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Genetic Diversity and Molecular Markers in Introduced and Thai Native Apple Snails (*Pomacea* and *Pila*)

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The genetic diversity and species-diagnostic markers in the introduced apple snail, *Pomacea canaliculata* and in the native Thai apple snails; *Pila ampullacea*, *P. angelica*, *P. pesmei*, and *P. polita*, were investigated by restriction analysis of COI and are reported for the first time. Twenty-one composite haplotypes showing non-overlapping distributions among species were found. Genetic heterogeneity analysis indicated significant differences between species ($P < 0.0001$) and within *P. pesmei* ($P < 0.0001$) and *P. angelica* ($P < 0.0004$). No such heterogeneity was observed in *Pomacea canaliculata* ($P > 0.0036$ as modified by the Bonferroni procedure), *P. ampullacea* ($P = 0.0824$-1.000) and *P. polita* ($P = 1.0000$). A neighbor-joining tree based on genetic distance between pairs of composite haplotypes differentiated all species and indicated that *P. angelica* and *P. pesmei* are closely related phylogenetically. In addition, the 16S rDNA of these species was cloned and sequenced. A species-specific PCR for *P. canaliculata* was successfully developed with a sensitivity of detection of approximately 50 pg of the target DNA template. The amplification of genomic DNA (50 pg and 25 ng) isolated from the fertilized eggs, and juveniles (1, 7, and 15 d after hatching) of *Pomacea canaliculata* was also successful, and suggested that *Pomacea canaliculata* and *Pila* species can be discriminated from the early stages of development.

Keywords: Apple snails, Genetic diversity, PCR-RFLP, *Pila*, *Pomacea*

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Introduction

The apple snails indigenous to Thailand are members of the genus *Pila*. The group comprises *P. ampullacea*, *P. angelica*, *P. gracilis*, *P. pesmei* and *P. polita* according to Keawjam (1986). Whereas *P. ampullacea*, *P. pesmei* and *P. polita* are sympatrically distributed in the north, northeast, and central regions, *P. angelica* and *P. gracilis* are found only on the Thai peninsula. Of these species, only *P. polita* is easily differentiated morphologically, the other four species present taxonomic difficulties and can be misidentified (Keawjam, 1986; 1987).

The golden apple snail, *Pomacea canaliculata* (Lamarck, 1822) was illegally introduced into Thailand to clean fish aquaria because of its ability to consume several kinds of aquatic plants and algae, and was first discovered in the wild in 1984 (Keawjam and Upatham, 1990). Because of its short maturation time (2-3 mo) and its potential to lay large numbers of eggs (Cowie, 2002), *Pomacea canaliculata* continues to have an increasing impact on freshwater communities. The severe observed decrease in the *Pila* spp. population may be the result of competition with *Pomacea canaliculata* (Lauhachinda *et al*., 1988). Moreover, the golden apple snail has been reported to have caused serious damage to rice crops since 1996 and it is now regarded as being the most important rice pest in Thailand (Janyapeth and Archawakom, 1999).

The golden apple snails in Thailand exhibit variability in shell color and banding pattern, which ranges from golden yellow to olive yellow and dark olive green, and the snails have numbers of small and large bands. Using shell morphology, male reproductive organ anatomy and allozymes, Keawjam and Upatham (1990) classified the golden apple

snails in Thailand into three different species; *Pomacea canaliculata*, *Pomacea insularum,* and *Pomacea* sp.

The taxonomy of *Pila and Pomacea* snails has been based principally on morphological characteristics. However, external characteristics (e.g. shell morphology) are influenced by habitat and the environment (Keawjam 1986 and 1987). Thus, the two sympatric species may be morphologically similar and misidentified as a single species. On the other hand, allopatric populations inhabiting different habitats may show ecomorphological variations and a questionable species status.

The objectives of this study were to determine the levels of genetic diversity of the introduced (*Pomacea canaliculata*) and the native apple snails (*P. ampullacea*, *P. angelica*, *P. pesmei,* and *P. polita*) in Thailand, and to identify molecular genetic markers capable of facilitating the taxonomic identification of *Pila* snails by using restriction analysis of cytochrome oxidase subunit I (COI). A *Pomacea canaliculata*-specific marker was also developed from 16S rDNA, to differentiate *Pomacea* and *Pila* snails at the early stages of development.

Materials and Methods

Sampling Three hundred and twenty-four apple snails; *Pomacea canaliculata* ($N = 180$), *P. ampullacea* ($N = 28$), *P. angelica* ($N = 27$), *P. pesmei* ($N = 63$) and *P. polita* ($N = 26$) were collected from their respective ranges in Thailand (Fig. 1 and Table 1). The identification of apple snails was carried out according to Keawjam (1986) and Keawjam and Upatham (1990). Foot tissue was dissected out and kept at −80°C until required.

DNA extraction The total DNA of each snail was extracted from a piece of foot tissue using a phenol-chloroform-proteinase K method (Winnepenninckx *et al.*, 1993). DNA concentration was determined spectrophotometrically (Maniatis *et al.*, 1982), and extracted DNA was stored at 4 °C until required.

PCR, restriction enzyme digestion, and agarose gel electrophoresis The mitochondrial cytochrome oxidase subunit I (COI) gene segment of each apple snail (710 bp) was amplified using primers LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer, *et al.*, 1994). Amplification was carried out in a 50 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 100 mM of each dNTP, 2.0 mM $MgCl_2$, 0.5 µM of each primer, 1 unit of DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, finland), and 25 ng of DNA template.

PCR was performed in a thermocycler (OmniGene, Hybaid, Franklin, USA) and consisted of predenaturation at 94°C for 3 minutes followed by 10 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute and extension at 72°C for 1 minute, and an additional 35 cycles at higher stringency using the same conditions except an annealing temperature of 53°C. Final extension was carried out at 72°C for 7 minutes.

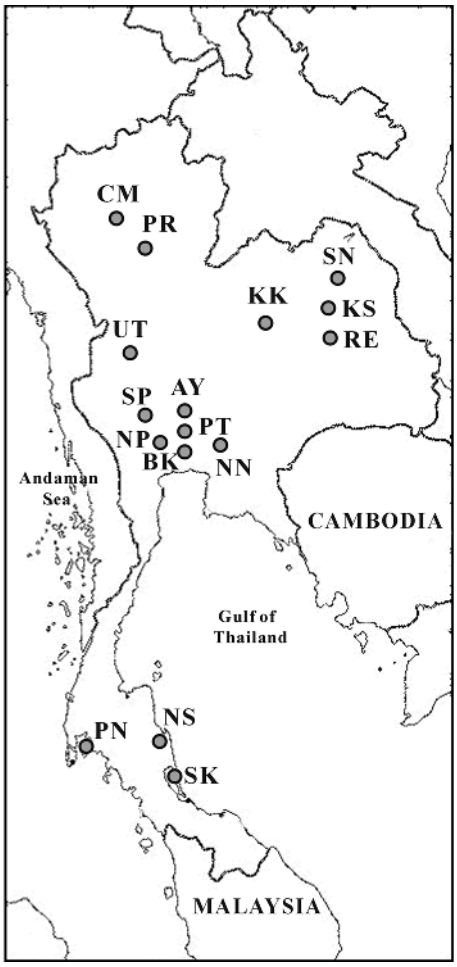

Fig. 1. Map of Thailand indicating the introduced apple snail (*Pomacea canaliculata*) and native apple snail (*P. ampullacea, P. polita*, *P. pesmei* and *P. angelica*) sample collection sites. Dots represent geographic locations from which at least one apple snail species was collected.

Five microliters of the amplified products were size-fractionated through 1.0% agarose gels to determine whether the expected fragment had been successfully amplified. Approximately 200 ng of the amplified COI was separately digested with *Dde* I, *Taq* I, and *Ssp* I. The digests were electrophoretically analyzed using 2.0% agarose gels and visualized under a UV transilluminator after ethidium bromide staining (Maniatis *et al.*, 1982).

Cloning and sequencing of 16S rDNA The 16S rDNA gene segments of native apple snails ($N = 1$ for each species) and of *Pomacea canaliculata* (PcKKNE4 and PcSNNE1) were amplified by PCR using the 16S primers; $16S_F$; 5'-CGC CTG TTT AAC AAA AAC AT-3', and $16S_R$, 5'-CCG GTC TGA ACT CAG ATC ATG T-3' (Palumbi *et al.*, 1991). The 560 bp product was gel-eluted and ligated to pGEM^R-T Easy vector (Hoelzel and Green, 1992). One-tenth of the volume of each ligation reaction was then electrotransformed into *E. coli* JM 109 (Dower *et al.*, 1988). Recombinant clones were selected using a *lac Z* system by following a standard protocol (Maniatis *et al.*, 1982). The insert

Table 1. Sampling collection sites and sample sizes of apple snails used in this study

Species	Localities	Abbreviation*	Sample size (*N*)
Pomacea canaliculata	Chiangmai	PcCMN	15
	Phrae[a]	PcPRN	11
	Khonkhen[b]	PcKKNE	13
	Sakhonnakhon[c]	PcSNNE	13
	Taling Chun[d]	PcTCBK	5
	Kasetsart University	PcKUBK	12
	Nakhonnayok[e]	PcNNC	9
	Nakhonpathom[f]	PcNPC	12
	Suphanburi	PcSPC	20
	Pathumthani	PcPTC	18
	Ayutthaya[g]	PcAYC	18
	Uthaithani	PcUTC	9
	Songkhla[h]	PcSKPT	13
	Phangnga[I]	PcPNPT	12
P. ampullacea	Don Maung	PaDMBK	5
	Taling Chun[d]	PaTCBK	9
	Nakhonnayok[e]	PaNNC	11
	Nakhonpathom[f]	PaNPC	3
P. polita	Sakhonnakhon[c]	PoSNNE	12
	Phrae[a]	PoPRN	10
	Phangnga[I]	PoPNPT	4
P. pesmei	Khonkhen[b]	PeKKNE	12
	Sakhonnakhon[c]	PeSNNE	10
	Roiet	PeRENE	11
	Kalasin	PeKSNE	9
	Nakhonpathom[f]	PeNPC	10
	Ayutthaya[g]	PeAYC	11
P. angelica	Songkhla[h]	PanSKPT	10
	Nakhonsithammarat	PanNSPT	6
	Phangnga[I]	PanPNPT	11

*Species names (Pc, Pa, Po, Pe or Pan) are followed by two capital letters to illustrate names of localities and one or two capital letters to reveal the regions (N, NE, C or PT).
Abbreviations: Pc , *Pomacea canaliculata*; Pa, *P. ampullacea*; Po, *P. polita*; Pe, *P. pesmei*; Pan, *P. angelica*; N, north; NE, northeast; C, Central region; PT, peninsular Thailand. Identical superscripts indicate that more than one species are co-occurred in that locality.

sizes were verified by colony PCR (Srisuparbh *et al*., 2003). Each insert was sequenced in both directions using an automated DNA sequencer (LI-COR, Lincoln, USA).

Primer design, species-specificity, and sensitivity tests The nucleotide sequences of 16S rDNA were aligned using Clustal W (Thompson et al., 1994), and species-specific primers for *Pomacea canaliculata* were designed using OLIGO 4.0 (National Biosciences Inc.). A specificity test was carried out against the genomic DNA of *Pomacea canaliculata* ($N = 131$) and of non-target species ($N = 32$, 6, 30, and 14 for *P. ampullacea*, *P. angelica*, *P. pesmei,* and *P. polita*, respectively).

PCR was performed in a 25 µl reaction volume using the reaction components described above. Thermal cycling involved predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min. Final extension was performed at 72°C for 7 min. The amplified product was analyzed electrophoretically in 1.6% agarose gels. The sensitivity of detection was determined using varying concentrations of *Pomacea canaliculata* genomic DNA (12.5 pg to 25 ng) using the conditions used for the species specificity test. The amplifications of total DNA (50 pg and 25 ng DNA template) isolated from a fertilized egg and from juveniles of *Pomacea canaliculata* 1, 7, or 15 days old were also examined.

Data analysis Restriction profiles of COI were alphabetically coded in order of appearance. Each apple snail was assigned a three letter code (that of COI digested with *Dde* I, *Taq* I and *Ssp* I, respectively) to describe the composite haplotypes. The genetic distance between mtDNA composite haplotype (d_{mtDNA}), haplotype diversity (h), and nucleotide diversity within samples (π), and nucleotide divergence between samples (d_A) were calculated (Nei and Li, 1979; Nei and Tajima, 1981; Nei, 1987) using REAP (McElroy *et al.*, 1991). A neighbor-joining tree (Saitou and Nei,

1987) based on the percent sequence divergence between pairs of composite haplotypes was constructed using Neighbor in PHYLIP (Felsenstein, 1993). Geographic heterogeneities of allele distribution frequencies were analyzed (Guo and Thompson, 1992). F_{ST} values between pairs of samples within species were tested to determine whether they differed significantly from zero (Weir and Cockerham, 1984) using Arlequin (Schneider *et al.*, 1997). The significance levels of multiple test results were adjusted following the sequential Bonferroni method (Rice, 1989).

Results

PCR-RFLP The COIs of the introduced (*Pomacea canaliculata*) and of the native apple snails (*P. ampullacea, P polita, P. pesmei* and *P. angelica*) were subjected to restriction analysis using *Dde* I, *Taq* I, and *Ssp* I and generated 11, 7, and 2 digestion profiles, respectively (Fig. 2 and Table 2). *Taq* I-digested COI provided a species-specific RFLP profile (D) for *P. ampullacea*. Moreover, *Pomacea canaliculata*, *P. ampullacea,* and *P. angelica* were unambiguously discriminated by digesting COI with *Dde* I (Table 2).

In total, 21 composite haplotypes were found. No composite haplotypes were shared among species. Six composite haplotypes (DAA, AAB, DAB, AFB, DFA, and IAA) were observed in *Pomacea canaliculata* whereas three (EDA, KDA, and JDA), one (BAA), seven (BBA, BCA, CBA, CCA, HCA, CGA, and CEA) and four (GBA, GCA, FBA, and FCA) were observed in *P. ampullacea, P polita, P. pesmei* and *P. angelica*, respectively (Table 3). The genetic distance between pairs of composite haplotypes ranged between 0.41-13.84%.

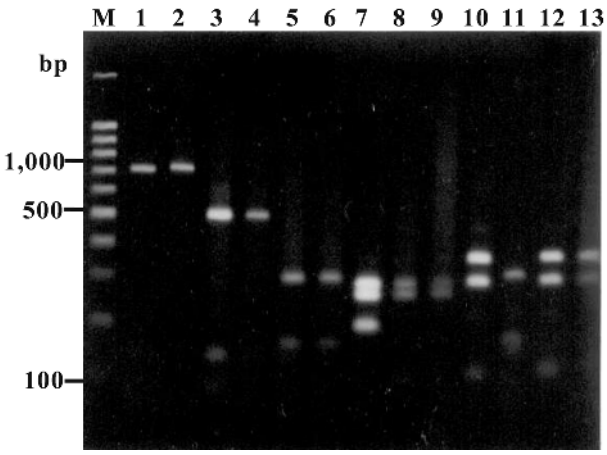

Fig. 2. Example of RFLP analysis of the amplified *Dde* I-digested COI of the apple snails with restriction pattern A (lanes 10, 12, 13), B (lanes 3-4), D (lanes 8-9), E (lanes 5-6), I (lane 7) and K (lane 11). Lanes 1 and 2 are of undigested COI gene segment. An 100 bp ladder (lane M) was used as a marker.

Haplotype diversity and nucleotide diversity within samples

Table 2. Restriction fragment patterns resulted from digestion of COI of *Pomacea canaliculata, P. ampullacea*, *P. polita*, *P. pesmei and P. angelica* with restriction endonucleases

Enzyme	Pattern observed (bp)	Species				
		Pc	Pa	Po	Pe	Pan
Dde I	A: 350, 275, 100	+	-	-	-	-
	B: 500, 140, 90	-	-	+	+	-
	C: 400, 140, 90	-	-	-	+	-
	D: 275, 260, 100	+	-	-	-	-
	E: 300, 160, 140, 90	-	+	-	-	-
	F: 410, 250, 90	-	-	-	-	+
	G: 680	-	-	-	-	+
	H: 410, 140, 90	-	-	-	+	-
	I: 275, 260, 200	+	-	-	-	-
	J: 325, 200, 100	-	+	-	-	-
	K: 325, 160, 140	-	+	-	-	-
Taq I	A: 640, 120	+	-	+	-	-
	B: 550, 120	-	-	-	+	+
	C: 550, 120, 110	-	-	-	+	+
	D: 350, 225, 130	-	+	-	-	-
	E: 425, 130, 120	-	-	-	+	-
	F: 640, 100	+	-	-	-	-
	G: 310, 280, 120	-	-	-	+	-
Ssp I	A: 710	+	+	+	+	+
	B: 490,220	+	-	-	-	-

+, found in investigated species.

Table 3. Distribution frequencies of composite haplotypes of the introduced golden apple snail (*Pomacea canaliculata*) and native apple snails (*P. ampullacea*, *P. polita*, *P. pesmei* and *P. angelica*) based on restriction analysis of COI with *Dde* I, *Taq* I, and *Ssp* I

Sample	Composite haplotype																					
	DAA	AAB	DAB	AFB	DFA	IAA	EDA	KDA	JDA	BAA	CBA	CCA	HCA	CGA	CEA	BBA	BCA	GBA	GCA	FBA	FCA
Pomacea canaliculata																					
PcCMN	3	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcPRN	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcKKNE	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcSNNE	-	8	4	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcTCBK	3	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcKUBK	3	7	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcNNC	2	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcNPC	2	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcSPC	-	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcPTC	7	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcAYC	3	14	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcUTC	5	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcSKPT	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PcPNPT	-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. ampullacea																					
PaDMBK	-	-	-	-	-	-	3	-	2	-	-	-	-	-	-	-	-	-	-	-	-
PaTCBK	-	-	-	-	-	-	8	1	-	-	-	-	-	-	-	-	-	-	-	-	-
PaNNC	-	-	-	-	-	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PaNPC	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. polita																					
PoSNNE	-	-	-	-	-	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-	-
PoPRN	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-
PoPNPT	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-
P. pesmei																					
PeKKNE	-	-	-	-	-	-	-	-	-	-	7	5	-	-	-	-	-	-	-	-	-
PeSNNE	-	-	-	-	-	-	-	-	-	-	3	3	4	-	-	-	-	-	-	-	-
PeRENE	-	-	-	-	-	-	-	-	-	-	5	6	-	-	-	-	-	-	-	-	-
PeKSNE	-	-	-	-	-	-	-	-	-	-	2	4	-	1	2						
PeNPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	1	-	-	-	-
PeAYC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	5	-	-	-	-
P. angelica																					
PanSKPT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	2	-	-
PanPNPT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	5
PanNSPT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6

of *Pomacea canaliculata* varied between 0.00-0.70 and 0.00-1.96% with overall diversities of 0.35 and 0.98%, respectively. Within *Pila* snails, *P. polita* and *P. ampullacea* exhibited lower genetic diversities than *P. pesmei* and *P. angelica* (Table 4). A large RFLP divergence was found between *Pomacea canaliculata* and *Pila* species (5.06-12.47%). The lowest nucleotide divergence among these species was 1.61% (*P. angelica* and *P. pesmei*).

A neighbor-joining tree constructed on the basis of genetic distance among pairs of composite haplotypes (Fig. 3), revealed clear species differentiation. Geographic heterogeneity analysis indicated significant differences between the composite haplotype distributions of the species ($P < 0.0001$). Of the *Pomacea canaliculata* samples, 91 (the exact test) and 84 (F_{ST} statistics) of 91 possible comparisons were not significantly different after applying the sequential Bonferroni procedure ($P > 0.0036$). Within *Pila*, the *P. ampullacea* ($P = 0.0881 - 1.000$ and $P = 0.0824 - 1.000$ for the exact test and the F_{ST} estimate, respectively) and the *P. polita* ($P = 1.0000$ for both analyses) gene pools were panmictic. In contrast, population differentiation was observed between *P. pesmei* originating from the northeast and central regions ($P < 0.0001$ for both analyses). Likewise, geographic population differentiation of *P. angelica* was observed

Table 4. Haplotype and nucleotide diversity within samples of *Pomacea canaliculata, P. ampullacea, P. pesmei, P. polita* and *P. angelica* determined by restriction analysis of COI

Sample	Haplotype diversity ($h \pm$ SE)	Nucleotide diversity (×100)
Pomacea canaliculata		
PcCMN	0.34 ± 0.13	1.04
PcPRN	0.00 ± 0.00	0.00
PcKKNE	0.00 ± 0.00	0.00
PcSNNE	0.56 ± 0.11	0.55
PcTCBK	0.70 ± 0.22	1.64
PcKUBK	0.62 ± 0.12	1.96
PcNNC	0.39 ± 0.16	1.18
PcNPC	0.30 ± 0.15	0.92
PcSPC	0.00 ± 0.00	0.00
PcPTC	0.50 ± 0.06	1.53
PcAYC	0.39 ± 0.13	1.24
PcUTC	0.56 ± 0.09	1.69
PcSKPT	0.00 ± 0.00	0.00
PcPNPT	0.00 ± 0.00	0.00
Overall	**0.35 ± 0.04**	**0.98**
P. ampullacea		
PaDMBK	0.60 ± 0.18	2.35
PaTCBK	0.22 ± 0.17	0.30
PaNNC	0.00 ± 0.00	0.00
PaNPC	0.00 ± 0.00	0.00
Overall	**0.20 ± 0.10**	**0.62**
P. polita		
PoSNNE	0.00 ± 0.00	0.00
PoPRN	0.00 ± 0.00	0.00
PoPNPT	0.00 ± 0.00	0.00
Overall	**0.00 ± 0.00**	**0.00**
P. pesmei		
PeKKNE	0.53 ± 0.08	0.22
PeSNNE	0.73 ± 0.08	0.69
PeRENE	0.51 ± 0.08	0.21
PeKSNE	0.78 ± 0.11	1.29
PeAYC	0.55 ± 0.07	0.23
PeNPC	0.20 ± 0.15	0.08
Overall	**0.79 ± 0.02**	**0.90**
P. angelica		
PanSKPT	0.36 ± 0.16	0.16
PanNSPT	0.55 ± 0.07	0.23
PanPNPT	0.00 ± 0.00	0.00
Overall	**0.68 ± 0.08**	**1.50**

between PanSKPT-PanNSPT (P = 0.0004 for both analyses) and PanSKPT-PanPNPT (P < 0.0001 for both analyses) but not between PanNSPT-PanPNPT (P = 0.0375 and P = 0.0053).

Species-specific PCR The length of the amplified 16S rDNA of the native apple snails (564-569 bp) was larger than that of *Pomacea canaliculata* (546 bp and 549 bp).

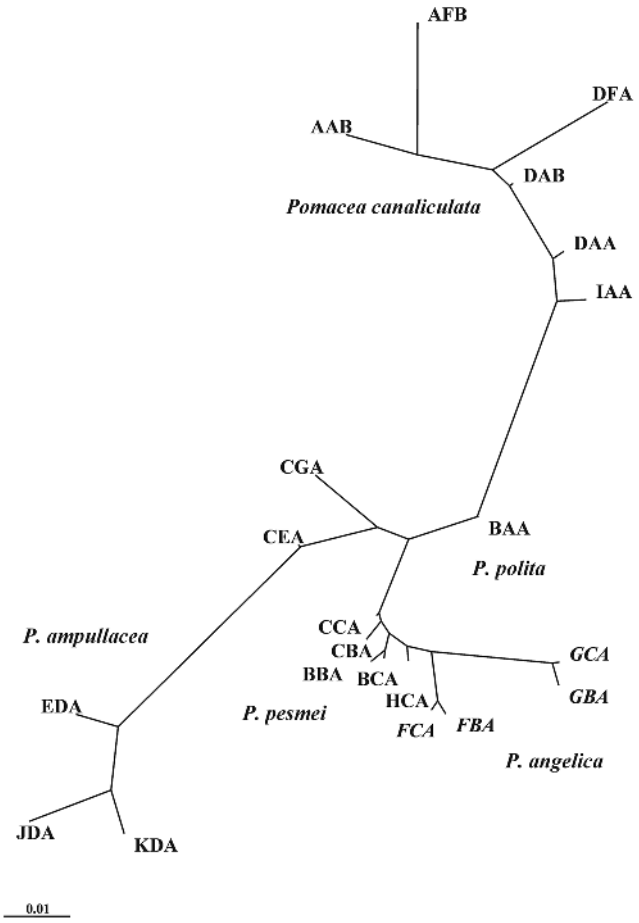

Fig. 3. A neighbor-joining tree summarizing the genetic relationships of *Pomacea canaliculata*, *P. ampullacea*, *P. polita*, *P. pesmei,* and *P. angelica*; constructed using the average genetic distance between pairs of composite haplotypes.

Divergence between the 16S rDNA sequences of *Pomacea canaliculata* and *Pila* species was 16.88-19.60% (calculated from the aligned sequences in Fig. 4). Within *Pila*, lower sequence divergences were found (6.53-11.54%). A species-specific PCR product (KUPC1, 211 bp) was successfully identified for *P. canaliculata* (Fig. 5). The sensitivity of detection was approximately 50 pg of the target genomic DNA template. Amplification of genomic DNA (50 pg and 25 ng) isolated from fertilized eggs and juveniles (1, 7 and 15 d old) was also consistently successful.

Dicussion

RFLP analysis and species-diagnostic composite haplotypes in apple snails Based on a single enzyme digestion, *Dde* I-digested COI could differentiate *Pomacea canaliculata* (patterns A, D and I) from native *Pila* species accurately. In addition, the non-overlapped composite haplotypes of the apple snails allows simple and reliable species identification.

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P.ampullacea    CCGGTCTGAACTCAGATCATGTAAAATT-TTAATGGTCGAACAGACCAACCCTCNNAAGC
P.pesmei        CCGGTCTGAACTCAGATCATGTAAAATT-TTAATGGTCGAACAGACCAACCCTTAAGAAC
P.polita        CCGGTCTGAACTCAGATCATGTAAAATT-TTAATGGTCGAACAGACCAACCATTAAGAGC
P.angelica      CCGGTCTGAACTCAGATCATGTAAAATT-TCAATGGTCGAACAGACCAACCCTTAAAAGC
PcKKNE4         CCGGTCTGAACTCAGATCATGTAGAATTATTAATGGTCGAACACACCAACCCTCAAAAGC
PcSKPT1         CCGGTCTGAACTCAGATCATGTAGAATTATTAATGGTCGAACAGACCAACCCTCAAAAAC
                ********************** **** * ******************** *    * *

P.ampullacea    TTCTACACCCTTAGGA-TATTTTAATCCAACATCGAGGTCACAAACCTTT-CTTTCGATT
P.pesmei        TTCTACACCCTTAGGA-TATTTTAATCCAACATCGAGGTCACAAACCTTT-CTTTCGATT
P.polita        TTCTACACCCTTGGGAATATTTTAATCCAACATCGAGGTCACAAACCTTTTCTTTCGATT
P.angelica      TTCTACACCCTTAGGA-TATTTTAATCCAACATCGAGGTCACAAACCTTT-CTTTCGATT
PcKKNE4         TTCTGCACCTTTAGGG-TATTCTAGTCCAACATCGAGGTCACAATCCTCT-CTTTCGATT
PcSNNE1         TTCTGCACCTTTAGGA-TATTCTAGTCCAACATCGAGGTCACAATCCTTT-CTTTCGATT
                **** **** ** **  **** ** ******************** *** * *********

P.ampullacea    AGAACTCTTAAGATAGATATGCTGTTATCCCTATGGTAACTTATTCTATTAATCAAAAT
P.pesmei        AGAACTCTTAAGATAGATATGCTGTTATCCCTATGGTAACTTATTCTATTAATCAAAAC
P.polita        AGAACTCTTAAGATAGATATGCTGTTATCCCTATGGTAACTTATTCTATTAATCAAAAT
P.angelica      AGAACTCTTAAGATAGATATGCTGTTATCCCTATGGTAACTTATTCTATTAATCAAAAT
PcKKNE4         AGAACTCCTAAGAAAGATATGCTGTTATCCATATGGTAACTAATTCTGATAATCAAAAA
PcSNNE1         AGAACTCCTAAGAAAGATATGCTGTTATCCCTATGGTAACTAATTCTGATAATCAAAAA
                ******* ***** ***************** ********** *****  *********

                                        KUPC1F
P.ampullacea    ATTTTGGATC-AATTTAAATAAATCAATAGTAAATACTACAAAAACTCTGGAAGTTTTAT
P.pesmei        ATTTTGGATC-AGTTTAAATAAGCTAATATTAAATATTATAAAA-CTCTGGAAGTTTCAT
P.polita        ATTTTGGATCCAATTCAAATAAATTAATACTAAATATTATAAAA--TCTGGAAGTTTTGT
P.angelica      ATTTTGGATCAAATTTAAATAAATTAATAACAAATAATTCGGTA--TATCGAAGTTTTAC
PcKKNE4         ATTTTGGATAAAA--CATA-AGAGTAACCTAA-TACTA----------GGAAGTTTTTT
PcSNNE1         ATTTTGGATAAAAAACATA-AAAGTAACTTTAG-TACTA----------GGAAGTTTTTT
                ********  *    * * *    ***   *  ** *           ********

P.ampullacea    TTGTTCCAATAGTCGCCCCAACTAAAAAGTT--AACAACCTATAATCTCATT-TTAATA
P.pesmei        TTGTTCCAATAGTCGCCCCAACTAAAAACTT--AATAACCTATAATCTTATT-CTAATA
P.polita        TTGTTCCAATAGTCGCCCCAACTAAAAAATTT-AATAACTCATAATTTTATTCTTAATA
P.angelica      CTATTCCA-TAGTCGCCCCAACTAAAAAGATTATAGTAATTTGAAATTTTGTTTTAAATA
PcKKNE4         TGATTCCTA-AGTCGCCCCAACTAAAAATTTA--AAAAGATTAGAATTCTCAT----ATA
PcSNNE1         TGATTCCTA-AGTCGCCCCAACTAAAAATTTA--AAAAGATTAGAATTTTTAT----ATA
                   ****   ******************  *   *  *       ***     *   ***

P.ampullacea    TAATTTATGTTGTTATAAATCCTTAAAGCTCAATAGGGTCTTCCTGTCTAATAGTGAAAT
P.pesmei        CATCGTATGCTATTATAGATCCTTAAAGCTCAATAGGGTCTTCCTGTCTATTAGTAAAAT
P.polita        TAATTCATGTCATTATAAATCTCTTAAGCTCAATAGGGTCTTCCTGTCTATTAGTAACAT
P.angelica      TAATTTATATTGCTATAGATTTTTAAAGCTCAATAGGGTCTTCCTGTCTATTAGTAATAT
PcKKNE4         TACCTTACTCCATAATAC-TTTTATAAGCTCAATAGGGTCTTCCTGTCTCTTAGTCATAT
PcSNNE1         TACTTTACTCTATAATAC-TTTTATAAGCTCAATAGGGTCTTCCTGTCTTTTAGTTATAT
                 *   *       ***  * *   ************************  **** * **

P.ampullacea    TTAAGCCTTTACACTTAAAAGTCAATTTCATTTTAATTTATAAGAGACAGGCTAATTCTC
P.pesmei        TTAAGCCTTTACACTTAAAAGTCAATTTCATTTTAATTTATAAGAGACAGGCTAATTCTC
P.polita        TTAAGCCTTTGCACTTAAAAGTCAATTTCATTTTAGTTAGTAAGAGACAAACTAATTCTC
P.angelica      TCAGGCCTTTGCACCTGAAGATCAATTTCATTTTAATTTGTAAAAGACAGGCTAATTCTT
PcKKNE4         ATAAGCCTTTGCACTTATAAGTTAATTTCATCTTATACAAAAAAAGACAGCCTAACTTTC
PcSNNE1         ATAAGCCTTTGCACTTATAAGTTAATTTCATTTTACACAAAAAAAGACAGCTTAACTTTC
                  * ****** *** *  *  * ******** ***      ** *****   *** * *
                                KUPC1R
P.ampullacea    GTCAAACCATTCATACAAGCCTTTAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
P.pesmei        GTCAAACCATTCATACAAGCCTTCAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
P.polita        GTTAAACCATTCATACGAGCCTTCAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
P.angelica      GTTAAACCATTCATACAAGCCTTCAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
PcKKNE4         GTTAAACCTCTCATACTAGCCTTCAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
PcSNNE1         GTCAAACCTTTCATACTAGCCTTCAATTATAAGGCAAATGATTATGCTACCTTTGCACGG
                ** *****  ****** ****** ********************************

P.ampullacea    TCAGAGTACCGCGGCCGTTAAA-TCTTTATCACTGGGCAGGTCAGGCTCTTTATGTA--T
P.pesmei        TCAGAGTACCGCGGCCGTTAAAATATATCACTGGGCAGGTTAGACTCTTTATAAAA-T
P.polita        TCAGAGTACCGCGGCCGTTAAA-TAAACATCACCGGGCAGGTTAGACTCTTTATAAAAAT
P.angelica      TCAAAGTACCGCGGCCGTTAAA---AAAATCACTGGGCAGGTTAGACTCTTTATATTAAC
PcKKNE4         TCAAAGTACCGCGGCCGTTAAA--AAAA-TCACTGGGCAGGCTAGACTCTTTATAATT-A
PcSNNE1         TCAAAGTACCGCGGCCGTTAAA--AAAAATCACTGGGCAGGCCAGACTCTTTATAATT-A
                *** ******************       **** *******  ** ********

P.ampullacea    CAGTANNAAGAGACATGTTTTTGTTAAACAGGCG
P.pesmei        AAAAACAAAGAGACATGTTTTTGTTAAACAGGCG
P.polita        ATAAACAAAGACACATGTTTTTGTTAAACAGGCG
P.angelica      GTAAACAAAGAGACATGTTTTTGTTAAACAGGCG
PcKKNE4         ATAAACAAAGAGCCATGTTTTTGTTAAACAGGCG
PcSNNE1         ATAAGCAAAGAGCCATGTTTTTGTTAAACAGGCG
                     ***** **********************
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Fig. 4. Sequences of 16S rDNA of *P. ampullacea*, *P. pesmei*, *P. polita*, *P. angelica*, and *Pomacea canaliculata* (PcKKNE4 and PcSNNE1). The locations and sequences of *Pomacea canalicalata*-specific forward primer (KUPC1F), and those complementary to a reverse primer (KUPC1R) appear in boldface and underlined.

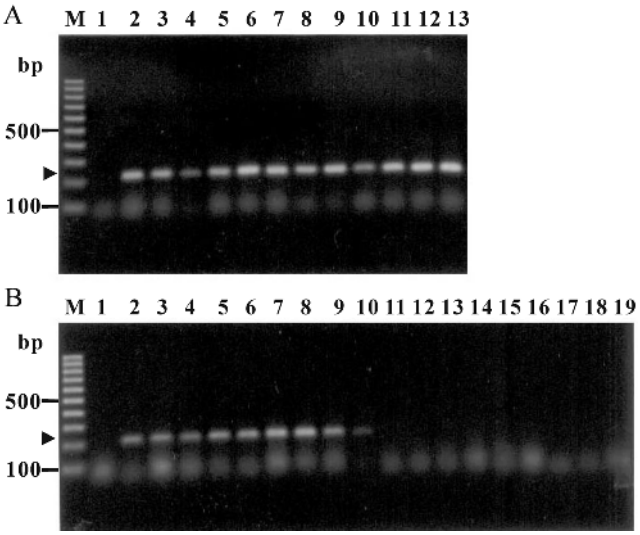

Fig. 5. Agarose gel electrophoresis showing the specificity of KUPC1F/R primers to the genomic DNA of *Pomacea canaliculata* (panels A lanes 2-13 and B lanes 2-10), *P. ampullacea* (B, lanes 11-12), *P. pesmei* (B, lanes 13-15), *P. polita* (B, lanes 16-17) and *P. angelica* (B, lanes 18-19). Lane 1 contains negative controls (without *Pomacea canaliculata* DNA). Arrowheads indicate a 211 bp PCR product. A 100 bp ladder (lanes M) was used as the DNA marker.

Brandt (1974) and Keawjam (1986) reported that *P. polita* is abundantly distributed in the north, northeast, and central regions of Thailand, but it is not found on the Thai peninsula. However, we found apple snails with the external characteristics of *P. polita* in a number of locations on the peninsula. Specimens from Phangnga ($N = 4$) were included in the analysis, and these possessed a BAA composite haplotype, which is found only in *P. polita*. This is the first report of *P. polita* on the Thai peninsula.

Only one or two common composite haplotypes were found in most populations of the apple snails. Non-overlapping distribution patterns of COI composite haplotypes of apple snails suggested a lack of hybridization at both the intergeneric and interspecific levels. Practically, copulation between male *Pomacea canaliculata* and female *P. pesmei* under laboratory conditions is possible but the females did not subsequently lay eggs (C. Janyapeth, personal communication). Nevertheless, the possibility of hybridization and gene introgression between these taxa cannot be ignored unless the nuclear genetic markers of these snails are scrutinized.

Although only a limited number of native apple snails were analyzed, specimens were collected from most parts of the species ranges and should have been representative. We did not include *Pila gracilis* in the present study, because it was not found, although this apple snail has been reported in provinces below Phangnga (Keawjam, 1986).

Genetic diversity and the differentiation of apple snails It has been reported that the cytochrome b (cyt*b*) gene accumulates nucleotide changes at a rate that is sufficient to resolve phylogenetic relationships among closely related species (Collins *et al*., 1996). However, when Tinti *et al*., (2002) analyzed the genetic stock structure of the sardine (*Sardina pilchardus*) originating from the Adriatic (10 geographic sample, $N = 285$) and Ionian Seas (1 geographic sample, $N = 22$) by sequencing a 307-bp cyt*b* gene segment, they found a lack of intraspecific genetic subdivision among

Table 5. Geographic heterogeneity analysis and F_{ST} estimate between pairs of geographic samples of native apple snails (*P. ampullacea*, *P. polita*, *P. pesmei*, and *P. angelica*) in Thailand

Species	Geographic heterogeneity analysis	F_{ST} estimate
P. ampullacea		
PaDMBK-PaTCBK	P = 0.1197^{ns}	P = 0.1120^{ns}
PaDMBK-PaNNC	P = 0.0881^{ns}	P = 0.0824^{ns}
PaDMBK-PaNPC	P = 0.4704^{ns}	P = 0.4575^{ns}
PaTCBK-PaNNC	P = 0.4475^{ns}	P = 0.4560^{ns}
PaTCBK-PaNPC	P = 1.0000^{ns}	P = 1.0000^{ns}
PaNNC-PaNPC	P = 1.0000^{ns}	P = 1.0000^{ns}
P. polita		
PoSNNE-PoPRN	P = 1.0000^{ns}	P = 1.0000^{ns}
PoSNNE-PoPNPT	P = 1.0000^{ns}	P = 1.0000^{ns}
PoPRN-PoPNPT	P = 1.0000^{ns}	P = 1.0000^{ns}
P. pesmei		
PeKKNE-PeSNNE	P = 0.0861^{ns}	P = 0.1394^{ns}
PeKKNE-PeRENE	P = 0.6890^{ns}	P = 0.6806^{ns}
PeKKNE-PeKSNE	P = 0.1264^{ns}	P = 0.2746^{ns}
PeKKNE-PeNPC	P < 0.0001*	P < 0.0001*
PeKKNE-PeAYC	P < 0.0001*	P < 0.0001*
PeSNNE-PeRENE	P = 0.0830^{ns}	P = 0.1402^{ns}
PeSNNE-PeKSNE	P = 0.1135^{ns}	P = 0.1886^{ns}
PeSNNE-PeNPC	P < 0.0001*	P < 0.0001*
PeSNNE-PeAYC	P < 0.0001*	P < 0.0001*
PeRENE-PeKSNE	P = 0.2547^{ns}	P = 0.4122^{ns}
PeRENE-PeNPC	P <0.0001*	P < 0.0001*
PeRENE-PeAYC	P <0.0001*	P < 0.0001*
PeKSNE-PeNPC	P < 0.0001*	P < 0.0001*
PeKSNE-PeAYC	P < 0.0001*	P < 0.0001*
PeNPC-PeAYC	P = 0.1451^{ns}	P = 0.0641^{ns}
P. angelica		
PanSKPT-PanNSPT	P < 0.0004*	P < 0.0004*
PanSKPT-PanPNPT	P < 0.0001*	P < 0.0001*
PanNSPT-PanPNPT	P = 0.0375^{ns}	P < 0.0053*

Significance levels were adjusted following the sequential Bonferroni procedure (Rice, 1989).

geographic samples. Conversely, we found both inter- and intraspecific genetic heterogeneity among indigenous oysters in Thailand based on the COI polymorphism (Klinbunga *et al.*, 2003). This gene segment was also successfully used to identify genetic differentiation and biased female gene flow in the giant tiger shrimp, *Penaeus monodon* (Klinbunga *et al.*, 2001). Therefore, we selected COI rather than cyt*b* for population genetic studies of apple snails in Thailand.

Haplotype and nucleotide diversity within species was relatively low in *Pomacea canaliculata* and *P. ampullacea,* and no COI polymorphism was found in *P. polita*. Higher genetic diversity was found in *P. pesmei* and *P. angelica*.

The estimation of haplotype and nucleotide diversity in the present study is based solely on haplotype frequencies and the band sharing of RFLP patterns (Graves and McDowell, 1994). Although the digestion of COI with 3 restriction enzymes was sufficient for species identification, more restriction enzymes would be needed to provide more accurate estimates of genetic diversity in these taxa.

Pomacea canaliculata was introduced to Thailand only about two decades ago (Keawjam and Upatham, 1990). The conventional rate of evolution for mitochondrial genes is about 1×10^{-8} substitutions per site per year per evolutionary line (Brown *et al.*, 1979). Therefore, the diversity of COI shown by this species is probably the result of multiple introductions to Thailand from different geographic origins rather than by COI substitutions through evolutionary time. In addition, the sequence divergence shown by the 16S rDNA gene segment among representative *Pomacea canaliculata* individuals (PcKKNE4 and PcSNNE1) was 3.74%, further suggesting a time lapse of approximately 3.74 million years since the last common ancestor.

Intraspecific genetic substructuring was observed in *P. pesmei* and *P. angelica*. The former is commonly distributed

in central and northeast Thailand but is rare in the north and absent from the Thai peninsula (Keawjam, 1986). RFLP analysis of COI indicates fragmentation of the *P. pesmei* gene pool into the northeast (PeKKNE, PeSNNE, PeRENE and PeKSNE) and the central (PeAYC and PeNPC) groups ($P < 0.0001$). Likewise, differentiation within *P. angelica* (PeSKPT -PeNSPT, $P < 0.0001$ and PeSKPT-PePNPT, $P = 0.0004$) was also observed.

Both *Pomacea canaliculata* and *Pila* snails are natural hosts of *Angiostrongylus cantonensis* (Chen, 1935), which causes eosinophilic meningoencephalitis in humans (Witoonpanich *et al.*, 1991; Keawjam *et al.*, 1993). Pipitgool *et al.* (1997) surveyed *Angiostrongylus* infection in snails collected from northeast Thailand and found 0.0% and 0.9% infection prevalences in *P. polita* ($N = 77$) and *P. ampullacea* ($N = 423$), respectively. Using RFLP markers, the species-origins of apple snails can be identified unambiguously, therefore, the parasite prevalence can be investigated in more detail (*i.e.,* the determination of host species, the evaluation of the susceptibilities of species to parasites, and the determination of relationships between the distributions of the different snail species and the prevalences of *A. cantonensis*). Moreover, the existence of population differentiation in *P. pesmei* and *P. angelica* allow the prevalence of infection in different snail populations to be evaluated.

Molecular taxonomy of apple snails *Pomacea* apple snails in Thailand have been identified as *Pomacea canaliculata*, *Pomacea insularum,* and *Pomacea* sp. (Keawjam and Upathum 1990). Although *Pomacea* apple snails were genetically examined using 9 monomorphic and 4 polymorphic (*Est*-3, *Hbdh*-1, *Idh* and *Pgm*-2) loci, qualitative data (band patterns) rather than quantitative data (allele frequencies, genetic distances, geographic heterogeneities, and *F* statistics) were reported. Therefore, the results were premature with respect to evaluating the species diversity of *Pomacea* in Thailand. Our results indicate that the gene pool of *Pomacea* snails in Thailand is panmictic ($P > 0.0036$). Accordingly, it should be recognized as a single species rather than 3 different species.

Annadale (1920) recognized seven species and two races of *Pila* snails in Thailand, whereas Brandt (1974) classified these snails into 5 species; *P. ampullacea*, *P. gracilis*, *P. pesmei*, *P. polita,* and *P. scutata,* and placed *P. angelica* under *P. pesmei*. Subsequently, Keawjam (1986) viewed *P. scutata* as a rare non-banded form of *P. gracilis,* but differentiated *P. angelica* from *P. pesmei*. Only *P. polita* can be easily distinguished morphologically, whereas *P. gracilis* can be misidentified as a small form of *P. pesmei,* and *P. angelica* as a large form of *P. pesmei*. In addition, small *P. angelica* is similar to young *P. ampullacea*.

Our results indicate that *P. pesmei* and *P. angelica* should not be regarded as a single species. The phylogenetically close relationship between *P. angelica* and *P. pesmei* supports the taxonomic difficulties reported by earlier morphological and geographic distribution studies. The ability to distinguish native apple snails using diagnostic composite haplotypes of COI resolves taxonomic problems in the genus *Pila* in Thailand.

***Pomacea canaliculata*-specific PCR based on the 16S rDNA polymorphism** A rapid and simple method for differentiating *Pomacea canaliculata* and *Pila* apple snails during the early stages of development is required. Moreover, a marker specific to *Pomacea canaliculata* could allow the detection of its gene introgression, if any, into the gene pools of native *Pila* snails. The ability to identify the species-origin of *Pomacea canaliculata*, without any false positives using as little as 50 pg of genomic DNA extracted from fertilized eggs, juveniles, or adults of *Pomacea canaliculata* demonstrates the high specificity and sensitivity of the KUPC1 marker. Distinguishing between *Pomacea canaliculata* and *Pila* apple snails by species-specific PCR is more cost-effective and convenient than typical PCR-RFLP analysis, and can be used to discriminate these species at the early stages of development.

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