Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of
Doctor of Natural Sciences

Presented by

Amin Eimanifar
Born in: Oroumieh, Iran
Oral-examination: 06.08.2014

Molecular phylogeography and population genetics of the brine shrimp *Artemia* (Crustacea, Branchiopoda, Anostraca)

Referees: Prof. Dr. Michael Wink

Prof. Dr. Thomas Braunbeck

Acknowledgements

FIRST OF ALL, I would like to express my great appreciation to my supervisor, Prof. Dr. Michael Wink. He opened a new window for me during my PhD study and his profound insights were invaluable. He helped me to deeper understand the relationships between ecology and phylogeography of *Artemia*. I am grateful to Prof. Wink for giving me a chance doing my PhD project in his research group, for all his advice, multi-faceted supports, encouragements, flexibility and patience throughout my study. My appreciation goes out to Prof. Dr. Thomas Braunbeck for his support of my PhD study.

This project could not have been conducted without the extensive collaboration with others who provided *Artemia* samples. I would especially like to thank Gilbert Van Stappen (Ghent University, Belgium), Francisco Amat (Instituto de Acuicultura de Torre de la Sal (IATS - CSIC), Spain), Brad Marden (Great Salt Lake *Artemia*, Inc., U.S.A), Alireza Asem (Institute of Evolution and Marine Biodiversity, Ocean University, China), Mark Coleman (Actis Environmental Services, Australia), Razia Sultana (Food and Marine Resources Research Center, Pakistan) and Vasudevan Sugumar (University of Madras, India).

My appreciation goes out to Theodor C. H. Cole and Fear Douglas who proofread and improved my manuscripts. Theodor C. H. Cole helped me a lot to improve my oral presentations. I am truly thankful to Dr. William Camargo (Private Aquaculture Company, Florida, USA) and Prof. Dr. James S. Clegg (University of California, USA) for their extensive proofreading of this thesis.

There are other people who deserve special thanks including Petra Fellhauer for her professional and logistic support, Astrid Backhaus, Hedwig Sauer-Gürth and Heidi Staudter for their technical assistance. I am indebted to Markus Santhosh Braun who helped me substantially during thesis preparation and also for the translation of the summary to German.Lastly, to all my colleagues in IPMB for their kind discussion during my study.

My parents stood behind me as I pursued my education, so to Dad and Mom, and my brother: thank you for your love and encouragement. My Dad's diligent efforts during my PhD study were inspiring, and improved well my activities here. He enriched me by his valuable consultations both in cultural and educational perspectives.

My PhD study was supported by a doctoral scholarship of the German Academic Exchange Service (DAAD) reference number A-10-97179. The Graduate Academy of Heidelberg University supported me to attend the EMBL (European Molecular Biology Laboratory) international symposium in Heidelberg.

I just want to say "THANK YOU SO MUCH" for everyone who I may have forgotten.

Amin Eimanifar

Spring 2014

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Summary

Brine shrimps *Artemia* (Crustacea, Anostraca) are a group of cosmopolitan extremophile microcrustaceans which are composed of a complex of six sexual species and numerous Eurasian Haplotype Complex (EHC) lineages. In the present study, we analyzed a partial sequence of the mitochondrial *cytochrome c oxidase subunit I* (*COI*) and nuclear *Internal transcribed spacer1* (*ITS1*), as well as genomic fingerprints by ISSR-PCR (inter-simple sequence repeats) for a large set of *Artemia* specimens (N ~ 600) from various geographical localities (N = 102) across Eurasia and America. Asian lineages have revealed a mixture of paraphyletic and polyphyletic groups of *Artemia*. *A. urmiana* and *A. tibetiana* represent a species complex with multiple genetic lineages. EHC lineages (Eurasian and Africa) showed a star-like haplotype pattern, which had more genetic similarities to other sexual Asian species, except *A. sinica*. Bayesian analysis of *COI* was used to estimate the time of divergence of Asian *Artemia* species. The Bayesian analysis indicated that Asian taxa are relatively young, particularly EHC lineages. *A. urmiana* recorded as the oldest species originated in the Pleistocene and could be considered as a major source of its expansion to its modern habitats in Eurasia. Molecular dating analyses using a relaxed bayesian molecular clock depicted that *A. sinica* has been diverged from other Asian lineages in the Miocene, around 20 million years ago (Mya).

A. urmiana was the dominant sexual species in Urmia Lake exhibiting a high genetic diversity with a low level of genetic structure in the entire lake. This species was essentially homogeneous indicative of panmixia. A correlation between population differentiation and geographical and ecological differences was not observed.

The invasive *A. franciscana* have permanently colonized 31 geographical localities along the southern and eastern coastal regions of Asia. EHC lineages were observed in 39 inland geographical localities in Asia. Asian *A. franciscana* generated a signature of haplotype diversity as compared to the source population from Great Salt Lake (GSL, USA). The high genetic diversity of Asian *A. franciscana* is probably attributed the numerous, human-mediated, dispersal events and multiple introductions from GSL & San Francisco Bay (SFB, USA) and eventually indirect introductions from other native localities in the Americas. Our results indicate that biological invasion do not necessarily lead to reduced genetic diversity, particularly if multiple source populations, each with distinctive genetic composition, contribute to the founding populations. EHC lineages showed low genetic diversity which is in contrast to the restricted geographic distribution, strong genetic structure, and regional endemism of sexual *Artemia* lineages in Asia.

A. franciscana in GSL revealed the same condition as observed in *A. urmiana*. It showed a high genetic diversity with an evidence of panmixia throughout the lake.

Zusammenfassung

Artemia (Crustacea, Anostraca) ist eine Gruppe kosmopolitischer, extremophiler Microcrustaceen, die sich aus einem Komplex sechs sexueller Arten und zahlreicher EHC (Eurasian Haplotype Complex) Abstammungslinien zusammensetzt. In dieser Studie wurden partielle Sequenzen der mitochondrialen Untereinheit I des *Cytochrom c Oxidase* Gens (*COI*), des nukleären Gens *Internal transcribed spacer1* (*ITS1*) und daneben DNA-Fingerprints mittels ISSR-PCR (inter-simple sequence repeats) eines umfangreichen Sets von Artemien (N ≈ 600) unterschiedlicher geographischer Lokalitäten (N = 102) innerhalb Eurasiens und Amerikas analysiert. Asiatische Abstammungslinien zeigten eine Mischung von para- und polyphyletischen *Artemia*-Gruppen. *A. urmiana* und *A. tibetiana* gehörten zum selben Artenkomplex mit mehrfachen genetischen Abstammungslinien. EHC Abstammungslinien (Eurasien und Afrika) zeichneten sich durch ein sternförmiges Haplotypenmuster aus, das – mit Ausnahme von *A. sinica* – eine höhere genetische Ähnlichkeit zu anderen sexuellen asiatischen Arten aufwies. Bayessche Analysen des *COI* Gens wurden verwendet, um den Zeitpunkt der Aufspaltung der asiatischen *Artemia* Arten abzuschätzen. Die Analysen weisen darauf hin, dass asiatische Taxa und EHC im Besonderen relativ jung sind. *A. urmiana* ist die älteste bekannte Art, entstammt dem Pleistozän und kann als eine Hauptquelle der Ausbreitung in die modernen Habitate Eurasiens angesehen werden. Molekulare, auf einer Bayesschen „relaxed molecular clock“ basierende Datierungsmethoden zeigten, dass sich *A. sinica* von den übrigen asiatischen Abstammungslinien im Miozän vor etwa 20 Millionen Jahren abspaltete.

A. urmiana ist die dominierende sexuelle Art im Urmiasee und von hoher genetischer Diversität mit einer geringen Populationsstruktur im gesamten See geprägt. Diese Art war genetisch homogen, was für eine panmiktische Population spricht. Eine Korrelation zwischen genetischer Differenzierung und geographischen und ökologischen Unterschieden konnte nicht festgestellt werden.

Die invasive Art *A. franciscana* hat bis heute 31 Regionen entlang der Süd- und Ostküsten Asiens permanent kolonialisiert. EHC Abstammungslinien wurden in 39 Gebieten des Festlandes Asiens beobachtet. Im Vergleich zur Ursprungspopulation des Großen Salzsees (USA) weist *A. franciscana* in Asien eine spezifische Haplotypendiversität auf. Seine hohe genetische Diversität geht wahrscheinlich auf mehrfache anthropogene Dispersion, die wiederholte Einführung der Art aus dem GSL & San Francisco Bay (SFB, USA), sowie die indirekte Einführung aus anderen Ursprungsgebieten Amerikas zurück. Unsere Ergebnisse lassen darauf schließen, dass biologische Invasionen nicht zwingend mit verminderter genetischer Diversität einhergehen müssen. Dies ist insbesondere dann der Fall, wenn Ursprungspopulationen unterschiedlicher Herkunft und verschiedener genetischer Zusammensetzung neue Populationen begründen. EHC Abstammungslinien zeigen eine

geringe genetische Diversität und stehen damit im Gegensatz zu sexuellen *Artemia*-Arten Asiens, welche durch ihre abgegrenzte geographische Verbreitung, die ausgeprägte Populationsstruktur und ihren regionalen Endemismus gekennzeichnet sind.

A. franciscana vom Great Salt Lake zeigte ein ähnliches Muster wie *A. urmiana*. Die Art war geprägt von hoher genetischer Diversität und es gibt Hinweise auf Panmixie im gesamten See.

Parts of the present thesis have already been published or are in preparation.

1. **Eimanifar, A.,** Wink, M., 2013. Fine-scale population genetic structure in *Artemia urmiana* Günther, 1890 from Lake Urmia based on DNA sequences of mtDNA and ISSR genomic fingerprinting. Organisms Diversity and Evolution 13, 531−543.

2. **Eimanifar, A.,** Van Stappen, G., Marden, B., Wink, M., 2014. *Artemia* biodiversity in Asia: phylogeography of the introduced American species *Artemia franciscana* Kellogg, 1906. Molecular Phylogenetics and Evolution (In revision)

3. **Eimanifar, A.,** Marden, B., Braun, M., Wink, M., 2014. Geographical variabilities and population genetic structure in *Artemia franciscana* Kellogg, 1906 based on mtDNA sequences, ISSR genomic fingerprinting and biometry (Submitted to Marine Biodiversity).

4. **Eimanifar, A.,** Van Stappen, G., Wink, M., 2014. Biogeographical structure and evolutionary divergence times among Asian brine shrimps *Artemia* (Crustacea, Anostraca) (In preparation).

Additional publication related to this project.

1. Asem, A., **Eimanifar, A.,** Djamali, M., De los Rios, P., Wink, M., 2014. Biodiversity of the hypersaline Urmia Lake National Park (NW Iran). Diversity 6, 102−132.

The following abstract has already been published at international conference.

1) **Eimanifar, A.,** Van Stappen, G., Marden, B., Wink, M., 2013. *Artemia* (Crustacea, Anostraca): A model organism for phylogeography and evolutionay ecology. *New Model Systems for Linking Evolution and Ecology. 1– 4 May 2013, EMBL Heidelberg, Germany* (poster presentation).

1. Introduction

1.1. Taxonomy of *Artemia*

The brine shrimp *Artemia* is a distinctive zooplankter which has continental distribution and can be found in over 500 geographical localities except Antarctica (Triantaphyllidis et al., 1997a,b, Muñoz and Pacios, 2010). It has been first described by Schlösser in 1755 (Sorgeloos, 1980a), who named it as *Cancer salinus* (Asem et al., 2010a). According to Martin and Davis, (2001), the taxonomic status of the genus *Artemia* can be defined as shown below:

Subphylum: Crustacea Brünnich, 1772

Class: Branchiopoda Latreille, 1817

Subclass: Sarsostraca Tasch, 1969

Order: Anostraca Sars, 1867

Family: Artemiidae Grochowski, 1896

Genus: *Artemia* Leach, 1819

Species:

Artemia franciscana Kellogg, 1906

Artemia persimilis Piccinelli & Prosdocimi, 1968

Artemia salina Linnaeus, 1758

Artemia urmiana Günther, 1899

Artemia sinica Cai, 1989

Artemia tibetiana Abatzopoulos et al., 1998

Eurasian Haplotype Complex (EHC) Barigozzi, 1974 (or *A. parthenogenetica*)

The phylogenetic tree of the most recognized *Artemia* species is depicted in Fig. 1.

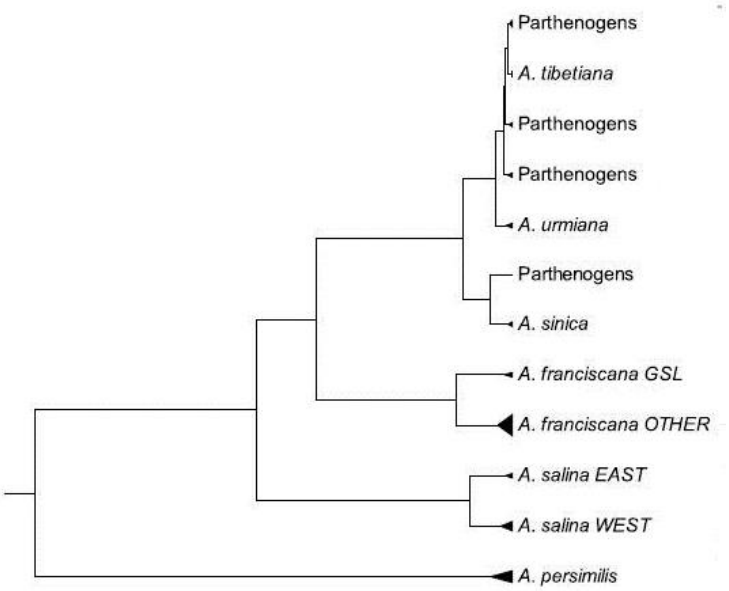


Fig. 1. *ITS1* phylogenetic tree of recognized *Artemia* species.

(**Source:** Baxevanis et al., 2006)

1.2. Morphology of *Artemia*

Artemia has a segmented body with leaf-like jointed appendages which suggest a bigger size. The total length of adult males is about 8 – 10 mm and an adult female is 10 – 12 mm (Fig. 2), depending on the species. The body is divided into head, thorax and abdomen (Fig. 3). The head is composed of six segments which are fused by median and compound eyes, labrum, appendages (antennae and maxillae) and development of muscular and skeletal systems (Fig. 4). The head includes excretory organs and a part of linear digestive tract. The compound eyes contain many *ommatidia* which developed from the lateral side of the compound eyes. The *labrum* is an elastic and muscular part, which developed from the bases of compound eyes during developmental stages. The labrum helps animal during mastication and swallowing processes.

The thorax consists of eleven segments, shaping the swimming legs or phyllopods. The main function of swimming legs is in locomotion, osmoregulation and respiration of the animal. The nauplius has three pairs of appendages including the first antennae (sensorial function), the second antennae (locomotory + filter-feeding function) and the mandibles (food uptake function). The first and eleventh segments of phyllopods have the smallest size but this would be increased toward the middle of the thorax. Female *Artemia* can easily be distinguished by the brood pouch or uterus located behind the 11th pair of thoracopods.

The abdomen extends behind the thorax and is composed of eight annular segments. The first abdominal segment specializes by genital segments. There include gonopods either the paired penes of the male or the ovisac of the femaleThe ovisac are structured into a single unit whereas, the penes are paired. Abdominal segments (two to seven) have no appendages but the last segment (eight) ends to pair of cercopods which is called furcal *rami* or telson (Cassel, 1937; Abatzopoulos et al., 2002).

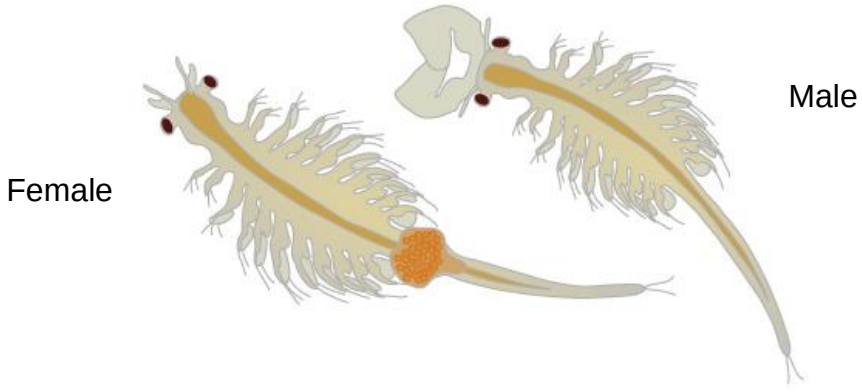


Fig. 2. External morphological *Artemia* features (male and female).

(**Source:** http://www.fao.org)

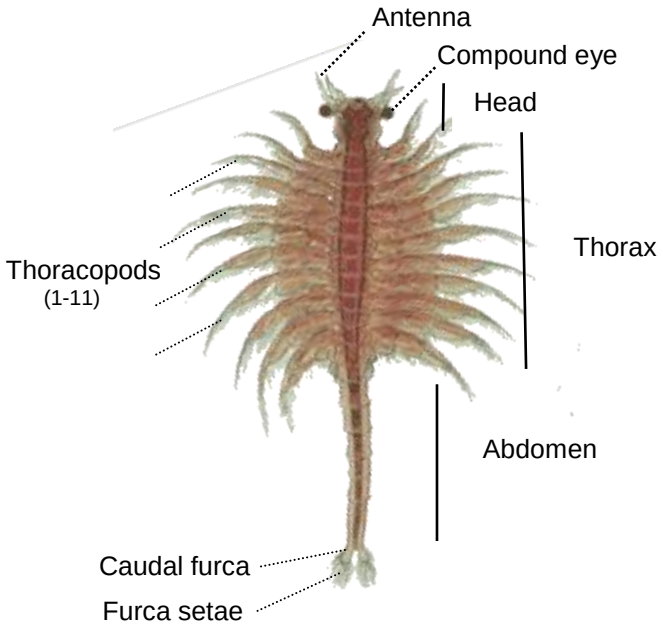
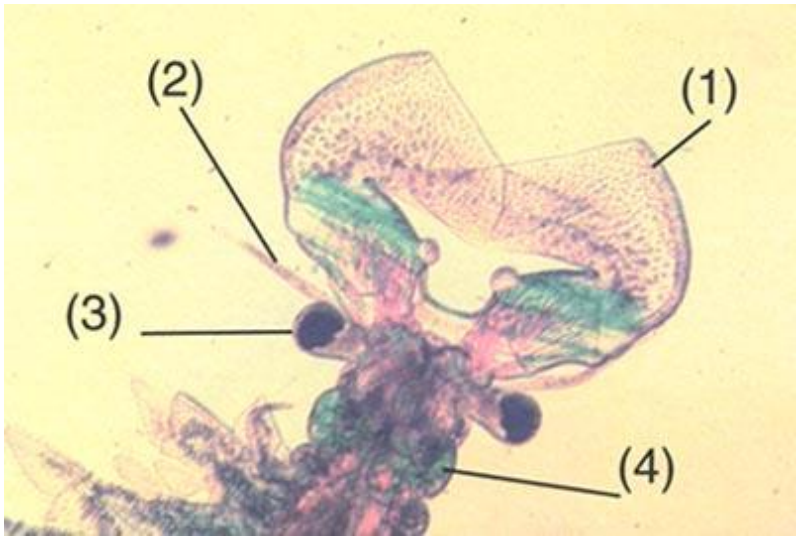


Fig. 3. The body structure of *Artemia*.

(**Source:** www. futurechimp.blogspot.com)



1: Antenna, **2:** Antennula, **3:** Lateral complex eye, **4:** Mandible

Fig. 4. A Schematic view of the head of *Artemia* (male).

(**Source:** http://www.fao.org)

1.3. Life cycle of *Artemia*

Artemia is composed of six recognized bisexual species and numerous parthenogenetic (asexual) populations which are variable according to ploidy levels (Van Stappen, 2002). Depending on sexual or parthenogenetic species, the development of *Artemia* cysts undergoes two developmental pathways including ovoviviparous *vs* viviparous. In the former mode, the fertilized eggs produce free-swimming nauplii by female. Females can produce about 300 nauplii every 4 days. In the extreme environmental condition (*e.g*. high salinity, low oxygen levels), encysted gastrula embryos (cysts) are released by female to the environment by mating male and female (Liang and MacRae, 1999). The larvae produced through viviparous reproduction appear to have the same morphological features but there are significant biochemical differences among them (Liang and MacRae, 1999).

The cysts are externally surrounded by a thick brown-color shell. Dry conditions cause the encysted embryo to enter a dormant state, which allows it to withstand complete drying, temperatures over 100 °C. Dormancy is terminated by a dehydration-rehydration cycle. The rehydrated cysts exist in a quiescent state which is termed anhydrobiosis (Browne and Bowen, 1991). The cyst floats across the column of the water and finally accumulates in saltpan or lake sediments forming diapausing egg banks, allowing the persistence of populations during unfavorable periods. The cysts hatch after 24-48 h under standard conditions (salinity: 35 – 40 $g.l^{-1}$, temperature: 26 – 28 °C, pH: 7.5 – 8.0, aeration: 4 ppm, illumination: 2000 lux). The fresh-hatched *Artemia* (nauplii) molts about 17 times and it takes two weeks to reach to adult stage (Fig. 5).

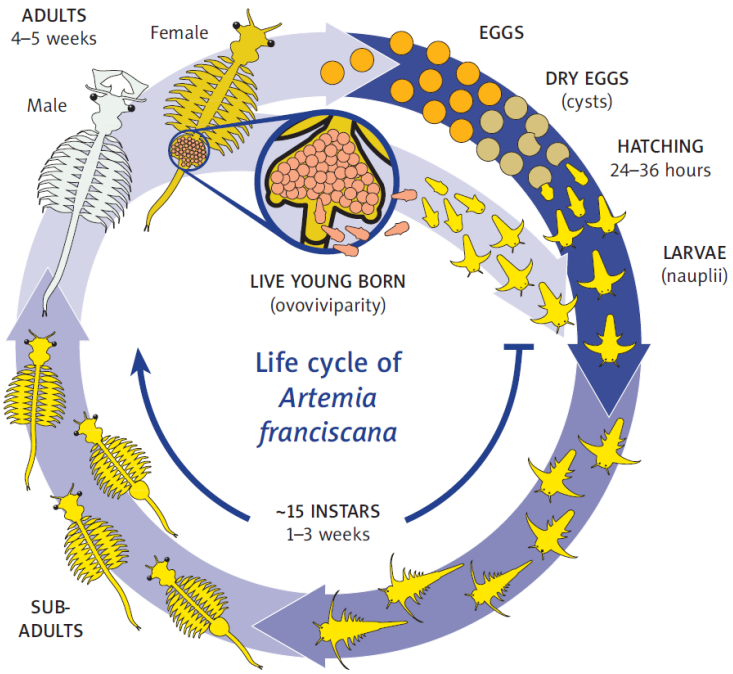


Fig. 5. Schematic representation of *Artemia* life cycle

(Source: http://www.eurovolvox.org/Protocols/PDFs/LifeCycle1.0_UK_eng.pdf)

1.4. Ecology of *Artemia*

The ecological conditions in the saline ecosystems where brine shrimp thrives are extreme, favoring the evolutionary development of one of the best osmoregulating systems known in the animal kingdom (Sorgeloss, 1980). *Artemia* can survive in highly saline environments including inland salt lakes, coastal lagoons and solar saltworks (Van Stappen, 2008) with salinity ranging from 35 to 350 $g.l^{-1}$ (Van Stappen, 2002; Eimanifar and Mohebbi, 2007; Asem et al., 2014). This striking physiological adaptation has brought to the conclusion that “the ionic composition of the waters inhabited by *Artemia* varies more than that of any other aquatic metazoan” (Cole and Brown, 1967). The common feature of all *Artemia* habitats is their high salinity. Salinity is a crucial abiotic factor limiting the geographical distribution of *Artemia* population across the world (Van Stappen, 2008). Other parameters such as temperature, light intensity, primary food production have an influence on the quantitative amount of *Artemia* population (Van Stappen, 2008). At high salinity level, predators and other food competitors are not found which could lead to develop *Artemia* in these extreme conditions (Camargo, 2002). In addition to salinity, the geographical distribution of *Artemia* populations depends on climatological conditions (Van Stappen, 2008).

So far, *Artemia* inhabits in the areas where yearly evaporation exceeds yearly precipitation (Vanhaecke et al., 1987). *Artemia* do not survive in the humid conditions but the occurrence of *Artemia* in the subhumid extreme southern part of Chile is quite evocative (Gajardo et al., 1999). There are several strains in America and Asia which are able to live in the extremely cold and warm conditions. In general, *Artemia* strains do not survive at temperatures below 5 °C but some populations in Chile (Atacama and Torres del Paine) challenges this general rule (Van Stappen, 2008).

Brine shrimp cysts float across the column of water and finally accumulate along coastal line of seawater by wind (Tackaert and Sorgeloos, 1993). There apparently inert golden-brown or sometimes whitish-brown minute particles between 200 to 300 µm in diameter and each weighing between 2.8 to 4.0 µg are actually dormant dry cysts (Clegg and Conte, 1980).

The cyst distribute via hydrological connectivity, wind, migratory birds or even human activities (Eimanifar and Mohebbi, 2013, Muñoz et al., 2014). The dispersal of *Artemia* cysts leads to colonization of indigenous species in non-indigenous regions across the continents. The displacement and colonization of an exotic invasive species such as *A. franciscana* jeopardizes the local biodiversity of *Artemia*, leading to extinction of most autochthonous *Artemia* populations (Amat et al., 2007).

1.5. Distribution of *Artemia*

Two bisexual species are native to the New World, namely *A. persimilis* Piccinelli and Prosdocimi 1968 (Argentina and Chile) and *A. franciscana* Kellogg 1906 (North, Central and South America). Four bisexual species live in the Old World: *A. salina* Linnaeus 1758 (Mediterranean basin), *A. urmiana* Günther 1899 (Lake Urmia, Iran, and Crimean salt lakes; Abatzopoulos et al., 2009), *A. tibetiana* Abatzopoulos et al., 1998 (Tibetan plateau), and *A. sinica* Cai 1989 (China and Mongolia).

Abatzopoulos et al., (2002) suggested that obligate parthenogenetic *Artemia* populations show different ploidy levels and that they can not readily be considered as belonging to a single species, for example "*A. parthenogenetica"*. They proposed using the general terms 'parthenogenetic populations' or 'parthenogenetic strains' as an alternative without taxonomic consequences. According to our experience, we would be even more cautious: in most cases we have only information of origin of samples and we are not sure whether animals were sexual or parthenogenetic. As a consequence, we have here introduced the term Eurasian Haplotype Complex (EHC) to describe a group of populations sharing the same basic haplotype. EHC includes documented parthenogenetic populations, but it needs to be established whether all EHC members are parthenogenetic.

EHC are widely distributed over Eurasia, extending from the Canary Islands to China, and they have been introduced into Australia (Maccari et al., 2013). EHC has even been found in Great Salt Lake in North America (probably introduced by humans) (Endebu et al., 2013). Geographical distribution of *Artemia* species is shown in Fig. 6.

The existence of sexual and EHC lineages reflects genetic flexibility of *Artemia* which shows exceptional adaptation ability to diverse ecological habitats. The distribution pattern of *Artemia* is uneven and mainly depends on ecological and physiological parameters (Van Stappen, 2008). So far, several studies have outlined the actual occurrence of *Artemia* in different geographical regions but extensive exploratory activities are required to update its distribution over the globe. The natural habitats of *Artemia* have seen climatological variations which could restrict its persistence in the environment. Some salt lands are dynamic, whereas others are unpredictable depending on seasonal variations (Gajardo and Beardmore, 2012). Salinity is a crucial abiotic factor which has a fundamental role in geographical distribution of *Artemia* lineages (Van Stappen, 2008). Some places are thalassohaline but some of them are athalassohaline (Gajardo and Beardmore, 2012). *Artemia* species has been distributed in the New vs the Old World which reveals an asymmetric pattern (Van Stappen, 2008).

1.5.1. New World *Artemia*

1.5.1.1. North and South America

There are two species which can be found in Americas: *A. franciscana* and *A. persimilis*. The former species is called 'superspecies', a 'case of evolution in progress' or a

‘cluster of incipient species or species *in statu nascendi*’. This species has been broadly distributed through its natural habitats in the Americas and particularly in non-indigenous regions across Eurasia and Australia (Amat et al., 2005, 2007; Muñoz et al., 2014). *A. franciscana* has distinctive features including physiological performance, phenotypic plasticity and highly repetitive heterochromatin (Gajardo et al., 2001; Vikas et al., 2012). It has a particular intraspecific genetic diversity which makes it an excellent model for evolutionary biology studies (Gajardo et al., 1995; 2002). There are two commercial sources of *A. franciscana* in North Americas: Great Salt Lake (GSL) and San Francisco Bay (SFB). These regions have been used as the main source of live food in aquaculture industry (Sorgeloos et al., 2001). There is one population from Mono Lake USA which shows an ecological isolation with regards to other American populations named *A. monica* Verrill 1869 (Van Stappen, 2008). *A. persimilis* has a narrow geographical distribution and is restricted to the southern latitudes in South America, particularly Argentina and Chile. It has an exclusive chromosomal rearrangement (2n = 44), which exhibits a very low chromocenter numbers with *A. salina* (Gajardo et al., 2001). The occurrence of *A. persimilis* in South America has raised zoogeographical questions about its natural distribution and adaptation behavior over this continent (Gajardo et al., 1998; 2001; 2004). The phylogenetic position of *A. persimilis* needs to be taken into account since it has different positions with regard to different genetic markers (Gajardo and Beardmore, 2012).

1.5.1.2. Australia

Australia has a unique biodiversity due to its Gondwanan history exhibiting a high level of endemic taxa. Australia is the home of two halobiont anostraceans namely *Artemia* and *Parartemia* (Geddes and Williams, 1987). The genus *Parartemia* is composed of at least eight species with wide distribution on this continent (Geddes, 1981; Timms, 2006). Dispersal of *Artemia* has been suggested through migratory birds (Austral-Asian avian flyway) or human alteration by inoculating an exotic *A. franciscana* into coastal Western of Australia (Van Stappen, 2008). EHC lineages are dispersed into the inland salt lakes along South-western of Western Australia (Bunn and Edward, 1984). Recent reviews reveal that *A. franciscana* may have been introduced from Great Salt Lake or San Francisco Bay into coastal Queensland regions such as Port Alma and Rockhampton (Clark and Bowen, 1976; Timms, 2006; Van Stappen, 2002; McMaster et al., 2007). Numerous literature indicates an expansion of *A. franciscana* into North-western and South-western coastal regions of Western Australia (Timms, 2006). More systematic analyses are required to characterize *Artemia* species composition on this continent.

1.5.1.3. Europe and Africa

A. salina Linnaeus 1758 is the only bisexual species distributed over the Mediterranean basin including North Africa (Van Stappen, 2008; Triantaphyllidis et al., 1997b). There was a

large hiatus concerning to species distribution in North and South Africa but this issue has been updated in a systematic study carried out by Baxevanis et al., (2014). EHC lineages are found in Eastern Europe and Mediterranean basin, coexisting with bisexual counterparts (Amat et al., 1995). A dramatic increase of salinity and habitat subdivision has led to migration of EHC to Mediterranean basin (Abreu-Grobois, 1987). It is believed that the Atlantic coast of Africa and East African rift valley are responsible for dispersing of *Artemia* cysts via migratory birds (Kaiser et al., 2006). In a recent study, the colonization and establishment of an exotic American species *A. franciscana* is observed in some localities across Europe. The existence of American species over Europe is mainly due to anthropogenic activities (Muñoz et al., 2014). The dispersal of *A. urmiana* from Urmia Lake (Iran) into the Koyashskoe Lake (Crimean Peninsula, Ukraine) has been reported so far (Abatzopoulos et al., 2009).

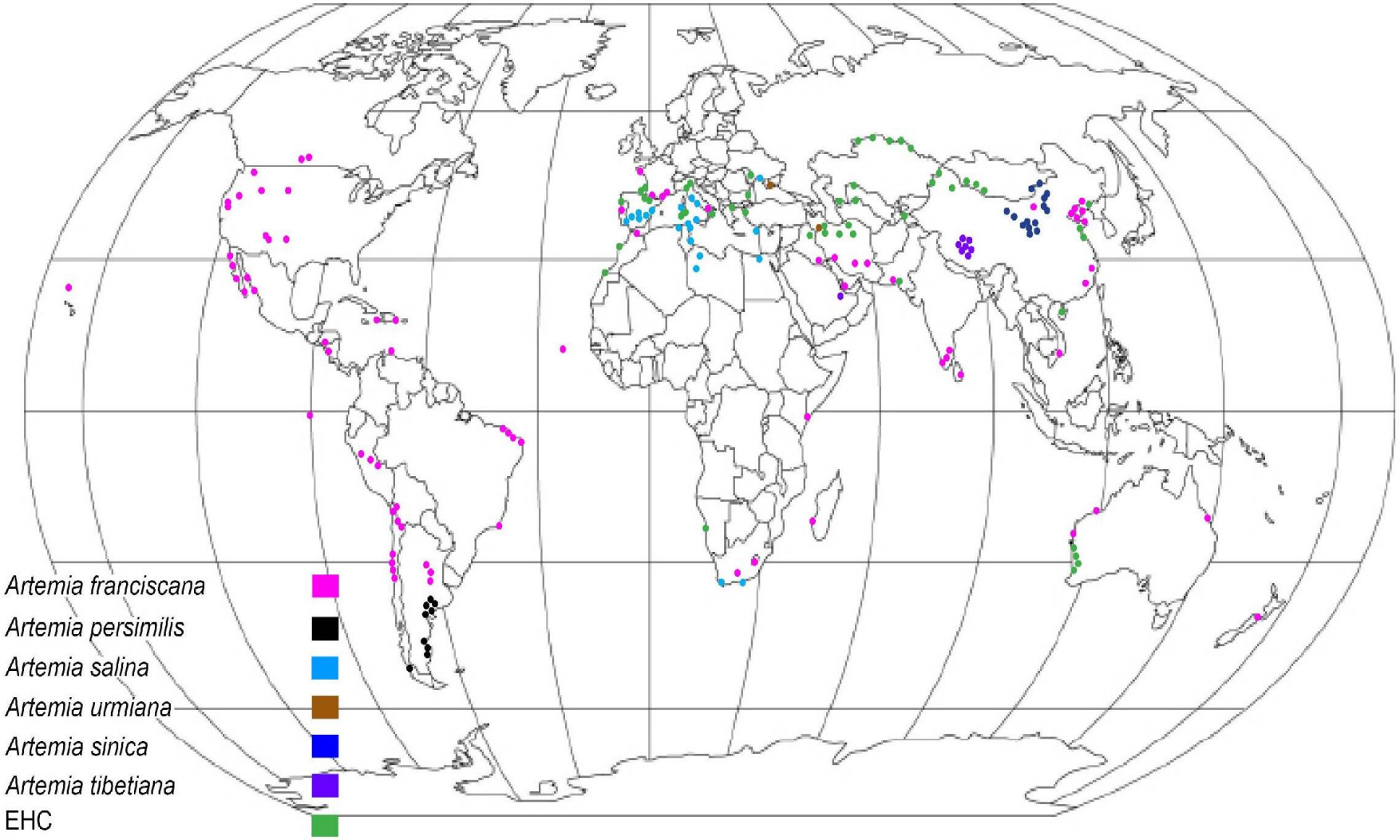


Fig. 6. Distribution map of *Artemia* species. Each species marked with different colours.

(**Source:** Muñoz, 2009 (modified)

1.5.1.4. Asia

Biodiversity of *Artemia* populations throughout America and Europe has been well documented, whereas knowledge of Asian *Artemia* and its distribution is less known (Muñoz et al., 2008, 2010, 2013; Maniatsi et al., 2009). Asia (Central and Eastern), a vast region with a pronounced topographical and climatological diversity harbors diverse *Artemia* species (Van Stappen, 2008). Three bisexual species (*A. urmiana* Günther 1899; *A. tibetiana* Abatzopoulos et al., 1998; *A. sinica* Cai 1989) and numerous EHC lineages inhabit in Asia. The geographic distribution and coexisting of EHC with sexual species has been a subject for evolutionary biologist (Van Stappen, 2008). Urmia Lake contains *A. urmiana* as a bisexual species and multiple EHC lineages which have coexisted with sexual counterpart around the lake (Agh et al., 2007; Asem et al., 2014). *A. tibetiana* from Lagkor Co (Tibet) is a newly characterized species found in Eastern Asia, which has a marked morphological diversity compared to other related Asian species (Abatzopoulos et al., 1998). Phylogenetic relationships of *A. tibetiana* with other native Asian lineages have opened a new window in *Artemia* biology (Van Stappen, 2008).

1.6. Importance of *Artemia*

Artemia has been widely used as a major live feed in shrimp hatcheries and commercial larviculture owing to its high quality of nutritional value (Sorgeloos et al., 1986). *Artemia* larvae have valuable elements and essential enzymes for predatory fish. It has a worldwide marketing and is exported as vacuum dried cysts into developing nations across Eurasia (Gajardo and Beardmore, 2012). *Artemia* harbors several striking biological features including: a) peculiar feeding ability (non-selective filter feeding) which offers a potential vector in delivering substances through bio-encapsulation technique (Sorgeloos et al., 2001), b) primitive easy culturing with various types of feed sources under axenic and gnotobiotic conditions (Verschuere et al., 1999), c) short generation times (maximum 2 weeks) (Van Stappen, 1996), d) abundance in vast geographical regions (Bossier et al., 2004), e) two mode of reproductions (sexual/asexual), f) large or small scale of production for aquaculture purposes (Abatzopoulos et al., 2002) and g) widest adaptability to ecological conditions and long-term survival by producing cysts (Gajardo and Beardmore, 2012).

1.7. Genetics

1.7.1. Mitochondrial genome organization of *Artemia*

The complete mitochondrial genome of North American species *A. franciscana* has been sequenced. It has 15,822 base pairs (bp) in total length and 35.5% G+C content (A = 4,899, 30.96%; T = 5,297, 33.48%; G = 2,798, 17.69%; C = 2,828, 17.87%). The percentage of A+T content in *Artemia* mtDNA (64.5 %) is lower in than other arthropods ranging 75.3 % (*Locusta*), 84.8% (*Apis*) and much more similar to *Daphnia* (62.3%). Mitochondrial genome size in *Artemia* is similar to *Strongylocentrotus purpuratus* (15,650 bp), *Paracentrotus lividus*

(15,697 bp), *Drosophila yakuba* (16,019 bp), *Apis mellifera* (16.343 bp), larger than in *Caenorhabditis elegans* (13,794 bp), and *Ascaris suum* (14,284 bp) and to somewhat similar to *Daphnia* (15,333 bp) (Valverde et al., 1994). The mitochondrial genome organization of nine arthropods is shown in Table 1.

Table 1 Mitochondrial genome organization of nine arthropods (**Sources:** Crease, 1999).

[a] Protein coding genes
[b] Values are approximate as exact 5′ aand 3′ ends have not been mapped in most taxa.
[c] Mean of the two control regions

Taxon	Total length (bp)	Total (A+T%)	No. of codons	PCG[a] (A+T%)	1rRNA[b]		srRNA[c]		Control region	
					Length	(A+T%)	Length	(A+T%)	Length	(A+T%)
Artemia	15822	64.5	3521	63.9	1153	64.0	712	61.4	1770	68.0
Daphnia	15333	62.3	3681	60.4	1314	68.3	753	67.2	689	67.1
Drosophila	16019	78.6	3728	76.7	1326	83.4	789	79.3	1077	92.9
Apis	16343	84.9	3676	83.2	1371	85.3	786	81.4	827	96.0
Locusta	15722	75.3	3714	74.1	1314	78.9	827	76.0	875	86.0
Ixodes	14539	72.6	3599	71.0	1229	76.9	712	78.7	359	71.9
Rhipicephalus	14710	77.9	3592	77.9	1190	81.4	693	79.1	263	64.1
Anopheles gambiae	15363	77.6	3734	75.9	1325	82.5	800	79.6	519	94.2
Anopheles quadrimaculatus	15455	77.4	3729	75.4	1321	82.2	794	80.5	625	93.5

Artemia encodes a total of 37 genes which are found in other vertebrate and invertebrate mitochondrial genomes. *Artemia* mtDNA includes two ribosomal RNAs (12S and 16S), 22 tRNAs, three subunits of cytochrome c oxidase (CO I, II and III), two subunits of the H+ATP synthase (ATPase 6 and ATPase 8), the cytochrome b (Cyt b), and seven subunits of the NADH dehydrogenase (ND 1 to 6 and 4L). The graphical mtDNA map of *A. franciscana* is depicted in Fig. 7. The position and orientation of ribosomal RNAs and tRNA genes in *Drosophila* is much more similar to *Artemia*, but some exceptions still exist. The tRNA ^{Q} and tRNA ^{I} genes are located in a different position.

1.7.2. Genetic code and codon usage in *Artemia* mtDNA genome

In *Artemia*, ATA specifies methionine instead of isoleucine, AGA and AGG specifies serine instead of arginine and TGA specifies tryptophan instead of termination code. This condition has been conserved in other arthropods such as *Drosophila* (Clary and Wolstenholme, 1985), *Apis mellifera* (Crozier and Crozier, 1993) and *Locusta migratoria* (McCracken et al., 1987). *Artemia* has a high content of A+T in codon usage. The most frequent codons are TTT and ATT. In *Artemia*, ATN and GTG are initiation codons whereas; TAA and TAG are termination codons.

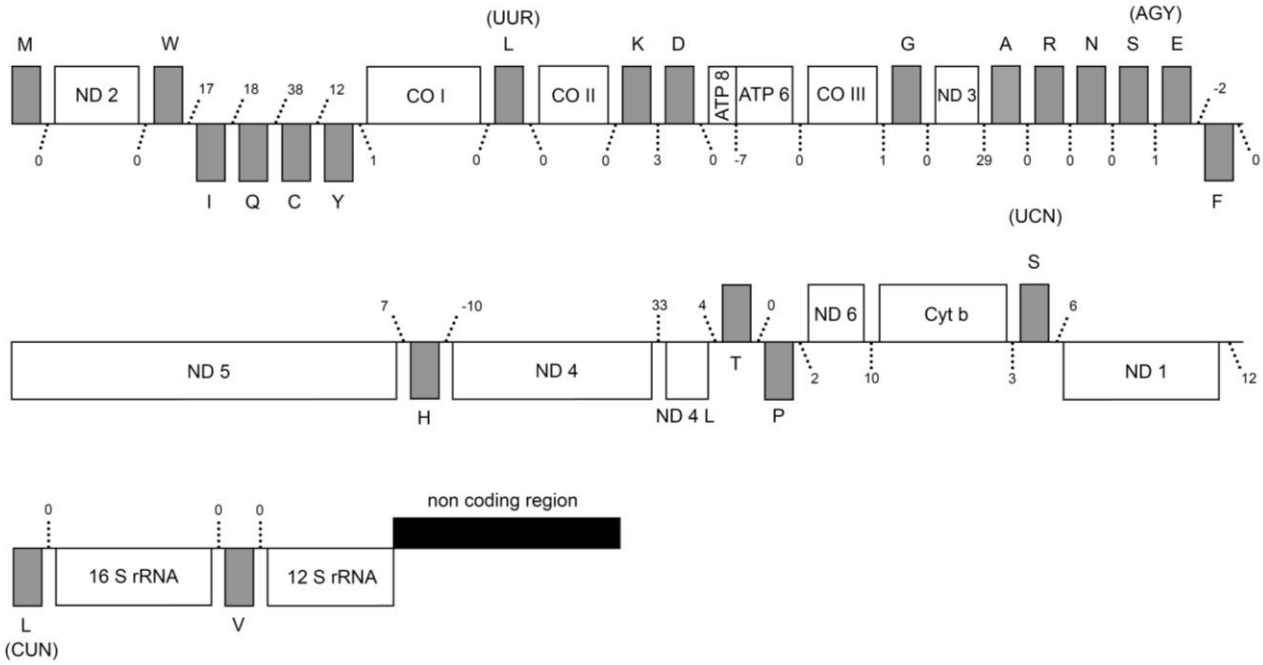


Fig. 7. A map of *Artemia franciscana* mitochondrial genome. Protein coding genes and two subunits of Ribosomal RNA genes are shown by rectangles followed by non-coding region by a wide black bar. Twenty-two tRNA genes are shown by grey-colored boxes denoted by corresponding amino acids by the one-letter code. The numbers indicates the nucleotides separating the different genes. The negative numbers indicate overlapping nucleotides between adjacent genes. Protein boxes above the line are encoded in the heavy strand of mitochondria. Protein boxes below the line are encoded in the light strand of mitochondria (**Source:** Valverde et al., 1994).

1.7.3. Molecular phylogeny

Particular attention has been focused in determining evolutionary relationships between organisms. A rapid advancement in DNA sequence technology has led researchers to employ more advanced techniques in the field of molecular phylogenetics. The main objective of phylogeny reconstruction is to define genealogical relationships among biological entities. In fact, it is an area of molecular evolution focusing on evolutionary history, divergence times and patterns from a common ancestor (Graur and Li, 2000). Evolutionary relationships among organisms are illustrated by constructing a phylogenetic tree. A tree is a graphical representation of evolutionary history of a group of organisms which consisted of nodes and branches. Branches are connected by adjacent nodes and each node represents a single taxonomic unit which characterized by species, populations or individuals (Graur and Li, 2000). There are three distinct patterns which could be observed in a phylogenetic tree including monophyly, paraphyly and polyphyly (Hennig, 1966). Monophyletic and paraphyletic have an independent single evolutionary origin but polyphyletic groups harbor a multiple

origin(s) depending on marker gene (Kitching et al., 1998). In the polyphyletic group, the evolutionary characters existed in the genome are absent in the most recent common ancestor (Kitching et al., 1998). Multiple alignments of homologous genes are input file to construct a phylogeny tree. Homologous genes have been evolved from a common ancestor whereas; paralogous genes have been diverged from a common ancestor by a duplication event (Nei and Kumar, 2000).

1.7.4. Phylogeography

Phylogeography is a new sub-discipline of combined biogeography, population genetics and phylogenetic analyses. It describes the processes related to geographical distribution of organisms particularly at the intraspecific level. It provides information about distribution of species with regard to historical aspects (Avise et al., 1987; 2000). The main aspect underlined in studies of phylogeography is the explanation of genetic variation within and between populations at different spatial scales (Avise, 2000). Phylogeography has several applications such as identification of cryptic species which has an indistinguishable morphological feature. It identifies geographical origins of species and in fact it is the fundamental basis for population genetic studies (Avise et al., 1987; Palumbi, 1996).

In phylogeography, there are two important mechanisms which have influence on the geographical distribution of organisms named vicariance and dispersal.

Vicariance happens by interrupting of geographical barriers to widespread the biotic distribution of populations. Through it, the most widespread distribution of organisms would lead to differentiate to multiple distinct populations (Sanmartin et al., 2007). Some organisms do have capability to transverse the physical barriers which means physical isolation could not cause to biotic isolation of organisms (Mathias et al., 2001). Vicariance would result in to divide populations; whereas dispersal allows isolated populations to interact with other adjacent geographically disjunct populations (Mathias et al., 2001).

Dispersal is occurred at a variety of life history stages which affect population structure of majority of organisms (McDowall, 2004, Sanmartin et al., 2007). Dispersal could be happened either active or passive (Bohonak and Jenkins, 2003). Active dispersal needs a substantial amount of energy for movement but passive dispersal is assisted by vectors (Bohonak and Jenkins, 2003). Meteorological effects such as ocean currents and winds are important vectors facilitating dispersal (Sanmartin et al., 2007). Ecological conditions, presence/absence of predators and competitors could inhibit or promote dispersal activity of organisms (Stephan, 2004). Vicariance and dispersal reflects the historical events of geographical range of population of species. In order to understand the population distribution patterns, we need to measure their effects in natural environmental condition (Bohonak, 1999).

Vicariance events occur at very large temporal scales and generally are measured in combination of geomorphological studies (Sanmartin et al., 2007). By combining the

biogeography and population genetic studies, we are able to infer the dispersal scale of natural populations.

1.7.5. Population genetics

Population genetics defines spatial structure of genetic variation between and within populations. It provides useful information at multiple population level. It includes information on the amount of gene flow, divergence times and pattern, biogeographical structure and degree of relatedness among individual of population (Bohonak and Jenkins, 2003; Feral, 2002; Palumbi, 1996, 1997; Scribner et al., 1994; Slatkin, 1987). Genetic structure studies could provide a powerful tool for understanding contemporary and historical processes among population over the evolutionary times (Bay et al., 2004; Slatkin, 1987). At population level studies, it is important to evaluate the concordance of Hardy-Weinberg equilibrium of expected populations in which random mating with no migration are occurred. There are several ways to calculate populations structure, in which F-statistic is the most widely index measuring genetic variation between sub-populations (Wright, 1951). F_{ST} value is an optimum index which measured based on F_{ST} = (Ht – Hs)/ Ht. Ht = heterozygosity of total population, Hs = average heterozygosity (Wright, 1951). Gene flow (Nm) is the average number of migrants transferring between populations over the generations. It is quantified based on Nm = $(1-F_{ST})/ 4F_{ST}$. Although gene flow has been understood the most recognized model so far, but it has some unrealistic assumptions (Whitlock and McCauley, 1999). The results obtained by F_{ST} calculations are compared to other estimates such as Nei's standard genetic distance and R_{ST} produced by microsatellite data (Slatkin, 1995).

There has been a long debate among different authors concerning F_{ST} or R_{ST} values which could reveal the desired estimates of gene flow (Gaggiotti et al., 1999, Balloux and Goudet, 2002). In theory, there is no difference between F_{ST} and R_{ST} since both of them estimates the similar assumptions, but in the more existing studies, F_{ST} is generally used for population genetic differentiation index. The sampling strategies could have a great influence on sub-populations analyses. Individuals from several sub-populations would lead to underestimate the degree of genetic structure between populations (Balloux and Lugon-Moulin, 2002).

1.7.6. Molecular clock

The number of polymorphic positions between sequences is not always useful since they could have emerged in recent or past evolutionary times. It would be very useful to determine the time when those differences are appeared. The idea that there is a linear correlation of evolutionary rate was first suggested by Zuckerland and Pauling in 1962 by comparing a protein sequence from different species. Later, they explained that the observed variance could arise from stochasticity of the process (Zuckerkandl and Pauling, 1965).

By applying this theory to DNA sequences, the observed variance across species is better explained by using per generation rather than per year time estimate (Laird et al., 1969). The number of discrepancies in molecular clock between or inside a taxonomic group is found and this led to relaxing conditions of the molecular clock. According to the suggested data, different species experienced different evolutionary pressure; some were evolving faster than others. By changing the rates over time, the use of molecular clock in the most evolutionary biology is needed (Kumar, 2005).

1.7.6.1. Molecular clock models

Several methods exist for estimating divergence times among lineages which all assume that the rate of amino acids or nucleotide substitution is approximately constant over evolutionary times (Zuckerkandl and Pauling, 1962, 1965). It is biologically unexpected to accept the identical evolutionary rates since this issue depends on different factors including natural selection, population size, generation time and mutation pattern. The “strict molecular clock” provides unreliable information about divergence times and phylogenetic inferences. The “relaxed molecular clock” models assume that each branch experienced an independent heterogeneous rate of molecular evolution. Under these models, lineages clustered in particular clades have the same evolutionary rates, because the most closely related lineages are evolved at similar evolutionary rates. The relaxed molecular clock models are explained as “autocorrelated” and basically includes the lognormal (Kishino et al., 2001) and the “CIR” processes (Lepage et al., 2007). Other researchers have refused the concept of identical evolutionary rates and they suggested applying independent rates for each lineage. The latter description is known as “uncorrelated” which includes gamma models (Drummond et al., 2006).

1.7.6.2. Molecular clock calibration

Estimation of divergence times within and among species would require the correct calibration points in a given phylogenetic tree. There are three approaches in order to calibrate the age of most common ancestor in a phylogenetic tree including fossils and biogeographic events (Bromham and Penny, 2003). Based on relaxed clock methods, multiple calibration points are used in the tree since they predict the accurate variation rate. This condition would share a divergence time for whole tree (Benton and Donoghue, 2007).

Some problems rise when using fossil data as calibration points. The fossil evidence contains many gaps and is largely incomplete. The fossil record usually does not give the actual evolutionary age for lineages, but defined minimum and maximum constraints may be used for the calibration of molecular clocks (Benton and Donoghue, 2007). The minimum constraints are hard bounds and include the oldest fossils belonging to a crown clade. The oldest fossil has always younger evolutionary age than the origin of the clade. The maximum constraints are soft bounds that may be represented by probability distributions that reflect the distribution of fossilliferous rocks around the same time (Benton et al., 2009).

According to Benton and Donoghue, (2007) “an older fossil deposit that ought to contain fossils of the clade in question, but does not, can mark an ultimate maximum bound”. The fossil data could have a great influence on the accuracy of the age of phylogenetic tree which actually needs a careful attention to use it.

Calibration dates could be based on biogeographical data, but the phylogenetic event must be associated with a major biogeographical event. The problem of the use of biogeographical data is the uncertainty of major events over time. Determining the correct calibration points for estimation divergence times require a specific evaluation of a geographical event in the context of the biology of the species.

1.7.7. Inter Simple Sequence Repeat (ISSR-PCR) genomic fingerprinting

During the last decades, several PCR-based techniques have been developed in the field of molecular marker technologies. Microsatellites or simple sequence repeats (SSR) are short and tandemly repeatable sequences which vary between one to six bases. SSR loci represent a powerful nuclear marker which is hypervariable and found more abundant in the entire genome (Liu and Wendel, 2001). Development and characterization of SSRs is considered to be time-consuming and expensive method in many experimental models. A modified method of SSR-based marker, ISSR-PCR (inter simple sequence repeat) was described by Zietkiewics et al., (1994) which has had a wide applicability in numerous studies. ISSR is a non-expensive method which generates highly reproducible and sufficient amount of polymorphisms in many systems. It works using single primer extended from 2 to 4 sequences, which in fact does not need any prior knowledge of DNA sequence information (Wang et al., 2005). This technique has been applied to understand molecular taxonomy, hybridization, sex determination, inter and intraspecific differentiation and phylogenetic reconstruction in a wide range of organisms (Wink et al., 1998, 2001; Eimanifar and Wink, 2013; Hundsdörfer et al., 2005). The amplified products are visualized by several approaches including agarose gel electrophoresis labelled with ethidium bromide, polyacrylamide gel electrophoresis (PAGE) stained with silver or radioactive (Wink et al., 1998). ISSRs are dominant markers which are assessed by the construction of a binary matrix defined by presence or absence bands (Ratnaparkhe et al., 1998). They have a relatively even distribution of microsatellites and are more informative than RAPD markers (Ratnaparkhe et al., 1998).

1.8. Aims and Scope

The present study attempts to unravel the phylogeographic structure and biogeographic history of *Artemia* lineages across its wide distribution in Eurasia, Africa and America. Through understanding the phylogenetic reconstruction of EHC lineages and their relationship with sexual species, this project aims to reveals the following objectives based on information generated by mitochondrial and nuclear DNA sequence variation and ISSR genomic fingerprinting methods.

1) Distribution pattern and biogeographic structure of Asian *Artemia* lineages.

2) Evolutionary history and estimation of divergence times of sexual and EHC lineages in Asia.

3) Intra and interspecific genetic variation of *A. urmiana* Günther, 1899 from Urmia Lake, Iran.

4) Colonization and establishment of an exotic American species *A. franciscana* Kellogg, 1906 in Asia.

5) Population genetic structure of *A. franciscana* Kellogg, 1906 from Great Salt Lake, USA.

2 Materials and methods

2.1. Sampling strategy

Artemia specimens have been collected as dried cysts by various scientists. All samples were subsequently transferred and vouchered according to the instruction in Institute of Pharmacy and Molecular Biotechnology (IPMB), Heidelberg University.

In total, samples were obtained from 102 geographical localities consisting of 600 individual cysts of *Artemia* across Eurasia, Africa and America. Samples information regarding IPMB vouchers numbers, country of origin, geographical coordinates, and GenBank accession numbers is given in the separated chapters.

2.2. Equipments

Lists of instruments and consumables used for laboratory analyses are depicted in Tables 2, 3, 4.

Table 2 Analytical instruments used in the present study.

Instruments	**Company**
Automated sequencer: ABI 310, ABI 3100	Applied Biosystems
Electrophoresis microcomputer power supply E452	Fröbel
Gel chambers for agarose gel	University Heidelberg
Gel dryer	Fröbel
Laboratory scale	Sartorius
Microcentrifuge-Biofuge 13R	Haereus
Microcentrifuge: Biofuge Fresco	Haereus
Fluorescence microscope BZ-9000E	Keyence, BioLabs
PCR machines: TRIO-Thermoblock and T Gradient	Biometra
PH meter; Pipetman P2, P20, P100, P1000	Gilson
UV-transiluminator II-200-M [312nm]	Bachofer
Vortex: Reax 2000	Heidolf
Incubator	Haereus

Photometer DU 640	Beckman
Vertical gel rig for PA gels: Base-Ace Sequencer	Stratagene

2.3. Solutions and chemicals

Table 3 Chemicals, enzymes and solutions used in the present study.

Chemicals, Enzymes and other Materials	**Company**
Agarose	HYBAID-AGS
Acrylamide, Long RangerTM	FMC/Biozym
Amonium sulfate	Gerbu
Amonium acetate	Merck
Big Dye Terminator kit	Applied Biosystems
Bovine serum albumin	Sigma
Ethanol	Merck and J.T Becker
EDTA	Roth
Ethidium bromide	Serva
Bromophenol blue	Serva
Guanidine thiocyanate	Roth
Isopropanol	Applichem
ß-Mercaptoethanol	Merck
Nucleotides	Sigma
Proteinase K	Merck
Chelex$^{@}$-100 Resin (100 – 200 mesh)	Bio-RAD
Reaction tubes (0.2, 0.5, 1.5, 2 ml)	Eppendorf
REDTaq^{TM} DNA polymerase	Sigma
Sodium dodecyl sulfate (SDS)	Applichem
^{33}P-α-ATP (3000 Ci/mmol)	Perkin Elmer, LAS, GmbH
Silane	Sigma

TEMED (N,N,N,N-Tetramethylendiamine)	Roth
Sodium acetate	Merck
Sterile filter, 0.22 µm	Sartorius
Taq DNA polymerase	Sigma
Tris-HCL	Roth
NaoCL (13%)	Güssing GmbH
Merk water	Merk
Mineral Oil	Sigma

Table 4 Buffers and solutions used in the present study.

Stock Solutions	**Components**
Agarose gel solution	1.3% agarose, 1 µg/ml ethidium bromide in water
Ammonium acetate	4 M ammonium acetate in water
Ammonium persulfate	10% solution in water
EDTA buffer	10% EDTA, 0.5% NaF, 0.5% thymol, 1% Tris (pH 7.5)
Guanidine thiocyanate buffer	4 M guanidine thiocyanate, 0.1 M Tris-HCL, 1% ß-mercapto-ethanol pH 5
λ-PST I size standard	DNA cut with PST I restriction enzyme
Lysis buffer	25 mM EDTA, 75 mM Nacl, 10 mM Tris-HCL, pH 7.0
Nucleotide mix	2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 dTTP
PCR buffer (10X)	100 mM Tris, 500 mM KCL, 5% Triton X-100, 15 mM $MgCl_2$, hydrochloric acid (pH 8.5)
SDS solution	20% solution in water
Sodium acetate solution	3 M sodium acetate, acetic acid (pH 4.6)
PA gel solution	362.5 g urea, 110 ml Long Ranger, 150 ml TBE buffer (10X), water to 1 L
TAE buffer	40 mM Tris, 1 mM EDTA, acetic acid (pH 8.0)
TE buffer	10 mM Tris, 1 mM EDTA, hydrochloric acid (pH 8.0)

2.4. DNA extraction, PCR amplification and sequencing

Detailed description of the methods is available in chapters 3-1, 3-2, 3-3 and 3-4.

2.5. Sequence alignment

Detailed description of the method is available in chapters 3-1, 3-2, 3-3 and 3-4.

2.6. Phylogenetic analyses

Detailed description of the methods is available in chapters 3-1, 3-2, 3-3 and 3-4.

2.7. Molecular dating analysis

Detailed description of the methods is available in chapter 3-1.

2.7.2. Bayesian analysis and divergence time estimation using BEAST

Detailed description of the methods is available in chapter 3-1.

2.8. Demographic history

Detailed description of the methods is available in chapter 3-1.

2.9. Genomic fingerprinting, inter-simple-sequence-repeats (ISSR)

Detailed description of the methods is available in chapters 3-2 and 3-4.

2.10. ISSR statistics

Detailed description of the methods is available in chapters 3-2 and 3-4.

2.11. Biometry of *Artemia* cysts

Detailed description of the methods is available in chapter 3-4.

2.11.1. Sample preparation

Detailed description of the methods is available in chapter 3-4.

2.11.2. *Artemia* cyst hydration

Detailed description of the methods is available in chapter 3-4.

2.11.3. Cyst measurements

Detailed description of the methods is available in chapter 3-4.

2.11.4. Diameter of decapsulated cysts

Detailed description of the methods is available in chapter 3-4.

2.11.5. Chorion thickness

Detailed description of the methods is available in chapter 3-4.

2.11.6. Statistical analysis

Detailed description of the methods is available in chapter 3-4.

3 Research projects

3.1 Biogeographical structure and evolutionary divergence times among Asian brine shrimps *Artemia* (Crustacea, Anostraca)

3.1.1. Abstract

We examined the biogeographic structure, evolutionary age and historical demography of the Asian *Artemia* lineages (*A. urmiana*, *A. sinica*, *A. tibetiana* and Eurasian haplotype complex, EHC) from 39 geographical localities using nucleotide sequences of the mitochondrial *cytochrome oxidase subunit I* (*COI*) gene. Asian *Artemia* cluster into four distinctive clades with a high nodal support consisting of 69 unique haplotypes. *A. sinica* and *A. tibetiana* have restricted geographical distribution whereas EHC lineages and especially *A. urmiana* show wider ranges. A star-like haplotype pattern was visible in EHC lineages which were genetically close to two sexual species, *A. urmiana* and *A. tibetiana*. The Bayesian approach of molecular clock indicated that *A. sinica* diverged already in the late Miocene (19.99 mya) whereas *A. urmiana*, *A. tibetiana* and EHC shared a common ancestor in the late Pliocene (5.41 mya). Both mismatch distribution and neutrality tests indicated a recent population expansion in *A. urmiana* and EHC lineages. The diversification within *A. urmiana* and EHC lineages occurred in the Pleistocene (1.72 mya) and Holocene (0.84 mya).

Keywords: Biogeographic structure, evolutionary age, Asian *Artemia*, mtDNA-*COI* marker

3.1.2. Introduction

Brine shrimps *Artemia* (Crustacea, Anostraca) are cosmopolitan extremophile microcrustaceans which are distributed in over 500 geographically isolated areas across the world except Antarctica (Abatzopoulos et al., 2002). *Artemia* is the most accomplished survivor in hypersaline environments with the two mode of reproduction including sexual and asexual (parthenogenetic) reproductions (Gajardo and Beardmore, 2012).

Three sexual species are native in Asia: *A. urmiana* Günther, 1899 (Lake Urmia, Ukraine), *A. tibetiana* Abatzopoulos et al., 1998 (Tibetan plateau) and *A. sinica* Cai, 1989 (China and Mongolia). Abatzopoulos et al., (2002) suggested that obligate parthenogenetic *Artemia* populations show different levels of ploidy and that they cannot readily be considered as belonging to a single species, for example *A. parthenogenetica* which had been introduced by Muñoz et al., (2010). Abatzopoulos et al., (2002) proposed using the general terms ‘parthenogenetic populations’ or ‘parthenogenetic strains’ as an alternative without taxonomic consequences. According to our experience, we would be even more cautious: in most cases we have only information on the origin of samples and not whether animals were sexual or parthenogenetic. As a consequence, we have introduced the term Eurasian Haplotype Complex (EHC) to describe a group of populations sharing the same basic haplotypes. EHC

includes documented parthenogenetic populations, but it needs to be established whether all EHC members are parthenogenetic. EHC lineages are widely distributed over Eurasia, extending from Canary Islands to China (Muñoz et al., 2010; Maccari et al., 2013). EHC has even been found in Great Salt Lake in North America (probably introduced by humans) (Endebu et al., 2013).

Evolutionary history of some European, African and Asian EHC lineages have been investigated based on nucleotide sequences from mtDNA and ncDNA by Baxevanis et al. (2006), Muñoz et al. (2010) and Maccari et al. (2013). Some Eurasian EHC lineages were generated through hybridization between closely related Asian species and additionally through a contagious parthenogenesis mechanism by occurrence of rare males within parthenogenetic EHC lineages (Xu et al., 2013; Maccari et al., 2013; 2014).

Divergence times of Asian *Artemia* lineages are widely unknown and therefore a more comprehensive phylogeny and evolutionary history of Asian lineages would be useful to understand the evolution and adaptation of brine shrimps. In order to explore divergence times of Asian *Artemia*, a complete set of *Artemia* samples was collected from 39 geographical areas across Asia. In addition, all available GenBank sequences from *COI* were included in the present study.

The purpose of the present study is twofold. Firstly, we expanded the phylogeographical studies of Muñoz et al. (2010) and Maccari et al. (2013) to include newly unexplored localities throughout the Asia. We used the mitochondrial *COI* gene (which is mostly employed by most authors working on brine shrimps) to further evaluate the phylogeography of Asian lineages. Secondly, evolutionary age and divergence times of all Asian *Artemia* were estimated based on a maximum parsimony molecular clock approach.

3.1.3. Materials and methods

Sampling collection and DNA extraction

We obtained 243 *Artemia* specimens collected from 39 geographical localities throughout Asia. Our sampling strategy covers most distribution areas of *Artemia* from Asia. A full list of *Artemia* samples, IPMB voucher numbers, species status and GPS coordinates of all localities are shown in Table 5. Additional sequences from Genbank were included to our dataset as shown in Table 6. Total genomic DNA was isolated from individual cyst of *Artemia* using a Chelex-based method, followed by proteinase K digestion at 56 °C for two hours (Eimanifar and Wink, 2013). Extracted DNA was stored –20 ˚C, and used further for genetic analysis.

PCR amplification and sequencing

A fragment of the mitochondrial *cytochrome c oxidase subunit I* (*COI)* was amplified and sequenced. PCR was carried out in a total volume of 50 µl reactions containing *Taq* DNA polymerase (Bioron, GmbH, Germany) as described before (Eimanifar and Wink, 2013). The

purified PCR products were directly sequenced in two reactions with the same primer used in PCR amplification as described in Eimanifar and Wink, (2013).

All sequences were aligned automatically using Bioedit 7.1.3.0 (Hall, 1999). In order to make sure that base calls were true at all polymorphic positions, we double checked whole dataset against the original chromatogram. The aligned sequences were converted into amino acids using MEGA6 in order to find a possible signal of nuclear pseudogenes (Tamura et al., 2013). Additional 277 *COI* sequences were retrieved from GenBank and added to our dataset. In total, the whole dataset includes 520 *COI* sequences. The phylogenetic analyses were rooted using *Daphnia tenebrosa* (HQ972028) as outgroup.

Phylogenetic analysis

Phylogenetic analyses were carried out using Maximum likelihood (ML) and Bayesian Inference (BI). 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 model for all dataset was TrN+I+G with the following parameters -lnL = 2514.30, (A = 0.24, C = 0.23, G = 0.18, T = 0.33), nst = 6, rate s= gamma, shape = 1.64, ncat = 4, pinvar = 0.53). A Maximum Likelihood (ML) tree was reconstructed using MEGA6 program with all proposed parameters (Tamura et al., 2013). In our dataset, the GTR model was used as a replacement for the suggested models because the suggested models were not implemented in MEGA package.

Genetic distances [p-distances and Kimura-2-parameter (K2P) nucleotide models] were calculated using MEGA6. Population genetic diversity parameters, including haplotype diversity (HD), nucleotide diversity (π), number of polymorphic sites (V), number of mutations (M) were calculated for each species using DnaSP 5.0 (Librado and Rozas, 2009). We performed two neutrality tests of Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) for each species which are based on allele frequency using Arlequin v. 3.5 (Excoffier and Lischer, 2010) with 10,000 bootstrap.

Interspecific phylogenies among *COI* haplotypes were reconstructed using median-joining network analysis, based on parsimony criteria (Bandelt et al., 1999), implemented in the software NETWORK 4.6.1.0 (Forster et al., 2004). Median joining algorithm with default settings was used for construction network (weight = 10, ε = 0). We constructed a *COI* haplotype map based on two criteria 1) all individuals sequenced in IPMB (Institute of Pharmacy and Molecular Biotechnology) and 2) Additional sequences from GenBank.

Mismatch analysis was conducted for the *COI* dataset in order to test historical demographic trends as implemented in Arlequin 3.5 (Excoffier and Lischer, 2010) with 10,000 bootstrap. Mismatch distributions (Rogers and Harpending, 1992) and the demographic parameters such as θ_0, θ_1 and τ were estimated for the mtDNA data set in Arlequin v. 3.5 (Excoffier and Lischer, 2010). Goodness of fit was assessed by the sum of square deviations

(SSD) and the Harpending's raggedness index (Harpending, 1994) between the observed and the expected mismatch with their significance determined by a parametric bootstrap. The Harpending's raggedness index quantifies the smoothness of the observed pairwise difference distribution and a non-significant result indicates an expanding population (Harpending, 1994).

Analysis of Molecular Variance (AMOVA) was performed to find out genetic variation among the complete *COI* dataset using Arlequin 3.5 (Excoffier and Lischer, 2010) with 10,000 permutations. AMOVA were grouped based on species identified in the phylogenetic tree.

Molecular dating analysis

Calibrations

There are no fossil records in *Artemia*, and we therefore 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 (Kotov and Taylor, 2011). The age of the most recent common ancestor (tMRCA) of all major clades is provided as mean ± standard deviation.

Bayesian analysis and divergence time estimation using BEAST

Bayesian tree reconstruction and divergence times of Asian *Artemia* lineages were determined using BEAST v 2.1.1.1 (Drummond and Rambaut, 2007) under following parameters: 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 run twice independently for 40 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 and 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 then inputted to FigTree v1.3.1 to visualize tree and divergence times of lineages.

3.1.4. Results

Sequence characterization

A total of 520 mitochondrial *COI* sequences were analysed. The mitochondrial alignment consisted an average of 560 nucleotides, 102 sites were polymorphic and 85 sites were parsimony-informative. The maximum genetic distance was observed within *A. tibetiana* (2%) and the lowest in *A. sinica* (0.5%). Pairwise genetic distances among *Artemia* lineages are summarized in Table 7.

Genetic diversity and phylogenetic relationships

COI haplotype diversity within sexual Asian species was higher in *A. tibetiana* and *A. urmiana* as compared to putative asexual EHC lineages. The amount of genetic diversity among EHC lineages was higher in Europe than Asia or Africa (0.71 ± 0.05, 0.55 ± 0.03 and 0.41 ± 0.09). Statistics of sequence polymorphisms are detailed in Table 8 and the distribution of haplotypes for *COI* dataset within localities are shown in Tables 9, 10.

Phylogenetic trees generated by ML and BI from *COI* sequences had concordant topologies and found four distinct well-supported clades (Fig. 8) which correspond to the recognized *Artemia* species. *A. sinica* clusters at the base of all Asian taxa. However, some specimen which had been associated with EHC lineages in previous publications (Muñoz et al., 2010; Maniatsi et al., 2011; Maccari et al., 2013) cluster with *A. urmiana*.

A *COI* phylogeny network from IPMB sequences showed 45 distinct haplotypes which are connected together with a maximum number of 105 mutational steps (Fig. 9). EHC lineages consisted of two major haplotypes (H3 and H1) from which other haplotypes derive with frequencies between 7 to 5. Numerous singleton haplotypes surrounded the major haplotypes. Haplotypes did delineate a genetic partition corresponding to species designation except H19 and H28. The two haplotypes (H19 and H28) came from Lake Urmia and had therefore been considered as *A. urmiana* but according to this analysis they belong to EHC. *A. urmiana* lineages consisted of two major haplotypes (H5, H15) with multiple singleton haplotypes.

The *COI* haplotype network of the complete dataset showed a more complex architecture, comprising 69 different haplotypes, 118 mutational steps and 4 major haplotypes which were exclusive to major species of *Artemia* (Fig. 10). EHC revealed a typical star-like topology and a short genealogy. The central haplotype H3 was the most abundant one (44%, 228 of 520), including individuals from Eurasia and Africa. Haplotype H52 consisted of individuals from Tibet which were considered as *A. tibetiana* before; according to this analysis they are part of EHC.

A. urmiana consists of a haplotype complex including two major haplotypes (H5 and H37) which are surrounded by several haplotypes with frequencies between 7 to 2. Haplotypes H2, H5, H7, H19, H46, H47, H48 and H50 correspond to individuals from Bulgaria, China, Greece, Ukraine, Tibet, Turkey and Turkmenistan indicating that *A. urmiana* has a much wider distribution than had previously been assumed.

A. sinica is represented by two major haplotypes (H30 and H32) with a strong geographical structure in Asia. *A. tibetiana* showed one major haplotype (H21) and several other haplotypes which has a close genetic relationship with *A. urmiana* and EHC.

AMOVA analysis indicated that most of the genetic variation was partitioned between lineages (94 %, $P<0.05$), whereas 6 % of genetic variation was attributed within each species

of *Artemia*. The average genetic differentiation index (*F*ST) for all lineages was calculated to be 0.94, respectively.

Historical demography

Tajima's *D* values and Fu's *Fs* estimates were negative and highly significant for *A. urmiana* and EHC (Asia), indicating the rejection of the null hypothesis of constant size (Table 8). Observed mismatch distributions for those species showed a sound match to the unimodal pattern reflecting a sudden expansion model. EHC lineages showed a clear matched pattern since their neutrality outcomes were negative and non-significant (Table 11; Fig. 11).

Estimation of divergence times based on *COI* marker

In the *COI* tree rooted with *Daphnia* the divergence between *A. sinica* and other Asian species took place in the late Miocene c. 19.99 (9.37 – 36.69) mya ago. The split between *A. urmiana* and EHC clades happened in the Pleistocene c. 2.03 (0.75 – 3.54) mya. The split between *A. tibetiana* and *A. urmiana* + EHC clades occurred in the late Pliocene c. 5.41 mya (2.19 – 9.99) (Table 12; Fig. 12). According to our calibration, diversification within *A. urmiana* and EHC lineages took place in the Pleistocene and Holocene (Table 13).

Table 5 Origin of *Artemia* samples from Asia. Samples are presented according to species designation and alphabetical order of country of origin. IPMB = Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany; ARC = Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium.

No.	IPMB voucher /ARC code number	Abbreviation for locality	Species		Locality, Province, State or District	Country	Geographic coordinates	GenBank accession numbers
			*A	B				
1	64745 /1206	XIE	S	S	Xiechi Lake, Shanxi	China	111°55'E – 35°44'N	KF691269 − KF691277
2	66311	YUN	S	S	Yuncheng, Shanxi	China	110°58'E – 34°59'N	KF691298 − KF691302
3	65829 /1524	JIN	T	T	Jingyu Lake, Xinjiang	China	89°09'E – 36°03'N	KF691215 − KF691218
4	57250	TIB1	T	T	Tibet area	China	30°46'N – 85°48'E	KF691245 − KF691249
5	57248	TIB2	T	T	Tibet area	China	31°37'N – 88°59'E	KF691316 − KF691318
6	57211	URM	U	U	Urmia Lake	Iran	45º28'E – 37º35'N	JX512748 – JX512808
7	55582 /1317	BAM	EHC	EHC	Bameng, Inner Mongolia	China	40°46'N – 107°27'E	KF691148 − KF691153
8	64756 /1233	CAN	EHC	EHC	Canghzhou, Hebei	China	38°32'N – 117°00'E	KF691166 − KF691169
9	64767 /1210	CHE	EHC	EHC	Chengkou, Shandong	China	117º43'E – 38º05'N	KF691170 − KF691172
10	64762 /1216	DON	EHC	EHC	Dongjiagou, Liaoning	China	121º 53'E –39º04'N	KF691187 − KF691189
11	64744 /1199	GAH	EHC	EHC	Gahai, Qinghai	China	97°37'E – 37°07'N	KF691199 − KF691204
12	65627 /1211	HAN	EHC	EHC	Hangu, Tianjin	China	117°50'E – 39°25'N	KF691208 − KF691214
13	64742 /1077	SHA	EHC	EHC	Shanyao, Fuijan	China	118°53'E – 25°08'N	KF691233 − KF691235

14	64764 /1073	YIN	EHC	EHC	Yingkou, Liaoning	China	122°13'E – 40°40'N	KF691287 − KF691290
15	57227	INC	EHC	EHC	Incheh Lake, Gonbad, Golestan	Iran	37º24'N – 54º36'E	KF691333 − KF691337
16	57223	LAGW	EHC	EHC	Lagoons around Urmia Lake, West Azerbaijan	Iran	37º15'N – 45º40'E	KF691338 − KF691342
17	57224	LAGE	EHC	EHC	Lagoons around Urmia Lake, Dasht-E-Tabriz, East Azerbaijan	Iran	37º47'N – 45º25'E	KF691343 − KF691345
18	57226	MIG	EHC	EHC	Mighan Salt Lake, Arak	Iran	34º20'N – 49º50'E	KF691357 − KF691361
19	57225	QOM	EHC	EHC	Qom Salt Lake, Qom	Iran	34º40'N – 51º52'E	KF691367 − KF691372
20	57255	ABG	EHC	EHC	Abu-Ghraib, Baghdad	Iraq	44º30'E – 33º20'N	KF691373 − KF691375
21	57232	ARS	EHC	EHC	Aral Sea	Kazakhstan	45º52'N – 58º43'E	KF691391 − KF691397
22	57233	ASS	EHC	EHC	Aral Sea (South)	Kazakhstan	44º43'N – 59º34'E	KF691398 − KF691403
23	57235	KYZ	EHC	EHC	Kyzylkak	Kazakhstan	53°26'N – 73°48'E	KF691404 − KF691408
24	57234	NCS	EHC	EHC	North Caspian sea	Kazakhstan	47°06'N – 51°55'E	KF691409 − KF691414
25	57236	PAV	EHC	EHC	Pavlodar	Kazakhstan	52º18'N – 76º57'E	KF691415 − KF691420
26	57231	TUZ	EHC	EHC	Tuz Lake, Pavlodar	Kazakhstan	51º19'N – 78º38'E	KF691421 − KF691434
27	57325 /1720	BYA	EHC	EHC	Bolshoye Yarovoye, Altayskiy	Russia	52º50'N – 78º41'E	KF691455 − KF691459
28	55586 /1702	EBE	EHC	EHC	Ebeyty, Omskaya	Russia	54º59'N – 73º23'E	KF691460 − KF691466
29	55581 /1641	GOR	EHC	EHC	Gorkoye Lake	Russia	55º21'N – 68º32'E	KF691467 − KF691471
30	64747 /1389	KUC	EHC	EHC	Kuchukskoye, Altayskiy	Russia	52°42'N – 79°46'E	KF691472 − KF691474
31	55579 /1528	KUL	EHC	EHC	Kulundinskoye, Altayskiy	Russia	53º10'N – 79º30'E	KF691475 − KF691477
32	64750 /1640	KUR	EHC	EHC	Kurgan area	Russia	55º29'N – 64º 27'E	KF691478 − KF691480

33	64752 /1705	MME	EHC	EHC	Maloye Medvezhye (Kurganskaya)	Russia	55°12'N – 67°57'E	KF691481 − KF691484
34	55585 /1735	MYA	EHC	EHC	Maloye Yarovoye (Altayskiy)	Russia	53º4'N – 79º10'E	KF691485 − KF691491
35	64749 /1507	MED	EHC	EHC	Medvezhye (Kurganskaya)	Russia	66°4'E – 54°55'N	KF691492 − KF691494
36	64751 /1642	VOS	EHC	EHC	Voskresenskoye (Kurganskaya)	Russia	55°32'N – 67°23'E	KF691495 − KF691497
37	57292 /1512	CAM	EHC	U	Çamalti Saltern, Izmir	Turkey	26º53'E – 38º39'N	KF691520 − KF691529
38	57258 /1371	KBG	EHC	U	Kara Bogaz Gol	Turkmenistan	53º33'E – 41º17'N	KF691530 − KF691534
39	57252 /1715	CAA	EHC	EHC	Cape Aktymsyk, Karakalpakstan	Uzbekistan	43°54'N – 59°30'E	KF691547 − KF691555

Species status is abbreviated by: **S** = *A. sinica*, **T** = *A. tibetiana*, **U** = *A. urmiana*, **EHC** = Eurasian Haplotype Complex.

*A: Species designation according to distribution; B: Species designation according to haplotypes.

Table 6 Sampling information retrieved from GenBank.

Locality, Province, state or district	Country	Species	GenBank No.
Aral Sea (ARA)	Uzbekistan	EHC	HM998996
Swakopmund (NAM)	Namibia	EHC	HM998995
Eilat (EIL)	Israel	EHC	HM998997
Ankiembe (MAD)	Madagascar	EHC	HM998999
Tanggu (TAG)	China	EHC	HM998995
Qarun (QAR)	Egypt	EHC	HM998998
Margherita di Savodia (MAS)	Italy	EHC	HM999001
Odiel (ODI)	Spain	EHC	HM999002
M. Embolon (MEM)	Greece	EHC	HM999003
M. Embolon (MEM)	Greece	EHC	HM999004
M. Embolon (MEM)	Greece	EHC	HM999005
Maharlu (MAH)	Iran	EHC	HM999000
Urmia Lake	Iran	U	JX512748–JX512808
Narte saltern (ALB)	Albania	EHC	KF707790-99
Atanasovko Lake (ATA)	Bulgaria	EHC	KF707720-26, KF707800-04
Oybuskoye Lake (OYB)	Ukraine	EHC	KF707810-19
Koyashskoe Lake (KOY)	Ukraine	EHC & U	KF707700-09, KF707805-09
Alexandria saltern (EGY)	Egypt	EHC	KF707785-89
Bagdad saltern (IRA)	Iraq	EHC	KF707727-45
Urmia Lake (URM)	Iran	EHC & U	KF707710-19, KF707765-74
Aral Sea (ARA)	Uzbekistan	EHC	KF707820-25
Maloje Jarovoe Lake (MAL)	W. Altai	EHC	KF707826-35
Bolshoe Jarovoe Lake (BOL)	W. Altai	EHC	KF707836-44
Moimishanskoe Lake (MOI)	W. Altai	EHC	KF707865-74
Korangi Creek saltern (PAK)	Pakistan	EHC	KF707775-84
Aibi Lake (AIB)	China	EHC	KF707746-54
Lagkor Co Lake (LAG)	Tibet	EHC & T	KF707845-54
Gahai Lake (GAH)	China	EHC	KF707755-64
Koyashskoe Lake (AUKOY)	Ukraine	U & EHC	KF707691-99
Urmia Lake (AUURM)	Iran	U & EHC	KF707681-90, KF707875-84
Gaize Lake (ATGAI)	Tibet	T	KF707895-99
Jingyu Lake (ATJIN)	Tibet	T	KF707909-18
Hayan Lake (ATHAY)	Tibet	T	KF707900-08
Yuncheng saltern (ASYUN)	China	S	KF707885-90
Odiel saltpan (ODI)	Spain	EHC	DQ426824
Cabo de Gata saltpan (GAT)	Spain	EHC	DQ426825
El Bosque saltpan (BOS)	Spain	EHC	DQ426826
Rio Maior saltpan (RIO)	Portugal	EHC	GU591380
Senitra saltpan (SEN)	Portugal	EHC	GU591381
Larache saltpan (LAR)	Morocco	EHC	GU591382
Margherita di Savodia saltpan (MAR)	Italy	EHC	GU591383
Bjurliv Lake (BJU)	Kazakhstan	EHC	GU591384
Vineta Swakopmund saltworks (NAM)	Namibia	EHC	
Atanasovko Lake (ATA)	Bulgaria	EHC	
Kujalnicsky Liman (KUJ)	Ukraine	EHC	
Wadi El Natrun (WAD)	Egypt	EHC	

Species status are abbreviated by: **S** = *A. sinica*, **T** = *A. tibetiana*, **U** = *A. urmiana,* **EHC** = Eurasian Haplotype Complex.

Table 7 Net nucleotide sequence divergence based on uncorrected p-distances (lower triangle) and K2P (upper triangle) for Asian *Artemia* species. 1=100%.

Species	***A. sinica***	***A. tibetiana***	***A. urmiana***	**EHC**
A. sinica		0.158	0.179	0.172
A. tibetiana	0.136		0.051	0.057
A. urmiana	0.153	0.048		0.018
EHC	0.148	0.052	0.018	

Table 8 Genetic diversity indices for *COI* from *Artemia* species.

Species	N	V	M	H	HD	π	K	*Tajima*	Fu´s Fu
A. sinica	24	7	7	6	0.7 ± 0.06	0.003 ± 0.002	1.38	– 0.81	–1.01
A. tibetiana	36	42	42	17	0.9 ± 0.02	0.01 ± 0.006	10.68	0.197	–0.22
A. urmiana	79	48	49	34	0.88 ± 0.03	0.006 ± 0.005	3.40	– 2.13*	–26.00**
EHC - Africa	38	9	9	7	0.41 ± 0.09	0.002 ± 0.001	1.22	– 1.26	–1.65
EHC - Asia	283	48	49	22	0.55 ± 0.03	0.004 ± 0.003	2.11	– 2.10*	–7.65*
EHC - Europe	58	24	24	13	0.71 ± 0.05	0.01 ± 0.003	7.25	1.27	2.20

Table 9 Data matrix of variable sites and distribution of unique haplotypes with their frequencies among 243 *Artemia* individuals using 560 nt of *COI*. H = haplotype, F = haplotype frequency and numbers = polymorphic sites. All sequences are analyzed only for IPMB dataset. GenBank sequences are marked with purple color.

H	F	Individuals & locations
H1	21	A.P._INC1_Iran A.P._INC2_Iran A.P._INC3_Iran A.P._INC4_Iran A.P._INC5_Iran A.P._KBG1_Turkmenist A.P._KBG2_Turkmenistan A.P._KBG3_Turkmenistan A.P._MIG1_Iran A.P._MIG2_Iran A.P._MIG3_Iran A.P._MIG5_Iran A.P._LAGE1_Iran A.P._LAGE3_Iran A.P._CAM2_Turkey A.P._GAH1_China A.P._GAH2_China A.P._GAH3_China A.P._GAH4_China A.P._GAH5_China A.P._GAH6_China
H2	1	A.P._KBG4_Turkmenistan
H3	121	A.P._KBG5_Turkmenistan A.P._LAGW1_Iran A.P._LAGW2_Iran A.P._LAGW3_Iran A.P._LAGW4_Iran A.P._LAGW5_Iran A.P._LAGE2_Iran A.P._ABG1_Iraq A.P._ABG2_Iraq A.P._ABG3_Iraq A.P._CAA2_Uzbekistan A.P._CAA3_Uzbekistan A.P._CAA4_Uzbekistan A.P._CAA5_Uzbekistan A.P._CAA6_Uzbekistan A.P._CAA7_Uzbekistan A.P._CAA9_Uzbekistan A.P._BYA1_Russia A.P._BYA2_Russia A.P._BYA4_Russia A.P._BYA5_Russia A.P._ASS1_Kazakhstan A.P._ASS2_Kazakhstan A.P._ASS3_Kazakhstan A.P._ASS4_Kazakhstan A.P._ASS5_Kazakhstan A.P._HAN1_China A.P._HAN2_China A.P._HAN3_China A.P._HAN4_China A.P._HAN5_China A.P._HAN7_China A.P._EBE1_Russia A.P._EBE2_Russia A.P._EBE4_Russia A.P._EBE6_Russia A.P._KUL1_Russia A.P._KUL3_Russia A.P._MED2_Russia A.P._KUR1_Russia A.P._KUR3_Russia A.P._KUR4_Russia A.P._YIN1_China A.P._YIN2_China A.P._YIN3_China A.P._YIN4_China A.P._SHA1_China A.P._SHA2_China A.P._SHA3_China A.P._CAN1_China A.P._CAN2_China A.P._CAN3_China A.P._CAN4_China A.P._CHE1_China A.P._CHE2_China A.P._CHE3_China A.P._VOS1_Russia A.P._VOS2_Russia A.P._VOS3_Russia A.P._ARS1_Kazakhstan A.P._ARS2_Kazakhstan A.P._ARS3_Kazakhstan A.P._ARS4_Kazakhstan A.P._ARS5_Kazakhstan A.P._ARS6_Kazakhstan A.P._ARS7_Kazakhstan A.P._NCS1_Kazakhstan A.P._NCS2_Kazakhstan A.P._NCS3_Kazakhstan A.P._NCS4_Kazakhstan A.P._NCS5_Kazakhstan A.P._NCS6_Kazakhstan A.P._GOR1_Russia A.P._GOR2_Russia A.P._GOR3_Russia A.P._GOR4_Russia A.P._GOR5_Russia A.P._BAM1_China A.P._BAM2_China A.P._BAM3_China A.P._BAM4_China A.P._BAM5_China A.P._BAM6_China A.P._TUZ1_Kazakhstan A.P._TUZ2_Kazakhstan A.P._TUZ3_Kazakhstan A.P._TUZ4_Kazakhstan A.P._TUZ5_Kazakhstan A.P._TUZ6_Kazakhstan A.P._TUZ7_Kazakhstan A.P._TUZ8_Kazakhstan A.P._TUZ9_Kazakhstan A.P._TUZ10_Kazakhstn A.P._TUZ11_Kazakhstan A.P._TUZ12_Kazakhstan A.P._TUZ13_Kazakhstan A.P._TUZ14_Kazakhstan A.P._PAV1_Kazakhstan A.P._PAV2_Kazakhstan A.P._PAV3_Kazakhstan A.P._PAV4_Kazakhstan A.P._PAV5_Kazakhstan A.P._PAV6_Kazakhstan A.P._KYZ1_Kazakhstan A.P._KYZ2_Kazakhstan A.P._KYZ3_Kazakhstan A.P._KYZ4_Kazakhstan A.P._KYZ5_Kazakhstan A.P._MYA1_Russia A.P._MYA2_Russia A.P._MYA3_Russia A.P._MYA4_Russia A.P._MYA5_Russia A.P._MYA6_Russia A.P._MYA7_Russia A.P._DON1_China A.P._DON2_China A.P._DON3_China A.P._KUC1_Russia A.P._KUC2_Russia A.P._KUC3_Russia
H4	7	A.P._MIG4_Iran A.P._QOM1_Iran A.P._QOM2_Iran A.P._QOM3_Iran A.P._QOM4_Iran A.P._QOM5_Iran A.P._QOM6_Iran
H5	7	A.P._CAM1_Turkey A.P._CAM3_Turkey A.P._CAM4_Turkey A.P._CAM5_Turkey A.P._CAM6_Turkey A.P._CAM9_Turkey A.P._CAM10_Turkey
H6	1	A.P._CAM7_Turkey
H7	1	A.P._CAM8_Turkey
H8	1	A.P._CAA1_Uzbekista
H9	1	A.P._CAA8_Uzbekistan
H10	1	A.P._BYA3_Russia
H11	1	A.P._HAN6_China
H12	5	A.P._MME1_Russia A.P._MME3_Russia A.P._EBE5_Russia A.P._EBE7_Russia A.P._KUL2_Russia
H13	5	A.P._MME2_Russia A.P._MME4_Russia A.P._MED1_Russia A.P._MED3_Russia A.P._KUR2_Russia
H14	1	A.P._EBE3_Russia
H15	23	A.U._NC2_1_Urmia A.U._NE2_Urmia A.U._NE5_Urmia A.U._NW3_Urmia A.U._ME1-1_Urmia A.U._ME2-2_Urmia A.U._ME2-4_Urmia A.U._MW1-1_Urmia A.U._MW1-3_Urmia A.U._MW1-4_Urmia A.U._MW1-5_Urmia A.U._MW2-1_Urmia A.U._MW2-4_Urmia A.U._SE2-4_Urmia A.U._SE2-6_Urmia A.U._SE3-3_Urmia A.U._SC1-2_Urmia

		A.U._SC1-3_Urmia A.U._SC3-1_Urmia A.U._SC3-2_Urmia A.U._SC3-4_Urmia A.U._SE1-1_Urmia A.U._SW2_Urmia
H16	1	A.U._NC2-3_Urmia
H17	1	A.U._NC1-2_Urmia
H18	1	A.U._NE1_Urmia
H19	3	A.U._NE3_Urmia A.U._NW5_Urmia A.U._SE3-2_Urmia
H20	1	A.U._NE4_Urmia
H21	1	A.U._ME2-1_Urmia
H22	1	A.U._ME2-6_Urmia
H23	3	A.U._MW1-2_Urmia A.U._SE3-1_Urmia A.U._SE1-2_Urmia
H24	1	A.U._MW1-6_Urmia
H25	1	A.U._MW2-2_Urmia
H26	1	A.U._SE2-2_Urmia
H27	1	A.U._SE2-3_Urmia
H28	1	A.U._SE2-5_Urmia
H29	1	A.U._SC2-2_Urmia
H30	1	A.U._SC3-3_Urmia
H31	1	A.U._SE1-3_Urmia
H32	1	A.T._JIN1_China
H33	5	A.T._JIN2_China A.T._JIN4_China A.T._TIB1-2_China A.T._TIB1-3_China A.T._TIB1-5_China
H34	1	A.T._JIN3_China
H35	1	A.T._TIB1-1_China
H36	1	A.T._TIB1-4_China
H37	1	A.T._TIB2-1_China
H38	1	A.T._TIB2-2_China
H39	1	A.T._TIB2-3_China
H40	1	A.SI._XIE1_China
H41	2	A.SI._XIE2_China A.SI._XIE3_China
H42	3	A.SI._XIE4_China A.SI._XIE7_China A.SI._YUN2_China
H43	1	A.SI._XIE5_China
H44	6	A.SI._XIE6_China A.SI._XIE8_China A.SI._XIE9_China A.SI._YUN1_China A.SI._YUN3_China A.SI._YUN5_China
H45	1	A.SI._YUN4_China

Note: Purple color reflects sequences from Eimanifar and Wink, 2013

Table 10 Data matrix of variable sites and distribution of unique haplotypes with their frequencies among 520 *Artemia* individuals using 560 nt of *COI*. H = haplotype, F = haplotype frequency and numbers = polymorphic sites. GenBank sequences are marked with different colors.

H	**F**	**Individuals & locations**
H1	79	A.P._INC1_Iran A.P._INC2_Iran A.P._INC3_Iran A.P._INC4_Iran A.P._INC5_Iran A.P._KBG1_Turkmenistan A.P._KBG2_Turkmenistan A.P._KBG3_Turkmenistan A.P._MIG1_Iran A.P._MIG2_Iran A.P._MIG3_Iran A.P._MIG5_Iran A.P._LAGE1_Iran A.P._LAGE3_Iran A.P._CAM2_Turkey A.P._GAH1_China A.P._GAH2_China A.P._GAH3_China A.P._GAH4_China A.P._GAH5_China A.P._GAH6_China A.P._Egypt_APD05 A.P._ELA1_Egypt A.P._ELA2_Egypt A.P._ELA3_Egypt A.P._ELA4_Egypt A.P._ELA5_Egypt A.P._ELA6_Egypt A.P._ELM1_Egypt A.P._ELM2_Egypt A.P._ELM3_Egypt A.P._ELM4_Egypt A.P._ELM5_Egypt A.P._ELM6_Egypt A.P._ELM7_Egypt A.P._ELM8_Egypt A.P._ELM9_Egypt A.P._SAI1_Egypt A.P._SAI2_Egypt A.P._SAI3_Egypt A.P._SAI4_Egypt A.P._SAI5_Egypt A.P._SAI6_Egypt A.P._ANK2_Madagascar A.P._ANK3_Madagascar A.P._ANK5_Madagascar A.P._ANK6_Madagascar A.P._ANK7_Madagascar A.P._HM998997_Israel A.P._HM998999_Madagascar A.P._HM998998_Egypt A.P._HM999001_Italy A.P._URM21_Urmia A.P._URM22_Urmia A.P._URM17_Urmia A.P._GAH3_China A.P._GAH10_China A.P._GAH5_China A.P._GAH4_China A.P._GAH1_China A.P._GAH14_China A.P._GAH6_China A.P._GAH7_China A.P._GAH8_China A.P._GAH9_China A.P._EGY1_Egypt A.P._EGY4_Egypt A.P._ALB5_Albania A.P._ALB9_Albania A.P._ALB1_Albania A.P._ALB2_Albania

		A.P._ALB4_Albania A.P._ALB6_Albania A.P._ALB7_Albania A.P._ALB10_Albania A.P._ARA5_Uzbekistan A.P._ARA6_Uzbekistan A.P._ARA8_Uzbekistan A.P._LAG7_Tibet
H2	1	A.P._KBG4_Turkmenistan
H3	228	A.P._KBG5_Turkmenistan A.P._LAGW1_Iran A.P._LAGW2_Iran A.P._LAGW3_Iran A.P._LAGW4_Iran A.P._LAGW5_Iran A.P._LAGE2_Iran A.P._ABG1_Iraq A.P._ABG2_Iraq A.P._ABG3_Iraq A.P._CAA2_Uzbekistan A.P._CAA3_Uzbekistan A.P._CAA4_Uzbekistan A.P._CAA5_Uzbekistan A.P._CAA6_Uzbekistan A.P._CAA 7_Uzbekistan A.P._CAA9_Uzbekistan A.P._BYA1_Russia A.P._BYA2_Russia A.P._BYA4_Russia A.P._BYA5_Russia A.P._ASS1_Kazakhstan A.P._ASS2_Kazakhstan A.P._ASS3_Kazakhstan A.P._ASS4_Kazakhstan A.P._ASS5_Kazakhstan A.P._ASS 6_Kazakhstan A.P._HAN-1_China A.P._HAN-2_China A.P._HAN-3_China A.P._HAN- 4_China A.P._HAN5_China A.P._HAN7_China A.P._EBE1_Russia A.P._EBE2_Russia A.P._EBE4_Russia A.P._EBE6_Russia A.P._KUL1_Russia A.P._KUL3_Russia A.P._MED2_Russia A.P._KUR1_Russia A.P._KUR3_Russia A.P._KUR4_Russia A.P._YIN1_China A.P._YIN2_China A.P._YIN3_China A.P._YIN4_China A.P._SHA 1_China A.P._SHA2_China A.P._SHA3_China A.P._CAN1_China A.P._CAN2_China A.P._CAN3_China A.P._CAN4_China A.P._CHE1_China A.P._CHE2_China A.P._CHE 3_China A.P._VOS1_Russia A.P._VOS2_Russia A.P._VOS3_Russia A.P._ARS 1_Kazakhstan A.P._ARS2_Kazakhstan A.P._ARS3_Kazakhstan A.P._ARS4_Kazakhstan A.P._ARS5_Kazakhstan A.P._ARS6_Kazakhstan A.P._ARS7_Kazakhstan A.P._NCS 1_Kazakhstan A.P._NCS2_Kazakhstan A.P._NCS3_Kazakhstan A.P._NCS4_Kazakhstan A.P._NCS5_Kazakhstan A.P._NCS6_Kazakhstan A.P._GOR1_Russia A.P._GOR2_Russia A.P._GOR3_Russia A.P._GOR4_Russia A.P._GOR5_Russia A.P._BAM1_China A.P._BAM2_China A.P._BAM3_China A.P._BAM4_China A.P._BAM5_China A.P._BAM 6_China A.P._TUZ1_Kazakhstan A.P._TUZ2_Kazakhstan A.P._TUZ3_Kazakhstan A.P._TUZ4_Kazakhstan A.P._TUZ5_Kazakhstan A.P._TUZ6_Kazakhstan A.P._TUZ 7_Kazakhstan A.P._TUZ8_Kazakhstan A.P._TUZ9_Kazakhstan A.P._TUZ10_Kazakhstan A.P._TUZ11_Kazakhstan A.P._TUZ12_Kazakhstan A.P._TUZ13_Kazakhstan A.P._TUZ 14_Kazakhstan A.P._PAV1_Kazakhstan A.P._PAV2_Kazakhstan A.P._PAV3_Kazakhstan A.P._PAV4_Kazakhstan A.P._PAV5_Kazakhstan A.P._PAV6_Kazakhstan A.P._KYZ 1_Kazakhstan A.P._KYZ2_Kazakhstan A.P._KYZ3_Kazakhstan A.P._KYZ4_Kazakhstan A.P._KYZ5_Kazakhstan A.P._MYA1_Russia A.P._MYA2_Russia A.P._MYA3_Russia A.P._MYA4_Russia A.P._MYA5_Russia A.P._MYA6_Russia A.P._MYA7_Russia A.P._DON1_China A.P._DON2_China A.P._DON3_China A.P._Spain_APD01 A.P._Spain_Portugal A.P._KUC1_Russia A.P._KUC2_Russia A.P._KUC3_Russia A.P._HM998996_Aral A.P._HM998995_Namibia A.P._HM998995_China A.P._HM999002_Spain A.P._HM999000_Iran A.P._KOY1_Ukraine A.P._KOY6_Ukraine A.P._KOY10_Ukraine A.P._KOY9_Ukraine A.P._KOY8_Ukraine A.P._KOY7_Ukraine A.P._KOY5_Ukraine A.P._KOY4_Ukraine A.P._KOY3_Ukraine A.P._KOY2_Ukraine A.P._KOY11_Ukraine A.P._KOY17_Ukraine A.P._KOY18_Ukraine A.P._KOY14_Ukraine A.P._KOY16_Ukraine A.P._URM8_Urmia A.P._URM15_Urmia A.P._URM14_Urmia A.P._URM13_Urmia A.P._URM11_Urmia A.P._URM10_Urmia A.P._URM7_Urmia A.P._URM6_Urmia A.P._URM5_Urmia A.P._URM4_Urmia A.P._URM20_Urmia A.P._URM23_Urmia A.P._URM24_Urmia A.P._URM16_Urmia A.P._URM18_Urmia A.P._URM19_Urmia A.P._URM25_Urmia A.P._ATA7_Bulgaria A.P._ATA6_Bulgaria A.P._ATA5_Bulgaria A.P._ATA4_Bulgaria A.P._ATA3_Bulgaria A.P._ATA2_Bulgaria A.P._IRA3_Iraq A.P._IRA4_Iraq A.P._IRA5_Iraq A.P._IRA12_Iraq A.P._IRA13_Iraq A.P._IRA2_Iraq A.P._IRA7_Iraq A.P._IRA8_Iraq A.P._IRA9_Iraq A.P._IRA6_Iraq A.P._IRA22_Iraq A.P._IRA15_Iraq A.P._IRA17_Iraq A.P._IRA18_Iraq A.P._IRA20_Iraq A.P._IRA14_Iraq A.P._IRA16_Iraq A.P._IRA19_Iraq A.P._IRA21_Iraq A.P._AIB9_China A.P._AIB6_China A.P._AIB4_China A.P._AIB2_China A.P._AIB1_China A.P._PAK14_Pakistan A.P._PAK11_Pakistan A.P._PAK12_Pakistan A.P._PAK13_Pakistan A.P._PAK2_Pakistan A.P._PAK3_Pakistan A.P._PAK4_Pakistan A.P._PAK5_Pakistan A.P._PAK6_Pakistan A.P._PAK7_Pakistan A.P._EGY5_Egypt A.P._EGY10_Egypt A.P._EGY9_Egypt A.P._ALB3_Albania A.P._ALB8_Albania A.P._ATA8_Bulgaria A.P._ATA10_Bulgaria A.P._ATA11_Bulgaria A.P._ATA12_Bulgaria A.P._ARA1_Uzbekistan A.P._ARA7_Uzbekistan A.P._MAL4_Russia A.P._MAL6_Russia A.P._MAL7_Russia A.P._BOL1_Russia A.P._BOL10_Russia A.P._BOL2_Russia A.P._BOL3_Russia_Mac A.P._BOL4_Russia_Mac A.P._BOL7_Russia A.P._BOL9_Russia A.P._LAG3_Tibet A.P._LAG2_Tibet A.P._LAG5_Tibet A.P._LAG1_Tibet A.P._MOI2_Russia A.P._MOI8_Russia
H4	7	A.P._MIG4_Iran A.P._QOM1_Iran A.P._QOM2_Iran A.P._QOM3_Iran A.P._QOM4_Iran A.P._QOM5_Iran A.P._QOM6_Iran
H5	24	A.P._CAM1_Turkey A.P._CAM3_Turkey A.P._CAM4_Turkey A.P._CAM5_Turkey A.P._CAM

		6_Turkey A.P._CAM9_Turkey A.P._CAM10_Turkey A.P._Bulgaria_APD07 A.P._Bulgaria_APD08 A.P._HM999004_Greece A.P._HM999005_Greece A.U._AUKOY12_Ukraine A.U._AUKOY9_Ukraine A.U._AUKOY5_Ukraine A.U._AUKOY3_Ukraine A.U._AUKOY2_Ukraine A.P._ATA1_Bulgaria A.P._OYB10_Ukraine A.P._OYB3_Ukraine A.P._OYB5_Ukraine A.P._OYB6_Ukraine A.P._OYB1_Ukraine A.P._OYB13_Ukraine A.P._OYB7_Ukraine
H6	1	A.P._CAM7_Turkey
H7	1	A.P._CAM8_Turkey
H8	1	A.P._CAA1_Uzbekistan
H9	1	A.P._CAA8_Uzbekistan
H10	4	A.P._BYA3_Russia A.P._MAL10_Russia A.P._MAL3_Russia A.P._BOL8_Russia
H11	1	A.P._HAN6_China
H12	11	A.P._MME1_Russia A.P._MME3_Russia A.P._EBE5_Russia A.P._EBE7_Russia A.P._KUL2_Russia A.P._MAL2_Russia A.P._MAL1_Russia A.P._MAL5_Russia A.P._MAL8_Russia A.P._MAL9_Russia A.P._BOL6_Russia
H13	5	A.P._MME2_Russia A.P._MME4_Russia A.P._MED1_Russia A.P._MED3_Russia A.P._KUR2_Russia
H14	8	A.P._EBE3_Russia A.P._MOI10_Russia A.P._MOI1_Russia A.P._MOI3_Russia A.P._MOI4_Russia A.P._MOI6_Russia A.P._MOI7_Russia A.P._MOI9_Russia
H15	1	A.P._Morocco_APD03
H16	1	A.P._Ukraine_APD04
H17	1	A.P._Bulgaria_APD06
H18	2	A.P._ANK1_Madagasca A.P._ANK4_Madagasca
H19	7	A.P._HM999003_Greece A.P._AIB10_China A.P._AIB7_China A.P._AIB8_China A.P._OYB4_Ukraine A.P._OYB9_Ukraine A.P._OYB8_Ukraine
H20	1	A.T._JIN1_China
H21	9	A.T._JIN2_China A.T._JIN4_China A.T._TIB1-2_China A.T._TIB1-3_China A.T._TIB1-5_China A.T._ATHAY3_China A.T._ATHAY6_China A.T._ATHAY9_China A.T._ATHAY10_China
H22	1	A.T._JIN3_China
H23	1	A.T._TIB1-1_China
H24	2	A.T._TIB1-4_China A.T._ATHAY5_China
H25	1	A.T._TIB2-1_China
H26	1	A.T._TIB2-2_China
H27	1	A.T._TIB2-3_China
H28	1	A.SI._XIE-1_China
H29	2	A.SI._XIE-2_China A.SI._XIE-3_China
H30	9	A.SI._XIE4_China A.SI._XIE7_China A.SI._YUN2_China A.SI._ASYUN3_China A.SI._ASYUN9_China A.SI._ASYUN10_China A.SI._ASYUN2_China A.SI._ASYUN4_China A.SI._ASYUN7_China
H31	1	A.SI._XIE5_China
H32	10	A.SI._XIE6_China A.SI._XIE8_China A.SI._XIE9_China A.SI._YUN1_China A.SI._YUN3_China A.SI._YUN5_China A.SI._ASYUN1_China A.SI._ASYUN5_China A.SI._ASYUN6_China A.SI._ASYUN8_China
H33	1	A.SI._YUN4_China
H34	1	A.U._AUURM10_Urmia
H35	4	A.U._AUURM9_Urmia A.U._NE3_Urmia A.U._NW5_Urmia A.U._SE3-2_Urmia
H36	1	A.U._AUURM8_Urmia
H37	30	A.U._AUURM3_Urmia A.U._AUURM4_Urmia A.U._AUURM16_Urmia A.U._AUURM18_Urmia A.U._AUURM20_Urmia A.U._AUURM21_Urmia A.U._AUURM22_Urmia A.U._NC2_1_Urmia A.U._NE2_Urmia A.U._NE5_Urmia A.U._NW3_Urmia A.U._ME1-1_Urmia A.U._ME2-2_Urmia A.U._ME2-4_Urmia A.U._MW1-1_Urmia A.U._MW1-3_Urmia A.U._MW1-4_Urmia A.U._MW1-5_Urmia A.U._MW2-1_Urmia A.U._MW2-4_Urmia A.U._SE2-4_Urmia A.U._SE2-6_Urmia A.U._SE3-3_Urmia A.U._SC1-2_Urmia A.U._SC1-3_Urmia A.U._SC3-1_Urmia A.U._SC3-2_Urmia A.U._SC3-4_Urmia A.U._SE1-1_Urmia A.U._SW2_Urmia
H38	1	A.U._AUURM12_Urmia
H39	1	A.U._AUURM11_Urmia
H40	1	A.U._AUURM7_Urmia
H41	1	A.U._AUURM5_Urmia
H42	1	A.U._AUURM2_Urmia
H43	5	A.U._AUURM17_Urmia A.U._AUURM24_Urmia A.U._MW1-2_Urmia A.U._SE3-1_Urmia A.U._SE1-2_Urmia

H44	2	A.U._AUURM19_Urmia A.U._AUURM23_Urmia
H45	2	A.U._AUURM25_Urmia A.U._MW2-2_Urmia
H46	4	A.U._AUKOY11_Ukraine_Maccari A.U._AUKOY8_Ukraine A.U._AUKOY4_Ukraine A.U._AUKOY1_Ukraine
H47	1	A.P._AIB3_China
H48	1	A.P._ATA15_Bulgaria
H49	1	A.P._ARA4_Uzbekistan
H50	5	A.P._LAG8_Tibet A.P._LAG4_Tibet A.P._LAG6_Tibet A.P._LAG10_Tibet A.P._LAG9_Tibet
H51	1	A.P._MOI5_Russia
H52	5	A.T._ATGAI4_China A.T._ATGAI1_China A.T._ATGAI5_China A.T._ATGAI2_China A.T._ATGAI3_China
H53	3	A.T._ATHAY1_China A.T._ATHAY2_China A.T._ATHAY4_China
H54	5	A.T._ATHAY8_China A.T._ATJIN1_China A.T._ATJIN3_China A.T._ATJIN5_China A.T._ATJIN10_China
H55	1	A.T._ATJIN2_China
H56	5	A.T._ATJIN4_China A.T._ATJIN6_China A.T._ATJIN7_China A.T._ATJIN8_China A.T._ATJIN9_China
H57	1	A.U._NC2-3_Urmia
H58	1	A.U._NC1-2_Urmia
H59	1	A.U._NE1_Urmia
H60	1	A.U._NE4_Urmia
H61	1	A.U._ME2-1_Urmia
H62	1	A.U._ME2-6_Urmia
H63	1	A.U._MW1-6_Urmia
H64	1	A.U._SE2-2_Urmia
H65	1	A.U._SE2-3_Urmia
H66	1	A.U._SE2-5_Urmia
H67	1	A.U._SC2-2_Urmia
H68	1	A.U._SC3-3_Urmia
H69	1	A.U._SE1-3_Urmia

Red: Muñoz et al., 2010, **Light blue:** Maniatsi et al., 2011, **Light green:** Maaccari et al., 2013, **Purple:** Eimanifar and Wink, 2013.

Table 11 Mismatch distribution analyses for Asian *Artemia* species using *COI* sequences.

Species	Mismatch distribution					
	M	θ_0	θ_1	**Tau**	**SSD (P-value)**	**Raggedness index (P-value)**
A. sinica	1.38	0.91	99999	0.51	0.01 (0.43)	0.08 (0.56)
A. tibetiana	2.24	0.53	17.22	1.93	0.001 (0.86)	0.02 (0.85)
A. urmiana	2.59	0.00	14.06	2.95	0.008 (0.13)	0.04 (0.28)
EHC	0.91	0.00	99999	0.75	0.005 (0.05)*	0.11 (0.01)*

***M*:** Observed mean mismatch, θ_0 and θ_1**:** effective population size before and after the population expansion, respectively; ***r*:** age of expansion; **SSD:** sum of the square deviations between the observed and the expected mismatch; ***Hri*:** Harpending raggedness index. Significant results are indicated by a star *P < 0.05.

Table 12 Estimated divergence time among major nodes of *Artemia* based on *COI* .

Nodes	Mean age (mya)[a]	95% HPD (mya)[b]
1	0.53	0.02 – 0.76
2	34.01	16.96 – 65.42
3	0.79	0.11 – 1.33
4	27	10.54 – 67.49
5	1.22	0.31 – 2.22
6	19.99	9.37 – 36.69
7	4.37	1.56 – 9.45
8	1.21	0.31 – 2.19
9	5.41	2.19 – 9.99
10	0.84	0.22 – 1.22
11	2.03	0.75 – 3.54
12	1.72	0.62 – 2.87

[a]Denotes that the unit of mean age is in million years.

[b]Refers to lower and upper 95% HPD intervals, and the units are in million years.

Table 13 Estimated divergence age within Asian *Artemia* species.

Species	Mean age (mya)[a]
A. sinica	1.22 (0.31 – 2.22)[b]
A. tibetiana	1.21 (0.31 – 2.19)
A. urmiana	1.72 (0.62 – 2.87)
EHC	0.84 (0.22 – 1.22)

[a]Denotes that the unit of mean age is in million years.

[b]Refers to lower and upper 95% HPD intervals, and the units are in million years.

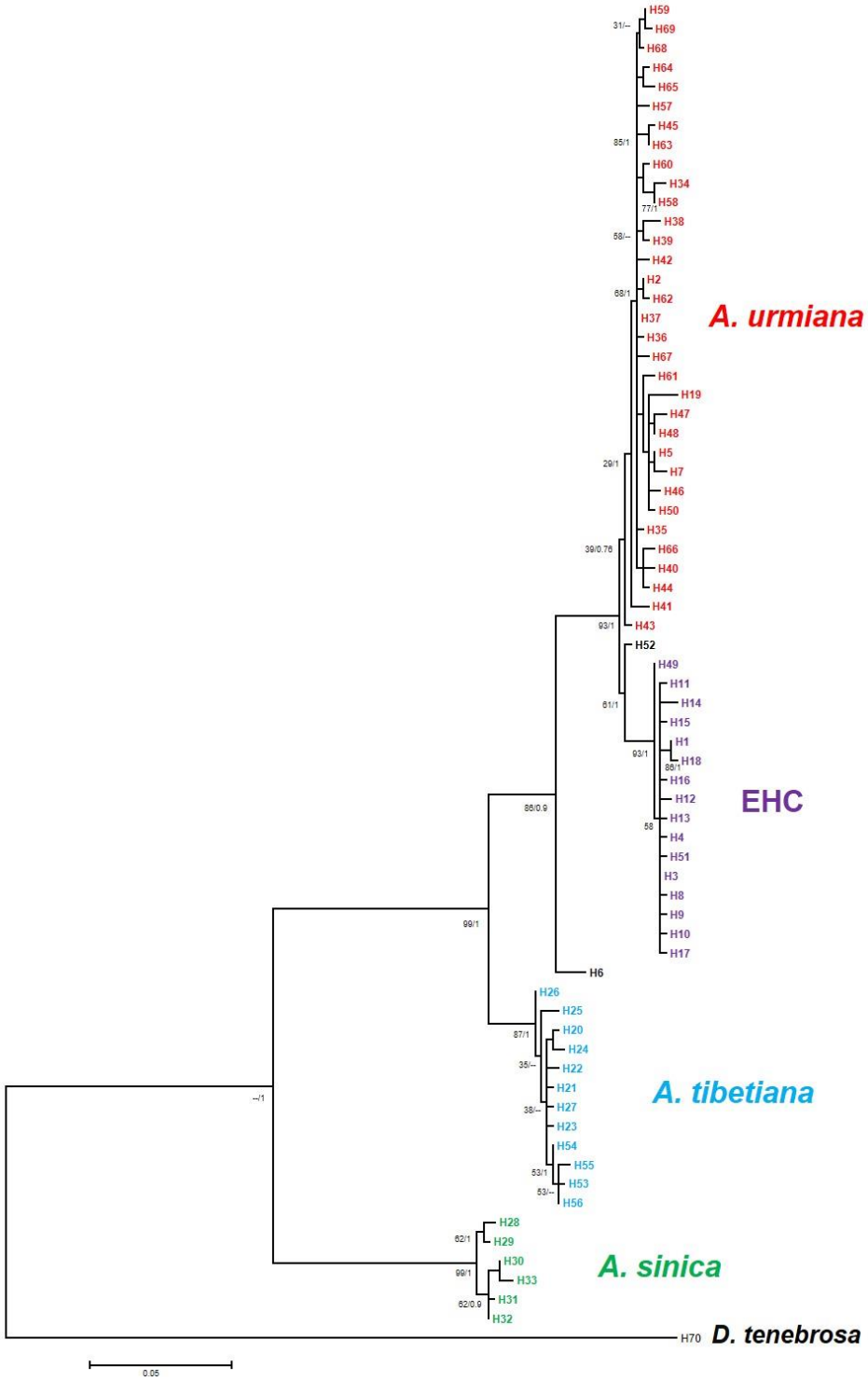


Fig. 8. Maximum likelihood phylogram for 70 unique haplotypes of Asian *Artemia* based on *COI* marker. The ML bootstrap vales and Bayesian supports are shown for each major nodes from left to right. Haplotypes found for each species corresponds to associated individuals listed in Table 10. Each species are illustrated with different colors. The tree is rooted with *Daphnia tenebrosa* (H70) as a outgroup.

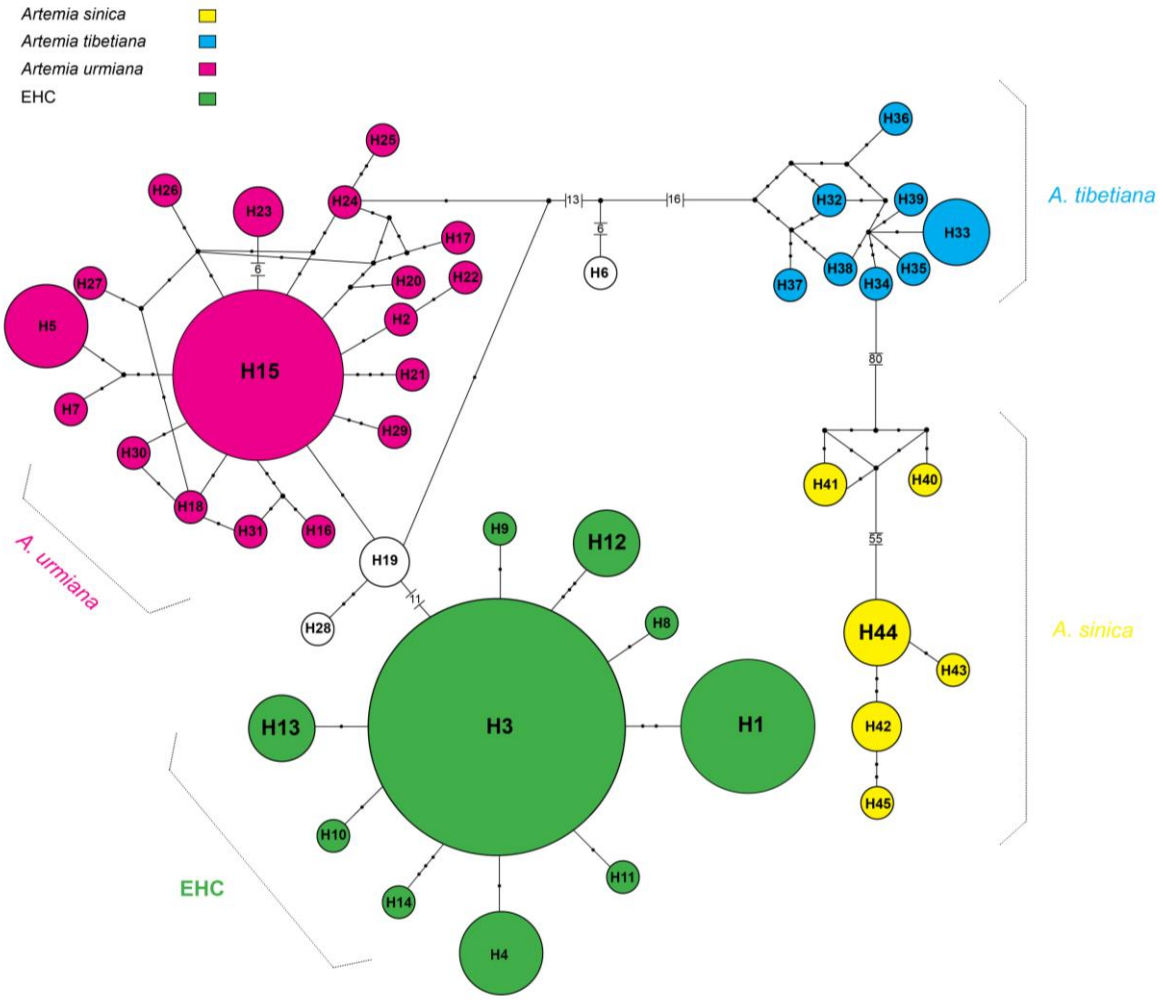


Fig. 9. *COI* haplotype network for Asian *Artemia* lineages (IPMB sequences), reconstructed by statistical parsimony. Haplotype frequencies are proportional to circle size. Circles are colored according to species description. A small black circle indicates number of mutational steps separating haplotypes. Associated individuals with their frequencies for each haplotypes are listed in Table 9.

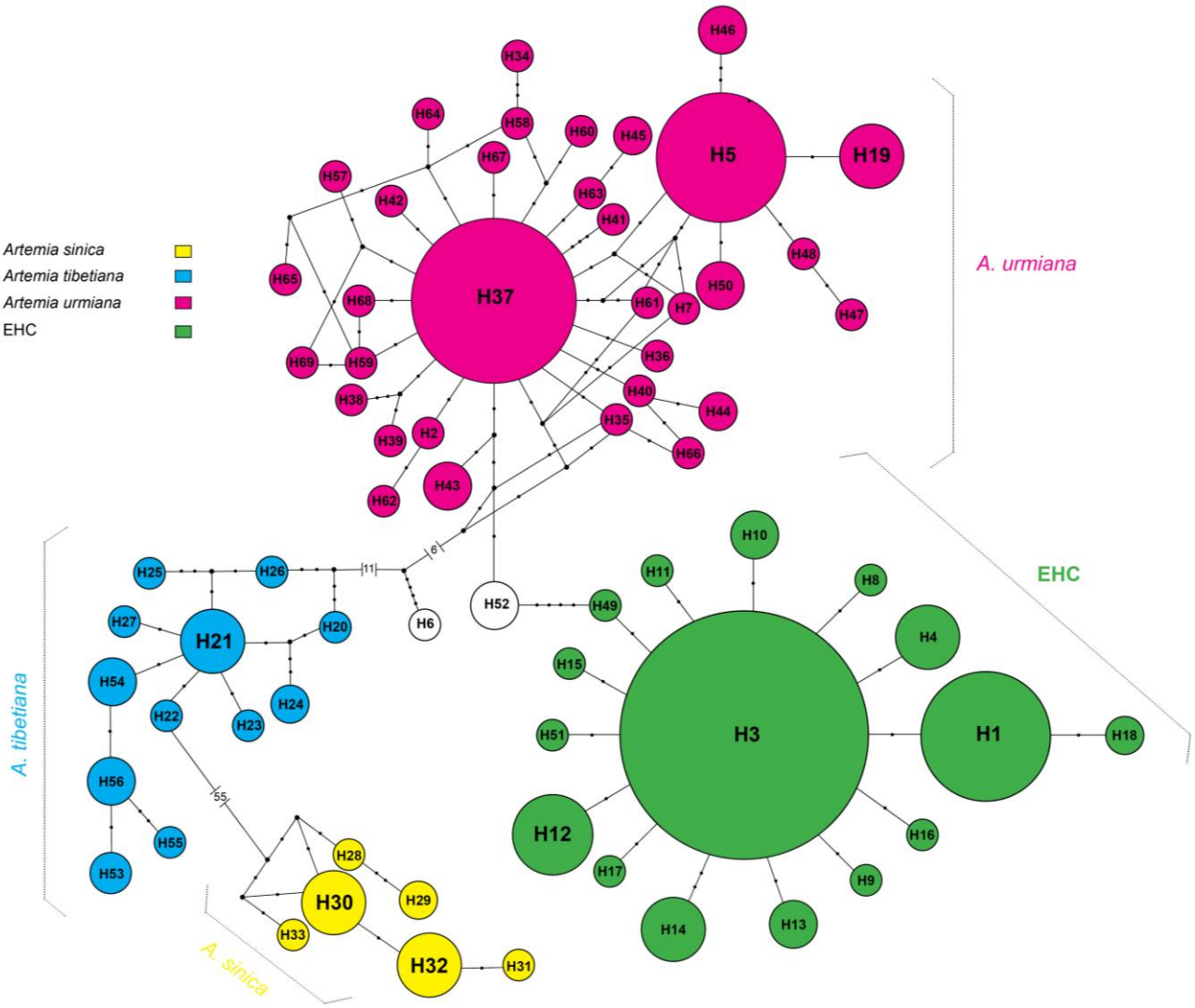


Fig. 10. Median-joining network of *COI* gene haplotypes of *Artemia* (IPMB and Genbank sequences). Each circle corresponds to haplotypes exhibiting the number of individuals. Circles are colored according to species description. A small black circle indicates number of mutational steps separating haplotypes. Associated individuals with their frequencies for each haplotypes are listed in Table 10.

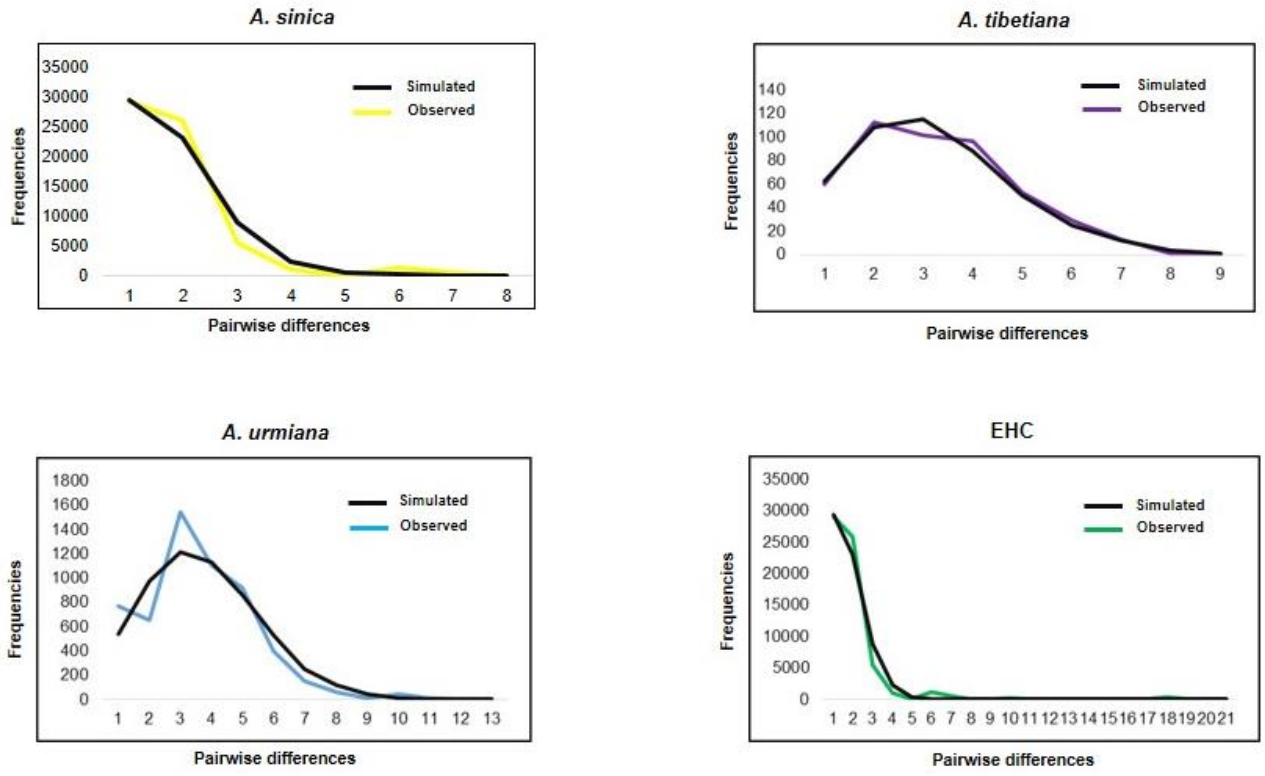


Fig. 11. Mismatch analysis showing the inferred demographic histories of *Artemia* individuals from each species. Black lines represent simulated data and colored lines represent observed data.

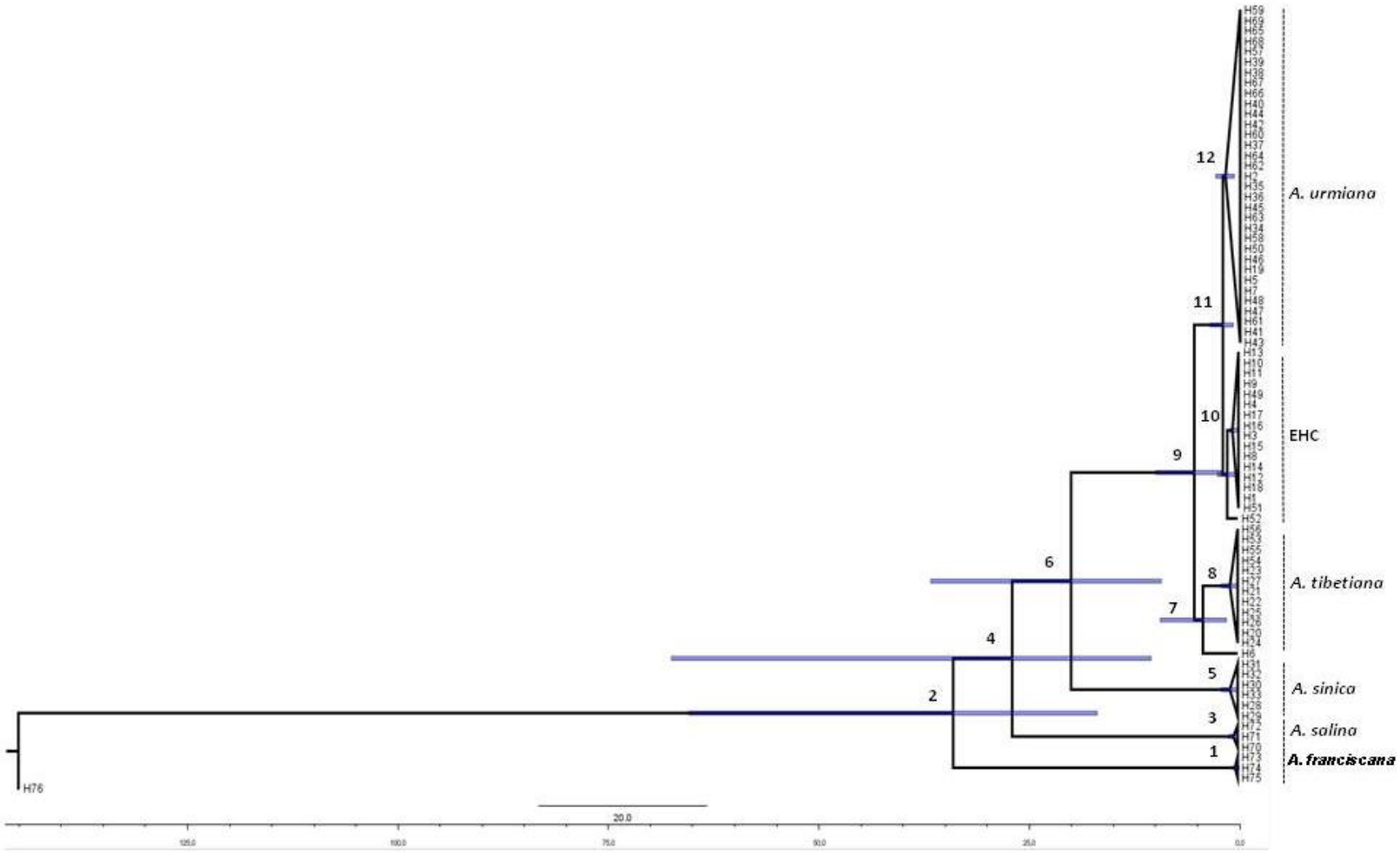


Fig. 12. A chronogram for the Asian *Artemia* lineages obtained under a relaxed clock model using *COI* gene. The blue node bars indicate 95% posterior probability intervals. The geological time scale is in million years. Mean divergence time for major nodes are shown by numbers. Each number corresponds to divergence times listed in Table 12. The tree is externally calibrated with fossil evidence.

3.1.5. Discussion

Asian *Artemia* phylogeography and haplotype network

The present study highlights the distribution pattern and phylogeography of brine shrimps throughout Asia. Our *COI* phylogeny based on ML and BI approaches delineated that Asian lineages cluster into 4 clades. Sexual species have pronounced genetic structure and are geographically isolated with the exception of *A. urmiana* which shows a much wider distribution outside Lake Urmia as previously assumed. EHC lineages showed a narrow genetic structure and broadly distributed across Eurasia and Africa (Lázaro et al., 2009; Maccari et al., 2013).

The interspecific sequence divergence based on p-distances was varied between 1.8–15.3 %. The *COI* interspecific values are within the range reported for other aquatic crustaceans such as fairy shrimp (0.012–0.058 %) (Reniers et al., 2013), Daphina (2.6–5.7 %) (Hebert et al., 2003), rotifers (0.2–13.1 %) and decapods (0.28–1.37 %).

A peculiarity in the *COI* haplotype network is the observation that some individuals corresponding to haplotypes H2, H5, H7, H19, H46, H47, H48 and H50 which had been considered to be part of EHC cluster within *A. urmiana*. According to our network, there are three hypotheses to explain this condition. Firstly, EHC lineages show a recent origin, since they might have originated from Asian sexual species (Baxevanis et al., 2006; Muñoz et al., 2010; Maccari et al., 2013). Assuming that they had recently expanded, some individuals would have not had sufficient time to diverge from their original sexual species (Law and Crespi, 2002). Secondly, *A. urmiana* might have dispersed to adjacent regions via migratory birds or human activities so that this taxon is no longer endemic in Asia (Abatzopoulos et al., 2009). A possible dispersal of *A. urmiana* into other localities has a parallel in the colonization of native American species, *A. franciscana* into non-indigenous regions across Eurasia and Australasia (Muñoz et al., 2014). In fact, the latter hypothesis needs to be carefully re-assessed, since we only deal with mitochondrial DNA sequence variation. So, more detailed systematic investigations regarding nuclear DNA markers and their life history are urgently required. Third, EHC lineages might have been mislabeled during sampling procedures or even sequencing. This opinion needs to be carefully addressed by re-checking of samples or even collecting more samples from other explored and unexplored localities in Eurasia and Africa.

Moreover, three haplotypes H19, H28, H52 which had been considered to represent *A. urmiana* and *A. tibetiana* according to their geographic distribution are apparently members of EHC (Fig. 9, 10). It needs to be analysed if the discrepancy is due to hybridization between EHC and sexual species in Asia (Baxevanis et al., 2006) or only wrongly identified specimens.

Genetic diversity of EHC

EHC showed an overall lower genetic diversity with recent evolutionary expansion (Muñoz et al., 2010). However, European EHC lineages revealed a higher genetic diversity as compared to those form Asia and Africa. Possible explanations are: Firstly, environmental heterogeneities (climate and hydrology) could influence extinction or colonization processes shaping genetic variation among lineages (Storfer et al., 2010; Maccari et al., 2013). Secondly, high frequencies of mutation and possibly presence of rare males in parthenogenetic European/African EHC lineages could enhance genetic diversity (Simon et al., 2003; Lo et al., 2009; Maccari et al., 2013, 2014). Contagious parthenogenesis has important evolutionary consequences at it results in the repeated generation of new asexual genotypes, increasing the genetic diversity in parthenogens. This counteracts the loss of asexual genotypes resulting from the accumulation of deleterious mutations (Muller's ratchet) of gene conversion (Tucker et al., 2013) and could contribute to the evolutionary success of parthenogenesis (Simon et al., 2003).

Divergence times between sexual/asexual Asian lineages

Dates of divergence among *Artemia* species are controversial. This is due to absence of fossil evidence in this genus. Our study is based on a secondary calibration with a *Daphnia* fossil whose evolutionary age is known. Based on *COI* New and Old world *Artemia* shared a common ancestor about 34 mya, whereas the divergence within Asian lineages started about 20 mya in the late Miocene which is partially in accordance other estimates (Baxevanis et al., 2006) based on nuclear genes. All EHC lineages and *A. urmiana* shared a common ancestor around 2.03 mya (Pleistocene). EHC lineages are young with a diversification within the last 0.84 million years (Holocene).

However, there are also other time estimates: Based on biogeographical evidence EHC lineages should have diverged from *A. urmiana* 11 mya and *A. sinica* from the rest of Asian species ca. 8 mya. The divergence time within EHC lineages was assumed to 3.5 mya (Baxevanis et al., 2006). As discussed before, DNA data implicate a much younger time scenario. Muñoz et al. (2010) emphasized that EHC from Africa and Europe are relatively a young lineages related to Holocene refugia. Manaffar et al. (2011) have argued that *Artemia urmiana* has diverged 11 mya, whereas Urmia Lake appears to have been formed later in the late Pleistocene. If these estimates are correct, *A. urmiana* must have originated elsewhere and was later introduced into Urmia Lake. Shadrin et al. (2012) supported this hypothesis because *Artemia* cysts extracted from sediment cores of Urmia Lake were roughly 5,000 years old and mostly likely parthenogenetic. Anufriieva and Shadrin (2012) even suggested that *A. urmiana* might have originated in Miocene salt lakes, however, this latter hypothesis appears to be very speculative as no *Artemia* fossils have so far been found.

3.2 Fine-scale population genetic structure in *Artemia urmiana* Günther, 1890 from Lake Urmia based on DNA sequences of mtDNA and ISSR genomic fingerprinting.

3.2.1. Abstract

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

Keywords: Genetic variability, Population structure, *Artemia urmiana*, Lake Urmia

3.2.2. Introduction

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

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

Lake Urmia is the largest non-coastal thalassohaline lake in the Middle East close to the Turkish border and the second largest permanent hypersaline lake in the world. It is located on a semiarid plateau in north-western Iran (37°20′ E – 45°40′ N) at 1278 m above sea level (Hassanzadeh et al., 2012). Lake Urmia shows many similarities to the Great Salt Lake in Utah (USA), including geographical topography, chemistry, and biological features (Kelts and Shahrabi, 1986; Eimanifar and Mohebbi, 2007). Apparently, Lake Urmia was

always hypersaline because it collects water from rivers but has no outlet to other areas (Kelts and Shahrabi, 1986). Within the lake there are 102 islands, and its water hosts diverse bacterial communities, hyperhalophilous phytoplankton, and notably the almost endemic brine shrimp *A. urmiana.* The lake is an international park and protected biosphere reserve as recognized by the United Nations.

Before 1995, Lake Urmia had a surface area of 5000–6000 km^2 (140×40–55 km; water depth 16 m). Annual average precipitation was 246 mm, average temperature 9.4 ºC, and water salinity 140–220 g/l (Manaffar et al., 2011; Delju et al., 2012; Hassanzadeh et al., 2012). Between 1997 and 2006, annual precipitation dropped to 204 mm and mean annual temperatures increased by 17% (Hassanzadeh et al., 2012). The progressing drought has caused fundamental changes in the physiochemical composition of the lake: currently the salinity exceeds >300 g/l. The surface area has decreased to less than 2366 km^2 and water volume was reduced from 42 in 1995 to 22 billion m^3 in 2010 (Hoseinpour et al., 2010; Manaffar et al., 2011; Pengra, 2012).

Artemia cyst production in the top 50 cm of the lake has been estimated at 4243 to 4536 t/year for 1995 (Asem et al., 2012). A considerable decline of cyst concentrations from 399 cysts/l in 1995 to 3 cysts/l in 2007 has been recorded; currently less than 1 cyst/l are assumed (Manaffar et al., 2011; Asem et al., 2012). Consequently, these alterations are already threatening the survival of fauna and flora. Eventually the *A. urmiana* will be driven to local extinction if the present conditions continue to reduce population densities even further.

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

In this study, *A. urmiana* was systematically collected from representative 15 sampling sites of Lake Urmia in order to determine its population structure and genetic variability. The mitochondrial *COI* gene was sequenced and ISSR-PCR fingerprinting was carried out to assess genomic variability and phylogeographic structure.

3.2.3. Material and methods

Sampling and DNA extraction

In order to achieve a good coverage of intraspecific genetic variability and population structure, we have collected encysted embryos from 15 sampling sites in Lake Urmia (Fig. 13). Table 14 provides information of the collection sites, voucher numbers, number of individuals studied and corresponding locality codes (used hereafter).

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

PCR amplification and sequencing protocols

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

Sequence alignment and phylogenetic analyses

All mtDNA sequences were edited using Bioedit sequence alignment vers. 7.0.9.0 (Hall, 1999). Forward and reverse sequences were inspected visually. Sequences were translated into amino acid sequences to ensure the absence of stop codons and that no pseudogenes were amplified. Sequences were aligned using Clustal W2 vers. 2.0 multiple sequence alignment (Larkin et al., 2007). The aligned nucleotide matrix, polymorphic sites, and distinct haplotypes with their frequencies were analyzed using Dnasp v. 5.00 (Librado and Rozas, 2009). All sequences were deposited in GenBank according to locality and related haplotypes (Accession Numbers JX512748–JX512808). Pairwise genetic distances were calculated using Kimura 2-parameter (K2P) (Kimura, 1980) model as provided in MEGA5 (Tamura et al., 2011). *K2P* was used because it allows for higher probability of transitional vs. transversional base substitution and has been employed in earlier phylogenetic study on bisexual *Artemia* populations (Van der Heijden et al., 2012; Hou et al., 2006). The phylogenetic trees were reconstructed using maximum likelihood (ML) in Phyml vers. 3.0 (Guindon et al., 2010) and Bayesian inference (BI) in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). The closely related *A. tibetiana* was chosen as an outgroup.

For parametric analysis, DNA sequence alignment was used to calculate the best fitting nucleotide substitution model of DNA sequence evolution, via jModelTest v. 0.1.1 (Posada, 2008) according to the corrected Akaike and Bayesian information criterion (AIC

and BIC) (Akaike, 1974). The codon-based partitioning model was used to reconstruct ML and BI tree for *COI* data set using all parameters of selected model. 1st, 2nd, and 3rd codon positions

Table 14 Sampled populations of *A. urmiana* with IPMB voucher number, geographical coordinates, total number of individuals, and molecular genetic diversity indices.

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

*__LC =__ locality code, ***N =*** number of individuals, ***HD =*** haplotype diversity, ***π =*** nucleotide diversity, ***H =*** number of haplotypes, ***P =*** number of segregating sites, ***M =*** total number of nucleotide substitutions, ***K =*** average number of nucleotide differences per population.

of *COI* gene were set up to allow each codon position to have its own rate.

Bayesian analyses were run under the following condition: two simultaneous runs, 8,000,000 generations, random starting tree with four independent Markov chains (MCMC), tree sampling every 500 generations, early-phase 0.5 million generations (50%) trees discarded as burn-in. Posterior clade probabilities (PP) were calculated from the post remaining tree in order to support branches reliability (Erixon et al., 2003). Inter- and intrapopulation genetic diversity parameters were calculated using Dnasp v. 5.00 (Librado and Rozas, 2009). The examined statistical indexes were as following: number of haplotypes (*H*), number of polymorphic sites (*P*), number of mutations (*M*), nucleotide diversity (π), haplotype diversity (*HD*), average number of nucleotide differences (*K*) per population. The

neutrality of mutations and signal of population expansion through neutral evaluation were tested by Tajima's *D* (Tajima, 1989) and Fu's *Fs* values (Fu, 1997). Genealogical relationships among haplotypes were reconstructed using Network program (Bandelt et al., 1999), based on a median joining algorithm. All sequences were collapsed to the unique haplotypes by the software Dnasp v. 5.00. The relationship between geographic and genetic distances was evaluated by Isolation-by-Distance (IBD) analysis which is included in IBDWS software v. 3.14 (Jensen et al., 2005), implementing 30,000 randomizations. IBDWS performs Mantel tests with Reduced Major Axis (RMA) regression analysis. Geographic distances (km) among localities were calculated using Google map distance calculator.

Genomic fingerprinting by ISSR-PCR

Genomic fingerprinting analysis was carried out by inter simple sequence repeat (ISSR)-PCR. Fifteen ISSR primers were initially evaluated to identify the population variability within and among 150 randomly selected individuals belonging to 15 geographically different regions of *A. urmiana* from Lake Urmia. Five out of fifteen screened primers were chosen because banding patterns could be unambiguously identified (Table 15, Fig. 14). All PCR amplifications were executed in a 25 µl final volume containing 40–50 ng of template DNA, 2.5 µl of 10× PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM TrisHCl pH 8.8, 0.1 % Tween-20, 25 mM $MgCl_2$), 10 pmol of primer, 2 µg/µl bovine serum albumin (BSA), 0.5 units of *Taq* DNA polymerase (Bioron, GmbH, Germany), 0.1 mM dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 µCi [α-^{33}P]-dATP (Perkin Elmer, LAS, GmbH, Germany). DNA amplifications were performed in a thermal cycler (Biometra, Tgradient, Germany) and started with 5 min at 94 °C followed by 35 cycles: 94 °C denaturation for 1 min, 48–54 °C annealing for 50 s and 72 °C extension for 2 min. The final cycle was followed by a 7 min extension at 72 °C. All amplified products were mixed with 8 µl of bromophenol blue and run by high-resolution denaturing polyacrylamide gels 6% (0.2 mm) for 3 h at 65 W (size 45×30 cm) containing 1× TBE buffer. The gels were dried and exposed for two days to X-ray hyperfilm (Kodak, Taufkirchen, Germany) and subsequently developed. We repeated PCR amplification for 30% of the individuals per locality to ensure reproducibility and repeatability of each marker. Eventually, the films were scanned and polymorphic bands identified for scoring.

ISSR statistics

Quality and quantity of amplified ISSR fragments were carefully checked visually. Ambiguous and smeared bands were excluded from the analysis. ISSR fragments are dominant makers which are inherited biparentally (Arafeh et al., 2002). The data matrix (1 = presence; 0 = absence of a band) was constructed to calculate genetic information of each population (Table 16). An average inbreeding level was calculated based on individual's

inbreeding coefficient F_{AFLP} (an analog to F_{IS}) in order to test the assumption of Hardy-Weinberg equilibrium (HWE) of populations using FAFLPcalc (Dasmahapatra et al., 2008).

The data matrix was then employed to determine the genetic diversity parameters for each population using AFLP SURV vers. 1.0 (Vekemans et al., 2002). The parameters were as following: number of polymorphic bands (*N*), percentage of polymorphic bands of total bands (*P*), average gene diversity (*Hw*), total gene diversity (*Ht*), and Nei's genetic diversity (Lynch and Milligan, 1994). Genetic diversity values were measured with different levels of

Table 15 List of primers screened for ISSR analysis, sequences (5'–3'), GC content, annealing temperature, amplification pattern, and total number of loci amplified in *A. urmiana*.

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

With *Y = C or T

inbreeding coefficients within populations (F_{IS} = 0, 0.05, 0.1, 0.15, 0.2, and 0.25). Genetic relationships were generated based on Jaccard's (Jaccard, 1908) similarity coefficient by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using Unweighted Pair Group Method with arithmetic average (UPGMA) (Sokal and Michener, 1958) algorithm which implemented in NTSYS-pc 2.02 software package (Rohlf, 1998). Principle Coordinate Analysis (PCA) was carried out for all ISSR genotypes in order to visualize relationships among individuals of *A. urmiana*. The programs DCENTER, EIGEN, and MOD3D were used to design PCA platform which included in NTSYS-pc 2.02 package. The final matrix was derived from the Nei genetic distance matrix in order to evaluate integrity of ISSR dendogram. Mantel's test (1967) was performed to find out any significant correlation between geographical (km) and genetic distance (Nei) using NTSYS-pc 2.02 software with 1000 random permutation. Analysis of Molecular Variances (AMOVA) was conducted to quantify genetic variability within and among regions using Arlequin v. 3.5 package (Excoffier and Lischer, 2010) with 10,000 permutations. The analysis was performed using binary matrix data sets. A total of 150 individuals were classified into 15 different groups corresponding to geographical localities. Overall population differentiation index (F_{ST}) was calculated between populations using Arlequin v. 3.5 package.

A Bayesian model-based clustering algorithm was implemented to determine the population structure using the program STRUCTURE v. 2.2 (Pritchard et al., 2000a; Falush et al., 2007). This algorithm assigns individuals into potential number of clusters (*K*). Simulations were performed by taking 10 independent runs using the admixture model with correlated allele frequencies of *K*= 1–20. ISSR genotypes were processed with a period of burn-in 50,000 and 20,000 MCMC repetitions (Jonathan et al., 2000). The most appropriate number of *K* was determined by calculating the likelihood of the posterior probability (Ln *P* (N/K) (Falush et al., 2007) and *ad hoc* quantity *DK* for each *K* partition. Posterior probability change with respect to *K* between different runs is assigned as a method for determination of true *K* value (Evanno et al., 2005).

3.2.4. Results

DNA sequence diversity & phylogeography

The DNA sequence alignment of the *COI* gene comprised a total of 561 bp. 61 *COI* sequences revealed 508 invariable and 53 variable sites of which 23 were parsimony informative. Pairwise genetic distances (K2P) for *COI* sequences differed by maximally 0.7% among all localities. *COI* sequences can be grouped into 36 haplotypes. Four haplotypes were shared by two or more individuals: Haplotype H1 is common and was found in 22 individuals (Table 17) occurring in all 15 populations. Haplotype diversity ranged from 0.6 to 1 and nucleotide diversity from 0.001 to 0.01. The overall nucleotide diversity was low (π =

0.005) but haplotype diversity (*HD* = 0.87) was highest among regions. *COI* sequences from NW, MW2, and SE2 localities showed the highest degree of variable characters (Table 14). Combining the localities from North, Middle, and South, both the highest and lowest values of *HD* and π were observed in the southern part of the lake. Tajima's *D* and Fu's *Fs* neutrality test yielded negative values with significant outcome (Tajima's $D = -2.355$, $P < 0.01$, Fu's $Fs = -37.029$, $P < 0.02$). ML and BI tree were reconstructed using one single model with all given parameters. In the present study, GTR model was used as a replacement for suggested TPM2uf+G model because suggested model was not implemented in PhyML package. We used GTR model since it is the most general neutral, independent, finite-sites model in molecular phylogenetic and has been applied in a phylogeography study on *Artemia* populations (Tavare, 1986; Muñoz et al., 2010).

The phylogenetic tree derived from *COI* sequences using ML and BI was based on 61 individuals which came from different localities. Only few distinct clades were recovered; however they were not supported by significant bootstrap values. Members within each clade usually came from different regions of the lake, indicating a high degree of connectivity between populations (Fig.15). This finding was corroborated by a haplotype-spanning network analysis which did not recover any distinct genetic structure (Fig. 16). By combining individuals into three main geographical regions, the most distribution of haplotypes is occurred in southern part of Lake Urmia (Fig. 17). Isolation by distance analysis revealed no significant correlation between geographical and genetic divergence ($Z = 3727958.2903$, $r = 0.1496$, one-sided $P = 0.93$). A value of $R^2 = 0.0224$ for RMA regression analysis was calculated.

Table 16 Construction of ISSR binary matrix for *A. urmiana* localities in Urmia Lake.

NE-5 1 0 0 1 0 0 1 0 1 0 1 1 0 0 0 0 1 0 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 1 1 1 0 1 1 1 0 1 1 0 0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 1 0 1 0 0 0 0 1 1 0 1 0 1 1 0 0 1 0 0 1 0 1 0 1 1 0 0 1 0 0 0 1 0 1 0 0 1 0 0 1 0 1 1 0 1 0 1 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 0 1 1 1

NE-6 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 1 0 0 1 1 0 1 1 0 1 1 1 1 0 1 0 0 0 1 0 0 0 0 0 1 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 1 1 1 0 0 1 0 1 0 1 1 0 1 0 1 0 0 0 1 0

NE-7 1 1 0 1 0 1 1 0 1 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0 1 1 0 0 0 1 0 1 0 0 0 0 1 0 0 0 0 0 1 1 0 0 1 1 1 0 0 1 1 0 0 0 0 0 1 1 0 0 1 0 1 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0

NE-8 1 1 0 1 0 1 1 1 1 0 0 1 0 0 0 0 1 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 0 1 0 1 1 1 1 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 1 1 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 1 1 0 1 1 0 0 1 0 1 0 1 1 0 1 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0

NE-9 1 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 0 1 0 0 0 1 0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 1 0 0 1 0 0 0 0 1 1 0 1 1 0 0 1 0 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 1 0 0 1 0 0 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 1 1 1 1 0 1 0 0 0 0 1 0 0 1 0 0 1 0 0 1 0 1 1 0 0 0 1 0 1 0 1 1 0 0 0 1 1

NE-10 1 0 0 1 0 0 1 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 0 1 1 0 0 1 0 0 1 0 0 1 0 1 0 1 1 0 1 1 0 0 1 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 1 1 0 1 1 1 1 0 1 0 0 1 1 0 1 0

NW-1 1 0 0 1 0 0 1 1 1 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 1 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 1 1 1 0 1 1 0 1 1 1 0 0 1 1 0 1 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 1 0 1 0 1 1 0 0 1 0 1 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0

NW-2 1 1 0 1 0 0 1 1 1 0 1 0 0 0 0 0 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 1 1 1 0 0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 0 1 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 1 1 0 0 1 1 0 1 1 0 0 1 1 1 0 0 0 1 0 0 0 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0

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NW-4 1 1 0 1 0 0 1 1 1 0 1 0 0 0 0 0 1 0 0 0 1 0 0 1 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 1 1 1 0 1 0 0 0 1 0 0 0 0 1 1 0 1 1 1 0 1 0 0 0 0 1 0 1 1 1 0 0 1 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 1 0 0 1 0 0 0 1 0 0 1 0 0 1 0 0 0 0 1 0 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 0 1 0 1 0 0 1 1 1 0 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 1

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NW-7 1 1 0 1 0 0 1 1 1 0 1 0 0 0 0 0 1 0 1 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 1 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 1 1 1 0 0 1 1 0 0 0 0 0 1 1 1 0 0 0 0 0 0 1 0 1 0 1 0 0 0 0 0 1 1 0 0 0 1 0 0 1 0 0 1 0 0 1 1 0 1 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 0 1 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 1 1 0 0 1 0 1 0 1 1 0 0 0 0

NW-8 0 0 1 0 1 1 0 1 0 0 0 1 1 0 0 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 1 1 1 0 1 1 1 1 0 0 0 1 0 0 0 1 0 1 0 0 1 0 0 0 0 1 1 0 0 1 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 1 1 1 0 0 0 1 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0

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ME1-1 0 0 1 0 1 1 0 1 0 0 0 1 1 0 0 0 0 1 0 1 0 1 0 0 0 0 0 1 0 0 0 1 1 0 1 1 1 1 1 1 0 0 0 1 1 0 1 0 1 0 0 1 1 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 0 1 1 1 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 1 1 1 1 1

ME1-2 0 0 1 0 1 1 0 1 0 0 0 1 1 0 0 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 1 1 1 0 1 1 1 1 0 0 0 1 0 0 0 1 0 1 0 0 1 0 0 0 0 1 1 0 0 1 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 1 1 1 0 0 0 1 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0

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ME1-5 1 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 1 1 0 0 1 0 0 1 0 0 0 1 0 1 0 1 0 1 0 0 0 1 1 1 0 1 1 1 0 0 0 1 0 1 0 0 0 0 1 0 1 1 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 0 0 1 0 0 0 0 0 0 1 1 0 0 1 0 1 1 0 0 0 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1 1 0 1 1 1

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ME1-7 1 1 0 1 0 0 1 1 1 0 0 0 0 0 0 0 1 0 1 0 0 1 0 1 1 0 1 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 1 1 1 0 0 1 0 0 0 1 0 1 1 0 1 1 0 1 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 0 0 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 1 0 0 1 1 1 1 1 0 1 0 0 1 1 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 1 1 1 1

ME1-8 1 1 0 1 1 1 1 0 1 1 1 1 0 0 1 0 1 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 0 0 1 0 0 0 0 0 0 1 0 1 1 0 1 1 1 0 0 0 0 1 1 0 0 1 0 1 1 0 1 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 0 1 1 1

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ME1-10 0 0 0 1 0 0 0 1 1 0 1 1 0 0 0 1 1 1 0 1 0 1 1 1 1 0 0 0 0 0 0 0 0 0 1 0 1 1 1 0 0 0 0 0 0 0 0 1 0 1 0 0 1 1 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1


ME2-1
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ME2-3
ME2-4
ME2-5
ME2-6
ME2-7
ME2-8
ME2-9
ME2-10
MW1-1
MW1-2
MW1-3
MW1-4
MW1-5
MW1-6
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MW1-8
MW1-9
MW1-10
MW2-1
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MW2-7
MW2-8
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SE1-1
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SC-1
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SC3-6
SC3-7
SC3-8
SC3-9
SC3-10
SW-1
SW-2
SW-3
SW-4
SW-5
SW-6
SW-7
SW-8
SW-9
SW-10

Table 17 Data matrix of polymorphic sites among 561 bp of *COI* for 61 individuals, number of unique haplotypes, and their frequencies. H indicates haplotype and F haplotype frequencies.

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

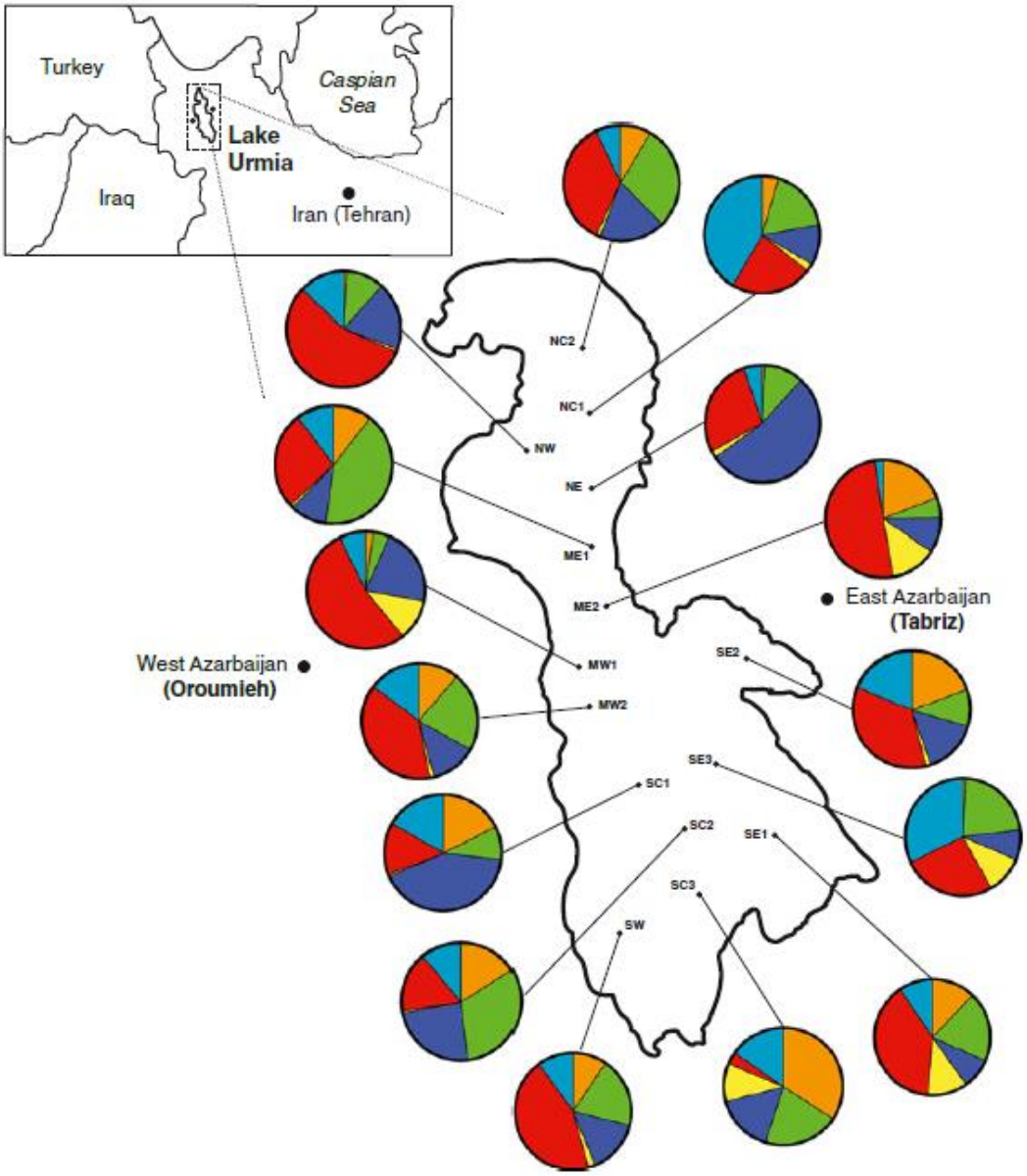

Fig. 13. Sampling sites with proportions of genetic subtypes within each locality based on Bayesian analysis of *A. urmiana* (STRUCTURE, *K* = 6). 1–6: distinct genetic clusters.

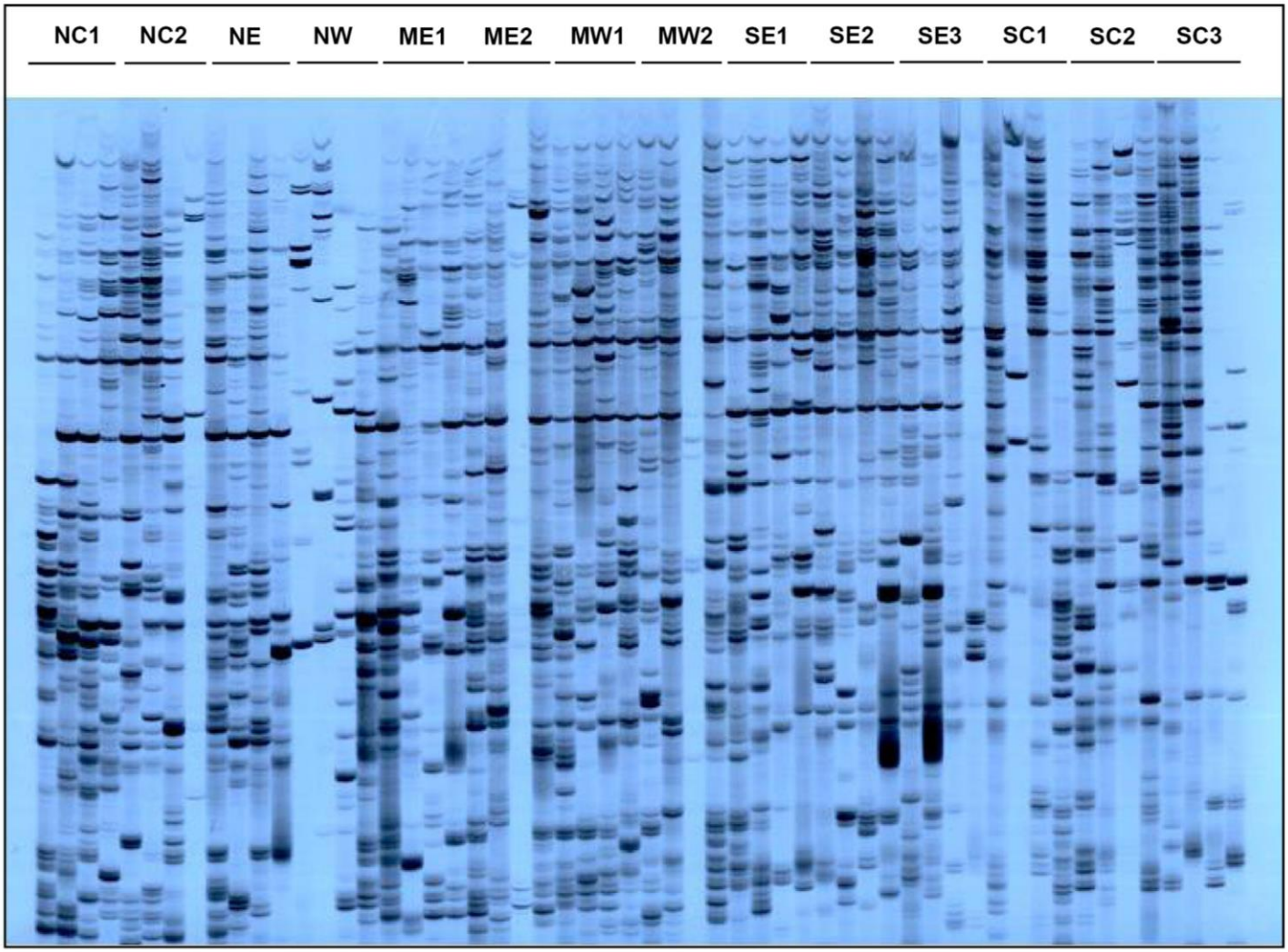

Fig. 14. A representative of ISSR gel for *A. urmiana* populations using $(AG)_8YT$. Geographical localities are shown above the gel.

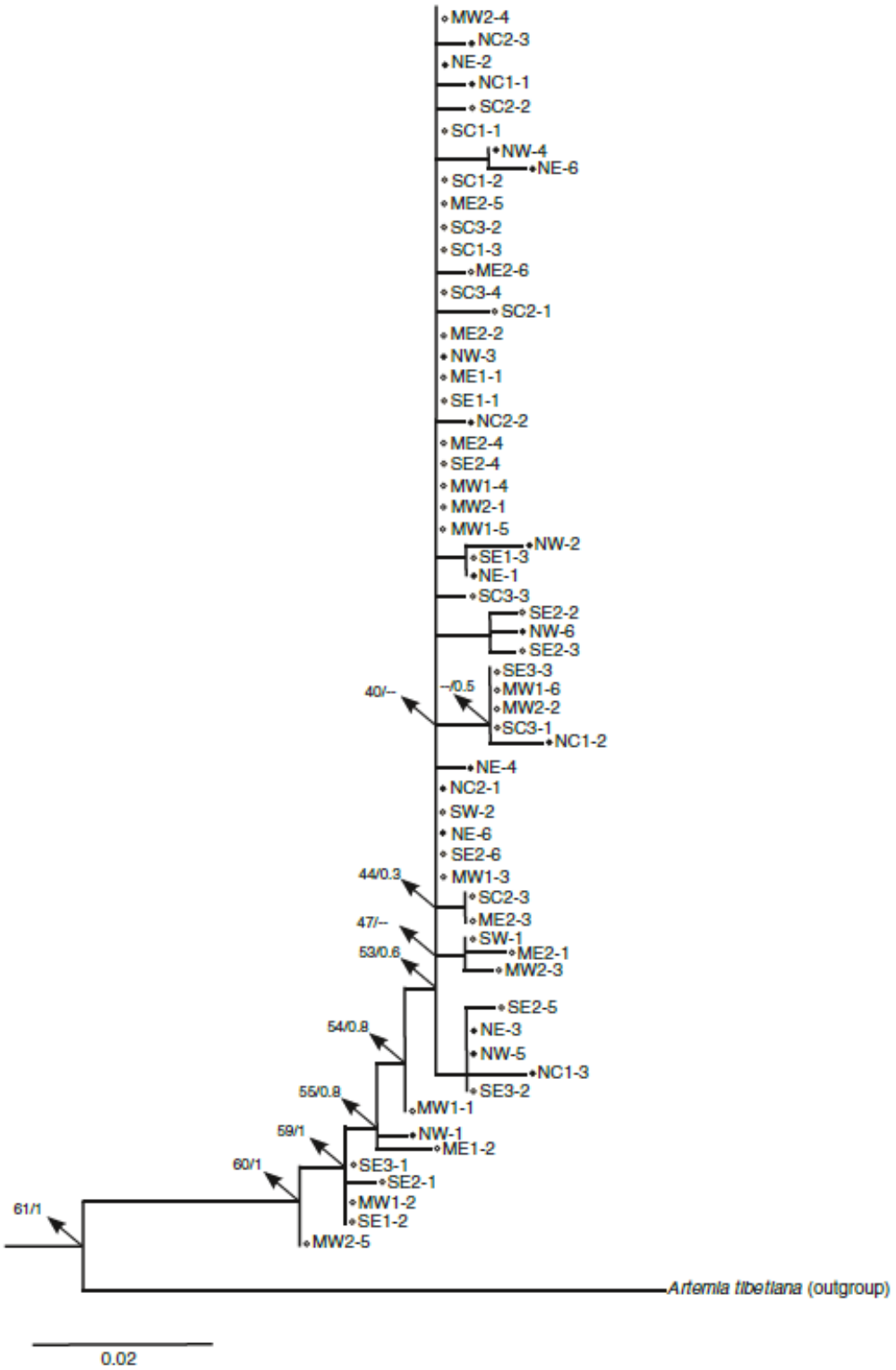

Fig. 15. Phylogeography of *A. urmiana* reconstructed from nucleotide sequences of *COI* based on GTR model from 61 individuals represented by ML tree. Bootstrap values for ML (1000 replicates), and Bayesian posterior probabilities are denoted for each major node from left to right. Northern, middle, and southern sampling sites are shown as *black*, *white* and *gray* circles.

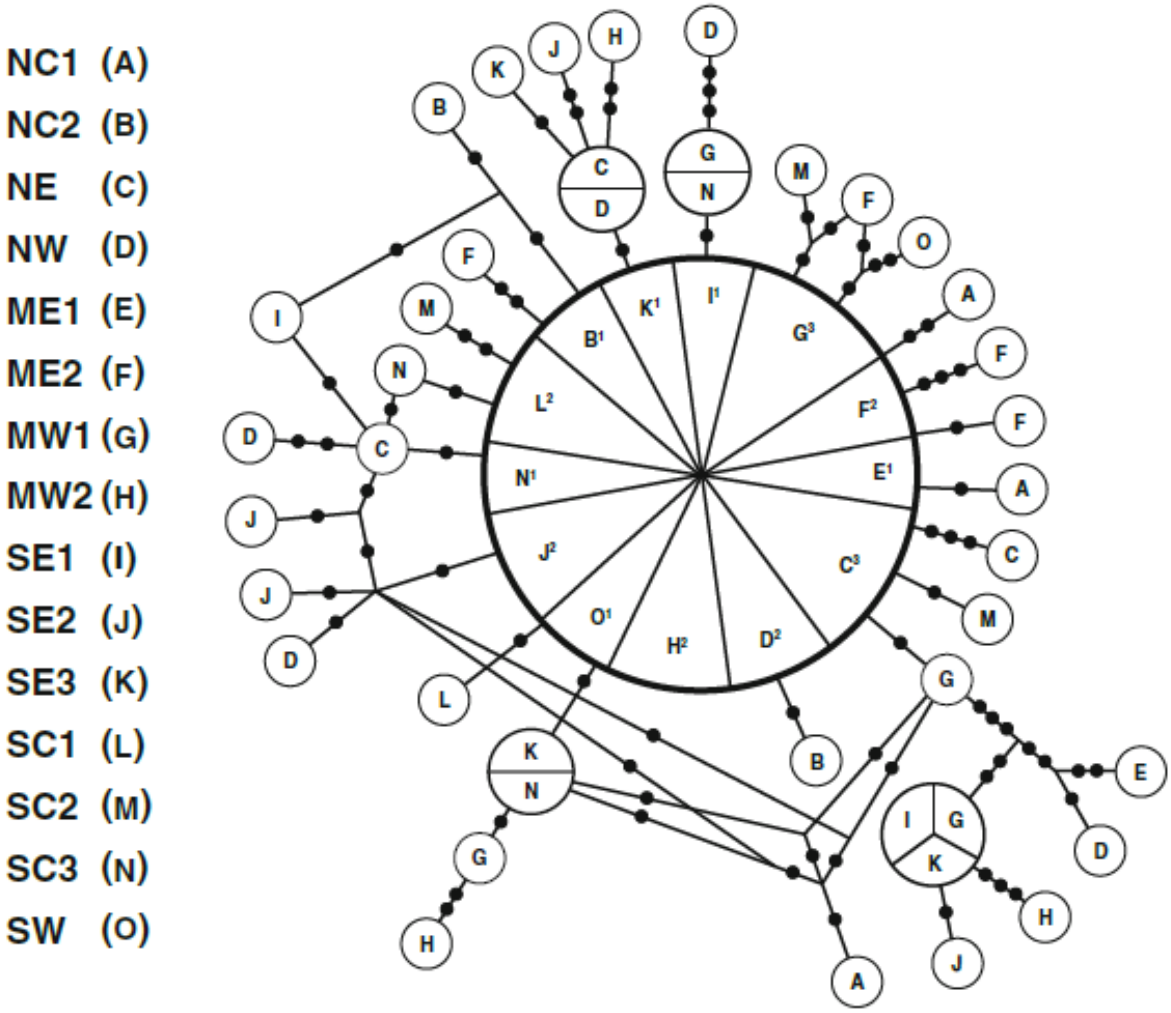

Fig. 16. Maximum parsimony haplotype network of 61 *COI* sequences. Frequency of the observed haplotypes is reflected by relative size of *circles*; *small circles* represent single haplotypes. *Bullets* depict mutational steps. A–O: abbreviations for individual sampling sites. Number of haplotype frequencies for major haplotype (center circle) indicated as superscripts for each locality; all others have frequency 1.

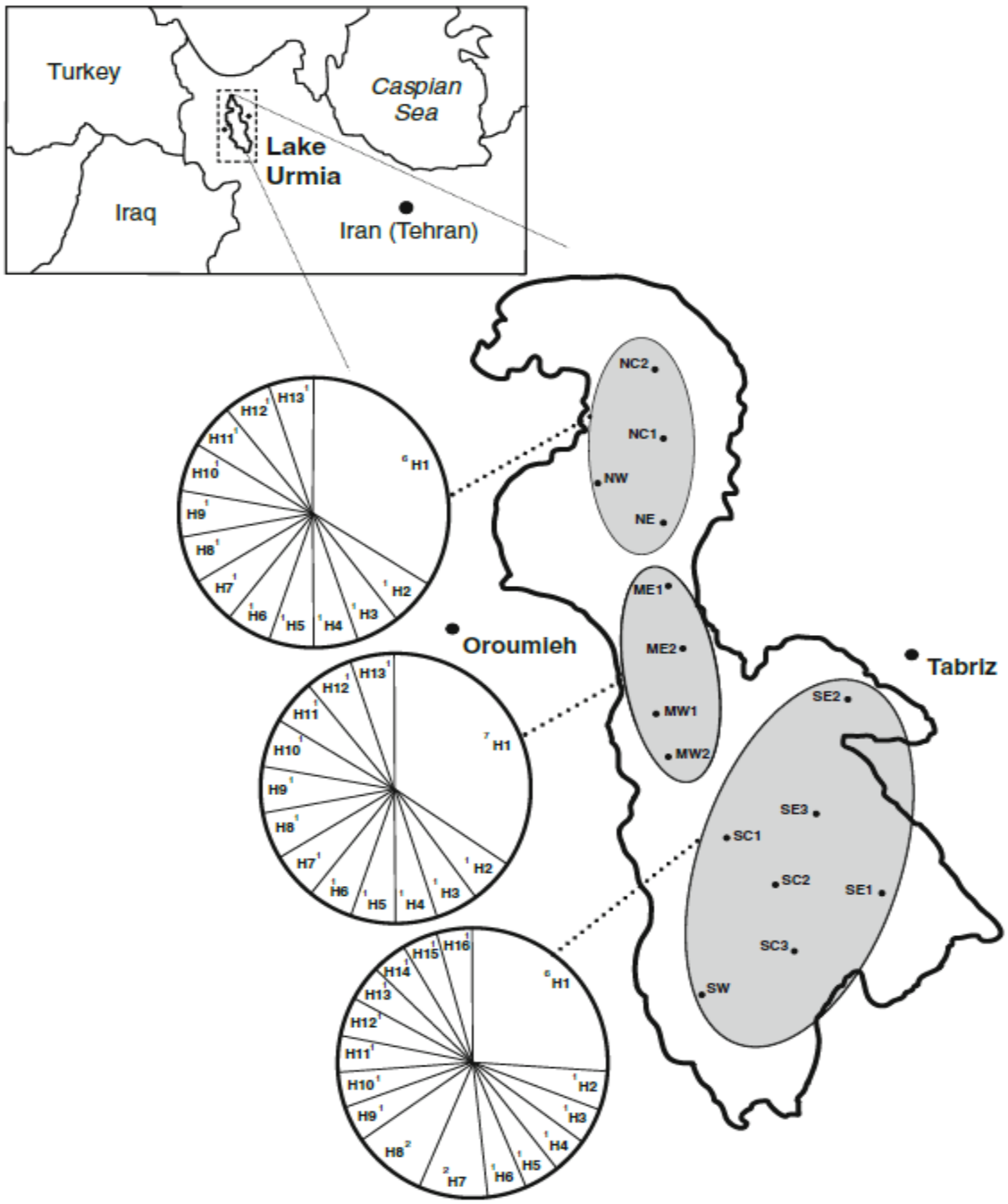

Fig. 17. Haplotype distribution map of *A. urmiana* individuals for northern, middle and southern geographical regions. Frequency of each haplotype is reflected by relative size of each segment. Number of individuals indicated as superscripts for each haplotype.

ISSR profiling

Similar to insects (Hundsdörfer et al., 2005), *Artemia* has a high number of microsatellite loci in its genome. As a consequence, ISSR-profiles can differ between individuals. In total, 172 reproducible and scorable ISSR bands were available for the analysis. The average number of polymorphic bands was 113.2 among all regions. The highest and lowest levels of genetic variability were observed in MW1, MW2 (*PPL* = 135, i.e. 78.5%, *Hj* = 0.29±0.014) and NC2 (*PPL* = 94, i.e. 54.7%, *Hj* = 0.22±0.017). The values of diversity statistics differed only slightly with an increasing F_{IS}.

Presuming a subtle deviation from HWE, all genetic diversity indexes were calculated based on calculated inbreeding coefficient level (F_{IS} = 0.25). Nei's gene diversity (*Hj*) ranged between 0.2 to 0.29, with an average of 0.24 ± 0.007, demonstrating a substantial level of genetic variability among all localities (Table 18). By grouping 15 sampling regions into three distinct areas North, Middle, and South, the highest amount of genetic variability was detected in the Middle (*PPL* = 90.7%, *Hw* (average gene diversity) = 0.26 ± 0.02, *Ht* (total gene diversity) = 0.3 and South regions of the lake (*PPL* = 95.9%, *Hw* = 0.24 ± 0.007, *Ht* = 0.3).

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

ISSR Genetic population structure

A hierarchical analysis of genetic differentiation based on ISSR data indicated that most of the genetic variation occurred within individual localities (89%). AMOVA test for ISSR data revealed low levels of differentiation between localities (F_{ST} = 0.1, P<0.05). Bayesian clustering analysis by STRUCTURE was carried out to determine the total genetic variability using the admixture model without prior information of sampling localities. The calculation of DK and *K* consistently revealed a peak which was considered to detect the best fit *K* value. Genetic composition of individuals per locality was assigned to several segments depending on genetic similarities of individuals. The first highest posterior probability was obtained for 6 segments (*K* = 6) (Table 19) represented by different colours in Fig. 13. Each individual is represented by a single vertical line which is allocated to one of the six different segments. Each colour represents one segment and proportion of each colour is based on individuals allele frequencies identified by STRUCTURE. Similar to the situation of *COI*, in each locality

individuals are present whose genotypes were not geographically coherent, indicating a random relationship of individuals across geographical localities in the lake (Fig. 18).

Table 18 Genetic variation among *A. urmiana* populations according to ISSR markers $(AG)_8C$, $(AG)_8YT$, $(GA)_9T$, $(TG)_8G$, and $(AC)_8C$.

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

N**= number of individuals examined, ***NPL = number of polymorphic loci, ***PPL*** = percentage of polymorphic loci, ***Hj*** **=** Nei's gene diversity (expected heterozygosity).

Table 19 Estimated posterior probabilities and delta *K* for each *K* partition.

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

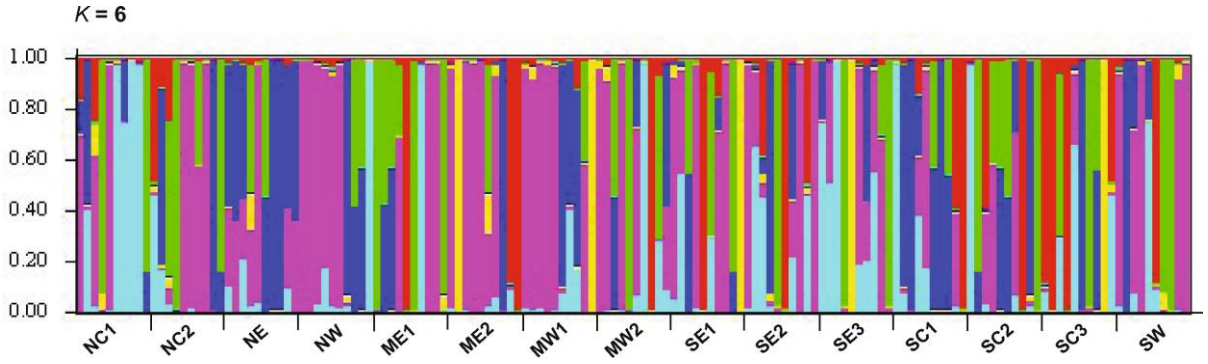

Fig. 18. Bar plot of *A. urmiana* specimens showing admixture proportion of individuals for each locality calculated by STRUCTURE (*K* = 6). Sampling sites are shown below bar plot. In bar plot, each individual corresponds to a thin vertical line and the proportion of each locality is represented by its corresponding colours.

3.2.5. Discussion

Genetic variability and population structure

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

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

Lack of a clear dispersal genetic structure among sampling regions is usually attributed to an excessive amount of gene flow which is sufficient to prevent genetic drift or natural selection (Maggio et al., 2009). *A. urmiana* showed a random relationship of individuals resulting in a large panmictic population. Panmixia in marine environments is due to an obvious lack of physical barriers to gene flow (Cowen et al., 2000). Panmixia has been observed in the water bloom-forming cyanobacterium *Microcystis aeruginosa* in which multilocus sequence analysis demonstrated five intraspecific lineages with a high frequency

of recombination (Tanabe and Watanabe, 2011). Absence of population structure with high amount of gene flow was observed in the crustacean *Aristeus antennatus* and the marine fish *Thunnus obesus* (Chiang et al., 2006; Maggio et al., 2009). Continental zooplankters such as *Artemia* appear to be panmictic due to passive transport of cysts through wind and waterfowl-mediated dispersal (Green et al., 2005; Maniatsi et al., 2009). High dispersal ability of *A. urmiana* via bird species, hydrological connectivity and anthropogenic influences (intentional and nonintentional) could explain the panmixia hypothesis also for *A. urmiana.* The short-distance dispersal of fairy shrimp *Ranchipodopsis wolfi* by wind and the long-distance dispersal of *Daphnia lumholtzi* by migratory waterfowl are other examples in this regard (Brendonck and Riddoch, 1999; Havel et al., 2000). Salt concentrations can shape different ecological habitats in saline lakes (Hontoria and Amat, 1992). However, Lake Urmia does not have significant differences in salinity between its northern and southern part (Agh, 2007). This implies a lack of distinct ecological zones in the lake. Our genetic data agree with the idea that a strong ecological differentiation does not exist in Lake Urmia. The evolutionary pattern of *A. urmiana* is consistent with morphological variation investigated by Asem et al., (2007) indicating random distribution of morphotypes across the lake.

Population genetic differentiation

Genomic analyses by ISSR fingerprinting have proven to be reliable and advantageous in their cost-benefit outcome for detection of DNA polymorphisms in different taxa (Hundsdörfer et al., 2005; Sarwat, 2012). The ISSR technique had been used by Hou et al., (2006) to examine 10 parthenogenetic *Artemia* strains from China. A similar population diversity was found in these Chinese populations (H = 0.29) as in our study. The F_{ST} parameter can vary between 0 (absence of genetic divergence) and 1 (fixation of alleles) within the population. High F_{ST} values are indicative of high genetic differentiation in populations (Ruiz et al., 2011). Multiple clades of *A. urmiana* individuals have been revealed by means of applying two clustering methods (UPGMA and STRUCTURE). The UPGMA dendrogram and PCA tree topology are congruent with morphological patterns indicating the presence of various genetic lineages of *A. urmiana* with random distribution of individuals in the lake (Asem et al., 2007).

Suggested conservation measures

Desiccation of Lake Urmia is severely progressing due to ecological changes and human activities. If these influences continue, they will cause a loss of genetic diversity as well as a dwindling of food resources for many organisms. Although *Artemia* is rather a hardy organism, the almost endemic *A. urmiana* population appears to be doomed to extinction unless effective conservation measures are developed and implemented enabling a sustainable exploitation of *Artemia* in the future. In the present drought conditions, annual

population density assessment of *A. urmiana* is urgently required to estimate the size of the actual cyst bank throughout Lake Urmia. Establishment of *ex situ* collections (e.g., a live cyst bank) from different geographical locations within Lake Urmia could be a first step to conserve the diversity of genetic resources of *A. urmiana*.

3.3 *Artemia* biodiversity in Asia with the focus on the phylogeography of the introduced American species *Artemia franciscana* Kellogg, 1906

3.3.1. Abstract

Asia harbors a diverse group of sexual and asexual *Artemia* species, including the invasive *Artemia franciscana*, which is native to the Americas. The phylogeny of Asian *Artemia* species and the phylogeography of the introduced *A. franciscana* from 81 sampling localities in Eurasia, Africa and America were elucidated using mitochondrial (*COI*) and nuclear DNA (*ITS1*) sequences. According to a *COI* phylogeny, 7 distinctive genetic groups were recognized, with a complex phylogeographic structure among Asian *Artemia*. A haplotype complex which includes parthenogenetic lineages is distributed in 39 inland geographical localities in Asia, illustrating a wide distribution with a narrow genetic structure on this continent. The invasive *A. franciscana* was discovered in 31 geographical localities along the southern and eastern coastal regions of Asia. Three sexual species (*A. sinica*, *A. tibetiana* and *A. urmiana*) have a restricted distribution in certain geographical localities in Asia. In contrast to *COI* phylogeny reconstruction, *ITS1* sequences showed 5 genetic clades which were not fully consistent with the *COI* tree, indicating gene flow and hybridization. Asian *A. franciscana* showed higher haplotype diversity as compared to the source population from the Great Salt Lake (USA), which could be attributed to multiple introductions by mass dispersal in Asia via human activities. The invasive success of *A. franciscana* in Asia could lead to a long-term biodiversity disturbance of the autochthonous *Artemia* species on the continent.

Keywords: Asian *Artemia*, biodiversity, mtDNA-*COI*, nDNA-*ITS1*, phylogeography, introduced species.

3.3.2. Introduction

Artemia (Crustacea, Anostraca) – a cosmopolitan halophilic microcrustacean – is the most conspicuous inhabitant of hypersaline lakes, salt ponds, lagoons, and man-made saltworks. The genus has a worldwide distribution and is composed of six well-described bisexual species and parthenogenetic lineages with diverse ploidy levels (Gajardo and Beardmore, 2012; Scalone and Rabet, 2013).

Two bisexual species are native to the New World, namely *A. persimilis* Piccinelli and Prosdocimi, 1968 (Argentina and Chile) and *A. franciscana* Kellogg, 1906 (North, Central and South America). Four bisexual species live in the Old World: *A. salina* Linnaeus, 1758 (Mediterranean basin), *A. urmiana* Günther, 1899 (Lake Urmia, Iran, and Crimean salt lakes, Abatzopoulos et al., 2009), *A. tibetiana* Abatzopoulos et al., 1998 (Tibetan plateau), and *A. sinica* Cai, 1989 (China and Mongolia). The asexual parthenogenetic populations are

sometimes termed "*A. parthenogenetica*" Barigozzi 1974 (Browne and Bowen, 1991), although this binomen is not generally accepted (Abatzopoulos et al., 2002). They are widely distributed over Eurasia, extending from the Canary Islands to China, and they have been introduced in Australia (Maccari et al., 2013b). Abatzopoulos et al., (2002) suggested that obligate parthenogenic *Artemia* populations show different levels of ploidy and they cannot readily be considered as belonging to a single species, for example *A. parthenogenetica*. They proposed using the general terms 'parthenogenetic populations' or 'parthenogenetic strains' as an alternative without taxonomic consequences. According to our experience, we would be even more cautious: in most cases samples were analyzed from which it was not known whether the animals were sexual or parthenogenetic. As a consequence, we have here introduced the term Eurasian Haplotype Complex (EHC) to describe a group of populations sharing the same basic haplotype. EHC includes documented parthenogenetic populations, but it needs to be established whether all EHC members are parthenogenetic. We have kept the term '*A. parthenogenetica*' only when literature is cited in which this term was used.

Artemia has been widely used as a live food in the aquaculture industry and the aquarium pet trade (Van Stappen, 2008). Since 1950, *A. franciscana* cysts have been exported intentionally from two sources in the USA, namely San Francisco Bay (SFB) and the Great Salt Lake (GSL), to the world aquaculture markets, which has caused a rapid worldwide dispersal of this American species (Van Stappen, 2008; Muñoz, 2009).

In the last three decades, *A. franciscana* has been introduced into the Western Mediterranean areas, including Portugal, Morocco, Spain, Italy, France, Tunisia, which eventually has led to co-existence with autochthonous *A. salina* and "*A. parthenogenetica*" (Amat et al., 2005; 2007; Ben Naceur et al., 2010; Muñoz and Pacios, 2010; Scalone and Rabet, 2013; Muñoz et al., 2014). In conditions of co-existence, the exotic species *A. franciscana* may outcompete local strains, which might bring about a serious biodiversity threat for native *Artemia* populations (Amat et al., 2007).

Biodiversity and phylogeographic patterns of *Artemia* populations throughout America and Europe have been well documented, whereas knowledge of Asian *Artemia* population genetics and phylogeography is fragmentary (Muñoz et al., 2008, 2010, 2013; Maniatsi et al., 2009). Asia (Central and Eastern), a vast region with a pronounced topographical and climatological diversity, harbours diverse *Artemia* species (Van Stappen, 2008). In Europe, *A. salina* and parthenogenetic populations occur, and the co-existence of both has been documented for a number of locations such as the Iberian Peninsula. America is dominated by *A. franciscana* throughout the continent, with the exception of *A. persimilis* in the extreme south of South America (Amat et al., 2007; Muñoz et al., 2013).

The aim of the present study was to assess species phylogeny and phylogeography of *Artemia* across Asia, and to determine population genetic diversity indices of introduced *A. franciscana* in Asia as compared with native *A. franciscana* populations in America. To achieve these aims, mitochondrial and nuclear genes were sequenced from a comprehensive sample set which had been systematically collected from 70 relevant areas across Asia. For comparison, the sample set also included 11 areas in America, Europe, and Africa with other *Artemia* species, including native *A. franciscana* and *A. persimilis* from the Americas, the introduced *A. franciscana* from Europe, and native *A. salina* from Europe and Africa.

3.3.3. Materials and methods

Origin of cyst samples and sample analysis

In total, 497 individuals of *Artemia* are collected from 81 geographical localities throughout Asia, Europe, Africa, and America. The sampling localities with their geographical coordinates, abbreviation, number of individuals analyzed, countries, and IPMB/ARC code numbers are documented in Table 20. Total genomic DNA was extracted according to standard protocols, which were described in our previous study (Eimanifar and Wink, 2013). All extracted DNA samples were stored at 4 ºC until needed.

We amplified and sequenced two genes: the mitochondrial *cytochrome c oxidase subunit I* (*COI*) and the nuclear *Internal Transcribed Spacer 1* (*ITS1*). Both genes have been extensively applied to deduce the phylogeographic structure of *Artemia* (Baxevanis et al., 2006; Muñoz et al., 2008). PCR amplifications were performed in a final reaction volume of 50 µl in a thermocycler (Biometra, Tgradient, Germany) with *Taq* DNA polymerase (Bioron, GmbH, Germany) according to conditions published previously (Eimanifar and Wink, 2013).

PCR products of about ~710 bp for *COI* and ~1500 bp for *ITS1* were clear and sharp according to agarose gel electrophoresis. Amplified products were purified according to standard procedures described by Eimanifar and Wink, (2013), before sequencing. Sequencing was done bidirectionally, using the same primers as in the PCR amplifications and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 by StarSEQ GmbH (Mainz, Germany). The sequencing protocol consisted of 33 cycles of initial denaturation at 95 ºC for 5 min, denaturation at 96 ºC for 10 s, annealing at 50 ºC for 5 s, and extension at 60 ºC for 4 min. The PCR products were analyzed on an ABI 3730 automated capillary sequencer (Applied Biosystems, Germany).

Complementary strands of each gene were checked manually and unambiguously aligned using the Bioedit sequence alignment vers. 7.0.9.0 (Hall, 1999). There was no evidence of premature stop codons and nuclear pseudogene copies. *COI* sequences were translated to amino acid sequences using MEGA6 (Tamura et al., 2013).

Our own DNA dataset includes 497 sequences for *COI;* additionally, 44 sequences from GenBank were included for genetic comparison. Only one single specimen of *Artemia* as a representative of each locality was used to sequence *ITS1*, because *ITS1*shows low variation within populations (Eimanifar and Wink, 2013). Our own *ITS1* dataset includes 80 sequences from localities examined, 15 sequences from 15 geographical localities of Urmia Lake, Iran (spread over the northern, middle, and southern parts of the lake), and finally 30 sequences from GenBank. In total, our DNA dataset thus includes 125 sequences for *ITS1* gene. The List of GenBank sequences used in the present study is shown in Table 21.

The reference *COI* sequences, retrieved from GenBank, included representatives from each species of *Artemia* (*A. franciscana* DQ119645, *A. persimilis* HM998992; DQ119647; EF615593–94, *A. salina* EU543467, *A. urmiana* JX512748–JX512808, *A. sinica* DQ119648, *A. tibetiana* EF615587 & "*A. parthenogenetica*" GU591380). Reference sequences for *ITS1* (GenBank) included *A. franciscana* DQ201297, *A. persimilis* DQ201263, *A. salina* DQ201306, *A. urmiana* DQ201276, *A. sinica* DQ201285, *A. tibetiana* DQ201270 and "*A. parthenogenetica*" DQ201272. *COI* datasets were rooted using *Daphnia pulex* (GQ466410) and *ITS1* datasets were rooted using *Streptocephalus proboscideus* (AY519840) as outgroups. The same zooplankton organisms have been used as outgroups in several phylogenetic studies of the genus *Artemia* (Baxevanis et al., 2006; Wang et al., 2008; Zhang et al., 2013). All deposited sequences are shown in Table 20.

Phylogenetic analyses

A statistical test for substitution saturation effect was carried out using the program DAMBE5 (Xia, 2013). 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 TIM3+G and GTR+G. In the *COI* dataset, the GTR model was used as a replacement for the suggested TIM3+G model because the suggested model was not implemented in the PhyML package. ML tree calculations were carried out with all proposed parameters using PhyML ver. 3.0 (Guindon et al., 2010). A Bayesian tree was determined using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001). Analysis was performed using two simultaneous runs, 10,000,000 generations, a random starting tree with four independent Markov chains (MCMC), and tree sampling every 500 generations. We discarded the first 20% of the generations as burn-in, and posterior probabilities were determined by constructing a 50% majority rule consensus for the remaining trees. The trees were visualized using FigTree v. 1.3.1 (Rambaut, 2009).

Genetic diversity indices were determined with the mtDNA-*COI* dataset for all species using DNAsp v. 5.00 (Librado and Rozas, 2009). These include number of haplotypes (*H*), number of variable sites (*V*), total number of nucleotide substitutions (*M*), nucleotide diversity (π), haplotype diversity (*HD*) and average number of nucleotide differences (*K*) per population.

Relationships among mitochondrial and nuclear haplotypes were reconstructed based on the median joining network implemented in the Network program (Bandelt et al., 1999). In order to find potential source populations of Asian *A. franciscana*, haplotype networks were constructed for *A. franciscana* individuals from Asia, Europe, and Americas. A phylogeny network was also constructed for closely related Asian species, including *A. urmiana*, *A. tibetiana*, *A. sinica*, and the Eurasian Haplotype Complex (EHC). Haplotype network was also constructed for all *ITS1* individuals.

Table 20 Origin of *Artemia* samples from Asia, Europe, Africa, and America. Samples are presented according to the alphabetical order of country of origin. IPMB = Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany; ARC = Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium.

No.	IPMB voucher /ARC code number	Abbreviation for locality	Sample size	Locality, Province, State or District	Country	Geographic coordinates	GenBank accession numbers
1	57283	REL	4	Relizane Sebkha	Algeria	00°39'E – 35°50'N	KF691133 − KF691136
2	57296	ING	6	Ingebright North	Canada	50°22'N – 109°19'W	KF691137 − KF691142
3	57286	PLU	5	Pedra de Lume, Sal Island	Cape Verde	16°46'N – 22°53'W	KF691143 − KF691147
4	55582 /1317	BAM	6	Bameng, Inner Mongolia	China	40°46'N – 107°27'E	KF691148 − KF691153
5	64755/1589	BEID	3	Beidaba, Shandong	China	117°57'E – 38°05'N	KF691154 − KF691156
6	64746 /1241	BEI	3	Beidachi, Inner Mongolia	China	107°25'E – 37°58'N	KF691157 − KF691159
7	57245	BBA	6	Bohai Bay area	China	119°30'E – 39°48'N	KF691160 − KF691165
8	64756 /1233	CAN	4	Canghzhou, Hebei	China	38°32'N – 117°00'E	KF691166 − KF691169
9	64767 /1210	CHE	3	Chengkou, Shandong	China	117º43'E – 38º05'N	KF691170 − KF691172
10	64754/1665	DAG	3	Da Gang, Shandong	China	120°10'E – 36°05'N	KF691173 − KF691175
11	57242	DLI	11	Dalian, Liaoning	China	121°36'E – 38°54'N	KF691176 − KF691186
12	64762 /1216	DON	3	Dongjiagou, Liaoning	China	121º 53'E –39º04'N	KF691187 − KF691189
13	64763 /1668	DOG	4	Dongying, Shandong	China	118°29'E – 37°27'N	KF691190 − KF691193
14	65831 /1577	ERY	5	Eryan, Shandong	China	117°53'E – 38°01'N	KF691194 − KF691198
15	64744 /1199	GAH	6	Gahai, Qinghai	China	97°37'E – 37°07'N	KF691199 − KF691204
16	64765 /1669	HAI	3	Haixing, Hebei	China	117°47'E – 38°11'N	KF691205 − KF691207
17	65627 /1211	HAN	7	Hangu, Tianjin	China	117°50'E – 39°25'N	KF691208 − KF691214

18	65829 /1524	JIN	4	Jingyu Lake, Xinjiang	China	89°09'E – 36°03'N	KF691215 − KF691218
19	64758 /1592	LEG	3	Leguantai, Shandong	China	117°54'E – 38°03'N	KF691219 − KF691221
20	57237	LUA	6	Luannan, Hebei	China	118º42'E – 39º30'N	KF691222 − KF691227
21	57240 /1694	NAN	5	Nanpu, Hebei	China	118º20'E – 39º05'N	KF691228 − KF691232
22	64742 /1077	SHA	3	Shanyao, Fuijan	China	118°53'E – 25°08'N	KF691233 − KF691235
23	64760/1578	SID	3	Sidao, Shandong	China	117°57'E – 38°01'N	KF691236 − KF691238
24	64766 /1597	SIK	6	Sikou, Shandong	China	120°40'E – 37°16'N	KF691239 − KF691244
25	57250	TIB1	5	Tibet area	China	30°46'N – 85°48'E	KF691245 − KF691249
26	57244	TTA	7	Tanggu, Tianjin	China	117º40'E – 39º01'N	KF691250 − KF691256
27	57239 /1696	WUD	5	Wudi, Shandong	China	117°56'E – 38°6'N	KF691257 − KF691261
28	64753 /1598	WUZ	3	Wuzhidui, Hebei	China	116°42'E – 39°18'N	KF691262 − KF691264
29	55580 /1262	XIA	4	Xiaocaidan, Qinghai	China	95º6'E – 37ºN	KF691265 − KF691268
30	64745 /1206	XIE	9	Xiechi Lake, Shanxi	China	111°55'E – 35°44'N	KF691269 − KF691277
31	64757 /1582	XIN	3	Xinhu, Shandong	China	117°56'E – 37°57'N	KF691278 − KF691280
32	64759 /1214	YAG	3	Yangkou, Shandong	China	120º10'E – 37°17'N	KF691281 − KF691283
33	64761 /1596	YANH	3	Yanhua, Shandong	China	117°48'E – 38°09'N	KF691284 − KF691286
34	64764 /1073	YIN	4	Yingkou, Liaoning	China	122°13'E – 40°40'N	KF691287 − KF691290
35	57246	YUA	7	Yuantong (or Jinshan), Shanghai	China	120°52'E – 30°42'N	KF691291 − KF691297
36	66311	YUN	5	Yuncheng, Shanxi	China	110°58'E – 34°59'N	KF691298 − KF691302
37	57243	ZHS	13	Zhan hua, Shandong	China	118°07'E – 37°42'N	KF691303 − KF691315
38	57248	TIB2	3	Tibet area	China	31°37'N – 88°59'E	KF691316 − KF691318
39	57308	SCE	5	Salina Cero	Colombia	09°27'N – 75°36'W	KF691319 − KF691323
40	62948	KEL	4	Kelambakkam, Madras	India	13º5'N – 79º7'E	KF691324 − KF691327
41	62949	TUT	5	Tuticorin, Tamil Nadu	India	8º55'N – 78º8'E	KF691328 − KF691332

42	57227	INC	5	Incheh Lake, Gonbad, Golestan	Iran	37º24'N – 54º36'E	KF691333 − KF691337
43	57223	LAGW	5	Lagoons around Urmia Lake, West Azarbaijan	Iran	37º15'N – 45º40'E	KF691338 − KF691342
44	57224	LAGE	3	Lagoons around Urmia Lake, Dasht-E-Tabriz, East Azarbaijan	Iran	37º47'N – 45º25'E	KF691343 − KF691345
45	57228	MAHR	5	Maharlu Lake, Shiraz, Fars	Iran	29º28'N – 52º47'E	KF691346 − KF691350
46	57230	MAH	6	Mahshahr port, Mahshahr	Iran	49º11'E – 30º33'N	KF691351 − KF691356
47	57226	MIG	5	Mighan Salt Lake, Arak	Iran	34º20'N – 49º50'E	KF691357 − KF691361
48	57229	NOG	5	Nough catchment, Nough, Kerman	Iran	30º60'N – 56º50'E	KF691362 − KF691366
49	57225	QOM	6	Qom Salt Lake, Qom	Iran	34º40'N – 51º52'E	KF691367 − KF691372
50	57211	URM	61	Urmia Lake	Iran	45º28'E – 37º35'N	JX512748 – JX512808
51	57255	ABG	3	Abu-Ghraib, Baghdad	Iraq	44º30'E – 33º20'N	KF691373 − KF691375
52	57256	GAA	8	Garmat Ali, Basrah	Iraq	47º49'E – 30º30'N	KF691376 − KF691383
53	57305	YAL	7	Yallash	Jamaica	17°51'N – 76°33'W	KF691384 − KF691390
54	57232	ARS	7	Aral Sea	Kazakhstan	45º52'N – 58º43'E	KF691391 − KF691397
55	57233	ASS	6	Aral Sea (South)	Kazakhstan	44º43'N – 59º34'E	KF691398 − KF691403
56	57235	KYZ	5	Kyzylkak	Kazakhstan	53°26'N – 73°48'E	KF691404 − KF691408
57	57234	NCS	6	North Caspian sea	Kazakhstan	47°06'N – 51°55'E	KF691409 − KF691414
58	57236	PAV	6	Pavlodar	Kazakhstan	52º18'N – 76º57'E	KF691415 − KF691420
59	57231	TUZ	14	Tuz Lake, Pavlodar	Kazakhstan	51º19'N – 78º38'E	KF691421 − KF691434
60	57301	BLO	4	Bahía de Lobos, Sonora	Mexico	27°18'N – 110°30'W	KF691435 − KF691438
61	57257	KOC	10	Korangi Creek, Karachi coast	Pakistan	67º10'E – 24º48'N	KF691439 − KF691448
62	57279	SLU	6	Santa Luzia, Tavira	Portugal	37°06'N – 07°38'W	KF691449 − KF691454
63	57325 /1720	BYA	5	Bolshoye Yarovoye, Altayskiy	Russia	52º50'N – 78º41'E	KF691455 − KF691459

64	55586 /1702	EBE	7	Ebeyty, Omskaya	Russia	54º59'N – 73º23'E	KF691460 − KF691466
65	55581 /1641	GOR	5	Gorkoye Lake	Russia	55º21'N – 68º32'E	KF691467 − KF691471
66	64747 /1389	KUC	3	Kuchukskoye, Altayskiy	Russia	52°42'N – 79°46'E	KF691472 − KF691474
67	55579 /1528	KUL	3	Kulundinskoye, Altayskiy	Russia	53º10'N – 79º30'E	KF691475 − KF691477
68	64750 /1640	KUR	3	Kurgan area	Russia	55º29'N – 64º 27'E	KF691478 − KF691480
69	64752 /1705	MME	4	Maloye Medvezhye (Kurganskaya)	Russia	55°12'N – 67°57'E	KF691481 − KF691484
70	55585 /1735	MYA	7	Maloye Yarovoye (Altayskiy)	Russia	53º4'N – 79º10'E	KF691485 − KF691491
71	64749 /1507	MED	3	Medvezhye (Kurganskaya)	Russia	66°4'E – 54°55'N	KF691492 − KF691494
72	64751 /1642	VOS	3	Voskresenskoye (Kurganskaya)	Russia	55°32'N – 67°23'E	KF691495 − KF691497
73	57272	CSR	5	Sanlucar, Cadiz	Spain	36º52'N – 06º19'W	KF691498 − KF691502
74	57254	SLA	6	Sri Lanka	Sri Lanka	7º52'N – 80º46'E	KF691503 − KF691508
75	57277	MEG	6	Mégrine saltworks	Tunisia	36°47'N – 10°14'E	KF691509 − KF691514
76	57278	SFA	5	Sfax saltworks	Tunisia	35°45'N – 10°43'E	KF691515 − KF691519
77	57292 /1512	CAM	10	Çamalti Saltern, Izmir	Turkey	26º53'E – 38º39'N	KF691520 − KF691529
78	57258 /1371	KBG	5	Kara Bogaz Gol	Turkmenistan	53º33'E – 41º17'N	KF691530 − KF691534
79	57299	GSL	12	Great Salt Lake, Utah	U.S.A.	40°45'N – 111°54'W	KF691535 − KF691546
80	57252 /1715	CAA	9	Cape Aktymsyk, Karakalpakstan	Uzbekistan	43°54'N – 59°30'E	KF691547 − KF691555
81	57253 /1719	VCH	13	Vinhchau, Soctrang	Vietnam	9º17'N – 105º55'E	KF691556 − KF691568

Note: For the *ITS1* gene, a subset of individuals, 1 for each locality, was sequenced.

All *ITS1* sequences are deposited under the following accession numbers: KF703762 − KF703854.

Table 21 List of *Artemia* GenBank samples with their accession numbers used in phylogenetic analyses.

Locality, Province, State, or District	Abbreviation	Country	Species	GenBank accession No.	References
Mar Chiquita, Córdoba Las Tunas, Santa Fe Santiago del Estero, Santiago del Estero	G_ARG	Argentina	*A. franciscana*	GU248362 GU248368 GU248367	Maniatsi et al., 2009
Macau, Rio Grande do Norte Galinhos, Rio Grande do Norte Areia Branca/Grossos, Rio Grande do Norte	G_BRA	Brazil	*A. franciscana*	GU248371 GU248370 GU248369	Maniatsi et al., 2009
Los Vilos, Choapa Cerro Las Cejas, Atacama region Chaxas, Antofagasta Salar Llamará, Antofagasta El Convento, Valparaíso Iquique, Iquique	G_CHI	Chile	*A. franciscana*	GU248379 – 80 GU248375 – 76 GU248377 – 78 GU248365 – 66 GU248363 – 64 GU248373 – 74	Maniatsi et al., 2009
Great Salt Lake, Utah (Gunnison Bay) Great Salt Lake, Utah (Gunnison Bay) Great Salt Lake, Utah (Gunnison Bay) Great Salt Lake, Utah (Gilbert Bay) Great Salt Lake, Utah (Gilbert Bay) Great Salt Lake, Utah (Gilbert Bay)	GSL (N1) GSL (N2) GSL (N3) GSL (S1) GSL (S2) GSL (S3)	U.S.A.	*A. franciscana*	KJ863440 − KJ863449 KJ863450 − KJ863459 KJ863470 − KJ863479 KJ863430 − KJ863439 KJ863480 − KJ863490 KJ863460 − KJ863469	Eimanifar et al., 2014 Unpublished
Celestun, Yucatan Real de las Salinas, Campeche Yavaros, Sonora Salinas de Hidalgo, San Luis Potosi Texcoco, Estado de Salinas Casa Blanca, Cuatro Cienagas de Carranza, Coahuila La Colorada lagoon, Oaxaca San Quintin, Baja California Faro San Jose, Baja California Frank Pais, Guantanamo Great Salt Lake, Utah San Francisco Bay, California Salina Fraternidad, Cabo Rojon Laguna de las Salinas Bastoncillo, Lajas Little Manitou Lake, Saskatchewan	MexCe Mex99 MexY MexH MexT MexCB MexLC MexSQ MexFSJ GUA GSL SFB USSF USSB MANW	Mexico Mexico Mexico Mexico Mexico Mexico Mexico Mexico Mexico Cuba USA USA Puerto Rico Puerto Rico Canada	*A. franciscana* *AF1 AF2 AF2, AF3 AF4, 5, 6, 7, 8, 9 AF7, 8, 0, 10, 11 AF12, 13, 14, 15, 16, 17 AF18, 19, 20, 21 AF10, 22, 23 AF1, 10, 24 AF10, 20, 25 AF10, 18, 20, 21, 26, 27 AF10, 18, 20, 25 AF28, 29 AF28, 30, 31, 32, 33, 34 AF35, 36	KF662951 – 3043	Muñoz et al., 2013

Muskiki Lake, Saskatchewan	CMUS	Canada	AF35, 37, 38		
Chaplin Lake, Saskatchewan	CHAP	Canada	AF39, 40, 41, 42, 43		
Meacham Lake, Saskatchewan	CMEA	Canada	AF35, 44, 45, 46, 47		
Mossoro, Grossos	BRM	Brazil	AF10		
Salinas de Galerazamba	CGZ	Colombia	AF48, 49, 50		
Salinas de Manaure	CM	Colombia	AF51, 52		
Salina Cero	CSC	Colombia	AF49, 50, 53, 54, 55		
Tayrona	CT	Colombia	AF56, 57, 58		
Playa Salinas, Ancash	PPS	Peru	AF59, 60, 61, 62, 63		
Los Chimus	PLC	Peru	AF59, 64		
Virrilla, Piura	PV	Peru	AF65		
Humedales de Ventanilla, Callao	PVe	Peru	AF59, 61, 66, 67		
La Milagrosa, Chilca	PLM	Peru	AF68, 69		
Salinas de Cumaraguas	VEC	Venezuela	AF51, 70, 71		
Yallahs Pond	JAYA	Venezuela	AF18, 19		
Salar de Llamara	CHSL	Chile	AF72, 73, 74, 75		
Laguna Cejas, Salar de Atacama	CHLC	Chile	AF18		
Los Vilos, Poza Palo Colorado	CHLV	Chile	AF76, 77		
Pichilemu Cahuil saltworks	CHPI	Chile	AF18, 78		
Poza Pampilla IV Region	CHP	Chile	AF79, 80		
Mar Chiquita, Cordoba	AMC	Argentina	AF81		
Salinas Grandes, Cordoba	ASG	Argentina	AF72, 73, 74, 82, 83, 84, 85		
Mono Lake	AMON	USA	AF87, 88, 89, 90, 91, 92		
Pedra de Lume, Sal Island	PLU	Cape Verde	AF86, 93		
Yuncheng	G_SIN	China	*A.sinica*	HM998990	Maniatsi et al., 2011
NaLin, Inner Mongolia				DQ119649	Hou et al., 2006
Shui Quan Zi, Inner Mongolia				DQ119650	Hou et al., 2006
Yimeng, Inner Mongolia				EF615591	Wang et al., 2008
Bameng, Inner Mongolia				EF615592	Wang et al., 2008
Nima, Tibet	G_TIB	China	*A. tibetiana*	EF615584	Wang et al., 2008
Yangnapengco, Tibet				EF615585	
Qixiang Lake, Tibet				EF615586	
Jingyu Lake, Xinjiang				EF615588	
Co Qen, Tibet				EF615589	
Odiel saltpan	G_PAR	Spain	"*A. parthenogenetica*"	DQ426824	Muñoz et al., 2010b
Cabo de Gata saltpan		Spain		DQ426825	
El Bosque saltpan		Spain		DQ426826	
Rio Maior saltpan		Portugal		GU591380	
Senitra saltpan		Portugal		GU591381	
Larache saltpan		Morocco		GU591382	
Margherita di Savoia saltpan		Italy		GU591383	
Bjurliv Lake		Kazakhstan		GU591384	

Vineta Swakopmund saltworks Atanasovko Lake Kujalnicsky Liman Wadi El Natrun		Namibia Bulgaria Ukraine Egypt			

3.3.4. Results

Genetic diversity in *Artemia*

Alignment of all *COI* sequences (Asia, Europe, Africa, and America) revealed a total of 112 variable sites, among which 24 characters were singleton and 88 characters were parsimony informative. Sequences of Asian *A. franciscana* had 17 variable sites (9 characters were singleton and 8 characters were parsimony informative). The Asian lineages of EHC showed a similar degree of sequence diversity with 19 variable sites (10 characters were singleton and 9 characters were parsimony informative). The results of the saturation test demonstrated that the *COI* data sets exhibit no substitution saturation; the *Iss* values (0.364) observed are significantly lower than *Iss.c* (0.711) ($P<0.05$).

All genetic diversity estimates are presented for all species in Table 22. The mean haplotype and nucleotide diversity for all *COI* sequences was 0.83 and 0.1, respectively. The mean haplotype diversity among *A. franciscana* sequences from the Great Salt Lake (USA) was higher than that of European *A. franciscana* populations (0.60 ± 0.10 *vs* 0.54 ± 0.07), but lower than in Asian *A. franciscana* populations (0.71 ± 0.01). The haplotype diversity of Asian *A. franciscana* (0.71 ± 0.01) was higher than that of Asian EHC (0.5 ± 0.04). The mean nucleotide diversity among *A. franciscana* lineages from America, Europe, and Asia was 0.0010 ± 0.0002, 0.020 ± 0.002 and 0.0040 ± 0.0001, respectively, and similar to that of Asian EHC (0.001 ± 0.0001).

Phylogeography in *Artemia*

The phylogenetic trees generated by ML and BI from *COI* sequences had concordant topologies and included 7 distinct, well-supported clades (Fig. 19). Clades IV to VII correspond to recognized *Artemia* species. *A. urmiana* is paraphyletic with respect to EHC in this reconstruction but not in the network (Fig. 22). *A. tibetiana* is represented by two clades and considered as a sister group with clade I. *A. sinica* constituted an independent clade at the base of Asian *Artemia* species. *A. salina* from the Old World clusters at the base of *Artemia*, followed by two American species (*A. persimilis* and *A. franciscana*).

The length of *ITS1* gene in all alignment was 1116 base pairs (bp), including a total of 212 nucleotide insertions or deletions (indels). The vast majority of indels is shared among species. Phylogenetic relationships based on *ITS1* sequences were not fully congruent to those recovered in the *COI* tree (Fig. 20). In the *ITS1* ML tree, 5 clades were identified and supported by high bootstrap values. In contrast with *COI* data, sequences of *A. tibetiana* were identical with those of EHC, indicating a close relationship to them. Both data sets agree that the Asian taxa were shared a common ancestry with *A. franciscana*. Whereas *A. salina* took a basal position in the *COI* tree, this position is taken by *A. persimilis* in the *ITSI*

tree. This finding should be regarded with care, as we had only several single sequences for *A. persimilis* which came from GenBank.

The inferred phylogeny from *COI* revealed a complex phylogeographic structure, especially among Asian *Artemia* populations. *A. sinica* and *A. tibetiana* are genetically divergent and are found in restricted geographical regions in Asia. *A. urmiana* has been described so far for Urmia Lake, Iran, and for the Crimean coast, Ukraine, but presumed parthenogenetic samples from Turkey and Turkmenistan fall under the same clade as *A. urmiana*. EHC and *A. franciscana* are two species with a wide distribution in Asia. Asian lineages of EHC were found in 39 geographical regions in 9 Asian countries (Turkey, Iraq, Iran, Pakistan, China, Russia, Kazakhstan, Turkmenistan, and Uzbekistan). Introduced *A. franciscana* was detected in 31 geographical localities in 7 Asian countries (Iraq, Iran, Pakistan, India, Sri Lanka, Vietnam, and China), with the majority (22 out of 31) scattered over 6 inland and coastal Chinese provinces. The list of individuals corresponding to each unique haplotype is presented in Tables 23 and 24.

Localities were pooled together based on geographical proximity, in order to increase the resolution of sample size. According to our classification, a total of 16 isolated geographical regions (I–XVI), represented by a minimum of 10 and a maximum of 117 individuals per region were observed in Eurasia. Fifteen regions were assigned for the whole of Asia, and one region, grouping Europe and North Africa together, as a reference. The diversity of *Artemia* species in each geographic region studied is documented as a pie chart in Fig. 21.

A *COI* phylogeny network of 299 individuals from *A. urmiana, A. tibetiana, A. sinica*, and the EHC generated 75 distinct haplotypes, including 9 main haplotypes with frequencies between 5 and 136 (Fig. 22). A summary of haplotypes and their origin is documented for each network in Tables 24, 25 and 26.

In the *COI* network, EHC is genetically close to *A. urmiana* and separated by 6 mutational steps (Fig. 22). Numerous singleton haplotypes have developed from the nine major haplotypes H1, H2, H3, H6, H7, H14, H15, H58, and H65. For *A. urmiana* and *A. tibetiana*, only three major haplotypes are apparent (H14, H15, and H58). *A. tibetiana* is sister (or basal) to the *A. urmiana*/EHC clade. *A. sinica* has diverged from other Asian species by 41 mutational steps (Fig. 22). Only a single major haplotype has been observed for *A. sinica*.

ITS1 network for all individuals revealed 95 segregation (polymorphic) sites, among which 43 characters were singleton and 52 characters were parsimony informative. A total 27 distinct haplotypes were observed (H1–H27). Among these haplotypes, two main haplotypes had the highest frequencies. Haplotype 1 was the most dominant haplotype and consisted of

a mixture of *A. urmiana*, *A. tibetiana* and EHC (52/124, 42% of individuals). Haplotype 3 was the second most dominant haplotype which included *A. franciscana* individuals (33/124, 26.6% of individuals). The other haplotypes were varied between 1–7 individuals (Fig. 23).

The *COI* haplotype network of 374 *A. franciscana* sequences from Eurasia and the other related American populations revealed 84 unique haplotypes, comprising four major haplotypes (H5, H6, H8 and H17), which were found in invaded populations in the Asia. Numerous haplotypes surrounded the major ones with different frequencies varied between 2 to 9. Both GSL and SFB haplotypes were shared the most common haplotypes among three major haplotypes with different frequencies. Moreover, Asian *A. franciscana* showed shared haplotypes with other available American populations from Chile, Colombia, Cuba, Brazil, Jamaica, Mexico and Venezuela. Haplotype H6 was the most common haplotype which showed a contribution (9 % of individuals) of European *A. franciscana* into Asian lineages (Fig. 6). Asian *A. franciscana* also revealed shared haplotypes with European *A. franciscana* including H5 (2 % of individuals) and H8 (1 % of individuals) (Fig. 24).

Table 22 Summary of *COI* population genetic statistics for all *Artemia* species, including *A. franciscana* from GSL, Europe, and Asia, and EHC lineages. N = number of samples, V = number of variable sites, M = total number of nucleotide substitutions, H = number of haplotypes, HD = haplotype diversity, π = nucleotide diversity, K = mean number of pairwise nuclear differences.

Species	N	V	M	H	HD	π	K
A. franciscana (GSL, USA)	12	2	2	3	0.6 ± 0.1	0.001 ± 0.0008	0.65
A. franciscana (Europe)	11	21	21	2	0.54 ± 0.07	0.02 ± 0.005	11.45
A. franciscana (Asia)	152	17	17	12	0.7 ± 0.01	0.004 ± 0.001	2.41
A. persimilis	4	10	10	4	1 ± 0.17	0.01 ± 0.006	5.16
A. salina	20	30	31	9	0.88 ± 0.04	0.01 ± 0.005	7.54
[∫]***A. urmiana***	71	59	61	40	0.9 ± 0.03	0.006 ± 0.006	3.8
A. sinica	15	27	27	8	0.86 ± 0.06	0.01 ± 0.008	6.49
A. tibetiana	12	17	17	11	0.98 ± 0.04	0.005 ± 0.004	3.24
[†]**EHC** (Asia)	181	101	103	14	0.5 ± 0.04	0.003 ± 0.007	1.77

[∫]*A. urmiana* JX512748 – JX512808 (Eimanifar and Wink, 2013).

[†]EHC refers to the Eurasian Haplotype Complex.

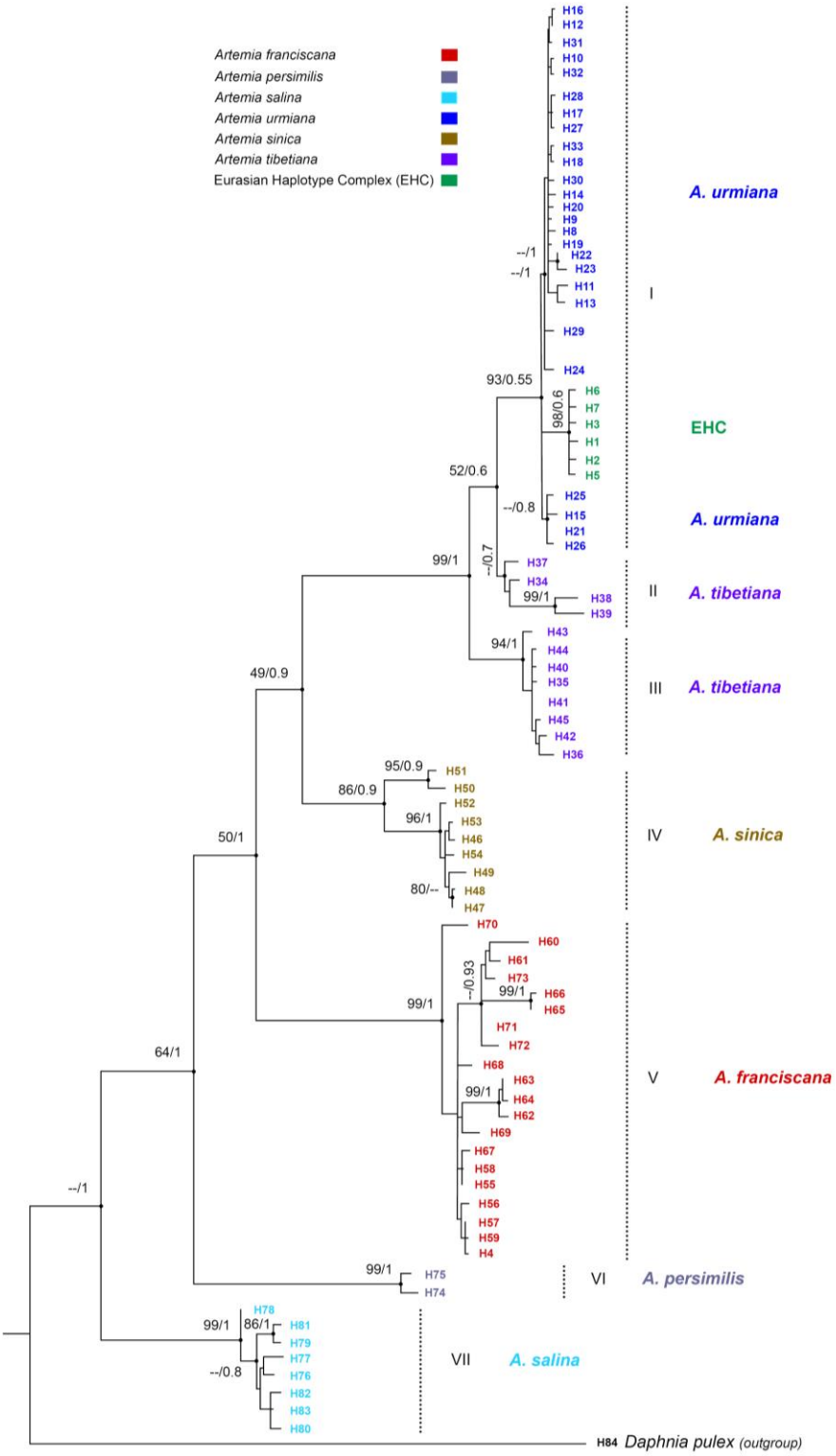

Fig. 19. *COI* phylogeny of *Artemia* based on ML and BI for 541 individuals. Numbers along nodes indicate bootstrap supports for ML and BI approaches. Geographical localities in each clade are shown by different haplotypes defined by different colors in the pie chart. Seven distinct clades are shown by Roman numerals (I − VII). *Daphnia pulex* was used as an outgroup.

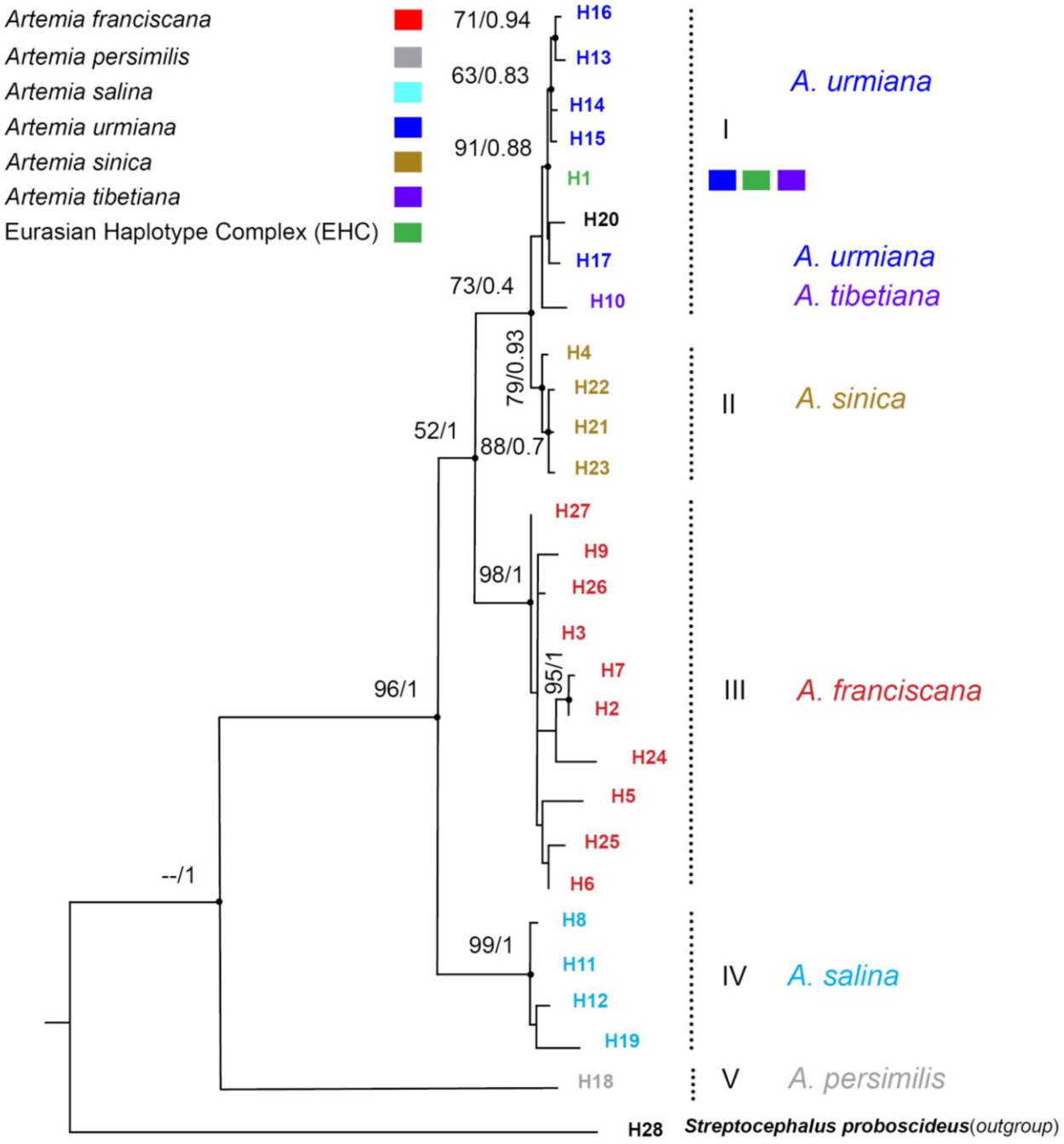

Fig. 20. *ITS1* Phylogeny of *Artemia* based on GTR model for 125 individuals. Bootstrap values for ML & BI are denoted for each major node. Geographical localities in each clade, grouped per country, are shown by different colors defined in the pie chart. Five distinct clades are shown by Roman numerals (I − V). *Streptocephalus proboscideus* was used as an outgroup.

Table 23 Data matrix of variable sites and distribution of unique haplotypes with their frequencies among 541 *Artemia* individuals using 579 bp of *COI*. H = haplotype, F = haplotype frequency and numbers = polymorphic sites. Forty-four sequences (bold) of *Artemia* from all species have been included from GenBank.

H	F	Individuals and Locations
H1	177	INC-1 INC-2 INC-3 INC-4 INC-5 LAGW-1 LAGW-2 LAGW-3 LAGW-4 LAGW-5 LAGE-1 LAGE-2 LAGE-3 MIG-1 MIG-2 MIG-3 MIG-4 MIG-5 QOM-1 QOM-2 QOM-3 QOM-4 QOM-5 QOM-6 ABG-1 ABG-2 ABG-3 ARS-1 ARS-2 ARS-3 ARS-4 ARS-5 ARS-6 ARS-7 ASS-1 ASS-2 ASS-3 ASS-4 ASS-5 ASS-6 KYZ-1 KYZ-2 KYZ-3 KYZ-4 KYZ-5 NCS-1 NCS-2 NCS-3 NCS-4 NCS-5 NCS-6 PAV-1 PAV-2 PAV-3 PAV-4 PAV-5 PAV-6 TUZ-1 TUZ-2 TUZ-3 TUZ-4 TUZ-5 TUZ-6 TUZ-7 TUZ-8 TUZ-9 TUZ-10 TUZ-11 TUZ-12 TUZ-13 TUZ-14 BYA-1 BYA-2 BYA-4 BYA-5 EBE-1 EBE-2 EBE-3 EBE-4 EBE-6 GOR-1 GOR-2 GOR-3 GOR-4 GOR-5 KUC-1 KUC-2 KUC-3 KUL-1 KUL-3 KUR-1 KUR-2 KUR-3 MME-2 MME-4 MYA-1 MYA-2 MYA-3 MYA-4 MYA-5 MYA-6 MYA-7 MED-1 MED-2 MED-3 VOS-1 VOS-2 VOS-3 BAM-1 BAM-2 BAM-3 BAM-4 BAM-5 BAM-6 CAN-1 CAN-2 CAN-3 CAN-4 CHE-1 CHE-2 CHE-3 DLI-8 DON-1 DON-2 DON-3 GAH-1 GAH-2 GAH-3 GAH-4 GAH-5 GAH-6 HAN-1 HAN-2 HAN-3 HAN-4 HAN-5 HAN-6 HAN-7 LUA-3 LUA-4 LUA-5 SHA-1 SHA-2 SHA-3 SID-1 SID-3 XIA-1 XIA-4 YIN-1 YIN-2 YIN-3 YIN-4 MAHR-1 MAHR-3 KOC-4 KOC-5 KOC-6 KOC-7 KOC-9 KOC-10 CAM-2 KBG-1 KBG-2 KBG-3 KBG-5 CAA-2 CAA-3 CAA-4 CAA-5 CAA-6 CAA-7 CAA-8 CAA-9 **G_PAR_1 G_PAR_2 G_PAR_5 G_PAR_6**
H2	1	BYA-3
H3	5	EBE-5 EBE-7 KUL-2 MME-1 MME-3
H4	2	BEI-3 WUD-1
H5	1	CAA-1
H6	1	**G_PAR_3**
H7	1	**G_PAR_4**
H8	44	**G_PAR_7 G_PAR_8** NC2-1 NC1-1 NC1-3 NE-2 NE-3 NE-5 NW-3 NW-4 NW-5 ME1-1 ME2-1 ME2-2 ME2-4 MW1-1 MW1-3 MW1-4 MW1-5 MW2-1 MW2-4 SE2-4 SE2-6 SE3-2 SE3-3 SC1-1 SC1-2 SC1-3 SC2-1 SC2-2 SC3-1 SC3-2 SC3-4 SE1-1 SW-2 CAM-1 CAM-3 CAM-4 CAM-5 CAM-6 CAM-8 CAM-9 CAM-10 KBG-4
H9	1	NC2-2
H10	1	NC2-3
H11	1	NC1-2
H12	1	NE-1
H13	1	NE-4
H14	1	NE-6
H15	2	NW-1 ME1-2
H16	1	NW-2
H17	1	NW-6
H18	1	ME2-3
H19	1	ME2-5
H20	1	ME2-6
H21	3	MW1-2 SE3-1 SE1-2
H22	1	MW1-6
H23	1	MW2-2
H24	1	MW2-3

H25	1	MW2-5
H26	1	SE2-1
H27	1	SE2-2
H28	1	SE2-3
H29	1	SE2-5
H30	1	SC2-3
H31	1	SC3-3
H32	1	SE1-3
H33	1	SW-1
H34	1	CAM-7
H35	2	**G_TIB_1 G_TIB_2**
H36	1	**G_TIB_3**
H37	2	**G_TIB_4 G_TIB_5**
H38	1	**G_TIB_6**
H39	1	**G_TIB_7**
H40	1	TIB1-1
H41	7	TIB1-2 TIB1-3 TIB1-5 TIB2-2 JIN-2 JIN-3 JIN-4
H42	1	TIB1-4
H43	1	TIB2-1
H44	1	TIB2-3
H45	1	JIN-1
H46	10	**G_SIN_1 G_SIN_3** XIE-4 XIE-6 XIE-7 XIE-8 XIE-9 YUN-1 YUN-2 YUN-3
H47	2	**G_SIN_2 G_SIN_5**
H48	1	**G_SIN_4**
H49	1	**G_SIN_6**
H50	1	BEI-1
H51	1	BEI-2
H52	3	XIE-1 XIE-2 XIE-3
H53	1	XIE-5
H54	1	YUN-4
H55	49	MAH-1 MAH-3 MAH-5 NOG-1 NOG-3 NOG-4 NOG-5 SLA-6 VCH-13 BEID-1 BEID-2 BEID-3 BBA-2 BBA-5 BBA-6 DAG-2 DAG-3 DLI-1 DLI-4 DLI-6 DLI-9 DLI-10 DLI-11 DOG-1 DOG-3 ERY-2 ERY-5 HAI-2 LUA-1 LUA-2 NAN-4 NAN-5 TTA-3 TTA-6 WUD-2 WUD-5 WUZ-3 XIA-2 XIA-3 YAG-1 YUA-2 ZHS-1 ZHS-13 KOC-1 KOC-2 KOC-3 KOC-8 **G_CHI_11 G_CHI_12**
H56	1	MAH-2
H57	129	MAH-4 MAH-6 NOG-2 SLA-1 SLA-2 SLA-3 SLA-4 SLA-5 VCH-1 VCH-2 VCH-3 VCH-4 VCH-5 VCH-6 VCH-7 VCH-8 VCH-9 VCH-10 VCH-11 VCH-12 KEL-1 KEL-2 KEL-3 KEL-4 TUT-1 TUT-2 TUT-3 TUT-4 TUT-5 GAA-1 GAA-2 GAA-3 GAA-4 GAA-5 GAA-6 GAA-7 GAA-8 BBA-1 BBA-4 DAG-1 DLI-2 DLI-3 DLI-5 DLI-7 DOG-2 DOG-4 ERY-3 ERY-4 HAI-1 HAI-3 LEG-1 LEG-2 LEG-3 LUA-6 NAN-1 NAN-2 NAN-3 SID-2 SIK-1 SIK-2 SIK-3 SIK-4 SIK-5 SIK-6 TTA-1 TTA-2 TTA-4 TTA-5 TTA-7 WUD-3 WUD-4 WUZ-1 WUZ-2 XIN-1 XIN-2 XIN-3 YAG-2 YAG-3 YANH-1 YANH-2 YANH-3 YUA-1 YUA-3 YUA-4 YUA-5 YUA-6 YUA-7 ZHS-2 ZHS-3 ZHS-4 ZHS-5 ZHS-6 ZHS-7 ZHS-8 ZHS-9 ZHS-10 ZHS-11 ZHS-12 MAHR-2 MAHR-4 MAHR-5 SLU-1 SLU-2 SLU-3 SLU-4 SLU-5 SLU-6 GSL-1 GSL-2 GSL-3 GSL-4 GSL-5 GSL-6 GSL-7 GSL-8 GSL-9 GSL-10 GSL-11 GSL-12 **G_BRA_1 G_BRA_2** SCE-1 YAL-1 YAL-2 YAL-3 YAL-4 YAL-5 YAL-6 YAL-7
H58	1	BBA-3
H59	1	ERY-1
H60	5	PLU-1 PLU-2 PLU-3 PLU-4 PLU-5
H61	1	**G_FRA**

H62	2	**G_CHI_1 G_CHI_2**
H63	2	**G_CHI_3 G_CHI_4**
H64	2	**G_CHI_5 G_CHI_6**
H65	6	**G_CHI_7 G_CHI_8 G_CHI_9 G_CHI_10 G_ARG_2 G_ARG_3**
H66	1	**G_ARG_1**
H67	3	SCE-2 SCE-4 SCE-5
H68	1	SCE-3
H69	3	BLO-1 BLO-3 BLO-4
H70	1	BLO-2
H71	4	ING-1 ING-2 ING-3 ING-5
H72	1	ING-4
H73	1	ING-6
H74	1	**G_PER_1**
H75	3	**G_PER_2 G_PER_3 G_PER_4**
H76	1	**G_SAL**
H77	7	REL-1 REL-2 REL-4 CSR-2 CSR-3 CSR-4 CSR-5
H78	1	REL-3
H79	1	CSR-1
H80	5	MEG-1 MEG-2 MEG-3 MEG-4 MEG-5
H81	1	MEG-6
H82	4	SFA-1 SFA-3 SFA-4 SFA-5
H83	1	SFA-2
H84	1	DAP

Table 24 Data matrix of variable sites and distribution of unique haplotypes with their frequencies among 125 *Artemia* individuals using 1116 bp of *ITS1*. H = haplotype, F = haplotype frequency and numbers = polymorphic sites. Thirty-one (bold) of *Artemia* from all species have been included from GenBank.

H	F	Individuals and Locations
H1	52	KOC LAGE QOM MIG CAM ABG MAHR INC LAGW KBG BYA TUZ CAA KYZ PAV EBE MYA TIB2 KUL GOR BAM TIB1 LUA ARS ASS NCS YIN KUR MME DON CAN SHA CHE VOS MED YAG ERY GAH KUC SID SE2 SC2 SC3 ME1 SW MW2 SC1 NC2 NW NE ME2 **G_TIB**
H2	2	GAA DLI
H3	33	NOG NAN ZHS YUA WUD KEL TUT WUZ BEID DAG YANH SIK DOG HAN MAH **G_FRA_1 G_SFB_1 G_CHI_1 G_CHI_2 G_CHI_3 G_CHI_4 G_CHI_5 G_CHI_6 G_CHI_7 G_CHI_8 G_CHI_9 G_CHI_10 G_ARG_2 G_ARG_3** BLO SCE YAL SLU
H4	7	XIA XIE BEI YUN **G_SIN_1 G_SIN_2 G_SIN_3**
H5	1	TTA
H6	5	VCH BBA HAI **G_BRA_1 G_BRA_2**
H7	1	SLA
H8	1	REL
H9	2	XIN LEG
H10	1	JIN
H11	2	MEG CSR
H12	1	SFA
H13	1	NC1
H14	1	SE3
H15	1	SE1
H16	1	MW1
H17	1	**G_AU**
H18	1	**G_PER**
H19	1	**G_SAL**
H20	1	**G_PAR**
H21	1	**G_SIN_4**
H22	2	**G_SIN_5 G_SIN_6**
H23	1	**G_SIN_7**
H24	1	**G_GSL_2**
H25	1	**G_CAN**
H26	1	**G_ARG_1**
H27	1	PLU
H28	1	STR

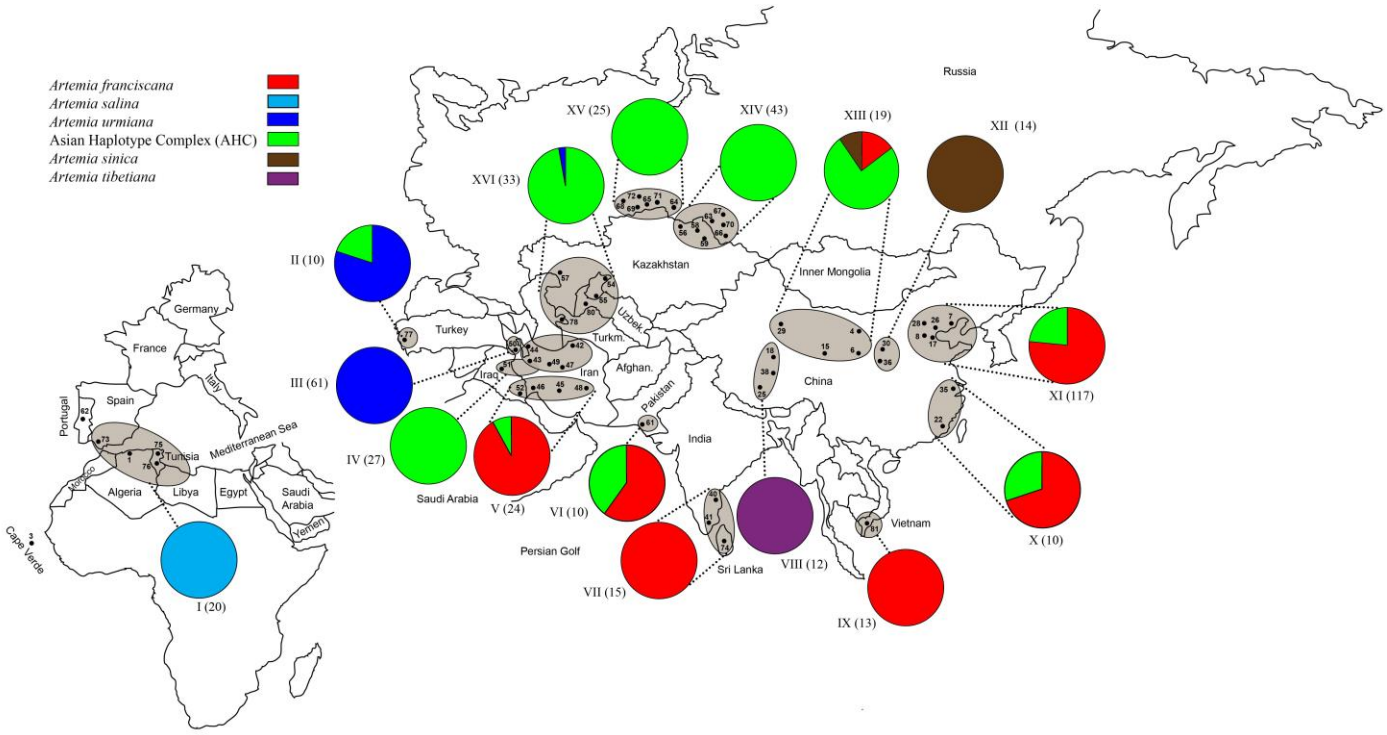

Fig. 21. Species diversity in Asian, European and African *Artemia* populations illustrated as a pie chart. Sixteen isolated gray-colored geographical regions were defined, based on minimum geographical proximity between adjacent localities. In region XI, only 5 out 24 localities are shown. *Artemia* species are represented by different colors. Values in parentheses in the center of the pie chart represent the number of individuals per region used for analysis.

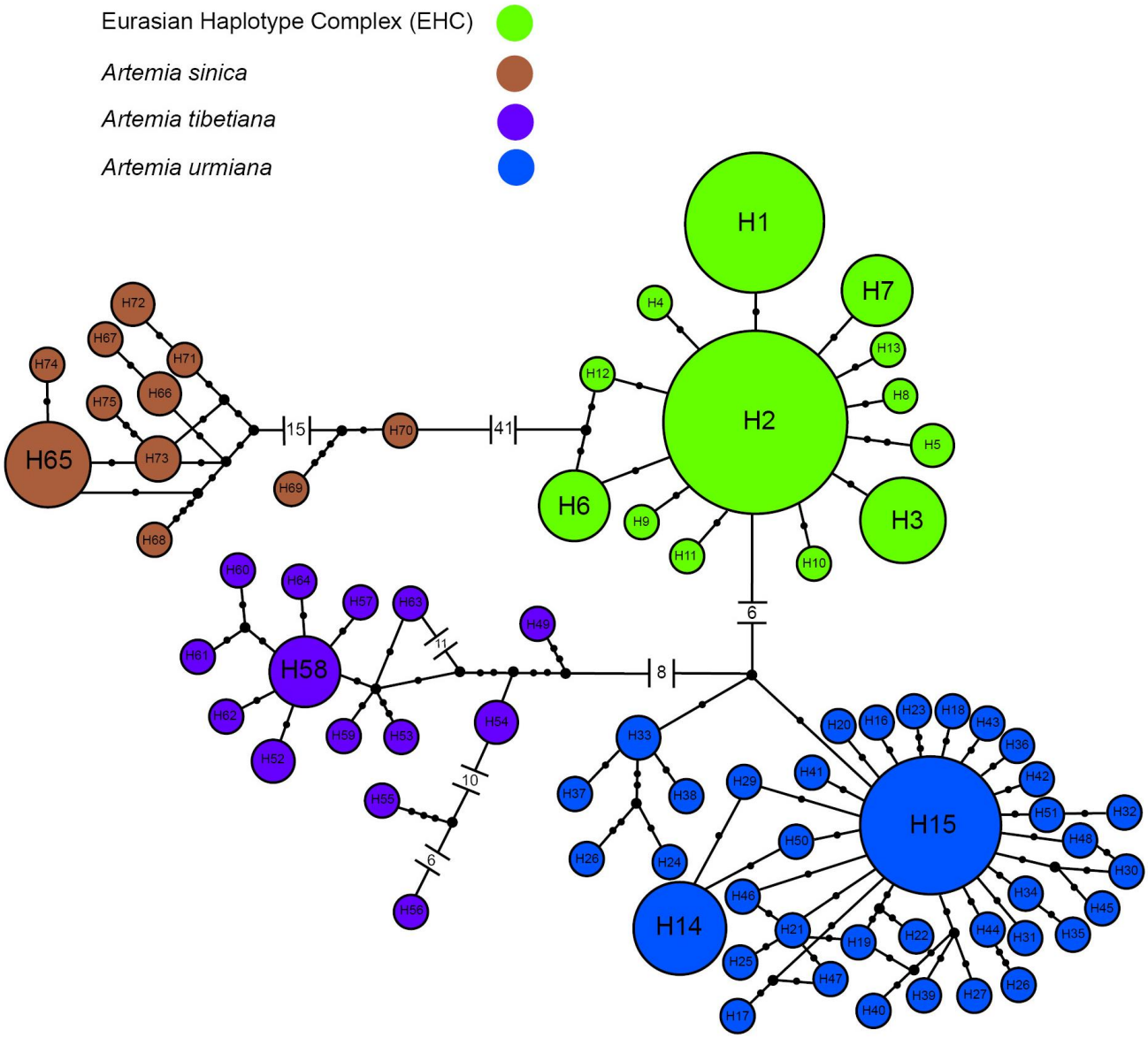

Fig. 22. The relationship between *COI* haplotypes, based on a minimum spanning network for *A. urmiana, A. tibetiana*, *A. sinica* and EHC sequences (N = 299). The diameter of each circle, characterized by a haplotype number, corresponds to the haplotype frequency. Black dots between haplotypes denote a point mutation. Species are shown by different colors. In total, 75 distinct haplotypes (H1 – H75) are recognized among lineages.

Table 25 Data matrix of 75 distinct haplotypes among 299 *A. urmiana*, *A. tibetiana*, *A. sinica* and the EHC individuals using 579 bp of *COI*. H = haplotypes, F = haplotype frequency. GenBank sequences are marked with bold.

H	F	Individuals and Locations
H1	24	INC-1 INC-2 INC-3 INC-4 INC-5 LAGE-1 LAGE-3 MIG-1 MIG-2 MIG-3 MIG-5 GAH-1 GAH-2 GAH-3 GAH-4 GAH-5 GAH-6 XIA-1 XIA-4 CAM-2 KBG-1 KBG-2 KBG-3 **G_PAR_5**
H2	136	LAGW-1 LAGW-2 LAGW-3 LAGW-4 LAGW-5 LAGE-2 ABG-1 ABG-2 ABG-3 ARS-1 ARS-2 ARS-3 ARS-4 ARS-5 ARS-6 ARS-7 ASS-1 ASS-2 ASS-3 ASS-4 ASS-5 ASS-6 KYZ-1 KYZ-2 KYZ-3 KYZ-4 KYZ-5 NCS-1 NCS-2 NCS-3 NCS-4 NCS-5 NCS-6 PAV-1 PAV-2 PAV-3 PAV-4 PAV-5 PAV-6 TUZ-1 TUZ-2 TUZ-3 TUZ-4 TUZ-5 TUZ-6 TUZ-7 TUZ-8 TUZ-9 TUZ-10 TUZ-11 TUZ-12 TUZ-13 TUZ-14 BYA-1 BYA-2 BYA-4 BYA-5 EBE-1 EBE-2 EBE-4 EBE-6 GOR-1 GOR-2 GOR-3 GOR-4 GOR-5 KUC-1 KUC-2 KUC-3 KUL-1 KUL-3 KUR-1 KUR-3 MYA-1 MYA-2 MYA-3 MYA-4 MYA-5 MYA-6 MYA-7 MED-2 VOS-1 VOS-2 VOS-3 BAM-1 BAM-2 BAM-3 BAM-4 BAM-5 BAM-6 CAN-1 CAN-2 CAN-3 CAN-4 CHE-1 CHE-2 CHE-3 DON-1 DON-2 DON-3 HAN-1 HAN-2 HAN-3 HAN-4 HAN-5 HAN-7 LUA-3 LUA-4 LUA-5 SHA-1 SHA-2 SHA-3 SID-1 SID-3 YIN-1 YIN-2 YIN-3 YIN-4 MAHR-1 MAHR-3 KOC-4 KOC-5 KOC-6 KOC-7 KOC-9 KOC-10 KBG-5 CAA-2 CAA-3 CAA-4 CAA-5 CAA-6 CAA-7 CAA-9 **G_PAR_1 G_PAR_2**
H3	7	MIG-4 QOM-1 QOM-2 QOM-3 QOM-4 QOM-5 QOM-6
H4	1	BYA-3
H5	2	EBE-3 DLI-8
H6	5	EBE-5 EBE-7 KUL-2 MME-1 MME-3
H7	5	KUR-2 MME-2 MME-4 MED-1 MED-3
H8	1	HAN-6
H9	1	CAA-1
H10	1	CAA-8
H11	1	**G_PAR_3**
H12	1	**G_PAR_4**
H13	1	**G_PAR_6**
H14	9	**G_PAR_7 G_PAR_8** CAM-1 CAM-3 CAM-4 CAM-5 CAM-6 CAM-9 CAM-10
H15	26	NC2-1 NE-2 NE-3 NE-5 NW-3 NW-5 ME1-1 ME2-2 ME2-4 MW1-1 MW1-3 MW1-4 MW1-5 MW2-1 MW2-4 SE2-4 SE2-6 SE3-2 SE3-3 SC1-2 SC1-3 SC3-1 SC3-2 SC3-4 SE1-1 SW-2
H16	1	NC2-2
H17	1	NC2-3
H18	1	NC1-1
H19	1	NC1-2
H20	1	NC1-3
H21	1	NE-1
H22	1	NE-4
H23	1	NE-6
H24	1	NW-1
H25	1	NW-2
H26	1	NW-4
H27	1	NW-6
H28	1	ME1-2
H29	1	ME2-1
H30	1	ME2-3
H31	1	ME2-5

H32	1	ME2-6
H33	3	MW1-2 SE3-1 SE1-2
H34	1	MW1-6
H35	1	MW2-2
H36	1	MW2-3
H37	1	MW2-5
H38	1	SE2-1
H39	1	SE2-2
H40	1	SE2-3
H41	1	SE2-5
H42	1	SC1-1
H43	1	SC2-1
H44	1	SC2-2
H45	1	SC2-3
H46	1	SC3-3
H47	1	SE1-3
H48	1	SW-1
H49	1	CAM-7
H50	1	CAM-8
H51	1	KBG-4
H52	2	**G_TIB_1 G_TIB_2**
H53	1	**G_TIB_3**
H54	2	**G_TIB_4 G_TIB_5**
H55	1	**G_TIB_6**
H56	1	**G_TIB_7**
H57	1	TIB1-1
H58	5	TIB1-2 TIB1-3 TIB1-5 JIN-2 JIN-4
H59	1	TIB1-4
H60	1	TIB2-1
H61	1	TIB2-2
H62	1	TIB2-3
H63	1	JIN-1
H64	1	JIN-3
H65	7	**G_SIN_1 G_SIN_3** XIE-6 XIE-8 XIE-9 YUN-1 YUN-3
H66	2	**G_SIN_2 G_SIN_5**
H67	1	**G_SIN_4**
H68	1	**G_SIN_6**
H69	1	BEI-1
H70	1	BEI-2
H71	1	XIE-1
H72	2	XIE-2 XIE-3
H73	3	XIE-4 XIE-7 YUN-2
H74	1	XIE-5
H75	1	YUN-4

Table 26 Data matrix of 84 unique haplotypes with their frequencies among 374 *A. franciscana* individuals using 579 bp of *COI*. H = haplotype, F = haplotype frequency. One hundred seventy-seven sequences (bold) of *A. franciscana* from two commercialized USA populations (GSL and SFB), other available American populations (North, Central and South) and European populations have been included from GenBank.

H	F	Individuals & locations
H1	2	**CHI-1 CHI-2**
H2	3	**CHI-3 CHI-4 AF78**
H3	2	**CHI-5 CHI-6**
H4	9	**CHI-7 CHI-8 CHI-9 CHI-10 ARG-2 ARG-3 AF85 AF73 AF72**
H5	51	**CHI-11 CHI-12** MAH-1 MAH-3 MAH-5 NOG-1 NOG-3 NOG-4 NOG-5 SLA-6 VCH-13 BEID-1 BEID-2 BEID-3 BBA-2 BBA-5 BBA-6 DAG-2 DAG-3 DLI-1 DLI-4 DLI-6 DLI-9 DLI-10 DLI-11 DOG-1 DOG-3 ERY-5 HAI-2 LUA-1 LUA-2 NAN-4 NAN-5 TTA-3 TTA-6 WUD-2 WUD-5 WUZ-3 XIA-2 XIA-3 YAG-1 YUA-2 ZHS-1 ZHS-13 KOC-1 KOC-2 KOC-3 KOC-8 **AF26 AF20 AF20 (EU)**
H6	68	BRA-1 BRA-2 SLU-1 SLU-2 SLU-3 SLU-4 SLU-5 SLU-6 SLA-1 SLA-2 SLA-3 SLA-4 SLA-5 VCH-1 VCH-2 VCH-3 VCH-4 VCH-5 VCH-6 VCH-7 VCH-8 VCH-9 VCH-10 VCH-11 VCH-12 KEL-1 KEL-3 GAA-3 GAA-4 GAA-5 GAA-7 BBA-4 DOG-2 DOG-4 HAI-1 SIK-1 SIK-2 SIK-4 SIK-5 SIK-6 TTA-1 TTA-2 TTA-4 TTA-5 WUD-3 WUD-4 WUZ-1 WUZ-2 XIN-1 XIN-3 YUA-4 YUA-5 YUA-6 YUA-7 ZHS-2 ZHS-3 ZHS-5 ZHS-6 ZHS-7 ZHS-8 ZHS-9 ZHS-10 ZHS-11 ZHS-12 **AF25 AF22 AF10 AF10 (EU)**
H7	2	**ARG-1 AF81**
H8	102	SCE-1 YAL-1 YAL-2 YAL-3 YAL-4 YAL-5 YAL-6 YAL-7 GSL-1 GSL-3 GSL-4 GSL-5 GSL-7 GSL-9 GSL-10 GSL-12 MAH-4 MAH-6 NOG-2 KEL-2 KEL-4 TUT-1 TUT-2 TUT-3 TUT-4 TUT-5 GAA-1 GAA-2 GAA-6 GAA-8 BBA-1 DAG-1 DLI-2 DLI-3 DLI-5 DLI-7 ERY-4 HAI-3 LEG-2 LEG-3 LUA-6 NAN-1 NAN-2 NAN-3 SID-2 SIK-3 TTA-7 XIN-2 YAG-2 YAG-3 YANH-1 YANH-2 YANH-3 YUA-1 YUA-3 MAHR-2 MAHR-4 MAHR-5 **AF90 AF87 AF49 AF18 GSL (S1-1) GSL (S1-3) GSL (S1-4) GSL (S1-5) GSL (S1-6) GSL (S1-8) GSL (N1-1) GSL (N1-2) GSL (N1-3) GSL (N1-5) GSL (N1-6) GSL (N1-7) GSL (N1-8) GSL (N1-9) GSL (N1-10) GSL (N2-2) GSL (N2-3) GSL (N2-4) GSL (N2-5) GSL (N2-7) GSL (N2-8) GSL (N2-9) GSL (N2-10) GSL (S3-2) GSL (S3-5) GSL (S3-9) GSL (S3-10) GSL (N3-1) GSL (N3-3) GSL (N3-4) GSL (N3-6) GSL (N3-7) GSL (N3-8) GSL (N3-9) GSL (S2-1) GSL (S2-4) GSL (S2-5) GSL (S2-9) GSL (S2-11) AF18 (EU)**
H9	5	SCE-2 SCE-4 SCE-5 **AF53 AF50**
H10	1	SCE-3
H11	3	BLO-1 BLO-3 BLO-4
H12	2	BLO-2 **AF3**
H13	5	ING-1 ING-2 ING-3 ING-5 **AF39**
H14	1	ING-4
H15	1	ING-6
H16	7	PLU-1 PLU-2 PLU-3 PLU-4 PLU-5 **AF86 AF86 (EU)**
H17	23	**GSL-2 GSL-6 GSL-8 GSL-11 GSL (S1-2) GSL (S1-7) GSL (S1-9) GSL (S1-10) GSL (N1-4) GSL (N2-6) GSL (S3-1) GSL (S3-3) GSL (S3-7) GSL (S3-8) GSL (N3-2) GSL (N3-5) GSL**

		(N3-10) GSL (S2-2) GSL (S2-3) GSL (S2-6) GSL (S2-8) GSL (S2-10) AF27
H18	3	MAH-2 **AF21 AF21 (EU)**
H19	1	BBA-3
H20	1	ERY-1
H21	1	ERY-2
H22	2	ERY-3 LEG-1
H23	3	WUD-1 **AF19 GSL (S3-4)**
H24	1	ZHS-4
H25	2	**AF93 AF93 (EU)**
H26	1	**AF92**
H27	2	**AF91 AF9**
H28	1	**AF89**
H29	1	**AF88**
H30	1	**AF84**
H31	1	**AF83**
H32	1	**AF82**
H33	1	**AF80**
H34	1	**AF79**
H35	1	**AF77**
H36	1	**AF76**
H37	2	**AF75 AF74**
H38	1	**AF71**
H39	3	**AF70 AF51 AF1**
H40	1	**AF69**
H41	1	**AF68**
H42	2	**AF67 AF61**
H43	2	**AF66 AF59**
H44	1	**AF65**
H45	1	**AF64**
H46	2	**AF63 AF60**
H47	1	**AF62**
H48	1	**AF58**
H49	1	**AF57**
H50	1	**AF56**
H51	1	**AF55**
H52	1	**AF54**
H53	1	**AF52**
H54	1	**AF48**
H55	1	**AF47**
H56	1	**AF46**
H57	3	**AF45 AF36 AF35**
H58	1	**AF44**
H59	1	**AF43**
H60	1	**AF42**
H61	2	**AF41 AF40**

H62	1	**AF38**
H63	1	**AF37**
H64	3	**AF34 AF32 AF28**
H65	1	**AF33**
H66	1	**AF31**
H67	1	**AF30**
H68	1	**AF29**
H69	1	**AF24**
H70	1	**AF23**
H71	1	**AF17**
H72	1	**AF16**
H73	1	**AF15**
H74	2	**AF14 AF13**
H75	1	**AF12**
H76	1	**AF11**
H77	2	**AF8 AF6**
H78	1	**AF7**
H79	1	**AF5**
H80	1	**AF4**
H81	1	**AF2**
H82	1	**GSL (N2-1)**
H83	1	**GSL (S3-6)**
H84	1	**GSL (S2-7)**

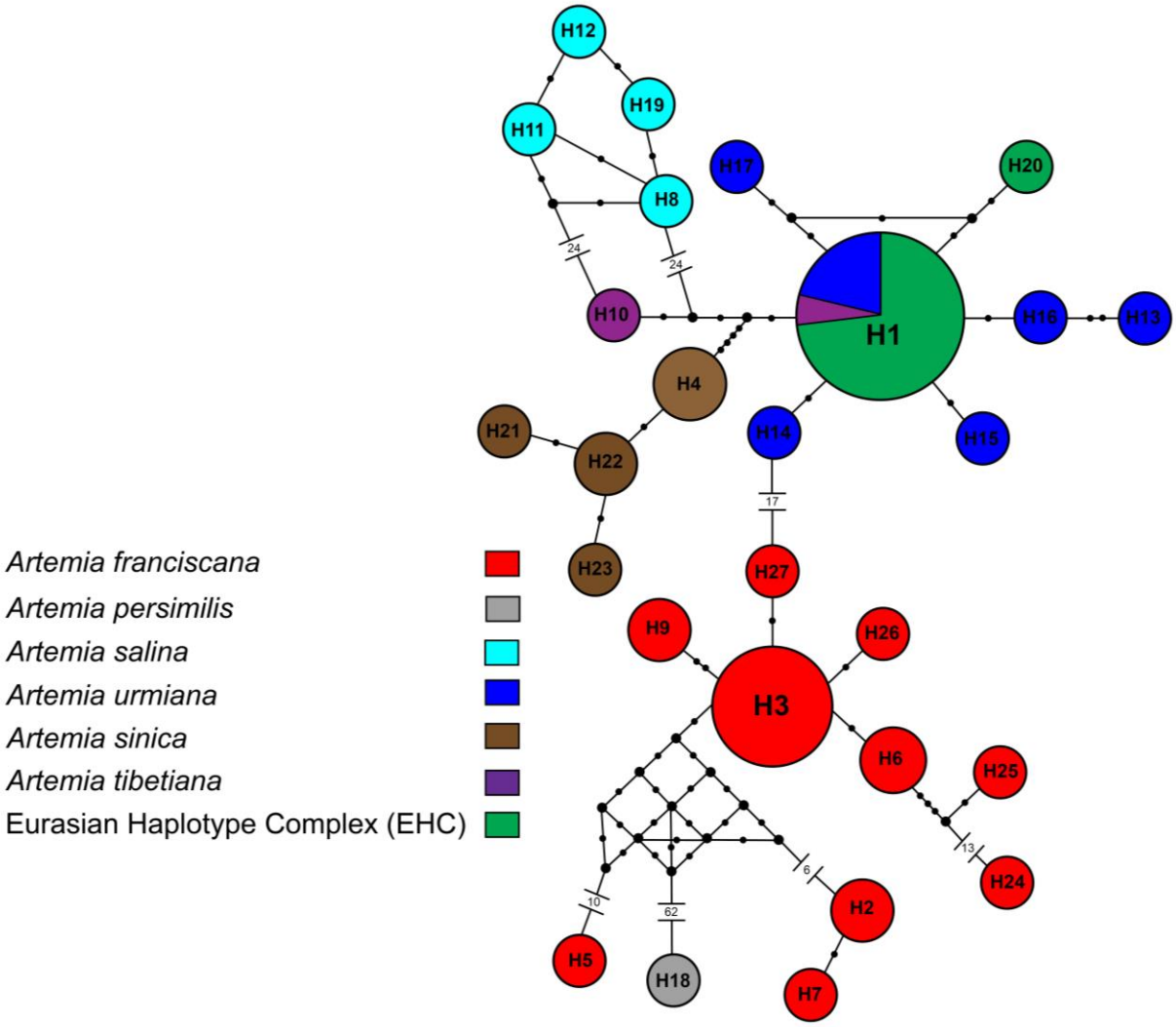

Fig. 23. The relationship between *ITS1* haplotypes, based on a minimum spanning network for all individuals (N = 124). The diameter of each circle, characterized by a haplotype number, corresponds to the haplotype frequency. Black dots between haplotypes denote a point mutation. Species are shown by different colors. In total, 27 distinct haplotypes (H1 – H27) are recognized among lineages.

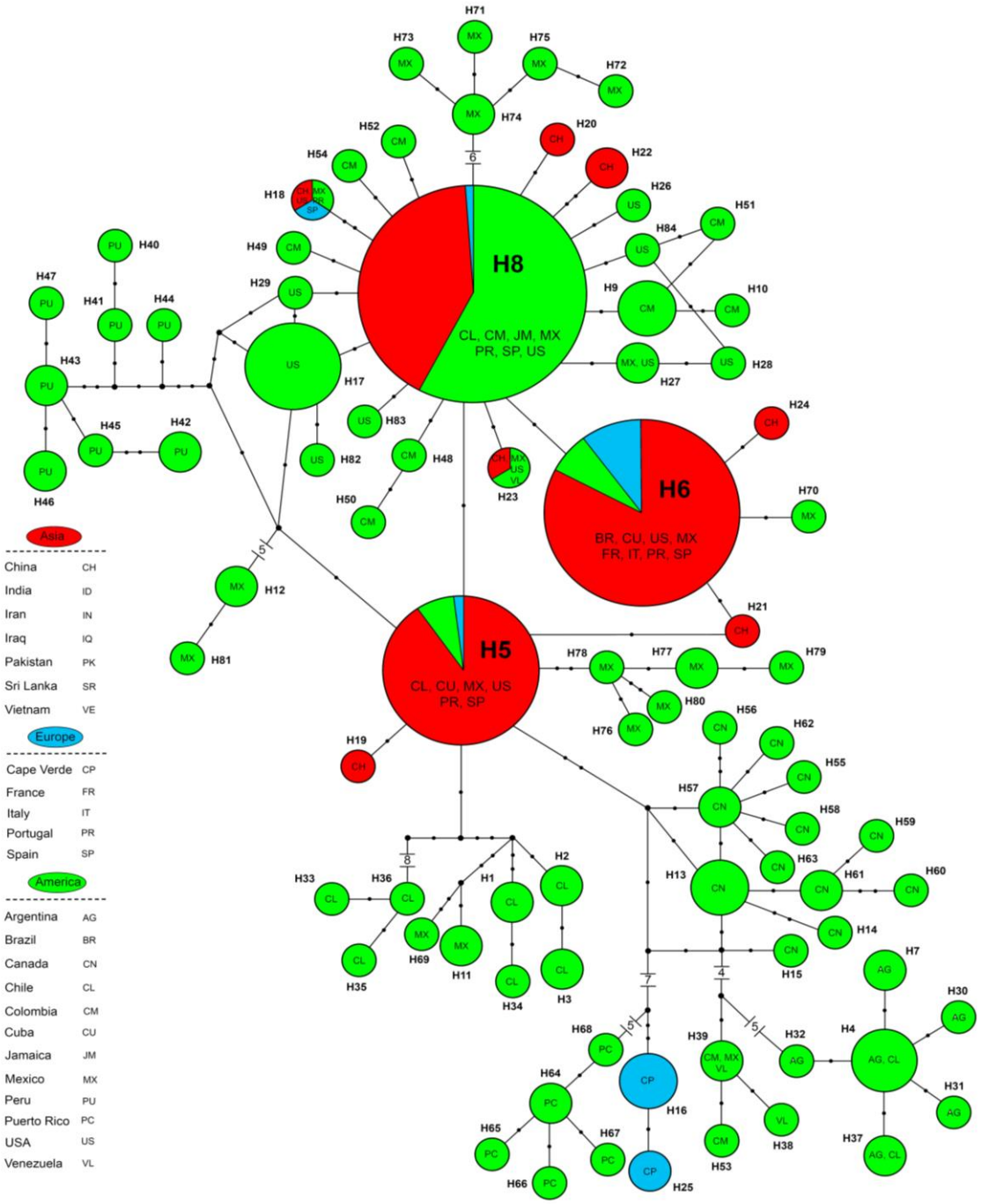

Fig. 24. *COI* Median-joining network constructed from 374 *A. franciscana* sequences. The sizes of the circles are proportional to the number of individuals containing a/the haplotype. Black dots between haplotypes denote a point mutation. Asia, Europe, and America are shown by red, blue, and green colors. Countries are shown by abbreviations. In total, 84 unique haplotypes (H1– H84) are recognized among lineages. One hundred seventy-seven sequences of *A. franciscana* from different localities including Americas and Europe have been included from GenBank. Haplotypes H5, H6 and H8 indicate sequences from GSL and SFB. Haplotypes H17, H18, H23, H82, H83 and H84 indicate sequences from GSL.

3.3.5. Discussion

Phylogeny of *Artemia*

The current study represents the most complete survey, to our knowledge, to focus on the phylogenetic relationships of Asian *Artemia* and the recent colonization of American species *A. franciscana* throughout Asia.

Phylogenetic trees (ML & BI) of *Artemia* based on mitochondrial sequences (*COI*) showed inconsistency with nuclear sequences (*ITS1*). This phylogeny presents several features. Firstly, the genus *Artemia* consists of seven or nine genetically distinct clusters whose robustness is strongly supported by bootstrap values higher than 75% and by Bayesian posterior probabilities higher than 90% in trees (Fig. 19 and 20). A group consists of geographically widely distributed, presumed parthenogenetic populations (EHC), which show a close genetic relationship to *A. urmiana* and *A. tibetiana*. Another cluster includes *A. sinica*, which constitutes a single distinct group. Reproductive incompatibility of *A. tibetiana* with Asian sister species has been addressed previously (Abatzopoulos et al., 1998, 2002). A low level of genetic divergence associated with the marked morphological diversity of *A. tibetiana* reflects slow rates of divergence or recent speciation (Kappas et al., 2011). In this case, recent speciation is more likely, although the estimated divergence time between *A. tibetiana* and *A. sinica* lies roughly at around 8 Mya (Baxevanis et al., 2006). In *COI* tree, *A. tibetiana* showed two distinct clades which necessitate further taxonomical re-evaluation for this species (Muñoz et al., 2010). Two genes (*COI* & *ITS1*) in all provided incongruent splitting evidence showing shared haplotypes between species in Asia. There is a substantial sequence divergence between newly established Asian *A. franciscana* haplotypes and Asian lineages which indicates a high number of mutations among *A. franciscana* and EHC lineages. This could be an indication of cryptic lineages in Asia (Muñoz et al., 2010; Havermans et al., 2011; Clare et al., 2013). However, our observations should be regarded carefully, since we could have to do with morphologically cryptic species without obvious different characteristics in nature. So, extensive genetic analyses in conjunction with morphological analyses are required to understand any cryptic diversity of *Artemia* in Asia. Secondly, there is the larger geographic range of EHC, which indicates their potential colonization abilities, related to their habitat conditions (Muñoz et al., 2010). In fact, EHC lineages may undergo a relaxed selection of dispersal, since they do not suffer from mating limitation when colonizing in new environments (Haag and Ebert, 2004). It has been suggested that the colonization performance of parthenogens is also related to habitat availabilities resulting from natural and human alterations (Muñoz et al., 2010).

A third feature is that two sibling species from the New World have evolved from a common sexual ancestor *A. salina* in the Mediterranean area (Gajardo and Beardmore,

2012). In our phylogenetic trees, *A. franciscana* has been broadly expanded into Asia and was much closer to Asian species rather *A. persimilis*.

Phylogeography of *A. franciscana* in Asia

The present study is a first attempt to characterize the colonization of an exotic American species in non-indigenous regions throughout Asia. The characterization of *Artemia* species depends on biochemical and genetic methods, because there are no reliable morphological characteristics to differentiate among them (Asem et al., 2010a).

Artemia franciscana is an American species that has been introduced throughout Europe and Asia and has established permanent populations along the southern and eastern coastal regions of Asia, as well as in the Mediterranean region (Zheng et al., 2004; Amat et al., 2007; Van Stappen et al., 2007; Ruebhart et al., 2008). *A. franciscana* from Great Salt Lake has been used for the production of *Artemia* cysts and biomass, combined with salt production in solar saltworks; but in aquaculture facilities worldwide, it is by far the dominant strain being used (Sorgeloos et al., 2001), and for this reason this strain was included in the analysis. *A. franciscana* has now colonized wide areas of Asia; it showed an overall high genetic variability in our study, compared to the GSL source population. Generally, invasive populations show a lower genetic diversity in non-indigenous locations compared to the source population (Golani et al., 2007). This has been demonstrated for the introduced Vinh Chau (Vietnam) *A. franciscana* and its source SFB population, intraspecific genetic differentiation and reduction of haplotype diversity having been demonstrated due to founder effects in the established VC strain within a year after inoculation (Kappas et al., 2004). In our study, *COI* genetic diversity is higher in the exotic Asian *A. franciscana* as compared with GSL and native Asian species. This phenomenon has been observed in some Mediterranean *A. franciscana* populations as well (Hontoria et al., 2012; Muñoz et al., 2014). An overall high genetic diversity among all Asian *A. franciscana* might be the result of human-mediated numerous dispersal events by multiple introductions from the two primary sources of *A. franciscana* (GSL and SFB) and eventually from non-documented introductions from Central American and European populations (Kolbe et al., 2004; Facon et al., 2006; Roman and Darling, 2007; Hontario et al., 2012; Li et al., 2012; Muñoz et al., 2014). Our findings support the introduction of *A. franciscana* from multiple sources into the Asia which has not been observed in Mediterranean regions using *COI* gene (Muñoz et al., 2014). In general, higher genetic diversity ensures an adaptive potential and physiological plasticity of the introduced species outside its native region (Dlugosch and Parker, 2008; Ruebhart et al., 2008; Vikas et al., 2012; Muñoz et al., 2014). The reduced *COI* genetic diversity of *A. franciscana* in several single Asian localities, however, can be due to population bottlenecks and founder effects during the colonization process.

Competitive superiority of *A. franciscana* over native Eurasian species has been observed following the sequence *A. franciscana* > "*A. parthenogenetica*" > *A. salina* (Van Stappen, 2002; Scalone and Rabet, 2013). According to our study, Asian EHC with lower genetic diversity are more susceptible to invasion than those with higher genetic diversity. However, in the case of invasion by *A. franciscana* of commercial origin, the intensity of nearby aquaculture operations is also a decisive factor determining invasion pressure. EHC contains both automictic and apomictic populations according to Baxevanis et al., (2006). Apomictic populations are genetically identical to their mothers, therefore providing less tolerance for invasion (Allendorf et al., 2013). Further study is required in order to establish a link between the invasive successes of *A. franciscana* in Asia, on the one hand, and apomixis of the local parthenogenetic populations, on the other. Consequently, biodiversity threats, e.g. the extinction of local species, might be assessed from a long-term evolutionary perspective. The widespread pattern of *A. franciscana* outside America is a relatively recent phenomenon and just a few decades old, due to the recent expansion of aquaculture. Thus, colonization of *A. franciscana* in Asia is at an initial stage (Van Stappen et al., 2007). More systematic investigation is thus required to document the post-inoculation micro-evolutionary changes of this species in its new environments.

If related species come into contact after artificial introductions, hybridization might occur (Ellstrand and Schierenbeck, 2000; Allendorf et al., 2013). Thus far, natural hybridization has been shown to occur among the three presumably oldest species (*A. franciscana*, *A. persimilis* and *A. salina*) through unidirectional mitochondrial introgression. Hybridization between *A. franciscana* and *A. salina* resulted in a maternally-derived haplotype, which clustered in the *A. salina* group (Kappas et al., 2009). In our *ITS1* tree, a possible hybridization event between *A. tibetiana* and EHC lineages could be estimated. There is also partial infertility when crossing between *A. franciscana* on the one hand and *A. sinica*, *A. urmiana*, and *A. salina* on the other (Zheng et al., 2004). Unlike terrestrial environments, the dynamics of hybrid zones in aquatic environments such as lakes and ponds have not yet been modeled appropriately (Kappas et al., 2009). Further studies are needed to determine potential hybrids among Old World *Artemia* and hybrids of *A. franciscana* with Asian species. Moreover, hybridization of rare males of diploid parthenogenetic populations with Asian sexual females produced functionally fertilized and viable diploid F1 hybrids. This observation would open up a new window on parthenogenesis through hybridization (Maccari et al., 2013a).

Asian *A. franciscana* showed no phylogeographic pattern, which is the result of natural (migratory birds, wind) and anthropogenic activities (use of *Artemia* nauplii as live food in hatcheries and *Artemia* production in solar salt works). Human activities have initiated

and accelerated the rate of colonization of American *A. franciscana* in Asia, for example the developing aquaculture industry. The passive dispersal of *Artemia* cysts by waterfowl has been indicated by Green et al. (2005). Maniatsi et al. (2011) indicated that the differences in genetic diversity among "*A. parthenogenetica*" populations were related to their ploidy level/reproductive mode (automixis *vs* apomixis). Although the ploidy levels of some Eurasian populations have been documented (Maniatsi et al., 2011), we conclude from our study that EHC is a mixture of clones with different, but as yet unknown, ploidy levels and sexual systems, which need to be further analyzed in detail.

3.4 Analysis of the Genetic Variability of *Artemia franciscana* Kellogg, 1906 from the Great Salt Lake (USA) Based on mtDNA Sequences, ISSR Genomic Fingerprinting and Biometry

3.4.1. Abstract

The genetic structure of the brine shrimp *Artemia franciscana*, an extremophile halophilic microcrustacean, from the Great Salt Lake (USA), was investigated by analysis of nucleotide sequences of the mitochondrial *cytochrome c oxidase subunit I* (COI), genomic fingerprinting by ISSR-PCR (inter-simple sequence repeats) and biometry. Samples from six different geographical localities of Great Salt Lake revealed 11 distinct haplotypes with a low level of haplotype diversity (*HD* = 0.68). Phylogenetic and genetic fingerprinting analyses supported the null hypothesis, that the brine shrimps of this lake form a panmictic gene pool with a low level of genetic differentiation index (F_{ST} = 0.05) and a high rate of gene flow (Nm= 2.8) between geographical areas. Moreover, morphological differences supported the presence of a panmictic population. Neutrality tests and mismatch distribution revealed that *A. franciscana* has undergone a recent population expansion. In spite of hypersaline conditions in the Northern (Gunnison Bay) regions of the GSL, the genetic population structures of different areas of the lake are not linked to ecological or limnological conditions. Therefore, *A. franciscana* from the Great Salt Lake should be considered as a single management unit for conservation.

Keywords: Genetic variability, Population structure, *Artemia franciscana*, Great Salt Lake, Panmixia

3.4.2. Introduction

Artemia (Crustacea, Anostraca) – a continental extremophile zooplankter − is a small macroscopic organism with a wide distribution across all continents except Antarctica (Maniatsi et al., 2009; Kappas et al., 2011). *Artemia* is extremely osmotolerant and can inhabit hypersaline lakes or lagoons, which are hostile environments for most other organisms (Gajardo and Beardmore, 2012).

The genus *Artemia* comprises six distinct sexual species and a Eurasian lineage complex containing parthenogenetic populations (Gajardo et al., 2002). Four established sexual species occur in restricted regions of Eurasia, including *A. salina* in the Mediterranean basin (Triantaphyllidis et al., 1997), *A. urmiana* Günther, 1899 in Lake Urmia (Iran) and Lake Koyashskoe, Ukraine (Abatzopoulos et al., 2009), *A. sinica* in China Cai, 1989, and *A. tibetiana* in Tibet (Abatzopoulos et al., 1998). An undescribed new species might exist in Kazakhstan Pilla and Beardmore, 1994. The two other sexual species are found in America

with *A. franciscana* throughout North and South America, and *A. persimilis* Piccinelli and Prosdocimi, 1968 in Argentina and Chile.

The Great Salt Lake (GSL) is the largest hypersaline lake in North America and the fourth largest lake in the world. GSL is located in the arid western United States (40°50′ N − 112°25′ W) and has an average elevation of 1,280 m above sea level, a size of 113×48 km with an average maximum water depth of 11.5 m (Moon et al., 2008). Historically, GSL is the vestige of the ancient large freshwater Lake Bonneville (51,000 km^2) which had begun to shrink at the end of Pleistocene to the present smaller size (4,200 km^2). Neither Lake Bonneville nor the Great Salt Lake had a natural outflow to oceans (Utah Geological Survey, Jones et al., 2009). Lake Bonneville has undergone numerous geological and hydrological alterations over the past century and subsequently is characterized by multiple sedimentary layers (Stephens, 1990; Post, 1977). The land within the GSL consists of 8 islands. The lake harbors a unique ecosystem with numerous bacterial taxa, halophytic phytoplankton, several protozoa and especially two keystone species: brine flies (*Ephydridae*) and brine shrimp *Artemia*. In addition, the GSL hosts more than 250 million migrating birds (Aldrich and Paul, 2002). *A. franciscana* of GSL has been exploited as a commercial species and is used as a high value live food in the international aquaculture industry (Sorgeloos et al., 2001).

Before 1955, Gunnison Bay and Gilbert Bay were part of a continuous lake area that had a typical salinity ranging between 20 to 27% (Stephens, 1990). In 1959, the construction of a semipermeable Southern Pacific Railroad Causeway (SPRC) divided the lake into two distinct bays: the Northern region (Gunnison Bay) and the Southern region (Gilbert Bay). Due to a lack of freshwater influx into Gunnison Bay, this region of GSL has become *de facto* a terminal lake and its salinity has reached saturation (salinity of approximately 28%) (Loving et al., 2002). According to recent assessments of water salinity, Gunnison Bay has a much higher salinity that Gilbert Bay (295 *vs* 140 ppt) (Marden, pers. com., 2014). Gilbert Bay receives inflow from several major freshwater rivers, it is less saline (16%) and supports a diverse fauna and flora (Wurtsbaugh, 1992; Stephens, 1998).

The question arises of whether the unequal salinity in Gunnison and Gilbert Bay has an influence on genetics and population structure of *A. franciscana.* In a previous study, we could show that *A. urmiana* in the hypersaline Lake Urmia (Iran) (which shows several similarities to GSL) constitutes a single panmictic population with a lack of apparent population structure across the lake (Eimanifar and Wink, 2013). The biological and ecological importance of *A. franciscana* for GSL has stimulated systematic research to determine its cyst size and population structure across the entire lake. The underlying genetic variation of *Artemia* in the GSL is relatively unknown and systematic studies have investigated a potential linkage between geographical and ecological differences within the

GSL and genetic variation of *A. franciscana*. In the present study we have investigated cyst diameter and genetic population structure of *A. franciscana* from 6 different geographical regions in the Great Salt Lake including the high-saline Gunnison Bay and the less saline Gilbert Bay. Nucleotide sequences of the mitochondrial *COI* gene and genomic fingerprinting by ISSR-PCR were carried out to understand morphological and genomic variability of *A. franciscana* in GSL and to test whether the separation of the lake into two parts already shows an influence.

3.4.3. Materials and methods

Sampling and DNA extraction

Sixty-one individuals of *A. franciscana* (cyst) were collected from 6 geographical regions distributed across Great Salt Lake (Fig. 25). Table 27 summarizes relevant information regarding sampling sites, voucher numbers, number of individuals studied, geographical coordinates, and corresponding locality codes (used hereafter). There is no specific permissions required for these locations/activities. Our field studies did not involve endangered or protected species which provided by the specific locations in our study (e.g. GPS coordinates). Total genomic DNA was extracted from single cysts using Chelex-100 (6%, Bio-Rad Laboratories, CA, USA) as described in (Eimanifar and Wink, 2013). Extracted DNA was stored at 4 °C until used for subsequent genetic analysis.

PCR amplification of marker genes and sequence alignment

The mitochondrial *cytochrome c oxidase subunit I* (*COI*) was amplified by polymerase chain reaction (PCR) using established primer pairs (Muñoz et al., 2008). Standard PCR conditions were performed in a total volume of 50 µl in a thermocycler (Biometra, Tgradient, Germany) with *Taq* DNA polymerase (Bioron GmbH, Germany) and sequencing was outlined in (Eimanifar and Wink, 2013). DNA sequences were edited using Bioedit sequence alignment version 7.0.9.0 (Hall, 1999). Both strands of DNA were inspected visually. In order to ensure the absence of stop codons, all sequences were translated into amino acids using MEGA6 (Tamura et al., 2013).

Phylogenetic analyses

Phylogenetic analyses included maximum likelihood (ML) in Phyml vers. 3.0 (Guindon et al., 2010) and Bayesian inference (BI) in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). The closely related *A. persimilis* from South America was selected as an outgroup. The best fitting nucleotide substitution model of DNA was selected based on Akaike Information Criterion model (AICc) as implemented in jModelTest v. 0.1.1 (Posada, 2008). 1000 bootstrap replications were run for the ML tree for statistical analysis.

A codon-based partitioned Bayesian analysis was conducted under the following conditions: two simultaneous runs, 8,000,000 generations, a random starting tree with four

independent Markov chains (MCMC), tree sampling every 500 generations, early-phase 0.5 million generations (50%) trees discarded as burn-in. Posterior clade probabilities (PP) were calculated from the post remaining tree in order to support branches reliability (Erixon et al., 2003).

Genetic diversity indices were computed with the Dnasp v. 5.00 (Librado and Rozas, 2009) based on polymorphic sites (V), number of haplotypes (H), haplotype diversity (*h*) and nucleotide diversity (π). Population demographic events were evaluated by calculating Tajima's *D* (Tajima, 1989) and Fu's *Fs* values (Fu, 1997) using the Arlequin 3.5 package.

Table 27 Origin of *A. franciscana* samples, including the IPMB voucher number, and their GenBank accession numbers.

IPMB/ Voucher Number	**Geographical locality**	**Locality code**	**Individuals**	**Geographical coordinates**	**GenBank accession numbers**
66314	Gunnison Bay	N1	10	41°37'122"N, 112°50'295"W	Pending
66315	Gunnison Bay	N2	10	41°27'119"N, 112°55'229"W	"
66316	Gunnison Bay	N3	10	41°24'100"N, 112°38'020"W	"
66317	Gilbert Bay	S1	10	40°59'116"N, 112°30'509"W	"
66318	Gilbert Bay	S2	11	41°06'888"N, 112°46'144"W	"
66319	Gilbert Bay	S3	10	40°50'837"N, 112°26'700"W	"

(Excoffier and Lischer, 2010). Mismatch distributions were determined according to the sudden expansion model in Arlequin 3.5. We used goodness-of-fit tests based on the sum of squared deviations (SSD) and raggedness index (Rag) to test the significance of fit of distribution.

Relationships among haplotypes were reconstructed using the NETWORK program (Bandelt et al., 1999), based on a median joining algorithm. The relationship between geographic and genetic distances was evaluated by Isolation-by-Distance (IBD) analysis, which is included in the IBDWS software v. 3.14 (Jensen et al., 2005), implementing 30,000 randomizations. IBDWS performs Mantel tests with Reduced Major Axis (RMA) regression analysis. Geographic distances (km) between localities were calculated using the Google map distance calculator.

Genomic fingerprinting by ISSR-PCR

Inter simple sequence repeat (ISSR)-PCR was carried out using fifteen primers. All primers were checked to identify variability within and among 60 randomly selected individuals from the Great Salt Lake. Five out of fifteen screened primers were chosen because banding patterns were sharp and unambiguous (Table 28, Fig. 26). PCR amplifications were carried out in a 25 µl final volume and properly labelled with radioactive [α-^{33}P]-dATP (Perkin Elmer, LAS, GmbH, Germany). PCR products were run through high-resolution denaturing polyacrylamide gels 6% (0.2 mm, 0.02 cm) and exposed to X-ray hyperfilm (Kodak, Taufkirchen, Germany) as elucidated by (Eimanifar and Wink, 2013). A significant number of individuals per locality (30%) were taken for repeated PCR amplification to ensure reproducibility and repeatability of each marker.

ISSR statistical analysis

ISSR bands were checked visually and only the unequivocally reproducible ones were scored for each individual as present (1) or absent (0). The binary data matrix (presence/absence) was constructed in MS Excel and used for genetic analyses (Table 29). All six localities were analyzed using FAFLPcalc (Dasmahapatra et al., 2008) and subsequently POPGENE v. 1.31 (Yeh et al., 1999) for the following parameters: Shannon's information index (I), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL).

In order to check the assumption of Hardy-Weinberg equilibrium (HWE) of populations, inbreeding coefficient F_{AFLP} (an analog to F_{IS}) was calculated using FAFLPcalc ((Dasmahapatra et al., 2008). Genetic diversity values were measured with different levels of inbreeding coefficients within populations (F_{IS} = 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35) using FAFLPcalc (Dasmahapatra et al., 2008).

Jaccard's coefficient index was used for calculating the similarity matrix among individuals of localities. The similarity coefficients were then employed to construct dendrograms using the Community Analysis Package (Seaby and Henderson, 2007). Principle coordinate analysis (PCA) was conducted to assess the relationships among individuals using the DCENTER and EIGEN modules of the NTSYS-pc 2.02 software package (Rohlf, 1998).

A non-parametric analysis of molecular variance (AMOVA) was performed in order to determine genetic variability within and among populations using the Arlequin v. 3.5 package (Excoffier and Lischer, 2010) with 10,000 permutations. All individuals were grouped into 6 different groups, corresponding to geographical localities. A Mantel test was applied to find out the correlation of genetic and geographic distances (km) between all location pairs using the NTSYS-pc 2.02 software package (Rohlf, 1998), with 1000 random permutations.

The genetic differentiation coefficient (G_{ST}) was calculated by POPGENE. The Wright's (F_{ST}) averaged over loci and total gene diversity (*Ht*) were calculated by using AFLP-SURV v. 1.0 (Vekemans, 2002). We applied a Bayesian model-based clustering algorithm using STRUCTURE v. 2.2 (Pritchard et al., 2000; Falush et al., 2007) in order to construct population structure for ISSR. This algorithm assigns individuals into a potential number of clusters (*K*) which is varied from 1 to 10. We performed 10 independent runs using the admixture model with correlated allele frequencies for each *K* value. MCMCs were processed for 500,000 cycles with the first 20% cycles discarded as burn-in. The most appropriate *K* was determined using Delta K explained by (Evanno et al., 2005).

Biometry of *Artemia* cysts

A small number of cysts from each locality were hydrated according to the method carried out by (Asem et al., 2007). A few drops of 1% Lugol's solution were added to prevent metabolic activity within the cysts during the hydration process. The diameter of 900 hydrated cysts from six localities was measured under a microscope equipped with an eyepiece calibration that is based on micrometer dimensions.

The hydrated cysts were decapsulated using an equal volume of NaOCl and a few drops of NaOH according to the method explained by (Bruggemann et al., 1980). A total quantity/number of 900 decapsulated cysts was randomly chosen and considered for subsequent measuring.

Chorion thickness (CT) was calculated according to the following formula:

CT = (Mean diameter of untreated cyst − Mean diameter of decapsulated cyst)/2

Data analysis

All diameters (capsulated, decapsulated, and chorion thickness) from six localities were analyzed by one-way ANOVA in order to determine mean values (Tukey test, $P < 0.05$) as implemented in the IBM SPSS v. 21 package (Brosius, 2013). Hierarchical cluster analysis and Principle Coordinate Analysis (PCA) were performed in order to find relationships among localities using SPSS v. 21 package (Brosius, 2013).

Table 28 List of primers screened for ISSR analysis, sequences (5'–3'), GC content, annealing temperature, amplification pattern, and total number of loci amplified in *A. franciscana*.

Primer	Motif (5'–3')	GC (%)	Annealing temperature (°C)	Amplification pattern	Total number of bands
ISSR1	(TG)8G	52.9	48–54	Smear	–
ISSR2	$(CAC)_5$	66.7	48–54	Smear	–
ISSR3	$(GACA)_4$	50	48–54	Smear	–
ISSR4	$(AG)_{12}$	50	48–54	Poor	–
ISSR5	$(TC)_9$	50	48–54	Poor	–
ISSR6	$(GT)_{10}$	50	48–54	Smear	–
ISSR7	$(CA)_{10}A$	47.6	48–54	Poor	–
ISSR8	$(GAA)_5$	33.3	48–54	No amplification	–
ISSR9	$(CAG)_6$	66.7	48–54	No amplification	–
ISSR10	(AC)8C	52.9	48–54	No amplification	–
ISSR11	$(AG)_8C$	52.9	48	Good & sharp	32
ISSR12	$(AG)_8YT$	50	48	Good & sharp	35
ISSR13	$(GA)_9T$	47.4	50	Good & sharp	28
ISSR14	(GTG)5	67	50	Good & sharp	17
ISSR15	(GA)9C	53	49	Good & sharp	35

3.4.4. Results

Genetic diversity and phylogeography

The mitochondrial *COI* sequence set had a total length of 649 nucleotides. A total of 11 haplotypes were detected among 39 substitutions from *A. franciscana* collected in 6 geographical localities (S1 to S3, N1 to N3). Among them, two haplotypes (H1, H2) were common and were shared by 16 and 36 individuals which came from all 6 localities (Table 30). A few singleton haplotypes were observed. Haplotype diversity varied from 0.2 to 0.86, being the highest in S2 and S3 localities (Table 31). The mean haplotype diversity was higher (HD = 0.6), whilst nucleotide diversity was lower among regions (π = 0.001). The mean haplotype and nucleotide diversity were higher in southern rather than northern localities (*HD* = 0.68, π = 0.001). Neutrality tests resulted in negative values with non-significant outcome (Tajima's *D* = – 0.29, *P* = 0.46, Fu's *Fs* = – 0.49, *P* = 0.25). The

mismatch distributions for COI sequences produced a unimodal pattern. A low and non-significant raggedness value (r = 0.22 ± 0.11, P = 0.34; SSD = 0.07 ± 0.12, P = 0.35) in all 61 sequences indicates that the species might have experienced a sudden population expansion. The most appropriate GTR model with all proposed parameters was used for the construction of ML and BI trees. This was due to the suggested HKY+G model and has not been implemented in ML and BI approaches. The GTR model has been employed before in phylogenetic studies on *Artemia* populations (Muñoz et al., 2010; Eimanifar and Wink, 2013).

ML and BI trees were reconstructed based on 61 individuals from six different geographical localities. The trees had almost identical topologies with some minor differences which were not supported by significant bootstrap values (Fig. 27). Individuals from sites S1 to S3 and N1 to N3 were not clustered but randomly distributed, implying the lack of population structure and high connectivity among localities. The pattern obtained was confirmed by a haplotype-spanning network analysis in which geographically separated haplotypes could not be identified (Fig. 28). According to our results, *A. franciscana* from southern localities have more haplotypes than those of the hyper-saline northern sites (Table 31). As expected, a Mantel test of unbiased Nei's genetic and geographic distances showed an absence of significant patterns of isolation by distance (Z = 152648.3116, r = − 0.2680, one-sided *P* = 0.85).

Table 29 Construction of ISSR binary matrix for *A. franciscana* localities in Great Salt Lake.

S3-1	1 1 0 0 1 0 1 1 1 1 0 1 1 0 1 0 0 0 0 1 1 1 1 1 0 1 1 1 1 1 1 1 0 0 0 1 0 0 0 1 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 0 1 1 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 0 0 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 1 0 1 0 0 0 0 1 0 0 1 1 1 1 0 0 0 1 0 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 0 0
S3-2	1 1 0 0 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 0 0 1 0 1 1 0 1 1 1 1 0 1 0 1 0 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 1 1 0 0 0 1 0 0 1 0 0 0 0 0 1 1 1 1 0 0 1 1 1 1 0 0 0 0 0 1 0 1 1 1 1 0 0 1 1 1 1 0 0 0 1 0 0 0 0 1 0 0 1 1 1 1 1 1 1 1 0 0 1 1 1 0 1 0 1 0 0 0 0 1 1 0 1 1 1 1 0 0 1 1 1 0
S3-3	1 1 0 0 1 1 1 1 1 1 0 1 1 1 1 0 0 0 0 1 0 1 1 0 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 0 0 0 1 0 1 0 1 1 1 0 1 1 0 1 0 0 0 1 0 0 0 0 0 0 0 1 1 1 1 1 1 0 1 1 1 1 0 0 0 1 0 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 0 0 1 1 1 1 1 1 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 0 1 1 1 0
S3-4	1 1 0 0 1 0 1 1 1 1 0 1 1 1 1 0 0 0 0 1 0 0 1 1 1 1 0 1 0 1 1 1 1 0 0 0 1 1 0 1 0 0 0 0 1 0 0 0 1 0 1 0 1 1 1 0 0 0 1 1 0 1 1 1 1 0 0 1 1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 0 1 0 0 0 1 1 1 0 1 1 1 1 0 1 1 0 1 1 1 0 0 1 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 0 0 0 0 0 0 0 1 1 1 0 0 1 1 1 0
S3-5	1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 0 1 0 1 0 0 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 1 1 1 0 1 0 1 1 0 0 0 1 1 1 0 0 1 1 1 1 1 1 1 1 0 0 0 1 0 0 0 0 0 0 0 0 1 1 1 1 0 0 1 1 1 1 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 0 0 0 0 0 1 1 0 1 0 1 0 1 1 1 1 1 1 1 0 1 1 0 0 1 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 0
S3-6	1 1 0 0 1 0 1 1 1 1 0 1 1 1 0 0 0 0 0 1 1 1 1 1 0 1 1 1 1 1 1 1 1 0 0 0 1 0 1 1 1 1 0 1 1 0 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 1 0 0 1 1 1 0 0 1 1 1 0 0 1 1 1 1 1 1 1 0 0 0 1 0 1 0 0 0 0 0 1 0 1 1 1 1 0 1 1 1 1 1 1 0 1 0 0 0 1 1 1 1 0 1 1 1 0 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 0
S3-7	1 1 0 0 1 0 1 1 1 1 0 1 1 0 0 1 0 0 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 0 1 1 1 0 1 1 0 0 1 1 1 0 0 0 1 1 1 0 0 1 1 1 0 0 1 1 0 1 1 1 0 0 0 0 1 0 1 0 0 0 1 1 1 1 0 0 1 1 1 1 0 0 0 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 0 0 0 0 0 1 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 0 1 1 1 0
S3-8	1 1 0 0 1 0 1 1 1 1 0 1 1 0 1 0 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0 1 1 0 1 0 0 1 0 1 1 1 0 1 0 0 0 1 1 1 0 0 0 1 1 1 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 1 1 1 1 1 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 0 1 0 1 1 1 0 0 0 0 0 1 1 1 1 0 0 0 0 0 1 1 1 0 0 1 1 1 0 1 1 1 1 0 0 0 0 0 1 1 1 0 0 1 1 1 0 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 1 0 0 1 0
S3-9	1 1 0 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 1 0 1 0 0 0 0 0 1 0 0 1 1 1 0 1 0 0 1 0 1 1 1 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 0 1 0 0 0 1 1 0 0 0 1 1 1 0 1 1 1 1 1 1 0 0 0 1 0 0 1 1 0 0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1
S3-10	1 1 0 0 1 0 1 1 1 1 0 1 1 0 0 1 0 0 1 1 1 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 0 0 0 1 0 0 0 0 1 0 0 0 1 1 0 0 1 0 1 0 0 1 1 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 1 1 1 0 0 0 1 1 1 0 0 0 1 1 0 1 0 1 1 1 0 1 1 0 0 1 0 1 0 1 0 0 1 1 0 0 1 1 0 1 1 0 0 0 0 1 1 1 1 1 1 0 0 0 0 1 1 1 0 1 1 1 1 0 1 1 1 0
N3-1	1 0 0 0 0 0 0 1 1 1 1 0 1 1 0 0 0 0 1 0 1 0 1 1 1 1 0 1 1 1 0 1 1 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 1 0 1 0 0 1 1 1 0 0 0 0 0 0 1 1 1 1 0 1 0 1 1 1 0 0 0 1 1 1 1 0 0 0 0 1 1 1 1 0 1 0 1 1 1 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 1 1 0 1 0 1 1 1 1 0 1 1 1 1 1 0 1 0 0 1 0 0 1 1 1 1 1 1 0 0 0 0 1 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1
N3-2	0 0 1 1 0 0 0 0 0 1 1 0 1 1 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1 0 1 1 0 1 1 0 1 1 1 0 0 1 0 1 1 1 0 0 0 0 1 1 1 1 1 0 0 0 1 0 0 0 1 1 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 0 1 1 1 1 0 1 0 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 0
N3-3	1 1 0 0 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 0 1 1 0 0 1 0 1 0 1 1 0 0 0 0 1 1 0 0 1 1 0 0 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 1 0 1 0 0 0 0 0 0 1 0 1 1 1 0 1 1 0 1 0 1 0 1 0 0 0 0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 1 0 0 1 1 0 1 1 1 1 0
N3-4	1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 0 0 0 1 0 1 0 0 1 1 1 1 0 1 1 1 0 0 1 1 1 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 1 1 1 0 0 0 1 0 0 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 1 0 1 1 1 1 0 0 0 1 0 1 1 1 0 0 0 0 0 0 1 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 0 1 0 0 0
N3-5	1 1 0 0 0 0 1 1 1 1 0 1 1 0 0 1 0 1 0 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0 0 1 0 1 0 0 1 0 0 1 0 0 1 1 1 0 0 0 1 1 1 0 0 0 1 0 1 1 1 0 0 1 1 1 0 0 1 1 1 0 0 0 1 0 1 0 0 0 1 1 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 1 0 1 0 1 1 0 1 0 0 1 1 1 0 0 0 1 0 1 1 1 0 1 0 0 1 1 1 0 1 1 0 1 0 1 0 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 1 1 0 0
N3-6	1 1 0 1 0 1 1 1 1 1 0 1 1 0 0 1 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 0 0 1 1 1 1 0 1 1 0 0 1 1 0 1 0 0 1 0 1 0 1 0 0 0 0 0 1 1 0 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 1 1 1 0 1 0 1 1 0 0 0 0 1 1 1 0 1 0 0 1 0 1 0 1 1 1 0 0 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0 1 1 1 0
N3-7	1 1 0 0 1 1 1 1 1 1 0 1 1 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 0 0 1 0 1 0 0 1 1 1 0 1 0 0 1 1 0 0 0 1 1 0 1 0 1 1 0 0 1 0 1 1 0 1 1 1 1 1 1 0 0 0 0 1 0 1 1 1 1 0 1 0 1 0 1 0 0 1 1 1 1 0 1 0 1 0 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 1 0 0 1 0 0 1 1 1 0 0 0 0 1 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 0 0
N3-8	1 1 0 0 0 0 1 1 1 1 0 1 1 0 1 1 0 1 0 1 0 0 1 0 1 1 1 1 0 1 1 1 0 0 0 1 1 1 0 0 1 0 0 1 0 0 0 1 1 0 1 0 1 1 1 0 0 1 0 0 0 0 0 1 1 1 1 0 0 0 1 0 1 0 1 0 0 0 1 1 0 0 0 0 0 1 1 1 0 0 1 1 0 1 0 0 0 1 0 1 0 0 1 1 0 0 0 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 1 0 0 1 1 1 0
N3-9	1 1 0 0 1 1 1 1 1 1 0 1 1 0 0 0 0 0 0 1 0 0 1 0 1 1 1 1 0 1 1 1 0 1 1 1 0 1 0 0 1 0 0 1 0 0 1 1 1 0 0 0 1 1 1 0 1 0 0 0 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 1 0 0 0 0 1 1 1 0 0 1 0 0 1 1 0 1 0 0 0 1 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 0 0
N3-10	1 1 1 0 1 0 1 1 1 1 0 1 1 1 0 0 0 0 0 1 0 0 0 0 1 0 1 1 0 1 0 1 1 1 1 0 1 0 0 1 1 0 0 0 0 1 0 1 0 0 0 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 1 1 1 0 0 0 0 0 1 1 1 0 1 1 0 1 0 0 1 1 0 1 1 1 1 0 1 1 0 0 1 1 0 1 0 1 0 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 0 1 1 1 0
S1-1	1 1 0 0 1 1 1 1 1 1 0 1 1 1 1 0 0 0 0 1 0 1 0 1 1 1 1 1 1 1 1 1 1 0 0 0 1 0 0 1 0 1 1 0 1 0 0 0 1 1 0 1 1 0 1 1 0 0 0 0 1 0 0 1 1 0 1 1 1 0 0 0 0 0 0 0 0 1 1 1 0 1 0 0 1 1 0 1 1 1 0 0 0 0 1 1 0 1 0 1 1 0 1 1 1 0 1 0 0 0 0 1 1 0 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 0 1 1 0 1 1 1 1 0 0 0 1 1 0 0
S1-2	0 1 0 0 1 1 1 1 1 1 0 0 0 0 1 0 0 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 0 1 0 1 0 1 1 1 0 1 0 0 1 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 0 0 0 0 0 1 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 1 0 1 0 1 1 0 1 1 0 0 1 0 1 1 1 1 0 0 0 0 0 1 1 1 0 1 0 0 0 1 1 1 1 0 0 0 0 1 1 1 0 1 1 1 0 0 1 0 0 0 0 1 1 1 0 0 0 0
S1-3	0 1 1 0 1 0 1 1 1 1 0 1 0 0 0 1 1 0 0 0 1 0 0 1 1 0 0 1 0 1 1 1 1 1 0 0 0 1 0 1 1 0 1 1 1 0 0 1 0 1 0 1 1 0 1 1 1 0 0 0 0 0 0 1 1 1 0 0 0 1 0 0 1 0 1 0 1 1 0 1 1 1 0 0 0 0 0 0 0 1 1 1 0 0 0 1 0 1 1 1 0 0 0 0 1 0 1 1 1 0 1 0 0 1 0 1 0 1 1 0 1 1 1 0 1 0 0 0 1 1 1 0 0 1 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 1 0 0
S1-4	1 0 0 1 1 0 0 1 0 1 0 1 0 0 1 1 1 0 0 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 1 1 0 0 0 1 1 1 0 0 0 1 0 1 1 0 0 0 1 1 1 0 0 0 1 0 1 1 0 0 0 1 1 1 0 0 0 0 1 0 0 0 0 1 1 1 1 0 0 1 1 1 0 0 0 0 0 1 1 1 0 1 1 0 1 1 0 0 0 1 0 1 0 0 0 0 1 0 0 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 0 0 0 0 1 0 0 0 0 1 1 0 0 1 1 0 0

S1-5 1000110101111111010101111011111100000000000100000000000101000001100011001100011110001110110010101101101101010011001001001110100111100000000111101111 0

S1-6 1000111111011001100100011111011100110010111101011000011101100110000010000000000110001110000000000110111110001000000001100111010111110111011111111011110

S1-7 1100111111101100110010000111101111111110010110101100011100000000110001000000000111100011100000000011011011000101000100010010101111111010010111110011111

S1-8 1100111111101100001011100111010110011001011011000100011100110011000010000000011110001010000110101101101001010001000001001100011111100000100111110110 0

S1-9 1100111111100100101000110111101110111001010010001100011100110001100010001101011110001110000011010110110110001011100100100101010011110110101111100110 0

S1-10 1100011110001000010000101111001101110000100100111001111000010000010100010010001100011100000010101101101110111111000000100111000111110000100111101111 0

S2-1 1100100110101101100100111111101111001000010010000110001100010001000010010000000110001111000110110011111100100010000001100111010011100000101001100111 0

S2-2 1100001111111100000100111001011110010000100101010101111100001110111100010100111101011100001000111011010010000110010110010100111111000001001111001110 0

S2-3 1100100111110110110010011111110111000100001101000010001010000000100111100000001111000111100001011111110100100001100000100111011111110100101111100111 0

S2-4 1000100111110110110010101111111011110010110000100110010001011100011000110000000111100011110001101001111010000001100110010011001001110000001011111011110

S2-5 1000101111101101100000100111111111100100001001000000000011001000100100100000001111100011110000101011101010000001000000110011001111111000001000011000000

S2-6 0000111111101101000010100111101100001010110010011010010100001001101100010001111110001100000010101101111110101000000001100110000111100000000111110001 11

S2-7 0000111111101101000010101111101100001000011010001100111011111000110111100000000011110001110000100001101001101010111010001001100111100000000111111110111 0

S2-8 1000111111101100110001100111111111000111001011010010000011000000100100000000001111000111000010100110110110101001100000100101000011100000011011110100 00

S2-9 1110111111011111000100111111111110001001011110011100100100110000001111000000101110001110000100011101001101010100000001001110011111100000110111110111 0

S2-10 1100101111111010010101101111111100110000110101000000001001100000001010000000001110001110000001011110110000000101110000010011000111110000001101111101110

N2-1 1100101111101110000010000111101110001011011100111000010100110000001111011100001110001110000011011110110100000011100000100111000011110010110111100111 0

N2-2 0001111111111101100010000111111111001100001001011100001011010010000110100110001111000111000001011110110010100001100100100101110011110000110111100111 0

N2-3 0100111111101100110010100111111111000100101001001100000101001100000001000000110001111000100000011111111011000000101110010110010111001110000011011110011 0

N2-4 1100101111101101110010101111111111000101110100100101000101111000000000100100000111100011100001101100010010000000000000110011101111010000011011110111 00

N2-5 0100101011011001010000100001011110010000000101010000101001010011010100000010111100011100000101111011111100000000000000100100000101110000110111110 1 0

N2-6 1100111111101100110010110111101110001001011010011010110110011000001010000000011110001110000111101101001001010100000001001000100111000001111111011110

N2-7 0000001010011001100101101111011100110111110101011100111000100010000100011010111100011100000111100010010000101110010010010101001111000011111111000010

N2-8 0100111111101101110010101111111111000101110110101011001101000000000000100011010111100011100001000111011010000101000010010010001111111000010001110011111

N2-9 1100111111101101101010011000111111001100101001110110000010000000110101110000101111100011100000101111011110000000000011001001100000011110000000000111111110

N2-10 1010011111100100110010111111110011101110101111010000101010001000110101000000001111000111000011011110101100001000101001100000000011100001110111111100 10

N1-1 1 0 1 1 0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 0 0 0 1 1 1 1 1 0 1 1 1 0 0 0 1 0 1 1 0 1 0 0 1 0 1 0 0 1 0 0 0 0 0 1 0 0 0 1 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 0 0 0 1 0 0 1 1 0 0 0 1 0 1 0 0 0 1 0 0 1 1 0 0 1 0 0 0 1 0 0 1 1 1 0 0 0 0 0 0 0 1 1 0 1 1 0 1 0 1 0 0

N1-2 1 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 1 1 0 1 0 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 1 1 1 1 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 0 1 0 0 0 1 1 0 0 0 1 0 0

N1-3 1 1 0 1 1 1 1 1 1 1 0 1 0 0 0 1 0 0 0 1 0 0 1 0 0 0 0 1 0 1 1 1 0 0 0 1 0 0 0 0 1 0 0 1 1 0 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 1 0 0 0 1 1 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 1 1 0 1 0 0 1 0 1 0 0 0 1 0 0 0 0 1 1 1 0 1 0 1 0 0 1 1 1 1 1 1 0 0 0 0 1 1 0 1 1 1 1 0 0 1 1 1 0

N1-4 0 1 0 0 1 0 1 1 1 1 1 1 1 0 0 1 1 0 0 1 0 0 0 0 1 1 1 1 0 1 1 1 1 1 0 1 0 0 0 0 1 1 0 1 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 1 1 0 1 1 0 1 1 0 0 0 0 0 1 0 1 0 0 0 0 0 1 0 0 1 0 1 0 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 1 1 1 0

N1-5 1 0 1 1 0 1 1 1 1 1 0 1 1 0 1 0 0 0 0 1 0 0 1 1 1 1 1 1 0 1 1 1 0 1 0 1 0 0 1 0 1 1 0 1 1 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 1 0 0 0 1 1 0 0 0 1 1 1 1 0 1 0 1 1 1 0 0 0 0 0 0 0 0 0 1 1 0 1 1 1 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 1 0 0 1 0 0 1 0 0 0 0 1 1 1 0 0 0 0 1 1 1 1 1 1 1 0 0 1 1 1 0

N1-6 1 1 0 0 1 1 1 1 1 1 0 1 1 1 1 1 1 0 0 1 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 0 0 0 0 1 1 0 1 1 0 1 1 1 0 0 0 0 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 1 1 0 1 0 1 1 1 1 0 1 1 1 1 0 0 0 0 1 1 1 0 0 0 0 1 1 0 0 0 1 1 0 0 1 0 0 0 0 1 1 1 1 1 1 0 0 0 0 1 1 1 0 0 1 1 1 1 1 1 0

N1-7 1 0 1 1 0 1 0 1 1 1 0 1 1 0 0 1 0 1 0 1 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0 0 0 1 0 1 1 1 0 1 1 0 1 1 0 1 0 0 0 0 0 0 1 1 0 0 0 1 0 0 1 0 0 0 0 0 1 1 0 1 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 0 1 0 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 1 1 0 0 1 1 1 0 0 0 0 0 1 1 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 1 1 1 0

N1-8 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 0 0 0 1 0 0 1 0 0 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 0 1 0 0 1 1 0 1 1 0 1 0 0 1 0 1 0 0 0 1 0 0 1 0 0 1 0 0 1 1 0 0 1 0 1 1 1 1 1 0 0 0 0 0 0 0 1 1 1 1 1 0 0 1 1 0

N1-9 1 0 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 0 1 1 0 0 1 1 0 0 1 0 1 0 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 1 0 0 0 0 1 1 0 1 0 0 0 1 1 0 1 0 1 1 0 1 0 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 1 1 0 1 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 1 1 1 0

N1-10 1 0 0 0 0 0 1 1 1 1 1 1 1 1 0 0 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 0 1 1 0 0 0 1 0 0 1 1 0 1 0 1 0 0 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 1 1 0 1 1 1 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1 0 0 1 1 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 0 0 0 0

ISSR profiling

Artemia exhibits a substantial amount of genetic diversity through the accumulation of a high number of repetitive STR sequences within its entire genome (Hundsdoerfer et al., 2005; Eimanifar and Wink, 2013). Due to this, ISSR-profiles can differ between individuals. All primers generated a total of 147 reproducible and distinguishable ISSR bands, of which 143 were polymorphic (98%). The highest and lowest levels of genetic variability were observed in S3, N3 (S3: NPL = 107, i.e. 73%, *Hj* = 0.34 ± 0.01 & N3: NPL = 119, i.e. 81%, *Hj* = 0.34 ± 0.01) and N1 (NPL = 99, i.e. 67%, *Hj* = 0.27 ± 0.01). There is a slight trend toward higher genetic diversity by increasing F_{IS}. Assuming a slight departure from HWE, all genetic diversity indexes were calculated based on the calculated inbreeding coefficient level (F_{IS} = 0.31). The intraspecific genetic variability for six localities of *A. franciscana* was estimated to be from 0.27 to 0.34, with a mean of 0.33 ± 0.02. The overall level of gene diversity indicates a significant level of genetic variability among all prospected localities (Table 32).

Six localities of *A. franciscana* were classified into two distinct geographical regions, north and south. The greater number of genetic polymorphisms was observed in southern localities (*PPL* = 73.3%, *Ht* (total gene diversity) = 0.32. An overall Nei's gene differentiation (G_{ST}) and a fixation index (F_{ST}) were calculated to be 0.15 and 0.05, indicating that nearly 92% of the genetic variation occurred within *A. franciscana* population (Table 33).

The relationships among 60 individuals were subjected to a cluster analysis based on Jaccard similarity coefficient (J) and the UPGMA algorithm. The UPGMA analysis clustered all individuals into five major groups, whose distribution of individuals was consistent with the *COI* phylogeny tree. The Mantel test for ISSR data did not reveal any significant correlation between genetic and geographic distances (r = 0.618, *P* = 1). The result of Principle Coordinate Analysis (PCA) scatterplot was in accordance with UPGMA clustering, indicating a random distribution of *A. franciscana* individuals in the Great Salt Lake.

ISSR profiles

The total genetic variability with an admixture ancestry model without any prior information was further analyzed, based on Bayesian clustering analysis using the STRUCTURE program. The best fit number of *K* was inferred based on the maximum statistic of *DK* when using 5 ISSR loci (*DK* = 16.60568). The optimum *K* was obtained at *K* = 3 and defined by three different colors, which reveals a relatively greater variability of maximum likelihood among different tested *K.* In the bar plot, each individual belonged to a single vertical line represented by one of the three different segments. The genetic composition of individuals per locality was assigned to several segments, depending on the genetic similarities of individuals. The spatial population structure of all individuals did not

correspond to geographic divisions, indicating a random genetic exchange among individuals across geographical localities in the lake (Fig. 25).

Morphological variation

The mean diameter value of non-decapsulated cysts showed the largest and smallest size in S2 (233.06 ± 9.83 µm) and N3 (216.17 ± 9.15 µm). The largest measures in decapsulated cysts were seen in S1 and S2 (217.58 ± 11.93 & 217.65 ± 10.90 µm) and smallest in S3 (210.81 ± 10.44 µm). All cyst diameters for each locality, as well as chorion thickness, are documented in Table 34.

Our results suggest that all 3 cyst parameters of *A. franciscana* exhibit sound variation between 6 geographical regions, but chorion thickness plays a considerable role in the differentiation of individuals from each locality. Chorion thickness in *A. franciscana* varied between 1.07 to 8.65 µm, showing variability among different cysts examined here. Hierarchical analysis clustered all individuals into two main groups, whose distribution showed no distinct geographical pattern, which was supported also by PCA analysis (Fig. 29).

Table 30 Data matrix of polymorphic sites among 61 *COI* sequences, number of unique haplotypes, and their frequencies. H indicates haplotype and F haplotype frequencies.

H	F	Individuals & locations
H1	37	S1-1 S1-3 S1-4 S1-5 S1-6 S1-8 N1-1 N1-2 N1-3 N1-5 N1-6 N1-7 N1-8 N1-9 N1-10 N2-2 N2-3 N2-4 N2-7 N2-8 N2-9 N2-10 S3-2 S3-5 S3-9 S3-10 N3-1 N3-3 N3-4 N3-6 N3-7 N3-8 N3-9 S2-1 S2-4 S2-9 S2-11
H2	16	S1-2 S1-7 S1-9 S1-10 N1-4 N2-6 S3-1 S3-7 S3-8 N3-2 N3-5 N3-10 S2-2 S2-3 S2-8 S2-10
H3	1	N2-1
H4	1	N2-5
H5	1	S3-3
H6	1	S3-4
H7	1	S3-6
H8	1	S2-5
H9	1	S2-6
H10	1	S2-7
H11	1	*A. persimilis* (outgroup)

Table 31 Summary of genetic diversity indices for *A. franciscana* from six geographical regions of GSL.

Regions	**N***	***V***	***M***	***H***	***HD***	***π***	***K***
N1	10	1	1	2	0.2 ± 0.15	0.0003 ± 0.0005	0.2
N2	10	4	4	5	0.66 ± 0.16	0.001 ± 0.001	0.95
N3	10	1	1	2	0.46 ± 0.13	0.0007 ± 0.0005	0.46
S1	10	1	1	2	0.53 ± 0.09	0.0008 ± 0.0005	0.53
S2	11	5	5	5	0.78 ± 0.09	0.002 ± 0.001	1.27
S3	10	7	7	7	0.86 ± 0.10	0.003 ± 0.002	1.88
Total	61	11	11	11	0.6 ± 0.05	0.001 ± 0.001	0.76

N: number of individuals, ***V***: number of polymorphic sites, ***M***: number of nucleotide substitutions, ***H***: number of haplotypes, ***HD***: haplotype diversity, ***π***: nucleotide diversity and ***K***: average number of nucleotide differences per locality.

Table 32 Summary of genetic variation statistics for all loci of ISSR among six geographical locations of *A. franciscana* in GSL.

Regions	**N**	**(*Hj*) ± SD**	***I* (mean ± SD)**	**NPL**	***PPL***
N1	10	0.27 ± 0.01	0.34 ± 0.26	99	67
N2	10	0.3 ± 0.01	0.36 ± 0.25	106	72
N3	10	0.34 ± 0.01	0.4 ± 0.23	119	81
S1	10	0.32 ± 0.01	0.38 ± 0.25	109	75
S2	10	0.31 ± 0.01	0.38 ± 0.27	106	72
S3	10	0.34 ± 0.01	0.38 ± 0.26	107	73

***Hj*,** Nei's gene diversity; ***I*,** Shannon's information index; **NPL,** Number of polymorphic loci; ***PPL*,** percentage of polymorphic loci.

Table 33 Overall genetic variability across all the 60 *A. franciscana* individuals based on ISSR analysis.

Marker	*I*	*Ht*	NPL	*PPL*	G_{ST}	F_{ST}	Nm
ISSR	0.45 ± 0.19	0.33 ± 0.02	143	98	0.15	0.05	2.8

I, Shannon's information index; ***Ht,*** total genetic diversity; **NPL,** no. of polymorphic loci; ***PPL,*** percentage of polymorphic loci; $\mathbf{G_{ST}}$**,** gene differentiation; $\mathbf{F_{ST}}$**,** Wright inbreeding coefficient; **Nm,** gene flow

Table 34 Mean ± SD of the cyst diameter, the decapsulated cyst diameter, and the chorion thickness for *A. franciscana* from six different geographical regions.

Regions	Untreated Cyst (µm) (Mean ± SD)	Decapsulated Cyst (µm) (Mean ± SD)	Chorion Thickness (µm)
N1	230.99 ± 11.32 a	213.69 ± 11.16 ab	8.65
N2	231.92 ± 11.80 a	216.32 ± 10.71 a	7.8
N3	216.17 ± 9.15 b	214.03 ± 10.01 ab	1.07
S1	232.71 ± 11.18 a	217.58 ± 11.93 a	7.56
S2	233.06 ± 9.83 a	217.65 ± 10.90 a	7.7
S3	218.30 ± 13.12 b	210.81 ± 10.44 b	3.74

Note: the same alphabetical indicates non-significant values.

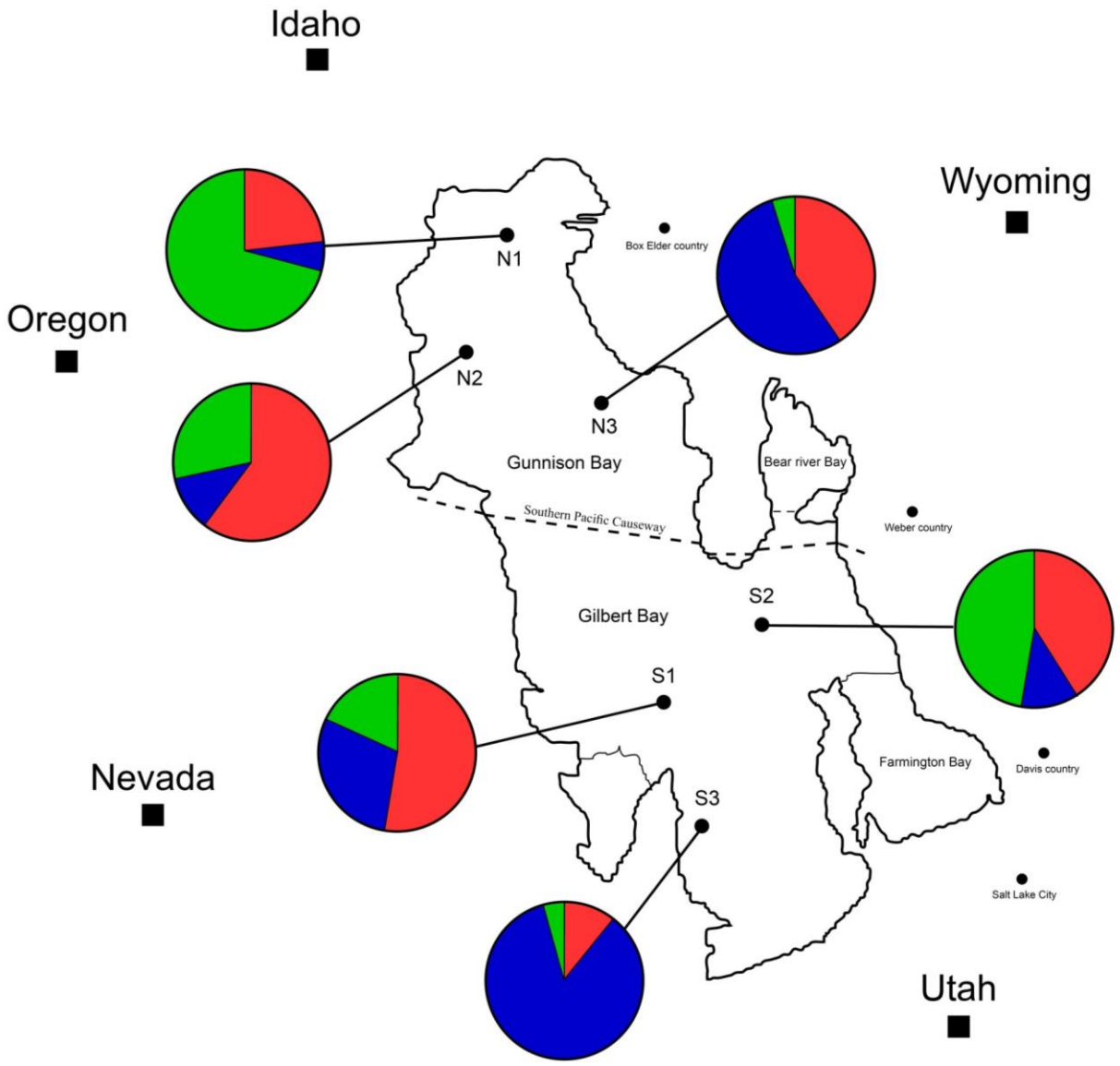

Fig. 25. ISSR fingerprinting schematic representation of sampling localities and proportion of genetic clusters for each locality, generated by Bayesian analysis of *A. franciscana* (STRUCTURE, *K* = 3). 1–3: distinct genetic clusters.

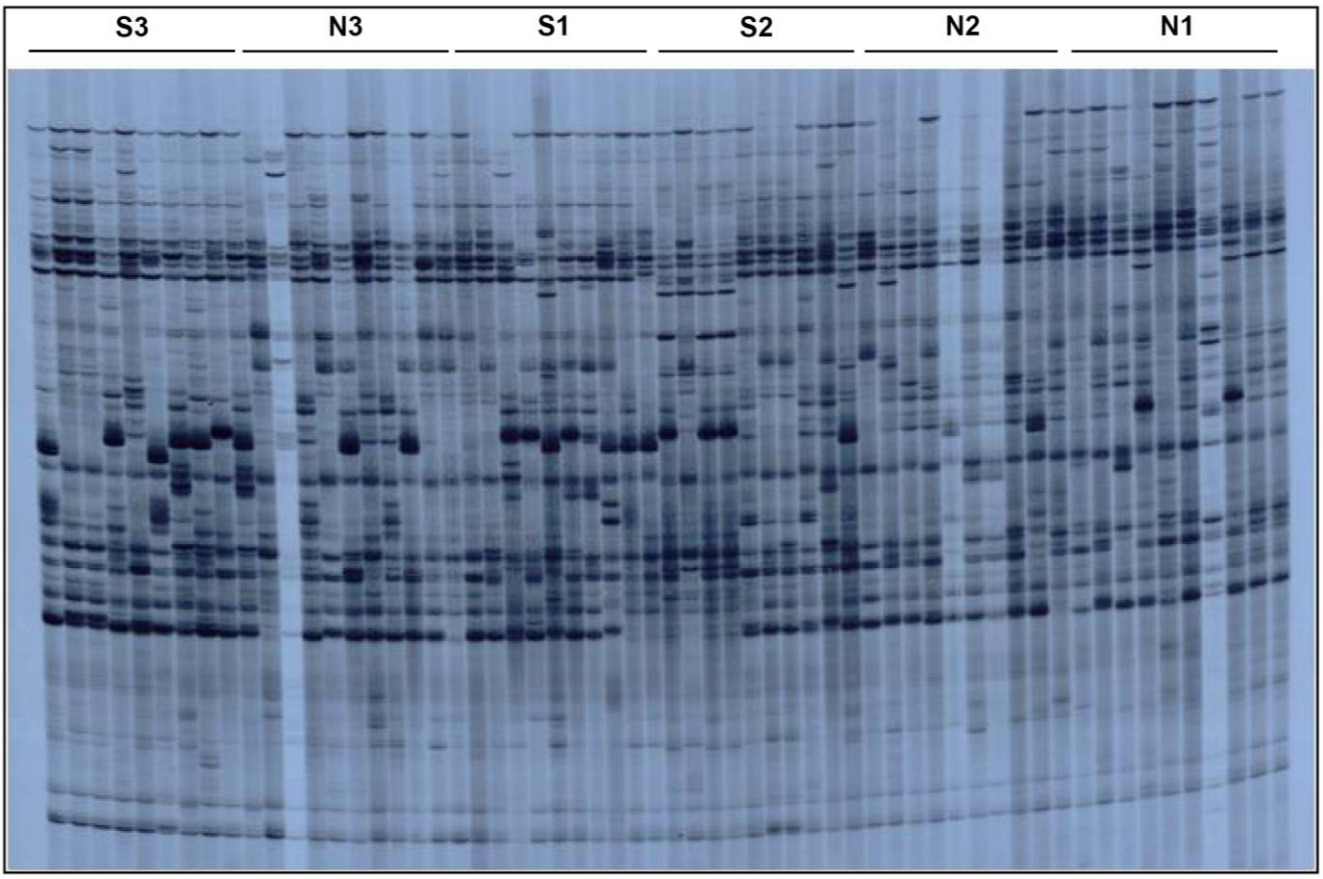

Fig. 26. A representative of ISSR gel for *A. franciscana* populations using $(AG)_8C$. Southern geographical localities are shown by S1, S2 and S3. Northern geographical localities are shown by N1, N2 and N3.

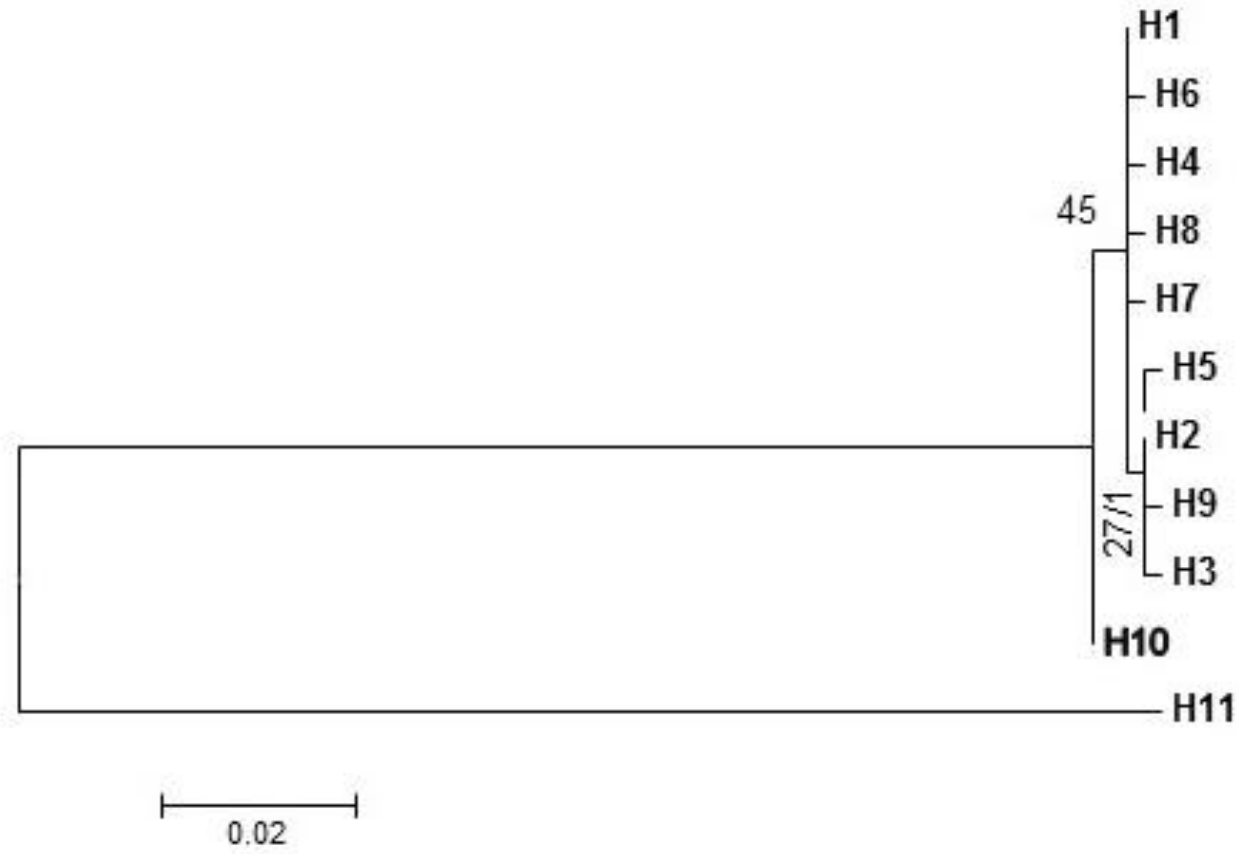

Fig. 27. Molecular phylogeny of *A. franciscana* based on the *COI* gene, generated by maximum likelihood using MEGA6 program. Major nodes are supported by bootstrap and posterior probabilities values from left to right. H1–H10 = distinct haplotypes.

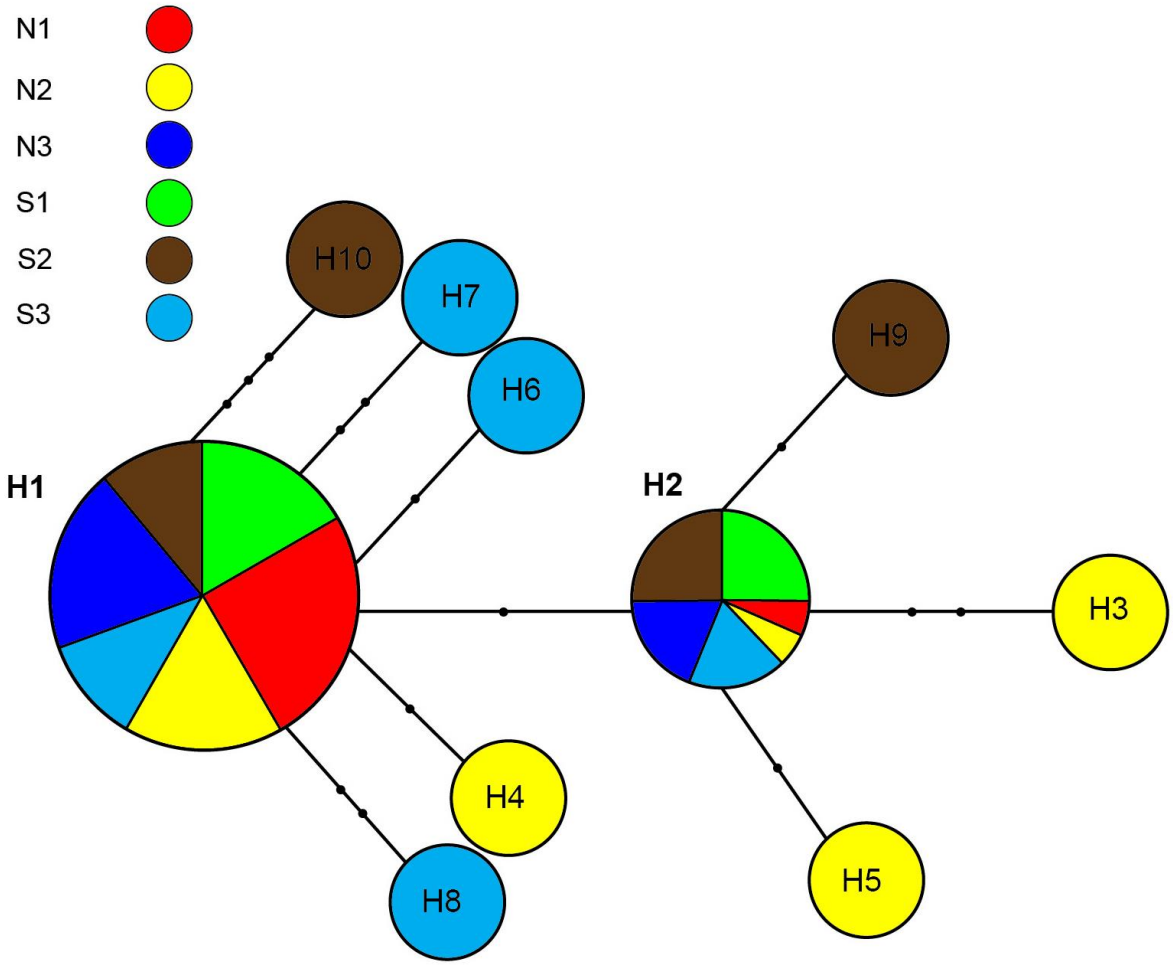

Fig. 28. Construction of median joining network for 10 *COI* haplotypes of *A. franciscana*. From GSL. Each circle denotes a haplotype, and the proportions of each circle correspond to haplotype frequencies (the smaller circles are based on a single sample). Small black dots represent the number of nucleotide substitutions between haplotypes. Geographical localities of *A. franciscana* in Great Salt Lake are illustrated by different colors.

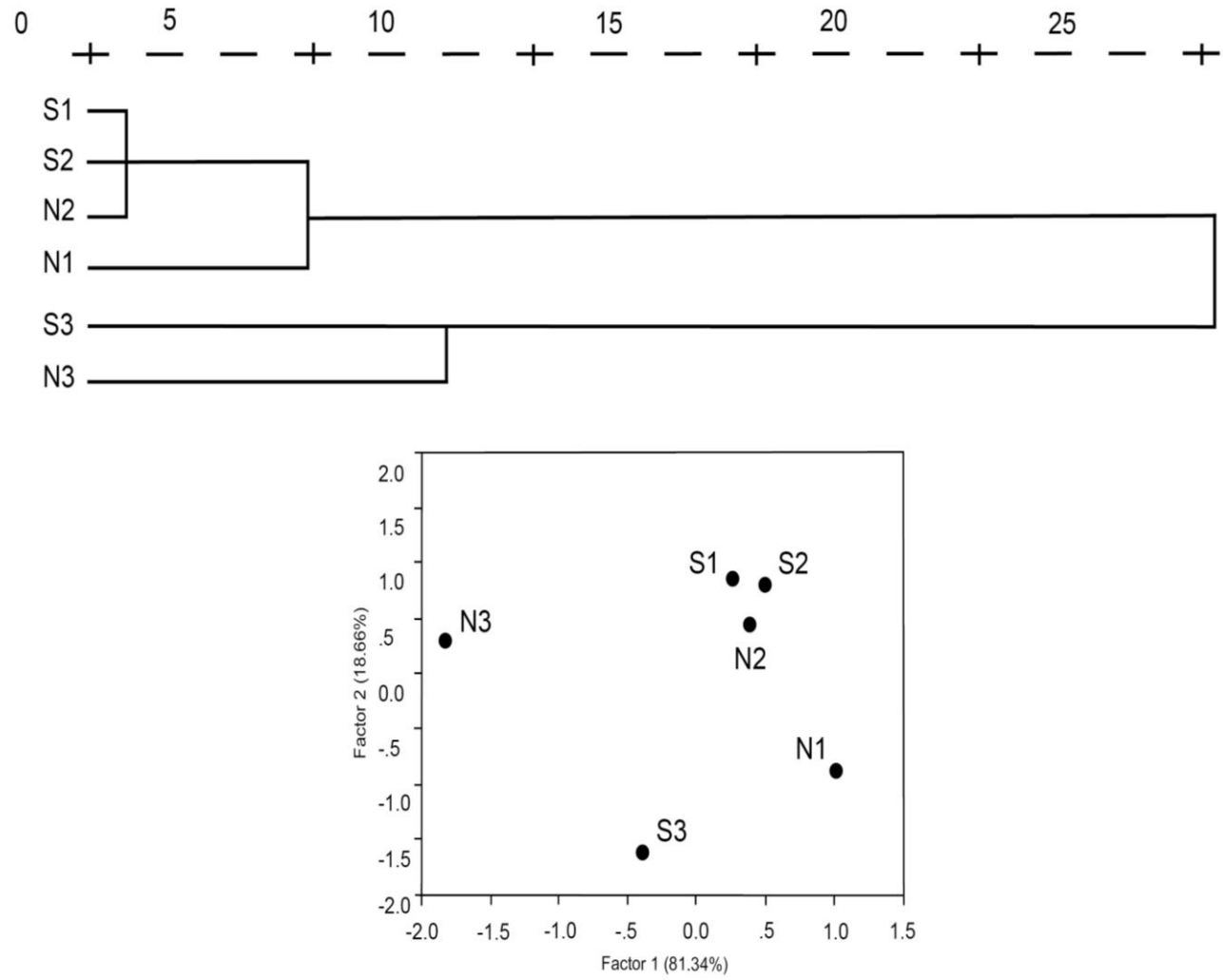

Fig. 29. Principal Component Analysis (PCA) of three morphological traits (N = 1800).

3.4.5. Discussion

Genetic diversity and population structure

The Great Salt Lake is the home of a commercially important species of *A. franciscana* in North America. In spite of its broad utilization, the genetic diversity and population structure have not been studied in detail. In the present study, *A. franciscana* showed an overall high genetic diversity (*COI*, ISSR data), particularly in southern regions of GSL, but a lower diversity in the hyper-saline and isolated northern part. Estimates of genetic differentiation for both mitochondrial DNA and ISSR did not show genetic structure among localities, even though a mean significant F_{ST} value was detected for ISSR loci. Considering the morphological data of the cyst size, again no common relationship between localities could be detected. These results support the hypothesis that *A. franciscana* of GSL still constitutes a single panmictic population, followed by a substantial amount of gene flow (Eimanifar and Wink, 2013). The GSL has been receiving inflow from several freshwater rivers from the Southern and Eastern regions, which presumably provides favorable conditions for the species to construct a relative abundance of numerous singleton haplotypes (Post, 1977; Eimanifar et al., 2006; Eimanifar and Wink, 2013), although the lake has experienced hydrological changes of water level in both regions, which basically limits the connectivity between both areas. Another crucial factor affecting the population diversity could be the hyper-saline conditions in the northern sites (Arnow and Stephens, 1990; Larson and Belovsky. 2013). Salt compositions and concentrations create different ecological conditions in saline lakes (Hontoria and Amat, 1992). Although the GSL shows significant differences in salinity between its northern and southern regions (Larson and Belovsky. 2013), our results did not reveal corresponding genetic clusters. A similar situation was observed among *A. urmiana* from Lake Urmia, implying that no strong ecological differentiation exists in the GSL (Eimanifar and Wink, 2013), or that these ecological happenstances are too recent to result in any genetic differentiation.

The high genetic diversity of *A. franicscana* could be attributed to sexual behavior and large population size in the GSL. A similar and higher level of haplotype diversity has been observed in *A. salina* (*HD* = 0.58), *A. urmiana* (*HD* = 0.87), and the decapod crustacean *Aristeus antennatus* (*H* = 0.8 – 0.9) (Muñoz et al., 2008; Maggio et al., 2009; Eimanifar and Wink, 2013). Interestingly, ISSR profiles were more complex in *A. franciscana* than in *A. urmiana*, indicating the presence of a high number of microsatellite sequences in the genome of *A. franciscana*.

It is noteworthy that differences in genetic diversity between *A. franciscana* from USA and *A. urmiana* from Iran might be correlated to habitat heterogeneities, geographical ranges, and the hydrological regimes of both lakes (Post, 1977; Eimanifar and Mohebbi, 2007). *A.*

franciscana is capable of maintaining a high genetic diversity by adapting to unfavorable environmental conditions such as high salinity ranges (Parmesan, 2006; Jump et al., 2009; Takahashi and Katano, 2010).

A. franciscana populations showed a significant negative value of neutrality with sound unimodal distribution, signifying a recent population expansion in the lake. The star-linked haplotype network and mismatch distribution parameters (SSD, raggedness index) also indicate a process of expansion, which has also been detected in *A. urmiana* in Lake Urmia (Eimanifar and Wink, 2013).

Zooplankters such as *Artemia* are easily dispersed via migratory waterfowl, wind (cysts), hydrological connectivity and anthropogenic influences (intentional and non-intentional introductions into new areas). Coupled with the absence of physical barriers across the lake, these mechanisms could facilitate extensive gene exchange among *A. franciscana* populations throughout the lake (Cowen et al., 2000; Green et al., 2005; Maniatsi et al., 2009). Similarly, our results, based on genetic and morphological evidence, revealed the lack of genetic structure, emphasizing a panmixia of *A. franciscana* in GSL, similar to the situation of *A. urmiana* in Lake Urmia. Panmixia has been observed among others in the Antarctic krill *Euphausia vallentini*, the crustacean *Aristeus antennatus* and a gill parasite *Gotocotyla sawara* (Chiang et al., 2006; Harkins et al., 2013; Shi et al., 2014).

Morphological variation

All three cyst traits revealed a smaller cyst size of *A. franciscana* in comparison to *A. urmiana*, which suggest its applicability in the aquaculture industry. In *A. urmiana*, most differentiation of the population was related to the diameter of decapsulated cysts and the chorion thickness (Bruggemann et al., 1980). *A. urmiana* showed that the widest variation of chorion thickness ranged between 1.2 – 9.3 µm (Abatzopoulos et al., 2006).

Although biometry differences were noticed between individuals of *A. franciscana* from GSL, they were not strong enough to suggest disjunctive populations. Instead, influences of physico-chemical parameters and food availability are the most plausible reasons to explain the differences in cyst sizes (Abatzopoulos et al., 2006). Once again, the morphological clustering pattern of *A. franciscana* is consistent with the pattern observed for *A. urmiana*, indicating a random distribution of morphotypes across the lake (Asem et al., 2007).

Conservation implications

The genetic and morphological data of *A. franciscana* from the GSL suggests the presence of a panmictic population with no apparent geographic differentiation. Therefore, *A. franciscana* from the GSL can be regarded as a single management unit for conservation.

4 General discussion and conclusions

4.1. Asian *Artemia* – species complex pattern

IN THIS THESIS, new information is given on the biogeographic history, population structure and genetic diversity of *Artemia* lineages from a wide range of geographical localities across Eurasia and America. Moreover, this thesis provides valuable information about the distribution pattern of *Artemia* lineages from unexplored regions throughout Asia.

Asia is a geographically diverse continent and has enriched hydrochemical and geological resources (Zheng, 2002). Phylogenetic studies on Asian *Artemia* have hardly been carried out. Our extensive phylogeny and phylogeographic studies have shown that Asian *Artemia* clustered into several genetically distinct clades, whose species distributions were inconsistent with each other. *A. tibetiana* shows a difference in genetic structure depending on the molecular markers. The distribution of *A. tibetiana* has been restricted to the Qinghai-Tibet Plateau due to this region having a relatively young geological age with a very diverse chemical and geological composition. In this study, putative parthenogenetic populations are nominated as “Eurasian Haplotype complexes” (EHC) according to the topology in their phylogenetic trees. The “EHC” term has not previously been used at all becuase (Baxevanis et al., 2006; Muñoz et al., 2010; Maccari et al., 2013) the reproductive mode of *Artemia* lineages has been has been established by rearing them under standard laboratory conditions. In the current study, we have used cysts as the material for all genetic analyses and we did not identify whether they were sexual or parthenogenetic (asexual). As a consequence, we have introduced the term Eurasian Haplotype Complex (EHC) to describe a group of populations sharing the same basic haplotype.

An analysis of *COI* haplotype revealed a species complex for *A. urmiana*. This species showed a reduced range of endemicity by sharing several haplotypes belonging to EHC lineages in Eurasia. A significant genetic diversity has been observed among sexual species consistent with our expectations based on their life history in Asia. EHC lineages revealed a low level of genetic diversity with the exception of European EHC. The highest level of genetic diversity observed among European EHC lineages is mostly due to the presence of rare males which has already been explained by Maccari et al. (2013).

The dispersion pattern of *A. urmiana* is barely recognizable since there are several hypersaline lakes and lagoons in Iran which have not been colonized by *A. urmiana*. In addition, several localities in Iraq (Maknoon, 2001; Salman et al., 2012), Ukraine (Shadrin and Batogova, 2008; Shadrin et al., 2012) and Russia (Shadrin and Anufriieva, 2012) have been colonized by *A. urmiana*, but their biosystematic and phylogenetic status has not yet been fully explored.

A. urmiana has been an endemic species in Urmia Lake over the years (Eimanifar and Mohebbi, 2007; Asem et al., 2007). In 2008, a new *Artemia* population from Koyashskoye Lake, Ukraine was attributed to *A. urmiana* (Shadrin et al., 2008) and its taxonomical status was promptly identified as *A. urmiana* by Abatzopoulos et al., (2009). Consequently, *A. urmiana* was not considered as an endemic species to Urmia Lake. Anufriieva and Shadrin, (2012) even suggested that a possible biogeographical origin of *A. urmiana* is the Miocene salt lakes, of which natural outcrops are found in Crimea. Migration of waterbirds has been proposed as an active mechanism for the scattering of *A. urmiana* cysts in the new habitat, Koyashskoye Lake (Khomenko and Shadrin, 2009; Shadrin et al., 2012). The absence of *A. urmiana* in other Crimean lakes puts into question the passive transport of *A. urmiana* by migratory waterbirds (Abatzopoulos et al., 2009). Abatzopoulos et al. (2009) suggests *A. urmiana* cysts from Urmia Lake could have been transferred via a preferential human trade route (salt trade). In this regard, both suggested mechanisms could be acceptable for the distribution of *A. urmiana* to its non-indigenous regions.

4.2. Evolutionary history between EHC lineages and their sexual ancestors

EHC lineages showed a broad geographical expansion in inland localities across Asia. EHC lineages were genetically similar to the two sexual species *A. urmiana* and *A. tibetiana*, indicating multiple evolutionary origins from two Asian sexual ancestors (Muñoz et al., 2010; Maccari et al., 2013).

The close phylogenetic relationship of EHC with Asian sexual species has been described by Beardmore and Abreu-Grobois, 1983; Abatzopoulos et al., 1997; Triantaphyllidis, 1997; Baxevanis et al., 2006; Maccari et al., 2013. Historical demography and the evolutionary age of Asian *Artemia* lineages indicate that *A. sinica* has diverged from other sexual species around Miocene times. Demographic analyses indicate that EHC lineages have undergone a recent evolutionary range expansion which might occur in the Holocene. The evolutionary age of EHC lineages has been estimated to be at least 3 Mya, revealing the possible divergence of EHC from its Asian sexual ancestor, (mostly *A. urmiana*), approximately 5.5 Mya (Beardmore and Abreu-Grobois, 1983).

The presence of numerous small salt water ecosystems associated with groundwater seepages, local springs and the topographic variations in delta environments has maintained the survival of *A. urmiana* populations during the Pleistocene. Such ecological conditions were most probably not found in Black Sea regions due to more severe climatic conditions in higher latitudes. Lithological and palynological evidence implies that Urmia Lake has had a considerable level of water fluctuations during the Pleistocene and Holocene (Kelts and Shahrabi, 1986; Djamali et al., 2008).

4.3. *A. urmiana* – a major sexual species in Urmia Lake, Iran

Urmia Lake is one of the largest oligotrophic lakes located in Northwestern Iran (Asem et al., 2014). It has a unique biodiversity including numerous valuable species which are living within and nearby regions of the lake (Asem et al., 2014). It has been suggested that saline lakes have a relatively fragile ecosystem undergoing noticeable environmental changes. In the case of Urmia Lake, a significant volume of water has been lost due to climatological and anthropogenic alterations. The recent desiccation of the lake is much more evident when compared to its previous shoreline (Manaffar, 2012). Under the current condition of the lake, the probability of species extinction is rather high, so more careful protection policies are needed in order to avoid the extinction of highly adapted crustacean species such as *Artemia urmiana*.

A high level of genetic variation has been observed within *A. urmiana* individuals all over the lake. Most of the genetic variations have been observed in Southern localities of the lake which is consistent with the previous findings carried out by Eimanifar et al. (2006). *A. urmiana* populations are genetically homogenous: no significant genetic structure was observed using different technical approaches such as mtDNA-*COI* sequencing, genome fingerprints and biometry.

The lake is vulnerable to environmental changes, possibly leading to the formation of newly diverse haplotypes in the lake. All individuals are potential contributors in the lake and could be able to freely transfer into adjacent localities via hydrological connectivity. Therefore, conservation management guidelines are urgently required in order to preserve the biological integrity of *A. urmiana* in Urmia Lake.

4.4. *A. franciscana*: a threat for local *Artemia* biodiversity

Artemia has been used as a model organism for studying invasion biology since the timing of the first introduction of an exotic American species *A. franciscana* into non-indigenous localities in the 1980s (Green et al., 2005). In Asia, local *Artemia* have been threatened by introducing *A. franciscana* from multiple sources (the Americas and Europe). This phenomenon indicates the great impact human activities have had on the dispersal of *Artemia* across the world (Amat et al., 2005; Van Stappen, 2008). The establishment of allochthonous populations in the non-indigenous regions has emerged as an economic opportunity rather than a threat to biodiversity (Van Stappen, 2008). This is due to the economic importance of *Artemia* as a major live food for commercially valuable aquatic species (Sorgeloos et al., 2001).

The present findings indicate that *A. franciscana* has been introduced into Southern and Eastern regions of Asia from two commercial sources in the U.S.A.: the Great Salt Lake, Utah (GSL) and salterns in the San Francisco Bay area (SFB), North & South America and

Europe. Establishment of an exotic *A. franciscana* in Eurasia has been documented since this species has distinctive physiological characteristics and is able to outcompete local species once it is established (Ruebhart et al., 2008; Vikas et al., 2012). Asian *A. franciscana* shows a high level of haplotype diversity compared to the original population source, which is a sign of multiple introductions with mass dispersal in Asia. This phenomenon has also been observed in some Mediterranean populations (Muñoz et al., 2014). *A. franciscana* shows a lack of genetic structure in Asia which is mostly due to human impacts on dispersal of *Artemia* across Eurasia.

4.5. *A. franciscana* – a super species in North America

A. franciscana is a major halophilic and commercialized *Artemia* species inhabiting both Southern and Northern arms of the Great Salt Lake, Utah. Inter- and intra-population studies of *A. franciscana* reveal substantial genetic diversity across the entire lake. The results show a lack of genetic structure among *A. franciscana* populations, indicative of panmixis in the lake. Despite significant differences of water salinity in both arms of the GSL, most genetic variation has occurred in the Southern region. This condition is mostly due to the presence of major rivers flowing into the lake in that area. *A. franciscana* in the GSL should be considered as a single management unit; however, I note that parthenogenetic populations have recently been reported from this lake (Endebu et al., 2013).

4.6. Conclusions and suggestions

The resolution of the phylogenetic trees could be improved by using additional nuclear markers (i.e. Microsatellite) for Asian *Artemia* lineages. Nevertheless, the DNA sequencing (*COI* and *ITS1*) and ISSR-PCR techniques used in this study have been informative in detecting phylogeographic and fine-scale population structure in Asian sexual species and EHC lineages throughout Eurasia.

In particular, sequencing of two genes have generated several distinct clusters for Asian *Artemia*. But the obtained patterns were not all consistent, indicating a complex structure in Asia. Taxonomic status of *A. tibetiana* needs further evaluation since it has been observed in two independent clades. According to our phylogenetic studies, *A. urmiana* has shared several haplotypes with EHC lineages in Eurasia. Spreading of *A. urmiana* cysts through natural means (migratory birds and wind) and physical ones (anthropogenic activities) could support dispersal into adjacent localities. More systematic analyses using multidisciplinary approaches are required to figure out the biogeographic structure of this species in Eurasia. Hybridization among Asian sexual species has been suggested as a possible mechanism for the origin and genetic diversity of EHC lineages in Eurasia (Maccari et al., 2013). Although our study has provided broad information on the population structure of EHC lineages in Eurasia, much remains to be done. The evolutionary origins and the

possible geographic origins of EHC lineages have been clearly indicated to be from the two sexual species in Asia including *A. urmiana* and *A. tibetiana* (Maccari et al., 2013). It is highly recommended that more sophisticated genomic tools be employed (such as Next Generation Sequencing and transcriptome analysis) for analysis of the sexual and EHC lineages to gain a deeper understanding of the evolutionary mechanisms of asexuality in the genus *Artemia*.

EHC is a mixture of clones with different, but as yet unknown, ploidy levels, which need to be further analyzed in detail. The estimation of ploidy levels using cytogenetic and flow cytometry approaches are needed for EHC lineages in Eurasia. The relationship of ploidy levels and phylogeographic structure among EHC lineages would be another next step to gain better understanding of the evolutionary history of these lineages.

The *A. franciscana* now colonized in Eurasia is a serious threat to the global biodiversity of *Artemia*. The colonization of this exotic species in non-indigenous regions leads to its outcompeting local *Artemia* populations. The current condition would result in species extinction jeopardizing the local biodiversity of *Artemia* (Amat et al., 2007). So far, the occurrence of *A. franciscana* has been reported for Mediterranean regions (Muñoz et al., 2014), South Africa (Kaiser et al., 2006; Baxevanis et al., 2014) and Australia (Vanhaecke et al., 1987). Australia is a place where very little study has been aimed at understanding the population genetic structure of *Artemia* populations. Further genetic investigations are required concerning the present distribution of the autochthonous and allochthonous *Artemia* populations in Africa and Australia. The successful colonization of an allochthonous species in non-native regions depends on multiple parameters such as environmental conditions and life-history traits (Amat et al., 2007; Van Stappen, 2008). The effect of human-mediated dispersal of *A. franciscana* in the new environments could be enhanced by numerous introductions either intentionally or accidentally. The occurrence of cryptic species suggested in this study could be further understood by using a combination of morphologic and genetic approaches.

A. franciscana from the GSL has a potential genetic diversity and its microevolutionary structure needs to be determined by including additional localities over its natural ranges. Characterization of life-span traits of *A. franciscana* populations across the GSL could provide reasonable clues about population genetic diversity and physiological adaptation of *A. franciscana* in GSL.

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