Phylogeography and taxonomy of the blue-spotted maskray (ex-Neotrygon kuhlii; ikan pari totol biru) : the intricate story of a widespread species complex. Seminar, LIPI Research Center on Oceanography, Ancol on 19 October 2018

Philippe Borsa, Irma S. Arlyza

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Friday 19 October 2018

Phylogeography and taxonomy of the blue-spotted maskray (ex-*Neotrygon kuhlii*; ikan pari totol biru): the intricate story of a widespread species complex

Speaker: Philippe Borsa, researcher at Institut de recherche pour le developpement (IRD), France
Authors: Philippe Borsa (IRD), Irma Arlyza (LIPI-RCO), and collaborators
Organizer: Muhammad Hafizt (LIPI-RCO)

Abstract: The blue spotted maskray was once thought to be a single species widely distributed in the Indo-West Pacific, from the Red Sea to Fiji and from southern Japan to New South Wales. Genetics have shown that it actually consists of at least 12 distinct lineages with parapatric distribution. The available phylogenies suggest that the evolutionary origin of the species complex lies in the southwestern Pacific in the Miocene (between 5 and 23 million years ago). The blue-spotted maskray progressively colonized the Indo-West Pacific from the East to the West – The lineages in the western Coral Triangle and the Indian Ocean are the most recently evolved. The taxonomy of the blue-spotted maskray species complex is still an unfinished job, of which a detailed chronological account will be presented. Major but neglected contributions to the early stages of the taxonomic work on *N. kuhlii* were from French naturalist Jean-Rene Constant Quoy in 1828 and from Japanese artist Kawahara Keiga around 1830. Genetics led to a complete taxonomic reshuffle, starting with the pioneering DNA barcoding work of Australian geneticist Bob Ward in 2008, followed by acrimonious competition between traditional taxonomists and molecular geneticists. For nearly two centuries, the taxonomy of the species complex has been marked by neglect, confusion, and error.

Philippe Borsa

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Population geneticist / phylogeography / molecular taxonomy

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IRD / LIPI PARI project:
Population genetics of el**A**smob**R**anchs in the **I**ndonesian seas

-dedicated to the population genetics of sharks and rays in the Indonesian seas. This is a joint IRD and LIPI project, with I.S. Arlyza and P. Borsa as principal investigators.

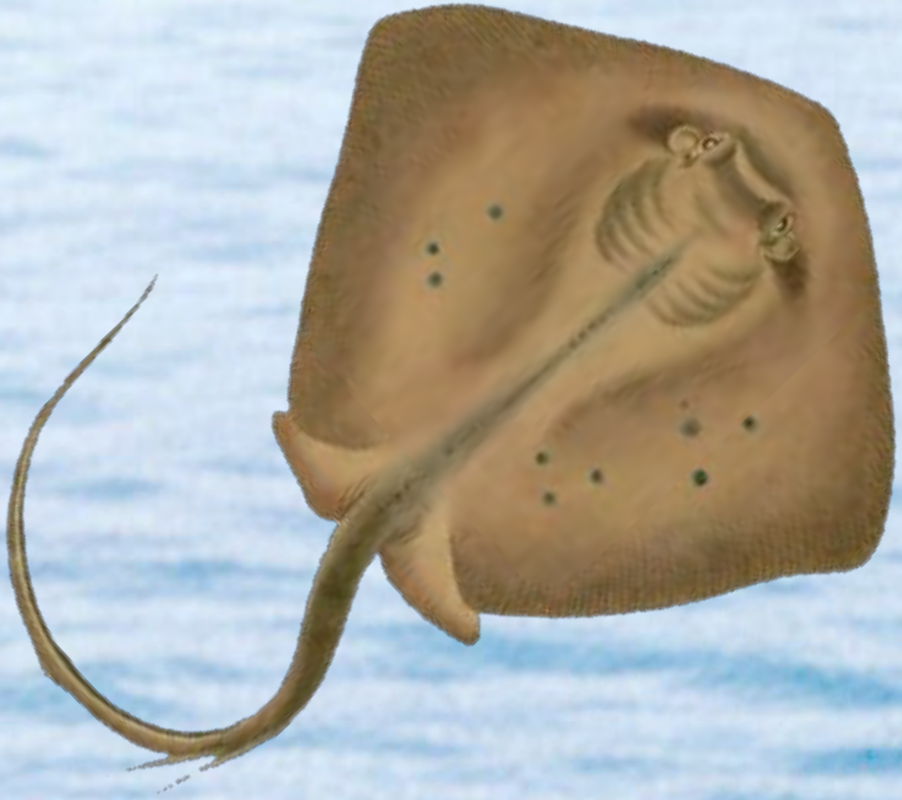
- the Indo-Malay-Papua region has the world's highest diversity of sharks and rays, many species of which still remain undescribed.

- meanwhile, Indonesia is the world's top "producer" of elasmobranchs. However, elasmobranch fisheries in Indonesia are unregulated, unmonitored and unmanaged. The few data available indicate that up to one third of the elasmobranch species of the Indonesian seas now face extinction.

- it is therefore urgent to acquire basic knowledge on sharks and rays in the Indonesian seas, so as to provide managers with information that may help stop the carnage *

* carnage" = pembunuh masa

Phylogeography and taxonomy of the blue-spotted maskray (ex-*Neotrygon kuhlii*; ikan pari totol biru): the intricate story of a widespread species complex



CONTENTS

PART I – Phylogeny and evolutionary history

- Phylogenetic trees enable us to reconstruct the evolutionary relationships among species. In a rooted phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of those descendants. Assuming some molecular clock, the depth of the node is interpreted as a time estimate.

Melody Puckridge and co-authors proposed a bayesian time-tree based on *CO1*, *16S* and *RAG-1* sequences of species of the stingray family (Dasyatidae). The tree was rooted by the Australian wedgefish *Rhynchobatus australiae*, and calibrated using the oldest known dasyatid fossil, which is dated at 136.4–130 million years ago (Mya), that is the early Cretaceous. It should be noted that only a single fossil was used here, to calibrate the whole tree.

Nevertheless, the authors were confident that the last common ancestor to the blue-spotted maskray was between 22 Mya and 8 Mya centered at 15.7 Mya, that is, the late Tertiary or Miocene. That date coincides with the development of modern coral reef ensembles in the central Indo-West Pacific, including the Coral Sea. The blue-spotted maskray consisted of several deep, distinct lineages [the nine "clades" mentioned on Puckridge et al.'s (2013) time tree], which justifies these authors' cautious use of the term "species complex" over that of mere "species".

- The blue-spotted maskray species complex has a wide geographic distribution as it occupies the entire tropical Indo-West Pacific, from the Red Sea to southeastern Africa, to Japan and Fiji in the southwestern Pacific Ocean. The distribution includes all the eastern African coast from Djibouti to Mozambique, Madagascar, the Arabian Sea, the Sea of Oman and the Persian Gulf, the periphery of the Indian peninsula, the Gulf of Bengal and the Andaman Sea, the whole Indo-Malay-Philippine archipelago, southeastern Asia, Taiwan, the Japanese archipelago, the Micronesian islands and the whole Melanesia including West Papua, Papua New Guinea, the Solomon Islands, New Caledonia, Vanuatu, and Fiji. Around Australia, the blue-spotted maskray occurs all along the tropical coastline, from Western Australia to New South Wales.

- Borsa et al. (2018) produced a maximum-likelihood mitochondrial tree based on partial *CO1* and *cytochrome b* gene sequences. The triangles at the extremities of the branches of the tree represent the coalescence of the sampled haplotypes within a lineage: the horizontally longer the triangle, the deeper the coalescence; the vertically broader the triangle, the larger the sample size of individual haplotypes. The tree features 10 distinct lineages, the deepest of which is *N. trigonoides* from the Coral Sea, which is here used to root the tree. What we see is that the next deeper lineage is the one sampled in West Papua, which is northwest to the Coral Sea. The second-next deeper lineage is the one sampled in the Moluccas, that is, immediately west of West Papua. Then we have a rattle-like sub-tree that includes all lineages west of the Moluccas.

- Remember that the deeper the lineage, the older the node at its base. So, we found that the older lineages are those in the easternmost part of the distribution of the species complex. In other words, the more westward we progress, the younger the lineages are. The movement that we can draw here recapitulates the way the blue-spotted maskray complex likely colonized the whole Indo-West Pacific.

- If one pushes further into the Indian ocean, the same general pattern was observed on the tree based on the *CO1* gene sequences currently available from BOLD, the Barcode of Life Datasystems database (http://www.barcodinglife.com/). At the root of the sub-tree are the lineages from India. The most recent lineage is the one from Tanzania. The Red Sea lineage is placed at an intermediate position. So the westward colonisation movement seemingly continued into the Indian Ocean.

- One thing is to describe the broad pattern of colonization of the Indo-West Pacific by the blue-spotted maskray. Another one is to understand how new species arose all along the way. The deep lineages I have presented earlier in this talk indeed correspond to different species: they are deep enough regarding the usual levels of genetic differentiation among separate species in Elasmobranchs. Each lineage is geographically separate from, albeit contiguous to its neighbour, a pattern that is called parapatry (see supplementary slide no. 1). The parapatric distribution of lineages indicates reproductive isolation, which in turn confirms that the different lineages are distinct species. Indeed, reproductive isolation is one of the most obvious properties of a species, that distinguishes it from another species.

The speciation hypothesis that we propose for the blue spotted maskray is one where populations in periodical geographic isolation from one another repeatedly crashed and expanded. During crashes, caused by the lowering of the sea level and the correlated diminution of available coastal habitat, the populations were smaller and confined to refuges. This situation was prone to genetic differentiation through the random erosion of genetic variability. During the following event of rising sea level, the favourable habitat expanded again, triggering population expansion where new mutations occurred. During the subsequent population crash, again only a random part of the genetic variability created during the population expansion phase was retained, the rest being eliminated. In the long term, this processus translated into an accumulation of genetic differences between populations from adjacent sea basins, leading to speciation. For the last two million years, cycles of diminishing sea level followed by rising sea level have occurred in a recurrent fashion approximately every 100,000 years, which is the period of the so-called Milankovitch cycle that affects both the Earth's orbit around the Sun and the Earth's rotation axis. Within just the last two million years, about 20 such cycles thus occurred, which we believe was sufficient to create new species in the blue-spotted maskray species complex.

Part I - Phylogeny and evolutionary history

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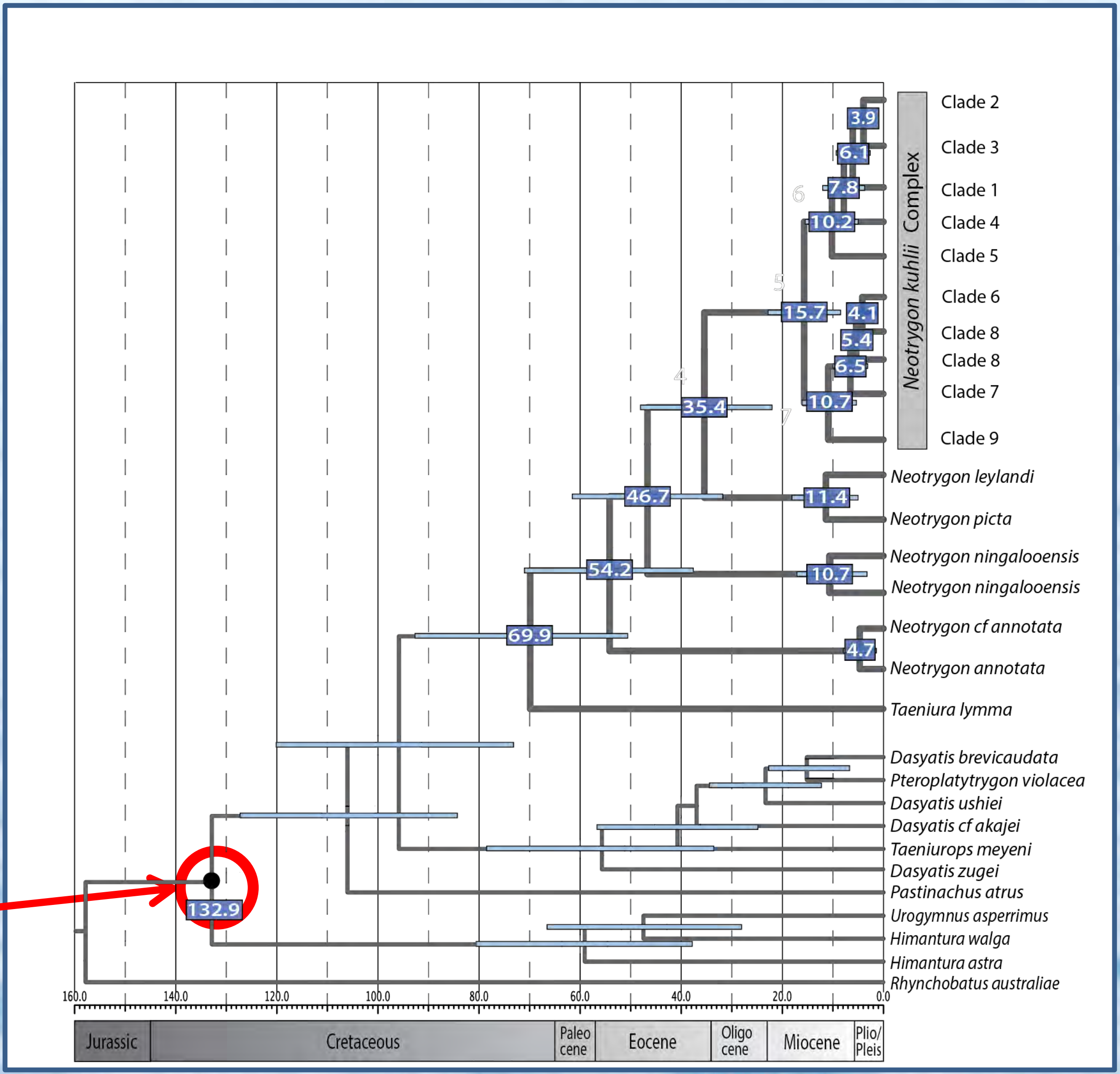
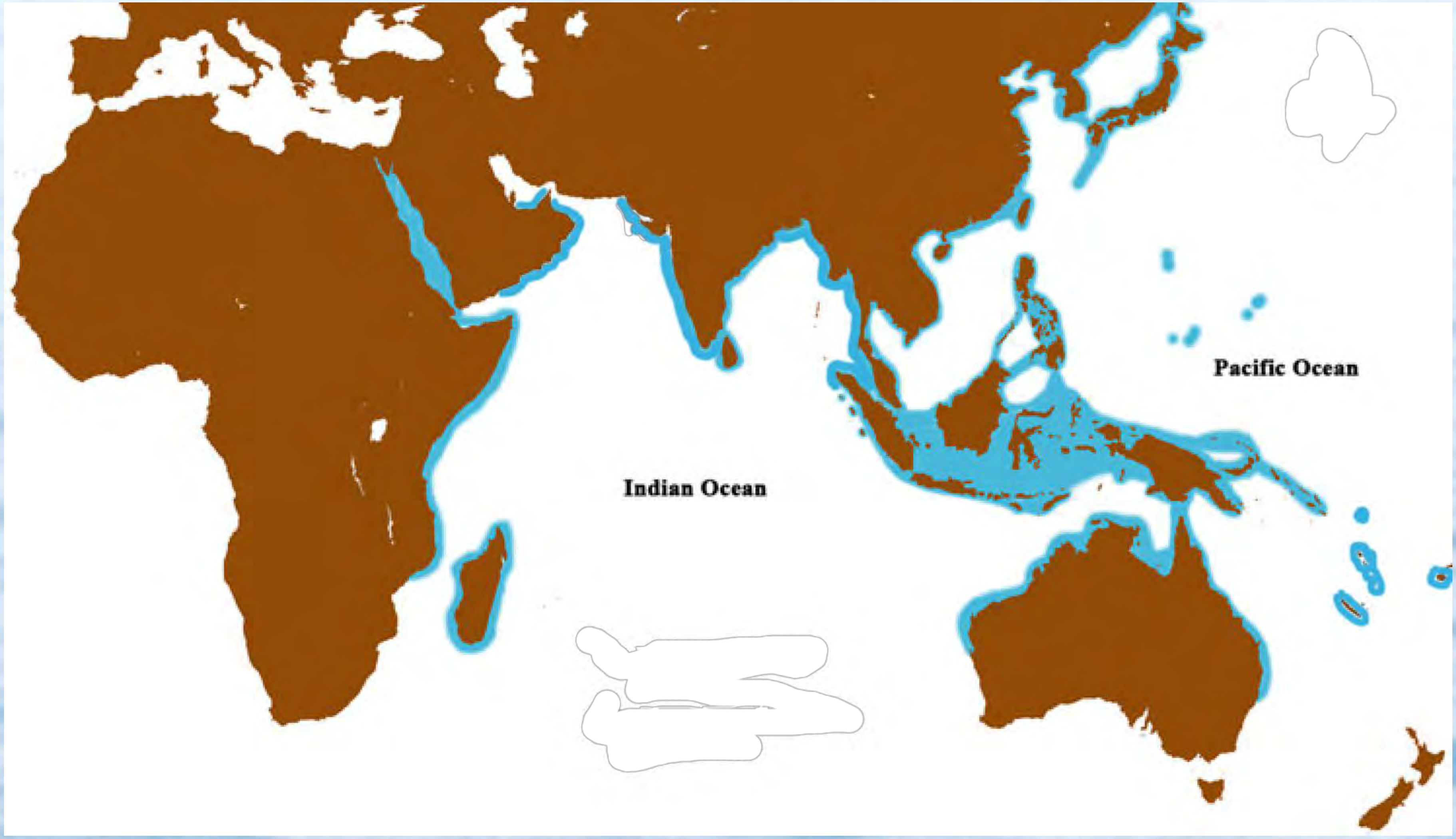


FOSSIL

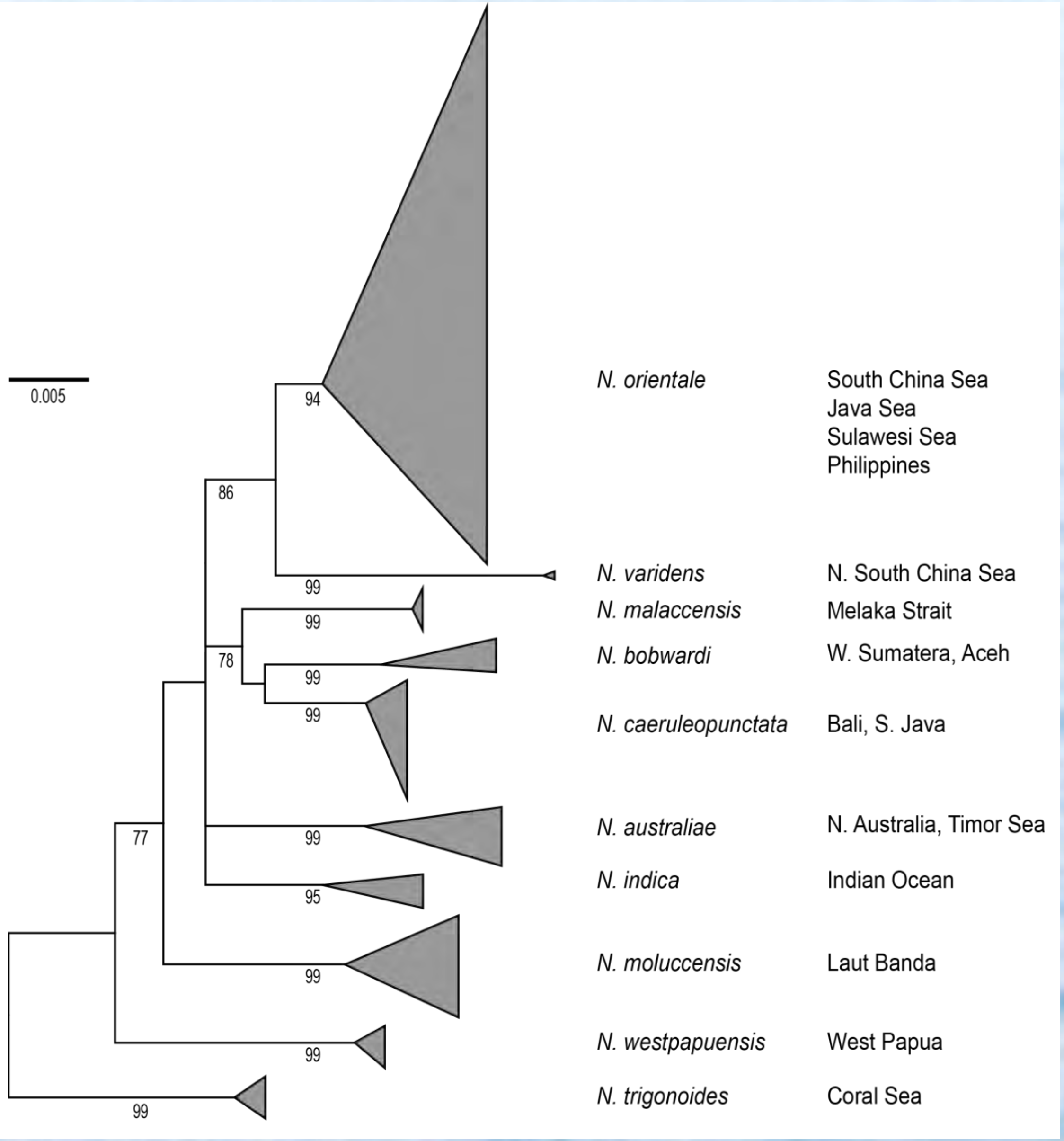
Bayesian time-tree of Puckridge et al. (*Ecol. Evol.*, 2013)

Part I - Phylogeny and evolutionary history



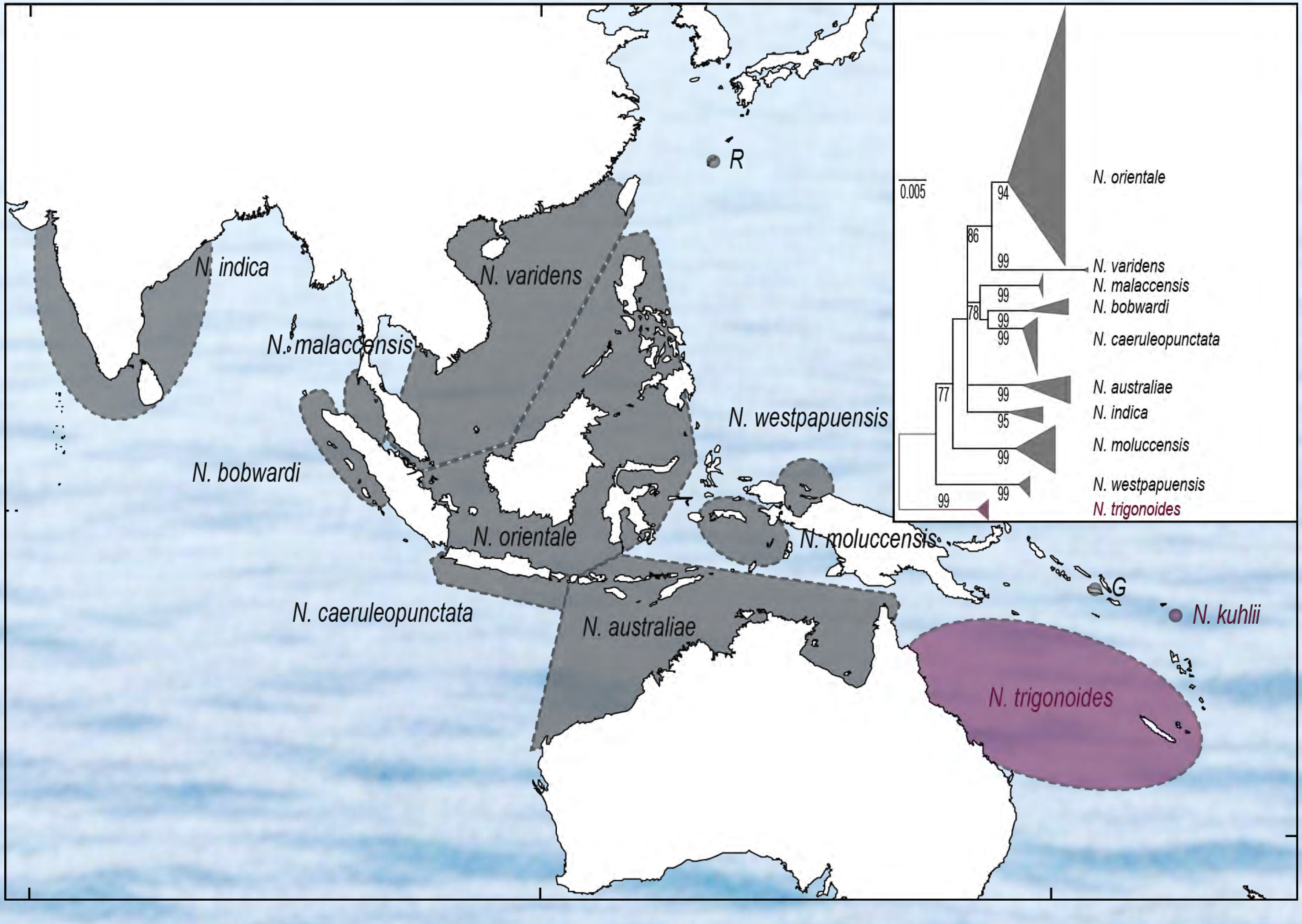
Distribution of the *Neotrygon kuhlii* species complex

Part I - Phylogeny and evolutionary history



Maximum-likelihood tree of Borsa et al. (*J. Oceanol. Limnol.*, 2018)

Part I - Phylogeny and evolutionary history



Distribution of the *Neotrygon kuhlii* species complex

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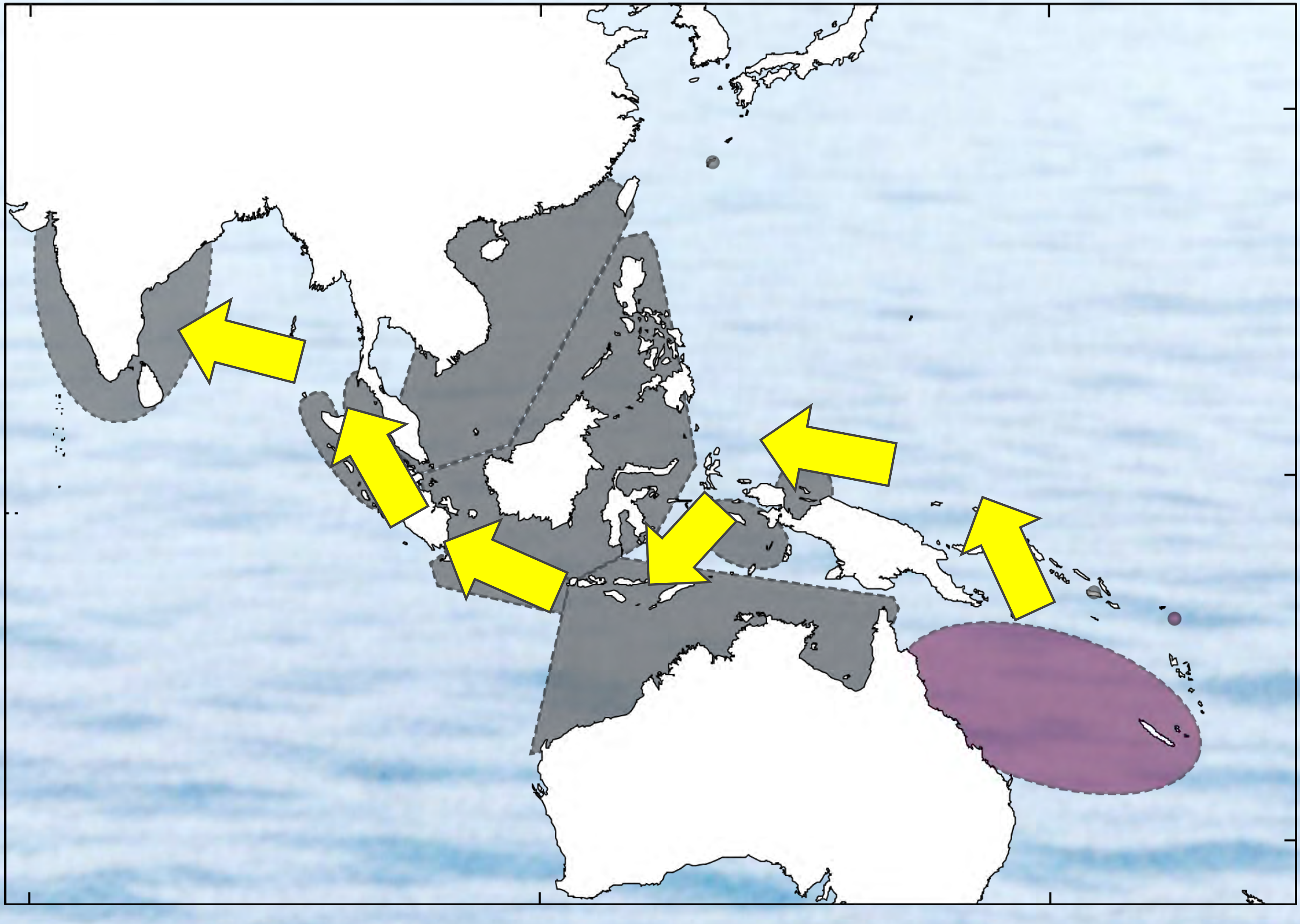
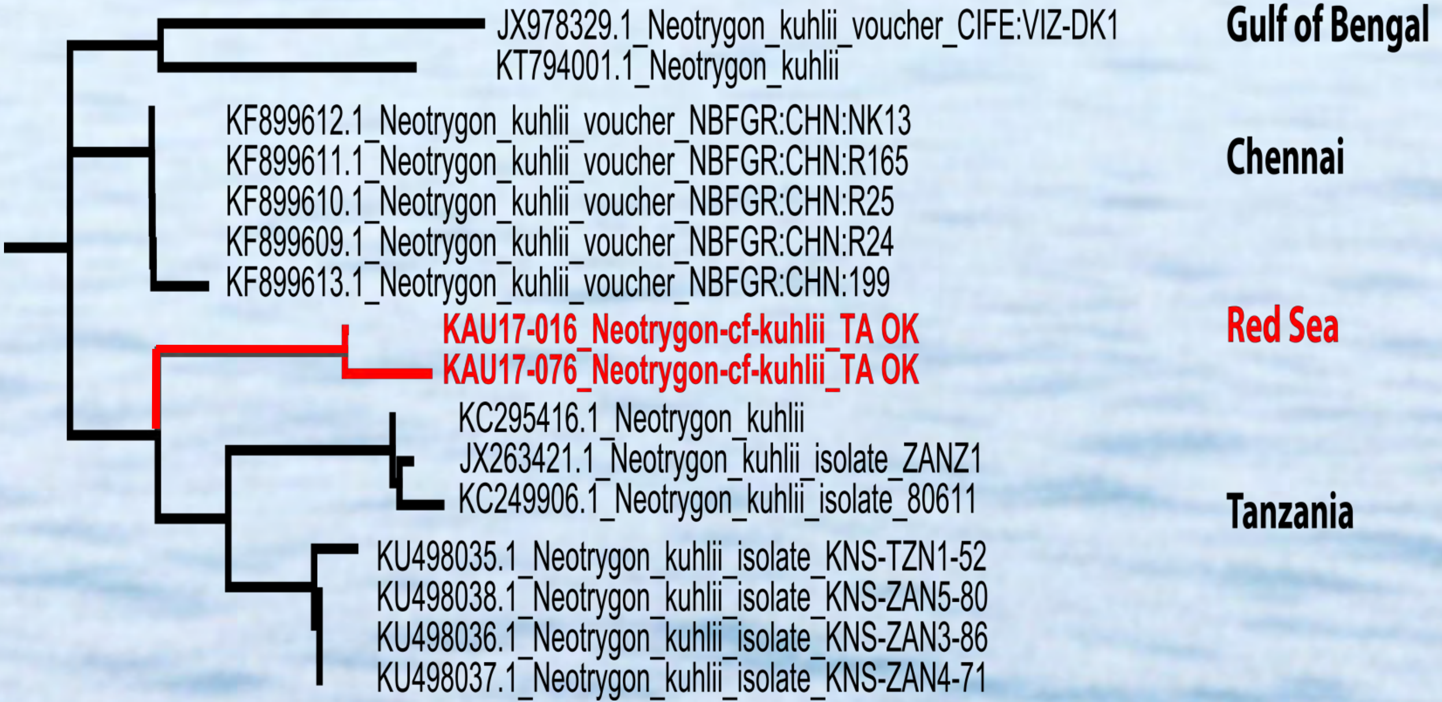


History of the *Neotrygon kuhlii* species complex

Part I - Phylogeny and evolutionary history



NJ tree of *CO1* gene sequences (BOLD)

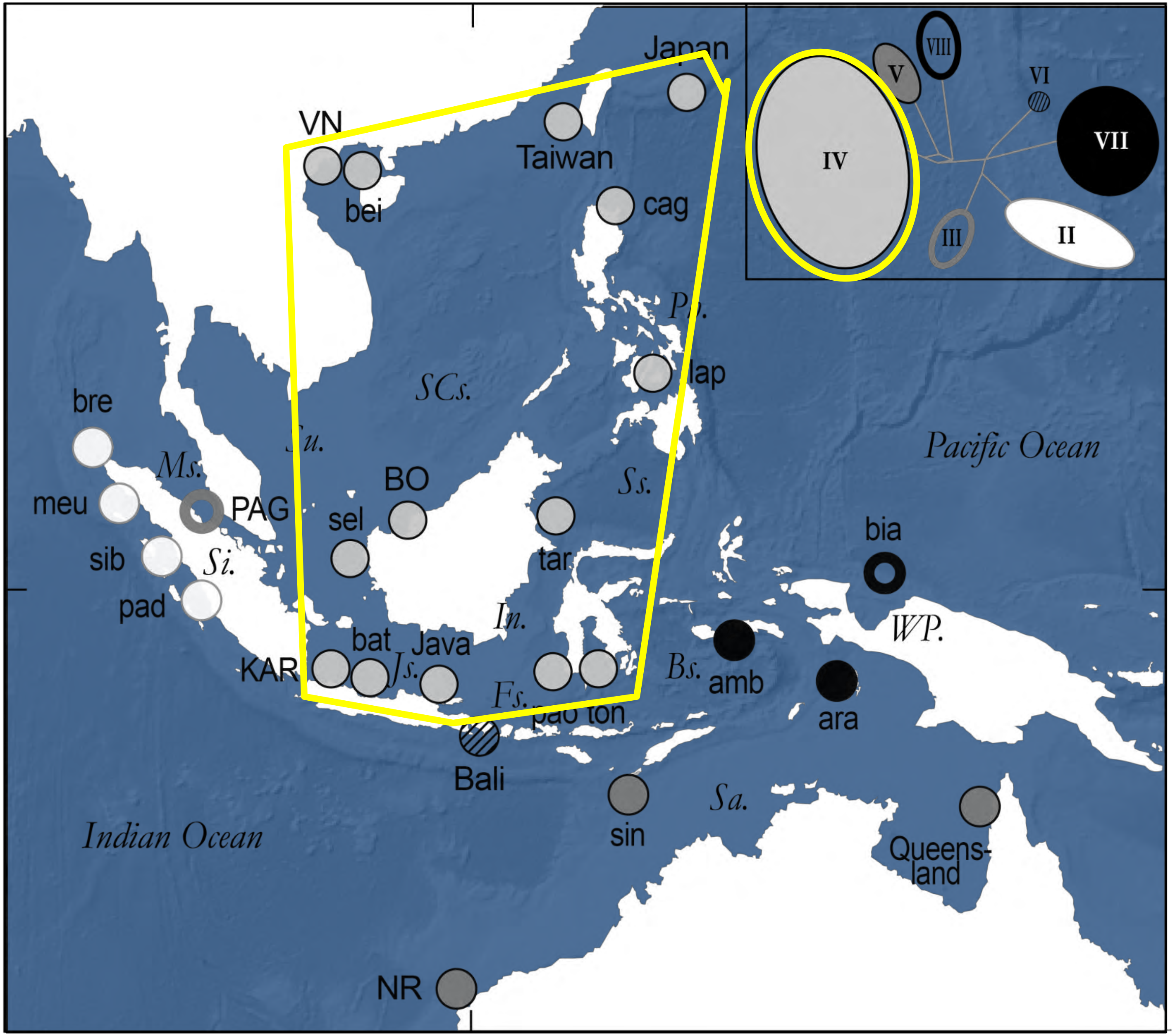
History of the *Neotrygon kuhlii* species complex

Part I - Phylogeny and evolutionary history

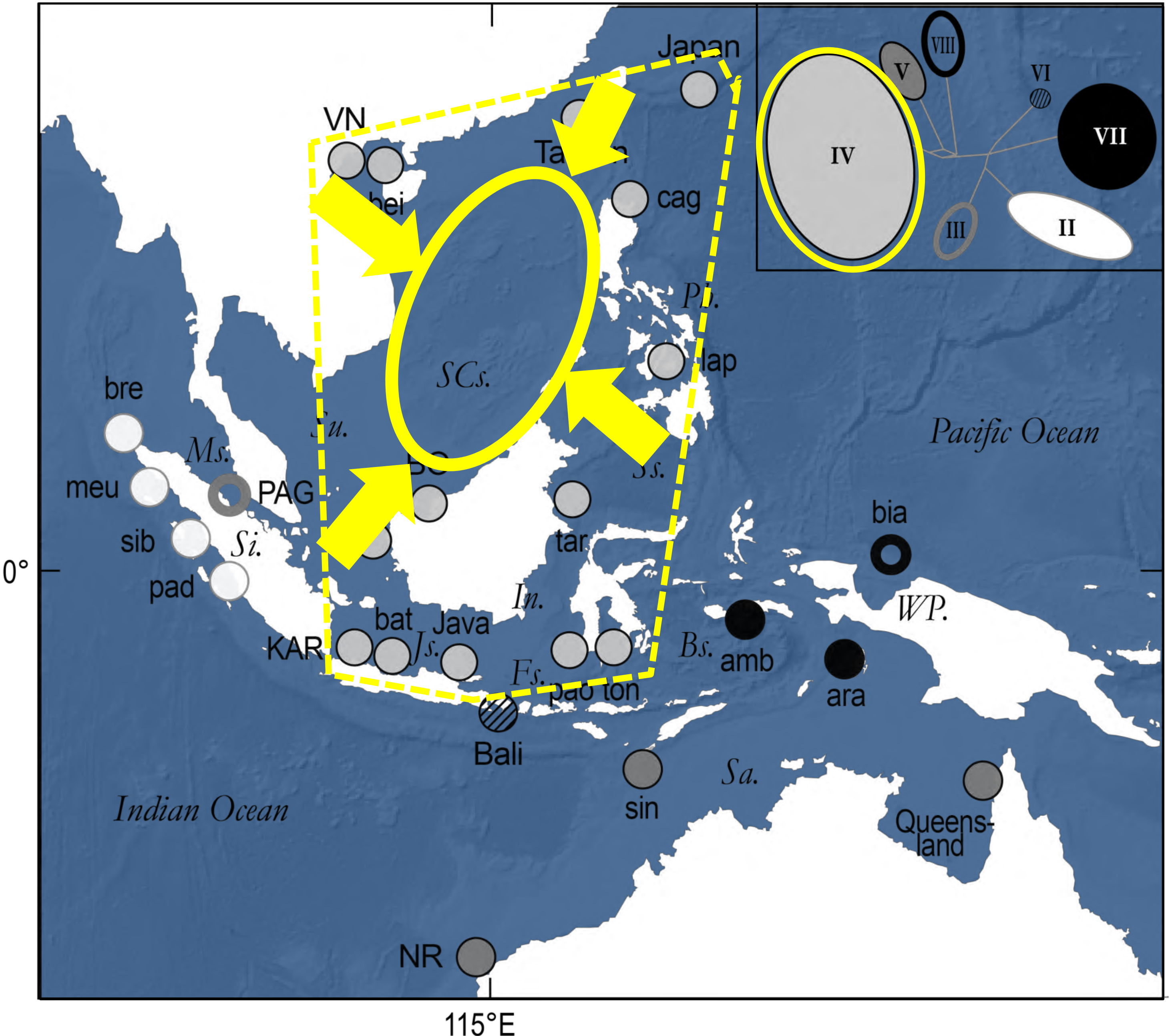
NJ tree of *CO1* gene sequences (BOLD)

History of the *Neotrygon kuhlii* species complex

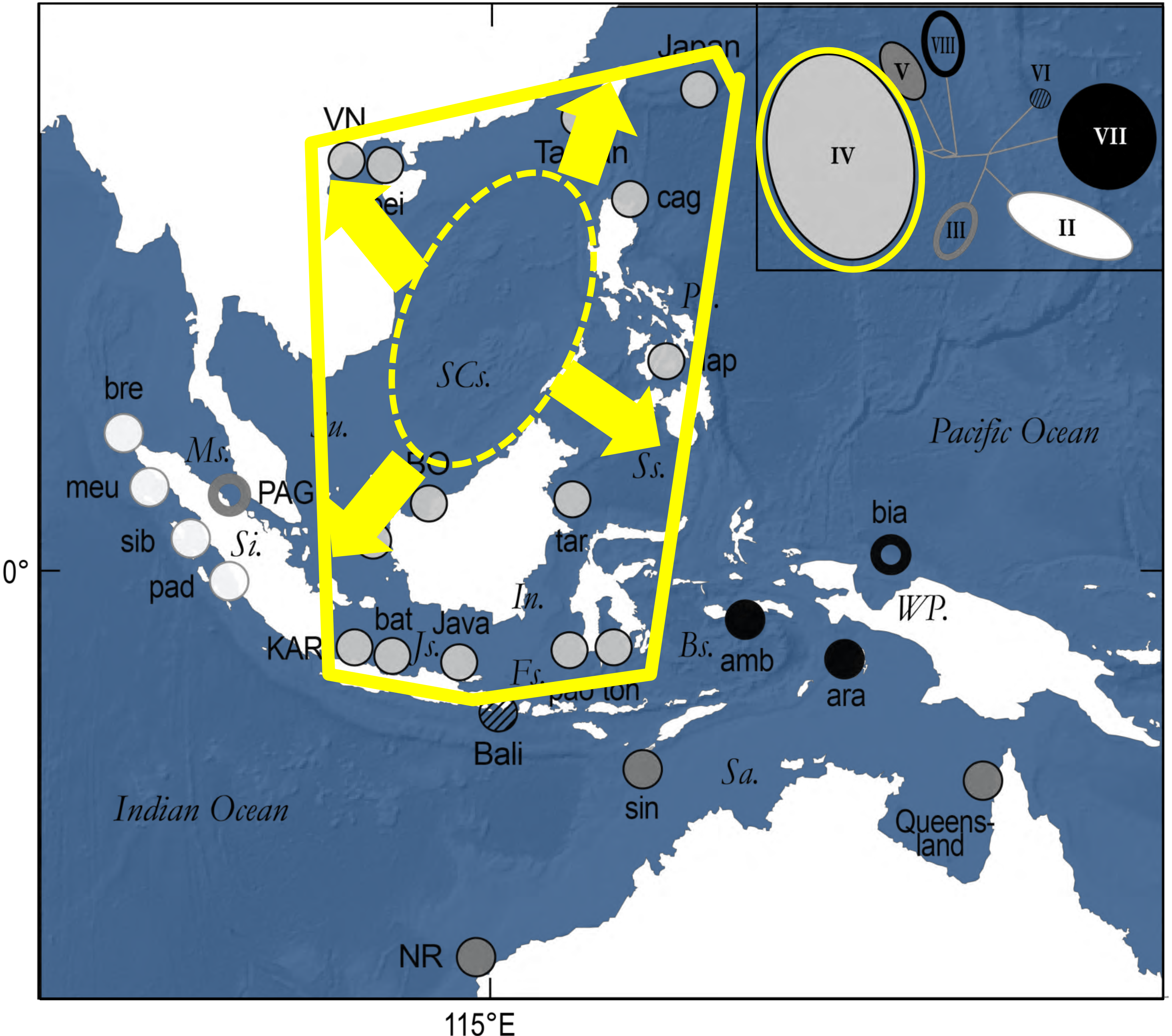
Speciation hypothesis



Speciation hypothesis



Speciation hypothesis



PART II – Taxonomy: a chronological account

- This story starts when French physician and naturalist Jean-René-Constant Quoy embarked together with Joseph-Paul Gaimard on Jules Dumont d'Urville's expedition to the southwestern Pacific on board French royal navy's vessel *Astrolabe* from 1826 to 1829 (Dumont d'Urville 1833 ; Quoy and Gaimard 1835).

When in late August and early September 1827 the *Astrolabe* called at Havre Dorei (00°51'S 131°39'E; now Manokwari in West Papua) the two naturalists collected a number of specimens, among which there was a juvenile of blue spotted maskray, now preserved in alcohol at the Museum national d'histoire naturelle (MNHN) in Paris. This is the oldest known voucher for that species in a museum collection.

- The two naturalists' field notes hold the first reference to the blue-spotted maskray, in late February or early March 1828, when they stopped over at Vanikoro in the Santa Cruz islands north of the Vanuatu archipelago.

Quoy and Gaimard's description of the Vanikoro maskray (as *Raia cœrulea*, the "raie bleue"; also locally named "foro") was unfortunately unknown to researchers who subsequently worked on the blue-spotted maskray, until Marie-Louise Bauchot from the MNHN mentioned it for the first time (Bauchot 1994). Quoy had made a visibly accurate drawing of one of the specimens from Vanikoro, which was published by Borsa and Béarez (2016). Quoy's aquarel features ocellated blue spots ($N = 14$), dark-brown spots ($N = 13$), dark speckles ($N = 3$), and lighter-brown scapular blotches ($N = 2$, one on each shoulder).

Two specimens preserved in alcohol were brought back from Vanikoro and deposited at the MNHN.

- At the same period, German naturalists Philipp Franz von Siebold and Heinrich Bürger, who were based in Nagasaki, had begun to make extensive collections of the fauna of Japan. Von Siebold and Bürger hired the services of aquarellist Kawahara Keiga who produced a number of extraordinarily elegant and sharp paintings. Pictures of some of Keiga's pencil and watercolour work are accessible through the Naturalis Biodiversity Center/Wikimedia Commons website (https://commons.wikimedia.org/wiki/Commons:Naturalis_Biodiversity_Center).

Curator Martien van Ooijen from Naturalis explains that it is on the basis of Bürger's collection, which includes 256 watercolours of fishes painted around 1830 by Keiga, that the Fish Volume of Temminck and Schlegel's (1845) *Fauna Japonica* was written (see https://science.naturalis.nl/en/people/scientists/).

- In 1841, German naturalists J. Müller and C.F.G. Henle published an extensive treaty about the systematics of elasmobranchs, entitled "*Systematische Beschreibung der Plagiostomen*", which included the description of a number of new species. Included there was the blue-spotted maskray.

The original description of the blue-spotted maskray *Trygon kuhlii* was accompanied by the lithography of a female specimen (Müller & Henle, 1841: pl. 51). The description of the colour patterns ("*einzene, kleine, runde blaue Flecken (3-6) auf jeder Brustflosse*" was obviously based on the accompanying lithography, which shows three ocellated blue spots on the left side and six on the right side.

- Müller and Henle (1841: xxii) declared that the drawing they reproduced had been done by "*Kuhl und Hasselt*". Müller and Henle referred to German zoologist Heinrich Kuhl and Dutch naturalist Johan Coenraad van Hasselt, who were sent to Java to collect specimens for the Leiden museum. Therefore, Müller and Henle (1841) believed that they reproduced the illustration of a blue-spotted maskray specimen form Java. What we can see actually, is that the lithography representing *T. kuhlii* (Plate 51 of Muller and Henle, 1841) was based on Keiga's painting of a specimen from the Nagasaki region. Apparently, Müller and Henle were totally unaware of the origin of the painting they had reproduced along with their description. This error was later corrected by Temminck and Schlegel (1843), Duméril (1865), Jordan and Fowler (1903) and Borsa and Béarez (2016), but not by Last et al. (2016) when the latter undertook to revise the taxonomy of the blue-spotted maskray.

- In 2008, Australian geneticist Robert D. Ward and co-authors from CSIRO in Hobart published a detailed paper on the DNA barcoding of Australasian elasmobranchs. They had sampled *N. kuhlii* from four distinct locations: the Gulf of Carpentaria, Bali, the Java Sea, and Taiwan. Ward and co-authors suspected the occurrence of cryptic species within *N. kuhlii*, because of the unusually high level of genetic divergence among geographic populations, at the *CO1* locus.

- By 2009, following Ward et al.'s (2008) findings, Irma Arlyza and I had decided to embark on the LIPI/IRD PARI project. We chose the blue-spotted maskray as the main model species to study, because it was then the most abundant elasmobranch species in the fish landings throughout Indonesia, therefore a species for which we were more likely to get adequate sample sizes for the study of genetic variation, than any other elasmobranch species in the Indo-Malay archipelago. We also already knew from Ward et al. (2008) that there were substantial genetic differences between geographically distant populations and potential cryptic species. In 2010, we published a first tree of mitochondrial haplotypes, based on extensive sampling from the Indo-Malay-Papua archipelago. This was presented as a poster at the ATBC meeting in Bali and communicated to Australian stingray taxonomist Peter R. Last from CSIRO, Hobart. The PARI project is still alive, as we are planning to continue to release data and many more questions have not yet been addressed properly.

- Mitochondrial DNA (mtDNA) sequences clearly distinguish the New Caledonian maskray lineage, originally *Raya trigonoides* Castelnau 1873 from all other lineages of the blue-spotted maskray (Borsa et al. 2013). Spot patterns are also distinct, as the New Caledonian maskray shows a high number of dark speckles that are absent or nearly absent in the other lineages, except the remote-located Indian-Ocean maskray. It also possesses a distinctive pair of darker blotches in the scapular region. The dark speckling and the pair of blotches were absent from Müller and Henle's (1841) description of *Trygon kuhlii* and from the figure that accompanies it. Hence the New Caledonian maskray was resurrected as *Neotrygon trigonoides* (Castelnau, 1873) by Borsa et al. (2013). We were eager to honour Castelnau's (1873) contribution and at the same time clarify some of the taxonomic issues concerning the blue-spotted maskray.

One thing that may introduce some trouble here is the nomenclature at the genus level: *Raia*, *Trygon*, *Raya*, *Dasyatis*, *Neotrygon*, … which name is the correct one? Morphological characters on which earlier taxonomic accounts were based were not unambiguous. Genetics revealed that the blue-spotted maskray and its closely-related maskray congeners *N. annotata*, *N. leylandi* and *N. picta* form a distinct clade in the molecular phylogeny of the family Dasyatidae (e.g., Puckridge et al. 2013). The genus *Neotrygon*, made available by Castelnau (1873) for the New Caledonian maskray is the one that is now widely accepted by stingray taxonomists, to the exclusion of the other names.

- With a pinch of bad faith, Last and co-authors later wrote: "*Borsa et al. (2013) … resurrected N. trigonoides, but failed to comprehend the need to characterise the type of N. kuhlii before making this decision. While some information on spotting was provided, they failed to understand the need to follow standard taxonomic practices used for the family group (stingrays), and provided no other descriptive information on the species. A more robust study of the taxonomy of this species is in progress*". These comments, taken from Last et al. (2016) failed to acknowledge that the spot patterns of the New Caledonian maskray were so strikingly different from those originally described by Müller and Henle (1841) that it was not conceivable that the two species be confused. In other words, Last et al. (2016) disregarded the evidence and dismissed our work based on the mere fact that we did not follow "*standard taxonomic practices for stingrays*", i.e. those which Last and co-authors have used for a number of years and want to impose to the community.

Within months after Last et al.'s (2016) announcement of "*a more robust study of the taxonomy of this species*", we started to realize there were flaws – some of which will be briefly exposed here – in their own descriptions of new species. The flaws were so serious that they actually question the fundamentals of the so-called "*standard taxonomic practices*" advertised by Last et al. (2016).

- In that same Last et al. (2016) paper, three new species were described (*N. australiae*, *N. caeruleopunctata* and *N. orientale*) after the authors had chosen one of the syntypes designated by Müller and Henle (1841) as the lectotype of *N. kuhlii*. They had chosen one of the two specimens collected at Vanikoro by Quoy and Gaimard. The three new species, which had been originally detected as distinct mitochondrial lineages by Ward et al. (2008) were here described, not based on mtDNA sequences, but based on a number of measurements on the body, such as the length of snout, the number of vertebrae, and many other measurements in all directions. Last et al. (2016) claimed that each of a number of such measurements was useful to diagnose the new species.

As we tried to verify this, we soon found that actually none of these measurements, taken one by one, was diagnostic of any of the species within the blue-spotted maskray species complex. And this is indeed a serious problem. We are still wondering how Last et al. (2016) were actually able to distinguish the new species from one another based on morphometrics and the only explanation we have is that they actually entirely relied on Ward et al's (2008) results – further confrmed by Arlyza et al. (2013), Borsa et al. (2013) and Puckridge et al. (2013) – without fully acknowledging it.

In our view, morphometric descriptions in this case should be undertaken a posteriori, i.e, once species of a complex of cryptic species have been delineated and described based on molecular genetics. Also, the morphometric descriptions of Last et al. (2016) should have taken into account both the non-independence of the measurements taken, and the natural variability within a species. Based on Last et al.'s (2016) series of measurements, it appears that there is a great deal of variation within a species. Hence, sample sizes should have been much larger than those used. And as many of the measurements taken were not independent from one another, geometric morphometrics or multivariate analyses should have been considered instead of an accumulation of linear measurements. In contrast to morphometrics, nucleotide sequences offer a profusion of potentially diagnostic characters, and comparatively small sample sizes already suffice to distinguish the different species from one another, so high is the level of genetic differentiation between them.

In passing, the one syntype of *N. kuhlii* that was chosen as lectotype by Last et al. (2016) was a specimen from Vanikoro in the Santa Cruz archipelago. The lectotype is, among a series of specimens originally proposed as type material (the so-called syntype series), the one which becomes the reference specimen for the species. One should be careful to choose as lectotype a specimen that best fits the intentions of the authors of the species. It happens that the pigmentation patterns of the Vanikoro maskray, thus now the typical *N. kuhlii*, do not fit those of the original description of the species by J. Müller and F.G.J. Henle (Borsa and Béarez, 2016). Moreover, in their re-description of *N. kuhlii*, Last et al. (2016) hastily included into that species a fresh specimen collected from Guadalcanal Island in the Solomon archipelago, over 800 km away from Vanikoro, the type-locality. We found that pigmentation patterns clearly distinguish the Guadalcanal maskray from *N. kuhlii* from Vanikoro (Borsa and Béarez 2016).

- The Guadalcanal maskray was subsequently described as *N. vali* by Borsa (2017). "*Vali*" simply means "stingray" in the Gela language, the one spoken by fishermen around Guadalcanal island.

- This was followed by the recognition of four more lineages as distinct species, and their description as, in alphabetical order: *N. bobwardi*, which occurs in Aceh and West Sumatera; *N. malaccensis* which occurs in the Melaka Strait; *N. moluccensis* from the Banda Sea; and *N. westpapuensis* from Biak (Borsa et al. 2016, 2018). These new species were formally described on the basis of DNA sequences exclusively.

- The Indian-Ocean maskray *N. indica* was formally described as well (Pavan-Kumar et al. 2018). Although this lineage is derived from a blue-spotted maskray stock, as we have seen earlier, the Indian-Ocean maskray typically exhibits numerous black speckles, a pattern that so far in this presentation we only observed in the phylogenetically distant New Caledonian maskray *N. trigonoides*. This apparent reversion in colour patterns possibly stems from selection pressure towards one of two extremes (i.e., either conspicuous ocellated blue spots with little or no speckling, or more-discreet ocellated spots with conspicuous speckling).

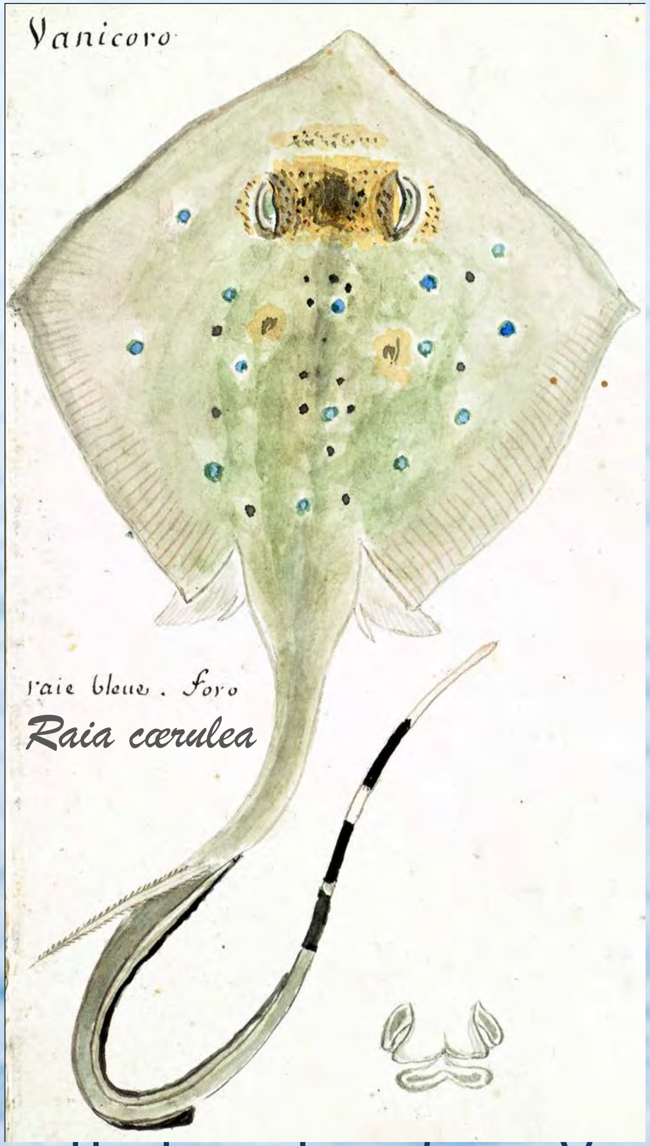
Part II - Taxonomy: a chronological account

1828

Jean Rene Constant Quoy
(1790-1869)

Jules Dumont d'Urville
(1790-1842)

1828

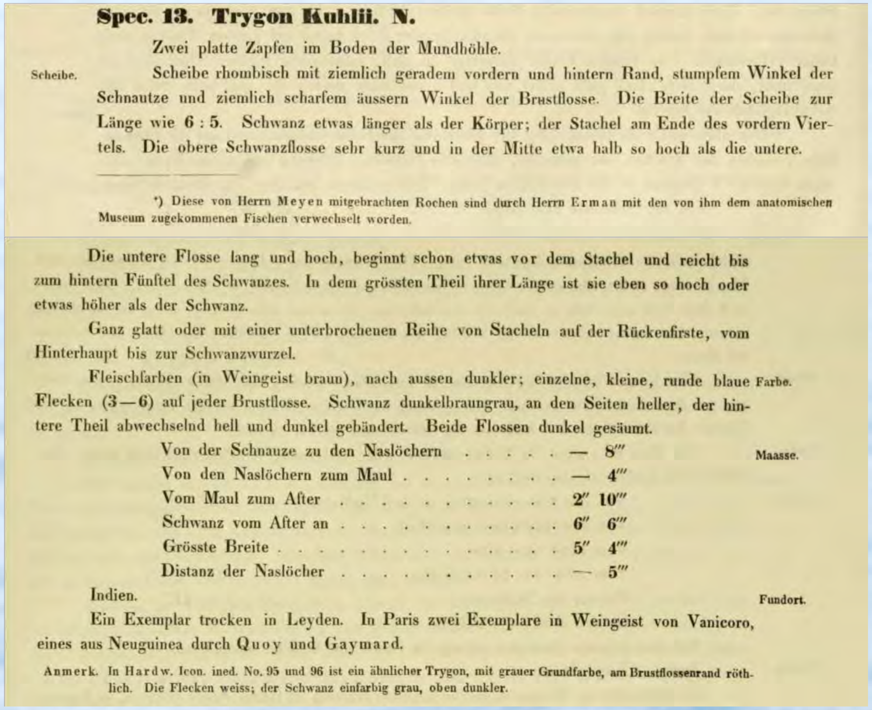


Aquarel of blue-spotted maskray from Vanikoro (Santa Cruz islands) by French naturalist J.R.C. Quoy

ca. 1830

Aquarel of blue-spotted maskray from Nagasaki
by Japanese artist K. Keiga

1841



Spec. 13. Trygon Kuhlii. N.

Zwei platte Zapfen im Boden der Mundhöhle.

Scheibe. Scheibe rhombisch mit ziemlich geradem vordern und hintern Rand, stumpfem Winkel der Schnautze und ziemlich scharfem äussern Winkel der Brustflosse. Die Breite der Scheibe zur Länge wie 6 : 5. Schwanz etwas länger als der Körper; der Stachel am Ende des vordern Viertels. Die obere Schwanzflosse sehr kurz und in der Mitte etwa halb so hoch als die untere.

---

*) Diese von Herrn Meyen mitgebrachten Rochen sind durch Herrn Erman mit den von ihm dem anatomischen Museum zugekommenen Fischen verwechselt worden.

Die untere Flosse lang und hoch, beginnt schon etwas vor dem Stachel und reicht bis zum hintern Fünftel des Schwanzes. In dem grössten Theil ihrer Länge ist sie eben so hoch oder etwas höher als der Schwanz.

Ganz glatt oder mit einer unterbrochenen Reihe von Stacheln auf der Rückenfirste, vom Hinterhaupt bis zur Schwanzwurzel.

Fleischfarben (in Weingeist braun), nach aussen dunkler; einzelne, kleine, runde blaue Flecken (3—6) auf jeder Brustflosse. Schwanz dunkelbraungrau, an den Seiten heller, der hintere Theil abwechselnd hell und dunkel gebändert. Beide Flossen dunkel gesäumt. Farbe.

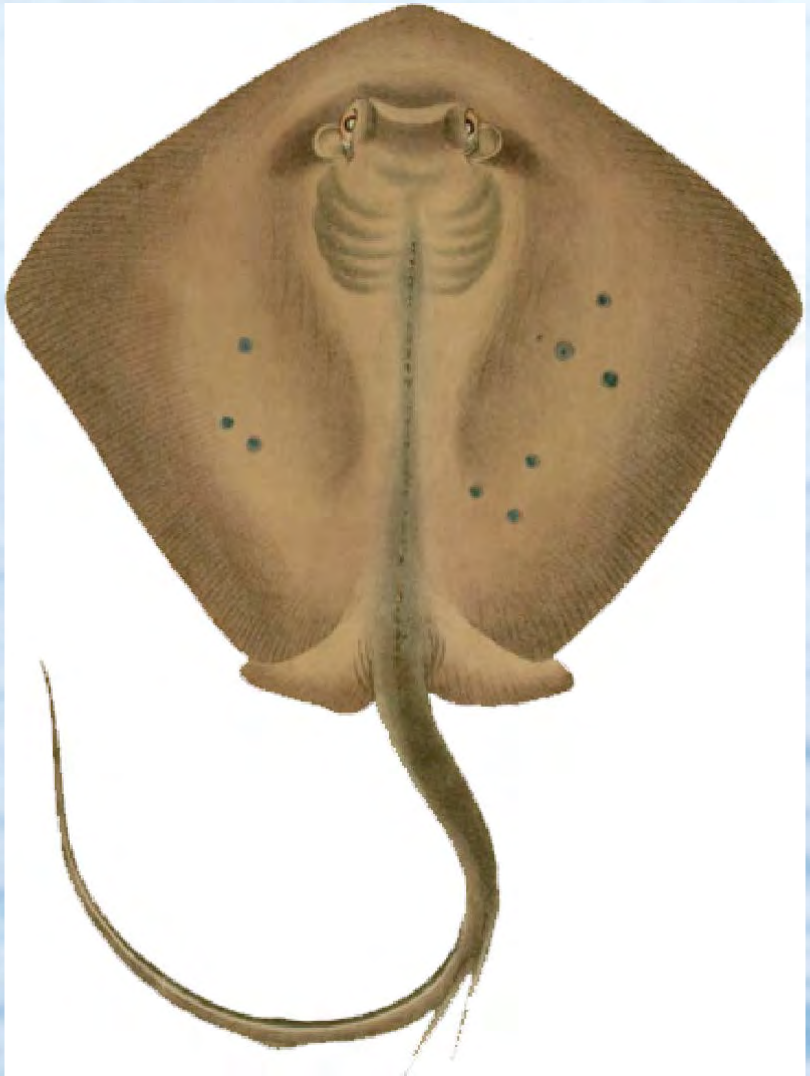
Maasse.

Von der Schnauze zu den Naslöchern	—	8‴
Von den Naslöchern zum Maul	—	4‴
Vom Maul zum After	2″	10‴
Schwanz vom After an	6″	6‴
Grösste Breite	5″	4‴
Distanz der Naslöcher	—	5‴

Indien. Fundort.

Ein Exemplar trocken in Leyden. In Paris zwei Exemplare in Weingeist von Vanicoro, eines aus Neuguinea durch Quoy und Gaymard.

Anmerk. In Hardw. Icon. ined. No. 95 und 96 ist ein ähnlicher Trygon, mit grauer Grundfarbe, am Brustflossenrand röthlich. Die Flecken weiss; der Schwanz einfarbig grau, oben dunkler.



Description of *Trygon kuhlii* by German naturalists J. Muller and F.G.J. Henle

Müller and Henle
(1841)

Keiga
(ca. 1830)

2008

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Marine and Freshwater Research, 2008, 59, 57–71

DNA barcoding Australasian chondrichthyans: results and potential uses in conservation

Robert D. Ward [A,B], Bronwyn H. Holmes [A], William T. White [A] and Peter R. Last [A]

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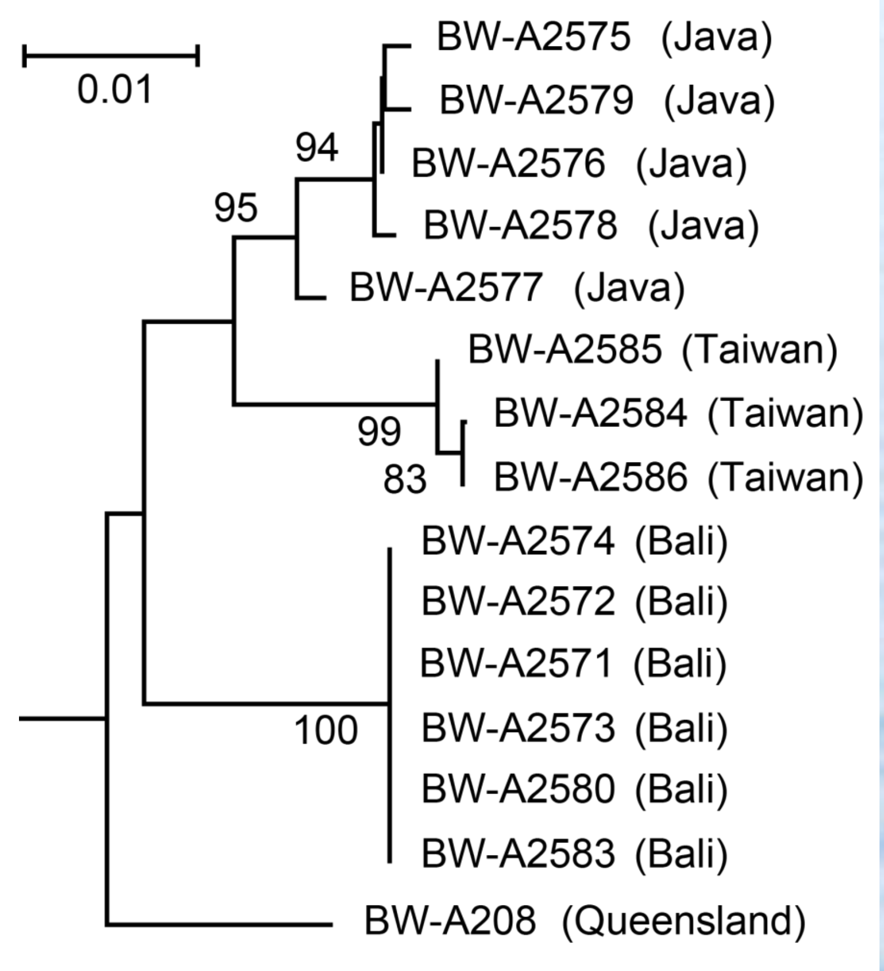
Hobart, Tasmania 7001, Australia.
[B] Corresponding author. Email: Bob.Ward@csiro.au

2008



Neotrygon kuhlii DNA barcoding: Ward et al. 2008

2010

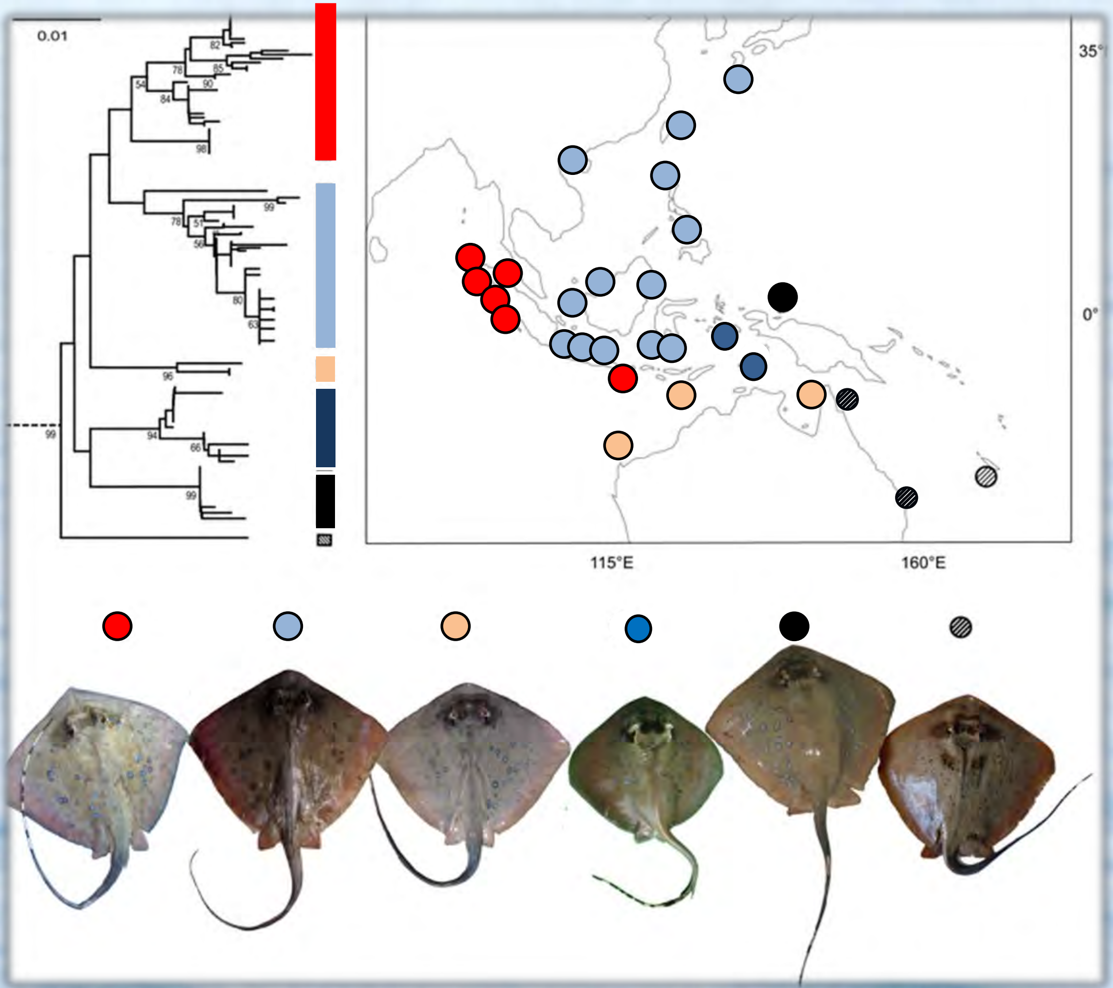
PARI project

Population genetics of Indonesian elasmobranchs

– IRD/LIPI 2010-2018 –

2010

PARI project



genetics

distributions

taxonomy

2011-2012

Irma S. Arlyza preparing a vertical polyacrylamide gel at P. Berrebi’s laboratory in Montpellier

2013

Contents lists available at SciVerse ScienceDirect

Comptes Rendus Biologies

www.sciencedirect.com

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

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

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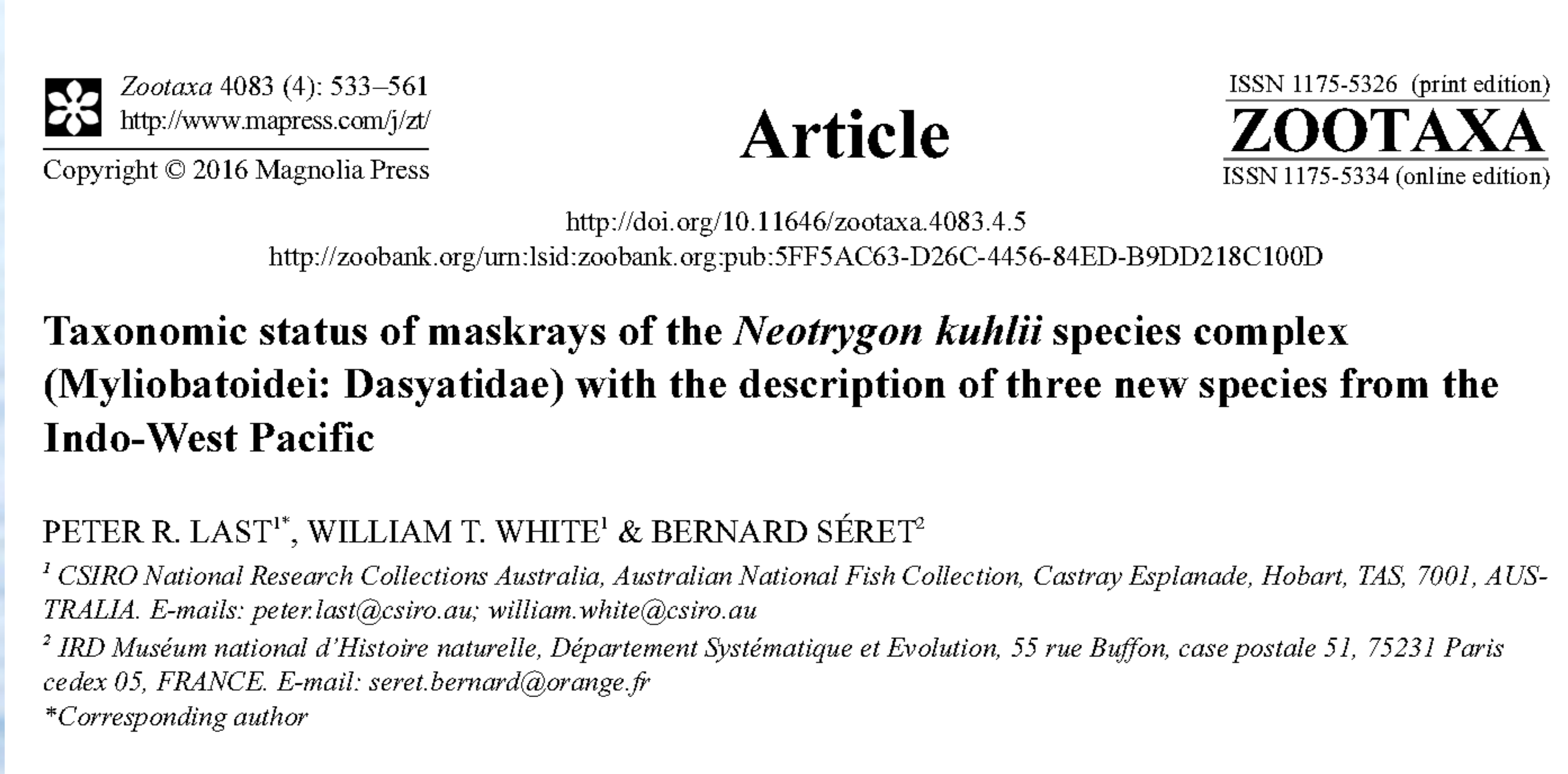
[g] *Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan*

2013

Neotrygon kuhlii

Neotrygon trigonoides

2016



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Taxonomic status of maskrays of the *Neotrygon kuhlii* species complex (Myliobatoidei: Dasyatidae) with the description of three new species from the Indo-West Pacific

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**Corresponding author*

2016

Character	Species (*N*)			
	N. australiae	*N. caeruleo-punctata*	*N. kuhlii*	*N. orientale*
	(9)	(10)	(3)[a]	(6)
Disc width (DW) (cm)	>45	>47	>30	>38
DW / disc length (DL)	1.2–1.3	1.2-1.3	~1.2	1.2–1.3
Angle of snout (°)	101–103	125–130	~107	124–129
Snout length / interorbital width	1.6–2.1	1.8–2.4	1.7–2.1	1.9–2.5
Length from snout tip to pectoral-fin insertion / distance from snout tip to DW	1.7–1.8	1.9–2.0	1.8–1.9	1.8–2.0
Disc width / distance from snout tip to DW	2.3–2.5	2.6–2.9	2.5–2.6	2.7–2.9
Preoral length / mouth width	1.8–2.3	1.6–2.3	2.4–2.8	1.8–2.2
Prenasal length / internasal distance	1.3–1.6	1.4–1.8	1.5–1.8	1.3–1.6
Interspiracular distance / DW (%)	14–16	13–14	13–15	13–16
Nostril length / DW (%)	3.4–4.0	2.5–3.5	2.8-4.1	3.1–3.8
Nasal curtain width / DW (%)	8.4–9.1	8.0–9.4	8.0-8.3	8.9–10.0
Mouth width / DW (%)	6.5–7.3	6.6–7.9	6.4–6.8	7.0–7.5
Horizontal distance from cloaca to caudal sting base / DL (%)	42–52	51–56	~55	48–56
Pectoral-fin radials	105–113	105– 110	113b	105–110
Total vertebral centra (including synarcual)	129–134	133–142	133b	125–141
Trunk centra (including synarcual)	37–41	38–43	39b	36–42
Largest spot on disc / eye width	0.7–1.2	0.5–0.8	0.3–0.5	0.5–0.9
Size of largest blue spot on medial belt / DW (%)	3.3–5.6	1.7– 2.7	~2.1	2.8–3.3
Number of blue spots on medial belt	2–17	0–3	0-6	0–6

Last et al. 2016

Table 1 Ranges of values for 19 morphological characters claimed to be diagnostic of four species in the blue-spotted maskray species complex (data compiled from Last et al., 2016). *Diagnosticity*: utility of character to diagnose at least one of the four species. *N*, sample size

Character	Species (*N*)				**Diagnosticity of character**
	N. australiae	*N. caeruleo-punctata*	*N. kuhlii*	*N. orientale*	
	(9)	(10)	(3)[a]	(6)	
Disc width (DW) (cm)	>45	>47	>30	>38	**NO**
DW / disc length (DL)	1.2–1.3	1.2-1.3	~1.2	1.2–1.3	**NO**
Angle of snout (°)	101–103	125–130	~107	124–129	**?**
Snout length / interorbital width	1.6–2.1	1.8–2.4	1.7–2.1	1.9–2.5	**NO**
Length from snout tip to pectoral-fin insertion / distance from snout tip to DW	1.7–1.8	1.9–2.0	1.8–1.9	1.8–2.0	**NO**
Disc width / distance from snout tip to DW	2.3–2.5	2.6–2.9	2.5–2.6	2.7–2.9	**NO**
Preoral length / mouth width	1.8–2.3	1.6–2.3	2.4–2.8	1.8–2.2	**?**
Prenasal length / internasal distance	1.3–1.6	1.4–1.8	1.5–1.8	1.3–1.6	**NO**
Interspiracular distance / DW (%)	14–16	13–14	13–15	13–16	**NO**
Nostril length / DW (%)	3.4–4.0	2.5–3.5	2.8-4.1	3.1–3.8	**NO**
Nasal curtain width / DW (%)	8.4–9.1	8.0–9.4	8.0-8.3	8.9–10.0	**NO**
Mouth width / DW (%)	6.5–7.3	6.6–7.9	6.4–6.8	7.0–7.5	**NO**
Horizontal distance from cloaca to caudal sting base / DL (%)	42–52	51–56	~55	48–56	**NO**
Pectoral-fin radials	105–113	105– 110	113[b]	105–110	**NO**
Total vertebral centra (including synarcual)	129–134	133–142	133[b]	125–141	**NO**
Trunk centra (including synarcual)	37–41	38–43	39[b]	36–42	**NO**
Largest spot on disc / eye width	0.7–1.2	0.5–0.8	0.3–0.5	0.5–0.9	**NO**
Size of largest blue spot on medial belt / DW (%)	3.3–5.6	1.7– 2.7	~2.1	2.8–3.3	**NO**
Number of blue spots on medial belt	2–17	0–3	0-6	0–6	**NO**

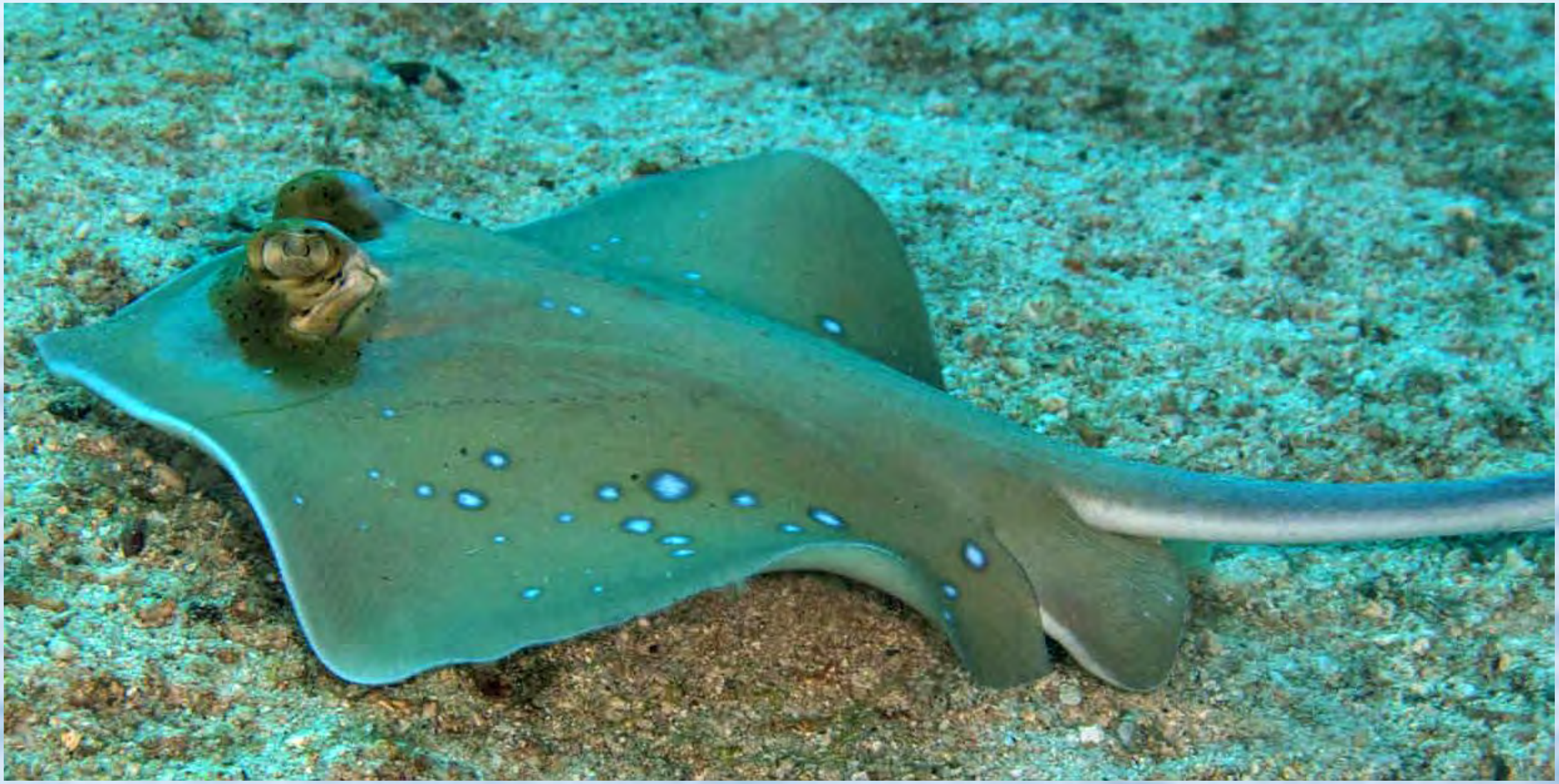
2016

FIGURE 4. Freshly caught specimen of *Neotrygon kuhlii* (CSIRO 7723-01, female 295 mm DW) from Honiara, Solomon Islands: (A) dorsal view; (B) ventral view.

TAXONOMIC STATUS OF MASKRAYS OF THE *NEOTRYGON KUHLII* *Zootaxa* 4083 (4) © 2016 Magnolia Press · 539

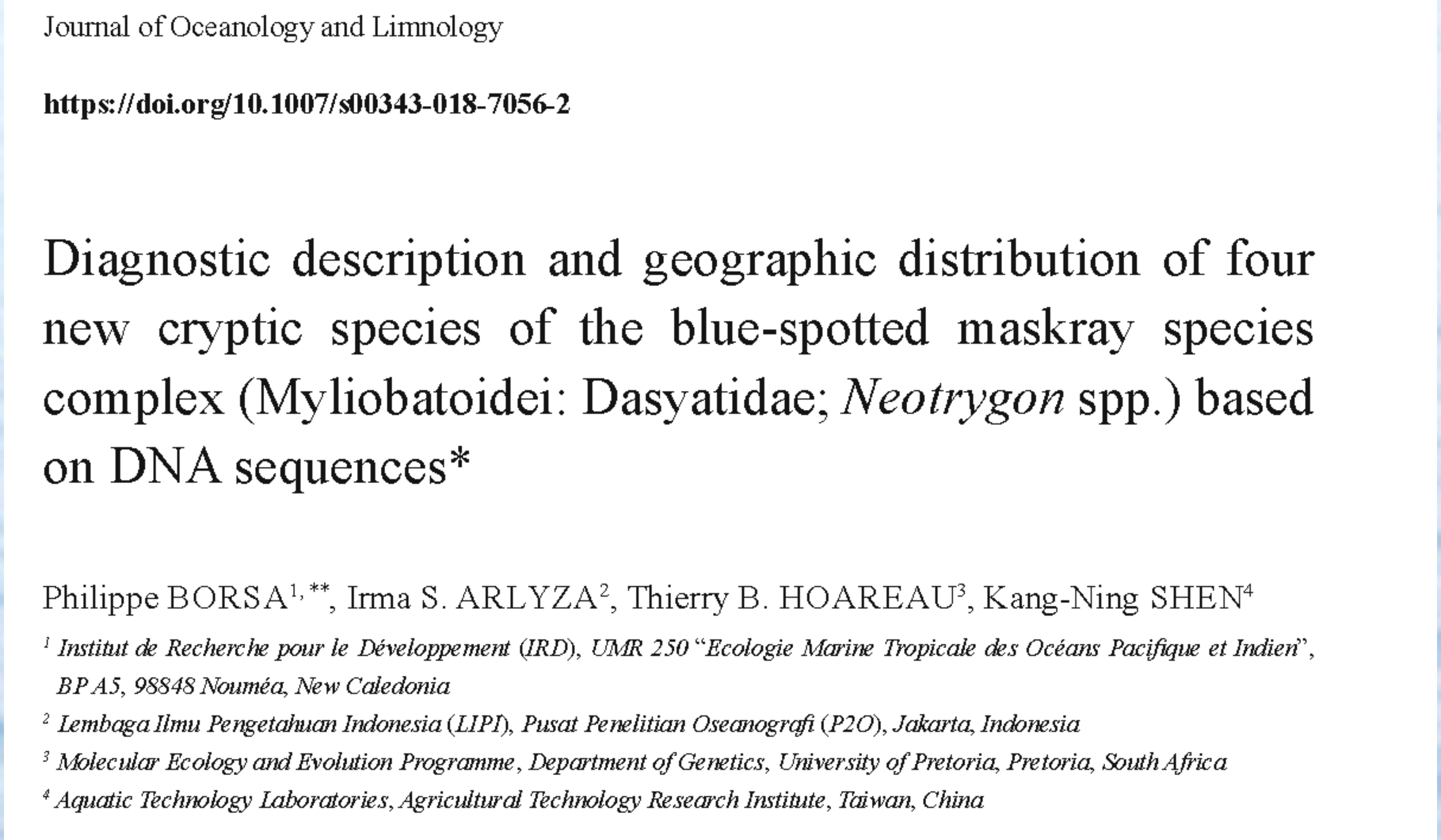
Guadalcanal maskray
misidentified as *Neotrygon kuhlii* by Last et al. 2016

2017



Guadalcanal maskray
described as *Neotrygon vali* by Borsa 2017

2018



Journal of Oceanology and Limnology

https://doi.org/10.1007/s00343-018-7056-2

Diagnostic description and geographic distribution of four new cryptic species of the blue-spotted maskray species complex (Myliobatoidei: Dasyatidae; *Neotrygon* spp.) based on DNA sequences*

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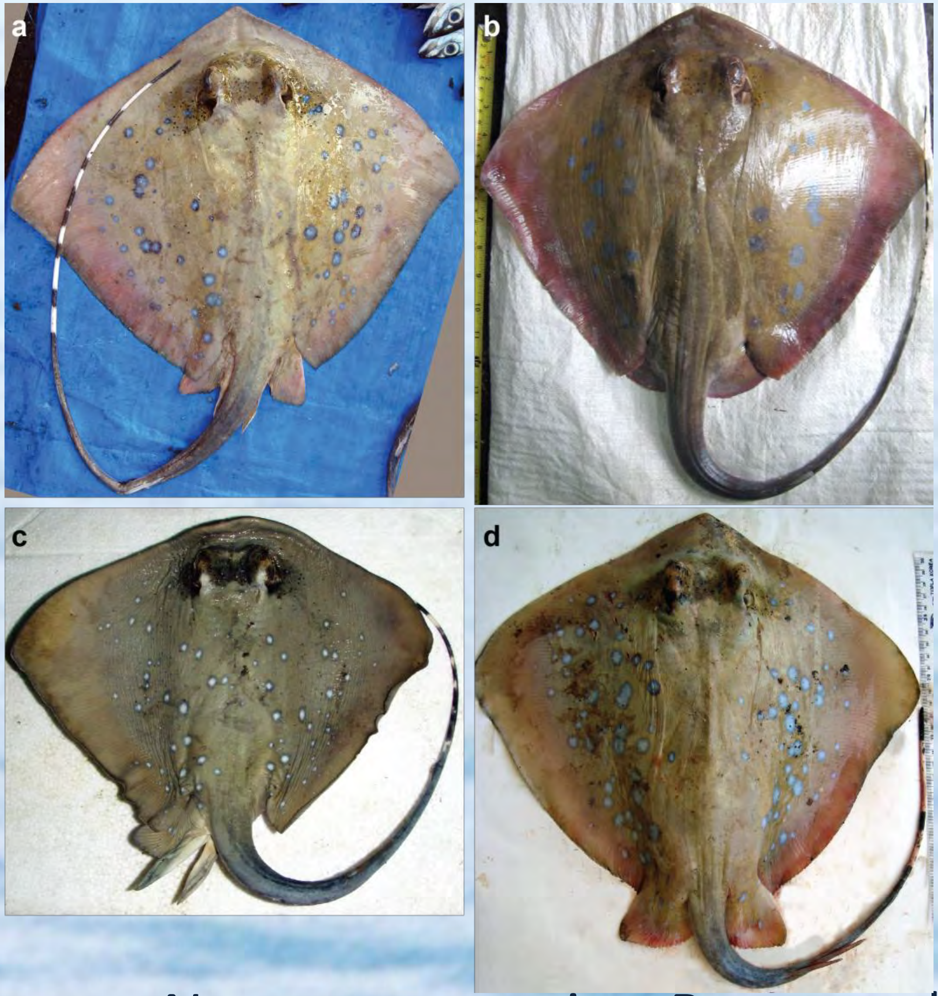
[2] *Lembaga Ilmu Pengetahuan Indonesia (LIPI), Pusat Penelitian Oseanografi (P2O), Jakarta, Indonesia*

[3] *Molecular Ecology and Evolution Programme, Department of Genetics, University of Pretoria, Pretoria, South Africa*

[4] *Aquatic Technology Laboratories, Agricultural Technology Research Institute, Taiwan, China*

Four new *Neotrygon* species: Borsa et al. 2018

2018



N. bobwardi
Aceh

N. malaccensis
Melaka Strait

N. moluccensis
Tual

N. westpapuensis
Biak

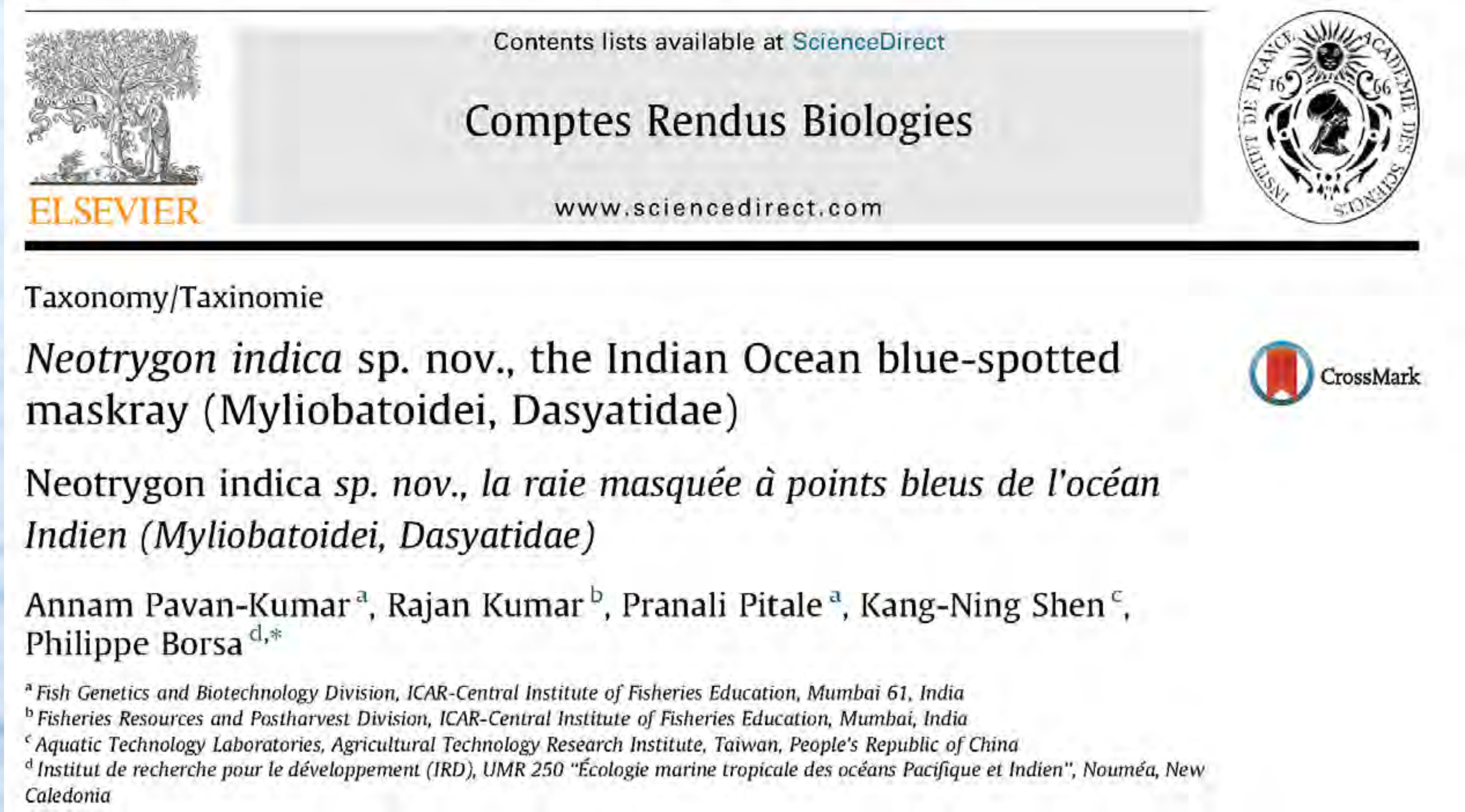
Four new *Neotrygon* species: Borsa et al. 2018

2018

5’-CTGGCCTCAGTTTACTTATCCGAACAGAACTAAGCCAACCAGGCGC
TTTACTGGGTGATGATCAAATTTATAATGTAATCGTCACTGCCCACGC
CTTCGTAATAATCTTCTTTATAGTAATGCCAATTATAATTGGTGGGTTT
GGTAATTGACTAGTACCCCTAATAATTGGGGCTCCGGACATAGCCTTT
CCACGAATGAACAACATAAGTTTTTGACTTCTGCCTCCCTCATTCCTAC
TACTGCTAGCCTCAGCAGGGGTAGAAGCCGGAGCCGGAACAGGTTGA
ACAGTTTATCCCCCATTAGCTGGTAATCTAGCACATGCCGGAGCTTCT
GTAGACCTTACAATCTTCTCTCTTCACCTAGCAGGTGTTTCCTCTATTC
TGGCATCCATCAACTTTATCACAACAATTATTAATATAAAACCACCTG
CAATCTCCCAGTATCAAACCCCATTATTCGTCTGATCTATTCTTGTTAC
AACTGTACTTCTCCTGCTATCCCTACCAGTCCTAGCAGCTGGCATTAC
TATACTCCTCACAGACCGAAATCTTAATACAACTTTCTTTGACCCAGCT
GGAGGGGGAGATCCCATTCTTTACCAACACCTC-3’.

New species description

2018



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Taxonomy/Taxinomie

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

Neotrygon indica *sp. nov., la raie masquée à points bleus de l'océan Indien (Myliobatoidei, Dasyatidae)*

Annam Pavan-Kumar [a], Rajan Kumar [b], Pranali Pitale [a], Kang-Ning Shen [c], Philippe Borsa [d,*]

[a] *Fish Genetics and Biotechnology Division, ICAR-Central Institute of Fisheries Education, Mumbai 61, India*
[b] *Fisheries Resources and Postharvest Division, ICAR-Central Institute of Fisheries Education, Mumbai, India*
[c] *Aquatic Technology Laboratories, Agricultural Technology Research Institute, Taiwan, People's Republic of China*
[d] *Institut de recherche pour le développement (IRD), UMR 250 "Écologie marine tropicale des océans Pacifique et Indien", Nouméa, New Caledonia*

Neotrygon indica: Pavan-Kumar et al. 2018

2018

Neotrygon indica: Pavan-Kumar et al. 2018

PART III - Research perspectives

- More taxonomic work ahead – The genetic relationship of *N. kuhlii* with *N. trigonoides* should be considered a priority for future taxonomic research on the blue-spotted maskray. The genetic analysis of the lectotype of *N. kuhlii* would be helpful in this respect, but genetically analyzing fresh specimens from Vanikoro could be an alternative.

  The current *N. orientale* lineage has not yet been clearly delimitated and it may comprise cryptic species. The blue-spotted maskray has so far been only loosely sampled in the eastern Coral Triangle and in Micronesia, which together represent a wide part of its range. Future research in this part of the Indo-West Pacific may uncover more cryptic lineages.

- Calibrating the molecular clock using the coalescence of mtDNA – Similarities and dissimilarities in a set of DNA sequences sampled from a species typically yield information relevant to the reconstruction of the genealogy of thesequences, hence part of the evolutionary history of the species (Hudson 1990). The genetic divergence among the sampled sequences correlates with the time elapsed since their most recent common ancestor. The process by which genetic sequences merge backwards to their most recent common ancestor is their coalescence. Within the last few decades, a theory of coalescence has developed, which predicts how sequences merge to their most common ancestor backwards in time. For transforming relative times to the most recent common ancestor into calendar times, one needs to apply mutation rates and calibrate what is called the molecular clock that is taxon-specific.

  Because no suitable mutation rates are available for elasmobranch populations, one of our objectives is to calibrate the molecular clock in the blue-spotted maskray by providing estimatesof mutation rates for its mtDNA. For this, we will extract the information provided by the star-like coalescence patterns visible within haplogroups of mtDNA sequences (see the supplementary slide no.2 at the end of this presentation). By assuming that these expansion events correspond to the post-glacial rise in sea-level, we will obtain mutation rates that will be more accurate than the one derived from fossils. Then, we should be able to estimate the demographic reconstruction of the species and the most recent speciation events.

- Dating the colonization history step by step – As we have seen, the colonization of the Indo-West Pacific by the blue-spotted maskray most likely occurred from the East to the West in a stepwise fashion. As at least the latest population expansions are recorded in the phylogeny of the DNA sequences, it should be possible to date the last steps of the colonization.

- Identifying selected genes, speciation genes – Each lineage of the blue-spotted maskray having evolved in geographic isolation from the other lineages, it is expected it has adapted to local environmental conditions. By comparing the composition of the genomes representative of different lineages, one should be able to pinpoint those adaptive alleles that have been selected. Moreover, in case of secondary contact between two geographically adjacent lineages, it is expected that the latter have maintained their selective advantage by reinforcement (Dobzhansky 1951). Reinforcement is the process through which reproductive isolation increases by the selection of alleles that prevent hybridization, or of those that lower hybrid fitness.

- In short, and this will be the conclusion of this seminar, we view the blue-spotted maskray as a model-species complex to study speciation in marine stingrays.

Acknowledgements

Our collaborators through the PARI project have included Philippe Béarez, Patrick Berrebi, Chih-Wei Chang, Wei-Jen Chen, Jean-Dominique Durand, Thierry B. Hoareau, Chung-Der Hsiao, Rajan Kumar, Martin Laporte, Mark G. Meekan, Annam Pavan-Kumar, Pranali Pitale and Kang-Ning Shen. Sergey Bogorodsky provided the NJ tree of *CO1* sequences for the Indian Ocean. Bob Ward provided a few comments based on the abstract; many thanks to him for kindly sharing his thoughts at different stages of the project. Thanks and congratulations to Muhammad Hafizt for organizing this seminar.

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Part III – Some research perspectives

* More taxonomic work ahead

* Calibrating the molecular clock using the coalescence of mtDNA

* Dating the colonization history step by step

* Identifying selected genes, speciation genes, ...

…

* Blue-spotted maskray as a **model for speciation** in stingrays

- Terima kasih

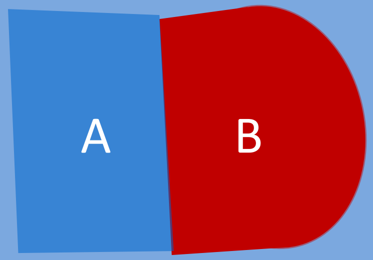
Supplementary slides

- Supplementary slide no. 1: Parapatric phylogeographic structure

- Supplementary slide no. 2 (Fig. 2 of Arlyza et al. 2013): Blue-spotted maskray, *Neotrygon kuhlii*. Median-joining parsimony network (Network: Bandelt et al. 1999) of the haplotypes (as determined from 519-bp nucleotide sequences at the *COI* locus) of 147 individuals (sampling details in Table 1). Clades (*II*-*VIII*) are delineated by ellipses. Branch length is proportional to the number of mutational steps; circles represent individual haplotypes, their area being proportional to their frequency in the total sample and their colour according with Figure 1; squares represent haplotypes from Tanzania and India. Numbers at the origin of each clade are bootstrap scores (1000 bootstraps) from, respectively, maximum parsimony (Mega5; Tamura et al. 2011) / Neighbour-Joining (Kimura-2 parameter distance; Mega5) / maximum likelihood (HKY+G mutation model; MEGA5) trees rooted by *N. trigonoides* (GenBank JQ765533-JQ765535, JX263420, JX304916, and JX304917) (see Supplementary Material, Fig. S1). Haplogroups *Ia* and *Ib* include haplotypes from Tanzania and India, respectively. Clades encompass haplotypes from different samples (abbreviations from Table 1) as following: *II*: bre, meu, sib, pad; *III*: PAG; *IV*: KAR, VN, bei, sel, bat, Java, BO, tar, pao, Taiwan, ton, cag, lap, Japan; *V*: NR, sin, Queensland; *VI*: Bali; *VII*: amb, ara; *VIII*: bia. Haplogroups *Ia* and *Ib* (present work) are included in 'Clade 8' of Puckridge et al. (2013); Clade *IV* is '*Neotrygon kuhlii* 1' of Naylor et al. (2012) and includes 'Clades 1-4' of Puckridge et al. (2013); Clades *V* and *VI* are, respectively, 'Clade 5' and 'Clade 6' in Puckridge et al. (2013). Scale bar: 1 mutational step.

Parapatric phylogeographic structure

Parapatry is the term used to describe a situation where “pairs of taxa present separate but contiguous geographic distributions”
(Bull 1991 *Annual Review of Ecology and Systematics*)



Parapatry

In the blue spotted maskray:
The geographic distributions of the lineages were mutually exclusive.
Therefore, we describe them as contiguous, thus parapatric.

Neotrygon kuhlii species complex: CO1 gene sequences