Evolving in the darkness: phylogenomics of *Sinocyclocheilus* cavefishes highlights recent diversification and cryptic diversity

Tingru Mao^{1,†}, Yewei Liu^{1,†}, Mariana M. Vasconcellos², Marcio R. Pie³, Gajaba Ellepola¹, Chenghai Fu¹, Jian Yang⁴, Madhava Meegaskumbura^{1,*}

¹Guangxi Key Laboratory for Forest Ecology and Conservation, College of Forestry, Guangxi University, Nanning, Guangxi, P.R.C.

²Programa de Pós-Graduação em Ecologia. Universidade Federal do Rio de Janeiro. Rio de Janeiro - RJ. Brazil

³Departamento de Zoologia, Universidade Federal do Paraná, Brazil, 81531-980

⁴Key Laboratory of Environment Change and Resource Use, Beibu Gulf, Nanning Normal University, Nanning, Guangxi, P.R.C.

[†] These authors contributed equally

Corresponding author *E-mail*: madhava_m@mac.com

ABSTRACT

Trogglomorphism—any morphological adaptation enabling life to the constant darkness of caves, such as loss of pigment, reduced eyesight or blindness, over-developed tactile and olfactory organs—has long intrigued biologists. However, inferring the proximate and ultimate mechanisms driving the evolution of troglomorphy in freshwater fish requires a sound understanding of the evolutionary relationships between surface and troglomorphic lineages. We use Restriction Site Associated DNA Sequencing (RADseq) to better understand the evolution of the *Sinocyclocheilus* fishes of China. With a remarkable array of derived troglomorphic traits, they comprise the largest cavefish diversification in the world, emerging as a multi-species model system to study evolutionary novelty. We sequenced a total of 120 individuals throughout the *Sinocyclocheilus* distribution. The data comprised a total of 646,497 bp per individual, including 4378 loci and 67,983 SNPs shared across a minimum of 114 individuals at a given locus. Phylogenetic analyses using either the concatenated RAD loci (RAxML) or the SNPs under a coalescent model (SVDquartets, SNAPP) showed a high degree of congruence with similar topologies and high node support (> 95 for most nodes in the phylogeny). The major clades recovered conform to a pattern previously established using Sanger-based mt-DNA sequences, with a few notable exceptions. We now recognize six major clades in this group, elevating the blind cavefish *S. tianlinensis* and the micro-eyed *S. microphthalmus* as two new distinct clades due to their deep divergence from other clades. PCA plots of the SNP data also supports the recognition of six major clusters of species congruent with the identified clades based on the spatial arrangement and overlap of the species in the PC space. A Bayes factor delimitation (BFD) analysis showed support for 21 species, recognizing 19 previously described species and two putative new cryptic ones. Two species whose identities were previously disputed, *S. furcodorsalis* and *S. tianeensis*, are supported here as distinct species. In addition, our multi-species calibrated tree in SNAPP suggests that the genus *Sinocyclocheilus* originated around 10.5 Mya, with most speciation events occurring in the last 2 Mya, likely favored by the uplift of the Qinghai-Tibetan Plateau and cave occupation induced by climate-driven aridification during this period. These results provide a firm basis for future comparative studies on the evolution of *Sinocyclocheilus* and its adaptations to cave life.

KEYWORDS: Phylogenomics, RADseq, diversification, cavefish, species delimitation, introgression

1. INTRODUCTION

Many animal lineages across the world, ranging from flatworms (Leal-Zanchet et al., 2014) to fish and amphibians (Hutchison, 1958), have evolved to live in caves. In order to occupy cave subterranean habitats, each of these lineages had to adapt to extreme conditions of low availability of food, oxygen, and light, leading to the repeated acquisition of one or more adaptations, including elongated appendages, lowered metabolism, specialized sensory systems, and loss of eyes and pigmentation (Jeffery, 2019). The concerted evolution of these traits across different taxa, giving rise to convergent troglomorphic forms, comprise a great example of the process of natural selection in response to cave-associated selective regimes (Borowsky, 2010; Jeffery et al., 2010; Klaus et al., 2013; Porter et al., 2003). Though large troglomorphic radiations are rare – mainly because of the limited extent of cave habitats – the cyprinid fish genus *Sinocyclocheilus* shows an exceptional diversity of cave adaptations including at least three independent origins of cave-adapted phenotypes (Mao et al., 2021).

With 75 known species, *Sinocyclocheilus* includes the largest radiation of cavefish in the world, which diversified across Karstic habitats associated with the Li River in the Guangxi,

Guizhou, and Yunnan provinces of China (Jiang et al., 2019; Zhao and Zhang, 2009). The troglomorphic adaptations of *Sinocyclocheilus* include the development of a specialized sensory system, such as degeneration or complete loss of eyes, degeneration of scales, loss of pigmentation, expansion of pectoral fins, evolution of ‘horns’ and enhancement of the neuromast system (He et al., 2013; Li et al., 2020; Ma et al., 2020; Meng et al., 2013). This wealth of troglomorphic adaptations makes *Sinocyclocheilus* an important multi-species model system to study evolutionary novelty in response to selection (Chen et al., 2009; Meng et al., 2013; Xiao et al., 2005; Yang et al., 2016). Yet, this requires a robust time-calibrated phylogeny for this group including several independent markers across their genome, which is currently missing.

Our current understanding of the phylogenetic relationships within *Sinocyclocheilus*, based solely on mitochondrial DNA (mtDNA), revolves around the recognition of four main clades, A, B, C, and D (Mao et al., 2021), sometimes also referred to as the “jii”, “angularis”, “cyphotergous”, and “tingi” groups (Li and He, 2009; Xiao et al., 2005; Zhao and Zhang, 2009). These are sequential clades rather than reciprocally monophyletic units. The earliest emerging clade (Clade A) is restricted to Guangxi, at the eastern fringes of the genus distribution. Clades B and C have overlapping distributions restricted to the middle of the genus distribution, and species of Clade D are found mostly in the lotic habitats associated with hills to the west. In addition, Clade B encompasses most species with extensive troglomorphic traits such as the complete loss of eyes (blind) and well-formed forehead protrusions (horns). The genus is thought to have originated during the Miocene-Pliocene with cave occupation taking place predominantly during the Pliocene-Pleistocene transition in response to an uplift event and aridification of the Qinghai-Tibetan Plateau (Mao et al., 2021).

Nearly all of the molecular studies to date on the phylogenetic relationships within *Sinocyclocheilus* have been based on mtDNA (Chen et al., 2018; Jiang et al., 2019; Liang et al., 2011), limiting some of the diversification-scale analyses as well as insights into the population-level processes that shape this remarkable radiation. This is an important limitation, given that the uniparental nature of mtDNA inheritance might obscure important events of admixture in the history of *Sinocyclocheilus* while hampering the inference of species limits given that it represents only a single genetic locus. With the advent of next-generation sequencing, particularly highly efficient methods for recovering thousands of orthologous loci such as Restriction-site associated DNA sequencing (Cariou et al., 2013), we can infer the relationships within *Sinocyclocheilus* with higher accuracy, as well as its species limits, ancestral admixture, and diversification events.

The main goals of our study were: (1) to infer the evolutionary relationships and divergence times within *Sinocyclocheilus* through phylogenomic methods; (2) to assess the level of genetic support for the currently recognized species in the genus using Bayesian species delimitation methods; and (3) to investigate possible ancestral admixture events among *Sinocyclocheilus* species. In short, we were able to build a well-resolved tree for the genus despite recognizing some introgression events, to confirm the distinctiveness of 19 previously described species, and to recognize two additional cryptic species. Finally, we estimate that the most recent common ancestor (MRCA) of *Sinocyclocheilus* originated around 10.5 Mya, which is considerably older than previous estimates based on mitochondrial data alone.

2. METHODS

2.1 Taxon sampling and laboratory work

To elucidate the phylogenetic relationships of *Sinocyclocheilus* using multiple nuclear genomic markers, we carried out a detailed phylogenomic analysis of the group. We collected samples of 120 individuals in Guangxi, Yunnan and Guizhou provinces of China, including 19 previously recognized species and 2 unidentified species of *Sinocyclocheilus* (Fig. S1). Given the rarity of these species, only fin-clips were collected from all individuals and frozen immediately at -85 °C until DNA extraction. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocols. Electrophoresis was performed to ensure DNA integrity of the samples prior to genomic library preparation.

We employed a RAD sequencing protocol (Baird et al., 2008), which involves the use of a single restriction enzyme to cut genomic DNA at specific sites, using molecular barcodes to identify each individual and build a genomic library of short fragments distributed across the entire genome of all individuals. Each DNA sample was digested using the EcoRI enzyme and ligated to a P1 Illumina adapter at the compatible ends of the fragments. This adapter allows forward amplification with Illumina sequencing primers containing a 6-bp long nucleotide barcode for sample identification. The barcoded adapter-ligated fragments from all individuals were subsequently pooled, randomly sheared, and size-selected. DNA was then ligated to a second P2 adapter, a Y-shaped adapter with divergent ends. Finally, fragment sizes of 200-400 bp and 400-600 bp were isolated for library construction using a MinElute Gel Extraction kit (Qiagen). We quantified DNA concentration in a Qubit2.0, and checked the quality (insert size) of genomic libraries in an Agilent 2100. qPCR was also performed to detect the effective concentration of libraries (if > 2nM) in the appropriate insert size. Final genomic libraries were then sequenced on an Illumina HiSeq2500 platform generating 125-bp paired-end reads.

2.2 RADseq data assembly

Our pre-processing bioinformatics of the sequenced genomic library consisted of the following: first, the raw reads of fastq format were filtered through in-house scripts to optimize read number and to reduce artifacts within the data assembly. In this step, clean reads were obtained by removing low-quality reads and reads containing adapter sequence or poly-N from the raw data. In addition, the number of reads from each RAD site were tracked, and RAD sequences above a certain threshold were removed since highly repetitive sequences most likely represent paralogs. Single mismatch derivatives of these highly repetitive RAD sequences were also removed. Finally, low frequency RAD sequences below a threshold (of ≤ 5) were also removed from further analysis, as the associated paired-end reads would lack sufficient coverage for accurate genotyping (SNP calling).

We processed the clean RADseq reads using the ipyrad 0.9.56 pipeline assembly (Eaton and Overcast, 2020), which is well suited for downstream phylogenetic analyses (Leaché et al., 2015). Since there was no suitable reference genome, we used a *de novo* method to assemble the R1 clean reads. During demultiplexing, restriction sites were trimmed from all reads. All individuals had deep sequencing and high-quality assembly, hence all individuals were retained for downstream analyses. Given the genus *Sinocyclocheilus* is tetraploid (Li and Guo, 2020), we increased the depth of coverage of retained loci to 10x, for accurate genotyping and unbiased heterozygosity. Thus, the maximum number of alleles in the individual consensus sequence was set to 4, to allow more allele combinations within each individual. We set the clustering threshold to 90% similarity and allowed trimmed reads of at least 75bp to proceed in the assembly. In addition, datasets with various minimum numbers

of individuals per locus were filtered (96, 102, 108, 114 and 120, corresponding to 80–100% of individual coverage). Other parameters in ipyrad were set to default values.

Demultiplexed, unfiltered reads for the RAD data pertaining to this study can be accessed through the NCBI GenBank Short Read Archive (PRJNA764266).

2.3 Phylogenomic analyses

We used the concatenated sequences from all loci of the m114 matrix (8.44% missing data), corresponding to at least 95% individual coverage across loci to infer phylogenetic relationships among all samples. Phylogenomic relationships of all *Sinocyclocheilus* samples were inferred based on the maximum likelihood analysis of a matrix with 1,560,414 characters under a GTRCAT model (Stamatakis, 2014). We also inferred the species tree using the unlinked SNP dataset of the m114 matrix (with a single SNP sampled from each locus) in SVDquartets (Chifman and Kubatko, 2014) implemented in PAUP* 4.0a (Wilgenbusch and Swofford, 2003). SVDquartets estimates the relationships among taxa under the coalescent model by inferring splits among quartets of randomly sampled taxa. All possible taxa quartets (8,214,570 quartets) were evaluated and node support was estimated with 100 bootstrap replicates. The species tree inferred in SVDquartets can assist in identifying incomplete lineage sorting (ILS) or introgression among species in nodes with low support. All phylogenies were visualized using FigTree v1.4.3.

2.4 Population cluster analyses

We used a principal component analysis (PCA) to downscale genomic differences among individuals into the first two principal components and visualize the differences between populations. The PCA was based on the .snps.hdf file generated by ipyrad after assembling the data using the ipyrad.pca tool (Eaton and Overcast, 2020). Prior to the analysis, we performed the following treatments: (1) Assigning samples to populations based on the phylogenetic relationships using the imap dictionary. (2) Filtering SNP data using the minmap dictionary to ensure that 50%, 60%, 70%, 80%, 90% of samples have data in each group. (3) Inputting missing data using the "sample" algorithm. Each RAD locus was randomly subsampled to a single SNP to reduce the effect of linkage on the results. After filtering, 3,459, 2,942, 1,993, 987, and 165 SNPs were included in the analysis.

2.5 Species delimitation method

We performed Bayes factor species delimitation analyses using BFD* (Leaché et al., 2014). This method computes the marginal likelihood of species trees inferred with the software SNAPP using the Path Sampling approach in BEAST2 (Bouckaert et al., 2014). The method can be used to compare different species delimitation models using genome-wide SNP data in a multispecies coalescent framework. Due to computational limitations, we did not analyze all 120 samples simultaneously. We conducted three independent analyses of species delimitation (Table 1): the first including *S. cf. guanyangensis* with the other three closely related taxa in clade A (26 samples), the second including *S. cf. longibarbatulus* with the other four closely-related taxa in clade C (28 samples), and the third including *S. furcodorsalis* with the other 4 closely-related taxa in clade B (26 samples). The data were filtered to include no missing data among selected samples (Table 1). The parameters for SNAPP were set following Leaché and Bouckaert (2018) for the BFD* analyses (Leaché and Bouckaert, 2018). We conducted a path sampling for a total of 48 steps (MCMC length = 100,000, pre-burnin = 10,000) to calculate the marginal likelihood estimation (MLE) for all models, ranking them by comparing the size of the Bayes Factors across different models.

2.6 Divergence time estimation

We inferred divergence times for the *Sinocyclocheilus* using the MSC model in SNAPP, building a species tree of unlinked SNPs following Stange et al (2018), as this algorithm can compensate for ascertainment bias introduced by the exclusion of invariable sites (Bryant et al., 2012; Stange et al., 2018). As running the complete data set was computationally challenging, we selected a single representative per species (21 terminal clades) and reassembled them in ipyrad (2,602 unlinked SNPs for 21 individuals). We then used the Ruby script `snapp_prep.rb` to prepare the XML input for SNAPP (available at https://github.com/mmatschiner/snapp_prep). For time calibration, we used the time inferred by Liang et al (2011) to the split between *S. donglanensis* and its sister group *S. lingyunensis* at approximately 1.32 Mya (SD:1.02, 95%CI: 0.08-3.91) (Liang et al., 2011). We then ran an independent SNAPP analysis with 20 million MCMC generations, sampling at every 2,000 steps. We checked for stationarity and convergence of chains in TRACER v1.7.1 (ESS>200). A maximum clade credibility tree was generated with TreeAnnotator discarding the first 10% of each MCMC chain as a burn-in. The program FigTree v1.4.3 was used to visualize the summary tree.

2.7 Tests of introgression

To understand the causes of conflicting phylogenetic signals in the species and gene trees of *S. tianlinensis*, *S. yishanensis*, and *S. macrophthalmus*, we used Treemix from the ipyrad analysis toolkit inferring instances of ancestral gene flow (Pickrell and Pritchard, 2012). Prior to the Treemix analysis, the dataset was processed and filtered in the same way as in the principal component analysis, obtaining 3,459 SNPs. We tested the number of migration edges (m) in the range of 1 to 10, estimating their likelihood score to determine the appropriate value for migration edge in this data. Adding additional admixture edges will always improve the likelihood score, but with diminishing returns as you add additional edges that explain little variation in the data (Kim et al., 2021; Popovic et al., 2020).

3. RESULTS

3.1. RADseq dataset

The RADseq genomic libraries of the 120 individuals of *Sinocyclocheilus* yielded approximately 9.8 million reads per individual on average, of which 99.91% were retained after the filtering step of the assembly (supplementary table S1). We selected the parameter combination Sino_m114 (supplementary table S2) for phylogenetic analyses as it had the optimal combination of loci-clustering parameters for *Sinocyclocheilus* that maximized the fraction of variable sites that were phylogenetically informative. The data comprised a total of 646,497 bp, including 4,378 loci and 67,983 SNPs shared across at least 114 individuals (95% individual coverage at a given locus), of which 61,023 SNPs were parsimony informative for phylogenetic analyses (Sino_m114; supplementary table S2).

3.2 Phylogenetic reconstruction of RADseq data

The maximum likelihood analysis of the concatenated RADseq loci in RAxML and the coalescent-based SVDquartets analysis of unlinked SNP data recovered similar phylogenetic relationships among the 19 known *Sinocyclocheilus* species with a high degree of support (> 95) for most nodes (Fig. 1, Fig. 2). As expected, the bootstrap support values at a few nodes of the tree obtained by the coalescent SVDquartets analysis were slightly lower than those obtained by the concatenated analysis (Fig. 1). The two nodes with low bootstrap support in the SVDquartets phylogeny were: the one separating *S. macrophthalmus* and *S. yishanensis* (= 74), and that corresponding to the MRCA of *S. macrophthalmus*, *S. yishanensis*, *S. lingyunensis*, *S. donglanensis* (= 55) (Fig. 2), which suggests some uncertainty in the placement of *S. macrophthalmus* and *S. yishanensis*. Our phylogenetic analyses (Figs. 1-2, A–F) recovered *Sinocyclocheilus* as consisting of six major clades, four of which has been recognized before (Clades A, B, C, and D – Mao et al. 2021). *Sinocyclocheilus tianlinensis*, recognized as a new Clade E, is recovered as the sister species to Clades B, C, D, and F, which is not consistent with the topology of the mtDNA phylogeny (Mao et al., 2021). Likewise, *S. microphthalmus*, recognized as a new Clade F, is now recovered as the sister species to Clades C and D, which is also not congruent with previous phylogenies. Additional incongruences to previous mt-DNA based studies in the Clade C include: *S. longibarbus* recovered as the sister species to *S. xunlensis*, and *S. yishanensis* as the sister species to *S. macrophthalmus* (Mao et al., 2021). Interestingly, one incongruence also occurred across our trees inferred by different methods: *S. tianlinensis* had a different relationship with a somewhat low bootstrap value in the SNAPP tree (Fig. 3). This suggests conflicting signal, possibly due to introgression. Other than that, relationships across all analyses were congruent for all the remaining species.

3.3 Population clustering

We used PCA plots to summarize and visualize in two dimensions the genomic differences across all samples of *Sinocyclocheilus*. The first two principal components explained between 32.2% and 24.9% of the variation using different thresholds for data completeness (50% – 90%) (Fig. 4). The first two axes showed that samples clustered into six subgroups congruent with the major clades identified in our phylogenies.

3.4 Species delimitation

The results for all models tested with BFD* method are summarized in Table 1. Models A1 and C1 support the recognition of *S. cf. guanyangensis* (MLE= -2043.41) and *S. cf. longibarbus* (MLE= -1581.81) as separate species. In addition, whether *S. furcodorsalis* and *S. tianeensis* are the same species is also controversial (Liang et al., 2011; Zhao and Zhang,

2009). However, using the BFD* method, the model representing the current taxonomy showed a high Bayes Factor value (MLE= -1564.99), suggesting that *S. furcodorsalis* and *S. tianeensis* are indeed distinct species, supporting the current taxonomy.

3.5 Divergence times estimates

Our time-calibrated tree in SNAPP suggests that the crown age for the genus *Sinocyclocheilus* is around 10.5 My old. In an MSC model, *S. tianlinensis* and Clade B show a sister-group relationship (with probability of 0.77). In Clade C, *S. yishanensis* and *S. macrophthalmus* (probability 1.00), *S. lingyunensis* and *S. donglanensis* (probability 0.77) formed sub-clades. (Fig. 3). Compared with the concatenated ML and SVDquartets trees, the SNAPP tree showed a different topology with 5 major clades, including *S. tianlinensis* in Clade B.

3.6 Treemix analysis

The likelihoods for different migration boundaries increased steadily as more edges are included in the model (from 0 to 10). However, the likelihood did not improve significantly when the number of migration edges increased above 5. Treemix analyses of $m=0-5$ suggests the possibility of ancestral gene flow in *S. tianlinensis* and the ancestral species of *S. furcodorsalis* and *S. tianeensis*. Moreover, the possibility of gene flow also exists in the ancestors of *S. yishanensis*, *S. macrophthalmus* and *S. lingyunensis*, *S. donglanensis* (Fig. 5).

4. DISCUSSION

4.1 Phylogeny and cryptic species of *Sinocyclocheilus*

A total of 75 species of *Sinocyclocheilus* fish from China has been described up to now (Jiang et al., 2019), mainly based on morphology and mtDNA data. Nevertheless, some authors have questioned the validity of a few of those species (Liang et al., 2011; Zhao and Zhang, 2009) mainly due to their morphological similarities and incongruences across mt-DNA based phylogenies. For example, the validity of *S. furcodorsalis* and *S. tianeensis* has been questioned by several authors due to their close morphological resemblance (Liang et al., 2011). Such controversies have hindered drawing general conclusions concerning the evolution, ecology, conservation, and biogeography of *Sinocyclocheilus*. Therefore, this genus is in urgent need of a resolved a phylogeny and a clear taxonomy to support robust comparative analyses of the evolution of cave-adaptations in this group.

To this end, we tested the species boundaries in *Sinocyclocheilus* using RAD sequencing-based molecular species delimitation, which had found wide utility in previous evolutionary research (Herrera et al., 2016; Leaché et al., 2014; Rancilhac et al., 2019; Razkin et al., 2016). In addition, *Sinocyclocheilus* had never been subjected to a rigorous molecular species delimitation analysis before. Furthermore, while morphological changes in *Sinocyclocheilus* occur rapidly, molecular divergence, especially in regions under neutral evolution, is assumed to be limited. Therefore, we presumed that mt-DNA based molecular delimitation would not reflect accurate phylogenetic relationships in this group as clearly as the current study, based on a genome-scale sampling that also includes regions under selection. This increased genomic sampling may reflect more accurately the species tree in which *Sinocyclocheilus* evolved their specialized morphology for cave dwelling. Nevertheless, the major conclusions based on the mtDNA phylogeny of Mao et al. (2021) - that blind species independently evolved at least three times - is supported by this RAD-marker based phylogeny as well (Mao et al., 2021).

Trees based on RAXML, SVDquartets and SNAPP were for the most part congruent comprising highly supported nodes with only two nodes on the species trees with low

bootstrap support. These species tree methods (i.e. SVDquartets or SNAPP) analyze each SNP separately based on the coalescent theory, so they are expected to show lower bootstrap support whenever there is a conflicting signal among different SNPs in the dataset (Leaché et al., 2015). The tree generated with SNAPP also had a few inconsistencies with the other trees. In the SNAPP tree, *S. tianlinensis* is a sister species to Clade B while *S. donglanensis* and *S. lingyunensis* are sister species to Clade C. However, in the concatenated RAXML tree, *S. tianlinensis* is a sister species to Clades B, C, D, F, while *S. donglanensis*, *S. lingyunensis* are a sister clade to *S. macrophthalmus* and *S. yishanensis* in Clade C. We assume that this conflicting phylogenetic signal is either due to ancestral gene flow, introgression or incomplete lineage sorting. Treemix analysis suggests introgressive gene flow.

Previous evolutionary studies based on mitochondrial genes have shown that *Sinocyclocheilus* is divided into four major clades (Liang et al., 2011; Ma et al., 2019; Romero et al., 2009; Zhao and Zhang, 2009). However, the phylogenetic relationship obtained with RAD-seq analysis in the present study suggests that this group can be further divided into two additional branches, comprising six major clades (Fig. 1). The principal component analysis further supports this division by clustering the samples into six clusters which correspond to the six clades (Fig. 4). Previous mt-DNA based phylogenies (Mao et al., 2021) recognized *S. tianlinensis* and *S. microphthalmus* as species within clade B, but our phylogenomic results indicate that those two species actually belong to two independent clades due to their deep divergences in Clades B and C respectively.

Our results also support that *S. guanyangensis* and *S. cf. guanyangensis*, which resemble each other morphologically, are in fact two distinct species, the latter being a putative new species. The same is true for *S. longibarbatatus* and *S. cf. longibarbatatus* (a putative new species) as well. Field sampling indicated that these species pairs occur in different caves, but in close proximity to each other (see map in Fig. S1). Based on morphological and molecular data, it appears that different species of cave fish have repeatedly invaded different cave waters and acquired their own independent troglomorphic characteristics. This may be the case for these species as well. Geographical isolation followed by strong genetic drift among populations seems to be the main mechanism for the rapid formation of species in the genus *Sinocyclocheilus* (Ma et al., 2019; Yang et al., 2021; Zhao and Zhang, 2009). However, especially in small, isolated populations such as *S. cf. guanyangensis* and *S. cf. longibarbatatus*, genetic differentiation and species formation may occur even more rapidly due to the lack of gene flow among isolated populations. This mechanism, occurring under strong selective pressure associated with cave dwelling, may accelerate and amplify the rapid evolution of adaptive traits in other species of *Sinocyclocheilus* as well, resulting in a particularly rich species diversity with frequent evolution to cave-adapted morphologies.

Our genome-wide SNP analyses identified two putative new species of *Sinocyclocheilus* out of the 120 individuals sampled across 21 localities. Considering that our study did not include samples for all 75 species described so far, we suspect that cryptic diversity in the group could be potentially higher, i.e., the species diversity of the *Sinocyclocheilus* complex may be vastly underestimated.

4.2 Divergence time estimation and gene flow

The age of the most recent common ancestor (MRCA) of *Sinocyclocheilus* fish, inferred by our RADseq data, was estimated to be around 10.5 Mya, similar to that inferred by

previous mtDNA-based studies (Li et al., 2008; Liang et al., 2011; Mao et al., 2021). We also found that most divergence events occurred relatively recent in the history of the group (in the last 2 Mya) (Fig. 3). The *Sinocyclocheilus* MRCA possibly lived on the Yunnan-Guizhou Plateau during the late Tertiary. During the Quaternary, the Qinghai-Tibetan Plateau underwent an abrupt upturn, with major changes in the geological environment and a dramatic transformation of the Yunnan-Guizhou Plateau (Li and Fang, 1999; Shi et al., 1999). At the same time, global temperature began to fall and Northern Hemisphere ice caps grew (Hewitt, 2000; Ma et al., 2019; Svendsen et al., 2004). The MRCA of the *Sinocyclocheilus* radiation may have adapted to live in underground caves due to dramatic changes in the environment. The relatively late tectonic uplift of the Tibetan Plateau 3.6 Mya may have affected population dynamics of *Sinocyclocheilus* as well. For example, three species (*Sinocyclocheilus grahami*, *S. rhinoceros*, and *S. anshuiensis*) experienced two episodes of population declines during the two intense uplift phases (Qingzang movement: 3.6 Mya~1.7Mya, Kunhuang movement: 1.1Mya~0.6Mya) of the third tectonic uplift of the Qinghai-Tibet Plateau (Yang et al., 2016). Changes in population size and gene flow may be related to enhanced Asian monsoon and precipitation, with large-scale glacial activity resulting from these two phases considerably affecting diversification in this group. Therefore, the intense late tectonic activity in the Qinghai-Tibet Plateau, combined with environmental changes, could have promoted the rate of diversification in these cavefish during the last 2 My.

SNAPP is a multispecies coalescent method that assumes no gene flow among species. This may lead to inaccurate inference of topology in the presence of gene flow and may explain the incongruences among the SNAPP tree and the other trees. In the Treemix analysis, we found possible ancestral gene flow between some species of *Sinocyclocheilus*, which is likely responsible for the inconsistent phylogenetic signals described previously (Fig. 5). Subterranean river-capture events may have influenced this phenomenon. The geographical locations of the species showing possible ancestral genetic admixture are in close proximity and were possibly connected through underground networks, especially during the Pleistocene. With the upliftment of the Tibetan plateau during the early Pleistocene, many cave systems within the basin, which were interconnected previously, would have become completely or periodically isolated, preventing gene flow among populations in close proximity, giving rise to the patterns among sister taxa that we see today. Interestingly, gene flow is more prominent around the karstic northwestern regions of the Guangxi plains, especially in Clades B and C. Therefore, we can assume that the deeper caves and the subterranean river systems associated with the Guangxi karst region (Zhao and Zhang, 2009) were periodically interconnected, allowing limited gene flow. The deeper phylogenetic relationship of *S. tianlinensis* and the species in Clade B, suggests that they diverged during the Miocene. Furthermore, introgression is not apparent among the species from the hilly terrains of Yunnan plateau, suggesting that they inhabit isolated subterranean systems in these hills.

5. CONCLUDING REMARKS

In summary, our study showed that: 1) instead of the 4 major clades of *Sinocyclocheilus* previously recognized by phylogenetic relationships, the genus can be now categorized into 6 major clades; 2) the MRCA of *Sinocyclocheilus* appeared around 10.5 Mya coinciding with cave formation due to upliftment and dry conditions associated with the aridification of China during the late Miocene and the Pliocene; 3) the BFD* analyses support the hypothesis that the two cryptic species found in this study (*S. cf. longibarbatulus* and *S. cf. guanyangensis*) and the morphologically similar *S. tianeensis* and *S. furcodorsalis* are distinct species. We further draw attention to the fact that the diversity of the *Sinocyclocheilus*

species may be substantially underestimated due to their cryptic nature. Future studies using novel genomic techniques (such as RADseq or whole genome sequencing) could potentially unravel the true diversity of this remarkable radiation.

Abbreviations

RADseq: Restriction site-associated DNA sequencing; mt-DNA: Mitochondrial DNA; BFD: Bayes factor delimitation; Mya: Million years ago; ILS: Incomplete lineage sorting; PCA: Principal component analysis; SNP: Single-nucleotide polymorphism; MSC: Multispecies coalescent; MLE: Marginal likelihood estimate; ML: Maximum Likelihood

Acknowledgements

We thank Rohan Pethiyagoda (Australian Museum) and Mario van Gastel for suggestions to improve the paper. We thank the following for assistance in the field: Shipeng Zhou, Bing Chen, Dan Sun, Jayampathi Herath and Amrapali Rajput.

Authors' contributions

MM, MMV, MRP, TRM, YWL, conceptualized the research and designed the methodology. YWL, CHF, MTR, MM, GE, JY conducted fieldwork and curated the data. TRM, MMV, YWL, MRP, GE, carried out formal analysis. TRM, MMV, MM, MRP, GE, YWL, wrote the original draft. MM, MMV, MRP, and JY supervised MTR & YWL. MM and JY acquired funding. TMR, GE and YWL made figures. All authors reviewed and edited the draft. All authors read and approved the final manuscript.

Funding

Funding for this study is provided by (1) Guangxi University Startup Funding to MM for fieldwork, lab work, analyses and supporting TRM, YWL, CHF (2) National Natural Science Foundation of China (#31860600) to JY for fieldwork (3) Guangxi Natural Science Foundation (#2017GXNSFFA198010) to JY for research work. These funding bodies played no role in the design of the study and collection, analysis, and interpretation of data or in the writing of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included as supplementary information and the genetic data (Genbank) can be accessed upon acceptance of the paper. Please see the materials and methods section for the SRA project name for RAD data.

Ethics approval and permission

Methods of sampling approval by the Ethics Committee of Guangxi University. Field sampling approval through the Guangxi Provincial Government.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

6. REFERENCES

- Baird, N.A., Etter, P.D., Atwood, T.S., Currey, M.C., Shiver, A.L., Lewis, Z.A., Selker, E.U., Cresko, W.A., Johnson, E.A.J.P.o., 2008. Rapid SNP discovery and genetic mapping using sequenced RAD markers. 3, e3376.
- Borowsky, R.J.B.S.F.E.S.P., 2010. The evolutionary genetics of Cave fishes: convergence, adaptation and pleiotropy. 141-168.
- Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J.J.P.c.b., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. 10, e1003537.
- Bryant, D., Bouckaert, R., Felsenstein, J., Rosenberg, N.A., RoyChoudhury, A.J.M.b., evolution, 2012. Inferring species trees directly from biallelic genetic markers: bypassing gene trees in a full coalescent analysis. 29, 1917-1932.
- Cariou, M., Duret, L., Charlat, S.J.E., evolution, 2013. Is RAD - seq suitable for phylogenetic inference? An in silico assessment and optimization. 3, 846-852.
- Chen, S., Zhang, R., Feng, J., Xiao, H., Li, W., Zan, R., Zhang, Y.J.J.o.F.B., 2009. Exploring factors shaping population genetic structure of the freshwater fish *Sinocyclocheilus grahami* (Teleostei, Cyprinidae). 74, 1774-1786.
- Chen, Y.Y., Li, R., Li, C.Q., Li, W.X., Yang, H.F., Xiao, H., Chen, S.Y., 2018. Testing the validity of two putative sympatric species from *Sinocyclocheilus* (Cypriniformes: Cyprinidae) based on mitochondrial cytochrome b sequences. *Zootaxa* 4476, 130-140.
- Chifman, J., Kubatko, L.J.B., 2014. Quartet inference from SNP data under the coalescent model. 30, 3317-3324.
- Eaton, D.A., Overcast, I.J.B., 2020. ipyrad: Interactive assembly and analysis of RADseq datasets. 36, 2592-2594.
- He, Y., Chen, X.-Y., Xiao, T.-Q., Yang, J.-X., 2013. Three-dimensional morphology of the *Sinocyclocheilus hyalinus* (Cypriniformes: Cyprinidae) horn based on synchrotron X-ray microtomography.
- Herrera, S., Shank, T.M.J.M.P., Evolution, 2016. RAD sequencing enables unprecedented phylogenetic resolution and objective species delimitation in recalcitrant divergent taxa. 100, 70-79.
- Hewitt, G.J.N., 2000. The genetic legacy of the Quaternary ice ages. 405, 907-913.
- Hutchison, V.H.J.E.M., 1958. The distribution and ecology of the cave salamander, *Eurycea lucifuga*. 28, 2-20.
- Jeffery, W.R., 2019. *Astyanax mexicanus*: A vertebrate model for evolution, adaptation, and development in caves. *Encyclopedia of Caves*. Elsevier, pp. 85-93.
- Jeffery, W.R., Strickler, A.G., Trajano, E., Bichuette, M., Kapoor, B.J.B.o.S.F.E.S.P., 2010. Development as an evolutionary process in *Astyanax* cavefish. 141-168.
- Jiang, W.-S., Li, J., Lei, X.-Z., Wen, Z.-R., Han, Y.-Z., Yang, J.-X., Chang, J.-B.J.Z.r., 2019. *Sinocyclocheilus sanxiaensis*, a new blind fish from the Three Gorges of Yangtze River provides insights into speciation of Chinese cavefish. 40, 552.
- Kim, D., Bauer, B.H., Near, T.J.J.S.B., 2021. Introgression and Species Delimitation in the Longear Sunfish *Lepomis megalotis* (Teleostei: Percomorpha: Centrarchidae).
- Klaus, S., Mendoza, J.C., Liew, J.H., Plath, M., Meier, R., Yeo, D.C.J.B.I., 2013. Rapid evolution of troglomorphic characters suggests selection rather than neutral mutation as a driver of eye reduction in cave crabs. 9, 20121098.
- Leaché, A.D., Banbury, B.L., Felsenstein, J., De Oca, A.N.-M., Stamatakis, A.J.S.b., 2015. Short tree, long tree, right tree, wrong tree: new acquisition bias corrections for inferring SNP phylogenies. 64, 1032-1047.

- Leaché, A.D., Bouckaert, R.R., 2018. Species trees and species delimitation with SNAPP: a tutorial and worked example. Workshop on Population and Speciation Genomics, Český Krumlov.
- Leaché, A.D., Fujita, M.K., Minin, V.N., Bouckaert, R.R.J.S.b., 2014. Species delimitation using genome-wide SNP data. 63, 534-542.
- Leal-Zanchet, A.M., de Souza, S.T., Ferreira, R.L., 2014. A new genus and species for the first recorded cave-dwelling Cavernicola (Platyhelminthes) from South America. Zookeys, 1-15.
- Li, C., Chen, H., Zhao, Y., Chen, S., Xiao, H.J.E., evolution, 2020. Comparative transcriptomics reveals the molecular genetic basis of pigmentation loss in Sinocyclocheilus cavefishes. 10, 14256-14271.
- Li, J., Fang, X.J.C.S.B., 1999. Uplift of the Tibetan Plateau and environmental changes. 44, 2117-2124.
- Li, X., Guo, B.J.P.o.t.R.S.B., 2020. Substantially adaptive potential in polyploid cyprinid fishes: evidence from biogeographic, phylogenetic and genomic studies. 287, 20193008.
- Li, Z., Guo, B., Li, J., He, S., Chen, Y.J.C.S.B., 2008. Bayesian mixed models and divergence time estimation of Chinese cavefishes (Cyprinidae: Sinocyclocheilus). 53, 2342-2352.
- Li, Z., He, S.J.H., 2009. Relaxed purifying selection of rhodopsin gene within a Chinese endemic cavefish genus Sinocyclocheilus (Pisces: Cypriniformes). 624, 139-149.
- Liang, X.-F., Cao, L., Zhang, C.-g.J.E.b.o.f., 2011. Molecular phylogeny of the Sinocyclocheilus (Cypriniformes: Cyprinidae) fishes in northwest part of Guangxi, China. 92, 371-379.
- Ma, L., Zhao, Y., Yang, J.-x., 2019. Cavefish of China. Encyclopedia of caves. Elsevier, pp. 237-254.
- Ma, Z., Herzog, H., Jiang, Y., Zhao, Y., Zhang, D.J.I.z., 2020. Exquisite structure of the lateral line system in eyeless cavefish Sinocyclocheilus tianlinensis contrast to eyed Sinocyclocheilus macrophthalmus (Cypriniformes: Cyprinidae). 15, 314-328.
- Mao, T.-R., Liu, Y.-W., Meegaskumbura, M., Yang, J., Ellepola, G., Senevirathne, G., Fu, C.-H., Gross, J.B., Pie, M.R.J.B.e., evolution, 2021. Evolution in Sinocyclocheilus cavefish is marked by rate shifts, reversals, and origin of novel traits. 21, 1-14.
- Meng, F., Braasch, I., Phillips, J.B., Lin, X., Titus, T., Zhang, C., Postlethwait, J.H.J.M.b., evolution, 2013. Evolution of the eye transcriptome under constant darkness in Sinocyclocheilus cavefish. 30, 1527-1543.
- Pickrell, J., Pritchard, J.J.N.P., 2012. Inference of population splits and mixtures from genome-wide allele frequency data. 1-1.
- Popovic, I., Matias, A.M.A., Bierne, N., Riginos, C.J.E.A., 2020. Twin introductions by independent invader mussel lineages are both associated with recent admixture with a native congener in Australia. 13, 515-532.
- Porter, M.L., Crandall, K.A.J.T.i.E., Evolution, 2003. Lost along the way: the significance of evolution in reverse. 18, 541-547.
- Rancilhac, L., Goudarzi, F., Gehara, M., Hemami, M.-R., Elmer, K.R., Vences, M., Steinfarz, S.J.M.p., evolution, 2019. Phylogeny and species delimitation of near Eastern Neurergus newts (Salamandridae) based on genome-wide RADseq data analysis. 133, 189-197.
- Razkin, O., Sonet, G., Breugelmans, K., Madeira, M.J., Gómez-Moliner, B.J., Bäckeljau, T.J.M.P., Evolution, 2016. Species limits, interspecific hybridization and

- phylogeny in the cryptic land snail complex *Pyramidula*: the power of RADseq data. 101, 267-278.
- Romero, A., Zhao, Y., Chen, X., 2009. The hypogean fishes of China. *Chinese Fishes*. Springer, pp. 211-278.
- Shi, Y., Li, J., Li, B.J.A.G.S.-C.E.-. 1999. Uplift of the Qinghai-Xizang (Tibetan) plateau and east Asia environmental change during late Cenozoic. 54, 20-28.
- Stamatakis, A.J.B., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. 30, 1312-1313.
- Stange, M., Sánchez-Villagra, M.R., Salzburger, W., Matschiner, M.J.S.b., 2018. Bayesian divergence-time estimation with genome-wide single-nucleotide polymorphism data of sea catfishes (Ariidae) supports Miocene closure of the Panamanian Isthmus. 67, 681-699.
- Svendsen, J.I., Alexanderson, H., Astakhov, V.I., Demidov, I., Dowdeswell, J.A., Funder, S., Gataullin, V., Henriksen, M., Hjort, C., Houmark-Nielsen, M.J.Q.S.R., 2004. Late Quaternary ice sheet history of northern Eurasia. 23, 1229-1271.
- Wilgenbusch, J.C., Swofford, D.J.C.p.i.b., 2003. Inferring evolutionary trees with PAUP. 6.4. 1-6.4. 28.
- Xiao, H., Chen, S.-y., Liu, Z.-m., Zhang, R.-d., Li, W.-x., Zan, R.-g., Zhang, Y.-p.J.M.P., Evolution, 2005. Molecular phylogeny of *Sinocyclocheilus* (Cypriniformes: Cyprinidae) inferred from mitochondrial DNA sequences. 36, 67-77.
- Yang, J., Chen, X., Bai, J., Fang, D., Qiu, Y., Jiang, W., Yuan, H., Bian, C., Lu, J., He, S.J.B.b., 2016. The *Sinocyclocheilus* cavefish genome provides insights into cave adaptation. 14, 1-13.
- Yang, N., Li, Y., Liu, Z., Chen, Q., Shen, Y.J.E.B.o.F., 2021. Molecular phylogenetics and evolutionary history of *Sinocyclocheilus* (Cypriniformes: Cyprinidae) species within Barbinae in China. 1-14.
- Zhao, Y., Zhang, C., 2009. Endemic fishes of *Sinocyclocheilus* (Cypriniformes: Cyprinidae) in China-species diversity, cave adaptation, systematics and zoogeography. Beijing: Science Press.

MAIN TEXT TABLES AND FIGURES

Table 1. Description of the BFD* species delimitation models for the *Sinocyclocheilus* groups, including the model tested, the number of species, the MLE, the BF between that model and the model of current taxonomy, the model rank and the clade to different species models belong. Since the unidentified species are sister groups with *S. guanyangensis* and *S. longibarbatus*, we herein label them as *S. cf. guanyangensis* and *S. cf. longibarbatus*.

Model	Numbe rs of Species	MLE	Ra nk	BF	Cla de
Current taxonomy: (<i>S. guanyangensis</i> , <i>S. huangtianensis</i>), <i>S. guilinensis</i>)	3	-2044.0811	2	-	A
ModelA1: (((<i>S. guanyangensis</i> , <i>S. cf.</i> <i>guanyangensis</i>), <i>S. huangtianensis</i>), <i>S.</i> <i>guilinensis</i>)	4	-2043.4052	1	1.3517	A
ModelA2: (<i>S. huangtianensis</i> , <i>S. guilinensis</i>)	2	-2220.3862	3	352.610	A
Current taxonomy: (<i>S. longibarbatus</i> , <i>S. xunlensis</i>), (<i>S.</i> <i>huanjiangensis</i> , <i>S. brevis</i>))	4	-1608.1623	2	-	C
ModelC1: (((<i>S. longibarbatus</i> , <i>S. cf. longibarbatus</i>), <i>S. xunlensis</i>), (<i>S. huanjiangensis</i> , <i>S.</i> <i>brevis</i>))	5	-1581.8094	1	52.7057	C
ModelC2: (<i>S. brevis</i> , <i>S. huanjiangensis</i>), <i>S.</i> <i>xunlensis</i>)	3	-1691.0134	3	165.702	C
ModelC3: (<i>S. brevis</i> , <i>S. xunlensis</i>)	2	-1783.1874	4	350.050	C
Current taxonomy: (((<i>S. mshanensis</i> , <i>S. brevibarbatus</i>), <i>S.</i> <i>altishoulderus</i>), (<i>S. tianeensis</i> , <i>S.</i> <i>furcodorsalis</i>))	5	-1564.9927	1	-	B
ModelB1: (((<i>S. mshanensis</i> , <i>S. brevibarbatus</i>), <i>S.</i> <i>altishoulderus</i>), <i>S. furcodorsalis</i>)	4	-1572.4045	2	14.8236	B

ModelB2:					
((<i>S. brevibarbatus</i> , <i>S. altishoulderus</i>), <i>S.</i>					
<i>furcodorsalis</i>)	3	-1596.9541	4	63.9227	B
-					
ModelB3:					
(<i>S. altishoulderus</i> , <i>S. furcodorsalis</i>)					
	2	-1678.6393	3	1	B

*MLE = Marginal likelihood estimate

*BF = Bayes factor

18

Fig 2. Topology estimated from SVDquartets species tree analysis indicating the phylogenetic relationships of all the 21 *Sinocyclocheilus* species and bloodlines identified in our study based on 4378 unlinked SNPs for 120 individuals.

Fig 3. Time-calibrated maximum clade credibility species tree of the all 21 *Sinocyclocheilus* species is inferred by SNAPP. Node support is indicated by the Bayesian posterior probability next to nodes.

Fig 4. The PCA plots based on the 3459, 2942, 1993, 987, 165 SNP dataset show the population clustering between the 120 samples. The projection of individual samples on the surface is defined by the first two axes of the principal components, the x-axis (PC1 = 24.3%, 22.2%, 21.5%, 21.8%, 16.4%) and the y-axis (PC2 = 7.9%, 8.1%, 7.8%, 8.9%, 8.5%). Different symbols corresponded to the different clades. Different colors corresponded to differentiate the species inside each clade.

Fig 5. Treemix trees with different admixture edges ($m=1-5$) and the plotting of the $\ln(\text{likelihood})$ for different values of admixture edges. Arrows indicate when migration occurred in the population tree. The thickness represents the strength of the migration weight.

SUPPORTING INFORMATION

Fig S1. Map of the sampling localities of *Sinocyclocheilus* species. *S.donglanensis* and *S.altishoulderus* are caught in the same cave.

Table S1. Summary of the RAD data

Species	Sample ID	reads_raw	reads_passed_filter	Retained reads (%)	clusters_tal_at_90%	clusters_hidepth	hetero_est	error_est	reads_consens	loci_in_assembly_m114
S. mashanensis	GX0025mashanensis	6147831	6142674	0.999161168	620708	192493	0.010226	0.003766	175189	4019
S. brevibratus	GX0053brevibratus	10574223	10566811	0.99929905	718281	294336	0.009814	0.002564	272278	4334
S. brevibratus	GX0082brevibratus	11322894	11314123	0.999225375	760656	330506	0.009505	0.002611	306178	4340
S. yishanensis	GX0089yishanensis	11666117	11658167	0.999318539	813824	351254	0.010757	0.002407	324895	4337
S. yishanensis	GX0090yishanensis	10898276	10886321	0.998903038	1086550	337981	0.011189	0.002539	311630	4244
S. microphthalmus	GX0091microphthalmus	12193551	12185058	0.999303484	739666	320505	0.007106	0.003299	298095	4340
S. xunlensis	GX0161xunlensis	14567416	14535854	0.997833384	714585	332393	0.007879	0.001922	310820	4363
S. xunlensis	GX0162xunlensis	8286436	8262346	0.997092839	636925	262650	0.009182	0.002243	243443	4309
S. xunlensis	GX0163xunlensis	10705744	10695277	0.999022301	655543	304495	0.008336	0.002137	283693	4356
S. xunlensis	GX0164xunlensis	10123039	10112284	0.998937572	702603	289488	0.008326	0.002259	269436	4361
S. xunlensis	GX0165xunlensis	7784571	7775855	0.998880349	599966	255365	0.00899	0.002364	236322	4279
S. tianensis	LIU001tianensis	6200451	6197355	0.999500681	611306	183928	0.010657	0.003789	167325	3995
S. tianensis	LIU002tianensis	7039053	7035295	0.999466121	652330	211343	0.010347	0.00397	192887	4137
S. tianensis	LIU003tianensis	11888599	11881038	0.999364013	742560	293098	0.008929	0.003864	271517	4324
S. tianensis	LIU004tianensis	14921317	14911227	0.999323786	826400	324984	0.008396	0.004097	301144	4336
S. furcodorsalis	LIU005furcodorsalis	10769061	10760550	0.99920968	694537	281720	0.008468	0.003948	260984	4312
S. furcodorsalis	LIU006furcodorsalis	10372715	10362650	0.999029666	667800	281437	0.009014	0.003599	259945	4230
S. furcodorsalis	LIU007furcodorsalis	9634251	9627253	0.999273633	686353	248630	0.008375	0.003833	230088	4308
S. furcodorsalis	LIU008furcodorsalis	9696241	9690370	0.999394508	681881	265864	0.00886	0.00373	245785	4319
S. tianlineensis	LIU009tianlineensis	8995569	8987159	0.999065095	650273	250974	0.010398	0.002293	230813	4128
S. tianlineensis	LIU010tianlineensis	11999075	11988071	0.999082929	672240	305230	0.009288	0.002118	283459	4338
S. tianlineensis	LIU011tianlineensis	12705218	12692693	0.999014185	717785	298209	0.008986	0.002306	276261	4319
S. tianlineensis	LIU012tianlineensis	9967212	9958416	0.999117506	663287	278613	0.010455	0.002181	256958	4268
S. tianlineensis	LIU013tianlineensis	9446480	9434427	0.998724075	657929	263438	0.010548	0.002229	243003	4247
S. tianlineensis	LIU014tianlineensis	14062403	14037489	0.998228326	666170	301330	0.009456	0.001876	279731	4139

S.tianline nsis	LIU015tianli nensis	98376 96	9828401	0.9990 55165	643895	275394	0.01028 3	0.0022 59	254319	4277
S.guanyan ngensis	LIU016guan yangensis	58354 16	5831140	0.9992 67233	575893	191385	0.01097 5	0.0024 85	175146	4081
S.guanyan ngensis	LIU017guan yangensis	74250 08	7420300	0.9993 65927	595404	247260	0.01015 9	0.0022 49	228375	4307
S.guanyan ngensis	LIU018guan yangensis	75993 28	7594728	0.9993 94683	596396	244371	0.00984 2	0.0024 45	225969	4302
S.guanyan ngensis	LIU019guan yangensis	55028 61	5498020	0.9991 20276	552348	183387	0.01242 1	0.0024 77	166198	3953
S.guanyan ngensis	LIU020guan yangensis	96183 18	9610904	0.9992 29179	644403	272336	0.00914 5	0.0028 4	251941	4292
S.guanyan ngensis	LIU021guan yangensis	97913 36	9784948	0.9993 47586	663951	277438	0.00968 7	0.0026 9	256631	4326
S.guanyan ngensis	LIU022guan yangensis	96419 47	9635302	0.9993 10824	657512	273533	0.00971 7	0.0027 58	253231	4340
S.guanyan ngensis	LIU023guan yangensis	86908 02	8685388	0.9993 77043	639812	263571	0.00977 3	0.0026 59	243131	4318
S.guanyan ngensis	LIU024guan yangensis	99292 01	9920990	0.9991 73045	726970	277238	0.00917 0	0.0035 04	256422	4331
S.guanyan ngensis	LIU025guan yangensis	86658 97	8658838	0.9991 85428	769636	237526	0.01096 0	0.0036 81	216446	4236
S.guanyan ngensis	LIU026huan gtianensis	79994 84	7993211	0.9992 15824	760252	238732	0.01148 4	0.0036 46	217323	4190
S.guanyan ngensis	LIU027huan gtianensis	11407 539	11398034	0.9991 66779	829908	291284	0.01038 5	0.0035 11	268354	4291
S.guanyan ngensis	LIU028huan gtianensis	11051 312	11042598	0.9992 11496	779445	287736	0.0102 0	0.0033 84	265541	4297
S.guanyan ngensis	LIU029huan gtianensis	74315 68	7423445	0.9989 0696	690005	229699	0.01108 9	0.0035 0	210050	4163
S.cfiguany angensis	LIU030guan yangensis	10367 199	10354231	0.9987 49132	706623	284698	0.00928 4	0.0030 26	263659	4309
S.cfiguany angensis	LIU031guan yangensis	15698 789	15686607	0.9992 24017	859130	332964	0.00937 4	0.0033 0	309120	4321
S.cfiguany angensis	LIU032guan yangensis	11610 896	11601005	0.9991 48128	739162	300310	0.00958 9	0.0028 4	278115	4302
S.cfiguany angensis	LIU033guan yangensis	13628 426	13613358	0.9988 9437	766450	329302	0.00959 1	0.0029 64	305771	4333
S.lingyun ensis	LIU034lingy unensis	10200 248	10193547	0.9993 43055	705087	275869	0.00830 2	0.0033 57	255620	4276
S.microph thalmus	LIU035micro phthalmus	96462 94	9640033	0.9993 50942	666565	247166	0.00799 5	0.0033 0	228746	4260
S.microph thalmus	LIU036micro phthalmus	11684 750	11676797	0.9993 19369	687515	278963	0.00791 4	0.0030 59	259306	4317
S.microph thalmus	LIU037micro phthalmus	72035 37	7197587	0.9991 74017	626951	214213	0.0087 0	0.0032 22	197446	4165
S.microph thalmus	LIU038micro phthalmus	10711 256	10703957	0.9993 18567	761746	274957	0.00838 4	0.0034 55	254268	4299
S.microph thalmus	LIU039micro phthalmus	13168 573	13157998	0.9991 96952	787637	315043	0.00762 6	0.0038 66	293031	4342
S.microph thalmus	LIU040micro phthalmus	10504 013	10495614	0.9992 00401	729223	280416	0.00851 2	0.0030 6	259710	4303
S.altishou lderus	LIU041altish oulderus	91159 42	9110083	0.9993 5728	712724	242381	0.00961 9	0.0033 67	223200	4279
S.altishou lderus	LIU042altish oulderus	92956 71	9290264	0.9994 18331	680978	253956	0.00941 6	0.0033 66	234614	4307
S.altishou lderus	LIU043altish oulderus	36866 72	3684018	0.9992 8011	530351	91735	0.01384 6	0.0039 29	80739	2676

S. altishou lderus	LIU044altish oulderus	88296 72	8823233	0.9992 70754	710418	238946	0.00937 3	0.0037 16	220079	4314
S. altishou lderus	LIU045altish oulderus	62536 30	6248873	0.9992 39322	626661	184394	0.01015 4	0.0036 1	168412	4110
S. altishou lderus	LIU046altish oulderus	10379 052	10371057	0.9992 29698	726338	267972	0.00944 3	0.0034 28	247545	4300
S. altishou lderus	LIU047altish oulderus	11990 325	11981108	0.9992 31297	761782	280146	0.00910 3	0.0034 54	259243	4301
S. altishou lderus	LIU048altish oulderus	90079 52	9000403	0.9991 61963	661993	235686	0.00918 7	0.0034 5	217407	4309
S. donglan ensis	LIU049dongl anensis	78637 31	7858427	0.9993 25511	650142	217966	0.00935 5	0.0034 1	200740	4269
S. donglan ensis	LIU050dongl anensis	60120 65	6008402	0.9993 90725	595334	182885	0.00964 4	0.0033 11	167463	4101
S. donglan ensis	LIU051dongl anensis	72704 22	7264479	0.9991 82578	645058	216115	0.00951 1	0.0032 88	198658	4257
S. donglan ensis	LIU052dongl anensis	51898 44	5185630	0.9991 8803	589539	158550	0.01020 8	0.0034	144164	3888
S. donglan ensis	LIU053dongl anensis	70491 42	7043507	0.9992 00612	639506	202634	0.00927 7	0.0033 51	186106	4245
S. donglan ensis	LIU054dongl anensis	71005 65	7092862	0.9989 15157	641196	208822	0.00954 5	0.0032 25	192016	4249
S. donglan ensis	LIU055dongl anensis	11642 270	11633291	0.9992 28759	778733	273183	0.00848 9	0.0033 1	252643	4319
S. donglan ensis	LIU056dongl anensis	99024 88	9896300	0.9993 75107	710436	261784	0.00864 8	0.0032 04	242095	4317
S. guilinen sis	LIU057guilin ensis	93329 71	9324079	0.9990 47249	709154	238640	0.00959 9	0.0035 27	220326	4267
S. guilinen sis	LIU058guilin ensis	85659 78	8558576	0.9991 35884	684331	234549	0.00920 4	0.0032 82	216566	4241
S. guilinen sis	LIU059guilin ensis	10775 383	10764841	0.9990 21659	789590	262483	0.00946 1	0.0039 97	242317	4297
S. guilinen sis	LIU060guilin ensis	95242 01	9514228	0.9989 52878	840547	246917	0.01039 5	0.0043 84	224668	4210
S. guilinen sis	LIU061guilin ensis	12009 748	11999083	0.9991 11971	831139	287828	0.00888 4	0.0039 79	265617	4309
S. guilinen sis	LIU062guilin ensis	11881 410	11869513	0.9989 98688	930238	285733	0.01081 9	0.0041 8	260322	4219
S. guilinen sis	LIU063guilin ensis	55730 70	5567744	0.9990 44333	716929	163633	0.01334 9	0.0044 89	144726	3700
S. guilinen sis	LIU064guilin ensis	64295 39	6421534	0.9987 54965	646217	196636	0.01055 4	0.0038 91	179618	4034
S. mashan ensis	LIU065mash anensis	12773 768	12759456	0.9988 79579	771966	302170	0.00883 3	0.0033 17	280230	4325
S. mashan ensis	LIU066mash anensis	18924 069	18912035	0.9993 6409	895580	348784	0.00779 5	0.0036 01	324401	4325
S. mashan ensis	LIU067mash anensis	87937 21	8787492	0.9992 91654	677670	255773	0.00923 9	0.0036 24	235973	4286
S. mashan ensis	LIU068mash anensis	96026 01	9593898	0.9990 93683	689748	255671	0.00845 5	0.0036 62	236501	4328
S. mashan ensis	LIU069mash anensis	18354 043	18341140	0.9992 96994	898018	367559	0.00877 1	0.0039 04	341854	4349
S. mashan ensis	LIU070mash anensis	93151 34	9308690	0.9993 08223	676300	251238	0.00878 6	0.0038 83	231899	4325
S. mashan ensis	LIU071mash anensis	11296 508	11288842	0.9993 21383	716126	281199	0.00872 7	0.0034 43	260513	4340
S. cf longib arbatus	LIU072longi barbatus	12556 306	12546046	0.9991 82881	819994	295806	0.00911 2	0.0034 07	273901	4323

S.longibar batus	LIU073longi barbatus	11256 884	11246465	0.9990 74433	779530	278082	0.00846	0.0041 72	257838	4334
S.cflongib arbatus	LIU074longi barbatus	86728 67	8665361	0.9991 34542	684535	244397	0.00991 8	0.0036 15	224987	4314
S.cflongib arbatus	LIU075longi barbatus	10392 345	10383838	0.9991 81417	757547	285335	0.00916 8	0.0036 03	263834	4317
S.longibar batus	LIU076longi barbatus	93493 51	9342131	0.9992 27754	729563	268365	0.00913	0.0035 31	247476	4312
S.cflongib arbatus	LIU077longi barbatus	81702 22	8163676	0.9991 98798	689396	232697	0.01014 5	0.0035 14	214146	4294
S.cflongib arbatus	LIU078longi barbatus	87409 67	8734259	0.9992 32579	666300	240461	0.01065 7	0.0024 64	222145	4324
S.longibar batus	LIU079longi barbatus	82385 75	8231454	0.9991 35651	684689	227551	0.01043 5	0.0027 7	209521	4305
S.macrop hthalmus	LIU080macr ophthalmus	87665 61	8758914	0.9991 27708	699118	245249	0.01089 3	0.0026 66	225910	4305
S.macrop hthalmus	LIU081macr ophthalmus	10822 267	10810591	0.9989 21113	778952	278653	0.01035 8	0.0025 71	257592	4315
S.macrop hthalmus	LIU082macr ophthalmus	11554 037	11542467	0.9989 98618	695695	289584	0.00991 3	0.0023 65	268091	4325
S.macrop hthalmus	LIU083macr ophthalmus	10235 217	10224387	0.9989 41889	653383	272724	0.01003 1	0.0021 86	252622	4335
S.macrop hthalmus	LIU084macr ophthalmus	10150 601	10141912	0.9991 43992	675414	273280	0.01029 4	0.0025 8	252910	4326
S.macrop hthalmus	LIU085macr ophthalmus	83879 02	8381373	0.9992 21617	627774	231344	0.01042 2	0.0025	213273	4295
S.huanjia ngensis	LIU086huanj iangensis	98606 68	9853911	0.9993 14752	638229	269510	0.00811 4	0.0024 47	250258	4355
S.huanjia ngensis	LIU087huanj iangensis	92165 30	9210709	0.9993 68417	651220	253423	0.00829 6	0.0025 63	235163	4333
S.huanjia ngensis	LIU088huanj iangensis	10088 282	10083081	0.9994 84451	635342	278161	0.00829 5	0.0025 75	258408	4324
S.huanjia ngensis	LIU089huanj iangensis	89707 82	8963190	0.9991 53697	639357	250469	0.00889	0.0025 24	231848	4322
S.huanjia ngensis	LIU090huanj iangensis	11690 809	11680339	0.9991 04425	778832	287701	0.00831 1	0.0034 94	266758	4322
S.huanjia ngensis	LIU091huanj iangensis	98120 08	9805062	0.9992 92092	698569	264968	0.00836 9	0.0034 67	245471	4337
S.huanjia ngensis	LIU092huanj iangensis	10110 052	10101594	0.9991 63407	729917	263698	0.00799 9	0.0036 59	244153	4337
S.huanjia ngensis	LIU093huanj iangensis	94901 44	9483135	0.9992 61444	704181	277437	0.00836 8	0.0034 08	257015	4332
S.huanjia ngensis	LIU094huanj iangensis	10331 954	10324059	0.9992 35866	678557	266485	0.00876 9	0.0027 02	247105	4349
S.yishane nsis	LIU095yisha nensis	11078 825	11070738	0.9992 70049	689417	278309	0.01088	0.0026 71	257159	4323
S.yishane nsis	LIU096yisha nensis	92096 44	9203319	0.9993 1322	668856	258976	0.01142 8	0.0026 87	238602	4297
S.yishane nsis	LIU097yisha nensis	11948 928	11939669	0.9992 25119	710819	280410	0.01059 5	0.0024 9	259199	4321
S.yishane nsis	LIU098yisha nensis	97179 02	9711389	0.9993 29794	627159	253618	0.01065 8	0.0022 5	234931	4316
S.yishane nsis	LIU099yisha nensis	82539 34	8245811	0.9990 15863	640514	237456	0.01134 3	0.0025 97	218144	4295
S.yishane nsis	LIU100yisha nensis	94725 30	9461442	0.9988 29457	626280	253619	0.01056 7	0.0023 46	234567	4315
S.xunlensi s	LIU101xunle nsis	87178 81	8708887	0.9989 68327	648178	239426	0.00896 8	0.0024 81	221900	4334

S.xunlensis	LIU102xunlensis	9176341	9169184	0.999220059	658452	256582	0.008473	0.00257	237853	4336
S.xunlensis	LIU103xunlensis	11154384	11142474	0.998932258	773286	301312	0.009184	0.002294	279735	4353
S.xunlensis	LIU104xunlensis	18736734	18717682	0.998983174	1091524	395736	0.007878	0.002228	369084	4356
S.angustiporus	LIU105angustiporus	5236660	5232391	0.999184786	605103	151880	0.016385	0.002671	135907	3236
S.angustiporus	LIU106angustiporus	6884510	6879551	0.999279687	684665	205307	0.016159	0.002812	185042	3688
S.angustiporus	LIU107angustiporus	6737620	6731762	0.999130554	663549	200421	0.014345	0.003028	181613	3597
S.brevis	LIU108brevis	6193310	6188183	0.999172171	599403	194962	0.010927	0.002586	178136	4078
S.brevis	LIU109brevis	9907760	9900681	0.99928551	664376	259661	0.009382	0.002423	240663	4294
Summary		983210.98	9823582.908	0.99913263	703204.1167	261033.9833	0.009693742	0.0030865	240994.6917	4241.525

Note: values in the last line are averages. Clusters_total: Clusters that passed filtering for 10x minimum coverage. Loci_in_assembly: Loci retained after passing coverage and paralog filters.

Table S2. Sequence information with different parameters

Matrix	Unlinked SNPs	Consensus sequences (bp)	VAR	PIS	Missing (%)	PIS/VAR(%)
Sino_m96	23,869	3,534,842	380,519	336,928	13.46	88.54
Sino_m102	17,187	2,544,628	272,838	242,335	10.89	88.85
Sino_m108	10,549	1,560,414	166,407	148,253	8.21	89.09
Sino_m114	4,378	646,497	67,983	61,023	5.25	89.76
Sino_m120	151	22,274	2,199	1,989	2.29	90.45

Note: Sequence information in the RAD data matrices (n=120) generated with different parameters of minimum number of individuals per locus (m) values. The data matrices shown in bold type were used for analyses.