Development of Species-Specific PCR Primers for the Rapid and Simultaneous Identification of the Six Species of Genus *Takifugu*

Chun Mae Dong, Yeon Jung Park, Jae Koo Noh, Eun Soo Noh, Cheul Min An, Jung-Ha Kang, Jung Youn Park, and [†]Eun-Mi Kim

Biotechnology Research Division, National Institute of Fisheries Science, Busan 46083, Korea

ABSTRACT : Pufferfish (*Takifugu* spp.) are economically important edible marine fish. Mistakes in pufferfish classification can lead to poisoning; therefore, accurate species identification is critical. In this study, we used the mtDNA cytochrome c oxidase subunit I gene (COI) to design specific primers for six *Takifugu* species among the 21 domestic or imported pufferfish species legally sold for consumption in Korea. We rapidly and simultaneously identified these pufferfish species using a highly efficient, multiplex polymerase chain reaction (PCR) system with the six species-specific primers. The results showed that species-specific multiplex PCR (multiplex species-specific polymerase chain reaction; MSS-PCR) either specifically amplified PCR products of a unique size or failed. MSS-PCR yielded amplification fragment lengths of 897 bp for *Takifugu pardalis*, 822 bp for *T. porphyreus*, 667 bp for *T. niphobles*, 454 bp for *T. poecilonotus*, 366 bp for *T. rubripes*, and 230 bp for *T. xanthpterus* using the species-specific primers and a control primer (ca. 1,200 bp). We visualized the results using agarose gel electrophoresis to obtain accurate contrasts of the six *Takifugu* species. MSS-PCR analysis is easily performed and provides identification results within 6 h. This technique is a powerful tool for the discrimination of *Takifugu* species and will help prevent falsified labeling, protect consumer rights, and reduce the risk of pufferfish poisoning.

Key words : Pufferfishes, Multiplex Species-Specific (MSS), Identification, PCR, Mitochondrial DNA COI

INTRODUCTION

Fish in the Tetraodontidae family are found in both freshwater and brackish waters of tropical and temperate coastal areas. Although many species in this family are economically important fishery resources, with unique flavors and high market value, they are often difficult to classify due to their wide variety of morphological variation (Kim & Lee, 1990; Tyler, 1980). Within Tetraodontidae, most pufferfish species in the genus *Takifugu* are highly toxic to humans, containing tetrodotoxins that affect a variety of organs including the liver and ovaries. Toxicity varies considerably among regions and seasons; therefore, inaccurate *Takifugu* species classification carries a risk of tetrodotoxin poisoning. The Ministry of Food and Drug Safety of Korea allows the trade of only 21 domestic and imported pufferfish species; however, some of these species have very similar morphologies and are difficult to distinguish. Trade disputes arising from problems with species identification and classification among six *Takifugu* species commonly consumed in Korea have motivated the search for a simple, rapid, and effective molecular genetic

[†] Corresponding Author : Eun-Mi Kim, Dr., Biotechnology Research Division, National Institute of Fisheries Science, Busan 46083, Korea. Tel: +82-51-720-2462, E-mail: ocean0629@korea.kr



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method for identification of these species.

Previous studies have applied morphological and component analyses to determine the species used in fishery products; recently, diagnostic polymorphic sites have been used to identify closely related species (Unseld et al., 1995; Kim et al., 2014). Molecular identification assays based on mtDNA sequences have been widely used to identify the origins of fishery resources and products for the prevention of fraud, mislabeling, and health risks (Rasmussen & Morrissey, 2008; Cohen et al., 2009; Acar et al., 2017). However, little effort has been devoted to molecular-based species identification of *Takifugu* pufferfish species (Hsieh & Hwang, 2004; Ishizaki et al., 2006; Hsieh et al., 2010; Luekasemsuk et al., 2015).

We developed a *Takifugu* species identification method that will contribute to the establishment of a safe fishery management and distribution system. We designed speciesspecific primers in the mtDNA cytochrome c oxidase subunit I (COI) gene region for the rapid and accurate identification of six *Takifugu* species, all of which are consumed in Korea: *Takifugu pardalis*, *T. porphyreus*, *T. niphobles*, *T. poecilonotus*, *T. rubripes*, and *T. xanthpterus*.

MATERIALS AND METHODS

For genetic analyses, we collected muscle tissue samples from *T. pardalis* (n=8), *T. porphyreus* (n=28), *T. niphobles* (n=8), *T. poecilonotus* (n=6), *T. rubripes* (n=23), and *T. xanthpterus* (n=198) stored by the National Institute of Fisheries Science (NIFS) and/or Marine Fish Resource Bank of Korea (MFRBK), and identified their morphological characteristics. These samples were collected in sterile tubes and preserved in 99.99% ethanol until DNA extraction. Total DNA from each sample was extracted using an automated DNA extraction system (MagExtractor MFX-6100, Toyobo, Osaka, Japan). Genomic DNA was quantified using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, USA) and stored at -20° C until genetic analyses.

We analyzed complete sequences of the mtDNA COI gene registered with the National Center for Biotechnology Information (NCBI) for the six pufferfish species: T. pardalis (GenBank accession no., AP009528.1), T. porphyreus (KY514076.1), T. niphobles (KY514069.1), T. poecilonotus (AP009539.1), T. rubripes (KP641572.1), and T. xanthpterus (KP641579.1) using the BioEdit v. 7.0.0 software to identify inter- and intra-species variation and conserved regions. Then we designed common primers (Taki-F35, Taki-R1232) specific to the six species (Fig. 1A). Polymerase chain reaction (PCR) was performed in a total volume of 20 µL, comprising 2 µL genomic DNA (20 ng), 0.6 µL dNTP (250 µM), 2 µL 1× PCR buffer containing 2 mM MgCl₂, 0.4µL 10 pmol forward primer, 0.4 µL 10 pmol reverse primer, 0.2 µL 0.5 U DNA Taq (Anti-HS Taq, TNT Research, Seoul, Korea). The primers used are listed in Table 1. PCR amplification was performed using an ABI 2720 Thermal Cycler under the following conditions: 10 min of initial denaturation at 95°C; 37 cycles of 45 s at 94°C, 45 s at 58°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. Amplified PCR products were sequenced using an ABI BigDye Terminator Cycle Sequencing Kit (ver. 3.1, Applied Biosystems, Foster, CA, USA), and analyzed using an ABI 3730XL DNA analyzer (Applied Biosystems).

We assembled forward and reverse sequences of the six *Takifugu* species with common primers using the SeqMan software (DNASTAR, USA), and searched for single nucleotide polymorphisms (SNPs) showing species specificity except for intraspecies point mutation. Then we designed species-specific forward primers for each target species in which the SNP was located at the 3' end (Fig. 2B(1)–(7), Table 1). We performed species-specific PCR analyses using the six forward primers (JB352, GB425, BS586, HJB796, JJB883, and GCB1028) and one reverse primer (Taki-R1232) under the PCR conditions described above for the common primer, but at an annealing temperature of

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		10	20	30	40 50
Takifugu niphobles					AATCACACGCTGAT
Takifugu poecilonotus	AACCACCC	CTTAACCC	CAGCCATTCT	ACCTGTGGG	AATCACACGCTGAT
Takifugu pardalis	AACCACCC	CTTAACCC	CAGCCATTCT	ACCTGTGGG	AATCACACGCTGAT
Takifugu porphyreus					AATCACACGCTGAT
Takifugu rubripes					AATCACACGCTGAT
Takifugu xanthopterus	AACCACCC	CTTAACCC	CAGCCATTCT		AATCACACGCTGAT
		60	70	80 (a) Taki-	
Takifugu niphobles			CAAAGATATCC		ACCTAGTTTTTGGT
Takifugu poecilonotus					ACCTAGTTTTTGGT
Takifugu pardalis					ACCTAGTTTTTGGT
Takifugu porphyreus	TTTTCTCA	ACCAATCA	CAAAGATATCC	GCACCCTAT	ACCTAGTTTTTGGT
Takifugu rubripes					ACCTAGTTTTTGGT
Takifugu xanthopterus	TTTTCTC	ACCAATCA	CAAAGATATCC	GCACCCTAT	ACCTAGTTTTTGGT
		110	120	130	140 150
Takifugu niphobles					CTTATTCGGGCCGA
Takifugu poecilonotus					CTTATTCGGGCCGA
Takifugu pardalis	GCCTGAG	CGGAATAG	TAGGCACTGCA	ACTAAGTCT'	C T T A T T C G G G C C G A
Takifugu porphyreus					I C T T A T T C G G G C C G A
Takifugu rubripes					I C T T A T T C G G G C C G A
Takifugu xanthopterus	GCCTGAG				I C T T A T T C G G G C C G A
		160	170	180	190 200
Takifugu niphobles	ACTCAGTO	AACCCGGT	GCACTCTTGGG	GCGATGACC	A GATCTACAATGTAA
Takifugu poecilonotus					GATTTACAATGTAA
Takifugu pardalis					AGATCTACAATGTAA
Takifugu porphyreus					GATCTACAATGTAA
Takifugu rubripes Takifugu xanthopterus					A G A T C T A C A A T G T A A A G A T T T A C A A T G T A A
	ACTUACIO		220	230	240 250
		210			
Takifugu niphobles					ATAGTAATACCAATC
Takifugu poecilonotus					ATAGTAATACCAATC
Takifugu pardalis					ATAGTAATACCAATC
Takifugu porphyreus Takifugu rubripes					A T A G T A A T A C C A A T C A T A G T A A T A C C A A T C
Takifugu xanthopterus					ATAGTAATACCAATC
	8	260	270	280	290 300
ST LONGER THE STREET					
Takifugu niphobles					ATAATCGGAGCCCC
Takifugu poecilonotus Takifugu pardalis					ATAATTGGAGCCCC
Takifugu paraans Takifugu porphyreus					ATAATCGGAGCCCC ATAATCGGAGCCCC
Takifugu rubripes					ATAATCGGAGCCCC
Takifugu xanthopterus					ATAATCGGAGCCCC
		310	320	330	340 350
]
Takifugu niphobles					CTGACTGCTTCCCC
Takifugu poecilonotus Takifugu pardalis					CTGACTGCTTCCCC CTGACTACTTCCCC
Takifugu porphyreus					CTGACTACTTCCCC
Takifugu rubripes					CTGACTGCTTCCCC
Takifugu xanthopterus					CTGACTGCTTCCCC
		360	370	380	390 400
Takifugu niphobles					GAAGCCGGAGCGGGT
Takifugu poecilonotus					GAAGCCGGAGCGGGT
Takifugu pardalis					GAAGCCGGAGCGGGT
Takifugu porphyreus					GAAGCCGGGAGCGGGT
Takifugu rubripes	CATCCTT	CTCCTTCT	GCTCGCATCCI	CTGGAGTA	GAAGCCGGAGCGGGT
Takifugu xanthopterus	CATCCTT	CTCCTTCT	GCTCGCATCC	CTGGAGTA	GAAGCCGGAGCGGGT
	(b) JB352	410	420	430	440 450
- 110 - 11 - 11					
Takifugu niphobles Takifugu poecilonotus					T C T T G C C C A C G C A G G T C T T G C C C A C G C A G G
Takifugu pardalis					TCTTGCCCACGCAGG
Takifugu porphyreus					TCTTGCCCACGCAGG
Takifugu rubripes					TCTTGCCCACGCAGG
Takifugu xanthopterus	ACGGGCT	GAACCGTTT	ACCCACCCT	AGCAGGAAA	TCTTGCCCACGCAGG
		460	470 (c) GB425		490 500
Takifugu niphobles					
Takifugu poecilonotus					T T G C A G G G G G T C T C T T T T G C A G G G G G T C T C C T
Takifugu pardalis					TIGCAGGGGTCTCCT
Takifugu porphyreus					TTGCAGGGGTCTCCT
Takifugu rubripes					TTGCAGGGGTCTCCT
Takifugu xanthopterus					TTGCAGGGGTCTCCT

Fig. 1. Nucleotide alignment and information from the COI gene of six *Takifugu* species for use in species identification. Red boxes indicate the designed primer sets. COI, c oxidase subunit I.

		510	520	530 540 550
Takifugu niphobles			AACTTCATCAC	
Takifugu poecilonotus Takifugu pardalis			AACTTCATCAC	AACTATCATTAACATGAAACCC
Takifugu porphyreus				AACTATCATTAACATGAAGCCC
Takifugu rubripes				AACTATCATTAACATGAAGCCC
Takifugu xanthopterus				AACTATCATTAACATAAAACCC
		560	570	580 590 600
Takifugu niphobles]]	• 1 • • • • 1 • • • • 1 • • • • 1 • • • • 1
Takifugu poecilonotus	CCAGCAA			TTTTCGTATGAGCCGTTTTAAT
Takifugu pardalis				TTTTCGTGTGAGCCGTCTTAAT TTTTCGTGTGAGCCGTTTTAAT
Takifugu porphyreus				TTTTCGTGTGAGCCGTTTTAAT
Takifugu rubripes				TTTTCGTGTGAGCCGTTTTAAT
Takifugu xanthopterus				TTTTCGTGTGAGCCGTTTTAAT
		610	620	630 (d) BS586 640 650
.				
Takifugu niphobles				CCAGTCCTTGCAGCAGGGATTA
Takifugu poecilonotus Takifugu pardalis				CCAGTACTTGCAGCAGGGATTA CCAGTCCTTGCAGCAGGAATTA
Takifugu porphyreus				CCAGTCCTTGCAGCAGGGATTA
Takifugu rubripes				CCAGTCCTTGCAGCAGGGATTA
Takifugu xanthopterus				CCAGTCCTTGCAGCAGGGATTA
		660	670	680 690 700
.				. 1 1 1 1 1
Takifugu niphobles	CAATGCT		CGAAACTTAAA	
Takifugu poecilonotus Tabifugu pardalis	CAATACT			TACAACCTTCTTTGACCCAGCA
Takifugu pardalis Takifugu porphyreus				TACAACCTTCTTTGACCCAGCA TACAACCTTCTTTGACCCAGCA
Takifugu rubripes				TACAACCTTCTTTGACCCAGCA
Takifugu xanthopterus				TACAACCTTCTTTGACCCAGCA
		710	720	730 740 750
Takifugu niphobles				ACTTATTCTGATTCTTTGGGCA
Takifugu poecilonotus				ACTTATTCTGATTCTTTGGGCA
Takifugu pardalis				ACTTATTCTGATTCTTTGGGCA
Takifugu porphyreus Takifugu porphyreus				ACTTATTCTGATTCTTCGGGCA ACTTATTCTGATTCTTTGGACA
Takifugu rubripes Takifugu xanthopterus				ACTTATTCTGATTCTTTGGGCA
lakinaga xananopieras				
		760 • • • • • • • •	770 • • • • • • • • •	780 790 800 • • • • • • • • • • • • •
Takifugu niphobles				G G C T T C G G G A T G A T T T C A C A T A
Takifugu poecilonotus				GGCTTCGGAATAATCTCGCACA
Takifugu pardalis				GGCTTCGGGATAATCTCACACA
Takifugu porphyreus				GGCTTCGGGATAATTTCACATA
Takifugu rubripes Takifugu xanthopterus				GGCTTCGGAATAATTTCACACA GGCTTCGGGATAATTTCACACA
Tuknugu xunulopterus	CCCTOAA			830 840 (e) HJB796 850
		810	820	
Takifugu niphobles				
	TCGTAGC			
Takifugu poecilonotus		C T A C T A C T C G		ACCATTCGGCTACATGGGTATG
Takifugu poecilonotus Takifugu pardalis	TCGTAGC	CTACTACTCG CTACTACTCG	GGCAAAAAAGA	ACCATTCGGCTACATGGGTATG ACCATTCGGTTACATGGGCATG
Takifugu pardalis Takifugu porphyreus	TCGTAGC TCGTAGC TTGTAGC TCGTAGC	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG		ACCATTCGGCTACATGGGTATG ACCATTCGGTACATGGGCATG ACCATTCGGCTATATGGGCATG ACCATTCGGCTACATGGGCATG
Takifugu pardalis Takifugu porphyreus Takifugu rubripes	T C G T A G C T C G T A G C T T G T A G C T C G T A G C T C G T A G C	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG		ACCATTCGGCTACATGGGCATG ACCATTCGGTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG
Takifugu pardalis Takifugu porphyreus	T C G T A G C T C G T A G C T T G T A G C T C G T A G C T C G T A G C	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG		ACCATTCGGCTACATGGGTATG ACCATTCGGTACATGGGCATG ACCATTCGGCTATATGGGCATG ACCATTCGGCTACATGGGCATG
Takifugu pardalis Takifugu porphyreus Takifugu rubripes	TCGTAGC TCGTAGC TTGTAGC TCGTAGC TCGTAGC TCGTAGC	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG	GCCAAAAAGA GCCAAAAGGA GCCAAAAGGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAGA	ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTATATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGTTATATGGGCATG
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC	CTACTACTCG S60	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG S80 890 900
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu niphobles	TCGTAGC TCGTAGC TTGTAGC TCGTAGC TCGTAGC TCGTAGC	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAAA	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG BSD 890 900 1 1 1 1 1
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu niphobles Takifugu poecilonotus	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTATGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCACTACTCG CCATGATGGC	GC A A A A A A G A GC A A A A A A G A GC A A A A A A A G A GC A A A A A A A G A STO 	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG S80 890 900
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu niphobles Takifugu poecilonotus	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTATGAG GTATGAG GTCTGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCACTACTCG CCATGATGGC CCATGATGGC	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA GCCAAAAAAGA CCACGGTCTTC CATCGGTCTTC	ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGCCATG B800 890 900 .
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu niphobles Takifugu poecilonotus Takifugu pardalis Takifugu porphyreus	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTCTGAG GTCTGAG GTCTGAG GTCTGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAAA	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG BSD 890 900 1
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu niphobles Takifugu poecilonotus Takifugu pardalis	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTCTGAG GTCTGAG GTCTGAG GTCTGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAAA	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG B80 890 900 1 TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu porbobles Takifugu porcilonotus Takifugu porphyreus Takifugu rubripes	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTCTGAG GTCTGAG GTCTGAG GTCTGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC	GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAGA GCCAAAAAAGA GCCAAAAAAAA	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG BSD 890 900 1
Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus Takifugu poecilonotus Takifugu poecilonotus Takifugu pardalis Takifugu porphyreus Takifugu rubripes Takifugu xanthopterus	TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC TCGTAGC GTCTGAG GTCTGAG GTCTGAG GTCTGAG	CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CTACTACTCG CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGGC CCATGATGCC	GCAAAAAGA GCAAAAAGA GCAAAAAGA GCAAAAAGA GCAAAAAAGA GCAAAAAAGA GCAAAAAAGA GCAAAAAAGA CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC CATCGGTCTTC	ACCATTCGGCTACATGGGTATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG ACCATTCGGCTACATGGGCATG BSO 900 900 I I I I I TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA TTGGCTTTATTGTATGAGCCCA TGGCTTTATTGTATGAGCCCA TGGCTTTATTGTATGAGCCCA TGGCTTTATTGTATGAGCCCA TGGCTTTATTGTATGAGCCCA
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Fig. 1. Continued.

The Rapid and Simultaneous Identification of Six Takifugu

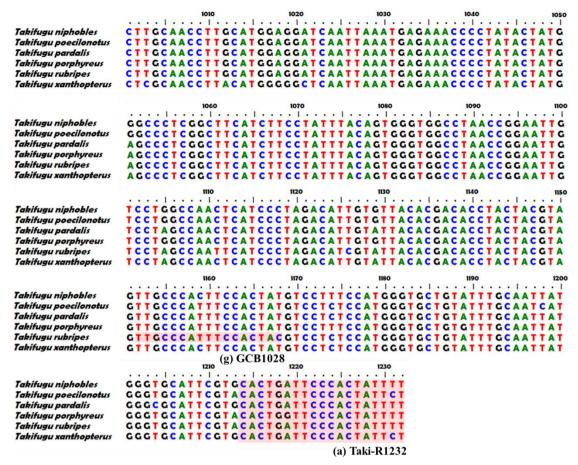


Fig. 1. Continued.

60°C. Amplified PCR products were subjected to 2% agarose gel electrophoresis (100 V, 40 min) with 1× RedSafe (iNtRon, Korea), and confirmed using the Gel Doc image analysis system (ATTO Corp., Japan).

RESULTS AND DISCUSSION

A region of the cytochrome COI is widely applied in genetic identification of animals including fishes and

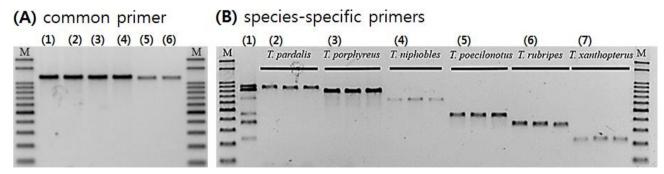


Fig. 2. Identification of species by multiplex polymerase chain reaction (PCR) using common primer and species-specific primers. Samples are identified as follows: (A) common primer; (1) *T. pardalis*, (2) *T. porphyreus*, (3) *T. niphobles*, (4) *T. poecilonotus*, (5) *T. rubripes*, (6) *T. xanthopterus*, (B) lane (1) template mixture, (2) *T. pardalis*, (3) *T. porphyreus*, (4) *T. niphobles*, (5) *T. poecilonotus*, (6) *T. rubripes*, (7) *T. xanthopterus*, (M) 100 bp DNA ladder (iNtRON, Korea).

Primers	Sequence $(5' \rightarrow 3')$	Product size (bp)	Specific species
JB352	TTCTGACTACTTCCCCCG	897	Takifugu pardalis
GB425	ACGGTTTACCCACCCT	822	T. porphyreus
BS586	GTACCAAACACCTCTTTTCGTA	667	T. niphobles
HJB796	CGGCTTCGGAATAATCTCG	454	T. poecilonotus
JJB883	GCCATCGGTCTTCTTGGT	366	T. rubripes
GCB1028	TCGCAACCTTACATGGG	230	T. xanthpterus
Taki-F35	GCAATCACACGCTGATT	1 107	Common primer
Taki-R1232	AYAATAGTGGGAARCAGTG	1,197	(Genus Takifugu)

Table 1. Species-specific primers used for multiplex polymerase chain reaction (PCR)

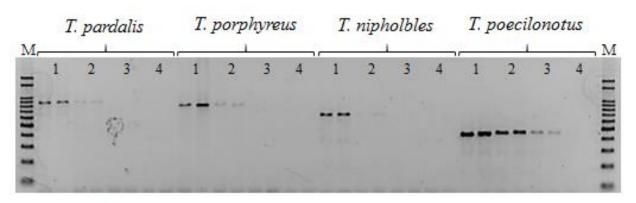
improved effectiveness in species identification on the previous report (Rasmussen & Morrissey, 2008; Cohen et al., 2009; Acar et al., 2017). PCR analyses using species-specific primers have excellent sensitivity and specificity, and can analyze large numbers of samples within a short time. These methods are widely used for species identification because they are simpler than DNA sequence analyses (Hwang et al., 2002). Also, MSS-PCR methods are economical in terms of time, effort, and cost compared to other DNA based methods, and the determination of the most important species can provide clear and repeatable results (Sezaki et al., 2005; Noh et al., 2017).

We successfully developed and applied a multiplex PCR assay based on species-specific variation for rapid and simultaneous identification of six target species: *T. pardalis*, *T. porphyreus*, *T. niphobles*, *T. poecilonotus*, *T. rubripes*, and *T. xanthpterus*. This is the first study to use conventional PCR amplification to identify six pufferfish species commonly imported into the Korean fishery market.

We obtained 1,252 bp sequences of the mtDNA COI region from the six species, and used a 1,197 bp common primer to confirm MSS-PCR amplification (Fig. 1). Sequences obtained using the common primer were analyzed using the DNA Sequence Polymorphism (DnaSP) v. 5.10.01

software. Haplotype analysis results for a total of 271 individuals among the six species indicated 4 haplotypes of *T. pardalis* (n=8), 4 of *T. porphyreus* (n=28), 7 of *T. niphobles* (n=8), 4 of *T. poecilonotus* (n=6), 6 of *T. rubripes* (n=23), and 32 of *T. xanthpterus* (n=198). Except for intraspecies genetic variation, species-specific SNPs were observed at 352 bp (JB352), 425 bp (GB425), 586 bp (BS586), 796 bp (HJB796), 883 bp (JJB883), and 1,028 bp (GCB1028) (Fig. 1).

MSS-PCR products containing the same amounts of forward primers were confirmed by agarose gel electrophoresis, with accurate DNA amplification of each species and clear distinction among amplified products by size (Table 1). Sequencing analyses confirmed that MSS-PCR products of the six species had 100% identity with the expected regions. Primer dimers and nonspecific amplification products were not observed, and no crossreactions were observed among species-specific amplifications in DNA mixtures of the six species (Fig. 2). The MSS-PCR assay sensitivity test showed that DNA template concentrations of stored products from the six species were 10 ng/µL, 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL. The PCR assay sensitivity of each species was detectable to a concentration of 1 ng/µL among all six



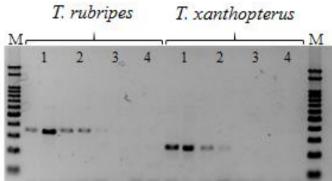


Fig. 3. Sensitivity for detection of the six *Takifugu* species using species-specific primer sets. Sensitivity analyses were performed using 10-fold amplification with serial dilution from 10 to 0.01 ng/μL genomic DNA from each of two individuals. Samples are identified as follows: lane (M) 100 bp DNA ladder (iNtRON, Korea). (1) 10 ng, (2) 1 ng, (3) 0.1 ng, (4) 0.01 ng.

species (Fig. 3).

In conclusion, the molecular method developed in this study was simple, rapid, and inexpensive compared to direct sequencing analyses, and did not require high-quality equipment. The MSS-PCR method is capable of clearly distinguishing and/or authenticating six *Takifugu* species in case of mislabeling via accident or fraud. This technique can be used as a regulatory tool to protect public health and enforce Korean fishery product import regulations.

ORCID

Chun Mae Dong https://orcid.org/0000-0001-7068-5939

Yeon Jung Park https://orcid.org/0000-0001-5363-7847 Jae Koo Noh https://orcid.org/0000-0002-9010-6225

Eun Soo Noh https://orcid.org/0000-0003-3880-5050

Cheul Min An https://orcid.org/0000-0002-5033-2498

Jung-Ha Kang https://orcid.org/0000-0002-6554-4581

Jung Youn Park https://orcid.org/0000-0002-6948-6059

Eun-Mi Kim https://orcid.org/0000-0003-3376-2995

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: Kim EM.

Data curation: Park YJ, Noh ES.

Formal analysis: Noh JK, Park YJ, Dong CM.

Methodology: Kim EM, Park YJ, Dong CM.

Software: Park YJ, Dong CM.

Validation: Kim EM, Kang JH, An CM.

Investigation: Kim EM, Park JY.

Writing - original draft: Kim EM, Dong CM.

Writing - review & editing: Kim EM, Park YJ, Dong CM.

ETHICS APPROVAL

This article does not require IRB/IACUC approval because there are no human and animal participants.

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