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Genetic variation and evolutionary origins of parthenogenetic *Artemia* (Crustacea: Anostraca) with different ploidies

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Using two nuclear (*ITS1* and *Na⁺/K⁺ ATPase*) and three mitochondrial (*COI*, *16S* and *12S*) markers, we determined the genetic variation and evolutionary relationship of parthenogenetic and bisexual *Artemia*. Our analyses revealed that mitochondrial genes had higher genetic variation than nuclear genes and that the *16S* showed more variety than the other mitochondrial genes in parthenogenetic populations. Triploid parthenogens showed lower genetic variation than diploid ones, whereas the tetra- and pentaploids had greater genetic distance than diploid parthenogens. No shared haplotype was found between individuals of parthenogenetic populations and Asian bisexual species with the exception of *Na⁺/K⁺ ATPase* (*Artemia tibetiana*). Only mitochondrial markers can demonstrate phylogenetic relationships, and showed that the parthenogenetic *Artemia* is a polyphyletic group in which the diploid lineages share a common ancestor with *Artemia urmiana* while tetraploids are closely related to *Artemia sinica*. The triploid and pentaploid linages are likely to be directly derived from diploid and tetraploid parthenogens, respectively. Subsequently, west Asia is origin for di-/triploids, and tetra-/pentaploids rose from East Asia.

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Introduction

The brine shrimp *Artemia* (Crustacea: Anostraca), a cosmopolitan halophilic crustacean, is the most conspicuous inhabitant of hypersaline lakes, salt ponds, salt lagoons and man-made saltworks. The genus *Artemia* consists of seven bisexual species and a large number of parthenogenetic populations (Van Stappen 2002; Eimanifar *et al.* 2014). Four bisexual species live in the Old World: *Artemia salina* (Linnaeus, 1758) (Mediterranean basin), *Artemia urmiana* Günther, 1899 (Lake Urmia, Iran and Crimean salt lakes, Russia), *Artemia sinica* Cai, 1989 (China and Mongolia) and *Artemia tibetiana* Abatzopoulos *et al.*, 1998 (Qinghai–Tibetan Plateau, China). The other three bisexual species are native to the New World, namely *Artemia monica* Verrill, 1869 (Mono Lake, USA), *Artemia franciscana* Kellogg, 1906 (North America, Central America and South America) and *Artemia persimilis* Piccinelli and Prosdocimi, 1968 (Argentina and Chile). Obligate parthenogenetic *Artemia* consists of di-, tri-, tetra-, penta- and heteroploid populations and populations with mixtures of different ploidies (Sun *et al.* 1999; Abatzopoulos *et al.* 2002, 2003). Cyclical parthenogenesis has never been evidenced in *Artemia* (Zhang & King 1992).

Parthenogenetic animals are thought to have a narrow genetic structure and broader geographical dispersion as compared with bisexual species (Johnson 2006; Muñoz *et al.*

2010; Eimanifar *et al.* 2014). That is also true for parthenogenetic *Artemia* which are found in many inland salt lakes, lagoons and coastal salterns in Eurasia, Africa and Australia (Van Stappen 2002; Eimanifar *et al.* 2014). Muñoz *et al.* (2010) have already revealed that diploid parthenogenetic *Artemia* have extensive geographical distributions with extremely low genetic variation. Diploid parthenogenetic *Artemia* are automictic and have a tendency to be polyclonal, while polyploids are apomictic producing identical (monoclonal) generations (Barigozzi 1974; Browne & Bowen 1991; Triantaphyllidis *et al.* 1998; Abatzopoulos *et al.* 2002, 2003). Therefore, it could be expected that automictic populations display higher genetic variability than apomictic monoclonal ones. This supposition was not supported strongly by data documented to date. Some studies have evidenced triploid and tetraploid parthenogenetic *Artemia* had lower genetic variation than diploids (Zhang & King 1992; Maniatsi *et al.* 2011), but the pentaploids from Dongfanghong Saltern (China) had higher genetic variability in comparison with its sympatric diploid and other allopatric diploids and tetraploids (Zhang & King 1992). Zhang & King (1992) proposed that the genetic differences among allopatric di- and polyploidy populations could be attributed to the interaction among environmental parameters, ploidy levels and origins, while the dissimilarities between sympatric diploids and polyploids could result from the influence of ecological isolation.

Four general hypotheses have been proposed for the origin of parthenogenesis: (i) spontaneously, (ii) interspecific hybridization, (iii) as a result of incomplete reproductive isolation between sexuals and parthenogens, and (iv) via infection by microorganisms such as *Wolbachia pipientis* and *Xiphinematobacter* spp. (Maniatsi *et al.* 2010). The origin of parthenogenesis has been puzzling in the genus *Artemia* although Maniatsi *et al.* (2010) have already proved that the *Wolbachia* did not play substantial role in the induction of parthenogenesis in *Artemia*. An earlier phylogenetic study based on the diversity of allozymes demonstrated that parthenogenetic *Artemia* was a monophyletic group originated from *A. salina* (see Abreu-Grobois & Beardmore 1982). This study also indicated that tri- and tetraploids were originated from diploids while pentaploids came from tetraploids. In the recent years, the maternal origins of diploid and triploid parthenogenetic lineages have been suggested to be from *A. urmiana* and an unknown bisexual *Artemia* population from Kazakhstan (Muñoz *et al.* 2010; Maniatsi *et al.* 2011; Maccari *et al.* 2013), while tetraploids are believed to have arisen from *A. sinica* (Maniatsi *et al.* 2011). No phylogenetic analysis has been documented for pentaploid *Artemia*.

Here we compare the genetic variation of nuclear and mitochondrial lineages among different ploidy degrees of parthenogenetic populations of *Artemia*, as well as investigate their possible phylogenetic relationships and evolutionary origins.

Materials and methods

Sampling strategy

Artemia cyst samples were obtained from five geographically isolated salt lakes and one coastal saltern from China (Table 1). Our sampling includes all reported ploidy levels known for parthenogenetic *Artemia*, that is di-, tri-, tetra- and pentaploidy. The cysts were hatched in 0.2 μm filtered seawater (salinity 32–33 g/L) under optimal laboratory conditions (Sorgeloos *et al.* 1986). Instar I nauplii were raised according to the standardized laboratory method of Coutteau *et al.* (1992). The Salinity was adjusted to 70 g/L by adding sea salt to seawater, temperature and photoperiod were 25 $\pm$ 1 °C and 12-h L/12-h D, respectively. A mixed diet of *Dunaliella salina* and LANSY ZM (INVE (Thailand) Ltd., Wachirabarami, Phichit, Thailand) was supplied following the feeding schedule given by Triantaphyllidis *et al.* (1995). When sexes can be determined, each female was moved to a new container and cultured separately to establish cloned cultures. All examined populations had been confirmed to be parthenogenetic. The ploidy level of each clone was then determined using newly hatched nauplii by conventional chromosome preparation (Abatzopoulos *et al.* 1986; Petrović 1991). Diapause cysts were collected from each clone and stored at −25 °C for subsequent phylogenetic analysis.

Other available sequences were retrieved from GenBank including parthenogenetic *Artemia* from Eurasia and Africa with known ploidy levels and Asian bisexual species. The detailed information for all samples analysed in the present study is shown in Table 1. The bisexual population from Kazakhstan was not included in this study, as its taxonomic status is unclear (see discussion).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from decapsulated cysts following the Chelex® 100 Resin method (Bio-Rad Laboratories, USA). Single decapsulated cysts were crushed using a sterilized pipette tip, incubated for 2.5–3 h at 60 °C, vortexed every 30 min and finally incubated for 10 min at 80 °C. Then the tubes were centrifuged at 12 300 ***g*** for 1 min and the supernatant phase was directly used for PCR (Montero-Pau *et al.* 2008; Eimanifar & Wink 2013). All extracted DNA were stored at −80 °C for further genetic experiments.

Two fragments of nuclear genes (*ITS1* and *Na^+/K^+ ATPase*) and three mitochondrial genes (*COI*, *16S* and *12S*) were amplified. PCR was carried out on a total volume of 20 μL containing 8 μL of ddH_2O, 10 μL Taq polymerase

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Table 1 Sampling information and labcodes of *Artemia* specimens used in the present study, including also the information of data downloaded from GenBank

Locality	Reproduction mode/Ploidy	Number of specimens	Lab codes	GenBank accession numbers
Barkol Lake, China	P/2n	5	BRK1-5 (2n)	*ITS1*: KU183800-04[a]
		5	BRK1-5 (2n)	*Na+/K+ ATPase:* KU183900-04[a]
		5	BRK1-5 (2n)	*COI*: KU183949-53[a]
		3	BRK1-3 (2n)	*16S*: KU183848-50[a]
		3	BRK1-3 (2n)	*12S*: KU183874-76[a]
	P/4n	5	BRK1-5 (4n)	*ITS1*: KU183805-09[a]
		5	BRK1-5 (4n)	*Na+/K+ ATPase:* KU183905-09[a]
		4	BRK1-4 (4n)	*COI*: KU183954-57[a]
		2	BRK1-2 (4n)	*16S*: KU183851-52[a]
		2	BRK1-2 (4n)	*12S*: KU183877-78[a]
	P/5n	5	BRK1-5 (5n)	*ITS1*: KU183810-14[a]
		5	BRK1-5 (5n)	*Na+/K+ ATPase:* KU183910-14[a]
		3	BRK2-3,7 (5n)	*COI*: KU183958-60[a]
		2	BRK1-2 (5n)	*16S*: KU183853-54[a]
		2	BRK1-2 (5n)	*12S*: KU183879-80[a]
Aibi Lake, China	P/2n	5	AB1-5 (2n)	*ITS1*: KU183815-19[a]
		5	AB1-5 (2n)	*Na+/K+ ATPase*: KU183938-42[a]
		5	AB1-5 (2n)	*COI*: KU183983-87[a]
		2	AB1-2 (2n)	*16S*: KU183867-68[a]
		2	AB1-2 (2n)	*12S*: KU183893-94[a]
	P/3n	5	AB1-5 (3n)	*ITS1*: KU183820-24[a]
		5	AB1-5 (3n)	*Na+/K+ ATPase*: KU183925-29[a]
		5	AB1-5 (3n)	*COI*: KU183988-92[a]
		2	AB1-2 (2n)	*16S*: KU183862-63[a]
		2	AB1-2 (2n)	*12S*: KU183888-89[a]
Ga Hai, China	P/2n	5	GAH1-5 (2n)	*ITS1*: KU183825-29[a]
		5	GAH1-5 (2n)	*Na+/K+ ATPase*: KU183915-19[a]
		7	GAH1-7 (2n)	*COI*: KU183961-67[a]
		3	GAH1-3 (2n)	*16S*: KU183855-57[a]
		3	GAH1-3 (2n)	*12S*: KU183881-83[a]
Aqqikkol Lake, China	P/2n	8	AQ1-8 (2n)	*ITS1*: KU183830-37[a]
		8	AQ1-8 (2n)	*Na+/K+ ATPase*: KU183930-37[a]
		5	AQ1-5 (2n)	*COI*: KU183978-82[a]
		3	AQ1-3 (2n)	*16S*: KU183864-66[a]
		3	AQ1-3 (2n)	*12S*: KU183890-92[a]
Hoh Lake, China	P/4n	5	HOH1-5 (4n)	*ITS1*: KU183838-42[a]
		5	HOH1-5 (4n)	*Na+/K+ ATPase*: KU183920-24[a]
		6	HOH1,3-7 (4n)	*COI*: KU183972-77[a]
		4	HOH1-4 (4n)	*16S*: KU183858-61[a]
		4	HOH1-4 (4n)	*12S*: KU183884-87[a]
Yinggehai Saltern, China	P/5n	5	YGH1-5 (5n)	*ITS1*: KU183843-47[a]
		5	YGH1-5 (5n)	*Na+/K+ ATPase*: KU183943-47[a]
		4	YGH1-4 (5n)	*COI*: KU183968-71[a]
		3	YGH1-3 (5n)	*16S*: KU183869-71[a]
		3	YGH1-3 (5n)	*12S*: KU183895-97[a]
Jiangsu, China	P/4n	2	JIA7,12 (4n)	*ITS1*: DQ201288[b], FJ004943[c]
		1	JIA (4n)	*COI*: HM998993[d]
Torre Colimena, Italy	P/2n	2	TCL1-2 (2n)	*ITS1*: DQ201278-9[b]
		1	TCL (2n)	*COI*: HM998995[d]
Ankiembe, Madagascar	P/3n	3	MAD2,4,6 (3n)	*ITS1*: FJ004944[c], DQ201280,84[b]
		1	MAD (3n)	*COI*: HM998999[d]
Swakopmund, Namibia	P/2n	2	NAM1,5 (2n)	*ITS1*: DQ201281-2[b]
		1	NAM (2n)	*COI*: HM998995[d]
Moimishanskoe Lake, W. Altai	P/2n	1	MOI5 (2n)	*ITS1*: KF736285[e]
		1	MOI5 (2n)	*COI*: KF707871[e]

Table 1 Continued

Locality	Reproduction mode/Ploidy	Number of specimens	Lab codes	GenBank accession numbers
Maloje Jarovoe Lake, W. Altai	P/2n	1	MAL6 (2n)	*ITS1*: KF736281[e]
		1	MAL6 (2n)	*COI*: KF707832[e]
Bolshoe Jarovoe Lake, W. Altai	P/2n	1	BOL8 (2n)	*ITS1*: KF736283[e]
		1	BOL (2n)	*COI*: KF707843[e]
Atanasovko Lake, Bulgaria	P/2n	1	ATA2 (2n)	*ITS1*: KF736259[e]
		1	ATA2 (2n)	*COI*: KF707725[e]
Bagdad Saltern, Iraq	P/2n	1	IRA3 (2n)	*ITS1*: KF736265[e]
		1	IRA3 (2n)	*COI*: KF707727[e]
Alexandria Saltern, Egypt	P/2n	1	EGY5 (2n)	*ITS1*: KF736269[e]
		1	EGY5 (2n)	*COI*: KF707786[e]
Korangi Creek Saltern, Pakistan	P/2n	1	PAK4 (2n)	*ITS1*: KF736273[e]
		1	PAK4 (2n)	*COI*: KF707781[e]
Narte Saltern, Albania	P/2n	1	ALB3 (2n)	*ITS1*: KF736275[e]
		1	ALB3 (2n)	*COI*: KF707794[e]
Oybuskoye Lake, Ukraine	P/2n	1	OYB7 (2n)	*ITS1*: KF736277[e]
		1	OYB7 (2n)	*COI*: KF707818[e]
Aral Sea, Uzbekistan	P/2n	1	ARA15 (2n)	*ITS1*: KF736279[e]
		1	ARA5 (2n)	*COI*: KF707822[e]
Citros, Greece	P/4n	1	CIT (4n)	*COI*: HM998993[d]
Kessani, Greece	P/4n	1	KES (4n)	*COI*: HM998993[d]
Mesi, Greece	P/4n	1	MES (4n)	*COI*: HM998993[d]
Tanggu, China	P/2n	1	TAG (2n)	*COI*: HM998995[d]
Messolonghi, Greece	P/3n	1	MEO (3n)	*COI*: HM998997[d]
Tuticorin, India	P/3n	1	TUT (3n)	*COI*: HM998997[d]
Puttalam, Sri Lanka	P/3n	1	PUT (3n)	*COI*: HM998997[d]
Incheh Lake, Iran	P/2n	1	INL (2n)	*Na+/K+ ATPase*: HM854367[f]
Lagoons of Urmia Lake, Iran	P/2n	1	LUL (2n)	*Na+/K+ ATPase*: HM854366[f]
MaharluLake, Iran	P/2n	1	MAL (2n)	*Na+/K+ ATPase*: HM854365[f]
Urmia Lake, Iran	B	1	*A. urmiana*	*ITS1*: DQ201276[b]
		1		*Na+/K+ ATPase*: HM854368[f]
		1		*COI*: HM998991[d]
		1		*16S*: NC_021382[g]
		1		*12S*: NC_021382[g]
Yuncheng, China	B	1	*A. sinica*	*ITS1*: DQ201285[b]
		1		*Na+/K+ ATPase*: HM854369[f]
		1		*COI*: DQ119650[d]
		2		*16S*: KU183872-73[a]
		2		*12S*: KU183898-99[a]
Lagkor Co, China	B	1	*A. tibetiana*	*ITS1*: DQ201270[b]
		1		*Na+/K+ ATPase*: KU183948[a]
		1		*COI*: EF615588[d]
		1		*16S*: JQ975178[g]
		1		*12S*: JQ975178[g]
Great Salt Lake, USA	B	1	*A. franciscana*	*ITS1*: DQ201300[b]
		1		*Na+/K+ ATPase*: HM854370[f]
		1		*COI*: GU248372[d]
		1		*16S*: NC_001620[h]
		1		*12S*: NC_001620[h]

P, Parthenogenetic; B, Bisexual

[a]This study. [b]Baxevanis *et al*., 2006. [c]Kappas *et al*., 2009. [d]Maniatsi *et al*., 2011. [e]Maccari *et al*., 2013. [f]Manaffar *et al*., 2011. [g]Zhang *et al*., 2013 (JQ975178- NC_021382). [h]Perez *et al*., 1994 (NC_001620).

(2 × Easy Taq® PCR SuperMix, Code# AS111 + dye; TransGen Biotech Co., Ltd., China), 0.4 μL of DNA solution and 0.8 μL of each primer.

A fragment of the nuclear DNA containing a partial sequence of the 18S ribosomal RNA (*18S*), the complete sequence of internal transcribed spacer 1 (*ITS1*) and a partial sequence of the 5.8S ribosomal RNA (*5.8S*) gene was PCR-amplified using the primers 18d-5′/R58 (Baxevanis *et al.* 2006). PCR amplification was performed under the following conditions: 4 min at 93 °C, 32 cycles of 40 s at

93 °C, 40 s at 62 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C.

A partial fragment of the nuclear gene, *Na^+/K^+ ATPase α-1 subunit*, was amplified using the primers suggested by Manaffar *et al.* (2011). The thermal cycler PCR conditions were as follows: 94 °C for 2 min, 32 cycles of 94 °C for 25 s and 56 °C for 25 s and 72 °C for 1 min, and final extension with 72 °C for 3 min.

Analysis of the mitochondrial cytochrome oxidase subunit 1 (*COI*) partial fragment was performed using the invertebrate universal primers L*COI*490/HC02198 (Folmer *et al.* 1994). PCR amplification was carried out under the following conditions: a cycle of 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 60 s at 45 °C, and 60 s at 72 °C, with a final step of 5 min at 72 °C (Muñoz *et al.* 2010). Both the universal primers (Folmer *et al.* 1994) and the specific *Artemia* primers (1/2*COI*_Fol-F and 1/2*COI*_Fol-R; Muñoz *et al.* 2008) failed for Hoh Lake tetraploids and Barkol Lake pentaploids, but the mixture of both primers gave weak bands. These samples were cloned by T-Vector pMD 19 (Cat. # 3271, Lot # K1701AA, Takara Bio Inc., Japan).

The partially combined fragments of 16S ribosomal RNA (*16S*) and 12S ribosomal RNA (*12S*) were amplified using the primers *16S*-SP/*12S*-SP (Bossier *et al.* 2004). PCR amplification was carried out using the following programme: 1 cycle of 94 °C for 2 min, 34 cycles of 1 min 15 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C and a final extension cycle of 72 °C for 4 min. Each partial fragment of *16S* (1034 bp) and *12S* (371 bp) was separated via aligning the combined sequences with complete sequence of *16S* ribosomal RNA, tRNA-Val and *12S* ribosomal RNA in *Artemia franciscana* (GenBank: NC_001620), *Artemia urmiana* (GenBank: NC_021382), *Artemia tibetiana* (GenBank: JQ975178).

Using these methods we obtained 199 sequences, including the *ITS1* sequences of 48 individuals, *Na^+/K^+ ATPase* sequences of 49 individuals, *COI* sequences of 44 individuals, *16S* sequences of 29 individuals and *12S* sequences of 29 individuals. The number of sequences retrieved from GenBank was 55 (Table 1). After alignment, we obtained a 1197-bp *ITS1* data set of 72 specimens, a 200-bp *Na^+/K^+ ATPase* data set of 52 specimens, a 579-bp *COI* data set of 72 specimens, a 1034-bp *16S* data set of 29 specimens, and a 371-bp *12S* data set of 29 specimens.

Sequence alignment and phylogenetic analyses

Sequences were aligned using MEGA ver. 6.00 with default parameters (Tamura *et al.* 2013). To estimate the genealogical relationships among haplotypes, a median network was generated using the median-joining algorithm in the NETWORK program v 4.610 (Bandelt *et al.* 1999). For each ploidy, number of segregating sites (P), the total number of nucleotide substitutions (M), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences per population (K) were computed using DNASP v 5.10 program (Librado & Rozas 2009).

Phylogenetic trees were reconstructed using maximum likelihood (ML) in MEGA ver. 6.00 (Tamura *et al.* 2013) and Bayesian inference (BI) as implemented in MRBAYES 3.2.2 on XSEDE (Miller *et al.* 2010), and *Artemia franciscana* was chosen as an outgroup. For ML, the robustness of branches was assessed by 1000 bootstrap replicates. For BI the best fitting nucleotide substitution model of DNA was selected based on MRMODELTEST 2.2 (Nylander 2004) (Table 2). Different genes required different number of generations to properly reach convergence as reported in Table 2. The trees were visualized using FIGTREE v 1.4.0 (Rambaut 2012). For the ML bootstraps, the values <70 were regarded as low, 70–94 as moderate, and ≥95 as high (Hillis & Bull 1993). For the BI posterior probabilities, the values <0.94 were considered as low, and ≥0.95 as high (Alfaro *et al.* 2003). Overall mean, within mean and also between mean distances were computed using uncorrected *p-distance* nucleotide model as implemented in MEGA ver. 6.00.

Results

Haplotype distribution and genetic diversity

The 67 *ITS1* sequences revealed 16 distinct haplotypes for Eurasian and African parthenogenetic *Artemia* (H1-H16). Among them, H1 was present in almost 45% (30/67) of individuals analysed, including all ploidy degrees (Fig. 1). The tetraploid JIA population had two different haplotypes (H1 and H16), but the other tetraploid populations, BRK and HOH, constituted the unique haplotypes H8 and H13, respectively. Pentaploid YGH population possessed two distinct haplotypes (H11 and H12) and shared H9 with other specimens with different ploidy degrees. Tetraploid JIA7 exhibited a unique haplotype (H16) which was genetically close to *A. sinica*. Triploid AB shared the same haplotype with diploid AB and GAH populations in Asia but there is no shared haplotype between triploid AB and the African MAD (3n) populations.

Table 2 Information of optimal nucleotide substitution models identified for each gene by MRMODELTEST

Gene	Model	nst	Rates	Generations	SD	Burn-in
ITS1	HKY+G	2	Gamma	4 000 000	0.004	10 000
Na^+/K^+ ATPase	JC	1	Equal	2 000 000	0.006	5000
COI	HKY+G	2	Gamma	4 000 000	0.003	10 000
16S	GTR+G	6	Gamma	1 000 000	0.004	2500
12S	GTR+G	6	Gamma	1 000 000	0.005	2500

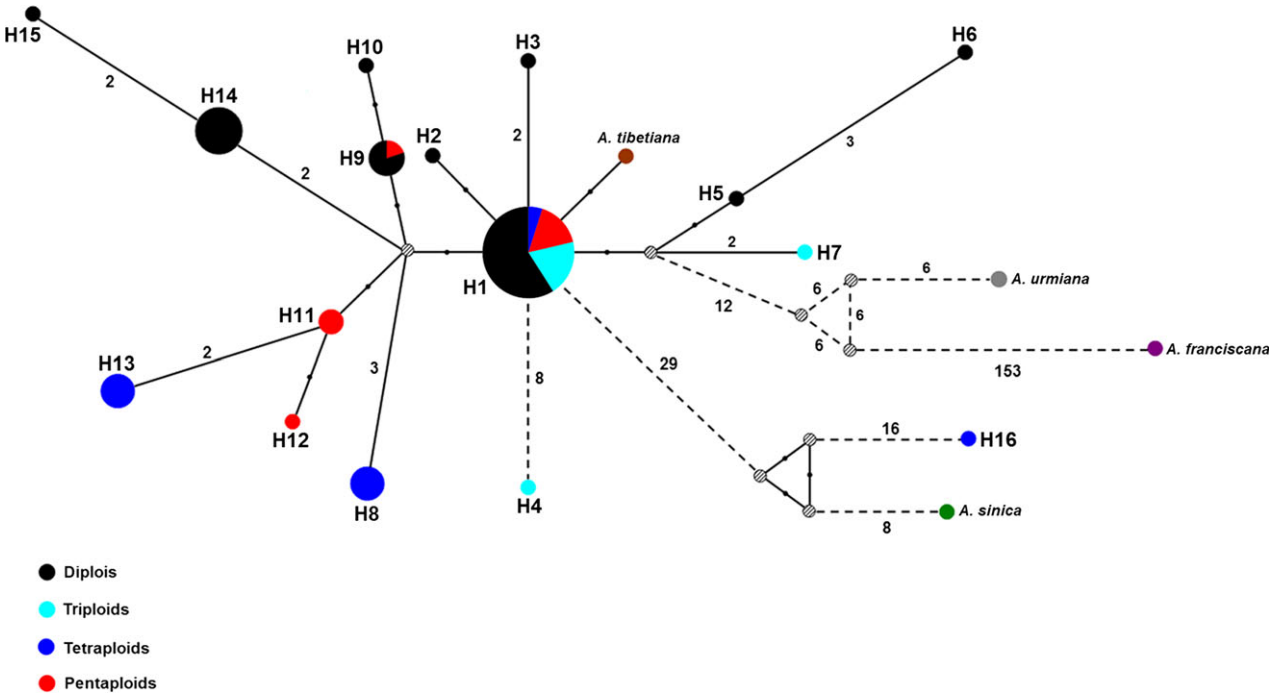

Fig. 1 Haplotype network of parthenogenetic *Artemia* and Asian bisexual *Artemia* based on ITS1. The size of each circle is proportional to the frequency of individuals. Hatched circles indicate intermediate or unsampled haplotypes. Black dots/numbers between haplotypes represent the number of nucleotide substitutions. A total 16 haplotypes (H1–H16) were observed. H1 (30 individuals): JIA12 (4n), MAD2 (3n), MOI5 (2n), MAL6 (2n), BOL8 (2n), BRK1-5 (2n), AB1-5 (2n), AB1-5 (3n), GAH1-5 (2n), BRK1-5 (5n); H2 (1 individual): ALB3 (2n); H3 (1 individual): EGY5 (2n); H4 (1 individual): MAD6 (3n); H5 (1 individual): TCL2 (2n); H6 (1 individual): TCL1 (2n); H7 (1 individual): MAD4 (3n); H8 (5 individuals): BRK1-5 (4n); H9 including 5 individuals: ATA2 (2n), IRA3 (2n), OYB7 (2n), ARA15, YGH4 (5n); H10 including 1 individual: PAK4 (2n); H11 (3 individuals): YGH1-2,5 (5n); H12 (1 individual): YGH3 (5n); H13 (5 individuals): HOH1-5 (4n); H14 (9 individuals): NAM1,5 (2n), AQ1-7 (2n); H15 (1 individual): AQ8 (2n); H16 (1 individual): JIA7 (4n).

The Na^+/K^+ *ATPase* sequences of 51 parthenogenetic specimens showed two haplotypes (H1 and H2). The Iranian diploids (INL, LUL and MAL) and BRK (2n) (China) were grouped in H2, while all other parthenogenetic individuals (including all ploidy degrees) shared the haplotype H1 with *A. tibetiana* (43/51, 84% of individuals) (Fig. 2).

The *COI* sequences for all 65 parthenogenetic individuals produced 27 haplotypes (H1–H27). Haplotype 1 was the major haplotype which was present in diploids and triploids (15/65, 23% individuals). Diploids, triploids, tetraploids and pentaploids were observed in 10, 3, 10 and 6 haplotypes, respectively. There were no shared haplotypes between parthenogenetic and sexual *Artemia* (Fig. 3).

The *16S* sequences for all parthenogens (24 individuals) displayed nine haplotypes (H1–H9), which seemed to be ploidy specific. Diploid haplotypes (H1, H2, H3 and H5) and triploid haplotypes (H4) were clearly clustered with *A. urmiana*. Tetraploid haplotype (H6) and pentaploid haplotypes (H7, H8 and H9) were distinctly grouped with *A. sinica* (Fig. 4).

The *12S* sequences for all parthenogens (24 individuals) revealed eight distinct haplotypes (H1–H8). Similar to the 16S gene, the diploid haplotypes (H1, H3 and H4) and triploid haplotype (H2) were clustered with *A. urmiana*. Tetraploid haplotypes (H5 and H7) and pentaploid haplotypes (H5, H6 and H8) were grouped with *A. sinica* (Fig. 5).

Results of the genetic statistics for the studied parthenogenetic *Artemia* are summarized in Table 3. Mitochondrial genes showed higher haplotype diversity than nuclear genes. In mitochondrial genes, *16S* displays highly dissimilarity ($P = 170$, $M = 174$, Hd = 0.91, $\pi = 0.083$, K = 85.46), although the highest haplotype diversity belongs to *12S* ($Hd = 0.98 \pm 0.03$). For mitochondrial genes, pentaploid and diploid parthenogens revealed high haplotype diversity (*Hd*). In two nuclear genes, the *ITS1* had higher haplotype diversity in pentaploids (0.71 ± 0.11) than in the others, while the Na^+/K^+ *ATPase* gene possessed the greatest haplotype diversity in diploids (0.42 ± 0.09).

Phylogenetic analyses

For each of the *COI*, *16S*, *12S* and *ITS1* data sets, ML and BI approaches generated concordant tree topology, whereas they generated different topologies for the Na^+/K^+ *ATPase* (Figs 6–10). In the Na^+/K^+ *ATPase* BI tree, all partheno-

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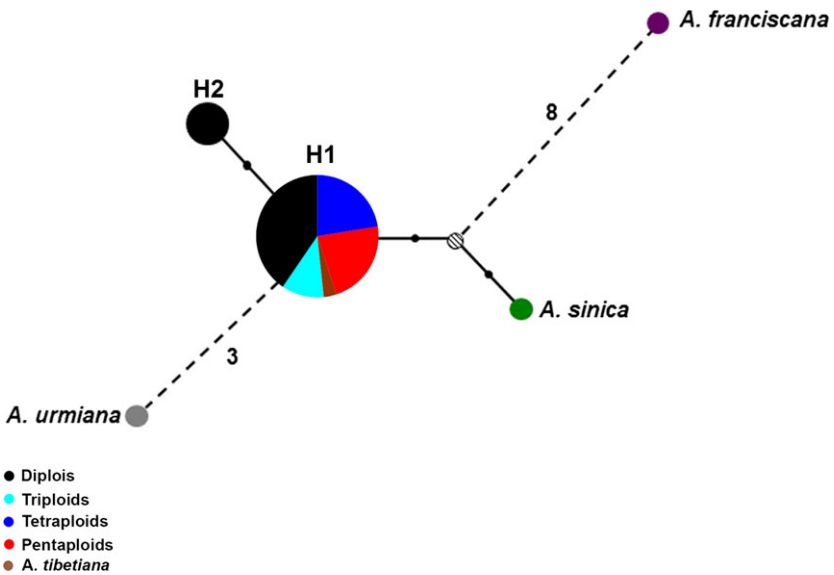

Fig. 2 Haplotype network of parthenogenetic *Artemia* and Asian bisexual *Artemia* based on Na+/K+ ATPase. The size of each circle is proportional to the frequency of individuals. Hatched circles indicate intermediate or unsampled haplotypes. Black dots/numbers between haplotypes represent the number of nucleotide substitutions. A total of 2 haplotypes (H1–H2) were observed. H1 (44 individuals): AB1-5 (2n), AB1-5 (3n), GAH1-5 (2n), AQ1-8 (2n), BRK1-5 (4n), BRK1-5 (5n), HOH1-5 (4n), YGH1-5 (5n), A. tibetiana; H2 (8 individuals): INL (2n), LUL (2n), MAL (2n), BRK1-5 (2n).

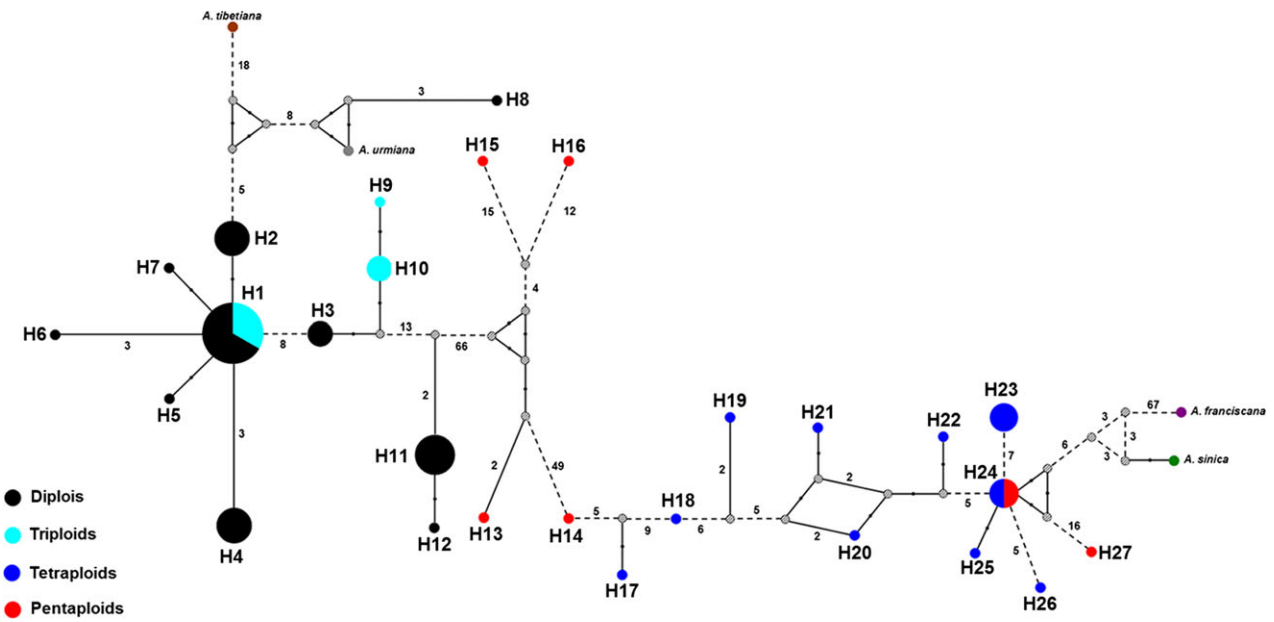

Fig. 3 Haplotype network of parthenogenetic *Artemia* and Asian bisexual *Artemia* based on COI. The size of each circle is proportional to the frequency of individuals. Hatched circles indicate intermediate or unsampled haplotypes. Black dots/numbers between haplotypes represent the number of nucleotide substitutions. A total of 27 haplotypes (H1–H27) were observed. H1 (15 individuals): IRA3 (2n), EGY5 (2n), PAK4 (2n), ALB3 (2n), MAL6 (2n), ATA2 (2n), BRK 1-2,4-5 (2n), AB1-5 (3n); H2 (5 individuals): AB1-5 (2n); H3 (3 individuals): TCL (2n), TAG (2n), NAM (2n); H4 (5 individuals): AQ1-5 (2n); H5 (1 individual): BOL (2n); H6 (1 individual): BRK3 (2n); H7 (1 individual): MOI5 (2n); H8 (1 individual): OBY7 (2n); H9 (1 individual): MAD (3n); H10 (3 individuals): MEO (3n), TUT (3n), PUT (3n); H11 (7 individuals): GAH1-7 (2n); H12 (1 individual): ARA5 (2n); H13 (1 individual): YGH3 (5n); H14 (1 individual): YGH2 (5n); H15 (1 individual): YGH1 (5n); H16 (1 individual): YGH4 (5n); H17 (1 individual): HOH4 (4n); H18 (1 individual): HOH3 (4n); H19 (1 individual): HOH1 (4n); H20 (1 individual): HOH6 (4n); H21 (1 individual): HOH7 (4n); H22 (1 individual): HOH5 (4n); H23 (4 individuals): JIA (4n), CIT (4n), KES (4n), MES (4n); H24 (4 individuals): BRK1-2 (4n), BRK3,7 (5n); H25 (1 individual): BRK4 (4n); H26 (1 individual): BRK3 (4n); H27 (1 individual): BRK2 (5n).

genetic populations have clustered with three Asian sexual species, while in the ML tree *A. sinica* and *A. urmiana* were assigned to unique clades (Fig. 6). The tree of *ITS1* gene assigned the studied specimens into two major groups, *A. sinica* and JIA7 (4n) in one clade and all the other specimens in the other one (Fig. 7).

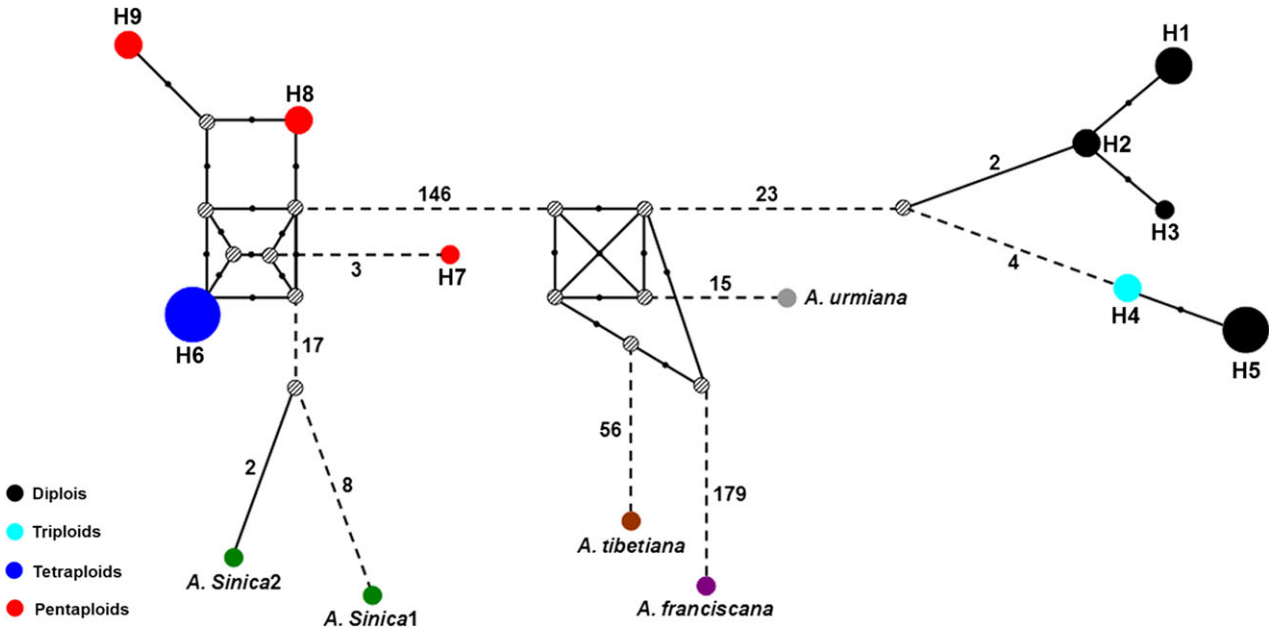

Fig. 4 Haplotype network of parthenogenetic *Artemia* and Asian bisexual *Artemia* based on 16S. The size of each circle is proportional to the frequency of individuals. Hatched circles indicate intermediate or unsampled haplotypes. Black dots/numbers between haplotypes represent the number of nucleotide substitutions. A total of 9 haplotypes (H1–H9) were observed. H1 (3 individuals): GAH1-3 (2n); H2 (2 individuals): AQ1-2 (2n); H3 (1 individual): AQ3 (2n); H4 (2 individuals): AB1-2 (3n); H5 including 5 individuals: BRK1-3 (2n), AB1-2 (2n); H6 (6 individuals): BRK1-2 (4n), HOH1-4 (4n); H7 (1 individual): YGH3 (5n); H8 (2 individuals): YGH1-2 (5n); H9 (2 individuals): BRK1-2 (5n).

Trees based on data sets of three mitochondrial genes (*COI*, *16S* and *12S* genes) were similar in topology and they classified the studied specimens into two major clades (Figs 8–10). In the first major clade, all di- and triploid parthenogens (with the exception of OYB-2n 7 in the COI trees; Fig. 8) have clustered in a clade sister to *A. urmiana*, and they together were sister to *A. tibetiana*. In the second major clade, all tetra- and pentaploid parthenogens were assigned to a clade that was sister to *A. sinica* (Figs 8–10).

The within-group genetic distances of five genes are shown in Table 4. All genes revealed a very low genetic divergence except the *COI* of YGH (5n) population, which showed a relatively high genetic distance of 0.058. The overall genetic divergences among all the studied individuals were higher in the three mitochondrial genes (0.070–0.090) than in the two nuclear genes (0.000) (Table 4).

The between-ploidy distances of nuclear genes are lower than those of mitochondrial genes (Table 5). The *ITS1* gene showed the highest genetic distance between tetraploids and other ploids (0.003), and the lowest genetic distance between triploids and pentaploids (0.000). No genetic variation was found for Na^+/K^+ *ATPase* between triploids and tetra-/pentaploids, as well as between tetraploids and pentaploids. In mitochondrial genes, the mean genetic distance between diploid parthenogens and *A. urmiana* was lower than those between diploid parthenogens and *A. tibetiana*/*A. sinica*. Tetraploid parthenogens showed much lower genetic distance with *A. sinica* than with the other Asian sexual species. Moreover, low genetic divergence was found between diploids and triploids and between tetraploids and pentaploids (Table 5).

Discussion

Phylogenetic analyses displayed a clear discordance between nuclear (*ITS1* and Na^+/K^+ *ATPase*) and mitochondrial (*COI*, *16S* and *12S*) gene trees (Figs 6–10). It has been suggested that a mismatch between mtDNA and nDNA trees could be a sign of incomplete lineage sorting among *Artemia* populations in Eurasia and Africa (Eimanifar *et al.* 2014). Nuclear genes, especially Na^+/K^+ *ATPase* had lower genetic diversity than mitochondrial ones (Tables 4 and 5). This is a major reason that the trees based on two nuclear genes could not gain high resolution in sorting the linage of parthenogenetic *Artemia* (Figs 6 and 7).

Parthenogenetic *Artemia* showed less genetic variation than bisexual relatives (Muñoz *et al.* 2010; Eimanifar *et al.* 2014). Although the genetic diversity varied considerably among all parthenogens (Zhang & King 1992; Maniatsi *et al.* 2011), apomictic polyploid parthenogens exhibited lower genetic variation than automictic diploid ones (Abatzopoulos *et al.* 2003; Maniatsi *et al.* 2011). This view

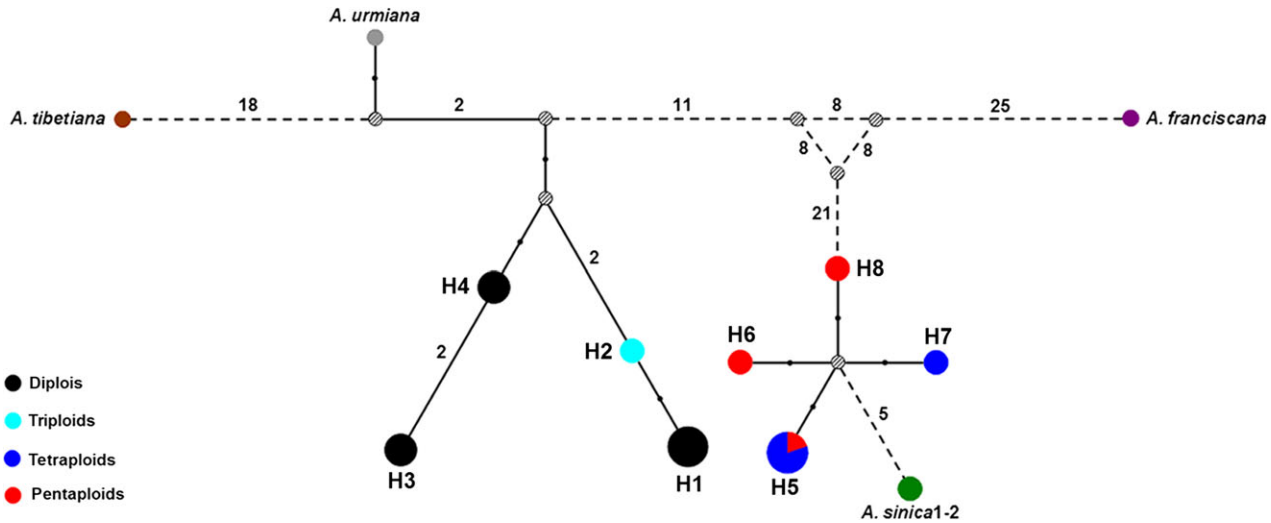

Fig. 5 Haplotype network of parthenogenetic *Artemia* and Asian bisexual *Artemia* based on 12S. The size of each circle is proportional to the frequency of individuals. Hatched circles indicate intermediate or unsampled haplotypes. Black dots/numbers between haplotypes represent the number of nucleotide substitutions. A total of 16 haplotypes (H1–H8) were observed. H1 (5 individuals): BRK1-3 (2n), AB1-2 (2n); H2 (2 individuals): AB1-2 (3n); H3 (3 individuals): AQ1-3 (2n); H4 (3 individuals): GAH1-3 (2n); H5 (5 individuals): HOH1-4 (4n), YGH3 (5n); H6 (2 individuals): YGH1-2 (5n); H7 (2 individuals): BRK1-2 (4n); H8 (2 individuals): BRK1-2 (5n).

Table 3 Population genetic diversity indices for parthenogenetic *Artemia* (all populations) with various ploidy levels

Gene	Ploidy degree	N	P	M	H	Hd ± SD	π ± SD	K
ITS1	Diploid	23	4	4	3	0.50 ± 0.08	0.0009 ± 0.0004	1.12
	Triploid	5	0	0	1	0	0	0
	Tetraploid	10	6	6	2	0.55 ± 0.07	0.0029 ± 0.0007	3.33
	Pentaploid	10	24	24	4	0.71 ± 0.11	0.0044 ± 0.0010	5.13
	Total	48	32	32	8	0.69 ± 0.06	0.0024 ± 0.0010	2.83
Na+/K+ ATPase	Diploid	18	1	1	2	0.42 ± 0.09	0.0021 ± 0.0010	0.43
	Triploid	5	0	0	1	0	0	0
	Tetraploid	10	0	0	1	0	0	0
	Pentaploid	10	0	0	1	0	0	0
	Total	48	1	1	2	0.19 ± 0.07	0.0009 ± 0.0010	0.19
COI	Diploid	22	7	7	5	0.79 ± 0.03	0.003 ± 0.0010	1.89
	Triploid	5	0	0	1	0	0	0
	Tetraploid	10	31	31	9	0.78 ± 0.05	0.018 ± 0.0030	10.88
	Pentaploid	7	90	91	6	0.95 ± 0.09	0.073 ± 0.0070	40.71
	Total	44	140	145	17	0.90 ± 0.22	0.081 ± 0.0040	45.00
16S	Diploid	11	9	9	4	0.74 ± 0.09	0.004 ± 0.0010	4.43
	Triploid	2	0	0	1	0	0	0
	Tetraploid	6	0	0	1	0	0	0
	Pentaploid	5	7	7	3	0.80 ± 0.16	0.003 ± 0.0010	3.20
	Total	24	170	174	9	0.88 ± 0.03	0.083 ± 0.0030	85.46
12S	Diploid	11	6	6	3	0.70 ± 0.08	0.008 ± 0.0020	3.05
	Triploid	2	0	0	1	0	0	0
	Tetraploid	6	2	2	2	0.53 ± 0.17	0.002 ± 0.0010	1.06
	Pentaploid	5	3	3	3	0.80 ± 0.16	0.004 ± 0.0020	1.60
	Total	24	49	50	8	0.98 ± 0.03	0.065 ± 0.0050	24.11

N = number of individuals, P = number of segregating sites, M = total number of nucleotide substitutions, H = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, K = average number of nucleotide differences per population.

was only partly supported by the present study, which found lower-than-diploid genetic variations for triploids (Table 4). However, the within-group genetic variations of tetraploids and pentaploids, particularly for the *ITS1* and *COI* genes, were usually higher than those of the diploids (Table 4).

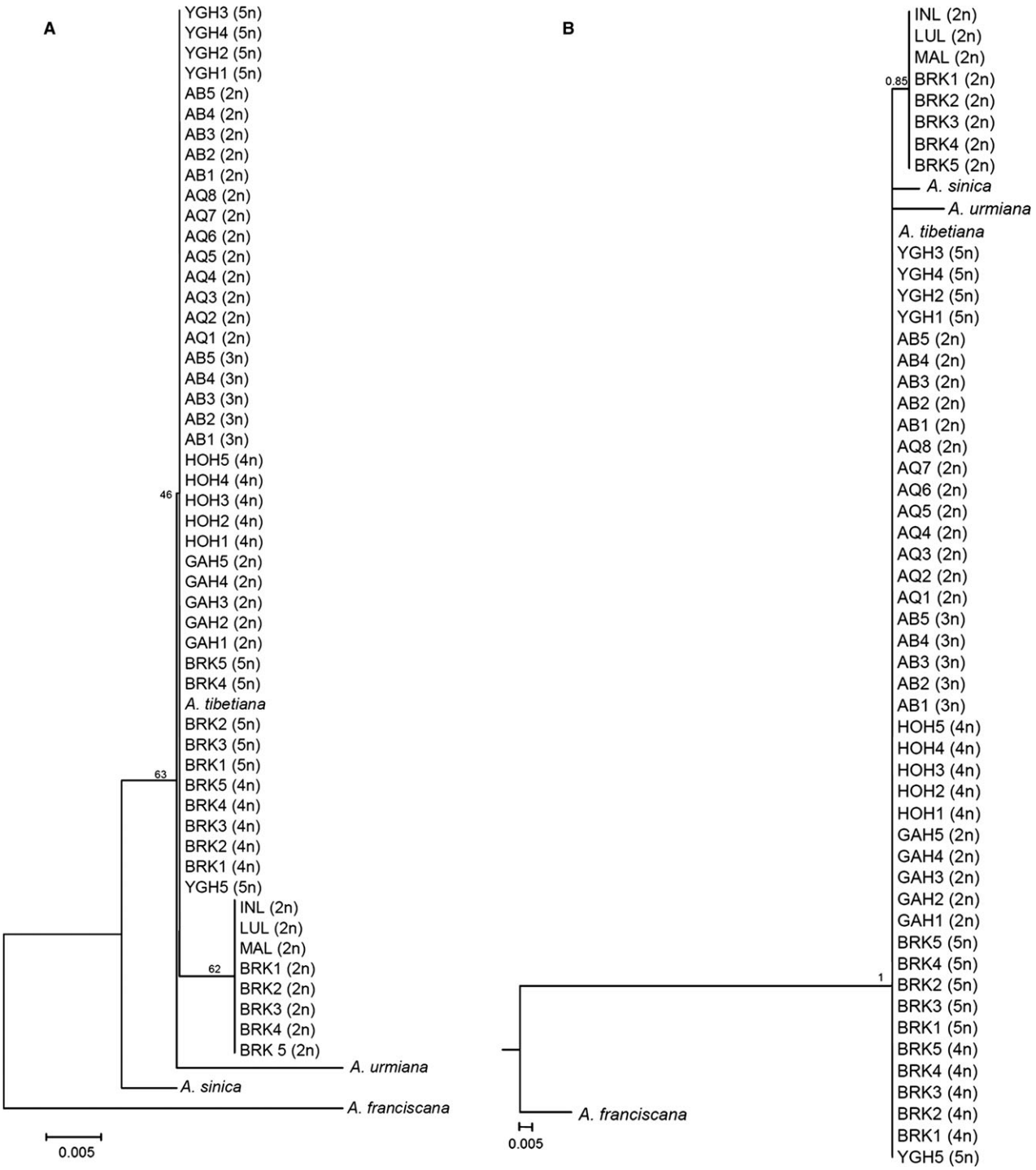

Fig. 6 Phylogenetic relationships of parthenogenetic and Asian bisexual *Artemia* based on Na+/K+ ATPase gene. —A. Maximum-likelihood tree. —B. Bayesian inference tree.

Abreu-Grobois & Beardmore (1982) claimed that parthenogenetic polyploid *Artemia* were monophyletic, but other studies have shown that the gene pool of *Artemia* parthenogens has arisen from different lineages (Bossier *et al.* 2004; Baxevanis *et al.* 2006; Maniatsi *et al.* 2011). Recent studies have indicated that diploid parthenogenetic *Artemia* originated from *A. urmiana* and *Artemia* sp. from Kazakhstan (Muñoz *et al.* 2010; Maniatsi *et al.* 2011;

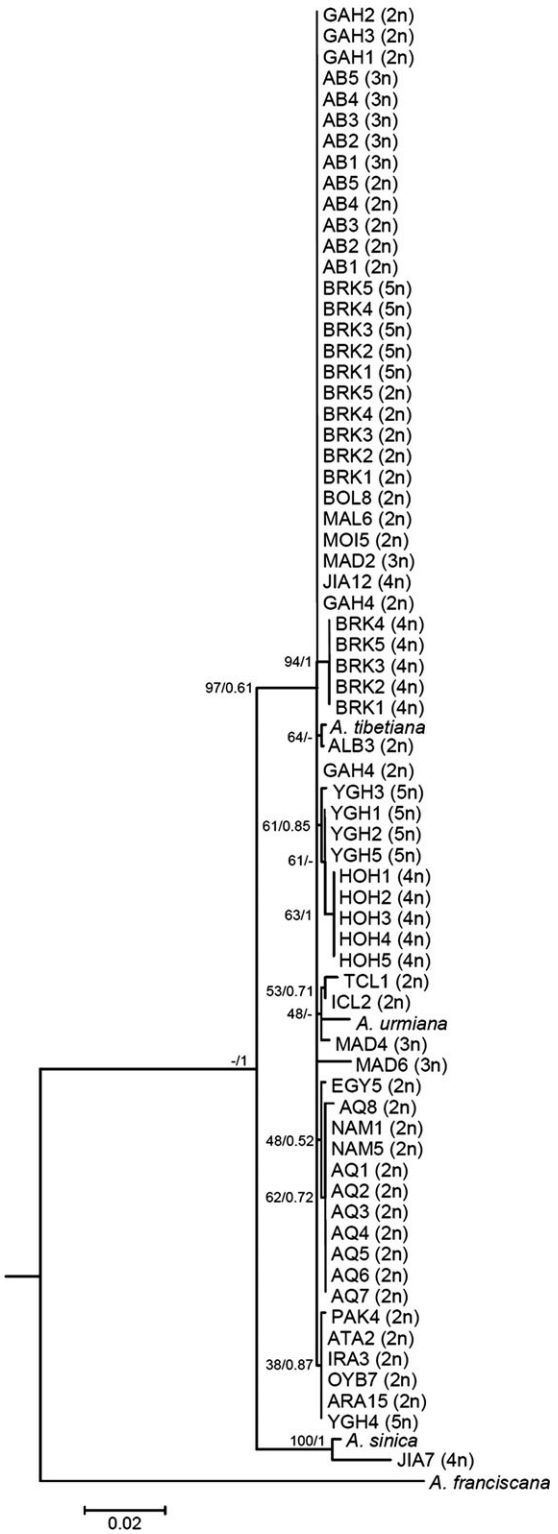

Fig. 7 Phylogenetic relationships of parthenogenetic and Asian bisexual *Artemia* based on ITS1 gene. The maximum-likelihood bootstrap (left) and Bayesian support values (right) are shown for each major node.

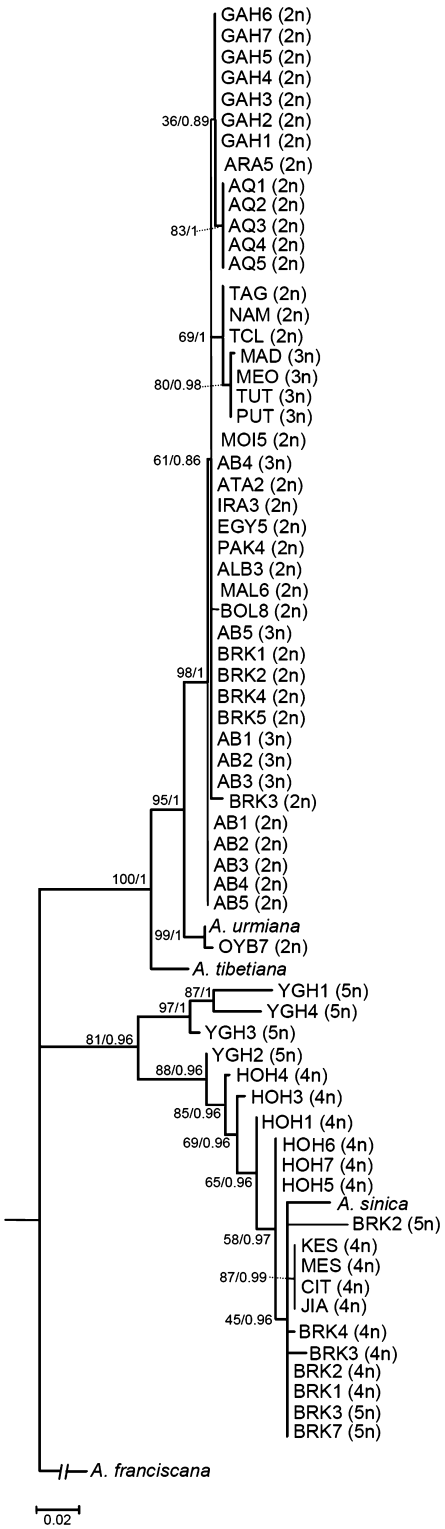

Fig. 8 Phylogenetic relationships of parthenogenetic and Asian bisexual *Artemia* based on COI gene. The maximum-likelihood bootstrap (left) and Bayesian support values (right) are shown for each major node.

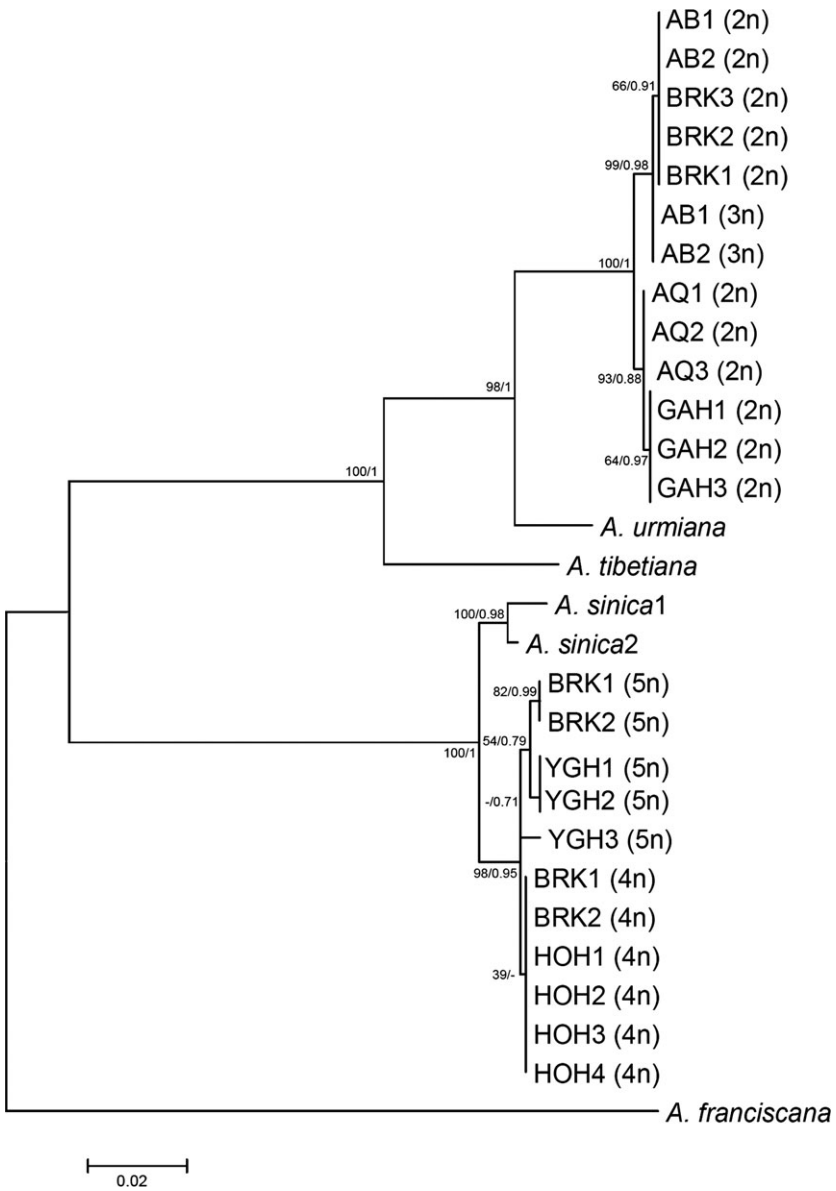

Fig. 9 Phylogenetic relationships of parthenogenetic and Asian bisexual *Artemia* based on 16S gene. The maximum-likelihood bootstrap (left) and Bayesian support values (right) are shown for each major node.

Maccari *et al.* 2013), while triploids and tetraploids have been derived from a diploid parthenogenetic or *A. urmiana* ancestor and *A. sinica*, respectively (Maniatsi *et al.* 2011). Our findings, based on three mitochondrial markers, support the close maternal relationship among diploid and triploid clones and together they constitute a clade sister to *A. urmiana* (Figs 8–10). Attributing the most recent common ancestor of diploid parthenogens to the bisexual *Artemia* from Kazakhstan (Muñoz *et al.* 2010; Maccari *et al.* 2013) is difficult as the holotype of this species is not clearly known (Van Stappen, pers. Comm.). In this regard, the biological species concept of this population has not been supported by phylogenetic studies (Hou *et al.* 2006). As a result, the concept of the evolutionary origin of diploid parthenogens would be different if accepting it as a species or a subspecies/semi-species. The close phylogenetic relationship of the sexual population from Kazakhstan and *A. urmiana* has been accepted (Pilla & Beardmore 1994; Hou *et al.* 2006; Muñoz *et al.* 2010). Therefore, the origin of diploid parthenogens should be rationally attributed to a common ancestor with *A. urmiana*, but this needs further systematic re-evaluation.

Two hypotheses have been proposed by Maniatsi *et al.* (2011) to explain the origins of triploid *Artemia* populations. First, hybridization of an unreduced ovum of diploid parthenogens with *A. urmiana* (see also Johnson

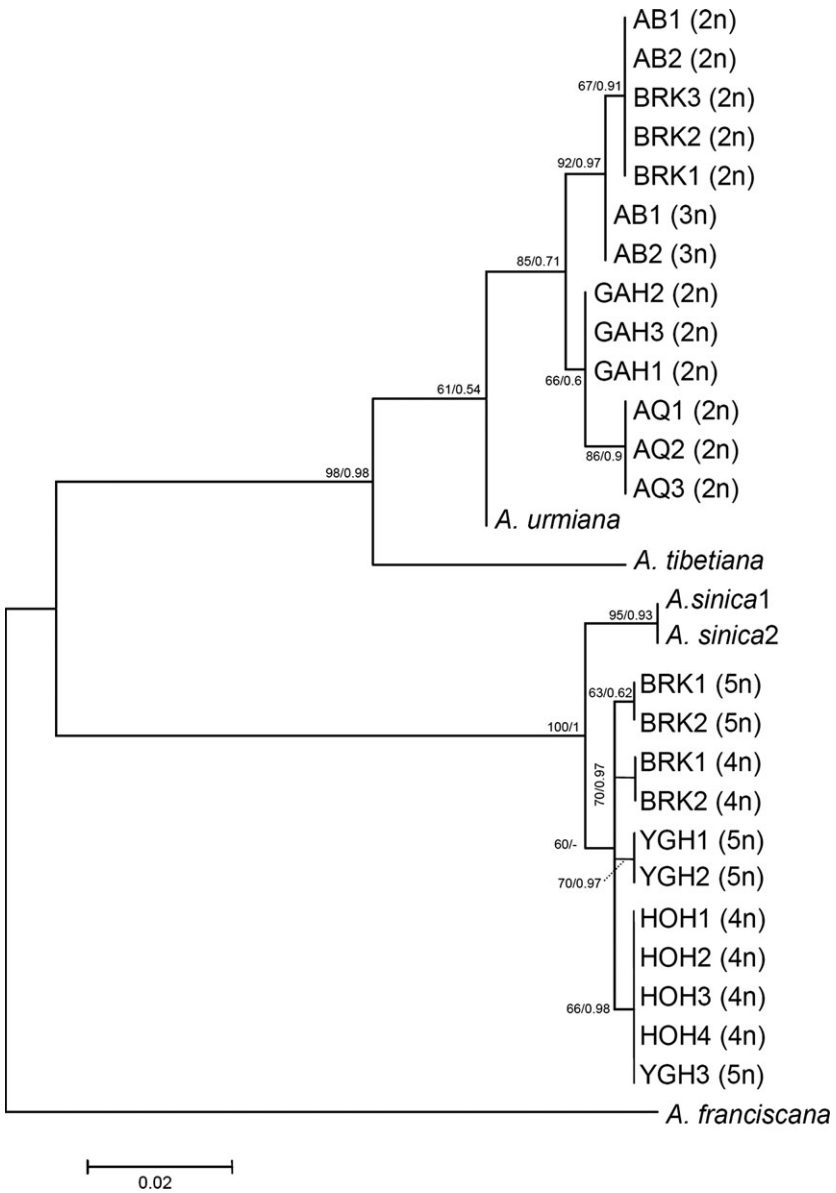

Fig. 10 Phylogenetic relationships of parthenogenetic and Asian bisexual *Artemia* based on 12S gene. The maximum-likelihood bootstrap (left) and Bayesian support (right) values are shown for each major node from left to right, respectively.

et al. 1999) and second, hybridization of an unreduced ovum of *A. urmiana* with a rare male of diploid parthenogens (see also Wallace 1992). However, triploids could theoretically arise by hybridization of an unreduced ovum of *A. urmiana* with a rare male of diploid parthenogens (as well as a normal male of *A. urmiana*), or by polyspermy of normal *A. urmiana* ova. But the probability of these evolutionary routes is likely to be lower than that of the first hypothesis (also see later). Molecular analyses have confirmed that the triploid parthenogens have a much lower genetic distance with diploid parthenogens than with *A. urmiana* (Table 5; see also Maniatsi *et al.* 2011), and to date almost no variation has been found for the maternally inherited mitochondrial genes among triploid *Artemia* linages (Table 4), which suggests that the mitogenome of the triploids should have been directly inherited from diploid parthenogens. Additionally, fusion of a reduced ovum of diploid parthenogen (automictic) with two reduced nuclei (polar bodies) could be another possible origin of triploid *Artemia*.

Mitochondrial genes have been suggested as definite markers to distinguish the maternal origin of species (Sinclair *et al.* 2010). Based on phylogenetic analyses of *CO1* mitochondrial genes, Maniatsi *et al.* (2011) concluded that tetraploid parthenogenetic *Artemia* should have originated from *A. sinica*. Our analyses of three mitochondrial genes

Table 4 The within-group (defined by population and ploidy level) genetic distances (uncorrected p-distance) calculated for five genes of the studied parthenogenetic *Artemia*

Population	*ITS1*	*Na+/K+ ATPase*	*COI*	*16S*	*12S*
BRK (2n)	0.000	0.000	0.002	0.000	0.000
BRK (4n)	0.000	0.000	0.005	0.000	0.000
BRK (5n)	0.000	0.000	0.018	0.000	0.000
AB (2n)	0.000	0.000	0.000	0.000	0.000
AB (3n)	0.000	0.000	0.000	0.000	0.000
GAH (2n)	0.000	0.000	0.000	0.000	0.000
AQ (2n)	0.000	0.000	0.000	0.001	0.000
HOH (4n)	0.000	0.000	0.012	0.000	0.000
YGH (5n)	0.010	0.000	0.058	0.003	0.004
All diploids	0.001	0.002	0.003	0.004	0.008
All triploids	0.000	0.000	0.000	0.000	0.000
All tetraploids	0.003	0.000	0.013	0.000	0.003
All pentaploids	0.001	0.000	0.074	0.003	0.004
Overall distance	0.000	0.000	0.080	0.090	0.070

showed that both tetra- and pentaploid parthenogens were clustered in a clade containing also *A. sinica* (Figs 8–10), and the genetic distance between pentaploids and tetraploids was lower than that between pentaploid and *A. sinica* (Table 5). These results suggest that tetraploids could arise maternally from *A. sinica* and pentaploids should have been straightly originated from tetraploid parthenogens.

The origins of tetraploidy have been ascribed to both autotetraploidy (via polyspermy or uncompleted mitotic/meiotic division) and allotetraploidy (via hybridization) (Bendiksby *et al.* 2011; Li *et al.* 2011; Tomiyoshi *et al.* 2012). Richard *et al.* (1995) have indicated that hybridization between a tetraploid and two different diploid species could be the origin of pentaploids. In *Artemia*, nuclear markers could not provide enough differentiation among tetraploids, pentaploids and *A. sinica*, and individuals of both polyploid forms are very similar to *A. urmiana* and *A. tibetiana* in terms of *ITS1* and Na^+/K^+ *ATPase* genes (Table 5; Figs 6 and 7). Microsatellite analyses, which were usually powerful in determining relationships of recently diversified linages, are currently unavailable in elucidating the origin of polyploid *Artemia* parthenogens, as the most tested loci have not been determined in sexual species (Maniatsi *et al.* 2011). According to our mitochondrial results the close genetic distance between tetraploids and pentaploids proves that pentaploids could be originated from the most common ancestor of apomictic tetraploids and it can be referred to autopentaploidy or hybridization of a tetraploid oocyte with a sperm of *A. sinica*.

Eimanifar *et al.* (2014) put all Eurasian parthenogenetic *Artemia* populations, which shared the basic *COI* haplotype but were significantly different from bisexual species, into the group EHC (Eurasian haplotype complex). According to those results, the EHC was more likely to consist of diploid and triploid parthenogenetic *Artemia* than tetraploid and pentaploids ones, as all the EHC members have been clustered with *A. urmiana*.

Table 5 Between-group genetic distances (uncorrected p-distance) calculated for five genes

Gene	Group	URM	TIB	SIN	Diploids	Triploids	Tetraploids
ITS1	TIB	0.008					
	SIN	0.030	0.024				
	Diploids	0.008	0.002	0.023			
	Triploids	0.007	0.001	0.023	0.001		
	Tetraploids	0.010	0.004	0.025	0.003	0.003	
	Pentaploids	0.007	0.001	0.023	0.001	0.000	0.003
Na^+/K^+	TIB	0.015					
ATPase	SIN	0.025	0.010				
	Diploids	0.016	0.001	0.011			
	Triploids	0.015	0.000	0.010	0.001		
	Tetraploids	0.015	0.000	0.010	0.001	0.000	
	Pentaploids	0.015	0.000	0.010	0.001	0.000	0.000
COI	TIB	0.042					
	SIN	0.157	0.148				
	Diploids	0.023	0.044	0.150			
	Triploids	0.022	0.043	0.150	0.002		
	Tetraploids	0.163	0.155	0.026	0.157	0.157	
	Pentaploids	0.152	0.144	0.066	0.148	0.148	0.056
16S	TIB	0.070					
	SIN	0.154	0.151				
	Diploids	0.042	0.081	0.160			
	Triploids	0.042	0.081	0.161	0.004		
	Tetraploids	0.151	0.152	0.020	0.158	0.158	
	Pentaploids	0.151	0.152	0.022	0.158	0.159	0.003
12S	TIB	0.049					
	SIN	0.119	0.128				
	Diploids	0.017	0.061	0.125			
	Triploids	0.016	0.060	0.125	0.007		
	Tetraploids	0.116	0.124	0.016	0.121	0.121	
	Pentaploids	0.117	0.122	0.016	0.120	0.120	0.005

In conclusion, nuclear genes (*ITS1* and Na^+/K^+ *ATPase*) could not clearly sort the lineages of *Artemia* species/populations, while mitochondrial genes (*COI*, *12S and 16S*) revealed that parthenogenetic *Artemia* is a polyphyletic group. Di- and tetraploid parthenogenetic *Artemia* originated from *A. urmiana* and *A. sinica*, and tri- and pentaploids from diploid and tetraploid *Artemia*, respectively. Consequently, west Asia is believed to be the origin of di-/triploids, while tetra-/pentaploids appear to have arisen from east Asia.

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