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ORIGINAL ARTICLE

The development of species-specific AFLP-derived SCAR and SSCP markers to identify mantis shrimp species

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Abstract

Mantis shrimp has become commercially valuable in many countries, while the commercially aquaculture still unsuccessful. The stable supply of the species-specific markers for precise identification can play a key role of foods authentication as well as restoring/enhancing mantis shrimp stocks in future. The aim of this research was to identify species-specific markers for Squillid and Harpiosquillid mantis shrimp taxa using Amplified fragment length polymorphism-Single strand conformation polymorphism (AFLP-SSCP) approaches. Selective amplification would be substituted as a total of 40 primer combinations was performed using either three-base (i.e., *Eco*RI+3 and *Mse*I+3 in 20 primer combinations) or two-base (i.e., *Eco*RI+2 and *Mse*I+2 in 20 primer combinations) selective primers. These had been size-fractionated via 6% denaturing polyacrylamide gel electrophoresis, ten AFLP fragments exhibiting species or genus-specific characteristics were cloned, sequenced, and GenBank interrogated. A primer pair was designed and their specificity was tested versus the genomic DNA of various species. Results show that the primer E_{+2}-13/M_{+2}-13Hr_{158} generated PCR products for just *H. harpax*, while E_{+3}-14/M_{+3}-2HhHr$_{151}$ and E_{+2}-13/M_{+2}-13Hh_{150} generated PCR products for both *H. harpax* and *H. raphidea* and not others (i.e., *M. nepa*, *O. oratoria*, and *E. woodmasoni*). SSCP was then applied in order to differentiate between *H. harpax* and *H. raphidea*. These SSCP results indicate that species can be differentiated based on polymorphic fragment nucleotides. Indeed, primers E_{+2}-13/M_{+2}-13Hr_{158}, E_{+3}-14/M_{+3}-2HhHr$_{151,}$ and E_{+2}-13/M_{+2}-13Hh_{150} were all successfully confirmed as present in processed mantis shrimp samples (i.e., saline-preserved and heat-dried). These results provide new species-specific markers for mantis shrimp identification.

Keywords Harpiosquillid · Squillid · AFLP · SSCP · SCAR · Species-specific markers

Introduction

Stomatopods, commonly known as mantis shrimps, are widely distributed across several parts of the world including tropical and subtropical regions [1]. These species reach highest diversity in the Indo-West Pacific [2]. Mantis shrimp is being one of the most potentials economic importance, mostly processed as by-catch aquatic animals in global crustacean trawl fisheries. Although a number of species are commercially exploited across the Mediterranean as well as in South Africa, America, China, Japan, and Thailand [2–6] information regarding the lifecycle of mantis shrimp remains limited. These animals are of economic importance as they are mainly dried and used as a dependable source of raw material for fishmeal and poultry feeds. Mantis shrimp meat is also consumed by humans and is reputed to possess medicinal value [3]. Recently, mantis shrimp (*Miyakella*

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nepa) muscle obtained from by-catch resources was even used as an alternative mammalian collagen source [7]. As stomatopod species within the genus *Harpiosquilla*, for example *H. raphidea* (Fabricius 1798) and *H. harpax* (de Haan 1844), are the largest known [1], they are commercially valuable in many countries including Thailand [2, 8].

The Food and Agriculture Organization of The United Nations (FAO) has noted that confidence in the safety and integrity of food supply is an important consumer requirement. Thus, the accurate labeling of food components avoids commercial deception, and also mitigates potential safety risks caused by the introduction of ingredients that might be harmful to human health [9]. DNA-based identification methods have been proposed as analytically powerful strategies for species identification, especially in crustacean processed products [10, 11]. Species-specific markers are particularly useful for identifying taxa that have similar morphologies, and can also be applied for food product species identification. However, although the complete mitochondrial DNA sequence for the mantis shrimp *H. harpax* (de Haan 1844) has been reported [12], no publications have appeared to date concerning species-diagnostic DNA markers for the important mantis shrimp species found in Thailand.

Polymerase chain reaction (PCR) approaches in combination with Restriction Fragment Length Polymorphism (RFLP) strategies or single-stranded conformation polymorphisms (SSCPs) have been mostly used for identifying the species origins of crustacean products because these are convenient and cost-effective [13, 14]. Indeed, in order to overcome weaknesses and combine the strengths of RFLP and Randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) have also been developed for the simultaneous analysis of numerous loci [15]. This technique reveals tremendously high genomic polymorphisms that can be used for fingerprinting as well as for the generation of high-resolution mantis shrimp linkage maps [16].

In earlier work, Maldini et al. [17] assessed the potential to use AFLP technology to determine fish and seafood species (i.e., mollusks and crustaceans) in both processed commercial products and domestic stocks. The ten primer combinations utilized in this analysis generated fragments within a size range between 70 base pairs (bp) and 600 bp; the total number of fragments reported varied between 14 and 90, and major differences were observed between different systematic groups. A comparison of informative markers between unknown frozen and fresh products as well as reference samples enabled the accurate identification of 32 different species, including 20 freshwater and marine fish, four crustaceans and eight mollusks. Taxonomic characterizations were performed at either the species or the population level depending on the number of available individuals. Similarly, AFLP fingerprints for six *Penaues* species, *P. monodon*, *P. chinensis*, *P. merguiensis*, *P. latisulcatus*, *P. canaliculatus* and *P. japonicas*, have also been reported [18]. Phylogenetic analyses reveal that these six species comprise two major clades, *P. monodon*, *P. chinensis*, and *P. merguiensis*, alongside *P. latisulcatus*, *P. canaliculatus*, and *P. japonicus*. This earlier work also showed that AFLP technology provides a viable approach for penaeid shrimp species identification and phylogenetics study.

The aim of this study was to develop a series of high-polymorphic DNA markers using AFLP technology that can then be applied for determining the species origins of five mantis shrimps, *H. raphidea* (Fabricius 1798), *H. harpax* (De Haan 1844), *M. nepa* (Latreille 1828), *Erugosquilla woodmasoni* (Kemp 1911), and *Oratosquilla oratoria* (De Haan 1844). Species-specific PCR-AFLP and SSCP markers for these taxa were successfully developed and applied practically in this research for tracing the species origin substituted by species-specific marker.

Materials and methods

Samples and DNA extraction

Adult mantis shrimp samples including the giant harpiosquillid *H. raphidea* (Fabricius 1798) (N = 14, 121.98 ± 8.32 g), the robber harpiosquillid *H. harpax* (De Haan 1844) (N = 30, 26.06 ± 3.55 g), the smalleyed squillid *M. nepa* (Latreille 1828) (N = 30, 24.46 ± 4.75 g), the smooth squillid *E. woodmasoni* (Kemp 1911) (N = 10, 13.10 ± 1.24 g), and the Japanese squillid *O. oratoria* (De Haan 1844) (N = 30, 11.14 ± 2.16 g) were collected from Thai and Chinese coastal fishing boats. The external biological taxonomy of all mantis shrimp samples was initially diagnosed using Ahyong [2] and Carpenter and Niem [8]. Body weights and total body lengths were measured in each case, and specimens were stored at − 30 °C until required for AFLP analyses (frozen samples).

Saline-preserved and heat-dried mantis shrimp samples were processed following the protocols outlined by the Department of Fisheries Products [19] but with minor adjustments. For saline processed, whole mantis shrimp were placed into boiling water and the cephalothorax, abdominal segments, and telson were removed after 20 min. Abdominal flesh was placed in a salt solution containing 1.5% NaCl and after 1 day the whole sample was steamed at 85 °C for 30 min. The cephalothorax, abdominal segments, and telson of raw whole-mantis shrimp were removed as part of the heat-dried process. Abdominal flesh was then heated in oven at 60 °C for 4 h, and all samples were stored at 4 °C ± 1 °C in a refrigerator prior to analysis.

High molecular weight genomic DNA (gDNA) was extracted from a pleopod piece of each mantis shrimp using the phenol–chloroform-proteinase K method [20]. The concentration of extracted DNA was estimated spectrophotometrically in each case and stored at 4 °C.

Pooled DNA samples for AFLP and denaturing polyacrylamide gel electrophoresis

Mantis shrimp gDNA from each species was pooled for two replicates of five individuals each to generate *H. raphidea* (Hr01–Hr05; N = 5 and Hr06–Hr10; N = 5), *H. harpax* (Hh01–Hh05; N = 5 and Hh06–Hh10; N = 5), *E. woodmasoni* (Ew01–Ew05; N = 5 and Ew06–Ew10; N = 5), *M. nepa* (Mn01–Mn05; N = 5 and Mn06–Mn10; N = 5), and *O. oratoria* (Oo01–Oo05; N = 5 each and Oo06–Oo10; N = 5 each).

AFLP analysis was then carried out as described previously [15]. Briefly, pooled gDNA (250 ng in total, 50 ng of each individual) was digested with 6 U of *Eco*RI and 5 U of *Mse*I (Promega, USA) at 37 °C for 5 h before overnight ligation at 16 °C with restriction site-specific adaptors (i.e., *Eco*RI adaptor 5′-CTCGTAGACTGCGTACC-3′/5′-AATTGGTACGCAGTCTAC-3′ and *Mse*I adaptor 5′-GACGATGAGTCCTGAG-3′/5′-TACTCAGGACTCAT-3′) using T4 DNA ligase (Promega, USA). Preamplification was then performed utilizing adaptor specific primers with a single selective base on each, either *Eco*RI (5′-GACTGCGTACCAATTCA-3′) or *Mse*I (5′-GATGAGTCCTGAGTAAC-3′). A 25-µl reaction mixture containing 1 µl ligated DNA, 1 µl of each *Eco*RI and *Mse*I preamplification primers (10 µM each), 2.5 µl 10 × PCR buffer, 1.5 µl $MgCl_2$ (1.5 mM), 0.5 µl dNTPs (0.2 mM) and 1.5 U i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea) was used. A Thermo Cycler PCR (Eppendrof Mastercycler, Germany) with the following, 20 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min; 72 °C for 5 min was used for preamplification. Preamplification products were then diluted fivefold with nano pure H_2O and selectively amplified with primers with an additional two or three selective bases at the 3′ terminus in each case. A 25-µl reaction mixture containing 3 µl diluted preamplification products, 1 µl each of *Eco*RI and *Mse*I selective primers (10 µM each), 2.5 µl 10 × PCR buffer, 1.5 µl $MgCl_2$ (1.5 mM), 0.5 µl dNTPs (0.2 mM) and 1.5 U i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea) was used. A Thermo Cycler PCR (Eppendrof Mastercycler, Germany) with the following, 2 cycles of 94 °C for 30 s, 65 °C for 45 s, and 72 °C for 1.30 min; 12 cycles of 94 °C for 30 s, 64 °C for 45 s, and 72 °C for 1.30 min, during which the annealing temperature was progressively decreased by 1 °C; 23 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 1.30 min, with a 72 °C final extension for 7 min. AFLP fragments were size-fractionated through 6% polyacrylamide sequencing gels under a constant 35 W for 1.5 h and visualized using silver staining.

Cloning and sequencing candidate species-specific AFLP fragments

Candidate AFLP fragments that were found restricted within specific species were excised, eluted from the gel, and reamplified using the same primers for selective amplification. A 50-µl reaction mixture contained 5 µl of the eluted DNA, 1 µl each of *Eco*RI and *Mse*I primers (2 µM each), 5 µl of 10 × PCR buffer, 1 µl of dNTPs (0.2 mM) and 1.5 U i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea). After agarose gel electrophoretically analyzed, these re-amplified products were then gel-eluted by illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GM Healthcare life sciences, USA), cloned into a pGEM®-T Easy vector (Promega, USA), and transformed into *E. coli* strain JM109 cells. Plates were then left until the cell suspension was fully absorbed before being further incubated at 37 °C overnight. Recombinant clones containing inserted DNA are white while those without inserted DNA are blue. Insert sizes were verified using colony PCR [20] and plasmid DNA was extracted from recombinant clones using illustra™ plasmidPrep Mini Spin Kit (GM Healthcare life sciences, USA) and unidirectional sequenced.

Nucleotide sequences were blasted against data in GenBank using the search tools Blast*N* and Blast*X* (https://www.ncbi.nlm.nih.gov/blast); similarity was considered significant when a probability (E) value recovered was less than 10^{-4}.

PCR, agarose gel electrophoresis, and SSCP analysis of sequence characterized amplified region (SCAR) markers

A pair of primers was designed from each insert using Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/). The criteria used for primer design were a length between 18 and 25 bases, a melting temperature between 55 and 70 °C, a random base distribution to avoid polypurine and polypyrimidine tracts, and differences of less than 5 °C in primer pair melting temperatures. Primers were tested against those from five mantis shrimp species in both fresh and preserved samples.

PCR reactions were performed in a 50 µl reaction volume containing 10 mM Tris–HCl at pH 8.8 for 25 °C. Reactions also contained 50 mM KCl, 0.1% Triton X - 100, between 1.5 mM and 2.0 mM $MgCl_2$, one unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland), 200 µM of dNTP, 0.25 µM of each primer, and 50 ng of gDNA. Amplification conditions involved predenaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s,

annealing at 50 °C, 53 °C or 55 °C for 30 s, and extension at 72 °C for 45 s. A final extension was performed at 72 °C for 7 min. Five microliters of amplification products were electrophoretically analyzed using an agarose gel between 1.5 and 1.8% to verify whether, or not, the process was successful. Marker polymorphism was further analyzed using SSCP.

Non-denaturating polyacrylamide gels were used for size-fractionation of both single- and double-stranded DNA in SSCP analyses. Thus, six microliters of amplification product from each mantis shrimp were mixed with four volumes of loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, and 10 mM NaOH), denatured in a boiling bath at 95 °C for 5 min and immediately cooled on ice for 3 min. Denatured PCR products were then electrophoretically analyzed through non-denaturing polyacrylamide gels (37.5:1 crosslink) at between 12.5 and 17.5% concentrations at 12.5 V/cm at 4 °C for between 16 and 24 h. Bands were then visualized using silver staining.

Results

AFLP analysis reveals candidate species-specific markers in Harpiosquillid and Squillid mantis shrimps

Over thirty of positive species-specific markers in both two replicates of pooled mantis shrimp gDNA were derived from AFLP analysis. A total of ten species-specific AFLP fragments identified from a screening of 40 primer combinations of selective amplification products were found in *Harpiosquilla* (six markers) and as well as in *Miyakella* and *Oratosquilla* (four markers) (Fig. 1). The selected AFLP fragments must clearly consistent in two replicates of pooled mantis shrimp gDNA from each species (data not shown). These fragments were cloned and sequenced. Comparison of DNA sequences derived from

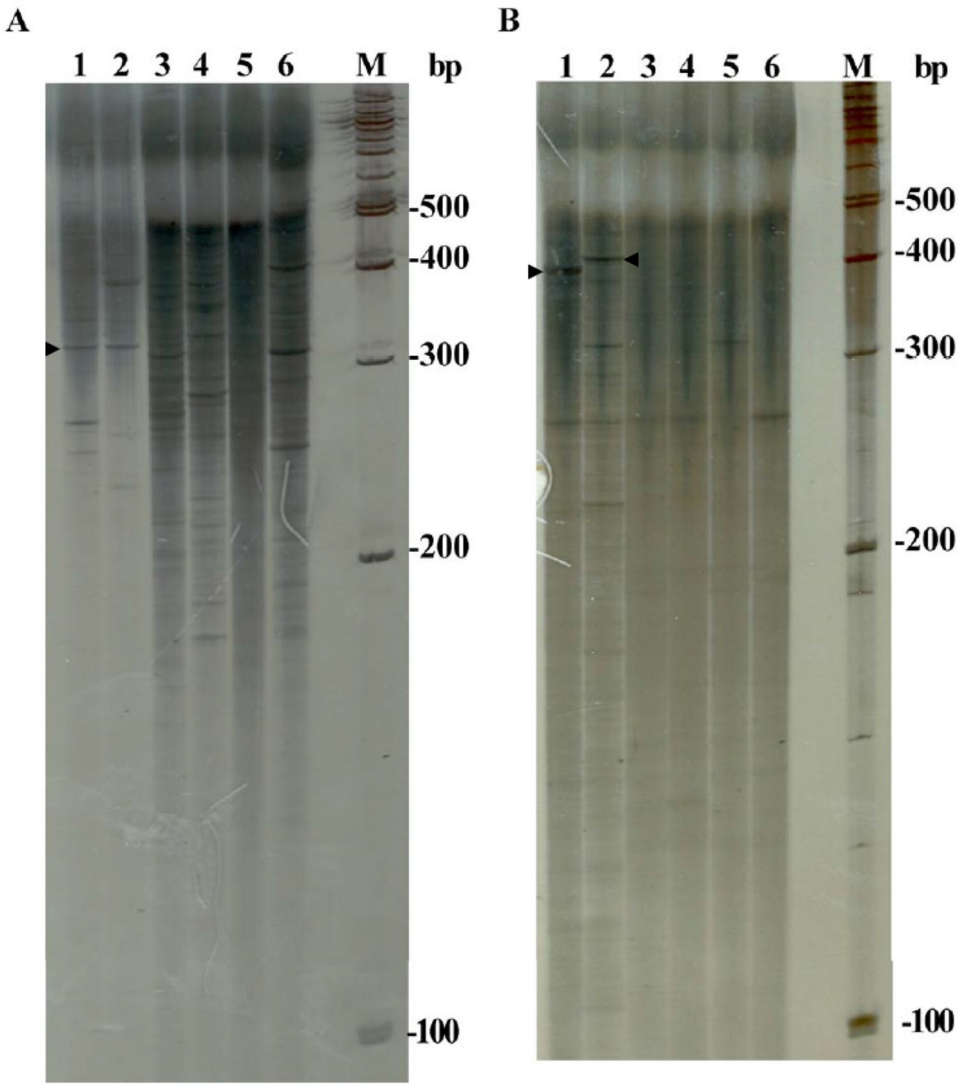

Fig. 1 A 4.5% denaturing polyacrylamide showing AFLP patterns of *H. harpax* (lane 1), *H. raphidea* (lane 2), *E. woodmasoni* (lane 3), two replicates of the pooled *M. nepa* (lanes 4 and 5) and *O. oratoria* (lane 6) using primer combinations *Eco* RI_{+3}-14/*Mse* I_{+3}-2 (**a**) and *Eco* RI_{+2}-13/*Mse* I_{+2}-13 (**b**). Lane M is 100 bp of DNA. The arrowheads point to selected candidate species-specific markers

candidate species-specific AFLP fragments with data in GenBank indicate that five markers comprise newly unidentified sequences (E-values greater than 10^{-4}).

Fragments E_{+3}-14/M_{+3}-2HhHr, E_{+2}-13/M_{+2}-13Hh, E_{+2}-13/M_{+2}-13Hr, E_{+3}-9/M_{+3}-1Hh, and E_{+3}-5/M_{+3}-1Ew all significantly match with hypothetical protein C7M84_009465 (*Penaeus vannamei*, E-value = 2×10^{-6}), as well as to the retrovirus-related Pol polyprotein from the opus transposon (*Portunus trituberculatus*, 7×10^{-13}), the CGG triplet repeat-binding protein 1 (*Aphis craccivora*, 1×10^{-30}), and the putative polyketide synthase 3 (*Symbiodinium microadriaticum*, E-value = 1×10^{-05}), respectively (Table 1).

Species-specific PCR of AFLP-derived SCAR markers

Primers were further designed for a species-specific AFLP-derived SCAR marker (Table 1). A preliminary species-specificity test revealed that primer E_{+2}-13/M_{+2}-13Hr_{158}F/R generated expected bands in just *H. harpax* but not in other species (*H. raphidea*, *M. nepa*, *O. oratoria*, and *E. woodmasoni*). In contrast, E_{+3}-14/M_{+3}-2HhHr$_{151}$F/R, E_{+2}-13/M_{+2}-13Hh$_{150}$F/R, and E_{+3}-9/M_{+3}-1Hh$_{209}$ generated expected bands in both *H. harpax* and *H. raphidea*, while E_{+3}-4/M_{+3}-12HhHr$_{171}$F/R, E_{+2}-4/M_{+2}-14MnOoII$_{107}$F/R, E_{+3}-1/M_{+3}-9Hr$_{244}$, and E_{+3}-5/M_{+3}-1Mn$_{208}$ generated expected bands in all species examined. No positive amplification product in any species was observed in the case of either E_{+2}-4/M_{+2}-14MnOo$_{228}$F/R or E_{+3}-5/M_{+3}-1Ew$_{200}$ (Fig. 2). PCR products of primers E_{+2}-13/M_{+2}-13Hh150, E_{+3}-14/M_{+3}-2HhHr$_{151,}$ and E_{+3}-5/M_{+3}-1Mn$_{208}$ were then selected for further identification in SSCP analysis. Results show that E_{+2}-13/M_{+2}-13Hr$_{158}$F/R were only seen in *H. harpax*; these primers were therefore further tested to encompass a larger sample size from processed samples.

SSCP genotyping of species-specific markers in mantis shrimp

A SSCP analysis was performed to identify whether, or not, the amplification products of developed SCAR markers contain single nucleotide polymorphisms (SNPs) for Harpiosquillid and Squillid mantis shrimp species. Results show that SNP patterns from both E_{+2}-13/M_{+2}-13Hh$_{150}$ and E_{+3}-14/M_{+3}-2HhHr$_{151}$ were polymorphic between *H. harpax* and *H. raphidea* (Fig. 3), while E_{+3}-5/M_{+3}-1Mn$_{208}$ elucidated polymorphic SSCP patterns among *H. harpax*, *H. raphidea*, *E. woodmasoni*, *M. nepa*, and *O. oratoria* (Fig. 3). These species-specific markers can also be used for other applications including the identification of species

Table 1 Summary of primer results

AFLP marker	Specificity	Size of fragment (bp)	SCAR primer sequence	Expected product (bp)	Blast analysis (closest species, e-value)
E_{+3}-4/M_{+3}-12	Hh, Hr	240	5′CTCAACTCgggTATggTAAgAC3′ 5′gATgAgTCCTgAgTAACggTAA3′	171	Unknown
E_{+3}-14/M_{+3}-2	Hh, Hr	323	5′CTCCTACTTCCAAAATgTCACC3′ 5′gCgTCAAgACCACgATTTACCC3′	151	Hypothetical protein C7M84_009465 (*Penaeus vannamei*, 2×10^{-6})
E_{+2}-4/M_{+2}-14I	Mn, Oo	478	5′CCCAgAACAACgAAAggAATAg3′ 5′AATAgTCCAAACCgACAAgAgg3′	228	Unknown
E_{+2}-4/M_{+2}-14II	Mn, Oo	240	5′gTCAAgTAAggTTgggATAgTg3′ 5′AAgCggTgTCTTCAgAgTggTA3′	107	Unknown
E_{+2}-13/M_{+2}-13	Hh	403	5′TTCAAACCAAACACgCTCCACA3′ 5′AgTAgTgAAgTCTggCTTgTgT3′	150	Retrovirus-related Pol polyprotein from the opus transposon (*Portunus trituberculatus*, 7×10^{-13})
E_{+2}-13/M_{+2}-13	Hr	420	5′ATTgACgAgACATCTgATTgCg3′ 5′AgggAATCgTTgAAAAAAgCgg 3′	158	CGG triplet repeat-binding protein 1 (*A. craccivora*, 1×10^{-30})
E_{+3}-1/M_{+3}-9	Hr	408	5′ ggTgTAAATgCgACTgTggC 3′ 5′ TTTCCCCCCTTTATTCCTTg 3′	244	Unknown
E_{+3}-9/M_{+3}-1	Hh	419	5′ TTgCgACgATAAAAACATTg 3′ 5′ TgACgAgACATCTgATTgCg 3′	209	Ribonuclease H-like domain, Domain of unknown function DUF659 (*Cinara cedri*, 3×10^{-34})
E_{+3}-5/M_{+3}-1	Mn	463	5′ GGCGAGAACTTGCTGCGAAC 3′ 5′ TATTTgAgACCACgACACgg 3′	200	Unknown
E_{+3}-5/M_{+3}-1	Ew	336	5′ TgCgTAAgAgTCTgTTCAAT 3′ 5′ TAACATTCAggTAggCAAgC 3′	208	Putative polyketide synthase 3 (*Symbiodinium microadriaticum*, 1×10^{-05})

Accession numbers MT321633-MT321637, MT294137 and MT321638-MT321641, respectively

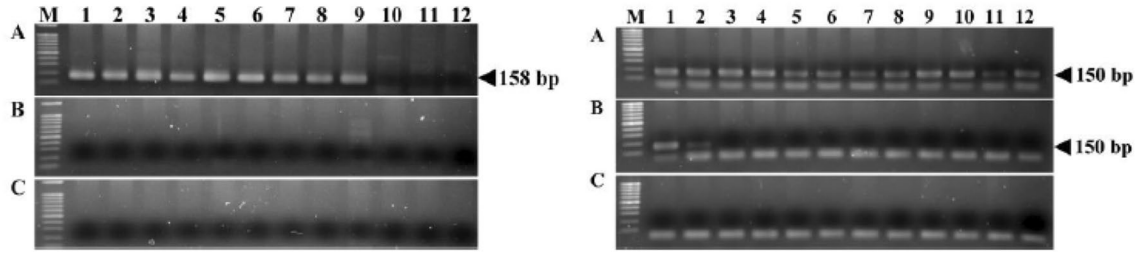

Fig. 2 A 1.8% ethidium bromide stained agarose gel showing PCR products of SCAR markers derived from *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h (158 bp) and *Eco* RI_{+2}-13/*Mse* I_{+2}-13-Hh (150 bp) tested against gDNA of *H. harpax* (lanes 1–9A), *H. raphidea* (lanes 10–12A and 1–2B), *M. nepa* (lanes 3–9B), *E. woodmasoni* (lanes 10–12B and 1–4C), and *O. oratoria* (lanes 5–11C). Lanes 12C and M contain a negative control (without DNA template) and a 100 bp DNA marker, respectively

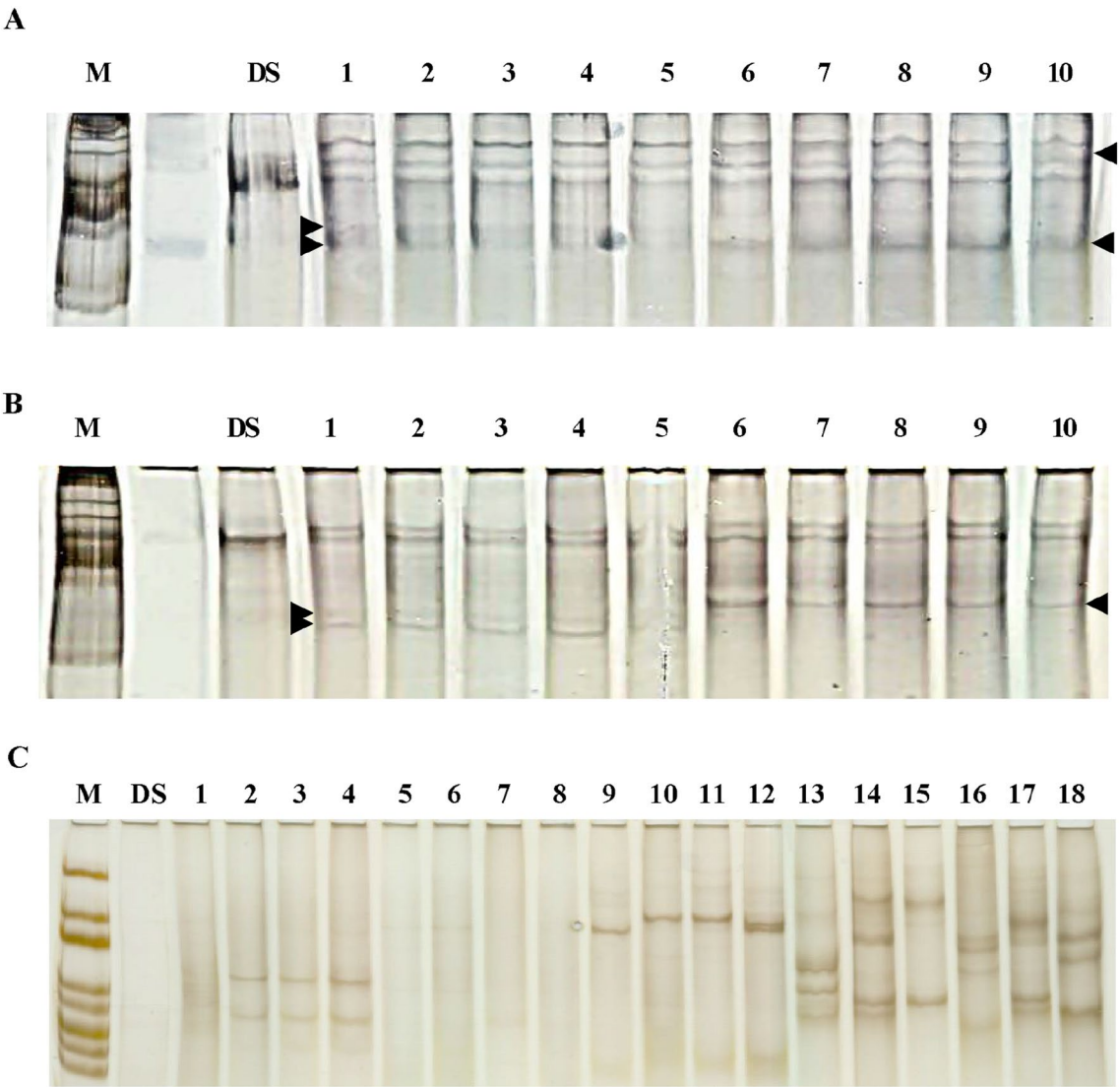

Fig. 3 A 17.5% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of amplification product of a SCAR marker derived from E_{+2}-13/M_{+2}-$13Hh_{150.}$ (**a**), E_{+3}-14/M_{+3}-$2HhHr_{151}$ (**b**) tested against gDNA of *H. harpax* (lanes 1–5**a** and **b**) and *H. raphidea* (lanes 6–10**a** and **b**). A 15% silver stained non-denaturing polyacrylamide gel (37.5:1) showing SSCP patterns of the amplification product of a SCAR marker derived from tested against gDNA of *H. raphidea* (lanes 1–4**c**), *H. harpax* (lanes 5–8**c**), *E. woodmasoni* (lanes 9–12**c**), *M. nepa* (lanes 13–15**c**), and *O. oratoria* (lanes 16–18**c**). Lanes M and DS contain a 100 bp DNA ladder as well as the non-denatured PCR product (double stranded DNA control), respectively

with similar morphologies as well as species identification in food products.

Stability of *H. harpax*-specific SCAR markers in processed mantis shrimp samples

Results show that PCR amplification using primers E_{+2}-13/M_{+2}-13Hr_{158} successfully confirmed consistently amplified gDNA of the five saline-preserved and heat-dried mantis shrimp samples. Data also show that only *H. harpax* DNA from both saline-preserved and heat-dried processes was successfully amplified (100%) (Fig. 4I, II); thus, primer E_{+2}-4/M_{+2}-14MnOoII$_{107}$ was used as the DNA quality control for amplification results (Fig. 4III). DNA extracted from *H. harpax* using phenol/chloroform-based methods indicate the sensitivity and reliability of species-specific SCAR markers. Results show that at least 0.5 ng of *H. harpax* DNA templates can be used for PCR amplification using *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h primer (data not shown).

Discussion

Species-specific markers gained from amplified fragment length polymorphism (AFLP) analysis

The AFLP method is a very useful tool for identifying multi-loci molecular markers [15]. Species-specific markers for mantis shrimp were developed from fixed polymorphisms in the gDNA of each species. BSA was applied during the screening step to reduce tedious and time-consuming processes; this was carried out by pooling an equal amount of DNA from several individuals of a particular species. Once candidate markers were identified, they were used to individually screen or test against a greater number of bulked DNA to confirm the accuracy of results [21]. The AFLP results reported here reveal that more than one nucleotide sequence was obtained from a single insert and indicates that one fragment represented co-migrating pieces that had different nucleotide sequences but similar sizes [22]. The

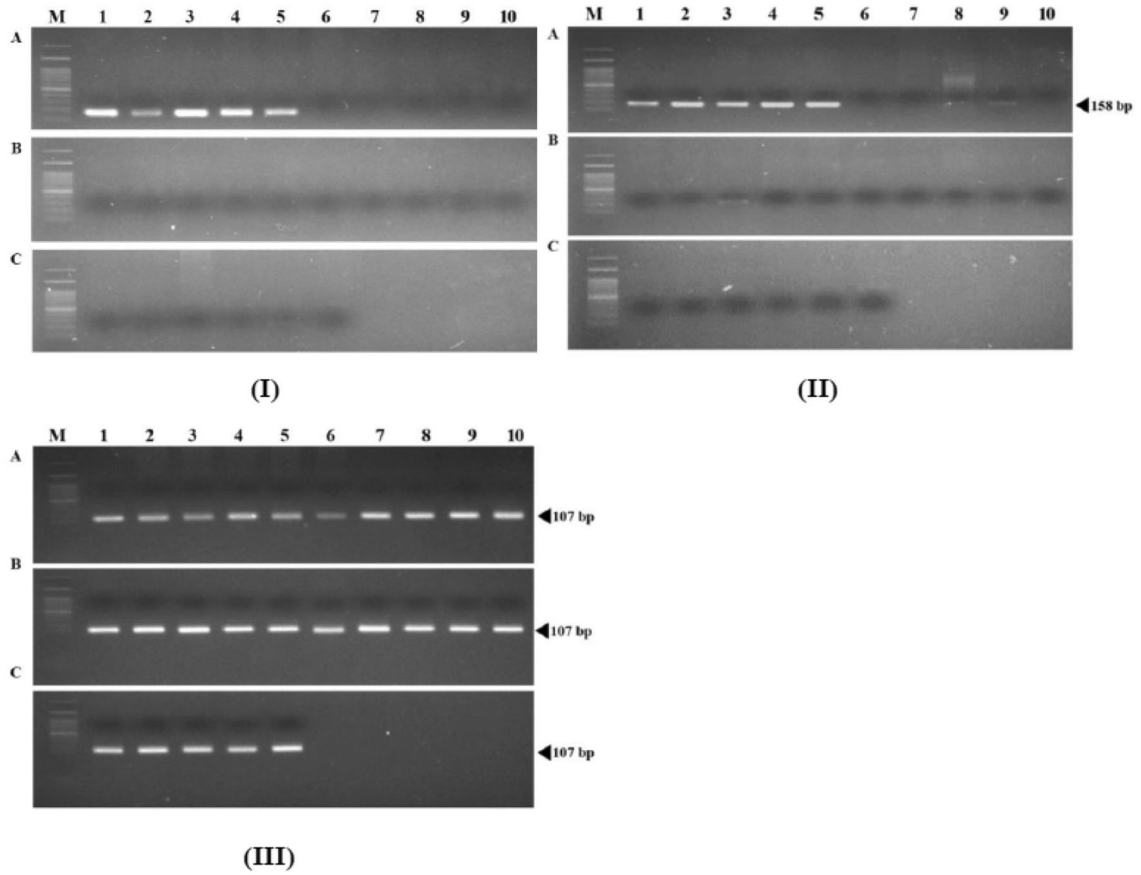

Fig. 4 A 1.8% ethidium bromide stained agarose gel showing PCR products of a SCAR markers derived from *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h (158 bp) tested against gDNA of saline-preserved (**I**) and heat-dried processes (**II**) *H. harpax* (lanes 1–5A), *H. raphidea* (lanes 6–10A), *E. woodmasoni* (lanes 1–5B), *M. nepa* (lanes 6–10B), and *O. oratoria* (lanes 1–5C). Lanes 6C and M contain a negative control (without DNA template) and a 100 bp DNA marker, respectively. Primer E_{+2}-4/M_{+2}-14MnOoII$_{107}$ was used for DNA quality control (**III**)

AFLP fingerprints generated here were highly reproducible and reveal genetic differentiation between genera and amongst mantis shrimp species.

The potential of AFLP approach was then used to determine fish and seafood species (e.g., *Penaeus monodon*, *Procambarus clarki*, and *Austropotamobius pallipes*) in processed commercial products and domestic stocks [17]. An AFLP-derived SCAR marker for specific mantis shrimp species has hitherto not been reported. We therefore present a low cost and convenient AFLP-based series of SNP markers that are highly efficient for developing species-specific tests without prior information. This approach has been especially useful within highly homozygous species in the same genus.

Results show that of the ten AFLP-derived SCAR markers, only the *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h primer generated *H. harpax*-specific PCR results. Two AFLP-derived SCAR markers (E_{+2}-13/M_{+2}-13Hh_{150} and E_{+3}-14/M_{+3}-2$HhHr_{151}$) generated amplification products in just the Harpiosquillid mantis shrimp, *H. raphidea* and *H. harpax*. Polymorphic SSCP genotypes of E_{+2}-13/M_{+2}-13Hh_{150} and E_{+3}-14/M_{+3}-2$HhHr_{151}$ therefore allow population genetic studies to be undertaken for these species and can therefore be applied for the conservation and selection of a particular genetic stock for aquacultural purposes.

Species-diagnostic markers of mantis shrimp based on SSCP analysis of AFLP-derived SCAR fragments

Results show that E_{+3}-5/M_{+3}-1Mn_{208} primers generated amplification products in all mantis shrimp species. Indeed, SSCP analysis, favored for identifying species origins due to convenience and cost-effectiveness, was then applied to verify whether, or not, nucleotide sequences from species were different [23]. Non-overlapping SSCP patterns among closely related mantis shrimp species were then observed; these polymorphic SSCP markers can therefore be used efficiently for species authentication in this economically important species as well as the previous studied in *P. pelagicus* [24] and many economically important fish species [13]. In this study, results reveal more than one pattern of E_{+3}-5/M_{+3}-1Mn_{208} in SSCP genotypes of *M. nepa* and *O. oratoria*; it is clear that SSCP analysis preferred short fragments to provide reduced incidences of conformation change and lower intraspecific DNA variability relative to longer sequence fragments.

In terms of morphological information, an ambiguous taxonomic diagnostic always happens in the mantis shrimp study [2, 8]. The cytochrome c oxidase subunit-I (COI) and the large ribosomal subunit (16S) rRNA have been applied in conservative studies of DNA barcoding to assess stomatopod larvae biodiversity in the Indo-Pacific and in waters around Hong Kong [25, 26]. Stomatopod species identification remains in an early stage of development, however, in terms of molecular analysis; species-specific markers can therefore play important roles in processed product quality control for this economically important species.

At present, DNA sequences for most stomatopod species are rare in databases. This means that high throughput DNA sequencing methodology, including next generation sequencing technology (NGS), will be useful for the large-scale genomic and transcriptomic sequencing of mantis shrimp species worldwide [27]. In earlier work, [28] determined gene expression profiles of hemocytes from *Harpiosquilla raphidea* using this approach and identified 93,344 assembly consensus sequences using the software Trinity [29]. A total of 219 differentially expressed transcripts were identified.

A *H. harpax*-specific PCR based on the *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h primer in processed samples

We have successfully developed species-specific SCAR markers for *H. harpax* in this analysis that can be applied in Thai waters. Amplification success for *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h was observed even using poor quality gDNA extracted from processed samples. The consistency and reproducibility of expected amplification products from *H. harpax* demonstrates that it is possible to develop markers for the simple and rapid authentication of *H. harpax* products as this is a valuable species. Species-diagnostic markers have been developed so far for five species of penaeid shrimp [14] as well as abalone [30], oysters [31], mud crab [32], and blue swimming crab [24] in Thailand. However, aquaculture technology for mantis shrimp lags behind, and has not been so successful. The species-specific SCAR markers presented here will prove useful for the study of new species as well as for the examination of the origins of new populations of Hapiosquillid mantis shrimps in the future.

Conclusions

In conclusion, this report is the first to describe the development of SCAR markers that can be used to identify species of mantis shrimps. Genera of harpiosquillid and squillid mantis shrimps can be clearly distinguished using E_{+3}-14/M_{+3}-2$HhHr_{151}$F/R, E_{+2}-13/M_{+2}-13Hh_{150}F/R, and E_{+3}-9/M_{+3}-1Hh_{209} primers and conventional PCR. Species identification between the largest stomatopods, *H. raphidea* and *H. harpax*, was possible using the E_{+2}-13/M_{+2}-13Hr_{158}F/R primer as well as E_{+2}-13/M_{+2}-13Hh_{150} and E_{+3}-14/M_{+3}-2$HhHr_{151}$ SSCP patterns. The species-specific identification of five economically important mantis shrimp was possible using E_{+3}-5/M_{+3}-1Mn_{208} SSCP patterns.

We were able to obtain a species-specific SCAR marker for mantis shrimp from AFLP fragments. Our PCR

amplification of *Eco* RI_{+2}-13/*Mse* I_{+2}-13-h primer clearly demonstrates the presence of this unique band in just *H. harpax* and not in the other four mantis shrimp species assessed. The SCAR markers revealed here will prove useful for the future rapid authentication of this economically important species in both natural stocks and commercial products.

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Compliance with ethical standards

Conflict of interest Authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval The main research project researcher of this study was approved for animal ethics by Institute of animals for scientific purposes development (IAD), THAILAND (Animal used license U1-03631-2559, National Level). This research was approved for biosafety by the Institutional Biosafety Committee of Burapha University Thailand.

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