Molecular Phylogeny of Asian Freshwater and Marine Stingrays Based on the DNA Nucleotide and Deduced Amino Acid Sequences of the Cytochrome *b* Gene

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To elucidate the phylogenetic relationship among stingrays of three freshwater and five marine species of the genera *Himantura*, *Dasyatis*, and *Pastinachus*, belonging to the family Dasyatidae distributed in Thailand, Laos, India, and Japan, we determined the nucleotide sequences of the mitochondrial DNA region encoding cytochrome *b*. Using two rayfish species belonging to the superfamily Myliobatoidea as out-groups, we found three clades for the collected stingrays in the family Dasyatidae from analyses using the neighbor-joining, maximum parsimony, and maximum likelihood algorithms. While species belonging to the genus *Himantura* formed one group, *Dasyatis laosensis* and *Dasyatis* sp. formed another group. The results obtained failed to show clear phylogenetic relationships of the genus *Pastinachus* with the genera *Dasyatis* and *Himantura*, since the corresponding bootstrap resampling values were 72, 55, and 65 in the neighbor-joining, maximum parsimony, and maximum likelihood trees, respectively.

Key words: cytochrome *b* gene, freshwater stingray, molecular phylogeny, mitochondrial DNA

Ichthyologists studying the elasmobranchs have noted that the interrelationships at lower taxonomic levels remain confusing and controversial.¹⁾ Recently, McEachran and Dunn²⁾ have presented a phylogenetic analysis of skates, which reviews and summarizes the anatomical studies published over the last 45 years. However, in terms of volume of publications, phylogenetic studies that dealt with freshwater and marine stingrays are meager. Thus, their phylogenetic relationships are still under debate, though the currently available information has been evaluated recently by Lovejoy.³⁾

According to Nelson,⁴⁾ elasmobranchs consist of nine orders, among which all rays are grouped into one order Rajiformes, which is subdivided into four suborders. Stingrays are assembled into the superfamily Dasyatoidea, which belongs to the suborder Myliobatoidei. The superfamily Dasyatoidea is categorized by Nelson⁴⁾ into two families: Dasyatidae and Urolophidae. The family Dasyatidae comprises marine, brackish, and freshwater stingrays,

which are distributed in the Atlantic, Indian, and Pacific regions. This family is further subdivided into two subfamilies, Dasyatinae (stingrays) and Potamotrygoninae (river stingrays). The family Urolophidae is represented by round stingrays which live in a marine habitat and are distributed in the western Atlantic, eastern Indian, and Pacific regions. This classification is mainly based on the fossil evidence and morphological characteristics exhibited by the extinct and extant representatives of the rayfishes.

From the analysis of mitochondrial cytochrome *b* gene from more than 100 animal species including mammals, birds, amphibians, fishes, and some invertebrates, Kocher *et al.*⁵⁾ inferred that during nearly 400 million years of evolution, only about four amino acids changes have occurred in the fish lineages, and that this rate of amino acid substitution is five times slower in comparison to bird and mammal lineages. Five species of shark (*Galeocerdo*, *Prionace*, *Sphyrna*, *Heterodontus*, and *Carcharhinus*) were included in this comparative study. In relation to this finding,

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Kitamura *et al.*⁶⁾ reported that there was no nucleotide variation in the cytochrome *b* gene segment (405 bp) among seven specimens of the bull shark *Carcharhinus leucas* collected from the Atlantic and Pacific Oceans and related river systems in Mexico and Nicaragua. Furthermore, DNA nucleotide sequences of the mitochondrial cytochrome *b* gene have also been used by Kitamura *et al.*⁷⁾ to clarify the molecular phylogeny of the sharks and rays of the superorder Squalea.

In this study, we determined the nucleotide sequences of mitochondrial DNA encoding cytochrome *b* to elucidate the molecular phylogenetic relationships of Asian freshwater and marine stingrays belonging to the genera *Dasyatis*, *Himantura*, and *Pastinachus*.

Fig. 1. A map showing sampling sites of the stingrays examined.

Materials and Methods

Materials

Nineteen sample specimens consisting of eight stingrays from Thailand, India, Laos, and Japan, and two rayfishes from Japan were examined (Fig. 1, Table 1).

Either fast skeletal muscle tissue or a segment of the skin tissue from each specimen was excised into pieces by scissors, immersed into absolute ethanol, and kept in a refrigerator before use.

DNA Preparation

The ethanol-treated muscles (20–40 mg) were put into 500 μ l of TNES (10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, and 0.5% SDS) containing 8 M urea and digested with 10–15 μ l of proteinase K (20 mg/ml) at 37°C overnight.⁷⁾ The samples were treated twice with phenol/chloroform/isoamylalcohol (25:24:1, v/v) and subsequently twice with chloroform/isoamylalcohol (24:1, v/v). To the aqueous layer containing DNA was added 50 μ l of 3 M sodium acetate (pH 5.2) and 1 ml of 99.5% ethanol. DNA pelleted by centrifugation at 16,000 $\times$ g for 15 min was washed with 70% ethanol, dried under vacuum, and dissolved in 500 μ l of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

DNA Amplification

DNA fragments encoding the mitochondrial cytochrome *b* gene were amplified by PCRs. Amplifying conditions were 93°C for 60 s in denaturation, 53°C for 90 s in annealing, and 72°C for 90 s in extension for 30 cycles, with a final polymerization step at 72°C for five min. PCR products were resolved by electrophoresis in a 1.5% agarose gel of a low melting point grade and stained with ethidium bromide to visualize DNA bands. The separated bands were excised from the gel and placed into 500

Table 1. Localities and habitats of Asian stingrays and ray fishes analyzed for the cytochrome *b* gene

Samples	Scientific name	Locality* ¹	Country	Habitat
1	<i>Himantura signifer</i>	Chaophraya R. (3)* ²	Thailand	Freshwater
2	<i>Himantura signifer</i>	Chaophraya R. (3)	Thailand	Freshwater
3	<i>Himantura signifer</i>	Chaophraya R. (3)	Thailand	Freshwater
4	<i>Himantura signifer</i>	Chaophraya R. (3)	Thailand	Freshwater
5	<i>Himantura chaophraya</i>	Chaophraya R. (3)	Thailand	Freshwater
6	<i>Himantura chaophraya</i>	Chaophraya R. (3)	Thailand	Freshwater
7	<i>Himantura chaophraya</i>	Ganges R. (1)	India	Freshwater
8	<i>Himantura imbricata</i>	Gulf of Thailand (4)	Thailand	Marine
9	<i>Himantura imbricata</i>	Gulf of Thailand (4)	Thailand	Marine
10	<i>Himantura imbricata</i>	Gulf of Thailand (4)	Thailand	Marine
11	<i>Himantura gerrardii</i>	Bay of Bengal (2)	India	Marine
12	<i>Pastinachus sephen</i>	Chaophraya R. (3)	Thailand	Freshwater/Marine
13	<i>Pastinachus sephen</i>	Chaophraya R. (3)	Thailand	Freshwater/Marine
14	<i>Pastinachus sephen</i>	Ganges R. (1)	India	Freshwater/Marine
15	<i>Dasyatis laosensis</i>	Mekong R. (5)	Laos	Freshwater
16	<i>Dasyatis laosensis</i>	Mekong R. (5)	Laos	Freshwater
17	<i>Dasyatis</i> sp.	Gulf of Thailand (4)	Thailand	Marine
18	<i>Dasyatis</i> sp.	Gulf of Thailand (4)	Thailand	Marine
19	<i>Dasyatis akajei</i>	Suruga Bay (6)	Japan	Marine
20	<i>Aetobatus narinari</i>	Suruga Bay (6)	Japan	Marine
21	<i>Gymnura japonica</i>	Suruga Bay (6)	Japan	Marine

*¹ R denotes river.

*² Numbers in the parentheses represent sampling locations shown in Fig. 1.

Fig. 2. A schematic diagram of an amplified region of stingray mitochondrial DNA and sites of oligonucleotides (arrows) used as primers for PCR and DNA sequencing.

μ l of TE buffer. The gel bands containing DNA were incubated at 65°C for 20 min and DNA was extracted by the phenol/chloroform method as described before. Initial amplifications of DNA fragments encoding the full length of cytochrome *b* employed versatile primers, GLU1L (5'CACCGTTGTTATTCAACTAC3') for the light strand and THR1H (5'GGATTACAAGACCGATGCTT3') for the heavy strand, which were originally synthesized in this study by reference to the homologous sequences of carp, lungfish, amphibia, and human (Fig. 2). Later, we designed two new primers RACB1L (5'AAC-TTATCCCAGCTAACCC3') and RACB2H (5'GAAG-GTAAGGCTTCGTTGTT3') for the light and heavy strands of the cytochrome *b* gene, respectively, in addition to the published primers L-15460 (5'ACAGCCTTCGTAGGTTATGTACT3') and CB2-H (5'CCCTCAGAATGATATTTGCTCTCA3').⁸⁾

DNA nucleotide sequences were determined for both strands with a Perkin Elmer Applied Biosystems model 373S DNA sequencer after labeling samples with *Taq* Dye Deoxy terminator cycle sequencing kits.

Phylogenetic Analysis

The alignments for DNA nucleotide and deduced amino acid sequences were carried out by package software SeqEd (Perkin Elmer). The neighbor-joining,⁹⁾ maximum parsimony,¹⁰⁾ and maximum likelihood¹¹⁾ methods were employed throughout for phylogenetic tree constructions and presentation of the results. The neighbor-joining analysis was performed with PHYLIP, using Kinuma's two parameters genetic distance modified by Felsenstein.¹²⁾ The maximum parsimony analysis was carried out with MacClade¹³⁾ and PAUP,¹⁴⁾ using heuristic search. The maximum likelihood analysis was performed with DNAML in PHYLIP.¹²⁾

Results

Sample Collection

Himantura signifer, *H. chaophraya*, and *Dasyatis laosensis* are identified as obligatory freshwater stingrays.¹⁵⁾ While specimens of *Pastinachus sephen* were collected from the Chaophraya and Ganges Rivers (Fig. 1), this species inhabits both freshwater and marine environments.¹⁵⁾ Other stingray species in Table 1 belong to the marine habitat: *H. imbricata* and *Dasyatis* sp. (unidentified) collected from the Gulf of Thailand off Bangkok, and *H. gerrardii* collected from the Bay of Bengal off Calcutta. Two marine stingray species, eagle ray *Aetobatus narinari* and butterfly ray *Gymnura japonica*, which belong to the superfamily Myliobatoidea, were collected from Suruga

Bay off Shimizu in Japan and used as out-groups in this study. All the samples were collected in 1996 and 1997.

DNA Nucleotide and Deduced Amino Acid Sequences of Cytochrome *b*

DNA fragments encoding the full length of cytochrome *b* were amplified by PCR and sequenced using 5'- and 3'-primers designed in the present study or previously reported.^{7,8)} The region amplified for most species examined contained 1,143 nucleotides including the stop codon TAA and encoded 380 amino acids. *G. japonica* exceptionally contained 1,149 nucleotides encoding 382 amino acid residues, six nucleotides being inserted upstream the stop codon. Consequently, nucleotides from 1,141 to 1,146 (381–382th amino acid residues) of this species were omitted from the data analysis. With such arrangements for the DNA nucleotide sequence analysis, there were 402 variable sites (data not shown), whereas in the sequences of 380 amino acids, 88 variable sites were observed (Fig. 3).

Intraspecies Variations in Nucleotide and Amino Acid Sequences

Pairwise distances of nucleotide and amino acid sequences between the 12 taxa calculated by PAUP¹¹⁾ are shown in Table 2. Intraspecies variations were observed between samples geographically isolated for two freshwater species, *H. chaophraya* and *P. sephen*. One specimen of *H. chaophraya* from the Ganges River showed variations in 23 nucleotides and five amino acids from the two specimens from the Chaophraya River. One specimen of *P. sephen* from the Ganges River also showed differences in 91 nucleotides and seven amino acids from that of the *P. sephen* specimen collected from the Chaophraya River. On the other hand, only one nucleotide substitution was observed among the three specimens of *H. imbricata* from the Gulf of Thailand. No genetic variation was observed among the specimens of *H. signifer*, *D. laosensis*, and *Dasyatis* sp. examined. Intraspecific variations described above for *H. chaophraya* and *P. sephen* were large enough to be reminiscent of those in different species. Therefore, phylogenetic analyses were performed on 12 taxa including ten species and two geographically isolated races of *H. chaophraya* and *P. sephen* as shown in Table 2.

Ratio of Transition to Transversion

Both transition and transversion substitutions increased linearly with increasing genetic distance until around 0.2 (Fig. 4). The average value was calculated to be 4.1 for pairwise ratio of each taxa. Therefore, the value of 8.2 was used for weighing transversion to transition in the following phylogenetic analyses.

Phylogenetic Trees of Cytochrome *b* Gene

Three phylogenetic trees based on DNA nucleotide sequences by the neighbor joining, maximum parsimony, maximum likelihood methods, were obtained for 12 taxa, where two rayfishes collected in Japan were included in order to root the trees (Fig. 5). The following inferences were obtained from all the trees obtained. First, stingrays belonging to the genus *Himantura* formed one group, where the first freshwater stingray species *H. signifer* collected in Thailand branched with two marine stingray spe-

<i>H. signifer</i>	MAMNIRKTHPLLLKIINNSLIDLPAPTNISTWWNFGSLLGLCLIIQILTGLFLAMHYTADISSAFSSVAHI	70
<i>H. chaophraya</i> (T)	. . T F T I	
<i>H. chaophraya</i> (I)	. . TT F I	
<i>H. imbricata</i>	 L	
<i>H. gerrardii</i>	 I . . V T . S . . I	
<i>P. sephen</i> (T)	 I T . S . . I	
<i>P. sephen</i> (I)	 I . . V T . S . . I	
<i>D. laosensis</i>	. . T F T . A . . I M	
<i>D. species</i>	. . T F . . . D T . A . . I M	
<i>D. akajei</i>	. . T F T I M	
<i>A. narinari</i>	. . VN F S . . S A . . A P . . T	
<i>G. japonica</i>	. . T S T	
<i>H. signifer</i>	CRDVNYGWLIRNTHANGASLFFICAYLHIARGFYYSYLYKEAWNIGVILVLLMATAFVGIVLPWGQMS	140
<i>H. chaophraya</i> (T)	 I I L T L	
<i>H. chaophraya</i> (I)	 I I L I T	
<i>H. imbricata</i>	 M V T VL	
<i>H. gerrardii</i>	 M V T	
<i>P. sephen</i> (T)	 M V L N T	
<i>P. sephen</i> (I)	 M V L N T	
<i>D. laosensis</i>	 I M V L N T V	
<i>D. species</i>	 I M V L N T V	
<i>D. akajei</i>	S I M V L N T V	
<i>A. narinari</i>	 M I I T IM	
<i>G. japonica</i>	 M S I L N T V	
<i>H. signifer</i>	FWGATVITNLLSALPYIGDMLVQWIWGGFSDNATLTRFFAFHFLPFIIAALTMVHLLFLHETGSNNPI	210
<i>H. chaophraya</i> (T)	 I T	
<i>H. chaophraya</i> (I)	 I T	
<i>H. imbricata</i>	 T V . V	
<i>H. gerrardii</i>	 T V . V	
<i>P. sephen</i> (T)	 T V . V A T	
<i>P. sephen</i> (I)	 S T V . V A T	
<i>D. laosensis</i>	 T I T F . . VT I T	
<i>D. species</i>	 T I T F . . V I T	
<i>D. akajei</i>	 T I T F . . V I T	
<i>A. narinari</i>	 F T I T V . V I D T	
<i>G. japonica</i>	 I YT F . . L DS T	
<i>H. signifer</i>	GLDSNMDDKIPFHPYYSYKDLLGFFILLILLTLTLFLPNLLGDAENFIPANPLVTPPHIKPEWYFLFAYA	280
<i>H. chaophraya</i> (T)	 L A . . M . . YS V	
<i>H. chaophraya</i> (I)	 L A . . M . . YS	
<i>H. imbricata</i>	 M LII . . A . . M T	
<i>H. gerrardii</i>	 M LI . . F . . A . . M T	
<i>P. sephen</i> (T)	. . I FF . . F . . A T	
<i>P. sephen</i> (I)	. . I FF . . F . . A T	
<i>D. laosensis</i>	. . T . . T FT V FL A . . T N L	
<i>D. species</i>	. . T . . T FT M FL A . . T N I	
<i>D. akajei</i>	. . T . . T FT V FL A . . T N	
<i>A. narinari</i>	. . P . . D F . . F . . I L T T	
<i>G. japonica</i>	. . T . . D F . . A . . A . . M T	
<i>H. signifer</i>	ILRSIPNKLGGVLALAFSILILLVPLLHTSKQRSITFRPITQTLFWLLIVNTIILTWWIGGPVEQPFIT	350
<i>H. chaophraya</i> (T)	 M N L VT V	
<i>H. chaophraya</i> (I)	 M N L VT	
<i>H. imbricata</i>	 M I I VA I	
<i>H. gerrardii</i>	 F M Q . . I I VA I	
<i>P. sephen</i> (T)	 T I S . . L VA	
<i>P. sephen</i> (I)	 I S . . L VA	
<i>D. laosensis</i>	 V I I VT	
<i>D. species</i>	 V I I VT	
<i>D. akajei</i>	 V I I VT	
<i>A. narinari</i>	 I . . M I M . . VT	
<i>G. japonica</i>	 I MA . . V	
<i>H. signifer</i>	IGQIASITYFSFFLILFPFIAGWVENKMLNL	380
<i>H. chaophraya</i> (T)	 L	
<i>H. chaophraya</i> (I)	 L	
<i>H. imbricata</i>	 A L K	
<i>H. gerrardii</i>	 L K	
<i>P. sephen</i> (T)		
<i>P. sephen</i> (I)		
<i>D. laosensis</i>		
<i>D. species</i>		
<i>D. akajei</i>	 V	
<i>A. narinari</i>	 L SP	
<i>G. japonica</i>	 T FL L K . . DP	

Fig. 3. Amino acid sequences of the full length of cytochrome *b* in stingrays and rayfishes.

Dots indicate residues matching with those of *Himantura signifer*. The letters "T" and "I" in parentheses added to *H. chaophraya* and *P. sephen* indicate Thailand and India, respectively, where samples were collected. The nucleotide sequence data for deducing the present amino acid sequences will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB021494-AB021505.

Table 2. Nucleotide substitutions and amino acid replacements between taxa which are calculated with PAUP

	1	2	3	4	5	6	7	8	9	10	11
	<i>H. signifer</i>	<i>H. chaophraya</i> (T)	<i>H. chaophraya</i> (I)	<i>H. imbricata</i>	<i>H. gerrardii</i>	<i>P. sephen</i> (T)	<i>P. sephen</i> (I)	<i>D. laosensis</i>	<i>D. species</i>	<i>D. akajei</i>	<i>A. narinari</i>
1 <i>H. signifer</i>	—										
2 <i>H. chaophraya</i> (T)* ¹	181(26)* ³	—									
3 <i>H. chaophraya</i> (I)* ¹	176(25)	23(5)	—								
4 <i>H. imbricata</i>	146(25)	171(31)	169(31)	—							
5 <i>H. gerrardii</i>	166(28)	176(29)	177(31)	67(13)	—						
6 <i>P. sephen</i> (T)	207(26)	196(28)	194(30)	193(30)	174(25)	—					
7 <i>P. sephen</i> (I)	193(29)	188(29)	188(30)	181(32)	137(23)	91(7)	—				
8 <i>D. laosensis</i>	203(39)	188(31)	191(32)	198(41)	186(40)	172(29)	160(29)	—			
9 <i>D. species</i> * ²	204(39)	181(31)	192(32)	197(41)	182(40)	174(29)	163(29)	27(5)	—		
10 <i>D. akajei</i>	205(38)	184(30)	185(31)	200(40)	193(40)	177(29)	167(29)	37(6)	45(6)	—	
11 <i>A. narinari</i>	245(42)	238(41)	239(40)	227(43)	214(42)	216(41)	218(41)	206(42)	208(42)	208(42)	—
12 <i>G. japonica</i>	253(40)	233(34)	238(36)	217(39)	218(43)	224(35)	223(39)	214(40)	214(39)	216(37)	215(50)

*¹ T and I in the parentheses represent Thailand and India, respectively.

*² Species unidentified.

*³ Numbers in the parentheses represent the replacements of amino acid.

Fig. 4. Relationship of transition and transversion substitutions to Kimura's two parameters genetic distance modified by Felsenstein.¹²⁾

The open circles and squares represent transition and transversion in the eight species of stingrays, respectively, whereas solid circles and squares represent substitution in 21 pairwise with the out-group species.

cies *H. imbricata* collected in Thailand and *H. gerrardii* collected in India, differing from two samples of the second freshwater stingray *H. chaophraya* collected in Thailand and India. However, the present two taxa of *H. chaophraya* yielded two separable lineages, suggesting the occurrence of different local races in this species. Secondly, the third freshwater stingray of *D. laosensis*, collected in Laos, a marine stingray of *Dasyatis* sp. collected in Thailand, and *D. akajei* collected in Japan, formed the same group. Thirdly, two local races were demonstrated for *P. sephen*. These relationships were well supported with high values in bootstrap analysis.

Although *D. laosensis*, the marine stingray of *Dasyatis* sp., and *P. sephen* formed the same group in the maximum parsimony and maximum likelihood trees, their respective bootstrap values, 55 and 65, were slightly low. On the other hand, *D. akajei*, *D. laosensis* and *Dasyatis* sp. were in the same group and these branched off from

other stingrays including the *Pastinachus* species in the neighbor-joining tree. The relationship within species belonging to the genus *Himantura* was the same irrespective of the phylogenetic trees constructed. In contrast to the maximum parsimony and maximum likelihood trees, however, the neighbor-joining tree provided a close relationship for the genus *Pastinachus* to that of the genus *Himantura* than to the genus *Dasyatis*, although their bootstrap value was not high.

Phylogenetic Trees with Amino Acid Sequences of Cytochrome b

The phylogenetic trees derived from the amino acid sequences of cytochrome *b* according to the neighbor-joining and maximum parsimony methods are shown in Fig. 6. These appear to be slightly different from those based on nucleotide sequences. Species belonging to the genera *Himantura*, *Dasyatis* and *Pastinachus* were in the distinct groups according to the data based on amino acid sequences, although their phylogenetic relationship was not well established.

The most remarkable difference between the phylogenetic trees with amino acid sequences and DNA nucleotide sequences was the placement of the marine rayfish *A. narinari* collected in Japan to an inner group, although this placement seems to be less reliable. The phylogenetic tree according to the maximum parsimony method indicated that the present out-groups might not be best.

Discussion

Nelson⁴⁾ has recorded that the subfamily Dasyatidae (stingrays) consists of six genera, with about 50 species. In this study, our attention was focused on three genera (namely *Himantura*, *Dasyatis*, and *Pastinachus*) of Asian stingrays. We analyzed eight species belonging to these three genera, among which *H. signifer*¹⁶⁾ and *D. laosensis*¹⁷⁾ and *H. chaophraya*¹⁸⁾ were reported as new species in 1982, 1987, and 1990, respectively.

The molecular phylogenetic analyses by the neighbor-joining, maximum parsimony and maximum likelihood methods based on DNA nucleotide sequences of the cytochrome *b* gene showed that the general topology of the trees remained mostly unaltered. The DNA nucleotide

Fig. 5. Molecular phylogenetic trees according to the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods for stingrays based on DNA nucleotide sequences of the cytochrome *b* gene.

Numbers in the phylogenetic trees indicate bootstrap resampling values from 100 replicates. The bar indicates genetic distance.

sequences of eagle ray *A. narinari* and butterfly ray *G. japonica*, were employed in order to root the molecular phylogenetic trees with the neighbor-joining, maximum parsimony, and maximum likelihood methods.

According to taxonomic grouping,^{4,19)} the genus *Dasyatis* branches with the genus *Himantura*, both belonging to the family Dasyatidae. In the present study on molecular phylogeny of stingrays, the genus *Himantura* formed one group with a high bootstrap value by the neighbor-joining, maximum parsimony, and maximum likelihood methods based on DNA nucleotide sequences. The giant stingray *H. chaophraya* collected in India, a synonym of *H. fluvialis*,^{15,16)} was closely related to the same species collected in Thailand. However, the DNA nucleotide and amino acid sequences of cytochrome *b* of *H. chaophraya* specimens collected in India and Thailand were clearly different

from each other. These two races probably diverged since their geographical isolation to different river systems during evolutionary time. It is interesting to investigate their detailed morphological characterizations. In spite of such an unsettled grouping for the genus *Himantura*, all stingrays in this genus formed a closely related group. The present study demonstrated that *H. gerrardii* was the closest relative of *H. imbricata*, whereas *H. signifer* was closely related to *H. imbricata* and *H. gerrardii*. Such relationship is not inferred by the data reported previously based on morphological characters.¹⁶⁻¹⁹⁾

The marine stingray of *Dasyatis sp.* is the closest relative of *D. laosensis*, the two species together forming one group in any molecular phylogenetic trees with high bootstrap values. Such a close relationship between *D. laosensis* and the marine stingray of *Dasyatis sp.* is interesting

Fig. 6. Molecular phylogenetic trees according to the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods for stingrays based on amino acid sequences of cytochrome *b*.

Numbers in the phylogenetic trees indicate bootstrap resampling values from 100 replicates. The bar indicates genetic distance.

from the ecological and physiological aspects as well, since *D. laosensis* of Laos inhabits freshwater, whereas *Dasyatis* sp. collected from Thailand lives in a marine habitat.

Judging from low bootstrap values, DNA nucleotide and deduced amino acid sequences data for cytochrome *b*, we find it difficult to confidently establish the phylogenetic relationship of *P. sephen* with other genera of stingray. The present phylogenetic trees constructed according to the maximum parsimony, and maximum likelihood methods suggest that it is the closest relative of the genus *Dasyatis*. But, the neighbor-joining method implies that it could be closely related to the genus *Himantura*. However their bootstrap values were both low. Bigelow and Schroeder²⁰⁾ reported a brief description that *Pastinachus* was once classified under the subgenus *Dasyatis*. In this study we infer that specimens of *P. sephen* collected in India²¹⁾ and Thailand are slightly different from each other in their DNA nucleotide and amino acid sequences of cytochrome *b* as in the case of *H. chaophraya*.

In conclusion, we have analyzed 12 taxa from Thailand, India, Laos, and Japan to elucidate phylogenetic links of freshwater stingrays and also their relationship with marine stingrays. The molecular phylogenetic trees derived from our analyses demonstrated some of their relations distinctly and others vaguely. The occurrence of local races in the two freshwater stingrays, *H. chaophraya* and *P. sephen* is also revealed. Further studies are required to satisfactorily correlate such differences in the same species with those in morphological characters.

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