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Identification of the hybrid between *Oryzias latipes* and *Oryzias curvinotus* using nuclear genes and mitochondrial gene region

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ABSTRACT

Commercialized transgenic fish should be sterilized to prevent their genetic effects on native wild fish in the event of their accidental release to the environment. Diploid and allotriploid hybrids between *Oryzias latipes* and *Oryzias curvinotus* are sterile, which contributes to the sterilization of transgenic *O. latipes* or *O. curvinotus* strains, for example, fluorescent aquarium fish. However, it is very difficult to distinguish these hybrids from their parental species by morphological measurements. Thus, we confirmed whether our previously developed species identification method for *O. latipes* and *O. curvinotus* by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products of nuclear DNA gene regions, namely, PCR-RFLP using *Hin*f I or *Hsp*92 II for the aromatase gene, *Rsa* I for the calmodulin gene, and *Hae* III for the caspase-6 gene, is useful for distinguishing the diploid and allotriploid hybrids from their parental *Oryzias* species. We found that the hybrids have maternal mitochondrial DNA by PCR-RFLP analysis using *Mse* I of the 16S rRNA gene.

1. Introduction

Oryzias latipes is an egg-laying freshwater fish native to Japan, Korea, and southwestern China. Its small size, ease of breeding, and relatively brief generation time make it a useful experimental animal and a good aquarium fish. It inhabits marshes, ponds, and brooks amidst rice fields in alluvial plains. Studies of allozymes encoded in the nuclear genome and mitochondrial DNA of *O. latipes* have shown that wild populations of this species consist of four genetically different groups: the Northern population from the coast of the Sea of Japan in eastern Japan; the Southern population from the Pacific coast of eastern Japan and from western Japan; the East Korean population from eastern Korea and southern Korea; and the China-West Korean population from China and western Korea (Sakaizumi et al., 1983; Sakaizumi, 1984, 1986; Sakaizumi and Jeon, 1987; Matsuda et al., 1997a, 1997b; Takehana et al., 2003, 2004a, 2004b). The Southern and China–West Korean populations are divided into nine and three subgroups, respectively (Takehana et al., 2004a, 2004b; Sakaizumi, 2000).

In recent years, green, orange, and red fluorescent transgenic fish of *O. latipes*, namely, TK-1, have been commercialized as aquarium fish in Taiwan. However, commercialized transgenic fish should be sterile to prevent their genetic effects on native wild fish in the event of their accidental release to the environment. On the other hand, *Oryzias curvinotus*, which is distributed from the North–East region of the Indochina Peninsula to the south region of China, is closely related to *O. latipes*. The F1 female offspring of the cross between *O. latipes* and *O. curvinotus* can lay diploid eggs but the F1 male offspring are sterile (Hamaguchi and Sakaizumi, 1992; Sakaizumi et al., 1992). The F2 offspring derived from the diploid eggs are sterile triploid hybrids (Hamaguchi and Sakaizumi, 1992; Sakaizumi et al., 1992, 1993; Kurita et al., 1995). The F1 male is completely sterile, but produces spermlike cells with a large head and an abnormal flagellum (Hamaguchi and Sakaizumi, 1992; Shimizu et al., 1997, 2000). The diploid and allotriploid hybrids between *O. latipes* and *O. curvinotus* are sterile (Kurita et al., 1995). Thus, these hybrids contribute to the sterilization of transgenic *O. latipes* or *O. curvinotus* strains, for example, fluorescent aquarium fish. However, it is very difficult to distinguish these hybrids from their parental species by morphological measurements, because of their morphological similarity.

Recently, DNA markers have been widely used for the species identification of many organisms including fish (Pérez et al., 2005; Michelini et al., 2007; Itoi et al., 2008). Identification methods based on DNA depend on simple and sensitive PCR techniques applicable to small and fixed amounts of samples. Thus, we previously developed a diagnostic

Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA.

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identification method for distinguishing *O. curvinotus* from *O. latipes* by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products of nuclear DNA gene regions (aromatase, calmodulin, and caspase-6 genes) and a mitochondrial DNA gene region (16S ribosomal RNA (rRNA) gene) (Masaoka et al., 2010).

In this study, we confirm the feasibility of PCR-RFLP analysis for distinguishing diploid and allotriploid hybrids from their parental *Oryzias* species.

2. Materials and methods

2.1. Specimens and DNA sample

The Northern and the Southern populations of *O. latipes*, *O. curvinotus*, and the F1 and F2 hybrids between *O. latipes* and *O. curvinotus* were analyzed for species-specific PCR-RFLP profiles (Table 1). Total DNA was extracted by phenol–chroloform and ethanol precipitation as in our previous study (Masaoka et al., 2010).

2.2. PCR amplification of partial 16S rRNA, aromatase, calmodulin, and caspase-6 genes

The amplifications of the partial aromatase, calmodulin, caspase-6, and 16S rRNA gene fragments of the two *Oryzias* species and their hybrids were carried out using the same PCR reaction mixtures and conditions used in our previous study (Masaoka et al., 2010). The electrophoresis of the PCR products was carried out using the same conditions used in our previous study (Masaoka et al., 2010).

2.3. PCR-RFLP analysis

The PCR products of the partial aromatase gene were digested with *Hin*f I (TAKARA) and *Hsp*92 II (Promega). The PCR products of the partial calmodulin and caspase-6 genes were digested with *Rsa* I (TOYOBO) and *Hae* III (TAKARA), respectively. The PCR products of the partial 16 S rRNA gene were digested with *Mse* I (New England BioLabs). The conditions for the restriction enzyme reactions and electrophoresis of the products were the same as those used in our previous study (Masaoka et al., 2010).

3. Results

We amplified DNA fragments of approximately 560, 800, 400, and 610 bp from *O. latipes* and *O. curvinotus* by PCR using the primers for the aromatase, calmodulin, caspase-6, and partial 16S rRNA genes, respectively.

The digestion of the PCR products of the partial aromatase gene of the two species with endonuclease *Hin*f I or *Hsp*92 II showed polymorphisms upon electrophoresis on agarose gel. The RFLP products of *O. latipes* were of fragment type A and those of *O. curvinotus* were of fragment type B (Fig. 1, Table 2). On the other hand, the RFLP products of the F1 and F2 hybrids were of fragment type AB (Fig. 1, Table 2). The digestion of the *Oryzias* species PCR products using primers for the partial calmodulin gene with endonuclease *Rsa* I showed that the Northern population of *O. latipes* were of fragment type A, the Southern population of *O. latipes* were of fragment type B, and the *O. curvinotus* were of fragment type C (Fig. 2, Table 2). The fragment types of the *O. latipes* Northern population ♀×*O. latipes* Southern population ♂, *O. latipes* Southern population ♀×*O. latipes* Northern population ♂, and F1 hybrid offspring were in agreement with the fragment types of both parents (Fig. 2, Table 2). Furthermore, the fragment type of the F2 hybrid offspring was ABC (Fig. 2, Table 2). The digestion of the PCR products of the partial caspase-6 gene of the two species with endonuclease *Hae* III showed polymorphism upon electrophoresis on agarose gel, namely, *O. latipes* was of fragment type A, *O. curvinotus* was of fragment type B, and F1 and F2 hybrids were of fragment type AB (Fig. 3, Table 2). The results of the RFLP analysis of the PCR products of the partial 16S rRNA gene of the two species with endonuclease *Mse* I showed polymorphism upon electrophoresis on agarose gel. The results of the PCR-RFLP analysis of the partial 16S rRNA gene with endonuclease *Mse* I showed that the Northern population of *O. latipes* was of fragment type A, the Southern population of *O. latipes* was of fragment type B, and the *O. curvinotus* was of fragment type C, respectively (Fig. 4, Table 2). Furthermore, the F1 and F2 generations include population hybrids that were in agreement with the maternal fragment type (Fig. 4, Table 2).

Table 1
Oryzias species and hybrids examined.

Species or hybrid	Location	Sample No.
O. latipes		
Northern population	Nanao, Ishikawa, Japan	16
Southern population	Tamaki, Mie, Japan	22
Northern population♀×Southern population♂	Nanao×Tamaki	20
Southern population♀×Northern population♂	Tamaki×Nanao	20
O. curvinotus	Hong Kong, China	36
F1 Hybrid (*O. latipes* ♀×*O. curvinotus* ♂)	Tamaki×Hong Kong	20
	Nanao×Hong Kong	20
F1 Hybrid (*O. curvinotus* ♀×*O. latipes* ♂)	Hong Kong×Tamaki	20
	Hong Kong×Nanao	20
F2 Hybrid (F1 hybrid (*O. latipes* ♀×*O. curvinotus* ♂) ♀×*O. latipes* ♂)	(Tamaki×Hong Kong)×Nanao	20

4. Discussion

The fragment types of the PCR-RFLP products of the partial genes differ between *O. latipes* and *O. curvinotus*. Moreover, the Northern population of *O. latipes* only has a specific fragment type of the PCR-RFLP products of the partial calmodulin and 16S rRNA genes obtained using the endonucleases *Alu* I and *Mse* I, respectively. The results of this study were in agreement with those of our previous study (Masaoka et al., 2010). Thus, the method we developed is useful in identifying *O. curvinotus* from *O. latipes*, and in distinguishing the Northern population of *O. latipes* from the other three populations of *O. latipes*.

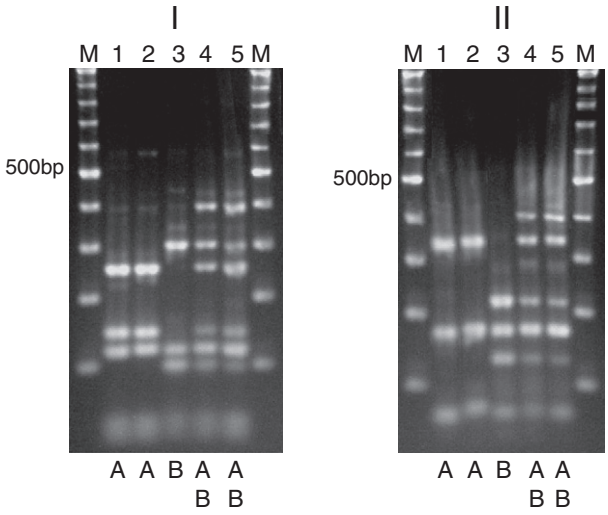

Fig. 1. Restriction analysis of PCR products of partial aromatase gene digested with *Hin*f I or *Hsp*92 II. I: *Hin*f I, II: *Hsp*92 II. Lane 1, *O. latipes* Northern population ♂; lane 2, *O. latipes* Southern population ♀; lane 3, *O. curvinotus* ♂; lane 4, F_1 hybrid (*O. latipes* Southern population ♀×*O. curvinotus* ♂) ♀; lane 5, F_2 hybrid (F_1 hybrid (*O. latipes* Southern population ♀×*O. curvinotus* ♂) ♀×*O. latipes* Northern population ♂); M, 100-bp DNA ladder. A, B, AB: Fragment types.