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Taxonomy/Taxinomie

Resurrection of New Caledonian maskray *Neotrygon trigonoides* (Myliobatoidei: Dasyatidae) from synonymy with *N. kuhlii*, based on *cytochrome-oxidase I* gene sequences and spotting patterns

Résurrection de la raie pastenague de Nouvelle-Calédonie Neotrygon trigonoides *(Myliobatoidei: Dasyatidae) de la synonymie avec* N. kuhlii, *sur la base des séquences du gène de la* cytochrome-oxydase I *et des patterns de taches*

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ABSTRACT

The maskray from New Caledonia, *Neotrygon trigonoides* Castelnau, 1873, has been recently synonymized with the blue-spotted maskray, *N. kuhlii* (Müller and Henle, 1841), a species with wide Indo-West Pacific distribution, but the reasons for this are unclear. Blue-spotted maskray specimens were collected from the Indian Ocean (Tanzania, Sumatra) and the Coral Triangle (Indonesia, Taiwan, and West Papua), and *N. trigonoides* specimens were collected from New Caledonia (Coral-Sea). Their partial *COI* gene sequences were generated to expand the available DNA-barcode database on this species, which currently comprises homologous sequences from Ningaloo Reef, the Coral Triangle and the Great Barrier Reef (Coral-Sea). Spotting patterns were also compared across regions. Haplotypes from the Coral-Sea formed a haplogroup phylogenetically distinct from all other haplotypes sampled in the Indo-West Pacific. No clear-cut geographic composition relative to DNA-barcodes or spotting patterns was apparent in *N. kuhlii* samples across the Indian Ocean and the Coral Triangle. The New Caledonian maskray had spotting patterns markedly different from all the other samples. This, added to a substantial level of net

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nucleotide divergence (2.6%) with typical *N. kuhlii* justifies considering the New Caledonian maskray as a separate species, for which we propose to resurrect the name *Neotrygon trigonoides*.

RÉSUMÉ

La raie pastenague à taches bleues, *Neotrygon kuhlii* (Müller et Henle, 1841), possède une large distribution indo-ouest pacifique. Une raie pastenague tachetée de Nouvelle-Calédonie, *Neotrygon trigonoides* Castelnau, 1873, a été récemment placée en synonymie de *N. kuhlii*, mais les raisons en sont obscures. Des spécimens de raie pastenague à taches bleues ont été collectés dans l'océan Indien (Tanzanie, Sumatra) et le triangle de Corail (Indonésie, Taiwan et Papouasie occidentale), tandis que des spécimens de *N. trigonoides* actuellement désignés comme *N. kuhlii* ont été collectés en Nouvelle-Calédonie (mer de Corail). Leurs séquences partielles du gène *COI* ont été produites pour augmenter la base de données de *barcodes* disponible sur cette espèce, laquelle comprend déjà des séquences homologues de Ningaloo Reef, du triangle de Corail et du récif de la Grande-Barrière (mer de Corail). Les patterns de taches ont été également analysés sur l'ensemble des régions échantillonnées. Les haplotypes de la mer de Corail forment un ensemble phylogénétiquement distinct du reste de l'Indo-Ouest pacifique. Nous n'avons pas observé de différences géographiques nettes entre les populations de *N. kuhlii* de l'océan Indien et celles du triangle de Corail, que ce soit au niveau des *barcodes* ADN ou des *patterns* de taches. La raie pastenague tachetée de Nouvelle-Calédonie s'avère posséder des *patterns* de taches très différents de ceux de tous les autres spécimens. Cela, ajouté à un niveau de divergence nucléotidique cohérent (2,6 %) avec les *N. kuhlii* typiques, justifie le statut d'espèce à part entière de la forme présente en Nouvelle-Calédonie. Nous proposons de rétablir le nom *N. trigonoides* pour cette espèce, dont la distribution semble concerner l'ensemble de la mer de Corail.

Mots clés :
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1. Introduction

Recent advances in the molecular phylogenetics of stingrays (Myliobatoidei: [1]) have challenged the previously accepted taxonomy of species in a number of genera [2–6]. Both a *cytochrome-oxidase I* gene (*COI*)-based barcoding survey of Australasian chondrichthyans [7], a recent *COI*-barcoding survey of northwestern Australian myliobatoids [8] and a recent systematic survey of elasmobranchs based on the nicotinamide-adenine dinucleotide dehydrogenase subunit two gene marker [3] have pointed to problematic cases relative to the definition of species boundaries, when divergent haplotypes are observed within a given nominal species. One of these cases is the blue-spotted maskray, *Neotrygon kuhlii* (Müller and Henle, 1841) [9].

The blue-spotted maskray under its current taxonomic definition has a wide Indo-Pacific distribution, from the Red Sea to southern Africa and from the western Indian Ocean to the western Pacific, reaching Japan in the North, Tonga in the South-East and New Caledonia in the South [10]. It is currently the most frequently landed stingray species in the Coral Triangle [11], hence it is a species of commercial interest but also one of conservation concern given the general vulnerability of elasmobranchs to fishing pressure [12,13]. *N. kuhlii* shows unusually high within-species diversity, with average nucleotide distances among haplotypes reaching 2.8%–3.0% at the *COI* locus [7,8]. An intron-marker based diversity study has confirmed the remarkably high degree of genetic differentiation among populations, but has failed to detect multiple cryptic species within Coral Triangle *N. kuhlii* [14]. One study [8] reported 3.5% nucleotide divergence at the *COI* locus between a sample from the Great Barrier Reef and samples from northwestern Australia and the Coral Triangle. A recent phylogeographic survey of *Neotrygon* spp. [15] has confirmed this preliminary result. Blue-spotted maskray from the Coral-Sea deserve particular scrutiny as argued in the following.

The New Caledonian maskray, *N. trigonoides* Castelnau, 1873 [16] is currently considered as a junior synonym of *N. kuhlii* [15,17,18]. In his description, F. de Castelnau [16] noted a large number of small black spots on the dorsal surface of the stingray whereas in their description of *N. kuhlii*, J. Müller and J. Henle [9] only mentioned "*einzene, kleine, runde blaue Flecken (3-6) auf jeder Brustflosse*" and provided a figure of *N. kuhlii* that illustrates this description (Fig. 1A). The two species, *N. kuhlii* and *N. trigonoides*, were synonymized by P.R. Last and W.T. White [17] who compared the holotype of Castelnau's *N. trigonoides* to what they identified as *N. kuhlii* from eastern Australia: "A close examination of the holotype of *Raya trigonoides* Castelnau, 1873 from New Caledonia. . . confirmed that it is conspecific with eastern Australian forms of *N. kuhlii*".

In the present work, we re-examine spotting patterns in Castelnau (1873)'s *N. trigonoides* [16] and compare them to Müller and Henle (1841)'s *N. kuhlii* [9] and to blue-spotted maskray specimens from a range of locations in the Indo-West Pacific. We examine *COI* gene sequences for samples of blue-spotted maskray from two distant sites in the Coral-Sea, and from a number of sites in the Indian Ocean and in the Coral Triangle. Finally, we provide a redescription of *N. trigonoides* based on the *COI* gene sequence.

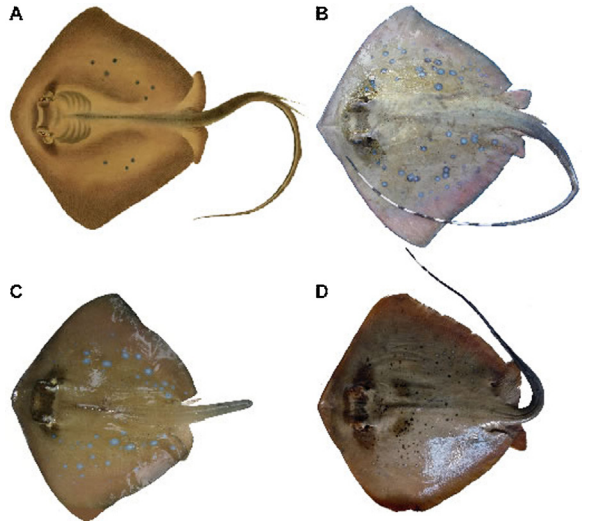

Fig. 1. Specimens of blue-spotted maskrays from various locations showing variation in pigmentation patterns. **A**. Original drawing of a specimen (presumably type material) of *Neotrygon kuhlii* (pl. 51 in [9]). **B**. Specimen MZB 20843 (male *N. kuhlii*; no. 2 in Table 1) from Meulaboh, Aceh (04°07′N 96°08′E; 28 April 2009; I.S.A.). **C**. Specimen MZB 20851 (male *N. kuhlii*, 260 mm DW; no. 8 in Table 1) from Pulau Pari, Java Sea (05°51′S 106°37′E; 14 December 2008). **D**. Specimen MNHN 2009-0823 (female *N. trigonoides*, 350 mm DW; no. 38 in Table 1) from Saint-Vincent Bay, New Caledonia (21°57′S 166°02′E; 07 March 2009; P.B.).

2. Materials and methods

2.1. Samples

Table 1 lists the blue-spotted maskray individuals sampled for DNA and barcoded for the present survey. This list includes 13 newly sampled specimens from the Indian Ocean (nos. 1–4 in Table 1), the Coral Triangle (nos. 7–9; 28; 30–32) and New Caledonia (nos. 37–39). All newly sampled specimens were deposited in zoological collections (Table 1) except no. 1 (from Pemba Island, Tanzania), which was not retained. Table 1 also includes references to sequence data retrieved from GenBank (http://www.ncbi.nlm.nih.gov/; accessed 25 November 2012) to which the new sequences were compared.

2.2. Nucleotide sequences

A tissue fragment ~0.05 cm^3 to ~1 cm^3 was removed, using surgical scissors, from the pelvic or pectoral fins, or the tail and was preserved in 95% ethanol at ambient temperature. DNA extraction was done using either the Viogene (Taiwan) tissue genomic DNA extraction protocol, or the DNEasy DNA extraction kit of Qiagen GmbH (Hilden, Germany). DNA was stored in 1X, pH 8.0 TE buffer (AppliChem, Darmstadt, Germany). Polymerase chain reaction (PCR) amplification of a fragment of the *COI* gene was done in a T-Gradient thermal cycler (Biometra, Göttingen, Germany) using 20 μL reaction mixture with the following concentrations: 0.05 units/μL Taq DNA polymerase in 2× DFS-Taq Mastermix (Bioron GmbH, Ludwigshafen, Germany), 16 mM $(NH_4)_2SO_4$, 65 mM TrisHCl, pH 8.8 at 25 °C, 0.01% Tween-20, 2.75 mM $MgCl_2$, 0.8 mM dNTP mix, 0.4 μM of each primer (AITBiotech, Singapore) and 2 μL DNA template. The primers were *FishF1* (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and *FishR1* (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) [19]. PCR parameters were an initial denaturation at 94 °C for 2 min, followed by 35 cycles of heating (94 °C for 1 min), annealing (47 °C for 1 min) and extension (72 °C for 1 min) with a final extension step at 72 °C for 10 min.

Individual MNHN 2009-0823 was also PCR-amplified for the complete *cytochrome b* gene, using primers *L14735* (5′-AAAAACCACCGTTGTTATTCAACTA-3′) and *CB7* (5′-CTCCAGTCTTCGGCTTACAAG-3′, slightly modified from *CB6ThrH-15930*) [20,21]. The reaction volume was 15 μL and the reaction mixture contained 0.2 mM dNTPs, 1.5 μL 10 × PCR buffer (Bioman, Taipei), 0.5 μM each of forward and reverse primer, 0.2 U Taq DNA polymerase (Bioman), and 1.0 μL template DNA. The PCR parameters were initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation (94 °C for 45 s), annealing (48 °C for 1 min), and extension (72 °C for 1 min), and a final extension step at 72 °C for 10 min.

PCR products were visualized on 1% agarose gels and their size was estimated as approximately 670 bp. After purification (by isopropanol precipitation), 1 μL 1/8-diluted PCR product was subjected to sequencing reaction, in both forward and reverse directions, using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA). Cycling conditions were according to the manufacturer's protocol. Sequencing reaction products were cleaned by removing dye-terminator (CleanSEQ kit, Beckman Coulter, Beverly, MA, USA) and loaded onto an ABI Prism 3100 DNA sequencer (Beckman Coulter).

2.3. Phylogenetic analysis

The phylogenetic analysis package Mega5 [22] was used to estimate nucleotide distance between major clades and nucleotide diversity within. Among the nucleotide substitution models proposed to this effect by Mega5, the most likely according to the Bayesian information criterion was the Tamura 3-parameter model [23] (T92) with non-uniform evolutionary rates among sites modelled by discrete gamma distribution (+G). Therefore, nucleotide distance and diversity were estimated according to the T92+G model.

Phylogenetic analyses were done using the Neighbour-joining (NJ) algorithm on T92+G-modelled genetic distances (MEGA5) and partitioned Maximum-likelihood (ML) as implemented in the RAxML-HPC [24] with its graphical interface raxmlGUI 0.93 [25]. For the partitioned ML search with the mixed model of nucleotide substitution, a GTR+G model (with four discrete rate categories) was used for each partition (respective to codon position) as RAxML only provides GTR [26] -related models of rate heterogeneity for nucleotide data [24]. ML tree search was done with 100 separate runs using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees in each run by comparing likelihood scores under the GTR+G model. Nodal support was assessed by bootstrapping the original

Table 1
Specimens of blue-spotted maskray (*Neotrygon kuhlii*) and New Caledonian maskray (*N. trigonoides*) analysed for nucleotide sequence variation at the *COI* gene locus.

Region (species), sampling location	Coordinates	Sampling date	*N*	Collector	Fig. 2 no.	Collection no.	Tissue no.	Photograph	GenBank accession no.
Indian Ocean (*N. kuhlii*)									
Pemba Island, Tanzania	05°21′S 39°38′E	May 2010	1	JDD	1	–	IRD zanz1	[b]	KC295416
Meulaboh, Aceh	04°07′N 96°08′E	April 2009	1	ISA	2	MZB 20843 (LIPI 4406)	ME3	Fig. 1B	JX304805
Perbaungan, Malacca Strait	03°39′N 98°59′E	December 2009	1	ISA	3	MZB 20847 (LIPI 4401)	MS_KL3	[b]	JX304818
Padang, Sumatera	00°56′S 100°21′E	August 2009	1	ISA	4	MZB 20845 (LIPI 4411)	PD1	-	JX304828
Ningaloo Reef	21°55′-22°35′S 113°39′-113°53′E	August–September 2010	2	O. O'Shea	5, 6	–	NKNR42, 43	-	JQ765536–JQ765537
Coral Triangle (*N. kuhlii*)									
Pulau Pabelokan, Java Sea	05°27′S 106°29′E	Aug. 2009	1	ISA	7	MZB 20852 (LIPI 4410)	PB2	[b]	JX304829
Pulau Pari, Java Sea	05°51′S 106°37′E	December 2008	1	Mumu	8	MZB 20851 (LIPI 4402)	PR	Fig. 1C	JX304836
Pulau Peniki, Java Sea	05°46′S 106°38′E	March 2009	1	ISA	9	MZB 20850 (LIPI 4399)	PN5	[b]	JX304840
Haiphong, Viet Nam	20°46′N 106°52′E	September 2010	2	-	10, 11	–	NKVN74, 75	-	JQ765561, JQ765562
Java Sea	-	April 2004	5	W.T. White	12–16	–	BW A2575–BW A2579	-	EU398737–EU398741
Tanjung Manis, South China Sea	02°07′N 111°19′E	April 2004	1	J. Caira, K. Jensen, C. Healy	17	–	GN3636 = BO424	[b]	JN184065
South China Sea	05°20′N 110°26′E	2011	1	-	18	–	FBBGC040-11	-	JQ681494
Bali	08°45′S 115°10′E	August 2002	1	W.T. White	19	CSIRO H 6124–01	BW A2580	-	EF609342
Bali	08°45′S 115°10′E	April 2004–March 2005	5	W.T. White	20–24	–	BW A2571–BW A2574, BWA2583	-	EU398736, EU398742–EU398745
Bali	08°44′S 115°11′E	January 2008	1	PB	25	–	NK_BL	[b]	JX304860
Penghu, Taiwan	~23°37′N ~119°36′E	May 2005	3	-	26–28	–	BW A2584–BW A2585	-	EU398733–EU398735
West coast, Taiwan	-	Oct. 2010	1	H.C. Ho	29	–	wjc627 = 20101017HBH	-	JX304868
Ishigaki-shima, Ryukyu Islands	~24°18′N ~124°10′E	November 2004	1	-	30	NSMT P–91858	-	-	AB485685
Ambon, Molucca Islands	03°40′S 128°11′E	October 2008	1	I.S. Arlyza, La Pay	31	MZB 20864 (LIPI 4400)	AM1	[b]	JX304892
Kei Island, Molucca Islands	~07°37′S ~135°20′E	April 2009	1	A. Kusnadi	32	MZB 20866 (LIPI 4405)	ARA1	[b]	JX304898
Biak, West Papua	00°58′S 136°16′E	May 2009	1	Alvi	33	MZB 20867 (LIPI 4408)	BK5	[b]	JX304909
Gulf of Carpentaria	12°28′S 141°29′E	March 1995	1	-	34	–	BW A208	-	DQ108184
Coral-Sea (*N. trigonoides*)									
Lizard I., Great Barrier Reef	14°41′S 145°27′E	December 2008	3	MGM	35–37	–	NKGBR39, 40; NKNR41	-	JQ765534, JQ765535
St Vincent Bay, New Caledonia	21°56′S 165°55′E	August 2008	1	P. Morlet	38	CSIRO uncat. [a] (NC 20080816)	Dkuh 20080816 (NC1)	[b]	JX304916
St Vincent Bay, New Caledonia	21°57′S 166°02′E	March 2009	1	P. Morlet	39	MNHN 2009–0823	Dkuh 20090307 (NC2)	Fig. 1D	JX263420
St Vincent Bay, New Caledonia	21°57′S 166°02′E	August 2008	1	P. Morlet	40	IRDN 20090816	Nkuh 20090816 (NC3)	[b]	JX304917

CSIRO: Commonwealth Scientific and Industrial Research Organisation, Hobart; *IRDN*: Institut de recherché pour le développement, Nouméa; *MNHN*: Muséum national d'histoire naturelle, Paris; *MZB*: Museum Zoologicum Bogoriense, Cibinong; *NSMT*: National Science Museum, Tokyo; *N*: sample size.

[a] Formalin/alcohol-preserved specimen (female, 22.5 cm disc width) sent by PB to Alistair Graham, CSIRO, Hobart, on 14 August 2009.

[b] Photograph posted in Supplementary Material, Fig. S1.

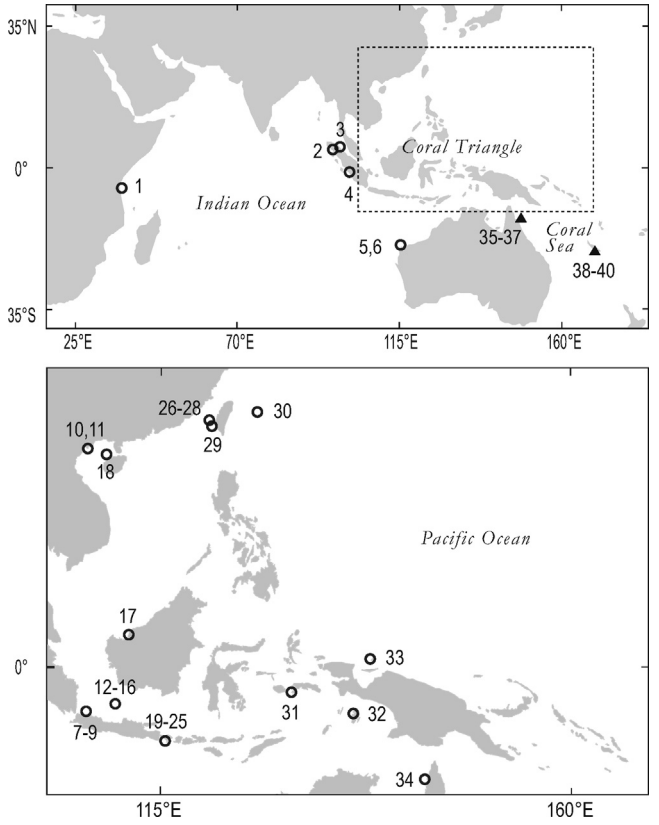

Fig. 2. Map of the central Indo-West Pacific region, with sampling sites for blue-spotted maskray, *Neotrygon kuhlii* and New Caledonian maskray, *N. trigonoides*. Specimen numbers as in Table 1.

matrix of sequences [27] with the NJ algorithm and ML criterion, based on 1000 pseudo-replicates.

2.4. Spotting patterns

The sample of individuals chosen for studying spotting patterns included the holotype of *N. trigonoides* (NMMV 51684), the specimen represented Plate 51 of [9] (presumably one of the three syntypes of *N. kuhlii*), and those specimens analysed at the *COI* locus of which we possessed a photograph of quality suitable to counting and sizing all spots present on the dorsal surface of the disk (Fig. 1 and Supplementary material, Figs. S1 and S2).

The number of ocellated spots on the dorsal side of an individual was counted and their diameter was measured relative to disk width (DW). The maximal diameter of an ocellated spot was then assigned to one of three classes chosen arbitrarily: *small*, when diameter $\leq$ 2% DW; *medium*, when diameter was comprised between 2% and 4% DW; and *large*, when diameter $>$ 4% DW. Dark-brown or black *spots* ($>$ 1% DW) and *speckles* ($\leq$ 1% DW) on the dorsal side of disk were also counted. Dark spots and speckles located in the mask area were not counted, because the quality of some of the pictures was not good enough to distinguish them from the brown background in this part of the head (Supplementary material, Figs. S1 and S2). The symmetrical pair of brown blotches in the scapular region was encoded 0 (when absent), 1 (when visible) or 2 (when conspicuous). Spotting patterns were compared among individuals through correspondence analysis (CA) [28]. CA was run using the FactoMineR package [29] under R [30]. Only dark spots and speckles are clearly visible on specimen NMV 51684. The ocellated white spots mentioned by Castelnau [16] have apparently faded in preservative, as noted previously [17]. Because its spotting patterns were characterized by three descriptor variables only (number of black speckles, number of black dots and presence/absence of brown scapular blotch), specimen NMV 51684 was included as active element in a second CA run based on these three descriptor variables.

3. Results

The NJ tree of *COI* haplotypes (Fig. 3) showed a main dichotomy separating maskrays from the Coral-Sea (northern Great Barrier Reef, New Caledonia) from all the other blue-spotted maskray sampled in the Indo-West Pacific. The net nucleotide divergence between the two clades was 0.026 whereas the nucleotide diversity within clades was 0.003 and 0.027, respectively. By comparison, the net nucleotide distance between *N. leylandi* and *N. picta* was 0.031. The ML tree constructed on the basis of the GTR+G model yielded a slightly different topology (not shown), where the Coral-Sea maskray haplotypes formed a haplogroup external to *N. kuhlii*, but whose monophyly was not supported enough statistically. In all other aspects,

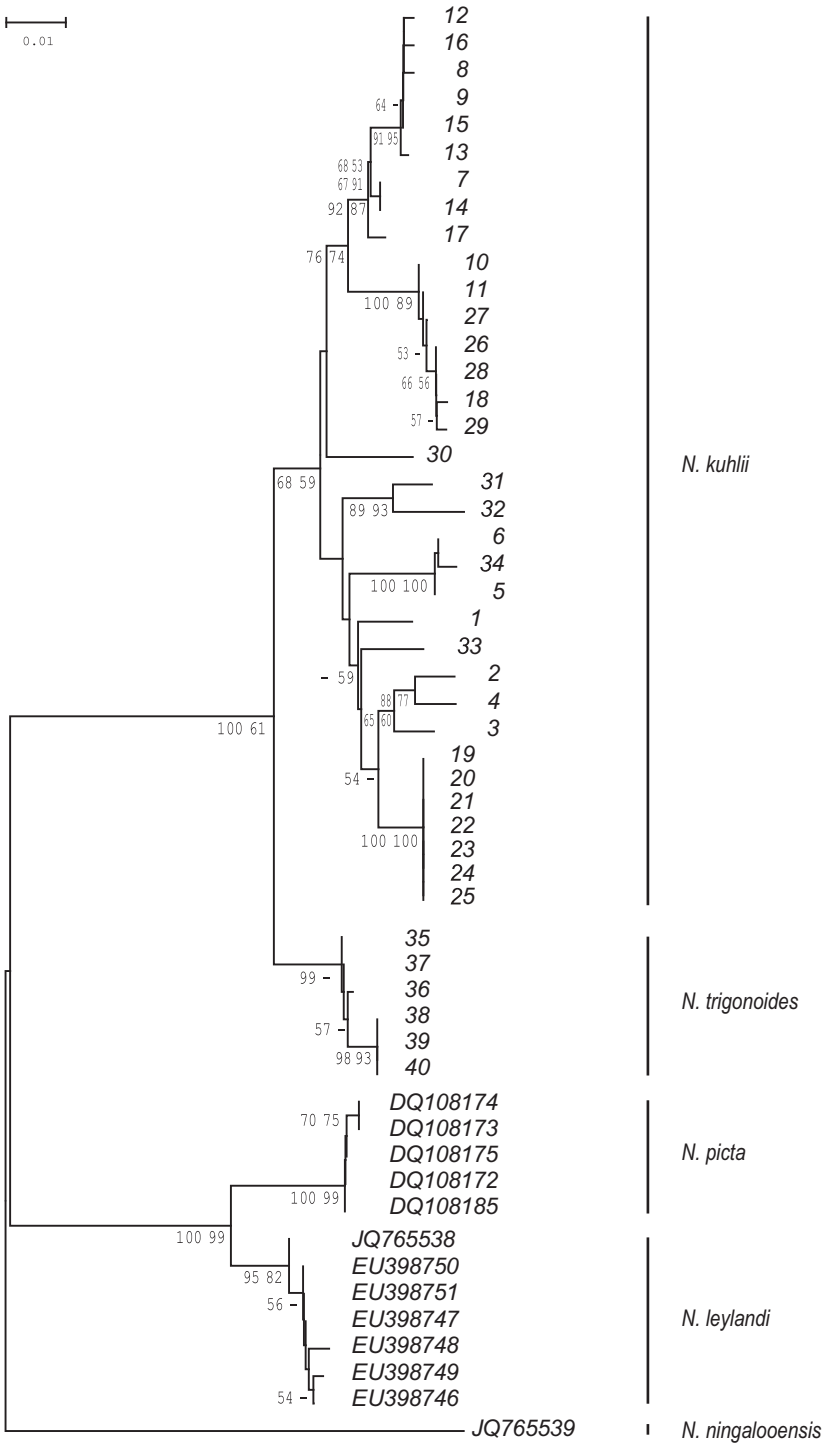

Fig. 3. Neighbour-joining (NJ) tree of partial *COI* gene nucleotide sequences (T92+G-modelled nucleotide distances; MEGA5 [22]) from 34 blue-spotted maskray, *Neotrygon kuhlii*, specimens collected distribution-wide and 6 New Caledonian maskrays, *N. trigonoides*, from the Coral-Sea. Tree rooted by homologous sequences from *N. leylandi*, *N. ningalooensis*, and *N. picta*. Distance scale bar, percent bootstrap values (> 50%; 1000 bootstrap resamplings; MEGA5) are given. The first percentage indicated at a node is the bootstrap score relative to the NJ tree; the second percentage concerns the maximum-likelihood phylogeny constructed under RAxML (GTR+G model; [26]). Specimens numbered as in Table 1 and Fig. 2.

the ML tree was similar to the NJ tree. Bootstrap scores for the ML tree were added on the NJ tree (Fig. 3). Thus, both the NJ tree and ML tree of *COI* haplotypes recovered monophyly of the blue-spotted maskray, but yielded different results on whether the Coral-Sea maskrays formed a strongly supported monophyletic subgroup. In spite of the slightly incongruent results based on two tree reconstruction methods, both clearly separated maskrays

Table 2
Neotrygon kuhlii and *N. trigonoides*. Matrix of individuals characterized by the numbers of ocellated spots [sorted into three size-classes: *small*: ≤ 2% disk width (DW); *medium*: > 2% DW and ≤ 4% DW; *large*: > 4% DW], the number of dark speckles (≤ 1% DW), the number of dark spots (> 1% DW), and the absence or presence of a scapular brown blotch (0: absent; 1: visible; 2: conspicuous) on the dorsal surface of left or right half-disk.

Species, individual no.		Sampling region	Side of disk	*N* ocellated spots			*N* dark speckles	*N* dark spots	Scapular blotch
Fig. 2 no.	Specimen no.			Small	Medium	Large			
Neotrygon kuhlii									
-	Pl. 51 of [9]	Unknown	Left	2	1	0	2	0	0
			Right	1	5	0	1	0	0
1	zanz 1	Tanzania	Left	44	29	2	10	0	0
			Right	51	21	2	15	0	0
2	MZB 20843	Aceh	Left	28	13	0	16	0	0
			Right	29	11	0	16	0	0
3	MZB 20847	Malacca Strait	Left	2	14	0	5	0	0
			Right	4	10	1	3	0	0
7	MZB 20852	Java Sea	Left	5	8	1	21	0	0
			Right	9	3	0	17	0	0
8	MZB 20851	Java Sea	Left	22	9	2	12	0	0
			Right	18	6	2	8	0	0
9	MZB 20850	Java Sea	Left	12	12	2	2	0	0
			Right	22	11	0	1	0	0
17	BO424	S. China Sea	Left	35	22	0	16	0	0
			Right	41	6	0	13	0	0
25	NK BL	Bali	Left	21	6	0	43	0	0
			Right	24	5	0	54	0	0
31	MZB 20864	Ambon	Left	9	7	0	5	0	0
			Right	2	12	0	2	0	0
32	MZB 20866	Kei Islands	Left	36	9	0	53	0	0
			Right	30	9	0	53	1	0
33	MZB 20867	Biak	Left	41	25	0	10	0	0
			Right	37	13	2	7	0	0
Neotrygon trigonoides									
-	NMV 51684	New Caledonia	Left	ND	ND	ND	24	4	1
			Right	ND	ND	ND	26	2	1
38	CSIRO uncat.	New Caledonia	Left	54	0	0	36	1	2
			Right	38	0	0	25	1	2
39	MNHN 2009-0823	New Caledonia	Left	33	0	0	85	10	2
			Right	28	0	0	91	8	2
40	IRDN 20090816	New Caledonia	Left	15	0	0	55	13	2
			Right	16	0	0	57	13	2

N: number of spots or speckles; ND: no data.

from the Coral-Sea from all the other blue-spotted maskrays sampled in the Indo-West Pacific.

Spotting patterns of New Caledonian maskray specimens were markedly different from all other *N. kuhlii* specimens examined (Table 2). Diagnostic differences included the absence of medium or large ocellated spots in New Caledonian maskray, whereas all other *N. kuhlii* specimens possessed a substantial proportion of these; the presence of a few dark spots in all specimens from New Caledonia, *vs.* absence in all other *N. kuhlii* specimens except one; and the presence of a pair of brown scapular blotches, exclusively in specimens from New Caledonia. Moreover, ocellated spots were rather pale grey in New Caledonian maskray specimens *vs.* pale-blue to blue in all other *N. kuhlii* specimens (Supplementary material, Figs. S1 and S2). Pictures of blue-spotted maskray taken underwater at Lizard Island (Supplementary material, Fig. S2), which could not be utilized for the present morphological analysis, revealed features similar to those characterizing New Caledonian specimens, i.e. numerous dark speckles, a few dark spots, and the scapular brown blotch.

Correspondence analysis of the matrix presented in Table 2 determined two main clusters (Fig. 4A, B). One cluster exclusively comprised the specimens from New Caledonia; the analysis placed the holotype of *N. trigonoides* within this cluster (Fig. 4B). The other cluster comprised all *N. kuhlii* specimens from the Indian Ocean and the Coral Triangle and also comprised the *N. kuhlii* specimen referred to by Müller and Henle [9] along their description of the species (Fig. 4A, B).

4. Discussion

Contemporary species concepts are diverse but all relate to the idea that species more or less can be defined as segments of lineages of the global genealogical network pertaining to the metapopulation level of organization [31]. Properties of species may include reproductive isolation from other species, the possession of fixed character state differences, and monophyly [31–34].

Neotrygon trigonoides was originally defined as a distinct species from its unique spotting patterns [16]. The reason for declaring *N. trigonoides* a synonym of the

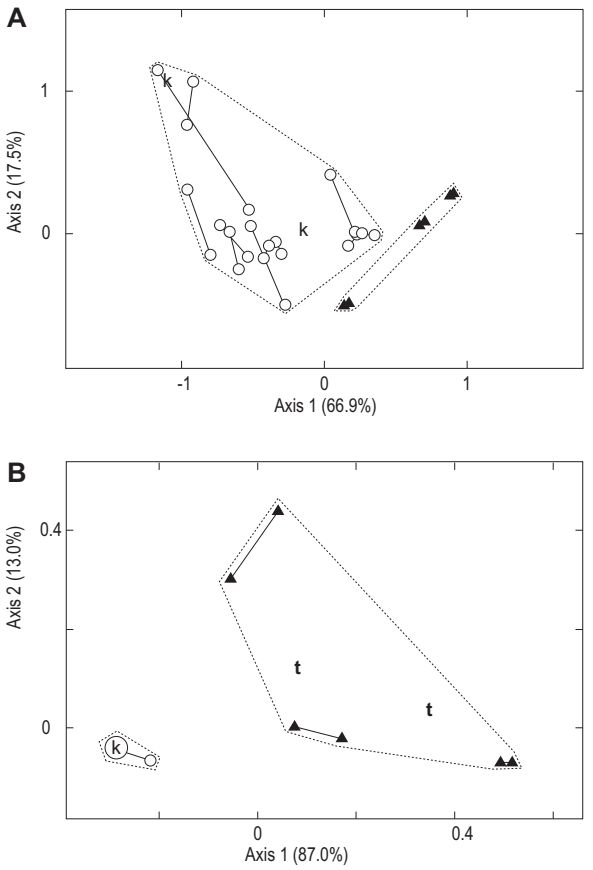

Fig. 4. *Neotrygon kuhlii* and *Neotrygon trigonoides*. Correspondence analysis (CA: [28]) of the matrix of individuals characterized by the size-frequencies of ocellated spots on the dorsal side of a half-disk (Table 2). CA was run using the FactoMineR package [29] under R [30]; percentages for each axis are their inertias [28]. *Open circles* (○): *N. kuhlii*; *closed triangles* (▲): New Caledonian maskrays, *N. trigonoides*; *k*: *N. kuhlii* specimen depicted in Plate 51 of [9]; *t*: holotype of *N. trigonoides*. Thin segments link data points representing the two half-disks of an individual. *Dotted lines* delineate groups of individuals grouped by hierarchical clustering analysis [29]. **A**. Individuals characterized by six variables; *N. trigonoides* holotype excluded. **B**. All 40 individuals characterized by three variables (number of black speckles, number of black dots and presence/absence of brown scapular blotch), to allow the placement of the holotype of *N. trigonoides* in the absence of ocellated spot data.

distinctly colour-patterned *N. kuhlii* originates from the similarity of the *N. trigonoides* holotype, from New Caledonia, with blue-spotted maskray specimens from eastern Australia [17] which for some unclear reason had been erroneously identified as *N. kuhlii*. As shown in this paper, *N. trigonoides* should be rehabilitated as a valid species and blue-spotted maskrays from eastern Australia are of this species.

Haplotypes from blue-spotted maskray specimens sampled from the northern Great Barrier Reef and from New Caledonia (Coral-Sea) clustered into a well-defined and statistically supported clade external to *N. kuhlii* in the NJ tree of genetic distances. Character-evolution based ML phylogeny also found Coral-Sea haplotypes to cluster externally to those of *N. kuhlii*, although it failed to group them within a truly monophyletic haplogroup. We interpret this minor difference with the topology of the NJ tree as an indication that the phylogenetic information from the single and short *COI* fragment sequenced in this study might be insufficient. M. Puckridge et al. [15] have recently produced a phylogeny of *Neotrygon* spp. based on concatenated partial *COI* and *16S* haplotypes, which confirms this hypothesis. All blue-spotted maskray mitochondrial haplotypes from the Coral-Sea (including new individuals from New Caledonia, from the northern Great Barrier Reef, from southeastern Queensland and from northeastern New South Wales) clustered as a well-defined clade in Puckridge et al.'s [15] phylogenetic reconstruction (their figure 2b). This was resolved as a clade sister to that formed by *N. kuhlii* mitochondria [15]. The *N. kuhlii* clade included a specimen from the Gulf of Carpentaria, which is geographically adjacent to the northern Barrier Reef where only *N. trigonoides* haplotypes have been sampled thus far ([8,15]; present results). This abrupt geographic discontinuity in mitochondrial genetic composition relative to the genetic continuity observed all across the Coral-Sea on the one side, and the Sahul shelf and even the whole Indian Ocean and Coral Triangle on the other side ([15], present results) is incompatible with the idea of a gradual genetic transition between *N. trigonoides* and *N. kuhlii*. Because no contemporary discontinuity of habitat suitable to *N. kuhlii* [10] is apparent across the Torres Strait region, this points to reproductive isolation between the two species.

Although reproductive isolation was here inferred between *N. trigonoides* and *N. kuhlii*, no firm proof of this has been presented. Relying on the single mitochondrial phylogeny may appear insufficient for assessing systematic relationships. However, nuclear (*RAG-1*) haplotypes from either side of the Torres Strait are also different [15]. Another line of evidence for two separate species is the large nucleotide distance between *N. trigonoides* and *N. kuhlii*, which was comparable to the distance between *N. leylandi* and *N. picta*. Last, the colour pattern of *N. trigonoides* distinguishes it from Müller and Henle's [9] *N. kuhlii* making it diagnosable on the basis of trivial external examination. Thus, *N. trigonoides* should be resurrected as a valid species because of its diagnosability when using both colour patterns and genetic characters, because of the monophyly of its mitochondrial DNA haplotypes, and because of the geographic distribution of haplotypes which highlights the genetic gap between *N. kuhlii* from the Gulf of Carpentaria (west of Torres Strait) and *N. trigonoides* immediately east of it. All the foregoing constitutes properties expected from separately evolving metapopulation lineages, i.e., distinct species [31].

The *COI* nucleotide sequences of *N. kuhlii* examined in this paper were sampled from 34 specimens from 18 locations spanning almost the full longitudinal range of the species, from the eastern coast of Africa to the West Pacific. Several well-supported sub-clades were visible within the *N. kuhlii* clade as observed previously [3,7,15]. The occurrence of deeply rooted clades within *N. kuhlii* has led authors to suggest that it consists of several cryptic species [3,15]. In the present study, as in other *COI*-based studies [7,8], we find no compelling, definitive evidence

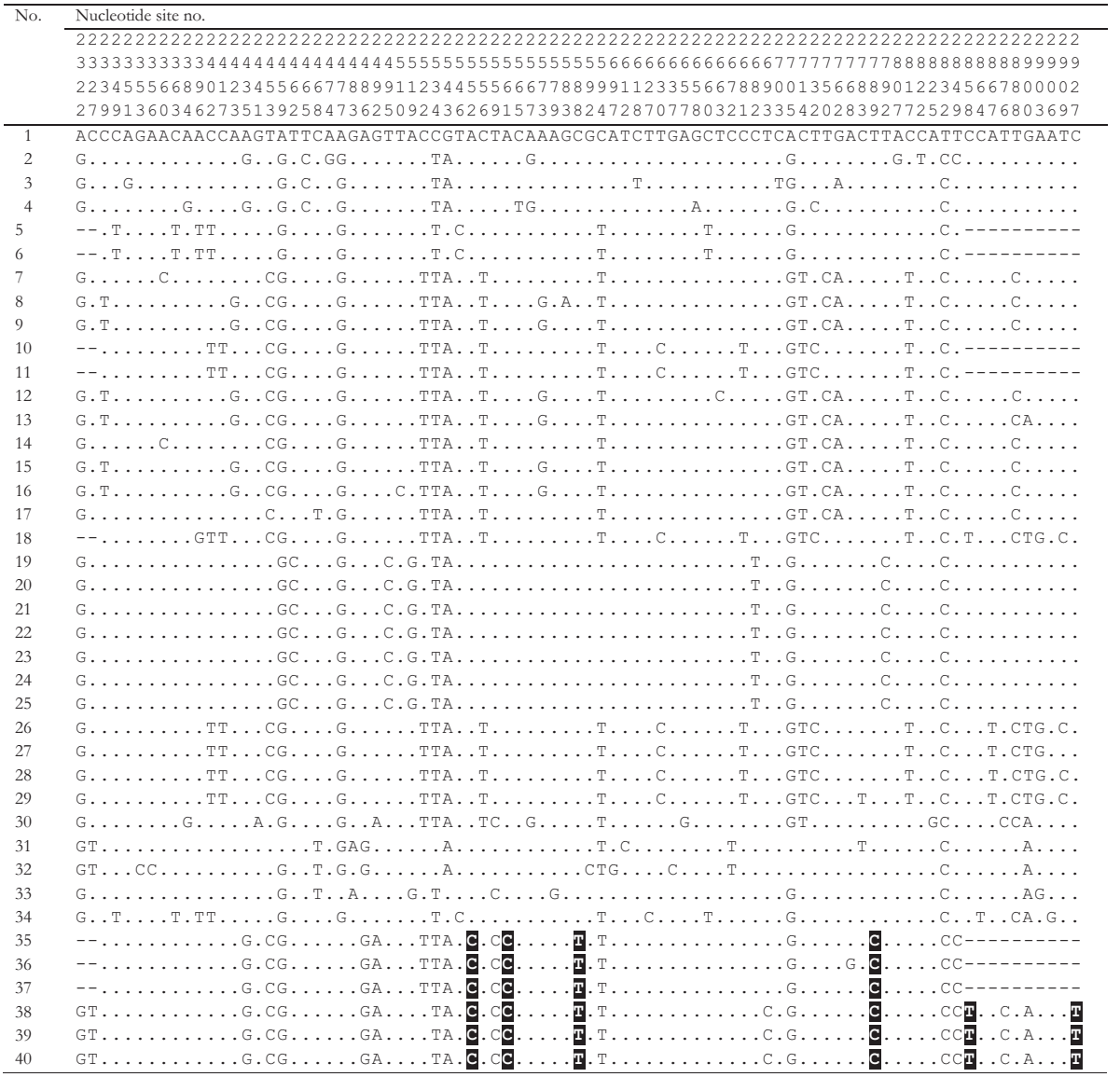

Fig. 5. Variable nucleotide sites in *COI* gene sequences of *Neotrygon kuhlii* and *Neotrygon trigonoides*. Numbers allocated to specimens as in Table 1 and Fig. 2. Nucleotide sites numbered according to [1] (GenBank JN184065). *Highlighted*: synapomorphies characterizing the New Caledonian maskray, *N. trigonoides*.

favouring this interpretation (Fig. 3). Likewise, spotting patterns as measured in our study did not allow a clear partition of *N. kuhlii* into distinct groups. However, more detailed nuclear-based investigations are needed, to test whether different mitochondrial clades within *N. kuhlii* may belong to reproductively isolated populations. The *RAG-1* marker has proven to be too conservative to this effect [15]. Size-polymorphic introns [14] might be more appropriate. The few intron data available thus far [14] are compatible with a discontinuous distribution of genetically differentiated, possibly isolated populations. However, denser geographic sampling is necessary to further investigate potential geographic boundaries or transition zones.

5. Resurrection of *Neotrygon trigonoides*

Family Dasyatidae Jordan, 1888 [35]; Dasyatidae belong to the superfamily Myliobatoidei recently redefined on the basis of mitochondrial genomes and nuclear genes [1]. Genus *Neotrygon* Castelnau, 1873 [16]. *N. trigonoides* Castelnau, 1873, resurrected species.

5.1. Previous references

Raya trigonoides [16]; *Dasyatis kuhlii* (non Müller & Henle, 1841) [36,37]; *N. kuhlii* (non Müller & Henle, 1841) [8,17,38]; *N. trigonoides* [5,16].

5.2. Material examined

The *N. kuhlii* and *N. trigonoides* material examined for nucleotide sequence variation at the *COI* locus is listed in Table 1. Specimens of *N. kuhlii* and *N. trigonoides* examined for spotting patterns are listed in Table 1 and their photographs are presented in Supplementary material, Figs. S1 and S2, respectively.

Three vouchers of *N. trigonoides* that were characterized by their nucleotide sequences at the *COI* locus were placed in ichthyological collections: CSIRO uncatalogued, female, 225 mm DW, from Saint-Vincent Bay, New Caledonia – this specimen was captured on 16 August 2008 by P. Morlet and deposited in the Australian National Fish Collection (CSIRO, Hobart) by P.B. on 14 August 2009 (Supplementary material, Fig. S1); MNHN 2009-0823, female, 350 mm DW, New Caledonia (Fig. 1D); IRDN 20090816, male, 302 mm DW, from Saint-Vincent Bay, New Caledonia, captured on 16 August 2009 by P. Morlet and deposited in the fish collections of the IRD centre in Nouméa, New Caledonia.

5.3. Redescription

Castelnau [16] has provided a qualitative description of the colour patterns of *N. trigonoides*: "Entirely of a light brown lilac colour, with a few faint white oscillated [sic] spots on the disk, and a larger number of smaller black ones dispersed in a most irregular way; posterior part of the tail annulated, black and orange; lower side of the body entirely of a light cream colour". The holotype of *N. trigonoides* (NMV 51684; Supplementary material, Fig. S1), a juvenile male, 182 mm DW, from New Caledonia, seems to have lost its ocellated spots in preservative. Live or freshly captured *N. trigonoides* are characterized by the presence of dark spots larger than $> 1\%$ DW and by a symmetrical pair of brown blotches in the scapular region (Supplementary material, Fig. S2).

Although spotting patterns are useful as a character to distinguish between *N. trigonoides* and *N. kuhlii* (Fig. 4), the mitochondrial sequence has proven to be an excellent diagnostic character (Fig. 3) and because of this fact, we consider it to be much more adequate than any of the morphological characters employed thus far, including spotting patterns (present work), to base our description on.

The present redescription of *N. trigonoides* is based on the nucleotide sequence of a 655-base pair (bp) fragment of the *COI* gene (hereafter abbreviated as 'partial *COI* gene') homologous to the portion of the mitochondrial genome of *N. kuhlii* comprised between nucleotide sites 2278 and 2932 (GenBank JN184065; [1]). The partial *COI* gene of specimen MNHN 2009-0823 has the following sequence (accession no. JX263420 in GenBank): 5′- C C T T T A C T T A G T C T T T G G T G C A T G A G C A G G G A T A G T A G G C A C T G G C C T T A G T T T A C T T A T C C G A A C A G A A C T A A G C C A A C C A G G C G C T T T A C T G G G T G A T G A T C A A A T T T A T A A T G T A A T C G T C A C T G C C C A C G C C T T C G T A A T A A T C T T C T T T A T G G T A A T G C C A A T T A T A A T C G G T G G G T T T G G T A A C T G A C T A G T A C C C C T G A T G A T T G G A G C T C C G G A C A T A G C C T T T C C A C G A A T A A A C A A C A T A A G T T T T T G A C T T C T A C C T C C C T C C T T C C T A C T C C T G C T A G C C T C A G C A G G A G T A G A A G C T G G A G C T G G A A C A G G T T G A A C A G T T T A T C C C C C A T T A G C T G G T A A T C T A G C A C A T G C C G G A G C T T C T G T A G A C C T T A C A A T C T T C T C T C T T C A C C T A G C A G G T G T C T C C T C T A T T C T G G C A T C C A T C A A C T T T A T C A C A A C A A T T A T T A A T A T A A A A C C A C C T G C A A T C T C C C A G T A T C A A A C C C C A T T A T T C G T C T G A T C C A T T C T T G T T A C A A C T G T A C T T C T C C T G C T A T C C C T A C C A G T C C T A G C A G C T G G C A T T A C C A T A C T C C T T A C A G A C C G A A A T C T T A A C A C A A C T T T C T T T G A C C C A G C T G G A G G A G G A G A T C C C A T T C T T T A C C A A C A T C T C T T C -3′. This sequence has accession No. KC295416 in GenBank.

In addition, the nucleotide sequence of the *cytochrome b* gene (1132-bp) of *N. trigonoides* MNHN 2009-0823 homologous to the portion of the mitochondrial genome of *N. kuhlii* comprised between nucleotide sites 10859 and 11992 (GenBank JN184065; [1]) is the following: 5′- A A C A T C C G T A A A A C A C A T C C C C T A T T C A A A A T T A T C A A C A A C T C A C T A A T T G A T C T A C C A G C T C C A A C C A A T A T T T C C A C C T G A T G A A A T T T T G G T T C C C T A C T A G G C C T T T G C C T A A T T A T C C A A A T C C T T A C A G G C C T A T T C C T A G C T A T A C A C T A C A C C G C A G A C A T C T C A T C A G C A T T C T C C T C A G T T G C C C A T A T C T G C C G A G A C G T T A A C T A C G G T T G A C T A A T C C G C A A T A T T C A C G C T A A C G G C G C C T C A A T A T T C T T C A T C T G T G T T T A T C T C C A T A T T G C T C G A G G A C T T T A C T A T G G C T C C T A C C T C A A T A A A G A A A C C T G A A A T A T C G G A G T A G T T A T C C T A G T G T T A C T A A T A G C C A C C G C A T T C G T A G G C T A T G T T C T C C C A T G A G G A C A A A T A T C A T T C T G A G G G G C A A C C G T T A T C A C C A A C T T A C T A T C A G C C C T C C C C T A T A T T G G A G A C A T G T T A G T T C A A T G A A T C T G A G G G G G C T T C T C A A T T G A C A A T G C A A C A T T A A C T C G A T T T T T C A C A T T T C A T T T T C T A T T T C C C T T T G T A A T T G C A G C T C T T A C T A T A A T T C A C C T T C T C T T C C T T C A T G A A A C A G G T T C T A A C A A C C C A A C C G G A C T C T C A T C T A A C A T A G A C A A A G T C C C G T T T C A T C C T T A T T A T A C A T A T A A A G A T C T A G T A G G C T T C T T C A T C C T T C T A A T A C T A C T A A C T C T A C T T G C C C T A T T T A C A C C A A A C C T C C T A G G G G A T A C A G A A A A C T T T A T T C C A G C C A A C C C C C T C G T C A C A C C T C C C C A T A T T A A A C C A G A G T G A T A C T T C T T A T T T G C C T A C G C T A T T C T A C G C T C T A T C C C C A A T A A A C T A G G A G G A G T C C T A G C C C T C G C C T T C T C A A T C T T T A T C C T G C T A C T A A T C C C C A T T C T T C A C A C C T C T A A A C A A C G A A G C C T T A C C T T C C G T C C A A T T A C A C A A C T C C T G T T C T G A C T C T T A G T G G C A A A C A C A A T C A T C C T A A C A T G A A T C G G C G G C C A A C C C G T A G A A C A G C C A T T C A T C A T T A T T G G C C A A A T C G C C T C A A T C A C C T A C T T C T C C T T C T T C C T C A T C C T A T T C C C A A T C G C T G G A T G A T G A G A A A A C A A A A T G T T A A A C C T T A -3′. This sequence has accession no. KC493691 in GenBank.

5.4. Vernacular names

N. trigonoides is apparently endemic to the Coral-Sea. As the holotype of the species is from New Caledonia, and as to our knowledge there is no *Neotrygon* species other than *N. trigonoides* occurring in New Caledonia, we propose as

the English vernacular name: New Caledonian maskray. We propose as the French vernacular name: *raie pastenague à points noirs et bleus*, as it is usually called in New Caledonia [39], even though *gris* (grey) might be a more accurate epithet than *bleus* (blue) to designate the colour of the ocellated spots.

5.5. Comparison with closely related species

Species of the genus *Neotrygon* (*N. kuhlii*, *N. leylandi*, *N. ningalooensis*, *N. picta* and *N. trigonoides*) show no diagnostic difference in the partial *COI* amino-acid sequence as translated from the 519-bp fragment analyzed in common in all five species (present study). Among species in the genus, *N. trigonoides* is most closely related to *N. kuhlii* (Fig. 3). *N. trigonoides* can be separated from *N. kuhlii* by the nucleotide synapormorphies highlighted on Fig. 5.

5.6. Geographic distribution

DNA-barcoded *N. trigonoides* specimens were collected in Lizard Island [8], off southeastern Queensland and northeastern New South Wales [15] and in New Caledonia ([15], present study). Therefore, based on the material genetically identified to species thus far, *N. trigonoides* exclusively occurs in the Coral-Sea. Blue-spotted maskrays from Moreton Bay (southeastern Queensland) in the southwestern Coral-Sea also possess dark spots and the symmetrical pair of scapular blotches on either side of the chord characteristic of *N. trigonoides* (although the latter are fainter than in specimens from New Caledonia); however, in contrast with New Caledonian specimens, their ocellated spots are pale-blue and are also larger in size relative to DW (from a series of photographs by S. Theiss; pers. comm.). Based on spotting patterns from specimens photographed underwater by F.R. McConnaughey (www.gettyimages.com), *N. trigonoides* is also present in Vanuatu at the eastern boundary of the Coral-Sea [40].

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2013.05.005.

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