

Phylogenetic relationships of Nemacheilidae cavefish (*Heminoemacheilus*, *Oreonectes*, *Yunnanilus*, *Paranemachilus*, and *Troglonectes*) revealed by analysis of mitochondrial genome and seven nuclear genes

DEAR EDITOR,

Cave loaches within the family Nemacheilidae are among the most diverse group of cavefish in southwestern China. Although certain species have been included in previous phylogenetic studies, the intergeneric and interspecific relationships of Chinese cave loaches in Nemacheilidae remain poorly investigated due to insufficient sampling. In this study, a total of 45 samples from 37 recognized species and two unidentified species of cave loaches were collected, accounting for 87.5% of the eight recognized genera of cave loaches within the family Nemacheilidae in China. The complete mitochondrial genome (mitogenome) and seven nuclear genes were sequenced, and the phylogenetic tree of Chinese cave loaches was reconstructed. Both Bayesian inference and maximum-likelihood analyses resolved the phylogenetic relationships at the generic and species levels, suggesting the effectiveness of this multilocus marker system in determining phylogenetic relationships in Chinese cave loaches. Phylogenetic analysis not only confirmed previous taxonomic hypotheses based on morphological data but also provided new insights into the relationships of many cave loaches at the genus and species levels as well as suggestions for the current taxonomy of cave loaches within the family Nemacheilidae.

The karst regions of southwestern China are considered the center of Chinese cavefish biodiversity, boasting the most concentrated distribution of cavefish in the world. However, many cavefish species in the region remain poorly known and highly threatened (IUCN, 2022). Nemacheilidae, which contains approximately 22 genera and 269 species, constitutes one of the largest and most diverse families, second only to Cyprinidae in terms of diversity (Zhang et al., 2020). These cave loaches, which include both stygophilic and stygobitic species, are restricted in their distribution to the karst regions of southwestern China and exhibit highly specialized morphologies (Lan et al., 2013; Li, 2018). This has led to inadequately resolved taxonomies and enigmatic phylogenetic relationships among cave groups. The presence of both subterranean and surface-dwelling phenotypes among

these genera, as well as the influence of convergent evolution, make it challenging to distinguish them based on morphological characteristics alone. The classification of Chinese cave loaches has been the subject of debate, with various taxonomic schemes proposed based on morphological and molecular analyses. However, classification remains controversial and unresolved, particularly with regards to the genera *Oreonectes* and *Troglonectes* (Supplementary Table S1) (Du et al., 2008; Tang, 2012; Zhang & Zhao, 2016). Initially, Du et al. (2008) divided the genus *Oreonectes* into the *platycephalus* group (rounded or truncated caudal-fin) and the *furcicaudalis* group (forked caudal-fin) based on caudal-fin shape. Kottelat (2012), with reference to the original description of the caudal-fin shape and dorsal-fin origin clearly in front of the pelvic-fin origin, suggested that species with a forked caudal fin should not belong to the genus *Oreonectes*. Tang (2012) suggested a new genus for species with a forked caudal-fin. This taxonomic suggestion was accepted by Zhang & Zhao (2016) and *Troglonectes* Zhang, Zhao & Tang, 2016 was established with *O. furcicaudalis* as the type species. Later, the classification of certain species was revised based on morphological and molecular data (Supplementary Table S1). Currently, Chinese cave loaches in this family include 83 species in eight genera, i.e., *Heminoemacheilus* (five species), *Homatula* (two species), *Oreonectes* (six species), *Paranemachilus* (three species), *Schistura* (two species), *Troglonectes* (19 species), *Triplophysa* (32 species), and *Yunnanilus* (14 species) (Supplementary Table S2). However, the representative samples used in these genetic marker-based classification studies were too small and restricted to the mitogenome, and their classification requires further investigation.

In this study, we revealed the phylogenetic relationships of Chinese cave loaches in the family Nemacheilidae based on the most extensive geographic and taxonomic sampling to date (Supplementary Figure S1) as well as phylogenetic analyses of the mitogenome and seven nuclear genes (Supplementary Tables S3, S4), which should improve our

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understanding of the taxonomy, diversity, and evolution of Chinese cave loaches. Phylogenetic trees were reconstructed using maximum-likelihood (ML) and Bayesian inference (BI) methods for the mitogenome and nuclear genes under best partitioning schemes and evolutionary models (Supplementary Table S5). Genetic distances between species were also calculated. Further details regarding molecular analysis are described in the Supplementary Materials and Methods.

Phylogenetic analysis indicated that the genera *Heminoemacheilus*, *Yunnanilus*, *Oreonectes*, *Paranemachilus*, and *Troglonectes* were not monophyletic (Figure 1; Supplementary Figures S2, S3). Within *Yunnanilus*, *Oreonectes*, *Triplophysa* (Tr.), *Troglonectes* (T.), and *Heminoemacheilus*, *Y. retrodorsalis* was nested within *Troglonectes*; *Y. jinxiensis* and *H. zhengbaoshani* were nested within *Paranemachilus*; and *O. acridorsalis*, *H. parvus*, and *O. anophthalmus* formed a monophyletic group with strong support. *Yunnanilus* and *Oreonectes* were each divided into two highly supported clades, namely, *Yunnanilus* (I), *Yunnanilus* (II), *Oreonectes* (I), and *Oreonectes* (II), respectively (Figure 1; Supplementary Figure S2).

Three species delimitation methods based on sequence differences, i.e., Assemble Species by Automatic Partitioning (ASAP), Bayesian implementation of the Poisson Tree Processes (bPTP), and Automatic Barcode Gap Discovery (ABGD), were used for *Micronemacheilus*, *Oreonectes* (I), *Oreonectes* (II), *Paranemachilus*, *Troglonectes*, *Triplophysa*, *Yunnanilus* (I), and *Yunnanilus* (II), resulting in the identification of 31, 31.27 (29–35), and 28 species, respectively (Supplementary Figures S4, S5 and Tables S6, S7). ASAP and bPTP merged several recognized species into a single species, i.e., *T. elongatus*, *T. jiarongensis*, and *T. dongganensis*, and *T. donglanensis*, *T. duanensis*, *T. translucens*, *T. macrolepis*, and *T. microphthalmus*, but split *O. platycephalus* and *M. pulcherrimus* into two species (Supplementary Figures S4, S5). Notably, ABGD merged *Y. jiuchiensis* and *Y. pleurotaenia* into a single species and *H. zhengbaoshani* and *P. pingguoensis* into a single species (Supplementary Figures S4, S5 and Table S6). Within the Chinese cave loaches, the genetic distances between several recognized species were very small, i.e., 0.25% (*T. jiarongensis* vs. *T. elongatus*), 0.41% (*T. jiarongensis* vs. *T. dongganensis*), and 0.84% (*T. macrolepis* and *T. microphthalmus*) (Supplementary Table S8).

Remarks: Based on the most comprehensive phylogenetic tree of cave loaches in the family Nemacheilidae to date, several suggestions are made for their classification. Within the family Nemacheilidae, *Heminoemacheilus* (type species *H. zhengbaoshani*) was originally considered a monotypic genus defined by the absence of head scalation and postcleithrum (vs. *Paranemachilus*), long barbels, and incomplete lateral line (vs. *Traccatichthys*) (Lan et al., 2013; Zhu & Cao, 1987). However, our phylogenetic results revealed only minimal genetic differences between *H. zhengbaoshani* and *Paranemachilus* samples and *H. parvus* and *Oreonectes* (I) samples. Furthermore, the type species of *Heminoemacheilus* (*H. zhengbaoshani*) was nested within the genus *Paranemachilus*, indicating that *Heminoemacheilus* may be invalid. Thus, according to Article 23 of the International Code of Zoological Nomenclature “Principle of Priority”, the validity of *Heminoemacheilus* Zhu & Cao, 1987 is negated, becoming a junior synonym of *Paranemachilus* Zhu, 1983 (ICZN, 2022). These findings suggest that the presence or absence of head

scalation is not a useful criterion for recognizing distinct genera. Currently, the revised genus *Paranemachilus* consists of four species, i.e., *P. genilepis*, *P. pingguoensis*, *P. jinxiensis*, and *P. zhengbaoshani*.

The monophyly of *Oreonectes* was rejected and needs to be revised. Genetically, *Oreonectes* was initially divided into three clades, i.e., Clade D (*Oreonectes* (II)), Clade G (*Oreonectes* (I)), and Clade A (*Troglonectes*). Clade D represents true *Oreonectes*, given the presence of the type species (*O. platycephalus*) in the genus. Within Clade G, *O. acridorsalis*, *H. parvus*, and *O. anophthalmus* were entangled with each other. To accommodate these species, we suggest that they be merged and established into a new genus named *Karstsinnectes* Zhou, Luo, Wang, Zhou & Xiao, gen. nov (Figure 1; Supplementary Figure S2 and Appendix I). However, this new genus cannot be diagnosed and described until more specimens are available. For the time being, *Oreonectes sensu stricto* contains *O. guananensis*, *O. guilinensis*, *O. luochengensis*, *O. platycephalus*, and *O. polystigmus*.

Both morphological and molecular data failed to support the monophyly of *Yunnanilus* (Figure 1; Du et al., 2021). In the phylogenetic tree, *Y. jiuchiensis* and *Y. pleurotaenia* from Yunnan formed a sister clade with (*Paranemachilus*+*Troglonectes*), named *Yunnanilus* (II). *Yunnanilus bailianensis* and *Y. longibarbatus* from Guangxi formed a distinct evolutionary clade, named *Yunnanilus* (I). The invalidation of *Heminoemacheilus* (see above) indicates that these two species no longer belong to *Yunnanilus* (Lan et al., 2013) or *Heminoemacheilus* (Du et al., 2021). Based on their distinct morphological characters, i.e., anterior and posterior nostrils closely set (vs. slightly separated in *Micronemacheilus*) and significant geographic segregation (Supplementary Figure S6), we propose the establishment of a new genus to accommodate *Y. bailianensis* and *Y. longibarbatus*. However, given the current confusing taxonomy of *Yunnanilus* (Du et al., 2021), additional samples are needed in future studies. We also note that the *Y. nigromaculatus* sample (GenBank accession No. MW532081) used by Du et al. (2021) may not belong to *Yunnanilus* (Supplementary Figure S3) but may instead be more closely related to the family Catostomidae. Thus, potentially misidentified samples should be considered when recognizing the monophyly of *Eonemachilus* to provide more accurate taxonomic suggestions.

Despite the identification of *Troglonectes* as monophyletic, its membership and species validity require further confirmation. Several taxonomic schemes have been proposed for *Troglonectes* and its species based on morphology (Du et al., 2008; Kottelat, 2012; Li, 2018; Tang, 2012; Zhang & Zhao, 2016; Supplementary Table S2). However, the placement of *Y. retrodorsalis* has raised questions concerning the validity and monophyly of *Troglonectes*. Based on the previous records (Lan et al., 1995; Du et al., 2008) and our phylogenetic tree, we propose that *Y. retrodorsalis* be merged into *Troglonectes* to preserve the monophyly of *Troglonectes* (Figure 1). While we concur with the assignments of *T. donglanensis*, *T. duanensis*, *T. lihuensis*, and *T. dongganensis* at the genus level by Li (2018), i.e., from the genera *Oreonectes* and *Triplophysa* to the genus *Troglonectes*, we propose a different taxonomy based on the phylogenetic tree (Figure 1), genetic distance (Supplementary Table S8), species delimitation

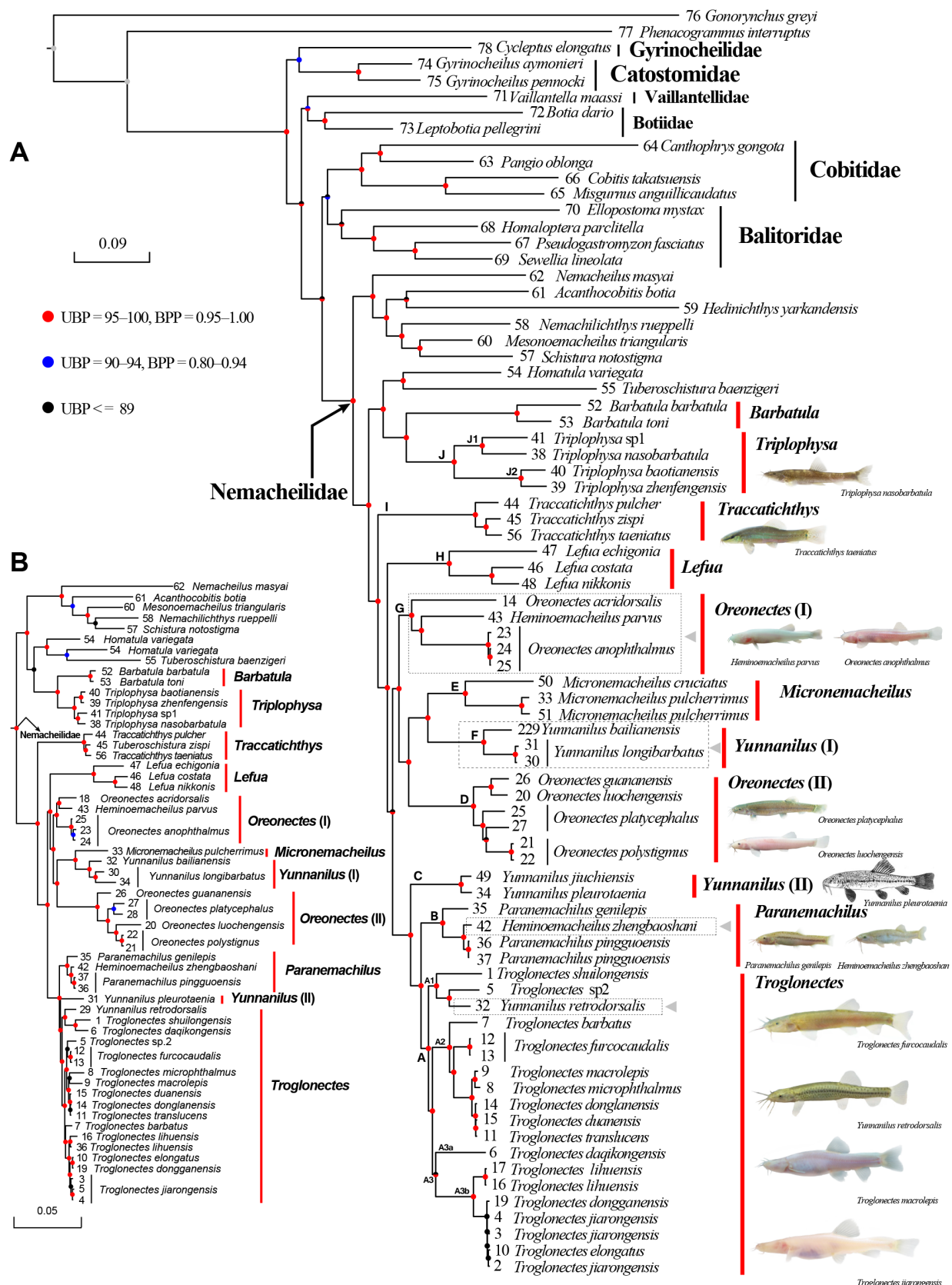


Figure 1 Phylogenetic relationships of Chinese cave loaches and outgroups

A, B: Bayesian inference (BI) and maximum-likelihood (ML) tree estimated from mitogenome (A) and combined nuclear data (B, seven genes). In this phylogenetic tree, Bayesian posterior probabilities (BPP) from BI analysis/ultrafast bootstrap support (UBP) from ML analysis are shown in different colored circles on nodes. Scale bar represents 0.09/0.05 nucleotide substitutions per site. Gray boxes and triangles indicate species to be reassigned. Numbers at terminals in A and B correspond to IDs in Supplementary Table S4. Photographs of loaches were taken by Tao Luo, Jia-Hu Lan, and modified from Zhu (1989).

(Supplementary Table S7), and morphological comparisons (Wang, 2022). Notably, we suggest that *T. donglanensis* and *T. duanensis* are either synonyms of *T. translucens* or two local populations. The original descriptions of *T. elongatus*, *T. jiarongensis*, and *T. dongganensis* were published in April 2012 (online electronic versions were available on 4 November 2011), July 2012, and July 2013, respectively. Therefore, in accordance with the priority guidelines of ICZN (2022), we suggest that *T. jiarongensis* and *T. dongganensis* are synonyms of *T. elongatus* based on the lack of significant genetic or morphological differences (Wang, 2022) (Figure 1; Supplementary Table S8). Given the morphological differences between *T. elongatus* and the remaining two species (Lan et al., 2013; Li, 2018), interspecific relationships may need further resolution. Our analysis also rejects the suggestion of Li (2018) that *T. daqikongensis* is a synonym of *Homatula maolanensis*. Based on the original publication information and examination of specimens, *T. daqikongensis* can be morphologically distinguished from *Homatula maolanensis* based on the following characters: dorsal-fin rays (iii, 9–10 vs. iii, 6), anal-fin rays (iii, 6–7 vs. ii, 4), pectoral-fin rays (i, 10–12 vs. i, 13), pelvic-fin rays (i, 6–7 vs. i, 5), and caudal-fin branched rays (13–16 vs. 15) (Li et al., 2006; Wang, 2022). Furthermore, *T. daqikongensis* is located in the Xiaoqikong west scenic area of the Zhangjiang River, while *Homatula maolanensis* is located in the Maolan National Nature Reserve east of the Zhangjiang River. Although bPTP, ABGD, and ASAP merged *T. macrolepis* and *T. microphthalmus* as a single species, we conservatively consider these two species as independent valid species given their distinct morphological characteristics and deep nuclear genetic differences. Currently, the revised genus *Troglonectes* consists of 15 species, i.e., *T. barbatus*, *T. daqikongensis*, *T. elongatus*, *T. furcocaudalis*, *T. hechiensis*, *T. huanjiangensis*, *T. lihuensis*, *T. lingyunensis*, *T. longibarbatus*, *T. macrolepis*, *T. maolanensis*, *T. microphthalmus*, *T. retradorsalis*, *T. shuilongensis*, and *T. translucens* (Li, 2018; Zhao et al., 2021).

For *Yunnanilus* (I) (including *Y. bailianensis* and *Y. longibarbatus* from Guangxi) and *Oreonectes* (I) (including *O. acridorsalis*, *O. anophthalmus*, and *H. parvus*), we propose the establishment of two new genera to accommodate these species. However, additional specimens need to be reviewed to obtain more detailed evidence, such as chromosomes, skeletons, and distinct morphologies.

In this study, we sequenced the mitogenome and seven nuclear genes from 45 samples representing 37 recognized species and two unidentified species of cave loaches within the family Nemacheilidae. We constructed the most comprehensive phylogenetic tree to date and resolved and estimated the phylogenetic relationships at the genus level as well as the taxonomy and validity of certain species of Chinese cave loaches. These results are important for further research on the origin, evolution, adaptative radiation, and conservation of Chinese cavefish.

Taxonomy

***Karstsinnectes* Zhou, Luo, Wang, Zhou & Xiao, gen. nov.**

Type species: *Oreonectes anophthalmus* Zheng, 1981.

Diagnosis : (1) body naked, scaleless, and body color pattern absent; (2) eyes absent; (3) lips with furrows but no papillae; (4) anterior and posterior nostrils slightly separated, anterior nostril tube long, without elongated short barbel-like tip;

(5) without longitudinal stripe; (6) dorsal fin with 7 branched rays, dorsal-fin origin slight posterior to ventral-fin origin; (7) caudal fin forked or rounded, caudal peduncle with adipose crests; (8) cheeks scaleless; (9) lateral line and cephalic lateral-line canals present; (10) bony capsule of swim bladder open posteriorly.

Etymology: The genus name *Karstsinnectes* is a combination of words. Karst is an English word meaning an area of water-eroded limestone; sin is an abbreviation of the Greek word Sino, refers to Chinese; nectes, a Greek word meaning swimmer. We suggest its English common name “Chinese Karst Loach Genus” and Chinese common name “Zhōng Huá Kā Qiū Shǔ (中华喀鳅属)”.

Distribution: Currently, this genus includes four species, i.e., *Karstsinnectes acridorsalis* (Lan, 2013) **comb. nov.**, *Karstsinnectes anophthalmus* (Zheng, 1981) **comb. nov.**, *Karstsinnectes hyalinus* (Lan, Yang & Chen, 1996) **comb. nov.**, and *Karstsinnectes parvus* (Zhu & Zhu, 2014) **comb. nov.**, all of which distributed in the Hongshuihe, Zuojiang, and Youjiang river basins in Guangxi, China.

NOMENCLATURAL ACTS REGISTRATION

The electronic version of this article in portable document format represents a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new name contained in the electronic version are effectively published under that Code from the electronic edition alone (See Articles 8.5–8.6 of the Code). This published work and the nomenclatural acts it contains have been registered in ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information can be viewed through any standard web browser by appending the LSID to the prefix <http://zoobank.org/>. Publication LSID:

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SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

All samples were obtained following Chinese regulations for the Implementation of the Protection of Terrestrial Wild Animals (State Council Decree (1992) No. 13) and the Guidelines for the Care and Use of Laboratory Animals by the Ethics Committee at Guizhou Normal University (Guiyang, Guizhou, China).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.Z. and T.L. conceived and designed the research; T.L., Y.L.W., Q.Y., N.X., J.J.Z., and H.Q.D. conducted field surveys and collected samples; T.L., Y.L.W., Q.Y., and L.W. performed molecular work; T.L., Y.L.W., Q.Y., J.J.Z., and J.Z. wrote, discussed, and revised the manuscript. All authors read and approved the final version of the manuscript.

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Tao Luo^{1,2,#}, Qin Yang^{2,#}, Li Wu^{2,#}, Ya-Li Wang¹, Jia-Jun Zhou^{3,4}, Huai-Qing Deng¹, Ning Xiao^{5,*}, Jiang Zhou^{2,*}

¹ School of Life Sciences, Guizhou Normal University, Guiyang, Guizhou 550025, China

² School of Karst Science, Guizhou Normal University, Guiyang, Guizhou 550001, China

³ Zhejiang Forest Resource Monitoring Center, Hangzhou, Zhejiang 310020, China

⁴ Zhejiang Forestry Survey Planning and Design Company Limited, Hangzhou, Zhejiang 310020, China

⁵ Guiyang Healthcare Vocational University, Guiyang, Guizhou 550081, China

[#]Authors contributed equally to this work

*Corresponding authors, E-mail: armiger@163.com; zhoujiang@ioz.ac.cn

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