



Identification of tuna species (Thunnini tribe) by PCR-RFLP analysis of mitochondrial DNA fragments

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ABSTRACT

Tunas are highly priced and the Atlantic bluefin tuna is most endangered trade fish in the world. For effective fishery management and protection of consumers' rights, it is important to develop a molecular method to identify the species of tuna products. In this study, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was developed to identify 10 tuna or tuna-like species. Primers were designed to amplify six mitochondrial DNA fragments from each specimen, and three restriction enzymes including *AfaI*, *DdeI*, and *AluI* were used to analyze the short length fragments produced from *ATPase* and control region (*CR*) genes. Besides these, the phylogenetic relationships among tested fish species were analyzed based on phylogenetic trees constructed using the amplified *COI* and *CR* sequences. The results indicated that the developed PCR-RFLP really provided a useful and academic technique to identify high-priced tuna species.

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1. Introduction

Tunas are commercially important fishery worldwide. Tunas and tuna-like fishes belong to six genera, namely *Auxis*, *Euthynnus*, *Katsuwonus*, *Thunnus* (tribe Thunnini) and the bonitos, *Sarda*, and *Gymnosarda* (tribe Sardini) (Pillai & Mallia, 2007). Among them, genera *Thunnus* is the largest one with seven species, and the species belonging to this genus are called true tunas (Kunal & Kumar, 2013).

Generally, morphological features are used to identify different tunas. But the gross morphologies of some tuna species are so similar, their separation requires very detailed morphological knowledge, known usually only to experts. Moreover, this method is impossible to identify fish fillets, semi-processed, and canned products, which nowadays represent a high proportion of seafood marketing (Bottero, Dalmaso, Cappelletti, Secchi, & Civera, 2007). However, identification of the species origin of processed as well as fresh or unprocessed tuna species secures the rights of the customer to be informed about the identity of purchased products. (Aranishi, Okimoto, & Izumi, 2005; Infante, Catanese, Ponce, & Machado, 2004).

Nowadays, several molecular genetic markers have been used for tuna species identification as well as phylogenetic relationship studies (Chow, Nakagawa, Suzuki, Takeyama, &

Matsunaga, 2006; Paine, McDowell, & Graves, 2007; Viñas & Tudela, 2009). Among them, analysis of mitochondrial genome (mtDNA) has been extensively used, as (1) mtDNA has a greater abundance (higher copy number) in sample extracts than nuclear genome; (2) mtDNA is maternally inherited and thus sequence ambiguities from heterozygous genotypes are theoretically avoided; and (3) the rate of base substitution of mtDNA is higher than that of genomic DNA, causing a rapid evolution (Pardo & Pérez-Villareal, 2004). For mtDNAs, the cytochrome oxidase subunit I (*COI*) and cytochrome b (*Cyt b*) genes are of ancient origin and show a relatively high mutation rate, thus they are often used to discriminate different tunas, even closely related tuna species belonging to the same families and genera (Aranishi et al., 2005; Jerome, Lemaire, Bautista, Fleurence, & Etienne, 2003; McClellan, 2000; Wang, Jiang, & Qiao, 2011; Yi, Ellsworth, & Li, 2002). Besides *COI* and *Cyt b* genes, other mtDNA fragments, such as the control region (CR) and *ATPase* gene, are also used to resolve evolutionary relationships among closely related tuna species (Pardo & Pérez-Villareal, 2004).

To analyze these genetic markers, many techniques derived from polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPD), PCR single-stranded conformational polymorphism (PCR-SSCP), and PCR restriction fragment length polymorphism (PCR-RFLP), have been developed (Lin & Hwang, 2007; Lin, Shiau, & Hwang, 2005). Among them, the PCR-RFLP technique is the most popular and widely used ones (Hsieh, Chai, & Hwang, 2005; Lin et al., 2005; Lin & Hwang, 2007). In this study, the PCR-RFLP was used to analyze the mitochondrial fragment of *Cyt b*, *COI*, CR, 12S rDNA, 16S rDNA, and *ATPase* genes, which enabled identification of 10 commercially important tunas or tuna-like species: *Thunnus alalunga*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus maccoyii*, *Thunnus tonggol*, *Katsuwonus pelamis*, *Euthynnus affinis*, *Auxis thazard*, *Auxis rochei* and *Sarda orientalis*. The phylogenetic trees based on *COI* and CR sequences were also constructed. This technique required neither expensive equipments nor costly nucleotide sequencing, and therefore could be routinely applied to discover fraudulent mislabeling of commercial tuna products in food inspection laboratories.

2. Materials and methods

2.1. Preparation of samples

The following fish samples were investigated in this study: genus *Thunnus*: *T. alalunga*, *T. albacares*, *T. obesus*, *T. maccoyii*, *T. tonggol*; genus *Katsuwonus*: *K. pelamis* (three separated populations); genus *Euthynnus*: *E. affinis*; genus *Auxis*: *A. thazard*, *A. rochei*; genus *Sarda*: *S. orientalis*; and genus *Tetrapturus*: *T. audax*. A list of species, geographical regions are provided in Table 1. All of the tunas were morphologically identified by their external characters by Zhoushan Entry-Exit Inspection and Quarantine Bureau (Zhoushan, China), or Zhejiang Huangguan Foodstuff Co., LTD (Taizhou, China). Three or more individuals of each species were used for analysis.

2.2. DNA extraction

Total DNA was extracted from 30 mg of frozen muscle tissue of each sample using the AxyPrep Multisource Genomic DNA Miniprep kit (Axygen Biotechnology, Hangzhou,

Table 1. Taxonomy, collection sites, and lengths of *Cyt b*, *COI*, *CR*, *ATPase*, 12S rDNA and 16S rDNA genes from 10 tuna species and 1 billfish species identified in this study.

Species	Collection site	Number of samples	Cyt b		COI		CR		ATPase		12S rDNA		16S rDNA	
			Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)
<i>T. alalunga</i>	Zhoushan Entry-Exit Inspection and Quarantine Bureau, Zhoushan, China	3	793	47.3	707	46.7	619	36.5	725	45.1	433	49.2	616	47.6
<i>Thunnus lbacares</i>	Zhoushan Entry-Exit Inspection and Quarantine Bureau, Zhoushan, China	3	792	46.3	707	46.8	618	35.9	725	45.0	433	49.2	617	47.1
<i>T. obesus</i>	Zhoushan Entry-Exit Inspection and Quarantine Bureau, Zhoushan, China	3	792	47.4	707	47.0	618	37.1	725	45.2	433	49.2	617	47.3
<i>T. maccoyii</i>	Zhoushan Entry-Exit Inspection and Quarantine Bureau, Zhoushan, China	3	792	47.1	707	47.0	581	37.9	725	44.8	433	49.2	617	47.5
<i>T. tonggol</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou, China	3	792	47.0	707	46.7	619	36.8	725	45.1	433	49.2	617	47.3
<i>K. pelamis</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou, China	3	792	46.1	707	46.4	571	37.7	725	46.8	434	50.0	573	48.0
<i>E. affinis</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou, China	3	792	48.7	707	47.5	569	37.4	725	47.6	393	48.9	572	47.7
<i>A. thazard</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou, China	3	806	46.4	707	47.1	555	37.8	725	48.3	434	49.5	616	47.6
<i>A. rochei</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou	3	800	47.6	707	46.5	566	35.7	725	48.0	434	49.8	617	47.7

(Continued)

Table 1. Continued.

Species	Collection site	Number of samples	Cyt b		COI		CR		ATPase		12S rDNA		16S rDNA	
			Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)	Length (bp)	G + C content (%)
<i>Sarda orientali</i>	Zhejiang Huangguan Foodstuff Co., Ltd, Taizhou, China	3	792	46.5	651	47.5	563	32.7	686	46.3	391	48.3	573	48.0
<i>T. audax</i>	Zhoushan Entry-Exit Inspection and Quarantine Bureau, Zhoushan, China	3	–	–	707	48.2	552	40.4	725	47.9	434	48.9	632	47.8

China) according to the manufacturer's instructions. DNA samples were dried and resuspended in 50 µL of elution buffer, their quantities were calculated using absorbance measurements at 260 nm wavelength on NanoDrop2000 (Thermo Fisher Scientific, Waltham, USA).

2.3. PCR amplifications

Firstly, the *Cyt b*, *COI*, *CR*, 12S rDNA, 16S rDNA, and *ATPase* genes of all the tested samples were amplified by a standard PCR, using the forward and reverse primer sets listed in Table 2. The primer sets of *CR*, 12S rDNA, 16S rDNA, *ATPase* genes and forward primer of *COI* gene were designed using the Primer 5.0 software, based on nucleotide sequences documented in NCBI database. Whereas primer sets of *Cyt b* and reverse primer of *COI* were taken from previous reports (Chiu et al., 2012; Terio et al., 2010).

PCR was carried out in a reaction volume of 25 µL containing 100 ng of template DNA, 2.5 U of Taq polymerase (Takara, Dalian, China), 2.5 µL of 10 × Taq polymerase reaction buffer, 200 mM of each dNTP, and 25 pmol of each primer. PCR was performed on a T-Gradient Thermoblock (Biometra GmbH, Göttingen, Germany) under the following conditions: 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57°C, 1 min at 72°C and a final step of 10 min at 72°C. Successful PCR products were purified using either the PCR clean-up kit or the gel purification kit (Axygen), and their lengths were estimated by 2.0% agarose gel electrophoresis.

2.4. Nucleotide sequencing

The purified PCR products were ligated into the pMD18-T vector (Takara) and transformed into competent cells of *Escherichia coli* DH5α. Recombinant clones containing the inserts of correct size were identified by restriction enzyme digestion of purified plasmids, or colony-direct PCR on randomly chosen white colonies using specific primer set. Three to five independent positive clones were selected and sequenced for each specimen (Invitrogen, Shanghai, China). All of the sequences obtained in this study have been deposited in the GenBank database under accession numbers KM055356-KM055424.

Table 2. The primers used in this study.

Target gene	Primer ^a	Sequence (5'–3')	Reference
<i>Cyt b</i>	FP	GCTCACTACTTGGCCTTTC	Terio et al. (2010)
	RP	TGGAGGCTAGGAGGGCTAGT	
<i>COI</i>	FP	TCAACCAACCAAYAAAGACATYGGCAC	Chiu, Su, Pai, & Chang (2012)
	RP	TAGACTTCTGGRTGKCCAAAGAATCA	
<i>CR</i>	FP	TACCCCAAACCTCCCAAAGCTA	JN635368, JN635369, AB097669, KC501712-KC501673, KC015952-KC015960
	RP	CGTKTGCACTSTGARATGYCA	
<i>ATPase</i>	FP	CTTCGACCAATTTATGAGCCC	AF115273-AF115278, AF260430, AB185022, GU256526
	RP	GTTARTGGTCAKGGGCTKGGRTC	
12S rDNA	FP	AAACTGGGATTAGATACCCCACTAT	AB106283-AB106311, AB176804, AB176805, HM003553-HM003555, JN007517-JN007526
	RP	GAGRGTGACGGGCGGTGTGT	
16S rDNA	FP	CGCCTGTTTAMCAAAACAT	GU256522-GU256526, GU256528, GU325784, JN086149, JN086150, JN086152-JN086154
	RP	CCGGTCTGAACCTCAGATCAYG	

^aFP: Forward primer; RP: Reverse primer.

2.5. Phylogenetic analysis

The sequences of amplified fragments were initially automatically aligned using the program Clustal X version 1.83 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins,

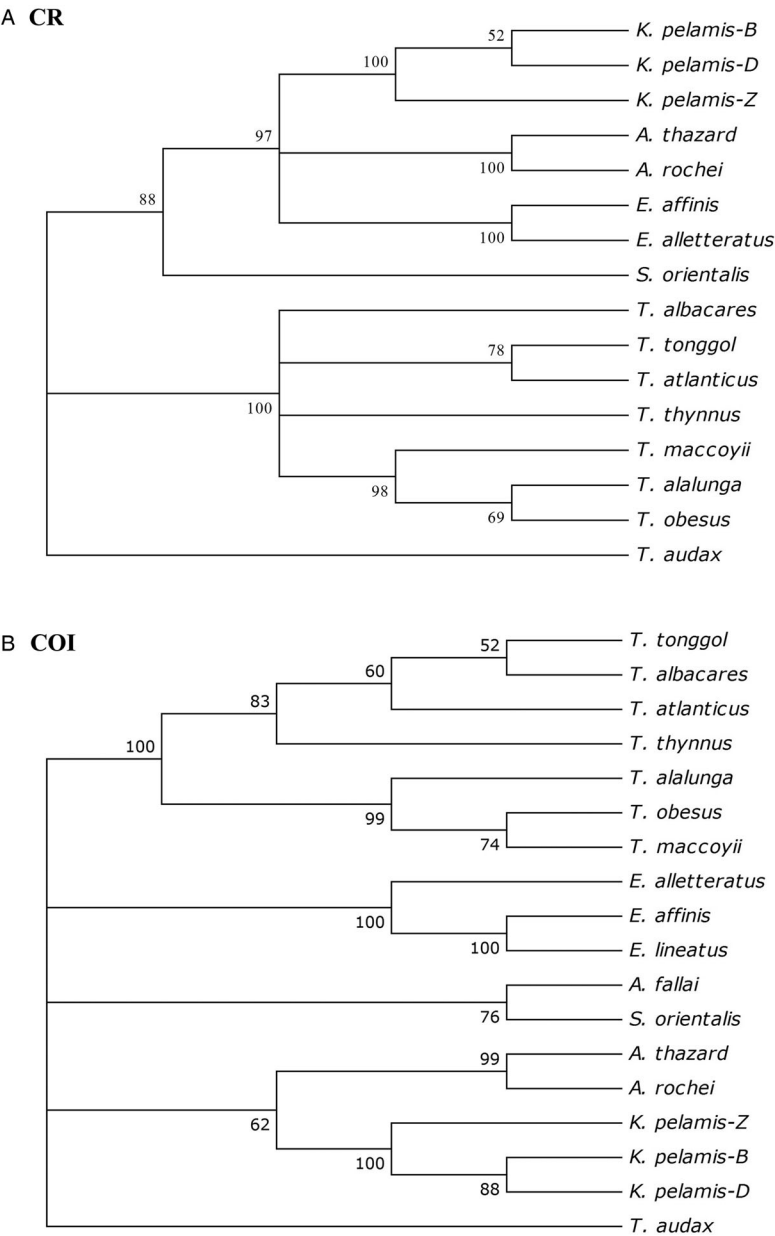


Figure 1. Neighbor-joining tree based on phylogenetic analysis of the fragments of *CR* (A) and *COI* (B) genes, showing the relationships of tuna species. Numbers at branching points represent the percentages of 1000 bootstrapped data sets supporting the specific internal branches. Branches without bootstrap numbers indicate that the bootstrap values are below 50%. *T. audax* was used as an out group. The abbreviations for species and locations are as in Table 1.

1997). Then, phylogenetic analysis of the aligned sequences was performed with the MEGA 4.0 software (Tamura, Dudley, Nei, & Kumar, 2007). The genetic distance matrix was calculated according to Kimura's (1980) two-parameter model, and was then used to construct a phylogenetic tree using the neighbor-joining method (NJ) (Saitou & Nei, 1987) with the interior branch tests of 1000 replicates (Sitnikova, Rzhetsky, & Nei, 1995) (Figure 1).

2.6. RFLP analysis

For RFLP analysis, a set of restriction enzymes was chosen according to the different tuna species studied here: *AfaI*, *DdeI*, *AluI* (Takara, Dalian, China). Each digestion was performed in a 20 μ L mixture that contains 200 ng PCR product, 10 U restriction endonuclease, 2 μ L 10 $\times$ digestion buffer. Digestive reactions were incubated at 37°C for 1 h, then the restriction fragments were separated on 2% agarose gel. Gels are stained with EtBr and then visualized with ChemiDoc XRS⁺ system (Bio-Rad Laboratories, Inc., Hercules, CA).

3. Results

3.1. PCR amplification and sequence analysis

Using the designed primer sets, the fragments of *Cyt b*, *COI*, *CR*, 12S rDNA, 16S rDNA, and *ATPase* genes were successfully amplified from all of the tested samples. For each gene, no variation in size was detectable among individual amplicons on agarose gel. The length and GC% content of each PCR product were analyzed. As shown in Table 1, a higher A/T base content compared to C/G base content was observed among the sequences examined, especially the *CR* fragment. This observation is consistent with previous findings that the *CR* is an A/T-rich region of the mitochondrial genome (Bernt, Braband, Schierwater, & Stadler, 2013; Li, Xu, Cheng, Sun, & Wang, 2012; Zhou, Zhuo, Zou, Chen, & Zou, 2014).

Over all, the examined *Cyt b*, *COI*, *CR*, 12S rDNA, 16S rDNA, and *ATPase* fragments showed 90–100% similarity to their corresponding sequences of each species documented in NCBI database. The *Cyt b* gene of *S. orientalis*, 12S rDNA, 16S rDNA, and *ATPase* fragments of *E. affinis* were the firstly reported in our study. When the amplified fragments were aligned among tested tuna species, we found the *CR* had the highest degree of divergence (6.9–46.7%), followed by the *ATPase* (0.9–22.3%) (Supplementary Table S1).

3.2. Phylogenetic analysis of tuna species

Phylogenetic analysis of tuna species was carried out based on the amplified sequences by the NJ method, using *T. audax* as an outgroup. The *COI* sequences of *E. alletteratus* (AB099716), *E. lineatus* (GU440322), *T. thynnus* (KF906720), *T. atlanticus* (DQ107588), and the *CR* sequences of *E. alletteratus* (AB099716), *T. thynnus* (KF906720), *T. atlanticus* (HQ630706) were retrieved from the GenBank database. The results showed that only the phylogenetic trees constructed based on *CR* and *COI* sequences were consistent with the evolutionary distance previously reported, using morphological characters or molecular

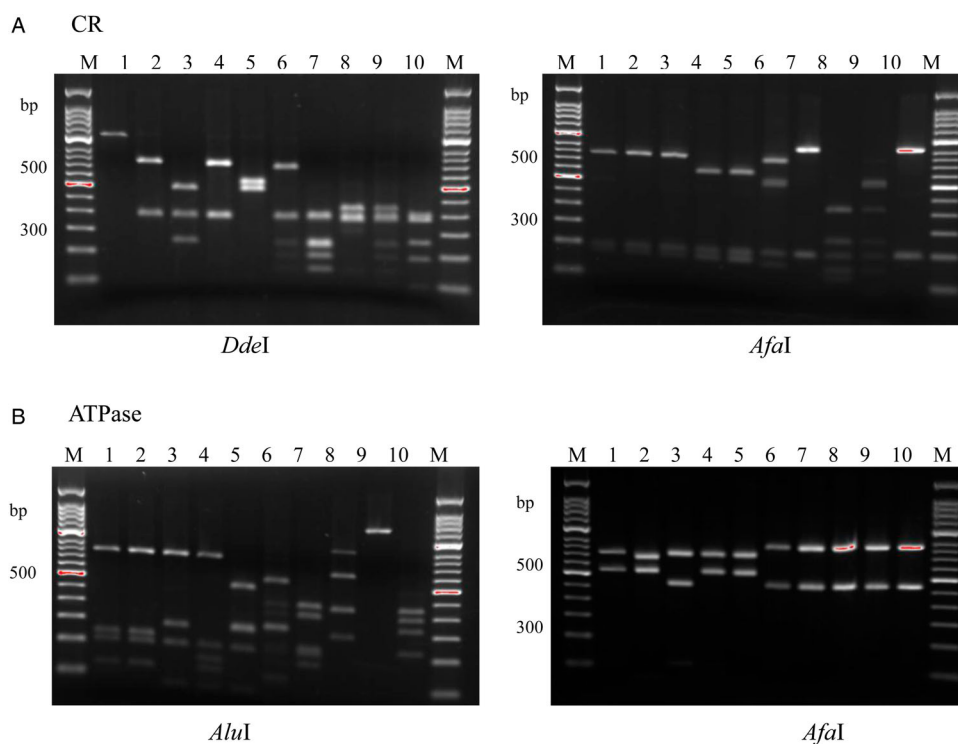


Figure 2. PCR-RFLP patterns of ten tuna and tuna-like species. The fragments of *CR* (A) and *ATPase* (B) genes amplified from different tunas were digested with restriction enzymes *AfaI*, *DdeI* and *AluI*. Lane 1, *T. obesus*; Lane 2, *T. alalunga*; Lane 3, *T. albacares*; Lane 4, *T. maccoyii*; Lane 5, *T. tonggol*; Lane 6, *S. orientalis*; Lane 7, *K. pelamis*; Lane 8, *A. rochei*; Lane 9, *A. thazard*; Lane 10, *E. affinis*; Lane M, 50 bp DNA ladder.

techniques (Collette, Reeb, & Block, 2001; Kunal & Kumar, 2013). As shown in Figure 2(a), the tuna species studied in this paper clustered into two obvious clades by *CR* sequences with a relatively strong bootstrap support. Genus *Thunnus* clustered into the first clade. In this clade, the species *T. obesus*, *T. alalunga*, and *T. maccoyii* were placed in a closely clustered and easily identifiable sub-clade, while *T. tonggol* and *T. atlanticus* were grouped together. The rest fish species clustered together, forming the second clade. In this clade, the genera *Auxis*, *Euthynnus*, *Katsuwonus* were recovered well (with 100% bootstrap value support), and *S. orientalis* was distantly placed. When *COI* sequences were used for phylogenetic relationship analysis, *S. orientalis* was firstly separated. The rest fish species clustered into three clades: the species in genus *Thunnus* clustered together, forming the first clade; *Euthynnus* genus recovered as the second clade; and the species in *Auxis* and *Katsuwonus* genera were grouped together, forming the third clade.

3.3. RFLP patterns of PCR amplicons from tested tuna species

To explore the feasibility of using the *Cyt b*, *COI*, *CR*, 12S rDNA, 16S rDNA, and *ATPase* as molecular markers in tuna species identification, many endonucleases (such as *AfaI*, *AluI*, *DdeI*, *EcoRI*, *EcoRV*, *HindIII*, *HinfI*, *MspI*, *SfiI*, and *PstI*) were applied to analyze

the expected products from the examined genes of each species. But according to the restriction map, no polymorphism in the restricted fragment length between species was observed for *Cyt b*, *COI*, 12S rDNA, and 16S rDNA genes. Therefore, restriction endonucleases analysis for these fragments was invalid, and no further effort was attempted to search inter-specific polymorphisms in these fragments. For *ATPase* and *CR* genes, different RFLP patterns were expected to generate when digested with *AfaI*, *DdeI*, and *AluI*. The sizes of restriction fragments expected for the selected enzymes are shown in Table 3. Because signals of fragments less than 50 bp are usually weak and not always perceptible in agarose gel electrophoresis analysis, they are not included in Table 3.

Figure 2 show the RFLP profiles generated from a single digest of each tuna species. As expected, the digestion of *CR* gene with *DdeI* allowed differentiation of all the tested tuna species by specific RFLP patterns except *T. alalunga* (Lane 2) and *T. maccoyii* (Lane 4). Then the restriction enzyme *AfaI* was used, and two fragments (longer than 50 bp) of 100 and 436 bp were produced for *T. alalunga*, whereas three fragments (longer than 50 bp) of 100, 104 and 351 bp were produced for *T. maccoyii*. For *ATPase* gene, all of the tuna species could be firstly differentiated by digestion with *AluI*, except *T. obesus* (Lane 1) and *T. alalunga* (Lane 2). When following digested with *AfaI*, these two tuna species were separated as *ATPase* of *T. obesus* was cut into two fragments of 319 plus 365 bp, whereas *T. alalunga* was cut into three fragments of 23, 319, and 345 bp (the 23 bp fragment was not included in Table 3). The results indicate using RFLP patterns produced by digestion of *ATPase* and *CR* fragments with *DdeI*, *AfaI* and *AluI*, the identification of certain tuna species was possible.

4. Discussion

In view of the increase in international seafood trade and the existing laws requiring products to be correctly labeled, there is a growing need for simple techniques that enable authentication of fish and seafood products.

The raw tuna species of *Thunnus* have been identified by many different DNA-based techniques (Abdullah & Rehbein, 2015; Dalmaso et al., 2007; Kunal & Kumar, 2013; Lin & Hwang, 2007; Terio et al., 2007; Wolf, Burgener, Hubner, & Luthy, 2000). PCR-RFLP was for the first time applied in authenticating two *Thunnus* and two bonito species in 1996, by amplifying two short fragments of mitochondrial *Cyt b* gene that

Table 3. Size of restriction fragments of 11 tuna species expected to be produced by the selected enzymes.

Sample	CR		ATPase	
	<i>DdeI</i>	<i>AfaI</i>	<i>AluI</i>	<i>AfaI</i>
<i>T. obesus</i>	581	100, 435	66, 107, 122, 384	319, 365
<i>T. alalunga</i>	196, 386	100, 436	66, 107, 122, 373	319, 345
<i>T. albacares</i>	127, 196, 256	100, 436	107, 147, 391	55, 265, 365
<i>T. maccoyii</i>	196, 385	100, 104, 351	66, 81, 107, 392	319, 365
<i>T. tonggol</i>	257, 324	84, 100, 352,	108, 147, 152, 226	320, 365
<i>S. orientalis</i>	195, 368	100, 425	61, 113, 161, 316	265, 420
<i>K. pelamis</i>	96, 120, 126, 197	258, 314	86, 107, 115, 153, 225	265, 420
<i>A. rochei</i>	139, 197, 219	73, 89, 101, 202	146, 222, 317	265, 420
<i>A. thazard</i>	150, 197, 219	74, 89, 101, 212	685	265, 420
<i>E. affinis</i>	96, 128, 148, 199	103, 466	114, 155, 165, 222	265, 420

were fewer than 123 bp (Ram, Ram, & Baidoun, 1996). Quinteiro et al. (1998) identified four *Thunnus* and two bonito species from non-commercially available canned samples by restriction patterns of a 126 bp sequence. However, the fragments sizes digested after restriction enzymes were too small, causing the RFLP patterns to be observed by complicated sodium dodecyl sulfate electrophoresis and silver stain (Quinteiro et al., 1998). After that, a 276 bp *Cyt b* fragment was utilized to differentiate five species (Pardo & Pérez-Villareal, 2004). Generally, for fish species belonging to the same genus, the PCR-RFLP is not always operated smoothly in the experiments, due to the high degree of homology of the sequences (Lin & Hwang, 2007). For this reason, 6 mitochondrial genes were tested in our study, their PCR-RFLP patterns among 10 tunas or tuna-like species were analyzed. The results indicated the *ATPase* and *CR* region were suitable for simultaneous and unambiguous identification of tunas and tuna-like species, after digestion with two restriction enzymes. Therefore, the developed PCR-RFLP method really provided a useful and academic technique to identify tuna species of high values.

When these amplified mitochondrial DNA fragments were analyzed, we found the tandemly repeated sequences were seldom presented. Tandemly repeated sequences, variable in number and length, have been reported in several fish species and appeared to be common among poikilotherms (Alvarado-Bremer, Naseri, & Ely, 1997). It has been suggested that the presence of tandemly repeated sequences in the mtDNA were correlated to low metabolic rates (Alvarado-Bremer et al., 1997). Our findings, and those obtained by others (Alvarado-Bremer et al., 1997; Cawthorn, Steinman, & Corli-Witthuhn, 2011; Qiu, Kitchen, Beerli, & Miyamoto, 2013), give additional support to this view, since tunas and related fish species have high metabolic rates and they lack tandemly repeated sequences almost entirely.

After a close inspection of the phylogenetic tree constructed based on the *CR* and *COI* fragments, both genes had excellent potential as genetic marker as they allowed a full discrimination of all detected species. Generally, the gene tree based on *CR* marker gave a higher bootstrap support, probably as a consequence of its greater genetic variability (Table S1). However, the *S. orientalis* (belonging to genus *Sarda*) was placed together with the genera *Auxis*, *Euthynnus*, *Katsuwonus* when *CR* was used for phylogenetic tree construction. Based on the evolutionary distance among tunas and related species proposed by Collette et al. (2001), the genera *Auxis*, *Euthynnus*, *Thunnus*, and *Katsuwonus* are parallel in the tribe Tuna Thunnini, whereas the genus *Sarda* was in the tribe Bonitos Sardini. Thus, it seems the *CR* fragment was less suitable to differentiate tunas at the genus level.

The *COI* phylogenetic tree we constructed was almost identical to one constructed by Kunal and Kumar (2013), which was based on *COI* sequences retrieved from NCBI nucleotide database. One minor difference was the *T. atlanticus*, *T. tonggol* and *T. albacares* formed a clearly sub-clade (with 53% bootstrap support) in our *COI* phenogram, whereas they were grouped together with *T. obesus* by a lower level of bootstrap support (35%) in results of Kunal and Kumar (2013). Collette (1978) firstly proposed a subgenus *Neothunnus* to separate *T. atlanticus*, *T. tonggol*, and *T. albacares* from other tuna species (proposed subgenus *Thunnus*). He regarded the three *Neothunnus* species differ from others in having central heat exchangers rather than lateral heat exchangers, and in being confined to more tropical waters (Collette, 1978; Ward, Zemlak, Innes, Last, & Hebert, 2005). Although this review has been challenged by several molecular

analysis (Alvarado-Bremer et al., 1997; Chow & Kishino, 1995; Chow et al., 2003; Elliott & Ward, 1995; Takeyama, Chow, Tsuzuki, & Matunaga, 2001; Ward, 1995), our *COI* phenogram seems to be concordant with this kind of subdivision. Nevertheless, several criticisms have risen mainly related to the use of a single genetic marker (Moritz & Cicero, 2004; Teletchea, Maudet, & Hanni, 2005). For example, Viñas and Tudela (2009) mentioned the *COI* is less efficient than the *CR* sequences in differentiating species in the *Neothunnus* subgenus, as the *T. albacares* sequences were polyphyletic. Although variation among *COI* sequences were not observed in our study (partially because of small sample number used in our study), we proposed both of the mtDNA *COI* and *CR* were used as genetic markers in phylogenetic studies of 10 tuna and tuna-like species.

5. Conclusion

In conclusion, we amplified six mitochondrial genes from tunas and their relative species, some of them were firstly reported. To test their application potential in species identification, all of these genes were used for PCR-RFLP analysis. Their PCR-RFLP patterns indicated that *ATPase* and *CR* were suitable for simultaneous and unambiguous species identification, after digestion with two restriction enzymes. Both of the *COI* and *CR* phylogenetic trees allowed a full discrimination of all tested species in our study. However, in the genus *Thunnus*, phylogenetic tree constructed based on *COI* fragments supports the differentiation of three *Neothunnus* species (*T. atlanticus*, *T. tonggol*, and *T. albacares*) from the other tuna species, whereas the *CR* phylogenetic tree supports the monophyletic origin of tuna species in the genus *Thunnus*. The results presented here can be used effectively in tuna species identification, and to investigate the molecular evolution and phylogenetics of tuna species.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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