National Marine Fisheries Service
NOAA

Fishery Bulletin
established in 1881

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Population structure of the ocellate spot skate (*Okamejei kenojei*) inferred from variations in mitochondrial DNA (mtDNA) sequences and from morphological characters of regional populations

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Abstract—The population structure of the ocellate spot skate (*Okamejei kenojei*), distributed in the western North Pacific, was assessed on the basis of genetic variations in the mitochondrial DNA control region (mtCR) and differences in morphological characters and features. Significant genetic differentiation in mtCR was observed among 6 regional populations along the Japanese Archipelago and Korean Peninsula. Unique haplotypes found in Osaka Bay and off the east coast of Kyushu Island suggested an absence of gene flow from the other 5 regions. In addition, comparisons of morphological characters and features, including measurements, nuchal thorn counts, differences in maturity size, and coloration, indicated that populations of ocellate spot skate from Osaka Bay and from off the Pacific coast of northern Japan were clearly distinguishable from 4 other regional populations. Together with molecular differentiation among the regional populations, they suggest that the straits, ocean currents, and limited migrations are significant barriers to gene flow between populations. Future fisheries management of the species is discussed on the basis of the present findings.

Manuscript submitted 6 July 2018.
Manuscript accepted 26 November 2018.
Fish. Bull. 117:24–36 (2019).
Online publication date: 4 December 2018.
doi: 10.7755/FB.117.1.4

Many elasmobranch fishes (sharks, rays, and skates) are particularly vulnerable to overfishing because of their large size, slow growth, late maturity, and low fecundity (Brander, 1981; Dulvy et al., 2000; Griffiths et al., 2010; Griffiths et al., 2011). These life-history traits translate into low rates of reproduction and low potential rates of population increase (Walker and Hislop, 1998). Elasmobranch fishes are also targeted by fisheries because their meat, fins, and liver oil are commercially valuable (Dulvy et al., 2014; Davidson et al., 2016). In addition, elasmobranch fishes are often caught as bycatch of fisheries that focus on more commercially valuable teleost fish species, such as tunas and demersal fishes (Dulvy et al., 2014). The fishing pressure on elasmobranchs is increasing and therefore their extinction risk and conservation interest are being discussed on a global scale (e.g., Dulvy et al., 2014; Davidson et al., 2016). Shallow-water elasmobranchs particularly have a higher risk of extinction or decrease in population because of their constant exposure to coastal fisheries (Dulvy et al., 2014). The ocellate spot (or spiny) skate *Okamejei kenojei* (family Rajidae) is widely distributed 1) in shallow coastal waters of 20–230 m depth (mainly 30–100 m) in the western North Pacific: 2) from the coastal waters off southern Hokkaido, Japan, southward to waters off northern Taiwan, including 3) along the Pacific coast of Japan; 4) within the Sea of Japan; 5) the East China Sea; and 6) the Yellow Sea (Ishihara, 1987; Hatooka et al., 2013; Last et al., 2016). The age at maturity of the species has been estimated to be about 3 years, and females produce at least 300 egg capsules during

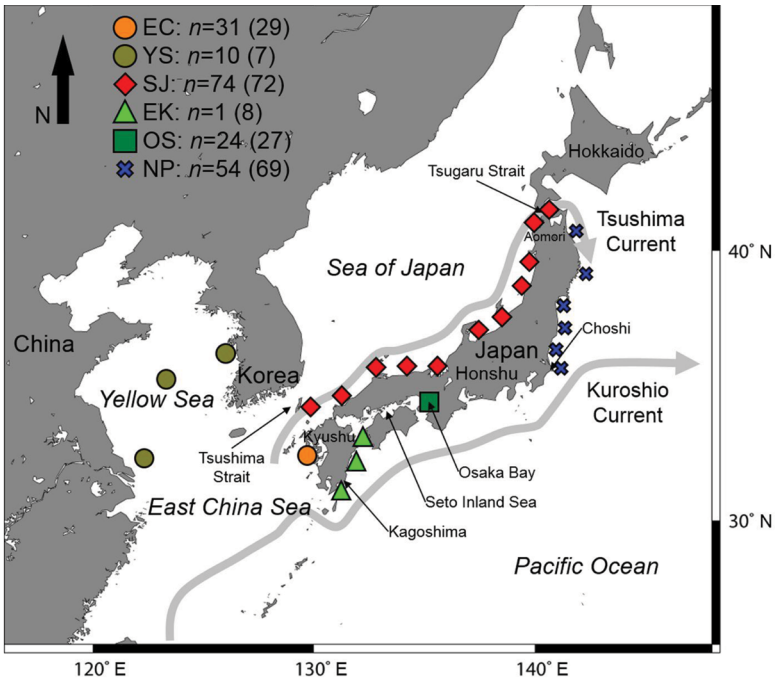

Figure 1

Sampling area and number of examined specimens of *Okamejei kenojei*. Each symbol represents a sampling site. *n*=number of specimens used in mtCR analysis and numbers in parentheses=number of specimens used in morphological comparisons. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

their lifetime, although some may produce up to 600 over 4 years (Ishihara et al., 2009). The egg capsules (43–59 mm length) have tendrils on each corner, which anchor the capsules to the seafloor (Ishiyama, 1958).

Skates generally have a low dispersal ability (Vargas-Caro et al., 2017). In fact, previous tagging studies have suggested that some skates have relatively small home ranges (e.g., Walker et al., 1997), and population genetics have also revealed evidence of a local population structure of skates (e.g., Chevolot et al., 2006). Similarly, *O. kenojei* was formerly recognized as two allopatric species, *Raja porosa*, known from the East China Sea, Yellow Sea, Sea of Japan, and the Pacific coast of southern Japan, and *R. fusca*, known from the Pacific coast of northern Japan (Ishiyama, 1967). According to Ishiyama (1967), the nominal forms could be distinguished by differences in external characters, such as snout length, interorbital width, and cranium shape. Later, Boeseman (1979) suggested that *R. porosa* was a junior synonym of *R. kenojei* (=*Okamejei kenojei*), and was followed by Ishihara (1987), who considered both *R. porosa* and *R. fusca* to be junior synonyms of *O. kenojei* owing to similarities in the distributional pattern of ventral sensory pores and similarities in clasper structure. Nevertheless, Ishihara (1987) recognized several morphological variants among local populations, in particular in reference to individuals from the Pacific coast of northern Japan, which were previously recognized as *R. fusca*, i.e., as the "northern form." Ishihara (1987) suggested that the "northern form" differed from forms in other areas in having a bluntly angled and extremely translucent snout, numerous nuchal thorns, and black spots or reticulated patterns scattered over the entire dorsal surface of the disc. Such morphological divergence among local populations has suggested the existence of a complex population structure within the species.

Okamejei kenojei has considerable economic value in Korea and Japan (Ishihara, 1990; Ishihara et al., 2009; Baeck et al., 2011). Domingues et al (2018) suggested that management and conservation policies for shark and ray fisheries should include information on genetic diversity. However, despite the potential population structuring and high economic value of *O. kenojei*, there have been no studies of genetic structure at the population level for this species—information that is crucial for fisheries management and conservation practices. In some skate species, molecular genetic studies have revealed both population structure and demographic history, as well as the existence of cryptic species based on mitochondrial DNA (mtDNA) sequences (e.g., Valsecchi et al., 2005; Chevolot et al., 2006; Griffiths et al., 2010; Griffiths et al., 2011; Spies et al., 2011; Dudgeon et al., 2012; Frodella et al., 2016; Im et al., 2017; Vargas-Caro et al., 2017). The mtDNA control region (mtCR) has been frequently used to infer population structures within species because of the high levels of nucleotide polymorphism evident in several skate species (Valsecchi et al., 2005). In this context, we examined the population structure of *O. kenojei* by analyzing mtCR sequences and morphological variations and present an outline of population boundaries and their connectivity for fisheries management.

Materials and methods

Samples

For the mtCR analysis and morphological comparisons, a total of 293 individuals of *Okamejei kenojei* were collected from six regions along the Japanese Archipelago (regional populations abbreviated as EC (East China Sea); SJ (Sea of Japan); EK (East coast of Kyushu Is.); OS (Osaka Bay); NP (Pacific coast of northern Japan); and YS (Yellow Sea) (Fig. 1; Suppl. Table). Of these, 194 individuals (EC=31; YS=10; SJ=74; EK=1; OS=24; NP=54) were used for mtCR sequence analysis, 212 individuals (EC=29; YS=7; SJ =72; EK=8; OS=27; NP=69) for morphological comparisons, and 113 individuals

(EC=22; YS=0; SJ=44; EK=1; OS=20; NP=26) for both mtCR analysis and morphological comparisons. The individuals for both mtCR analysis and morphological comparisons only partly overlapped because some of the individuals were in poor condition and could not be used for either of the mtCR analysis or morphological comparisons.

mtCR analysis

Total DNA was extracted from muscle tissue preserved in 99.5% ethanol by using the DNeasy Tissue Kit (Qiagen, Japan) or Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI[1]), according to the manufacturer's protocols. Polymerase chain reaction (PCR) was performed with the primers ElDloopF (5′-TCC CAA AGC CAA GAT TCT GC-3′) and RajinaeP7r (5′-AAA CTG GGA GGG CTG GAA ATC TTG A-3′) (Valsecchi et al., 2005), which amplified 597 base pairs (bp) of mtCR. The fragment was amplified by using the Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) with 15.1 µL PCR reaction mix, including 8.3 µL of distilled water, 1.5 µL of 10× PCR buffer, 1.2 µL of 2.5 mM dNTPs, 1.5 µL of 5 mM each primer, 0.1 µL of EX-Taq polymerase (TaKaRa Bio Inc., Shiga, Japan) and 1 µL of DNA template or 10.2 µL PCR reaction mix, including 2.2 µL of distilled water, 5 µL of KAPA2G Robust HotStart Ready Mix PCR Kit (KAPA Biosystems, Wilmington, MA), 1 µL of 5 mM of each primer and 1 µL of DNA template. The cycling conditions consisted of an initial denaturation cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 15 s (denaturation), 56°C for 15 s (annealing) and 72°C for 30 s (extension), and a single final extension cycle at 72°C for 7 min. Successful amplification was confirmed by electrophoresis of the PCR products on 1% agarose gel, stained with RedSafe (iNtRON Biotechnology, Seoul, South Korea). The PCR products were purified with ExoSAP-IT (Affymetrix Inc., Santa Clara, CA). DNA sequencing was performed with BigDye Terminator Cycle Sequencing Kit,vers. 1.1, and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences determined here were deposited in INSDC (International Nucleotide Sequence Database Collaboration) under accession numbers LC386653–LC386846 (Suppl. Table).

The mtCR sequences were edited with BioEdit, vers. 7.2.5 (Hall, 1999), and aligned with ClustalW interface, available in MEGA 6 (Tamura et al., 2013). A haplotype network was inferred by using the median-joining network method with Network, vers. 5.0.0.1 (Fluxus Technology Ltd, Sudbury, Suffolk, UK). Haplotype diversity (h) and nucleotide diversity (ϖ) were calculated by using Arlequin, vers. 3.5.1.3 (Excoffier and Lischer, 2010). The pairwise F_{ST} values that included information on haplotype frequencies were used for indices of genetic differentiation. The null hypothesis of genetic homogeneity was assessed by 10,000 replications with Arlequin and sequential Bonferroni corrections (Rice, 1989).

Morphological comparisons

Morphological characters were examined after fixation in 10% formalin and preservation in 70% ethanol or 50% isopropanol. For comparisons of coloration among regional populations, photographs of the fresh color of most specimens (before fixation) were taken by digital photography, except for specimens from YS and EK. For morphological comparisons, 13 measurements, those of Last et al. (2008) and Ishiyama (1958), were taken: 1) total length (TL); 2) disc length; 3) disc width; 4) tail length; 5) head length; 6) dorsal snout length; 7) eye diameter; 8) distance between orbits; 9) ventral head length; 10) ventral snout length; 11) prenasal snout length; 12) distance between nostrils; and 13) distance between 1st gill openings. The numbers of nuchal thorns were also counted. Differences in measurements among regional populations were assessed for respective males and females by analysis of covariance (ANCOVA) by using TL as a covariate. A covariance matrix of arcsine-transformed morphometric ratios (with TL as denominator) was prepared for ANCOVA. If significant differences among the regional populations were observed in ANCOVA (P<0.05), post-hoc pairwise comparisons with Holm's adjustment were used to establish differences between individual regional populations. All statistical analyses for morphological comparisons were conducted with R language, vers. 3.1.2 (R Core Team, 2014).

Differences in maturity size or developmental stage among regional populations were estimated from clasper length in males; claspers elongate with growth and are indicative of maturity size and developmental stage (Stehmann, 2002). Following Stehmann (2002), we considered males to be mature if they had both alar and malar thorns and a hard clasper skeleton. The number of rows of tail thorns in females was also compared, the number increasing with growth to a maximum of 5 in the genus *Okamejei* (Ishiyama, 1967; Ishihara, 1987). The number of rows of tail thorns was therefore considered to be indicative of developmental stage. The estimated maturity size in males and females was also assessed by post-hoc pairwise comparisons with Holm's adjustment.

Results

mtCR analyses

The mtCR sequences of 597 base pairs revealed 11 variable nucleotide sites with 10 transitions and 1 transversion, without any deletions and insertions, and a total of 14 haplotypes (haplotype codes were defined as Ok1–14) in 194 individuals. Among the 5 regional

[1] Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1

Genetic diversity indices calculated from mtCR 597 base pairs from 6 regional populations within *Okamejei kenojei*. *n*=number of individuals sampled; *H*=number of haplotypes; *h*=haplotype diversity; π=nucleotide diversity. Range is followed by mean ±standard deviation.

Population	Area	*n*	*H*	*h*	π
EC	East China Sea	31	5	0.6989 ±0.0498	0.0016 ±0.0013
YS	Yellow Sea	10	3	0.5111 ±0.1643	0.0011 ±0.0011
SJ	Sea of Japan	74	7	0.5050 ±0.0654	0.0011 ±0.0010
EK	East coast of Kyushu Is.	1	1	0	0
OS	Osaka Bay	24	2	0.0833 ±0.0749	0.0001 ±0.0003
NP	Pacific coast of northern Japan	54	2	0.2013 ±0.0667	0.0003 ±0.0005
Total		194	14	0.8191 ±0.0137	0.0036 ±0.0022

Table 2

Pairwise F_{ST} (below diagonal) and associated significance (above diagonal) of mtCR 597 base pairs from 5 regional populations (except EK) of *Okamejei kenojei*. +=significant at $P<0.001$ level (after Bonferroni correction). Population abbreviations are given in Table 1.

	EC	YS	SJ	OS	NP
EC		+	+	+	+
YS	0.372		+	+	+
SJ	0.416	0.493		+	+
OS	0.584	0.778	0.634		+
NP	0.592	0.727	0.631	0.838	

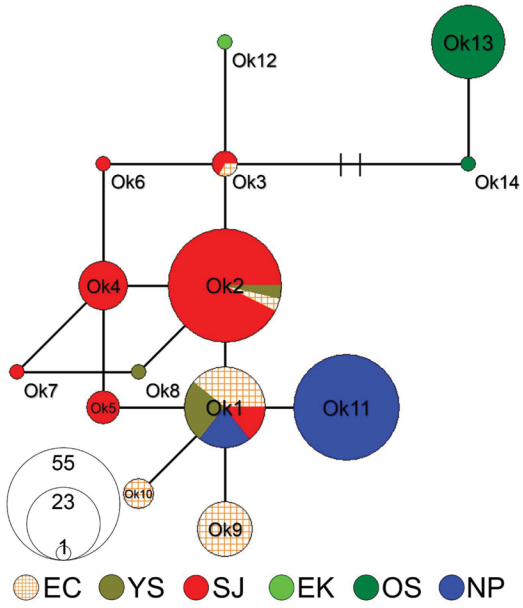

Figure 2

Haplotype network constructed from the mtCR (597 bp) variations of *Okamejei kenojei* from 6 regional populations. Each circle represents a single haplotype. Circle size represents number of individuals. Each bar represents one substitution. Numerals indicate haplotype codes. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

populations, except for EK, the number of haplotypes ranged from 2 to 7, and haplotype (*h*) and nucleotide (π) diversity ranged from 0.0833 to 0.6989 and from 0.0001 to 0.0016, respectively (Table 1). Genetic diversity indices were highest in the EC population, and lowest in the OS population.

Among a total of 14 haplotypes, 11 haplotypes were unique to a regional population (i.e., were private haplotypes), and 5 singletons seen in only one individual (having an unshared haplotype) (Fig. 2). Three haplotypes (Ok1–3) were shared among different regional populations and placed in central positions in the network; one among EC, YS, SJ and NP populations (Ok1), another among EC, YS and SJ (Ok2), and the others between EC and SJ (Ok3). Although most of the haplotypes differed from each other by single substitutions, two unique haplotypes found in the OS population (Ok13 and 14) were characterized by 2 and 3 substitutions, respectively. The frequency of private haplotypes was high, which accounted for 45% (88/194) of all observations (EC=55%, 17/31; YS=10%, 1/10; SJ: 23%, 17/74; EK=100%, 1/1; OS=100%, 24/24; NP=52%, 28/54).

The pairwise F_{ST} values among the five regional populations (except that of EK) ranged from 0.372 to 0.838, and were statistically significant after Bonferroni corrections in all cases ($P<0.001$) (Table 2). The F_{ST} values between OS or NP and the other populations were especially high (OS: 0.584–0.838; NP: 0.592–0.838).

Table 3

Proportional measurements of males (in % of total length) from 6 regional populations of *Okamejei kenojei* and their morphological differentiation significance among populations. Ranges are followed by mean (±standard deviation in parentheses). Numbers under "significance" indicate *P* values in ANCOVA. Populations separated by < are significantly different. NS=not significant. Population abbreviations are given in Table 1.

	EC *n*=13	YS *n*=3	SJ *n*=36	EK *n*=3	OS *n*=10	NP *n*=30	Significance
Total length (mm)	97–408 (258 ±99)	428–455 (441 ±11)	104–453 (334 ±97)	185–390 (271 ±87)	323–435 (409 ±31)	98–566 (376 ±154)	
Disc length	54.0–58.4 (56.7 ±1.2)	54.2–59.0 (56.2 ±2.1)	54.0–61.4 (57.3 ±1.7)	56.6–61.7 (59.1 ±2.1)	55.1–61.1 (57.6 ±1.8)	54.5–60.2 (58.5 ±1.6)	NS
Disc width	63.5–69.9 (67.9 ±2.1)	64.4–70.7 (67.1 ±2.7)	64.2–73.4 (68.1 ±2.7)	65.6–73.5 (69.6 ±3.2)	61.5–72.7 (67.0 ± 3.0)	66.9–74.3 (70.6 ±2.0)	NS
Tail length	48.5–54.4 (50.1 ±1.5)	46.9–50.2 (49.0 ±1.5)	46.2–51.4 (48.5 ±1.2)	45.6–50.8 (47.4 ±2.4)	45.6–49.1 (47.3 ±0.8)	45.7–54.1 (48.1 ±2.5)	NS
Dorsal head length	19.4–23.3 (21.5 ±1.3)	19.0–20.5 (19.8 ± 0.6)	18.9–24.5 (21.1 ±1.5)	21.6–24.5 (22.7 ±1.3)	20.4–23.4 (21.3 ±0.8)	18.0–22.6 (20.6 ±1.0)	NS
Dorsal snout length	12.9–16.8 (15.1 ±1.3)	13.0–13.7x (15.1 ±1.3)	12.5–17.9 (14.4 ±1.3)	14.3–18.1 (15.8 ±1.7)	13.8–16.4 (14.5 ±0.7)	11.9–16.1 (14.2 ±0.9)	NS
Eye diameter	3.0–5.0 (3.7 ±0.6)	2.6–3.4 (2.9 ±0.4)	3.1–4.3 (3.6 ±0.3)	3.4–3.6 (3.6 ±0.1)	2.8–3.5 (3.2 ±0.2)	2.6–4.4 (3.3 ±0.4)	NS
Distance between orbits	4.1–5.2 (4.6 ±0.4)	4.7–5.0 (4.9 ±0.1)	4.3–5.2 (4.8 ±0.2)	3.7–5.1 (4.6 ±0.6)	4.8–5.6 (5.1 ±0.3)	4.9–5.8 (5.3 ±0.2)	< 0.001 [EC, YS, SJ<NP] [SJ<OS]
Ventral head length	26.4–30.5 (29.3 ±1.1)	27.2–29.2 (28.1 ±0.8)	27.2–31.5 (29.5 ±1.1)	29.0–32.8 (29.5 ±1.1)	28.5–31.0 (29.6 ±0.7)	26.0–30.7 (29.0 ±1.1)	NS
Ventral snout length	14.0–17.8 (15.7 ±1.4)	13.0–14.2 (13.5 ±0.5)	12.5–19.1 (14.9 ±1.5)	14.4–18.5 (16.4 ±1.7)	14.2–16.7 (14.7 ±0.7)	11.9–16.5 (14.4 ±1.2)	NS
Prenasal snout length	10.2–14.2 (12.3 ± 1.4)	10.0–10.9 (10.6 ± 0.4)	9.6–15.6 (11.8 ±1.5)	11.5–15.0 (13.4 ±1.4)	11.1–13.3 (11.6 ±0.6)	9.4–13.5 (11.6 ±1.0)	NS
Distance between nostrils	7.0–8.4 (7.8 ±0.4)	7.2–8.2 (7.6 ±0.4)	6.9–8.8 (7.9 ±0.4)	7.6–8.4 (8.0 ±0.3)	7.2–8.3 (7.8 ±0.3)	7.6–9.4 (8.6 ±0.4)	NS
Distance between 1st gill openings	14.1–16.1 (15.0 ±0.6)	13.9–14.8 (14.2 ±0.4)	13.4–17.0 (15.1 ±1.0)	14.1–15.7 (15.1 ±0.7)	13.1–15.1 (14.2 ±0.7)	14.8–17.5 (16.2 ±0.7)	NS

Morphological comparisons

Measurements and count Excluding TL, measurements of 12 characters (1 in males and 11 in females) differed significantly among some of the regional populations (Tables 3 and 4); for example, distance between orbits in males, NP individuals was characterized by a more widely separated orbits than those of other populations (Table 3; Fig. 3C). NP females were easily distinguished from females of other populations in having a broader disc, smaller eye, shorter head and snout, and more widely separated nostrils and gill openings (Table 4; Fig. 3, B, F, H). Furthermore, OS females were also characterized by a smaller eye, and by a longer head and snout than the corresponding features of females of other populations (Table 4; Fig. 3, B and F). Among 4 regional populations (EC, YS, SJ, and EK), EC females had longer tails than females of SJ, SJ females had a shorter head and snout than females of EK, and YS females had a smaller eye than females of EC and SJ (Table 4; Fig. 3, B and F).

Individuals (>ca. 300 mm TL) from NP usually had more than 4 nuchal thorns (range: 3–12) in both sexes, but similar-size individuals from the other 4 populations (except OS) usually had 2 or 3 (range: 2–4) (Fig. 4, A and B). Individuals of both sexes from OS (>ca. 300 mm TL) usually had 2 nuchal thorns (range: 1–3), which were somewhat lower numbers overall than those of other populations. Among smaller specimens of males and females (< ca. 200 mm TL), NP individuals had 2 nuchal thorns, those of other populations having only 1.

Maturity size Clasper length of males fitted a single sigmoid curve against TL (Fig. 5). Specimens with clasper length >24% TL were considered mature because they possessed both alar and malar thorns and hard claspers. The size of mature NP males ranged from 454 to 566 mm TL (average: 493 ±32 mm TL), but between 343 and 455 mm TL (average: 410 ±25 mm TL) in other populations. The estimated maturity size in males differed significantly between NP and other three populations, excluding EK and YS ($P<0.05$).

Number of rows of tail thorns in females also fitted a single sigmoid curve against TL (Fig. 6, A and B) and were similar to clasper growth. Individuals from

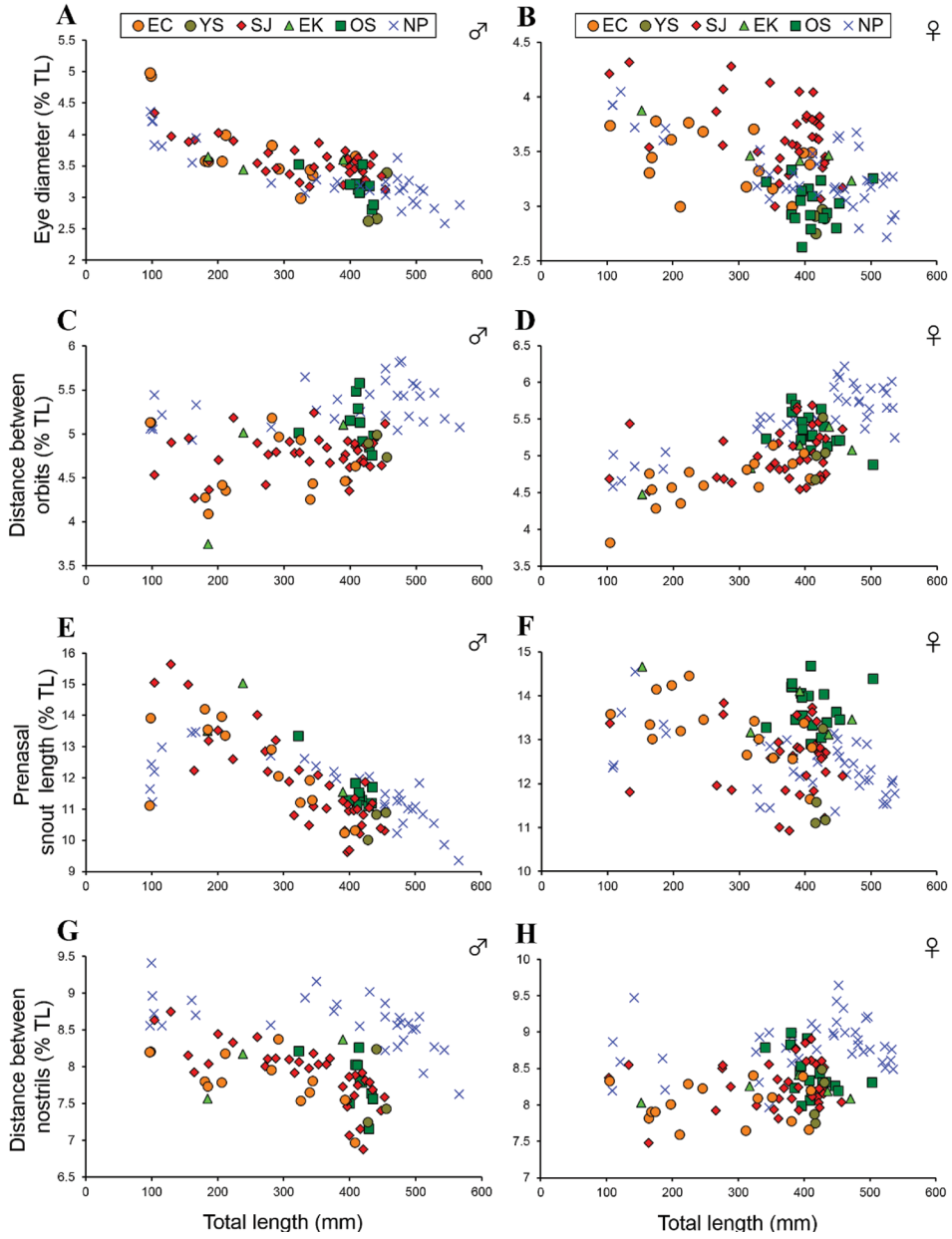

Figure 3

Plots of selected proportional morphometric characters (in % of total length) for 6 regional populations of *Okamejei kenojei*. (**A**, **B**) eye diameter, males and females, (**C**, **D**) distance between orbits, males and females, (**E**, **F**) prenasal snout length, males and females, (**G**, **H**) distance between nostrils, males and females. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

NP that had 5 rows of tail thorns ranged between 450 and 536 mm TL (average: 495 ±28 mm TL), but similar individuals from other populations ranged between 288 and 503 mm TL (400 ±37 mm TL), mostly 350–430 mm TL. Given that the specimens with 5 rows of tail thorns were mature, these estimated maturity sizes for females differed significantly between NP and the other 4 populations, excluding EK ($P<0.05$).

Coloration Typical fresh body coloration of 4 regional populations (except YS and EK) observed in the present study was as follows: EC—dorsal surface dark

Table 4

Proportional measurements of females (in % of total length) from 6 regional populations of *Okamejei kenojei* and their morphological differentiation significance among populations. Ranges are followed by mean ±standard deviation in parentheses. Numbers under Significance indicate *P* values in ANCOVA. Populations separated by < are significantly different. NS=nonsignificant. Population abbreviations are given in Table 1.

	EC *n*=16	YS *n*=4	SJ *n*=36	EK *n*=5	OS *n*=17	NP *n*=39	Significance
Total length (mm)	104–410 (275 ±97)	415–430 (422 ±6)	103–457 (362 ±83)	153–471 (354 ±113)	341–503 (411 ±35)	108–536 (398 ±125)	
Disc length	55.3–59.0 (57.4 ±1.1)	55.7–60.8 (57.9 ±2.0)	54.4–61.2 (57.8 ±1.8)	57.1–61.7 (59.6 ±1.4)	56.5–61.1 (59.4 ±1.2)	54.3–64.5 (59.2 ±1.8)	0.001 [EC, SJ<OS, NP]
Disc width	67.8–72.2 (69.8 ±1.5)	67.5–72.7 (70.5 ±2.1)	64.5–73.7 (69.4 ±2.4)	66.7–74.4 (70.9 ±2.7)	67.2–73.0 (69.6 ±1.7)	65.3–78.1 (71.8 ±2.4)	< 0.001 [EC, SJ, OS<NP]
Tail length	45.4–52.1 (48.7 ±1.7)	42.5–47.4 (45.8 ±1.9)	42.0–51.1 (45.8 ±2.3)	43.2–49.9 (45.4 ±2.4)	41.1–46.4 (44.3 ±1.1)	42.9–52.5 (45.8 ±2.3)	< 0.001 [SJ, OS, NP<EC]
Dorsal head length	20.6–24.1 (22.6 ±0.9)	19.3–22.7 (20.5 ±1.3)	19.8–23.9 (21.9 ±0.9)	22.9–24.0 (23.4 ±0.4)	22.0–24.0 (23.0 ±0.7)	19.8–23.7 (21.4 ±0.9)	< 0.001 [EC, EK, OS<NP] [EK, OS<SJ]
Dorsal snout length	14.3–17.6 (15.9 ±0.8)	14.1–16.2 (14.7 ±0.9)	13.4–16.8 (15.3 ±0.9)	16.1–17.3 (16.6 ±0.4)	16.0–17.7 (16.7 ±0.6)	13.8–16.9 (15.1 ±0.7)	< 0.001 [EC, SJ, NP<OS] [EC, EK<NP] [SJ<EK]
Eye diameter	3.0–3.8 (3.4 ±0.3)	2.8–3.0 (2.9 ±0.1)	3.0–4.3 (3.6 ±0.3)	3.2–3.9 (3.5 ±0.2)	2.6–3.3 (3.0 ±0.2)	2.7–4.1 (3.3 ±0.3)	< 0.001 [EC, SJ, NP<YS, OS] [SJ<NP] [EK<OS]
Distance between orbits	3.8–5.1 (4.7 ±0.3)	4.8–5.5 (5.2 ±0.3)	4.5–5.7 (5.0 ±0.3)	4.5–5.4 (5.0±0.3)	4.9–5.8 (5.4 ±0.2)	4.6–6.2 (5.5 ±0.4)	NS
Ventral head length	27.2–31.4 (29.8 ±1.1)	27.9–32.2 (29.2 ±1.8)	27.3–32.0 (29.8 ±1.0)	30.2–31.4 (30.8 ±0.4)	29.6–32.1 (30.7 ±0.7)	26.7–31.7 (29.5 ±1.1)	0.001 [SJ, NP<OS]
Ventral snout length	14.5–18.2 (16.6 ±0.9)	14.0–16.6 (14.9 ±1.0)	13.6–17.7 (15.9 ±0.9)	16.7–18.8 (17.4 ±0.8)	15.8–17.7 (16.8 ±0.5)	13.9–17.5 (15.4 ±0.8)	0.001 [SJ, NP<OS]
Prenasal snout length	11.7–14.5 (13.2 ±0.7)	11.1–13.3 (11.8 ±0.9)	10.9–13.8 (12.6 ±0.7)	13.1–14.7 (13.7 ±0.6)	12.9–14.7 (13.7 ±0.5)	11.4–14.5 (12.4 ±0.7)	< 0.001 [YS, SJ, NP<OS] [EC, EK<NP] [SJ<EK]
Distance between nostrils	7.6–8.4 (8.0 ±0.3)	7.8–8.5 (8.1 ±0.3)	7.5–8.9 (8.3 ±0.3)	8.0–8.4 (8.2 ±0.1)	8.0–9.0 (8.4 ±0.3)	8.0–9.6 (8.8 ±0.3)	< 0.001 [EC, YS, SJ, EK, OS<NP] [EC<OS]
Distance between 1st gill openings	14.8–16.5 (15.6 ±0.5)	14.7–15.9 (15.5 ±0.4)	14.8–18.0 (16.1 ±0.7)	15.2–16.2 (15.6 ±0.4)	14.9–16.1 (15.5 ±0.3)	15.2–18.5 (16.7 ±0.6)	< 0.001 [EC, YS, SJ, EK, OS<NP] [SJ<OS]

brown or chocolate brown with a pair of pale ocelli and several small paler spots, these markings sometimes indistinct (Fig. 7A); SJ—dorsal surface dark, light, or yellowish brown with 2 pairs of pale or whitish ocelli, and several paler or yellowish spots (Fig. 7B); OS —dorsal surface dark brown or chocolate brown with a pair of pale ocelli and several lighter spots, these markings somewhat indistinct (Fig. 7C); NP—dorsal surface dark brown or grayish brown with 2 pairs of light brown and whitish ocelli, and numerous small black specks and several irregular whitish and yellowish spots, and pairs of whitish spots close together on dorsal tail (Fig. 7D).

Discussion

Population structure of ocellate spot skate

Both nucleotide and haplotype variation seen in mtCR sequences occurred at lower levels in the 5 populations of *O. kenojei* (Table 1), than those seen in other demersal bony fishes (Heyden et al., 2010). Low levels of genetic diversity have been frequently reported in other elasmobranchs (e.g. Hoelzel et al., 2006; Cannas et al., 2010; Daly-Engel at al., 2010; Veríssimo et al., 2010; Vargas-Caro et al., 2017), because they exhibit slower rates of genetic evolution than other vertebrates (Martin et al., 1992; Martin, 1999). In addition, Feutry et al. (2014) suggested that mtCR in elasmobranchs could be under higher evolutionary constraints than those of other mitochondrial regions. In the present study, however, the significant F_{ST} indices among the regional populations revealed a fine-scale population structure within *O. kenojei*, clearly indicating the limits of gene flow (Table 2). In fact, 11 of a total of 14 haplotypes were unique to each regional population (Fig. 2, Ok4–14), and the frequency of private haplotypes was high, which accounted for 45% of all observations. The results of the morphological comparisons, including measurements (Tables 3 and 4; Fig. 3), nuchal thorn counts (Fig. 4), sizes at maturity in males and females (Figs. 5 and 6), and coloration (Fig. 7) also almost corresponded with the results from the mtCR

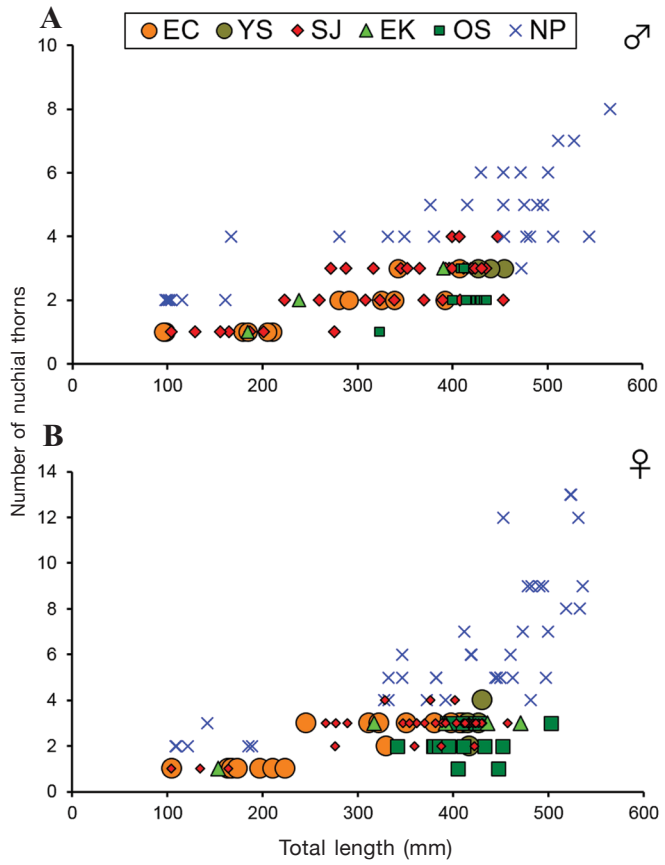

Figure 4

Plots of nuchal thorn numbers from 6 regional populations of *Okamejei kenojei*: (**A**) males, (**B**) females. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

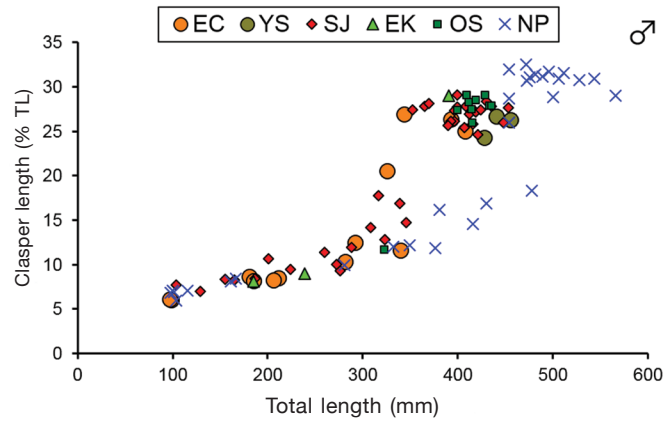

Figure 5

Plots of clasper length (as % of total length) of males from 6 regional populations of *Okamejei kenojei*. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

analysis; our results supported the uniqueness of the regional populations and suggested limits to migration. Such fine-scale population structures in other skate species have already been well documented (e.g., Chevolot et al., 2006; Griffiths et al., 2010, 2011; Dudgeon et al., 2012; Im et al., 2017; Vargas-Caro et al., 2017) and are considered to have been derived from the constraints of low dispersal because of large benthic egg capsules and the absence of a pelagic larval stage. Vargas-Caro et al. (2017) suggested that smaller skates inhabiting coastal areas had well-defined population structures than larger skates inhabiting offshore areas because body size and habitat are related to dispersal potential. Maximum size of members of the family Rajidae vary from 33 cm to more than 2 m TL, and most members occur from continental shelves to more than 4000 m depth (Last et al., 2016). Studies on migration and habitat preference of *O. kenojei* are few; however, the species is a typical small skate maturing at ca. 400–500 mm TL and inhabits coastal areas shallower than 150 m depth (Ishihara et al., 2009; Hatooka et al., 2013; Last et al., 2016).

In comparison with the OS population, however, morphological divergence of the NP population was somewhat remarkable against genetic differentiation; both genetic and morphological data sets did not agree completely. Among 12 measurements of characters (excluding TL), as well as nuchal thorn counts, maturity size, and coloration, NP individuals were distinguished by 8 of the 15 characters (disc width, dorsal head length, distance between orbits, distance between nostrils, distance between 1st gill openings, maturity size, nuchal thorn, and coloration), but OS individuals were distinguished by 6 of the 15 characters (dorsal snout length, eye diameter, ventral head length, ventral snout length, prenasal snout length, and nuchal thorn) (see Tables 3–4; Figs. 3–7). It should be noted that we examined a single maternally inherited gene (mtCR) which cannot be considered derived from male-mediated gene flow. This single gene may also explain the partial mismatch between our mtCR analysis and our morphological comparisons. Furthermore, genetic differences may also be random to some extent; Spies et al. (2006) indicated that genetic differences at mtDNA cytochrome c oxidase subunit I (COI) were not observed even among different species. In another case, incongruence between genetic and morphological variation may be due to adaptations to different environments. For example, adaptive evolutionary changes in life history, physiology, and phenotype in the winter skate (*Leucoraja ocellata*) have been associated with epigenetic regulation that causes changes in gene expression for adaptation to different environments without obvious genetic change (Kelly and Hanson, 2013; Lighten et al.,

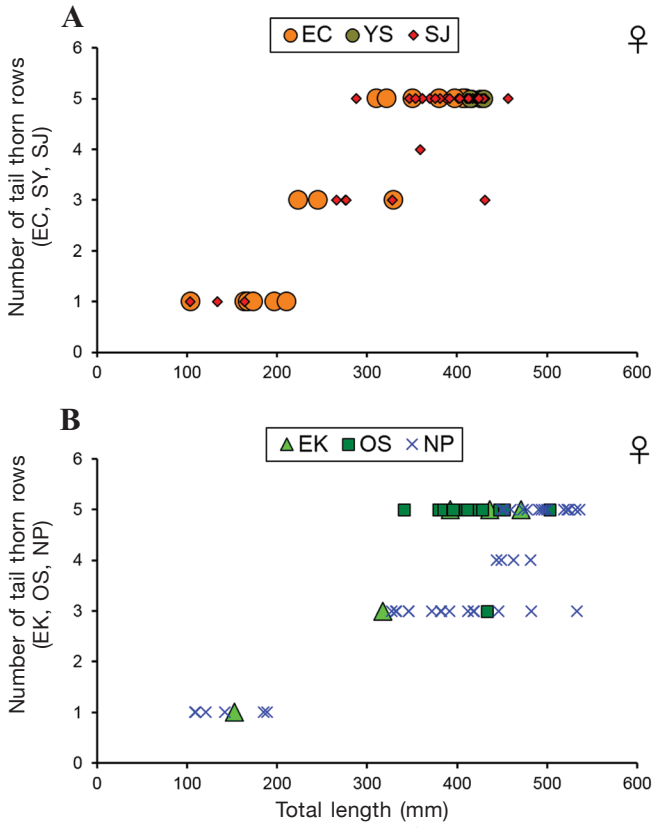

Figure 6

Plots of number of tail thorn rows of females within 6 regional populations of *Okamejei kenojei*. (**A**) EC, YS, and SJ, (**B**) EK, OS, and NP. EC=East China Sea; YS=Yellow Sea; SJ=Sea of Japan; EK=East coast of Kyushu Is.; OS=Osaka Bay; NP=Pacific coast of northern Japan.

2016). Therefore, further study of the species based on nuclear DNA or other mtDNA markers, or both, would reveal more details in population structure and associated morphological variations.

In the haplotype network for ocellate spot skate, the OS haplotypes (Ok13 and Ok14) were distant from others, but these two haplotypes are closely related to each other in the network (Fig. 2). In addition, OS individuals were characterized by a smaller eye, longer head and snout, and fewer nuchal thorns than those in other regional populations (Tables 3 and 4; Figs. 3 and 4), clearly suggesting significant isolation of the OS population from the other populations. In fact, the OS individuals were collected only from Osaka Bay, situated at the innermost part of the Seto Inland Sea (Fig. 1), and *O. kenojei* were not collected from other marginal areas of Osaka Bay. This finding may suggest that this species is inhabiting patchy areas and exhibiting discontinuity in around the Seto Inland Sea, and its distributional area is also isolated from other areas.

Although one of two haplotypes of the ocellate spot skate in NP was shared with other regional populations (Fig. 2, Ok1), NP individuals clearly differed in morphological characters from other populations, by having a broader disc, smaller eye, shorter head and snout, widely separated orbits, nostrils and gill openings, a greater number of nuchal thorns and numerous small black specks present on its dorsal surface (Figs. 3, 4, and 7). The size at maturity of males and females was also greater than that in other regional populations (Figs. 5 and 6). Such morphological divergence of NP from the other regional populations agreed well with the findings of Ishiyama (1967) and Ishihara (1987). Although the former recognized NP individuals as a separate species, the sharing of a haplotype with other regional populations (Ok1) indicates the absence of reproductive isolation between them. Although gene flow between NP and other regional populations has been limited, as suggested by a significant F_{ST}, we treated the NP as the area of a local population within *O. kenojei*, as suggested by Ishihara (1987). Gene flow between it and the other populations seems to be restricted by the Tsugaru Strait between the Sea of Japan and Pacific Ocean (Fig. 1)—further evidence of a phylogeographical break across the Tsugaru Strait (Kai et al., 2014; Kai and Yamanaka[2]).

Size at maturity was clearly larger in NP individuals than in other regional populations (Figs. 5 and 6). Such differences between Sea of Japan, Sea of Okhotsk, and Pacific Ocean populations have been noted for several fish species. For example, Tamate (2012) showed that the body size of the anadromous masu salmon (*Oncorhynchus masou*) returning to the coast from the Sea of Okhotsk was significantly smaller than that of the same species from the Pacific and Sea of Japan coasts. In contrast, Tohkairin et al. (2014) showed that the maximum body size of the marbled snailfish (*Crystallichthys matsushimae*) was much smaller in the Sea of Japan population than in the Pacific and Sea of Okhotsk population. Because the genetic divergence between NP and other populations was at the intraspecific level, some other evolutionary factors may have shaped the geographic size variations in *O. kenojei*.

It should be noted that the NP haplotypes were similar to those of SJ, YC and EC, but distantly related to those of OS, suggesting that NP was colonized from SJ through the Tsugaru Strait, not from OS along the Pacific coast of Japan. Although no obvious geographic barriers are known between OS and NP, the absence of a major population of *O. kenojei* from the Pacific coast of central Japan suggests that the strong Kuroshio Current, originating from tropical waters, may have prevented dispersal of the species. In fact, the main

[2] Kai, Y., and T. Yamanaka. 2017. Tsugaru Straight hybrid zone between two Japanese marine sculpins (genus *Cottiusculus*). [Available at website, accessed May 2018]

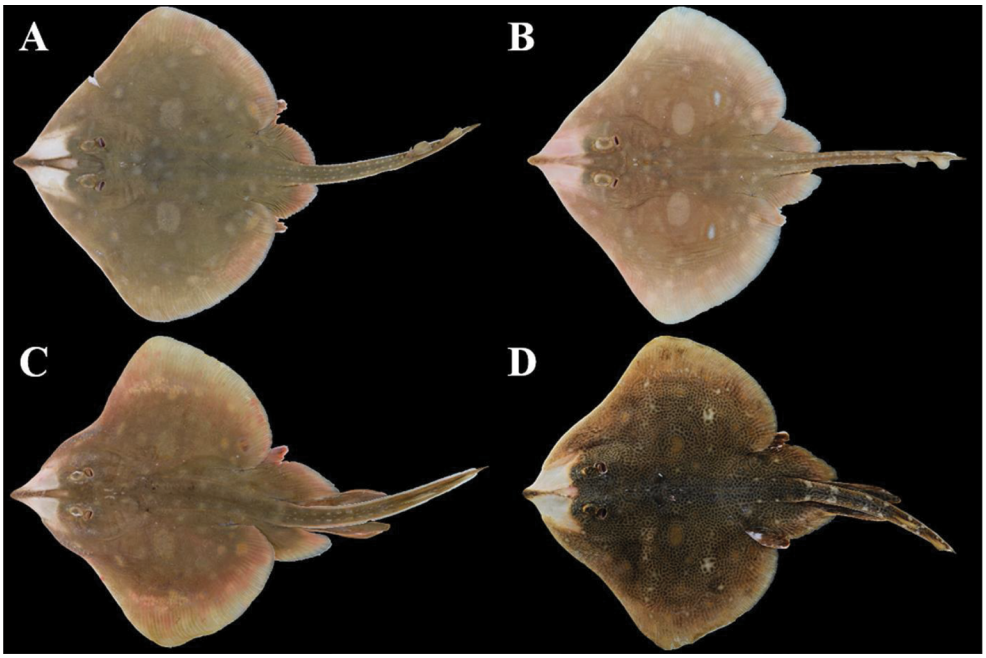

Figure 7

Typical coloration of 4 regional populations of *Okamejei kenojei*. (**A**) FAKU 140518, 384 mm total length (TL), adult female, East China Sea (**B**) BSKU 116603, 289 mm TL, immature male, Sea of Japan, (**C**) FAKU 140521, 430 mm TL, adult male, Osaka Bay, (**D**) BSKU 112173, 462 mm TL, adult male, Pacific coast of northern Japan.

route of the Kuroshio Current has been stable at least since the last glacial period (Kojima et al., 2000), shaping the distributional range of various coastal species along the Japanese Archipelago (Matsuura, 2012).

The genetic and morphological divergence of ocellate spot skate populations between the Sea of Japan and East China Sea, suggested by significant F_{ST} values (Table 2) and measurements (Table 4), is mirrored in several coastal marine species. For example, Liu et al. (2007) disclosed 3 mtDNA lineages in redlip mullet (*Chelon haematocheilus*)—lineages that diverged among the Sea of Japan and the East and South China seas during Pleistocene glaciations. In addition, genetic and morphological divergence was also detected between EC and YS, which are separated by the Tsushima Strait and Tsushima Current (Fig. 1). Similarly, genetic divergence between the Yellow Sea and Japanese coast of the East China Sea has been noted in several marine fishes, including the white croaker (*Pennahia argentata*), spotted halibut (*Verasper variegatus*), and gizzard shad (*Konosirus punctatus*) (see Han et al., 2008; Sekino et al., 2011; Gwak et al., 2015). As noted in the above examples with *C. haematocheilus* and *K. punctatus*, 3 regional populations (EC, YS, and SJ) of *O. kenojei* also diverged on account of Tsushima Strait and the Tsushima Current, which act as geographical barriers.

Implications for fisheries management Small and benthic (or benthopelagic) elasmobranch species tend to exhibit more distinct population structures at smaller spatial scales than those exhibited by large pelagic species (Larson et al., 2017). In fact, movement patterns of some skate species investigated in tagging studies, have shown that most had a small home range (within a 100-km^2 area) (Walker et al., 1997; Hunter et al., 2005; King and McFarlane, 2010; Neat et al., 2015; Farrugia et al., 2016; Vargas-Caro et al., 2017) in spite of their potential ability to migrate hundreds of kilometers (King and McFarlane, 2010). Regarding *O. kenojei*, a lack of tagging studies has meant no insights on migratory range, although the extensive population structure found in this study suggests that the species has a small home range.

Because fish populations have a unique set of dynamics, such as recruitment, growth, and mortality that influence current and future status, it is important, even within the same species (Pope et al., 2010), to define management units (MUs) based on population structure and to execute subsequent assessment and management of stocks according to each MU. Clearly, MUs for *O. kenojei* need to be set according to the population structure of the species because of the genetic and morphological differentiation of populations among the investigated regions. We suggest at least 6 MUs for

O. kenojei: East China Sea (EC), Yellow Sea (YS), Sea of Japan (SJ), east coast of Kyushu Is. (EK), Osaka Bay (OS), and Pacific coast of northern Japan (NP). In particular, careful monitoring of population (stock) abundance of local populations characterized by low genetic diversity and geographical isolation, such as the abundance of OS and NP populations of *O. kenojei,* is necessary. Tagging studies, which may reveal some aspects of migratory behavior, may also help to clarify factors contributing to the population structure of *O. kenojei*.

Acknowledgments

We are very grateful to N. Nakatsuka, S. Ikeguchi, T. Kintaka, T. Mochizuki, T. Imaizumi, T. Mukai, R. Komoto, Y. Uchida, S. Suzuki, D. Noguchi, J.-K. Kim, and K. Sakai for collecting samples. We sincerely thank the captains, officers and crews of the RV *Wakataka-maru* and RV *Taka-maru* (Fisheries Research and Education Agency), and RV *Shimane-maru* (Shimane Prefectural Fisheries Technology Center) for assistance in the field. We also thank G. S. Hardy (Ngunguru, New Zealand) for his critical reading of the manuscript.

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