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# DNA Barcoding Reveals Cryptic Diversity in the Genus Triplophysa (Cypriniformes: Cobitidae, Nemacheilinae) from the Northeastern Qinghai-Tibet Plateau

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# Abstract

**Background:** The northeastern part of the Qinghai-Tibet Plateau (QTP) is one of the areas where the number of species of plateau loach is the largest. As one of the three major groups of fishes distributed on the QTP, plateau loach have very important ecological value. However, their taxonomy and systematics are still controversial, and a large number of new species have been reported. The reason for this phenomenon is that the degree of morphological variation is low, the phylogenetic information provided by morphological and anatomical features used for species identification is relatively poor, and there are many cryptic species. Based on the high-density sampling points from the biodiversity hotspots surveyed, this study aims to evaluate the taxonomic characteristics of the plateau loach by means of morphology, DNA barcoding and multiple species demarcation methods to accurately describe species and allocate taxonomic units to unknown specimens.

**Results:** After careful identification and comparison of the morphology and DNA barcoding of 1,630 specimens, 22 species were identified, 20 of which were considered valid local species and two of which were new species that had not been described. Based on the combination of morphological and molecular methods, a total of 24 native species have been found, two of which are cryptic species: *Triplophysa robusta sp1* and *Triplophysa minxianensis sp1*. Fourteen of the 24 species form clusters of barcodes, which allow them to be reliably identified. The remaining cases involved 10 closely related species, some of which were rapidly differentiated, had a disputed taxonomic status, or showed introgressions.

**Conclusions:** The results highlight the need to combine traditional taxonomies with molecular methods to correctly identify species, especially in closely related species such as the plateau loach. This study provides a basis for protecting the biodiversity of plateau loach.


# Background

With problems such as global climate change, issues related to populations, the ecological environment, energy and food are becoming increasingly serious, and sustainable anthropogenic development and the ability to understand and meet the requirements of biodiversity is becoming urgent (Loreau et al, 2001; Isbell et al, 2011; Cardinale et al, 2012). There is a major global demand for accurate and rapid identification of species for the protection and sustainable use of biodiversity resources. Species identification and classification is a basic requirement for biological research. Based on morphological characteristics, classical taxonomy has made great contributions to species classification; however, due to morphological plasticity, traditional taxonomy cannot accurately distinguish all species, in particular, some forms of similar, related species (Robinson and Parsons, 2002; Pigliucci, 2005). Therefore, there is a need for a new way to support species identification with classical taxonomy methods. Tautz et al. (2002) first suggested using DNA sequencing, namely, DNA taxonomy, as the main platform for biological classification. Then, Professor Paul Hebert from the University of Guelph in Canada introduced the concept of DNA barcoding, highlighting its significance to the field of biological taxonomy and species

identification (Hebert et al., 2003; Remigio et al., 2003) and suggesting the use of the mitochondrial cytochrome C oxidase subunit I (COI) gene as the basis for animal DNA barcoding. The applicability of DNA barcoding to the identification of marine and freshwater fish species has been shown by using a short fragment of approximately 650 bp from the mitochondrial COI gene to identify species based on sequence differences (Ward et al., 2005; Zhang et al., 2012; Zhang et al., 2013; Valdez-Moreno et al., 2012; Lakra et al., 2011; Bhattacharjee et al., 2012; Hubert et al., 2008). A growing number of studies show that DNA barcodes are widely used in animal species identification, classification, cryptic species detection, phylogenetic research, etc. (Smith et al., 2008; Swartz et al., 2008; Rock et al., 2008; Almerón-Souza et al., 2018), and to construct barcode databases, such as the Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org), in which approximately 96,425 fish specimens belonging to 10,267 species have been barcoded. DNA barcoding, as a compliment to traditional species identification, can be used to automate and standardize the process of specimen identification, reducing the dependence on the experience of taxonomists (Sales et al., 2018; Burrows et al., 2019).

The Qinghai-Tibet Plateau (QTP), known as “the roof of the world”, is rich in biodiversity and is a relatively unique area with many endemic species (Khan, et al., 2005). The native fish living in the Qinghai-Tibet region belong to three orders: Salmoniformes, Siluriformes and Cypriniformes (Wu and Wu, 1992). *Triplophysa*, which belong to the family Nemacheilinae (Cypriniformes) are widely distributed on the QTP and in its adjacent regions (Wang et al., 2016). It is a special group adapted to the climatic characteristics of the QTP, such as cool temperatures and oxygen shortages (Zhu and Wu, 1981; Wu and Wu, 1992). In 1992, there were 33 *Triplophysa* species identified. However, over time, a large number of new species have been described; so far there are a total of 140 valid species (He et al., 2008; Li et al.,2017;). Although there may be some synonyms species (He et al., 2008; Prokofiev et al., 2007), these studies show that a large amount of unknown biodiversity exists in the *Triplophysa*, and many species have not been recognized or described. The phenomenon of many new species being reported is mainly caused by the existence of cryptic species or the lack of careful classification review. The simple body structure and relatively conservative morphological evolution of the plateau loach fish, coupled with their weak migration ability due to the restrictions of the water system, have led to limited gene exchange between different populations. Over time, although morphologically imperceptible, the process of species differentiation, including genetic structural differentiation and reproductive isolation, may have occurred, and many hidden taxa may have been ignored. Therefore, the genus *Triplophysa* should be considered in the study of cryptic diversity.

Classical morphological classification has always played a dominant role in species identification, but it has limitations. In particular, for the fish of the genus *Triplophysa*, the phenotype is easily affected by biological factors and the external environment and there is morphological plasticity; therefore, morphological differences are not easily detected (He et al., 2008). Moreover, some species were named many years ago, and their morphological descriptions were relatively simple. All these factors have led to difficulties in the subsequent identification of species and taxonomic research. Due to the difficulty in obtaining detailed data for comparisons, it is possible that the distribution of some species is artificially expanded and mistakenly divided into different geographical populations (Ding et al., 1996; Prokofiev,

2007). Such a long-term, complex classification history is significant to the genus. Many studies have shown that species identification results based on DNA barcoding, which is one of the effective means to identify morphologically indistinguishable cryptic species, have a high degree of matching with the current classification system (Barrett et al., 2005; Dincă et al., 2011; Dhar et al., 2017). *Triplophysa* species can also be identified by DNA barcoding (Li et al., 2017). To date, there are no studies on the identification or evaluation of cryptic biodiversity within the genus *Triplophysa* using DNA barcoding in northeastern QTP. Herein, samples obtained from northeastern QTP, a hotspot for biodiversity, were identified using DNA barcoding. Furthermore, the uncovered cryptic lineage was analysed in combination with morphological features.

# Results

A total of 1,630 native specimens were collected from the northeastern edge of the QTP (Table S1), and 22 morphospecies were identified including two undetermined species (*Triplophysa sp1* and *Triplophysa sp2*). Among the specimens, the endemic species *T. robusta* (n = 413) had the largest number of individuals, followed by *T. minxianensis* (n = 253). The undetermined species *T. sp1* (n = 3) and *T. bleekeri* (n = 5) had the lowest number of specimens, with 68 specimens per species on average (Table 1). A total of 1,630 COI sequences were obtained. The size of the sequences obtained was 606 bp after trimming to a consensus length. No stop codons were observed, and the mean nucleotide composition within the complete data set was 30.6% thymine (T), 26.7% cytosine (C), 24.3% adenine (A) and 18.4% guanine (G). There were 393 conserved sites, 213 variable sites, 178 parsimonious sites and 35 singleton sites. A total of 230 unique haplotypes were generated in the 1623 COI sequences. The haplotype number of *T. robusta* was the largest (N*h* = 46), followed by that of *T. obscura* (N*h* = 27) and *T. stoliczkai* (N*h* = 25). The haplotype numbers of *T. bleeker* and *T. orientalis* were the smallest (N*h* = 1). Correspondingly, the haplotype diversity of *T. robusta* is the highest (*h* = 0.9360 ± 0.006). The nucleotide diversity is the highest for *T. obscura* ($\pi$ = 0.00777 ± 0.00145).

Table 1
Sampling information number of individuals and diversity parameters for the specimens included in this study.

| Species | Collection site (River) | Numbure of specimens(N) | Numbure of haplotypes (Nh) | Haplotype diversity (h) | Nucleotide diversity (π) |
|---|---|---|---|---|---|
| *Triplophysa bleekeri* (Sauvage *et* Dabry, 1874) | Jialing River | 5 | 1 | —— | —— |
| *T. dalaica* (Kessler, 1876) | Jinghe River | 55 | 9 | 0.469 ± 0.083 | 0.00114 ± 0.00102 |
| *T. hsutschouensis* (Rendahl, 1933) | Heihe River, Shulehe River, Shiyanghe River | 46 | 8 | 0.731 ± 0.041 | 0.00440 ± 0.00125 |
| *T. leptosoma* (Herzenstein, 1888) | Shulehe River | 7 | 3 | 0.667 ± 0.160 | 0.00126 ± 0.00095 |
| *T. minxianensis* (Wang et zhu, 1979) | Yellow River, Jinghe River | 253 | 21 | 0.385 ± 0.040 | 0.00074 ± 0.00001 |
| *T. minxianensis sp1* | Yellow River, Weihe River | 20 | 2 | 0.526 ± 0.036 | 0.00087 ± 0.00047 |
| *T. obscura* (Wang, 1987) | Jialing River, Weihe River, Taohe River | 234 | 27 | 0.877 ± 0.012 | 0.00777 ± 0.00145 |
| *T. orientalis* (Herzenstein, 1888) | Taohe River | 19 | 1 | —— | —— |
| *T. papillosolabiatus* (Kessler, 1879) | Heihe River, Shulehe River | 95 | 13 | 0.603 ± 0.036 | 0.00201 ± 0.00112 |
| *T. pappenheimi* (Fang, 1935) | Yellow River, Weihe River | 21 | 4 | 0.610 ± 0.114 | 0.00196 ± 0.00011 |
| *T. polyfasciata* (Ding, 1996) | Jialing River | 10 | 3 | 0.600 ± 0.131 | 0.00121 ± 0.00082 |
| *T. pseudoscleroptera* (Zhu & Wu, 1981) | Yellow River, Xiahe River | 9 | 4 | 0.583 ± 0.183 | 0.00138 ± 0.00105 |
| *T. robusta* (Kessler, 1876) | Yellow River, Jialing River, Shiyanghe River | 219 | 46 | 0.936 ± 0.006 | 0.00588 ± 0.00182 |
| *T. robusta sp1* | Jinghe River | 194 | 15 | 0.591 ± 0.020 | 0.00133 ± 0.00012 |

| Species | Collection site (River) | Numbure of specimens(N) | Numbure of haplotypes (Nh) | Haplotype diversity (h) | Nucleotide diversity (π) |
|---|---|---|---|---|---|
| *T. scleroptera* (Herzenstein, 1888) | Yellow River, Taohe River | 44 | 3 | 0.090 ± 0.059 | 0.00015 ± 0.00005 |
| *T. sellaefer* (Nichols, 1925) | Jinghe River | 22 | 5 | 0.338 ± 0.128 | 0.00089 ± 0.00101 |
| *T. shiyangensis* (Zhao & Wang, 1983) | Shiyang River | 25 | 11 | 0.770 ± 0.086 | 0.00367 ± 0.00180 |
| *T. siluroides (Herzenstein, 1888)* | Yellow River, Xiahe River, Taohe River | 28 | 5 | 0.529 ± 0.105 | 0.00108 ± 0.00095 |
| *T. sp1* | Yellow River, Jialing River | 3 | 2 | 0.667 ± 0.314 | 0.00660 ± 0.00269 |
| *T. sp2* | Jialing River | 67 | 6 | 0.172 ± 0.062 | 0.00029 ± 0.00007 |
| *T. stoliczkai (Steindachner, 1866)* | Yellow River, Xiahe River, Taohe River, Jinghe River, Jialing River, Shiyanghe River | 129 | 25 | 0.812 ± 0.028 | 0.00285 ± 0.00149 |
| *T. strauchii* (Kessler, 1874) | Heihe River | 11 | 2 | 0.509 ± 0.101 | 0.00084 ± 0.00056 |
| *T. tenuis* (Day, 1877) | Heihe River, Shulehe River | 97 | 11 | 0.378 ± 0.061 | 0.00082 ± 0.00010 |
| *T. wuweiensis* (Li & Chang, 1974) | Shiyanghe River | 17 | 3 | 0.404 ± 0.130 | 0.00090 ± 0.00085 |
| Total | | 1630 | 230 | | |

The phylogenetic tree was constructed by neighbor-joining method (NJ), maximum likelihood method (ML) and Bayesian inference method (BI).With *Homatula variegata* as the outclass group (Gen Bank no. : MF953219), the topological structure of the phylogenetic trees obtained by the three analysis methods is basically the same, and only the topological structure of NJ tree is retained here, and the values at the node represent support values of NJ/ML/BI tree is added respectively. The PTP analysis with a maximum likelihood partition and Bayesian implementation resulted in 17 MOTUs (Fig. 3). The GMYC analysis resulted in the same 17 MOTUs as those obtained in the PTP analysis (likelihood ratio = 76.41, $P$< 0.0001). The ABGD analysis resulted in 19 MOTUs with Kimura (K80) TS/TV = 2.0. The BOLD system distinguished 19 MOTUs for the 22 morphological species. *T. strauchii*, *T. orientalis*, *T. tenuis*, *T. wuweiensis*, *T. polyfasciata*, *T. bleekeri*, *T. sp1*, *T. sellaefer*, *T. minxianensis sp1*, *T. hsutschouensis* and *T.*

*robusta* showed correspondence between the morphological species and MOTUs. The MOTUs of *T. minxianensis*, *T. pappenheimi*, *T. siluroides*, *T. pappenheimi* and *T. robusta sp1* cannot be distinguished by the PTP, GMYC, ABGD or BOLD analyses. The same phenomenon occurs between *T. stoliczkae* and *T. dalaica* and between *T. scleroptera* and *T. pseudoscleroptera*. *T. leptosome* and *T. papilloso-labiatus* cannot be distinguished by the PTP or GMYC analyses, but they can be distinguished by the ABGD and BOLD analyses. The same is true of *T. shiyangensis*.

The average K2P intraspecific distance ranged between 0 and 3.10% (Table 2). The maximum observed average K2P intraspecific distance was that of *T. robusta*. The maximum intraspecific K2P distance ranged from 0 to 7.90%. The largest K2P intraspecific distance was observed for *T. robusta*, followed by *T. minxianensis* with a value of 7.40%. The nearest neighbour distance ranged between 0 and 8.57%. For *T. robusta*, *T. minxanensis*, *T. siluroides* and *T. pappenheimi*, a nearest neighbour distance of 0% was observed. The nearest neighbour distance of 18 species was lower than the maximum K2P intraspecific distance. Only the nearest neighbour distance of *T. scleroptera* and *T. pseudoscleroptera* was less than 1%, at 0.40%. The distributions of the maximum K2P intraspecific distances and the nearest neighbour K2P genetic distances reflected the overlap; in addition, no barcode gap was found (Fig. 4).

Table 2
Genetic K2P distances of the *Triplophysa* species.

| species | OTU | Mean intra- | Maximum intra- | NN Dist | NN |
|---|---|---|---|---|---|
| *Triplophysa bleekeri* | OTU-1 | 0.0000 | 0.0000 | 0.0731 | *T. papillosolabiatus* |
| *T. shiyangensis* | OTU-3 | 0.0069 | 0.0130 | 0.0265 | *T. stoliczkae* |
| *T. strauchii* | OTU-4 | 0.0017 | 0.0020 | 0.0271 | *T. stoliczkae* |
| *T. orientalis* | OTU-5 | 0.0000 | 0.0000 | 0.0598 | *T. stoliczkae* |
| *T. tenuis* | OTU-7 | 0.0039 | 0.0070 | 0.0613 | *T. pseudoscleroptera* |
| *T. wuweiensis* | OTU-8 | 0.0033 | 0.0050 | 0.0751 | *T. obscura* |
| *T. sp2* | OTU-9 | 0.0028 | 0.0030 | 0.0264 | *T. obscura* |
| *T. obscura* | OTU-10 | 0.0101 | 0.0200 | 0.0264 | *T. sp2* |
| *T. polyfasciata* | OTU-11 | 0.0022 | 0.0030 | 0.0857 | *T. bleekeri* |
| *T. sp1* | OTU-12 | 0.0099 | 0.0100 | 0.0290 | *T. papillosolabiatus* |
| *T. leptosoma* | OTU-13 | 0.0022 | 0.0030 | 0.0147 | *T. papillosolabiatus* |
| *T. papillosolabiatus* | OTU-14 | 0.0053 | 0.0130 | 0.0147 | *T. leptosoma* |
| *T. sellaefer* | OTU-16 | 0.0036 | 0.0070 | 0.0440 | *T. minxianensis* sp1 |
| *T. hsutschouensis* | OTU-18 | 0.0063 | 0.0130 | 0.0320 | *T. robusta* |
| *T. scleroptera* | OTU-6 | 0.0030 | 0.0050 | 0.0040 | *T. pseudoscleroptera* |
| *T. pseudoscleroptera* | OTU-6 | 0.0040 | 0.0070 | 0.0040 | *T. scleroptera* |
| *T. stoliczkae* | OTU-2 | 0.0060 | 0.0120 | 0.0130 | *T. dalaica* |
| *T. dalaica* | OUT-2 | 0.0040 | 0.0070 | 0.0130 | *T. stoliczkae* |
| *T. robusta* | | 0.0310 | 0.0790 | | |
| | OTU-19 | 0.0082 | 0.0250 | 0.0320 | *T. hsutschouensis* |

The mean and the maximum of intra-group distances, the nearest neighbor (NN), and the minimum distance to the NN for the Nominal species.

| species | OTU | Mean intra- | Maximum intra- | NN Dist | NN |
|---|---|---|---|---|---|
| | OTU-15 | 0.0077 | 0.0180 | 0.0000 | *T. pappenheimi, T. siluroides* |
| *T. minxianensis* | | 0.0160 | 0.0740 | 0.0110 | *T. siluroides* |
| | OTU-17 | 0.0016 | 0.0020 | 0.0430 | *T. robusta* |
| | OTU-15 | 0.0050 | 0.0100 | 0.0000 | *T. siluroides*, *T.pappenheimi* |
| *T. siluroides* | OTU-15 | 0.0030 | 0.0050 | 0.0000 | *T. pappenheimi*, *T. robusta* |
| *T. pappenheimi* | OTU-15 | 0.0020 | 0.0030 | 0.0000 | *T. siluroides*, *T. minxianensis* |

The mean and the maximum of intra-group distances, the nearest neighbor (NN), and the minimum distance to the NN for the Nominal species.

Most species form very good evolutionary branches in the NJ tree, and these main branches represent different taxonomic species. Monophyletic clades have also been observed for *T. stoliczkae* and *T. dalaica*, and *T. scleroptera* and *T. pseudoscleroptera*. Neither *T. minxianensis* nor *T. robusta* formed an independent monophyletic clade, but they formed two larger branches according to geographic distribution. Because of a shared haplotype between *T. minxianensis*, *T. pappenheimi*, *T. siluroides* and *T. robusta*, these four species form a larger clade. The trend of mixed genealogies was confirmed by the examination of the haplotype networks. Two pairs of species (*T. stoliczkae* and *T. dalaica* (Fig. 5A) and *T. scleroptera* and *T. pseudoscleroptera* (Fig. 5B)) cannot be distinguished by the four algorithms used for MOTU delimitation, and there is no shared haplotype between them. Four haplotypes were shared among *T. minxianensis*, *T. pappenheimi*, *T. siluroides* and *T. robusta* (Fig. 5C).

# Discussion

In this study, a total of 24 species were reported, including two new species: a cryptic species in the *T. minxianensis* population and a cryptic species in the *T. robusta* population. The morphological and molecular data were consistent in 14 of the 22 species identified. The results show that there are two cryptic species that can be described in the biodiversity hotspot area, which reinforces the general view that there is still a large amount of unrecorded diversity in the plateau loach. A single sequence forms a single branch for *T. bleekeri* and *T. orientalis*. It is necessary to collect more specimens and add sequences, but we do not rule out the possibility of identifying more cryptic species.

Different numbers of MOTUs were identified in the four DNA barcode analysis methods: 17 different MOTUs were identified using the PTP and GMYC models and 19 MOTUs were identified using the ABGD and BOLD methods. *T. shiyangensis* and *T. leptosoma* cannot be distinguished by the PTP or GMYC

models, but the ABGD and BOLD methods allow different MOTUs to be assigned to each species (Fig. 3). The inconsistent results of the four methods may be due to different threshold values used for the identification of species; the BOLD system method defaults to 2.2%, ABGD to 2.7%, PTP to 1%, and GMYC to 2%. Although it has been pointed out that the RESL in the BOLD system has a stronger taxonomic performance than that in the ABGD system, showing better species identification and MOTU assignment results (Ratnasingham and Hebert, 2013), the two methods in this study achieved the same results, which may be related to the identified species. A key aspect implicit in DNA barcoding analysis is the genetic distance threshold values used to define the MOTUs. COI genetic distance values from 1% (Hubert et al., 2008) to 2% (Keskin et al., 2013) have been considered the threshold values for fish DNA barcoding analysis. However, these values are derived from comparative analyses of species diversity in different aquatic ecosystems. For example, 2% is used to represent the DNA barcodes for the community of fish in certain rivers (Pereira et al., 2013). However, when DNA barcoding analysis was used for a group of closely related species (e.g., the same genus), a lower genetic distance value has been reported (Carvalho et al., 2011; Pereira et al., 2011, 2013). In particular, a low threshold value of 0.92% is needed to distinguish MOTUs in the genus *Laemolyta* (Anostomidae) (Ramirez and Galetti, 2015). Although most of the values obtained in this paper are above 1.47% (14 out of 18 MOTUs, Table 2), the maximum threshold value of related species detected between the obtained MOTUs is 0.40%, and some species have shared haplotypes. The phenomenon of sharing haplotype among the plateau fishes may be related to the complicated mechanism of speciation and differentiation or the convergent evolution of local adaptation (Shen et al., 2019; Chen et al., 2020). A relatively low threshold of genetic distance may be obtained when the intraspecies relationships within a genus are analysed. It is clear that the different DNA barcoding analysis methods are largely related to the target species analysed (Pentinsaari et al., 2017).

The difference in the number of MOTUs detected by the different analysis methods was mainly seen in two pairs of MOTUs: the genetic distance between *T. shiyangensis* and *T. stoliczkae* was relatively low (2.65%), as was the genetic distance between *T. leptosoma* and *T. papilloso-labiatus* (1.47%). These relatively low genetic distance values may be related to the late differentiation of these MOTUs. Notably, the MOTUs of relatively recent origin had less time than species of distant origin to accumulate genetic differences, which hindered their correct identification, even though the species differ greatly in their morphological characteristics. *T. papilloso-labiatus* has obvious swim bladder, while *T. leptosoma* does not (Zhao, 1984). The characteristics of the genetic diversity of these species are the same: there is a relatively high level of haplotype diversity (> 0.5) and relatively low levels of nucleotide diversity (< 0.5%) (Table 1). This indicates that after the differentiation of these species, influenced by the founder effect and environmental heterogeneity caused by water system changes, the population rapidly accumulated variation, resulting in a high haplotype diversity index. The accumulation time of the nucleotide diversity index was much longer than that of the haplotype diversity index. In terms of geographical distribution, these two species are mainly distributed in the Shulehe River and Heihe River. The possibility of sympatric speciation exists, but this needs to be confirmed by further analysis.

An example of incompletely separated species was also found. *T. minxianensis*, *T. robusta*, *T. pappenheimi* and *T. siluroides* are not sufficiently differentiated by COI gene differences, and there are

also shared haplotypes among the four species (Fig. 5). These phenomena can be explained as frequent gene infiltration events before species differentiation (Feng et al., 2018) or phenotypic plasticity in fish (Robinson and Parsons, 2002; Thibert-Plante and Hendry, 2011). Another species that together with *T. minxianensis*, *T. robusta*, *T. pappenheimi* and *T. siluroides* on a larger branch is *T. hsutschouensis*. The morphological characteristics of *T. hsutschouensis*, which was identified as an independent species isolated from *T. robusta*, include bare and scaleless bodies and a relatively low ratio of body length to body height (Wang, 1991). *T. robusta* only has residual scales in specific parts of its body. The Jinghe River population of *T. robusta* has scales along the lateral line from the caudal fin to the front of the dorsal fin. Moreover, the Jinghe River population and other populations of *T. robusta* were clustered into two branches (Fig. 3), and the genetic distance between the populations reached 7.9% (Table 2). These phenomena suggested the existence of cryptic species of *T. robusta*. There was no difference between *T. minxianensis* and *T. minxianensis sp1* in the degradation of the swim bladder, whether the end of the pelvic fin reached the anus, the starting point of the dorsal fin and the pelvic fin relative to each other or the morphological measurement data. But the scales of *T. minxianensis sp1* were only found in the caudal peduncle and this is quite different from *T. minxianensis*, in which all the body parts except the head have obvious round scales. The genetic distance between the two populations was 7.4% (Table 2), which indicated that there were cryptic species in *T. minxianensis*. Similar to this example of incomplete species separation, Wang (1991) argued that the plateau loach groups without scales (*T. hsutschouensis*) come from scaly groups (*T. minxianensis*) following the degeneration of scales. The groups with remnant body scales (*T. robusta*) are the intermediate species between the two types. Whether scales degenerate marks a leap in the evolution of plateau loach populations. The cryptic species found in this study provide more evidence for this speculation.

The morphological characteristics and molecular characteristics were inconsistent in *T. pseudoscleroptera* and *T. scleroptera*. The two species have similar appearances but different internal anatomical structure. The anterior and posterior segments of the swim bladder of *T. pseudoscleroptera* were the same size, with a long pouch or oblong oval shape and no pyloric caecum. The posterior chamber of the swim bladder of *T. scleroptera* is developed, the anterior segment is thin and the posterior segment is enlarged into a long pouch (Zhu et al., 1981). Without the comparison of internal anatomical structure, these species are easy to misidentify and morphological identification may be incorrect (He et al., 2008). However, due to the low interspecific distance between the two species (0.40%), the two MOTUs cannot be correctly distinguished. This inconsistency was also found between *T. dalaica* and *T. stoliczkai*. The posterior chamber of *T. dalaica*'s swim bladder was oval, while the posterior chamber of *T. stoliczkai* 's swim bladder was degraded; this feature can be used to accurately distinguish the two species.

As shown by the two cases reported here, the DNA barcoding did not show enough difference to distinguish similar species because the lineages were not completely divided into different branches. Similar phenomena have been found in *Psorophora* (Chan-Chable et al., 2016), Syngnathidae (Zhang et al., 2017) and *Laemolyta* (Ramirez et al., 2015), and mixed lineage cases are particularly common in plateau fish (Shen et al., 2018). In this sense, to find evidence of reproductive isolation, it is important to

combine nuclear genetic and ecological data for further research (Mardulyn et al., 2011; Versteirt et al., 2015; Beebe, 2018).

It is easy to identify species with morphological characteristics that are not significantly different as a single species. For example, *T. bleekeri* and *T. polyfasciata* have very similar morphological characteristics, there is no significant difference in the quantitative traits in different proportions of their bodies, and they have been identified as the same nominal species. Ding et al. (1996) believed that they should be divided into two different species based on molecular data and pointed out that the main distinguishing feature was that there were 10–12 wide, dark brown horizontal stripes on the side of the body. However, even among *T. bleekeri* individuals collected from the same site, the horizontal stripes on the side of its body can range from 0–10. Of the specimens collected from Wenchuanhe River in Sichuan Province, most had 5–7 horizontal stripes, and almost none had more than 10. It was concluded that the validity of *T. polyfasciata* was still questionable (He et al., 2008). In this study, the numbers of these two species of plateau loach collected were relatively small, with 10 *T. bleekeri* and 5 *T. polyfasciata*, and 7–9 horizontal stripes were observed on the sides of the fish bodies. The division into two different species was also not supported by morphology, but the genetic distance between the two species reached 8.57%, far exceeding the threshold of genetic distance within the species of 2% (Pereira et al., 2013). Therefore, it is speculated that these two species have undergone genetic differentiation in terms of genetic material, but due to the small size of the individual (the length of the collected sample is 5–8 cm), the morphological difference is not obvious, so they have historically been regarded as one species. Obviously, the body colour or body markings of the plateau loach may not be an effective classification feature for the identification of species and cannot be used as the main basis for identification.

Herzenstein (1891) identified *T. papilloso-labiatus* as a subspecies of *T. strauchii*; this finding was also supported by Zugmeyer (1910). *T. strauchii* lack a developed mastoid process similar to that of *T. papilloso-labiatus*. Instead, they have only a strong, naked fold, while the mastoid process on the upper lip of the plateau loach living in the Hexi corridor is obviously a double line, and that on the lower lip is blurred double line. Characteristics such as the mastoid process and strong, naked crease are continuously transitive in a geographical distribution without obvious boundaries. However, the appearance of significant double lines on the mastoid marks discontinuity in the variation, and there are relatively stable differences in a series of other morphological traits. Thus, *T. papilloso-labiatus* should be regarded as an independent species (Li and Chang, 1974; Zhao, 1984). This is also supported in the phylogenetic tree constructed in this study (Fig. 3). *T. strauchii* and *T. papilloso-labiatus* are clustered into two different branches and should be independent species.

There is little difference in the morphological characteristics between *T. wuweiensis* and *T. scleroptera*. Li and Chang (1974) regarded *T. wuweiensis* as an independent species based on 7 morphological traits. Zhu and Wu (1975, 1981) believed that there was a certain continuity in the identification characteristics of these two species. However, after collecting specimens of *T. scleroptera* distributed in the Datonghe River, only one mountain away from the *T. wuweiensis* specimens, Zhao (1984) believed that there were significant differences between the two species in the number of pectoral fin rays, intestinal shapes and

gill rakers, supporting *T. wuweiensis* as an independent species. In this study, *T. wuweiensis* and *T. scleroptera* clustered in different branches, and the two species were greatly differentiated, which also supported the idea that *T. wuweiensis* is an independent species. The low genetic diversity of *T. wuweiensis* may be due to the short time since species differentiation and the low haplotype diversity and nucleotide diversity may be caused by the founder effect and the narrow distribution area (the species is only distributed in the east and west Shiyanghe River tributaries).

*T. shiyangensis*, *T. papilloso-labiatus* and *T. hsutschouensis* are distributed in three inland river systems in the Hexi corridor. The maximum intra-species genetic distance of these three species is more than 1%. This may be mainly due to the wide geographic distribution of the three species and the large population differentiation caused by the barriers created by the water systems. This phenomenon also appears in the sympatric distribution of *Gymnocypris chilianensis*, in which each geographic population is clustered into a single branch, with a large genetic differentiation (Zhao et al., 2011).

The different geographic populations of some widespread species are identified as different species or subspecies due to some more significant morphological differences. For example, *T. stoliczkae* was divided into 7 subspecies (Herzenstein, 1891) due to the differences in the number of gill rakers, the proportion of quantitative traits and the number of spiral loops of intestinal tubes with changes in altitude or water system. In this study, the samples were collected in three drainage systems (Yellow River, Jialing River and the inland rivers in the Hexi corridor). The maximum genetic distance within the population was greater than 1.2% (Table 2). However, the samples of different water systems have shared haplotypes. This indicated that the population differentiation of *T. stoliczkae* was low in the surveyed area.

The membranous swim bladder of *T. obscura* is very developed with a constriction in the middle, and its length accounts for approximately 2/3 of the abdominal cavity. Compared with *T. orientalis*, its body surface has obvious spines. It is regarded as an independent new species (Li, 2017). In this study, a relatively large number of samples (n = 234) were collected in the distribution area. The phylogenetic tree showed that the samples from different water systems were clustered into different branches, the maximum genetic distance within the species was 2%, and the nucleotide diversity and haplotype diversity were relatively high (h = 0.887, π = 0.00777). These findings indicate that there is a large differentiation between the two geographically separated populations of *T. obscura* and the possibility of allopatric speciation. *T. obscura* and *T. orientalis* are also divided into two different monophyletic lines in the phylogenetic tree, which is consistent with the results of the analysis of Wu (2017).

Although only 3 specimens of *T. sp1* were collected in the Liangdang section of the Jialing River, there are obvious differences in morphological characteristics from other species of plateau loach. It should be identified as a new species that has not been reported, but more specimens should be collected for further confirmation. *T. sp2* was collected in the Jialing River, and showed degeneration of the membranous swim bladder, leaving only a small chamber, an anus near the start of the anal fin, the end of the pelvic fin adjacent to the anus, a large spot on the back of the body, a spot on the side of the body

and other morphological characteristics which were obviously different from those of the closely related species *T. obscura*. A detailed description of these newly discovered species is necessary to make it possible to record the relationship between morphology and molecular identification criteria (Versteirt et al., 2015).

## Conclusions

This study is the first comprehensive assessment of plateau loach species in a biodiversity hotspot using standard DNA barcoding. A high-density sample collection was carried out in this area to collect all known nominal species of plateau loach in this region. Although 14 of the 24 taxonomic species can be easily identified by DNA barcoding and classical morphological classification, 10 species pose serious challenges to standardized and automated molecular identification through mitochondrial DNA. Newly discovered species and cryptic species identified through DNA barcoding technology revealed the need for a taxonomic revision of the genus. If combined with the MOTUs identified here, the study of morphological features can be facilitated to support the delimitation of species and classification. At the same time, the use of single gene regions is an imperfect tool because their low sequence differences are ignored (Ratnasingham and Hebert, 2013). It is necessary to combine nuclear markers with ecological and biological data (Ajamma et al., 2016; Waldir et al., 2018; Durand et al., 2017; Wang et al., 2018) and expand the survey area and number of species to evaluate the species boundary of the plateau loach genus. This study provides a basis for protecting the biodiversity of plateau loach.

## Methods

# Sample collection

The samples were collected at 114 sampling sites in two exorheic rivers (Jialing River, which is the largest branch of the Yangtze River, and the upstream of the Yellow River) and three inland water bodies (Shiyanghe River, Heihe River and Shulehe River) located on the northeastern edge of the QTP from 2015 to 2018 (Fig. 1, Fig. 2). The specimens were caught using gill nets and cage nets. To accurately identify the fish based on taxonomic books, the fresh specimens were examined for specific morphological characters (Zhu and Wu, 1981; Wu and Wu, 1992; Wang, 1991). The muscle tissue of each specimen was preserved in 95% ethanol for DNA extraction, and the voucher specimens were stored in 10% formaldehyde solution for further examination of specific morphological characters (Table S1).

# Dna Extraction, Amplification And Sequencing

Total genomic DNA was extracted from the muscle tissue using the high-salt method, and a segment of 651 bp from the cytochrome oxidase I gene (COI) was amplified using the published primers FishF1 (5’ TCAACCAACCACAAAGACATTGGCAC3’) and FishR1 (5’ TAGACTTCTGGGTGGCCAAAGAATCA3’) (Ward et al., 2005). The PCR amplifications were performed in 30 μL, including 21.25 μL of molecular grade water,

3.0 μL of 10 × PCR buffer, 1.5 μL of each primer (10 mM), 1.5 μL of dNTPs (10 mM), 0.375 μL of Taq polymerase, and 1 μL of template DNA. The PCR conditions consisted of initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min followed by a hold at 4 °C. The PCR products were analysed in 1% agarose gels containing ethidium bromide stain and bidirectionally sequenced using sequencing primers. The purified PCR products were sequenced on an ABI 3730 XL DNA System.

# Genetic Distance Analyses

The sequencing chromatogram were checked by Chromas 1.45 software, and the forward and reverse sequences were assembled and edited with the SeqMan program (DNASTAR Inc., WI, USA). The sequences of each specimen generated in this study were compared and aligned using the ClustalW program. Haplotype number, haplotype diversity and nucleotide diversity were calculated with DnaSP 5.0 (Librado and Rozas, 2009). A neighbour-joining (NJ) tree, intraspecific and interspecific genetic distances were constructed based on the Kimura 2-parameter (K2P) distance model using MEGA, version 5.0with the bootstrap support values calculated with 1000 replicates (Tamura et al., 2011). Mr Bayes 3.2.5 software was used for Bayesian inference analysis (Ronquist et al. 2003). The posterior probability represents the credibility of each branch. In the BI method, the random tree is taken as the starting tree, and a GTR + G substitution model. Fifty million MCMC generations with a 10% burn-in were run independently. The maximum likelihood method was analysed with PhyML 3.0 software (Guindon et al. 2010), the substitution model was defined as GTR + I + G, and the number of substitution rate categories was set as 6, 100 times of self-guided method to test the confidence of each branch. These values were used to calculate the maximum, minimum and mean intraspecific and interspecific molecular operational taxonomic unit (MOTU) distances (Ramirez et al., 2017), and the barcoding gap was checked by the intraspecific and interspecific genetic distances (Shen et al., 2018). Finally, the DNA barcodes of species displaying haplotype sharing or mixed genealogies with close relatives were examined using Network 4.6 software (Bandelt et al., 1999).

# Species Delimitation

The species identified based on morphological characters were referred to as valid species, and species delimited by DNA sequences were referred to as MOTUs (Hutama et al., 2017; Shen et al., 2018). Four MOTU delimitation algorithms were used to delimit species. A Poisson tree processes (PTP) model was used to delimit species through the bPTP server (http://species.h-its.org/ptp/), including a Bayesian likelihood PTP (Zhang et al., 2013). The general mixed Yule-coalescent (GMYC) model in the R package Splits 1.0–19 (Fujisawa and Barraclough, 2013) was used to infer the MOTUs. An ultrametric tree was used as the input file for the PTP and GMYC and was reconstructed using Beast 2.4.8 (Bouckaert et al., 2014) based on a strict clock, a birth and death model and a GTR + G substitution model. Fifty million

MCMC generations with a 10% burn-in were run independently. The barcode index number (BIN) system was used to delimit MOTUs automatically in the BOLD workbench (http://v4.boldsystems.org/) (Ratnasingham and Hebert, 2013), and Automatic Barcode Gap Discovery (ABGD) was used via a web interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) (Puillandre et al., 2012).

# Abbreviations

QTP
the Qinghai-Tibet Plateau; COI:the mitochondrial cytochrome C oxidase subunit I; BOLD:the Barcode of Life Data Systems; NJ:the neighbor-joining method;ML:maximum likelihood method; BI:Bayesian inference method; K2P:Kimura 2-parameter; MOTU:molecular operational taxonomic unit; PTP:A Poisson tree processes model; GMYC:the general mixed Yule-coalescent model; BIN:the barcode index number system; ABGD:Automatic Barcode Gap Discovery.

# Declarations

### Ethics approval and consent to participate

All the sampling sites were not privately-owned or protected, and field sampling did not involve protected species. The collection of fishes complied with the guidelines of Gansu Fisheries Research Institute. The study was approved by the Laboratory Animal Ethics Committee of Gansu Fisheries Research Institute. All animals and experiments were conducted in accordance with the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, China).

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### Authors’ contributions

LZ and DYY conceived and designed the experimental plan. WT, LZ performed experiments. WT, ZYP and YZY analyzed and interpreted the sequence data. WT, DYY drafted the manuscript. All authors read and approved the final manuscript.

### Consent to publish

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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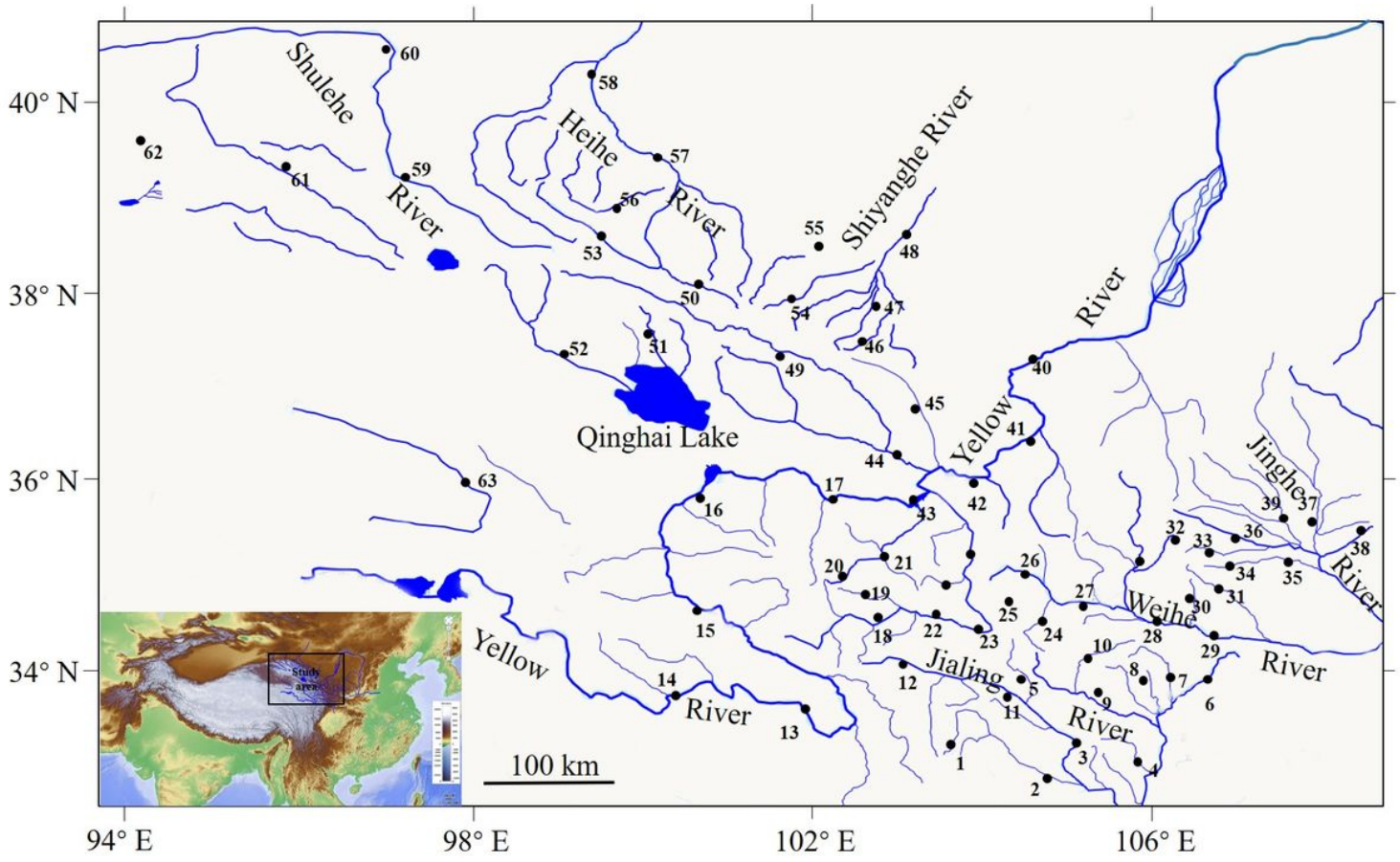
# Figures



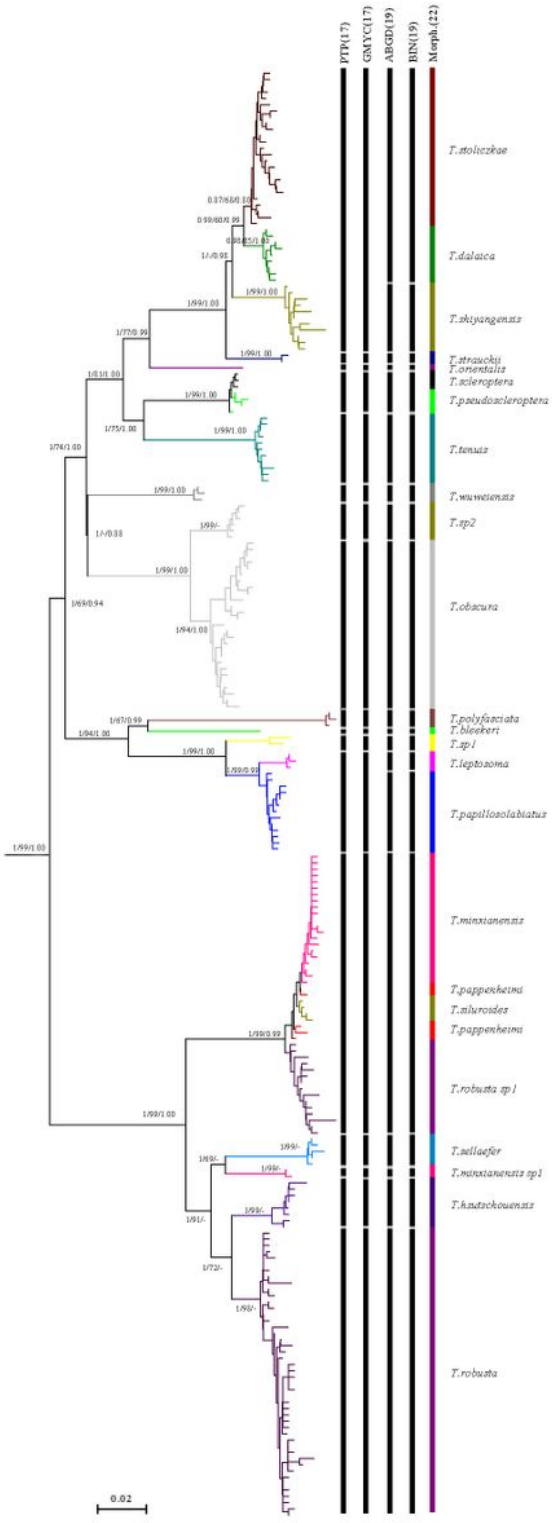

## Figure 1

Collection sites. Details of the 111 sites and collected specimens are provided in Table S1. (The sectors name of sampling sites: 1. Jiuzhai; 2.Wenxian; 3.Wudou; 4.Kangxian; 5.Tanchang; 6.Liangdang; 7.Huixian; 8.Chengxian; 9.Xihe; 10.Lixian; 11.Zhouqu; 12.Diebu; 13.Maqu; 14.Hongyuan; 15.Henan; 16.Longyangxia; 17.Jishishan; 18.Luqu; 19.Hezuo; 20.Xiahe; 21.Linxia; 22.Zhuoni; 23.Minxian; 24.Wushan; 25.Zhangxian; 26.Weiyuan; 27.Gangu; 28.Qinzhou; 29.Maiji; 30.Qingshui; 31.Zhangjiachuan; 32.Jingning; 33.Chongxin; 34.Huating; 35.Lingtai; 36.Kongtong; 37.Xifeng; 38.Ningxian; 39.Zhenyuan; 40.Wufo; 41.Pingchuan; 42.Lanzhou; 43.Yongjing; 44.Minhe; 45.Yongdeng; 46.Zhuanglang; 47.Liangzhou; 48.Minqin; 49.Menyuan; 50.Arou; 51.Gangcha; 52.Tianjun; 53.Qilian; 54.Huangcheng; 55.Jinchang; 56.Sunan; 57.Linze; 58.Gaotai; 59.Yumen; 60.Guazhou; 61.Subei; 62.Akesai. This base map is from 91 Vita Assistant software http://www.91weitu.com/index.htm, edited in Adobe Photoshop CS5 software.). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.


## Figure 2

Studied specimens of Triplophysa. 1. T. dalaica G003; 2. T. stoliczkai G0070; 3. T. polyfasciata G0187; 4. T. bleekeri G0195 ;5. T. robusta G0531; 6. T. obscura G0822; 7. T. pappenheimi G0852; 8. T. siluroides G0873; 9. T. hsutschouensis G0915; 10. T. minxianensis GS0213; 11. T. pseudoscleroptera GS0216; 12. T. scleroptera GS0230;13. T. strauchii GS0273; 14. T. papillosolabiatus GS0305; 15. T. wuweiensis GS0381;

16. T. orientalis GS0400; 17. T. shiyangensis GS0432; 18. T. leptosoma GS0441; 19. T. tenuis GS0500; 20. T. sellaefer GS0560; 21. T. sp1 GS562; 22. T. sp2 GS565. Scale bars equal 1 cm




## Figure 3

The phylogenetic tree showing the clustering of the OTUs obtained by the four MOTUs delimitation algorithms. (The values at the node represent support values in NJ/ML/BI analysis respectively. The

length of branch indicates the percentage of divergence.) Figure 4 Relationship between maximum genetic distance within species and nearest neighbor genetic distance among species.

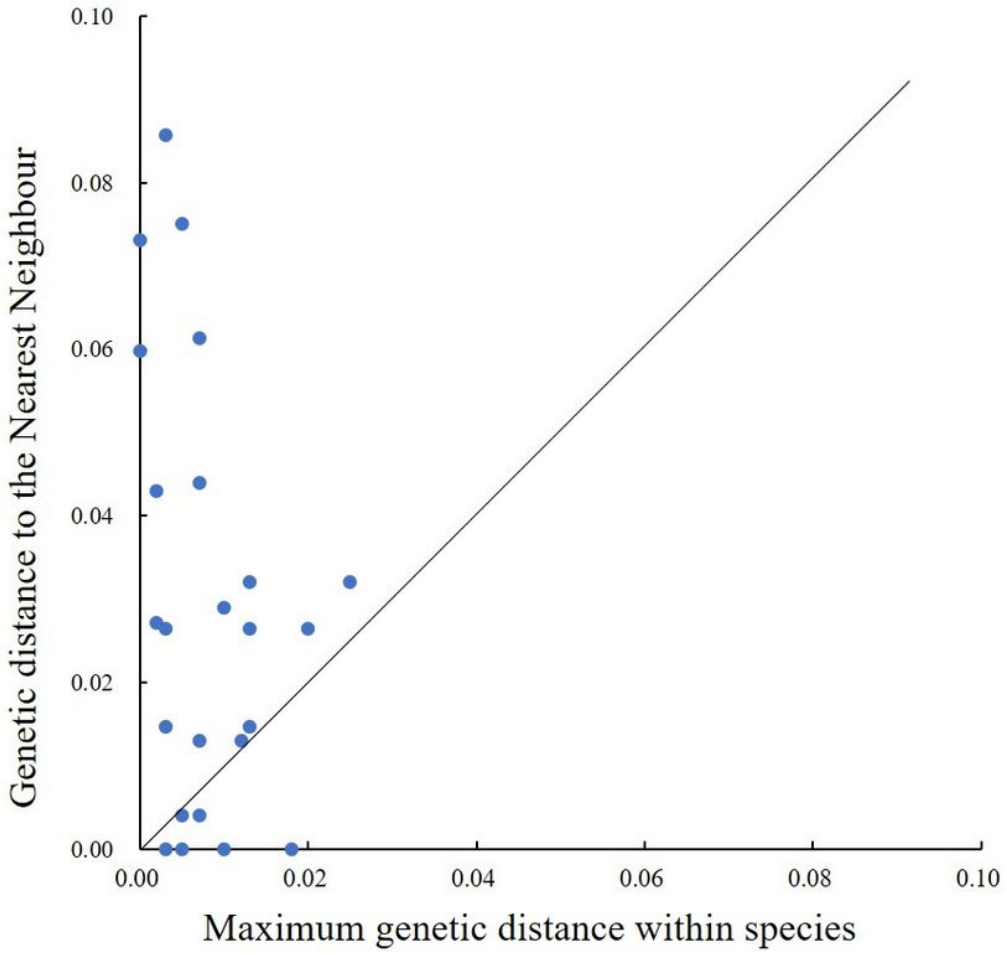



**Figure 4**

Relationship between maximum genetic distance within species and nearest neighbor genetic distance among species.

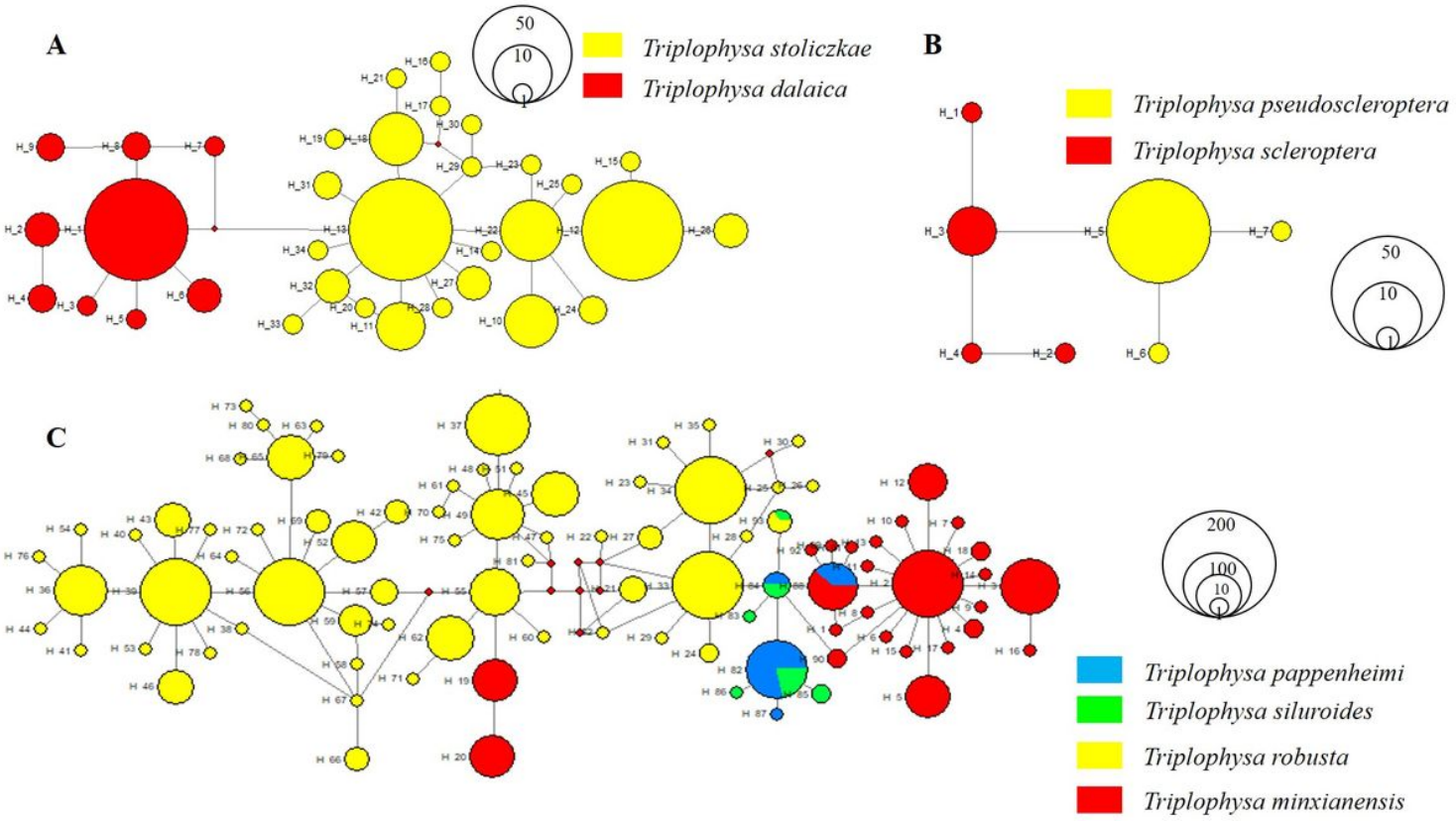



**Figure 5**

The Haplotype networks for the species group involved in mixed genealogies.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx