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Morphological conservation of rays in the genus *Rhinoptera* (Elasmobranchii, Rhinopteridae) conceals the occurrence of a large batoid, *Rhinoptera brasiliensis* Müller, in the northern Gulf of Mexico

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Abstract

In 2007, three rays identified as *Rhinoptera brasiliensis* based on tooth series counts were captured in the northern Gulf of Mexico, a region far outside their accepted range of the coastal waters of southern Brazil. Genetic analyses confirmed that these individuals were distinct from *R. bonasus*, the only recognized indigenous rhinopterid in the Gulf of Mexico. Further analyses of over 250 specimens confirmed the widespread occurrence of two species in the northern Gulf of Mexico and revealed that the anomalous individuals related most closely to vouchered specimens of *R. brasiliensis* from Brazil. Discriminant function analyses of morphological data identified several potential discriminating characters, but the degree of overlap of the measurements and counts between the two species rendered most impractical for identification purposes. However, the shape of the supracranial fontanelle appeared to be consistently reliable in differentiating between the two species. Tooth series counts (*R. bonasus* = 5 to 15, *R. brasiliensis* = usually 7 to 13) were significantly different between the two species but exhibited considerable overlap. This is the first study to verify the occurrence of *R. brasiliensis* in the northern Gulf of Mexico; however, the close genetic relationships to other rhinopterid species, as well as the morphological similarity of the group as a whole, require additional research.

Key words: taxonomy, phylogeny, systematics, cownose rays, Batoidea

Introduction

The assessment and management of highly migratory fish populations can be a challenging task. This is especially true when the species under consideration form part of a morphologically conservative species complex and distributions of one or more species overlap. Many batoid genera (e.g. *Manta* Bancroft; *Aetobatus* Blainville) contain species complexes that are so morphologically similar that until recent revisions proved otherwise (Marshall *et al.* 2009; White *et al.* 2010), they were considered to be single wide-ranging species (White and Last

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2012). The rays comprising the genus *Rhinoptera* Cuvier, morphologically resemble each other so closely that the only characters that have been used to differentiate among species are the number and shape of their tooth series (Bigelow and Schroeder 1953). The resemblance of these species to one another, and the fact that several of their ranges overlap, has led some authors to question the validity of several proposed species (Schwartz 1990; Compagno 1999, 2005).

In 2007, three cownose rays (*Rhinoptera* sp.) were captured during a Mississippi Deep Sea Fishing Tournament in Biloxi, Mississippi (MS). The three specimens resembled *Rhinoptera bonasus* (Mitchill), which is the only species reported to occur in the region, except that the numbers of tooth series in the upper and lower jaws (9–11 tooth series per jaw) were inconsistent with descriptions of this species (≤8 tooth series per jaw) (Garman 1913; Bigelow and Schroeder 1953; McEachran and de Carvalho 2002). Preliminary genetic analyses (J. Quattro, unpublished data) indicated these specimens harbored variants with genetic distances (uncorrected p-distance ~ 6%) that far exceeded those expected for intraspecific variation within *R. bonasus* collected in the same area (e.g., see Dudgeon *et al.* 2012). Several rhinopterids are described as having dentition matching that found in these "anomalous" specimens (e.g. the Brazilian cownose ray, *R. brasiliensis* Müller) but none are reported to occur in the western North Atlantic Ocean (Bigelow and Schroeder 1953).

Rhinoptera brasiliensis has been proposed to be restricted to the continental shelf waters of an approximately 1800 kilometer stretch of coastline from Rio de Janeiro to Rio Grande do Sul in southern Brazil (Vooren and Lamónaca 2004). There have been few reports of *R. brasiliensis* occurring in waters outside of this proposed native range. Bigelow and Schroeder (1953) indicated that a rhinopterid with nine tooth series in the upper jaw and eight tooth series in the lower jaw was captured off Beaufort, North Carolina on August 26, 1881. They concluded that since this was the only capture of an individual with tooth series resembling *R. brasiliensis* outside of Brazil on record at the time, it must have been an aberrant *R. bonasus*. Records from the Museum of Comparative Zoology, Harvard University (MCZ) indicate that a specimen, identified as *R. brasiliensis*, was collected off Nantucket, Massachusetts in August of 1959. Reports of *R. brasiliensis* being captured off the coast of Colombia (Acero and Garzon 1982) and in the southern Gulf of Mexico off Veracruz, Mexico (Isaís and Dominguez 1996) were likewise seemingly rare occurrences with only a few individuals reported in each case. As such, the accepted range of *R. brasiliensis* has remained restricted to a relatively small area off the coast of southern Brazil. However, recent captures of multiple specimens with dentition characteristic of *R. brasiliensis* in the northern Gulf of Mexico (hereafter referred to as GOM) brings into question the validity of the 'aberrant *R. bonasus*' hypothesis, as well as the utility of tooth series counts for differentiating between *R. bonasus* and *R. brasiliensis*. Confounding the issue further, a study by Naylor *et al.* (2012) suggested that several rhinopterid specimens collected from the western North Atlantic off the east coast of the United States (hereafter referred to as WNA) and in the GOM genetically relate most closely to *R. steindachneri* Evermann & Jenkins. However, *R. steindachneri* are proposed to be indigenous to the eastern Pacific Ocean and were previously described (Evermann and Jenkins 1891, Garman 1913) as possessing seven tooth series, as in *R. bonasus*, although recent accounts indicate that they can possess as many as nine (McEachran and Notarbartolo di Sciara 1995). However, Naylor *et al.* (2012) did not have access to genetic material from a verified specimen of *R. brasiliensis* from its proposed native range, so the relationships among the individuals in question, *R. steindachneri* and *R. brasiliensis,* could not be examined.

Given the presence and taxonomic uncertainty of the second species of cownose ray in the GOM and WNA, it was the purpose of this study to determine the identity of this species (tentatively identified as *R.* cf. *brasiliensis*) and to further examine the validity of *R. brasiliensis*. Currently both *R. brasiliensis* and *R. bonasus* are considered species of concern for conservation purposes (Vooren and Lamónaca 2004, Barker 2006). The International Union for the Conservation of Nature (IUCN) Shark Specialist Group performed an assessment of *R. brasiliensis* in its accepted range of southern Brazil in 2004 and determined the species is endangered, and perhaps critically endangered (Vooren and Lamónaca 2004). This determination was based upon the species having undergone heavy exploitation off the Brazilian coast from the 1980's to present. *Rhinoptera bonasus* was assessed by the IUCN in 2006 and found to be near threatened (Barker 2006). The range of *R. bonasus* extends from the southern portions of New England off the United States through Central America and down to southern Brazil in South America. The only reported overlap of distribution of these two species is along the Brazilian coast. However, if the ranges of these two species overlap to a much greater extent and *R. brasiliensis* is prevalent in the GOM and WNA, the conservation assessments for both species might warrant reconsideration.

Materials and methods

Specimen Collection. *Rhinoptera* specimens were collected opportunistically from 2007 to 2012 from coastal and estuarine waters of Florida, Alabama, Mississippi, Louisiana, and Texas in the GOM (Fig. 1). Specimens (n=257) were collected during monthly or annual trawl and gillnet surveys conducted by, the Florida Fish and Wildlife Conservation Commission (FWC), the Dauphin Island Sea Laboratory (DISL), the University of Southern Mississippi's Gulf Coast Research Laboratory (GCRL), and the National Marine Fisheries Service (NMFS) Mississippi Laboratories. Additional Louisiana specimens were collected from a spearfishing tournament in 2012. A reference specimen of *R. brasiliensis* was collected in 2012 off Aracaju, State of Sergipe, in northeastern Brazil [Fig. 1(a)] and deposited in the Ichthyological collection of the Museu de Zoologia da Universidade de Sao Paulo (MZUSP 113721). Additionally, a single mitochondrial cytochrome oxidase subunit I (COI) sequence (JX124888.1) for a *R. brasiliensis* collected from the Bacia de Santos, Sao Paulo, Brazil in 2009 and deposited in the collection of the Universidade Estadual Paulista, Lab de Biologia e Genetica de Peixes was obtained from Genbank as a second reference for this species. These two specimens were identified as *R. brasiliensis* based on tooth series counts as well as the shape of the supracranial fontanelle, as described in Gallo *et al.* (1997). Tissue samples from specimens of *R. bonasus* (n=12) were collected from the waters of the Chesapeake Bay at the 2nd Annual Chesapeake Bay Stingray Tournament in June 2011 by researchers at Hood College. These specimens were used as references for *R. bonasus* since there is no current genetic evidence of a second species of *Rhinoptera* occurring in the Chesapeake Bay (Carney *et al.* 2017, J. McDowell, pers comm). As previous research has suggested that the second species present in the GOM related most closely to *R. steindachneri* from the Gulf of California (Naylor *et al.* 2012), representative tissue samples of this species (n=21) were obtained from Mazatlan, Sinaloa, Mexico. Although it has been suggested that a cryptic species may be present within the range of *R. steindachneri* (Sandoval-Castillo and Rocha-Olivares 2011), *R. steindachneri* is the only currently described species from the eastern Pacific, so all specimens from Mazatlan were assumed to represent this species. Representative COI sequences for the only other rhinopterids currently available in GenBank, *R. javanica* (Müller & Henle) and *R. jayakari* Boulenger, were obtained for inclusion in phylogenetic analyses (three sequences from Lim *et al.* 2015).

Genetic analyses. Tissues collected from individual rays were placed immediately and stored in 95% ethyl alcohol until lysis. Total genomic DNA was extracted from tissues using the DNeasy Blood & Tissue Kit (QIAGEN Inc, Valencia, California, USA) following the manufacturer's protocol. The presence and quality of total genomic DNA was visualized on 1% agarose gels stained with ethidium bromide.

The Polymerase Chain Reaction (PCR) was used to characterize a 540 basepair (bp) fragment of the mitochondrial (mtDNA) cytochrome-oxidase I (COI) locus commonly used for DNA barcoding (Hebert *et al.* 2003). Primers employed (Forward—VF2_t1, Reverse—FishR2_t1) and PCR amplification conditions followed those found in Ivanova *et al.* (2007). The presence of all amplicons was visually confirmed on ethidium bromide stained agarose gels prior to DNA sequencing. PCR products were precipitated with a 20% polyethylene glycol/2.5 M NaCl mixture and washed twice with 70% cold ethanol. The forward and reverse PCR primers were used as sequencing primers in separate reactions using the ABI BigDye® Terminator version 3.1 Cycle Sequencing Kit. Sequencing reactions were then read on an ABI 3130 automated sequencer. Sequence files were exported into Sequencher™ (Gene Codes Corporation, Ann Arbor, Michigan, USA) and contiguous sequences made of forward and reverse sequences from each individual. The accuracy of base calls for all contiguous sequences was checked by eye. Contiguous sequences were exported from Sequencher™ as text files for analyses.

Maximum Parsimony (MP) and Bayesian Inference (BI) criteria were used to reconstruct phylogenetic hypotheses. Included in all analyses (as outgroups) were sequences from five species of *Mobula* Rafinesque (taken from Poortvliet *et al.* 2015), the sister group to *Rhinoptera* (e.g., Lim *et al.* 2015). As no length variation was observed in this coding fragment of COI, all sequences were aligned 'by eye' without ambiguity. MP phylogenetic analyses utilized algorithms in PAUP*4.0 (version 4b10; Swofford 2002). For MP analyses, characters were treated as unordered and searches used the 'Random Stepwise Addition' option with 10 replicates per heuristic search. Bootstrapping (Felsenstein 1985) was used to estimate the reliability of individual clades in phylogenetic reconstructions (1,000 replicates) using the search strategy described above. Partitioned (by codon position) BI analyses utilized algorithms available in MrBayes (version 3.2.1, Ronquist *et al.* 2011). Prior to BI analyses, PartitionFinder (v1.1.1, Lanfear *et al.* 2012) was used to find the optimal partitioning scheme and models of

nucleotide substitution for the COI data partitioned by codon position with the AICc criterion for model selection. The selected models for all subsequent BI analyses were K80, F81, and GTR+I for first, second and third codon positions, respectively. For BI analyses, we ran six independent MCMC iterations (three heated and one cold chain(s) per iteration) for 50,000,000 generations and sampled topologies every 100 generations. Posterior probabilities (PP) were estimated by sampling trees from the PP distribution after discarding the first 25% of the trees from each independent run as ''burn-in''. All branches that received less than 80% PP were collapsed. Since networks can be a more appropriate representation of relatedness among groups of closely related haplotypes (e.g., shallow intraspecific divergences) than a strictly bifurcating phylogeny, we augmented traditional phylogenetic approaches by constructing a minimum spanning network where appropriate (Bandelt *et al.* 1999). Networks were constructed using the algorithms available in PopART (version 1.7.2, http://popart.otago.ac.nz).

In addition to the phylogenetic analyses, we also assigned 'unknown' haplotypes to species using the barcoding approach and criterion of 'Best Close Match' as suggested by Meier *et al.* (2006). To calculate the 95% threshold for intraspecific variation, we constructed a dataset that included intraspecific variation observed in *Rhinoptera*, including *R. bonasus* (12 vouchered animals from Virginia), *R. brasiliensis* (our voucher plus the unique sequence from GenBank), *R. steindachneri* (GC), *R. steindachneri* (PCBC) and five sequences from *Mobula kuhlii* (Valenciennes)(taken from Steinke *et al.* 2016). We then used these data and the program 'Species Identifier' (version 1.8) to calculate the 95% threshold for intraspecific variation using pairwise K2P distances (0.55% for this dataset). Individual haplotypes were used as queries against this intraspecific dataset to calculate percent sequence divergence (as a K2P distance) and then the identity of the 'Best Close Match' that fell within this intraspecific threshold.

Morphological analyses. Identifications of *Rhinoptera* specimens collected from the GOM resulting from phylogenetic analyses (i.e. *R. bonasus* or *R.* cf. *brasiliensis*) were used as the basis for all further analyses. In addition to disc width (DW), a suite of 23 morphometrics (based primarily on Aguiar *et al*. 2001, Table 1, Fig. 2 A–F) and top and bottom jaw tooth series counts (Fig. 2 F) were recorded from a subset of genetically identified specimens. All measurements were recorded to the closest 0.1 millimeter (mm). Morphometric measures were standardized in an attempt to remove the effect of allometric growth using the methods of Elliot *et al.* (1995):

$$M_{adj} = M(L_s/L_o)^b$$

Where:
M_{adj} = the size adjusted measurement
M = the original measurement
L_s = overall mean of DW for all fish from all samples
L_o = DW of individual fish
b = slope of regression of log M on log L_o

To determine the effectiveness of these adjustments, correlation coefficients were calculated for the relationship between standardized measurements (M_{adj}) and DW. Correlation coefficients above 0.5 were considered to indicate the effect of size had not been adequately removed and those characters were removed from further analyses.

Mann-Whitney tests were performed to determine if there was a significant difference between the two proposed species for top and bottom jaw tooth counts. Discriminant analysis of morphometric data using backwards stepwise selection procedures, with a probability of entry of 0.05 and a probability of removal of 0.10, was utilized to assess the ability to separate species based solely on morphological characters. In addition, original measurements (M) were standardized as a percentage of DW and individually examined by t-test to determine the efficacy of each character in identifying the species, given that the assumptions of normality and homoscedasticity were met. When character data did not conform to these assumptions, a Mann-Whitney test was used to determine if statistically significant differences existed.

In addition to the morphometric analyses, the shape of the supracranial fontanele (Fig.3) was documented for a subset of individuals that were genetically identified in an effort to further examine the utility of this character to differentiate between the two species inhabiting the northern Gulf of Mexico.

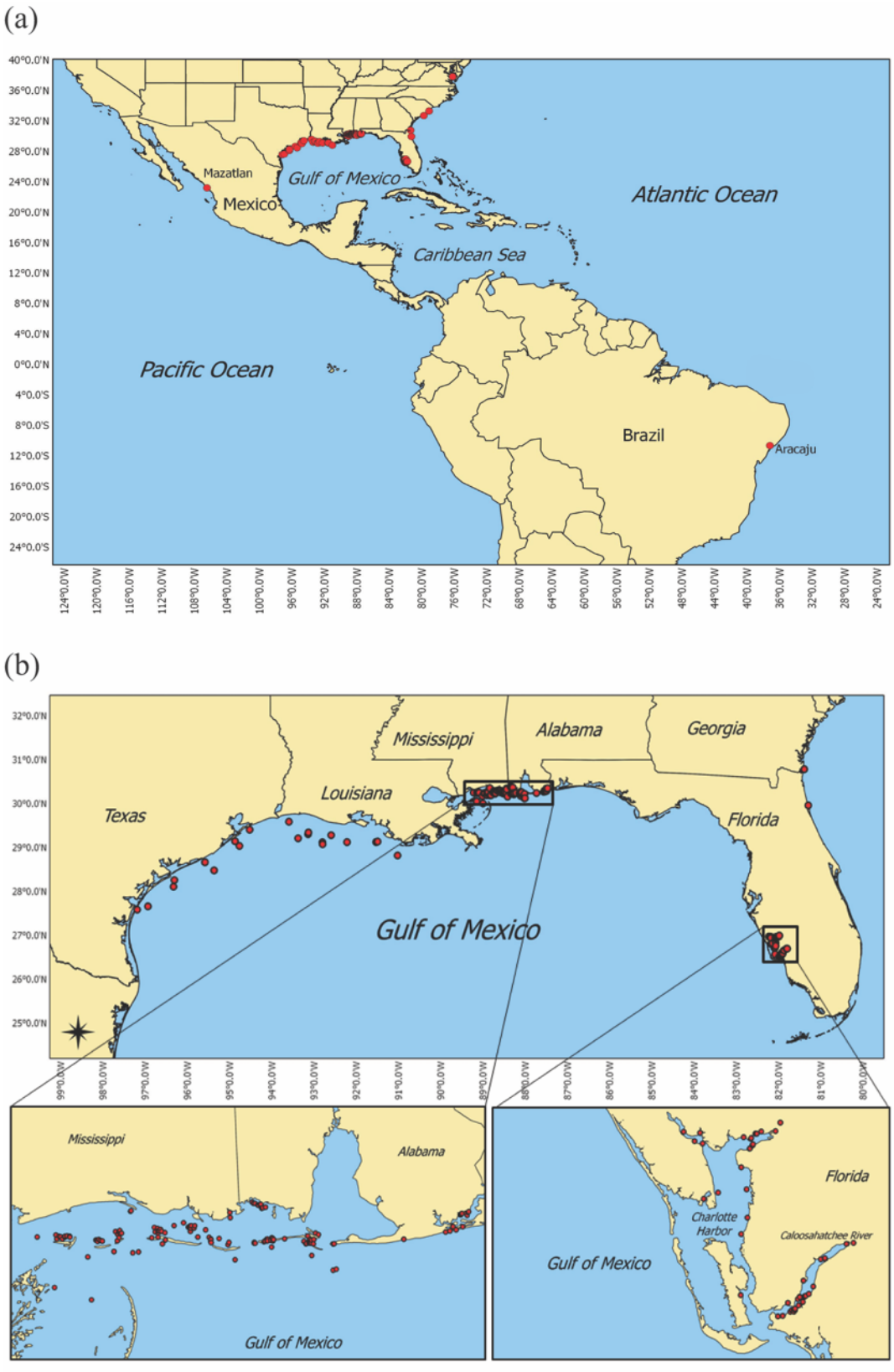

FIGURE 1. Capture locations (red circles) of *Rhinoptera* spp. examined during this study. A. All specimens examined. B. Detailed view of the Gulf of Mexico including insets of the coastal waters of Mississippi and Alabama and the Charlotte Harbor region of Florida.

TABLE 1. Explanation of abbreviations of external measurements. Measurements are illustrated in Figures 2.

Measurement	Abbreviation
External morphometrics	
First gill slit length	1BL
First inter-branchial distance	1ID
Fifth gill slit length	5BL
Fifth inter-branchial distance	5ID
Anterior projection length	APL
Cranial length	CL
Dorsal fin height	DFH
Dorsal fin length	DFL
Disk length	DL
Disk width	DW
Distance from anterior groove to 1st gill slit	G1B
Distance from anterior groove to 5th gill slit	G5B
Inter-nasal distance	IND
Inter-orbital distance	IOD
Inter-spiracle distance	ISD
Length of top central tooth	LCT
Pre-cloacal length	PCL
Pre-dorsal length	PDL
Pre-oral length	POL
Pelvic fin width	PW
Width of top central tooth	WCT

Results

Genetic analyses. Fragments of the mitochondrial COI locus (540 bp in length) were obtained for 298 specimens (257 *Rhinoptera* sp. from the GOM, 12 reference *R. bonasus*, one vouchered *R. brasiliensis*, and 21 *R. steindachneri*) and were included in a phylogenetic analysis in addition to sequences obtained from Genbank (a single *R. brasiliensis* and five species within the genus *Mobula*). A total of 25 distinct COI haplotypes (Table 2) were uncovered in our material and included four haplotypes in *R. steindachneri* and 21 haplotypes including vouchered individuals of *R. bonasus* or *R. brasiliensis* as well as the *Rhinoptera* sp. collected from the GOM. Phylogenetic analyses utilizing the parsimony (MP) criterion returned two equally parsimonious trees of length 221 steps (Consistency Index = 0.6652, Retention Index = 0.8511). The two topologies differed primarily in the placement of *R. steindachneri* haplotypes *Rs.*COI. 3 and *Rs.*COI.2, either as a clade within a group containing haplotypes *Rb.*COI.13—*Rb.*COI.19, or as a paraphyleAc group outside of haplotypes *Rb.*COI.13—*Rb.*COI.19. ParAAoned Bayesian Inference (BI) analyses suggested the laGer grouping of haplotypes, that is, a paraphyleAc *Rs.*COI.3 and *Rs.*COI.2 outside of haplotypes *Rb.*COI.13—*Rb.*COI.19. Given this agreement, we present the BI topology in Figure 4, but cauAon that bootstrap support and posterior probabiliAes for either alternaAve were poor.

The MP and BI analyses were largely congruent in both topology and in relative support for various groupings of taxa and haplotypes. The only appreciable difference among the phylogenies supported by each analysis, in addition to that discussed above, involved the placement of *R. javanica*, either as a clade outside of all other sampled *Rhinoptera* (MP), or as a sister group to *R. jayakari* and *Rs.*COI.3 and *Rs.*COI.2 plus *Rb.*COI.13—*Rb.*COI.19 (BI). However, neither bootstrap analyses from the MP or posterior probabilities from the BI analysis confidently supported either alternative and therefore this branch was collapsed in Figure 4.

TABLE 2. COI haplotypes observed in *Rhinoptera*; for clarity, only polymorphic sites differentiating haplotypes are shown. Two-letter codes preceding haplotype designations signify the putative taxon sampled: Rb - *R. bonasus*, Rbr - *R. brasiliensis*, Rs (PCBC) - *R. steindancheri*, Rs (GC) - *R. steindancheri* Gulf of California. Abbreviations for sampling sites: TX - Texas, Gulf of Mexico (GOM); LA - Lousiana, GOM; MS - Mississippi, GOM; AL - Alabama, GOM; FL - Florida, VA – Virginia (Chesapeake Bay), BR – Brazil, MA – Mazatlan, Sinaloa, Mexico.

Haplotype	Polymorphic Sites	Collection Sites							
		TX	LA	MS	AL	FL	VA	BR	MA
Rb.COI.1	TGGTATACGT CGTACTTGTT ATCACTTCAA AGGTTAGACC TTGGCTACAT GCGATC	2		7	12	15	10		
Rb.COI.2	.... T......... ....T. ..A.....		1	7	3	18	2		
Rb.COI.3	....A.....			4	2	5			
Rb.COI.5	.... T......... ....A.....		4	39	9	27			
Rb.COI.6	....TG.....			5	1	1			
Rb.COI.7	....C....				3				
Rb.COI.8	....G..			1					
Rb.COI.9	.......T.. T......... ....A.....			1					
Rb.COI.10	.... T......... ....A..G....			1					
Rb.COI.11	....C.... ....G.			2		1			
Rb.COI.12	....A.. ....A.....					5			
Rs.COI.1 (GC)	C......... ......C... .....C.... ...C...G.. ...T.....C .....T								9
Rs.COI.4 (GC)	C.A....... ......C... .....C.... ...C...G.. ...T.....C .....T								1
Rs.COI.3 (PCBC)	CAAC.CGTAC .AC.....CC G...TC..CC GAAC.GT..T ....T.CT.C A.AGC.								9
Rs.COI.2 (PCBC)	CAAC.CGTAC .AC.....CC G...TC..CC GAAC..T..T ....T.CT.C A.AGC.								2
Rbr.COI.13	CAAC.CGTAC .AC.....CC GC..TCC.CC GAAC.GT..T .C..T.CT.C A.AGC.			1					
Rbr.COI.14	CAAC.CGTAC .AC.....CC GC..TC..CC GAAC.GT..T .C..T.CT.C A.AGC.	8	5	22	8				
Rbr.COI.15	CAAC.CGTAC .AC.....CC GC..TC..CC GAAC.GT..T ....T.CT.C A.AGC.	5	7	19	5			2	
Rbr.COI.17	CAAC.CGTAC .AC.....C. GC..TC..CC GAAC.GT..T ....T.CT.C A.AGC.			1					

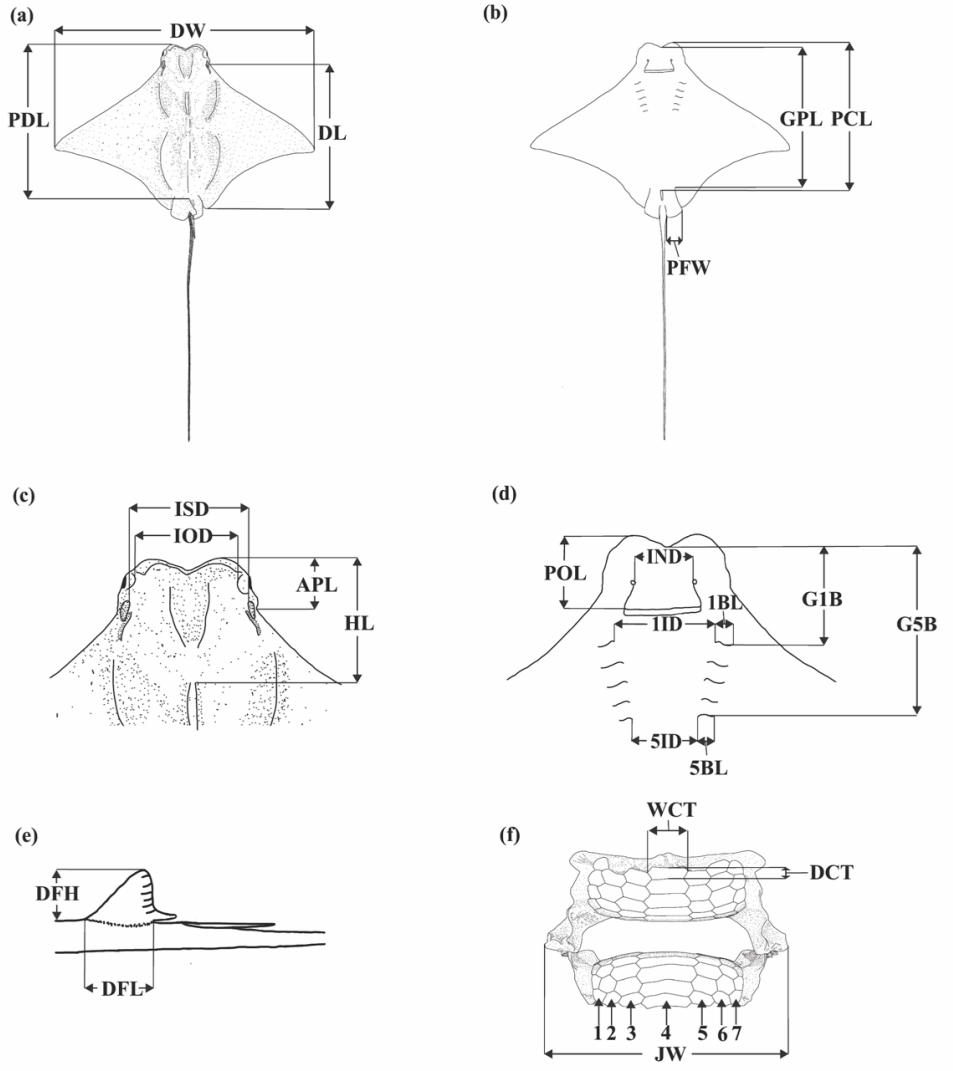

FIGURE 2. Illustrations depicting morphological measurements and counts taken from *Rhinoptera* spp. In this study. Descriptions of abbreviations of counts and measurements are listed in Table 1. A. Dorsal view. B. Ventral view. C. Cephalic region in dorsal view. D. Cephalic region in ventral view. E. Lateral view of dorsal fin. F. Labial view of jaw.

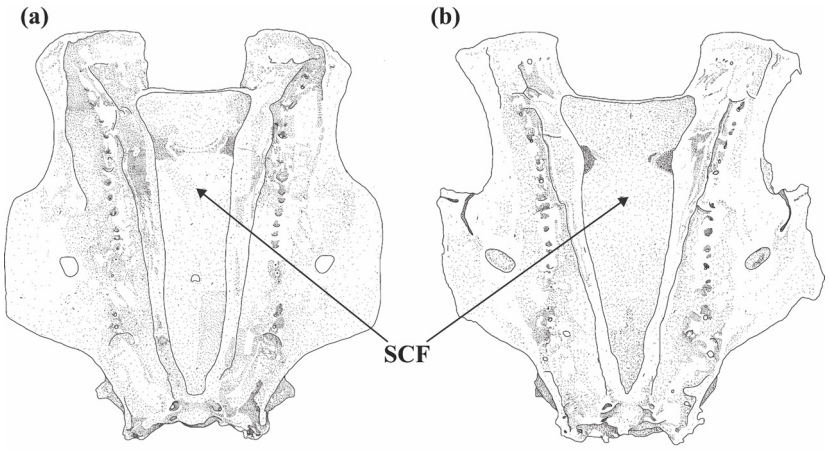

FIGURE 3. Chondrocrania in dorsal view illustrating the interspecific difference in the shape of the supra-cranial fontanelle (SCF). This difference was identified as a potentially useful field characteristic. A. Chondrocranium of *Rhinoptera* bonasus. B. Chondrocranium of *Rhinoptera brasiliensis*.

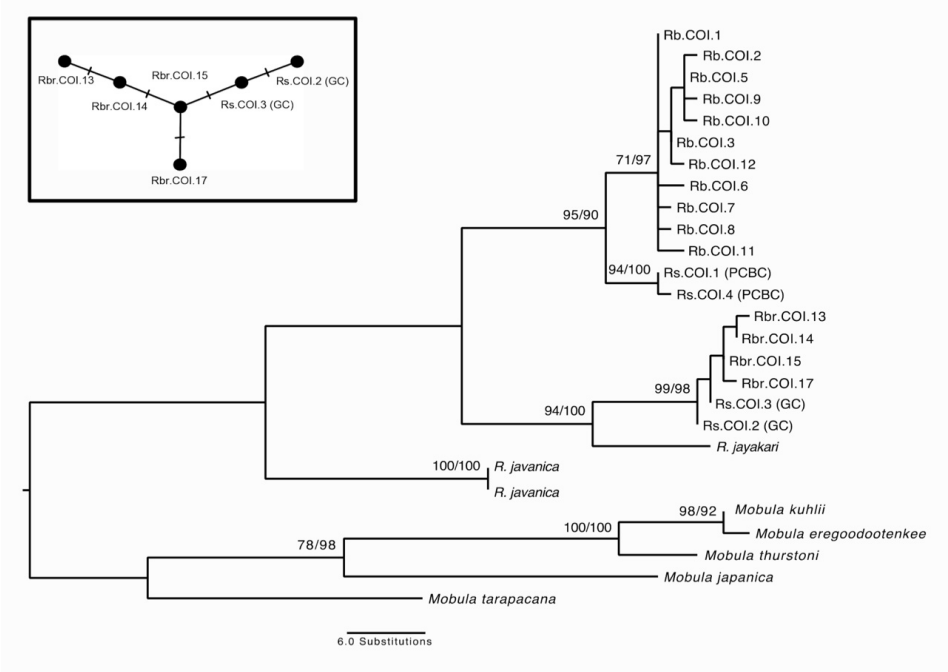

FIGURE 4. Molecular hypothesis relating COI haplotype diversity observed in cownose rays (*Rhinoptera*). Homologous sequences from five species of *Mobula* were used as outgroups. Shown is a topology based on Bayesian inference where nodes with less than 80% posterior probability (PP) are collapsed. PP and bootstrap values from Bayesian inference (BI) and maximum parsimony (MP) analyses are indicated (PP/MP). Only nodes that received strong support (>90% (PP) in addition to >70% (MP)) are indicated. Codes for individual haplotypes are those found in Table 2. Haplotypes from *R. jayakari* and *R. javanica* (Lim *et al.* 2015) and all *Mobula* (Poortvliet *et al.* 2015) were retrieved from GenBank. Inset—a minimum spanning network that relates haplotypes observed in *R. brasiliensis* and *R. steindachneri* (GC), see text for explanation.

Of interest in these topologies is a polyphyletic *R. steindachneri*, where two haplotypes (*Rs.*COI.1, *Rs.*COI.4) sampled from this species form a monophyletic group with haplotypes sampled from putative *R. bonasus* from the GOM and WNA, and two haplotypes (*Rs.*COI.2, *Rs.*COI.3) were nested within a grouping that includes haplotypes putatively identified as *R.* cf. *brasiliensis* from the GOM and WNA, as well as the vouchered *R. brasiliensis* specimens from Brazil. Previous research suggested the potential for cryptic speciation in *R. steindachneri* (Sandoval-Castillo and Rocha-Olivares 2011), and, given the location of capture and phylogenetic position suggested in Naylor *et al.* (2012), we provisionally assign haplotypes *Rs.*COI.2 and *Rs.*COI.3 to *R. steindachneri*, Gulf of California (GC—*Rs.*COI.2 (GC) and *Rs*.COI.3 (GC), and haplotypes *Rs*.COI.1 and Rs.COI.4 to *R. steindachneri*, Pacific Coast of Baja California (PCBC—*Rs*.COI.2 (PCBC) and *Rs*.COI.3 (PCBC); these designations are consistent with Sandoval-Castillo and Rocha-Olivares (2011).

Overall tree topology showed that haplotypes surveyed from various species of *Rhinoptera* formed two large, well supported clades. One included haplotypes characterized in our reference *R. bonasus* from the Chesapeake Bay (n=12) plus individuals sampled from the GOM (n=176). Sister to this group was the well-supported clade containing two haplotypes sampled from *R. steindachneri* (PCBC) (*Rs*.COI.2 (PCBC) and *Rs*.COI.3 (PCBC)). Support for this grouping was strong, greater than 90% for both the bootstrap (MP) and posterior probability (BI) analyses, as was the support for monphyly of the PCBC haplotypes, our vouchered *R. bonasus* and individual unassigned haplotypes from the GOM contained within this larger clade. For the unassigned haplotypes, the 'best close match' criterion invariably indicated *R. bonasus* and no other species fell within the 95% threshold for intraspecific divergence. Based on these results, counts and measures of diagnostic morphological characters (see below), and geographic distribution (GOM and WNA), we assigned haplotypes *Rb*.COI.1—*Rb*.COI.12 as intraspecific variants within *R. bonasus*.

A second, larger clade contained COI haplotypes sampled from a vouchered specimen of *R. brasiliensis* (GenBank accession JX124888.1), the single *R. brasiliensis* specimen from Sergipe state, Brazil (MZUSP 113721), and putative *R.* cf. *brasiliensis* collected from the GOM (n=81). One of the topologies recovered under the MP criterion was largely concordant with that hypothesized by the BI analyses and placed the two haplotypes sampled

from *R. steindachneri* (GC) as a monophyletic group nested within haplotype variation uncovered in *R.* cf. *brasiliensis*/*R. brasiliensis*. However, calculated bootstrap and posterior probability values indicated poor support for this and other alternatives, although the monophyly of the entire 'clade' was strongly supported by both analyses. Despite no significant support for reciprocal monophyly of haplotypes sampled from *R. steindachneri* (GC) and *R.* cf. *brasiliensis/R. brasiliensis* a single fixed difference differentiated all surveyed *R.* cf. *brasiliensis*/*R. brasiliensis* haplotypes from the two haplotypes sampled in *R. steindachneri* (GC). A minimum spanning network, that is more likely appropriate for depicting relationships among closely related haplotypes as observed here, clearly defines this single nucleotide substitution as differentiating haplotypes observed in *R. steindachneri* (GC) and *R.* cf. *brasiliensis/R. brasiliensis* (inset, Fig. 4). A barcoding approach using the 'Best Close Match' criterion of Meier *et al.* (2006), in all cases, assigned haplotypes *Rb*.COI.13—*Rb*.COI.19 (excluding the vouchered *Rb*.COI.15) as *R. brasiliensis*. Therefore, given the geographic distribution of samples collected and diagnostic morphological characters (see below), we assign haplotypes *Rb*.COI.13—*Rb*.COI.19 as intraspecific variants within *R. brasiliensis* despite the lack of any significant support for monophyly in independent phylogenetic analyses.

Morphology. There were statistically significant differences in the numbers of both upper (U = 526.000, p < 0.001) and lower (U = 429.500, p < 0.001) tooth series between the two species, although there was overlap between the counts (Fig. 5). *Rhinoptera bonasus* was found to possess from 5 –13 (mean = 7.5, S.D. = 1.2) tooth series in the upper jaw and 6 –13 (mean = 7.7, S.D. = 1.1) tooth series in the lower jaw. *Rhinoptera brasiliensis* was found to possess from 7—14 (mean = 10.0, S.D. = 1.4) and 7 –15 (mean = 10.9, S.D. = 1.8) tooth series in the upper and lower jaw, respectively. Of the 207 specimens collected from the WNA and GOM identified as either *R. bonasus* or *R. brasiliensis*, 186 individuals were genetically assigned identifications consistent with those assigned from tooth series counts, resulting in an 89.9% rate of agreement. Nineteen of the 21 individuals for which there were discrepancies between the two identifications, had tooth series counts consistent with descriptions of *R. brasiliensis*, while two had tooth series counts consistent with descriptions of *R. bonasus*.

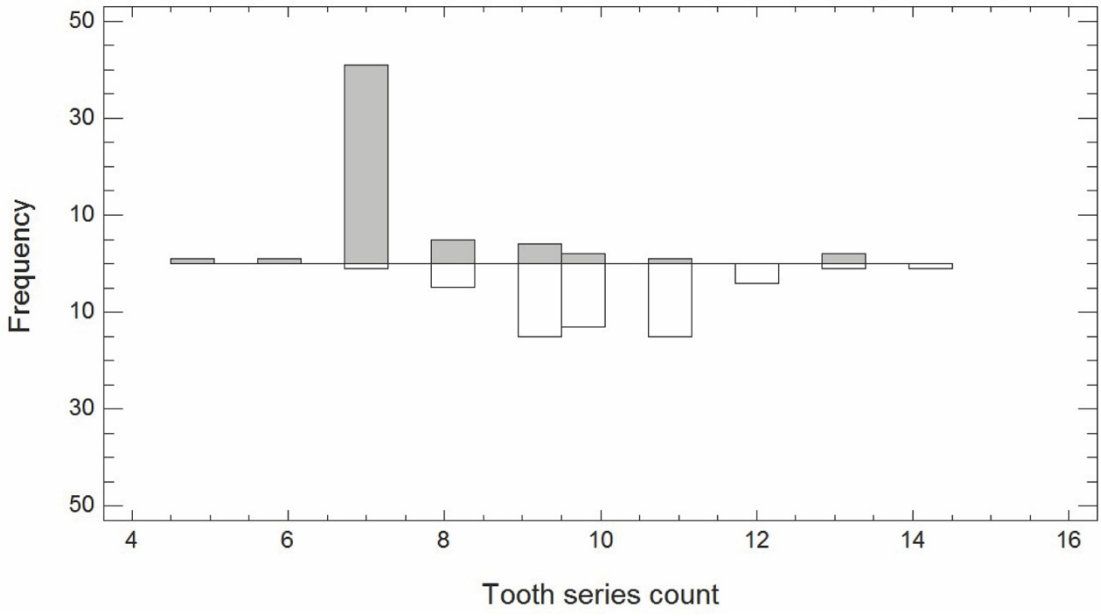

FIGURE 5. Frequency histogram of upper jaw tooth series counts for *R. bonasus* (gray) and *R. brasiliensis* (white) examined during this study.

Morphological measurements were analyzed for 89 (41 *R. bonasus*, 48 *R. brasiliensis*) of the individuals for which genetic sequences were available. Specimens included in morphological analyses ranged from 346 to 965 mm DW for *R. bonasus* and 468 to 1019 mm DW for *R. brasiliensis*. Using discriminant analysis with backward stepwise selection procedures, 93.3% (Wilks Lambda = 0.329; F = 23.608; d.f. = 7,81 p < 0.001) of rays were assigned identifications based on morphology consistent with identifications based on genetic analyses. The area under the receiver operating characteristic (ROC) curve was 0.974. Under cross validation, 87.6% of individuals

were correctly identified. By this method, seven characters were retained by the model: disc length, pre-dorsal distance, interorbital distance, internasal distance, distance from anterior groove to mid-point of fifth gill slit, distance of anterior grove to mid-point of first gill slit, and width of top central tooth in bottom jaw (Table 3). Mann-Whitney and t-test results indicated that there were significant differences between the two species for 17 of the 23 measurements (Table 3). Despite significant differences in the mean values of each of these measures, the ranges of values exhibited by the two species overlapped for all.

TABLE 3. Summary statistics for assignments of intraspecific variation observed in cownose rays (*Rhinoptera* sp.). Distances for the 'Best Close Match' criterion (Meier *et al.* 2006) are percent sequence divergence calculated as K2P distances (95% cutoff = 0.55).

Haplotype	K2P Distance to BCM (%)	Taxon - BCM
RbCOI.1	---	*R. bonasus* (Voucher)
RbCOI.2	---	*R. bonasus* (Voucher)
RbCOI.3	0.19	*R. bonasus*
RbCOI.4	0.19	*R. bonasus*
RbCOI.5	0.19	*R. bonasus*
RbCOI.6	0.37	*R. bonasus*
RbCOI.7	0.19	*R. bonasus*
RbCOI.8	0.19	*R. bonasus*
RbCOI.9	0.37	*R. bonasus*
RbCOI.10	0.19	*R. bonasus*
RbCOI.11	0.37	*R. bonasus*
RbCOI.12	0.37	*R. bonasus*
RbCOI.13	0.37	*R. brasiliensis*
RbCOI.14	0.19	*R. brasiliensis*
RbCOI.15	---	*R. brasiliensis* (Voucher)
RbCOI.17	0.19	*R. brasiliensis*

In addition to the differences noted above, the shape of the supracranial fontanelle (Fig. 3) was found to be distinctly different between the two species for all specimens examined (19 individuals: 7 *R. bonasus*, 12 *R. brasiliensis*). In *R. bonasus*, the supracranial fontanelle tapered gradually posterior to the nasal capsules, with the lateral margins being nearly straight. In *R. brasiliensis*, the margins of the supracranial fontanelle taper sharply inward directly posterior to the nasal capsules and immediately flared back out at the level of the orbits, whereafter it tapers gradually, with the margins being more rounded than straight.

Discussion

Phylogeny. The geographic range of *R. brasiliensis* has long been assumed to be limited to a relatively small area off the coast of southern Brazil (Bigelow & Schroeder 1953, Vooren and Lamónaca 2004). In addition, the validity of the species has long been in question with many suggesting that specimens representing this species are aberrant *R. bonasus* (Schwartz 1990; Compagno 1999). However, it is apparent from the data presented herein that two species of cownose ray inhabit the GOM. Based on genetic identifications paired with morphological analyses, the anomalous individuals tentatively identified as *R.* cf. *brasiliensis* relate most closely to the *R. brasiliensis* collected from Brazil and should therefore be considered conspecific. These specimens are genetically and morphologically distinct from individuals we assign as *R. bonasus*. The results of this study suggest that the current range of *R. brasiliensis* extends from southern Brazil to at least the northern GOM in the United States. The extent of the species occurrence into the WNA appears to be limited based on the results of recent research finding only a single individual off the coast of North Carolina (Naylor *et al.*, 2012) as well as four individuals captured off the coast of Georgia (J. Quattro, unpublished data). There has been no evidence of a second species of *Rhinoptera* in the Chesapeake Bay (Carney *et al.* 2017, J. McDowell, pers comm) which supports a large seasonal population of cownose rays.

TABLE 4. External morphological measurements for *Rhinoptera bonasus* and *Rhinoptera brasiliensis* utilized in discriminant function analyses as well as t, and Mann-Whitney tests. *t* indicates Student's t-test and *U* indicates Mann-Whitney test. Mean and range values presented as a ratio of disc width.

Measurement	*R. bonasus* Mean (Range)	*R. cf. Brasiliensis* Mean (Range)	Statistic	Significance
Disc length	0.535 (0.472–0.661)	0.603 (0.488–0.775)	*U*=302.0	*p*<0.001
Length of the anterior projection	0.084 (0.047–0.132)	0.083 (0.062–0.130)	*U*=1010.0	*p*=0.834
Dorsal fin length at base	0.066 (0.050–0.081)	0.071 (0.049–0.089)	*U*=672.0	*p*=0.010
Dorsal fin height	0.037 (0.022–0.054)	0.040 (0.030–0.051)	*U*=558.0	*p*<0.001
Pre-dorsal distance	0.573 (0.542–0.609)	0.586 (0.509–0.637)	*U*=637.0	*p*=0.004
Interorbital distance	0.154 (0.109–0.172)	0.150 (0.107–0.177)	*U*=944.5	*p*=0.748
Cranial length	0.157 (0.090–0.185)	0.160 (0.126–0.203)	*U*=856.0	*p*=0.294
Interspiracle distance	0.148 (0.125–0.170)	0.154 (0.138–0.171)	*U*=631.5	*p*=0.004
Pre-oral distance	0.109 (0.090–0.136)	0.116 (0.083–0.130)	*U*=465.0	*p*<0.001
Internasal distance	0.082 (0.070–0.113)	0.088 (0.071–0.094)	*U*=246.0	*p*<0.001
Pre-cloacal distance	0.527 (0.495–0.573)	0.548 (0.491–0.582)	*U*=414.0	*p*<0.001
Distance from anterior groove to pelvic fin	0.518 (0.485–0.549)	0.551 (0.491–0.666)	*U*=293.5	*p*<0.001
Pelvic fin width	0.068 (0.048–0.140)	0.075 (0.049–0.138)	*U*=731.5	*p*=0.038
Left fifth gill slit length	0.019 (0.015–0.023)	0.017 (0.013–0.024)	*t*=2.767	*p*=0.007
Fifth interbranchial distance	0.118 (0.105–0.126)	0.117 (0.101–0.136)	*t*=0.886	*p*=0.378
Distance from anterior groove to mid-point of fifth gill slit	0.235 (0.221–0.259)	0.250 (0.221–0.270)	*U*=334.0	*p*<0.001
Left first gill slit length	0.026 (0.021–0.032)	0.027 (0.021–0.036)	*U*=791.0	*p*=0.113
First interbranchial distance	0.168 (0.150–0.189)	0.166 (0.155–0.185)	*U*=1170.0	*p*=0.127
Distance of anterior grove to mid-point of first gill slit	0.138 (0.119–0.172)	0.147 (0.121–0.164)	*U*=506.0	*p*<0.001
Width of top central tooth in upper jaw	0.005 (0.003–0.008)	0.004 (0.002–0.007)	*U*=1571.0	*p*<0.001
Width of top central tooth in lower jaw	0.005 (0.001–0.007)	0.004 (0.002–0.006)	*U*=1527.5	*p*<0.001
Length of top central tooth in upper jaw	0.030 (0.022–0.042)	0.027 (0.022–0.036)	*U*=1454.0	*p*<0.001
Length of top central tooth in lower jaw	0.024 (0.017–0.031)	0.022 (0.015–0.027)	*U*=1351.5	*p*=0.003

Data presented by Naylor *et al.* (2012) suggested that the aberrant individuals present in the GOM were polyphyletic with regard to *R. steindachneri*, and haplotypes were shared between the unknown GOM animals and *R. steindachneri* sampled in the Gulf of California. We, likewise, found a very close relationship between *R. steindachneri* (GC) and *R. brasiliensis*, but also found a single fixed difference that differentiates haplotype variation surveyed in the two species. As single fixed differences from two large samples are unlikely due to sampling alone (e.g., Hey 1991), we believe the pattern uncovered from COI is intriguing and not due to insufficient sampling. However, while further genetic and morphological comparisons are required to fully elucidate the relationships among the North American rhinopterids, data presented in this study suggest that although closely related, *R. brasiliensis* and *R. steindachneri* (GC) are genetically distinct. In addition, the genetic divergence between the two clades of *R. steindachneri* sampled from Mazatlán would appear to indicate cryptic speciation, as was previously suggested by Sandoval-Castillo and Rocha-Olivares (2011). Recent research by Barreto (2016) provides additional support of the results presented in this paper and further examines the evolutionary relationships among the North American rhinopterids. The author's research, utilizing both nuclear and mitochondrial DNA analyses, indicates similar patterns of distribution and phylogenetic relationships as our own, lending independent validation of these results. Their results indicate the widespread presence of *R. brasiliensis* in the southern GOM as well as the close genetic relationship between *R. steindachneri* and *R. brasiliensis*. However, as in the current study, they maintain *R. steindachneri* and *R. brasiliensis* as separate species based on the genetic differences they exhibit.

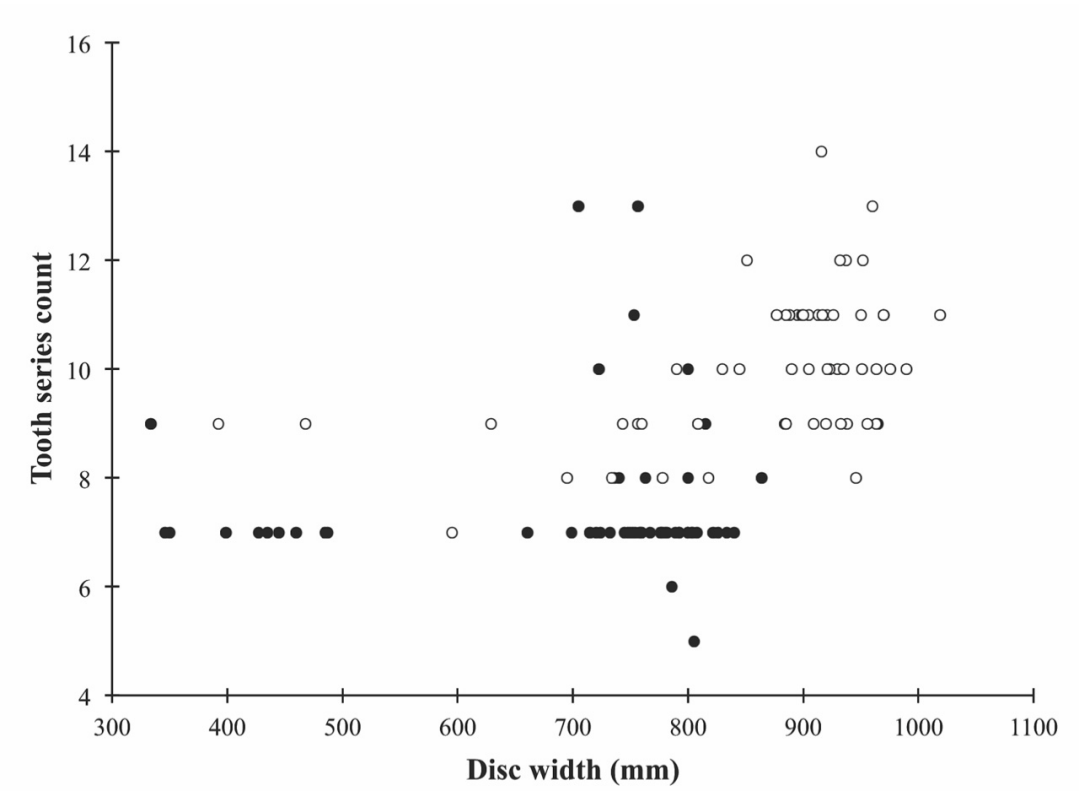

FIGURE 6. The relationships between disc width (mm) and upper jaw tooth series counts for *Rhinoptera bonasus* (black) and *Rhinoptera brasiliensis* (white) examined in this study.

Morphology. There was a high rate of agreement between the species assignments suggested by tooth series counts and sequence information; however, there were some discrepancies. There appears to be substantial overlap in tooth series counts between the two species, as previously suggested by Schwartz (1990). Not only were tooth series counts variable within each species but at the individual level as well, with tooth series regularly splitting and coalescing. This has the potential to result in increased tooth counts with an increase in size, as teeth may be added as the individual grows. Seven of a subset of 27 specimens (26.9%) examined during the course of this study were found to have individual teeth splitting into two separate series (n=2), or separate teeth coalescing into a single series (n=1). In some cases these events were witnessed to occur in the same individual (n=4). The

segmentation of tooth series had been previously discussed by both Bateson (1894) and Gudger (1933) based upon a single specimen. Despite the potential for variability, tooth counts were still a relatively accurate (~90%) method for discriminating between the two species.

Discriminant analyses of external morphometrics indicated significant differences in numerous characters, several of which agree with the findings of both Bigelow and Schroeder (1953) (internasal distance and tooth plate conformation) and Aguiar *et al.* (2001) (internasal distance and distance from anterior groove to midpoint of fifth gill slit). However, the ranges of values for the two species overlap, indicating that when taken individually they are of little use for identification purposes. A character that could prove diagnostic in field surveys is the shape of the supracranial fontanelle (Fig. 3). Gallo *et al.* (1997) indicated that there are differences in the shape of this structure between the two species, and that the shape is fairly consistent within a species. This was confirmed by material, collected from different localities, examined in the current study. Although skeletal, this character can occasionally be observed externally, potentially lending itself to use for identification in the field or laboratory.

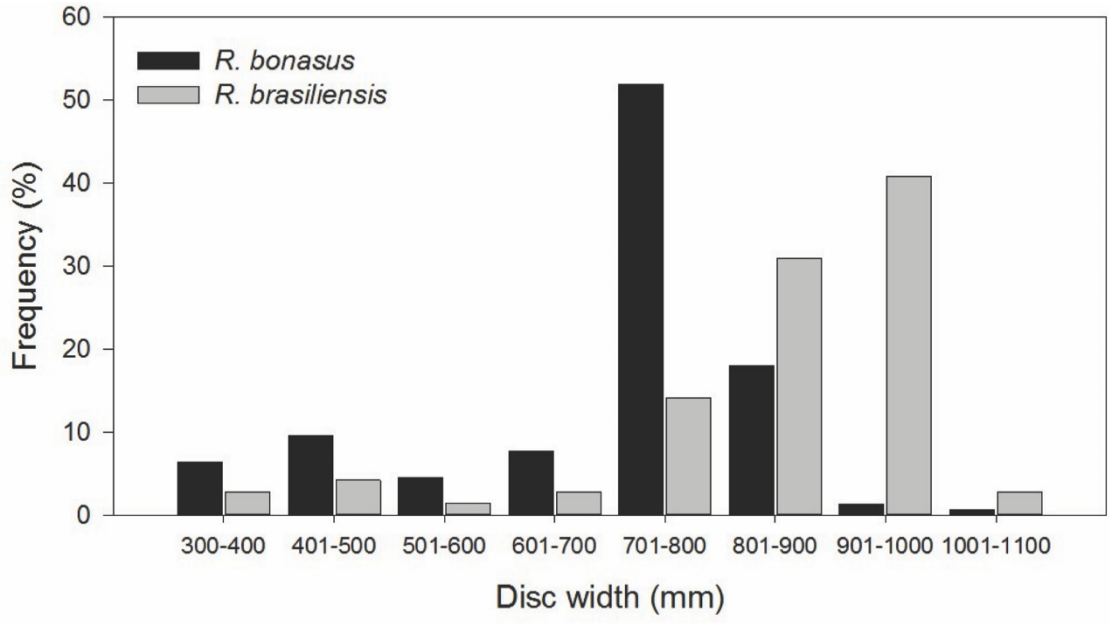

FIGURE 7. Disc width (mm) frequencies of *Rhinoptera bonasus* and *Rhinoptera brasiliensis* examined during this study.

Conclusions

It is impossible to estimate how long and to what extent *R. brasiliensis* has been present in the GOM, but the reports of Bigelow and Schroeder (1953), Schwartz (1990), and the MCZ ledger would all seem to indicate a presence in the greater WNA potentially dating at least to the 1950's. Holdings at the Biodiversity Research and Teaching Collections at Texas A & M University (TCWC) include 6 *R. brasiliensis* (based on tooth counts and supracranial fontanelle shape), originally identified as *R. bonasus*, which were collected from the waters off of Freeport, Texas (5) and between Cedar and Seahorse Keys, Florida (1) in the GOM. All were collected between 1979 and 1981, providing further evidence of a longstanding presence in the GOM. While the historical prevalence of *R. brasiliensis* in this region is unknown, their presence brings into question the results of any research, particularly recent research, that focuses on *R. bonasus*, unless all individuals included were either genetically or morphologically identified to the species level. It is unclear whether there are any differences in the life history and ecology of the two species but the data from the current study suggests that *R. brasiliensis* regularly attains a larger overall size than *R. bonasus* (Fig. 7), as well as being larger at birth. Any study that incidentally includes members of both species would not accurately reflect these parameters in either species, nor accurately reflect any other differentiated morphological parameters, if they exist. Therefore, care should be taken in applying the results of these studies until it can be determined if any such differences exist between the two species.

Another important issue that arises from the evidence of a larger range for *R. brasiliensis* concerns its endangered status. Because the IUCN assessment placed so much weight on the supposition that the species has

such a limited range and was heavily fished within that range for decades, the findings of the current study suggest that a reassessment is warranted. However, much more information is needed. It is important to note that because of the federally mandated use of turtle excluder devices in U.S. waters, bycatch in the trawl fishery could be expected to be minimal, and the fact that currently there is no directed fishery, the species could thrive in the coastal waters of the GOM.

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