Keywords Genetic variability · Population structure · *Artemia urmiana* · Lake Urmia

Introduction

Artemia (Crustacea, Anostraca)—a cosmopolitan macrozooplankter—is a tiny nonselective filter-feeding invertebrate that is highly adapted to hypersalinity. It occurs in over 600 locations across the world, except Antarctica (Van Stappen 2002). The genus *Artemia* includes seven sexual species and a parthenogenetic species complex, *A. parthenogenetica*, whose species status is under discussion.

Five sexual species are found in Eurasia including *A. salina* in the Mediterranean basin (Triantaphyllidis et al. 1997), *A. urmiana* (Gunther 1890) in Lake Urmia (Iran) and Lake Koyashskoe, Ukraine (Abatzopoulos et al. 2009), *A. sinica* in Yuncheng Lake, China (Cai 1989), *A. tibetiana* in Tibet (Abatzopoulos et al. 1998), and an undescribed new species in Kazakhstan (Pilla and Beardmore 1994). The other two species are *A. franciscana* (Kellogg 1906) distributed throughout North and South America, and *A. persimilis* (Piccinelli and Prosdocimi 1968) restricted to specific sites in Argentina and Chile.

Lake Urmia—the largest non-coastal thalassohaline lake in the Middle East—is close to the Turkish border and is the second largest permanent hypersaline lake in the world (Karbassi et al. 2010). It is located on a semiarid plateau in north-western Iran (37°20′ E–45°40′ N) at 1,278 m above sea level (Hassanzadeh et al. 2012). Lake Urmia shows many similarities to the Great Salt Lake in Utah (United States), including geographical topography, chemistry, and biological features (Kelts and Shahrabi 1986; Eimanifar and Mohebbi 2007). Apparently, Lake Urmia was always hypersaline because it collects water from rivers but has no outlet to other areas (Kelts and Shahrabi 1986). Within the lake, there are 102 islands, and its water hosts diverse bacterial communities, hyperhalophilous phytoplankton, and notably the almost endemic brine shrimp *A. urmiana.* The lake is an international park and protected biosphere reserve as recognized by the United Nations.

Before 1995, Lake Urmia had a surface area of 5,000–6,000 km^2 (140×40–55 km; water depth 16 m). Annual average precipitation was 246 mm, average temperature

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A. Eimanifar (✉) · M. Wink
Institute of Pharmacy and Molecular Biotechnology,
Heidelberg University, Im Neuenheimer Feld 364,
69120 Heidelberg, Germany
e-mail: A.Eimanifar@uni-heidelberg.de

9.4 °C, and water salinity 140–220 g/l (Manaffar et al. 2011; Delju et al. 2013; Hassanzadeh et al. 2012). Between 1997 and 2006, annual precipitation dropped to 204 mm and mean annual temperatures increased by 17 % (Hassanzadeh et al. 2012). The progressing drought has caused fundamental changes in the physiochemical composition of the lake: currently the salinity exceeds >300 g/l. The surface area has decreased to less than 2,366 km^2 and water volume was reduced from 42 billion m^3 in 1995 to 22 billion m^3 in 2010 (Hoseinpour et al. 2010; Manaffar et al. 2011; Pengra 2012).

Artemia cyst production in the top 50 cm of the lake has been estimated at 4,243 to 4,536 t/year for 1995 (Asem et al. 2012). A considerable decline in cyst concentrations from 399 cysts/l in 1995 to 3 cysts/l in 2007 has been recorded; currently less than 1 cyst/l are assumed (Manaffar et al. 2011; Asem et al. 2012). Consequently, these alterations are already threatening the survival of fauna and flora. Eventually, *A. urmiana* will be driven to local extinction if the present conditions continue to reduce population densities even further.

So far, the genetic variability and population structure of *A. urmiana* in its main area, Lake Urmia, are hardly known. This is due partly to insufficient sampling from different regions of the lake. For *A. urmiana*, emphasis had been placed on morphological and initial genetic (RFLP) studies to infer population structure and geographical variability (Eimanifar et al. 2006; Asem et al. 2007, 2010).

In this study, *A. urmiana* was collected systematically from 15 representative sampling sites of Lake Urmia in order to determine its population structure and genetic variability. The mitochondrial *COI* (mtDNA *cytochrome c oxidase subunit I*) gene was sequenced and inter-simple sequence repeats (ISSR)-PCR fingerprinting was carried out to assess genomic variability and phylogeographic structure.

Materials and methods

Sampling and DNA extraction

To achieve good coverage of intraspecific genetic variability and population structure, we collected encysted embryos from 15 sampling sites in Lake Urmia (Fig. 1). Table 1 provides information on the collection sites, voucher numbers, number of individuals studied and corresponding locality codes (used hereafter).

Extraction of total DNA was carried out from single cysts using Chelex-100 (6 %, Bio-Rad Laboratories, Hercules, CA) (Van Stappen et al. 2007; Montero-Pau et al. 2008). Extracted DNA was stored at 4 °C until further genetic analysis.

PCR amplification and sequencing protocols

The mitochondrial *COI* gene, which is informative for phylogeographic studies of *Artemia* (Muñoz et al. 2008) was amplified and sequenced (710 bp). Standard PCR was carried out in a total volume of 50 μl in a thermocycler (Biometra, Tgradient, Germany) with *Taq* DNA polymerase (Bioron, Ludwigshafen am Rhein, Germany) according to published protocols. PCR products were precipitated in 2 mol/l ammonium acetate and 360 μl absolute ethanol (Merck, Darmstadt, Germany) followed by centrifugation for 20 min (Gonzalez and Wink 2010). PCR products were sequenced in both directions using the same primers as for PCR. Cycle sequences were performed under the following conditions: 5 min initial denaturation 95 °C, 10 s denaturation at 96 °C, 5 s annealing at 50 °C, and 4 min extension at 60 °C, repeated for 33 cycles. Sequencing was carried out on an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (StarSEQ, Mainz, Germany).

Sequence alignment and phylogenetic analyses

All mtDNA sequences were edited using Bioedit sequence alignment vers. 7.0.9.0 (Hall 1999). Forward and reverse sequences were inspected visually. Sequences were translated into amino acid sequences to ensure the absence of stop codons and that no pseudogenes were amplified. Sequences were aligned using Clustal W2 ver. 2.0 multiple sequence alignment (Larkin et al. 2007). The aligned nucleotide matrix, polymorphic sites, and distinct haplotypes with their frequencies were analysed using Dnasp v. 5.00 (Librado and Rozas 2009). All sequences were deposited with GenBank according to locality and related haplotypes (accession numbers: JX512748–JX512808). Pairwise genetic distances were calculated using the Kimura 2-parameter (K2P) (Kimura 1980) model as provided in MEGA5 (Tamura et al. 2011). *K2P* was used because it allows for higher probability of transitional vs transversional base substitution and has been employed in earlier phylogenetic study on bisexual *Artemia* populations (Van der Heijden et al. 2012; Hou et al. 2006).

Phylogenetic trees were reconstructed using maximum likelihood (ML) in Phyml vers. 3.0 (Guindon et al. 2010) and Bayesian inference (BI) in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). The closely related *A. tibetiana* was chosen as an outgroup. For parametric analysis, DNA sequence alignment was used to calculate the best fitting nucleotide substitution model of DNA sequence evolution, via jModelTest v. 0.1.1 (Posada 2008) according to the corrected Akaike and Bayesian information criterion (AIC & BIC) (Akaike 1974). The codon-based partitioning model was used to reconstruct ML and BI trees for the *COI* data set using all parameters of the selected

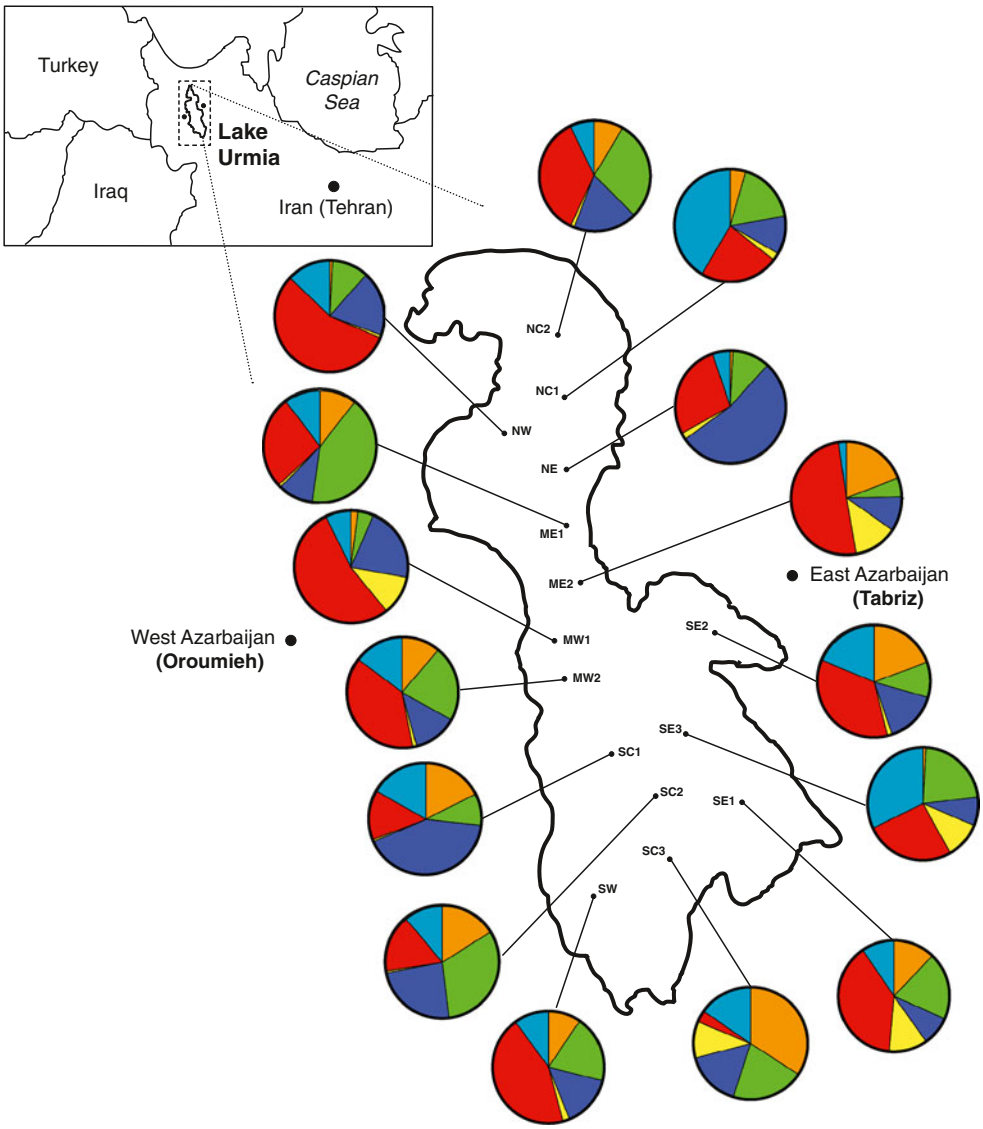

Fig. 1 Sampling sites with proportions of genetic subtypes within each locality based on Bayesian analysis of *Artemia urmiana* (STRUCTURE, K=6). *1–6* Distinct genetic clusters

model. The 1st, 2nd, and 3rd codon positions of the *COI* gene were set up to allow each codon position to have its own rate.

Bayesian analyses were run under the following conditions: two simultaneous runs, 8,000,000 generations, random starting tree with four independent Markov chains (MCMC), tree sampling every 500 generations, early-phase 0.5 million generations (50 %) trees discarded as burn-in. Posterior clade probabilities (PP) were calculated from the post remaining tree in order to support branch reliability (Erixon et al. 2003). Inter- and intrapopulation genetic diversity parameters were calculated using Dnasp v. 5.00 (Librado and Rozas 2009). The examined statistical indexes were as following: number of haplotypes (H), number of polymorphic sites (P), number of mutations (M), nucleotide diversity (π), haplotype diversity (HD), average number of nucleotide differences (K) per population. The neutrality of mutations and signal of population expansion through neutral evaluation were tested by Tajima's D (Tajima 1989) and Fu's Fs values (Fu 1997). Genealogical relationships among haplotypes were reconstructed using the Network program (Bandelt et al. 1999), based on a median joining algorithm. All sequences were collapsed to the unique haplotypes by the software Dnasp v. 5.00. The relationship between geographic and genetic distances was evaluated by isolation-by-distance (IBD) analysis, which is included in IBDWS software v. 3.14 (Jensen et al. 2005), implementing 30,000 randomisations. IBDWS performs Mantel tests with reduced major axis (RMA) regression analysis. Geographic distances (km) among localities were calculated using the Google map distance calculator.

Genomic fingerprinting by ISSR-PCR

Genomic fingerprinting analysis was carried out using inter simple sequence repeat (ISSR)-PCR. Fifteen ISSR primers were initially evaluated to identify the population variability

Table 1 Sampled populations of *Artemia urmiana* with IPMB voucher number, geographical coordinates, total number of individuals, and molecular genetic diversity indices. *LC* Locality code, *N* number of individuals, *HD* haplotype diversity, π nucleotide diversity, *H* number of haplotypes, *P* number of segregating sites, *M* total number of nucleotide substitutions, *K* average number of nucleotide differences per population, *IPMB* Institute of Pharmacy and Molecular Biotechnology

IPMB voucher	Geographic locality	Geographic coordinates	LC	*N*	*HD*	π	*H*	*P*	*M*	*K*
57210	North-Central	45°36 E–37°99 N	NC1	10	1±0.27	0.008±0.002	3	7	7	4.66
57223	North-Central	45°34 E–38°12 N	NC2	10	1±0.27	0.003±0.001	3	3	3	2
57211	North-East	45°42 E–37°93 N	NE	10	0.93±0.12	0.004±0.001	5	7	7	2.33
57209	North-West	45°18 E–37°99 N	NW	10	1±0.09	0.009±0.002	6	17	16	5.53
57212	Middle-East	45°40 E–37°83 N	ME1	10	1±0.5	0.01±0.007	2	8	8	8
57213	Middle-East	45°41 E–37°75 N	ME2	10	0.93±0.12	0.004±0.001	5	8	8	2.66
57214	Middle-West	45°28 E–37°71 N	MW1	10	0.93±0.12	0.005±0.002	5	9	9	3.2
57215	Middle-West	45°29 E–37°64 N	MW2	10	0.9±0.16	0.01±0.003	4	16	15	6.2
57217	South-East	45°72 E–37°40 N	SE1	10	1±0.272	0.009±0.003	3	8	8	5.33
57218	South-East	45°73 E–37°66 N	SE2	10	0.93±0.12	0.008±0.002	5	15	14	4.86
57220	South-East	45°68 E–37°54 N	SE3	10	1±0.27	0.008±0.002	3	8	7	4.66
57219	South-Central	45°44 E–37°51 N	SC1	10	0.6±0.31	0.001±0.0005	2	1	1	0.66
57221	South-Central	45°55 E–37°43 N	SC2	10	1±0.272	0.005±0.001	3	5	5	3.33
57222	South-Central	45°58 E–37°29 N	SC3	10	1±0.177	0.002±0.0006	4	3	3	1.5
57216	South-West	45°36 E–37°26 N	SW	10	1±0.5	0.005±0.002	2	3	3	3
Total					0.87±0.042	0.005±0.0007	36	53	50	3.2

within and among 150 randomly selected individuals belonging to 15 geographically different regions of *A. urmiana* from Lake Urmia. Out of 15 screened primers, 5 were chosen because banding patterns could be identified unambiguously (Table 2). All PCR amplifications were executed in a 25 μl final volume containing 40–50 ng template DNA, 2.5 μl 10× PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1 % Tween-20, 25 mM $MgCl_2$), 10 pmol primer, 2 μg/μl bovine serum albumin (BSA), 0.5 units *Taq* DNA polymerase (Bioron), 0.1 mM dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 μCi [α-^{33}P]-dATP (Perkin Elmer, LAS, Rodgau, Germany). DNA amplifications were performed in a thermal cycler (Tgradient, Biometra, Goettingen ,Germany) and started with 5 min at 94 °C

Table 2 List of primers screened for ISSR analysis, sequences (5′–3′), GC content, annealing temperature, amplification pattern, and total number of loci amplified in *A. urmiana*

Primer	Motif (5′–3′)	GC (%)	Annealing temperature (°C)	Amplification pattern	Total number of bands
ISSR1	$(AC)_8T$	47.1	48–54	Smear	–
ISSR2	$(CAC)_5$	66.7	48–54	Smear	–
ISSR3	$(GACA)_4$	50	48–54	Smear	–
ISSR4	$(AG)_{12}$	50	48–54	Poor	–
ISSR5	$(TC)_9$	50	48–54	Poor	–
ISSR6	$(GT)_{10}$	50	48–54	Smear	–
ISSR7	$(CA)_{10}A$	47.6	48–54	Poor	–
ISSR8	$(GAA)_5$	33.3	48–54	No amplification	–
ISSR9	$(CAG)_6$	66.7	48–54	No amplification	–
ISSR10	$(GCCG)_4$	100	48–54	No amplification	–
ISSR11	$(AG)_8C$	52.9	48	Good and sharp	19
ISSR12	$(AG)_8YT$[a]	50	48	Good and sharp	84
ISSR13	$(GA)_9T$	47.4	50	Good and sharp	17
ISSR14	$(TG)_8G$	52.9	50	Good and sharp	21
ISSR15	$(AC)_8C$	52.9	49	Good and sharp	31

[a] Y = C or T

followed by 35 cycles: 94 °C denaturation for 1 min, 48–54 °C annealing for 50 s and 72 °C extension for 2 min. The final cycle was followed by a 7-min extension at 72 °C. All amplified products were mixed with 8 μl bromophenol blue and run on a high-resolution denaturing polyacrylamide gels 6 % (0.2 mm) for 3 h at 65 W (size 45×30 cm) containing 1× TBE buffer. The gels were dried and exposed for 2 days to X-ray hyperfilm (Kodak, Taufkirchen, Germany) and subsequently developed. We repeated PCR amplification for 30 % of the individuals per locality to ensure reproducibility and repeatability of each marker. Eventually, the films were scanned and polymorphic bands identified for scoring.

ISSR statistics

The quality and quantity of amplified ISSR fragments were carefully checked visually. Ambiguous and smeared bands were excluded from the analysis. ISSR fragments are dominant makers that are inherited biparentally (Arafeh et al. 2002). The data matrix (1 = presence; 0 = absence of a band) was constructed to calculate genetic information of each population. An average inbreeding level was calculated based on individual's inbreeding coefficient F_{AFLP} (an analog to F_{IS}) in order to test the assumption of Hardy-Weinberg equilibrium (HWE) of populations using FAFLPcalc (Dasmahapatra et al. 2008).

The data matrix was then employed to determine the genetic diversity parameters for each population using AFLP SURV vers. 1.0 (Vekemans 2002). The parameters were as follows: number of polymorphic bands (*N*), percentage of polymorphic bands of total bands (*P*), average gene diversity (*Hw*), total gene diversity (*Ht*), and Nei's genetic diversity (Lynch and Milligan 1994). Genetic diversity values were measured with different levels of inbreeding coefficients within populations (F_{IS}=0, 0.05, 0.1, 0.15, 0.2, and 0.25). Genetic relationships were generated based on Jaccard's (Jaccard 1908) similarity coefficient by sequential agglomerative hierarchical and nested (SAHN) clustering using the unweighted pair group method with arithmetic average (UPGMA) (Sokal and Michener 1958) algorithm implemented in the NTSYS-pc 2.02 software package (Rohlf 1998). Principle coordinate analysis (PCA) was carried out for all ISSR genotypes in order to visualise relationships among individuals of *A. urmiana*. The programs DCENTER, EIGEN, and MOD3D used to design the PCA platform were included in NTSYS-pc 2.02 package. The final matrix was derived from the Nei genetic distance matrix in order to evaluate integrity of ISSR dendogram. Mantel's test (1967) was performed to identify any significant correlation between geographical (km) and genetic distance (Nei) using NTSYS-pc 2.02 software with 1,000 random permutations. Analysis of molecular variances (AMOVA) was conducted to quantify genetic variability within and among regions using Arlequin v. 3.5 package (Excoffier and Lischer 2010) with 10,000 permutations. The analysis was performed using binary matrix data sets. A total of 150 individuals were classified into 15 different groups corresponding to geographical localities. An overall population differentiation index (F_{ST}) was calculated between populations using the Arlequin v. 3.5 package.

A Bayesian model-based clustering algorithm was implemented to determine the population structure using the program STRUCTURE v. 2.2 (Pritchard et al. 2000; Falush et al. 2007). This algorithm assigns individuals into potential number of clusters (*K*). Simulations were performed by taking ten independent runs using the admixture model with correlated allele frequencies of *K*=1–20. ISSR genotypes were processed with a period of burn-in 50,000 and 20,000 MCMC repetitions (Jonathan et al. 2000). The most appropriate number of *K* was determined by calculating the likelihood of the posterior probability (Ln *P* (N/K) (Falush et al. 2007) and *ad hoc* quantity *DK* for each *K* partition. Posterior probability change with respect to *K* between different runs is assigned as a method for determination of true *K* value (Evanno et al. 2005).

Results

DNA sequence diversity and phylogeography

The DNA sequence alignment of the *COI* gene comprised a total of 561 bp. The 61 *COI* sequences revealed 508 invariable and 53 variable sites, of which 23 were parsimony informative.

Pairwise genetic distances (K2P) for *COI* sequences differed by maximally 0.7 % among all localities. *COI* sequences can be grouped into 36 haplotypes. Four haplotypes were shared by two or more individuals: haplotype H1 is common and was found in 22 individuals (Table 3) occurring in all 15 populations. Haplotype diversity ranged from 0.6 to 1 and nucleotide diversity from 0.001 to 0.01. The overall nucleotide diversity was low (π=0.005) but haplotype diversity (*HD*=0.87) was highest among regions. *COI* sequences from NW, MW2, and SE2 localities showed the highest degree of variable characters (Table 1). Combining the localities from North, Middle, and South, both the highest and lowest values of *HD* and π were observed in the southern part of the lake. Tajima's *D* and Fu's *Fs* neutrality test yielded negative values with significant outcome (Tajima's *D*=–2.355, *P*<0.01, Fu's *Fs*=–37.029, *P*<0.02). ML and BI trees were reconstructed using one single model with all given parameters. In the present study, the GTR model was used as a replacement for the suggested TPM2uf+G model because the suggested model was not implemented in the PhyML package. We used the GTR model as it is the most general neutral, independent, finite-sites model in molecular phylogenetic and has been applied successfully in previous phylogeography studies on *Artemia* populations (Tavaré 1986; Muñoz et al. 2010).

Table 3 Data matrix of polymorphic sites among 561 bp of *COI* (mtDNA *cytochrome c oxidase subunit I*) for 61 individuals, number of unique haplotypes, and their frequencies. *H* Haplotype, *F* haplotype frequencies

H	1111 1111122222 2223333334 4444444455 5566 1235780123 4568912345 6781245681 2345678923 5904	F	Individuals & locations
			NC2-1, NE-2, NE-3, NE-5 NW-3, NW-5, ME1-1, ME2-2 ME2-4, MW1-1, MW1-3, MW1-5 MW2-1, MW2-4, SE2-4, SE2-6 SE3-2, SC1-2, SC1-3, SC3-4
H1	GCCATCTCGT TATCCATGGA GCCCAACACT GGATAATGCC TTGG	22	SE1-1, SW-2
H2	....T...	1	NC2-2
H3	.... C.......T.	1	NC2-3
H4	....G.......	1	NC1-1
H5	.T........ ....T....... ....A..	1	NC1-2
H6	...G...... ....G.....	1	NC1-3
H7	....T.	1	NE-1
H8	.... .G........ ....A..	1	NE-4
H9	....G.... .A......T.	1	NE-6
H10	......G..C .... A.......T. C...	1	NW-1
H11	.....T.... ......G.T.	1	NW-2
H12	A......... ....C.T. ..A.	1	NW-4
H13	....A.. ..T.......	1	NW-6
H14	......G..C .... A..C.....T C...	1	ME1-2
H15	..T....... ....G....	1	ME2-1
H16	....T.....G	1	ME2-3
H17	.... C.........	1	ME2-5
H18	....C....... .... C...	1	ME2-6
H19	......G... .... A.........	3	MW1-2, SE3-1, SE1-2
H20	A.........	2	MW1-4, SC3-2
H21	.T........ .....G....	1	MW1-6
H22	.T..C..... .....G.... ...T......	1	MW2-2
H23	....G.....	1	MW2-3
H24	.T....G... .... .T........ A.........	1	MW2-5
H25	......G... ....T.. A.........	1	SE2-1
H26	.......T.. ....T.......	1	SE2-2
H27	....T. ..T....... .... .C..	1	SE2-3
H28	.... .T........ ....A	1	SE2-5
H29	.T........	2	SE3-3, SC3-1
H30	....C	1	SC1-1
H31	....C......	1	SC2-1
H32	........A. ....C...	1	SC2-2
H33	....T....G	1	SC2-3
H34	....A.	1	SC3-3
H35	....T. ....T.	1	SE1-3
H36	....T......	1	SW-1

The phylogenetic tree derived from *COI* sequences using ML and BI was based on 61 individuals from different localities. Only a few distinct clades were recovered; however, they were not supported by significant bootstrap values. Within each clade, members usually came from different regions of the lake, indicating a high degree of connectivity between populations (Fig. 2). This finding was corroborated by a haplotype-spanning network analysis that did not recover any distinct genetic structure (Fig. 3). By combining individuals into three main geographical regions, the main distribution of haplotypes occurs in the southern part of Lake Urmia (Fig. 4). IBD analysis revealed no significant correlation between geographical and genetic divergence (Z=3727958.2903, r=0.1496, one-sided P=0.93). A value of R^2=0.0224 for RMA regression analysis was calculated.

ISSR profiling

Similar to insects (Hundsdörfer et al. 2005), *Artemia* has a high number of microsatellite loci in its genome. As a consequence, ISSR-profiles can differ between individuals. In total, 172 reproducible and scorable ISSR bands were available for analysis. The average number of polymorphic

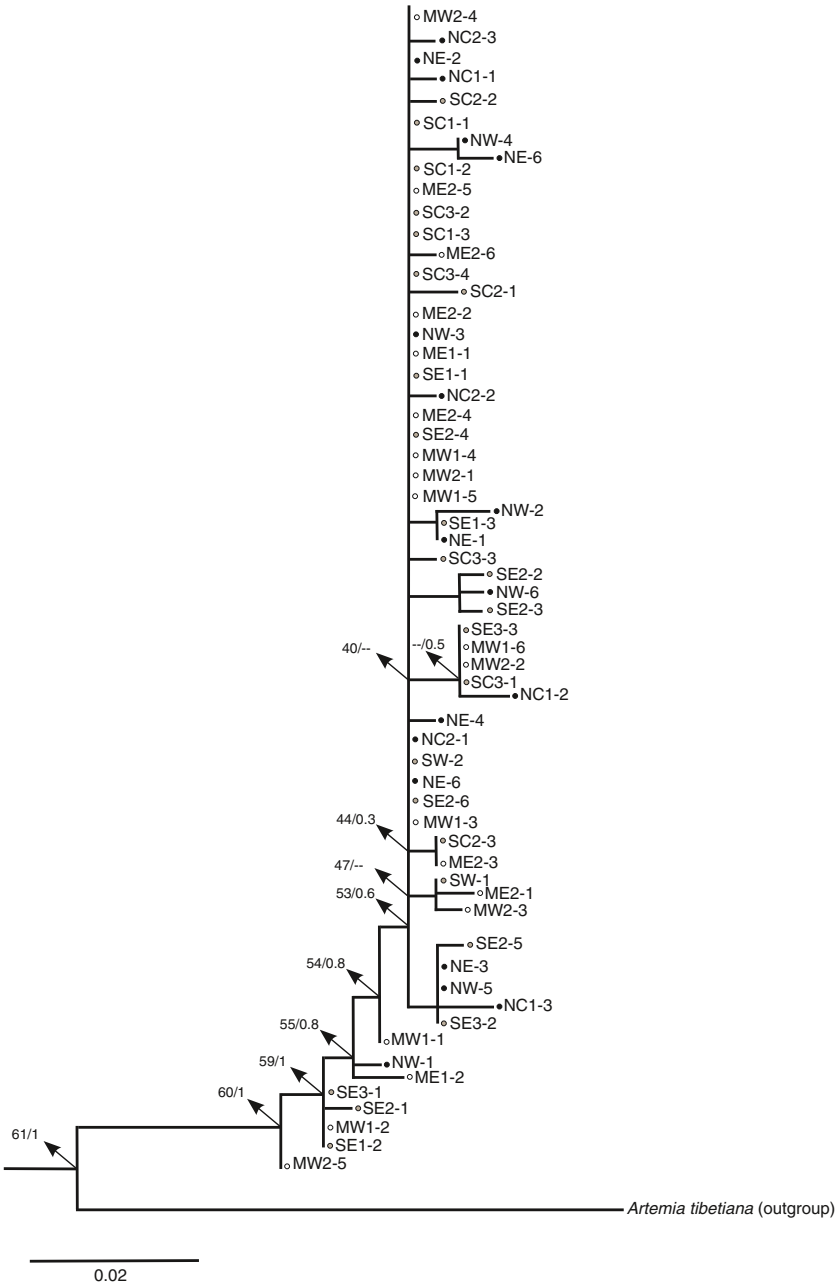

Fig. 2 Phylogeography of *A. urmiana* reconstructed from nucleotide sequences of *COI* (mtDNA *cytochrome c oxidase subunit I*) based on GTR model from 61 individuals represented by a maximum likelihood (ML) tree. Bootstrap values for ML (1,000 replicates), and Bayesian posterior probabilities are denoted for each major node from *left* to *right*. Northern, middle, and southern sampling sites are shown as *black*, *white* and *gray* circles

bands was 113.2 among all regions. The highest and lowest levels of genetic variability were observed in MW1, MW2 (*PPL*=135, i.e. 78.5 %, *Hj*=0.29±0.014) and NC2 (*PPL*=94, i.e. 54.7 %, *Hj*=0.22±0.017). The values of diversity statistics differed only slightly with an increasing F_{IS}.

Presuming a subtle deviation from HWE, all genetic diversity indexes were calculated based on calculated inbreeding coefficient level (F_{IS}=0.25). Nei's gene diversity (*Hj*) ranged from 0.2 to 0.29, with an average of 0.24± 0.007, demonstrating a substantial level of genetic variability among all localities (Table 4). By grouping 15 sampling regions into three distinct areas North, Middle, and South, the highest amount of genetic variability was detected in the Middle [*PPL*=90.7 %, *Hw* (average gene diversity)=0.26± 0.02, *Ht* (total gene diversity)=0.3] and South regions of the lake (*PPL*=95.9 %, *Hw*=0.24±0.007, *Ht*=0.3).

Intraspecific relationships were computed based on Jaccard similarity coefficients, and an actual cluster analysis was subsequently performed based on genetic distances by the UPGMA method. The UPGMA analysis recovered four separate groups with random distribution of individuals in each group. The Mantel test for ISSR data did not reveal any significant correlation between genetic and geographic distances (*r*=0.083, *P*=0.75). Relationships among individuals were illustrated by PCA, which confirmed the random distribution of individuals in Lake Urmia.

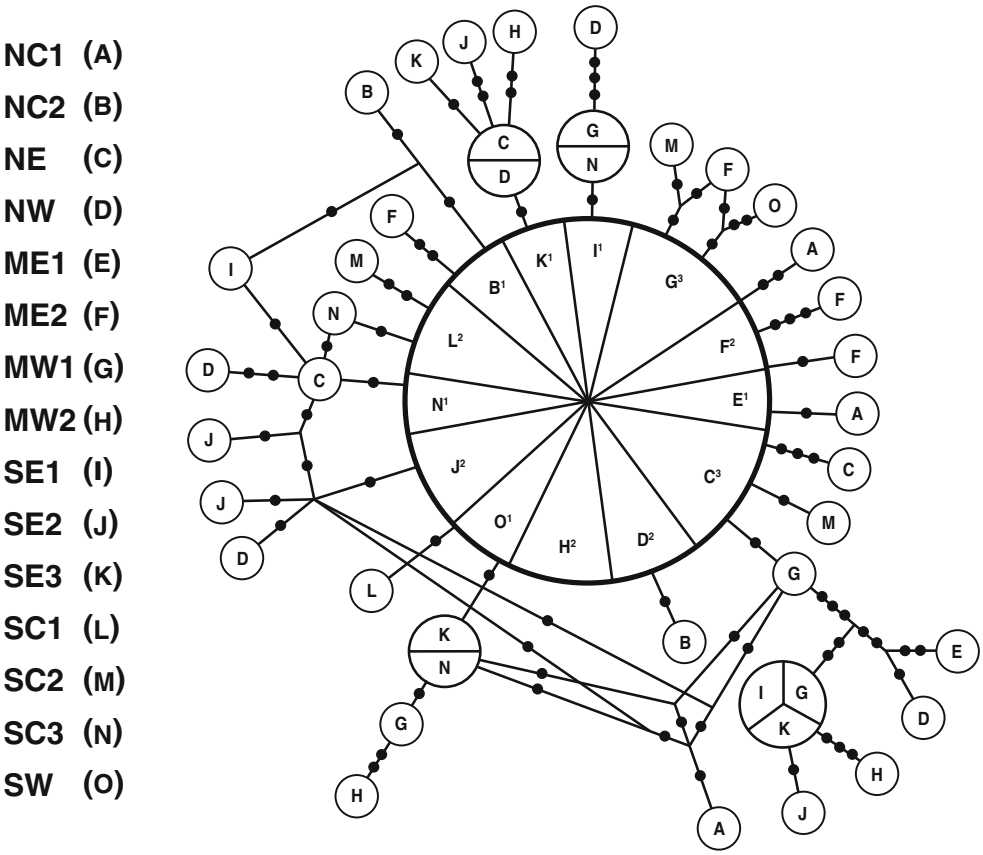

Fig. 3 Maximum parsimony haplotype network of 61 *COI* sequences. Frequency of observed haplotypes is reflected by relative size of *circles*; *small circles* represent single haplotypes. *Bullets* depict mutational steps. *A–O* Abbreviations for individual sampling sites. Number of haplotype frequencies for major haplotype (*centre circle*) indicated as superscripts for each locality; all others have frequency 1

ISSR Genetic population structure

A hierarchical analysis of genetic differentiation based on ISSR data indicated that most of the genetic variation occurred within individual localities (89 %). AMOVA test for ISSR data revealed low levels of differentiation between localities (F_{ST}=0.1, P<0.05). Bayesian clustering analysis by STRUCTURE was carried out to determine the total genetic variability using the admixture model without prior information of sampling localities. The calculation of DK and K consistently revealed a peak that was considered to detect the best fit K value. Genetic composition of individuals per locality was assigned to several segments depending on genetic similarities of individuals. The first highest posterior probability was obtained for six segments (K=6) (Table 5) represented by different colours in Fig. 1. Each individual is represented by a single vertical line, which is allocated to one of the six different segments. Each colour represents one segment, and the proportion of each colour is based on individuals allele frequencies identified by STRUCTURE. Similar to the situation with *COI*, in each locality individuals are present whose genotypes were not geographically coherent, indicating a random relationship of individuals across geographical localities in the lake (Fig. S1).

Discussion

Genetic variability and population structure

The present study was performed to determine genetic variability and population structure of endemic *A. urmiana* with a comparatively restricted distribution in Lake Urmia. Sequences of *COI* are useful to assess evolutionary changes and phylogeographic structure in closely related species as well as within single species (Hebert et al. 2003). Although narrow geographic distributions are usually associated with low genetic diversity (Hamrick and Godt 1989), *A. urmiana* showed an unexpectedly high level of haplotype diversity (*HD*= 0.87), which is similar to that of another decapod crustacean, *Aristeus antennatus* (*H*=0.8–0.9) (Maggio et al. 2009); whereas a lower haplotype diversity had been observed in *Artemia salina* (*H*=0.58) (Muñoz et al. 2008). The high level of haplotype diversity might be due to the large population size, and particular life-history traits of *A. urmiana* populations in the lake. The haplotype network shows a star-like topology, with many rare haplotypes originating from a central haplogroup. In agreement with an earlier RFLP analysis (Eimanifar et al. 2006), the majority of variation was found in the southern part where most of the rivers discharge into the lake.

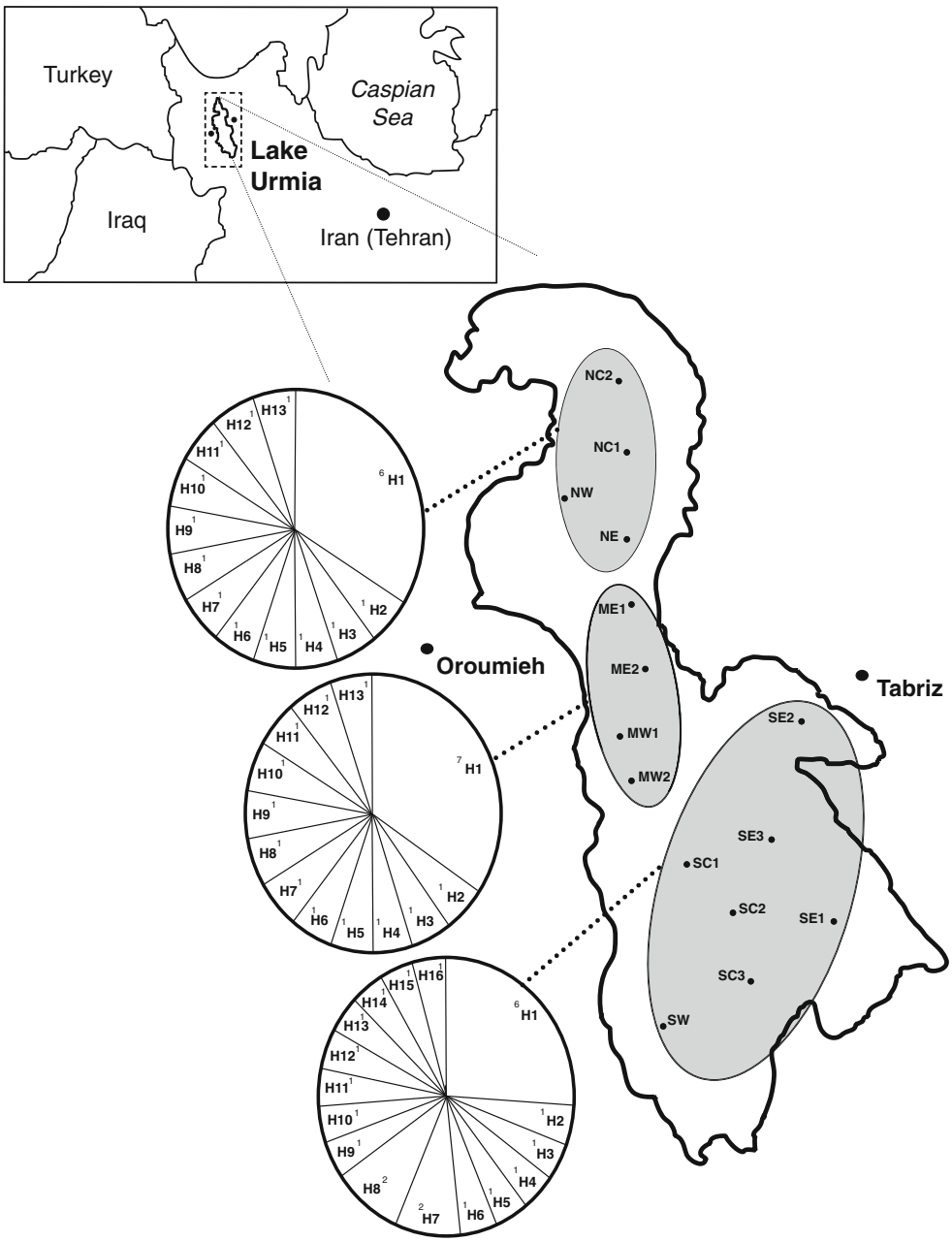

Fig. 4 Haplotype distribution map of *A. urmiana* individuals for northern, middle and southern geographical regions. Frequency of each haplotype is reflected by relative size of each segment. Number of individuals indicated as superscripts for each haplotype

It has been postulated that high haplotype diversity associated with low nucleotide diversity is a possible sign of a genetic bottleneck, usually followed by recent population expansion (Alves et al. 2001). The marked haplotype diversity could be interpreted as an indication for an allopatric speciation process. This would demand that Lake Urmia must have been fragmented into several small lakes during an earlier stage of its history, which would allow the genetic differentiation of allopatric populations. When sea level came back to the present level, this fragmentation stopped and the existing haplotypes became mixed all over the lake. Indeed, the lake has experienced substantial changes in ecological conditions such as water level, salinity and temperature (Kelts and Shahrabi 1986; Djamali et al. 2008). A significant negative value for neutrality, and the occurrence of star-shaped haplotype networks suggests a recent population expansion of *A. urmiana* in the lake (Chenoweth and Hughes 2003), which would agree with our assumption. The potential of bottleneck existing in *A. urmiana* could be assessed by studying samples dating back to 1990 and earlier.

Lack of a clear dispersal genetic structure among sampling regions is usually attributed to excessive gene flow, which is sufficient to prevent genetic drift or natural selection (Maggio et al. 2009). *A. urmiana* showed a random relationship of individuals resulting in a large panmictic population. Panmixia in marine environments is due to an obvious lack of physical barriers to gene flow (Cowen et al.

Table 4 Genetic variation among *A. urmiana* populations according to ISSR markers $(AG)_8C$, $(AG)_8YT$, $(GA)_9T$, $(TG)_8G$, and $(AC)_8C$. *N* Number of individuals examined, *NPL* number of polymorphic loci, *PPL* percentage of polymorphic loci, *Hj* Nei's gene diversity (expected heterozygosity)

Location	*N*	(*NPL*)	*PPL* (%)	(*Hj*) ± SD
NC1	10	122	70.9	0.26±0.014
NC2	10	94	54.7	0.22±0.017
NE	10	104	60.5	0.22±0.015
NW	10	116	67.4	0.21±0.014
ME1	10	109	63.4	0.24±0.015
ME2	10	107	62.2	0.2±0.014
MW1	10	135	78.5	0.29±0.014
MW2	10	135	78.5	0.29±0.014
SE1	10	108	62.8	0.25±0.016
SE2	10	104	60.5	0.22±0.015
SE3	10	105	61	0.23±0.016
SC1	10	115	66.9	0.25±0.015
SC2	10	122	70.9	0.26±0.014
SC3	10	108	62.8	0.21±0.014
SW	10	114	66.3	0.26±0.016
Total (mean)	150	113.2	65.8	0.24±0.007

2000). Panmixia has been observed in the water bloom-forming cyanobacterium *Microcystis aeruginosa*, in which multilocus sequence analysis demonstrated five intraspecific lineages with a high frequency of recombination (Tanabe and Watanabe 2011). Absence of population structure with a high amount of gene flow was observed in the crustacean *Aristeus antennatus* and the marine fish *Thunnus obesus* (Chiang et al. 2006; Maggio et al. 2009).

Continental zooplankters such as *Artemia* appear to be panmictic due to passive transport of cysts through wind- and waterfowl-mediated dispersal (Green et al. 2005; Maniatsi et al. 2009). The high dispersal ability of *A. urmiana* via bird species, hydrological connectivity and anthropogenic influences (intentional and nonintentional) could also explain the panmixia hypothesis for *A. urmiana.* The short-distance dispersal of the fairy shrimp *Ranchipodopsis wolfi* by wind and the long-distance dispersal of *Daphnia lumholtzi* by migratory waterfowl are other examples in this regard (Brendonck and Riddoch 1999; Havel et al. 2000).

Salt concentrations can shape different ecological habitats in saline lakes (Hontoria and Amat 1992). However, Lake Urmia does not have significant differences in salinity between its northern and southern part (Agh 2007). This implies a lack of distinct ecological zones in the lake. Our genetic data agree with the idea that a strong ecological differentiation does not exist in Lake Urmia. The evolutionary pattern of *A. urmiana* is consistent with morphological variation investigated by Asem et al. (2007) indicating random distribution of morphotypes across the lake.

Table 5 Estimated posterior probabilities and delta *K* for each *K* partition

K	Posterior probabilities (Pp)	Delta *K* (*DK*)
1	2.098174	12860.9
2	2.002665	308.7586
3	19.02741	4.984388
4	25.08456	0.05607
5	35.32541	0.08571
6	229.7114	1.237647
7	80.87119	0.823651
8	96.37787	0.188944
9	115.4721	0.340862
10	45.23025	3.497659
11	177.4118	0.63626
12	119.7991	0.235144
13	90.53564	3.124294
14	707.6359	0.421149
15	245.3703	0.374414
16	172.037	2.328511
17	1332.544	0.472307
18	695.4783	0.077544
19	194.8597	0.809865
20	93.64482	2.49325

Population genetic differentiation

Genomic analyses by ISSR fingerprinting have proven to be reliable and advantageous in their cost-benefit outcome for detection of DNA polymorphisms in different taxa (Hundsdörfer et al. 2005; Sarwat 2012). The ISSR technique had been used by Hou et al. (2006) to examine ten parthenogenetic *Artemia* strains from China. A similar population diversity was found in these Chinese populations (H=0.29) as in our study. The F_{ST} parameter can vary between 0 (absence of genetic divergence) and 1 (fixation of alleles) within the population. High F_{ST} values are indicative of high genetic differentiation in populations (Ruiz et al. 2011)

Multiple clades of *A. urmiana* individuals have been revealed by means of applying two clustering methods (UPGMA and STRUCTURE). The UPGMA dendrogram and PCA tree topology are congruent with morphological patterns indicating the presence of various genetic lineages of *A. urmiana* with random distribution of individuals in the lake (Asem et al. 2007).

Suggested conservation measures

Desiccation of Lake Urmia is progressing severely due to ecological changes and human activities. If these influences continue, they will cause a loss of genetic diversity as well

as a dwindling of food resources for many organisms. Although *Artemia* is a rather hardy organism, the almost endemic *A. urmiana* population appears to be doomed to extinction unless effective conservation measures enabling a sustainable exploitation of *Artemia* in the future are developed and implemented. In the present drought conditions, annual population density assessment of *A. urmiana* is urgently required to estimate the size of the actual cyst bank throughout Lake Urmia. Establishment of ex situ collections (e.g. a live cyst bank) from different geographical locations within Lake Urmia could be a first step towards conserving the diversity of genetic resources of *A. urmiana*.

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