

Rapid Species Identification of Elasmobranch Fish (Skates and Rays) using Oligonucleotide Microarray

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Abstract

Correct identification and classification of fish species is important for conservation and management of the fish resources. However, previous species identification in skates and rays based on morphological similarities and differences is sometimes misleading. All over the world, there are 485 species in Rajiformes. Despite high species diversity, the species in this family share similar morphological features. Therefore, an accurate species identification system is necessary in this family. In the present study, we developed an oligonucleotide microarray for species identification of skates in the waters of Korea. We verified genetic variation of skates by sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI). All microarray results corroborated the species-specific sequences and allowed simple, fast and cost-effective discrimination of large number of samples. These results indicate that the oligonucleotide microarray can be a useful tool for rapid species identification of skates.

Keywords: Skate, COI, Microarray, Rajiformes, Elasmobranch

Introduction

The majority of skates (family Rajidae) are marine

demersal cartilaginous fishes that inhabit continental and insular margins at depths shallower than 3,000 m¹. They have flat pectoral fins continuous with their head, two dorsal fins and a short, spineless tail. All over the world, there are 485 species, 61 genera in Rajiformes and 227 species, 25 genera in Rajidae (<http://sp2000.org>). In the ocean systems surrounding Korea, 11 species of skates belonging to 4 genera have so far been identified. These are *Bathyraja isotrachys*, *Bathyraja bergi*, *Dipturus kwangtungensis*, *Dipturus macrocauda*, *Dipturus tengu*, *Okamejei kenoei*, *Okamejei acutispina*, *Okamejei boesemani*, *Okamejei meerdervoortii*, *Raja pulchra* and *Raja koreana*².

Skates are commercially important fish species in Korea. Especially, *Raja pulchra* enjoys a higher consumer preference and fetches a higher price than other raja species because of its better meat quality. In many cases, skates are served in the market as fresh or fermented fillets. Since the distinguishing morphological features are no longer detectable in the fillets of the skates, consumers can sometimes get deceived for the species and make a payment more than the normal. Despite high species diversity, species of the Rajidae family share similar morphological features. Historically, skate species have been identified by morphological differences. However, this method has been problematic as some species display similar morphological features, making them extremely difficult to be distinguished³. Therefore, a reliable identification method for the skate species is necessary for proper conservation of the skate resources and fair trade of the skate products.

Recently, molecular techniques including DNA sequencing and polymerase chain reaction have been used in fishery science to improve species identification^{4,5}. Furthermore, DNA barcode using mitochondrial cytochrome c oxidase subunit I as a robust method for determining species identity has been proposed for a number of vertebrate and invertebrate taxa⁵⁻⁸. Such a method has provoked an international consortium to establish an international standard for monitoring and screening a species and also for accelerating the discovery of new species^{5,9}. The DNA barcode method was used in discriminating commercial species in Australia and confirmed that unambiguous identification of individually isolated fish eggs, larvae, fillets and fins from each species was possi-

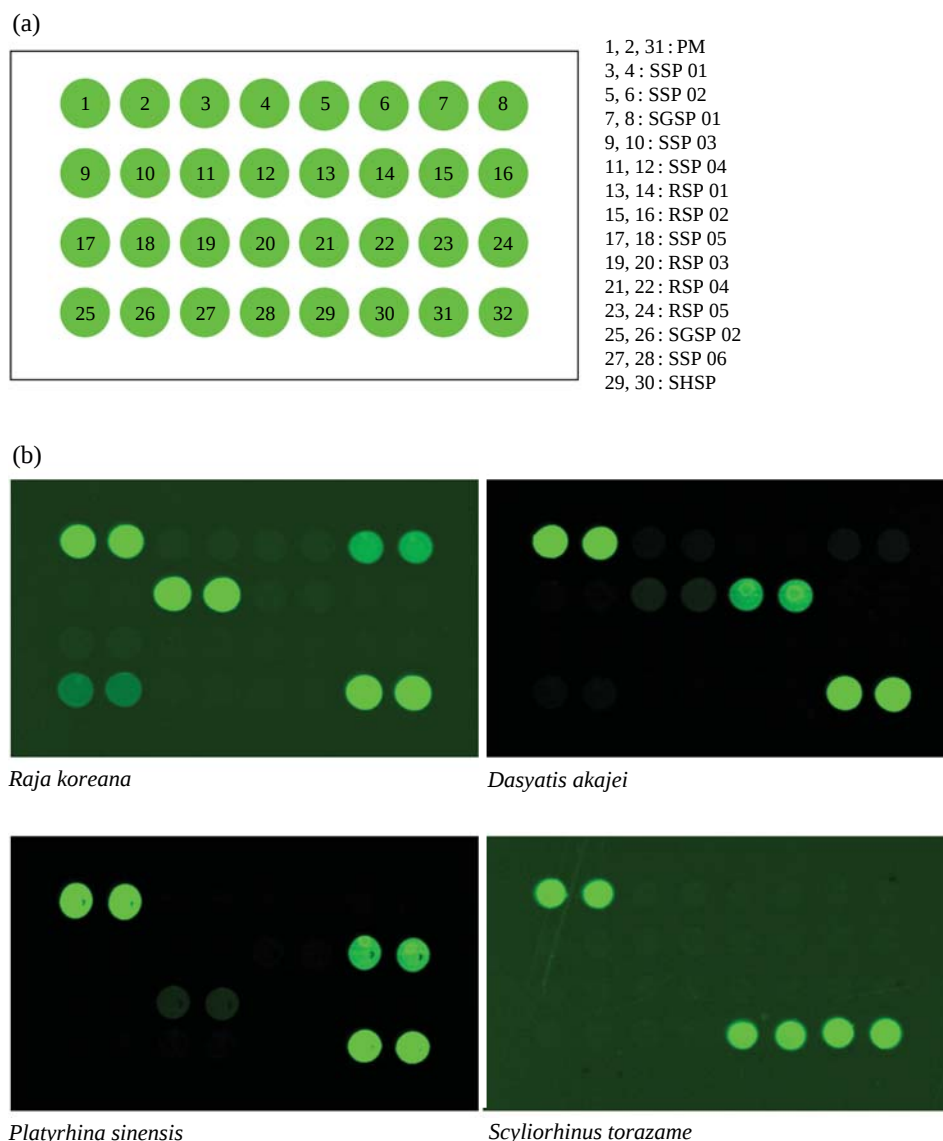


Figure 1. (a) Layout of microarray and (b) typical hybridization patterns for species identification of skates. All species-specific probes were printed in duplicate.

ble. In addition, DNA barcode based on single nucleotide polymorphisms (SNPs) has already been applied to a number of species¹⁰⁻¹⁴.

In the present study, we developed an oligonucleotide microarray for species identification of skates in Korean waters. We first determined genetic variation and designed species-specific probes for skates, rays, and sharks using a mitochondrial gene, cytochrome c oxidase subunit I (COI). The present oligonucleotide microarray contains six species of skates, five rays, and a shark. All microarray results were consistent with the species-specific sequences. The oligonucleotide microarray provides a sensitive and rapid method of identification for each species of skates.

Results and Discussion

Phylogenetic Analysis

A total of 32 specimens were successfully analyzed. A 654 bp fragment of the COI gene was consistently sequenced. All the new sequences have been deposited in GenBank (Table 1).

For the COI sequence, 255 of the 654 positions were variable among the 32 different sequences, with 245 positions judged informative for parsimony analysis (Figure 2). Most (209 of 255) substitutions occurred in third base codon positions, the remainder being in the first codon position (44 of 255), except for 2, which was in the second position. There were no deletions

Table 1. The list of skates, rays and sharks analyzed for COI sequences.

Order	Family	Genus	Scientific Name	Voucher specimen	Genbank acc.
Rajiformes	Rajidae	Okamejei	<i>Okamejei kenojei</i>	FOken050308-1	EF150858
			<i>Okamejei kenojei</i>	FOken050308-2	EF150859
			<i>Okamejei kenojei</i>	FOken050308-3	EU310786
			<i>Okamejei kenojei</i>	FOken050308-4	EU310787
			<i>Okamejei kenojei</i>	FOken050317-1	EU310788
			<i>Okamejei kenojei</i>	FOken050317-2	EU310789
			<i>Okamejei kenojei</i>	FOken050402-1	EU310794
			<i>Okamejei kenojei</i>	FOken050402-2	EU310795
			<i>Okamejei kenojei</i>	FOken050513-1	EU310798
			<i>Okamejei acutispina</i>	FOacu050513-1	EU334808
			<i>Okamejei acutispina</i>	FOacu050513-2	EU334809
			<i>Okamejei acutispina</i>	FOacu050718-1	EU334810
			<i>Okamejei acutispina</i>	FOacu050718-2	EU334811
			<i>Okamejei meerdervoortii</i>	FOmee050915-1	EU334814
	Raja	Raja	<i>Raja pulchra</i>	FRpul050326-3	EU327176
			<i>Raja pulchra</i>	FRpul050402-1	EU327178
			<i>Raja pulchra</i>	FRpul050506-1	EU327170
			<i>Raja pulchra</i>	FRpul050506-2	EU327171
			<i>Raja koreana</i>	FRkor050818-1	EU339348
			<i>Raja koreana</i>	FRkor050818-2	EU339349
	Dipturus	Dipturus	<i>Dipturus kwangtungensis</i>	FDkwa050511-1	EU339344
			<i>Dipturus kwangtungensis</i>	FDkwa050721-1	EU339346
			<i>Dipturus kwangtungensis</i>	FDkwa050721-2	EU339347
	Rhinobatidae	Platyrrhina	<i>Platyrrhina sinensis</i>	FPsin050818-1	EU339357
			<i>Platyrrhina sinensis</i>	FPsin050818-2	EU339358
	Dasyatidae	Dasyatis	<i>Dasyatis matsubarae</i>	FDmat050818-1	EU339363
			<i>Dasyatis akajei</i>	FDaka050513-1	EU339356
	Urolophidae	Urolophus	<i>Urolophus aurantiacus</i>	FUaur050818-1	EU339352
			<i>Urolophus aurantiacus</i>	FUaur050818-2	EU339353
	Myliobatidae	Aetobatus	<i>Aetobatus flagellum</i>	FAfla050620-1	EU339362
Carcharhiniformes	Scyliorhinidae	Scyliorhinus	<i>Scyliorhinus torazame</i>	FStor050417-1	EU339365
			<i>Scyliorhinus torazame</i>	FStor050417-2	EU339366

or insertions, and all sequences could be translated according to the standard vertebrate mtDNA code. Descriptive statistics for phylogenetic analysis were as follows: nucleotide base frequencies (given in percent) were A=24.6%, C=27.9%, G=17.6%, and T=29.9%. The average A+T content was 54.5% overall and 60% in the third codon positions. The proportion of G at the third codon position (8.7%) was very low. Skates had higher G+C contents than rays and the shark (46.4%, 44.3%, 39.7%, respectively).

Molecular information indicated that *O. kenojei* and *O. acutispina* were the closest relatives with a sequence divergence of 9% and 97 bootstrap replicate support (Figure 2, Table 3). *Okamejei meerdervoortii* was the sister taxon of this clade, with sequence divergences of approximately 10% to either of the species (Table 3).

Raja pulchra and *R. koreana* were grouped as sister taxa and together formed a clade, with sequence di-

vergence of 10% and 63 bootstrap that was sister to *Dipturus kwangtungensis*. Average intergeneric sequence divergence was 12% between *Okamejei* and *Dipturus* species, 13% between *Okamejei* and *Raja*, and 10% between *Dipturus* and *Raja*. Intrageneric levels of divergence were 4.8% among the three *Okamejei* species and averaged 5.3% across congeneric pairs within *Raja* (Only Korean species). These values are congruous with those reported in other chordate species, where divergence in a 710 bp fragment of COI ranged from 4 to 32% in 94% of congeneric pairs (3, 20). A well-supported clade was observed containing the *Okamejei*, *Raja*, and *Dipturus* genera. Consequently, Rajidae is considered monophyletic and is distinct from the other 4 families (3 rays and shark) with 1,000 bootstrap replicate support. Within the Rajidae family, average sequence divergence was 11% among 6 species.

Dasyatis matsubarae and *D. akajei* were grouped as

Table 2. Pair-wise nucleotide differences (below diagonal) and proportion of sequence divergence (above diagonal) in 654 bp of cytochrome c oxidase subunit I from each species (*Okamejei kenojei*, *Okamejei acutispina*, *Okamejei meerdervoortii*, *Raja pulchra*, *Raja koreana*, *Dipturus kwangtungensis*, *Platyrrhina sinensis*, *Dasyatis matsubarae*, *Dasyatis akajei*, *Urolophus aurantiacus*, *Aetobatus flagellum*, *Scyliorhinus torazame*).

	O. <i>kenojei</i>	O. <i>acutispina</i>	O. <i>meerdervoortii</i>	R. <i>pulchra</i>	R. <i>koreana</i>	D. <i>kwangtungensis</i>	P. <i>sinensis</i>	D. <i>matsubarae</i>	D. <i>akajei</i>	U. <i>aurantiacus</i>	A. <i>flagellum</i>	S. <i>torazame</i>
<i>Okamejei kenojei</i>	–	0.09	0.10	0.14	0.11	0.12	0.27	0.24	0.23	0.26	0.25	0.26
<i>Okamejei acutispina</i>	51.90	–	0.10	0.13	0.11	0.12	0.26	0.24	0.24	0.26	0.24	0.26
<i>Okamejei meerdervoortii</i>	61.10	62.00	–	0.12	0.11	0.10	0.26	0.24	0.24	0.24	0.23	0.23
<i>Raja pulchra</i>	81.15	76.00	72.50	–	0.10	0.10	0.24	0.24	0.24	0.25	0.24	0.27
<i>Raja koreana</i>	65.90	68.00	68.00	58.50	–	0.10	0.26	0.24	0.23	0.25	0.24	0.27
<i>Dipturus kwangtungensis</i>	70.70	70.00	60.00	60.50	58.00	–	0.25	0.23	0.23	0.27	0.24	0.26
<i>Platyrrhina sinensis</i>	143.30	141.50	140.50	129.75	140.50	135.17	–	0.27	0.28	0.26	0.28	0.28
<i>Dasyatis matsubarae</i>	130.60	134.00	131.00	130.25	130.00	129.00	145.50	–	0.08	0.22	0.21	0.26
<i>Dasyatis akajei</i>	127.40	132.00	131.00	130.25	129.00	127.00	148.50	46.00	–	0.23	0.22	0.26
<i>Urolophus aurantiacus</i>	142.80	142.00	133.00	138.50	136.00	143.67	139.50	21.00	125.00	–	0.22	0.28
<i>Aetobatus flagellum</i>	138.70	133.00	129.00	132.00	131.00	133.67	151.50	14.00	121.00	120.00	–	0.25
<i>Scyliorhinus torazame</i>	140.50	144.00	126.00	146.75	144.00	141.00	148.00	40.00	141.00	148.00	137.00	–

sister taxa and together formed a clade, with sequence divergence of 8% and 100 bootstrap.

Each species showed unique sequences and the intergeneric variations were much greater than the intrageneric variations. Using the sequences we constructed the phylogenetic tree of the rays and skate. As you examined the Figure 3, Each species genus *Okamejei* and *Raja* showed a distinct group each other with high bootstrap value (> 50). To bring greater reliability for grouping of rajidae in the phylogenetic tree, molecular information of specimens about genus *Bathyrja* might be needed. Generally, rajidae is considered a separate cluster from other families with no overlap or sharing among them.

Species-specific Probes

To identify species among skates and rays, probes were designed based on species-specific variation sites in the mitochondrial COI gene. COI gene serves species-specific variation sites, which is enough to identify species. In analysis of COI gene variation, we detected variations within individuals, which prohibited from selecting species specific probes. In order for precise identification, individual variation had to be avoided.

In a previous study, probes containing more than two base variations showed difference of melting

temperature, which was enough to discriminate between positive and negative targets¹⁵. In this study, all selected probes contained more than two base variations. Also, the position of probes within target products was an important factor in determining signal intensity^{16,17}. Therefore, species-specific probes were selected as close as possible to fluorescent labeled positions. Each probe included the variation sites at central region¹⁵. To relieve steric hindrance, 10 mer of oligo dTTP was added at 5' end of the probes.

Specific probe for *Okamejei kenojei* was located in 381-400 of the mitochondrial COI gene. When compared with other fishes, numbers of mismatched sequence was minimum 2 to 7 bases. Mismatched bases of specific probe for *Raja koreana* were minimum 2 to 5 bases. In order to obtain more clear results, counts of mismatched sequence of target fish were more than two bases different to other fishes, and position of specific probes were near by 3' direction of reference¹⁵⁻¹⁷. Detailed target variation sites and specific probes sequence are shown in Table 1 and 2.

Hybridization Patterns of Skates, Rays and Shark using DNA Chip

For identification of specific species, target sequence was labeled with cyanine-3 by asymmetric PCR. During amplification, asymmetric condition of pri-

[illegible]

Figure 2. Variable nucleotide sites in 654 bp sequences of cytochrome c oxidase subunit I gene in skates, rays and sharks. Dots indicate nucleotide identity with the *Okamejei kenojei* (FOken050308-1) sequence.

mers allowed higher target products than opposite non-targeting, which included same sequence of pro-

bes. As a result, during hybridization, single strand formed target products were able to bind probes. Re-

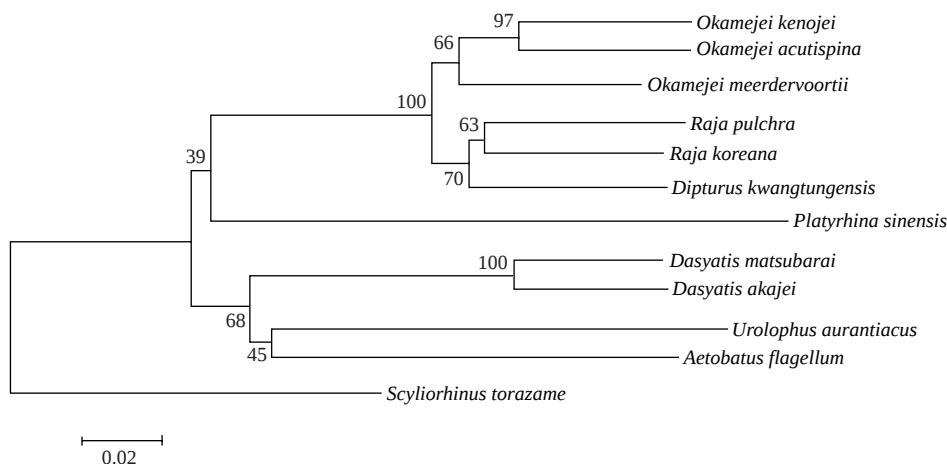


Figure 3. A phylogenetic tree of 12 taxa of skates, rays, and sharks based on the 654 nucleotide long mitochondrial COI sequences. The tree was produced by the Neighbor-joining method. Results of the bootstrap analysis are indicated in number. The scale bar indicates numbers of nucleotide substitution per site.

Table 3. Species-specific probe list for species identification.

Probe name	Sequence	Species
PM	5'-NH ₂ -T(10)GTTTCGCTCCTGATCCCTGG-3'	—
SSP 01	5'-NH ₂ -T(10)TAGACCTGACAATTTTCTCC-3'	<i>Okamejei kenojei</i>
SSP 02	5'-NH ₂ -T(10)TATGGCCCTCCCAGTCCTA-3'	<i>Raja pulchra</i>
SSP 03	5'-NH ₂ -T(10)CATCACTATATTACTACGGATCG-3'	<i>Dipturus kwangtungensis</i>
SSP 04	5'-NH ₂ -T(10)TTTGATCCAGCTGGAGGAG-3'	<i>Raja koreana</i>
SSP 05	5'-NH ₂ -T(10)CCCTGCCAGTACTAGCAGC-3'	<i>Okamejei acutispina</i>
SSP 06	5'-NH ₂ -T(10)ATCACCATATTACTTACAGATCG-3'	<i>Okamejei meerdervoortii</i>
RSP 01	5'-NH ₂ -T(10)CATTACTATGCTTCTCACAGATCG-3'	<i>Dasyatis akajei</i>
RSP 02	5'-NH ₂ -T(10)CCTCTCGTTACCTGTATTAGCAG-3'	<i>Platyrrhina sinensis</i>
RSP 03	5'-NH ₂ -T(10)GCTCTCTCTTCCTGTTTTCAGC-3'	<i>Urolophus aurantiacus</i>
RSP 04	5'-NH ₂ -T(10)TCCATCCTCATTACAACAATC-3'	<i>Dasyatis matsubarae</i>
RSP 05	5'-NH ₂ -T(10)CAGCAGGAATCACAATACTCCT-3'	<i>Aetobatus flagellum</i>
SHSP	5'-NH ₂ -T(10)CCTGTCCCTTGACGCC-3'	<i>Scyliorhinus torazame</i>
SGSP 01	5'-NH ₂ -T(10)TTAACTTCATCACCACAATTAT-3'	Skate group specific
SGSP 02	5'-NH ₂ -T(10)TGATCAATTCTTGTTACAAC-3'	Skate group specific

*PM: Position Marker, non-homologous sequence for skates, rays and shark

*SSP: Skate Specific Probe, RSP: Ray Specific Probe, SHSP: Shark specific probe

*SGSP 01: Skate Group Specific Probes, 438-459 region at COI gene

*SGSP 02: Skate Group Specific Probes, 509-531 region at COI gene

sults of hybridization showed clear pattern, because of melting temperature difference between perfect match sequences and mismatches when specific probe showed more than two bases mismatched¹⁷. In case of *Raja koreana*, specific signal intensity of DNA microarray was more than 15,000 arbitrary units (Figure 4a). Because each specific probe included a minimum of 2 base differences in target region, the hybridization patterns of other fishes also showed clear species identity. In experiments on *Dasyatis akajei*, *Platyrrhina sinensis* and *Scyliorhinus torazame*, hybridization signal intensity were more than 11,000 (Figure 4b, c, d). The fluorescence hybridization patterns of other fishes on a DNA chip are shown in Figure 1 and 2.

Conclusions

Skates are marine demersal fishes that inhabit the oceans of the world. In terms of evolution, skate is an elasmobranch like rays and sharks, being genetically very close to each other. In order to establish efficient management and conservation policies, reliable species identification of skates is necessary. Mitochondrial genome of animals has been highlighted as an appropriate target for identification, compared to the nuclear genome. The mitochondrial genome lacks introns, has limited exposure to recombination, and possesses haploid mode of inheritance¹⁸. High number of mitochondria in a cell and the short standardized

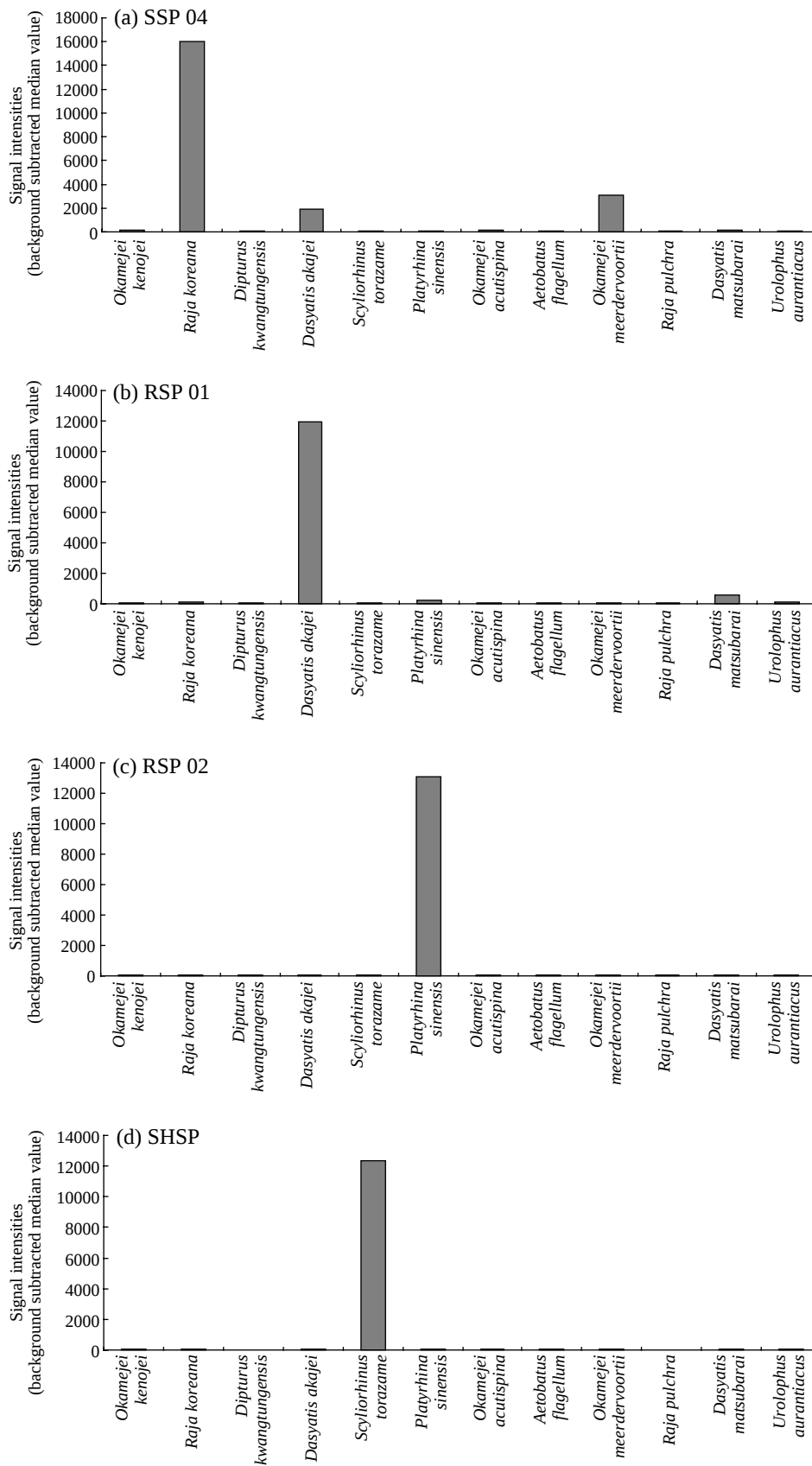


Figure 4. Hybridization signal intensities for each skate, ray and shark species. (a) *Raja koreana*, (b) *Dasyatis akajei*, (c) *Platyrrhina sinensis* and (d) *Scyliorhinus torazame*

sequences in the COI and Cytochrome b (CYTB) genes enable to distinguish individuals of a species based on high variable sequences among representative specimens. In many vertebrates and invertebrate taxa, COI gene has been used in species identification and also serves as a DNA barcode, an inventory of DNA sequences from a standardized genomic region. Furthermore, the COI gene is conserved enough to be amplified with broad-range primers and divergent enough to allow species discrimination, as the utility of barcoding relies on the assumption that genetic variation within a species is much smaller than variation between species. Although phylogenetic analysis using only COI gene is not sufficient enough to solve the evolutionary relationships between skates and rays, it is adequate for identification of species.

Recently, DNA microarray techniques have become efficient, practical and accurate tools for species identification^{19-21,26}. A microarray for species identification allows simple, fast and cost-effective discrimination of large number of samples simultaneously.

In this study, we developed a microarray for species identification of skates and rays. We determined genetic variation of skates by sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) and designed the species-specific probes. Species identification using the oligonucleotide microarray showed that it is a highly useful and practical tool for such a purpose. This method will also be applicable to management and conservation of other over-fished or endangered skate species.

Materials and Methods

Specimens and DNA Extraction

Specimens were purchased from fish markets during 2005. We made a concerted effort to collect multiple samples from each of the three ocean basins, including the Western sea, the Southern Sea and the East Sea. Taxonomic identification of samples are provided in Table 1. Specimens used for molecular analyses were retained as vouchers and deposited in the KORDI (Korea Ocean Research & Development Institute). A portion of the musculature was excised from fresh specimens of each species and immediately preserved in 95% ethanol. In total, 32 genomic DNA were extracted using the Qiagen DNeasy tissue kit following the manufacturer's protocol. DNA sample quality and quantity was analyzed by spectrophotometer and gel electrophoresis.

DNA Amplification and Sequencing

To amplify mitochondrial COI gene of skate, we

designed COI primers for skate family. The amplification reaction mixture consisted of 0.5 U of Taq polymerase (PROMEGA, USA), 1X GO Taq buffer, 1.5 mM magnesium chloride, 0.5 μ M of COI universal primers: Raja-COI F 5'-TCAGCCATCTTACC TGTGGC-3', Raja-COI R1, 5'-GGGTGTCCGAAG AATCAGAA-3', 200 mM each dNTP and 10 ng of genomic DNA of skates, rays and shark. PCR was carried out in a DNA-ENGINE (MJ Research, USA); 35 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 45 s and extension at 72°C for 1 min. To confirm the amplification process, PCR products were analyzed by electrophoresis with 1.5% agarose gels containing 0.5 μ g/mL ethidium bromide. Amplified COI fragments were isolated using the QIAquick PCR purification kit (QIAGEN, Germany) and used for PCR direct sequencing. Sequencing was carried out using the fluorescent dye-termination methods (Big Dye, Applied Biosystems, USA) and a genetic analyzer (ABI PRISM 3100 Avant, Applied Biosystems) according to the manufacturer's instructions. Chromatograms were visually checked and sequences aligned manually. MEGA 4.0 was used to produce a Nexus-compatible text file of all sequences for phylogenetic analysis²².

Sequence and Phylogenetic Analysis

DNA sequences were analyzed and aligned using ClustalW in MEGA4.0 under the following parameters: pairwise alignment parameters=gap opening 15, gap extension 1, DNA weight matrix IUB; multiple alignment parameters=gap opening 15, gap extension 1, delay divergent sequences 30%, DNA weight matrix IUB. The resulting sequence alignment was exported to Nexus format. The aligned sequences were analyzed with Distance Method using MEGA 4.0.

Scyliorhinus torazame was chosen as the designated out-group for COI gene analyses (due to its distant relationship to the Rajiformes). Sequence divergence was calculated using the Kimura-2-parameter (K2P) model²³. Phylogenetic analyses were performed by Neighbor-Joining method²⁴ with K2P distances, using the MEGA 4.0 program²². Bootstrap analysis (1,000 replicates) was used for Distance Method to assess to relative robustness of branches²⁵.

DNA Amplification for Hybridization

To obtain amplified COI gene fragments from skates, rays and a shark, amplification process was carried out using asymmetric PCR strategy. Concentration of reverse primer was ten times higher than forward primer. In order to differentiate between COI gene variations, reverse primer was labeled with cyanine 3 fluorescent dye during primer synthesis. Asymmetric

PCR products were directly hybridized to microarray without additional purification process.

Microarray for Species Identification

A total of 15 amino-modified oligonucleotide probes were located in duplicate on the slide, including one position marker probe and two universal family Rajidae probes. Universal Skate group specific probes were designed based on conserved region of skates COI gene to distinguish between rays and the shark. Synthesized oligonucleotide probes were resuspended at 50 μ M in Spot buffer (3X SSC, 1.5 M betaine) and then stored in 384 wells-microtitre plates. Specific probes were printed onto silylated slides (Cell Associate, USA) using the robotic microarrayer (Cartesian Technology, USA) with four sub-arrays, allowing parallel testing of different samples (Figure 1). After fabrication of microarray, spotted arrays were incubated in a humidity chamber with a controlled environment of 25°C and 60% humidity. Slides were washed with 0.1% SDS solution to remove remaining probes and avoid reacting and immobilizing by sodium borohydride solution (NaBH₄ 1 g : PBS 300 mL : Ethanol 100 mL). Remaining solution of treated slides were washed three times using nuclease free water and dried with centrifugation for 5 min at 800 rpm. Amplified asymmetric PCR products were separated from each other with the help of perfusion chamber (GraceBio, USA) and applied to microarray. Hybridization process was performed in 100 μ L of 3X SSC, 0.3% SDS for one hour at 55°C. Hybridization chamber was filled with distilled water in order to prevent evaporation of reaction solution. For elimination of non-reacting probes and hybridization components, the microarrays were washed with 1X SSC plus 0.1% SDS at 3 min, 1X SSC at 3 min and 0.1X SSC at one min, and dried with centrifugation. Washed and dried microarrays were stored at room temperature before scanning.

Scanning of Microarray

Hybridization signals of microarrays were scanned using Genepix 4000B (Axon instrument, USA). Scanning conditions were laser power 99% and PMT gain 600. After scanning, the fluorescence intensity of probes was quantified using the Genepix 4.1 software. In order to ascertain positive or negative result of each probe, image pixel intensities were calculated and the local background intensity was subtracted from median value of each spot.

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