A peer-reviewed version of this preprint was published in PeerJ on 7 November 2013.

View the peer-reviewed version (peerj.com/articles/200), which is the preferred citable publication unless you specifically need to cite this preprint.

Muñoz J, Amat F, Green AJ, Figuerola J, Gómez A. 2013. Bird migratory flyways influence the phylogeography of the invasive brine shrimp *Artemia franciscana* in its native American range. PeerJ 1:e200 https://doi.org/10.7717/peerj.200

Bird migratory flyways influence the phylogeography of the invasive brine shrimp *Artemia franciscana* in its native American range

Since Darwin's time, waterbirds have been considered an important vector for the dispersal of continental aquatic invertebrates. Bird movements have facilitated the worldwide invasion of the American brine shrimp *Artemia franciscana*, transporting cysts (diapausing eggs), and favouring rapid range expansions from introduction sites. Here we address the impact of bird migratory flyways on the population genetic structure and phylogeography of *A. franciscana* in its native range in the Americas. We examined the sequence variation for two mitochondrial gene fragments (COI and 16S for a subset of the data) in a large set of population samples representing the entire native range of *A. franciscana.* Furthermore, we performed Mantel tests and redundancy analyses (RDA) to test the role of flyways, geography and human introductions on the phylogeography and population genetic structure at a continental scale. *A. franciscana* mitochondrial DNA was very diverse, with two main clades, largely corresponding to Pacific and Atlantic populations, mirroring American bird flyways. There was a high degree of regional endemism, with populations subdivided into at least 12 divergent, geographically restricted and largely allopatric mitochondrial lineages, and high levels of population structure (Φ_{ST} of 0.92), indicating low ongoing gene flow. We found evidence of human-mediated introductions in nine out of 39 populations analysed. Once these populations were removed, Mantel tests revealed a strong association between genetic variation and geographic distance (i.e., isolation-by-distance pattern). RDA showed that shared bird flyways explained around 20% of the variance in genetic distance between populations and this was highly significant, once geographic distance was controlled for. The variance explained increased to 30% when the factor human introduction was included in the model. Our findings suggest that bird-mediated transport of brine shrimp propagules does not result in substantial ongoing gene flow; instead, it had a significant historical role on the current species phylogeography, facilitating the colonisation of new aquatic environments as

PeerJ PrePrints | https://peerj.com/preprints/79v1/ | v1 received: 9 Oct 2013, published: 9 Oct 2013, doi: 10.7287/peerj.preprints.79v1

they become available along their main migratory flyways.

Joaquín Muñoz, Francisco Amat, Andy J. Green, Jordi Figuerola, Africa Gómez

Joaquín Muñoz (quini@ebd.csic.es), Andy J. Green (ajgreen@ebd.csic.es), Jordi Figuerola (jordi@ebd.csic.es). Department of Wetland Ecology, Estación Biológica de Doñana (CSIC), Isla de la Cartuja, Av. Américo Vespucio, s/n. 41092, Seville, Spain

Francisco Amat (amat@iats.csic.es). Instituto de Acuicultura de Torre de la Sal (CSIC), 12595, Ribera de Cabanes, Castellón, Spain

Africa Gómez (a.gomez@hull.ac.uk). Department of Biological Sciences, University of Hull, Cottingham Rd, Hull, HU6 7RX, U.K. (corresponding author)

Introduction

Since Darwin (1859), the role of birds as dispersal vectors for the diapausing propagules of continental aquatic organisms has been recognized (Carlquist 1983; Bilton *et al.* 2001; Green & Figuerola 2005). A range of studies have emphasized the importance of dispersal at short and long range by waterbirds for both passively dispersed aquatic invertebrates – through their diapausing eggs or other dispersing stages - and plants – through their seeds – (Figuerola & Green 2002; Green & Figuerola 2005; Green *et al.* 2008; Brochet *et al*. 2010a, 2010b; Van Leeuwen *et al*. 2012a, 2012b). Such long-distance, bird-mediated dispersal between aquatic habitats should result in high population gene flow and reduced population or phylogeographic structure. In stark contrast to this prediction, ongoing gene flow between populations has consistently been found to be low (Boileau *et al.* 1992; De Meester *et al.* 2002) and phylogeographic structures quite marked, with high levels of endemism (Gómez *et al.* 2000; Gómez, Montero-Pau, *et al.* 2007; De Gelas & De Meester 2005; Muñoz *et al.* 2008). This paradox has been explained through a combination of high population growth rates, rapid local adaptation and a buffering effect of large egg banks accumulated in sediments, resulting in a monopolisation of resources by the few initial founders, reducing the impact of further immigrants on population structure – what was termed the "monopolisation hypothesis" (De Meester *et al.* 2002). Consistent with this hypothesis, several studies failed to uncover any relationship between the geographic distribution of genetic lineages and bird migration patterns (Gómez, Montero-Pau, *et al.* 2007; Mills *et al.* 2007; Muñoz *et al.* 2008). In contrast, the perceived similarity between bird migratory pathways and the distribution of passively dispersed invertebrate genetic lineages suggests that waterfowl are important dispersal vectors (Taylor *et al.* 1998; Freeland *et al.* 2000; Hebert *et al.* 2003). In fact, Figuerola et al. (2005) tested explicitly the

relationship between bird movements and aquatic invertebrate population genetic structure, revealing a significant association between historical ringing data – used as a proxy of bird-mediated dispersal between populations – and population genetic distances for two cladocerans and a bryozoan in North America, concluding that birds significantly contributed to effective dispersal.

Given that continental aquatic invertebrates are unlikely to be in migration-drift equilibrium (Boileau *et al.* 1992; Gómez, Montero-Pau, *et al.* 2007), recent studies have interpreted population isolation-by-distance (IBD) patterns as a signature of historical patterns due to sequential colonisation events, as newly available habitats are more likely to be colonised by nearby populations, with little further impact of gene flow (Gómez, Montero-Pau, *et al.* 2007; Mills *et al.* 2007; Muñoz *et al.* 2008). Therefore, associations between bird movements and genetic distance in aquatic invertebrates based on mitochondrial markers could result from bird-mediated historical colonisation of newly available habitats, instead of ongoing gene flow (Figuerola *et al.* 2005). Shedding light on the role of bird movements on the geographic distribution of genetic lineages would help to understand the structuring of genetic diversity and phylogeography in passively dispersed aquatic invertebrates.

Artemia franciscana (Kellogg, 1906)(Crustacea: Anostraca), the most widely distributed brine shrimp in America, occurs in hypersaline habitats from Canada to Chile and many Atlantic islands (Hontoria & Amat 1992). It is found in a wide diversity of isolated water bodies, including coastal rock pools and lagoons, inland playas and high mountain salt lakes, permanent prairie salt lakes and commercial salt works (Van Stappen 2002), spanning an extreme range of water chemistry compositions and salinity from high carbonate, or high sulphate athalasic waters to seawater salterns (Bowen *et al.* 1988). It is a sexual species, and females produce two types of eggs: subitaneous

eggs in benign environmental conditions suitable for population growth, and diapausing eggs (i.e., cysts) during adverse conditions. *Artemia* cysts are amongst the most resistant animal life forms, surviving extreme environmental stresses including UV radiation, desiccation, thermal extremes and anoxia (Clegg 2005). Cysts accumulate at the shoreline and in egg banks in lake sediments (Moscatello & Belmonte 2009), and are readily dispersed by birds, which are the main vectors between catchments. Wind dispersal occurs but over much shorter distances (<1 km, Vanschoenwinkel *et al*. 2009). Many migratory bird species, especially shorebirds, use *Artemia* habitats and adult brine shrimp - often carrying viable cysts - can make up a substantial component of their diet (Anderson 1970; Sánchez *et al.* 2005; Varo *et al.* 2011; Vest & Conover 2011). Birds can disperse cysts between habitats either externally - attached to their feathers or feet - or internally in their digestive tract (Brochet et al. 2010b; Green *et al.* 2005, 2013; Sánchez *et al.* 2007, 2012). Research showing the internal transport of viable *A. franciscana* cysts in the field by the American Avocet, *Recurvirostra americana* (AJG, unpublished data), confirms shorebirds as an effective agent of dispersal in North America (see also Green *et al*. 2005). Recently, Viana *et al*. (2013) estimated the maximum dispersal for *Artemia* cysts via wildfowl as between 230 and 1209 km based on gut passage times of cysts ingested by captive ducks and the distances moved by wild ducks.

Populations of *A. franciscana* have substantial levels of genetic (Abreu-Grobois & Beardmore 1982; Gajardo *et al.* 1995; Maniatsi *et al.* 2009) and morphological variation (Hontoria & Amat 1992), and are locally adapted to the ionic composition of their habitats (Bowen *et al.* 1988). Indeed, effective reproductive isolation between some populations is due to different ranges of tolerance to ionic compositions (Bowen *et al.* 1988), and so this taxon is regarded by some authors as a “superspecies” (Bowen *et al.* 1988). Nevertheless, despite half a century of research for aquaculture and

ecotoxicology, comprehensive large-scale phylogeographic surveys of *A. franciscana* are lacking.

Cysts from *A. franciscana* – harvested mainly from populations in San Francisco Bay saltworks and the Great Salt Lake in the U.S.A. – have been used globally as a food source in aquaculture and in the pet fish trade for decades (Abatzopoulos 2002; Amat *et al.* 2005, 2007). Effluents from fish farms are likely to contain cysts that can potentially colonise nearby natural wetlands. In addition, the introduction of *A. franciscana* has been and still is promoted worldwide to increase salt production or to generate local sources of cysts until as recently as 1993 (Tackaert & Sorgeloos 1993; Sui et al. 2012). As a result of such accidental and intentional inoculations, *A. franciscana* has become an invasive species in saline and hypersaline wetlands worldwide (Muñoz & Pacios 2010; J Muñoz, A Gómez, J Figuerola, F Amat, C Rico, AJ Green, 2013 unpublished). For instance, this invasion has led to rapid local extinction of native *Artemia* species in the Mediterranean region (Amat *et al.* 2005). Commercial strains of *A. franciscana* were also introduced in various American sites in the 1970s (Camara 2001; Amat *et al.* 2004). In Brazil, further spreading of the species, probably via bird movements, was noticed within a few years of its introduction in areas where it was previously absent (Camara 2001). However, the impact of these introductions on the genetic diversity and structure of native American populations has yet to be investigated.

Artemia franciscana represents a very interesting model to test the effect of bird movements on the geographic distribution of genetic lineages and patterns of genetic variation in aquatic invertebrates since (1) its distribution encompasses three continental-scale bird migratory flyways spanning both North and South America (i.e., the Pacific, Central and Atlantic flyways), but is highly fragmented due to its habitat

requirements (hypersaline lakes), (2) its habitats are frequented by migratory shorebirds; *Artemia* is an important prey of these and other waterbirds and its cysts can be readily quantified in waterbird excreta (Green *et al.* 2005; Sánchez *et al.* 2007), and (3) the intentional or accidental inoculations outside the native range may be affecting its natural population genetic structure.

Here, we carry out the first comprehensive phylogeographic study of *A. franciscana* throughout its known native range (i.e., from Central Canada to southern Chile and Argentina, including the Caribbean islands) using sequence variation for two mitochondrial genes (COI and 16S). Our results indicate a high level of genetic structure and endemism at a continental scale, identify the impact of human introductions and suggest a direct link between bird migratory routes (i.e., flyways) and the historical colonization of *A. franciscana* throughout the Americas, revealing a key role for birds in initial founder events.

Methods

Samples, laboratory procedures, and sequences

We obtained samples from 39 *A. franciscana* populations across its American geographical distribution, from Canada to Chile and Argentina, including Caribbean islands and a population from Cape Verde (Table 1 and Figure 1). Most samples were cysts obtained from the 'cyst-bank' of the Instituto de Acuicultura de Torre de la Sal (CSIC, Castellón, Spain), collected between 1984 and 2000. Four Canadian samples were collected in the field in 2009, two of them (MANW and CHAP) as adults, which were preserved in absolute ethanol until needed. An additional cyst sample from Mono Lake (U.S.A.), collected in the 1970s was kindly provided by the Artemia Reference Centre (ARC 270). A few cyst samples that yielded poor quality DNA extractions were

subject to hatching and the resulting nauplii used for DNA extractions (i.e., MexCB and GUA samples).

DNA extractions were carried out on individual cysts (previously rinsed in distilled water), whole nauplii or partial adults using a HotSHOT protocol (Montero-Pau *et al.* 2008). We used *Artemia*-specific primers 1/2COI_Fol-F and 1/2COI_Fol-R (Muñoz *et al.* 2008) to amplify and sequence a 709 bp fragment of the mitochondrial Cytochrome *c* Oxidase Subunit I gene (COI). We also amplified and sequenced a 535 bp fragment of the 16S ribosomal RNA gene for a subset of individuals carrying different COI haplotypes to facilitate comparison with other published sequences using primers 16Sar-5'/16Sbr-3' (Palumbi 1996). PCRs were performed in 20 µL total volume containing 1x reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 units *Taq* DNA polymerase (Bioline, London, UK) and 0.5 µM of each primer. PCR conditions were as follows: 94ºC for 3 min, followed by 35 cycles of 45 s at 94ºC, 60 s at 45ºC (60-64ºC for 16S locus), and 60 s at 72ºC, followed by 5 min at 72 ºC. PCR products were purified for sequencing using ExoSAP-IT® (Exonuclease I and Shrimp Alkaline Phosphatase in buffer; USB Corp., Ohio, USA), cleaned with Sephadex®-G50 (GE Healthcare Corp.), and labelled using the BigDye Terminator Sequencing Ready Reaction v3.1 kit (Applied Biosystems). The resulting fragments were separated on an ABI 3130xl genetic analyzer. Sequences were checked, edited, and aligned using Sequencher™ v4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). All sequences were deposited in GenBank [accession numbers KF662951 - KF663043]. Available published sequences of the same gene fragments, to which we could assign a geographic origin, were also included in our phylogenetic analyses [GenBank: DQ401259–DQ401278, GU248382-GU248387, FJ007820-FJ007834, AF202735-AF202753].

Genetic analyses

Neighbour Joining (NJ) and Maximum Likelihood (ML) phylogenetic trees were inferred for both COI and 16S gene fragments. NJ trees were constructed using evolutionary distances computed using the Maximum Composite Likelihood method and 1000 bootstrap replicate tests in MEGA5 (Tamura *et al.* 2011). The best-scoring ML trees for COI were estimated using RAxML-VI-HPC v. 7.2.8 (Stamatakis 2006) on the CIPRES portal at the San Diego Supercomputer Center (http://www.phylo.org), optimising free model parameters and executing 1000 rapid bootstraps. Average genetic distances between the main COI lineages – corrected by the K2P + G substitution model – were carried out using MEGA5. Additionally, to identify lineages in our COI dataset, we used the Automatic Barcode Gap Discovery (ABGD) approach (Puillandre *et al*. 2012) using the webtool (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html).

Intra-population gene diversity *Hs* (standardized haplotype diversity) for COI was computed using the program RAREFAC (Petit 1998) to account for population differences in sampling size. Nucleotide diversity and pairwise Φ_{ST} values for COI from all 39 sampled populations were estimated using ARLEQUIN v. 3.1. (Excoffier *et al.* 2005).

Testing isolation-by-distance patterns and effect of bird flyways

The significance of correlations between pairwise genetic and geographic distances (isolation-by-distance or IBD patterns) was tested using Mantel tests on IBDWS v.3.21 (Jensen *et al.* 2005). Prior to analyses in this section, populations inferred to be introduced intentionally by humans (see Table 1) were removed. For all sampled locations, precise decimal longitude and latitude coordinates were obtained using

Google Earth (http://earth.google.com). A geographic distance matrix was then computed using Geographic Distance Matrix Generator v.1.2.3 (Ersts, American Museum of Natural History, Center for Biodiversity and Conservation, http://biodiversityinformatics.amnh.org/open_source/gdmg). We used a population geographic distance matrix (Table S1) and a population genetic distance matrix for COI data (Φ_{ST} values using Kimura 2-Parameter as the evolutionary model, Table S2). The 99% confidence intervals for the slope and intercept were estimated using Reduced Major Axis (RMA) regression with 30,000 bootstrap randomizations using log km geographical distance.

We used canonical Redundancy Analysis (RDA) in CANOCO (ter Braak & Smilauer 2002) to estimate the relative contribution of geographic distances, migratory flyways and human introductions on genetic distance between populations. RDA, a multiple linear regression method widely used in community ecology, has recently been applied to infer the role of spatial versus environmental variables in structuring population genetics data (e.g. Orsini et al 2013). As the dependent matrix we used the sample loadings of a Principal Components Analysis calculated on Φ_{ST} values using Kimura 2-Parameter as the evolutionary model for COI data. Environmental variables were whether the flyway overlapped with the *Artemia* population (0 or 1 depending on the presence of birds from the Atlantic, Central or Pacific flyways in the area) and introduction history (0 or 1). We modelled spatial variables using latitude and longitude (x and y).

We used the overlap of sampled populations with the three main migratory flyways in America based on two sources (1) Boere and Stroud (2006) for shorebirds and (2) Birdlife International (extracted from

http://www.birdlife.org/datazone/userfiles/file/sowb/flyways/). These data were used as a proxy for bird movements between locations. This approach is likely to be a rough approximation to the probability of bird migration between locations, given that more precise estimates cannot be obtained due to the absence of sufficient shorebird ringing data for the whole of the Americas. Even if extensive ringing data was available, this would only estimate current bird movements, whereas climate changes since the Pleistocene are likely to have strongly affected bird movements (Alerstam 1993).

RDA analyses were carried out using each set of migratory flyway data (i.e., Boere and Stroud (2006), and Birdlife International information) and the variance partitioning calculated according to Borcard et al. (1992) when the model was significant. All environmental variables contributed to the full model, so we constructed two additional RDAs considering only flyway or introduction history as environmental variables (data files used in RDA are deposited in Dryad doi:xxxxx).

Results

Phylogenetic relationships and geographic distribution of lineages

Once PCR primers were removed and sequences trimmed to the same length, the 604 bp COI alignment contained 603 individuals newly sequenced in this study (see Table 1), which collapsed into 93 haplotypes. No ambiguities, insertions, deletions or stop codons were present in the alignment. There were 121 variable sites, 86 of them parsimony informative, and 104 synonymous and 15 non-synonymous substitutions.

Using the default parameters, ABGD did not find any partitions in our dataset, so we reduced X, (i.e., the minimum barcode gap width) as suggested by the program, to 1.0 and used Kimura 80, identifying 12 groups with a prior intraspecific distance of = 0.0028 (we use the term 'lineages' thereafter, see below for their geographic distribution). When

the prior intraspecific distance increases to 0.0046 (not high by any standards) then the number of partitions reduces again to 1.

Both phylogenetic approaches ML and NJ were highly consistent, with two highly supported main branches displaying a geographic distribution along the continent, one mainly Atlantic (lineages 9-12) and the other split between two sub-branches along the Pacific Rim (lineages 1-7) and in Central Canada (lineage 8). Overall, there were at least 12 mtDNA lineages, most of them well supported (Figure 2). With the exception of lineage 1, each of the lineages showed a restricted geographic distribution indicating a high level of regional endemism (Figure 3 for the geographic distribution of the lineages). Lineage 2 was found in a single coastal site in NE Mexico, Lineage 3 in the five locations in Peru, Lineage 4 in two high altitude populations from Central Mexico, lineages 5 and 7 in Central Chile, lineage 6 in a single sulphate-rich location in NE Mexico, lineage 8 in four locations in Saskatchewan, Canada, lineage 9 in Puerto Rico, lineage 10 in Cape Verde, lineage 11 in several locations around the Caribbean, Baja California and SW Mexico, and lineage 12 in Argentina and Chile. In stark contrast to the rest, lineage 1 was genetically diverse and geographically widespread, found across a large geographical area across both sides of the continent, including Brazil, Chile, Cuba, Jamaica, Mexico, USA and Colombia. Out of the 27 haplotypes in lineage 1, seven haplotypes were detected in SFB and GSL, the two commercialised populations in the USA that have been sources for the invasion in the Mediterranean (J Muñoz, A Gómez, J Figuerola, F Amat, C Rico, AJ Green, 2013 unpublished)(see section *Introduced populations* below). In addition, lineage 1 contained six closely related haplotypes only found in Mono lake – which harbours a population often considered as a separate species, *A. monica* – and haplotypes found in three Colombian populations (CSC, CT and CGZ), five Mexican populations, and the Jamaican population. Two other lineages

were distributed across the continental E-W divide, creating contact zones between lineages. In lineage 12, most of the haplotypes were found in Argentinean populations, but four of these haplotypes (three of them shared across populations) were found in a Chilean Altiplano population (Salar de Llamara). Lineage 11, mainly Caribbean, has a few haplotypes in two Mexican populations from the Pacific side (Las Coloradas and Faro San José, where they coexist with lineage 1). Finally, although both lineages 1 and 11 are found in Colombia, they were not found together in any of the populations sampled. The genetic divergence between the 12 main lineages ranged from 1.8% (between lineages 1 and 4) to 6.0% (between lineages 2 and 9) (Table 2).

The 16S alignment contained 408 bp from 122 individuals, which collapsed into 59 haplotypes. There were two singleton indels, 63 variable sites and 43 parsimony informative sites. In contrast to the COI analysis, the NJ and ML reconstructions were poorly resolved, especially the basal branches, but most of the lineages recovered by the COI analyses were also recovered for the 16S data, with variable levels of support (see Figure 4). COI Lineages 3, 5, 7 and 9 were highly supported for both ML and NJ analyses in the 16S analysis, whereas lineages 1 and 2 on the one hand and lineages 4 and 6 on the other, collapsed into poorly supported branches. The 16S analyses allowed us to assign several previously sequenced populations, which we were unable to sample, to COI lineages, particularly in NW America and the Caribbean. In addition, the 16S analysis revealed the presence of two new lineages in the Caribbean, one in the Virgin Islands, related to lineage 9 (Puerto Rico) and another in Inague Island (Bahamas) related to Lineage 12 (Argentina, Chile). Note that these maintain the relationship with Atlantic lineages. Regarding North-Western American populations - extensively sampled by Prof Sarane Bowen's group - in New Mexico, Nevada and British Columbia, they hold private haplotypes which appear in a poorly supported

branch with Mono Lake and Mexican haplotypes. Other populations (Clinton, Basque Lake, Baja California and Carrizo Soda lake) also appear in the composite lineage 1 and 2, underscoring the diversity of U.S.A. *A. franciscana* populations. Interestingly, 16S haplotypes from Jesse Lake (Nebraska) belong to the Central Canadian lineage (lineage 8) together with Little Manitou, Muskiki, Meacham and Chaplin haplotypes.

Intra- and inter-population genetic diversity in COI

The number of individuals sequenced per population ranged between 4 and 37, depending on available material (average 15.97; see Table 1 for estimates of π and H, and details of the haplotypes present in each population). The number of haplotypes per population ranged from one to seven. The highest standardized gene diversity (*Hs*) was found in the Mexican population MexH, whereas five populations (MexCe and Mex99 from Mexico, BRM from Brazil, PV from Peru, AMC, from Argentina, and CHLC from Chile) were fixed for a single haplotype. Most haplotypes were found within single countries, except for several haplotypes shared between some countries and the two commercial U.S.A. populations SFB and GSL (see Table 1).

Populations were strongly structured genetically (global Φ_{ST} = 0.92; 0.94 when putative introduced populations were removed), with Φ_{ST} being highly significant between all populations except for three lakes from Central Canada, plus one pair from Chile/Argentina (see Table S2).

Identification of introduced populations

We found genetic evidence for putative non-native populations originating from the commercialised SFB or GSL in nine sites from Mexico, Cuba, Jamaica, Brazil and Chile.

In these populations, at least one sampled individual shared a haplotype with SFB and/or GSL populations (see Table 1). Those populations showed three patterns: (1) all individuals sampled shared haplotypes with SFB and/or GSL (BRM, GUA, and CHLC); (2) populations had haplotypes shared with the commercialised populations and a further haplotype (Af19 for MEXLC and JAYA) which differs from Af18 (a common haplotype in SFB and GSL) by a single substitution; and (3) populations sharing some haplotypes with SFB and/or GSL, but which also had unrelated additional/private haplotypes (MexT, MexSQ, MexFSJ, and CHPI). For four of the nine putative introduced populations, the occurrence of intentional introductions had been previously reported either in the same or nearby sites (see references in Table 1). Note that introduced *A. franciscana* populations are likely to further expand into nearby suitable habitats due to passive dispersal by birds.

Isolation-by-distance pattern and the role of American migratory flyways

Mantel tests on pairwise genetic and geographic distances for populations ranging from Chile-Argentina to Canada, excluding those inferred to be introduced, revealed a strong IBD pattern (Figure 5) with a highly significant correlation between pairwise geographic and genetic distances, indicating that geographic distance between populations explains a large proportion of the genetic variability in the sampled area (R^2-value= 0.323, *p*-value <0.001).

RDA showed that both flyway and introduction history were significantly associated to population genetic distance ($p<0.02$ for all the correlations with genetic distance, whether or not geographical distance was controlled for). The effect of using different flyway data was quite small. Flyway explained 18.7-21.2% of variance in population genetic distance, depending on the flyway dataset used (Table 3). Genetic

distance was also affected by historical anthropogenic introductions (15.6% of variance explained) and both factors together (i.e., flyway and anthropogenic introduction) explained 30.6-31.2% of variance in genetic distance. In comparison, geographic distance only explained 8.2-8.7% of variance.

Discussion

Our analyses revealed that *A. franciscana* has a strong regional genetic structure in its native distribution range throughout the Americas, with twelve largely allopatric endemic lineages. Such high level of population structure, supported by a very high overall Φ_{ST} value, high number of private haplotypes and significant IBD patterns, indicate that the populations studied are not connected by high ongoing gene flow, pointing instead to the effects of genetic drift and persistent founder effects during historical colonisation processes and development of local adaptation (i.e., the Monopolisation hypothesis; De Meester et al. 2002). The few population pairs with non-significant population differentiation (three lakes from Central Canada, plus one pair from Chile/Argentina) are likely to reflect recent colonisation, rather than ongoing gene flow. Our data also reveals the impact of *A. franciscana* introductions on the phylogeography of the species, as the lineage including the commercialised SFB and GSL populations has now achieved the widest distribution across the continent, in some cases coexisting – and presumably hybridizing – with pre-existing native populations. In addition, our results suggest that migratory birds have an important role in the colonisation of new habitats and are associated with range expansions both in the history of *Artemia* colonisation across the Americas, and also at a local scale, where birds facilitate the expansion of introduced lineages.

358

359 *The role of bird migratory flyways*

360 Our study provides new evidence supporting the key historical role of waterbirds as the
361 main factor shaping the population genetic structure of continental aquatic invertebrates
362 at an intra-continental scale. The patterning of the main phylogenetic lineages, with an
363 Atlantic, Central and Pacific distribution – instead of a North American (Nearctic) *vs*.
364 South American (Neotropical) division reflecting the recognized zoogeographic regions
365 and the long isolation of the continents (Lomolino *et al.* 2010; Holt et al. 2013) - strongly
366 suggests that historical bird migratory flyways, which occur alongside both the major
367 coasts of this continent, determined the historical spread of *A. franciscana* genetic
368 lineages. Bird movements also might have allowed the subsequent persistence of this
369 structure by facilitating colonisation along each migratory flyway, which shaped the main
370 East-West division in mitochondrial lineages. RDA showed that the effect of migratory
371 flyways was highly significant, and accounted for 20% of the genetic variation between
372 populations once geographic distance was taken into account, suggesting that the
373 distribution of genetic lineages in *A. franciscana* is likely to reflect the impact of historical
374 bird flyways on native phylogeographic patterns. In addition, the strong detected IBD
375 pattern suggests that the chances of bird-mediated colonisation are highly distance
376 dependent (see Viana *et al*. 2013), although instances of long-distance dispersal and
377 colonisation, for example from Argentina to Chile, or to Colombia from northern
378 Caribbean populations, are also apparent from our data. A corollary of our results is that
379 bird movements must have shown some stability, forming parallel N-S flyways during the
380 time frame of *A. franciscana* population diversification (i.e., throughout the Pleistocene)
381 extending into new breeding areas becoming available in the north and new wintering
382 sites in the southern extreme of the continent (Buehler *et al.* 2006). These results are no

surprise given the high transport rates of *Artemia* cysts by waterbirds (Green *et al*. 2005; Sánchez *et al.* 2007) and the lack of other dispersal vectors. Migratory waterbirds have existed since the Early Cretaceous (Lockley *et al*. 2012). Although now-extinct migratory mammals were once major vectors of plant dispersal in the Americas (Janzen 1984), the hypersaline habitats used by *Artemia* are not conducive to dispersal by large mammals.

Our results for *A. franciscana* agree with other studies in N America pointing to an effect of bird movements on the genetic structure of passively dispersed aquatic invertebrates (Figuerola *et al.* 2005), but extends these findings to the whole of the Americas, and emphasizes the major role of bird movements in facilitating colonisation into new suitable habitats – in agreement with the patterns found in *A. franciscana* in the invaded Mediterranean range (J Muñoz, A Gómez, J Figuerola, F Amat, C Rico, AJ Green, 2013 unpublished), and those for *A. salina* in its native range (Muñoz *et al.* 2008). Although at a continental scale our results suggest that bird movements do not promote contemporary gene flow between *Artemia* populations, such gene flow may still have a role at a local scale or when new areas suitable for colonization become available.

Phylogeographic patterns

The high level of endemism and population structure in native *A. franciscana* populations, with low ongoing gene flow and occasional long-distance migration resembles the patterns found in *Artemia salina* (Muñoz *et al.* 2008), a sexual native Mediterranean brine shrimp species, and in a range of other aquatic passively dispersed taxa including sexual reproduction in their life cycles (Gómez *et al.* 2000; Edmands 2001; De Gelas & De Meester 2005; Mills *et al.* 2007; Ketmaier *et al.* 2008; Korn *et al.* 2010). Our results also expand and confirm the deep phylogenetic breaks found by

Maniatsi *et al*. (2009) in a mtDNA and nDNA study of a more limited number of populations revealing only three lineages. As populations of these passively dispersed organisms can be founded by a small number of propagules, followed by rapid population growth and establishment of large diapausing egg banks, this favours the presence of persistent/long term founder effects, thus reducing the effect of gene flow, possibly reinforced by the development of local adaptation, according to the Monopolisation hypothesis (De Meester *et al.* 2002). Cysts are undoubtedly still regularly dispersed between suitable habitats by waterbirds, but they are unlikely to become established owing to the Monopolisation effects.

Given the range of genetic divergence between lineages (from 2 to 6%) the time frame of their fragmentation can be approximated roughly using a COI molecular clock for other shrimp taxa (1.4% sequence divergence per million year; Knowlton & Weigt 1998), which translates into 1.4-4.3 million years of divergence (reaching the Pliocene), between *A. franciscana* lineages. Even a faster rate of 2% per million years will result on pre-Pleistocene divergence times between the main lineages. Therefore, a contribution of Pliocene/Pleistocene climatic oscillations to population fragmentations after range expansions across the continent can be inferred from our data, possibly allowing survival of lineages in separate geographical areas including Caribbean islands and areas in N and S America. Mexico has the highest lineage richness, with five out of the 12 COI lineages being native to this country - including lineages from both Pacific and Atlantic clades. These findings suggest that this region is likely to have supported separate refugia during climatically adverse periods. The occurrence of a highly divergent Central Canadian prairie lineage was unexpected, as an ice sheet covered this area during the last glacial maximum (Ehlers & Gibbard 2004). However, the 16S data from GenBank suggest that this lineage also occurs in more southern areas in

central USA (Nebraska), where it may have survived south of the ice sheets, and then undergone postglacial colonisation of Central Canada.

Following climate-driven turnover of hypersaline habitats, migratory shorebirds would be involved in expanding the lineages into newly appearing suitable habitats and the chances of successful spread would be strongly distance dependent. However, long-distance colonisation events must have also occurred. For example, assuming that the ancestor of lineages 9 and 10 inhabited the Caribbean area, the colonisation of Argentina by the ancestor of lineage 12 must have entailed successful transfer of some cysts between these distant areas (see Figure 2). The genetic composition of lineage 12, where most of the haplotypes were found in Argentinean populations (see also Figure 3) but four of them (three shared across populations) were found in a Chilean Altiplano population (Salar de Llamara), suggests recent colonisation from Argentina to Chile. Finally, the colonisation of Cape Verde islands, with private haplotypes distantly related to Caribbean lineages, must have involved long-distance transport, possibly from birds accidentally landing there after storms sent them off course, although we cannot rule out the possibility of an unreported human-mediated introduction involving cysts from a Caribbean population not included in our study. Shorebirds are likely vectors for such long-distance dispersal events, and have often been implicated in the dispersal of plants between North and South America, or to oceanic islands (Cruden 1966; Proctor 1968).

The 16S analysis also shows higher richness of lineage 12 in Argentina with two Chilean populations (Convento and Salar de Llamara), for which evidence of nuclear DNA (nDNA) introgression among lineages exists (Maniatsi *et al.* 2009). Furthermore, as migratory flyways overlap on some areas, this could have occasionally resulted in

transfers from the Atlantic to the Pacific coasts, as has been suggested for other passively dispersed aquatic species (Miura *et al.* 2011).

Natural spread of lineages from refugial areas is likely to result in contact zones between lineages, which we expect to be sharp, as we found between lineages 1 and 11 in Colombia, where despite two lineages being present in the area, there are no sites where both co-occur.

Taxonomic considerations

The COI gene is one of the most widely used tools for species delineation (Hebert, Penton, *et al.* 2004; Costa *et al.* 2007; Gómez, Wright, *et al.* 2007). Sequence divergence of 3% have been proposed as a threshold for species delimitation in crustaceans (Costa *et al.* 2007, but see Lefébure *et al.* 2006), but other approaches are also used, such as GMYC (Pons *et al.* 2009) or automatic barcode delimitation, which we used here (Puillandre *et al.* 2012). Our analysis revealed 12 lineages in *A. franciscana*, some of them, like lineages 9 (Puerto Rico) plus 10 (Cape Verde) compared to all the others, or lineages 11 (Circum Caribbean) plus 12 (Argentina-Chile) compared to all the others, have genetic divergences of over 5%. Surprisingly, reproductive isolation – due to ecological specialisation and local adaptation – has only been reported between Mono Lake and San Francisco Bay populations (Bowen *et al.* 1988) due to the inability of individuals of each of these populations to survive in each others' ecological conditions, but our data show they are very closely related. Indeed cross-fertility has been observed in the laboratory between the San Francisco Bay population and 15 other populations from the whole range of the species, including some populations included in our study that belong to very divergent mtDNA lineages such as Inague Saltern (Bahamas), Little Manitou (Canada, lineage 8), and Puerto Rico

(lineage 9) (Clark & Bowen 1976). Therefore, we concur with Bowen *et al.* (1988) in regarding *A. franciscana* as a very diverse "superspecies", where reproductive isolation mediated by habitat adaptation might occur, but populations in intermediate habitats could act as venues for genetic exchange between ecological isolates.

Impact of introductions on native population structure and management implications

Given the strong phylogeographic structure of *A. franciscana*, and the high level of private haplotypes found, we used haplotype sharing between the commercialised populations (SFB and GSL) and distant populations as a criterion of recent human mediated introduction. Using this criterion, we identified nine populations where genetic evidence pointed to putative human introductions into Mexico, Cuba, Jamaica, Brazil and Chile. For four of these populations, the occurrence of intentional introductions in the same or nearby sites during the 1970s could be documented. Therefore, our genetic data confirms that the established *A. franciscana* populations in these locations are, at least partially, of introduced origin, and validates our criterion. As for the impact of introductions on native populations, four Mexican populations contained additional private haplotypes not closely related to the introduced ones, which suggests the presence of a pre-existing population before the introduction and the likely introgression of both populations with persistence of native haplotypes, although nuclear loci would be necessary to confirm this. In two populations, we found a discrepancy between our genetic results and previously published ones. The first case is the MEXLC population, which we regard as introduced (lineage 1), whereas in Tizol-Correa *et al*. (2009) the haplotype found is shared with populations from our lineage 11. The second case is CHLC (Laguna Cejas, Chile), which we regard as introduced whereas Maniatsi *et al*. (2009) found that their mtDNA was most closely related to native populations from

Central Chile (which belong to our lineage 7). While these authors concluded that the discrepancies between their nDNA and mtDNA data from the latter population pointed to incomplete lineage sorting, an alternative explanation is that they are due to the population being admixed with native and introduced ancestry, as introduced *A. franciscana* is known to hybridize even with the genetically divergent *A. persimilis* (Kappas *et al.* 2009). This failure to detect introduced mtDNA lineages in this population might arise from reduced sample sizes, or indicate real temporal changes in these populations reflecting recent introductions, as samples are likely to have been obtained in different years (unfortunately, no collection dates for these population is reported in Maniatsi *et al.* 2009). Despite human introductions, haplotypes presumably from pre-existent populations have survived and coexist with introduced ones, although a possible loss of genetic diversity due to introductions cannot be ruled out, and should be investigated in the future, perhaps using sediment cores (a method widely used in the crustacean model organism *Daphnia*) from populations where commercialised strains were introduced.

The high genetic richness found in our COI analyses, and the presence of private haplotypes belonging to lineage 1 in distant populations away from SFB and GSL, including three Colombian and five Mexican populations likely to be native, indicates that the natural distribution of lineage 1 extended further than the two commercialized populations before any human introductions took place. This was also in evidence from our 16S analyses, which included more extended geographic coverage of NW America, and revealed further sites within lineage 1 in the W USA and British Columbia harbouring private haplotypes not found in SFB or GSL. This is also consistent with the presence of private and closely related haplotypes at Mono Lake. The peculiarity and fragmentation of the habitats used by the species, the potential of salinity and varying

ionic composition to act as a strong selective agent, the capacity to produce massive quantities of resting eggs that can be readily dispersed by birds, combined with the apparent limitation in modern gene flow, makes this group an ideal system for further studies testing the role of local adaptation and mass effects on reducing gene flow between populations.

Given the impact of the invasive *A. franciscana* across the world, and the high genetic and ecological richness of its native populations, further population translocations should be highly discouraged, and the use of native strains as a source of cysts should be encouraged even within the Americas.

Conclusions

Our analyses suggest that *A. franciscana* phylogeography in its native range was shaped by (1) Pliocene/Pleistocene climate fluctuations, which contributed to changes in the areas available to the species, (2) historical bird-mediated colonization along migratory flyways, which shaped the East-West population division, (3) strong and persistent founder events, facilitated by high population growth rates and large population sizes, preventing further gene flow despite ongoing bird-mediated dispersal, and (4) human introductions coupled with regional bird dispersal, explaining the large but localised geographic range of the lineages derived from the commercially exploited North American populations. Our findings suggest that, at a continental scale, bird-mediated transport of invertebrate propagules does not result in substantial ongoing gene flow, but instead determines species phylogeography, facilitating the colonisation of newly available aquatic environments along bird flyways.

Acknowledgements

We thank G. Van Stappen (Artemia Reference Centre) for providing the Mono Lake cyst sample and Joachim Mergeay, who read a previous version of this manuscript, and provided many constructive comments including suggesting using RDA. M.T. Bidwell helped with sampling in Canada.

References

Abatzopoulos TJ (2002) *Artemia: basic and applied biology*.

Abreu-Grobois FA, Beardmore JA (1982) Genetic differentiation and speciation in the brine shrimp *Artemia*. In: *Mechanisms of speciation* (ed. Barigozzi C), pp. 345–376. Alan R. Liss, New York.

Alerstam T (1993) *Bird Migration*. Cambridge University Press.

Amat F, Cohen RG, Hontoria F, Navarro C (2004) Further evidence and characterization of *Artemia franciscana* (Kellogg, 1906) populations in Argentina. *Journal of Biogeography*, 1735–1749.

Amat F, Hontoria F, Navarro JC, Vieira N, Mura G (2007) Biodiversity loss in the genus *Artemia* in the Western Mediterranean Region. *Limnetica*, **26**, 387.

Amat F, Hontoria F, Ruiz O Sanchez MI, Figuerola J, & Hortas F (2005) The American brine shrimp as an exotic invasive species in the western Mediterranean. *Biological Invasions*, **7**, 37–47.

Anderson W (1970) A preliminary study of the relationship of salt ponds and wildlife-South San Francisco Bay. *Calif Fish Game 56, 240-252. ILLUS. 1970*, **56**.

Bilton DT, Freeland JR, Okamura B (2001) Dispersal in freshwater invertebrates. *Annual Review of Ecology and Systematics*, **32**, 159–181.

Boere GC, Stroud DA (2006) The flyway concept: what it is and what it isn't. In: *Waterbirds around the world.* (ed G.C. Boere, C.A. Galbraith & D.A. Stroud), pp. 40–47. The Stationery Office, Edinburgh, UK.

Boileau MG, Hebert PDN, Schwartz SS (1992) Non-equilibrium gene frequency divergence : founder effects in natural populations. *Journal of Evolutionary Biology*, **5**, 25–39.

Borcard D, Legendre P, Drapeau P (1992) Partialling out the spatial component of ecological variation. *Ecology*, **73**, 1045-1055.

Bowen ST, Buoncristiani MR, Carl JR (1988) *Artemia* habitats: Ion concentrations tolerated by one superspecies. *Hydrobiologia*, **158**, 201–214.

Brochet, AL, M. Gauthier-Clerc, M. Guillemain, H. Fritz, A. Waterkeyn, A. Baltanas, and A. J. Green (2010b). Field evidence of dispersal of branchiopods, ostracods and bryozoans by teal (*Anas crecca*) in the Camargue (southern France). Hydrobiologia **637**:255-261.

Brochet, A. L., M. Guillemain, H. Fritz, M. Gauthier-Clerc, and A. J. Green. 2010a. Plant dispersal by teal (*Anas crecca*) in the Camargue: duck guts are more important than their feet. Freshwater Biology **55**:1262-1273.

598 Buehler DM, Baker AJ, Piersma T (2006) Reconstructing palaeoflyways of the late
599 Pleistocene and early Holocene Red Knot *Calidris canutus*. *Ardea*, **94**, 485–498.
600 Camara M (2001) Dispersal of *Artemia franciscana* Kellogg (Crustacea; Anostraca)
601 populations in the coastal saltworks of Rio Grande do Norte, northeastern Brazil.
602 *Hydrobiologia*, **466**, 145–148.
603 Carlquist S (1983) Intercontinental dispersal. *Sonderbd. naturwiss. ver Hamburg*, **7**, 37–
604 47.
605 Castro B (1993) Biologia y cultivo de *Artemia franciscana* en el Ex Lago de Texcoco, de
606 Ecatepec, Estado de México. Universidad Nacional Autónoma de México.
607 Castro BT, M.J. Castro, V.R.E. Marín, G.R. Young, D. Jenoure, M.G. Castro, S.A.
608 Malpica, & A.R. De Lara (2000) Calidad de quistes y morfometria de la poblacion
609 de *Artemia franciscana* (Kellogg, 1906) de la laguna pequenya de Yallahs,
610 Jamaica. *Ciencias Marinas*, **26**, 201–214.
611 Castro J, T Castro, J Sánchez, G Castro, A Castro, J Zaragoza, R de Lara, and M. del C
612 Monroy (2006) Cysts and nauplii biometry characteristics of seven *Artemia*
613 *franciscana* (Kellog, 1906) populations from Mexico. *Revista de biología marina y*
614 *oceanografía*, **41**, 187 – 193.
615 Clark LS, Bowen ST (1976) The genetics of *Artemia salina*. VII. Reproductive isolation.
616 *The Journal of heredity*, **67**, 385–8.
617 Clegg JS (2005) Desiccation tolerance in encysted embryos of the animal extremophile,
618 *Artemia*. *Integrative and comparative biology*, **45**, 715–24.
619 Costa FO, deWaard, J R, Boutillier, J, Ratnasingham, S, Dooh, RT, Hajibabaei, M &
620 Hebert, PD (2007) Biological identifications through DNA barcodes: the case of the
621 Crustacea. *Canadian Journal of Fisheries and Aquatic Sciences*, **64**, 272–295.
622 Cruden, RW (1966) Birds as Agents of Long-Distance Dispersal for Disjunct Plant
623 Groups of Temperate Western Hemisphere. Evolution 20:517-532.
624 Darwin CR (1859) On the origin of species by means of natural selection. John Murray,
625 London.
626 Edmands S (2001) Phylogeography of the intertidal copepod *Tigriopus californicus*
627 reveals substantially reduced population differentiation at northern latitudes.
628 *Molecular ecology*, **10**, 1743–50.
629 Ehlers J, Gibbard PL (2004) *Quaternary glaciations: extent and chronology, Part 2*.
630 Elsevier.
631 Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software
632 package for population genetics data analysis. *Evolutionary Bioinformatics Online*,
633 **1**, 47.
634 Figuerola J, Green AJ (2002) Dispersal of aquatic organisms by waterbirds: a review of
635 past research and priorities for future studies. *Freshwater Biology*, **47**, 483–494.
636 Figuerola J, Green AJ, Michot TC (2005) Invertebrate eggs can fly: evidence of
637 waterfowl-mediated gene flow in aquatic invertebrates. *The American Naturalist*,
638 **165**, 274–80.
639 Freeland JR, Romualdi C, Okamura B (2000) Gene flow and genetic diversity: a
640 comparison of freshwater bryozoan populations in Europe and North America.
641 *Heredity*, **85 Pt 5**, 498–508.
642 Frisch D, Green AJ, Figuerola J (2007) High dispersal capacity of a broad spectrum of
643 aquatic invertebrates via waterbirds. *Aquatic Sciences*, **69**, 568–574.
644 Gajardo GGM, Colihueque N, Parraguéz M, Sorgeloos P (1998) International Study on
645 *Artemia* LVIII. Morphologic differentiation and reproductive isolation of *Artemia*

populations from South America. *International Journal of Salt Lake Research*, **7**, 133–151.
Gajardo GGM, Conceicao M, Weber L, Beardmore JA (1995) Genetic variability and interpopulational differentiation of *Artemia* strains from South America. *Hydrobiologia*, **302**, 21–29.
Gelabert F, Solis L (1994) La selección del tamaño de particulas alimenticias por *Artemia* de Guantánamo, Cuba. *Rev. Invest. Mar*, **15**, 141–144.
De Gelas K, De Meester L (2005) Phylogeography of *Daphnia magna* in Europe. *Molecular ecology*, **14**, 753–64.
Gómez A, Carvalho GR, Lunt DH (2000) Phylogeography and regional endemism of a passively dispersing zooplankter: mitochondrial DNA variation in rotifer resting egg banks. *Proceedings of the Royal Society B, Biological sciences*, **267**, 2189–97.
Gómez A, Montero-Pau J, Lunt DH, Serra M, Campillo S (2007) Persistent genetic signatures of colonization in *Brachionus manjavacas* rotifers in the Iberian Peninsula. *Molecular Ecology*, **16**, 3228–3240.
Gómez A, Wright PJ, Lunt DH, Cancino JM, Carvalho GR, & Hughes, RN (2007) Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. *Proceedings of the Royal Society B, Biological sciences*, **274**, 199–207.
Green AJ, Figuerola J (2005) Recent advances in the study of long-distance dispersal of aquatic invertebrates via birds. *Diversity and Distributions*, **11**, 149–156.
Green AJ, Sánchez MI, Amat F, Figuerola J, Hontoria F, Ruiz O, & Hortas, F (2005) Dispersal of invasive and native brine shrimps *Artemia* (Anostraca) via waterbirds. *Limnology and Oceanography*, **50**, 737–742.
Green, A. J., D. Frisch, T. C. Michot, L. K. Allain, and W. C. Barrow. 2013. Endozoochory of seeds and invertebrates by migratory waterbirds in Oklahoma, USA. Limnetica **32**:39-46.
Green, A. J., K. M. Jenkins, D. Bell, P. J. Morris, and R. T. Kingsford. 2008. The potential role of waterbirds in dispersing invertebrates and plants in arid Australia. Freshwater Biology **53**:380-392.
Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 14812–7.
Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA Barcodes. *PLoS biology*, **2**, e312.
Hebert PDN, Witt JDS, Adamowicz SJ (2003) Phylogeographical patterning in *Daphnia ambigua*: regional divergence and intercontinental cohesion. *Limnology and Oceanography*, **48**, 261–268.
Holt, B. G. Lessard J-P, Borregaard MK, Fritz SA, Araújo MB, Dimitrov D, Fabre P-H, Graham CH. Graves GR, Jønsson KA, Nogués-Bravo D, Wang Z, Whittaker RJ, Fjeldså J, Carsten Rahbek 2013. An Update of Wallace's Zoogeographic Regions of the World. Science 339: 74-78.
Hontoria F, Amat F (1992) Morphological characterization of adult *Artemia* (Crustacea, Branchiopoda) from different geographical origins. American populations. *Journal of Plankton Research*, **14**, 1461–1471.
Janzen, D. H. 1984. Dispersal of Small Seeds by Big Herbivores - Foliage Is the Fruit. *American Naturalist* 123:338-353.

Jensen JL, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. *BMC genetics*, **6**, 13.
Kappas I, Baxevanis AD, Maniatsi S, Abatzopoulos TJ (2009) Porous genomes and species integrity in the branchiopod *Artemia*. *Molecular phylogenetics and evolution*, **52**, 192–204.
Ketmaier V, Pirollo D, Matthaeis E, Tiedemann R, Mura G (2008) Large-scale mitochondrial phylogeography in the halophilic fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840) (Branchiopoda: Anostraca). *Aquatic Sciences*, **70**, 65–76.
Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society B: Biological Sciences*, **265**, 2257–2263.
Korn M, Green AJ, Machado M, García-de-Lomas J., Cristo M, Cancela da Fonseca L., Frisch D., Pérez-Bote JL, & Hundsdoerfer AK (2010) Phylogeny, molecular ecology and taxonomy of southern Iberian lineages of *Triops mauritanicus* (Crustacea: Notostraca). *Organisms Diversity & Evolution*, **10**, 409–440.
Lefébure T, Douady CJ, Gouy M, Gibert J (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and evolution*, **40**, 435–47.
Lockley, M. G., Lim, J. D., Kim, J. Y., Kim, K. S., Huh, M., and Hwang, K. G. 2012. Tracking Korea's Early Birds: A Review of Cretaceous Avian Ichnology and Its Implications for Evolution and Behavior. *Ichnos-an International Journal for Plant and Animal Traces* 19:17-27
Lomolino MV, Riddle BR, Whittaker R, Brown JH (2010) *Biogeography*. Sinauer Associates.
Maniatsi S, Kappas I, Baxevanis AD, Farmaki T, Abatzopoulos TJ (2009) Sharp phylogeographic breaks and patterns of genealogical concordance in the brine shrimp *Artemia franciscana*. *International Journal of Molecular Sciences*, **10**, 5455–5470.
De Meester L, Gómez A, Okamura B, Schwenk K (2002) The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. *Acta Oecologica*, **23**, 121–135.
Mills S, Lunt DH, Gómez A (2007) Global isolation by distance despite strong regional phylogeography in a small metazoan. *BMC Evolutionary Biology*, **7**, 225.
Miura O, Torchin ME, Bermingham E, Jacobs DK, Hechinger RF (2011) Flying shells: historical dispersal of marine snails across Central America. *Proceedings of the Royal Society B: Biological sciences*, **279**, 1061–1067.
Montero-Pau J, Gómez A, Muñoz J (2008) Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnology and Oceanography- Methods*, **6**, 218–222.
Moscatello S, Belmonte G (2009) Egg banks in hypersaline lakes of the South-East Europe. *Saline systems*, **5**, 3.
Muñoz J, Gómez A, Green AJ, Figuerola, J, Amat, F. Rico, C (2008) Phylogeography and local endemism of the native Mediterranean brine shrimp *Artemia salina* (Branchiopoda : Anostraca). *Molecular Ecology*, **17**, 3160–3177.
Muñoz J, Pacios F (2010) Global Biodiversity and Geographical Distribution of Diapausing Aquatic Invertebrates: The Case of the Cosmopolitan Brine Shrimp, *Artemia* (Branchiopoda, Anostraca). *Crustaceana*, **83**, 465–20.

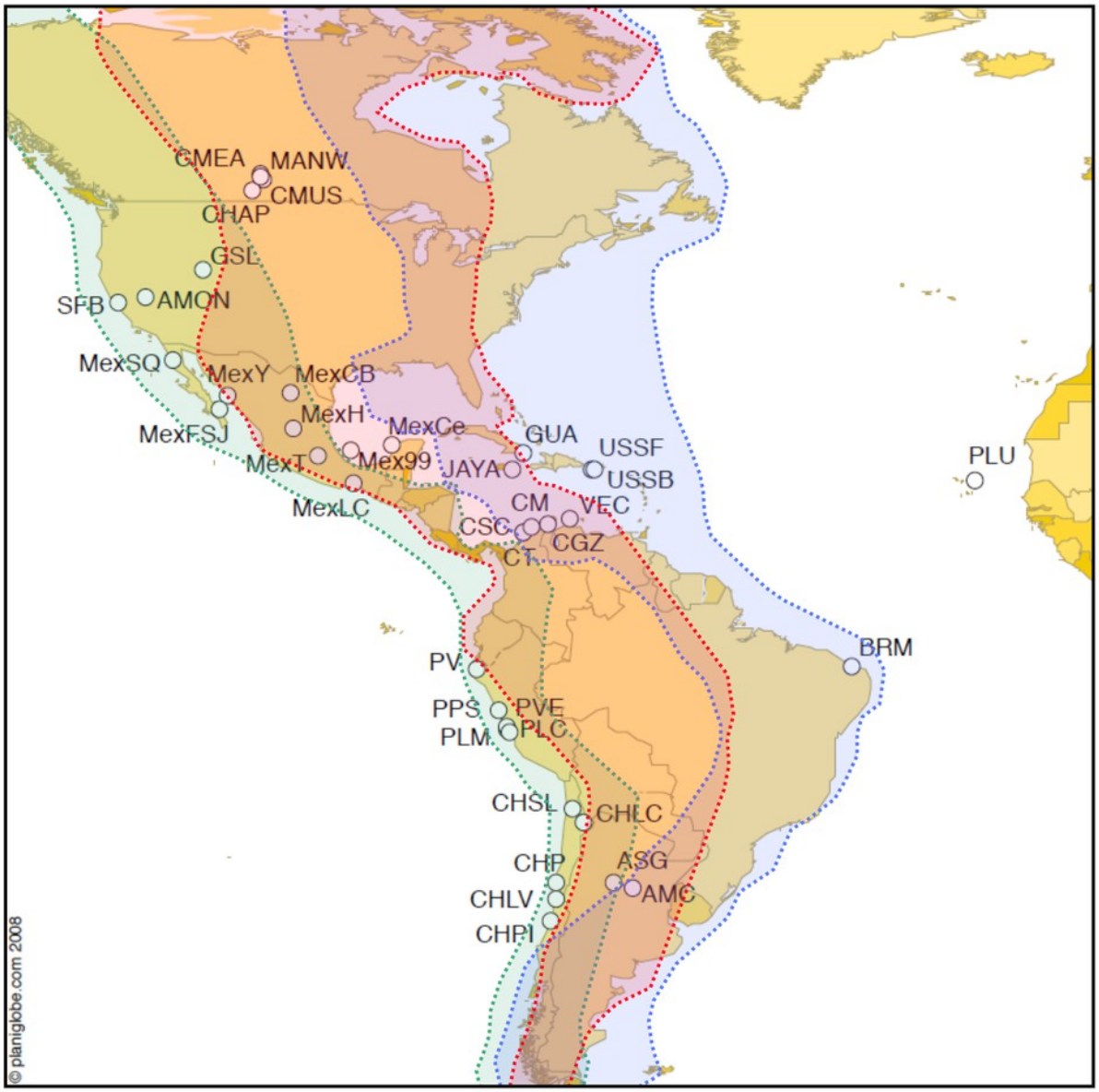
743 Palumbi S (1996) Nucleic acids II: the polymerase chain reaction. *Molecular*
744 *systematics*, **2**, 205–247.
745 Petit R (1998) Identifying populations for conservation on the basis of genetic markers.
746 *Conservation Biology*, **1**, 844–855.
747 Pons, Joan, Timothy G. Barraclough, Jesus Gomez-Zurita, Anabela Cardoso, Daniel P.
748 Duran, Steaphan Hazell, Sophien Kamoun, William D. Sumlin, and Alfried P. Vogler.
749 (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed
750 insects. *Systematic biology* **55**, 595-609.
751 Proctor, V. W. (1968). Long-distance dispersal of seeds by retention in digestive tract of
752 birds. *Science*, **160**, 321-322.
753 Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode
754 Gap Discovery for primary species delimitation. *Molecular Ecology*, ***21***, 1864-1877.
755 Sánchez MI, Green AJ, Amat F, Castellanos EM (2007) Transport of brine shrimps via
756 the digestive system of migratory waders: dispersal probabilities depend on diet and
757 season. *Marine biology*, **151**, 1407–1415.
758 Sánchez MI, Green AJ, Castellanos EM (2005) Seasonal variation in the diet of
759 Redshank *Tringa totanus* in the Odiel Marshes, southwest Spain : a comparison of
760 faecal and pellet analysis. *Statistica*, **52**, 210–216.
761 Sánchez MI, Hortas F, Figuerola J, Green AJ (2012) Comparing the dispersal potential
762 of a native and an invasive brine shrimp via waterbirds. *Freshwater Biology*, **50**,
763 737-742.
764 Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses
765 with thousands of taxa and mixed models. *Bioinformatics*, ***22***, 2688-2690.
766 Sui, L. Y., Q. Zhang, J. Wang, H. He, P. Sorgeloos, and G. Van Stappen. 2012. High-
767 Temperature Adapted *Artemia Franciscana* (Kellogg, 1906) from Vietnam Is a
768 Suitable Strain for Inoculation in Temperate Conditions of Bohai Bay Saltworks,
769 China. Crustaceana, **85,** 1709-1723.
770 van Leeuwen, C. H. A., G. van der Velde, J. M. van Groenendael, and M. Klaassen.
771 2012a. Gut travellers: internal dispersal of aquatic organisms by waterfowl.
772 Journal of Biogeography, **39,** 2031-2040.
773 van Leeuwen, C. H. A., G. van der Velde, B. van Lith, and M. Klaassen. 2012b.
774 Experimental Quantification of Long Distance Dispersal Potential of Aquatic
775 Snails in the Gut of Migratory Birds. Plos One **7,** e32292.
776 Van Stappen G (2002) Zoogeography. In: *Artemia: basic and applied biology* (ed.
777 Abatzopoulos ThJ, Beardmore JA, Clegg JS SP), pp. 171–224. Kluwer Academic
778 Publishing.
779 Tackaert W, Sorgeloos P (1993) Use of brine shrimp to increase salt production.
780 *Seventh Symposium on Salt*, **1**, 617–622.
781 Tamura K, Peterson D, Peterson N (2011) MEGA5: molecular evolutionary genetics
782 analysis using maximum likelihood, evolutionary distance, and maximum parsimony
783 methods. *Molecular Biology and Evolution*, **28**, 2731–273.
784 Taylor DJ, Finston TLTL, Hebert PDN (1998) Biogeography of a widespread freshwater
785 crustacean: pseudocongruence and cryptic endemism in the North American
786 *Daphnia laevis* complex. *Evolution*, **52**, 1648–1670.
787 ter Braak CJF, Šmilauer P (2002) CANOCO Reference Manual and CanoDraw for
788 Windows User's Guide: Software for Canonical Community Ordination (version 4.5).
789 Ithaca, NY, USA (www.canoco.com): Microcomputer Power.
790 Tizol-Correa R (2009) Caracterización morfométrica del camarón de salmuera
791 (Artemia), de Cuba. *Revista cubana de investigaciones pesqueras*, **26**, 25–30.

792 Tizol-Correa R, Maeda-Martínez AM, Weekers PHH, Torrentera L, Murugan G (2009)
793 Biodiversity of the brine shrimp Artemia from tropical salterns in southern México
794 and Cuba. *Current Science*, **96**, 81–87.
795 Vanschoenwinkel, B., S. Gielen, M. Seaman, and L. Brendonck. 2009. Wind mediated
796 dispersal of freshwater invertebrates in a rock pool metacommunity: differences in
797 dispersal capacities and modes. *Hydrobiologia* **635**:363-372.
798 Varo N, Green A, Sánchez M, Ramo C (2011) Behavioural and population responses to
799 changing availability of *Artemia* prey by moulting black-necked grebes, *Podiceps*
800 *nigricollis*. *Hydrobiologia* **664**, 163-171.
801 Vest JL, Conover MR (2011) Food Habits of Wintering Waterfowl on the Great Salt Lake,
802 Utah. *Waterbirds*, **34**, 40–50.
803 Viana DS, Santamaría L, Michot TC, Figuerola J (2013) Migratory strategies of
804 waterbirds shape the continental-scale dispersal of aquatic organisms. *Ecography*,
805 **36**, 430-438.
806

Figure 1

Map of *Artemia franciscana* sampled sites and American bird migratory flyways.

The sampled populations are shown, with indication of the main American migratory flyways following Birdlife International (see text for details). Green shading: Pacific flyway, red shading: Central flyway, blue shading, Atlantic flyway.



PeerJ PrePrints | https://peerj.com/preprints/79v1/ | v1 received: 9 Oct 2013, published: 9 Oct 2013, doi: 10.7287/peerj.preprints.79v1

Figure 2

Phylogenetic relationships of native *Artemia franciscana* COI haplotypes

The tree topology is the one obtained in the NJ analysis, with bootstrap values shown for NJ (below branches) and ML (above branches). Haplotypes found in the commercialised populations SFB and GSL are marked in red. Haplotype numbers and populations where these were found are noted at the tips. Each lineage label indicates which countries it is found in and its overlap with the Pacific, Atlantic or Central migratory flyways (P, A or C respectively).

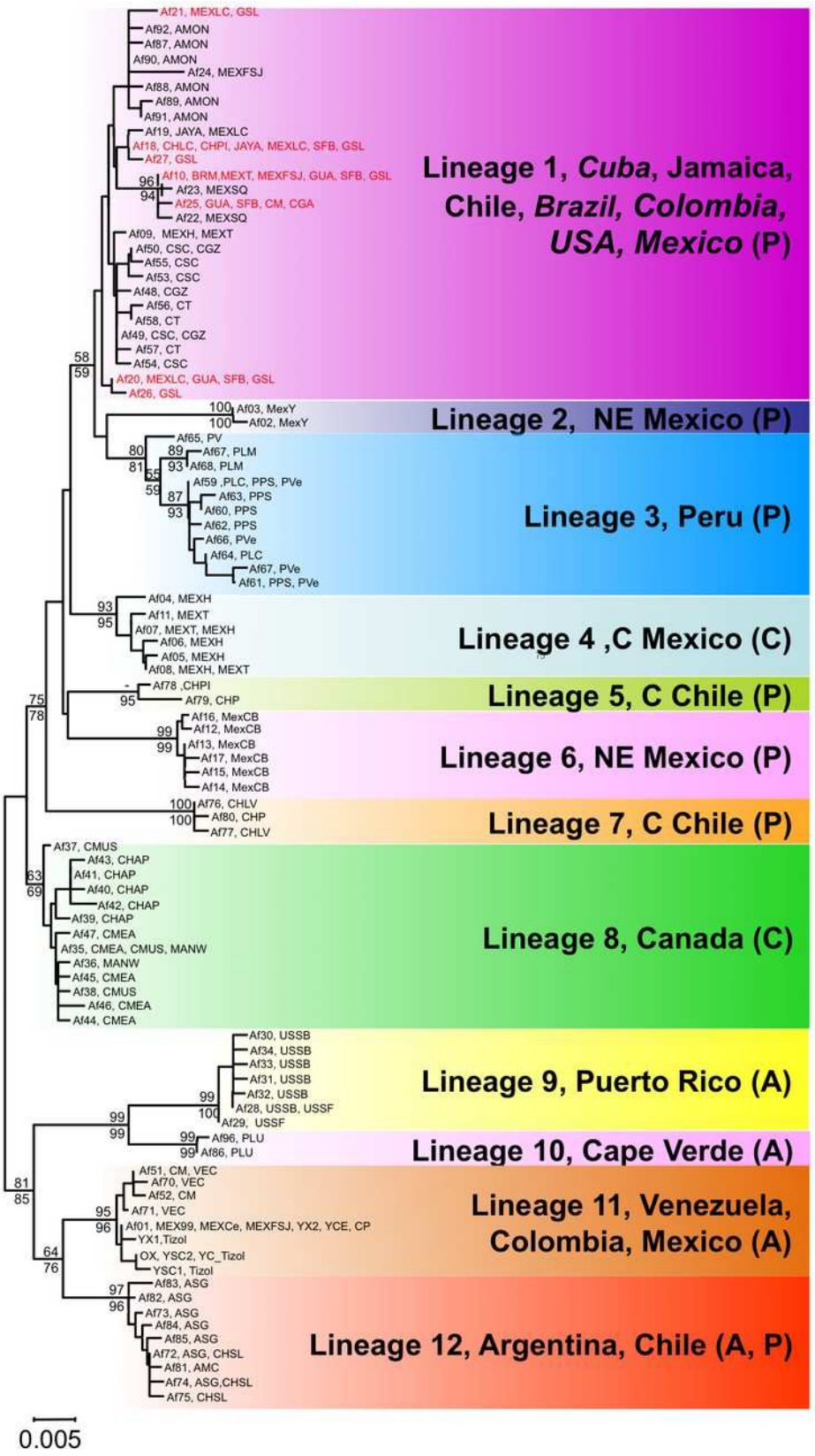


Figure 3

Geographic distribution of *Artemia franciscana* mtDNA lineages.

The distribution of each COI lineage is shown as areas with the same colour coding as in Figure 2. Disjunct areas are linked by lines. Introduced populations are denoted by a grey bucket. Only populations sampled for this study are included. Empty circles denote unsampled *A. franciscana* populations.

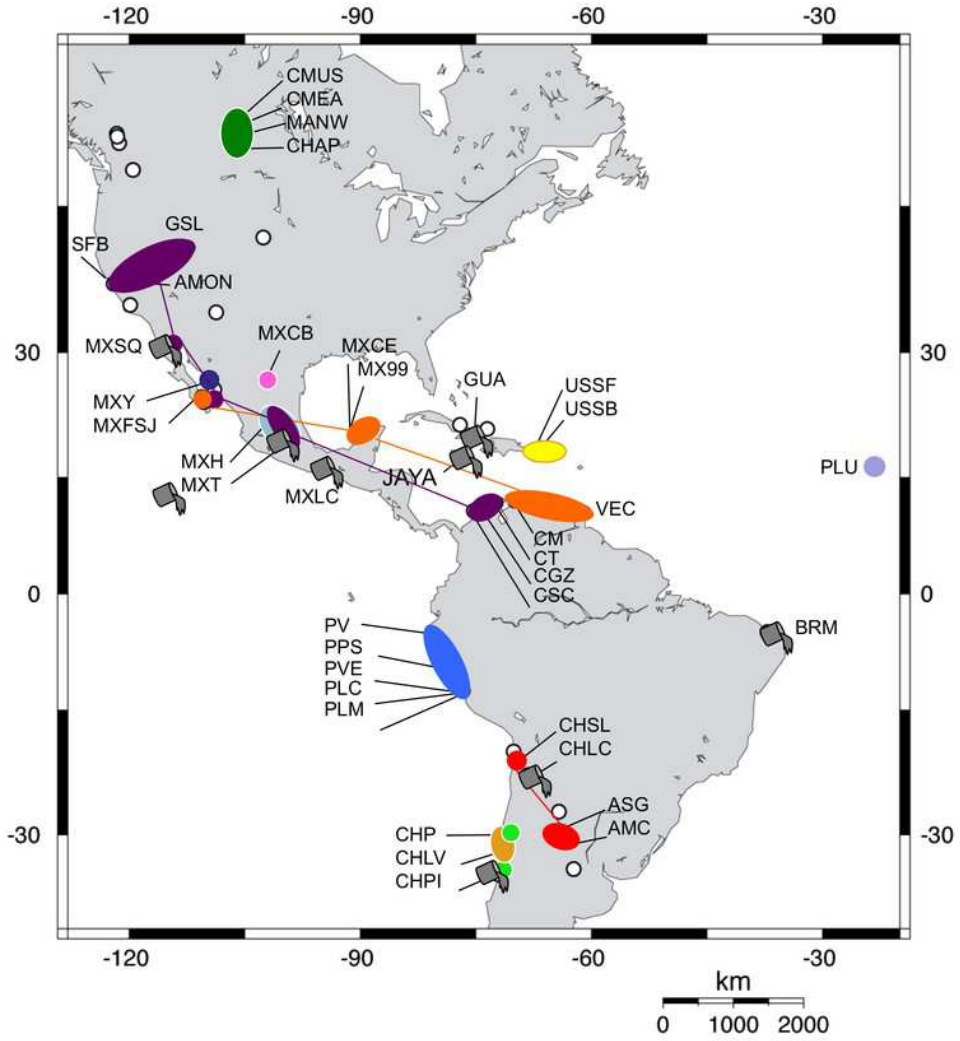


Figure 4

Phylogenetic relationships for native *Artemia franciscana* 16S.

The tree topology is the one obtained in the NJ analysis, with bootstrap values shown for NJ (below branches) and ML (above branches). Haplotypes found in the commercialised populations SFB and GSL are marked in red.

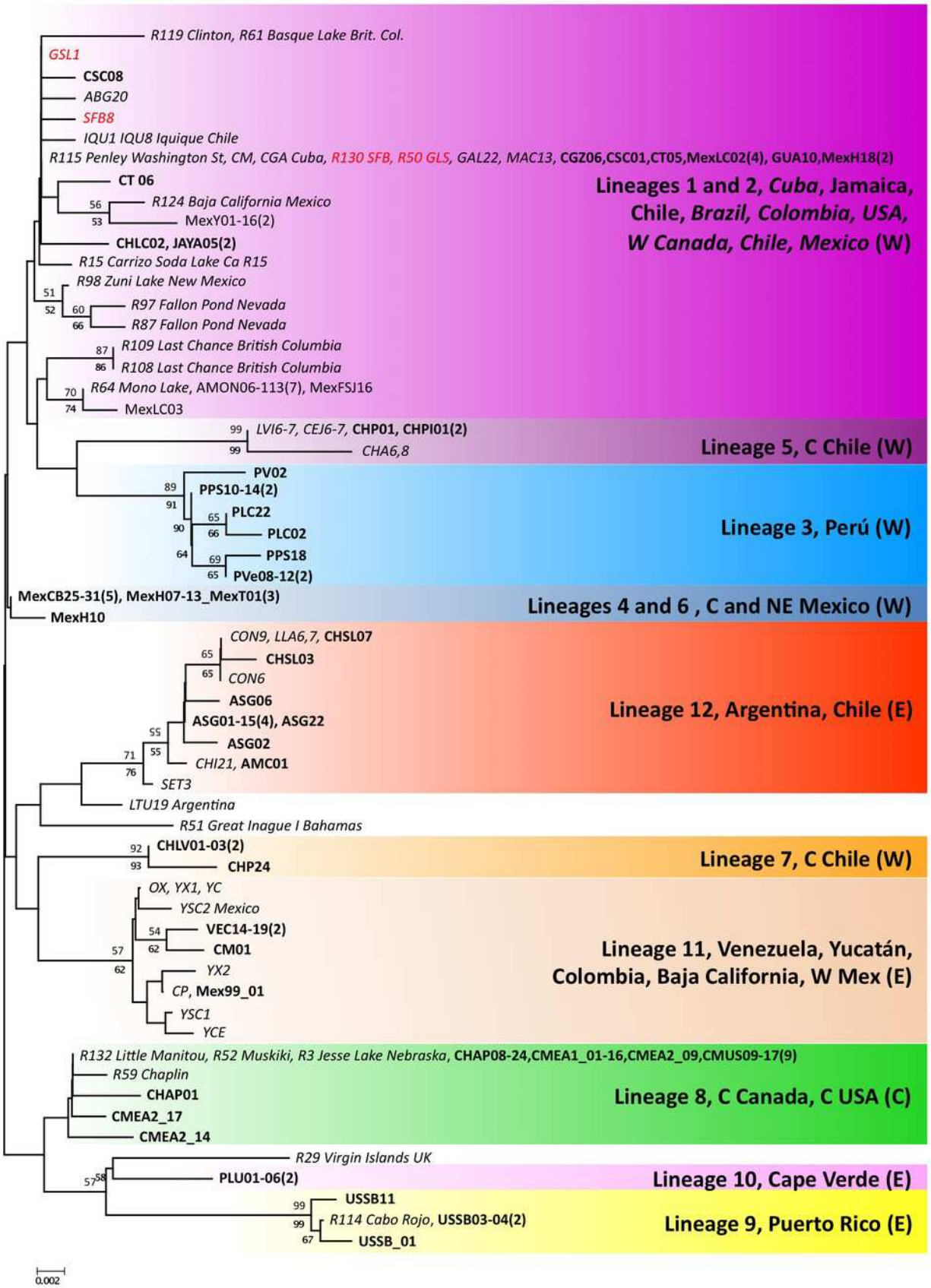


Figure 5

Isolation by distance in native *Artemia franciscana* populations.

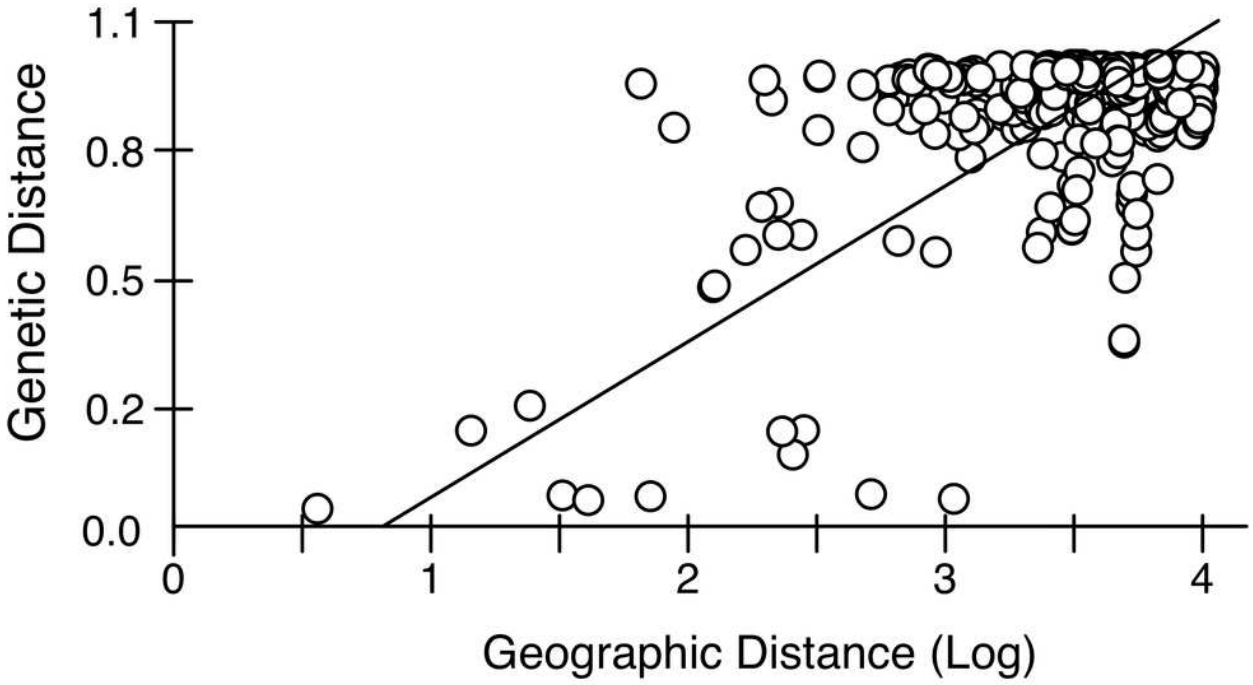


Table 1 (on next page)

Artemia franciscana populations sampled

Population code, year of sampling, COI haplotype code, number of individuals per haplotype per population (N_{HAP}) and number of individuals sequenced per population (N_{TOTAL}), are given for each sampling site. *Hs* = Standard gene diversity; π = Nucleotide diversity; N.A. = Insufficient data to calculate *Hs* (the minimum sample size per population performed by RAREFAC was 11). The main commercialised U.S.A. populations (i.e., SFB and GSL) and haplotypes from these populations shared with other populations are shown in red. Populations considered as introduced are indicated in bold. Only populations collected as part of this study are included.

Code	Population (year)	COI haplotypes	N_{HAP}	N_{TOTAL}	*Hs*	π	Refs. for introduction
MexCe	Celestún, Yucatán, México (1984)	Af01	15	15	0.000	0.0000	-
Mex99	Real de las Salinas, Campeche, México (1999)	Af01	14	14	0.000	0.0000	-
MexY	Yavaros, Sonora, México (1993)	Af02; Af03	1; 11	12	0.167	0.0003	-
MexH	Salinas de Hidalgo, San Luis Potosí, México (1989)	Af04; Af05; Af06; Af07; Af08; Af09	1; 1; 1; 2; 3; 3	11	0.873	0.0091	-
MexT	Texcoco, Estado de México, México (1989)	Af07; Af08; Af09; Af10; Af11	1; 2; 7; 1; 2	13	0.705	0.0099	Introduced from SFB in 1975 (Castro 1993) cited in (Castro *et al.* 2006)
MexCB	Salinas Casa Blanca, Cuatro Ciénagas de Carranza, Coahuila, México (1995)	Af12; Af13; Af14; Af15; Af16; Af17	2; 6; 1; 3; 3; 1	16	0.817	0.0023	-
MexLC	La Colorada lagoon, Oaxaca, México (1993)	Af18; Af19; Af20; Af21	3; 1; 8; 2	14	0.648	0.0041	-
MexSQ	San Quintín, Baja California, México (??)	Af10; Af22; Af23;	12; 1; 1	14	0.275	0.0005	-
MexFSJ	Faro San José, Baja California, México (1991)	Af01; Af10; Af24	1; 2; 1	4	N.A.	0.0226	-
GUA	Frank País, Guantánamo, Cuba (1994)	Af10; Af20; Af25	6; 1; 9	16	0.575	0.0019	Introduced in the 70's in 7 saltworks, (Gelabert & Solis 1994) cited in (Tizol-Correa 2009).
GSL	Great Salt Lake, Utah, USA (??)	Af10; Af18; Af20; Af21; Af26; Af27	1; 2; 21; 2; 2; 1	29	0.475	0.0028	-
SFB	San Francisco Bay, California,USA (??)	Af10; Af18; Af20; Af25	26; 6; 4; 1	37	0.480	0.0033	-
USSF	Salina Fraternidad, Puerto Rico, Cabo Rojo, USA (2000)	Af28; Af29	12; 4	16	0.400	0.0007	-
USSB	Laguna de las Salinas Bastoncillo, Lajas, Puerto Rico, USA (2000)	Af28; Af30; Af31; Af32; Af33; Af34	6; 4; 1; 1; 1; 1	14	0.769	0.0017	-
MANW	Little Manitou Lake, Saskatchewan, Canada (2009)	Af35; Af36	8; 1	9	N.A.	0.0004	-
CMUS	Muskiki Lake, Saskatchewan, Canada (2009)	Af35; Af37; Af38	12; 1; 1	14	0.275	0.0005	-
CHAP	Chaplin Lake, Saskatchewan, Canada (2009)	Af39; Af40; Af41; Af42; Af43	11; 1; 1;	16	0.533	0.0027	-
CMEA	Meacham Lake, Saskatchewan,Canada (2009)	Af35; Af44; Af45; Af46; Af47	2; 1 26; 1; 1; 1; 1	30	0.253	0.0005	-
BRM	Mossoro, Grossos, Brazil (1994)	Af10	11	11	0.000	0.0000	Introduced in 1977 from SFB (Camara 2001)
CGZ	Salinas de Galerazamba, Colombia (1985)	Af48; Af49; Af50	1; 8; 7	16	0.592	0.0017	-
CM	Salinas de Manaure, Colombia (1999)	Af51; Af52	15; 1	16	0.125	0.0002	-
CSC	Salina Cero, Colombia (1999)	Af49; Af50; Af53; Af54; Af55	8; 4; 1; 1; 1	15	0.676	0.0015	-
CT	Tayrona, Colombia (1999)	Af56; Af57; Af58	9; 4; 2	15	0.590	0.0023	-
PPS	Playa Salinas, Ancash, Perú (1995)	Af59; Af60; Af61; Af62; Af63	6; 2; 1; 3; 1	13	0.756	0.0023	-
PLC	Los Chimus, Perú (1992)	Af59; Af64	14; 1	15	0.133	0.0002	-

PV	Virrilla, Piura, Perú (1996)	Af65	16	16	0.000	0.0000	-
PVe	Humedales de Ventanilla, Callao, Perú (1996)	Af59; Af61; Af66; Af67	8; 1; 6; 1	16	0.642	0.0020	-
PLM	La Milagrosa, Chilca, Perú (1993)	Af68; Af69	14; 2	16	0.233	0.0004	-
VEC	Salinas de Cumaraguas, Venezuela (1994)	Af51; Af70; Af71	10; 4; 1	15	0.514	0.0009	-
JAYA	Yallahs Pond, Jamaica (1998)	Af18; Af19	15; 1	16	0.125	0.0002	Known since 1992, morphology extremely similar to SFB (Castro *et al.* 2000)
CHSL	Salar de Llamará, Chile (1994-lab)	Af72; Af73; Af74; Af75	5; 1; 1; 2	9	N.A.	0.0014	
CHLC	Laguna Cejas, Salar de Atacama, Chile (1995-lab)	Af18	16	16	0.000	0.0000	Maniatsi et al (Maniatsi *et al.* 2009) found different haplotypes, which were native.
CHLV	Los Vilos, Poza Palo Colorado, Chile (1997)	Af76; Af77	6; 10	16	0.500	0.0008	
CHPI	Pichilemu Cahuil saltworks, Chile (??-lab)	Af18; Af78	8; 8	16	0.533	0.0103	Reportedly introduced by artisanal workers (Gajardo *et al.* 1998) no details as to when.
CHP	Poza Pampilla IV Region, Chile (1997)	Af79; Af80	14; 1	15	0.133	0.0048	-
AMC	Mar Chiquita, Córdoba, Argentina (1997)	Af81	16	16	0.000	0.0000	-
ASG	Salinas Grandes, Córdoba, Argentina (2000)	Af72; Af73; Af74; Af82; Af83; Af84; Af85	7; 1; 1; 1; 1	13	0.731	0.0018	-
AMON	Mono Lake (1970s, ARC270)	Af87; Af88; Af89; Af90; Af91; Af92	1; 1; 1; 7; 1; 1	12	0.682	0.0016	-
PLU	Pedra de Lume, Sal Island, Cape Verde (??)	Af86; Af93	15; 1	16	0.125	0.0002	-

1

Table 2(on next page)

Genetic divergence between *Artemia franciscana* mtDNA lineages using COI data

Genetic Distance K2P+G estimated with MEGA between lineages. Genetic distances higher than (or equal to) 4% (0.04) are marked in bold.

Lineage (distribution)	1	2	3	4	5	6	7	8	9	10	11
1 (USA + introduced)	-										
2 (NE Mexico)	0.024 (0.006)	-									
3 (Perú)	0.020 (0.005	0.029 (0.007)	-								
4 (C Mexico)	0.018 (0.005)	0.031 (0.007)	0.027 (0.006)	-							
5 (C Chile)	0.023 (0.005)	0.033 (0.008)	0.031 (0.007)	0.023 (0.006)	-						
6 (NE Mexico)	0.026 (0.006)	0.038 (0.008)	0.038 (0.008)	0.028 (0.007)	0.028 (0.007)	-					
7 (C Chile)	0.032 (0.007)	**0.043 (0.009)**	0.032 (0.007)	0.032 (0.008)	0.032 (0.007)	0.039 (0.009)	-				
8 (Canada)	0.020 (0.005)	0.034 (0.008)	0.029 (0.007)	0.019 (0.005)	0.025 (0.007)	0.029 (0.007)	0.031 (0.008)	-			
9 (Puerto Rico)	**0.048 (0.010)**	**0.060 (0.012)**	**0.058 (0.011)**	**0.048 (0.010)**	**0.049 (0.010)**	**0.055 (0.011)**	**0.052 (0.011)**	**0.040 (0.009)**	-		
10 (Cape Verde)	**0.044 (0.009)**	**0.059 (0.012)**	**0.051 (0.010)**	**0.044 (0.010)**	**0.047 (0.010)**	**0.050 (0.010)**	**0.045 (0.010)**	0.032 (0.008)	0.023 (0.006)	-	
11 (Yucatán, Colombia…)	0.035 (0.008)	**0.044 (0.010)**	**0.043 (0.009)**	0.033 (0.008)	**0.041 (0.009)**	**0.045 (0.010)**	**0.043 (0.010)**	0.023 (0.006)	0.038 (0.008)	0.034 (0.008)	-
12 (Argentina, Chile)	0.037 (0.008)	**0.048 (0.010)**	**0.046 (0.009)**	0.037 (0.008)	**0.043 (0.009)**	**0.047 (0.010)**	**0.044 (0.010)**	0.025 (0.006)	**0.044 (0.009)**	0.036 (0.008)	0.020 (0.005)

Table 3(on next page)

Redundancy analyses (RDA) assessing the contribution of spatial (geographical coordinates of populations) and environmental factors to the genetic distance between *Artemia franciscana* populations

Explained variance (%) for three RDAs with different environmental variables is given in separate columns. The first RDA included flyway and human introduction as environmental variables, while the others considered only flyway or introduction. Results are given for flyway assignments made according to Boere & Stroud (2006) and Birdlife international (http://www.birdlife.org/datazone/userfiles/file/sowb/flyways/) (see Table S2 for matrix details).

Source of variation	Flyway + introduction	Flyway	Introduction
Space	8.2 / 8.7	8.3 / 10.2	10.8
Environment	31.2 / 30.6	21.2 / 18.7	15.6
Environment/Space interaction	2.4 / 1.8	2.3 / 0.4	0.0

Figure 6

Artemia franciscana adults and cysts

Possible photo for Journal homepage. Author Andy J Green, released with a CC BY 3.0 licence.