Molecular analyses revealed three morphologically similar species of non-native apple snails and their patterns of distribution in freshwater wetlands of Hong Kong

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Abstract

Aim: Effective control of invasive species and conservation of native biodiversity requires accurate species identification. Several species of apple snails (Ampullariidae: Pomacea) from South America have become widespread pests in Asia since their introduction in the early 1980s, but their taxonomic uncertainty has hindered our understanding of the invasive processes. We aim to determine the identity and distribution of Pomacea species in Hong Kong, which has been known as a stepping stone of species invasion.

Location: Hong Kong.

Methods: We collected 162 apple snails from five freshwater wetlands of Hong Kong, sequenced the mitochondrial COI, genotyped the nuclear EF1α and built a COI data set of 1378 sequences of apple snails from mainland China, Malaysia, Argentina and Brazil. We identified the species, determine the introgression pattern and analysed their population structures.

Results: We found the co-occurrence of P. canaliculata, P. maculata and P. occulta in Hong Kong for the first time. P. canaliculata represented the dominant species (85% of collected specimens). The three Pomacea species from Hong Kong had low genetic diversity. High genetic similarity existed between P. canaliculata populations of Hong Kong and Malaysia, P. occulta populations of Hong Kong and mainland China and P. maculata populations of mainland China and Malaysia. Our samples contained a high proportion of hybrids.

Main conclusions: Apple snails may have been introduced into Hong Kong multiple times: both P. canaliculata and P. occulta likely came from Argentina; P. maculata came from both Argentina and Brazil. Genetic drift largely explains the differentiation of Pomacea spp. among regions. Introgressive hybridization may have contributed to population fitness by increasing genetic variation to neutralize the founder effect. The knowledge on species identity, distribution and genetic diversity of apple snails...
1 | INTRODUCTION

Biological invasion represents one of the key drivers of biodiversity change by impacting native species and altering the structure of species interaction (Diaz et al., 2019; Sentis et al., 2021). Effective control of invasive species and conservation of native biodiversity demands reliable species taxonomy (Ely et al., 2017; Shaik et al., 2016). Accurate species identification is essential for pest quarantine and early detection of invasive species. Poor taxonomy may lead to confusion of the invasive species and their native relatives, and thus delay the control against invasive species and even endanger the conservation of natives (Hayes, 2021). Moreover, correct species identification serves as a promise to the knowledge of the distribution patterns and population genetic patterns, which contribute to the management of biological invasion. Understanding the distribution patterns is crucial for range prediction and risk prevention of invasion (Zhang et al., 2020). Furthermore, knowledge of the patterns of genetic diversity and genetic structure can help elucidating evolutionary processes and invasive histories (Jackson et al., 2015).

Several apple snail species of the genus Pomacea (Caenogastropoda: Ampullariidae) have successfully invaded tropic and subtropic habitats globally and become the widespread agricultural and environmental pests (Cowie et al., 2017). High morphological symmetry and plasticity make Pomacea species difficult to identify (Hayes, 2021). Taxonomic studies integrating morphological characterization and molecular phylogenetics using mitochondrial genes over the last decade have clarified some of the taxonomic confusion in Pomacea spp. (Hayes et al., 2012; Rawlings et al., 2007; Yang et al., 2019; Yang & Yu, 2019). The accurate taxonomy of apple snails has improved our knowledge on their distributions in their introduced regions around the world, such as in North America (Pierre et al., 2017; Rawlings et al., 2007), Asia (Hayes et al., 2012) and Pacific islands (Tran et al., 2008). Nevertheless, chance of introduction as well as differences in physiological tolerance to environmental factors such as temperature and hypoxia may determine their distribution and dominance patterns in different regions (Matsukura et al., 2016; Mu et al., 2015, 2018; Seuffert & Martin, 2021). In many of their invaded regions, the identities of apple snails remain unknown which hinders our understanding of their ecological impacts, and the development of effective management measures (Kwong et al., 2009, 2010; Yang et al., 2019).

At least five non-native Pomacea species (P. canaliculata, P. maculata, P. occulta, P. diffusa and P. scalaris) occur across South-East Asia, among which the first three have caused great economic and ecological impacts (Hayes et al., 2008; Yang et al., 2019; Yang & Yu, 2019). In mainland China, P. canaliculata occurs in 12 southern provinces, but P. maculata only distributes in several locations in the southwestern regions, such as Sichuan Province and Chongqing Municipality, and one population in the eastern Zhejiang Province (Lv et al., 2013; Yang et al., 2018). Pomacea occulta, mis-identified as P. maculata in a previous study (Song et al., 2010), previously considered as a cryptic clade (Lv et al., 2013; Yang et al., 2019), is now recognized as a distinct species (Yang & Yu, 2019). Pomacea occulta exhibits a wide distribution in 11 provinces, often co-existing with P. canaliculata in China (Yang et al., 2019). Both P. diffusa and P. scalaris have been considered to possibly have a smaller ecological impact, but their non-native populations have occurred in some regions (Li, 2008; Rawlings et al., 2007). Hayes et al. (2008) reported non-native populations of P. diffusa in Sri Lanka as a result of the aquarium trade. Although P. diffusa serves as a popular pet for aquarium trade, this species has not yet invaded the wetlands of China (Yang, Liu, Song et al., 2018). The fifth species, P. scalaris, however, have been reported only from southern Taiwan outside its native range (Hayes et al., 2008; Wu et al., 2011).

The Pomacea spp. invaded Hong Kong in the early 1980s (Kwong et al., 2008). Results from two territory-wide surveys showed that non-native apple snails, initially found in the paddy fields and drainage channels of the northern New Territories, had spread to southern New Territories and Tsing Yi Island (Kwong et al., 2008; Yipp et al., 1991). Yipp et al. (1991) reported the presence of Ampullaria levior and Ampullaria gigas in the northern New Territories. Both Ampullaria species are junior synonyms of P. canaliculata (Hayes et al., 2012). However, no specimens collected by Yipp et al. (1991) remain for confirmation of their species identities. In a study mainly aiming to determine the distribution of apple snails in Hong Kong, Kwong et al. (2008) selected 16 most morphologically distinct individuals based on shell size and head-foot colours from 34 freshwater habitats for determination of mitochondrial cytochrome c oxidase subunit I (COI) sequences, which led to the identification of these specimens as P. canaliculata. Nevertheless, due to the small sample size used and considerable intra-specific plasticity in shell morphology in Pomacea, it remains undetermined how many species of apple snails have invaded the freshwater wetlands of Hong Kong.

In the present study, we collected apple snails from five populations in Hong Kong to clarify their species identities and composition through sequencing the COI gene of at least 30 specimens from each population. Because several studies have extensively investigated the species identities of invasive Pomacea spp. in mainland China and
compared with the global introduced and native populations (Hayes et al., 2008; Lv et al., 2013; Yang, Liu, He, et al., 2018), we compared the genetic diversity of apple snails in Hong Kong with those in mainland China. We included sequences of apple snails from Malaysia because it provides the only country with recent COI gene data on apple snails (Kannan et al., 2020; Rama Rao et al., 2018). Moreover, because introgressive hybridization between Pomacea species has been detected in both Asia and their native ranges (Glasheen et al., 2020; Matsukura et al., 2013; Yang et al., 2020; Yoshida et al., 2014), we also analysed the hybridization patterns of apple snails collected from Hong Kong through genotyping the nuclear elongation factor 1-alpha (EF1α) gene.

2 | METHODS

2.1 | Specimen collection

We sampled 162 apple snails from five localities of New Territories, Hong Kong, during September 2019 to March 2020, including 30 snails from Lok Ma Chau (LMC, 22°30′49″N, 114°0′23″E), Hong Kong Wetland Park (WP, 22°28′13″N, 114°0′23″E), and Lam Tsuen (LT, 22°26′35″N, 114°8′3″E), 32 snails from Ho Sheung Heung (HSH, 22°30′33″N, 114°0′6″E) and 40 snails from Kam Tin (KT, 22°24′54″N, 114°4′13″E, and 22°26′19″N, 114°3′41″E) (Figure 1). These sites covered the diversity of habitats inhabited by apple snails in Hong Kong’s freshwater environments: two vegetable gardens (HSH and LT), a reconstructed wetland as a compensation for the loss of wetland due to construction of a rail road (KT), a wetland park created as a compensation for the wetland loss due to new town development (WP), and a lotus pond (LMC). We observed a strong sex bias (female/male = 8/1) in adults and only a few egg clutches in HSH. Sex ratios in other populations were close to 1:1. We deposited the tissue samples used in this study in Zhejiang Museum of Natural History (catalog numbers A50000001M1–A5000000162M1) as voucher specimens.

2.2 | DNA extraction, PCR and sequencing of mitochondrial COI

We extracted total DNA from ~10 mg of foot muscle tissue of each snail using the TIANamp Genomic DNA Kit (TIANGEN) following the manufacturer’s protocol, and finally eluted them in 200 μl ddH2O. We amplified the 5′-end portions of the mitochondrial COI from each individual with the universal primer pair LCO1490/HCO2198 (Folmer et al., 1994; Appendix S1: Table S1) in 25 μL PCR reaction, which contained 0.625 U TaKaRa Ex Taq, 1× Ex Taq Buffer, 5 mM dNTP mixture, 10 μM each primer and 1 μL total DNA template. We performed PCR by initial denaturation at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C and 60 s at 72°C, then final extension at 72°C for 8 min and termination at 4°C. We visualized and checked for specificity of the PCR products via gel electrophoresis. We sent single product amplicons to Sangon Biotech for sequencing using the forward primer by using an ABI3730 DNA Analyzer (Applied Biosystems), and checked all the fluorescent peaks of the sequences for errors using the program Geneious v11.1.5 (Kearse et al., 2012). If the sequence had low quality with multiple peaks, we sequenced the amplicon again with the reverse primer. We obtained the target region after removing the primers and error bases at both ends using Geneious.
We obtained 162 COI sequences from the five apple snail populations in Hong Kong. After trimming the primers and ambiguous bases at the ends, the sequences were 619 bp in length (GenBank accession numbers MT806196–MT806357).

2.3 | COI datasets and haplotypes

A preliminary BLAST search (Johnson et al., 2008) of the GenBank nucleotide database suggested that the sequences of apple snails from Hong Kong generated in the present study matched those of *P. canaliculata*, *P. maculata* and *P. occulta*. Therefore, we included the published COI sequences of the three species that covered the known geographical ranges of populations from mainland China and Malaysia, and also the representative sequences from Argentina and Brazil to form a combined dataset (Appendix S1: Table S2). Altogether, the dataset comprised 1378 sequences, including 162 sequences from Hong Kong, 1066 sequences of *P. canaliculata*, *P. maculata* and *P. occulta* from mainland China, and 76 sequences of *P. canaliculata* and *P. maculata* from Malaysia, 45 sequences of *P. canaliculata* and *P. maculata* from Argentina and 29 sequences of *P. maculata* from Brazil.

We performed global alignment of the dataset using Geneious v11.1.5 (Kearse et al., 2012) and trimmed the sequences to a consensus sequence dataset to generate haplotypes after alignment. Because the 521 sequences from Lv et al. (2013) were 503 bp in length, which was the shortest among the sequences from various studies, we trimmed the dataset with a total of 1378 sequences to 503 bp. To evaluate any trade-off of shorter COI sequence on the studies, we trimmed the dataset with a total of 1378 sequences to length, which was the shortest among the sequences from various sensus sequence dataset to generate haplotypes after alignment.

We identified the apple snails from Hong Kong based on the phylogenetic analysis. For the apple snails from Hong Kong, we further determined the nuclear EF1α gene for each snail collected from Hong Kong and diagnosed as *P. canaliculata* or *P. maculata* using the primer-specific-multiplex PCR described by Yang et al. (2020). Although a previous study based on the EF1α gene indicated incomplete lineage sorting between *P. occulta* and *P. canaliculata* and *P. maculata* (Yang et al., 2019), we also genotyped the EF1α gene of the snails with a COI type of *P. occulta* to check the hybridization patterns among these species. The multiplex PCR assay included two pairs of specific primers (Appendix S1: Table S1), including EF3Fc/EF3Rc targeting on a 125 bp of canaliculata-EF1α, and EF1Fm/EF1Rm targeting on a 125 bp of maculata-EF1α.
EF3Rm targeting on a 151 bp of maculata-EF1α (Yang et al., 2020). We performed PCR in 25 μl solution containing 12.5 μl Premix Taq™ (Takara), 1 μl each primer (10 mM), 1 μl template DNA, and 7.5 μl ddH2O. The PCR programme included 94°C for 3 min followed by 35 cycles of 94°C for 3 min, 56°C for 30 s and 72°C for 20 s, and a final elongation at 72°C for 5 min. We distinguished the genotypes of the nuclear EF1α by applying 5 μl ampiclons in 3.5%–4.0% (w/v) agarose gels.

3 | RESULTS

3.1 | Sequences, haplotypes and similarity

Analysis of 503 bp COI sequence in 1378 apple snails from Asia (China, Hong Kong and Malaysia) and South America (Argentina and Brazil) revealed a total of 88 haplotypes (Table 1; Appendix S1: Table S3). The COI dataset of 578 bp with 857 sequences revealed five additional haplotypes from the one or two base pair variations of the regions flanking the 503 bp core region: two additional haplotypes from Hap1 with sequences KR020969, KR021005 and KT852706 from the mainland Chinese populations, one additional haplotype from Hap6 from the Malaysian population (MG230774), one additional haplotype from Hap38 from the Argentine population (AB728577), and one additional haplotype from Hap67 from the Brazilian population (EU528568). Despite a slight increase in haplotype diversity from the dataset of longer sequence length, we only reported the results with the sequences trimmed to 503 bp to cover sequences from more regions from mainland China.

Among the 88 haplotypes, 36 of them were identified from Hong Kong, mainland China, and Malaysia (Hap1–Hap36; Table 1). Comparing between Asia and South America populations, we only identified the sharing of P. canaliculata Hap1 among Argentina and the introduced ranges of Hong Kong, mainland China and Malaysia. There were 27 haplotypes of P. canaliculata (Hap37-Hap54) and P. maculata (Hap55-Hap63) exclusively from the Argentinian populations (Appendix S1: Table S3). Hap64–Hap88 of P. maculata were generated only from the Brazilian populations (Appendix S1: Table S3).

The 162 sequences from Hong Kong belonged to 10 haplotypes (Hap1–Hap10, Table 2). The two most common haplotypes were Hap1 (61.1% of total sequences) and Hap2 (19.8%), which were identified as P. canaliculata and presented in all the five populations. Hap8, the third top abundance haplotype (6.8%), was identified as P. occulta and detected from four populations except the northern vegetable garden in Ho Sheung Heung (Table 2). The other haplotypes were rare (<5%), distributed in only one or two populations and represented by one to eight individuals. In addition, BLAST analysis against the GenBank nucleotide database identified four unique haplotypes in Hong Kong (Table 1), including Hap5 (P. canaliculata, 1 bp different from the closest haplotype Hap1), Hap7 (P. maculata, 7 bp different from Hap6) and Hap9–Hap10 (P. occulta, 3 bp and 1 bp different from Hap8, respectively). Both Hap5 and Hap 9 were found in Lok Ma Chau; Hap7 and Hap10 were found in Ho Sheung Heung and Wetland Park, respectively (Table 2).

Apple snails from mainland China were more genetically diverse, with 21 haplotypes of P. canaliculata, 3 haplotypes of P. maculata and 6 haplotypes of P. occulta (Table 3). In contrast, the Malaysian Pomacea had only four haplotypes of P. canaliculata and one haplotype of P. maculata (Table 3). Hap1 was the most common haplotype of P. canaliculata from Hong Kong, but it was the second largest haplotype of P. canaliculata from mainland China. Hap2 that had been identified in 507 specimens was the most dominant haplotype in China (Table 3), but it was absent from the Malaysian population.

3.2 | Phylogenetic relationships and genetic divergence

The NJ, ML and BI trees of COI sequence all supported the monophyletic clades of P. canaliculata, P. maculata and P. occulta (Figure 2, Appendix S1: Figure S1). Although the posterior probability value for distinguishing P. canaliculata sequences into its own monophyletic clade was lower than 0.7 on the BI tree, both the ML and NJ trees showed high bootstrap values (100) at the major clades (Figure 2, Appendix S1: Figure S1). Both the P. canaliculata and P. maculata clades were further clearly split into two clades each in the phylogenetic trees (Figure 2). Among the Hong Kong haplotypes, Hap1 and Hap5 of P. canaliculata were in Clade A, and Hap2–Hap4 were in Clade B. Hap6–Hap7 were in Clade C (P. maculata), while haplotypes in Clade D (P. occulta) were all from specimens collected in Brazil. Hap8–Hap10 were nested in the P. occulta clade.

The phylogenetics of haplotypes further confirmed the presence of three species of Pomacea in Hong Kong: P. canaliculata (Hap1–Hap5), P. maculata (Hap6–Hap7) and P. occulta (Hap8–Hap10). All five locations contained P. canaliculata, accounting for 85.8% of the studied specimens. We detected P. occulta (8.0%) in all the populations except Ho Sheung Heung, while P. maculata (6.1%) was limited to Ho Sheung Heung and Kam Tin (Table 1; Figure 1). The three species co-existed in Kam Tin. In other locations P. canaliculata either co-existed with P. maculata (Ho Sheung Heung) or with P. occulta (Lok Ma Chau, Wetland Park and Wetland Park) (Table 1; Figure 1).

The intra-specific K2P distances of P. canaliculata, P. maculata and P. occulta each exhibited a dumbbell shape with high probabilities at both ends (Figure 3). The genetic variations of P. canaliculata and P. maculata were high with a maximum intra-specific K2P distance of 0.069 and 0.060, respectively (Figure 3). The inter-specific K2P distances of P. maculata and P. occulta were of broad ranges from 0.070 to 0.133 and 0.062 to 0.125, respectively (Figure 3). Obvious genetic distance gaps of the maximum intra-specific and the minimum inter-specific K2P distance (i.e. DNA barcoding gaps) (Meyer & Paulay, 2005) existed in both P. canaliculata and P. occulta, indicating the effective of molecular identification. Although the maximum intra-specific and the minimum inter-specific genetic distances were close in P. maculata, the DNA barcoding gap was also formed (Figure 3).
### TABLE 1 Distribution of the haplotypes generated from the COI dataset containing sequences of apple snails from Hong Kong, mainland China and Malaysia. Haplotypes starting with Lv are from Lv et al. (2013) with the numbers of sequences included in brackets. ‘/’ means no representative sequences for the haplotype

| Species         | Haplotype | Total number | Hong Kong | Mainland China | Malaysia |
|-----------------|-----------|--------------|-----------|----------------|----------|
| *P. canaliculata* | Hap1      | 345          | 99 (MT806196, 198-202, 204-5, 207-10, 214-7, 219, 221-3, 225, 233, 238-52, 255-63, 266-7, 268-74, 276-9, 281-6, 288-9, 291-5, 297, 300, 306, 311, 319, 321-2, 325, 328, 331, 333, 336, 338-44, 347-8, 350-1, 353-4, 356-7) | 224 [KP310366-435, KR020962-78, KR020994-1013, KT852706-22, KT852758-62, MH293333, MH293336-7, MH293345, MH293353-4, MH293356-8, MH293360-4, MH293366-70, MH293372-3, MH293375, MH293388, LvHap6 (72)] | 22 (MG230753-62, MG230781-2, MN623427, MN623429-31, MN623433-4, MN623436-9) |
| *P. canaliculata* | Hap2      | 539          | 32 (MT806213, 228-32, 234-7, 264-5, 296, 298-9, 302-5, 310, 314, 316-7, 324, 326-7) | 507 [KP310264-89, KP310291-365, KR020942-61, KR020980, KR020983-93, KT852727-56, MH293322-32, MH293334, MH293338-44, MH293346-52, MH293377-86, MH293389-90, MH293392-422, MH293424-9, MH293431-8, MH293440-1, MH293443-7, MH293449-50, LvHap12 (252)] | / |
| *P. canaliculata* | Hap3      | 22           | 6 (MT806307, 309, 313, 315, 320, 323) | 1 (KP310290) | 15 (MG230742-52, MG230763, MG230767, MN623435, MN623440) |
| *P. canaliculata* | Hap4      | 26           | 1 (MT806211) | 25 [KP310436, KP310439-41, KP310445, KR020979, KR020981, KR021014, KT852723-4, LvHap21 (15)] | / |
| *P. canaliculata* | Hap5      | 1            | 1 (MT806280) | / | / |
| *P. maculata*   | Hap6      | 74           | 8 (MT806197, 203, 212, 218, 220, 224, 226-7) | 29 (KT852782-5, KR021027, KR02103-4-40, KP310480-96) | 37 (MG230786-93, MG230783-4, MG230768-80, MG230764-6, MN623417-26, MN623432) |
| *P. maculata*   | Hap7      | 2            | 2 (MT806206, 253) | / | / |
| *P. occulta*    | Hap8      | 191          | 11 (MT806254, 275, 290, 301, 308, 312, 318, 335, 349, 352, 355) | 180 [MH293335, MH293430, MH293423, MH293439, MH293442, MH293448, KP310446-50, KP310452-73, KP310474-9, KT852763-81, KT852787-9, KR021021-6, KR021028-33, LvHap1 (107)] | / |
| *P. occulta*    | Hap9      | 1            | 1 (MT806287) | / | / |
| *P. occulta*    | Hap10     | 1            | 1 (MT806332) | / | / |
| *P. canaliculata* | Hap11     | 9            | / | 9 (KP310438, KP310437, KR020982, KR021015-20) | / |
| *P. canaliculata* | Hap12     | 47           | / | 47 [KP310442-4, KT852725-6, MH293355, MH293359, MH293365, MH293371, MH293374, MH293376-7, MH293391, LvHap18 (34)] | / |

(Continues)
| Species          | Haplotype | Total number | Hong Kong | Mainland China   | Malaysia |
|------------------|-----------|--------------|-----------|------------------|----------|
| P. canaliculata  | Hap13     | 1 /          |           | 1 (KT85275)      | /        |
| P. canaliculata  | Hap14     | 1 /          |           | 1 [LvHap7 (1)]   | /        |
| P. canaliculata  | Hap15     | 1 /          |           | 1 [LvHap8 (1)]   | /        |
| P. canaliculata  | Hap16     | 1 /          |           | 1 [LvHap9 (1)]   | /        |
| P. canaliculata  | Hap17     | 1 /          |           | 1 [LvHap10 (1)]  | /        |
| P. canaliculata  | Hap18     | 1 /          |           | 1 [LvHap11 (1)]  | /        |
| P. canaliculata  | Hap19     | 1 /          |           | 1 [LvHap13 (1)]  | /        |
| P. canaliculata  | Hap20     | 1 /          |           | 1 [LvHap14 (1)]  | /        |
| P. canaliculata  | Hap21     | 1 /          |           | 1 [LvHap15 (1)]  | /        |
| P. canaliculata  | Hap22     | 1 /          |           | 1 [LvHap16 (1)]  | /        |
| P. canaliculata  | Hap23     | 1 /          |           | 1 [LvHap17 (1)]  | /        |
| P. canaliculata  | Hap24     | 3 /          |           | 3 [LvHap19 (3)]  | /        |
| P. canaliculata  | Hap25     | 1 /          |           | 1 [LvHap20 (1)]  | /        |
| P. canaliculata  | Hap26     | 1 /          |           | 1 [LvHap22 (1)]  | /        |
| P. canaliculata  | Hap27     | 1 /          |           | 1 [LvHap23 (1)]  | /        |
| P. canaliculata  | Hap28     | 1 /          |           | /                | 1 (MG230785) |
| P. canaliculata  | Hap29     | 1 /          |           | /                | 1 (MN623428) |
| P. maculata      | Hap30     | 1 /          |           | 1 (KT852786)     | /        |
| P. maculata      | Hap31     | 1 /          |           | 1 [LvHap24 (1)]  | /        |
| P. occulta       | Hap32     | 1 /          |           | 1 [LvHap3 (1)]   | /        |
| P. occulta       | Hap33     | 21 /         |           | 21 [LvHap5 (21)] | /        |
| P. occulta       | Hap34     | 1 /          |           | 1 [LvHap2 (1)]   | /        |
| P. occulta       | Hap35     | 1 /          |           | 1 (KP310451)     | /        |
| P. occulta       | Hap36     | 1 /          |           | 1 [LvHap4 (1)]   | /        |
3.3 | Population genetic diversity and structure

The three descriptive indices of population genetic diversity (i.e. $Hd$, $\pi$, and $k$) revealed polymorphisms in *Pomacea* spp. from Hong Kong, mainland China and Malaysia, except for *P. maculata* from Malaysia (Table 3). In general, *P. canaliculata* showed higher genetic diversity with two times higher mean $Hd$ and more than ten times higher mean $\pi$ and $k$ than *P. maculata* and *P. occulta*. Among the three regions, the genetic diversity of *P. canaliculata* was the highest in mainland China followed by Malaysia and Hong Kong. These results indicate that *P. canaliculata* has a more complex introduction history than the other two species, especially in mainland China. For *P. maculata*, the Hong Kong population has approximately three to seven times higher of $Hd$, $\pi$ and $k$ values than those from mainland China. In addition, *P. occulta* population between Hong Kong and mainland China exhibited a similar trend of genetic diversity, with less than three times difference between populations in these two regions.

The population analysis revealed high levels of intra-specific gene flow in the population pairs (Figure 4). *Pomacea canaliculata* and *P. maculata* in mainland China had a significant differentiated population genetic structure than the populations in Hong Kong with $F_{ST} = 0.289$ ($p < .01$) and $F_{ST} = .213$ ($p < .05$), respectively. However, the Hong Kong and mainland China populations of *P. occulta* showed low $F_{ST}$ values ($<0.1$) with the corresponding high $Nm$ values ($>1$), which implicates a high gene flow between the populations. The Malaysia and the mainland China population of *P. canaliculata* had a significant differentiation ($F_{ST} = 0.260; p < .01$), while the Malaysia and Hong Kong population of *P. maculata* had a significant differentiation ($F_{ST} = 0.333; p < .05$) (Figure 4; Appendix S1: Table S4). In contrast, the Malaysia and mainland China populations of *P. maculata* and the Malaysia and Hong Kong populations of *P. canaliculata* did not show obvious genetic variance with $F_{ST}$ values <0.1.

Analysing the haplotypes with 95% and 90% parsimony connection limits for reconstructing the networks revealed the same structures in *P. canaliculata* and *P. occulta*. The haplotypes of *P. canaliculata* recovered three unconnected networks (A–C) (Figure 5). Two of the networks (A–B) distributed with the haplotypes from the populations of Hong Kong, mainland China, Malaysia and Argentina. Network A contained 20 haplotypes with Hap1 placed as a predominant central, which is also the only haplotype shared.
by the populations of Hong Kong, mainland China, Malaysia and Argentina. All the other haplotypes surrounded Hap1 with one or several mutational steps. Network B contained 16 haplotypes, with only one haplotype from Argentina (Hap43) and several missing haplotypes. Hap3 was shared by the populations of Hong Kong, mainland China and Malaysia, and Hap2 and Hap4 were shared by the populations of Hong Kong and mainland China. Network C of *P. canaliculata* contained only the haplotypes from Argentinean populations.

All the haplotypes of *P. occulta* connected on network D (Figure 5). Despite a couple of haplotypes of *P. occulta* occurred in Hong Kong and mainland China, the two populations shared only Hap8 with the other haplotypes surrounding it with one or several steps away (Figure 5).

For the haplotypes of *P. maculata*, applying the 90% parsimony limit resulted in three un-linked networks (E–G) (Figure 5). Network E included the haplotypes of the populations in Hong Kong, mainland China, Malaysia, Argentina and Brazil. Networks F and G were comprised haplotypes of the Brazilian population (Figure 5). Network E contained 22 haplotypes with Hap6 being the only haplotype shared by the populations in Hong Kong, mainland China and Malaysia. Hap30–Hap31 of mainland China population were one or two steps away from Hap6, while Hap7 of Hong Kong population was five steps away from the Hap6–Hap30–Hap31 cluster. The Hap6–Hap30–Hap31 cluster was closer to the haplotypes of
Brazil than Argentina. However, Hap7 shared a triplet joint with both the populations of Argentina than Brazil with one step closer to the Argentinean population than to the Brazilian population (Figure 5). Network G was split into five subnetworks when using the 95% parsimony limit: three unconnected haplotypes (Hap64, Hap70 and Hap77), Hap75 and Hap76 connecting with eight missing haplotypes and the remaining haplotypes connecting in the same patterns as in Network G.

3.4 | Genetic introgression

Overall, 49.6% of snails were ‘pure’ *P. canaliculata* and there was only one ‘pure’ *P. maculata* individual out of the ten maculata-COI specimens. ‘Pure’ *P. canaliculata* dominated the Kam Tin, Ho Sheung Heung and Wetland Park populations, while the hybrids of *P. canaliculata*-COI and *P. maculata*-EF1α dominated the Lok Ma Chau and Wetland Park populations (Figure 1). Among the *P. maculata*-COI specimens, the *P. canaliculata*-EF1α was the most common nuclear genotype, followed by the genotype of mixed-EF1α from Ho Sheung Heung; only one individual of pure *P. maculata* was detected from Kam Tin (Figure 1).

Due to incomplete lineage sorting or recent hybridization, we were not able to distinguish *P. occulta* from *P. canaliculata* and *P. maculata* based on the nuclear EF1α sequences (Yang et al., 2019). The *P. canaliculata*-EF1α, *P. maculata*-EF1α and mixed-EF1α snails of EF1α genotyped were all detected from *P. occulta* in Hong Kong (Figure 1).

4 | DISCUSSION

Based on analysis of the mitochondrial COI barcoding region, we identified three non-native apple snail species, *P. canaliculata*, *P. maculata* and *P. occulta*, from various freshwater wetlands in the New Territories of Hong Kong, but they could not be readily distinguished by shell morphology (see Appendix S1: Figure S2). To our knowledge, our study represents the first report of Pomacea spp. other than *P. canaliculata* in natural habitats of Hong Kong, which has enhanced our knowledge on the distribution ranges of *P. maculata* and *P. occulta* in Asia (e.g. Hayes et al., 2008; Yang et al., 2019; Yang, Liu, He, et al., 2018). *Pomacea maculata* was restricted to several populations in mainland China (Yang, Liu, He, et al., 2018) and has not been reported from Shenzhen, Guangdong Province of China, which is physically connected with Hong Kong through a network of drainage channels in the same catchment (Yang, Liu, He, et al., 2018). Moreover, there has been no report of *P. occulta* as an invasive species other than in the mainland and Hong Kong. Whether *P. maculata* and *P. occulta* are truly absent on the wetlands require confirmation based on more apple snail samples. Nevertheless, our current data indicate a risk for *P. maculata* and *P. occulta* to spread to the above-mentioned areas in future. As Lu et al. (2018) suggested Hong Kong as one of the major stepping-stone for invasive species to the mainland of China, the delineation and identification of invasive species in Hong Kong are essential for early warning and control of species introduction to China and other Asian regions.

*Pomacea canaliculata* was the most widespread species, being recorded from all of the five sampled natural populations in Hong Kong. *Pomacea maculata*, however, was restricted to two locations (a vegetable garden in Ho Sheung Heung and an artificial wetland in Kam Tin) in our study, and in each population, this species accounted for the smallest proportion of *Pomacea* individuals at the sites. These results are in agreement with Hayes et al. (2008) which showed that *P. canaliculata* is often the dominant species in Asia, and *P. maculata* is distributed in fewer locations and often co-occurring with *P. canaliculata*. In contrast, the native range of *P. canaliculata* is mainly Northern Argentina and Southern Uruguay, while *P. maculata* occurs in a much larger range than *P. canaliculata* in South America, extending from most western Brazil to Paraná and Uruguay river basins of Argentina and Uruguay (Glasheen et al., 2020; Hayes et al., 2008, 2012; Martín et al., 2001). It is difficult to determine how the native habitat breadth may have affected the ecological impacts and thus driven the relatively success between the two closely related *Pomacea* species, except perhaps in more high latitude areas (Matsukura et al., 2013).
Hong Kong, the differences in the apple snail species composition among the sampling sites may have largely relied on the numbers of introductions that have been made, although their differences in tolerance to environmental stressors may have also contributed.

Our phylogenetic analysis showed that the invasive populations of *P. canaliculata* in Hong Kong were likely the results of repeated introductions from various locations in Argentina (Hayes et al., 2012). The same conclusion can be applied to *P. canaliculata* populations in mainland China and Malaysia. We found a novel haplotype of *P. canaliculata* in Hong Kong. There were also unique *P. canaliculata* haplotypes to the Malaysian populations. The other haplotypes of *P. canaliculata* from Hong Kong and Malaysia were likely a subset of those that have been reported from mainland China (Lv et al., 2013; Yang, Liu, He, et al., 2018). However, the dominant haplotypes of *P. canaliculata* were different among Hong Kong, mainland China and Malaysia populations, which indicated that *P. canaliculata* was with a complex invasion history in the three regions.

The *P. maculata* populations of mainland China, Malaysia, Vietnam, Singapore and Cambodia likely came from Brazil (Hayes et al., 2008; Yang, Liu, He, et al., 2018); the Korean populations likely came from Argentina (Hayes et al., 2008), while the populations in Japan and Thailand likely came from either Argentina or Brazil.
(Hayes et al., 2008; Matsukura et al., 2008). Our study identified two haplotypes of *P. maculata* from Hong Kong: the major haplotype (Hap6) shared between the populations of mainland China and Malaysia indicating the same Brazilian origin, whereas the novel minor haplotype (Hap7) may have come from Argentina. Despite the different introduction histories of the Asian *P. maculata* populations, haplotype diversity in each of three regions was low with only one or two COI haplotypes. Notably, *P. maculata* is now widely distributed in southern coastal states of the United States from Texas to South Carolina, which was speculated to have resulted from multiple and separate introductions from Argentina and Brazil (Byers et al., 2013; Rawlings et al., 2007; Underwood et al., 2019).

In this study, we discovered *P. occulta* for the first time in Hong Kong and revealed its invasion history similar to that in mainland China. *Pomacea occulta* co-exists with *P. canaliculata* in four of the five populations with a lower abundance. The widely overlapped distribution of *P. occulta* with *P. canaliculata* indicated their co-introduction from Argentina, as was indicated by Yang et al. (2019) in an analysis of apple snail distribution in mainland China. *Pomacea occulta* was absent from the vegetable garden in the most northern sampling locality (Ho Sheung Heung), where the sex ratio of the apple snail population was strongly bias. Apple snail populations have shown extremely variable brood sex ratios that were determined genetically but equal sex ratios in populations (Yusa, 2006; Yusa & Suzuki, 2003). Moreover, we did not observe obvious correlation between the species distribution pattern and the habitat type. The bias sex ratio indicates that the apple snail population in Ho Sheung Heung was disrupted, which could be caused by various factors, such as environmental contaminants and conditions (Ciparis et al., 2012). Alternatively, it could be due to sampling bias.

Compared to the native apple snail populations in Brazil and Argentina (total of 53 haplotypes), those in the introduced ranges in Hong Kong, mainland China and Malaysia have lower haplotype diversity. This result can be explained by the founder effect, which is the loss of genetic variation due to the small invasive population. Nucleotide diversity (*π*), which is relatively independent of sample size and sequence length, is a more stable index of genetic divergence (Li, 1997). For *P. occulta*, the genetic diversity of the Hong Kong populations (*π* = 0.00122) was close to that of mainland China (*π* = 0.00126). The genetic diversity indices showed that the genetic composition of *P. canaliculata*, *P. maculata* and *P. occulta* populations of these species in Hong Kong, mainland China and Malaysia were not identical. Considering at least two generations a year of apple snails in most of the introduced ranges in Asia, genetic variation has been accumulated for four decades since their first introduction. However, there seemed to be high genetic similarities between the Hong Kong and Malaysia populations of *P. canaliculata*, the Hong Kong and mainland China populations of *P. occulta* and the mainland China and Malaysia populations of *P. maculata*, which can probably be attributed to the low variability of the COI sequences. The pairwise *F_{ST}* and *N_{m}*, values among the Hong Kong, mainland China and Malaysia populations of *P. maculata* and *P. canaliculata* showed that their differentiation was more likely due to genetic drift resulted from the small subset of introduced populations from the native ranges.

Hybridization can enhance the chances of local adaptation and range expansion into new habitats (Mesgaran et al., 2016; Pfennig et al., 2016). Glasheen et al. (2020) first reported the pattern of hybridization of apple snails in their native ranges. Our results of multiple EF1α genotypes in *P. occulta* supports the idea that some of the founder individuals might have been hybrids. Hybrids of *P. canaliculata* and *P. maculata* in their native areas in Uruguay and Brazil were high, reaching up to 30% (Glasheen et al., 2020). The degree of hybridization of the two species in Hong Kong was even higher, reaching 53%. Similarly, Yang et al. (2020) found more hybrids than pure apple snails in southern coastal areas of mainland China. The higher percentage of hybrids indicated the hybridization has intensified in the introduced ranges through their co-occurrence in habitats, which could have probably promoted the invasion through benefiting from the more intensive genetic exchanges.

The apple snail populations in Hong Kong contained hybrids with different genetic introgression patterns. To our knowledge, the 'pure' *P. maculata* individual in our study is the first report of 'pure' *P. maculata* in Asia. No 'pure' *P. maculata* has been reported from other Asian regions including mainland China, Japan and Korea (Matsukura et al., 2016; Yang et al., 2020), but it is not known whether this conclusion will change when more sequences of the EF1α gene from these regions become available. The genetic introgression among populations may be affected by natural selection imposed through environmental stressors. For example, Matsukura et al. (2013), Matsukura et al. (2016) reported differential cold tolerance abilities among the 'pure' species and hybrids, and suggested that such physiological differences might have determined the distribution of these apple snails in Japan. Hybridization between species can contribute to population fitness by increasing genetic variation to compensate the reduced genetic variation resulted from the founder effect (Pfennig et al., 2016). The enhancement of local adaptation might result the range expansion in new habitats, and thus, the high hybridization rate in Hong Kong will pose invasion risk to China and nearby regions. However, a comprehensive understanding of genetic benefits through hybridization for range expansion in apple snails requires further population genomic research.

In summary, we found *P. maculata* and *P. occulta* in Hong Kong for the first time. Our results showed that there were likely multiple introductions of apple snails in Hong Kong, with *P. canaliculata* and *P. occulta* having been introduced from Argentina, and *P. maculata* having been introduced separately from Argentina and Brazil. The low genetic diversity indicated the apple snail populations in Hong Kong indicated the presence of founder effect. Both the repeated introduction and high hybridization might compensate and can enhance genetic variation and thus pose further invasion risk to nearby regions. In other invaded regions where only *P. canaliculata* has been reported to cause great economic impact (i.e. Taiwan of China, Philippines, Myanmar, Laos; Cowie et al., 2017), molecular studies of *Pomacea* should be conducted to determine their true species identities, species composition and patterns of hybridization. Our results
emphasize the importance of strengthening quarantine measures to prevent the further spread of these apple snails, not only to prevent their further spread, such as P. maculata to the southern provinces of China (i.e. Guangdong, Guangxi and Fujian) and P. occulta to other Asian countries, but also to prevent the gene flow among the different populations of the same species.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT
All data obtained in this study have been deposited to GenBank under the accession numbers MT806196–MT806357.

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REFERENCES
Byers, J. E., McDowell, W. G., Dodd, S. R., Haynie, R. S., Pintor, L. M., & Wilde, S. B. (2013). Climate and pH predict the potential range of the invasive apple snail (Pomacea insularum) in the Southeastern United States. *PLoS One*, 8(2), e56812. https://doi.org/10.1371/journal.pone.0056812
Ciparis, S., Henley, W. F., & Voshell, J. R. (2012). Population sex ratios of pleurocerid snails (Leptoxis spp.): Variability and relationships with environmental contaminants and conditions. *American Malacological Bulletin*, 30(2), 287–298. https://doi.org/10.4003/006.030.0208
Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: A computer program to estimate genealogies. *Molecular Ecology*, 9(10), 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x
Cowie, R. H., Hayes, K. A., Strong, E. E., & Thiengo, S. C. (2017). Non-native apple snails: systematics, distribution, invasion history and reasons for introduction. In R. C. Joshi, R. H. Cowie, & L. S. Sebastian (Eds.), *Biological and Management of Invasive Apple Snails* (pp. 3–32). The Philippine Rice Research Institute.
Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). *jModelTest 2*: More models, new heuristics and parallel computing. *Nature Methods*, 9(8), 772. https://doi.org/10.1038/nmeth.2109
Diaz, S., Settele, J., Brondizio, E., Ngo, H. T., Güeze, M., & Zayas, C. (2019). *Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services*. IPBES Secretariat.
Ely, C. V., Bordignon, S. A. D., Trevisan, R., & Boldrini, I. I. (2017). Implications of poor taxonomy in conservation. *Journal for Nature Conservation*, 36, 10–13. https://doi.org/10.1016/j.jnc.2017.01.003
Excoffier, L., & Lischer, H. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3), 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x
Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3(5), 294–299.
Frankham, R., Ballou, J. D., & Briscoe, D. A. (2002). Introduction to conservation genetics. Cambridge University Press.
Glaseen, P. M., Burks, R. L., Campos, S. R., & Hayes, K. A. (2020). First evidence of introgressive hybridization of apple snails (Pomacea spp.) in their native range. *The Journal of Molluscan Studies*, 86(2), 96–103. https://doi.org/10.1093/mollus/eyz035
Hayes, K. A. (2021). Taxonomic shortcuts lead to long delays in species discovery, delineation, and identification. *Biological Invasions*, 23(4), 1285–1292. https://doi.org/10.1007/s10530-020-02438-8
Hayes, K. A., Cowie, R. H., Thiengo, S. C., & Strong, E. E. (2012). Comparing apples with apples: Clarifying the identities of two highly invasive Neotropical Ampullariidae (Caenogastropoda). *Zoological Journal of the Linnean Society*, 166(4), 723–735. https://doi.org/10.1111/j.1096-3642.2012.00867.x
Hayes, K. A., Joshi, R. C., Thiengo, S. C., & Cowie, R. H. (2008). Out of South America: Multiple origins of non-native apple snails in Asia. *Diversity and Distributions*, 14(4), 701–712. https://doi.org/10.1111/j.1472-4642.2008.00483.x
Huelsenbeck, J. P., Ronquist, F., Nielsen, R., & Bollback, J. P. (2001). Bayesian inference of phylogeny and its impact on evolutionary biology. *Science*, 294(5550), 2310–2314. https://doi.org/10.1126/science.1065889
Jackson, H., Strubbe, D., Tollington, S., Prys-Jones, R., Matthysen, E., & Groombridge, J. J. (2015). Ancestral origins and invasion pathways in a globally invasive bird correlate with climate and influences from bird trade. *Molecular Ecology*, 24(16), 4269–4285. https://doi.org/10.1111/mec.13307
Johnson, M., Zaretskaya, I., Raytsev, Y., Merezhuk, Y., McGinnis, S., & Madden, T. L. (2008). NCBI BLAST: A better web interface. *Nucleic Acids Research*, 36(Suppl. 2), W5–W9. https://doi.org/10.1093/nar/gkn201
Kannan, A., Rama Rao, S., Ratnayeke, S., & Yow, Y. Y. (2020). The efficiency of universal mitochondrial DNA barcodes for species discrimination of Pomacea canaliculata and Pomacea maculata. *PloS One*, 8(12), e8755. https://doi.org/10.7717/plosone.8755
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199
Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2), 111–120. https://doi.org/10.1007/BF01731581
Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547-1549. https://doi.org/10.1093/molbev/msy096

Kwong, K. L., Chan, R. K., & Qiu, J. W. (2009). The potential of the invasive snail *Pomacea canaliculata* as a predator of various life-stages of five species of freshwater snails. *Malacologia*, 51(2), 343-356. https://doi.org/10.4002/004-050.012008

Kwong, K. L., Dudgeon, D., Wong, P. K., & Qiu, J. W. (2010). Secondary production and diet of an invasive snail in freshwater wetlands: Implications for resource utilization and competition. *Biological Invasions*, 12(5), 1153-1164. https://doi.org/10.1007/s10530-009-9537-x

Kwong, K. L., Wong, P. K., Lau, S. S., & Qiu, J. W. (2008). Determinants of the distribution of apple snails in Hong Kong two decades after their initial invasion. *Malacologia*, 50(1-2), 293-302. https://doi.org/10.4002/0076-2997.50.1.293

Li, M. C. (2008). *Ecological comparisons of growth and feeding between Pomacea canaliculata and Pomacea scalaris* (p. 56). National Sun Yat-sen University.

Li, W. H. (1997). *Molecular evolution*. Sinauer Associates.

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25(11), 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Lu, J., Li, S.-P., Wu, Y., & Jiang, L. (2018). Are Hong Kong and Taiwan stepping-stones for invasive species to the mainland of China? *Ecology and Evolution*, 8, 1966-1973. https://doi.org/10.1002/ece3.3818

Lv, S., Zhang, Y., Liu, H. X., Hu, L., Liu, Q., Wei, F. R., & Utzinger, J. (2013). Phylogenetic evidence for multiple and secondary introductions of invasive snails: *Pomacea* species in the People’s Republic of China. *Diversity and Distributions*, 19, 147-156. https://doi.org/10.1111/j.1472-6462.2012.00924.x

Martin, P. R., Estebenet, A. L., & Cazzaniga, N. J. (2001). Factors affecting the distribution of *Pomacea canaliculata* (Gastropoda: Ampullariidae) along its southernmost natural limit. *Malacologia*, 43(1), 13-23. https://doi.org/10.1017/S002587420002090X

Matsukura, K., Izumi, Y., Yoshida, K., & Wada, T. (2016). Hybridization as a facilitator of invasive apple snail (*Pomacea canaliculata*) in Florida, USA. *Biological Invasions*, 19(9), 2647-2661. https://doi.org/10.1007/s10530-017-1474-5

Meyer, C. P., & Paulay, G. (2005). DNA barcoding: Error rates based on comprehensive sampling. *PloS Biology*, 3(12), e422. https://doi.org/10.1371/journal.pbio.0030422

Müller, K., Sun, J., Cheung, S. G., Fang, L., Zhou, H., Luan, T., Zhang, H., Wong, C. K. C., & Qiu, J.-W. (2018). Comparative proteomics and codon substitution analysis reveal mechanisms of differential resistance to hypoxia in congeneric snails. *Journal of Proteomics*, 172, 36-48. https://doi.org/10.1016/j.jprot.2017.11.002

Mu, H., Sun, J., Fang, L., Luan, T., Williams, G. A., Cheung, S. G., Wong, C. K. C., & Qiu, J.-W. (2015). Genetic basis of differential heat resistance between two species of congeneric freshwater snails: Insights from quantitative proteomics and base substitution rate analysis. *Journal of Proteome Research*, 14(10), 4296-4308. https://doi.org/10.1021/acs.jproteome.5b00462

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, 32(1), 268-274. https://doi.org/10.1093/molbev/msu300

Pfenning, K. S., Kelly, A. L., & Pierce, A. A. (2016). Hybridization as a facilitator of species range expansion. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20161329. https://doi.org/10.1098/rspb.2016.1329

Pierre, S. M., Quintana-Ascencio, P. F., Boughton, E. H., & Jenkins, D. G. (2017). Dispersal and local environment affect the spread of an invasive apple snail (*Pomacea maculata*) in Florida, USA. *Biological Invasions*, 19(9), 2647-2661. https://doi.org/10.1007/s10530-017-1474-5

Rama Rao, S., Liew, T. S., Yow, Y. Y., & Ratnayeke, S. (2018). Cryptic diversity: Two morphologically similar species of invasive apple snail in Peninsular Malaysia. *PloS One*, 13(5), e0196582. https://doi.org/10.1371/journal.pone.0196582

Rambaut, A., & Drummond, A. (2010). FigTree v1.3.1. Institute of Evolutionary Biology, University of Edinburgh.

Rawlings, T. A., Hayes, K. A., Cowie, R. H., & Collins, T. M. (2007). The identity, distribution, and impacts of non-native apple snails in the continental United States. *BMC Evolutionary Biology*, 7, 97. https://doi.org/10.1186/1471-2148-7-97

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19(12), 1572-1574. https://doi.org/10.1093/bioinformatics/btg180

Senti, A., Montoya, J. M., & Lurgi, M. (2021). Warmonging increasingly invasion success in food webs. *Proceedings of the Royal Society B: Biological Sciences*, 288(1947), 20202622. https://doi.org/10.1098/rspb.2020.2622

Seuffert, M. E., & Martin, P. R. (2021). Exceeding its own limits: Range expansion in Argentina of the globally invasive apple snail *Pomacea canaliculata*. *Hydrobiologia*, 848(2), 385-401. https://doi.org/10.1007/s10750-020-04447-z

Shaik, R. S., Zhu, X., Clements, D. R., & Weston, L. A. (2016). Understanding invasion history and predicting invasive niches using genetic sequencing technology in Australia: Case studies from Cucurbitaceae and Boraginaceae. *Conservation Physiology*, 4(1), cow030. https://doi.org/10.1093/conph/cow030

Song, H., Hu, Y., Wang, P., Mu, X., Li, X., Wang, X., & Luo, J. (2010). Sequencing cytochrome oxidase subunit 1 of mitochondrial DNA and the taxonomic status of apple snails. *Chinese Journal of Zoology*, 45(1), 1-7. https://doi.org/10.3724/SJSP.1101.2010.01138

Tran, C. T., Hayes, K. A., & Cowie, R. H. (2008). Lack of mitochondrial DNA diversity in invasive apple snails (Ampullariidae) in Hawaii. *Malacologia*, 50(1-2), 351-357.

Underwood, E. B., Darden, T. L., O’Donnell, T. P., & Kingsley-Smith, P. R. (2019). Population genetic structure and diversity of the invasive island apple snail *Pomacea maculata* (Perry, 1810) in South Carolina and Georgia. *Journal of Shellfish Research*, 38(1), 163-175. https://doi.org/10.2983/033.038.0115

Wright, S. (1943). Isolation by distance. *Genetics*, 28, 114-138. https://doi.org/10.1093/genetics/28.2.114

Wu, J.-Y., Wu, Y.-T., Li, M.-C., Chiu, Y.-W., Liu, M.-Y., & Liu, L.-L. (2011). Reproduction and juvenile growth of the invasive apple snails *Pomacea canaliculata* and *P. scalaris* (Gastropoda: Ampullariidae) in Taiwan. *Zoological Studies*, 50(1), 61-68.

Yang, Q.-Q., He, C., Liu, G.-F., Yin, C.-L., Xu, Y.-P., Liu, S.-W., Qiu, J.-W., & Yu, X.-P. (2020). Introggressive hybridization between two non-native apple snails in China: Widespread hybridization and homogenization in egg morphology. *Pest Management Science*, 76(12), 4231-4239. https://doi.org/10.1002/ps.5980

Yang, Q. Q., Liu, S., He, C., Cowie, R. H., Yu, X., & Hayes, K. A. (2019). Invisible apple snail invasions: Importance of continued vigilance.
and rigorous taxonomic assessments. Pest Management Science, 75(5), 1277–1286. https://doi.org/10.1002/ps.5241
Yang, Q. Q., Liu, S. W., He, C., & Yu, X. P. (2018). Distribution and the origin of invasive apple snails, Pomacea canaliculata and P. maculata (Gastropoda: Ampullariidae) in China. Scientific Reports, 8(1), 1185. https://doi.org/10.1038/s41598-017-19000-7
Yang, Q. Q., Liu, S. W., Song, F., Liu, G. F., & Yu, X. P. (2018). Comparative mitogenome analysis on species of four apple snails (Ampullariidae: Pomacea). International Journal of Biological Macromolecules, 118(Pt A), 525–533. https://doi.org/10.1016/j.ijbiomac.2018.06.092
Yang, Q. Q., & Yu, X. P. (2019). A new species of apple snail in the genus Pomacea (Gastropoda: Caenogastropoda: Ampullariidae). Zoological Studies, 58, e13. https://doi.org/10.6620/ZS.2019.58-13
Yipp, M. W., Cha, M. W., & Liang, X. Y. (1991). A preliminary impact assessment of the introduction of two species of Ampullaria (Gastropoda: Ampullariidae) into Hong Kong. In C. Meier-Brook (Ed.), Proceedings of the Tenth International Malacological Congress (pp. 393–397). UNITAS Malacologia.
Yoshida, K., Matsukura, K., Cazzaniga, N. J., & Wada, T. (2014). Tolerance to low temperature and desiccation in two invasive apple snails, Pomacea canaliculata and P. maculata (Caenogastropoda: Ampullariidae), collected in their original distribution area (northern and central Argentina). Journal of Molluscan Studies, 80(1), 62–66. https://doi.org/10.1093/mollus/eyt042
Yusa, Y. (2006). Genetics of sex-ratio variation inferred from parent-offspring regressions and sib correlations in the apple snail Pomacea canaliculata. Heredity, 96(1), 100–105. https://doi.org/10.1038/sj.hdy.6800758
Yusa, Y., & Suzuki, Y. (2003). A snail with unbiased population sex ratios but highly biased brood sex ratios. Proceedings of the Royal Society B-Biological Sciences, 270(1512), 283–288. https://doi.org/10.1098/rspb.2002.2226
Zhang, Z., Capinha, C., Karger, D. N., Turon, X., Maclsaac, H. J., & Zhan, A. (2020). Impacts of climate change on geographical distributions of invasive ascidians. Marine Environmental Research, 159, 104993. https://doi.org/10.1016/j.marenvres.2020.104993

BIOSKETCH
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Author contributions: QQY, JCI, XPY and JWQ conceived the project; QQY and JCI conducted field surveys; XXZ, JNL and YJJ conducted laboratory analyses with input from all co-authors; QQY led the writing with assistance from JCI and JWQ; all authors contributed to revision.

SUPPORTING INFORMATION
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