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Research Article

Multilocus Molecular Phylogeny Brings Insight Into Systematics and Evolution of Genus *Tridentiger* (Teleostei: Gobiiformes)

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The genus *Tridentiger* Gill, 1859 comprises barbeled and nonbarbeled species, which is considered a key character to further divide the genus. Our phylogenetic tree based on 5026-bp sequence data of six genes indicated a well-supported sister relationship between the nonbarbeled *T. nudicervicus* and the barbeled *T. barbatus* and *T. microsquamis*, which rejected monophyly of the nonbarbeled species. Therefore, genus *Tridentiger* should not be further divided into nonbarbeled and barbeled genera. Due to the presence of the common diagnosis (the outmost row of jaw teeth in tricuspid form), *Papuligobius*, the closest sister genus, should be assigned as a junior synonym of *Tridentiger*. Moreover, 12S ribosomal RNA (12S rRNA) Kimura-2-parameter (K2P) distance (0.0000–0.0016) between *T. microsquamis* and *T. radiatus* falls in the interval of intraspecific distances (0.0000–0.0030), much smaller than the interspecific distances (0.0122–0.1383). Therefore, *T. radiatus* should be treated as a synonym of *T. microsquamis*. K2P distances between the southern and northern lineages of *T. barbatus* fall in the interval of interspecific distances in 12S rRNA, cytochrome *b* (Cyt*b*), and recombination activating gene 1 (RAG1) genes, which were delineated as different species in the species delimitations. The mean divergence time between *T. kuroiwae* in the Ryukyu Islands and its congener, *T. brevispinis* and *T. obscurus* in the Japan Archipelago, was dated back to 1.56 million years ago (mya) and close to the formation of the Tokara Gap (~1.5 mya), which might be a barrier driving divergence of the three species. However, as many *Tridentiger* species show different salinity and habitat preferences, ecological factors might also play important roles in the evolution of the genus *Tridentiger*.

Keywords: *Papuligobius*; species delimitation; the Tokara Gap; *Tomiyamia*; *Triaenopogon*

1. Introduction

The genus *Tridentiger* Gill, 1859 of the family Oxudercidae Günther, 1861 [1, 2], is diagnosed by the presence of tricuspid teeth in the outermost row on both the upper and lower jaws [3, 4]. The genus is now comprised of eight valid species [5]: *Tridentiger barbatus* (Günther, 1861), *T. brevispinis* Katsuyama, Arai & Nakamura, 1972, *T. bifasciatus* Steindachner, 1881, *T. kuroiwae* Jordan & Tanaka, 1927, *T. nudicervicus* Tomiyama, 1934, *T. obscurus* (Temminck & Schlegel, 1845), *T. radiatus* Cui, Pan, Yang & Wang, 2013, *T. trigonocephalus* (Gill, 1859). These species are euryhaline fishes widely found in sediment-rich freshwater and coastal environments in the East Asia [6]. Some species of *Tridentiger* are well-known invasive species,

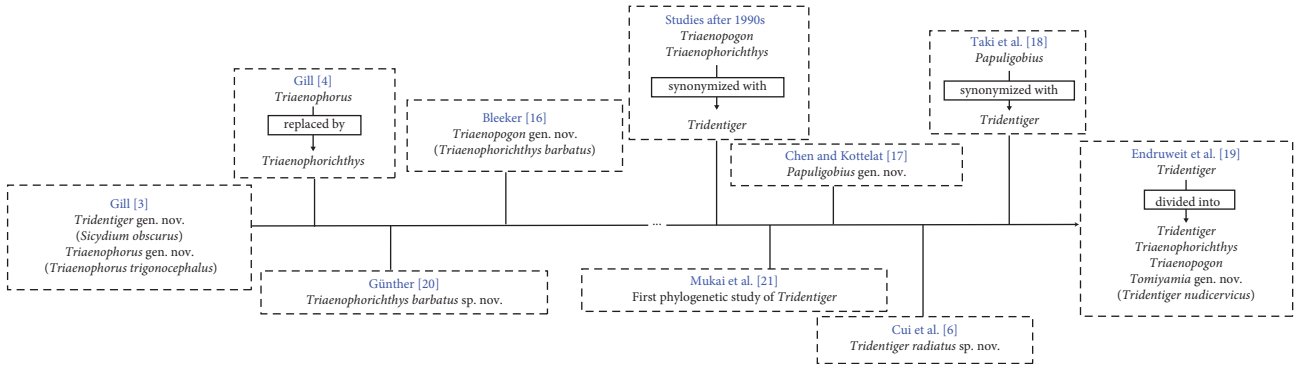

FIGURE 1: A brief taxonomic history of the genus *Tridentiger* and its synonyms. Species name within brackets is the type species for the corresponding genus.

including *T. barbatus*, *T. bifasciatus*, and *T. trigonocephalus* [7]. Invasion of *T. bifasciatus* can be traced back to the 1980s, which is presumably related to international transportation of oysters [8] and ballast water [9] in North America. In recent years, its invasion into lakes via the routes of the South-to-North Water Transfer Project in China, and its impact posed to the local biodiversity, are gaining growing attention [10, 11]. *Tridentiger trigonocephalus* has also experienced a great success since the 1990s, whose populations are now broadly found in marine and brackish water of the United States, Australia, the Mediterranean, and Black Sea [7]. Comparatively, invasion of *T. barbatus* is lack of extensive reports since its exotic population was first found in the United States [12]. However, the populations of *T. barbatus* and *T. nudicervicus* in Japan are considered near-threatened due to increasing coastal infrastructure and resultant reduction of intertidal mudflat habitats [13, 14].

The genus *Tridentiger* was erected by Gill [3], typified by *Sicydium obscurus* Temminck & Schlegel, 1845, characterized by the presence of tricuspid teeth at the outermost row of jaw teeth (Figure 1). Simultaneously, the genus *Triaenophorus* was established for the new species *Triaenophorus trigonocephalus* Gill, 1859 under the *Tridentiger* category, and the former was replaced by *Triaenophorichthys* Gill, 1859 due to preoccupation [15]. Later, *Triaenopogon* Bleeker, 1874, was proposed for the barbeled species *Triaenophorichthys barbatus* Günther, 1861 [16]. These three genus names are currently considered synonyms of *Tridentiger* based on their tricuspid teeth [4, 6].

Despite this synonymy, further division of *Tridentiger* has been frequently proposed in recent studies. Mukai et al. [21], using allozyme polymorphism, reconstructed phylogenetic relationships of seven *Tridentiger* species (excluding *T. radiatus* described later) and found a closer relationship between *T. barbatus* and *T. nudicervicus*. Mukai et al. [21] suggested dividing *Tridentiger* into two groups: one with *T. barbatus* and *T. nudicervicus*, and another with the other five species. However, Cui et al. [6] presented a different perspective based on a 12S ribosomal RNA (12S rRNA) phylogeny, which grouped *T. nudicervicus* with *T. bifasciatus* and *T. trigonocephalus*, contradicting Mukai et al.'s [21] findings. Consequently, Cui et al. [6] proposed a dichotomy of *Tridentiger* into nonbarbeled and barbeled species, and suggested the possible resurrection of *Triaenopogon* for barbeled species. More recently, Taki et al. [18] proposed the merge of *Papuligobius* Chen & Kottelat, 2003 with the genus *Tridentiger* due to its presence of tricuspid teeth. Endruweit [19], synthesizing Mukai et al. [21] and Cui et al.'s [6] idea, split the genus into *Tridentiger*, *Triaenophorichthys*, *Triaenopogon*, and *Tomiyamia* Endruweit, 2024, which further complicated their taxonomy. Therefore, a re-evaluation on systematics of the genus *Tridentiger* based on multiloci phylogeny is necessary.

Taxonomies of the *Tridentiger* species are also contentious. Numerous cases of introgression between *T. brevispinis* and *T. obscurus* were reported in Japan, suggesting weak reproductive isolation between the two species [22, 23]. The two species showed low genetic differentiations in mitochondrial genes, but can be distinguished by several nuclear genes [24]. The validity of these two species needs further evaluation. Cui et al. [6] described a new barbeled *Tridentiger* species, *T. radiatus*, as the sister species of *T. barbatus*, which is genetically distinct from the other seven valid species in 12S rRNA. This species was diagnosed by its sparser barbels on the cheek, denser scales covered on the body, and unique blurred color pattern [6]. These characters, nevertheless, were similar to the description of an earlier, but rarely mentioned species, *Triaenopogon microsquamis* Wu, 1931, that has long been considered a synonym of *T. barbatus* [25]. Wei et al. [26] presented the first exploration on the genetic structure of *T. barbatus* along the coast of China and uncovered a north-south differentiation within the species. This implies a greater species diversity for the barbeled *Tridentiger* species.

In this study, we presented systematic research of the genus *Tridentiger* based on the phylogeny of multiple mitochondrial and nuclear genes. We aimed at (1) seeking cryptic species diversity within this genus; (2) verifying the interrelationship between *T. microsquamis* and *T. radiatus*; (3) clarifying phylogenetic relationships of the eight valid species and providing proper suggestions on taxonomy of *Tridentiger* and its related genera, including *Triaenophorichthys*, *Tomiyamia*, *Triaenopogon*, and *Papuligobius*.

2. Materials and Methods

2.1. Sampling. From 2014 to 2016, 83 specimens of eight species in the genus *Tridentiger*, that is, *T. obscurus*, *T. brevispinis*, *T. kuroiwae*, *T. trigonocephalus*, *T. bifasciatus*, *T. nudicervicus*, *T. barbatus*, and *T. microsquamis*, were collected from local

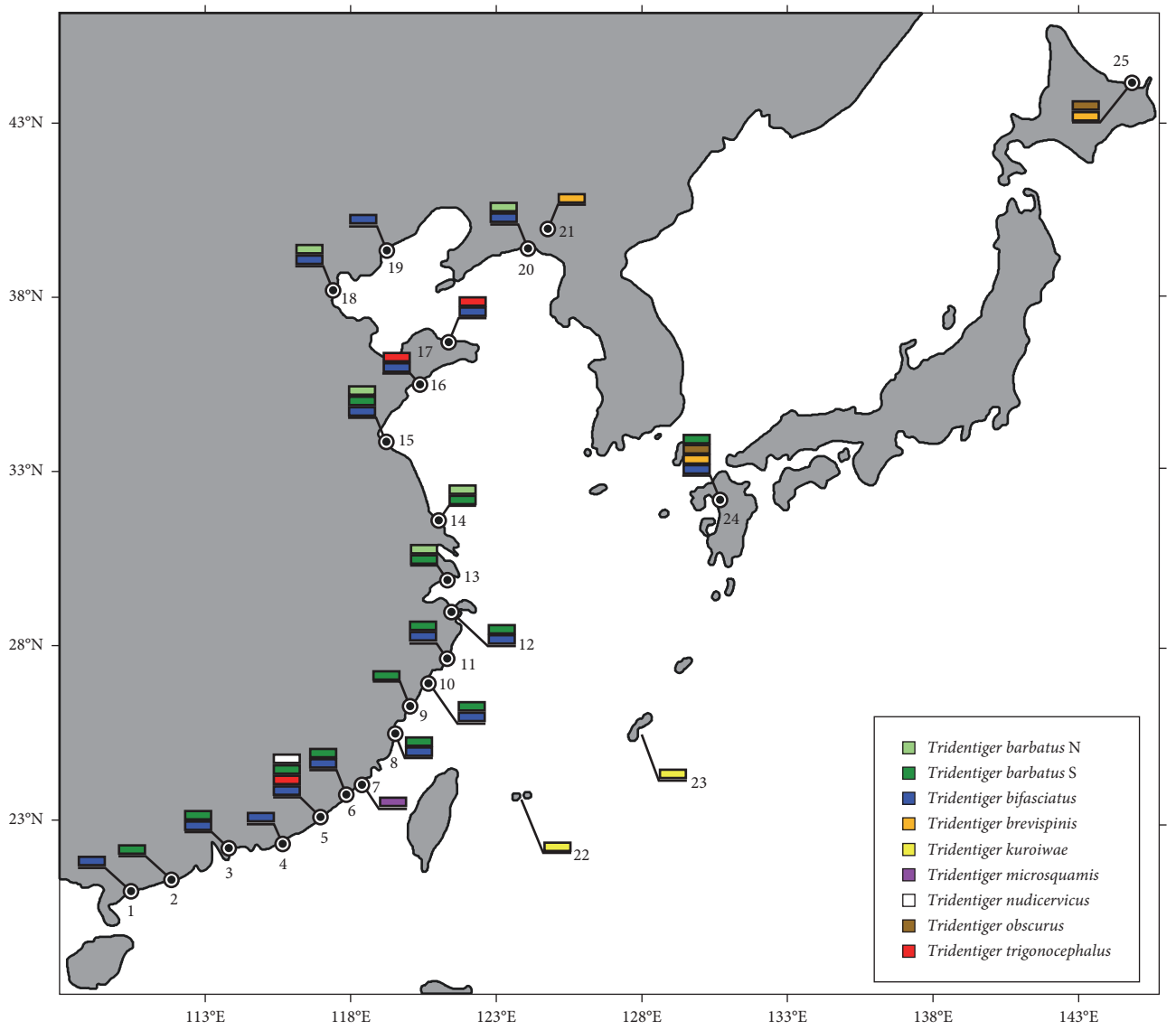

FIGURE 2: Map of East Asia indicating 25 sample sites of the eight *Tridentiger* species. Sample sites are labeled with numbers: 1. Zhanjiang; 2. Yangjiang 3. Shenzhen; 4. Shanwei; 5. Raoping; 6. Zhangzhou; 7. Xiamen; 8. Fuding; 9. Rui'an; 10. Dongtou; 11. Taizhou; 12. Ningbo; 13. Shanghai; 14. Rudong; 15. Lianyungang; 16. Qingdao; 17. Yantai; 18. Tianjin; 19. Qinhuangdao 20. Dandong; 21. Kuandian; 22. Iriomote; 23. Okinawa; 24. Kyushu; 25. Hokkaido.

fish markets and rivers of China and Japan. The sampling sites of these specimens are shown in Figure 2. Among our collections, most *T. barbatus* specimens were derived from Wei et al. [26], randomly selected from each sampling locality of the southern and northern lineages. An additional *T. barbatus* specimen was collected from Kyushu, Japan (Figure 2). Two specimens of *T. microsquamis* were collected from Xiamen (= Amoy, type locality of *T. microsquamis*). The other specimens were carefully identified following Akihito et al. [4]. Based on the latest Gobiiformes phylogenetic studies [2], specimens of *Acanthogobius flavimanus* (Temminck & Schlegel, 1845), *Rhinogobius similis* Gill, 1859, and *Papuligobius uniporus* Chen & Kottelat, 2003 were collected as outgroups for phylogenetic tree reconstructions. Additionally, sequences of *Papuligobius ocellatus* (Fowler, 1937) were downloaded from NCBI as another outgroup. All the specimens were fixed by 95% ethanol and stored in the Department of Oceanography, National Sun Yat-sen University, Kaohsiung (DOS).

2.2. DNA Extraction, Amplification, and Sequencing. Total genomic DNA of the specimens was isolated from fin clips and/or muscle by Micro-Elute DNA Clean/Extraction Kit (GMbiolab Co., Ltd.), following the manual instructions. Six gene markers were amplified, including 12S rRNA, cytochrome *b* (Cyt*b*), Rhodopsin (Rh), recombination activating gene 1 (RAG1), super conserved receptor expressed in brain 2 (Sreb2), and zinc finger of the cerebellum 1 (Zic1). Protocols, cycling conditions, and primers for polymerase chain reactions (PCRs) of these genes are summarized in Supporting Information S1. PCR products were purified using the SAP-Exo Kit

Table 1: Information of datasets used in this study.

Dataset	Usage	Genes	Number of sequences	Alignment length
Dataset 1	Seeking cryptic species diversity within the genus *Tridentiger*	Cyt*b*	87	681 bp
Dataset 2	Verifying synonymy of *T. microsquamis* and *T. radiatus*	12S rRNA	30	644 bp
Dataset 3	Reconstructing the reliable phylogeny of *Tridentiger*	Cyt*b*, 12S rRNA, Rh, RAG1, Sreb2, Zic1	26	5026 bp

(Jena Bioscience GmbH) according to the manual instructions. After purification, all samples were sent for sequencing by a commercial company. For 12S rRNA and Cyt*b*, the PCR products were only sequenced using the corresponding forward primers. For RAG1, Rh, Sreb1, and Zic1, the products were sequenced at both ends with the corresponding forward and reversed primers. All the sequences were assembled with Seqman of Larsergene v7.10.0 (DNA star Inc., Madison, WI, USA), aligned using the Clustal W method in MEGA11 with default settings, and trimmed to obtain uniform length [27, 28]. Any obvious misalignment would be revised by eyes.

2.3. Datasets for Phylogenetic Analyses and Saturation Test. The sequence data of the six genes were collated to form three different datasets for genetic analysis (Table 1, Supporting Information S2, datasets 1–3). Cyt*b* sequences of all the *Tridentiger* specimens and the outgroups (i.e., dataset 1 in Table 2) were used to study the genetic diversity of the *Tridentiger*. Two individuals from each clade identified in the Cyt*b* tree were selected as representatives, and their 12S rRNA, Rh, RAG1, Sreb2, and Zic1 were sequenced for phylogenetic analyses. Due to potential introgression between *T. obscurus* and *T. brevispinis*, we included sequences of all specimens of the two species, and a total of 22 *Tridentiger* specimens were used in datasets 2–3 (Table 3). Due to only 12S rRNA sequences of *T. radiatus* were available from GenBank [6], dataset 2 is composed of the 22 aforementioned specimens, including *T. microsquamis* from Xiamen, to verify the interrelationship between *T. microsquamis* and *T. radiatus*. Finally, 12S rRNA, Cyt*b*, Rh, RAG1, Sreb2, and Zic1 of the 22 specimens were concatenated in SequenceMatrix v2.10.0 [29] to form dataset 3 for phylogenetic analyses, species delimitation, and molecular dating, with the six genes treated as six independent partitions in the analyses.

The nonconservative regions in 12S rRNA alignments were removed using online Gblocks v0.91b with the default parameters [30, 31] (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks). Substitution saturation test of each gene was performed using the method of Xia et al. [32] in DAMBE v.7.0.28 [33]. Levels of substitution saturation of 12S rRNA were testified using cured alignment files generated by Gblocks v0.91b, while saturation test of the protein-coding genes, that is, Cyt*b*, Rh, RAG1, Sreb2, and Zic1, were carried out on the three codon positions, respectively (Supporting Information S2). Saturated genes or codon positions were excluded from the phylogenetic analyses. Best-fit models were selected based on the Bayes Information Criterion using jModelTest2, which were summarized in Supporting Information S2 [34, 35].

2.4. Phylogenetic Analyses and Species Delimitation. Phylogenetic analyses of the datasets 1–3 were performed using maximum likelihood (ML) and Bayesian inference (BI) methods. All the phylogenetic trees were rooted by *Acanthogobius flavimanus*, with best-fit models selected by jModeltest2. ML trees were reconstructed in IQ-TREE [36]. Bootstrap values were estimated with 1,000 bootstrap replicates [37]. BI trees were reconstructed in MrBayes v3.2 [38]. Two independent Markov Chain Monte Carlo with four chains were performed for 5,000,000 generations, sampling every 100 generations and discarding the first 25% samples as burn-in. Sufficient convergence of runs was estimated by summary statistics implemented in MrBayes v3.2 (estimated sample size (ESS) > 200, potential scale reduction factor ~1). Additionally, we performed phylogenetic analyses on each partition to ascertain congruence of their gene tree topologies; any poorly supported (PP < 0.95 and BS < 75) contradictory nodes would be considered congruent.

Genetic distances were calculated using the Kimura-2-parameter (K2P) model in MEGA 11 [28, 39]. K2P distance of 12S rRNA was calculated using dataset 2 to compare genetic differences between *T. radiatus* and *T. microsquamis*, while those of the other five genes were calculated using partitions of dataset 3. The interspecific and infraspecific distances were visualized in GraphPad Prism v8.00.2 (GraphPad Software Inc.) using box and scatter plots. Species delimitations were performed with dataset 3 in the itaxoTools package v 0.1 [40], including a distance-based method, the Assemble Species by Automatic Partitioning (ASAP; [41]) and two tree-based methods, the single threshold Bayesian Poisson tree processes (bPTPs; [42]) and the generalized mixed Yule coalescent (GMYC; [43]). ASAP was performed on the partitions of dataset 3 using the default settings. The ML tree of the dataset 3 generated from IQ-TREE was used as phylogram input for bPTP. Ultrametric tree input for GMYC was generated with dataset 3 in BEAST v2.70.4 [44] using the constant population size model and the best-fit substitution models from jModeltest2, following the instructions of GMYC [43]. Two 10,000,000-generation MCMC runs were conducted, and sufficient convergence and burn-ins of the runs were checked in Tracer v1.7 (http://tree.bio.ed.ac.uk/software/tracer), with effective sampling sizes of all parameters ascertained over 200. The ultrametric tree was then generated with maximum clade credibility and discarding the first 10% samples as burn-in in TreeAnnotator of the BEAST2 package [44].

Table 2: The *Tridentiger* and outgroup specimens used in this study.

Sampling site code	Sampling site	Accession number of Cyt*b*	Specimen voucher
T. bifasciatus			
1	Zhanjiang	MT215264	DOS01583
1	Zhanjiang	MT215265	DOS01584
3	Shenzhen	MT215258	DOS01222
3	Shenzhen	MT215259	DOS01223
4	Shanwei	MT215266	DOS01649
4	Shanwei	MT215267	DOS01653
5	Raoping	MT215268	DOS01655
5	Raoping	MT215269	DOS01657
6	Zhangzhou	MT215252	DOS01043
6	Zhangzhou	MT215253	DOS01044
8	Fuding	MT215261	DOS01456
10	Dongtou	MT215256	DOS01112
10	Dongtou	MT215257	DOS01114
11	Taizhou	MT215262	DOS01491
11	Taizhou	MT215263	DOS01494
12	Shanghai	MT215254	DOS01047
12	Shanghai	MT215255	DOS01050
15	Qingdao	MT215247	DOS00786
17	Yantai	MT215243	DOS00631
17	Yantai	MT215244	DOS00640
18	Tianjin	MT215250	DOS00956
18	Tianjin	MT215251	DOS00960
19	Qinhuangdao	MT524720	DOS01009
19	Qinhuangdao	MT524721	DOS01015
20	Dandong	MT215245	DOS00721
20	Dandong	MT215246	DOS00724
24	Kyushu	MT215270	DOS03853-1
24	Kyushu	MT215271	DOS03853-2
T. trigonocephalus			
5	Raoping	MT215321	DOS03986-2
16	Qingdao	MT215322	DOS00871
17	Yantai	MT215323	DOS00893
17	Yantai	MT215324	DOS00894
T. nudicervicus			
5	Raoping	PQ878622	DOS03051-1/IOCAS000345-1
5	Raoping	PQ878623	DOS03051-2/IOCAS000345-2
T. barbatus southern lineage			
2	Yangjiang	MT215291	DOS01536
2	Yangjiang	MT215292	DOS01540
3	Shenzhen	MT215287	DOS01417
3	Shenzhen	MT215288	DOS01420
4	Shanwei	MT215297	DOS01666
4	Shanwei	MT215298	DOS01667
5	Raoping	MT215293	DOS01646
5	Raoping	MT215294	DOS01647
8	Fuding	MT215279	DOS01292
8	Fuding	MT215280	DOS01293
9	Rui'an	MT215277	DOS00792
9	Rui'an	MT215278	DOS00796
10	Dongtou	MT215281	DOS01337
10	Dongtou	MT215282	DOS01343
11	Taizhou	MT215289	DOS01438

Table 2: Continued.

Sampling site code	Sampling site	Accession number of Cyt*b*	Specimen voucher
11	Taizhou	MT215290	DOS01441
12	Ningbo	MT215295	DOS01659
12	Ningbo	MT215296	DOS01660
13	Shanghai	MT215283	DOS01359
13	Shanghai	MT215284	DOS01360
14	Rudong	MT215303	DOS02907-1
14	Rudong	MT215304	DOS02907-12
15	Lianyungang	MT215301	DOS02899-8
15	Lianyungang	MT215302	DOS02899-9
24	Kyushu	OR067083	DOS03223
T. barbatus northern lineage			
13	Shanghai	MT215285	DOS01362
13	Shanghai	MT215286	DOS01367
14	Rudong	MT215305	DOS02907-34
14	Rudong	MT215306	DOS02913-15
15	Lianyungang	MT215299	DOS02899-1
15	Lianyungang	MT215300	DOS02899-13
18	Tianjin	MT215273	DOS00727
18	Tianjin	MT215274	DOS00730
20	Dandong	MT215275	DOS00761
20	Dandong	MT215276	DOS00762
T. microsquamis			
7	Xiamen	MT215317	DOS03052-1
7	Xiamen	MT215318	DOS03052-2
T. obscurus			
24	Kyushu	MT215319	DOS03294-1
24	Kyushu	MT215320	DOS03294-2
T. brevispinis			
21	Kuandian	MT215312	DOS03861
24	Kyushu	MT215307	DOS03224
24	Kyushu	MT215308	DOS03229-1
24	Kyushu	MT215309	DOS03229-2
25	Hokaido	MT215310	DOS03273
25	Hokaido	MT215311	DOS03274
T. kuroiwae			
22	Iriomote	MT215313	DOS03134-2
22	Iriomote	MT215314	DOS03134-3
23	Okinawa	MT215315	DOS03148-1
23	Okinawa	MT215316	DOS03148-2
Acanthogobius flavimanus			
19	Qinhuangdao	MT215240	DOS00609
Rhinogobius similis			
—	Pingtung	MT215241	DOS00425-1
Tridentiger uniporus			
—	Thành Phố Huế	MT215242	DOS03541-1
Tridentiger ocellatus			
—	—	KF415628	NMBE 1066454

2.5. Molecular Dating. We inferred the divergence time of the genus *Tridentiger* using the Birth-Death process model in Beast v2.7.4 with concatenated data of the six genes (i.e., the dataset 3, [44]). Because introgression of mitochondrial gene between *T. brevispinis* and *T. obscurus* accounts for low genetic differentiation in their 12S rRNA and Cyt*b* sequences, which might underestimate their divergence time. Only the nuclear genes (i.e., RAG1, Rh, Sreb2, and Zic1) were used for their

Table 3: Accession numbers of sequences used in datasets 2 (12S) and 3 (6 genes).

Species	12S rRNA	Cyt*b*	RAG1	Rh	Sreb2	Zic1	Voucher
Acanthogobius flavimanus	MT210646	MT210671	MT210694	MT210719	MT210744	MT210769	DOS00609
Rhinogobius similis	MT210648	MT210673	MT210696	MT210721	MT210746	MT210771	DOS00425-1
Tridentiger uniporus	MT210647	MT210672	MT210695	MT210720	MT210745	MT210770	DOS03541-1
Tridentiger ocellatus	KF415434	KF415628	KF415833	—	KF416255	KF416040	NMBE 1066454
Tridentiger barbatus northern lineage	MT210649	MT210674	MT210697	MT210722	MT210747	MT210772	DOS00727
Tridentiger barbatus northern lineage	MT210650	MT210675	MT210698	MT210723	MT210748	MT210773	DOS00730
Tridentiger barbatus southern lineage	MT210651	MT210676	MT210699	MT210724	MT210749	MT210774	DOS01417
Tridentiger barbatus southern lineage	MT210652	MT210677	MT210700	MT210725	MT210750	MT210775	DOS01420
Tridentiger bifasciatus	MT210653	MT210678	MT210701	MT210726	MT210751	MT210776	DOS01047
Tridentiger bifasciatus	MT210654	MT210679	MT210702	MT210727	MT210752	MT210777	DOS01050
Tridentiger brevispinis	MT210655	MT210680	MT210703	MT210728	MT210753	MT210778	DOS03861
Tridentiger brevispinis	MT210656	MT210681	MT210704	MT210729	MT210754	MT210779	DOS03224
Tridentiger brevispinis	MT210657	MT210682	MT210705	MT210730	MT210755	MT210780	DOS03229-1
Tridentiger brevispinis	MT210658	MT210683	MT210706	MT210731	MT210756	MT210781	DOS03229-2
Tridentiger brevispinis	MT210659	MT210684	MT210707	MT210732	MT210757	MT210782	DOS03274
Tridentiger brevispinis	MT210660	MT210685	MT210708	MT210733	MT210758	MT210783	DOS03273
Tridentiger kuroiwae	MT210661	MT210686	MT210709	MT210734	MT210759	MT210784	DOS03134-2
Tridentiger kuroiwae	MT210662	MT210687	MT210710	MT210735	MT210760	MT210785	DOS03148-1
Tridentiger microsquamis	MT210663	MT210688	MT210711	MT210736	MT210761	MT210786	DOS03052-1
Tridentiger microsquamis	MT210664	MT210689	MT210712	MT210737	MT210762	MT210787	DOS03052-2
Tridentiger nudicervicus	MT210665	PQ878622	MT210713	MT210738	MT210763	MT210788	DOS03051-1
Tridentiger nudicervicus	MT210666	PQ878623	MT210714	MT210739	MT210764	MT210789	DOS03051-4
Tridentiger obscurus	MT210667	MT210690	MT210715	MT210740	MT210765	MT210790	DOS03294-1
Tridentiger obscurus	MT210668	MT210691	MT210716	MT210741	MT210766	MT210791	DOS03294-2
Tridentiger trigonocephalus	MT210669	MT210692	MT210717	MT210742	MT210767	MT210792	DOS00893
Tridentiger trigonocephalus	MT210670	MT210693	MT210718	MT210743	MT210768	MT210793	DOS00894

divergence time estimation. Although Wei et al. [26] proposed a fast pedigree substitution rate (9.60%/million years) for *T. barbatus*, the pedigree rate might underestimate the age of the elder divergence events due to the power-law decay of the substitution rate over time [45]. We calibrate the substitution rate of Cyt*b* using a Gobiidae case of transisthmian divergences between the Pacific species *Bathygobius ramosus* Ginsburg, 1947, and its Atlantic sister lineage of *B. antiliensis* Tornabene, Baldwin & Pezold, 2010, and *B. brasiliensis* Carvalho-Filho & de Araújo, 2017, assuming that their divergence occurred during the uplift of the Isthmus of Panama (2.8–6.0 million years ago, mya [46–48]). The datasets and detail settings for Cyt*b* rate inference were given in Supporting Information S3. The calibrated Cyt*b* substitution rates for *Bathygobius* were 3.21%/million years (95% highest probability density interval [HPD95]: 1.66%–4.95%/million years). We applied the calibrated Cyt*b* rate to the genus *Tridentiger* using a normal distribution (mean = 0.033, standard deviation = 0.0084), covering the HPD95 of the calibrated Cyt*b* rate. The best-fit substitution model selected by jModeltest2 and ML tree of the dataset 3 were also applied in BEAST2. The uncorrelated relaxed lognormal clock and birth–death process were selected as the best-fit clock and tree models based on the Bayes factor (Supporting Information S3). Two MCMC runs were performed with 100,000,000 generations, sampling every 1000 generations. Sufficient convergence and burn-in of the runs were checked with Tracer v1.7 (http://tree.bio.ed.ac.uk/software/tracer), and all effective sampling sizes are ascertained over 200. The tree files of the two runs were combined in LogCombiner of the BEAST2 package. A consensus tree was then generated with maximum clade credibility and discarding the first 10% samples as burn-in in TreeAnnotator of the BEAST2 package [44].

3. Results

3.1. Genetic Datasets and Phylogenetic Analyses. In this study, a total of 87 Cyt*b* sequences were obtained (Table 2), with their sequence lengths range from 681 to 807 bp. The Cyt*b* sequences were aligned and trimmed into the 681-bp dataset 1. The dataset 1 contains no indel, and 274 polymorphic sites were recovered. The best-fit model for dataset 1 is HKY + I + G. ML and BI trees of dataset 1 congruently identify eight well-supported clades (PP = 1, BS = 99–100) in the genus *Tridentiger*, including *T. barbatus* northern lineage, *T. barbatus* southern lineage, *T. bifasciatus*, a mixed clade of *T. brevispinis* and

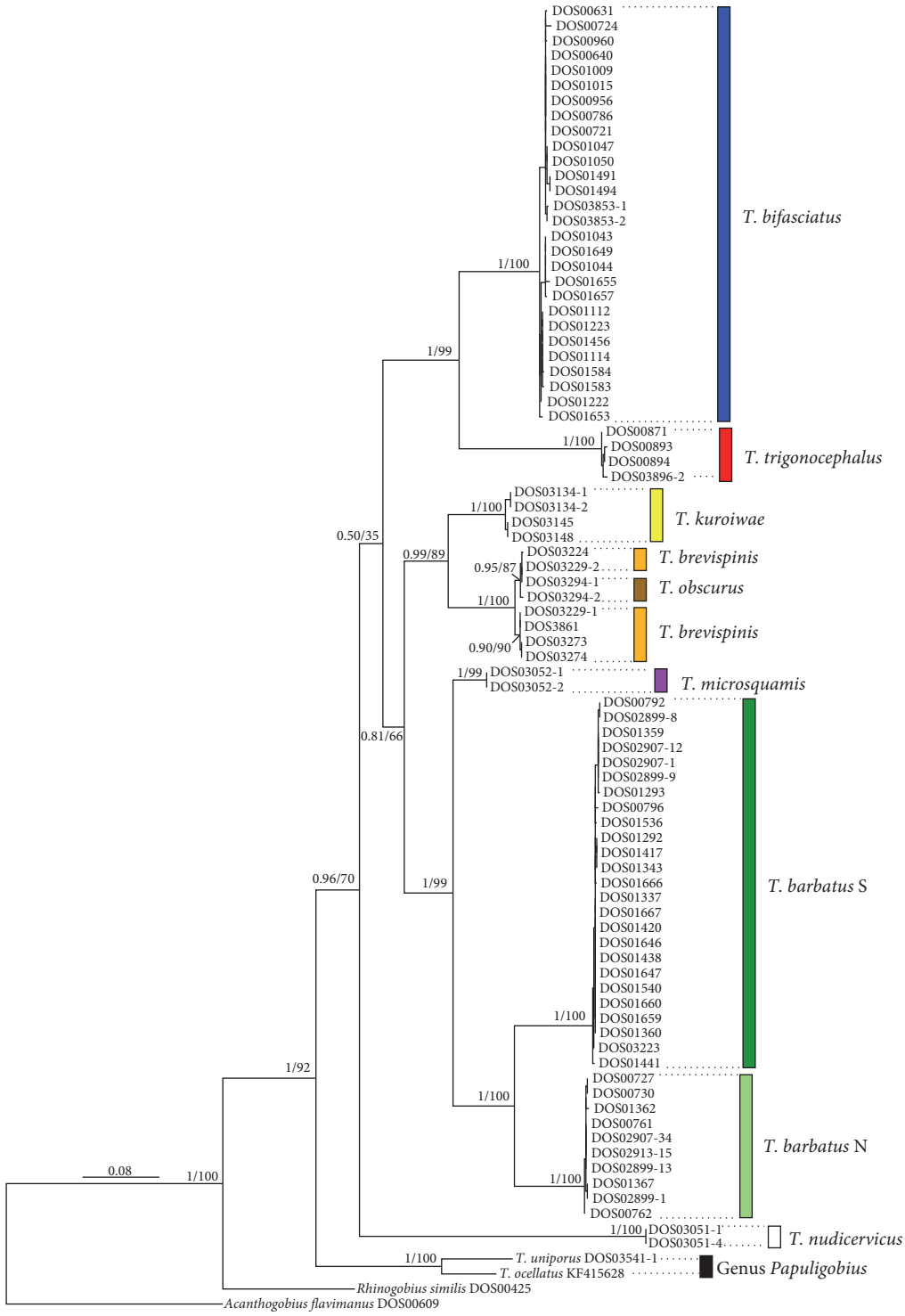

FIGURE 3: The ML tree reconstructed from the dataset 1 (681-bp Cyt*b*). The tree is rooted by *Acanthogobius flavimanus*. Posterior probabilities (before diagonal) and bootstrap values (after diagonal) obtained in BI and ML analyses are shown on each node.

T. obscurus, *T. kuroiwae*, *T. microsquamis*, and *T. trigonocephalus* based on their morphology (Figure 3). Therefore, we selected sequences of all the *T. brevipsinis* and *T. obscurus* species, the two longest sequences in each of the other clades, and the outgroups to form the Cyt*b* partition of the dataset 3. Their corresponding 12S rRNA, RAG1, Rh, Sreb2, and Zic1 sequences were obtained to form datasets 2 and 3.

The 644-bp dataset 2 of 12S rRNA includes five extra sequences of *T. radiatus* from Cui et al. [6], which contain 202 polymorphic sites composed of 33 singleton sites and 169 parsimony informative sites. The best-fit model for dataset 2 was TIM2 + G. The BI and ML trees of dataset 2 also identify eight well-supported (PP = 0.97–1, BS = 79–100) clades in the *Tridentiger* (Figure 4). Sequences of *T. radiatus* and

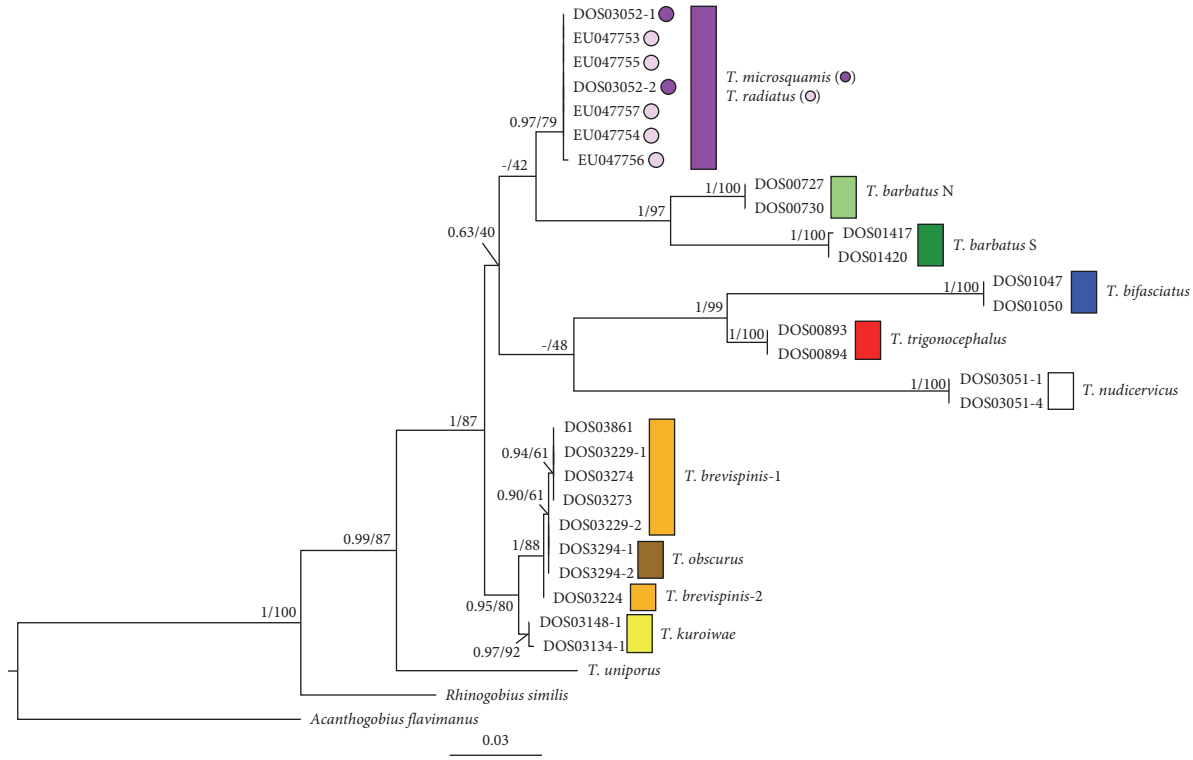

FIGURE 4: The ML tree of the dataset 2 (644-bp 12S rRNA) to compare genetic differentiation between *T. radiatus* from Cui et al. [6] and the topotypes of *T. microsquamis* collected in this study. The tree is rooted by *A. flavimanus*. Posterior probabilities (before diagonal) and bootstrap values (after diagonal) obtained in BI and ML analyses are shown on each node.

T. microsquamis are nested within a clade, supported by BI and ML trees with PP and BS values of 0.97 and 79, respectively.

The dataset 3 (Table 3) was comprised of six partitions, that is, 12S rRNA (664 bp), Cyt*b* (807 bp), RAG1 (1190 bp), Rh (691 bp), Sreb2 (958 bp), zic1 (716 bp), and their best-fit models for the six genes were TIM2 + G, TrN + G + I, TrNef + G, HKY + I, HKY + I, and TrN + I, respectively. Numbers of indels, polymorphic sites, parsimony informative sites, and substitution saturation test results of each partition were summarized in Supporting Information S2. No saturated codon position or gene was identified. Gene trees of the six partitions in the dataset 3 were summarized in Supporting Information S2, and no well-supported contradictory node was identified in the gene trees. In contrast, the ML and BI trees of dataset 3 showed identical and well-supported topologies (Figure 5). Most nodes were well supported by PP and BS values of 1 and 100, respectively. *Papuligobius uniporus* and *P. ocellatus* are resolved as the closest outgroup for the genus *Tridentiger* (PP = 1, BS = 100, Figure 5). The phylogenetic trees indicate three well-supported (PP = 1, BS = 91–100) groups within the *Tridentiger*, viz. the *T. obscurus* group, including *T. brevispinis*, *T. kuroiwae*, and *T. obscurus*; the *T. trigonocephalus* group, comprising *T. bifasciatus* and *T. trigonocephalus*; and the *T. barbatus* group, composed of *T. nudicervicus*, *T. microsquamis*, and the southern and northern lineages of *T. barbatus*. The *T. barbatus* group is at the basal node, sister to the clade composed of the *T. trigonocephalus* and *T. obscurus* groups. In contrast to the contentious relationships recovered from the datasets 1 and 2 (Figures 3 and 4), the *T. obscurus* specimens formed a clade separated from *T. brevispinis* in BI and ML trees of the dataset 3 (Figure 5), supported by PP and BS values of 1 and 100, respectively. The reciprocal monophyly of *T. obscurus* and *T. brevispinis* is also indicated in gene trees of RAG1, Rh, and Sreb2 (Supporting Information S2).

3.2. Genetic Distance and Species Delimitation. Pairwise K2P distance of the *Tridentiger* sequences is summarized in Table 4 and Figure 6. Interspecific distances (herein not including those between *T. brevispinis* and *T. obscurus*, *T. barbatus* northern and southern lineages, and *T. microsquamis* and *T. radiatus*) are obviously larger than intraspecific distances in 12S rRNA, Cyt*b*, and RAG1, but show overlap with the latter in Rh, Sreb2, and Zic1 (Figure 6). K2P distances of 12S rRNA between *T. microsquamis* and *radiatus* fall in the interval of intraspecific distances, which are obviously smaller than the 12S rRNA interspecific distances (Figure 6). K2P distances between *T. brevispinis* and *T. obscurus* also fall in the interval of intraspecific distances in 12S rRNA, Cyt*b*, Rh, Sreb2, and Zic1, but their RAG1 distances are close to the interspecific distance.

The results of species delimitations are shown in Figure 5. Most species delimitating analyses (i.e., the ASAP, bPTP, GMYC methods) delineated *T. barbatus* southern lineage, *T. barbatus* northern lineage, *T. microsquamis*, *T. nudicervicus*, *T. bifasciatus*, and *T. trigonocephalus* as different operational taxonomic units (OTUs, Figure 5). However, the ASAP on

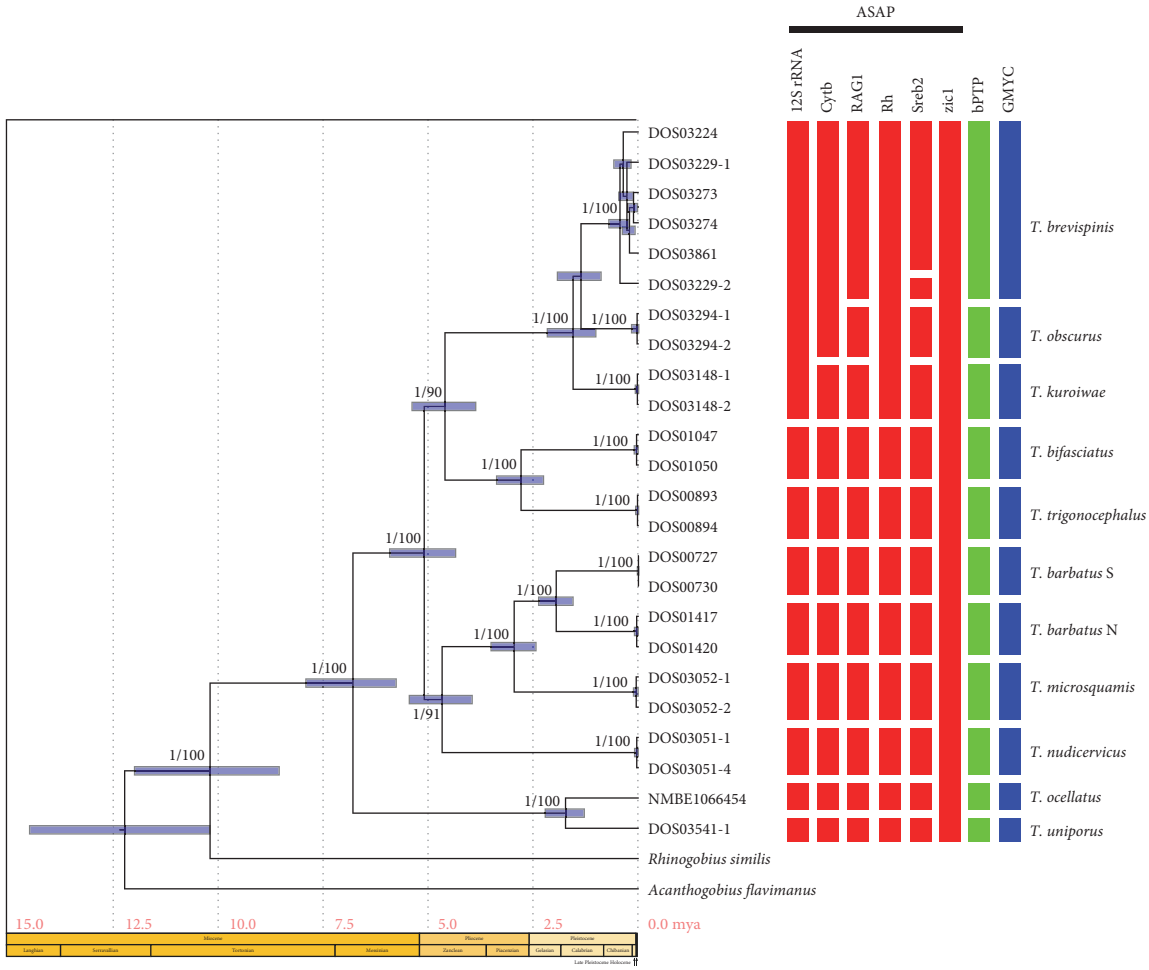

FIGURE 5: Phylogeny, divergence time, and species delimitations of the genus *Tridentiger* using from dataset 3 (5028-bp concatenated dataset of 12S rRNA, Cyt*b*, RAG1, Rh, Sreb2, and Zic1). The intervals of the geographic epochs are plotted based on Steinthorsdottir et al. [49]. Posterior probabilities (before diagonal) and bootstrap values (after diagonal) obtained in BI and ML analyses are shown on each node.

Zic1 delimitated all the *Tridentiger* species as a single OTU, which might be due to the low variation in Zic1. Although the bPTP and GMYC supported *T. brevispinis*, *T. kuroiwae*, and *T. obscurus* as different OTUs, the ASAP analyses of the six genes indicate different solutions for the three species, viz., in Cyt*b*, *T. brevispinis* and *T. obscurus* were delimitated as a single OTU; in RAG1, the three species were delimitated as three different OTUs; in 12S rRNA, Rh and Zic1, the three species were delimitated as a single OTU; in Sreb2, the species were delimitated as different OTUs, but suggested an extra OTU for DOS03229-2 (*T. brevispinis*).

3.3. Divergence Time of the Tridentiger Species. The divergence time inference suggested that the time to most recent ancestor (tMRCA) of the genus *Tridentiger sensu lato* is 5.10 mya (HPD95: 4.35–5.92 mya, Figure 5), which has been separated from *P. uniporus* and *P. ocellatus* since 6.79 mya (HPD95: 5.77–7.91 mya). The divergence of the *T. obscurus* and *T. trigonocephalus* groups was dated back to 4.61 mya (HPD95: 3.88–5.39 mya), while that between *T. nudicervicus* and the barbeled species (*T. barbatus* and *T. microsquamis*) was inferred back to 4.67 mya (HPD95: 3.96–5.45 mya). The divergence time of *T. bifasciatus* and *T. trigonocephalus* was dated back to 2.80 mya (HPD interval: 2.26–3.38 mya). In the *T. obscurus* group, the mean divergence time between *T. brevispinis* and *T. obscurus* is 1.37 mya (HPD95 interval: 0.89–1.93 mya), while *T. kuroiwae* separated from these two species since 1.56 mya (HPD95 interval: 1.02–2.17 mya). The divergence between *T. microsquamis* and *T. barbatus* was inferred back to 2.96 mya (HPD95 interval: 2.44–3.51 mya), while the tMRCA for the northern and southern lineages in *T. barbatus* is 1.96 mya (HPD95 interval: 1.56–2.38 mya).

4. Discussions

4.1. Systematics of the Genus Tridentiger. Our phylogenetic tree based on six genes (Figure 5) supports a sister relationship between the genera *Papuligobius* and *Tridentiger*, as well as the trichotomy within *Tridentiger sensu lato*, aligning with Endruweit's [19] taxonomic opinion. *Papuligobius* and the *T. barbatus* group differ from the *T. obscurus* and *T. trigonocephalus* groups by having only one infraorbital papilla cp, compared to four in the latter. Additionally, *Papuligobius* is distinguished by a longer row b, with 23–26 papillae, versus fewer

Table 4: Interspecific and intraspecific K2P distances of genus *Tridentiger*.

Gene	12S rRNA	Cyt*b*	RAG1	Rh	Sreb2	Zic1
Intraspecific	0.0000–0.0030	0.0000–0.0201	0.0000–0.0034	0.0000–0.0058	0.0000–0.0042	0.0000–0.0028
T. radiatus and *T. microsquamis*	0.0000–0.0016	—	—	—	—	—
The northern and southern lineages of *T. barbatus*	0.0710–0.0727	0.1298–0.1300	0.0085	0.0131–0.0146	0.0052	0.0000
T. brevispinis and *T. obscurus*	0.0000–0.0015	0.0000–0.0201	0.0059–0.0093	0.0058–0.0073	0.0052–0.0084	0.0014–0.0028
Interspecific (other species pairs)	0.0122–0.1529	0.1065–0.2506	0.0076–0.0346	0.0029–0.0557	0.0031–0.0212	0.0000–0.0141

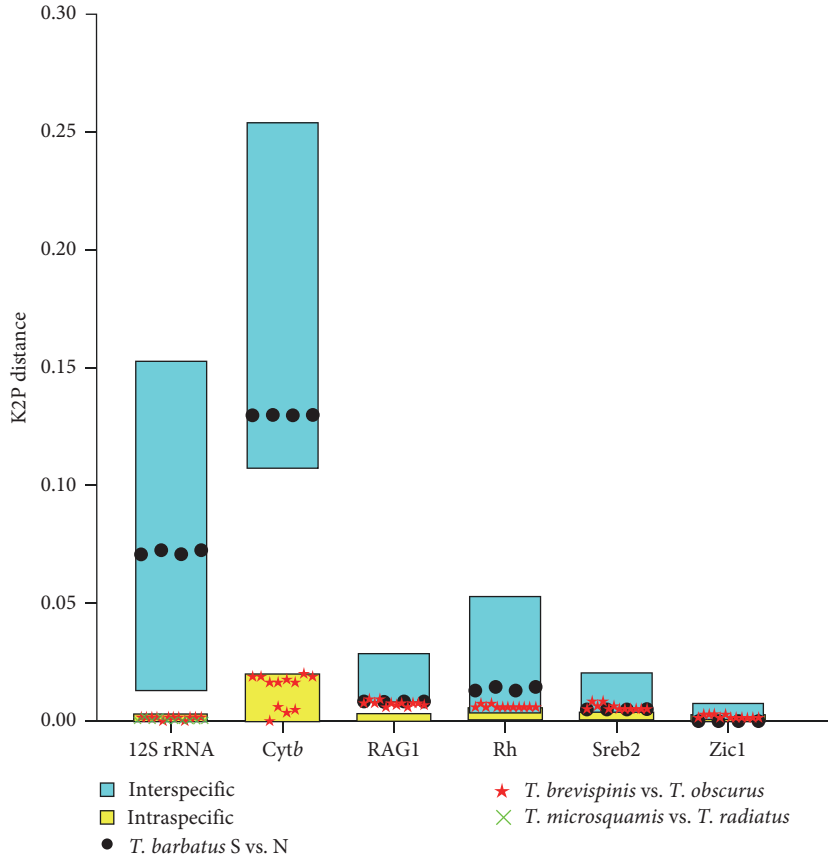

FIGURE 6: Boxplot and scatterplot showing pairwise K2P distances of the *Tridentiger* gene sequences. Pairwise distances between the northern and southern lineages of *T. barbatus*, *T. brevispinis*, and *T. obscurus*, and *T. microsquamis* and *T. radiatus* are shown as scatter plots. Interspecific K2P distances of other species pairs are shown as blue boxplot.

than 10 papillae in the *T. barbatus* group [17, 50]. Papilla patterns are key for characterizing gobiiform genera, but the number of papilla lines is rather significant than the number of papillae in each line when diagnosing genera. For example, Hoese [51] noted that longitudinal papilla line 4 (LA4) is consistently absent in *Acentrogobius* but present in *Glossogobius*. Conversely, the differences between *Papuligobius* and *Tridentiger* relate primarily to papilla counts in some lines [17]. Both genera share tricuspid jaw teeth as a diagnostic trait, which thereby has been proposed as synonyms by Taki et al. [18]. These phylogenetic and morphological evidence supports that *Papuligobius* is a synonym of *Tridentiger*.

Our study revealed a sister relationship between the nonbarbeled *T. nudicervicus* and the barbeled *T. barbatus* and *T. microsquamis* (Figure 5, PP = 1; BS = 94), which clearly rejected the dichotomy of barbeled and nonbarbeled *Tridentiger* species [6]. *Tridentiger nudicervicus* resembles *T. barbatus* and *T. microsquamis* by having united oculoscapular canals and fused H' + K' postoculoscapular sensory pores (vs. separated in their congeners). While conspicuous sexual dimorphism is noted in the *T. trigonocephalus* and *T. obscurus* groups, no significant sexual difference has been observed for the *T. barbatus* group [4, 6]. The two characters may support a closer affinity between *T. nudicervicus* and the barbeled species. However, the barbeled species can be easily distinguished from *T. nudicervicus* by barbels and skin flaps. Sensory pores C, D, and E are absent in *T. barbatus* and *T. microsquamis*, but present in its nonbarbeled congeners, including species formerly placed in *Papuligobius* [6]. Chen and Kottelat [17], thus, proposed the resurrection of *Triaenopogon* for *T. barbatus* and *T. microsquamis*, while the nonbarbeled *T. nudicervicus* should be assigned to a new genus (i.e., *Tomiyamia*, [19]).

Nevertheless, these variations in cephalic papilla and sensory pore patterns can be considered interspecific, which are commonly found in many gobiiform genera (e.g., *Mugilogobius*, *Luciogobius*, and *Rhinogobius*, [4]). Sensory pore H' and K' are also fused in *T. uniporus* while remain separated in its sister, *T. ocellatus* [17], supporting interspecific modification of sensory pores within *Tridentiger*. Although cephalic barbels were used to divide the *Tridentiger*, barbels of *T. barbatus* and *T. microsquamis* share a similar spatial arrangement with cephalic papillae of the nonbarbeled *Tridentiger* species [6]. The transverse opercular papillae row (*ot*) developed into barbels in *T. barbatus*, but retained a normal papilla row in *T. microsquamis* [6]. These evidences strongly imply continuous modification of cephalic papillae into barbels in the barbeled species, which is not a single synapomorphy to define *Triaenopogon*. Furthermore, Endruweit [19] proposed that

Tomiyamia differs from *Tridentiger sensu stricto* by having reduced nuchal squamation and free uppermost pectoral fin ray, which should also be considered as interspecific-level differentiation. The uppermost pectoral fin ray was free in *T. trigonocephalus*, but attached to other rays in its sister *T. bifasciatus*. *Tridentiger barbatus* and *T. microsquamis* are also characterized by the reduction of nuchal squamation; scales are absent in the majority of nape, except for a narrow area of predorsal scales [6]. Additionally, many gobiiform genera also include species with and without scales in the nape region (e.g., *Amblyeleotris*, *Rhinogobius*, and *Cryptocentrus*, [4]). Therefore, a combination of these characters alone could be considered interspecific variation instead of diagnostic characters for delimiting a genus.

Conclusively, the proposed diagnoses (i.e., cephalic papillae arrangement, sensory pores, and barbels) to differentiate *Tridentiger sensu stricto*, *Triaenopogon*, *Tomiyama*, and *Papuligobius* are more appropriately considered as interspecific variations. The five genera can be well united by the synapomorphy of tricuspid outermost jaw teeth. This suggests that they could be merged into a single genus. Furthermore, Endruweit's [19] proposal to assign five genera for only 10 species may be over-classification and hinder effective biodiversity assessment and conservation efforts. Therefore, we proposed *Papuligobius* as a junior synonym of *Tridentiger* Gill, 1958, and rejected the resurrection of *Triaenopogon* and the validity of *Tomiyama*.

4.2. Taxonomy of the Barbeled Tridentiger Species. *Triaenopogon microsquamis* was first described based on a specimen from Xiamen, China [52], but was later synonymized with *T. barbatus* by Wu [25]. Later, *T. radiatus* was published as a new barbeled species with sparser head barbels and smaller scales compared to *T. barbatus* [6]. In fact, Wu [52] stated that *T. microsquamis* differs from *T. barbatus* by having smaller scales and unique coloration, which is consistent with the diagnoses of *T. radiatus*. The transverse scale count of *T. microsquamis* and *T. radiatus* was 22 and 17–21, respectively, while that of *T. barbatus* is much fewer, 12–14 [6, 52]. The body color of *T. microsquamis* is blackish brown, inferior part lighter ("*La couleur est d'un brun noiratre, la face inferieure est plus Claire*," [52]), while that of *T. radiatus* is "*Dorsal brown to blackish brown, ventral pale brown*" [6]. In contrast, *T. barbatus* is olive green or yellowish and covered with multiple black bands on the body [6]. In this research, phylogenetic analyses suggested genetic homogeneity of *T. radiatus* and *T. microsquamis*; two topotypes of *T. microsquamis* share the same haplotype with four *T. radiatus* (Figures 4 and 6). The only difference between *T. radiatus* and *T. microsquamis* might be their longitudinal scale count. The longitudinal scale of *T. microsquamis* is 38 [52], which is marginally deviated from *T. radiatus* (40–50) and closer to *T. barbatus* (34–38, [6]). We noticed that the anterior body scales of the *T. microsquamis* topotypes are aligned irregularly and difficult to count. It might account for the difference in longitudinal scale counts between Wu [52] and Cui et al. [6]. *Tridentiger radiatus* is genetically and morphologically identical to *T. microsquamis*, and thus should be treated as a junior synonym of the latter.

Our previous research, Wei et al. [26] reported a deep divergence of two lineages within *T. barbatus*, whereas our results suggested that the two lineages might actually be two different species. All the phylogenetic analyses well support reciprocal monophyly of the northern and southern lineages of *T. barbatus* (Figures 3–5), and most delimitation analyses delineated the two lineages as different OTUs (except in the ASAP of Zic1 with the lowest variation, Figure 5). 12S rRNA, Cyt*b*, and RAG1 showed clear differences between interspecific and intraspecific distances (i.e., barcoding gap, [53]; Figure 6), suggesting that the three genes have sufficient information to distinguish interspecific and intraspecific differentiation in most *Tridentiger* species. K2P distances between the southern and northern lineages fall in the interval of interspecific distances of these three genes, suggesting that the divergence of the two lineages is a case of speciation. The type locality of *Tridentiger barbatus* is stated as "probably from China" [20], whereas our researches indicated two *T. barbatus*-like species in China (this study; [26]). *Triaenopogon japonicus* Rendahl, 1926 is another nominal species described under the genus *Triaenopogon* based on specimens from the Tokyo Bay, Japan, which can be a candidate name for the two lineages. However, we found a specimen of the southern lineage from Kyushu, Japan (DOS03223, Table 2), indicating that either both lineages or only the southern lineage are distributed in Japan (Figure 2). The exact species names for the two lineages need to be determined by further revision.

4.3. Evolution of the Tridentiger Species. The *Tridentiger* is one of the dominant gobiiform species in waters of the East Asia; most of its members can be found through China to Japan and Russia, spanning an extraordinary latitude gradient (e.g., *T. barbatus*, *T. bifasciatus*, *T. trigonocephalus*, and *T. nudicervicus*, [4]). Given their wide range of distributions, the *Tridentiger* species might have undergone a complicated population history, which could be ideal materials for population genetics studies [26, 54]. The complicated population history and wide range of distribution might make it difficult to trace the origin of *Tridentiger*, but evidences might still exist to associate their divergence with a geographical event. *Tridentiger uniporus* and *T. ocellatus* at the basal node of genus *Tridentiger* are reported in the Southeast Asia (e.g., the Mekong basins in Laos, Thailand, and Cambodia, [18]), while their congeners dominate waters of China, Korea, Japan, and Russia, with their southernmost record found in Northern Vietnam (Hải Phòng and Quảng Ninh in the Beibu Gulf, [55]). Their difference in spatial distribution implies a north–south divergence during the early evolution of genus *Tridentiger*. *Tridentiger kuroiwae* is an endemic species in the Ryukyu Islands, which separates from its closest congeners *T. obscurus* and *T. brevispinis* in the Japan Archipelago by the Tokara Gap, a well-known biogeographic barrier formed in around 1.5 mya [56], isolating species and populations between the Ryukyu Islands and the Japan Archipelago (e.g., species of *Rhinogobius*, [57]). Similarly, the separation of *T. kuroiwae* from its sisters (*T. obscurus* and *T. brevispinis*) was also dated back to 1.56 mya (HPD95 interval: 1.02–2.17 mya), implying that their allopatric divergence might also be associated with the formation of the Tokara Gap [56]. The divergence of the southern and northern lineages

of *T. barbatus* is proposed as a case of speciation related to glacial isolation. Although we adopted a slower substitution rate (3.3%/million years) than Wei et al. [26], the divergence time of the two lineages (1.96 mya, HPD95:1.56–2.38 mya) was still dated back to early Pleistocene (the Gelasian and Calabrian; [58]), which is considered as the start of the Quaternary Glacial Cycles [59]. Nevertheless, as the inferred HPD95 of divergence time of the two lineages covers a wide period, it is difficult to associate their divergence with the exact glacial events.

Ecological factors might also play an important role in the differentiation of the *Tridentiger* species. *Tridentiger bifasciatus* and *T. trigonocephalus* have different habitat preferences. *Tridentiger bifasciatus* prefers dilute waters, and *T. trigonocephalus* tends to inhabit more saline waters [60]. Therefore, niche separation might be a cause for divergence between the two species. Similar habitat segregation is also reported between *T. brevipsinis* (freshwater) and *T. obscurus* (brackish water, [23]). However, *T. brevispinis* and *T. obscurus* only show a clear interspecific level of differentiation in the RAG1 gene (Figure 6). Multiple hybridization cases between the two species are frequently reported from waters of the Japan Archipelago [22, 23], whereas their unique mitochondrial genotypes with species-level differentiation are never reported. Their validities and level of differentiation need to be further investigated with genome-wise tools (e.g., UCEs, RAD-seqs, and whole genome resequencing). The two lineages of *T. barbatus* with denser head barbels can be broadly found in coastal mudflats and estuarine in China, Korea, Japan, and Russia, whereas the sparsely-barbeled *T. microsquamis* is rarely found, only reported in Zhuhai and Xiamen, China [6, 26]. Their differences in distributions and population sizes imply that the well-developed head barbels might bring selective advantages to *T. barbatus* in the competition with *T. microsquamis*.

5. Conclusion

This study presented the first multilocus phylogeny for the genus *Tridentiger* of the Oxudercidae. Our phylogenetic tree revealed a well-supported sister relationship between the non-barbeled *T. nudicervicus* and the barbeled *T. barbatus* and *T. microsquamis*. This clearly rejected the dichotomy of *Tridentiger* into barbeled and nonbarbeled species. Therefore, *Triaenopogon*, representing the barbeled *Tridentiger* species, should not be resurrected. *Papuligobius* is resolved as the sister of *Tridentiger*, which is only different from the latter in two papillae rows. Given that *Papuligobius* possesses the diagnosis of *Tridentiger* (i.e., the first row of jaw teeth in tricuspid form), we proposed *Papuligobius* as the junior synonym of *Tridentiger*, which agrees with Taki et al.'s [18] opinion. Furthermore, Endruweit [19] set up a new genus, *Tomiyamia*, for *T. nudicervicus*, defined by having reduced nuchal squamation and the uppermost pectoral fin ray free. Nevertheless, these diagnoses can be commonly found as interspecific variation within not only *Tridentiger* (between *T. bifasciatus* and *T. trigonocephalus*) but also other gobiiform genera (e.g., *Amblyeleotris*, *Rhinogobius*, and *Cryptocentrus*). *Tomiyama* should be thereby considered a junior synonym of *Tridentiger*. Our results also provided new perspectives on the species taxonomy of *Tridentiger*. *Tridentiger radiatus* is morphologically identical to a rarely mentioned species, *T. microsquamis*, by having denser scales than *T. barbatus*. Our study indicated genetic homogeneity between these two species, supporting that *T. radiatus* should be treated as a junior synonym of *T. microsquamis*. Conversely, the southern and northern lineages of *T. barbatus* were delineated as different species in most species delimitations (except ASAP of Zic1), suggesting that these two lineages might be two species. Although the phylogeny of RAG1, Sreb2, and the concatenated dataset suggests separation of *T. brevispinis* and *T. obscurus*, interspecific level differentiation in their mitochondrial genes has never been found. Introgressions in both mitochondrial and nuclear genomes have been repeatedly reported in previous studies. These imply reproductive isolation between *T. brevispinis* and *T. obscurus* might be incomplete. The validity of these two species needs to be evaluated with genome-wide tools in the future. Taxonomy of the genus *Tridentiger* is contentious. The taxonomic revision of the present study may facilitate their identification. In addition, although not of large economical value, species of *Tridentiger* are common in fish markets of the Chinese coastal area; correct identification may improve fishery data.

Data Availability Statement

The gene sequences used in this study were deposited on NCBI Genbank with accession numbers of MT210646–MT210793, MT215240–MT215324, MT524720–MT524721, OR067083, and PQ878622–PQ878623.

Disclosure

All authors read, revised, and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Fan Li and Te-Yu Liao conceived and designed the study. Akihiko Koyama, Fan Li, Yuichi Kano, Kuidong Xu, and Te-Yu Liao conducted the investigation and sampling. Jiehong Wei and Wei-Cheng Jhuang performed the molecular experiments. Jiehong Wei performed the analyses and wrote the manuscript draft. Jing Liu and Te-Yu Liao provided funding support.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section and figshare database (doi: https://doi.org/10.6084/m9.figshare.28190423).

Supporting Information 1. PCR protocols, cycling conditions, and primers of the six genes used in this study.

Supporting Information 2. Alignment files, saturation test, and best-fit model of datasets 1–3 used in this study.

Supporting Information 3. Methodologies for calibrated substitution rate and model selection of molecular dating.

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