Species boundaries in the Himantura uarnak species complex (Myliobatiformes: Dasyatidae)

Irma Arlyza, Kang-Ning Shen, Dedy Duryadi Solihin, Dedi Soedharma, Patrick Berrebi, Philippe Borsa

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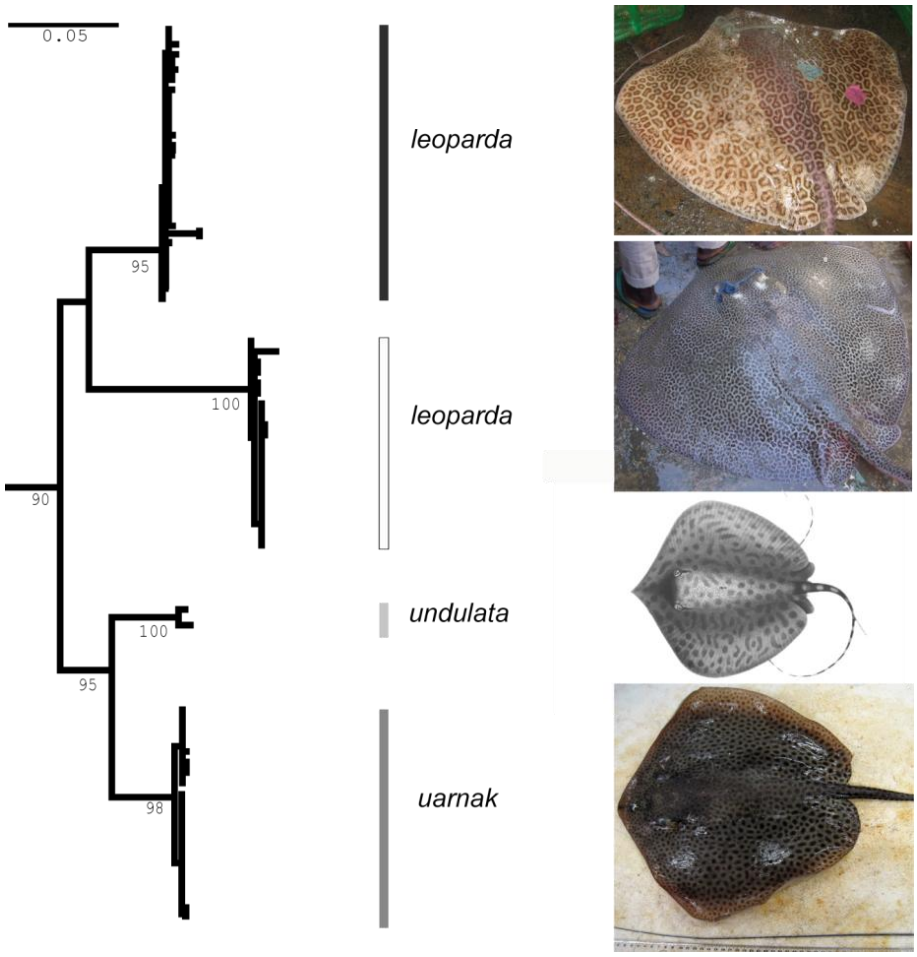
Species boundaries in the *Himantura uarnak* species complex (Myliobatiformes: Dasyatidae)

I.S. Arlyza [a,b], K.-N. Shen [c], D.D. Solihin [b], D. Soedharma [b], P. Berrebi [d], P. Borsa [e*]

[a] Lembaga Ilmu Pengetahuan Indonesia (LIPI), Pusat Penelitian Oseanografi (P2O), Jakarta, Indonesia
[b] Institut Pertanian Bogor, Bogor, Indonesia
[c] Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan
[d] Institut des sciences de l'évolution à Montpellier (ISE-M), UMR 5554 UM2-CNRS-IRD, Université Montpellier 2, Montpellier, France
[e] Institut de recherche pour le développement (IRD), UR 227 "Biocomplexité des écosystèmes récifaux", Montpellier, France

* Corresponding author, present address: Indonesian Biodiversity Research Center, Universitas Udayana, Jl Sesetan Gang Markisa 6, Denpasar, Indonesia. E-mail: philippe.borsa@ird.fr (P. Borsa)



Highlights

► Stingrays of the *Himantura uarnak* species complex were sampled throughout the Indo-West Pacific.
► Four reproductively isolated entities were detected by nuclear markers.
► Cryptic species were detected within the recently-described *H. leoparda*
► Four mitochondrial clades were uncovered, each specific to a species

Abstract

Samples of the '*Himantura uarnak*' species complex (*H. leoparda*, *H. uarnak*, *H. undulata* under their current definitions), mostly from the Coral Triangle, were analyzed using nuclear markers and mitochondrial DNA sequences. Genotypes at five intron loci showed four reproductively isolated clusters of individuals. The *COI* sequences showed four major mitochondrial lineages, each diagnostic of a cluster as defined by nuclear markers. No mitochondrial introgression was detected. The average Kimura-2 parameter nucleotide distance separating clades was 0.061-0.120 (net: 0.055-0.114), while the distance separating individuals within a clade was 0.002-0.008. Additional, partial cytochrome-*b* gene sequences were used to link these samples with previously published sequences of reference specimens of the three nominal species. One of the clusters was identified as *H. undulata* and another one, as *H. uarnak*, while two cryptic species were uncovered within the recently-described *H. leoparda*, challenging the current morphology-based taxonomy of species within the *H. uarnak* species complex.

Keywords: intron-size polymorphism, cytochrome oxidase I, cytochrome *b*, phylogeny, cryptic species, taxonomy, DNA barcoding

1. Introduction

Taxonomic accuracy is fundamental for correctly identifying species, hence for assessing their habitats and distributions, which in turn is important to conservation and fisheries management. Within the last two decades, much concern has been expressed regarding the conservation status of elasmobranchs (Stevens et al., 2000; Garcia et al., 2008; Lam and Sadovy de Mitcheson, 2011; White and Kyne, 2010). Elasmobranchs generally mature slowly and have low fecundity (Stevens et al., 2000). Shallow-water, bottom-dwelling elasmobranch species are particularly at risk of extinction because of their exposure to overfishing. This includes stingrays (Myliobatiformes: Dasyatidae), which are subject to intensive and unregulated exploitation in Southeast Asia (Blaber et al., 2009; White and Kyne, 2010) and elsewhere. As a consequence, several stingray species from the Australasian region are now listed as vulnerable (White and Kyne, 2010).

Molecular surveys conducted for the fish-Barcode of Life project have helped flagging cryptic species in stingrays (Last and White, 2008), underlining a still-incomplete taxonomy of species in the family. About one third of the species in the family Dasyatidae belong to the genus *Himantura* (25 species in this genus, some of them still undescribed; Manjaji, 2004; Last et al. 2008). *Himantura* species are widely distributed in the marine, brackish, and freshwater habitats of the tropical and subtropical Atlantic, Indian and Pacific Oceans (Manjaji, 2004). Here, we focus on stingrays of the '*Himantura uarnak*' species complex, which have received recent attention from morphological taxonomists. Species of the *H. uarnak* species complex have often been confused with one another, as all have a "similar overall disc shape and a dorsal disc pattern consisting of large spots in juveniles that form a complex pattern of spots, reticulations and/or ocelli in adults" (Manjaji-Matsumoto and Last, 2008). Four species are currently recognized in this group (Eschmeyer, 2012): *H. fava* (Annandale, 1909), *H. leoparda* (Manjaji-Matsumoto and Last, 2008), *H. uarnak* (Forsskål, 1775) and *H. undulata* (Bleeker, 1852). However, Manjaji-Matsumoto and Last (2008) consider *H. fava* as a junior synonym of *H. undulata*.

Taxonomic confusion may arise when morphological characters used to define species overlap. This may occur, in particular, between pairs of species that hybridize (e.g. Borsa and Quignard, 2001). Because fertilization in elasmobranchs is internal, hybridization is likely to be mostly absent in this group (Ward et

al., 2008; Dudgeon et al., 2012). The recent report of hybridization in a whaler shark species (Morgan et al., 2012) might be an exception.

The objectives of the present work were: (1) to investigate the phylogenetic species boundaries in the *H. uarnak* species complex, and clarify the systematics of this group using both mitochondrial and nuclear markers; (2) to assess the reliability of molecular markers for identification purposes in this group.

2. Materials and methods

Tissue samples of 115 individuals of the *Himantura uarnak* species complex were collected from Zanzibar, from Taiwan, and from 8 fish landing sites throughout Indonesia (Table 1). The tissue samples consisted of 0.01-0.5 cm3 of skin or flesh or tail extremity, dissected with surgical scissors and preserved in 90%-96% ethanol. DNA was extracted using the DNEasy® Tissue Kit (Qiagen GmbH, Hilden, Germany). The DNA extracts were stored at -30°C until required for laboratory analyses.

The individuals sampled were genotyped at a total of 5 size-polymorphic intron loci , which were scored using the exon primer pairs (*Act-2-F*, *Act-2-R*) and (*Cam-3-F*, *Cam-3-R*) (Atarhouch et al., 2003) following the approach and protocols detailed in Borsa et al. (in press). Annealing temperatures were 54°C for the amplification of the two *Act-2* introns and 55°C for that of the three *Cam-3* introns.

A 620-bp fragment of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified by polymerase chain reaction (PCR) using the *FishF1* and *FishR1* primers of Ward et al. (2005). PCR amplifications were performed in 50 µL reaction mixture containing 25µL 2X ReadyMix KapaTaq DNA polymerase mix (0.05 U/µL, 3 mM Mg^{2+}, 0.4mM each dNTP) (Kapa Biosystems, Boston, U.S.A.), 1µL 0.2µM of each primer (1st BASE, Singapore), 2µL DNA template and 21µL water (Qiagen). In addition, a 420-bp fragment of the mitochondrial cytochrome *b* (*cytb*) gene was similarly PCR-amplified using primers *GLU1L* and *CB2H* of Sezaki et al. (1999). The PCR mix was the same as for *COI*, except that primer concentration was quadrupled. Cycling parameters were an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation (95°C for 30 s), annealing (53°C for 30 s), and extension (72°C for 1 min) with the final extension step at 72°C for 2 min in an Applied Biosystems (Foster City, CA, U.S.A.) Veriti™ thermocycler. PCR-amplified DNAs were visualized on 1% agarose gels. Prior to sequencing, excess dNTPs and oligonucleotides were eliminated from the PCR product using shrimp alkaline phosphatase and exonuclease I (Exo-SAP-IT kit; Affymetrix, Santa Clara CA, U.S.A.) following the manufacturer's protocol. Sequence reactions were performed in both directions using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems), 8-10 µL purified PCR product, and 4-5 µL of either primer (3 µM) per reaction. Sequence-reaction products were loaded into an ABI 3130xl automated sequencer (Applied Biosystems) at the Hawaii Institute of Marine Biology EPSCoR sequencing facility. Forward and reverse sequences were proofread, aligned and edited using MEGA 5 (Tamura et al., 2011) and BIOEDIT (Hall, 1999). Edited sequences were deposited in GENBANK (http://www.ncbi.nlm.nih.gov/).

Correspondence analysis (Benzécri, 1982) implemented in GENETIX 4.02 (Belkhir et al., 2000) was performed to visualize clusters of individuals characterized by their genotypes at 5 polymorphic intron loci, by reducing the multidimensional allelic frequency space to a bidimensional space. Hardy-Weinberg equilibrium and two-locus linkage equilibrium were tested using random permutations under GENETIX. Bayesian structure analysis (STRUCTURE 2.3.1; Falush et al., 2007) was used to delineate clusters of individuals by determining the number of populations present in the total sample of the *Himantura uarnak* species complex and assigning individuals to these populations using the model-based program. The Markov-Chain Monte Carlo algorithm was run under the admixture model with correlated allele frequencies for 100,000 steps after 50,000 initial burn-in steps, without any a priori information on an

individual's sampling location, to estimate individual admixture proportions and assign individuals to populations. Five independent runs were performed (to check the stability of the assignation among runs) for each number of populations (*K*, ranging from 3 to 5) to determine the number of populations a posteriori by visually comparing the output diagrams.

The *COI* sequence dataset comprised 114 ingroup sequences and 27 outgroup sequences aligned over 620 bp. All new sequences were deposited in GENBANK under accession nos. JX263306-JX263424. The best substitution model according to the Bayesian information criterion (MEGA5) was HKY+G+I and this model was used to produce a maximum-likelihood (ML) phylogeny of the *COI* sequences. The *cytb* sequence dataset comprised 130 ingroup sequences, including 14 sequences (239 bp) from Manjaji (2004) here used as references, new sequences deposited in GENBANK (JX274304- JX274334), and one outgroup sequence (*H. signifier*; Manjaji, 2004), aligned over 239 bp. Twelve specimens of the *Himantura uarnak* species complex that were analyzed here belong to museum collections (Table 1). For ML analysis of the *cytb* dataset, the best substitution model according to the Bayesian information criterion (MEGA5) was K2+G. In both phylogenetic treatments, nodal support was assessed by bootstrapping (Felsenstein, 1985), based on 1000 pseudo-replicates.

3. Results

Four main clusters of individuals ('Clusters' *1-4*: Fig. 1) were detected from correspondence analysis of multiple-locus nuclear genotypes (matrix of genotypes presented as Supplementary material, Table S1). Three of them (*1*, *3*, *4*) were also detected from Bayesian structure analysis.

Four outliers were observed, including three individuals (ir024, ir037 and ir083) positioned between Cluster *1* and Cluster *3* and one individual (ir053) positioned between Cluster *1* and Cluster *4* (Fig. 1). Their proportion in the total sample was 4/109, using as denominator of this ratio the number of individuals genotyped at all 5 nuclear loci (Supplementary material, Table S1). Individuals ir024, ir037, and ir083 were doubly heterozygous for alleles quasi-diagnostic between Cluster *1* and Cluster *3* at the two loci (*Cam-3 A* and *Cam-3 B*) that differentiate these two clusters, whereas individual ir053 was doubly heterozygous for alleles quasi-diagnostic between Cluster *1* and Cluster *4* at 2/4 loci that differentiate the two latter clusters (Supplementary material, Table S1). Let us include into Cluster *1* those three individuals that possess Clade-*I* mitochondria and which were classed as intermediate between Cluster *1* and Cluster *3* by correspondence analysis (Fig. 1). The frequencies of alleles *Cam-3 A*^{175} and *Cam-3 B*^{165} in Cluster *1* as defined here were, respectively, 0.089 and 0.107. Under the hypothesis of panmixia and linkage equilibrium, the expected frequencies of the heterozygotes would be, respectively, 0.163 and 0.191, and the expected frequency of the double-heterozygotes would be 0.031. Here, the actual frequencies of the heterozygotes were, respectively, 5/28=0.179 and 6/28=0.214 (not significantly above the expectations from panmixia). However, the frequency of double-heterozygotes was 3/28=0.107 which is three times more than the expected frequency. The linkage disequilibrium between the two loci was D=0.032 and its associated probability (Black and Krafsur, 1985) was P=0.079. So, the hypothesis of non random association of alleles at the two loci was somewhat supported by the data, although the null hypothesis of linkage equilibrium could not be formally rejected either.

If this linkage disequilibrium is real, a sensible explanation for it is hybridization between the two species, where *Cam-3 A*^{175} – *Cam-3 B*^{165} di-locus haplotypes are introduced from Cluster *3* into Cluster *1*, which is characterized by *Cam-3 A*^{178} – *Cam-3 B*^{163} haplotypes. We assume that the effective population size of *Himantura* Cluster *1* is large enough to neglect the eventuality that linkage disequilibrium be caused by genetic drift and we discard the eventuality that linkage disequilibrium between *Cam-3 A* and *Cam-3 B*

arose by mutation at either one of the two loci. Another possibility for creating linkage disequilibrium is selection. The selection hypothesis would suppose some epistasy between the two intron loci (this is unlikely) or between loci closely linked to them, but then, one has to provide an explanation for the linkage disequilibrium between the two intron loci and the two putatively selected loci. Therefore, hybridization remains the most likely explanation and in this case, the three Clade-*I* outliers might be F1 hybrids. Similar reasoning leads to suspecting Individual ir053 (the single Clade-*IV* outlier) of possibly being a Cluster *4* x Cluster *1* backcross offspring. An alternative explanation would be shared polymorphism between the species, and the putative excess of double-heterozygotes in Cluster *1* would be caused by residual physical linkage between the two loci examined. This would imply that recombination between the two loci within a species would be slower than allele sorting between the two species, which is hard to conceive.

The ML phylogeny of mitochondrial lineages, based on partial *COI* gene sequences (Fig. 2) supported the monophyly of the *H. uarnak* species complex. The ingroup haplotypes were clustered into four main clades (*I-IV*) (Fig. 2). Clades were separated by an estimated 0.061-0.120 (net: 0.055-0.114) nucleotide distance (Kimura-2 parameter distance; MEGA5). Intra-clade nucleotide diversity was comparatively low (0.002-0.008). Clusters *1-4* were characterized by mitochondrial Clades *I-IV*, respectively (Fig. 1), with no mismatch observed. Albeit less resolved, the phylogeny based on *cytb* sequences (Fig. 3) provided the taxonomic information necessary to identify to species each of the previously defined Clusters (*1-4*), from the placement of reference specimens (Table 1). Clade *II* included sequences of reference *H. undulata* specimens and Clade *III* included those of *H. uarnak* (Fig. 3). Both Clades *I* and *IV* clustered with sequences of reference *H. leoparda* specimens. This included, respectively, specimens CSIRO H5284.05 and CSIRO H5478.01, both chosen as paratypes of *H. leoparda* by Manjaji-Matsumoto and Last (2008).

Thus, both Cluster *1* and Cluster *4* were identified as *H. leoparda* under its current definition, Cluster *2* was unambiguously identified as *H. undulata* and Cluster *3* was identified as *H. uarnak*. The outliers on Fig. 1 possessed the mitochondrial haplotype of the cluster geometrically closest to them.

4. Discussion

The present results challenge the current perception of species boundaries in the *Himantura uarnak* species complex, which are based on morphology (Manjaji-Matsumoto and Last, 2008).

Four distinct clusters of individuals defined by their multiple-locus genotypes were observed within the *H. uarnak* species complex. Each cluster was further characterized by a distinct mitochondrial clade, and no mitochondrial introgression was observed between clusters. Although some putative hybridization between clusters was inferred from the observation of heterozygous individuals interpreted as possible hybrids and/or backcross offspring, their low proportion (<4%) demonstrates that hybridization, if it actually occurs, is likely to occur only rarely. Thus, genetic homogeneity across large geographic distances (Table 1) is maintained within each cluster despite the eventuality of inter-cluster crosses. These observations altogether demonstrate the genetic isolation or quasi-isolation of the four clusters from one another, hence the occurrence of four biological species within the *H. uarnak* species complex. One of these species was *H. uarnak*, another one was *H. undulata*, while two cryptic species were uncovered within *H. leoparda* under its current definition. Considering that the two latter are sympatric over a broad geographic area, the low incidence of possible hybrids and back-crossed individuals, if confirmed, would not call into question their full qualification as biological species (Mayr, 1942).

The specimen identified as *H. fava* (Table 1), represented by a *COI* sequence deposited in the Barcoding of Life Database (http://bins.boldsystems.org) was here identified as *H. undulata*, supporting Manjaji (2004) who had since then synonymised the two species. Manjaji-Matsumoto and Last (2008) chose as paratypes of *H. leoparda* two individuals whose mitochondrial haplotypes indicate that they belong to different species. As no genetic data are currently available to determine the mitochondrial haplogroup of the holotype of *H. leoparda* (CSIRO H2903.01), it is not possible from the present results to determine which of Clusters *1* or *4* is the actual *H. leoparda*. Manjaji (2004) mentioned that two forms co-occurred within her *Himantura* sp. A (now *H. leoparda*) samples based on spotting patterns: a typical leopard-like spotted form and an atypical form with finer spots. Further research is warranted to determine whether differences in spotting patterns coincide with the distinction of the two cryptic species.

Given the continued confusion in the morphology-based taxonomy of species within the *H. uarnak* species complex, which contrasts with the sharp distinction provided by the partial *COI* sequences (present work), we advocate the use of the universal *COI* marker for species identification (Ward et al., 2008; 2009) in this group. Moreover, notwithstanding eventual rare cases of mitochondrial introgression, which yet would remain to be documented, it is sensible to propose that the *COI* marker be used as diagnostic character in a future taxonomic revision of the *H. uarnak* species complex.

If at all occurring, the rarity of individuals with mixed-species ancestry in the *H. uarnak* species complex supports the general view that hybridization is a rare phenomenon in elasmobranchs (Ward et al., 2008; Dudgeon et al., 2012). Since the species in the *H. uarnak* species complex have overlapping geographic ranges (Table 1) and share habitats, one suspects isolating mechanisms to operate. We speculate that interspecific differences in male clasper distal length and shape (Manjaji, 2004) and possible matching differences in the morphology of the female urogenital sinus may contribute to preventing successful mating between species in the *H. uarnak* species complex.

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Table 1 List of samples of the *Himantura uarnak* species complex used in the present study, with sampling location, voucher specimen numbers and GENBANK accession numbers. Specimens were sorted to species (using current nomenclature: Manjaji-Matsumoto and Last, 2008) following the results of the present study. *BOLD*: Barcoding of Life Database (Ratnasingham and Hebert, 2007); *CSIRO*: Commonwealth Scientific and Industrial Research Organisation, Hobart; *MZB*: Museum Zoologicum Bogoriense; *UMS*: Universiti Malaysia Sabah, Kota Kinabalu.

Species, Individual no.	Sampling location	Sampling date	Voucher	GENBANK *COI*	GENBANK *cyth*
Himantura leoparda [a]					
A1, A4 (Manjaji, 2004)	Kota Kinabalu, South China Sea	1999	-	-	-
A2 (Manjaji, 2004)	Sandakan, Sulu Sea	1999	UMS MMSK [c4]	-	-
A5 (Manjaji, 2004)	Sandakan, Sulu Sea	1999	-	-	-
A6 (Manjaji, 2004)	Kota Kinabalu, South China Sea	1999	CSIRO H5284.05 (paratype)	-	-
A9 (Manjaji, 2004)	Cochin, Laccadive Sea	1999	UMS MMPL11	-	-
zan6	Zanzibar, W Indian Ocean	May 2010	-	JX263335	JX274333
ir001, ir002, ir005, ir006, ir024, ir026, ir029, ir037, ir043, ir044, ir045, ir047, ir049, ir052, ir055, ir056, ir073, ir076, ir079-085	Batang, Java Sea [d]	Jun. 2006-Jan. 2008	-	JX263306-JX263330	JX274304-JX274328
ir086, ir088	Sunda Strait	Oct. 2010	-	JX263331, JX263332	JX274329, JX274330
ir112	Bali Sea	Feb. 2010	-	JX263333	JX274331
ir113	Southern coast of Java off Jogyakarta	Oct. 2010	-	JX263334	JX274332
Himantura leoparda [b]					
A7 (Manjaji, 2004)	Kota Kinabalu, South China Sea	1999	CSIRO H5478.01 (paratype)	-	-
BOLD TZMSC232-05 'H. uarnak'	Cape Vidal, Kwazulu-Natal, SW Indian O.	Nov. 2004	ADC 30.10-1 (tissue voucher)	JF493652	-
BOLD TZMSC474-05 'H. uarnak'	Cape Vidal, Kwazulu-Natal, SW Indian O.	May 2005	Smith 30.10-2	JF493651	-
ir003, ir004, ir008-023, ir025, ir027, ir028, ir030-036, ir038-042, ir046, ir048, ir050, ir051, ir053, ir054, ir057-072, ir074, ir075, ir077, ir078	Batang, Java Sea [d]	Jun. 2006-Jan. 2008	-	JX263361- JX263417	JX274358-JX274416
ir087	Sunda Strait	Oct. 2010	-	JX263418	JX274417
Himantura uarnak					
H. uarnak 1 (Manjaji, 2004)	Kota Kinabalu, South China Sea	1999	CSIRO H5476.03	-	-
H. uarnak 2 (Manjaji, 2004)	Kota Kinabalu, South China Sea	1999	CSIRO H5477.01	-	-
H. uarnak 3 (Manjaji, 2004)	Semporna, Celebes Sea	1999	CSIRO H5484.01	-	-
H. uarnak 6 (Manjaji, 2004)	West Papua	1999	EM330 (tissue voucher)	-	-
H. uarnak 8 (Manjaji, 2004)	West Papua	1999	MMPK7	-	-
wjc 637	Taiwan, China Sea	2010	NMMBP 015601	JX263360	JX274357
ir 089-099	Makassar Strait	Nov. 2009	-	JX263337-JX263347	JX274335-JX274345
ir100-106	Bone Basin off Selayar Island, Banda Sea	Nov. 2009	MZB 20875	JX263348-JX263354	JX274346-JX274352
ir107-109	Kendari, Banda Sea	Oct. 2010	-	JX263355-JX263357	JX274353-JX274355
ir110, ir111	Labuan Bajo, Flores Sea	Oct. 2010	-	JX263358-JX263359	JX274355-JX274356
Himantura undulata					
H. undulata 2 (Manjaji, 2004)	Sipitang, South China Sea	1999	CSIRO H5483.01	-	-
H. undulata 3 (Manjaji, 2004)	Sandakan, Sulu Sea	1999	CSIRO H5481.01	-	-
BOLD AAF0692 'H. fava' [c]	Sandakan, Sulu Sea	April 1996	BW-A221 (tissue voucher)	DQ108167	-
ir007	Batang, Java Sea [d]	Jun. 2006-Jan. 2008	-	JX263336	JX274334

[a] one of two cryptic species under *H. leoparda*, characterized by Cluster-*1* genotypes at 5 nuclear loci and by Clade-*I* mitochondria

[b] one of two cryptic species under *H. leoparda*, characterized by Cluster-*4* genotypes at 5 nuclear loci and by Clade-*IV* mitochondria

[c] original identification as *H. fava* by G. Yearsley and M.B. Manjaji; *H. fava* was then synonymised with *H. undulata* by Manjaji (2004)

[d] The approximate area fished from Batang on the southern shore of the Java Sea has ca. 1000 km radius and encompasses the Java Sea, the southernmost part of the South China Sea and the southwestern Makassar Strait; fishing vessels from Batang reach Natuna Island to the North-West and the Makassar Strait to the East (Adrim, 2008).

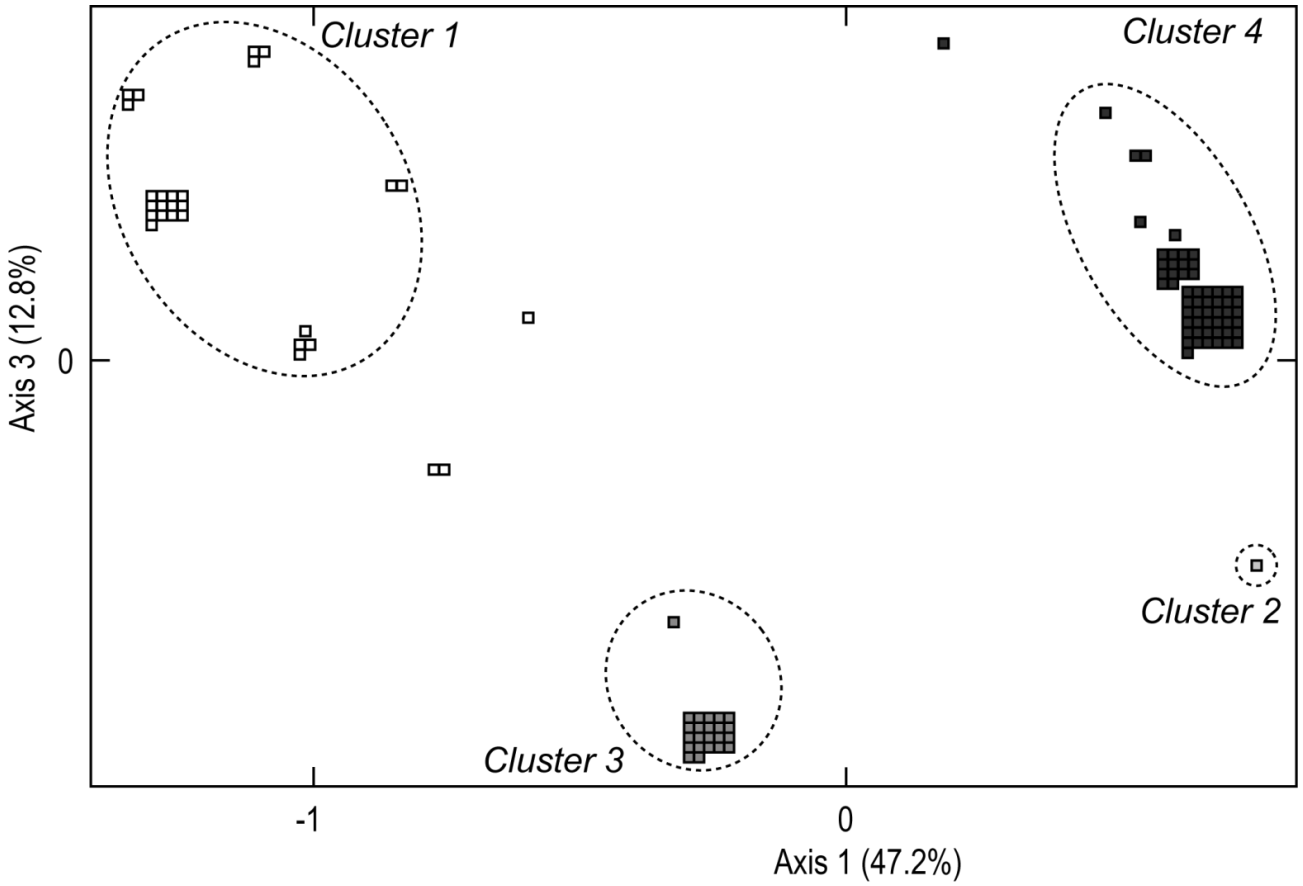

Fig. 1 *Himantura uarnak* species complex. Correspondence analysis (CA) (GENETIX: Belkhir et al. 2000) on individuals (squares) defined by their genotypes at five polymorphic intron loci. Ellipses delineate clusters of individuals with assignment >90% to either one of three clusters (*1, 3, 4*) obtained from Bayesian structure analysis (STRUCTURE: Falush et al., 2007). 'Cluster *2*' corresponds to a single individual, which was singled out on Axis 2 of CA (not shown). Individuals geometrically intermediate between Cluster *1* and either Cluster *3* or Cluster *4* were interpreted as backcross offspring (see text and Appendix 1). Mitochondrial-DNA clade of each individual (see Fig. 2) is indicated by a distinct tone of grey: *open*, Clade *I*; *light grey*, Clade *II*; *dark grey*, Clade *III*; *charcoal black*, Clade *IV*.

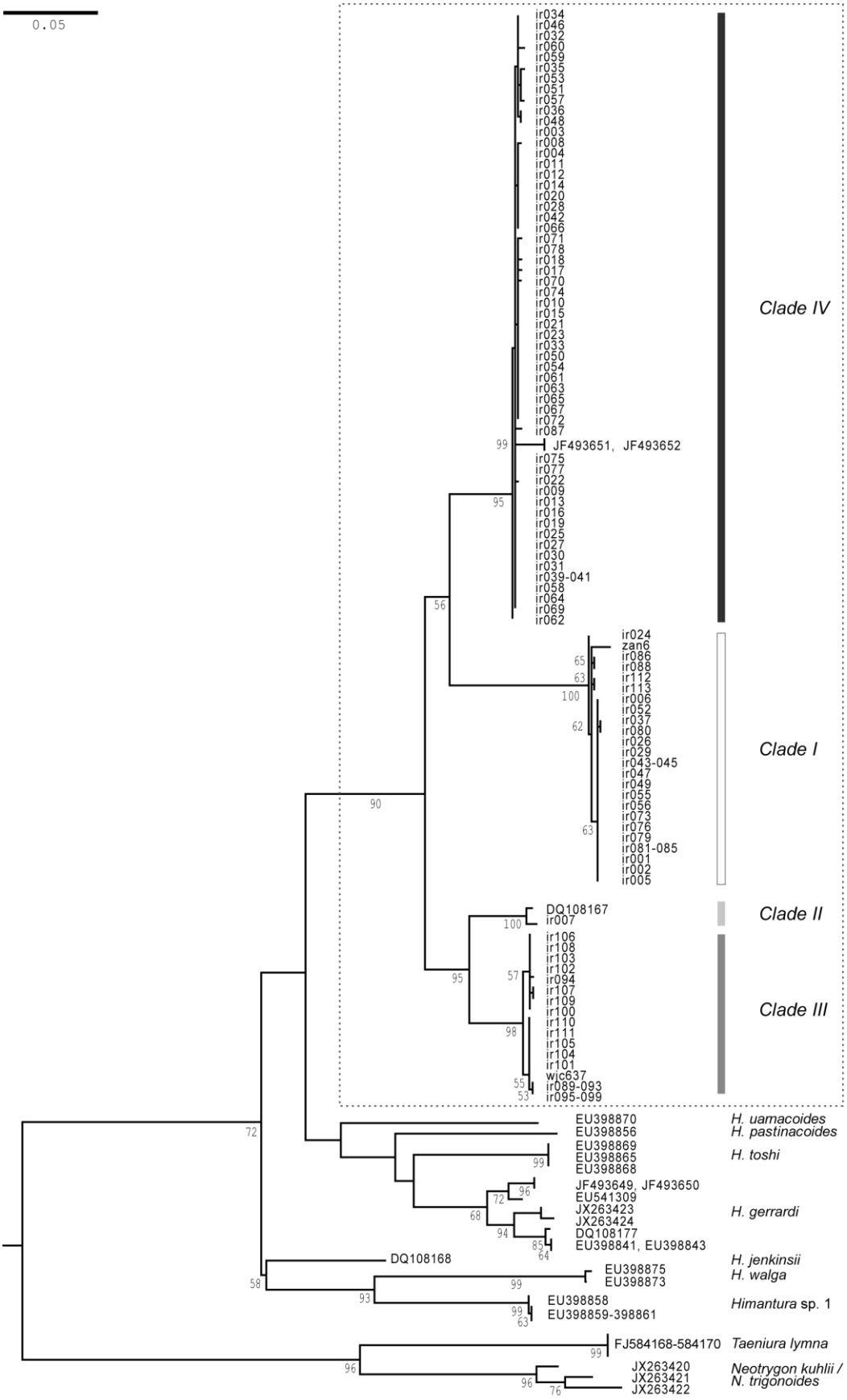

Fig. 2 *Himantura uarnak* species complex. Maximum-likelihood phylogeny of partial *COI* haplotypes including all homologous sequences from *Himantura* spp. retrieved from GENBANK (http://www.ncbi.nlm.nih.gov/) [sequences aligned over 620 bp; Hasegawa-Kishino-Yano+G+I substitution model; 1000 bootstrap resamplings; MEGA5 (Tamura et al., 2011)]. The tree was rooted by the homologous sequence in *Rhynchobatus djiddensis* (GENBANK JX263419). Only bootstrap scores >50% are presented. Dotted rectangle delimitates ingroup haplotypes.

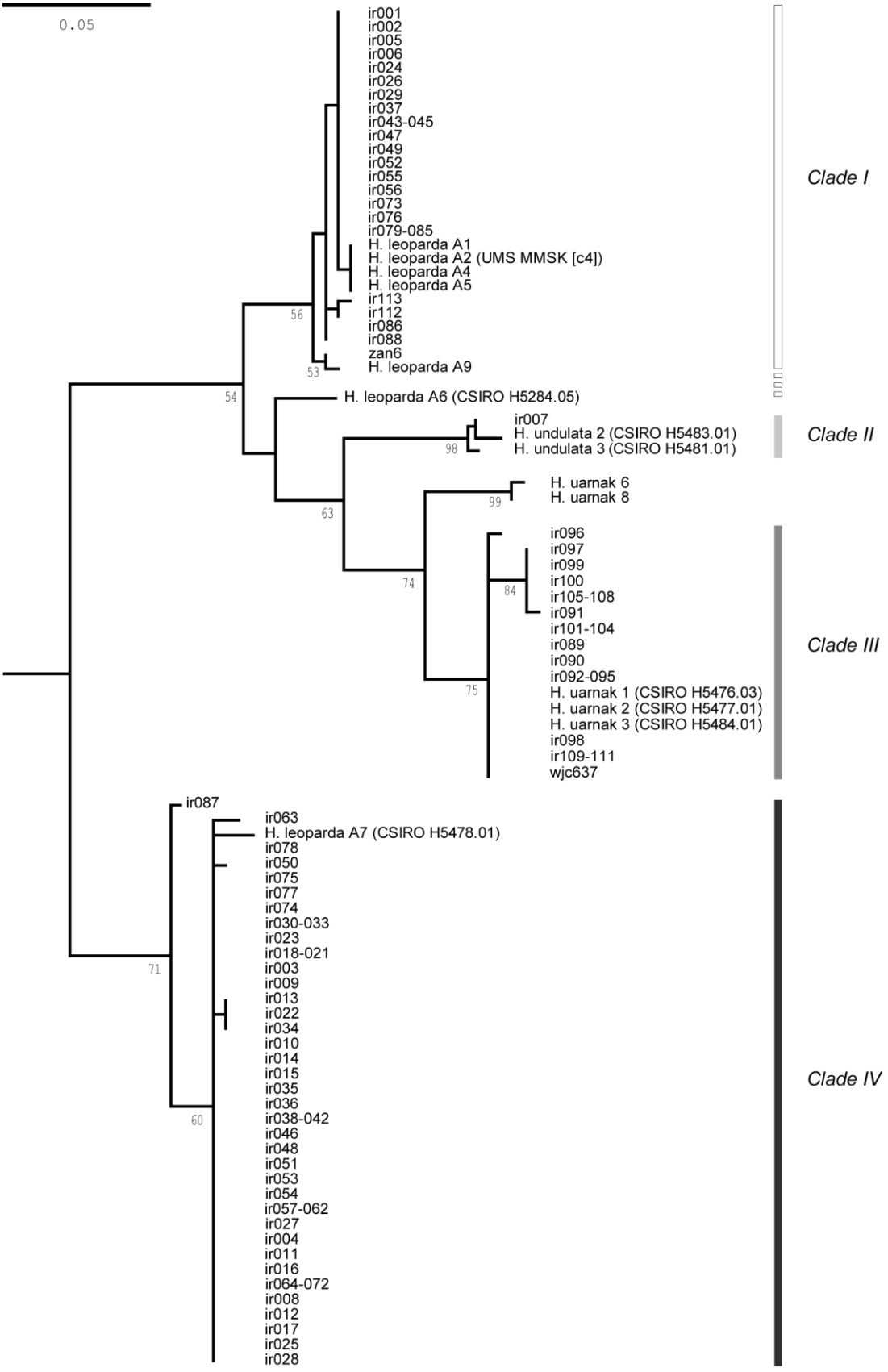

Fig. 3 *Himantura uarnak* species complex. Maximum-likelihood tree of partial *cytb* sequences including homologous sequences from Manjaji (2004) [sequences aligned over 239 bp; Kimura-2 parameter model with gamma-distributed evolutionary rates among sites; 1000 bootstrap resamplings; MEGA5 (Tamura et al., 2011)]. The tree was rooted by the homologous sequence in *H. signifer* specimen ZRC42547 (Manjaji, 2004). Only bootstrap scores >50% are presented.

Table S1. *Himantura uarnak* species complex. Genotypes and mitotype of 114 individuals, as determined from their size-alleles at 5 Mendelian-like intron loci and their nucleotide sequences at the *COI* and/or the *cytb* loci. *Batang* Batang, Java Sea; *Sunda* Sunda Strait; *Makassar* Makassar Strait; *Selayar* Bone Basin off Selayar Island, Banda Sea; *Kendari* Kendari, Banda Sea; *Labuanbajo* Labuan Bajo, Flores Sea; *Bali* Bali Sea; *Jogyakarta* off Jogyakarta, Indian Ocean coast of Java; *Zanzibar* Zanzibar, West Indian Ocean; *Taiwan* Taiwan, northwestern Pacific. *Assignment* of each individual to one of three clusters (*1*, *3*, *4*) was determined from Bayesian structure analysis [STRUCTURE: Falush et al., Mol. Ecol. Resour. 7 (2007) 574–578] of multiple-locus nuclear genotypes. The Markov-Chain Monte Carlo algorithm implemented in STRUCTURE was run under the admixture model with correlated allele frequencies for 100,000 steps after 50,000 initial burn-in steps, without any a priori information on an individual's sampling location, to estimate individual admixture proportions and assign individuals to populations. Five independent runs were performed for each a priori number of parental clusters (*K*), ranging from 2 to 6 to infer the number of clusters by visually comparing the output diagrams. The most informative diagram was produced with *K*=3 (Fig. S1).

Sample,	Nuclear locus					Assignment probability			Mitotype
Individual no.	*Act-2 A*	*Act-2 B*	*Cam-3 A*	*Cam-3 B*	*Cam-3 C*	Cluster *1*	Cluster *3*	Cluster *4*	
Batang									
ir001	186	100	178/175	163	125	0.934	0.053	0.013	*I*
ir002	186	100	178	163	125	0.987	0.009	0.004	*I*
ir003	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir004	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir005	186	100	178	163	125	0.988	0.008	0.004	*I*
ir006	186	100	178/175	163	125	0.933	0.054	0.012	*I*
ir007	190/187	100/097	175	165	144/129	0.008	0.016	0.976	*II*
ir008	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir009	190	100/098	175	165	144/129	0.006	0.008	0.987	*IV*
ir010	190	100/098	175	165	129	0.006	0.008	0.986	*IV*
ir011	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir012	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir013	190	100	175	-	144	0.004	0.008	0.988	*IV*
ir014	190	100	175	-	144	0.004	0.008	0.988	*IV*
ir015	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir016	190	100	175	-	144	0.004	0.008	0.988	*IV*
ir017	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir018	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir019	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir020	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir021	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir022	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir023	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir024	186	100	178/175	165/163	125	0.697	0.277	0.026	*I*
ir025	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir026	186	100	178	163	125	0.988	0.008	0.004	*I*
ir027	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir028	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir029	186	100	178	163	125	0.988	0.008	0.004	*I*
ir030	190	100	175	165	144	0.004	0.007	0.988	*IV*
ir031	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir032	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir033	-	100	-	-	-	-	-	-	*IV*
ir034	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir035	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir036	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir037	186	100	178/175	165/163	125	0.700	0.275	0.024	*I*
ir038	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir039	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir040	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir041	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir042	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir043	186	100	178	163	129/125	0.988	0.007	0.005	*I*
ir044	186	100	178	165/163	129/125	0.947	0.033	0.020	*I*
ir045	186	100	178	163	129/125	0.988	0.006	0.005	*I*
ir046	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir047	186	100	178	165/163	129/125	0.946	0.034	0.020	*I*
ir048	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir049	186	100	178	163	125	0.988	0.009	0.004	*I*
ir050	190	100	175	165	144/129	0.004	0.008	0.987	*IV*
ir051	190	100	175	165	144/129	0.005	0.008	0.988	*IV*
ir052	186	100	178	163	125	0.988	0.008	0.004	*I*

ir053	190	100	178/175	165/163	144	0.255	0.006	0.740	*IV*
ir054	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir055	186	100/098	178	163	125	0.989	0.007	0.004	*I*
ir056	186	100/098	178	163	125	0.989	0.007	0.004	*I*
ir057	190	100	175	165	129	0.005	0.008	0.987	*IV*
ir058	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir059	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir060	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir061	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir062	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir063	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir064	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir065	190	100/098	175	165	144/129	0.006	0.008	0.987	*IV*
ir066	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir067	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir068	-	100	-	-	-	-	-	-	*IV*
ir069	192/190	100	175	165	144/129	0.006	0.012	0.982	*IV*
ir070	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir071	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir072	190	100	175	165	144/129	0.004	0.008	0.987	*IV*
ir073	186	100	178	163	125	0.988	0.008	0.004	*I*
ir074	190	100	175	165	144/129	0.004	0.008	0.988	*IV*
ir075	-	100/098	175	165	-	-	-	-	*IV*
ir076	-	100	178	163	125	0.988	0.008	0.004	*I*
ir077	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir078	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir079	186	100	178/175	163	125	0.934	0.053	0.013	*I*
ir080	-	100	-	-	-	-	-	-	*I*
ir081	186	100	178	163	129/125	0.988	0.007	0.005	*I*
ir082	186	100	178	165/163	125	0.935	0.053	0.012	*I*
ir083	186	100	178/175	165/163	129/125	0.789	0.150	0.061	*I*
ir084	186	100	178	163	125	0.988	0.008	0.004	*I*
ir085	-	100	-	-	-	-	-	-	*I*
Sunda									
ir086	186	100	178	163	125	0.987	0.008	0.004	*I*
ir087	190	100	175	165	144	0.004	0.008	0.988	*IV*
ir088	186	100	178	163	125	0.988	0.008	0.004	*I*
Makassar									
ir089	186	100	175	165	125	0.008	0.984	0.008	*III*
ir090	186	100	175	165	125	0.008	0.984	0.008	*III*
ir091	186	100	175	165	125	0.008	0.984	0.008	*III*
ir092	186	100	175	165	125	0.008	0.984	0.008	*III*
ir093	186	100	175	165	125	0.008	0.984	0.008	*III*
ir094	186	100	175	165	125	0.008	0.984	0.008	*III*
ir095	186	100	175	165	125	0.008	0.984	0.008	*III*
ir096	186	100	175	165	125	0.008	0.984	0.008	*III*
ir097	186	100	175	165	125	0.008	0.984	0.008	*III*
ir098	186	100	175	165	125	0.008	0.984	0.008	*III*
ir099	186	100	175	165	125	0.008	0.984	0.008	*III*
Selayar									
ir100	186	100	175	165	125	0.008	0.984	0.008	*III*
ir101	186	100	175	165	125	0.008	0.984	0.008	*III*
ir102	186	100	175	165	125	0.008	0.984	0.008	*III*
ir103	186	100	175	165	125	0.008	0.984	0.008	*III*
ir104	186	100	175	165	125	0.008	0.984	0.008	*III*
ir105	186	100	175	165	125	0.009	0.984	0.008	*III*
ir106	186	100/098	175	165	125	0.050	0.924	0.026	*III*
Kendari									
ir107	186	100	175	165	125	0.008	0.984	0.008	*III*
ir108	186	100	175	165	125	0.008	0.984	0.008	*III*
ir109	186	100	175	165	125	0.008	0.984	0.008	*III*
Labuanbajo									
ir110	186	100	175	165	125	0.008	0.984	0.008	*III*
ir111	186	100	175	165	125	0.008	0.984	0.008	*III*
Bali									
ir112	186	100	178	163	125	0.988	0.008	0.004	*I*
Jogyakarta									
ir113	186	100/098	178	163	125	0.989	0.007	0.004	*I*
Zanzibar									
zan6	186	100	178	163	125	0.988	0.009	0.004	*I*
Taiwan									
wjc637	-	-	-	-	-	-	-	-	*III*

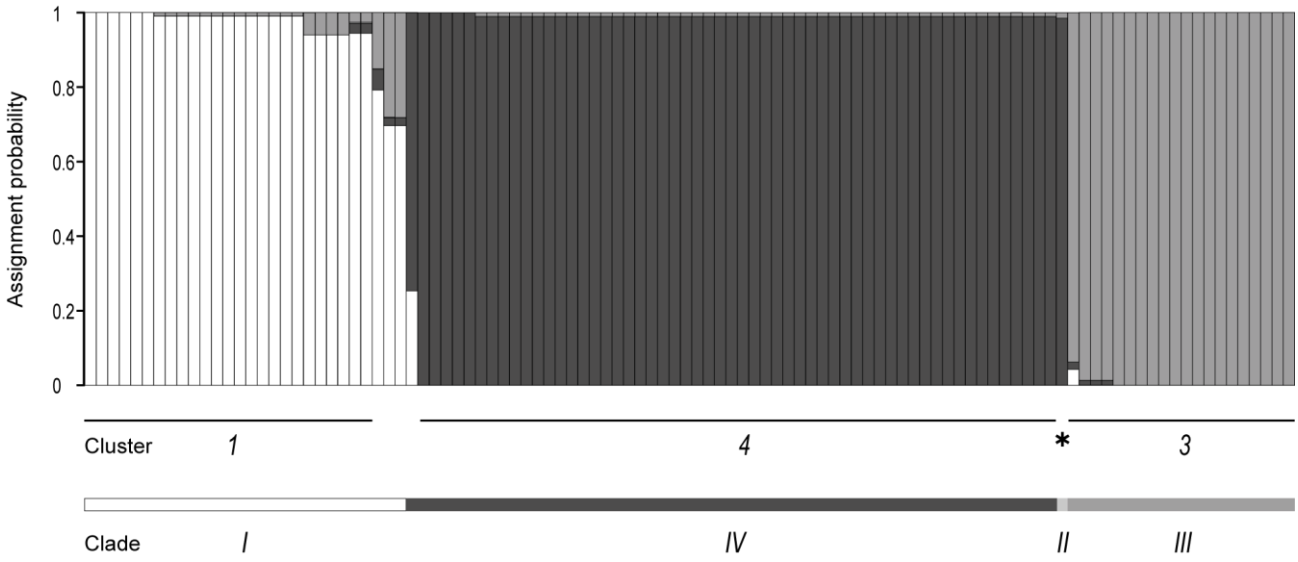

Fig. S1. *Himantura uarnak* species complex. Output diagram of Bayesian population structure analysis [STRUCTURE: Falush et al., Mol. Ecol. Resour. 7 (2007) 574–578] when the number of parent populations is *K*=3. Individual probabilities of assignment are shown on the y-axis. Individuals are represented by vertical bars and sorted by mitotype (Clades *I-IV*). Individual ir007, of a distinct cluster according to correspondence analysis [GENETIX: http://www.genetix.univ-montp2.fr/], is marked by an asterisk. Horizontal black traits underline individuals with >90% assignment probability to a cluster (*1*, *3*, or *4*).