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Mitochondrial phylogeography of the land snail *Cornu aspersum*: tracing population history and the impact of human-mediated invasion in austral South America

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ABSTRACT

Aim: Study one of the most widespread biological invasions by reconstructing the molecular phylogeographic history of non-native populations of the land snail *Cornu aspersum* in austral South America. Specifically, we wished to: (1) examine the genetic diversity of native vs. non-native populations of *C. aspersum*; (2) analyse the species' history of dispersal and colonization in austral South America; (3) compare the biogeographic patterns of native and introduced populations; and (4) identify signs of population bottlenecks and/or multiple independent introductions that might explain the current genetic diversity.

Locations: North Africa, northwest Europe, North America (California, USA), and South America (Chile).

Methods: We obtained sequences of the mitochondrial cytochrome b (Cytb) gene from *C. aspersum* individuals collected from two localities subject to recent introductions (Californian and Chilean populations in North and South America, respectively). We compared these sequences with previously published data from the species' native range (northeast and northwest Africa), as well as with data from a previous introduction (northwest Europe). We measured genetic variation within and among groups. We inferred genetic structure by analysis of molecular variance (AMOVA). We reconstructed phylogeographic patterns using phylogenetic and genetic diversity analyses.

Results: Among the 204 sequences from native and non-native populations, we identified 111 haplotypes. Of these, only three were shared between American and European populations, and no haplotypes were shared between Africa and the introduced populations. The analysis of genetic distances placed European populations between American and African populations. The AMOVAs showed that most of the genetic variance is present within populations rather than among populations or among localities. Haplotype network and phylogenetic analyses consistently demonstrated the existence of two major clades: the Tunisian and the American.

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Conclusions: Genetic diversity and genealogical relationships support the hypothesis of multiple introductions of *C. aspersum* into austral South America. At least a portion of the current genetic diversity of Chilean populations derives from North American and European (French, Italian, and Spanish) populations.

Keywords: agricultural pests, alien species, biogeography, bottlenecks, molluscs, multiple introductions

INTRODUCTION

Biological invasions are now widely recognized as a major component of global change, with profound impacts on biodiversity, agriculture, and public health (Lee, 2002; Sax *et al.*, 2005; Yoshida *et al.*, 2007). There is no doubt that human activities promote biological invasions by fortuitous or intentional dispersal events (Lockwood *et al.*, 2005), with humans acting as efficient dispersal agents worldwide, and facilitating the establishment of introduced species by altering native habitats, hence making them more prone to invasions (Genton *et al.*, 2005). The invasiveness and wide geographical range extension that some alien species exhibit in short periods of time is well documented, particularly in organisms associated with agrosystems and rural or urban settlements (Allendorf and Lundquist, 2003; Xu *et al.*, 2003; Schneider *et al.*, 2004; Philibert *et al.*, 2011). In this regard, molluscs are one of the most efficient invaders on a global scale due to their life-history characteristics (i.e. high fecundity), variability in morphology and physiology, and great capacity to endure a variety of environments (Madec *et al.*, 2000; Barker, 2001; Jørgensen and Sørensen, 2008). For example, Johnson (1988) found a rapid colonization and spread of non-native populations of the land snail *Theba pisana* in western Australia, despite the loss of genetic diversity and recent introduction. On the other hand, Astanei *et al.* (2005) found that rapid colonization of most of northwestern Europe and North America by invasive populations of the zebra mussel, *Dreissena polymorpha*, was characterized by high levels of genetic diversity and low genetic differentiation. Similarly, Dupont *et al.* (2003) found high levels of genetic diversity in alien populations of the hermaphroditic gastropod *Crepidula fornicata* in Europe. In all of the aforementioned cases, the dispersion of aquatic and land mollusc populations was enhanced by sea surface traffic.

Historical reconstructions of the successful establishment and spread of species outside their native range constitute one of the most interesting scenarios from which to study biological invasions from an ecological and evolutionary perspective (Facon *et al.*, 2006). In fact, a key aspect in the analysis of biological invasions is the understanding of the history of the invasion process, including the geographical pathways followed by the founders of the invading populations (Estoup and Guillemaud, 2010). This information provides insight into the origin and genetic composition of the invading populations (Dlugosch and Parker, 2008), as well as the role of such factors as the number of founders, population bottlenecks, migrations, and genetic diversity (Gray *et al.*, 1986; Frankham, 2005; Ficetola *et al.*, 2008).

According to population genetic theory, founding events often establish only a fraction of the genetic variants that occur in native populations (Nei *et al.*, 1975), and the severe bottlenecks associated with such founder events should decrease genetic variation, as well as produce rapid change in allelic frequencies (Johnson, 1988). In this regard, invasion biology has been surrounded by a genetic paradox: how do introduced populations – whose genetic variation has probably been depleted by population bottlenecks, resulting in low evolutionary potential and perhaps low reproductive fitness – persist and spread in new

conditions? (Sakai *et al.*, 2001; Allendorf and Lundquist, 2003; Kolbe *et al.*, 2004; Lambrinos, 2004; Frankham, 2005). Some authors argue that adaptation in newly colonized areas will be favoured by high levels of genetic variability (Genton *et al.*, 2005; Dlugosch and Parker, 2008). However, empirical studies have shown contradictory results, where some non-native species populations are able to rapidly adapt to the new conditions despite their exceedingly low genetic diversity (Hollingsworth, 2000; Tsutsui *et al.*, 2000; Xu *et al.*, 2003; Dlugosch and Parker, 2007, 2008; Puillandre *et al.*, 2007; Ficetola *et al.*, 2008), while other invasive populations show high genetic diversity despite frequent bottlenecks (Stepien *et al.*, 2002; Allendorf and Lundquist, 2003; Kolbe *et al.*, 2004; Genton *et al.*, 2005; Voisin *et al.*, 2005; Dlugosch and Parker, 2008). In this regard, Frankham (2005) highlights four explanations that could resolve this genetic paradox: (1) adaptation may not always be a prerequisite for the successful establishment of introduced populations; (2) rapid population expansion sometimes occurs just after introduction, retaining genetic diversity and facilitating later adaptation to the local environment; (3) polyploidization and hybridization phenomena may rapidly produce novel diversity in the introduced area; and (4) multiple introductions may generate a high level of genetic diversity in the introduced range. This last explanation has frequently been suggested as the main reason for the successful establishment and spread of alien species (Allendorf and Lundquist, 2003; Kolbe *et al.*, 2004; Astanei *et al.*, 2005; Genton *et al.*, 2005; Facon *et al.*, 2006; Dlugosch and Parker, 2008; Estoup and Guillemaud, 2010).

In this study, we undertook a mitochondrial phylogeographic analysis of a biological invasion using the land snail *Cornu aspersum* as a model. We analysed a wide geographical range based on its native range (North Africa), a previous introduction range (northwest Europe), and two recent introductions (California, USA in North America and Chile in South America). We used mtDNA (i.e. cytochrome b, Cytb) with the aims of: (1) examining the genetic diversity of native vs. non-native populations of *C. aspersum*; (2) analysing the species' history of dispersal and colonization in austral South America; (3) comparing the biogeographical patterns of native and introduced populations; and (4) identifying signs of population bottlenecks and/or multiple independent introductions that could explain the current genetic diversity. We addressed two mutually exclusive hypotheses that potentially explain the invasion process of *C. aspersum* in Chile. First, Chilean populations were recently introduced from European or North American populations in a single introduction event. Hence, we would expect to find lower genetic diversity and fewer signs of population bottlenecks in Chilean populations compared with the source populations and native range of *C. aspersum.* Second, Chilean populations are the result of recent multiple introductions from both European and North American populations. Hence, we would expect to find a high level of genetic diversity in the introduced range, probably higher than in the source populations.

METHODS

Study species and samples

The land snail *Cornu aspersum* Müller (Gastropoda: Pulmonata: Helicidae) is native to the Western Mediterranean, and has successfully colonized all continents, except Antarctica, since the Holocene (Guiller *et al.*, 2001). *Cornu aspersum* is currently widespread in temperate and subtropical regions of the world, dwelling in agricultural areas, parks and domestic gardens within cities, where the species develops abundant populations, and is considered a serious pest in some regions (Barker, 2001; Roth and Sadeghian, 2006; Guiller and Madec, 2010). According

to the literature, the first population introduction in North America was established *c*. 1859 (Selander and Kaufman, 1975), whereas the first population introduction in South America was in Chile *c*. 1900 (Artacho *et al.*, 2006). Data were collected from four different localities (Table 1). The analysis involved two localities that experienced recent introductions (Californian and Chilean populations in North and South America, respectively), together with published data from its native range (northeast and northwest Africa) and a previous introduction (northwest Europe) (Guiller and Madec 2010) (see online appendix at evolutionary-ecology.com/data/2826Appendix.pdf). Individuals from the Californian and Chilean populations were used for DNA analysis. These specimens were preserved in ethanol (95%) until analysis in the laboratory at Universidad de Chile, Santiago, Chile. For phylogenetic relationships, species from the GenBank database were included (see accession numbers in 2826Appendix.pdf).

PCR and sequencing

Before DNA extraction, individuals from the Californian and Chilean populations were soaked in double-distilled water for a minimum of one hour. A total DNA extraction of a single individual was performed from foot muscle following the protocol of Guiller and Madec (2010). A cytochrome b (Cytb) fragment of 555 base pairs (bp) was amplified using the Cytb1 (5′-TTATT-GAGGCGCTACGGTTAT-3′) and Cytb2 (5′-GCAAGC-GAAATATAAGGTTCT-3′) primers. This gene has been widely used in invertebrate species, including snails, allowing comparison of our data with data from the database. The PCR reaction (25 µL total volume) consisted of 3–5 µL of DNA template, 10× PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl), 0.20 µm of each primer, 1.5 mM $MgCl_2$, 200 µm of each dNTP, and 1.5 units of *Taq* DNA polymerase. The PCR regime was as follows: an initial cycle of denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Finally, the PCR was terminated by a final extension of 72°C for 10 min. The PCR products were purified using the QIAquick purification kit (QIAGEN). Sequences were developed at Macrogen, Seoul, Korea (http:www.macrogen.com). All sequences were deposited under the accession numbers JQ388107 to JQ388195. Sequencing reads from each amplicon were compared with the GenBank database using a BLASTN similarity search to reduce the possibility of including contaminated sequence data.

DNA analysis and phylogenetic reconstructions

DNA sequences were edited visually and manually using MacClade 4.06 (Madison and Madison, 2005). Sequences were aligned using the TranslatorX multiple sequence alignment program (Abascal *et al.*, 2010). Variable positions were confirmed by visual inspection and verified on both strands. We also screened genetic diversity in each population. The following standard genetic diversity indices were calculated using the software DnaSP v.5.1 (Librado and Rozas, 2009): polymorphic sites (*S*), number of haplotypes (*h*), haplotype diversity (*Hd*), average number of differences (*K*), nucleotide diversity (*p*), and Tajima's *D* test. To test the hypothesis of recent population growth, mismatch analyses were performed using the program Arlequin v.3.5.1.2 (Excoffier *et al.*, 2005), as sudden changes in population size generate unimodal distributions (Rogers and Harpending, 1992). The number of observed differences between pairs of mtDNA sequences was compared with the expected distribution of differences under a specified demographic model (i.e. constant population size or population growth).

Table 1. Geographic location and *Cornu aspersum* sample size and haplotypes found for each locality analysed

Locality	Country	Population	*N*	Haplotypes
South America	Chile	Viña del Mar	35	Hap 1, Hap 2, Hap 3, Hap 4, Hap 5, Hap 6, Hap 7,
		Concepción	27	Hap 8, Hap 9, Hap 10, Hap 11, Hap 12, Hap 13,
		Valdivia	19	Hap 14, Hap 15, Hap 16, Hap 17, Hap 18, Hap 19, Hap 20, Hap 21
North America	USA	California	8	Hap 4, Hap 22, Hap 23, Hap 24
Northwest Europe	Spain	Cap Finisterre	5	Hap 25, Hap 26, Hap 27, Hap 28, Hap 29, Hap 30,
		Muxia	6	Hap 31, Hap 32, Hap 33, Hap 34, Hap 35, Hap 36,
		Laxe	5	Hap 37, Hap 38, Hap 39, Hap 40, Hap 41, Hap 42
		Ribadeo	1	
		Llanes	5	
		Onton	1	
	Italy	Valledonia	2	Hap 4, Hap 51, Hap 52, Hap 53, Hap 54, Hap 55,
		Suni	2	Hap 56
		Terralba	1	
		Ala dei Sardi	2	
	Greece	Ayios Nikolaos	5	Hap 57, Hap 58, Hap 59, Hap 60
	Croatia	Pula	6	Hap 74, Hap 75, Hap 76, Hap 77
	France	Cancale	6	Hap 1, Hap 4, Hap 35, Hap 43, Hap 44, Hap 45,
		Baguer-Pican	5	Hap 46, Hap 47, Hap 48, Hap 49, Hap 50
		Pacé	6	
		Corsica	2	
North Africa	Algeria	Cap Larès	3	Hap 78, Hap 79, Hap 80, Hap 81, Hap 82, Hap 83,
		Cherchell	3	Hap 84, Hap 85, Hap 86, Hap 87, Hap 88, Hap 89,
		Tipasa	5	Hap 90, Hap 91, Hap 92, Hap 93, Hap 94, Hap 95,
		Bainem	1	Hap 96, Hap 97, Hap 98, Hap 99, Hap 100,
		Draria	1	Hap 101, Hap 102, Hap 103, Hap 104, Hap 105,
		Miramar	1	Hap 106, Hap 107, Hap 108, Hap 109, Hap 110,
		Médéa	1	Hap 111
		Berrouaghia	1	
		Mandoura	1	
		Dellys	1	
		Azzefoun	1	
		Tichi	6	
		Djémila	1	
		El Ancer	1	
		El Hédaïck	1	
		El Arrouch	1	
		Hammam Meskoutine	1	
		Seraïdi	1	
		Cap de Garde	1	
		Annaba	1	
		El Bouni	1	
		Bouteldja	1	
		Cap Rosa	1	
		Tazmalt	2	
	Tunisia	Tabarka	5	Hap 61, Hap 62, Hap 63, Hap 64, Hap 65, Hap 66,
		Bulla Régia	5	Hap 67, Hap 68, Hap 69, Hap 70, Hap 71, Hap 72,
		Thibar	1	Hap 73
		Dougga	1	
		Utique	5	
Total			**204**	

Note: GenBank accession numbers can be found at evolutionary-ecology.com/data/2826Appendix.pdf.

To determine genetic differences, we calculated pairwise Φ_{ST} and the distance matrix among populations using the program MEGA v.5 (Tamura *et al.*, 2011). The K2P model was first used to construct the mean divergences matrix among populations. To test and assess geographical division and population genetic structure, we performed a hierarchical analysis of molecular variance [AMOVA (Excoffier *et al.*, 1992)], with 1000 permutations (α set at 0.05), using the Arlequin program v.3.5.1.2 (Excoffier *et al.*, 2005) and the Tamura and Nei model of DNA sequence evolution. In this analysis, genetic variance was divided into three components: variations among localities (Φ_{CT}), variations among populations within localities (Φ_{SC}), and variations within populations (Φ_{ST}). Analysis of differentiation among populations was conducted using an exact test (based on haplotype frequency distributions) and pairwise Φ_{ST} statistics (based on haplotype frequencies and molecular divergence).

The genealogical relationship between groups was assessed with a haplotype network using the program Network 4.6 (Fluxus Technology Ltd.), followed by the median joining (MJ) algorithm to resolve intermediate nodes (Bandelt *et al.*, 1999). Phylogenetic relationships were estimated by maximum likelihood (ML) and Bayesian approaches (BI), as implemented in Treefinder, October 2008 version (Jobb *et al.*, 2004) and MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003), respectively. The best fitting models were estimated separately for each gene segment, as well as for each codon position within each segment, using the 'propose model' routine from Treefinder, October 2008 version (Jobb *et al.*, 2004) according to the Akaike Information Criterion. Using maximum likelihood, we estimated the best tree for each alignment, and support for the nodes was estimated with 1000 bootstrap pseudoreplicates. Model parameter values of the Bayesian inference were treated as unknown and were estimated during the run. Eight Markov chains were used, and each chain was started from a random tree. The 'temperature' parameter was set at 0.2. The Monte Carlo Markov chain length was 10 million generations and trees were sampled every 1000 generations. To establish if the Markov chains had reached a steady state, we plotted the –Ln likelihood scores of sampled trees against generation time. Trees generated before the steady phase were discarded as burn-in (first 10% of the sampled trees). Support for the nodes and parameter estimates were derived from a majority rule consensus of the last 100,000 trees sampled after convergence.

RESULTS

Sequence characteristics and genetic diversity

In total, 204 sequences of the land snail *C. aspersum* from native and non-native populations were aligned for Cytb (including 116 sequences retrieved from GenBank). For the Cytb dataset (555 bp), there were 275 polymorphic sites, of which 241 were parsimony informative. The total number of mutations identified was 401. No codon insertions or deletions were detected. Total nucleotide and haplotype diversities were high (0.081 ± 0.011 and 0.965 ± 0.01 respectively), defining 111 different haplotypes, of which only three were shared between the American and European populations; no haplotypes were shared between the African and introduced populations (Table 1 and Fig. 1). Analysis of the genetic diversity among localities showed that populations from North Africa (i.e. Algeria and Tunisia) and northwest Europe (i.e. Spain, France, and Italy) have higher values of nucleotide and haplotype diversity than populations from America. However, the number of polymorphic sites in Chilean populations was similar to that reported for the species'

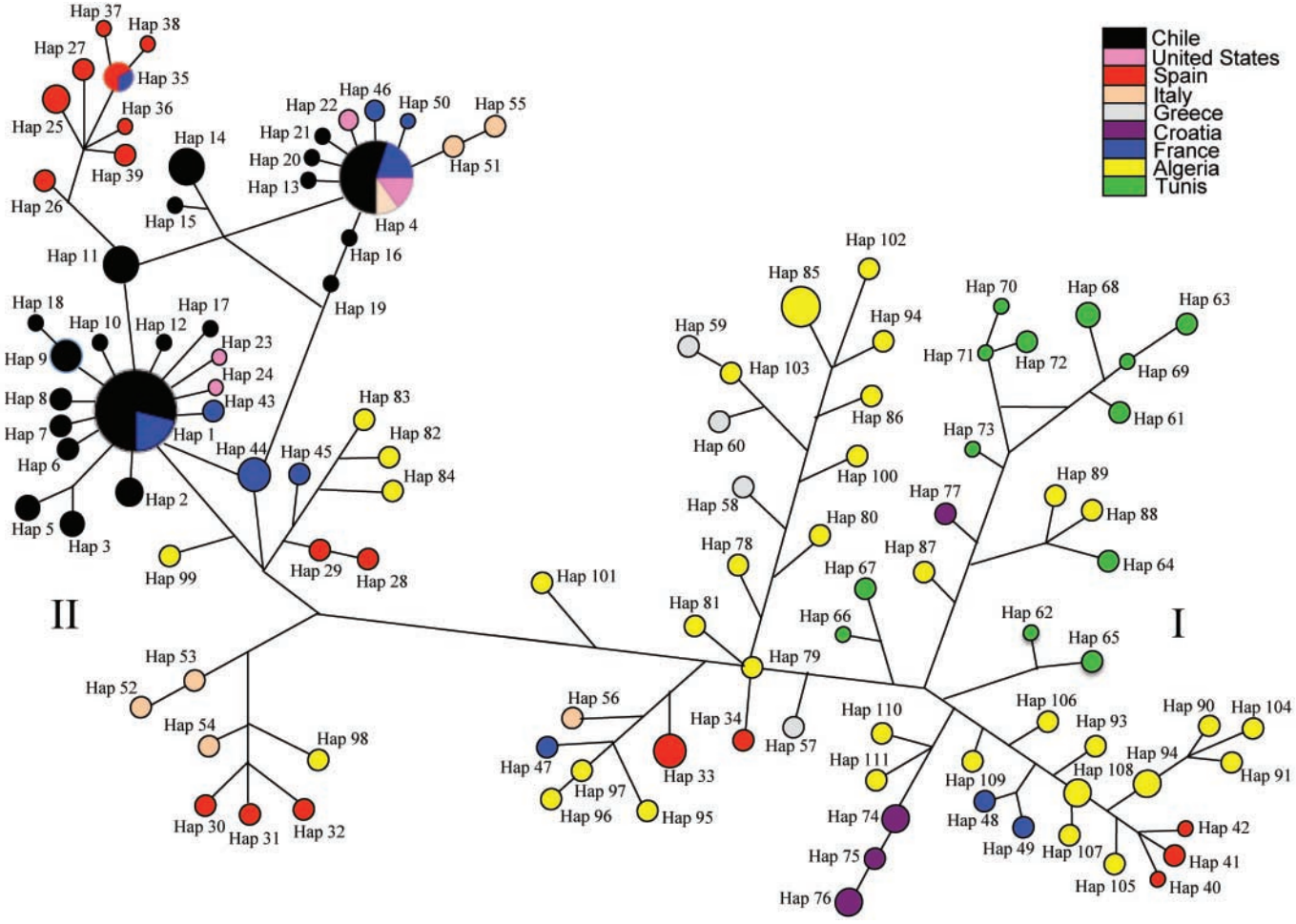

Fig. 1. Median-joining network of the 111 *Cornu aspersum* Cytb haplotypes (555-bp sequence) found in North Africa, northwest Europe, North America, and South America. Node sizes are proportional to haplotype frequencies. Haplotype network line lengths are proportional to the number of changes.

Table 2. Genetic diversity indices and test of neutrality among native and non-native populations of *C. aspersum*

Locality	Country	*n*	*h*	*Hd*	*S*	*K*	*p*	Tajima's *D*
South America	Chile	81	21	0.864	81	6.644	0.012	– 1.97728*
North America	USA	8	4	0.750	7	2.643	0.005	– 0.10024
Northwest Europe	Spain	23	18	0.976	138	43.367	0.078	0.64328
	Italy	7	7	1.000	65	23.000	0.041	– 0.77297
	Greece	5	4	0.900	73	41.000	0.076	1.50558
	Croatia	6	4	0.886	41	14.133	0.025	– 1.35896
	France	19	11	0.906	114	22.807	0.041	– 0.12265
North Africa	Algeria	38	34	0.993	196	63.650	0.115	1.38851
	Tunisia	17	13	0.963	110	40.007	0.072	0.97834
	Total	204	111	0.965	275	44.959	0.081	– 1.08392

Note: *n*, number of sampled individuals; *h*, number of haplotypes; *Hd*, haplotype diversity; *S*, polymorphic sites; *K*, average number of differences; *p*, nucleotide diversity.

*$P < 0.05$.

native range (e.g. Tunisia) and in some populations of the previous introduction range (e.g. France and Greece) (Table 2).

Demographic analyses and genetic differentiation

When considering the 204 sequences as a single population, the neutrality test (i.e. Tajima's *D* test) revealed no significant deviation from the standard neutral model (Table 2). However, based on the geographic information of the population distribution, Tajima's *D* test only rejects the hypothesis of neutrality for Chilean populations, with significant negative values (Table 2). Similarly, the mismatch distribution for American populations was close to the theoretically expected unimodal distribution for population expansion, whereas for the European and African populations, it followed a multimodal pattern (Fig. 2).

According to the haplotype network, there are two major haplogroups. The first is formed mainly by native African populations (I; Fig. 1) and is characterized by long branches without a star-like form. This haplogroup revealed that populations from Greece and Croatia do not share haplotypes with American populations (Fig. 1). The second haplogroup is formed by a fraction of Algerian populations, some of the European populations (i.e. Spain, France, and Italy), and all of the haplotypes from American populations (II; Fig. 1). This haplogroup is dominated by two common haplotypes that are shared between the Chilean and French populations (Hap 1) and between the Chilean, French, Italian, and Californian populations (Hap 4; Table 1 and Fig. 1), giving in both cases a star-like topology for the most recently introduced population (i.e. Chile). This group is most influenced by Spanish and French populations, and to a lesser extent by Algerian populations (Fig. 1).

The total molecular variance, as determined by the hierarchical AMOVA, revealed no significant genetic heterogeneity between localities of the native and introduced populations (Table 3). Comparison of molecular variance among all localities showed that genetic variation is partitioned mainly within populations (52%) and among populations within localities (33%) rather than among localities (14%; Table 3). Similarly, two-level AMOVAs

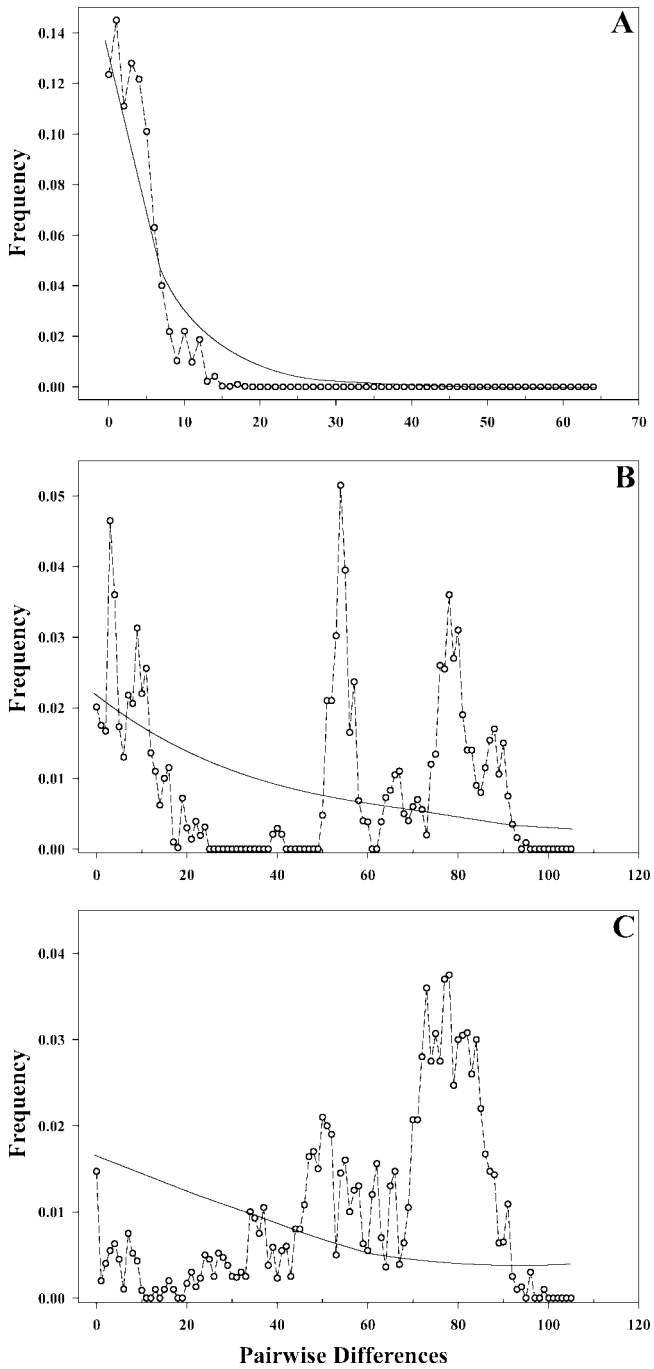

Fig. 2. Mismatch distribution established for (A) recently introduced populations, (B) previously introduced populations, and (C) native populations of *Cornu aspersum*. The solid line represents the expected mismatch distribution of a stationary population. The dotted line represents the observed mismatch distribution from segregating sites of the aligned Cytb sequences.

Table 3. Analysis of molecular variance (AMOVA) for native and non-native populations of *Cornu aspersum*

Source	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistic
Native vs. introduced populations					
Among localities: NAf vs. SA vs. NAm vs. NE	3	1521.461	4.16731	14.28	$\Phi_{CT} = 0.14280$
Among populations/within localities	5	732.064	9.81782	33.64	$\Phi_{SC} = 0.39247$*
Within populations	195	2963.577	15.19783	52.08	$\Phi_{ST} = 0.47922$*
Native vs. recently introduced populations					
Between localities: NAf vs. SA + NAm	1	1463.322	17.09869	45.91	$\Phi_{CT} = 0.45909$
Among populations/within localities	2	256.046	5.98442	16.07	$\Phi_{SC} = 0.29706$*
Within populations	140	1982.584	14.16131	38.02	$\Phi_{ST} = 0.61977$*
Native vs. previously introduced populations					
Between localities: NAf vs. NE	1	517.116	4.70846	12.2	$\Phi_{CT} = 0.12196$
Among populations/within localities	5	731.975	9.09820	23.57	$\Phi_{SC} = 0.26841$*
Within populations	108	2678.278	24.79887	64.24	$\Phi_{ST} = 0.35763$*
Previously vs. recently introduced populations					
Between localities: NE vs. SA + NAm	1	260.023	−0.78743	−4.97	$\Phi_{CT} = -0.04974$
Among populations/within localities	5	490.499	7.70208	48.65	$\Phi_{SC} = 0.46344$*
Within populations	142	1266.252	8.91727	56.32	$\Phi_{ST} = 0.43675$*

Note: d.f., degree of freedom; NAf, North Africa; SA, South America; NAm, North America; NE, northwest Europe.

*$P < 0.001$.

comparing North African and European populations (i.e. native versus previously introduced populations) showed that most of the genetic variation resided within populations (64%). However, in the comparison between the North African and American populations (i.e. native versus recently introduced populations), the greatest genetic variation was mainly found between localities (45%) followed by within populations (38%). However, despite the high variation between localities, the Φ_{CT} was not significant ($P = 0.43$; Table 3). Finally, the two-level AMOVA for previously versus recently introduced populations revealed similar values of genetic variation among populations within localities (56%) and within populations (48%), suggesting a significant degree of genetic differentiation in both cases ($\Phi_{SC} = 0.46$ and $\Phi_{ST} = 0.43$, $P < 0.001$; Table 3). Similarly, pairwise Φ_{ST} comparisons and exact tests indicated significant differentiation among populations. Pairwise Φ_{ST} values were higher among populations from the Americas (i.e. Chile and USA) and Africa, Greece and Croatia, ranging from 0.339 to 0.913 ($P < 0.05$; Table 4). All pairwise comparisons between introduced populations and native populations resulted in high Φ_{ST} values, with Algerian populations being less differentiated than Tunisian populations compared with introduced populations (Table 4).

Table 4. Pairwise differentiation tests for native and invasive populations of *Cornu aspersum*

	Ch	Cal	Sp	Fr	It	Gr	Cr	T	Al
Chile (Ch)	–	0.010 ± 0.003*	0.000 ± 0.000*	0.001 ± 0.002*	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*	0.000 ± 0.000*
USA (Cal)	0.076*	–	0.007 ± 0.001*	0.085 ± 0.005	0.163 ± 0.005	0.024 ± 0.002*	0.012 ± 0.001*	0.009 ± 0.001*	0.062 ± 0.009
Spain (Sp)	0.302*	0.103*	–	0.001 ± 0.000*	0.090 ± 0.005	0.090 ± 0.004	0.043 ± 0.004*	0.002 ± 0.001*	0.003 ± 0.001*
France (Fr)	0.125*	0.019	0.037	–	0.062 ± 0.006	0.016 ± 0.002*	0.007 ± 0.001*	0.000 ± 0.000*	0.001 ± 0.000*
Italy (It)	0.294*	0.141*	0.009	0.005	–	0.477 ± 0.012	0.219 ± 0.006	0.097 ± 0.005	0.276 ± 0.013
Greece (Gr)	0.829*	0.689*	0.281*	0.502*	0.444*	–	0.102 ± 0.002	0.089 ± 0.005	0.284 ± 0.009
Crotatia (Cr)	0.913*	0.908*	0.535*	0.710*	0.766*	0.670*	–	0.034 ± 0.001*	0.187 ± 0.010
Tunisia (T)	0.848*	0.694*	0.500*	0.617*	0.613*	0.537*	0.392*	–	0.009 ± 0.002*
Algeria (Al)	0.562*	0.339*	0.178*	0.272*	0.248*	0.122*	0.228*	0.224*	–

Note: Pairwise Φ_{ST} comparisons (below the diagonal) using Tamura and Nei's distance and *P*-values for exact tests of pairwise haplotype frequency comparisons (above the diagonal).
Ch, Chile; Cal, California (USA); Sp, Spain; Fr, France; It, Italy; Gr, Greece; Cr, Croatia; T, Tunisia; Al, Algeria.
*$P < 0.05$.

Phylogenetic analyses

The best-fit model of sequence evolution for the dataset was HKY with Γ-distributed rate variation for site mutation rate (Yang *et al.*, 1998). This model was used for both Bayesian and maximum likelihood analyses, and produced identical topologies (Fig. 3), although with differing levels of nodal support for some clades. Most clades and subclades were strongly supported. Phylogenetic reconstruction (Fig. 3) showed two major clades from which Tunisia and Croatia were monophyletic in Clade I, while Chile, the USA, Italy and Greece were monophyletic in Clade II. On the other hand, Algeria, Spain and France were polyphyletic, being divided in both clades. The comparison of the haplotype network (Fig. 1) with the phylogenetic tree (Fig. 3) revealed the two representations were quite similar, and the two groups identified in the haplotype network were also found within the phylogenetic tree.

DISCUSSION

Genetic diversity of native and introduced populations

Land snails are suitable organisms for studying phylogeography, since they tend to preserve phylogeographic patterns (Pfenninger *et al.*, 1996) due to their limited dispersal capacity and particular habitat requirements (Fiorentino *et al.*, 2010). Land snails have been described as unusual biological models, since some mitochondrial genes may vary by 10–30% within a species (Terrett *et al.*, 1996), and they tend to show particularly high mutation and substitution rates, potentially resulting in high mtDNA diversity (Davison, 2002). Here we found that *C. aspersum* follows this pattern, showing high haplotype diversity in all populations, of which the native populations from Africa show the highest diversity, followed by the Spanish and Italian populations (Table 2). In contrast, recently introduced populations (i.e. North and South America) are characterized by the lowest nucleotide diversity, while previously introduced populations have intermediate values of diversity, and native populations have the highest values (Table 2). The high haplotype diversity and low nucleotide diversity in introduced populations could be explained by recent divergence and by a high substitution rate, which could arise when haplotypes become 'trapped' in structured subpopulations (Davison, 2002). This means that during the invasion process, heterogeneous habitats would have promoted multiple isolated subpopulations, and the dynamic of repeated range fragmentation and re-expansion of *C. aspersum* as a result of human activities and multiple introductions, would have given origin to new haplotypes as well as driven some older haplotypes to extinction. These processes facilitated intraspecific diversification and population genetic differentiation.

Another interesting finding in the present study is the pattern in the average number of differences between native and introduced populations (Allendorf and Lundquist, 2003; Tollenaere *et al.*, 2010; Betancur *et al.*, 2011): the American populations of *C. aspersum* showed the lowest values, while the African populations had the highest values (Table 2). The Algerian and Tunisian populations have been subjected to greater evolutionary time, resulting in greater and older genetic divergence (Guiller *et al.*, 2006). Nevertheless, despite the aforementioned, both populations exhibit some contrasting features in genetic diversity that can be explained by: (1) a more recent divergence within the Tunisian region (Guiller *et al.*, 2001); (2) transitory and prolonged bottlenecks due to glacial/interglacial cycles during the Pleistocene; and (3) the lack of gene flow between eastern and western populations after the appearance of the

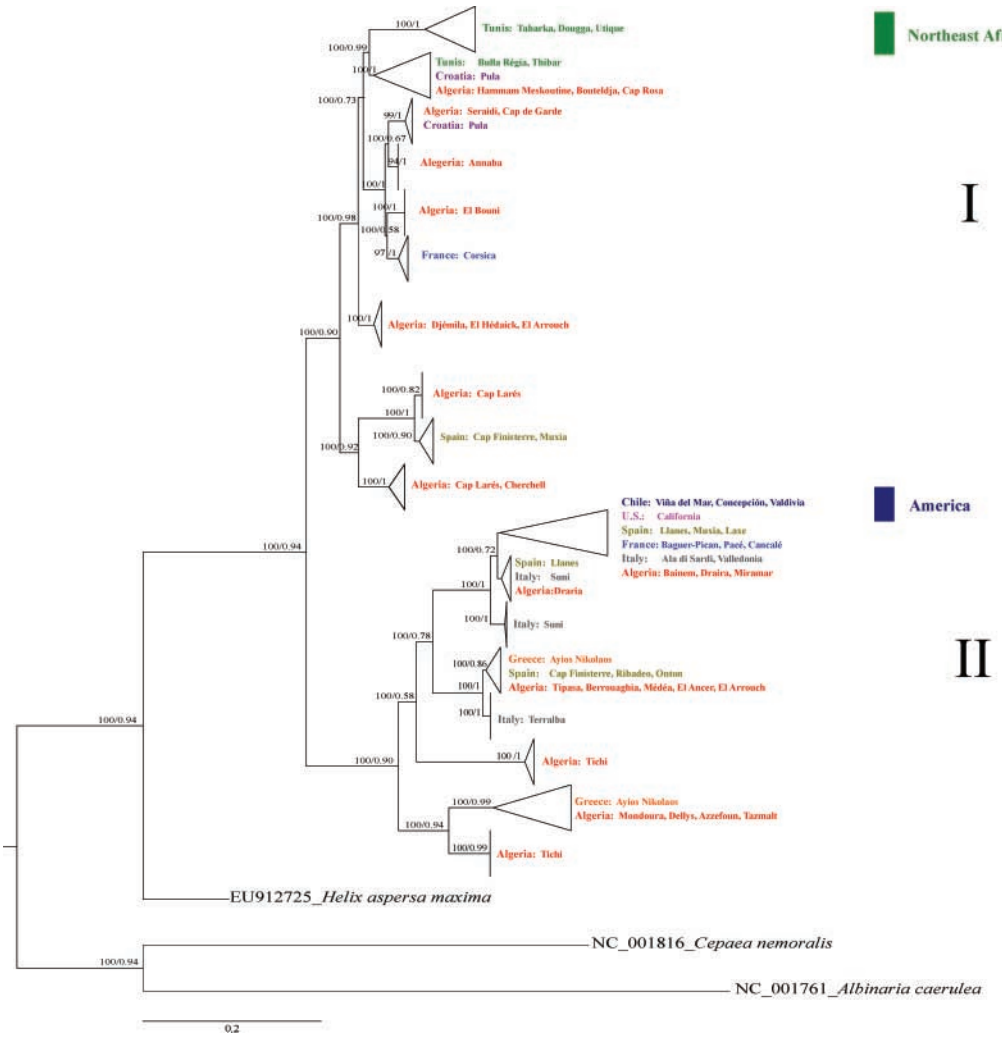

Fig. 3. Bayesian 50% majority-rule consensus tree of *C. aspersum* inferred from Cytb. Posterior probabilities from BI and bootstrap support values from ML analysis (where >50%) are given adjacent to the respective nodes for clades and subclades. The number of individuals sampled for each population is given in Table 1.

Tellian Atlas Mountain Chain, which isolated the Tunisian populations (Guiller *et al.*, 2001, 2006; Guiller and Madec, 2010).

Demographic analyses and genetic differentiation

The neutrality test showed that most of the populations of *C. aspersum* are in a constant-size neutral equilibrium [i.e. Tajima's *D* is expected to be nearly zero (Innan and Stephan, 2000)]. However, the austral South American populations showed a deviation from this neutral model (Table 2), since they reflect an excess of low-frequency variants (i.e. rare

polymorphisms), probably as a result of a recent selective sweep, which is consistent with either positive selection or an increase in population size (Akey *et al.*, 2004). In this regard, it is worth noting that land snails from Chile are the most recently introduced populations [i.e. *c*. 1900 (Artacho *et al.*, 2006)], hence the most likely explanation for this deviation from neutrality is a rapid expansion in the recent past, which is also supported by the mismatch distribution (Slatkin and Hudson, 1991; Guiller and Madec, 2010). In fact, austral populations from Chile closely resemble the theoretically unimodal distribution for population expansion.

Consistent with previous studies, our results confirm the marked differences between native and non-native *C. aspersum* populations, with non-native populations being less variable than native ones. As expected for a recent colonization of America, the differentiation among American populations was low, and among American and European populations lower than among African populations, but statistically significant (Table 3). Although the analysis of genetic distances grouped European populations between American and African populations, there was a small discrepancy with some European populations (i.e. France, Italy, and Spain), which did not show genetic differentiation among them and had very low genetic distances when compared with American populations. Similarly, within African populations, Algeria showed half the Tunisian genetic distance of American populations. This apparent discrepancy strongly suggests that American snails were derived from European populations (i.e. France, Italy, and Spain), but all populations were derived from Algerian populations (Guiller and Madec, 2010). The observed patterns of genetic diversity and differentiation suggest that the expansion of *C. aspersum* in America is likely the result of dispersal from the previously introduced populations.

On the other hand, comparisons between native versus introduced populations revealed that most of the genetic variance is present within populations rather than among populations within localities or among localities. Nevertheless, significant genetic differentiation was found among populations within localities. There was no significant locality (i.e. continent) level variance component, which is unsurprising, given that Africa is the source of European populations (Guiller and Madec, 2010), and in turn, European populations are the source of the American invasion (Selander and Kaufman, 1975; Artacho *et al.*, 2006). When we compared variance partitioning between previously and recently introduced populations, we found a similar distribution of genetic variability among populations and within populations, which in the first case is likely due to the great genetic distances between some European (i.e. Greece and Croatia) and American populations. All of these results are consistent if we consider that *C. aspersum* is an anthropochorous species with a world distribution influenced by humans (Guiller and Madec, 2010) and, like other land snail species, obeys a stepping-stone model since migration between populations is low, in accordance with their sedentary behaviour (Schilthuizen and Lombaerts, 1994).

Finally, the genealogical relationships between groups assessed by the haplotype network suggest that populations of *C. aspersum* in Chile could be the result of multiple introductions. In fact, the presence of two shared haplotypes with France, Italy, and the USA, as well as the close relationship with Spain, implies that at least some proportion of the current genetic diversity of Chilean populations derives from North American and European populations. Moreover, the fact that Chilean populations have a high number of haplotypes and similar values of haplotype diversity compared with populations from the native and previously introduced range, further supports the scenario of multiple introduction events, where slight bottlenecks in each introduction did not necessarily reduce overall levels of

genetic diversity (Allendorf and Lundquist, 2003; Kolbe *et al.*, 2004; Astanei *et al.*, 2005; Genton *et al.*, 2005; Facon *et al.*, 2006; Dlugosch and Parker, 2008; Estoup and Guillemaud, 2010).

Phylogenetic analyses

Despite network approaches being more effective than classical phylogenetic procedures for representing intraspecific evolution (Posada and Crandall, 2001), we found a similar pattern in both analyses. In general, our analyses of mtDNA genetic variation among populations of *C. aspersum* revealed substantial sequence divergence between samples from different geographic regions. Phylogenetic analyses consistently demonstrated the existence of two major clades and revealed a complex evolutionary history, with the Algerian populations a paraphyletic lineage, supporting the close genealogical relationship with all other populations. Clade I is characterized by the presence of populations from northeast Africa (i.e. Tunisia), clustered Croatian populations, and some populations from France (i.e. Corsica), Spain (i.e. Cap Finisterre, Muxia), and Algeria. Clade II includes all the recent introductions but also some of the European (i.e. Greece, Italy, France, and Spain) and Algerian populations. In this regard, the maximum likelihood (ML) and Bayesian (BI) analyses strongly support the phylogenetic relationship of American populations with European populations, and generate additional evidence for the multiple introduction scenarios.

In conclusion, multiple introductions of *C. aspersum* appear to have occurred in austral South America. Despite the genetic diversity within introduced and invasive species often being low due to populations being initiated with just a few individuals from the native range (Sakai *et al.*, 2001), there are some examples of non-native populations that have high genetic diversity as a result of multiple introductions (e.g. Allendorf and Lundquist, 2003; Kolbe *et al.*, 2004; Suehs *et al.*, 2004), as is shown by the results of the present study. If Chilean populations had come from a single source population in North America, Europe or Africa, one would expect a reduced number of alleles in Chile compared with native populations. However, austral Chilean populations exhibit a high number of alleles and haplotype diversity, suggesting, in contrast, the existence of multiple sources for introduced populations of this land snail species in South America. Since our findings only rely on mitochondrial DNA, further investigation using nuclear markers is warranted.

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