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Colonization and dispersal patterns of the invasive American brine shrimp Artemia franciscana (Branchiopoda: Anostraca) in the Mediterranean region

--Manuscript Draft--

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Abstract:	Cysts of the brine shrimp Artemia franciscana are harvested from the Great Salt Lake (GSL) and San Francisco Bay saltworks (SFB) in the U.S.A, and marketed worldwide to provide live food for aquaculture. This species has become invasive across several countries. We investigated (1) if the introduced populations in the Mediterranean region could have originated from these U.S.A. populations, (2) how the genetic diversity of Mediterranean compares with that at GSL and SFB, and (3) if genetic patterns in the Mediterranean can shed light on colonization routes. We sequenced a fragment of the Cytochrome c Oxidase Subunit I and screened microsatellites loci from Mediterranean populations and the two putative U.S.A. sources. Haplotypes from Mediterranean populations were identical or closely related to those from SFB and GSL, and not related to other available American populations. Microsatellite analyses showed a reduced population diversity for most Mediterranean populations suggesting bottleneck effects, but few populations showing similar or higher genetic diversity than native ones, which are likely to be admixed from both GSL and SFB due to multiple introductions. Results suggest natural dispersal via flamingos between two Spanish populations. Our analyses show that all invaded populations could have originated from those commercialised U.S.A. populations.
Response to Reviewers:	Dear Editor (Mr. Deepan Selvaraj), Please find below our reply to the reviewer's comments on our manuscript (minor changes) "Colonization and dispersal patterns of the invasive American brine shrimp Artemia franciscana (Branchiopoda: Anostraca) in the Mediterranean region" by Muñoz et al. submitted to Hydrobiologia.

We believe that we have addressed the minor changes suggested in the current revised version.

With kind regards,
Joaquin Munoz.

Comments for the Author:
Two reviewers found the manuscript interesting and well written. They suggest only minor changes that should be considered in a revised version of the manuscript.

Reviewer #1:
The Manuscript Number: HYDR-D-13-00660 "Colonization and dispersal patterns of the invasive American brine shrimp Artemia franciscana (Branchiopoda: Anostraca) in the Mediterranean region" is in scope of the journal Hydrobiologia. It regards sufficient range of relevant material; the aim of the paper is clear and the results are confirmed by good statistical base. The used literature is relevant, including recent research papers. I suggest that the manuscript could be improved adding data from other north-american populations not included in the present manuscript (see page 13, lines 12-15 from Discussion.
I conclude that this work can be published after the suggested corrections.

REPLY: We have re-analysed the phylogenetic relationship including a representative number of COI haplotypes from our study published in PeerJ 1: e200. http://dx.doi.org/10.7717/peerj.200, coming from Mono Lake (U.S.A.), Mexico, Cuba, Jamaica, Puerto Rico and others. The results stand the same. Additionally, we have rewritten the corresponding parts in the main text.

Reviewer #2:
The authors describe the genetic structure of invasive Artemia franciscana populations in the Mediterranean in relation to the genetic structure of potential source populations. I think this is an interesting study with an appropriate design. It is well written and the data are analysed according to the current state of the art. It is a mature manuscript that will only require minor revisions before it can be accepted for publication.
I have added my comments directly in a pdf file attached in this review. (and I can assure the editor that the fact that these comments are few in number is not the result of personal laziness). I am looking forward to see this manuscript out in print.

REVIEWER #2 PDF FILE'S COMMENTS
We followed most of the suggestions of this reviewer.
1.- We tried it and found heaps of loci for Branchinella longirostris but because of complex variation in ploidy levels we did not work further on it for now. We had mixed populations of di tri and tetraploids. Can you say something about the ploidy level of A. franciscana? I heard from some sources that Artemia might also have weird ploidy levels. But perhaps not franciscana. Did you ever get more than two microsat peaks?

REPLY: Artemia franciscana is a sexual diploid organism. Although within the Artemia genus there are asexual lineages with different ploidy levels, they are easy to differentiate morphologically and are not included in our current study.

TITLE PAGE

Colonization and dispersal patterns of the invasive American brine shrimp *Artemia franciscana* (Branchiopoda: Anostraca) in the Mediterranean region

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Keywords: Aquatic ecosystems, biological invasion, human- and bird-mediated dispersal, microsatellites, mtDNA, population structure

Running title: *Artemia franciscana* invasion in the Mediterranean

Abstract

Cysts of the brine shrimp *Artemia franciscana* are harvested from the Great Salt Lake (GSL) and San Francisco Bay saltworks (SFB) in the U.S.A, and marketed worldwide to provide live food for aquaculture. This species has become invasive across several countries. We investigated (1) if the introduced populations in the Mediterranean region could have originated from these U.S.A. populations, (2) how the genetic diversity of Mediterranean compares with that at GSL and SFB, and (3) if genetic patterns in the Mediterranean can shed light on colonization routes. We sequenced a fragment of the Cytochrome *c* Oxidase Subunit I and screened microsatellites loci from Mediterranean populations and the two putative U.S.A. sources. Haplotypes from Mediterranean populations were identical or closely related to those from SFB and GSL, and not related to other available American populations. Microsatellite analyses showed a reduced population diversity for most Mediterranean populations suggesting bottleneck effects, but few populations showing similar or higher genetic diversity than native ones, which are likely to be admixed from both GSL and SFB due to multiple introductions. Results suggest natural dispersal via flamingos between two Spanish populations. Our analyses show that all invaded populations could have originated from those commercialised U.S.A. populations.

Introduction

Aquatic environments are especially vulnerable to biological invasions (Sakai et al., 2001; Grosholz, 2002). The introduction and rapid spread of invasive species in both marine and freshwater ecosystems are of worldwide concern (Ruiz et al., 2000; Roman & Darling, 2007; Gherardi, 2007). For instance, the human-mediated dispersal rate for crustacean zooplankton at an intercontinental scale has been estimated to be up to 50,000 times greater than the natural dispersal rate (Hebert & Cristescu, 2002). Aquatic invertebrate invasions often remain undetected for decades due to the difficulty of identifying cryptic species (Knowlton, 1993; Knowlton & Weigt, 1997; Lee, 2000), and are often only discovered using molecular approaches (Mergeay et al., 2005; Mergeay et al., 2007). Non-marine aquatic ecosystems contain many passively dispersed taxa such as copepods, rotifers, ostracods, bryozoans and branchiopods, which produce resting eggs (i.e., encysted embryos in arrested state of development) that allow survival during unfavourable environmental conditions and facilitate dispersal (Hairston, 1996), and are often the stages involved in accidental anthropogenic introductions (Bailey et al., 2003; Gray et al., 2005).

The anostracan *Artemia franciscana* Kellogg, 1906, is a sexual brine shrimp native to the Americas (Amat et al., 2004) that inhabits hypersaline ecosystems such as lakes, lagoons and salt ponds. Since the 50s, *A. franciscana* commercially harvested cysts (i.e., diapausing or resting eggs) have been exported worldwide from two U.S.A. populations, San Francisco Bay (SFB) and the Great Salt Lake (GSL) in Utah for use as live food in aquaculture and the aquarium pet trade (Lavens & Sorgeloos, 2000, and references therein), leading to accidental or deliberate introductions into ecosystems outside the native range. *Artemia franciscana* was also intentionally inoculated into salterns worldwide to provide local sources of cysts (e.g., Camara, 2001). Thus, by the 80s many other commercial sources of cysts became available (Vanhaecke &

Sorgeloos, 1983), and notably from coastal China (Van Stappen et al., 2007). However, Bengtson et al. (1991) estimated that over 70% of marketed *Artemia* cysts originated from the GSL population. In addition to anthropogenic introductions, *Artemia* cysts can be effectively dispersed by migratory birds (Sánchez et al., 2007; Sánchez et al., 2012) and over short and mid distances by wind and motor vehicles (Vanschoenwinkel et al., 2008a, b; Waterkeyn et al., 2010). In particular, there is potential for spread of this invasive anostracan to hypersaline sites unaffected by aquaculture facilities via flamingos and other waterbirds.

The recent expansion of aquaculture in the Mediterranean region has led to the release of *A. franciscana* into sites previously occupied by native *Artemia* species. *Artemia franciscana* was first reported in Portugal around the 1980's (Hontoria et al., 1987), and later in France (Thiery & Robert, 1992), Spain, Italy, and Morocco (Amat et al., 2007, and references therein; Mura et al., 2006). Its establishment was followed by rapid local extinctions of the native *A. salina* and *A. parthenogenetica*. A combination of habitat loss and the establishment of *A. franciscana* have resulted in the loss of 55 - 74% of native *Artemia* populations across Spain, Portugal and France (Amat et al., 2007). Although the initial colonization of Mediterranean habitats is assumed to have originated from GSL and SFB in the U.S.A., this hypothesis has not been tested yet using genetic markers. Furthermore, although genetic studies can shed light on the role of aquatic birds as effective dispersal vectors of invertebrates (Figuerola et al., 2005), no such studies have yet been carried out on *A. franciscana*.

Artemia franciscana's high genetic diversity, phenotypic plasticity, high fecundity, and a large native geographic range could explain its high invasiveness (Amat et al., 2007; Ruebhart et al., 2008, and references therein). This invasive anostracan outcompetes the native parthenogenetic *Artemia* strain in laboratory experiments (Browne, 1980). In addition, propagule pressure can be extremely high.

Shrimp and finfish hatchery effluent leading to accidental releases and intentional inoculations involving large number of nauplii (i.e., first larval stage) may regularly have taken place in the form of multiple introductions, not necessarily from the same geographic source. Enemy release may also be involved in the ability of *A. franciscana* to outcompete native species, as compared to native Mediterranean *Artemia*, *A. franciscana* experiences reduced levels of parasitism by avian cestodes, which reduce the fecundity of brine shrimps and increase bird predation (Georgiev et al., 2007; Sánchez et al., 2009). Despite its detrimental effects on native *Artemia* biodiversity (Muñoz et al., 2008) and calls for import control and management (Ruebhart et al., 2008; Amat et al., 2005a), no specific actions have been carried out to contain the spread of *A. franciscana*. However, a better knowledge of the introduction sources, mode and patterns of invasion and colonisation in *A. franciscana* could assist the development of future strategies for the management of aquaculture and the conservation of hypersaline ecosystems.

The relationship between the levels of genetic diversity found in source populations and those found in the established populations after invasion is not straightforward (Roman & Darling, 2007; Darling et al., 2008; Dlugosch & Hays, 2008). Genetic diversity of invasive populations has generally been assumed to be reduced compared to those from the native range. Indeed, punctual introductions can result in strong population bottlenecks (Golani et al., 2007). Many invasive species suffer an associated reduction in genetic diversity due to founder effects (e.g., Muñoz-Fuentes et al., 2006), while large increases are rare (Dlugosch & Parker, 2008). However, similar or higher genetic diversity to the native range has been reported in some aquatic invasive species (Roman & Darling, 2007), due to introduction events from multiple sources and/or large propagule pressure lead to admixture in the non-native range (Roman & Darling, 2007; Wilson et al., 2009). Unlike natural extra-range

dispersal events, human-mediated biological invasions are often the result of multiple introductions from different sources to non-indigenous locations (Wilson et al., 2009). Therefore, new populations established directly by human-mediated introductions might have different genetic signatures to those established by natural dispersal from pre-existing populations within the introduced range, providing a valuable tool for understanding the colonisation routes of invasive species. For instance, human-mediated dispersal and multiple introductions should result in a lack of correlation between genetic and geographic distances (e.g., Elderkin et al., 2004), whereas the opposite (i.e., an isolation-by-distance pattern) or a random genetic distribution (e.g., Dupont et al., 2003) would be more likely under equilibrium conditions (but see Herborg et al., 2007).

The invasive history and biological traits of *A. franciscana* represent a rare opportunity to investigate the interplay between genetic patterns and the relative role that human- and waterbird-mediated dispersal have on population expansion. Here we investigate the origin, mechanisms, and patterns of *A. franciscana* invasion in the Mediterranean by screening populations with mitochondrial and nuclear markers, using combined phylogenetic, phylogeographic, and population genetic analyses. Specifically, we investigated whether (1) all Mediterranean populations originated from the marketed GSL and SFB populations, and (2) whether current patterns of genetic diversity and differentiation in the non-native range are consistent with scenarios of human- and bird mediated dispersal.

Materials and methods

Samples and data collection

We collected cyst samples from 16 invaded Mediterranean solar salterns and salt lakes, from a total of 26 populations identified to date, covering the four European countries

(i.e., Portugal, Spain, France, and Italy - Hontoria et al., 1987; Amat et al., 2005a) where *A. franciscana* has been detected. Additionally, we obtained cyst samples from the two native commercially exploited U.S.A. populations (SFB and GSL), and a population of unknown origin in Sal Island in the Cape Verde archipelago (Amat et al., 2010) (see Table 1). All samples were obtained from the 'cyst-bank' of the Instituto de Acuicultura de Torre de la Sal (CSIC, Castellón, Spain). Cysts were preserved in 100% ethanol until needed. In addition, for phylogenetic analyses we downloaded all available *A. franciscana* COI sequences from GenBank for which we could obtain geographic information, either directly from the GenBank record or from the original publication, including samples from U.S.A., and the rest of its native range in the Americas, as well as India, Vietnam and China.

Laboratory procedures

DNA was isolated from individual cysts (5-37 individuals per population for mitochondrial analyses, and 29-47 individuals for nuclear analyses), previously rinsed in distilled water, using an alkaline lysis protocol optimized for zooplanktonic diapausing eggs (Montero-Pau et al., 2008). We used specific *Artemia* primers in the same position as primers LCO1490/HCO2198 from (Folmer et al., 1994) to amplify a fragment of the Cytochrome *c* Oxidase Subunit I (COI) mitochondrial gene. PCR/sequencing protocols were performed following (Muñoz et al., 2008). Sequences were edited and aligned using Sequencher™ version 4.5 (Gene Codes Corp., © 1991-2005). All different sequences (i.e., haplotypes) found in the present study were deposited in DNA Data Bank of Japan (DDBJ) database (Accession No AB859230-AB859239 – see Table 2 for details and link to GenBank Acc Nos). Samples were genotyped for four microsatellite loci (Af_A108, Af_B10, Af_B9, and Af_B11) following Muñoz et al. (2009).

Mitochondrial DNA analyses

Identical sequences were collapsed into haplotypes prior to phylogenetic and phylogeographic analyses using FaBox (http://users-birc.au.dk/biopv/php/fabox/). We reconstructed the evolutionary history of all *A. franciscana* haplotypes (i.e., from Mediterranean and out of this area) using Neighbor-Joining (NJ) and Maximum Likelihood (ML) approaches in MEGA v.5.2.2. (Tamura et al., 2007) using the evolutionary model best fitting the data. The robustness of the branches was assessed with 1000 pseudo-replicates. DnaSP v.4.90 (Rozas et al., 2003) was used to compute the number of polymorphic sites and of non-synonymous substitutions.

TCS v.1.21 (Clement et al., 2000), which follows the statistical parsimony algorithm to generate a haplotype network, was used to display the genealogical relationships among our Mediterranean samples and some public available *A. franciscana* COI haplotypes. Standard intra-population diversity parameters, haplotype diversity (H) and nucleotide diversity (π), and inter-population pairwise ϕ_{ST} values (corrected by a K2-P evolutionary model) were obtained using Arlequin v.3.11 (Excoffier et al., 2005). Because differences in sampling can bias genetic diversity comparisons among different populations, a rarefaction analysis adapted for population genetic data conducted by the program RAREFAC v. 1.02 (available from R. Petit at http://www.pierroton.inra.fr/genetics/labo/Software/Rarefac/index.html) was used to calculate standardized allelic richness (A) for each sampled population. RAREFAC requires a rarefaction size (see Petit et al., 1998), which was set to ten in our case (n = 10). Thus, three populations with n<10 (i.e., CBU, FVO, and RFR) were not used in such analyses. All those estimates (i.e., H, π, A and ϕ_{ST}) were used to assess the population genetic diversity after introduction and to identify the likely origin of each invaded population.

Microsatellite analyses from U.S.A. and Mediterranean invaded sites

Arlequin was used to compute observed (H_O) and expected (H_E) heterozygosity, number of alleles (Na), linkage disequilibrium (LD) between loci, Hardy-Weinberg equilibrium (HWE) of each locus. The *Fst*-statistic may not be appropriate for assessment of genetic structure and differentiation among populations (Jost, 2008; Dupont et al., 2009), therefore, we calculated both *Fst* and *Dest* pairwise values using GenAlEx ver.6.5 (Peakall & Smouse, 2012). In addition, we used a Bayesian multi-locus method (with a non-equilibrium method, individual-based admixture analysis) implemented in BAPS v.5.2 (Corander et al., 2003; Corander et al., 2008) to infer population structure and to group the data by a stochastic optimization model to infer the posterior probability of the number of distinct clusters, *K*. In particular, we used the spatial model for genetic discontinuities, running five replicates with upper bound values of K = 5, 10, 20 and 25. Furthermore, to assess the most likely grouping of individuals in clusters, we used Principal Component Analysis (PCA) as a different clustering approach. PCA-GEN software (http://www2.unil.ch/popgen/softwares/pcagen.htm) is a program that does not require assumptions of equilibrium within populations, correlates genotypes and allele frequencies among all individuals using no information regarding population identification, and plots genetic structure among populations.

Results

Global mitochondrial phylogeography of invasive Artemia franciscana

The sequence alignment used in both phylogenetic and phylogeographic analyses were trimmed to 477 bp. The COI sequences aligned (including 274 generated in the present study – collected from 19 sites from GSL, SFB, Cape Verde and Mediterranean region; see Table 1) collapsed into 71 haplotypes. Overall, 94 variable sites and 62 parsimony

informative sites were revealed, with no indels or stop codons. Five non-synonymous substitutions were found in positions.

Both phylogenetic reconstruction methods (ML and NJ) recovered a virtually identical tree topology and support values, so only the ML reconstruction is shown (Fig. 1), with geographically concordant branches and over ten lineages, similarly to results from Muñoz et al (2013). All Mediterranean and other invasive populations had identical haplotypes to SFB and UTAH populations or highly related haplotypes to these.

The median-joining haplotype network showed three disjoined networks. One included the Cape Verde haplotypes, another for the Mexican and Chilean/Argentinean phylogenetic subclades (data not shown, but see Fig. 1), and a major network formed by the rest of haplotypes encompassing U.S.A., invasive and some Chilean haplotypes. In this latter network (see Fig. 2), when excluding relatively divergent Chilean haplotypes, a total of 12 closely related haplotypes, no more than five substitutions apart, were detected. A total of ten haplotypes were present in invaded populations in the Mediterranean, six of them found also in GSL and SFB (Fig. 2). The remaining four haplotypes were only found in invaded populations, although they were closely related to the most common haplotypes in GSL and SFB (1 or 2 substitutions apart). Both GSL and SFB shared the three most common haplotypes (i.e., HAf01, HAf02, and HAf04), but they were found at different frequencies. Haplotype HAf02 was the most common in GSL (79.3% of individuals), whereas haplotype HAf04 was the most common in SFB (70.3% of individuals). Furthermore, HAf02 and HAf04 were the most common haplotypes in the invaded populations. Amongst the 16 Mediterranean populations analysed, HAf04 was present at 14 sites, while HAf02 was present at six.

Mitochondrial genetic diversity in U.S.A. and Mediterranean populations

Intra-population haplotype diversity, *A* (Table 2; note that for three populations, the value *A* was not estimated due to low sample sizes), and inter-population pairwise genetic diversity ϕ_{ST} (Table 4B) indicated: 1) A high and significant level of population differentiation between both native populations SFB and GSL (ϕ_{ST} value of 0.546); 2) GSL had lower haplotype diversity than SFB; 3) Ten Mediterranean populations appeared to be related to SFB with non-significant ϕ_{ST} values and lower diversity values than SFB, except for FPI, which had similar diversity values; 4) Only one population, TRI, showed a non-significant ϕ_{ST} value when compared to GSL; 5) Four populations (ESM, GER, LTA, and SPA) were significantly different to both U.S.A. populations as indicated by ϕ_{ST} values, but of these four only LTA contained a haplotype not found in SFB or GSL.

Nuclear genetic diversity, regional structure and demographic patterns in the Mediterranean

All loci used to screen Mediterranean and North American samples (714 cysts) were unlinked (results not shown) and only the Af_108 locus was in Hardy-Weinberg disequilibrium for most populations, with significant homozygote excesses probably due to null alleles (Muñoz et al., 2009). Nevertheless, no population was found to be under disequilibrium for all loci. Af_108 was monomorphic for two populations, BFI and RFR, and RFR population could not be genotyped for the Af_B9 locus (see Tables 1 and 3 for details). The number of alleles per locus ranged from 13 (Af_A108) to 40 (Af_B11). The mean number of alleles (Na) and gene diversity (H_E) was similar in both commercialised native populations (13.5 and 14.0, and 0.753 and 0.847 in SFB and GSL, respectively, with SFB showing two private alleles in Af_B10). However, Na and H_E showed wide differences in the Mediterranean, ranging from 3.0 to 17.0 and 0.256 to

0.840, respectively, with private alleles in nine populations. Several introduced populations showed equal or higher Na than native ones.

Most pairwise F_{ST} and *Dest* values were highly significant (see Table 4A), even between both native populations SFB and GSL (0.076 and 0.593, respectively). Contrary to the results for mitochondrial ϕ_{ST} values, all Mediterranean populations showed significantly high pairwise F_{ST} and *Dest* values with SFB and GSL except ESM population, which showed non-significant values compared to GSL. Four Mediterranean populations (ESM, SLU, GER and CBU) showed no genetic differentiation between them based on their F_{ST} and *Dest* pairwise values.

Bayesian clustering analysis (BAPS) and Principal Component Analysis (PCA) gave similar population structure, but produced different numbers of clusters. BAPS analysis resulted in ten clusters with a probability higher than 0.97 (see Fig. 3 for details). Four clusters contained more than one population, while six populations were identified as single clusters. As expected, two multi-population clusters included the two native populations. The cluster containing GSL had five populations, and the cluster containing SFB had one. However, two clusters made up of two populations each were inferred with independence from the native populations (BMA-FVO, and LTA-FPI). The first two axes of the PCA explained 70.24% of the total variation. Unlike BAPS, PCA analysis did not consider SPA as belonging to the GSL genetic group, and did not group ALC with SFB. Both population structure analyses clearly show that most populations group around SFB and GSL, or in the space between them, indicating introductions from single sources or a range of admixture. However, three of the populations (BFI, RFR or AIG) were outside this admixture gradient.

Discussion

Our results strongly suggest that *A. franciscana* invasive populations across the Mediterranean region and other parts of the world originate from the commercialised populations at GSL and/or SFB in the U.S.A. Other genetic lineages in the native range are geographically restricted and genetically divergent, and have clearly played no part in the Mediterranean and the non-native range included in our study. Although we included all the available populations in U.S.A., we want to highlight that GSL and SFB are the only ones of importance for exporting cysts on the world market. In addition, Muñoz et al. (2013) have recently confirmed our results by surveying a continental phyogeography for *A. franciscana* including additional American haplotypes present on this study. The low frequency of private alleles (between 1.1% and 7.9% for microsatellites, data not shown) in the invasive populations, also suggests that SFB and GSL could be the original source populations.

However, we cannot rule out that some of the Mediterranean populations were established by secondary introductions from Asia, given the dominance of SFB and GSL haplotypes in China, India and Vietnam (Fig. 2) and the commercial availability of *A. franciscana* cysts from Bohai Bay in China on the world market (Van Stappen et al. 2007, http://www.bhb-artemia.com/). Surprisingly, Cape Verde haplotypes form a highly supported independent mitochondrial clade, even though this region has previously been assumed to be part of the invasive range of this species due to its isolation from the Americas (see Muñoz & Pacios, 2010). Our results suggest that *A. franciscana* may be native in the Cape Verde islands.

The three most common haplotypes from SFB and GSL (HAf01, HAf02, and HAf04) are extremely similar, indicating that either: 1) both populations were formed very recently; 2) one of them was used to 'seed' the other one (e.g., *A. franciscana* colonizing the salt ponds created at SFB may have originated from GSL, these sites

being connected via migratory waterbirds); or 3) after some relatively recent population differentiation there has been a lot of admixture. Despite the fact that our analyses included only a few nuclear loci, our results indicate a significant genetic divergence between these two populations (see also Muñoz et al., 2009). Although there are 10 microsatellites developed for this species, only the four used in this study amplified consistently and provided repeatable banding patterns. We recognize that this small number gives little power in our PCA and BAPS analyses. However, developing microsatellite markers for Anostraca is notoriously difficult, and we are not aware of any other studies that have used them for these crustaceans (but see Deiner et al., 2013), despite a range of studies using mitochondrial markers.

Mitochondrial markers have been very useful in inferring the origin and invasion pathways of introduced vertebrate and invertebrate species (Kelly et al., 2006; Ashton et al., 2008; Ficetola et al., 2008; Mabuchi et al., 2008; Gaubert et al., 2009). However, since the same three commonest haplotypes in *A. franciscana* are shared by GSL and SFB, but with different relative frequencies, the resolution offered by mtDNA is insufficient to make clear conclusions on which of these U.S.A. populations is involved as the ultimate source of invasions or estimate the level of admixture. The most common mtDNA haplotype from SFB (HAf04) is present in most invaded populations (see Table 2), and genetic drift is likely to be involved in changing haplotype frequencies of the invaded populations. In addition, many Mediterranean populations were not significantly different from the SFB population as measured with ϕ_{ST} (see Table 4B).

Different colonization and dispersal patterns can be expected to leave specific genetic signatures across the invaded range (Dupont et al., 2009; Willson et al., 2009). For instance, under a scenario of mass introduction, high genetic diversity is expected in the invaded populations. In addition, homogenization of the gene pool of invaded

populations or low population differentiation between invaded populations and the source population can be expected due to continuous or frequent introduction events, which is likely for easily accessible geographic areas. Examples of this mass introduction pattern occurs in our microsatellite analyses where we found one Mediterranean *A. franciscana* population (ESM) is not significantly different than the GSL population as measured with the G-statistics *Dest* and F_{ST} (see Table 4A). Furthermore, this Mediterranean population does not show significant differentiation with SLU or GER, and SLU is not differentiated from CBU. These four populations from different parts of the Iberian Peninsula also cluster together with GSL in the BAPS population structure analyses (Fig. 3), and do not show signs of loss of genetic diversity when compared with native populations (see Table 3).

On the other hand, as expected given the relatively reduced number of mitochondrial haplotypes in the native populations (and the smaller effective population size of mtDNA), most invasive populations showed reduced mtDNA diversity likely due to population bottlenecks, founder effects, and genetic drift during the colonisation process, which might reflect habitat monopolisation by a few highly successful individuals (see De Meester et al., 2002).

Our microsatellite results could fit with punctual human introductions resulting in population bottlenecks for at least three Mediterranean populations (BFI, AIG and MSA), which show the lowest genetic diversity (i.e., expected heterozygosity and number of alleles), but non-significant differentiation with the native population SFB at mitochondrial level (see Table 4B). All of these populations were strongly differentiated genetically with the rest of the populations according to the microsatellite analyses (Table 4A). BFI and AIG also appear as single population clusters in the PCA analysis, away from the admixture gradient between SFB and GSL where the rest of the populations are distributed (Fig. 3).

In addition, our results suggest natural dispersal between two populations in South Spain, La Tapa salt ponds (LTA) and Fuente de Piedra lagoon (FPI). Both mitochondrial and nuclear data show a close relationship between these populations (e.g., no significant F_{ST} value, clustering analyses, both share a unique mitochondrial haplotype – HAf05 in Table 2). LTA is a coastal saltpan population in Cadiz Bay with an intensive aquaculture industry and where *A. franciscana* was fully established around 2002 (Amat et al., 2005a; Amat et al., 2007). In contrast, FPI is a natural inland closed-basin lake situated 140 km away from LTA, and where the native *A. salina* occurred until *A. franciscana* was detected in 2005 (Amat et al., 2007). FPI holds the most important breeding colony of the greater flamingo *Phoenicopterus ruber* in Spain, is a highly protected Nature Reserve and has no influence from the aquaculture industry, but flamingos breeding there regularly fly to LTA to feed (Amat et al., 2005b). Flamingos are the most abundant waterbirds in saltpans along the Iberian coast by biomass (Rodríguez-Pérez & Green, 2006; Sánchez et al., 2013), and are effective dispersers of *Artemia* cysts (MacDonald, 1980; Sánchez et al., 2012. In addition, a mechanistic model of dispersal of *Artemia* cysts by waterbirds estimated that ducks may disperse them over distances of 230-1209 Km (Viana et al., 2013). Although anostracan cysts can also be dispersed a short distance by wind, this appears to be limited to a maximum of a few hundred metres Vanschoenwinkel et al., 2008a, b). Therefore, the most likely explanation for the colonization of FPI by *A. franciscana* is through natural dispersal via birds, rather than by direct human intervention. Unfortunately, our dataset does not have the necessary resolution to shed light into the invasion patterns of the other Mediterranean populations such as SPA, TRI from Spain; SGU from France; and ALC, BMA, FVO, RFR from Portugal, which likely involve a combination of several patterns described and also including admixture.

In conclusion, our results confirm previous indications that the worldwide invasion of *A. franciscana* is based on the spread of cysts originally from two commercially exploited U.S.A. populations (i.e., SFB and GSL). As in other aquatic invaders (Rius et al., 2008), high genetic diversity found in several Mediterranean populations point to an establishment as a result of multiple introductions from different populations of origin and/or high propagule pressure (see Wilson et al., 2009). Furthermore, high genetic diversity is usually linked to both adaptive potential and physiological plasticity, helping an introduced species to success as an invader (Dlugosch & Parker, 2008). Our results, and previous studies (Browne & Wanigasekera, 2000), indicate that *A. franciscana* possesses high genetic diversity, and high adaptive potential and plasticity, facilitating the successful colonisation of suitable habitats through the world. Future research using genomics approaches is desirable to provide better information on the relationships between populations in the native and non-native ranges and the role of local adaptation in the invasive process (e.g. to variation in water chemistry or temperature).

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Figure Legends

Figure 1: Tree showing the evolutionary history of *Artemia franciscana* inferred from the Cytochrome Oxidase Subunit I dataset obtained from the total geographic range analysed. Topology shown was obtained using Maximum Likelihood (see text for details). The haplotypes found in this study are labelled HAf01 to HAf10 (see Table 2). Only tips of the U.S.A.-invasive and Cape Verde lineages are shown for simplicity. For further information about the remaining lineages see Muñoz et al (2013). Tips from U.S.A.-invasive clade include geographical information in parenthesis. Haplotypes found in invaded non-American sites are indicated in red, and those exclusive from the U.S.A. commercialised populations analysed (i.e., GSL and SFB) are indicated in bold. Bootstrap supports over 50, after 1000 pseudo-replicates, are shown for the main branches.

Figure 2: Haplotype network displayed by TCS software. Circles (i.e., haplotypes) are scaled to the number of individuals observed with that haplotype. Grey circles indicate haplotypes exclusive to Mediterranean invaded populations. Higher divergent Chilean, Cape Verde, Argentinean, and Mexican haplotypes were removed from the network analysis, as they were not included in the 95% Confidential Interval of the parsimony algorithm in TCS software. Only the closest Chilean haplotypes are shown. Each connection represents a single nucleotide difference. Black circles correspond to unsampled or missing haplotypes. Haplotypes obtained in this study are labelled HAf01 to HAf10. GenBank haplotypes are labelled with their ARC code or corresponding Accession number. The geographic origin is indicated next to each haplotype. For population codes see Table 1. Haplotypes sharing one or more Mediterranean populations are labelled as Med.

Figure 3: Principal Component Analysis computed by PCA-GEN software plotted with the two main axes. Populations enclosed within lines were identified to have NO significant F_{ST}/*Dest* values in genetic differentiation analyses, but they group into the same cluster in a mutation-migration-drift equilibrium model (i.e., BAPS). Population codes (in the left column next to the figure) and points (inside the figure) sharing the same colour come from the cluster analysis computed by BAPS. A map of sampling sites is also included (see Table 1 for population codes and geographic coordinates).

Tables

Table 1: *Artemia franciscana* populations sampled for this study, population codes, geographic coordinates, and sampling date. Population codes are listed by alphabetical order within each country. Sequences for additional populations from the Americas range used in the phylogenetic analyses were obtained from GenBank (see methods).

Locality	Code	Latitude	Longitude	Sampling date
Native				
San Francisco Bay, California	SFB	37º39' N	122º25' W	--
Great Salt Lake, Utah	GSL	40º45' N	111º54' W	--
Non-native				
Alcochete, Portugal	ALC	38º44' N	08º58' W	2004
Esmolas, Aveiro, Portugal	ESM	40º39' N	08º41' W	1991
Bonfim, Portugal	BFI	38º24' N	08º34'01" W	1996
Bella Mandil, Portugal	BMA	37º01' N	07º52' W	2005
Cerro Bufo, Portugal	CBU	37º13' N	07º26' W	2002
F.M. Vontade, Portugal	FVO	37º00' N	07º54' W	1987
Rio Frio, Portugal	RFR	38º24' N	08º34' W	1993
Santa Luzia, Tavira, Portugal	SLU	37º06' N	07º38' W	2004
Fuente de Piedra, Málaga, Spain	FPI	37º06' N	04º45' W	2007
Gerri de Sal, Lleida, Spain	GER	42º20' N	01º04' E	2004
La Tapa, Cádiz, Spain	LTA	36º36' N	06º13' W	2004
San Pascual, Cádiz, Spain	SPA	36º30' N	06º09' W	2003
Trinitat, Ebro Delta, Tarragona, Spain	TRI	40º35' N	00º40' E	2004
Aigües Mortes, France	AIG	43º34' N	04º11' E	2002
Saillé-Guérande, France	SGU	47º20' N	02º26' W	2007
Margherita di Savoia, Italy	MSA	41º22' N	16º05' E	2004
Pedra de Lume, Sal Island, Cape Verde	PLU	16º46' N	22º53' W	2005

Table 2: Mitochondrial diversity for *Artemia franciscana* for a 477 bp COI fragment from the two native, Cape Verde (PLU), and the 16 non-native Mediterranean populations utilized in this study. Note that PLU† population has an uncertain origin. (n) = number of haplotypes per population; *H* = gene diversity; π = nucleotide diversity; *A* = standardized allelic richness; N = number of individuals analyzed per population. Bold numbers indicate the two main native haplotypes. Bold and italic numbers indicate haplotypes found exclusively in non-native Mediterranean populations. Asterisks indicate those non-native populations with higher standardised mitochondrial diversity than native ones. DDBJ Acc. No = DNA Data Bank of Japan Accession Number.

	Haplotype #										Diversity			
Locality (n)	**HAf01**	**HAf02**	**HAf03**	**HAf04**	**HAf05**	**HAf06**	**HAf07**	**HAf08**	**HAf09**	**HAf10**	***H***	***π***	***A***	**N**
Native														
SFB (4)	6	4	0	**26**	0	0	0	0	1	0	0.48	0.0019	1.877	37
GSL (5)	2	**23**	2	1	0	0	0	0	0	1	0.37	0.0022	1.847	29
Non-native														
ALC (2)	0	1	0	11	0	0	0	0	0	0	0.17	0.0007	0.833	12
ESM (3)	6	3	1	0	0	0	0	0	0	0	0.60	0.0033	2.000*	10
BFI (1)	0	0	0	10	0	0	0	0	0	0	0.00	0.0000	0.000	10
BMA (1)	0	0	0	12	0	0	0	0	0	0	0.00	0.0000	0.000	12
CBU (1)	0	0	0	6	0	0	0	0	0	0	0.00	0.0000	N.C.	6
FVO (2)	0	0	0	7	0	0	0	***1***	0	0	0.25	0.0005	N.C.	8
RFR (1)	0	0	0	5	0	0	0	0	0	0	0.00	0.0000	N.C.	5
SLU (1)	0	0	0	12	0	0	0	0	0	0	0.00	0.0000	0.000	12
FPI (3)	0	1	0	7	5	0	0	0	0	0	0.60	0.0025	1.759	13
GER (2)	16	0	0	1	0	0	0	0	0	0	0.12	0.0005	0.588	17
LTA (4)	2	4	0	3	3	0	0	0	0	0	0.80	0.0033	2.985*	12
SPA (3)	9	4	1	0	0	0	0	0	0	0	0.54	0.0029	1.713	14
TRI (2)	0	13	0	5	0	0	0	0	0	0	0.42	0.0018	0.993	18
AIG (1)	0	0	0	11	0	0	0	0	0	0	0.00	0.0000	0.000	11
SGU (1)	0	0	0	16	0	0	0	0	0	0	0.00	0.0000	0.000	16
MSA (1)	0	0	0	16	0	0	0	0	0	0	0.00	0.0000	0.000	16
PLU† (2)	0	0	0	0	0	***15***	***1***	0	0	0	0.12	0.0003	0.625	16
DDBJ Acc. No	*AB859230	*AB859231	*AB859232	*AB859233	AB859234	*AB859235	*AB859236	AB859237	*AB859238	*AB859239				

Due to shutdown occurred in U.S.A. and the stop of PubMed service, we followed the *Hydrobiologia* Editor's suggestion to send our sequences to DDBJ database. Asterisks (*) correspond to haplotypes with identical nucleotide sequence, but different length, to GenBank Acc No as follow: AB859230 = KF662968; AB859231 = KF662970; AB859232 = KF662971; AB859233 = KF662960; AB859235 = KF663036; AB859236 = KF663043; AB859238 = KF662975; and AB859239 = KF662977.

Table 3: Genetic characteristics of each native and Mediterranean sampled site for the four *Artemia franciscana* microsatellites used. N = number of individuals; H_O = observed heterozygosity; H_E = expected heterozygosity; P = p-value of exact test using Markov Chain Monte Carlo with a confidence interval of 95%; Na = number of alleles (pa = number of private alleles). Bold numbers indicate significant departure from HWE after sequential Bonferroni correction (p-value = 0.0083). Non-native populations marked with the symbol ‡ indicate those with higher mean Na values than native populations.

	Loci																					
Locality	**Af_A108**					**Af_B10**					**Af_B9**					**Af_B11**					**Mean**	
	N	H_O	H_E	P	Na (pa)	N	H_O	H_E	P	Na (pa)	N	H_O	H_E	P	Na (pa)	N	H_O	H_E	P	Na (pa)	H_E	Na
Native																						
SFB	27	0.296	0.797	**0.000**	7	42	0.357	0.355	0.204	8 (2)	38	0.579	0.923	**0.000**	20	34	0.882	0.935	0.861	19	0.753	13.5
GSL	44	0.705	0.724	0.225	9	44	0.818	0.817	0.598	9	40	0.825	0.903	0.285	17	37	0.865	0.944	0.015	21	0.847	14.0
Non-native																						
ALC	35	0.486	0.813	**0.000**	7	43	0.558	0.694	0.232	7	37	0.676	0.929	**0.001**	18 (1)	41	0.805	0.922	0.152	14	0.840	11.5
ESM	45	0.667	0.655	0.924	9	44	0.750	0.737	0.659	10	45	0.889	0.930	0.366	21 (1)	44	0.841	0.948	0.029	24 (1)	0.818	16.0‡
BFI	30	Monomorphic			1	44	0.477	0.463	0.872	3	32	0.531	0.833	0.009	9	42	0.738	0.734	0.242	9	0.507	5.2
BMA	46	0.630	0.568	0.258	6	46	0.761	0.709	0.384	7	46	0.870	0.875	0.937	21 (1)	46	0.804	0.855	0.235	15	0.752	12.2
CBU	41	0.610	0.655	0.576	8	40	0.725	0.681	0.807	8	32	0.875	0.890	0.854	13	38	0.868	0.938	**0.007**	22	0.791	12.7
FVO	45	0.578	0.537	0.611	6	45	0.444	0.709	**0.000**	5	42	0.929	0.928	0.138	21 (1)	43	0.605	0.828	0.000	10	0.751	10.5
RFR	36	Monomorphic			1	44	0.386	0.446	0.195	3	0	N.A.	N.A.	N.A.	0	44	0.659	0.615	0.799	9	0.265	3.0
SLU	42	0.476	0.601	0.032	7	42	0.786	0.749	0.519	9	38	0.947	0.934	0.165	22 (1)	41	0.829	0.924	0.281	20	0.802	14.5‡
FPI	39	0.590	0.803	**0.000**	8	40	0.675	0.652	0.819	6	40	0.800	0.871	0.671	12	38	0.842	0.925	0.118	18	0.813	11.0
GER	43	0.814	0.751	0.954	12 (1)	43	0.651	0.718	0.628	9	37	0.811	0.924	0.039	20	34	0.823	0.942	0.008	22	0.834	15.7‡
LTA	38	0.500	0.780	**0.000**	8	39	0.769	0.686	0.504	7	37	0.784	0.850	0.827	14	30	0.900	0.905	0.398	15	0.805	11.0
SPA	42	0.643	0.745	0.669	8	43	0.674	0.703	0.385	10 (1)	44	0.727	0.936	0.027	23 (1)	41	0.805	0.954	0.056	27 (1)	0.835	17.0‡
TRI	42	0.357	0.692	**0.000**	5	47	0.468	0.553	0.499	5 (1)	42	0.476	0.812	**0.000**	9	45	0.844	0.910	0.058	17	0.742	9.0
AIG	29	0.000	0.133	**0.000**	3	36	0.472	0.469	1.000	3	33	0.788	0.868	0.070	13	32	0.812	0.8649	0.369	12 (1)	0.583	7.7
SGU	28	0.321	0.675	**0.000**	4	29	0.034	0.034	1.000	2	23	0.304	0.907	**0.000**	11	29	0.828	0.829	0.202	9	0.611	6.5
MSA	32	0.125	0.569	**0.000**	3	41	0.195	0.182	1.000	3	37	0.784	0.860	0.308	12	40	0.775	0.781	0.521	9	0.598	6.7
TOTAL	**684**				**13**	**752**				**15**	**643**				**37**	**699**				**40**		

Table 4: A) Pairwise population matrix of G-Statistics Analysis for *Dest* and *Fst* values from nuclear loci above and below the diagonal, respectively, calculated by GenAlEx for 17 *Artemia franciscana* populations (two native from U.S.A. and 15 non-native from the Mediterranean. Values for the Portuguese population, RFR, could not be calculated, as only three loci were available. Values with NO statistical significance (*p*-value >0.05) are shown in bold and italics. **B)** ϕ_{ST} values from mitochondrial COI locus for 18 *A. franciscana* populations (two native from U.S.A. and 16 non-native from the Mediterranean). Population codes are those indicated in Table 1. Values in bold are statistically significant (*p*-value < 0.05).

A)

	SFB	**GSL**	**ALC**	**ESM**	**BFI**	**BMA**	**CBU**	**FVO**	**RFR**	**SLU**	**FPI**	**GER**	**LTA**	**SPA**	**TRI**	**AIG**	**SGU**	**MSA**
SFB	-	0.593	0.092	0.703	0.644	0.700	0.761	0.573	NA	0.708	0.427	0.666	0.501	0.228	0.143	0.333	0.115	0.119
GSL	0.076	-	0.390	***0.021***	0.863	0.140	0.085	0.140	NA	0.034	0.315	0.036	0.236	0.173	0.564	0.786	0.720	0.782
ALC	0.020	0.042	-	0.052	0.636	0.066	0.508	0.431	NA	0.057	0.049	0.047	0.056	0.020	0.030	0.082	0.055	0.062
ESM	0.094	***0.008***	0.462	-	0.911	0.120	0.046	0.197	NA	***0.014***	0.051	***0.006***	0.042	0.212	0.083	0.162	0.787	0.158
BFI	0.166	0.177	0.140	0.194	-	0.190	0.886	0.870	NA	0.187	0.181	0.185	0.191	0.177	0.195	0.123	0.200	0.213
BMA	0.106	0.023	0.497	0.022	0.767	-	0.086	0.086	NA	0.014	0.053	0.021	0.046	0.336	0.107	0.159	0.158	0.170
CBU	0.108	0.016	0.061	0.012	0.200	0.019	-	0.173	NA	***0.009***	0.045	0.011	0.038	0.042	0.094	0.175	0.158	0.174
FVO	0.094	0.024	0.059	0.032	0.210	0.020	0.032	-	NA	0.022	0.049	0.034	0.046	0.038	0.094	0.165	0.141	0.151
RFR	NA	NA	NA	NA	NA	NA	NA	NA	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
SLU	0.099	0.010	0.486	***0.008***	0.840	0.054	***0.021***	0.106	NA	-	0.046	0.012	0.036	0.275	0.092	0.164	0.811	0.162
FPI	0.064	0.038	0.422	0.418	0.820	0.356	0.323	0.315	NA	0.340	-	0.049	***0.012***	0.315	0.064	0.132	0.571	0.113
GER	0.087	0.010	0.423	***-0.000***	0.885	0.116	0.035	0.213	NA	0.050	0.4115	-	0.312	0.236	0.079	0.158	0.752	0.154
LTA	0.074	0.031	0.476	0.321	0.861	0.292	0.253	0.285	NA	0.250	0.041	0.040	-	0.292	0.075	0.146	0.645	0.132
SPA	0.036	0.023	0.132	0.028	0.838	0.048	0.316	0.250	NA	0.036	0.039	0.030	0.038	-	0.037	0.118	0.070	0.083
TRI	0.032	0.074	0.172	0.591	0.773	0.665	0.630	0.570	NA	0.632	0.425	0.579	0.501	0.229	-	0.130	0.243	0.065
AIG	0.085	0.142	0.393	0.862	0.313	0.723	0.887	0.752	NA	0.841	0.662	0.864	0.730	0.612	0.550	-	0.352	0.369
SGU	0.037	0.126	0.251	0.143	0.600	0.761	0.828	0.658	NA	0.152	0.112	0.134	0.126	0.343	0.064	0.116	-	0.075
MSA	0.036	0.137	0.293	0.872	0.641	0.816	0.914	0.699	NA	0.863	0.572	0.871	0.670	0.418	0.251	0.121	0.213	-

B)

	SFB	GSL	ALC	ESM	BFI	BMA	CBU	FVO	RFR	SLU	FPI	GER	LTA	SPA	TRI	AIG	SGU	MSA
SFB	-	**0.546**	0.017	**0.433**	0.088	0.100	0.046	0.078	0.027	0.100	0.149	**0.556**	**0.241**	**0.446**	**0.415**	0.094	**0.312**	0.100
GSL		-	**0.653**	**0.265**	**0.717**	**0.728**	**0.689**	**0.697**	**0.680**	**0.728**	**0.486**	**0.624**	**0.217**	**0.300**	0.049	**0.723**	**0.233**	**0.728**
ALC			-	**0.611**	-0.016	-0.000	-0.069	-0.017	-0.093	-0.000	0.224	**0.828**	**0.365**	**0.620**	**0.544**	-0.008	**0.380**	-0.000
ESM				-	**0.705**	**0.728**	**0.643**	**0.658**	**0.622**	**0.728**	**0.337**	0.209	0.084	-0.091	**0.289**	**0.717**	**0.294**	**0.728**
BFI					-	0.000	0.000	0.029	0.000	0.000	0.308	**0.921**	**0.460**	**0.702**	**0.643**	0.000	**0.469**	0.000
BMA						-	0.000	0.053	0.000	0.000	0.335	**0.927**	**0.489**	**0.721**	**0.661**	0.000	**0.493**	0.000
CBU							-	-0.040	0.000	0.000	0.237	**0.907**	**0.387**	**0.652**	**0.596**	0.000	0.408	0.000
FVO								-	-0.069	0.053	0.269	**0.892**	**0.417**	**0.665**	**0.610**	0.042	**0.433**	0.053
RFR									-	**0.000**	0.212	**0.903**	0.362	**0.636**	**0.581**	0.000	0.387	0.000
SLU										-	0.335	**0.927**	**0.489**	**0.721**	**0.661**	0.000	**0.493**	0.000
FPI											-	**0.555**	0.053	**0.368**	**0.361**	0.322	**0.267**	0.335
GER												-	**0.394**	0.172	**0.649**	**0.924**	**0.579**	**0.927**
LTA													-	0.119	0.122	**0.475**	0.137	**0.489**
SPA														-	**0.324**	**0.711**	**0.331**	**0.721**
TRI															-	**0.652**	0.117	**0.661**
AIG																-	**0.481**	0.000
SGU																	-	**0.493**
MSA																		-

Figure 1

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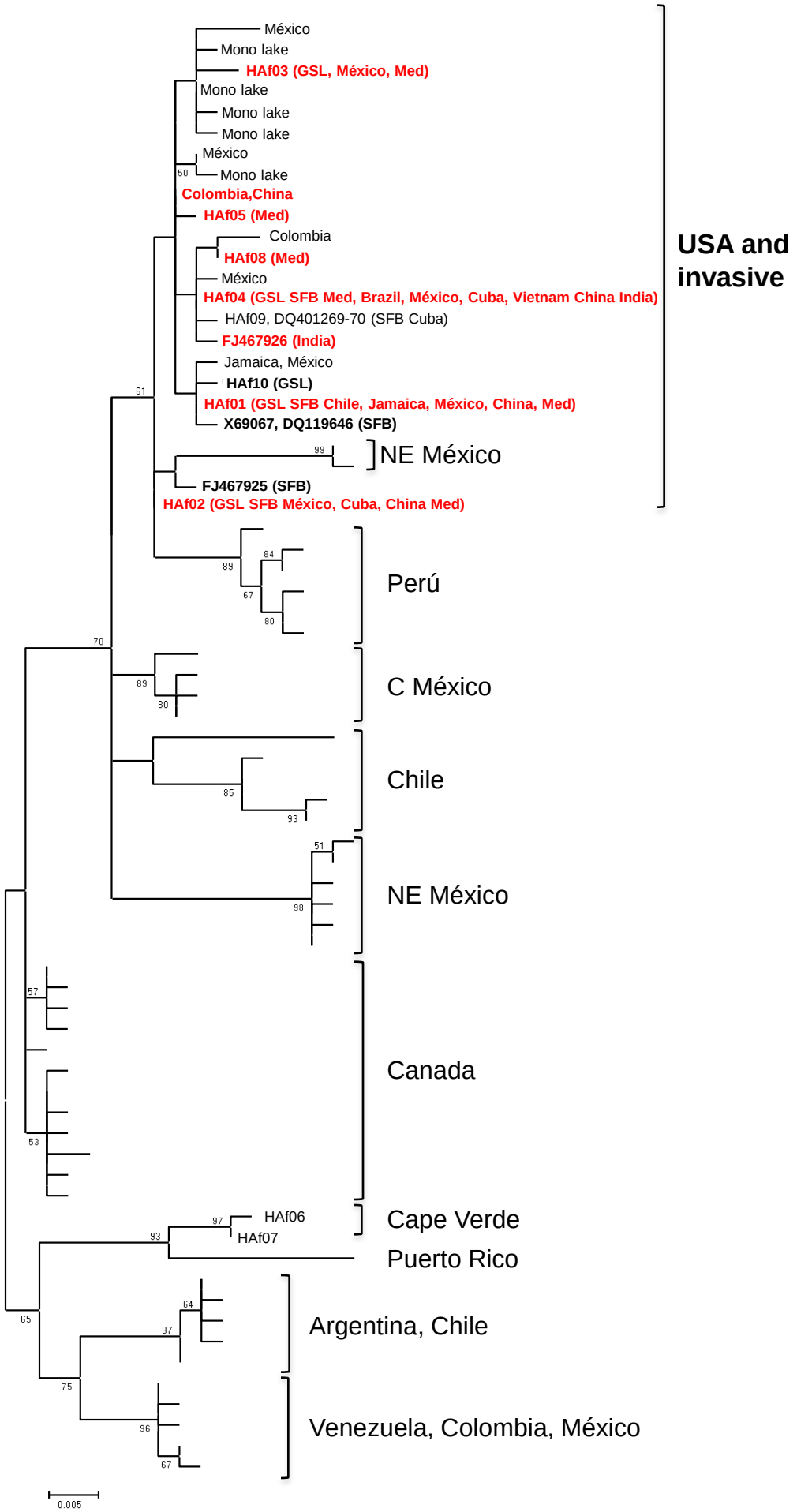

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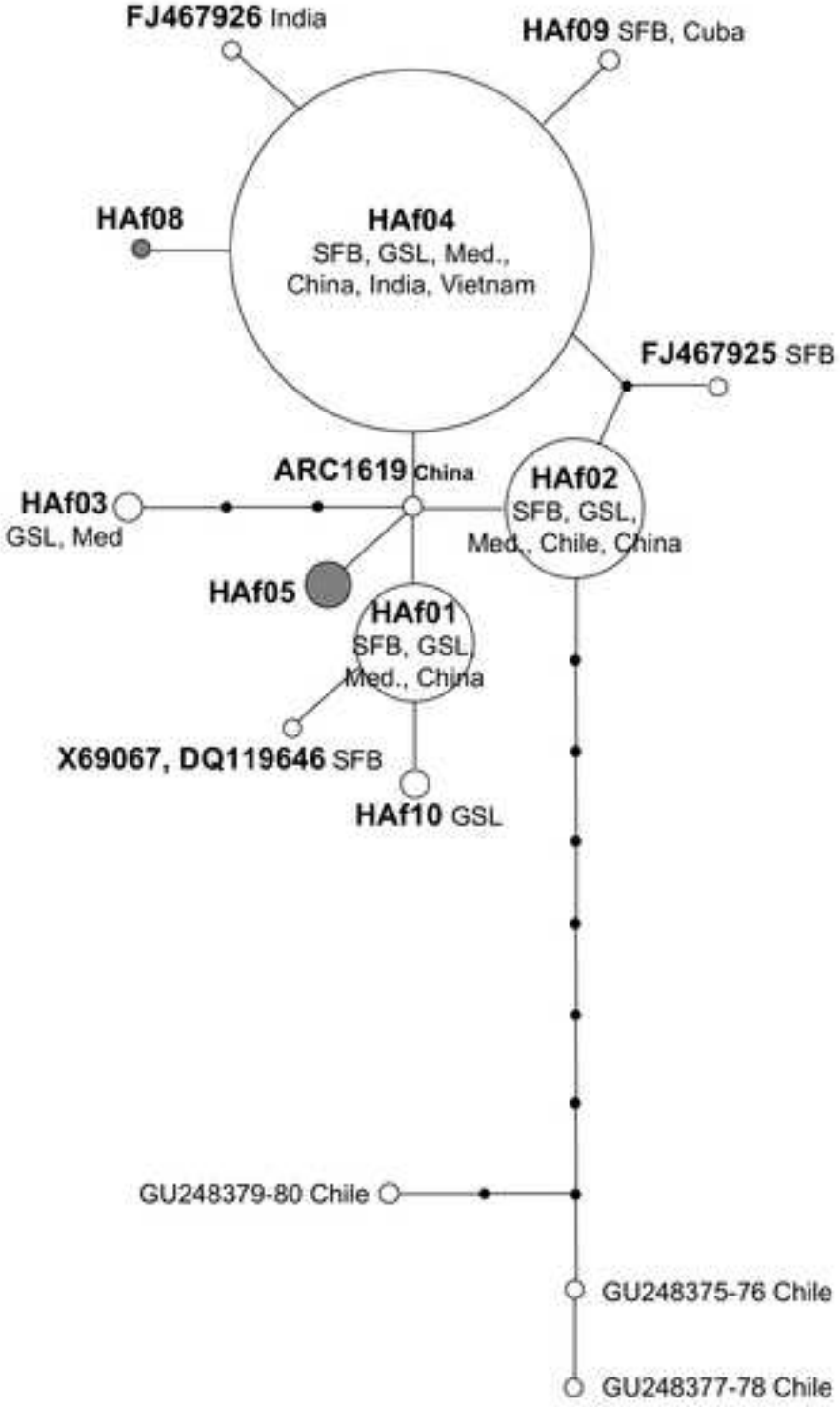

Figure 3
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