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Molecular systematics of bisexual *Artemia* populations

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Abstract

To help resolve phylogenetic relationships among bisexual *Artemia* populations, phylogenetic analysis was conducted using DNA sequences from the nuclear DNA internal transcribed spacer 1 (ITS-1) and portions of the mitochondrial genome corresponding to the cytochrome oxidase I (COI). DNA sequences were generated for nine bisexual *Artemia* populations living in different regions of the world. Phylogenetic trees based on ITS-1 and COI sequences indicated that bisexual *Artemia* populations consist of four groups. The bisexual *Artemia* populations from Tibet and Kazakstan always clustered with *Artemia urmiana* in the same group; there is small sequence divergence and genetic distance among them. Therefore, we deduced that bisexual *Artemia* populations from Tibet and Kazakstan may belong to the *A. urmiana* group. Our study did not support that bisexual *Artemia* populations from Tibet are a new, separate species *A. tibetiana*. We also found that *A. sinica* and *A. urmiana* have a small genetic distance. Based upon these findings, we conclude that *A. urmiana* may have played an important role in the evolution of *A. sinica*.

Keywords: bisexual *Artemia*, systematics, rDNA ITS-1, mtDNA COI

Introduction

The genus *Artemia* (Crustacea, Anostraca) comprises a complex of bisexual species and superspecies defined by the criterion of reproductive isolation, and of a large number of parthenogenetic populations (Sun, Song, Zhong, Zhang, Abatzopoulos & Chen 1999). Eight species have been documented in the scientific literature, including three species from the New World and five species from the Old World. The three species from the New World are *Artemia franciscana* (Kellogg 1906), widely distributed in North and South America, *Artemia persimilis* (Piccinelli & Prosdocimi 1968) from the saltworks of San Bartolomeo, Cagliari (Sardinia) or Hidalgo (Argentina) and *A. monica* (Lenz 1980), probably restricted to Mono Lake, CA, USA. In the Old World, the following five bisexual species were found: *A. salina* Leach (1819) from Lymington, England and the Mediterranean basin (Triantaphyllidis, Griel, Abatzopoulos, Thomas & Peleman 1997); *Artemia urmiana* (Günther 1890) in lake Urmia, Iran; *Artemia sinica* (Cai 1989) from Yuncheng lake, China; *Artemia* sp.? from Kazakhstan (Pilla & Beardmore 1994) and *A. tibetiana* (Abatzopoulos, Zhang & Sorgeloos 1998) from the high plateaux of Tibet, China.

Zoologists have long been interested in the phylogenetic relationships and taxonomic status of bisexual *Artemia* populations living in different regions of the world. Large numbers of research projects have been carried out concerning the phylogenetic relationships of bisexual *Artemia* (Barigozzi, Badaracco, Plevani, Baratelli, Profeta, Ginelli & Meneveri 1984; Cruces, Wonenburger, Diaz-Guerra, Sebastiàn & Renart 1986; Badaracco, Baratelli, Ginelli, Meneveri, Plevani, Valsasnini & Barigozzi, 1987; Browne & Bowen, 1991; Landsberger, Cancelli, Carettoni, Barigozzi & Badaracco 1992; Perez, Valverde, Batuecas, Amat, Marco & Garesse 1994; Badaracco, Bellorini & Landsberger 1995), but the phylogenetics of the bisexual *Artemia* has not yet been resolved. Of particular interest is the taxonomic status of *A. sinica* and *A. tibetiana*. Sorgeloos (1991) suggested reconsidering the bisexual species categorization of *A. sinica*. By using isozyme electrophoresis and analysis of reproductive

isolation, Hou, Cai, Zou and Yang (1997) showed that *A. sinica* is different from *A. urmiana*, and 11 different bisexual *Artemia* strains from China belong to *A. sinica*. However, Abatzopoulos *et al.* (1998) described *A. tibetiana* as a new species from Tibet. Hou, Qu, Zou and Zheng (2003) suggested that the taxonomic status of bisexual *Artemia* populations living in Tibet needs to be further studied based on the analyses of cysts and nauplii biometrics, morphometry of adults, cytogenetic and allozyme analyses and cross-breeding/fertility tests.

Molecular genetic information has been increasingly used to construct the phylogenetic relationships of species and to distinguish among morphologically similar species. Of the many molecular approaches available today, analyses of ribosomal DNA and mitochondrial DNA are among the simplest. Ribosomal DNA sequences (16S rDNA) have been used to establish the phylogentic relationship in Crustacea including *Artemia* (Cunningham, Blackstone & Buss 1992) and mitochondrial DNA sequences (cytochrome oxidase I (COI) and cytochrome b) have also been used to determine bisexual and parthenogenetic *Artemia* strains (Perez *et al.* 1994). Mitochondrial DNA is also used to detect the relationship between *A. franciscana* and *A. persimilis* (Gajardo, Crespo, Triantafyllidis, Tzika, Baxevanis, Kappas & Abatzopoulos 2004). In the present study, we used nuclear DNA of internal transcribed spacer 1 (ITS-1), located between 18S and 5.8S genes, and mitochondrial COI gene as molecular markers to estimate the genetic relatedness of bisexual *Artemia* populations living in different regions of the world to provide more detailed information on the origin of *A. sinica*, and to determine the taxonomic status of the two bisexual *Artemia* strains from Tibet and Kazakstan (Fig. 1).

Materials and methods

Sample collection

All nine bisexual *Artemia* strains (Table 1) used in this study were provided by the World *Artemia* Reference Center, Belgium. The geographic origin of the nine *Artemia* populations investigated in this work, followed by their code abbreviations (used hereafter), is as follows: *A. franciscana*, Great Salt Lake, USA (GSL); *A. franciscana* San Francisco Bay, USA (SF); *A. persimilis*, Buenos Aires, Argentina (BA); *A. sinica*, YunChen, ShanXi, China (YC); *A. sinica*, Shui Quan Zi, Inner Mongolia, China (SQZ); *A. sinica*, Na Lin, Inner Mongolia China (NL); *A. urmiana*, Urmia Lake, Iran (UM); QXC: *Artemia* sp.? Qi Xiang Cuo, Tibet, China (QXC) and *Artemia* sp.? Kazakstan (KZ). The animals were cultured in seawater (25 °C) at the Laboratory of Life Science College at Liaoning Normal University following the procedures described in Chen, Hou, Lin, Xu & Wang (2005).

DNA isolation

Internal transcribed spacer 1 (ITS-1) sequence variations have been used previously in many animals for phylogenetic analysis (e.g. Vogler & De Salle 1994; Caporale, Beal, Roxby & Van Beneden 1997; Odorico & Miller 1997; Van Herwerden Blair & Agatsuma 1998; Hugall, Stanton & Moritz 1999; Harris & Crandall 2000; Chu, Li & Ho 2001; Tang, Zhou, Song, Yang & Dai 2003). However, *Artemia* nauplii were too small (the length was only 3 mm) to extract a sufficient amount of DNA from single individuals for subsequent molecular analysis. Therefore, the *Artemia* nauplii were hatched from the cysts of different strains according to the methods described by Sorgeloos, Lavens, Leger, Tackaert and Versichele (1986).

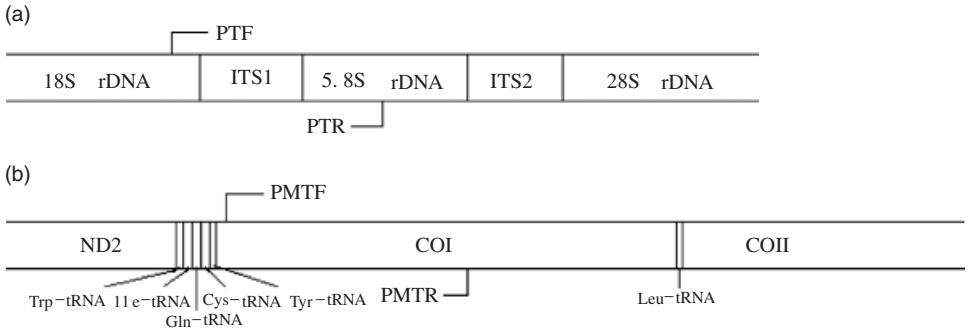

Figure 1 (a) Schematic presentation of the nuclear rDNA region used for amplification of the internal transcribed spacer (ITS) region of *Artemia* using PCR primers as indicated. (b) Schematic presentation of the mitochondrial DNA region used for the amplification of the COI gene of *Artemia* using PCR primers as indicated.

Table 1 *Artemia* populations (reproduction mode: bisexual) used in this study with collection locality and GenBank accession number

Species and populations	Collection location	Collection date	Chromosome numbers	Abbreviation	Genbank Accession No.	
					ITS-1	COI
A. franciscana	Great Salt Lake, USA	1996	42	GSL	DQ069924 DQ084191	DQ119645
	San Francisco Bay, USA	1996	42	SF	DQ069923 DQ084190	DQ119646
A. persimilis	Buenos Aires, Argentina	1996	44	BA	DQ069925 DQ084192	DQ119647
A. sinica	Yun Chen, Shan Xi, China	2004	42	YC	DQ069929 DQ084196	DQ119648
	Shui Quan Zi, Inner Mongolia, China	1993	42	SQZ	DQ069931 DQ084198	DQ119650
	NaLin, Inner Mongolia China	1993	42	NL	DQ069930 DQ084197	DQ119649
A. urmiana	Urmia Lake, Iran	2003	42	UM	DQ069926 DQ084193	DQ119651
A. sp.?	Qi Xiang Cuo, Tibet, China	2000	42	QXC	DQ069928 DQ084195	DQ119652
	Kazakstan	1996	42	KZ	DQ069927 DQ084194	DQ119653

A., *Artemia;* ITS-1, internal transcribed spacer 1; COI, cytochrome oxidase I.

Table 2 Primers used for amplifying the nuclear rDNA internal transcribed spacer 1 (ITS-1) region and mtDNA cytochrome oxidase I (COI) gene fragments

Region	Name	Sequence 5′-3′	Reference
ITS-1	PTF	GGAAGTAAAAGTCGTAACAAGG	Tang *et al.* (2003)
ITS-1	PTR	AGCCAAGTGATCCATCCTCCAG	Present study
COI	PMTF	GGTCAACAAATCATAAGATATTGG	Folmer *et al.* (1994)
COI	PMTR	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* (1994)

The total DNA was extracted from 200 nauplii selected randomly from culture plate after hatching. The nauplii were ground in an ice field and incubated at 55 °C for 4 h in a 500 μL TE buffer (10 mM L^{-1} Tris-HCl, 1 mM L^{-1} EDTA) containing 50 μL 10% SDS and 10 μL 20 mg mL^{-1} proteinase K. Total DNA was isolated using a standard phenol–chloroform extraction protocol according to Barigozzi *et al.* (1984) and Sun, Zhong, Song, Zhang & Chen (1999). After isolation, DNA was stored in 200 μL TE solution.

PCR and sequencing

The PCR primers for amplification of the ITS region were referred to as PTF and PTR for forward and reverse PCR primers (Table 2). The forward primer was adopted from Tang *et al.* (2003), and the reverse primer was designed based on the 5.8S rDNA sequence of *Artemia* salina (Ursi, Vandenberghe & De Wachter 1982; Vaughn, Sperbeck & Hughes 1984). In both cases, the primers were designed to be within the conserved flanking sequences of the 18S and 5.8S genes. The forward primer (PTF) was located in the 18S (corresponding to sites 5419 to 5441 in the reference sequence L07948), and the reverse primer (PTR) in the 5.8S rDNA (corresponding to sites 186–207 in the reference sequence M33097, Fig. 2*a*).

PCR amplification of the ITS-1 region was performed using the forward and reverse primers in a 25 μL reaction volume containing 2.5 μL 10 × buffer, 1.5 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, 1 μL of each of the primers at 10 μM, 5–10 ng DNA template and 0.5 μL of *Taq* DNA polymerase (Takara, Shiga, Japan) and water to 25 μL. An initial 3 min denaturation at 95 °C was followed by 35 cycles of 95 °C denaturation for 60 s, 50.5 °C annealing for 50 s, 72 °C extension for 1.5 min.

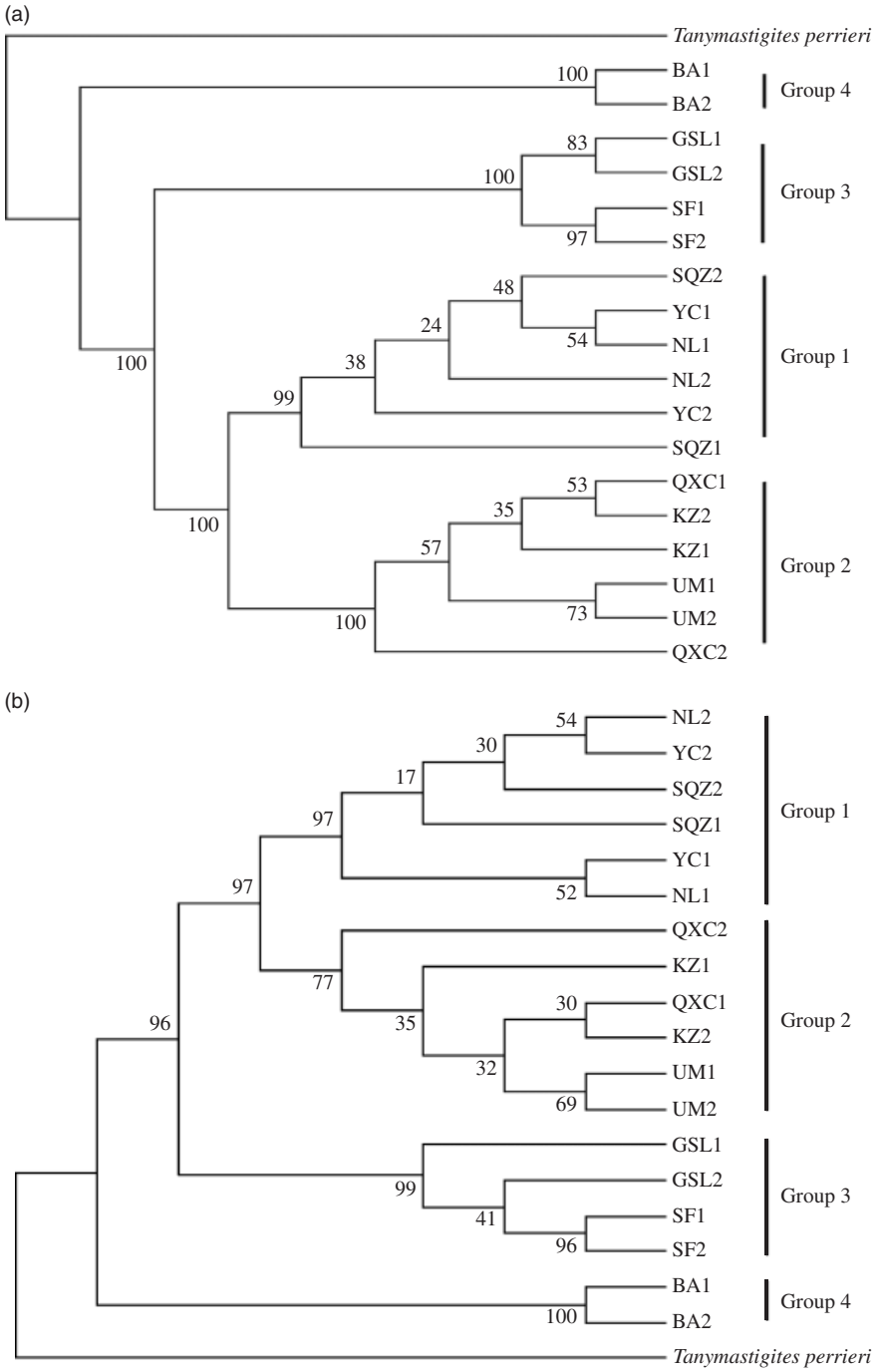

Figure 2 Phylogenetic tree conducted by the UPGMA (a) and NJ (b) analysis based on the internal transcribed spacer 1 (ITS-1) regions of nine different bisexual *Artemia* populations. Thousand replications of bootstrapping. GSL: *Artemia franciscana*, Great Salt Lake, USA; SF: *A. franciscana* San Francisco Bay, USA; BA: *A. persimilis*, Buenos Aires, Argentina; YC: *Artemia sinica*, Yun Chen, Shan Xi, China; SQZ: *A. sinica*, Shui Quan Zi, Inner Mongolia, China; NL: *A. sinica*, Na Lin, Inner Mongolia, China; UM: *Artemia urmiana*, Urmia Lake, Iran; QXC: *Artemia* sp.? Qi Xiang Cuo, Tibet, China KZ: *Artemia* sp.? Kazakstan. The number after each abbreviation means different clones of the same popultions. Group 1: East Asia bisexual group (SQZ, NL and YC); Group 2: Middle Asia bisexual group (KZ, UM and QXC); Group 3: North America bisexual group (GSL and SF) and Group 4: South America bisexual groups (BA).

Amplification cycles were followed by a final 7 min extension at 72 °C. Mitochondrial COI segments were amplified by the primers PMTF and PMTR (Table 2 and Fig. 2*b*) under similar conditions as used for the amplification of ITS region with modifications. Specifically, 10–45 ng template DNA was used, and the cycling conditions were as follows: 95 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s. All PCR amplifications were carried out with a GeneAmp 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The size and quality of PCR products were determined on 1.5% agarose gels. PCR products were purified with the Wizard PCR Prep DNA Kit (Takara) and then cloned into TA cloning vector (pMD 18-T Vector), before automated sequencing using the MegaBace 1000 DNA sequencer. Internal transcribed spacer 1 (ITS-1) and COI fragments were sequenced from both strands to obtain accurate sequences. Two clones were sequenced from each PCR to confirm sequences. All sequences were deposited in GenBank using a web-based browser to NCBI (http://www.ncbi.nlm.nih.gov).

Data analysis

Sequences were aligned using CLUSTALW and per cent divergences were calculated using the computer program MEGALIGN (DNASTAR, Madison, WI, USA). Genetic distances were estimated using the 'Molecular Evolutionary Genetic Analysis' (MEGA 3) software package based on the Kimura-2 parameter model (transition and transversion). UPMGA and Neighbour-Joining (NJ) methods in MEGA3 were used to construct phylogentic trees based on ITS-1 region and COI gene sequences. Statistical significance of groups within inferred trees was evaluated using the bootstrap method with 1000 replications. The ITS-1 of *Tanymastigites perrieri* (Branchiopoda, Anostraca, Branchipodidae, AJ307679) and mitochondrial COI gene fragment of *Eiocheir japoncia* (Malacostraca; Eumalacostraca; Decapoda; AF105246) were used as outgroup sequences respectively. Confidence levels for the phylogenetic trees were assessed by 1000 bootstrapping replications in MEGA3 under kimura's two-parameter model (below diagonal) of Aremidae ITS-1 regions.

Results

Analysis of ITS-1 region data set

Nuclear ITS1 rDNA of nine *Artemina* populations were amplified by PCR and followed by DNA sequencing. All sequences have been deposited at GenBank with accession numbers as listed in Table 1. The lengths of ITS-1 in the *Artemia* species, except *A. persimilis* (BA), are longer than all gene sequences of ITS-1 that have been reported, ranging from 764 bp in the *A. persimilis* (BA) to 924 bp in the *A. franciscana* (GSL and SF) (Genbank Accession No. DQ069923, DQ069931, DQ084190 and DQ084198). So the lengths of ITS-1 regions are highly variable in Artemiade, but little length polymorphism was found in the same species of *A. sinica* (903–906 bp) and among six sequences (897 bp) in group 2. The average sequence divergence between the three populations of *A. sinica* is only 0.9%. Our results are consistent with those found for crustaceans where ITS-1 was found to be highly polymorphic in length across species, but highly conserved within the same group (Chu *et al.* 2001). The genetic distance and divergence of ITS-1 are displayed in Table 3. Sequences of ITS-1 were aligned using CLUXTAL W (EMBL Accession No. ALIGN.000903). The aligned 1069 of ITS-1 regions includes 326 sites as variable, and 308 sites as parsimony informative sites, suggesting that the ITS-1 region is highly variable. Phylogentic trees were constructed by using UPMGA and NJ methods based on the ITS-1 region (Fig. 2). The topologies of the phylogenetic trees of *Artemia* populations constructed using ITS-1 are very similar, indicating that they support each other. Nine *Artemia* populations are all divided into four major groups. Group 1: East Asia bisexual group (SQZ, NL and YC); Group 2: Middle Asia bisexual group (KZ, UM and QXC); Group 3: North America bisexual group (GSL and SF) and Group 4: South America bisexual groups (BA). All the bootstrap values for the branches separating the four groups are high (>77), but the bootstrap values within every branch separating different population are not consistent (17–97). However, the low bootstrap values within every branch in each group did not affect the relationship of the four groups. This may be explained by the reason that intrapopulational variations in some populations are greater than variations among populations of the same species. For example, the intrapopulational variations in ITS-1 of Yun Cheng population and Na Lin population are 0.8% and 1.0%, respectively, whereas the sequences divergences between the two popualtions are 0.3–1.2%. These results show that the sequence variations in ITS-1 regions of *Artemia* are not a potential molecular marker for phylogenetic studies at the population level, but is very effective at the level of species. This view is identical to the result from Asian mitten crabs (Tang *et al.* 2003).

Table 3 Per cent divergence (above diagonal) and genetic distances under kimura's 2-parameter model (below diagonal) of *Aremidae* internal transcribed spacer 1 (ITS-1) regions

	GSL1	GSL2	SF1	SF2	UM1	UM2	KZ1	KZ2	QXC1	QXC2	NL1	NL2	YC1	YC2	SQZ1	SQZ2	BA1	BA2
GSL1	***	0.2	0.5	0.5	14.0	14.0	13.8	13.7	14.1	14.3	14.4	14.7	14.1	14.4	14.7	14.7	51.1	51.8
GSL2	0.00	***	0.8	0.8	14.1	14.1	14.0	13.8	14.2	14.4	14.5	14.8	14.3	14.6	14.8	14.8	51.2	51.9
SF1	0.01	0.01	***	0.0	14.5	14.5	14.4	14.2	14.6	14.8	14.9	15.2	14.7	15.0	15.2	15.2	51.7	52.4
SF2	0.01	0.01	0.00	***	14.5	14.5	14.4	14.2	14.6	14.8	14.9	15.2	14.7	15.0	15.2	15.2	51.7	52.4
UM1	0.14	0.14	0.15	015	***	0.0	0.2	0.2	0.2	0.6	2.9	2.7	2.5	2.6	2.9	3.1	48.0	48.0
UM2	0.14	0.14	0.15	0.15	0.00	***	0.2	0.2	0.2	0.6	2.9	2.7	2.5	2.6	2.9	3.1	48.0	48.0
KZ1	0.14	0.14	0.14	0.14	0.00	0.00	***	0.2	0.6	0.6	2.9	2.7	2.5	2.6	2.9	3.1	48.0	48.0
KZ2	0.14	0.14	0.14	0.14	0.00	0.00	0.00	***	0.2	0.6	2.9	2.7	2.5	2.6	2.9	3.1	48.0	48.0
QXC1	0.14	0.14	0.15	0.15	0.00	0.00	0.00	0.00	***	0.3	2.9	2.8	2.5	2.6	2.9	3.1	48.8	48.8
QXC2	0.14	0.14	0.15	0.15	0.01	0.01	0.01	0.01	0.00	***	3.0	2.9	2.6	2.7	3.0	3.2	48.4	48.4
NL1	0.14	0.15	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	***	1.0	0.3	1.2	1.0	0.9	49.6	49.6
NL2	0.15	0.15	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	0.01	***	0.9	0.8	1.1	1.2	50.4	50.4
YC1	0.14	0.14	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	0.00	0.01	***	0.8	0.7	0.6	49.4	49.4
YC2	0.14	0.15	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01	0.01	***	1.0	1.1	49.7	49.7
SQZ1	0.15	0.15	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	***	1.0	49.5	49.5
SQZ2	0.15	0.15	0.15	0.15	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	***	49.4	49.4
BA1	0.51	0.51	0.52	0.52	0.48	0.48	0.48	0.48	0.49	0.48	0.50	0.50	0.50	0.50	0.49	0.49	***	0.1
BA2	0.52	0.52	0.52	0.52	0.48	0.48	0.48	0.48	0.49	0.48	0.50	0.50	0.50	0.50	0.49	0.49	0.00	***

BA, Buenos Aires; GSL, Great Salt Lake; KZ, Kazakstan; NL, Na Lin; QXC, Qi Xiang Cuo; SF, San Francisco Bay; SQZ, Shui Quan Zi; UM, Urmia Lake and YC, Yun Chen.

Table 4 Per cent divergences (above diagonal) and Genetic distances under kimura's 2-parameter model (below diagonal) of *Aremida* cytochrome oxidase I (COI) gene fragments

	GSL	SF	UM	KZ	QXC	YC	NL	SQZ	BA
GSL	***	1.9	18.9	19.1	17.2	18.2	18.6	18.4	22.3
SF	0.02	***	18.9	19.3	17.7	16.8	17.2	17.0	22.5
UM	0.19	0.19	***	2.0	6.1	17.9	18.5	17.9	23.6
KZ	0.19	0.19	0.02	***	6.8	16.9	17.5	16.9	22.3
QXC	0.17	0.18	0.06	0.07	***	16.9	16.2	15.6	22.1
YC	0.17	0.17	0.17	0.17	0.17	***	1.4	1.2	17.6
NL	0.18	0.17	0.17	0.17	0.16	0.01	***	0.5	18.4
SQZ	0.18	0.17	0.17	0.17	0.16	0.01	0.00	***	17.8
BA	0.22	0.22	0.22	0.22	0.21	0.17	0.18	0.18	***

BA, Buenos Aires; GSL, Great Salt Lake; KZ, Kazakstan; NL, Na Lin; QXC, Qi Xiang Cuo; SF, San Francisco Bay; SQZ, Shui Quan Zi; UM, Urmia Lake and YC, Yun Chen.

Cytochrome oxidase subunit I data set

The nucleotide sequences of COI gene fragments in each population were based on results from both strands. The aligned 658 bp of COI gene fragments of the nine *Artemia* populations consists of (EMBL Accession No. ALIGN.000911) 198 variable sites and 149 parsimony informative sites. Pairwise sequence divergences and genetic distances of COI gene fragments are summarized in Table 4. The degree of sequence divergence of COI fragment among different *Artemia* groups is high, ranging from 15.6% to 23.6%, which is identical to the data (15–21%) of the whole COI gene among two *Artemia* bisexual species and parthenogenetic species (Perez *et al.* 1994). So we can deduce that different groups should be at the level of the species base on the sequence divergence of COI gene fragments. Phylogentic trees were constructed by using UPMGA and NJ methods based on COI gene fragments (Fig. 3). The topology of UPMGA and NJ trees of *Artemia* species (Figs 3*a* and *b*) inferred from mitochondrial COI sequences is similar to those of Fig. 2. Although the bootstrap value supporting the relationship between groups 1, and 2 with group 3 is not high (49), the bootstrap values are very high (100, 100, 100) within each group. Thus, it has no impact on the relationship of each group within itself. The low bootstrap value (49) between groups 1 and 2 with

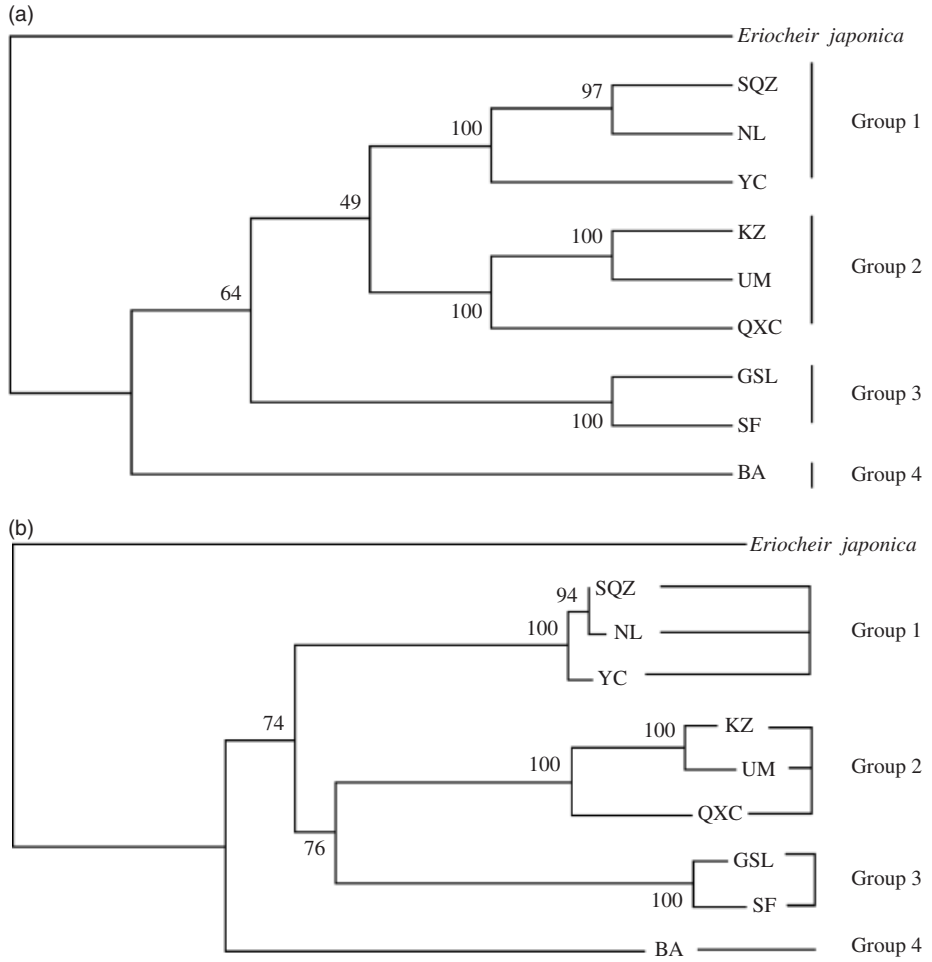

Figure 3 Phylogenetic tree conducted by the UPGMA (a) and NJ (b) analysis based on the COI gene fragments of nine different bisexual *Artemia* populations. Thousand replications of bootstrapping. GSL: *Artemia franciscana*, Great Salt Lake, USA; SF: *A. franciscana* San Francisco Bay, USA; BA: *A. persimilis*, Buenos Aires, Argentina; YC: *Artemia sinica*, Yun Chen, Shan Xi, China; SQZ: *A. sinica*, shui quanzi, Inner Mongolia, China; NL: *A. sinica*, NaLin, Inner Mongolia China; UM: *Artemia urmiana*, Urmia Lake, Iran; QXC: *Artemia* sp.? Qi Xiang Cuo,Tibet,China KZ: *Artemia* sp.? Kazakstan. Group 1: East Asia bisexual group (SQZ, NL and YC); Group 2: Middle Asia bisexual group (KZ, UM and QXC); Group 3: North America bisexual group (GSL and SF) and Group 4: South America bisexual groups (BA).

group 3 can be explained by the fact that the sequence length of COI fragments that we used was limited. The whole sequence of cytochrome oxidase subunit I can detect more exactly the relationship of bisexual and parthenogenetic *Artemia* strains to obtain a satisfactory result (Perez *et al*. 1994).

Discussion

Badaracco *et al*. (1987) assumed that *A. salina*, *A. persimilis* and an unknown *Artemia* strain identified as *A. sinica* by Cai (1989) could belong to a primitive group of species from which *A. franciscana* was derived. However, compared with *A. sinica*, *A. urmiana* and *A. franciscana*, *A. persimilis* seems to be closer to the ancestral group of species that evolved in the Mediterranean basin based upon the four trees in Figs 2 and 3. This result is similar to the conclusion that *A. persimilis* could belong to a primitive group of species from which *A. sinica* and *A. urmiana* and *A. franciscana* originated at different times (Badaracco *et al*. 1995). This hypothesis is supported by the fact that *A. persimilis* had, at least in the recent past, a wider geographical distribution (Barigozzi, 1989). Additionally, the per cent divergences of *A. persimilis*

with *A. sinica*, *A. urmiana* and *A. franciscana* in Tables 3 and 4 are especially high (48.0–52.4% in ITS-1 and 17.6–23.6% in COI genes) compared with other data (0.3–15.2% in ITS-1 and 1.2–19.3% in COI genes), especially in genetic distance. All data reveal that *A. persimilis* have a high divergence with *A. sinica*, *A. urmiana* and *A. franciscana*. From the results of Pilla and Beardmore (1994), no significant reproductive isolation was found among Eastern Old World populations. However, the dendrograms of cluster analysis based on allozyme electrophoresis in bisexual strains of *Artemia* showed that *A. sinica* and *A. urmiana* are two different groups (Hou *et al*. 1997), and the data from RAPD also support the same conclusion that *A. sinica* does not belong to the species of *A. urmiana* or any other bisexual species (Sun, Song, Zhong, Zhang, Abatzopoulos & Chen 1999). We obtained the same conclusion on this point. The average genetic distance between *A. sinica* and *A. urmiana* is 0.03 using ITS-1 sequences and 0.17 using COI sequences, suggesting that they have a very close relationship, confirming results obtained with allozymes (Hou *et al*. 1997) and AFLP analyses (Sun *et al*. 1999) in bisexual strains of *Artemia*. *Artemia persimilis* had a wider geographical distribution (Barigozzi 1989) and the habitat of *A. urmiana* just exist in the diffuse pathway of *Artemia* from Europe to Asia, suggesting that *A. urmiana* played an important role in the evolution of *A. sinica*.

The phylogentic trees in the present study all show the Chinese populations, except the bisexual *Artemia* population from Tibet (QXC), and are assembled together in the *A. sinica* clade. Meanwhile, the genetic distances between *A. sinica* and QXC (0.03 in ITS-1 and 0.16–0.17 in COI gene) are both at the interspecific level. So it is clear that the bisexual *Artemia* population from Tibet does not belong to *A. sinica*. Our results support the view that the Tibet and inner Mongolia groups can be clearly divided into two group (Hou *et al*. 2003). From the results of biometrics of cysts and nauplii, morphometry of adults, cytogenetics, allozyme and DNA analyses and cross-breeding/fertility tests, the species *A. tibetiana* was first reported by Abatzopoulos *et al.* (1998), and was supported by the results of analysis of AFLP markers (Sun, Song, *et al*. 1999). However, our results show that the bisexual *Artemia* in Tibet, Kazakstan and Iran cluster very closely in all four dendrograms, and they have a very small genetic distance (0.00–0.01 in ITS-1 and 0.02–0.07 in COI). Moreover, the sequence divergences of the nuclear DNA ITS-1 region between *A. urmiana* and the bisexual *Artemia* from Tibet and Kazakstan are very low (0.2–0.6%). This level of sequences divergence are even smaller than that observed within the species of *A. sinica* (0.6% to 3.0%), suggesting a difference within the range of the intraspecific level. The same conclusion can also be obtained from the COI data set. Although ITS1 have been reported to have a high degree of length polymorphism, the lengths of the bisexual *Artemia* from Tibet, Kazakstan and Iran are identical to each other (897 bp), but exhibit a large difference from other groups (903–906 bp in group 1, 924–925 bp in group 3 and 764 bp in group 4). Based on all the above data, we can deduce that bisexual *Artemia* in Tibet and Kazakstan belong to the species *A. urmiana*. This result is similar to the study of molecular phylogentic analysis on the relationship of 11 bisexual *Artemia* populations using the *abdA* gene (L. Hou, Unpubl. data). Of the cross-breeding/fertility tests in the Abatzopoulos experiments, the bisexual *Artemia* in Tibet and *A. urmiana* species have a proportion (40–60%) of interspecific crosses that were fertile and not significantly lower in all cases, and even had F2 and F3 generations, which demonstrates that there is no strict reproductive isolation between the bisexual *Artemia* populations from Tibet and *A. urmiana*. The reason for the hybrid breakdown at F2 and F3 generations may be due to lack of a proper environment and as a result, the reproductive system of *Artemia* cannot develop properly and therefore alters the proportion of successful inter-specific crosses. The bisexual *Artemia* from Tibet and *A. sinica* are geographically related by a common location in China, but they belong to different *Artemia* species. This may due to the special environment in Tibet, and the fact that the habitat of bisexual *Artemia* in Tibet and Iran are similar, resulting in a large gene flow between the bisexual *Artemia* between Tibet and Iran. Thus, our results, using analysis of DNA sequences of ITS-1 region and COI gene, do not support the view that the bisexual *Artemia* from Tibet is a new *Artemia* species (*A. tibetiana*).

Analysis of DNA sequences is extensively used in studies of systematics and evolution of *Artemia*. The molecular markers of ITS-1 and COI were effective in detecting the genetic relationships among different bisexual *Artemia* populations at the level of species in the present study. ITS1 is an intron that does not restrict to code protein has a high evolutionary speed, so compared with COI, ITS1 represents a more informative molecular marker and exhibits a high degree of high length polymorphism among different groups. However, we obtained a similar result from

the molecular markers of ITS-1 and COI. Based on the data presented above, we consider that *Artemia* from Tibet belongs to *A. urmiana*, and *A. urmiana* must play an important role in the evolution of *A. sinica*.

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