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Development of RAPD-SCAR and RAPD-generated PCR-RFLP Markers for Identification of Four *Anguilla* eel Species

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Abstract: Discriminating between eel species of the genus *Anguilla* using morphological characteristics can be problematic, particularly in the glass eel and elver stages. In this study, sequence-characterized amplified region (SCAR) and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) markers were developed for the identification of *Anguilla japonica, Anguilla bicolor bicolor, Anguilla rostrata*, and *Anguilla anguilla*. Random amplified polymorphic DNA (RAPD) fragments from *A. japonica* (362 bp), *A. bicolor bicolor* (375 bp), *A. rostrata* (375 bp), and *A. anguilla* (375 bp) were isolated, sequenced, and converted to SCAR markers. The principal difference between the SCARs of *A. japonica* and the three other species is the absence of a 13 bp deletion in the *A. japonica* SCAR. Specific PCR primers amplified a 290 bp fragment for *A. japonica* and 303 bp fragments for *A. bicolor bicolor, A. rostrata*, and *A. anguilla*. Restriction enzyme digestion with *TaqI*, *MaeI*, and *Tru9I* yielded PCR–RFLP patterns with differences that, when analyzed together, are sufficient for distinguishing each of the four eel species. In addition, RAPD fragments for *A. japonica* (577 bp), *A. bicolor bicolor* (540 bp), *A. rostrata* (540 bp), and *A. anguilla* (509 bp) were also isolated and sequenced. The *A. japonica, A. bicolor bicolor, A. rostrata*, and *A. anguilla* PCR products contain ten, nine, nine, and eight tandem repeats, respectively, of a 37 bp sequence. These results suggest that SCAR and PCR–RFLP markers and repeat numbers for specific loci will be useful for the identification of these four *Anguilla* eel species.

Key words: *Anguilla*, SCAR, PCR–RFLP, species identification

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INTRODUCTION

Eel is one of the most prized sea delicacies in Eastern Asia and is an excellent source of vitamin A, taurine, and unsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic acid. Eel farming techniques were developed in Korea in the 1960s, and Korea exported eel seedlings of medium size to Japan until the late 1980s. One domestic species, *Anguilla japonica*, is the most commercially valuable species of its genus in the Korean fisheries market. Because techniques for eel breeding have not yet been established, and the natural supply of *A. japonica* seedlings is insufficient, seedlings (glass eels and elvers) of *A. japonica* are imported for aquaculture from China and southeast Asian countries. The four *Anguilla* species most commonly imported for the Korean market are *A. japonica, A. bicolor bicolor, A. rostrata*, and *A. anguilla*. Generally the price of domestic *A. japonica* in Korean markets is much higher than that of other *Anguilla* species because of the superior freshness and safety of the domestic product.

The traditional eel taxonomy described by Ege (1939) and modified by Castle and Williamson (1974) divides the genus *Anguilla* into 15 species, three of which are further subdivided into two sub-species. These species exhibit very few species-specific features; all are snake-like in form, and their ecology and morphology is relatively uniform (Tesch, 1999). This leads to difficulties in distinguishing species, and only a few can be identified clearly by external morphological characteristics (Tesch, 1977; Kang et al., 2000). However, morphological feature-based species identification remains a difficult task due to overlapping of morphological characteristics especially in case of leptocephalus and elver. The development of new methods such as polymerases chain reaction (PCR)-based techniques

solved several taxonomic as well as management issues. A more simple, rapid, and robust PCR-based techniques for *Anguilla* species differentiation are needed.

The PCR technique permits the amplification of DNA fragments of specific or non-specific sequence (Mullis and Faloona, 1987). Recently, several PCR-based technologies have been employed for species identification. Although *A. rostrata* and *A. anguilla* are so closely related that they are difficult to differentiate, even by mitochondrial DNA analysis (Avise et al., 1986), they can be distinguished by PCR using 5S rDNA primers (Nieddu et al., 1998). Single-nucleotide polymorphism (SNP)-based PCR has also been used to differentiate *A. japonica* from *A. anguilla* (Itoi et al., 2005). However, no method clearly distinguishes between *A. japonica*, *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla* simultaneously. In addition, most of the species identification primers rely on mitochondrial markers, which do not contain paternal information. Although restriction fragment length polymorphism (RFLP) analysis of PCR-amplified mitochondrial DNA fragments can be effectively used for the discrimination of eel species (Lin et al., 2002), these methods can still lead to misidentification because of underestimation of intraspecific polymorphism (Gagnaire et al., 2007) or possible natural hybridization (Albert et al., 2006; Frankowski et al., 2008).

Random amplified polymorphic DNA (RAPD)-PCR using random primers does not require prior sequence knowledge, and has been used successfully to reveal DNA polymorphisms between genomes (Williams et al., 1990), it also overcomes the maternal inheritance disadvantage of mitochondrial DNA. Generating information from a RAPD fragment for the design of a specific primer is a technique used when amplification of conserved sequences is not desirable (Garner and Slavicek, 1996). Converting a RAPD marker into a sequence-characterized amplified region (SCAR) marker has been successfully used to authenticate organisms (Adinolfi et al., 2007), and for other genetic applications, including marker-assisted selection (Araneda et al., 2005). In the present study, we developed a sensitive RAPD–SCAR and RAPD-generated PCR–RFLP method for distinguishing among four commercially important eel species, *A. japonica*, *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla*. SCAR and PCR-RFLP markers were developed as an enhancement to overcome the limitations of morphological discrimination in the genus *Anguilla*.

MATERIALS AND METHODS

Preparation of samples and DNA extraction

Four individuals each of four eel species [Japanese eel (*A. japonica*), Indonesian shortfin eel (*A. bicolor bicolor*), European eel (*A. anguilla*), and American eel (*A. rostrata*)] were obtained from the Southern Inland Fisheries Research Institute in Korea and used in this study. Entire glass eels or elvers were stored in 95% ethanol for genetic analyses. Genomic DNA was extracted from muscles or fins using the TNES-urea buffer method (Asahida et al., 1996).

DNA amplification

The primers and PCR conditions used in our RAPD analysis were adapted from those reported by Kang et al. (2002). The sequence of universal rice primer (URP) 4 was 5'-AGGACTCGATAACAGGCTCC-3'. PCR was performed in 50-μL reaction mixtures containing 50 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, 200 ng URP4 primer, and 0.5 unit of *Taq* polymerase (Takara, Otsu, Japan). Amplification was performed in a PTC-220 thermal cycler (MJ Research, Waltham, USA) programmed for an initial 5-min denaturation step at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C; and a final 7-min extension at 72°C. PCR reactions using sequence-specific primers were performed in 20-μL reaction mixtures containing 50 ng genomic DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 5 pmol primers, and 0.2 unit *Taq* DNA polymerase (Takara, Otsu, Japan). PCR was performed in a thermal cycler programmed for an initial 5-min denaturation step at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C (primer pair A1F/A1R), 60°C (A2F/A2R), or 55°C (A3F/A3R), and 30 s at 72°C, and a final 10-min extension at 72°C. The specific primers were A1F (5'-TTTCAGGCACCCAATCTACCA-3') and A1R (5'-CGTG TATAGCCATTCAACTCC-3'); A2F (5'-ACAGAAAGCA GCATTTGAAG-3') and A2R (5'-TACGTGTATAGCCATT CAAC-3'); and A3F (5'-CAGCAAACCGCCAAACAA-3') and A3R (5'-TTTGGAGTGAGATAACTGAG-3'). Successful amplification was verified by 1.5% agarose gel electrophoresis.

Cloning and sequencing of RAPD fragments

Selected amplified fragments from RAPD assays were recovered from agarose gels using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The recovered DNA fragments were cloned into the pGEM-T vector using pGEM-T Vector System II (Promega, Madison, WI, USA) according to the manufacturer's instructions. After transformation into *E. coli* DH5α competent cells, white colonies were selected, and the plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The plasmids were sequenced using T7 forward and SP6 reverse primers with a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI 3100*xl* automated sequencer (Applied Biosystems, Foster City, CA, USA).

Restriction digests of PCR products

Three specific primer sets (A1F/A1R, A2F/A2R, and A3F/A3R) were designed to convert the cloned RAPD markers into single-locus PCR markers for SCAR. After PCR as described above, the PCR products were digested with 10 units of *TaqI*, *MaeI*, or *Tru9I* (Roche, Mannheim, Germany) restriction enzyme at 65, 45, or 65°C, respectively, for 2 h in a final volume of 25 μL. The digested PCR products were electrophoresed on 3% MetaPhor agarose gels (BMA, Rockland, ME, USA), stained with ethidium bromide, and visualized under ultraviolet light.

RESULTS AND DISCUSSION

RAPD patterns obtained from four eel species using the URP4 primer

RAPD fragments have been successfully used to characterize degrees of polymorphism both within and between species based on the number of polymorphic bands (Ruzainah et al., 2003). RAPD has also been used to confirm the taxonomic status of morphologically similar species in inter- and intraspecies level clarification (Bardakci and Skibinski, 1994; Callejas and Ochando, 1998). Here, 12 URPs were screened for the presence of polymorphic DNA bands in RAPD reactions, and the RAPD patterns were found to be distinct for each species. When PCR was performed with primer URP4 using DNA from each of four isolates of *A. japonica*, *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla*, polymorphic DNA band patterns were obtained for all four species (Fig. 1). PCR amplicons of approximately 370 and 540 bp were subsequently selected for their ease of cloning and different sizes among species.

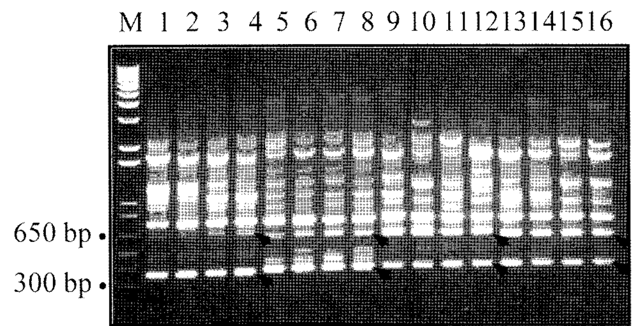

Fig. 1. RAPD profiles of four *Anguilla* eel species' genomes randomly amplified with universal rice primer 4 (URP 4). Lane M, 1-kb plus DNA ladder; lanes 1–4, *A. japonica*; lanes 5–8, *A. bicolor bicolor*, lanes 9–12, *A. rostrata*; lanes 13–16, *A. anguilla*. Arrows indicate the ~370- and ~540 bp fragments that were isolated and sequenced. Selected bands of the 1-kb DNA ladder are identified at left.

Characterization and SCAR amplification of ~370 bp RAPD fragments

Although RAPD polymorphism analysis is fast, simple, and inexpensive and has been used in research involving systematics (Smith et al., 1997), phylogenetics (Puterka et al., 1993), and population differentiation (Caccone et al., 1997), it has some disadvantages. Specifically, its low reproducibility has hindered its extensive utilization. However, the use of URP primer (20 mers) used in this study resulted in the amplification of specific bands leading to high reproductivity. We isolated, cloned, and sequenced the 362 bp RAPD fragment from *A. japonica* and the 375 bp RAPD fragments from *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla*. An alignment of the four RAPD sequences showed differences among the fragments (Fig. 2). Compared to the *A. japonica* sequence, the *A. bicolor bicolor* sequence had 8 transitions, 2 transversions, and a 13 bp insertion; the *A. rostrata* sequence had 7 transitions, 3 transversions, and a 13 bp insertion; and the *A. anguilla* sequence had 7 transitions, 2 transversions, 1 deletion, and a 13 bp insertion. Thus, the *A. japonica* RAPD fragment is easily distinguished from the RAPD fragments of the other species by its 13 bp deletion.

PCR of the ~370 bp RAPD fragments using the specific primers A1F and AIR amplified a 290 bp product for *A. japonica* and a 303 bp fragment for the other three species (Fig. 3A). In addition, PCR using the specific primers A2F and A2R amplified no band for *A. japonica* but amplified a 243 bp fragment for the other three species (Fig. 3B). A BLAST search (National Center for Biotechnology Information) of the ~370 bp fragment sequences revealed no obvious similarities with any other sequences in the GenBank database.

The use of more specific, sensitive, and reproducible markers such as RAPD-based SCARs has been explored for its application to the authentication of plant (Dnyaneshwar et al., 2006; Adinolfi et al., 2007) and animal species (Araneda et al., 2005). Its potential advantages could increase the industrial use of molecular techniques for commercially important samples (Dnyaneshwar et al., 2006). SCAR markers developed from RAPD fragments derived from genomic regions of unknown sequence could be useful for the unambiguous identification of four eels of the genus *Anguilla*.

PCR-RFLP analysis

The 303 bp *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla* PCR fragments and the 290 bp *A. japonica* fragment amplified using primers A1F and A1R were digested with the restriction enzymes *TaqI* and *MaeI*. As expected, *TaqI* digestion generated DNA fragments of 171 and 132 bp from the 303 bp fragment but not from the 290 bp fragment because the latter lacks a *TaqI* recognition site [T/CGA] (Fig. 4A). *MaeI* digestion yielded products of 274 and 29 bp from the *A. bicolor bicolor* PCR fragment, which contains a *MaeI* site [C/TAG], but not from the other PCR fragments (Fig. 4B). Finally, when the 130 bp PCR

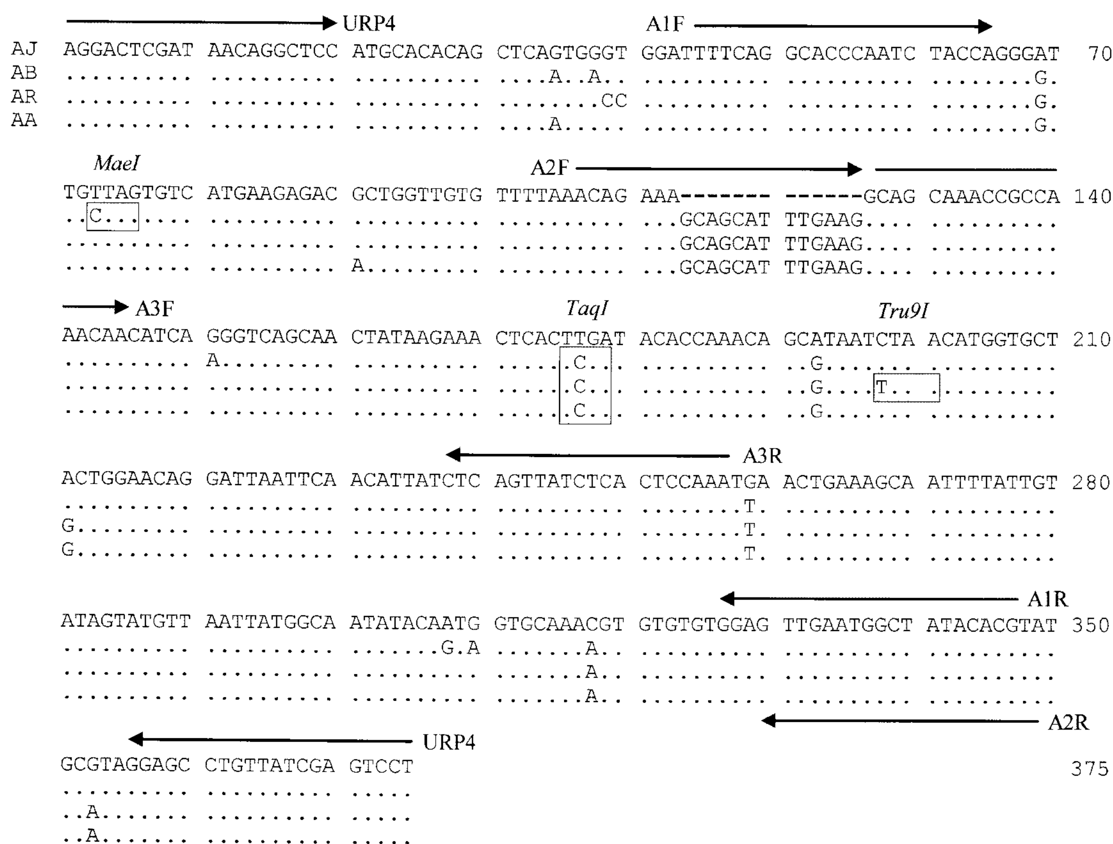

Fig. 2. Alignment of the ~370 bp RAPD marker sequences from four *Anguilla* eel species. The randomly amplified fragments were purified from gels, cloned, and sequenced. Dots indicate matches among the four species, and dashes indicate gaps. The primers used in this study are indicated by arrows. Boxes indicate restriction enzyme sites allowing the identification of *A. japonica* (AJ), *A. bicolor bicolor* (AB), *A. rostrata* (AR), and *A. anguilla* (AA).

fragments amplified from the RAPD products using primers A3F and A3R were digested with the restriction enzyme *Tru9I* [T/TAA], *A. rostrata* was distinguished by its *Tru9I* digestion products of 71, 34, and 25 bp; *Tru9I* digestion of the *A. japonica*, *A. bicolor bicolor*, and *A. anguilla* fragments generated products of 96 and 34 bp (Fig. 4C).

The morphological similarities between *A. japonica* and *A. anguilla* during grass eel and elver stages are a major hindrance in distinguishing these species, and several molecular techniques have been used in attempts to discriminate between them (Itoi et al., 2005). In addition, finding suitable markers for natural hybridization between *A. anguilla* and *A. rostrata* is crucial to the maintenance of the natural species composition and management of sustainable fisheries (Frankowski et al., 2008). Our method provides a rapid, simple, and inexpensive advancement for the identification of these species because it does not require sequencing; only band patterns from amplicon digestion are required.

Several studies have suggested that enzyme digestion of amplicons might be a useful strategy for eel identification because SNPs could alter recognition or cleavage sites

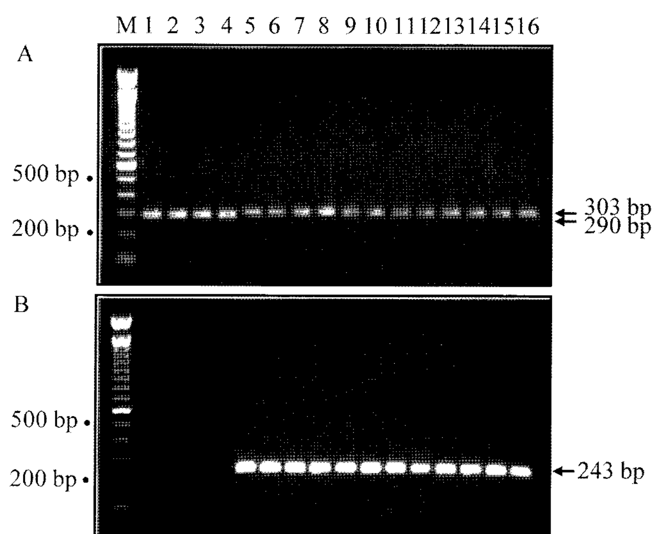

Fig. 3. PCR patterns obtained from amplification of the ~370 bp RAPD marker sequences from four *Anguilla* eel species using primer pairs A1F/A1R (A) and A2F/A2R (B). Lane M, 100 bp DNA ladder; lanes 1–4, *A. japonica*; lanes 5–8, *A. bicolor bicolor*; lanes 9–12, *A. rostrata*; lanes 13–16, *A. anguilla*. In (A), the *A. japonica* PCR products are 290 bp, and the *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla* PCR products are 303 bp. Selected bands of the 100 bp DNA ladder are identified at left.

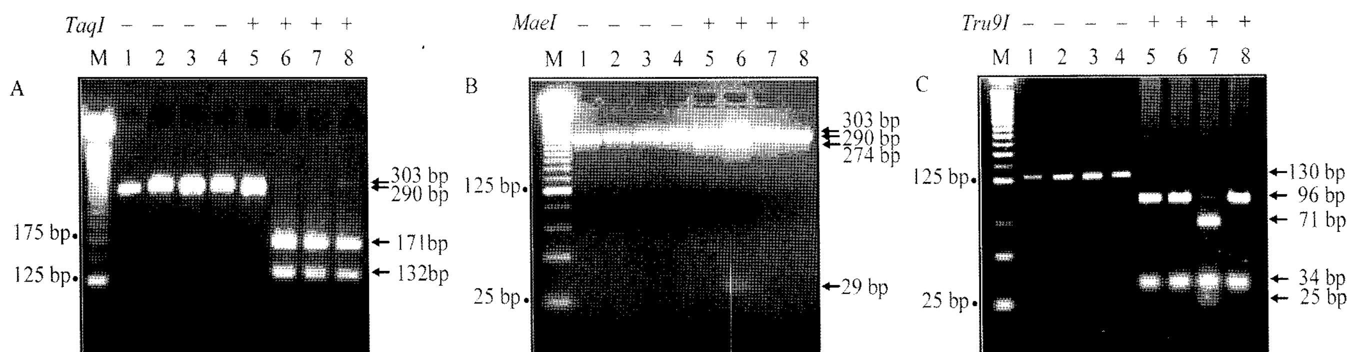

Fig. 4. RFLP patterns generated by restriction enzyme digestion of PCR amplicons. (A) *TaqI* digestion of A1F/A1R fragment. (B) *MaeI* digestion of A1F/A1R fragment. (C) *Tru9I* digestion of A3F/A3R fragment. Lane M, 25 bp DNA ladder; lane 1, *A. japonica* (undigested); lane 2, *A. bicolor bicolor* (undigested); lane 3, *A. rostrata* (undigested); lane 4, *A. anguilla* (undigested); lane 5, *A. japonica* (digested); lane 6, *A. bicolor bicolor* (digested); lane 7, *A. rostrata* (digested); lane 8, *A. anguilla* (digested). Selected bands of the 25 bp ladder are identified at left.

(Gagnaire et al., 2007; Frankowski et al., 2008). However, the problem of RFLP profiles has been studied with a primary focus on mitochondrial fragments (Rehbein et al., 2002), which assumes a single evolutionary history from only the maternal side. The pitfall of matrilineal discrimination inferred from mitochondrial data is that it can yield only a fraction of the genealogical information (Ballard and Whitlock, 2004). Natural hybridization between *A. anguilla* and *A. rostrata* cannot be detected by mitochondrial markers alone (Frankowski et al., 2008). The practical advantage of the SCAR and PCR-RFLP approach using genomic RAPD fragments is that it is sufficient for the evaluation of eel species despite the occurrence of hybridization and despite their similar morphological characteristics. Our results show that PCR-RFLP using three enzymes, *TaqI*, *MaeI*, and *Tru9I*, is sufficient to allow discrimination between the four *Anguilla* eel species.

Characterization of ~540 bp RAPD fragments

We also isolated, cloned, and sequenced the 577 bp *A. japonica* RAPD fragment, the 540 bp *A. bicolor bicolor* and *A. rostrata* RAPD fragments, and the 509 bp *A. anguilla* RAPD fragment. We found that the 577 bp *A. japonica* fragment contains ten repeats of the 37 bp sequence 5'-TACAC ACTCA CTATA TAACA CATGG AGCCT GGTCA GA-3', whereas the *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla* fragments contain nine, nine, and eight repeats of this sequence, respectively. These results suggest that analysis of the ~540 bp RAPD fragments obtained with the URP4 primer will prove useful for the identification of eel species and, particularly, for distinguishing *A. rostrata* from *A. anguilla*. Characterized variable-number tandem repeat (VNTR) regions have been used for evolutionary comparisons and genotyping assays in several bacterial (Vergnaud and Pourcel, 2006) and plant species (Blasko et al., 1998) and in humans (Buchmayer et al., 1996). Therefore, the localization of this VNTR polymorphism in the genomic locus of *Anguilla* species should provide a useful tool for the identification of these closely related species. In addition, we are developing highly specific SCAR markers from these RAPD fragments to reduce the probability of misidentification arising from the morphological confusion of samples, including eel species.

Evaluation of eel species identification methods

The straightforward differentiation among eel species has been hampered by the relatively recent speciation of eels, which has resulted in a low genetic distance between species (Minegishi et al., 2005) and overlap in morphological parameters (Lecomte-Finiger, 2003). Several molecular techniques have been applied to the problem of eel species discrimination, such as RAPD analysis (Lehmann et al., 2000), PCR-RFLP (Aoyama et al., 2000), single-strand conformation polymorphisms (Rehbein et al., 2002), PCR with species-specific primers (Pichiri et al., 2006), SNP analysis (Itoi et al., 2005), real-time PCR (Watanabe et al., 2004), and semi-multiplex PCR analysis (Gagnaire et al., 2007). Of these approaches, those based on DNA sequence differences have been recognized as the most accurate and economical methods to date, and their utility is widely acknowledged (Avise et al., 1986). Although amplified fragment length polymorphism and RFLP techniques are viable methods for detecting hybridization between *A. anguilla* and *A. rostrata* (Albert et al., 2006), use of these techniques for hybridization analysis has been hindered by the problem of mitochondrial background (Frankowski et al., 2008). Pichiri et al. emphasized the implementation of specific nuclear markers to discriminate among possible eel species hybrids, and our study suggests the possibility for future research related to this purpose.

We have shown that the *A. japonica*, *A. bicolor bicolor*, *A. rostrata*, and *A. anguilla* eel species can be clearly distinguished using a combination of SCAR and PCR-RFLP methods with genomic DNA. In addition, we

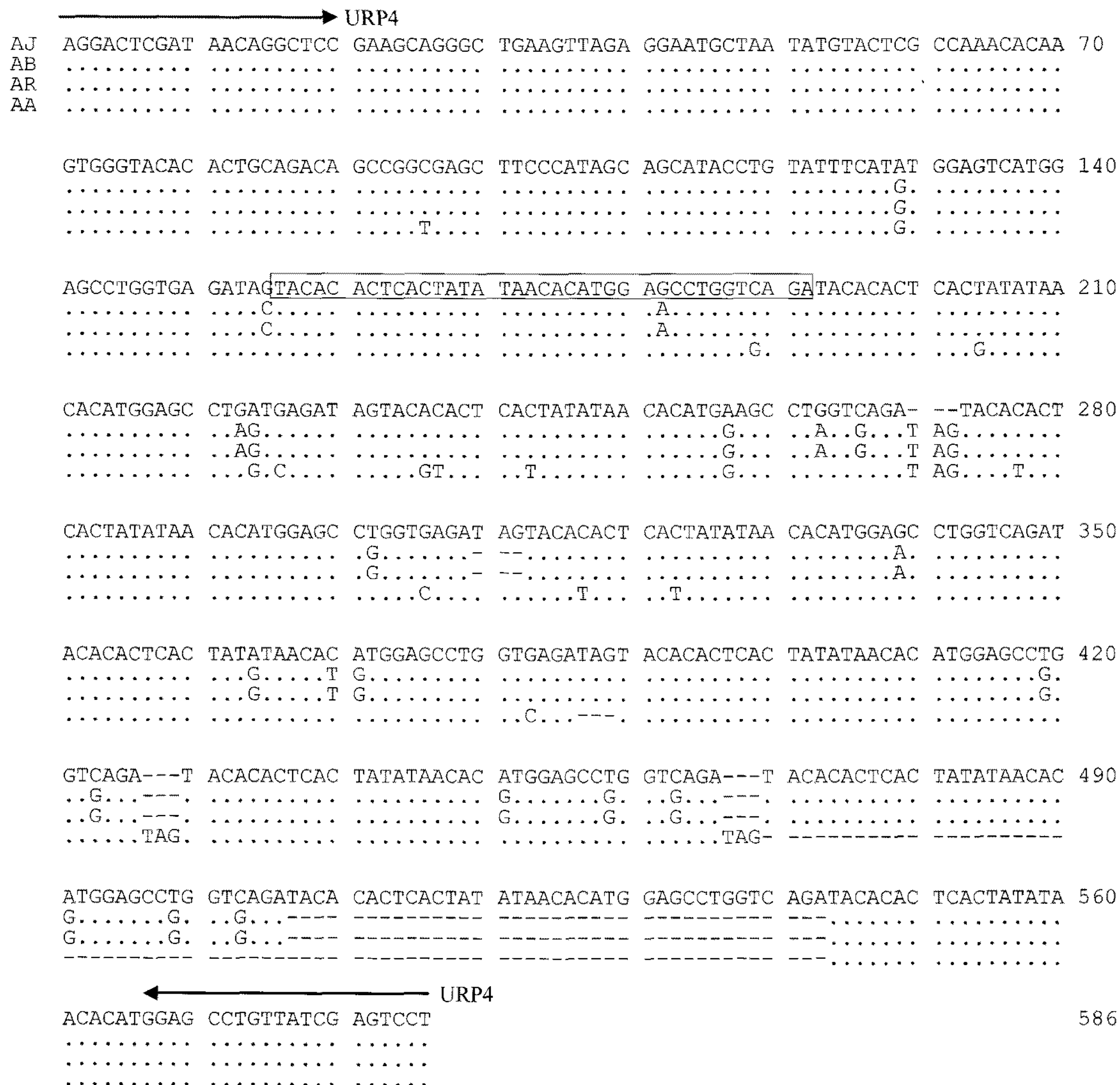

Fig. 5. Alignment of ~540 bp RAPD marker sequences. The randomly amplified fragments were purified from gels, cloned, and sequenced. Dots indicate matches among the four species, and dashes indicate gaps. The URP4 primer positions are indicated by arrows. Box indicates a 37 bp tandem repeat sequence. AJ, *A. japonica*; AB, *A. bicolor bicolor*; AR, *A. rostrata*; AA, *A. anguilla*.

performed the first characterization of tandem repeat polymorphisms for a 37 bp sequence found in ~540 bp RAPD fragments from these eel species and showed that tandem repeat polymorphisms can be applied effectively to the identification of species of the genus *Anguilla*. VNTRs have been successfully used as DNA markers for molecular typing studies of several phyla ranging from human to bacteria (Chang et al., 2006; Pourcel et al., 2007). Our characterization of two RAPD fragment regions of approximately 370 and 540 bp may provide a valuable genetic tool for differentiating the variability among species.

Sequencing and PCR analysis (SCAR and PCR-RFLP) of RAPD-generated fragments can provide useful species-specific markers for the simple, rapid, and routine identification of eel species in the genus *Anguilla*. Therefore, when used in combination with species-specific markers, SCAR and VNTR analyses are powerful and reliable tools for improving *Anguilla* species identification. This approach must be further tested for its practical utility in authenticating natural hybridization among eel species.

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