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Research Article

A test of the utility of DNA barcoding in the radiation of the freshwater stingray genus *Potamotrygon* (Potamotrygonidae, Myliobatiformes)

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Abstract

DNA barcoding is a recently proposed global standard in taxonomy based on DNA sequences. The two main goals of DNA barcoding methodology are assignment of specimens to a species and discovery of new species. There are two main underlying assumptions: *i)* reciprocal monophyly of species, and *ii)* intraspecific divergence is always less than interspecific divergence. Here we present a phylogenetic analysis of the family Potamotrygonidae based on mitochondrial cytochrome *c* oxidase I gene, sampling 10 out of the 18 to 20 valid species including two non-described species. Potamotrygonidae systematics is still not fully resolved with several still-to-be-described species while some other species are difficult to delimit due to overlap in morphological characters and because of sharing a complex color patterns. Our results suggest that the family passed through a process of rapid speciation and that the species *Potamotrygon motoro*, *P. scobina*, and *P. orbignyi* share haplotypes extensively. Our results suggest that systems of identification of specimens based on DNA sequences, together with morphological and/or ecological characters, can aid taxonomic studies, but delimitation of new species based on threshold values of genetic distances are overly simplistic and misleading.

Key words: Potamotrygon, barcoding, radiation, phylogenetics, COI, mtDNA.

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Introduction

The Consortium for the Barcode of Life was created in order to develop DNA barcoding as a global standard (http://barcoding.si.edu). The objective of DNA barcoding is the use of one or more genes to *i)* assign unknown specimens to its species, and *ii)* increase the discovery of new species (Moritz and Cicero, 2004). The proponents of barcoding argue that there exists an overwhelming number of undescribed species and the number of traditional taxonomists is too few to handle this diversity; morphologically cryptic species are overlooked by traditional morphological methods; and larval stages of some species groups often cannot be assigned to the correct species based on morphology (Hebert *et al.*, 2003). The DNA barcoding supposedly would be a fast, efficient, and globally accessible method for delimiting and identifying new species (Hebert *et al.*, 2003). The mitochondrial gene cytochrome *c* oxidase I (COI) was chosen as the standard gene for DNA barcoding because it shows a conserved amino acid sequence that facilitates the design of universal primers applicable to a diverse group of organisms; COI also apparently functions well to discriminate species (Hebert *et al.*, 2003, 2004; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006).

There are two basic assumptions that underpin DNA barcoding methodology; 1) monophyly of species with respect of the molecular marker used, and 2) intraspecific genetic divergence is much smaller than genetic differences among species, thus justifying the use of divergence thresholds to assign individuals to correct species based on genetic divergence. In a seminal publication on barcoding,

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Hebert *et al.* (2004) found in a survey of 260 North American bird species that COI divergences among close relatives were on average 18 times higher than intraspecific divergences. The authors proposed a 10x threshold divergence between clades to within clades as a way to accelerate the discovery of new species, *i.e.* divergences over the 10x threshold most likely represent divergences among species rather than within species. Using this approach Hebert *et al.* (2004) identified four possible new species of birds nesting in North America. Hajibabaei *et al.* (2006) found distinct COI barcodes for 97.9% out of 521 lepidopteran species surveyed in Costa Rica, albeit no threshold value was proposed to assign specimens to described species or to identify new species, an important component of barcoding.

However, critics of DNA barcoding argue that many of the barcoding studies do not compare sister species, the primary candidates to share haplotypes and to show low interspecific divergence. Additionally, because these studies do not sample diversity within the geographical distribution of the species, *i.e.* they are typological in character, current DNA barcoding studies tend to underestimate intraspecific genetic variability (Moritz and Cicero, 2004). Indeed, Johnson and Cicero (2004) found results that sharply contrast those of Hebert *et al.* (2004) when comparing sister species of birds from North America. They found average interspecific genetic values much lower and intraspecific genetic variability much higher than those found by Hebert *et al.* (2004), and they also found many cases of paraphyly.

In the present study we test the efficiency of the mitochondrial gene COI for delimiting species of freshwater stingrays of the family Potamotrygonidae, using the methodology proposed by the DNA barcoding consortium. The family Potamotrygonidae is currently composed of three genera, *Paratrygon* Duméril, 1865, *Plesiotrygon* Rosa, Castello and Thorson, 1987, and *Potamotrygon* Garman, 1877. The first two genera are considered monotypic while *Potamotrygon* contains 16 (Carvalho *et al.*, 2003) to 18 (Rosa, 1985) taxonomically valid species. However, the taxonomy of the family is not well resolved with some species being considered taxonomically dubious, and several species yet to be described (Carvalho *et al.*, 2003).

Earliest taxonomic recognition of potamotrygonid stingrays dates to the colonial period (Castex *et al.*, 1963 and references therein); however, it was not until the 1960's and 1970's that taxonomist became interested in this group. Studies from this period were based on few individuals (*e.g.* Castex *et al.*, 1963; Castex, 1964; Achenbach and Achenbach, 1976) and were unable to encompass the phenotypic variation observed in nature. Rosa (1985) provided the first taxonomic revision of the family, generating the first genus-level phylogenetic hypothesis. Rosa (1985) also produced a species key for the Potamotrygonidae; the key was based primarily on coloration patterns, although his study relied primarily on specimens already deposited in museums. We used the key of Rosa (1985) and characters in original descriptions to classify our specimens.

Starting in the late 1990's, several groups of researchers initiated field studies and collections in the Amazon basin focusing particularly on the Negro River (Araújo, 1998), the Amazon River (Charvet-Almeida, 2004), especially its mouth and lower portion (Charvet-Almeida, 2001; Almeida, 2003), the Xingu River (Charvet-Almeida, 2006) and Tocantins River (Rincon, 2006). These studies resulted in the discovery of new and what appear to be new species, but also demonstrated very high degrees of polychromatism in some species pointing out the difficulty of delimiting species using the key proposed by Rosa (1985). An especially high degree of polychromatism was observed in *Potamotrygon motoro* and *Potamotrygon orbignyi* in the Negro River (Araújo, 1998), *Potamotrygon motoro* in the Amazon River (Charvet-Almeida, 2004), *Potamotrygon scobina* in the mouth of the Amazon River (Almeida, 2003), and *Potamotrygon orbignyi* in the Paranã-Tocantins River (Rincon, 2006), and in the Xingu River (Charvet-Almeida, 2006). Despite their high degree of polychromatism, the taxonomic validity of these three species of *Potamotrygon* is uncontested by taxonomists specializing in chondrichthyans (Carvalho *et al.*, 2003; Rosa and Carvalho, 2007).

To test the efficacy of DNA barcoding in delimiting potamotrygonid species, we generated the first comprehensive hypothesis of intra-familial phylogenetic relationships for the freshwater stingray family Potamotrygonidae and also evaluated the intraspecific genetic diversity of sampled species. We survey 10 out of the 18 valid species using a 522 bp portion of the COI gene, to infer phylogenetic relationships within the family, to test alternate phylogenetic hypotheses, and to test the utility of DNA barcoding in this family.

Material and Methods

Sampling of taxa

We sampled six valid species and two non described species of the genus *Potamotrygon* with at least two individuals per species, for a total of 36 specimens (Table 1).

We analyzed the species *Potamotrygon motoro* (n = 10), *P. orbignyi* (n = 6), *P. scobina* (n = 2), *P. leopoldi* (n = 2), *P. falkneri* (n = 3), *P. schroederi* (n = 4), *Potamotrygon cf. motoro* (n = 4), and *Potamotrygon* sp.1 (n = 5). *Potamotrygon* sp.1 and *Potamotrygon cf. motoro* are endemic to the Amazonian Negro River and Tapajós River, respectively. Both species are well characterized morphologically and ecologically (Araújo, 1998; Carvalho, pers. com.), but have yet to be scientifically described. We also included sequences of *P. henlei* (n = 1), and *Plesiotrygon iwamae* (n = 1) from Marques (2000). As outgroup, we included the shark *Heterodontus francisci* (GenBank# AJ310141), and the Myliobatiformes stingray species *Hexatrygon bickelli* (GenBank# AY597334), and

Table 1 - Analyzed species and localities of collection.

Species	*N*	Site of collection	Reference
Potamotrygon motoro	1	Aripuanã River, AM, Brazil (06° 00' S, 60° 12' W)	Present study
	2	Janauacá Lake, AM, Brazil (03° 28' S, 60° 17' W)	
	1	Parguaza River, AM, Venezuela (06° 24' N, 67° 10' W)	
	3	Juruá River, AM, Brazil (04° 51' S, 66° 51' W)	
	1	Jarauá River, AM, Brazil (02° 76' S, 64° 88' W)	
	1	Itu River, AM, Brazil (00° 29' S, 63° 15' W)	
	1	Demini River, AM, Brazil (00° 47' S, 62° 56' W)	
Potamotrygon orbignyi	2	Demini River, AM, Brazil (00° 31' S, 62° 53' W)	Present study
	4	Aripuanã River, AM, Brazil (06° 07' S, 60° 13' W)	
Potamotrygon scobina	1	Pará River, PA, Brazil (00° 54' S 48° 17' W)	Present study
	1	Aripuanã River, AM, Brazil (06° 07' S, 60° 13' W)	
Potamotrygon leopoldi	1	Xingu River, PA, Brazil (03° 15' S, 52° 04' W)	Present study
	1	Xingu River, PA, Brazil (03° 34' S, 51° 52' W)	
Potamotrygon falkneri	3	Paraná River, MS, Brazil (20° 45' S, 51° 40' W)	Present study
Potamotrygon schroederi	1	Caura River, BO, Venezuela (06° 50' S, 64° 47' W)	Present study
	3	Demini River, AM, Brazil (00° 46' S, 62° 56' W)	
Potamotrygon sp. 1	2	Aiuanã River, AM, Brazil (00° 52' S, 65° 15' W)	Present study
	3	Itu River, AM, Brazil (00° 27' S, 63° 37' W)	
Potamotrygon cf. *motoro*	4	Tapajós River, PA, Brazil (04° 33' S, 56° 15' W)	Present study
Potamotrygon henlei	1	Tocantins River	Marques (2000)
Plesiotrygon iwamae	1	Solimões River	Marques (2000)
Himantura pacifica	1	Panama	Marques (2000)
Hexatrygon bickelli	1		GenBank: AY597334
Heterodontus francisci	1		GenBank: AJ310141
Total	41		

Himantura pacifica. The genus *Himantura* is considered the most likely sister taxon of Potamotrygonidae (Lovejoy, 1996; McEachran *et al.*, 1996; Lovejoy *et al.*, 1998; Dunn *et al.*, 2003).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from muscle tissues preserved in alcohol by the standard protocol of Sambrook *et al.* (1989). We amplified the mitochondrial COI gene by the polymerase chain reaction (PCR) with the primers COIf 5'-ctgcaggaggagaycc-3' (forward) and COIa 5'-agtataagcgtctgggtagtc-3' (reverse), described by Palumbi and Benzie (1991). The PCR reactions were performed in 25 µL reaction volume, containing 2.5 µL of dNTPs (2.5 mM); 2.5 µL of 10X buffer (100 mM Tris-HCl, 500 mM KCl); 2 µL from each primer (2 µM), 3 µL of $MgCl_2$ (25 mM); 1 µL of DNA (ca. 10 ng) e 1 U of Taq DNA polymerase. The amplification cycles were carried out as follows: 35 cycles of denaturing at 92 °C for 1 min; annealing at 52 °C for 35 s; and extension at 72 °C for 90 s. A final extension was carried out at 72 °C for 5 min. PCR products were visualized on 1% agarose gel. PCR products were then purified with the *GFX PCR DNA Kit* (GE Healthcare), and eluted in 20 µL of elution buffer. Amplification primers were used as cycle sequencing primers, and cycle sequencing reactions were carried out at 52 °C following the protocol recommended by the manufacturer of the kit *ET Terminator Cycle Sequencing Kit* (GE Healthcare). Cycle sequencing reactions were carried out in a final volume of 10 µL and contained 4 µL of DNA, 2 µL of 0.2 µM primer, 2 µL of buffer supplied in the *ET* kit, and 2 µL of *ET* mix. Amplified products were precipitated using standard ammonium acetate/ethanol precipitation. Cycle sequencing products were resuspended in Hi-Di Formamide, and resolved on the MegaBACE 1000 (GE Healthcare) automatic sequencer.

Alignment

Sequences were aligned in ClustalW (Thompson *et al.*, 1996) using default setting and edit by eye. ClustalW is implemented in the program BioEdit (Hall, 1999). Variable sites were checked in MEGA 3.0 (Kumar *et al.*, 2004), and the complete alignment was translated into putative amino acids. The 522 bp alignment did not show insertions or deletions, and conceptual translation did not reveal any unexpected stop codons.

Phylogenetic analyses and hypotheses testing

Phylogenetic analyses under neighbor-joining (NJ), Maximum-likelihood (ML), and Bayesian-inference (BI) optimality criteria were performed using PAUP* 4.0b10

(Swofford, 2002). The NJ methodology is the standard method of phylogenetic inference in DNA barcoding studies (Hebert *et al.*, 2003); its use in DNA barcoding studies is in part due to its strong track record in being able to rapidly analyze large species assemblages (Kumar and Gadagkar, 2000). The chosen molecular substitution model was the computationally simple Kimura-two-parameter (K2P) (Kimura, 1980) which is the standard model of molecular evolution used in DNA barcoding studies (Hebert *et al.*, 2003). Robustness of the NJ topology was assessed using 2,000 bootstrap replicates.

We used the software Modeltest 3.7 (Posada and Crandall, 1998) to determine the best suited model of sequence evolution and the accompanying evolutionary parameter values for the data. The General Time Reversible (GTR) model of molecular evolution (Rodríguez *et al.*, 1990), with rate homogeneity and a portion of sites treated as invariable, was determined to be the most likely model of sequence evolution. The GTR + inv model of sequence evolution was implemented in ML and BI analyses. Maximum-likelihood topology was also estimated in PAUP* 4.0b10 (Swofford, 2002) with 25 heuristic searches using random addition of sequences, and implementing the tree bisection and reconnection (TBR) algorithm. Statistical robustness of the ML topology was accessed using 200 bootstrap replicates.

Bayesian-inference analysis was performed in the program MrBayes 3.01 (Ronquist and Huelsenbeck, 2003). The data were partitioned into three categories representing first, second, and third positions of the COI gene, each following the GTR + inv model of sequence evolution. We ran 2,000,000 generations using default long and short chain and heating parameters, sampling trees, and branch-length every 100 generations. Log likelihoods stabilized within the first 10% of the run, and therefore we discarded these initial 200,000 trees as burnin steps in the computation of a 50% majority rule consensus tree.

Pair-wise distances under Kimura-two-parameter (K2P), and maximum likelihood GTR + inv models of molecular evolution were generated in PAUP* 4.0b10 (Swofford, 2002).

Alternate phylogenetic hypotheses were tested within the ML and NJ frameworks. Four hypotheses were tested: monophyly of *P. motoro*, monophyly of *P. orbignyi*, monophyly of *P. scobina*, and the reciprocal monophyly of the three species. We found the most likely or the best NJ trees that satisfied these constrains, and compared them against the most likely and best NJ topologies using the Kishino-Hasegawa (K-H) test (Kishino and Hasegawa, 1989), and Shimodaira-Hasegawa (S-H) tests (Shimodaira and Hasegawa, 1999). Assessment of significance was tested using the RELL bootstrap (Kishino *et al.*, 1990). We also tested the hypothesis that our data follows a clock-like tempo of molecular evolution by enforcing the constraint of a clock-like mode of molecular evolution, and testing if this constraint resulted in a significantly less likely phylogenetic hypothesis using the likelihood-ratio test (Huelsenbeck and Rannala, 1997). To test if alleles of species were under natural selection, we used the McDonald-Kreitman test (McDonald and Kreitman, 1991) implemented in the program DnaSP 3.1 (Rozas *et al.*, 2003).

Results

Phylogenetic analyses

The COI sequence alignment comprises 522 sites of which 180 were variable and 121 were parsimony informative (GenBank accession numbers EF532644-EF532683). Within Potamotrygonidae, the dataset contained 111 variable sites and 79 parsimony informative sites. All three methods of phylogenetic reconstruction indicated that the three most broadly distributed species in the Amazon basin, *P. motoro*, *P. orbignyi*, and *P. scobina*, are not reciprocally monophyletic (Figures 1-3). Monophyly of the other species including *Potamotrygon* sp. 1, and *Potamotrygon cf. motoro*, is statistically well supported in all analyses. The species *P. motoro*, *P. orbignyi*, *P. scobina*, *P. leopoldi*, *P. falkneri*, *Potamotrygon cf. motoro*, and *P. henlei*, hereafter called the rosette-spot clade, form a well defined and well supported clade; however, relationships among the species of the rosette-spot clade remain unresolved in all three methods of phylogenetic reconstruction (Figures 1-3).

The phylogenetic position of *Plesiotrygon iwamae* conflicts among NJ, ML, and BI analyses. In the NJ topology, *Plesiotrygon iwamae* appears as sister to all species of *Potamotrygon* (Figure 1). In the ML phylogeny, *Plesiotrygon iwamae* is sister to *Potamotrygon schroederi*, and *Potamotrygon* sp.1 appears as sister to the rest of the species of the family Potamotrygonidae (Figure 2). In the BI phylogeny, *Plesiotrygon iwamae* appears nested within *Potamotrygon* with *P. schroederi* as sister to other species in family (Figure 3). The internal tree branches within the Potamotrygonidae phylogeny are relatively short, especially within the rosette-spot clade.

Maximum parsimony analysis was not carried out since we have multiple individuals represented by the same haplotype which in turn resulted in a large number of equally parsimonious topologies. The consensus of these equally parsimonious topologies had the same phylogenetic pattern obtained in other analyses, but statistical tests could not be carried out efficiently.

Consistent with expectation, pair-wise genetic distances presented similar values for closely related species under both Kimura-two-parameter (K2P) and maximum likelihood GTR + inv models of molecular evolution. However, relatively smaller distances were found for distantly related species in K2P distance than in the GTR + inv model due to the inability of the K2P model to compensate for the accumulation of homoplaseous changes at deeper phylogenetic divergence (Tables 2 and 3).

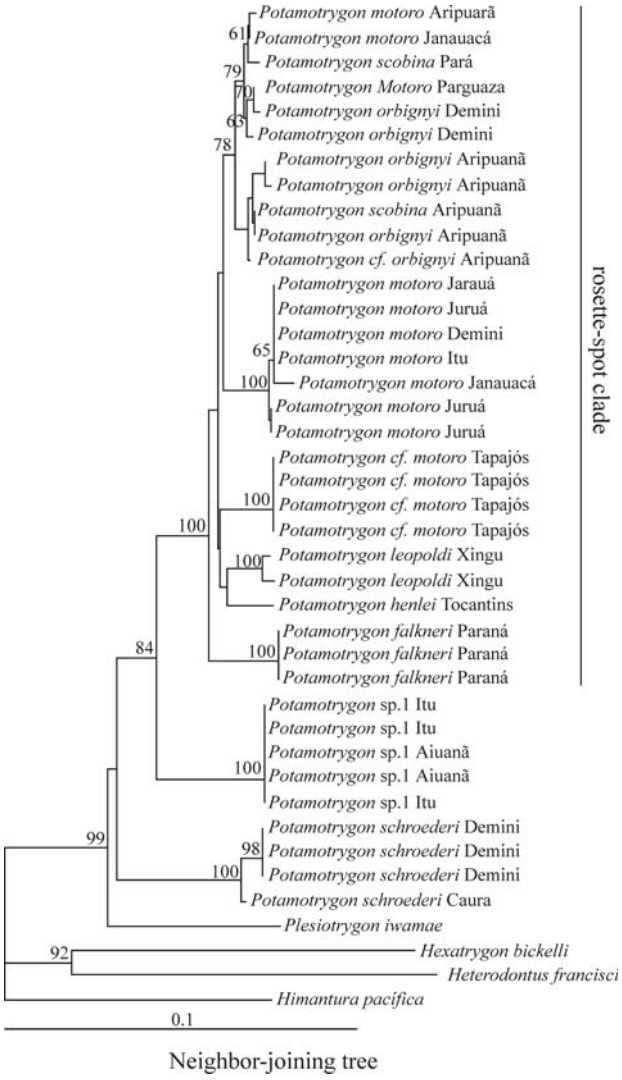

Figure 1 - Phylogenetic relationships of the potamotrygonids based on a Neighbor-Joining analyses of mitochondrial COI sequence data using K2P substitution model. Bootstrap values less than 50% were not shown. *Plesiotrygon iwamae* appears sister to all species of *Potamotrygon*. *P. motoro*, *P. scobina*, and *P. orbignyi* are non-monophyletic. The relationships among species of rosette-spot group are not well supported and branch lengths are short, evidence of a radiation.

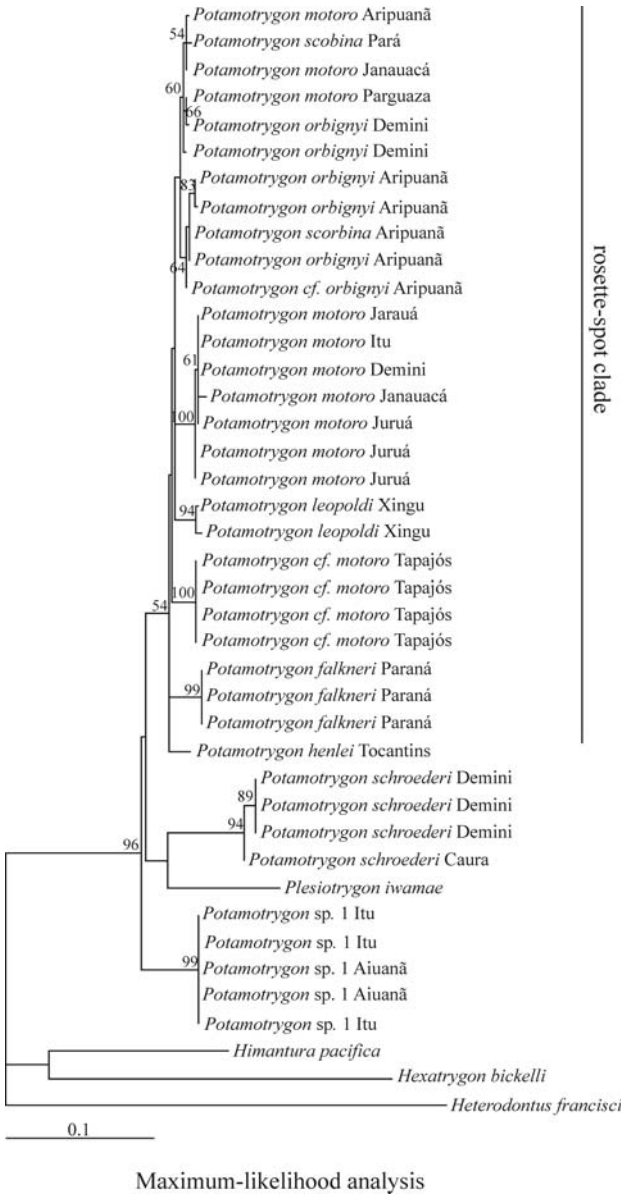

Figure 2 - Phylogenetic relationships of the potamotrygonids based on a Maximum-Likelihood phylogenetic analyses of mitochondrial COI sequence data using GTR + inv substitution model. *Plesiotrygon iwamae* is sister to *Potamotrygon schroederi* and *Potamotrygon* sp.1 appears as the sister species of the family Potamotrygonidae. *P. motoro*, *P. scobina*, and *P. orbignyi* are non-monophyletic. The relationships among species of rosette-spot group are not well supported and branch lengths are short, evidence of a radiation.

Genetic distances among the species *P. motoro*, *P. scobina*, and *P. orbignyi* can not be considered true measures of divergence, since these species are not reciprocally monophyletic. Because of their lack of reciprocal monophyly, intraspecific genetic distances within these species show similar levels of divergence as among species measure of genetic distance (Table 2).

Hypotheses testing

Testing of alternate ML and NJ topological hypotheses under the Kishino-Hasegawa (1989) and Shimodaira-Hasegawa (1999) frameworks resulted in essentially the same conclusions (Tables 4 and 5). The monophyly of *Potamotrygon motoro* was not statistically rejected in the ML topology and was only rejected by the K-H test in the NJ topology. Monophyly of *P. orbignyi* was rejected by both tests in the ML topology and the NJ topology. Likewise, monophyly of *P. scobina* was rejected in the ML topology by the K-H test, but not by the S-H test, and was rejected by both tests in the NJ topology. Enforcing the reciprocal monophyly of all three species resulted in significantly worse phylogenetic hypotheses under the ML criterion, as well as under the NJ phylogenetic estimate. Results of the McDonald-Kreitman test indicated that natural selec-

tion is or was acting in *P. orbignyi* ($p < 0.05$), however, overall sequence data evolve in a clock-like fashion (LTR test $p > 0.05$).

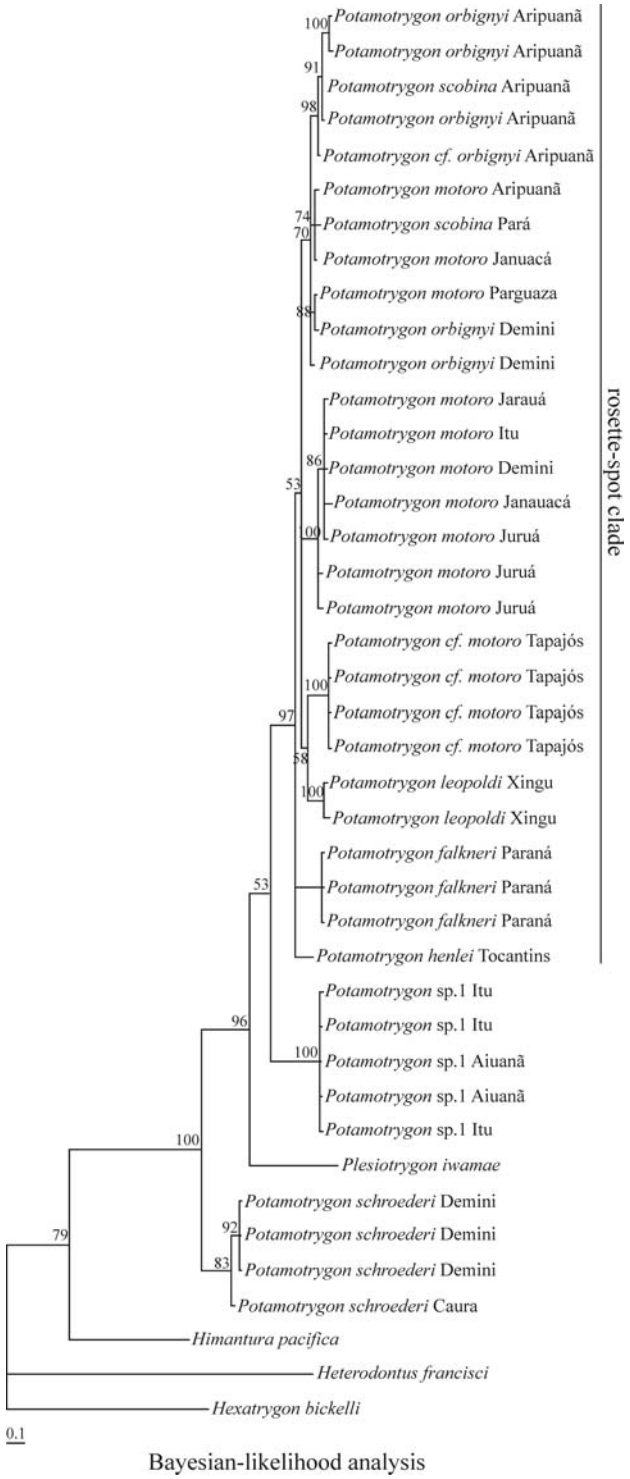

Figure 3 - Phylogenetic relationships of the potamotrygonids based on a Bayesian-inference phylogenetic analyses of mitochondrial COI sequence data using GTR + inv substitution model. *Plesiotrygon iwamae* appears nested within *Potamotrygon* with *Potamotrygon schroederi* as sister species of the family. *P. motoro*, *P. scobina*, and *P. orbignyi* are non-monophyletic. The relationships among species of rosette-spot group are not well supported and branch lengths are short, evidence of a radiation.

Discussion

Potamotrygonidae is the only family of the Class Chondrichthyes that is considered completely adapted to living in fresh water (Thorson *et al.*, 1983). Initial phylogenetic studies of Rosa (1985), Lovejoy (1996), Lovejoy *et al.* (1998), and Marques (2000) aimed to test the monophyly of this family, to infer the phylogenetic relationships among its three described genera, and to establish its sister group. These studies supported the monophyly of the Potamotrygonidae and most of them concluded that colonization of fresh water in South America is the result of a single colonization event. The majority of authors also considered the marine genus *Himantura* to be the sister taxon of Potamotrygonidae. Relationships within the Potamotrygonidae were hypothesized as (*Paratrygon* (*Plesiotrygon* (*Potamotrygon*))), although Marques (2000) and Quijada (2003) found *Plesiotrygon* nested within *Potamotrygon*. The first attempt to define species relationships within the genus *Potamotrygon* was made by Marques (2000), although his study was based on only few specimens per species and species were not sampled throughout their geographical distribution. This sampling design assumes that species are monophyletic with respect to the alleles at the studied locus, and that they show no or only very limited intraspecific variation (Funk and Omland, 2003). In turn, this assumption requires that nominal study species represent genetically and reproductively independent lineages whose boundaries have been accurately identified by taxonomists and whose reconstructed gene trees are accurate approximations of organismal history, *i.e.* species trees (Funk and Omland, 2003). This scenario also does not accommodate species that are paraphyletic or polyphyletic, cases extensively reported in the literature (Funk and Omland, 2003, and references therein). Non-reciprocal monophyly of species can, for example, be caused by the retention of ancestral polymorphism or introgression following a hybridization event (Avise, 2000; Moritz and Cicero, 2004). In our phylogenetic analysis we sampled four species in at least two different localities, and majority of species are represented by at least four individuals. Even with this sampling scheme, we find extensive haplotype sharing, and lack of monophyly in three out of the eight investigated species with more than one specimen sampled.

Potamotrygon motoro, Potamotrygon scobina, and *Potamotrygon orbignyi*

We found that three of the four species with broadest distribution in the Amazon basin (the fourth species *Paratrygon aiereba* was not included in this study), species which also are highly polymorphic, were not reciprocally monophyletic, and extensively shared haplotypes among species. Haplotype sharing may indicate that *i)* taxonomic validity of these species should be reassessed; *ii*) lineages did not have enough time to reach reciprocally monophyly; *iii*) hybridization; or *iv*) combination of these factors (Moritz and Cicero, 2004).

Table 2 - Maximum and minimum genetic distances between species under K2P (below diagonal) and GTR + inv (above diagonal) substitution model. Distances among the polyphyletic taxa (sensu Funk and Omland, 2003) *Potamotrygon motoro*, *P. scobina*, and *P. orbignyi* are indicated in bold. Diagonal elements are K2P intraspecific divergences. Note that distances in bold are within range of intraspecific variability and cannot be used to discriminate these species based on thresholds. The proposed 10x threshold also cannot be used to discriminate *P. leopoldi* from all other *Potamotrygon* species.

			1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Himantura pacifica*	min	-	0.36039	0.48298	0.26358	0.26033	0.24875	0.27669	0.23774	0.35266	0.27973	0.25546	0.24636	0.25665
		max	-	0.36039	0.48298	0.26358	0.26033	0.26676	0.27669	0.25798	0.35266	0.27973	0.25547	0.25238	0.25665
2	*Hexatrygon bickelli*	min	0.21571	-	0.51363	0.35694	0.38597	0.39116	0.42223	0.38737	0.38315	0.41943	0.38833	0.40410	0.40817
		max	0.21571	-	0.51363	0.35694	0.38737	0.44773	0.42223	0.41301	0.38315	0.41095	0.39262	0.41228	0.40817
3	*Heterodontus francisci*	min	0.24499	0.23618	-	0.41983	0.41221	0.42093	0.45371	0.41221	0.49989	0.41095	0.41654	0.42701	0.42450
		max	0.24499	0.23618	-	0.41983	0.42460	0.45796	0.45371	0.43947	0.49989	0.07734	0.42063	0.43396	0.42450
4	*Potamotrygon* sp.1	min	0.17137	0.21119	0.22852	0.00000	0.06078	0.06341	0.07865	0.06078	0.11505	0.07734	0.10454	0.07343	0.07628
		max	0.17137	0.21119	0.22852	0.00000	0.06823	0.08589	0.07865	0.07130	0.11505	0.07734	0.10455	0.07624	0.07628
5	*Potamotrygon scobina*	min	0.16130	0.2139	0.23171	0.05652	0.01359	**0.00387**	0.02979	**0.00000**	0.11358	0.02906	0.08812	0.02649	0.03540
		max	0.16380	0.22209	0.23728	0.06297	0.01359	**0.03558**	0.03212	**0.02026**	0.11578	0.03135	0.10079	0.03099	0.03750
6	*Potamotrygon motoro*	min	0.16380	0.21661	0.23171	0.05866	**0.00385**	0.00000	0.02766	**0.00193**	0.11360	0.02696	0.08814	0.02444	0.03541
		max	0.17118	0.24373	0.23937	0.07572	**0.03350**	0.03147	0.04445	**0.03137**	0.13901	0.04348	0.11578	0.04294	0.04764
7	*Potamotrygon henlei*	min	0.17354	0.22436	0.23127	0.06926	0.02747	0.02546	-	0.02767	0.12176	0.03503	0.10008	0.02775	0.03922
		max	0.17354	0.22436	0.23127	0.06926	0.02949	0.03954	-	0.03710	0.12176	0.03503	0.11118	0.03008	0.03922
8	*Potamotrygon orbignyi*	min	0.16130	0.21661	0.22620	0.05652	**0.00000**	**0.00192**	0.02546	0.00192	0.11578	0.02697	0.09339	0.02444	0.03541
		max	0.16865	0.23016	0.23984	0.06514	**0.01950**	**0.03549**	0.0335	0.01751	0.12734	0.03152	0.11113	0.03115	0.04493
9	*Plesiotrygon iwamae*	min	0.20114	0.20563	0.23034	0.09735	0.09508	0.09508	0.09708	0.09735	-	0.12770	0.12883	0.11998	0.13176
		max	0.20114	0.20563	0.23034	0.09735	0.09735	0.11072	0.09708	0.10408	-	0.12770	0.12885	0.12280	0.13176
10	*Potamotrygon* cf. *motoro*	min	0.17629	0.23577	0.22641	0.06938	0.02751	0.02550	0.03147	0.02550	0.10408	0.00000	0.09216	0.03145	0.04293
		max	0.17629	0.23577	0.22641	0.06938	0.02954	0.03959	0.03147	0.02949	0.10408	0.00000	0.10293	0.03379	0.04293
11	*Potamotrygon schroederi*	min	0.16923	0.21437	0.21804	0.09126	0.07807	0.07807	0.08449	0.08254	0.10624	0.08016	0.00000	0.09510	0.10136
		max	0.16923	0.21437	0.22346	0.09126	0.08931	0.09796	0.09354	0.09600	0.10624	0.08915	0.00772	0.10987	0.11204
12	*Potamotrygon leopoldi*	min	0.16631	0.23043	0.23171	0.06732	0.02555	0.02353	0.02546	0.02353	0.09963	0.02954	0.08254	0.00578	0.04485
		max	0.16884	0.23043	0.23728	0.06951	0.02959	0.03965	0.02747	0.02954	0.09963	0.03157	0.09617	0.00578	0.04734
13	*Potamotrygon falkneri*	min	0.21437	0.22486	0.22894	0.06951	0.03368	0.03368	0.03560	0.03368	0.10887	0.03979	0.08931	0.04194	0.00000
		max	0.21985	0.22486	0.06951	0.06951	0.03573	0.04379	0.03560	0.04186	0.10887	0.03979	0.09848	0.04403	0.00000

Table 3 - Distances among outgroup taxa and an average of Potamotrygonidae species under K2P and GTR + inv model of evolution.

	K2P distance	ML distance
Himantura x Potamotrygonidae	0.17713	0.27001
Hexatrygon x Potamotrygonidae	0.22182	0.39907
Heteredontus x Potamotrygonidae	0.22241	0.43416

The phylogenetic results are consistent with the possibility that these species can actually represent one highly polychromatic species with broad geographical distribution within the Amazon basin. There is overlap in some morphometric characters (Rosa, 1985), although this author mostly measured juveniles, and there is also a large amount of color and pattern variation within *P. scobina* from the Marajó Bay (Almeida, 2003), and *P. orbignyi* from the Tocantins River (Rincon, 2006). There also appears to be some overlap in color pattern among the species *P. motoro*, *P. orbignyi*, and *P. scobina* from various places in the Amazon basin (Araújo and Toffoli, unpublished data). For examples of color patterns see electronic appendix at http://www.evoamazon.net/publications. However, there are also several morphological as well as ecological characters that have been argued to separate these species. Differences between *P. scobina* and *P. orbignyi* include presence/absence of a labial furrow (Almeida, 2003), color of the ventral portion of tail, teeth cuspid, and dorsal color patterns (Rincon, 2006).

Another hypothesis is that *P. motoro*, *P. orbignyi*, and *P. scobina* are true evolutionary species, and *P. motoro* is the ancestor. Although non-monophyletic, very little haplotype sharing among geographic localities of *P. scobina* and *P. orbignyi* occurs, and both species are phylogenetically nested within *P. motoro*. *Potamotrygon scobina* and *P. orbignyi* may be in the process of incipient speciation with not yet well defined species borders. The observed selection acting on *P. orbignyi* supports this scenario, as does a phylogeographic analysis (Toffoli, 2006), but additional data including ecological, behavioral, and populational studies are needed to test this hypothesis rigorously. The potential role of hybridization in the diversification of this group is unknown.

Phylogenetic relationships within the rosette-spot clade - "The Radiation"

Phylogenetic relationships among species of the rosette-spot clade are not resolved. With the exception of *Potamotrygon motoro*, *P. orbignyi* and *P. scobina*, the monophyly of all other species is well supported, but no statistical support exists for phylogenetic relationships among them. This pattern suggests that the rosette-spot clade underwent or is still in the process of phylogenetic radiation, resulting in a rapid increase in number of species (Schluter, 2000). A phylogenetic signature of this process is generally a polytomy, due to extremely short evolutionary periods that separate successive speciation events. This prevents the accumulation of synapomorphic characters at internode branches, leading to the failure of all phylogenetic methods to reconstruct a statistically supported bifurcating topology. Radiations are not rare or restricted in time or space, and usually fill a previously unoccupied niche. Some examples include the Cambrian radiation (Philippe *et al.*, 1994; Bromham *et al.*, 1998; Conway-Morris, 1999), *Anolis* lizards from the Caribbean (Jackman *et al.*, 1999),

Table 4 - Results of ML constraint tests of monophyly of species *Potamotrygon motoro*, *P. orbignyi*, and *P. scobina*. * $p < 0.05$.

Topology type			KH-test	SH-test
	-ln L	Diff -ln L	p	p
Unconstrained topology	2267.274	(best)		
Monophyly of *P. motoro*	2287.596	20.322	0.057	0.078
Monophyly of *P. orbignyi*	2293.598	26.323	0.022*	0.017*
Monophyly of *P. scobina*	2287.120	19.846	0.037*	0.059
Reciprocal monophyly of *P. motoro, P. orbignyi*, and *P. scobina*	2318.833	51.559	0.003*	0.003*

Table 5 - Results of NJ constraint tests of monophyly of species *Potamotrygon motoro*, *P. orbignyi*, and *P. scobina*. *$p < 0.05$.

Topology type			KH-test	SH-test
	-ln L	Diff -ln L	p	p
Unconstrained topology	2270.696	(best)		
Monophyly of *P. motoro*	2289.381	18.685	0.034*	0.123
Monophyly of *P. orbignyi*	2297.192	26.496	0.010*	0.011*
Monophyly of *P. scobina*	2293.614	22.918	0.013*	0.037*
Reciprocal monophyly of *P. motoro, P. orbignyi*, and *P. scobina*	2322.812	52.116	0.009*	0.009*

the Hawaiian silversword alliance (Baldwin and Sanderson, 1998), cichlid fishes of the east African lakes (Avise, 1990; Turner *et al.*, 2001), and sciaenid fishes of the western Atlantic (Vinson *et al.*, 2004), among others.

Rooting the Potamotrygonidae

In the NJ topology, *Plesiotrygon iwamae* appears sister to all species of *Potamotrygon* (Figure 1), in the ML phylogeny, *Plesiotrygon iwamae* is sister to *Potamotrygon schroederi* (Figure 2), and in the BI phylogeny, *Plesiotrygon iwamae* appears nested with *Potamotrygon* (Figure 3). The phylogenetic relationship of *Plesiotrygon iwamae* in the ML topology is not statistically supported by a high bootstrap value. Despite these conflicts, the unrooted ingroup topology among *Plesiotrygon iwamae*, *Potamotrygon schroederi*, *Potamotrygon* sp.1, and the rosette-spot clade of *Potamotrygon* remains the same in all three methods of phylogenetic inference (Figure 4). Correct placement of the root is especially difficult in phylogenies with relatively short internal branches rooted by a distantly related or highly divergent outgroup. Classic example involves the placement of the root of birds whose closest relatives are the distantly related crocodilians, and vice versa (Mindell *et al.*, 1999; García-Moreno and Mindell, 2000).

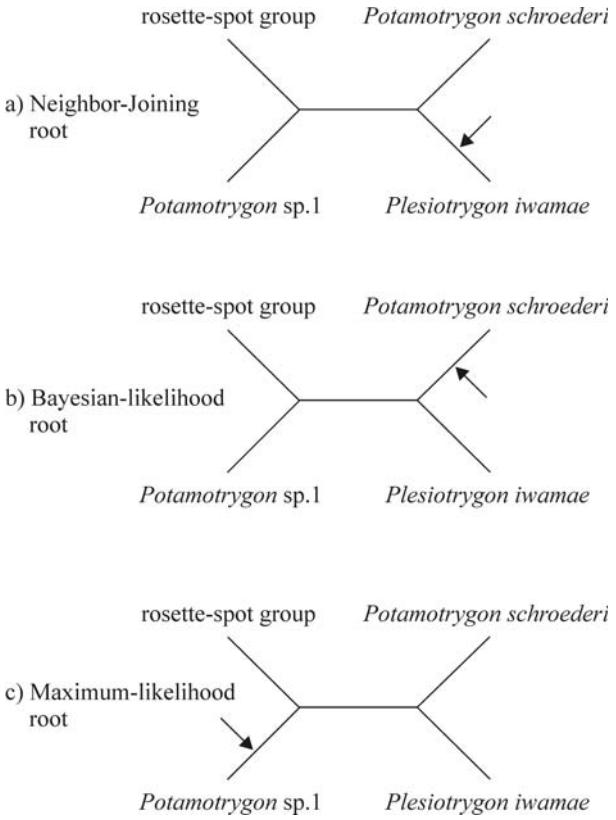

Figure 4 - Schematic representation of the ingroup topology which is recovered by all three methods of phylogenetic inference. Arrows represent rooting points of the different analyses: a) Neighbor-joining root; b) Bayesian-inference root; c) Maximum-likelihood root. See text for discussion.

The average maximum likelihood within Potamotrygonidae distance is 5.9%, while the average maximum likelihood distance observed between Potamotrygonidae and the *Himantura* sister group is 27.0%, and higher to other cartilaginous outgroup species (Table 3). Therefore, it is not entirely surprising that the root of the Potamotrygonidae is difficult to place. However, we suspect that *Plesiotrygon iwamae* is phylogenetically nested within *Potamotrygon,* as suggested by the ML and BI topologies (Figures 2 and 3). This supposition is derived from the use of a more complex and more appropriate model of molecular evolution in the ML and BI analyses, and the consistently better performance of likelihood-based methods of phylogenetic inference when proper models of molecular evolution are used (Huelsenbeck and Hillis, 1993; Hillis *et al.*, 1994). Eliminating third codon positions or translating the sequence data into putative amino acids, and analyzing these datasets using NJ also places *Plesiotrygon iwamae* within the genus *Potamotrygon* (results not shown). Furthermore, the inference drawn from the ML and BI topology is supported by Marques (2000) and Quijada (2003) who also hypothesize the paraphyly of *Potamotrygon* and the nested position of *Plesiotrygon* with *Potamotrygon*.

DNA barcoding

Studies supporting DNA barcoding often do not compare sister species which are the natural candidates to share haplotypes (Moritz and Cicero, 2004). Assuming that *Potamotrygon motoro, P. scobina*, and *P. orbignyi* are valid species in the sense of being a natural kind, our results showed that the use of DNA sequences to assign unknown specimens to any of these three species would invariably fail. Correct assignment would only be possible with the addition of other data types such as morphological, ecological, and behavioral characters, or even haplotype frequency data if non-random distribution of haplotypes across geography existed. Methodologies such as DNA barcoding which rely solely on one data type and one criterion will inevitably have higher failure rates than those incorporating diverse sources of information. Substituting one character-type philosophical system (*e.g.*, based on meristic characters) of taxonomy for another one character-type system (*e.g.*, based on COI DNA barcoding) will not solve existing taxonomic problems. One system may clarify some taxonomic questions, but it will also obscure other questions. Thus, unless a holistic approach to taxonomy and systematics is used we will inevitably fail to understand important aspects of biological diversity.

With the above *caveat* in mind, and assuming our sampling is representative of the genetic diversity present in the remaining analyzed *Potamotrygon* species, the remaining species surveyed in this study form monophyletic and well supported groups separated by reasonably large genetic distances. This pattern would allow the use of the COI phylogeny to assign unknown specimens to their cor-

rect species, as proposed by the Barcoding Consortium. However, this assignment is critically dependent on sufficiently dense genetic sampling of the group studied, *i.e.* it requires populational rather than typological sampling. The case of specimens from the Caura River, Venezuela, and specimens from Negro River, Brazil, is an illustrative example. As recognized morphologically, *P. schroederi* occurs both in the Negro and Orinoco River basins; the type specimen was from the Orinoco, but after being lost, a neotype was designated from the Negro River (Carvalho, 2001).

Our analyses indicate that the Venezuelan Caura River specimen is sister to the Negro River specimens, and separated by 0.8% sequence divergence. The genetic divergence of 0.8% is relatively low compared to the average 2.5% among rosette-spot clade divergence, and would indicate, based on barcoding assumptions, that fishes from the Caura and the Negro Rivers represent a single species. This barcoding-based inference is predicated on the assumption that the clades from which these individuals were sampled are sister, an assumption which critically hinges on taxonomic sampling. On the other hand, the color pattern of the Orinoco basin *P. schroederi* is quite different when compared to animals from Negro River. The Negro/Orinoco watershed divide represents a major barrier to gene flow for a number of species, with numerous instances of sister species occurring on either side of the divide (Reis *et al.*, 2003), and therefore it is possible that *P. schroederi* from the Negro and Caura Rivers represent different species. We, therefore, can neither accept nor reject that *P. schroederi* occurring on either side of the Negro/Orinoco watershed divide represents more than one species. These results pose serious questions about the exclusive reliance on the DNA barcoding methodology in species identification, at least for the freshwater stingrays of the family Potamotrygonidae.

Assuming that species are well characterized, are monophyletic, and that intraspecific divergence is much smaller than interspecific divergence, criteria that can only be verified through populational rather than the currently prevalent typological sampling and analyses, DNA barcoding should be powerful in assigning unknown samples to species. However, DNA barcoding is not powerful in discovering new species using the proposed threshold methods as advocated by Hebert *et al.* (2003). For example, in a comprehensive study of the adoption of threshold values for three thoroughly sampled groups of invertebrates, Meyer and Paulay (2005) found a significant overlap of intraspecific genetic variability and interspecific distance, providing strong evidence against the adoption of thresholds. Furthermore, the use of thresholds implicitly assumes that molecular sequences evolve in a clock-like manner. While we cannot reject a clock-like mode of molecular evolution in the potamotrygonid COI dataset, a clock-like mode of molecular evolution is rare in all groups of organism (for a review see Li, 1997) which resulted in the development of multiple algorithms specifically taking this fact into account in the calculation of divergence times (Sanderson, 1997, 2002; Thorne and Kishino, 2002).

DNA barcodes have been proposed as a fast, efficient, and inexpensive way to catalogue all biodiversity (Hebert *et al.*, 2003). However, most studies that advocate usefulness of barcoding actually test its assumptions in already predefined taxonomic groups based on previous works, whereas true barcoding consists of broad, essentially blind and random surveys of communities, with little or no background taxonomic information (Rubinoff, 2006a). The study of Hajibabaei *et al.* (2006) is one of the most striking positive results of barcoding, identifying 98% of previously well characterized species of tropical Lepidoptera. However, Funk and Omland (2003) found species level paraphyly and polyphyly in 23% of 2,319 vertebrate species whose mitochondrial genomes were surveyed. The Lepidoptera results (Hajibabaei *et al.*, 2006) should be seen as an exception rather than norm. The study does not report the intraspecific sampling scheme used.

The more geographically widespread a species is, the higher is the probability of finding elevated levels of intraspecific variability if the species is sampled across its distributional range. Consequently, such a study would have a higher probability of finding overlaps between genetic distance among closely related species and variability within a species, making even more questionable the establishment of thresholds, as well as increasing the likelihood of observing paraphyly and polyphyly. If, however, all specimens of a particular species were sampled from the same locality, one is likely to observe much less intraspecific variability than if sampling was done across a distributional range of the species. This consequently would lead to a reduction in the variation within species, and to a reduction in the number of observed cases of paraphyly and polyphyly. The degree of difference between intraspecific and interspecific divergence would also be overestimated.

The fundamental problem with using thresholds lies in the fact that species are a natural kind that embody an evolutionary process. Species are subject to demographic and selective processes that will act to increase or decrease genetic diversity and coalescent depth of individual species (*e.g.*, Avise, 2000; Hey, 2001; Coyne and Orr, 2004). Species may not be morphologically distinct from other species, yet be genetically distinct (*e.g.*, Hrbek *et al.*, 2006), and conversely species may be morphologically distinct without being genetically distinct (*e.g.*, Verheyen *et al.*, 2003; Duda Jr. and Rolán, 2005). Species are real evolutionary groups and not categories which are created as a direct function of perceived distinction (Hey, 2001). Species are not categories defined by criteria, such as amount of sequence divergence. Therefore, the use of thresholds in "discovering" new species is overly simplistic, misleading, and invalid.

Freshwater stingrays are a taxonomic group similar to several other tropical biota in the sense that much taxonomy is yet to be done and therefore a good exercise for testing usefulness of barcoding in discovery life on Earth. We found that four out of 10 species surveyed (40%) could not be discriminated by barcoding criteria (we observed sharing of haplotypes among *Potamotrygon motoro*, *P. orbignyi*, and *P. scobina*, and small sequence divergence between *P. aff. schroederi* from Venezuela and *P. schroederi* from Negro River). DeSalle (2006) advocates that DNA barcoding should focus mainly on the identification of specimens rather than the discovery of new species, which should be essentially the domain of taxonomy. However, this radically changes the main goal of barcoding, which is identification of life on Earth in a cost-effective fashion. This mission makes it obligatory to deal with taxonomic groups in which previous taxonomic efforts are virtually nonexistent. The results of the current and other studies pose serious doubts of the appeal of designating billions of dollars to the barcoding enterprise (Cameron *et al.*, 2006; Rubinoff, 2006a,b). So should one trust and rely on barcoding? By any standard, 23% (Funk and Omland, 2003) or even higher (40% - present study) failure rates to identify taxonomically valid species are not acceptable, and the political, economical, and environmental consequences of using DNA barcoding as currently proposed can be enormous.

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