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High haplotype variability in established Asian populations of the invasive Caribbean bivalve *Mytilopsis sallei* (Dreissenidae)

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Abstract *Mytilopsis sallei* is one of a small number of tropical estuarine organisms known to have successfully invaded habitats outside their native range in the Caribbean. This bivalve now occurs in several major ports in East Asia, which suggests the transport of larvae and/or adults by vessels. However, little work has been done to determine transfer pathways because direct evidence is difficult to obtain. Here we test whether there is sufficient genetic variability in a mitochondrial marker of established Asian populations of *M. sallei* to allow for future reconstruction of invasion history. We sampled a 376-base-pair fragment of the mitochondrial cytochrome oxidase I (*COI*) of *M. sallei* for 254 individuals representing 11 populations from Singapore, India, Hong Kong and Taiwan. We found high variability with 24 positions distinguishing 15 haplotypes. Haplotype diversity ranged between 0.6 and 0.8 in eight Singapore populations, and an analysis of molecular variance showed that there was no significant genetic segregation in these populations. Observed haplotype diversity was also high in a population from Visakhapatnam, India, but was slightly lower in samples from Taiwan and Hong Kong. Preliminary data also indicate that Singapore, India, Hong Kong, and Taiwan populations may have different dominant haplotypes. These results suggest that there is sufficient genetic variability to use mitochondrial markers for reconstructing the invasion history of *Mytilopsis sallei*, when larger sample sizes become available.

Keywords Dreissenidae · *Mytilopsis* · Alien invasive species · COI gene · Southeast Asia

Introduction

There are only a small number of documented cases of marine invasions by invertebrates in tropical coastal areas (Crisci et al. 2003; Hutchings et al. 2002; Lewis et al. 2006; but see Carlton and Eldredge 2009). Arguably the best known is the establishment of the estuarine Caribbean dreissenid bivalve *Mytilopsis sallei* (Récluz) in and near major international ports

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and marinas in Australasia (e.g., Furuse and Hasegawa 1984; Karande and Menon 1975; Morton 1981, 1989; Nuttall 1990a, b; Stepien et al. 2001; Tan and Morton 2006; Wangkulangkul and Lheknim 2008, as *M. adamsi*; Willan et al. 2000) and the Mediterranean (Galil and Bogi 2009). *Mytilopsis sallei* is a highly adaptable New World relative of the freshwater Asian zebra mussel *Dreissena polymorpha* (Pallas), the latter being on the top 100 list of the world's worst invasive alien species as classified by the Global Invasive Species Database (see http://www.issg.org/). Given their ecological resemblance, *M. sallei* is likely to become similarly notorious because it is capable of surviving in a wide range of salinities (Ramachandra Raju et al. 1975) and temperatures (Tan, unpubl. obs.). The dispersal of this versatile and fecund species from the Caribbean to Southeast Asia has generally been attributed to shipping, either by way of the Atlantic to West Africa (Oliver et al. 1998) or via the Pacific through the Panama Canal (Morton 1981). The most likely modes of transportation are in cargo vessel ballast water (Chu et al. 1997; Williams et al. 1988) and/or through hull fouling (Willan et al. 2000). Unfortunately, a direct identification of the pathways and frequencies of invasion would require detailed and frequent examination of ballast water and ship hulls that are difficult to sample adequately, even in the best of circumstances. A potential alternative technique is to develop genetic markers that can identify invasive populations from different locations. This approach can potentially also determine the origin, direction and intensity of gene flow (Avise 2000; Chandler et al. 2008; Wang et al. 2009, 1999; Zardus and Hadfield 2005). Using these techniques for *M. sallei* is currently not feasible because too few sequences are available and it is thus unknown whether the species harbours sufficient genetic variability for reconstructing invasion pathways. Here we describe the genetic variability of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) of *M. sallei* from established populations in Singapore, India, Hong Kong, and Taiwan.

Materials and methods

Sampling

To assess the extent of genetic variation of *Mytilopsis* across different spatial scales, eight populations in Singapore (Kranji, Sembawang, Punggol, Serangoon, Potong Pasir, Geylang, Kim Seng, Pandan) and three populations elsewhere in South and East Asia (Visakhapatnam on the east coast of India, Tolo Harbour in Hong Kong, and Kaohsiung in Taiwan) were sampled (see Fig. 1). Unfortunately, several attempts to obtain specimens from its native Caribbean region were unsuccessful. Animals were collected by hand during low tide in the period between July 2004 and March 2005. Prior to tissue extraction, live individuals were preserved immediately in 100% ethanol for at least a few days with their valves held apart. Between 20 and 28 animals from each site were used for mtDNA extraction and subsequent analyses. As initial tests failed to detect sex-linked haplotype specificity (Geller 1994, 1996; Stepien et al. 1999), no attempt was made to distinguish sexes in samples.

mtDNA extraction, purification and amplification

DNA was extracted from posterior mantle tissue using a commercial kit (DNEasy, Qiagen) according to manufacturer's instructions. Extracted genomic DNA (1–2 μl) was initially subjected to the polymerase chain reaction (PCR) using COI (cytochrome c oxidase I) universal primers LCO1490 and HCO2198 (Folmer et al. 1994). Sequence quality was dissatisfactory and new primers internal to the universal primers were designed (forward: 5′-GGAGCTTAGTGCTCCTGGA-3′ and reverse: 5′AAGCATTGTCAGCCCACCA-3′). These primers amplified a ~492-base pair (bp) fragment of the COI gene. Each 25 μL reaction comprised 2.5 μL 10 × PCR buffer (Qiagen), 1.2 μL each of the oligonucleotide primers (10 μM), 1 μL dNTP's (0.2 mM), 0.5 U Taq polymerase (Takara Ex Taq Hotstart version) and autoclaved distilled water to volume. The thermal profile used an initial denaturation cycle at 95°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 57°C (30 s), and extension at 72°C (90 s). A 2 min extension at 72°C was added at the end of the cycle to increase copy number. After verification via gel electrophoresis, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and cycle-sequenced twice in the reverse direction using the Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). The cycle-sequenced product underwent a

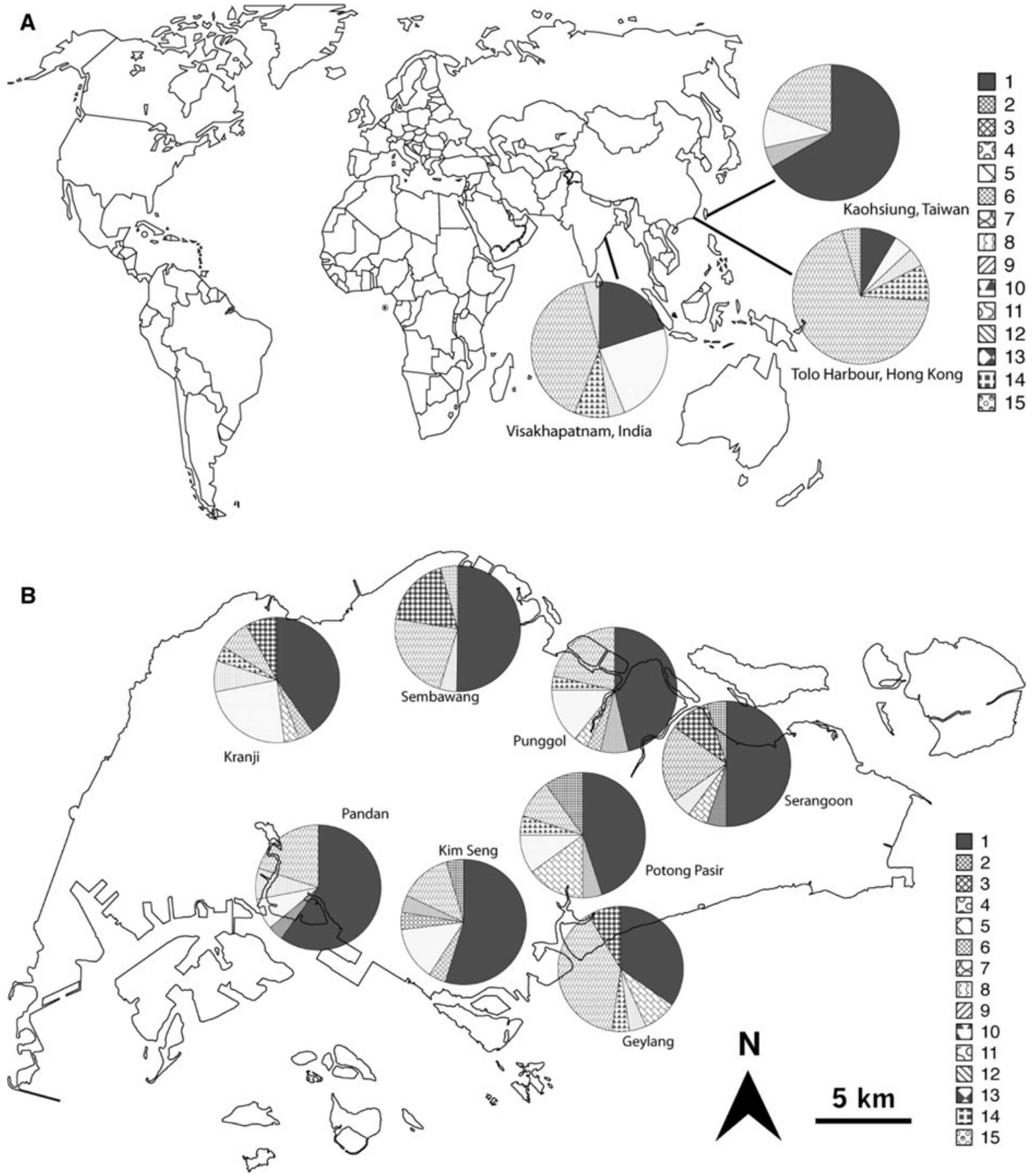

Fig. 1 Sampling localities and composition of COI haplotypes among (**A**) East Asian (India, Hong Kong and Taiwan) and (**B**) Singapore populations of *Mytilopsis sallei*. Pie charts indicate haplotype proportions. In (**B**), pie charts are centred approximately over actual collecting locations, except Geylang

final purification step using CleanSEQ Dye Terminator Removal kit (Agencourt Bioscience Corporation). Sequencing with the new primers was carried out on an ABI Prism 3,100 Genetic Analyzer at the Department of Biological Sciences, National University of Singapore. Sequences representing the different haplotypes were deposited in GenBank (accession numbers DQ078480–078494).

Analysis

Sequences were contigued with SEQUENCHER 3.1 (Gene Codes) and manually edited before creating consensus sequences. The alignment of the consensus sequences was indel-free and translatable into amino acids. Haplotype diversity (*h*) was estimated according to Nei (1987) and analyses of molecular variance (AMOVA) were performed for the relatively well sampled Singaporean populations using ARLEQUIN 2.0 (Excoffier et al. 1992; Schneider et al. 2000) to determine if any genetic structure can be detected. Pairwise ϕ_{ST}s were employed to provide preliminary estimates of genetic distances between sampled populations.

Results

DNA from 254 individuals in Singapore, India, Hong Kong and Taiwan were extracted, amplified and sequenced. A total of 15 haplotypes with variation across 24 nucleotide positions of the 376-bp COI gene fragment were identified. Most base changes occurred in the third position and were silent. Changes in the first and second positions were rare but resulted in amino-acid changes. Fourteen haplotypes were observed in Singapore, six in India and Hong Kong, and four in Taiwan (Table 1). Between four and eight haplotypes were obtained from the 20–28 individuals sampled at each location (Table 1). Four haplotypes (the lowest number) were obtained from a population in Kaohsiung, Taiwan, whilst twice the number of haplotypes was observed in a population sampled from Kranji, Singapore (see also Fig. 1).

With one exception all common haplotypes (found in >5 specimens) occurred in at least two countries. At each site in Singapore, the number of haplotypes varied between five and eight. Haplotypes 1 and 11 were dominant in Singapore and Taiwan, but were also found in all other East Asian populations. Haplotypes 1 and 11 occurred in relative proportions of up to 70% (Table 1), and contributed between 48 and 86% at all localities. In the majority of cases, haplotype 1 was more common than haplotype 11, except for the samples from Tolo Harbour (Hong Kong), Visakhapatnam (India) and Geylang (Singapore) where the reverse proportion was observed. Haplotype 6 was also present in nearly all localities except for two Singapore sites. About half the haplotypes sequenced are relatively rare (10% or less). We also found four unique haplotypes (nos 3, 7, 9 and 12), i.e., haplotypes represented by a single individual, in populations from Singapore and India (Table 1). Haplotype diversity (*h*) ranged between 0.518 and 0.790, while nucleotide diversity (π) was between 0.00522 and 0.00988 (Table 2). In general, populations from Singapore and India had higher

Table 1 Proportions of the 15 *Mytilopsis sallei* mtCOI haplotypes in 11 populations sampled in Hong Kong, Taiwan, India and Singapore

Location/Haplotype	*n*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total no. of haplotypes
Hong Kong: Tolo Harbour	23	8.7	0	0	0	0	4.3	0	4.3	0	8.7	69.6	0	0	0	4.3	6
Taiwan: Kaohsiung	21	66.7	4.8	0	0	0	9.5	0	0	0	0	19.0	0	0	0	0	4
India: Visakhapatnam	25	20.0	0	0	0	0	24.0	0	4.0	0	8.0	40.0	4.0*	0	0	0	6
Singapore: Sg Sembawang	22	50.0	0	0	0	0	0	0	4.5	0	0	22.7	0	18.9	0	4.5	5
Singapore: Sg Serangoon	20	50.0	5.0	0	0	5.0	5.0	0	0	0	0	20.0	0	10.0	5.0	0	7
Singapore: Sg Punggol	28	46.4	7.1	0	3.6	3.6	14.3	0	0	0	3.6	21.4	0	0	0	0	7
Singapore: Potong Pasir	20	45.0	5.0	0	0	15.0	10.0	0	0	0	5.0	10.0	0	0	10.0	0	7
Singapore: Sg Geylang	23	34.8	0	0	0	8.7	0	0	4.3	0	4.3	39.1	0	8.7	0	0	6
Singapore: Kim Seng	22	54.5	0	0	4.5	0	13.6	4.5*	0	4.5*	0	13.6	0	0	4.5	0	7
Singapore: Sg Pandan	25	60.0	0	4.0*	0	0	8.0	0	8.0	0	0	20.0	0	0	0	0	5
Singapore: Kranji	25	40.0	0	0	4.0	4.0	24.0	0	8.0	0	4.0	8.0	0	8.0	0	0	8

* denotes unique haplotypes; *n* = sample size. See Fig. 1 for locations of populations sampled

Table 2 Haplotype diversity (h) and nucleotide diversity (π) of the mitochondrial COI gene in *Mytilopsis sallei* populations from Hong Kong, Taiwan, India and Singapore

Location	h ± SE	π ± SE
Hong Kong: Tolo Harbour	0.518 ± 0.1218	0.00774 ± 0.00450
Taiwan: Kaohsiung	0.533 ± 0.1114	0.00522 ± 0.00343
India: Visakhapatnam	0.763 ± 0.0544	0.00988 ± 0.00575
Singapore: Sg Sembawang	0.693 ± 0.0764	0.00801 ± 0.00485
Singapore: Sg Serangoon	0.726 ± 0.0917	0.00752 ± 0.00462
Singapore: Sg Punggol	0.735 ± 0.0671	0.00802 ± 0.00480
Singapore: Potong Pasir	0.779 ± 0.0820	0.00875 ± 0.00525
Singapore: Sg Geylang	0.739 ± 0.0612	0.00877 ± 0.00522
Singapore: Kim Seng	0.688 ± 0.0982	0.00816 ± 0.00492
Singapore: Sg Pandan	0.610 ± 0.0948	0.00693 ± 0.00428
Singapore: Kranji	0.790 ± 0.0621	0.00956 ± 0.00559

genetic diversity than those from Hong Kong and Taiwan.

The 15 haplotypes were relatively diverse, with uncorrected genetic distances ranging between 0.27 and 3.19%. The maximum genetic distance recorded was between haplotypes 3 and 10 (Table 3). An AMOVA showed that there was little or no genetic structure among the eight Singapore populations, i.e., 99% of the variation was accounted for within each population ($P = 0.196$, df $= 184$). However, pairwise analyses of pooled Singapore samples with populations from Taiwan, Hong Kong and India revealed significant differences ($P < 0.05$) between those of Singapore and Hong Kong, and those of Singapore and India (Table 4). There was no significant difference between pooled Singapore and Taiwan populations (Table 4).

Discussion

In this study we identified a surprisingly large number of different haplotypes (15) that are distinguished by 24 variable sites within a relatively short 376 bp segment of COI gene. This variability was found in 254 individuals sampled from Singapore, India, Hong Kong, and Taiwan. This suggests that the gene has sufficient genetic variability for studying invasion history and gene flow in *Mytilopsis sallei.* At least four haplotypes were identified from each of the 11 populations sampled. Within Singapore, *Mytilopsis* populations had a total of 14 different haplotypes in eight populations located not more than 15 km apart, i.e., gene flow between the populations appear

Table 3 Uncorrected “p” distance (%) matrix between two haplotypes indicative of sequence divergence of 15 haplotypes (H) in *Mytilopsis sallei*

H	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	0.27	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	0.80	0.53	–	–	–	–	–	–	–	–	–	–	–	–	–
4	0.53	0.27	0.27	–	–	–	–	–	–	–	–	–	–	–	–
5	0.80	1.06	1.60	1.33	–	–	–	–	–	–	–	–	–	–	–
6	1.06	1.33	1.86	1.60	0.27	–	–	–	–	–	–	–	–	–	–
7	1.33	1.60	2.13	1.86	0.53	0.27	–	–	–	–	–	–	–	–	–
8	1.33	1.60	2.13	1.86	0.53	0.80	1.06	–	–	–	–	–	–	–	–
9	1.86	2.13	2.66	2.39	1.06	1.33	1.60	1.60	–	–	–	–	-	–	–
10	2.39	2.66	3.19	2.93	1.60	1.86	2.13	2.13	0.53	–	–	–	–	–	–
11	1.06	1.33	1.86	1.60	0.80	1.06	1.33	1.33	1.86	2.39	–	–	–	–	–
12	1.33	1.60	2.13	1.86	1.06	1.33	1.60	1.60	2.13	2.66	0.27	–	–	–	–
13	1.33	1.60	2.13	1.86	1.06	1.33	1.60	1.60	2.13	2.66	0.80	1.06	–	–	–
14	1.33	1.60	2.13	1.86	1.06	1.33	1.60	1.60	2.13	2.66	0.80	1.06	1.06	–	–
15	1.33	1.60	2.13	1.86	1.06	1.33	1.60	1.60	2.13	2.66	0.80	1.06	1.06	0.53	–

Note the high divergence (>3%) between H3 and H10

Table 4 Population pairwise ϕ_{ST} values for 11 populations of *Mytilopsis sallei* from Singapore (SB: Sungei Sembawang; SR: Sungei Serangoon; PG: Sungei Punggol; KR: Kranji; PL: Sungei Pandan; PP: Potong Pasir; KS: Kim Seng Canal; GY: Geylang Canal), Taiwan (TW: Kaohsiung), Hong Kong (HK: Tolo Harbour) and India (IN: Visakhapatnam)

Site	SB	SR	PG	KR	PL	PP	KS	GY	TW	HK
SB										
SR	−0.0328									
PG	0.0288	−0.0164								
KR	0.0560*	0.0252	0.0016							
PL	0.0286	−0.0140	−0.0230	0.0249						
PP	0.0334	−0.0109	−0.0248	−0.0203	−0.0037					
KS	0.0367	−0.0135	−0.0343	−0.00767	−0.0245	−0.0346				
GY	−0.0053	0.0070	0.0399	0.0511	0.0646	0.0358	0.0586*			
TW	0.0511	−0.0041	−0.0143	0.0623	−0.0310	0.0187	−0.0172	0.1070*		
HK	0.1780*	0.2120*	0.2340*	0.2260*	0.2840*	0.2240*	0.2610*	0.0589*	0.3450*	
IN	0.0931*	0.0918*	0.0851*	0.0541	0.1340*	0.0663*	0.0984*	0.0051	0.1850*	0.0349

An asterisk (*) indicates significant difference ($P < 0.05$)

unstructured, which may indicate that gene exchange occurs among the populations either naturally or assisted by anthropogenic shipping activities. Interpreting the comparative data for Singapore, India, Hong Kong, and Taiwan is more difficult. Despite several attempts, we were not able to obtain samples from the native range, and the relatively small number of individuals available for the populations from India, Hong Kong, and Taiwan precluded a more robust analysis. Nevertheless, our preliminary data suggest that *Mytilopsis* populations in India and Hong Kong have different dominant haplotypes from those in Singapore and Taiwan. Whilst high genetic diversity is not unexpected for invasive species (Stepien et al. 2005) and marine organisms (Dupont et al. 2003; Crandall et al. 2008), the very high genetic variability here observed for invasive *Mytilopsis* populations is surprising. The data can either be explained by a small number of invasions through a genetically unusually variable population or the high genetic diversity is due to multiple invasions. We favour the latter explanation given that high diversity is found in four countries and it appears unlikely that single invasions would consistently produce such a pattern. Multiple founders and/or multiple founding sources have also been observed for the Chinese mitten crab *Eriocheir sinensis* that is now established in Europe (Wang et al. 2009), and the European green crab *Carcinus maenas* in Canada (Roman 2006). This is in contrast to the marine gastropod *Rapana venosa*, which lacks genetic diversity in introduced populations compared to those in the native range (Chandler et al. 2008).

The maximum haplotype diversity for the COI gene, $h = 0.79$, was recorded for the Kranji-Singapore population. This haplotypic diversity is significantly higher than reported for populations of other surface-dwelling dreissenoid relatives such as *Congeria kusceri* ($h = 0.66$; Stepien et al. 2001). The sequence divergence of COI within *Mytilopsis sallei* populations yielded a range of genetic distance from 0.276 to 3.19%. Intraspecific COI variability in *M. sallei* was higher than in other members of the Dreissenidae (up to 1.1%; Quaglia et al. 2008; Therriault et al. 2004). Given their high genetic variability, there may be inherent difficulties in controlling invasive populations of these bivalves. However, since this study was carried out, a barrage built across Marina estuary in 2009 has effectively isolated what was perhaps the largest *Mytilopsis* community (including Kim Seng, Potong Pasir and Geylang populations sampled in this study) in Singapore from the sea. It remains to be seen if these genetically variable populations can survive in what will eventually be a freshwater reservoir fed by rain and urban runoff.

Our study unfortunately also demonstrates why the use of gene sequences for reconstructing the invasion history of species faces serious sampling issues when source and invasive populations are found in disparate geographic areas. Obtaining samples from all relevant areas is very expensive and time-consuming and the number of samples that needs to be analysed remains unknown until a preliminary assessment of the genetic variability within the populations is completed. In the case of *M. sallei*, our preliminary assessment revealed that the COI gene has sufficient variability for reconstructing the invasion history of this species. In addition, we have also shown that a large number of individuals and locations need to be sampled in order to obtain a good coverage of haplotype diversity.

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