

Molecular evidence of hybridisation in two invasive species of *Pomacea* (Gastropoda: Ampullariidae) in Peninsular Malaysia

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Abstract. Hybridisation has played an important role in generating evolutionary novelty and diversification in plants and animals. During invasions, hybridisation may contribute to lineages with improved genotypes and greater invasive potential. Two morphologically cryptic species of invasive apple snails, *Pomacea canaliculata* and *P. maculata*, are known to hybridise in their native and invaded ranges. These two species are widespread in Peninsular Malaysia and occur in sympatry in several sites. We asked whether hybrid lineages of *Pomacea* existed in Peninsular Malaysia and whether genetic exchange was ongoing in nine populations. We generated mitochondrial and nuclear genealogies to assess patterns of interspecific genetic exchange and subsequently, hybrid diagnosis. First, we conducted a restriction enzyme analysis based-preliminary screening using the nuclear elongation factor 1-alpha (EF1 α) amplicons of 90 *Pomacea* specimens from nine locations. Next, we reconstructed phylogenies of the nuclear EF1 α and mitochondrial cytochrome c oxidase subunit I (COI) to validate the restriction analysis data. The molecular data provided evidence of interspecific hybridisation at a rate of 42.2% where (i) 18 heterozygous individuals possessed both EF1 α sequences of *P. canaliculata* and *P. maculata* and (ii) 20 individuals exhibited EF1 α -COI mito-nuclear incongruences. Our study provides the first molecular evidence of introgression and ongoing hybridisation in Peninsular Malaysia with potential implications for the acquisition of traits that enhance invasiveness in hybrid lineages.

Key words. apple snails, nuclear elongation factor 1-alpha, mitochondrial cytochrome c oxidase subunit I, genetic exchange, phylogeny

INTRODUCTION

Biological invasions have resulted in disastrous impacts on the economy and environmental health of nations leading to an increased interest in invasion processes, correlates of invasive success, and the evolution of greater invasiveness. For recently introduced species, sufficient genetic variation may be crucial for establishing in novel environments; thus, the transition from a few founders to a successfully established invasive population has presented a ‘genetic paradox’ in the field of invasion biology. Reticulate evolutionary events such as hybridisation and, subsequently, genetic introgression are potentially important for the acquisition of pre-adapted advantageous alleles and novel genetic variation in introduced populations, aiding with establishment, but potentially acting as catalysts for increased invasiveness (Ellstrand & Shierenbeck, 2000; Pfennig et al., 2016). With the worldwide spread of biological invasions expected to rise concomitantly with climate change (Fournier et al., 2019), and the presence

of external reticulate evolutionary forces that potentially foster invasion (Klonner et al., 2017), invasion-mediated damages are likely to be exacerbated.

Pomacea canaliculata (Lamarck, 1822) and *P. maculata* (Perry, 1810) (Ampullariidae) are two species of South American gastropods that have gained international notoriety for their invasion of multiple freshwater ecosystems in tropical and subtropical regions including North America, Europe, Oceania, and Asia. Their adaptability to a wide range of environments, rapid growth rate, high fecundity, voracious appetites, and polyphagous feeding habits have accounted largely for their rapid spread and consequent ecological and economic impacts in the invaded range (Carlsson et al., 2004; Cowie et al., 2006; Kwong et al., 2009; Lv et al., 2009; Teem et al., 2013). In Peninsular Malaysia, *Pomacea* was first reported in Selangor in 1991 (Chang, 1992; Mat Hassan & Abdul Kadir, 2003) and the invasion has since spread to agricultural wetlands throughout the peninsula, causing massive infestations and extensive damage (Salleh et al., 2012; Arfan et al., 2014, 2016; Yahaya et al., 2017). Analyses of mitochondrial DNA revealed the existence of *P. canaliculata* and *P. maculata*, although interspecific similarities in external morphology, even in traits previously described as species-specific (e.g., Hayes et al., 2012), suggest that hybrid lineages may occur in the peninsula (Rama Rao et al., 2018).

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Hybridisation between *Pomacea canaliculata* and *P. maculata* is reported in both native and introduced ranges such as Japan, South Korea, China, and the Philippines (Matsukura et al., 2013; Glasheen et al., 2020; Yang et al., 2020). The potential for interspecific hybridisation is especially high in freshwater species, where isolated and fragmented habitats promote allopatric speciation and high phylogeographic structure within taxa, but premating isolating barriers fail to develop (Streit et al., 1994; Seehausen & Wagner, 2014). These processes have been considered largely responsible for the high levels of hybridisation detected in numerous families of freshwater fish (Wallis et al., 2018) and Cerithioidean gastropods (Glaubrecht & von Rintelen, 2008) following secondary contact, from geological or climate-induced changes. In *P. canaliculata* and *P. maculata*, hybridisation may have facilitated range expansion in native populations and also contributed to invasion success (Glasheen et al., 2020). Recurrent backcrossing of hybrids and parent populations tends to retain beneficial introgressed alleles while simultaneously removing maladaptive alleles to generate introgressive hybrids with enhanced physiological traits (Pfennig et al., 2016). In *Pomacea*, putative hybrid *P. maculata* COI haplotypes in Argentina have greater tolerances to cold and desiccation stresses (an adaptive trait of pure *P. canaliculata* lineages) as compared to individuals from pure *P. maculata* lineages (Yoshida et al., 2014). In China, *P. canaliculata* COI haplotypes, including those with some degree of hybrid ancestry, have a wider distribution than hybrid populations of *P. maculata* (Yang et al., 2020). The absence of pure *P. maculata* lineages in China suggests that the acquisition of adaptive traits from *P. canaliculata* through hybridisation may have played a role in *P. maculata*'s establishment and spread.

Our objective was to establish whether hybrid lineages of *Pomacea* existed in Peninsular Malaysia, which would serve as an initial step toward investigating the implications of hybrid populations and, ultimately, appropriate control efforts. Molecular studies in the peninsula have relied, thus far, on the cytochrome c oxidase subunit I (COI) and 16S ribosomal deoxyribonucleic acid (rDNA) barcoding markers for assessing species identity of *Pomacea* (Phoong et al., 2018; Rama Rao et al., 2018; Kannan et al., 2020); however, these markers are maternally inherited and cannot by themselves assess genetic signatures of hybridisation. Biparentally inherited nuclear markers are required for hybrid diagnosis because genetic material from both parents are integrants in the hybrid's genome. In fact, a combination of both mitochondrial and nuclear markers provides multiple genealogies to infer cryptic evolutionary events and demographic processes (Seixas et al., 2018; Brito et al., 2020). The nuclear elongation factor 1-alpha (EF1 α) marker possesses sufficient variation and species-discriminating qualities to assess ampullariid evolutionary relationships and population structure (Jørgensen et al., 2008; Hayes et al., 2009). Additionally, the EF1 α marker has revealed evidence of genetic exchange between *P. canaliculata* and *P. maculata* when used in combination with the COI marker (Matsukura et al., 2013; Glasheen et al., 2020; Yang et al., 2020). In this study, we used a combination of the mitochondrial COI

and nuclear EF1 α markers within a phylogenetic framework to assess genetic exchange between *P. canaliculata* and *P. maculata* in Peninsular Malaysia.

MATERIAL AND METHODS

Sample collection and processing. The use of *Pomacea* specimens and the experimental procedures were approved by the Sunway University Research Ethics Committee (Approval code: PGSUREC 2018/044). We used 9 to 15 individuals of *Pomacea* spp. collected from nine geographic locations (Fig. 1, Table 1) in Peninsular Malaysia (February 2016–September 2019). Specific permissions for sample collection were not required (with the exception Taman Wetlands Putrajaya, Approval reference number: PPj/R/A/TWH/69[14]) as no protected species were involved. Sampling history of the nine locations and the subsequent rapid multiplex COI screening indicated the presence of four sympatric populations of *P. canaliculata* and *P. maculata* (Guar Cempedak, Putrajaya, Sekinchan, and Temoh), where each site varied in species density. Species composition of *Pomacea* in the remaining sites consisted entirely of either *P. canaliculata* (Limbat Lembu and Subang Jaya) or *P. maculata* (Tasik ChinChin, Kuantan, and Pasir Gudang) (unpublished data). Fifty-two previously processed specimens were used in this study where 24 (MN623417–MN623440), 18 (MG230742–MG230748, MG230759–MG230767, MG230771, and MG230773) and 10 (MG230780–MG230781, MG230783–MG230785, MG230788–MG230789, and MG230791–MG230793) COI sequences deposited into the National Center for Biotechnology Information (NCBI) database (Table 1) were incorporated into the COI phylogenetic analyses. For the remaining individuals, we heat-shocked the snails with hot water (100°C) to facilitate the removal of the soft tissues at the columellar muscle using forceps to keep the shells intact (Fukuda et al., 2008). A piece of foot tissue of approximately 1–5 mg from each individual was immersed in distilled water for two hours to soften the fibres, then chopped and homogenised.

Genomic DNA extraction and amplification. We extracted total genomic DNA from foot tissue using the NucleoSpin®Tissue extraction kit (Macherey-Nagel, Germany). We measured the concentration and purity of the eluted DNA using Biodrop ulite (Biodrop, United Kingdom) and stored the DNA at –20°C for future use. Barcoding regions of the EF1 α and COI genes were amplified using the (i) forward F7 (5'-TGTGAATAAGATGGACAGGA-3') and reverse 5R primers (5'-AATCCTAACCTCCAATTGT-3') (Hayes et al., 2009) for a 520 bp EF1 α fragment and (ii) forward LCO1490 (5'-GGTCAACAAATCATAAAGATATTG-3') and reverse HCO2198 primers (5'TAAACTTCAGGGTGAC CAAAAAATCA-3') (Folmer et al., 1994) for a 650 bp COI fragment. DNA amplifications were performed in a final volume of 25 μ L containing 12.5 μ L Prime Taq Premix (2 \times) (GENETBIO Inc., Korea), 1 μ L of 10 pmol/ μ L forward and reverse primers, 1 μ L extracted DNA sample, and 9.5 μ L distilled water. Amplifications were carried out using T100® Thermal Cycler (Bio-Rad Laboratories Inc., USA)

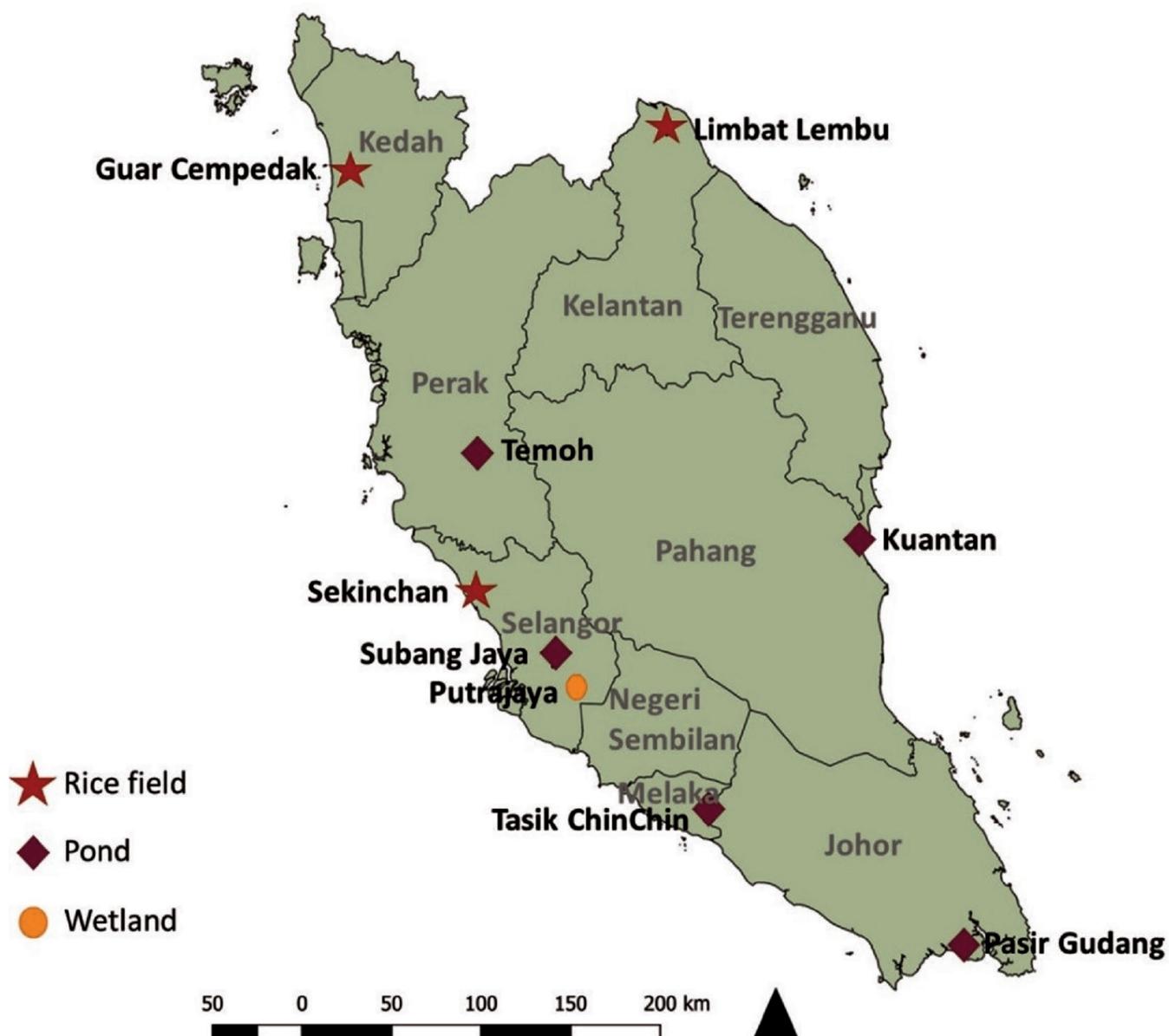


Fig. 1. Geographical location and habitat type of sampling sites of *Pomacea* species in Peninsular Malaysia.

with PCR parameters described by Hayes et al. (2009) and Folmer et al. (1994) and the amplicons were quantified via electrophoresis on 1% agarose gel.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis, TOPO-TA Cloning and sequencing. Restriction enzyme analysis conducted by Matsukura et al. (2013) incorporated the *ApaLI* restriction enzyme to obtain species-specific RFLP profiles of the 520 bp EF1 α amplicons. We employed this approach as a rapid preliminary screening method for all 90 individuals. Briefly, both species produced two different RFLP profiles, where (i) *P. canaliculata* produced a single fragment of 520 bp, (ii) *P. maculata* produced two fragments of 330 bp and 190 bp, and (iii) putative hybrids had a combination of all three fragments. We conducted restriction digests in a final volume of 50 μ L containing 10 μ L EF1 α amplicons, 2 μ L *ApaLI* restriction enzyme (New England Biolabs, USA), 5 μ L 1 \times cutsmart buffer (New England Biolabs, USA) and 33 μ L distilled water. We incubated digestion mixtures for two

hours at 37°C. Restriction fragments of digested products were electrophoresed on a 1% agarose gel. Based on RFLP profiles, we cloned heterozygous EF1 α amplified products, which were snails with RFLP profiles of all three fragments originating from both *P. canaliculata* and *P. maculata*, using the TOPO-TA Cloning Kit for Sequencing (Invitrogen, USA), and the isolates were extracted using the ZymoPURETM Plasmid MiniPrep Kit (Zymo Research Corp., USA). EF1 α amplicons with the *P. canaliculata* and *P. maculata* RFLP profiles along with the cloned plasmid extracted DNAs were subsequently sequenced via cycle sequencing (MyTACG Bioscience Enterprise).

Phylogenetic analysis. We examined, edited and merged forward and reverse DNA sequencing chromatograms of the EF1 α and COI amplicons into contiguous sequences using ChromasPro2.0 (Technelysium Pty. Ltd., Australia) and BioEdit 7.0.5.3 (Hall, 1999). We analysed a total of 108 EF1 α and 90 COI sequences from 90 *Pomacea* specimens, of which all EF1 α and 38 COI sequences were generated in this

Table 1. List of *Pomacea* individuals obtained from this study with their sampling locations, clade ID, and GenBank accession numbers for the EF1 α and COI sequences. Accession numbers of EF1 α and COI reference sequences of *Pomacea* spp. from native and invaded regions used in the analyses are provided. ‘a’, ‘b’, ‘c’, ‘d’, ‘e’, ‘f’, and ‘g’ represent cloned sequences isolated from heterozygous individuals in the present study. ‘*’, ‘†’, ‘‡’ indicate references sequences used in the median-joining network, phylogenetic, and both analyses, respectively. ‘—’ indicate unavailable data.

Location	Latitude/ Longitude	Species/Clade	Sequence ID	GenBank Accession Number	
				EF1 α	COI
Kuantan, Pahang, Malaysia	103.296668/ 3.835439	<i>P. maculata</i>	AN1	MW715317	MN623417
		<i>P. maculata</i>	AN2	MW715318	MN623418
		<i>P. maculata</i>	AN3	MW715319	MN623419
		<i>P. maculata</i>	AN4	MW715320	MN623420
		<i>P. maculata</i>	AN5	MW715321	MN623421
		<i>P. maculata</i>	AN6	MW715322	MW721183
		Hybrid	AN7	d MW715323 e MW715324	MW721184
		<i>P. maculata</i>	AN8	MW715325	MW721185
		<i>P. maculata</i>	AN9	MW715326	MW721186
		<i>P. maculata</i>	AN13	MW715327	MW721187
Tasik ChinChin, Melaka, Malaysia	102.481937/ 2.275085	<i>P. maculata</i>	CC1	MW715328	MN623422
		<i>P. maculata</i>	CC2	MW715329	MN623423
		<i>P. maculata</i>	CC3	MW715330	MN623424
		<i>P. maculata</i>	CC4	MW715331	MN623425
		<i>P. maculata</i>	CC5	MW715332	MN623426
		<i>P. maculata</i>	CC6	MW715333	MW721188
		<i>P. maculata</i>	CC7	MW715334	MW721189
		<i>P. maculata</i>	CC8	MW715335	MW721190
		<i>P. maculata</i>	CC9	MW715336	MW721191
		Hybrid	CC12	a MW715337 b MW715338	MW721192
Subang Jaya, Selangor, Malaysia	101.5977/ 3.0796	<i>P. canaliculata</i>	SJ2	MW715340	MG230743
		<i>P. canaliculata</i>	SJ5	MW715341	MG230744
		<i>P. canaliculata</i>	SJ7	MW715342	MG230745
		<i>P. canaliculata</i>	SJ12	MW715343	MW721193
		<i>P. canaliculata</i>	SJ13	MW715344	MG230746
		Hybrid	SJ14	a MW715345 c MW715346	MG230747
		Hybrid	SJ15	a MW715347 c MW715348	MW721194
		<i>P. canaliculata</i>	SJ16	MW715349	MW721195
		Hybrid	SJ17	a MW715350 c MW715351	MG230748
Limbat Lembu, Kelantan	102.2460/ 6.0459	<i>P. canaliculata</i>	LL2	MW715353	MN623427
		<i>P. canaliculata</i>	LL5	MW715356	MN623428
		<i>P. canaliculata</i>	LL6	MW715357	MN623429
		<i>P. canaliculata</i>	LL9	MW715360	MW721201
Putrajaya, Selangor, Malaysia	101.6970/ 2.9630	Hybrid	PJ1	MW715361	MG230763
		Hybrid	PJ2	MW715362	MG230764
		Hybrid	PJ3	MW715363	MG230765
		Hybrid	PJ4	MW715364	MG230766
		Hybrid	PJ5	e MW715365 d MW715366	MG230767
		Hybrid	PJ11	MW715367	MG230771
		Hybrid	PJ13	a MW715368 b MW715369	MN623435

Location	Latitude/ Longitude	Species/Clade	Sequence ID	GenBank Accession Number	
				EF1α	COI
Putrajaya, Selangor, Malaysia	101.6970/ 2.9630	Hybrid	PJ14	a	MW715370
				b	MW715371
			PJ15		MW715372
		Hybrid	PJ16	g	MW715373
				c	MW715374
			PJ20	a	MW715375
				b	MW715376
		<i>P. canaliculata</i>	PJ29		MW715377
			PJ36	a	MW715378
				b	MW715379
		<i>P. canaliculata</i>	PJ45		MW715380
Guar Cempedak, Kedah, Malaysia	100.4481/ 5.8545	Hybrid	GC1		MW715381
			GC2		MW715382
			GC5		MW715383
		<i>P. canaliculata</i>	GC6		MW715384
			GC7		MW715385
		<i>P. canaliculata</i>	GC8		MW715386
			GC9		MW715387
			GC11	b	MW715388
				f	MW715389
		<i>P. canaliculata</i>	GC14		MW715390
			GC18		MW715391
			GC20		MW715392
Pasir Gudang, Johor, Malaysia	103.900952/ 1.475061	Hybrid	PD1	c	MW715393
				g	MW715394
			PD2	a	MW715395
		Hybrid	PD3		MW715396
			PD4	c	MW715397
			PD5	f	MW715398
		<i>P. maculata</i>	PD6		MW715399
			PD7	a	MW715400
			PD9	c	MW715401
		<i>P. maculata</i>	PD10	a	MW715402
				b	MW715404
Sekinchan, Selangor, Malaysia	101.1451/ 3.5067	<i>P. canaliculata</i>	SK2		MW715405
			SK3		MW715406
			SK4		MW715407
		<i>P. canaliculata</i>	SK5		MW715408
			SK6		MW715409
			SK7		MW715410
		<i>P. canaliculata</i>	SK8		MW715411
			SK9		MW715412
			SK10		MW715413
		Hybrid	SK11		MW715414
			SK12	c	MW715415
				g	MW715416
Temoh, Perak, Malaysia	101.1998/ 4.2403	Hybrid	TM1		MW715417
			TM2		MW715418
		<i>P. canaliculata</i>	TM4		MW715419
			TM5		MW715420
			TM6		MW715421

Location	Latitude/ Longitude	Species/Clade	Sequence ID	GenBank Accession Number	
				EF1 α	COI
Temoh, Perak, Malaysia	101.1998/ 4.2403	<i>P. canaliculata</i> Hybrid	TM8	MW715425	MW721221
			TM9	MW715426	MW721222
			TM10	MW715427	MW721223
			TM11	MW715428	MW721224
			TM12	MW715429	MW721225
			TM14	MW715430	MW721226
			TM15	MW715431	MW721227
			TM17	MW715432	MW721228
			—	MN590313*	—
			—	MN590314*	—
Parque Rodo, Montevideo, Uruguay	—	<i>P. canaliculata</i> Hybrid	—	MN590317*	—
			—	MN590318*	—
			—	MN590320*	—
			—	MN590321*	—
			—	MN590323*	—
			—	MN590325*	—
			—	MN590326*	—
			—	MN590327*	—
			—	MN590396*	—
			—	MN590313*	—
Magallanes, Uruguay	—	<i>P. canaliculata</i>	—	MN590314*	—
			—	MN590317*	—
			—	MN590318*	—
			—	MN590320*	—
			—	MN590321*	—
			—	MN590323*	—
			—	MN590325*	—
			—	MN590326*	—
			—	MN590327*	—
			—	MN590392*	—

Location	Latitude/ Longitude	Species/Clade	Sequence ID	GenBank Accession Number	
				EF1α	COI
Magallanes, Uruguay	—	<i>P. canaliculata</i>	—	MN590382*	—
			—	MN590383*	—
			—	MN590384*	—
			—	MN590385*	—
			—	MN590386*	—
			—	MN590401*	—
			—	MN590402*	—
Tala Creek, Uruguay	—	<i>P. canaliculata</i>	—	MN590368*	—
			—	MN590410*	—
			—	MN590407*	—
			—	MN590373*	—
			—	MN590372*	—
			—	MN590371*	—
			—	MN590367*	—
			—	MN590366*	—
			—	MN590363*	—
			—	MN590362*	—
Punta Gorda, Uruguay	—	<i>P. maculata</i>	—	MN590357*	—
			—	MN590370*	—
			—	MN590404*	—
			—	MN590405*	—
			—	MN590408*	—
			—	MN590409*	—
			—	MN590406*	—
			—	MN590398*	—
			—	MN590397*	—
			—	MN590369*	—
			—	MN590365*	—
			—	MN590364*	—
			—	MN590356*	—
Careiro Castanho, Brazil	—	<i>P. maculata</i>	—	MN590355*	—
			—	MN590354*	—
			—	MN590352*	—
			—	MN590349*	—
			—	MN590341*	—
			—	MN590353*	—
			—	MN590351*	—
			—	MN590350*	—
			—	MN590348*	—
			—	MN590347*	—
			—	MN590346*	—
			—	MN590345*	—
			—	MN590344*	—
Campo Grande, Brazil	—	<i>P. maculata</i>	—	MN590343*	—
			—	MN590342*	—
			—	MN590338*	—
			—	MN590340*	—
			—	MN590339*	—
			—	MN590337*	—

Location	Latitude/ Longitude	Species/Clade	Sequence ID	GenBank Accession Number	
				EF1α	COI
Barro Alto, Brazil	—	<i>P. maculata</i>	—	MN590336*	—
			—	MN590334*	—
			—	MN590333*	—
			—	MN590332*	—
			—	MN590331*	—
Campinorte, Brazil	—	<i>P. maculata</i>	—	MN590335*	
Mato Grosso, Brazil	—	<i>P. maculata</i>	—	FJ710336‡	EU528559†
			—	FJ710337‡	EU528568†
		<i>P. scalaris</i>	—	FJ710350†	FJ710316†
Buenos Aires, Argentina	—	<i>P. canaliculata</i>	—	FJ710344‡	EU528529†
		<i>P. scalaris</i>	—	FJ710345‡	FJ710315†
La Leonesa, Argentina	—	<i>P. canaliculata</i>	—	FJ710346‡	FJ710314†
Maldonado, Uruguay	—	<i>P. canaliculata</i>	—	—	FJ710313†
Philippines	—	<i>P. canaliculata</i>	—	—	EU528483†
Florida, USA	—	<i>P. paludosa</i>	—	FJ710347†	EU528590†
			—	FJ710348†	EU528591†
			—	—	EF514960†
Amazonas, Brazil	—	<i>P. diffusa</i>	—	FJ710351†	FJ710317†
Para, Brazil	—	<i>P. diffusa</i>	—	FJ710352†	EU528547†
			—	—	EU528553†

study and deposited into the NCBI database with the accession numbers MW715317–MW715432 and MW721183–MW721228, respectively (Table 1). We included COI data from Phoong et al. (2018), Rama Rao et al. (2018) and Kannan et al. (2020) for 18 (MG230742–MG230748, MG230759–MG230767, MG230771, and MG230773), 10 (MG230780–MG230781, MG230783–MG230785, MG23088–MG230789 and MG230791–MG230793) and 24 (MN623417–MN623440) specimens, respectively (Table 1). We retrieved 10 EF1α and 15 COI GenBank reference sequences of *Pomacea* spp. generated by Rawlings et al. (2007) and Hayes et al. (2008, 2009) from the NCBI database (Table 1) as taxonomic references to aid in the phylogenetic interpretation. All sequences were aligned using ClustalX (Larkin et al., 2007) to generate EF1α and COI datasets. The COI data matrix was partitioned using the 1st, 2nd, and 3rd codon positions. We conducted phylogenetic analyses using Bayesian inference (BI) and maximum likelihood (ML) methods of tree reconstruction. The best-fit BI DNA substitution models were selected using Kakusan version 3.0 (Tanabe, 2007) based on the Bayesian Information Criterion (BIC). Two independent runs with four Markov Chain Monte Carlo (MCMC) for 12,000,000 (EF1α) and 15,000,000 (COI) generations were run in MrBayes v3.2.1 (Huelsenbeck et al., 2001) and convergence diagnostics were calculated every 5,000th generation. Trees in each chain were sampled every 500th generation. We discarded the first 25% of the topologies

as burn-in after evaluating the convergence in Tracer version 1.5.0 (Rambaut & Dummond, 2009). We reconstructed EF1α and COI ML phylogenetic trees via IQ-Tree (Trifinopoulos et al., 2016) with 1,000 bootstrap replicates based on the best fit DNA substitution models from jModelTest 2.1.10. (Darriba et al., 2012) using the AIC. Phylogenetic trees were viewed using FigTree v.1.4.0 (Rambaut, 2012). We included *P. scalaris* and *P. diffusa* as outgroup taxa.

Median-Joining Network (MJN) analysis. A haplotype network was reconstructed with NETWORK® 10.2.0.0 (Bandelt et al., 1999) via the median-joining (MJ) method using a 409 bp alignment of EF1α sequences, consisting of specimens in the present study (N=108) and in the native range (N=105). Native EF1α sequences from Hayes et al. (2009) and Glasheen et al. (2020) were retrieved from the NCBI database (Table 1). The relationship between the haplotypes was assessed to establish and confirm possible origins of hybrid lineages from the native regions into Peninsular Malaysia.

RESULTS

PCR-RFLP screening. The PCR-RFLP screening successfully discriminated the *Pomacea* individuals in all nine sites with distinct RFLP profiles for *P. canaliculata* and

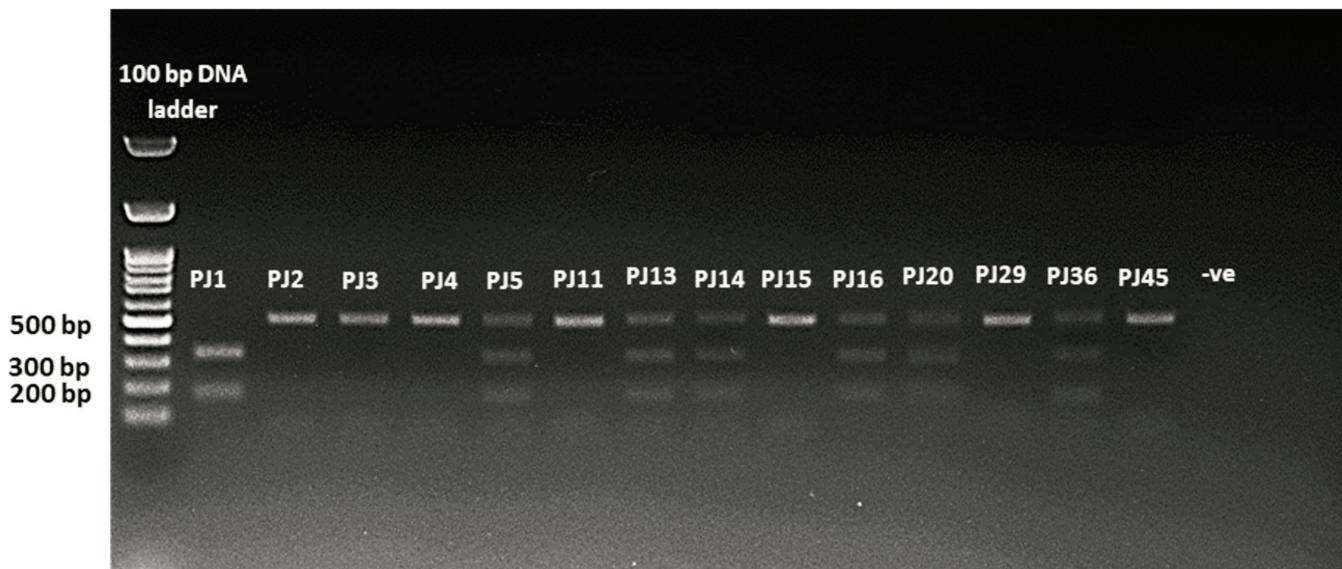


Fig. 2. Representative agarose gel electrophoresis image showing the *ApaLI*-digested *EF1 α* amplicons for 14 specimens from Putrajaya. The single band, two-band, and three-band RFLP profiles indicate *Pomacea canaliculata*, *P. maculata*, and interspecific heterozygous hybrids, respectively.

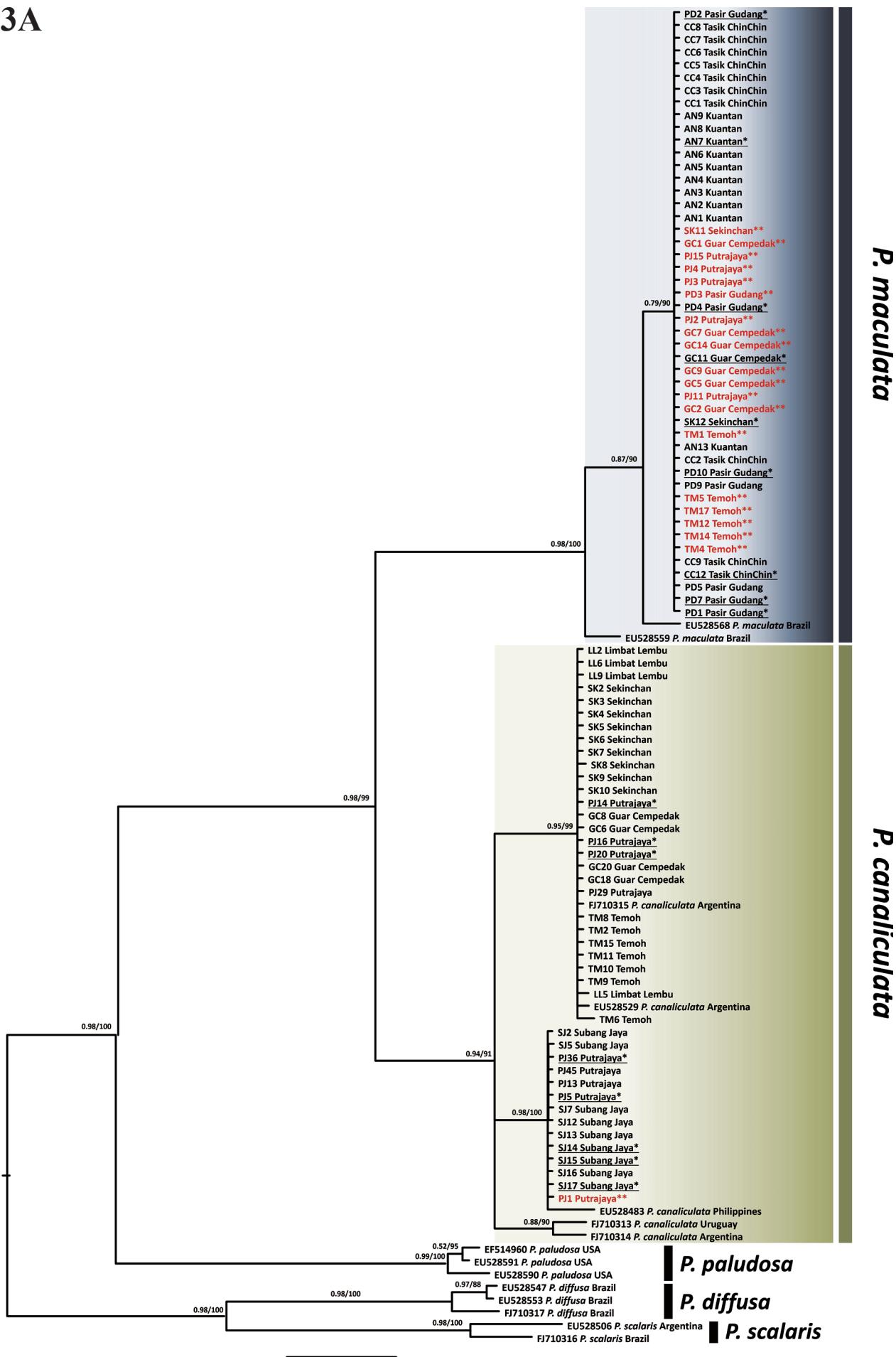
P. maculata (Fig. 2; Supplementary Fig. S1, <https://figshare.com/s/61b79734906fd3178244>). The largest proportion of screened samples consisted of 51 individuals that possessed a single fragment of 520 bp and were identified as *P. canaliculata*. Twenty-one individuals were represented by two fragments of sizes 330 bp and 190 bp. These individuals were identified as *P. maculata*. The remaining 18 individuals represented the smallest proportion and had a combination of all three restriction fragments, confirming the presence of heterozygotic alleles inherited from both species. Thus, these individuals were identified as putative heterozygous hybrids. Based on the nuclear RFLP profiles alone, these heterozygous hybrids were present in all sites except Limbat Lembu and Temoh with frequent occurrences in Pasir Gudang, Subang Jaya, and Putrajaya.

Phylogenetic analyses for evidence of genetic exchange. Mitochondrial and nuclear genealogies were phylogenetically analysed from 90 COI and 108 *EF1 α* sequences obtained from 90 *Pomacea* specimens to reveal patterns of genetic exchange between *P. canaliculata* and *P. maculata* (Fig. 3A, B). The COI phylogenetic trees were reconstructed using the Generalised time reversible model with a gamma shape parameter (GTR + G) (-1940.82), Felsenstein 1981 (F81) + G (-935.01) and Hasegawa, Kishino and Yano (HKY) + G (-3876.78) models for the 1st, 2nd, and 3rd codon positions respectively in the BI analysis and the Jukes-Cantor (JC) model for all three partitions with a log likelihood value of -1705.18 in the ML analysis. For the *EF1 α* phylogenetic trees, the GTR + G (-2426.23) and Kimura 1980 including invariable sites with a gamma shape parameter (K80 + I + G) (BI = -1866.59 , ML = -1866.44) models were used for the BI and ML analyses, respectively. Species-specific monophyletic clades were revealed in both phylogenies where specimens in our study were found exclusively in two, the *P. canaliculata* and *P. maculata* clades, which

were homologous to the reference sequences. Putative *P. canaliculata* (N=32) and *P. maculata* (N=20) individuals were identified based on their concordant identification in both phylogenies. However, two conflicting genetic patterns in the mitochondrial and nuclear genealogies were observed for the remaining 38 individuals. One pattern was the mito-nuclear incongruence observed in 20 individuals where individuals with mitochondrial COI *P. canaliculata* sequences possessed nuclear *EF1 α* *P. maculata* sequences and vice versa. These individuals were identified as introgressive putative hybrids. The second pattern was observed in 18 individuals which were heterozygous at the *EF1 α* locus, possessing both *P. canaliculata* and *P. maculata* sequences.

MJN for evaluation of hybrid origin. The MJN based on the alignment of 213 *EF1 α* sequences of *Pomacea canaliculata* and *P. maculata* from the study sites in the peninsula (N=108 sequences from 90 individuals) and native sites (N=105) consisted of 99 haplotypes, of which 38 were comprised of *Pomacea* specimens in the present study (Fig. 4). Two distinct groups separated by 15 mutations were detected—*P. canaliculata* and *P. maculata* groups consisting of 62 and 37 haplotypes, respectively, which corresponded to the *EF1 α* phylogeny (Fig. 3). Native *Pomacea* spp. hybrids found in three regions, Parque Rodo Montevideo, Punta Gorda (Uruguay), and Careiro Castanho (Brazil) shared a close relationship to *Pomacea* spp. in the peninsula, where in the *P. maculata* group, haplotypes from Tasik ChinChin and Pasir Gudang were closely related (separated by one mutation) to hybrid haplotypes from Punta Gorda and Careiro Castanho. In the *P. canaliculata* group, a haplotype group consisting of specimens from Limbat Lembu, Guar Cempedak, Subang Jaya, and Putrajaya was shared with a hybrid haplotype from Parque Rodo Montevideo whereas a haplotype group from Temoh, Sekinchan, and Putrajaya was shared with a hybrid haplotype from Punta Gorda.

3A



3B

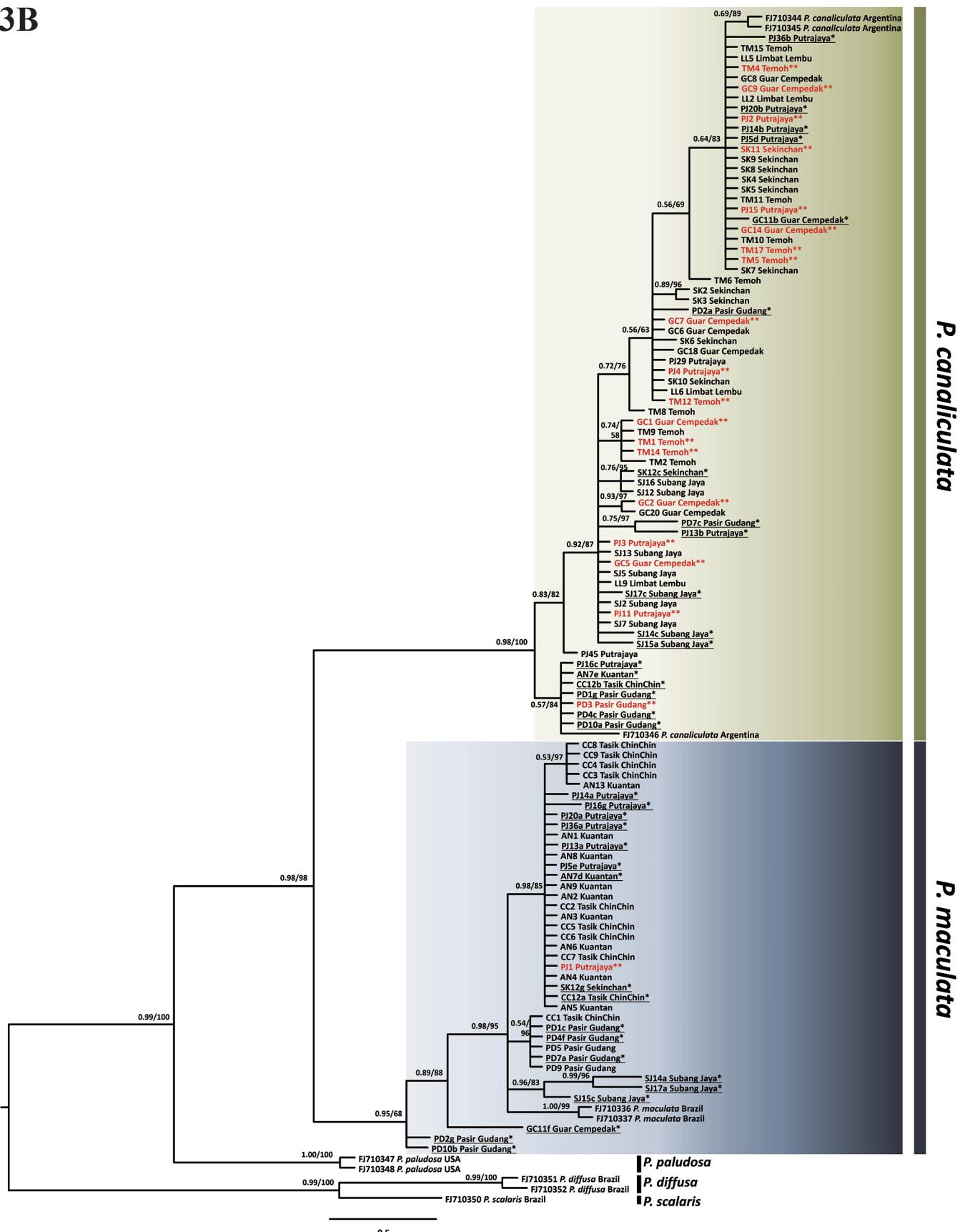
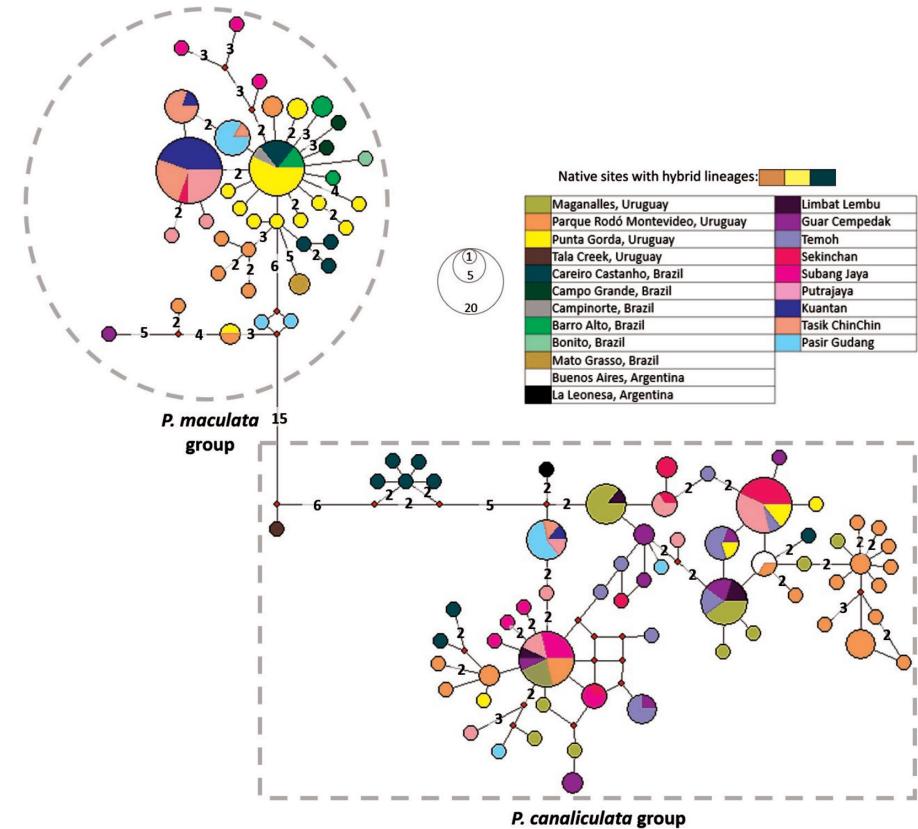


Fig. 3. Bayesian inference phylogenograms depicting relationship of *P. canaliculata* and *P. maculata* from Peninsular Malaysia and *Pomacea* spp. from other regions based on the (A) mitochondrial COI and (B) nuclear EF1 α markers. Kuantan, Tasik ChinChin, Limbat Lembu, Subang Jaya, Putrajaya, Guar Cempedak, Pasir Gudang, Sekinchan, and Temoh refer to geographic locations in Peninsular Malaysia where specimens in this study were collected. Bayesian posterior probabilities/maximum likelihood bootstrap supports are indicated by nodal values. *Pomacea difussa* and *P. scalaris* were used to root the phylogenies. *Pomacea canaliculata* and *P. maculata* clades are highlighted in green and blue, respectively. Underlined taxa marked with '*' indicate interspecific heterozygous individuals whereas taxa in red and marked with '**' are COI-EF1 α mito-nuclear incongruences.

4A



4B

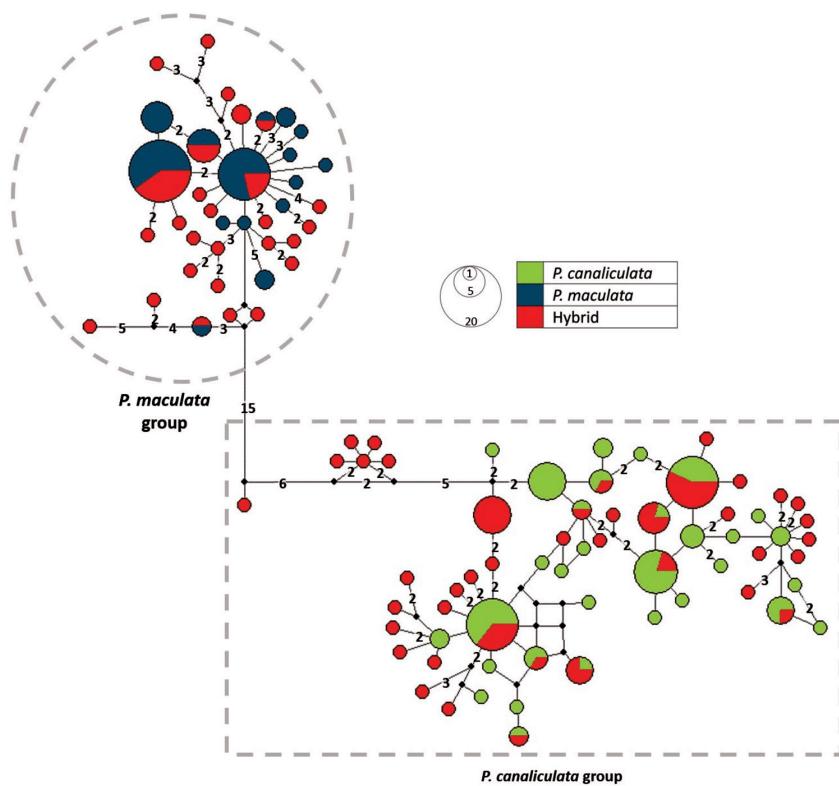


Fig. 4. Median-joining haplotype network of *Pomacea canaliculata* and *P. maculata* sequences from Peninsular Malaysia (N=108 from 90 individuals) and the native ranges (N=105) based on 409 nucleotides of the EF1 α gene. The network shows the relationship between haplotypes from different geographic regions based on sequence similarity. Unique sequences within each individual were included in the alignment (sequences for homozygotes were not doubled). Node colours represent the (A) geographic location and (B) species identity of the sequences (see legends). Each node represents a unique haplotype and node size is proportional to the haplotype frequency. Branches between nodes indicate a single nucleotide substitution unless denoted by numerical values for multiple nucleotide substitutions. Red (A) and black (B) nodes represent hypothetical ancestors or unsampled haplotypes. Two major groups are framed in grey dotted lines; *P. canaliculata* and *P. maculata*.

DISCUSSION

The present study provides the first molecular evidence of introgression and ongoing hybridisation in Peninsular Malaysia. A common underlying presumption would be that ongoing hybridisation events are more likely to occur in the sympatric sites. However, the nuclear EF1 α data detected the presence of several heterozygous hybrid individuals in all sites except for Limbat Lembu and Temoh (although further sampling and screening may be required). These snails have both *P. canaliculata* and *P. maculata* parental lineages detected at the nuclear locus and suggest ongoing hybridisation in these sites, particularly Putrajaya, Pasir Gudang, and Subang Jaya (the latter two were previously considered to consist of only one species of *Pomacea*). The observed interspecific heterozygosity at the EF1 α locus may indicate either first generation (F_1) or multiple generation hybridisation events; thus, the extent of hybridisation remains uncertain because paternal and maternal ancestry of the specimens is unknown.

Apart from the heterozygous sequence data, we further examined the topology of the mitochondrial COI and nuclear EF1 α phylogenies to infer possible signals of interspecific genetic exchange. Molecular incongruences in species identity between both phylogenies were observed in 22.2% of individuals. Discrepancies in phylogenetic interpretation of mitochondrial and nuclear genealogical bases of identification may result from several mechanisms such as introgressive hybridisation, incomplete lineage sorting and the existence of cryptic species (Glaubrecht & Köhler, 2004; Glaubrecht & von Rintelen, 2008; Kim et al., 2010). Although the cause of the mito-nuclear incongruences in our study is unclear, we postulate that multiple episodes of introgression rather than incomplete lineage sorting is the most plausible explanation because (i) the latter has been ruled out in the native range (Glasheen et al., 2020), (ii) there is high conchological ambiguity associated with *P. canaliculata* and *P. maculata* in Peninsular Malaysia (Rama Rao et al., 2018) and (iii) heterozygous hybrid genotypes are present in the peninsula, representing a proportion of F_1 hybrids in these sites, and a source for the generation of introgressive hybrids via recurrent backcrossing.

Because genetic exchange between *Pomacea canaliculata* and *P. maculata* is reported in both native and invaded ranges (Matsukura et al., 2013; Glasheen et al., 2020; Yang et al., 2020), previous hybridisation events may have preceded the introduction of hybrid lineages into Peninsular Malaysia. Several snails analysed in this study share similar (Limbat Lembu, Guar Cempedak, Putrajaya, and Subang Jaya) and closely related (Temoh, Sekinchan, Kuantan, Tasik ChinChin, and Pasir Gudang) EF1 α haplotypes to *Pomacea* individuals from Parque Rodo Montevideo, Punta Gorda (Uruguay), and Careiro Castanho (Brazil), which are sites with confirmed hybridisation events (Fig. 4). This might explain the possible existence of introgressive hybrids in Subang Jaya, where no pure *P. maculata* lineage has been detected for recurrent backcrosses from a large number of screened individuals (N=64; unpublished data).

Introgressive hybrids from native regions could have led to extensive hybridisation events in Subang Jaya, leading to its current state represented by extensive recurrent ongoing hybridisation. Aside from native hybrid lineage sources, as mentioned, heterozygous hybrids could constitute a proportion of F_1 hybrids particularly in sympatric sites in Peninsular Malaysia and lead to frequent introgressive hybridisation among hybrid and parental lineages. These occurrences, in tandem with native hybrid lineage derived-introgression, correlate with high incidences of hybridisation events in Putrajaya and Guar Cempedak where haplotypes of *P. canaliculata* and *P. maculata* were found. The low incidence of hybridisation in Sekinchan suggests a recent introduction of *P. maculata*, which based on our sampling history (COI screening) represents only 2.3% of our sampled specimens (detected in 2019; unpublished data). Putative pure populations of *P. maculata* in Kuantan and Tasik ChinChin reported the least occurrences of hybridisation (10%) with only a single heterozygous hybrid, indicating possible ongoing hybridisation involving *P. canaliculata* lineages that were not found in our samples. An interesting finding was obtained from the genetic structure of the specimens from Pasir Gudang, where the rate of hybridisation was reported at 75.0%, despite only *P. maculata* being represented in current and previous samples (N=20; unpublished data). An important caveat is that species composition of single-species populations are at best assumptions based on COI screening to date and additional sampling could prove otherwise.

A significant fraction of introgressive hybrids (21.1%) in this study had conflicting mitochondrial (*Pomacea maculata*) and nuclear (*P. canaliculata*) haplotypes, also frequently observed in the native South American regions (Glasheen et al., 2020). This frequent directional pattern of introgression potentially confers a selective advantage to the genome of the recipient species; for instance, in China, *P. maculata* populations possessed nuclear genotypes of *P. canaliculata* to a certain extent and have acquired improved dessication and cold tolerances, thus expanding their distribution into temperate regions (Yang et al., 2020). The findings in the present study revealed that different regions are impacted by varying degrees of introgression and may require region-specific control measures, depending on the local environmental pressures. Hah et al. (2021) reported a strong association between *P. canaliculata* mitochondrial haplotypes and rice field habitats in Peninsular Malaysia. *Pomacea maculata* haplotypes were rarely documented in rice fields, but were prevalent in aquatic habitats such as rivers, lakes, and ponds. Whether *P. maculata* haplotypes are more vulnerable to irrigation regimes and pest management in rice fields, and whether hybridisation with *P. canaliculata* enhances the resilience and invasiveness of *P. maculata* in rice fields is unknown and merits investigation. It is noteworthy that all *P. maculata* individuals in Guar Cempedak, a rice field in the northern region of the peninsula (Figs. 1, 4), had hybrid ancestry via genetic exchange with *P. canaliculata*. Future studies that assess environmental and physiological associations of hybrids will provide better insights into control measures.

The results presented in this study cannot provide a comprehensive interpretation of the extent and type of DNA introgression, because it is unclear if introgression occurred at mitochondrial or nuclear loci. Bidirectional gene introgression was observed in the COI and EF1 α phylogenies from *P. canaliculata* to *P. maculata* and vice versa. Morphological assessments are required to complement the molecular data in order to reflect the current phylogenetic patterns of introgressed loci. Multiple animal studies have reported that introgressions are more likely to occur at mitochondrial rather than nuclear loci (Toews & Brelsford, 2012; Pons et al., 2014, which includes several caenogastropods (Kim et al., 2010; Köhler, 2016; Hirano et al., 2019). Moreover, the phenomenon of interspecific paternal leakage derived mitochondrial heteroplasmy in minute moss beetles (*Ochthebius quadricollis* and *O. urbanellia*) via mitochondrial introgression (Mastrantonio et al., 2019) has also been observed in an individual in this study (GC7) as reported in a previous study utilising the mitochondrial COI and 16S rDNA loci (Kannan et al., 2020). These suggest that mitochondrial introgression has occurred, at least to a certain extent, between *P. canaliculata* and *P. maculata* in Peninsular Malaysia, yet the possibility of nuclear introgression remains uncertain.

A significant caveat in this study is that the use of two independent markers in our study is insufficient to assess the extent of hybridisation. Also, we cannot confidently rule out the trace of hybrid ancestry in the putative ‘pure’ lineages because species identities of paternal and maternal ancestors were obscure. Multi-locus approaches, encompassing various regions in the genome with different evolutionary rates, provide a wider scope of analyses to overcome these limitations. Although microsatellite data have been developed for *P. canaliculata* and *P. maculata*, alleles for the two species are mostly shared and thus, uninformative for this question (Matsukura et al., 2016). Single nucleotide polymorphisms (SNPs) serve as a better option, providing reliable analyses for extensive hybridisation studies (Melville et al., 2017; Matucci et al., 2019) and may be a useful tool to address hybridisation in *Pomacea* spp. The development of these powerful biallelic panels with multiple informative loci across the genome, provides better species-discriminating resolution for delineating pure and hybrid snails. In addition, these panels enable the detection of distant hybrid ancestry, thus distinguishing the number of generations of hybrid backcrosses and the proportion of introgressed genes in each generation.

CONCLUSIONS

Our findings augment our understanding of introgression and ongoing hybridisation of *Pomacea canaliculata* and *P. maculata* in Peninsular Malaysia. The EF1 α gene is an effective nuclear marker for delineating *P. canaliculata* and *P. maculata*. The combined mitochondrial and nuclear-based phylogenetic analyses have successfully revealed widespread occurrences of interspecific hybridisation between *P. canaliculata* and *P. maculata* in all locations (except Limbat

Lembu) as well as cryptic introgression in Putrajaya, Guar Cempedak, Pasir Gudang, Sekinchan, and Temoh. Frequent hybridisation occurrences in our study suggest possible acquisition of enhanced phenotypic traits via introgression and thus, provide new insights into controlling the spread of these apple snails and their hybrids. However, empirical data are required to elucidate the selective phenotypic and physiological advantages acquired by these hybrids. Application of next-generation sequencing (NGS) approaches to provide multi-locus data across the genome is needed to explore the extent of hybridisation. In addition, these markers should be incorporated into an in-depth study assessing more populations of *Pomacea* across the peninsula along with the phenotypic and genetic impacts of hybridisation, which would reveal the extent of invasion as well as evolutionary consequences of hybridisation. Hybrid populations with elevated fitness, for instance those with enhanced phenotypic and physiological traits, could lead to further spread and damage, particularly in economically vulnerable areas (rice fields, etc.). Coordinated surveillance and a better understanding of underlying invasion mechanisms will be needed to effectively manage *Pomacea* populations in these areas.

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