J Appl Genet 46(4), 2005, pp. 381-385

Molecular identification of hairtail species (Pisces: Trichiuridae) based on PCR-RFLP analysis of the mitochondrial 16S rRNA gene

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Abstract. A rapid PCR-RFLP analysis was designed to identify 3 closely related species of hairtails: *Trichiurus lepturus*, *T. japonicus*, and *Trichiurus* sp. 2, basing on partial sequence data (600 bp) of the mitochondrial DNA encoding the 16S ribosomal RNA (16S rRNA) gene. Restriction digestion analysis of the unpurified PCR products of these 3 species, using *Eco*RI and *Vsp*I endonucleases, generated reproducible species-specific restriction patterns showing 2 fragments (250 bp and 350 bp) for *T. lepturus* in *Eco*RI digestion and 2 fragments (196 bp and 404 bp) for *T. japonicus* in *Vsp*I digestion, whereas no cleavage was observed for *Trichiurus* sp. 2 in both *Eco*RI and *Vsp*I digestions. The PCR-RFLP technique developed in this study proved to be a rapid, reliable and simple method that enables easy and accurate identification of these 3 closely related species of the genus *Trichiurus*.

Key words: 16S rRNA, hairtails, species identification, PCR-RFLP, Trichiuridae.

Introduction

The family Trichiuridae (hairtails or cutlassfishes), which comprises 9 genera and 32 species, are generally benthopelagic on continental shelves and slopes and distributed in tropical to warm temperate waters (Nakamura and Parin 1993). The taxonomic classification of members of the genus *Trichiurus* is confusing, because of their similar body shape and coloration. They have long been considered as "one variable species" (Tucker 1956), and *Trichiurus lepturus* (type locality: South Carolina, North America) was considered a single and valid circum-global species in the most recent FAO review by Nakamura and Parin (1993). Two species of hairtails – *Trichiurus japonicus* and *Trichiurus* sp. 2 – are most frequently and abundantly caught hairtails in Japanese waters (Nakabo 2002) and our recent genetic study involving the genetic divergence and phylogenetic analysis of the partial 16S rRNA gene showed that *T. lepturus*, *T. japonicus* and *Trichiurus* sp. 2 are indeed 3 separate valid species (Chakraborty et al. in press). Morphologically, these 3 species look very similar except for coloration of the dorsal fin (yellow in *Trichiurus* sp. 2 and white in both *T. lepturus* and *T. japonicus*) and length of the tail (very long in *T. japonicus*, medium in *T. lepturus* and short in *Trichiurus* sp. 2). Although morphological features are sufficient for species identification in fresh specimens, it is difficult to identify the species origin in processed products, such as fresh and frozen fillets, since many of the distinguishing morphological features are no longer detectable after processing. In Japan, hairtail fillets are mainly marketed as fresh or frozen; the fresh ones are usually used for raw consumption called "sashimi", while the frozen fillets are used for either boiled or grilled products (Mochijuki 1997). *Trichiurus* sp. 2 enjoys a higher consumer prefer-

Received: July 12, 2005. Revised: September 14, 2005. Accepted: September 15, 2005.
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ence and commercially fetches a higher price than *T. japonicus* because of its better meat quality.

In recent years, DNA-based studies involving PCR amplification of nuclear and mitochondrial DNA, followed by estimation of genetic divergences from the sequence data, are being used to discriminate between closely related species (Bourdy et al. 2003; Karaiskou et al. 2003; Mabuchi et al. 2003), with regard to their taxonomic and phylogenetic classification. However, in the case of processed products, most molecular biological methods used to verify species identity are based on PCR amplification of a polymorphic portion of DNA due to the simplicity, specificity and sensitivity (Palumbi 1996) and one such promising method is restriction fragment length polymorphism analysis of PCR products (PCR-RFLP). This method provides a simpler alternative to costly DNA sequencing techniques and has been widely used for species identification of many closely related species of fish (Quinteiro et al. 1998; Sebastio et al. 2001; Aranishi et al. 2005a, b).

The present study was undertaken to develop a simple, rapid and accurate method based on PCR-RFLP analysis of the 600-bp fragment encoding the mitochondrial 16S rRNA gene, for identification of 3 closely related and morphologically similar species of hairtails.

Materials and methods

Hairtail samples

Tissue samples of *T. japonicus* and *Trichiurus* sp. 2 from various parts of Japan were collected from 10 individual specimens each, while for *T. lepturus*, tissue samples were obtained from 15 individual specimens caught off the western Atlantic coast of America and Brazil. Details of the samples, indicating the collection date and localities, are given in Table 1.

Table 1. Specimens used for RFLP analysis, with date of collection and localities

Species	Locality	Date of collection	Catalogue number
Trichiurus sp. 2	Miyazaki, Japan	07.04.1999	MUFS 17608
	Okinawa, Japan	20.12.1999	MUFS 17756
	Okinawa, Japan	20.12.1999	MUFS 17757
	Okinawa, Japan	20.12.1999	MUFS 17758
	Okinawa, Japan	20.12.1999	MUFS 17759
	Okinawa, Japan	07.11.1999	MUFS 17760
	Miyazaki, Japan	07.12.2002	MUFS 21660
	Miyazaki, Japan	17.09.1999	MUFS 22242
	Miyazaki, Japan	17.09.1999	MUFS 22243
	Okinawa, Japan	11.11.2000	MUFS 22247
T. japonicus	Nagasaki, Japan	18.10.1999	MUFS 18237
	Nagasaki, Japan	18.10.1999	MUFS 18240
	Nagasaki, Japan	18.10.1999	MUFS 18242
	Nagasaki, Japan	18.10.1999	MUFS 18245
	Nagasaki, Japan	18.10.1999	MUFS 18397
	Chiba, Japan	20.08.2002	MUFS 20254
	Chiba, Japan	20.08.2002	MUFS 20256
	Chiba, Japan	20.08.2002	MUFS 22093
	Miyazaki, Japan	07.12.2002	MUFS 22094
	Miyazaki, Japan	28.10.2002	MUFS 22246
T. lepturus	off Atlantic coast, USA	12.09.1994	KU 1206
	off Atlantic coast, USA	12.09.1994	KU 1224
	off Atlantic coast, USA	10.03.1995	KU 1529
	off Pascagoula, Gulf of Mexico, USA	16.11.2001	KU 3900
	off Brownsville, Gulf of Mexico, USA	17.06.2002	KU 5078
	off Brazil	28.10.2004	MUFS(T) 01
	off Brazil	28.10.2004	MUFS(T) 02
	off Brazil	28.10.2004	MUFS(T) 03
	off Brazil	28.10.2004	MUFS(T) 04
	off Brazil	28.10.2004	MUFS(T) 05
	off Brazil	28.10.2004	MUFS(T) 06
	off Brazil	28.10.2004	MUFS(T) 07
	off Brazil	28.10.2004	MUFS(T) 08
	off Brazil	28.10.2004	MUFS(T) 09
	off Brazil	28.10.2004	MUFS(T) 10

MUFS = Miyazaki University, Fishery Sciences; T = Tissue Collection; KU = Kansas University

DNA extraction, PCR amplification and sequencing

Total DNA was extracted using a DNeasy Tissue kit (Qiagen, Tokyo, Japan) from muscle tissues preserved in 99.5% ethanol, according to the manufacturer's protocols. The partial 16S rRNA gene (600 bp) was amplified using the following primers: L2510 (5'-GCCTGTTTA ACAAAAACAT-3') and H3059 (5'-CGGTCTG AACTCAGATCACGT- 3') (Miya and Nishida, 1996). PCR amplification was carried out in a 25-μL reaction volume containing 1X Taq DNA polymerase reaction buffer (Bioneer, Korea), 5 μM of each dNTP (Bioneer, Korea), 0.40 μM of each primer, 0.125 U *Taq* DNA polymerase (Bioneer, Korea), and 1 μL of DNA template in a Techgene thermocycler (TC 312, Techne, UK). The thermal cycling profile was as follows: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, with a final extension for 5 min at 72°C. The PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide (0.5 μg mL^{-1}), and visualized under a UV transilluminator in a Digi Doc-It System (UVP, BioImaging system, USA).

Double-stranded DNA products were purified by Microcon 100 (Millipore, USA), and nucleotide sequences were determined by direct sequencing using a Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in a PRISM 310 Genetic Analyzer (Applied Biosystems, USA), following manufacturer's instructions. Primers used were the same as those for PCR amplification.

Identification of restriction sites

The sequence analysis data showed unique sites for 2 restriction enzymes, *Eco*RI and *Vsp*I among the 3 species. The restriction enzyme *Eco*RI produced 2 fragments of 250 bp and 350 bp in *T. lepturus*, whereas its restriction site was not present in *Trichiurus* sp. 2 and *T. japonicus*. The other restriction enzyme *Vsp*I produced 2 fragments of 196 bp and 404 bp in *T. japonicus*, but its restriction site was not present in *Trichiurus* sp. 2 and *T. lepturus*. All the individual specimens from each of the 3 species were used to verify the sequence polymorphism of the mitochondrial 16S rRNA gene, and no mutation was observed at the recognition sites of both restriction enzymes.

RFLP analysis

Restriction digestion of the PCR products for the partial 16S rRNA gene from the 3 species was carried out using *Eco*RI (Fermentas, Lithuania) and *Vsp*I (Fermentas, Lithuania) endonucleases separately in a 10-μL reaction mixture containing 1 × Buffer O^+ (Fermentas, Lithuania), 5 μL of unpurified PCR products, and 5 units of each endonuclease at 37°C for 1 h. A 5-μL sample of the reactant was run on a 2.0% agarose gel and visualized as described above.

Results and discussion

Figure 1 compares the aligned sequences of the partial 16S rRNA gene of *Trichiurus* sp. 2 (AB200990), *T. japonicus* (AB200989) and *T. lepturus* (AB201821), which enables identification of these 3 species. Direct digestion of the PCR products separately with *Eco*RI and *Vsp*I resulted in species-specific restriction patterns. They corresponded to the expected profiles as described above and using *Eco*RI, fragments of around 250 bp and 350 bp were seen in *T. lepturus* while an uncut fragment of around 600 bp was seen in both *Trichiurus* sp. 2 and *T. japonicus* (Figure 2). In the case of *Vsp*I, fragments of around 200 bp and 400 bp were seen in *T. japonicus* while an uncut fragment of around 600 bp was seen in *Trichiurus* sp. 2 and *T. lepturus* (Figure 2).

In order to verify the usefulness of this PCR-RFLP technique, fillet samples of hairtails imported from various countries (like Indonesia, Oman, Pakistan, Thailand and Canada) were purchased from various supermarkets in Japan and analyzed for species identification by this technique. Restriction digestion with both *Eco*RI and *Vsp*I showed that the samples obtained from Indonesia, Oman, Pakistan and Thailand belonged to *Trichiurus* sp. 2 (an uncut fragment of around 600 bp seen after restriction digestion with both *Eco*RI and *Vsp*I), while the samples from Canada belonged to *T. lepturus* (fragments of 250 bp and 350 bp seen after *Eco*RI digestion but an uncut fragment after *Vsp*I digestion). Representative results of the PCR-RFLP analysis of the fillets are shown in Figure 3.

The RFLP results for the commercial fillets were further verified by direct sequencing of the PCR products from representative fillet samples. The fillet samples from Indonesia, Oman, Pakistan and Thailand, identified as *Trichiurus* sp. 2, showed a high homology of more than 99% with *Trichiurus* sp. 2 (AB200990), with mutations at

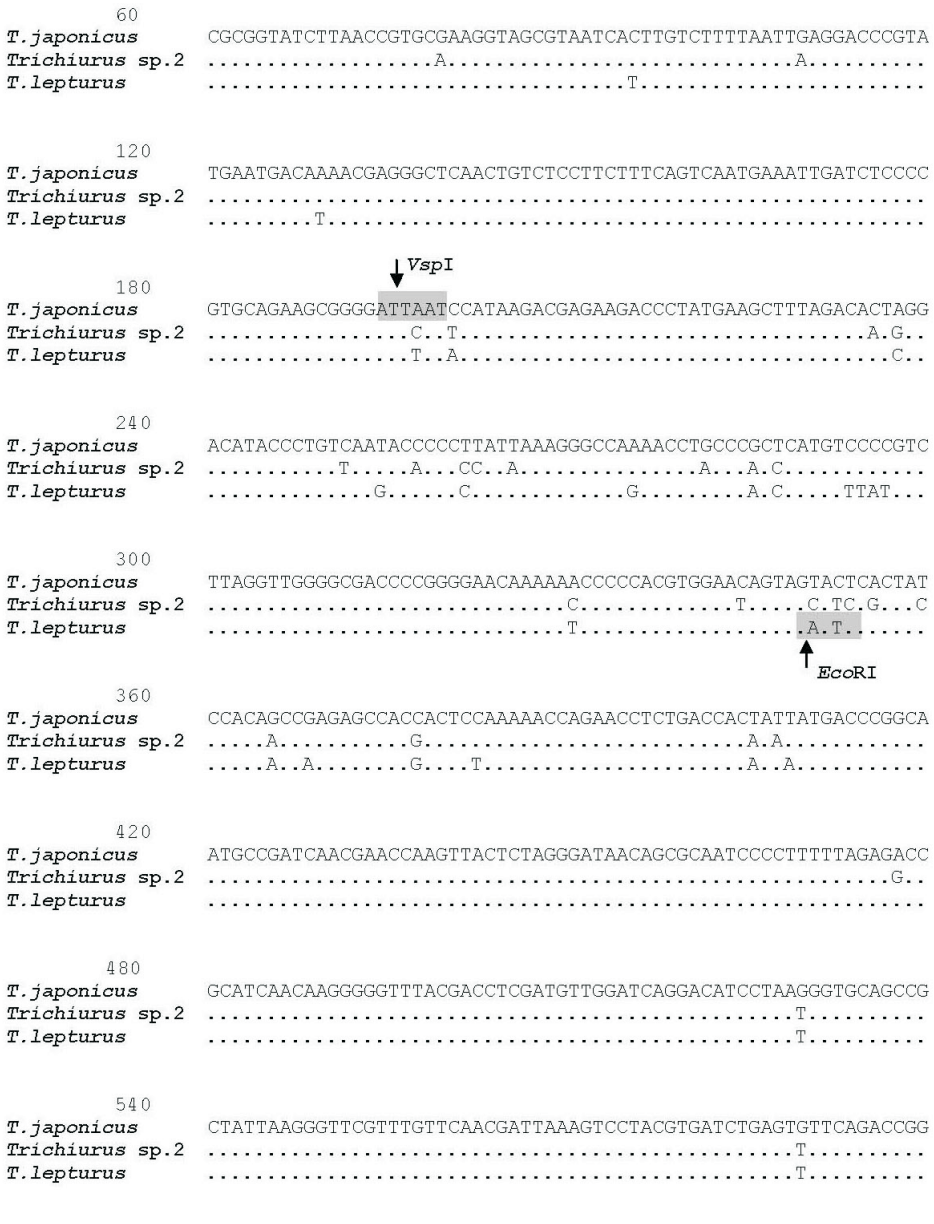

Figure 1. Alignment of DNA sequences of the partial mitochondrial 16S rRNA gene from *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus*. *Vsp*I and *Eco*RI sites are shown in boxes.

both *Eco*RI and *Vsp*I sites. In the case of fillet samples from Canada, sequencing of the representative samples showed a high homology of more than 99% with *T. lepturus*, with presence of the *Eco*RI site and absence of the *Vsp*I site.

The RFLP data obtained in this study attest to the mistaken identity of hairtail species in sequence data submitted to the GenBank. The database shows 3 partial 16S rRNA sequences (AY16492, AY216493 and AY216494) submitted as *T. lepturus* haplotypes. However, the restriction pattern for these 3 submitted sequences (AY216492, AY216493 and AY216494) was identical to that of *T. japonicus* (presence of the *Vsp*I site but absence of the *Eco*RI site). Moreover, the sequence similarity of these 3 sequences to *T. japonicus* was higher than 99%. The similar restriction pattern and high sequence similarity of these 3 submitted sequences with *T. japonicus* sequences proved that the submitted sequences were in fact from *T. japonicus*, and not from *T. lepturus*. The PCR-RFLP analysis using *Eco*RI and *Vsp*I restriction enzymes can easily and effectively identify the 3 species without any need of sequencing or phylogenetic analysis. The PCR-RFLP protocol developed in this study proved to be a simple, reliable, rapid and cost-effective method, requiring minimum experimental setup and enabling accu-

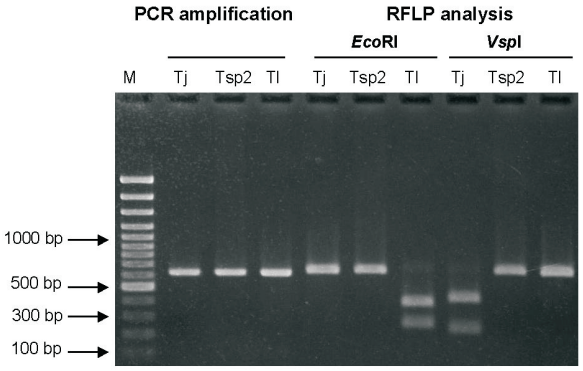

Figure 2. PCR-RFLP analysis of the partial 16S rRNA gene from *Trichiurus* sp. 2 (Tsp.2), *T. japonicus* (Tj), and *T. lepturus* (Tl). PCR amplification and RFLP analysis indicate the PCR products and those after restriction digestion using *Eco*RI and *Vsp*I, respectively. M = molecular weight ladder.

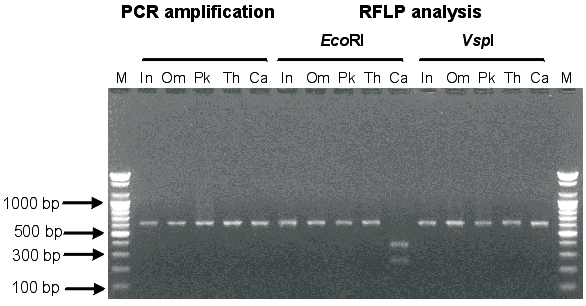

Figure 3. PCR-RFLP analysis of the partial 16S rRNA gene from imported fillet samples from Indonesia (In), Oman (Om), Pakistan (Pk), Thailand (Th) and Canada (Ca). PCR amplification and RFLP analysis indicate the PCR products and those after restriction digestion using *Eco*RI and *Vsp*I, respectively. M = molecular weight ladder.

rate identification of these closely related species of hairtails.

Acknowledgements. We are most grateful to Andrew C. Bentley and Edward O. Wiley (Biodiversity Research Center and Natural History Museum, University of Kansas, USA) and J.K. Dooley (Department of Biology, Adelphi University, USA) for providing tissue samples of *Trichiurus lepturus* specimens. This study was supported in part by Sasakawa Research Grant from the Japanese Science Society (No. 17–215), awarded to the first author.

REFERENCES

Aranishi F, Okimoto T, Izumi S, 2005a. Identification of gadoid species (Pieces, Gadidae) by PCR-RFLP analysis. J Appl Genet 46: 69–73.

Aranishi F, Okimoto T, Okhubo M, Izumi S, 2005b. Molecular identification of commercial spicy Pollack roe products by PCR-RFLP analysis. J Food Sci 70: 235–238.

Bourdy P, Heurtebise S, Lapegue S, 2003. Mitochondrial and nuclear DNA sequence variation of presumed *Crassostrea gigas* and *Crassostrea angulata* specimens: A new oyster species in Hong Kong? Aquaculture 228: 15–25.

Chakraborty A, Aranishi F, Iwatsuki Y, 2006. Genetic differences among three species of the genus *Trichiurus* (Perciformes: Trichiuridae) based on mitochondrial DNA analysis. Ichthyol Res (in press).

Karaiskou N, Apostolidis AP, Triantafyllidis A, Kouvatsi A, Triantaphyllidis C, 2003. Genetic identification and phylogeny of three species of the genus *Trachurus* based on mitochondrial DNA analysis. Mar Biotechnol 5: 493–504.

Mabuchi K, Okuda N, Kokita T, Nishida M, 2003. Genetic comparison of two color-morphs of *Apogon properuptus* from southern Japan. Ichthyol Res 50: 293–296.

Miya M, Nishida M, 1996. Molecular phylogenetic perspective on the evolution of the deep-sea fish genus *Cyclothone* (Stomiiformes: Gonostoma- tidae). Ichthyol Res 43: 375–398.

Mochijuki K, 1997. Pictorial encyclopedia of fish and shellfish. Kashiwa-Shobo Publ: 247-248.

Nakamura I, Parin NV, 1993. FAO Species Catalogue. Vol. 15. Snake mackerels and cutlassfishes of the world (families Gempylidae and Trichiuridae). An annotated and illustrated catalogue of the snake mackerels, snoeks, escolars, gemfishes, sackfishes, domine, oilfish, cutlassfishes, scabbardfishes, hairtails and frostfishes known to date. FAO, Rome.

Nakabo T, 2002. Family Trichiuridae. In: Nakabo T, ed. Fishes of Japan with pictorial keys to the species. Tokai University Press: 1749–1750.

Palumbi SR, 1996. Nucleic acid II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK, eds. Molecular Systematics. Sunderland, Sinauer Associates.

Quinteiro J, Sotelo CG, Rehbein H, Pryde SE, Perez-Martin RI, Rey-Mendez M, Mackie IM, 1998. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. J Agric Food Chem 46: 1662–1669.

Sebastio P, Zanelli P, Neri TM, 2001. Identification of anchovy (*Engraulis encrasicholus* L.) and gilt sardine (*Sardinella aurita*) by polymerase chain reaction, sequence of their mitochondrial cytochrome *b* gene and restriction analysis of polymerase chain reaction products in semipreserves. J Agric Food Chem 49: 1194–1199.

Tucker DW, 1956. Studies on trichiuroid fishes. A preliminary revision of the family Trichiuridae. Bull Brit Mus Nat Hist Zool 4: 73–130.