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Multiplex Polymerase Chain Reaction Method with Species-specific Primers for Differentiation of Two Closely Related Fish Species, *Beryx splendens* and *B. mollis* (Actinopterygii: Beryciformes)

Kazuya NISHIDA[1,2*], Satoru N. CHIBA[3], Kay SAKUMA[4], Ryouichi HIGASHI[5], Nobuaki SUZUKI[6], Mai MIYAMOTO[1,7], Shiroh YONEZAKI[8], Kouichi HOSHINO[9] and Kota SAWADA[1]

[1] Highly Migratory Resources Division, Fisheries Stock Assessment Center, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Yokohama, Japan
[3] Demersal Fish Resources Division, Fisheries Stock Assessment Center, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Kushiro, Japan
[4] Demersal Fish Resources Division, Fisheries Stock Assessment Center, Fisheries Research Institute, Japan Fisheries Research and Education Agency, Niigata, Japan
[5] Arai High School, Kosai, Japan
[6] Fisheries Data Sciences Division, Fisheries Stock Assessment Center, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Yokohama, Japan
[8] Socio-Ecological Systems Division, Fisheries Stock Assessment Center, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Yokohama, Japan
[9] Aquatic Organisms Collection Management Section, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Nagasaki, Japan

Abstract

Distinguishing between *Beryx splendens* and *Beryx mollis* (Actinopterygii: Beryciformes) on the basis of external morphology is difficult, and a reliable method of differentiation must be established. In this study, we developed a multiplex polymerase chain reaction (PCR) method with species-specific primers for distinguishing *B. splendens* from *B. mollis*. In total, 146 specimens were collected in the North Pacific Ocean, East China Sea, and Southwest Indian Ocean. Provisionally, 115 specimens were identified on the basis of the number of pyloric caeca. Phylogenetic analysis was also performed by using the 146 partial sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene and sequences from a DNA database. Identification by the number of pyloric caeca was consistent with the result of the COI phylogeny, and we surmised that the use of the COI sequences was effective for the differentiation of the two species. Multiplex PCR with species-specific primers was subsequently developed on the basis of the partial COI sequences. All of the specimens used in the molecular analyses were successfully identified as *B. splendens* or *B. mollis* via electrophoresis of the PCR products amplified using the species-specific PCR primers.

Discipline: Fisheries
Additional key words: commercial fish, cytochrome *c* oxidase subunit I (COI), DNA-based species differentiation, mitochondrial DNA, pyloric caeca

Introduction

The genus *Beryx* currently comprises three species—*Beryx splendens* Lowe, 1834; *B. mollis* Abe, 1959; and *B. decadactylus* Cuvier, 1829 (Busakhin 1982, Hayashi 2013). It is a benthopelagic fish genus, family

Present addresses:
[2] Lake Biwa Branch Office, National Institute for Environmental Studies, Otsu, Japan
[7] Environmental Management Unit, Environmental Consulting Department, JAPAN NUS Co., Ltd., Shinjuku, Japan
*Corresponding author: nishida.kaz@gmail.com
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Berycidae (Actinopterygii: Beryciformes), and inhabits the slopes of continental shelves and seamounts in tropical and subtropical areas (Paxton 1999, Shotton 2016). These species attract considerable attention as they are important resources for bottom fisheries; 14,500 metric tons were captured each year between 2010 and 2014 worldwide (mainly in the Northwest and South Pacific Ocean, West Indian Ocean, and Southeast Atlantic Ocean) by Japanese, Korean, Spanish, and Chilean fishing vessels (Watari et al. 2017). Although the current stock status of *Beryx* species is mostly unclear, the number of *B. splendens* catches have been depleted in some waters, such as in the high seas off Chile, probably because of a rapid increase in fishing pressure (Wiff et al. 2012). Additionally, the impacts of trawl fisheries on both deep-sea ecosystems and individual species, including *Beryx* species, have been widely recognized. Therefore, fisheries management procedures have recently been applied to some regional stocks of *Beryx* species (Watari et al. 2017, Fisheries Agency of Japan 2018, Sawada et al. 2021).

Morphological differences among the three *Beryx* species have been previously reported (Table 1). *Beryx decadactylus* is distinguishable from the other two species by its greater body depth and greater number of soft rays on the dorsal fin, but *B. splendens* and *B. mollis* are morphologically similar regarding both morphometrics and meristics. For instance, the number of soft dorsal-fin rays is considered to be a distinctive numeric character in each of them, but it overlaps between the two species (Yoshino et al. 1999, Yoshino & Kotlyar 2001, Hayashi 2013, Ikeda & Nakabo 2015; Table 1). Therefore, *Beryx mollis* specimens may have been misidentified as *B. splendens* specimens in previous surveys (Shishidou & Kamino 2010, Yanagimoto & Kobayashi 2012). By contrast, the number of pyloric caeca does not overlap between the two species (Yoshino et al. 1999, Yoshino & Kotlyar 2001; Table 1) and can be useful for species distinction. However, this character cannot be assessed without dissection. Moreover, it may be misleading in young individuals that may not have attained a full number of pyloric caeca, and it is not available in gutted samples. Previous studies have shown that the shape of the posterior nostril and shape of the posterior edge of the scales on the dorsal portion of the body can be used as identification keys (Yoshino et al. 1999, Hayashi 2013, Ikeda & Nakabo 2015; Yanagimoto & Chow 2020; Table 1), but the use of nonnumeric characters can lead to misidentification in degraded specimens. Moreover, these methods may not be effective in processed specimens.

Molecular techniques are promising for identifying closely related, morphologically similar species. Further, they are useful for species identification of various types of specimens (e.g., fish larvae and eggs; Chow et al. 1993, Akimoto et al. 2002) and processed products, such as gutted or even canned fish (Infante et al. 2004). The differences in the mitochondrial DNA (mtDNA) sequence between *B. splendens* and *B. mollis* have been reported, and species identification based on molecular techniques, such as restriction fragment length polymorphism (RFLP) and sequencing, has been developed (Akimoto et al. 2002, 2003, 2005, 2006, Yanagimoto & Kobayashi 2012).

Multiplex polymerase chain reaction (PCR) using

Table 1. Main morphological differences among the three *Beryx* species

	Beryx splendens	*B. mollis*	*B. decadactylus*
Shape of posterior nostril [a, c, d, e]	Slit	Ellipse	Ellipse
Number of soft dorsal-fin rays [a, b, c, d]	13-15	12-13	18-20
Posterior edge of scales on dorsal portion of the body [a, c, d]	Smooth	Serrated	?
Number of pyloric caeca [a, b]	27-36	12-20	70 or more
Standard length/body depth [c, d]	2.5 or more	2.5 or more	2.2 or less

[a] Yoshino et al. 1999, [b] Yoshino & Kotlyar 2001, [c] Hayashi 2013, [d] Ikeda & Nakabo 2015,
[e] Yanagimoto & Chow (2020)

species-specific primers for mtDNA polymorphisms has been used for species identification (Hare et al. 2000, Rocha-Olivares et al. 2008, Ravago-Gotanco et al. 2010, Cooke et al. 2012, Higashi et al. 2016). This identification method requires only a single PCR and agarose gel electrophoresis; it is faster and more convenient compared with other DNA-based species identification methods, such as RFLP and sequencing. However, no multiplex PCR method for distinguishing between *B. splendens* and *B. mollis* is presently available. A singleplex PCR method to differentiate *B. mollis* from *B. splendens* by using the mtDNA cytochrome *c* oxidase subunit I (COI) gene has been developed (Yanagimoto 2012). However, the PCR primers and protocol used were not described in detail, and the results of the study are currently not reproducible. Moreover, a singleplex PCR without an internal positive control carries the possibility of false negativity (amplification failure in *B. mollis*).

In this study, we designed novel primers on the mtDNA COI region for amplifying species-specific DNA fragments and optimized a protocol that can reliably distinguish between *B. splendens* and *B. mollis*. The validity of our novel method was tested by using tissue specimens obtained by research and commercial vessels. Further, we assessed variations in the number of pyloric caeca in the two *Beryx* species according to body size or geographic region to evaluate the usefulness of this trait in differentiating the two *Beryx* species. Our DNA-based species distinction method will potentially contribute to the management of *Beryx* species.

Materials and methods

1. Specimen collection

In total, 146 specimens were collected from the Emperor Seamount waters in the North Pacific Ocean, East China Sea, and Southwest Indian Ridge and Madagascar Ridge in the Southwest Indian Ocean (Table 2, Fig. 1). Of these, 82 specimens were sampled as *B. splendens* between 2013 and 2016 from the high seas of the North Pacific Ocean and Southwest Indian Ocean by Japanese commercial vessels and Research Vessel *Kaiyo-Maru*. Forty-three specimens were collected as *B. splendens* in 2014 from Japan's exclusive economic zone in the East China Sea by a fishing vessel. Additionally, five *B. splendens* specimens from the East China Sea and 16 *B. mollis* specimens from the East China Sea, registered at the Fisheries Technology Institute, Japan Fisheries Research and Education Agency (Registration Numbers: *B. splendens*, SNFR14675-78 and 16411; *B. mollis*, SNFR14461-63, 14474-76, 14498, 14499, 14505, 14506, 14565, 14858-60, 16414, and 16415), were used.

2. Morphological analysis

The pyloric caeca of 105 *B. splendens* (fork length [FL]: 166.1 mm-339.6 mm) and 10 *B. mollis* (FL: 134.8 mm-140.2 mm) specimens were counted onboard or in laboratories to confirm the species identification. The results were compared with those for *B. splendens* and *B. mollis* in literature (Yoshino et al. 1999, Yoshino & Kotlyar 2001; Table 1). In general, the number of pyloric caeca increases with growth in many fish species (Northcote & Paterson 1960, Costalago & Palomera 2014). In *B. splendens*, because the numbers of anal-fin

Table 2. Sampling regions and number of specimens of *Beryx splendens* and *B. mollis*

Sampling regions	Species name	
	Beryx splendens	*Beryx mollis*
North Pacific Ocean	40	
East China Sea [a]	48	16
Southwest Indian Ocean	42	
Total	130	16

[a] Five *B. splendens* specimens and all *B.mollis* specimens are registered at the Fisheries Technology Institute, Japan Fisheries Research and Education Agency.

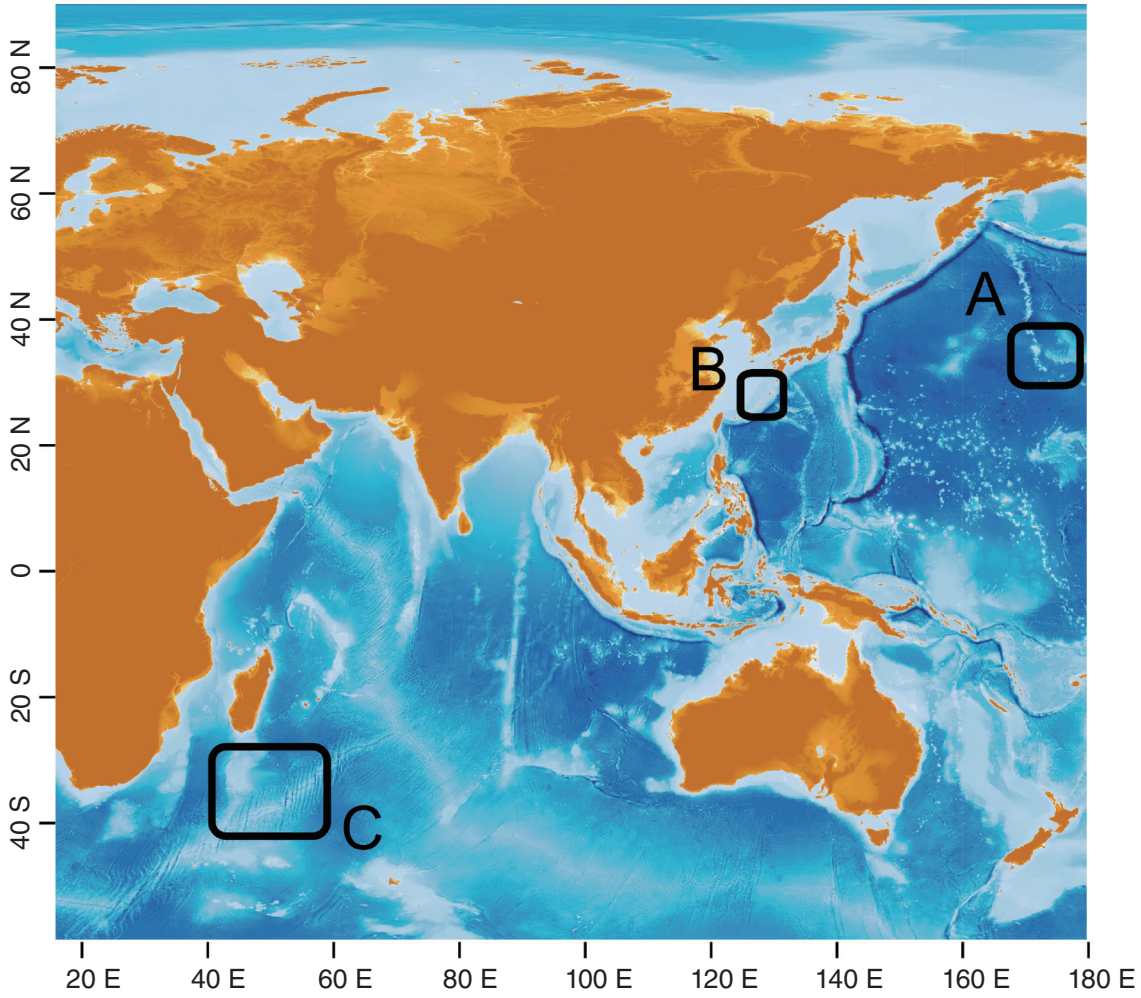

Fig. 1. Map of sampling locations
Squares indicate sampling locations in the Emperor Seamount waters of the North Pacific Ocean (A), the East China Sea (B), and the Southwest Indian Ridge and Madagascar Ridge in the Southwest Indian Ocean (C).

rays and lower gill rakers on the first gill arch show geographic variation (Yoshino & Kotlyar 2001, Yanagimoto 2004), we hypothesized that the number of pyloric caeca would also vary according to body size or geographic region, or both. Therefore, poisson regression models were constructed using the *B. splendens* data to test the influence of FL and geographic region on the number of pyloric caeca (Table 3). The number of pyloric caeca was set as a response variable, and FL and geographic region were added as explanatory variables. Akaike's information criterion (AIC) was used for model selection. A lower AIC of the null model than that of the models would suggest that neither geographic variation nor ontogenetic change were observable for the number of pyloric caeca, at least in the water bodies and size range included in the present study. These statistical analyses were conducted with R ver. 4.0.3 (R Development Core Team 2020). This analysis was not performed in *B. mollis* because of the insufficient sample size (10 specimens) and the narrow range of FL (134.8 mm-140.2 mm).

3. Sequencing and phylogenetic analysis

Total DNA was extracted from each muscle tissue sample or lysate of muscle tissues in TNES 6M-urea buffer (Asahida et al. 1996) using a Gentra Puregene Core Kit (Qiagen, Hilden, Germany). PCR conditions comprised 30 cycles of denaturation (94°C, 30 s), annealing (63°C, 60 s), and extension (72°C, 90 s). PCR was conducted in a TaKaRa Dice Standard TP650 thermal cycler (TaKaRa Bio, Shiga, Japan). Two primers, L5956 (Yanagimoto & Kobayashi 2012) and COI722_R, were used to amplify approximately 690 bp from the mtDNA COI region (Table 4). The latter was

Table 3. Poisson regression models of the relationship between the characteristics (number of pyloric caeca and fork length) of *Beryx splendens* specimens and the water bodies from which these specimens were collected

Models	Formulae [a]	β_1	β_2	$\beta_{3\ sio}$ [b]	$\beta_{3\ npo}$ [b]	$\beta_{4\ FL:A\ sio}$ [c]	$\beta_{4\ FL:A\ npo}$ [c]	AIC [d]
1. Null	$y_i = \exp(\beta_1)$	3.36** (0.02)						581.1
2. Size dependent	$y_i = \exp(\beta_1 + \beta_2 x_i)$	3.34** (0.09)	0.00 (0.00)					583.0
3. Area dependent	$y_i = \exp(\beta_1 + \beta_3 z_i)$	3.37** (0.03)		−0.06 (0.05)	−0.01 (0.04)			583.7
4. Area and size dependent	$y_i = \exp(\beta_1 + \beta_2 x_i + \beta_3 z_i)$	3.24** (0.21)	0.00 (0.00)	−0.05 (0.05)	0.04 (0.08)			585.3
5. Area, size and interaction	$y_i = \exp(\beta_1 + \beta_2 x_i + \beta_3 z_i + \beta_4 x_i \cdot z_i)$	3.24** (0.33)	0.00 (0.00)	−0.12 (0.50)	0.06 (0.41)	0.00 (0.00)	−0.00 (0.00)	589.2

[a] y_i is the number of pyloric caeca in each specimen i. β_1 is the intercept; β_2, β_3, and β_4 are slopes of each explanatory variable; x_i is the fork length of each specimen i, z_i is the water body where each specimen i was collected; $x_i \cdot z_i$ is the interaction between x_i and z_i.
[b] β_3 of the Southwest Indian Ocean ($\beta_{3\ sio}$) and β_3 of the North Pacific Ocean ($\beta_{3\ npo}$) are the values when β_3 of the East China Sea is zero.
[c] $\beta_{4\ FL:A\ sio}$ and $\beta_{4\ FL:A\ npo}$ indicate interactions between fork length and water body (Southwest Indian Ocean or North Pacific Ocean) when β_4 of the East China Sea is zero. Values in parentheses indicate standard errors.
[d] AIC: Akaike information criterion. Double asterisks indicate $P < 0.01$ by the Wald-test.

Table 4. Primers used in this study for amplification and sequencing of the mitochondrial gene COI of *Beryx splendens* and *B. mollis*

Methods	PCR primers (5′-3′) Forward		Reverse	Target species	Expected product size (bp)
PCR and Sequencing	L5956 (CACAAAGACATT GGCACCCT)	×	COI722_R (GGATGGCCAAA GAATCAGAA)	Both	689
Multiplex PCR	L1803-16S (AGTACCGCAAGG GAAAGCTGAAA)	×	H2590-16S (ACAAGTGATTG CGCTACCTT)	Both	906
	COI255LBmMod (GGAAACTGACTA ATCCCCATG)	×	BeryxCOIR02 (CGGGGTCAAA GAAGGTTGTA)	*Beryx molllis*	433
	COI360LBsMod (CCTCTTCTGGGG TAGAATCA)	×	BeryxCOIR02	*B.splendens*	327

newly designed for this PCR in the present study. PCRs were performed in 20 μL reaction volumes containing 0.1 μL of 1 U *Taq* DNA polymerase (TaKaRa Ex *Taq*, TaKaRa Bio), 2.0 μL of 10× *Taq* buffer, 1.6 μL of each dNTP mixture (2.5 mM each), and 1.0 μL of each 10 μM primer. The PCR products were individually purified using an Exo-SAP-IT PCR product cleanup reagent (Affymetrix Inc., Santa Clara, CA, USA). Cycle sequencing reactions were performed with a BigDye Terminator Kit 3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The reaction products were then purified via ethanol precipitation and then directly sequenced using a PRISM 310 Genetic Analyzer (Thermo Fisher Scientific).

DNA sequences were assembled using ATGC ver. 8 (GENETYX, Tokyo, Japan) and collapsed into haplotypes by using DNaSP ver. 5.1 (Librado & Rozas 2009). Sequences of the three *Beryx* species were obtained from the DNA database (INSDC; International Nucleotide Sequence Collaboration) (*B. splendens*: AB679231 and JF492952; *B. mollis*: AB679215; *B. decadactylus*: JF492951 and AB679219; Table 5) and added to the dataset. These sequences were aligned using MAFFT ver. 7 with default settings (Katoh & Standley 2013). Phylogenetic relationships among the haplotypes were examined by using the maximum likelihood method implemented in RAxML ver. 8.0.0 (Stamatakis 2014). For the outgroup taxon, *B. decadactylus* was used. We used a general time-reversible model with sites following a discrete gamma distribution (GTR+Γ), recommended by the author of the program (Stamatakis 2016). The robustness of the maximum likelihood tree was assessed using a rapid bootstrap analysis with 1,000 replications.

4. Development of species-specific primers and protocol for multiplex PCR

Species-specific primers for *B. splendens* and *B. mollis* were designed using Primer 3 Plus (Untergasser et al. 2007) on the basis of the COI sequences in the DNA database and those obtained in this study (Table 5). The 255th and 360th base pairs of the COI sequences in *B. splendens* and *B. mollis* were A and G, respectively (Fig. 2). Thus, two species-specific forward primers (COI255LBmMod and COI360LBsMod; Table 4 and Fig. 2), the base pairs of which at the 3′-end matched the species-specific bases complementarily, were designed. Mismatch bases were installed in the third position from the 3′-end to decrease the melting temperature on

Table 5. Mitochondrial COI sequences that were obtained from the DNA database (INSDC) for the three *Beryx* species and used in this study

Registered species name	Number of sequences	Accession numbers	Water bodies where specimens were collected
Beryx splendens	21	EF609297	Tasmanian coastal waters, South Pacific Ocean
		JF492952-6	Agulhas Bank, Southwest Indian Ocean
		JF952688	Japanese coastal waters, Northwest Pacific Ocean,
		MF414880	South China Sea
		AB679227-36, AP002939, DQ027987, DQ996312	Unknown
B. mollis	30	AB679215-18	Eastern Indian Ocean
		AB860129-42, DQ993168, KP244504-11, KU892805, KU943271-2	Unknown
B. decadactylus	2	JF492951	Agulhas Bank, Southwest Indian Ocean
		AB679219	Eastern Indian Ocean

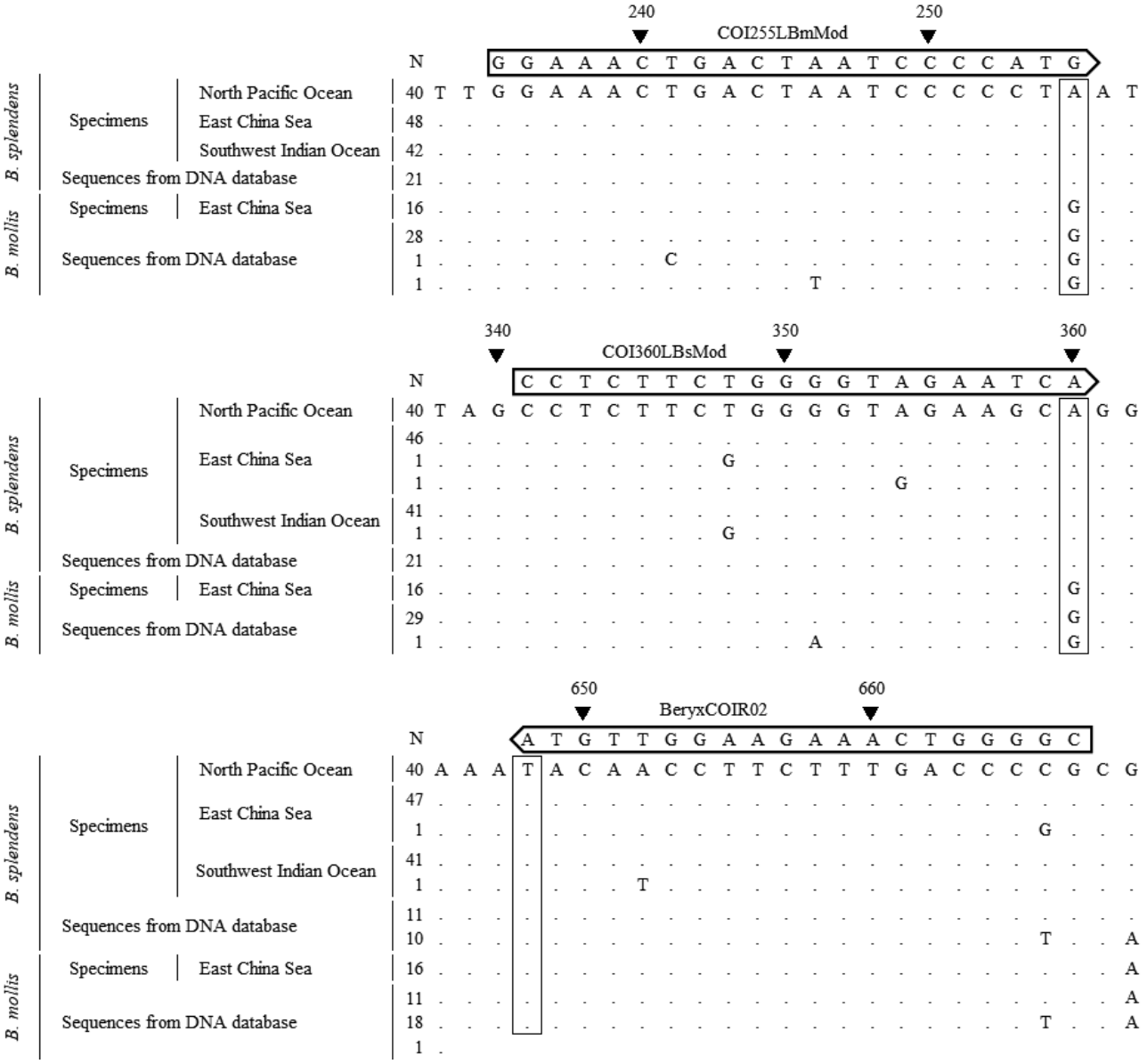

Fig. 2. Design of species-specific PCR primers (COI255LBmMod, COI360LBsMod, and BeryxCOIR02) on the mitochondrial DNA COI region of *Beryx splendens* and *B. mollis*

Numerals above black inverted triangles show the nucleotide position from the 5′-end of the COI region. Periods indicate the same bases as in the sequence on the first line. N indicates the number of sequences. COI: cytochrome *c* oxidase subunit I

nontarget species and increase the specificity of PCR amplification, according to Sommer et al. (1992). Moreover, a single reverse primer (BeryxCOIR02) was designed (Table 4 and Fig. 2). These primer sets amplified fragments of 327 bp for *B. mollis* and 433 bp for *B. splendens*. Besides these primers, universal primers L1803-16S and H2590-16S (Miya & Nishida 1999; Table 4) for the partial mtDNA 16SrRNA region (906 bp) were used as an internal positive control.

TaKaRa *Taq* (TaKaRa Bio), which has no proofreading function in DNA chain extension, was used in the species-specific multiplex PCR. These PCRs were performed in 20 μL reaction volumes containing 0.1 μL of 1 U *Taq* DNA polymerase, 2.0 μL of 1× *Taq* buffer, 1.6 μL each dNTP mixture (2.5 mM each), 0.5 μL of 10 μM L1803-16S, 0.5 μL of 10 μM H2590-16S, 0.25 μL of 10 μM COI360LBsMod, 0.25 μL of 10 μM COI255LBmMod, and 0.5 μL of 10 μM BeryxCOIR02. PCR was conducted on a TaKaRa Dice Standard TP650 thermal cycler (TaKaRa Bio) under the following

conditions: 35 cycles of denaturation (94°C, 30 s), annealing (48°C, 30 s), and extension (72°C, 90 s). PCR products were fluorescently labeled with Midori Green (Nippon Genetics, Tokyo, Japan), electrophoresed (100 V, 30 min) in 2.0% agarose gel, and detected on an LED transilluminator (LB-17, MaestroGen, Hsinchu, Taiwan).

To evaluate the effectiveness of the multiplex PCR for species differentiation, we compared the results of multiplex PCR with those of the morphological and molecular species differentiations.

Results

1. Morphological and genetic differentiation of *Beryx* species

The numbers of pyloric caeca of the specimens captured as *B. splendens* from the North Pacific Ocean, East China Sea, and Southwest Indian Ocean were in the ranges of 23-36, 24-45, and 24-31, respectively (Fig. 3). The numbers ranged from 15 to 20 for *B. mollis* specimens. The null model was chosen as the best model (Table 3), and including geographic region and body length did not improve the model. Moreover, all slopes (β_2 to β_4) were approximately 0 in the full model.

Nucleotide sequences of 613 bp of the mtDNA COI gene were determined, and 38 haplotypes were detected. All newly generated sequences were deposited in the DDBJ under accession numbers LC600240-77.

The phylogenetic analysis detected two clades, A and B (Fig. 4), which respectively corresponded to the specimens identified as *B. splendens* and *B. mollis* on the basis of the numbers of pyloric caeca.

2. Results of species-specific multiplex PCR

The species-specific PCR products for *B. splendens* (Clade A) were 300-400 bp long, and those for *B. mollis* (Clade B) were 400-500 bp long (Fig. 5); these were within the expected amplified fragment size ranges (Table 4). No amplification failure or misidentification of the species-specific products was recognized in a total of 146 PCRs. A positive control of 900-1,000 bp was obtained for all specimens, and a weak fragment of 100-200 bp was unexpectedly amplified from some specimens.

Discussion

1. Effectiveness of species-specific multiplex PCR

Differentiation of *B. splendens* and *B. mollis* is difficult, and the misidentification of *B. mollis* species as *B. splendens* is still frequent due to the resemblance of external morphologies of the two species (Yanagimoto & Kobayashi 2012). Accurate species differentiation tools for these species are therefore needed for effective fisheries management. In this study, we showed the concordance between morphological and molecular

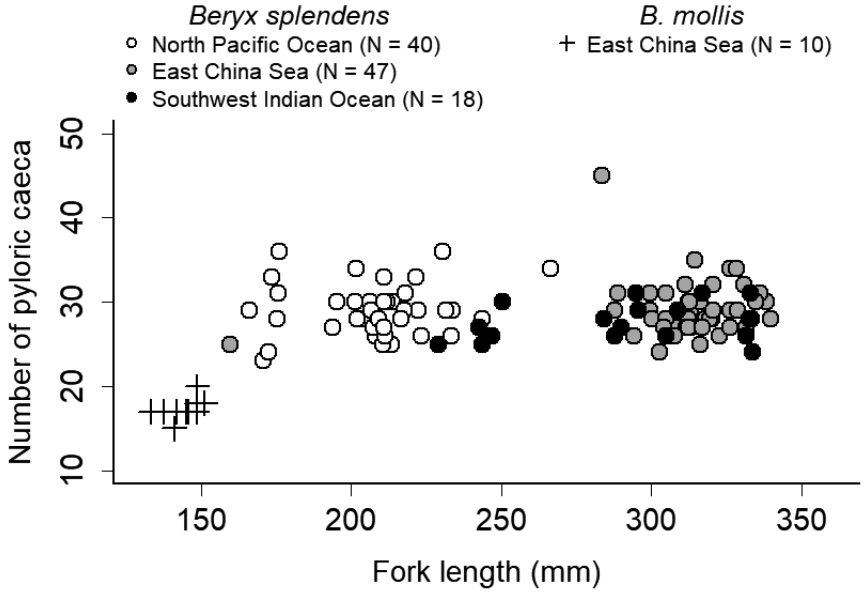

Fig. 3. Relationship between fork length and number of pyloric caeca in specimens captured as *Beryx splendens* and *B. mollis* from the East China Sea, North Pacific Ocean, and Southwest Indian Ocean

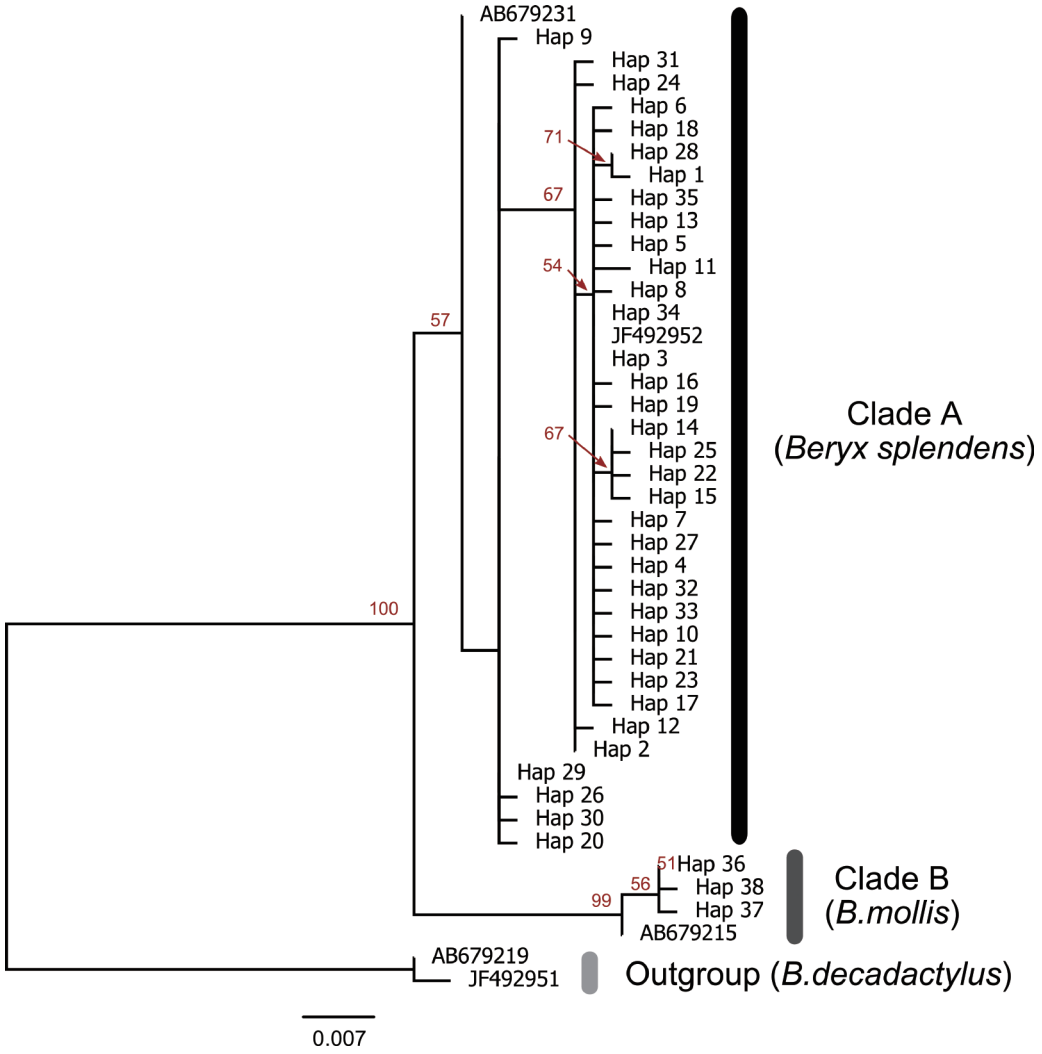

Fig. 4. Maximum likelihood tree of mitochondrial DNA COI sequences from the three *Beryx* species
Numerals beside each internal branch indicate bootstrap values; only values over 50% are shown. JF492952 and AB67231 are registered as *Beryx splendens*, AB679215 as *B. mollis*, and AB679219 and JF492951 as *B. decadactylus* in the INSDC database. COI: cytochrome *c* oxidase subunit I

differentiation of the two *Beryx* species and reconfirmed the validity of the two as separate species. Then, we developed species-specific primers and optimized a multiplex PCR protocol for robust species differentiation. Below, we discuss the precision of our species-specific primers and potential sources of uncertainty.

In our validation, all the species-specific bands were successfully amplified. Yanagimoto (2012) reported a false positive rate of 2% (1/50 specimens) for *B. splendens* specimens by using the singleplex PCR differentiation method. Moreover, a previous species-specific PCR method used for other species had some unintentional amplification, probably because of the existence of unknown haplotypes or nonspecific priming (e.g., 5% in Rocha-Olivares 1998, 4.5% in Higashi et al. 2016). We therefore consider that our multiplex PCR method is precise enough for species differentiation. Unexpected bands of 100-200 bp were found in some specimens, but these bands did not overlap with the expected fragments and did not influence the precision of the multiplex PCR method.

Lack of comprehensive coverage of geographic regions was one of the potential sources of uncertainty in the present study. Lévy-Hartmann et al. (2011) and Yanagimoto et al. (2015) reported genetic differences in

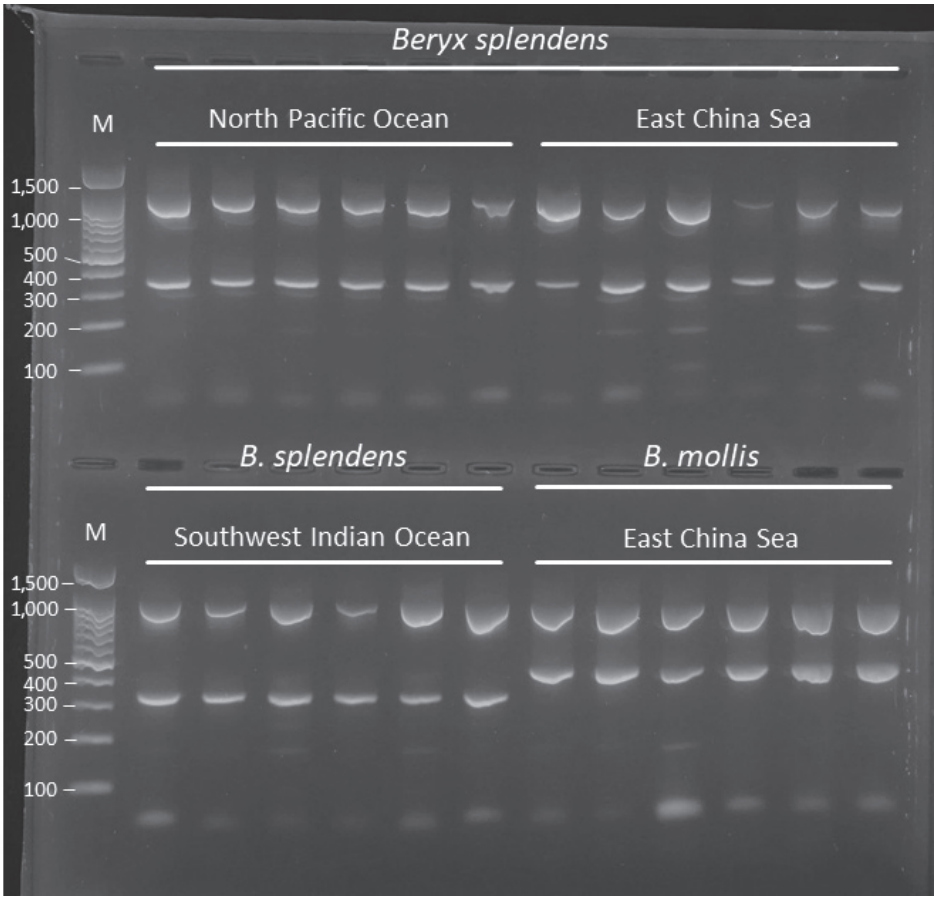

Fig. 5. Results of species-specific multiplex PCR for differentiation of *Beryx splendens* and *B. mollis*
M indicates the size marker.

B. splendens among oceans. Despite the circumglobal distribution of *B. splendens*, we were unable to analyze specimens from various areas including the Atlantic Ocean, and the distributions of the two species were not covered comprehensively in this study. Additionally, sequences of *B. splendens* from the Atlantic Ocean were not obtained from the DNA database. Thus, it is possible that genetic diversity among the species was not fully covered. Additional testing of samples from different geographic regions, such as the Atlantic Ocean, should help to confirm the robustness of the method.

2. Differentiation based on the number of pyloric caeca

Our findings suggest the potential usefulness of the number of pyloric caeca as a trait to differentiate *Beryx splendens* and *B. mollis*. The number of pyloric caeca in some *B. splendens* specimens was lower than the values previously reported in literature; nevertheless, these numbers were not low enough to overlap with those in *B. mollis*. The results of model selection indicated that the number of pyloric caeca in *B. splendens* specimens did not vary geographically or ontogenetically, at least within the geographic regions and size range (FL 166 mm-340 mm) included in this study. The number of pyloric caeca in our *B. mollis* specimens (FL 135 mm-140 mm) had probably reached its full count because our results agreed with the findings of previous studies (Yoshino et al. 1999, Yoshino & Kotlyar 2001; Table 1) that counted the number of pyloric caeca in larger *B. mollis* specimens (maximum standard length: 308 mm).

Beryx species individuals with FLs under the minimum sizes in this study are rarely captured by the bottom fishery (Shotton 2016, Watari et al. 2017); thus, species distinction using the number of pyloric caeca may be useful for most commercial samples, provided that the samples are not gutted.

Conclusion

In the present study, we successfully developed a novel species-specific multiplex PCR method for differentiating two morphologically similar and commercially important species of the genus *Beryx*. Implementation of conservation measures for *Beryx splendens* stocks is being considered in some regions (Takeuchi 2014, Watari et al. 2017). Our species-specific multiplex PCR may help to improve the precision of assessments of *B. splendens* stocks by making it easier to determine the bycatch rates of *B. mollis*. Additionally, little is known about the early life histories of *B. splendens* (Takeuchi 2014, Watari et al. 2017, Yanagimoto & Chow 2020, Sawada et al. 2021, Sawada & Okuda 2021) and the other two Beryx species. Furthermore, this new method can be used to distinguish larvae and juvenile individuals of *Beryx* species, which are likely to be difficult to discriminate morphologically.

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