Eastern spread of the invasive *Artemia franciscana* in the Mediterranean Basin, with the first record from the Balkan Peninsula

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Abstract

In the last 30 years since its first appearance in Portugal, the North-American *Artemia franciscana* has successfully invaded hypersaline habitats in several Mediterranean countries. Here, we review its spread in the Mediterranean Basin since its first occurrence in the 1980s and report its first occurrence in Croatia, based on both morphological identification (adults) and genetic evidence (cysts). The haplotypes we found in the population from this new locality (two of which were new to both the native and invaded ranges of *A. franciscana*) suggest either direct or secondary introduction from the main harvested cyst sources (Great Salt Lake or San Francisco Bay, USA) and indicate that some genetic native diversity in the species has not yet been captured by existing studies. Our finding means that the species has reached the eastern shores of the Adriatic Sea and therefore is now present on the Balkan Peninsula. We detected that its eastward spread is still continuing, posing a fundamental threat to remaining populations of native *Artemia* species in Eastern Europe.

Keywords

invasive species, *Artemia franciscana*, salt works, Mediterranean

Introduction

The Nearctic-Neotropical brine shrimp *Artemia franciscana* has invaded several other parts of the world in the last decades. In the Mediterranean Basin, its spread is relatively well-documented. Since its first finding in the 1980s in Portugal, it has reached Morocco and Tunisia in North-Africa and several European countries along the western shores of the Mediterranean Sea, even spreading northwards along the Atlantic coast of France (Fig. 1a; Amat et al., 2005; Mura et al., 2006; Muñoz, 2009; Ben Naceur et al., 2010; Scalone & Rabet, 2013). The arrival of *A. franciscana* in a new habitat has drastic consequences for the native communities. Since it is a better competitor than its native European counterparts, *A. salina* and *A. parthenogenetica* (Browne & Halanych, 1989), it rapidly displaces them (Amat et al., 2005, 2007), leading to the loss of genetically-unique populations (Muñoz et al., 2008). Its displacement of native *Artemia* is largely due to its higher reproductive rate (Amat et al., 2007) and can be further fostered by the higher prevalence of avian cestode parasites in the native species (Redón et al., 2015). *A. franciscana* is furthermore a more efficient filter-feeder than the native species, and hence its replacement of native *Artemia* might influence the entire food web by increasing grazing pressure on phytoplankton communities (Sánchez et al., 2016). Therefore, its spread represents a threat to the central and eastern Mediterranean region, where hypersaline habitats are still inhabited by the native species (Mura et al., 2006; Fig 1b).

A. franciscana cysts are commercially available worldwide almost everywhere, originating (directly or indirectly) mainly from two locations in the USA: San Francisco Bay (SFB) and Great Salt Lake (GSL). According to a recent genetic study (Muñoz et al., 2014), it is most likely that its current Mediterranean distribution is a result of multiple (human) introductions of strains from SFB and GSL, but in some cases, natural dispersal by waterbirds could have also occurred from already established populations (Green et al., 2005).

In this study, we present the first appearance of *A. franciscana* in Croatia, compare haplotypes with other Mediterranean and commercially used populations, and review the current distribution of the species in the Mediterranean Basin, demonstrating its further eastward invasion.

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Materials and methods

Artemia samples were collected from the Nin salt works in Croatia in August 2011. The Nin salt works (Solana Nin, N 44.24254, E 15.192166, Fig. 1) are one of the oldest and still operational salinas on the Adriatic seashore of Croatia. They are located in the Nin bay, on the northernmost part of the Zadar peninsula, in the centre of Dalmatia, and cover 55 ha. They are so-called solar salt works, where only solar energy is used for the concentration of salt in the evaporation pans. We found *Artemia* populations in two neighbouring pans. One of them had deeper water and much higher *Artemia* densities (we refer to this one as no. 1, while the other we name no. 2). They had similar conductivity and total phosphorus (TP) values (no. 1: 204 mS/cm, TP: 153.7 µg/l; no. 2: 210 mS/cm, 100.3 µg/l). TP from water samples was measured spectrophotometrically as molybdate reactive phosphorous after persulphate digestion in the lab (Eaton et al., 2005).

Animals were collected from both pans with a plankton net and were preserved in 70% ethanol. Free-swimming cysts were also collected and stored in 96% ethanol. Morphological identification of the first 100 adults was based on Hontoria & Amat (1992) and Mura & Brecciaroli (2004).

Genomic DNA was extracted from randomly chosen individual cysts using the HotSHOT protocol (Montero-Pau et al., 2008). The *Artemia*-specific primers 1/2COI Fol-F and 1/2COI Fol-R (Muñoz et al., 2008) were used to amplify and sequence a fragment of the mitochondrial Cytochrome *c* Oxidase Subunit I gene (COI). PCRs were performed using the same protocol as in Muñoz et al. (2008). PCR cycling consisted in a first cycle of 3min at 94°C, followed by 35 cycles of 45s at 94°C, 1min at 55°C and 1min at 72°C, then a final elongation step of 5min at 72°C. PCR products were checked for amplification on a 1% agarose gel using SYBR Safe DNA gel stain, then directly sequenced by the company Macrogen. A total of 34 individual sequences from Nin (Croatia) were trimmed and aligned using BIOEDIT v7.0.9 (Hall, 1999), and all new haplotypes were deposited in Genbank (Genbank accession numbers: MH197108-MH197111, see also Table S2 in Supplementary Material). Our COI data set was combined with some previously published data sets from Muñoz et al. (2013, 2014) and Eimanifar et al. (2015) (see Table XXX for accession numbers). Basic population statistics for the Nin population were generated using DNAsp (Librado & Rozas, 2009). A statistical

parsimony haplotype network (Clement et al., 2000) with a parsimony connection limit of 95% was generated using Popart (Leigh & Bryant, 2015).

We also compiled available data on the observation of *A. franciscana* in Mediterranean waters to visualise its spread over the last 30 years (Amat et al., 2005; Mura et al., 2006; Muñoz, 2009; Ben Naceur et al., 2010; Scalone & Rabet, 2013; Sánchez et al., 2017). If multiple observations were reported from the same location, we used the earliest one as the first year of observation for the given location.

Results

The two populations of the Nin salt works were both bisexual (females:males ratio was 56:44 in pan no. 1, and 65:35 in pan no. 2), consisting either entirely of adults (pan. no. 2) or with a very few pre-adults (pan no. 1). Based on adult morphology, individuals were all identified as *Artemia franciscana*. The spherical frontal knob on the head of males was clearly visible. Females had sharply pointed ovisacs, short antennules, and lateral lobules before the ovisacs.

After sequencing and trimming, a COI fragment of 548bp was obtained for 34 cysts sequenced from the Nin salt works. An analysis of local similarity between sequences (BLAST) has unambiguously confirmed the morphological identification of the Nin salt work cysts as *A. franciscana* (Blast-n identity = 99%, e-value = 0.0). Overall, 5 variable sites, including 4 parsimony informative sites, were found for the Nin sequences, with no indels or stop codons. No non-synonymous substitutions were found among the sequences.

We found a total of four haplotypes (hereafter referred as NIN_01, NIN_02, NIN_03 and NIN_14) over 28 sequenced individuals. Two of them were very common in our dataset (43% and 46% of cysts for NIN_01 and NIN_02, respectively) while NIN_03 and NIN_14 showed low frequency (4% and 7% of cysts, respectively). The haplotype diversity (h) and nucleotide diversity (π) were 0.616 and 0.00327, respectively for the Nin sampling point. The haplotype network (Fig. 2) clearly shows a star-like structuring among four haplotypes (Af49, Af90, NIN_02, Haf04). The two most frequent Nin haplotypes, NIN_01 and NIN_02, were identical for our 548bp COI fragment to haplotypes reported worldwide and originating from the native American range, which includes the

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two commercial SFB and GSL populations and China (Fig. 2 and Table S1 in Supplementary Material). They are also well established in the invaded Mediterranean range (Table S1 in Supplementary Material). The low-frequency Croatian haplotypes NIN_03 and NIN_14 were directly linked to the highly connected and common haplotype NIN_02, but are private (i.e. found only at the sampling location) to the Nin salt works and had never been recorded before (Fig. 2 and Table S1 in Supplementary Material).

Discussion

For the first time, we have demonstrated the occurrence of *Artemia franciscana* in Croatia, its easternmost occurrence in the Mediterranean region. Previously, no *Artemia* species, either native or introduced, have been recorded from the Nin salt works, or in the vicinity of Nin (Petrović, 1991). This suggests that this alien species has colonized a habitat that was unoccupied by native species. In fact, our finding is the first recent *Artemia* occurrence in Croatia. Although there are data in the literature listed as Croatian (e.g. Triantaphyllidis et al., 1998; Muñoz & Pacios, 2010), these are errors: the locations reported are Montenegrin (Ulcinj) and Slovenian (Sečovelje, Strunjan; see also Fig. 1b), suggesting incoherent usage of country data and names after the breakdown of former Yugoslavia. These data from today's Slovenia and Montenegro represent the nearest populations of *Artemia* spp. in the Balkans. The sampling campaign carried out in the 1980s proved them all to be reproducing parthenogenetically, indicated clonal populations typically named as *A. parthenogenetica* (Petrović, 1991). Since then, we have no information as to whether *A. franciscana* has colonized these locations.

During the years immediately after its initial appearance in Portugal in the 1980s, there were only a few observations of *A. franciscana* in new locations (Fig. 3). Altogether, there are only seven new occurrences before 2000, while most of the reports we have identified (25) are from 2000-2005. This was the period when the species invaded Spain, spread along the coasts of France, and first appeared in Morocco and Italy (Fig. 3). After 2005, there are only **five** new locations, including our own data (Croatia, 2011), **two** in Spain (2007, **2016**), one in France (2007), and the first record in Tunisia (2009).

The genetic analysis of this newly introduced *A. franciscana* population in Croatia provides some evidence as to the possible origin and vector of introduction of this species. The four different haplotypes found in the Nin salt work are identical (NIN_01 and NIN_02) or closely related (NIN_03 and NIN_14) to widespread haplotypes all over both the native and introduced range of *A. franciscana* (Table S1 in Supplementary Material). This suggests an introduction through commercial cysts (mainly used in aquaculture and the aquarium trade) as it has been previously demonstrated for most of the introduced range of *A. franciscana* (Amat et al., 2005; Muñoz et al., 2013, 2014). Combined with previous results from the literature, our results suggest an introduction from the main harvested cysts sources from the Great Salt Lake (GSL) or San Francisco Bay (SFB), which have been historically the two most commercially distributed native populations (Amat et al., 2005).

On the other hand, as in the population genetics study of Muñoz et al. (2014) on *A. franciscana* in the western basin of the Mediterranean, we cannot exclude secondary introductions from Asia into locations like the Croatian Nin salt works. SFB and GSL haplotypes (here referred as NIN_01 and NIN_02; see Table S1 in Supplementary Material for more details) have high frequencies in introduced populations of *A. franciscana* in different Asian countries like China, India and Vietnam (Muñoz et al., 2014). Moreover, *A. franciscana* cysts from Bohai Bay in China are readily available on the world market (Van Stappen et al., 2007; Muñoz et al., 2014). The introduction of *A. franciscana* in Croatia may be explained by a direct introduction of commercially available cysts originating from North America or China.

Interestingly, we found two haplotypes (NIN_03 and NIN_14; so far private to Croatia) new to both the native and invaded ranges of *A. franciscana*, supporting the possibility of a novel introduction from a commercial source. This indicates that continued commercialization of this highly invasive crustacean continues to promote its spread at the expense of native *Artemia* populations, and that stopping such commercialization is likely to reduce the spread. This finding also suggests the full genetic diversity of *A. franciscana* has not yet been sampled in either its invaded or its native range. High haplotype diversity in alien crustacean populations is not unusual (Lejeune et al., 2014). It is very likely that in the large and intensively harvested population Great Salt Lake, some genetic native diversity has not yet been captured by existing studies. These private haplotypes are likely to originate

in the two cited USA populations, but we cannot rule out the possibility that some other native locations (e.g. Muñoz et al., 2013) may have contributed to the invasion process of *A. franciscana*. Further study of the genetic diversity in the native range would help improve the understanding of the invasion history process of this species.

An alternative explanation for the arrival of *A. franciscana* in Croatia is dispersal by migratory waterbirds, which can be efficient dispersers of *A. franciscana* cysts through endozoochory (Green et al., 2005; Sánchez et al., 2012; Muñoz et al., 2013; Reynolds et al., 2015). Even though there are no known *A. franciscana* localities in the close Balkan neighbourhood, the Italian locality of Margherita di Savoia is only 300 km from Nin. Although most cysts dispersed by waterbirds will be transported within 100 km, successful dispersal events are also expected over several hundreds of km (Sánchez et al., 2012; Viana et al., 2013). However, our analysis did not show the presence in the Nin salt works (see Table S1 in Supplementary Material and Fig. 2) of any haplotype previously reported from Margherita di Savoia (haplotypes HAF04, HAF07 and AF10; Muñoz et al., 2013, 2014). This suggests there is no direct connection between the Nin population and the geographically closest known population of *A. franciscana*. However, we cannot rule out the possibility of an introduction via waterbird movement of the American brine shrimp to Nin from some other more remote, or as yet unknown, Mediterranean location. Furthermore, the general pattern of eastward spread of the species in the Mediterranean Basin (Fig. 3) implies an important role for stepwise secondary dispersal events, instead of a series of independent direct human introductions of cysts originating directly from commercial sources. Birds may play a major role in such stepwise spread, although human vectors may also be important (Waterkeyn et al., 2010).

When contemplating merely the frequency of finding *A. franciscana* in new localities around the Mediterranean Sea, one might conclude that the fastest spread of the species has occurred around year 2000, and that since then it has slowed down somewhat. However, if we consider not only the number of new populations but also their location (Fig. 3), we can detect that the eastward spread is still continuing and that the gaps in the data are more likely to be attributable to a sampling effect. The ongoing eastward spread poses a fundamental threat to the eastern Mediterranean and Black Sea populations of native *Artemia* species in countries such as Greece, Turkey, Bulgaria or Israel (Muñoz

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& Pacios, 2010). The arrival of *A. franciscana* to Croatia increases the chances of its further spread to all nearby salt works in Italy and the Balkan Peninsula that are still known to host native (Fig. 1b) or no *Artemia*. At the same time, most of these data come from the 1980s and 90s, which emphasises that more effort is needed to update our knowledge on the ~~actual~~-current distribution of all *Artemia* species in the central and eastern Mediterranean. While it is unlikely that the commercialization of *A. franciscana* within the Mediterranean region could be efficiently prevented in order to safeguard remaining native *Artemia* biodiversity, stricter regulation of aquaculture effluents and aquarium trade might help to slow down the spread of the species, e.g. through regionally adopted legislation that only enables the use of native species in local aquaculture and salt works, preferentially originating from the nearest native population. Harvesting and storing substantial quantities of cysts in gene banks would also be an important conservation effort for preserving the gene pool of native populations still existing in the region. Furthermore, improvement in the communication between conservation authorities and local stakeholders is ~~crucially~~-vitally important to reduce the risk of direct introduction events of the invasive *A. franciscana*.

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Figure captions

Fig. 1 a Current distribution of *Artemia franciscana* in the Mediterranean Basin (black), with the new Croatian location (red). **b** – Native Mediterranean *Artemia* populations (green) in the vicinity of the easternmost occurrences of *Artemia franciscana* (based on Abatzopoulos et al., 1987; Vanhaecke et al., 1987; Petrović, 1991; Hontoria & Amat, 1992; Moscatello et al., 2002; Amat et al., 2005; Mura et al., 2005)

Fig. 2 Statistical parsimony network of *Artemia franciscana* based on a 548bp COI gene fragment. Haplotypes are labelled according to their original labels from the literature (see Table S1 in Supplementary Material for accession numbers). Black points represent inferred haplotypes not sampled in our dataset. Am. NC: American non-commercial populations; SFB: San Francisco Bay; GSL: Great Salt Lake; MED: Mediterranean (see Table S1 in Supplementary Material); CRO: Croatia (Nin)

Fig. 3 Eastward spread of *Artemia franciscana* in the Mediterranean Basin, showing the date of the first record from different locations in seven countries. Locations in France, Spain and Portugal are grouped in three boxes