Journal of Biochemistry and Molecular Biology, Vol. 37, No. 2, March 2004, pp. 213-222

JOURNAL OF Biochemistry and Molecular Biology

Species Identification of the Tropical Abalone (*Haliotis asinina, Haliotis ovina,* and *Haliotis varia*) in Thailand Using RAPD and SCAR Markers

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Received 12 May 2003, Accepted 7 July 2003

A randomly amplified polymorphic DNA (RAPD) analysis was used to identify the species- and population-specific markers of abalone; *Haliotis asinina, H. ovina,* and *H. varia* in Thai waters. Fifteen species-specific and six population-specific RAPD markers were identified. In addition, an 1650 bp band (UBC195) that was restricted to *H. ovina* from the Gulf of Thailand (east) was also found. All of the specific RAPD markers were cloned and sequenced. Twenty pairs of primers were designed and specificity-tested ($N = 12$ and 4 for target and non-target species, respectively). Seven primer pairs (CUHA1, 2, 4, 11, 12, 13, and 14) were specifically amplified by *H. asinina* DNA, whereas a single pair of primers showed specificity with *H. ovina* (CUHO3) and *H. varia* (CUHV1), respectively. Four primer pairs, including CUHA2, CUHA12, CUHO3, and CUHV1, were further examined against 216 individuals of abalone ($N = 111$, 73, and 32, respectively). Results indicated the species-specific nature of all of them, except CUHO3, with the sensitivity of detection of 100 pg and 20 pg of the target DNA template for CUHA2 and CUHA12 and CUHV1, respectively. The species-origin of the frozen, ethanol-preserved, dried, and boiled *H. asinina* specimens could also be successfully identified by CUHA2.

Keywords: Abalone, PCR, RAPD, SCARs, Species-specific markers

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Introduction

Abalones are economically important archeogastropods that are currently cultured worldwide. There are over 15 species of abalone, which are being farmed and are commercially important (Jarayabhand and Paphavasit, 1996). The total world production of abalone was approximately 13,000 metric tons in 1999, 7,165 tons of which (55%) were produced on farms. The major producers of abalone are China and Taiwan. They annually contribute approximately 75% of the cultured production (Gordon, 2000).

Three species of tropical abalone (*Haliotis asinina*, *H. ovina*, and *H. varia*) are found in Thai waters (Jarayabhand and Paphavasit, 1996). Both *H. asinina* and *H. ovina* are distributed along the east coast of the upper Gulf of Thailand, and all three species occur in the Andaman Sea (Tookvinas *et al*., 1986; Nateewatana and Bussarawit, 1988). Among these abalone, *H. asinina*, provides the highest percentage (85%) between the meat weight and total weight when compared to *H. ovina* (40%) and *H. varia* (30%), respectively (Singhagraiwan and Doi, 1993). Accordingly, *H. asinina* is presently being initially commercially cultured in Thailand.

Appropriate genetic markers can be used to elevate the cultural and management efficiency of abalone in Thailand (Jarayabhand *et al*., 2002). The sustainable success of aquacultural activity of commercially important species requires a basic knowledge on stock structure as well as the use of suitable molecular genetic markers to establish broodstock management programs of exploited species (Avise, 1994; Calvalho and Hauser, 1994). In addition, species-specific markers play important roles in preventing the supply of incorrect abalone larvae for the industry as well as

quality control of abalone from Thailand. These markers are also necessary for the development of monospecific farming of *H. asinina* in Thailand.

Species-specific sequences of lysin were reported in the pink (*H. corrugata*) and red (*H. rufescens*) abalone (Vacquier *et al*., 1990). Partial sequences of 18S rDNA were also used to differentiate the closely related abalone, *H. discus discus* and *H. discus hannai*. The amplified 18S rDNA was directly sequenced and multiple-aligned with that of *H. madaka*, *H. gigantea*, and a land gastropod (*Limicolaria kambeul*). The inferred 18S rDNA phylogeny indicated that *H. discus discus* and *H. discus hannai* are closely related but distinguishable at the subspecies level (Naganuma *et al*., 1998). In addition, a tandemly-repeated satellite DNA (290-291 bp in length) was identified by *Sal* I digestion of genomic DNA of five Eastern Pacific (California) abalone species, including the red (*H. rufescens*), white (*H. sorenseni*), flat (*H. walallensis*), pinto (*H. kamtschatkana*), and pink (*H. corrugata*) abalone. Sequences of satellite DNA were determined by direct sequencing and revealed species-specificity in these abalone (Muchmore *et al*., 1998). For identification of the species origins of abalone tissue from South Africa, species-specific PCR was developed to distinguish *H. midae* from *H. spadicea* using a portion of lysin cDNA sequences (Lee and Vacquier, 1995). PCR specifically amplified a 1,300 bp fragment of the genomic DNA from dried, cooked, and fresh *H. midae* tissue. A smaller fragment of the 146 bp product was successfully amplified and used for the identification of canned *H. midae*. Additionally, PCR-RFLP revealed interspecific polymorphism that differentiated these species unambiguously (Sweijd *et al*., 1998).

Recently, species-diagnostic markers of *H. asinina*, *H. ovina*, and *H. varia* were developed using PCR-RFLP of 16S rDNA (Jarayabhand *et al*., 2002). Restriction of the amplified 16S rDNA with *Alu* I could differentiate these abalone unambiguously (patterns A and E in *H. asinina*, $N = 115$; B in *H. ovina*, $N = 71$ and C, D, F, and G in *H. varia*, $N = 23$). Further digestion of 16S rDNA with *Bam* HI, *Eco* RI, and *Hae* III yielded non-overlapping composite haplotypes in these abalones; AAAA and AAAE in *H. asinina*, ABBB, AAAB and AABB in *H. ovina* and BABG, BABC, BABD, BABF and AABG in *H. varia*, respectively. Species-specific PCR, based on 16S rDNA polymorphism, was successfully developed in *H. asinina* and *H. varia* (100% amplification success with no false positive) but not in *H. ovina* (68.75% amplification success with extensive false positives from other species) (Klinbunga *et al*., 2003).

Interspecific hybridization and gene introgression between *H. rubra* and *H. laevigata* was reported, based on an allozyme analysis (Brown, 1995). Theoretically, hybridization between the male *H. asinina* and/or *H. varia* with females of different species could have eliminated the accuracy of the species identification, based on matriarchally inherited markers like 16S rDNA. As a result, *H. asinina*- and *H. varia*-specific nuclear DNA markers need to be developed.

Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) is a relatively simple method for the identification of genetic markers at different taxonomic levels, without the need for knowledge of sequences of the genome under investigation (Welsh and McClelland, 1990; Williams *et al*., 1990; Hadrys *et al*., 1992). The objective of this study was to develop reproducible species-specific markers of *H. asinina* in Thailand. Candidate species- and population-specific RAPD markers were cloned and sequenced. The primers were designed. Specificity, sensitivity, and stability of the selected markers were examined. The sequence-characterized amplified region (SCAR) markers that offered an accurate discrimination of *H. asinina* from *H. ovina* and *H. varia* were developed.

Materials and Methods

Sampling Specimens of three abalone species [*H. asinina* ($N = 111$), *H. ovina* ($N = 73$), and *H. varia* ($N = 32$)] were collected (Table 1 and Fig. 1) and individually kept at −30ºC until required. Alternatively, the foot muscle of each specimen was dissected and kept at −80ºC until further use for the DNA extraction.

DNA Extraction The total DNA was extracted from a piece of the foot muscle of each abalone using the phenol-chloroform-proteinase K method (Klinbunga *et al*., 1996). The concentration of the extracted DNA was spectrophotometrically estimated. The DNA was stored at 4ºC until needed.

RAPD-PCR One hundred and thirteen primers that were purchased from Operon Technologies Inc. (Alameda, USA) and the University of British Columbia (Canada) were screened for amplification success against 2-3 representatives of each abalone species (Fritsch *et al*., 1993). Five primers (OPB11, UBC101, UBC195, UBC197, and UBC271) were tested against different populations of *H. asinina* ($N = 5$ per location), *H. ovina* ($N = 7$-8 per location), and *H. varia* ($N = 15$ and 3 for HVPHUW and HVPHAW).

RAPD-PCR was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2-3 mM of $MgCl_2$, 100 µM of each dNTP, 0.2 µM of a primer, 1 unit of Ampli*Taq* DNA Polymerase (Perkin-Elmer, Cetus, USA), and 25 ng of a DNA template. The amplification was performed in an Omnigene thermal cycler (Hybaid, Middlesex, UK) according to Klinbunga *et al.* (2001). Five microlitres of the amplification reaction were electrophoresed through 1.6% agarose gels and visualized under a UV transilluminator after ethidium bromide staining (Maniatis *et al*., 1982).

Cloning and sequencing of abalone RAPD markers Twenty-two RAPD fragments, showing population-, region-, or species-specificity for each abalone (Table 2), were gel-eluted using a Prep-A-Gene DNA purification kit (Bio-Rad Ltd, Hercules, USA) and reamplified with the original primer. The target band was eluted and ligated to the pGEM-T Easy vector (Promega, Madison, USA). One-tenth of the volume of each ligation reaction was

Table 1. Sample collection sites and sample sizes of abalone (*H. asinina*, *H. ovina* and *H. varia*) used in this study

Geographic origin	Abbreviation	Sample size (*N*)
H. asinina		
Samet Island, Rayong (Gulf of Thailand)	HASAME	10
P_0 stock, Rayong (Gulf of Thailand)	HARAYE	15
Cambodia (east of peninsular Thailand)	HACAME	20
P_0 stock, Cambodia (east of peninsular Thailand)	HACAMHE	12
Talibong Island, Trang (Andaman Sea)	HATRAW	23
F_1, Philippines	HAPHIE	19
Indonesia	HAINDW	12
H. ovina		
Sichang Island, Chon Buri (Gulf of Thailand)	HOCHOE	20
Samet Island, Rayong (Gulf of Thailand)	HOSAME	19
Churk Island, Trang (Andaman Sea)	HOTRAW	18
Similan Island, Phangnga (Andaman Sea)	HOPHAW	16
H. varia		
L-Island, Phuket (Andaman Sea)	HVPHUW	29
Similan Island, Phangnga (Andaman Sea)	HVPHAW	3
Total (*N*)		216

Fig. 1. Map of Thailand illustrating sampling collection sites for abalone (*H. asinina*, *H. ovina and H. varia*) used in this study. Dots represent geographic locations (excluding *H. asinina* from Indonesia and Philippines) for which at least one abalone species was sampled. Note that Samet Island (SAM) is located in Rayong (RAY) province.

electrotransformed to *E. coli* XL1-BLUE (Dower *et al*., 1988). Recombinant clones were selected by the *lacZ* system following standard protocols (Maniatis *et al*., 1982). DNA sequences of 2-6 clones of each insert were examined from both directions using an automated DNA sequencer (Li-Cor, Lincoln, USA).

Species-specific PCR, sensitivity, and stability tests Twenty pairs of primers were designed using OLIGO 4.0. They were preliminarily tested for specificity against genomic DNA of the target ($N = 12$) and non-target species ($N = 4$) using PCR conditions that are described by Klinbunga *et al*. (2000), except that the annealing temperature was increased to 65°C. Four pairs of primers (CUHA2, CUHA12, CUHO3, and CUHV1) were further examined against larger specimens ($N = 216$, Table 1). The amplification product was electrophoretically analyzed through 1.6% agarose gels.

Sensitivity of the detection was examined against varying concentrations of the target DNA template (10 pg to 25 ng), using the same conditions as were used for the specificity test. The stability of CUHA2 and CUHA12 was tested against the poor genomic DNA template that was extracted from 10 individuals of the ethanol-preserved larvae (approximately 1 mm in size stored at 4°C for 6 mo) and frozen (3 yr at −30°C), dried (80°C for 72 h and kept at room temperature for 2 wk), and boiled (10 min) *H. asinina* broodstock using the Chelex-based method (Walsh *et al*., 1994).

Results and Discussion

RAPD-PCR has widely been used for population genetic studies as well as the identification of molecular markers for various applications in several organisms (Heipel *et al*., 1998; Tassanakajon *et al*., 1998; Klinbunga *et al*., 2001). An

Table 2. Population-, region-, and species-specific markers of tropical abalone in Thailand (*H. asinina*, *H. ovina*, and *H. varia*) revealed by RAPD analysis using primers OPB11, UBC101, UBC195, UBC197, and UBC271

Species/population	Primer	RAPD marker (bp)	Name of Clones
H. asinina	OPB11	1400	pCUHA14 and pCUHA16
		800	pCUHA17 and pCUHA18
	UBC101	1700	pCUHA19
		1325	pCUHA20
		590	pCUHA13
	UBC195	760	pCUHA4, pCUHA21 and pCUHA22
	UBC197	1400	pCUHA23
		710	pCUHA1
	UBC271	1000	pCUHA24
		650	pCUHA2, pCUHA3, pCUHA5, pCUHA6, pCUHA12 and pCUHA25
/HATRAW	UBC195	650	pCUHA10 and pCUHA11
	UBC271	850	pCUHA7 and pCUHA8
		450	pCUHA9
/HAPHIE	UBC195	1000	pCUHA26
		680	pCUHA27
	UBC197	415	pCUHA15
H. ovina	OPB11	475	pCUHO1, pCUHO2 and pCUHO3
	UBC195	950	pCUHO4
/Gulf of Thailand	UBC195	1650	pCUHO5
H. varia	OPB11	690	pCUHV4, pCUHV5, pCUHV6 and pCUHV7
	UBC195	700	pCUHV1 and pCUHV2
		550	pCUHV3

analysis of the genetic diversity and population differentiation of *H. asinina*, *H. ovina*, and *H. varia* using PCR-RFLP of 16S and 18S rDNA (Jarayabhand *et al*., 2002; Klinbunga *et al*., 2003) revealed a lack of genetic heterogeneity of *H. asinina* in Thailand ($P>0.05$). The analysis, however, did indicate significant genetic differences between *H. asinina*, *H. ovina*, and *H. varia* ($P<0.0001$). This suggested the possibility of identifying a large number of species-specific markers in *H. asinina*.

Among the 113 RAPD primers that were screened, 27 primers (OPA1, 2, 10, 15, 10, and 20, OPB11, 16, and 17, UBC101, 119, 160, 168, 174, 193, 195, 197, 200, 210, 220, 264, 267, 271, 272, 456, 457, and 459) yielded successful amplification results in three abalone. Five primers (OPB11, GTAGACCCGT; UBC101, GCGCCTGGAG; UBC195, GATCTCAGCG; UBC197, TCCCCGTTCC and UBC271, GCCATCAAGA) were selected for the identification of candidate species-specific (or population-specific) markers of each abalone.

In total, the 10, 2, and 3 fixed RAPD markers were found in *H. asinina*, *H. ovina*, and *H. varia* (Table 2 and Fig. 2). Three candidate population-specific RAPD markers were observed in *H. asinina* that originated from Talibong Island (HATRAW) and the Philippines (HAPHIE), respectively. In addition, an 1,650 bp RAPD marker that was generated from UBC195 was specifically found in *H. ovina*, originating from the Gulf of Thailand (east), but not in the Andaman Sea sample (west).

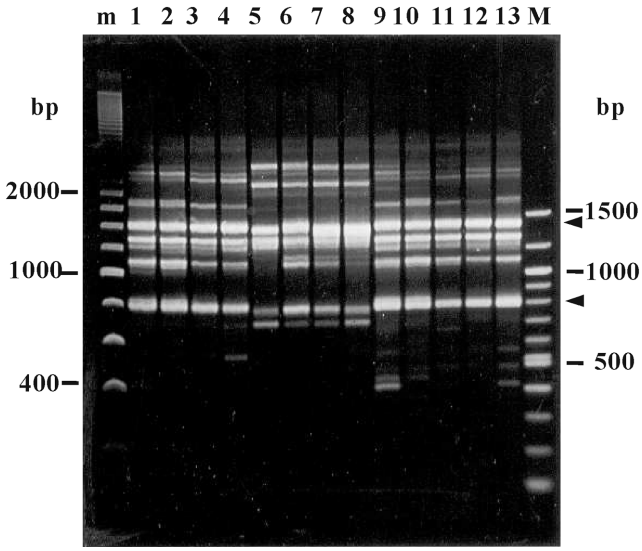

Fig. 2. An example of species-specific RAPD markers resulted from amplification of total DNA of *H. asinina* from HARAYE (lanes 1-4), HATRAW (lanes 5-8) and HACAMHE (lanes 9-13) with OPB11. Arrowheads indicate species-specific RAPD bands found in *H. asinina*. Lanes M and m are 100 bp and 200 bp DNA markers, respectively.

RAPD-PCR is sensitive to reaction conditions, including

Table 3. Sequences of primers designed from population-specific (CUHA11) and species-specific RAPD markers of *H. asinina*, *H. ovina*, and *H. varia*

Primer	Sequence
CUHA1	F: 5'-GAATCCAACATGCGTCAAAG-3'
	R: 5'-CTGGAAACAATCGCAGGTCA-3'
CUHA2	F: 5'-TTGTTCAGCATTCTGTGGCAGTTCT-3'
	R: 5'-CTTCTTTTTTGCTGACCCTTTGGAG-3'
CUHA4	F: 5'-TCAGCGAAACCAACCAACAC-3'
	R: 5'-TTGGACGCAGCTATTCACAT-3'
CUHA11	F: 5'-CCCCGAGGAGTATACAACTCTTCC-3'
	R: 5'-TCGAGTTCTTTTCCACAATGCACC-3'
CUHA12	F: 5'-CTAATCCCACACAGCCATCACCAG-3'
	R: 5'- AAGAAGTGACGAAGAGGTAGGCAG-3'
CUHA13	F: 5'-TGACCTGTGTTGAGACTCTACGGA-3'
	R: 5'-TGAGGGGAGATGGAGTAGCCGC-3'
CUHA14	F: 5'-CGTGAAGACAGTTACTGAAAGTGG-3'
	R: 5'-ATCGTTTGTGTTATGTCTCCTCTG-3'
CUHO3	F: 5'-GGGTATCTTCCCACAACAGC-3'
	R: 5'-GCACTTGCCTACATCCTTTCAC-3'
CUHV1	F: 5'-CCCCTTGTTTCTCCTTCTTG-3'
	R: 5'-CGATGACGCAGGCGGTTTGA-3'

Table 4. Specificity of SCAR markers developed from population-specific or species-specific RAPD markers of *H. asinina*, *H. ovina*, and *H. varia*

Primer	Expected product (bp)	*H. asinina*				*H. ovina*	*H. varia*
		HACAMHE	HACAME	HATRAW	HAPHIE		
CUHA1-F/R	292	+	+	+	+	-	-
CUHA2-F/R*	168	+	+	+	+	-	-
CUHA3-F/R[a]	368	NS	NS	NS	NS	NS	NS
CUHA4-F/R	290	+	+	+	+	-	-
CUHA5-F/R	264	+	+	+	+	+	+
CUHA6-F/R	103	+	+	+	+	+	+
CUHA7-F/R[a]	554	NS	NS	NS	NS	NS	NS
CUHA8-F/R[a]	114	NS	NS	NS	NS	NS	NS
CUHA9-F/R[a]	142	+	+	+	+	+	+
CUHA10-F/R[a]	472	NS	NS	NS	NS	NS	NS
CUHA11-F/R[a]	417	+	+	+	+	-	-
CUHA12-F/R*	312	+	+	+	+	-	-
CUHA13-F/R	296	+	+	+	+	-	-
CUHA14-F/R	473	+	+	a 515 bp band	a 515 bp band	-	-
CUHA15-F/R[b]	171	+	+	+	+	+	+
CUHO1-F/R	414	+	+	+	+	+	+
CUHO2-F/R	146	+	+	-	-	+ and a 215 bp band	-
CUHO3-F/R*	328	-	-	-	-	+	-
CUHO4-F/R	619	NS	NS	NS	NS	NS	NS
CUHV1-F/R*	229	-	-	-	-	-	+

Abbreviations: +, successful amplification with the expected product; -, no amplification product; NS, non-specific fragments obtained; *, primers further tested against a large sample size (*N*=216); [a], primers derived from HATRAW-specific RAPD markers; [b], primers derived from a HAPHIE-specific RAPD marker.

(A)

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GCCATCAAGA TGTGACATGT TCATGGGCAA CTAAAATGGA TTGAAAAAAC
GTTACAAGTA TTTCACAAAC CTTTCCCTTT ATATATTTAT CCTGAATTAA
ATTGAACTCT GCTGAAAAGT GGCATATCAT TAATCAAAAT ATAGACTGGT
CAAACGTAAT GACTTCTTCA TGACTAAATA TAACTGAAGA TCAATGAACA
AGGTAAATAT TGCTGTAAAC AATAAACAGG CACATGTTTC AAAACAATTC
CACACAAAGG TAACTTTTGC TATTTGGTTA TGTAAACAAG CTGCAGAGCC
CTCTTATGAA AGTTCACCTC CCAATGACTT CATTTATCAG TAATGTGTGA
                pCUHA2-F
TCATATCCAG GATGCTTGTT CAGCATTCTG TGGCAGTTCT TGACAACTTG
ATTTCAGTAC TGAATGAACA AGAAAGACTG ACAATTTTGT TCCATGTTGC
CCCCAAGTTA TTCTTGACAG ATGTAGATTC TGCATTAGTG ACAAAGATTT
                              pCUHA2-R
TTATATATCT CCAAAGGGTC AGCAAAAAAG AAGTACAGTA TACCCTGGTG
ATATCTTCCA CTGTTTGTCA GAGGGAACAT ATGGTACTGT ATCCAGACAC
ACGTTATAAC CAGGGTGCAC TGTACATGCC CACATGGAAG CATGAATCAT
GACAGTCAAT ACCATAACTG TATTTAATTT ACAAAAAAAA ATATATATGA
TCTTGATGGC
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(B)

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GCCATCAAGA ATAAGCGTTA TGAAAACACT AACTCAAATC AGCCAAATAA
TCCCACTAGT GATATCCTCT TTGGACTGAA GTTGTTTGTT TTAAGAGTAA
                                              pCUHA12-F
GAGGATTATA AGGCATGCTC AACTGGGTTT GGTCGTTTAA CTAATCCCAC
ACAGCCATCA CCAGCAGAGG GAATGATTTA CACATCTCAT TTTTTGTGCT
TAATTATTGT GTTTGTTTTT TATTGAAAAA AAAATGAAAT GGGAGTGTGA
ACAGCTTTAC AGTTTATATC GACACTGGTA CCATACAACA TACAATACAA
AATTTAAATG CAGCATGTGT TGTCACATAT GTATATAAAT TTGAATTACA
AACATGGGTA AAGCTTGAAG ACAATCTTAT ATGGAAATAG TGAATTATCT
                                             pCUHA12-R
GATCTTGATA ATCTGTCCAT CTTAAGATCT GCCTACCTCT TCGTCACTTC
TTCTTTTAGA TTTGATACTA AACATGATTG AAGAATTAAC CTCGTGGTTT
GTCATATGAC AATCTTGTAT TAGAACTAGG TTAGGTTTAT TTACCTTTGT
AAAGTGCATT ACACACATAC AAACTAACTG TATTAATATA GAGCATTTAT
GAGAATGGTA TTGGTAAGAN AATTATTTTT GGTGTCTTGA TGGC
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(C)

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GATCTCAGCG CATACATAAT CATATTCATT ACAAAGGATA TAACACCAGA
AATGTCGCGC CATAATTCTT ATGACATACA GAAACCGGGG TAATTTTATC
GGCATCGCTG CTATATAATT TCCCCTCCTG AAACTAATAG AATTTCCTAA
                                    pCUHV1-F
TTGGGGtTGT CATGGTAGGT TCTTTGCCTA ACCCCTTGTT TCTCCTTCTT
GTCGACATGA AAAGACCACT TTATCAAATA TTTCATCCTC ACTTCATTCA
CAATAATCTC TTGATGAGCC CAACATTCGT CTGAAACATC CGCACGAGGC
AGCCGGAACC ACCAGGCTGT TGGCAACTTA TTCAAAGTCT CTTCTCTTCA
CCCACAGAGA TTTTTTCACA ATACCAACAA ACAATATCCA TCAAACCGCC
  pCUHA1-R
TGCGTCATCG TTTGTACTCA AGCCTCAATC GAACTTCGCC AGTGCACTAT
GGACGTGCTT CGACGGtCGG CTGCAAACCT TTGCAACAAA ACCACCGATT
CTAGAAGTTT CGTTCATTGC ATTCCCCCGA CAACTGCTAT TATAAAACAT
TTAGTTAGTG TAATTGTAAC TGGTCTATCT CGAGTCTTCG TCTTCATTAC
GGATAATTAG CCCGTCGCTG AGGGGGGcAC TTTCTACGCT CATCCATCCC
GCTGAGATC
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Fig. 3. Sequences of CUHA2 (A), CUHA12 (B) and CUHV1 (C). The locations and sequences of *H. asinina*- and *H. varia*-specific forward primers and those complementary to reverse primers are labeled in boldface and underlined.

the requirement of a good quality DNA template for consistent results. False negatives may possibly occur. We then converted the candidate species-specific (and population-specific) RAPD fragments to sequence-characterized amplified region (SCAR) markers (Weising *et al*., 1995).

Twenty-two RAPD fragments were cloned (Table 2). Seventy-two clones were sequenced. Thirty-nine different sequences were found. These indicated that the RAPD

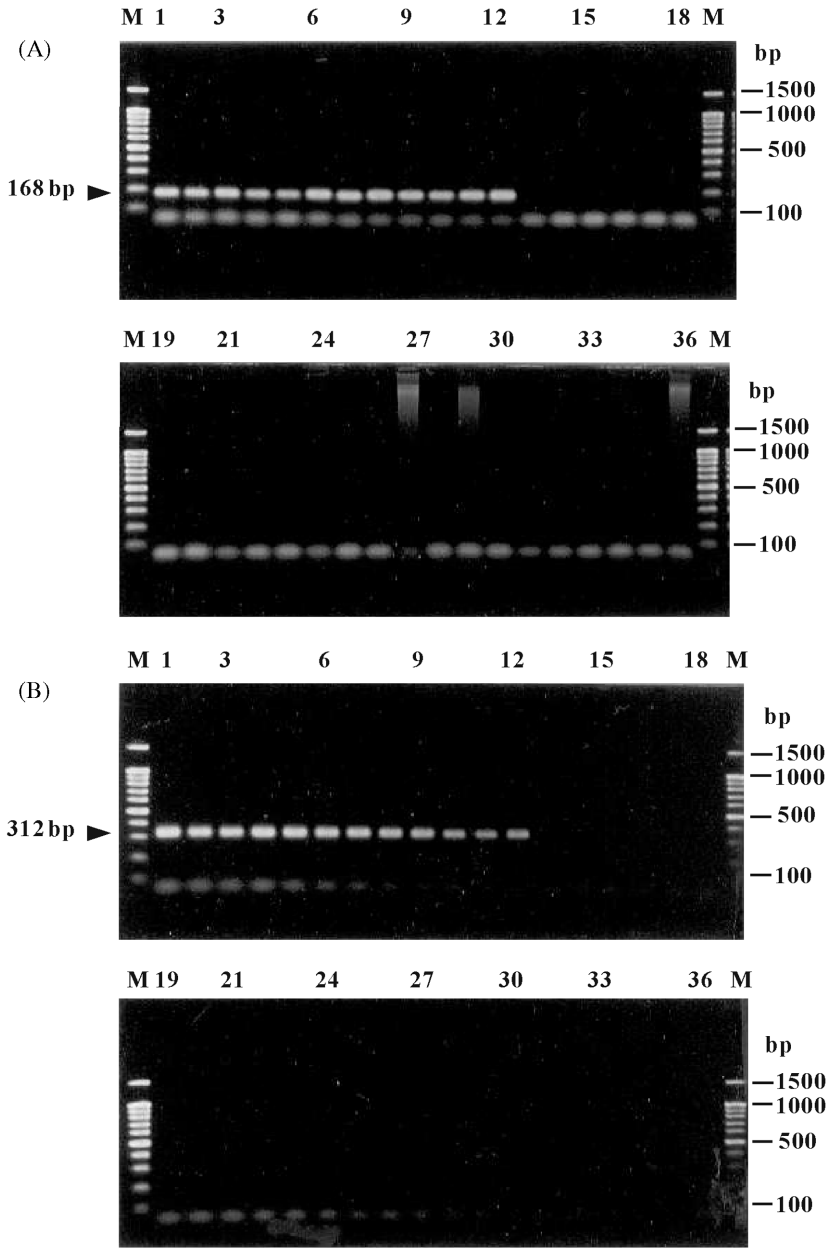

Fig. 4. Agarose gel elctrophoresis illustrating species-specificity of CUHA2 (A), CUHA12 (B) and CUHV1 (C) against genomic DNA of *H. asinina* (panels A, B and C, lanes 1-12), *H. ovina* (panels A, B and C, lane 13-24), and *H. varia* (panels A, B and C, lanes 25-36). An 100 bp ladder (lanes M) was used as a DNA marker.

fragments represented co-migrating fragments that had different nucleotide sequences but similar sizes. Almost all of the RAPD markers (33/39 accounting for 85%) were unknown sequences when compared with the data in the GenBank using Blast*N* and Blast*X* (E values $>10^{-4}$).

Twenty pairs of primers were designed from those unknown sequences (pCUHA1-pCUHA15, pCUHO1-pCUHO4 and pCUHV1; Table 2). Originally, seven primer sets were designed for the development of population-specific markers in *H. asinina*. Four of these (CUHA3, CUHA7, CUHA8, and CUHA10) provided non-specific amplification results; CUHA9 and CUHA15 provided positive amplification in all of the abalone species and CUHA11 yielded a *H. asinina*-specific rather than a HATRAW-specific nature (Table 3). Therefore, population-specific SCAR markers were not found in *H. asinina* (Table 4).

In addition, the CUHA5, CUHA6 and CUHO1 primers also showed positive amplification bands in all three abalone species. We further tested the specificity of CUHA5 (264 bp) and CUHA6 (103 bp) against gastopods (the giant African snail, *Achatina fulica*, and the apple snails, *Pomacea canaliculata*, *Pila ampullacea*, *P. angelica*, *P. pesmei*, and *P.*

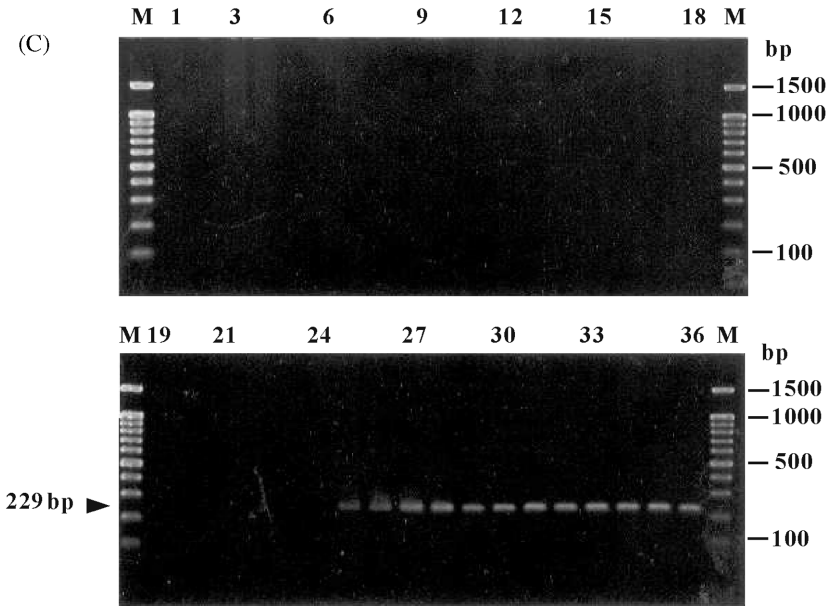

Fig. 4. Continued.

polita), oysters (*Crassostrea belcheri*, *C. iredalei*, *Saccostrea cucullata*, *S. forskali*, and *Striostrea mytiloides*), mussel (*Perna viridis*), and crustaceans (the black tiger prawn, *Penaeus monodon* and the giant freshwater prawn, *Macrobrachium rosenbergii*) (data not shown). These primers provided no positive amplification fragments in the non-abalone species and may be used as genus-diagnostic markers of abalone in this study.

Based on the preliminary screening, seven pairs of primers (CUHA1, CUHA2, CUHA4, CUHA11, CUHA12, CUHA13, and CUHA14) revealed species-specificity in *H. asinine*, while the CUHO3 and CUHV1 primers exhibited the expected product in *H. ovina* and *H. varia*, respectively (Tables 3 and 4). We did not select CUHA14 for the analysis against larger specimens of *H. asinina* because different sizes of the amplification products were observed (expected 473 bp in HACAMHE and HACAME and a larger 515 bp in HATRAW and HAPHIE, respectively).

Four sets of primers (CUHA2, CUHA12, CUHO3, and CUHV1, Fig. 3) were further examined against a large sample size of abalone ($N = 216$, Table 1). Species-specificity was observed from CUHA2, CUHA12, and CUHV1 (100% without false positive/negative results, Fig. 4). Although CUHO3 yielded a strong amplification product in the target species (100%), a very faint product was also observed in some individuals of *H. asinina* and *H. varia*, which suggests that problems may arise from the non-specific amplification of this primer pair (data not shown). No heterozygotes that exhibited two different sizes (alleles) of the amplification products were observed across the overall specimens that were analyzed by these primers. This implies the retention of a dominant segregated fashion of the original RAPD markers.

A limited sample size of *H. varia* was included in this study ($N = 29$ and 3 for HVPHAW and HVPHUW, respectively). This was due to a lack of *H. varia* that was found in our sampling sites. Accordingly, a *H. varia*-specific SCAR marker (229 bp from CUHV1) should be further tested against specimens that cover a larger geographic distribution of *H. varia* before practical implementation of this marker for the species-identification purpose.

The sensitivity of CUHA2, CUHA12, and CUHV1 was tested using different concentrations of the target DNA template (10 pg-25 ng). These primer pairs revealed a good correlation between the amount of DNA template and intensity of the PCR product. The detection sensitivity was approximately 100 pg of the target DNA template for CUHA2 and CUHA12, but there was a greater sensitivity with CUHV1 (20 pg). The sensitivity levels of the species-specific PCR that was developed in this study were sufficient for the identification of the species-origins of abalone, beginning with the early development stages.

For rapid species-identification of *H. asinina*, the tedious and time-consuming phenol/chloroform extraction method was simplified to a rapid 5% Chelex-based method. The positive fragment (168 bp) of CUHA2 was still consistently obtained from frozen, ethanol-preserved, dried and boiled specimens of *H. asinina*. All but a single dried specimen was successfully amplified by CUHA12 (Fig. 5).

In the present study, we demonstrate the successful development of species-specific SCAR markers in *H. asinina* and *H. varia*. These markers can be used to verify species-origins of various forms of abalone products from Thailand and prevent supplying incorrect abalone larvae for the culture industry. In the future, a large number of the remaining clones, possessing *H. asinina*-specific RAPD inserts, guarantee that additional SCAR markers could be developed if diagnostic

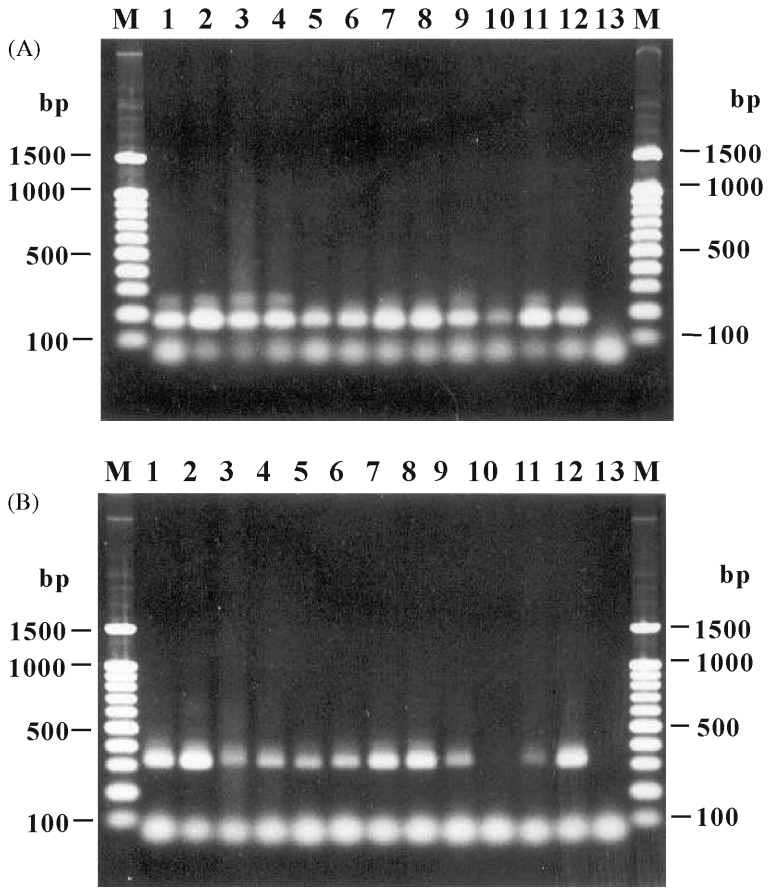

Fig. 5. Agarose gel elctrophoresis showing results from amplification of total DNA extracted with a phenol/chloroform (lanes 1-2) and a 5% Chelex extraction methods (lanes 3-12) of frozen (lanes 1-4), ethanol-preserved larvae (lanes 5-8), dried (lanes 9-10) and boiled (11-12) broodstock of *H. asinina* with CUHA2 (panel A) and CUHA12 (panel B). Lanes 13 are negative controls (without DNA template). A 100 bp ladder (lanes M) was used as a DNA marker.

markers that are described here fail to provide species-specific results when used to examine the species-origins of new populations of *H. asinina*.

Acknowledgments We thank the two anonymous referees for their useful comments. This work was supported by the Thailand Research Funds (TRF) Project, 4320015, that was awarded to SK and AT and was partially supported by JSPS (DNA sequencing). The Royal Golden Jubilee Ph.D. Program, TRF, granted a student fellowship to PA.

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