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Scale-dependent post-establishment spread and genetic diversity in an invading mollusc in South America

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ABSTRACT

Aims Our study aimed to characterize the dispersal dynamics and population genetic structure of the introduced golden mussel *Limnoperna fortunei* throughout its invaded range in South America and to determine how different dispersal methods, that is, human-mediated dispersal and downstream natural dispersal, contribute to genetic variation among populations.

Location Paraná–Uruguay–Río de la Plata watershed in Argentina, Brazil, Paraguay and Uruguay.

Methods We performed genetic analyses based on a comprehensive sampling strategy encompassing 22 populations (N = 712) throughout the invaded range in South America, using the mitochondrial cytochrome c oxidase subunit I (COI) gene and eight polymorphic nuclear microsatellites. We employed both population genetics and phylogenetic analyses to clarify the dispersal dynamics and population genetic structure.

Results We detected relatively high genetic differentiation between populations (F_{ST} = −0.041 to 0.111 for COI, −0.060 to 0.108 for microsatellites) at both fine and large geographical scales. Bayesian clustering and three-dimensional factorial correspondence analyses consistently revealed two genetically distinct clusters, highlighting genetic discontinuities in the invaded range. Results of all genetic analyses suggest ship-mediated 'jump' dispersal as the dominant mode of spread of golden mussels in South America, while downstream natural dispersal has had limited effects on contemporary genetic patterns.

Main conclusions Our study provides new evidence that post-establishment dispersal dynamics and genetic patterns vary across geographical scales. While ship-mediated 'jump' dispersal dominates post-establishment spread of golden mussels in South America, once colonies become established in upstream locations, larvae produced may be advected downstream to infill patchy distributions. Moreover, genetic structuring at fine geographical scales, especially within the same drainages, suggests a further detailed understanding of dynamics of larval dispersal and settlement in different water systems. Knowledge of the mechanisms by which post-establishment spread occurs can, in some cases, be used to limit dispersal of golden mussels and other introduced species.

Keywords

Biological invasions, dispersal mechanism, genetic differentiation, golden mussel *Limnoperna fortunei*, non-indigenous species, population structure.

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INTRODUCTION

Knowledge of dispersal dynamics of non-indigenous species, and the genetic patterns defined by these dynamics, is crucial to our understanding of evolutionary aspects of the colonization process and management of biological invasions (Hampton *et al.*, 2004; Wilson *et al.*, 2009). However, studying these issues remains challenging in aquatic animals, as information pertaining to dispersal dynamics is often incomplete. Life history characteristics including modes of reproduction (i.e. asexual versus sexual), the production and duration of free-swimming larval stages (e.g. veligers) and varying propensity for human-mediated dispersal all may influence the distribution and genetic structure of introduced populations (e.g. Darling & Folino–Rorem, 2009; Dupont *et al.*, 2009; Dybdahl & Drown, 2010). These factors and interactions among them may result in rapid range expansions coupled with complicated dispersal dynamics at different geographical scales (e.g. Hastings *et al.*, 2005; Bock *et al.*, 2011). Consequently, we have limited knowledge of how patterns of dispersal and colonization vary across geographical scales and the factors that may contribute to these patterns in aquatic non-indigenous species.

Use of appropriate species with intensive sampling strategies and robust genetic analyses based on polymorphic molecular markers may advance our understanding of both dispersal dynamics and related population genetic structure (e.g. Darling & Folino–Rorem, 2009; Zhan *et al.*, 2010). Introduced freshwater mussel species offer promising models for examining factors driving dispersal and determining genetic variation at different geographical scales (e.g. Müller *et al.*, 2002; Hughes *et al.*, 2004). Compared with active dispersers such as fish, veliger-producing mussels have limited abilities to swim against water currents to effect long-distance upstream dispersal, mainly owing to limited mobility of sessile adults. Natural dispersal of these mussels occurs primarily by passive spread of larvae advected by currents, resulting in mainly downstream spread (Griffiths *et al.*, 1991; Ricciardi, 1998). Therefore, natural dispersal is typically constrained to a specific drainage system and gene flow occurs mainly in an upstream-to-downstream direction. Physical barriers limit range expansion of freshwater mussels across drainages, although human-mediated transport of propagules can affect both local and long-distance 'jump' dispersal (Oliveira *et al.*, 2006; Hickey, 2010). It may be possible to clarify these dispersal mechanisms by examining the relationship between population genetic structure, the spatial arrangement of watersheds, and the movement of human vectors between them.

The golden mussel (*Limnoperna fortunei*) is native to freshwater systems in China and other Southeast Asian countries including Laos, Cambodia, Vietnam, Indonesia and Thailand (Ricciardi, 1998). This mussel has been introduced to and become a pest species in both East Asia and South America (Ricciardi, 1998; Boltovskoy *et al.*, 2006). It was likely introduced in 1991 to South America in ballast water discharged into the Río de la Plata estuary by a transoceanic vessel (Pastorino *et al.*, 1993). Following its initial introduction, golden mussels spread upstream extremely quickly. The species has spread more than 3000 km from its original point of entry and now inhabits almost the entire Río de la Plata basin (Boltovskoy *et al.*, 2006; Oliveira *et al.*, 2006). In these invaded areas, the mussels have caused large-scale biofouling on both natural and artificial hard substrates, sometimes attaining population densities of approximately 200,000 individuals/m^2 (Boltovskoy *et al.*, 2006). The species is also a nuisance to industrial and power plants and has altered both the structure and function of invaded ecosystems (Boltovskoy *et al.*, 2006; Paolucci *et al.*, 2007). The huge economic and ecological consequences of the golden mussel urge a detailed understanding of the post-establishment spread dynamics.

Human-mediated dispersal of golden mussels in South America has likely occurred both by discharge of ballast water containing larvae and by hull fouling by adults on recreational or commercial vessels (Ricciardi, 1998; Boltovskoy *et al.*, 2006). The predominant upstream pattern of spread, and spread across drainages, implicates human-mediated dispersal throughout South America (Ricciardi, 1998; Oliveira *et al.*, 2006). Meanwhile, downstream dispersal is expected to occur along drainages, mainly owing to the high fecundity (approximately 29,800 eggs per female; Karatayev *et al.*, 2007), extended spawning season (6–10 months; Boltovskoy *et al.*, 2009) and long free-swimming larval phase (approximately 20 days; Cataldo *et al.*, 2005). Its occurrence in different river drainages and life history characteristics (i.e. free-swimming larvae and sessile adults) suggest that the species exists as several large population groups defined by drainage systems, with extensive gene flow occurring within, but limited between, drainages. Here, we use the mitochondrial cytochrome c oxidase subunit I (COI) gene and eight polymorphic nuclear microsatellites to characterize dispersal dynamics and genetic structure of 22 golden mussel populations collected throughout the invaded range in South America and to determine how different dispersal methods (i.e. human-mediated dispersal and downstream movement of larvae) contribute to genetic variation among populations. In particular, we test the hypothesis that the spread among different drainages is owing to ship-mediated 'jump' dispersal, while that within a drainage is mainly a result of downstream natural dispersal.

METHODS

Sample collection

We conducted comprehensive sampling in the Paraná–Uruguay–Río de la Plata watershed encompassing the invaded range in Argentina, Brazil, Paraguay and Uruguay (Fig. 1; Table 1). Intensive sampling was conducted in the Paraná–Uruguay delta and the Río de la Plata estuary (populations EC, CR, TI, SF, BA, QU, PL, SL and MA), where the invasions began and also the southernmost range in South America. Following the historic invasion pathway to the north,

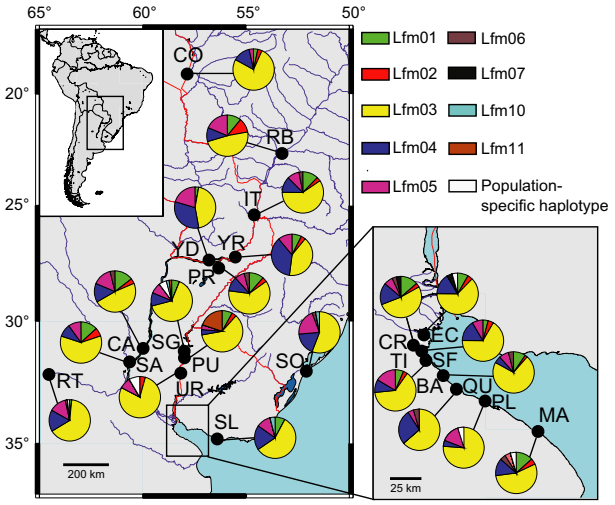

Figure 1 Sampling sites and distribution of mitochondrial cytochrome c oxidase subunit I (COI) haplotypes for the introduced golden mussel *Limnoperna fortunei* in South America. Site IDs as per Table 1. Pie charts indicate the proportion of haplotype groups observed at each site, with different colours referring to different haplotypes.

we sampled along the Paraná River as far as the upper Paraná in Brazil (populations SA, CA, RB, IT, YR, YD and PR). In the Uruguay River, we sampled up to the northern range limit (populations SG, PU). We also sampled one population (RT) along the western range limit in the Río Tercero reservoir in Argentina, and one population (CO) from the upper Paraguay River in Brazil, the northernmost range limit. Additionally, we included one sample (population SO) from the Guaíba basin on the coastal plain of Rio Grande do Sul in Brazil. In total, we collected 712 individuals from 22 locations (Fig. 1).

Molecular markers, DNA amplification and data collection

We performed genetic analyses based on both the mitochondrial cytochrome c oxidase subunit I (COI) gene and eight nuclear microsatellites. The COI gene was amplified using the primers LIMNO–COIF1 (Pie *et al.*, 2006) and HCO2198 (Folmer *et al.*, 1994). Microsatellite markers were developed from microsatellite-enriched libraries using the method modified by Zhan *et al.* (2007). After an annealing temperature optimization, polymorphism pre-assessment and linkage disequilibrium test, eight microsatellite markers (Table S1) were selected for genetic analyses. The effectiveness of these markers, including occurrence of null allele(s) and large allele dropout, was tested using MICRO-CHECKER version 2.2.0 (van Oosterhout *et al.*, 2004). Because the detection of null allele using MICRO-CHECKER relies on Hardy–Weinberg Equilibrium (HWE), and Wahlund effects and inbreeding in recently established populations may cause biased results, we also performed a correlation between PCR amplification failure rate and null allele frequency reported by MICRO-CHECKER using the Mantel test (Mantel, 1967).

Genomic DNA was extracted from posterior adductor muscles using the protocol described by Elphinstone *et al.* (2003). The PCR amplification and sequencing of COI were performed based on the protocols used by Bock *et al.* (2011). The doubly uniparental inheritance (DUI) of mitochondrial genome observed in other Mytilidae species was tested by sorting and analysing two different sexes using the method by Gillis *et al.* (2009). The PCR amplification of microsatellite loci was performed using a three-primer protocol (Schuelke, 2000) and the locus-specific annealing temperatures (Table S1). Amplified fragments were separated on an ABI 3130XL automated sequencer with GeneScan™–500 LIZ™ internal size standard. To confirm genotyping accuracy, we reran approximately 5% of the samples randomly chosen from each DNA plate.

Phylogenetic and demographic analyses

Bayesian inference (BI) and neighbour-joining (NJ) phylogenetic analyses were conducted using the brown mussel *Perna perna* as outgroup (GenBank accession number: EF493941). The BI and NJ analyses were essentially performed using methods of Zhan *et al.* (2010). To further resolve haplotype relationships within shallow phylogenies, a statistical parsimony haplotype network was generated at the 95% connection limit using TCS version 1.21 (Clement *et al.*, 2000).

Demographic history was investigated using Tajima's D (1989), Fu's F_s (1997) and mismatch distributions of pairwise differences (Rogers & Harpending, 1992) based on the COI sequences. Tajima's D and Fu's F_s implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005) were used to test whether the COI fragment evolved under neutrality or not. Negative values of these statistics indicate non-neutral evolution (i.e. selection and/or hitchhiking associated with selective sweeps) and/or population expansion. To test the population expansion hypothesis, we performed the mismatch distribution analysis implemented in ARLEQUIN. The fit between observed and expected distributions was tested using sum of squared deviations (SSD) for the estimated stepwise expansion models (Schneider & Excoffier, 1999). Significance was assessed with a permutation test under the null hypothesis that sudden population expansion cannot be rejected.

Population genetic analyses

Genetic diversity within sampling sites was calculated based on the COI gene and eight microsatellites. Intra-population genetic diversity for the COI gene was characterized by the number of haplotypes (n), haplotype diversity (h) and nucleotide diversity (π) using DNASP version 5.00.07 (Rozas *et al.*, 2003). Microsatellite-based intra-population diversity was measured by the number of alleles (A), allelic frequency (F), allelic richness (A_r), as well as the observed heterozygosity (H_O) and the expected heterozygosity (H_E) using FSTAT version 2.9.3.2 (Goudet, 2001). The Markov chain method

Table 1 Sampling details and genetic diversity indices for mitochondrial cytochrome c oxidase subunit I (COI) gene and microsatellite markers for the golden mussel *Limnoperna fortunei* in South America. *N*, sample size for different molecular markers in different populations; *n*, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity; *A*, average number of alleles; A_r: allelic richness; H_O and H_E, mean observed heterozygosity and expected heterozygosity computed at eight microsatellite loci.

				COI					Microsatellite				
ID	Region/State and Country	Latitude Longitude	Approximately date detected	*N*	*n*	Haplotype Code	*h*	π	*N*	*A*	A_r	H_O	H_E
CO	Corumbá, Brazil	−18.997° −57.654°	2000	29	5	Lfm01-05	0.416	0.0021	30	6.8	6.0	0.2214	0.5343
RB	Río Baía, Alto Rio Paraná, Brazil	−22.686° −53.253°	2002	27	5	Lfm01-05	0.724	0.0059	33	6.9	5.9	0.2285	0.5387
IT	Itaipú Hydroelectric Power Reservoir, Brazil	−25.408° −54.590°	2000	32	6	Lfm01-06	0.625	0.0033	30	7.8	6.8	0.2802	0.5916
YR	Yabebiry River, Misiones, Argentina	−27.297° −55.543°	1996	27	5	Lfm01-05	0.704	0.0037	28	6.8	6.1	0.1446	0.5329
YD	Yaciretá Dam, Brazil, Paraguay and Argentina	−27.471° −56.704°	1996	34	4	Lfm01, Lfm03-05	0.677	0.0029	29	6.3	5.8	0.1283	0.5387
PR	Paraná River, Santa Tecla, Corrientes, Argentina	−27.605° −56.385°	1996	26	6	Lfm01-06	0.609	0.0033	29	6.4	5.9	0.1930	0.4925
CA	Cayastá, Santa Fe, Argentina	−31.187° −60.033°	1996	27	6	Lfm01-06	0.726	0.0051	28	5.7	5.3	0.1764	0.5376
SG	Salto Grande Dam, Uruguay	−31.195° −57.905°	1996	48	8	Lfm01, Lfm03-05, Lfm09-12	0.570	0.0031	28	7.0	6.2	0.2814	0.5390
PU	Puerto Luis, Salto Grande Lake, Argentina	−31.257° −57.907°	2001	26	7	Lfm01-05, Lfm11, Lfm18	0.643	0.0064	40	7.8	6.4	0.2331	0.5781
SA	Setubal Lagoon, Santa Fe, Argentina	−31.635° −60.681°	2001	30	5	Lfm01-05	0.618	0.0042	34	7.1	6.1	0.2295	0.5196
SO	Sao Gonçalo Channel, Brazil	−31.811° −52.388°	1999	34	5	Lfm03-06, Lfm10	0.631	0.0034	34	6.8	5.9	0.2190	0.5596
UR	Uruguay River, Colón, Argentina	−32.152° −58.188°	1996	23	4	Lfm02-03, Lfm05, Lfm17	0.387	0.0025	26	5.3	5.1	0.2046	0.5483
RT	Río Tercero Dam, Córdoba, Argentina	−32.213° −64.473°	1998	59	6	Lfm01, Lfm03-06, Lfm13	0.546	0.0022	30	7.5	6.4	0.1795	0.5288
EC	Del Este Channel, Buenos Aires, Argentina	−34.346° −58.519°	1994	24	6	Lfm01-03, Lfm05, Lfm07, Lfm14	0.594	0.0041	40	7.4	6.2	0.1711	0.5758
CR	Carapachay River, Buenos Aires, Argentina	−34.397° −58.594°	1994	29	7	Lfm01-07	0.700	0.0035	30	6.6	5.0	0.1404	0.5130
TI	Luján River, Tigre, Buenos Aires, Argentina	−34.415° −58.578°	1994	24	5	Lfm01-05	0.540	0.0068	40	8.0	6.0	0.1893	0.5282
SF	Luján River, San Fernando, Argentina	−34.428° −58.552°	1994	30	5	Lfm01-05	0.575	0.0064	30	6.0	5.4	0.1399	0.4847
BA	Buenos Aires city, Argentina	−34.606° −58.346°	1991	52	6	Lfm01-06	0.483	0.0056	30	7.4	6.6	0.2188	0.6205
QU	Quilmes, Buenos Aires, Argentina	−34.716° −58.214°	1991	22	4	Lfm03, Lfm05-07	0.541	0.0028	40	7.6	6.6	0.2027	0.5566
PL	Punta Lara, Buenos Aires, Argentina	−34.782° −58.011°	1991	21	4	Lfm03-05, Lfm15	0.414	0.0027	39	7.9	6.7	0.1829	0.5760
SL	Santa Lucía River, Canelones, Uruguay	−34.522° −56.394°	1994	26	5	Lfm01, Lfm03-05, Lfm10	0.634	0.0038	30	6.9	6.1	0.2245	0.5565
MA	Magdalena, Buenos Aires, Argentina	−35.013° −57.536°	1991	22	7	Lfm01-06, Lfm16	0.688	0.0036	34	6.5	5.5	0.2162	0.5390
Total				672	17		0.597	0.0034	712	205	6.0	0.2002	0.5450

implemented in the program GENEPOP version 3.4 (Raymond & Rousset, 1995) was employed to estimate the probability of significant deviation from HWE. Significance criteria were adjusted for the number of simultaneous tests using sequential Bonferroni's corrections (Rice, 1989). Population genetic differentiation was determined by F_{ST} based on microsatellites and Φ_{ST} based on mtDNA with the Tamura–Nei substitution model using ARLEQUIN. A total of 10^4

permutations were performed to allow for significance after sequential Bonferroni's correction.

To detect hierarchical genetic structure among sampling sites, we performed an analysis of molecular variance (AMOVA) using ARLEQUIN. We divided all samples into six groups based on different river basins: group I Upper Paraguay (population CO), group II Paraná River (populations RB, IT, YR, YD, PR, CA and SA), group III Uruguay River (populations PU, SG and UR), group IV Río Tercero (population RT), group V Sao Gonçalo (population SO) and group VI Paraná Delta–Río de la Plata (populations EC, CR, TI, SF, BA, QU, PL, SL and MA). Molecular variance was partitioned into three levels: between groups, among populations within groups and within populations. To illustrate dispersal patterns along drainages, isolation-by-distance (IBD) was examined by testing the correlation between genetic distance [$F_{ST}/(1 - F_{ST})$ for microsatellite data and $\Phi_{ST}/(1 - \Phi_{ST})$ for COI data] and geographical distance using the Mantel test with 10^4 permutations implemented in GENEPOP. We performed IBD analyses on both the whole invaded area and separated drainages where more than four populations were sampled. We measured the geographical distance between sites using the shortest waterway with GOOGLE EARTH version 5.

To further assess population genetic structure, we performed the Bayesian clustering analysis implemented in STRUCTURE version 2.3.1 (Pritchard *et al.*, 2000). The Bayesian clustering method assigns individuals into genetic clusters to maximize the fit of the data to theoretical expectations derived from HWE. For *Limnoperna* populations in South America, possible Wahlund effects and inbreeding may violate the assumption of HWE (see Discussion). We performed a three-dimensional factorial correspondence analysis (3D-FCA) without such an assumption using GENETIX version 4.05 (Belkhir *et al.*, 2004) to see whether both methods can provide consistent results or not. For STRUCTURE analyses, inference for partitioning all individuals into an estimated number of sub-populations (K) was based on 10^6 Markov chain Monte Carlo iterations, after discarding the initial 10^5 iterations as burn-in. We assessed likelihoods for models with the number of clusters ranging from one to the total number of sites (i.e. 22). Ten independent runs were performed for each specified K-value to verify convergence. The number of clusters was estimated using the method of Evanno *et al.* (2005).

RESULTS

Phylogenetic pattern and population demography

A total of 672 individuals from 22 populations were successfully sequenced for the mitochondrial COI gene. Analysis of the whole 510-bp alignment revealed 24 variable sites, resulting in 17 haplotypes (GenBank accession numbers: HQ843794–HQ843810, Table 1). After sorting and analysing two sexes, we did not detect any evidence for doubly uniparental inheritance.

Phylogenetic reconstruction using both Bayesian inference and neighbour-joining methods revealed one shallow group (Fig. 2a). This finding was confirmed by the 95% parsimony haplotype network (Fig. 2b). The network exhibited a star-shape pattern with the most dominant haplotype (Lfm03) at the centre and the others connected to it by a limited number of mutation steps (Fig. 2b).

Both neutral evolution tests, Tajima's D and Fu's F_s, resulted in non-significant negative values ($D = -1.22$, $P = 0.09$; $F_s = -3.40$, $P = 0.18$). Further mismatch distribution analysis did not support sudden population expansion hypothesis ($\tau = 0.28$, $P_{SSD} < 0.0001$).

Genetic diversity within populations

When intra-population genetic diversity was assessed using the COI gene, haplotype number per population varied from four to eight with an average of 5.5, while haplotype diversity ranged from 0.387 to 0.726 with an average of 0.593 (Table 1). A relatively high genetic diversity was detected in the most recently established populations (Table 1), for instance, population SG (Salto Grande Dam, Uruguay), where golden mussels were detected in 2001, exhibited the highest number of haplotypes ($n = 8$).

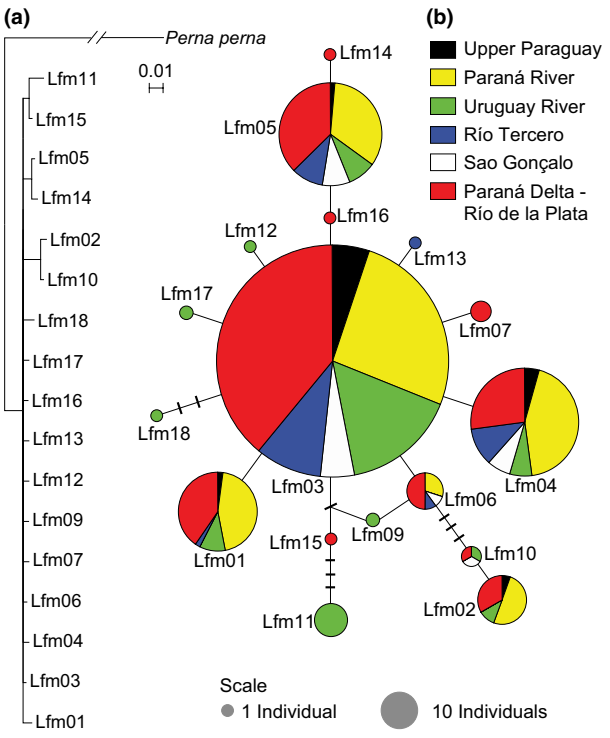

Figure 2 Bayesian inference tree (a) based on mitochondrial cytochrome c oxidase subunit I (COI) haplotypes, and COI haplotype network (b) generated with TCS for the introduced golden mussel *Limnoperna fortunei* in South America. Haplotype names as per Table 1. For the TCS network, sampled haplotypes are indicated by circles and missing or unsampled haplotypes are indicated by hashes. Haplotypes are painted according to different geographical regions from which the sample was collected. Circle size is proportional to observed haplotype frequency.

A total of 712 golden mussel individuals were genotyped at eight microsatellite loci. We did not detect large allele dropout at all loci in all populations. No correlation ($P > 0.87$) was detected between PCR amplification failure rate and null allele frequency reported by MICRO-CHECKER. In addition, we observed high success rates of PCR amplification (> 90%). Although we cannot totally rule out the presence of null alleles, all available evidence suggests that null alleles occurred at relatively low frequencies.

In total, we identified 205 different alleles across all populations examined, with the mean number of alleles per population ranging from 5.3 to 8.0 (Table 1). Mean expected heterozygosity (H_E) ranged from 0.4847 to 0.6205, while mean allelic richness (A_r) varied from 5.0 to 6.8 alleles (Tables 1 and S2). Generally, we observed little difference in microsatellite diversity in all populations including both historic and recently established ones (Tables 1 and S2). Deviations from HWE were observed at multiple loci and sampling locations after sequential Bonferroni correction. All the deviated cases showed significant heterozygote deficiency ($P < 0.001$).

Genetic structure among populations

Population genetic differentiation (pairwise Φ_{ST}) based on COI data varied from −0.041 to 0.111, with an average of 0.007. Twenty-five population pairs were significantly different, although these patterns were not upheld after sequential Bonferroni correction (Table 2). We found a higher level of genetic differentiation (Pairwise F_{ST}) when analysing microsatellite data. Pairwise F_{ST} values ranged from −0.060 to 0.108, with an average of 0.039, and after sequential Bonferroni correction, 85 of 231 comparisons (36.8%) remained significantly different (Table 2). Surprisingly, high genetic differentiation was observed not only between distant populations, but also between neighbouring ones in the same drainages. For example, pairwise F_{ST} was 0.100 between populations YD and PR, situated only 40 km apart in the Paraná River. Fine-scale genetic differentiation was also detected in the Río de la Plata estuary, for instance $F_{ST} = 0.053$ between populations EC and SF, which are only 9 km apart. In contrast, some geographically isolated populations had very low pairwise F_{ST} values, for instance between populations CO and QU ($F_{ST} = 0.004$), which are separated by approximately 2000 km (Table 2; Fig. 1). This finding was confirmed by AMOVA, which revealed that only 0.50% and 0.04% of genetic variance at the COI and microsatellite loci, respectively, was explained by the among-group component (Table 3).

Three-dimensional factorial correspondence analysis (3D-FCA) and Bayesian clustering provided a largely consistent view of population genetic structure (Fig. 3a,b). Factorial correspondence analysis grouped all populations into two clusters. Component 1 explained 11.13% of genetic variance and separated the two clusters (Fig. 3a). Interestingly, these two clusters showed genetically discontinuous distributions in the invaded range in South America (Fig. 3c). Populations sampled from the same drainages were not assigned to a single cluster; while geographically isolated populations were often assigned into the same clusters (Fig. 3a,b). More interestingly, populations from the initial invasion area, the Río de la Plata estuary, were grouped into different clusters. Compared with cluster 2, cluster 1 has a wider geographical coverage. Besides the Río de la Plata estuary, cluster 1 was also detected in the upper Paraguay River, upstream and downstream of the Paraná River, Río Tercero, the Uruguay River and Sao Gonçalo channel, whereas cluster 2 was limited to the mid-reach of the Paraná River (Fig. 3c). This pattern was confirmed by Bayesian clustering analysis (Fig. 3b). Bayesian clustering analysis also suggests a two-cluster model as the most parsimonious possibility (Fig. S1). Consistent with factorial correspondence analysis, the majority of individuals from the two different clusters defined by 3D-FCA were also assigned to two different clusters with high membership coefficients (Q) in Bayesian clustering analysis (Fig. 3b).

We observed no correlation between genetic and geographical distances for either the whole invaded range or for separated drainages using either mtDNA or microsatellite data (Fig. 4), suggesting that isolation-by-distance was not characteristic of golden mussel populations in South America.

DISCUSSION

Analyses of golden mussel populations based on microsatellites support two principal genetic clusters in South America. Interestingly, these two clusters were also detected in the initial invasion region of the Río de la Plata estuary (Fig. 1). Sites EC, CR and TI are geographically close to others (SF, BA, QU, PL and MA; Fig. 1), yet populations were genetically separated into distinct clusters. Direct evidence in support of this conclusion derives from both Bayesian clustering and 3D-FCA analyses (Fig. 3). Three possible processes could be responsible for the two-cluster pattern in the initial invasion region: (1) the two clusters were seeded by two genetically distinct sources; (2) rapid selection owing to strong local adaptation; and/or (3) genetic drift owing to demographic changes. The Río de la Plata estuary region receives a large amount of international shipping, and golden mussels have a very broad native range in East and Southeast Asia; hence, opportunities for multiple introductions are not insignificant. Moreover, key environmental factors, including average water temperature (20.0 °C versus 22.2 °C; E. Paolucci, personal communication), differ within the Río de la Plata estuary. Different temperatures may lead to different spawning seasons and survival rates of molluscs (e.g. Cudney–Bueno *et al.*, 2008; Travers *et al.*, 2009), resulting in limited gene flow between these two areas. Indeed, different spawning seasons were detected in different populations in the Río de la Plata (Boltovskoy *et al.*, 2009). Owing to the short invasion history in South America, it is unlikely that the pattern observed here has been originally generated from rapid selection associated with local adaption in the Río de la Plata estuary. However, genetic divergence derived from multiple introductions from genetically distinct sources could be maintained owing to

Table 2 Estimates of population genetic differentiation based on microsatellite markers (pairwise F_{ST}, below diagonal) and mitochondrial cytochrome oxidase c subunit I (COI) gene (pairwise Φ_{ST}, above diagonal) for the golden mussel *Limnoperna fortunei* across the introduced range in South America. Underlined and bold numbers indicate statistical significance before and after sequential Bonferroni corrections, respectively. Population IDs as per Table 1./span>

Pop	CO	RB	IT	YR	YD	PR	CA	SG	PU	SA	SO	UR	RT	EC	CR	TI	SF	BA	QU	PL	SL	MA
CO	****	0.024	−0.013	0.014	0.061	−0.022	0.005	−0.011	0.073	−0.008	0.033	−0.006	0.010	0.037	−0.015	−0.032	0.005	−0.009	0.111	0.021	−0.017	−0.020
RB	**0.055**	****	−0.005	0.014	0.032	−0.009	−0.021	0.008	0.042	−0.021	0.001	0.007	0.037	−0.019	−0.001	−0.006	−0.015	0.021	0.020	0.009	−0.009	−0.010
IT	0.037	**0.056**	****	0.003	0.033	−0.035	−0.029	−0.018	0.058	−0.029	0.012	−0.001	0.005	−0.003	−0.031	−0.027	−0.019	−0.020	0.054	0.003	−0.028	−0.036
YR	0.037	**0.107**	**0.042**	****	−0.008	0.003	−0.005	0.016	0.082	0.007	0.010	0.053	0.014	0.043	−0.006	−0.013	0.010	0.041	0.090	0.042	−0.020	−0.001
YD	0.032	**0.071**	**0.057**	**0.085**	****	0.040	0.012	0.033	0.109	0.042	−0.011	0.074	0.007	0.034	0.036	0.017	0.008	0.069	0.042	0.025	−0.001	0.047
PR	0.033	**0.080**	**0.044**	0.014	**0.100**	****	−0.029	−0.021	0.051	−0.033	0.014	−0.008	0.007	−0.002	−0.034	−0.032	−0.018	−0.024	0.061	0.005	−0.030	−0.041
CA	−0.044	0.013	−0.007	−0.060	0.042	−0.023	****	−0.013	0.051	−0.029	−0.004	0.008	0.005	−0.018	−0.026	−0.020	−0.026	−0.008	0.026	−0.001	−0.029	−0.029
SG	0.032	0.028	**0.039**	**0.076**	**0.052**	**0.068**	**0.056**	****	0.050	−0.013	0.005	−0.009	−0.002	0.000	−0.012	−0.023	−0.017	−0.012	0.040	−0.015	−0.021	−0.016
PU	0.036	**0.071**	**0.039**	**0.042**	**0.057**	**0.054**	0.047	**0.034**	****	0.045	0.079	0.056	0.109	0.051	0.060	0.053	0.056	0.083	0.090	0.055	0.055	0.050
SA	0.031	0.036	0.031	0.048	0.045	0.043	0.015	0.001	0.023	****	0.014	−0.006	0.019	−0.010	−0.027	−0.024	−0.018	−0.014	0.048	0.006	−0.024	−0.035
SO	**0.059**	0.029	**0.049**	**0.083**	**0.087**	**0.080**	**0.053**	0.033	**0.045**	0.039	****	0.018	−0.007	−0.008	0.023	−0.004	−0.020	0.033	−0.006	−0.017	−0.012	0.024
UR	0.045	0.045	**0.047**	**0.064**	**0.063**	0.038	0.029	0.019	**0.053**	0.038	0.029	****	0.017	−0.002	0.011	−0.015	−0.009	−0.005	0.046	−0.013	−0.001	0.001
RT	0.032	**0.038**	**0.050**	**0.069**	0.025	0.034	0.035	0.022	**0.046**	0.018	0.029	0.016	****	0.019	0.014	−0.013	−0.011	0.015	0.040	−0.013	−0.015	0.019
EC	0.028	**0.071**	0.027	0.024	**0.054**	**0.059**	0.039	**0.046**	**0.033**	0.030	**0.082**	**0.072**	**0.056**	****	0.012	0.003	−0.028	0.010	−0.022	−0.021	−0.004	0.006
CR	0.029	**0.108**	**0.043**	0.000	0.049	0.041	0.035	**0.053**	0.015	0.036	**0.088**	0.047	0.047	0.027	****	−0.026	−0.008	−0.013	0.076	0.020	−0.027	−0.038
TI	**0.044**	**0.065**	**0.039**	0.030	**0.056**	0.020	0.014	**0.044**	0.026	0.019	**0.046**	0.040	0.037	0.035	0.021	****	−0.022	−0.015	0.056	−0.009	−0.036	−0.030
SF	0.015	**0.050**	**0.045**	0.059	0.045	**0.058**	**0.060**	0.009	**0.054**	0.009	**0.060**	0.020	0.003	**0.053**	0.052	0.033	****	−0.006	−0.002	−0.027	−0.026	−0.011
BA	0.028	0.039	0.023	0.036	0.045	0.033	0.031	0.019	0.020	0.009	0.014	0.025	0.021	0.017	0.040	0.024	0.024	****	0.072	0.006	−0.010	−0.021
QU	0.004	**0.050**	0.024	0.032	0.033	0.031	0.031	0.027	0.028	0.022	**0.059**	0.048	0.029	0.015	0.012	0.020	0.012	0.009	****	−0.006	0.040	0.077
PL	0.016	**0.044**	0.021	0.021	0.034	0.033	0.028	0.023	0.013	0.010	0.034	**0.047**	0.037	0.015	0.006	0.017	0.024	0.006	0.005	****	−0.010	0.021
SL	**0.073**	0.024	0.046	**0.090**	**0.080**	**0.074**	0.040	0.024	**0.051**	0.029	0.008	**0.052**	0.040	**0.085**	**0.090**	**0.043**	**0.059**	0.022	**0.063**	**0.052**	****	−0.028
MA	0.029	**0.073**	0.029	**0.047**	**0.076**	0.031	0.024	0.036	**0.058**	**0.049**	0.037	0.034	0.025	**0.061**	0.041	**0.045**	0.028	0.007	0.037	**0.048**	**0.066**	****

Table 3 Results of the analysis of molecular variance (AMOVA) for the golden mussel *Limnoperna fortunei* across the invaded range in South America. All samples were divided into six groups based on geographical distributions: group I Upper Paraguay (population CO), group II Paraná River (populations RB, IT, YR, YD, PR, CA and SA), group III Uruguay River (populations PU, SG and UR), group IV Río Tercero (population RT), group V Sao Gonçalo (population SO) and group VI Paraná Delta–Río de la Plata (populations EC, CR, TI, SF, BA, QU, PL, SL and MA).

Source of variation	Sum of square	Variance components	Percentage variation	*P*-value
mtDNA				
Among groups	7.31	0.004	0.50	0.0469
Among populations within groups	15.90	0.005	0.53	0.2659
Among individuals within populations	560.86	0.863	98.98	0.1887
Total	584.07	0.872		
Microsatellite				
Among groups	43.63	0.001	0.04	0.0000
Among populations within groups	135.13	0.112	4.85	0.0000
Among individuals within populations	2696.01	2.186	95.11	0.3930
Total	2874.76	2.299		

limited gene flow between different locations in the Río de la Plata estuary. Genetic drift can increase genetic differentiation between populations by randomly changing allele frequencies, especially when effective population size decreases. Although our genetic surveys did not support sudden population expansions during establishment and post-establishment spread, related studies in bivalves showed that larvae were produced by a limited number of adults even in large populations, resulting in spatial differentiation associated with substantial genetic drift (e.g. Li & Hedgecock, 1998). Consequently, the influence of genetic drift cannot be ruled out without further investigation.

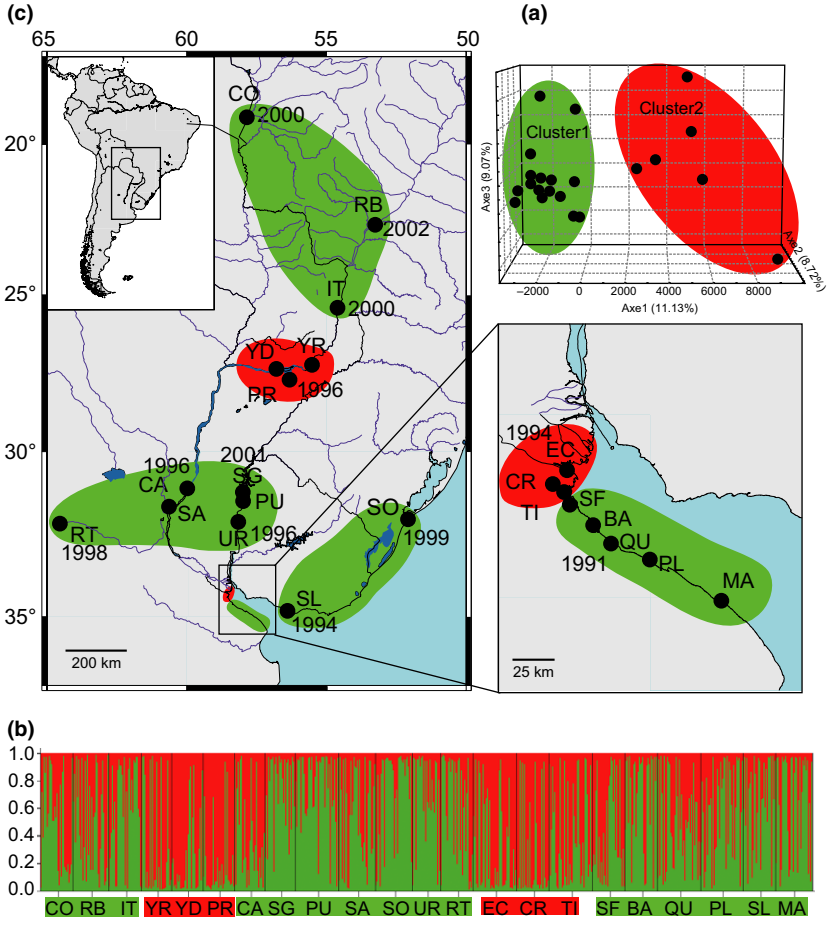

Figure 3 Three-dimensional factorial correspondence analysis (3D-FCA) (a) and Bayesian clustering (b) of the golden mussel *Limnoperna fortunei* based on eight polymorphic microsatellites in 22 populations collected from South America, and geographical distribution (c) of two genetically distinct clusters defined by 3D-FCA and Bayesian clustering analyses. The year that golden mussels were reported at each major geographical site is provided. For Bayesian clustering analysis, each genotype is represented by a thin vertical line, with proportional membership in different clusters indicated by colours. Bold vertical lines separate collection sites, with site IDs indicated below the plot. Site IDs as per Table 1.

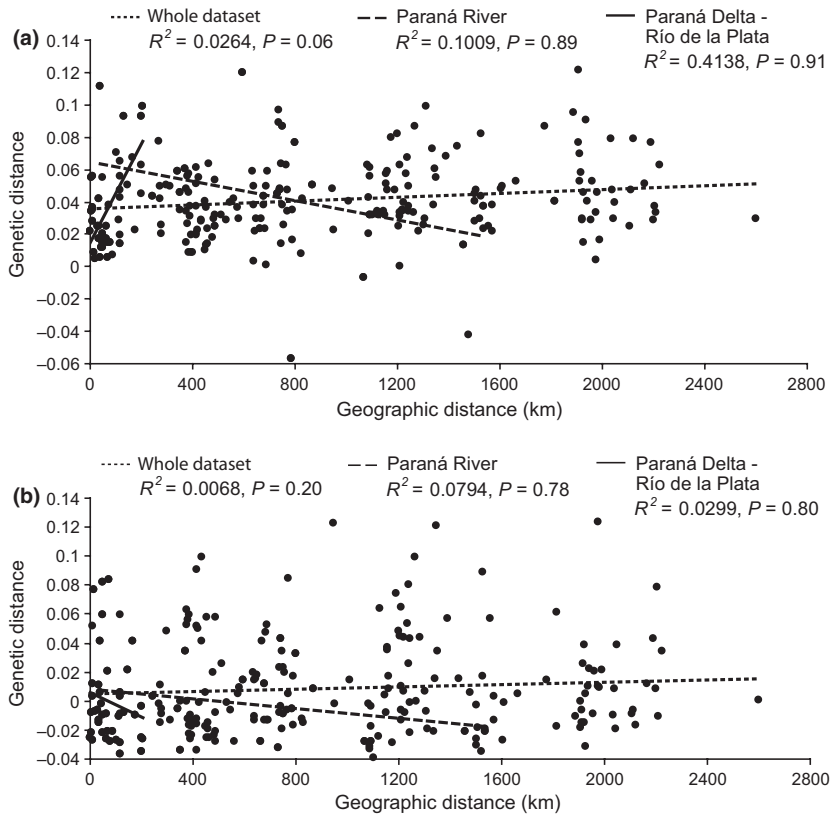

Figure 4 Isolation-by-distance (IBD) assessment based upon the correlation between geographical and genetic distance [$F_{ST}/(1 - F_{ST})$ for microsatellite data (a) and $\Phi_{ST}/(1 - \Phi_{ST})$ for the mitochondrial cytochrome c oxidase subunit I (COI) data (b)].

The two principal genetic clusters we identified exhibit a discontinuous patchy distribution pattern in the invaded range in South America (Fig. 3). This finding provides a striking contrast to the cases of zebra and quagga mussel invasions in North America, where very limited or no significant genetic differentiation has been reported (Table 4). Compared with zebra and quagga mussels, we generally detected lower overall genetic diversity and higher population genetic differentiation for golden mussels (Table 4). Our results are, however, similar to the pattern of zebra mussel range expansion in Europe, which exhibits a genetic discontinuity along rivers (Müller *et al.*, 2002). For the zebra mussel invasions, genetic admixture among initial colonies prior to further spread could explain the absence of genetic differentiation in North American populations, while slower invasion process during the initial stages of spread produced the contrasting pattern in Europe (Müller *et al.*, 2002). For the golden mussel in South America, it was first detected in the Río de la Plata estuary in 1991 (Fig. 3). After a few years, golden mussels 'jumped' to upstream drainages (Fig. 3). A relatively short invasion history, coupled with possible multiple introductions, could contribute to the discontinuous patchy distribution pattern.

Our genetic analyses suggest that there have been no apparent demographic changes during the post-establishment spread of golden mussels in South America. The newly colonized sites, including the upper Paraguay River and upstream of the Paraná River, exhibited a similar level of genetic diversity to relatively historic populations (Tables 1 and S2). All these results suggest large numbers of propagules were transported to found these populations. Although molluscs with high fecundity can colonize a large geographical area from only several individuals (e.g. Chandler *et al.*, 2008; Dybdahl & Drown, 2010), we did not observe genetic bottlenecks in any of the newly established golden mussel populations as would be expected to have only a small number of colonists been involved in the range expansions. A similar pattern has been observed not only in molluscs such as zebra mussels (e.g. Marsden *et al.*, 1995; Astanei *et al.*, 2005) and quagga mussels (e.g. Wilson *et al.*, 1999; Imo *et al.*, 2010), but also in other non-indigenous species such as ascidians (e.g. Zhan *et al.*, 2010). Biological characteristics common to these species, such as high fecundity, an extended spawning season and high larval density, may be responsible for this pattern. For mussels, juveniles and adults fouled on external hull surfaces, and veliger larvae contained in ballast water, can quickly and effectively be transported to areas where new invasions, or population supplementation of existing colonists, may occur (Ricciardi, 1998; Boltovskoy *et al.*, 2006). In addition, a number of invasive freshwater mussels including zebra, quagga and golden mussels have high tolerance to aerial exposure and desiccation, which can assist in overland spread. Aerial exposure tolerance tests in zebra and quagga mussels showed that, given summer conditions, adults can survive overland transport for about 3–5 days (Ricciardi *et al.*, 1994; McMahon, 1996), and golden mussel adults can survive approximately 120 h at 25 °C (Darrigran *et al.*, 2004).

We detected a massive heterozygote deficiency relative to HWE, representing 76% of all cases analysed (Table S2). A

Table 4 Comparison of microsatellite-based genetic features of the three highly invasive freshwater mussels, zebra mussel *Dreissena polymorpha*, quagga mussel *Dreissena bugensis* and golden mussel *Limnoperna fortunei*

Items	Zebra mussel *Dreissena polymorpha*	Quagga mussel *Dreissena bugensis*	Golden mussel *Limnoperna fortunei*
Native range (Reference)	Ponto-Caspian Region (Son, 2007)	Dnieper Delta (Son, 2007)	China and southeast Asia (Ricciardi, 1998)
Native pop. surveyed (genetic diversity)	Romania (H_E = 0.894)	Black and Caspian Seas region (H_E = 0.778–0.893)	N/A
Introduced pop. surveyed (genetic diversity)	Great Lakes (H_E = 0.878–0.888) Europe (H_E = 0.790–0.940)	Great Lakes (H_E = 0.802–0.931) Black and Caspian Seas region (H_E = 0.796–0.931) German (H_E = 0.873–0.931)	South America (H_E = 0.485–0.621)
Genetic differentiation within native pop.	F_{ST} = 0.051	F_{ST} = 0.008 to 0.030	N/A
Genetic differentiation within introduced pop.	F_{ST} = −0.007 to 0.032	F_{ST} = 0 to 0.026	F_{ST} = −0.060 to 0.108
Genetic differentiation between introduced and native pop.	F_{ST} = 0.008 to 0.054	F_{ST} = 0.002 to 0.035	N/A
Dispersal dynamics suggested by genetic analyses in invaded range	Ship-mediated dispersal & downstream movement of larvae	Ship-mediated dispersal	Ship-mediated 'jump' dispersal
References	Müller *et al.* (2002) and Astanei *et al.* (2005)	Wilson *et al.* (1999), Therriault *et al.* (2005) and Imo *et al.* (2010)	This study

deficit of heterozygotes has been reported in both marine (e.g. Hedgecock *et al.*, 2004; Zhan *et al.*, 2009) and freshwater bivalves (e.g. Wilson *et al.*, 1999; Astanei *et al.*, 2005; Therriault *et al.*, 2005; Imo *et al.*, 2010). The departure ratios in zebra and guagga mussels, 77.1 and 78.3%, respectively (Astanei *et al.*, 2005; Imo *et al.*, 2010), are slightly higher than that obtained in golden mussels here. Biological factors including inbreeding, Wahlund effect and selection, as well as the presence of null alleles can account for the heterozygote deficiency. Although we cannot totally rule out the presence of null alleles, the high success rate of PCR amplification and the lack of correlation ($P > 0.87$) between PCR amplification failure rate and null allele frequency suggests that null alleles are likely not a major cause of the observed heterozygote deficiency. Conversely, cohorts comprising kin and related individuals can be presented spatially in bivalves, increasing the probability of recurrent inbreeding (e.g. Li & Hedgecock, 1998). For golden mussels, two-thirds of populations consist of females (Ricciardi, 1998). Fewer males also increase the probability of recurrent inbreeding. In addition, frequent translocation of golden mussel propagules by commercial or recreational boating activities (Boltovskoy *et al.*, 2006) supports temporal and/or spatial Wahlund effects as another possible major cause. Frequent translocation of propagules could result in several cohorts coexisting at a single location. Changes in genetic composition in a single bay in association with different genetic composition of larvae have been reported in other bivalves with similar biological characteristics, including the Pacific oyster *Crassostrea gigas* (Li & Hedgecock, 1998). Practically, the departure from HWE can influence results derived from analyses assuming HWE such as STRUCTURE. However, multiple analyses with (i.e. STRUCTURE) and without HWE assumptions (i.e. 3D-FCA) provided largely consistent results, suggesting that departure from HWE has minor effects on STRUCTURE results in the present study.

Dispersal of golden mussels in South America, as characterized by genetic analyses, appears to be dominated by 'jump' dispersal dynamics. This hypothesis is supported by all genetic analyses including pairwise F_{ST} analysis (Table 2), Bayesian clustering analysis and 3D-FCA (Fig. 3), isolation-by-distance (Fig. 4) and AMOVA (Table 3). The congruent pattern confirmed by different analyses adds additional strength to the hypothesis of 'jump' dispersal. Jump dispersal also dominates dispersal dynamics in other invasive aquatic non-indigenous species, including zebra and quagga mussels (Table 4 and references therein), hydrozoans (Darling & Folino–Rorem, 2009) and macrophytes (Mineur *et al.*, 2010). A high rate of human-mediated transportation of propagules, likely due to ballast water and/or ship hull fouling, could account for such a pattern (Ricciardi, 1998; Boltovskoy *et al.*, 2006). The Paraná River is the second largest in South America and is used extensively for transcontinental and regional commercial shipping. Overland dispersal to new watersheds could be effected by juvenile and/or adult mussels fouled on recreational boats or trailers (Johnson *et al.*, 2001; Boltovskoy *et al.*, 2006).

Our genetic analyses suggest downstream dispersal has had limited effects on contemporary genetic patterns of golden mussels in South America. After golden mussel populations established in upstream areas, gene flow appears not to have extensively occurred in the downstream direction, at least not between distant populations in the same drainages. Although extensive downstream gene flow has been demonstrated in zebra mussels over large geographical scales such as the

Shannon–Erne drainage in Ireland (Astanei *et al.*, 2005), our genetic analyses in golden mussel populations provide little evidence of such downstream gene flow. This conclusion is supported by multiple lines of evidence from genetic analyses, especially by the genetic discontinuities along the same drainages (Fig. 3). For example, Bayesian clustering and 3D-FCA analyses consistently grouped populations collected from the same rivers into genetically distinct clusters (Fig. 3). In addition, both analyses consistently separated middle stream populations (YR, PR, YD) from those both upstream (IT) and downstream (CA, SA) in the Paraná River (Fig. 3). This finding was also confirmed by pairwise F_{ST} values. We observed high F_{ST} values between neighbouring populations along rivers, with some values exceeding those for populations collected from different rivers (Table 2). Similarly, significant microgeographical genetic differentiation in the same water bodies was also observed in other invasive freshwater mussels such as quagga mussels (Wilson *et al.*, 1999). Some studies estimated that free-swimming veliger larvae can travel more than 300 km downstream (Stoeckel *et al.*, 1997), and such downstream movement of larvae might have occurred in golden mussels. However, difficulties in directly tracking dispersal of individual larvae result in uncertainties about whether or not larvae can settle in downstream areas owing to competition with local populations and/or availability of suitable substratum, and if some can settle eventually, it can be challenging to determine their origin. Therefore, a more detailed understanding of dynamics of larval dispersal and settlement in different water systems is essential to clarify why genetic patterns of mussels vary in different geographical scales.

CONCLUSIONS

Compared with very limited or no genetic structuring during invasions by zebra and quagga mussels in some invaded areas such as North America, we observed that golden mussel populations in South America exhibit relatively high population structuring at both fine and large geographical scales. Although free-swimming veliger larvae can be advected along with water currents to distant downstream areas, our genetic analyses showed such downstream dispersal has had limited effects on genetic patterns of golden mussel populations. Significant genetic structuring, especially within the same drainages, suggests that ecological and/or evolutionary factors may influence larval settlement and spread, and further limit gene flow between populations.

Our study provides new evidence of scale-dependent post-establishment spread in aquatic non-indigenous species. The results of our study suggest that 'jump' dispersal, most likely due to ship-mediated transportation of propagules, dominates post-establishment spread of golden mussels in South America. However, once colonies become established at upstream locations, larvae produced may be advected downstream to infill patchy distributions. Although human-mediated 'jump' dispersal appears to dominate spread of golden mussels in South America, natural downstream dispersal should not be discounted. Our sampling covered several newly colonized areas, shortly after golden mussels were first reported. This time interval may be too brief to permit golden mussels to spread widely from upstream-to-downstream areas, although such spread appears likely in the near future.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Values of K (A) and ΔK (B) calculated as in Evanno *et al.* (2005) for detecting the biologically relevant clusters of the golden mussel *Limnoperna fortunei* collected from 22 locations in South America.

Table S1 Microsatellite markers and their corresponding primers developed and used in the present study for the golden mussel *Limnoperna fortunei*. T_a, optimal annealing temperature (°C); *S*, allele size range (bp). M13, universal M13 tail (5′ -CACGACGTTGTAAAACGAC-3′) was added to the 5′ end of each forward primer to allow fluorescent labelling.

Table S2 Genetic diversity at eight microsatellite loci for the golden mussel *Limnoperna fortunei* sampled from 22 locations across the invaded range in South America.

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BIOSKETCHES

Aibin Zhan has broad research interests in conservation genetics, evolutionary genetics and molecular ecology of aquatic species.

Hugh J. MacIsaac's research group is interested in a variety of questions pertaining to invasion biology, particularly with respect to aquatic ecosystems.

Author contributions: H.J.M., F.S. and A.Z. conceived the project; P.V.P., E.P., F.S. and P.S. collected the specimens. A.Z., P.V.P., S.G., E.P. performed the laboratory work. A.Z., P.V.P., S.G. and M.E.C. analysed the data. A.Z. wrote the manuscript, and all authors contributed to revising the manuscript; All authors contributed equally in discussing research strategy and development.

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