Available online at www.sciencedirect.com

ScienceDirect

Molecular Phylogenetics and Evolution 40 (2006) 724–738

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Molecular phylogenetics and asexuality in the brine shrimp *Artemia*

Athanasios D. Baxevanis, Ilias Kappas, Theodore J. Abatzopoulos [*]

Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece

Received 7 October 2005; revised 13 April 2006; accepted 18 April 2006

Abstract

Explaining cases of long-term persistence of parthenogenesis has proven an arduous task for evolutionary biologists. Interpreting sexual–asexual interactions though has recently advanced owing to methodological design, increased taxon sampling and choice of model organisms. We inferred the phylogeny of *Artemia*, a halophilic branchiopod genus of sexual and parthenogenetic forms with cosmopolitan distribution, marked geographic patterns and ecological partitioning. Joint analysis of newly derived ITS1 sequences and 16S RFLP markers from global isolates indicates significant interspecific divergence as well as pronounced diversity for parthenogens, matching that of sexual ancestors. Maximum parsimony, maximum likelihood, and Bayesian methods were largely congruent in reconstructing the phylogeny of the genus. Given the current sampling, at least four independent origins of parthenogenesis are deduced. Molecular clock calibrations based on biogeographic landmarks indicate that the lineage leading to *A. persimilis* diverged from the common ancestor of all *Artemia* species between 80 and 90 MYA at the time of separation of Africa from South America, whereas parthenogenesis first appeared at least 3 MYA. Common mitochondrial DNA haplotypes delineate *A. urmiana* and *A. tibetiana* as possible maternal parents of several clonal lineages. A novel topological placement of *A. franciscana* as a sister clade to all Asian *Artemia* and parthenogenetic forms is proposed and also supported by ITS1 length and other existing data.

Keywords: *Artemia*; Molecular phylogeny; Parthenogenesis; Brine shrimp; ITS1 region; Molecular rate; MtDNA; Geological time

1. Introduction

Evolution has solved the problem of reproduction in various ways. Strictly asexual organisms though—those that reproduce without fertilization—are, in the long-term, something of an evolutionary paradox. They are barred not only from the benefits of sexual reproduction (outcrossing and meiotic recombination) but also from the resulting genetic variance needed for evolutionary change. Current theories attempting to explain the dominance of sex (Judson and Normark, 1996; Normark et al., 2003) are largely unsuccessful in accounting for parthenogenetic lineages. Most notably, the inability of such taxa to purge deleterious mutations is thought of as the prime factor driving them to evolutionary degradation (Hurst and Peck, 1996). Nevertheless, parthenogens attain short-term persistence and, occasionally, provocative longevity (Schön et al., 1996; Mark Welch and Meselson, 2000). For this reason, the origin, genetic diversity and phylogenetic history of asexual systems have been atop evolutionary biologists' list of inquiries.

Mutational and ecological models provide more or less specific predictions regarding the genetic and life history architecture of unisexual taxa, the patterns of variation in nuclear and organelle DNA and the timing of consequences due to loss of sexuality (Normark and Moran, 2000; Normark et al., 2003; Simon et al., 2003). In addition, the rate and mode of origin of parthenogenesis may be crucial in determining levels of genetic diversity, the geographic distribution and ecological success of asexuals (Sandoval et al., 1998; Vrijenhoek, 1998; Schön et al., 2000; Law and Crespi, 2002; Paland et al., 2005). Phylogenetic approaches to the evolution and lifespan of asexuality are especially powerful since typical signatures of the loss of sex

---

[*] Corresponding author. Fax: +30 2310 998256.
E-mail address: abatzop@bio.auth.gr (T.J. Abatzopoulos).

1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.04.010

(Delmotte et al., 2001), rates of molecular change (Schön et al., 2003) and hybridization (Delmotte et al., 2003) can be readily detected on reconstructed trees. Methodologically, such investigations are largely dependent on adequate sampling of extant taxa and sufficient screening of genomic regions. The former may seriously influence both the estimated timeframe of asexual lineages and the number of transition events. The latter can help determine whether the observed clonal divergence is mutationally generated or recurrently captured from sexual ancestors (Crease et al., 1989; Chaplin and Hebert, 1997). It is thus obvious that more robust interpretations are made from model systems where the alternative modes of reproduction are confounded within a single organismal lineage and additional biogeographic and ecological data can be related to patterns of divergence. Recent work has emphasized the need for this integration (Simon et al., 2003). It is paradoxical though that, for *Artemia*, admittedly the most accomplished survivor of hypersaline settings and a confusing case of ancient asexuality, similar critical and detailed assessments are overdue.

Artemia is a genus (Crustacea, Anostraca) of sexual and parthenogenetic forms with a global distribution in inland salt lakes and coastal lagoons (Triantaphyllidis et al., 1998; Van Stappen, 2002). The organism has featured in the literature extensively, by virtue of its importance in aquaculture and as a model system for varied research. From an evolutionary perspective in particular, different adaptations, distinctive genetic features and marked biogeographic patterns are all found in the genus, thus offering unique opportunities for studies on phylogeny and the interaction between sexuality and parthenogenesis (Abatzopoulos et al., 2002a). Currently, six bisexual species (Abatzopoulos et al., 2002b) and a heterogeneous group of obligate parthenogens are recognized, which are either apomictic or automictic. A sharp geographic boundary separates the New World bisexuals (*A. franciscana*, *A. persimilis*) from their Old World relatives (*A. salina*, *A. urmiana*, *A. sinica*, and *A. tibetiana*). Similarly, parthenogenetic populations are restricted to the Old World, where they comprise the majority. Bisexuals are diploid with $2n = 42$ (except *A. persimilis* where $2n = 44$), while parthenogens range in ploidy from $2n$ to $5n$ (Abatzopoulos et al., 1986). Morphometric and/or morphological, life history and genetic divergences are widely partitioned both within and between the different reproductive modes (for review see Browne, 1992). Allopatric divergence and ecological specialization are believed to have shaped *Artemia* evolution, while the influence of dispersal on contemporary regional distributions has only recently been explored (Green et al., 2005).

A historical aspect of *Artemia* phylogeny has been mainly obtained through allozyme studies. Beardmore and Abreu-Grobois (1983) outlined the series of phylogenetic events in the genus. Evaluation of the degree and patterns of interspecific divergence have indicated that the primal evolutionary event has been the separation of New and Old World bisexual lineages. This was followed by the separation of *A. franciscana* and *A. persimilis* in the New World and the divergence of *A. salina* and *A. urmiana* lines in the Old World (see tree in Fig. 1). Based on an allozymi-

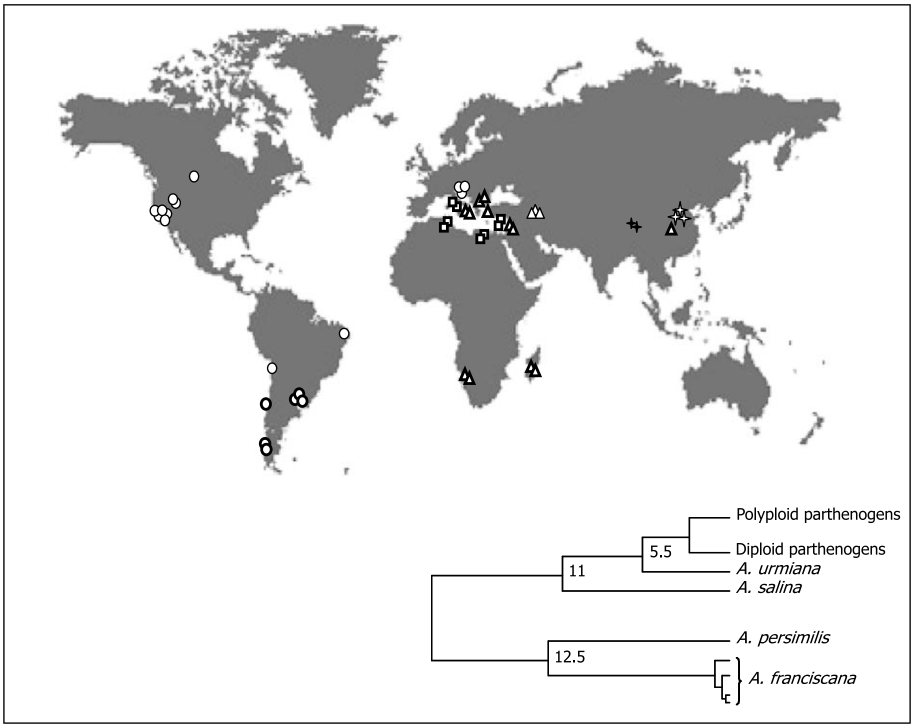

Fig. 1. The 46 *Artemia* individuals (sequenced for ITS1 region) as distributed in the world map. ○, *A. franciscana*; **○**, *A. persimilis*; **□**, *A. salina*; △, *A. urmiana*; +, *A. tibetiana*; ✦, *A. sinica*; and **△**, parthenogenetic *Artemia* strains. Inserted tree shows currently accepted phylogenetic relationships in the genus as determined by allozyme markers (see also Beardmore and Abreu-Grobois, 1983). Numbers at nodes are divergence times in million years.

cally calibrated molecular clock, Abreu-Grobois (1987) speculated on a Mediterranean genesis of parthenogenetic *Artemia*. Accordingly, the parthenogenetic clade branched from an Old World sexual ancestor (possibly *A. urmiana*) approximately 5.5 million years ago (MYA). Unreliable as it may be from a molecular marker perspective (see Avise, 1994) this estimate strikingly accords with geological evidence suggesting a synchronous (5.96–5.33 MYA, Krijgsman et al., 1999) dramatic increase in salinity and subdivision of habitats in the Mediterranean basin (Hsü, 1972). In those conditions, selection favoured enhanced colonization potential granted by reproductive assurance (i.e. parthenogenetic reproduction, Baker, 1965). Subsequent events involved rapid radiation of asexual forms in a wide range of habitats coupled with ploidy shifts. More recently though, a substantially more ancient origin of parthenogenetic *Artemia* (30–40 MYA) was claimed by Perez et al. (1994) based on mtDNA sequence divergence.

Despite extensive recording of *Artemia* biodiversity (Abatzopoulos et al., 2002a) and recurrent evidence for eccentric sequence change (Maley and Marshall, 1998; Crease, 1999; Sáez et al., 2000; Coleman et al., 2001; Hebert et al., 2002), full-scale phylogenetic analyses of the genus are lacking. Based on a global sampling and using sequences of the ITS1 nuclear region, we have tried to infer the phylogenetic relationships and investigate the biogeographic history and ages of all recognized *Artemia* species and asexual forms. To assess congruence between nuclear and mtDNA markers, a subset of populations were also screened for 16S mtDNA restriction patterns.

Throughout this study we have chosen to refer to asexual forms of *Artemia* as populations. The indiscreetly used term *A. parthenogenetica* is at odds with the biological species concept, since obligate parthenogens existing in numerous clones at several different ploidy levels cannot readily be considered as belonging to a single species (Abatzopoulos et al., 2002a).

2. Materials and methods

2.1. Populations analyzed

Artemia exhibits two types of parturition. Embryos in the brood pouch of the female either develop to free-swimming nauplii (ovoviviparity) or are released as cysts (oviparity), forming resting propagule banks which may sequester a substantial proportion of variability from the active population. Therefore, due to the significance of resting cyst banks to evolutionary change (Hairston and De Stasio, 1988; De Meester et al., 2002) and to sample overall genetic diversity more adequately, we have used encysted embryos as starting material for the analyses. Sampling was on a global scale (Fig. 1) and included all major biogeographical regions and, when possible, multiple representatives from the different localities (Table 1). Cyst hatching (hatching percentage >85%) and rearing of nauplii (survival >90%) followed procedures described in Baxevanis et al. (2005). Samples with lower values for these two indices were excluded. With the onset of sexual maturity, examination of sex ratios enabled the unambiguous confirmation of parthenogens, in conjunction with published data on these populations.

2.2. DNA extraction, PCR, and sequencing

DNA was extracted from single specimens (starved for at least 24 h) using 150–200 μl of Chelex suspension (6%, Bio-Rad Laboratories, CA, USA) with proteinase K (15 μl of 20 mg/ml initial stock) pre-treatment (Estoup et al., 1996). "No-DNA" extractions were also conducted to check for contamination. PCR and sequencing of the complete ITS1 region was performed using the universal primer 18d-5′ by Hillis and Dixon (1991) and a newly designed primer R58 (5′ ACCCACGAGCCAAGTG ATCC 3′) based on deposited 5.8S rDNA sequences of *Penaeus vannamei* (AF121132). Amplifications were performed on a PTC-100® Peltier thermal cycler (MJ Research) with the following temperature profiles and 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. Total reaction volumes of 25 μl consisted of 2.5 μl template DNA, 5 μl 5× PCR buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 0.001 mM of each primer and 1.25 U of *Taq* DNA polymerase (Expand High Fidelity^{PLUS} PCR System, Roche). PCR products were excised from agarose gels (2%) and purified using the NucleoSpin Extract kit (Macherey-Nagel). Cycle sequencing was performed with Excell II DNA sequencing kit (EPICENTRE) in both directions. Due to the size of the PCR product (see Section 3), an additional set of internal primers were also designed (sequences available upon request) and used for sequencing. Reactions were electrophoresed on a PRISM 3730xl DNA analyzer (Applied Biosystems).

2.3. Sequence alignment and phylogenetic analyses

The total data set consisted of 47 sequences (outgroup included) (see Table 1). BLAST searches confirmed the identity of the PCR product on the basis of detected similarities with 18S and 5.8S rRNA genes from a number of taxa as well as *A. franciscana* AJ238061, *A. salina* X01723 (see Weekers et al., 2002), *Artemia* sp. M33097 (see Vaughn et al., 1984). A fairy shrimp sequence (*Streptocephalus proboscideus*, AY519840, 680 bp), spanning part of 18S, the complete internal transcribed spacer 1 and part of 5.8S (Daniels et al., 2004), was used to root the phylogeny (used sequence length = 612 bp). For alignment we used the program MEME (Multiple Em for Motif Elicitation, Bailey and Elkan, 1994) in combination with MACAW (Schuler et al., 1991) to identify regions of statistically significant local similarity. In total, six highly conserved regions in both the ingroup taxa and the outgroup were chosen to guide the final alignment. Two of them were

Table 1
List of the studied individuals, with their abbreviations, species designation and GenBank accession numbers for ITS1 sequences

Individual	Population	Species	Accession No.
SFB1	San Francisco Bay, USA	*A. franciscana*[a]	DQ201298
SFB2	San Francisco Bay, USA	*A. franciscana*[a]	DQ201297
SFB5	San Francisco Bay, USA	*A. franciscana*[a]	DQ201293
SFB8	San Francisco Bay, USA	*A. franciscana*[a]	DQ201292
SFB9	San Francisco Bay, USA	*A. franciscana*[a]	DQ201294
GSL1	Great Salt Lake, USA	*A. franciscana*[b]	DQ201300
GSL3	Great Salt Lake, USA	*A. franciscana*[b]	DQ201301
IQU1	Iquique, Chile	*A. franciscana*[c]	DQ201296
ING3	Ingebright, Canada	Unknown[b]	DQ201299
MAS1	Margherita di Savoia, Italy	*A. franciscana*[d]	DQ201295
MAS6	Margherita di Savoia, Italy	*A. franciscana*[d]	DQ201291
MAS7	Margherita di Savoia, Italy	*A. franciscana*[d]	DQ201290
BAI7	Buenos Aires, Argentina	*A. persimilis*[a]	DQ201263
BAI8	Buenos Aires, Argentina	*A. persimilis*[a]	DQ201265
BAI9	Buenos Aires, Argentina	*A. persimilis*[a]	DQ201264
PIC7	Pichilemu, Chile	*A. persimilis*[c]	DQ201266
TDP2	Torres del Paine, Chile	*A. persimilis*[c]	DQ201268
TDP3	Torres del Paine, Chile	*A. persimilis*[c]	DQ201267
SFA4	Sfax, Tunisia	*A. salina*[a]	DQ201302
SFA9	Sfax, Tunisia	*A. salina*[a]	DQ201305
SAN4	Sant' Antioco, Italy	*A. salina*[e]	DQ201304
SAN6	Sant' Antioco, Italy	*A. salina*[e]	DQ201303
WNA4	Wadi El-Natrun, Egypt	*A. salina*[b]	DQ201306
WNA5	Wadi El-Natrun, Egypt	*A. salina*[b]	DQ201307
LAR8	Larnaca, Cyprus	*A. salina*[b]	DQ201308
LAR9	Larnaca, Cyprus	*A. salina*[b]	DQ201309
URM1	Urmia Lake, Iran	*A. urmiana*[a]	DQ201276
URM2	Urmia Lake, Iran	*A. urmiana*[a]	DQ201277
URM3	Urmia Lake, Iran	*A. urmiana*[a]	DQ201275
SIN1	Yuncheng, P.R. China	*A. sinica*[a]	DQ201285
SIN2	Yuncheng, P.R. China	*A. sinica*[a]	DQ201286
SIN3	Yuncheng, P.R. China	*A. sinica*[a]	DQ201287
TIB3	Lagkor Co, Tibet, P.R. China	*A. tibetiana*[f]	DQ201270
TIB7	Lagkor Co, Tibet, P.R. China	*A. tibetiana*[f]	DQ201269
MEM4	Megalon Embolon, Greece	Parthenogenetic[b]	DQ201274
MEM9	Megalon Embolon, Greece	Parthenogenetic[b]	DQ201273
JIA7	Jiangsu, P.R. China	Parthenogenetic[b]	DQ201288
POL14	Polychnitos, Greece	Parthenogenetic[g]	DQ201283
EIL1	Eilat, Israel	Parthenogenetic[b]	DQ201272
EIL2	Eilat, Israel	Parthenogenetic[b]	DQ201271
TCL1	Torre Colimena, Italy	Parthenogenetic[h]	DQ201279
TCL2	Torre Colimena, Italy	Parthenogenetic[h]	DQ201278
MAD4	Ankiembe, Madagascar	Parthenogenetic[i]	DQ201280
MAD6	Ankiembe, Madagascar	Parthenogenetic[i]	DQ201284
NAM1	Swakopmund, Namibia	Parthenogenetic[i]	DQ201281
NAM5	Swakopmund, Namibia	Parthenogenetic[i]	DQ201282
OUTGROUP		*Streptocephalus proboscideus*[j]	AY519840

[a] Baxevanis et al. (2005).
[b] Van Stappen (2002) and references therein.
[c] Gajardo et al. (2004).
[d] Mura et al. (2004).
[e] Mura and Brecciaroli (2004).
[f] Abatzopoulos et al. (2002b).
[g] Triantaphyllidis et al. (1993).
[h] Mura et al. (2005).
[i] Triantaphyllidis et al. (1996).
[j] Daniels et al. (2004).

located within the partial regions of 18S and 5.8S rRNA genes. The obtained similarities in these two regions are justified by the fact that the primers used in our study and those of Daniels et al. (2004) (for the outgroup sequence) amplify a similar region (partial 18S, ITS1, partial 5.8S). Subsequently, sequences were loaded into ClustalX 1.8 (Thompson et al., 1997) and several alignments were performed under a range of gap opening (7–20) and extension (1.5–12) parameters aiming to find an optimized multiple alignment, which preserved the structure and position of the previously identified conserved regions. This yielded a final alignment in ClustalX (slow-accurate mode) with a gap opening penalty of 15, a gap extension penalty of 2 and a delay divergent sequence percentage of 15. All sequences from this study were deposited in GenBank (see Table 1).

Phylogenetic analysis was implemented in PAUP* 4.0b10 (Swofford, 1998) under maximum parsimony (MP) and maximum likelihood (ML) and in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) under Bayesian methods. We have tried not to dismiss from our analysis the historical information contained within gaps (see Giribet and Wheeler, 1999). For cladistic analysis, gaps were treated both as missing data and as a fifth state (according to Bena et al., 1998). In a third approach, they were also coded using GapCoder (Young and Healy, 2003) according to the proposed method ("simple indel coding") by Simmons and Ochoterena (2000) under the assumption that adjacent gap positions are caused by a single indel event. In this context, each indel of a particular length and position was coded as a separate binary character (presence/absence) thus creating a matrix (266 coded indels in total) which was implemented along the original data matrix into phylogenetic analyses. For MP, trees were generated using heuristic searches with TBR (tree-bisection-reconnection) branch swapping and 500 random taxon additions. Nodal support was assessed by 1000 bootstrap replicates. For ML (heuristic search; TBR branch swapping; and 100 bootstrap replicates) the best-fit substitution model employed was the HKY85 + G (Hasegawa et al., 1985), as determined by Modeltest 3.7 (Posada and Crandall, 1998). The parameters of this model were: unequal base frequencies ($A = 0.24$, $C = 0.20$, $G = 0.28$, $T = 0.28$), number of substitution types $Nst = 2$, $T_S/T_V = 0.99$, γ-shape parameter $\alpha = 1.94$. For Bayesian analysis (two simultaneous runs; 1,000,000 generations; four chains; sampling every 100 generations; first 2000 trees discarded as burn-in) the HKY85 + G model was implemented by adjusting likelihood settings to $Nst = 2$ and rates $= \gamma$. For model-based phylogenetic methods gaps were treated as missing data.

The assumption of molecular rate constancy was tested according to Takezaki et al. (1995). To infer dates of splitting events, molecular clock calibrations were based on well-supported major as well as regional biogeographic landmarks. In reconstructing the history of the loss of sex, we have assumed that sexual reproduction is the ancestral state and transitions to asexuality are irreversible.

2.4. RFLP screening

Total genomic DNA was separately extracted from 30 individuals (for each population, individuals sequenced for ITS1 were also scored for mtDNA analysis) from the populations SFB, BAI, SFA, SAN, WNA, LAR, URM, SIN, TIB, MEM, JIA, POL, EIL, TCL, MAD, and NAM (abbreviations of populations are given in Table 1) using the procedures described previously. Part of the 16S rRNA gene was amplified with the universal primers L_{2510} and H_{3080} (Palumbi, 1996). The protocols for PCR, including reaction conditions and amplification profiles, are described in Baxevanis and Abatzopoulos (2004). PCR products were digested with nine restriction endonucleases (*Alu*I, *Bfa*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I, and Taq^{α}I), electrophoretically separated in 1.5% agarose gels, stained with ethidium bromide and photographed under UV light. Data were coded as composite mtDNA genotypes based on site differences, inference of which was aided by the published sequence of the whole mtDNA of *A. franciscana* (Valverde et al., 1994). Nucleotide divergence between populations and their component haplotypes was computed in REAP 4.0 (McElroy et al., 1992) according to Nei and Tajima (1981), Nei (1987) and Nei and Miller (1990). A Neighbor Joining phylogram was constructed using the Neighbor module of the PHYLIP 3.6b package (Felsenstein, 2004).

3. Results

3.1. ITS1 sequence characteristics

The ITS1 data set for the ingroup sequences ranged from 983 to 1438 bp (excluding gaps in the alignment). The alignment length (including the outgroup) was 1579 bp. Transition/transversion versus divergence plots (not shown) did not show any signs of sequence saturation. The disparity index statistic (Kumar and Gadagkar, 2001) was used to test for similarity in nucleotide composition across sequences and identify rogue taxa with atypical patterns of substitution. Such patterns were most conspicuous for WNA5, EIL1, EIL2, ING3, and SIN3 sequences. Regarding the length of the whole ITS1 region, a clade-specific pattern was revealed with three well-defined groups. The first corresponded to *A. persimilis* sequences (bearing a length of 983–984 bp), the second to *A. salina* (1436–1438 bp) while the third was common for *A. franciscana*, *A. sinica*, *A. urmiana*, *A. tibetiana* and all parthenogens (1133–1181 bp). This characteristic length variation was also further verified through sequencing of a number of representatives from each taxon with a second set of internal primers.

3.2. Nuclear sequence variation

Uncorrected pairwise distances ranged from zero to 28.1% for the ingroup taxa. Minimum and maximum uncorrected pairwise divergence values for the outgroup were 39.9 and 44.3%, respectively. Using the HKY85 + G model, the same estimates reached a maximum of 39.5% with an overall mean of 15.2% (83.8 and 17.5% including the outgroup, respectively). A summary of mean inter- and intraspecific sequence divergences is shown in Table 2. The interspecific maximum is 34.7% for *A. persimilis* vs

Table 2
Average inter- and intraspecific ITS1 sequence divergence estimates based on the HKY85 + G model of nucleotide substitution

	A. persimilis (3)	*A. franciscana* (5)	*A. salina* (4)	*A. sinica* (1)	*A. urmiana* (1)	*A. tibetiana* (1)	Parthenogens (7)
A. persimilis (3)	—	*0.242*	*0.254*	*0.227*	*0.215*	*0.217*	*0.224*
A. franciscana (5)	0.327	—	*0.163*	*0.113*	*0.111*	*0.108*	*0.115*
A. salina (4)	0.347	0.199	—	*0.132*	*0.135*	*0.129*	*0.137*
A. sinica (1)	0.300	0.128	0.154	—	*0.031*	*0.026*	*0.031*
A. urmiana (1)	0.280	0.126	0.159	0.032	—	*0.008*	*0.016*
A. tibetiana (1)	0.282	0.123	0.150	0.026	0.008	—	*0.012*
Parthenogens (7)	0.293	0.132	0.162	0.032	0.017	0.012	—
Intraspecific divergence	0.013	0.018	0.024	0.004	0.006	0.000	0.020

Uncorrected estimates are shown above the major diagonal. The number of populations examined per species is indicated in parentheses.

A. salina while the minimum is 0.8% for *A. tibetiana* vs *A. urmiana*. Within species divergence estimates ranged from zero (*A. tibetiana*) to 2.4% (*A. salina*). Sequence diversity within parthenogenetic *Artemia* (2.0%) is considerably higher compared with all remaining bisexuals (except *A. salina*). In fact, certain isolates from Israel (EIL1 and EIL2) and China (JIA7) display divergence values from other asexuals (3.6–7.2%, data not shown) that greatly exceed interspecific comparisons among *A. sinica*, *A. urmiana* and *A. tibetiana* (see Table 2).

3.3. MtDNA divergence

The PCR-amplified 16S rRNA fragment was 536 bp. The mtDNA analysis only included populations with corresponding ITS1 sequences. RFLP screening revealed a total of 30 cleavage sites, corresponding to 122 nucleotides (restriction patterns are available upon request). Overall, nine composite genotypes were identified. Sequence divergence estimates between populations and between haplotypes (designated as A–I) are given in Table 3. Nucleotide divergence estimates between populations ranged from zero to 20.2%. Similar to the results of the nuclear assay, *A. persimilis* is the most divergent lineage separated from all others by a distance range between 13 and 20.2%. *Artemia urmiana* and *A. tibetiana* are mitochondrially indistinguishable, sharing the same and most abundant haplotype (C) with five parthenogenetic populations (EIL, TCL, MAD, NAM, and POL). Parthenogens from POL differed from the previous cluster by a mean divergence of 1%, owing to the presence of a second haplotype (interclonal distance for C vs G is 5.6%, see Table 3). Parthenogens from MEM also displayed two haplotypes (D and F), one of them shared with *A. sinica* (F).

3.4. Phylogenetic inference

The full ITS1 data set (including the outgroup) consisted of 448 parsimony informative sites when gaps were treated as missing data (544 with indel coding). Reconstructed trees using MP, ML, and Bayesian methods were generally congruent and variably resolved for shallow branches. The 50% majority rule MP tree based on indel coding (number of steps 1093) is shown in Fig. 2a, while that based on gaps

Table 3
Mean nucleotide divergence values among populations (sample size = 30 individuals) and their component haplotypes (A–I) based on 16S RFLP profiles with nine restriction endonucleases

	Populations																
	MEM^{D,F}	JIA^{E}	TCL^{C}	POL^{C,G}	LAR^{H}	EIL^{C}	NAM^{C}	MAD^{C}	WNA^{H}	SFB^{B}	BAI^{A}	SIN^{F}	URM^{C}	TIB^{C}	SFA^{I}	SAN^{I}	
							Composite genotypes										
MEM^{D,F}	—																
JIA^{E}	0.037	—					I	H	G	F	E	D	C	B	A		
TCL^{C}	0.022	0.051	—				—	*0.009*	*0.112*	*0.121*	*0.131*	*0.121*	*0.111*	*0.101*	*0.174*	I	
POL^{C,G}	0.007	0.024	0.010	—				—	*0.101*	*0.117*	*0.121*	*0.117*	*0.101*	*0.092*	*0.202*	H	
LAR^{H}	0.103	0.121	0.101	0.087	—				—	*0.011*	*0.022*	*0.035*	*0.056*	*0.072*	*0.147*	G	
EIL^{C}	0.022	0.051	0.000	0.010	0.101	—				—	*0.010*	*0.046*	*0.066*	*0.082*	*0.157*	F	
NAM^{C}	0.022	0.051	0.000	0.010	0.101	0.000	—				—	*0.056*	*0.051*	*0.092*	*0.134*	E	
MAD^{C}	0.022	0.051	0.000	0.010	0.101	0.000	0.000	—				—	*0.020*	*0.082*	*0.157*	D	
WNA^{H}	0.103	0.121	0.101	0.087	0.000	0.101	0.101	0.101	—				—	*0.101*	*0.142*	C	
SFB^{B}	0.074	0.092	0.101	0.074	0.092	0.101	0.101	0.101	0.092	—				—	*0.101*	B	
BAI^{A}	0.148	0.134	0.142	0.130	0.202	0.142	0.142	0.142	0.202	0.165	—				—	A	
SIN^{F}	0.027	0.010	0.066	0.028	0.112	0.066	0.066	0.066	0.112	0.082	0.157	—					
URM^{C}	0.022	0.051	0.000	0.010	0.101	0.000	0.000	0.000	0.101	0.101	0.142	0.066	—				
TIB^{C}	0.022	0.051	0.000	0.010	0.101	0.000	0.000	0.000	0.101	0.101	0.142	0.066	0.000	—			
SFA^{I}	0.113	0.131	0.110	0.097	0.009	0.110	0.110	0.110	0.009	0.101	0.174	0.121	0.110	0.110	—		
SAN^{I}	0.113	0.131	0.110	0.097	0.009	0.110	0.110	0.110	0.009	0.101	0.174	0.121	0.110	0.110	0.000	—	

treated as missing data (number of steps 930) is shown in Fig. 2b. *Artemia persimilis* representatives cluster into two groups, with sequences TDP2 and TDP3, from the Chilean side of the Andes, being more separated from the remaining Argentinean populations (bootstrap value 100%). Clear separation of *A. salina* is evident into eastern (WNA4, WNA5, LAR8, and LAR9) and western (SFA4, SFA9, SAN4, and SAN6) populations with a nodal support of 100%. Indel coding (Fig. 2a) has yielded a more robust separation of the *A. salina* clade (bootstrap value 100%) as opposed to the MP tree based on missing data (84%, Fig. 2b) or fifth state (tree not shown). This is presumably a result of putatively informative phylogenetic signals contained within the larger ITS1 region of *A. salina* as opposed to all other species. *A. urmiana* and *A. tibetiana* are invariably grouped with the cluster of parthenogenetic populations, while *A. sinica* is more closely related to parthenogens from China (JIA7) than to all remaining Asian bisexuals and parthenogenetic strains. Within parthenogens, three distinctive clusters are evident: the first one includes clones MAD4, TCL1 and TCL2 (closely related to *A. urmiana*), the second one includes the other representative from Madagascar (MAD6) together with the two representatives from Namibia (NAM1 and NAM5) and one isolate from Greece (POL14), while the third cluster includes the remaining Greek isolates (MEM4 and MEM9) and parthenogens from Israel (EIL1 and EIL2).

All previous groupings are faithfully retained irrespective of the tree building method. Maximum likelihood and Bayesian inferred topologies are shown in Figs. 3 and 4, respectively. Both methods yield a significantly better separation between *A. franciscana* from Great Salt Lake (GSL1 and GSL3) and the remaining sequences of this cluster compared with MP. All phylogenetic algorithms

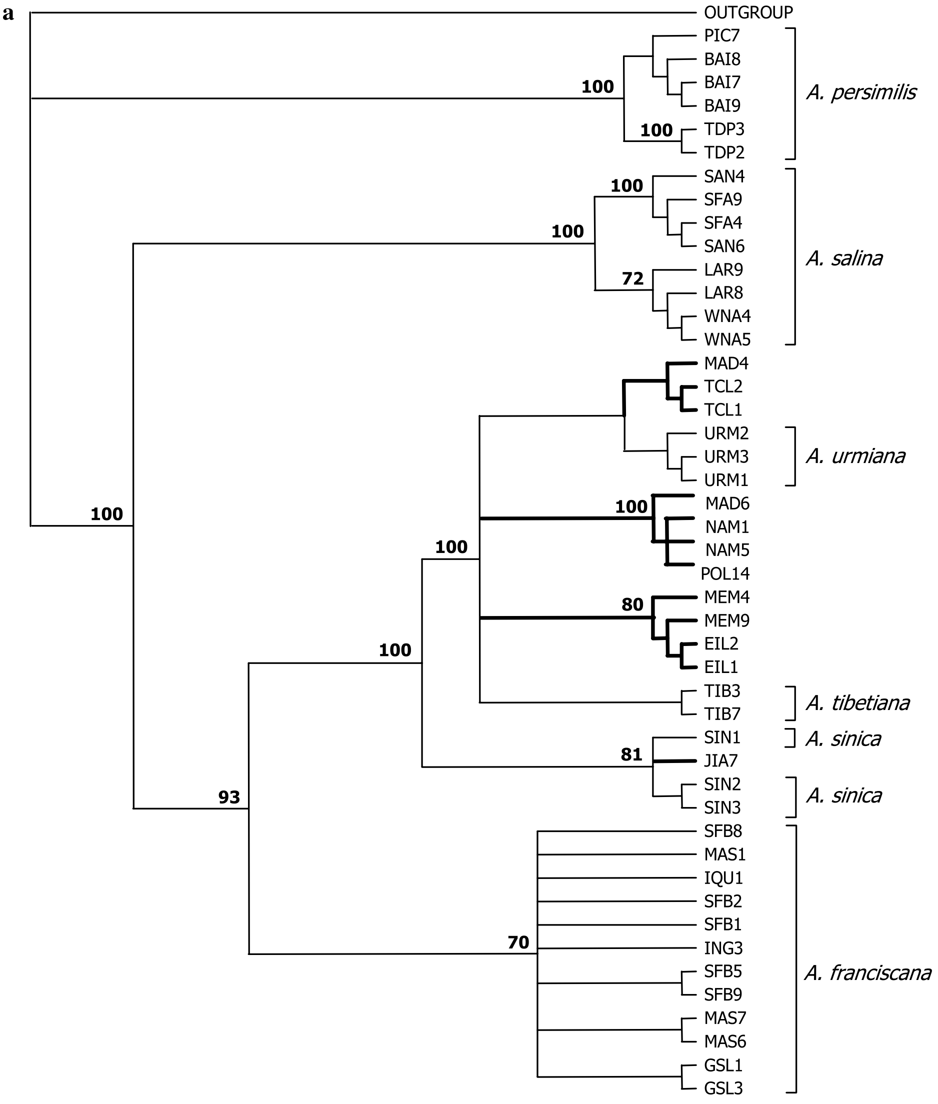

Fig. 2. Majority rule (50%) maximum parsimony trees derived from the ITS1 sequence data showing phylogenetic relationships among *Artemia* species. (a) Gaps were treated as separate binary characters (see section 2.3) according to "simple indel coding" by Simmons and Ochoterena (2000). (b) Gaps were treated as missing data. Nodal support is indicated above branches. Bootstrap values (1000 replicates) of less than 60% for internal nodes are not shown. Thickened lines indicate parthenogenetic lineages. The topology is rooted with the outgroup *Streptocephalus proboscideus*.

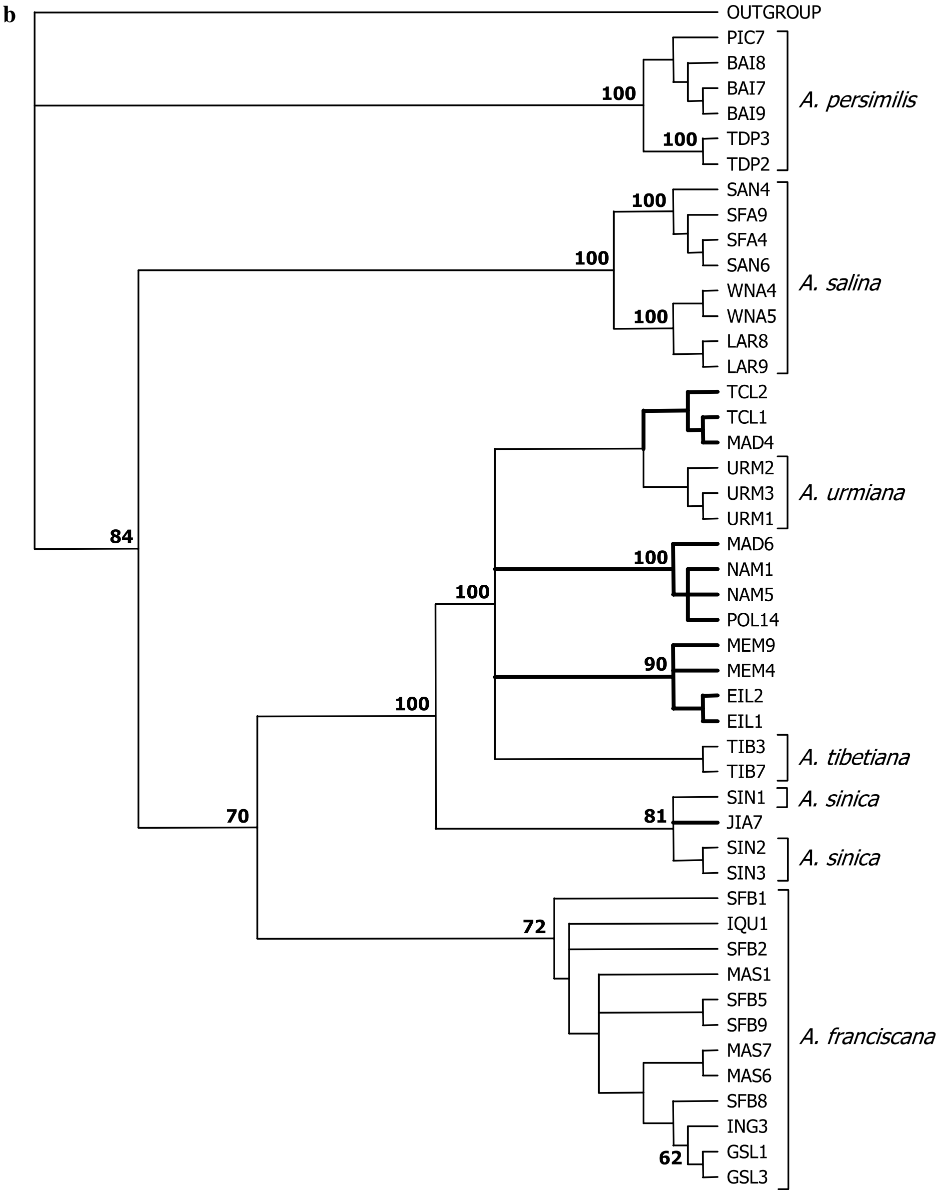

Fig. 2 (*continued*)

(MP, ML, and Bayesian) returned the same topology with *A. persimilis* as significantly differentiated from all the remaining species, *A. franciscana* invariably resolved as the sister group to Asian bisexuals and parthenogenetic *Artemia* and *A. sinica* as clearly divergent from the remaining Asian sexuals and the parthenogens (see Figs. 2a and b, 3 and 4).

The mtDNA phylogeny of a subset of taxa with corresponding ITS1 sequences is shown in Fig. 5. Although *A. persimilis*, *A. franciscana*, *A. salina*, and *A. sinica* are markedly differentiated, most parthenogens display identical mtDNA profiles with *A. urmiana* and *A. tibetiana*. Also, Greek parthenogenetic populations (POL and MEM) are grouped separately as they possess additional clones, two of which are private (D and G, see Table 3). Results of a Mantel test (Mantel, 1967) reveal a lack of association ($r = 0.56$, $P > 0.05$) between nuclear and mtDNA divergences for clones with complete ITS1 and 16S rRNA data. Thus, parthenogens with identical mtDNA clones (e.g. NAM, TCL, MAD, and EIL) are separated by ITS1 divergences ranging from 0.5 to 3.9% (data not shown). The reverse is also true: the ITS1 distance between MEM and POL isolates is 0.6% while the haplotype divergences of their component clones range from 1.1 to 6.6% (Table 3).

3.5. Age estimates

Rate constancy for the entire ITS1 data set was evaluated following the method by Takezaki et al. (1995). The branch length test was applied to an initial neighbor joining topology, based on the HKY85 + G model of substitution. The assumption of constant rates of molecular evolution

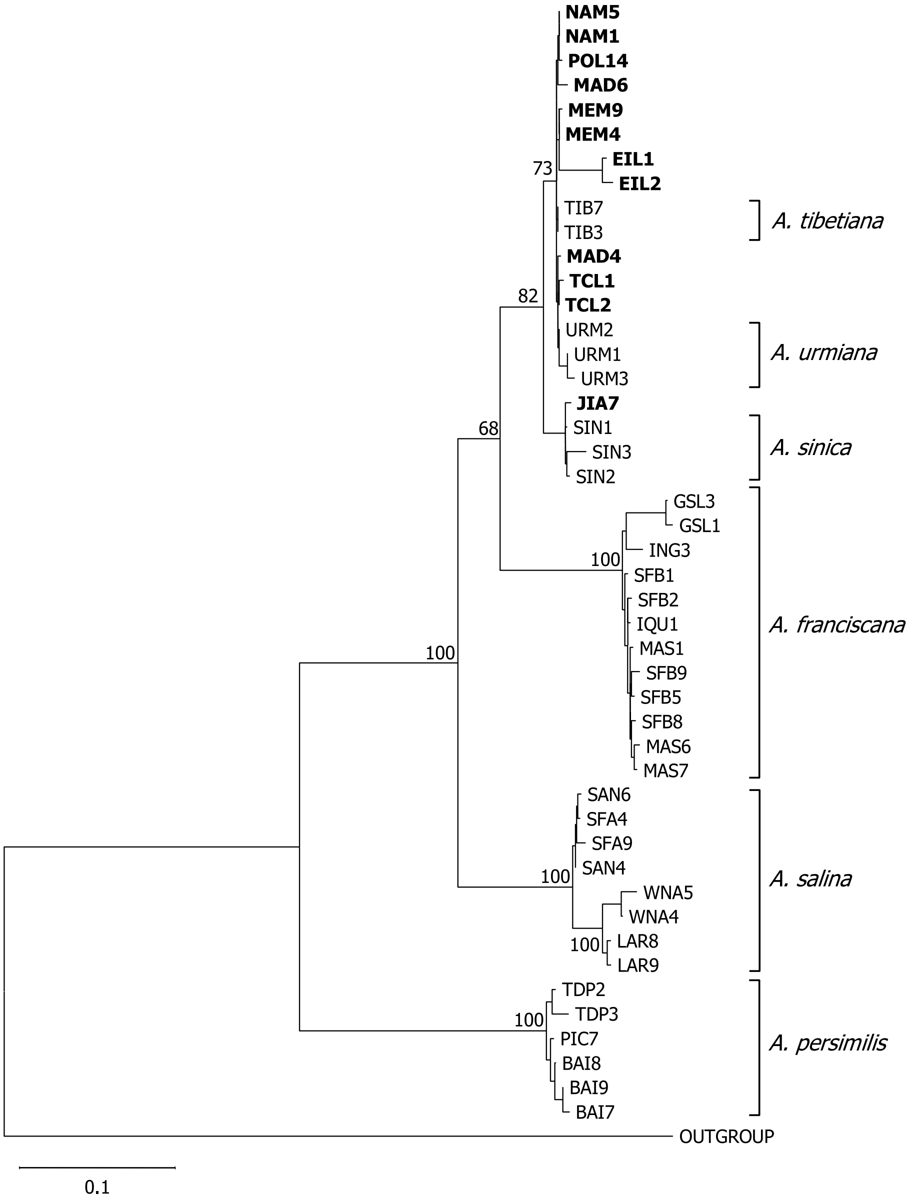

Fig. 3. Maximum likelihood phylogram ($-\ln L = 6720.69$) of *Artemia* species based on the HKY85 + G model of nucleotide substitution ($-\ln L = 6752.37$; $A = 0.24$, $C = 0.20$, $G = 0.28$, $T = 0.28$; number of substitution types $Nst = 2$; $T_S/T_V = 0.99$; γ-shape parameter $\alpha = 1.94$). Bootstrap values (100 replicates) of less than 60% for internal nodes are not shown. Boldface acronyms indicate parthenogenetic lineages. The topology is rooted with the outgroup *Streptocephalus proboscideus*.

was rejected ($U = 78.42$, $df = 45$, $P < 0.05$). Inspection of branch lengths resulted in exclusion of 10 sequences (WNA5, WNA4, EIL1, EIL2, SIN3, MAD6, ING3, TCL2, URM2, and TDP3) which invariably evolved significantly different than the average rate. It has to be noted that, a number of these sequences had already been recognized as suspect outliers with the disparity index test (see section 3.1). The U statistic for the remaining sequences was 32.53 ($P > 0.05$, $df = 35$) and therefore, the hypothesis of clocklike evolution was justified. A linearized tree was then constructed for the pruned data set (Fig. 6).

Estimates of times of divergence were based on a combination of phylogenetic signal within the data matrix and major/regional biogeographic events. The former included the extreme differentiation and restricted presence of *A. persimilis* in Argentina and Chile, the clustering of *A. salina* into eastern and western Mediterranean groups, and the partitioning of the whole genus on the basis of ITS1 length. The latter were related to the breakup of Gondwana (see Nei, 1987; Mayr, 2001), to geological and hydrographic events in the Mediterranean basin during the Messinian salinity crisis (Penzo et al., 1998; Hrbek and Meyer, 2003; Huyse et al., 2004), and to the geological history of the Tibetan plateau. Hence, the calibration of *A. persimilis* divergence from the common ancestor was set to the time corresponding to the severance of South America from Gondwana at the middle Cretaceous (80–90 MYA). This yields a rate of sequence divergence of 0.2%/MYA within

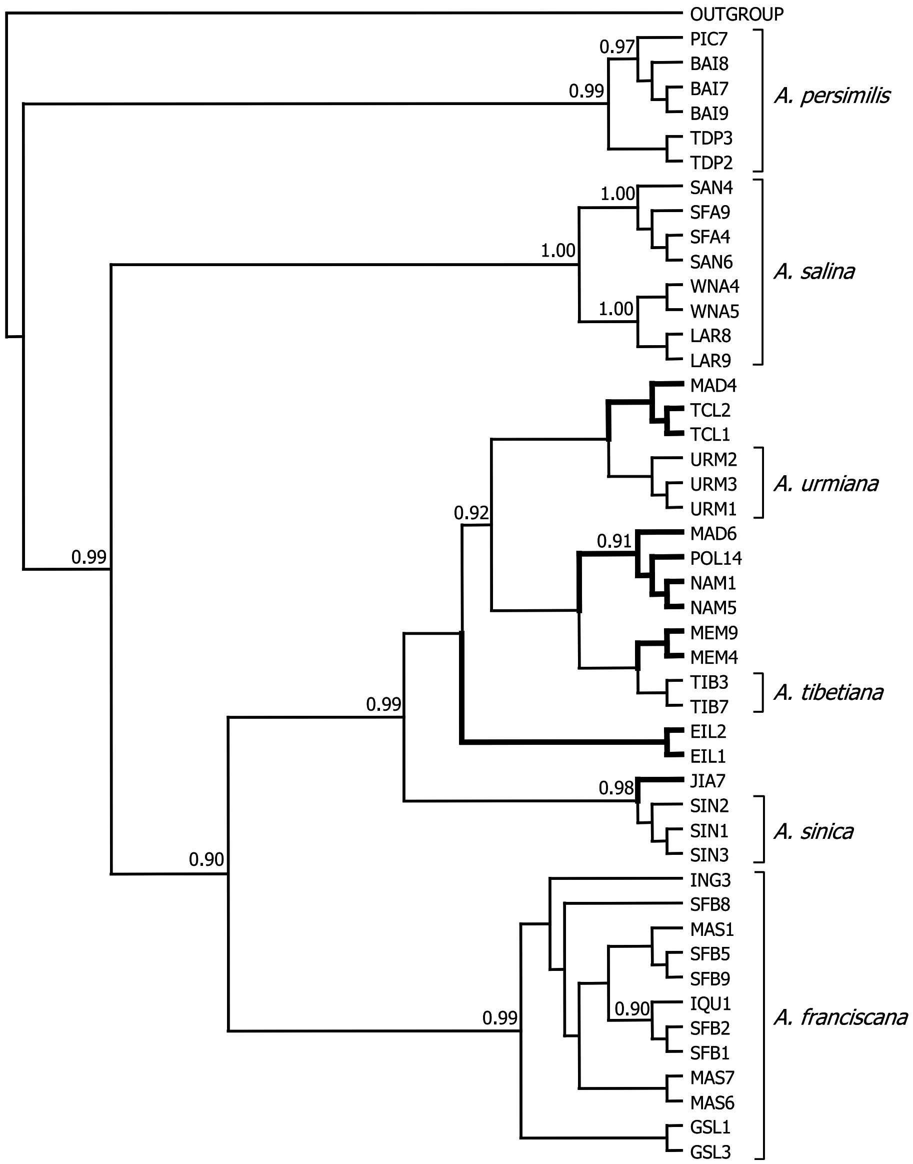

Fig. 4. Bayesian inference topology of phylogenetic relationships in the genus *Artemia*. Sequence evolution is based on the HKY85 + G model. In two simultaneous runs, four Markov chains (three heated and one cold) were started from a random tree and run for 1,000,000 generations with sampling frequency every 100 generations. First 2000 trees were discarded as burn-in. Posterior probabilities higher than 0.90 are shown. Thickened lines indicate parthenogenetic lineages. The topology is rooted with the outgroup *Streptocephalus proboscideus*.

each lineage. *Artemia salina* seems to have diverged ca. 40 MYA, at about the time of emergence of the Mediterranean Sea, while its separation into eastern and western groups (ca. 7 MYA) roughly coincides with the beginning of the salinity crisis and subsequent fragmentation of the Mediterranean. Asian bisexuals and parthenogens last shared a common ancestor with *A. franciscana* 32 MYA, at the timeframe (early Tertiary) of a broad land connection between North America and Europe (Mayr, 2001). Within the Asian clade of bisexuals, *A. sinica* branched off the remaining groups 8 MYA, within a period for which geological and thermochronological evidence (Clark et al., 2005; Molnar, 2005) demarcate the elevation of the Tibetan plateau and subsequent separation of eastern and western landscapes. Considering our sampling effort, at least four independent origins of parthenogenesis are deduced, the most ancient of which dates at 3.5 MYA (*A. sinica* vs JIA7). The ages of the remaining parthenogenetic lineages could not be estimated since monophyletic arrangements were uncertain.

4. Discussion

The current study is the first robust, global phylogeny of the genus *Artemia*. Vicariance and pronounced ecological diversification seem to have shaped current biogeographic patterns to a great extent. The parthenogenetic gene pool is a heterogeneous amalgam of clones, including both narrow endemics and widespread lineages of distinctive spatial and temporal origins and genetic diversity. Considering the

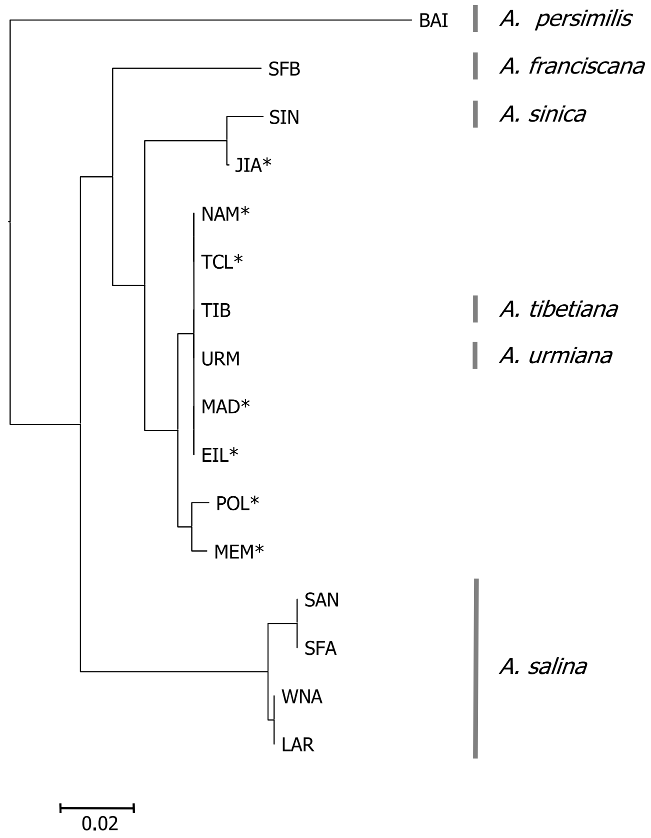

Fig. 5. NJ phylogram of *Artemia* populations assayed for 16S mtDNA RFLP patterns. The tree is based on nucleotide divergence estimates from restriction sites data. Asterisks indicate parthenogenetic populations.

diversity of life cycles and modes of reproduction in zooplanktonic invertebrates, our study provides additional insights into patterns of distribution in the continental aquatic realm and, in particular, for halophilic lineages that have made the transition to asexuality. It also corroborates the antiquity of anostracan members at the lower to middle Cretaceous (see Remigio and Hebert, 2000 and references therein; Daniels et al., 2004) and yields evidence that their historical biogeography may have been affected by common geological landmarks.

The phylogenetic signal present in ITS1 has proven sufficient in delineating *Artemia* species and, in some cases, detecting substantial differentiation at the intraspecific level. The observed interspecific divergence estimates (zero to 39.5%) are within the range reported for other continental aquatic crustaceans of Gondwana origin. For example, in the fairy shrimp genus *Streptocephalus*, interspecific values based on 5.8S–ITS1–18S sequence divergence vary from 0.90 to 90% (Daniels et al., 2004). However, lower ITS1 estimates, in the range of 15–21%, have been obtained for *Darwinula* (Schön et al., 1998). Regarding the intraspecific level, our estimate of 2.0% sequence diversity within parthenogenetic *Artemia* is striking when compared with *Darwinula stevensoni*. This ostracod is believed to be reproducing asexually for millions of years, however no ITS1 variability was detected in a sampling range spanning from Finland to South Africa (Schön et al., 1998). A slow down in the rate of molecular evolution for ITS1 has been hypothesized to explain such extensive invariability. In

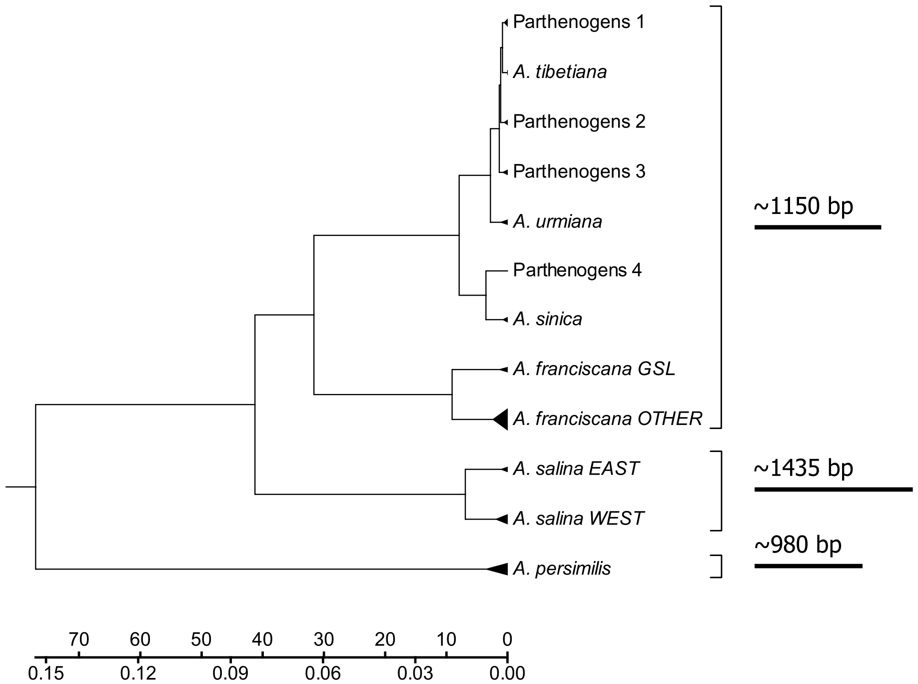

Fig. 6. Linearized tree of *Artemia* ITS1 sequences. Ten sequences evolving significantly different than the average rate at the 0.05 level were excluded (WNA5, WNA4, EIL1, EIL2, SIN3, MAD6, ING3, TCL2, URM2, and TDP3) and branch lengths were re-estimated following Takezaki et al. (1995). Triangles at the tips of the tree denote grouping of sequences for the purposes of illustration. The size of triangles is proportional to within-group divergence. Brackets indicate ITS1 length variation among clades. Values above and below the scale bar are MYA and sequence divergence, respectively. Parthenogens 1, POL14; NAM1; NAM5; Parthenogens 2, MEM4; MEM9; Parthenogens 3, MAD4; TCL1; and Parthenogens 4, JIA7.

our study, the obtained rate of 0.2%/MYA is generally higher than that of most Darwinulidae lineages with an exceptional fossil record (Schön et al., 2003) and may be a consequence of brine life as suggested by Hebert et al. (2002) for a number of halophilic lineages including *Artemia*. Apart from its mode and rate of evolution, ITS1 has been used for estimating phylogenies at different hierarchical levels (Harris and Crandall, 2000; Weekers et al., 2001; Chaw et al., 2005). Although ITS1 length variation has been reported in a number of organisms (Schlötterer et al., 1994; Von der Schulenburg et al., 2001), our results represent an unusual case of both increased size (compared with the ITS1 length of other anostracans, see Daniels et al., 2004) and clade-specific length variation within a single organismal lineage. Thus, apart from its phylogenetic utility, ITS1 may also serve as a crude marker for identification of unknown *Artemia* isolates. For example, isolates MAS1, MAS6, and MAS7 from Italy were confirmed as belonging to *A. franciscana* introduced samples (see Mura et al., 2004) while the species status (also *A. franciscana*) of the unknown ING3 isolate from Ingebright, Canada (see Table 1) was clarified. Furthermore, detailed investigations of ITS1 structure should provide useful insights into the origin, nature and possible association to high sequence change of ITS1 elements.

The clade of *A. persimilis* diverged from the ancestral stock of Gondwana populations as a result of the tectonic separation of South America from Africa. The intraspecific component of diversity within *A. persimilis* is in agreement with previous analyses based on mtDNA markers (Gajardo et al., 2004). The evolutionary route of *A. salina* seems to be closely related to the geologic history of the Mediterranean basin. The substantial differentiation into eastern and western *A. salina* groups, also strongly supported by AFLP analysis (Triantaphyllidis et al., 1997), is in accordance with the expansion of land and extensive desiccation of the Mediterranean Sea during the Messinian salinity crisis (see Penzo et al., 1998). The area of land exposure between southern Turkey and Egypt maps well with the eastern *A. salina* cluster, while that between the Gulf of Sidra and the Strait of Sicily coincides with the western *A. salina* cluster. Unlike previous studies (Abreu-Grobois, 1987) indicating *A. franciscana* as the most variable and sub-structured species, our results point out that genetic diversity within *A. salina* may be at least 30% higher (see Table 2). Considering the island-like nature and abiotic variability of *Artemia* habitats, interpopulational differences may be further enhanced and established rapidly, thus generating geographic population structure and leading to evolutionary groups with distinct ecological and life history profiles (Kappas et al., 2004).

Although the reconstructed phylogeny is concordant with well-established geological events, a degree of uncertainty exists over the topological placement of *A. franciscana*, which is unconventional to current phylogenetic theory in *Artemia*. The affinity of *A. franciscana* with all Asian *Artemia* and parthenogens is geologically problematic, yet recurring. RAPD analysis (Sun et al., 1999) has shown that the genetic distance between *A. sinica* and parthenogenetic *Artemia* is equal to that between *A. franciscana* and coastal Chinese parthenogens. Phylogenetic reconstruction based on COI and Cytb sequences (Perez et al., 1994) has indicated that *A. franciscana* shares a more recent relationship with parthenogenetic *Artemia* than with *A. salina*. More importantly, though, aspects of phenotypic evolution in the genus lend credence to the observed relationship between *A. franciscana* and Asian-parthenogenetic lineages. Analysis of morphological characters (Triantaphyllidis et al., 1997) has shown that a pair of characteristic spine-like outgrowths at the basal part of each penis is present in all bisexual *Artemia* except *A. salina*. This character is also absent from male specimens of the extinct population from Lymington, UK (collection of the Natural History Museum of London). Most remarkably, these structures are present in rare males (Mura and Brecciaroli, 2002) known to originate from asexual forms. The same groupings are also revealed by examination of the bulges at either side of the base of the ovisac (absent in *A. salina*) (Amat, 1980) and the morphology of frontal knobs (subconical in *A. salina* as opposed to spherical in all other species) (Mura and Brecciaroli, 2004). These phenotypic patterns cannot be interpreted as primitive absences but rather as secondary losses or modifications in conjunction with a parallel ecological shift (low temperature tolerance, see Browne, 1992) in *A. salina*. The latter two characters, in particular, are relevant to reproductive morphology as they may determine the mechanical configuration of the couple during pairing. Our hypothesis and evolutionary timescales imply that the conflicting relationship between *A. franciscana* and Asian-parthenogenetic lineages may be resolved by invoking phylogenetic explanations analogous to other zooplanktonic invertebrates (see Gómez et al., 2002; De Queiroz, 2005) and as yet unconsidered in *Artemia*. Although our results for *A. franciscana* may be regarded as provisional, they strongly suggest that current thinking on the primary separation of New and Old World lines may require a substantial re-evaluation.

The present study highlights the benefits of *Artemia* as a model organism for investigating the interaction between sexuality and parthenogenesis. On the basis of nuclear DNA sequences and cytoplasmic markers, a genetically diverse assemblage of parthenogens is inferred, in close affinity with Asian bisexuals (*A. sinica*, *A. urmiana*, and *A. tibetiana*). Although bootstrap values within the Asian-parthenogenetic clade are relatively wide for ITS1 (<60 to 100%), both nuclear and mtDNA phylogenies are consistent with a polyphyletic origin of parthenogens and suggest that hypotheses of a single origin (Beardmore and Abreu-Grobois, 1983) are incorrect. All Asian bisexuals are genetically distinct, at least for nuclear DNA (see also Abatzopoulos et al., 2002b), and are implicated in transitions to asexuality. For example, the presence of a common haplotype (C, see Table 3) indicates that a number of clones may have captured mtDNA from *A. urmiana* or *A. tibetiana*. This implies that, for some genetic or ecological reasons, loss of sex is more frequent in the Old World. Due to the high nodal support from their closest sexual progenitor (*A. sinica*), only Chinese parthenogens (JIA7) could be confidently dated, bearing an age of 3.5 MYA. Real antiquity can be debatable (Martens et al., 2003) and several authors (Law and Crespi, 2002 and references therein) have proposed that generation time may be biologically more relevant than chronological time in assessing the evolutionary implications of asexual reproduction. Taking into account that *Artemia* completes an average of 10 generations per year, a conservative estimate of 30,000,000 generations is obtained. Escaping mutational meltdown (Lynch et al., 1993) and extinction in such a generation-span may be mediated by a combination of three mechanisms, all present in *Artemia*: large population sizes, protection provided by the dormant stage and polyploidy. It is therefore conceivable that apomictic clones of *Artemia* form monophyletic groups with their closest sexual ancestors that are at least as old as the estimated divergence between *A. sinica* and the parthenogenetic population from China. A possibility exists, however, that this timeframe is not accurately estimated as sampling of Chinese parthenogens was rather limited. Overestimation seems more unlikely though, since intraspecific diversity in *A. sinica* is known to be insignificant (Naihong et al., 2000) and at the order of magnitude determined here, unless of course a more recent sexual progenitor has gone extinct.

A conclusion that can, with a certain degree of cautiousness, be extended to all parthenogenetic representatives, regardless of their sources of origin and ages, is that their observed genetic diversity of 2.0% (see Table 2) is not a reflection of the diversity of their closest bisexual ancestors. At least for a common group of clones tested here, matrix comparison of nuclear and mtDNA divergences strengthens the above reasoning. The lack of association between the diversities found within parthenogens and their nearest sexuals relatives, if verified, may provide clues to the mode of origin of parthenogenetic populations and designate intra- or interspecific hybridization as possible candidates (Schön et al., 2000; Delmotte et al., 2001, 2003). In the first case, the role of parthenogenetically produced rare males will be crucial and concrete data on their functionality and reproductive potential will be needed (Browne, 1992; Simon et al., 2003). On the other hand, interspecific hybridization in the strict sense (crosses between bisexual species) would likely provide new insights into the distribution of parthenogenetic lineages, since their occurrence should, to a greater or a lesser extent, be associated with the distributions of their sexual parentals. In the long term, however, physiological and ecological tolerances as well as genetic architecture (ploidy level, parthenogenetic mechanism of reproduction, i.e. apomixis or automixis) may also determine niche breadth of asexuals (Zhang and Lefcort, 1991). Although studies have reported marked ecological diversification among natural clones of *Artemia* (Browne and Hoopes, 1990; Baxevanis and Abatzopoulos, 2004), there is no evidence favouring either a general purpose or a specialist genotype. Our data also point to no specific direction, but a prediction made by Vrijenhoek (1998) for the possibility of intermediate clones occupying a narrow but universally available niche seems very promising in this case. This may also better explain competition between asexuals and their sexual relatives as well as the enigmatic absence, or at least unverified existence, of parthenogens from the New World.

Acknowledgments

This research was partly supported by EU project ICA4-CT-2001-10020 (INCO) on *Artemia* biodiversity. We are grateful to Nadia Fahmi for fast and accurate text editing. We thank Dr. G. Giribet, Dr. D. Mark Welch and an anonymous reviewer for constructive comments that improved substantially the manuscript.

References

Abatzopoulos, T.J., Kastritsis, C.D., Triantaphyllidis, C.D., 1986. A study of karyotypes and heterochromatic associations in *Artemia*, with special reference to two N. Greek populations. Genetica 71, 3–10.

Abatzopoulos, T.J., Beardmore, J.A., Clegg, J.S., Sorgeloos, P., 2002a. *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Abatzopoulos, T.J., Kappas, I., Bossier, P., Sorgeloos, P., Beardmore, J.A., 2002b. Genetic characterization of *Artemia tibetiana* (Crustacea: Anostraca). Biol. J. Linn. Soc. 75, 333–344.

Abreu-Grobois, F.A., 1987. A review of the genetics of *Artemia*. In: Sorgeloos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), *Artemia* Research and its Applications, vol. 1. Morphology, Genetics, Strain Characterization, Toxicology. Universa Press, Wetteren, Belgium, pp. 61–99.

Amat, F., 1980. Differentiation in *Artemia* strains from Spain. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), The Brine Shrimp *Artemia*, vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Universa Press, Wetteren, Belgium, pp. 19–39.

Avise, J.C., 1994. Molecular Markers Natural History and Evolution. Chapman and Hall, New York.

Bailey, T.L., Elkan, C., 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In: Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, California, pp. 28–36.

Baker, H.G., 1965. Characteristics and modes of origin of weeds. In: Baker, H.G., Stebbins, G.L. (Eds.), Genetics of Colonizing Species. Academic Press, New York, pp. 147–172.

Baxevanis, A.D., Abatzopoulos, T.J., 2004. The phenotypic response of ME_2 (M. Embolon, Greece) *Artemia* clone to salinity and temperature. J. Biol. Res. 1, 107–114.

Baxevanis, A.D., Triantaphyllidis, G.V., Kappas, I., Triantafyllidis, A., Triantaphyllidis, C.D., Abatzopoulos, T.J., 2005. Evolutionary assessment of *Artemia tibetiana* (Crustacea, Anostraca) based on morphometry and16S rRNA RFLP analysis. J. Zool. Syst. Evol. Res. 43, 189–198.

Beardmore, J.A., Abreu-Grobois, F.A., 1983. Taxonomy and evolution in the brine shrimp *Artemia*. In: Oxford, G.S., Rollinson, D. (Eds.), Protein Polymorphism: Adaptive and Taxonomic Significance. Academic Press, London, pp. 153–164.

Bena, G., Prosperi, J.-M., Lejeune, B., Olivieri, I., 1998. Evolution of annual species of the genus *Medicago*: a molecular phylogenetic approach. Mol. Phylogenet. Evol. 9, 552–559.

Browne, R.A., 1992. Population genetics and ecology of *Artemia*: insights into parthenogenetic reproduction. Trends. Ecol. Evol. 7, 232–237.

Browne, R.A., Hoopes, C.W., 1990. Genotypic diversity and selection in asexual brine shrimp (*Artemia*). Evolution 44, 1035–1051.

Chaplin, J.A., Hebert, P.D.N., 1997. *Cyprinotus incongruens* (Ostracoda): an ancient asexual? Mol. Ecol. 6, 155–168.

Chaw, S.-M., Walters, T.W., Chang, C.-C., Hu, S.-H., Chen, S.-H., 2005. A phylogeny of cycads (Cycadales) inferred from chloroplast *matK* gene, *trnK* intron, and nuclear rDNA ITS region. Mol. Phylogenet. Evol. 37, 214–234.

Clark, M.K., House, M.A., Royden, L.H., Whipple, K.X., Burchfiel, B.C., Zhang, X., Tang, W., 2005. Late Cenozoic uplift of southeastern Tibet. Geology 33, 525–528.

Coleman, M., Matthews, C.M., Trotman, C.N.A., 2001. Multimeric hemoglobin of the Australian brine shrimp *Parartemia*. Mol. Biol. Evol. 18, 570–576.

Crease, T.J., 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). Gene 233, 89–99.

Crease, T.J., Stanton, D.J., Hebert, P.D.N., 1989. Polyphyletic origins of asexuality in *Daphnia pulex* II. mitochondrial-DNA variation. Evolution 43, 1016–1026.

Daniels, S.R., Hamer, M., Rogers, C., 2004. Molecular evidence suggests an ancient radiation for the fairy shrimp genus *Streptocephalus* (Branchiopoda: Anostraca). Biol. J. Linn. Soc. 82, 313–327.

Delmotte, F., Leterme, N., Bonhomme, J., Rispe, C., Simon, J.-C., 2001. Multiple routes to asexuality in an aphid species. Proc. R. Soc. Lond. B 268, 2291–2299.

Delmotte, F., Sabater-Muñoz, B., Prunier-Leterme, N., Latorre, A., Sunnucks, P., Rispe, C., Simon, J.-C., 2003. Phylogenetic evidence for hybrid origins of asexual lineages in an aphid species. Evolution 57, 1291–1303.

De Meester, L., Gómez, A., Okamura, B., Schwenk, K., 2002. The monopolization hypothesis and the dispersal-gene flow paradox in aquatic organisms. Acta Oecol. 23, 121–135.

De Queiroz, A., 2005. The resurrection of oceanic dispersal in historical biogeography. Trends Ecol. Evol. 20, 68–73.

Estoup, A., Largiadèr, C.R., Perrot, E., Chourrout, D., 1996. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. Mol. Mar. Biol. Biotech. 5, 295–298.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package). Version 3.6b. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Gajardo, G., Crespo, J., Triantafyllidis, A., Tzika, A., Baxevanis, A.D., Kappas, I., Abatzopoulos, T.J., 2004. Species identification of Chilean *Artemia* populations based on mitochondrial DNA RFLP analysis. J. Biogeogr. 31, 547–555.

Giribet, G., Wheeler, W.C., 1999. On gaps. Mol. Phylogenet. Evol. 13, 132–143.

Gómez, A., Serra, M., Carvalho, G.R., Lunt, D.H., 2002. Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). Evolution 56, 1431–1444.

Green, A.J., Sánchez, M.I., Amat, F., Figuerola, J., Hontoria, F., Ruiz, O., Hortas, F., 2005. Dispersal of invasive and native brine shrimps *Artemia* (Anostraca) via waterbirds. Limnol. Oceanogr. 50, 737–742.

Hairston, N.G., De Stasio Jr., B.T., 1988. Rate of evolution slowed by a dormant propagule pool. Nature 336, 239–242.

Harris, D.J., Crandall, K.A., 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. Mol. Biol. Evol. 17, 284–291.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hebert, P.D.N., Remigio, E.A., Colbourne, J.K., Taylor, D.J., Wilson, C.C., 2002. Accelerated molecular evolution in halophilic crustaceans. Evolution 56, 909–926.

Hillis, D.M., Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66, 411–453.

Hrbek, T., Meyer, A., 2003. Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). J. Evol. Biol. 16, 17–36.

Hsü, K.J., 1972. When the Mediterranean dried up. Sci. Am. 227, 26–36.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Hurst, L.D., Peck, J.R., 1996. Recent advances in understanding of the evolution and maintenance of sex. Trends Ecol. Evol. 11, 46–52.

Huyse, T., Van Houdt, J., Volckaert, F.A.M., 2004. Paleoclimatic history and vicariant speciation in the "sand goby" group (Gobiidae, Teleostei). Mol. Phylogenet Evol. 32, 324–336.

Judson, O.P., Normark, B.B., 1996. Ancient asexual scandals. Trends Ecol. Evol. 11, 41–46.

Kappas, I., Abatzopoulos, T.J., Van Hoa, N., Sorgeloos, P., Beardmore, J.A., 2004. Genetic and reproductive differentiation of *Artemia franciscana* in a new environment. Mar. Biol. 146, 103–117.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655.

Kumar, S., Gadagkar, S.R., 2001. Disparity index: a simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. Genetics 158, 1321–1327.

Law, J.H., Crespi, B.J., 2002. Recent and ancient asexuality in *Timema* walkingsticks. Evolution 56, 1711–1717.

Lynch, M., Bórger, R., Butcher, D., Gabriel, W., 1993. The mutational meltdown in asexual populations. J. Hered. 84, 339–344.

Maley, L.E., Marshall, C.R., 1998. The coming of age of molecular systematics. Science 279, 505–506.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.

Mark Welch, D., Meselson, M., 2000. Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science 288, 1211–1215.

Martens, K., Rossetti, G., Horne, D.J., 2003. How ancient are ancient asexuals? Proc. R. Soc. Lond. B 270, 723–729.

Mayr, E., 2001. What Evolution Is? Basic Books, New York.

McElroy, D., Moran, P., Bermingham, E., Kornfield, I., 1992. REAP—the restriction enzyme analysis package. J. Hered. 83, 157–158.

Molnar, P., 2005. Mio-Pliocene growth of the Tibetan plateau and evolution of east Asian climate. Palaeontologica Electronica 8.1.2 <http://palaeo-electronica.org/paleo/2005_1/molnar2/issue1_05.htm>.

Mura, G., Brecciaroli, B., 2002. Use of morphological characters as a tool for species separation. Regional Workshop on *Artemia* Biodiversity (INCO Concerted Action). Beijing, China.

Mura, G., Brecciaroli, B., 2004. Use of morphological characters for species separation within the genus *Artemia* (Crustacea, Branchiopoda). Hydrobiologia 520, 179–188.

Mura, G., Amat, F., Abatzopoulos, T.J., Moscatello, S., 2004. First record of *Artemia franciscana* in an Italian saltwork. Fifth International Large Branchiopod Symposium. Toodyay, Western Australia.

Mura, G., Baxevanis, A.D., Lopez, G.M., Hontoria, F., Kappas, I., Moscatello, S., Fancello, G., Amat, F., Abatzopoulos, T.J., 2005. The use of a multidisciplinary approach for the characterization of a diploid parthenogenetic *Artemia* population from Torre Colimena (Apulia, Italy). J. Plank. Res. 27, 895–907.

Naihong, X., Audenaert, E., Vanoverbeke, J., Brendonck, L., Sorgeloos, P., De Meester, L., 2000. Low among-population genetic differentiation in Chinese bisexual *Artemia* populations. Heredity 84, 238–243.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M., Tajima, F., 1981. DNA polymorphism detectable by restriction endonucleases. Genetics 97, 145–163.

Nei, M., Miller, J.C., 1990. A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. Genetics 125, 873–879.

Normark, B.B., Moran, N.A., 2000. Testing for the accumulation of deleterious mutations in asexual eukaryote genomes using molecular sequences. J. Nat. Hist. 34, 1719–1729.

Normark, B.B., Judson, O.P., Moran, N.A., 2003. Genomic signatures of ancient asexual lineages. Biol. J. Linn. Soc. 79, 69–84.

Paland, S., Colbourne, J.K., Lynch, M., 2005. Evolutionary history of contagious asexuality in *Daphnia pulex*. Evolution 59, 800–813.

Palumbi, S.R., 1996. Nucleic acids II: the polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics. Sinauer Associates, Sunderland, MA, pp. 205–247.

Penzo, E., Gandolfi, G., Bargelloni, L., Colombo, L., Patarnello, T., 1998. Messinian salinity crisis and the origin of freshwater lifestyle in western Mediterranean gobies. Mol. Biol. Evol. 15, 1472–1480.

Perez, M.L., Valverde, J.R., Batuecas, B., Amat, F., Marco, R., Garesse, R., 1994. Speciation in the *Artemia* genus: mitochondrial DNA analysis of bisexual and parthenogenetic brine shrimps. J. Mol. Evol. 38, 156–168.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Remigio, E.A., Hebert, P.D.N., 2000. Affinities among Anostracan (Crustacea: Branchiopoda) families inferred from phylogenetic analyses of multiple gene sequences. Mol. Phylogenet. Evol. 17, 117–128.

Sáez, A.G., Escalante, R., Sastre, L., 2000. High DNA sequence variability at the α1 Na/K-ATPase locus of *Artemia franciscana* (brine shrimp): polymorphism in a gene for salt-resistance in a salt-resistant organism. Mol. Biol. Evol. 17, 235–250.

Sandoval, C., Carmean, D.A., Crespi, B.J., 1998. Molecular phylogenetics of sexual and parthenogenetic *Timema* walking-sticks. Proc. R. Soc. Lond. B. 265, 589–595.

Schlötterer, C., Hauser, M.-T., Von Haeseler, A., Tautz, D., 1994. Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. Mol. Biol. Evol. 11, 513–522.

Schön, I., Martens, K., Rossi, V., 1996. Ancient asexuals: scandal or artefact? Trends Ecol. Evol. 11, 296–297.

Schön, I., Butlin, R.K., Griffiths, H.I., Martens, K., 1998. Slow molecular evolution in an ancient asexual ostracod. Proc. R. Soc. Lond. B. 265, 235–242.

Schön, I., Gandolfi, A., Di Masso, E., Rossi, V., Griffiths, H.I., Martens, K., Butlin, R.K., 2000. Persistence of asexuality through mixed reproduction in *Eucypris virens* (Crustacea, Ostracoda). Heredity 84, 161–169.

Schön, I., Martens, K., Van Doninck, K., Butlin, R.K., 2003. Evolution in the slow lane: molecular rates of evolution in sexual and asexual ostracods (Crustacea: Ostracoda). Biol. J. Linn. Soc. 79, 93–100.

Schuler, G.D., Altschul, S.F., Lipman, D.J., 1991. A workbench for multiple alignment construction and analysis. Proteins: Structure, Function, and Genetics 9, 180–190.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Simon, J.-C., Delmotte, F., Rispe, C., Crease, T., 2003. Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. Biol. J. Linn. Soc. 79, 151–163.

Sun, Y., Zhong, Y.-C., Song, W.-Q., Zhang, R.-S., Chen, R.-Y., 1999. Detection of genetic relationships among four *Artemia* species using randomly amplified polymorphic DNA (RAPD). Int. J. Salt Lake Res. 8, 139–147.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Takezaki, N., Rzhetsky, A., Nei, M., 1995. Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. 12, 823–833.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Triantaphyllidis, G.V., Abatzopoulos, T.J., Sandaltzopoulos, R.M., Stamou, G., Kastritsis, C.D., 1993. Characterization of two *Artemia* populations from two solar saltworks of Lesbos island (Greece): biometry, hatching characteristics and fatty acid profile. Int. J. Salt Lake Res. 2, 59–68.

Triantaphyllidis, G.V., Abatzopoulos, T.J., Miasa, E., Sorgeloos, P., 1996. International Study on *Artemia*. LVI. Characterization of two *Artemia* populations from Namibia and Madagascar: cytogenetics, biometry, hatching characteristics and fatty acid profiles. Hydrobiologia 335, 97–106.

Triantaphyllidis, G.V., Criel, G.R.J., Abatzopoulos, T.J., Thomas, K.M., Peleman, J., Beardmore, J.A., Sorgeloos, P., 1997. International Study on *Artemia*. LVII. Morphological and molecular characters suggest conspecificity of all bisexual European and North African *Artemia* populations. Mar. Biol. 129, 477–487.

Triantaphyllidis, G.V., Abatzopoulos, T.J., Sorgeloos, P., 1998. Review of the biogeography of the genus *Artemia* (Crustacea, Anostraca). J. Biogeogr. 25, 213–226.

Young, N.D., Healy, J., 2003. GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4, 6 <http://www.biomedcentral.com/1471-2105/4/6>.

Valverde, J.R., Batuecas, B., Moratilla, C., Marco, R., Garesse, R., 1994. The complete mitochondrial DNA sequence of the crustacean *Artemia franciscana*. J. Mol. Evol. 39, 400–408.

Van Stappen, G., 2002. Zoogeography. In: Abatzopoulos, T.J., Beardmore, J.A., Clegg, J.S., Sorgeloos, P. (Eds.), *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 171–224.

Vaughn, J.C., Sperbeck, S.J., Hughes, M.J., 1984. Molecular cloning and characterization of ribosomal RNA genes from the brine shrimp: nucleotide sequence analysis and evolution of the 5.8S rRNA gene region and its flanking nucleotides. Biochim Biophys Acta 783, 144–151.

Von der Schulenburg, J.H.G., Hancock, J.M., Pagnamenta, A., Sloggett, J.J., Majerus, M.E.N., Hurst, G.D.D., 2001. Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol. Biol. Evol. 18, 648–660.

Vrijenhoek, R.C., 1998. Animal clones and diversity. BioScience 48, 617–628.

Weekers, P.H.H., De Jonckheere, J.F., Dumont, H.J., 2001. Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographic patterns in representatives of the genus *Calopteryx* (Insecta: Odonata) of the west Mediterranean and adjacent west European zone. Mol. Phylogenet. Evol. 20, 89–99.

Weekers, P.H.H., Murugan, G., Vanfleteren, J.R., Belk, D., Dumont, H.J., 2002. Phylogenetic analysis of anostracans (Branchiopoda: Anostraca) inferred from nuclear 18S ribosomal DNA (18S rDNA) sequences. Mol. Phylogenet. Evol. 25, 535–544.

Zhang, L., Lefcort, H., 1991. The effects of ploidy level on the thermal distributions of brine shrimp *Artemia parthenogenetica* and its ecological implications. Heredity 66, 445–452.