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Genotyping of Toxic Pufferfish Based on Specific PCR-RFLP Products As Determined by Liquid Chromatography/Quadrupole-Orbitrap Hybrid Mass Spectrometry

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ABSTRACT: A method based on liquid chromatography−electrospray mass spectrometric analysis of the enzymatically digested amplicons derived from the mitochondrial 16S rRNA gene was established for the discrimination of toxic pufferfish. A MonoBis C18 narrow-bore silica monolith column (Kyoto Monotech) and a Q Exactive mass spectrometer (Thermo Fisher) were employed for separation and detection, respectively. Monoisotopic masses of the oligonucleotides were calculated using Protein Deconvolution 3.0 software (Thermo Fisher). Although a lock mass standard was not used, excellent accuracy (mass error, 0.83 ppm on average) and precision (relative standard deviation, 0.49 ppm on average) were achieved, and a mass accuracy of <2.8 ppm was maintained for at least 180 h without additional calibration. The present method was applied to 29 pufferfish samples, and results were consistent with Sanger sequencing.

KEYWORDS: *pufferfish, fugu, polymerase chain reaction, restriction fragment length polymorphism, electrospray ionization mass spectrometry*

INTRODUCTION

According to an official bulletin from the Japanese government, 21 species of pufferfish are categorized as edible; however, not all parts (organs/tissues) are edible. The edible parts that are free of tetrodotoxin vary by species. Other pufferfish are prohibited for sale because of the presence of toxins throughout the whole body (e.g., *Lagocephalus lunaris*[1]) or because there is a lack of toxicity data for a particular species. Therefore, species-based labeling of pufferfish is officially required in the Japanese market; mislabeling, which could lead to serious food poisoning, is a criminal offense.

Differentiation and discrimination of pufferfish are not just a challenge in Japan. In the United States, the distribution of pufferfish is prohibited except for limited importation from Japan of edible parts of *Takifugu rubripes*. However, a tetrodotoxin poisoning occurred in Chicago in 2007 because of an illegal importation of frozen pufferfish (Lagocephalus family) from China that had been mislabeled as monkfish.[2]

Discrimination of pufferfish species is carried out in the Japanese market morphologically; however, inedible *L. lunaris* is at risk of misidentification as edible *Lagocephalus wheeleri*, owing to the species having similar morphologies.[1] Moreover, morphological examination of pufferfish is virtually impossible in the case of filleted or minced fish. A tetrodotoxin poisoning outbreak due to consumption of dried fillets of *L. lunaris* occurred in Minneapolis in 2014.[3] A discrimination capability is also needed in the context of food fraud investigations because *Takifugu rubripes* is far more expensive than other kinds of pufferfish.

For DNA-based differentiation of pufferfish species, the genes of cytochrome *b*, 12S rRNA, 16S rRNA, and cytochrome *c* oxidase I in mitochondrial DNA have been analyzed by Sanger sequencing, PCR restriction fragment length polymorphism (RFLP) analysis, and real-time PCR analysis.[2,4−8] Whereas Sanger sequencing is accurate and well-established, PCR-RFLP and real-time PCR analysis are simple and rapid for analyzing DNA polymorphism. However, because detection of DNA in the latter two methods is usually carried out by non-sequence-based techniques, which typically involve using a DNA-binding fluorescent dye, the reliability of discrimination is inferior to that of Sanger sequencing.

Mass spectrometry (MS) is one of the most reliable technologies for identifying and characterizing biomolecules. Although matrix-assisted laser desorption/ionization−time-of-flight MS is frequently used for the analysis of proteins and short oligonucleotides (< ∼25 nucleotides (nt)), it is not applicable for long oligonucleotides owing to the subsequent fragmentation, adduct formation, and low ionization efficiency.[9−11] Instead, electrospray ionization (ESI) can produce many charge states of multiply charged ions ($[M - nH]^{n-}$) from biomacromolecules and, therefore, the mass of the analyte can exceed the upper limit of the scan range of the spectrometer. This requires transformation of a charge state series into the corresponding mass via deconvolution. In the context of ESI-MS, whereas dissociation of a DNA duplex, purification, and desalting are required for direct measurement to overcome ionization issues such as ion suppression and adduct formation,[12−17] liquid chromatography (LC) provides an online separation of the analytes from coexisting substances instead of laborious sample purification and desalting.[18,19] Whereas LC/ESI-MS has been used for amplicon-based genotyping including differentiation of pathogen species, single-nucleotide polymorphism, and short tandem repeat,[9,12,15,20−24] discrimination of species other than pathogens by LC/ESI-MS has not been reported.

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Table 1. DNA Sequences Used for the PCR Templates

Species	DNA sequences[a]	Length (nt)[b]	Reference[c]	Accession No.
Takifugu rubripes	CCATGTGGAATGAAAACACCCTT**TTTAAA**CCCAAGAGTCACCACTCTAGGATACAGAACATCTGACCAAT-AATGAT**CCGG**CT-AAAGCCGATTAACGAACCGAGTTACCCTAGGG	114	(26)	AP006045
Takifugu porphyreus	CCATGTGGAATGAAAACACCCTT**TTTAAA**ACCAAGAGTCACCACTCTAGGATACAGAACATCTGACCAAT-AATGAT**CCGG**CT-AAAGCCGATTAACGAACCGAGTTACCCTAGGG	114	(26)	AP009529
Takifugu chrysops	CCATGTGGAATGAAGACACCCTCTCTAAAACCAAGAGTCACCACTCTAAGTTACAGAACATCTGACCAAT-AATGAT**CCGG**CT-AAAGCCGATTAACGAACCGAGTTACCCTAGGG	114	(26)	AP009525
Takifugu vermicularis	CCATGTGGAATGAAAACACCCTT**TTTAAA**ACCAAGAGTCACCACTCTAAGTTACAGAACATCTGACCAATTAATGAT**CCGG**CT-AAAGCCGATTAACGAACCGAGTTACCCTAGGG	115	–	AB741999
Takifugu pardalis	CCATGTGGAATGAAAACACCCTT**TTTAAA**ACCAAGAGTCACCACTCTAAGTTACAGAACATCTGACCAAT-AATGAT**CCGG**CT-AAAGCCGATTAACGAACCGAGTTACCCTAGGG	114	(26)	AP009528
Takifugu niphobles	CCATGTGGAATGAAAACACCCTT**TTTAAA**ACCAAGAGTCACCACTCTAAGTTACAGAACATCTGACCAAT-AATGAT**CCGG**CTTAAAGCCGATTAACGAACCGAGTTACCCTAGGG	115	(26)	AP009526
Takifugu poecilonotus	CCATGTGGAATGAAAACACCTT**TTTAAA**ACCAAGAGTCACCACTCTAAGTTACAGAACATCTGACCAGT-AATGAT**CCGG**CTT-AAGCCGATTAACGAACCGAGTTACCCTAGGG	114	(26)	AP009539
Lagocephalus wheeleri	AAAAAC-AAGAGCCACAGCTCTAATGAGCAGAACATCTGACCTACCA--GAT**CCGG**C--ATAGCCGATCAACGAACCGAGTTACCCTAGGG	86	(26)	AP009538
Lagocephalus gloveri	AAAAAC-AAGAGCCACAGCTCTAATGAACAGAACATCTGACCTACCA--GAT**CCGG**C--ATAGCCGATCAACGAACCGAGTTACCCTAGGG	86	–	AB742032
Lagocephalus lunaris	AAAAAC-AAGAGCCACAGCTCTAATAAACAGAACATCTGACCAGCCA--GAT**CCGG**C--CTAGCCGATCAACGAACCGAGTTACCCTAGGG	86	(25)	Not available
Lagocephalus inermis	AAAAAC-AAGAGCCACAGCTCTAATAAACAGAACATCTGACCCACCA--GAT**CCGG**C--ACAGCCGATCAACGAACCGAGTTACCCTAGGG	86	–	AB742030

[a]Underline indicates primer-binding site, and bold indicates recognition sites of the restriction enzymes. [b]nt, nucleotide. [c]–, unpublished.

In this study, LC/ESI-MS is applied for the first time for PCR-RFLP analysis of fish samples to permit rapid and accurate discrimination of toxic pufferfish species.

MATERIALS AND METHODS

Chemicals and Materials. 1,1,1,3,3,3-Hexafluoro-2-propanol and triethylamine were "eluent additive for LC-MS" grade and supplied by

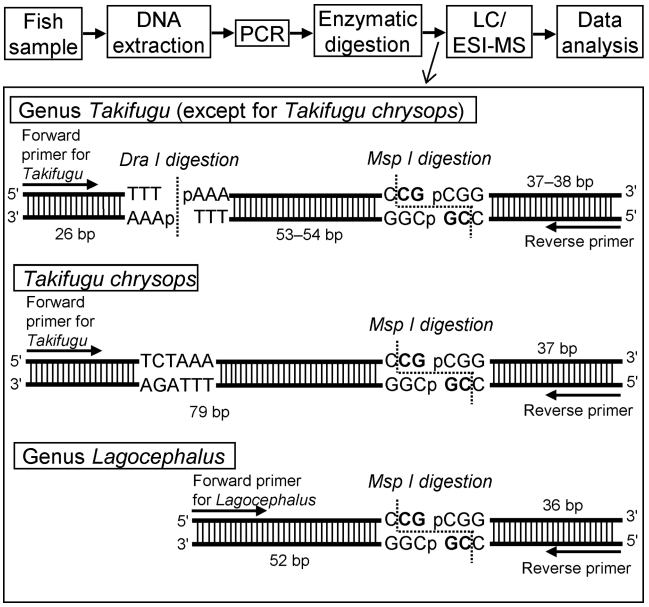

Figure 1. Method summary. Bold indicates the nucleotides filled with the remaining DNA polymerase. p indicates a phosphate group.

Sigma-Aldrich (St. Louis, MO, USA). Synthetic DNA templates (86–115 nt, sequences are shown in Table 1) were purchased from Eurofins Genomics (Tokyo, Japan).

Preparation of Digested Amplicons. The procedure of sample preparation is summarized in Figure 1. PCR reactions were performed on a 25 μL reaction scale and consisted of 0.625 U of Pfu-X DNA polymerase (Jena Bioscience, Jena, Germany), 0.2 mM of each dNTP (Jena Bioscience), 1× detergent-free Phusion HF buffer (New England Biolabs, Ipswich, MA, USA), either 12.5 ng of an extracted fish DNA or 0.5 pmol of the synthetic DNA template, and 0.4 μM of each primer. Two forward primers (5′-CCATGTGGAATGAAAACACC-3′ for *Takifugu* and 5′-AAAAACAAGAGCCACAGCTCTAA-3′ for *Lagocephalus*) and one reverse primer (5′-CCCTAGGGTAACTCGGTTCG-3′) were designed with the help of Primer3Plus software (http://www.bioinformatics.nl/primer3plus/) and synthesized by Fasmac (Atsugi, Japan). These primers were used to amplify 114–115 and 86 bp amplicons of the mitochondrial 16S rRNA gene of *Takifugu* and *Lagocephalus* species, respectively (Figure 1).[25,26] Thermocycling was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 95 °C, 2 min, then 30 cycles at 95 °C, 30 s, 56 °C, 30 s, 72 °C, 30 s, 72 °C, 7 min, and 12 °C hold. After PCR, 2 μL of the 10× universal buffer M (Takara Bio, Otsu, Japan, containing 100 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM dithiothreitol, and 500 mM NaCl) and 1 μL of each FastDigest restriction enzyme (*Dra*I and *Msp*I, Thermo Fisher Scientific) were added to the tube and incubated for 30 min at 37 °C, followed by additional incubation for 5 min at 72 °C. A portion of the reaction solution was analyzed by polyacrylamide gel electrophoresis (PAGE) using the staining dye Gel Green (Biotium, Hayward, CA, USA), if necessary. The remaining solution was filtered with a Nanosep MF centrifugal device containing a Bioinert membrane (0.45 μm, Pall Corp., Port Washington, NY, USA) before LC/ESI-MS analysis. The filtrates were stored at −20 °C until analysis.

LC/ESI-MS Analysis. The LC/ESI-MS was performed using an Ultimate 3000 liquid chromatograph and a Q Exactive quadrupole–Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific). A reverse-phase silica monolith column (MonoBis C18, Kyoto Monotech, Kyoto, Japan; 2.0 × 50 mm, mesopore size = 30 nm, product no. 2050H30ODS) was used for the separation at 20 °C with a MonoBis C18 guard column (Kyoto Monotech). The mobile phase, delivered at a flow rate of 0.4 mL/min, was 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol, 15 mM triethylamine (pH 7.9) (A)–methanol (B).[27] The proportion of B in the mobile phase was changed over a linear gradient as follows: 0–0.5 min, 5%; 0.5–1 min, 5–30%; 1–3.5 min, 30–40%; 3.5–5 min, 40–98%; 5–6 min, 98%; 6–6.05 min, 98–5%; and 6.05–8 min, 5%. The sample injection volume was 1.0 μL except for the tests of the stability of mass accuracy and the temperature of the column (0.5 μL each). The postcolumn flow was connected to the mass spectrometer for 3.5–6 min after sample injection. A heated ESI probe (HESI-II, Thermo Fisher) was used in negative ion mode and with the following parameters: spray voltage, 2.5 kV; vaporizer temperature, 350 °C; sheath gas, 50 arbitrary units (au); auxiliary gas, 15 au. The parameters for the desolvation at the entrance of the mass spectrometer were as follows: capillary temperature, 350 °C; sweep gas pressure, 1 au; S-lens radiofrequency level, 100. High-resolution mass analysis was performed using the following parameters: scan range, m/z 700–3500; nominal resolution (at m/z 200), 140,000; auto gain control target, 1×10^6; microscans, 3; maximum ion time, 50 ms; in-source collision-induced dissociation, 15 eV. The C-trap pressure, which can be adjusted with the valve inside the chassis, was reduced to 0.2 MPa to improve the sensitivity for long oligonucleotides. A lock mass was not assigned. Mass calibration was performed daily using sodium trifluoroacetate clusters (n = 5–24, m/z 792.85908–3376.38045).[28] The calibration solution was 0.5 mg/mL sodium trifluoroacetate (pH 3.5) in water/acetonitrile (1:1, v/v) and was infused into the mass spectrometer at the flow rate of 10 μL/min. The in-source collision-induced dissociation voltage was set to 60 eV to develop the cluster ions at higher mass range.

Peak detection and deconvolution from the raw data using the Xtract algorithm[29,30] were carried out automatically with the Protein Deconvolution version 3.0 software (Thermo Fisher). The parameters were as follows: fit factor, 90%; charge range, 3–50.

Analysis of Pufferfish and Other Fish Species. Twenty-nine pufferfish samples (1 boiled skin of *T. rubripes*, 3 raw livers, and 1

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ovary of *Takifugu porphyreus*, 1 raw muscle tissue of *Takifugu vermicularis*, 2 fresh whole bodies of *Takifugu pardalis*, 1 dried fillet of *Takifugu snyderi*, 19 fresh whole bodies of *Takifugu poecilonotus*, and 1 dried fillet of *Lagocephalus wheeleri*) and 8 raw muscle tissues of other fish (Atlantic mackerel (*Scomber scombrus*), Japanese horse mackerel (*Trachurus japonicus*), Japanese pilchard (*Sardinops melanostictus*), skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), Japanese amberjack (*Seriola quinqueradiata*), Atlantic salmon (*Salmo salar*), and flying fish (*Cypselurus pinnatibarbatus japonicus*)) were obtained from fish wholesalers and retail markets in Japan. Total genomic DNA in fish tissue (~50 mg) was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) in accordance with the protocol of the manufacturer. In the case of whole-body samples, fresh fins were collected for extraction. The species of the pufferfish samples being studied were verified using the official method of the Office of Import Food Safety, Inspection and Safety Division, Department of Food Safety, Ministry of Health, Labour and Welfare, Japan (2011), which was based on Sanger sequencing of the 16S rRNA gene.[6] The same method was also used to confirm the common names of other fish samples with the aid of the BLAST search (DNA Data Bank of Japan). Amplification and LC/ESI-MS analysis were performed as described above.

RESULTS AND DISCUSSION

Optimization of LC/ESI-MS Conditions. Past studies dealing with LC/ESI-MS analysis of long oligonucleotides have

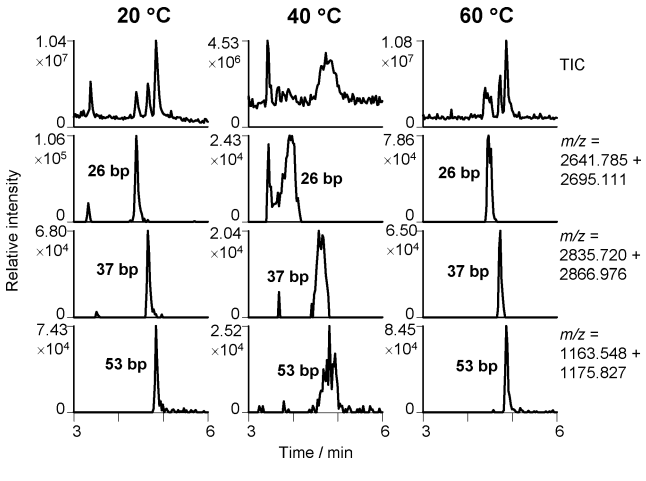

Figure 2. Chromatograms of the PCR products of the synthetic DNA of *Takifugu rubripes* for comparison of temperatures for the MonoBis C18 column. TIC, total ion current.

mostly been carried out using a poly(styrene-divinylbenzene) (PS-DVB) capillary monolith column (0.2 mm i.d.) at low flow rate (2 μL/min).[18−24,31] Instead, we tested a narrow-bore type (2.0 mm i.d.) reverse-phase silica monolith column for the separation of long oligonucleotides employing a much higher flow rate (400 μL/min), which enabled the use of a heated sprayer at the interface. Accordingly, the DNA duplexes were denatured, regardless of column temperature, when the mobile phase was sprayed into the ion source with the aid of heated nitrogen (350 °C).

Column temperature was optimized (Figure 2). Signals for the three pairs of DNA duplexes (26, 37, and 53 bp) and the primers were separated from one another at 20 and 60 °C, whereas distorted peaks were observed at 40 °C. These differences may be related to the formation of the double-helix structure during chromatographic separation. Oberacher et al. reported that a DNA duplex longer than 25−50 bp could be denatured in the analytical column at a temperature between 50

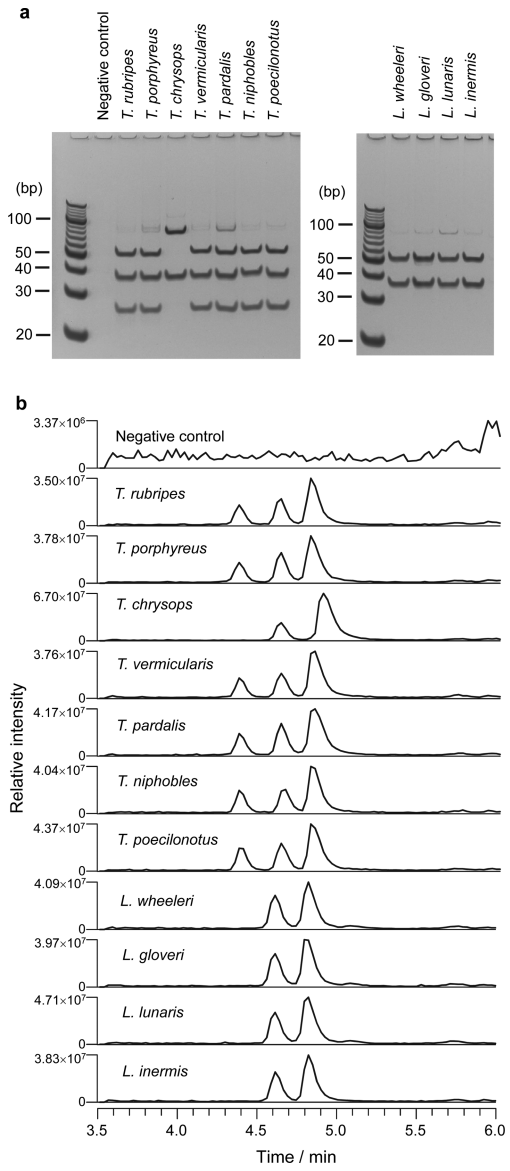

Figure 3. Results for PCR-RFLP analysis of synthetic pufferfish DNA: (a) PAGE; (b) LC/ESI-MS.

and 80 °C.[24] Accordingly, possible explanations for this behavior are that the DNA duplexes maintained their rigid structure at 20 °C, denatured partially at 40 °C, and denatured completely at 60 °C. Taking the separation behavior and the maximum column temperature recommended by the manufacturer (40 °C) into consideration, the column temperature was adjusted to 20 °C. Despite the low column temperature, the maximum back pressure of the system (12.5 MPa) was below the upper limit of the column (30 MPa) recommended by the manufacturer, owing to the inherently large channel size of the silica monolith.[32]

Discrimination of Synthetic DNA by PCR-RFLP Coupled with LC/ESI-MS. The PCR-RFLP method was used to discriminate 11 synthetic DNA (range, 86−115 bp) samples (Table 1), which served as the positive control templates for the pufferfish. The list for the target pufferfish was compatible with the method published by the Japanese government based on the Sanger sequencing. The exceptions to this were *T. pardalis* and *T. snyderi* and, given that the target sequences for these two species are identical, *T. pardalis* was

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Table 2. LC/ESI-MS Analysis of the Amplicons Digested with the Endonucleases

species	F/R[a]	length (nt[b])	expected mol formula	monoisotopic mass, n = 5 (interday) theor value (Da)	measured value (Da, av ± SD)	rel standard deviation (ppm)	av mass error[c] (ppm)
T. rubripes	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.366 ± 0.003	0.37	0.57
	F	53	$C_{515}H_{647}O_{307}N_{208}P_{53}$	16296.750	16296.763 ± 0.006	0.34	0.80
	F	37	$C_{360}H_{452}O_{218}N_{147}P_{37}$	11466.909	11466.920 ± 0.002	0.18	0.93
	R	37	$C_{360}H_{456}O_{225}N_{132}P_{36}$	11341.885	11341.890 ± 0.003	0.25	0.45
	R	53	$C_{522}H_{659}O_{331}N_{186}P_{53}$	16468.655	16468.661 ± 0.004	0.25	0.42
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.352 ± 0.001	0.13	1.44
T. porphyreus	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.367 ± 0.004	0.44	0.73
	F	53	$C_{516}H_{647}O_{306}N_{210}P_{53}$	16320.762	16320.764 ± 0.017	1.05	0.78
	F	37	$C_{360}H_{452}O_{218}N_{147}P_{37}$	11466.909	11466.919 ± 0.006	0.50	0.85
	R	37	$C_{360}H_{456}O_{225}N_{132}P_{36}$	11341.885	11341.892 ± 0.004	0.33	0.60
	R	53	$C_{522}H_{660}O_{332}N_{183}P_{53}$	16443.648	16443.651 ± 0.010	0.62	0.53
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.350 ± 0.002	0.28	1.19
T. chrysops	F	79	$C_{768}H_{965}O_{458}N_{303}P_{78}$	24173.107	24173.129 ± 0.017	0.70	0.92
	F	37	$C_{360}H_{452}O_{218}N_{147}P_{37}$	11466.909	11466.918 ± 0.006	0.56	0.84
	R	37	$C_{360}H_{456}O_{225}N_{132}P_{36}$	11341.885	11341.891 ± 0.006	0.52	0.58
	R	79	$C_{779}H_{980}O_{489}N_{283}P_{79}$	24566.979	24567.021 ± 0.016	0.64	1.69
T. vermicularis	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.367 ± 0.004	0.46	0.69
	F	54	$C_{526}H_{661}O_{314}N_{209}P_{54}$	16599.801	16599.821 ± 0.009	0.56	1.21
	F	37	$C_{360}H_{452}O_{218}N_{147}P_{37}$	11466.909	11466.922 ± 0.006	0.49	1.11
	R	37	$C_{360}H_{456}O_{225}N_{132}P_{36}$	11341.885	11341.890 ± 0.005	0.48	0.42
	R	54	$C_{533}H_{672}O_{336}N_{190}P_{54}$	16780.717	16780.724 ± 0.004	0.21	0.40
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.349 ± 0.005	0.65	1.06
T. pardalis	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.367 ± 0.002	0.30	0.64
	F	53	$C_{516}H_{648}O_{307}N_{207}P_{53}$	16295.755	16295.764 ± 0.020	1.21	1.11
	F	37	$C_{360}H_{452}O_{218}N_{147}P_{37}$	11466.909	11466.922 ± 0.007	0.60	1.08
	R	37	$C_{360}H_{456}O_{225}N_{132}P_{36}$	11341.885	11341.889 ± 0.006	0.49	0.51
	R	53	$C_{523}H_{660}O_{331}N_{185}P_{53}$	16467.659	16467.671 ± 0.011	0.66	0.76
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.350 ± 0.003	0.36	1.21
T. niphobles	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.367 ± 0.005	0.64	0.82
	F	53	$C_{516}H_{648}O_{307}N_{207}P_{53}$	16295.755	16295.764 ± 0.012	0.76	0.80
	F	38	$C_{370}H_{465}O_{225}N_{149}P_{38}$	11770.955	11770.971 ± 0.005	0.41	1.36
	R	38	$C_{370}H_{468}O_{230}N_{137}P_{37}$	11654.943	11654.951 ± 0.004	0.30	0.74
	R	53	$C_{523}H_{660}O_{331}N_{185}P_{53}$	16467.659	16467.666 ± 0.006	0.34	0.39
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.353 ± 0.003	0.38	1.57
T. poecilonotus	F	26	$C_{254}H_{321}O_{154}N_{94}P_{25}$	7925.362	7925.369 ± 0.005	0.61	0.91
	F	53	$C_{516}H_{648}O_{308}N_{207}P_{53}$	16311.750	16311.730 ± 0.014	0.84	1.21
	F	37	$C_{360}H_{453}O_{220}N_{144}P_{37}$	11457.898	11457.910 ± 0.003	0.23	1.10
	R	37	$C_{360}H_{455}O_{223}N_{135}P_{36}$	11350.897	11350.892 ± 0.007	0.62	0.53
	R	53	$C_{522}H_{659}O_{330}N_{186}P_{53}$	16452.660	16452.664 ± 0.007	0.45	0.42
	R	26	$C_{256}H_{322}O_{157}N_{98}P_{26}$	8085.340	8085.349 ± 0.003	0.33	1.07
L. wheeleri	F	52	$C_{505}H_{632}O_{296}N_{209}P_{51}$	15937.744	15937.761 ± 0.008	0.48	1.04
	F	36	$C_{349}H_{439}O_{212}N_{143}P_{36}$	11138.852	11138.844 ± 0.010	0.87	0.90
	R	52	$C_{511}H_{647}O_{327}N_{179}P_{52}$	16131.586	16131.595 ± 0.006	0.39	0.61
	R	36	$C_{350}H_{443}O_{219}N_{130}P_{35}$	11053.834	11053.844 ± 0.004	0.38	0.88
L. gloveri	F	52	$C_{505}H_{632}O_{295}N_{209}P_{51}$	15921.750	15921.765 ± 0.007	0.46	0.94
	F	36	$C_{349}H_{439}O_{212}N_{143}P_{36}$	11138.852	11138.848 ± 0.006	0.50	0.49
	R	52	$C_{512}H_{648}O_{328}N_{178}P_{52}$	16146.585	16146.580 ± 0.009	0.54	0.51
	R	36	$C_{350}H_{443}O_{219}N_{130}P_{35}$	11053.834	11053.849 ± 0.010	0.89	1.33
L. lunaris	F	52	$C_{505}H_{631}O_{293}N_{212}P_{51}$	15930.761	15930.776 ± 0.006	0.36	0.96
	F	36	$C_{348}H_{439}O_{213}N_{141}P_{36}$	11114.841	11114.846 ± 0.006	0.58	0.51

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Table 2. continued

species	F/R[a]	length (nt[b])	expected mol formula	monoisotopic mass, n = 5 (interday) theor value (Da)	measured value (Da, av ± SD)	rel standard deviation (ppm)	av mass error[c] (ppm)
	R	52	$C_{512}H_{649}O_{330}N_{175}P_{52}$	16137.574	16137.583 ± 0.007	0.42	0.59
	R	36	$C_{350}H_{442}O_{218}N_{133}P_{35}$	11078.841	11078.836 ± 0.003	0.24	0.37
L. inermis	F	52	$C_{504}H_{631}O_{293}N_{210}P_{51}$	15890.755	15890.773 ± 0.006	0.38	1.10
	F	36	$C_{348}H_{438}O_{211}N_{144}P_{36}$	11123.852	11123.851 ± 0.004	0.35	0.26
	R	52	$C_{513}H_{649}O_{330}N_{177}P_{52}$	16177.580	16177.590 ± 0.005	0.33	0.64
	R	36	$C_{350}H_{443}O_{220}N_{130}P_{35}$	11069.829	11069.836 ± 0.008	0.71	0.84

[a]F, forward strand; R, reverse strand (see Figure 1). [b]nt, nucleotide. [c]Mass error is defined as the absolute deviation of a measured monoisotopic mass from the theoretical one.

selected as being a representative example. The result obtained by PAGE is shown in Figure 3a, and that obtained by LC/ESI-MS is given in Figure 3b and Table 2. Using PAGE, the band pattern could be divided into only three groups: *Takifugu chrysops*, another species of genus *Takifugu*, and genus *Lagocephalus* (Figure 3a). Each band, which corresponded to the DNA duplex detected by PAGE, was also detected by LC/ESI-MS as a peak (Figure 3b) and where each peak in the chromatogram represented the mass spectra of the forward and reverse oligonucleotides. Because the digestion with the endonucleases was performed just after PCR amplification without purification, the sticky end generated by the *Msp*I endonuclease was filled with the remaining DNA polymerase during incubation (Figure 1).[12]

Method Validation. Accuracy and precision data for the LC/ESI-MS analyses are presented in Table 2. Although an internal mass standard was not used, excellent accuracy (mass error = 0.83 ppm on average) and precision (standard deviation = 0.49 ppm on average) were achieved. Although this performance reflects the inherent performance of the Orbitrap analyzer, deconvolution also contributed to an improved accuracy and precision. Because dozens of peaks having different charge states and isotopic compositions are taken into account for the calculation of a monoisotopic mass, the monoisotopic mass calculated by deconvolution has a reduced observational error and must have been more accurate than any molecular mass information determined from an individual peak.[30,33] In addition, the use of the monoisotopic mass for mass characterization is preferable to that of the average mass for achieving enhanced accuracy and precision.[33] Because the monoisotopic mass is, for all practical purposes, independent of the isotope ratio,[34] the acquired data can be directly compared with the theoretical monoisotopic mass calculated from the molecular formula. An alternative mass spectrometry metric for a biomolecule is the average mass, which is defined as the centroid of the isotopic distribution of the biomolecule. However, according to a calculation by Zubarev et al., the accuracy of the average mass measurement is limited to 10 ppm for biomolecules larger than 10000 Da because of natural variations of isotopic abundances of the elements. Furthermore, the nonsymmetric shape of the isotopic distribution causes negative bias.[33] Manduzio et al. reported an average mass deviation of 9.15 ± 7.11 ppm as a result of analyzing a 114 bp PCR product despite an Orbitrap analyzer being used.[12] Kullolli et al. used an LTQ-Orbitrap Veros for analyzing microRNA ranging from 22 to 25 nt.[35] A mass accuracy of 1.3 ppm on average was reported; however, these studies compared the monoisotopic *peaks* of the multiply charged ions with the corresponding theoretical ones instead of the deconvoluted monoisotopic *mass* of the molecule. Because the monoisotopic peak disappears from a mass spectrum with increasing molecular mass,[33] the approach involving the monoisotopic *peaks* is applicable only for short oligonucleotides.

Periodic analyses were carried out to evaluate the stability of mass accuracy. Digested amplicons derived from the synthetic DNA of *Takifugu chrysops* were analyzed every 10 h for 180 h. As a result, all of the monoisotopic masses of the digested PCR amplicons were successfully determined within a mass error of 2.8 ppm without any additional mass calibration. Consequently, a daily calibration can be replaced by a weekly calibration.

As a result of the above findings and observations, a flowchart for the discrimination of the pufferfish is proposed (Figure 4). The target pufferfish always yields two or three peaks in the TIC chromatogram at a retention time of 4−5.5 min; otherwise, the sample is not the target pufferfish. If two peaks are observed in the range, a pair of the monoisotopic masses of the DNA duplex corresponding to the latter peak will be compared to the theoretical values to determine if any of the species is *T. chrysops*, *L. inermis*, *L. gloveri*, *L. lunaris*, or *L. wheeleri*. The mass tolerance for the determination is 3 ppm, which does not cause overlap. If three peaks are observed in the range, a similar matching procedure is required for the second peak in some cases as well as for the third peak (Figure 4).

Mixed Template. The synthetic DNA template of *L. lunaris* was mixed with that of *L. wheeleri* at ratios of 1:1, 1:4, and 1:19, and the mixtures were analyzed as described above. As the result of the preparation, four pairs of DNA duplexes would have existed in the sample solution, and the success of the discrimination relies on the difference of the masses between each oligonucleotide. The results are shown in Figure 5. In the case when the mass difference was about 25 Da (Figure 5a, single base substitution) or 7 Da (Figure 5b, triple base substitution), each strand could be analyzed independently for the mixing ratio of 1:1; however, minor constituents could not be calculated properly. When the difference was about 6 Da (Figure 5c, the complementary sequence of Figure 5b) because of an overlapping of clusters of peaks, the isotopic mass could not be calculated even for a mixing ratio of 1:1, although this could be compensated for by the results for the complementary strand. These observations are basically consistent with the study of Manduzio et al., who investigated the discrimination ability for an equal mixture of templates with single base substitution.[12] They reported that a mass difference of 16 Da (A→G) could be discriminated, although that of 9 Da (T→A) could not be discriminated for analysis of 114 bp DNA. This was overcome by emphasizing the difference of the m/z values

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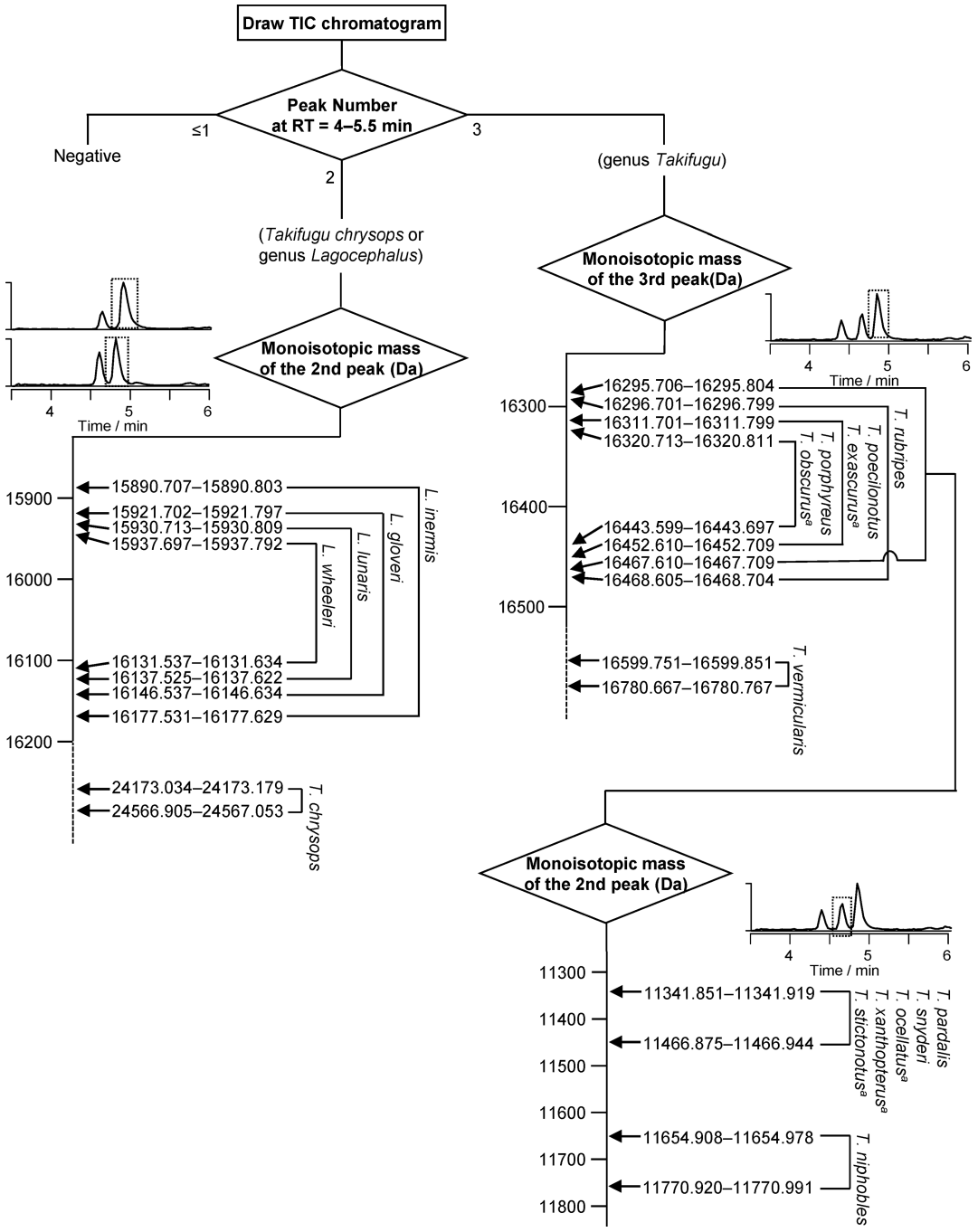

Figure 4. Flowchart for the discrimination of pufferfish. TIC, total ion current; RT, retention time. [a] Not included to the target of the Japanese official method.

using endonuclease digestion, which is consistent with the present strategy.

As an alternative to calculating the monoisotopic mass, the extracted ion chromatograms (XICs) of the most abundant isotopic ions were examined as a means to discriminate *T. rubripes* from *T. pardalis*. However, it was impossible to discriminate the two species using XIC because of the overlapping of the peaks (Figure 6), although they could be discriminated using a deconvolution approach (Table 2). Therefore, deconvolution was the preferred approach for species identification, especially when the sample was considered to be derived from a single source.

Application for Small Samples. A small amount of pufferfish alone or its presence in another fish was analyzed by the present methods. As a result of the analysis of a raw muscle tissue homogenate of *T. vermicularis* (0.1 mg equivalent, $n = 2$), all of the expected DNA fragments were successfully identified with an average mass error of 1.50 ppm. In addition, raw muscle tissue of *T. vermicularis* mixed with that of Korean black scraper (*Thamnaconus modestus*) (2.5 wt % of *T. vermicularis* in 50 mg of mixture) could be identified with an average mass error of 1.15 ppm ($n = 2$). Therefore, the present method is considered applicable to small sample amounts as long as PCR amplification proceeds successfully.

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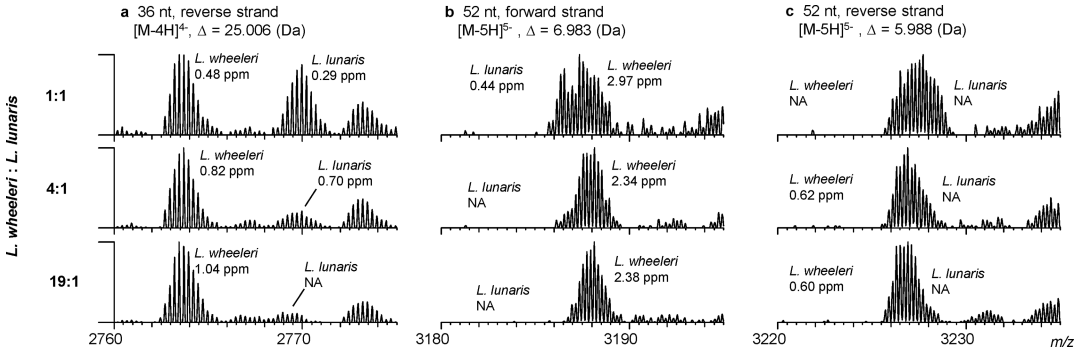

Figure 5. Total ion current chromatograms obtained from the mixed templates. nt, nucleotides. NA, not available.

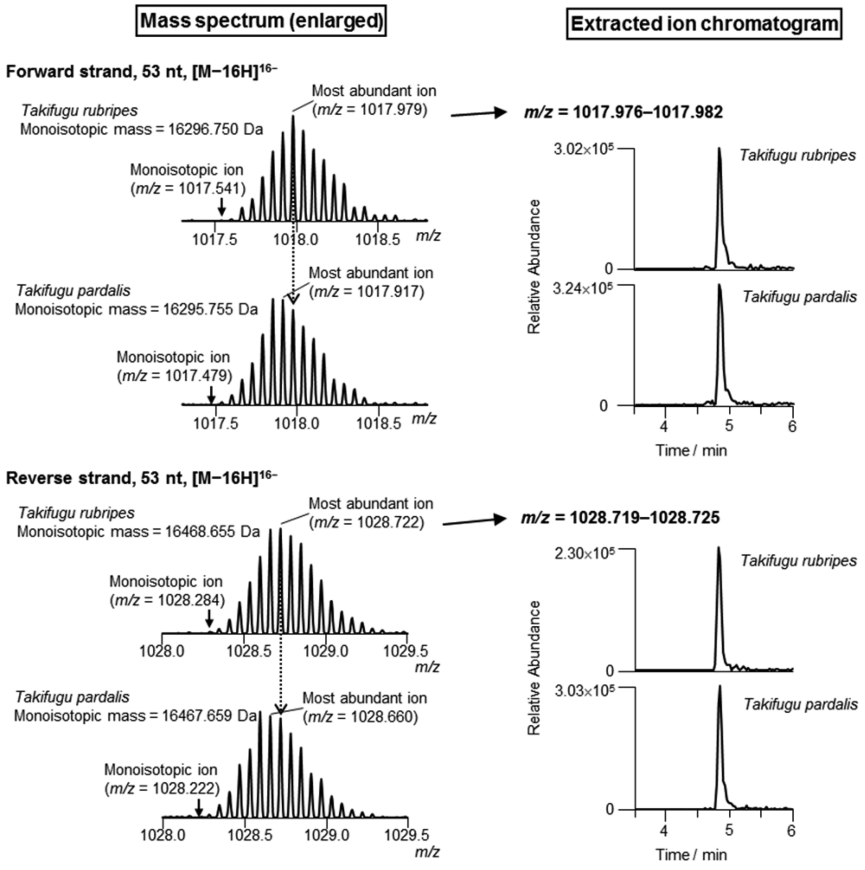

Figure 6. Example of failure of discrimination between *T. rubripes* and *T. pardalis* using individual isotopic peaks. nt, nucleotides.

Discrimination of Pufferfish and Other Fish Samples. The present method was applied to 29 pufferfish samples and 8 other fish species (Figure 7). The results for discrimination based on the present criteria (Figure 4) were consistent with the official method based on Sanger sequencing, although the present method could not discriminate *T. pardalis* from *T. snyderi* because these species contain the same amplicon sequence (data not shown). Additionally, no deconvolution value was calculated from the chromatograms of fish samples other than pufferfish.

As mentioned above, the use of LC/ESI-MS for fish genotyping has been demonstrated for the first time. A remaining problem is the interpretation of data obtained from a mixed template at a closer ratio as mentioned above. Using multiplex PCR with another pair of pufferfish-specific or species-specific primers may be an effective way for resolving analytes in a mixture.

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Notes

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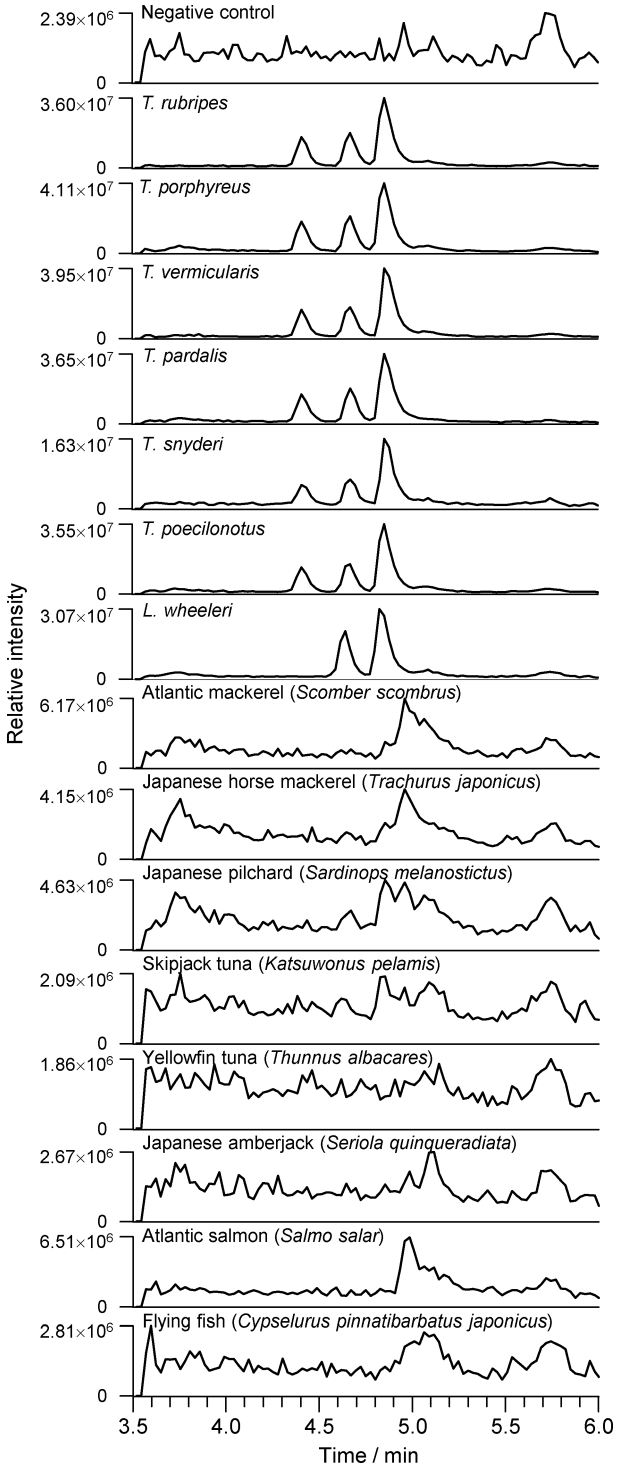

Figure 7. Total ion current chromatograms derived from actual fish samples.

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