ブリ属魚類のBenedenia seriolaeとNeobenedenia girellaeのPCR-RFLP法による種判別

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Short communication

Distinction of the Skin Flukes *Benedenia seriolae* and *Neobenedenia girellae* Infecting *Seriola* spp. by PCR-RFLP Assay

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ABSTRACT—We developed a PCR-RFLP method to distinguish *Benedenia seriolae* and *Neobenedenia girellae*, both infecting amberjacks *Seriola* spp. more precisely than a morphological method. PCR products of 28S rRNA and COX1 were digested by *Cla* I and *Eco*R I, respectively. For all individuals of *B. seriolae*, the digestions split each of the PCR products into two fragments. On the other hand, although the products of most of the *N. girellae* samples were digested by neither the enzymes, only PCR products of 28S rRNA gene of three of the individuals were split into three fragments, suggesting they were the hybrid between the two species.

Key words: capsalid, monogenea, PCR-RFLP, *Seriola* spp., skin fluke

Capsalid skin flukes are major parasites of amberjacks *Seriola* spp. worldwide (Ogawa *et al.*, 1995; Ogawa, 2015). They attach to the fish body surface using a haptor and ingest skin tissues. In heavy infections, fish rub their body against the culture nets, which causes skin wounds and facilitates secondary infections with bacterial and viral pathogens, and thus induces massive mortality.

Two capsalids, namely *Benedenia Seriolae* (Yamaguti, 1934) and *Neobenedenia girellae* (Hargis, 1955), the latter of which was a synonym of *N. melleni* (MacCallum, 1927) according to Whittinton and Horton (1996), co-parasitize *Seriola* spp. cultured in Japan. *Benedenia seriolae* was first described as *Epibdella seriolae* from wild amberjacks *S. dumerilii* in Japan (Yamaguti, 1934; Ogawa and Shirakashi, 2017). *Neobenedenia girellae* has been reported in Japanese aquaculture only since the 1990s. It is assumed that *N. girellae* were introduced to Japan (Ogawa *et al.*, 1995). Identifying skin flukes is important because the use of hydrogen peroxide preparations and praziquantel preparations for cultured *Seriola* spp. are approved only against *Benedenia serilae* in Japan. These two skin flukes are generally distinguished by morphological characters (Ogawa and Shirakashi, 2017). However, it is difficult to morphologically distinguish immature specimens of them, as well as poorly preserved adult specimens.

PCR-restriction fragment length polymorphism (RFLP) of 28S rRNA gene has been used to distinguish parasite species (Spano *et al.*, 1998; Marcilla *et al.*, 2002; Su *et al.*, 2006). The PCR-RFLP method for identification is simple and cost-effective compared with DNA sequencing (Su *et al.*, 2006). In this study, a PCR-RFLP method to distinguish *B. seriolae* and *N. girellae* was developed using 28S rRNA gene and cytochrome c oxidase 1 (COX1) gene sequences.

Materials and Methods

Parasites

Adult skin flukes were collected from cultured amberjacks *S. dumerili* at the following institutes in Japan: Shirahama Station, Aquaculture Research Institute, Kindai University, Wakayama Prefecture; Nagasaki Prefectural Institute of Fisheries, Nagasaki Prefecture; Kagoshima Prefectural Fisheries Technology and Development Center, Kagoshima Prefecture. Parasite specimens were initially distinguished by morphological characters at each institute, primarily by body shape and by the shape of the anterior midline section of the body between the anterior attachment organs according to Kinami *et al.* (2005). The numbers of *B. seriolae* and *N. girellae* specimens morphologically distinguished were 1 and 13 from Wakayama, 5 and 14 from Nagasaki, and 1 and 21 from Kagoshima, respectively. The samples were placed in RNA*later* after morphological distinction and stored at –80°C till further processing.

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DNA extraction

DNA was extracted from parasites with the phenol-chloroform method. Briefly, each parasite was homogenized in DNA extraction buffer (10 mM Tris [pH8.0], 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 5 M urea) and treated with proteinase K (20 μg) overnight at 37°C. DNA samples were extracted by phenol-chloroform, and purified by ethanol precipitation. DNA pellets were air dried completely and dissolved in distilled water.

PCR and digestion by restriction enzymes

PCR primer sets for 28S rRNA, C1 (5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (5′-TGGTCCGTGTTTCAAGAC-3′), and COX1, JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and COX1 (5′-AATCATGATGCAAAAGGTA-3′), were used and DNA fragments were amplified as described by Sepúlveda and González (2014). Each PCR reaction had a final volume of 50 μL. PCR amplicons were purified with phenol-chloroform and dissolved in 17 μL distilled water. The PCR products of the 28S rRNA were cloned into pGEM T Easy Vector (Promega) and sequenced. The COX1 sequences were obtained by direct sequencing. Restriction enzymes for distinction of the two species were chosen according to the nucleotide sequences of the amplicons. For *Cla* I digestion of 28S rRNA gene, the PCR product was mixed with 2 μL 10 × M buffer and 1 μL *Cla* I, and incubated at 30°C overnight. For *Eco*R I digestion of COX1 gene, the PCR amplicon was mixed with 2 μL 10 × H buffer and 1 μL *Eco*R I and incubated at 37°C overnight. The DNA fragments were analyzed by 2% agarose gel electrophoresis.

Results and Discussion

Schematic illustrations for the amplicons and digested fragments were shown in Fig. 1. The sizes of the amplicons expected from the sequences for 28S rRNA amplicons of *B. seriolae* and *N. girellae* were 891 and 885 bp respectively, while those for COX1 amplicons were 960 and 958 bp, respectively. For *N. girellae*, digestion of the PCR amplicons of 28S rRNA and COX1 with *Cla* I and *Eco*R I were expected not to produce any cleavage products. On the other hand, for *B. seriolae*, *Cla* I digestion of the 28S rRNA amplicon was expected to produce a 677 bp and 214 bp fragments and *Eco*R I digestion of the COX1 amplicon was to produce a 314 bp and 646 bp fragments.

Before digestion, single PCR amplicons were detected for both 28S rRNA gene and COX1 gene (Fig. 2A, C and E) for both species. For morphologically distinguished *B. seriolae* from all three localities, the digestions split each of the PCR products into two fragments. Meanwhile, although most of morphologically distinguished *N. girellae* samples were not digested (Fig. 2B, D and F), only 28S rRNA PCR amplicons of three *N. girellae* individuals from Wakayama Prefecture were split into three fragments. (Fig. 2B, lane 4, 5 and 10). The 28S rRNA PCR product was subcloned and 22 clones were sequenced. The sequences of 9 out of 22 clones were identical to the known sequence of *N. girellae*, and the other 13 clones were identical to that of *B. seriolae*. All sequences of the COX1 clones of the specimens were identical to that of *N. girellae*. These individuals might be hybrid between the two species possessing heterogeneous 28S rRNA gene derived from *B. serioale* and *N. girellae*, while the mtDNA was inherited from *N. girellae*. There have been reports on hybrids in several parasite taxa (King *et al.*, 2015), including the protozoan *Leishmania* (Jennings *et al.*, 2014), the trematode *Schistosoma* (Southgate *et al.*, 1976; Tchuem Tchuenté *et al.*, 1997; Huyse *et al.*, 2009) and the monogenean *Macrogyrodactylus* (Barson *et al.*, 2010). However, to our knowledge, there is no report on hybrid of capsalid monogenean and for between different genus. Although *B. seriolae* and *N. girellae* often co-exist on the same host individual, their mode of copulation differs (Ogawa *et al.*, 2014) and it is unlikely that the two species can copulate with each other. Further study is needed to explain the presence of the heterogeneous genome.

The results of PCR-RFLP of the Nagasaki Prefecture samples were consistent with the morphological identifications (Figs. 2C and D). On the other hand, two morphologically distinguished *N. girellae* samples from

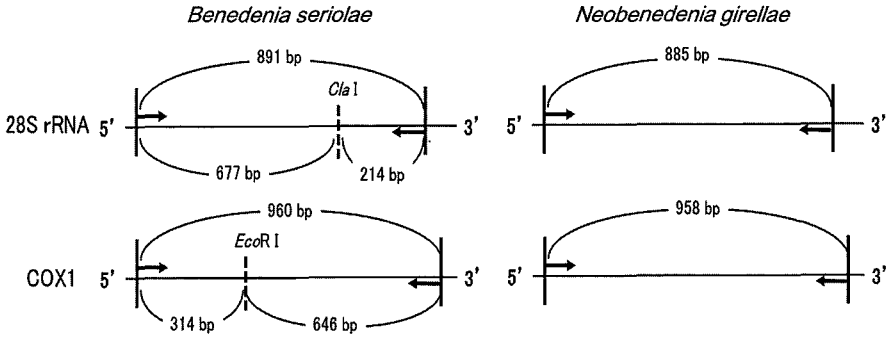

Fig. 1. Schematic figures for the digestion patterns of 28S rRNA and COX1 PCR products by *Cla* I and *Eco*R I, respectively.

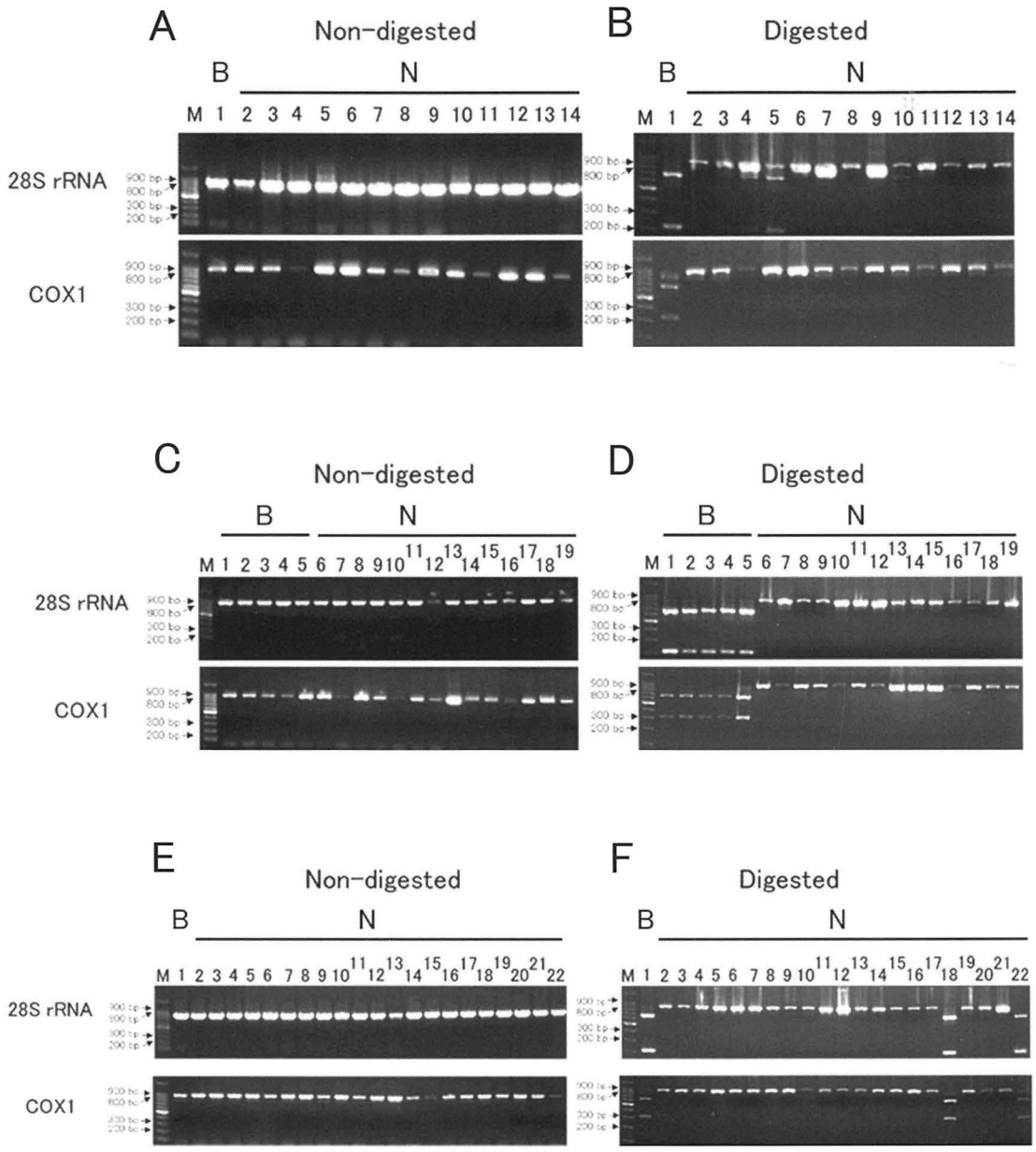

Fig. 2. Agarose gel electrophoresis of 28S rRNA and COX1 non-digested and digested PCR products of Wakayama (A and B, 1; *Benedenia seriolae*, 2–14; *Neobenedenia girellae*), Nagasaki (C and D, 1–5; *B. seriolae*, 6–19; *N. girellae*) and Kagoshima (E and F, 1; *B. seriolae*, 2–22; *N. girellae*) prefectures samples that identified morphologically. 28S rRNA PCR products were digested with *Cla* I and COX1 were digested with *Eco*R I. M; 100 bp ladder marker.

Kagoshima Prefecture were identified as *B. seriolae* by PCR-RFLP (Fig. 2F, lane 18 and 22), suggesting the inaccuracy or difficulty in the morphological identification method used in the present study.

In summary, this PCR-RFLP method is useful for distinction of *N. girellae* and *B. seriolae*. This method likely allows us to confirm morphological characterization of the species, not only for adults but also young worms, larvae or eggs. Furthermore, the unexpected PCR-RFLP results from some individuals from Wakayama Prefecture require further investigation to clarify if this is indeed hybrid of the two species. Further analyses on the temporal and spatial distribution of *B. seriolae* and *N. girellae* in cultured amberjacks by using this method will provide further biological information on the lifecycles of these skin flukes.

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ブリ属魚類の *Benedenia seriolae* と *Neobenedenia girellae* の PCR-RFLP 法による種判別

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2 種のハダムシ *B. seriolae* および *N. girellae* のゲノム DNA 中の 28S rRNA およびミトコンドリア DNA 中の COX1 遺伝子配列を用いた PCR-RFLP による種判別法を開発した。和歌山県，長崎県および鹿児島県のカンパチから採取した *B. seriolae* および *N. girellae* より DNA を抽出し，PCR により増幅した 28S rRNA および COX1 遺伝子をそれぞれ *Cla* I および *Eco*R I で消化した。どちらの PCR 産物も *B. seriolae* では 2 本の断片に消化され，*N. girellae* では消化されなかった。形態的に *N. girellae* と同定された一部の個体が，本手法により *B. seriolae* であると確認された。さらに，和歌山県で採取した *N. girellae* のうち 3 個体は，28S rRNA においては 3 本の断片が生じ，COX1 は消化されなかった。この 3 個体は両種の交雑個体であることが示唆された。

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