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Philippe Borsa, Irma S. Arlyza, Wei-Jen Chen, Jean-Dominique Durand, Mark Meekan, et al.. Resurrection of New Caledonian maskray *Neotrygon trigonoides* (Myliobatoidei: Dasyatidae) from synonymy with *N. kuhlii*, based on cytochrome-oxidase I gene sequences and spotting patterns. *Comptes Rendus Biologies*, 2013, 336, pp.221-232. ird-00847459

HAL Id: ird-00847459

<https://ird.hal.science/ird-00847459>

Submitted on 23 Jul 2013

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To be cited as:

Borsa P, Arlyza IS, Chen W-J, Durand J-D, Meekan MG, Shen KN (2013) Resurrection of New Caledonian maskray *Neotrygon trigonoides* (Myliobatoidei: Dasyatidae) from synonymy with *N. kuhlii*, based on cytochrome-oxidase I gene sequences and spotting patterns. *Comptes Rendus Biologies* 336, 221-232.

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

Philippe Borsa ^{a,*}, Irma S. Arlyza ^b, Wei-Jen Chen ^c, Jean-Dominique Durand ^d, Mark G. Meekan ^e, Kang-Ning Shen ^{f,g}

^a Institut de recherche pour le développement (IRD), UR 227 « Biocomplexité des écosystèmes récifaux », Montpellier, France

^b Lembaga Ilmu Pengetahuan Indonesia (LIPI), Pusat Penelitian Oseanografi (P2O), Ancol, Jakarta, Indonesia

^c Marine Phylogenomics Laboratory, Oceanography Institute, National Taiwan University, Taipei, Taiwan

^d IRD, UMR 5119 Ecosym, Université Montpellier 2, Montpellier, France

^e Australian Institute of Marine Science, Perth, Australia

^f Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan

^g Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

* Corresponding author, present address: IR UR 227 c/o Indonesian Biodiversity Research Center, Universitas Udayana, Jl Sesean Gang Markisa 6, Denpasar, Indonesia. Tel. +62 812 37453473; E-mail: philippe.borsa@ird.fr (P. Borsa)

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 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*.

Key words: Indo-West Pacific; Coral Triangle; Coral Sea; New Caledonia; molecular systematics.

RESUME

La raie pastenague à taches bleues, *Neotrygon kuhlii* (Müller and 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 de cet acte 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) et 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. Ceci, ajouté à un niveau de divergence nucléotidique consistant (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 : Indo-Ouest Pacifique ; Triangle de Corail ; Mer de Corail ; Nouvelle-Calédonie ; systématique moléculaire.

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 2 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 kuhlî* (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]. *Neotrygon kuhlî* 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. kuhlî* [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. kuhlî* [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. kuhlî*, J. Müller and J. Henle [9] only mentioned “*einzelne, kleine, runde blaue Flecken (3-6) auf jeder Brustflosse*” and provided a figure of *N. kuhlî* that illustrates this description (Fig. 1A). The two species, *N. kuhlî* 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. kuhlî* 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. kuhlî*”.*

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. kuhlî* [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 re-description of *N. trigonoides* based on the *COI* gene sequence.

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 $\sim 0.05 \text{ cm}^3$ to $\sim 1 \text{ cm}^3$ 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 2x DFS-Taq Mastermix (Bioron GmbH, Ludwigshafen, Germany), 16 mM $(\text{NH}_4)_2\text{SO}_4$, 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'- T C A A C C A A C C A C A A G A C A T T G G C A C -3') and *FishR1* (5'- T A G A C T T C T G G G T G G C C A A A G A A T C A -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'- A A A A A C C A C C G T T G T T A T T C A A C T A -3') and *CB7* (5'- C T C C A G T C T T C G G C T T A C A A G -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 $\times$ PCR buffer (Biomann, Taipei), 0.5 μM each of forward and reverse primer, 0.2 U Taq DNA polymerase (Biomann), 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, U.S.A.). Cycling conditions were according to the

manufacturer's protocol. Sequencing reaction products were cleaned by removing dye-terminator (CleanSEQ kit, Beckman Coulter, Beverly, MA, U.S.A.) 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 Neighbor-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 4 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]. Maximum-likelihood 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 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. kuhlîi*), 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 an haplogroup external to *N. kublii*, but whose monophyly was not supported enough statistically. In all other aspects, 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 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. kublii* specimens examined (Table 2). Diagnostic differences included the absence of medium or large ocellated spots in New Caledonian maskray, whereas all other *N. kublii* 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. kublii* 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. kublii* 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. kublii* 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 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 and co-authors [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, *Neotrygon 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 constitute 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 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]. *Neotrygon trigonoides* Castelnau, 1873, resurrected species.

5.1. Previous references

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

5.2. Material examined

The *N. kublii* and *N. trigonoides* material examined for nucleotide sequence variation at the *COI* locus is listed in Table 1. Specimens of *N. kublii* 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 center in Nouméa, New Caledonia.

5.3. Redescription

Castelnau [16] has provided a qualitative description of the colour patterns of *Neotrygon 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 the two species *N. trigonoides* and *N. kublii* (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. kublii* 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 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

TACAATCTTCTCTCTTCACCTAGCAGGTGTCTCCTCTATTCTGGC
 ATCCATCAACTTTATCACAACAATTATTAATATAAAACCACCTGCA
 ATCTCCCAGTATCAAACCCCATTTATTCGTCTGATCCATTCTTGTTA
 CAACTGTACTTCTCCTGCTATCCCTACCAGTCCTAGCAGCTGGCA
 TTACCATACTCCTTACAGACCGAAATCTTAACACAACCTTTCTTTGA
 CCCAGCTGGAGGAGGAGATCCCATTTCTTTACCAACATCTCTTC-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. kublii* 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 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 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 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 [39], 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 [36], 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. kublii*, *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 5 species (present study). Among species in the genus, *N. trigonoides* is most closely related to *N. kublii* (Fig. 3). *N. trigonoides* can be separated from *N. kublii* by the nucleotide synapomorphies highlighted in 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

We have no conflicts of interest concerning this article.

Acknowledgements

We are grateful to Susan Theiss (University of Queensland, Brisbane) for sharing excellent pictures of blue-spotted maskrays from Moreton Bay, and to Hans Ho (NMMB, Pingtung) for sub-sampling tissue from a specimen from Taiwan. Gavin Naylor and Zhong-Duo Wang kindly provided sampling details for

some of the *Neotrygon* spp. material referred to in the present study. We are grateful to Patrick Morlet, fisherman in Bouraké, New Caledonia, for fishing and preserving *N. trigonoides* specimens for us. We thank Catherine Picq (IRD, Montpellier) for her help in getting a copy of the original description by Castelnau; Renny Hadiaty (MZB, Cibinong) and Patrice Pruvost (MNHN, Paris) for allocating collection numbers to our specimens; and Dianne Bray (Museum Victoria, Melbourne) for details on the holotype of *N. trigonoides*. The opus of Müller and Henle was consulted online from the Biodiversity Heritage Library Internet site (<http://www.biodiversitylibrary.org>). Conceived and designed the experiments: PB. Contributed reagents/materials/analysis tools: PB, WJC, ISA, JDD, MGM, KNS. Did the experiments: PB, ISA, KNS. Analyzed the data: PB, WJC. Wrote the paper: PB. Funded by AIRD, IRD, ISE-M, LIPI-P2O, and National Taiwan University-Institute of Fisheries Science; the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/xxx>.

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

Region (species),	Coordinates		Sampling date	N	Collector	Fig. 2 no.	Collection no.	Tissue no.	Photo-	GenBank accession no.
Sampling location									graph	
<i>Indian Ocean (N. kuhlii)</i>										
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
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		August –	2	O. O'Shea	5, 6	-	NKNR42, 43	-	JQ765536-JQ765537
	113°39'-113°53'E		September 2010							
<i>Coral Triangle (N. kuhlii)</i>										
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
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
<i>Coral Sea (N. trigonoides)</i>										
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

^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

Table 2. *Neotrygon kublii* and *N. trigonoides*. Matrix of individuals characterized by the numbers of ocellated spots [sorted into three size-classes: *small*: $\leq 2\%$ disk width (DW); *medium*: $> 2\%$ DW and $\leq 4\%$ DW; *large*: $> 4\%$ DW], the number of dark speckles ($\leq 1\%$ 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. *N*: number of spots or speckles. *ND*: no data.

Species, individual no. Fig. 2 Specimen no. no.		Sampling region	Side of disk	<i>N</i> ocellated spots			<i>N</i> dark speckles	<i>N</i> dark spots	Scapular blotch
				Small	Medium	Large			
<i>Neotrygon kublii</i>									
-	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
<i>Neotrygon trigonoides</i>									
-	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

CAPTIONS TO FIGURES

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.).

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.

Fig. 3. Neighbour-Joining (NJ) tree of partial *COI* gene nucleotide sequences (I92+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, per cent 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.

Fig. 4. *Neotrygon kuhlii* and *N. 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*; *dosed 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 6 variables; *N. trigonoides* holotype excluded. **B.** All 40 individuals characterized by 3 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.

Fig. 5. Variable nucleotide sites in *COI* gene sequences of *N. kuhlii* and *N. 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*.

Fig. 1

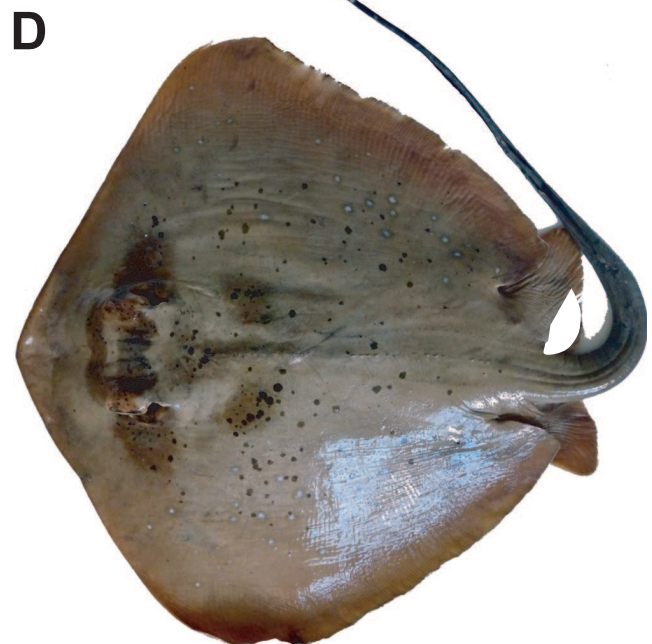
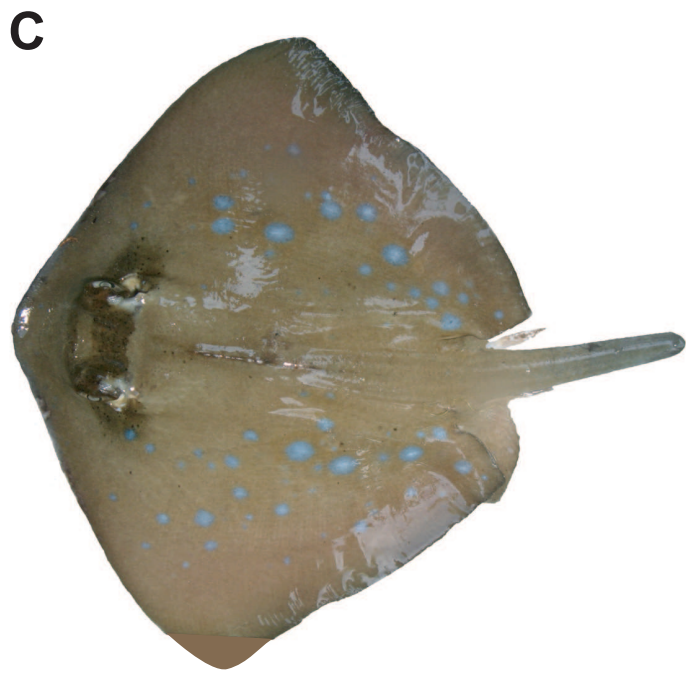
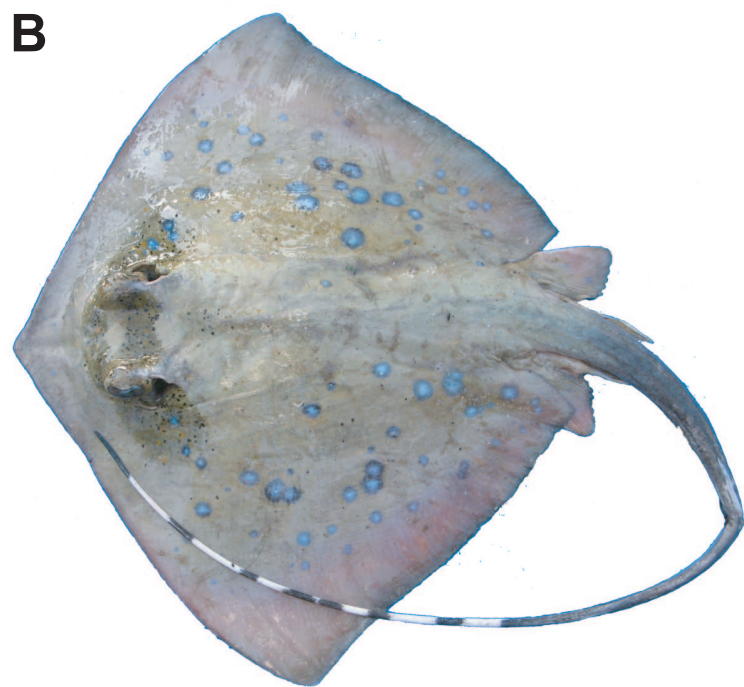
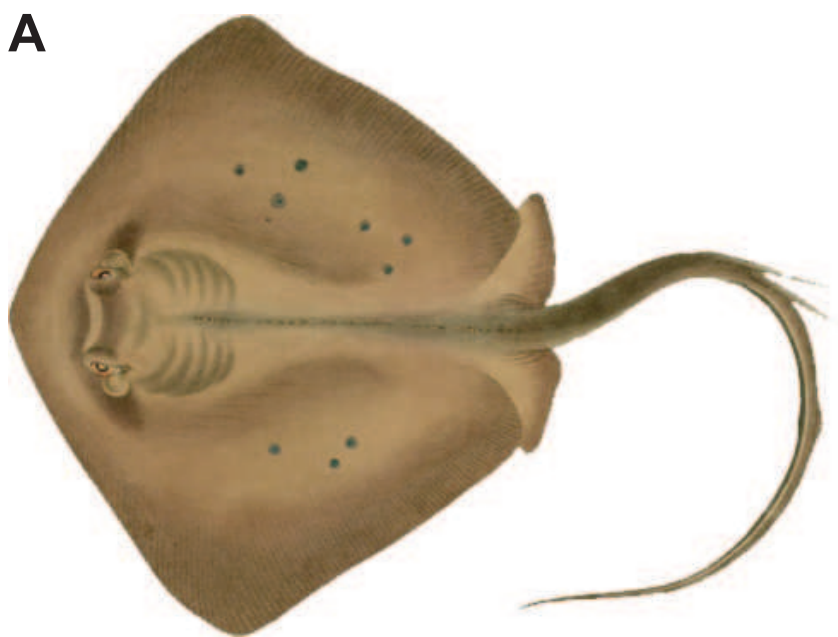


Fig. 2

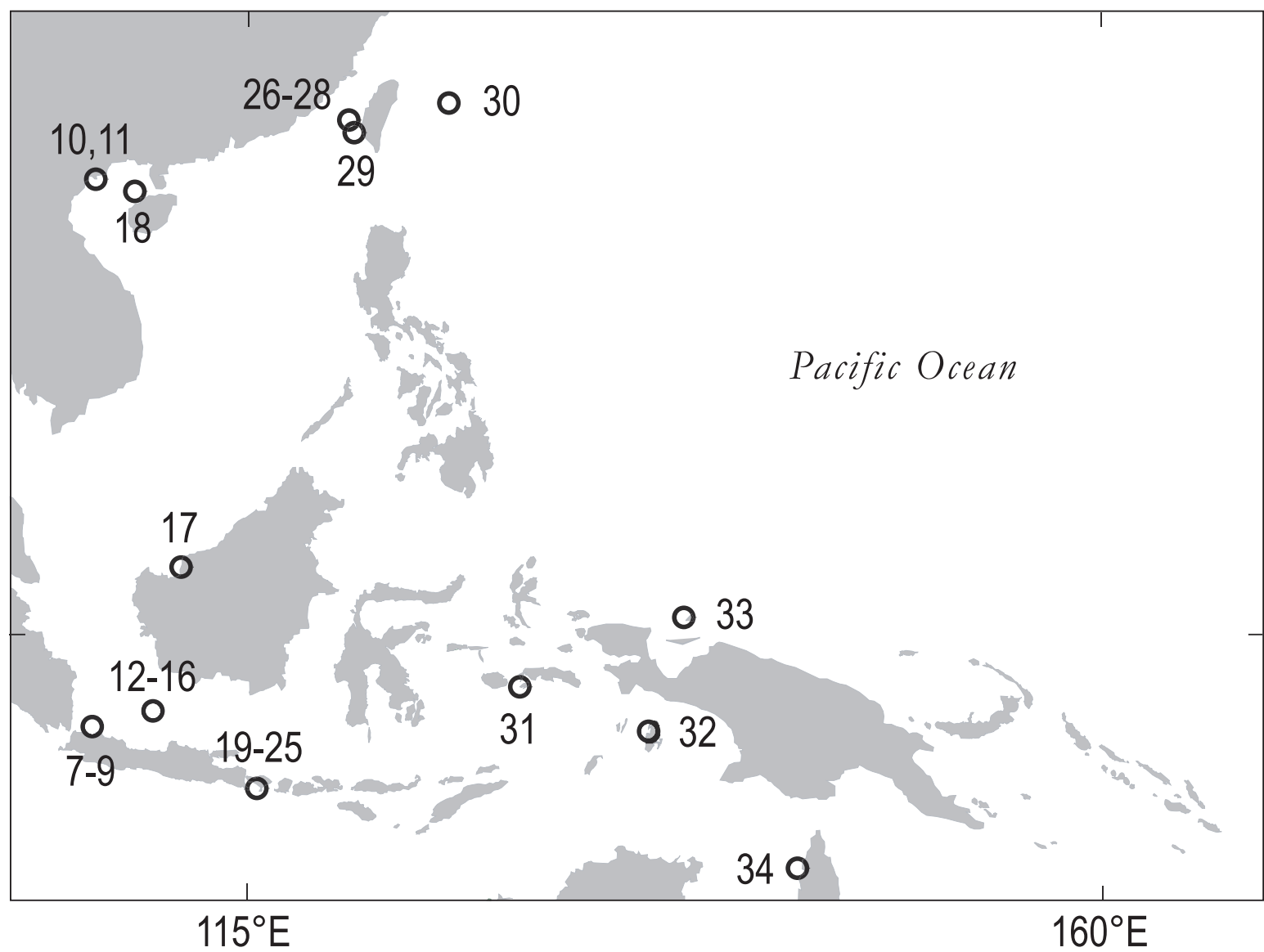
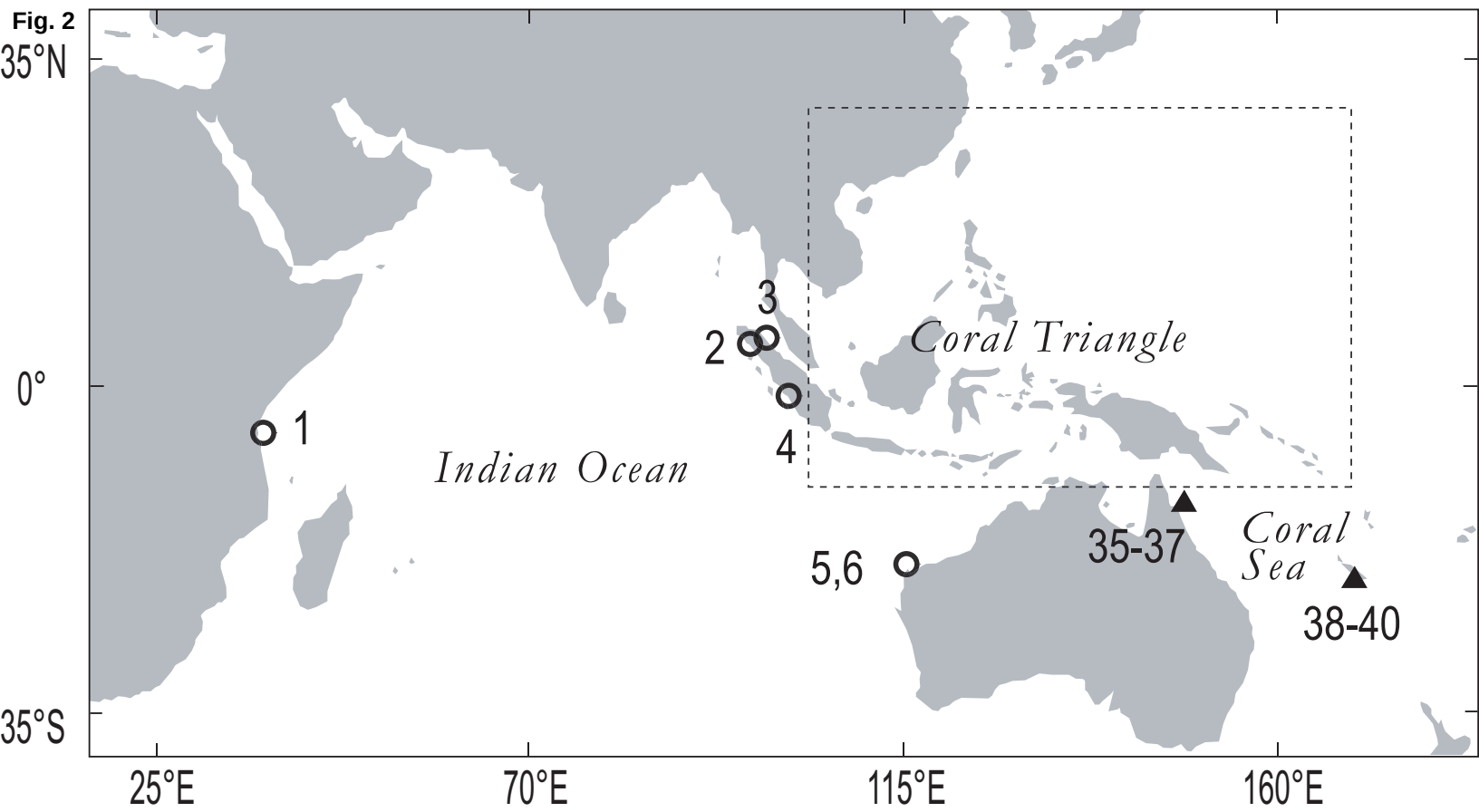
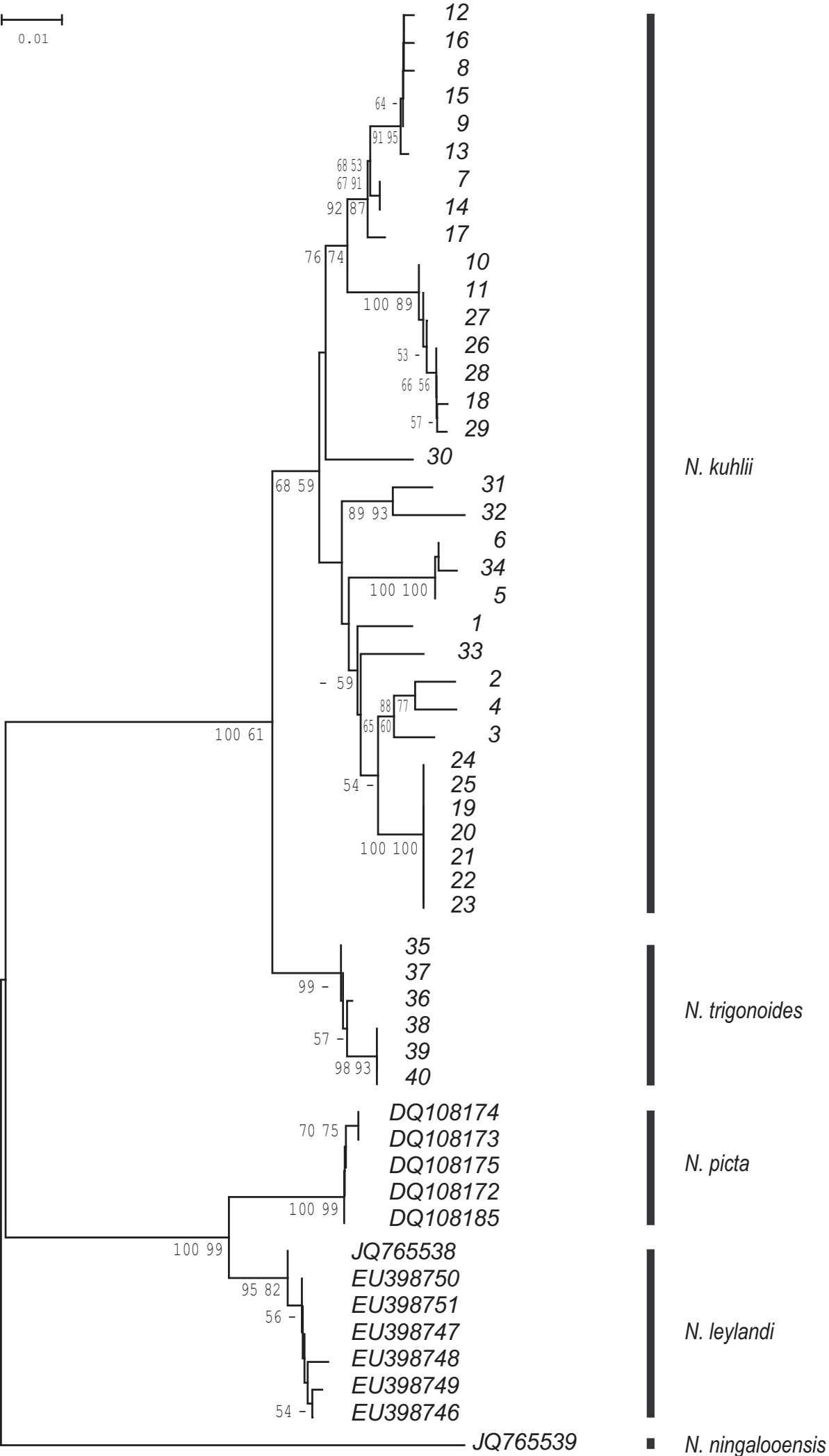


Fig. 3



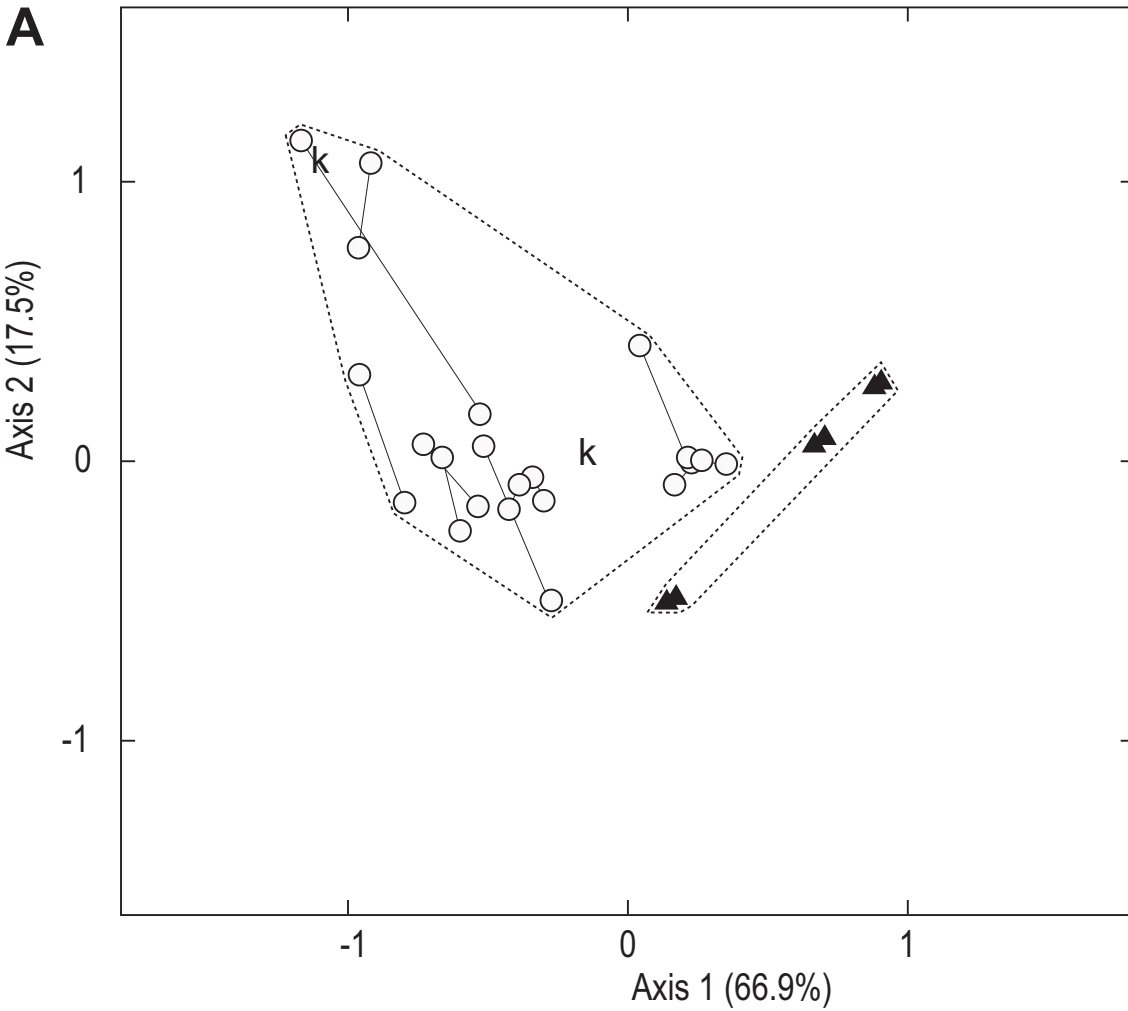
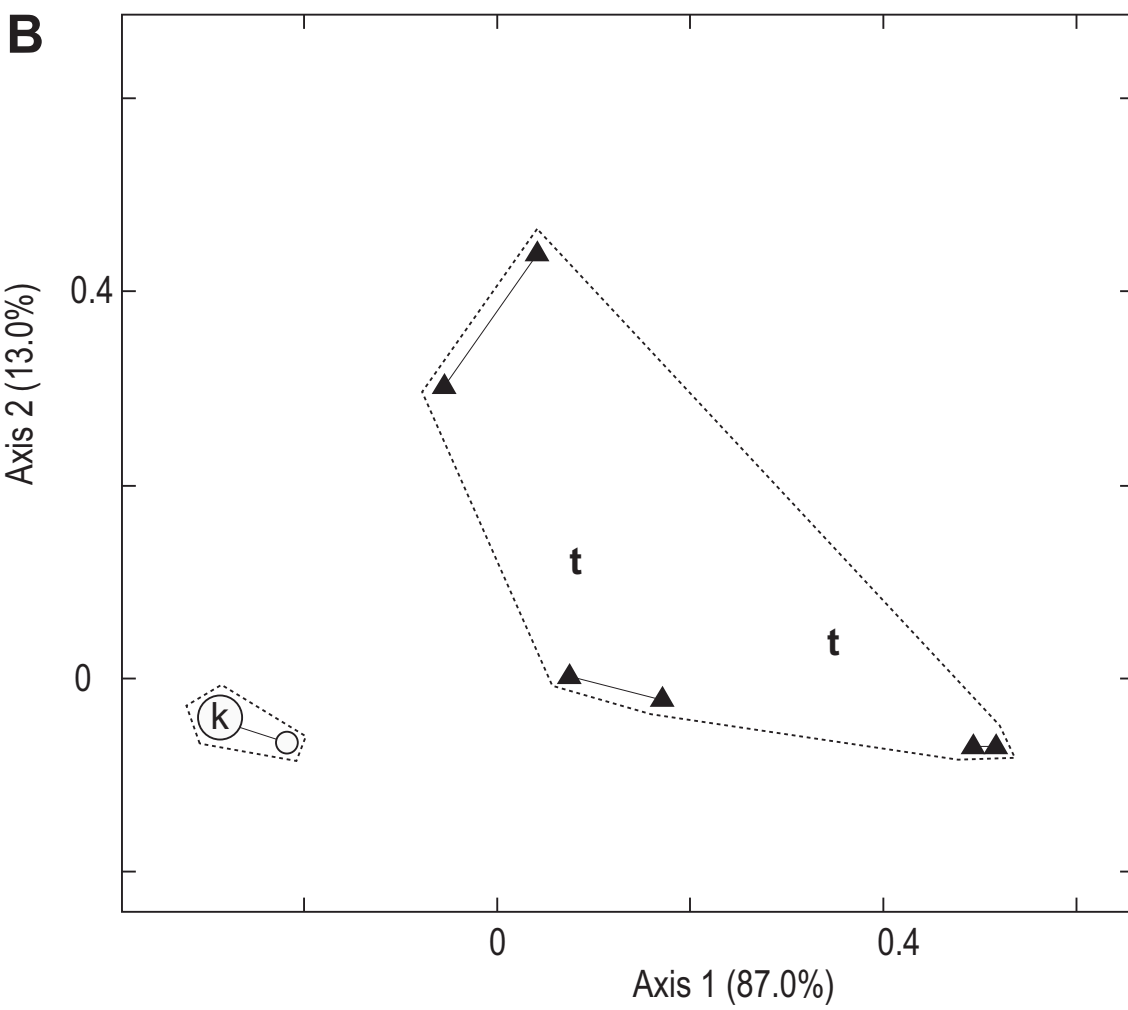
A**B**

Fig. 5

[illegible]

Supplementary material to:

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

by Philippe Borsa, Wei-Jen Chen, Irma S. Arlyza, Jean-Dominique Durand, Mark G. Meekan and Kang-Ning Shen

Fig. S1 here appended

Fig. S2 here appended

References

Castelnau F. de (1873) Contribution to the ichthyology of Australia, Proc. Zool. Acclim. Soc. Victoria 2, 37-158.

Last P.R., White W.T. (2008) Resurrection of the genus *Neotrygon* Castelnau (Myliobatoidei: Dasyatidae) with the description of *Neotrygon picta* sp. nov., a new species from northern Australia. In Last P.R., White W.T., Pogonoski J.J. (eds.), Descriptions of new Australian Chondrichthyans. CSIRO Mar. Atm. Res. Pap. 22, CSIRO, Hobart, pp. 315-325.

Müller J, Henle J. 1841. Systematische Beschreibung der Plagiostomen, mit sechzig Steindrucktafeln. Veit und Comp., Berlin, xxii+200 p., 60 pl.



A



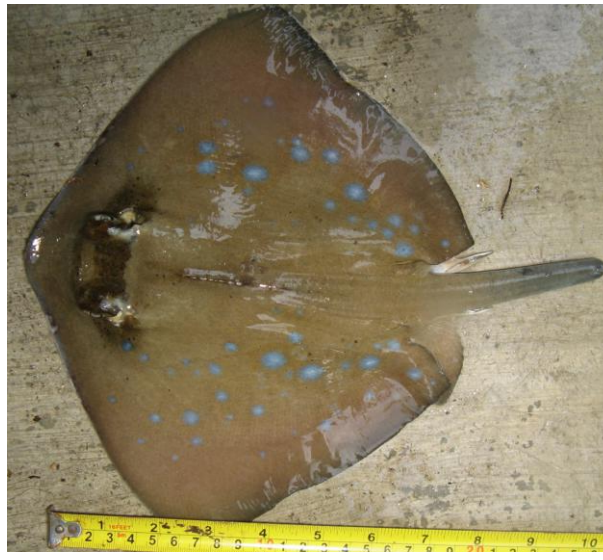
B



C



D



E



F

Fig. S1 Blue-spotted maskray, *Neotrygon kuhlii* specimens examined for variation in spotting patterns. **A.** *N. kuhlii*, specimen presented Pl. 51 of Müller and Henle (1841), possibly one of the three syntypes (from India or New Guinea or Vanikoro). **B.** *N. kuhlii*, specimen Zan 1 from Mkoani, Pemba Island, Tanzania (05°21'S 39°38'E), 26 May 2010 (J.-D.D.). **C.** *N. kuhlii*, specimen ME-3 (MZB 20843 = LIPI 4406) from Meulaboh, Aceh (04°07'N 96°08'E), April 2009 (I.S.A.). **D.** *N. kuhlii*, specimen MSKL 3 (MZB 20847 = LIPI 4401) from Perbaungan, Malacca Strait (03°39'N 98°59'E), December 2009 (I.S.A.). **E.** *N. kuhlii*, specimen DK-PR (MZB 20851 = LIPI 4402) from Pulau Pari, Java Sea, December 2008. **F.** *N. kuhlii* specimen PB 2 (MZB 20852 = LIPI 4410) from Pulau Pabelokan, Java Sea (05°27'S 106°29'E), August 2009 (I.S.A.).



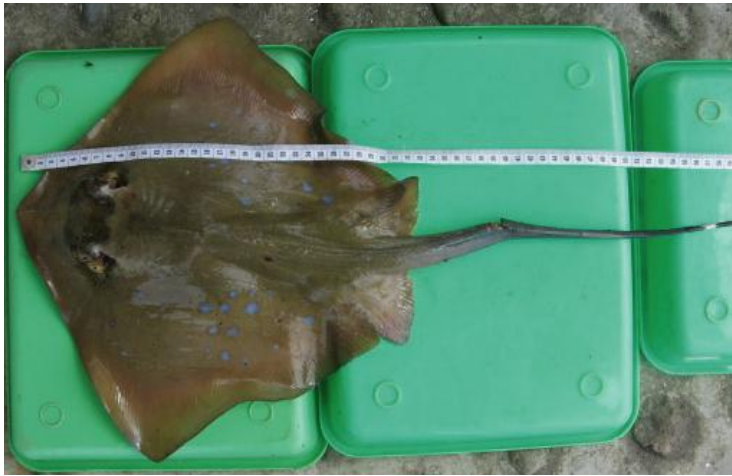
G



H



I



J



K



L

Fig. S1 (continued). **G.** *N. kuhlii* specimen PN 5 (MZB 20850 = LIPI 4399) from Pulau Peniki, Java Sea ($05^{\circ}46'S$ $106^{\circ}38'E$), March 2009 (I.S.A.). **H.** *N. kuhlii* specimen BO-424 from Tanjung Manis, Sarawak, South China Sea ($02^{\circ}07'N$ $111^{\circ}19'E$), April 2004 (photograph downloaded from: http://tapewormdb.uconn.edu/index.php/hosts/specimen_details/elasmobranch/1483/0). **I.** *N. kuhlii* specimen NK_K_BL from Kedonganan Bay, Bali ($08^{\circ}44'S$ $115^{\circ}11'E$), 31 January 2008 (P.B.). **J.** *N. kuhlii*, specimen AM-1 (MZB 20864 = LIPI 4400) from Ambon, Banda Sea ($03^{\circ}40'S$ $128^{\circ}11'E$), October 2008. **K.** *N. kuhlii*, specimen ARA-1 (MZB 20866 = LIPI 4405) from Kei Islands in the Arafura Sea ($\sim 07^{\circ}37'S$ $\sim 135^{\circ}20'E$), 08 April 2009 (A. Kusnadi). **L.** *N. kuhlii*, specimen BK-5 (MZB 20867 = LIPI 4408) from Biak Island, West Papua ($00^{\circ}58'S$ $136^{\circ}16'E$), May 2009 (Alvi).

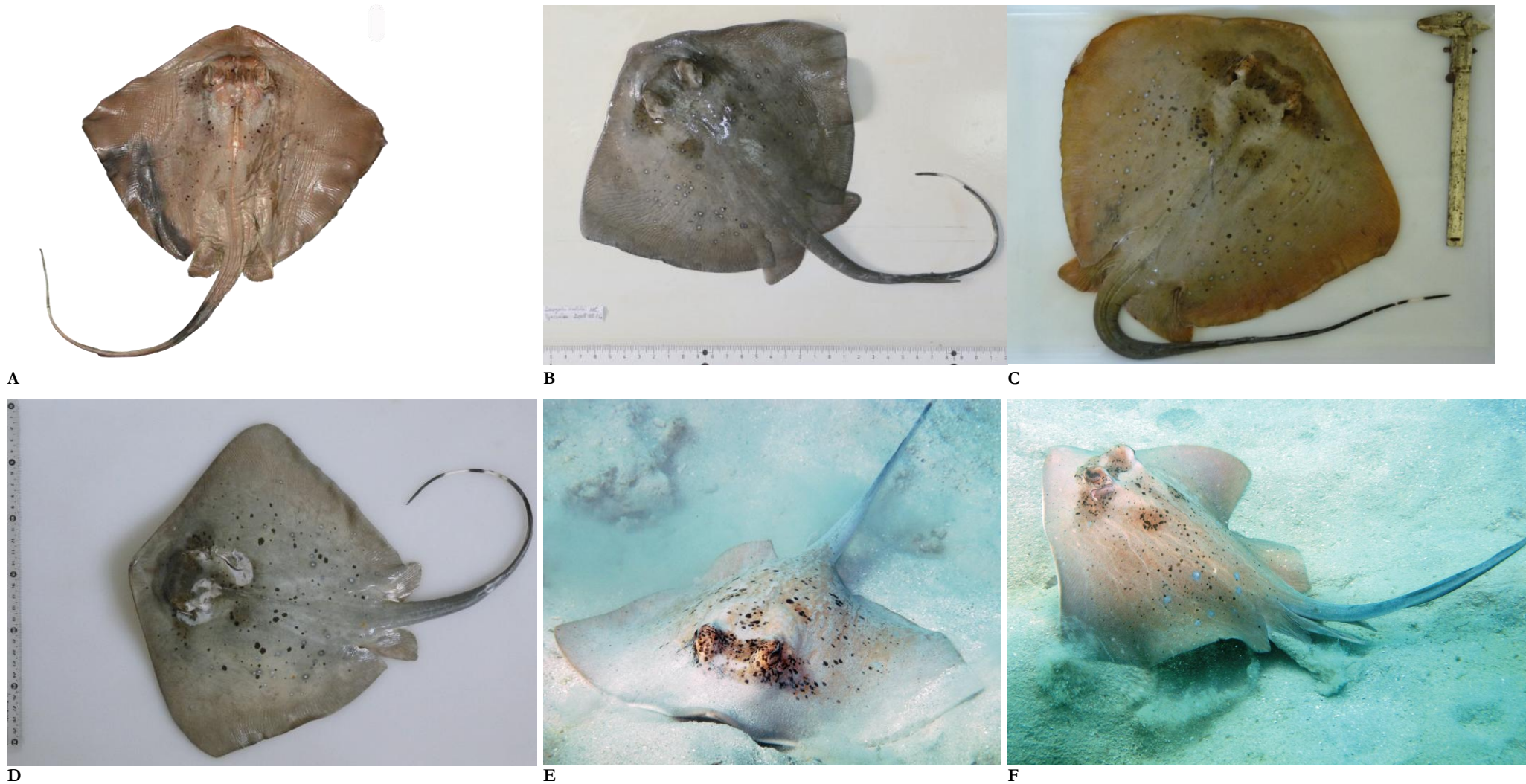


Fig. S2. *Neotrygon trigonoides*. Photographs of New Caledonian specimens analyzed for spotting patterns and additional underwater photographs of individuals sighted underwater at Lizard Island, northern Great Barrier Reef. **A.** Holotype of *Neotrygon trigonoides* (NMV 51684 = A5255): male, 182 mm DW, New Caledonia, examined by F. de Castelnau (Castelnau 1873) (Fig. 5B of Last and White 2008). **B.** *N. trigonoides* specimen deposited at the Australian National Fish Collection, CSIRO (uncat.; female, 225 mm disc width), from Saint Vincent Bay (21°56'S 165°55'E) 16 August 2008; fisherman: Patrick Morlet; formalin/alcohol-preserved specimen sent by PB to Alistair Graham, CSIRO, Hobart, on 14 August 2009 (P.B.). **C.** *N. trigonoides* specimen MNHN 2009-0823 (female, 350 mm DW) from Saint Vincent Bay (21°57'S 166°02'E), 07 March 2009; fisherman: Patrick Morlet (P.B.). **D.** *N. trigonoides* specimen IRDN 20090816 (male, 302 mm DW) from Saint Vincent Bay (21°57'S 166°02'E) 16 August 2009; fisherman: Patrick Morlet (P.B.). **E, F.** Underwater photographs of two males sighted at Lizard Island (14°41'S 145°27'E), 01 January 2013 (M.G.M.).