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Deep Mitochondrial Divergence in Baja California Populations of an Aquilopelagic Elasmobranch: The Golden Cownose Ray

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Abstract

Assessing the realized effect of dispersal in the genetic makeup of a species has significant evolutionary, ecological, and economical consequences. Here, we investigate the genetic diversity and population differentiation in the aquilopelagic golden cownose ray *Rhinoptera steindachneri* from the Gulf of California (GC) and the Pacific coast of Baja California (PCBC) using the mitochondrial NADH2 gene. Low levels of genetic diversity were found with only 4 polymerase chain reaction-restriction fragment length polymorphism haplotypes among 76 specimens. Pacific coast organisms were fixed for a unique haplotype not shared with rays from the gulf; 92% of GC rays possessed a single NADH2 haplotype not found in the Pacific. This produced significant differentiation between the GC and the PCBC ($\Phi_{CT} = 0.972$, $P < 0.001$). A pronounced phylogeographic pattern was found in which GC haplotypes were reciprocally monophyletic relative to a very divergent Pacific lineage ($d = 10\%$). Our results indicate that despite high dispersal potential, GC and PCBC golden cownose ray populations are characterized by highly divergent mitochondrial lineages. Although more evidence is needed to corroborate the genetic isolation and systematic status of PCBC and GC golden cownose rays, our results suggest a possible cryptic species in the region.

Key words: *aquilopelagic marine ray, Baja California, cryptic divergence, genetic structure, Gulf of California, Rhinoptera steindachneri*

Species mobility is a conspicuous feature and has often been used as a predictor of gene flow (Slatkin 1987). Nevertheless, the assumption of elevated gene flow based on high potential mobility can lead to spurious inferences and underestimations of intraspecific and interspecific genetic differentiation leading to unnoticed biological diversity. Unraveling the existence of unforeseen genetic heterogeneity in presumably mobile organisms is of fundamental consequence not only for the assessment of biodiversity but also for ascertaining the underlying mechanisms. Understanding the relation between mobility and genetic structure is of considerable interest because of its multiple implications. From an evolutionary perspective, genetic differentiation in the face of high mobility may reflect unsuspected mechanisms of reproductive isolation. From an ecological perspective, population differentiation in the presence of migration may reflect adaptation to local environments. And from a conservation and economical perspective, understanding population connectivity patterns may help identify population segments requiring special management considerations (Avise 1994).

The distribution of the golden cownose ray *Rhinoptera steindachneri* extends from Baja California Sur, Mexico, to northern Peru, including the Gulf of California (GC). This coastal batoid fish feeds over the bottom in shallow water (~30 m) but usually swims near the surface (McEachran and Notarbartolo di Sciara 1995). As an aquilopelagic ray, it is capable of reaching high speeds and swimming great distances (Compagno 1990). The null expectation is that high mobility would be conducive to elevated gene flow and genetic homogeneity among distant populations. However, Bizzarro et al. (2007) have reported considerable size

differences in *R. steindachneri* between the Pacific Coast of Baja California (PCBC) and the GC. Should this phenotypic difference correlate with genetic differentiation among allopatric populations, it would reflect limited genetic interchange. Alternatively, in the absence of genetic heterogeneity, it may reflect phenotypic plasticity within a panmictic population in response to environmental differences between PCBC and the GC. *Rhinoptera steindachneri* has trophonemata viviparity and extreme low fecundity, giving birth to only one large pup per year. Evidence suggests localized breeding and nursery grounds in the GC and the PCBC (Villavicencio Garayzar 1995); where *R. steindachneri* is a significant component in local multispecific fisheries that are economically important for the region (Villavicencio Garayzar 1995; Márquez-Farias 2002; Bizzarro et al. 2007). In light of its susceptibility to overexploitation owing to its K-strategy, management decisions will benefit from increased understanding of the patterns of geographic genetic variation.

A previous study (Sandoval-Castillo et al. 2004) revealed significant levels of cryptic genetic differentiation and phylogeographic signal between PCBC and GC shovelnose guitarfish (*Rhinobatos productus*), suggesting the presence of diversifying forces in the region and the possible existence of cryptic speciation. Consequently, the aim of this paper is to address the influence of these putative forces in the levels of genetic diversity and population differentiation of the more vagile aquilopelagic golden cownose ray *R. steindachneri*. In so doing, we will be testing the prediction of genetic homogeneity suggested by its high mobility and providing the basis for stock identification in the species.

Materials and Methods

Tissue samples ($n = 76$) were collected from the commercial catch landed in Bahía Kino, Sonora (lat 28°50′N; long 111°58′W) and San Felipe, Baja California (lat 31°11′N; long 114°48′W) in the GC and in San Ignacio (lat 26°45′N; long 113°12′W) and Bahía Almejas (lat 24°24′N; long 111°39′W), Baja California Sur, in the PCBC (Figure 1). Samples were stored in 95% ethanol. Total genomic DNA was isolated using proteinase K digestion followed by a salting out protocol with lithium chloride (Gemmell and Akiyama 1996). The mitochondrial (mt) NADH2 gene was amplified using primers ND2Met47 (TTT TGG GCC CAT ACC) and ND2Trp18 (GCT TTG AAG GCT TTT GGT) designed for this study. Each 50 µl reaction containing 0.18 mM of each dNTP, 1X PCR buffer, 0.2 µM each primer, 2 U *Taq* DNApol (NEB, Ipswich, MA), and 2 µl of template DNA was amplified with a thermal cycling profile of: one cycle 2 min at 95 °C; 35 cycles 15 s at 94 °C, 1 min at 56 °C, 2 min at 72 °C; and a final cycle of 7 min at 72 °C. The amplified segment was digested with 5 restriction endonucleases (*Alu*I, *Cfo*I, *Hae*III, *Mse*I, and *Rsa*I) following manufacturer's protocols (NEB). Restriction fragments were separated using 6% polyacrylamide gel electrophoresis. Endonuclease digestions were used to identify distinct haplotypes. Subsequently, 2 individuals from each haplotype were sequenced using BigDye terminator 1.1 (Applied Biosystems, Foster City, CA). Sequencing was performed in an automatic sequencer ABI 3100 Gene Analyzer (Applied Biosystems).

Sequences were aligned using CLUSTAL X (Thompson et al. 1997). Haplotype and nucleotide diversities (Nei 1987), interhaplotype genetic distance (Kimura 1980) and divergence (Nei and Tajima 1981), were calculated with REAP 4 (McElroy et al. 1992) from DNA sequence data. We conducted an analysis of molecular variance (AMOVA) to analyze the hierarchical partitioning of genetic variation using the program Arlequin 3.1 (Excoffier et al. 2005). We adjusted significance levels of multiple tests with the sequential Bonferroni correction (Rice 1989). Finally, haplotype evolutionary relationships were assessed with a neighbor-joining (NJ) reconstruction using Kimura (1980) distance with the program MEGA 4.0 (Tamura et al. 2007), in which a sequence of *Myliobatis longirostris* was used as outgroup.

Results

Only 4 composite haplotypes were found among 76 golden cownose rays (GenBank accession HQ540559–62), resulting in low average population haplotype ($h = 0.077$) and nucleotide ($\pi = 0.255\%$) diversities (Table 1). Two haplotypes (Rs1 and Rs4) were found in 96% of the specimens. Rs1 predominated among GC fish, whereas all PCBC organisms were fixed for Rs4 (Figure 1), resulting in a marked difference in diversity between Pacific and gulf rays (Table 1). As expected, DNA sequence analyses showed that RFLP underestimated nucleotide diversity indices (Table 1).

AMOVA revealed the absence of genetic differentiation between localities in the GC or in the Pacific coast ($\Phi_{SC} = 0.000$, $P = 0.99$). However, differentiation between GC and Pacific coast rays was extreme and highly significant ($\Phi_{CT} = 0.972$, $P < 0.001$).

The NJ phylogeny shows that GC and PCBC lineages are reciprocally monophyletic (Figure 1) and highly divergent (10.03%, Table 1). Molecular clock calibrations for elasmobranch mtDNA range between 0.4% and 0.95% My^{-1} (Duncan et al. 2006; Quattro et al. 2006; Richards et al. 2009). Assuming a conservative average rate of 0.8% My^{-1}, the most divergent cownose ray lineages may have evolved in isolation for 12.5 My, which dates back to oldest evidence of the emergence of the Baja California Peninsula (Henry and Aranda-Gomez 2000).

Discussion

Genetic Diversity and Differentiation

Levels of mitochondrial diversity of the golden cownose rays in Baja California were low but not atypical of cartilaginous fishes (Table 4, Heist 2004; Sandoval-Castillo et al. 2004; Hoelzel et al. 2006). Elasmobranchs possess

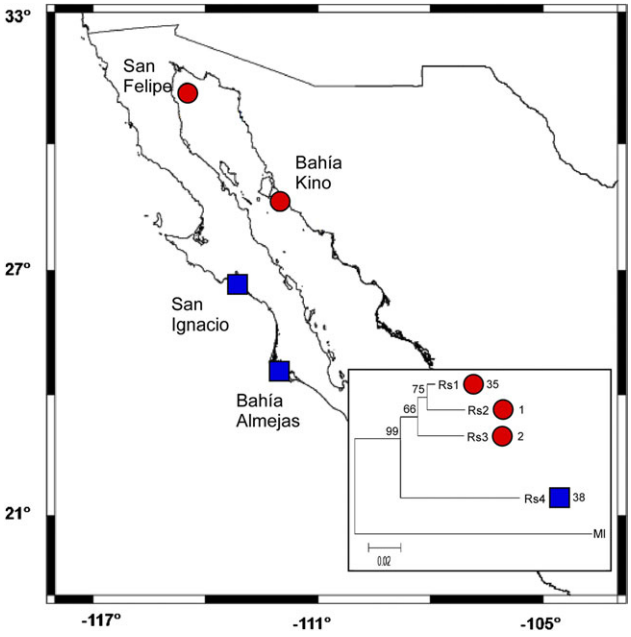

Figure 1. Collection locations of *Rhinoptera steindachneri* from the GC (circles) and the PCBC Peninsula (squares). Insert: phylogenetic NJ reconstruction of NADH2 sequences of *R. steindachneri* from the GC (circles) and PCBC Peninsula (squares). *Myliobatis longirostris* (MI) was used as outgroup. Nonparametric bootstrap support values are indicated next to nodes and haplotype frequencies next to symbols.

slower rates of molecular evolution than other vertebrates (Martin and Palumbi 1993), which may contribute to depressed intraspecific genetic diversities as found in other slowly evolving vertebrates such as turtles (Avise et al. 1992). Perhaps the low molecular diversity in the golden cownose ray reflects intrinsic slow rates of NADH2 evolution. For instance, the guitarfish *R. productus* has high levels of mtDNA control region (CR) diversity (Sandoval-Castillo et al. 2004) consistent with a high fecundity and relative abundance. However, NADH2 variation produced a pattern of depressed diversity like the one observed in *R. steindachneri* (Sandoval-Castillo JR, Rocha-Olivares A, unpublished data), reflecting differential rates of evolution between the noncoding CR and the structural NADH2 gene. Unfortunately, the amplification of CR in Myliobatiform rays (including *R. steindachneri*) has proven more difficult than in other elasmobranchs (e.g., see Stoner et al. 2003), apparently due to several long insertions in the locus (personal observations).

In addition to slow evolutionary rates, low haplotype and nucleotide diversities may reflect high levels of genetic drift due to the presence of reduced effective population sizes. This may appear paradoxical given that *R. steindachneri* is among the 5 most abundant rays present in commercial catches from the GC (Márquez-Farias 2002). On the other hand, historical demographic fluctuations or selective sweeps could account for the marked fixation of a private haplotype among Pacific fish. Cownose ray populations from the PCBC and GC are the northernmost representatives of the species (McEachran and Notarbartolo di Sciara 1995). As peripheral populations they may experience stronger selective pressures than more tropical core populations (Segelbacher and Storch 2002) and may be more susceptible to bottlenecks driven by climate changes, such as those attributed to Pleistocene glaciations (Jacobs et al. 2004).

The strong genetic structure in *R. steindachneri* is surprising given the high potential mobility and the lack of evident barriers to dispersal (Waples 1987; Doherty et al. 1995; Chenoweth et al. 1998). However, gene flow depends not only on dispersal but also on successful breeding. Different aspects of a species reproductive behavior may lead to genetic differentiation in geographic scales much smaller than predicted by their dispersal potential (Palumbi 1994). Long distance movements and recurrence in specific reproductive and feeding areas have been documented in other aquilopelagic rays, such as *Rhinoptera bonasus* (Smith and Merriner 1987) and *Myliobatis californica* (Gray et al.

Table 1 Mitochondrial NADH2 haplotype and nucleotide diversities of *Rhinoptera steindachneri* and levels of genetic divergence

	Sample size *n*	Haplotype diversity *h*	Nucleotide diversity π (%)		Genetic divergence *d* (%)	
Localities			Sequence[a]	RFLP	Sequence	RFLP
Bahia Kino	19	0.205	0.659	0.141		
San Felipe	19	0.105	0.366	0.088		
Bahia Almejas	19	0.000	0.000	0.000		
San Ignacio	19	0.000	0.000	0.000		
Regions						
GC	38	0.152	0.503	0.114	<0.001[b]	<0.001[b]
Pacific Coast	38	0.000	0.000	0.000	0.00[b]	0.00[b]
Average		0.077	0.255	0.057	6.68[c]	5.44[c]
Total	76	0.544	5.335	4.168	10.03[d]	8.16[d]

[a] 1061 base pairs.

[b] Between localities in each region.

[c] Among all localities.

[d] Between GC and Pacific coast.

1997). The existence of recurrent localized nursery areas may reflect some level of female philopatry, which has been suggested as a possible cause of the genetic structure in some elasmobranchs (Sandoval-Castillo et al. 2004; Hueter et al. 2005; DiBattista et al. 2008; Schultz et al. 2008). This has also been hypothesized for *R. steindachneri* in the GC and PCBC Peninsula, based on their use of specific and localized reproductive areas (Villavicencio Garayzar 1995). Tagging experiments are necessary to assess the extent of realized mobility and hypothesized philopatry in *R. steindachneri*.

Phylogeography and Taxonomic Implications

Delimiting sibling species on the basis of genetic divergence may be fraught with caveats (Ferguson 2002), but the mtDNA divergence between PCBC and GC cownose rays represents one of the largest intraspecific divergence reported for an elasmobranch, even exceeding interspecific divergences in batoid fishes (Table 2, Heist 1999; Sandoval-Castillo et al. 2004; Quattro et al. 2006; Richards et al. 2009). We argue that this extreme divergence suggests the existence of a cryptic species of *Rhinoptera* in the PCBC because the type locality of *R. steindachneri* is within the GC. Nevertheless, samples from a wider geographic range are necessary to both locate the position of the phylogeographic break and to reject the hypothesis of intermediate haplotypes. In the absence of junior synonyms for the PCBC rays, comparative life history and morphological studies are necessary to identify phenotypic diagnostic differences allowing the description of the cryptic species.

Compagno (1999) suggested that only 5 of the 11 nominal species of *Rhinoptera* are valid. These 5 species are strongly allopatric with widespread geographic ranges believed to reflect their high mobility (Schwartz 1990). However, the extreme differentiation in *R. steindachneri* at a regional geographic scale may be evidence of more than 5 valid species in the genus *Rhinoptera*. Moreover, the lack of evident morphological differentiation in the face of such extreme genetic differentiation between golden cownose rays reveals that genetic divergence can occur without concurrent evident morphological differentiation in this genus.

Conservation and Management Perspective

Our results indicate that GC and Pacific *R. steindachneri* belong to independent evolutionary lineages that should be managed independently following Moritz (1994). In addition, the extreme K-selected reproductive strategy of the species, with late maturation and only one pup per

Table 2 Intraspecific and interspecific genetic divergence in elasmobranch species using mitochondrial DNA gene sequences or whole genome RFLP

Intraspecific Species	Localities	*n*	Gene	Divergence *d* (%)	References
Gymnura marmorata	GC/PC	120	NADH2	<0.01	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Narcine entemedor	GC/PC	80	NADH2	0	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Rhinobatos productus	GC/PC	136	NADH2	1.2	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Myliobatis californica	GC/PC	75	NADH2	0.3	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Rhizoprionodon terraenovae	GM/AC	52	CR	<0.01	Heist (1999)
Isurus oxyrinchus	PC/AC	110	mtDNA-RFLP	0.9	Heist (1999)
R. productus	GC/PC	64	CR	2.5	Sandoval-Castillo et al. (2004)
Carcharhinus plumbeus	GM/AC	95	mtDNA-RFLP	<0.01	Heist (1999)
Sphyrna lewini	PC/WA	76	CR	51.0[a]	Quattro et al. (2006)[a]
Aetobatus narinari	WP/AC	36	Cytochrome *b*	3.3[a]	Richards et al. (2009)[a]
Rhinoptera steindachneri	GC/PC	76	NADH2	10.0[a]	This study
Interspecific					
Species 1	Species 2	Gene		Divergence *d* (%)	References
Narcine entemedor	*N. vermiculata*	NADH2		4.0	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
R. productus	*R. glaucostigma*	NADH2		5.0	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
M. californica	*M. longirostris*	NADH2		8.8	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Dasyatis dipterura	*D. longa*	NADH2		3.3	Sandoval-Castillo JR and Rocha-Olivares A, unpublished data
Himantura pacifica	*H. schmardae*	Cytochrome *b*		6.5	Richards et al. (2009)
Potamotrygon motoro	*P. castexi*	Cytochrome *b*		4.1	Richards et al. (2009)

PC, Pacific coast; GM, Gulf of Mexico; AC, Atlantic Coast; WA, Western Atlantic; WP, Western Pacific.

[a] Considered cryptic species.

litter, makes them very sensitive to overexploitation. The ecological strategy of *R. steindachneri* and the low genetic diversity in the studied populations reveals the urgent need of a suitable management strategy to avoid overfishing. Even though the "species" is considered abundant, it may be the case that catches may actually be composed of more than one demographical entity, which may differ in relevant life-history attributes. Consequently, we need to ascertain with additional genetic and nongenetic data the evolutionary and taxonomic nature of these nominally "intraspecific" lineages in order to shed more light on the appropriate measures for their management and conservation.

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