**GENETIC DIVERSITY OF INTRODUCED
(*POMACEA CANALICULATA*)
AND NATIVE (*PILA*) APPLE SNAILS IN THAILAND
REVEALED BY RANDOMLY AMPLIFIED
POLYMORPHIC DNA (RAPD) ANALYSIS**

B. Thaewnon-ngiw

Program of Bioscience, Graduate School of Kasetsart University,
Bangkok 10900, Thailand

S. Klinbunga*

Marine Biotechnology Research Unit, National Center for
Genetic Engineering and Biotechnology (BIOTEC),
National Science and Technology Development Agency (NSTDA),
113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

K. Phanwichien

Department of Zoology, Faculty of Science, Kasetsart University,
Bangkok 10900, Thailand

N. Sangduen

Department of Genetics, Faculty of Science, Kasetsart University,
Bangkok 10900, Thailand

N. Lauhachinda

Department of General Science Faculty of Science, Kasetsart University,
Bangkok 10900, Thailand

P. Menasveta

Department of Marine Science, Faculty of Science,
Chulalongkorn University, Bangkok 10330, Thailand

Received 21 May 2003, Accepted 25 July 2003

ABSTRACT

Genetic diversity of the introduced golden apple snail, *Pomacea canaliculata* (Lamarck, 1822) and four native apple snails; *Pila ampullacea* (Linnaeus, 1758), *P. angelica* (Annandale, 1920), *P. pesmei* (Morelet, 1889) and *P. polita* (Deshayes, 1830) in Thailand were studied by RAPD analysis. Two hundred and two polymorphic fragments (180-1500 bp in length) were generated across overall investigated samples ($N = 254$) using three informative primers (OPA07, OPB10 and UBC122). The percentages of polymorphic bands were 98.86%, 94.56%, 90.91%, 96.94% and 95.51% for *Pomacea canaliculata*, *P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*, respectively. This indicated high genetic polymorphism of these taxa. A neighbor-joining tree between pairs of geographic samples within *Pomacea canaliculata* suggested a lack of phylogeography in this species. Moreover, candidate species-specific RAPD markers (pKUSCARPILA-F/R) found in *Pomacea canaliculata* (340 bp, OPB10), *P. ampullacea* (640 bp, OPA07), *P. angelica* (380 bp, UBC122) and *Pila* snails (430 bp, OPA07) were cloned and sequenced. Locus-specific primers were designed and tested against the target and non-target species. A 259 bp SCAR marker was found in 95.0% of *Pila* apple snails ($N = 163$) but not in *Pomacea canaliculata* ($N = 30$). Therefore, this SCAR marker could be used in coupling with a *Pomacea canaliculata*-specific RAPD marker to unambiguously differentiate the introduced and native apple snails in Thailand.

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

1. INTRODUCTION

The golden apple snail, *Pomacea canaliculata* (Lamarck, 1822) is found in the new world; South America, Central America, the West Indies and the Southern USA (Pain 1972). It was illegally introduced into Thailand by the fish trade for cleaning fish aquaria because of its ability to consume several kinds of aquatic plants and algae. The golden apple snail was first discovered naturally in Thailand in 1984 (Keawjam and Upatham 1990). Owing to its short period of time to reach maturation stages (2-3 months) and the potential to lay large amounts of eggs, the number of *Pomacea canaliculata* consistently increases affecting ecological impact of the freshwater communities in Thailand.

The golden apple snail exhibits variability of shell colors and banding patterns ranged from golden yellow, olive yellow to dark olive with different 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 3 different species including *Pomacea canaliculata*, *Pomacea insularum* and *Pomacea* sp..

Apple snails indigenous to Thailand were taxonomically recognized as members of the genus *Pila*. Brandt (1974) classified native apple snails in Thailand to *P. ampullacea*, *P. scutata*, *P. gracilis*, *P. pesmei* and *P. polita* based on shell characteristics, color and sculpture and geographic distributions.

Keawjam (1986) revised a taxonomic identification key of indigenous apple snails in Thailand and morphologically recognized these species as *P. ampullacea*, *P. angelica*, *P. gracilis*, *P.*

pesmei and *P. polita*. While *P. angelica* was further differentiated from *P. pesmei*, *P. scutata* was taxonomically identified as a non-banded form of *P. gracilis*. Among these species, only *P. polita* are easily differentiated morphologically whereas other four species exhibit taxonomic difficulties and can be misidentified. For instance, *P. gracilis* can be misidentified as the small form of *P. pesmei* and *P. angelica* can be misidentified as the large form of *P. pesmei*. Apparently, *P. ampullacea*, *P. pesmei* and *P. polita* are sympatrically distributed in the north, north-east and central region whereas *P. angelica* and *P. gracilis* are restrictively found in peninsular Thailand.

Severe decreasing of *Pila* snails may have resulted from ecological competition with *Pomacea canaliculata* (Lauhachinda et al., 1988). Serious destructive damages of rice crops by the golden apple snail have been reported since 1996. It is regarded as the most important pest for rice crops in Thailand at present (Janyapeth and Archawakom, 1999).

Classification of *Pila* and *Pomacea* snails has been carried out based principally on morphological characters. However, the external characteristics (e.g. shell morphology) are influenced by a variety of habitats and environmental conditions (Keawjam, 1986 and 1987). Accordingly, 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 variation and have questionable species status.

The basic information on numbers of species and/or populations in a particular area is of importance for conservation programs (Carvalho and Hauser, 1994). Knowledge on genetic variation levels of apple snails in Thailand is, therefore, important for the construction of appropriate management schemes of these species. However, information about inter- and intraspecific genetic diversity of *Pila* and *Pomacea* snails in Thailand is not available at present.

RAPD-PCR is a conceptually simple technique for examination of genetic diversity of organisms (Williams et al., 1990; Welsh and McClelland, 1990). Typically, a short oligonucleotide primer (e.g. 10-12 mer) with the G+C content > 50% is used therefore, prior knowledge of the genome under investigation is not required. RAPD analysis essentially scans part of the genome containing priming sites close to one another that are located in an inverted orientation. Polymorphism is treated in a dominant fashion (Williams et al., 1990; Weising et al., 1995). RAPD analysis considerably requires less tedious and time-consuming than other molecular genetic techniques (e.g. restriction fragment length polymorphism, RFLP; single-stranded conformation polymorphism, SSCP and amplified fragment length polymorphism, AFLP) based on fragment polymorphism.

The objectives of this study were to determine genetic diversity of the introduced (*Pomacea canaliculata*) and native apple snails (*P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*) in Thailand and to identify molecular genetic markers to assist taxonomic identification of *Pila* and *Pomacea canaliculata* apple snails using RAPD analysis. Candidate genus- and/or species-specific RAPD markers were converted to sequence-characterized amplified region (SCAR) markers through cloning and sequencing. A *Pila*-specific SCAR marker was successfully developed.

2. MATERIALS AND METHODS

Sampling

Two hundred and fifty four individuals of apple snails constituting of the introduced golden apple snail, *Pomacea canaliculata* ($N = 136$) and local apple snails, *P. ampullacea* ($N = 21$), *P. polita* ($N = 25$), *P. pesmei* ($N = 47$) and *P. angelica* ($N = 25$) were collected from geographically different locations in Thailand (Fig. 1 and Table 1). Taxonomic identification of apple snails was carried out according to Brandt (1974), Keawjam (1986) and Keawjam and Upatham (1990).

Figure 1 : Map of Thailand indicating sample collection sites of the introduced (*Pomacea canaliculata*) and native apple snails (*P. ampullacea*, *P. polita*, *P. pesmei* and *P. angelica*) used this study. Dots represent geographic locations from which at least one species were sampling. Detailed information and abbreviations of sample sites are shown in Table 1.

Table 1 : Sample collection sites and sample sizes of apple snails used in this study

Species	Geographic origin	Abbreviation	Sample size (N)
<i>Pomacea canaliculata</i>	Chiangmai	PcCMN	10
	Phrae	PcPRN	8
	Khonkhen	PcKKNE	8
	Sakhonnakhon	PcSNNE	10
	Taling Chun	PcTCBK	3
	Kasetsart University	PcKUBK	9
	Nakhonnayok	PcNNC	4
	Nakhonpathom	PcNPC	11
	Suphanburi	PcSPC	16
	Pathumthani	PcPTC	16
	Ayutthaya	PcAYC	16
	Uthaithani	PcUTC	7
	Songkhla	PcSKPT	11
	Phangnga	PcPNPT	7
<i>Pila ampullacea</i>	Don Maung	PaDMBK	2
	Taling Chun	PaTCBK	9
	Nakhonnayok	PaNNC	8
	Nakhonpathom	PaNPC	2
<i>Pila polita</i>	Sakhonnakhon	PoSNNE	11
	Phrae	PoPRN	10
	Phangnga	PoPNPT	4
<i>Pila pesmei</i>	Khonkhen	PeKKNE	9
	Sakhonnakhon	PeSNNE	9
	Roiet	PeRENE	8
	Kalasin	PeKSNE	5
	Nakhonpathom	PeNPC	7
	Ayutthaya	PeAYC	9
<i>Pila angelica</i>	Songkhla	PanSKPT	9
	Nakhonsithammarat	PanNSPT	6
	Phangnga	PanPNPT	10

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

DNA extraction

Total DNA was extracted from a piece of the foot tissue of each snail using a phenol-chloroform-proteinase K method (Winnepeninckx et al., 1993). DNA concentration was spectrophotometrically determined and further adjusted by a mini-gel method (Maniatis et al., 1982). DNA was stored at 4°C until required.

Randomly amplified polymorphic DNA (RAPD) analysis and agarose gel electrophoresis

Fifty decanucleotide primers were screened for the amplification success against a single individual of each species. RAPD-PCR was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, Triton X-100, 2 mM 0.1% mgCl_2 , 100 mM of each dNTP, 0.4 µM of each primer, 1 unit of DyNAzymeTM II DNA Polymerase (Finnzymes) and 25 ng of DNA template. PCR was performed in a thermal cycler (Omnigene, Hybaid) for 40 cycles including denaturation at 94°C for 10 seconds, annealing at 36°C for 30 seconds and extension at 72°C for 90 seconds. The final extension was performed at 72°C for 5 minutes. Three primers (OPA07, OPB10 and UBC122) were chosen for population genetic studies of apple snails.

RAPD analysis using OPA07 and OPB10 was carried out using the conditions described above whereas amplification using UBC122 were performed for 35 cycles where denaturation and annealing steps were extended to 45 seconds and 60 seconds, respectively. The amplification products were electrophoretically analyzed through 1.6% agarose gels and visualized under a transilluminator after ethidium bromide staining (Maniatis *et al.*, 1982).

Data analysis

Each RAPD fragment is treated as an independent character. Sizes of RAPD bands were estimated by comparing with a 100 bp ladder and recorded in a binary matrix to represent the absence (0) or presence (1) of a particular band.

The similarity index between individuals was calculated by $S_{xy} = 2n_{xy} / n_x + n_y$, where n_x and n_y represent the number of RAPD bands in individuals x and y, and n_{xy} represents the number of shared bands between individuals (Nei and Li, 1979).

Within samples similarity (S_i) is calculated as the average of S_{xy} across all possible comparisons between individuals within a geographic sample.

Between sample similarity corrected by within sample similarity (S_i and S_j of geographic samples i and j, respectively) is also calculated between pairs of individuals across samples i and j using the equation; $S'_{ij} = 1 + S_{ij} - (S_i + S_j)/2$. Genetic distance between paired samples was then calculated as $D'_{ij} = 1 - S'_{ij}$ (Lynch, 1990).

Unrooted Neighbor-joining trees (Saitou and Nei, 1987) were constructed from genetic distances between pairs of geographic samples within *Pomacea canaliculata* and between apple snail species using Neighbor in PHYLIP 3.56c (Felsenstein, 1993).

Development of a *Pila*-specific SCAR marker

Candidate species-specific RAPD markers successfully amplified in >95% of the target species (Fig. 2 and Table 5) were excised from the gel and re-amplified. The eluted product was separately ligated to pGEM-T Easy vector (Promega) overnight at 4°C before transformed into *E. coli* XL1-BLUE (Maniatis *et al.*, 1982). Sizes of inserts were verified by

Figure 2 : Agarose gel electrophoresis illustrating a candidate species-specific marker for *P. ampullacea* (640 bp, panel A lanes 1–3 and 7–9) and *Pila apple snails* (430 bp, panel A lanes 1–9 where lanes 4–6 and 10–12 were *P. pesmei* and *Pomacea canaliculata*, respectively), *Pomacea canaliculata* (340 bp, panel B lanes 7–12 where lanes 1–6 were *Pila apple snails*) and *P. angelica* (380 bp, panel C lanes 6–12 where lanes 1 and 2–5 were *Pomacea canaliculata* and *P. pesmei*, respectively). Arrowheads indicated species-diagnostic RAPD markers described in Table 5.

colony PCR using primers pUC1 (5'-TCCGGCTCGTATGTTGTGTGGA-3') and pUC2 (5'-GTGCTGCAAGGCGATTAAGTTGG-3'). Recombinant clones representing those inserts were unidirectional sequenced using an automated sequencer (LI-COR 4100). Nucleotide sequences were blasted against data in the GenBank using BlastN and BlastX. Significant matching was considered when the probability (E) value was $< 10^{-4}$.

Four pairs of primers (Table 6) were designed using OLIGO 4.0 and tested against representative individuals of the target ($N = 10$) and non-target species ($N = 2-5$). Primers pKURAPDPA2-F/R were further tested against 163 individuals of native snail species ($N = 38, 27, 30$ and 68 for *P. ampullacea*, *P. polita*, *P. angelica* and *P. pesmei*, respectively) and 30 individuals of *Pomacea canaliculata*.

PCR was performed in a 25 μ l reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 100 μ M of each dNTP, 0.2 μ M of each primer, 1.0 unit of DyNAzymeTM II DNA Polymerase (Finnzymes) and 25 ng of DNA template. Amplification was composed of predenaturation at 94°C for 3 minutes followed by 10 cycles of 94°C for 30 seconds, annealing at 65°C-1°C per cycle for 1 minute and extension at 72°C for 1 minute and additional 35 cycles at the annealing temperature of 53°C. The final extension was performed at 72°C for 7 minutes. The resulting product was electrophoretically analyzed through 1.8% agarose gels.

3. RESULTS

Genetic diversity and differentiation of apple snails in Thailand revealed by RAPD analysis

Among 50 investigated primers, 28 primers could amplify genomic DNA of both *Pila* and *Pomacea canaliculata* successfully. Three primers (OPA07, OPB10 and UBC122) exhibiting reproducible and easy scoring results were selected for analysis of genetic diversity and identification of molecular markers of apple snails in Thailand (Fig 2).

High genetic polymorphism was observed in all snail species. A total of 202 RAPD fragments ranging from 180 bp and 1500 bp in length were generated (Table 2). The number of scored

Table 2 : Sequences of RAPD primers, sizes and number of amplified bands and the percentage of polymorphic bands resulted from RAPD analysis of the introduced (*Pomacea canaliculata*) and native (*P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*) apple snails in Thailand using OPA07, POB10 and UBC122

Primers	Sequences	Size-range (bp)	No. of RAPD bands	Polymorphic band (%)
OPA07	GAAACGGGTG	190–1500	67	100
OPB10	CTGCTGGGAC	200–1500	71	100
UBC122	GTAGACGAGC	180–1500	64	100
Overall		180–1500	202	100

Table 3 : Total number of bands, percentage of polymorphic and monomorphic bands of each apple snail revealed by RAPD analysis using primers OPA07, OPB10 and UBC122

Primer No.	<i>Pomacea canaliculata</i> (N= 136)			<i>P. ampullacea</i> (N= 21)			<i>P. angelica</i> (N= 25)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA07	35	35	0	29	32	1	27	25	2
OPB10	28	28	0	33	32	0	24	23	1
UBC122	25	24	1	30	21	3	26	22	4
Total	88	87 (98.86%)	1 (1.36%)	92	87 (94.56%)	5 (5.43%)	77	70 (90.91%)	7 (9.09%)

Primer No.	<i>P. pesmei</i> (N=47)			<i>P. polita</i> (N= 25)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA07	30	29	1	33	32	1
OPB10	31	29	2	32	32	0
UBC122	37	37	0	24	21	3
Total	98	95 (96.94%)	3 (3.06%)	89	85 (95.51%)	4 (4.49%)

bands across all investigated species was 67, 71 and 64 bands for primers OPA07, OPB10 and UBC122, respectively. All of these were polymorphic (found in less than 95% of overall investigated specimens) indicating extremely high genetic diversity levels of apple snails (Table 3).

The average number of bands per primer was 29.33 for *Pomacea canaliculata* and 30.67, 25.67, 32.67 and 29.67 for *P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*, respectively. The percentage of polymorphic bands of respective species was 98.86%, 94.56%, 90.91%, 96.94% and 95.51% (Table 3) indicating that genetic diversity of the introduced and native apple snails with the exception of *P. angelica* was comparable. While *P. angelica* exhibited the highest level of the similarity index within species (0.7184 ± 0.0385), *P. pesmei* showed the lowest within species similarity index (0.5909 ± 0.0232).

The average genetic distance between geographic samples within species across all primers of *Pomacea canaliculata*, *P. ampullacea*, *P. angelica*, *P. pesmei*, and *P. polita* ranged from - 0.0213 - 0.0543, 0.0378 - 0.1622, 0.0546 - 0.2495, 0.0003 - 0.1056 and 0.0174 - 0.0948, respectively. The average genetic distances between different species of apple snails was 0.2527 (*P. polita*-*P. pesmei*) - 0.3540 (*P. polita*-*Pomacea canaliculata*) (Table 4).

Table 4 : Average genetic distances (D'_{ij} , below diagonal) and similarity indices (S'_{ij} , above Subscripts diagonal) between species of apple snails in Thailand based on RAPD analysis using OPA07, OPB 10 and UBC122

	<i>Pomacea canaliculata</i>	<i>P. ampullacea</i>	<i>P. angelica</i>	<i>P. pesmei</i>	<i>P. polita</i>
<i>Pomacea canaliculata</i>	-	0.6629	0.6695	0.6765	0.6460
<i>P. ampullacea</i>	0.3372	-	0.6488	0.7142	0.6505
<i>P. angelica</i>	0.3305	0.3512	-	0.6762	0.6558
<i>P. pesmei</i>	0.3235	0.2859	0.3239	-	0.7473
<i>P. polita</i>	0.3540	0.3495	0.3442	0.2527	-

A neighbor-joining (NJ) tree constructed from the average RAPD genetic distance between pairs of geographic samples within *Pomacea canaliculata* did not show phylogeography in this species (Fig. 3) but divided the introduced *Pomacea canaliculata* into 4 closely related groups; I (PcSPC, PcPTC, PcAYC and PcTCBK), II (PcSNNE, PcPNPT and PcNNC), III (PcUTC, PcCMN, PcNPC and PcKUBK) and IV (PcPRN, PcKKNE and PcSKPT) disregarding their geographic origins. At the interspecific levels, a neighbor-joining tree indicated large genetic differences between apple snail species (Fig. 4).

Figure 3 : A neighbor-joining tree illustrating genetic relationships of *Pomacea canaliculata* in Thailand based on RAPD genetic distance between pairs of geographic samples using primers OPA07, OPB10 and UBC122.

Figure 4 : A neighbor-joining tree illustrating genetic relationships of *Pomacea canaliculata*, *P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita* based on the average RAPD genetic distance between apple snail species using primers OPA07, OPB10 and UBC122.

Development of a sequence-characterized amplified region (SCAR) marker for detection of *Pila* apple snails in Thailand

Although high polymorphic levels were observed from RAPD analysis, candidate genus- and/or species-specific RAPD markers observed in greater than 95.0% of the target apple snail species were also found (Fig. 2 and Table 5). These promising RAPD bands were cloned and further characterized.

Two different recombinant clones from 640 bp (pKURAPDPA1 and pKURAPDPA2) and 340 bp (pKURAPDPC1 and pKURAPDPC2) and a single clone from 380 bp (pKURAPDPAN1)

Table 5 : Candidate species-specific RAPD markers of apple snails in Thailand revealed by RAPD analysis

Primer	RAPD marker	Species/Genus	Specificity in target species (%)
OPB10	340 bp	<i>Pomacea canaliculata</i>	95.0
OPA07	640 bp	<i>P. ampullacea</i>	100
UBC122	380 bp	<i>P. angelica</i>	100
OPA07	430 bp	<i>Pila</i>	96.5

Table 6 : Sequences of locus-specific primers designed from recombinant clones possessing a species-specific RAPD fragment of *Pomacea canaliculata*, *P. ampullacea* and *P. angelica* and *Pila* snails

Clone	Primer sequence	Amplification results
pKURAPDPC1 ^a	ND	ND
PKURAPDPC2 ^a	ND	ND
pKURAPDPA1 (a 640 bp insert)	F: 5'-CCC GCC GTG ACA ACA GTA AG-3' R: 5'-AGA TGA ACT GAG CAG CAG AAA CA-3'	52.63% in <i>P. ampullacea</i> but not in other species
pKURAPDPAN1 (a 380 bp insert)	F: 5'-TCT TTT CTT GTA TTA CTG TGC TAT G-3' R: 5'-GCC TTA CCA GGG TTA GGA TTT A-3'	-
pKURAPDPII (a 430 bp insert)	F: 5'-CAG TCT CAG GAT GGG ATT CAG G-3' R: 5'-TCA GGG TCG TGT TCC GCT TT-3'	-
pKURAPDPA2 ^b (a 640 bp insert)	F: 5'-CCG TGC AAA AGG CAA ACT GAA-3' R: 5'-AGG GAA CGA TGG GTT GGG ATT-A 3'	95% in <i>Pila</i> apple snails but not in <i>Pomacea canaliculata</i>

^a SCAR markers were not developed from these sequences because a simple and reliable PCR-based method for identification of *Pomacea canaliculata* was already developed based on 16S rDNA polymorphism (Thaewnon-ngiw, 2003).

^b Subsequently, pKURAPDPA2-F/R were called as pKUSCARPILA-F/R.

-, amplification was not successful; ND, not determined.

```

GAAACGGGTG GCGAAAAGGA AAAAGGTGGC CTGGAATAAA CAAAGAACCA 50
                pKUSCARPILA-F
CGAGTTATCA CTCAGTGCCG TGCAAAAGGC AAACTGAAAT TCCAGCCACA 100
TAGATCATAA TACTTTAAAT TCATTTTAAA ACAAATACA ATGAGACAAT 150
TTTAAATTCA AACACATTTT ACATTAACAC TAGAACCTTC AACACGCCTA 200
CCACATTAC ATGGGAGACA ACCCCACATG TCGGCAATCA AAATGGCGGC 250
AGCCTCGTGC TTCACATGTG CCTGGTAGGC GAACAAATC CGTTCAAATC 300
                pKUSCARPILA-R
GTTTTTAATCC CAACCCATCG TTCCCTGCAT TTTATATCCC CTACAAGATT 350
TACCCTTAAA CTTTTTATCC TAAAGCATTT TCTTAAAAAG TACTATCTTA 400
TCACCATGAA CATGGCAGGG AAAAAACATC CTAAAAATGC ACAATAGCAT 450
CACGCTTTTC GCTACCATTA CAACATATCT TTTTAGACAC AAGGCAAGTA 500
ACCATTCAAC TAAACANTT AACAAAAACC ATATTCATAC CTGGTTCAAA 550
CCAGACACGC CNTTGTGAG AGCTGTGGCC TTTTCATGCTG AACGCTGAAC 600
TCGAAACTAA ACTTCCAACC GCANGNTCCT CCACCCNNT TTC 643

```

Figure 5 : Nucleotide sequences of pKUSCARPILA (formerly called pKURAPDPA2). The locations and sequences of a *Pila*-specific forward primer (pKUSCARPILA-F) and those complementary to a reverse primer (pKUSCARPILA-R) are labeled in boldface and underlined.

and 340 bp (pKURAPDPI1) inserts were unidirectional sequenced. Results from blasted analysis did not show significant matching of these sequences with any sequence in the GenBank (E-value > 0.0001). Therefore, these RAPD fragments were regarded as unknown sequences.

Primers pKURAPDPAN1-F/R and pKURAPDPI1-F/R did not provide the products even though amplification conditions were further adjusted (e.g. thermal profiles, MgCl₂ concentration). A preliminary result using pKURAPDPA1 primers revealed 100% successful amplification in *P. ampullacea* ($N = 10$). Nevertheless, only 52.63% of overall *P. ampullacea* specimens ($N = 38$) were successfully amplified by this primer set (Table 6).

Surprisingly, pKURAPDPA2-F/R (hereafter called pKUSCARPILA primers) did not only amplify *P. ampullacea* DNA (the amplification success = 35/38 individuals) but also provided the expected amplification product in *P. angelica* (30/30), *P. pesmei* (65/68) and *P. polita* (24/27) but not in *Pomacea canaliculata* ($N = 30$). The amplification success was accounted for 95.09% (155/163) across overall investigated *Pila* apple snails (Fig. 6).

4. DISCUSSION

Genetic diversity of apple snails in Thailand

Using RAPD analysis, genetic diversity of *Pomacea* and *Pila* apple snails in Thailand was elucidated. Molecular genetic markers for differentiation of *Pila* and *Pomacea* apple snails were

Figure 6 : Agarose gel electrophoresis showing specificity of *pKUSCARPILA-F/R* primers against genomic DNA of *Pila* apple snails (lanes 2 - 13) and *Pomacea canaliculata* (lanes 14 - 18). Arrowheads indicate a 259 bp PCR product. Lane 1 was a negative control (without DNA template). A 100 bp ladder (lanes M) was used as the DNA marker.

identified. Simplification for detection of *Pila* apple snails by a PCR-amplified SCAR marker was successfully developed.

All scored RAPD bands generated by each primer across overall species were polymorphic. The average number of polymorphic bands of each species was nearly identical where a lower level of polymorphic bands (90.91%) was observed in *P. angelica*. This suggested the potential of RAPD analysis for determination of inter- and intraspecific genetic differences of apple snails in Thailand. Similar circumstances on high genetic polymorphism detected by RAPD analysis across investigated species were also found in the mud crabs; *Scylla serrata*, *S. oceanica* and *S. tranquebarica* (Klinbunga et al., 2000) and the cupped oysters of genera *Saccostrea*; *S. cucullata*, and *S. forskali* and *Striostrea*; *Striostrea* (*Parastristrea*) *mytiloides* (Klinbunga et al., 2001).

Although limited numbers of native apple snails were included in our experiments, specimens were collected from major parts of their geographic ranges and should have represented the gene pool of a particular species. We did not include *P. gracilis* in the present study because it was not found during sampling collection even though *P. gracilis* was previously reported to be common in the further south of Phangnga (Keawjam, 1986).

Recently, genetic diversity and molecular diagnostic markers of *Pomacea canaliculata* and four native apple snails (*P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*) in Thailand were also studied by PCR-RFLP of cytochrome oxidase subunit I, COI (Thaewnon-ngiw, 2003). Twenty-one mitochondrial composite haplotypes showing non-overlapped distribution between species were found. Geographic heterogeneity analysis indicated significant genetic differences among species ($P < 0.0001$) and within *P. pesmei* ($P < 0.0001$) and *P. angelica* ($P < 0.0004$). Nevertheless, a panmictic gene pool was observed in *Pomacea canaliculata* ($P > 0.05$), *P. ampullacea* ($P = 0.0824 - 1.000$) and *P. polita* ($P = 1.0000$). Neighbor-joining trees constructed from divergence

between pairs of composite haplotypes, geographic samples and species revealed clear differentiation among snail species.

Notably, genetic diversity resulted from RAPD analysis was contradictory to that from PCR-RFLP of COI (Thaewnon-ngiw, 2003). Restriction analysis of COI revealed a lack of genetic polymorphism in *P. polita* but the present study indicated the high level of polymorphism in that species. This should have resulted from the female founder effects in *P. polita*. Moreover, the former approach revealed that *P. angelica* exhibited the highest level among investigated species. Nevertheless, this species showed the lowest diversity level when analyzed by RAPD-PCR. This should have resulted from limited sample sizes of native apple snails in the present study.

Contradictory results on genetic diversity levels of the same investigated samples analyzed by different molecular markers have been previously reported. Sihanuntavong et al. (1999) and Sittipraneed et al. (2001a) examined genetic diversity and population differentiation of the honey bee (*Apis cerana*) in Thailand by restriction analysis of small subunit (s) rDNA, large subunit (lr) rDNA and the intergenic COI-COII with *Dra* I. Low haplotype and nucleotide diversity levels were found in the north-to-central, peninsular Thailand and Phuket Island populations while the highest diversity was observed in bees from Samui Island. Geographic heterogeneity analysis and F_{ST} estimate differentiated *A. cerana* in Thailand into 3 populations including northern Thailand (A), Peninsular Thailand (B) and Samui Island (C). Using microsatellite analysis, high levels of heterozygosity were observed in bees from the north-to-central (north, north-east and central region) and peninsular Thailand ($H_e = 0.52 - 0.59$) but limited heterozygosity was found in the Samui Island sample ($H_e = 0.28$). The north-east sample could be further differentiated from the north and the central regions (Sittipraneed et al., 2001b).

On the basis of RAPD analysis, genetic distance between geographically different samples of *Pomacea canaliculata* was small (-0.3021 - 0.0543) implying low degrees of genetic differentiation in this species. In addition, phylogeny of *Pomacea canaliculata* did not illustrate phylogeography in this species but rather reflected effects of multiple introduction of *Pomacea canaliculata* from different geographic origins to Thailand. The lack of geographic heterogeneity of *Pomacea canaliculata* in Thailand analyzed by COI polymorphism (Thaewnon-ngiw, 2003) supported results from RAPD analysis in this study.

Differentiation between juvenile *Pomacea canaliculata* and *Pila* apple snails based on external morphology is difficult. Moreover, it has been suspected that intergeneric (between *Pomacea canaliculata* and each *Pila* species) and intrageneric (between species within the genus *Pila*) hybridization and gene introgression of apple snails in Thailand may be occurred. Phylogenetic analysis based on RAPD analysis (this study) and restriction analysis of COI (Thaewnon-ngiw, 2003) unambiguously suggested that the occurrence of hybridization and introgression of apple snails in Thailand at both inter- and intrageneric levels was unlikely.

Notably, some pairwise comparisons of genetic distance between *Pomacea canaliculata* and *Pila* apple snails were lower than those within the genus *Pila* implying that genetic distance estimated from RAPD analysis rapidly reached a plateau of divergence among these taxa. This may saturate phylogenetic data of apple snails in this study. Moreover, genetic diversity based

on RAPD analysis relied entirely on sharing of RAPD-amplified fragments after electrophoresis. The possibility of comigration of RAPD fragments having similar sizes but different sequences cannot be excluded (Klinbunga *et al.*, 2001). Therefore, homology of comigrating fragments should be further verified by Southern blotting hybridization.

***Pomacea canaliculata*-specific RAPD and *Pila*-specific SCAR markers**

Several useful genetic markers were identified in this study. An intense 340 bp RAPD fragment was found in approximately 95.0% of overall investigated *Pomacea canaliculata* specimens ($N = 136$) and could be simply used as *Pomacea canaliculata*-diagnostic markers.

Although species-specific RAPD markers of apple snail allow direct examination of snail species, RAPD-PCR is sensitive to reaction conditions due to low reproducibility of this technique (Hadrys *et al.*, 1992). Moreover, RAPD-PCR also requires good quality DNA template for reliable and consistent results which may not be possible for field specimens. This may significantly cause false negative results from positive specimens (Weising *et al.*, 1995).

Concurrently, Thaewnon-ngiw *et al.*, (2003) developed species-diagnostic markers of *Pomacea canaliculata* in Thailand. The 16S rDNA of representative individuals of each apple snail species were cloned and sequenced. Species-specific PCR for *Pomacea canaliculata* was successfully developed ($N = 131$ and 82 for the target and non-target species) with the sensitivity of detection approximately 50 pg of the target DNA template. Amplification of genomic DNA (50 pg and 25 ng) isolated from the fertilized egg, and juveniles (1, 7 and 15 days after hatching) of *Pomacea canaliculata* was also successful suggesting that discrimination of *Pomacea canaliculata* and *Pila* species could be carried out since the early stages of development.

Using an identical approach, candidate *Pila*-specific (430 bp from OPA07), *P. ampullacea*-specific (640 bp from OPA07) and *P. angelica*-specific (380 bp from UBC122) RAPD fragments were cloned and sequenced. SCAR markers were then developed. Only, pKUSCARPILA-F/R (formerly called pKURAPDPA2-F/R) primer pair provided specific amplification results in 95.09% of *Pila* (*P. ampullacea*, *P. angelica*, *P. pesmei* and *P. polita*) but not in *Pomacea canaliculata*. This indicated the successful development of a specific SCAR marker for detection of *Pila* apple snails in Thailand. On the basis of the original data, a 640 bp RAPD band was *P. ampullacea*-specific. Therefore, *Pila*-specific nature of the derived SCAR marker may have explained by the occurrence of an alternative allele of this locus in *P. angelica*, *P. pesmei* and *P. polita* but complexity of RAPD patterns obviated the ability to identify that alternative RAPD band after gel electrophoresis.

RAPD-PCR is one of the potential approaches for identification of molecular genetic markers in various taxa (Hadrys *et al.*, 1992; Stothard and Rolinson, 1996; Klinbunga *et al.*, 2001 and 2002). In the present study, we used this technique to examine genetic diversity of apple snails in Thailand and to identify diagnostic RAPD and SCAR markers for *Pomacea canaliculata* and *Pila* apple snails, respectively. These markers could be used together for unambiguously discriminate the introduced and native apple snails in Thailand.

ACKNOWLEDGMENTS

We thank the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for providing facilities required by our experiments. Our sincere appreciation goes to Piti Amparyup for technical assistance.

REFERENCES

1. Brandt, R.A.M. (1974). The non-marine aquatic mollusca of Thailand. *Archiv fur Molluskenkunde*. 105: 1-423.
2. Carvalho, G.R. and Hauser, L. (1994). Molecular genetics and the stock concept in fisheries. *Rev. Fish Biol. Fisheries* 4: 326-350.
3. Felsenstein, J. (1993). *Phylip (Phylogenetic Inference Package) version 3.5c*. Distributed by the author. Department of Genetics, University of Washington, Seattle.
4. Hadrys, H., Balick, M. and Schierwater, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1: 55-63.
5. Janyapeth, S. and Archawakom, T. (1999). Biology of the alien apple snails. In: Thai Ministry of Agriculture and Cooperatives: Conference Report on the Alien Apple Snail. Pailin Hotel, Phitsanulok, pp. 1.1-1.15.
6. Keawjam, R.S. (1986). The Apple snails of Thailand: distribution habitat and shell morphology. *Malacol. Rev.* 19: 61-81.
7. Keawjam, R.S. (1987). The Apple snails of Thailand: aspects of comparative anatomy. *Malacol. Rev.* 20: 69-89.
8. Keawjam, R.S. and Upatham, E.S. (1990). Shell morphology, reproductive anatomy and genetic patterns of three species of apple snails of the genus *Pomacea* in Thailand. *J. Med. Appl. Malacol.* 2: 45-57.
9. Klinbunga, S., Boonyapakdee, A. and Pratoomchat, B. (2000). Genetic diversity and species diagnostic markers of mud crabs (Genus *Scylla*) in eastern Thailand determined by RAPD analysis. *Mar. Biotechnol.* 2: 180-187.
10. Klinbunga, S., Ampayup, P., Tassanakajon, A., Jarayabhand, P. and Yoosukh, W. (2001). Genetic diversity and molecular markers of cupped oysters (Genera *Crassostrea*, *Saccostrea* and *Striostrea*) in Thailand revealed by Randomly Amplified Polymorphic DNA analysis. *Mar. Biotechnol.* 3: 133-144.
11. Klinbunga, S., Ampayup, P., Khamnamtong, N., Tassanakajon, A., Jarayabhand, P. and Yoosukh, W. (2002). Molecular genetic markers for taxonomy of oysters in Thailand. *Fisheries Sci.* 68 (suppl II) 1087-1090.
12. Lauhachinda, N., Duangswasdi, S. and Sidhi, S. (1988). South America golden apple snails (*Pomacea canaliculata*): the new pest of aquatic plants. In: 26th Kasetsart University Conference Report, Kasetsart University, Bangkok, Thailand. pp. 108-115.
13. Lynch, M. (1990). The similarity index and DNA fingerprinting. *Mol. Biol. Evol.* 7: 478-484.
14. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York

15. Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76: 5269-5273.
16. Pain, T. (1972). The Ampullariidae, an historical survey. *J. Conchol.* 27: 453-462.
17. Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
18. Sihanuntavong, D., Sittipraneed, S. and Klinbunga, S. (1999) Mitochondrial DNA diversity and population structure of the honey bee, *Apis cerana*, in Thailand. *J. Apic. Res.* 38: 211-219.
19. Sittipraneed, S., Laoaroon, S., Klinbunga, S. and Wongsiri, S. (2001a) Genetic differentiation of the honey bee (*Apis cerana*) in Thailand: evidence from microsatellite polymorphism. *J. Apic. Res.* 40: 9-16.
20. Sittipraneed, S., Sihanuntavong, D. and Klinbunga, S. (2001b) Genetic differentiation of the honey bee (*Apis cerana*) in Thailand revealed by polymorphism of a large subunit of mitochondria ribosomal DNA. *Insectes soc.* 48: 266-272.
21. Stothard, J. R. and Rollinson, D. (1996). An evaluation of random amplified polymorphic DNA (RAPD) for the identification and phylogeny of freshwater snails of the genus *Bulinus* (Gastropoda : Planorbidae). *J. Moll. Stud.* 62: 165-176.
22. Thaewnon-ngiw, B. (2003): Species diversity and molecular markers of alien apple snails (*Pomacea* sp.) and native apple snails (*Pila* sp.) in Thailand. PhD Thesis, Interdisciplinary Graduate Program, Kasetsart University. 226 pp.
23. Weising, K., Hybom, H., Wolff, K. and Meyer, W. (1995). DNA Fingerprinting in Plants and Fungi. CRC Press, Boca Raton, FL.
24. Welsh, J. and McClelland, M. (1990). Fingerprinting genome using PCR with arbitrary primers. *Nucleic Acids Res.* 18: 7213-7218.
25. Williams, J.G.K., Kubelik, A.R., Lipak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.
26. Winnepenninckx, B., Backeljau, T. and De Wachter, R. (1993). Extraction of high molecular weight DNA from molluscs. *TIG.* 9: 407.