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Genetic Divergence between Gopher Rockfish (*Sebastes carnatus*) and Black and Yellow Rockfish (*Sebastes chrysomelas*)

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The genus *Sebastes* contains over 110 species, an unusually high level of species diversity for marine fishes. Many of the 70 species that occupy the northeast Pacific Ocean coexist during parts or all of their life cycle. Although allopatric speciation explains much of this diversity, the presence of many closely related species within the same locations suggests that other isolating mechanisms may play a role. *Sebastes carnatus* and *Sebastes chrysomelas* are a sympatrically distributed pair of sister species having no morphologically distinguishing characteristics other than color: *S. carnatus* has pinkish spots on a brown background, and *S. chrysomelas* has yellow spots on a black background. In this study, seven nuclear microsatellite DNA loci were employed to assess relatedness of 111 *S. carnatus* and 91 *S. chrysomelas* sampled from three locations spanning the species' range. Analysis of the seven microsatellite loci provided evidence of genetic divergence between color morphs ($F_{ST} = 0.046$, $P < 0.0001$). Furthermore, magnitude of genetic divergence between the color morphs was consistent among geographic locations. Divergence between *S. carnatus* and *S. chrysomelas* was low relative to that detected among other species within the genus, suggesting that the two morphs represent reproductively isolated incipient species.

ROCKFISHES (genus *Sebastes*) represent a rare case of a marine species flock (Johns and Avise, 1998), with more than 110 species worldwide and over 70 species inhabiting the continental shelf of the northeast Pacific Ocean (Chen, 1971). On a global scale, diversification in *Sebastes* is characterized by long-distance dispersal and allopatric speciation between distant locations (e.g., Rocha-Olivares et al., 1999). On a more local scale, however, coexistence of many closely related *Sebastes* species with similar life histories, habitat requirements, and high dispersal capabilities suggests that reproductive isolating mechanisms other than geographic separation may be important. Assortative mating may result in reproductive isolation in marine animals without geographic barriers (Palumbi, 1994). In *Sebastes*, internal fertilization, complex mating behaviors (e.g., Gingras et al., 1998), and pronounced color polymorphism between sister taxa (e.g., Orr et al., 2000; Orr and Blackburn, 2004) raise the possibility that assortative mating by color may play a role in initiating or reinforcing reproductive isolation within this group.

Sebastes carnatus and *Sebastes chrysomelas* are distinguished primarily by color (Seeb, 1986; Love et al., 2002). Both color morphs are benthic inhabitants of rocky reefs and kelp forests that typically live in less than 30 m depth and commonly coexist in temperate waters from northern California to central Baja California (Love et al., 2002). The two species are found sympatrically, but *S. carnatus* typically inhabits slightly deeper water than does *S. chrysomelas*, with transition between 11 to 14 m (Larson, 1980a). *Sebastes chrysomelas* has a black body with yellow patches, and *S. carnatus* has a dark brown body with pink patches. Although *S. chrysomelas* has significantly fewer gill rakers than *S. carnatus*, all other morphological and meristic characters overlap (Chen, 1986). Because color pattern develops after settlement from the planktonic stage, it has been suggested that *S. carnatus* and *S. chrysomelas* are merely color morphs, where color is determined for life by an environmentally induced developmental switch occurring at metamorphosis (Carr, 1991; R. Larson, pers. comm., 2001).

Previous genetic studies addressing the genetic relatedness and species status of *S. carnatus* and *S. chrysomelas* were inconclusive. Allozyme analysis (Seeb, 1986) and restriction fragment length polymorphisms (RFLPs; Hunter, 1994) detected slight but significant frequency differences between *S. carnatus* and *S. chrysomelas*, and mitochondrial control region sequences (Alesandrini and Bernardi, 1999) did not display significant genetic divergences between the two color morphs. The main goal of this study was to more fully assess the degree of genetic divergence between the two color morphs collected from the same locations as an indicator of reproductive isolation, using highly sensitive microsatellite DNA molecular markers. Specifically, we aimed to address the following ques-

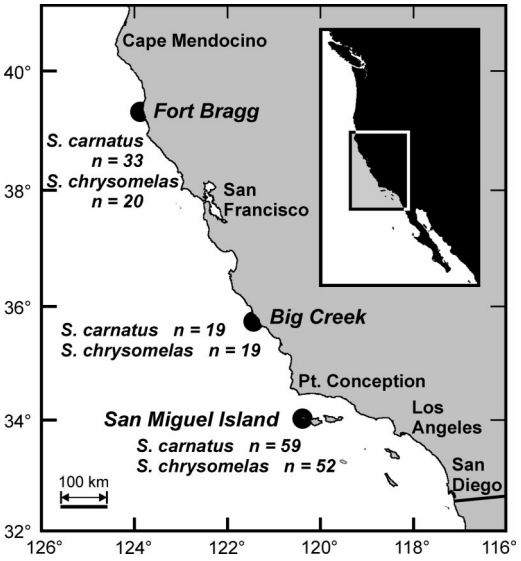

Fig. 1. Sample sites of tissue collections for microsatellite DNA analysis on the coast of California (Fort Bragg = FB; Big Creek = BC; San Miguel Island = SM) including sample sizes of *Sebastes carnatus* and *Sebastes chrysomelas* from each site. Inset map shows general sampling location on the west coast of North America.

tions: (1) Is there evidence for genetic divergence between the color morphs collected from the same locations, indicating reproductive isolation? (2) Is there a latitudinal difference in the level of intermorph genetic divergence as suggested by Hunter (1994)?

Materials and Methods

Sampling.—Samples of *S. carnatus* and *S. chrysomelas* were collected along the California coastline, from Fort Bragg (FB; *S. carnatus* = 33, *S. chrysomelas* = 20), Big Creek Reserve (BC; *S. carnatus* = 19, *S. chrysomelas* = 19), and San Miguel Island (SM; *S. carnatus* = 59, *S. chrysomelas* = 52; Fig. 1). Fin clips from a total of 111 *S. carnatus* and 91 *S. chrysomelas* were collected from commercial live-fish fisheries (hook-and-line; 26% of all samples) and scientific collection operations (hook-and-line, pole spear; 74% of all samples) aboard NOAA vessels David Starr Jordan and McArthur II during 1998 and 1999 (See Acknowledgments for permit information). When possible, samples were collected by pole spear and SCUBA after direct observation of the fish in mixed populations. Individuals were classified to species by color. Fin clips from each fish were immediately placed in 95% ethanol. Individuals exhibited a wide range of lengths, indicating that the collection contained several year classes. However, length data were not available for all individuals.

Laboratory analysis.—Approximately 50 mg of tissue was digested with proteinase K in CTAB extraction buffer (3 h at 55 C). Total genomic DNA was purified with phenol/chloroform extractions and ethanol precipitation using standard protocols (Sambrook et al., 1989). The Polymerase Chain Reaction (PCR) was used to amplify seven microsatellite loci designed from *Sebastes rastrelliger* (M. Westerman unpublished data; GenBank accession numbers AF269052–AF269061). Four loci (*Sra.* 5–9, *Sra.* 7–2, *Sra.* 7–7, and *Sra.* 7–25) showed dinucleotide, one (*Sra.* 11–103) trinucleotide, and two (*Sra.* 15–8 and *Sra.* 16–5) tetranucleotide repeats. PCR was performed on a Perkin Elmer 9600 DNA thermal cycler using the BRL PCR Reagent System (Gibco BRL) following manufacturer's protocols, with approximately 150 ng template DNA in 15 μl total volume. Typical cycling conditions included an initial denaturation of 2 min at 95 C, followed by 36 cycles of 1 min at 92 C, 1 min at 57 C, and 1 min at 72 C. Final extension was carried out for 7 min at 72 C. Annealing temperature was adjusted to optimize PCR conditions (See GenBank submission for details). Forward primers were fluorescently labeled (Applied Biosystems), and PCR products were electrophoresed on 36 cm, 6% Long-Ranger denaturing polyacrylamide gels (FMC BioProducts) and detected using an ABI 377 scanner. Internal molecular weight standards were included in each lane, and products were sized using GeneScan v.3.7 software (Applied Biosystems).

Statistical analysis.—Exact-significance testing methods were used to evaluate each locus and population for conformance to Hardy-Weinberg equilibrium using a Markov chain algorithm as implemented in GENEPOP v. 3.3 (Raymond and Rousset, 1995). Corrections were made against Type I error in exact tests using the sequential Bonferroni method (Rice, 1989). Tests for linkage disequilibrium between all pairs of loci were also performed using the Markov chain method in GENEPOP.

To estimate the level of within-population genetic diversity, expected heterozygosity (H_E; eq. 8.4 Nei, 1987) and observed heterozygosity (H_O) were calculated for each locus using Arlequin v. 2.001 (S. Schneider, unpubl.; http://lgb.unige.ch/arlequin). Total and mean number of alleles per locus were calculated using FSTAT (Goudet, 1995). The significance of the differences in expected heterozygosity and av-

erage alleles per locus were evaluated between color morphs using the Wilcoxon signed ranks test, implemented in StatView v. 4.5 (www.statview.com).

The inbreeding coefficient (F_{IS}; Weir and Cockerham, 1984) was calculated to test for nonrandom mating with four arrangements of the data: pooling all individuals into a single collection, pooling all individuals of each species into separate collections, pooling *S. carnatus* and *S. chrysomelas* at each of the three sample sites, and single values for each of the six separate collections (*S. carnatus* at BC, FB, and SM and *S. chrysomelas* at BC, FB, and SM). Sequential Bonferroni corrections were used to adjust for multiple tests (Rice, 1989).

Genetic variance among collections was calculated using allele frequencies (F_{ST}; Weir and Cockerham, 1984), and mean repeat number (ρ_{ST}; Michalakis and Excoffier, 1996). Pairwise F_{ST} estimates and unbiased estimators of exact significance probabilities were determined using the Markov-chain algorithm as implemented in GENEPOP v. 3.3 (Raymond and Rousset, 1995; http://wbiomed.curtin.edu.au/genepop). Significance was adjusted for multiple tests with the sequential Bonferroni method. Pairwise estimates of ρ_{ST} were calculated and significance was determined using permutation-based resampling implemented by Arlequin v. 2.001 (10,000 permutations).

To further investigate how distinct each individual was from the alternative color morph, an assignment test was performed (Paetkau et al., 1995). Assignment probabilities were generated using the program Doh (J. Brzustowski; http://www2.biology.ualberta.ca/jbrzusto/doh.php). Asymmetric assignment distances were calculated as a measure of how much more likely genotypes of individuals sampled in population x, belong to population x than in population y.

Results

Genotypic frequencies did not differ significantly from Hardy-Weinberg equilibrium expectations. Average F_{IS} for each population across all loci was not significant ($\alpha = 0.008 = 0.05/6$ tests) for *S. carnatus* (FB $P = 0.22$, BC $P = 0.83$, SM $P = 0.47$) or *S. chrysomelas* (FB $P = 0.38$, BC $P = 0.28$, SM $P = 0.65$). Average F_{IS}-values for each locus across all populations were also not significant ($\alpha = 0.007 = 0.05/7$ tests) at any of the seven loci. Further, all single locus F_{IS}-values for each collection were not significant after corrections for multiple tests ($\alpha = 0.0012 = 0.05/42$ tests). Tests for linkage disequilibrium were not significant.

The seven microsatellite loci employed in the analysis had three to 34 alleles (*Sra.* 5–9 = 3, *Sra.* 7–2 = 20, *Sra.* 7–7 = 19, *Sra.* 7–25 = 34, *Sra.* 11–103 = 6, *Sra.* 15–8 = 15, *Sra.* 16–5 = 32) totaling 129 alleles. The average number of alleles per locus was 16.4 (SE ± 3.85) for *S. carnatus* and 14.3 (SE ± 3.83) for *S. chrysomelas* (Wilcoxon signed rank $P = 0.059$). Average heterozygosity was $H_E = 0.75$ (SE ± 0.07), $H_O = 0.75$ (SE ± 0.07) for *S. chrysomelas* and $H_E = 0.80$ (SE ± 0.06), $H_O = 0.78$ (SE ± 0.07) for *S. carnatus*. The difference in H_E was not significant (Wilcoxon signed rank $P = 0.091$). *Sebastes carnatus* and *S. chrysomelas* shared 67.6% of alleles (88/130). Private alleles (alleles only found in one species) comprised 25.4% of *S. carnatus* alleles (30/118) and 12.0% of *S. chrysomelas* alleles (12/100).

Evidence for genetic divergence between color morphs was inferred from F_{IS} pooling analyses. Nonsignificant F_{IS}-values ($\alpha = 0.004 = 0.05/12$ tests) were detected within each of the six collections (all *P*-values > 0.22) and when collections were pooled by species (*P*-values = 0.55 and 0.59). In contrast, F_{IS}-values were significant ($\alpha = 0.004 = 0.05/12$ tests) when both color morphs at each sample site were pooled (all *P*-values < 0.003) and when all samples were grouped as one collection (*P*-value < 0.0001). The fact that F_{IS}-values were significant only when individuals of both species were pooled is evidence for separate taxa (Wahlund effect; Hartl, 1988).

Differences in allele frequencies and repeat numbers between *S. carnatus* and *S. chrysomelas* were consistently detected at each sample location. Three of seven loci had F_{ST}-values that were significant after Bonferroni correction ($\alpha = 0.007 = 0.05/7$ tests; *Sra.* 5–9 $P = 0.004$, *Sra.* 7–2 $P < 0.0001$, *Sra.* 16–5 $P = 0.0003$). The mean F_{ST} over all loci, over all populations, was also significant ($F_{ST} = 0.046$; $P < 0.0001$). Locus *Sra.* 7–2 was the most divergent with an F_{ST} of 0.241 ($P < 0.00001$) and accounts for most of the genetic differentiation observed in this study. The ρ_{ST} over all loci was significant ($\rho_{ST} = 0.307$; $P < 0.0001$) but was not significant when locus *Sra.* 7–2 was removed from the analysis ($\rho_{ST} = -0.0014$; $P = 0.18$). Pairwise tests ($\alpha = 0.003 = 0.05/15$ tests) reveal that levels of divergence between species were consistently significant ($P < 0.0001$) at each sampling location (FB, BC, and SM), with F_{ST}-values of 0.048, 0.037, and 0.050, respectively, and ρ_{ST}-values of 0.323, 0.242, and 0.323, respectively (Table 1). High polymorphism at some loci (up to 34 alleles) in combination with sample sizes ranging from 19–59 may have resulted in incomplete

TABLE 1. PAIRWISE TESTS OF GENETIC DIVERGENCE. F_{ST} and ρ_{ST} between each sample population (Fort Bragg = FB, Big Creek = BC, and San Miguel Island = SM) of *Sebastes chrysomelas* and *Sebastes carnatus*. F_{ST}-values below the diagonal, ρ_{ST}-values above the diagonal as calculated from seven microsatellite loci. Sample sizes are provided in Figure 1.

		Sebastes chrysomelas			*Sebastes carnatus*		
		FB	BC	SM	FB	BC	SM
S. chrysomelas	FB	—	0.044	0.021	0.323*	0.377*	0.351*
	BC	−0.007	—	−0.005	0.228*	0.242*	0.278*
	SM	0.004	0.003	—	0.297*	0.314*	0.323*
S. carnatus	FB	0.048*	0.042*	0.056*	—	−0.021	−0.007
	BC	0.042*	0.040*	0.052*	0.001	—	−0.015
	SM	0.041*	0.039*	0.050*	−0.002	−0.001	—

* Indicates significant values at the $P < 0.0001$ level.

representation of all alleles at some loci included in this study. However, simulations of genetic distance and sample size (S. Kalinowski unpubl. data) with F_{ST} of 0.05 (mean F_{ST} over all loci for this study = 0.046) revealed that sample sizes of 20 May be adequate to estimate genetic distance (θ) even at highly polymorphic loci.

Tests to determine population structure among locations within either species were not significant. No pairwise tests of F_{ST} or ρ_{ST} were significant ($\alpha = 0.003 = 0.05/15$ tests) between sampling locations within species (Table 1). Mean F_{ST} (-0.001) and mean ρ_{ST} (0.012) values within *S. carnatus* were somewhat lower than within *S. chrysomelas* (0.002 and 0.013, respectively), but neither the mean nor single locus values were significant.

An assignment test for all individuals of *S. carnatus* and *S. chrysomelas* is shown in Figure 2. All but four individuals (two *S. carnatus* and two *S. chrysomelas*) of the total 202 were assigned to the correct color morph, yielding an assignment fidelity of 98%. The four incorrectly assigned individuals did not strongly misassign, but fell within a single log-likelihood of assigning into the correct color morph. A total of 21% (19/91) of the *S. chrysomelas* and 12% (13/111) of the *S. carnatus* morphs demonstrated either ambiguous (correctly assigned, but likelihood less than two orders of magnitude over equal assignment) or misassignments (Fig. 2). Asymmetric distances showed that *S. carnatus* individuals were somewhat more distant from *S. chrysomelas* (3.8), than vice-versa (3.5; Mann Whitney *U*-test, $P = 0.11$), indicating that *S. carnatus* were less likely to misassign than were *S. chrysomelas*.

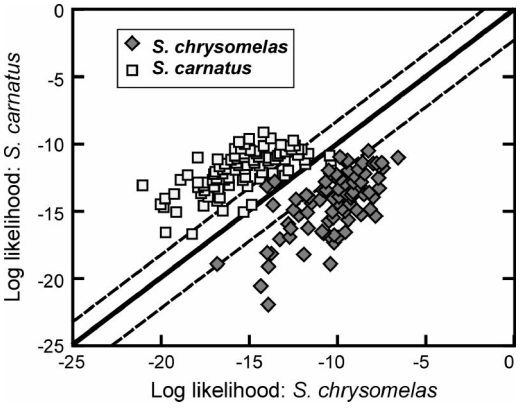

Fig. 2. Results of assignment tests designating the probability of each individual as belonging to *Sebastes chrysomelas* ($n = 91$) or *Sebastes carnatus* ($n = 111$) as determined by seven microsatellite loci. Probability values are plotted on a log scale. Dark diagonal line indicates the line of equal probability. Individuals outside the dotted lines are 100 times more likely to belong to one species than the other.

DISCUSSION

Detection of consistent genetic divergence between color morphs at each location indicates that phenotypic plasticity is not likely the cause of coloration differences between *S. carnatus* and *S. chrysomelas*. Furthermore, because divergence occurred at multiple microsatellite loci (i.e., presumably random samples of neutral genomic variation), genes other than those simply controlling color appear to show differentiation. Therefore, we infer that the color morphs represent reproductively isolated species. Although significant, the overall F_{ST}-values between *S. carnatus* and *S. chrysomelas* (0.046) were about an order of magnitude lower than comparisons between other rockfish species within the closely related subgenus *Pteropodus* for the same microsatellite loci (V. Buonaccorsi, unpubl. data). The low level of divergence suggests that the two color morphs have recently diverged and may be in the process of speciating (i.e., comprising incipient species).

The results of the present study add to the previous genetic studies on the species complex. Based on nuclear ITS RFLPs, Hunter (1994) detected significant genetic divergence between *S. chrysomelas* and *S. carnatus* only in northern regions and concluded that the morphs should be grouped as a single species. Latitudinal variation in interspecies divergence was not detected in the current study. However, it is possible that reproductive isolation may have begun in the northern portion of the range; thus, the more slowly evolving nuclear ITS-1 region mirrored our microsatellite data only in the north.

The high rate of correct designation in assignment analyses further corroborates genetic distinctiveness between the two species. However, assignment tests also revealed a low frequency of ambiguous or misclassified individuals. Such nondiagnostic genotypes may be best explained by incomplete lineage sorting rather than hybridization. Given that genetic divergence between species was slight, some overlap in assignment probability may be expected. In addition, hybridization could quickly homogenize the two gene pools unless there was strong selection against hybrid individuals. Interspecies hybridization and phenotypic intermediates have been reported among rockfishes of the Puget Sound (Seeb, 1998) and the northwest Atlantic Ocean (Roques et al., 2001). However, as with Alesandrini and Bernardi (1999), we did not observe phenotypically intermediate color patterns to the extent that species designation could not be made with confidence.

The main findings of this study are genetic distinctiveness between *S. carnatus* and *S. chrysomelas* and likely recent divergence of these species. Historical allopatry or exclusion-recolonization events are common in northern habitats (e.g., copper rockfish in Puget Sound) that are subject to glaciation-deglaciation cycles (Buonaccorsi et al., 2002), but these phenomena are less likely to influence populations along the California coastline. Pleistocene sea level change (Cutler et al., 2003) did influence the availability of habitat in southern California (Graham et al., 2003) but never fully isolated this region. Results presented here show no intraspecific genetic structure along a latitudinal gradient. The lack of divergent mitochondrial DNA lineages (Alesandrini and Bernardi, 1999; Narum, 2000) further supports the hypothesis that morphs were not separated for an extensive period prior to secondary contact. Alternatively, assortative mating may act to reinforce isolating mechanisms or initiate reproductive isolation in sympatry. Behavioral observations (Larson, 1980b) along with genetic divergence detected in this study indicate reproductive isolation of *S. carnatus* and *S. chrysomelas*, but quantitative observations of mating behavior (i.e., Fischer, 1980) are needed to demonstrate the mechanism for recent divergence in these species.

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