



A note on the biogeographical origin of the brine shrimp *Artemia urmiana* Günther, 1899 from Urmia Lake, Iran

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Abstract

The brine shrimp *Artemia urmiana*, an abundant inhabitant of the hypersaline Urmia Lake in northwestern Iran, has recently been described from Lake Koyashskoe, also a shallow hypersaline lake that is located on the Black Sea coast of the Crimean Peninsula (Ukraine). This discovery has questioned the endemism of *A. urmiana* in Urmia Lake and has also brought into question the biogeographical origin of this species. In the present study, we combined recent genetic divergence data (mtDNA-*COI*) with palaeoecological evidence to address the biogeographical origin of *A. urmiana*. Calibration of the molecular clock of the *COI* region was set by assigning the age of the micro-crustacean *Daphnia pulex* minimally at 145 Mya. The divergence age of *A. urmiana* in Urmia Lake dates back to 383,000 years, whereas Ukrainian *Artemia* reflects a very young populations that diverged about 196,000 years ago. Palaeoecological evidence suggests that the age of the major habitat of *A. urmiana* i.e. Urmia Lake goes back to the Tertiary Period while the Ukrainian habitats of the species are very young, by virtue of geological features of the Holocene age. We conclude that the biogeographical origin of *A. urmiana* is outside of Europe and the current state of knowledge strongly suggests that Urmia Lake has been the major source of its expansion into its modern habitats in Europe.

Key words: Biogeographical, origin, *Artemia urmiana*, Iran, Ukraine

Introduction

Brine shrimps in the genus *Artemia* (Anostraca, Crustacea) are cosmopolitan zooplankton that inhabit a wide geographical range across the world except Antarctica (Van Stappen 2002). The genus includes two bisexual species, *A. franciscana* and *A. persimilis*, which are distributed in the New World, and four bisexual species, *A. salina*, *A. urmiana*, *A. sinica* and *A. tibetiana*, what are found in the Old World (Asem *et al.* 2010). Also parthenogenetic populations exist which are exclusively restricted to the Old World and Australia (McMaster *et al.* 2007; Van Stappen 2007).

The bisexual species from the Eastern Old World may hybridize with each other producing intermediate phenotypes, which can be fertile for several generations (Abatzopoulos *et al.* 2009). Among all bisexual species, *A. urmiana* is thought to have originated from a single geographic region, Urmia Lake in northwestern Iran (Eimanifar & Mohebbi 2007; Eimanifar & Wink 2013).

Urmia Lake is the site of the first biogeographical record of *Artemia*, ca. 982 A.D., by an anonymous unknown geographer (Asem 2008). Later, this species was nominated as *A. urmiana* by Günther 1899 (Asem & Rogers 2012) and finally, Clark and Bowen 1976 demonstrated its reproductive isolation and eventually confirmed its taxonomical status as an independent biological species. *A. urmiana* was long considered to be an endemic species in Urmia Lake (Eimanifar & Mohebbi 2007; Asem *et al.* 2007; 2009). In 2008, a new *Artemia* population from

Koyashskoye Lake, Ukraine was assigned to the species *A. urmiana* (Shadrin *et al.* 2008) and its taxonomical status was promptly corroborated as being *A. urmiana* by Abatzopoulos *et al.* (2009). Since then, *A. urmiana* was no longer considered to be an Urmia Lake endemic. Shadrin *et al.* (2012) even suggested that the possible biogeographical origin of *A. urmiana* was the Miocene salt lakes of which natural outcrops are found in Crimea. Recent genetic analyses based on mitochondrial *COI* sequences suggest that *A. urmiana* has an even wider distribution in Eurasia (Eimanifar *et al.* 2015).

Migrating waterbirds were proposed as an active disperser of *A. urmiana* cysts to new habitats such as Koyashskoye Lake (Khomenko & Shadrin 2009; Shadrin *et al.* 2012). On the other hand, *A. urmiana* is absent in other Crimean lakes which would demand another dispersal mechanism (Abatzopoulos *et al.* 2009). Abatzopoulos *et al.* (2009) suggested that cysts of *A. urmiana* from Urmia Lake could have been transferred via human salt trade routes. In this regard, both suggested dispersal routes may be responsible for the distribution of *A. urmiana* to non-native regions. The dispersion pattern of *A. urmiana* is hardly underestimated since there are several hypersaline lakes and lagoons in Iran which have not been colonized by *A. urmiana*. In addition, several localities in Iraq (Maknood 2011; Salman *et al.* 2012), Ukraine (Shadrin & Batogova 2009; Shadrin *et al.* 2012) and Russia (Shadrin & Anufrieva 2012) and other sites (Eimanifar *et al.* 2015) have been colonized by *A. urmiana*, but more systematic analyses are required to confirm the wider distribution of *A. urmiana* elsewhere. Thus, a comparative study of the genetic status of the supposed recently colonizing populations attributed to *A. urmiana* and the populations living in their center of geographic diversity, i.e., Urmia Lake, can help to unravel the dispersal puzzle of the species.

The present study is aimed at understanding the biogeographical origin of *Artemia urmiana* outside its nominal range by combining genetic divergence data and available palaeoecological evidences for Urmia Lake and Black Sea area.

Material and methods

Sampling and DNA extraction. We obtained 23 individuals of *Artemia* specimens that were collected from 3 geographical localities in Ukraine. The sampling information, IPMB voucher numbers, species status and GPS coordinates of three localities are shown in Table 1. In addition, *A. urmiana* sequences were included to our dataset from the previous studies. Total genomic DNA was isolated from one individual cyst of three Ukrainian *Artemia* using a Chelex-based method, followed by proteinase K digestion at 56 °C for two hours (Eimanifar & Wink 2013). Extracted DNA were stored, and used further for genetic analysis.

TABLE 1. Geographical localities of *Artemia*, IPMB voucher, geographical localities and GIS coordinates from Ukraine.

No.	IPMB voucher number	Abbreviation for locality	Locality, Province, state or district	Geographic coordinates	GenBank accession numbers
1	57259	DJA	Djarylpach	45°57'N – 32°59'E	KU053797-KU053802
2	57260	SAK	Sakskoye Lake	45°10'N – 33°30'E	KU053803-KU053807
3	75210	DZH	Dzharylhach	45°34'N – 32°52'E	KU053808-KU053819
4	57211	NE, NW, NC1, NC2	Urmia Lake	45°28'E – 37°35'N	Eimanifar & Wink 2013

PCR amplification and sequencing. A fragment of mitochondrial *cytochrome c oxidase subunit I (COI)* was chosen and amplified according to published procedures (Eimanifar & Wink 2013). The amplified products were purified and directly sequenced in two reactions with the same primers that were used in PCR amplification. The purification method and sequencing condition has been described elsewhere (Eimanifar & Wink 2013).

All sequences were aligned automatically using Bioedit 7.1.3.0 (Hall 1999) and, prepared for exploratory analyses. In order to make sure that all base calls were true at all polymorphic positions, we double checked whole dataset against the original chromatogram. The aligned sequences were converted into amino acid sequence using MEGA6 in order to find possible pseudogenes in the dataset (Tamura *et al.* 2013).

Additional *COI* sequences belonging to different species of *Artemia* were retrieved from GenBank and added

to the alignment dataset. The entire dataset consisted of 47 *COI* sequences. The data were rooted using *Daphnia tenebrosa* (HQ972028) as an outgroup. All new sequences have been submitted to GenBank (KU053797–KU053819).

Phylogenetic analysis. Phylogenetic analyses were carried out using Maximum likelihood (ML) and Bayesian inference (BI) approaches. The best-fit nucleotide substitution model based on Akaike's information criterion (AIC) was used to construct ML and BI trees using jModelTest v. 0.1.1 (Posada 2008). The best fit models for the mitochondrial and nuclear DNA dataset were TIM2+I+G and TIM1+G. The ML tree was reconstructed using MEGA6 program with all proposed parameters (Tamura *et al.* 2013). In our dataset, the GTR model was used as an alternative for the suggested models because these latter models were not implemented in MEGA package.

Molecular dating analysis. Calibrations. There are no fossil records of *Artemia*, and therefore we resorted to secondary calibration of our clock models. Divergence time was set at 145 Mya, based on *Daphnia* O. F. Mueller (Crustacea, Cladocera), a fossil from Jurassic/Cretaceous period (Kotov & Taylor 2011). The age of the most recent common ancestor (tMRCA) of all major clades were assigned a normally distributed prior with a given age and a standard deviation of 1 Ma.

Bayesian analysis and divergence time estimations using Beast. Bayesian tree reconstruction and divergence time of all sequences were determined using BEAST v1.6.2 (Drummond & Rambaut 2007), under the following parameters: the nucleotide substitution model = GTR with four rate categories, gamma heterogeneity among species, molecular clock model = an uncorrelated lognormal relaxed model, and tree reconstruction = Birth-Death model. XML files for all BEAST runs were created using BEAUTi v1.7.4 (Drummond *et al.* 2012). The analysis was performed twice independently for 30 million generations, taking samples every 1,000 generations. Posterior probability distributions of parameters were obtained by MCMC sampling. All runs were then combined after a burn-in of 10% using LogCombiner v1.7.2. Tracer v1.5 was used to verify stationary distribution of acceptable mixing of the MCMC steps to ensure that each parameter had been appropriately sampled (i.e., effective sampling size >200). The maximum clade credibility tree using median heights was annotated using TreeAnnotator v1.7.2 and was then input into FigTree v1.3.1 to visualize the tree and divergence times of lineages.

Results

The phylogenetic trees generated by ML and BI approaches had concordant topologies and found distinct well-supported clades, which correspond to recognized *Artemia* species (Fig. 1). The *COI* tree showed the existence of a solid monophyletic clade, subdivided into individuals of *A. urmiana* from Iran and Ukraine. In the rooted *COI* tree with *Daphnia*, *A. urmiana* in Urmia Lake, Iran, has diverged earlier than the Ukrainian populations. According to our findings, the divergence time for Ukrainian *Artemia* was roughly estimated to be 196,000 years, whilst it was ca. 383,000 years for *A. urmiana* in Urmia Lake (Fig. 1).

Discussion

Palaeoenvironmental history of Urmia Lake and the Ukrainian coastal salt lakes. A key element needed to understand the diversification events in *A. urmiana* was revealed in the phylogenetic tree (Fig. 1), which allows the comparison of the geological origin and evolution of their two main natural environments i.e., Lake Urmia in NW Iran, and the littoral salt lakes in Ukrainian Black Sea coasts.

Urmia Lake. When a causeway was constructed that connected the eastern and western shores of Lake Urmia in the early 2000s, a geophysical exploration of the sediment infills was undertaken. The seismic profiles (unpublished internal report, Iran Ministry of Roads and Transportation) suggested that a minimum of 300 meters of fluvio-lacustrine sediments were in central part of the lake basin. Later, a core of 100 meters length, recovered from central part of the lake was dated by palynostratigraphy and a ²³⁰Th absolute age determination. These analyses suggested an age of about 200,000 years for the base of the core (Djamali 2008; Djamali *et al.* 2008a). A minimum age of 600,000 years can thus be assumed for the 300 meters of sediments in the lake basin. However, the true age of Urmia Lake might be much older, dating back to the early Pleistocene and even Pliocene.

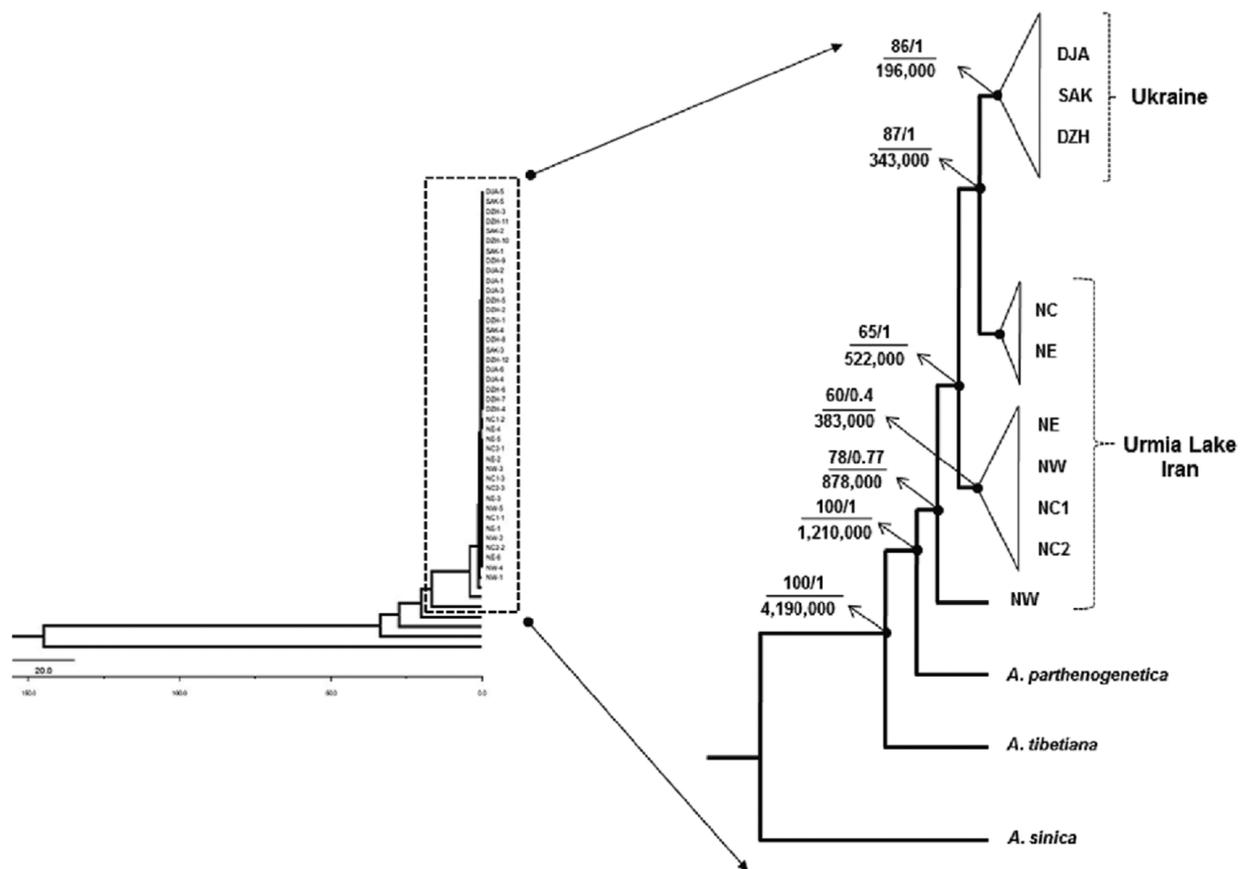


FIGURE 1. Bayesian inference chronogram with estimated divergence times for *Artemia* individuals from Urmia Lake and Ukraine Lake. Numbers above dash shows bootstrap support (BS) for maximum likelihood and Bayesian posterior probabilities (BPP). Numbers at in the bottom dashes indicates divergence times for major clades. The divergence times are shown in scales (KYA).

The water level of Urmia Lake has undergone many oscillations during the last 200,000 years as revealed by sedimentological, geochemical and palynological investigations (Djamali *et al.* 2008b; Stevens *et al.* 2012). However, the lake has remained saline to hypersaline for most of its history. Some very short episodes of nearly brackish water conditions have also been reported (Djamali *et al.* 2008b). It seems that the lake has not undergone a complete desiccation even during the Last Glacial Maximum (LGM) when it became a seasonal playa lake. Microscopic observations indicate that *Artemia* cysts have been continuously present in the lake sediment for at least since 200,000 years (Djamali *et al.* 2010). This fact indicates that even if the overall lake size has greatly changed, the presence of smaller ecosystems of different physico-chemical characteristics associated with delta complexes of entering river systems and springs has guaranteed the existence of the smaller micro-habitats for salt water aquatic organisms such as *Artemia* in the lake basin.

Ukrainian black sea littoral salt marshes. Palaeoecological investigations suggest that these salt marshes are very young geological features and formed at the mid-Holocene following the end of a marine transgression phase (Subetto *et al.* 2007). The transition from marine conditions to saline lake conditions occurred about 5340-5610 years ago at Lake Saks koye or Saki (45° 06.81' N; 33° 33.19'E) and about 500 years later about 5200 years ago at Lake Dzharylgach (45° 34.68' N; 32° 51.67' E) (Subetto *et al.* 2007). The very young geological age of these littoral salt lakes/marshes is not surprising because the Black Sea has undergone dramatic sea level changes during the Quaternary which have completely reshaped the coastal geomorphology during the episodes of lowstands and highstands (Winguth *et al.* 2000).

A sequential stratigraphical analysis of sediments in the Danube river delta in northwestern part of the Black Sea has revealed at least 12 episodes during which the Sea level has dropped >100 meters below present-day levels during the last 900,000 years (Winguth *et al.* 2000). During the LGM (18,000 years ago), the lake levels dropped 150 meters (Winguth *et al.* 2000). During the late glacial to early Holocene, the Black Sea was a large freshwater

lake with many fluctuations in water level. Its water levels fell 90 meters about 8500 years ago (Lericolais *et al.* 2007; 2009) and marine conditions dominated between 8000 to 7000 years ago (about 7150 year ago according to Lericolais *et al.* 2007) when the Black Sea became connected to the Mediterranean Sea (Martin *et al.* 2007).

Implications for the diversification of *Artemia urmiana*. The above-mentioned palaeo-environmental account indicates that unlike the Urmia Lake which has continuously existed since Pliocene or early Pleistocene, the Ukrainian sites represent very young ecosystems from mid-Holocene (about 5200 to 5600 years ago). The very dynamic nature of the Black Sea coastal areas caused by past dramatic sea level changes prevented the formation and stability of these littoral salt marshes, the latter environments being of a geologically transient nature. The divergence time for Ukrainian *A. urmiana* populations dating back to the beginning of the late Pleistocene (196,000 years) strongly suggests that these populations have originated from older populations, such as those from Urmia Lake. Further, the divergence time for the clade comprising all Iranian and Ukrainian populations estimated at 878,000 years ago may indicate that *A. urmiana* have evolved sometime during the early Pleistocene (2.59 – 0.78 Ma).

Manaffar *et al.* (2011) have argued that *A. urmiana* diverged 11 million years ago, whereas the time frame for the formation of Urmia Lake is estimated to be the late Pliocene-early Pleistocene, according to our estimation. If these estimates are correct, *A. urmiana* must have originated elsewhere and later dispersed to Urmia Lake. Shadrin *et al.* (2012) supported this hypothesis with regard to findings of Manaffar *et al.* (2011), because *Artemia* cysts extracted from sediment cores of Urmia Lake (5,000 years old) were parthenogenetic. Anufrieva & Shadrin (2012) even suggested that *A. urmiana* might have possibly originated from Miocene salt lakes of which natural outcrops are found in Crimea. However, this latter hypothesis is very speculative since no *Artemia* fossils have so far been described from these deposits. Furthermore, such saline lake deposits can also be found elsewhere in southwestern Asia, including the Iranian plateau.

Since the formation of Urmia Lake, *A. urmiana* populations have been isolated in this hydrologically closed inland basin. The presence of numerous small salt water ecosystems associated with groundwater seepage, local springs and the topographic variations in delta environments of entering river systems during periods of both lake highstands and lowstands has promoted the survival of *A. urmiana* populations during the Pleistocene glaciations. Such ecological conditions were most probably not found in the Black Sea region due to more severe climatic conditions in higher latitudes, particular physiography characterized by steeper basin slopes, stronger erosional processes, and periodic freshwater incursions.

As conclusion of this work Urmia Lake is apparently much older than the Ukrainian lakes supporting an expansion of *A. urmiana* populations from NW Iran into Ukraine.

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