DNA Barcoding, Identification and Validation of the puffer fish (Order: Tetraodontiformes) in China Coastal Waters

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Abstract

Tetraodontiformes is a special group of higher teleosts, with a long and problematic taxonomic history due to their wide distribution and species diversity. It is a difficult task for both professionals and nonprofessionals to accurately identify all species only according to morphological characteristics. DNA barcoding can identify species at the molecular level. In this study, we collected 616 specimens of Tetraodontiformes and their DNA barcodes from the coastal waters of China. According to the morphological characteristics, 50 species were preliminarily identified, belonging to 23 genera, 6. Among them, DNA barcoding analysis showed that Takifugu pseudommus and Takifugu chinensis are the synonyms of Takifugu rubripes. And Lagocephalus wheeleri is the synonym of Lagocephalus Spadiceus. The third important finding is that the species of Takifugu have close genetic relationship. If T. rubripes, T. pseudommus and T. chinensis are taken as one species, the average interspecific genetic distance of Takifugu is 6.21 times of the average intraspecific genetic distance, which does not reach the DNA barcode threshold of more than 10 times proposed by Hebert. Among them, the genetic distance between T. oblongus and T. stictonotus is the largest, 0.045; And between T. bimaculatus and T. flavidus is the smallest, only 0.013. However, species can be clustered into separate clades in the NJ tree. In conclusion, this study provided molecular basis for solving the problem of confusion in the classification of Tetraodontiformes, it found that there are synonym phenomena in the order, and provided molecular evidence for clarifying the valid species names of Lagocephalus Spadiceus and Takifugu rubripes. The results can provide reliable DNA barcoding information for the identification and classification of Tetraodontiformes, and also provide technical support for the development and utilization of puffer fish resources and the identification of the original components of related commodities on the aquatic product market.

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Abstract

Tetraodontiformes is a special group of higher teleosts, with a long and problematic taxonomic history due to their wide distribution and species diversity. It is a difficult task for both professionals and nonprofessionals to accurately identify all species only according to morphological characteristics. DNA barcoding can identify species at the molecular level. In this study, we collected 616 specimens of Tetraodontiformes and their DNA barcodes from the coastal waters of China. According to the morphological characteristics, 50 species were preliminarily identified, belonging to 23 genera, 6. Among them, DNA barcoding analysis showed that *Takifugu pseudommus* and*Takifugu chinensis* are the synonyms of *Takifugu rubripes* . And *Lagocephalus wheeleri* is the synonym of *Lagocephalus Spadiceus* . The third important finding is that the species of*Takifugu* have close genetic relationship. If *T. rubripes* ,*T. pseudommus* and *T. chinensis* are taken as one species, the average interspecific genetic distance of *Takifugu* is 6.21 times of the average intraspecific genetic distance, which does not reach the DNA barcode threshold of more than 10 times proposed by Hebert. Among them, the genetic distance between *T. oblongus* and*T. stictonotus* is the largest, 0.045; And between *T. bimaculatus* and *T. flavidus* is the smallest, only 0.013. However, species can be clustered into separate clades in the NJ tree. In conclusion, this study provided molecular basis for solving the problem of confusion in the classification of Tetraodontiformes, it found that there are synonym phenomena in the order, and provided molecular evidence for clarifying the valid species names of*Lagocephalus Spadiceus* and *Takifugu rubripes* . The results can provide reliable DNA barcoding information for the identification and classification of Tetraodontiformes, and also provide technical support for the development and utilization of puffer fish resources and the identification of the original components of related commodities on the aquatic product market.

1. Introduction

Tetraodontiformes is a special group of higher teleost fishes, famous for the name of "puffer fish". It is believed that puffer fish is the general name of fishes in family Tetraodontidae of order Tetraodontiformes. However, there is also a view that all species in the order Tetraodontiformes are called as puffer fish (Wu & Chen, 1981). The species in this order are very abundant and distributed in various habitats, such as freshwater, brackish and coastal waters, open seas and so on (Yamanoue et al., 2008). So far, FishBase (https://www.fishbase.de/)has recorded 446 species of Tetraodontiformes, belonging to 106 genera and 10 families. According to the records of "Fauna Sinica", there are 131 species in 61 genera of 10 families in China (Su & Li, 2002), one of them is freshwater fish. Tetraodontiformes are distributed in a considerable amount along the coast of China, and occupy an important economic position in Chinese fishery (Su & Li, 2002). Among them, *Thamnaconus* ,*Takifugu* and *Lagocephalus* are with high economic value.

The puffer fish has a long edible history in China and Japan, and it is regarded as a precious aquatic product for its high protein content and extremely delicious taste. Although most of the puffer fishes taste delicious and have high economic value, many of them contain toxic tetrodotxin (TTX) in the ovary, liver, kidney, eyes and blood. If they are not handled properly or eaten by mistake, people will be poisoned or even die. Therefore, it is urgent to screen the species of edible puffer fish and avoid the occurrence of puffer fish poisoning. The taxonomy and identification of puffer fish is the key to solve the above problems.

In the traditional taxonomy, the classification and identification of Tetraodontiformes are mainly based on the morphological characteristics of stripes, spots, body color, and mouth shape, etc. However, the morphology

of closely related species of this order is very similar and difficult to distinguish. Whether professionals or non-professionals, it is an extremely difficult task to identify Tetraodontiformes fishes only according to their morphological characteristics. So far, there are a large number of synonyms in the order Tetraodontiformes (Liu et al., 1999; Song et al., 2001; Cui et al., 2005; Reza et al., 2008; Matsuura, 2010; Sakai, Sakamoto, & Yoshikawa, 2021), and the classification and identification of species are confused, which makes it difficult to accurately count the number of species of this order (Chen & Zhang, 2015; Su & Li, 2002). Moreover, most of the commodities of the puffer fish in the market have been processed and lost their original morphological characteristics, which makes it impossible to identify species. DNA barcoding is used to identify species at molecular level, which is not affected by time, region and individual morphology (Hebert, Ratnasingham & Waard, 2003). Combining the morphological characteristics of species and DNA barcodding technology to identify species will make the identification results more objective and reliable.

At present, there have been many reports on the study of puffer fish at the molecular level. However, these studies only focus on one genus or several species of Tetraodontiformes (Song et al., 2001; Elmerot et al., 2002; Ishizaki et al., 2006; Reza et al., 2008; Zhang & He, 2008; Santini, 2013; Chen, 2019), and there is no study on the species identification of the whole order Tetraodontiformes. Therefore, it is urgent to carry out accurate classification and identification research of order Tetraodontiformes, so as to improve technical support for food safety of fishery biological sources and trade regulation of aquatic products.

In order to solve the difficulty of morphological classification of Tetraodontiformes, this study used DNA bar code technology and morphological methods to identify 616 samples of Tetraodontiformes collected from the coast of China, clarified the synonyms of some species of this order, and confirmed the effective species names of these species Building a perfect classification system of DNA barcode for the order Tetraodontiformes, will not only enrich the database of fish DNA barcode, but also promote the rapid development of puffer fish taxonomy and systematics, and provide technical support for aquatic product quality safety and trade supervision.

Materials and methods

2.1 Sample collection

Since 2011, our team has collected Tetraodontiformes specimens from the coastal waters of China. In the past 11 years, a total of 616 specimens of Tetraodontiformes have been collected (Table 1). After rough classification according to morphologicalcharacteristics, they were preserved in 95% ethanol for subsequent DNA barcode identification. All voucher specimens are deposited in the National Marine Fishery Biological Germplasm Resource Bank.

2.2 Morphological identification

The body length, weight and other measurable traits of the specimen were measured. The morphological taxonomic characteristic of Tetraodontiformes is mainly base on the body shape, fin number, stripes, spots, body color and mouth shape, etc described by Chen & Zhang (2015) and Su & Li (2002).

2.3 DNA extraction and quality inspection

DNA was extracted from the muscle of fish by using TIANamp Marine Animals DNA Kit (TIANGEN BIOTECH (BEIJING) CO.,LTD.). For detailed steps of DNA extraction, refer to the kit manual. DNA samples were stored at -20 .

Nano-300 micro-spectrophotometer (Allsheng) was used to detect the concentration and purity of DNA samples, and the quality of the DNA samples was detected by 1% agarose gel.

2.4 PCR amplification and DNA sequencing

The published universal primers for fish DNA barcodes (Ward et al., 2005) were used for PCR amplification and sequencing, and the length of the amplification product was 707bp. The primers were as follows:

COIF: 5'-TCAACCAACCACAAAGACATTGGCAC-3'

COIR: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'

PCR amplification was in total volume of 25 μL, containing 12.5 μL Taq Mixture (2x), 2 μL DNA template, 1 μL of each primer (10 μM) and 8.5 μL ddH2O. Thermo cycling were carried out as follows: 94 for 5 minutes, 94 for 30 seconds, 53˜56 for 30 seconds, 72 for 1 minute, cycle 35 times from step 2 to step 4, finally 72 for 10 minutes. PCR products were detected by 1% agarose gel, and the qualified samples were sent to BGI in Qingdao for direct bidirectional sequencing.

2.5 Data analysis

The raw sequences were assembled by seqman of Lasergene software package (Swindell & Plasterer, 1997; Burland, 1999) and aligned by Cluster W in BioEdit version 7.0.9 (Hall, 1999). After correction and alignment, the effective sequence length was 687 bp. The haplotypes were analyzed by DNASP version 5.10.01 (Librado & Rozas, 2009). Each haplotype sequence was compared for similarity in NCBI (https://www.ncbi.nlm.nih.gov/) and BOLD (*http://www.barcodinglife.org/*). The top matches showing [?] 98% similarity were used as the preliminary identification results. Then based on Kimura- 2- parameter (K2P) mode (Kimura, 1980), the genetic distance of different taxonomic levels of Tetraodontiformes was calculated.

Bothus myriaster and *Glyptocephalus stelleri* were selected as the outgroups, based on the haplotypes of COI gene of Tetraodontiformes, the Neighbor-joining tree (NJ tree) of Tetraodontiformes was constructed in Mega 7.0 (Kumar, 2015).

3. Result

3.1 Morphological identification

According to the morphological characteristics, 616 samples were identified as 50 species, belonging to 23 genera, 6 families and 4 Suborder (Table 1). Among them, 612 samples can be identified to the level of species, 4 samples can only be identified to the level of genus because the sample is juvenile fish and the morphological characteristics are not obvious.

Among the 23 genera of Tetraodontiformes collected in this study, the morphology of the related species of *Takifugu* is very similar and difficult to distinguish. For example, the morphologic features of *T. alboplumbeus* and *T. niphobles* are so similar that they are difficult to distinguish. It is generally believed that *T. niphobles* have obvious chest spots, while the chest spots of *T. alboplumbeus* are not obvious, and there are 4-6 dark bands on the back of *T. alboplumbeus* . Of the 616 specimens collected in this study, 20 specimens were identified as the *T. niphobles* , and 11 specimens had the characteristics of the *T. alboplumbeus* , but they were different from the *T. alboplumbeus* , because they had only two dark bands. Furthermore, it is also difficult to distinguish *T. rubripes* , *T. pseudommus* and *T. chinensis* , some study suggest *T. rubripes* has irregular round black spots and white patterns in front of the caudal fin on the sides of the body, *T. chinensis* has no such black marks while *T. pseudommus* has white spots scattered on a black background on the dorsal and lateral sides of the body (Baek et al., 2018; Reza et al., 2008). In this study, 8 samples were identified as *T. chinensis* , 4 as *T. pseudommus* , and 27 as *T. rubripes* .

Additionally, the morphological characteristics can distinguish *Lagocephalus wheeleri* (Abe, Tabeta & Kitahama, 1984) from *Lagocephalus spadiceus* (Richardson, 1845) The former has elliptical dorsal spinule patch,

and the latter has a rhomboidal patch with a posterior extension. In this study, 82 specimens were identified as *L. wheeleri* ,10 were identified as *L. spadiceus* , and 4 were the intermediate individuals of the two species, that is they have discontinuous patch.

3.2 DNA Barcoding of the Tetraodontiformes

3.2.1 Characteristics of DNA Barcode Sequences of Tetraodontiformes

The results showed that among the 616 COI gene sequences of Tetraodontiformes with a length of 687 bp, there were 370 conserved sites, 62 transition sites (si), 43 transversional site (sv), and the si/sv ratio was 1.4. The results of DNA base content analysis showed that the average DNA base composition of 687 bp COI gene of Tetraodontiformes was: T: 27.7%, C: 29.9%, a: 23.8%, G: 18.6%, and the content of A+T was 51.5%, and the content of C+G was 48.5%. Moreover, the average G+C content of the first base of codon was the highest reaching 56.9%. Additionally, it shows that the sequence similarity of COI gene among the species of *Takifugu* is very high, and the number of conservative sites was 561 in 236 sequences of*Takifugu* with a length of 687bp.

3.2.2 Haplotype analysis of DNA barcodes in Tetraodontiformes

Haplotype analysis showed that 616 COI gene sequences of Tetraodontiformes were divided into 171 haplotypes. Among them, 253 COI gene sequences of *Takifugu* were divided into 65 haplotypes, and Hap 99 is the same hyplotype of *T. pseudommus, T. rubripes*and *T. chinensis* , Hap 136 is the same hyplotype of*T.rubripes* and *T. pseudommus* and Hap 88 is same hyplotype of *T. alboplumbeus* and *T. niphobles* . 121 COI gene sequences of *Lagocephalus* were divided into 36 hapltypes, and Hap 50 and Hap 53 is the same hyplotype of *L. wheeleri* and *L. Spadiceus* .

3.2.3 Genetic distance of Tetraodontiformes

The intraspecific genetic distance ranged from 0 to 0.009, and the average intraspecific genetic distance was 0.003. The interspecific genetic distance ranged from 0.001 to 0.298, the average interspecific genetic distance was 0.2, and the ratio of the average interspecific genetic distance to the average intraspecific genetic distance was 70.63. The average genetic distance within genus was 0.016. The genetic distance between genera ranged from 0.12 to 0.298, and the average genetic distance between genera was 0.223. The average genetic distance within the family was 0.106. The genetic distance between families ranged from 0.192 to 0.247, and the average genetic distance between families was 0.229.

The results showed that the interspecific genetic distance between*T. pseudommus, T. rubripes,* and *T. chinensis* was very small, and the range of interspecific genetic distance between them was 0.001 to 0.002, the average interspecific genetic distance was 0.002, and the interspecific genetic distance between*T. alboplumbeus* and *T. niphobles* is 0.002. Additionally, the interspecific genetic distance between *L. wheeleri* and *L. Spadiceus* is also very small, only 0.003.

Moreover, the results showed that the species of *Takifugu* have close genetic relationship. If *T. rubripes* , *T. pseudommus*and *T. chinensis* are taken as one species, *alboplumbeus*and *T. niphobles* are considered as one species, there are 14 species of this genus in this study. And the average interspecific genetic distance is 6.21 times of the average intraspecific genetic distance, which does not reach the DNA barcode threshold of more than 10 times proposed by Hebert. Among them, the genetic distance between*T. oblongus* and *T. stictonotus* is the largest, 0.045; The interspecific genetic distance of several species in the genera is less than 0.02 (as shown in Table 2), the genetic distance between *T. bimaculatus* and *T. flavidus* is the smallest, only 0.013.

3.2.4 Phylogenetic relationships of Tetraodontiformes

As shown in Figure 1, in the Neighbor-joining tree based on the haplotype of COI gene of Tetraodontiformes, the species of 6 families collected in this study are divided into two clades, Diodontidae and Tetraodontidae clustered into a clade, Monacanthidae, Balistidae, Ostraciidae and Triacanthidae clustered into a clade, as shown in Figure 1. Moreover, the results showed that Ostraciidae and Triacanthidae clustered in separate clades, as did Diodontidae and Tetraodontidae.

In the Neighbor-joining tree, *T. rubripes* , *T. pseudommus*and *T. chinensis* clustered into the same clade, and *T. alboplumbeus* and *T. niphobles* clustered into the same clade, as did *L. wheeleri* and *L. Spadiceus* . If taken the species clustered into the same clade as one species. In the Neighbor-joining tree, each species can gather into a separate clade except for the species that may be synonyms, although the interspecific genetic distance of some species of *Takifugu* is less than 0.02(as shown in Table 2).

3.2.5 DNA barcode classification and identification of Tetraodontiformes

The DNA barcoding identification result shows that 616 samples were identified as 46 species, belonging to 23 genera, 6 families and 4 Suborder (Table 1). Among them, DNA barcoding analysis showed that the specimens identified as *T. rubripes* , *T. pseudommus* , and *T. chinensis* had the same haplotype, the range of interspecific genetic distance between them was 0.001 to 0.002, and the average interspecific genetic distance was 0.002. In additional, in the NJ tree, they clustered into one clade. According to the DNA barcode identification results, *T. rubripes* , *T. pseudommus* and *T. chinensis* should be the synonyms. According to the principle that the first published species name is the valid name, for the three species, *T. rubripes* is the valid species name. Furthermore, the same results were found in *L. wheeleri* and *L. Spadiceus* . It showed that the specimens identified as *L. wheeleri* and *L. Spadiceus* had the same haplotype, the interspecific genetic distance between them was only 0.003, and they clustered into one clade in NJ tree. The results showed that *L. wheeleri* and *L. Spadiceus*should be synonyms, and *L. Spadiceus* is valid.

For *T. alboplumbeus* and *T. niphobles* , the DNA barcode identification results also showed that the two species may be synonyms. However, since the 11 specimens identified as *T. alboplumbeus* do not completely have the morphological characteristics of *T. alboplumbeus* , it is not certain that these specimens are hybrids of*T. alboplumbeus* and *T. niphobles* . Or that *T. alboplumbeus* and *T. niphobles* are the same species, but they will undergo a morphological transformation process.

4. Discussion

4.1 DNA barcoding threshold of Tetraodontiformes

DNA barcoding is used to identify species at molecular level, which is independent of time, region and individual morphology (Hebert, Ratnasingham & Waard, 2003). This study shows that DNA barcoding can quickly and accurately identify the species of Tetraodontiformes, without being affected by key factors such as the integrity and development stage of the sample and the taxonomic expertise of the appraiser. Besides, for those closely related species, the COI gene sequence may have high homology, but they can still be distinguished by phylogenetic tree. For example, the sequence similarity between *T. flavidus* (Hap 100) and *T. bimaculatus* (Hap 91) has reached 98.7% (678 conserved sites in the 687 bp COI gene sequence). And in *Takifugu* , the average interspecific genetic distance is 6.21 times of the average intraspecific genetic distance, which does not reach the DNA barcode threshold of more than 10 times proposed by Hebert. Among them, the genetic distance between *T. oblongus* and *T. stictonotus* is the largest, 0.045; The interspecific genetic distance of several species in the genera is less than 0.02 (as shown in Table 2), the genetic distance between *T. bimaculatus* and *T. flavidus* is the smallest, only 0.013. Therefore, the genetic distance threshold of 0.02 proposed by Hebert et al. is not applicable to the species of this genus.

4.2 Quality control of sequences downloaded from databases

NCBI's GenBank and BOLD are the two most abundant databases of DNA barcode resources. In recent years, the number of DNA barcode sequences submitted to NCBI and BOLD has increased explosively. When we use these two databases for DNA barcode sequence alignment analysis, we sometimes encounter inaccurate matching results. It has been reported that there are many errors in the sequences of NCBI database (Shen et al., 2013; Liu et al., 2020). Some studies also believe that BOLD database has conducted more rigorous review and screening on the submitted sequences, so the data in this database is more reliable (Wang et al., 2009; Macher, Macher & Leese, 2017). In fact, there are also wrong sequences in BOLD database (Lis, Lis & Ziaja, 2016).

In this study, all sequences were run BLAST in NCBI and BOLD databases. When BLAST is run on the sequence of most samples in the database, the sequences with the top similarity match are only the sequences of one species, which is consistent with the identification result. However, sometimes in the highly similar sequences of the results, in addition to the sequences of the species that are consistent with the identification results, there will also be a few sequences of the species that are inconsistent with the identification results. For instance, two published COI gene sequences of *T. porphyreus* (KT951818, KT951819) in the database are 100% similar to the sequences of *T. alboplumbeus* samples. When these two sequences were run BLAST in the database, the highly similar sequences in the result are all sequences of *T. alboplumbeus* , except for the two sequences of *T. porphyreus* , and these two sequences cannot match other sequences of*T. porphyreus* in the database. Therefore, the information of the two sequences in the database is incorrect. In addition, the sequence of*T. chinensis* , *T. pseudommus* and *T. rubripes* showed 99.56% similarity to the two mitochondrial genome sequences (NC_024199, KJ562276) of *T. flavidus* in BOLD and NCBI. After checking, it was found that the information of these two sequences was also wrong.Therefore, there are some incorrect sequences of Tetraodontiformes in the NCBI and BOLD databases.

The unreliable data in the databases will directly lead to the misidentification of species. In order to reduce the interference of the wrong sequence in the database on the analysis results, it is suggested that the relevant sequences in the database should be strictly screened when using DNA barcode technology to identify species. In particular, if the information in NCBI and BOLD databases is found to be inaccurate when the voucher specimens with complete morphological characteristics are available, the database or data submitter shall be contacted in time to correct the sequence information.

4.3 Synonym phenomenon in the order of Tetraodontiformes

For reasons such as untimely information exchange, and the morphological characteristics of fish are not only susceptible to subjective factors, but also will change significantly in different developmental stages and environmental conditions, the same species may have two or more different Latin names, which is also called synonym.

This study shows that there are a lot of synonyms in Tetraodontiformes. For example, sequences of the samples morphologically identified as *P. leiurus*were identified as *M. Leiurus* in NCBI, and there is no information about *P. leiurus* can be found in NCBI. In the FishBase (https://www.fishbase.de/), *P. leiurus* and *M. Leiurus* are synonyms, and *P. leiurus* is the valid name for this species. What's more, the samples which morphologically identified as*D. Nigroviridis* , in NCBI and BOLD databases, they were identified as *T. Nigroviridis* . In fact, in the FishBase *D. Nigroviridis* and *T. nigroviridis* are synonyms, and the name of*D. nigroviridis* is valid. The variety of species names in the database will affect the molecular identification results of species.We suggest that the unsynchronized information in the database is also one of the reasons for the confusion of species identification of Tetraodontiformes.

In addition, in the FishBase, it is considered that both *L. wheeleri* from *L. spadiceus* are effective species. And the morphological characteristics, can distinguish *Lagocephalus wheeleri* (Abe, Tabeta & Kitahama, 1984) from *Lagocephalus spadiceus* (Richardson, 1845) The former has elliptical dorsal spinule patch, and the latter has a rhomboidal patch with a posterior extension. Some studies have suggested that these

morphological features cannot be used to distinguish *L. wheeleri* from *L. spadiceus* , and that *L. wheeleri* may be a form of *L. spadiceus* , so the two species are actually the same species (Matsuura, 2010; Sakai, Sakamoto, & Yoshikawa, 2021). In this study, the results of haplotype analysis showed that they had the same haplotype. And in the Neighbor-joining tree, they clustered on the same clade, and the interspecific genetic distance was only 0.003. It was supported the view that *L. wheeleri* is the synonym of *L. spadiceus* .

Moreover, there are many reports believe that *T. rubripes* ,*T. chinensis* and *T. pseudommus* may be different phenotypes of the same species, *T. chinensis* and *T. pseudommus* are the synonyms of *T. rubripes* (Liu et al., 1999; Song et al., 2001; Cui et al., 2005; Reza et al., 2008; Park et al., 2020). However, it is considered that all three species are are valid in the FishBase, and they can be distinguished with morphological characteristics. T. rubripes has irregular round black spots and white patterns in front of the caudal fin on the sides of the body, T. chinensis has no such black marks while T. pseudommus has white spots scattered on a black background on the dorsal and lateral sides of the body (Baek et al., 2018; Reza et al., 2008). In this study, 8 samples were identified as*T. chinensis* , 4 as *T. pseudommus* , and 27 as *T. rubripes* , and it was found that the samples morphologically identified as the three species had the same haplotype, the range of interspecific genetic distance between them was 0.001 to 0.002, and the average interspecific genetic distance was 0.002. In additional, in the NJ tree, they clustered into one clade. It was proved that the *T. chinensis* and *T. pseudommus* are the synonyms of *T. rubripes* , and *T. rubripes* is the valid name.

And the morphologic features of*T. alboplumbeus* and *T. niphobles* are so similar that they are difficult to distinguish. It is generally believed that *T. niphobles* have obvious chest spots, while the chest spots of *T. alboplumbeus* are not obvious, and there are several dark bands on the back of *T. alboplumbeus* . In this study, the DNA barcode identification results also showed that the two species may be synonyms. However, since the 11 specimens identified as *T. alboplumbeus* do not completely have the morphological characteristics of *T. alboplumbeus* , it is not certain that these specimens are hybrids of*T. alboplumbeus* and *T. niphobles* . Or that *T. alboplumbeus* and *T. niphobles* are the same species, but they will undergo a morphological transformation process. Matsuura (2017) regarded *T. niphobles* and *T. alboplumbeus* as synonyms based on the same color patterns and no differences in morphological characteristics between the type specimens. Some researchers support this view as well (Okabe et al., 2019), and these two species are also considered to be synonyms in NCBI. However, in the FishBase, it is considered that both *T. alboplumbeus* and *T. niphobles* are effective species. Zhang & He (2008) showed that 12s and cytb genes could separate *T. alboplumbeus* and *T. niphobles* , but the study did not describe the morphological characteristics of the two species. Zhou et al. (2020) Showed that the mitochondrial genome can separate *T. alboplumbeus*and *T. niphobles* effectively. However, the study did not describe the morphological characteristics of the two species as well. Therefore, whether *T. alboplumbeus* and *T. niphobles* are synonyms has not been determined.

4.4 Reconstruction of Tetraodontiformes phylogenetic relationships

Many researchers have studied the relationship between families of Tetraodontiformes based on different methods (Breder & Clark, 1947; Winterbottom, 1974; Rosen, 1984; Leis, 1984; Tyler & Sorbini, 1996; Santini & Tyler,2003; Holcrof, 2005; Alfaro, Santini & Brock, 2007; Yamanoue et al., 2007; Yamanoue et al., 2008;). In their study, there were more or less changes in the phylogenetic tree of Tetraodontiformes. Similarly, they agreed that Balistidae and Monacanthidae were closely related, and Diodontidae and Tetraodontidae were closely related as well.

In this study, the Neighbor-joining tree based on the haplotype of COI gene of Tetraodontiformes shows that the species of 6 families are splited into two clades. And Diodontidae and Tetraodontidae clustered into a clade, Monacanthidae, Balistidae, Ostraciidae and Triacanthidae clustered into a clade, as shown in Figure 1. The result of that Diodontidae and Tetraodontidae clustered in a separate clade, is consistent with the research results of others. However, unlike other studies, Balistidae and monacanthidae did not cluster into a clade in this study. The reason for this result may be that there are not enough samples collected in this study. For example, only one sample of Triacanthidae was collected. It is also possible that the COI gene sequence is not suitable for the systematic relationship study of families and higher taxonomic levels.

In the Neighbor-joining tree based on the haplotype of COI gene of Tetraodontiformes, the species of the same genus clustered together, and the same species are clustered into a clade, which is basically consistent with the results of morphological identification. Although the interspecific genetic distance of some species of Takifugu is less than 0.02(as shown in Table 2), in the Neighbor-joining tree, except for *T. pseudommus* , *T. rubripes* and *T. chinensis* , each species can gather into a separate clade. It indicates that the COI gene sequence is suitable for the classification and identification of genera and lower taxonomic levels.

5. Conclusion

In this study, 616 samples of Tetraodontiformes were identified by using morphological characteristics and DNA barcoding technology. It revealed that DNA barcoding can be effectively used in the identification of Tetraodontiformes.

Moreover, it is suggested that DNA barcoding can provide molecular evidence for clarifying the problem of species synonyms and confirming valid species names. It provided molecular evidence for clarifying the valid species names of *L. Spadiceus* and *T. rubripes* .

In addition, this study reconfirms that there are some incorrect sequences in both NCBI and BOLD databases. It is suggested that synonyms and the unreliable data are also the reasons for the confusion of taxonomic identification of Tetraodontiformes. We suggest that when using DNA barcode technology to identify species, the sequence in the database needs to be strictly screened. When the sample has complete morphological characteristics, the final identification results of species should be determined by combining the morphological characteristics.

Conflict of Interest

We wish to draw the attention of the Editor to the following facts may be considered as potential conflicts of interest and significant financial contributions to this work.

All authors agreed to this submission and the Corresponding author has been authorized by co-authors. This manuscript has not been published before and is not concurrently being considered for publication elsewhere. This manuscript does not violate any copyright or other personal proprietary right of any person or entity and it contains no abusive, defamatory, obscene or fraudulent statements, nor any other statements that are unlawful in any way.

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Data Availability Statement

DNA sequences have been deposited in GenBank under Accession numbers OQ700230–OQ700845. Details regarding individual samples are available in Table 1.

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Table. 1 Sample collection information and identification results

Suborder	family	Genus	Morphological identification	Molecular identification	Number of
Balistoidei	Balistidae	*Balistoides*	*Balistoides conspicillum*	*B. conspicillum*	4
		Canthidermis	*Canthidermis maculata*	*C. maculata*	1
		Melichthys	*Melichthys vidua*	*M. vidua*	6
		Rhinecanthus	*Rhinecanthus aculeatus*	*R. aculeatus*	4
		Sufflamen	*Sufflamen chrysopterum*	*S. chrysopterum*	3
			Sufflamen fraenatum	*S. fraenatum*	3
		Xanthichthys	*Xanthichthys auromarginatus*	*X. auromarginatus*	1
	Monacanthidae	*Aluterus*	*Aluterus monoceros*	*A. monoceros*	1
			Aluterus scriptus	*A. scriptus*	13
		Cantherhines	*Cantherhines pardalis*	*C. pardalis*	1
		Chaetodermis	*Chaetodermis penicilligerus*	*C. penicilligerus*	35
		Monacanthus	*Monacanthus chinensis*	*M. chinensis*	7
		Stephanolepis	*Stephanolepis cirrhifer*	*S. cirrhifer*	24
		Thamnaconus	*Thamnaconus hypargyreus*	*T. hypargyreus*	16
			Thamnaconus modestus	*T. modestus*	23
Ostracioidei	Ostraciidae	*Lactoria*	*Lactoria cornuta*	*L. cornuta*	7
		Ostracion	*Ostracion cubicus*	*O. cubicus*	1
			Ostracion rhinorhynchos	*O. rhinorhynchos*	8
Tetraodontoidei	Diodontidae	*Diodon*	*Diodon holocanthus*	*D. holocanthus*	19
			Diodon hystrix	*D. hystrix*	9
			Diodon liturosus	*D. liturosus*	5
	Tetraodontidae	*Arothron*	*Arothron hispidus*	*A. hispidus*	3
			Arothron mappa	*A. mappa*	1
			Arothron stellatus	*A. stellatus*	4
		Dichotomyctere	*Dichotomyctere nigroviridis*	*D. nigroviridis*	11
		Lagocephalus	*Lagocephalus gloveri*	*L. gloveri*	17
			Lagocephalus inermis	*L. inermis*	8
			Lagocephalus spadiceus	*Lagocephalus spadiceus*	14
			Lagocephalus wheeleri	*Lagocephalus spadiceus*	82
		Pao	*Pao leiurus*	*P. leiurus*	21
		Sphoeroides	*Sphoeroides pachygaster*	*S. pachygaster*	1
		Takifugu	*Takifugu alboplumbeus*	*T. alboplumbeus*	11
			Takifugu bimaculatus	*T. bimaculatus*	35
			Takifugu chinensis	*T. rubripes*	8
			Takifugu flavidus	*T. flavidus*	22
			Takifugu niphobles	*T. alboplumbeus*	20
			Takifugu oblongus	*T. oblongus*	26
			Takifugu obscurus	*T. obscurus*	14
			Takifugu ocellatus	*T. ocellatus*	36
			Takifugu pardalis	*T. pardalis*	2
			Takifugu poecilonotus	*T. poecilonotus*	5
			Takifugu porphyreus	*T. porphyreus*	3
			Takifugu pseudommus	*T. rubripes*	4
			Takifugu rubripes	*T. rubripes*	27
			Takifugu snyderi	*T. snyderi*	2
			Takifugu stictonotus	*T. stictonotus*	1
			Takifugu vermicularis	*T. vermicularis*	4

Suborder	family	Genus	Morphological identification	Molecular identification	Number of
			Takifugu xanthopterus	*T. xanthopterus*	33
		Torquigener	*Torquigener hypselogeneion*	*T. hypselogeneion*	9
Triacanthoidei	Triacanthidae	*Triacanthus*	*Triacanthus biaculeatus*	*T. biaculeatus*	1
Total	6	23	50	46	616

Table. 2 Interspecific genetic distance of some *Takifugu*

	T. alboplumbeus	*T. bimaculatus*	*T. rubripes*	*T. flavidus*	*T. oblongus*	*T. obscurus*	*T. ocellatus*
T. alboplumbeus		0.0063	0.0061	0.0062	0.0076	0.0060	0.0068
T. bimaculatus	0.0357		0.0061	0.0034	0.0065	0.0055	0.0068
T. rubripes	0.0318	0.0314		0.0056	0.0067	0.0057	0.0068
T. flavidus	0.0323	**0.0127**	0.0259		0.0065	0.0049	0.0064
T. oblongus	0.0432	0.0325	0.0372	0.0321		0.0063	0.0078
T. obscurus	0.0303	0.0255	0.0250	**0.0196**	0.0329		0.0063
T. ocellatus	0.0379	0.0382	0.0343	0.0331	0.0447	0.0299	
T. pardalis	0.0220	0.0295	0.0251	0.0253	0.0336	0.0207	0.0310
T. poecilonotus	0.0300	0.0316	0.0300	0.0255	0.0400	0.0263	0.0204
T. porphyreus	0.0343	0.0283	0.0281	0.0242	0.0408	0.0231	0.0349
T. snyderi	0.0299	0.0410	0.0312	0.0354	0.0438	0.0314	0.0373
T. stictonotus	0.0351	0.0430	0.0327	0.0401	0.0454	0.0329	0.0420
T. vermicularis	0.0359	0.0338	0.0358	0.0292	0.0436	0.0298	0.0350
T. xanthopterus	0.0236	0.0283	0.0243	0.0224	0.0337	**0.0199**	0.0242

NOTE: Above the diagonal is the standard deviation, below the diagonal is the interspecific genetic distance of *Takifugu* , and bold indicates the interspecific distance less than 0.02.

Figure 1. The phylogenetic tree of Tetraodontiformes based on 171 COI gene haplotypes

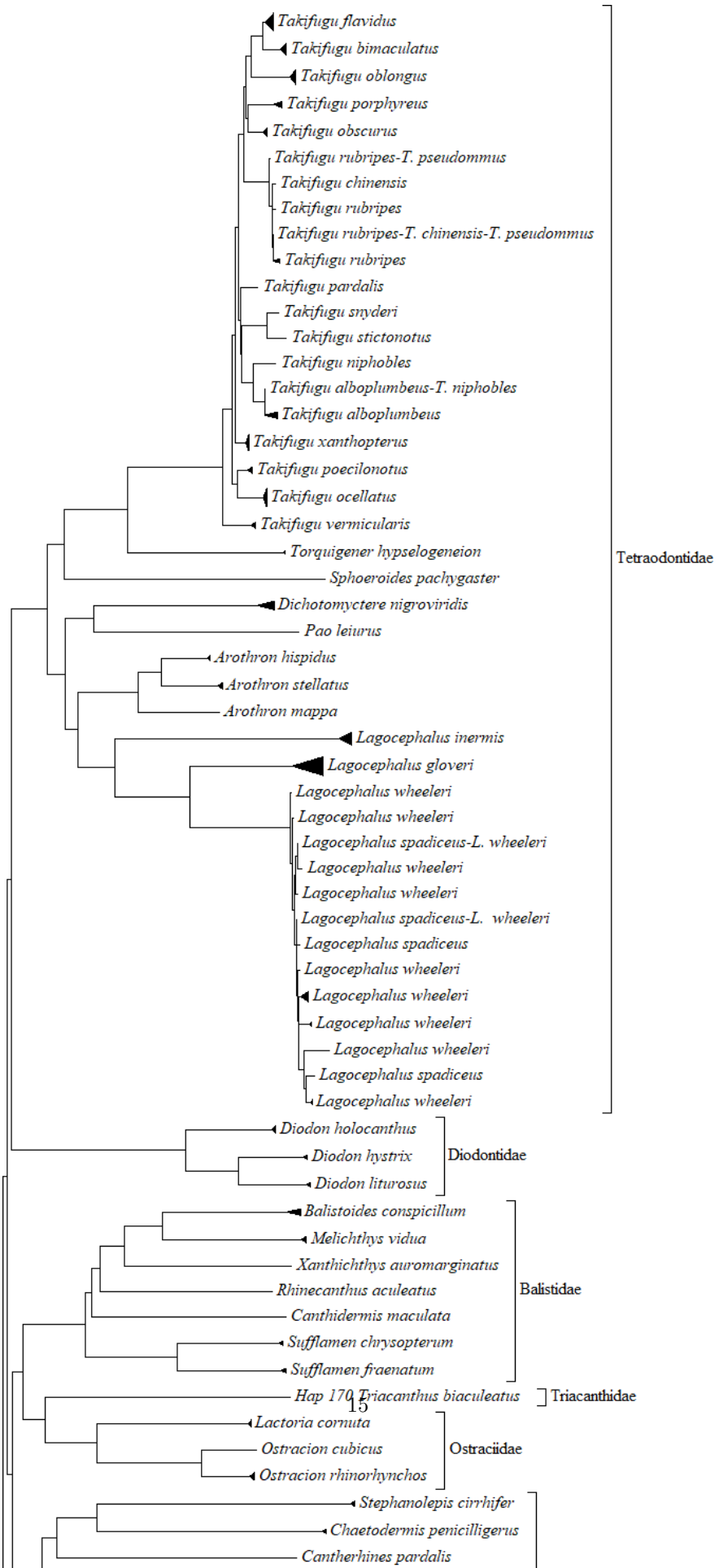

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Table 1.docx available at https://authorea.com/users/601544/articles/632700-dna-barcoding-identification-and-validation-of-the-puffer-fish-order-tetraodontiformes-in-china-coastal-waters

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