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Analysis of individual year-classes of a marine fish reveals little evidence of first-generation hybrids between cryptic species in sympatric regions

Martha O. Burford · Giacomo Bernardi · Mark H. Carr

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Abstract As settled juveniles and adults, blue rockfish (*Sebastes mystinus*) are nonmigratory inhabitants of kelp and rocky reef habitats along the California coast, USA, and prior to settlement, they possess a pelagic larval and juvenile stage lasting 3–5 months. A previous study of adults revealed two cryptic species within *S. mystinus* and evidence of reproductive isolation in a region where both cryptic adults co-occur. Given this pattern of reproductive isolation, we investigated the degree of hybridization or introgression in individual year-classes shortly after juvenile settlement in two different years (2001 and 2002). Using microsatellite markers, we found little indication of hybridization in new juvenile year-classes despite an adult population that comprised both cryptic species. However, we found an average of two percent of hybrid or introgressed individuals in regions with a low frequency of one of the two species. Therefore, while the lack of hybrids or introgression supports the hypothesis of reproductive isolation between the cryptic species within *S. mystinus*, the age-structured analysis also revealed a spatial pattern of low-frequency differences in the number of introgressed individuals. These results suggest that reproductive barriers may breakdown when one of the two species predominates the regional adult gene pool.

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M. O. Burford · G. Bernardi · M. H. Carr
Department of Ecology and Evolutionary Biology, University of California Santa Cruz, 100 Shaffer Road, Santa Cruz, CA 95060, USA

Present Address:
M. O. Burford (✉)
Department of Biology, University of Central Oklahoma, 100 North University Drive, Edmond, OK 73034, USA
e-mail: mob8@cornell.edu

Introduction

Many marine species are sedentary as adults, but possess the potential for dispersal over large distances via larvae that are pelagic for weeks–months. With the high dispersal potential of many of these marine organisms and with the lack of obvious mechanisms for vicariant speciation, such as those found in terrestrial environments, speciation events or genetic structure in populations would seem unlikely or rare. Until recently, theoretic predictions and empirical evidence (Waples 1987) suggested an inverse relationship between the duration of the pelagic phase and genetic differentiation. Therefore, the prevailing belief was that marine species with extended pelagic larval phases should be panmictic. However, evidence exists for species flocks or rapid speciation (Johns and Avise 1998; McCafferty et al. 2002; Palumbi and Lessios 2005), cryptic speciation (Hyde et al. 2008; Burford and Bernardi 2008; Burford 2009), and population differentiation (e.g., Buonaccorsi et al. 2002; Miller et al. 2005) in marine taxa with protracted pelagic phases. The evidence also suggests that physical (Hyde et al. 2008) or reproductive isolation in place prior to secondary contact (Burford 2009) between cryptic species prevents introgressive hybridization. The absence of introgressive hybridization maintains distinct species during secondary contact. Given the potential for widespread dispersal for many marine species, it is important to assess the degree of reproductive isolation and whether prezygotic (e.g., assortative mating, ecological segregation) or post-zygotic barriers (e.g., hybrid breakdown, gametic

inviability) maintain closely related species. Knowledge of the nature of isolation, whether it is due to pre- or post-zygotic barriers between newly formed species, will provide evidence of the events that shaped and mechanisms that maintain the two species (e.g., Crow et al. 2010).

Recently, researchers have focused on how marine species are maintained in the face of high potential for larval dispersal and gene flow (Johannesson et al. 1995; Palumbi and Lessios 2005; Hyde et al. 2008; Burford and Bernardi 2008; Butlin et al. 2008; Johannesson 2009). Investigating both the degree of geographic isolation and the phylogenetic relationships, Palumbi and Lessios (2005) analyzed speciation patterns in the marine environment and provided an excellent demonstration of Mayr's (1942 and 1963) reconstruction of geographic speciation in the sea. In this analysis, they reported two closely related sister-taxa within the sea urchin genus *Echinometra* that had a sympatric distribution and reproductive isolation. The sister-taxa within *Echinometra* showed lower genetic divergence than sympatric populations of sister-taxa within the genus *Diadema* and indicated recent speciation and rapid reproductive isolation (Palumbi and Lessios 2005). Furthermore, a recent study on a genus of marine fish, *Hexagrammos*, concluded that complete reproductive isolation between two species that overlap in their distribution was due to the rapid formation of pre-zygotic barriers during a sympatric speciation event (Crow et al. 2010). This suggests that understanding the degree and nature of reproductive isolation between two sympatrically distributed sister species will provide more information on their progress to distinct species than an analysis of geographic distributions of the two species alone.

The blue rockfish, *Sebastes mystinus*, is a long-lived (approximately 45 years) species in which adults are relatively sedentary but pelagic larvae may disperse for 3–5 months (Love et al. 2002). An extensive study of the adult population of *Sebastes mystinus* throughout a large portion of its range (southern California to northern Washington, USA) revealed two genetically distinct lineages, cryptic species, that segregated latitudinally, except for an approximately 450 km range of overlap from central Oregon to northern California (Burford 2009). The two cryptic species did not form a gradual cline in frequency in the overlap region, but instead a marked, abrupt step-cline where both species co-occur at approximately equal numbers and then in a short geographic distance north and south of this zone both species drop in frequency (Burford 2009). In addition, there was little evidence of introgression in the adult population throughout this region of co-occurrence, suggesting the existence of reproductive isolating mechanisms, such as assortative mating, differences in the timing of mating, or habitat partitioning. There are several examples of marine invertebrates in the eastern Pacific with a geographic distribution and similar pattern of overlap to that of *S. mystinus*, such as the acorn barnacle (*Balanus glandula*) or the blue mussel (*Mytilus* spp.) (Sotka et al. 2004; Rawson et al. 1999). However, these groups form hybrid zones or genetic clines between two species or two populations and therefore, unlike *S. mystinus* (Burford and Bernardi 2008; Burford 2009), do not appear to be reproductively isolated. In the study of adult *S. mystinus*, it was not conclusive whether pre-zygotic or post-zygotic barriers supported reproductive isolation in this group (Burford 2009). For example, if F1 hybrids die before they reach reproductive age (approximately 5 years of age), they would have been missed in the previous study of adults alone. Therefore, the analysis of young-of-the-year *S. mystinus* provides an opportunity to confirm whether these two species produce settling hybrids within their range. In particular, by investigating juveniles and adults where both species of *S. mystinus* overlap, such as in northern California, and adjacent regions where they do not coexist, such as central California, we can unravel the mechanism of reproductive isolation in this group.

To test the hypothesis of reproductive isolation within *S. mystinus*, we conducted an age-structured study in which we first compared the degree of genetic divergence between the two cryptic species in new year-classes with that in the adult population. If the two cryptic species are reproductively isolated and maintain this isolation, we predict a similar degree of genetic distinctiveness between the two species in multiple generations, including each of the two settlement year-classes of juveniles and the adult population, which presumably comprises multiple year-classes. Second, to test whether juveniles were produced at different times in regions where both adult species exist, we compared length frequency differences between the two species. A finding of differences in reproductive timing would suggest a pre-zygotic barrier, limiting the ability of the two species to mate and produce hybrids. Finally, focusing on just the juvenile sample to understand the degree of reproductive isolation, we tested for each of three patterns of variation: first-generation hybrids (F1), back-crossed individuals (introgression), or a Wahlund effect (i.e., admixture of genetically distinct juvenile species).

To determine the conditions that promote hybridization, we compared juveniles from each of the two different settlement year-classes to regional adults where both species co-occur and in adjacent regions where one of the two species predominates. In the northern region (Fort Bragg) where both species coexist in the adult population, samples of the juvenile year-class can have F1 or introgressed individuals or no intermediate types (i.e., no hybridization). A lack of intermediate types would suggest reproductive isolation between the two species due to barriers that are either pre-zygotic or act during the pelagic phase. In the absence of juveniles that are genetically intermediate, we

would expect to find a signature of a Wahlund effect, a deficiency of heterozygotes due to admixture of two distinct species, in the juvenile sample similar to that found in the previous study of adults (Burford 2009). Alternatively, the existence of hybrid juveniles in regions where adults of both cryptic species co-occur would mean that there are no pre-zygotic barriers to hybridization. However, this would also indicate the existence of post-zygotic barriers acting on hybrids between the juvenile and adult stage, because the previous adult sample lacked F1 individuals (Burford 2009). In regions where adults of one of the two cryptic species predominate, we would predict little or no hybrids because of low encounter rates with the rarer species (i.e., an Allee effect). However, an increase in frequency of F1 or introgressed hybrids in these regions would suggest a breakdown of pre-zygotic barriers when the less common adult breeds with the more common one. Differences in the frequency of hybridization between regions where both species co-occur as adults and where one species predominates should provide evidence of the nature of reproductive isolation and reveal mechanisms that maintain the two species despite the large range of overlap.

With the exception of the review of speciation in sea urchins by Palumbi and Lessios (2005), there are few examples of temperate marine species with a high dispersal potential that are recently diverged and reproductively isolated despite a sympatric distribution. Moreover, there are few investigations of first-generation (F1) or back-crossed hybrids (introgression) in the offspring (new year-classes) of natural populations of these sympatrically distributed cryptic species. Our lack of knowledge about the frequency of hybrid juveniles in the wild limits our ability to identify the degree of reproductive isolation and whether pre-zygotic barriers or post-zygotic barriers caused the isolation. Therefore, an analysis of the genetic structure of year-class formation between these species provides the first opportunity to assess the degree reproductive isolation between the species and to confirm the existence of F1 hybrids. Our goal here was not to investigate geographic distribution differences in the frequency of the two cryptic species, but to first investigate the degree of reproductive isolation, compare any evidence of hybridization between regions that differ in the frequency of adult species, and then assess the nature of the isolation between the two recently diverged cryptic species of *S. mystinus*.

Materials and methods

Sampling

We sampled juveniles from locations within and outside areas of co-occurrence of the two cryptic species of *S. mystinus* (Type 1 and Type 2) along 680 km of coast between southern California (Santa Barbara) to northern California (Fort Bragg). The northernmost sample location (Fort Bragg) is at the southern end of the adult admixture zone that extends to central Oregon. The sampling area in this study covers the main core of the geographic range of *S. mystinus*. We designated regions, north, central, and southern to follow management regions for *S. mystinus* and because major upwelling zones and/or biogeographic boundaries divide these regions (Fig. 1; e.g., Point Reyes, Wing et al. 1995; Point Conception, Wares et al. 2001). In addition, a previous study using microsatellite loci found evidence of genetic differentiation between adults south of Point Conception with adults in the central region (Burford 2009). Within these regions, there was variation in co-occurrence of the two cryptic species in the adults (Burford 2009), with the highest in the northern region at Fort Bragg. We sampled on average 100 juveniles at each sampling location at the end of the settlement period in 2001 and 2002 (Table 1) and sampled more locations in the 2002 year-class. To collect a diverse sample of all size juvenile blue rockfish, while on scuba, we sampled juveniles using large underwater handheld 4.8-mm square mesh BINCKE nets (Anderson and Carr 1998; Ammann 2004). We froze whole individual juveniles from each sampling location for later processing, which included positive identification using diagnostics (e.g., coloration, dorsal fin ray counts, and gill raker counts; Miller and Lea 1972; Laroche and Richardson 1981), obtaining standard lengths, and then preserving whole individuals in 95% ethanol for subsequent genetic analyses. We compared species composition of juveniles to that of adults, as determined in previous studies (Burford and Bernardi 2008; Burford 2009) in each region of sampled juveniles (adult sample dates and locations, Appendix 1) (Fig. 1).

Size frequency differences

We tested for significant differences in standard length between the two species across the sampling regions using a general linear model (GLM) with standard length as the dependent variable and the main effects of type and location as the independent variables, and interaction effect of type-by-location in SYSTAT (v.10.2). We focused this analysis on juveniles collected in 2002, because we had more widespread sampling and more locations with both types than in the 2001 year-class, thus increasing our power to detect any differences. To identify which locations or types influenced the pattern, we conducted a *post-hoc* contrast using the Tukey HSD Multiple Comparisons in SYSTAT, which generated significance values after corrections for multiple comparisons. For all GLM and *t* tests, we tested the assumptions of homoscedasticity and normality.

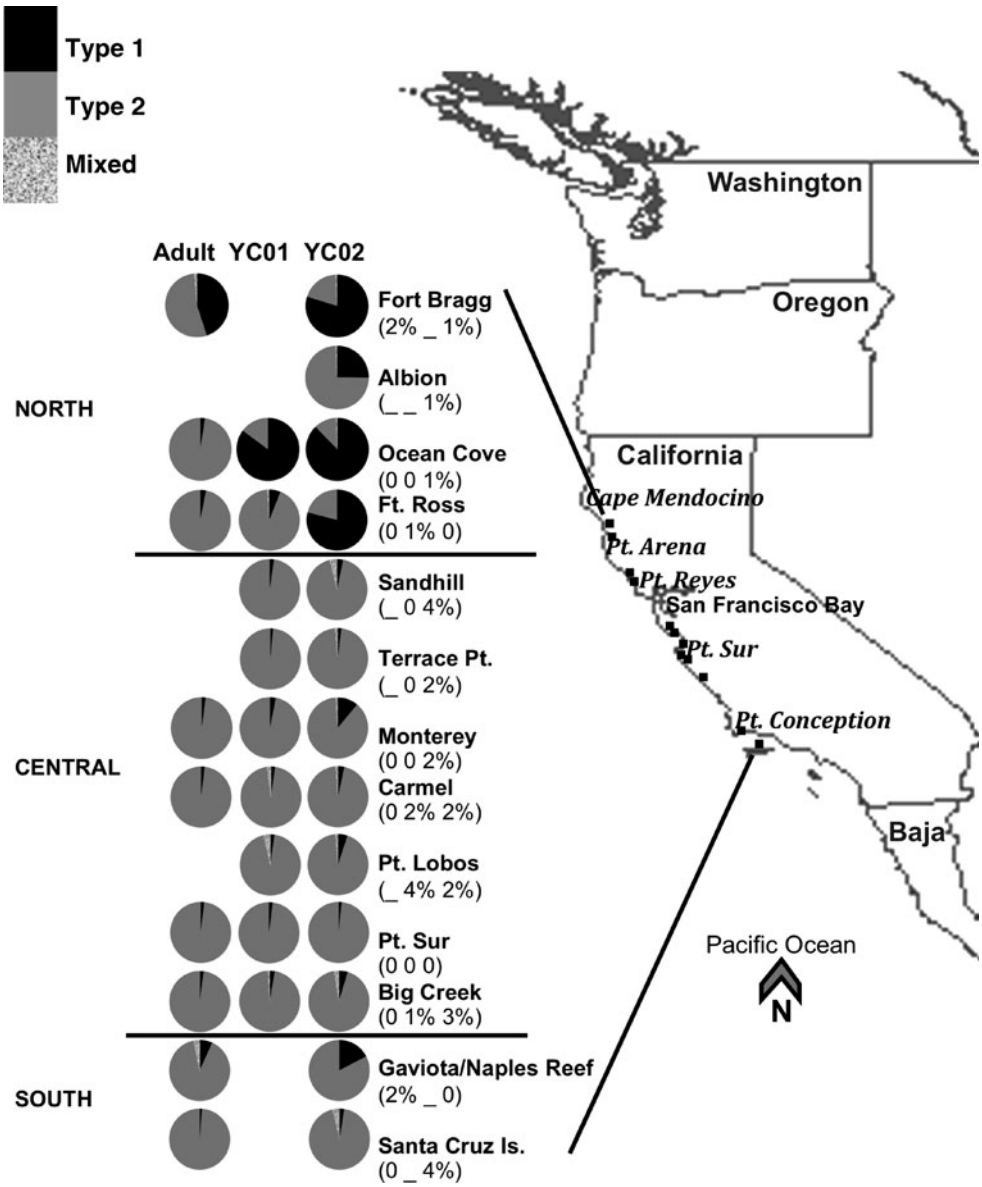

Fig. 1 Distribution of sample locations and genetic structure of adult and juvenile *Sebastes mystinus*. Geographic features are depicted in italics. *Pie charts* are the summary of the STRUCTURE assignment using microsatellite loci and from *left* to *right* represent individuals from the adult, and juveniles from the 2001 and 2002 year-classes (YC01 and YC02, respectively). The sampling range within California is divided into three regions, north, central, and south, which are depicted on the *left-hand* side of the figure. Below each sampling location is the percentage of mixed individuals for each sample with underscores indicating no sample

DNA extraction and amplification

For the microsatellite analyses, we extracted genomic DNA from the caudal fin of each specimen using a Qiagen DNA Extraction Kit (Valencia, CA) following the manufacturer's protocol. From the extracted genomic DNA, we amplified nine microsatellite loci designed from *S. rastrelliger* (Buonaccorsi et al. 2004; Westerman et al. 2005) using the polymerase chain reaction (PCR) and used six of these nine loci for the microsatellite analysis (*Sra.7-2, Sra.7-7, Sra.7-25, Sra.6-52, Sra.15-8, Sra.16-5,* GenBank Ass. No.: AF269054-57, 59, 61, respectively). We followed the protocol outlined in Burford and Larson (2007) for fragment amplification and scoring of the microsatellite loci. In total, we analyzed 760 individuals from 9 locations for the 2001 year-class and 1,478 individuals from 13 locations for the 2002 year-class (Fig. 1; Table 1).

Statistical analyses

Genetic differences between juvenile species

To identify the differences between the two genetic species (Type 1 and Type 2) and to compare juveniles to the corresponding adult samples, we analyzed the frequency distribution of alleles per locus, including differences in the distribution spread (frequency and range of alleles), differences in the dominant allele, and differences in the number of unique alleles among juvenile samples. We tested for differences in allele frequency distributions using a nonparametric two-sample Kolmogorov–Smirnov (KS)

Table 1 Sample data and summary statistics of *Sebastes mystinus* juveniles, including sample location, sample date, sample size (*N*), mean standard length (SL) in mm for Type 1 (T1) and Type 2 (T2), allele number (#), allelic richness (*A*), observed (H_O), and expected (H_E) heterozygosity

Year	Sample location	Collection date	*N*	SL T1 (mm)	SL T2 (mm)	Allele #	*A*	H_O	H_E	*HW*	*LE*
2001	Ocean Cv.	23-Aug	84	63.83	59.95	17.17	15.86	0.74	0.81	*	NS
	Ft. Ross	22-Aug	78		63.55	15.33	14.53	0.80	0.81	NS	NS
	Sandhill	10-Jul	72		52.47	15.50	14.84	0.79	0.80	NS	NS
	Terrace Pt.	10-Jul	89		54.87	16.50	15.07	0.76	0.78	NS	NS
	Monterey	16-Jul	61		59.40	14.00	13.98	0.75	0.81	NS	NS
	Carmel	16-Jul	93		55.10	16.17	14.68	0.75	0.80	NS	NS
	Pt. Lobos	11-Jul	109		51.24	17.83	15.43	0.80	0.80	NS	NS
	Pt. Sur	12-Jul	75		53.04	15.17	14.40	0.78	0.79	NS	NS
	Big Creek	11-Jul	99		54.11	16.17	14.44	0.77	0.79	NS	NS
2002	**Ft. Bragg**	1-Aug	116	54.30	53.89	18.50	11.86	0.77	0.82	*	NS
	Albion	1-Aug	133	53.97	51.44	20.17	12.52	0.78	0.83	*	NS
	Ocean Cv.	31-Jul	147	54.19	53.77	20.67	12.43	0.76	0.81	*	NS
	Ft. Ross	31-Jul	129	54.77	55.14	20.67	12.55	0.76	0.83	*	NS
	Sandhill	24-Jul	97		54.50	17.67	11.27	0.77	0.78	NS	NS
	Terrace Pt.	24-Jul	128		55.04	18.67	11.38	0.78	0.79	NS	NS
	Monterey	27-Jul	164		57.33	19.33	11.90	0.77	0.81	*	NS
	Carmel	27-Jul	132		59.89	18.67	11.50	0.79	0.80	NS	NS
	Pt. Lobos	25-Jul	126		53.73	18.17	11.56	0.76	0.79	*	NS
	Pt. Sur	26-Jul	98		55.32	17.33	11.06	0.76	0.78	NS	NS
	Big Creek	25-Jul	125		55.68	19.83	11.78	0.78	0.81	*	NS
	Santa Cruz Is.	13-Aug	26		70.47	10.67	10.50	0.78	0.80	NS	NS
	Naples Reef	28-Aug	57	71.16	74.00	16.67	12.47	0.80	0.83	NS	NS
2001/2002[a]	Type 1	NA	466	54.77		21.83	21.74	0.77	0.79	NS	NS
	Type 2	NA	1,752		57.48	28.17	23.17	0.77	0.79	*	NS

Bolded locations were collected within days of each other, and nonbolded locations were sampled at dates later in the season

P values for Hardy–Weinberg (*HW*) and Linkage Equilibrium (*LE*) violations (*NS* not significant, * <0.05; implemented in GENEPOP using 10,000 iterations). The standard length for each type at each sample location is provided for those locations that had several individuals of each. Allelic richness measures based on 60 and 24 diploid individuals for the 2001 and 2002 sample locations (respectively) and 439 diploid individuals for types. [a]The standard length for the two types is from the 2002 data

and for the degree of genetic divergence using *F*-statistics (Weir and Cockerham 1984) as implemented in FSTAT (Goudet 1995). General microsatellite analysis of *S. mystinus* collections included: (1) an estimate of genetic diversity (Nei 1987) and allelic richness (at equivalent sample sizes) using FSTAT version 2.9.3.2 (Goudet 1995), (2) an estimate of expected and observed heterozygosity (H_E and H_o) using ARLEQUIN 2.000, and (3) deviations from Hardy–Weinberg (HW) genotypic expectations and independence of microsatellite loci (linkage equilibrium) 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) based on 10,000 iterations. We 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). To test for significant differences in genetic diversity between species, we used a two-tailed paired *t* test (Sokal and Rohlf 1995).

Hybrid analysis

To test for admixture of individual juvenile samples and to identify any first-generation hybrids or introgressed individuals (mixed ancestry), we conducted a Bayesian assignment test in STRUCTURE (Pritchard et al. 2000). We used this analysis to fit the structure of the juvenile samples to an appropriate number of clusters or groups by using several grouping priors (*K*), an admixture model, and allowing correlation among allele frequencies (Falush et al. 2003). We ran all juveniles from both year-classes and all adults together and generated posterior probabilities in STRUCTURE using 100,000 iterations of the Markov

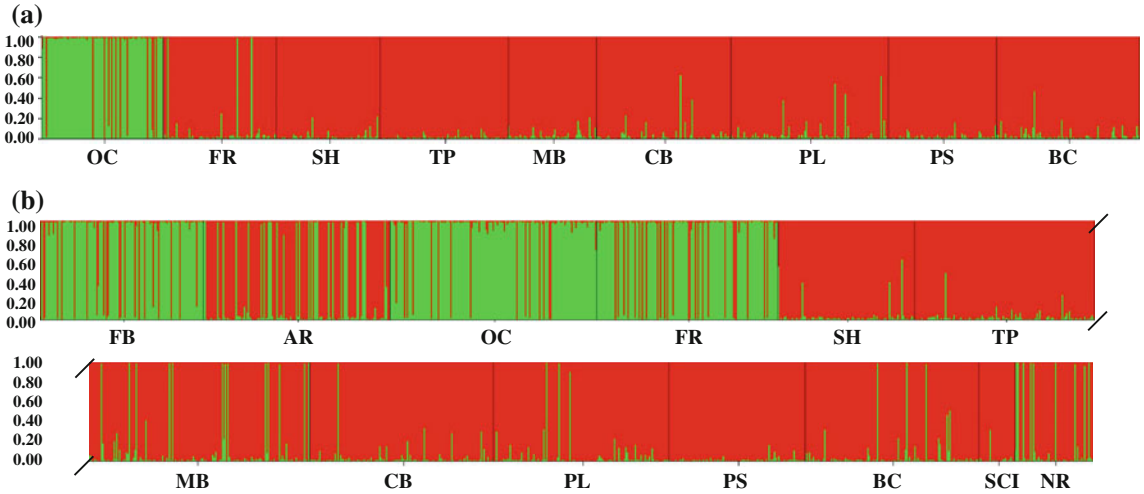

Fig. 2 Results of the assignment model of *Sebastes mystinus* juveniles in the **a** 2001 year-class and **b** the 2002 year-class using STRUCTURE. Sample locations are depicted on the *x* axis and posterior probabilities are depicted on the *y* axis. The sample locations are Fort Bragg (*FB*), Albion (*AR*), Ocean Cove (*OC*), Fort Ross (*FR*), Sandhill (*SH*), Terrace Point (*TP*), Monterey (*MB*), Carmel (*CB*), Point Lobos (*PL*), Point Sur (*PS*), Big Creek (*BC*), Naples Reef (*NR*), and Santa Cruz Island (*SCI*)

chain Monte Carlo (MCMC) method after an initial burn-in period of 40,000. We verified *K* and the stability of other model parameters by running the model with 5 replicates and *K*-values ranging from 1 to 5 and analyzing the log-likelihood, the data estimate [Ln(Pr(X/K))], and variance structure of the estimated log (natural) probability for different *K*s (Appendix 2). We confirmed *K* by using a *post-hoc* analysis of ΔK following Evanno et al. (2005). We calculated final posterior probabilities using all five runs. To confirm posterior probabilities generated by the STRUCTURE analysis, and to calculate posterior probabilities of F1 and F2 hybrids, and backcrossed individuals, we used the program NewHybrids (Anderson and Thompson 2002). We ran all juveniles from both year-classes and generated posterior probabilities using 100,000 iterations of the MCMC after an initial burn-in period of 40,000. In contrast to previous analyses (Burford and Bernardi 2008; Burford 2009), we considered individuals with a posterior probability assignment of 0.75 or greater as "pure" types. Those individuals that were admixtures of both species (introgressive hybridization) had posterior probabilities between 0.55 and 0.75, whereas posterior probabilities of F1 hybrids ranged between 0.45 and 0.55.

Results

Size frequency differences

At five sample locations with substantial frequencies of both species (Fort Bragg, Albion, Fort Ross, Ocean Cove, and Naples Reef in 2002), the mean standard length of Type 1 individuals was greater than Type 2 individuals at Fort Bragg, Albion, and Ocean Cove, while the opposite was true at Naples Reef (Table 1). The differences in standard length suggest larger juveniles within a sampling location had either a greater growth rate or an earlier birth date than the smaller individuals. We found a significant type-by-location effect in the GLM for the analysis restricted to these locations (Table 2a). The *post-hoc* pairwise comparisons of the interaction between type and location indicated that Type 1 juveniles were significantly larger than Type 2 juveniles at Albion River (Type 1 = 53.97 mm, Type 2 = 51.44 mm; $P < 0.05$). While

Table 2 Results of the general linear model (GLM), with the dependent variable of length, and categorical values of location, type, and the interaction type-by-location of 2002 year-class of juvenile *S. mystinus* at (a) all locations with two juvenile genetic types, and (b) all locations with two genetic types sampled in July (note Naples Reef was removed from this analysis, see text)

Source	Sum-of-the-squares	*df*	Mean square	F-ratio	*P*
(a)					
Type	0.044	1	0.044	0.003	0.957
Location	9,889.681	4	2,472.420	165.950	**0.0001**
Type × location	199.43	4	49.861	3.347	**0.010**
Error	8,492.173	570	14.899		
(b)					
Type	39.698	1	39.698	3.093	0.079
Location	235.618	3	78.539	6.119	**0.0001**
Type × location	104.231	3	37.744	2.707	**0.045**
Error	6,610.412	515	12.836		

Bolded *P* values are significant (alpha = 0.05)

the Type 2 juveniles were larger than Type 1 juveniles at Naples Reef, this was not significant in the *post-hoc* pairwise comparison (Type 1 = 71.16 mm, Type 2 = 74.00 mm, $P > 0.05$). The juveniles collected at Naples Reef were collected at a later date, but we still found significant location and type-by-location effects when we removed this location from a subsequent analysis (Table 2b). We did not find significant pairwise comparisons of standard length between the two types at Fort Bragg and Ocean Cove.

Genetic differences between juvenile species

There was significant genetic divergence between the species (both year-classes combined $F_{ST} = 0.119$; $P < 0.05$), which was consistent in both year-classes ($F_{ST} = 0.12$ in 2001, $F_{ST} = 0.12$ in 2002). When we divided all juveniles into two distinct types, there was a difference in the most common allele between the Type 1 and the Type 2 individuals at 5 of 6 loci. In addition, there were 14 and 52 unique alleles for Type 1 and Type 2 juveniles, respectively. However, the large difference in sample size, with a sample size of 453 for Type 1 and 1,724 for Type 2 may have contributed to the discrepancy in the number of unique alleles. In general, we found the same common alleles and number of unique alleles at each locus within a species between the 2 years, with the exception of a change in the most common allele at *Sra.16-5* for the Type 1 species. The results from the two-sample KS test showed significant differences in the distribution of alleles between the two species ($P < 0.05$, both year-classes) at 2 of 6 loci (*Sra.7-2* $P < 0.001$, *Sra.7-25* $P < 0.01$).

The analysis of genotype frequencies (all loci combined) supported a Wahlund effect for the juveniles. We found significant violation of HW expectations due to lower than expected heterozygote frequencies when we analyzed locations in both the 2001 and 2002 year-classes. However, genotype frequencies were not significantly different from HW expectations when we analyzed separate types per location. Specifically, there were significant violations of HW expectations at Ocean Cove in the 2001 year-class and at 8 sample locations in the 2002 year-class ($P < 0.05$; Table 1). The groups of individual types at these locations did not violate HW with the exception of Type 1 at Ocean Cove in 2002, which remained out of HW equilibrium. In addition, there was no significant linkage disequilibrium between loci at individual sample locations for either year-class once we corrected for multiple comparisons (Table 1). Despite these overall trends in the allele and genotype frequency data from both year-classes, there were no significant differences in genetic diversity between the two types using measures of both genetic diversity and allelic richness (Table 1).

Hybrid analysis

Running both year-classes and adults in a STRUCTURE model, we found support for two groups (K) based on the value of ΔK (Fig. 1; Table 3; Appendix 3). For this analysis, only 9% of the 2,238 individual juveniles run in this analysis had missing microsatellite data. We found a low frequency of individuals with posterior probabilities that indicated mixed assignment in many of the samples, with a slightly higher frequency in the 2002 year-class and in the central and southern regions (Figs. 1, 2; Table 3). The percentage of individuals of mixed ancestry ranged from 0 to 4% across sampling locations and year (Table 3). Only 1 individual of all the mixed-ancestry individuals had missing data (16% missing data, 1 juvenile from 2002 year-class at Ocean Cove); the remaining mixed-ancestry individuals had no missing data. While we found a higher frequency of Type 1 juveniles in the northern region, we did not find a higher frequency of mixed-ancestry juveniles at locations where there were approximately equal numbers of both species in the adult sample (Fort Bragg). At sample locations where one type was predominate, we found a different frequency of mixed-ancestry juveniles depending on whether the less represented type at that location had a frequency of at least 12% or less than 11% (Fig. 2; Table 3). Specifically, there was a lack of mixed individuals at locations with a frequency of 12% or more of the under-represented juvenile or adult Type (Type 2 at Ocean Cove in 2001, Type 2 at Fort Bragg and Fort Ross, and Type 1 at Albion and Naples Reef in 2002), with few exceptions (1 juvenile at Fort Bragg and at Albion). At locations with a frequency of 11% or less of the under-represented Type, we found a higher frequency of mixed-ancestry individuals. For example, we found the highest frequency of mixed-ancestry individuals at Carmel and Point Lobos (2.2 and 3.7%, respectively) in the 2001 year-class, and Sandhill, Big Creek, and Santa Cruz Island (4.1, 2.6, and 3.8%, respectively) in the 2002 year-class. Of these mixed-ancestry individuals, only eight were F1 hybrids and the remaining 19 individuals were backcrossed or introgressed individuals. Based on the STRUCTURE analysis of these 19 individuals, only four (one individual from Carmel and Point Lobos in 2001 and Ocean Cove and Sandhill Bluff in 2002) were backcrossed or introgressed into the Type 1 lineage, indicated by a higher frequency of the Type 1 in the assignment test, and the remaining 15 were backcrossed into the Type 2 lineage (Fig. 2; Table 4).

The results of individual juvenile posterior probabilities in the NewHybrids compared with the STRUCTURE analysis were similar. Specifically, we found 99% agreement in the individual juvenile posterior probabilities of assignment to one of the two types. There were some exceptions, including individual juveniles that classified as

Table 3 Results of the STRUCTURE admixture assignment of *Sebastes mystinus* juveniles and adults using microsatellite loci and a *K* of 2

Location	*N*	Frequency			Number
		Type 1	Type 2	Mixed	Mixed individuals (F1)
2001 Year-class					
Ocean Cove	84	0.852	0.148		
Ft. Ross	78	0.060	0.927	0.013	1
Sandhill	72	0.023	0.977		
Terrace Pt.	89	0.015	0.985		
Monterey	61	0.031	0.969		
Carmel	93	0.022	0.956	0.022	2
Pt. Lobos	109	0.022	0.941	0.037	4 (2)
Pt. Sur	75	0.020	0.980		
Big Creek	99	0.027	0.963	0.010	1 (1)
2002 Year-class					
Ft. Bragg	116	0.798	0.193	0.009	1
Albion	133	0.252	0.740	0.008	1
Ocean Cove	147	0.881	0.116	0.007	1
Ft. Ross	129	0.791	0.209		
Sandhill	97	0.034	0.966	0.041	4 (1)
Terrace Pt.	128	0.020	0.964	0.016	2 (1)
Monterey	164	0.111	0.877	0.012	2 (1)
Carmel	132	0.034	0.951	0.015	2
Pt. Lobos	126	0.051	0.933	0.016	2
Pt. Sur	98	0.016	0.984		
Big Creek	125	0.047	0.927	0.026	3 (2)
Naples Reef	57	0.174	0.826		
Santa Cruz Is.	26	0.025	0.937	0.038	1
Total/Avg	2,238	0.196	0.794	0.019	27 (8)
Adults[a]					
Ft. Bragg	66	0.450	0.535	0.015	1
Ocean Cove	41	0.024	0.976		
Ft. Ross	60	0.031	0.969		
Monterey	47	0.021	0.979		
Carmel	86	0.019	0.981		
Pt. Sur	52	0.018	0.982		
Big Creek	56	0.020	0.980		
Gaviota	59	0.068	0.898	0.034	2
Santa Cruz Is.	15	0.013	0.987		

The table includes the sample size (*N*) and proportional membership to each group (Type 1 or Type 2) from the 5 model runs, frequency of mixed-ancestry individuals, and the number of mixed individuals and of these, the number of F1 hybrids in parenthesis

[a] Adult data are from Burford (2009)

mixed in STRUCTURE (either mixed or F1), but had pure Type 2 posterior probabilities of 0.75 or greater in the NewHybrids analysis (5 individuals, Table 4), suggesting a slight over estimate of introgression in STRUCTURE. The remaining individuals that were mixed in the STRUCTURE analysis had highest posterior probabilities in pure Type 2, F2, or backcrossed to Type 2 categories in the NewHybrids analysis (Table 4). In contrast, a few individuals that were either Type 1 or Type 2 in the STRUCTURE analysis had higher posterior probabilities in one of the mixed categories in the NewHybrids analysis (7 individuals, Table 4). Of these individuals, three of the 7 had 0.75 or greater posterior probability in the combined mixed category in the NewHybrids analysis, suggesting that these three individuals may actually be of mixed ancestry. Finally, in the NewHybrids analysis, we did not find any individuals with high posterior probabilities in the mixed F1 category. The individuals that we classified as F1 in the STRUCTURE analysis were mostly classified as F2 or backcrossed to Type 2 in the NewHybrids analysis. Overall, both analyses showed a low level of introgression, with few F1 or F2 hybrids, and there was agreement in classification of individuals to one of the two "pure" types.

Table 4 Comparison of posterior probabilities of individual *Sebastes mystinus* juveniles with differences in posterior probabilities between assignment in STRUCTURE and NewHybrids

Year	Location	STRUCTURE posterior probabilities			NewHybrids posterior probabilities							
		Probability		Class	Pure		Mixed				Combined	Class
		Type 1	Type 2		Type 1	Type 2	F1	F2	Bck T1	Bck T2		
2001	Ocean Cv.	**0.980**	0.020	Type 1	0.226	0.002	0.001	**0.443**	0.315	0.014	**0.773**	**Mixed**
	Ft. Ross	0.250	**0.750**	**Mixed**	0.001	**0.740**	0.000	0.165	0.007	0.087	0.259	
	Carmel	**0.630**	0.370	**Mixed**	0.318	0.069	0.000	**0.467**	0.108	0.038	0.613	
	Carmel	0.385	**0.615**	**Mixed**	0.000	**0.575**	0.000	0.326	0.001	0.097	0.424	
	Pt. Lobos	0.374	**0.626**	**Mixed**	0.000	**0.517**	0.000	0.330	0.005	0.147	0.483	
	Pt. Lobos	0.542	0.458	**F1**	0.001	0.330	0.000	**0.540**	0.004	0.125	0.670	
	Pt. Lobos	0.446	0.554	**F1**	0.000	**0.901**	0.000	0.073	0.002	0.024	0.098	**Type2**
	Pt. Lobos	**0.617**	0.383	**Mixed**	0.000	0.189	0.016	**0.418**	0.162	0.216	**0.811**	**Mixed**
	Big Creek	0.466	0.534	**F1**	0.000	0.281	0.000	**0.558**	0.010	0.152	0.719	
2002	Ft. Bragg	0.350	**0.650**	**Mixed**	0.001	**0.533**	0.002	0.255	0.030	0.178	0.465	
	Ft. Bragg	0.126	**0.874**	Type 2	0.000	0.298	0.000	0.156	0.000	**0.545**	0.702	
	Albion	0.123	**0.877**	Type 2	0.000	0.062	0.013	0.292	0.009	**0.625**	**0.938**	**Mixed**
	Albion	0.348	**0.652**	**Mixed**	0.000	**0.728**	0.003	0.148	0.039	0.082	0.272	
	Ocean Cv.	**0.889**	0.111	Type 1	0.273	0.011	0.006	0.329	**0.352**	0.030	0.717	
	Ocean Cv.	**0.874**	0.126	Type 1	0.342	0.005	0.002	**0.344**	0.297	0.012	0.654	
	Ocean Cv.	**0.714**	0.286	**Mixed**	0.343	0.168	0.000	**0.397**	0.036	0.056	0.489	
	Ft. Ross	**0.885**	0.115	Type 1	0.034	0.017	0.013	**0.496**	0.351	0.088	**0.949**	**Mixed**
	Sandhill	0.536	0.464	**F1**	0.003	0.247	0.021	**0.354**	0.132	0.243	**0.750**	**Mixed**
	Sandhill	0.373	**0.627**	**Mixed**	0.000	**0.701**	0.005	0.142	0.037	0.114	0.299	
	Sandhill	0.371	**0.629**	**Mixed**	0.000	**0.755**	0.000	0.161	0.002	0.082	0.245	**Type2**
	Sandhill	**0.612**	0.388	**Mixed**	0.152	0.008	0.008	**0.482**	0.176	0.174	**0.839**	**Mixed**
	Terrace Pt.	0.479	0.521	**F1**	0.098	0.310	0.008	**0.316**	0.101	0.167	0.592	
	Terrace Pt.	0.256	**0.744**	**Mixed**	0.000	0.043	0.032	0.358	0.068	**0.498**	**0.957**	**Mixed**
	Monterey	0.414	0.586	**F1**	0.002	**0.379**	0.015	0.224	0.036	0.344	0.619	
	Monterey	0.287	**0.713**	**Mixed**	0.000	**0.812**	0.001	0.080	0.007	0.100	0.188	**Type2**
	Carmel	0.335	**0.665**	**Mixed**	0.000	**0.738**	0.001	0.120	0.012	0.129	0.262	
	Carmel	0.308	**0.692**	**Mixed**	0.000	**0.769**	0.002	0.088	0.007	0.133	0.231	**Type2**
	Pt. Lobos	0.306	**0.694**	**Mixed**	0.000	**0.632**	0.002	0.183	0.015	0.167	0.368	
	Pt. Lobos	0.323	**0.677**	**Mixed**	0.000	**0.568**	0.007	0.162	0.022	0.240	0.432	
	Pt. Lobos	0.141	**0.859**	Type 2	0.000	0.379	0.000	0.176	0.000	**0.444**	0.621	
	Big Creek	0.321	0.679	**Mixed**	0.007	**0.785**	0.000	0.152	0.001	0.055	0.208	**Type2**
	Big Creek	0.456	0.544	**F1**	0.000	0.038	0.007	**0.566**	0.129	0.261	**0.962**	**Mixed**
	Big Creek	0.513	0.487	**F1**	0.000	**0.400**	0.008	0.307	0.056	0.228	0.600	
	Santa Cruz Is.	0.320	0.68	**Mixed**	0.000	**0.441**	0.008	0.188	0.018	0.345	0.559	

The table includes juvenile year-class, location of the individual, posterior probabilities, and classification (see text) generated by STRUCTURE, and posterior probabilities generated by NewHybrids analysis including pure Type 1, pure Type 2, and mixed F1, F2, backcross to Type 1 and backcross to Type 2. We also included the combined mixed posterior probability; the individual was not a pure type and classifications based on the greater than 0.75 posterior probability. Bolded numbers are the highest probability per analysis

Discussion

This study revealed little evidence of first-generation (F1) hybrids or genetic mixing (introgression) between the Type 1 and Type 2 species of *S. mystinus* in regions where both types co-occur in roughly equal numbers in either the adult or juvenile samples (northern California). The genetic analysis revealed a Wahlund effect (admixture signature) at the locations sampled in this region, indicating the co-occurrence of juveniles as separate types, which was also supported by the high posterior probabilities in individual assignments. With the exception of inviability of hybrids

during the pelagic phase, these data confirmed reproductive isolation between the two species within the first year of life after settlement, which could not be ruled out in the previous study of adults (Burford 2009), as F1 hybrids may not survive from settlement to reproductive age. With the proximity to the admixture region (450-km region, Fort Bragg to Central Oregon) and current trajectories (see Sotka et al. 2004), we believe it is more likely that settling juveniles in the Fort Bragg region were either regionally produced or produced in the extended admixture region to the north and not from the northern Oregon or Washington where Type 1 adults predominate or from central/southern California where Type 2 adults predominate. Therefore, these juveniles would represent reproduction from areas that had both adult species. Given that these two species recently diverged (approximately 500 Kya ± 200 K; Burford 2009), the degree of reproductive isolation, and partially sympatric distribution of both the adults and juveniles, it appears that speciation in *S. mystinus* was similar to the sympatric distribution and the rapid speciation found in *Echinometra* reported by Palumbi and Lessios (2005). The lack of evidence of hybrids in this region combined with the Wahlund effect suggests that reproductive barriers prevent the formation of hybrids (i.e., pre-zygotic barriers) and not selection against or reduced viability of hybrids (i.e., post-zygotic barriers), similar to the reproductive isolation reported between the sympatric species found in the genus *Hexagrammos* (Crow et al. 2010). We also found evidence of breakdown of these reproductive barriers in regions with a low frequency of the Type 1 species in either the adults or settling juveniles, suggesting the reproductive isolation in this group is complicated and not complete.

In contrast to the Fort Bragg region with high frequencies of both species and where we would expect greater hybridization due to increased probability of encounters between the two species, there was evidence of low-level introgression at locations with low numbers of Type 1 individuals in the central/southern region. At these locations, most individuals with mixed assignment had a higher percentage of Type 2 alleles than Type 1 in both the STRUCTURE and NewHybrids analyses. This indicated that introgression was mostly due to the rarer Type 1 mixing with the more common Type 2 adults, and producing F1 hybrids that likely backcrossed to the higher frequency Type 2 in the regional adults (i.e., breakdown of pre-zygotic barriers). This result contrasts with previous studies on hybrid zones in this region, including a phylogeographic study on the acorn barnacle, which found a steep hybrid cline between two genetically differentiated populations in northern California (Sotka et al. 2004) and blue mussel, which found greater hybridization in a region where both coexist (Rawson et al. 1999).

Pre-zygotic barriers may include mechanical barriers (e.g., mismatch of reproductive structures), physical segregation (e.g., depth, habitat associations), or assortative mating (e.g., reproductive timing, courtship rituals). One or a combination of pre-zygotic barriers may maintain the genetic species in regions of overlap and may break down in regions where one of the two types is at low frequency. A pre-zygotic barrier of depth segregation supported the maintenance of cryptic species of the vermilion rockfish (*S. miniatus*; Hyde et al. 2008). In contrast, in this study there was no apparent habitat or depth difference in settled juveniles *of S. mystinus* sampled or between adult types within a location in the study of adults (Burford 2009). However, there may be microgeographic partitioning within a reef (e.g., segregation to different parts of the kelp bed). To confirm, this requires careful sampling within a reef and observation of associated habitat type and depth with collected individuals.

Reinforcement during secondary contact or differences in local adaptation when the groups were separated may contribute to differences in reproductive timing between the two species. Evidence of potential differences in reproductive timing included the significant differences in length frequency in the northern juvenile settlement region. Here, Type 1 individuals were larger than Type 2, while at Naples Reef, the Type 2 individuals were significantly larger than Type 1. Because there was a switch in length and type between the two locations, this pattern suggests an adaptive difference in reproductive timing or growth rather than a fixed difference between the two species. If there were differences in growth rate or reproductive timing difference between the two types, it would suggest that these traits are ecologically linked such that Type 2 individuals do better in the central and southern regions than in the north. Unfortunately, the differences in length frequency between the two types were not found at all sample locations, and with only one site in the southern region with Type 1 juveniles, it is difficult to determine whether differences in standard length can be attributed to adaptive differences between the species or whether it was idiosyncratic. To distinguish differences in growth rate versus birth dates requires an analysis of the birth-date distribution and growth rate using juvenile otolith microstructure or controlled aquarium studies. While there may be either one or multiple pre-zygotic barriers maintaining the two cryptic species where both types co-occur, pinpointing the exact barrier or barriers will require detailed sampling combined with ecological and depth data and observations of mating behavior. For example, assortative mating may be due to chemical or sound cues, or mating behavior differences, which may break down when encounter rates are low. Given that adult *S. mystinus* form aggregations in the

rocky reef habitats, it is also possible that in regions where both species are abundant, they form single-species aggregations, while in regions where one species predominates the rarer species forms aggregations with the more abundant species. This would increase reproductive encounter rates between the two cryptic species. Based on this study, the barriers are most likely pre-zygotic, but the potential exists that it may be due to post-zygotic barriers, such as selection against hybrids during the pelagic phase.

Analyzing the *S. mystinus* year-classes for signs of admixture or introgression between the species facilitated a greater understanding of the genetic distinctiveness of the two species. First, it confirmed little or no introgression between the two species as observed with the adults (Burford 2009) and suggest that isolation is maintained by pre-zygotic barriers. Second, it provided evidence of possible low level or rare introgressive hybridization in regions where one type was at much lower frequencies than the other (e.g., either forced to mate or increased encounter rates), which suggests that there were not intrinsic barriers to mating between the two species. Finally, it provided evidence of regional differences in the behavior of the species. For example, there were differences in size modes of the Type 1 lineage in the north versus the south, and there was a higher frequency of admixed individuals in the central/southern region for both year-classes than that of the adults. Therefore, it appears that the two species potentially interbreed, but remain isolated in most cases, which, barring selection during the pelagic phase, provides the first clear evidence that ecological or behavioral mechanisms maintain the two cryptic species of *S. mystinus*. Understanding how oceanographic processes affect hybridization or the lack of it in the different regions is an important next step in explaining the reproductive barriers in this group. Using an age-structured analysis of these cryptic species of marine fishes, we confirmed that these two species do not readily produce hybrids in regions where both adult species coexist and that instead, they showed a high degree of reproductive isolation between the two species. We found evidence of pre-zygotic barriers in this group that may break down when one of the two species is at a low frequency. Therefore, studies that include the progeny of an adult population can provide more critical information on the complicated dynamics of speciation and secondary contact in groups that are sympatrically distributed than those studies of adults alone.

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Appendix 1

See Table 5.

Table 5 Adult *Sebastes mystinus* sample locations summarized from Burford (2009)

Location	Collection #	*N*	Date	Year	Collection method	Within-site divergence *P*
Ft. Bragg	3	69	Aug/Sep	2006	Port Samples	>0.05
Ocean Cove	2	41	Sep/Oct	2002	CENCAL/Spear	>0.05
Ft. Ross	3	71	Sep/Oct	2002	Hook&Line	>0.05
Monterey	4	52	May/Aug	2003/04	Spear/Hook&Line	>0.05
Carmel	3	93	Feb/Jul	2002	CENCAL/Hook&Line	>0.05
Pt. Sur	2	55	Feb	2002/03	Hook&Line	>0.05
Big Creek	2	78	Jul/Aug	2002/03	Hook&Line	>0.05
Gaviota	1	60	Oct	2004	Hook&Line	>0.05
Santa Cruz Is.	1	15	Sep	2002	Hook&Line	>0.05

We use these locations in California for comparison to the juvenile samples. This table includes the sample location, number of collections at that location, N, collection month, year, and method, and whether there is any significant genetic divergence between collections within a sampling location

Appendix 2

See Table 6.

Table 6 Results of parameter estimates in the STRUCTURE assignment model using groupings (K) of 1–5, average log-likelihood, variance, data estimates for K, and estimates of ΔK

Grouping priors	Average	Variance	K data estimate	ΔK
K1	−8,4045.10	0.01	0.000	
K2	−7,7442.66	11.13	0.000	1,904.62
K3	−7,7195.20	457.73	0.602	13.48
K4	−7,7236.08	1,729.31	0.400	6.34
K5	−7,7540.46	9,091.03	0.000	

These numbers were generated using 5 runs of the admixture model in STRUCTURE

Appendix 3

See Table 7.

Table 7 Standard error of the combined 5 runs of STRUCTURE analysis of juvenile *Sebastes mystinus*

Location	N	SE type1	SE type2
2001 Year-class			
Ocean Cove	84	5.27E-09	5.27E-09
Ft. Ross	78	0.000	7.45E-09
Sandhill	72	2.24E-04	2.24E-04
Terrace Pt.	89	0.000	0.000
Monterey	61	0.000	0.000
Carmel	93	0.000	0.000
Pt. Lobos	109	0.000	0.000
Pt. Sur	75	0.000	0.000
Big Creek	99	0.000	0.000
2002 Year-class			
Ft. Bragg	116	1.32E-09	1.32E-09
Albion	133	0.000	0.000
Ocean Cove	147	2.24E-04	2.24E-04
Ft. Ross	129	2.24E-04	2.24E-04
Sandhill	97	0.000	0.000
Terrace Pt.	128	1.65E-10	1.65E-10
Monterey	164	2.74E-04	2.74E-04
Carmel	132	0.000	0.000
Pt. Lobos	126	2.74E-04	2.74E-04
Pt. Sur	98	2.24E-04	2.24E-04
Big Creek	125	2.24E-04	2.24E-04
Santa Cruz Is.	26	1.65E-10	1.65E-10
Naples Reef	57	1.32E-09	1.32E-09
Adults[a]			
Ft. Bragg	66	2.24E-04	2.24E-04
Ocean Cove	41	1.65E-10	1.65E-10
Ft. Ross	60	2.24E-04	2.24E-04
Monterey	47	2.24E-04	2.24E-04
Carmel	86	2.74E-04	2.74E-04
Pt. Sur	52	0.000	0.000
Big Creek	56	0.000	0.000
Avila	44	2.74E-04	2.74E-04
Gaviota	59	2.74E-04	2.74E-04
Santa Rosa Is.	53	2.74E-04	2.74E-04
Santa Cruz Is.	15	0.000	0.000

[a] Adult data are from Burford (2009)

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