Evolution, 54(2), 2000, pp. 652–659

RESTRICTED GENE FLOW AND INCIPIENT SPECIATION IN DISJUNCT PACIFIC OCEAN AND SEA OF CORTEZ POPULATIONS OF A REEF FISH SPECIES, *GIRELLA NIGRICANS*

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Abstract.—Population disjunctions have been proposed to play an important role in speciation processes. In this study, we have examined the possible role of the Pacific Ocean–Sea of Cortez disjunction as a contributing factor to cryptic speciation in a reef fish, the opaleye, *Girella nigricans.* Mitochondrial control region (D-loop) sequences (380 bp) of 117 individuals completely separated opaleye populations from the Pacific Ocean and the Sea of Cortez. Although opaleye exhibit pelagic larval stages that remain in the water column for several months, gene flow between the Pacific Ocean and the Sea of Cortez was found to be extremely limited (F_{ST} = 0.84, Nm = 0.10). Whereas limited gene flow and reciprocal monophyly suggest that the observed physical and genetic disjunction are potentially contributing to the incipient speciation of Pacific and Sea of Cortez opaleye, moderate levels of D-loop sequence divergence (3.3%) and the absence of fixed allozyme markers challenge this idea. Pacific Coast populations also exhibited restricted gene flow levels (F_{ST} = 0.25, Nm = 1.49) across Punta Eugenia, a recognized oceanographic boundary along the Baja California coast. Thus, opaleye individuals grouped into three clades: one clade in the Sea of Cortez, one Pacific clade south of Punta Eugenia, and one Pacific clade north of Punta Eugenia. Future work in this region will determine if our results can be generalized to other disjunct populations.

Key words.—Biogeography, *Girella,* mitochondrial DNA, population structure.

Received May 4, 1999. Accepted October 11, 1999.

''The phenomenon of disjunction, or complete geographic isolation, is of considerable interest because it is almost universally believed to be a fundamental requirement for speciation.'' Since this statement by Endler (1977), relatively few studies have evaluated the role of population disjunction on speciation mechanisms for marine organisms (e.g., Stepien and Rosenblatt 1991; Palumbi 1994; Burton 1998; Hellberg 1998). The main reason for the paucity of studies in this area is that only in rare circumstances are the combination of biological and chronological data available. Such a combination is found in the Baja California region. Geological and biological studies have been established in this region in the past 30 years and provide an essential framework to our study (Brusca 1973).

The geological formation of the Baja California Peninsula, which was associated with the opening of the Sea of Cortez, has created disjunct populations of fish and invertebrates. These populations are present in the northern (upper) region of the Sea of Cortez (also called the Gulf of California) and along the northern Pacific Coast of the Baja California Peninsula. In contrast, they are rare or absent from the warmer southern waters of the peninsula. The presence of disjunct populations gives us an opportunity to understand the potential role of disjunction in speciation events, by evaluating the level of gene flow and genetic divergence between these populations. Approximately 30 fish species have been classified as disjunct (Present 1987). Among these species, the most abundant nearshore species is the opaleye, *Girella nigricans* (Girellidae, Perciformes; Thomson et al. 1979).

The opaleye is a shallow-water, herbivorous fish whose range extends along the California coast from San Francisco Bay to near the southern tip of Baja California, Mexico, and north of La Paz into the Sea of Cortez (Norris 1963; Miller and Lea 1972). The species has been historically subdivided into two species, *G. nigricans* along the Pacific Coast and *G. simplicidens* in the Sea of Cortez. However, this division, which is based on differences in tooth morphology and color patterns, was challenged after careful morphological examination of a larger sample size (Norris and Prescott 1959; Orton 1989). Furthermore, using allozymes, Orton and Buth (1984) could find no fixed genetic differences between these two forms and therefore grouped them in a single species, *G. nigricans.*

The habitat range of *G. nigricans* encompasses several biogeographic regions and traditionally recognized oceanographic barriers. These barriers are Point Conception, Punta Eugenia, and Cabo San Lucas, which define the Oregonian (north of Point Conception), Californian (Point Conception to Cabo San Lucas), and Panamic (Sea of Cortez) biogeographic provinces (Briggs 1974). The Californian province is further divided at Punta Eugenia into two subregions (Briggs 1974). The northernmost portion of the *G. nigricans* range, from San Francisco Bay to Point Conception, is in the Oregonian biogeographic province, where few individuals are found. These individuals have been suggested to be the result of larval transport during unusual events such as El Niños, where ocean currents and temperatures allow southern California fish ranges to shift northward. From Point Conception to Punta Eugenia (Baja California, Mexico), *G. nigricans* is a very abundant component of the reef fish community from the intertidal zone to approximately 25-m depth. South of Punta Eugenia, opaleye are found as far south as Bahia Tortugas (Baja California), but rarely further south. It is likely that they are not present between Bahia Magdalena and Cabo

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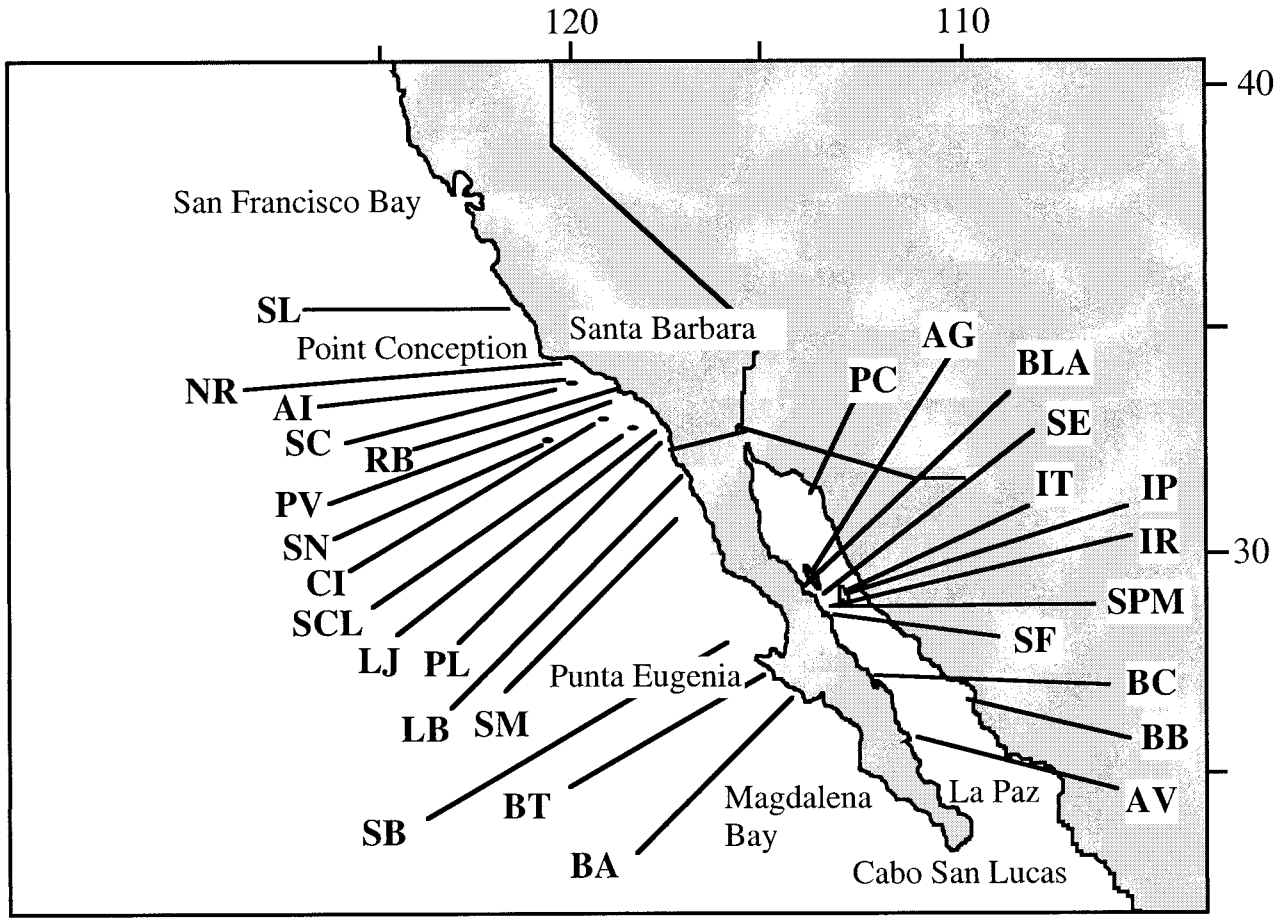

FIG. 1. Sampling localities of *Girella nigricans* on the Pacific Coast of California and Baja California and in the Gulf of California (Sea of Cortez). See Figure 2 for list of abbreviations.

San Lucas (de la Cruz-Aguero et al. 1994). On the Sea of Cortez side of the Baja California Peninsula, no individuals were sighted between Cabo San Lucas and La Paz. Opaleye are abundant north of Bahia Agua Verde on the west coast of the Sea of Cortez and north of Guaymas on the east coast. Thus, populations of *Girella* are termed ''disjunct'' (Present, 1987).

The goal of our study was to use a phylogeographic approach to evaluate the role of disjunction, combined with biogeographic barriers, on potential incipient speciation events that may have occurred in *G. nigricans.*

MATERIALS AND METHODS

Samples and DNA Extraction

Girella nigricans individuals were collected in tidepools with handnets (Point Loma, La Jolla, and La Bufadora individuals) or by spear while we were free or scuba diving. A total of 117 individuals were sampled along the range of the species (Fig. 1). Repeated sampling in the south, on the west coast of the Baja California Peninsula, between Bahia Magdalena and Cabo San Lucas, and on the east coast of the peninsula, between Cabo San Lucas and La Paz, yielded no fish. Interviews with local fishermen indicate that individuals may occasionally have been caught there, however, no collections reflect it. Samples of the Australian congeneric *Girella tricuspidata* were collected by B. Page (Canberra, Australia) and used as an outgroup following Orton (1989).

Liver tissue was extracted immediately after collection and preserved in 95% ethanol at ambient temperature in the field, then stored at 4°C in the laboratory. Tissues were digested overnight at 55°C in 500 μl of extraction buffer (400 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% SDS, Proteinase K). Then DNA was purified by standard chloroform extraction and isopropanol precipitation (Sambrook et al. 1989).

Polymerase Chain Reaction Amplification

Amplification of the mitochondrially encoded control region (also called D-loop) was accomplished with the universal PRO-L and D-Loop primers (Meyer 1993). Both strands of 10 individuals were sequenced. Consensus terminal sequences were used to design specific internal primers that were subsequently used for the remainder of the samples: GIR-DL-F 5′ATT ATT GCC CCT CAC CTT 3'; GIR-DL-R 5′ TAC ATA TAT GTA WTA TCA CCA 3′. Both strands of 10 individuals were then sequenced with the new set of primers. Because no differences were observed between the sequenced strands, the remaining samples were only sequenced with the forward primer. Each 100 μl reaction con-

TABLE 1. Summary of sequence variation within and among populations of *Girella nigricans*. Number of haplotypes, number of variable sites, and mean sequence divergence were calculated using DNAsp (Rozas and Rozas 1997) for populations with $n > 3$. Diversity was calculated according to the equation: $h = (1 - \Sigma x_i^2)n/(n - 1)$, where x_i is the frequency of the ith mtDNA type (Nei 1987).

Region name	Locality name	n	Number of haplotypes	Diversity	No. of variable sites	Mean percent divergence
Pacific Coast and Gulf of California		107	87	0.99	93	6.3
Gulf of California		43	38	0.96	47	2.7
	Punta Cholla	5	5	1.00	19	2.7
	Bahia de los Angeles	9	9	1.00	28	2.8
	Angel de la Guarda	6	6	1.00	11	1.5
	San Pedro Martir	6	6	1.00	32	4.0
Pacific Coast		64	48	0.97	66	5.6
South of Punta Eugenia		20	17	0.97	45	4.4
	Bahia Asuncion	11	10	0.98	37	3.8
	Bahia Tortuga	9	8	0.97	40	5.4
North of Punta Eugenia		44	33	0.97	57	5.1
	Naples Reef	13	6	0.82	8	0.8
	San Diego	11	11	1.00	24	2.1
	Punta Banda	4	3	0.83	6	1.0
	Santa Cruz Island	4	4	1.00	12	2.1
Clade I		43	38	0.96	47	2.7
Clade II		28	17	0.89	21	1.0
Clade III		36	30	0.98	38	1.6

tained 10–100 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), 150 mM of each dNTP, and 0.3 mM of each primer and was amplified with a cycling profile of 45 sec at 94°C, 45 sec at 48°C, 1 min at 72°C, for 35 cycles. After purification following the manufacturers' protocols (Perkin-Elmer; Applied Biosystems, Foster City, CA), sequencing was performed with the primers used in the polymerase chain reaction (PCR) amplification on an ABI 373 automated sequencer.

Sequence Analysis

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the mitochondrial control region sequences. Phylogenetic relationships were assessed using the neighbor-joining method (Nei 1987) implemented by the Software package PAUP (vers. 4.0; Swofford 1998). Maximum parsimony using common algorithms could not be used due to prohibitive computer time. Thus, topological confidence was evaluated with 500 bootstrap replicates (Felsenstein 1985) for neighbor joining and also with 500 replicates using the fast-step method for maximum parsimony (only one tree kept at each replicate). In both neighbor joining and maximum parsimony, bootstrapping analyses were performed with equal weighting of transitions and transversions, as well as with transversions weighted three times as much as transitions. Alternative tree topologies were tested using the Kishino and Hasegawa (1989) method implemented by PAUP. Gene flow (F_{ST}) and haplotype diversity were calculated using the software package DNAsp (Rozas and Rozas 1997) following Hudson et al. (1992). Population structure was evaluated by an analysis of molecular variance (AMOVA; Excoffier et al. 1992) as implemented in Arlequin (vers. 1.1; Schneider et al. 1997). We used AMOVA to test for significant partitions of total genetic variance (Φ_{CT}) among previously defined regions. The significance of individual Φ_{CT} statistics was tested by comparison to null distributions constructed from 1000 random permutations of the original data matrix.

RESULTS

Sequence Analysis and Phylogenetic Reconstructions

A total of 117 sequences were obtained from individuals spanning the entire range of the species (Fig. 1). The portion of mitochondrial control region that was aligned comprised 344 characters (the amplified portion was approximately 380 base pairs long), 102 were variable, and 71 were phylogenetically informative (sequences are available from the author). Haplotype diversity, number of variable sites, and mean percent sequence divergences are given in Table 1. No insertions or deletions were observed in our dataset. As expected, transitions were more common than transversions (ratio 3.1). In all cases (neighbor joining, maximum parsimony, no weights, 3:1 transversion:transition weights) the tree topologies remained unchanged.

The phylogenetic reconstruction obtained with the neighbor-joining method is presented in Figure 2. Three major clades were found. The first clade (clade I) included all the individuals collected in the Sea of Cortez. This clade was well supported by bootstrap analysis (81% to 89% according to which method was used; see Fig. 2 legend). This clade included 43 individuals (38 different haplotypes), which shared three synapomorphies. Individuals collected on the Pacific Coast were divided into two clades. One clade (clade II) comprised 30 individuals (21 different haplotypes, seven synapomorphies), which included 15 (of 20) individuals collected south of Punta Eugenia (BA and BT; Fig. 2). Clade II also contained two individuals collected in Santa Barbara Harbor and all the individuals collected at Naples Reef, off the coast of Santa Barbara, California (SBH, NR; Fig. 2). This clade was well supported by bootstrap analysis (98% to

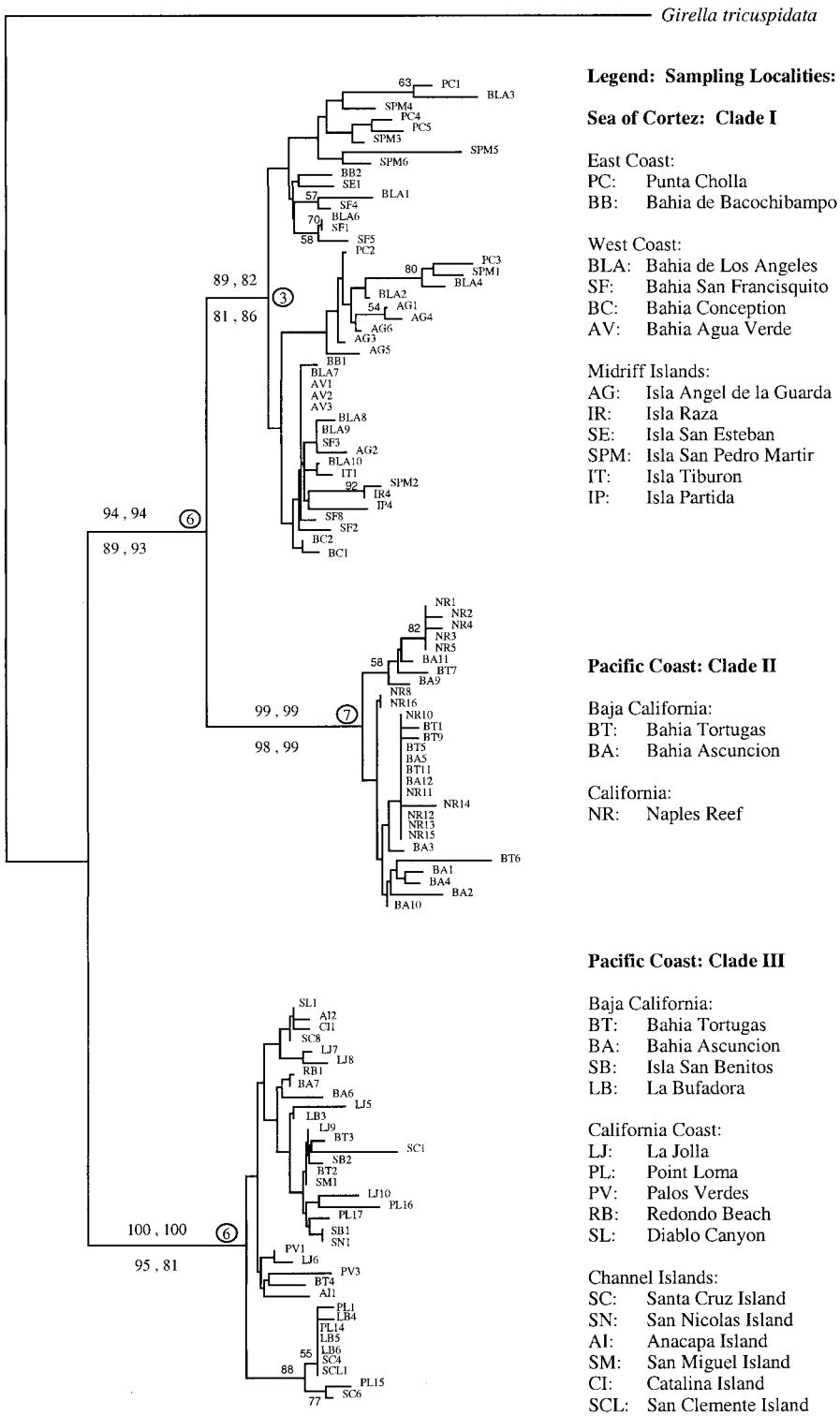

FIG. 2. Phylogenetic relationships of *Girella nigricans* individuals using the neighbor-joining method. The right panel indicates a list of abbreviations used in the tree and in Figure 1. Numbers above the major nodes indicate bootstrap support using the neighbor-joining method with equal weights for transitions and transversions (first figure), and when transversions are weighted three times as much as transitions (second figure). Numbers below the major nodes correspond to bootstrap support using the maximum-parsimony method with equal weights and 3:1 weights for transversions and transitions. In all cases, 500 bootstrap replicates were used. Other numbers indicate bootstrap support using the neighbor-joining method, with equal weights for transitions and transversions (only values above 50% are shown). Circled numbers next to the nodes indicate the number of synapomorphies for the corresponding node. *Girella tricuspidata* was used as an outgroup.

TABLE 2. Average sequence divergence and presumed substitution rate observed between and within clades of *Girella nigricans* (Fig. 2). Data are compared with values found in the literature for the same mitochondrial region (D-loop).

Taxa	Sequence divergence range		Mutation rate per million years	Reference
	Intraspecific	Interspecific		
Clade I–clade II	3.3%			this study
Clade I–clade III	4.4%			this study
Clade II–clade III	4.9%			this study
Clade I	1.2%			this study
Clade II	0.7%			this study
Clade III	1.9%			this study
Melanochromis sp.	0.5–1.1%	1.1–4.9%	20.0%	Bowers et al. 1994
Rhinogobius spp.	0.8–1.8%	11.3–11.7%		Chen et al. 1998
Anguilla spp.	1.1%	21.8%		Sang et al. 1994
Acipenser spp.	2.3%	0–40%	2.0%	Brown et al. 1993, 1996
Xiphias gladius	3.4%		6.9%	Bremer et al. 1995;
	3.8%			Rosel and Block 1996

99% of the replicates). The other clade (clade III), which was supported in 81% to 100% of the bootstrap replicates, included 44 individuals with 34 different haplotypes. This clade comprised all individuals collected north of Punta Eugenia (except all Naples Reef and two SBH individuals) from the Pacific Coast and the remaining five individuals collected south of Punta Eugenia (Baja California; Fig. 2). Individuals from clade III shared six synapomorphies.

The phylogeny presented in Figure 2 placed clade I and clade II as sister clades. Individuals from these two clades shared six synapomorphies. In contrast, clade II and clade III only shared four synapomorphies. Although the grouping of clade I with clade II is well supported by bootstrap analysis (89% to 94% of the bootstrap replicates), the alternate topology (clade II + III), which would correspond to placing all individuals collected on the Pacific Coast of California and Baja California together, could not be rejected using a Kishino and Hasegawa test ($P = 0.15$).

Sequence Divergences

The sequence divergence between clade I and clade II was 3.3%, which is among the highest intraspecific sequence divergences found in the fish literature for D-loops (Table 2; Grant and Bowen 1998). *Girella nigricans* individuals collected on the Pacific Coast separate into two clades, clade II and clade III. This separation is well supported, as discussed above, and the sequence divergence between these two clades (4.9%) was similar to the divergence between these clades and clade I (4.4% and 3.3%).

Population Structure and Gene Flow

Populations were grouped into three regions: Sea of Cortez, Pacific Coast south of Punta Eugenia, and Pacific Coast north of Punta Eugenia. Population structure within these regions was found to be low. For example, gene flow between the east and west coast of the Sea of Cortez (within clade I) was high ($F_{ST} = 0.13$, $Nm = 3.39$). Although islands have been shown to be effective barriers to gene flow in some fish populations with limited dispersal (Planes et al. 1996; Chubb et al. 1998), in our case gene flow between the mainland and the midriff islands in the Sea of Cortez ($F_{ST} = 0.02$, $Nm = 18.97$) and between the California Channel Islands and mainland California ($F_{ST} = 0.01$, $Nm = 31.76$) indicated that there was little restriction to gene flow.

In contrast, gene flow between regions was found to be limited. Gene flow across Cabo San Lucas was found to be extremely low ($F_{ST} = 0.84$, $Nm = 0.10$; see Hedrick 1999 for theoretical maximum F_{ST}/G_{ST} values). Although gene flow across Punta Eugenia was not as restricted as what was observed across Cabo San Lucas, it was still found to be very limited ($F_{ST} = 0.25$, $Nm = 1.49$). These results were also reflected in an AMOVA. Approximately 31.9% of the total variance could be attributed to the variation between populations from the Sea of Cortez and the Pacific Ocean ($\Phi_{CT} = 0.31$, $P < 0.005$). When considering the three regions (Sea of Cortez, north and south of Punta Eugenia), approximately 24.6% of the total variance could be attributed to the variation between these regions ($\Phi_{CT} = 0.24$; $P < 0.02$).

DISCUSSION

Dispersal Capabilities and Gene Flow

In marine systems, dispersal capabilites have been generally associated with gene flow levels (e.g., Waples 1987; Palumbi 1992; Doherty et al. 1995; Shulman and Bermingham 1995; but see Cunningham and Collins 1998; Shulman 1998). Dispersal in *G. nigricans* may occur at the larval, juvenile, and adult stage. *Girella nigricans* releases pelagic larvae in the summer (Stevens et al. 1989). The larval pelagic phase, which remains near the surface (Stevens et al. 1989), lasts several months (Waples 1987), thus allowing larvae to disperse efficiently. Typical juveniles spend several months to a year in tidepools. However, studies of giant kelp (*Macrocystis pyrifera*) rafts have shown that juveniles may also be found associated with these floating structures (Mitchell and Hunter 1970; Kingsford 1995). Calculations show that juveniles may disperse over approximately 200 nautical miles while associated with these structures (Waples 1986). Finally adults may be able to disperse, although to a much lesser extent than juveniles or larvae. It is therefore likely that currents and oceanographic barriers have the potential to play an important role in the dispersion of *G. nigricans*, and ultimately to have a significant impact on gene flow levels.

Punta Eugenia as a Barrier to Gene Flow

Phylogenetic inferences have been used to correlate biogeographic patterns and evolutionary relationships. This correlation, called phylogeography, was developed by Avise (1994) and is now widely used (e.g., Bowen and Grant 1997). Although Avise has presented compelling evidence that barriers to gene flow coincide with biogeographic boundaries in the southeastern United States (reviewed in Avise 1992), it is likely that this is not a general phenomenon, as was recently discussed by Burton (1998) and Cunningham and Collins (1998). Burton (1998) showed that Point Conception, California, is a definite biogeographic boundary for species, but is not an effective barrier to gene flow for populations.

Punta Eugenia has traditionally been considered a biogeographic boundary (Briggs, 1974), but has never been tested as a barrier to gene flow for populations of marine organisms. We propose two explanations for the limited gene flow that was observed across Punta Eugenia for *G. nigricans*: (1) current patterns at Punta Eugenia may limit larval dispersal across the point, thus reducing gene flow levels; and (2) the region north of Punta Eugenia (Guerrero Negro to Punta Banda) is not a suitable habitat for *Girella. Girella* needs rocky substrates to graze on algae, and this region is generally composed of sandy habitats that do not allow *Girella* to survive. It is tempting to suggest that currents at Punta Eugenia are responsible for the effective barrier to gene flow. However, it is as likely that the very large ecological barrier to adult dispersal, north of Punta Eugenia, is actually responsible for the limited gene flow.

Santa Barbara/Naples Reef Samples

Puzzling results were obtained with individuals from Naples Reef (a reef off the coast of Santa Barbara, CA) and two individuals from Santa Barbara. Whereas no individuals collected between Punta Eugenia and the Santa Barbara area were found in Clade II, all individuals from Naples Reef and two of 10 individuals from Santa Barbara were members of that clade. Sequencing and sampling errors were ruled out because individuals were sequenced twice independently, several months apart, and samples were collected during three different field trips, four years apart. Mislabeling or accidental release of experimental subjects was also checked and discarded as a possible explanation (S. Anderson, pers. comm.). One possible explanation is that these individuals are the results of recruitment bouts over long geographic distances. As described above, *Girella* exhibits a long pelagic larval stage (up to a few months) and also long juvenile stages. The regular life cycle of *G. nigricans* includes an intertidal juvenile phase (Norris 1963), and Naples Reef is an offshore subtidal reef that lacks the juvenile intertidal habitat. Thus, larvae are recruited from other regions. It is possible that recruitment is fairly rare and that individuals sampled in our study were the product of an infrequent event such as an El Niño, where currents from the south are more frequent. Subsequent gene flow between Naples Reef and Santa Barbara would account for some clade II individuals found there. Alternatively, Cunningham and Collins (1998) have argued that extinction and recolonization phenomena are an underestimated phenomenon that could explain several unusual phylogeographic patterns. Yet another explanation is that clade II and clade III correspond to incipient or cryptic species, which have overlapping distributions. Additional work will be needed to determine which of these hypotheses apply to opaleye.

Origin of Disjunct Populations

The presence of disjunct populations of temperate fish in the Sea of Cortez may be explained by two alternative scenarios. Brusca (1973) proposed that the northern Gulf–Pacific Coast disjunct distributions are due to post glacial warming of waters around the tip of the peninsula, which restricted movement of temperate fish to more northern waters. Glacial events may have resulted in multiple invasions/separations in the Sea of Cortez by allowing fish to round the Cabo San Lucas region when water temperatures were cooler. This hypothesis would predict a separation between populations of approximately 10,000 years. Alternatively, Durham and Allison (1960) proposed that approximately 1 million years ago, the Baja California peninsula was restricted to a small area in the north and the southern region of the Baja California peninsula was composed of several islands. The resulting seaways may be responsible for the genetic differentiation observed between populations of lizards north and south of such presumed seaways (Upton and Murphy 1997). Temperate fish could have moved freely in and out of the area that is now the Gulf of California. When the generally accepted molecular clock for fish mitochondrial DNA (2% per million years to 10% per million years) is used, the sequence divergence observed between Sea of Cortez and Pacific fishes (3.3% to 4.4%) corresponds to a divergence time of approximately 0.3–2.2 million years ago. Although molecular clocks should be used with caution, these values point toward an early divergence between Sea of Cortez and Pacific fishes that favors the midpeninsular seaway rather than the glacial period hypothesis. Our data therefore suggest that *Girella* populations in the Sea of Cortez diverged in allopatry once the seaways across the Baja California Peninsula were closed.

Disjunct Populations and Incipient Speciation

As mentioned in the introduction, disjunction has been proposed as a possible mechanism for speciation. In the case of *G. nigricans* populations, physical and genetic disjunction was observed across Cabo San Lucas, at the southern tip of the Baja California Peninsula. How does this disjunction relate to a possible speciation event?

Individuals from the Sea of Cortez formed a well-supported monophyletic clade, which did not include any Pacific individuals. Although no morphological characters can separate Pacific opaleye from Gulf opaleye, our results suggest that these forms may correspond to cryptic species. Cryptic species are organisms morphologically indistinguishable but genetically distinct, which are not uncommon in marine systems (Knowlton 1993). Generally, marine species tend to exhibit extensive gene flow, which should theoretically slow down the process of population divergence and speciation (Hansen 1980, 1982; Shaklee et al. 1982; Palumbi 1992, 1994; Shulman 1998). However, the number of cryptic species in marine systems, which have been described in several taxa including

invertebrates, plants, sea turtles, cetaceans, and fishes (reviewed in Knowlton 1993), are likely to have been vastly underestimated due to the difficulty of studying them.

Although our data may be interpreted as evidence for the separation of Gulf and Pacific opaleyes into two newly formed cryptic species due to population disjunction, some data challenge this hypothesis. The average sequence divergence between Gulf and Pacific opaleye is high (3.3% to 4.4%), but not outside the range of previously observed intraspecific sequence divergences (Table 2; Grant and Bowen 1998). Furthermore, previous results, based on allozymes, showed no fixed differences between these two populations (Orton and Buth 1984). This indicates that nuclear and mitochondrial lineages may present conflicting results. This inconsistency underscores the importance of using several loci, both nuclear and mitochondrial, to have a comprehensive understanding of the evolutionary history of the taxa under consideration.

Disjunction has had a major impact on the dispersal of *G. nigricans*, which in turn resulted in a disruption of gene flow associated with high genetic divergence. Although this separation may have lasted for over two million years, it does not seem to have been sufficient for allopatric speciation to occur. Future data on *G. nigricans*, as well as other disjunct species, should shed light on the importance of the Pacific–Sea of Cortez disjunction on local speciation events.

Acknowledgments

This paper is dedicated to the memory of our friend and colleague Ken Norris, who, before becoming a world expert on marine mammals, devoted many years to the study of opaleye. We would like to thank all the people who helped us collect fish samples: S. Alesandrini, S. Anderson, D. Reed, D. Canestro, W. Toller, N. Crane, A. Marsh, M. Kenner, B. Page, D. Huang, C. L. Ortiz, and the crew of the *Don Jose*. We would also like to thank A. Marsh, M. Marsh, and D. Canestro for logistical support. D. Swofford provided support and several beta versions of the software package PAUP. This research was funded by a UCMEXUS grant and UCSC faculty research funds granted to GB and a Myers Grant to AT.

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