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RESEARCH ARTICLE

Incipient speciation within a subgenus of rockfish (*Sebastosomus*) provides evidence of recent radiations within an ancient species flock

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Abstract The high frequency of speciation events associated with species flocks (i.e., radiations of closely related species) provides invaluable insight into the speciation process. Investigations of the speciation process in the marine environment are rare, and therefore, the genetic analysis of the rockfish genus *Sebastes*, considered an ancient marine species flock, provides an opportunity to investigate this process in the sea. Using both mitochondrial and nuclear markers, we analyzed five closely related species within the rockfish subgenus *Sebastosomus*. Our goal was to understand the evolutionary history and genetic relationships among species within this group and to provide evidence of recent speciation events within the subgenus. In the genetic analysis of the subgenus, we found different stages of the speciation process, with greater genetic divergences among three of the five species, evidence of recent divergence between two of the five species, *Sebastes entomelas* and *S. mystinus*, and significant genetic divergence between two lineages within S. *mystinus* revealing a signature of incipient speciation. We also found frequency differences of the two *S. mystinus* lineages among sample locations and found no evidence of introgression between the lineages at the location where both coexist. Although *Sebastes* is an example of an ancient species flock, this study provides evidence of ongoing speciation within the genus and reveals stages of this process from incipient to distinct species.

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Introduction

Investigations of "species flocks," described as adaptive radiations within a genus, reveal critical information on the process of speciation. These investigations provide a rare opportunity to test mechanisms of speciation, because this series of radiations captures several replicates of this process within a given environment (e.g., within an island chain) or over a relatively short-period of time or both. While these radiations can occur relatively rapidly, they may occur within an environment over a period of time such that members of the flock may reveal different stages of the speciation process, from divergent populations, to incipient species, to well defined and distinct species. Directly investigating these different stages may reveal both recent and historic time-scale processes, such as changes in geology, ecology, or dispersal patterns that contribute to deep internal divisions within populations and to speciation. Therefore, the unique circumstances that contribute to these rapid radiations provide an opportunity to investigate the role of recent events or processes on speciation. There are many examples of species flocks in terrestrial and freshwater systems (e.g., Galápagos finches, Hawaiian silverswords, Malawi cichlids, and West Indian Anolis lizards; Schluter 2000), but few examples in the marine environment (e.g., Antarctic notothenioids, Eastman and McCune 2000; McCartney et al. 2003).

Along the west coast of North America, the rockfishes of the genus *Sebastes* comprise approximately 60 species and are thought to exemplify an ancient species flock, which

initially diverged in a series of rapid speciation events approximately 5 million years ago (Johns and Avise 1998). While extant species within *Sebastes* may represent ancient speciation events, it is unclear whether more recent or ongoing speciation occurs within the genus. It is possible that the characteristic of rapid speciation that defines the evolutionary history of *Sebastes* may occur on a contemporary time-scale. Recent studies on the genetic structure of species within this genus provide empirical evidence of high genetic differentiation among populations and low genetic differentiation among closely related species, suggestive of incipient speciation or recent speciation events (Seeb 1998; Alesandrini and Bernardi 1999; Rocha-Olivares and Vetter 1999; Buonaccorsi et al. 2002, 2005; Narum et al. 2004; Miller et al. 2005; Burford and Larson 2007). Thus, while Johns and Avise (1998) provided evidence for an ancient radiation within *Sebastes* and despite the characteristically long pelagic phase (ca. 3–6 months) and high dispersal potential for many taxa within the genus, these more recent studies provide evidence of both genetic divergence within species and recent speciation events.

Sibling cryptic species, with little or no morphological differences and high genetic divergence, are common in the marine environment (Knowlton 1993). Early in the speciation process (at the incipient species stage), these cryptic species may have lower genetic divergences or be genetically distinct only at specific genetic markers (e.g., Knowlton 2000). There is evidence for this discrepancy within the genus *Sebastes* for a sibling pair, the gopher rockfish, *Sebastes carnatus*, and the black and yellow rockfish, *S. chrysomelas*. Two different comparative studies of this sibling pair found little or no morphological differences with moderate to high genetic divergence (Alesandrini and Bernardi 1999; Narum et al. 2004, respectively). Alesandrini and Bernardi (1999) concluded that the moderate genetic divergence at the mtDNA control region was due to incomplete lineage sorting, which provides evidence of a recent divergence between the sibling pair. The observed maintenance of either moderate or high genetic divergences combined with little or no morphological differences and similar ecological roles, could be due to the sister-taxa inhabiting different depth ranges (i.e., ecological segregation; Larson 1980).

An ideal group of rockfishes to address the question of sibling species and recent genetic divergence are closely related species within the subgenus *Sebastosomus* (Gill 1864). The subgenus includes a monophyletic group of five species of rockfish, *S. mystinus* (blue rockfish), *S. entomelas* (widow rockfish), *S. serranoides* (olive rockfish), *S. flavidus* (yellowtail rockfish), and *S. melanops* (black rockfish). *Sebastes ciliatus* (dusky rockfish) is also a member of the subgenus (Kendall 2000), but does not genetically group with the other five species (Johns and Avise 1998; Rocha-Olivares et al. 1999a; Hyde and Vetter 2007), and we confirmed this same pattern using both mitochondrial and nuclear sequence data (Burford and Bernardi, unpublished data). In this study, we investigate the genetic relationships within the subgenus to determine the timing of the radiation using a molecular clock and coalescence methods, and to determine whether closely related yet morphologically distinct species show predicted greater levels of genetic differentiation than morphologically similar species or populations within species. For example, in the absence of unequal substitution rates an ancient and rapid radiation would predict relatively similar aged species with a similar degree of genetic divergence, whereas a complex pattern of speciation, including ancient radiations and more recent speciation, predicts different ages and degree of divergence among species within the subgenus. In particular, we were interested in both the relationship between *S. mystinus* and *S. entomelas*, which form a sister group in all previous phylogenetic analyses (Johns and Avise 1998; Rocha-Olivares et al. 1999a; Hyde and Vetter 2007), and in the level of genetic divergence within *S. mystinus*. While both *S. mystinus* and *S. entomelas* have overlapping ranges, they have a population center and depth distribution difference, and are morphologically distinct (see Love et al. 2002). Therefore, we wanted to understand whether ecological and distributional differences were the mechanisms that led to speciation or reinforcement between the species. Two previous studies on *S. mystinus* found significant genetic structure among populations of adults and juveniles (Cope 2004; Burford and Larson 2007, respectively). However, the degree and geographic extent of this divergence was unclear due to limited sample locations and conflicting results between the genetic analysis of juveniles versus adults; Burford and Larson (2007) found a genetically divergent juvenile population south of Cope's (2004) proposed geographic break at Cape Mendocino for adult *S. mystinus*.

The specific goals of this study were to understand the evolutionary relationships and history of closely related species within the subgenus *Sebastosomus* based on both mitochondrial and nuclear sequence data. First, we analyzed five species within *Sebastosomus* and in so doing, we tested two hypotheses: (1) species within the subgenus *Sebastosomus* are very divergent (e.g., greater than found in the subgenus *Sebastomus*: Rocha-Olivares et al. 1999b), and (2) individuals identified as *S. mystinus* and *S. entomelas* group in distinct clades (reciprocally monophyletic). Second, we characterized and compared the genetic structure of adult *S. mystinus*, at three sample locations in different parts of its range, using microsatellite markers to further analyze genetic homogeneity within *S. mystinus* and test a third hypothesis: (3) the long pelagic duration and predicted high dispersal potential of *S. mystinus* maintains one genetically homogenous group. Previous studies on *Sebastes*

species used either sequence or microsatellite data, which allows us to compare the degree of genetic divergence within *S. mystinus* to the known divergences between other species within the genus. The phylogenetic reconstruction, using sequence data from other closely related species within the subgenus *Sebastosomus*, allowed us to quantify the relative degree of genetic divergence among groups. By investigating genetic relationships of closely related taxa within the subgenus *Sebastosomus*, we provide evidence for recent and incipient speciation.

Materials and methods

Main study species

Sebastes entomelas and *S. mystinus* are closely related species that differ in both distribution (depth and range) and morphology (Love et al. 2002). *Sebastes entomelas* has a northern distribution (Kodiak Island to northern Baja California) with the largest population numbers from British Columbia to northern California and tends to school at depths of 140–210 m. Whereas the *S. mystinus* has a more southern distribution, with largest population numbers along the central and northern California coast (approximately 700 km; Fig. 1). In addition, *S. mystinus* is found at shallower depths than *S. entomelas* (surface to 90 m; Leet et al. 2001; Love et al. 2002). *Sebastes entomelas* is a slender bodied rockfish that differs from *S. mystinus* in body shape and in coloration, but only slightly in meristics. After the protracted pelagic phase (up to 6 months), *S. mystinus* juveniles settle approximately from April through July (Wyllie-Echeverria 1987) and adults tend to show high site fidelity (Miller and Geibel 1973; Jorgensen et al. 2006). *Sebastes entomelas* also has a protracted larval phase, up to 5 months, and juveniles appear to settle in nearshore areas (Love et al. 2002). Finally, *S. mystinus* feeds predominately on plankton, which is distinct from other members of the subgenus and uncommon within the genus.

Sampling

We analyzed samples from three key portions of the range of *S. mystinus*: Neah Bay, Washington, Fort Bragg, California, and Gaviota, near Santa Barbara, California (see Fig. 1). Neah Bay and Gaviota are near the northern and southern extremes of the range of *S. mystinus*, and Fort Bragg is located in the northern part of the distribution center described above. In addition, Fort Bragg is located just south of Cape Mendocino the proposed site for a geographic break between *S. mystinus* populations (Cope 2004). We sampled approximately 50 *S. mystinus* individuals from these three sites (Appendix A; Fig. 1) and our goal with these samples was to quantify genetic differentiation within *S. mystinus*. For the phylogenetic analyses, we also sampled 20 *S. entomelas* from Newport, Oregon, 8 samples of *S. melanops* from Ocean Cove, Carmel Bay and Santa Cruz Island in California, and 14 samples of *S. serranoides*

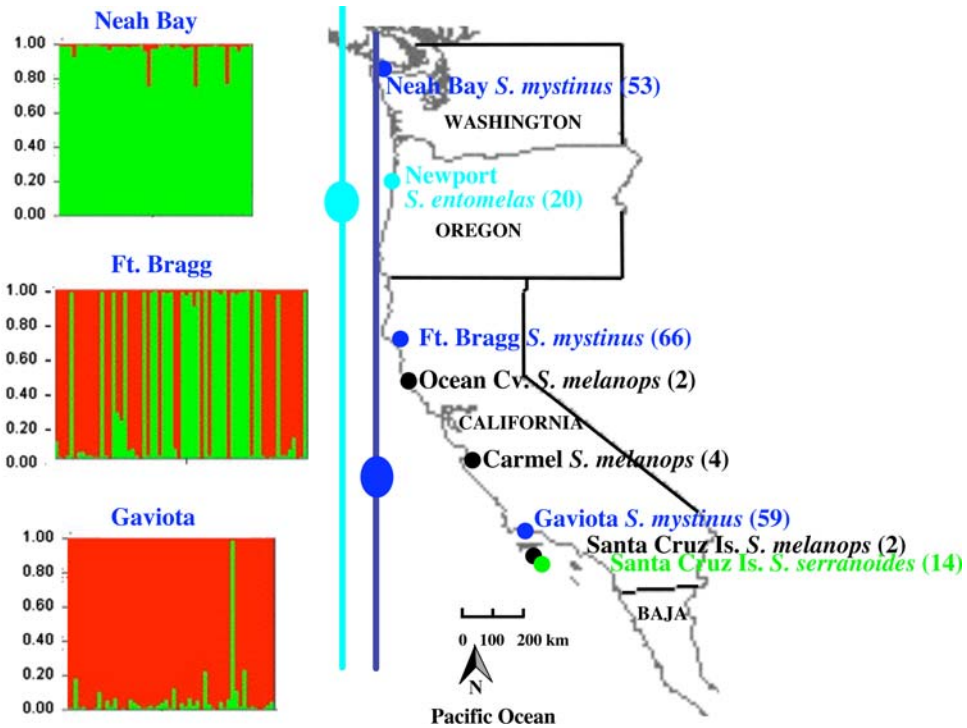

Fig. 1 Map of sampling locations for *S. mystinus, S. melanops*, *S. serranoides*, and *S. entomeals* and results of the *S. mystinus* assignment test in STRUCTURE. The assignment test results show a graph of the posterior probabilities for individuals at each sampling location as described in the text using $K = 2$, correlation among allele frequencies, and an admixture model. The *two lines* show the distribution of *S. entomelas* (*light blue*) and *S. mystinus* (*dark blue*), indicating both the center of distribution (*circles*) and relative distance from the shoreline

from Santa Cruz Island, California. Because we were unable to obtain our own samples, we used a sample of *S. flavidus* (GenBank accession nos. **DQ678548**) provided by Hyde and Vetter (2007). We used published sequences of *S. marinus* and *S. alutus* (GenBank accession nos. **U12065** and **DQ678519**) for the calibration of the molecular clock.

We obtained specimens predominately from port samples of recreational fishing boats by the California Department of Fish and Game and the Washington Department of Fish and Wildlife and by hook and line catches from commercial passenger fishing vessels (CPFV). We chose these three sampling locations for *S. mystinus* because samplers collected individuals using similar methods and individual samples came from a wide range of depths within a location. We used reproductive size males and females for analysis (over 200 mm standard length) and obtained fin-clips from all adults and preserved the fin-clips in 95% ethanol for subsequent genetic analyses.

DNA extraction and amplification

We extracted genomic DNA from caudal fin of each specimen using a Qiagen DNA Extraction Kit (Valencia, CA, USA) following the manufacturer's protocol. We sequenced the 5′ end of the mitochondrial control region (CR) from the extracted DNA and using the L15725rv (5′-ARRCCYGARTGRTAYTTYYTNTTYGC-3′; revised from Sorenson et al. 1999) and CR-E primers (5′-CCTGAAG TAGGAACCAGATG-3′; Lee et al. 1995). The cycling conditions included an initial denaturation of 5 min at 94°C, 45 s at 94°C, 1 min at 52°C, 1 min at 72°C, for 35 cycles, and a final extension of 5 min at 72°C. We generated CR sequences that were 313 bp long from 151 individuals. In addition, we sequenced 97 individuals in both the reverse and forward direction, to confirm heterozygotes, of the nuclear recombination activating gene 1 (RAG1) using the RAG1F and RAG8R primers (Quenouille et al. 2004). We generated 750 bp sequences from the nuclear RAG1 gene. We excluded all individuals that had more than one heterozygous site (35 individuals), where *cis*/*trans* situations could not be unambiguously determined. When a single heterozygous site was present, we used sequences with each alternative nucleotide score for that site.

For the microsatellite analysis of *S. mystinus*, we amplified six microsatellite loci designed from *S. rastrelliger* (Buonaccorsi et al. 2004; Westerman et al. 2005) that showed moderate polymorphism and were easy to score (Appendix B). We followed the protocol in Burford and Larson (2007) for PCR reactions, fragment amplification and scoring of these microsatellite loci and conducted fragment analysis on an ABI3100 automated sequencer. For the microsatellite analysis, we analyzed 178 individual *S. mystinus* (Fig. 1).

Data analyses

DNA sequence analyses

For the phylogenetic reconstruction, we aligned sequences of the 5′ end of the CR and RAG1 gene using the program Clustal V (Applied Biosystems) in Sequence Navigator. We generated measures of haplotype number and diversity using the software package ARLEQUIN v3.0 (Excoffier et al. 2005) and tested for the most appropriate substitution model with Modeltest v.3.7 (Posada and Crandall 1998). We assessed phylogenetic relationships within *Sebastosomus* using CR sequence data and a random and balanced (approximately two individuals per species) sample of each species using Neighbor-Joining, Maximum Parsimony, and Maximum Likelihood (with a molecular clock enforced) methods implemented in PAUP* v.4.0b10 (Swofford 1998). We used 1,000 bootstrap replicates to test the topology of each tree. We also used Bayesian inference (BI) methods generated in MrBayes v.3.1 (Huelsenbeck and Ronquist 2001) and based on a substitution model generated in MrModeltest v.2.2 (Nylander 2004; Nylander et al. 2004). For the expanded phylogeny, using CR sequence data and all sample locations and species, we used the Neighbor-Joining and BI methods following similar procedures as described above. Due to computational limitations, we could not use either the Maximum Likelihood or Maximum Parsimony methods for the expanded data set. Finally, using the RAG1 sequence data we assessed relationships within the subgenus using the Neighbor-Joining and BI methods. The RAG1 sequence data was only used to assess patterns within the subgenus. To test for significant differences between alternative tree topologies, we used the Shimodaira-Hasegawa (SH) likelihood optimality criterion (1,000 replicates) as implemented in PAUP.

To analyze genetic structure, we calculated average sequence divergence between groups [(% between − (%within$_1$ + %within$_2$))] and generated pairwise F_{ST} values. We also conducted an AMOVA (Excoffier et al. 1992) following the groupings of samples in the Neighbor-Joining and BI results and generated significance values by analyzing the expected ϕ_{ST} and Va (among group variance) and for pairwise F_{ST} values using 1,000 permutations (ARLEQUIN).

Microsatellite analyses of Sebastes mystinus

For the microsatellite analysis we analyzed each locus for scoring errors due to null alleles, large allele dropout or stutter, using Micro-Checker v.2.2.1 (Van Oosterhout et al. 2004). General microsatellite analysis of *S. mystinus* collections included: (1) an estimate of genetic diversity (Nei 1987, Eq. 7.39) using FSTAT v.2.9.32 (Goudet 1995),

(2) an estimate of expected and observed heterozygosity (H_E and H_o) using ARLEQUIN, (3) an analysis of allelic richness that measures diversity at equivalent sample sizes implemented in FSTAT, and (4) deviations from Hardy-Weinberg (HW) expectations and independence of the microsatellite loci using an exact test (Guo and Thompson 1992) as implemented in GENEPOP version 3.2 (Raymond and Rousset 1995b). For all exact tests, we generated significance probabilities using the Markov chain method as described in Guo and Thompson (1992) and implemented in GENEPOP (using 10,000 iterations), used a sequential Bonferonni (Rice 1989) to correct for multiple comparisons and to avoid type 1 error, and used Fisher's method of combining probabilities (Raymond and Rousset 1995a, b; Sokal and Rohlf 1995). For comparisons of diversity measures among populations and between types, we used a one-tailed paired *t* test (Sokal and Rohlf 1995). To assess genetic divergence among individual sample locations, we measures of F_{ST} (Weir and Cockerham 1984) and pairwise F_{ST} as implemented in FSTAT and used 10,000 permutations to generate *P* values.

We used the program STRUCTURE v.2.1 (Pritchard et al. 2000) to test the clustering of individuals within populations and regions and to test the natal origin of individuals of mixed ancestry. In these tests, we used several grouping priors (*K*), used an admixture model, and allowed for correlation among allele frequencies (Falush et al. 2003). We generated posterior probabilities in STRUCTURE using 30,000 iterations of the Markov chain Monte–Carlo method after an initial burn-in period of 30,000. We verified *K* and confirmed stability of the other model parameters by running the model with *K* values ranging from 1 to 5 and confirmed appropriate groupings by analyzing the average log-likelihood and data estimate [Ln(Pr(*X*/*K*))] for different *K*s (Pritchard et al. 2000). We confirmed *K* by measuring ΔK following Evanno et al. (2005). Similar to the analysis of genetic structure using sequence data, we used the results of the individual assignments to place individuals into separate groups and test genetic divergences between these groups using an AMOVA and pairwise differences as implemented in ARLEQUIN and FSTAT and used 10,000 permutations to generate *P* values for both analyses.

Historical demography

To estimate the time of coalescence for *S. mystinus* and *S. entomelas* lineages, we assumed that coalescence was reached when population size was 1% of the estimated present value (Wares and Cunningham 2001). We used CR sequence data for this analysis, because there was a greater number of individuals and number of species for this gene than for the RAG1 gene. We based coalescence time on two parameters: mutation rate (μ) and population growth (g). We used a *Sebastes* specific μ for the mitochondrial control region, which we estimated using a molecular clock calibrated by the split between the Atlantic and Pacific sister-taxa, *Sebastes marinus* and *S. alutus* (see Rocha-Olivares et al. 1999b). We generated specific mutation rates for these lineages with the estimated divergence dates of 3.0 and 3.5 million years ago (Mya) for a *trans*-Artic exchange between the Pacific and North Atlantic taxa (Vermeij 1991; Briggs 1995; Rocha-Olivares et al. 1999b; Wares and Cunningham 2001). The opening of the Bering strait occurred approximately 3.5 Mya (Briggs 1995), which coincides with the first fossil evidence of large-scale invasion of Pacific fauna into the north Atlantic (Vermeij 1991). The end of the exchange occurred approximately 3.0 Mya (Briggs 1995). We used the 3.0 Mya divergence following Rocha-Olivares et al. (1999b) and Hyde and Vetter's (2007) phylogenetic analyses of *Sebastes*. We also used the 3.5 Mya divergence following several previous studies on other marine taxa (Wares and Cunningham 2001; Riginos and Cunningham 2005). We estimated the maximum likelihood of parameters θ ($\theta = 2N_t\mu$) and population growth (g) using unconstrained exponential growth (exponential growth parameter in units in μ^{-1}) in FLUCTUATE v.1.4 (Kuhner et al. 1998). We used randomly generated seeds and ran the estimates 10 times per region to check the stability of the parameter estimates and measure mean and standard error for each parameter. The final analysis included 10 short Monte Carlo chains of 200 steps each and 10 long chains of length 20,000, with a sample increment of 20. We used this method to test whether these lineages were present prior to the last glacial maximum (LGM) approximately 19,000 years ago and to analyze whether they were expanding or contracting populations.

Results

Sebastosomus

Interspecific phylogeny and historical demography

For the CR sequence data, we used the substitution model and rate results generated in Modeltest for the phylogenetic analysis, pairwise comparisons, and in the AMOVA for the ingroup (smaller and expanded phylogeny: hLRT best-fit model = HKY + G and K81uf + I+G; gamma shape = 0.1298 and 0.6298 and pinvar = 0.00 and 0.4548, respectively). For the BI test using all individuals, we used the results from MrModeltest to generated BI trees and bootstrap values (smaller and expanded phylogeny: hLRT best-fit model = HKY + G and GTR + I+G; gamma shape = 0.1564 and 0.6633 and pinvar = 0.00 and 0.4539, respectively). For the CR sequence analysis using the smaller data

set, we found similar topologies with all methods, and present the topology generated by the Maximum Likelihood method with a molecular clock enforced in Fig. 2a. For the expanded CR sequence analysis, we found similar topologies for both trees generated by Neighbor-joining and Bayesian methods, which we present in Fig. 2b. In all trees, *Sebastes melanops*, *S. flavidus*, and *S. serranoides* individuals grouped together in distinct groups following common nomenclature. For both trees (Fig. 2a, b), we found two statistically equivalent trees (SH test: $P = 0.23$ and 0.24, respectively). Both trees presented showed *S. entomelas* and *S. mystinus* as sister-groups, and another tree (not presented) showed *S. entomelas* embedded within the Type 1 group of *S. mystinus*. For the phylogenetic analysis using the smaller data set (Fig. 2a), we found 5 fixed differences between *S. melanops* and *S. serranoides*, 18 fixed differences at the node between the assemblage that included *S. melanops, S. flavidus,* and *S. serranoides* (Group 1) and the other assemblage that included *S. entomelas* and *S. mystinus* (Group 2), 1 fixed difference between *S. entomelas* and *S. mystinus*, and 3 fixed differences between *S. mystinus* Type 1 and Type 2. For the expanded tree (Fig. 2b), we found unexpected results in Group 2 that comprised *S. mystinus* and *S. entomelas*. First, we found no fixed differences between *S. entomelas* and *S. mystinus* or within *S. mystinus*.

The trees generated using RAG1 sequence data and substitution model and rates generated by both Modeltest and MrModeltest (hLRT best-fit model = HKY + G; gamma shape = 0.009; pinvar = 0) further confirmed the relationship among all five species in the phylogeny (Fig. 2c). We found one fixed transition at position 624 of the RAG1 sequence between *S. entomelas* and *S. mystinus* and this difference was a synonymous change. Similar to the CR analysis, we found two statistically equivalent trees (SH test: $P = 0.08$). As with the previous trees, we found one tree presented in Fig. 2c that showed *S. entomelas* and *S. mystinus* as sister-groups, and another tree (not presented) that showed *S. entomelas* embedded within *S. mystinus.*

We estimated coalescence times for mtDNA sequence data using a μ of 8.744×10^{-8} to 10.2×10^{-8} estimated from 363 bp of the control region (Table 1). Using the net nucleotide divergence, *d* (Nei and Li 1979) between the sister-taxa *S. marinus* and *S. alutus* ($d = 0.0765$), estimates of the North Atlantic invasion of Pacific taxa (3.5–3.0 Mya), and generation time of 50% female maturity in *S. alutus* (8 years; Love et al. 2001), we estimated the mutation rate per generation year ($\mu = [1/2(d)(\text{years/gen})^{-1}]$). Using μ and g (exponential growth), the coalescence time for *S. mystinus* and *S. entomelas* were both greater than the time to the last glacial maximum (LGM; approximately 19 Kya) and close to an order of magnitude greater in *S. mystinus* (Table 1). These results revealed that *S. entomelas* was an older lineage than *S. mystinus*, which supported *S. entomelas* as a distinct group from *S. mystinus*. The divergence date generated by sequences divergences and represented in the Maximum Likelihood tree (Fig. 2a) placed the divergence date of the sister-taxa also beyond the LGM at approximately 0.94–1.10 Mya.

Interspecific genetic differentiation

We report genetic divergences within the subgenus using both the CR and RAG1 sequences in Table 2. In general, we found higher variation in the CR sequences than the RAG1 sequences. Using the sequences generated with the CR, we found 4.93% (11.8% for expanded tree) divergence between the Group 1 and Group 2 and 1.08% (3.3% for expanded tree) divergence between *S. serranoides* and *S. melanops*. For *S. entomelas* and *S. mystinus*, we found three fixed differences in the smaller versus no fixed differences in the expanded sample analyses, respectively (Fig. 2b). For both phylogenetic analyses using CR sequence data, we found the sequence divergence between individuals of each species was similar to the divergence of individuals within each species (within *S. mystinus* 2.9%; within *S. entomelas* 3.0%; between 4.0%), and found lower than expected F_{ST} values ($F_{ST} = 0.33$) given their current taxonomic status as distinct species (Table 2). For the RAG 1 sequence data we found 0.5% sequence divergence between the Group 1 and 2 assemblages, 0.2% sequence divergence between *S. mystinus* and *S. entomelas*.

Sebastes mystinus

Intraspecific phylogeny and historical demography using CR sequence data

Using CR sequence data and all *S. mystinus* sample locations (expanded data set), we did not find any fixed differences that characterized the three locations. However, *S. mystinus* individuals partitioned into two assemblages with the expanded CR sequence data (Type 1 and 2; Fig. 2b). There was a group of 8 *S. mystinus* individuals (basal) from all three locations (Fig. 2b; Table 3). We found a fixed transition between the two *S. mystinus* assemblages with the exception of three samples, Fort Bragg 29 and Neah Bay 305 in Type 1 and Fort Bragg 8 in Type 2 that reversed this difference (position 213 in CR sequence). Due to low variability at the RAG1 gene, there was no resolution of the two assemblages using the RAG1 sequence data. We grouped the samples by population and then by their phylogenetic groupings for subsequent analyses.

We found different coalescence times for the Type 1 and Type 2 groups using the CR sequence data. For the Type 1

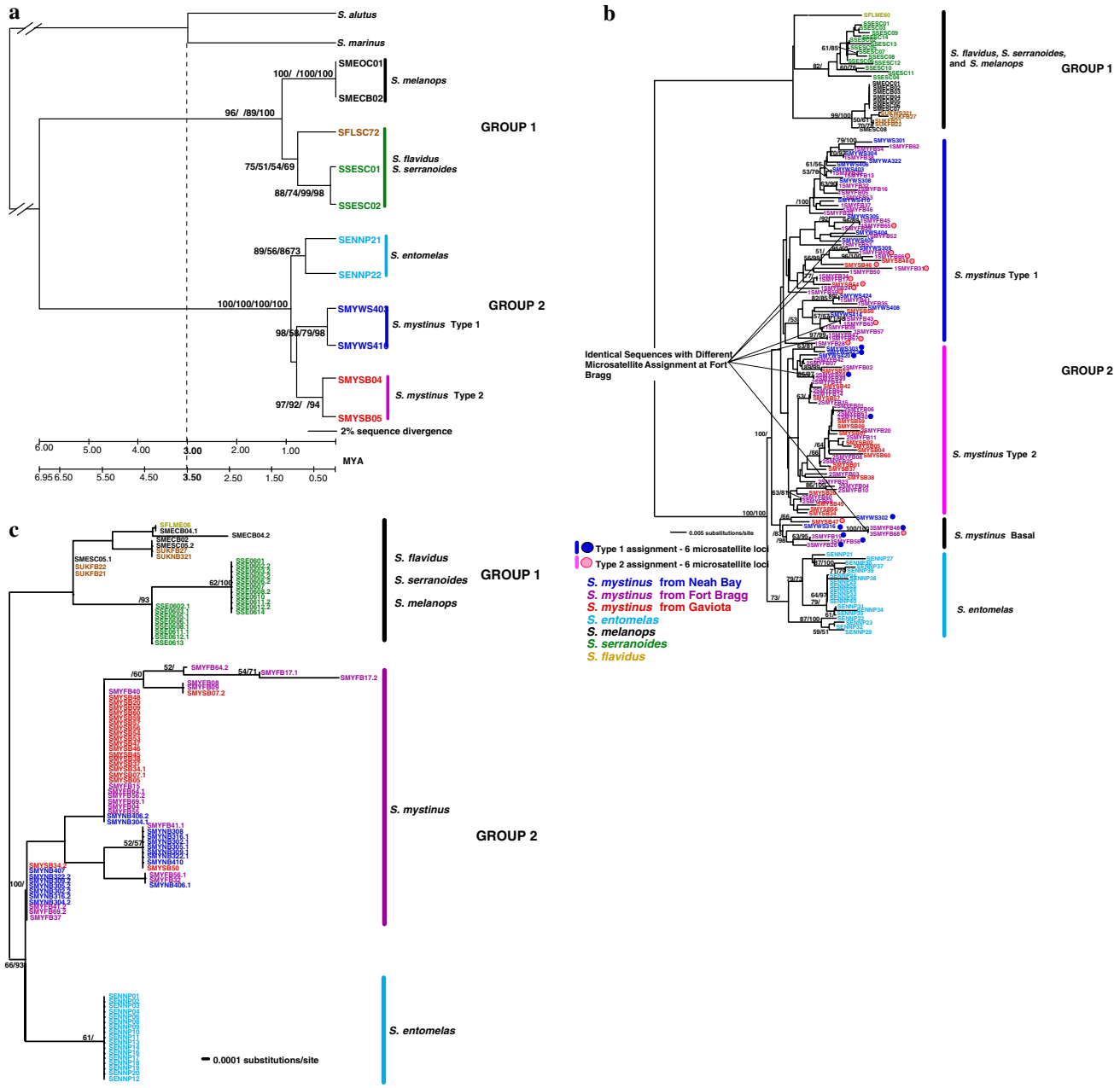

Fig. 2 Subgenus *Sebastosomus* phylogenetic relationships using **a** the Maximum Likelihood method (HKY + G) with molecular clock enforced, including *Sebastes alutus* and *S. marinus* (North Pacific vs. North Atlantic sister-taxa) as the outgroup for 313 bp of 5′ mtDNA CR and numbers at the major nodes indicating bootstrap support using the Neighbor-joining, Maximum Parsimony, posterior probabilities using Baysian Inference (1,000 replicates), **b** Neighbor-Joining method (Kimura 3-parameter) for 313 bp of the 5′ mtDNA CR, with *blue* and *red* color-coded *circles* and *bars* indicating the microsatellite assignment to type based on the STRUCTURE assignment (*blue* for Type 1; *red* for Type 2), and **c** Neighbor-Joining method (Kimura 2-parameter) for 750 bp of the nuclear gene RAG1. For both **b** and **c**, numbers at major nodes indicate boostrap support using the Neighbor-joining method (1,000 replicates) and posterior probabilities using the Bayesian Inference. The *left hand* legend indicates the list of species groups used in the phylogenetic reconstruction. In all cases, we only report bootstrap values of 50% or greater. We indicate major assemblages on the *right hand* side of each tree. The four individuals in brown were originally collected as *S. mystinus*, but group with *S. melanops* and were likely misidentified

group, we found a slightly longer coalescence time than the Type 2 group. In addition, we found relatively greater growth rate in the Type 2 compared to the Type 1 group (Table 1). The coalescence time for the Type 1 group, at high frequencies (approximately 96%) in the Northern location, was approximately an order of magnitude greater than the LGM (19 Kya). The divergence date calculated using sequence divergences and represented in the Maximum Likelihood tree (Fig. 2b), placed a divergence date of the two lineages between 780 and 920 Kya.

Table 1 Demographic parameters for *Sebastes entomelas, S. mystinus*, and both types of *S. mystinus* (Type 1 and 2) based on CR sequence data and generated using estimates of θ ($\theta = 2N_E\mu$) column 2 and g (exponential growth parameter) column 3

Species or type	θ (growth)	g	Coalescence time (ya)	
			3.5 Mya	3.0 Mya
S. entomelas All	0.07 (±0.006)	127.87 (±4.99)	412,408 (±15,269)	353,539 (±13,090)
S. mystinus All	1.70 (±0.09)	265.20 (±8.78)	198,787 (±6,487)	179,411 (±5,561)
S. mystinus Type 1	1.16 (±0.28)	241.22 (±21.61)	220,038 (±21,317)	188,628 (±18,274)
S. mystinus Type 2	1.01 (±0.40)	588.59 (±79.89)	90,763 (±10,610)	77,807 (±9,096)

Coalescence time is shown in columns 4 and 5 for both the 3.5 and 3.0 Mya divergence dates, respectively. The standard deviation is in parentheses after each parameter estimate. We averaged the parameters and calculated standard deviations over ten runs of the model

Table 2 Pairwise F_{ST} values for adult *Sebastosomus* and *Sebastes mystinus* at sample locations: (A) Pairwise F_{ST} values using sequence data from CR and RAG1; (a) five recognized species of subgenus *Sebastosomus*, (b) three S. *mystinus* populations and *S. entomelas*, (c) three groups, *S. mystinus* Type 1 and 2, and *S. entomelas*. (B) Pairwise F_{ST} values for *S. mystinus* using CR and microsatellite data; (a) all three populations, (b) four groups, Fort Bragg divided between the two types, and (c) two Types of *S. mystinus*

A.

(a)	*S. mystinus*	*S. entomelas*	*S. serranoides*	*S. flavidus*	*S. melanops*
S. mystinus	*	0.623	0.739	NA	0.621
S. entomelas	0.334	*	0.924	NA	0.912
S. serranoides	0.753	0.850	*	NA	0.735
S. flavidus	0.716	0.831	0.676	*	
S. melanops	0.767	0.864	0.797	0.969	*
(b)	*S. mystinus* NB	*S. mystinus* FB	*S.mystinus* GA	*S. entomelas*	
S. mystinus NB	*				
S. mystinus FB	0.017	*			
S.mystinus GA	0.139	0.038	*		
S. entomelas	0.342	0.339	0.453	*	
(c)	*S. mystinus* T1	*S. mystinus* T2	*S. entomelas*		
S. mystinus T1	*				
S. mystinus T2	0.211	*			
S. entomelas	0.307	0.528	*		

B.

(a)	mtDNA CR		Microsatellite loci			
	Neah Bay	Fort Bragg	Neah Bay	Fort Bragg		
Fort Bragg	0.017		0.037			
Gaviota	0.139	0.038	0.119	0.026		
(b)	mtDNA CR			Microsatellite loci		
	Neah Bay	Fort Bragg T1	Fort Bragg T2	Neah Bay	Fort Bragg T1	Fort Bragg T2
Fort Bragg T1	0.000			0.000		
Fort Bragg T2	0.216	0.210		0.122	0.124	
Gaviota	0.139	0.136	0.002	0.119	0.120	0.0030
(c)	mtDNA CR	Microsatellite loci				
	S. mystinus T1	*S. mystinus* T1				
S. mystinus T2	0.211	0.123				

The RAG1 sequence data was only used to make interspecific comparisons

NB Neah Bay, *FB* Fort Bragg, *GA* Gaviota, *T1* Type 1, *T2* Type 2

Bolded numbers were significant after corrections for multiple comparisons at the alpha level of 0.05

Intraspecific Hardy–Weinberg equilibrium and genetic diversity using microsatellite loci

The results from the Micro–Checker analysis indicated that there were no scoring errors at any of the loci or populations due to problems in the PCR reactions such as large allele dropout or stutter, which confirmed that the above results were not due to inherent problems with the individual microsatellite loci. At one location, Fort Bragg, there was evidence for a null allele (i.e., an allele that is present but is not captured due to mutation in the primer sequence) at one locus (*Sra.6-52*). However, when we analyzed each

Table 3 Results of adult *Sebastes mystinus* groupings or assignment between markers (CR and microsatellite loci)

Sample location	mtDNA CR				Microsatellites			Both markers	
	N	Basal (%)	Type 1 (%)	Type 2 (%)	*N*	Type 1 (%)	Type 2 (%)	*N*	Inconsistent (%)
Neah Bay, WA	18	10	74	16	53	97.5	2.5	17	16
Fort Bragg, CA	61	8	39	52	66	45.4	54.6	56	25
Gaviota, CA	21	5	19	76	59	5.3	94.7	20	20

Table includes sample size (*N*) for CR sequence data, microsatellite data, and for both marker comparisons and percentage of individuals that assigned to each of the groups

type separately at Fort Bragg, there was no evidence of a null allele. We found significant violations of HW genotypic expectations at Neah Bay and Fort Bragg ($P < 0.05$, after corrections) within *S. mystinus*. However, when we analyzed each sample collection per location separately (see Appendix A), the Neah Bay sample was in HW equilibrium. The Fort Bragg sample was in HW equilibrium after dividing the sample into the two types. In comparison to the results on HW expectations, we only found one significant violation of linkage equilibrium between locus *Sra.7-25* and *Sra.6-52* at Fort Bragg ($P < 0.05$ after corrections). This was also resolved when we conducted a second analysis with separate types at this location. Therefore, the null allele, HW and linkage equilibrium violations resolved when we analyzed the two types separately per location.

Genetic diversity was not significantly different among samples for the microsatellite data for all measures of genetic diversity (Appendix A, paired *t* test, $P > 0.05$). In addition, we found similar genetic diversity and allelic richness between the two types of *S. mystinus*. We found difference in the dominant (highest frequency) allele at 5 of 6 loci between the two types and 38 and 26 private alleles within the Type1 and Type 2 groups (Appendix B).

Intraspecific genetic differentiation

To better understand the degree of genetic distinctiveness between the two types and any differences between locations within *S. mystinus*, we used the findings of the phylogeographic reconstruction to organize our subsequent analyses by, (1) comparing the three locations (Neah Bay, Fort Bragg, and Gaviota), (2) separating the two types within Fort Bragg and then comparing four groups (Neah Bay, Fort Bragg 1, Fort Bragg 2, and Gaviota), and (3) grouping all *S. mystinus* Type 1 individuals for either the CR sequences and microsatellite loci and compared them to Type 2 individuals.

For both CR and microsatellite data, there was significant genetic differences in pairwise F_{ST} values between sample locations at Neah Bay and Fort Bragg combined versus Gaviota (Table 2). In general, we found significant pairwise differences for comparisons between the two *S. mystinus* types for all groupings. This was true for comparisons between types at Fort Bragg or for all individuals in Type 1 versus Type 2 regardless of geographic location ($P < 0.05$; CR $F_{ST} = 0.21$ and microsatellite $F_{ST} = 0.12$). With the microsatellite loci, we also found significant pairwise F_{ST} values between Neah Bay and Fort Bragg. For both the sequence and microsatellite data, the highest percent among group variance (Va) in the AMOVA confirmed the significant groupings of the two groups, Fort Bragg Type 1 and Neah Bay versus Fort Bragg Type 2 and Gaviota (Table 4).

Intraspecific assignment test using microsatellite data

Employing both results from the AMOVA and number of sampling locations as priors to designating *K* (groupings) for the program STRUCTURE, we found similar main genetic break for both a *K* of 2 and 3. However, but the analysis of the data estimates [Ln(Pr(*X*/*K*))] and Δ*K* among the five runs for each *K* supported a grouping of 2 (Fig. 1; Appendix C). The assignment test showed a strong break between the two types, with a 100, 56 and 1.69% of the individuals assigned to Type 1 at Neah Bay, Fort Bragg and Gaviota, respectively. We found that individuals generally assigned to one or the other type and were not admixtures of both types.

Combined sequence and microsatellite analyses

We found corroboration between the two molecular markers, CR sequence versus microsatellite loci, when we overlaid the assignment test results on the Neighbor-joining trees generated from CR sequence data (see Fig. 2c). However, there were a few exceptions, including samples that assigned to the opposite microsatellite type within a CR assemblage (e.g., nuclear microsatellite markers assigned the individual to Type 1 whereas it was found in the Type 2 CR group; Fig. 2c; Table 3).

Looking specifically at the CR sequences in comparison to the microsatellite loci because they represent differences in genomic versus organelle DNA, inheritance, effective population size (mtDNA $1/4N_E$), and mutation rate, we

Table 4 Analysis of Molecular Variance for adult *Sebastes mystinus*: (a) Results of the hierarchical analysis of variance (AMOVA) of mtDNA control region (313 bp) with groupings based on the Neighbor-Joining tree, (b) Results of the hierarchical AMOVA using microsatellite data and with groupings based on the microsatellite assignments

Number of groupings	Populations	PSI_{SC}	PSI_{St}	PSI_{ct}	Variance among groups (%)	
					(Va)	*P*
(a)						
2	NB vs. (FB, GA)	0.0365	0.0511	0.0152	1.52	0.3187
2	(NB, FB) vs. GA	0.0191	0.0604	0.0421	4.21	0.3363
2	(NB, GA) vs. FB	0.1310	0.0285	−0.1179	−11.79	0.6501
Groupings						
2	NB vs. (FB1, FB2,GA)	0.1428	0.0958	−0.0548	−5.48	0.7840
2	**(NB, FB1) vs. (FB2, GA)**	**−0.0084**	**0.1756**	**0.1825**	**18.25**	**0.0000***
2	(NB, FB1, FB2) vs.(GA)	0.1387	0.1060	−0.0380	−3.8	0.6061
Number of groupings	Populations	PSI_{SC}	PSI_{St}	PSI_{ct}	Variance among groups (%)	
					(Va)	*P*
(b)						
2	NB vs. (FB, GA)	0.0276	0.0775	0.0514	5.14	0.0000*
2	(NB, FB) vs. GA	0.0380	0.0701	0.0334	3.34	0.3324
2	(NB, GA) vs. FB	0.1181	0.0325	−0.0970	−9.7	0.6618
Groupings						
2	NB vs. (FB1, FB2, GA)	0.0730	0.0943	0.0230	2.30	0.0000*
2	**(NB, FB1) vs.(FB2, GA)**	**0.0000**	**0.1278**	**0.1208**	**12.08**	**0.0000***
2	(NB, FB1, FB2) vs. GA	0.0817	0.0877	0.0066	0.66	0.2473
2	(NB, GA) vs. (FB1, FB2)	0.1206	0.064	−0.0644	−6.44	0.6852
2	(NB, FB2) vs. (GA, FB1)	1,208	0.0679	−0.0601	−6.01	0.3128

NB Neah Bay, *FB* Fort Bragg, *GA* Gaviota, *FB1* Fort Bragg Type 1, *FB2* Fort Bragg Type 2

Asterisks indicate significant *P* values (after a sequential Bonferroni correction) and bold values indicate the arrangement with the highest % Va

found six instances within the Fort Bragg sample where identical sequences assigned to opposite Types (as indicated in Fig. 2c). In general, we found the highest frequency of this inconsistency between marker types at Fort Bragg and Gaviota (25%; Table 3).

Discussion

While the genus *Sebastes* is an example of an ancient species flock, in this study, we show that recent and rapid speciation produced closely related species within the subgenus *Sebastosomus*. Moreover, we found evidence of incipient speciation within *S. mystinus*. Specifically, the major results generated by this study indicate that (1) five closely related species within the subgenus *Sebastosomus* exhibit average CR sequence divergence, for example we found 12% sequence divergence when we compared Group 1 and 2 using a single individual per taxonomic group, (2) there was either no or a low number of fixed differences between *S. entomelas* and *S. mystinus* with CR and RAG1 sequence data, and (3) both the CR and microsatellite data split *S. mystinus* into two types and revealed significant genetic divergence between them (using F_{ST} and AMOVA analyses). Averaging over both markers, we also found an uneven distribution of the two types among sample locations, with Type 1 individuals comprising 96% of the northern sample, 55% of Fort Bragg, and 10% of the southern sample and indications of admixture within the sample location where both types co-existed. Thus, the combined results reveal the close genetic relationship between *S. entomelas* and *S. mystinus* and that the species described as *S. mystinus* consists of two distinct lineages. To understand the pattern of speciation within the subgenus, we first discuss the relationship between *S. entomelas* and the *S. mystinus* assemblages and then discuss the pattern found within *S. mystinus*.

Relationship between *S. entomelas* and *S. mystinus*

Comparing the genetic relationship between *S. mystinus* and *S. entomelas* to other members of the subgenus provides evidence for the recent divergence between these sister-taxa, which suggests both historic and recent speciation

events formed this subgenus. Using both CR and RAG1 sequence data, we found higher genetic divergence between the three species within Group 1 (*S. serranoides*, *S. flavidus*, and *S. melanops*) and between Group 1 and 2 (*S. mystinus* and *S. entomelas*). In contrast, there was little or no sequence divergence between *S. mystinus* and *S. entomelas*. We were surprised to find low genetic differentiation between *S. entomelas* and *S. mystinus* given the differences in body shape (slender vs. round) and coloration (brownish vs. blue/black). Whereas these two species may appear morphologically different, the similarity in their meristics is consistent with a close genetic relationship.

Sebastes entomelas was the sister-taxon to *S. mystinus* in previous phylogenetic analyses using sequence data from both cytochrome b and the CR (Johns and Avise 1998; Rocha-Olivares et al. 1999a; Hyde and Vetter 2007). However, in an analysis using cytochrome *b* sequence data and multiple individuals from both species, Mesa and Bernardi (unpublished data) found no fixed differences between *S. entomelas* and *S. mystinus*. Similar to their results and using CR sequence data, we did not find any fixed differences between *S. entomelas* and *S. mystinus* individuals. We believe the discrepancy between our results and those of the previously published phylogenies was due to using many individuals in our phylogenetic reconstruction. In contrast, we found one fixed difference between *S. mystinus* and *S. entomelas* at the RAG1 gene, which supported two distinct groups. There are two possible explanations for the finding of low genetic divergence between the sister-taxa, (1) incomplete lineage sorting supported by the more recent coalescence time for *S. entomelas* and *S. mystinus* or, (2) post-speciation introgression. Because sorting occurs faster in mitochondrial markers, our finding of greater genetic differentiation in the nuclear and the absence of such in the mitochondrial sequence data is inconsistent with a simple incomplete lineage sorting explanation.

Inconsistent results between markers could be due to incipient species hybridizing early in the speciation process or before complete reproductive isolation (see Coyne and Orr 2004). Several studies reported introgressive hybridization among closely related species within the genus *Sebastes* (Seeb 1998; Roques et al. 2002; Buonaccorsi et al. 2005), which may mean that hybridization is frequent within the genus. While there were no fixed differences between *S. entomelas* and *S. mystinus* using the expanded CR sequence data, there were no indications of current hybridization at either marker (e.g., *S. entomelas* individuals form a distinct group with both markers). Therefore, we found a discrepancy in the degree of divergence between the RAG1 and CR sequence data, consistent with historic, not recent, introgression between these two groups (e.g., *S. mystinus* breeding with *S. entomelas* early in the speciation process) and subsequent delayed lineage sorting of the mitochondrial markers.

The relationship between *S. mystinus* and *S. entomelas* provide an example of morphologically distinct species, in coloration and body-shape, which are genetically similar and not reciprocally monophyletic at all genes. While the genetic similarity of *S. mystinus* and *S. entomelas* individuals, indicative recent speciation, was higher than other genetically divergent members of the subgenus, the two species do show ecological partitioning (e.g., depth, latitude, and diet), which may maintain the two groups. Therefore, ecological partitioning between the sister-taxa, combined with the absence of evidence for incomplete lineage sorting or contemporary introgression, suggests the possibility of ecological speciation, a mechanism proposed by Larson (1980) in his study on black and yellow and gopher rockfish (*S. chrysomelas* and *S. carnatus*, respectively).

Relationship between the two types of *S. mystinus*

The combined results (six microsatellite loci and CR sequences) support the existence of at least two genetic types of *S. mystinus* and further confirm that the pattern of speciation within the subgenus contains both recent and historic events. We were surprised to find this level of genetic divergence within *S. mystinus* given its long pelagic larval phase (4–6 months) and previous findings of panmixia (from San Miguel to Fort Bragg) in earlier microsatellite and mtDNA sequence analyses of adult *S. mystinus* (Burford and Larson 2007; Cope 2004, respectively). Therefore, the high dispersal potential does not maintain one homogeneous population. In this study, we found high genetic divergence within a short geographic distance at Fort Bragg south of Cape Mendocino, which was originally proposed by Cope (2004) as a geographic break between two populations of *S. mystinus*. There are two alternative explanations for the degree of reduced gene flow and genetic divergence between the types of *S. mystinus,* (1) incipient speciation, produced in allopatry or sympatry, and maintenance of genetic isolation due to assortative mating, reproductive barriers, or ecological separation, or (2) historic separation and genetic differentiation of two populations of *S. mystinus,* due to either genetic drift or local adaptation, and subsequent range expansion into an area of overlap (i.e., not incipient species but distinct populations).

The level of genetic divergence between the genetic types of *S. mystinus* was greater than most previous intraspecific studies within the genus. Using the microsatellite data, we found greater genetic divergence between the two types ($F_{ST} = 0.12$) than that found between the sister-taxa *S. chrysomelas* (black and yellow rockfish) and *S. carnatus*

(gopher rockfish) (F_{ST} = 0.056; Narum et al. 2004). In addition, it was greater than the highest level of genetic divergence found between adult samples of *S. melanops* (F_{ST} = 0.032; Miller et al. 2005), samples of adult *S. caurinus* from the coast versus the Puget Sound (F_{ST} = 0.098; Buonaccorsi et al. 2002), and comparable to pairwise comparisons between *S. auriculatus* from the Puget Sound and Mexico (F_{ST} = 0.138; Buonaccorsi et al. 2005). Finally, the microsatellite data revealed diagnostic differences between Type 1 and 2 individuals, which further supports the conclusion that these two lineages have greater genetic distinctiveness than that found between populations.

Unlike the previous intraspecific genetic studies mentioned above, we found the high degree of genetic divergence between types of *S. mystinus* within kilometers of each other at one location. Despite their sympatric distribution at Fort Bragg and potential for interbreeding, these groups remain genetically isolated. We found clear signs of admixture at the Fort Bragg sample (violations of HW and linkage equilibrium) that resolved when we analyzed the types separately. Violations of HW and linkage equilibrium indicated a lack of intermediate individuals (F1 hybrids or introgression), which would cause the sample to conform to equilibrium expectations, suggests reproductive isolation between the two types (i.e., no hybrid population). The results of high individual assignment to types also confirmed this conclusion. The evidence of admixture and lack of introgression between the two types, support the conclusion that these types remain separated in proximity due to an isolating mechanism and are not just genetically differentiated populations. For this reason, it is critical to confirm the consistency of this pattern at other locations where both types might coexist.

Analyses using historical demographic data further confirmed the uniqueness of the two types. While there are many assumptions inherent in estimating coalescence time, we find it compelling that there was such a large relative difference in coalescence time between the Type 1 and 2 lineages and that both lineages had coalescence times beyond the LGM. This combined with evidence of an expanding population, and similar genetic diversity and high number of private alleles of the two types, indicated that both the Type 1 and 2 individuals came from a large population that existed and persisted beyond the LGM. While the divergence data using traditional molecular clock methods showed a much earlier date than the coalescence method, this was expected given the recent divergence and potentially large effective population size of the two lineages (see Edwards and Beerli 2000). Importantly, both methods provided evidence that this date exceeds the LGM, which means the process of divergence preceded, yet likely was enhanced by, physical separation during this time. The geographic distribution and the demographic data provide evidence that these two lineages diverged and expanded in different geographic areas.

Previous studies found similar patterns to those reported here on other marine taxa and provide further evidence of northern population persistence during the LGM (Marko 2004; Hickerson and Cunningham 2005). This would explain our finding of lower frequency of both mixed assignments and lack of Type 2 in the northern sample at Neah Bay. A recent range expansion from the northern part of the distribution, which is predominately comprised of Type 1 *S. mystinus*, into the southern range would maintain a signature of genetic distinctiveness between the two types. Moreover, this signature would persist if introgression occurs at a low-frequency. In their study of *Balanus glandula*, Sotka et al. (2004) found a steep cline in central California explained by secondary contact and low-frequency introgression between historically isolated populations. If a similar pattern without isolating mechanisms (i.e., the two types form viable offspring) exists with *S. mystinus*, we would predict a loss of genetic distinctiveness between the two types with time. However, in this study both the lack of F1 hybrids and the degree of genetic distinctiveness in an area of high frequency of both types (Fort Bragg), support the alternative conclusion, reproductive isolation.

Reproductive isolation, asymmetric distribution due to adult habitat or depth preferences, or assortative mating, which would reduce the frequency of interbreeding and maintain the two types, could explain the sympatric distribution of genetically distinct types. The lack of genetic distinctiveness typical of species boundaries or between the species within Group 1 of the subgenus, could be due to inadequate time for complete lineage sorting between the two types at the CR gene. A genetically diverse or a large effective size of the ancestral population could maintain genetic similarities between two distinct lineages until lineage sorting was complete and produce the pattern we observed in this study. Previous studies on incipient speciation and species flocks proposed this explanation when genetic divergence between groups was lower than recognized species boundaries, yet higher than genetically differentiation populations (Avise et al. 1984; Edwards and Beerli 2000; Knowlton 2000; McCartney et al. 2003). The similar genetic diversity of the types for both markers, indicative of a large ancestral population, the high degree of divergence between the two lineage using microsatellite markers, and signature of incomplete lineages sorting combined with the lack of introgression between the two types, supports incipient species within *S. mystinus*. By analyzing relationships within the subgenus, we uncovered the early phase of the speciation process within a member of this group, in particular within a species where the long pelagic phase would predict genetic homogeneity. Moreover, the

comparison and low sequence divergence of interspecific (between sister-taxa) and intraspecific lineages (within *S. mystinus*) provides further evidence of the recent speciation rate within the subgenus (McCune and Lovejoy 1998).

The species flock of the genus *Sebastes*, as discussed by Johns and Avise (1998), Rocha-Olivares et al. (1999a), and Alesandrini and Bernardi (1999), is probably replete with closely related yet morphologically distinguishable species such as *S. chrysomelas* and *S. carnatus* (Alesandrini and Bernardi 1999; Narum et al. 2004) and *S. entomelas* and *S. mystinus* (this study). The question remains whether closely related species that are morphologically similar, yet genetically distinct, such as our finding between the two types of *S. mystinus* is characteristic of the genus. The idea of sibling or cryptic species in the marine environment is not a new one (Knowlton 1993), and it is certainly not surprising to find it in the geographically widespread and species rich genus *Sebastes*. Our study provides compelling evidence that speciation within the genus may be more recent and an ongoing than previously assumed and that the long pelagic phase of *S. mystinus* does not predict genetic homogeneity throughout the range. Given the results of morphologically and genetically distinct freshwater cichlids in both Eastern Africa and Nicaraguan crater lakes (Schliewen et al. 2001; Barluenga et al. 2006), a thorough analysis of species within the genus *Sebastes* that includes multiple markers and individuals from populations spanning the species range, will provide an opportunity to further investigate the mechanisms of speciation in the marine environment.

Conclusion

We found evidence of recent divergence and incipient speciation within the subgenus *Sebastosomus*. Within the subgenus, we found different degrees of genetic distinction, with greater genetic divergences among *S. melanops*, *S. flavidus*, and *S. serranoides* and lower genetic distinction between S. *mystinus* and *S. entomelas* and between the two types of *S. mystinus*. As such, the subgenus reveals different stages of the speciation process and provides evidence of ecological speciation between *S. entomelas* and *S. mystinus* and incipient speciation within *S. mystinus*. In addition, within *S. mystinus*, the genetically distinct types did not show signs of introgression or hybridization at a location where we sampled equal frequencies. While resolving the question of the spatial extent of overlap between these types and the degree of admixture versus introgression will require larger spatial sampling of adults throughout the range, our study confirms that there are two unique lineages of *S. mystinus* that do not readily interbreed at one location where they coexist.

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Appendix A

Table 5

Appendix B

Table 6

Table 5 Adult sample locations and summary statistics for *Sebastes mystinus*, *S. entomelas*, *S. serranoides*, and *S. melanops*

Location	Collection#	*N* total	Year	Mean SL (cm)	Collection method	Microsatellite data						mtDNA CR				ncDNA RAG1					
						H_O	H_E	F_{IS}	A#	Private Allels	AR (N = 50/pop.; N = 29/FB Type; N = 79/Type)	Sequence #	S	H#	Hd	pi	Sequence #	S	H#	Hd	Pi
Sebastes mystinus																					
Washington																					
Neah Bay	1	25	2003	33.0	Port sample																
	2	28	2004	30.3	Port sample																
	Pooled	**53**				0.74	0.79	0.06	**15.57**	19	17.06	**19**	38	19	1.000	0.031	**17**	2	4	0.684	0.001
California																					
Fort Bragg		**66**	2006	30.2	Port sample	0.76	0.84*	0.09	17.83	14	17.43	**62**	64	50	0.992	0.030	**18**	5	8	0.830	0.002
Fort Bragg Type1		**29**				0.74	0.79	0.06	12.50	8	12.50	**34**	51	30	0.993	0.030		NA	NA	NA	NA
Fort Bragg Type2		**37**				0.78	0.78	0.00	12.33	6	11.38	**23**	26	16	0.957	0.017		NA	NA	NA	NA
Fort Bragg Basal												**5**	13	4	0.900	0.024					
Gaviota	1	**60**	2004	23.5	Hook & Line/Port sample	0.80	0.79	−0.01	13.83	5	13.33	**22**	40	21	0.996	0.025	**20**	3	4	0.284	0.001
Type 1		**83**				0.74	0.78*	0.06	18.17	38	18.06	**52**	60	44	0.994	0.030		NA	NA	NA	NA
Type 2		**95**				0.79	0.79	0.00	16.17	26	15.47	**43**	44	33	0.976	0.018		NA	NA	NA	NA
Basal						NA	NA	NA	NA		NA	**8**	21	7	0.964	0.028					
Sebastes entomelas						NA	NA	NA	NA		NA										
Oregon																					
Newport		20	2006		Port sample							**20**	25	13	0.884	0.018	**17**	0	1	0.000	0.000
Sebastes serranoides						NA	NA	NA	NA		NA										
California																					
Santa Cruz Island		**14**	2006		Hook & Line							**14**	19	12	0.967	0.014	**19**	1	2	0.515	0.001
Sebastes melanops						NA	NA	NA	NA		NA										
California																					
Ocean Cove					CENCAL spear Fishing meet																
Carmel					CENCAL spear Fishing meet																
Santa Cruz Island					Hook & Line	All loci combined															
	Pooled	**8**										**8**	2	2	0.250	0.002	**5**	2	4	0.900	0.002

NA not applicable or not available

The table provides the expected and observed heterozygosity (H_E and H_O), allele number (A#), and private alleles for microsatellite analysis and sequence number (#), number of segregating sites (S), haplotype number (H#), haplotype diversity (Hd), and estimate of nucleotide diversity (Pi) for 313 bps of mtDNA control region and 750 bps of RAG1 sequence data. See text for details

Table 6 Microsatellite summary statistics for *Sebastes mystinus*

Population			Neah Bay	Ft. Bragg	Gaviota
Locus	A (all pops)				
Sra.7-2	20	N	53	66	59
		A#	8	13	14
		AR	7.830	12.210	13.500
		F_{IS}	0.173	0.227	−0.083
		H_O	0.491*	0.636*	0.966
		H_E	0.592	0.823	0.893
		PA#	3	2	4
Sra.7-7	10	N	53	64	59
		A#	7	10	9
		AR	6.94	9.5	8.84
		F_{IS}	0.008	0.068	0.047
		H_O	0.736	0.719	0.729*
		H_E	0.742	0.771	0.765
		PA#	0	0	0
Sra.7-25	48	N	52	64	59
		A#	36	36	20
		AR	35.53	31.89	18.69
		F_{IS}	0.094	0.037	−0.006
		H_O	0.865*	0.860*	0.763
		H_E	0.955	0.892	0.758
		PA#	10	4	0
Sra.16-5	34	N	50	65	59
		A#	27	27	23
		AR	27.00	25.19	22.30
		F_{IS}	0.109	−0.008	0.046
		H_O	0.840	0.954	0.898
		H_E	0.943	0.946	0.942
		PA#	5	4	1
Sra.15-8	13	N	53	66	59
		A#	9	12	9
		AR	8.83	11.57	8.82
		F_{IS}	−0.092	0.087	−0.024
		H_O	0.774	0.697	0.763
		H_E	0.709	0.763	0.745
		PA#	1	3	0
Sra.6-52	10	N	53	64	59
		A#	7	9	8
		AR	7	8.99	7.85
		F_{IS}	0.056	0.155	−0.042
		H_O	0.736*	0.719*	0.678
		H_E	0.779	0.850	0.651
		PA#	0	1	0

The table provides the number of individuals sampled (N), allele number (A#), allelic richness (AR), inbreeding coefficient (F_{IS}), expected and observed heterozygosity (H_E and H_O), and total number of private alleles (PA#). Significant values for violations of HW expectations ($P < 0.05$) are indicated with asterisks (10,000 iterations)

Appendix C

Table 7

Table 7 Results of the grouping analysis for *Sebastes mystinus* using six microsatellite loci

K	Ln(Pr(X/K))	Bayes Theorem	Δ*K*
1	−4903.46	0.00	
2	−4462.80	0.98	298.57
3	−4466.80	0.02	8.24
4	−4554.80	0.00	3.06
5	−4530.30	0.00	

The analysis includes, the data output, the analysis using Bayes Theorem, and Δ*K*

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