

Not in the Least Concern: anthropogenic influences on a South-east Asian apple snail *Pila scutata* (Ampullariidae)

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Abstract South-east Asian apple snails, *Pila* spp., have been declining since the introduction of globally invasive, congeneric South American *Pomacea* spp., yet *Pila* ecology remains poorly studied, with most occurrence records unconfirmed. *Pila scutata*, a previously widespread species, presumed native to the Malay peninsula and assessed as Least Concern in the IUCN Red List, was formerly harvested for food, and may have experienced anthropogenic translocations. We surveyed the Malay peninsula (specifically Peninsular Malaysia and Singapore) to investigate the current distribution and genetic diversity of *P. scutata*. Six populations were found in Singapore, but only one in Peninsular Malaysia. Mitochondrial COI and 16S sequencing revealed that the Malaysian population shared a single haplotype of both genes with the Singapore populations (500 km distant). This low genetic diversity could stem from a recent anthropogenic introduction, which brings into question the true native range of *P. scutata* and, coupled with poorly resolved taxonomy of the genus, necessitates a reassessment of its IUCN Red List status. Introduced populations pose a dilemma, and the lack of genetic diversity is of concern in light of *Pila* decline throughout South-east Asia. Our results highlight that conservation management of *P. scutata* and its congeners must therefore be better informed by greater taxonomic resolution and more comprehensive investigations of their ecology, both in native and introduced ranges.

Keywords Cryptogenic species, freshwater snails, human introductions, Peninsular Malaysia, *Pila scutata*, Singapore, threat status

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Introduction

Although invertebrates comprise 80% of known species, the conservation status of < 1% is known (Collen et al., 2012). Amongst invertebrates the freshwater gastropods are most at risk: at least 51% of assessed species are considered threatened, with the potential threat level ranging from 33% (assuming no Data Deficient species are threatened) to 68% (assuming all Data Deficient species are threatened; Collen et al., 2012). Hotspots of freshwater gastropod diversity include the ancient lakes of Sulawesi, Indonesia and the Mekong basin (Strong et al., 2008). Despite the high diversity of freshwater molluscs in South-east Asia, research has mostly focussed on the zoonotic parasites they host, with little other emphasis beyond inventories of the molluscan hosts (e.g. Palmieri et al., 1977; Burch & Lohachit, 1983; Attwood, 2010).

Among the known parasite hosts are Ampullariidae, the largest freshwater snails in South-east Asia (Komalamisra et al., 2009; Hayes et al., 2015). Up to 12 ampullariid species of the genus *Pila*, and multiple synonyms, have been recorded from the region (Cowie, 2015). The confused taxonomy has complicated the understanding of species distributions, and other than a few important studies in the 1980s and 1990s (Keawjam, 1986, 1987a,b; Pagulayan & Remigio, 1993), the current status of *Pila* in South-east Asia remains largely unknown. In contrast, the introduced South American congenerals, *Pomacea* spp., have been well studied because of their impacts in paddy fields (Isnainingsih & Marwoto, 2011; Nghiem et al., 2013; Joshi et al., 2017). There have been numerous anecdotal accounts of the decline of *Pila* species since the introduction of *Pomacea* spp. (e.g. Halwart, 1994; Marwoto et al., 2011), but the neglect of *Pila* taxonomy has made it difficult to ascertain the status of the species. Although population trends of South-east Asian *Pila* species are unknown, seven species have been assessed as Least Concern (IUCN, 2017).

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One such species is *Pila scutata* (Sri-Aroon & Richter, 2012), with which various synonyms have been associated (Low et al., 2013; Ng et al., 2014; Cowie, 2015). It is reported to be widely distributed across South-east Asia (Low et al., 2013), but some occurrences are unconfirmed and others are probably misidentifications. For example, Keawjam (1986) concluded from extensive surveys of Thailand ampullariids that previous records (Brandt, 1974) of *P. scutata* were *Pila gracilis* that lacked bands. Hayes et al. (2009b) reported on a previous misidentification of *P. scutata* (as *Pila conica*) from Viet Nam. Further south, the distribution of *P. scutata* in Peninsular Malaysia has not been mapped, but it has been reported to be common across the peninsula (van Benthem Jutting, 1931; Berry, 1974). In Singapore, *P. scutata* was formerly widespread and common in ponds and ditches (e.g. Ponniah, 1962; Johnson, 1973; H.E. Ng, pers. comm.), but by the early 2000s the species remained extant in only a small number of localities (Clements et al., 2006). It was also reportedly declining in Peninsular Malaysian rice fields (Joshi et al., 2017).

Early records of *P. scutata* were from streams and rivers (e.g. de Morgan, 1885, as *Ampullaria wellesleyensis*), but later surveys failed to find it in natural, undisturbed habitats (Tan et al., 2013; D.S. Johnson, unpubl. manuscript, based on work in the 1960s and early 1970s). Records from the 1960s indicated that *P. scutata* was associated with anthropogenic habitats, such as fishponds, throughout the Malay peninsula (Johnson, 1973). Up to the mid 20th century the species was a common food item for local communities, and for livestock feed (Ponniah, 1962; Lim et al., 1978). Its popularity as a protein source suggests that it was either naturally widespread (and abundant) or widely translocated by people, or both. This species has also been introduced to Pacific islands (Cowie, 1997, 2002; Smith, 2003) purportedly as food, via the Philippines (Cowie, 1998; Tran et al., 2008). The introduced population in Hawaii has very low mitochondrial DNA diversity (Tran et al., 2008), as has been recorded for many non-native taxa that experience a genetic bottleneck during the introduction stage (Dlugosch & Parker, 2008).

Despite being considered indigenous to Singapore (Ng, 1991; Chan, 1996; Tan et al., 2012), considering the near-exclusive occurrence of *P. scutata* in man-made or disturbed habitats, it is possible that its current distribution reflects past anthropogenic translocations. Such history would complicate efforts to resolve the taxonomy and biogeography of *Pila* in South-east Asia. With the apparent decline of *Pila* populations throughout the region, it is imperative that any anthropogenic influence be uncovered and considered when examining their biogeography. Thus, we aimed to document the current distribution of *P. scutata* in the Malay peninsula (specifically Peninsular Malaysia and Singapore) and to provide an assessment of the status of this cryptogenic species (assumed native, but possibly introduced) in Singapore.

Methods

Surveys and data collection

Records of *P. scutata* and other congeners from Singapore and Peninsular Malaysia (collectively referred to here as the Malay peninsula) were obtained from available literature (Supplementary Table 1). Specimens in the Zoological Reference Collection (ZRC) of the Lee Kong Chian Natural History Museum at the National University of Singapore were examined. Records and photographs of type material of *P. scutata* and its synonyms (following Cowie, 2015), and of other *Pila* species, were also obtained from the following collections: the Australian Museum, Sydney (AMS), the Academy of Natural Sciences, Philadelphia (ANSP), the Field Museum, Chicago (FMNH), the Forest Research Institute Malaysia, Kuala Lumpur, Malaysia (FRIM), the Natural History Museum, London (NHMUK), the Zoological Survey of India, National Zoological Collection India, Kolkata (NZSI), the Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt (SMF), the University of Michigan Museum of Zoology, Ann Arbor (UMMZ), and the Zoologisches Museum Hamburg (ZMH).

Based on records from these sources, we surveyed known ampullariid localities in the Malay peninsula from August 2013 to March 2015. Opportunistic sampling was also carried out at other potentially suitable habitats, including water bodies around limestone outcrops, ditches, rice fields and ponds. Samples from one additional population in Singapore were obtained from Daniel J.J. Ng. Comparative material from Java in Indonesia was obtained from the collection of the Zoologisches Museum Berlin, Germany (the type locality of *P. scutata* is Pardana in Java; Mousson, 1848), and other material was collected from the Philippines and Viet Nam (regions where *P. scutata* has previously been recorded; Pagulayan & Remigio, 1993; Jørgensen et al., 2008).

DNA analysis

We extracted total genomic DNA from the foot tissue of at least 20 individuals of each *P. scutata* population, and at least one individual of other *Pila* species obtained. We added 900 µl CTAB buffer (0.1 M Tris pH 8; 1.4 M NaCl; 0.02 M EDTA; 20 g/l CTAB) and 20 µl Proteinase K (Invitrogen) to tubes containing the tissue samples. The samples were then incubated for 3 h at 55 °C. We extracted the DNA using 25 : 24 : 1 phenol : chloroform : isoamyl alcohol (Biozol), and precipitated with 100% ethanol. After washing the DNA pellets in 70% ethanol, we dissolved the pellets in 50 µL of water. Mitochondrial COI and 16S rRNA genes were amplified in polymerase chain reactions (PCR) with a total volume of 23–24 µl PCR reaction mixture (2.5 µl of BioReady rTaq 10 × buffer, 2 mM dNTPs, 1 µl each of 10 µM primers, 0.25 µl of BioReady rTaq DNA Polymerase

(Bulldog Bio), and DNase-free sterile water), at 95 °C for 5 min, 34 cycles of 95 °C for 30 s, 45–48 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min. Amplicons of each gene were obtained using different pairs of primers depending on the quality of tissue obtained (Supplementary Table 2). The primer pairs LCO1490/HCO2198 and dg_LCO1490/dg_HCO2198 amplified approximately 600 base pairs (bp) in the COI barcode region, and mlCOIntF/jgHCO2198 amplified a shorter fragment (313 bp) in the 3' region of the COI. The latter pair was used in specimens that failed to amplify the standard barcode region. The size of 16S fragments amplified ranged from 320 to 476 bp. The PCR products were checked visually on a 1% agarose gel. Post PCR clean-ups were performed on successfully amplified products using SureClean reagent (Bioline Inc., London, UK) following the manufacturer's recommendations. The purified products were sequenced with BigDye Terminator reactions and analysed on the ABI PRISM 3130XL sequencer (Applied Biosystems, Foster City, USA) at the DNA Sequencing Laboratory of the National University of Singapore.

We visually inspected and trimmed sequences using *Sequencher 4.6* (Genecodes, Ann Arbor, USA). The COI and 16S genes were aligned using *MAFFT v. 7* (Kato & Standley, 2013) with default settings. Aligned COI sequences were checked for translatability into amino acids and were gap free. DNA sequences were inspected using objective clustering in *SpeciesIdentifier v. 1.7.9* (Meier et al., 2006). Objective clustering was performed using the *SpeciesIdentifier (TaxonDNA 1.6.2; Meier et al., 2006)* to determine molecular operational taxonomic units (mOTUs) at a range of thresholds (0–6%), to test stability of the results. In objective clustering sequences are grouped according to uncorrected pairwise distances (Meier et al., 2006, 2008; Srivathsan & Meier, 2012); i.e. members of a set of putative conspecific sequences have at least one match to a sequence in the set that falls within a given threshold distance. Previous studies have shown the intraspecific genetic distance for the majority of gastropods is < 2% for COI (Meier et al., 2008; Layton et al., 2014). Sequences were checked against the GenBank database (Benson et al., 2013) under default parameters in *MegaBLAST* (OMICtools, Le-Petit-Quevilly, France). Further analysis was not carried out because of the low variation within and among the populations (see Results). All sequences were deposited in GenBank (KY081728–KY081759, KY087525) and BOLD (SEAM001–023).

Results

Historical distribution of *P. scutata* in the Malay peninsula

Two *Pila* species are known from Singapore, *P. scutata* and *Pila ampullacea*, with the latter species recorded as a single population that is now considered extirpated (Ng et al.,

2014). *Pila scutata*, however, was widely distributed prior to the 1990s, and found in fishponds and reservoirs throughout Singapore (Fig. 1; Supplementary Table 1). In Peninsular Malaysia *P. scutata* was similarly recorded from human-disturbed habitats such as artificial ponds and paddy fields (Fig. 1; Supplementary Table 1). These records of *P. scutata* from the peninsula were from the north, close to the border with Thailand (e.g. Kangar, Perlis, ANSP388826: Sow-Yan Chan, pers. comm.; Machang, Kelantan: Lim et al., 1978; Boo Liat Lim, pers. comm.), and along the western coast of the peninsula. The southernmost record for the peninsula was from a stream in central Johor (ZRC.MOL.6955), c. 80 km from Singapore.

Current distribution of *P. scutata* in the Malay peninsula

We collected *P. scutata* from six localities in Singapore: Kranji, Tampines, Sarimbun, Seletar, Pandan and Jurong (Fig. 1, Plate 1). *Pomacea* spp. were syntopic with *P. scutata* at four of these sites (Kranji, Tampines, Sarimbun, Seletar). Only one population of *P. scutata* was found in Peninsular Malaysia, in Ipoh (Fig. 1, Plate 1). In contrast, *Pomacea* spp. were found at multiple sites where *Pila* spp. were previously recorded, although not at the Ipoh site where *P. scutata* was found (Fig. 1).

Identification of *P. scutata* and congeners from South-east Asia

Based on examination of *Pila* spp. collected for this study (with the exception of *Pila* from Java that was loaned from ZMB, and collected in 2015) from the known range of *P. scutata*, we identified *P. scutata* from Java, Indonesia (ZMB106543), and from Neuva Ecija, Philippines (ZRC.MOL.7018; Plate 1). From the states of Kelantan, Terengganu, Perak, Selangor and Perlis in Peninsular Malaysia three other congeners were identified (Plate 2): *P. ampullacea*, *Pila angelica* and *P. gracilis*. Ampullariids collected from Viet Nam were identified as *Pila erythrochila*, *P. gracilis*, and *Pila virescens* (Plate 2).

COI and 16S from 128 *P. scutata* individuals were successfully sequenced. From Singapore, these included 20 each from Kranji, Tampines, Sarimbun and Pandan, six from Seletar, and one from Jurong; from Peninsular Malaysia, 20 from Ipoh; from the Philippines, 20 from Neuva Ecija; from Indonesia, one from Java. Among the other species, both COI and 16S were sequenced from *P. ampullacea* and *P. gracilis* from Peninsular Malaysia, whereas only COI was sequenced from *P. angelica*, and only 16S was sequenced from *P. gracilis* and *P. virescens* from Viet Nam (all one individual each).

Sequence clustering of COI using objective clustering (Meier et al., 2006) produced five mOTUs that were mostly

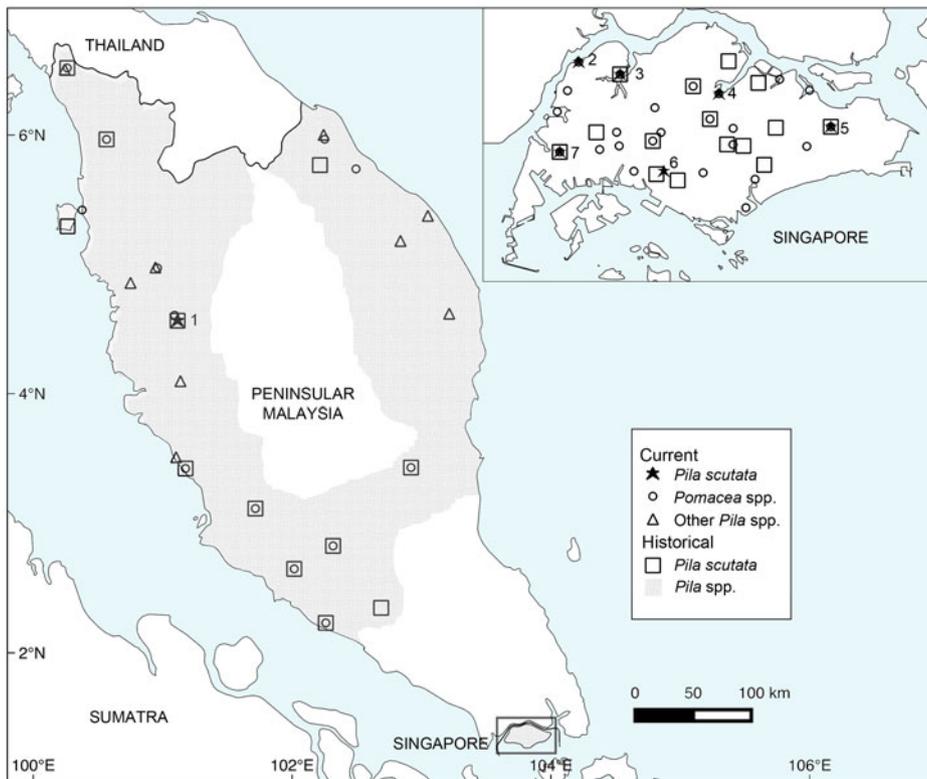


FIG. 1. Historical and current distribution of *Pila scutata* and other ampullariids in the Malay Peninsula (Peninsular Malaysia and Singapore). Sites with extant *P. scutata* populations: 1, Ipoh; 2, Sarimbun; 3, Kranji; 4, Seletar; 5, Tampines; 6, Pandan; 7, Jurong. Records within a 10 km radius in Peninsular Malaysia, and within a 1 km radius in Singapore were treated as single localities.

congruent with the morphologically-identified species over 1–5% thresholds (Table 1). Across these thresholds, the numbers of clusters remained stable, with no specimens involved in mOTU reassignment between thresholds. Over the same threshold range *P. scutata* sequences separated into two mOTUs: a Javan specimen on its own, and the other 127 sequences as a single mOTU (Table 1). At 6% threshold, the Javan *P. scutata* sequence merged with the larger *P. scutata* cluster. Results for 16S were similar to those for COI; the Javan specimen remained a separate mOTU up to the 4% threshold. The mOTUs for COI for other *Pila* spp. remained stable across 1–6% thresholds, corresponding to the morphologically-identified species.

All specimens that were identified as *P. scutata* based on morphology were matched to GenBank COI sequences of *P. scutata* collected from Hawaii (as *Pila conica* in Rawlings et al., 2007; Tran et al., 2008) with 100% identity, except for the Javan *P. scutata*, which was a 95% match (Supplementary Table 3). Other than *P. scutata*, only *P. gracilis* (Peninsular Malaysia and Viet Nam) and *P. virescens* (Viet Nam) had high matches (99–100% identity) to available sequences on GenBank (Supplementary Table 3).

Mitochondrial DNA diversity of *P. scutata* populations

All 107 *P. scutata* individuals from Singapore and Peninsular Malaysia shared a single COI haplotype (0% uncorrected

pairwise distance). Pairwise distances were 0.78% between the Malay peninsula population and that of the Philippines, 0–0.7% between the Philippines population and individuals from Hawaii (Rawlings et al., 2007; Tran et al., 2008), and 5.4–7.1% between the Javan individual and all other *P. scutata*. For 16S, *P. scutata* from Singapore, Peninsular Malaysia, the Philippines, and Hawaii shared a single 16S haplotype (0% uncorrected pairwise distances), whereas the pairwise distances between the Javan individual and all other *P. scutata* were 4.7–4.8%.

Discussion

Historical and current distribution of *P. scutata* in the Malay peninsula

The first record of an ampullariid in Singapore (as *Ampullaria* sp.) by Traill (1847) lacks figures or specimens, preventing identification of the species. We found that museum and literature records corroborate historical accounts of *P. scutata* being formerly widespread and common in Singapore (Johnson, 1973) and Peninsular Malaysia (Berry, 1974). Yet in our exhaustive surveys we discovered only six populations in Singapore, and only one population in Peninsular Malaysia. Five of these populations overlapped with previous records (Supplementary Table 2), with only Sarimbun and Pandan being newly reported localities. All

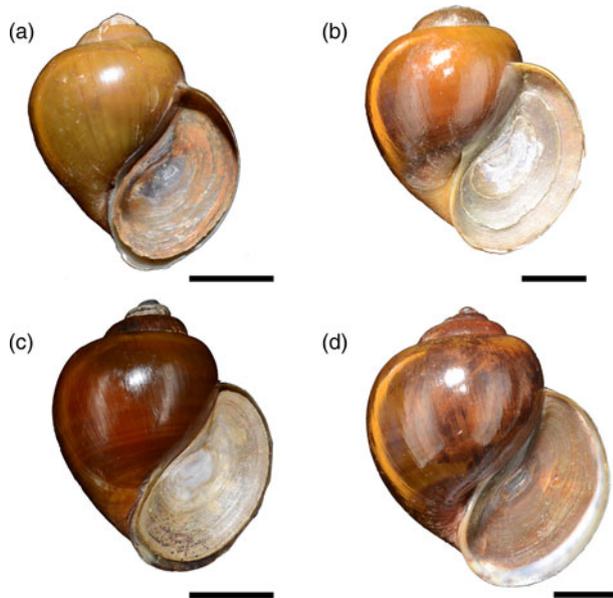


PLATE 1 *Pila scutata* from South-east Asia: (a) Bogor, Java, Indonesia (ZMB106543), (b) Neuva Ecija, Philippines (ZRC.MOL.7018), (c) Ipoh, Malaysia (ZRC.MOL.6961), (d) Pandan, Singapore (ZRC.MOL.7062). Scale bars = 10 mm. Photographs by T.H. Ng.

seven sites were at former village areas, which included plantations and ponds (Ponniah, 1962; Yeap, 2015; National Archives of Singapore, 2016).

The decline of *P. scutata* in Singapore has been attributed to habitat loss and canalization of open country streams (Ng et al., 1993; Chan, 1996). Many previously known *P. scutata* localities no longer exist (Ng et al., 2014). A similar situation was encountered in Peninsular Malaysia, where many former sites have been modified or have disappeared altogether. Lack of habitat alone, however, does not completely explain the decline of *P. scutata*, as this resilient species was known to be common even in degraded habitats such as stocked fishponds and septic ponds (Ponniah, 1962; Johnson, 1973). Furthermore, some concrete canals around Singapore with banks that had recently been naturalized or softened for aesthetic and recreational reasons (Lee et al., 2010) now resemble freshwater habitats of the past that were ideal for snails (Johnson, 1973), and paddy fields where the species thrived are still present in rural areas of Peninsular Malaysia.

These remaining suitable areas and newly-created habitats, however, are being rapidly colonized by South American *Pomacea* spp. Throughout the Malay peninsula, *Pomacea* spp. appear to have replaced *P. scutata* in areas where the latter were once common (Fig. 1). Thus, a plausible alternative hypothesis for the decline of *P. scutata* may be competition from the confamilial *Pomacea* spp. (but see Ng et al., 1993; Chan, 1996; Tan et al., 2012). The decline in *P. scutata* in Singapore appears to have coincided

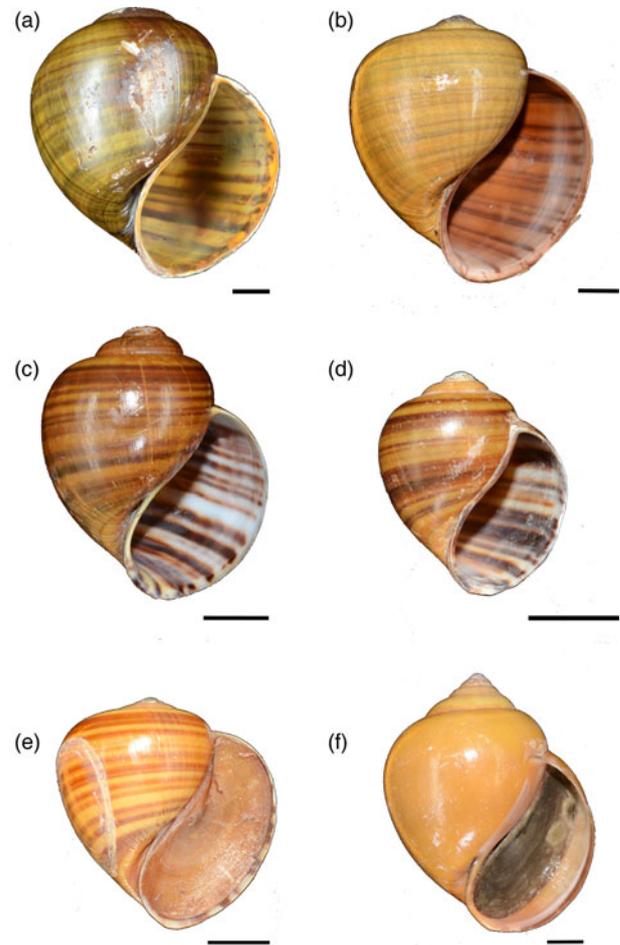


PLATE 2 Other ampullariids recorded from Peninsular Malaysia and Viet Nam: (a) *Pila ampullacea*, Malaysia (ZRC.MOL.7001), (b) *Pila angelica*, Malaysia (ZRC.MOL.6971), (c) *Pila gracilis*, Malaysia (ZRC.MOL.6992), (d) *P. gracilis*, Viet Nam (ZRC.MOL.7006), (e) *Pila erythrochila*, Viet Nam (ZRC.MOL.7016), (f) *Pila virescens*, Viet Nam (ZRC.MOL.7014). Scale bars = 10 mm. Photographs by T.H. Ng.

with the introduction and spread of *Pomacea* spp. since the late 1980s (Ng et al., 2014). *Pomacea* spp. were first collected from a single reservoir in 1988, but are now found in most reservoirs and many canals and ponds (Ng et al., 2014). The possible displacement of native apple snail species has also been reported in other regions invaded by *Pomacea* spp. (e.g. Halwart, 1994; Thaewnon-ngiw et al., 2004; Marwoto et al., 2011). Efforts by collaborators from the Philippines and Indonesia to find comparative material of *Pila* for this study were largely unsuccessful as they encountered an overwhelming presence of *Pomacea* instead (Roberto Pagulayan & Ristiyanti M. Marwoto, pers. comms). *Pomacea* was also established at the same locality in the Philippines where *P. scutata* was obtained for this study.

It is uncertain if the extant populations of *P. scutata* in the Malay peninsula will survive in the long-term. Two of the localities with no *Pomacea* present, Pandan (Singapore) and Ipoh (Malaysia) have experienced recent

TABLE 1. Number of *Pila* species identified based on morphology compared to number of molecular operational taxonomic units (mOTU) at objective clustering thresholds between 1 and 6% (uncorrected pairwise distances) for COI and 16S genes.

| No. of morphospecies (by gene) | No. of mOTUs at clustering threshold | | | | | |
|---|---|---|----|----|----|---|
| | 1% | 2% | 3% | 4% | 5% | 6% |
| COI | | | | | | |
| 4 species: <i>P. scutata</i> (SGP, MYS, PHL, IDN), <i>P. ampullacea</i> (MYS), <i>P. angelica</i> (MYS), <i>P. gracilis</i> (MYS) | 5 mOTUs: <i>P. scutata</i> (SGP, MYS, PHL), <i>P. scutata</i> (IDN), <i>P. ampullacea</i> (MYS), <i>P. angelica</i> (MYS), <i>P. gracilis</i> (MYS) | | | | | 4 mOTUs: <i>P. scutata</i> (SGP, MYS, PHL, IDN), <i>P. ampullacea</i> (MYS), <i>P. angelica</i> (MYS), <i>P. gracilis</i> (MYS) |
| 16S | | | | | | |
| 4 species: <i>P. scutata</i> (SGP, MYS, PHL, IDN), <i>P. ampullacea</i> (MYS), <i>P. gracilis</i> (MYS, VNM), <i>P. virescens</i> (VNM) | 6 mOTUs: <i>P. scutata</i> (SGP, MYS, PHL), <i>P. scutata</i> (IDN), <i>P. ampullacea</i> (MYS), <i>P. gracilis</i> (MYS, VNM), <i>P. virescens</i> (VNM) | 5 mOTUs: <i>P. scutata</i> (SGP, MYS, PHL), <i>P. scutata</i> (IDN), <i>P. ampullacea</i> (MYS), <i>P. gracilis</i> (MYS, VNM), <i>P. virescens</i> (VNM) | | | | 4 mOTUs: <i>P. scutata</i> (SGP, MYS, PHL, IDN), <i>P. ampullacea</i> (MYS), <i>P. gracilis</i> (MYS, VNM), <i>P. virescens</i> (VNM) |

SGP, Singapore; MYS, Malaysia; PHL, Philippines; IDN, Indonesia; VNM, Viet Nam.

habitat modification (THN, pers. obs.), and the third, in Jurong (Singapore) is connected by a canal to Tengeh Reservoir (Daniel J.J. Ng, pers. comm.), where *Pomacea* is established. The faster growth, higher reproductive capacity, and voracious appetite for aquatic macrophytes of *Pomacea* spp. (Cowie, 2002; Carlsson et al., 2004; Morrison & Hay, 2011; Chaichana & Sumpun, 2014) are invasive traits that threaten the continued survival of *P. scutata* in the remaining four localities where it co-occurs with *Pomacea* (i.e. Kranji, Sarimbun, Tampines, Seletar).

Anthropogenic influences and taxonomic confusion

Another factor that may have contributed to the decline of *P. scutata* in the Malay peninsula is the unexpected lack of mitochondrial variation we found across all extant populations. Although the species is considered native to the Malay peninsula, the current low genetic diversity and lack of geographical structure may have stemmed from anthropogenic introduction, and a resulting founder effect. Although non-native species may overcome the limitations of genetic bottlenecks experienced during introduction (Roman & Darling, 2007), low genetic diversity is known to affect the fitness of populations (Kinziger et al., 2011).

The low level of variation in the Malay peninsula *P. scutata* mitochondrial genes mirrors that of the introduced population in Hawaii (as *P. conica* in Tran et al., 2008). Clustering analysis showed the Hawaiian populations to be closely linked to the Philippines population, strongly indicating the Philippines as the origin of *P. scutata* in Hawaii (as *P. conica* in Cowie, 1995; Tran et al., 2008), with the Philippines population showing some slight variation (0.3–0.6% uncorrected pairwise distances). The extremely low mitochondrial diversity among Malay peninsula populations of *P. scutata* is thus not likely to be natural. By comparison, preliminary analysis of COI genes of *P. gracilis* from the Malay peninsula (four sites 60–250 km apart) showed that individuals from each population had distinct haplotypes.

Comparisons of genetic diversity between the native range and introduced localities, or among non-native regions, can uncover introduction pathways (Hayes et al., 2008; Wong et al., 2010). Attempts to obtain comparative specimens from the type locality of *P. scutata* (i.e. Java) and other countries for this study were largely unsuccessful. It is thus impossible to draw definitive conclusions regarding the true native distribution of *P. scutata*, nor is it certain that the lack of genetic diversity in Malay peninsula *P. scutata* is the result of a historical genetic bottleneck experienced by a native population (rather than of a founder effect caused by anthropogenic introduction). To address such biogeographical questions it would be necessary first to conduct a comprehensive revision of the genus in

South-east Asia with a wider range of samples from throughout the region, using multiple molecular markers, including nuclear genes (Meier, 2008).

All extant populations of *P. scutata* identified in our study, however, were found in human-disturbed areas. The pre-modified natural inland waters of Singapore were probably too soft and acidic to sustain a high diversity of molluscs (Johnson, 1967; Yeo & Lim, 2011). The historical absence from natural habitats, and invariably close association of *P. scutata* with human-modified habitats, supported by the lack of genetic diversity across the populations in this study, therefore strongly indicate that present-day populations of *P. scutata* in the Malay peninsula have been introduced.

Further complicating matters, our results also suggest that the species thus far identified as *P. scutata* may be a species complex. *Pila scutata* of Java was separated from *P. scutata* of the Malay peninsula and the Philippines up to a threshold of > 4% in both COI and 16S. Intraspecific pairwise difference for the majority of gastropods is < 2% (Meier et al., 2008; Layton et al., 2014), although there are exceptions: e.g. a terrestrial snail, *Cepaea nemoralis*, has > 10% intraspecific divergence (Thomaz et al., 1996). Interspecific relationships for some *Pila* species have been investigated previously (Hayes et al., 2009b), but intra- and interspecific differences for the genus, especially in South-east Asia, are poorly known. We are unable to determine conclusively whether the Javan snails on the one hand and the Malay peninsula and Philippine snails on the other hand represent two distinct species or a single species with high intraspecific variation. Apple snails are notoriously difficult to distinguish by conchological characters (Hayes et al., 2012), and possible hybridization sometimes confounds genetic analysis (Matsukura et al., 2013). Thus, a taxonomic revision of South-east Asian ampullariids is overdue (Hayes et al., 2009a, 2015).

Consequences for conservation

Outreach campaigns in Peninsular Malaysia over the past 20 years successfully raised awareness of the threat of *Pomacea* among farmers, who actively remove apple snails from paddy fields but are unable to tell the genera apart (Joshi et al., 2017). Older literature, including medically-related studies, identified *P. scutata* as the most common apple snail on the Malay peninsula (e.g. Berry, 1974; Lim et al., 1978). However, we found only one population of *P. scutata*, in Ipoh, compared to multiple populations of *P. gracilis*, along both the east (Kelantan, Terengganu) and west (Perak, Selangor) coasts of Peninsular Malaysia. Given the morphological similarity between different *Pila* spp., especially juveniles (Keawjam, 1986), it is possible that some of the historical records of *P. scutata* in the Malay peninsula were of *P. gracilis* or juveniles of *P. ampullacea* (Plate 2).

Many of the historical records could not be verified because of the lack of clear photographs and voucher specimens. The example here of poorly resolved taxonomy affecting conservation is not unique to ampullariids, it is symptomatic of a wider issue across understudied invertebrate groups in general (Collen et al., 2012), and requires more attention.

Conclusions

Our findings strongly suggest that present-day populations of *P. scutata* in the Malay peninsula were probably introduced by people. The questionable origins of the South-east Asian apple snail *P. scutata* in its presumed native range necessitates a reassessment of the species' current IUCN Red List status. These extant populations pose a dilemma, and the lack of genetic diversity is of concern in light of *Pila* decline throughout South-east Asia. Conservation management of *P. scutata* and its other South-east Asian congeners must therefore be better informed by greater taxonomic resolution, and more comprehensive investigations of their ecology, both in native and introduced ranges.

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Conflicts of interest None.

Ethical standards All animal handling procedures were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the National University of Singapore and comply with the journal's Code of Conduct.

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