Journal of Heredity 2009:100(3):273–283
doi:10.1093/jhered/esp005
Advance Access publication March 20, 2009

© The American Genetic Association. 2009. All rights reserved.
For permissions, please email: journals.permissions@oxfordjournals.org.

Species Delineation and Evolutionary History of the Globally Distributed Spotted Eagle Ray (*Aetobatus narinari*)

Vincent P. Richards, Marcy Henning, Wayne Witzell, and Mahmood S. Shivji

From the National Coral Reef Institute, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004 (Richards and Shivji); the Save Our Seas Shark Center, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004 (Richards and Shivji); the Guy Harvey Research Institute, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004 (Henning and Shivji); and the National Marine Fisheries Service, Southeast Fisheries Science Center, Miami, FL 33149 (Witzell).

Address correspondence to Mahmood S. Shivji at the address above, or e-mail: mahmood@nova.edu.

Abstract

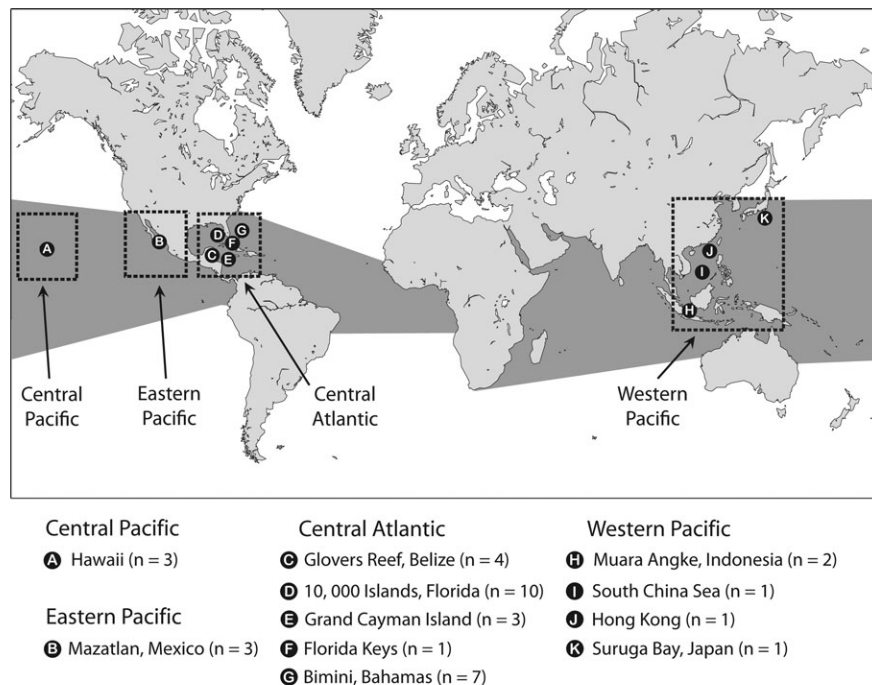
The spotted eagle ray (*Aetobatus narinari*), a large coral reef–associated batoid of conservation concern, is currently described as a single, circumglobally distributed species. However, geographic differences in its morphology and parasite diversity have raised unconfirmed suspicions that *A. narinari* may constitute a species complex. We used 1570 bp of mitochondrial and nuclear sequence data (cytochrome *b*, cytochrome *c* oxidase subunit I, and internal transcribed spacer 2) to assess the validity of *A. narinari* as a single cosmopolitan species and infer its evolutionary history. Specimens from 4 major geographic regions were examined: the Central Atlantic, Eastern Pacific, Western Pacific, and Central Pacific. Phylogenies described 3 distinct, reciprocally monophyletic lineages with no genetic exchange among regions. Based on combined genealogical concordance and genetic distance criteria, we recommend that the Western/Central Pacific lineage be recognized as a distinct species from lineages in the Central Atlantic and Eastern Pacific. The latter 2 lineages, separated by the Isthmus of Panama, are proposed as subspecies. A basal position in phylogenetic analyses and statistical parsimony results support an Indo-West Pacific origin for the *A. narinari* species complex, with subsequent westerly dispersal around the southern tip of Africa into the Atlantic and then into the Eastern Pacific.

Key words: *batoid, conservation, evolutionary history, speciation*

Molecular analyses are resolving many taxonomic uncertainties, including revealing that species once thought to cover wide geographic areas are often complexes comprising 2 or more genetically distinct species (Knowlton 2000; Vogler et al. 2008; Wang et al. 2008). In addition to providing taxonomic clarity and more accurate biodiversity assessments, such findings have management and conservation ramifications as many of the newly identified species may have limited ranges and relatively small population sizes rendering them more vulnerable to human impact. Consequently, the accurate delimitation of species boundaries is an important conservation need (Avise 1998; Frankham et al. 2002), especially for exploited species.

The spotted eagle ray (*Aetobatus narinari*) is a large, reef-associated, and mainly coastal batoid with an apparent circumtropical distribution (Figure 1) (Compagno and Last 1999). Although currently designated a single species throughout its range, geographic differences in its morphology and the distribution of 7 tapeworm species parasitic to *A. narinari* have led to unconfirmed suggestions that this batoid probably comprises a species complex (Compagno and Last 1999; Compagno et al. 2005; Marie and Justine 2005; Kyne et al. 2006). *Aetobatus narinari* is subject to intense and unregulated inshore fisheries particularly in parts of Southeast Asia and is considered highly vulnerable to sustained harvest due to its relatively low fecundity (Compagno and Last 1999; Kyne et al. 2006). Consequently, *A. narinari* is listed on the IUCN Red List of Threatened Species as “Vulnerable” in Southeast Asia and “Near Threatened” globally (IUCN 2006 Assessment). However, these listings are based on the current designation of *A. narinari* as a single globally distributed species. Given long-standing suspicions that it may be a species complex with its component taxa having narrower geographic distributions and population sizes, clarifying these uncertainties is a high priority for guiding conservation efforts (Kyne et al. 2006)

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024



Central Pacific
A Hawaii (n = 3)

Eastern Pacific
B Mazatlan, Mexico (n = 3)

Central Atlantic
C Glovers Reef, Belize (n = 4)
D 10, 000 Islands, Florida (n = 10)
E Grand Cayman Island (n = 3)
F Florida Keys (n = 1)
G Bimini, Bahamas (n = 7)

Western Pacific
H Muara Angke, Indonesia (n = 2)
I South China Sea (n = 1)
J Hong Kong (n = 1)
K Suruga Bay, Japan (n = 1)

Figure 1. World map showing location of animals sampled. Shading indicates northern and southern extents of *Aetobatus narinari's* geographic range (from Compagno and Last 1999). Sample sizes per location shown in parentheses.

and providing an informed perspective on the evolutionary diversity and history of this lineage.

To provide this clarification, we took a multilocus genetic approach examining signals of evolutionary history in DNA sequences from the mitochondrial cytochrome *b* (cyt *b*), cytochrome *c* oxidase subunit I (COI) genes, and the nuclear ribosomal internal transcribed spacer 2 (ITS2) locus. Our results reveal that *A. narinari* is composed of at least 3 distinct lineages with no genetic exchange among individuals from the Central Atlantic, Eastern Pacific, and Western/Central Pacific regions. These findings support the recognition of at least 2 distinct species within "*A. narinari*" and 2 lineages that at a minimum should be provided subspecies status. Our results also indicate an Indo-Pacific origin for the *Aetobatus* lineage with subsequent westerly dispersal and diversification into the Atlantic and then eastern Pacific.

Materials and Methods

Specimens Analyzed

Tissue samples were obtained from 36 *A. narinari* individuals from globally distributed regions (Central Atlantic, Eastern Pacific, Western Pacific, and Central Pacific; Figure 1). In addition to *A. narinari*, the genus *Aetobatus* contains 3 other species, any of which would provide a valid outgroup for our intraspecific phylogenetic analysis. We utilized *Aetobatus flagellum* obtained from Ariake Bay, Japan, as the outgroup. All samples were preserved in 95% ethanol at 4 °C.

Polymerase Chain Reaction and Sequencing

Genomic DNA was extracted from approximately 25 mg of tissue using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The following primer pairs were designed for polymerase chain reaction (PCR) amplification and sequencing. Cyt *b* gene: AnarCBF1 (5′-GAGGGGCAACTGTCATCACTAACC-3′) and AnarCBR1 (5′-CGATTGGGAAAAGGAGGAGGAA-3′); ITS2 locus: Bat5.8SF1 (5′-GCTACACCTGTCT-GAGGGTCGCC-3′) and Bat28SR1 (5′-ACAGGCTAGGC-CTCGATCAGAAGG-3′). The primer pair FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR2 (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) (Ward et al. 2005) was used for COI PCR and sequencing. The above primer pairs produced 596 bp of nucleotide sequence from the 3′ end of cyt *b*, 571 bp from the 5′ end of COI, and 403 bp of the entire ITS2 locus. The ITS2 primers also gave 280 bp from the 5′ end of the flanking ribosomal 28S gene.

Total PCR volumes were 50 μl and contained 1 μl of the extracted genomic DNA (unquantified), 5 μl 10× PCR buffer, 50 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer, and 0.75 units of HotStar Taq DNA polymerase (Qiagen Inc.). PCR was performed in a Mastercycler gradient (Eppendorf Inc., Westbury, NY) thermal cycler as follows: 95 °C initial heating for 15 min to activate the hot start DNA polymerase, followed by 35 cycles of 94 °C for 1 min, 50 °C (55 °C for ITS2) for 1 min, 72 °C for 1 min, and a 5-min final extension step at 72 °C. For a few individuals that did not amplify well under these conditions, the *Taq* polymerase was increased to 1.75 units, number of cycles increased to 41, and the 72 °C extension step

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

increased to 2 min. A negative control (all reaction components without genomic DNA) was included in each PCR set to check for reagent contamination. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced completely in both directions using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit. Products were separated using an Applied Biosystems 3130 Genetic Analyzer and bases called using Applied Biosystems Sequencing Analysis Software version 5.2. Individual sequences are available from GenBank (accession numbers FJ812178-FJ812206).

Data Analysis

CLUSTAL X version 1.81 (Thompson et al. 1997) and MacCLADE version 4.03 (Maddison and Maddison 2001) were used to align and edit individual sequences. Cyt *b* and COI DNA sequences were translated using GENEDOC version 2.6.02 (Nicholas et al. 1997) and checked for correct vertebrate mitochondrial DNA (mtDNA) amino acid coding and aberrant start/stop codons.

Genealogical Analyses

Evolutionary relationships among DNA sequences were estimated by constructing unrooted statistical parsimony networks for each locus using the Templeton et al. (1992) method as implemented in the software package TCS version 1.13 (Clement et al. 2000). Ambiguous loops in the networks were resolved using criteria based on coalescent theory (Crandall and Templeton 1993), summarized by Pfenninger and Posada (2002) as follows: 1) frequency criterion: haplotypes are more likely to be connected to haplotypes with higher frequency than to singletons; 2) topological criterion: haplotypes are more likely to be connected to interior haplotypes than to tip haplotypes; and 3) geographical criterion: haplotypes are more likely to be connected to haplotypes from the same population or region than to haplotypes occurring in distant populations.

To further explore evolutionary relationships among the *A. narinari* sequences, we conducted phylogenetic analyses on concatenated mitochondrial and nuclear sequence data sets. The practice of combining sequence data from different loci continues to be debated (see Cunningham 1997 and references therein). The partition homogeneity test is often used to determine whether data should be combined, with Bull et al. (1993) recommending that data partitions showing significant heterogeneity ($P < 0.05$) not be combined. However, this level of significance may be too conservative. Farias et al. (2000) and Sullivan (1996), for example, showed that increased phylogenetic accuracy was obtained by combining apparently heterogeneous mitochondrial and nuclear data sets (partition homogeneity test P values of 0.01 and 0.016, respectively). In addition, Cunningham (1997) demonstrated that combined data sets with P values >0.01 improved or did not decrease phylogenetic accuracy. We performed a partition homogeneity test in PAUP version 4.0b10 (Swofford 2002) on concatenated cyt *b* and COI sequences (excluding the *A. flagelum* outgroup sequence), and no significant heterogeneity between the data partition was detected ($P = 1.0000$; 100 replicates). Another partition homogeneity test on an *A. narinari* and *A. flagellum* alignment of concatenated cyt *b*, COI, and ITS2 sequences produced a P value of 0.01 (100 replicates). The ITS2 section of the alignment contained numerous indels: 2 among *A. narinari* ingroups (3 and 6 bp) and 5 between the ingroups and the *A. flagellum* outgroup (11–15 bp). Removal of these indels did not affect the outcome of the partition homogeneity test. In light of the prior studies mentioned, we feel that the partition homogeneity test failed to show sufficient heterogeneity among the data partitions to reject their combination. Consequently, we considered combining all sequence data sets (total evidence) to be a valid approach.

PAUP was used to perform maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ) phylogenetic reconstructions on the combined mitochondrial–nuclear data set (with *A. flagellum* as the outgroup). Although numerous studies have shown indels to be reliable phylogenetic characters (Lloyd and Calder 1991; Baldwin and Markos 1998; Prather and Jansen 1998; Simmons et al. 2001; Coleman and Vacquier 2002), they may not evolve according to the more complex substitution models commonly implemented in ML phylogenetic approaches (Golenberg et al. 1993). Consequently, 2 approaches to the combined mitochondrial–nuclear data set were employed: 1) a 1548-bp alignment with all indel sites removed and 2) a 1621-bp alignment containing the indels. Using the Akaike information criterion (AIC) in MODELTEST 3.06 (Posada and Crandall 1998), the transversion model with invariable sites (TVM + I) was selected for the ML analysis of the 1548-bp data set. To account for the uncertainty that indels may not evolve according to the models implemented in MODELTEST, we used the Jukes–Cantor model, which assumes equal base frequencies and all substitutions equally likely for the ML analysis on the indel-containing 1621-bp data set, with indels weighted as separate presence/absence characters using the "simple gap coding" method of Simmons and Ochoterena (2000).

ML and MP heuristic searches obtained starting trees via stepwise addition using 10 random addition sequence replicates, and branch swapping was performed using tree bisection–reconnection. The MP analysis was unweighted. For the NJ analysis, the TVM + I model was used with the 1548-bp data set and uncorrected *p* distance with the 1621-bp data set. Statistical support for branch nodes was assessed using nonparametric bootstrap analyses (100 replicates for ML, 500 replicates for MP, and 2000 replicates for NJ) (Felsenstein 1985). Nodes with bootstrap values ≥70% were considered well supported (Hillis and Bull 1993). The ML heuristic bootstrap searches obtained starting trees via stepwise addition using the as-is option, and branch swapping was performed using nearest neighbor interchange.

PAUP was also used for ML, MP, and NJ phylogenetic reconstructions (with *A. flagellum* as the outgroup) on each locus independently. Tree searches and bootstrap analyses were performed according to the procedures outlined

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

above. Identifying the basal clade (ancestral population) was of major interest in our analyses for inferring the evolutionary history of *A. narinari*. Therefore, we statistically evaluated alternative tree topologies with each of the 3 major phylogeographic lineages (clades; see Results) constrained to be basal using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa 1999) in PAUP. Significance was determined via a 1-tailed test comparing the test statistic to a null distribution generated from 1000 bootstrap replicates. The bootstrap was performed using the resampling estimated log-likelihood method (Kishino et al. 1990). This procedure gives a good approximation of ML bootstrap probabilities without having to reestimate branch lengths for each replicate (Kishino et al. 1990).

Assessment of Comparative Genetic Divergence

To assess genetic divergence thresholds that might be informative for species delineation, we compared divergence between the 3 major *A. narinari* phylogeographic lineages obtained (i.e., Western/Central Pacific, Eastern Pacific, and Central Atlantic; see Results) with that between other taxonomically uncontested batoid and shark congener pairs. Using sequence data available in GenBank, we selected 6 closely related batoid congener pairs (representing 4 families) and 1 shark subspecies pair that had good sequence overlap with our data. Genetic distances for cyt *b* and COI were calculated using the following sequences (accession numbers in parentheses)—cyt *b*: *Himantura imbricatus* (AB021497), *Himantura gerrardii* (AB021498), *Himantura pacifica* (AF110638), *Himantura schmardae* (AF110639), *Potamotrygon motoro* (AF110626), *Potamotrygon castexi* (AF110627), *Dasyatis akejei* (D50027), *Dasyatis laosensis* (AB021504), *Dasyatis* sp. (AB021496), *Sphyrna tiburo tiburo* (L08042), and *Sphyrna tiburo vespertina* (L08043). COI: *Raja cerva* (DQ108189) and *Raja australis* (DQ108188). Although the *Dasyatis* sp. cyt *b* GenBank sequence was from a *Dasyatis* stingray not identified to species, the possibility that it is actually the same species as its comparison congener (*D. laosensis*) is highly unlikely; *D. laosensis* is an obligate freshwater species (Roberts and Karnasuta 1987) collected in the Mekong River, Laos (Sezaki et al. 1999), whereas *Dasyatis* sp. was collected in salt water (Gulf of Thailand) (Sezaki et al. 1999).

To facilitate comparison with other published studies, mean pairwise uncorrected *p* and Kimura 2-parameter (K2P)–corrected sequence distances were calculated using MEGA3 (Kumar et al. 2004). Although these distances are useful for comparative purposes, patristic distances calculated from the branch lengths of a phylogeny constructed using the correct model of sequence evolution offer the advantage of eliminating underestimates of genetic distance due to substitution saturation (Lefébure et al. 2006). Therefore, we calculated patristic distances from cyt *b* and COI ML phylogenies, which included all *A. narinari* sequences and the congeners obtained from GenBank, using the program PATRISTICv1.0 (Fourment and Gibbs 2006). We also wished to gain an accurate estimate of genetic distance among the 3 *A. narinari* lineages so that divergence time could be calculated. The best estimate would be patristic distances obtained from an ML phylogeny of all 3 loci. However, due to the uncertain nature of indel evolution as discussed earlier, the ITS2 data were excluded and distances calculated from the combined cyt *b* and COI data sets.

Inclusion of the batoid congener pairs *H. pacifica*–*H. schmardae*, *P. motoro*–*P. castexi*, and the shark subspecies pair *S. tiburo tiburo*–*S. tiburo vespertina* in the comparative pairwise genetic distance calculations required a cropped cyt *b* alignment (432 bp) as the GenBank coverage of these sequences was slightly upstream of our *A. narinari* sequence alignment. MODELTEST (using AIC) selected TrN+I as the best fit model for the COI and combined mtDNA alignments and the TVM + I + G model for the 432-bp cyt *b* alignment. Heuristic tree searches used the same parameters stated earlier.

Estimating Divergence Time

A likelihood ratio test (LRT) (Huelsenbeck and Rannala 1997) was performed to test for the existence of a molecular clock on the combined COI/cyt *b* *A. narinari* data set and the 432-bp cyt *b* *A. narinari* plus congeners data set. The LRT test was made more stringent by reducing the number of taxa as follows: only unique haplotypes were included in the combined COI/cyt *b* analysis and only 1 (most divergent) *A. narinari* haplotype from each of the 3 major lineages was included in the 432-bp cyt *b* analysis.

Aetobatus narinari is restricted to tropical and subtropical waters (Compagno and Last 1999); therefore, it is likely that Central Atlantic and Eastern Pacific populations became isolated from one another when the Isthmus of Panama closed approximately 3.1 million years ago (Ma) (Coates and Obando 1996). However, the land bridge may have still been passable as recently as 2 Ma (Cronin and Dowsett 1996). Therefore, mutation rates for the combined COI/cyt *b* data were calculated for both isthmus time intervals using the patristic distance between the Central Atlantic and Eastern Pacific lineages.

Results

Statistical Parsimony

We identified 11 cyt *b* haplotypes, 8 COI haplotypes, and 7 ITS2 sequence types in the 36 globally distributed *A. narinari* individuals. At the 95% probability level, TCS produced 2 distinct subnetworks for COI: Western and Central Pacific haplotypes formed 1 network; the Eastern Pacific and Central Atlantic haplotypes formed the other. For cyt *b*, TCS produced 3 distinct networks comprised of Western/Central Pacific, Eastern Pacific, and Central Atlantic haplotypes. The distinct networks for each mitochondrial gene could only be joined at the 91% probability level (Figure 2). Haplotype connections within the distinct (95%) networks remained unchanged in the joined (91%) networks.

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

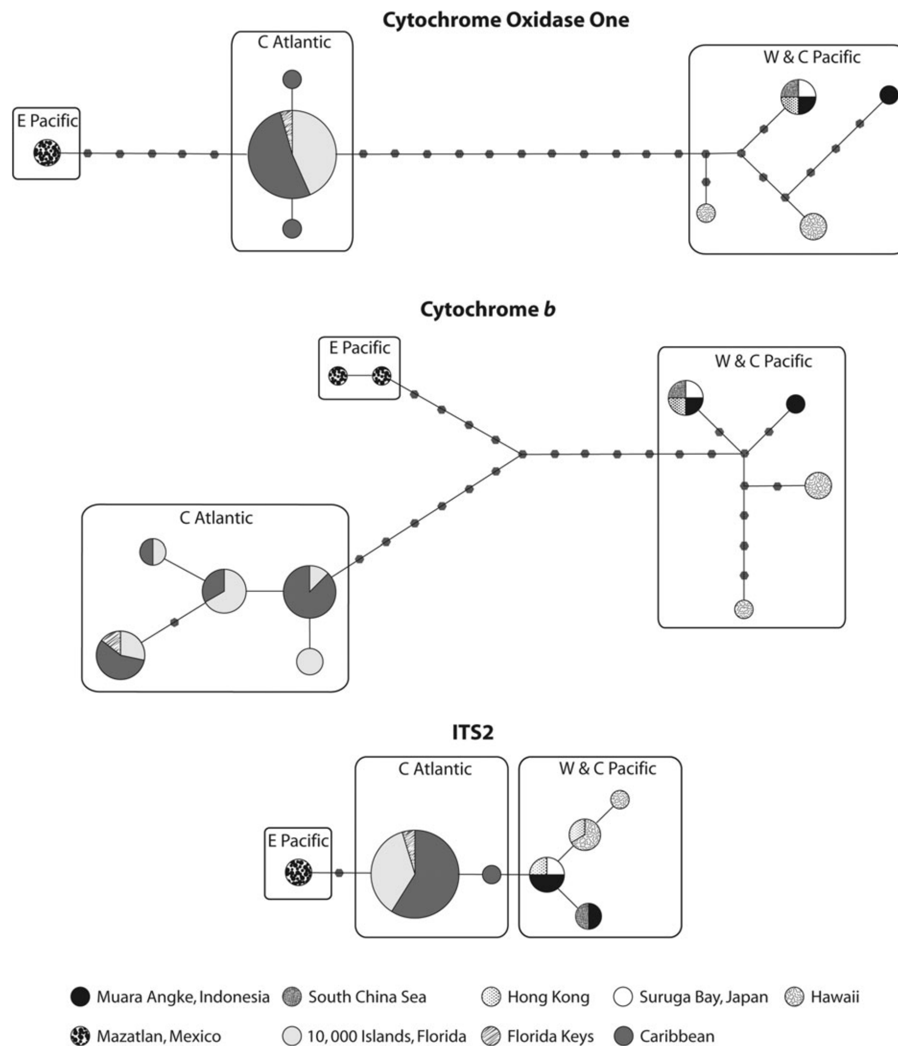


Figure 2. TCS networks depicting relationships among mitochondrial haplotypes and ITS2 sequence types. Circle size is proportional to haplotypes/sequence-type frequency, connecting lines represent single mutational steps, and small black hexagons represent hypothetical missing haplotypes/sequence types not sampled.

Although the nuclear ribosomal ITS2 locus occurs in multiple copies within an individual (Long and Dawid 1980), the process of concerted evolution often homogenizes all copies (Hillis and Dixon 1991). However, 3 of the 36 *A. narinari* individuals sequenced (1 from Hong Kong, 1 from Grand Cayman Island, and 1 from Indonesia) demonstrated intraindividual heterogeneity at 3 distinct nucleotide positions (i.e., there was a single heterogeneous site per individual). These heterogeneous positions were clearly identified as double peaks on both forward and reverse sequencing electropherograms. Consequently, each of these 3 individuals generated 2 distinct ITS2 sequences types. Two separate TCS analyses were performed on the ITS2 data: the first excluded the 3 heterogeneous individuals and the second included all individuals. All 3 individuals from the Eastern Pacific possessed identical sequence types and contained 2 indels (3 and 6 bp) separated by a single base pair. Indels were not present in the ITS2 of individuals from any other region. The 2 indels were weighted as separate presence/absence characters (simple gap coding; Simmons and Ochoterena 2000).

Results of both ITS2 TCS analyses were single 95% probability networks where sequence types formed 3 distinct lineages (Eastern Pacific, Central Atlantic, and Western/Central Pacific) (Figure 2). Although branching patterns in both ITS2 networks were identical, in the second network (all individuals) (Figure 2), one of the sequence types from the heterogeneous Cayman Island individual replaced a hypothetical/missing sequence type that had previously connected the solitary Central Atlantic sequence type to the Western/Central Pacific sequence types. The ITS2 networks produced the only alternative connection: a connection between the 2 tip sequence types from Hawaii and Indonesia/China, which was broken in accordance with criteria (1) and (3) outlined in the Materials and Methods.

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

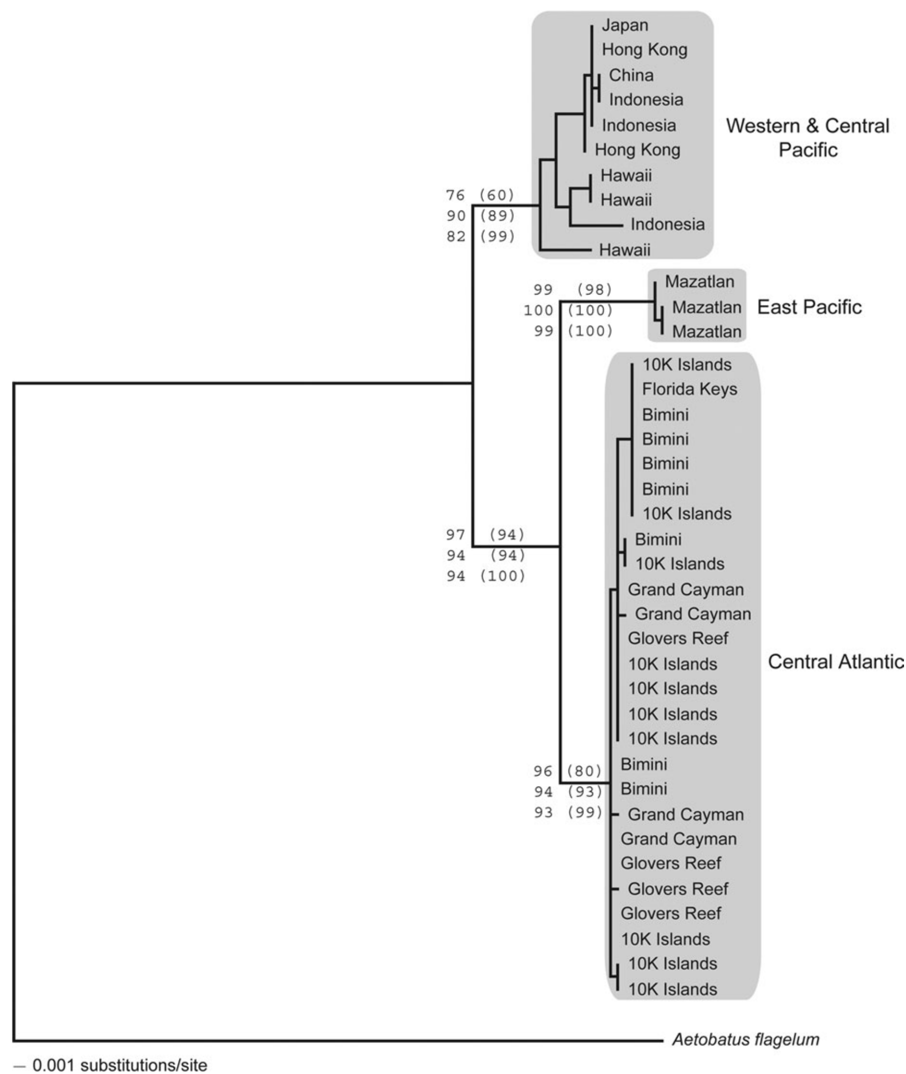


Figure 3. ML phylogeny of the combined COI/cyt *b*/ITS2 1621-bp data set. Bootstrap values are indicated over branches in descending order as follows: ML, MP, and NJ (values in parentheses are for the 1548-bp data set).

Phylogenetic Relationships

Analyses of the combined mitochondrial–nuclear 1548-bp (ITS2 indels removed) and 1621-bp (ITS2 indels retained) data sets by ML, MP, and NJ methods each produced 3 virtually identical, well-supported topologies, which grouped the *A. narinari* sequences into 3 reciprocally monophyletic lineages, that is, Western/Central Pacific, Eastern Pacific, and Central Atlantic, with the Western/Central Pacific clade as basal (Figure 3). The only low bootstrap support (60% with ML) occurred for the monophyly of Western/Central Pacific lineage when using the 1548-bp data set. Alternative hypotheses of basal Eastern Pacific or Central Atlantic clades were rejected by the SH test (P = 0.041 and P = 0.021, respectively). Independent phylogenetic analyses of each locus also recovered the same 3 major clades (data not shown, but available from the author on request).

Comparative Genetic Distances

Genetic distances among the 3 *A. narinari* lineages based on COI and cyt *b* exceeded or were close to distances between several taxonomically uncontroversial batoid congeners (Table 1). For example, all pairwise measures (*p*, K2P, and ML patristic) of *A. narinari* COI genetic distance for Western/Central Pacific versus Central Atlantic lineages and Western/Central Pacific versus Eastern Pacific lineages exceeded (or in 1 case equaled) the COI genetic distance between the batoid congener pair *R. cerva* versus *R. australis*. For cyt *b*, genetic distances for Western/Central Pacific versus Central Atlantic *A. narinari* lineages exceeded that of the *D. laosensis* versus *Dasyatis* sp. comparison and were very close to (i.e., just less than [*p* and K2P distances] or just more than [ML patristic distances]) that of the *H. imbricatus* versus *H. gerrardii* comparison. The cyt *b* genetic distance between the Western/Central Pacific and Eastern Pacific

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

Table 1. Comparison of COI and cyt *b* genetic distances (%) among *Aetobatus narinari* lineages and between elasmobranch congeners

Comparison	*p*[a]	K2P[b]	ML[c]
COI			
A. narinari lineages			
Central Atlantic versus Eastern Pacific	1.1	1.1	1.1
Western/Central Pacific versus Eastern Pacific	3.8	3.9	4.0
Western/Central Pacific versus Central Atlantic	2.8	2.8	2.9
Elasmobranch congeners			
Raja cerva versus *Raja australis* (batoids)	2.6	2.7	2.9
cyt *b*			
A. narinari lineages			
Central Atlantic versus Eastern Pacific	2.5	2.5	2.6
Western/Central Pacific versus Eastern Pacific	2.7	2.7	2.9
Western/Central Pacific versus Central Atlantic	2.9	3.0	3.3
Elasmobranch congeners			
Dasyatis laosensis versus *Dasyatis* sp. (batoids)	2.6	2.6	2.9
Himantura imbricatus versus *Himantura gerrardii* (batoids)	3.0	3.1	3.2
Dasyatis akejei versus *Dasyatis laosensis* (batoids)	3.3	3.4	3.5
Potamotrygon motoro versus *Potamotrygon castexi* (batoids)	3.7	3.8	4.1
Himantura pacifica versus *Himantura schmardae* (batoids)	5.6	5.8	6.5
Sphyrna tiburo tiburo versus *Sphyrna tiburo vespertina* (sharks)	5.8	6.1	6.6

[a] Mean pairwise uncorrected sequence distance.

[b] Mean pairwise Kimura 2-parameter sequence distance.

[c] Mean pairwise patristic distance generated using the TrN + I (COI) and TVM + I + G (cyt *b*) models.

lineages similarly exceeded the *D. laosensis* versus *Dasyatis* sp. comparison and were only slightly lower than the *H. imbricatus* versus *H. gerrardii* comparison. Notably, however, all COI and cyt *b* distances between the Central Atlantic and Eastern Pacific *A. narinari* lineages were lower than those of the batoid congeners compared.

Aetobatus Lineage Divergence Times

The LRT on the combined COI/cyt *b* mitochondrial data indicated that the nucleotide substitution rate was homogeneous across lineages (test statistic = 9.56; P = 0.57). The calculated mutation rates based on patristic distances between the Central Atlantic and Eastern Pacific lineages were 0.58% per million years (My) assuming an isthmus closure 3.1 Ma, and 0.90% per My assuming a final isthmus closure 2 Ma. These mutation rates, respectively, produced *A. narinari* divergence times of 5.7 and 3.7 My for the Western/Central Pacific versus Eastern Pacific lineage comparison and 5.4 and 3.4 My for the Western/Central Pacific versus Central Atlantic lineage comparison.

Rates of Molecular Evolution

Nucleotide substitution rate in the 432-bp cyt *b A. narinari* plus congeners data set was not homogeneous across lineages (test statistic = 23.44; P = 0.02). Accelerated mitochondrial substitution rates have been detected previously in some batoids. For example, using the 12S rRNA, tRNA, and NADH dehydrogenase subunits 1 and 2 loci, Dunn et al. (2003) showed a rate increase for *Potamotrygon*. Our phylogenetic analysis indicated a similar rate increase for the *Potamotrygon* congeners and a rate slow down for *H. imbricatus* and *H. gerrardii*. These species were subsequently removed from the analysis, and an LRT on the remaining congeners showed the substitution rate to be homogeneous (test statistic = 4.02; P = 0.86).

Discussion

Is *A. narinari* a Species Complex?

The increasing studies proclaiming discovery of new species based on divergent, single-locus (typically mitochondrial) sequences have prompted debates about the validity of such species designations and concerns about "taxonomic inflation" potentially misdirecting the allocation of limited conservation resources (Rubinoff 2005; Meiri and Mace 2007). To avoid this conundrum, we took a multilocus (mitochondrial and nuclear) and multianalytical (genealogical and comparative genetic distances) approach to investigate if the globally distributed spotted eagle ray comprises more that 1 species, as has long been suspected based on morphological and parasite diversity grounds.

Our phylogenetic analyses identified 3 well-supported, reciprocally monophyletic lineages each restricted to the Western/Central Pacific, Eastern Pacific, and Central Atlantic regions, respectively. In its simplest form, the phylogenetic species concept (PSC) defines species as minimum diagnosable units (Cracraft 1989), and although numerous variations of this concept have been proposed (see Avise 2000; Wiens and Penkrot 2002; and references therein), they all essentially agree that species are in the first instance evolutionarily distinct lineages (De Queiroz 1998). Under this "distinct lineage" definition, our results suggest that *A. narinari* as currently taxonomically described is actually comprised of at least 3 species. However, the PSC does not emphasize reproductive isolation as a delineating criterion, a fundamental component of the "biological species concept" (Mayr 1963). In an attempt to combine speciation concepts, Avise and Ball (1990) advocated the use of multiple independent loci in species delineation. They proposed that the lengthy period of time required for major phylogenetic distinctions to accumulate concordantly at independent loci was probably also sufficient for intrinsic reproductive barriers to form. Populations that showed concordant, reciprocally monophyletic patterns over independent loci were therefore worthy of formal taxonomic recognition. Our mitochondrial and nuclear data clearly show genealogical concordance in lineage identification. However,

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

Avise and Ball (1990) also suggested that full species status only be given to populations showing genealogical concordance in sympatry as populations held in reproductive isolation by allopatric barriers could potentially interbreed if such barriers break down. Consequently, they recommended a designation of subspecies for phylogenetically distinct, allopatric populations. Under these more stringent guidelines, the 3 allopatric *A. narinari* lineages identified in our study should be regarded at the minimum as subspecies.

A statistically based PSC standard that has been recently proposed for designating operational species based on DNA sequence data is the 95% connection limit in statistical parsimony networks (Hart and Sunday 2007). By this standard, nonrecombining loci (e.g., mtDNA sequences) from Linnaean species typically form distinct (i.e., unconnected) subnetworks, whereas sequences from single species typically remain in a single cohesive network at the 95% statistical parsimony limit. Using this limit, our COI statistical parsimony analysis identified 2 unconnected subnetworks, 1 formed by the Western/Central Pacific lineage and the other by the joined Eastern Pacific and Central Atlantic lineages. In contrast, the cyt *b* results showed 3 unconnected subnetworks comprising the Western/Central Pacific, Eastern Pacific, and Central Atlantic lineages. These networks could only be joined by lowering the statistical parsimony limit to 91%. Based on this quantitative PSC standard, *A. narinari* is comprised of at least 2 (COI as a marker) or 3 (cyt *b* as a marker) distinct species deserving of Linnaean status. Although popular, genealogical approaches are not the only genetic tools available for species delineation. Several workers have stressed the value of comparative measures of genetic divergence or distance (Highton 1990; Good and Wake 1992; Fraser and Bernatchez 2001; Buckley-Beason et al. 2006; Lefébure et al. 2006). Assuming that intrinsic barriers to gene flow between recognized congeneric species are well established, the genetic distance between these species can be used as thresholds for species delineation in related taxa. Using such genetic distance thresholds as a comparative criterion, the following observations support delineating at least the Western/Central Pacific versus Eastern Pacific/Central Atlantic *A. narinari* lineages as 2 species: 1) the genetic distance (*p*, K2P, and ML patristic) accumulated for both mitochondrial genes between the Western/Central Pacific versus Eastern Pacific and Central Atlantic *A. narinari* lineages was close to or exceeded that of several taxonomically uncontroversial elasmobranch congeners (Table 1) and the COI genetic distances between *A. narinari* lineages exceed those reported in the literature for several other uncontroversial batoid (e.g., genus *Bathyraja*) and shark (e.g., genus *Carcharhinus*) congeners (*Bathyraja* [*p* distance]: *Bathyraja maculata* vs. *Bathyraja lindbergi* = 0.5%; *Bathyraja violacea* vs. *Bathyraja interrupta* = 0.6%; *Bathyraja aleutica* vs. *Bathyraja abyssicola* = 0.9%; *Bathyraja mariposa* vs. *B. interrupta* = 1.1%; *Bathyraja parmifera* vs. *Bathyraja smirnovi* = 1.2% [Spies et al. 2006], and *Carcharhinus* [K2P distance]: *Carcharinus altimus* vs. *Carcharinus plumbeus* = 0.51%; *Carcharinus limbatus* vs. *Carcharinus tilstoni* = 0.45% [Ward et al. 2008]); and 2) the K2P COI distances between the *A. narinari* lineages (2.8–3.9%) was substantially higher than the average, intraspecific K2P COI genetic distances determined for 171 elasmobranch species (0.37%; Ward et al. 2008).

The genealogical distinctiveness and comparative genetic distance results considered together lend strong support to according full species status to the Western/Central Pacific *A. narinari* lineage. However, despite the concordant (nuclear and mitochondrial) genealogical distinctiveness of the Eastern Pacific lineage, the comparatively smaller genetic distances between this and the Central Atlantic lineage tempers the case for providing full species recognition to both these lineages. We therefore recommend listing Central Atlantic and Eastern Pacific lineages together as a separate single species distinct from the Western/Central Pacific species. We also recommend that the Central Atlantic and Eastern Pacific lineages each be provided separate subspecies status in accordance with the genealogical concordance criteria.

It is worthy of note that our findings are concordant with previous speculation on the taxonomic status of *A. narinari* based on the distribution of parasitic worms; these distributions had led to suggestions that Atlantic *A. narinari* were distinct from those in the Indo-Pacific (Marie and Justine 2005).

Isolation and the Panamanian Isthmus

The 432-bp cyt *b* data set included *H. schmarde* and *S. tiburo tiburo* from the Caribbean and *H. pacifica* and *S. tiburo vespertina* from the East Pacific. It has been proposed that these species/subspecies were isolated when the Isthmus of Panama closed (Martin et al. 1992; Lovejoy 1996). Interestingly, the closely matched cyt *b* genetic distances (5.6–6.6%) across the Isthmus for *Himantura* and *Sphyrna* were more than twice that for *A. narinari* (2.5–2.6%) (Table 1). A homogeneous mutation rate among lineages in the reduced 432-bp data set allowed us to apply the same cyt *b* mutation rate among these species. Consequently, if we assume that the *Himantura* and *Sphyrna* taxa were isolated by the isthmus closure 3.1 or 2 Ma, it would date (using patristic distance) the *A. narinari* isolation across the same divide at 1.2 or 0.8 Ma, respectively—an improbable timing because it is well after even the most recent date (2 My) for isthmus closure. As a result, if we assume *A. narinari* populations were isolated at even the most recent isthmus closure date of 2 Ma (Cronin and Dowsett 1996), it would date isolation of *Himantura* and *Sphyrna* taxa at approximately 5 Ma. These results suggest that both the *Himantura* and *Sphyrna* taxa were probably isolated prior to closure. Numerous studies have shown that many transisthmian taxa probably diverged prior to closure (Tringali et al. 1999; Marko 2002; Banford et al. 2004).

Evolutionary History

In addition to *A. narinari* with its circumtropical distribution, there are 3 other described *Aetobatus* species, *A. flagellum*, *Aetobatus guttatus*, and *Aetobatus ocellatus*, all with distributions restricted to the East Indies Triangle (Compagno and Last

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

1999). This region is proposed to be a center of speciation and subsequent radiation for many marine taxa within the Indo-West Pacific (Briggs 2000, 2005; and references therein). Thus, a credible hypothesis for the biogeographic pattern observed for *Aetobatus* is that this genus originated in the Indo-West Pacific, with *A. narinari* subsequently radiating throughout the Pacific and into the Atlantic Ocean. Phylogenetic analyses of the combined mitochondrial–nuclear data set using *A. flagellum* as an outgroup reveals an ancestral position for the Western/Central Pacific lineage, corroborating the hypothesis of an Indo-West Pacific origin for *A. narinari*. A similar Pacific ancestral bias has also been found for the globally distributed olive ridley turtle (*Lepidochelys olivacea*) and the scalloped hammerhead shark (*Sphyrna lewini*) (Bowen et al. 1998; Duncan et al. 2006).

Dispersal Pathways

Assuming an Indo-West Pacific origin for *A. narinari*, there are 2 plausible dispersal pathways into the Atlantic: 1) east across the east Pacific barrier (EPB) and then through the isthmus prior to closure (a third potential pathway, dispersal around the southern tip of South America, is considered highly unlikely due to the extremely cold water) or 2) west through the Indian Ocean and then around the southern tip of Africa. Both routes contain formidable barriers to dispersal and gene flow. The EPB is an expanse of deep open water without islands stretching 4000–7000 km from the west coast of the Americas to the Central Pacific; the southern tip of Africa represents a dramatic transition between the warm waters of the Aghulhas current along the southeast coast and the colder waters off the southwest coast created by the Benguela upwelling system. However, it seems that neither barrier is impassable. For example, the EPB appears to have been breached numerous times by reef fish and urchin larvae (Lessios et al. 1998, 2001; Lessios and Robertson 2006) and also by migrations of the olive ridley turtle and the scalloped hammerhead shark (Bowen et al. 1998; Duncan et al. 2006). The South African coastline has been widely proposed as a pathway for species invading the Atlantic from the Indian Ocean (Briggs 1974; Bowen et al. 1994, 2001; Rocha et al. 2005).

The mitochondrial–nuclear phylogenetic analyses combined with the COI and ITS2 statistical parsimony results support an *A. narinari* western dispersal into the Atlantic via the southern tip of Africa. For example, using the ancestral position of the Western/Central Pacific lineage to polarize the TCS networks (Templeton 1998), both COI and ITS2 networks showed the Eastern Pacific lineage as most derived, with the Central Atlantic intermediate between the Eastern Pacific and Western/Central Pacific lineages. The cyt *b* TCS network is ambiguous regarding this issue with either plausible dispersal pathway possible, accompanied by a subsequent sundering of a combined Central Atlantic and Eastern Pacific lineage by the isthmus closure. The ambiguity of the cyt *b* results notwithstanding, the TCS networks suggest a dispersal path westward from the Pacific, through the Atlantic, and into the Caribbean, with the Eastern Pacific subsequently colonized from the Caribbean prior to the closure of the Isthmus of Panama.

Further support for the South African dispersal pathway is provided by the history of the Benguela upwelling system. Marlow et al. (2000) showed that the water temperature of the region was previously warmer than today and started to cool approximately 3.2 Ma due to intensification of wind-driven upwelling. Therefore, it seems possible that *A. narinari* individuals may have been able to disperse around the southern tip of Africa prior to the onset of cooling. The date reported for the onset of cooling is remarkably close to the divergence time estimated for the Western/Central Pacific and Central Atlantic lineages of 3.4 Ma (using the mutation rate of 0.90%). Therefore, as also suggested for trumpetfishes (Bowen et al. 2001), the eventual speciation between these *A. narinari* populations may not have been the result of a rare migration but rather a vicariant sundering of a warm water corridor between ocean basins. Clearly, the examination of *A. narinari* samples from the northwest coast of Africa would provide further insight to the evolutionary history of this species.

Conclusion

Utilizing the combination of genealogical concordance and genetic distance criteria, we have delineated the globally distributed species of *A. narinari* (Euphrasen 1790) into at least 2 distinct species. One species ranges through the Western and Central Pacific and the other through the Central Atlantic and the Eastern Pacific. The latter species is further divided into 2 subspecies separated by the Isthmus of Panama. Our data support an Indo-West Pacific origin for the *A. narinari* species complex, followed by radiation into the Atlantic and Eastern Pacific, probably via the southern tip of Africa. In addition to providing taxonomic clarification and insight into evolutionary history, these findings have direct management implications, as the reduced ranges and population sizes of the delineated species reinforce concerns about the already threatened and vulnerable status of these batoids.

Funding

NOAA Center for Sponsored Coastal Ocean Science award #NA04NOS4260065 to the National Coral Reef Institute; the Save Our Seas Foundation Project grant 71; the Guy Harvey Research Institute operational funds. This is NCRI publication No. 111.

Acknowledgments

We thank J. Bizzaro, D. Chapman, M. Corcoran, K. Duncan, D. Gilliam, D. Grubbs, A. Hebard, K. Longenecker, F. Marquez, J. Ovenden, W. Smith, S. Watabe, A. Yamaguchi, and the Hong Kong Aquarium staff for tissue samples and R. Vargo for laboratory assistance. We are grateful to the associate editor Steve Karl and 3 anonymous reviewers for comments that improved the manuscript.

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

References

Avise JC. 1998. Conservation genetics in the marine realm. J Hered. 89:377–382.

Avise JC. 2000. Phylogeography: the history and formation of species. Cambridge (MA): Harvard University Press.

Avise JC, Ball RM. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. In: Futuyma D, Antonovies J, editors. Oxford surveys in evolutionary biology. New York: Oxford University Press. p. 45–67.

Baldwin BG, Markos S. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S–26S rDNA: congruence of ETS and its trees of *Calycadenia* (Compositae). Mol Phylogenet Evol. 10:449–463.

Banford HM, Bermingham E, Collette BB. 2004. Molecular phylogenetics and biogeography of transisthmian and amphi-Atlantic needlefishes (Belonidae: *Strongylura* and *Tylosurus*): perspectives on New World marine speciation. Mol Phylogenet Evol. 31:833–851.

Bowen BW, Bass AL, Rocha LA, Grant WS, Robertson DR. 2001. Phylogeography of the trumpetfishes (*Aulostomus*): ring species complex on a global scale. Evolution. 55:1029–1039.

Bowen BW, Clark AM, Abreu-Grobois FA, Chaves A, Reichart HA, Ferl RJ. 1998. Global phylogeography of the ridley sea turtles (*Lepidochelys* spp.) as inferred from mitochondrial DNA sequences. Genetica. 101:179–189.

Bowen BW, Kamezaki N, Limpus CJ, Hughes GR, Meylan AB, Avise JC. 1994. Global phylogeography of the loggerhead turtle (*Caretta caretta*) as indicated by mitochondrial DNA haplotypes. Evolution. 48:1820–1828.

Briggs JC. 1974. Marine zoogeography. New York: McGraw-Hill Book Co.

Briggs JC. 2000. Centrifugal speciation and centers of origin. J Biogeogr. 27:1183–1188.

Briggs JC. 2005. The marine East Indies: diversity and speciation. J Biogeogr. 32:1517–1522.

Buckley-Beason VA, Johnson WE, Nash WG, Stanyon R, Menninger JC, Driscoll CA, Howard J, Bush M, Page JE, Roelke ME, et al. 2006. Molecular evidence for species-level distinctions in clouded leopards. Curr Biol. 16:2371–2376.

Bull JJ, Huelsenbeck JP, Cunningham CW, Swofford DL, Waddell PJ. 1993. Partitioning and combining data in phylogenetic analysis. Syst Biol. 42:384–397.

Clement M, Posada D, Crandall AK. 2000. TCS: a computer program to estimate gene genealogies. Mol Ecol. 9:1657–1659.

Coates AG, Obando JA. 1996. The geological evolution of the Central American Isthmus. In: Jackson J, Budd A, Coates A, editors. Evolution and environment in tropical America. Chicago (IL): The University of Chicago Press. p. 21–56.

Coleman AW, Vacquier VD. 2002. Exploring the phylogenetic utility of its sequences for animals: a test case for abalone (*Haliotis*). J Mol Evol. 54:246–257.

Compagno LJV, Last PR. 1999. Myliobatidae. Eagle rays. In: Carpenter KE, Niem V, editors. FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Rome (Italy): Food and Agricultural Organization. p. 1511–1519.

Compagno LJV, Last PR, Stevens JD, Alava MNR. 2005. Checklist of Philippine chondrichthyes. CSIRO marine laboratories report 243. Tasmania, Australia.

Cracraft J. 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: Otte D, Endler J, editors. Speciation and its consequences. Sunderland (MA): Sinauer. p. 28–59.

Crandall KA, Templeton AR. 1993. Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics. 134:959–969.

Cronin TM, Dowsett HJ. 1996. Biotic and oceanographic response to the Pliocene closing of the Central American Isthmus. In: Jackson J, Budd A, Coates A, editors. Evolution and environment in tropical America. Chicago (IL): University of Chicago Press. p. 76–104.

Cunningham CW. 1997. Can three incongruence tests predict when data should be combined? Mol Biol Evol. 14:733–740.

De Queiroz K. 1998. The general lineage concepts of species, species criteria, and the process of speciation. In: Howard D, Berlocher S, editors. Endless forms: species and speciation. New York: Oxford University Press. p. 57–75.

Duncan KM, Martin AP, Bowen BW, De Couet HG. 2006. Global phylogeography of the scalloped hammerhead shark (*Sphyrna lewini*). Mol Ecol. 15:2239–2251.

Dunn KA, McEachran JD, Honeycutt RL. 2003. Molecular phylogenetics of myliobatiform fishes (Chondrichthyes: Myliobatiformes), with comments on the effects of missing data on parsimony and likelihood. Mol Phylogenet Evol. 27:259–270.

Euphrasen BA. 1790. Raja (narinari). Kongl Vet Acad Handl. 11:217–219.

Farias IP, Ortí G, Meyer A. 2000. Total evidence: molecules, morphology, and the phylogenetics of cichlid fishes. J Exp Zool. 288:76–92.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783–791.

Fourment M, Gibbs MJ. 2006. PATRISTIC: a program for calculating patristic distances and graphically comparing the components of genetic change. BMC Evol Biol. 6:1. doi: 10.1186/1471-2148-1186-1181.

Frankham R, Ballou JD, Briscoe DA. 2002. Introduction to conservation genetics. Cambridge (UK): Cambridge University Press.

Fraser DJ, Bernatchez L. 2001. Adaptive evolutionary conservation: towards a unified concept for defining conservation units. Mol Ecol. 10:2741–2752.

Golenberg EM, Clegg MT, Durbin ML, Doebley J, Ma DP. 1993. Evolution of a noncoding region of the chloroplast genome. Mol Phylogenet Evol. 2:52–64.

Good DA, Wake DB. 1992. Geographic variation and speciation in the torrent salamanders of the genus *Rhyacotriton* (Caudata: Rhyacotritonidae). Univ Calif Publ Zool. 126:1–91.

Hart MW, Sunday J. 2007. Things fall apart: biological species form unconnected parsimony networks. Biol Lett. 3:509–512.

Highton R. 1990. Taxonomic treatment of genetically differentiated populations. Herpetologica. 46:114–121.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol. 42:182–192.

Hillis DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q Rev Biol. 66:411–453.

Huelsenbeck JP, Rannala B. 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. Science. 276:227–232.

Kishino H, Miyata T, Hasegawa M. 1990. Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. J Mol Evol. 31:151–160.

Knowlton N. 2000. Molecular genetic analyses of species boundaries in the sea. Hydrobiologia. 420:73–90.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5:150–163.

Kyne PM, Ishihara H, Dudley SFJ, White WT. 2006. Aetobatus narinari. 2007 IUCN red list of threatened species. Available from: URL http://www.iucnredlist.org. [cited 2008 May 22].

Lefébure T, Douady CJ, Gouy M, Gibert J. 2006. Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. Mol Phylogenet Evol. 40:435–447.

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024

Lessios HA, Kessing BD, Pearse JS. 2001. Phylogeography of the pantropical sea urchin *Tripneustes*: contrasting patterns of population structure between oceans. Evolution. 57:2026–2036.

Lessios HA, Kessing BD, Robertson DR. 1998. Massive gene flow across the world's most potent marine biogeographic barrier. Proc R Soc Lond Ser B Biol Sci. 265:583–588.

Lessios HA, Robertson DR. 2006. Crossing the impassable: genetic connections in 20 reef fishes across the Eastern Pacific Barrier. Proc R Soc Lond B Biol Sci. 273:2201–2208.

Lloyd DG, Calder VL. 1991. Multi-residue gaps, a class of molecular characters with exceptional reliability for phylogenetic analyses. J Evol Biol. 4:9–21.

Long EO, Dawid IB. 1980. Repeated genes in eukaryotes. Annu Rev Biochem. 49:727–764.

Lovejoy NR. 1996. Systematics of myliobatoid elasmobranchs: with emphasis on the phylogeny and historical biogeography of neotropical freshwater stingrays (Potamotrygonidae: Rajiformes). Zool J Linnean Soc. 117:207–257.

Maddison DR, Maddison WP. 2001. MacClade 4: analysis of phylogeny and character evolution. Version 4.03. Sunderland (MA): Sinauer Associates.

Marie AD, Justine J. 2005. Monocotylids (Monogenea: Monopisthocotylea) from *Aetobatus* cf. *narinari* off New Caledonia, with a description of *Decacotyle elpora* n. sp. Syst Parasitol. 60:175–185.

Marko PB. 2002. Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the isthmus of panama. Mol Biol Evol. 19:2005–2021.

Marlow JR, Lange CB, Wefer G, Rosell-Mele A. 2000. Upwelling intensification as part of the Pliocene-Pleistocene climate transition. Science. 290:2288–2291.

Martin AP, Naylor GJP, Palumbi SR. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature. 357:153–155.

Mayr E. 1963. Animal species and evolution. Cambridge (MA): Harvard University Press.

Meiri S, Mace GM. 2007. New taxonomy and the origin of species. PLoS Biol. 5:1835–1836.

Nicholas KB, Nicholas HB Jr, Deerfield DW II. 1997. Genedoc: analysis and visualization of genetic variation. Embnew News. 4:14.

Pfenninger M, Posada D. 2002. Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migration, and secondary contact. Evolution. 56:1776–1788.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics. 14:817–818.

Prather LA, Jansen RK. 1998. Phylogeny of Cobaea (Polemoniaceae) based on sequence data from the ITS region of nuclear ribosomal DNA. Syst Bot. 23:57–72.

Roberts TR, Karnasuta J. 1987. *Dasyatis laosensis*, a new whiptailed stingray (family *Dasyatidae*), from the Mekong river of Laos and Thailand. Environ Biol Fishes. 20:161–167.

Rocha LA, Robertson DR, Rocha CR, Van Tassell JL, Craig MT, Bowen BW. 2005. Recent invasion of the tropical Atlantic by an Indo-Pacific coral reef fish. Mol Ecol. 14:3921–3928.

Rubinoff D. 2005. Utility of mitochondrial DNA barcodes in species conservation. Conserv Biol. 20:1026–1033.

Sezaki K, Begum RA, Wongrat P, Srivastava MP, SriKantha S, Kikuchi K, Ishihara H, Tanaka S, Taniuchi T, Watabe S. 1999. Molecular phylogeny of Asian freshwater and marine stingrays based on the DNA nucleotide and deduced amino acid sequences of the cytochrome *b* gene. Fish Sci. 65:563–570.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol. 16:1114–1116.

Simmons MP, Ochoterena H. 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst Biol. 49:369–381.

Simmons MP, Ochoterena H, Carr TG. 2001. Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analyses. Syst Biol. 50:454–462.

Spies IB, Gaichas S, Stevenson DE, Orr JW, Canino MF. 2006. DNA-based identification of Alaska skates (*Amblyraja*, *Bathyraja* and *Raja*: Rajidae) using cytochrome c oxidase subunit I (COI) variation. J Fish Biol. 69(Suppl B): 283–292.

Sullivan J. 1996. Combining data with different distributions of among-site rate variation. Syst Biol. 45:375–380.

Swofford DL. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland (MA): Sinauer Associates.

Templeton AR. 1998. Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. Mol Ecol. 7:381–397.

Templeton AR, Crandall KA, Sing CF. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics. 132:619–633.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.

Tringali MD, Bert TM, Seyoum S, Bermingham E, Bartolacci D. 1999. Molecular phylogenetics and ecological diversification of the transisthmian fish genus *Centropomus* (Perciformes: Centropomidae). Mol Phylogenet Evol. 13:193–207.

Vogler C, Benzie J, Lessios H, Barber P, Worheide G. 2008. A threat to coral reefs multiplied? Four species of crown-of-thorns starfish. Biol Lett. 4:696–699.

Wang JY, Frasier TR, Yang SC, White BN. 2008. Detecting recent speciation events: the case of the finless porpoise (genus *Neophocaena*). Heredity. 101:145–155.

Ward RD, Holmes BH, White WT, Last PR. 2008. DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. Mar Freshw Res. 59:57–71.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. 2005. DNA barcoding Australia's fish species. Philos Trans R Soc Lond B Biol Sci. 360:1847–1857.

Wiens JJ, Penkrot TA. 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Syst Biol. 51:69–91.

Received November 4, 2008; Revised January 20, 2009; Accepted February 18, 2009

Corresponding Editor: Stephen Karl

Downloaded from https://academic.oup.com/jhered/article/100/3/273/868985 by guest on 23 April 2024