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Phylogenetic relationship and toxic variations  
between Turkish Mediterranean and Japanese  
puffer fish species

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**Doctoral Dissertation**

**PHYLOGENIC RELATIONSHIP AND TOXIC  
VARIATIONS BETWEEN TURKISH  
MEDITERRANEAN AND JAPANESE PUFFER  
FISH SPECIES**

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**Graduate School of Marine Science and Technology**

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## ABSTRACT

Biological diversity has been negatively affected by invasive species and has become a global problem against protection efforts. The Mediterranean Sea and the Red Sea were connected after the opening of the canal Suez Canal in 1869. Therefore, both the Red Sea and Mediterranean Sea were exposed to the invasion of organism. However, the extensive majority of migrational movement has occurred from the Red Sea to the Mediterranean Sea and this bioraid is termed “Lessepsian migration”. The puffer fishes belonging to the family Tetraodontidae are highly abundant teleosts, with 184 species belonging to 27 genera and represented with six Lessepsian species in the Mediterranean coasts of Turkey. *Lagocephalus sceleratus*, *L. suezensis* and *L. spadiceus* have been detected as the most abundant puffer fish species among those Lessepsian puffer fishes and the economical and ecological effects in the eastern Mediterranean system are highly concerned. *Lagocephalus sceleratus* is regarded to be among the worst invasive species in the Mediterranean Sea with a significant impact on the surrounding ecosystem and on the fisheries sector.

*L. sceleratus* is considered to be a serious hazard to consumers since it contains a strong marine toxin, tetrodotoxin (TTX), which can be lethal to humans. Therefore, the studies in Chapter 2 focused on confirmation and comparison of toxicity of *L. sceleratus*. Firstly, TTX contents of twenty specimens caught from Marmaris and Iskenderun Bay, Turkey, and twelve specimens caught from Okinawa Island, Japan were determined by LC-MS/MS. Individual and tissue depending TTX distribution were clarified. As a result, all of the specimens examined in this study contained detectable amount of TTX. Although testis, muscle and skin tissues relatively contained less toxicity comparing with the ovary and other internal organs, TTX contents were individual and tissue dependent. The results of the study

in Chapter 2 indicates that *L. sceleratus* is a potential risk for human consumption and needed to be eliminate.

Whole mitogenome sequences from many teleost lineages have been determined and used for phylogenetic analyses. Thus, in Chapter 3, the complete mitochondrial DNA (mtDNA) of *L. sceleratus*, *L. suezensis* and *L. spadiceus* were determined according to primer-walking strategy and the gene structures were also analyzed. Afterwards, phylogeny within the family Tetraodontidae were clarified. Since these puffer fish species have migrated, it is needed to understand the zoogeographical and evolutionary origin. Both the gene structure and phylogenetic trees showed that *L. sceleratus* and *L. suezensis* were closely related species, although morphological characteristics were unique of each species. In addition, the members of genus *Lagocephalus* can be distinguished by using molecular markers based on their mtDNA. Thus, molecular identification of three puffer fish species by polymerase chain reaction (PCR) were carried out in Chapter 4.

PCR-based identification methods have been widely used to identify and authenticate of fish species and seafood products. Species-specific PCR amplification was conducted based on the species-specific nucleotide sequences of mtDNA of three Lessepsian puffer fish species, *L. sceleratus*, *L. suezensis* and *L. spadiceus*. Analysis of the alignment of the eight reference sequences obtained from GenBank showed that the bases in 119 position of NADH dehydrogenase subunit 2 (ND2) region in mtDNA could differentiate *L. sceleratus*. On the other hand, the bases in 123 and 138 position of cytochrome oxidase subunit 1 (COI) region in mtDNA could successfully differentiate *L. suezensis* and *L. spadiceus*, respectively. The method constructed in this Chapter is a useful tool for authentication and identification of *Lagocephalus* species. And also, the method can be used to verify the labeling issues of seafood products.

In Chapter 2, TTX contents of *L. sceleratus* were confirmed. However, the accumulation mechanism of TTX was not discussed. It has been recently reported that TTX binds with high molecular weight substances and the substances neutralize the lethal effects of TTX. Hence, in Chapter 5, the binding protein from the ovary of *L. sceleratus* were purified. The results of the analysis showed that TTX-binding ability was weak and destroyed during the gel filtration purification process. Therefore, the puffer fish saxitoxin and tetrodotoxin binding protein (PSTBP) was cloned from cDNA constructed from *L. sceleratus* liver. The characteristics of the protein was highly similar with those of plasma binding proteins of *Takifugu pardalis*, which are known as PSTBP1 and PSTBP2. This result indicates that PSTBP might have an important role on TTX accumulation in genus *Lagocephalus* but not only *Takifugu*. Experiment on TTX-binding protein of *L. sceleratus* is now in progress.

As a conclusion in the present, overall data collected from this study will be fundamental for the eastern Mediterranean fishery and the handling of the problems caused by Lessepsian puffer fish species. Moreover, the identification method will give a great advantage to local authorities and consumers to eliminate these species and the PSTBP of *L. sceleratus* will be guided to better understanding of the TTX accumulation mechanism in the puffer fish.

## Table of Contents

Acknowledgements	ii
Abstract	iv
List of Tables	xii
List of Figures	xiii
Chapter 1	1
General introduction	
1.1. Impact of Lessepsian puffer fish species on the Mediterranean fisheries	2
1.2. Tetrodotoxin	3
1.3. Impacts of Lessepsian puffer fish species on public health	4
Objectives and outline of thesis	5
Chapter 2	6
Confirmation and comparison of toxicity of <i>Lagocephalus sceleratus</i> caught from Turkish Mediterranean coasts and Okinawa Island, Japan	
2.1. Introduction	10
2.2. Materials and methods	12
2.2.1. Samples and materials	12
2.2.2. Toxin extraction	12
2.2.3. LC-MS/MS and LC-ESI/MS analysis	12

2.3. Results and discussion	13
2.3.1. Toxicity of <i>Lagocephalus sceleratus</i>	13
2.3.2. Conclusions	15
Tables and figures	
Chapter 3	22
Determination and characteristics of the complete mitochondrial DNA of <i>Lagocephalus sceleratus</i> , <i>L. suezensis</i> and <i>L. spadiceus</i> species and their phylogeny within the family Tetraodontidae	
3.1. Introduction	22
3.2. Materials and methods	24
3.2.1. Samples and materials	24
3.2.2. Total DNA extraction	25
3.2.3. Design of PCR primers	25
3.2.4. PCR amplification and electrophoresis	26
3.2.5. Purification of PCR products	26
3.2.6. DNA sequencing	26
3.2.7. Genome annotation and phylogenetic analyses	27
3.3. Results and discussion	28
3.3.1. Mitochondrial genome summary	28
3.3.2. Protein-coding genes	29

3.3.3. Transfer and ribosomal RNA genes	30
3.3.4. A + T rich regions	31
3.3.5. Phylogenetic relationships within the family Tetraodontidae	31
3.3.6. Conclusions	33
Tables and figures	
Chapter 4	46
The species authentication of <i>L. sceleratus</i> , <i>L. suzensis</i> and <i>L. spadiceus</i> by rapid PCR amplification method	
4.1. Introduction	46
4.2. Materials and methods	48
4.2.1. Samples and materials	48
4.2.2. Total DNA extraction	48
4.2.3. Species-specific primer construction	49
4.2.4. Species-specific PCR amplification	49
4.2.5. DNA sequencing	50
4.2.6. Method validation and selectivity	50
4.3. Results and discussion	51
4.3.1. DNA extraction	51
4.3.2. Species-specific PCR amplifications	51

4.3.3. Validation of method	52
4.3.4. Conclusions	54
Tables and figures	
Chapter 5	60
Purification, cDNA cloning and recombinant expression of TTX-binding protein from <i>Lagocephalus sceleratus</i>	
5.1. Introduction	60
5.2. Materials and methods	61
5.2.1. Samples and materials	61
5.2.2. Protein extraction and purification	62
5.2.3. Detection of toxin related substances by LC-MS	62
5.2.4. Total RNA extraction	62
5.2.5. cDNA synthesis	63
5.2.6. Rapid amplification of cDNA ends (RACE)	63
5.2.7. Amplification and cloning of internal PSTBP cDNA fragment	64
5.2.8. Sequence analysis	64
5.2.9. Tissue distribution of PSTBP mRNA	65
5.2.10. Construction of the recombinant expression vector	66
5.2.11. Recombinant LsPSTBP expression, refolding and purification	66

5.2.12. Circular dichroism spectroscopy analysis	68
5.2.13. TTX binding ability assay	68
5.3. Results and discussion	69
5.3.1. Protein purification	69
5.3.2. cDNA sequence of LsPSTBP	69
5.3.3. The distribution of LsPSTBP mRNA in different tissues	70
5.3.4. Construction of the recombinant expression vector	71
5.3.5. Recombinant LsPSTBP expression, refolding and purification	72
5.3.6. Conformational characterization of recombinant LsPSTBP	73
5.3.7. TTX binding assay	73
5.3.8. Conclusions	74
Tables and figures	
Chapter 6	93
General conclusions	93

## List of Tables

<b>Table 2-1</b>	The analytical conditions of LC-MS/MS for TTX quantification	17
<b>Table 2-2</b>	Toxicological results of <i>Lagocephalus sceleratus</i> specimens	18
<b>Table 3-1</b>	Oligonucleotide primers for PCR amplifications	41
<b>Table 3-2</b>	Fish species studied in this research with NCBI Genbank accession numbers	35
<b>Table 3-3</b>	Percentage nucleotide identities of complete mitogenomes among <i>Lagocephalus</i> species	36
<b>Table 3-4</b>	A + T content, AT-skew, and GC-skew of three mitochondrial genomes from <i>Lagocephalus sceleratus</i> , <i>L. suezensis</i> and <i>L. spadiceus</i>	37
<b>Table 3-5</b>	Amino acid composition of protein-coding genes from <i>Lagocephalus sceleratus</i> , <i>L. suezensis</i> , and <i>L. spadiceus</i>	38
<b>Table 3-6</b>	The start and stop codons of protein-coding genes from <i>Lagocephalus sceleratus</i> , <i>L. suezensis</i> , and <i>L. spadiceus</i>	39
<b>Table 4-1</b>	Species-specific primer sequences and their optimum annealing temperatures of three puffer fish species	55
<b>Table 5-1</b>	List of primers	75
<b>Table 5-2</b>	Amino acid identity of <i>L. sceleratus</i> TBT-p protein among other puffer fish species	76
<b>Table 5-3</b>	TTX binding ability of recombinant LsPSTBP	77

## List of Figures

<b>Fig. 1-1</b>	Distribution maps of three Lessepsian puffer fish species along the Turkish Mediterranean coasts	6
<b>Fig. 1-2</b>	Impacts of Lessepsian fish species on the eastern Mediterranean fisheries	7
<b>Fig. 1-3</b>	Proposed mechanism of TTX accumulation in marine animals	8
<b>Fig. 1-4</b>	The structures of TTX and its analogues	9
<b>Fig. 2-1</b>	The examined <i>L. sceleratus</i> specimens in this study	19
<b>Fig. 2-2</b>	LC-ESI/MS profile of TTX standard (bottom) and TTX extract of ovary sample of specimen no. 16. TTX and analogues are marked with arrows on the top of the peaks	20
<b>Fig. 2-3</b>	MS/MS fragmentation pattern of TTX and analogues ( <i>m/z</i> 272, <i>m/z</i> 304 and <i>m/z</i> 320)	21
<b>Fig. 3-1</b>	Puffer fish species used in this study	40
<b>Fig. 3-2</b>	PCR amplification region and sequencing strategy for the detection of complete mtDNA genome of three <i>Lagocephalus</i> species	41
<b>Fig. 3-3</b>	Organization of mitochondrial genomes from three <i>Lagocephalus</i> species	42

<b>Fig. 3-4</b>	AT content and AT/GC-skew values of each codon position in each protein-coding gene (PCG) from three <i>Lagocephalus</i> species	43
<b>Fig. 3-5</b>	Phylogenetic reconstruction among puffer fishes based on concatenated nucleotide and amino acid sequences from twelve protein coding genes	44
<b>Fig. 3-6</b>	The maximum parsimony tree among puffer fish species	45
<b>Fig. 4-1.</b>	The alignment results and species-specific primer regions of three puffer fish species	56
<b>Fig. 4-1</b>	Continued	57
<b>Fig. 4-2</b>	Agarose gel electrophoresis of amplification products obtained by a species-specific PCR	58
<b>Fig. 4-3</b>	Agarose gel electrophoresis of sensitivity of the species-specific PCR method	59
<b>Fig. 5-1</b>	The purification and TTXs content of ovary protein extraction	78
<b>Fig. 5-1</b>	Continued	79
<b>Fig. 5-2</b>	Nucleotide and deduced amino acid sequences of LsPSTBP cDNA	80
<b>Fig. 5-3</b>	Multiple sequence alignment of deduced amino acid sequences of LsPSTBP with those of the homologous proteins	81
<b>Fig. 5-4</b>	Neighbor-joining phylogenetic tree of TBT-p and PSTBP amino acid sequences from 8 puffer fish species	82

<b>Fig. 5-5</b>	Melt-curve analysis of the qPCR products	83
<b>Fig. 5-6</b>	Tissue-specific expression of LsPSTBP mRNA in different tissues from <i>L. sceleratus</i> by real-time RT-PCR.	84
<b>Fig. 5-7</b>	Amplification results of PSTBP cDNA with restriction sites	85
<b>Fig. 5-8</b>	Digested PSTBP and pET-16b expression vector with <i>Nde</i> I and <i>BamH</i> I restriction enzymes	85
<b>Fig. 5-9</b>	Screening of constructed expression vector by direct ligation screening and colony PCR	86
<b>Fig. 5-10</b>	SDS-PAGE analysis of different expression combinations	87
<b>Fig. 5-11</b>	SDS-PAGE analysis of different expression combinations with/without urea treatment	88
<b>Fig. 5-12</b>	Purification strategy and SDS-PAGE analysis	89
<b>Fig. 5-12</b>	Continued	90
<b>Fig. 5-13</b>	Far-UV CD spectrum of recombinant LsPSTBP	91
<b>Fig. 5-14</b>	Schematic diagram of separation of bound-free TTX	92



# Chapter 1

## *General Introduction*

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Biological diversity has been negatively affected by invasive species and has become a global problem against protection efforts (Bax *et al.*, 2003). Serious zoogeographic and ecological rebounds have been a concern since the opening of the Suez Canal in 1869. Two faunistically and hydrographically varied areas of water with great capacity, the Mediterranean Sea and the Red Sea, were connected by the canal. Therefore, both the Red Sea and Mediterranean Sea were exposed to the invasion of organism. However, the vast majority of migrational movement has occurred from the Red Sea to the Mediterranean Sea, and this influx of biota is termed “Lessepsian migration” (Por, 1978).

‘The study of the Lessepsian migration presents many advantages to scientists as the date of the opening of the Suez Canal is known as is the origin of the invaders. Therefore, extensive research has been, and is still being carried out to monitor the arrival of invasive species, study their impact on the marine ecosystem as a whole and to provide predictions. Given that it is predicted that invasions of species of the Mediterranean will continue and since changes in ecosystems as well as in coastal fisheries have already been reported, this becomes an ecological and economic issue which must be addressed and constantly studied and monitored (Corsini-Foka, 2005, 2006; IUCN 2008; Oral 2010).’

The highest number of Lessepsian species detected belong to the family Tetraodontidae, which comprised five species (Akyol *et al.*, 2005; Turan, 2010). The puffer fishes belonging to the family Tetraodontidae are highly abundant teleosts, with 184 species belonging to 27 genera (Matsuura, 2015). *Lagocephalus lagocephalus*, *L. sceleratus*, *L.*

*spadiceus*, *L. suezensis*, *Sphoeroides pachygaster* and *Torquigener flavimaculosus* are widely distributed puffer fish species in the Mediterranean Sea. The distribution maps of three puffer fish species are shown in Fig. 1-1.

According to size of populations, three types of introduced species by Suez Chanel can be distinguished in Turkish waters: Rare species, species having established stable populations, very common and abundant migrant species. On the other hand, there are harmful effects of the lessepsian species. These can be categorized as: (a) health problem (poisoning, pain) for fishermen, swimmers, divers, tourism, (b) net damages, (c) mesh clogging, (d) fouling and (e) extra labour for fishermen. There are three types of consequences of introduction of lessepsian immigrants in Turkey: ecological consequences, economical consequences, economical and ecological consequences (Turan, 2010).

### **1.1 Impacts of Lessepsian puffer fish species on the Mediterranean fisheries**

In the Mediterranean, this invasive species, is being caught as by-catch in relatively significant numbers, has no actual economic value and is therefore directly discarded at sea (EastMed 2010a). It was concluded that this fish has been able to successfully establish itself due to its rapid growth, reproduction at an early age, adaptation ability, absence of predators and/or competitors, and the fact that it is not a targeted species. It is agreed that the socio-economic impact of this alien species on the local fisheries of the Eastern Mediterranean countries is significant. Complaints from local fishers have become frequent in Egypt, Lebanon, Cyprus, Turkey and Greece amongst others where the destruction of nets due to entangling or to predation by *L. sceleratus* on already entangled fish is common (Kalogirou, 2010).

*L. sceleratus* is considered a major nuisance by fishers since it damages fishing gear by attacking fish caught in nets and lines, along with reducing local stocks of squids

and octopus through predation (Figure 1-2). This species can easily cut lines and nets using its strong teeth. Fishers have had to change their methods and gears of fishing and even their fishing grounds in order to reduce the negative impacts of this invasive on their livelihoods (EastMed 2010b). In addition, puffer fish species can accumulate a high amount of the fatal neurotoxin, tetrodotoxin (TTX).

## **1.2 Tetrodotoxin**

Tetrodotoxin (TTX) is water soluble, heat resistant, and it can be absorbed through mucous membranes and the small intestine, and also is a well-studied neurotoxin known for its distribution in pufferfish (Yokoo, 1950) and marine invertebrates such as snails (Noguchi *et al.*, 1981), crabs (Yasumura *et al.*, 1986), starfish (Maruyama *et al.*, 1985), blue-ringed octopus (Sheumack *et al.*, 1978) and sea slugs (McNabb *et al.*, 2010). Generally, TTX blocks the voltage-gated sodium ion channels, incapacitating nerve conduction and muscle action potentials, causing progressive paralysis and death due to failure of the respiratory system (Kao *et al.*, 1963; Moore *et al.*, 1966; Puilingi *et al.*, 2015). The minimum lethal dose and minimum acute dose of TTX to human (wt. 50 kg) are estimated to be around 2 mg and 0.2 mg, respectively. Depending upon the amount of the toxin ingested, symptoms usually appear within 10–45 min of exposure, though some cases have reported being asymptomatic until as much as 3–6 h after exposure. Oral paresthesia is usually the initial symptom, which gradually spreads to the extremities and trunk. Other early symptoms include taste disturbance, dizziness, headache, diaphoresis, and pupillary constriction. These may or may not be accompanied by gastrointestinal symptoms of salivation, hypersalivation, nausea, vomiting, hyperemesis, hematemesis, hypermotility, diarrhea, and abdominal pain (Noguchi and Ebesu, 2001). TTX, as the primary agent of pufferfish poisoning, is reported to be produced by intestinal bacteria of TTX-bearing animals and other marine bacteria (Hashimoto *et al.*, 1990). The main

mechanism of TTX accumulation in puffer fish was determined as being via a food web (Noguchi *et al.*, 2006) (Fig. 1-2). In last decades, various TTX analogues has been established (Fig. 1-3) which can be further classified into (1) hemilactal type analogues, (2) 5-deoxy-10,7-lactone type analogues, (3) 4,9- and 4,4a-anhydro type analogues and (4) tetrodonic acid type analogue (Yotsu-Yamashita *et al.*, 2001 and 2013).

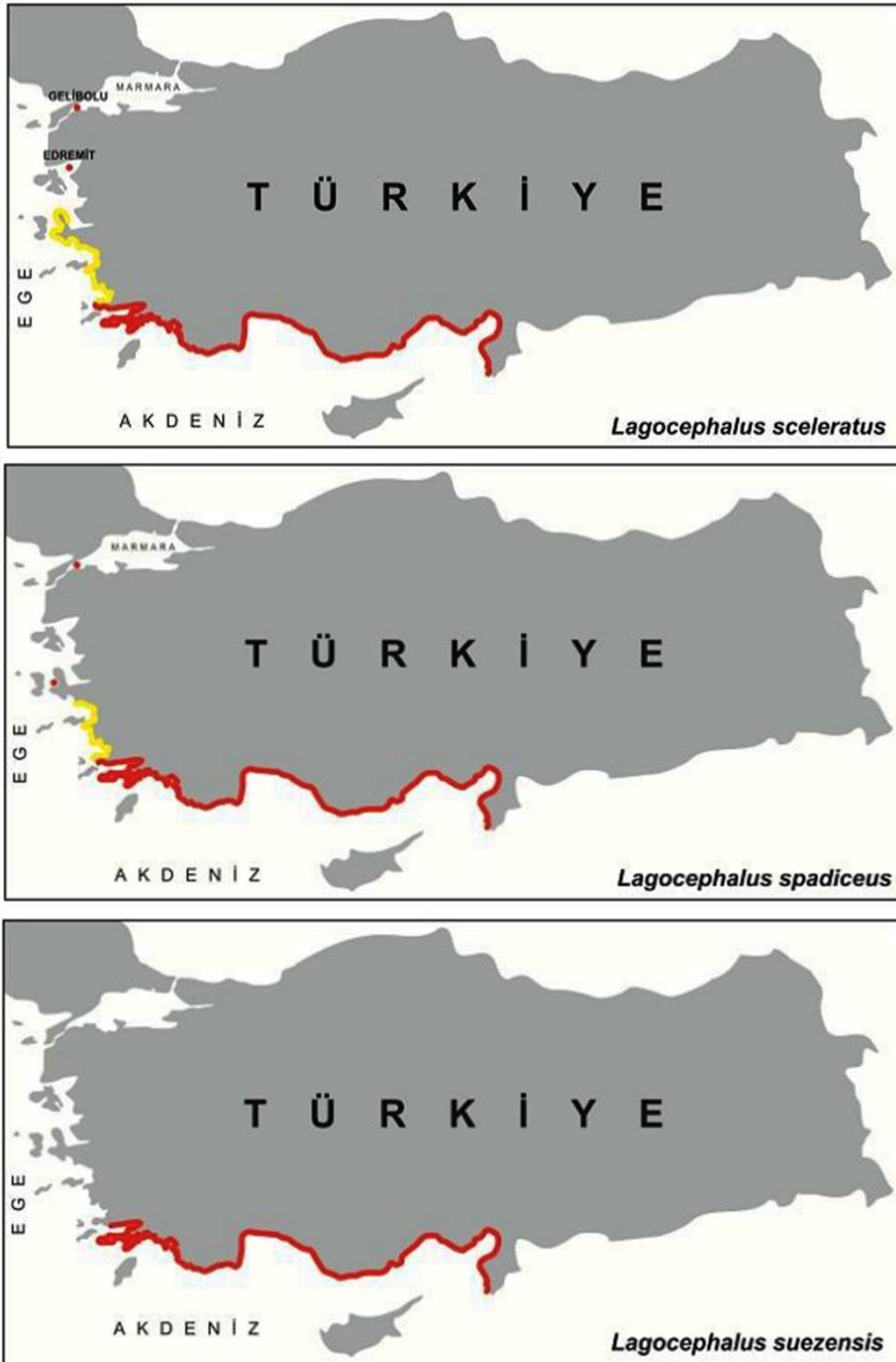
### **1.3 Impacts of Lessepsian puffer fish species on public health**

In the Mediterranean, several cases of poisoning have been recorded as *L. sceleratus* is marketed regardless of the risk it poses to public health. Its large size might be one of the reasons behind this species being sold. There have already been 13 recorded cases of death in the Eastern Mediterranean as well as other cases of intoxication (Chamandi *et al.*, 2009; Kalogirou, 2010). From the time *L. sceleratus* settled in the Mediterranean, it is being sold and consumed in Egypt, where it is now considered a delicacy irrespective of its ban by Egyptian law. In Turkey, where landings have also been banned, fishers still sell it illegally and Turkish fishers readily consume it (Aydin 2011). Moreover, this species is also consumed in Lebanon by some fishers and a small number of consumers ignorant of the health threats it poses where several cases of unofficial intoxication have been reported in that country after eating *L. sceleratus*. The only official record was in 2008 when a 68 year old woman complaining of limb weakness and dyspnea was brought to a hospital in Beirut. The family revealed after questioning that she had eaten a half-cooked liver of *L. sceleratus* (Chamandi *et al.*, 2009). Even though in that particular case the woman survived, the local media records seven cases of death in the past few years in Lebanon due to consumption of puffer fishes. As a result, the Lebanese authorities banned in 2011 the fishing, selling and consuming of all puffer fishes including *L. sceleratus*. Fish consumers in the

eastern Mediterranean are becoming increasingly concerned about the availability of those fishes in the market since it is hard to separate small-sized individuals from other commercial species of the same size resulting in accidental consumption.

### **Objectives and outline of thesis**

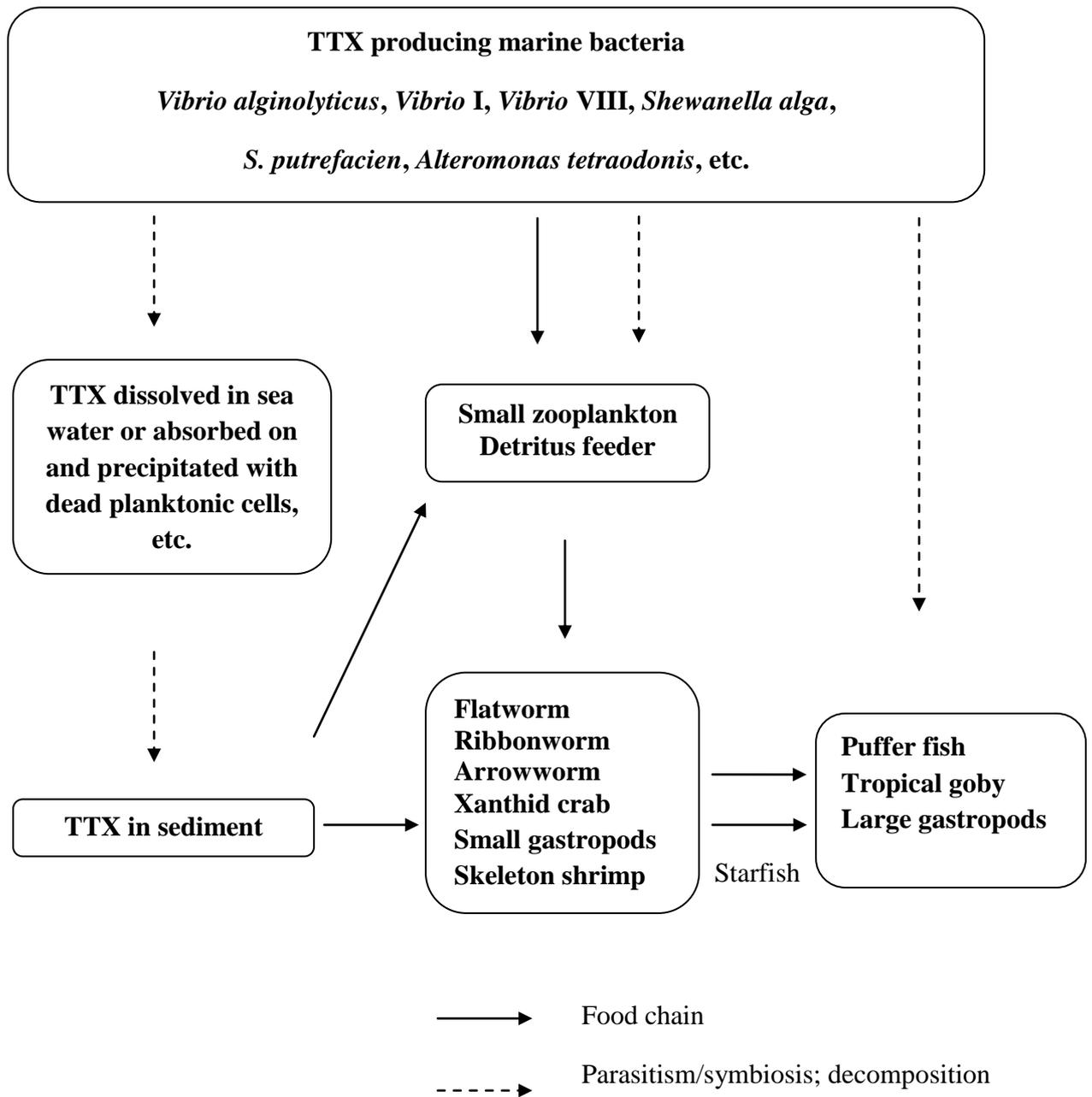
- 1-** The current situation and impacts of Lessepsian puffer fish species on the Mediterranean fishery was explained and potential harmful effects were clarified after the determination of toxicity.
- 2-** The evolutionary origin of three Lessepsian species were determined after the sequencing of complete mtDNA of these species, differentiation was clarified by molecular phylogeny. And also, phylogeny of genus *Lagocephalus* re-constructed with other members of family Tetrodontidae.
- 3-** The quick identification method was constructed in order to authenticate, eliminate or detect the labeling issues from seafood market. The method described in this study, will provide a great advantage for the local authorities and consumers.
- 4-** Finally, the accumulation mechanism of TTX in *L. sceleratus* was studied. One of the TTX binding protein was cloned and characteristics of this protein was discussed within the same protein family. The recombinant expression was carried out in *E. coli* protein expression system and the binding ability of this protein with TTX was determined in order to better understanding of the accumulation mechanism of TTX.



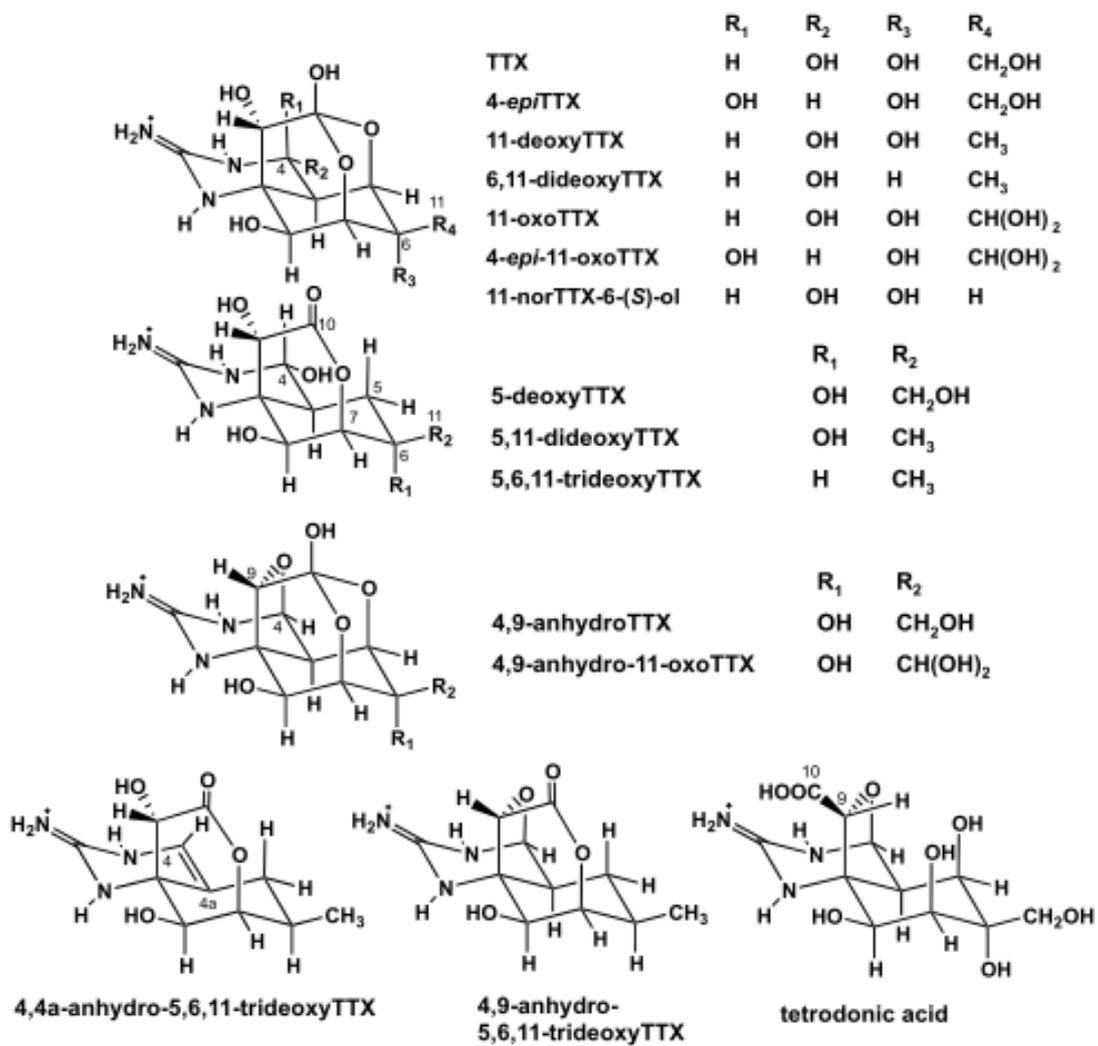
**Fig. 1-1** Distribution maps of three Lessepsian puffer fish species along the Turkish Mediterranean coasts (Irmak, 2012). Red, yellow and red dots indicate wide distribution, rare distribution and local records of three puffer fish species, respectively.



**Fig. 1-2** Impacts of Lessepsian fish species on the eastern Mediterranean fisheries. A: Damaged nets and long lines and caught Lessepsian fish species (Turan, 2010); B: Fishing hook and nets found in the stomach of *L. sceleratus* and attacked fishes by *L. sceleratus* (Irmak, 2012).



**Fig. 1-3** Proposed mechanism of TTX accumulation in marine animals (Noguchi *et al.*, 2006)



**Fig. 1-4** The structures of TTX and its analogues (Puilingi *et al.*, 2015).

## Chapter 2

### *Confirmation and comparison of toxicity of *Lagocephalus sceleratus* caught from Turkish Mediterranean coasts and Okinawa Island, Japan*

---

#### **2.1 Introduction**

*L. sceleratus* was first collected in the Mediterranean Sea on February 2003 from Gokova Bay (Northeren Mediterranean Sea, Turkey) (Akyol *et al.*, 2005), and on November 2004 from Jaffa along the Israeli coast (Golani and Levy, 2005). In Greek waters, *L. sceleratus* was first recorded from the Cretan Sea (Aegean Sea) in July 2005 (Kasapidis *et al.*, 2007). Since then, *L. sceleratus* has been recorded with an increasing frequency, in many areas of Mediterranean Sea and for this reason it is considered as one of the faster expanding Lessepsian immigrants. This fast expansion rate indicates a better ability to adapt to different environmental conditions and may also affect diversity and/or abundance of native species in the near future (Peristeraki *et al.*, 2006). The collection of numerous juvenile *L. sceleratus* fish, together with picarel, bogue and smelt in islands of the Southeast Aegean Sea, which resulted in confusion to both fishermen and consumers was one of the most important incidents reported. A wide range of specimens of different sizes have also been caught, indicating that these species are well adapted to the area. *L. sceleratus* is regarded to be among the worst invasive species in the Mediterranean Sea with a

significant impact on the surrounding ecosystem and on the fisheries sector. This increased publicity has resulted in familiarization of the fishermen with the species' characteristics, and subsequent contribution to over-reporting, since all specimens are being delivered to local authorities.

*Lagocephalus sceleratus* is another Lessepsian puffer species, which is originally from the Indo-Pacific region and a source of food poisoning with a high associated risk of mortality, as it commonly accumulates TTX. (Zenetos *et al.*, 2005; Peristeraki, 2006; Streftaris and Zenetos, 2006; Ozturk, 2010). Several cases of intoxication have been reported following the consumption of *L. sceleratus* from the eastern Mediterranean (Bentur *et al.*, 2008), 13 cases of death were also recorded in the Eastern Mediterranean also as well as other cases of intoxication (Chamandi *et al.*, 2009; Kalogirou, 2010). The toxicity has also been determined in specimens obtained from the Aegean sea and Egypt (Sabrah *et al.*, 2006; Katikou *et al.*, 2009; Ali *et al.*, 2011). Hereby, the current European legislative requirements are banned the (Regulation 853/2004/EC; Regulation 854/2004/EC) poisonous fish of the family Tetraodontidae and products derived from them and those type of products must not be placed on the market. In accordance with the present Turkish Commercial Fisheries Notification 3/1 (RG-17/5/2013-28650), *L. sceleratus* and *L. spadiceus* must not be landed and sold on to market because of their toxicity.

Therefore, in this chapter toxicity of *L. sceleratus* caught in Turkish coasts was determined, toxin profiles were clarified and toxicity was compared with *L. sceleratus* specimens caught in Okinawa Island, Japan.

## **2.2 Materials and methods**

### **2.2.1 Samples and materials**

Twenty specimens of *L. sceleratus* caught by longline fishing in the Marmaris and Iskenderun Bay, Turkey on September 2012, and April and May 2013. All specimens were immediately placed on ice, frozen, and transported to Japan. And also twelve specimens of *L. sceleratus* caught by longline fishing in the Okinawa Island, Japan on June 2015 and transported to Laboratory of Marine Biomaterial and Functional Biochemistry. All of specimens kept frozen at -70 °C prior to use. The pictures of studied specimens are provided in Fig. 2-1.

### **2.2.2 Toxin extraction**

All specimens were partially thawed and dissected into six parts: muscle, liver, gonad, kidney, intestine and skin. The tissues were homogenized with 0.1% acetic acid, and TTX was extracted after heating in a boiling water for 10 min (Kodamo and Sato, 2005). Acidic tissue extracts were ultra filtered through a 3K Amicon® Ultra Centrifugel Device (MWCO 3000). The filtrate was used as the sample solution for liquid chromatography/tand mass spectrometry (LC-MS/MS), liquid chromatography/electron spray ionization-mass spectrometry (LC-ESI/MS) and HPLC-post column derivative fluorescent detection analysis.

### **2.2.3 LC-MS/MS and LC-ESI/MS analysis**

LC-MS/MS was performed as reported previously (Nakagawa *et al.*, 2006). Briefly, the LC was operated with the Waters Acquity™ Ultra Performance LC pump system equipped by a TSKgel Amide-80 column (2.0-i.d. x 150 mm, 3 μm, Toso, Tokyo, Japan). The mobile phase was an aqueous solution containing 16 mM ammonium formate buffer

(pH 5.5) and acetonitrile (4:6, v/v) and eluted at a flow rate of 0.2 mL/min at 25 °C. The mass spectrometer was operated in MRM, detecting in positive ion mode, and two product ions,  $m/z$  162 and  $m/z$  320 were detected. The LC-ESI/MS was performed under the same conditions as that used for the LC-MS/MS with an exception of MRM mode, single ion monitoring (SIM) was used (Nagashima et al., 2011). The ions  $m/z$  320 for TTX,  $m/z$  302 for 4,9-anhydroTTX,  $m/z$  304 for 5-deoxyTTX and 11-deoxyTTX,  $m/z$  290 for 11-norTTX-6,  $m/z$  288 for 6,11-dideoxyTTX, and  $m/z$  272–162 for 5,6,11-trideoxyTTX were detected in SIM mode. The analytical conditions of LC-MS/MS for TTX shown in Table 2-1.

## **2.3. Results and discussion**

### **2.3.1. Toxicity of *Lagocephalus sceleratus***

Toxicity of the puffer fish *L. sceleratus* is shown in Table 2-2. All of specimens examined in this study showed toxicity. TTX contents were individual and tissue dependent. In general, testis, muscle and skin tissues contained less toxicity comparing with the ovary and other internal organs. Similar toxin distribution among tissues of *L. sceleratus* were previously reported (Kano et al., 1984; El-Sayed et al., 2003; Sabrah et al., 2006; Katikou et al., 2009; Ali et al., 2011; Rodríguez et al., 2012).

The female specimen, No: 9 (body weight 1210 g, body length 44.0 cm) collected from Marmaris Bay, Turkey showed the highest toxicity among other specimens from the Mediterranean Sea. The ovary was the most toxic as high as 80.0 µg TTX/g. The most toxic fish were found to be in their spawning stage during the months of April, May, and June between 2002 and 2003 from the Gulf of Suez and 24% of specimens were strongly toxic (>1000 MU/g tissue) (Sabrah et al., 2006). The minimum lethal dose of TTX for humans is estimated to be approximately 10000 MU (≈2 mg) (Noguchi and Ebesu, 2001; Noguchi and Arakawa, 2008). The mass chromatograms and organ distribution of toxins in these

Lagocephalus species from Okinawa Island, Japan, were very similar to those from the Turkish coasts. However, the male specimen, No: 31 was detected as a strongest TTX containing sample in the liver (109.63  $\mu\text{g TTX/g}$ ) among the specimens collected from Okinawa Island, Japan. The accidentally consumption of 25 g of ovary of specimen 9 or liver of specimen 31 would be enough to cause death humans. Itoi *et al.*,(2012) reported that the amount of toxin in the ovary was significantly higher than in the testes (non-toxic) from *Takifugu niphobles* during spawning after the detection of the immunoreactivity of TTX against anti-TTX monoclonal antibody from various tissues. The second highest toxicity detected from the male specimen, No: 19 which caught from Iskenderun Bay. The intestine was the most toxic as high as 48.8  $\mu\text{g TTX/g}$ , followed by the kidney (34.0  $\mu\text{g TTX/g}$ ), and the liver (25.4  $\mu\text{g TTX/g}$ ), estimated as ‘strongly toxic levels’. It is notable that the flesh and testis of specimen 19 and 31 had detectable amounts of TTX at 3.4, 7.20, 10.15 and 2.6  $\mu\text{g TTX/g}$ , respectively. Katikou *et al.*, (2009) reported similar toxicity from muscle tissue (10.16  $\mu\text{g TTX eq/g}$ ) of *L. sceleratus* caught in the Aegean Sea.

LC-ESI/MS profile of the ovary extract of specimen no. 16 (collected from Iskenderun Bay, Turkey) is shown in Fig. 2-2. The mass chromatograms were scanned at SIM as described above. In the selected ion mass chromatogram at  $m/z$  320, the peak at a retention time of 16.05 min was consistent with that of TTX standard at a retention time of 16.38 min (Figure 2-2, the bottom). The peaks at a retention time of 7.66 min at  $m/z$  272 and that of 12.39 min at  $m/z$  302 were estimated to be trideoxyTTX and anhydroTTX; respectively, although TTX analogues could not be identified and amount could not be estimated because of lack of the standard of TTX analogues. Total ion current mass chromatogram demonstrated that trideoxyTTX was a major toxic substance in the ovary extract (Figure 2-2, the top). Jang *et al.*, (2010) reported that TTX, 5,6,11-trideoxyTTX and 4,9-anhydroTTX showed daughter ion at  $m/z$  162 after multiple reaction monitoring mode

(MRM). Thus, TTX and analogues were confirmed after MRM scan (Fig. 2-3).Rodríguez *et al.*, (2012) noted that 5,6,11-trideoxyTTX was the major TTX analogue in *L. sceleratus* specimens and followed by 11-deoxyTTX and 11-norTTX-6(S)-ol. While, TTX was the second main substance after the 5,6,11-trideoxyTTX in this study. Similar toxin profile was reported in other puffer fish species as the Japanese *Fugu pardalis*, in which the non toxic 5,6,11-trideoxyTTX was the major toxin, while 11-deoxyTTX and 5-deoxyTTX were only detected in the ovaries and liver with 10% and 1% of TTX, respectively (Jang and Yotsu-Yamashita, 2006). These deoxy analogues were also present in the Korean *Fugu niphobles* and in *T. nigroviridis* and *T. biocellatus* but in lower amounts than TTX and 5,6,11-trideoxyTTX (Jang, *et al.*, 2010). In addition, 11-deoxyTTX and 4,9-anhydroTTX were detected in the Bangladesh *Takifugu oblongus*, mostly in the ovary, even though 5,6,11-trideoxy was the toxin dominant in this organ (Diener, *et al.*, 2007). Data published so far suggest that the deoxy analogues of TTX are commonly present in puffer fishes, although the different ratios between them are specific of the species and/or of the regions where the fish are collected. For this reason, it is possible that the relation of toxins in the puffer fish *L. sceleratus* was different (Rodriguez *et al.*, 2012). So, 5,6,11-trideoxyTTX was the main toxin and followed by TTX. Further studies would be needed to verify if the toxin profile of the *L. sceleratus* caught in Turkish waters is similar to that obtained here or if instead other TTX related substances are detected in this species.

### **2.3.2 Conclusion**

The concentrations of TTX and its analogues in the ovary was generally higher in *L. sceleratus* both from the Marmaris and Iskenderun Bay, Turkey and Okinawa Island, Japan, and relatively lower in the liver, testis, intestine, and flesh. Because of high concentration of TTX in the ovary and liver of these two species from the Turkish coasts (80.0 and 25.4 µg/g at highest) and Okinawa Island, Japan (42.98 and 109.63 µg/g at highest), their consumption

should be cause to fatal poisoning. 11-OxoTTX, which is considered a more potent analogue than TTX, was not detected from any of examined specimens. In this chapter, the toxicity of *L. sceleratus* confirmed and quantified. There was no significant differences of the toxicity between the samples caught from Turkish and Japanese waters. Although, a few samples showed strong toxicity, the potential hazardous of this species confirmed and it is needed to eliminate from the retail seafood market.

**Table 2-1.** The analytical conditions of LC-MS/MS for TTX quantification.

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Apparatus: Waters Aquality UPLC LC-MS system

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LC conditions

Column	TSKgel Amide-80 column( $\Phi$ 2.0 x 150mm、3 $\mu$ m、Tosoh)
Mobile phase	16mM Ammonium formate(pH5.5):acetonitrile(4:6、v/v)
Flow rate	0.2 ml/min
Column temperature	25 °C
Injection volume	10 $\mu$ l

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MS conditions

Ionization mode	Electrospray ionization、positive ion mode
Detective mode	Selected ion recording
Capillary voltage	3.50 kV
Cone voltage	48 V
Source temperature	120 °C
Desolvation temperature	350 °C
Cone gas (N <sub>2</sub> )	50 l/h
Desolvation gas (N <sub>2</sub> )	600 l/h

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**Table 2-2.** Toxicological results of *Lagocephalus sceleratus* specimens (TTXequivalents expressed as  $\mu\text{g/g}$ ). Samples collected from the Mediterranean Sea are boxed.

Sample No	Date of sampling	Sex	Body weight (g)	Body length (mm)	Toxicity ( $\mu\text{gTTX/g}$ )					
					Muscle	Liver	Gonad	Kidney	Intestine	Skin
<u>Samples collected from Marmaris Bay, Turkey</u>										
1	May, 2013	M	308	265	0.35	0.41	0.46	0.25	1.7	0.41
2	April, 2013	M	255	244	0.35	0.40	0.40	0.53	1.64	0.45
3	May, 2013	M	248	247	0.16	0.13	0.26	0.21	0.16	0.21
4	May, 2013	F	193	221	0.62	1.61	1.65	0.82	1.12	1.28
5	May, 2013	M	155	199	0.64	0.76	0.76	0.56	0.59	0.80
6	May, 2013	M	145	196	1.46	3.40	1.59	1.75	1.15	2.08
7	May, 2013	M	980	405	0.79	0.84	0.75	0.90	1.00	0.80
8	May, 2013	N/A	220	260	0.77	0.90	N/A	0.86	1.10	0.91
9	April, 2013	F	1210	440	0.29	2.18	80.0	1.77	0.62	0.52
10	April, 2013	F	750	405	0.10	0.33	27.6	0.51	0.85	0.35
11	April, 2013	M	625	360	0.07	0.12	0.19	0.09	0.13	0.10
12	September, 2012	M	335	290	0.21	0.19	0.19	0.22	0.32	0.24
13	September, 2012	N/A	274	280	0.29	0.69	0.83	0.40	0.57	0.25
14	September, 2012	M	231	270	0.24	0.33	0.73	0.21	0.32	0.15
15	September, 2012	M	273	275	0.26	0.25	0.34	0.16	0.18	0.26
<u>Samples collected from Iskenderun Bay, Turkey</u>										
16	May, 2013	F	1965	450	0.17	0.23	7.56	0.29	0.30	0.37
17	May, 2013	M	1670	430	0.14	0.61	0.30	1.99	0.77	0.33
18	May, 2013	M	1220	385	0.18	0.17	0.17	0.16	0.17	0.30
19	May, 2013	M	1380	480	3.42	25.4	2.58	34.0	48.8	3.30
20	May, 2013	M	1300	440	0.76	0.75	0.73	0.74	0.76	0.76
<u>Samples collected from Okinawa Island, Japan</u>										
21	June, 2015	F	4700	580	N/A	N/A	0.48	N/A	N/A	0.13
22	June, 2015	F	5400	660	0.05	0.15	2.53	0.35	0.26	0.21
23	June, 2015	F	4400	600	N/A	N/A	0.22	N/A	N/A	N/A
24	June, 2015	M	1900	510	0.14	0.45	0.25	0.38	0.42	0.16
25	June, 2015	F	4900	620	N/A	N/A	3.26	0.06	0.02	0.17
26	June, 2015	M	2100	520	N/A	0.20	0.07	0.16	0.17	0.09
27	June, 2015	F	950	430	0.37	1.70	6.60	0.76	0.84	0.45
28	June, 2015	M	2000	520	0.10	3.50	0.17	1.22	0.53	0.20
29	June, 2015	F	3800	650	0.42	3.12	9.01	0.54	1.11	0.47
30	June, 2015	F	4100	720	1.15	1.08	42.98	1.59	1.33	1.78
31	June, 2015	M	2350	530	187.20	109.63	10.15	7.45	13.90	4.13
32	June, 2015	M	2000	610	2.70	9.54	2.11	5.49	11.89	6.18

**A**

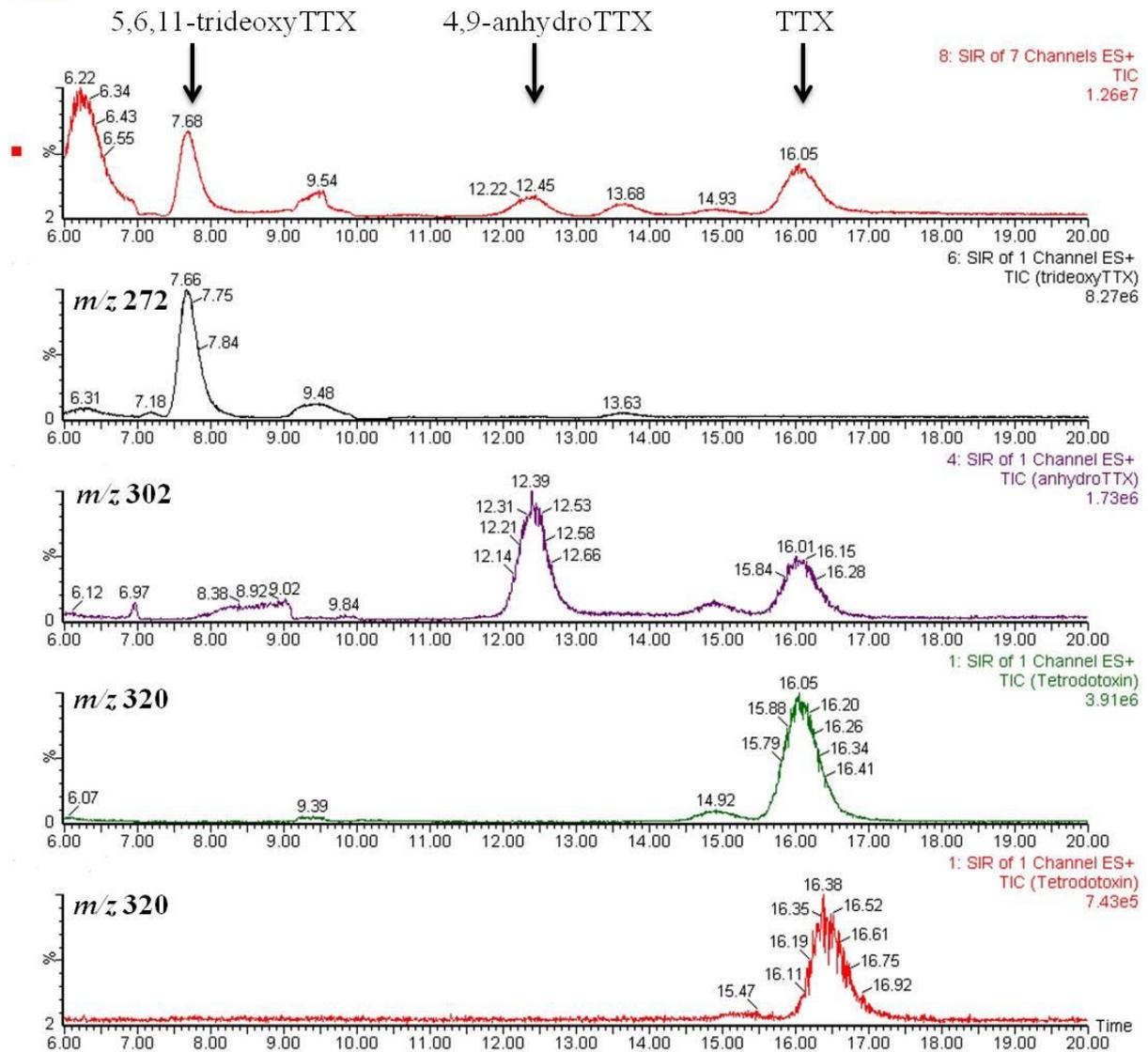


**B**



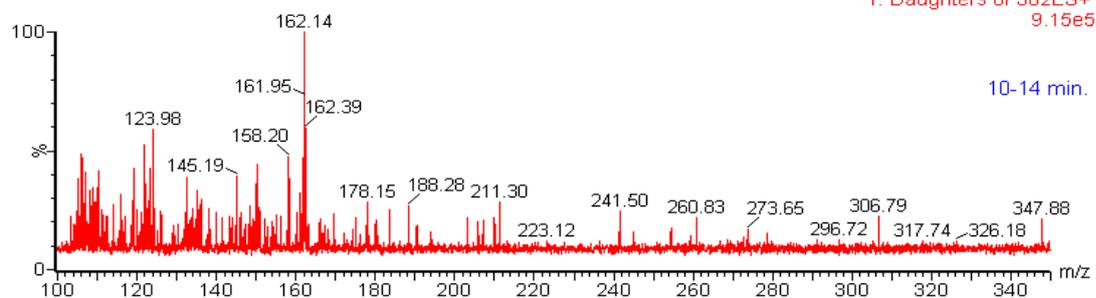
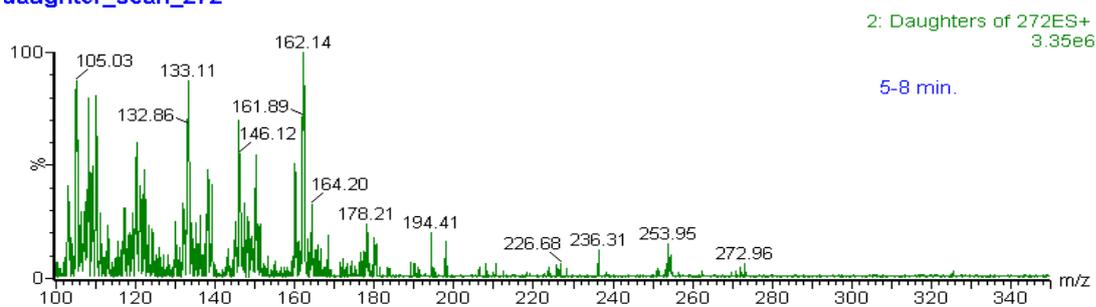
**Fig. 2-1** The examined *L. scleratus* specimens in this study. A: Specimens were caught in Marmaris Bay, Turkey; B: Specimens were caught in Okinawa Island, Japan.

Ovary

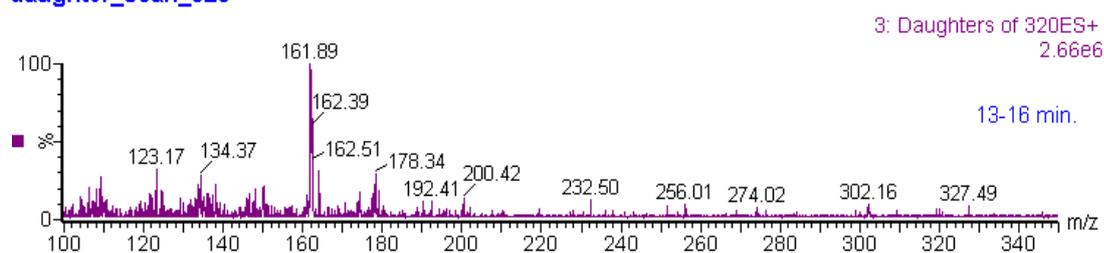


**Fig. 2-2** LC-ESI/MS profile of TTX standard (bottom) and TTX extract of ovary sample of specimen no. 16. TTX and analogues are marked with arrows on the top of the peaks.

daughter\_scan\_272



daughter\_scan\_320



**Fig. 2-3.** MS/MS fragmentation pattern of TTX and analogues ( $m/z$  272,  $m/z$  304 and  $m/z$  320).

# Chapter 3

## *Determination and characteristics of the complete mitochondrial DNA of Lagocephalus scleratus, L. suezensis and L. spadiceus species and their phylogeny within the family Tetraodontidae*

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### 3.1 Introduction

Puffer fishes of the family Tetraodontidae usually occur in shallow warm, tropical seas, and freshwaters of the world, although some species may be found at depths over 350 m (Matsuura, 1982; Tyler and Matsuura, 1997). They differ externally from other families of the order Tetraodontiformes by the following combination of characters: head large and blunt; jaws modified to form a beak of four heavy, powerful teeth, two above and two below; eyes high on head; gill opening, a simple slit in front of pectoral fins; dorsal and anal fins located far posteriorly, containing seven to 15 soft rays; caudal fin truncate, rounded, or emarginate to somewhat lunate; pelvic fins absent; lateral line (when present) often indistinct, forming an interconnected pattern on sides of the head and body, but quite distinct in some genera (e.g., *Lagocephalus* and *Torquigener* Whitley 1930c); typical scales absent, but many spinules often present on back and/or belly, and sometimes on sides (Matsuura, 2001). The Tetraodontidae is the most speciose family in the Tetraodontiformes, including 184 species. Consequently, they have been poorly studied

and taxonomic confusion remains. The correct interpretation of any kind of comparative biological data requires an evolutionary frameworks (Matsuura, 2015).

The mitochondrial DNA (mtDNA) of vertebrates is a self-replicating, approximately 15–20 kb long, circular duplex molecule. It usually encodes for 13 proteins, 22 tRNAs, and two rRNAs. In addition, most vertebrate mtDNAs examined have a single, large, noncoding region, highly variable in size among same or different lineages, that contains signals for its replication and transcription (Boore, 1999). Recently, large numbers of complete mtDNA sequences of fish species including puffer fish can be obtained from NCBI Genbank database.

Mitochondrial DNA represents a useful marker system for use in population and phylogenetic studies due to its maternal mode of inheritance and relative lack of recombination. It also has an effective population size that is one-quarter that of single-copy nuclear DNA, making it more likely to exhibit differentiation due to effects of genetic drift. Puffer fishes have a compact genome size compared to other vertebrates. Thus, two puffer fishes, *Takifugu rubripes* and *Tetraodon nigroviridis*, have been used as a model of the evolution of vertebrate genomes (Brenner et al., 1993; Crnogorac-Jurcevic *et al.*, 1997). Furthermore, mitochondrial (mt) genomes of the genus *Takifugu* were applied to a model system of evolutionary biology (Yamanoue *et al.*, 2009), and freshwater invasions by puffer fishes were clarified from a mitogenomic perspective after the complete mitochondrial nucleotide sequence of family Tetraodontidae was analyzed (Yamanoue *et al.*, 2011). Lastly, the effect of habitat shifts on teleost fish diversity was investigated after the molecular phylogeny of eight different genes of puffer fishes were analyzed (Santini *et al.*, 2013).

Although the taxonomy of puffer fishes has been progressing, the family Tetraodontidae has been poorly studied and taxonomic confusions has never been comprehensively reviewed (Matsuura, 2015).

*L. sceleratus*, *L. suezensis*, and *L. spadiceus* are most abundant Lessepsian puffer fish species in the eastern Mediterranean biota, which are originally migrated from Indo-Pacific. The information on *Lagocephalus* species from the Mediterranean Sea, especially for mtDNA and phylogenetic relationships are lacking. On the other hand, classification at the species level in *Lagocephalus* species has not yet been studied in depth, causing confusion in the status of several species (Matsuura *et al.*, 2011).

Therefore, in this study, firstly the complete mtDNA sequences of three Lessepsian puffer fish species, *L. sceleratus*, *L. suezensis*, and *L. spadiceus* were determined. The relationship between morphological and molecular biological aspects was also analyzed. Furthermore, the structure, characteristics, and the differentiation of mt genes among the species were determined. Lastly, the phylogeny of 35 puffer fish species were re-constructed according to their mtDNA protein-coding genes.

## **3.2 Materials and methods**

### **3.2.1 Samples and materials**

Specimens of *L. sceleratus* and *L. suezensis* were collected from Marmaris Bay, Turkey, while a specimen of *L. spadiceus* was collected from Gökova Bay, Turkey. All specimens were immediately placed on ice, frozen, and transported to Japan. Specimens were kept at  $-70^{\circ}\text{C}$  until DNA extraction. Fish then were identified by morphological characteristics as previously reported (Bilecenoglu *et al.*, 2002; Akyol *et al.*, 2005; Tuncer *et al.*, 2008). The information of the studied species as well as their pictures are provided in Fig. 3-1.

### 3.2.2 Total DNA extraction

Total DNA from three species was extracted using TNES–urea buffer (8 M urea, 10 mM Tris–HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, and 1% SDS) (Asahida *et al.*, 1996). Briefly, 50 mg of muscle tissue from each individual was homogenized in 500  $\mu$ L TNES-urea buffer containing 20  $\mu$ L Proteinase K (20 mg/mL). After the mixture was incubated at 55°C for 8 h, 500  $\mu$ L of Tris buffer-saturated phenol was added and the mixture was centrifuged at 1250 g for 15 min at 20°C. The supernatant was transferred to a new tube and phenol extraction was repeated to improve sample purity. Then, 500  $\mu$ L of a cold mixture of chloroform/isoamyl alcohol (24:1) was added and the mixture was centrifuged at 1250 g for 15 min at 20°C. The supernatant was transferred to a new tube, and 50  $\mu$ L of 3M sodium acetate (NaOAc) (pH 7.0) and 1000  $\mu$ L of cold 100% ethanol were added. After precipitation at –20°C for 8 h, the mixture was centrifuged at 1250 g for 15 min at 20°C to pellet the total DNA. Finally, the pellet was washed with 75% chilled ethanol, dried, and re-suspended in 200  $\mu$ L TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). Total extracted DNA was kept at 4 °C until use.

### 3.2.3 Design of PCR primers

Seven pairs of PCR primers were constructed to amplify overlapping segments of the entire mitogenomes of three *Lagocephalus* species after the alignment of well-known puffer fish mt genomes as well as on the partial results of ongoing sequencing. The PCR amplification region and sequencing strategy for the detection of complete mtDNA genome are shown in Fig. 3-2 and list of primers are provided in Table 3-1. 100  $\mu$ M primers were purchased from Operon, and an aliquot was further diluted with TE buffer to a concentration of 20  $\mu$ M. The stock primers (100  $\mu$ M) were kept at –25 °C while low-concentration primers (20  $\mu$ M) were used for PCR amplifications and kept at 4 °C.

### **3.2.4 PCR amplification and electrophoresis**

Seven fragments were amplified using *Ex Taq*® DNA polymerase (Takara, Tokyo, Japan). Each PCR was carried out in a volume of 50 µL containing 25–75 ng of template DNA, 5 µL of 10× PCR buffer containing 25 mM MgCl<sub>2</sub>, 4 µL of dNTPs (10 mM), 20 µM of each primer, and 0.4 µL of *Ex Taq*® DNA polymerase. The PCR amplification was carried out in a Veriti®-200 Thermal Cycler (Applied Biosystems) under the following conditions: 30 cycles of 10 s at 98°C for denaturation, 60–64°C for annealing, and 1–4 min at 72°C for extension, and a final extension at 72°C for 10 min. PCR products were stored at 4 °C until use.

Agarose gel electrophoresis was used to evaluate PCR amplifications. In brief, 5 µl of PCR product was mixed with 1 µl of 6x dye buffer and loaded in 1% agarose gels containing Gel Green™ (Biotium). Electrophoresis was carried on at 100 V for 30 min in a Mupid®-2 plus (Advance, Japan) sub-marine type electrophoresis system and gels were visualized in a ImageQuant™ LAS-4000 mini transilluminator (GE Healthcare).

### **3.2.5 Purification of PCR products**

Double-stranded PCR products were purified with illustra™ ExoProStar™ (GE Healthcare) to remove the unincorporated primers and dNTPS according to manufacturer's instructions.

### **3.2.6 DNA sequencing**

Purified PCR products were subsequently used for direct sequencing with dye-labeled terminators using the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Grand Island, NY, USA). The primers used were the same as those used for PCR amplification, and newly constructed gene-specific internal primers were used. The primer

walking strategy was applied to sequence the entire mt genomes of three *Lagocephalus* species. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were then analyzed using an ABI 3130 automated DNA sequencer (Applied Biosystems, Japan). Sequence data were finally manipulated using SeqEd v1.3 and assembled with Sequencher® v4.8 sequence analysis software (<http://www.genecodes.com>).

### 3.2.7 Genome annotation and phylogenetic analyses

Protein-coding genes (PCGs) and rRNA genes were identified by sequence comparison with the complete mt genomes of puffer fishes deposited in GenBank. The tRNA genes were identified and initially acquired by using both tRNAscan-SE (Lowe and Eddy, 1997) and ARWEN (Laslett and Canback, 2008) search servers. The typical tRNAs, which were not estimated by tRNAscan-SE, were aligned with other puffer fish sequences.

Nucleotide base compositions and base skew values of each PCG in three puffers were calculated with MEGA 6 software (Tamura *et al.*, 2013). All PCGs were aligned at the amino acid level using the default settings in ClustalW (MEGA 6), and the alignments were back-translated to the corresponding nucleotide sequences. Composition skew was calculated according to formulas with an AT skew =  $[A - T] / [A + T]$  and a GC skew =  $[G - C] / [G + C]$  (Perna and Kocher, 1995). Total nucleotides within the A + T-rich region were subjected to Tandem Repeats Finder software (Benson, 1999) and secondary structures of tandem repeats were deduced from the mfold web server (Zuker, 2003) (<http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form>).

The newly determined mt genomes were analyzed together with the available complete mtDNA sequences of 33 other puffer fish species (Table 3-2). Trees were constructed based on the concatenated nucleotide and amino acid sequences. The analytical

analogy, maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) were used to infer phylogenetic trees with 1000 bootstrap replicates. The best substitution model was also determined based on the lowest Bayesian information criterion (BIC) using MEGA 6 with partial deletion. The GTR + G + I and JTT + G + I + F model were the appropriate models for the nucleotide dataset and the amino acid sequence dataset, respectively.

### 3.3 Results and discussion

#### 3.3.1 Mitochondrial genome summary

The complete mt genomes of *L. sceleratus*, *L. suezensis*, and *L. spadiceus* were 16,442, 16,494, and 16,508-bp in length, respectively. The three mitogenomes have been stored in NCBI GenBank database under the following accession numbers: KP013618 (*L. sceleratus*), KP013619 (*L. suezensis*), and KM667972 (*L. spadiceus*). They all displayed 37 typical animal mt genes (13 PCGS, 22 tRNAs, and 2 rRNAs) and a control region (A-T-rich region). The gene order and orientation were similar to those of a typical vertebrate genome (Fig. 3-3). All genes were encoded on the heavy strand (H-strand), except for the ND6 gene and eight tRNAs, and all genes were similar in length to those in other teleosts. Additionally, 11 instances of gene overlap involving 26 bp were observed in all three *Lagocephalus* mitogenomes. These overlaps were located between the ATP8 and ATP6 genes, ND4L and ND4 genes, ND5 and ND6 genes, and between  $tRNA^{Ile}$ ,  $tRNA^{Gln}$ , and  $tRNA^{Met}$ , and  $tRNA^{Thr}$  and  $tRNA^{Pro}$ . The A + T compositions of the three *Lagocephalus* species were normal among the other puffers (A + T > 51%). Nucleotide identity of complete mitogenomes among *Lagocephalus* species are shown in Table 3-3. The nucleotide identity percentage between *L. spadiceus* and *L. wheeleri* was detected the most highest '91.45%' while the nucleotide identity between *L. sceleratus* and *L. suezensis* was 87.17%.

### 3.3.2 Protein-coding genes

The average A + T content of all PCGs was about 50% (Table 3-4). The A + T content of the second codon in PCGs was higher than that of the first and third codons in PCGs (Fig. 3-4A). Similar A + T profiles have been reported in tiger puffer fish (Elmerot *et al.*, 2002). The lowest A + T content was detected in the first codon of the PCGs ATPase6 and ND3, whereas the highest A + T content was observed in the second codon of the Cytb region (Fig. 3-4A). Leu, Pro, Ser, and Thr were the most abundant amino acids in the mtDNA PCGs of these three puffer fish, which comprise approximately 45% of the total amino acids (Table 3-5).

An AT/GC-skew for all codons in each PCG of the three *Lagocephalus* species has been calculated (Table 3-4). The genes encoded by the N-strand and the J-strand showed obvious negative GC-skew values, whereas the third codon positions in most genes showed positive AT-skew values, except for the COII gene (Fig. 3-4B). In general, the AT-skew value was positive or negative for each of PCGs and the AT-skew values showed noticeable variation in both codon positions and genes. The AT-skew of the second codon was negative for all PCGs except for the ND6 region, which was quite different from the first and third codons. The bias against G was particularly remarkable at the third codon and was larger than that in the first and second codons. In contrast, even in the same gene, AT/GC-skew values were different among the three puffer species (Fig. 3-4B). Francino and Ochman (1997) reported that distinct differences in the base composition of the same group of homologous genes mainly arises due to asymmetric mutations during gene replication, and that this phenomenon makes it more vulnerable to DNA damage. Mutation progression is case dependent and similar mutation bias for closely related species can be determined by their mtDNA similarity (Jia and Higgs, 2008). However, how such bias occurred in different codons, and in the same gene in closely related species is still unclear.

The common start codon ATG was observed in all mt PCGs except for the COI gene, which uses GTG rather than ATG (Table 3-6). Stop codons present in PCGs were TAA in the ND1, ATPase8, ATPase6, ND4L, ND5, ND6 (except *L. spadiceus*, which was AGA), and *Cytb* genes, TA in the ND2 and COIII genes, AGG in the COI (except *L. spadiceus*) and ND6 genes, and T in the COII, ND3, and ND4 genes. The use of an incomplete stop codon to terminate protein translation has also been observed in other fish species (Yamanoue *et al.*, 2006; Peng *et al.*, 2006; Kartavtsev *et al.*, 2007 and Wang *et al.*, 2008). The incomplete stop codon would be the modified form of the complete termination signal UAA occurring by polyadenylation after cleavage of the polycistronic RNA (Ojala *et al.*, 1980).

### 3.3.3 Transfer and ribosomal RNA genes

The 22 traditional tRNA genes of three puffer species range from 64 to 76-bp in size, which are sufficient to encode the cloverleaf secondary structure of typical tRNAs, and were determined with to have an A + T content of about 54% (Table 3-4). Their anticodons were identical to those observed from other Tetraodontidae species. With the exception of *tRNA<sup>Ser</sup>* (AGN), which lacks a stable stem-loop structure in the D-loop (DHU arm), all tRNA genes showed the typical cloverleaf secondary structures (Wolstenholme, 1992 and Yamanoue *et al.*, 2006).

Two rRNA genes, 12S rRNA (*rrnS*) and 16S rRNA (*rrnL*), were found to be located between *tRNA<sup>Phe</sup>* and *tRNA<sup>Val</sup>*, and between *tRNA<sup>Val</sup>* and *tRNA<sup>Leu</sup>*. The lengths of the three *rrnS* were 948, 949, and 947 bp respectively, while those three of *rrnL* were 1,673, 1,678, and 1,671 bps. The A + T content of 16S rRNA was slightly higher compared with that of 12S rRNA (Table 3-4). The rRNA mt genome of puffer fish has been widely used for molecular phylogenetic and species identification (Song *et al.*, 2001; Holcroft, 2005; Ishizaki *et al.*,

2006; Alfaro *et al.*, 2007; Reza *et al.*, 2008). Our results also indicate that the mt genes *rrnS* and *rrnL* can be used for species discrimination.

### 3.3.4 A + T rich regions

The A + T-rich regions of *L. sceleratus*, *L. suezensis*, and *L. spadiceus* were located between *tRNA<sup>Pro</sup>* and *tRNA<sup>Phe</sup>*. Total gene clusters were 811, 855, and 879-bp in length, respectively, with a high A + T content of 65.60, 63.86, and 66.10%, respectively (Table 3-4). Clayton (1991) reported that A + T-rich regions have functional importance, although they do not encode any known functional genes. Variation in the cluster size of control regions among these three *Lagocephalus* species was likely due to the variable number of tandem repeats. Furthermore, small numbers of insertions/deletions in the flanking sequences of the repeat region could cause size variations. Conserved structural elements exist in the A + T-rich region due to the control of genome replication and gene expression during transcription (Clayton, 1982; Fernandez-Silva *et al.*, 2003). The consensus pattern motifs found in three *Lagocephalus* species are shown in Fig. 3. While the long polythymine stretch (T-stretch) has been reported in several animal mt genomes, the three *Lagocephalus* species studies here did not contain any poly stretches. Several studies have shown that the A + T-rich region consists of variable polymorphic sites and provides a great opportunity to study population genetics and to reconstruct phylogenies of closely related species (Grabowski *et al.*, 2004; Kim *et al.*, 2007; Wan *et al.*, 2011; Yang *et al.*, 2013). Therefore, the A + T-rich region present in the mt genome of the three puffer fishes could be used for phylogenetic analyses, population genetics, and species identification of Lessepsian *Lagocephalus* species.

### 3.3.5 Phylogenetic relationships within the family Tetodontidae

Puffer fish species have been widely studied by researchers investigating their morphology, toxicity, mechanism of toxin accumulation, and molecular biology, but their

phylogeny remains an on-going area of research (Yamanoue *et al.*, 2011). The maximum likelihood (ML) and neighbor-joining (NJ) trees showed the same topology. The ML tree (-lnL: 155,770.3566) shown in Fig. 3-5 was constructed by concatenated nucleotide sequences from PCGs while the NJ tree was constructed by concatenated amino acid sequences with all codon positions except ND6 region. The light strand encoded ND6 was not included because of the deviating nucleotide and amino acid composition of this gene compared to the heavy strand encoded genes. Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5408)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 10880 nucleotide and 3445 amino acid positions in the final dataset. The family Tetraodontidae was divided into four major clades. The Tetraodontid species were divided into sixteen genera by their morphology and osteology as proposed by Tyler (1980). The phylogeny was also studied using mitochondrial 12S and 16S rRNA sequences and nuclear RAG1 gene and included 19 puffer fish species (Holcroft, 2005; Alfaro *et al.*, 2007). Yamanoue *et al.* (2011) reconstructed the phylogeny of puffer fish species and four major clades were detected among members of the family Tetraodontidae and the genus *Lagocephalus* was the most basal genus. The genera *Tetraodon*, *Torquigener*, and *Spheroides*, are non-monophylic, although the other genera are monophylic (Fig. 3-5). Santini *et al.* (2013) determined the phylogeny of tetraodontiform fishes after the analyzing of the concatenated dataset of two

mitochondrial and twenty nuclear genes with maximum likelihood and Bayesian analyses and also, 16S rRNA and 15 nuclear markers were used to evaluate fossil tip-dating versus node-age calibrations in tetraodontiform fishes (Arcila *et al.*, 2015). The results of both studies showed high similarities of the phylogeny of family Tetraodontidae. Therefore, results from previous reports are supported by our study. The genus *Lagocephalus* was divided into two sub-groups. *L. sceleratus* and *L. suezensis* were found to be more closely related species, although *L. spadiceus* was located in the second sub-group.

The phylogeny was also constructed using the maximum parsimony (MP) tree with concatenated amino acid sequences (Fig. 3-6). MP trees are based on a simpler hypothesis and the lowest number of changes in the data was sufficient to explain the common ancestor of the characteristics. The MP tree showed similar topography with other ML and NJ trees with one major exception. Although our results and previous reports have showed four major clades in the phylogeny of the family Tetraodontidae, five clades were obtained after MP analysis using the subtree-pruning-regrafting (SPR) algorithm (Nei and Kumar, 2000). *Sphoeroides pachygaster* was separated after the tree re-branched with outlier *Triodon macropterus*. There were no significant differences in the position of Lessepsian puffers.

Single nucleotide polymorphic sites were detected from the entire mt genomes of Turkish and Japanese *L. sceleratus*, although there was no significant differences. Thus, the biogeographical origin of sample could not be discriminated by PCR reaction.

### **3.3.6 Conclusion**

In conclusion, the complete mitogenome sequences of the three Lessepsian puffers' were determined in this chapter, which are the most effective invasive species in eastern Mediterranean biota and fisheries. A comparative analysis of three Lessepsian *Lagocephalus* mitogenomes was performed, and our results show that gene content, gene arrangement, base

composition, codon usage, and RNA and tRNA structures are highly conserved in Tetraodontidae, especially among closely related species. Phylogenetic relationships within Tetraodontiformes that are based on mitogenomic data are consistent with the traditional morphological classification, suggesting that mitogenome sequences are useful for resolving phylogenetic relationships at various taxonomic levels. The sequence trees of concatenated mt PCGs were constructed using different computational algorithms and differences. Since these species are migrants, their molecular relationship with the other puffers needed to be determined. Although their biogeographical origin was Indo-Pacific, the mutation ratio of mt genomes and the molecular phylogeny were not clear. Moreover, these results prove that mtDNA genomes of puffers fish can be used to determine their phylogeny and provide a great advantage for species discrimination.

**Table 3-2.** Fish species studied in this research with NCBI Genbank accession numbers

Species	Accession No.	Species	Accession No.
<b>Family Triodontidae</b>		<i>Lagocephalus wheeleri</i>	AP009538
<i>Triodon macropterus</i>	AP009170	<b><i>Lagocephalus spadiceus*</i></b>	<b>KM667972</b>
<b>Family Tetraodontidae</b>		<b><i>Lagocephalus suzezensis*</i></b>	<b>KP013619</b>
<i>Arothron firmamentum</i>	AP006742	<i>Marilyna darwinii</i>	AP011937
<i>Arothron manilensis</i>	AP011929	<i>Omegophora armilla</i>	AP011936
<i>Auriglobus modestus</i>	AP011917	<i>Polyspina piosae</i>	AP011913
<i>Canthigaster jactator</i>	AP011911	<i>Pelagocephalus marki</i>	AP011938
<i>Canthigaster valentini</i>	AP011912	<i>Sphoeroides annulatus</i>	AP011915
<i>Carinotetraodon lorteti</i>	AP011918	<i>Sphoeroides pachygaster</i>	AP006745
<i>Carinotetraodon salivator</i>	AP011919	<i>Takifugu oblongus</i>	AP009535
<i>Chelonodon patoca</i>	AP009541	<i>Takifugu rubripes</i>	AP006045
<i>Chelonodon pleurospilus</i>	AP011928	<i>Takifugu synderi</i>	AP009531
<i>Colomeus asellus</i>	AP011909	<i>Tetractenos glaber</i>	AP011935
<i>Colomeus psittacus</i>	AP011910	<i>Tetraodon biocellatus</i>	AP011921
<i>Lagocephalus laevigatus</i>	AP011934	<i>Tetraodon nigroviridis</i>	AP006046
<i>Lagocephalus lagocephalus</i>	AP011933	<i>Tetraodon mbu</i>	AP011923
<i>Lagocephalus lunaris</i>	GQ461750	<i>Torquigener brevipinnis</i>	AP009537
<i>Lagocephalus sceleratus</i> (J)	AP011932	<i>Torquigener pleurogramma</i>	AP011926
<b><i>Lagocephalus sceleratus</i> (T)*</b>	<b>KP013618</b>	<i>Tylerius spinosissimus</i>	AP011939

\*Sequences newly determined in this study. ‘J’ and ‘T’ refer to sample collected from Japanese and Turkish coasts, respectively.

**Table 3-3.** Percentage nucleotide identities of complete mitogenomes among *Lagocephalus* species

%	<i>Lsce</i> (J)	<i>Lsue</i>	<i>Lspa</i>	<i>Llag</i>	<i>Lwhe</i>	<i>Llun</i>	<i>Llae</i>
<i>L. sceleratus</i> (T)	99.10	87.17	83.78	81.65	82.37	81.93	80.76
<i>L. suezensis</i>	87.22	100	85.61	82.25	82.96	82.51	81.60
<i>L. spadiceus</i>	83.82	85.61	100	87.86	91.45	83.76	87.54

Note: *Lsce*, *Lsue*, *Lspa*, *Llag*, *Lwhe*, *Llun* and *Llae* refer to *L. sceleratus*, *L. suezensis*, *L. spadiceus*, *L. lagocephalus*, *L. wheeleri*, *L. lunaris* and *L. laevigatus*, respectively. ‘J’ and ‘T’ refer to sample collected from Japanese and Turkish coasts, respectively.

**Table 3-4.** A + T content, AT-skew, and GC-skew of three mitochondrial genomes from *Lagocephalus sceleratus*, *L. suezensis* and *L. spadiceus*

Region	A + T content %			AT-skew			GC-skew		
	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>
Whole genome	51.40	52.64	52.39	0.07	0.06	0.05	-0.29	-0.28	-0.31
Protein-coding	49.87	51.65	51.08	0.03	0.02	0.00	-0.37	-0.35	-0.38
1st codon position	49.46	49.06	48.53	0.10	0.08	0.08	-0.34	-0.32	-0.35
2st codon position	52.81	53.27	53.23	-0.09	-0.10	-0.11	-0.33	-0.31	-0.35
3rd codon position	50.35	52.34	51.47	0.08	0.07	0.05	-0.44	-0.43	-0.44
tRNA genes	53.26	54.20	54.99	0.13	0.13	0.10	-0.12	-0.11	-0.12
16S rRNA	54.39	54.29	53.68	0.25	0.23	0.22	-0.09	-0.09	-0.11
12S rRNA	49.77	49.95	50.48	0.22	0.19	0.22	-0.08	-0.08	-0.13
A + T rich region	65.60	63.86	66.10	0.03	0.04	0.00	-0.18	-0.14	-0.17

Note: *Lsce*, *Lsue* and *Lspa* refer to *L. sceleratus*, *L. suezensis*, and *L. spadiceus*, respectively.

**Table 3-5.** Amino acid composition of protein-coding genes from *Lagocephalus sceleratus*, *L. suezensis*, and *L. spadiceus*

Amino acid	Content (%)		
	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>
Ala	6.46	6.12	6.66
Cys	1.74	1.50	1.61
Asp	2.10	2.05	1.88
Glu	1.99	2.10	1.96
Phe	3.68	4.43	4.07
Gly	4.69	4.51	3.82
His	4.90	4.83	4.61
Ile	4.71	5.03	4.86
Lys	2.97	3.03	3.14
Leu	14.85	15.02	15.33
Met	3.43	2.98	3.30
Asn	3.92	4.02	4.09
Pro	11.58	10.65	11.46
Gln	4.06	3.61	3.68
Arg	3.65	3.55	3.49
Ser	9.15	9.67	9.71
Thr	7.49	7.95	7.34
Val	3.19	3.11	3.03
Trp	2.42	2.51	2.67
Tyr	3.02	3.33	3.27

Note: *Lsce*, *Lsue* and *Lspa* refer to *L. sceleratus*, *L. suezensis*, and *L. spadiceus*, respectively.

**Table 3-6.** The start and stop codons of protein-coding genes from *Lagocephalus sceleratus*, *L. suezensis*, and *L. spadiceus*

Gene	Start codon			Stop codon		
	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>	<i>Lsce</i>	<i>Lsue</i>	<i>Lspa</i>
ND1	ATG	ATG	ATG	TAA	TAA	TAA
ND2	ATG	ATG	ATG	TA	TA	TA
COI	GTG	GTG	GTG	AGG	AGG	TAA
COII	ATG	ATG	ATG	T	T	T
ATPase8	ATG	ATG	ATG	TAA	TAA	TAA
ATPase6	ATG	ATG	ATG	TAA	TAA	TAA
COIII	ATG	ATG	ATG	TA	TA	TA
ND3	ATG	ATG	ATG	T	T	T
ND4L	ATG	ATG	ATG	TAA	TAA	TAA
ND4	ATG	ATG	ATG	T	T	T
ND5	ATG	ATG	ATG	TAA	TAA	TAA
ND6	ATG	ATG	ATG	AGG	AGG	AGA
Cytb	ATG	ATG	ATG	TAA	TAA	TAA

Note: *Lsce*, *Lsue* and *Lspa* refer to *L. sceleratus*, *L. suezensis*, and *L. spadiceus*, respectively.



*Lagocephalus sceleratus* (Silver-cheeked toadfish)

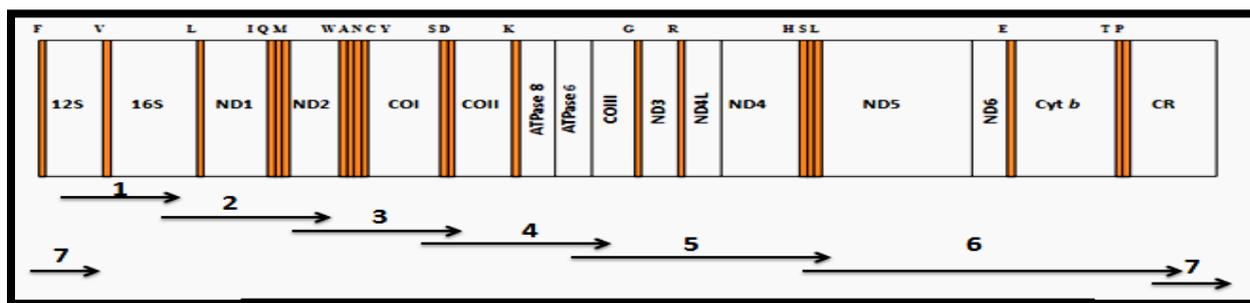


*Lagocephalus suezensis* (Suez puffer)



*Lagocephalus spadiceus* (Half-smooth golden puffer fish)

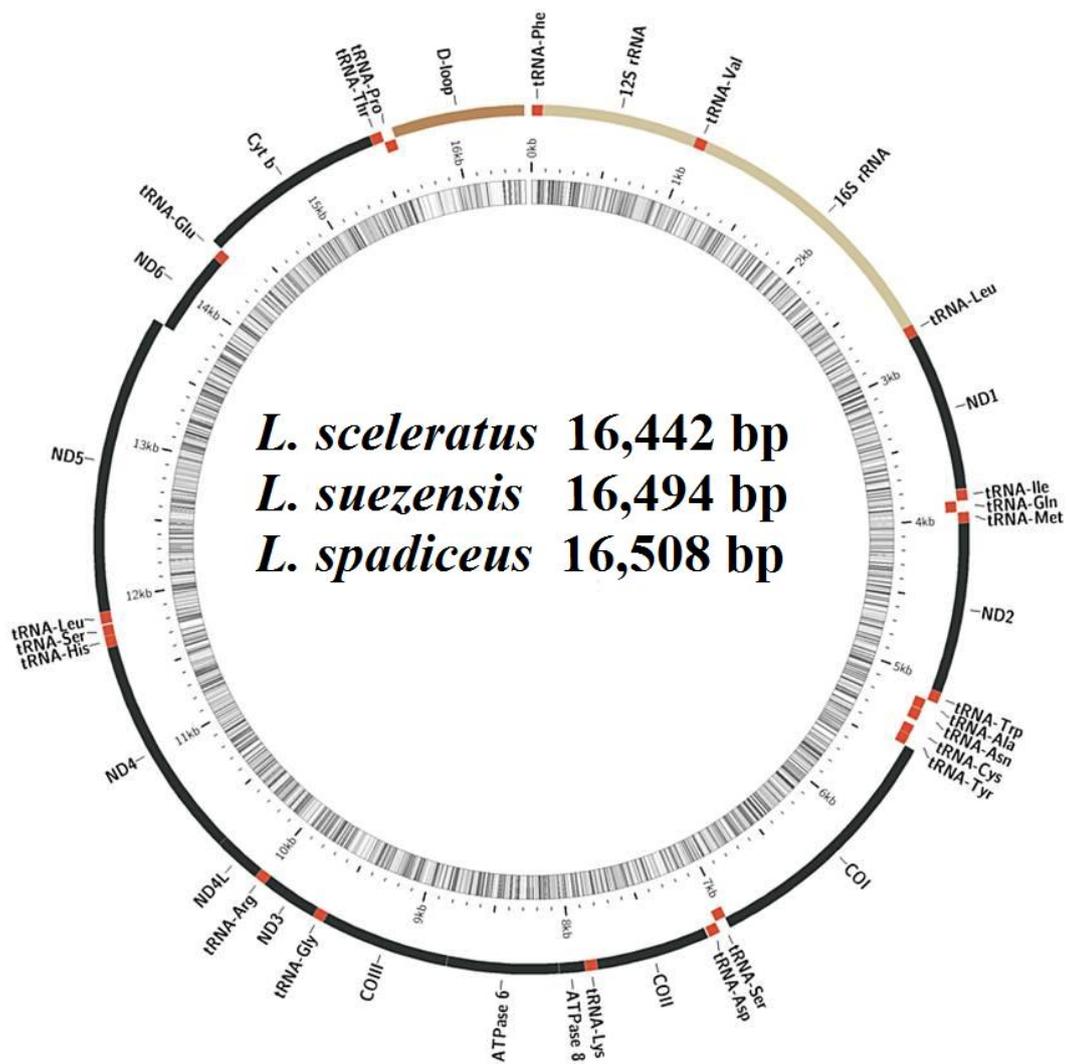
**Fig. 3-1** Puffer fish species used in this study.



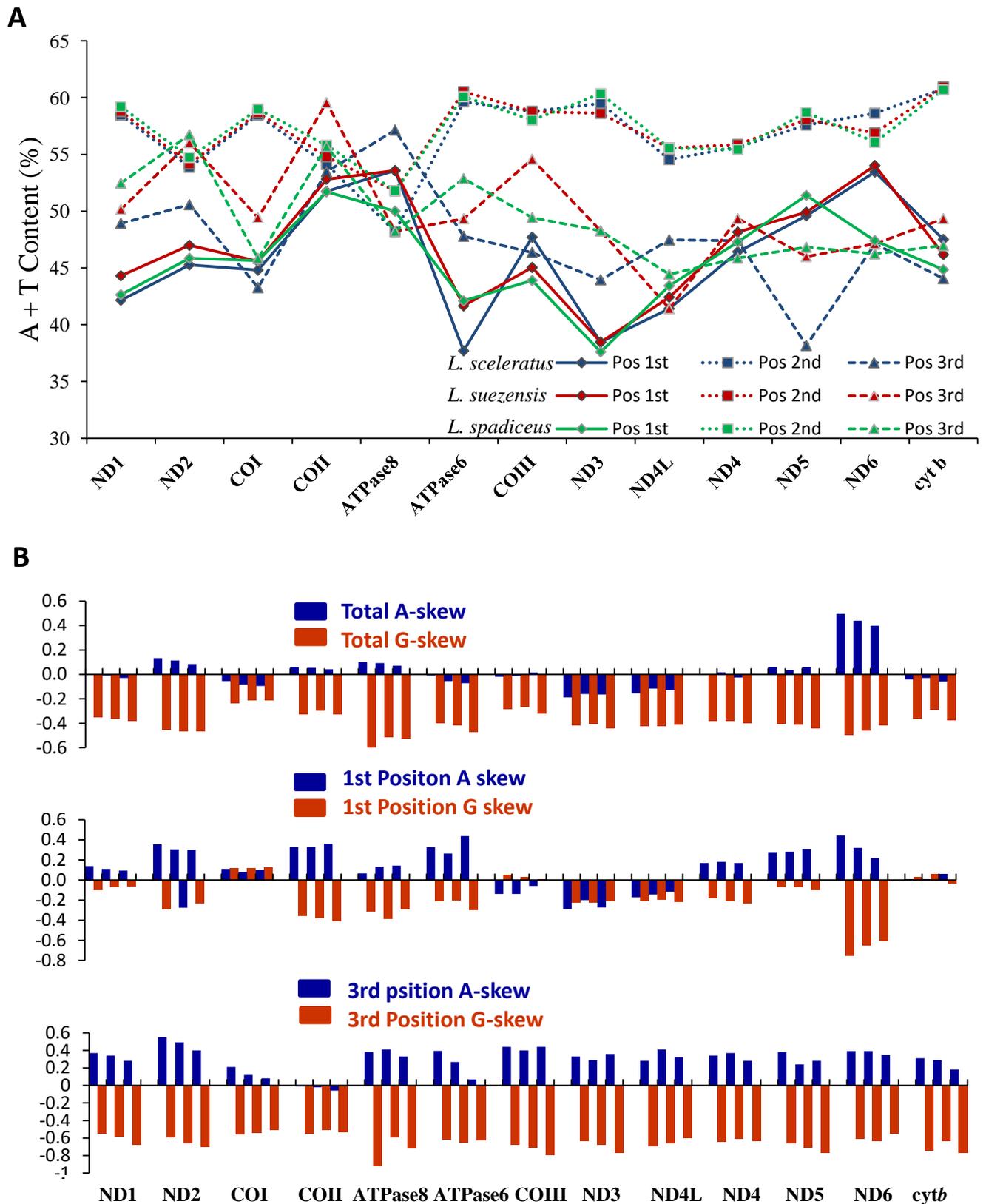
**Fig. 3-2** PCR amplification region and sequencing strategy for the detection of complete mtDNA genome of three *Lagocephalus* species.

**Table 3-1** Oligonucleotide primers for PCR amplification

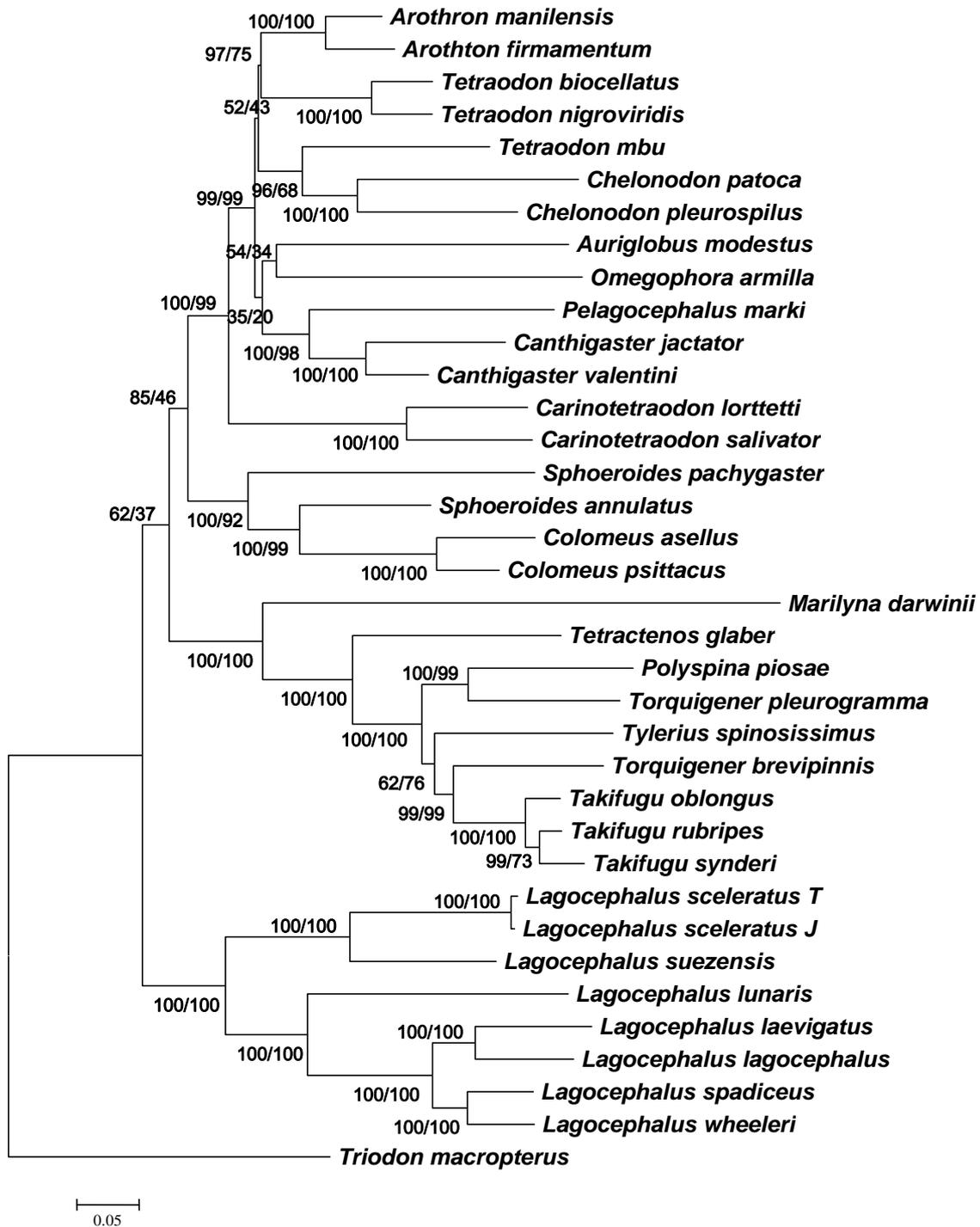
Region	Primers	Annealing Temperature	Sequence		bp
			5'	3'	
1	LSAF	64 °C	TTA AGC CAT AAG TGA AAA CTT GAC TTA G	28	
	LSAR		GAA CTC AGA TCA CGT AGG ACT TTA ATC GTT	30	
2	LSBF	62 °C	CAT AAG ACG AGA AGA CCC TAT GGA GCT TTA GA	32	
	LSBR*		AAT CTA AAG ARG CAG ATT GAC GTC TGC C	28	
3	LSCF	62 °C	AGA CCA AGG GCC TTC AAA GCC CTC AG	26	
	LSCR		GCA CCT TTY TTT AGC TTA AAA GGC TAA	27	
4	LSDF	62 °C	CAA YTA GGA TTT CAA GAC GCA GCW TCA CC	29	
	LSDR		TGA TAC TAG AAA GAT TAT GAG CCT CAT CAG	30	
5	LSEF	60 °C	TCT AAT GGC ACA TCA AGC ACA CGC ATA CC	29	
	LSER		GCA TAG CTT TTR CTT GGA GTT GCA CCA AC	29	
6	LSFF	62 °C	CAT TAG ATT GTG ATT CTA AAA ACA GGG	27	
	LSGR		TCG GCA TGT GGR RTT ATC TCG CTT ATG	27	
7	LSHF	60 °C	ATA CTC TTA TTG AAG GTG AGG GAC AAT	27	
	LSHR		CCA GTT TGT GYC TTA GCT TTC GTG GG	26	



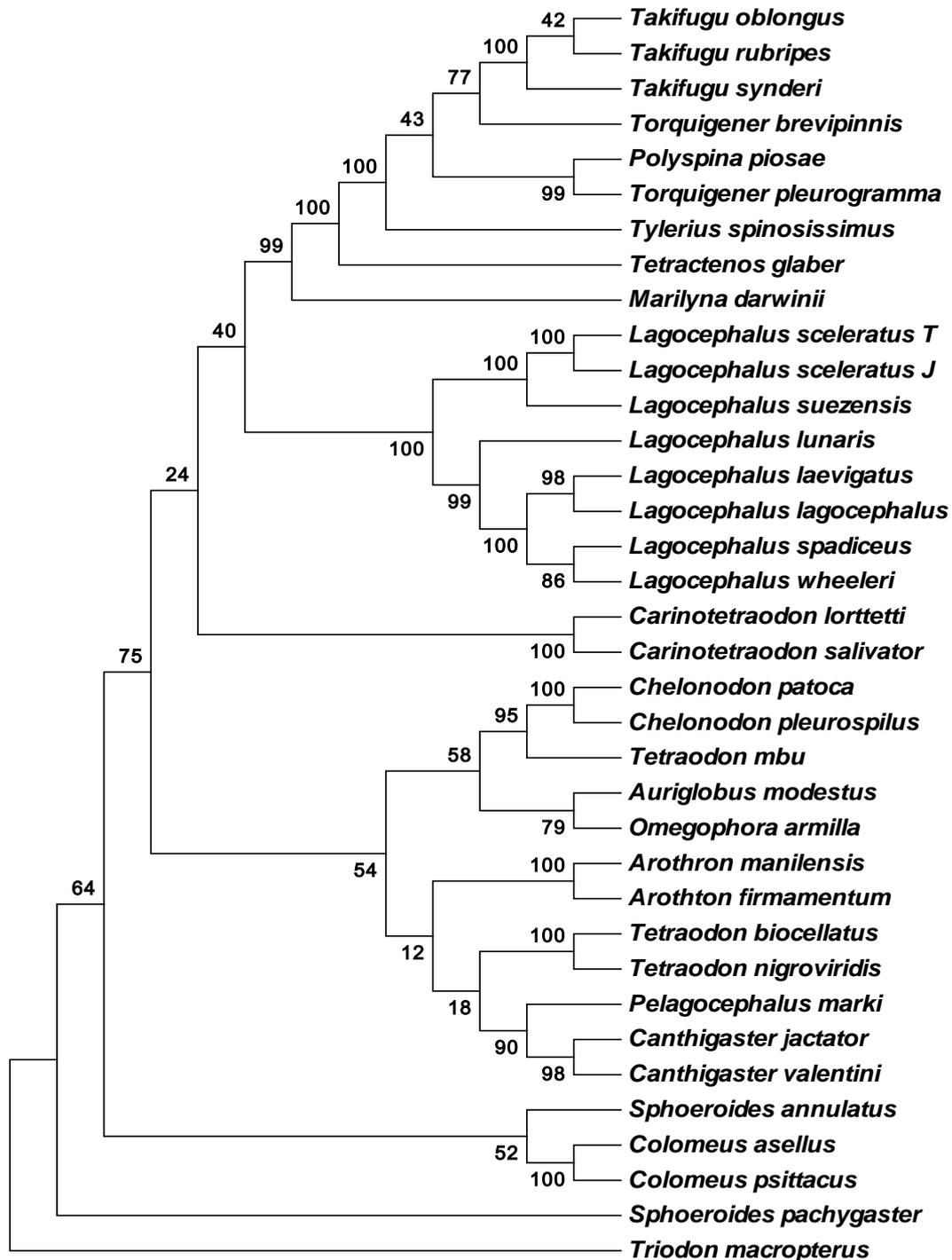
**Fig. 3-3** Organization of mitochondrial genomes from three *Lagocephalus* species. Genes coded by the N-strand (counter clockwise) are in the inner circle. The 22 tRNA genes are indicated by red. The AT-rich region is shown in brown. Gene sizes are roughly estimated. Gene model was constructed with MitoAnnotator online tool (<http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html>).



**Fig. 3-4** AT content and AT/GC-skew values of each codon position in each protein-coding gene (PCG) from three *Lagocephalus* species. (A) AT contents. (B) AT/GC-skew values. Three sets of bars (from left to right) in each PCG represent the corresponding AT/GC-skew values for *Lagocephalus sceleratus*, *L. suezensis*, and *L. spadiceus*. Pos 1st, Pos 2nd and Pos3rd indicate each codon position in the PCGs.



**Fig. 3-5** Phylogenetic reconstruction among puffer fishes based on concatenated nucleotide and amino acid sequences from twelve protein coding genes. Numbers at each node represent bootstrap support for ML (1000)/NJ (1000 replicates) inference. *T. macropterus* was used as an outlier group. ‘J’ and ‘T’ refer to sample collected from Japanese and Turkish coasts, respectively.



**Fig. 3-6** The maximum parsimony tree among puffer fish species. The most parsimonious tree with the length = 9,382 is shown. The consistency index is (0.379264), the retention index is (0.510572), and the composite index is 0.209519 for all sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *T. macropterus* was used as an outlier group. ‘J’ and ‘T’ refer to sample collected from Japanese and Turkish coasts, respectively.

## Chapter 4

### *The species authentication of *L. sceleratus*, *L. suezensis* and *L. spadiceus* by rapid PCR amplification method*

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#### 4.1 Introduction

Fish species identification is traditionally based on external morphological features, including body shape, pattern of colors, scale size and count, number and relative position of fins, number and type of fin rays, or various relative measurements of body parts (Strauss and Bond 1990). Traditional and official methods used in species identification, including fish, are based chiefly on the separation and characterization of specific proteins using electrophoretic techniques, such as isoelectric focusing: IEF (Rehbein, 1990) and capillary electrophoresis: CE (Kvasnicka, 2005), high performance liquid chromatography: HPLC (Hubalkova *et al.*, 2007), or immunoassay systems, such as Enzyme-Linked Immuno Sorbent Assay: ELISA (Asensio *et al.*, 2008).

In the chapter 2, potential hazardous of Lessepsian puffer fish species were discussed. Hereby, the current European legislative requirements are banned the (Regulation 853/2004/EC; Regulation 854/2004/EC) poisonous fish of the family Tetraodontidae and products derived from them and those type of products must not be placed on the market. In accordance with the present Turkish Commercial Fisheries Notification 3/1 (RG-17/5/2013-28650), *L. sceleratus* and *L. spadiceus* must not be landed and sold on to market because of their toxicity. In addition, *L. suezensis* is now very common at the eastern

Mediterranean coast of Turkey and frequently caught by bottom trawlers at depths down to 40 m (Bilecenoglu *et al.*, 2002).

Therefore, authentication of harmful species is of high importance for the residents of the Mediterranean coast. Although the morphological appearance of each species is unique, after they are mixed and processed with other seafood it is impossible to identify them by morphological characteristics alone. Recently, DNA-based identification methods of seafood have been developed, and mtDNA has been widely used as a tool to identify the origin of fish and seafood products and fraud. (Rasmussen and Morrissey, 2008; Teletcha, 2009). Teletcha (2009) noted that the following three main reasons of common usage of mtDNA: (1) the abundance of mtDNA comparing with nuclear genes, (2) the compact size of the genome and its maternal heritage, and (3) evolution of mtDNA is much faster than that of genomic DNA and provides a key advantage of differentiation between closely related species. PCR-RFLP, PCR-sequencing and PCR-specific primers have been widely used in the past decades. The identification of puffer fish species have been studying in last decades. Hsieh and Hwang (2004), developed a PCR-RFLP assay to identify 16 puffer fishes (*Lagocephalus* spp., *Takifugu* spp.) from Taiwanese seawaters, Ishizaki *et al.*, (2006) constructed a PCR-RFLP method for authentication of nine puffer fish species based on mitochondrial cytochrome *b* region. Seventeen puffer fish species were identified from commercial samples after the digestion of mtDNA cytochrome *b* region with , BsaJ I, Aci I, Hinf I, Taq I, and Sap I endonucleases (Hsieh *et al.*, 2010). A TaqMan probe-based quantitative PCR method was also established for the detection of toxic puffer fish belong to four genera of Tetraodontidae (Luekasemsuk *et al.*, 2015). Nevertheless, the proper quick identification method of common Lessepsian puffer fish species has not been established yet.

In this chapter, species-specific PCR method was performed based on the species-specific nucleotides of mtDNA of three Lessepsian puffer fish species, *L. sceleratus*, *L.*

*suezensis* and *L. spadiceus*. Method sensitivity and selectivity was determined and method validation was confirmed.

## **4.2 Materials and Methods**

### **4.2.1 Samples and materials**

For authentication method and specificity test, the total DNA extracted from three *Lagocephalus* species in the previous chapter for mtDNA sequence determination were used, as well as DNA from following species: *L. wheeleri*, *L. lunaris*, *L. gloveri*, *L. inermis*, *L. oceanicus*, *Takifugu rubripes*, *T.porphyreus*, *T. niphobles*, *T. exasurus*, *T. synderi*, *T. obscurus*, *T. chrypsops*, *T. stictonotum*, *Sphoeroides pachygaster*, *Ostracion immaculatus*, *Chilomycterus reticulatus*, *Diodon holacantus*, *D. liturosus*, yellow tail, jack mackerel, red sea bream, Atlantic bonito, saury, mackerel, bigeye tuna, white shrimp, salmon, conger, grater amberjack, swordfish, red sunfish, bluefin tuna, pacific oyster and yellowfin tuna.

### **4.2.2 Total DNA extraction**

Total genomic DNA was extracted from muscle tissue of *L. sceleratus*, *L. suezensis* and *L. spadiceus* with a QuickGene DNA tissue kit by following the manufacturer's instructions. In short, 15 mg muscle tissue was mixed with 180  $\mu$ L Buffer MDT and 20  $\mu$ L proteinase K (Buffer EDT) solution, incubated at 55 °C for overnight, and centrifuged at 8,000  $\times$ g for 3 min at room temperature. The supernatant was transferred to a new tube and mixed with 180  $\mu$ L Buffer LDT, incubated at 70 °C for 10 min and then 240  $\mu$ L 99.5% ethanol was added. DNA was purified with a cartridge. The preparations were subjected to the cartridge, washed three times with each 500  $\mu$ L Buffer WDT, and finally eluted with 200  $\mu$ L CDT, elution buffer. The purity and concentration of extracted DNA was measured using

a Biospec Nano spectrophotometer (Shimadzu Biotech, Tokyo, Japan). Total extracted DNA was kept at 4 °C until use.

#### **4.2.3 Species-specific primer construction**

The primers used in this work were designed from the following complete mtDNA sequences obtained in the National Center for Biotechnology Information (NCBI) database: KP013618, AP011932 (*L. sceleratus*); KP013619 (*L. suezensis*); KM667972 (*L. spadiceus*); AP011934 (*L. laevigatus*); AP011933 (*L. lagocephalus*); GQ461750 (*L. lunaris*) and AP009538 (*L. wheeleri*). These sequences were aligned using the default settings in ClustalW package in the MEGA 6 software (Tamura *et al.*, 2013). The alignment results as well as species-specific primer regions showed in Fig. 4-1a-c. Species-specific primer pairs for *L. scelaratus*, *L. suezensis* and *L. spadiceus* were constructed based on their species-specific nucleotide polymorphic sites. The nucleotide sequences of the designed primers are shown on Table 4-1.

#### **4.2.4 Species-specific PCR amplification**

All PCR reactions were carried out in a volume of 25 µL containing 0.1 - 25 ng of template DNA, 2.5 µL of 10× PCR buffer containing 25 mM MgCl<sub>2</sub>, 2 µL of dNTPs (10 mM), 10 µM of each primer, and 0.2 µL of *Ex Taq*® DNA polymerase. The PCR amplification was carried out in an Eppendorf Mastercycler® ep thermal cycler (Hamburg, German) in the following conditions: 30 cycles of 10 s at 98°C for denaturation, 52–58°C for 30 s for annealing, and 1 min at 72°C for extension, and a final extension at 72 °C for 10 min. The amplified PCR products were analyzed by electrophoresis with 2% agarose gel in TAE buffer containing GelGreen™ Nucleic Acid Stain (Biotium, Hayward, California, USA) and visualized with a luminescent image analyzer (LAS-4000 mini, Fujifilm Coop., Tokyo, Japan).

#### 4.2.5 DNA sequencing

The PCR products were purified using the Quantum Prep™ Freeze‘N Squeeze DNA Gel Extraction Spin Column (Biorad) according to the manufacturer’s instructions. After that subsequently were sequenced on an ABI 3130 automated DNA sequencer (Applied Biosystems, Japan) using the primers of the PCR amplification and the BigDye Terminator Cycle Sequencing Ready Reaction Kit v 3.1 (Applied Biosystems) following the supplier’s recommendations.

#### 4.2.6 Method validation and selectivity

The sensitivity of constructed PCR methods were detected by using six different concentrations (0.1, 0.5, 1, 2.5, 5 and 25 ng) of template DNA in the each species-specific PCR. Each three species muscle tissues were also sterilized in a steel retort at 121 °C for 40 min, with 1.2 bars overpressure. Total genomic DNAs were also extracted from sterilized samples following the same procedure as mentioned above and subjected to PCR.

The selectivity of species-specific identification methods were analyzed after the amplification of each species-specific primer pairs with DNA samples of other eighteen puffer fish species belong to same and different genus of family Tetraodontidae (*L. wheeleri*, *L. lunaris*, *L. gloveri*, *L. inermis*, *L. oceanicus*, *Takifugu rubripes*, *T. porphyreus*, *T. niphobles*, *T. exasurus*, *T. synderi*, *T. obscurus*, *T. chrypsops*, *T. stictonotum*, *Sphoeroides pachygaster*, *Ostracion immaculatus*, *Chilomycterus reticulatus*, *Diodon holacantus*, *D. liturosus*). The amplification results were also analyzed by agarose gel electrophoresis.

After the testing of all three species-specific PCR primers, the total genomic DNA of sixteen different fish species (yellow tail, jack mackerel, red sea bream, Atlantic bonito, saury, mackerel, bigeye tuna, white shrimp, salmon, conger, grater amberjack, swordfish, red

sunfish, bluefin tuna, pacific oyster and yellowfin tuna) was also used with the same species-specific primer pairs and method validation was determined among the other fish species.

### **4.3 Results and discussion**

#### **4.3.1 DNA extraction**

The quantity of DNA in the solution was calculated from the absorbance of 260 nm and the purity was calculated with the ratio A260/A280. Recovery of total DNA from puffer fish samples varied between 15.2 and 60.8 ng/μl in raw flesh. However, recovery of DNA drastically decreased after sterilization procedure at 121 °C(1.51–6.54 ng/μl). The adsorption rate of the recovered DNAs were detected to be in range between 1.7–2, that indicated a high quality of extracted DNA and samples were suitable for further PCR amplification.

#### **4.3.2 Species-specific primer construction and amplification**

Analysis of the alignment of the eight reference sequences obtained from GenBank showed that the bases in 119 position of NADH dehydrogenase subunit 2 (ND2) mtDNA region could differentiate *L. sceleratus*, however the bases in 123 and 138 position of cytochrome oxidase subunit 1 (COI) could successfully differentiate *L. suezensis* and *L. spadiceus*, respectively. These two mtDNA regions were widely used as genetic markers for identification of species in many fields (Broughton and Gold, 2000; Kleet and Meyer, 2002; Ivanova *et al.*, 2007; Battisti *et al.*, 2014; Cabellero *et al.*, 2015; Cutarelli *et al.*, 2014; Pappalardo and Ferrito, 2015). The selection of mitochondrial molecular markers bring several advantages. Mitochondrial molecular markers, generally, evolve much faster than nuclear markers, and besides there are several copies of mt DNA inside a cell (Mackie *et al.*, 1999) and so that the abundance of stand is higher allowing more effective PCR amplifications in comparison to nuclear DNA. Primer set LSCSP-F/LSCSP-R could amplify

a specific 119 bp fragment for *L. sceleratus*, and would not have any reaction with other twenty Tetraodontidae species. Similarly, primer sets of LSUSP-F/LSUSP-R and LSPSP-F/LSPSP-R could amplify a 123 bp fragment for *L. suezensis* and 139 bp fragment for *L. spadiceus*, respectively. Every species-specific primer set was routinely investigated by using twenty specimens of other Tetraodontidae species. The results are shown in Fig. 4-2. Three unexpected PCR products were detected after the analyzing by agarose gel electrophoresis on Lane 17 (*Sphoeroides pachygaster*) and Lane 21 (*Diodon liturosis*) from species-specific PCR of *L. spadiceus*, nevertheless those unexpected PCR products were not identical molecular size with targeted PCR product. Therefore, primer specificity was increased by using mismatched sequences. The mismatching sequences were underlined on the Table 4-1. The range of PCR annealing temperatures for each primer set is wide. If the annealing temperature set too low, the miss-paired situation would be found in non-specific species. For that reason, the suitable annealing temperature for each primer set listed in Table 4-1.

Although, species-specific PCR have been widely used for identification of various kinds of seafood (Lin and Hwang, 2007; Fernández-Tajes et al., 2010; Freire *et al.*, 2011; Tognoli *et al.*, 2011; Michelini *et al.*, 2007), this study is the first survey of identification of potentially harmful Lessepsian puffer fish species.

#### **4.3.3 Method validation and selectivity**

A PCR authentication method strategy was developed for the authentication of three Lessepsian *Lagocephalus* species in fresh, frozen, processed or thermal treated products, based on the amplification of the short mtDNA fragments. DNA extracted from fish which is submitted to transformation process, especially those involving a severe thermal treatment, it is not suitable for amplification with large fragments, because the thermal treatment generates DNA fragmentation. Santaclara *et al.*, (2007) amplified a maximum fragment size

of 208 bp to ensure amplification from canned products DNA. Hereby, the PCR was designed to amplified 119, 123 and 139 bp fragment sizes, respectively. The sensitivity of PCR method was screened by using several (0.1 ng – 25 ng) concentrations of the *L. sceleratus*, *L. suezensis* and *L. spadiceus* total genomic DNAs. The amplification results of LSCSP-F/LSCSP-R, LSUSP-F/LSUSP-R and LSPSP-F/LSPSP-R showed on Fig. 4-3. All of the examined species-specific PCR amplifications could sufficiently conducted even at a DNA concentration of as low as 0.1 ng in the total volume of 25 µl reaction. The PCR sensitivity test was also conducted with each genomic DNAs extracted after the sterilization process at 121 °C for 40 min, with 1.2 bars overpressure. The species-specific PCR method was constructed in this study was not only highly selective but also sensitive.

Depending on the type of PCR instrument, the whole reaction can be finished in around 1 hour and also electrophoresis of PCR product for detection can be finished in 30 min. Together with total DNA extraction all authentication procedure can be performed within 4 hours. This analysis time is significantly short compared with other methods, like RFLP, which requires additional time for restriction enzyme digestion or gene sequencing methods.

The results obtained here corroborate the usefulness of the ND2 and COI mtDNA regions for species identification. Numerous studies have been published in the past few years using species-specific PCR primers to identify seafood species, including dolphinfish, sharks, flatfish, bonitos, sturgeon, oysters, small pelagic fish, mackerels, razor clams, and many more. Species-specific multiplex PCR assays based on DNA barcodes for sharks, guitarfish, and salmon and trout have successfully developed (Hellberg and Morrissey, 2011). Sangthong *et al.*, (2014) applied species-specific multiplex PCR to identify to puffer fish species: *L. lunaris*, *L. spadiceus* and *L. inermis*, with a fish positive primer pair, that

produced a 289 bp fragment while *L. lunaris*, *L. spadiceus* and *L. inermis*-specific primers produced 123, 196 and 493-bp fragments, respectively.

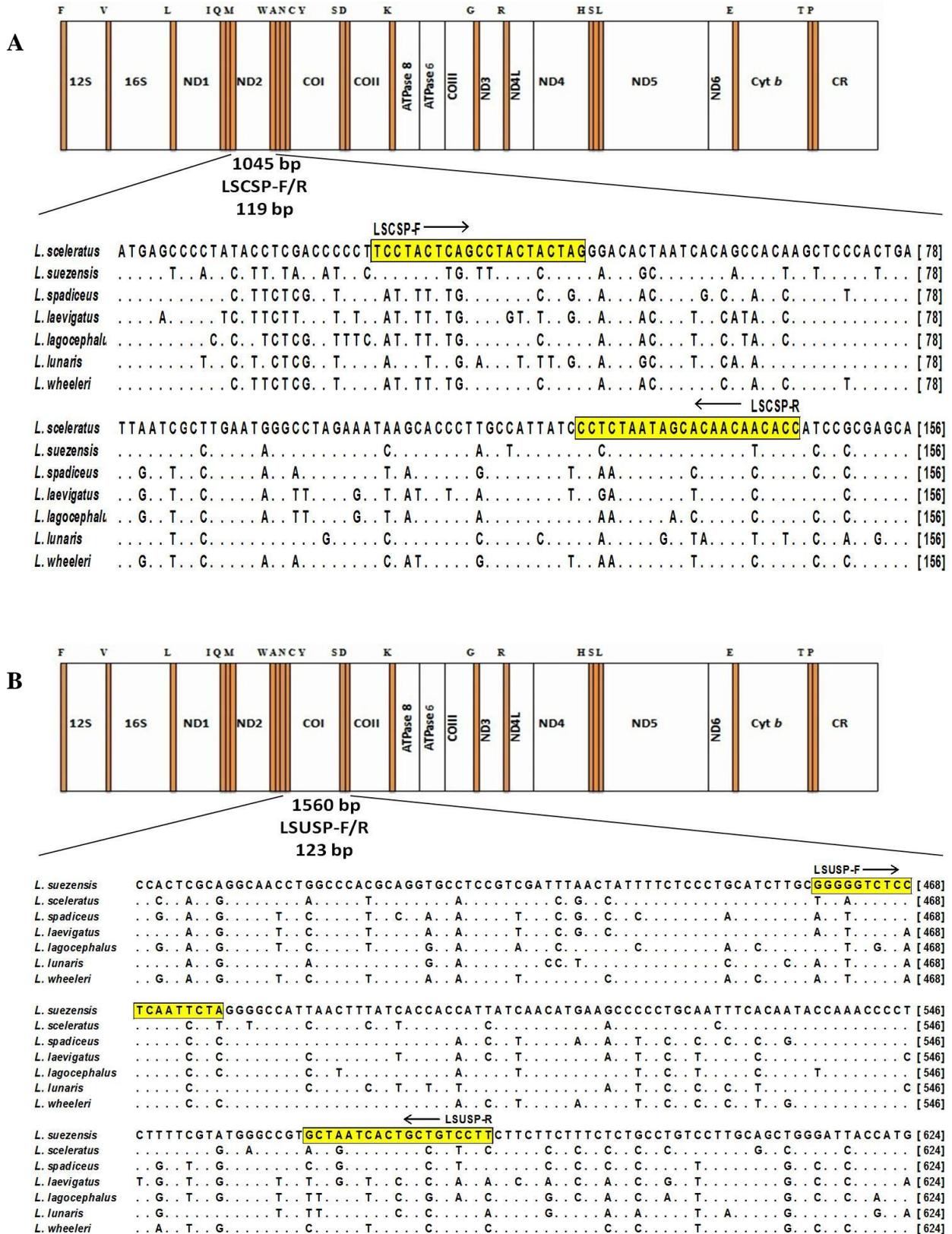
#### **4.3.4 Conclusion**

Lessepsian puffer fish species is brought high risk to eastern Mediterranean fishery. Hence, PCR amplification of selected mtDNA fragments, ND2 and COI by species-specific primers are a powerful and convenient technique for the identification of fish species, because of its simplicity, specificity, sensitivity and inexpensiveness. With constructed method in this chapter the identification relies not only on the different size of the amplicons obtained, but also on the presence of the target sequences specific for each species studied. Detection of mislabelling or fraud with this approach is possible also for processed food, such as frozen fillets, precooked seafood and patties or sticks where different fish species are mixed.

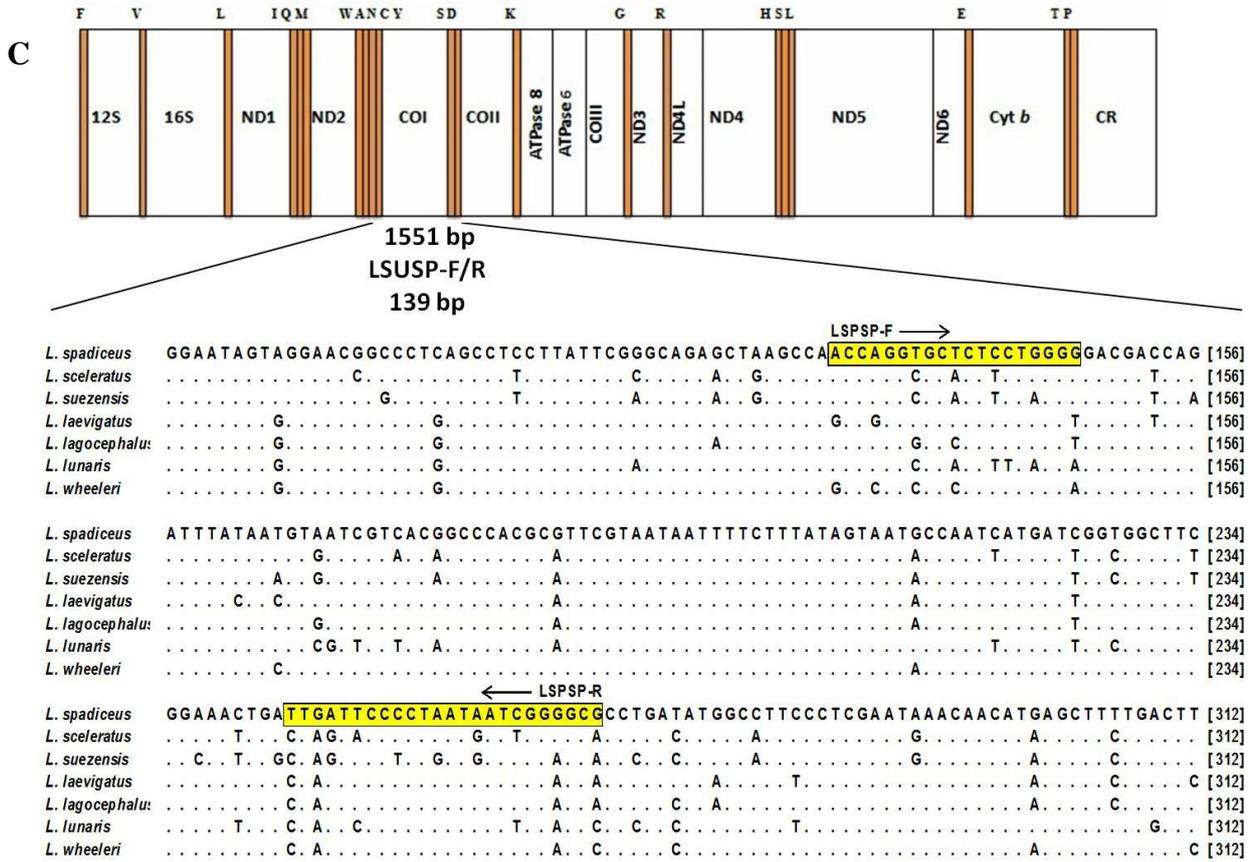
**Table 4-1.** Species-specific primer sequences and their optimum annealing temperatures of three puffer fish species.

<b>Primers</b>	<b>Sequence (5' 3')</b>	<b>Annealing Temperature (°C)</b>	<b>Targeted Species</b>
<b>LSCSP-F</b>	TCCTACTCAGCCTACTACTAG	58	<i>Lagocephalus sceleratus</i>
<b>LSCSP-R</b>	GGTGTGTTGTGCTATTAGAGC		
<b>LSUSP-F</b>	GGGGTCTCCTCAATTCTA	52	<i>Lagocephalus suezensis</i>
<b>LSUSP-R</b>	AAGGACAGCAGTGATTAGC		
<b>LSPSP-F</b>	ACCAGGTGCTCTCCTGG <u>CG</u>	53	<i>Lagocephalus spadiceus</i>
<b>LSPSP-R</b>	CGCC <u>TC</u> GATTATT <u>CG</u> GGGAATCAA		

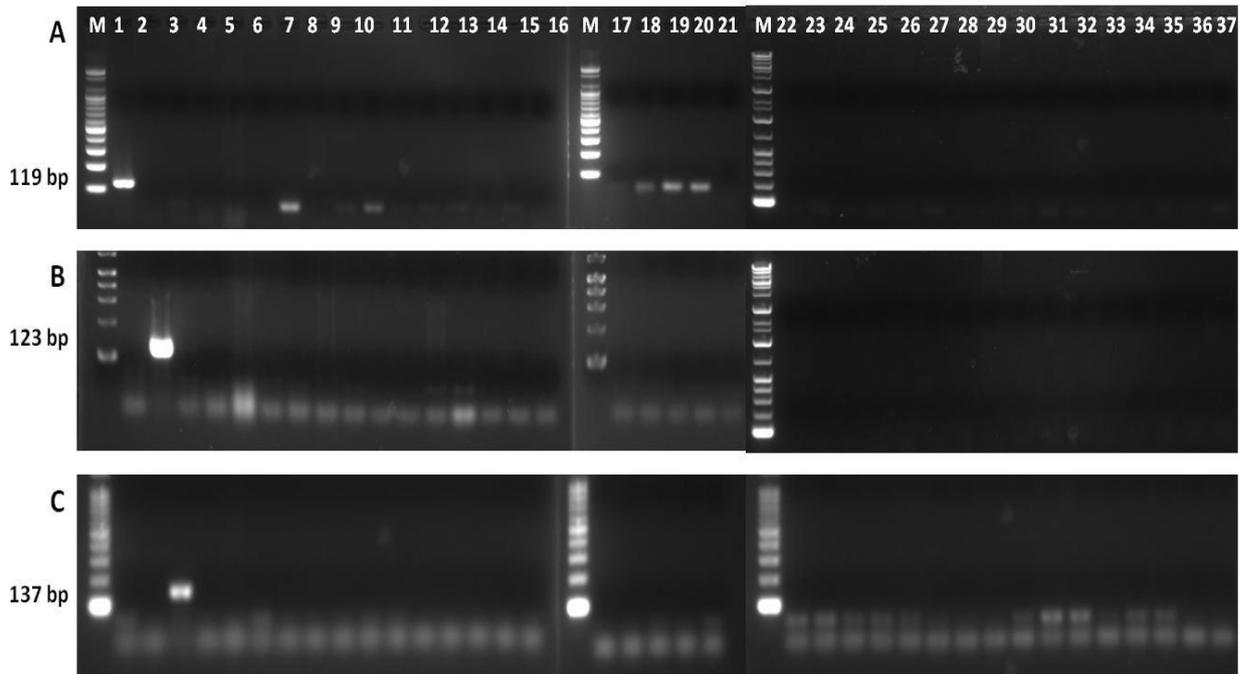
\*Mismatched sequences are underlined.



**Fig. 4-1.** The alignment results and species-specific primer regions of three puffer fish species. The nucleotide sequences of *L. sceleratus* (A), *L. suezensis* (B) and *L. spadiceus* (C) were aligned with other six *Lagocephalus* species.

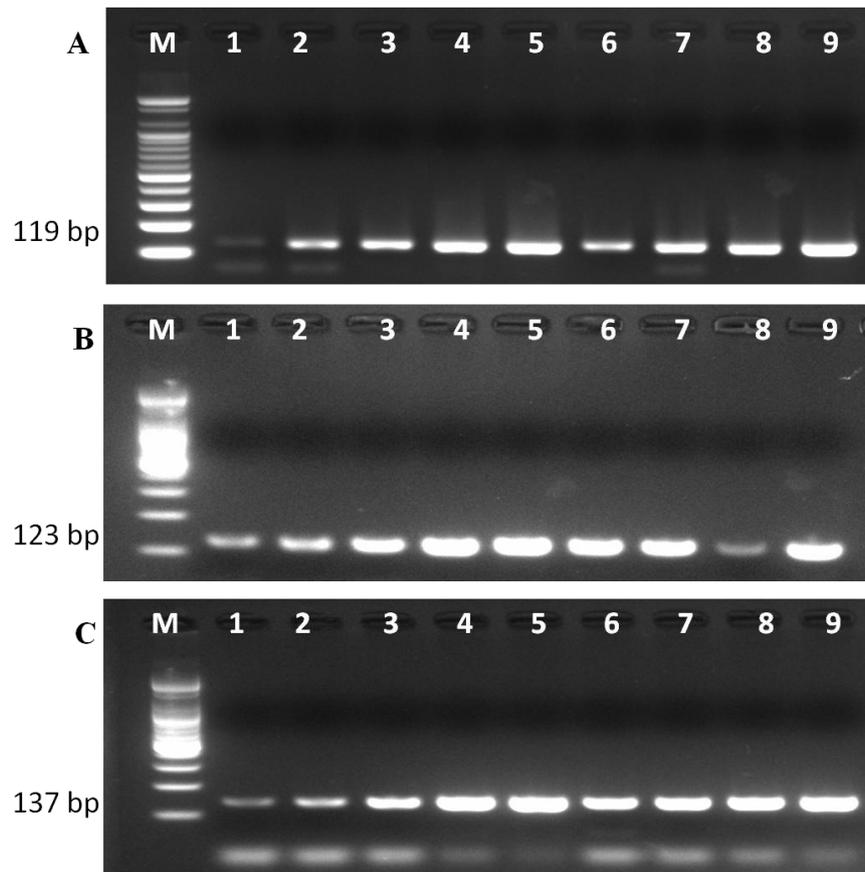


**Fig. 4-1** Continued.



**Fig. 4-2.** Agarose gel electrophoresis of amplification products obtained by a species-specific PCR.

(A), (B) and (C) indicate amplification results of LSCSP-F/LSCSP, LSUSP-F/LSUSP-R and LSPSP-F/LSPSP-R species-specific primer pairs and other twenty puffer fish species, respectively. Lane M: 100-bp marker, A1/B2/C2-*Lagocephalus sceleratus*, B1/A2/C3-*L. suezensis*, C1/A3/B3-*L. spadiceus*, 4-*L. wheeleri*, 5-*L. lunaris*, 6-*L. gloveri*, 7-*L. inermis*, 8-*L. oceanicus*, 9-*Takifugu rubripes*, 10-*T. porphyreus*, 11-*T. niphobles*, 12-*T. exasurus*, 13-*T. synderi*, 14-*T. obscurus*, 15-*T. chrypsops*, 16-*T. stictonotum*, 17-*Sphoeroides pachygaster*, 18-*Ostracion immaculatus*, 19-*Chilomycterus reticulatus*, 20-*Diodon holacantus*, 21-*D. liturosus*, 22-yellow tail, 23-jack mackerel, 24-red sea bream, 25-Atlantic bonito, 26-saury, 27-mackerel, 28-bigeye tuna, 29-white shrimp, 30-salmon, 31-conger, 32-grater amberjack, 33-swordfish, 34-red sunfish, 35-bluefin tuna, 36-pacific oyster, 37-yellowfin tuna.



**Fig. 4-3.** Agarose gel electrophoresis of sensitivity of the species-specific PCR method.

(A), (B) and (C) indicate amplification results of total genomic DNA of *L. sceleratus* amplified with LSCSP-F/LSCSP, total genomic DNA of *L. suezensis* amplified with LSUSP-F/LSUSP-R and total genomic DNA of *L. spadiceus* amplified with LSPSP-F/LSPSP-R species-specific primer pairs, respectively. Lane M: 100-bp marker, DNA concentration was as follows: 1-0.1 ng, 2-0.2 ng, 3-0.5 ng, 4-5 ng, 5-25 ng, 6-1 ng, 7-2.5 ng, 8-5 ng and 9-25 ng. Template DNA was added in the reactions between 1 to 5 was extracted from frozen tissue, while 6 to 9 was extracted after sterilization at 121 °C for 40 min, with 1.2 bars overpressure.

# Chapter 5

## *Purification, cDNA cloning and recombinant expression of TTX-binding protein from *Lagocephalus sceleratus**

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### 5.1 Introduction

Puffer fish and many other marine animals aggregate tetrodotoxin (TTX) and saxitoxin (STX) in their tissues with their analogs (Kodama *et al.*, 1983; Nakamura *et al.*, 1984; Jang and Yotsu-Yamashita, 2006). TTX has been turned out to be outside cause (Noguchi *et al.*, 1986; Yasumoto *et al.*, 1986), transferred to puffer fish body by the food chain and to be gathered in puffer fish (Kono *et al.*, 2008a,b). On the other hand, the transportation and collection frameworks of the toxins in puffer fish have not been clearly understood yet. Matsui *et al.*, (2000) reported TTX binding protein from the plasma of *Fugu niphobles* and the molecule mass of this protein was assessed to be 116 kDa by SDS-PAGE. Moreover, Yamashita *et al.*, (2001, 2002) likewise reported a soluble STX and TTX binding protein from the plasma of the puffer fish, *Fugu pardalis*, and named as the puffer fish STX and TTX binding protein (PSTBP). PSTBP possesses a Kd of 14.6 nM for [<sup>3</sup>H]STX in equilibrium binding assays. [<sup>3</sup>H]STX (10 nM) binding to PSTBP was half-inhibited by the presence of TTX and STX at 12 mM and 8.5 nM, respectively. From the consequences of gel filtration chromatography (200 kDa) and SDS-PAGE (104 kDa), PSTBP was proposed to comprise of noncovalently connected dimers of a solitary subunit. PSTBP monomer was made out of 42 kDa protein part and 62 kDa N-glycan. Two exceedingly homologous cDNAs

to one another coding PSTBP (PSTBP1, PSTBP2, the anticipated amino-acid identity 93%) were cloned from a cDNA library of *F. pardalis* liver. PSTBPs share high sequence homology (47%) with a tributyltin-binding protein 2 (TBT-bp2) found in Japanese flounder *Paralichthys olivaceus* (Oba *et al.*, 2007). TBT-bps are known as fish alpha 1-acidic glycoprotein (AGP)- like lipocalin proteins (Fournier *et al.*, 2000; Gutierrez *et al.*, 2000) that bind to tributyltin (TBT), which is exceedingly dangerous to marine organisms (Shimasaki *et al.*, 2002). The binding ability assay of recombinant TBT-bp shows that TBT-bp may reduce the toxic activity of TBT by binding (Nassef *et al.*, 2011; Satone *et al.*, 2011). For the study on the behaviours of PSTBP in puffer fish, we have to know whether PSTBP is a particular protein to puffer fish or not, and how this protein is appropriated among the tissues of puffer fish.

In this chapter, TTX-binding proteins from *L. sceleratus* were purified, TTX-protein interactions were clarified after the determining of TTX and its analogues from ovary proteins and PSTBP was cloned from *L. sceleratus*. The characterization of PSTBP of *L. sceleratus* was studied. Furthermore, the recombinant PSTBP protein was expressed and purified in *E. coli*. The physicochemical properties of recombinant LsPSTBP were characterized by a combination of mass and circular dichroism and TTX binding ability assay was carried out.

## **5.2 Materials and methods**

### **5.2.1 Samples and materials**

The toxicity results of *L. sceleratus* were explained in chapter 1. Ovary tissue (7.56 µg TTX/g) of specimen No: 16 was chosen for protein extraction. After the fish dissected, tissue was partially thawed, 2 g tissue was collected for TTX quantification and rest was kept at -25 °C until protein extraction.

### 5.2.2 Protein extraction and purification

10 g ovary from female specimen mixed with 20 ml 0.01M Tris acetate-0.15M NaCl (pH 7.5) buffer and homogenized for 1 min (IKA ULTRA-TURRAX T25 basic, 11000 r/min). After that, centrifuged at 13000 rpm for 15 min. Supernatant have collected and subjected to gel filtration column chromatography. 5 ml protein extraction was subjected onto Sephacryl S-400 gel filtration column chromatography (2.6\*75cm), with 0.01M Tris acetate-0.15M NaCl (pH 7.5) buffer at a flow rate 0.5 ml/min, monitored by absorbance at 280nm, and each 5 ml fractions were collected. TTX content was determined from each protein fractions detected by absorbance at 280 nm. After the determining of protein-TTX interactions, TTX-bearing fraction was subjected to HiPrep Q XL 16/10 anion exchange column chromatography. 1 ml protein extraction was gradient eluted with 1 M NaCl- 50mM Tris-acetate (pH 7.50) at a flow rate 0.5 ml/min, monitored by absorbance at 220 and 280nm, and unabsorbed and absorbed fractions collected. TTXs have determined from collected fractions.

### 5.2.3 Detection of toxin related substances by LC-MS

Tetrodotoxin and its analogues were detected as previously described in chapter 2. Briefly, single ion monitoring (SIM) was used (Nagashima *et al.*, 2011). The ions  $m/z$  320 for TTX,  $m/z$  302 for 4,9-anhydroTTX,  $m/z$  304 for 5-deoxyTTX and 11-deoxyTTX,  $m/z$  290 for 11-norTTX-6,  $m/z$  288 for 6,11-dideoxyTTX, and  $m/z$  272–162 for 5,6,11-trideoxyTTX were detected in SIM mode.

### 5.2.4 Total RNA extraction

Total RNA was extracted with TRIzol. Briefly, 0.5 g tissue was homogenized with 5 ml TRIzol reagent and precipitated with 2.5 ml isopropanol. After washed with 75% pre-cold

ethanol, re-suspended in 500µl RNAase free distilled water. Total RNA concentration was measured by Biospec Nano and quality was determined by denaturing electrophoresis. Total RNA was kept at -80 °C until use.

### **5.2.5 cDNA synthesis**

Single-stranded cDNA was constructed with PrimeScript Reverse Transcriptase with oligo dT primers. 1µg total RNA was mixed with 50 pmol oligo dT primer, 1 µl dNTP in total volume of 10 µl and heated at 65 °C for 5 min and cooled immediately on ice. After that reaction mixture was mixed with 4µl 5X PrimeScript buffer, 1µl reverse transcriptase and filled to total volume of 20 µl with RNase free dH<sub>2</sub>O. Incubated at 42 °C for 45 min, heated at 70 °C for 15 min and cooled on ice. Single-stranded cDNA was directly subjected to double-strand cDNA construction. 10 µl first-stranded cDNA reaction was mixed with 48.4 µl dH<sub>2</sub>O, 16 µl 5X second-strand buffer, 1.6 µl dNTP mix (10 mM) and 4 µl 20X second-strand enzyme mix in the total volume of 80 µl and incubated at 16 °C for 90 min. After that 6 units T4 DNA polymerase was added, incubated at 16 °C for 45 min and second-strand synthesis was terminated by mixing 4 µl of the EDTA/Glycogen mixture. Double-stranded cDNA was purified with phenol-chloroform and precipitated with 4 M ammonium acetate – 95% ethanol mixture. Double-stranded cDNA was kept at -25 °C until use.

### **5.2.6 Rapid amplification of cDNA ends (RACE)**

Rapid amplification of cDNA ends were conducted with Advantage 2 Polymerase Mix Kit according to manufacturer's instructions. 18 µl dH<sub>2</sub>O, 5 µl 10X cDNA PCR Reaction Buffer, 1 µl dNTP Mix (10mM), 1 µl Advantage 2 Polymerase Mix (50X), 1 µl AP1 primer (10mM), 1 µl LagoPSTBP-F/R primers (10mM) were mixed with 5 µl 1/250 dilutions of adaptor-ligated ds-cDNA. RACE PCR was carried out under the following

conditions: a cycle of 30 s at 94°C for denaturation and 30 cycles of 5 s at 94°C for denaturation, and 3 min at 72°C for extension. The amplified RACE PCR products were analyzed by electrophoresis with 1.2% agarose gel electrophoresis. The list of primers used in RACE PCR is shown on Table 5-1.

### **5.2.7 Amplification and cloning of internal PSTBP cDNA fragment**

PCR amplification was carried out by using Smart cDNA as template, and two primers Lago-PSTBP-F and Lago-PSTBP-R were designed on the basis of the cDNA conserved regions of PSTBP coding regions sequences from different puffer fish species. The PCR reaction volume contained H<sub>2</sub>O 17.3 µl, 10x PCR buffer 2.5 µl, dNTPs 2.0 µl, primer F 1 µl, primer R 1 µl, cDNA 1 µl, ExTaq (Takara, Japan) 0.2 µl and the cycling parameters: 30 cycles of heat denaturation at 98 °C for 10 s, annealing at 64 °C for 30 s, polymerization at 72 °C for 1 min, and a 10 min final extension at 72 °C. The PCR product was then cloned into the pGEM-T Easy vector (Promega). All sequencing reactions were performed according to the manufacturer's instructions using the T7 and SP6 promoter primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit v 3.1 (Applied Biosystems). Labeled fragments were then analyzed using an ABI 3130 automated DNA sequencer (Applied Biosystems, Japan) sequenced.

### **5.2.8 Sequence analysis**

The LsPSTBP cDNA sequence was analyzed using the BLAST algorithm at NCBI web site (<http://www.ncbi.nlm.nih.gov/blast>), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). Protein prediction was performed using software at the ExPASy Molecular Biology Server (<http://expasy.pku.edu.cn>). Multiple alignments were generated in Muscle (MEGA v. 6.0) with using the default settings.

The signal peptide and cleavage site of LsPSTBP were determined using the software SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP>). A phylogenetic tree was constructed based on the deduced amino acid sequences using the Neighbor-Joining (NJ) algorithm within MEGA version 6.0. Reliability of the tree was assessed by 1000 bootstrap repetitions.

### **5.2.9 Tissue distribution of PSTBP mRNA**

The mRNA expression of LsPSTBP in different tissues, including muscle, liver ovary and gill were determined by quantitative real-time PCR. Total RNA extraction and cDNA synthesis were described above. The cDNAs were diluted 100 times with RNA-free water for next step. The quantitative real-time PCR was performed on an Applied Biosystem 7300 Real-Time PCR system. Gene-specific primers Lago-PSTBP-F/R for LsPSTBP were used to amplify a product of 100 bp from cDNA. Two beta actin primers, Fugu-Actin-S-F/R were used to amplify a 87 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding samples. The qPCR amplifications were carried out using SYBR® Premix EX Taq™ II (Tli RNaseH Plus) Kit (Takara, Shuzo, Japan) in total volume of 20 µL containing 10 µl of 2×SYBR® Premix Ex Taq, 2 µL aliquot of single-stranded cDNA, 0.4 µM of each primer, and 0.4 µl of 50×ROX reference dye. The thermal profile for real-time PCR was 95 °C for 30 sec, 40 cycles of 94 °C for 5 sec and 60 °C for 31 sec. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Data were analyzed with the ABI 7300 SDS software (Applied Biosystems, Foster City, CA, USA), the baseline was set automatically by the software to maintain consistency. The relative gene expression fold change was determined by the  $2^{-\Delta \Delta C_t}$  method. All data were expressed as the mean  $\pm$  s.d.

and were analyzed by an unpaired Student's t-test after normalization. Level of expression among different tissues was compared using Tukey's test.

#### **5.2.10 Construction of the recombinant expression vector**

The cDNA fragment encoding the mature protein of PSTBP was amplified with specific primers Lago-PSTBP-M-NdeI and Lago-PSTBP-M-BamHI. Two restriction sites, *Nde* I and *Bam*H I, were added to the 5' end of these primers, respectively. PCR reaction was executed in a 25 µl volume containing 2.5 µl of 10x Ex Taq Buffer, 2.0 µl of dNTP, 1 µl of each primer and 1 µl of plasmid cDNA template, 17.3 µl of dH<sub>2</sub>O, 0.2 µl of Ex Taq polymerase (5 U/ m L) (Takara). The PCR cycling conditions were 20 cycles of 98 °C for 10 s, 65 °C for 30 s and 72 C for 1 min, and then a final elongation step at 72 C for 10 min. The prokaryotic expression plasmid pET-16b (Novagen, Madison, WI) was digested with *Nde* I and *Bam*H I and then ligated with the same digested and recovered PSTBP cDNA fragment, the LsPSTBP recombinant expression vector was constructed, which was entitled to pET16-LsPSTBP was then transformed to Nova blue non-expression host cells and plated on LB agar containing 12.5 mg/ml tetracycline. After the over night incubation at 37 °C, white colonies were picked and screened by direct colony PCR with T7 promoter and T7 terminator primers. And also, ligation was screened with combination of vector-specific and insert specific primers after the 1:10 dilution of ligation reaction mixture.

#### **5.2.11 Recombinant LsPSTBP expression, refolding and purification**

A single colony of successfully transformed *E. coli* was cultured overnight at 37 °C in Luria broth (LB) medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The overnight culture was inoculated in fresh LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown at 37 °C until absorbance at 600 nm reached 0.6–0.8. Expression of recombinant LsPSTBP was induced with 0.3 mM IPTG

(isopropyl  $\beta$ -D-thiogalactoside) after 4h incubation at 30 °C by shaking. The induced cells were harvested by centrifugation at 6000 $\times$ g for 10 min at 4 °C, then frozen at -80 °C. The extraction and refolding of recombinant LsPSTBP was carried out as previously described by Gu *et al.*, (2013) with slightly modifications. Briefly, frozen cell pellets were thawed and resuspended in 3 ml buffer A (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 5% glycerol, pH 8.0), then suspension was incubated on ice for 30 min, frozen at -80 °C.

The frozen suspension was thawed by immersing the tube into the water. After the solution became viscous, 3  $\mu$ l DNase I was added from stock solution of 5  $\mu$ g/ml DNase I and incubated at room temperature by rotating for 30 min. The cell lysate was centrifuged at 10,000 $\times$ g for 30 min to pellet insoluble inclusion bodies and both cells and supernatant was saved for further analysis. The inclusion bodies were resuspended in wash buffer, 0.5% (v/v) Triton X-100, 2M NaCl, incubated on ice for 30 min, and pelleted by centrifugation at 10,000 $\times$ g, 4 °C for 10 min. The washing step was repeated twice. Afterwards, inclusion bodies were mixed with 5 ml buffer B (8 M Urea, 50 mM Tris/HCl, pH 8.2), incubated on ice for 2 h for denaturing and solubilize the inclusion bodies. The denatured proteins were refolded by dialysis at 4 °C against 350 ml buffer C (50 mM Tris/HCl, 50 mM NaCl, 100 mM sucrose, 1 mM EDTA, 1 mM GSH, 0.1 mM GSSG, pH 8.2). After 36 h the buffer was changed to 1 L of buffer D (50 mM Tris/HCl, 50 mM NaCl, pH 8.2).

The refolded recombinant proteins were purified and analyzed by fast protein liquid chromatography (FPLC) on a Superdex-75 column (GE Healthcare, Piscataway, NJ) with 0.05 M Sodium phosphate – 0.15 M NaCl buffer (pH 7.0) at a flow rate of 0.5 mL/min. The column was calibrated with bovine serum albumin (66.2 kDa), carbonic anhydrase (29.0 kDa), cytochrome C (12.4 kDa) and aprotinin (6.5 kDa) in the same buffer (pH 7.0).

Fractions were collected and reconcentrated by Amicon Ultra-4 centrifugal filter device after centrifugation at 7500 g for 40 min. Then, recovered protein fractions were

further purified with HisTrap HP affinity chromatography. Firstly, column was equilibrated with 10 column volume of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), sample was injected and washed with binding buffer until the absorbance reached a steady baseline. Finally, proteins were eluted with elution buffer (20mM sodium phosphate, 0.5 M NaCl. 250 mM imidazole, pH 7.4) using a one-step linear gradient. All the peaks were collected and kept at -80 °C for the further analysis.

The final purification was conducted with Superdex-75 column (GE Healthcare, Piscataway, NJ) as described above. The presence of refolded LsPSTBP was confirmed by SDS–PAGE analysis. The protein concentrations were determined using a BCA protein assay kit.

#### **5.2.12. Circular dichroism spectroscopy analysis**

Circular dichroism (CD) spectra of recombinant LsPSTBP were collected using a Jasco J-720 Spectropolarimeter dichrograph (Japan Spectroscopic; Tokyo, Japan). The far-UV CD spectra were generated at 24 °C using the LsPSTBP protein at a concentration of approximately 7  $\mu$ M in 0.05 mM sodium phosphate buffer at pH 7. The assays were performed using a quartz cuvette with a path length of 2 mm, and 3 determinations within the range of 260–190 nm, at a rate of 50 nm/min, were recorded for each sample and averaged. The deconvolution and statistical analysis of the CD spectra were performed using the Dichroweb server (Whitmore and Wallace, 2004).

#### **5.2.13. TTX binding ability assay**

TTX (Wako Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in distilled water. Equal volumes of 6  $\mu$ M crude LsPSTBP (refolded, unpurified) solution and 3.3  $\mu$ M TTX (1000  $\mu$ l total), 16  $\mu$ M purified LsPSTBP and 60  $\mu$ M and 130  $\mu$ M TTX (400  $\mu$ l total)

were mixed in a glass test tube and incubated for 24 h at 4 °C. On the other hand, TTX content of recombinant LsPSTBP and TTX by itself were also detected after incubation at 4 °C for 24 h. The bound and free fractions were separated after ultra filtered through a 3K Amicon® Ultra Centrifugal Device (MWCO 3000). TTX was quantified by LC-MS/MS, calculated by following formula and expressed by the percentage. The schematic diagram of separation is shown on Figure 5-14.

$$\text{Bound TTX} = \text{Total TTX} - \text{Free TTX}$$

## **5.3 Results and discussion**

### **5.3.1 Protein purification**

The purification strategy of TTX binding protein is shown on Fig. 5-1. According to the Sephacryl S400 gel filtration column chromatograph three main protein fractions were determined. The TTX was mainly detected from Fr. III. Therefore, fractions between 72-79 was collected and further purified with HiPrep Q anion exchange chromatograph. Five fractions were collected and TTXs contents was confirmed by LC-ESI/MS. However, sample solutions did not show any similar peak at the retention of 13.48 time with the TTX standard. This results indicated that TTX was mainly found in the free form but not bound with high molecule weight substance. Hence, it was not be able to conduct research by purification method and known PSTBP protein was cloned, recombinant protein was expressed in *E. coli* and in vitro TTX binding assay was carried out.

### **5.3.2 cDNA sequence of LsPSTBP**

The LsPSTBP cDNA open reading frame was 639 bp in length. The complete nucleotide and deduced amino acid sequence of LsPSTBP were shown in Fig. 5-1. The ORF coded for a 212 amino acid protein which included a 20 amino acid signal peptide at the N-

terminal and a mature polypeptide of 192 amino acid residues. The calculated average molecular mass of the predicted mature protein was 21.3 kDa with an isoelectric point of 4.53.

The results of multiple alignment indicated that the amino acid sequence of LsPSTBP had significant sequence similarities with the putative PSTBP sequences of *L. gloveri* TBT-p Type 2\_2 (66.6 % identity), *L. wheeleri* TBT-p Type 2\_2 (66.1 % identity), *T. pardalis* PSTBP2 (51.0 % identity), *T. snyderi* PSTBP2 (50.90 % identity) and *T. rubripes* PSTBP2 (53.1 % identity) (Table 5-1). The alignment results were shown in Fig. 5-3. In the phylogenetic tree, tributyl binding proteins of genus *Lagocephalus* were clustered, respectively. The evolution of TBT-p or PSTBP proteins were genus dependent according to the phylogenetic tree (Fig. 5-4). According to the amino acid identity and alignment *L. scleratus* PSTBP protein was identified as ‘TBT-p Type 2\_2’.

### **5.3.3 The distribution of LsPSTBP mRNA in different tissues**

The LsPSTBP transcript and the beta actin transcript were readily detected from cDNA of muscle, liver, ovary and gill by Real-Time quantitative RT-PCR (Fig.5-4). The specific products are shown with a melting temperature ( $T_m$ ) of 80 °C, and unexpected products or primer dimer were not detected (Fig. 5-5). Results indicated that, the RNA amplification were highly sensitive and specific detection of transcripts from all of the cDNA were successful. The highest level of expression was found in liver (Fig. 5-6.)

The results showed high similarities with previous reports. Yotsu-Yamashita *et al.*, (2010) reported that PSTBP-like glycoproteins are commonly present in many species of puffer fish and PSTBP was detected from heart, liver, kidney, spleen, intestine, skin, muscle and ovary tissues. However, Tatsuno *et al.*, (2013) could not detected TR2 and T4 type of

TBT proteins from any tissues of non-toxic cultured *T. rubripes*. TBT type 2\_2 was detectable from all examined tissues and tissue distribution was confirmed.

#### **5.3.4 Construction of the recombinant expression vector**

The amplification results of mature PSTBP with restriction enzyme sites are shown in Fig. 5-7. The expected products of 596 and 576-bp were clearly detected. The results indicated that targeted fragment was successfully amplified from plasmid DNA of LsPSTBP open reading frame. PCR products were directly purified with FastGene PCR purification kit and subjected to digestion with same restriction enzymes, in parallelly pET-16b expression vector was also digested (Fig. 5-8).

After the digestion, the bands were cut and products were recovered from agarose gel. The concentration of products were determined by Nano drop and converted to fmol. The concentration of PSTBP and pET-16b vector after recovering were 58.83 and 9.56 fmol/ $\mu$ l, respectively. According to T4 DNA ligase protocol, the amounts were sufficient and ligation reaction was conducted. Firstly, ligation was screened with gene and vector-specific primer combinations (Fig. 5-9). After that, transformation was carried out with freshly harvested Nova blue competent cells and white colonies were screened with vector-specific primer pair (Fig. 5-9). According to colony PCR results, positive colony was inoculated with LB broth containing ampicilin and tetracycline. Plasmid DNA of LsPSTBP was purified with GE Healthcare Plasmid Mini Prep Kit. Finally, plasmid of pET16-LsPSTBP was screened with direct PCR amplification containing gene and vector-specific primers. The results proved that the pET16-LsPSTBP expression vector was successfully constructed and it can be used for further expression and purification analyses.

### 5.3.5 Recombinant LsPSTBP expression, refolding and purification

Recombinant proteins expressed in *E. coli* are often produced as aggregates called inclusion bodies. Growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubation at 30°C leads to soluble, active protein (Schein and Noteborn, 1989). Growth and induction at 25°C or 30°C may be optimal to export the target using the signal sequence leaders present in a number of pET vectors. In some cases, prolonged (e.g., overnight) induction at low temperatures (15°–20°C) may prove optimal for the yield of soluble protein. Therefore, different growth and induction conditions were carried out, in order to enhance the solubility of targeted recombinant LsPSTBP. The bacteria liquid before and after inducing were centrifuged at 3000g for 5 min at 4 °C, the bacterial cultures were lysed by lysis buffer and collected as described above. 10µl supernatant was mixed with 10µl 2×SDS-PAGE protein sample buffer, in parallel precipitated cells were re-suspended in 1×SDS-PAGE protein sample buffer and samples were incubated at 100 °C for 3min. The total weight of fusion protein molecular is about 25 kDa. The SDS-PAGE analysis of different expression combinations are shown on Fig. 5-10. The predicted recombinant protein was determined from the insoluble fraction, while no predicted expression was detected in the control group (Fig. 5-10). These results indicated that target gene LsPSTBP can be expressed in this protein expression system after IPTG induction. Therefore, proteins were extracted under the denaturing conditions with 8M Urea. This protocol, which consisted of an initial step of 2 hours washing of the LsPSTBP-rich inclusion bodies with 8 M urea (according to a described methodology), allowed the recovery of approximately 3 mg of total protein per 0.25 liter of bacterial culture (Fig. 5-11). Moreover, denatured proteins were refolded as described and purified.

After protein solubilization, LsPSTBP was purified by affinity chromatography, resulting in approximately 1.52 mg of purified LsPSTBP per 250 ml of bacterial culture. The

purified protein has a predicted mass of 21.3 kDa, corresponding to the 192-amino acid sequence encoded by mature ORF LsPSTBP plus the N-terminal His 6 –Tag added by the pET16b vector. The three step purification results and purity of the final protein was assessed by 14% SDS–PAGE (Fig.5-12).

### **5.3.6 Conformational characterization of recombinant LsPSTBP**

Far-UV CD spectra of LsPSTBP display a minimum near 217 nm and a positive peak below 200 nm (Fig. 5-13), which obviously reveals the typical secondary structure of a predominantly  $\beta$ -sheet protein. LsPSTBP contains abundant  $\beta$ -sheet structure (>60%), indicating that recombinant LsPSTBP was a well-refolded  $\beta$ -rich protein. The prediction of the secondary structure for LsPSTBP using the PSIPRED server produced results similar to those obtained by the deconvolution of the experimental data obtained by CD. These results are in accordance with known elements of secondary structure for family members of lipocalin.

### **5.3.7 TTX binding assay**

A binding assay indicated that LsPSTBP bound TTX. TTX was not detectable from purified recombinant LsPSTBP. The result indicates that recombinant protein did not contain any TTX. Moreover, TTX content did not decrease after the incubation for 24 h from only-TTX solution. After incubation of the LsPSTBP solution (6 and 16  $\mu$ M) with TTX solution (3.3, 60 and 130  $\mu$ M ) at 4 °C for 24 h, the mixture was fractionated by ultra centrifugation. TTX binding ability of recombinant LsPSTBP is shown on Table 5-3. The highest binding ability was detected with 37.9% between refolded crude protein and TTX. It might be due to the missed binding with other *E. coli* originated proteins. The binding ability of purified LsPSTBP was approximately 10%. These results indicated that different molar ratio between protein and TTX was effected differently. Satone *et al.*, (2011) reported

tributyltin-binding ability of recombinant TBT-p Type\_1 protein. The 38  $\mu$ M recombinant rTBT-bp1 solution was mixed with 380  $\mu$ M TBT solution and incubated at 15 °C for 16 h after fractionated by gel filtration column chromatography two protein fractions contained TBT with molecular ratio of 6:1 and 4:1, respectively. However, the binding ability could not calculate by the meaning of molar ratio, this study is the first report of TTX binding ability of TBT-p protein.

### 5.3.8 Conclusions

TBT-bps in fish may generally respond to exposure of chemicals. Thirteen putative TBT-bp-like proteins have been found in eight species of teleost including medaka, zebrafish (*Danio rerio*), and spotted green pufferfish (*Tetraodon nigroviridis*) (Satone *et al.*, 2008). In the brain of Japanese medaka, the expression levels of two types of genes encoding TBT-bp-like proteins are altered by polychlorinated biphenyls (Volz *et al.*, 2005). The expression levels of mRNA for TBT-bp1 were up regulated in medaka liver exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Nakayama *et al.*, 2008).

In conclusion, I have established an efficient approach for expression and purification of the TBT-p Type\_2 protein. Moreover, TTX binding ability of TBT-p protein was confirmed for the first time. Further experiments are required to reveal the binding ability of TBT-bps to TTX and other chemicals.

**Table 5-1** List of primers

<b>Name</b>	<b>Sequence (5' to 3')</b>	<b>Targeted gene</b>
Beta actin-F	AGAGCTACGAGCTGCCTGAC	Long $\beta$ -actin
Beta actin-R	GCTGGAAGGTGGACAGAGAG	
Lago-PSTBP-F	CTGGCGGTGCTTGGCACCAGAGCAGC	Partial TBT-bp
Lago-PSTBP-R	TTCTGATGAAGACCATCCCTCCTGTAG	
Fugu-actin-S-F	CTCTTCCAGCCATCCTTCCTT	Short $\beta$ -actin
Fugu-actin-S-R	GGATGTCGACGTCGCACTT	
Lago-PSTBP-S-F	ACACCGATATTGGGACATGGA	Short PSTBP
Lago-PSTBP-S-R	TCCGGAAACAAGCTCCTCTCT	
Lago-PSTBP-ORF-F	ATGGGTGTTGGACAACCACTGG	PSTBP-ORF
Lago-PSTBP-ORF-R	TCAGGCCTGGTCAGGCTTTGCA	
Lago-PSTBP-M-F	GCACCGAGTGCAGAAGACTGTT	Mature PSTBP
Lago-PSTBP-M-R	TCAGGCCTGGTCAGGCTTTGCA	
Lago-PSTBP-M-NdeI	TCGT <u>CATATG</u> GCACCGAGTGCAGAAG ACTGTT	Mature PSTBP with restriction sites
Lago-PSTBP-M-BamHI	AGCC <u>GGATCCT</u> CAGGCCTGGTCAGGCT TTGCA	
T7 promoter	TAATACGACTCACTATAGGG	Vector specific
T7 terminator	GCTAGTTATTGCTCAGCGG	

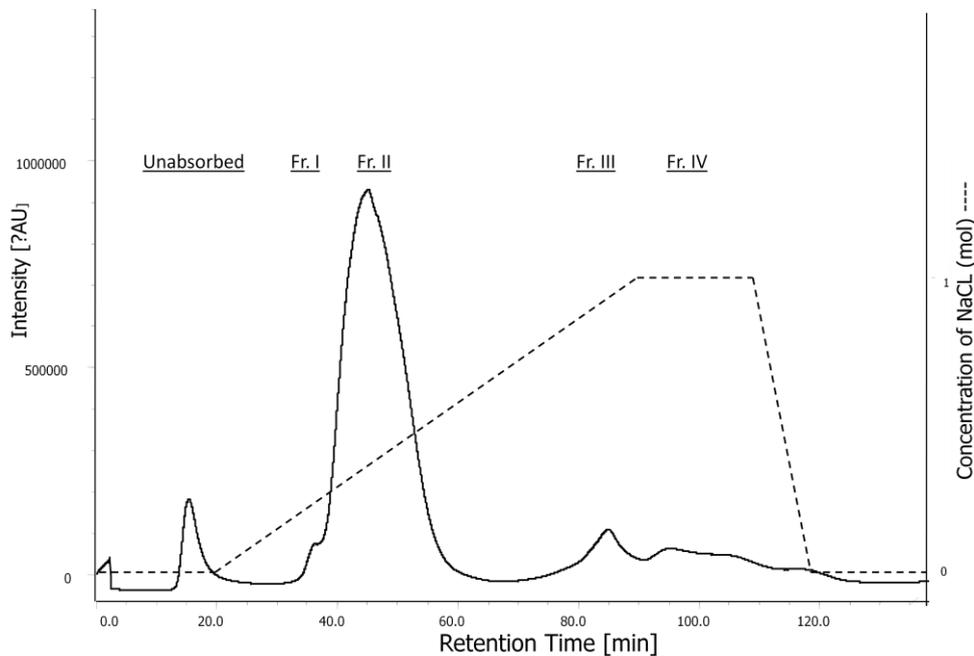
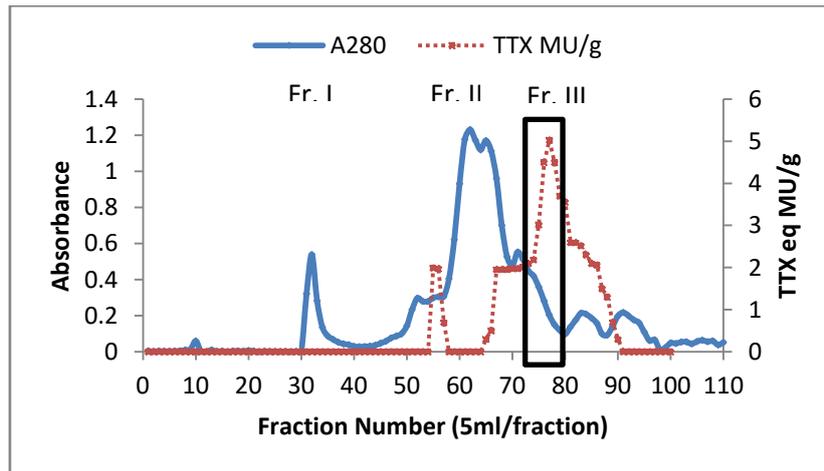
\*The restriction enzyme sites are underlined.

**Table 5-2** Amino acid identity of *L. sceleratus* TBT-p protein among other puffer fish species

Species	Identical amino acid number	Amino acid identity %
<i>L. gloveri</i> TBT-p Type 2_2	140/210	66.6
<i>L. wheeleri</i> TBT-p Type 2_2	139/210	66.1
<i>L. gloveri</i> TBT-p Type 2_1	139/210	66.1
<i>L. wheeleri</i> TBT-p Type 2_2	138/210	65.7
<i>T. pardalis</i> PSTBP 2	98/192	51.0
<i>T. snyderi</i> PSTBP 2	107/210	50.9
<i>T. rubripes</i> PSTBP 2	101/190	53.1

**Table 5-3** TTX binding ability of recombinant LsPSTBP

<b>Purity of protein</b>	<b>Molar ratio Protein/TTX</b>	<b>Binding %</b>
<b>Crude</b>	<b>1.8 (6 <math>\mu</math>M/3.3 <math>\mu</math>M)</b>	<b>37.9</b>
<b>Purified</b>	<b>0.26 (16 <math>\mu</math>M/60 <math>\mu</math>M)</b>	<b>9</b>
<b>Purified</b>	<b>0.12 (16 <math>\mu</math>M/130 <math>\mu</math>M)</b>	<b>11.6</b>



**Fig. 5-1.** The purification and TTXs content of ovary protein extraction. A: Sephacryl S-400 gel filtration column elution profile of ovary protein extraction. 2.6\*75cm with 0.01M Tris acetate-0.15M NaCl (pH 7.5) as eluent at a flow rate of 0.5ml/min, 5 ml/fraction. B: HiPrep Q XL HPLC column elution profile of Fr. III by Sephacryl S-400 chromatography. Mobile phase: A) 50mM Tris-acetate buffer (pH 7.5); B) 1.0 M NaCl-50mM Tris-acetate buffer (pH 7.5). Flow rate: 0.5 ml/min. Retention time: 140 min. C: LC-MS-SIR profiles of Fr. III by HiPrep Q XL 16/10 HPLC column.

TTXstd\_1ng/10mL

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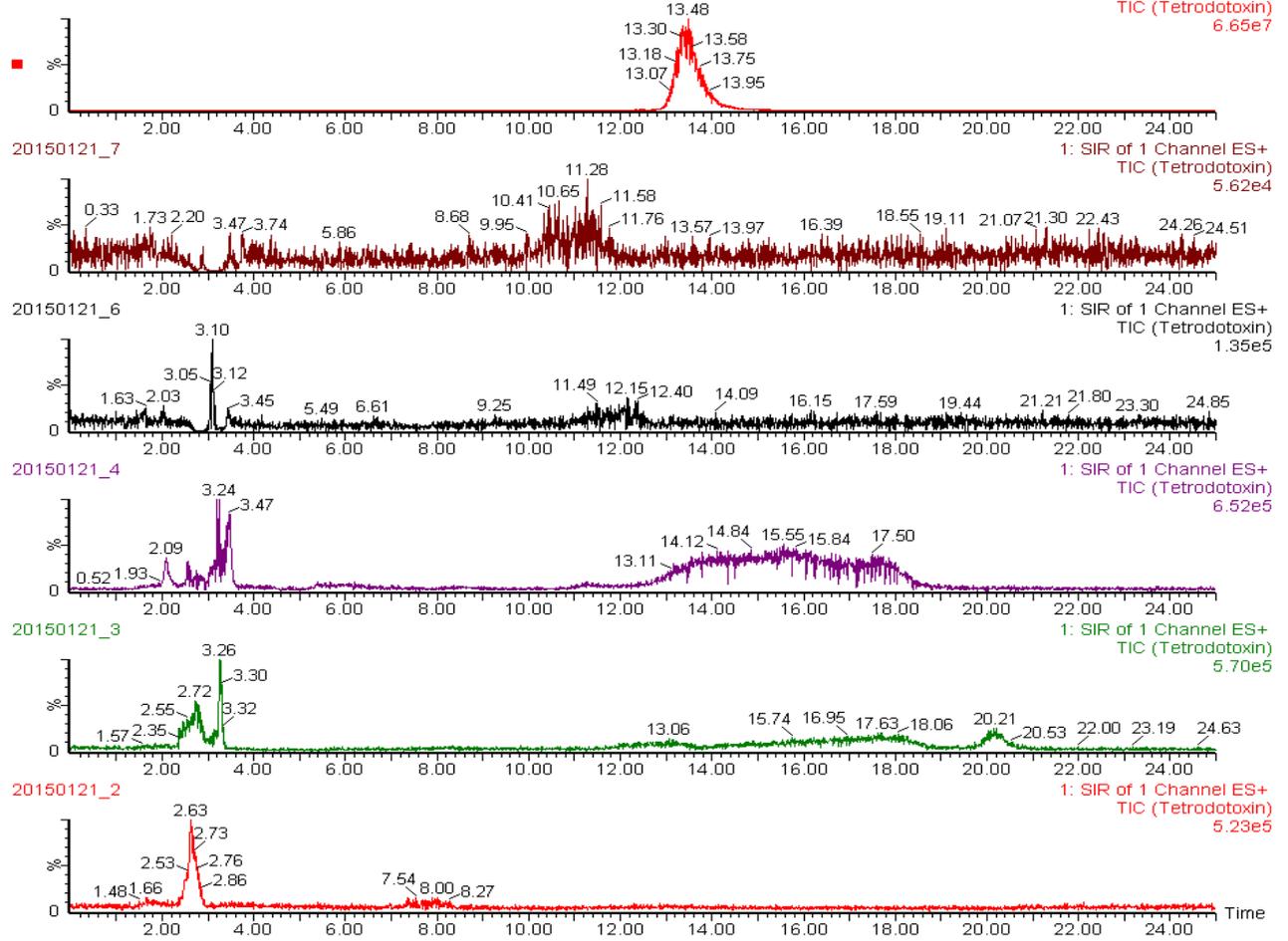


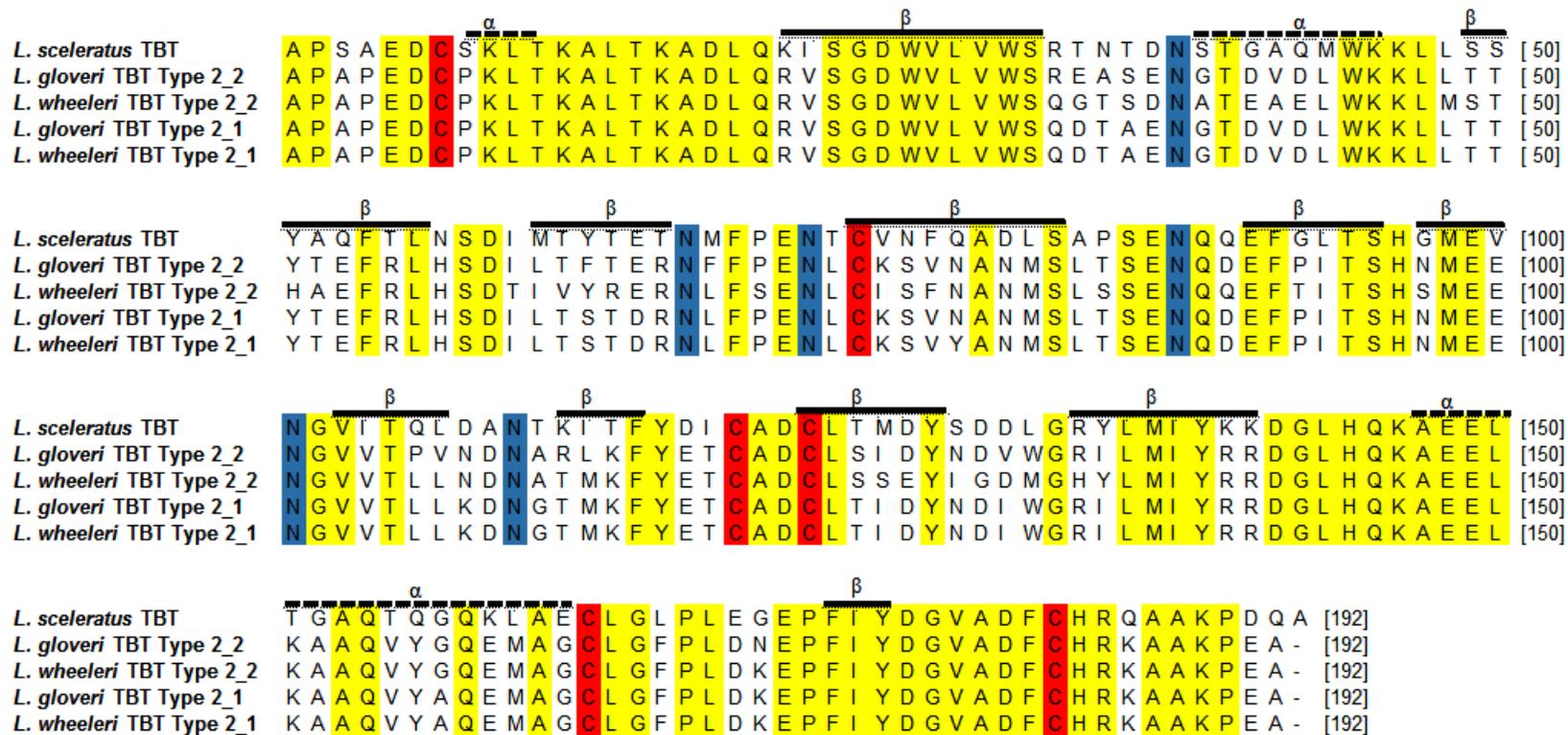
Fig. 5-1 Continued.

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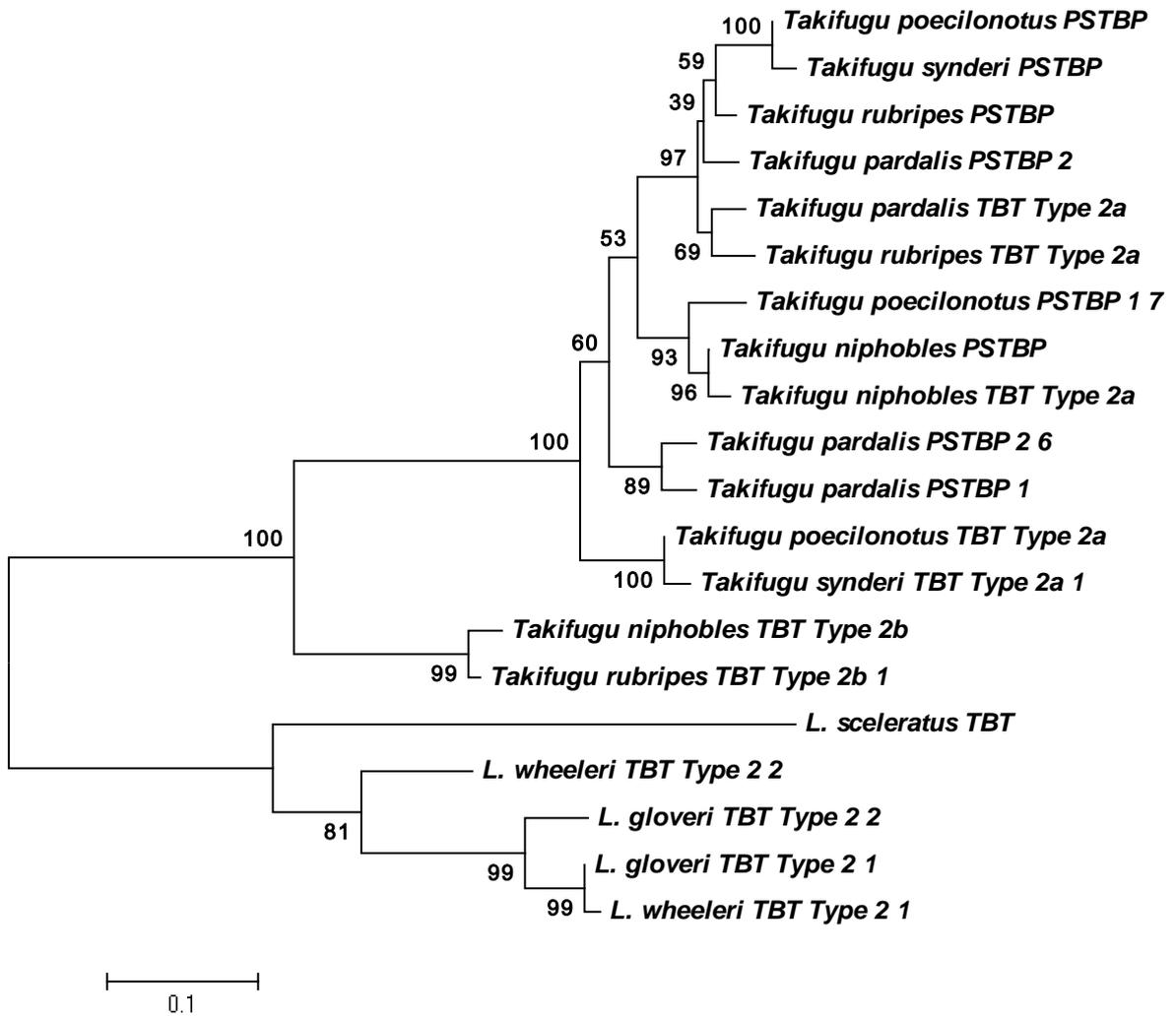
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    *
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    L G T R A A P S A E D C S K L
91  accaaagcattgaccaagcagatctgcagaagatctctgggtgac
    T K A L T K A D L Q K I S G D
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    W V L V W S R T N T D N S T G
181 gctcagatgtggaagaagctgctgagctcctacgctcagttcaca
    A Q M W K K L L S S Y A Q F T
226 ctcaactctgacatcatgacgtacacagagacgaatatgtttccg
    L N S D I M T Y T E T N M F P
271 gaaaacacctgtgtgaacttccaggcagacctgtcagcaccctct
    E N T C V N F Q A D L S A P S
316 gaaaaccagcaagagtttggccttacatcacacggaatggagggtg
    E N Q Q E F G L T S H G M E V
361 aacggagtcatcacacagttagatgccaaactaagattacgttc
    N G V I T Q L D A N T K I T F
406 acgacatctgtgctgattgtttgacaatggactacagtgatgat
    Y D I C A D C L T M D Y S D D
451 ttgggccggttacctgatgatctacaagaaggatggtctccatcag
    L G R Y L M I Y K K D G L H Q
496 aaggcagaagaactgacggggcgccaaactcagggtcagaaactg
    K A E E L T G A Q T Q G Q K L
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    A E C L G L P L E G E P F I Y
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    Q A *

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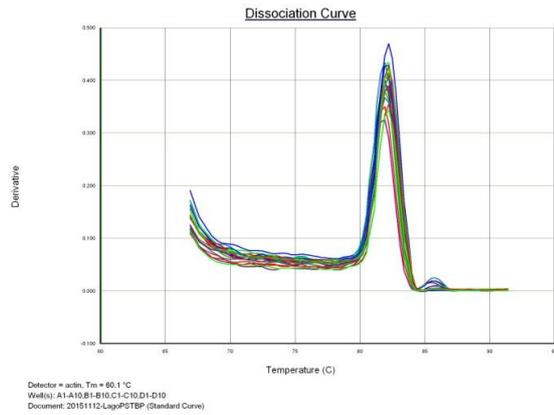
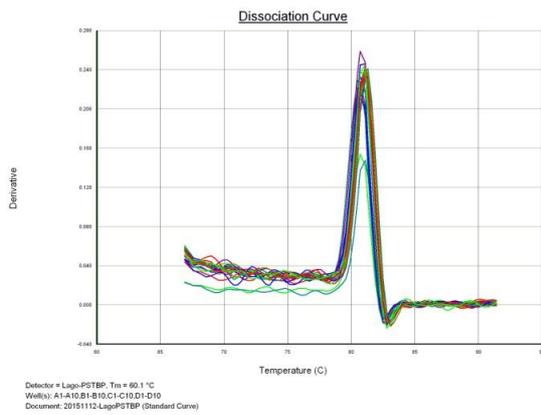
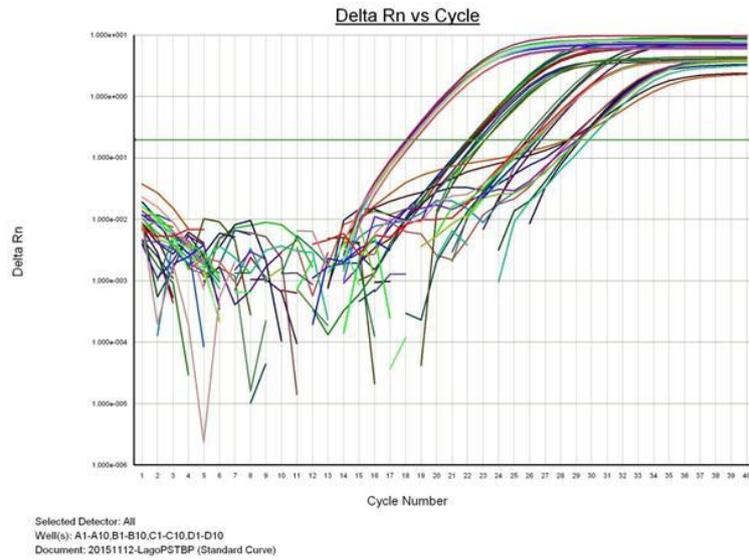
**Fig. 5-2** Nucleotide and deduced amino acid sequences of LsPSTBP cDNA. The letters in boxes (Asterisk) represented initiation codon (ATG) and termination codon (TGA). Solid lined boxes and underlined amino acids indicate potential N-linked glycosylation and half-Cys sites, respectively. Signal peptide was underlined and showed with italic letters.



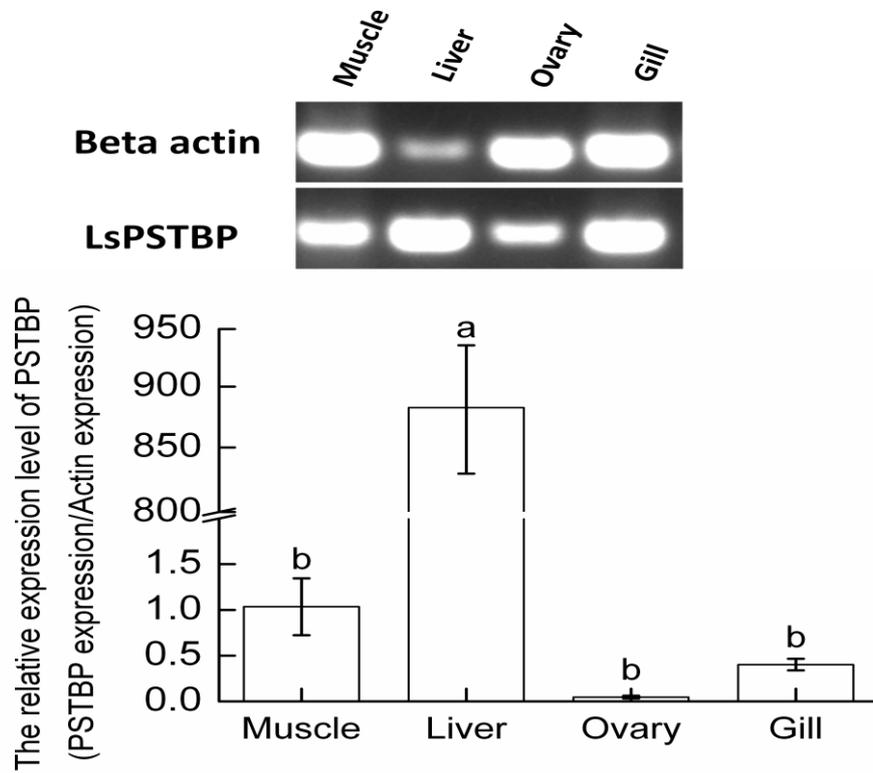
**Fig. 5-3** Multiple sequence alignment of deduced amino acid sequences of LsPSTBP with those of the homologous proteins. Multiple sequence alignments were performed using CLUSTAL W. The positions of conserved amino acid residues marked with yellow. Potential N-glycosylation sites showed with blue. Predicted secondary structures showed on the top of the residues. Half-Cys sites showed with red.



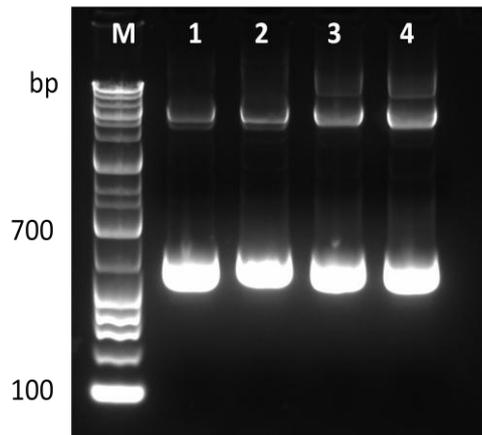
**Fig. 5-4** Neighbor-joining phylogenetic tree of TBT-p and PSTBP amino acid sequences from 8 puffer fish species. Note: Numbers represent the bootstrap values.



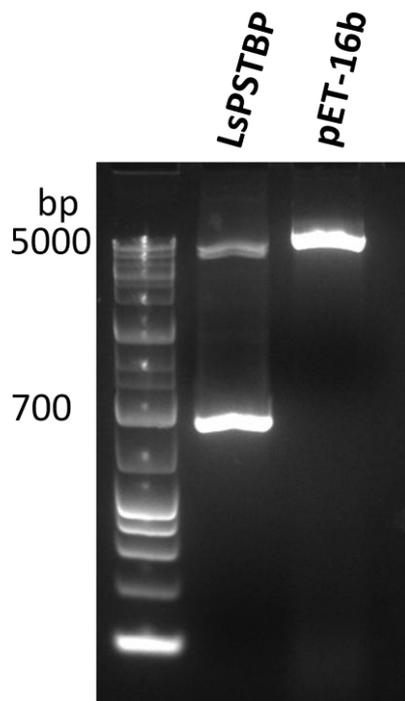
**Fig. 5-5** Melt-curve analysis of the qPCR products showed that the cDNA produced correct amplicons. Beta actin (left) and PSTBP (right).



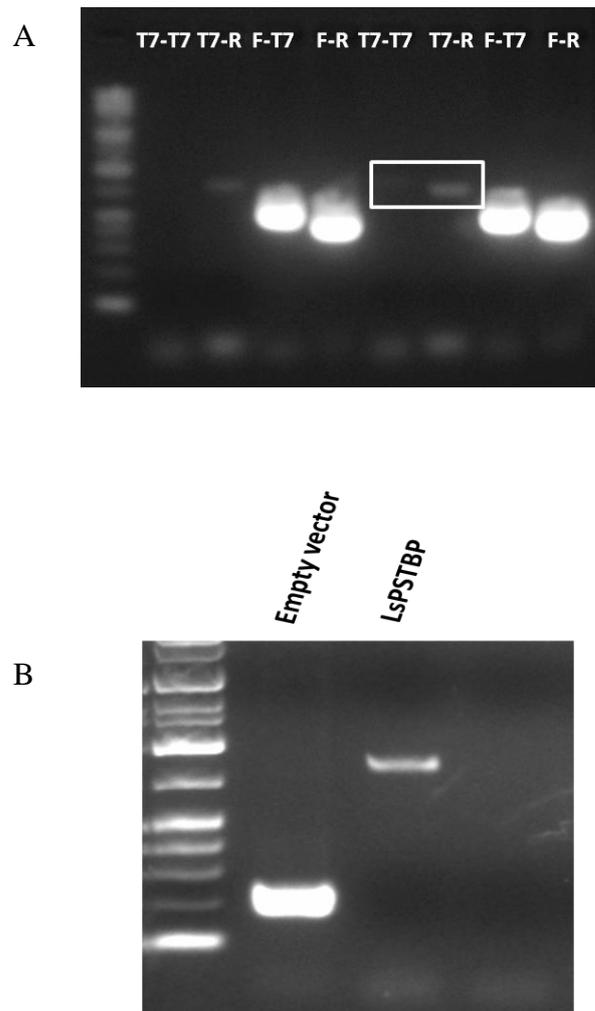
**Fig. 5-6** Tissue-specific expression of LsPSTBP mRNA in different tissues from *L. sceleratus* by real-time RT-PCR. Relative expressions were determined by  $2^{-\Delta\Delta Ct}$  method using beta actin as reference gene and presented as relative expression ratio. Vertical bars represented the mean  $\pm$  s.d. (n=5). Bars with different letters on their top indicate significant difference in expression level over the others when analyzed by Tukey's test (P<0.001).



**Fig. 5-7** Amplification results of PSTBP cDNA with restriction sites. 1 & 2: PSTBP with restriction sites; 3 & 4: PSTBP with gene-specific primers.



**Fig. 5-8** Digested PSTBP and pET-16b expression vector with *Nde* I and *BamH* I restriction enzymes.



**Fig. 5-9** Screening of ligation reaction and colony direct PCR. A: Ligation screening with vector (T7 promoter/T7 terminator) and gene-specific (Lago-PSTBP-M-F/R) primer pairs. B: PCR amplification of empty vector and constructed plasmid, LsPSTBP with vector specific primer pair.

## IPTG induction and optimization of protein expression

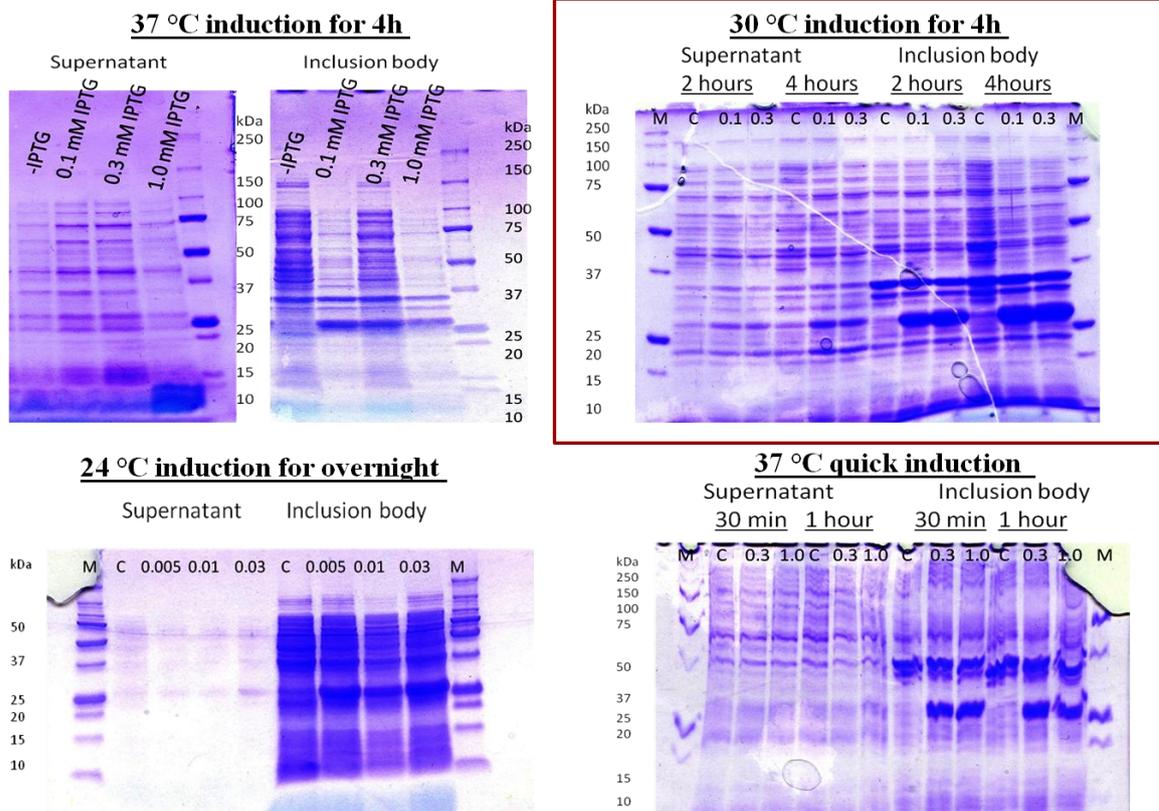
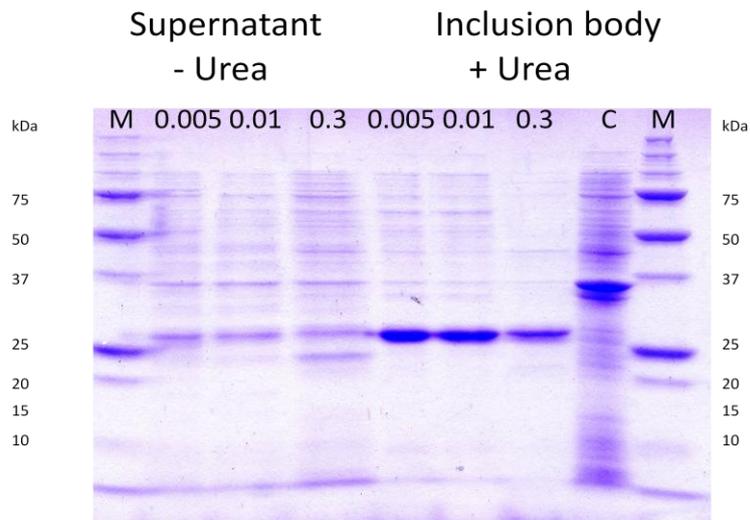
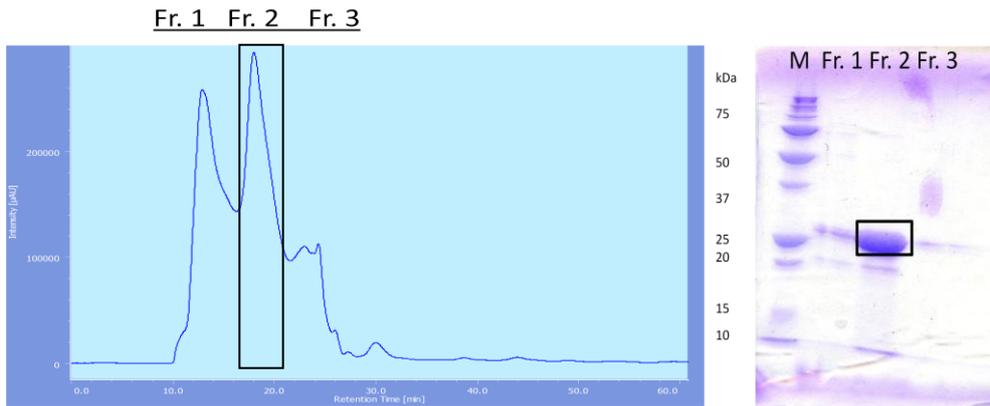


Fig. 5-10. SDS-PAGE analysis of different expression combinations. IPTG concentrations , temperature and different induction times are shown on the top of the each lanes. 30 °C for 4 h was chosen as an optimum condition and boxed with red square.

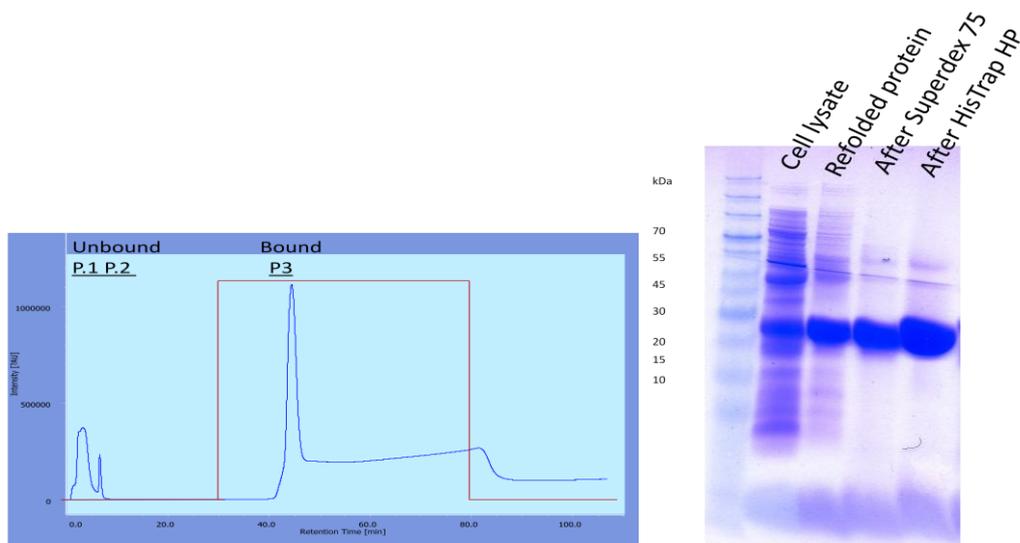


**Fig. 5-11.** SDS-PAGE analysis of crude protein extracts. Proteins were extracted from differently induced cells with/without urea treatment. IPTG concentrations are shown on the top of the each lanes. M: Molecular marker, C: Control, without IPTG induction.

1. Separation: Superdex 75 HPLC column elution profile of recombinant LsPSTBP. Mobile phase: 50mM sodium phosphate – 0.15 M NaCl (pH 7.0); Flow rate: 0.5 ml/min. Retention time: 60 min.



2. Purification: HisTrap HP column elution profile of Fr. 2 from Superdex 75 gel filtration chromatograph. Eluted with 250 mM imidazole; Flow rate: 0.5 ml/min. Retention time: 100 min.



**Fig. 5-12** Purification strategy and SDS-PAGE analysis of each purification step of recombinant LsPSTBP.

3. Separation: Superdex 75 HPLC column elution profile of recombinant LsPSTPB. Mobile phase: 50mM sodium phosphate – 0.15 M NaCl (pH 7.0); Flow rate: 0.5 ml/min. Retention time: 60 min.

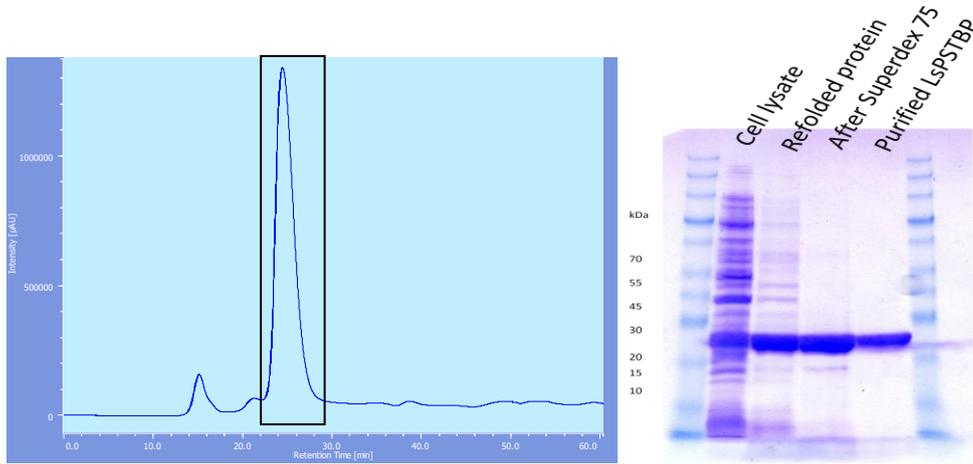
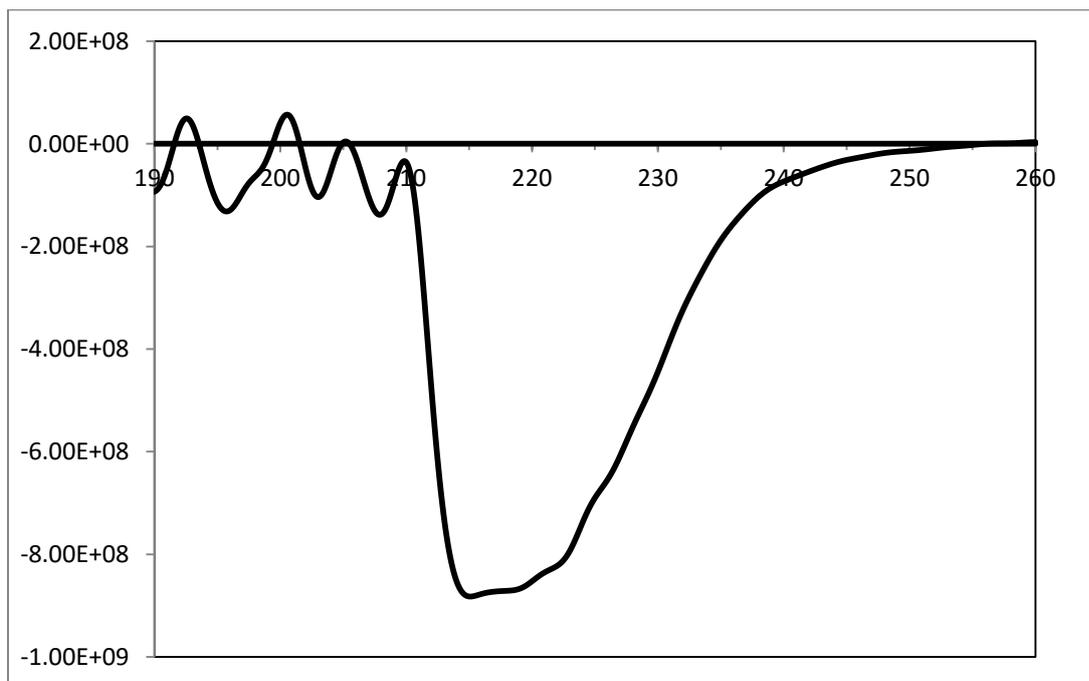


Fig. 5-12 Continued.



**Fig. 5-13.** Far-UV CD spectrum of recombinant LsPSTBP in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C.

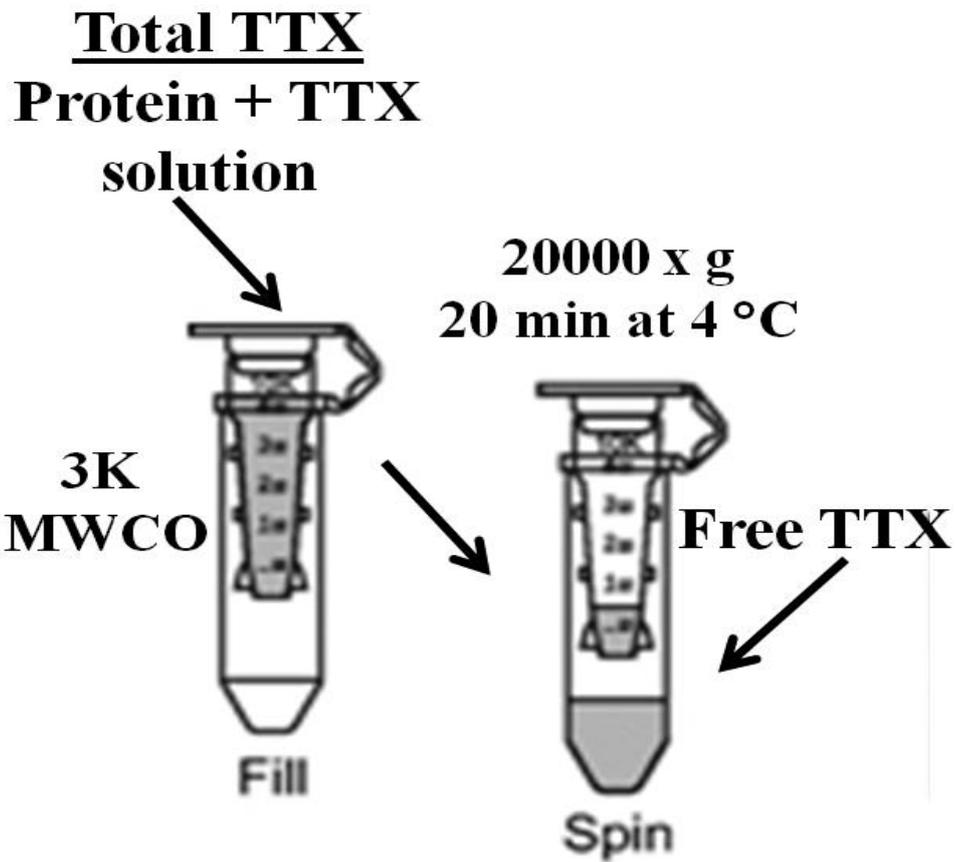


Fig. 5-14 Schematic diagram of separation of bound-free TTX.

# Chapter 6

## *General conclusion*

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Biodiversity was negatively effected by human hand and several problems have been recorded. Natural differences has been contrarily influenced by intrusive species and has turned into a worldwide issue against security endeavors. After the opening of Suez Canal in 1869, migrational development has happened from the Red Sea to the Mediterranean Sea. At present more than 300 aquatic species were relocated in the eastern Mediterranean system including 6 different puffer fish species. Several food safety issues have been caused after the consumption of tetrodotoxin-bearing puffer fish species such as *L. sceleratus*. It is therefore very important to clarify the potential risks, constructing the eliminating tools and understanding the toxin accumulation mechanism. In this study, toxicity of *L. sceleratus*, phylogeny and molecular identification of *L. sceleratus*, *L. suezensis* and *L. spadiceus* and TTX-binding assay of tributyltin-binding Type 2\_2 protein were studied. By combining molecular and chemical approach methods like LC-MS/MS, one step PCR and recombinant protein expression system, potential hazardous of puffer fish from Turkish Mediterranean coasts were elucidated and elimination methods were constructed. In addition, the relationship between TTX and TBT-p protein were clarified.

The results of toxicity of *L. sceleratus* have supported the public and international concerns related with food hygiene and safety. Here, I showed that the majority of the examples inspected in this study contained recognizable amount of TTX and TTX analogues.

In spite of the fact that testis, muscle and skin tissues moderately contained less toxicity comparing with the ovary and other internal organs, TTX contents were individual and tissue dependent. Although habitat of samples was completely different, there were no significant differences on toxicity between samples caught from Turkey and Japan. It is interesting to point out that, the profile of TTX analogues of different samples were highly concerned from both different sampling area. These findings have provided additional and important knowledge of toxin contents and accumulation of *L. sceleratus*.

The complete mitochondrial DNA (mtDNA) of *L. sceleratus*, *L. suezensis* and *L. spadiceus* were determined as 16,442, 16,494, and 16,508-bp in length, respectively. The structure of mtDNA of three species were typical among the puffer fish species. Furthermore, the phylogeny was reconstructed within the family Tetraodontidae. *L. sceleratus* and *L. suezensis* were more closely related species. The phylogenetic trees were highly similar with current molecular works. The results of characterization of mtDNA and phylogenetic analysis proved that genus *Lagocephalus* was evaluated within the separate clade. Therefore, the molecular identification of puffer fish species by mtDNA was clarified.

I have also provided the PCR based quick identification method. The differences of mtDNA nucleotide sequences were introduced to construct species-specific primer pairs, the one step PCR amplification method was determined. The bases in 119 position of NADH dehydrogenase subunit 2 (ND2) mtDNA region could differentiate *L. sceleratus*, however the bases in 123 and 138 position of cytochrome oxidase subunit 1 (COI) could successfully differentiate *L. suezensis* and *L. spadiceus*, respectively. These two mtDNA regions were widely used as genetic markers for identification of species in many fields. The selection of mitochondrial molecular markers bring several advantages. Mitochondrial

molecular markers, generally evolve much faster than nuclear markers, and besides there are several copies of mt DNA inside a cell and so that the abundance of stand is higher allowing more effective PCR amplifications in comparison to nuclear DNA. Hence, PCR amplification of selected mtDNA fragments, ND2 and COI by species-specific primers is a powerful and convenient technique for the identification of fish species, because of its simplicity, specificity, sensitivity and inexpensiveness.

The accumulation mechanism of TTX was also studied. The purification of binding protein from the ovary of *L. sceleratus* showed that that TTX-binding ability was weak and destroyed during the gel filtration purification process. At present, the exact reasons of binding ability of TTX to protein are not clear. Hence, puffer fish saxitoxin and tetrodotoxin binding protein (PSTBP) was cloned, from cDNA obtained from *L. sceleratus* liver. The characteristics of the protein (LsPSTBP) showed high similarity with those other known PSTBP and TBT-p binding proteins. Moreover, recombinant LsPSTBP protein was expressed and purified in *E. coli*. The secondary structure of refolded and purified recombinant protein was conserved among the other lipocalin family members. TTX binding ability assay was proved that recombinant TBT-p protein had binding ability to TTX and purified LsPSTBP binding ability was approximately 10%. This result indicates that PSTBP might have an important role on TTX accumulation in genus *Lagocephalus* but not only *Takifugu*.

In conclusion, the studies conducted provided on the essential role of risk assessment and elimination of toxic puffer fish species for the eastern Mediterranean fishery. The toxicity results revealed in this study was highly concerned with previous reports, and also supported to public concerns about toxic Lessepsian puffer fish species. It could be concluded from the present research that consumption of Lessepsian puffer fish species can be caused a high risk for human. Further studies of toxin accumulation mechanism is still needed.

## References

- Akyol, O., Ünal, V., Ceyhan, T., Bilecenoglu, M., 2005. First record of the silverside blaasop, *Lagocephalus sceleratus* (Gmelin, 1789), in the Mediterranean Sea. *Journal of Fish Biology* 66, 1183–1186.
- Alfaro, M.E., Santini, F., Brock, C.D., 2007. Do reefs drive diversification in marine teleosts? Evidence from the pufferfishes and their allies (order Tetraodontiformes). *Evolution* 61, 2104–2126.
- Ali, A.E., Gomaa, M., Othman, H., 2011. Toxicological Studies on Puffer Fishes, *Lagocephalus sceleratus* and *Amblyrhynchotes hypeslogenion* in Suez Gulf, Red Sea, Egypt. *Egyptian Journal of Aquatic Research*, 37, 123-130.
- Arcila, D., Pyron, R.A., Tyler, J.C., Orti, G., Betancur-R, R., 2015. An evaluation of fossil tip-dating versus node-age calibrations in tetraodontiform fishes (Teleostei: Percomorphaceae). *Molecular Phylogenetics and Evolution* 82, 131-145.
- Asahida, T., Kobayashi, T., Saitoh, K., Nakayama, I., 1996. Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing of urea. *Fisheries Science* 62, 727–730.
- Bax, N., Williamson, A. Agüero, M., Gonzalez, E., Geeves, W., 2003. Marine invasive alien species: a threat to global biodiversity. *Marine Policy* 27, 313–323.
- Asensio, L., González, I., García, T., *et al.*, 2008. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control* 19, 1–8. doi:10.1016/j.foodcont.2007.02.010.

Aydin, M., 2011. Growth, Reproduction and Diet of puffer fish (*Lagocephalus sceleratus* Gmelin, 1789) from Turkey's Mediterranean Sea Coast. Turkish Journal of Fisheries and Aquatic Sciences., 11, 589-596.

Battisti, C.D., Marciano, S., Magnabosco, C., Busato, S., Arcangeli, G., Cattoli, G., 2014. Pyrosequencing as a tool for rapid fish species identification and commercial fraud detection. J. Agric Food Chem, 62, 1985-205.

Bax, N., Williamson, A. Agüero, M., Gonzalez, E., Geeves, W. 2003. Marine invasive alien species: a threat to global biodiversity. Marine Policy 27, 313–323.

Benson, G., 1999. Tandem Repeats Finder: a program to analyze DNA sequences. Nucleic Acids Research 27, 573–580.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. Toxicon 52, 964–968.

Bilecenoglu, M., Taskavak, E., Kunt, KB., 2002. Range extension of three lessepsian migrant fish (*Fistularia commersoni*, *Sphyræna flavicauda*, *Lagocephalus suezensis*) in the Mediterranean Sea. J Mar Biol Ass UK 82, 525–526.

Boore, J.L., 1999. Animal mitochondrial genomes. Nucleic Acids Res. 27, 1767–1780.

Brenner, S., Elgar, G., Sandford, R., Macrae, A., Venkatesh, B., Aparico, S., 1993. Characterization of the pufferfish (fugu) genome as a compact model vertebrate genome. Nature 366, 265–268.

Broughton, R.E., Gold, J.E., 2000. Phylogenetic relationships in the North American Cyprinid genus *Cyprinella* (Actinopterygii: Cyprinidae) based on sequences of the mitochondrial ND2 and ND4L genes. *Copeia*, Vol. 2000, 1-10.

Caballero, S., Duchene, S., Garavito, M.F., Slikas, B., Baker, C.S., 2015. Initial evidence for adaptive selection on the NADG subunit two of freshwater dolphins by analyses of mitochondrial genomes. DOI: 10.1371/journal.pone.0123543

Chamandi, S.C., Kallab, K., Mattar, H., Nader, E., 2009. Human Poisoning after Ingestion of Puffer Fish Caught from Mediterranean Sea. Case Report - USEK Medical School - Lebanon.

Clayton, D.A., 1982. Replication of animal mitochondrial DNA. *Cell* 28, 693–705.

Corsini-Foka, M., Margies, P., Kondilatos, G., Economidis, P., 2006. *Torquigener flavimaculosus* Hardy and Randall, 1983 (Pisces: Tetraodontidae) off Rhodes island marine area: a new alien fish in the Hellenic waters. *Mediterranean Marine Science*, 7, 73-76.

Corsini-Foka, M., Margies, P., Kondilatos, G., Economidis, P.S., 2005. Lessepsian migration of fishes to the Aegean Sea: First record of *Tylerius spinosissimus* (Tetraodontidae) from Rhodes. *Cybium* , 347-354.

Crnogorac-Jurcevic, T., Brown, JR., Lehrach, H., Schalkwyk, LC., 1997. *Tetraodon fluviatilis*, a new puffer fish model for genome studies. *Genomics* 41, 177–184.

Cutarelli, A., Amoroso, M.G., De Roma, A., Girardi, S., Galiero, G., Guarino, A., Corrado, F., 2014. Italian market fish species identification and commercial frauds revealing by DNA sequencing. *Food Control*, 37, 46-50.

Diener, M., Christian, B., Ahmed, M.S., Luckas, B., 2007. Determination of tetrodotoxin and its analogues in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. *Analytical and Bioanalytical Chemistry*, 389, 1997–2002.

EastMed, 2010a. Report of the Sub-Regional Workshop on Collection and Organization of Data. Scientific and Institutional Cooperation to Support Responsible Fisheries in the EasternMediterranean. GCP/INT/041/EC – GRE – ITA/TD-03.

EastMed, 2010b. Report of the Sub-Regional Technical meeting on the Lessepsian migration and its impact on Eastern Mediterranean fishery. GCP/INT/041/EC – GRE – ITA/TD-04.

EastMed, 2010a. Report of the Sub-Regional Workshop on Collection and Organization of Data. Scientific and Institutional Cooperation to Support Responsible Fisheries in the EasternMediterranean. GCP/INT/041/EC – GRE – ITA/TD-03.

EastMed, 2010b. Report of the Sub-Regional Technical meeting on the Lessepsian migration and its impact on Eastern Mediterranean fishery. GCP/INT/041/EC – GRE – ITA/TD-04.

Elmerot, C., Arnason, U., Gojobori, T., Janke, A., 2002. The mitochondrial genome of the pufferfish, *Fugu rubripes*, and ordinal teleostean relationships. *Gene* 295, 163–172.

El-Sayed, M., Yacout, G. A., El-Samra, M., Ali, A., & Kotb, S. M., 2003. Toxicity of the Red Sea pufferfish *Pleuranacanthus sceleratus* ‘‘El-Karad’’. *Ecotoxicology and Environmental Safety*, 56, 367–372.

- Fernandez-Silva, P., Enriquez, J.A., Montoya, J., 2003. Replication and transcription of mammalian mitochondrial DNA. *Experimental Physiology* 88, 41–56.
- Fernández-Tajes, J., Freire, R., Méndez, J., 2010. A simple one-step PCR method for the identification between European and American razor clams species. *Food Chemistry*, 118, 995-998.
- Fournier, T., Medjoubi-N, N., Porquet, D., 2000. Alpha-1-acid glycoprotein. *Biochem. Biophys. Acta*, 1482, 157 – 171.
- Francino, M.P., Ochman, H., 1997. Strand asymmetries in DNA evolution. *Trends in Genetics* 13, 240–245.
- Freire, R., Arias, A., Méndez, J., Insua, A., 2011. Identification of European commercial cockles (*Cerastoderma edule* and *C. glaucum*) by species-specific PCR amplification of the ribosomal DNA ITS region. *Eur Food Res Technol*, 232, 83-86.
- Golani, D., Levy, Y., 2005. New records and rare occurrences of fish species from the Mediterranean coast of Israel. *Zoology in the Middle East* 36, 27–32.
- Grabowski, M., Grater, W.D., Stuck, K.C., 2004. A novel polymorphic mtDNA marker for population studies of the pink shrimp, *Farfantepenaeus duorarum* (Crustacea, Penaeidae). *Oceanologia* 46, 147–151.
- Gu, Y., Liu, Q., Chen, P., Guo, C., Liu, Y., Zhao, Y., Zhang, Y., Lin, D., 2013. Characterization of the oligomerization and ligand-binding properties of recombinant rat lipocalin 11. *Biochem. Biophys. Acta*, 1834, 1-7.
- Gutierrez, G., Ganfornina, M.D. & Sanchez, D. 2000. Evolution of the lipocalin family as inferred from a protein sequence phylogeny. *Biochem. Biophys. Acta*, 1482, 35 – 45.

- Hashimoto, K., Noguchi, T., Watabe, S., 1990. New aspect of tetrodotoxin. In: Pohland, A.E., *et al.*, (Eds.), *Microbial Toxins in Foods and Feeds*. Plenum Press, New York, pp. 159– 172.
- Hellberg, R.S.R., Morrissey, M.T., 2011. Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. *JALA*, 16, 308-321.
- Holcroft, N.I., 2005. A molecular analysis of the interrelationships of tetraodontiform fishes (Acanthomorpha: Tetraodontiformes). *Molecular Phylogenetics and Evolution* 34, 525–544.
- Hsieh, C.H., Chang, W.T., Chang, H.C., Hsieh, H.S., Chung, Y.H., Hwang, D.F., 2010. Puffer fish-based commercial fraud identification in a segment of cytochrome *b* region by PCR–RFLP analysis. *Food Chemistry*, 121, 1305-1311.
- Hsieh, H.S., Hwang, D.F., 2004. Molecular phylogenetic relationships of puffer fish inferred from partial sequences of cytochrome *b* gene and restriction fragment length polymorphism analysis. *J Agric Food Chem* 52, 4159–4165.
- Hubalkova, Z., Kralik, P., Tremlova, B., *et al.*, 2007. Methods of gadoid fish species identification in food and their economic impact in the Czech Republic: a review. *Vet Med* 52, 273–292.
- Irmak, Erhan, 2012. *Lessepsian puffer fishes on the Turkish coasts and their feeding properties*. PhD dissertation, Graduate School of Çanakkale Onsekiz Mart University. 84 pages.
- Ishizaki, S., Yokoyama, Y., Oshiro, N., Teruya, N., Nagashima, Y., Shiomi, K., Watabe, S., 2006. Molecular identification of pufferfish species using PCR amplification and restriction analysis of a segment of the 16S rRNA gene. *CBP, Part D* 1, 134-144.

Itoi, S., Yoahikawa, S., Tatsuno, R., Suzuki, M., Asahina, K., Yamamoto, S., Takanashi, S., Takatani, T., Arakawa, O., Sakakura, Y., Sugita, H., 2012. Difference in the localization of tetrodotoxin between the female and male pufferfish *Takifugu niphobles*, during spawning. *Toxicon* 60, 1000-1004.

IUCN - ISSG, 2008. *Aliens* 27, 1-30.

Ivanova, N.V., Zemalk Tyler, S., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, 7, 544-548.

Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. *Toxicon* 48, 980–987.

Jang, JH., Lee, JS., Yotsu-Yamashita, M., 2010. LC/MS Analysis of tetrodotoxin and its deoxy analogs in the marine puffer fish *Fugu niphobles* from the southern coast of Korea, and in the brackishwater puffer fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from southeast Asia. *Mar Drugs* 8, 1049–1058.

Jia, W., Higgs, P.G., 2008. Codon usage in mitochondrial genomes: distinguishing context-dependent mutation from translational selection. *Molecular Biology and Evolution* 25, 339–351.

Kalogirou, S., Corsini-Foka, M., Sioulas, A., Wennhage, H., Pihl, L., 2010. Diversity, structure and function of fish assemblages associated with *Posidonia oceanica* beds in an area of the Eastern Mediterranean Sea and the role of nonindigenous species. *Journal of Fish Biology*, 77, 2338-2351.

Kanoh, S., Noguchi, T., Maruyama, J., & Hashimoto, K., 1984. Toxicity of a pufferfish, “senninfugu” *Pleuranacanthus sceleratus*. *Nippon Suisan Gakkaishi*, 50, 2139.

Kao, C.Y., Furman, F.A., 1963. Pharmacological studies on tetrodotoxin, a potent neurotoxin. *J. Pharmacol. Exp. Ther.*, 140, 31–40.

Kartavtsev, Y.P., Jung, S.O., Lee, Y.M., Byeon, H.K., Lee, J.S., 2007. Complete mitochondrial genome of the bullhead torrent catfish, *Liobagrus obesus* (Siluriformes, Amblycipididae): Genome description and phylogenetic considerations inferred from the *Cytb* and 16S rRNA genes. *Gene* 396, 13–27.

Kasapidis, P., Peristeraki, P., Tserpes, G., Magoulas, A., 2007. First record of the Lessepsian migrant *Lagocephalus sceleratus* (Gmelin 1789) (Osteichthyes: Tetraodontidae) in the Cretan Sea (Aegean, Greece). *Aquatic Invasions* 2, 71–73.

Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A., Fotaras, T., 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon* 54, 50–55.

Kim, I., Cha SY., Lee, Ky., Lee KS., Choi, YS., Hwang, JS., Jin, BR., Han, YS., 2007. Polymorphism and genomic structure of the A + T-Rich region of mitochondrial DNA in the oriental mole cricket, *Gryllotalpa Orientalis* (Orthoptera: Gryllotalpidae). *Biochemical Genetics* 45, 589–610.

Klett, V., Meyer, A., 2002. What, if Anything, is a Tilapia?—Mitochondrial ND2 Phylogeny of Tilapiines and the Evolution of Parental Care Systems in the African Cichlid Fishes. *Mol. Bol. Evol.*, 19, 865-883.

Kodama, M., and Sato, S., 2005. Standard methods of analysis in food safety regulation. Chemistry, Ministry of Health, Labour and Welfare Ed., Japan Food Hygiene Association, Tokyo, Japan.

- Kodama, M., Ogata, T., Noguchi, T., Maruyama, J., Hashimoto, K., 1983. Occurrence of saxitoxin and other toxins in the liver of the pufferfish *Takifugu pardalis*. *Toxicon* 21, 897–900.
- Kono, M., Matsui, T., Furukawa, K., Takase, T., Yamamori, K., Kaneda, H., Aoki, D., Jang, J.H., Yotsu-Yamashita, M., 2008b. Examination of transformation among tetrodotoxin and its analogs in the living cultured juvenile puffer fish, kusafugu, *Fugu niphobles* by intramuscular administration. *Toxicon* 52, 714–720.
- Kono, M., Matsui, T., Furukawa, K., Yotsu-Yamashita, M., Yamamori, K., 2008a. Accumulation of tetrodotoxin and 4,9-anhydrotetrodotoxin in cultured juvenile kusafugu *Fugu niphobles* by dietary administration of natural toxic komonfugu *Fugu poecilonotus* liver. *Toxicon* 51, 1269–1273.
- Kvasnicka, F., 2005. Capillary electrophoresis in food authenticity. *J Sep Sci*, 28, 813–825.
- L, Whitmore., B.A., Wallace, 2004. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data, *Nucleic Acids Research*, 32, 668–673.
- Laslett, D., Canbäck, B., 2008. ARWEN, a program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. *Bioinformatics* 24, 172–175.
- Lin, W., Hwang, D., 2008. Application of species-specific PCR for the identification of dried bonito product (Katsuobushi). *Food Chemistry*, 106, 390-396.
- Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research* 25, 955–964.

Luekasemsuk, T., Panvisavas, N., Chaturongakul, S., 2015. TaqMan qPCR for detection and quantification of mitochondrial DNA from toxic pufferfish species. *Toxicon*, 102, 43-47.

Mackie, I. M., Pryde, S. E., González-Sotelo, C., Medina, I., Perez-Martín, R., Quinteiro, J., Rey-Mendez, M., & Rehbein, H. (1999). Challenges in the identification of species of canned fish. *Trends in Food Science & Technology*, 10(1), 9–14.

Maruyama, J., Noguchi, T., Narita, H., Jeon, J.K., Otsuka, M., Hashimoto, K., 1985. Occurrence of tetrodotoxin in a starfish, *Astropecten scoparius*. *Agric. Biol. Chem.*, 49, 3069–3070.

Matsui, T., Yamamori, K., Furukawa, K., Kono, M., 2000. Purification and some properties of a tetrodotoxin binding protein from the blood plasma of kusahogai, *Takifugu niphobles*. *Toxicon* 38, 463–468.

Matsuura, K., Tyler J.C., 1997. Tetraodontiform fishes, mostly from deep waters, of New Caledonia. No. 9. In: B. Séret (ed) Résultats des Campagnes MUSORSTOM, 17. Mémoires du Muséum National d'Histoire Naturelle, Paris (N. S.) (Sér A) Zool 174, 173-208.

Matsuura, K., 1982. A new triacanthodid fish, *Triacanthodes indicus* from the Indian Ocean. *Jpn J Ichthyol* 28, 385-392.

Matsuura, K., 2001. Triacanthodidae, Triacanthidae, Balistidae, Ostraciidae, Aracanidae, Triodontidae, Tetraodontidae. In: Carpenter K, Niem (eds) FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Vol 6. Bony fishes part 4 (Labridae to Latimeriidae). FAO, Rome, pp 3902-3928, 3948-3957.

Matsuura, K., 2015. Taxonomy and systematic of tetraodontiform fishes: a review focusing primarily on progress in the period from 1980 to 2014. *Ichtyol. Res.* 62, 72-113

Matsuura, K., Golani, D., Bogorodsky, SK., 2011. The First Record of *Lagocephalus guentheri* Miranda Ribeiro, 1915 from the Red Sea with notes on previous records of *L. lunaris* (Actinopterygii, Tetraodontiformes, Tetraodontidae). *Bull Natl Mus Sci Ser A* 37, 163–169.

McNabb, P., Selwood, A.I., Munday, R., Wood, S.A., Taylor, D.I., MacKenzie, A.L., van Ginkel, R., Rhodes, L.L., Cornelisen, C., Heasman, K., et al., 2010. Detection of tetrodotoxin from the grey sided-gilled sea slug-*Pleurobranchaea maculata*, and associated dog neurotoxicosis on beaches adjacent to the Hauraki Gulf, Auckland, New Zealand. *Toxicon*, 56, 466–473.

Michelini E, Cevenini L, Mezzanotte L *et al.*, 2007. One-step triplexpolymerase chain reaction assay for the authentication of yellowfin (*Thunnus albacares*), bigeye (*Thunnus obesus*), and skipjack (*Katsuwonus pelamis*) tuna DNA from fresh, frozen, and canned tuna samples. *J Agric Food Chem* 55:7638–7647.

Moore, J., Anderson, N., Narahashi, T., 1966. Tetrodotoxin blocking early conductance channel on sodium. *Fed. Proc. Am. Soc. Exp. Biol.*, 25, 569–572.

Nagashima, Y., Matsumoto, T., Kadoyama, K., Ishizaki, S., Terayama, M., 2011. Toxicity and molecular identification of green toadfish *Lagocephalus lunaris* collected from Kyushu Coast, Japan. *J. Toxicol.* Article ID 801285.

Nakagawa, T., Jang, J., Yotsu-Yamashita, M., 2006. Hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry of tetrodotoxin and its analogs. *Anal. Biochem.* 2006, 352, 142–144.

- Nakamura, M., Oshima, Y., Yasumoto, T., 1984. Occurrence of saxitoxin in puffer fish. *Toxicon* 22, 381–385.
- Nakayama, K., Sei, N., Oshima, Y., Tashiro, K., Shimasaki, Y., Honjo, T., 2008. Alteration of gene expression profiles in the brain of Japanese medaka (*Oryzias latipes*) exposed to KC-400 or PCB126. *Mar. Pollut. Bull.* 57, 460–466.
- Narahashi, T., 2001. Pharmacology of tetrodotoxin. *J. Toxicol. Toxin Rev.*, 20, 67–84.
- Nassef, M., Tawaratsumita, T., Oba, Y., Satone, H., Nakayama, K., Shimasaki, Y. *et al.*, 2011. Induction of tributyltin-binding protein type 2 in Japanese flounder, *Paralichthys olivaceus*, by exposure to tributyltin-d27. *Mar. Poll. Bull.*, 62, 412 – 414.
- Nei, M., Kumar, S., 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.
- Noguchi, T., Arakawa, O., 2008. Tetrodotoxin – distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar. Drug.* 6, 220-242.
- Noguchi, T., Arakawa, O., Takatani, T., 2006. TTX accumulation in pufferfish. *Comparative Biochemistry and Physiology, Part D* 1, 145–152.
- Noguchi, T., Ebesu, J.S.M., 2001. Puffer poisoning: epidemiology and treatment. *Journal of Toxicology – Toxin Reviews* 20, 1–10.
- Noguchi, T., Jeon, J.K., Arakawa, O., Sugita, H., Deguchi, Y., Shida, Y., Hashimoto, K., 1986. Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestine of a xanthid crab, *Atergatis floridus*. *J. Biochem.* 99, 311–314.

Noguchi, T., Maruyama, J., Ueda, Y., Hashimoto, K., Harada, T., 1981. Occurrence of tetrodotoxin in the Japanese ivory shell *Babylonia japonica*. Bull. Japan. Soc. Sci. Fish., 47, 901–913.

Oba, Y., Shimasaki, Y., Oshima, Y., Satone, H., Kitano, T., Nakao, M. *et al.*, 2007. Purification and characterization of tributyltin-binding protein type 2 from plasma of Japanese flounder, *Paralichthys olivaceus*. J. Biochem, 142, 229 – 238.

Ojala, D., Merkel, C., Gelfand, R., Attardi, G., 1980. The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. Cell 22, 393–403.

Oral, M., 2010. Alien Fish Species in the Mediterranean - Black Sea Basin. Journal of Black Sea/Mediterranean Environment. 16, 87-132.

Ozturk, B., 2010. Draft document on the alien species in the Mediterranean and the Black Sea. General Fisheries Commission for the Mediterranean, Scientific Advisory Committee. GFCM-SAC 12.

Pappalardo, A.M., Ferrito, V., 2015. DNA barcoding species identification unveils mislabeling of processed flatfish products in southern Italy markets. Fisheries Research, 164, 153-158.

Peng, Z., Wang, J., He, X., 2006. The complete mitochondrial genome of the helmet catfish *Cranoglanis boudierius* (Siluriformes: Cranoglanididae) and the phylogeny of otophysan fishes. Gene 376, 290–297.

Peristeraki, P., Lazarakis, G., Skarvelis, C., Georgiadis, M., Tserpes, G., 2006. Additional Records on the occurrence of alien fish species in the eastern Mediterranean Sea. Mediterranean Marine Science. 7/2:61-66.

Perna, N.T., Kocher, T.D., 1995. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. *Journal of Molecular Evolution* 41, 353–358.

Por, F., D., 1978. Lessepsian migration: the influx of Red Sea biota into the Mediterranean by way of the Suez Canal. *Ecological studies*. Berlin: Springer-Verlag: 23: 228.

Puilingi, C.G., Kudo, Y., Cho, Y., Konoki, K., Yotsu-Yamashita, M., 2015. Tetrodotoxin and its analogues in the pufferfish *Arothron hispidus* and *A. nigropunctatus* from the Solomon Islands: A comparison of their toxin profiles with the same species from Okinawa, Japan. *Toxins*, 7, 3436-3454; doi:10.3390/toxins7093436.

Rasmussen, R. S., & Morrissey, M. T., 2008. DNA-based methods for the identification of commercial fish and seafood species. *Comprehensive Reviews in Food Science and Food Safety*, 7, 280-295.

Rehbein, H., 1990. Electrophoretic techniques for species identification of fishery products. *Z Lebensm Unters Forsch* 191, 1–10.

Reza, MD. S., Furukawa, S., Mochizuki, T., Matsumura, H., Watabe, S., 2008. Genetic comparison between torafugu *Takifugu rubripes* and its closely related species karasu *Takifugu chinensis*. *Fisheries Science* 74, 743–754.

Rodríguez, P., Alfonso, A., Otero, P., Katikou, P., Georgantelis, D., Botana, L.M., 2012. Liquid chromatography–mass spectrometry method to detect Tetrodotoxin and Its analogues in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters. *Food Chemistry*, 132, 1103-1111.

Sabrah, M., El-Ganainy, A., Zaky, M., 2006. Biology and toxicity of the Puffer fish *Lagocephalus sceleratus* From the Gulf of Suez. Egyptian Journal of Aquatic Research, 32, 283-297.

Sangthonga, P., Ngernsiria, L., Sangthong, D., 2014. Identification of puffer fish of the genus *Lagocephalus*: *L. lunaris*, *L. spadiceus* and *L. inermis*, using multiplex PCR. Food Biotechnology, 28, 216-231.

Santaclara, F.J., Espiñeira, M., Vieites, J.M., 2007. Genetic identification of squids (families *Ommastrephidae* and *Loliginidae*) by PCR-RFLP and FINS methodologies. Journal of Agricultural and Food Chemistry, 55, 9913–9920.

Santini, F., Nguyen, M.T.T., Sorenson, L., Waltzek, T.B., Lynch Alfaro, J.W., Eastman, J.M., Alfaro, M.E., 2013. Do habitat shifts drive diversification in teleost fishes? An example from the pufferfishes (Tetraodontidae). Journal of Evolutionary Biology 26, 1003–1018.

Santini, F., Sorenson, L., Alfaro, M.E., 2013b. A new phylogeny of tetraodontiform fishes (Tetraodontiformes, Acanthomorpha) based on 22 loci. Molecular Phylogenetics and Evolution 69, 177-187.

Satone, H., Lee, J.M., Oba, Y., Kusakaba, T., Akahoshi, E., Shizuho, M., Suzuki, N., Sasayama, Y., Nassef, M., Shimasaki, Y., Kawabata, S., Honjo, T., Oshima, Y., 2011. Tributyltin-binding protein type 1, a lipocalin, prevents inhibition of osteoblastic activity by tributyltin in fish scales. Aquat. Toxicol. 103, 79–84.

Satone, H., Oshima, Y., Shimasaki, Y., Tawaratsumida, T., Oba, Y., Takahashi, E., Kitano, T., Kawabata, S., Kakuta, Y., Honjo, T., 2008. Tributyltin-binding protein type 1 has a distinctive lipocalin-like structure and is involved in the excretion of tributyltin in Japanese flounder, *Paralichthys olivaceus*. Aquat. Toxicol. 90, 292–299.

Schein, C.H. and Noteborn, M.H.M, 1989. *Bio/Technology* 7, 1141–1148.

Sheumack, D.D., Howden, M.E.H., Spence, I., Quinn, R.J. 1978. Maculotoxin: A Neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science*, 199, 188–189.

Shimasaki, Y., Oshima, Y., Yokota, Y., Kitano, T., Nakao, M., Kawabata, S.-I. *et al.*, 2002. Purification and identification of a tributyltin-binding protein from serum of Japanese flounder, *Paralichthys olivaceus*. *Env. Toxicol. Chem*, 2, 1229 – 1235.

Song, L., Liu, B., Xiang, J., Qian, P., 2001. Molecular phylogeny and species identification of pufferfish of the genus *Takifugu* (Tetraodontiformes, Tetraodontidae). *Marine Biotechnology* 3, 398–406.

Strauss, R.E., Bond, C.E., 1990. Taxonomic methods: morphology. In: Schreck CB, Moyle PB (eds) *Methods for fish biology*. American Fisheries Society, Maryland, pp 109–140.

Streftaris, N., Zenetos, A., 2006. Alien marine species in the Mediterranean - The 100 'Worst invasives' and their impact. *Mediterranean Marine Science*, 7, 87-118.

Tamura, K., Stecher, G., Peterson, D., Filipiński, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30, 2725–2729.

Tatsuno, R., Yamaguchi, K., Takatani, T., Arakawa, O., 2013. RT-PCR and MALDI-TOF mass spectrometry-based identification and discrimination of isoforms homologues to pufferfish saxitoxin and tetrodotoxin-binding protein in the plasma of non-toxic cultured pufferfish (*Takifugu rubripes*). *Biosci. Biotechnol. Biochem.*, 77, 208-213.

Teletcha, F., 2009. Molecular identification methods of fish species: reassessment and possible applications. *Reviews in Fish Biology and Fisheries* 19, 265–293.

- Tognoli, C., Saroglia, M., Terova, G., Gornati, R., Bernardini, G., 2011. Identification of fish species by 5S rRNA gene amplification. *Food Chemistry*, 129, 1860-1864.
- Tuncer, S., Cihangir, H.A., Bilecenoglu, M., 2008. First record of the Lessepsian migrant *Lagocephalus spadiceus* (Tetraodontidae) in the Sea of Marmara. *Cybius*, 32, 347-348.
- Turan, C., 2010. Status and trend of Lessepsian species in marine waters of Turkey. *FAO EastMed Technical Documents*. 04, 109–118.
- Tyler, J.C., 1980. Osteology, phylogeny, and higher classification of the fishes of the order Plectognathi (Tetraodontiformes). *NOAA Technical Report NMFS Circular 434*, 1–422.
- Volz, D.C., Bencic, D.C., Hinton, D.E., Law, J.M., Kullman, S.W., 2005. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces organ-specific differential gene expression in male Japanese medaka (*Oryzias latipes*). *Toxicol. Sci.* 85, 572–584.
- Wan, X., Li, J., Kim, M.J., Kang, T.H., Jin, B.R., Kim, I., 2011. Population genetic structure of the migratory rice leaf roller, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), inferred from the mitochondrial A + T-rich region and nuclear ITS2 sequences. *Genetic and Molecular Research* 10, 273–294.
- Wang, C., Chen, Q., Lu, G., Xu, J., Yang, Q., Li, S., 2008. Complete mitochondrial genome of the grass carp (*Ctenopharyngodon idella*, Teleostei): Insight into its phylogenetic position within Cyprinidae. *Gene* 424, 96–101.
- Wolstenholme, D.R., 1992. Animal mitochondrial DNA: structure and evolution. *International Review of Cytology* 141, 173–216.

Yamanoue, Y., Miya, M., Doi, H., Mabuchi, K., Sakai, H., Nishida, M., 2011. Multiple invasions into freshwater by pufferfishes (Teleostei: Tetraodontidae): A mitogenomic perspective. PLoS ONE 6: e17410. doi:10.1371/journal.pone.0017410.

Yamanoue, Y., Miya, M., Inoue, J.G., Matsuura, K., Nishida, M., 2006. The mitochondrial genome of spotted green pufferfish *Tetraodon nigroviridis* (Teleostei: Tetraodontiformes) and divergence time estimation among model organisms in fishes. Genes and Genetic Systems 81, 29–39.

Yamanoue, Y., Miya, M., Matsuura, K., Miyazawa, S., Tsukamoto, M., Doi, H., Takahashi, H., Mabuchi, K., Nishida, M., Sakai, H., 2009. Explosive speciation of *Takifugu*: Another use of fugu as a model system for evolutionary biology. Molecular Biology and Evolution 26, 623–629.

Yang, F., Du, Y., Cao, J., Huang, F., 2013. Analysis of three leafminers' complete mitochondrial genomes. Gene 529, 1–6.

Yasumoto, T., Yasumura, D., Yotsu, M., Michishita, T., Endo, A., Kotaki, Y., 1986. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. Agric. Biol. Chem. 50, 793–795.

Yasumura, D.; Oshima, Y.; Yasumoto, T.; Alcala, A.C.; Alcala, L.C., 1986. Tetrodotoxin and paralytic shellfish toxins in Philippine crabs. Agric. Biol. Chem., 50, 593–598.

Yokoo, A., 1950. Chemical studies on tetrodotoxin Rept. III. Isolation of spheroidine. J. Chem. Soc. Japan, 71, 591–592.

Yotsu-Yamashita, M., 2001. Chemistry of puffer fish toxin. J. Toxicol. Toxin Rev., 20, 51–66.

Yotsu-Yamashita, M., Abe, Y., Kudo, Y., Ritson-Williams, R., Paul, V.J., Konoki, K., Cho, Y., Adachi, M., Imazu, T., Nishikawa, T., *et al.*, 2013. First identification of 5,11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. *Mar. Drugs*, 11, 2799–2813.

Yotsu-Yamashita, M., Shoji, Y., Terakawa, T., Yamada, S., Miyazawa, T., Yasumoto, T., 2002. Mutual binding inhibition of tetrodotoxin and saxitoxin to their binding protein from the plasma of the puffer fish, *Fugu pardalis*. *Biosci. Biotechnol. Biochem.* 66, 2520–2524.

Yotsu-Yamashita, M., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T., Yasumoto, T., 2001. Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, *Fugu pardalis*. *Eur. J. Biochem.* 268, 5937–5946.

Zenetos, A., Cinar, M., Papadopoulou, M. P., Harmelin, J., Furnari, G., Andaloro, F., *et al.*, 2005. Annotated list of marine alien species in the Mediterranean with records of. *Mediterranean Marine Science*, 6, 63-118.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31, 3406–3415.