

Research Article

Molecular Systematics and Divergence of *Tor* and *Neolissochilus* Fishes (Cypriniforms: Cyprinidae) from Southeast Asia and South China

Ling Wu ^{1,2,3,4} Tao Qin ^{1,2,3,4} Huy Duc Hoang ^{5,6} Thaung Naing Oo,⁷
Xiao-Ai Wang ² and Xiao-Yong Chen ^{1,2,3}

¹Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences, Nay Pyi Taw 05282, Myanmar

²Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China

³Yunnan International Joint Laboratory of Southeast Asia Biodiversity Conservation, Mengla 666303, China

⁴University of Chinese Academy of Sciences, Beijing 100049, China

⁵Department of Ecology and Evolutionary Biology, University of Science, Ho Chi Minh City, Vietnam

⁶Vietnam National University, Ho Chi Minh City, Vietnam

⁷Forest Department, Ministry of Natural Resources and Environmental Conservation, Nay Pyi Taw 15015, Myanmar

Correspondence should be addressed to Xiao-Ai Wang; wangxiaoi@mail.kiz.ac.cn and Xiao-Yong Chen; chenxy@mail.kiz.ac.cn

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In Southeast Asia, mahseer fishes, such as the species of *Tor* and *Neolissochilus*, are significant native commercial fish. Their phylogeny and categorization have a convoluted history. In this study, the molecular systematics and divergence of *Tor* and *Neolissochilus* fishes from Southeast Asia and South China were examined using the partial or complete sequences of four mitochondrial genes (cytochrome oxidase I, cytochrome *b*, 16S, and ND4). This study substantiated the monophyly of *Tor* and supported *N. benasi* as an independent genus. The result supported *T. laterivittatus* as a synonym of *T. sinensis* and *T. dongnaiensis* as a synonym of *T. tambra*. In addition, we presented the high diversity and species crypticity of these two taxa in Southeast Asia. The divergent time estimation indicated *Tor* and *Neolissochilus* species originated in the early Miocene (about 16.73 Ma), and the divergence of the genus *Tor* and other species of the genus *Neolissochilus* began at about 12.86 Ma.

1. Introduction

The cyprinid fishes belonging to the genera *Tor* and *Neolissochilus* are commonly known as mahseer, which have been classified under the subfamily Torinae of Cyprinidae after extensive discussion on the phylogenetics of Cypriniformes [1, 2, 3, 4]. They are widely distributed in Pakistan, India, Bhutan, Bangladesh, Nepal, Sri Lanka, Myanmar, Laos, Vietnam, Thailand, Cambodia, and other countries in South Asia and Southeast Asia. In China, it is only distributed in Yunnan and Tibet. They live in a bunch of drainages, from the Yarlung Tsangpo River, Irrawaddy River, Salween River, and Mekong River to the Red River, as well as drainages in the Malay Peninsula, Sumatra, Java, Borneo, etc. They are omnivorous fish in the middle and lower layers. The adult mostly

resides in the calm areas of the main river, such as backwater bays, large tributaries, or deep-water regions with sluggish currents. On the other hand, subadults or juveniles are generally found in the higher-altitude water bodies of the tributaries. They exhibit anadromous movement as part of their reproductive behavior. The majority of these species have significant economic value within their respective geographical regions.

Currently, 17 species are valid in the genus *Tor* Gray and 32 species in the genus *Neolissochilus* Rainboth [2]. Phylogenetic relationships, morphological classification, and geographical distribution are closely related between the two fish genera. The main differences in morphology of the two genera are scale size, number of lateral-line scales, number of gill rakers, postlabial groove, presence or absence of a median lobe and its length, which is enlarged to form a median lobe and with a

continuous postlabial groove in *Tor* species, while with no median lobe and interrupted postlabial groove in *Neolissochilus* species. The species classification of these two genera is still confusing because the shape, size, and length of the median lobe are often highly variable [5], which is related to habitat and the ability to adhere in rapids [6, 7]. Subsequently, tropic polymorphisms were reported in the *Neolissochilus* species [8, 9]. Laskar et al. [10] emphasized the importance of the number of gill rakers on the first arm of the gill arch in distinguishing the two genera. The two genera have some overlapping morphological and meristic characters and share almost the same distribution area. Simultaneously, the description and identification of the two genera may have greater uncertainty due to the phenotypic plasticity and intraspecific variation affected by different geographical and environmental conditions, as well as the different selection and description of characters by different researchers. Because of these, there has not yet reached a consensus on the classification of species among taxonomists.

Most recent molecular phylogenetic studies on mahseer only used DNA barcoding to look at the variety of species and the validity of *Tor* and *Neolissochilus*. Hoang et al. [8] described two new species, *T. mekongensis* and *T. dongnaiensis*, based on the cytochrome oxidase I (COI) gene with a genetic distance of 0.7%. Subsequently, a genetic analysis by Walton et al. [11] showed that *T. dongnaiensis* could not be significantly distinguished from *T. tambra* in peninsular Malaysia and western Java. While Pinder et al. [12] supported the validity of *T. dongnaiensis* in their review of global *Tor* fish, but they considered that *T. mekongensis* may be a synonym of *T. dongnaiensis*. Hoang et al. [8] also discovered a new species record in South Vietnam, *N. stracheyi*, and showed that it was clustered with specimens from Laos; and considering that characters such as body color and median lobe have many variations, it is believed that the individuals identified by previous studies [13, 14] could correspond to the *Tor*-like morph of *N. stracheyi*. Lalramliana et al. [15] used DNA barcoding to describe a new species, *N. kaladanensis*, distributed in northeast India, and discovered that the conclusions supported by the morphological and molecular data of the relationship between *N. baoshanensis* and *N. stracheyi* were inconsistent; reasons for this inconsistency may include the fact that the species itself is synonymous, species identification errors, or limitations of DNA barcoding. Nguyen et al. [16] explored the phylogeny, evolution, and biogeography of six species of *Tor* and five species of *Neolissochilus* using three mitochondrial genes and showed that these two genera are sister groups of each other. Subsequent studies found that *T. hemispinus* and *T. qiaojienensis* were embedded with a clade containing *Neolissochilus* species, which did not support the monophyly of these two genera [15, 17]. It should be noted that both the molecular markers and the number of species involved in these studies, while the geographical coverage is small and phylogenetic relationships may not be very accurately inferred, are likely to change significantly as the species coverage or the strength of the molecular markers increases.

Currently, few studies focus on the biogeography of *Tor* and *Neolissochilus*. Only Nguyen et al. [16] proposed two hypotheses on the present distribution of mahseers: vicariance fragmentation and range expansion and speculated that the division between *Neolissochilus* and *Tor* probably occurred 7.6–3.8 Ma, or during the Miocene epoch. Most studies focused on higher-scale taxa and did not discuss these fishes. For example, Yang et al. [4] reconstructed the phylogenetic relationship of the Cyprininae sensu lato based on the RAG1 gene and mitochondrial genes and subsequently deduced that the tetraploidization and hexaploidization events of *Torini* likely occurred before the diversification (32.5 Ma) of tetraploid *Torini* and after the origin (18.1 Ma) of the now hexaploid *Torini*, respectively [18]. Phylogenetic trees based on different datasets revealed different topologies for the relationships between *Tor* and *Neolissochilus*. Therefore, it is necessary to construct a stable phylogenetic tree on a small scale and focus on the phylogenetic relationships and evolution of the study taxa.

The species belonging to the genera *Tor* and *Neolissochilus* have a broad distribution in Southeast Asia and South Asia. This research specifically focused on Southeast Asia and the Yunnan Province of South China, with a comprehensive examination of the species belonging to the *Tor* and *Neolissochilus*. By performing a molecular systematic study and divergence time estimation, we aim to inspect the validity of the species, understand their phylogenetic relationships, and explore their origin and evolution.

2. Materials and Methods

2.1. Sample Collection. Sampling and fieldwork in Yunnan province and Southeast Asia were carried out from 2005 to January 2023 by the Kunming Institute of Zoology, CAS. All specimens were collected as part of routine surveys. All fish handling and experiments complied with the relevant laws of the Chinese Laboratory of Animal Welfare and Ethics (GB/T 35892-2018). All fished individuals were rapidly euthanized by 10–100 mg/L anesthetic clove oil. The right pelvic fin was severed and preserved with pure alcohol and stored in the refrigerator at -80°C . Specimens were preserved by immersion in 75% alcohol or 10% formalin. General measurements and counts refer to Rainboth [19] and Laskar et al. [10]. A total of 153 individuals identified as *Tor* and *Neolissochilus* in the wild were selected for PCR; the genus *Tor* includes 41 specimens (5 valid plus 3 undescribed), and *Neolissochilus* includes 112 specimens (10 valid plus 4 undescribed). A further 53 sequences of the genera *Tor* and *Neolissochilus* were downloaded from the NCBI GenBank database and used as the ingroup together with the sequences obtained in this study. The ingroup taxa finally included 13 valid species and 3 undescribed species of genus *Tor*, 17 valid species, and 4 undescribed species of *Neolissochilus*. We retrieved reference sequences of 45 species from the NCBI GenBank database to be used as an outgroup in this study. Sample and sequence information used in the current study can be seen in *Supplementary table 1*.

2.2. DNA Extraction, PCR Amplification, and Sequencing. Genomic DNA was extracted from the fin clips that were preserved in pure ethanol. COI, cytochrome *b* (Cyt *b*), 16S ribosomal RNA (16S), and NADH dehydrogenase subunits 4 (ND4) were used in this study. DNA was extracted using the DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd.). PCR was carried out in a total volume of 25 μ L, which contained 1 μ L (10 μ M) of each Primer, 1 μ L Template DNA, 9.5 μ L ddH₂O, and 12.5 μ L 2 $\times$ Taq Master Mix (Vazyme Biotech Co., Ltd.). Primers used for sequencing are provided in *Supplementary table 2*. Two pair primers of 16S were used to amplify and assemble the full-length gene sequence, and primers for the ND4 gene were the universal primers designed for this study by Sangon Biotech (Shanghai) Co., Ltd. PCR conditions for COI and Cyt *b* fragment were: initial denaturing at 92°C for 3 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min, then hold at 10°C; for 16 S: initial denaturing at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58–62°C for 30 s, and 72°C for 60–90 s, and a final extension at 72°C for 5 min, then hold at 10°C; for ND4 encompassed initial denaturing at 95°C for 5 min, 30 cycles of 94°C for 45 s, 55°C for 45 s, extension at 72°C for 90 s, then followed by a 10 min final extension at 72°C. Purification and sequencing in both forward and reverse directions were undertaken at Sangon Biotech (Shanghai) Co., Ltd.

2.3. Sequence Alignment and Molecular Systematics. Sequences were manually proofread and assembled using SeqMan in the DNASTAR Lasergene v7.1 (DNASTAR, Inc., USA) and aligned by MUSCLE in MEGA7 [20]. To evaluate the phylogenetic performance of each gene and concatenated sequences, we used Lamp in the IQ-TREEv2.2.0 [21] to perform Likelihood Mapping analysis [22], and the Incongruence Length Difference Test was used to test the homogeneity in PAUP*4.0 [23]. Sequence datasets were analyzed in DnaSP v6 [24] to get the sequence characteristic information. The genetic distance between the species groups was calculated by averaging pair-wise comparisons of sequence across all individuals as implemented using MEGA 7.0.

Three methods were used to construct the phylogenetic tree for the concatenated dataset of four genes: maximum likelihood (ML), Bayesian inference (BI), and maximum clade credibility tree (MCC). All operations of ML and BI were done in PhyloSuite v1.2.2 [25]. Node support was determined by setting 1,000 bootstrap (BS) replicates in ML and 20,000,000 generations of Markov chains, sampling every 2,000 generations to yield 10,000 trees in BI. MCC analysis was performed in BEAST V1.10.4 [26]. The best partition scheme and evolutionary models were selected using PartitionFinder2 [27] with all algorithms and AICc criteria.

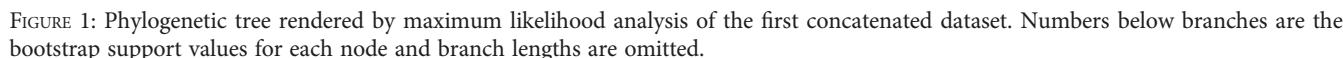
2.4. Divergence Time Estimation. Divergence time analysis used *Opsariichthys bidens* and six species of genus *Labeo* as outgroups in this study, and the tree topology yielded by ML analysis was used for inferring the divergence time. Two fossil calibrating points were selected to calibrate the divergence time. One was the separation of subfamilies Cyprininae

and Leuciscinae in the mid-Oligocene (27.7 Ma) [28]; one was the oldest African *Labeo*-like fossil from Loperot, Kenya, dating to the early Miocene (17.0 Ma). The filtered sequence dataset was parameterized using BEAUTi in the BEAST package to generate the input file. The analysis was performed using Unlink Subst. Models are carried out under a relaxed clock lognormal model. Yule model was selected as a prior. The Markov chain was run for 20,000,000 generations and sampled every 2,000 generations, then the first 10% of the trees were discarded as burn-in. The log results were examined in Tracer 1.7.2 [29]. The maximum credibility tree was summarized using TreeAnnotator in BEAST and visualized in FigTree 1.4.4 and iTOL (<https://itol.embl.de/>).

3. Results

3.1. Gene Characteristics and Variation. The first dataset was assembled for phylogeny, including all the sequences obtained in this study, 251 sequences in total (45 outgroup and 206 ingroup sequences). The aligned length of the first concatenated dataset (COI + Cyt *b* + ND4 + 16S) was 4,196 bp, with 1657 parsimony-informative sites, 308 singleton sites, and 2,231 constant sites. The average base composition was 26.6% T, 23.0% C, 29.5% A, and 20.9% G, and the estimated Ti/Tv was 4.0. The second dataset included the concatenated sequences of *O. bidens*, genus *Labeo* fishes, and selected representatives from the ingroup for divergence time analysis (*Supplementary table 1*). It included 53 sequences, and the aligned sequence length was 4,224 bp, with 1,000 parsimony-informative sites, 382 singleton sites, and 2,842 constant sites. The average base composition was 26.4% T, 23.1% C, 29.4% A, and 21.0% G, and the estimated Ti/Tv was 4.6.

3.2. Phylogenetic Relationships. The topologies of the reconstructed phylogenetic trees in ML and BI were similar. The result illustrated the species clustering situation and the genera *Tor* and *Neolissochilus* formed a paraphyletic group located at the top of the phylogenetic tree, which, together with *Labeobarbus* and *Varicorhinus* in Africa, constituted a clade with high support (Figure 1). The samples of *Tor* and *Neolissochilus* have been divided into three strongly supported evolutionary clades (BS = 100). The Clade I (red branch) located at the base of both genera was composed only of *Neolissochilus benasi*, but all other species in *Neolissochilus* were clustered into Clade II (blue branch), which is the sister group to Clade III (green branch) that contained all the samples from *Tor*. All phylogenetic analyses corroborated *Tor* as a monophyletic group with no obvious geographical structure; some species, including *T. malabaricus*, *T. khudree*, and *T. barakae* confined to India were not well elucidated, and they appeared to be multi-branched in the BI tree (not shown in this article). Meanwhile, the phylogenetic position of *T. remadeviae* may be changed due to outgroup selection. Among the two species in the Dongnai River, *T. mekongensis* had a clear phylogenetic branch, while *T. dongnaiensis* and a part of *T. tambra* clustered together. Furthermore, *T. tambra* and *T. tambroides* of Sunda Island are obviously mixed in the phylogenetic tree, excluding the Mekong basin. *T. putitora* from India and *T. yingjiangensis* from the Irrawaddy River basin were the most closely related,



and both had clear phylogenetic status (BS = 100). Phylogenetic reconstructions did not support the monophyly of *Neolissochilus*. It was obviously divided into two clades (BS = 100), one of which was Clade II and contained the type species of *Neolissochilus*. Clade II can be further divided into two subclades, one containing species closer to South Asia, i.e., India, Motuo in Tibet, and the region bordering India in Myanmar, located at the base of the phylogenetic tree of this genus; the other contained only Southeast Asian species, ranging from Yunnan in China to the island countries of Southeast Asia. The species identified as *N. stracheyi* downloaded from NCBI were scattered across the branches of this genus, with individuals from the Mekong Basin at the top and having the closest evolutionary relationship to *N. baoshanensis*. It was notable that an individual named *N. blanci* was mixed in, but it was actually more likely to be *N. stracheyi*. *Neolissochilus hemispinus* contained the Nujiang River, Longchuanjiang River, and Dayingjiang River individuals, which were resolved to be most closely related to *N. qiaojienensis*. The phylogenetic tree derived from the second dataset (Figure 2) displayed interspecific relationships more concisely and accurately.

3.3. Divergence Time Estimates. Divergence time estimates for major nodes are provided in Figure 3. This analysis revealed that the 95% highest posterior density (HPD) interval is 13.25–20.61 Ma, indicating that the common ancestor of *Tor* and *Neolissochilus* most likely diverged approximately 16.73 Ma of the mid-Miocene (Supplementary figure 1). *N. benasi* had a separate evolutionary pathway, while *Tor* and other *Neolissochilus* species began to differentiate around 12.86 Ma. The common ancestor of *Tor* likely diverged around 9.92 Ma. The main speciation event occurred in the Pliocene–Pleistocene.

4. Discussion

4.1. Taxonomic Classifications. Based on molecular data, most species' status was highly consistent with previous studies. The results presented herein encompassed 13 nominal species of *Tor* with three undescribed species, as well as 17 nominal species of *Neolissochilus* with four undescribed species. Due to the inadequate genetic data and morphological descriptions, the undescribed species are labeled as *Tor* sp.1, *Tor* sp.2, *Tor* sp.3, *Neolissochilus* cf. *dukai*, *Neolissochilus* cf. *stracheyi*, *Neolissochilus* cf. *vittatus*, and *Neolissochilus* cf. *baoshanensis* in the present study. Given taxonomic ambiguities, some species validity must be cautiously interpreted.

Within the genus *Tor*, a new species, *T. laterivittatus*, was described from the Nanla River, a tributary of the Lancang River, and a dark longitudinal stripe along the side of body being used as a key character to distinguish it from *T. sinensis* [14]. Subsequently, Roberts [13] examined and described the adults and subadults of *T. sinensis* with a deep, dark mid-lateral stripe from the Mekong basin in Laos and placed *T. laterivittatus* as a synonym of *T. sinensis*. In the present study, topotypic material (Figure 4(a)) from the Menglun, Nanla River, matched the original description of *T. laterivittatus* well and was genetically identical to that of *T. sinensis* from the Lancang River in Yunnan and the Mekong River in Laos (Figure 4(b)), six of which were collected in the same

small area as the type locality. Therefore, our result supported the assertion of Roberts [13] that *T. laterivittatus* is a synonym of *T. sinensis*.

The taxonomic identification between *T. tambra* and *T. tambroides* has been confusing. Current knowledge indicates the main difference between them is the length and shape of the median lobe; one with a short square median lobe was identified as *T. tambra*, while *T. tambroides* had a longer median lobe [30]. The addition of two more newly described species, *T. dongnaiensis* and *T. mekongensis*, has intensified the taxonomic discussion. Less conspicuous morphological characteristics, such as the length of the median lobe, set these two species apart from *T. tambra* and *T. tambroides* [8]. In our phylogenetic tree (Figure 1), *T. dongnaiensis* was nested in *T. tambra* from the Mekong basin, and phylogeny does not support its validity. The result was consistent with Walton et al. [11], who revealed a mixture of long-lobe and short-lobe morphotypes of the “*T. tambroides*” and considered the validity of *T. mekongensis* and *T. dongnaiensis* require further confirmation. We supported *T. dongnaiensis* as a synonym of *T. tambra*, but the monophyly and validity of *T. mekongensis* were obvious. The phylogenetic tree also showed that *T. tambra* had distinct geographic differentiation between the Mekong basin from China to Vietnam and the Sunda Islands. A similar result was mentioned by Nguyen et al. [16], which showed *T. douronensis* (= *T. tambra*) formed three distinct lineages, these being the Mekong river system from China to Vietnam, Sumatra Island in Indonesia, and Sarawak and Sabah (Borneo Island) in East Malaysia. However, species identification and geographic distribution of *T. tambra* are still confusing. What is noteworthy is the classification of *T. tambra* and *T. tambroides* in the Sunda Islands. Roberts [13] considered that the length and size of the median lobe are highly variable at sexual maturity and treated *T. tambroides* as junior synonyms of *T. tambra* in the Malay Peninsula, Sumatra, Borneo, and Java. Nguyen et al. [16] showed that samples from the Pahang River have a longer median lobe and that *T. tambroides* is a valid species. Recent studies based on mitochondrial genomes showed that species sequences are mixed with topologies similar to this study [31, 32]. Our morphological study observed that individuals of *T. tambra* in the Mekong basin had a short median lobe, except for *T. dongnaiensis* (= *T. tambra*), which has a long median lobe. Additionally, the samples from Java and Malaysia came together to form a single clade that included *T. tambroides*. The explanation may require careful consideration of the possibility of identical species, as well as mitochondrial genetic confusion and morphological neutralization caused by hybridization due to the proliferation of aquaculture and trade in Southeast Asia. Although the location of *T. tambroides* CBMZFI1758 was unknown, this study stated that it's grouped with *Tor* sp. 2 from the Irrawaddy River. Genetic divergence separates this clade from other *T. tambroides* and *T. tambra* species. Lim et al. [31] postulated that either this fish has been misidentified as *T. tambroides* or is possibly a cryptic species. We consider it to be a cryptic species, similar to the *Tor* sp. 3. The finding of cryptic species implies that there is likely more complexity to the species diversity of *Tor* in Southeast Asia.

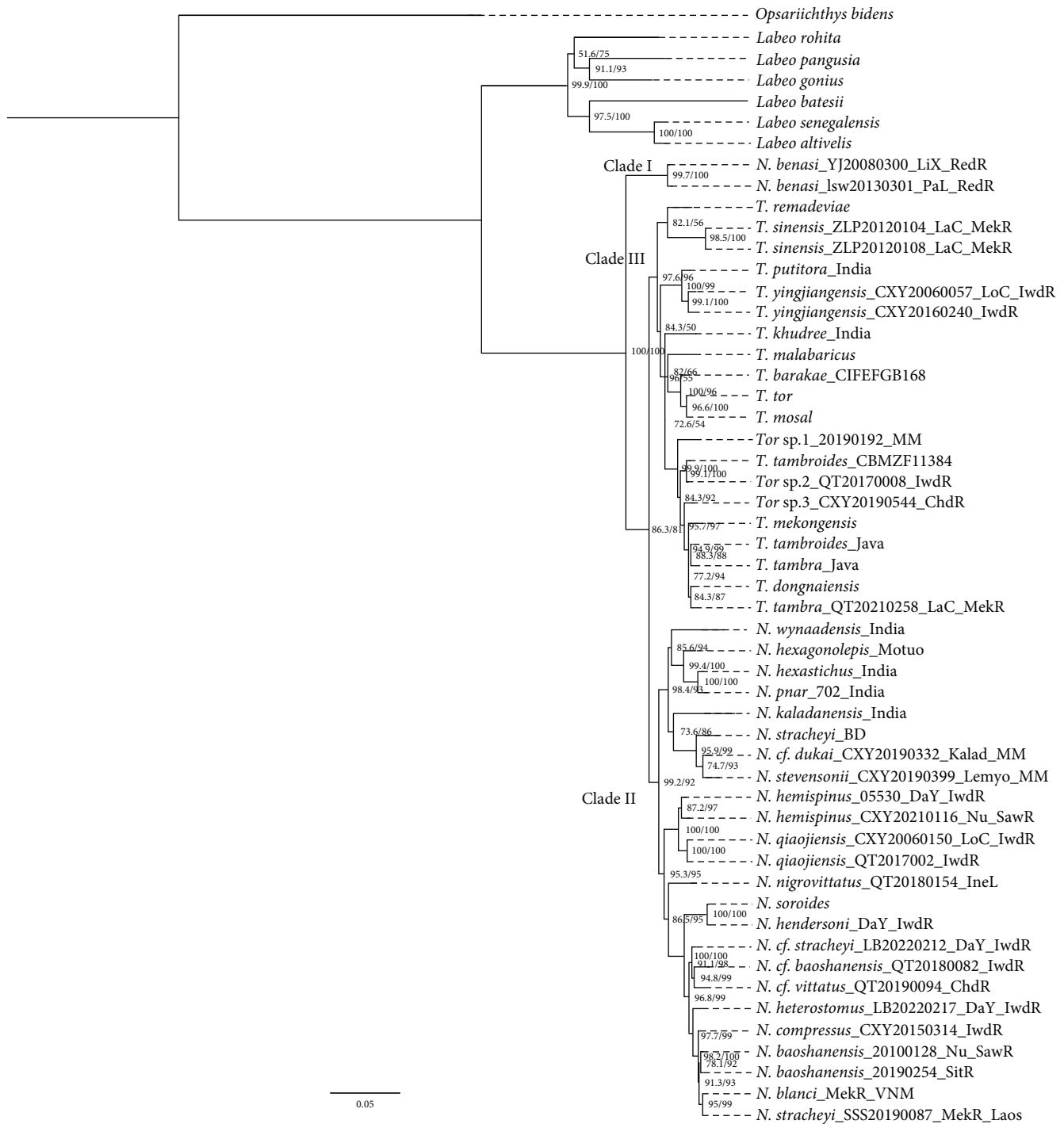


FIGURE 2: Phylogenetic tree rendered by maximum likelihood analysis of the second concatenated dataset. Numbers after the nodes are the bootstrap support values and branch lengths have been given.

Some studies have suggested that the species diversity within *Neolissochilus* may be severely underestimated with multiple undescribed species [33]. Such as the discovery of five new species confined to the Western Ghats [34]. Our analysis also showed that there are more clades in Southeast Asia that have not been clearly identified, including *Neolissochilus* cf. *duhai*, *Neolissochilus* cf. *stracheyi*, *Neolissochilus* cf. *vittatus*, and *Neolissochilus* cf. *baoshanensis*, which required

more detailed morphological analysis and molecular phylogeny to identify species, due to the roughly primitive description of species and the lack of sequence information in the NCBI database. At the same time, *Neolissochilus* species are reported to have phenotypic polymorphism and intraspecific variation. Trophic polymorphism was reported in the *N. soroides* from the Malay Peninsula, *N. hendersoni* from Penang Island, and *N. stracheyi* from the Mekong River, mainly

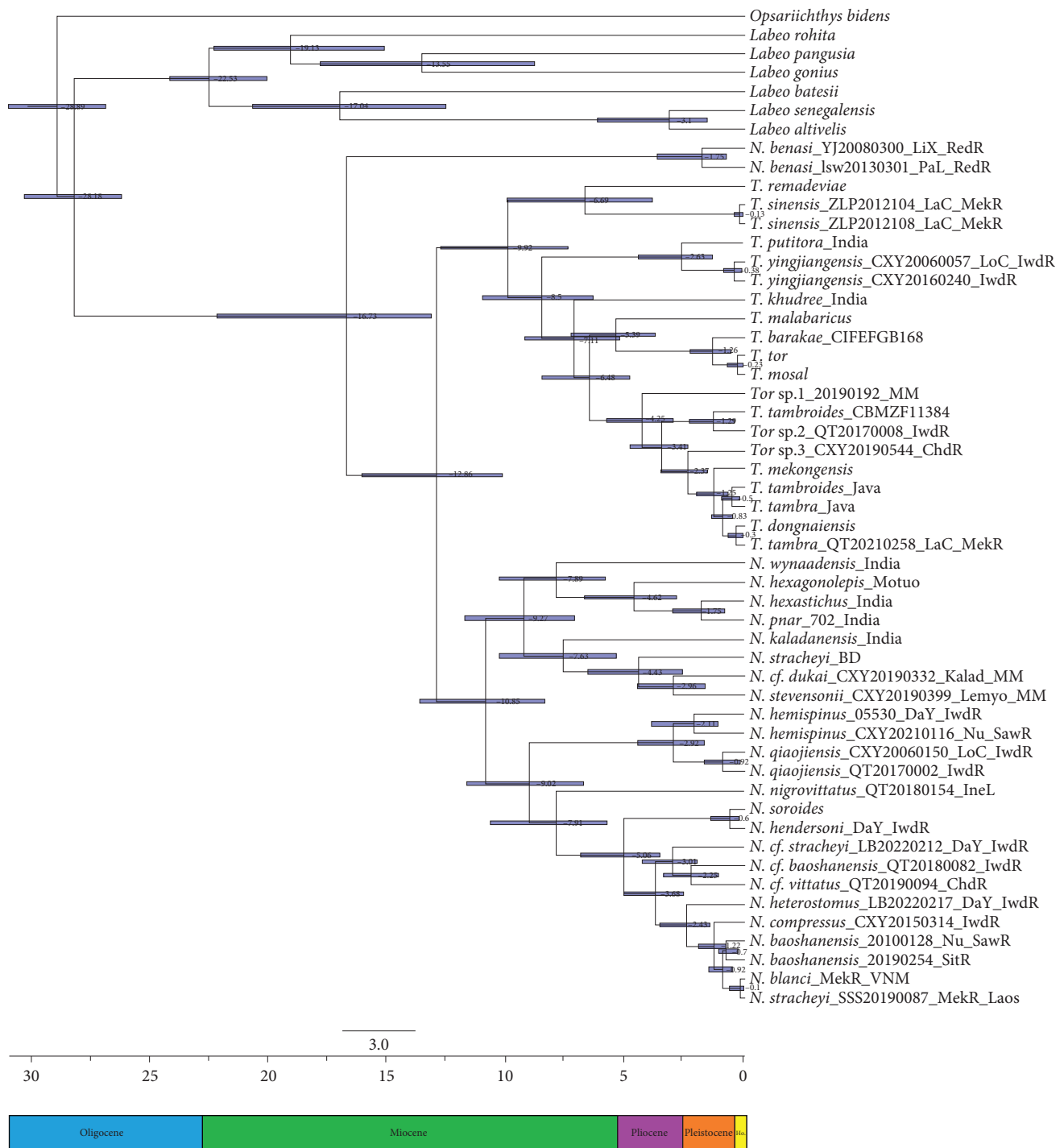


FIGURE 3: Divergence time tree rendered by maximum likelihood analysis of the second concatenated dataset. Numbers after the nodes represent the years of divergence, unit: million years.

manifested in the degree of lip or median lobe development [8, 9]. Obvious intraspecific variations can also be observed about head shape and median lobe size in *N. qiaojiensis* for the first time in this study. According to the first description, the median lobe of *N. qiaojiensis* was underdeveloped, being just slightly broader than the sides of the lower lip or forming a transversely membranous lobe [35]. Nevertheless, our study indicates that within this group, there are individuals who

possess a surprisingly long median lobe (Figure 5(b)), as well as those who closely resemble the original description (Figure 5(a)). Another species with intraspecific differentiation is *N. hemispinus*, which was only distributed in the Nujiang River from Yunnan before. However, we identified some individuals collected from the Dayingjiang River and Longchuanjiang River of the Irrawaddy drainage based on the present molecular systematic results (Figure 1). The

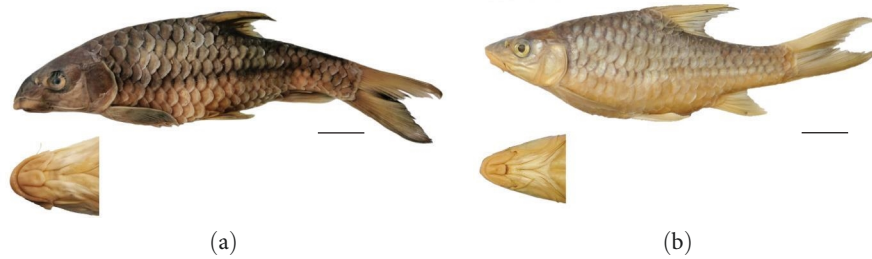


FIGURE 4: *Tor sinensis*. (a) KIZ-ZLP20120103, collected from Menglun, Yunnan Prov., China, 2012, 23.5 cm SL. (b) KIZ-YKF20140030, collected from Menghan, Yunnan Prov., China, 2014, 20.7 cm SL. The photos are from the author's own. Scale bars: 1 cm.

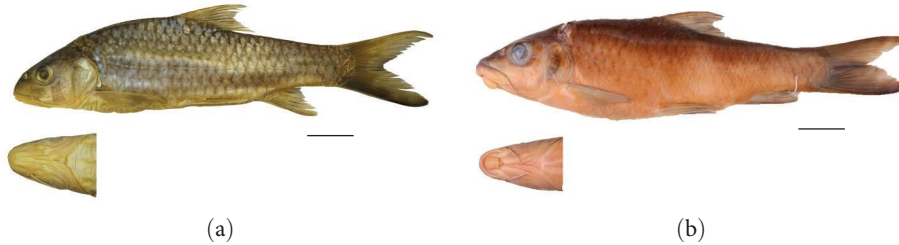


FIGURE 5: *Neolissochilus qiaojiensis*. (a) KIZ200311050, collected from Mengdian River, Yingjiang, Yunnan Prov., China, 2003, 18.7 cm SL. (b) KIZ-QT20170001, collected from Putao Market, Myanmar, 2017, 19.4 cm SL. The photos are from the author's own. Scale bars: 1 cm.

morphological differences between Nujiang drainage and Irrawaddy drainage were mainly manifested in body shape, the number of scales and fins, etc., such as the number of lateral-line scales (33–35 vs. 27–28), predorsal scales (11–12 vs. 9), the degree of ossification of the last unbranched dorsal-fin rays (less than 1/2 vs. more than 2/3) and transverse scales (27 vs. 20). It is a newly recorded fish in the Dayingjiang River and Longchuanjiang River, which could easily be misidentified as *N. qiaojiensis* from the same drainage basin. The difference in morphology may be due to natural selection pressures such as habitats and food resources in different water systems, which have led to divergent evolution of species morphological characteristics, but it has not reached the species level. After increasing the sample size of *N. hemispinus* in the Nujiang River, the morphological differences may be reduced.

Among the genus *Neolissochilus*, *N. stracheyi*, as the type species, initially appeared in Mawlamine and Sittwe, Myanmar, at the mouth of the Salween and Kaladan rivers, respectively [36, 37]. Later literature recorded that it was widely distributed throughout major river systems in Southeast Asia, such as the Mekong basins that include Laos, Thailand, Cambodia, and Vietnam; the Chao Phraya and Salween basin; the Tennasserim basin to the Mekong River, the Chindwin River in western Myanmar; and the Nanla River in Yunnan, China [19, 38, 39, 40, 41, 42]. Rahman et al. [43] have used DNA barcoding to identify *N. stracheyi* (GenBank No. MK572365–67) from Chittagong, Bangladesh, without further explanation. However, this sample, *N. stracheyi* Bangla, was clustered with *Neolissochilus* species but far away from *N. stracheyi* in Laos based on our phylogenetic tree (Figure 1). Since no distribution of *Neolissochilus* species has ever been documented in Bangladesh, we assumed that the population there represented a distinct species that has not yet been

described. Another individual used in Barman et al. [44], *N. stracheyi* India, was grouped with samples from Myanmar and identified as *Neolissochilus* cf. *vittatus*, but no matched location information was given. We considered it to be a misidentification of *N. stracheyi* and may collect from the Chindwin River basin in Manipur, India, which was consistent with the distribution of *Neolissochilus* cf. *vittatus* in Myanmar. The three nominal *Neolissochilus* species, *N. compressus*, *N. stracheyi*, and *N. baoshanensis*, needed to be discussed with caution because of their very low genetic differences from each other. Previous studies also reported that evidence of genetic distance differences based on COI does not support morphological results about some *Neolissochilus* species, like *N. stracheyi* and *N. baoshanensis* [15, 45]. Our research implied that these three species are very closely related and may be the same species. Populations in different drainages may represent different lineages and diverged from each other, but the degree of differentiation has not reached the level of independent species. Further analysis of the geographical distribution, *N. compressus* was a new species described based on one specimen by Day [46], whose type locality was corrected to the upper Irrawaddy River in Burma, rather than the erroneous Kashmir [47]. Wei et al. [48] published the mitochondrial genome of *N. stracheyi* (GenBank No. OM203155), which was collected from the Dayingjiang River in Yunnan, whereas according to our phylogenetic tree, this individual is more closely related to *N. baoshanensis* from the Nujiang River and Sittang River, whose type locality was the Longchuanjiang River and the Nujiang River [49]. The geographical distribution of these three species can be considered to be sympatric, which supported our hypothesis to some extent. Furthermore, it also reminded us to be cautious about the taxonomic status of these *N. stracheyi*, as the existing phylogenetic inferences

were almost based on individuals from the Mekong River, not from the type locality or even type watersheds.

It is well known that molecular-based species delimitation might present challenges in establishing distinct clades for divergence events that occurred recently, as evolving lineages may not have accumulated sufficient mutational differences. [50, 51]. Meanwhile, some studies have shown that the species of *Torinae* have a hybrid origin [52, 53]. It is difficult to distinguish allopolyploidy from homologous polyploidy based on tree topology when polyploidy occurs in two very close parents [4, 18]. Due to the maternal inheritance of mitochondria, different species may have similar or identical maternal parents, resulting in small genetic differences in mitochondrial genes between species. Hybridization plays an important role in the distribution pattern of genetic diversity, taxon differentiation, and speciation [54]. Among fish, evidence of hybridization is more prevalent in the fresh waters, especially between carps and minnows (Cypriniformes), owing to their high diversity, sympatry, and breeding behaviors [55, 56]. Studies such as high-throughput sequencing and population genetic analysis can further detect hybridization and explain these issues [57, 58].

4.2. Monophyly of *Tor* and *Neolissochilus*. The present study represents the most comprehensive phylogenetic study of the genus *Tor* and *Neolissochilus* in terms of sample size and molecular information to date. Since the genus *Neolissochilus* was split off from fish like *Tor* and *Barbodes*, it has been thought to be closely related to *Tor* [19, 59, 60], but later studies have called their phylogenetic positions into question [15, 16, 17]. The topology of the phylogenetic tree in this study is consistent with the results of Zheng et al. [17], who proposed that the *N. benasi* in Red River might represent a new genus. *Tor* is a monophyletic group, whereas the monophyly of *Neolissochilus* is not supported because *N. benasi* forms a separate clade. The divergence time results further proved that it has an independent evolutionary pathway earlier than other species. It should be emphasized that the validity of its status needs to be supported by detailed morphological work.

4.3. Pattern of Distribution Implications. All currently known species of *Tor* and *Neolissochilus* are endemic to the tropical and subtropical areas between the trans-Himalayan region in the northwest and the islands of Southeast Asia. Our calibrated divergence time analysis estimated that these two genera of fish may have originated at 16.73 Ma, well matched with the rapid uplift of the Himalayas (23–19 Ma) [61, 62], and species mainly began to differentiate in the Pleistocene, then speciation in the late Miocene. *N. benasi*, restricted to the Red River basin, was isolated alone at 16.73 Ma, which coincides with the timing of the formation of the Red River zone, which commenced at 27 Ma and ended at 21–17 Ma [63, 64, 65]. It has been hypothesized that the Red River zone at this stage acted as a potential geographic barrier for species dispersal; any change in the runoff regime could have caused a stronger isolation effect by the Red River at that time [66]. Two major events have been suggested as driving the formation and distribution of various species in Asia since the late

Miocene [67]. The first is the oscillation of climate and glacial and interglacial periods that occurred regularly in the Pliocene and Pleistocene. Since the middle Miocene, repeated changes in sea level due to climate change and glaciation have led to significant speciation and faunal succession in Southeast Asia [68, 69]. The second is tectonic activity, including rifting, uplifts of plateaus, and earthquakes. For example, studies have shown that the uplift of the Qinghai–Tibet Plateau and Himalayas may have important effects on the formation, divergence, and distribution of species around the Himalayan area [67, 70]. Meanwhile, these activities also affected the hydrological changes in Southeast Asia. River rearrangement–capture is considered to be an important factor for the isolation, diversification, and cross-basin distribution of fish [71, 72]. The species distribution in the present study was similarly consistent with the above assumptions, and the results supported that land connectivity due to climate fluctuations since the Middle Pleistocene led to the widespread distribution of *T. tambra*. Moreover, the new distribution of *N. baoshanensis* in the Sittang River may further hint at runoff changes in the Irrawaddy River, where the two were interconnected in the ancient drainage system. The isolation and divergence brought about by frequent geological and climatic changes in Southeast Asia may be one of the reasons to explain such a rich genetic diversity of the fish of the genera *Tor* and *Neolissochilus*.

5. Conclusions

This study is the most taxon-rich phylogenetic investigation of *Tor* and *Neolissochilus*, with 76% and 53% of species diversity, respectively. We evaluated the phylogenetic relationships of *Tor* and *Neolissochilus*, revealed the rich species diversity in Southeast Asia, and explored the species differentiation and distribution patterns. Broad trophic polymorphism, overlapping distribution zones, and minor interspecific genetic variations further complicate species identification and classification. Comprehensive sampling and precision analysis of morphological as well as omics data can better provide a perspective for further research on the origin, distribution and evolution of these taxa in Southeast Asia.

Data Availability

The new sequences of COI, Cyt *b*, 16S, and ND4 that support the findings of this study are deposited in the GenBank database.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Ling Wu was responsible for investigation, methodology, data curation, software, formal analysis, visualization, and writing—original draft. Tao Qin was responsible for conceptualization, investigation, and writing—review and editing. Huy Duc Hoang was responsible for resources and writing—review and editing. Thauang Naing Oo was responsible for

project administration and funding acquisition. Xiao-Ai Wang was responsible for validation and resources. Xiao-Yong Chen was responsible for conceptualization, methodology, project administration, supervision, validation, and funding acquisition, resources.

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Supplementary Materials

Supplementary 1. Table 1: information of sequences obtained from NCBI and samples used in the current study.

Supplementary 2. Table 2: primers used for PCR of molecular marker genes in this study.

Supplementary 3. Figure 1: divergence time tree indicated by HPD.

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