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RESEARCH ARTICLE

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Cryptic isolation of Gulf of California shovelnose guitarfish evidenced by mitochondrial DNA

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Abstract The shovelnose guitarfish *Rhinobatos productus* is an evolutionarily, ecologically, and economically important ray, with a continuous distribution from San Francisco, California (USA), to Mazatlan, Sinaloa, and in the Gulf of California (Mexico). Regional studies have revealed morphometric differences between shovelnose from the Gulf of California and the Pacific coast of Baja California, which may result from phenotypic plasticity in the presence of high levels of gene flow or from a degree of genetic differentiation in the presence of cryptic isolation within a continuous distribution. We used PCR-RFLP of the mitochondrial control region to assess the degree of genetic differentiation between Gulf of California and Pacific shovelnose guitarfish. We found very high levels of molecular diversity (averages: $h = 0.77$, $\pi = 1.19\%$), which may be associated with historically large and stable populations, as well as very significant levels of genetic differentiation between gulf and Pacific samples ($\chi^2 = 64$, $P < 0.0001$; $\Phi_{ST} = 0.63$, $P < 0.0001$, mean nucleotide divergence $d = 2.47\%$). We found a deep phylogeographic break between haplotypes from the gulf and the Pacific, which may suggest the existence of cryptic species but clearly indicates more than one evolutionarily significant unit of *R. productus*. Our results show a pattern of genetic structure and levels of differentiation consistent with the geological history of the region. Furthermore, these findings have wide-ranging implications for the management and conservation of cartilaginous fish in Mexico, as they reveal the existence of biological diversity that will go unnoticed without the genetic scrutiny of intraspecific variation and that is highly relevant for much needed management and conservation efforts.

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Introduction

Determining the mechanisms by which new species arise is a fundamental problem in evolutionary biology (Howard and Berlocher 1998). Population disjunctions are of considerable interest, because cessation of gene flow is almost universally believed to be a fundamental requirement for allopatric speciation (Endler 1989). Population subdivision leading to speciation in a continuous and seemingly unstructured environment like the ocean has been regarded as paradoxical (Palumbi 1992); however, population subdivision in marine organisms may result from mechanisms such as vicariant events (Bernardi et al. 2003), limited dispersal capabilities (Palumbi 1992), and fidelity to breeding areas (Ruzzante et al. 1998).

Analyses of high-resolution genetic markers to study geographic distribution patterns of genetic diversity have shown that widespread marine populations, once considered to be genetically homogeneous, actually possess genetic signatures that reflect historic and present barriers to gene flow (Bowen and Grant 1997; Palumbi 1999; Stepien et al. 2001). Failure to account for cryptic variation and structure in the genetic architecture of widespread organisms has far-reaching implications in evolution, where the connections between the geologic history and the geography of species formation will go unnoticed; in ecology, where causal connections between environmental and climatic changes and species distri-

butions and associations are a central issue; and in conservation biology, where the identification and delineation of significant units for management and conservation is of fundamental importance (Riddle et al. 2000).

The emergence of the Baja California peninsula and the associated creation of the Gulf of California have produced disjunct geographic distributions in several species of marine fishes and invertebrates over the last 5 million years (Myr) (Holt et al. 2000). The northern end of the gulf is older than the lower portion and was formed during the late Miocene (Larson et al. 1968). Geological evidence has revealed the historical existence of several seaways along the peninsula (Holt et al. 2000) that connected, intermittently, the Gulf of California and the Pacific Ocean, isolating and bringing into contact terrestrial and marine faunas, respectively (Upton and Murphy 1997; Riddle et al. 2000). Present biogeographic conditions in the Gulf of California have prevailed since about 10,000 years ago. Salinity and surface temperature in the gulf are higher than on the Pacific coast of Baja California, which is under the influence of the cold California current. In general, there is a marked environmental shift from warm tropical conditions in the lower gulf to warm temperate conditions in the upper gulf, which features localized permanent upwelling (Maluf 1983). The coasts surrounding the Gulf of California and along Pacific Baja California have a diversity of substrates and habitats that support very high levels of biodiversity (Dawson 1960).

The shovelnose guitarfish *Rhinobatos productus* (“shovelnose” henceforth) is a ray with a continuous distribution from San Francisco, California, USA, to Mazatlan, Sinaloa, Mexico, including the Gulf of California (Beeve and Tee-Van 1941). It is common throughout its range and may be the most abundant batoid in some areas. On the Pacific coast of Baja California and in the Gulf of California the shovelnose is actively fished; it is the predominant ray in the catches; and it has the best selling price, being an important source of income for regional fishers. Evolutionarily, guitarfishes are living representatives of the most primitive batoid lineage from which all living rays are derived. Thus, organisms in this group are considered important evolutionary links between sharks and rays (Compagno 1977).

There is evidence of morphometric differentiation between populations of guitarfish from the Pacific coast and the Gulf of California (Sandoval-Castillo and Villavicencio-Garayzar, unpublished data). However, this variation may reflect a phenotypic plastic response to differences in food availability and environmental regimes of both regions in the presence of high levels of gene flow between them. On the other hand, this variation may be associated with differences in the genetic makeup of allopatric populations and, hence, may be a consequence of cryptic genetic isolation in the face of a continuous distribution. Analyses of population structure by means of molecular tools have led to important advances in marine species management and conservation efforts (Nielsen and Powers 1995) and to an increased understanding of relevant evolutionary processes (Bernardi et al. 2003). Unfortunately, as is the case with most elasmobranchs (Feldheim et al. 2001), the genetic structure of *R. productus* remains completely unknown. Because of its ecological, economic and evolutionary importance, a study of the shovelnose population structure has been long overdue. In this paper we establish levels of genetic differentiation and exchange of *R. productus* from the Pacific and the Gulf of California to address the issue of phenotypic plasticity or cryptic isolation within the gulf.

Materials and methods

Tissue liver samples were dissected from the commercial catch of shovelnose (*Rhinobatos productus*) in Bahía Kino, Sonora (28°49′N; 111°57′W), in the Gulf of California, and in Bahía Almejas (24°24′N; 111°39′W), on the Pacific coast of southern Baja California. Samples were preserved in 95% ethanol and stored frozen until laboratory analyses. Approximately 10–50 mg of tissue was digested in 500 µl of DNAzol (Molecular Research Center, Cincinnati, Ohio) and proteinase K (60–100 ng μl^{-1}). Genomic DNA was purified using DNAzol and ethanol precipitation following the recommended protocol (Molecular Research Center, Cincinnati, Ohio). PCR amplifications were carried out using species-specific primers CbRhino1163 (5′-CYT ACT TCT CAT TAT TCC TNA TCC TCC TAC C-3′) and 12Srev326 (5′-ACT CGT ATA ACC GCG GTG GCT-3′). These primers were designed from known *R. productus* sequences of the cytochrome *b* and 12S rDNA genes. Each 25 µl reaction (0.18 mM dNTPs, 1×PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM each primer, 2 U *Taq* DNApol) was amplified with a thermal cycle profile of one cycle 5 min at 94°C; 35 cycles 15 s at 94°C, 120 s at 63°C, 45 at 72°C; and a final extension of 5 min at 72°C. In all amplifications negative controls containing deionized water instead of template DNA were used. Aliquots of amplified segments were digested using six restriction enzymes. Four digestions were made separately (*Rsa*I, *Hsp92*II, *Hae*III, and *Taq*I) and two were combined (*Mbo*I/*Alu*I) following manufacturer’s protocols. Fragments were separated by electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 µg μl^{-1}) and photographed.

Restriction fragment patterns were scored for each enzyme, and individual composite haplotypes were constructed using data from all enzymes. Haplotype diversity was calculated following Nei (1987), and nucleotide diversity, following Nei and Tajima (1981). Chi-squared significance values of differences in haplotype frequencies between regions were computed by using the Monte Carlo approach of Roff and Bentzen (1989). All calculations were made with the REAP statistical analysis package (McElroy et al. 1992). In addi-

tion, a neighbor-joining (NJ) reconstruction was made with Nei and Li's (1979) distance metric using the program Mega 2.0 (Kumar et al. 2001). Wright's fixation index (F_{ST}) and its molecular analog (Φ_{ST} Excoffier et al. 1992) were calculated using haplotype frequencies and the number of pairwise differences at restriction sites. Their significance was estimated with 10,000 permutations using the program Arlequin 2.0 (Schneider et al. 1999). Finally, gene flow (N_m) was estimated indirectly from linearized fixation indices (Cockerham and Weir 1993; Slatkin 1995).

Results and discussion

Genetic variability

Two of the enzymes (*Taq*I and *Hae*III) were monomorphic in the 64 shovelnose (*Rhinobatos productus*) analyzed (32 from each locality). Seventeen composite haplotypes were found (Table 1), resulting in a mean haplotype diversity of 0.77 and a mean nucleotide diversity of 1.19%. Haplotype diversity was higher than the one found by Grijalva-Chon et al. (2002) in the Pacific angel shark (0.14), but similar to those found by Heist et al. (1996a, 1996b) in shortfin mako (0.75) and sharpnose (0.64) sharks. On the other hand, nucleotide diversity (Table 2) was higher than the mean of six shark species (0.00–0.35%) reviewed by Heist (1999) and in the Pacific angel shark (0.08%) (Grijalva-Chon et al. 2002). Considering the higher number of restriction enzymes (>9) and the larger sample sizes involved in most of these studies, the genetic diversity of shovelnose may be considered much higher (i.e. obtained with only six enzymes). Moreover, elasmobranchs are known to have lower molecular evolutionary rates than other vertebrates (Martin and Palumbi 1993). However, mitochondrial haplotype diversities in this study were not unlike those found in many other marine teleosts (Grant and Bowen 1998; Rocha-Olivares and Sandoval-Castillo 2003). The high genetic variability observed in the shovelnose, an animal with a low molecular evolutionary rate, may be related to demographic events (Avise et al. 1984). Alternative combinations of small and large values of haplotype and nucleotide diversities may reflect different demographic histories; large values of both may be attributed to secondary contact between previously differentiated lineages or to a long evolutionary history in a large and stable population (Grant and Bowen 1998). Given the elevated fecundity of *R. productus* (Villavicencio Garayzar 1993), the hypothesis of a stable population through evolutionary time appears plausible and, in light of the results below, the influence of secondary contact cannot be ruled out.

Table 1 *Rhinobatos productus*. Haplotype frequencies of allopatric populations of shovelnose guitarfish

Haplotype	Gulf of California	Pacific coast
AAAAA	9	0
AAABA	1	0
AABAA	1	0
AACAA	2	0
AACBA	2	0
BAAAA	14	0
BAABA	1	0
BACAA	1	0
BACBA	1	0
BABAA	0	13
BABBA	0	3
BABCA	0	3
CABAA	0	6
CABBA	0	2
CABCA	0	2
DABAA	0	2
DABBA	0	1

Genetic differentiation and phylogeography

The morphometric differentiation in maximum size and first maturity size, between Pacific coast and gulf shovelnose from Bahía Kino, Sonora, and from Bahía Almejas, southern Baja California (Sandoval-Castillo and Villavicencio-Garayzar, unpublished data), may be interpreted as evidence of population isolation and differentiation. However, growth and reproduction are known to be under strong environmental influence in many species of fish (Brown 1957; Trexler and Travis 1990; Atkinson 1994; Imsland et al. 1995; Carlson 1997) and thus may result from the different environmental conditions in both regions. To the extent that this phenotypic differentiation is correlated with a genetic component, it will be possible to infer the existence of cryptic isolation in the continuous distribution of shovelnose ranging from the Pacific coast into the gulf. Average nucleotide divergence between regions was 2.47% (Table 2). Because no haplotypes were shared between Pacific and gulf samples, highly significant levels of genetic heterogeneity and differentiation were found ($\chi^2 = 64$, $P < 0.0001$; $F_{ST} = 0.23$, $P < 0.0001$; $\Phi_{ST} = 0.63$, $P < 0.0001$). These results indicate that there has not been sufficient historical gene flow between these two populations, one in the Gulf of California and the other on the Pacific coast of Baja California, to prevent the accumulation of genetic divergence and lineage sorting

Table 2 *Rhinobatos productus*. Mitochondrial diversity and divergence of allopatric populations of shovelnose guitarfish

	Haplotype diversity (h)	Nucleotide diversity (π, %)	Nucleotide divergence (d, %)
Gulf of California	0.749	1.06	
Pacific coast	0.794	1.31	
Average	0.767	1.19	2.47

in each region. Estimated levels of gene flow were less than one individual per generation ($N_m = 0.84$ and 0.15 for F_{ST} and Φ_{ST}, respectively). The hypothesis that shovelnose rays collected from both regions are members of the same panmictic population can be clearly rejected, albeit the evidence of secondary contact (see below).

Significant differences in mtDNA haplotype frequencies could be produced by the combination of limited mobility and geographic barriers. However, significant heterogeneity has been detected in very mobile species, and, in some cases, genetic structure in these species may be attributed to fidelity to breeding areas (e.g. Feldheim et al. 2001). Even though it is believed that *R. productus* performs extended reproductive migrations (Villavicencio Garayzar 1993), tag and recapture studies have shown some degree of site fidelity to specific nursery areas for parturition as well as fidelity to breeding areas for copulation (Dubois 1981). Moreover, the Baja California peninsula and the oceanographic differences between the Gulf of California and the Pacific coast are known barriers to gene flow for some marine species (e.g. Stepien et al. 2001). Molecular clock calibrations for the elasmobranch control region are unavailable, but whole mtDNA may evolve at a rate of 0.31% Myr^{-1} (mtDNA-RFLP Martin and Palumbi 1993) and the cytochrome *b* third codon silent rate of substitution may be ca. 0.71% Myr^{-1} (Martin 1995). Assuming a conservative rate of 0.8% Myr^{-1} for the control region, the nucleotide divergence of 2.47% of guitarfish suggests that the major lineages found in Bahía Kino and Bahía Almejas populations evolved in isolation for ca. 3.1 Myr. Divergence time between gulf and Pacific populations has been estimated between 0.12 and 2.3 Myr for some teleosts (Terry et al. 2000; Huang and Bernardi 2001; Stepien et al. 2001). These divergences are compatible with the time of closure of ancient seaways that joined the gulf and the Pacific during the emergence of the Baja California peninsula (Upton and Murphy 1997; Riddle et al. 2000).

The fact that 63% of the molecular variance is explained by inter-population differentiation is consistent with the phylogenetic analysis. The NJ reconstruction shows two divergent lineages that, with the exception of one individual, place gulf and Pacific haplotypes in reciprocal monophyly (Fig. 1). This can be recognized as a very pronounced phylogeographic structure of category I, in which the lineages are deeply allopatric (Avise et al. 1987). The presence in the Gulf of California of a haplotype more closely related to those in the Pacific may be interpreted as evidence of secondary contact. The non-negligible frequency (~3%) of this "Pacific" haplotype in Bahía Kino may be indicative of high levels of contemporary unidirectional gene flow into the gulf. The data at hand clearly show a deep phylogenetic break between one population in the gulf (Bahía Kino) and one in the Pacific (Bahía Almejas), suggesting a long evolutionary history in allopatry. The level of mitochondrial divergence separating these two lineages falls within the range of divergence estimated for allopatric speciation in a wide range of teleost species (McCune and Lovejoy 1998). Whether the remaining guitarfish populations in the gulf and Pacific will fall into these two significantly differentiated lineages (i.e. gulf and Pacific phylogroups); whether additional phylogroups are discovered; and/or whether these lineages are found to be partially sympatric (i.e. indicative of sympatric cryptic species) can only be speculated. What is certain is that a more complete geographic sampling is necessary both to estimate more robust patterns of gene flow and to locate the position of the phylogeographic break(s) along the coastal distribution of the shovelnose guitarfish.

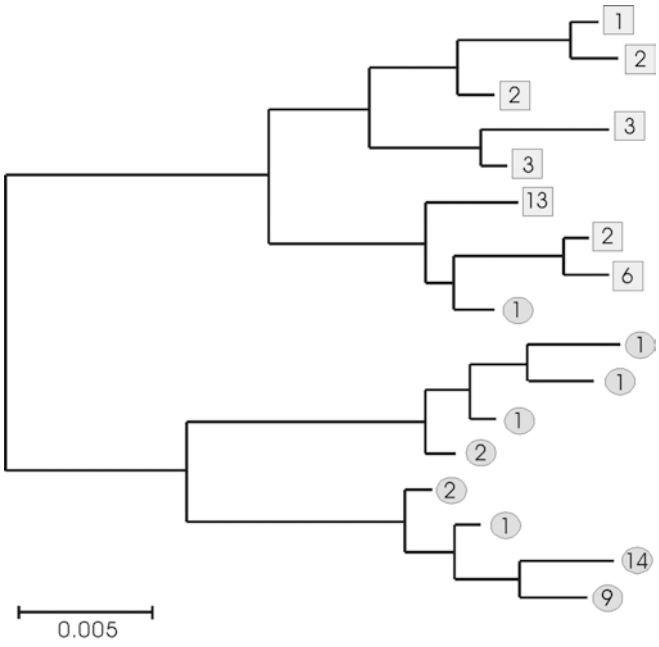

Fig. 1 *Rhinobatos productus*. Phylogenetic neighbor-joining reconstruction based on control region haplotypes of shovelnose guitarfish from the Gulf of California (*circles*) and the Pacific coast of Baja California (*squares*). *Figures in symbols* represent the number of fish sharing the haplotype

Taxonomic and conservation implications

How differentiated should populations be to deserve species status? The level of intra-specific genetic divergence in the present investigation is the highest that we are aware of from the literature for an elasmobranch (Heist 1999; Grijalva-Chon et al. 2002). However, it is within the intra-specific range found in many teleosts (Grant and Bowen 1998; Terry et al. 2000). From a genetic perspective, the line separating two incipient species from two differentiated populations may be fuzzy. However, considering the lower rate of molecular evolution of chondrichthyans relative to other vertebrates (Martin and Palumbi 1993), the genetic divergence found in shovelnose may reflect the existence of cryptic species. These results point to the need for closer and more thorough scrutiny of phenotypic and genetic

variation of gulf and Pacific shovelnose populations. Even if gulf and Pacific populations of shovelnose are co-specific, they qualify for conservation purposes as different evolutionarily significant units (ESU) following Moritz (1994) criteria. These ESUs should be considered in resource management initiatives such as the official norm NOM-029-PESC, which regulates the exploitation and conservation of cartilaginous fish in Mexico. Moreover, the finding of cryptic geographic isolation has wide-ranging implications for other elasmobranch species, in which comparable trends in phenotypic variation have been found in Pacific and Gulf of California populations.

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