***Neotrygon indica* sp. nov., the Indian-Ocean blue spotted maskray (Myliobatoidei, Dasyatidae)**

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Abstract: The blue-spotted maskray, previously *N. kuhlii*, consists of up to eleven lineages representing separate species. Nine of these species (*N. australiae*, *N. bobwardi*, *N. caeruleopunctata*, *N. malaccensis*, *N. moluccensis*, *N. orientale*, *N. vali*, *N. varidens, N. westpapuensis*) have already been formally described and two (Indian-Ocean maskray and Ryukyu maskray) remain undescribed. Here the Indian-Ocean maskray is described as a new species, *Neotrygon indica* sp. nov. Specimens of the new species were generally characterized on their dorsal side by a moderately large number of small ocellated blue spots, a low number of medium-sized ocellated blue spots, the absence of large ocellated blue spots, a high number of dark speckles, a few dark spots, and a conspicuous occipital mark. The new species formed a distinct clade in the tree built from concatenated nucleotide sequences at the *CO1* and *cytochrome-b* loci. The distribution of *N. indica* sp. nov. includes the Indian coast of the Bay of Bengal, the Indian coast of the Laccadives Sea, and Tanzania.

Keywords: New species; Mitochondrial DNA; Diagnosis; Distribution

Introduction

The blue-spotted maskray, previously *N. kuhlii* (Müller and Henle 1841), has a wide Indo-West Pacific distribution, from eastern Africa to Japan [Müller and Henle 1841; Annandale 1909; Puckridge et al. 2013; Borsa et al. 2017]. It was hypothesized to be a species complex after a barcoding survey revealed several deeply divergent mitochondrial lineages [Ward et al. 2008]. The hypothesis of a species complex was subsequently validated [Borsa et al. 2016b]. This species complex consists of up to eleven parapatrically-distributed lineages representing separate species of which nine have already been formally described [Borsa et al. 2017]. These are *N. australiae* Last, White and Séret 2016, *N. bobwardi* Borsa, Arlyza, Hoareau and Shen 2017, *N. caeruleopunctata* Last, White and Séret 2016, *N. malaccensis* Borsa, Arlyza, Hoareau and Shen 2017, *N. moluccensis* Borsa, Arlyza, Hoareau and Shen 2017, *N. orientale* Last, White and Séret 2016, *N. vali* Borsa 2017, *N. varidens* (Garman 1885), and *N. westpapuensis* Borsa, Arlyza, Hoareau and Shen 2017. Two other species in the genus, the nominal *N. kuhlii* from Vanikoro and *N. trigonoides* possess distinctive spot patterns [Borsa et al. 2013; Borsa and Béarez 2016; Borsa 2017a] that tell them apart from the blue-spotted maskray as it was originally described by J. Müller and F.G.J. Henle [1841]. Based on the only available information on colour patterns, one cannot exclude that *N. kuhlii* as it has been redefined by Last and co-authors [Last et al. 2016] and *N. trigonoides* are synonyms [Borsa and Béarez 2016]. The genetically distinctive Indian-Ocean maskray reported in the recent phylogeographic literature [Arlyza et al. 2013; Borsa et al. 2017] remains undescribed. The objective of the present paper is to formally describe the Indian-Ocean maskray, based on the mitochondrial DNA sequences of fresh specimens from the eastern coast of India and of previously-reported material from India and Tanzania.

Materials and Methods

Material examined

Seven specimens of the new species that were examined for spot patterns are listed in Table 1. Specimen NKGMF-3 from the Gulf of Mannar, to be designated as the holotype of the new species, was deposited at the Marine Biology Regional Centre (MBRC) in Chennai, India. The MBRC is part of the network of museums managed by, and an official repository of the Zoological Survey of India. Specimen NKGMF-1 from the Gulf of Mannar, to be chosen as the paratype of the new species, was deposited at the Fish Genetics and Biotechnology laboratory, ICAR-Central Institute of Fisheries Education in Mumbai, India. Voucher material of the new species also includes a specimen collected by APK and colleagues in Visakhapatnam,

Andhra Pradesh, Bay of Bengal on 15 August 2011. The specimen was discarded but a sub-sample of tissue was registered at the Central Institute of Fisheries Education , Mumbaiunder no. VIZNK-01. A photograph of this individual is available from the Barcoding of Life Data systems database (BOLD; http://www.barcodinglife.com/) under accession no. BOLD:ACB9305. Photographs of blue-spotted maskrays from India deposited in FishBase [Froese and Pauly 2016] were not retained for the analysis of spot patterns because of insufficient resolution. Comparative material for the characterization of spot patterns consisted of specimens from *N. australiae* (*N* = 2, including holotype), *N. bobwardi* (*N* = 5, including holotype), *N. caeruleopunctata* (*N* = 2, including a paratype), *N. malaccensis* (*N* = 1, holotype), *N. moluccensis* (*N* = 2, including holotype and single paratype), *N. orientale* (*N* = 8, including holotype), *N. vali* (*N* = 3, including holotype), *N. varidens* (*N* = 6), and *N. westpapuensis* (*N* = 1, holotype) (Table 1).

Spot pattern analysis

The diameter of ocellated blue spots on the dorsal side of the left and right pectoral fins, relative to disk width (DW), was measured from photographs. Ocellated blue spots were qualified as "small" when their maximum diameter was ≤ 2% DW, "medium" when ≤ 4% DW and "large" when > 4% DW [15Borsa et al. 2013]. Dark speckles (≤ 1% DW) and dark spots (> 1% DW) were also counted on the dorsal surface of the disk. The counts did not include those speckles and spots located within the dark band around eyes that forms the mask. The presence or absence of a darker occipital mark at the rear end of the neurocranium was also checked and categorized as "absent", "weak", or "conspicuous".

Analysis of nucleotide sequences

The DNA of six specimens of the new species was extracted using the phenol-chloroform protocol. A fragment of each the *CO1* and the *cytochrome b* genes was amplified by polymerase chain reaction according to Borsa et al. [2016b]. Nucleotide sequencing was done in both forward and reverse directions using the Sanger method. The consensus sequences were obtained after assembling the direct and reverse traces under BIOEDIT v. 7.1.11 [Hall 1999]. Sequences were then edited and aligned under BIOEDIT. We chose as reference for numerotating nucleotides the complete mitogenome sequence of *Neotrygon orientale*; Shen et al. 2016), accessible from GenBank (http:// www.ncbi.nlm.nih.gov) under no. KR019777. Five out of eight *N. indica* sp. nov. individuals characterized by their spot patterns (Table 1) had their nucleotide sequences at loci *CO1* and/or *cytochrome b* examined. The phylogeny of concatenated *CO1+cytochrome b* gene haplotypes was inferred by using the maximum-likelihood (ML) method under MEGA6 [Tamura et al. 2013]. The most likely nucleotide-substitution model, which was determined according to the Bayesian information criterion, was the Tamura-Nei model [Tamura and Nei 1993] (TN93) where a discrete Gamma distribution ($\Gamma = 0.76$) was used to model evolutionary rate differences among nucleotide sites and invariable sites were allowed. The ML tree was rooted by choosing New Caledonian maskray *N. trigonoides* as outgroup. The robustness of nodes in the tree was tested by bootstrap resampling.

Mean nucleotide distances within and between species were estimated from 57-individual *CO1* and 48-individual cytochrome-b sequence datasets compiled from the present work and from previous reports [Borsa et al. 2016b] using MEGA6. According to the Bayesian information criterion, the best substitution model for the CO1 gene sequence dataset was K2+G ($\Gamma = 0.07$). The best substitution model for the cytochrome b sequence data was HKY+G ($\Gamma = 0.05$), but the model used for the calculations was the second-best (TN93+G) which, contrary to the former, is available in MEGA6.

Notice

The present article in portable document (.pdf) format is a published work in the sense of the International Code of Zoological Nomenclature [International Commission on Zoological Nomenclature, 2012) or Code and hence the new names contained herein are effectively published under the Code. This published work and the nomenclatural acts it contains have been registered in ZooBank (http://zoobank.org/), the online registration system for the International Commission on Zoological Nomenclature. The ZooBank life science identifier (LSID) for this publication is urn:lsid:zoobank.org:pub:10551DF9-4B93-40ED-8910-012C5DCF8B96. The information associated with this LSID can be viewed through any standard Internet browser by appending the LSID to the prefix "http://zoobank.org/'. The online version of the present paper is archived and available from the *bioRxiv*, Cold Spring Harbor NY, U.S.A. (http://www.biorxiv.org/) and haL-IRD, France (http://www.hal.ird.fr/) repository websites .

Results and Discussion

Spot patterns

Spot patterns of eight *N. indica* sp. nov. specimens were summarized and compared to nine other species of the blue-spotted maskray complex (Table 1). *N. indica* sp. nov. specimens from India were characterized by a moderately large number of small ocellated blue spots (n = 15-57), a low number of medium-sized ocellated blue spots (n = 0-16), a total absence of large ocellated blue spots, a high number of dark speckles (n = 23-176), generally a few dark spots (n = 0-11; only exceptionally present in the other species examined), and a conspicuous occipital mark. These features were visible on the holotype (Fig. 1) and the paratype of the new species. As shown by our results, some overlap in spot patterns was observed between some pairs of species. Also, one expects that with increased geographic sampling, more variation will show up within a species, hence further blurring the distinction between species and further relativizing diagnoses based on spot patterns.

Genetic distinctness of the Indian-Ocean blue-spotted maskray

Neotrygon indica sp. nov. haplotypes clustered into a strongly supported, distinct subclade on the maximum-likelihood phylogenetic tree of concatenated *CO1+cytochrome b* gene sequences (Fig. 2), confirming previous results [Borsa et al. 2016b]. Estimates of genetic distance between pairs of species in the Indian Ocean blue-spotted maskray group ranged from 0.020 to 0.025 at the *CO1* locus and from 0.020 to 0.033 at the *cytochrome b* locus (Table 2). These values were all well above the highest estimates of genetic distance within a species (respectively, 0.012 and 0.014; Table 2). Therefore, the four species albeit closely related remained clearly distinct from one another using either the standard *CO1* barcode or the *cytochrome-b* marker.

Taxonomy

Neotrygon indica sp. nov. urn:lsid:zoobank.org:act:99F4F1A4-D5F6-4379-BC4E-7FB1E74CFA58. *Trygon kuhlii* [Annandale 1909]; presumably clade *Neotrygon kuhlii 3* of Naylor et al. [2012]; *Neotrygon kuhlii* [Borsa et al. 2013]; *Neotrygon kuhlii* haplogroup *I* [Arlyza et al. 2013; Borsa et al. 2016a]; *Neotrygon kuhlii* clade *8* [Puckridge et al. 2013]; Clade *I* [Borsa et al. 2016b]; Indian-Ocean maskray [Borsa et al. 2017]. Also BIN number BOLD:AAA5611 in BOLD.

Material examined

Table 1 lists all specimens examined to characterize spot patterns. The list of specimens characterized by their nucleotide sequence at mitochondrial loci *CO1* and *cytochrome b* is provided as appendix A of [Borsa et al. 2016b].

Types

A female specimen 174 mm disk length (Fig. 1) from the Gulf of Mannar, Tamil Nadu (9.12°N 79.46°E), collected at the Inico Nagar, Tuticorin fish landing centre by RK and Satish on 13 January 2017 is here designated as the holotype of *Neotrygon indica* sp. nov. The specimen was deposited by APK at the MBRC in Chennai, India on 12 May 2017. The specimen has registration no. ZSI/MBRC/F.1495. A female specimen 182 mm disk length fished from the Gulf of Mannar, Tamil Nadu (9.12°N 79.46°E) using bottom-set gillnets, collected by RK on 05 May 2017 is here designated as the single paratype of the new species. This specimen, labelled CIFEFGB/NKGM1 was subsequently deposited by APK at the Fish Genetics and Biotechnology laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India. Morphometric measurements on the holotype are presented in Table 3, along witth those of the paratype.

Diagnosis

Neotrygon indica sp. nov. from India is differentiated from all species of the blue-spotted maskray described so far (*N. australiae*, *N. bobwardi*, *N. caeruleopunctata*, *N. malaccensis*, *N. moluccensis*, *N. orientale*, *N. vali*, *N. varidens* and *N. westpapuensis*) by a combination of low number of medium-sized ocellated blue spots, total absence of large ocellated blue spots, high number of dark speckles, frequent occurrence of a few dark spots, and conspicuous occipital mark. However, this diagnosis did not apply to the single specimen sampled from Zanzibar. *Neotrygon indica* sp. nov. is distinct from *N. kuhlii* and *N. trigonoides* by the absence of the pair of conspicuous scapular blotches characteristic of these two species [Borsa et al. 2013; Borsa and Béarez 2016], although specimens of *N. indica* sp. nov. often possess a pair of diffuse darker marks in the scapular region. Individuals of *N. indica* sp. nov. sequenced at the *CO1* locus possessed T at nucleotide site 607 of the *CO1* gene, a character that was otherwise present in only two out of 130 *N. orientale* individuals and absent in all other cryptic species of the blue-spotted maskray (supplementary table S1 of Borsa et al. [2017]).

Distribution

The type locality of *N. indica* sp. nov. is the Gulf of Mannar in Tamil Nadu on the eastern coast of the Indian sub-continent. Based on the collection of voucher specimens from present study, the distribution of *N. indica* sp. nov. includes the coasts of Andhra Pradesh and Tamil Nadu states of India, from approximately 17.7°N to 8.8°N. Based on the material genetically examined thus far, the distribution of *N. indica* sp. nov. includes the Indian coast of the Bay of Bengal (Visakhapatnam, Chennai), the Indian coast of the Laccadives Sea (Kerala), and Tanzania ([Arlyza et al. 2013; Puckridge et al. 2013; Borsa et al. 2016b, 2017]; present study).

Etymology and proposed vernacular names

Epithet *indica* is the latin feminine adjectival form of the name of the country of type locality, India. Indian-Ocean blue-spotted maskray (English); Neeli Nishan Pakat (Hindi); Pulli Thirukhai (Tamil); Raie pastenague masquée à points bleus de l'océan Indien (French).

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Table 1. Spot patterns on the dorsal side of 33 blue-spotted maskray (*Neotrygon* spp.) specimens sorted by species. Specimens from Padang and Kota Kinabalu, as well as ASIZP0073604, ASIZP0073605 and ASIZP0078458 were identified to species based on sampling location [9Borsa et al. 2016b]; all other specimens were identified to species based on their *CO1* gene sequence [9Borsa et al. 2016b]. *L*, left pectoral fin; *R*, right pectoral fin. *Occipital mark*, diffuse dark blotch at rear end of neurocranium that was categorized as either absent (*0*), weak (*1*) or conspicuous (*2*).

Species, Specimen no. (field no.)	Locality of collection, country	*N* ocellated blue spots			*N* dark speckles (<1% DW)	*N* dark spots (>1% DW)	Occipital mark
		1. Small L, R	2. Medium L, R	3. Large L, R	L, R	L, R	
N. australiae							
CSIRO 7016-01 [a]	Weipa, Australia	14, 12	17, 20	3, 0	8, 9	0, 0	0
MZB-20863 (ENTTJS2)	Tanjung Sulamo, Indonesia	25, 25	28, 32	1, 0	13, 6	0, 0	0
N. bobwardi							
MZB-20843 (ME3) [a]	Meulaboh, Indonesia	28, 29	13, 11	0, 0	16, 16	0, 0	0
20150524-D	Padang, Indonesia	5, 11	4, 2	0, 0	6, 6	0, 0	0
20150524-E	Padang, Indonesia	16, 11	9, 8	0, 0	8, 15	0, 0	2
20150524-F	Padang, Indonesia	20, 36	14, 8	0, 0	3, 0	0, 0	1
20150524-11	Padang, Indonesia	8, 8	2, 1	0, 0	6, 13	0, 0	2
N. caeruleopunctata							
CSIRO H 7850-01 [b]	Sadeng, Indonesia	25, 22	4, 1	0, 0	17, 30	0, 0	1
20080131_BL (NK-K-BL)	Bali, Indonesia	21, 24	6, 5	0, 0	43, 54	0, 0	1
MZB-22131 (BAL-S)	Bali, Indonesia	31, 22	5, 9	0, 1	0, 1	0, 0	1
N. indica sp. nov.							
ZSI/MBRC/F.1495 (NKGMF-3) [a]	Gulf of Mannar, India	9, 10	0, 0	0, 0	19, 34	3, 2	2
CIFEFGB/NKGM1 (NKGMF-1) [b]	Gulf of Mannar, India	27, 26	0, 0	0, 0	37, 22	4, 1	2
VIZNK-01 (NKVSKP-1)	Visakhapatnam, India	3, 12	1, 9	0, 0	13, 10	0, 0	2
NKTTK-1	Tuticorin, India	30, 27	3, 3	0, 0	90, 86	0, 1	2
NKTTK-2	Tuticorin, India	20, 8	0, 0	0, 0	24, 32	0, 1	2
NKGMM-2	Gulf of Mannar, India	24, 26	7, 4	0, 0	30, 33	4, 1	2
NKGMM-4	Gulf of Mannar, India	14, 18	8, 4	0, 0	40, 38	5, 6	2
ZAN 1	Zanzibar, Tanzania	44, 51	29, 21	2, 2	10, 15	0, 0	0
N. malaccensis							
MZB-20847 (MSKL3) [a]	Kuala Lama, Indonesia	2, 4	14, 10	0, 1	5, 3	0, 0	0
N. moluccensis							
MZB-20866 (ARA1) [a]	Tual, Indonesia	36, 30	9, 9	0, 0	53, 53	0, 1	0
MZB-20864 (AM1) [b]	Ambon, Indonesia	9, 2	7, 12	0, 0	5, 2	0, 0	1
N. orientale							
CSIRO H7858-01 [a]	Muara Kintap, Indonesia	20, 14	15, 17	1, 1	2, 1	0, 0	0
MZB-20852 (PB2)	P. Pabelokan, Indonesia	5, 9	8, 3	1, 0	21, 17	0, 0	0
MZB-20851 (PR)	Pulau Pari, Indonesia	22, 18	9, 6	2, 2	12, 8	0, 0	0
MZB-20850 (PN5)	Pulau Peniki, Indonesia	12, 22	12, 11	2, 0	2, 1	0, 0	1
BO424	Tanjung Manis, Malaysia	35, 41	22, 6	0, 0	16, 13	0, 0	0
WJC-5367	Kota Kinabalu, Malaysia	17, 19	10, 8	0, 1	8, 4	0, 0	0
WJC-5368	Kota Kinabalu, Malaysia	19, 20	6, 12	0, 0	6, 5	0, 0	0
WJC-5369	Kota Kinabalu, Malaysia	20, 23	14, 10	0, 0	3, 2	0, 0	0
N. vali							
CSIRO H7723-01 [a]	Honiara, Solomon Is.	2, 4	1, 1	0, 0	3, 6	0, 0	0
Randall ([2005]: 18) [c]	Solomon Is.	11	4	0	6	0	0
Rosenstein [c]	Mbike wreck, Solomon Is.	21	6	0	7	0	0
N. varidens							
ASIZP0073604	Taiwan	0, 1	0, 0	0, 0	0, 0	0, 0	1
ASIZP0073605	Taiwan	3, 2	0, 0	0, 0	0, 0	0, 0	1
ASIZP0073535	Taiwan	3, 1	0, 0	0, 0	0, 0	0, 0	1
ASIZP0078458	Taiwan	7, 16	0, 0	0, 0	0, 0	0, 0	1
ASIZP0805866	Taiwan	4, 3	5, 6	0, 0	2, 7	0, 0	1
N. westpapuensis							
MZB-20867 (BK5) [a]	Biak, West Papua	41, 37	25, 13	0, 2	10, 7	0, 0	1

[a] Holotype; [b] Paratype; [c] Left side only

Table 2. Blue-spotted maskray *Neotrygon* spp. from the Indian Ocean and geographically adjacent Andaman Sea. Estimates (mean ± *SD*) of nucleotide distances between pairs of, and within blue-spotted maskray species at loci *CO1* and *cytochrome b* .

Marker, Species	Within-species	Species		
		N. indica	*N. bobwardi*	*N. caeruleopunctata*
CO1				
N. indica	0.012±0.003	-		
N. bobwardi	0.009±0.003	0.025±0.006	-	
N. caeruleopunctata	0.002±0.001	0.022±0.007	0.024±0.007	-
N. malaccensis	0.001±0.001	0.020±0.006	0.020±0.006	0.022±0.008
cytochrome b				
N. indica	0.014±0.004	-		
N. bobwardi	0.006±0.002	0.033±0.007	-	
N. caeruleopunctata	0.002±0.001	0.020±0.005	0.026±0.006	-
N. malaccensis	0.000±0.000	0.026±0.006	0.026±0.009	0.021±0.007

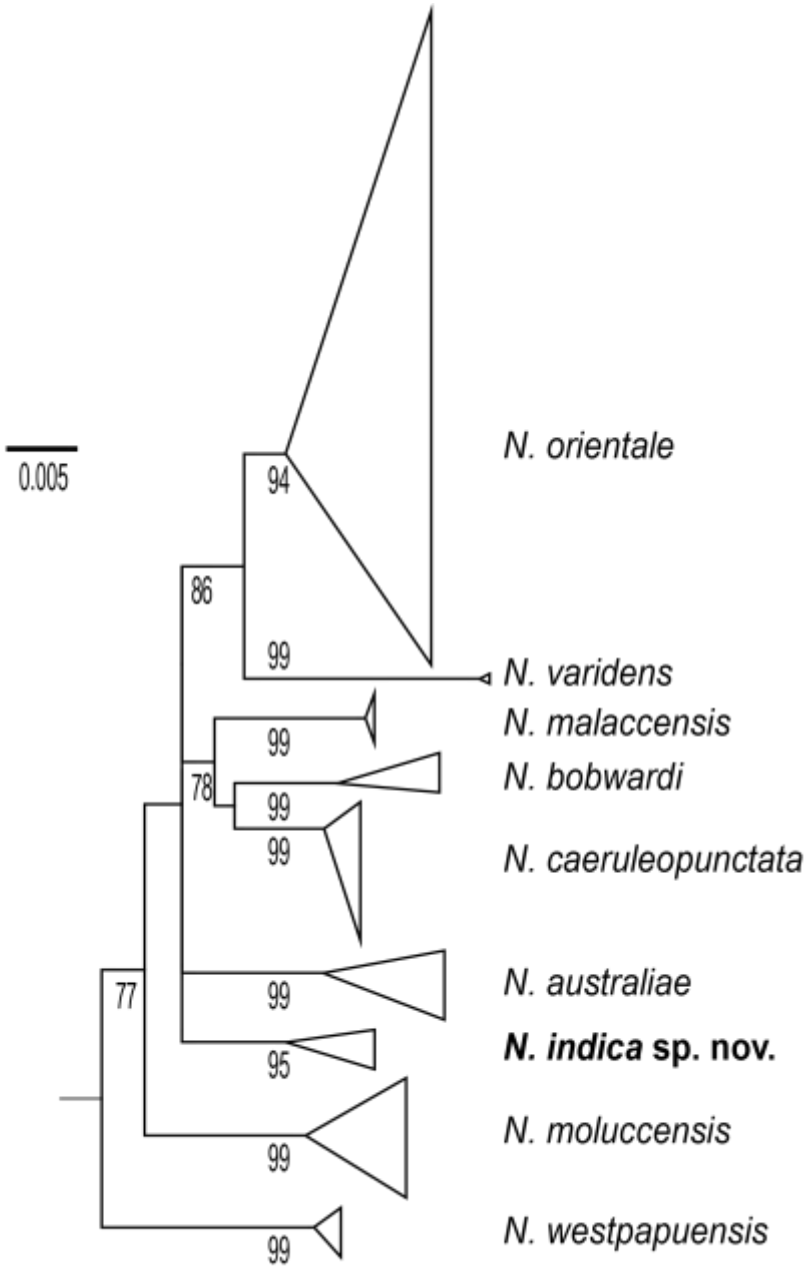
Table 3. Morphometric measurements for the type specimens of *Neotrygon indica* sp. nov. All measurements except disc width and number of caudal stings expressed as percentage of disc width.

Parameter	Holotype	Paratype
Disc width (mm)	240	213
Total length	166.6	183.0
Disc length	70.8	84.5
Distance from tip of snout to pectoral fin insertion	68.8	70.4
Distance from tip of snout to eye	10.4	9.4
Mouth width	7.5	8.5
Nostril length	6.6	4.7
Distance between nostrils	11.6	14.1
Head length	39.5	39.9
Snout pre orbital lengh (to lower jaw)	15.0	16.0
Eye length	6.3	6.6
Orbit diameter	10.0	9.4
Inter orbital width	7.5	7.0
Inter ocular width	16.6	18.8
Spiracle length	7.5	8.0
Interspiracular width	14.5	15.0
Orbit and spiracle length	9.16	14.08
Orbit to pectoral fin insertion	55.0	46.9
Nasal curtain length	5.8	4.7
Nasal curtain width	9.2	8.5
Width of 1st gill slit	4.2	3.3
Width of 3rd gill slit	2.9	3.3
Width of 5th gill slit	1.6	2.3
Distance between 1st and 5th gill slits (left)	13.3	14.1
Distance between 1st and 5th gill slits (right)	13.3	14.1
Distance between 1st gill slits	22.5	23.5
Distance between 5th gill slits	14.2	14.1
Pre-nasal length of snout	9.2	5.7
Distance from tip of snout to eye	14.2	14.1
Distance from tip of snout to maximum width	60.4	61.0
Distance from tip of snout to cloaca origin	61.7	67.6
Distance from pectoral fin insertion to base of 1st sting	43.8	37.6
Distance between axil of pelvic fin and base of 1st sting	43.3	37.6
Distance from tip of snout to origin of cloaca	66.3	68.1
Distance from origin of cloaca to base of 1st sting	41.7	44.6
Distance from origin of cloaca to tail tip	105.0	113.1
Cloaca length	7.1	6.6
Tail width at axil of pelvic fin	8.3	9.4
Tail width at base of 1st sting	2.3	6.6
Tail heighth at axil of pelvic fin	5.4	6.6
Tail heighth at base of 1st sting	2.6	2.3
Pelvic fin (embedded) length	14.2	13.6
Width across pelvicfin base	19.6	18.8
Greatest width across pelvic fin	33.3	42.3
Clasper length from pelvic axil	(female)	(female)
Post-cloacal clasper length	(female)	(female)
Tail width at base of sting	3.3	3.8
Tail height at base of sting	2.5	2.8
Disc thickness	12.5	14.0
Number of caudal stings	1	1
Length of sting	(removed)	14.1

Fig. 1. Holotype of *Neotrygon indica* sp. nov.: female specimen, 174 mm disk length from the Gulf of Mannar, Tamil Nadu, India (09.12°N 79.46°E) registered under no. ZSI/MBRC/F.1495 at the Marine Biology Regional Centre in Chennai, India (photograph by RK).

Fig. 2. Blue-spotted maskray *Neotrygon* spp. Simplified maximum-likelihood tree of concatenated *CO1+ cytochrome b* gene fragments, rooted by *N. trigonoides* [Borsa et al. 2013] showing the placement of the *N. indica* sp. nov. lineage. Terminal branches were collapsed into triangles whose width is proportional to the number of individuals sequenced and whose depth represents the genetic variability within a species. Numbers at a node are bootstrap scores, from 600 bootstrap resampling runs under MEGA6 [Tamura et al. 2013].

3


0.005
94
86
99
99
78
99
99
77
99
95
99
99
N. orientale
N. varidens
N. malaccensis
N. bobwardi
N. caeruleopunctata
N. australiae
N. indica sp. nov.
N. moluccensis
N. westpapuensis