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Abstract: Pufferfish may be responsible for human intoxications due to the accumulation of a potentially lethal neurotoxin, called tetrodotoxin (TTX). While traditionally some species of Pufferfish are consumed in Japan, their marketing is banned in the EU. However, their illegal presence in mislabelled products has been reported. Moreover, some species of the genus Lagocephalus spread in the Mediterranean Sea during the last decades due to the Lessepsian migration phenomenon and they may represent a significant emerging risk within the European seafood chain. This study aimed at finding a suitable molecular marker for quickly identifying Lagocephalus species in fresh and processed products. All the available sequences of COI and cytb mitochondrial genes were used to create different length datasets (long and short fragments) to be used to produce NJ trees depicting genetic relationships for Lagocephalus spp. The cytb was selected as molecular target and 17 new complete sequences of 6 Lagocephalus species, deriving from reference samples, were produced and included in the datasets. Then, a primer pair for amplifying a ~130bp cytb polymorphic fragment from all the Lagocephalus spp. was projected for identifying sixteen mislabelled commercial products with degraded DNA containing pufferfish. Cytb dataset's phylogenetic analysis supported the most recent species classification of the Lagocephalus genus and highlighted the presence of the toxic L. spadiceus in the products. The analysis of the proposed short fragment could represent a reliable tool to protect European consumers from emerging risk associated to toxic Lagocephalus spp.

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2 ***cephalus* spp. in fresh and processed products**

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Abstract

Pufferfish may be responsible for human intoxications due to the accumulation of a potentially lethal neurotoxin, called tetrodotoxin (TTX). While traditionally some species of Pufferfish are consumed in Japan, their marketing is banned in the EU. However, their illegal presence in mislabelled products has been reported. Moreover, some species of the genus *Lagocephalus* spread in the Mediterranean Sea during the last decades due to the Lessepsian migration phenomenon and they may represent a significant emerging risk within the European seafood chain. This study aimed at finding a suitable molecular marker for quickly identifying *Lagocephalus* species in fresh and processed products. All ~~the~~ available sequences of *COI* and *cytb* mitochondrial genes were used to create different length datasets (long and short fragments) to be used to produce NJ trees depicting genetic relationships for *Lagocephalus* spp. On the basis of its higher variability, ~~The~~ *cytb* gene was selected as the molecular target and 17 new complete sequences of 6 *Lagocephalus* species, deriving from reference samples, were produced and included in the datasets. Then, a primer pair for amplifying a ~130bp *cytb* polymorphic fragment from all of the *Lagocephalus* spp. was ~~projected~~ designed for identifying sixteen mislabelled commercial products with degraded DNA containing pufferfish. *Cytb* dataset's phylogenetic analysis supported the most recent species classification of the *Lagocephalus* genus and highlighted the presence of ~~the~~ toxic *L. spadiceus* in the products. The analysis of the proposed short fragment could represent a reliable tool to protect European consumers from the emerging risk associated to toxic *Lagocephalus* spp.

Keywords

Pufferfish; *cytb* gene; *COI* gene; species identification; mislabelling; toxic species; TTX

53 **1. Introduction**

54 Pufferfish (Tetraodontidae) have been known for a long time to be toxic to humans, as they
55 naturally harbour a heat-stable neurotoxin, called tetrodotoxin (TTX), which is potentially lethal if
56 ingested in sufficient quantities (Bane, Lehane, Dikshit, O'Riordan, & Furey, 2014). In addition,
57 some species are even reported to accumulate saxitoxin as well (Landsberg et al., 2006). Despite
58 this, some species of pufferfish (*fugu*) represent a traditional delicacy in Asian countries, especially
59 in Japan, where their consumption is allowed by cutting the head and separating the most hazardous
60 parts of the fish (liver, ovaries, intestine and skin), where the TTX is typically concentrated
61 (Noguchi, Onuki, & Arakawa, 2011). Whereas in Japan a specific training for preparing puffer fish
62 is required, non-toxic species are often freely consumed without such training in other countries. In
63 Taiwan, only *Lagocephalus gloveri* and *L.~~agocephalus~~ wheeleri* can be marketed and used for the
64 preparation of dry-dressed fish fillets (Hsieh et al., 2010). In China, the sale of fresh pufferfish is
65 banned, and ~~the~~ national aquaculture generally feeds the export market (Mouquan, 2015). However,
66 the latest regulations allow the species *Takifugu rubripes* and *Takifugu obscurus* to be farmed by
67 certified companies and sold after being processed, with a code on the package to track the
68 products' origin (Mouquan, 2015; Ningning, 2017). In the United States (US), only the importation
69 of *T. rubripes* from Japan is allowed, even if regulated by a specific set of conditions ~~(Cohen et al.,~~
70 ~~2009)~~ , while all other species from all other countries are not allowed for importation. Domestic
71 harvest is allowed, but only for non-toxic species (Cohen et al., 2009). Contrariwise, the European
72 Union (EU) states that fishery products derived from species of the family Tetraodontidae must not
73 be placed on the market at all (Regulation (EC) n. 854/2004). However, consumers can still be at
74 risk of TTX poisoning through the consumption of mislabelled products illegally sold on the
75 internal market.

76 Globally, the largest number of mislabelling cases have been reported in Asia (Hwang &
77 Noguchi, 2007) due to the fraudulent use of pufferfish in products labelled with more appealing
78 names such as "*gadus*" or "cod" (Li et al., 2015; Xiong et al., 2016; Xiong et al., 2017), "mullet

79 roe" (Hsieh, Hwang, Pan, Chen, & Hwang, 2003) and "angelfish" (Li et al., 2015). Cases of
80 fraudulent substitution occurred also in the US and in the EU market. In 2007, two individuals from
81 Chicago developed symptoms consistent with TTX poisoning after eating frozen fish labelled as
82 "gutted and head-off monkfish" (*Lophius* spp.) which was instead firstly molecularly identified as
83 *Lagocephalus spp.* based on a COI analysis ~~*L.agocephalus* spp.*sceleratus*~~ (Cohen et al., 2009) and
84 then morphologically ~~confirmed~~ considered to be *L. lunaris* (Cole et al., 2015). In 2015, a molecular
85 study conducted on ethnic seafood collected from the Italian market revealed the presence of
86 *Lagocephalus* spp. in products labelled as "squid" (Armani et al., 2015a). In both cases, the
87 involved products originated from China. In 2009, during an official control carried out by the
88 Italian Local Health Unit (LHU) of Empoli (Italy), one batch of processed fish imported in Italy
89 from Spain and labelled as *Uranoscopus sp.* (stargazer) was ~~found~~ molecularly identified as
90 composed by the puffer fish *L. laevigatus* and *S. pachygaster* ~~during an official control carried out~~
91 ~~by the Italian Local Health Unit (LHU) of Empoli (Italy)~~ (Barontini, Bossù, Campagna, &
92 Lorenzetti, 2010).

93 Currently, European consumers could also be at risk if toxic lessepsian species that are by-caught
94 during commercial fishing accidentally enter the food chain ~~(Andaloro, Falautano, Perzia,~~
95 ~~Maricchiolo, & Castriota, 2012)~~, as already reported in other countries. In fact, episodes of
96 intoxication due to the unaware consumption of toxic *L. sceleratus* locally fished in Egypt ~~(Zaki,~~
97 ~~2004)~~, Israel ~~(Bentur et al., 2008; Eisenman, Rusetski, Sharivker, Yona, & Golani, 2008; Kheifets,~~
98 ~~Rozhavsky, Solomonovich, Z., Marianna, & Soroksky, 2012),~~ Lebanon ~~(Chamandi, Kallab, Mattar,~~
99 ~~& Nader, 2009)~~ and Turkey ~~(Beköz, Beköz, Yilmaz, Tüzün, & Beköz, 2013)~~ have been reported
100 during the last fifteen years (Guardone et al., 2018). In addition to the spread of *L. sceleratus*, the
101 diffusion in the Mediterranean Sea of other *Lagocephalus* species is known (Acar, Ishizaki, &
102 Nagashima, 2017; Farrag, El-Haweet, & Moustafa, 2016).

103 Although each *Lagocephalus* species has a set of distinctive morphological features, a proper
104 identification could be difficult to untrained eyes, since some of them appear very similar (Matsuura,

Golani, & Bogorodsky, 2011). For instance, a recent study (Giusti et al., ~~under review~~2018) reported that the species *L. spadiceus* and *L. guentheri* inhabiting the Mediterranean waters were sometimes confused. Even the taxonomic classification of this genus still appears confused (Tuney, 2016) and its systematics is not entirely clear yet. The official finfishes' database (www.fishbase.org) factually lacks up-to-date and exact information, although updated scientific literature is currently available (Matsuura, 2010; Matsuura & Satoh, 2017). The major issue concerns which *Lagocephalus* species should be considered as valid. While eleven species (*L. cheesemanii, L. gloveri*, *L. guentheri*, *L. inermis*, *L. laevigatus*, *L. lagocephalus*, *L. lunaris*, *L. sceleratus*, *L. spadiceus, L. suezensis, L. wheeleri*) are listed in the database, according to some studies the actual species number may be reduced to nine. In fact, a study by Matsuura (2010), based on morphological recognition, argued that *L. wheeleri* is a junior synonym of *L. spadiceus,* while a recent work applying both morphological and genetic comparisons proved that *L. cheesemanii* is a senior synonym of *L. gloveri* (Matsuura & Satoh, 2017). Species identification is impossible in processed products, where the morphological features entirely lack.

DNA-based methods are nowadays the most applied techniques for seafood species identification in processed products. The most used molecular markers are the mitochondrial genes 16S ribosomal RNA (*16SrRNA*), cytochrome b (*cytb*) and cytochrome oxidase subunit I (*COI*) (Armani, Castigliego, & Guidi, 2012), which have already been used ~~also~~ for pufferfish species identification. PCR and sequencing of the *16SrRNA* was applied by Song, Liu, Xiang, & Qian (2001) and Tuney (2016), to discriminate some species belonging to the genus *Takifugu* and *Lagocephalus.* However, the high conservation degree of the *16SrRNA* hardly qualified it as a suitable marker in pufferfish inter-species discrimination. As regard the COI gene, ~~Similarly,~~ a low sequences variability was observed ~~for the *COI* gene~~ by ~~(~~Huang et al., (2014~~.), which was also recently~~ In other cases the DNA-barcoding approach, based on the BOLD database, ~~shown as~~ resulted poorly efficient in discriminating between *Lagocephalus* spp. ~~using DNA-barcoding analysis~~ (Armani et al., 2015a; Cohen et al., 2009; Xiong et al., 2016; Xiong et al., 2018).

Contrariwise, *cytb* is nowadays reported as the most suitable target given its proven high mutation rate between closely related pufferfish species. Huang et al. (2014) found that a short polymorphic *cytb* fragment was able to discriminate among some Taiwanese pufferfish species (almost all different from those present in the Mediterranean Sea), in processed products. To the best of our knowledge, no studies aimed at discriminating all the *Lagocephalus* species are reported. The most comprehensive one is the work of Santini et al. (2013) in which a multi-genic approach (*cytb* and *COI* coupled with other mtDNA and nDNA genes) was used for discriminating eight *Lagocephalus spp.*, coupled with many other puffer fish species. However, the use of several molecular targets undoubtedly makes the analysis more laborious in term of both time and costs.

Since workflow's speeding up and costs' decreasing are key factors in food control analysis, the present study aimed at discriminating all the species belonging to the genus *Lagocephalus* using a single molecular marker. Initially, since neither *COI'*s nor *cytb'*s gene inter-species discrimination capability on all the *Lagocephalus* spp. had been tested yet, both genes were selected to conduct a preliminary phylogenetic analysis using all the available sequences retrieved from the official databases. The average inter-species variability among the collected sequences of both the target genes was calculated to select the most suitable for this purpose. Then, the ability of a short *cytb* fragment located in the pufferfish polymorphic region was tested to assess the actual species identity in ~~some~~ processed products (characterized by degraded DNA) that were previously identified at genus level (*Lagocephalus* spp.) using the *COI* gene DNA-barcoding approach~~marker~~. Considering the toxicity degree varies according to the species, a proper identification method is in fact essential to protect consumers from emerging risks. In addition, this method could also allow monitoring the spread~~ing~~ of invasive *Lagocephalus* species throughout the Mediterranean Sea.

2. Materials and Methods

2.1 Preliminary phylogenetic analysis of *COI* and *cytb* sequences available on the databases and selection of the molecular marker

2.1.1 Sequences retrieval~~ing~~. All ~~the~~ complete and partial sequences (when available) of both

COI and *cytb* of all *Lagocephalus* species were retrieved from the official database GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and, for the *COI*, also from BOLD (http://www.boldsystems.org/). Since the reclassification of the genus *Lagocephalus* is relatively recent (Matsuura, 2010; Matsuura & Satoh, 2017), all species reported as valid in FishBase, although obsolete (*L. wheeleri* and *L. cheesemanii*), were searched for the sequences retrievaling. Among the available sequences of *L. spadiceus* only those proved as actually belonging to this species during a parallel study (Giusti et al., under review2018) were used. As regard *L. guentheri,* the considered *cytb* sequences were solely those produced in the previously mentioned study, as no other *L. guentheri* valid sequences are available yet on the database. All the sequences used for the preliminary analysis are reported in Table 1SM.

2.1.2 Data sets preparation. COI and *cytb* sequences were opportunely aligned with Clustal W in BioEdit version 7.0.9. (Hall, 1999) to obtain distinct data sets for each molecular marker. Two data sets were obtained for each gene: one for a long fragment and one for a short fragment. In details, *COI* aligned sequences were trimmed to achieve the standard ~655bp barcode fragment by Hebert, Cywinska, & Ball (2003) (long *COI* data set) and the ~139bp mini-barcode fragment proposed by the protocol of Armani et al. (2015b) (short *COI* data set). For the *cytb,* all the sequences ≥ 1089bp were included in the first data set (long *cytb* data set) while, to obtain the short *cytb* data set, all the aligned sequences were trimmed to achieve a ~100bp fragment, identified as a highly variable region in some pufferfish species by Huang et al. (2014).

2.1.3 Phylogenetic analysis and selection of the molecular marker. The four aligned data sets were used to produce neighbour-joining dendrograms (Saitou and Nei, 1987) using MEGA version 6.0 (Tamura, Dudley, Nei, & Kumar, 2013). Distances were computed using the Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap re-samplings. Both long *COI* and long *cytb* datasets were even used to calculate the average inter-species divergence among *Lagocephalus spp.* At the end of this phase the *cytb* gene was selected as molecular target for this study (detailed in section 3.1).

2.2 Collection and analysis of reference and market samples

2.2.1 Reference samples (RS).

Reference samples (RS) belonging to specimens of valid *Lagocephalus* species were collected. In particular, ethanol-preserved tissue samples from 38 pufferfish specimens belonging to the species *L. gloveri* (n=2) *L. laevigatus* (n=4)*, L. lunaris* (n=3)*, L. sceleratus* (n=3)*, L. spadiceus* (n=3) and *L. suezensis* (n=23) identified at the species level by morphological analysis, were kindly provided by Research Institutes and Museum collections or retrieved in this study (Table 1). The current availability of *COI* and *cytb* sequences from the three remaining *Lagocephalus* species (*L. guentheri, L. inermis* and *L. lagocephalus*) was considered sufficient for the achievement of equally informative data (Table 1SM). Moreover, 13 pufferfish specimens belonging to other six species (*Sphoeroides pachygaster* and *Takifugu* genus) were also collected. Each sample was labelled with an internal code (Table 1).

2.2.2 Market samples (MS). Sixteen DNA samples belonging to mislabelled market products, molecularly identified as *Lagocephalus* spp. using the *COI* gene DNA barcoding approach in the studies of Armani et al. (2015a), Xiong et al. (2016) and Xiong et al. (2018) were also analysed (Table 2).

2.2.3 Total DNA extraction from RS. All the ethanol-preserved tissue samples from identified specimens were washed in 100mM TRIS-base, pH 7.8 for 30 min[2] at room temperature on a thermo-shaker (T-Shaker ambient, Euroclone, Siziano, Pavia, Italy). Total DNA extraction was performed starting from ~10 mg of tissue following the protocol described by Armani et al. (2014).

2.2.4 RS and MS DNA evaluation. The quality and quantity of the DNA from both the RS and MS were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US). One thousand nanograms of DNA was electrophoresed on 1% agarose gel GellyPhorLE (Euroclone,Wetherby, UK), stained with GelRed™Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized via ultraviolet transillumination. DNA fragment size was estimated by comparison with the standard marker SharpMass™50-DNA ladder and Sharp-

209 Mass™1-DNA ladder (Euroclone S.p.A-Life Sciences Division, Pavia, Italy). Each DNA sample
210 was stored at -20 °C until further analysis.

211 *2.2.5 RS samples amplification*. The primer pair Glu-PUF (5'-
212 AACCACCGTTGTGATTCHACTACAA-3') and THR-PUF (5'-CGGCATCCGGYTTACAAGAC-
213 3'), designed by modifying those proposed by Sevilla et al. (2007) was used to amplify the
214 complete *cytb* sequence from all the RS. The following PCR protocol was applied: 20 ml reaction
215 volume containing 2 ml of a 10X buffer (BiotechRabbit GmbH, Berlin, Germany), 100 mM of each
216 dNTP (Euroclone Spa, Milano), 200 nM of forward primer, 200 nM of reverse primer, 1.0 U
217 PerfectTaq DNA Polymerase (BiotechRabbit GmbH, Berlin, Germany), 100 ng of DNA and DNase
218 free water (Euroclone Spa, Milano) with the following cycling program: denaturation at 95 °C for 3
219 min; 40 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 50 s; final extension at 72 °C for 7
220 min. In case of amplification failures, DNA samples were reamplified by coupling the primers Glu-
221 PUF and THR-PUF with the primers 4R and 7F (Sevilla et al., 2007), respectively, to finally obtain
222 the required full *cytb* fragment. PCR protocol followed that reported by Sevilla et al. (2007). Five
223 microliters of each PCR products were checked by gel electrophoresis on a 2% agarose gel. The
224 amplification of fragments of the expected length was assessed by making a comparison with the
225 standard marker SharpMass™ 50-DNA ladder (Euroclone Spa, Milano) and the concentration of
226 PCR products by making a comparison with the intensity of the bands of the DNA ladder. A
227 concentration of 10 ng/μl was used as a threshold to destine the samples to the following
228 sequencing phase. A variable number of PCR products was sequenced for each species according
229 the availability of previously deposited sequences on the databases (Table 1SM). Overall, 17
230 selected PCR products were purified with EuroSAP PCR Enzymatic Clean-up kit (EuroClone Spa,
231 Milano) and sequenced by the Ictiopathology and Aquaculture Laboratory of the Experimental
232 Zooprophylactic Institute of Lazio and Tuscany (Pisa, Italy). All the sequences were deposited in
233 GenBank (accession numbers are reported in Table 1).

234 *2.2.6 Primers design~~ing~~ and MS amplification*. The electrophoretic analysis showed that DNA

235 from MS was more degraded than those obtained from RS, since the fragments size never exceed
236 ~400 bp in length. Given the impossibility to amplify the complete *cytb* fragment from the MS, a
237 shorter molecular marker was therefore selected for the analysis of these samples. The primer pair
238 PUF/for-short (5'-CAGACAAAATCCCMTTCCACCC-3') and PUF/rev-short (5'-
239 AYCATTCTGGTTTGATGTGGGC-3'), was designed on all the available *Lagocephalus* spp.
240 sequences (both retrieved from the databases and obtained in this study) to amplify a ~130bp region
241 located on the polymorphic region proposed by Huang et al. (2014) from all the MS. The primer
242 pair was initially tested on all the RS (*Lagocephalus* spp., *Sphoeroides pachygaster* and *Takifugu*
243 *spp.*) to assess its ability in amplifying the target species using the following cycling program:
244 denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 30 s, 56 °C for 20 s, and 72 °C for 5 s; final
245 extension at 72 °C for 7 min. Then, the same PCR protocol was used for the amplification of the
246 DNA from MS. Five microliters of each PCR products were checked by gel electrophoresis on a 2%
247 agarose gel. The amplification of fragments and the concentration of PCR products were evaluated
248 as described above for the long fragment. All the PCR products from the 16 MS were sequenced as
249 described in *section 2.2.5*.

250 *2.2.7 Phylogenetic analysis using RS and MS sequences produced in this study.* All the obtained
251 RS complete sequences were aligned using Clustal W in BioEdit version 7.0.9. (Hall, 1999) and
252 included in the "long fragment *cytb* data set". All the short sequences obtained from the MS were
253 included in a new aligned data set (short fragment MS *cytb* data set), jointly with the sequences of
254 all the target species retrieved from GenBank, which were opportunely trimmed to achieve the new
255 ~130 bp barcode fragment analysed in this study. Both of the newly aligned data sets were used to
256 produce two new neighbour-joining dendrograms as descripted in *section 2.1.3.*

257 *2.2.8 Species identification in MS.* The identification of the species used in the MS production
258 was established both on the basis of the phylogenetic analysis of the "short fragment MS *cytb* data
259 set" (*section 2.2.7*) and through a NCBI BLAST analysis (with 100% sequences identity value). The
260 most recent *Lagocephalus* classification (which includes nine valid species) was considered for

261 interpreting the results.

262 3. Results and discussion

263 3.1 Phylogenetic analysis and selection of the molecular target

264 *3.1.1 COI gene.* With the development and the success of the DNA-Barcoding technique
265 proposed by Hebert, Cywinska, & Ball (2003), ~~the~~ *COI* was proposed as the unique barcode region
266 for animals. This region is nowadays recognized by many authors as the marker of choice for
267 numerous fish species discrimination, with more than 300,~~.~~280 *COI* records, belonging to more than
268 19,~~.~~790 species of actinopterygians, deposited in the Barcode of Life Database
269 (http://www.boldsystems.org/). DNA barcoding targeting the *COI* gene has however shown some
270 limitations in marine species identification, such as low resolutions in the cases of recently diverged
271 species or~~,~~ species complexes ~~and hybrids~~ (Trivedi, Aloufi, Ansari, & Ghosh, 2016). For example,
272 limits in tuna's~~s'~~ identification using the *COI* barcode are known (Viñas & Tudela, 2009). ~~Its poor~~
273 ~~discriminatory effectiveness has been reported also for pufferfish species identification.~~As regards
274 pufferfish Huang et al. (2014) performed a phylogenetic analysis comparing the complete *cytb* and
275 *COI* capability in discriminating between *L. inermis, L. lunaris, L. wheeleri, L. gloveri, S.*
276 *pachygaster* and many other *Takifugu* spp. and proved ~~that the *COI* sequences divergence among~~
277 ~~the analysed species was lower than among~~ *cytb* gene to be more variable ~~performed better~~ than
278 *COI* genen ~~for this purposes~~for these species, ~~sequences and therefore selected the latter as~~
279 ~~molecular marker~~. Recently, the ~~DNA-Barcoding~~ analysis of both the standard 655 *COI* barcode
280 and mini *COI* barcodes from processed products (which were the MS analysed in the present study)
281 collected on both European and Chinese market (Armani et al., 2015a; Xiong et al., 2016; Xiong et
282 al., 2018), ~~further confirmed~~ highlighted issues in using the DNA barcoding approach ~~the~~
283 ~~ineffectiveness of this marker~~ in discriminating among *Lagocephalus* species (Table 2).
284 Nevertheless, we ~~believe~~ suppose it is related to –the presence of sequences belonging to
285 misidentified specimens on the official databases rather than to ~~appropriate to underline that the~~
286 ~~reported unsuitability of the~~ the inability of the *COI* –gene itself in discriminating ~~between the~~

287 species among species of this genus. -In fact, the phylogenetic analysis performed in -the present
288 study using both the long and the short data set showed a number of “wrongly clustering” cases
289 such as *L. lunaris* grouping with *L. sceleratus* or both *L. inermis* and *L. gloveri* grouping with *L.*
290 *spadiceus.* This theory was recently supported in a study focusing on *L. spadiceus* and *L. guentheri*
291 species has been resolved through the analytical approach reported by Giusti et al. (under
292 review2018), that revealed the presence of sequences belonging to actual misidentified specimens
293 on the official databases. Despite this, tThe phylogenetic analysis performed in this latterthe present
294 study however showed a number of “wrongly clustering” cases such as *L. lunaris* grouping with *L.*
295 *sceleratus* or both *L. inermis* and *L. gloveri* grouping with *L. spadiceus* (data not shown). The
296 phylogenetic tree obtained with the short *COI* data set showed similar results (data not shown). This
297 aspect was attributable to the presence of wrongly deposited sequences on the official databases that
298 unavoidably lowered the analysis reliability.
299 *3.1.2 Selection of the cytb as molecular marker.Cytb gene*. *XX* The phylogenetic trees obtained
300 by both long and short *cytb* data set appeared each other similar. Contrarily from the *COI*, eEach
301 sequence of every *Lagocephalus* species clustered together with the same species’ group with
302 highly supported bootstrap (range 89%-100%) (Fig. 1a and 1b), the only exceptions were repre-
303 sented by the sequence EF126108 (deposited as *L. gloveri*), clustering with the group of *L.*
304 *spadiceus/L. wheeleri*, and the sequence KT833744 (deposited as *L. lagocephalus*), clustering with
305 the group of *L. inermis*. The average inter-species divergence among *Lagocephalus spp.* sequences
306 was higher in *cytb* (17.4%) than in *COI* (14.8%).
307 *3.2.3 Selection of the cytb as molecular marker*. The preliminary analysis of the four data sets
308 produced different topologies regarding the level of species relationship among *COI* and *cytb*. The
309 average inter-species divergence among *Lagocephalus spp.* sequences was higher in *cytb* (17.4%)
310 than in *COI* (14.8%). Overall, these results substantially confirmed those reported by the current
311 scientific literature, that indicated the *cytb* as a more suitable target than the *COI* in puffer fish spe-
312 cies identification (Huang et al., 2014) and, for this reason, it was selected as molecular marker for

313 this analysis. ~~.~~Moreover, even ~~In fact,~~ hypothesizing that the "wrongly clustering" sequences of
314 both genes' phylogenetic trees had been incorrectly deposited and removing them from the analysis,
315 the *cytb* sequences overall clustered better than the *COI* ones in both long and short fragment *cytb*
316 data sets. Given that the sequences EF126108 and KT833744 appeared problematic, their intra-
317 species and inter-species divergence was assessed in MEGA6 (Tamura, Dudley, Nei, & Kumar,
318 2013) using the Kimura 2-parameter model (Kimura, 1980). For the sequence EF126108 (deposited
319 as *L. gloveri*) the divergence with the other *L. gloveri/ L. cheesemani* sequences (intra-species vari-
320 ability) and with *L. spadiceus/L. wheeleri* sequences (inter-species variability) was calculated, while
321 for the sequence KT833744 (deposited as *L. lagocephalus*) the divergence with the other *L. lago-*
322 *cephalus* sequences (intra-species variability) and with *L. inermis* sequences (inter-species variabil-
323 ity) was calculated. Regarding the EF126108 sequence, the average intra-species variability (16.7%)
324 was proved much higher than the average inter-species variability (0.2%) so that we could certainly
325 state that the sequence had been wrongly deposited as *L. gloveri.* Analogously, the average intra-
326 species variability (19.9%) of the KT833744 sequence was proved much higher than the average in-
327 ter-species variability (1.5%) and it therefore could be considered as wrongly deposited. Thus, these
328 sequences were removed from the subsequent analysis. These results highlighted the actual dis-
329 crimination power of the ~100 bp polymorphic *cytb* fragment proposed by Huang et al. (2014) also
330 for the *Lagochepalus* genus.

331 Our findings overall showed that wrongly deposited sequences can distort the reliability of a
332 phylogenetic analysis. Probably, even though the *COI* was discarded, an in-depth investigation
333 aimed at analysing and properly removing all the *COI* sequences wrongly deposited on the official
334 database could make the standard barcode fragment and the mini barcode fragment suitable for the
335 *Lagocephalus spp.* identification in both fresh and processed products. ~~Thus, the inability in species~~
336 ~~identification reported in previous studies (Cohen et al., 2009; Armani et al., 2015a; Xiong et al.,~~
337 ~~2016; Xiong et al., 2018) may be due to problematic sequences rather than to a low discrimination~~
338 ~~power of the *COI* gene.~~

3.3 RS analysis

The complete *cytb* fragment was successfully amplified from all the RS specimens, with an average amplicon concentration of 40 ng/μl (data not shown). The new neighbour-joining dendrogram constructed by including the RS sequences in the "long fragment *cytb* data set" (*Section 2.5.1*) showed that all the new sequences correctly clustered with the relative species group with high bootstrap values (Fig. 2a).

3.4 MS identification

3.4.2 Primers designing and amplification of MS. In this study, the same *cytb* region investigated by Huang et al. (2014) was used for identifying the *Lagocephalus* species in all the investigated MS. However, since the forward primer presented numerous mismatches with the sequences of the target species of this study, another forward primer was designed on a conserved region, slightly moved towards the 5' end respect to the original primer. Contrariwise, the original reverse primer was maintained, although some base pairs were degenerated to allow a better matching with the sequences of the target species. The selected new ~130 bp fragment ranged from bps 665 to 795. This primer pair was proved as performant in both all RS and MS samples collected in this study, with an average amplicon concentration of 30 ng/μl.

3.4.3 MS identity assessment. In the studies conducted by Armani et al. (2015a), Xiong et al. (2016) and Xiong et al. (2018), that relied to the use of the *COI* gene, DNA samples from M1 to M11 showed a 99-100% species identity with *L. spadiceus/L. wheeleri, L. gloveri* and *L. inermis* by using the IDs analysis on BOLD and a 99-100% species identity with the species *L. spadiceus/L. wheeleri* and *L. gloveri* by using NCBI BLAST~~. The maximum identity value (100%) was not obtained through~~ while the analysis of the samples M12 - M16, which showed a 98-99% species identity with *L. spadiceus/L. wheeleri* and *L. inermis* by using both the IDs analysis on BOLD and NCBI BLAST.

On the contrary, in the final phylogenetic analysis of the "short fragment MS *cytb* data set" (*section 2.5.1*) all the sequences clustered with the group of *L. spadiceus/L. wheeleri* (Fig. 2b). As pre-

365 dictable, the NCBI BLAST analysis showed that the MS sequences had a 100% identity value with
366 the species *L. spadiceus/L. wheeleri* (Table 2).

367 The presence of this species in mislabelled market products not only represents an illegal prac-
368 tice in both European and Chinese countries but could even involve a health hazard in case of un-
369 aware consumers since *L. spadiceus* was reported as toxic (Chulanetra et al. 2011; Sangthong,
370 Ngernsiri, & Sangthong., 2014), as better detailed in the following section.

371 **3.5 *Lagocephalus* species identification as tool for safeguarding public health**

372 A considerable issue involving the official finfishes' database (www.fishbase.org) concerns the
373 toxicity degree attributed to the different *Lagocephalus* species. In this regard, *L. cheesemanii*, *L.*
374 *gloveri*, *L. inermis*, *L. lunaris* and *L. scelaratus* are reported as "poisonous to eat" and *L. guentheri*,
375 *L. suezensis, L. spadiceus* and *L. wheeleri* as "harmless", while no data are reported for the species
376 *L. laevigatus* and *L. lagocephalus* (www.fishbase.org). The poisonous species can be considered as
377 properly categorized since both *L. sceleratus* and *L. lunaris* are well-known as naturally -containing
378 high amount of TTX in their muscles, making these species impossible to prepare safely
379 (Chulanetra et al. 2011; Kosker at al., 2016; Rodriguez et al., 2012; Sabrah, El-Ganainy, & Zaky,
380 2006), *L. inermis* is also known as toxic, although to a lesser degree (Ghosh, Hazra, Banerjee, &
381 Mukherjee, 2004) and *L. gloveri,* even though commonly reported as non-toxic (Hsieh, Shiu, Cheng,
382 Chen, & Hwang, 2002; Hsieh et al., 2010; Huang et al., 2014), was occasionally demonstrated to be
383 weakly poisonous in some fishing seasons (Noguchi & Arakawa, 2008). On the contrary, the
384 classification of the "harmless" species is not always endorsed by the scientific literature. *L.*
385 *wheeleri* has been reported as "*almost* non-toxic" (Noguchi, Onuki, & Arakawa, 2011) or "weakly
386 toxic" (Noguchi & Arakawa, 2008). Besides, *L. spadiceus*, which according to the study of
387 Matsuura (2010) and the present results could be considered a synonymous of *L. wheeleri*, has been
388 reported as toxic (Chulanetra et al., 2011). Toxicity studies on *L. lagocephalus* clearly indicate the
389 potential danger of using this fish as food (Saoudi et al., 2008; Saoudi et al., 2011). These
390 inconsistencies can be explained by the fact that pufferfish act as hosts of TTX-producing bacteria

391 that live symbiotically in their bodies and are accumulated through the food chain, so that the
392 toxicity degree of a species is largely environment-dependent. According to this evidence, although
393 a predisposition of some species to accumulate more TTX than others is known, each *Lagocephalus*
394 spp. could become toxic if inhabiting suitable environment. It has been in fact proved ~~that pufferfish~~
395 ~~reared in net cages or land aquaria for a year became non-toxic because of the prevention of~~
396 ~~invasion of TTX bearing organisms and, by the same assumption,~~that when non-toxic species are
397 fed with diet containing TTX, they become toxic (Noguchi, Arakawa, & Takatani, 2006).
398 Concerns about European consumers' risk of TTX poisoning have emerged since some pufferfish
399 species, originally inhabiting the tropical Indian and Pacific Oceans or the sub-tropical Eastern
400 Atlantic Ocean, from which they originate, have invaded the Mediterranean Sea (Guardone et al.,
401 2018). It has been suggested that the Mediterranean conditions are becoming more and more
402 suitable for the survival of tropical species migrating from the Red Sea through the Suez Canal,
403 offering the alien species various advantages when competing with native species (Galil et al.,
404 2015). To date human intoxication cases in the Mediterranean basin were only associated to the
405 consumption of *L. sceleratus* ~~(Beköz, Beköz, Yilmaz, Tüzün, Beköz, 2013; Bentur et al., 2008~~;
406 ~~Chamandi et al., 2009; Eisenman, A., Rusetski, V., Sharivker, D., Yona, Z., & Golani, 2008;~~
407 ~~Kheifets, Rozhavsky, Solomonovich, Marianna, & Soroksky, 2012; Zaki, 2004~~) (Guardone et al.,
408 2018). The presence of this lessepsian species in the Mediterranean Sea, was reported in 2003 by
409 Akyol, Ünal, Ceyhan, & Bilecenoglu (2005). It is interesting to note that climatic change has
410 favoured the propagation of TTX-producing bacteria in the Mediterranean waters through the Suez
411 Canal (Saoudi et al., 2008). In fact, TTX had not been found in the Mediterranean at least until 2003
412 (Poletti, Milandri, & Pompei, 2003). Therefore, it cannot be excluded that other *Lagocephalus*
413 species inhabiting the Mediterranean Sea and considered as non-toxic or weakly toxic may
414 accumulate the TTX through the trophic chain in the future as recently occurred in gastropods from
415 Portugal and in shellfish from Greece (EFSA Panel, 2017; Rambla-Alegre et al., 2017).
416 An analytical method aimed at discriminating each *Lagocephalus* species represents a valuable

417 tool to evaluate the TTX exposure level of the European citizens and to assess the spreading of
418 pufferfish in the Mediterranean Sea. In this respect, our method is completely in agreement with the
419 Regulation (EU) No 1143/2014, which stated that "*the competent authorities should be prepared to*
420 *identify invasive species at an early stage, to evaluate the associated risks and to activate*
421 *appropriate management responses aimed at protecting marine ecosystems and human health*".

422 **4. Conclusions**

423 Unaware consumption of toxic pufferfish or products derived from them represents an emerging
424 risk for European consumers both due to the globalization of the fish supply chain and to the
425 spreading of toxic pufferfish in the Mediterranean Sea. Recently, potentially toxic *Lagocephalus*
426 species have been found in imported mislabelled commercial product. In addition, the current
427 spreading of some of these species also along the European coasts further increase the risk they
428 enter into the seafood chain. The molecular target selected in this study was proven to be effective
429 in identifying *Lagocephalus* species in both fresh and processed samples. Therefore, it represents a
430 reliable instrument for the official control aimed at preventing commercial and health frauds. In
431 addition, this approach can be useful in monitoring the spreading of toxic pufferfish in the
432 Mediterranean Sea and serve as support to the proper management of the marine ecosystem.

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Figure captions

Figure 1. Preliminary neighbour-joining dendrograms produced with **a)** the long *cytb* data set and with **b)** the short *cytb* data set (100bp fragment by Huang et al., 2014). All the retrieved sequences' accession numbers were reported. Different species clusters were highlighted in different colours. Wrongly clustering sequences were circled in black. L. GUE: *L. guentheri;* L. SPA: *L. spadiceus;* L. LAE: *L. laevigatus;* L. GLO: *L. gloveri;* L. LAG: *L. lagocephalus;* L. INE: *L. inermis;* L. LUN: *L. lunaris;* L. SUE: *L. suezensis;* L. SCE: *L. sceleratus;* (L. CHE): *L. cheesemanii* (synonymous of *L. gloveri* according to Matsuura and Satoh, 2017); (L. WHE): *L. wheeleri* (synonymous of *L. spadiceus* according to Matsuura, 2010).

Figure 2. Neighbour-joining dendrograms produced with RS (**a**) and MS (**b**) sequences. In figure 2a different species clusters were highlighted in different colours. In figure 2b MS are highlighted in red. All the retrieved sequences' accession numbers were reported. *Sequences produced in this study from RS. L. GUE: *L. guentheri;* L. SPA: *L. spadiceus;* L. LAE: *L. laevigatus;* L. GLO: *L. gloveri;* L. LAG: *L. lagocephalus;* L. INE: *L. inermis;* L. LUN: *L. lunaris;* L. SUE: *L. suezensis;* L. SCE: *L. sceleratus;* (L. CHE): *L. cheesemanii* (synonymous of *L. gloveri* according to Matsuura and Satoh, 2017); (L. WHE): *L. wheeleri* (synonymous of *L. spadiceus* according to Matsuura, 2010).

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Table

Table 1. List of puffer fish reference samples (RS) collected in this study. Sequenced samples were highlighted grey boxes and the relative sequence's GenBank accession number was reported in the last right column.

Species	code	Research Institute	Collection Area	
L. gloveri	L. GLO-1	National Museum of Nature and Science, Tokyo (Japan)	Japan	MG967655
	L. GLO-2	National Museum of Nature and Science, Tokyo (Japan)	Japan	MG967656
Lagocephalus laevigatus	L. LAE-1	Museum of Natural Science - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	USA (Gulf of Mexico)	MG817084
	L. LAE-2	Florida Fish and Wildlife Conservation Commission, Tallahassee, Florida (USA)	Florida (USA)	MG817085
	L. LAE-3	Florida Fish and Wildlife Conservation Commission, Tallahassee, Florida (USA)	Florida (USA)	
	L. LAE-4	Florida Fish and Wildlife Conservation Commission, Tallahassee, Florida (USA)	Alabama (USA)	MG817086
Lagocephalus lunaris	L. LUN-1	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Kuwait (Persian Gulf)	MG793380
	L. LUN-2	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Republic of Singapore	MG817075
	L. LUN-3	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Republic of Singapore	MG817076
Lagocephalus sceleratus	L. SCE-1	Ministry of Productive Reconstruction, Environment and Energy Directorate of Veterinary Centre of Thessaloniki, Greece	Thasos islands, North Aegean Sea	MG817077
	L. SCE-2	This study	Ashdod (Israel)	MG878890
	L. SCE-3	This study	Italy	MG878891
Lagocephalus spadiceus	L. SPA-1	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Vietnam	
	L. SPA-2	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Vietnam	MG817078
	L. SPA-3	Museum of Natural Sciences - Section of Ichthyology, Louisiana State University, Bâton Rouge, Louisiana (USA)	Vietnam	MG817079
Lagocephalus suezensis	L. SUE-1	This study	Ashdod (Israel)	MG817080
	L. SUE-2	This study	Ashdod (Israel)	MG817081
	L. SUE-3	This study	Ashdod (Israel)	MG817082
	L. SUE-4	This study	Ashdod (Israel)	MG817083
	L. SUE-5	This study	Ashdod (Israel)	
	L. SUE-6	This study	Ashdod (Israel)	
	L. SUE-7	This study	Ashdod (Israel)	
	L. SUE-8	This study	Ashdod (Israel)	
	L. SUE-9	This study	Ashdod (Israel)	

	L. SUE-10	This study	Ashdod (Israel)
	L. SUE-11	This study	Ashdod (Israel)
	L. SUE-12	This study	Ashdod (Israel)
	L. SUE-13	This study	Ashdod (Israel)
	L. SUE-14	This study	Ashdod (Israel)
	L. SUE-15	This study	Ashdod (Israel)
	L. SUE-16	This study	Ashdod (Israel)
	L. SUE-17	This study	Ashdod (Israel)
	L. SUE-18	This study	Ashdod (Israel)
	L. SUE-19	This study	Ashdod (Israel)
	L. SUE-21	This study	Ashdod (Israel)
	L. SUE-21	This study	Ashdod (Israel)
	L. SUE-22	This study	Ashdod (Israel)
	L. SUE-23	This study	Ashdod (Israel)
Sphoeroides pachygaster	S. PAC-1	Ministry of Productive Reconstruction, Environment and Energy Directorate of Veterinary Centre of Thessaloniki, Greece	Thasos islands, North Aegean Sea
Takifugu pardalis	T. PAR-1	Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan	Northwest Pacific
Takifugu porphyreus	T. POR-1	Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan	Northwest Pacific
Takifugu poecilonotus	T. POE-1	Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan	Northwest Pacific
Takifugu rubripes	T. RUB-1	Fisheries Laboratory, Graduate School of Agricultural and Life Sciences, University of Tokio, Japan	Northwest Pacific
	T. RUB-2	Fisheries Laboratory, Graduate School of Agricultural and Life Sciences, University of Tokio, Japan	Northwest Pacific
	T. RUB-3	Graduate School of Agricultural Sciences, Tohoku University, Japan	NR
	T. RUB-4	Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan	Northwest Pacific
	T. RUB-5	Department of Applied Biological Science, University of Miyazaki, Japan	Northwest Pacific
	T. RUB-6	Department of Applied Biological Science, University of Miyazaki, Japan	Northwest Pacific
	T. RUB-7	Department of Applied Biological Science, University of Miyazaki, Japan	Northwest Pacific
	T. RUB-8	Department of Applied Biological Science, University of Miyazaki, Japan	Northwest Pacific
Takifugu	T. VER-1	Graduate School of Fisheries and Environmental Sciences,	Northwest

vermicularis		Nagasaki University, Japan	Pacific

Table

Table 2. Market samples (MS) tested in this study. [a] (Chinese name, pinyin, English translation); [b] obtained through the analysis of the standard 655 *COI* barcode fragment (Hebert et al., 2003) or the ~139 mini-barcode fragment (Armani et al., 2015b) conducted by Xiong et al. (2016) (from samples MS-1 to MS-9), Armani et al. (2015a) (samples MS-10 and MS-11) and Xiong et al. (2018) (from samples MS-12 to MS-13); [c] obtained through the analysis of the ~130 bp *cytb* fragment tested in this study.

Code	Product presentation and state	Commercial[a] or scientific name in the ingredient list	Previous studies results[b]		Present study results[c]	
			BOLD	GenBank	Raw results (GenBank)	Final identification
MS-1	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-2	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-3	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-4	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-5	pre-packaged roasted	鳕鱼 Xue Yu Cod *Plecoglossus altivelis*	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-6	pre-packaged roasted	鳕鱼 Xue Yu Cod *Plecoglossus altivelis*	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*

MS-7	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.78% *L. inermis* 100-99.84% *L. gloveri* 100-91.01% *L. wheeleri* 100-91.23%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-8	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 100-93.43% *L. inermis* 100-99.83% *L. gloveri* 100-90.91% *L. wheeleri* 100-91.08%	*L. wheeleri* 100-99% *L. spadiceus* 100-99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-9	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. wheeleri* 100-99.53% *L.* cf *spadiceus* 99.84% *L. gloveri* 99-68% *L. spadiceus* 99.68-93.46% *L. inermis* 99.51%	*L. wheeleri* 100-99% *L. spadiceus* 99% *L. gloveri* 99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-10	pre-packaged baked	"*brandello del calamaro*" (english label: marine fish fillet)	*L. spadiceus* 100-93.51% *L. inermis* 100-99.82% *L. gloveri* 100% *L. wheeleri* 100-99.3% *L.* cf *spadiceus* 99.82%	*L. spadiceus* 100-99% *L. gloveri* 100% *L. wheeleri* 100-99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-11	pre-packaged baked	"*brandello del calamaro*" (english label: marine fish fillet)	*L. spadiceus* 100-93.93% *L. inermis* 100-99.82% *L. gloveri* 100% *L. wheeleri* 100-99.38% *L.* cf *spadiceus* 99.66%	*L. spadiceus* 100-99% *L. gloveri* 100% *L. wheeleri* 100-99%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-12	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 98-99.42% *L. wheeleri* 99.87% *L. inermis* 98.22%	*L. spadiceus* 98-99% *L. wheeleri* 99% *L. inermis* 98%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-13	pre-packaged roasted	鳕鱼 Xue Yu	*L. spadiceus* 98.33-99.4% *L. wheeleri* 99.27% *L. inermis*	*L. spadiceus* 98-99% *L. wheeleri* 99% *L. inermis*	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*

			98.48%	98%		
MS-14	pre-packaged roasted	鳕鱼 Xue Yu *Plecoglossus altivelis*	*L. spadiceus* 98-99.37% *L. wheeleri* 99.17% *L. inermis* 98.22%	*L. spadiceus* 98-99% *L. wheeleri* 99% *L. inermis* 98%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-15	pre-packaged roasted	鳕鱼 Xue Yu Cod	*L. spadiceus* 98-99.74% *L. wheeleri* 99.86% *L. inermis* 98.28%	*L. spadiceus* 98-99% *L. wheeleri* 99% *L. inermis* 98%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*
MS-16	pre-packaged roasted	鳕鱼 Xue Yu	*L. spadiceus* 98-99.02% *L. wheeleri* 99.57% *L. inermis* 98.72%	*L. inermis* 98% *L. spadiceus* 98% *L. wheeleri* 98%	*L. spadiceus* 100% *L. wheeleri* 100%	*L. spadiceus*

Figure

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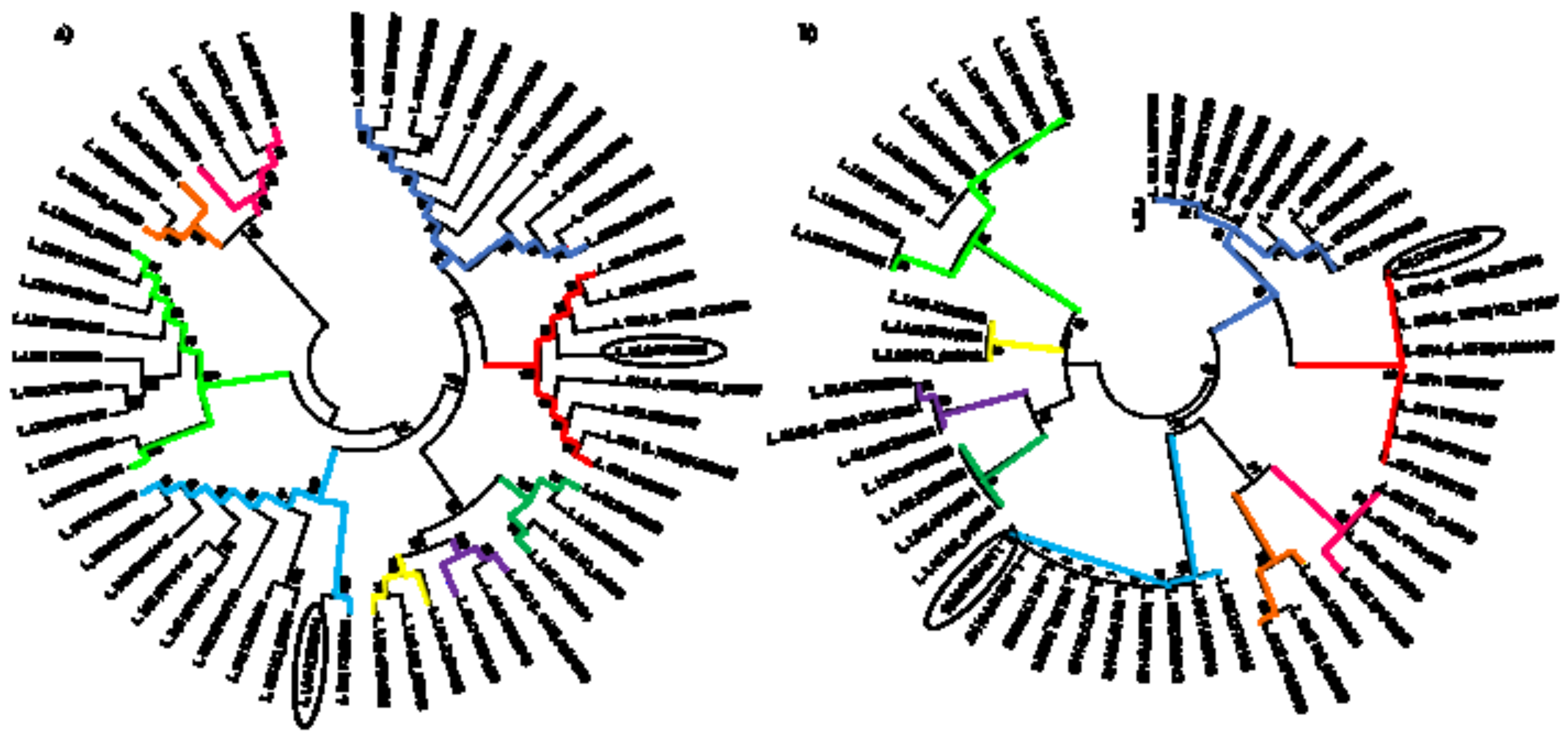

Figure

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