Application of molecular genetic methods to rockfish predation and habitat association research efforts in Central California

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Abstract

The Sanctuary Integrated Monitoring Network (SIMoN) supported efforts to use previously developed genetic methods to enhance ongoing research projects in the Monterey Bay National Marine Sanctuary by identifying unknown rockfish (*Sebastes* spp.) samples to the species level. The approach compares the genotype of an unknown individual at six nuclear microsatellite loci to a reference data set of genotypes from 33 *Sebastes* species commonly found in Central California. This method was applied to both newly-settled rockfish juveniles as part of a habitat association study and to rockfish remains recovered from the stomachs of jumbo squid off the Central California coast. In the California Current, jumbo squid (*Dosidicus gigas*) have been shown to feed on juvenile and adult groundfish, including rockfish *(Sebastes* spp.), Pacific hake, and small flatfish. However, many of the rockfish prey items cannot be identified to the species level, as squid often do not consume the heads, and consequently the otoliths, of larger prey. As the continued presence of squid has the potential to effect substantial change on California Current food webs, identification of those species most vulnerable to predation will improve the estimation of the impacts of this predator on the ecosystem. Although degradation prevented identification of all recovered samples, the genetic data provided increased taxonomic resolution of rockfish prey in jumbo squid diets, thus enhancing the information content of ongoing food habits and ecosystem modeling efforts to better understand the consequences of the ongoing presence of these predators.

Introduction

Widespread concerns by both commercial and recreational fishermen over the potential impacts of jumbo squid on fisheries resources in California prompted the initiation of food habits studies by workers at the Fisheries Ecology Division of the Southwest Fisheries Science Center in Santa Cruz. Results demonstrate that *D. gigas* commonly feeds on adult groundfish; *Sebastes* species, Pacific hake, and several species of small flatfish account for ~25% of individual prey items, and a greater proportion of their diet by volume (Field et al. 2007). Although identification of most prey items using hard parts (otoliths, beaks, scales) is relatively straightforward, squid often do not consume the heads of larger prey (Dawe et al. 1997). Consequently, many of the larger individual rockfish (*Sebastes* spp.) cannot be identified to the species level. The application of molecular genetic approaches to complement traditional food habits studies provides a means to better understand trophic interactions of jumbo squid in the California Current ecosystem.

Genotypic assignment tests, typically used to identify the population-of-origin in within-species studies (Pearse & Crandall 2004), can be applied to between-species identification when suitable markers can be identified. Pearse et al. (2007) evaluated more than 50 microsatellite loci for cross-species amplification and variation, and chose six loci to create a reference data set for 33 *Sebastes* species commonly found off of Central California. Using these data, unknown samples of *Sebastes* spp. can be assigned to the species from which their genotype most likely originated based on the allele frequencies in the reference data set. The method has been demonstrated to accurately assign individuals to the species level, and provides a simple and cost effective approach to identify early life history stages, archived, or forensic samples of *Sebastes* spp. (Pearse et al. 2007).

Materials and Methods

Samples

Unknown *Sebastes* samples were obtained from recently settled juvenile rockfish (K. Stierhoff) and from recovered vertebrae and skin tissue collected from jumbo squid stomach samples (J. Field). Juvenile rockfish were collected in trapping, trawling, and SCUBA surveys in the Monterey Bay. Squid were collected off of Cordell Bank (CB), Half Moon Bay (HMB), Monterey Bay (MB), and Nine Mile Bank off of San Diego (SD). A total of 46 putative *Sebastes* samples recovered from squid guts were analyzed. This included 8 cases in which some apparent rockfish tissue was recovered from the stomach along with a *Sebastes* spp. vertebrae. In addition, there were also four pairs of samples, each taken from a single squid, but suspected to be from two different rockfish individuals. For each of these, separate DNA extractions were completed for the bone and/or tissue samples, and these DNA extracts were analyzed separately. Two samples of pure water were also included in the DNA extraction process and used as negative controls for all subsequent analyses.

Laboratory Analysis

DNA was extracted from bone or tissue, and used for polymerase chain reaction (PCR) amplification of the six microsatellite loci. To extract the DNA from recovered vertebrae, each bone was wrapped in aluminum foil and briefly immersed in liquid nitrogen. The frozen sample was immediately crushed, and the resulting powder placed in a 1.5mL Eppendorf tube. All samples were then processed as normal for DNA extraction using Qiagen DNeasy 96 tissue extraction kits (Qiagen, Inc.).

Polymerase chain reaction (PCR) amplification of the suite of six microsatellite loci was attempted on each DNA sample. Because DNA extracted from samples recovered from squid stomachs was expected to be degraded, three separate PCR trials were done on each of these samples. In two of the trials, the PCR was conducted directly on the DNA extract, undiluted in one trial, and diluted 1:10 with diH_2O in the second trial. In the third trial, PCR products from the second trial were diluted and used as template for an additional round of PCR. These three trials provided both replication and complementary approaches to obtain accurate genotypes from the partially degraded DNA. Genotype scores from the three attempts were then compared for consistency before being used for assignment (see below).

Genetic Data

To ensure that accurate genetic results were obtained, a two-stage scoring process was used for the genetic data from all degraded DNA samples. First, the genotype from each PCR trial for each individual for each locus was determined independently by two people, and discrepancies between the two scores were resolved or deleted. This created three separate sets of genotype data, each based on one of the three attempted PCR amplifications. Second, these finalized genotypes from the three PCR trials were compared for each individual and each locus to identify consistent results. In this stage, each individual's genotype for each locus was categorized as either "OK", indicating that at least two of the three PCR trials produced an identical, usable, genotype, and that the third trial produced no genotype; "Single", indicating that one of the three PCR trials produced a usable genotype while the other two attempts produced no final genotype; "All Failed", indicating no usable genotypes for that locus for that individual; or finally "Discrepancy", indicating that at least two of the PCR trials produced a finalized genotyped, but that the allele calls were not identical. Genotype data for individual "Discrepancy" scores were then re-checked and resolved into "OK" when the true consensus score was clear, or single when one of the genotypes was determined to be of low quality. Finally, two composite data sets were created based on the above genotype categories. The first data set, designed to retain as much data as possible, is refered to as *all*, and includes all genotypes in both the "OK" and "Single" catagories. The second data set, limited to only high quality, replicated genotype data, included only genotypes from "OK" category, and is referred to as *replicated*.

Species Assignment

Genetic assignment of all putative *Sebastes* individuals was accomplished using the program GENECLASS2 (Piry et al. 2004), which calculates the likelihood that a tested genotype was derived from each reference species sample. Although the species identification method is based on the use of six microsatellite loci (Pearse et al. 2007), based

on an evaluation of assignment power we determined that a minimum of three loci are required to provide acceptable power for species identification (~80%, D. Pearse, unpublished data). Thus, only assignments of individuals for which at least three loci produced usable genotypes in the *all* or *replicated* data sets were considered valid. Little or no confidence was given to assignments based on less than three loci, regardless of replication.

Results

Among the samples recovered from squid stomachs, 11 out of 47 DNA samples (23%) were assigned with at least three loci using the *replicated* data set, and another 26 (55%) were included and assigned based on three *all* data loci, for a total of 37 DNA samples assigned (79%; Table 1). Due to the degradation of the sample DNA recovered from squid stomachs, failure to amplify some or all loci was common; 105 (37%) of the genotypes failed in all three PCR attempts. In addition, two negative controls (water) were run for all six loci in all three PCR attempts, for a total of 36 attempted genotype controls. Five of these resulted in weak but scorable genotypes, giving a raw false positive rate of 13.8%. However, three of the genotype were "singles" and one locus was scored in two attempts, resulting in a discrepancy, so neither negative control produced enough data to result in an assignment. Thus, although the repeated PCR amplifications and two-stage scoring process greatly increased the effort per sample involved in generating the genetic data, these quality control measures helped to ensure that accurate species assignments were obtained.

In all cases in which a DNA sample was included in both the *all* and *replicated* data sets with at least three loci, the assigned species was the same for both data sets (Table 1). These results support the use of the single-score genotype data for individuals with fewer than three replicated loci, because it does not appear that the single-score data are misleading. Furthermore, of the eight pairs of tissue and bone samples thought to originate from the same individual fish, six pairs produced usable genotypes for both samples for least three loci in the *all* and/or *replicated* data sets, and in all cases were assigned to the same species. The remaining two pairs of tissue and bone sample failed to produced enough usable genetic data. Nonetheless, one sample from which the DNA was extracted twice (HMB9-6), was produced three single genotypes from each extract that resulted in the DNA samples being assigned to different species. Thus, although we observed few discrepancies in assignment between the single and replicated data, some caution should be used in interpreting results base only on the *all* data set.

Of the four pairs of samples recovered from single squid but thought to represent multiple individual fish, sufficient genotype data for assignment was obtained for both individuals in three of the pairs. Genotype assignments from two of the pairs are consistent with both samples coming from the same species (although not necessarily the same individual), while the two samples of the third pair assign to different species (2-25 and 2-25B, Table 1).

The samples recovered from squid stomachs were identified based on the *replicated* genetic data as chilipepper (*Sebastes goodei*), shortbelly (*S. jordani*), widow (*S. entomelas*) and splitnose (*S. diploproa*) rockfish (Table 1). All four of these are among the most abundant species in the areas in which the samples were collected, and

all are strongly associated with semipelagic habitats. Chilipepper and widow rockfish are among the most important commercial rockfish species in California fisheries, each accounting for 20-30% of historical landings of rockfish; the chilipepper population is currently considered to be healthy and above target levels, widow rockfish is a rebuilding stock that is rapidly approaching target biomass levels. Shortbelly rockfish are historically unfished, but are an important forage species for piscivorous fishes, seabirds and marine mammals. Splitnose rockfish are caught and landed incidentally but are not a primary fisheries target and are generally less frequently encountered as a forage species to many predators. Both splitnose and shortbelly rockfish have also been confirmed as prey of giant squid based on otolith identification, as have bocaccio (*S. paucispinis*), bank (*S. rufus*), and aurora (*S. aurora*) rockfish, all of which are also known or thought to have semipelagic habitat associations. Although the sizes of the individuals identified here are unknown, vertebrae were typically 6 to 10 mm in length, suggesting adult fishes in the 30 to 50 cm size classes for most individuals, consistent with the size range of other documented large prey items of *Dosidicus* in the California Current.

The samples of recently settled juvenile rockfish yielded high-quality DNA extracts, and all samples produced scorable genotypes for at least three loci. Assignments were made as for the squid stomach samples, except that the genotypes were not replicated due to the reduced concern of contamination and low quality DNA. Most assignments were made with high confidence (>80%, Table 2), and low confidence assignments were due to conflict with closely related species (e.g. black-and-yellow versus gopher rockfish). Interestingly, only a single individual of one species (shortbelly rockfish) identified in the collection of newly-settled nearshore rockfish was also identified as prey of giant squid. This supports the observation that *Dosidicus* is primarily a predator on the semipelagic rockfish species assemblage, and does not target nearshore species.

Discussion

All of the samples identified with both the *all* and *replicated* data sets received the same identification in both cases, as did all pairs of extracts from the different samples (bone and tissue) of the same fish, although their scored genotypes were not always identical. Genetic identifications of most samples were consistent with identification based on otolith IDs (e.g. known shortbelly rockfish). However, genetic assignments of four individuals, all with only *all* data set genotype scores, did not match their otolith identifications. Some of these genetic identifications are unlikely, such as grass rockfish (*S. rastrelliger*), a shallow, nearshore species, and darkblotched rockfish (*S. crameri*), which is uncommon in central California. However, all other assignments were consistent with known species distributions. Based on the frequency of encountering *Sebastes* remains in earlier samples, a greater sample size of specimens to apply this method on was anticipated, but not realized in the samples processed so far. Additional samples have since been collected which will ultimately be analyzed with this approach.

Despite some inconsistencies, the general trends in prey species composition can be clearly interpreted based on this analysis. Small and medium-size semipelagic species in shelf and slope habitats are the most vulnerable to jumbo squid predation, while larger

adults and more bottom-oriented species are less often consumed. Although DNA degradation prevented the identification of all samples, this approach provides a means to better understand trophic interactions of jumbo squid by improving the taxonomic resolution of difficult to identify or closely related prey items, in this case *Sebastes* species, when morphological approaches are not possible.

Dissemination of results and future directions

These results were presented as a poster at the California Cooperative Oceanic and Fisheries Investigations (CalCOFI) Symposium on jumbo squid invasions in the Eastern Pacific Ocean in November of 2007 (of which Field was an organizer), and were of great interest to many conference participants. A similar poster was presented at the MBNMS 2008 Sanctuary Currents Symposium in April of 2008. Results have also been shared with commercial fishermen and fisheries managers over email and word of mouth, including many fishermen who are or have actively assisted in sample collections on their own time to support this effort (e.g., T. Mattusch, F/V Huli Cat, Princeton, CA). We anticipate the analysis of another approximately 40 to 50 samples recovered from *Dosidicus* stomachs, after which time we expect to publish the results in a peer-reviewed journal. Future posters or presentations in scientific forum are possible, and the results will continue to inform ongoing efforts to monitor jumbo squid food habits and model ecosystem impacts based on an ongoing California Sea Grant supported research effort on jumbo squid led by Prof. William Gilly (Hopkins Marine Station), of which Field is a co-investigator.

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Table 1: Species assignments by sample based on all clean genotype data (left) and based only on identical replicated data (right). A minimum of three loci are needed for an accurate assignment to species.

	ALL		REPLICATED	
sample	assignment	# loci	assignment	# loci
CB5-5	Shortbelly (*S. jordani*)	3		0
CB5-8		2		1
HMB5-6	Splitnose (*S. diploproa*)	5		1
HMB5-8	Rosy (*S. rosaceus*)	3		0
HMB7-3vert	Shortbelly (*S. jordani*)	3		2
HMB7-3tail	Shortbelly (*S. jordani*)	6	Shortbelly (*S. jordani*)	5
HMB8-9	Shortbelly (*S. jordani*)	3		1
HMB9-5vert	Splitnose (*S. diploproa*)	4	Splitnose (*S. diploproa*)	3
HMB9-5tis	Splitnose (*S. diploproa*)	4		2
HMB9-10vert	Splitnose (*S. diploproa*)	3		2
HMB9-10tis	Splitnose (*S. diploproa*)	6	Splitnose (*S. diploproa*)	3
CB3-3	Splitnose (*S. diploproa*)	4		2
CB3-4	Vermillian (*S. miniatus*)	3		0
CB3-28	Splitnose (*S. diploproa*)	3		1
HMB7-1	Chilipepper (*S. goodei*)	3		0
HMB7-2vert	Chilipepper (*S. goodei*)	5	Chilipepper (*S. goodei*)	4
HMB7-2tis	Chilipepper (*S. goodei*)	3	Chilipepper (*S. goodei*)	3
HMB7-4vert		2		0
HMB7-4tis		1		0
HMB7-6vert	Chilipepper (*S. goodei*)	4	Chilipepper (*S. goodei*)	3
HMB7-6tis	Chilipepper (*S. goodei*)	6	Chilipepper (*S. goodei*)	4
HMB7-11vert	Chilipepper (*S. goodei*)	6	Chilipepper (*S. goodei*)	4
HMB7-11tis	Chilipepper (*S. goodei*)	5	Chilipepper (*S. goodei*)	5
HMB7-11B	Chilipepper (*S. goodei*)	6	Chilipepper (*S. goodei*)	4
HMB7-2B	Chilipepper (*S. goodei*)	3		0
HMB7-4B		2		0
HMB8-5vert		1		1
HMB8-5tis		2		0
HMB8-7	Halfbanded (*S. semicinctus*)	3		0
HMB8-8		2		0
HMB9-3	Chilipepper (*S. goodei*)	3		0
HMB9-6	Aurora (*S. aurora*)	3		1
HMB9-6reex	Grass (*S. rastrelliger*)	3		0
SD3-3	Splitnose (*S. diploproa*)	3		1
CB1-1	Widow (*S. entomelas)*	5	Widow (*S. entomelas)*	4
HMB1-27	Splitnose (*S. diploproa*)	3		2
HMB2-1	Splitnose (*S. diploproa*)	3		2
HMB2-2	Splitnose (*S. diploproa*)	3		2
HMB2-12	Flag (*S. rubrivinctus*)	3		2
HMB2-20	Splitnose (*S. diploproa*)	5		2
HMB2-25	Darkblotched (*S. crameri*)	3		2
HMB2-25B	Starry (*S. constellatus*)	3		1
HMB4-5	Splitnose (*S. diploproa*)	3		1
HMB4-10		1		0
MBAY1-17		1		0
MBAY1-18		0		0
HMB2-10	Splitnose (*S. diploproa*)	4		2

Table 2: Juvenile rockfish assignments, with confidence scores as estimated using GENECLASS2. The second most likely assigned species selected by the program is also shown.

sample	most likely assignment	%	second most likely assignment	%
juvenile-86	Vermilion (*S. miniatus*)	99.83	Aurora (*S. aurora*)	0.17
juvenile-88	Starry (*S. constellatus*)	98.81	Rosy (*S. rosaceus*)	0.61
juvenile-95	Kelp (*S. atrovirens*)	67.32	Vermilion (*S. miniatus*)	9.41
juvenile-104	Black-and-Yellow (*S. chrysomelas*)	19.12	Aurora (*S. aurora*)	18.91
juvenile-112	Kelp (*S. atrovirens*)	58.60	Black-and-Yellow (*S. chrysomelas*)	28.21
juvenile-121	Vermilion (*S. miniatus*)	99.99	Aurora (*S. aurora*)	0.01
juvenile-127	Copper (*S. caurinus*)	98.78	Gopher (*S. carnatus*)	0.77
juvenile-133	Rosy (*S. rosaceus*)	50.26	Greenspotted (*S. chlorostictus*)	34.72
juvenile-135	Gopher (*S. carnatus*)	54.65	Black-and-Yellow (*S. chrysomelas*)	22.39
juvenile-138	Greenspotted (*S. chlorostictus*)	84.27	Shortbelly (*S. jordani*)	14.63
juvenile-139	Starry (*S. constellatus*)	98.20	Stripetail (*S. saxicola*)	1.77
juvenile-140	Olive (*S. serranoides*)	97.11	Greenspotted (*S. chlorostictus*)	2.37
juvenile-141	Greenspotted (*S. chlorostictus*)	97.48	Rosy (*S. rosaceus*)	2.50
juvenile-142	Greenspotted (*S. chlorostictus*)	77.04	Rosy (*S. rosaceus*)	19.92
juvenile-144	Vermilion (*S. miniatus*)	99.62	China (*S. nebulosus*)	0.21
juvenile-150	Olive (*S. serranoides*)	80.88	Black (*S. melanops*)	10.31
juvenile-151	Greenspotted (*S. chlorostictus*)	99.60	Splitnose (*S. diploproa*)	0.30
juvenile-152	Rosy (*S. rosaceus*)	70.43	Black (*S. melanops*)	27.79
juvenile-153	Rosy (*S. rosaceus*)	99.50	Black (*S. melanops*)	0.46
juvenile-162	Black-and-Yellow (*S. chrysomelas*)	75.02	Gopher (*S. carnatus*)	23.32
juvenile-163	Black (*S. melanops*)	62.92	Yellowtail (*S. flavidus*)	17.74
juvenile-165	Rosy (*S. rosaceus*)	91.37	Black-and-Yellow (*S. chrysomelas*)	7.10
juvenile-164	Shortbelly (*S. jordani*)	98.89	Bocaccio (*S. paucispinus*)	0.91