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Full Length Research Paper

Novel family- and genus-specific DNA markers in Mugilidae

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In this study, we identified novel family- and genus-specific DNA markers in Mugilidae fish. Genomic DNA was isolated from the blood of fish of 15 families and eighty (80) random primers were used for random amplified polymorphic DNA (RAPD) fingerprinting. When the primer OPAV04 was employed, a novel specific PCR product was observed in the Mugilidae family. In addition, another novel specific PCR product was also observed in the *Liza* genus while using primer OPAV10. Sequencing analysis revealed that the novel family- and genus-specific DNA fragments were 857 and 419 bp, respectively, and no similar sequences were found in GenBank. Two primers sets were designed based on the family- and genus-specific sequences to confirm the RAPD results and the 571 and 187 bp predicted bands were successfully amplified by PCR. Intriguingly, these two novel specific DNA markers were also effectively used for terrestrial and aquatic animal discrimination. Therefore, the novel family- and genus-specific DNA markers identified in this study can be used as an effective tool for rapid and accurate determination of the Mugilidae family and *Liza* genus, and even for cross-species identification.

Key words: Mugilidae, family- and genus-specific sequences, DNA markers.

INTRODUCTION

The Mugilidae family fish, referred to as mullets or grey mullets, are ray-finned fish inhabiting coastal and brackish waters of all tropical and temperate regions worldwide. The Mugilidae family includes 17 genera and a total of 72 valid species, most classified in the genera *Mugil* and *Liza*, which have 18 and 24 species, respectively (Thomson 1997; Nelson 2006). Along the Taiwan coast, 12 species of 7 genera of Mugilidae have been recorded in “The Fish Database of Taiwan” (http://fishdb.sinica.edu.tw/).

Mugil cephalus is a member of the Mugilidae family that migrates to the Taiwan coast and spawns in winter every year. Its fry tend to group in the estuary and are easily captured as a pond culture source. Currently, the food fish of *M. cephalus* are almost all pond-cultivated in Taiwan. The *M. cephalus* is an important source of income for the aquaculture industry in Taiwan: “karasumi” is the processed product of the eggs obtained from female *M. cephalus*, and has a high economic value.

Traditionally, morphological identification of fish is made according to the appearance, anatomy and useful taxonomic characteristics, such as hylogenetics, osteology, morphometrics, etc. (Harrison et al., 2007; Rossi et al., 1998a; Trewavas and Ingham, 1972). It is difficult to distinguish between the genera *Mugil* and *Liza* (Rossi et al., 1998a) by appearance and morphology, and the economic value of *M. cehpalus* is quite a bit higher than that of *Liza affinis*. Therefore, a molecular technique must be developed to distinguish the genera *Liza* in the

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Mugilidae family for necessary identification purposes. So far, studies on fish species identification in Mugilidae have included karyotype analysis (Nirchio et al., 2009; Rossi et al., 2005) using *in situ* hybridization techniques, genetic distance distribution by mt-DNA analysis (Papasotiropoulos et al., 2002, 2007), analysis of evolutionary relationships by allozyme electrophoresis (Rossi et al., 1998b, 2004; Turan et al., 2005), nucleic acid data (Fraga et al., 2007; Rossi et al., 2004), and 16S r-RNA mt-DNA (Liu et al., 2010; Rossi et al., 2004) methods for phylogeny verification.

The RAPD technique is applied for genetic analysis, analysis of phylogenic relationships and gender and species identification in fish (Barman et al., 2003; Chen et al., 2009; Elo et al., 1997; Govindaraju and Jayasankar, 2004; Horng et al., 2006; Wu et al., 2007). In this study, due to the characteristics of RAPD technology of simple manipulation, rapidity, and low cost, we employed this technique to identify food fish species in Taiwan. We expect to find a bio-marker for use as an adjuvant tool for early fish species identification to help minimize morphological discrimination errors in aquaculture.

MATERIALS AND METHODS

Sample collection

A total of 15 families and 63 fish were sampled from traditional markets, supermarkets and pond cultures in the central region of Taiwan. Blood samples were collected from the hearts of the fish of the families *Mugilidae*, *Cichlidae*, *Elopidae*, *Polynemidae*, *Sillaginidae*, *Cyprinidae*, *Sparidae*, *Trichiuridae*, *Sciaenidae*, *Chanidae*, *Epinephelus*, *Moronidae*, *Nemipteridae*, *Siganidae* and *Latidae*. The gender of the fish for the 15 families was not considered in this study.

Genomic DNA preparation

Extraction of genomic DNA from fish blood cells was performed as described previously (Huang et al., 2003). Each whole blood sample was washed in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA) by centrifugation at 2000×rpm for 5 min and the process was repeated several times until the supernatant was clear. The pellet was then resuspended in TNE buffer and stored at -20°C for frozen and thawed treatment. The pre-treated sample was mixed with 300 µl of 10% NH_4Cl, 75 µl proteinase K (10 mg/ml), 25 µl collagenase (3.8 IU/µl) and 200 µl of 10% SDS, and incubated at 55°C for 24 h with gentle agitation in a water bath. Genomic DNA was purified using phenol/chloroform extraction and isopropanol precipitation. Isolated genomic DNA was then dried and dissolved in a suitable volume of $2dH_2O$ ready for use. The terrestrial animal genomic DNAs of Brown Tsaiya ducks, Beijing ducks, angus, goats, pigeon, landrace, duroc and Yorkshire pigs prepared in our laboratory previously were used for species comparison.

RAPD-PCR analysis

The RAPD-PCR protocol followed was as described previously (Horng and Huang, 2003). Briefly, amplification was performed in a final volume of 15 µl containing 100 mM Tris-HCl (pH8.0), 1.5 mM $MgCl_2$, 50 mM KCl, 100 mM dNTPs, 0.14 mM primers (Operon Technologies, Inc., Alameda, CA, USA), 100 ng of template DNA and 0.5U *Taq* polymerase (DyNAzyme, Finnzymes Oy., Keilaranta, Espoo, Finland). Eighty (80) random primers (OPAA, OPAV, OPAO and OPC series) were used for RAPD-PCR. The reaction was carried out in a thermal cycler (HYBAID OminGrid) with the following amplification condition: 94°C for 5 min followed by 45 cycles of 1min at 94°C, 1 min at 36°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. The amplicons were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide (1.5 µg/ml) via UV light.

Specific fragment isolation, cloning and sequencing

The family- and genus-specific fragments were purified from agarose gel using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and cloned into pCR II-TOPO vector using a TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The confirmed construct, containing a specific fragment was sequenced using an ABI Prime BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA), was used for the analysis.

Identification of family- and genus-specific fragments by PCR

Primers were designed from the family- and genus-specific fragment sequences using GCG sequence analysis software (Genetic Computer Group, Madison, WI, USA) as follows: family-specific primers- MugilAV04SpeF1, 5'-aacacctctcatttctcaaacc-3' and MugilAV04SpeR1, and 5'-ttctgccatccaaattgatcc-3'; genus-specific primers- LizaAV10SpeF1, 5'-cgaacacccctacttttgatg-3' and LizaAV10SpeR1, and 5'-ttctgccatccaaattgatcc-3'. The 18S ribosomal gene was used as an internal control (18S-F: 5'-ctcccctcccgttacttgga-3' and 18S-R: 5'-ttggttttggtctgataaatgca-3') (Suchyta et al., 2003). The PCR conditions were the same as those used for RAPD-PCR, while the annealing temperature was raised to 62°C. The sizes of the PCR products were predicted as follows: family-specific length, 571 bp; genus-specific length, 187 bp and 18S, 100 bp, and were subjected to electrophoresis on 2% agarose gel as described previously.

RESULTS

Finding the family- and genus-specific bands by RAPD-PCR

Four random primers series (OPAA, OPAV, OPAO and OPC series) were used for RAPD-PCR to search for a specific DNA marker among ten families of fish bought from traditional markets, supermarkets and pond cultures in Taiwan. Most random primers yielded multiple bands representing polymorphism of RAPD fingerprinting between fishes. One of these primers, OPAV04 (TCTGCCATCC), amplified a major fragment in the RAPD fingerprints of all Mugilidae tested, but not in the other families (Figure 3). For further investigation, the specific DNA fragment was purified from agarose gel and inserted into the pCR II-TOPO vector for sequencing. A sequence length of 857 bp was obtained (Figure 1) and

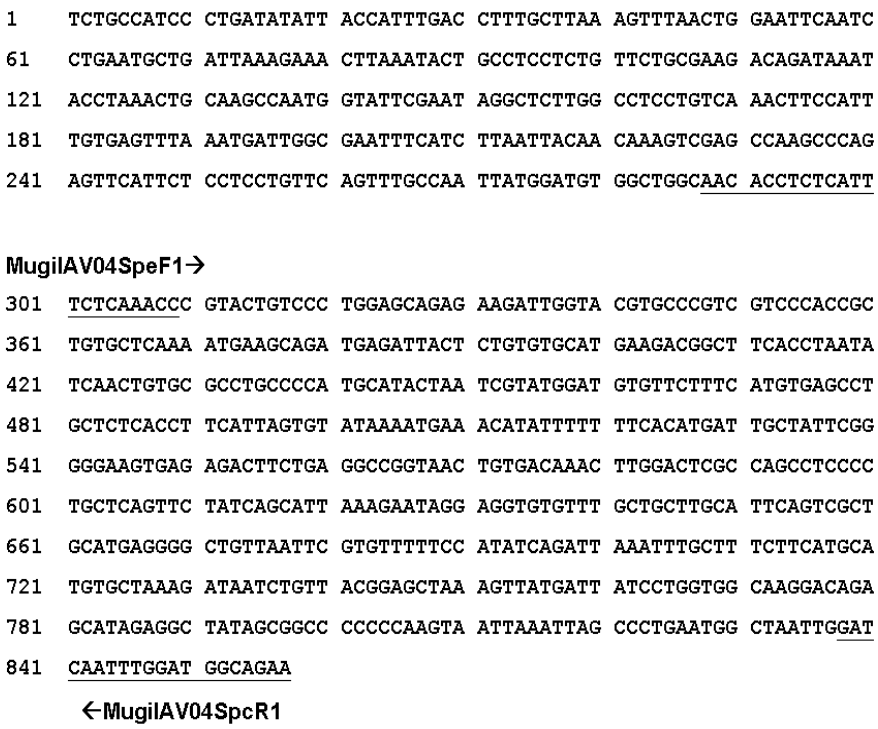

Figure 1. A novel family-specific DNA sequence (857 bp) of Mugilidae cloned from the RAPD fingerprints. Two primers, MugilAV04SpeF1 and MugilAV04SpeR1 (underlined), were designed based on the specific sequence for easy Mugilidae family identification by PCR.

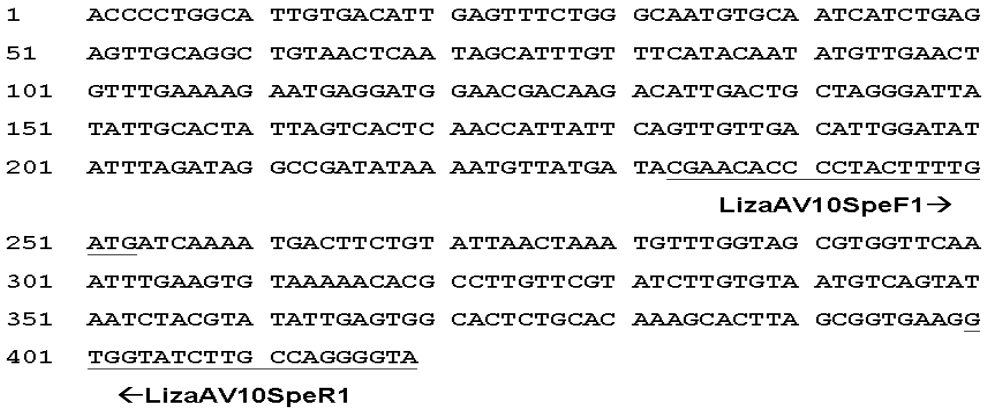

Figure 2. A novel genus-specific DNA sequence (419 bp) of *Liza* cloned from the RAPD fingerprints. Two primers, LizaAV10SpeF1 and LizaAV10SpeR1 (underlined), were designed based on the specific sequence for easy genus identification in Mugilidae by PCR.

has been submitted to GenBank (Accession Number, HM991290).

Intriguingly, we also found that the primer OPAV10 (GGACCTGCTG) amplified a significant band only in the RAPD fingerprints of the genera *Liza* from the Mugilidae tested (Figure 4). At the same time, we also purified the genus-specific fragment, cloned and sequenced it: its sequence length was 419 bp (Figure 2), and it has also been submitted to GenBank (Accession Number, DQ641039).

BLAST analysis revealed that these two specific fragments had no homologous sequences aligned with the nucleotide database. Thus, the cloned sequences could be considered novel family- and genus - specific

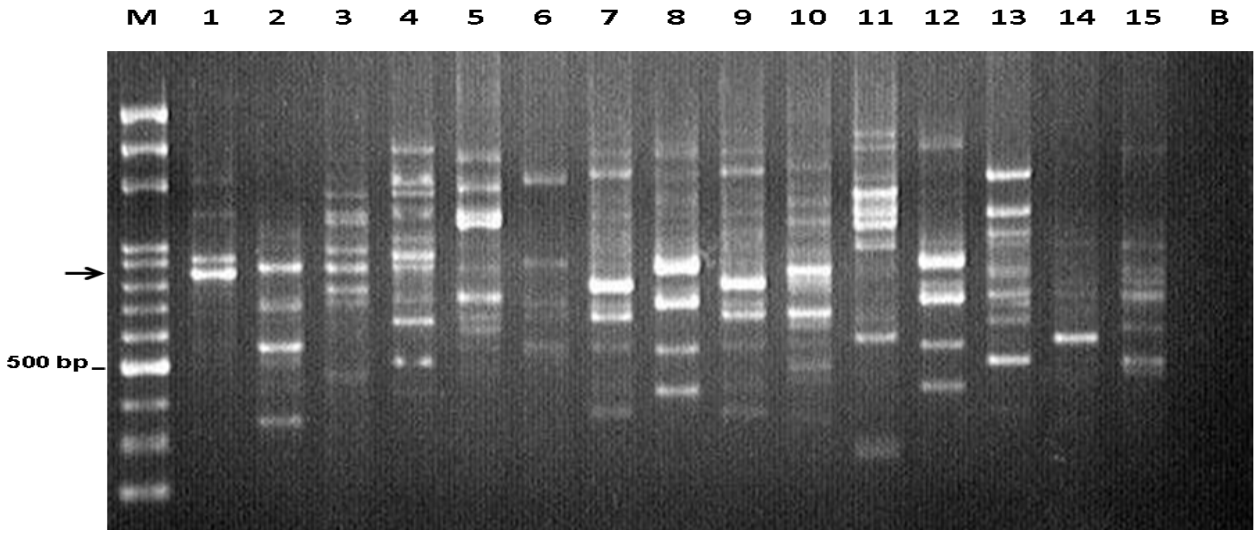

Figure 3. RAPD fingerprints of 15 popular food fish families in Taiwan. Genomic DNA isolated from fish blood was amplified with random primer OPAV04, which produced a specific band on the RAPD fingerprints only in the Mugilidae family (black arrow indicated). M: Bio-100 bp ladder markers, 1. *Liza spp.*, 2. *Liza affinis*, 3. *Liza haematocheilus*, 4. *Mugil cephalus*, 5. *Nemipterus virgatus*, 6. *Chanos chanos*, 7. *Siganus guttatus*, 8. *Trichiurus lepturus*, 9. *Epinephelus spp.*, 10. *Lateolabrax japonicu*, 11. *Lates calcarifer*, 12. *Elops machnata*, 13. *Tilapia zillii*, 14. *Aristichthys nobilis*, 15. *Eleutheronema rhadinus* and B is blank.

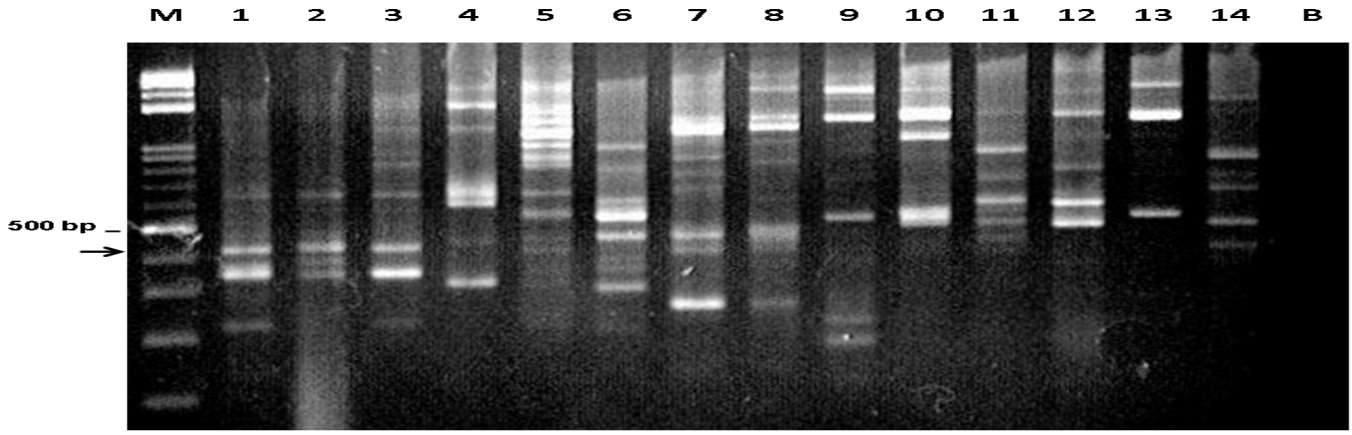

Figure 4. RAPD fingerprints of 10 popular food fish families in Taiwan. Genomic DNA isolated from fish blood was amplified with random primer OPAV10, which produced a specific band on the RAPD fingerprints only in the *Liza* genus (black arrow indicated). M: Bio-100 bp ladder markers, 1. *Liza spp.*, 2. *Liza affinis*, 3. *Liza haematocheilus*, 4. *Mugil cephalus*, 5. *Chanos chanos*, 6. *Pennahia argentata*, 7. *Siganus spp.*, 8. *Trichiurus spp.*, 9. *Evynnis spp.*, 10. *Pennahias macrocephalus*, 11. *Psenopsis spp.*, 12. *Sillago spp.*, 13. *Tilapia spp.*, 14. *Eleutheronema spp.* and B is blank.

sequences for Mugilidae and *Liza* spp. identification, respectively.

Validation of family- and genus-specific DNA fragments by PCR analysis

Two sets of primers, MugilAV04SpeF1/R1 and LizaAV10SpeF1/R1, were designed based on the family- and genus-specific sequences, respectively (Figures 1 and 2). The 18S ribosomal gene was used as the internal control. As predicted, the PCR results showed a 571 bp clear band using the family-specific primer set only in the Mugilidae family (Figure 5A, lanes 1~4), whereas the 18S gene product was observed in all fish. On the other hand, using the genus-specific primer set revealed a 187 bp band only in the *Liza* genus (Figure 5B, lanes 1~3). To confirm the accuracy and confidence limits of the PCR method, other individual fish were tested, and the family- and genus-specific bands were amplified in all Mugilidae

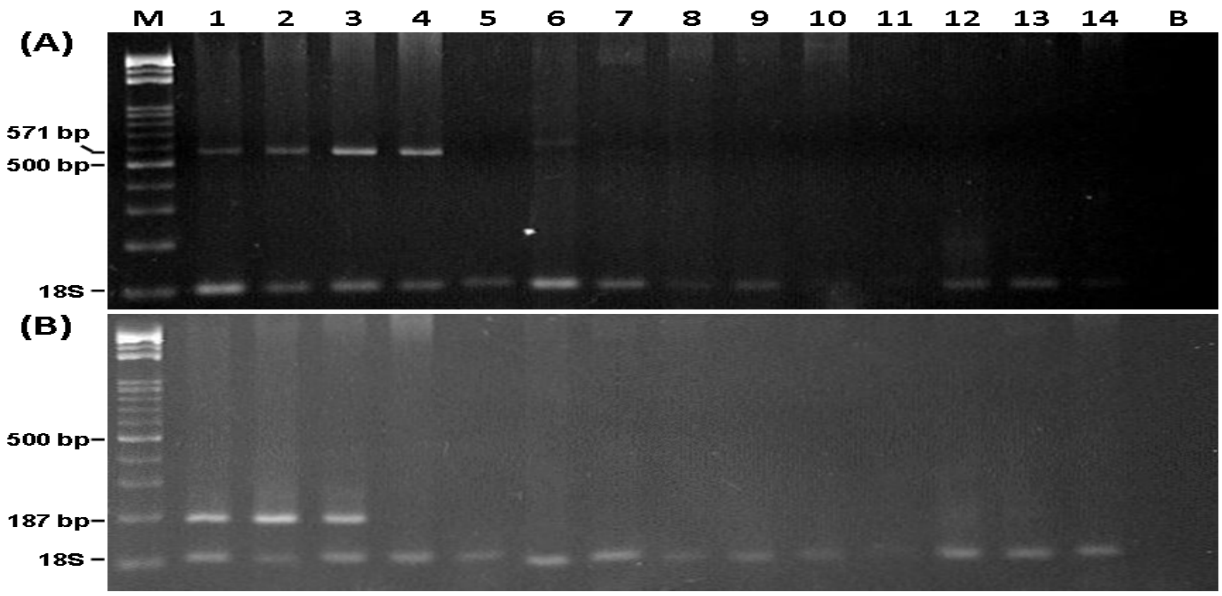

Figure 5. PCR analysis for family and genus identification in 10 popular food fish families in Taiwan. Genomic DNA isolated from fish blood was further amplified with family-specific primer, genus-specific primer and control 18S ribosomal gene primer sets. The family-specific PCR product (571 bp) was present only in Mugilidae (A), whereas the genus-specific PCR product (187 bp) was visualized only in Liza (B). The internal control 18S ribosomal gene presented a 100 bp band in all samples tested. M: Bio-100 bp ladder markers, 1. *Liza spp.*, 2. *Liza affinis*, 3. *Liza haematocheilus*, 4. *Mugil cephalus*, 5. *Chanos chanos*, 6. *Pennahia argentata*, 7. *Siganus spp.*, 8. *Trichiurus spp.*, 9. *Evynnis spp.*, 10. *Pennahias macrocephalus*, 11. *Psenopsis spp.*, 12. *Sillago spp.*, 13. *Tilapia spp.*, 14. *Eleutheronema spp.* and B is blank.

and *Liza* spp., respectively (data not shown). Thus, these results indicated that our family- and genus-specific primer sets could indeed be used as an accurate and efficient PCR-based method for family and genus identification in aquaculture.

Identification of the difference between terrestrial and aquatic animals

To further validate the uniqueness and specificity of the novel Mugilidae family-specific primer set, genomic DNA samples from terrestrial animals, such as bovine, porcine, goat, chicken, duck DNA etc., were tested by PCR. As shown in Figure 6, no 571 bp clear band was observed in any terrestrial samples, only in the aquatic sample Mugilidae. Similarly, the *Liza* spp. genus-specific primer set also amplified a significant signal (187 bp) and was detected in the aquatic and not the terrestrial animals tested (data not shown). These results strongly indicated that the novel Mugilidae family- and *Liza* genus-specific fragments can be used for cross-species identification.

DISCUSSION

The original purpose of this study was to find a unique DNA marker for the discrimination of terrestrial and aquatic animals in Taiwan. Firstly, we collected popular families of food fish in Taiwan and isolated genomic DNA from blood as described in materials and methods. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique has been successfully applied for species identification and sexing of animals (Bardakci and Skibinski, 1994; Chen et al., 2009; Horng et al., 2006; Kovács et al., 2000; Partis; Wells, 1996; Wu et al., 2007). In this study, the RAPD fingerprints of fish were amplified using random primers, and multiple major and minor bands in the fingerprints of the samples could be observed in each lane. This indicated that some DNA sequences were homologous and/or conserved in individuals. Using the OPAV04 primer, a specific fragment was found to be present only in the Mugilidae family tested (Figure 3). After specific fragment purification, cloning, sequencing and PCR verification, a Mugilidae family-specific 871 bp fragment was obtained. BLAST analysis of this specific sequence revealed that no nucleotide sequence was similar to the family-specific fragment, which suggested that it could be used as a novel identification marker for the Mugilidae family.

In addition, we found that the RAPD fingerprints obtained using the OPAV10 primer contained a significant band in the *Liza* genus of the Mugilidae family (Figure 4). After further investigation by fragment purification, cloning, sequencing and PCR confirmation, a *Liza* genus-specific 419 bp fragment was obtained. As with the family-specific fragment, there were no homologous sequences aligned with the nucleotide database.

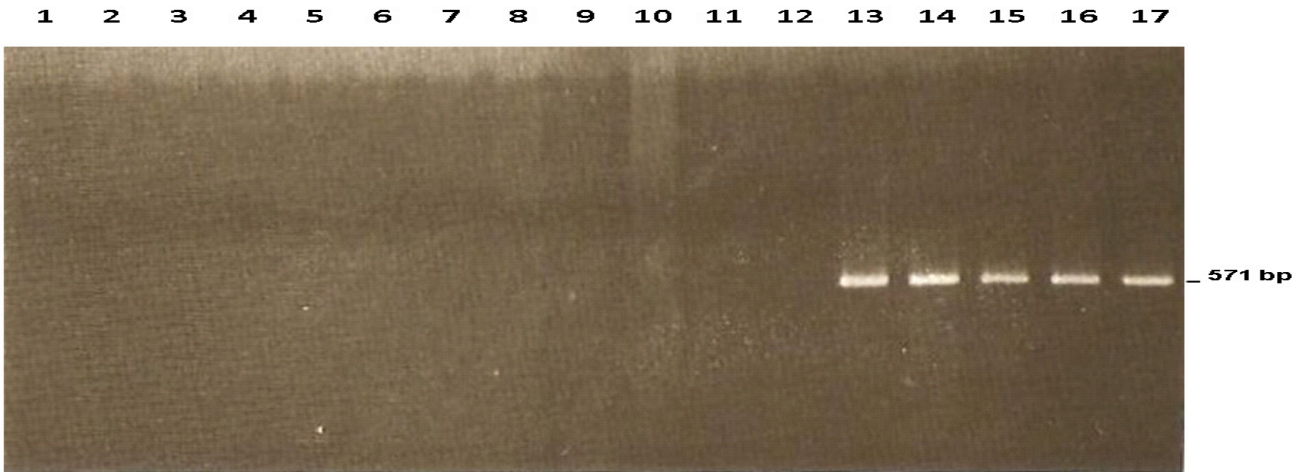

Figure 6. Discrimination of terrestrial and aquatic animals by PCR. Genomic DNA isolated from terrestrial and aquatic animals was further amplified using the family-specific primer set. A significant band (571 bp) was clearly displayed only in the aquatic but not in the terrestrial animals tested. Lanes 1 and 2: Brown Tsaiya duck, 3 and 4: Beijing duck, 5 and 6: Angus, 7 and 8: Goat, 9: Pigeon, 10: Landrace, 11: Duroc, 12: Yorkshire, 13~17: Mugilidae Liza spp..

Fortunately, no significant similarity was found between the Mugilidae family-specific and *Liza* genus-specific fragments by BLAST alignment. This result suggested that both the family- and genus-specific fragment may be used as novel discrimination markers in aquaculture fisheries.

The Mugilidae family consists of 17 genera and a total of 72 valid species, most of which are classified in the genera *Mugil* and *Liza*, which contain 18 and 24 species, respectively (Thomson 1997; Nelson 2006). Traditionally, morphological identification of fish is made according to appearance, anatomy and useful taxonomic characteristics (Rossi et al., 1998a). Unfortunately, the appearances of the genera *M. cephalus* and *L. affinis* are very similar and it is difficult to distinguish between the two. *M. cephalus* is an important source of income for the aquaculture industry in Taiwan; “karasumi” is the processing product of eggs obtained from female *M. cephalus*, with a high economic value, while *L. affinis* is of a relatively low economic value. In this study, we developed a novel *Mugil* molecular marker (Figure 1), and a *Liza* genus-specific fragment (Figure 2), to distinguish between the genera *Mugil* and *Liza* (Figure 5). The *Liza* genus-specific DNA marker provides a rapid, simple, accurate and useful tool for distinguishing between genera in *M. cephalus* fry and *Liza affinis,* preventing *Liza affinis* fry contamination at an early stage of classification.

Recently, vegetarian food has become more popular for reasons of health and religion. As the name implies, vegetarian food does not contain any terrestrial or aquatic animal products. Some merchants add animal products to vegetarian foods to raise the flavor and umami – the common approach to raise the umami in vegetarian foods is via aquatic components supplementation. The same also occurs in meat processing products. It is therefore necessary to develop a molecular technique for identification of the components of processing products. RAPD-PCR analysis is commonly used for specific identification in meat and seafood products (Bossier, 1999; Martinez and Yman, 1998). Our finding of a family-specific DNA marker that can be used to distinguish between terrestrial and aquatic animals is important (Figure 6), and indicates that the novel family-specific fragment can be used as a DNA marker for cross-species identification.

In conclusion, we have developed novel Mugilidae family- and *Liza* genus-specific DNA markers from RAPD fingerprints. Two primer sets, MugilAV04SpeF1/R1 and LizaAV10SpeF1/R1, were accurately and rapidly used for family and genus determination in aquaculture and even for cross-species identification by PCR.

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