A molecular phylogeny of Schizothoracinae (Teleostei: Cypriniformes: Cyprinidae) based on 12 protein-coding mitochondrial genes and RAG1 gene analysis

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Abstract The ever-increasing interest in the investigation of origin and speciation of schizothoracine fishes can be dated to 20th century. However, molecular phylogeny of Schizothoracinae and their phylogenetic relationships, as well as the divergence times still remain controversial. In this study, two DNA sets consisting of 12 protein-coding mitochondrial genes from 254 individuals and RAG1 gene from 106 individuals were used to reconstruct the phylogenetic relationships and calculate the divergence times among the subfamily schizothoracinae. Our results indicated that both of the data sets supported a non-monophyletic relationship due to involving of species of *Barbinae*. However, the phylogenetic relationships based on mtDNA genes were more reliable than that inferred from RAG1 gene. The highly specialized grade formed a monophyletic group, together with *Ptychobarbus* as a sister group of *Diptychus* and *Gymnodiptychus*, which was belonging to specialized grade, indicating that *Ptychobarbus* may be transition species to involve to highly specialized schizothoracianae. In addition, the primitive grade clustered with *Percocypris pingi*, a species of *Barbinae*. Based on mtDNA gene, the speciation time of Schizothoracinae was 66 Ma, and the divergence time of the primitive grade and *Percocypris pingi* was 64 Ma. The speciation times of the three grades Schizothoracinae were 57 Ma, 51 Ma and 43 Ma, respectively; and the divergence time of specialized and highly specialized grade was 46 Ma. The divergence times of three grades were not consistent with the three stages of uplift of Qinghai-Tibet Plateau, which is older than the times.

Keywords Schizothoracinae, protein-coding mtDNA, RAG1, phylogeny, divergence

77 Qinghai-Tibet Plateau, with an average altitude of 4000 meters, is the largest,
78 youngest and highest Plateau in the word (Zhang Ti-Cao et al., 2013), which is called
79 the roof of the world (Cao et al., 1981). The environment and climate of
80 Qinghai-Tibet Plateau had changed drastically due to the intensive uplift of the
81 Plateau since Quaternary. Its extreme environment (hypothermia and hypoxia) has
82 been pregnant with rich and colorful life, making it to be one of the world's
83 biodiversity hotspots. The uplift of Qinghai-Tibet Plateau had experienced three
84 stages in the Eocene, the Oligocene-Miocene, and the Pliocene-Quaternary,
85 respectively (Paul et al., 2001). The first stage of uplift, making Tibet Plateau reached
86 3000m elevation, happened in late Eocene when India began to wedge into Eurasia
87 (Dewey et al., 1989). From 30Ma to 10Ma, the second phase of uplift occurred.
88 However, at this stage, the Qinghai-Tibet Plateau proceeded with pediplanation cycle
89 (an equilibrium of gradual uplifting, faulting and erosion) as a plain (Dewey et al.,
90 1989). In the late Miocene, pediplanation cycle was ended with faulting and uplifting
91 of the Tibetan Plateau excessive erosion, and at the beginning of Pliocene, the Plateau
92 resumed uplift until Quaternary. At present, there is no accurate time or process of the
93 Qinghai-Tibet Plateau uplift because of the complexity of the process.

94 Schizothoracine fishes, commonly known as "mountain carps" and characterized
95 by low growth rate, low fecundity and late sexual maturity (Chen Z.M. & Chen, 2001),
96 belong to Cyprinidae，Cypriniformes, Teleostei and are endemic, typical to the
97 Qinghai-Tibet Plateau and its circumjacent areas (Cao et al., 1981). The name of
98 Schizothoracinae came from their characters of the two rows of large and ordered anal

scales, locating on both sides of the anus and anal fin that made the blank between the two rows of anal scales like a crack (Wu Y.F. & Wu, 1992). This subfamily includes 15 genera and more than 100 species all over the world (Mirza, 1991), and most of Schizothoracine fishes, 11genera and at least 76 species and subspecies distribute in the whole of China (Chen Y.F. & Cao, 2000; Wu Yun-Fei & Tan, 1991). As their major habitat, nine genera and more than half of species and subspecies distribute in the main drainages of Qinghai-Tibet Plateau of China such as Yellow River, Yangtze River, Yarlung Zangbo River, Irrawaddy River and Qinghai Lake (Cao et al., 1981).

As the extreme environment of Qinghai-Tibet Plateau, the mitochondrial genome is well suited for analysis the phylogenetic relationships for that energy metabolism, depending on the mitochondria, is the most apparent response to the adaption to hypothermia and hypoxia. And then, length of the sequence is necessary for maximum likelihood analysis to clarify interrelationships amongst long-diverged groups on account of that ML trees converge to the true tree as the number of sites approaches infinity (Rogers, 2001), and the general length of mitochondrial genomes is 1.6kb, which is sufficient for the phylogenetic tree. The problem of limitation in the number of sequence sites can be settled by the entire mitochondrial genomes (Inoue J.G. et al., 2003; Inoue Jun G. et al., 2004; Ioue et al., 2001; Ishiguro et al., 2003; Kawahara et al., 2008; Lavoue et al., 2008; Lavoue et al., 2005; Masaki et al., 2004; Minegishi et al., 2005; Miya Masaki & Nishida, 1999; Miya M. & Nishida, 2000; Miya Masaki et al., 2003; Saitoh et al., 2003; Saitoh et al., 2000; Saitoh et al., 2006). However, using mtDNA alone as the molecular marker can only reveal a single linkage group with

121 one history while the utilization of multiple nuclear sequences often shows a conflict
122 among gene trees due to hybridization, lineage sorting, paralogy or selection (Graham
123 et al., 2017). Hence, both the mitochondrial genome and the nuclear gene were used
124 in this study to avoid the possible differences.

125 Their special living environment makes them form the characters which are
126 highly adapted to hypothermia, hypoxia and high radiation. As for those characters,
127 Schizothoracine fishes have become a good model of Qinghai-Tibet Plateau to study
128 the mechanism adapting to the extreme environment. Based on the morphological
129 characters, Cao (Cao et al., 1981) divided the schizothoracinae into three grades
130 named morphological primitive schizothoracine fishes, morphological specialized
131 schizothoracine fishes, and morphological highly specialized schizothoracine fishes.
132 The primitive fishes, including *Racina*, *Schizothorax* and *Aspiorhynchus*, are covered
133 by fine scales and these fishes have three rows of pharyngeal teeth and two pairs of
134 barbell. Most of the specialized fishes' scales tend to degenerate and have two rows of
135 pharyngeal teeth and one pair of barbell, and *Ptychobarbus*, *Diptychus*, and
136 *Gymnodiptychus* are included in specialized grade. The highly specialized fishes'
137 scales completely disappear and have only one row of pharyngeal teeth and have no
138 barbell; highly specialized group consists of six genera named *Gymnocypris*,
139 *Oxygymnocypris*, *Schizopygopsis*, *Platypharodon*, *Chuanchia*, and *Herzenstein*. The
140 statistical studies showed a relationship between species richness and elevation that
141 the primitive groups peaked in the low elevation areas of 1250m to 2500m; the
142 specialized fishes mainly distributed in the elevation of 2750m to 3750m and highly

specialized grade fishes primary occupied the elevation zone of 3750m to 4750m (Cao et al., 1981). Some researches revealed that the speciation of three grades schizothoracine fishes resulted from the three stages of uplift of Qinghai-Tibet Plateau, which caused the branch of river basins.

By now, there are still many controversies on the phylogeny of Schizothoracinae and their close species. A large body of researches showing that Schizothoracinae originate from the primitive genera of Barbinae, such as *Barbodes, Varicorhinus, Barbus,* based on molecule or morphology (Cao et al., 1981; Chen Y.F. & Cao, 2000; Wu Xianwen et al., 1981), is widely accepted. Gaubert et al. (Gaubert et al., 2009) repute that Schizothoracinae's closest relatives to be the Barbinae fishes, which is consistent with He (He et al., 2004) and Qi (Qi et al., 2013; Qi et al., 2012), and the subfamily Schizothoracinae is not a monophyly with supertree from the Cyprinidae family level (Gaubert et al., 2009), which is the same to Li (Li Y.L. et al., 2013), but contradict the results of He (He et al., 2004) and Qi (Qi et al., 2012). In the study of Ruber in 2007 indicate that Barbine sensu stricto is closely related to Schizothoracinae, but it's an unresolved trifurcation between Barbine sensu stricto and two lineages of Schizothoracinae (Ruber et al., 2007). In the research of Li, however, Barbus barbus is embedded in Schizothoracinae that between the specialized group and the primitive group as the sister clade of specialized fishes with the method of mitochondrial genomes of three species of Schizothoracinae and single cytb gene (Li Y.L. et al., 2013). It's clear that Percocypris pingi, which belongs to *Percocypris* genus and Barbinae subfamily, cluster with the primitive clade in Wang's study (Wang

et al., 2013). Many reasons could explain the inconsistency between morphological phylogeny and molecular phylogeny. For example, He considers the morphological characters may don't fellow the evolutionary trace of the groups but are shaped by adapting to their survival environmental condition (He & Chen, 2007), such as convergent evolution (Qi et al., 2012). In addition, the low level of sequence divergence and ancestral polymorphism will make it difficult to discriminate species (He & Chen, 2007). The number of samples and the length or quantity of genes also play an important role in the reconstruction of phylogeny. For above reasons, using the molecular method, sufficient samples and appropriate gene to analysis the phylogenetic relationships of Schizothoracinae is becoming more and more urgent.

The overwhelming majority of experts agree with that the speciation of three grades Schizothoracinae fishes results from the uplift of Qinghai-Tibet Plateau that causing the branch of those watersheds. At the middle Tertiary-Dingqing group(late Miocene or early Pliocene) , with the movement of the Himalayas, Barbinae, being adapted to the warm climate, gradually tend to adapted to cold weather, which lead to speciate the Plesioschizothorax macrocephalus, which is the only fossil of Schizothoracinae in the world (Wu Y.F. & Wu, 1992). Based on geology and paleontology, Wu deduced that in the first phase of the Himalaya movement, as the early stage of Schizohtoracinae, Plesioschizothorax macrocephalus occurred. In the second phase of the Himalaya movement, the environment was so suitable for freshwater that Plesioschizothorax macrocephalus became the main fishes of those lakes, making this phase to be boom period. However, in the third phase of Himalaya

187 movement, in which the crust began to lift and lakes began to shrink and separate,
188 radical changes of environment resulted in the Plesioschizothorax macrocephalus'
189 differentiation, migration and extinction. Except Li used the partial mitochondrial
190 genome of *Cytb* gene to declare that the primitive clade divided in the late Eocene and
191 the Rapid speciation events of each clade from the Late Miocene to the Pliocene,
192 corresponding to the time of the geologic acceleration of the Qinghai-Tibetan Plateau
193 (Li Y.L. et al., 2013), there is no researches about the divergence time of
194 Schizothoracine fishes from the subfamily level. Hence it's essential to deduce the
195 divergence time of three grades of Schizothoracinae on the subfamily level to make
196 up the blank of this area.

197 In order to better resolve the problems of phylogenetic relationships of
198 Schizothoracinae and calculate the divergence times of these genera, we used 12
199 protein-coding mitochondrial genes from 254 individuals, which were belonging to
200 115 species, 11 genera, and RAG1 genes from 106 individuals, which were belonging
201 to 42 species, 8 genera, to reconstruct the phylogenetic tree of the subfamily
202 Schizothoracinae, and inferred the divergence times among Schizothoracine fishes,
203 and analyzed the relationships between the differentiation of Schzithoracinae and the
204 uplift of Qinghai-Tibet Plateau.

205 Materials and methods

206 *Taxon sampling and DNA extraction*

207 We employed 254 mtDNA sequences (table 1) belonging to 11genera of subfamily

208 Cyprinidae, 32 sequences among which were previous work of our team (GenBank
209 accession number: KT833082-KT833113). 4 sequences of Ictiobus were selected as
210 outgroups. The other mitochondrial genomes were downloaded in NCBI
211 (http://www.ncbi.nlm.nih.gov/) and the Genbank accession numbers were shown in
212 Supplementary Material S1.

213 For the RAG1 gene, 38 individuals were collected by ourselves from the rivers
214 of Qinghai Province and their tributary. We took the muscles or fins and then
215 immediately preserved in 95% ethanol stored at −20 °C for DNA extraction. Total
216 genomic DNA was isolated by using phenol-chloroform extraction (Sambrook et al.,
217 1989) and adjusted to 100ng/mL after testing its concentration on a NanoDrop 2000
218 supermicro spectrophotometer (Thermo Fisher Scientific Inc., USA). Meanwhile, 68
219 sequences of RAG1 gene were obtained from GenBank (Accession numbers and
220 species name were showed in Supplementary Material S2).

221 ***PCR Amplification and Sequencing***

222 The RAG1 gene was amplified using the pair of primers RF (5'-CTG AGC TGC AGT
223 CAG TAC CAT AAG ATG T-3') and RR (5'-TGA GCC TCC ATG AAC TTC TGA
224 AGR TAY TT-3') (Saitoh & Chen, 2008). The PCR (polymerase chain reaction)
225 reacted at 50uL total volume as follows: 15uL H_2O, 25uL 2 × PCR Master Mix buffer
226 (Novoprotein Science and Technology Ltd., Shanghai, China), 2.5uL each primer
227 (10mM), and 5uL genomic DNA. And the PCR reaction performed at initial
228 denaturation step at 94 °C for 4 min followed by 35 cycles at 94 °C for 40s, 59°C for
229 40s, and 72 °C for 90s; with a final extension at 72 °C for 10min. 2 μl of amplified

DNA was fractionated by electrophoresis through 1% agrose gels. And those PCR products with clear single band were sent to sequence in both directions in Beijing Biomed Company (Beijing, China).

Sequence editing and analysis

The RAG1 gene sequences were assembled using Contigexpression v9.1.0 software and aligned by online MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/), and then carefully checked by eye and edited manually in MAGE 6.0.

We used 12 protein-coding regions of mtDNA for sequence analysis, which the genes were encoded on the heavy-strand. Then the indices of substitution saturation for these sequences were estimated using DAMBE 5 software with the GTR model (http://dambe.bio.uottawa.ca/software.asp).

Phylogenetic analysis

There were 254 sequences of mitochondrial genomes using in phylogenetic analysis and 4 common sucker mitochondrial genomes were designed as outgroups (Saitoh et al., 2011b)（Supplementary Material S1）. Meanwhile, a total of 106 sequences of RAG1 gene was used and 1 common sucker RAG1 sequence was designed as the outgroup (Supplementary Material S2).

We used 12 protein coding regions to phylogenetic analysis, which the genes were encoded on the heavy-strand. The ND6 wasn’t included in the analysis because this gene was encoded on the light-strand that was different from the 12 genes. All of these sequences were aligned by online MAFFT version 7, and corrected by eyes. A few parts were ambiguous that were excluded. We also removed start codons, stop

252 codons and the overlapping regions between the coding genes ATP6-ATP8,
253 ATP6-COXIII, ND4L-ND4, and ND5-ND6 (Li Y.L. et al., 2013).

254 The model selection was implemented in Modeltest v3.7. GTR+I+G, as the best
255 model of mtDNA sequences, was used to construct the phylogenetic trees, while
256 SYM+I+G was selected as the best model of RAG1 sequences. GTR+I+G was used
257 as the best model of RAG1 for subsequent analysis because there was no SYM+I+G
258 model in the analysis software. We reconstructed the phylogenetic trees with
259 maximum likelihood (ML) method and Bayesian inference (BI) method. The ML
260 analysis was accomplished by RAxML v7.2.8 (Stamatakis et al., 2008), with
261 GTRGAMMA and GTRCAT models. The BI method was used MrBayes v3.2.4
262 (Huelsenbeck & Ronquist, 2005; Ronquist & Huelsenbeck, 2003). We used the GTR
263 model to run 2,000,000 generations of 4 simultaneous Monte Carlo Markov chains
264 (MCMC). The sampling frequency and pint frequency were set 100, the diagnostic
265 frequency was set 1000, and the genes were divided into 12 partitions and the
266 likelihood scores lower than those at saturation (burn-in = 500) trees were discarded
267 from the analysis. The posterior probabilities (BBP) of nodes were estimated based on
268 the 50% majority rule consensus of the trees.

269 ***Divergence time estimation***

270 Based on mtDNA genes, we used Beast v2.3.0 (Bouckaert et al., 2014) to calculate
271 the divergence times of three grades of Schizothoracinae with the lognormal incorrect
272 relaxed clock and GTR+G+I model. We used “Speciation: Yule Process” to run
273 500,000,000 chains. The calibrated nodes and constraint were as followed: basal to

Cyprinus (33.9Ma) (Saitoh et al., 2011a), and *Labeo bata* / *Labeo senegalensis* (49.1 Ma – 75.1 Ma) (Li Y.L. et al., 2013). And then convergence diagnostics were examined with Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/).

Results

Mitochondrial genomes features

A total of 254 mtDNA and 106 RAG1 sequences were obtained after manually editing, which were 10791bp and 1314bp in length, respectively. The average base composition of 12 protein-coding genes was A=28.0%, T=27.8%, G=16.4% and C=27.8%. The base composition of schizothoracinae showed the same characters with teleost fishes that A+T content is significantly higher than the G+C content with an obvious anti-G bias (Jiang et al., 2009; Jondeung et al., 2007; Tzeng et al., 1992). Additionally, the average base composition of RAG1 gene was A=25.3%, T=24.3%, G=26.5% and C=23.9%. There was no obvious base bias among RAG1 gene sequences.

Saturation analyses showed that neither of the mtDNA sequences nor RAG1 sequences was saturated and variable sites of RAG1 sequences were obviously distributed at codon 3 (Figure 1.). Thus, all sequences could be used in reconstructing phylogenetic trees for schizothoricine fishes.

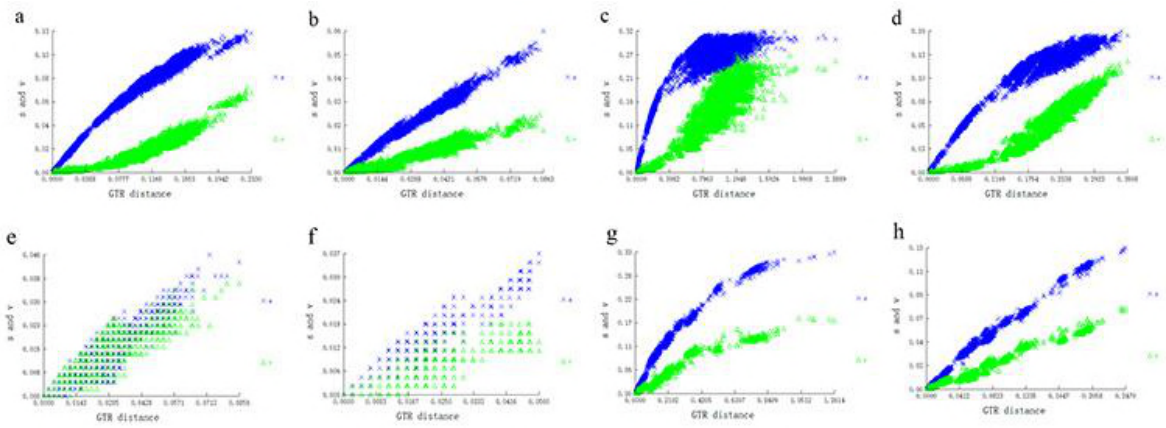


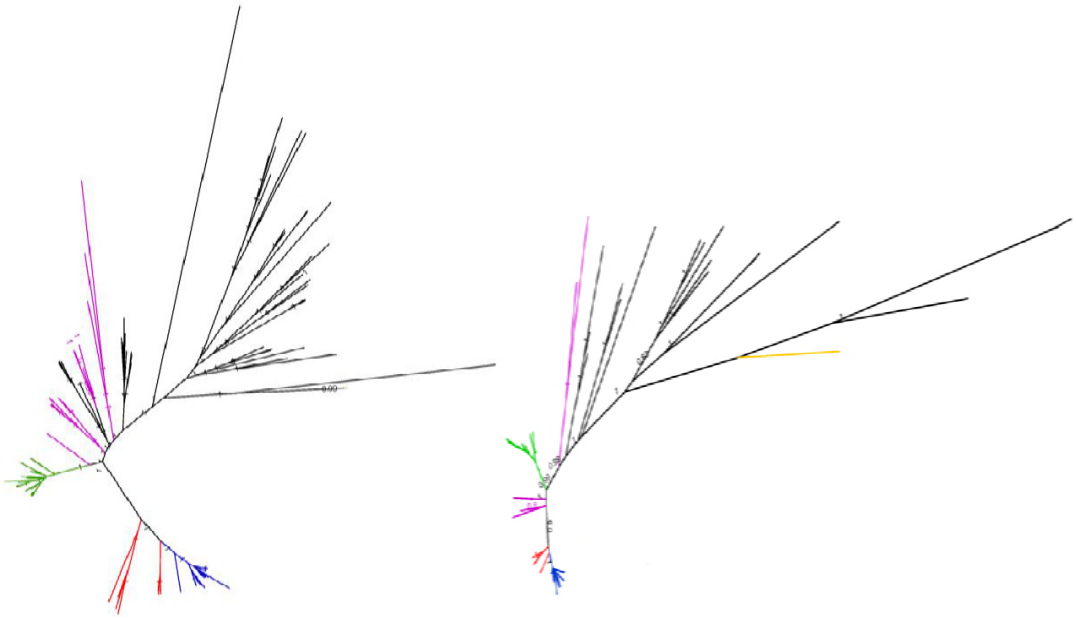
Figure 1 The saturation analysis of both the mitochondrial protein-coding genes (excluding ND6 and 12SrRNA) sequences and RAG1 gene sequences based on the GTR model. Figure a, b, c, and d represent saturation of codon1, codon2 codon3 and complete codon of 12 protein-coding genes, respectively; Figure e, f, g, and h represent saturation of the same part of RAG1 gene.

Phylogenetic analysis

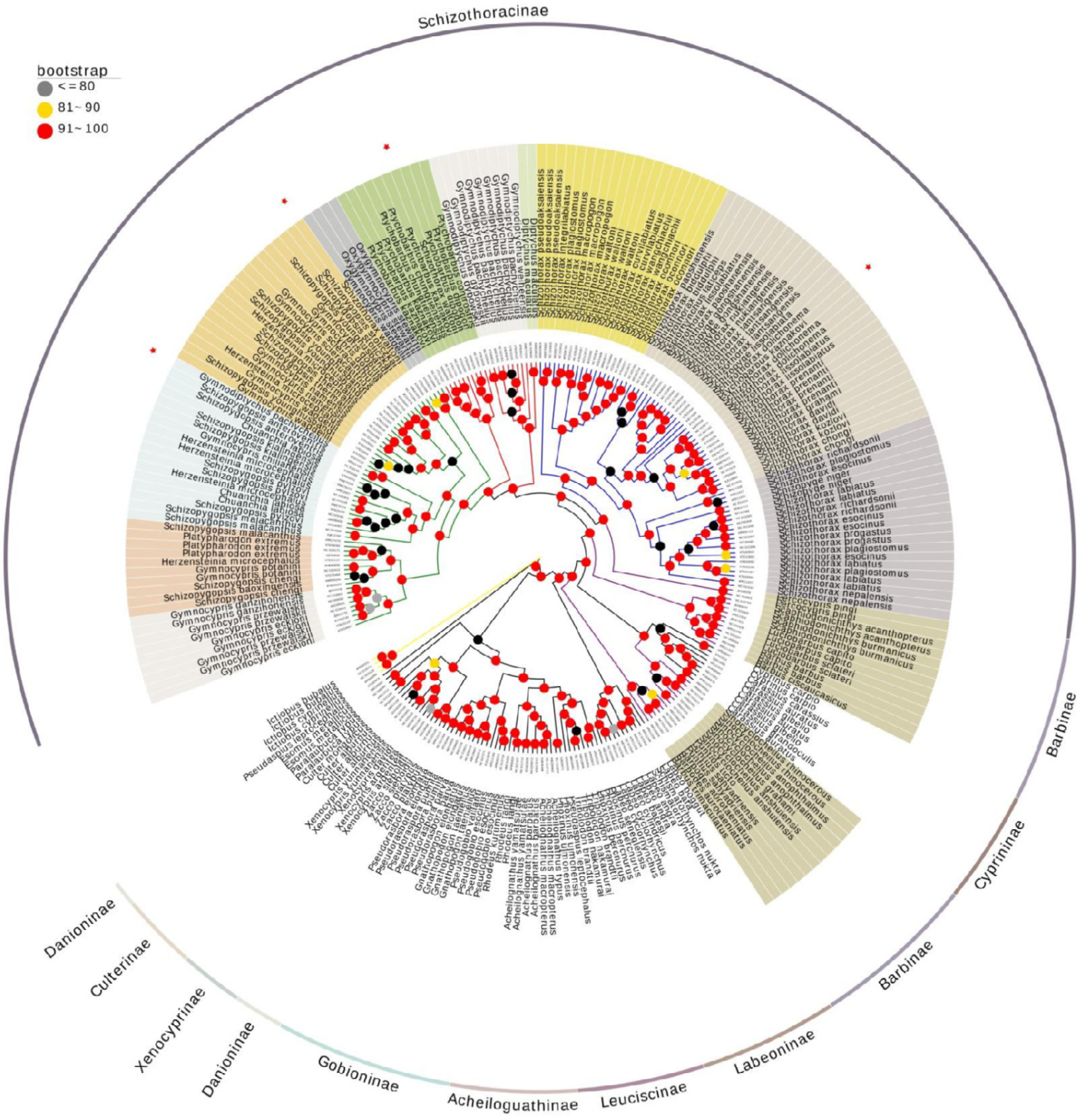
The maximum likelihood trees inferred from mitochondrial genomes of Cyprinidae were almost the same, only ML tree based on GTRCAT model was shown in Fig 2 (C), which was consistent with the Bayes tree Fig 2 (A). Similarly, the ML trees of RAG1 gene were almost the same, ML tree based on GTRCAT model showed in Fig 2 (D) was also consistent with BI tree(Fig 2 B). The trees based on RAG1 gene indicated that the Schizothoracinae was not a monophyletic groups, which the primitive grade clustered into a big branch with node support ratio of 98 and clade that include *Barbus barbus* and *Scaphiodonichthys acanthopterus* clustered with specialized and highly specialized clade with node support ratio of 37, indicating that the specialized and highly specialized schizothoracinae, as well as the species of Barbinae, as well as the species of *Barbinae* had most recent common ancestor, which

was clearly shown in Fig 3 F, that consistent with Wang (Wang et al., 2013) and Li (Li Y.L. et al., 2013). However, slightly different results were obtained from trees based on 12 protein-coding mtDNA genes. The fact that the subfamily schizothoracinae was not a monophyletic group only resulted from that the *Percocypris pingi* as the sister clade of the primitive grade (100). The clade of specialized grade schizothoracine fishes were separated from the clade that included primitive grade schizothorcine fishes and *Percocypris pingi* with strong support (96), and then the highly specialized schizothoracine fishes were differentiated from the specialized grade with stronger support (100).

A B



C



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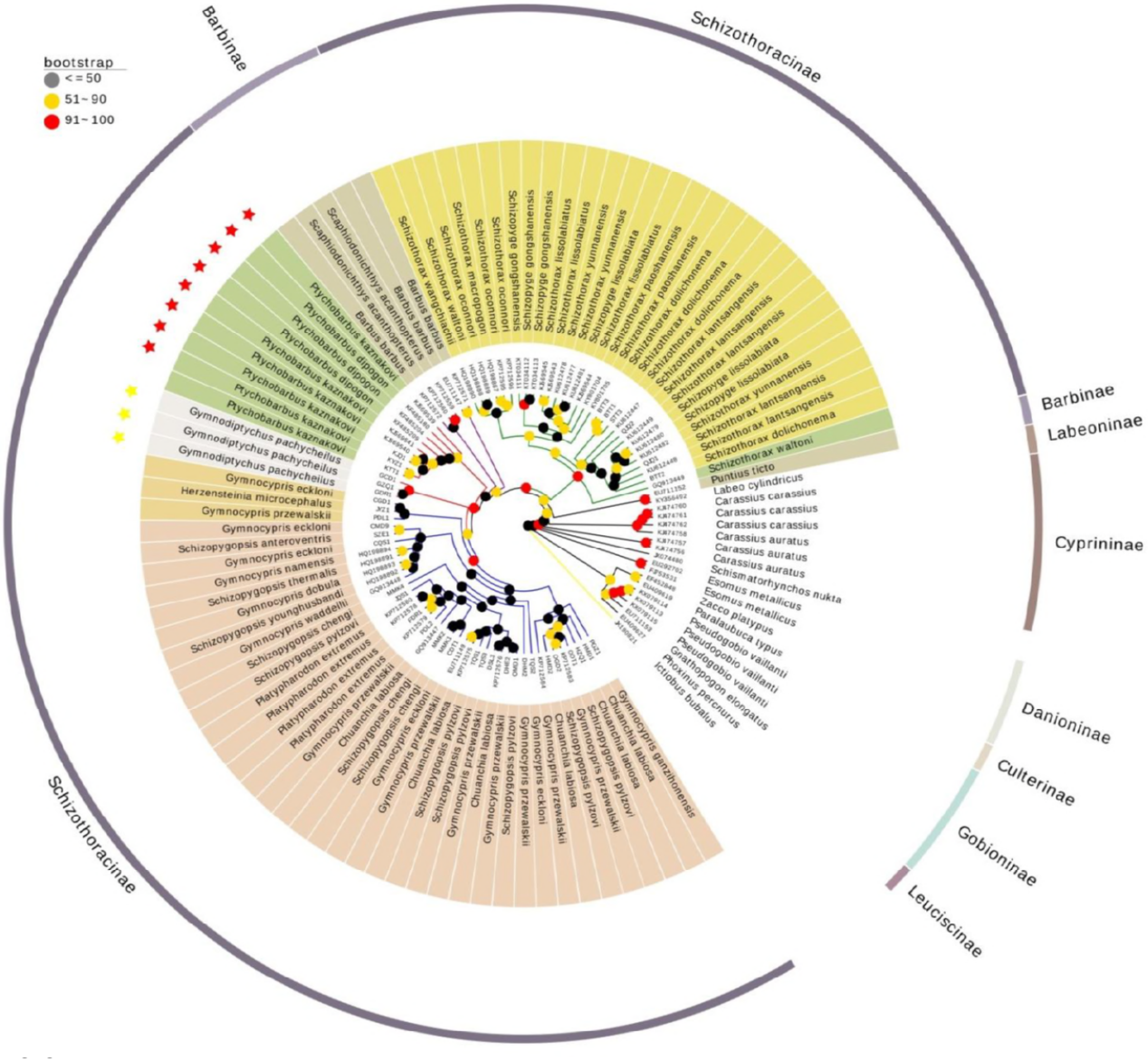
328 D



330 **Figure 2** The phylogenetic tree of Cyprniformes as inferred from the mitochondrial protein-coding genes

331 (excluding ND6 and 12SrRNA). Yellow branch represents the outgroup species, green branches

332 represent the primitive grade of schizothoracinae, red branches represent the specialized grade of

333 schizothoracinae and blue branches represent the highly specialized grade of schizothoracinae.

334 A The Bayes Inferred Tree of 12 protein-coding genes based on the GTR+I+G model, Ictiobus cyprinellus

335 was used as the outgroup. The nodal numbers indicated Bayesian posterior probability with the mcmc

336 ngen = 2000000.

337 B The Bayes Inferred Tree of RAG1 gene based on the GTR+I+G model, only the Ictiobus cyprinellus

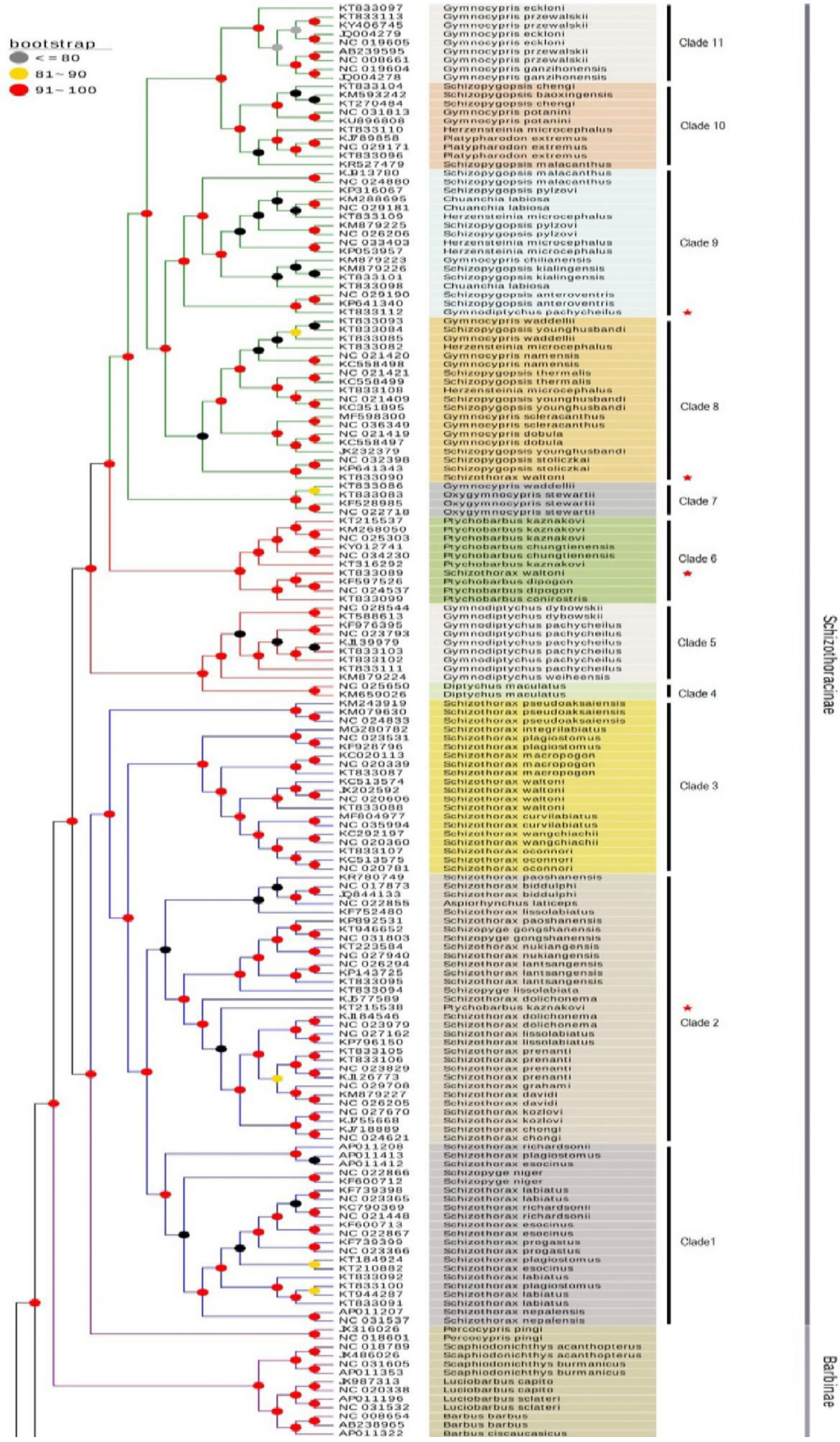
was used as the outgroup. The nodal numbers indicated Bayesian posterior probability with the mcmc ngen = 2000000.

C The overview of Maximum Likelihood Tree based on 12 protein-coding genes with the GTRCAT model, Ictiobus were used as outgroups. The branch label numbers indicated the bootstrap probabilities (rapid bootstrap set as 1000 replications).

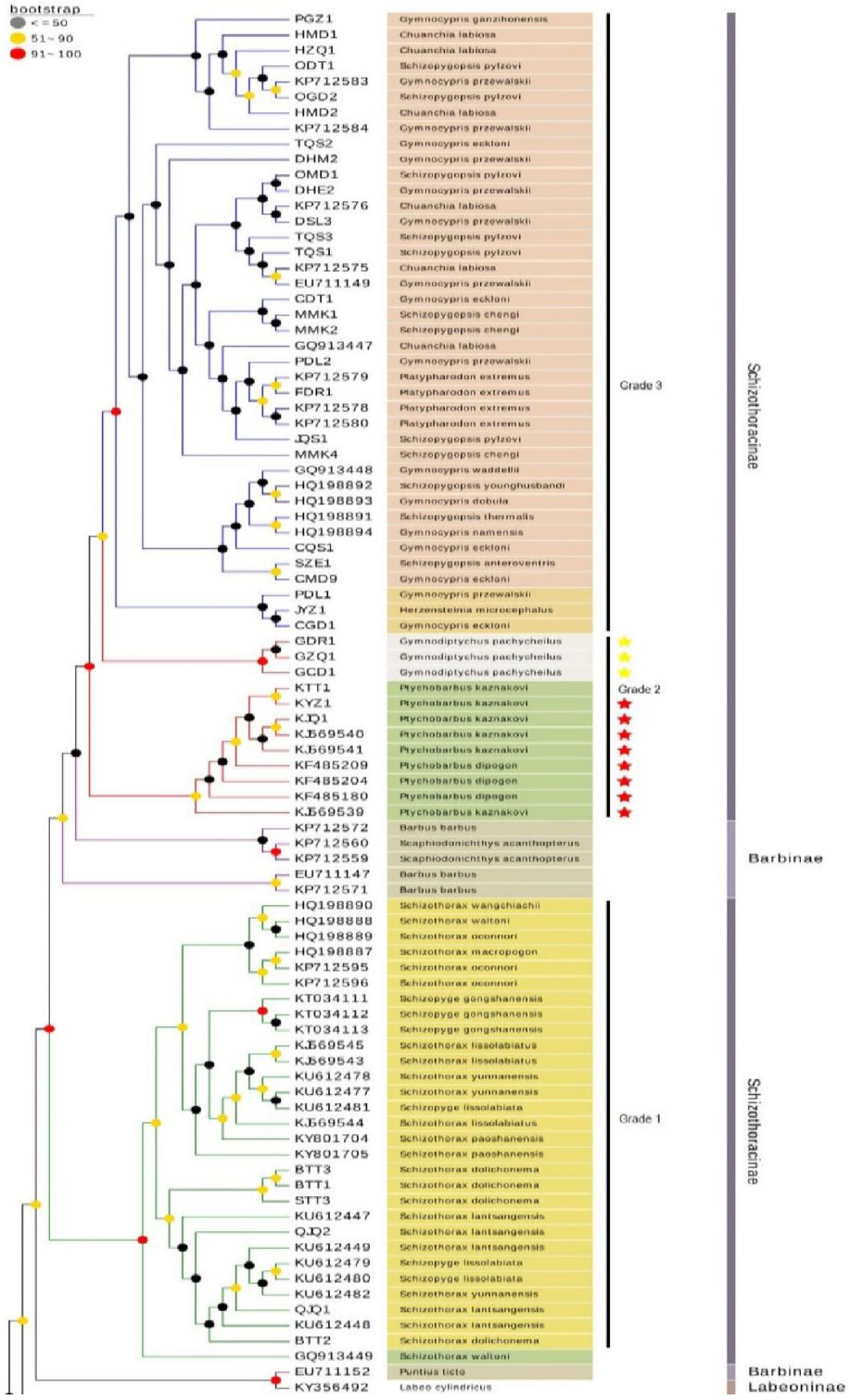
D The overview of Maximum Likelihood Tree based on RAG1 gene with the GTRCAT model, Ictiobus were used as outgroups. The branch label numbers indicated the bootstrap probabilities (rapid bootstrap set as 1000 replications).

Both two molecular datasets were inconsistent with the conclusion of morphology, which indicated that the specialized grade originated from the primitive schizothoracinae, and the highly specialized schizothoracinae originated from the specialized schizothoracinae. Meanwhile, in phylogenetic trees based on both genes, the Schizothoracinae was divided into two major clades, indicating the subfamily schizothoracinae might have two different origins. Specialized and highly specialized Schizothoracine fishes form one clade while primitive Schizothoracine fishes made up the other major clade that indicated primitive clade was single origin and specialized and highly specialized originated from another ancestor. The primitive clade located at the bottom of the tree and the highly specialized grade lay at the top of the tree, which was in accordance with the result of He (He et al., 2004).

E



F



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361 **Figure 3** The partial enlargement of Maximum Likelihood Tree only included the subfamily

362 schizothoracinae based on 12 protein-coding genes (E) and RAG1 gene (F). Green branches represent

363 the primitive grade of schizothoracinae, red branches represent the specialized grade of

364 schizothoracinae, blue braches represents the highly specialized grade of schizothoracinae, and purple

365 branches represent the species of Barbinae. Shadows with the different color indicated different clades.
366 Red stars in figure E represented individuals that might be incorrectly classified, while in figure F red
367 stars and yellow stars represented genera *Ptychobarbus* and *Gymnodiptychus*.

368 The Schizothoracinae was comprised of 11 clades based on 12 protein-coding
369 genes, showing in Fig 3 E, each with strong support. Clade 1 to 3 was included in
370 primitive schizothoracinae and clade 4, 6 was the part of specialized schizothoracinae,
371 clade 7 to 11 was involved in highly specialized schizothoracinae. It was noteworthy
372 that the clade 6 (*Ptychobarbus*) which belonged to specialized schizothoracinae
373 clustered with highly specialized schizothoracinae with strong support (100) that
374 might lead us to speculate that *Ptychobarbus* was the transitional taxa that the
375 specialized schizothoracinae evolved to highly specialized schizothoracinae and
376 making the result be inconsistent with the monophyly of specialized schizothoracinae
377 (Chen Z.M. & Chen, 2001), and *Diptychus* was the basal clade as monophyly.
378 *Gymnodiptychus* clustered together as monophyly that originated from *Diptychus*.
379 Primitive schizothoracinae was monophyletic group whereas these species of three
380 genera tangled up with each other that strongly supposed that genus *Racoma* and
381 genus *Schizopyge* should be merged into genus *Schozothorax*. Highly specialized
382 schizothoracinae, Clade 7 to 11, was monophyly clustered with *Ptychobarbus* as the
383 sister group, and *Oxygymnocypris* was the primordial genus, and phylogenetic
384 relationships among the other genera including *Schizopygopsis, Platypharodon,*
385 *Gymnocypris*, *Chuanchia,* and *Herzensteinia* were ambiguous. *Gymnocypris* possibly
386 originated from *Oxygymnocypris. Platypharodon* clustered together as the sister group

of *Gymnocypris przewalskii*. The other species of *Gymnocypris* and species of *Chuanchia* and *Herzensteinia* were embedded in genus *Schizopygopsis*. The results above were highly in line with the conclusion that members of the specialized schizothoracine group and the genera *Schizothorax*, *Schizopygopsis*, and *Gymnocypris* were paraphyletic based on complete mitochondrial genomes (Zhang J. et al., 2016). On the other hand, based on RAG1 gene (Fig 3 F), genera in highly specialized grade schizothoracinae were clustered together as a monophy clade that strongly supported (96), while genera in primitive grade and specialized grade schizothoracinae could distinguish each other clearly with unreasonable topology, which was obviously different from the results based on mtDNA genes.

Divergence times

Our estimated divergence times of schizothoracinae based on the mitochondrial genes were much older than the estimates of He (He et al., 2004) and Ruber (Ruber et al., 2007), but was consistent with Yang (Li Y.L. et al., 2013). The divergence times of three grades Schizothoracinae were not completely consistent with three stages of the uplift of Qinghai-Tibet Plateau. The speciation time of Schizothoracinae is 66 Ma, which is consistent with the first stage of the uplift of Qinghai-Tibet Plateau. The divergence time of the primitive grade and *Percocypris pingi* was 64 Ma, while the speciation time of the primitive grade was 57 Ma. And the divergence time of specialized and highly specialized Schizothoracinae is 46 Ma, which is much older than the second stage of uplift of Qinghai-Tibet Plateau. The speciation times of

specialized and highly specialized Schizothoracinae are 51 Ma and 43 Ma, respectively.

Discussion

Phylogeny analysis

The phylogeny of schizothoracinae is controversial all the time. Our phylogeny relationships of schizothoracinae, based on RAG1 gene, showed that the primitive grade schizothoracinae fishes clustered into a single branch that strongly supported and the specialized and highly specialized schizothoracinae clustered with some species of *Barbinae*, while phylogenetic of schizothoracinae based on 12 protein-coding mtDNA genes indicated that the specialized and highly specialized schizothoracinae as a sister clade directly clustered with clade that included the primitive grade schizothoracinae and *Percocyoris* rather than clustered with *Barbinae*. Subfamily schizothoracinae split into two clades into both molecular data, indicating that Schizothoracine fishes have two different origins, as the same as other molecular data (He et al., 2004; Li Y.L. et al., 2013; Qi et al., 2015), were inconsistent with morphological phylogenies that the schizothoracinae was monophyly and the specialized and highly specialized schizothoracinae was originated from primitive Schizothoracine fishes. In morphological phylogenies of schizothoracinaea, the trophic morphologies mainly were selected as criterions of taxonomy, such as the rows of pharyngeal teeth, lower jaws horn, pharyngeal bone, and the skull. To some extent, those morphological characters were determined by their habits and foraging ways. Convergent evolution, the same environment prompt to form the similar

morphologies, enforces the different species to cluster together, making the traditional taxonomy of the schizothoracinae was different with the molecular results (Qi et al., 2012). The convergent evolution is a common phenomenon such as the lower lip of *Labeoninae* (Li Jun-bing et al., 2005), eye and pigment degeneration and well developed projection of frontal bones of the cave species (Xiao et al., 2005), the morphological similarities of ground tits and ground jays result from convergent evolution (Qu et al., 2013). Those characters of morphologies are non-homologous but are similar that are meaningless to phylogenies. And then the low level of sequence divergence make the molecular mutations don't have enough time to stabilize and accumulate that result in the rapid differentiation of morphology don't synchronously reflect on the molecular variations. And ancestral polymorphism, rapid evolution, expansion and diversification process, mitochondrial introgressive hybridization may also are the main factors to the inconsistence between molecular phylogenies and morphological phylogenies (He & Chen, 2007). RAG1 gene, related to immune system, has the common disadvantages of nuclear genes as molecular markers, such as heterozygous ambiguity and paralogy, few available variable sites resulting from sequences conservation (Chen W.J. et al., 2008; Li Chenghong et al., 2007; Saitoh & Chen, 2008). Therefore, the phylogenetic trees reconstructed based on the two molecular data were different, and we speculated that the phylogenetic relationships of subfamily schizothoracinae based on 12 protein-coding genes were more reliable.

Our analysis of phylogeny of schizothoracinae was also different from other

some molecular phylogeny. The most prominent was that the closest relative species of schizothoracinae is *Percocypris pingi*, which was consistent with Yang (Yang et al., 2015). The genera of specialized grade are different from the other phylogeny. The *Diptychus* as the basal clade of specialized grade schizothoracinae that *Gymnodiptychus* was evolved from genus *Diptychus*, but *Ptychobarbus* was advanced genus which was clustered together with highly specialized grade schizothoracinae that were inconsistent with the result that *Gymnodiptychus* was advanced genus that evolved from *Ptychobarbus* based on Cyt *b* (Chen Z.M. & Chen, 2001). Maybe they sampled only minority species and individuals or only used Cyt *b* mitochondrial gene resulting in different conclusions. In the resolution of interrelationships amongst long-diverged groups, the length of the sequence was particularly important (Rogers, 2001). Therefore, just using the Cyt *b* gene may be inappropriate to speculate the phylogeny of schizothoracinae. Sampling as well plays a speculate role in reconstruct phylogenic relationships that we need to choose sufficient species and individuals.

Divergence times of schizothoracinae

The divergence times of three grades of Schizothoracinae were not completely consistent with the three stages of uplift of Qinghai-Tibet Plateau, which was contradicted with the morphology results. In our data, the speciation of Schizothoracinae was 66 Ma, which was the time of the first stage of uplift of Qinghai-Tibet Plateau, but the speciation times of primitive Schizothoracinae and the specialized Schizothoracinae and highly specialized Schizothoracinae was much older than the second and third stage of uplift of Qinghai-Tibet Plateau. The speciation time

of primitive clade and the specialized - highly specialized clade was very close. So the result of the divergence times was consistent with the phylogeny result, supporting that Schizothoracinae has two independent origins. The primitive clade was originated from the original genus of *Barbinae*, while the specialized and highly specialized clade, as a sister group with the clade consist of the primitive clade and *Percocypris pingi*, originated from the other genus of genus of Barbinae.

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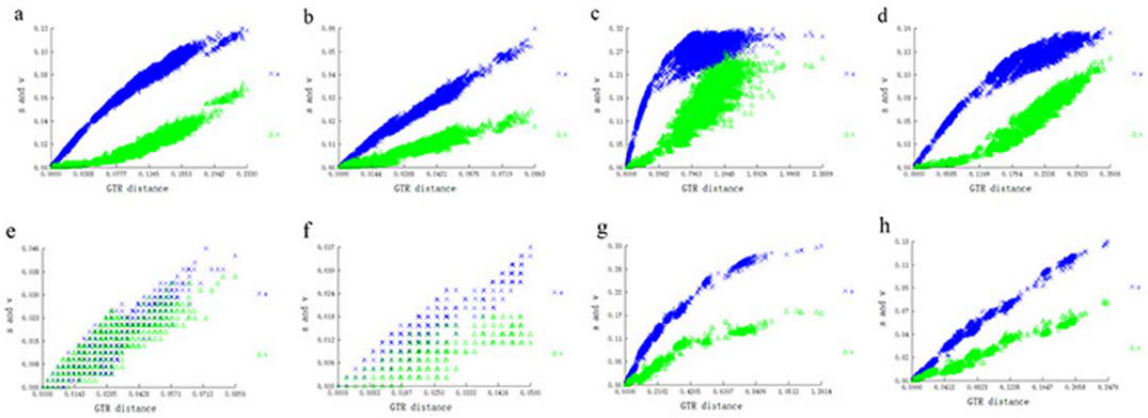
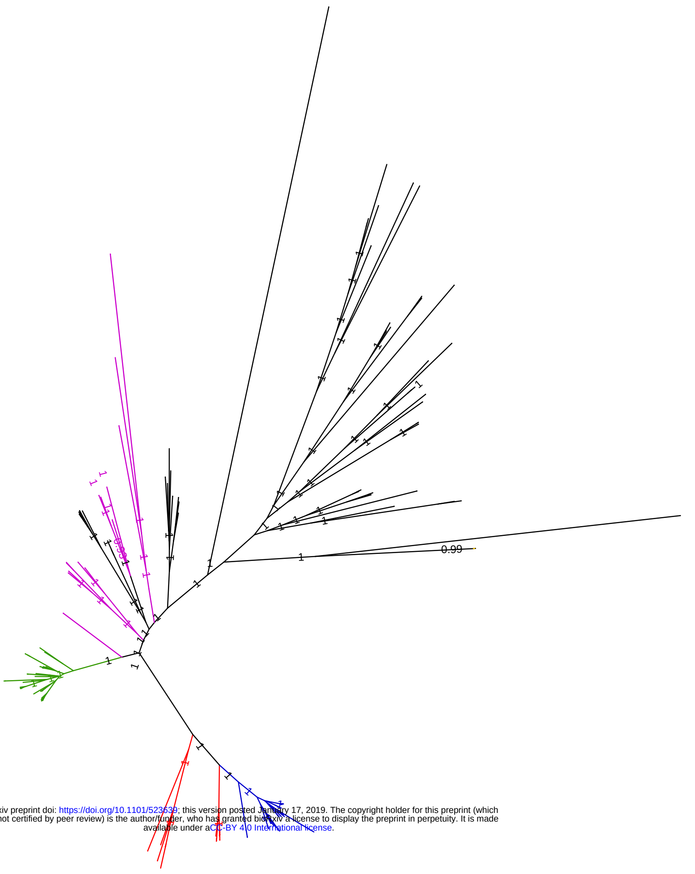
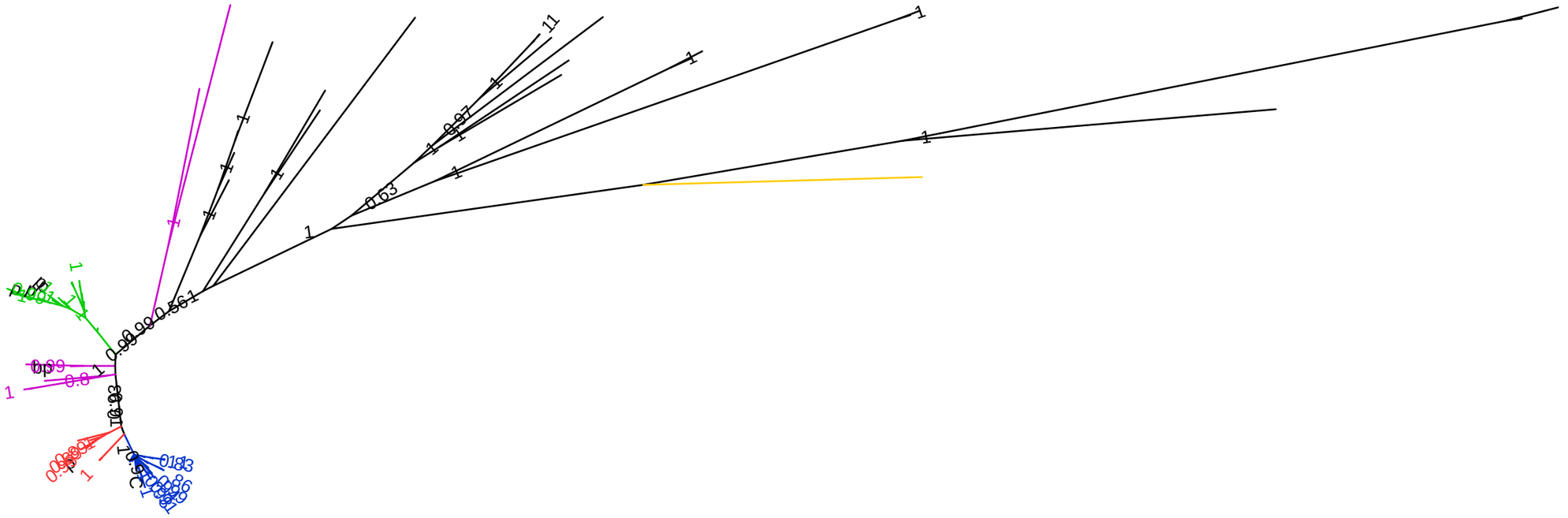
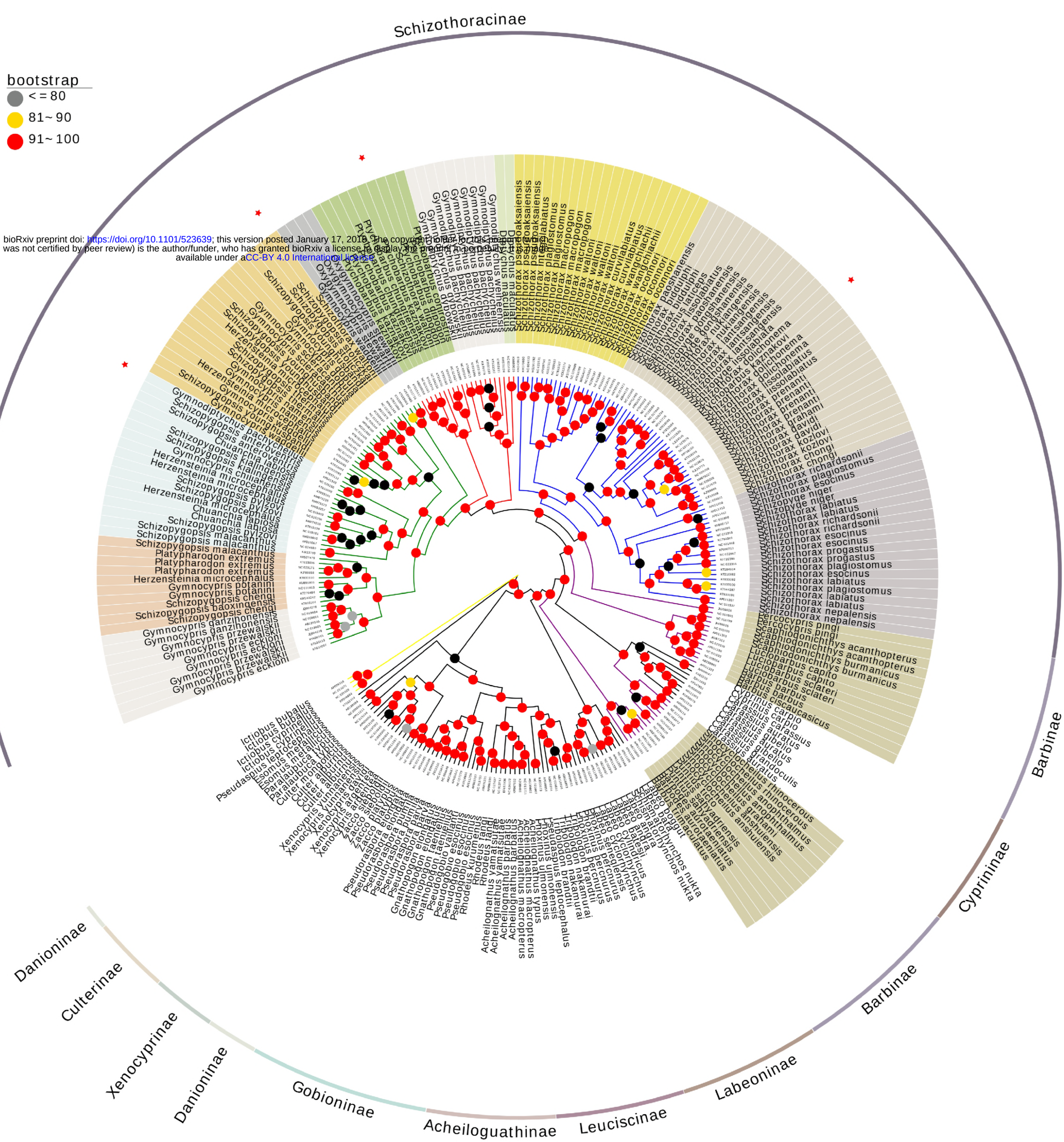
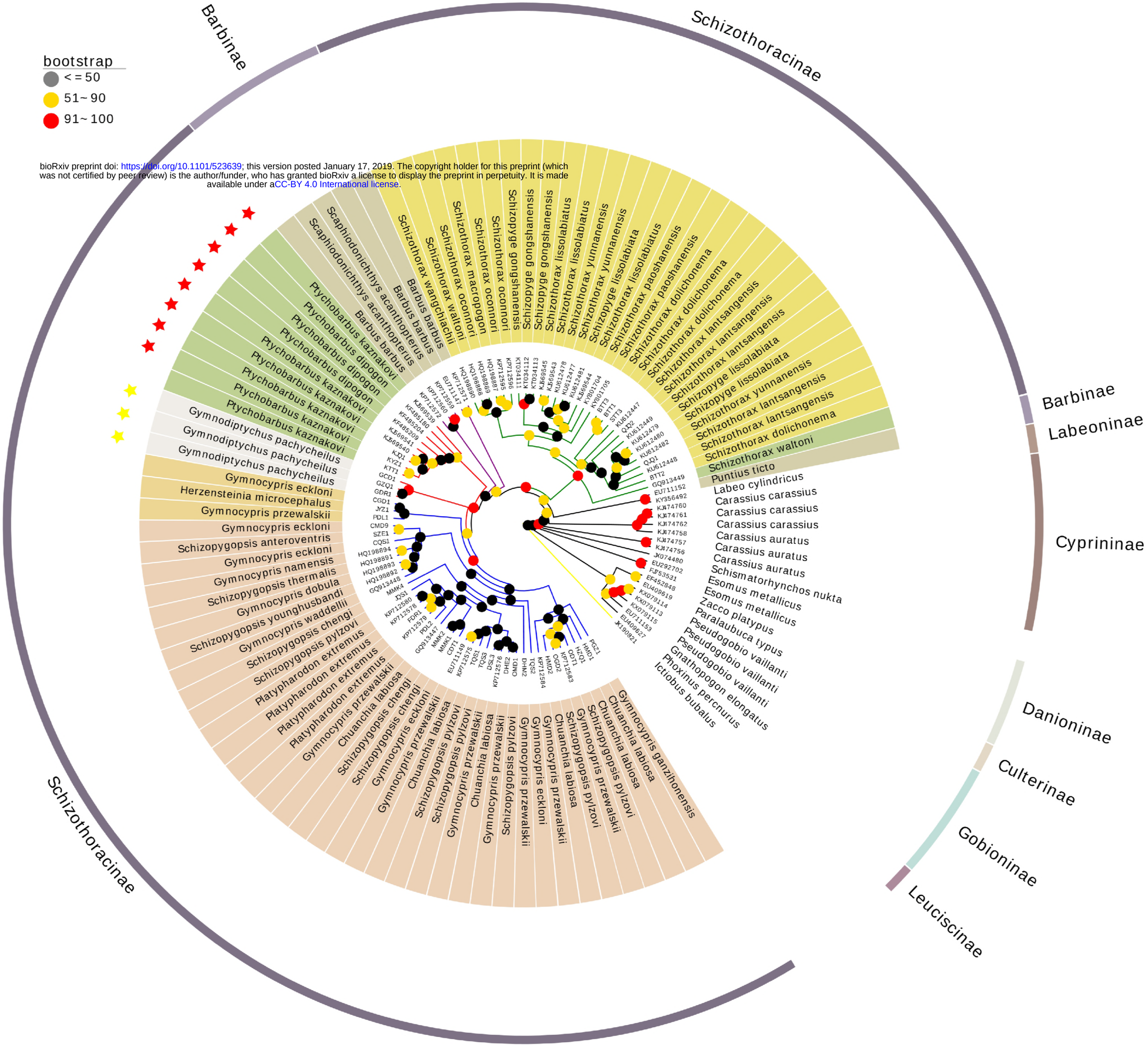


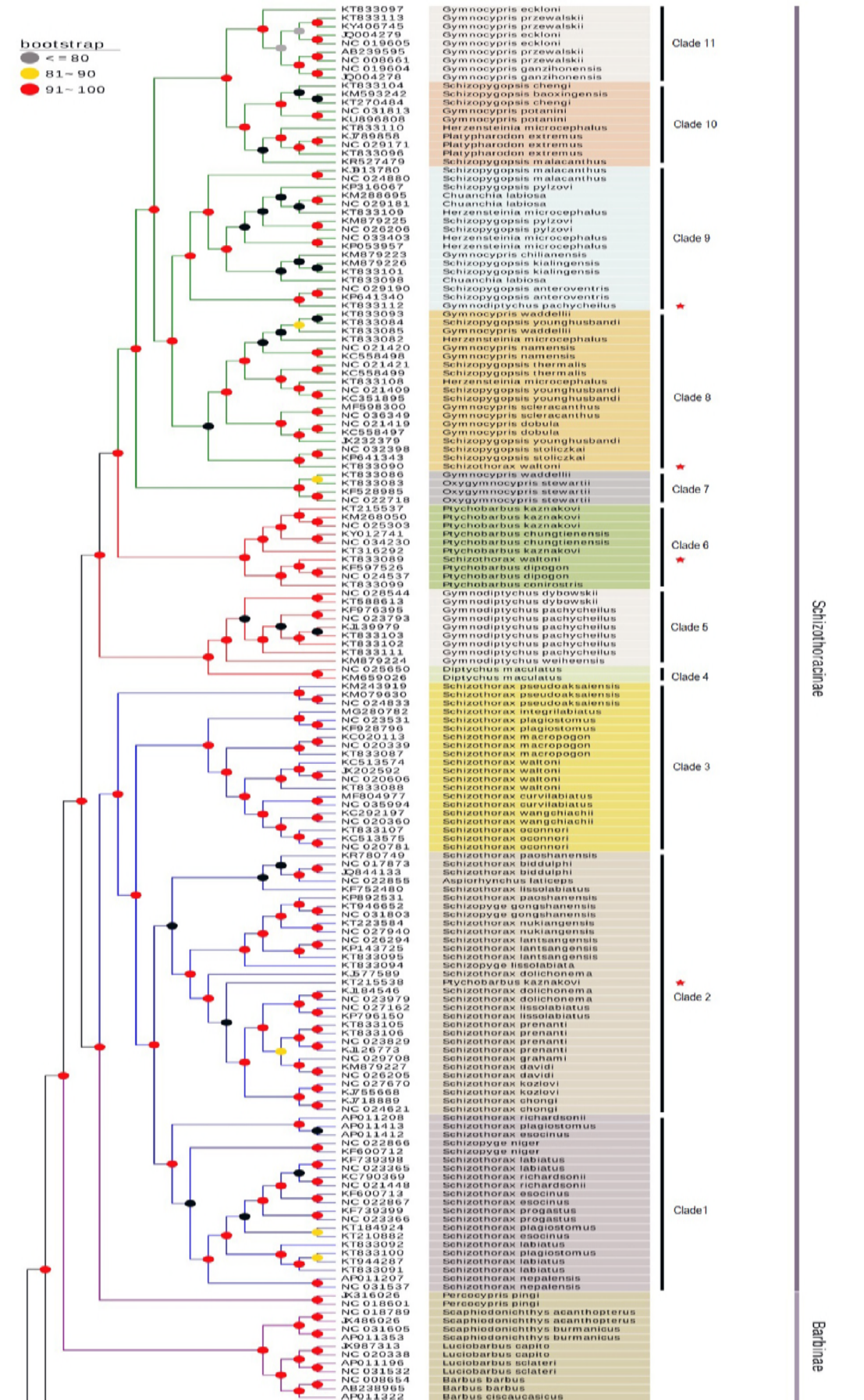
Fig 1 The saturation analysis of both the mitochondrial protein coding genes (excluding ND6 and 12SrRNA) sequences and RAG1 gene sequences based on GTR model. Blue part represent the , green part represent the . Fig a, b, c, and d represent saturation of codon1, codon2 codon3 and complete codon of 12 protein-coding genes, respectively; Fig e, f, g, and h represent saturation of the same part of RAG1 gene.










bootstrap
<= 80
81~90
91~100
KT833097 Gymnocypris eckloni
KT833113 Gymnocypris przewalskii
KY406745 Gymnocypris przewalskii
JQ004279 Gymnocypris eckloni
NC 019605 Gymnocypris eckloni
AB239595 Gymnocypris przewalskii
NC 008661 Gymnocypris przewalskii
NC 019604 Gymnocypris ganzihonensis
JQ004278 Gymnocypris ganzihonensis
KT833104 Schizopygopsis chengi
KM593242 Schizopygopsis baoxingensis
KT270484 Schizopygopsis chengi
NC 031813 Gymnocypris potanini
KU896808 Gymnocypris potanini
KT833110 Herzensteinia microcephalus
KJ789858 Platypharodon extremus
NC 029171 Platypharodon extremus
KT833096 Platypharodon extremus
KR527479 Schizopygopsis malacanthus
KJ913780 Schizopygopsis malacanthus
NC 024880 Schizopygopsis malacanthus
KP316067 Schizopygopsis pylzovi
KM288695 Chuanchia labiosa
NC 029181 Chuanchia labiosa
KT833109 Herzensteinia microcephalus
KM879225 Schizopygopsis pylzovi
NC 026206 Schizopygopsis pylzovi
NC 033403 Herzensteinia microcephalus
KP053957 Herzensteinia microcephalus
KM879223 Gymnocypris chilianensis
KM879226 Schizopygopsis kialingensis
KT833101 Schizopygopsis kialingensis
KT833098 Chuanchia labiosa
NC 029190 Schizopygopsis anteroventris
KP641340 Schizopygopsis anteroventris
KT833112 Gymnodiptychus pachycheilus
KT833093 Gymnocypris waddellii
KT833084 Schizopygopsis younghusbandi
KT833085 Gymnocypris waddellii
KT833082 Herzensteinia microcephalus
NC 021420 Gymnocypris namensis
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NC 021421 Schizopygopsis thermalis
KC558499 Schizopygopsis thermalis
KT833108 Herzensteinia microcephalus
NC 021409 Schizopygopsis younghusbandi
KC351895 Schizopygopsis younghusbandi
MF598300 Gymnocypris scleracanthus
NC 036349 Gymnocypris scleracanthus
NC 021419 Gymnocypris dobula
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JX232379 Schizopygopsis younghusbandi
NC 032398 Schizopygopsis stoliczkai
KP641343 Schizopygopsis stoliczkai
KT833090 Schizothorax waltoni
KT833086 Gymnocypris waddellii
KT833083 Oxygymnocypris stewartii
KF528985 Oxygymnocypris stewartii
NC 022718 Oxygymnocypris stewartii
KT215537 Ptychobarbus kaznakovi
KM268050 Ptychobarbus kaznakovi
NC 025303 Ptychobarbus kaznakovi
KY012741 Ptychobarbus chungtienensis
NC 034230 Ptychobarbus chungtienensis
KT316292 Ptychobarbus kaznakovi
KT833089 Schizothorax waltoni
KF597526 Ptychobarbus dipogon
NC 024537 Ptychobarbus dipogon
KT833099 Ptychobarbus conirostris
NC 028544 Gymnodiptychus dybowskii
KT588613 Gymnodiptychus dybowskii
KP976398 Gymnodiptychus pachycheilus
NC 023793 Gymnodiptychus pachycheilus
KJ139979 Gymnodiptychus pachycheilus
KT833103 Gymnodiptychus pachycheilus
KT833102 Gymnodiptychus pachycheilus
KT833111 Gymnodiptychus pachycheilus
KM879224 Gymnodiptychus weiheensis
NC 025650 Diptychus maculatus
KM659026 Diptychus maculatus
KM243919 Schizothorax pseudoaksaiensis
KM079630 Schizothorax pseudoaksaiensis
NC 024833 Schizothorax pseudoaksaiensis
MG280782 Schizothorax integrilabiatus
NC 023531 Schizothorax plagiostomus
KF928796 Schizothorax plagiostomus
KC020113 Schizothorax macropogon
NC 020339 Schizothorax macropogon
KT833087 Schizothorax macropogon
KF513574 Schizothorax waltoni
JX202592 Schizothorax waltoni
NC 020606 Schizothorax waltoni
KT833088 Schizothorax waltoni
MF804977 Schizothorax curvilabiatus
NC 035994 Schizothorax curvilabiatus
KC292197 Schizothorax wangchiachii
NC 020360 Schizothorax wangchiachii
KT833107 Schizothorax oconnori
KC513575 Schizothorax oconnori
NC 020781 Schizothorax oconnori
KR780749 Schizothorax paoshanensis
NC 017873 Schizothorax biddulphi
JQ841133 Schizothorax biddulphi
NC 022855 Aspiorhynchus laticeps
KF752480 Schizothorax lissolabiatus
KP892531 Schizothorax paoshanensis
KT946652 Schizopyge gongshanensis
NC 031803 Schizopyge gongshanensis
KT223584 Schizothorax nukiangensis
NC 027940 Schizothorax nukiangensis
NC 026294 Schizothorax lantsangensis
KP134725 Schizothorax lantsangensis
KT833095 Schizothorax lantsangensis
KT833094 Schizopyge lissolabiata
KJ577589 Schizothorax dolichonema
KT215538 Ptychobarbus kaznakovi
KJ184546 Schizothorax dolichonema
NC 023979 Schizothorax dolichonema
NC 027162 Schizothorax lissolabiatus
KP796150 Schizothorax lissolabiatus
KT833105 Schizothorax prenanti
KT833106 Schizothorax prenanti
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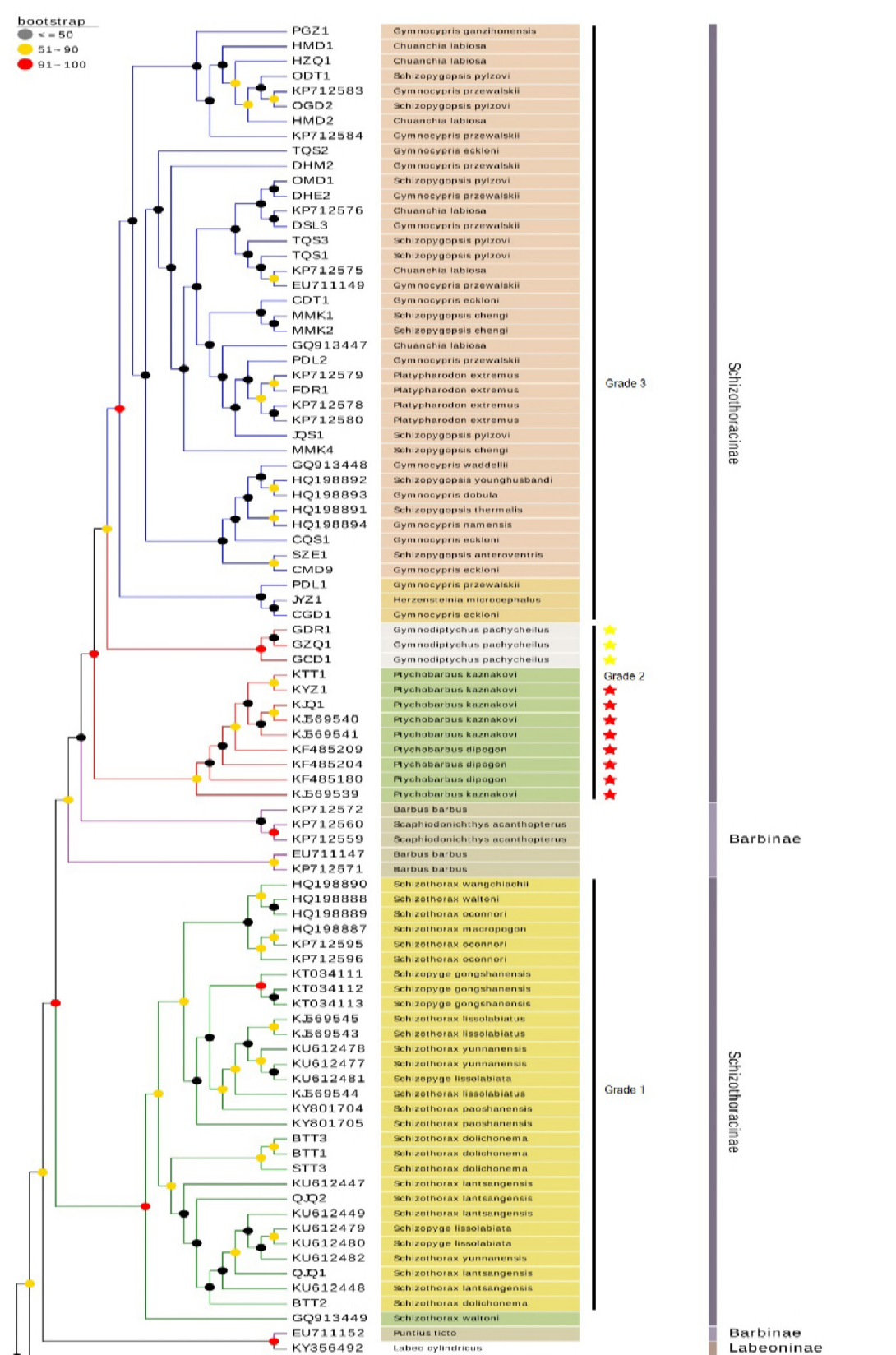
KJ126773 Schizothorax prenanti
NC 029708 Schizothorax grahami
KM879227 Schizothorax davidi
NC 026205 Schizothorax davidi
NC 027670 Schizothorax kozlovi
KJ755668 Schizothorax kozlovi
KJ718889 Schizothorax chongi
NC 024621 Schizothorax chongi
AP011208 Schizothorax richardsonii
AP011413 Schizothorax plagiostomus
AP011412 Schizothorax esocinus
NC 022866 Schizopyge niger
KF600712 Schizopyge niger
KF739398 Schizothorax labiatus
NC 023365 Schizothorax labiatus
KC790369 Schizothorax richardsonii
NC 021448 Schizothorax richardsonii
KF600713 Schizothorax esocinus
NC 022867 Schizothorax esocinus
KF739399 Schizothorax progastus
NC 023366 Schizothorax progastus
KT184924 Schizothorax plagiostomus
KT210882 Schizothorax esocinus
KT833092 Schizothorax labiatus
KT833100 Schizothorax plagiostomus
KT944287 Schizothorax labiatus
KT833091 Schizothorax labiatus
AP011207 Schizothorax nepalensis
NC 031537 Schizothorax nepalensis
JX316026 Percocypris pingi
NC 018601 Percocypris pingi
NC 018789 Scaphiodonichthys acanthopterus
JX486076 Scaphiodonichthys acanthopterus
NC 031605 Scaphiodonichthys burmanicus
AP011353 Scaphiodonichthys burmanicus
JX987313 Luciobarbus capito
NC 020338 Luciobarbus capito
AP011196 Luciobarbus sclateri
NC 031532 Luciobarbus sclateri
NC 008654 Barbus barbus
AB238965 Barbus barbus
AP011222 Barbus ciscaucasicus
Clade 11
Clade 10
Clade 9
Clade 8
Clade 7
Clade 6
Clade 5
Clade 4
Clade 3
Clade 2
Clade1
Schizothoracinae
Barbinae


bootstrap
<= 50
51 - 90
91 - 100
PGZ1 Gymnocypris ganzihonensis
HMD1 Chuanchia labiosa
HZQ1 Chuanchia labiosa
ODT1 Schizopygopsis pylzovi
KP712583 Gymnocypris przewalskii
OGD2 Schizopygopsis pylzovi
HMD2 Chuanchia labiosa
KP712584 Gymnocypris przewalskii
TQS2 Gymnocypris eckloni
DHM2 Gymnocypris przewalskii
OMD1 Schizopygopsis pylzovi
DHE2 Gymnocypris przewalskii
KP712576 Chuanchia labiosa
DSL3 Gymnocypris przewalskii
TQS3 Schizopygopsis pylzovi
TQS1 Schizopygopsis pylzovi
KP712575 Chuanchia labiosa
EU711149 Gymnocypris przewalskii
CDT1 Gymnocypris eckloni
MMK1 Schizopygopsis chengi
MMK2 Schizopygopsis chengi
GQ913447 Chuanchia labiosa
PDL2 Gymnocypris przewalskii
KP712579 Platypharodon extremus
FDR1 Platypharodon extremus
KP712578 Platypharodon extremus
KP712580 Platypharodon extremus
JQS1 Schizopygopsis pylzovi
MMK4 Schizopygopsis chengi
GQ913448 Gymnocypris waddellii
HQ198892 Schizopygopsis younghusbandi
HQ198893 Gymnocypris dobula
HQ198891 Schizopygopsis thermalis
HQ198894 Gymnocypris namensis
CQS1 Gymnocypris eckloni
SZE1 Schizopygopsis anteroventris
CMD9 Gymnocypris eckloni
PDL1 Gymnocypris przewalskii
JYZ1 Herzensteinia microcephalus
CGD1 Gymnocypris eckloni
GDR1 Gymnodiptychus pachycheilus
GZQ1 Gymnodiptychus pachycheilus
GCD1 Gymnodiptychus pachycheilus
KTT1 Ptychobarbus kaznakovi
KYZ1 Ptychobarbus kaznakovi
KJQ1 Ptychobarbus kaznakovi
KJ569540 Ptychobarbus kaznakovi
KJ569541 Ptychobarbus kaznakovi
KF485209 Ptychobarbus dipogon
KF485204 Ptychobarbus dipogon
KF485180 Ptychobarbus dipogon
KJ569539 Ptychobarbus kaznakovi
KP712572 Barbus barbus
KP712560 Scaphiodonichthys acanthopterus
KP712559 Scaphiodonichthys acanthopterus
EU711147 Barbus barbus
KP712571 Barbus barbus
HQ198890 Schizothorax wangchiachii
HQ198888 Schizothorax waltoni
HQ198889 Schizothorax oconnori
HQ198887 Schizothorax macropogon
KP712595 Schizothorax oconnori
KP712596 Schizothorax oconnori
KT034111 Schizopyge gongshanensis
KT034112 Schizopyge gongshanensis
KT034113 Schizopyge gongshanensis
KJ569545 Schizothorax lissolabiatus
KJ569543 Schizothorax lissolabiatus
KU612478 Schizothorax yunnanensis
KU612477 Schizothorax yunnanensis
KU612481 Schizopyge lissolabiata
KJ569544 Schizothorax lissolabiatus
KY801704 Schizothorax paoshanensis
KY801705 Schizothorax paoshanensis
BTT3 Schizothorax dolichonema
BTT1 Schizothorax dolichonema
STT3 Schizothorax dolichonema
KU612447 Schizothorax lantsangensis
QJQ2 Schizothorax lantsangensis
KU612449 Schizothorax lantsangensis
KU612479 Schizopyge lissolabiata
KU612480 Schizopyge lissolabiata
KU612482 Schizothorax yunnanensis
QJQ1 Schizothorax lantsangensis
KU612448 Schizothorax lantsangensis
BTT2 Schizothorax dolichonema
GQ913449 Schizothorax waltoni
EU711152 Puntius ticto
KY356492 Labeo cylindricus
Grade 3
Grade 2
Grade 1
Schizothoracinae
Barbinae
Schizothoracinae
Barbinae
Labeoninae