Genetic and morphological differences between *Sebastes vulpes* and *S. zonatus* (Teleostei: Scorpaeniformes: Scorpaenidae)

Nozomu Muto[1]
Yoshiaki Kai[2]
Tetsuji Nakabo[3]

Email address for contact author: muto@inet.museum.kyoto-u.ac.jp

[1] Division of Applied Biosciences
Graduate School of Agriculture
Kyoto University
c/o The Kyoto University Museum
Kyoto University
Yoshida, Sakyo, Kyoto, 606-8501 Japan

[2] Maizuru Fisheries Research Station
Field Science Education and Research Center
Kyoto University
Nagahama, Maizuru, Kyoto, 625-0086 Japan

[3] The Kyoto University Museum
Kyoto University
Yoshida, Sakyo, Kyoto, 606-8501 Japan

Abstract—The taxonomic status of *Sebastes vulpes* and *S. zonatus* were clarified by comprehensive genetic (amplified fragment length polymorphisms [AFLP] and mitochondrial DNA [mtDNA] variation) and morphological analyses on a total of 65 specimens collected from a single locality. A principal coordinate analysis based on 364 AFLP loci separated the specimens completely into two genetically distinct groups that corresponded to *S. vulpes* and *S. zonatus* according to body coloration and that indicated that they are reproductively isolated species. Significant morphological differences were also evident between the two groups; 1) separation by principal component analysis based on 31 measurements, and 2) separation according to differences in counts of gill rakers and dorsal-fin spines without basal scales, and in the frequencies of specimens with small scales on the lower jaw. Restriction of gene flow between the two groups was also indicated by the pairwise Φ_{ST} values estimated from variations in partial sequences from the mtDNA control region, although the minimum spanning network did not result in separation into distinct clades. The latter was likely due to incomplete lineage sorting between *S. vulpes* and *S. zonatus* owing to their recent speciation.

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The genus *Sebastes*, containing the live-bearing rockfishes, is the most species-rich scorpaenid genus, comprising over 110 species worldwide, over 30 of which are known from the western North Pacific (Kai et al., 2003; Nelson, 2006; Hyde and Vetter, 2007). Exhibiting a relatively high diversity in number of closely related species in contrast to other genera of marine fishes, the genus has long attracted the attention of evolutionary biologists (e.g., Love et al., 2002). The greater part of such diversity has been ascribed to an ancient explosive speciation event and subsequent adaptive radiation. These species have been interpreted as representing an "ancient species flock"—an occurrence rarely seen in marine fishes (Johns and Avise, 1998; Rüber and Zardoya, 2005). However, an increasing number of recent studies have documented recently diverged sibling species pairs that are indicative of ongoing speciation within the genus (Kai et al., 2002a; Narum et al., 2004; Hawkins et al., 2005; Hyde et al., 2008; Burford, 2009; Stefánsson et al., 2009). These events present a series of "snapshots" of the speciation process, providing us with unique insights into evolutionary processes in the marine realm (Sobel et al., 2009).

Sebastes vulpes, S. ijimae, and *S. zonatus* are closely related and morphologically similar western North Pacific species (Chen and Barsukov, 1976; Nakabo, 2002b; Kai et al., 2003; Hyde and Vetter, 2007) that are subject to some taxonomic confusion. Döderlein in Steindachner and Döderlein (1884) first described *Sebastes vulpes*; Jordan and Metz (1913) subsequently listed *S. vulpe*s as valid and described a new species, *S. ijimae*. Two color variants within *S. vulpe*s recognized by Matsubara (1943) were later considered to represent separate species (*S. vulpes* and a new species, *S. zonatus*) by Chen and Barsukov (1976). They characterized *S. vulpes* as having a dark gray body with distinct dense white spots and *S. zonatus* as having a white to pinkish body with three distinct vertical dark bands. In addition, Chen and Barsukov (1976) also recognized *S. ijimae* as a distinct species, noting that the three species shared almost the same distributional range, being known from southern Hokkaido

southward to central Honshu on the Pacific coast of Japan and to the western coast of Honshu. Amaoka (1984) and Nakabo (2002b) recognized the three species as valid, following Chen and Barsukov (1976). Several authors recognized only *S. vulpes*—*S. ijimae* and *S. zonatus* being considered as synonyms (Kanayama and Kitagawa, 1983; Nagasawa, 2000; Kitagawa et al., 2008; Ishida, 2009). However, because comprehensive genetic or morphological studies have been lacking to date, taxonomic status of the three species remains uncertain.

Failure to recognize reproductively isolated populations within an exploited stock can introduce critical errors in management (Carvalho and Hauser, 1994). *Sebastes vulpes* and *S. zonatus* are both abundant across northern Japan, together representing an important fisheries component, whereas *S. ijimae* is relatively rare (Sekigawa et al., 2003). Because of their high commercial value, some Japanese fisheries organizations have attempted to enhance the stocks of *S. vulpes* and *S. zonatus* through aquaculture (Sasaki, 2003; Sekigawa et al., 2003). However, without reliable taxonomic information for both species, it is unlikely that enhancement of the fishery will be realized. In this context, as a first step toward fully resolving the taxonomic status of *S. vulpes*, *S. ijimae*, and *S. zonatus*, we focused on *S. vulpes* and *S. zonatus*, using comprehensive genetic and morphological analyses.

Mitochondrial DNA (mtDNA) has been used successfully as a primary marker to infer species boundaries among species of *Sebastes* (e.g., Alesandrini and Bernardi, 1999; Kai et al., 2002b), although recently evolved sibling species pairs that are nonmonophyletic with respect to the mtDNA gene tree have been frequently reported in *Sebastes* (e.g., Kai et al., 2002a; Narum et al., 2004; Burford and Bernardi, 2008). The delimitation of such pairs requires data from multiple independent loci (e.g., Nichols, 2001; Avise, 2004). In fact, *S. vulpes* and *S. zonatus* are primarily distinguished by body coloration, which has been demonstrated as a good indicator of recent speciation in *Sebastes* (e.g., Kai et al., 2002a; Narum et al., 2004; Hyde et al., 2008; Orr and Hawkins, 2008). Accordingly, a technique called AFLP (amplified fragment length polymorphisms) (Vos et al., 1995) has also been used, because it is a multilocus approach that produces hundreds of highly replicable independent dominant markers (Bensch and Åkesson, 2005) and therefore estimates genetic divergence across the whole genome. Such an approach has successfully resolved the species boundaries and phylogenetic relationships among recently diverged species complexes in various organisms, in which mtDNA sequencing alone was less informative (e.g., Seehausen et al., 2003; Mendelson and Shaw, 2005). Moreover, evaluating a mtDNA gene tree against the background of a multilocus approach allows further discussion on the evolutionary relationships and histories among closely related species (e.g., Kai et al., 2002a; Hyde et al., 2008; Burford, 2009).

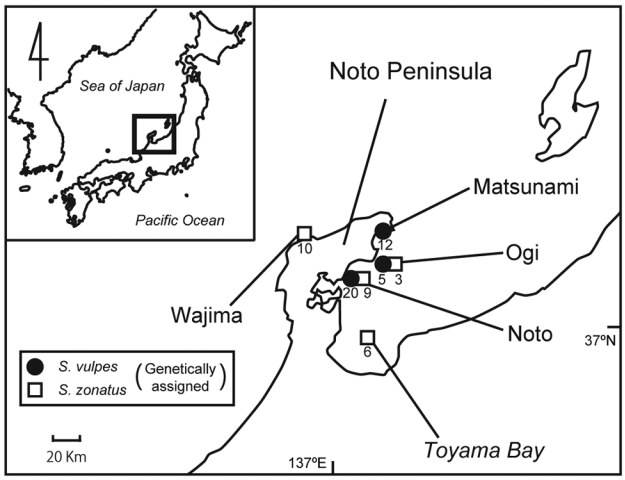

Figure 1

Collection sites around the Noto Peninsula, Island of Honshu, Japan, for the 65 *Sebastes* spp. specimens examined for genetic and morphological differences. Numbers below symbols represent sample sizes.

Materials and methods

Samples

In order to clearly demonstrate intrinsic reproductive isolation between *S. vulpes* and *S. zonatus*, a total of 65 specimens were collected from single locality around Noto, Ishikawa Prefecture, Japan (Fig. 1), thereby eliminating any geographical variations. The body coloration of each specimen was recorded with a photograph taken while the fish was alive or soon after death. Thirty-nine specimens with a grayish body were identified as *S. vulpes,* and the remaining 26 (with a brownish body) as *S. zonatus* (Fig. 2), generally by following the methods of Chen and Barsukov (1976). The two species are not usually caught together; *S. vulpes* is caught with set nets at ~60 m depth and *S. zonatus* with gill nets at ~150 m around the sampling locality (K. Sakai[1]). Muscle tissue was taken from each specimen before fixation and preserved in 99.5% ethanol. The specimens examined here were deposited in the Kyoto University Fish Collection (FAKU) (see Appendix for catalog numbers and collection data).

Genetic analysis

Genomic DNA was extracted from the preserved muscle tissue, by using the DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocols.

AFLP profiles were generated with the AFLP Plant Mapping Kit (Applied Biosystems, Foster City, CA) by following the manufacturer's protocol slightly modified by Kai et al. (2002a). For the selective amplification

[1] Sakai, K. 2011. Personal commun. Noto Marine Center, 3-47 Ossaka, Noto, Ishikawa 927-0552, Japan.

step, 12 randomly chosen primer pairs were used (Mse I + Eco RI [ACA + CAA, AAG + CAG, ACA + CTT, ACA + CAC, ACT + CTA, ACA + CTG, AAG + CAC, AGG + CAT, ACC + CTT, ACG + CAC, AAG + CAT, AGG + CTT]). Selective amplification products were analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems) together with a GeneScan-500 Rox size standard (Applied Biosystems). Fragment data were collected with Peak Scanner software, vers. 1.0 (Applied Biosystems). Electropherograms were scored for the presence (1) or absence (0) of fragments between 90 base pairs (bp) to 450 bp in size, so as to create binary matrices. Fragments were inferred as homologous if they differed by not more than 0.5 bp from the median. Euclidean pairwise genetic distances (Huff et al., 1993) were calculated from the binary matrices in GenAlEx, vers. 6.41 (Peakall and Smouse, 2006). Principal coordinate analysis (PCoA) with a covariance matrix and the data standardization method was performed on the basis of the Euclidean pairwise distance matrix, as implemented in GenAlEx, vers. 6.41 (Peakall and Smouse, 2006). By means of PCoA, we explored the genetic population structure among all 65 specimens without *a priori* grouping information.

The mitochondrial DNA sequence comprising 452 bp extending from the threonine transfer RNA ($tRNA^{Thr}$) gene to the middle conserved region of the control region (mtCR) was amplified with the primers L15876 (5′-AAG CAC TTG AAT GAG CTT G-3′) (Rocha-Olivares et al., 1999) and H16498 (5′-CCT GAA GTA GGA ACC AGA TG-3′) (Meyer et al., 1990). The polymerase chain reaction (PCR) proceeded for 30 cycles, with denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, PCR products being purified with USB® ExoSAP-IT® (Affymetrix, Santa Clara, CA). DNA sequencing was performed with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 310 genetic analyzer (Applied Biosystems). The DNA sequences were edited with the sequence alignment editor BioEdit 7.0.5.3 (Hall, 1999) and aligned with the program CLUSTAL X, vers. 2.1 (Larkin et al., 2007). Estimation of mitochondrial genetic structuring among specimens based on haplotype frequency and uncorrected genetic distances between haplotypes (Φ_{ST}) was performed by Arlequin, vers. 3.5 (Excoffier and Lischer, 2010). The significance of the Φ_{ST} value was tested by 10,000 random permutations. Arlequin 3.5 was also used to construct the minimum spanning network (MSN) of the haplotypes on the basis of minimum sequence differences. The sequences determined in this study have been deposited in GenBank (accession numbers AB614522–AB614526 and AB615270–AB615329).

Morphological analysis

Morphological characters were examined after fixation in 10% formalin and preservation in 70% ethanol. Measurements were made on 31 morphological characters, including standard length, which generally followed those described by Nakabo (2002a) except for the following: body depth 1 and 2 as defined by Kai and Nakabo (2002); upper peduncle length, lower peduncle length, spinous dorsal-fin base length, soft dorsal-fin base length, prepelvic length, and gill raker length as defined by Chen (1971); body width was taken as the distance between the uppermost bases of the right and left pectoral fins; pelvic-to-anal-fin length was taken as the distance from the anteriormost base of the pelvic fin to the origin of the anal fin.

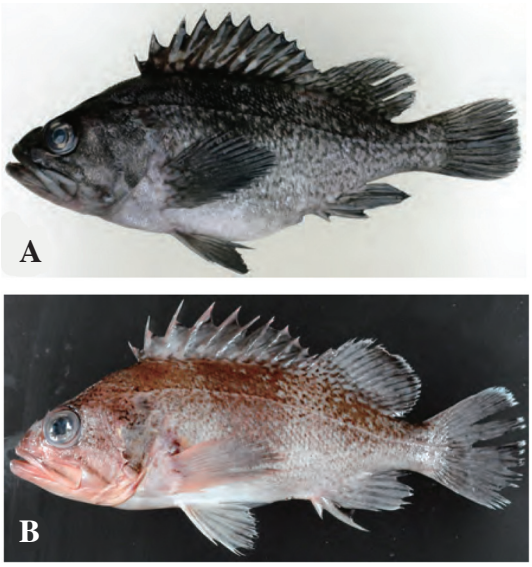

Figure 2

Coloration of fresh specimens of *Sebastes vulpes* and *S. zonatus*. (**A**) *S. vulpes*, FAKU 96090, 194.0 mm standard length; (**B**) *S. zonatus*, FAKU 97082, 167.9 mm standard length. Photo by N. Muto.

Analysis of covariance (ANCOVA) of log10 transformed measurements (with standard length as a covariate) was used to assess differences in morphometric characters between *S. vulpes* and *S. zonatus* when assumptions of normality and homogeneity of slopes were satisfied. The following characters met the assumptions required for ANCOVA: head length, snout length, orbit length, interorbital width, postorbital length, upper jaw length, body depth 1, body depth 2, body width, caudal peduncle depth, upper peduncle length, pectoral-fin length, pelvic-fin length, dorsal-fin base length, spinous dorsal-fin base length, soft dorsal-fin base length, preanal length, predorsal length, prepelvic length, pelvic-to-anal-fin length, 2nd dorsal-fin spine length, 3rd dorsal-fin spine length, and gill raker length. To provide an objectively defined score that summarizes the major components of variable measurements between the specimens, a principal component analysis (PCA) was conducted on the basis of all measurements. Raw measurement data were standardized by log transformation before PCA.

Counts were made on the dorsal-fin rays, anal-fin rays, pectoral-fin rays, pored lateral line scales, gill rakers, and dorsal-fin spines without basal scales. Significant differences in these characters were tested with the Mann-Whitney *U*-test. The presence or absence of small scales on the lower jaw was noted and the difference in frequencies between species assessed by Fisher's exact test. All statistical analyses were conducted using R language, vers. 2.11.1 (R Development Core Team, 2010). Differences were considered significant at $P<0.01$.

Results

Genetic analysis

The 12 primer sets yielded 364 AFLP fragments, of which 127 (34.9%) were polymorphic. All specimens tested displayed unique AFLP fragment patterns, indicating a high level of genetic variability. A PCoA based on the pairwise distance matrix clearly separated the specimens into two groups along the first principal coordinates (PCo) axis (accounting for 41.83% of the total variance) with no overlap (Fig. 3), which corresponded well with the initial identifications of *S. vulpes* and *S. zonatus* based on body coloration, except for two specimens (FAKU 82515 and FAKU 130236). The latter were initially identified as *S. vulpes*, but genetically assigned to *S. zonatus*. Because *S. vulpes* and *S. zonatus* were clearly distinguished by the PCo1 scores without any intermediate specimens, we regarded the above specimens as *S. zonatus*. Comparisons below were made between the two genetically assigned species (*S. vulpes*: 37 specimens, *S. zonatus*: 28 specimens). In contrast, the PCo2 and PCo3 scores (accounting for 13.52% and 12.56% of the total variance, respectively) did not result in separation of the two species (not shown). Despite the high polymorphism evident in the AFLP fragments, no diagnostic differences in fragment patterns were observed between genetically assigned *S. vulpes* and S. *zonatus*. Mean (± standard deviation) pairwise genetic distances estimated by the algorithm of Huff et al. (1993) were 22.63 ±3.95 within *S. vulpes*, 28.69 ±5.16 within *S. zonatus,* and 32.21 ±5.52 between them.

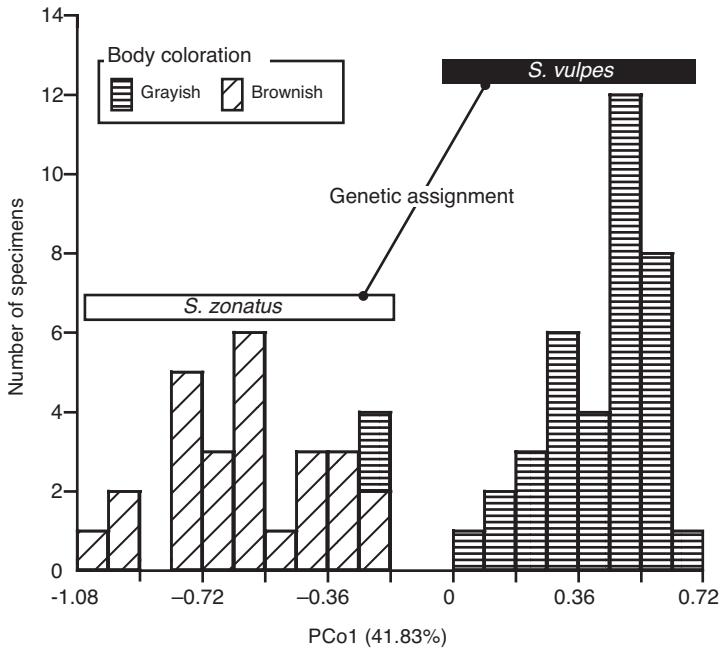

Figure 3

Distribution of first principal coordinate (PCo) scores based on 364 amplified fragment length polymorphisms fragments for *Sebastes zonatus* and *S. vulpes*. The amount of variance explained by PCo1 is given in parentheses. The body coloration of each specimen is designated by the fill pattern of the bars (grayish specimens, lateral stripes; brownish specimen, diagonal stripes). The present specimens were separated completely into two genetically distinct groups.

Within the amplified region of mtDNA, continuous sequences of part of the $tRNA^{Thr}$ gene (24 bp), the proline transfer RNA ($tRNA^{Pro}$) gene (70 bp), and part of the mtCR (358 bp) were aligned. The sequences contained 45 variable sites with three indels among 65 specimens, 29 of which were parsimony informative, defining a total of 41 haplotypes. Twenty-one haplotypes were found in *S. vulpes* and 22 in *S. zonatus*, two being shared by the two species. No transversions were observed. The nucleotide composition was AT-biased (A=38.4%, C=19.4%, G=13.6%, T=28.6%), as is common for fish mtDNA (McMillan and Palumbi, 1997). Pairwise sequence divergences between *S. vulpes* and *S. zonatus* varied from 0% to 3.1% (mean 1.5%). The haplotype diversities for *S. vulpes* and *S. zonatus* were 0.94 ±0.03 and 0.98 ±0.02, respectively, and nucleotide diversities (in %), 1.41 ±0.76 and 1.47 ±0.80, respectively. In the MSN inferred from mtDNA sequence variations, 41 haplotypes were connected to each other by one to six mutational steps, revealing a rather expanded topology in the network (Fig. 4). *Sebastes vulpes* and *S. zonatus* were not clearly separated in the network, but restricted gene flow between them was indicated by the low but significant pairwise Φ_{ST} value at $\alpha=0.05$ level ($\Phi_{ST}=0.053$, $P=0.011$ ±0.001).

Morphological analysis

Measurements of *S. vulpes* and *S. zonatus* and results of the ANCOVA are shown in Table 1. Among measurements meeting the statistical assumptions for ANCOVA, four characters out of 23 differed significantly between the two species.

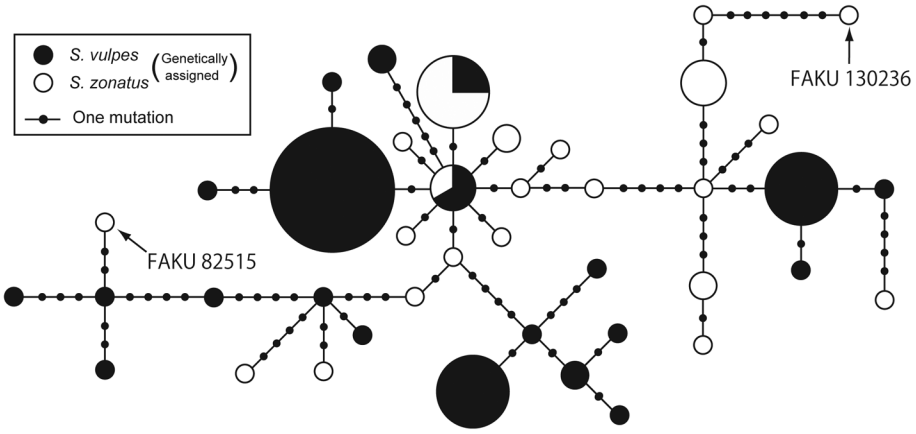

Figure 4

Minimum spanning network of mitochondrial DNA sequence haplotypes of *Sebastes vulpes* (filled circles) and *S. zonatus* (open circles). Each circle represents a unique haplotype (size proportional to number of specimens). The arrow indicates the specimen initially identified as *S. vulpes* by body coloration but genetically assigned to *S. zonatus* in the principal coordinate analysis of amplified fragment length polymorphisms. Black dots along branches represent nucleotide changes. The two species shared two haplotypes and did not exhibit clear separation in the network.

In the PCA, seven specimens were eliminated because they lacked one or more measurements, such as dorsal-fin spine length and anal-fin spine length. Nevertheless, plots of the PCA scores revealed marked differences between *S. vulpes* and *S. zonatus*. The first principal component (PC1) accounted for 87.5% of the variations. Because all loadings were negative, PC1 was considered a size component. PCs 2 and 3 were shape components, with both positive and negative loadings, and together accounted for an additional 4.9% of variation. These components were then visually assessed as dimensions of shape (Fig. 5). PC2 was heavily loaded on body width, 1st anal-fin spine length and 2nd anal-fin spine length (Table 2), providing separation between *S. vulpes* and *S. zonatus* with a narrow overlap. PC3 was heavily loaded on orbit length, prepelvic length, and 1st dorsal-fin spine length (Table 2), with the clusters of the two species broadly overlapping along PC3.

The results of countable characters are shown in Table 3. *Sebastes vulpes* had fewer gill rakers than *S. zonatus*. Counts of dorsal-fin spines without basal scales also differed significantly between *S. vulpes* and *S. zonatus*, as did the frequencies of specimens with small scales on the lower jaw (0.16 and 0.82, respectively). Other countable characters did not differ significantly between the species.

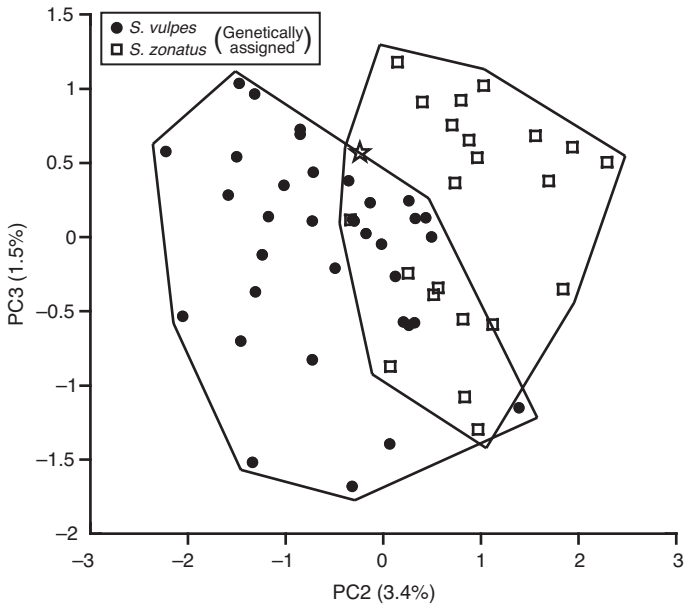

Figure 5

Plots of principal component (PC) scores based on 31 measurements for *Sebastes vulpes* (filled circles) and *S. zonatus* (open squares). The open star indicates one of the two specimens (FAKU 82515) initially identified as *S. vulpes* by body coloration but genetically assigned to *S. zonatus* in the principal coordinate analysis of amplified fragment length polymorphisms (FAKU 130236 was removed from analysis because it lacked a measurement for gill raker length). The two species were separated with a narrow overlap along the PC2 axis, the primary shape component.

Table 1

Measurements in proportion to standard length (SL) for *Sebastes vulpes* and *S. zonatus*. Data indicate ranges, means (in parentheses), and sample sizes (*n*). X indicates statistically a significant difference between the two species, demonstrated by analysis of covariance (ANCOVA) with standard length (SL) as a covariate. The specimens initially identified as *S. vulpes* by body coloration but genetically assigned to *S. zonatus* in the principal coordinate analysis of amplified fragment length polymorphisms are shown separately (FAKU 82515 and FAKU 130236). ns=not significant.

		S. zonatus (*n*=28)			
	S. vulpes (*n*=37)		FAKU 82515	FAKU 130236	ANCOVA
Standard length (mm)	156.4–249.9	137.3–286.4	189.0	154.8	
As % of SL					
Head length	38.0–41.1 (39.6, 37)	37.7–41.3 (39.3, 26)	41.4	40.4	ns
Snout length	10.1–12.4 (11.3, 37)	9.3–11.9 (10.7, 26)	11.2	11.6	X
Orbit length	8.9–11.3 (9.7, 37)	9.0–12.0 (10.4, 26)	10.7	10.2	X
Interorbital width	6.5–8.2 (7.4, 37)	6.6–8.3 (7.4, 26)	7.0	7.2	ns
Postorbital length	19.1–21.8 (20.3, 37)	18.8–21.9 (20.2, 26)	21.5	20.6	ns
Upper jaw length	19.1–21.6 (20.5, 37)	19.5–21.2 (20.1, 26)	20.3	21.6	X
Body depth 1	35.9–40.7 (38.3, 37)	35.8–41.2 (37.9, 26)	38.6	43.0	ns
Body depth 2	27.5–33.9 (30.3, 37)	29.3–33.4 (31.1, 26)	30.6	34.5	ns
Body width	17.5–24.0 (21.1, 37)	16.1–23.5 (18.7, 26)	18.9	22.2	X
Caudal peduncle depth	10.2–12.4 (11.5, 37)	10.4–12.3 (11.4, 26)	12.2	12.1	ns
Upper peduncle length	10.0–13.2 (11.7, 37)	10.4–13.0 (11.7, 26)	10.7	11.5	ns
Lower peduncle length	16.4–20.6 (18.6, 37)	16.6–19.8 (18.4, 26)	18.5	18.2	
Pectoral-fin length	24.9–31.2 (27.9, 37)	26.7–31.1 (28.8, 26)	28.4	27.8	ns
Pelvic-fin length	20.2–24.1 (22.1, 37)	20.9–24.4 (22.6, 26)	22.8	23.7	ns
Dorsal-fin base length	56.2–65.2 (60.3, 37)	57.2–65.4 (60.8, 26)	61.5	65.4	ns
Spinous dorsal-fin base length	34.0–43.1 (38.0, 37)	34.2–40.9 (37.7, 26)	38.2	41.5	ns
Soft dorsal-fin base length	19.5–25.2 (22.9, 37)	19.8–25.6 (22.8, 26)	23.4	24.7	ns
Preanal length	64.6–74.3 (68.3, 37)	63.7–72.2 (67.1, 26)	66.5	66.4	ns
Predorsal length	34.0–38.1 (35.8, 37)	33.0–40.0 (35.8, 26)	35.9	38.4	ns
Prepelvic length	39.1–52.5 (43.2, 37)	39.5–55.8 (43.5, 26)	43.4	44.1	ns
Anal-fin base length	12.9–16.7 (14.7, 37)	13.7–16.7 (14.9, 26)	13.8	14.4	
Pelvic-to-anal-fin length	26.6–40.5 (33.4, 37)	26.4–37.1 (33.0, 26)	33.5	32.9	ns
1st dorsal-fin spine length	5.3–8.6 (6.8, 37)	5.6–8.1 (7.0, 25)	6.8	7.1	
2nd dorsal-fin spine length	10.1–13.5 (11.5, 37)	10.6–13.9 (11.8, 25)	11.2	12.3	ns
3rd dorsal-fin spine length	13.5–18.2 (15.3, 36)	14.3–17.4 (15.7, 26)	15.8	18.0	ns
1st anal-fin spine length	5.0–8.2 (6.7, 37)	6.3–8.5 (7.3, 26)	7.4	7.8	
2nd anal-fin spine length	12.1–15.5 (13.4, 37)	13.1–17.0 (15.6, 26)	13.2	14.3	
3rd anal-fin spine length	12.2–15.8 (14.1, 37)	13.0–16.7 (14.8, 25)	13.7	15.1	
Pelvic-fin spine length	12.4–15.9 (13.6, 36)	13.2–15.6 (14.6, 26)	13.3	14.8	
Gill raker length	3.3–4.5 (3.8, 37)	3.0–4.7 (4.1, 25)	4.0	—	ns

Discussion

Genetic and morphological differentiation

Variations in AFLP loci across the whole genome revealed marked genetic structure among the specimens examined. The PCoA with AFLP disclosed two genetically distinct groups, which corresponded well with initial *S. vulpes* and *S. zonatus* identifications that were based on body coloration (Fig. 3). Because the present specimens were collected from a single sampling locality, the clear genetic differences between *S. vulpes* and *S. zonatus* indicated that they are reproductively isolated from each other and should be recognized as separate species. Notwithstanding, two specimens with grayish body coloration reminiscent of *S. vulpes* were clearly genetically assigned to *S. zonatus* on the basis of the PCoA with AFLP. Such discordance may be indicative of some intraspecific variation in body coloration in *S. zonatus,* or historical hybridization between *S. vulpes* and *S. zonatus*, as discussed below. Significant morphological differences also supported the validity of the two species. A PCA of body measurements resulted in clusters of *S. vulpes* and *S. zonatus* being almost completely separated, apart from a narrow overlap along the PC2 axis, the primary shape component (Fig. 5). Some countable characters also differed significantly between the two species. In addition, a restriction of gene flow between *S.*

Table 2

Factor loadings for principal component (PC) analysis of measurements of *Sebastes vulpes* and *S. zonatus* in specimens examined with all characters available for multivariate analysis.

	PC1	PC2	PC3		PC1	PC2	PC3
Standard length	−0.9895	−0.1027	0.0409	Soft dorsal-fin base length	−0.9445	−0.0692	0.1436
Head length	−0.9852	−0.1065	0.0703	Preanal length	−0.9567	−0.1952	−0.0632
Snout length	−0.9128	−0.2459	0.0786	Predorsal length	−0.9725	−0.0474	0.1441
Orbit length	−0.8875	0.1683	0.2684	Prepelvic length	−0.9080	−0.2030	−0.2424
Interorbital width	−0.9700	−0.0066	−0.0215	Anal-fin base length	−0.9390	−0.0077	−0.1620
Postorbital length	−0.9800	−0.0695	0.0980	Pelvic-to-anal-fin length	−0.8711	−0.1179	0.2136
Upper jaw length	−0.9783	−0.1367	0.0339	1st dorsal-fin spine length	−0.8878	0.2150	−0.2948
Body depth 1	−0.9768	−0.1375	−0.0715	2nd dorsal-fin spine length	−0.9371	0.1776	−0.0960
Body depth 2	−0.9727	−0.0285	−0.0632	3rd dorsal-fin spine length	−0.9357	0.2025	−0.0797
Body width	−0.8952	−0.3316	−0.1898	1st anal-fin spine length	−0.8194	0.3814	0.0470
Caudal peduncle depth	−0.9615	−0.1364	−0.0182	2nd anal-fin spine length	−0.8001	0.4780	0.0565
Upper peduncle length	−0.9401	−0.1069	0.0622	3rd anal-fin spine length	−0.9116	0.2521	−0.0827
Lower peduncle length	−0.9446	−0.0344	−0.0497	Pelvic-fin spine length	−0.9467	0.2192	−0.0177
Pectoral-fin length	−0.9686	0.0310	−0.0073	Gill raker length	−0.8520	0.1947	−0.0462
Pelvic-fin length	−0.9768	0.0100	−0.0085				
Dorsal-fin base length	−0.9792	−0.0444	0.1357				
Spinous dorsal-fin base length	−0.9644	−0.0373	0.1107				

Table 3

Distributions of countable characters in *Sebastes vulpes* and *S. zonatus*. Superscripts [a] and [b] indicate the counts of the two specimens initially identified as *S. vulpes* by body coloration but genetically assigned to *S. zonatus* (FAKU 82515 and FAKU 130236, respectively).

	Dorsal-fin rays		Anal-fin rays			Pectoral-fin rays (total)			Pectoral-fin rays (unbranched)						
Species	12	13	5	6	7	16	17	18	6	7	8	9	10	11	*n*
S. vulpes	19	18	—	36	1	5	31	1	1	—	11	22	3	—	37
S. zonatus	9	19[a,b]	1	26[a,b]	1	—	24[a,b]	4	—	1	4	17[b]	5[a]	1	28

	Pored lateral line scales									Gill rakers						
Species	28	29	30	31	32	33	34	35	36	24	25	26	27	28	29	*n*
S. vulpes	—	1	7	8	12	8	1	—	—	2	9	22	4	—	—	37
S. zonatus	1	—	4	5	6[a,b]	5	5	1	1	—	1	9[a,b]	9	8	1	28

	Dorsal-fin spines without basal scales												
Species	1	2	3	4	5	6	7	8	9	10	11	12	*n*
S. vulpes	3	–	1	1	1	3	3	3	9	4	—	5	33
S. zonatus	21	1[b]	2	1	1	—	—	1	—	—	1[a]	—	28

vulpes and *S. zonatus* was also indicated by the pairwise Φ_{ST} value based on mtDNA sequence variation although the MSN inferred from the mtDNA sequences did not clearly separate the two species and therefore indicated incomplete lineage sorting in mtDNA due to their recent speciation and the occurrence of introgression between them, or both (see below).

The difference in depth ranges between *S. vulpes* and *S. zonatus* also adds support to the recognition of two species. In the general sampling area, the different fish-

ing nets are indicative of the different depth habitats for the two species: adult *S. vulpes* are usually caught with set nets laid around 60 m depth, whereas *S. zonatus* are usually caught with gill nets laid around 150 m depth around the sampling locality (Sakai[1]); Similar habitat separation (by depth) is common for other closely related (sister) species of *Sebastes* (Narum et al., 2004; Orr and Blackburn, 2004; Burford and Bernardi, 2008; Hyde et al., 2008; Orr and Hawkins, 2008; Stefánsson et al., 2009)—ecologically based reproductive isolation having often been invoked for *Sebastes*. For example, Hyde et al. (2008) showed that *S. miniatus* and "S. crocotulus" were segregated by habitat depth and they hypothesized a speciation model for closely related species pairs of *Sebastes* in which truncation of depth-related ontogenetic migration may have led to speciation. More detailed ecological studies may provide further insights into the maintenance of independent gene pools by *S. vulpes* and *S. zonatus* and eventually provide clues for understanding the mechanisms underlying the considerable diversity within *Sebastes* (Ingram, 2011).

Incomplete lineage sorting and introgression

Although *S. vulpes* and *S. zonatus* are reproductively isolated from each other and should be treated as two distinct species, two specimens of *S. zonatus* had typical *S. vulpes* coloration. In addition, the two species shared two mtDNA haplotypes and did not exhibit clear separation in the MSN inferred from the mtDNA sequences (Fig. 4). This feature can be explained by 1) incomplete lineage sorting in mtDNA due to recent speciation, and 2) interspecific mtDNA gene flow mediated by hybridization and backcrossing (introgression), or both (Avise, 2000; Funk and Omland, 2003). Incomplete lineage sorting is a source of nonmonophyletic relationship among rapidly radiating species in a mtDNA gene tree (Funk and Omland, 2003) because newly diverged species are initially expected to be nonmonophyletic with respect to any gene tree owing to allelic separations predating the species split, thereafter progressing to reciprocal monophyly over time as ancestral haplotypes are sorted and unique mutations acquired (Avise, 2000). On the other hand, a mtDNA gene tree is also particularly susceptible to the effects of introgression because mtDNA is inherited maternally and does not recombine (Funk and Omland, 2003). In fact, both incomplete lineage sorting and introgression have been frequently reported within *Sebastes* (Roques et al., 2001; Kai et al., 2002a; Narum et al., 2004; Buonaccorsi et al., 2005; Hyde et al., 2008; Burford, 2009).

A rigorous statistical framework accounting for the stochastic variance of genetic processes is generally required to distinguish incomplete lineage sorting from introgression (Peters et al., 2007), although an *ad hoc* explanation can be given without the statistical rejection of alternative hypotheses (Avise, 2000; Donnelly et al., 2004). In this study, two lines of observations appeared to better support incomplete lineage sorting as the cause of the observed nonmonophyly of mtDNA, although the two processes are difficult to distinguish unequivocally and are not necessarily mutually exclusive.

First, the present MSN of mtDNA showed no distinct clades that corresponded with each species. The topology of the gene tree has often been used as an heuristic approach to determine the cause of lack of separation (e.g., Baker et al., 2003; Omland et al., 2006; Zakharov et al., 2009). A shallow genetic divergence between species without distinct clades, as observed in the present study, is generally interpreted as indicative of recent speciation and incomplete lineage sorting (e.g., Baker, 2003; Donnelley et al., 2004). The relatively small pairwise sequence divergences between *S. vulpes* and *S. zonatus*, corresponding closely to those of intraspecific variations found in some other species of *Sebastes* (Rocha-Olivares et al., 1999; Higuchi and Kato, 2002; Kai et al., 2002a; Burford and Bernardi, 2008), also indicate recent speciation between the two species. In addition, haplotype and nucleotide diversities within both *S. vulpes* and *S. zonatus* were relatively high compared with those of other species of *Sebastes* (Rocha-Olivares et al., 1999; Higuchi and Kato, 2002; Kai et al., 2002a), as well as other marine fishes (Grant and Bowen, 1998), indicating that the two species evolved from a large, genetically diverse ancestral population, thereafter maintaining large effective population sizes without recent bottlenecks (Grant and Bowen, 1998; Avise, 2000). Because the probability of complete sorting of ancestral haplotypes is a function not only of stochastic processes and time since speciation but also effective population sizes (Funk and Omland, 2003), it seems plausible that large, stable effective population sizes of those two species delayed lineage sorting, resulting in their present-day sharing of ancestral haplotypes.

Second, in the PCoA of AFLP, no specimens occupied positions intermediate between two clusters (=*S. vulpes* and *S. zonatus*) (Fig. 3). Because fragments detected in AFLP are inherited according to Mendelian expectations (Kakehi et al., 2005), hybridized specimens (F1) are generally expected to have intermediate fragment patterns between parental species (e.g., Congiu et al., 2001; Young et al., 2001). Therefore, the absence of intermediate specimens in PCoA indicated a lack of ongoing hybridization between the two species, although the possibility of historical introgression (including backcross) cannot be completely excluded. In fact, two specimens assigned to *S. zonatus* in the PCoA of AFLP had been initially identified as *S. vulpes* on the basis of body coloration (Fig. 3). One of those specimens (FAKU 82515) was plotted near the *S. vulpes* cluster in the PCA based on measurements (Fig. 5), the count of 11 dorsal-fin spines without basal scales for that specimen also being indicative of *S. vulpes* (usually more than six) rather than *S. zonatus* (usually one) (Table 3). Such equivocal morphological characters may be explained by traces of historical hybridization between *S. vulpes* and *S. zonatus*, which may have resulted in mtDNA introgression between them.

Conclusions

The recognition of *S. vulpes* and *S. zonatus* as two distinct species is the first step toward establishing a biologically based, species-specific management scheme for these commercially and recreationally important species. In order to demonstrate more detailed evolutionary relationships between the two species, specimens sampled throughout their overall distributional range are currently under examination.

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Appendix

Materials examined

Sebastes vulpes (genetically assigned) 37 specimens. FAKU 82514; Noto, Ishikawa Prefecture, Japan, 30 May 2002, 200.2 mm SL. FAKU 83188, 83189, 83193, 83195–83197; Noto, Ishikawa Prefecture, Japan, 31 May 2002, 200.9–243.2 mm SL. FAKU 96073; Noto, Ishikawa Prefecture, Japan, 8 April 2008, 203.5 mm SL. FAKU 96074–96078; Noto, Ishikawa Prefecture, Japan, 1 May 2008, 156.4–218.5 mm SL. FAKU 96082–96088, 96090–96094; Matsunami, Ishikawa Prefecture, Japan, 1 May 2008, 159.8–230.9 mm SL. FAKU 96097, 96099, 96100, 131566, 131567, 131569; Noto, Ishikawa Prefecture, Japan, 2 May 2008, 170.0–249.9 mm SL. FAKU 130099; Noto, Ishikawa Prefecture, Japan, 13 May 2004, 182.1 mm SL. FAKU 131533–131537; Ogi, Ishikawa Prefecture, Japan, 10 April 2006, 161.0–176.6 mm SL. *Sebastes zonatus* (genetically assigned) 28 specimens. *FAKU 82515; Wajima, Ishikawa Prefecture, Japan, 30 May 2002, 189.0 mm SL. FAKU 82516–82519, 82521; Wajima, Ishikawa Prefecture, Japan, 30 May 2002, 137.3–165.1 mm SL. FAKU 83185–83187; Noto, Ishikawa Prefecture, Japan, 30 May 2002, 252.1–286.4 mm SL. FAKU 85799; Noto, Ishikawa Prefecture, Japan, 11 February 2003, 257.5mm SL. FAKU 96095; Toyama Bay, Japan, 1 May 2008, 234.4 mm SL. FAKU 96096, 96098; Noto, Ishikawa Prefecture, Japan, 2 May 2008, 184.8–210.3 mm SL. FAKU 97077, 97080, 97082, 97083; Wajima, Ishikawa Prefecture, Japan, 7 July 2009, 147.9–170.3 mm SL. FAKU 129995, 130103–130105, 130349; Toyama Bay, Japan, 7 July 2004, 206.5–232.7 mm SL. FAKU 130100–130102; Ogi, Ishikawa Prefecture, Japan, 13 May 2004, 160.1–176.2 mm SL. FAKU 130235; Noto, Ishikawa Prefecture, Japan, 7 June 2004, 202.8 mm SL. *FAKU 130236; Noto, Ishikawa Prefecture, Japan, 12 May 2004, 154.8 mm SL. FAKU 131568; Noto, Ishikawa Prefecture, Japan, 2 May 2008, 204.2 mm SL. * indicates the specimen was initially identified as *S. vulpes* by body coloration but genetically assigned to *S. zonatus* in the principal coordinate analysis of amplified fragment length polymorphisms.