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Himantura tutul sp. nov. (Myliobatoidei: Dasyatidae), a new ocellated whipray from the tropical Indo-West Pacific, described from its cytochrome-oxidase I gene sequence

Himantura tutul *sp. nov. (Myliobatoidei : Dasyatidae), une nouvelle raie pastenague ocellée de l'Indo-ouest Pacifique tropical, décrite à partir de la séquence du gène de la cytochrome-oxydase I*

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ABSTRACT

It has been previously established that the Leopard Whipray, *Himantura leoparda*, consists of two genetically isolated, cryptic species, provisionally designated as 'Cluster 1' and 'Cluster 4' (Arlyza et al., Mol. Phylogenet. Evol. 65 (2013) [1]). Here, we show that the two cryptic species differ by the spotting patterns on the dorsal surface of adults: Cluster-4 individuals tend to have larger ocellated spots which also more often have a continuous contour than Cluster-1 individuals. We show that *H. leoparda*'s holotype has the typical larger-ocellated spot pattern, designating Cluster 4 as the actual *H. leoparda*. The other species (Cluster 1) is described as *Himantura tutul* sp. nov. on the basis of the nucleotide sequence of a 655-base pair fragment of its cytochrome-oxidase I gene (GENBANK accession no. JX263335). Nucleotide synapomorphies at this locus clearly distinguish *Himantura tutul* sp. nov. from all three other valid species in the *H. uarnak* species complex, namely *H. leoparda*, *H. uarnak*, and *H. undulata*. *Himantura tutul* sp. nov. has a wide distribution in the Indo-West Pacific, from the shores of eastern Africa to the Indo-Malay archipelago. *H. leoparda* under its new definition has a similarly wide Indo-West Pacific distribution.

Keywords: Himantura leoparda; new species; molecular taxonomy; *COI*; cytochrome *b*

RESUME

Il a été établi antérieurement que la raie léopard, *Himantura leoparda*, comprenait deux espèces cryptiques génétiquement isolées et provisoirement désignées comme 'Cluster 1' et 'Cluster 4' (Arlyza et al., Mol. Phylogenet. Evol. 65 (2013) [1]). Ici, nous montrons que les deux espèces cryptiques se distinguent l'une de l'autre par les patrons de taches sur la surface dorsale des individus adultes : en général, les individus du Cluster 4 possèdent des taches plus larges et au contour plus continu que les individus du Cluster 1. Nous montrons que l'holotype de *H. leoparda* présente le patron typique à larges taches ocellées, ce qui désigne le Cluster 4 comme étant réellement l'espèce *H. leoparda*. L'autre espèce (Cluster 1) est ici décrite comme *Himantura tutul* sp. nov. à partir de la séquence nucléotidique d'un fragment (655 paires de bases) du gène de la cytochrome oxydase I (GENBANK no. JX263335). Les synapomorphies nucléotidiques à ce locus permettent de distinguer sans ambiguïté *Himantura tutul* sp. nov. des trois autres espèces valides du complexe d'espèces *H. uarnak* : *H. leoparda*, *H. uarnak* et *H. undulata*. *Himantura tutul* sp. nov. possède une large distribution indo-ouest pacifique, depuis les côtes de l'Afrique de l'est jusqu'à l'archipel indo-malais. *H. leoparda*, telle que redéfinie à la suite du présent travail, présente une distribution géographique comparable à celle d'*Himantura tutul* sp. nov.

Mots-clés: Himantura leoparda; nouvelle espèce; taxonomie moléculaire; *COI*; cytochrome *b*

1. Introduction

Chondrichthyan systematics and taxonomy has gone through a recent upsurge in new species discovery [2,3], although a factor that may soon hamper the endeavour of describing Chondrichthyan biodiversity could be the trend towards local extinction of species in this phylum [4]. This is especially crucial in poorly-surveyed, species-rich regions of the world like the Indo-Malay-Papua archipelago [2,5,6], where fishing is mostly unregulated and vulnerable Chondrichthyan populations are unmonitored [6-8]. Stingrays (Myliobatoidei, including Dasyatidae [9]) are particularly at risk of population depletion: they grow and mature slowly, have low fecundity, and most of the species in the Dasyatidae family dwell in the shallow coastal habitats which are particularly exposed to overfishing and habitat degradation [7,10-13].

A recent taxonomic revision of ocellated whiprays in the '*Himantura uarnak*' species complex (Dasyatidae) has helped clarify the nomenclature in this group [14]. In this revision, the authors showed that a widespread leopard-spotted species formerly, erroneously identified as *H. undulata* [15,16] was in fact a distinct, undescribed species, *H. leoparda* Manjaji-Matsumoto and Last, 2008 [14]. The authors also synonymized *H. fava* (Annandale, 1909) [17] with *H. undulata* (Bleeker, 1852) [18] and maintained *H. uarnak* (Forsskål, 1775) [19] as another valid species in this group. The three species have been said to "differ subtly" in squamation and body shape, and by the ontogeny of colour patterns [14]. Subsequently, nuclear genetic markers have helped demonstrate that ocellated whiprays within the *H. uarnak* species complex comprised at least four biological species sensu [20]: *H. uarnak*, *H. undulata*, and two cryptic species within *H. leoparda* [1]. Four mitochondrial clades were also observed (Fig. 2 of [1]), that fully correlated with the four clusters.

The objectives of the present paper were (1) to investigate whether the two cryptic species currently under *H. leoparda* could or could not be distinguished by their spotting patterns; (2) to determine which of the two species should retain the name *H. leoparda*; (3) to provide a diagnostic description of the other species. In the process, we determined the nucleotide synapomorphies in the partial cytochrome-oxidase I (*COI*) gene sequence, the universal barcode in fishes [21], that distinguish species within the *H. uarnak* species complex.

2. Materials and Methods

2.1. Nucleotide sequences

Arlyza et al. [1] have provided two phylogenetic trees of the *H. uarnak* species complex, one based on the nucleotide sequence of a 620-base pair (bp) fragment of the *COI* gene, and the other one based on the nucleotide sequence of a 239-bp fragment of the cytochrome *b* (*cytb*) gene. Most of the *COI* gene sequences of [1] were of individuals also scored at nuclear loci; the *cytb* gene sequence dataset also included a number of reference specimens from the literature that could not be directly assigned to a nuclear cluster.

At the time Arlyza et al.'s paper [1] was in press, additional *COI* gene sequences of an undetermined species of the *H. uarnak* species complex (eventually identified as *H. uarnak* by the authors) were produced within the frame of a barcoding study of stingrays from the continental shelf off northwestern Australia [22]. Table 1 lists the specimens characterized by their *COI* gene sequences that were used for the present work, including those of [22].

Here, Individual zan6 (Table 1) was sequenced on a longer fragment of the *cytb* gene, as it is to be the holotype of the new species. The polymerase chain reaction used 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*) [24,25]. Fifteen µL reaction volume containing 0.2 mM dNTPs, 1.5 µL 10× PCR buffer (Bioman, Taipei), 0.5 µM each of forward and reverse primer, 0.2 U Taq DNA polymerase (Bioman), and 1.0 µL template DNA was subjected to cyclic temperature variation in a Biometra TGradient thermocycler (Biometra, Göttingen). 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-amplified DNAs were visualized under ultraviolet light on 1% agarose gel stained with ethidium bromide. After purification (by isopropanol precipitation), 1 µL 1/8-diluted PCR product was subjected to sequencing, in both forward and reverse directions, using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City CA). Cycling conditions were according to the manufacturer's protocol. Sequencing reaction products were cleaned by removing dye-terminator (CleanSEQ kit, Beckman Coulter, Beverly MA) and loaded onto an ABI Prism 3100 DNA sequencer (Beckman Coulter).

2.2. Phylogenetic analysis

We performed a new phylogenetic analysis based on the partial *COI* gene sequences of [1] and homologous sequences accessible from GENBANK (http:// www.ncbi.nlm.nih.gov/) including those of Cerutti-Pereyra et al. [22] (Table 1). Partial *COI*-gene sequences of *H. gerrardi* and *H. toshi* (Table 1) were chosen as the outgroup [1]. The best substitution model according to the Bayesian information criterion (using the MEGA5 software: [26]) was the Tamura-3 parameter substitution model [27] with gamma-distributed evolutionary rates (T92+G) and this model was used to produce a maximum-likelihood phylogeny of the partial *COI* gene sequences.

2.3. Determination of nucleotide synapomorphies

All complete nucleotide sequences of the 620-bp *COI* gene fragment, and only these, were aligned using the BIOEDIT sequence editing software [28]. The sequences were sorted by mitotype and nucleotide synapomorphies were then assessed visually.

2.4. Description of spotting patterns

We possess the pictures of the dorsal side of a number of individuals larger than 1000 mm disk width (DW) (Supplementary material, Fig. S1) identified to species ('Cluster 1' or 'Cluster 4' of [1]) from their genotypes at 5 intron loci. We analyzed the pictures of 27 individuals of quality good enough to distinguish the detail of spotting patterns on at least one side of the disk. In addition, we analyzed the picture of specimen CSIRO H2903.01, the holotype of *H. leoparda* (Fig. 1A of [14]). The analysis consisted of measuring the size of spots (ocellae) along a linear transect running from the mid-scapula to the lateral extremity of the disk (Fig. 1). Each of the specimens examined had to be sufficiently well preserved to have its spotting patterns intact along the transect. Ocellated spots with continuous contour (Fig. 2A) were distinguished from spots with interrupted contour (Fig. 2B, C). Three arbitrarily chosen size-classes were considered for the diameter of an ocella: 'small' (≤15 mm); 'medium' (>15mm and ≤25 mm); and 'large' (>25 mm). Each individual was thus characterized by 6 factors, which were the numbers of ocellated spots along a half-disk transect of each of the three size-classes with, respectively, continuous and interrupted contours. Some individuals whose pictures were of quality good enough to allow both right and left transects were represented twice in the matrix. Thus, we were able to produce a matrix of 37 entries, where each entry corresponded to the half-disk transect of an individual.

This matrix was then subjected to correspondence analysis (CA) [29] to visualize statistical differences, if any, in the spotting patterns of the two cryptic species. CA was run using the FACTOMINER package [30] under R [31].

3. Results

The phylogenetic analysis of *COI* gene sequences (Fig. 3) included 16 new sequences from the literature [22] in addition to the dataset we analyzed previously [1]. All *H. uarnak* sequences from Ningaloo

Reef [22] formed a distinct subclade within Clade III (*H. uarnak*) (Fig. 3), the other subclade consisting of *H. uarnak* haplotypes sampled in the Indo-Malay archipelago. The net nucleotide divergence (MEGA5: [26]) between the two sub-clades was 0.020 whereas the nucleotide diversity within subclades (MEGA5: [26]) was 0.002 to 0.003. Comparatively, the net nucleotide divergence between zan6, the only Clade-I individual sampled in the western Indian Ocean and the rest of Clade I, which consisted of haplotypes sampled from the Indo-Malay archipelago, was 0.010 and the nucleotide diversity within the latter group was 0.002. Similarly, the net nucleotide divergence between the two individuals of Clade IV from the Kwazulu-Natal in the western Indian Ocean (Table 1) and the rest of Clade IV, which consisted of haplotypes sampled from the Indo-Malay archipelago, was 0.015 and the nucleotide diversity within a subclade was 0 to 0.003.

Supplementary material, Table S1 details the nucleotide variability observed in the whole sequence dataset, which comprised 30 sequences of Clade I of [1], two of Clade II, 25 of Clade III, and 60 of Clade IV. Nucleotide synapomorphies were observed for each clade (Table 2), hence could be used as diagnostic characters of each of the corresponding species, i.e., respectively, Clusters 1-4 [1].

The results of the morphological analysis based on spotting patterns (Table 2) are presented in Fig. 4. All Cluster-1 individuals were positioned outside a convex envelope that would group all Cluster-4 individuals, indicating some segregation of Cluster 1 vs. Cluster 4 on the sole basis of spotting patterns. However, hierarchical cluster analysis provided overlapping clusters (represented by dotted lines on Fig. 4). The two datapoints representing Individual CSIRO H2903.01, the holotype of *H. leoparda*, were clearly positioned within the envelope delimitating Cluster-4 specimens to the exclusion of Cluster-1 specimens. The two data points representing Individual zan6 (the holotype of *Himantura tutul* sp. nov., see below) were positioned within the bulk of the Cluster-1 specimens. The main factors differentiating the two clusters were the number of large ocellated spots, characteristic of Cluster 4 exclusively, and the number of medium-sized ocellated spots, which were almost absent in Cluster-1 individuals (the only exception being the only presumed hybrid present in the sample). Small interrupted spots were most frequent in Cluster-1 individuals (Table 2).

4. Discussion

The sequencing of genomic DNA provides a powerful tool to characterize genetic variation within and among species [32, 33]. DNA sequences are increasingly commonly used to distinguish species boundaries and to identify individuals to species [21,32,34-36]. DNA sequences are also useful to diagnose new species, and given this, "we find no compelling evidence to exclude DNA-only descriptions" [35].

We were confronted with the problem of determining which of the two cryptic species then under *H. leoparda* was the true *H. leoparda*. No mitochondrial sequence of *H. leoparda*'s holotype (CSIRO H2903.01) seems to exist [37]. However, a picture of the dorsal side of *H. leoparda*'s holotype was available from the literature [14,37] thus allowing its characterization on the basis of spotting pattern. In the present work, a strong statistical relationship was demonstrated between multiple-locus nuclear genotype [1] and spotting pattern within the ocellated stingrays currently grouped under *H. leoparda*. This allowed us to assign the holotype of *H. leoparda* to Cluster 4 of [1]. Therefore, it is the other species (Cluster 1) that remained undescribed.

Each of Clades I, III and IV had representatives from both the Indian Ocean and the Indo-Malay archipelago. In all three cases, two subclades were detected, within which the nucleotide diversity was ~5 to ~10 times less than the net nucleotide distance between subclades. Pending more detailed sampling, we provisionally ascribe these differences to geographic variation. These differences may have arisen from past geographic isolation between the Indian Ocean and Pacific Ocean populations of each species, or may result from isolation by distance. To test these two hypotheses will require new, extensive geographic sampling. Isolation by distance has been identified as a possible mechanism of geographic differentiation in another stingray species of the family Dasyatidae, *Neotrygon kuhlii* [38].

The confusion in the taxonomy of ocellated whiprays of the *H. uarnak* species complex originates in part from the mediocrity of traditional morphological characters for separating biological species in this group [37]. The recent taxonomic revision of [14], based on morphology, helped clarify the nomenclature of species in this group but fell short of distinguishing two of the four species subsequently scored using nuclear markers [1]. In sharp contrast with morphological characters, the nucleotide sequence of a portion of the *COI* gene provides a clear distinction of the different species that were uncovered by nuclear markers [1]. It is therefore advisable to now base our description of *Himantura tutul* sp. nov. on the nucleotide sequence of the *COI* gene. It is sensible to employ this marker instead of the five nuclear markers used by [1] because the *COI* gene also provides the universal barcode in fishes [21] which has been proven to be very effective for identification to species in Elasmobranchs [39]. An alternative mitochondrial marker in Elasmobranchs could be the *NADH2* gene, now sequenced in 574 species representative of the genetic diversity of the whole phylum [36]. However, it is not yet possible to relate the five main *NADH2* haplogroups observed in the *H. uarnak* species complex (Fig. 52 of [36]) to Clusters 1-4 of [1].

5. Diagnostic description of *Himantura tutul* sp. nov.

5.1. Previous references

Himantura sp. A, pro parte: [37]; *Himantura leoparda*, pro parte: [14]; *Himantura leoparda* Cluster 1: [1].

5.2. Type material and vouchers

We chose as holotype a female individual, ~1150 mm DW, sampled by J.-D.D. at the Mkoani fish landing site on Pemba Island, Tanzania, 26 May 2010 (Fig. 5). A sample of tissue of this individual (zan 6) has been deposited by P. Borsa and J.-D.D. at the Museum national d'histoire naturelle (MNHN), Paris under registration no. MNHN-ICTI5184. This material consists of two sections of the tail, 25.1 and 28.5 mm long and 4.4 mm and 4.6 mm diameter, respectively. Since DNA can be re-extracted from this tissue sample and its *COI* sequence can be verified independently, and any other portion of the genome can be sequenced to complete the description of the species or to allow comparisons with other materials in the future, we consider that there was no necessity to deposit the whole dead specimen as holotype. Article 72.5.1 of the International Code of Zoological Nomenclature (ICZN) stipulates as eligible as name-bearing type "any part of an animal" [40]. It would have been a difficult task to preserve this specimen, given its size and the remoteness of the fishing location. We were also unprepared to face the administrative red tape, the cost, and the logistical problems that would arise in attempting to transfer a large, formalin-preserved specimen from its sampling site in remote Pemba Island to a museum overseas. Besides, several species in the genus *Himantura* including *H. leoparda*, *H. uarnak* and *H. undulata* are already classified as "vulnerable" in the IUCN Red List [40-43] and it is likely that *Himantura tutul* sp. nov. similarly will be placed on the list. Therefore, we deem it somewhat unethical to sacrifice an adult individual to be preserved as dead specimen in a collection. Buying an already dead specimen from a fish landing site would similarly send a contradictory message in this respect. .

We chose as paratypes of *Himantura tutul* sp. nov. two specimens previously determined as *H. leoparda* by Manjaji-Matsumoto and Last [14]: CSIRO H5284.05 (female 805 mm DW, from Kota Kinabalu fish market, Kota Kinabalu, eastern South China Sea, 1999; dissected jaws, denticle band and pelvic girdle retained), and UMS MMSK-c4 (from Sandakan, Sulu Sea, 1999). CSIRO H5284.05 is at the Australian National Fish Collection, Hobart, and UMS MMSK-c4 is in the fish collection of Universiti Malaysia Sabah in Kota Kinabalu. The rationale for choosing these specimens as paratypes of *Himantura tutul* sp. nov. is the following: both specimens have been registered in museum collections and both possess the mitotype I characteristic of *Himantura tutul* sp. nov. [1] based on the partial nucleotide sequences (239 bp of the *cytb* gene) provided by Manjaji [37]. One of these specimens (UMS MMSK-c4)

has the "fine-leopard" pattern mentioned in [37] which is the general pattern exhibited by individuals of *Himantura tutul* sp. nov. (present work). Although CSIRO H5284.05 has already been designated as a paratype of *H. leoparda* [14], Article 72.6 of the ICZN stipulates that "the fact that a specimen is already the name-bearing type ... of one nominal species-group taxon does not prevent its being the name-bearing type ... of another" [40].

The other material examined included all other specimens analyzed at both the *COI* locus, the *cytb* locus, and five size-polymorphic intron loci [1] (Table 1), at the *COI* locus only [22] (Table 1), and at the *cytb* locus only [1,37].

5.3. Description

A morphological description of *Himantura tutul* sp. nov. has already been provided (under *H. leoparda*) by Manjaji-Matsumoto and Last [14]. These authors did not refer to spotting patterns in their definition nor in their diagnosis of *H. leoparda*. The present results showed that the 'atypical fine leopard form' [37] of *H. leoparda* sensu [14] was characteristic of *Himantura tutul* sp. nov. while it was mostly absent in *H. leoparda* under its new definition (Table 2; Fig. 4; Supplementary material, Fig. S1). Therefore, spotting patterns are useful as a character to distinguish between the two species (Fig. 4). However, the mitochondrial sequence has proven to be totally diagnostic ([1]; present study) and because of this fact, we consider it to be much more an adequate character than any of the morphological characters employed thus far [14,37], including spotting patterns (present work), to base our description on.

The present description of *Himantura tutul* sp. nov. is based on the nucleotide sequence of a 655-base pair (bp) fragment of the *COI* gene (hereafter abbreviated as 'partial *COI* gene') homologous to the portion of the mitochondrial genome of *N. kuhlii* comprised between nucleotide sites 2278 and 2932 (GenBank JN184065; [9]). The partial *COI* gene of the holotype of *Himantura tutul* sp. nov. has the following sequence: 5'- C C T T T A T C T G A T C T T C G G T G C A T G A G C A G G G A T A G T G G G T A C T G G C C T T A G C C T G C T T A T T C G G A C A G A G C T A A G T C A A C C A G G C G C A C T A C T G G G T G A T G A T C A G A T C T A T A A T G T A A T T G T C A C T G C C C A T G C C T T C G T A A T A A T C T T T T T T A T G G T A A T G C C C A T T A T A A T T G G T G G T T T T G G T A A T T G A C T C G T T C C C C T A A T A A T T G G C G C C C C T G A T A T A G C T T T T C C T C G A A T A A A C A A C A T A A G T T T C T G G C T T C T C C C T C C A T C C T T C T T G C T A C T T T T G G C C T C T G C T G G A G T A G A A G C T G G A G C T G G A A C C G G T T G A A C A G T C T A C C C C C C A T T A G C T G G C A A T C T A G C A C A C G C A G G G G C T T C A G T A G A C T T A G C A A T C T T T T C G C T A C A T C T A G C C G G T G T A T C T T C T A T C T T G G C C T C C A T T A A T T T T A T T A C C A C A A T C A T T A A C A T A A A A C C A C C A G C A A T T T C A C A G T A T C A A A C A C C C C T C T T T G T C T G A T C G A T T C T C A T C A C T G C T G T A C T C C T C T T G T T A T C C C T T C C T G T T C T G G C A G C A G G C A T T A C A A T A C T T T T A A C A G A C C G T A A C C T C A A T A C A A C C T T C T T T G A C C C T G C A G G A G G A G G T G A C C C A A T T C T C T A T C A A C A T C T C T T C -3'. This sequence has accession no. JX263335 in GENBANK.

In addition, the nucleotide sequence of a 829-bp fragment of the *cytb* gene of the holotype of *Himantura tutul* sp. nov. is the following: 5'- A T A C C G C A G A C A T C T C C T C A G C A T T C T C C T C A G T T G C A C A T A T C T G C C G A G A T G T A A A C T A T G G C T G A C T A A T C C G C A A C A T C C A C G C T A A C G G C G C C T C C A T G T T C T T T A T C T G C A T T T A C C T T C A C A T T G C T C G A G G T T T T T A C T A T G G T T C C T A T C T T T A T A A A G A G A C C T G A A A C A T C G G A G T A A T C A T C T T A A T G C T A C T A A T A G C T A C T G C C T T T G T A G G T T A C G T C C T C C C A T G A G G A C A A A T A T C A T T C T G A G G A G C A A C C G T T A T T A C C A A C C T A T T A T C A G C C T T T C C C T A T A T T G G A G A T A T G C T A G T T C A G T G A A T C T G G G G T G G T T T T T C A G T G G A T A A C G C A A C A C T A A C T C G A T T C T T C A C A T T T C A C T T C C

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G C G T C C T T G C A C T T G C C T T C T C A A T T C T T A T T C T C C T T C T A G T C C C
A A T A C T A C A C A C C T C A A A A C A A C G A A G C C T T A C C T T C C G C C C A A T
C A C A C A A C T C C T T T T C T G A C T T C T A G T A A C A A A -3'. This sequence has accession no. JX274333 in GENBANK. Partial (239-bp) *cytb* gene sequences of other specimens of *Himantura tutul* sp. nov. have been published previously under *Himantura* sp. A [37] or under *H. leoparda* Cluster 1 [1]. These include paratypes CSIRO H5284.05 and UMS MMSK-c4, voucher specimen UMS MMPL11, and three other specimens labelled A1, A4 and A5 [37], and all other *Himantura tutul* sp. nov. individuals of Table 1.

5.4. Etymology

We chose as epithet of the new species the Malay word *tutul* which means "spotted" and which designates the spots of the leopard, *Panthera pardus* ("*macan tutul*" in Malay language). Thus, the new species was named after the leopard-like markings on the dorsal surface of large specimens (>1000 mm DW). We propose as the English vernacular name: Fine-spotted Leopard Whipray, to distinguish it from the Leopard Whipray, *Himantura leoparda*, which has larger spots (Fig. 1; Table 2). We propose as the French vernacular name *raie léopard à petites taches* and as the Malay vernacular name *pari tutul kecil*.

5.5. Comparisons with closely related species

Himantura tutul sp. nov. is closely related to *H. leoparda*, *H. uarnak*, and *H. undulata*: all four species cluster as a single clade in the phylogeny of the genus *Himantura* [1]. *Himantura tutul* sp. nov. and *H. leoparda* cannot be distinguished by their partial *COI* amino-acid sequences as translated from the 620-bp fragment analyzed in [1]. *H. uarnak* differs from the former two by a single, quasi-diagnostic amino-acid change (V to I) at the amino-acid position determined by the nucleotide triplet starting at position 171 of the 620-bp *COI* gene fragment sequence (Supplementary material, Table S1).

Himantura tutul sp. nov. can be separated from all three other species of the *H. uarnak* species complex by the nucleotide synapormorphies highlighted in Supplementary material, Table S1. All three other species also possess a number of nucleotide synapomorphies that allow their separation from one another (Supplementary material, Table S1).

5.6. Geographic distributions

Based on the material genetically identified to species thus far [1], the distribution of *Himantura tutul* sp. nov. comprises the coast of Tanzania in the western Indian Ocean (Pemba Island being the type locality of the new species), the Laccadive Sea, and part of the Indo-Malay archipelago including the Sunda Strait area, the southern coast of Java Island, the Bali Sea, the eastern South China Sea (Sabah), and the Sulu Sea (Sabah). The precise geographic origin of the *Himantura tutul* sp. nov. individuals sampled from the Batang fish landing place is unknown. However, the approximate area fished from Batang has ca. 1000 km radius, includes the whole Java Sea and reaches Natuna Island in the southernmost part of the South China Sea and Makassar Strait to the East [44]. A recent survey of the biodiversity of Myliobatoidei on Ningaloo Reef, northwestern Australia [22] failed to sample *Himantura tutul* sp. nov. there, suggesting the species may be absent from this region.

Now that *H. leoparda* has been redefined as a species distinct from *Himantura tutul* sp. nov. (present work), it is necessary to draw its distribution more precisely than previously done [14]. Based on the holotype and on the material genetically identified to species thus far [1], the distribution of *H. leoparda* under its present new definition comprises the coast of Kwazulu-Natal in the western Indian Ocean, and part of the Indo-Malay-Papua archipelago including the Sunda Strait area, the eastern South China Sea (Sabah), and the Gulf of Carpentaria which is also the type locality of the species [14]. The precise geographic origin of the *H. leoparda* individuals sampled from the Batang fish landing place is unknown (but see here above). *H. leoparda* is possibly absent from Ningaloo Reef [22].

Disclosure of interest

We have no conflicts of interest concerning this article.

Acknowledgements

We thank N. Hubert for sharing ideas on molecular taxonomy, and A. Yusmalinda and Rumini for advice on the use of the Malay word "*tutul*" as species name. We are grateful to A. Dettai and P. Pruvost (MNHN) and R. Hadiaty (MZB) for providing collection numbers to our material. Designed the study: P Borsa. Contributed reagents or materials or analysis tools: P Borsa, JDD, KNS, ISA, P Berrebi. Performed the experiments: KNS. Analyzed and interpreted the data: P Borsa, JDD, P Berrebi. Wrote the paper: P Borsa. A 6-month fellowship was allocated to ISA under the BEST program of Agence inter-organismes de recherche pour le développement (AIRD), France, to work with P Borsa and P Berrebi at ISE-M. Funded by AIRD, IRD UR227, 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.

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Table 1. *Himantura uarnak* species complex. Material examined for nucleotide variation at the *COI* locus. Nucleotide sequences and ancillary information were retrieved from [1,22] and from the BOLD [23] and GENBANK (http://www.ncbi.nlm.nih.gov/) databases. Tissue samples of Individuals ir001-ir037, ir039-ir067, and ir069-ir113 are currently preserved at LIPI-P2O laboratories in Jakarta. *Cluster* as defined in [1]

Species (Cluster), Individual no.	Sampling location	Sampling date	Voucher	GENBANK no.
H. leoparda (Cluster 4)				
BOLD TZMSC232-05 (misidentified as '*H. uarnak*')	Cape Vidal, Kwazulu-Natal, SW Indian O.	November 2004	ADC 30.10-1 (tissue voucher)	JF493652
BOLD TZMSC474-05 (misidentified as '*H. uarnak*')	Cape Vidal, Kwazulu-Natal, SW Indian O.	May 2005	Smith 30.10-2 (tissue voucher)	JF493651
ir003, ir004, ir008-023, ir025, ir027, ir028, ir030-036, ir039-042, ir046, ir048, ir050, ir051, ir053, ir054, ir057-067, ir069-072, ir074, ir075, ir077, ir078	Batang, Java Sea [d]	June 2006-January 2008	-	JX263361- JX263417
ir087	Sunda Strait	October 2010	-	JX263418
Himantura tutul sp. nov. (Cluster 1)				
zan6	Pemba Island, W Indian Ocean	May 2010	MNHN-ICTI5184	JX263335
ir001, ir002, ir005, ir006, ir024, ir026, ir029, ir037, ir043-045, ir047, ir049, ir052, ir055, ir056, ir073, ir076, ir079-085	Batang, Java Sea [d]	June 2006-January 2008	-	JX263306-JX263330
ir086, ir088	Sunda Strait	October 2010	-	JX263331, JX263332
ir112	Bali Sea	February 2010	-	JX263333
ir113	Southern coast of Java off Jogyakarta	October 2010	-	JX263334
Himantura uarnak (Cluster 3)				
wjc 637	Taichung, Taiwan, China Sea	January 2011	NMMBP 015601	JX263360
ir 089-099	Makassar Strait	November 2009	-	JX263337-JX263347
ir100-106	Bone Basin off Selayar Island, Banda Sea	November 2009	MZB 20875 (LIPI 4419)	JX263348-JX263354
ir107-109	Kendari, Banda Sea	October 2010	-	JX263355-JX263357
ir110, ir111	Labuan Bajo, Flores Sea	October 2010	-	JX263358-JX263359
16 individuals [22]	Ningaloo Reef, NW Australia	-	-	JQ765509, JQ765519-JQ765530 JQ815394, JQ815395, JQ929047
Himantura undulata (Cluster 2)				
BOLD AAF0692 (labelled '*H. fava*')	Sandakan, Sulu Sea	April 1996	BW-A221 (tissue voucher)	DQ108167
ir007	Batang, Java Sea [d]	June 2006-January 2008	-	JX263336
Himantura gerrardi (outgroup)				
wjc623, wjc624	Taichung, Taiwan, China Sea	June-July 2009-	-	JX263423, JX263424
BOLD TZMSC473-05.COI-5P	Cape Vidal, Kwazulu-Natal, SW Indian O.	March 2005	Smith 30.9-2 (tissue voucher)	JF493649
BOLD TZMSC230-05.COI-5P	Cape Vidal, Kwazulu-Natal, SW Indian O.	January 2005	ADC 30.9-1 (tissue voucher)	JF493650
INAPKKD-SIFT-3 (misidentified as '*H. uarnak*')	Kakinada, Bay of Bengal	-	-	EU541309
BOLD:FOA217-04	Sandakan, Sulu Sea	April 1996	BW-A217 (tissue voucher)	DQ108177
BOLD FOAD621-05	Muara, Indonesia	February 2005	BW-2181 (tissue voucher)	EU398841
BOLD FOAD619-05	Muara, Indonesia	February 2005	BW-2179 (tissue voucher)	EU398843
Himantura toshi (outgroup)				
BOLD FOAE256-06	Moreton Bay, Queensland	July-2005	BW-A2468 (tissue voucher)	EU398865
BOLD FOAD657-05	Hervey Bay, Queensland	June 2002	BW-2217 (tissue voucher)	EU398868
BOLD FOAD656-05	Hervey Bay, Queensland	June 2002	BW-2216 (tissue voucher)	EU398869

Table 2. *Himantura leoparda* and *Himantura tutul* sp. nov.. Matrix of individuals > 1000 mm disk width, characterized by the numbers of ocellated spots with, respectively, continuous and interrupted contours in each of three size-classes (*small*: ≤15 mm; *medium*; >15 mm and ≤25 mm; *large*: >25 mm). *Cluster* and *Mitotype* according to [1]. *L* left half-disk transect; *R* right half-disk transect; *DW* disk width. *1x3*, presumed F1 hybrid between Clusters 1 and 3 [1].

Species,	DW	Cluster	Mitotype	Continuous contour			Interrupted contour		
Individual no.	(mm)			Small	Medium	Large	Small	Medium	Large
Himantura tutul sp. nov.									
ir037 (L)	1180	1x3	I	1	3	-	2	9	-
ir044 (L)	1240	1	I	-	-	-	2	5	-
ir045 (R)	1280	1	I	-	-	-	7	4	-
ir047 (L)	1170	1	I	-	-	-	-	6	-
ir047 (R)	1170	1	I	-	-	-	-	6	-
ir049 (R)	1240	1	I	-	-	-	2	-	-
ir052 (L)	1360	1	I	-	-	-	-	3	-
ir076 (R)	1160	1	I	4	-	-	8	1	-
ir084 (L)	1200	1	I	3	-	-	1	-	-
zan6 (L)	1150	1	I	-	-	-	5	8	-
zan6 (R)	1150	1	I	-	-	-	-	8	-
Himantura leoparda									
ir030 (L)	1120	4	IV	6	6	-	4	3	-
ir030 (R)	1120	4	IV	10	2	1	-	2	2
ir031 (L)	1165	4	IV	-	4	2	1	7	3
ir031 (R)	1165	4	IV	-	8	-	-	7	1
ir032 (L)	1070	4	IV	2	-	-	1	12	3
ir034 (R)	1525	4	IV	-	1	-	-	9	8
ir036 (L)	1070	4	IV	-	5	2	1	4	4
ir036 (R)	1070	4	IV	-	3	1	-	5	5
ir048 (R)	1045	4	IV	-	1	-	-	8	1
ir050 (L)	1260	4	IV	-	9	2	-	4	1
ir050 (R)	1260	4	IV	-	6	6	-	1	-
ir051 (L)	1140	4	IV	-	9	-	1	5	2
ir059 (R)	1440	4	IV	-	-	-	-	6	7
ir061 (R)	1460	4	IV	-	-	5	-	2	9
ir062 (R)	1360	4	IV	-	2	7	-	-	3
ir062 (L)	1360	4	IV	-	3	9	-	2	3
ir063 (L)	1530	4	IV	-	1	-	-	6	5
ir064 (L)	1430	4	IV	-	-	-	-	4	4
ir065 (L)	1090	4	IV	-	3	1	-	1	4
ir065 (R)	1090	4	IV	-	1	3	-	7	3
ir069 (R)	1030	4	IV	6	-	-	6	2	-
ir070 (R)	1110	4	IV	1	3	-	4	6	-
ir071 (L)	1420	4	IV	1	4	4	-	5	3
ir077 (R)	1320	4	IV	-	-	4	1	3	7
H2903.01 (L)	1105	-	-	-	3	2	-	5	5
H2903.01 (R)	1105	-	-	-	5	1	2	7	-

CAPTIONS TO FIGURES

Fig. 1. *Himantura leoparda* and *Himantura tutul* sp. nov. Measurement of the diameter of ocellae along a transect spanning disk half-width, running from mid-scapular point to extremity of disk. The individual represented on this picture is *H. leoparda* ir030 (female, 1120 mm DW, landed in Batang, August 2007; I.S.A.)

Fig. 2. *Himantura leoparda* and *Himantura tutul* sp. nov. Characteristic spotting patterns on dorsal surface of adults, with varying degrees of interruption in the contour of ocellae. *Scale bar*: 5 cm. **A.** Most ocellae on this picture are closed polygons (from *H. leoparda*, individual no. ir062, female, 1360 mm DW, Batang, November 2007; I.S.A.). **B.** The contour of a percentage of the ocellae is interrupted (from *H. leoparda*, individual no. ir061, female, 1460 mm DW, Batang, November 2007; I.S.A.). **C.** All ocellae have interrrupted contour; the ocella shape is recognizable in some instances (from *Himantura tutul* sp. nov., individual no. ir055, male, 1130 mm DW, Batang, November 2007; I.S.A.). **D.** Ocella shape generally cannot be inferred (from *Himantura tutul* sp. nov., individual no. ir052, female, 1360 mm DW, Batang, November 2007; I.S.A.).

Fig. 3. *Himantura uarnak* species complex. Maximum-likelihood phylogeny of partial *COI* haplotypes including homologous sequences from *H. leoparda*, *H. uarnak* and *H. undulata* retrieved from GENBANK (http://www.ncbi.nlm.nih.gov/; Table 1) (sequences aligned over 620 bp; T92+G model, selected according to Bayesian information criterion; pairwise deletion; 1000 bootstrap resamplings; MEGA5 [26]). The tree was rooted by the homologous sequences in *H. gerrardi* and *H. toshi* (Table 1). Only bootstrap scores >80% are indicated.

Fig. 4. *Himantura leoparda* and *Himantura tutul* sp. nov. Correspondence analysis (CA: [29]) of the matrix of individuals characterized by the size-frequencies of ocellated spots along half-disk transect of the dorsal side (Table 2). CA was run using the FACTOMINER package [30] under R [31]; percentages for each axis are their inertias [29]. *Closed circles* (●): Cluster-4 individuals, now assigned to *H. leoparda*; *open squares* (□): Cluster-1 individuals, now assigned to *Himantura tutul* sp. nov.; *open circle* (○): Individual ir037, a presumed F1 hybrid between ♀ *Himantura tutul* sp. nov. and ♂ *H. uarnak* [1]; *shaded*: points representing the holotypes of *H. leoparda* (circles) and *Himantura tutul* sp. nov. (squares). *Dotted lines* delineate groups of individuals grouped by hierarchical clustering analysis [30].

Fig. 5. *Himantura tutul* sp. nov. Photograph of Individual zan6 (MNHN-ICTI5184; from Mkoani, Pemba Island, Tanzania (05°21'S 39°37'E), 26 May 2010; J.-D.D.) chosen as holotype. *Scale bar*: 10 cm

Figure 1

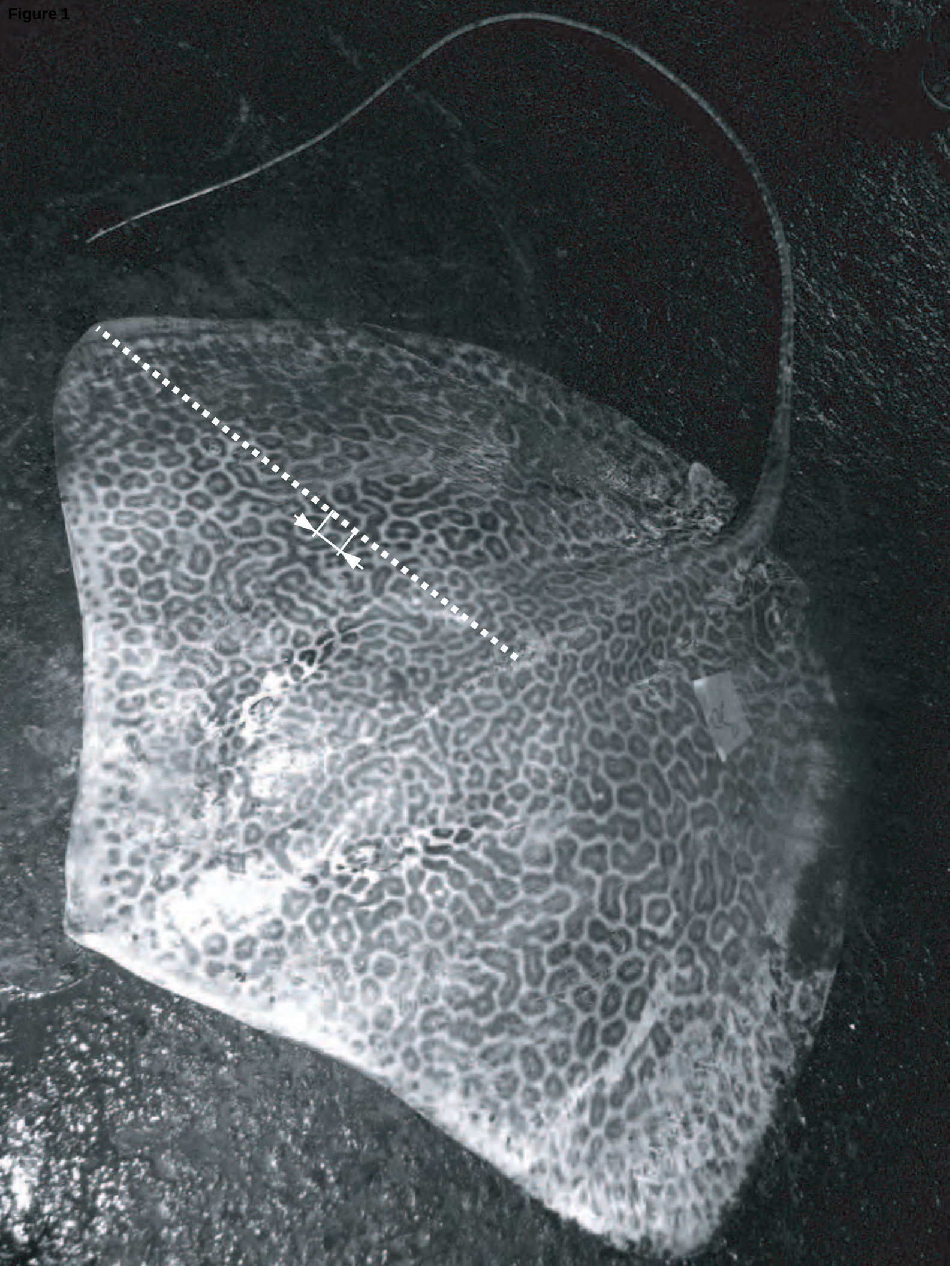


Figure 2

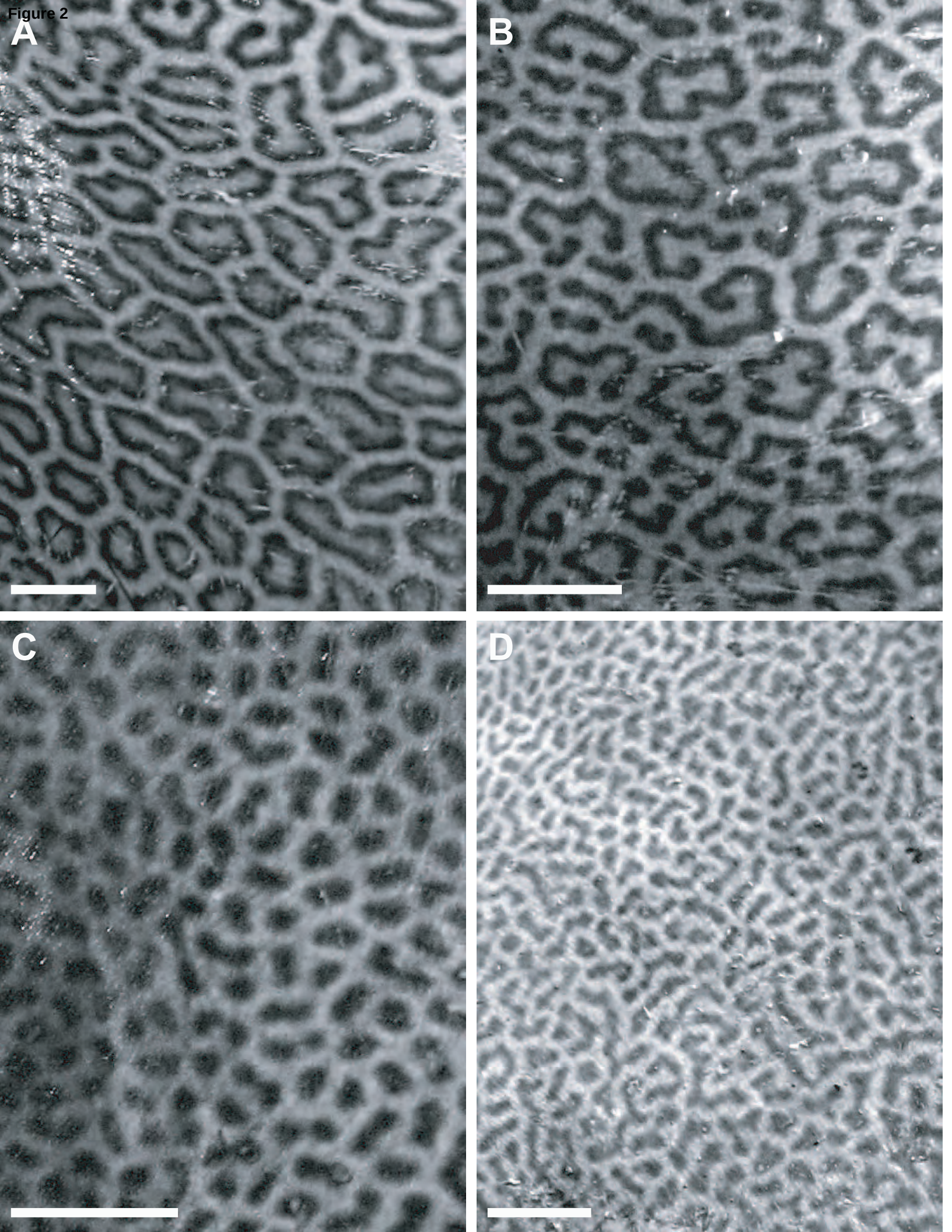
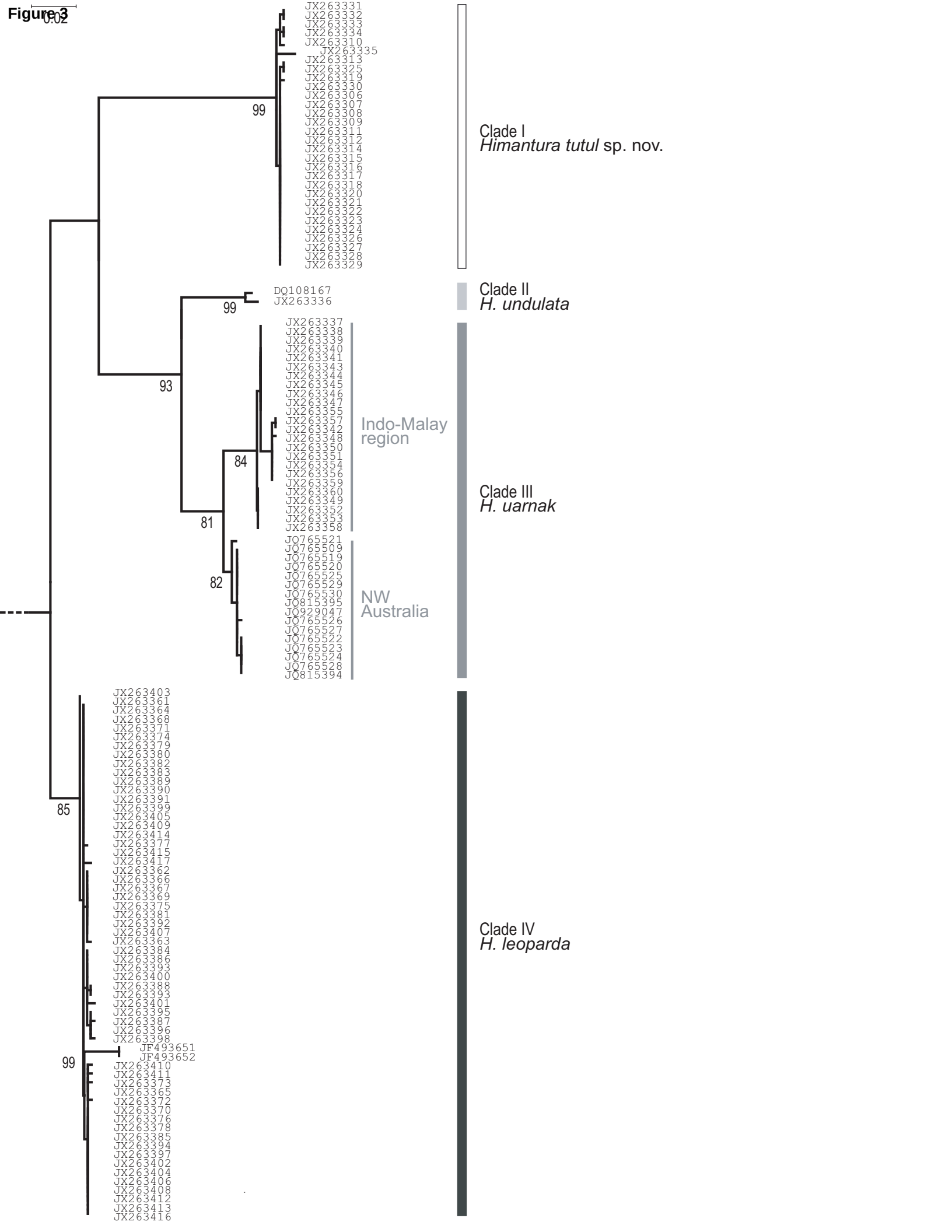


Figure 3


0.02
JX263331
JX263332
JX263333
JX263334
JX263310
JX263335
JX263313
JX263325
JX263319
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JX263306
JX263307
JX263308
JX263309
JX263311
JX263312
JX263314
JX263315
JX263316
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JX263320
JX263321
JX263322
JX263323
JX263324
JX263326
JX263327
JX263328
JX263329
99
Clade I
Himantura tutul sp. nov.
DQ108167
JX263336
99
Clade II
H. undulata
JX263337
JX263338
JX263339
JX263340
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JX263344
JX263345
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JX263355
JX263357
JX263342
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JX263350
JX263351
JX263354
JX263356
JX263359
JX263360
JX263349
JX263352
JX263353
JX263358
Indo-Malay region
93
84
81
82
JQ765521
JQ765509
JQ765519
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JQ765525
JQ765529
JQ765530
JQ815395
JQ929047
JQ765526
JQ765527
JQ765522
JQ765523
JQ765524
JQ765528
JQ815394
NW Australia
Clade III
H. uarnak
JX263403
JX263361
JX263364
JX263368
JX263371
JX263374
JX263379
JX263380
JX263382
JX263383
JX263389
JX263390
JX263391
JX263399
JX263405
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JX263417
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JX263367
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JX263375
JX263381
JX263392
JX263407
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JX263397
JX263402
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99
Clade IV
H. leoparda

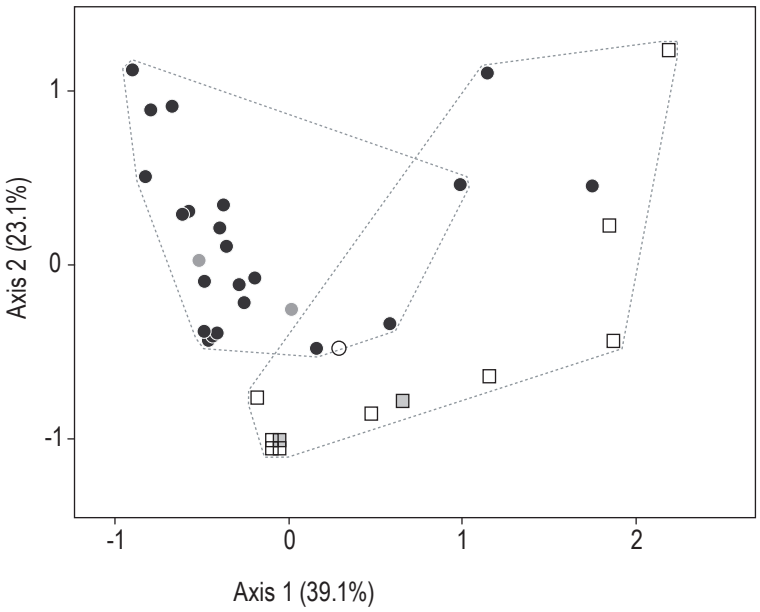

1
0
-1
Axis 2 (23.1%)
-1
0
1
2
Axis 1 (39.1%)

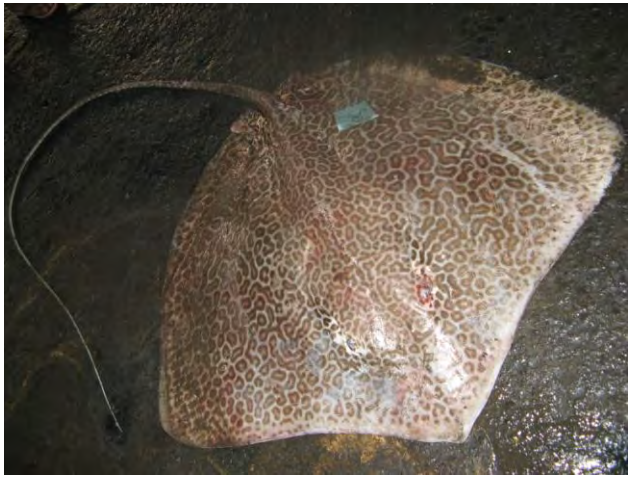
Figure 5

Table S1. *Himantura uarnak* species complex. Variable nucleotide sites along the 620-bp fragment of the *COI* gene. Nucleotide sites are numbered from 1 to 620, homologous to sites 2304-2923 of the mitochondrial genome of *Neotrygon kuhlii* (GENBANK JN184065; [9]). *Highlighted*: synapomorphies diagnosing *Himantura tutul* sp. nov.

| Clade (species), Individual no. | Nucleotide position | | |

Table S1 (continued)

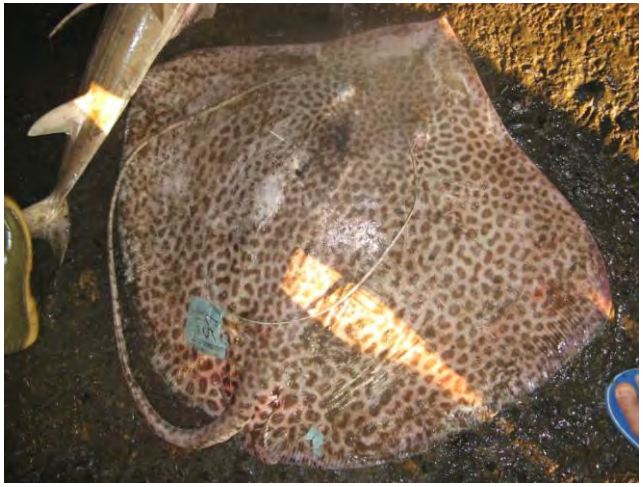
| Clade (species), Individual no. | Nucleotide position | | |



IR 030 (female, 1120 mm DW, cluster 4)

IR 031 (male, 1165 mm DW, cluster 4)

IR 032 (male, 1070 mm DW, cluster 4)



IR 033 (female, 880 mm DW, cluster 4)

IR 034 (female, 1525 mm DW, cluster 4)

IR 035 (female, 1240 mm DW, cluster 4)

Fig. S1. *Himantura uarnak* species complex. Pictures of specimens analyzed in the present study, with details on sex, disk width (DW), and multiple-locus nuclear genotype [Cluster 1, Cluster 3, or Cluster 4 according to Arlyza et al. (2012)]. Sampling locations (fish landing sites) and dates were: Batang, Java Sea, August 2007 (IR 030 – IR 042; IR 044-IR 049); Batang, November 2007 (IR 050 – IR 062); Batang, January 2008 (IR 063 – IR 085); Selayar, Banda Sea, November 2009 (IR 102); Singaraja, Bali Sea, 03 February 2010 (IR 112); Pemba Island, Zanzibar, 26 May 2010 (Zanz 6); Taiwan, East China Sea, 2010 (wjc 637); north-west of Weipa, Gulf of Carpentaria, November 1991 (CSIRO H2903.01).,

IR 036 (male, 1070 mm DW, cluster 4)

IR 037 (female, 1180 mm DW, presumed F1cluster 1 x cluster 3)

IR 038 (female, 660 mm DW, cluster 4)

IR 039 (female, 1430 mm DW, cluster 4)

IR 040 (female, 670 mm DW, cluster 4)

IR 041 (female, 620 mm DW, cluster 4)

IR 042 (female, 680 mm DW, cluster 4)

IR 044 (male, 1240 mm DW, cluster 1)

IR 045 (female, 1280 mm DW, cluster 1)

Fig. S1. (continued)

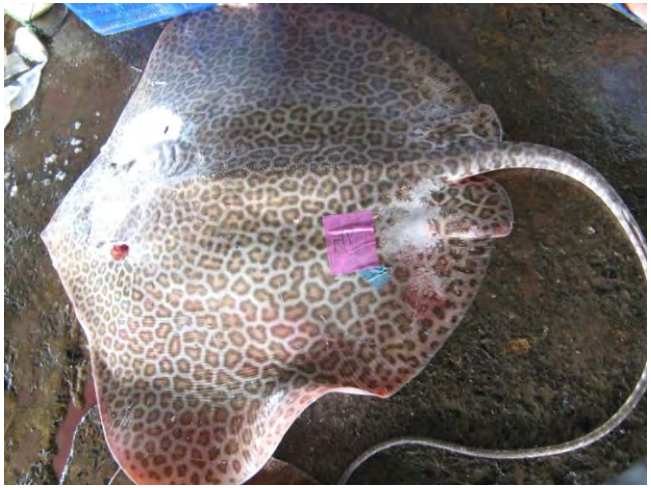
IR46 (female, 1260 mm DW, cluster 4)

IR 047 (male, 1170 mm DW, cluster 1)

IR 048 (male, 1045 mm DW, cluster 4)

IR 049 (male, 1240 mm DW, cluster 1)

IR 050 (female, 1260 mm DW, cluster 4)


IR 051 (female, 1140 mm DW, cluster 4)

IR 052 (female, 1360 mm DW, cluster 1)

IR 053 (female, 1400 mm DW, cluster 4)

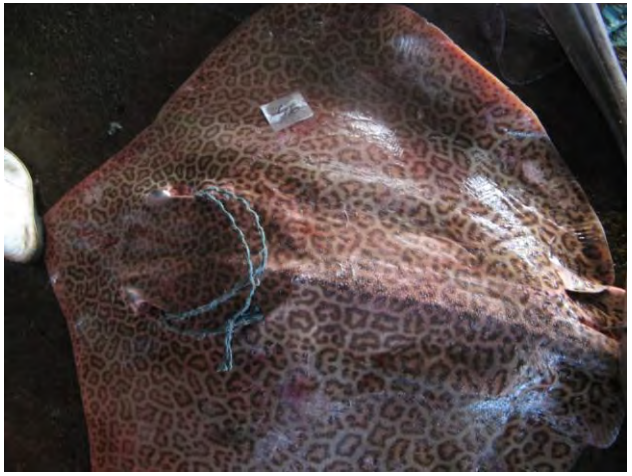
IR 054 (female, 1330 mm DW, cluster 4)

Fig. S1. (continued)

IR 055 (male, 1130 mm DW, cluster 1)

IR 056 (female, 1070 mm DW, cluster 1)

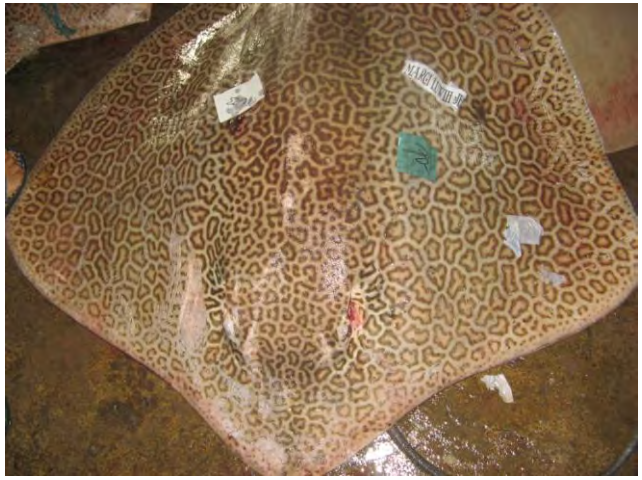
IR 057 (female, 1360 mm DW, cluster 4)


IR 058 (female, 1310 mm DW, cluster 4)

IR 059 (female, 1440 mm DW, cluster 4)

IR 060 (female, 1410 mm DW, cluster 4)

IR 061 (female, 1460 mm DW, cluster 4)


IR 062 (female, 1360 mm DW, cluster 4)

IR 063 (female, 1530 mm DW, cluster 4)

Fig. S1. (continued)

IR 064 (female, 1430 mm DW, cluster 4)

IR 065 (male, 1090 mm DW, cluster 4)

IR 066 (female, 940 mm DW, cluster 4)

IR 067 (male, 1120 mm DW, cluster 4)

IR 068 (female, 1400 mm DW, cluster 4)

IR 069 (female, 1030 mm DW, cluster 4)

IR 070 (male, 1110 mm DW, cluster 4)

IR 071 (female, 1420 mm DW, cluster 4)

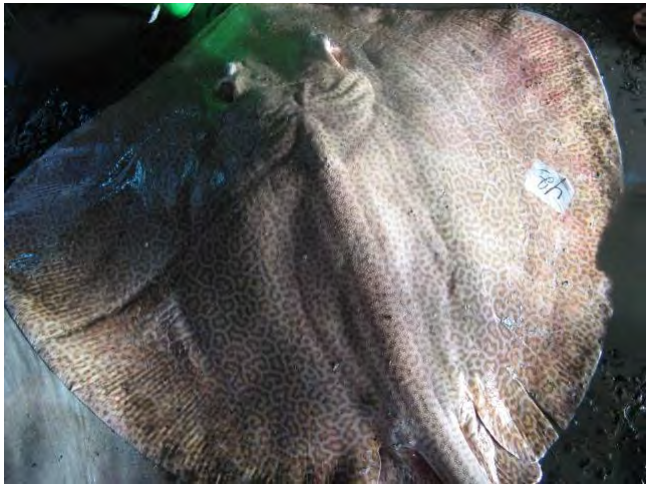
IR 072 (female, 1260 mm DW, cluster 4)

Fig. S1. (continued)

IR 073 (male, 1160 mm DW, cluster 1)

IR 074 (female, 1220 mm DW, cluster 4)

IR 075 (female, 1300 mm DW, cluster 4)


IR 076 (male, 1160 mm DW, cluster 1)

IR 077 (female, 1320 mm DW, cluster 4)

IR 078 (male, 490 mm DW, cluster 4)

IR 079 (female, 1260 mm DW, cluster 1)

IR 080 (male, 1150 mm DW, cluster 1)

IR 081 (female, 1300 mm DW, cluster 1)

Fig. S1. (continued)

IR 082 (female, 1210 mm DW, cluster 1)

IR 083 (female, 1020 mm DW, cluster 1)

IR 084 (female, 1200 mm DW, cluster 1)

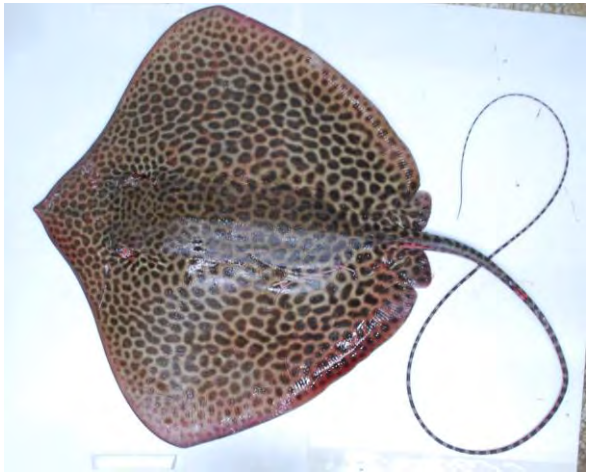
IR 085 (male, 1150 mm DW, cluster 1)

IR102=LIPI 4419 (male, 360 mm DW, cluster 3)

IR 112 (280 mm DW, cluster 1)

Zanz 6 (female, ~1150 mm DW, cluster 1)

CSIRO H2903.01 (female, 1105 mm DW)



wjc637= NMMBP 015601 (cluster 3)

Fig. S1. (continued)

Zanz 6, ventral side

Fig. S1. (continued)