Short communication

Identification of *Girella punctata* and *G. leonina* by PCR-RFLP analysis

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Two *Girella* species, *Girella punctata* and *G. leonina*, are sympatric sister species with an extensive overlap in their respective distributions on shallow rocky reefs from Hong Kong to the south of the Japanese Islands. Juveniles of the two species cannot be discriminated easily on the basis of external characters. In this study, after morphological identification of the species, sequencing analysis was carried out for the partial 16S ribosomal RNA gene and for the D-loop region in mitochondrial DNA. A total of 109 specimens were examined. Restriction site mapping of the sequences suggested that the electrophoretic patterns of polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis of the gene products would produce a species-specific banding pattern. Subsequently, the PCR-RFLP analysis showed that the method was as effective for separating the two morphologically similar species of the genus *Girella* as the sequencing analysis.

Keywords: D-loop, *Girella leonina*, *Girella punctata*, mtDNA, PCR–RFLP, 16S rRNA.

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Introduction

Marine teleosts belonging to the genus *Girella* are widely distributed in the Indo-Pan Pacific and eastern Atlantic Ocean (Yagishita and Nakabo, 2003). Among the 15 known species of *Girella*, three species, i.e. *Girella punctata*, *G. leonina*, and *G. mezina*, coexist in East Asia between Hong Kong and the southern Pacific Ocean off the Japanese Islands. The three species are closely related, although their food habits are slightly different (Yagishita and Nakabo, 2000, 2003). Kanda and Yamaoka (1995) suggested that *G. mezina* is herbivorous and *G. leonine* is omnivorous, whereas *G. punctata* overlaps the other two species in respect of its food preferences. Recently, we estimated that the divergence time between *G. mezina* and *G. punctata* + *G. leonina* was about 7.3 million years ago, whereas that between *G. punctata* and *G. leonina* was about 6.0 million years ago (Itoi *et al.*, 2006). We also studied the phylogenetic relationship among the species of the genus *Girella* and demonstrated that *G. punctata* and *G. leonina* in East Asia are sister species.

Identification of *G. punctata* and *G. leonina* juveniles on the basis of morphological characters alone is often confusing, whereas the morphology of *G. mezina* can easily be separated from the other two species (Okuno, 1962, 1963, 1971). Therefore, the phylogenetic relationship between *G. punctata* and *G. leonina* has always been a subject of considerable interest with regard to finding distinct features that could discriminate the two species at the juvenile stage. Generally, the 16S ribosomal RNA (rRNA) gene is the region used to investigate the relationship between intergeneric and interfamily phylogenies (Nishida *et al.*, 1998), whereas the D-loop region is adequate for resolving the relationships among closely related taxa, such as local races, subspecies, and sibling species (Moritz *et al.*, 1987).

Here, we establish a simple and highly sensitive method using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis for *G. punctata* and *G. leonina*, to serve as an important tool for ecological research.

Material and methods

A total of 95 specimens of *G. punctata* was collected from the seashore at Joetsu, Niigata ($n = 28$, 168–478 g); Kashima, Ibaraki ($n = 5$, 77–291 g); Kawasaki, Kanagawa ($n = 5$, 58–139 g); Shimoda, Shizuoka ($n = 12$, 74–116 g); Muroto, Kochi ($n = 29$, 550–1100 g); Izumi, Kagoshima ($n = 9$, 269–448 g); and Goto, Nagasaki ($n = 7$, 401–800 g) in Japan (Figure 1). In addition, 14 specimens of *G. leonina* were collected from the seashore at Shimoda, Shizuoka ($n = 9$, 24–168 g); Goto, Nagasaki ($n = 4$, 388–900 g); and Izumi, Kagoshima ($n = 1$, 394 g) in Japan (Figure 1). Immediately after collection, the fish were transported to the laboratory and kept at −20°C until analysis.

The fish were examined for morphological characters, including the numbers of lateral line scales with pores, the numbers of spines and soft rays in the dorsal fin and soft rays in the anal fin, the depth of emargination of the caudal fin, and the colour of the

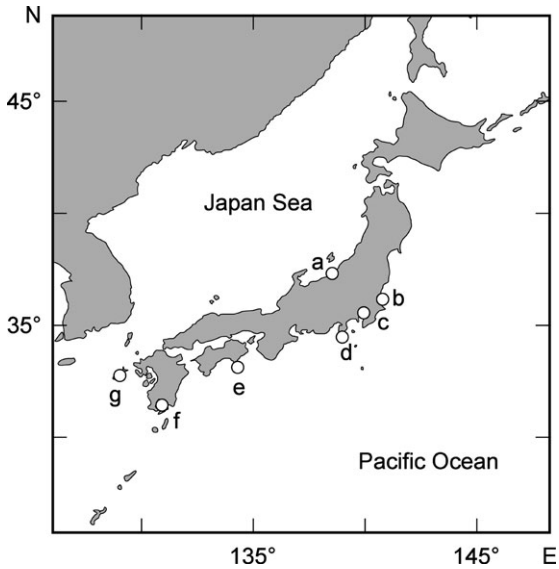

Figure 1. Sampling locations of *G. punctata* and *G. leonina*. Open circles represent sampling locations for the genus *Girella*: a, Joetsu; b, Kashima; c, Kawasaki; d, Shimoda; e, Muroto; f, Izumi; g, Goto.

opercular flap, as described by Yagishita and Nakabo (2000). Differences in morphology between *G. punctata* and *G. leonina* were as follows. The number of lateral line scales with pores in *G. punctata* is 50–56, whereas in *G. leonina*, it is 60–65. The pigmentation of the opercular flap is blackish in *G. leonina*, whereas in *G. punctata*, it is not black. The shape of the caudal fin in *G. punctata* is shallowly emarginated, whereas in *G. leonina*, it is deeply emarginated, with acute upper and lower lobes.

DNA extraction, PCR amplification, and PCR product sequencing

Total genomic DNA was extracted from the skeletal muscle, liver, or caudal fin of all 109 *G. punctata* and *G. leonina*, using the method of Sezaki *et al.* (1999). DNA fragments corresponding to the D-loop region and the 16S rRNA gene in mitochondrial DNA (mtDNA) were amplified by PCR. Primers 16SAR-L (5′-CGCCTG TTTATCAAAAACAT-3′) and 16SBR-H (5′-CCGGTCTGAACT CAGATCACGT-3′) used to amplify the partial 16S rRNA gene fragment (Figure 2) were obtained from Palumbi *et al.* (1991). Primers fDloop_F (5′-TTCCTGGCATTTGGTTCCTACTTCAG-3′) (Itoi *et al.*, 2006) and ftRPhe_R (5′-CCATCTTAACATCTTCA GTGTTATGC-3′) for amplifying the partial D-loop region flanking by a part of tRNA (transfer RNA) gene (Figure 2) were designed by referring to the corresponding regions in *Pagrus major* (GenBank database accession number NC_003196), *Takifugu rubripes* (AJ421455), and *Tranchurus tranchurus* (A B108498). PCR amplification was performed using a reaction mixture containing genomic DNA as a template, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA), 2 μl of 10× *Taq* DNA polymerase buffer (Promega), 0.8 μl of 10 μM primers, 1.6 μl of 2.5 mM dNTPs, and 1.6 μl of 25 mM $MgCl_2$, and the total volume was brought to 20 μl with sterile water. The thermal cycling profile of PCR consisted of initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 45 s, with a final extension step at 72°C for 2 min. PCR products were cloned into the TA site of pGEM–T Easy vector (Promega) according to Marchuk *et al.* (1991), using the *Escherischia coli* strain DH5α as a host bacterium.

Sequencing of PCR products was performed for both strands with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Alignment of nucleotide sequences partially coding for 16S rRNA and D-loop regions in the mtDNA of *G. punctata* and *G. leonina* was carried out using CLUSTAL W (Thompson *et al.*, 1994).

PCR-RFLP analysis

The PCR conditions employed for RFLP analysis were the same as described previously. The RFLP analysis of the gene products was performed by digesting 5 μl of each amplified product with 10 units of restriction enzyme; *Hin*fI (Nippongene, Tokyo, Japan) for 16S rRNA and *Dde*I (Toyobo, Osaka, Japan) or *Xba*I (Nippongene) for the D-loop region. The reaction was carried out at 37°C for 2 h in the reaction buffer supplied with the kit. The digested samples were subjected to electrophoresis on a 3% agarose gel and stained with ethidium bromide.

Results

Sequencing analysis

Partial regions of the 16S rRNA gene and the D-loop region in the mtDNA from 14 *G. punctata* and 5 *G. leonina* were amplified by PCR with appropriately designed oligonucleotide primers. A BLAST search of ∼600 bp nucleotide sequence of the 16S rRNA

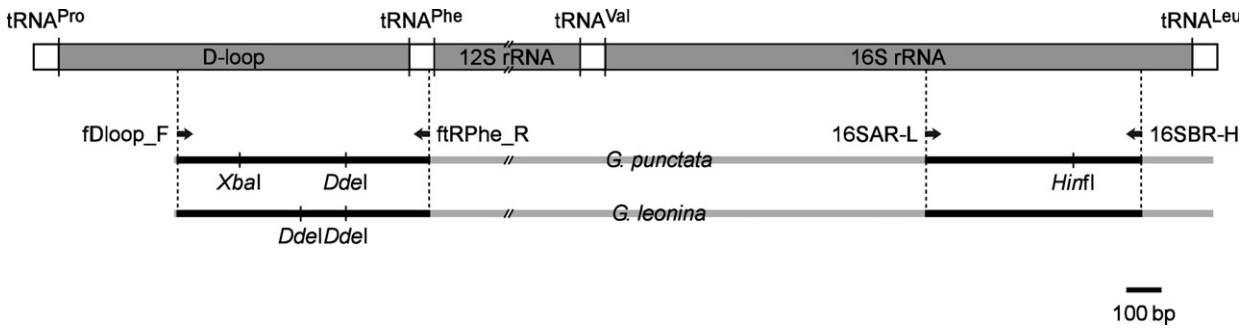

Figure 2. Restriction sites and locations of primers in the D-loop region and rRNA genes in mtDNA from *G. punctata* and *G. leonina*. Arrows represent the locations and orientation of primer recognition sequences. Restriction sites for *Xba*I and *Hin*fI are shown in the D-loop region and the 16S rRNA gene of *G. punctata*, whereas those for *Dde*I are shown in the D-loop region of both *G. punctata* and *G. leonina*.

gene amplified with primers 16SAR-L and 16SBR-H for each of the two *Girella* species matched exactly the corresponding sequences deposited in the databases (*G. punctata*, AB208648, AB233479–AB233491; *G. leonina*, AB208649, AB233475–AB233478; data not shown). Similarly, ~500 bp fragment of the D-loop region amplified with primers fDloop_F and ftRPhe_R also corresponded to the species-specific sequences in the databases (data not shown). Comparison of the partial mtDNA sequences from *G. punctata* and *G. leonina* revealed that these two *Girella* spp. could be distinguished from each other using the RFLP pattern of the 16S rRNA gene generated with *Hin*fI and that of the D-loop region with *Dde*I or *Xba*I.

Species identification

A unique restriction site for *Hin*fI was found in the 613 bp 16S rRNA gene product amplified from *G. punctata* (Figure 2), which resulted in two restriction fragments measuring 490 and 123 bp (Figure 3). In the case of the 497 bp D-loop region amplified with primers fDloop_F and ftRPhe_R, one restriction site for *Xba*I was found in *G. punctata*, but not in *G. leonina*. In contrast, the D-loop region of *G. punctata* and *G. leonina* contained one and two sites recognizable by the restriction enzyme *Dde*I, respectively (Figure 2). As a result, RFLP with *Dde*I produced fragments of 308 and 189 bp in *G. punctata* and of 307, 123, and 66 bp in *G. leonina* (Figure 3). Therefore, the two *Girella* species, *G. punctata* and *G. leonina*, can easily be discriminated by the PCR-RFLP analysis with these three restriction enzymes (Figure 3).

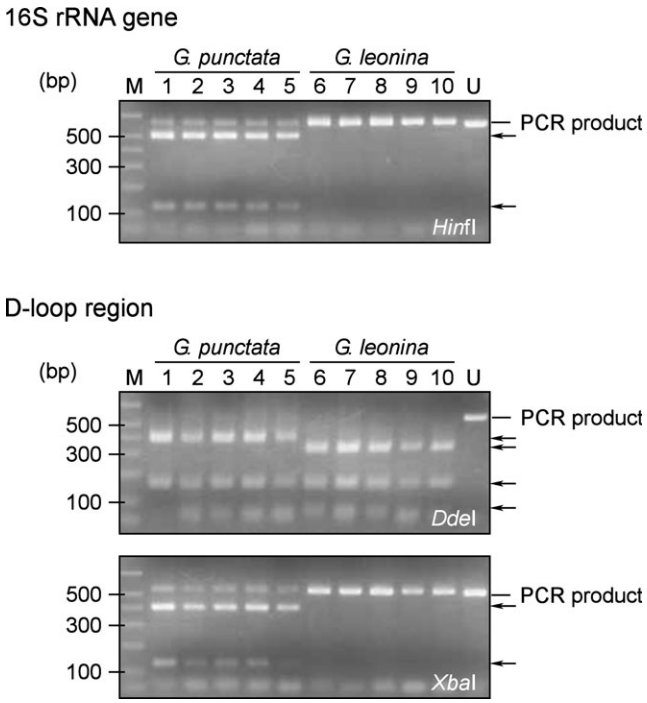

Figure 3. Typical PCR-RFLP profiles of the 16S rRNA gene and the D-loop region obtained from *G. punctata* and *G. leonina*. PCR products for the 16S rRNA gene were treated with *Hin*fI (upper panel), whereas those for the D-loop region were digested with *Dde*I or *Xba*I (lower panels). The restriction fragments are marked by arrows. Lanes 1–5, *G. punctata*; lanes 6–10, *G. leonina*; lane M, the molecular size marker (100 bp ladder); and lane U, untreated PCR product.

Discussion

Girella punctata and *G. leonina* are widely distributed, living on coastal rocky reefs from southern Japan to Hong Kong, and exhibit considerable intraspecific variation with respect to their morphology. Many authors, including Jordan and Thompson (1912), Matsubara (1955), and Araga (1984), described *G. punctata* as characteristically having 15 dorsal fin spines, one more than found in *G. leonina*. However, Nakabo (1993) found considerable intraspecific variation in the number of dorsal fin spines in both species. In a recent study, Yagishita and Nakabo (2000) found that the opercular flap of *G. punctata* was blackish dorsally in some specimens, a feature not described in the previous studies. Owing to such confusion, *Girella* has always been a subject of controversy with regard to the taxonomic status of its species, particularly those found in East Asia. Specimens were classified variously as *Crenidens melanichthys* (Richardson, 1846) or *G. melanichthys*; this name was placed in varying junior synonym with *G. punctata* or *C. leoninus* (the original combination for *G. leonina*; Richardson, 1846; Yagishita and Nakabo, 2000), and *G. melanichthys* was incorrectly used as the valid name for *G. leonina* (Yagishita and Nakabo, 2000). Yagishita and Nakabo (2000) recently revised the classification of the genus *Girella* in East Asia.

In this study, after identification of *G. punctata* and *G. leonina* by morphological analysis and nucleotide sequencing, samples were subjected to PCR-RFLP analysis. The results using partial mtDNA sequences (16S rRNA gene and D-loop region) showed that the technique was as effective for separating the constituent species of the genus *Girella* as the sequencing analysis (Figure 3). The results demonstrate that the phylogeny derived using mtDNA corresponds with the grouping obtained using the morphological characters of *G. punctata* and *G. leonina*.

Analytical methods to distinguish two *Girella* species, *G. punctata* and *G. leonina*, from tissues with no morphological determination are of great methodological importance for ecological research on coastal fish. We have shown that the DNA-based method that discriminates between the two *Girella* species without sequencing their genes can be applied easily in the field. Although it is not possible to discriminate the juveniles of the two species on the basis of their morphological characters alone, the same would be even more difficult at the larval stage. For example, the larvae of *Girella* species found at Tanoura inlet, Shimoda, Shizuoka, Japan were thought to be *G. punctata* (Yoshihara, 1998; Yoshihara *et al.*, 1998, 1999). However, after rearing in tanks for several months, the larvae appeared to be *G. punctata* and *G. leonina* (unpublished data). Therefore, the PCR-RFLP analysis reported in this study would potentially prove to be a useful tool for identifying the larvae of the two species.

In conclusion, we have been able to distinguish between *G. punctata* and *G. leonina* not only on the basis of differences in their morphological characters, but also on the basis of their nucleotide sequences of both partial 16S rRNA gene and the D-loop region in mtDNA. The present study established a molecular biological method for distinguishing between the two *Girella* species, *G. punctata* and *G. leonina*, by PCR-RFLP analysis based on the mtDNA sequences.

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