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Phylogeographical and Morphological Analyses of *Triplophysa stenura* (Cypriniformes: Nemacheilidae) from the Three Parallel Rivers Region, China

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Qiu Ren, Jun-Xing Yang, and Xiao-Yong Chen (2018) *Triplophysa stenura* is an indigenous and widely distributed loach in the Qinghai-Tibet plateau and adjacent Three Parallel Rivers region of China. Morphological and phylogenetic analyses were performed in order to explore the genetic and morphological variation in *T. stenura* populations from different geographic regions and infer the divergence time and relationships between populations from the three rivers (Jinsha River, Nu River and Lancang River). Two mitochondrial genes (Cyt *b*, D-Loop) and 35 morphological characters were selected for genetic and morphological analyses, respectively. Phylogenetic and network analyses reveal that *T. stenura* is a single lineage with three well supported clades strictly corresponding to specific river systems. Divergence time analysis suggests that the divergence of *T. stenura* and formation of the Three Parallel Rivers are closely associated with the Kun-Huang Movement (1.1-0.6 MYA), which lead to the uplift of the Qinghai-Tibet plateau. AMOVA reveals that there is moderate genetic differentiation among populations. Results from ANOVA suggest that several morphological characters show significant variation among populations and drainages. Descriptive morphological variation (e.g., color pattern) in different populations of *T. stenura* clarifies a set of characters that can be used to accurately identify members of this group in the future. We conclude that *T. stenura* has moderate population genetic structure and character variation in this study area and the divergence and evolution of *T. stenura* is associated with the uplift of the Qinghai-Tibet plateau.

Key words: *Triplophysa stenura*, Phylogeny, Morphology, Biogeography, Three Parallel Rivers region.

BACKGROUND

The Three Parallel Rivers region of China is located in northwest Yunnan Province, and represents the eastern part of area that resulted from the collision between the Indian and Eurasian plates. With the continental collision, three rivers (Jinsha, Lancang and Nu rivers) along with three mountain ranges (Gaoligong, Nu and Yunlong) formed north-southwards (Fig. 1). These ranges demonstrate one of the most unique natural landscapes on Earth and have been recognized as World Heritage locations (Ming and Shi 2006; Ming 2007). Some research has suggested that the formation of the Three Parallel Rivers area was closely correlated with the sharp uplift of the Qinghai-Tibet plateau in the late tertiary (3.6 Million Years Ago, MYA), and this major change in the topography of the Qinghai-Tibet plateau area has had profound impacts on speciation, phylogeographic structure and genetic patterns of species endemic to China (Ren et al. 1959; Li et

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al. 1995; Li and Fang 1998; Zheng et al. 2006). Therefore, the phylogeographic investigations of endemic fish species can reveal important information as to the origin and relationships between faunas of the Three Parallel Rivers area.

The genus *Triplophysa* Rendahl, 1933 includes approximately 142 species, making it one of the largest loach groups in the family Nemacheilidae, and 85% of this diversity is known from China (He 2008; Ren et al. 2012; Froese and Pauly 2017). The genus is wide spread in the Qinghai-Tibet plateau and adjacent areas (Wu and Wu 1991; Zhu 1989). Due to the similar appearance of *Triplophysa* species and limited variation between characters currently being examined, it can be difficult to distinguish taxa and geographic variants of species. This is especially true for widespread species whose morphological features (*i.e.* mouth structure, color pattern, intestine type) vary from different localities or river drainages.

Triplophysa stenura (Herzenstein 1888) is a small to medium sized loach, which is widely distributed in the Qinghai-Tibet plateau and upper reaches of Yangtze, Lancang (upper Mekong River) and Nu (upper Salween River) Rivers of China (Zhu 1989; Chen 1998). This species lives in swift-flowing streams, where it moves amongst rocky crevices by swimming in a saltatory manner. It mainly scrapes surfaces in streams for algae and sometimes feeds on some small aquatic invertebrates (Wu and Wu 1991). *T. stenura* is diagnosed by a combination of characters: caudal peduncle nearly round, depth reduced sharply

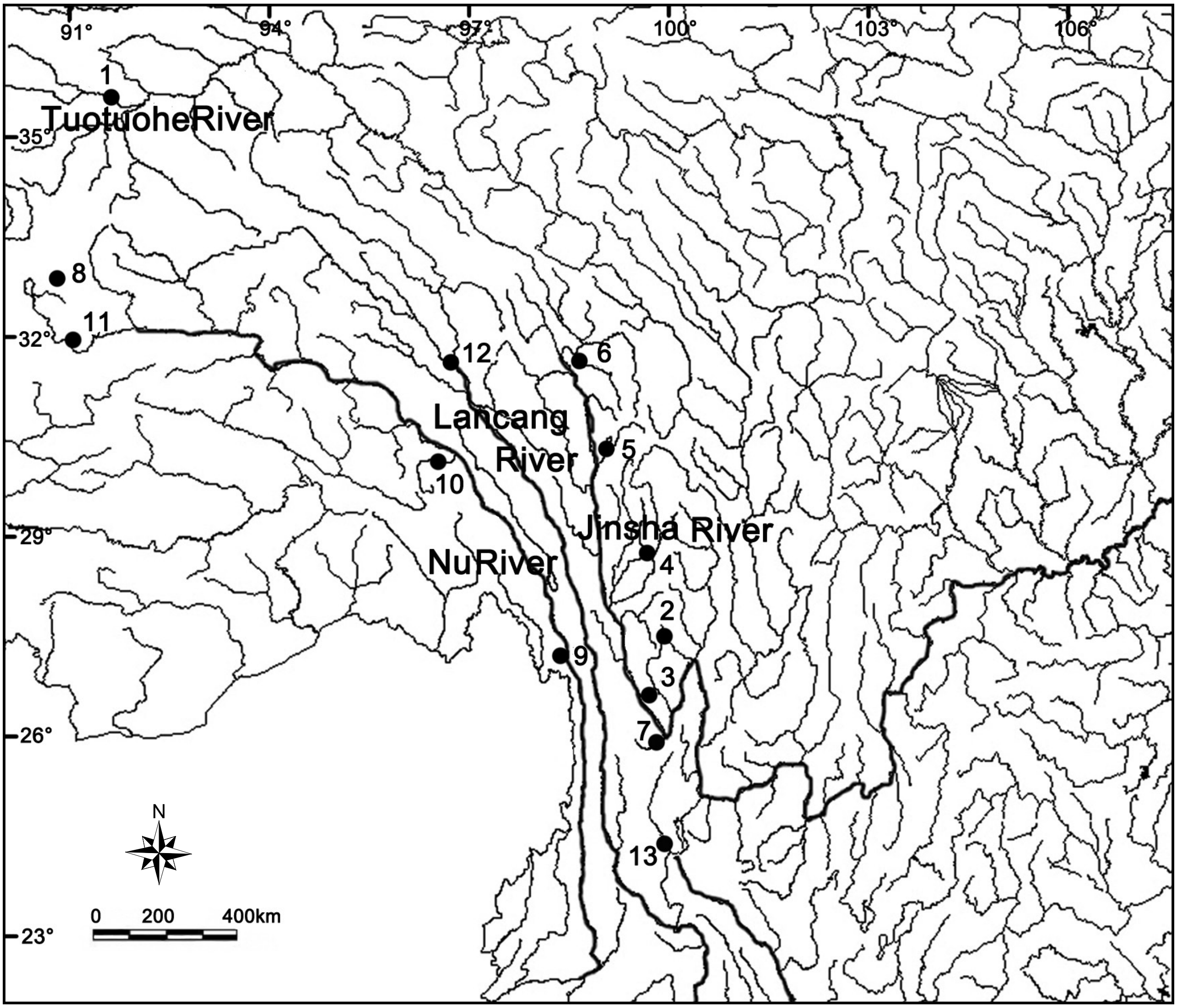

Fig. 1. Map showing the collection localities of *Triplophysa stenura* in this study (numbers correspond to name of localities in Table 1).

Table 1. Information on the sequences and specimens used in this study

Species	Location (abbr. and site number)	Drainage	Haplotype number	Voucher number	Genbank accession number		Specimens examined Catalog & total number
					Cytb	D-Loop	
Triplophysa stenura	Tuotuohe, Qinghai (TTH) (1)	Jinsha R.	H5	20040803001	KJ650345	KJ650374	2004002175-2004002195 (21)
			H6	20040803002	KJ650370	KJ650394	
			H7	20040803003	KJ650369	KJ650393	
			H8	20040803007	KJ650368	-	
			H4	20040803008	KJ650367	KJ650392	
	Xiaozhongdian, Yunnan(XZDH) (2)	Jinsha R.	H1	20040620001	JN837657	KJ650397	
			H2	20040620002	KJ650353	KJ650379	
			H3	20040620003	KJ650371	KJ650395	
			H4	20040620004	KJ650352	KJ650378	
	Yongsheng, Yunnan (YS) (3)	Jinsha R.	H11	041106046	KJ650365	KJ650390	
			H12	041106047	KJ650364	KJ650389	
			H12	041106048	KJ650363	KJ650388	
			H11	041106049	KJ650362	KJ650387	
	Xiangcheng, Sichuan (XC) (4)	Jinsha R.	H13	YJ2009033	KJ650361	KJ650386	
			H13	YJ2009034	KJ650360	-	
			H14	YJ2009035	KJ650359	KJ650385	
			H13	YJ2009036	KJ650358	KJ650384	
	Batang, Sichuan (BT) (5)	Jinsha R.	H17	YJ2009079	KJ650357	KJ650383	2009003584-2009003590 (7)
			H16	YJ2009080	KJ650356	KJ650382	
			H6	YJ2009081	KJ650373	-	
			H15	YJ2009082	KJ650350	KJ650376	
	Baiyu, Sichuan (BY) (6)	Jinsha R.	H18	2006009504	KJ650344	-	2006009504-2006009508 (5)
			H16	2006009505	KJ650372	KJ650396	
			H19	2006009507	KJ650355	KJ650381	
	Shigu, Yunnan (SG) (7)	Jinsha R.	-		-	-	2011000001-2011000008 (12)
	Anduoqu, Tibet (ADQ) (8)	Nu R.	H9	20040804001	KJ650366	KJ650391	2004002201-2004002213 (13)
			H10	20040804002	KJ650351	KJ650377	
	Gongshan, Yunnan (GS) (9)	Nu R.	H21	-	KJ650348	-	2000198-2000199 (2)
			H20	CJF1806	KJ650354	KJ650380	
	Baxiu, Tibet (BX) (10)	Nu R.	H22	-	KJ650347	-	
	Naqu, Tibet (NQ) (11)	Nu R.	H23	-	KJ650349	-	
	Changdu, Tibet (CD) (12)	Lancang R.	H24	-	KJ650346	KJ650375	20100042-20100048 (8)
	Yangbi, Yunnan (YB) (13)	Lancang R.	-		-	-	1974000595-1974000617 (23)
	GB1*		H25		DQ105247	DQ105319	
	GB2*		H26		DQ105246	DQ105318	
T. stewarti	Milin, Tibet				DQ105248		
T. orientalis	Xining, Qinghai				DQ105251		
T. stoliczkaeas	Gangca, Qinghai				DQ105249		

*Sequence obtained from Genbank.

towards the caudal-fin base; caudal peduncle length greater than head length; posterior chamber of air bladder degenerate; intestine bending in one to three coils behind stomach; depressed pelvic-fin with tip extending beyond anus.

Mitochondrial DNA is widely used to infer phylogenetic relationships among many species or groups at different taxonomic levels. Cytochrome *b* (Cyt *b*) and control region gene (D-Loop) have proven useful in resolving lower level relationships (interspecific and intraspecific) between fishes (Saka et al. 2003; He et al. 2006; Tang et al. 2006; Liu et al. 2007). In this study, these two genes are used as molecular markers to reconstruct the phylogenetics and geographic relationships within *T. stenura* and explore genetic variation and biogeographic patterns. With a phylogenetic resolution within *T. stenura*, the estimated age of the clades using a molecular clock inference provides an opportunity to examine the age of the species. Our analysis thus focuses on exploring genetic variation and evolution of *T. stenura*, and geographic distributions of forms geographically across the Three Parallel Rivers area. We also hypothesize geological and geographical events that occurred in the plateau that may be related to observed divergences. Morphological data and analyses serve as a parallel analysis to explore morphological variation and divergences among different populations of *T. stenura*. Finally, diagnostic characters typically used in the identification of *Triplophysa* species are discussed, which provides a reference to the taxonomy and identification of species in the genus for future studies of biodiversity.

MATERIALS AND METHODS

Sampling

Thirty-one tissue samples were collected from 14 localities in the Three Parallel Rivers area for Cyt *b* and D-Loop analyses. Tissues were from the base of the right pelvic fin of fresh specimens and were preserved in 99.5% ethanol. A total of 91 specimens were collected from eight localities for morphological examination and analyses. These specimens were fixed in 8-10% formalin, rinsed thoroughly and preserved in 75% ethanol for long-term storage. Specimens are deposited in the collection of Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences. *Triplophysa stewarti* which is thought to be closely related to *T. stenura*, and two other *Triplophysa* species - *T. orientalis*, and *T. stoliczkaeas* - were chosen as outgroups. Identification of specimens follows Zhu (1989). Detailed information regarding samples is provided in figures 1, 2 and table 1.

Genetic analyses

Total genomic DNA was extracted from fin tissues following standard methods (Sambrook et al. 1989). The Cyt *b* and D-Loop genes were amplified by polymerase chain reaction (PCR). Primer sets L14724 (5'-GACTTGAAAAACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Xiao et al. 2001) were used to amplify the Cyt *b* gene, and DL1 (5'-ACC CCT GGC TCC CAA AGC-3') and DH2 (5'-ATC TTA GCA TCT TCA GTG-3') were used to amplify D-Loop gene (Liu et al. 2002). A total volume of 50 μL in each reaction included: 37.75 μL double distilled water, 5 μL of 10 × reaction buffer (Mg^{2+} Plus), 3 μL of dNTP (each 2.5 mM), 2 μL BSA, 0.25 μL (5U/μL) taq polymerase, 1 μL (10 μm) of each oligonucleotide primer and 1 μL of total genomic DNA template. The PCR amplification was performed at an initial denaturation step at 95°C for 3 mins, followed by denaturing at 94°C for 1 min, annealing at 52°C for 1 min and extending at 72°C for 1 min for a total of 40 cycles; the final cycle was a 10 min extension at 72°C, deposited at 4°C. Primers used for sequencing were the same for PCR amplifications. The unpurified fragments were sequenced by Kunming Shuoyang Technology Co., Ltd. All sequences obtained are deposited in GenBank; accession numbers are listed in table 1.

Nucleotide sequences were initially assembled using SeqMan 6.0 and Editseq 6.0 in the DNA Star package (DNAStar Inc., U.S.), with manual correction if necessary. Sequences were aligned using Clustal W in MEGA 5 (Tamura et al. 2011) and translated into amino acids for confirmation of alignment and assignment of codon positions and detection of stop codons. DnaSP 4.10 (Rozas et al. 2003) and DAMBE 5.1.2 (Xia 2013) were used to identify haplotypes among all sequences. Aligned sequence data were imported into MEGA 5 for nucleotide composition analysis. The Kimura two-parameter model (Kimura 1980) was used to calculate nucleotide divergence (genetic distances). Nucleotide saturation was analyzed using DAMBE 5.1.2, and a plot of absolute number of transitions (Ti) and transversions (Tv) against TN93 distance revealed saturation in substitutions. Hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) was implemented using Arlequin 3.5 (Excoffier et al. 2010) to assess genetic structure and differentiation among populations. A haplotype network was constructed with Network 4.6.0.0 (Bandelt et al. 1999) using median-joining method (Weights = 10; epsilon = 10; Weighting transition/transversion = 1:3). Maximum Likelihood (ML) was implemented by PAUP*4.0b10 (Swofford 2003), the best-fit model of nucleotide evolution (GTR + I + G) was selected by Akaike Information Criterion (AIC) (Akaike 1974) in Modeltest 3.7. Support for recovered clades was measured using bootstrap analysis with 100 total pseudoreplicates. Bayesian tree resolution and determined posterior probabilities of recovered clades was completed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The most appropriate model of nucleotide substitution was selected by Modeltest 3.7. Analyses included 10,000,000 generations of Markov chains, which sampled every 1000 generations, to yield 10,000 trees. The first 25% of the trees were discarded as burn-in. A majority rule consensus tree calculated from the remaining 7,501 trees was used to determine posterior probabilities.

Cyt *b* was used to estimate divergence times in this analysis as we had more data for this particular gene than the D-Loop gene. The average substitution rate of Cyt *b* for cyprinids, 1.05% per million years (Dowling et al. 2002), was used. Divergence time was estimated using a Bayesian approach with BEAST 2.1.2 (Drummond and Rambaut 2007) and the input file was created using BEAUTi. Analysis was performed using the GTR + I + G model. The coalescent process, which allows for the estimation of population-level parameters, was chosen as the prior to generate patterns of lineage divergences. MCMC process was set as two independent runs of 10,000,000 generations. Trees were sampled every 1,000 generations and the first 10% of trees were discarded as burn-in. The log results were examined in Tracer 1.6 (Rambaut et al. 2013). The maximum credibility tree was summarized using TreeAnnotator in BEAST unit and visualized in Figtree 1.31 (Rambaut 2009).

Morphological analyses

A total of 24 morphometric characters (Table S1) were measured on the left side of specimens using a digital caliper system. Measurements

Fig. 2. Lateral views of *Triplophysa stenura* individuals from different localities. (A) Changdu, Tibet. KIZ 20100045 138 mm SL; (B) Batang, Sichuan KIZ 2009003584 113 mm SL; (C) Baiyu, Sichuan KIZ 2006009508 121 mm SL; (D) Shigu, Yunnan KIZ 201100003 86.6 mm SL; (E) Gongshan, Yunnan KIZ 20007198 78.4 mm SL; (F) Tuotuohe, Qinghai KIZ 2004002141 68.9 mm SL; (G) Anduoqu, Tibet KIZ 2004002201 79.9 mm SL; (H) Yangbi,Yunnan KIZ 1974000595 60.1 mm SL. Scale bar = 1 cm.

were recorded to the nearest 0.01 mm following methods of Chu and Chen (1989) and He (2008). A total of 11 descriptive characters (Table 2) were carefully examined using a microscope. Preliminary statistical analysis of all morphometric data was performed using Microsoft Excel. To alleviate the influence of allometry and body-size differences and normalize the data, measurements taken on the head were divided by head length and taken on the rest of body were divided by standard length, and all measurements were log10 transformed. To investigate whether there is significant character variation among populations and drainages and where the variation is, one-way analyses of variation (ANOVA) and post-hoc Tukey's test were conducted using R (R Core Team 2013). Principle component analysis (PCA) was performed using SPSS 20.0 for Windows

Table 2. Morphological character comparisons between populations of *Triplophysa stenura*

	Gongshan,Yunnan	Anduoqu, Tibet	Shigu, Yunnan	Batang, Sichuan
SL (mm)	69.3-78.4	72.5-92.7	61.9-86.6	48.6-113.7
Body type	medium, cylindrical	medium, slim	medium, slim	robust, cylindrical
Ground color	brownish yellow (ethanol)	pale grey (ethanol)	pale grey (ethanol)	light yellow (ethanol)
Mouth	thick with strong furrow	medium thick with slight furrow	medium thick with slight furrow	thick with strong furrow
Lower jaw	spade-like	spade-like	spoon-like	spade-like and spoon-like
Snout	blunt	pointed	pointed	blunt
Color pattern	6-8 blotches along dorsal midline	6-8 blotches along dorsal midline	colors mottled, no regular blotches along dorsal midline	6-10 blotches along dorsal midline
Dorsal fin origin	opposite to pelvic-fin origin	opposite to pelvic-fin origin	slightly posterior to pelvic-fin origin	slightly anterior to pelvic-fin origin
Intestine type	one coil	three coils	two coils	two coils
Caudal fin	slight concave	slight concave	deep concave	slight concave
Caudal peduncle	round	round	round	round
The tip of pelvic fin	extending beyond anus but not reaching the origin of anal fin	extending beyond anus but not reaching the origin of anal fin	extending beyond anus and reaching the origin of anal fin	extending beyond anus but not reaching the origin of anal fin

	Baiyu, Sichuan	Tuotuohe, Qinghai	Changdu, Tibet	Yangbi, Yunnan
SL (mm)	106.5-141.2	57.5-78.1	91.4-140.6	51.8-81.9
Body type	robust, cylindrical	medium, slim	slim, cylindrical	medium, cylindrical
Ground color	light yellow (ethanol)	pale grey (ethanol)	light yellow (ethanol)	brownish yellow (formalin)
Mouth	thick with strong furrow	medium thick with slight furrow	medium thick with slight furrow	thick with strong furrow
Lower jaw	spade-like and spoon-like	spade-like	spade-like	spoon-like
Snout	blunt	pointed	blunt	blunt
Color pattern	6-8 blotches along dorsal midline	6-8 blotches along dorsal midline	colors mottled, no regular blotches along dorsal midline	6-8 blotches along dorsal midline
Dorsal fin origin	slightly posterior to pelvic-fin origin	slightly posterior to pelvic-fin origin	slightly posterior to pelvic-fin origin	slightly anterior to pelvic-fin origin
Intestine type	three coils	three coils	two coils	one coil
Caudal fin	slight concave	slight concave	slight concave	slight concave
Caudal peduncle	round	round	round	slightly depressed
The tip of pelvic fin	extending beyond anus but not reaching the origin of anal fin	extending beyond anus and reaching the origin of anal fin	extending beyond anus but not reaching the origin of anal fin	extending beyond anus but not reaching the origin of anal fin

(SPSS, Chicago, IL, USA).

RESULTS

Sequence variation

In this study, 35 Cyt *b* sequences (33 from the present study and two from GenBank) yielded 26 unique haplotypes (Table 1). Cyt *b* gene was 1140bp in length, of which 89 sites were variable. Among the variable sites, 56 were parsimony informative polymorphic sites and 33 were singleton polymorphic sites. The average base composition of the 26 haplotypes was A = 25.5%, T = 29.7%, G = 17.5%, C = 27.3%. The mean base composition of Cyt *b* sequences have a low G content and are almost equal A, T and C contents, which is consistent with other Cobitid studies (He et al. 2006; Tang et al. 2006). The 26 sequences of D-Loop (plus 2 from GenBank) yielded 18 haplotypes. D-Loop sequences were 928bp, of which 801 sites were constant and 110 were variable. Among the variable sites, 39 were parsimony informative polymorphic sites and 71 were singleton polymorphic sites. The average base compositions of the 18 haplotypes were A = 33.7%, T = 31.8%, G = 13.6%, C = 20.9%. Haplotypes were shared by individuals within the same population or among different populations from the same river drainage. Individuals from different drainages, however, did not share haplotypes.

Phylogenetic relationships and divergence times

Independent phylogenetic analyses of 33 Cyt *b*, 26 D-Loop sequences, and the concatenated Cyt *b* and D-Loop data using ML and BI methods yielded similar topologies. All analyses resolved three strongly supported clades (Fig. 3). The haplotype network analysis yielded a phylogenetic network (Fig. 4) consistent with the phylogenetic trees resolved. The phylogenetic resolution of these genes supported the gene trees of *T. stenura* as being monophyletic and suggested that individuals within the same drainage but from different populations formed a common lineage. Populations distributed in Jinsha River drainage formed clade A; populations from Nu River drainage formed clade B; and clade C included individuals from the Lancang River drainage. Divergence time analysis estimated that *T. stenura* diverged about 3.88 MYA. The Jinsha River clade separated from the Nu and Lancang river clade about 1.39 MYA, and Lancang River and Nu River clades diverged about 0.61 MYA.

Genetic distances and structures

Genetic distances among individuals (including outgroup individual) ranged from 0.0009 to 0.0450; among populations ranged from 0.0169 to 0.0363; and between ingroups and outgroups ranged from 0.0940 to 0.1488. AMOVA (Table 3) revealed that most of the genetic variation (87.98%) was found within populations (F_{ST} = 0.120, $P <$ 0.001); significant genetic differentiation was also found among populations within drainages (F_{SC} = 0.119, $P < 0.001$); the least genetic variation was present among drainages, which also had no significant differentiation (F_{CT} = 0.001, $P > 0.05$).

Morphological variation

Comparisons of the 11 morphological characters among eight populations are shown in table 3. These results indicate that some characters, such as body shape, color pattern, mouth structure and intestine structure, demonstrated variation among populations of the widespread *T. stenura*. PCA and ANOVA analyses included 24 morphometric characters from eight geographic populations of the three drainages. Results of the PCA indicate that the first seven principal components (PCs) accounted for 84.6% of total variation. Scatter plots of the first two PCs, which accounted for 53.8%, illustrate that individuals were not clearly distinguishable by drainage (Fig. 5A), but they could be distinguishable by population within drainage separately (Fig. 5B, C and D). ANOVA results indicate that all of characters except for vent-anal distance (VAD) showed significant or extremely significant differences among populations. Sixteen characters demonstrated significant or extremely significant differences among drainages. The post-hoc Tukey's test identified where the differences are. The detailed ANOVA results are summarized in table S1.

DISCUSSION

Molecular phylogeny and biogeography

Results of the haplotype network and phylogenetic analyses support the hypothesis that

T. stenura forms a monophyletic group, including three clades following river drainages. Haplotypes are all unique to river drainages; none are shared among populations from different river drainages. These findings indicate that *T. stenura* exhibits clear geographical structure in the Three Parallel Rivers region. We assume that geographic barriers are the main factors hindering gene exchange between different drainages and leading to genetic differentiation. Once the independent drainages were formed following the uplift, gene exchange among these drainages must have been impeded, providing the opportunity for divergence in isolation.

The phylogeography of *T. stenura* reflects relationships between the three rivers, wherein populations from the Nu River were more closely related to those from the Lancang River than to those from the Jinsha River. These findings agree with results from Chu and Chen (1990), which is based on broad research of the ichthyofauna of this region. Chen (1998) and Chu and Chen (1989) proposed that the Nu and Lancang Rivers may have connected during the early-middle Pleistocene. Divergence time results suggest that the Jinsha River clade was first divided from the remaining clades at about 1.39 MYA, and then the Lancang River and Nu River clades split at around 0.61 MYA; both findings are consistent with Ming and Shi (2006) and consistent with the formation of the three rivers in early-middle Pleistocene. Using evidence from quaternary sediment characters, Zhang et al. (1998) suggested the formation of the Jinsha River and its development to its present state dated 1.54 ± 0.178 MYA, and the Nu River dated back to the early-middle Pleistocene (He et al. 1992). Phylogenetic and biogeographic research by Guo et al. (2005) on sisorid catfishes

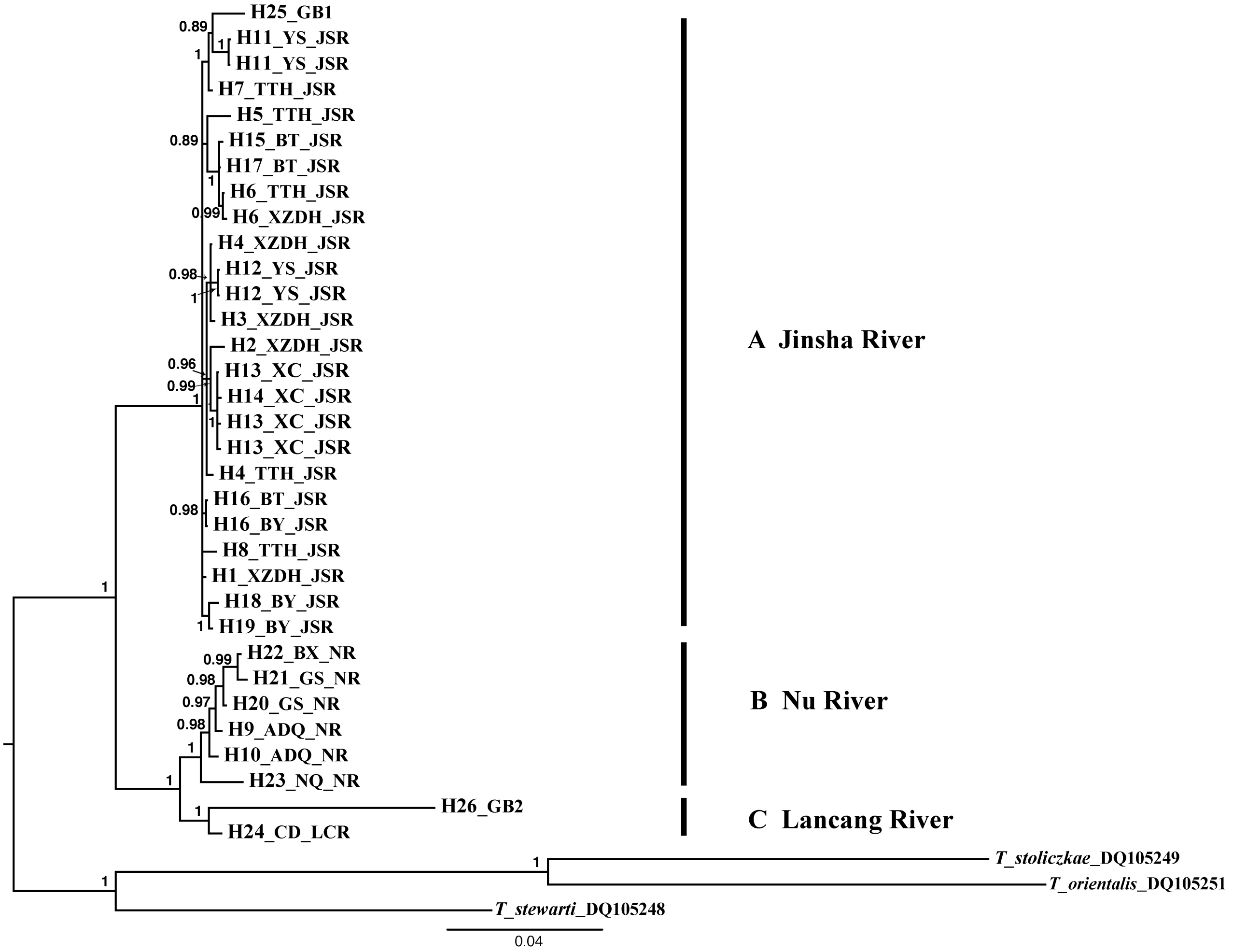

Fig. 3. Phylogenetic relationships within *Triplophysa stenura* as inferred by Bayesian analysis of combined Cytochrome *b* and D-Loop sequences. (Posterior probabilities are shown on nodes).

revealed that the Jinsha River was the first formed and isolated, followed by the Lancang River, then the Nu and Irrawardy Rivers; these results are partially consistent with findings supported in the present study.

Some geographic and geological linked research (Li and Fang 1998; Zheng et al. 2006) has shown that the uplift of the Qinghai-Tibetan plateau included four movements: Qinghai-Tibetan Movement (3.6-1.7 Ma), Kun-Huang Movement (1.1-0.6 Ma), Gonghe Movement (0.15 Ma) and Huaxi Movement (30 Ka). This relatively rapid uplift was initiated about 3.4 MYA, and included three phases: A, B and C, at the estimated ages of 3.6 MYA, 2.5 MYA and 1.7 MYA, respectively. Estimates herein identified the time of isolation and origin of the three rivers at approximately the middle period of the Kun-Huang Movement. According to Ming and Shi (2006), the Three Parallel Rivers region located in the northwestern Yunnan Province was formed and influenced by the uplift of the Qinghai-Tibetan and Yunnan

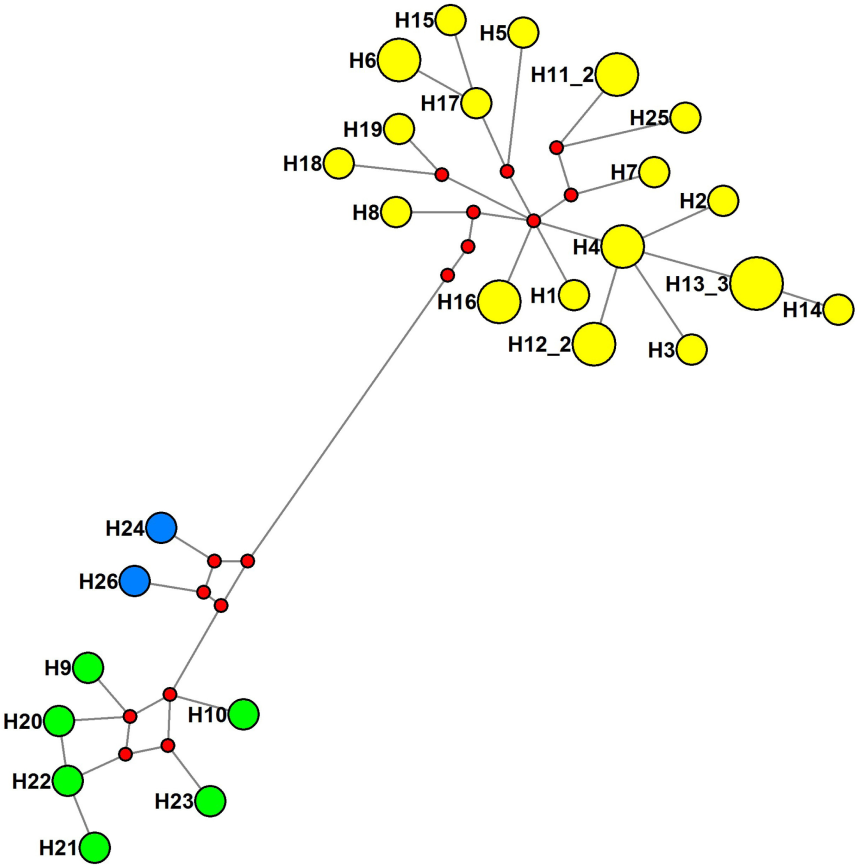

Fig. 4. The haplotype network constructed by median-joining method. Haplotypes are named as in table 1. Red dots are median vectors, which are hypothesized sequences to connect existing sequences within the network under maximum parsimony. The yellow circles are haplotypes from the Jinsha River drainage, blue circles are haplotypes from the Lancang River drainage, and green circles represent haplotypes from the Nu River drainage. The size of the circles is proportional to haplotype frequency.

Table 3. Hierarchical analysis of molecular variance (AMOVA) for *Triplophysa stenura*

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation (%)	*P*	Fixation indices
Among drainages	2	1.08	0.00066	0.13	$P > 0.05$	$F_{CT} = 0.00132$
Among populations/ within drainages	10	5.93	0.05914	11.89	$P < 0.001$	$F_{SC} = 0.11908$
Within populations	20	8.75	0.43750	87.98	$P < 0.001$	$F_{ST} = 0.12025$
Total	32	15.76	0.49730	100.00%		

Plateaus to the northwest from the Kun-Huang Movement and headwater erosion and incision of river systems and tributaries. The Kun-Huang Movement was the most transformative event of the northwest Lengthways Range and Gorge region as well as the Landform-River development of China, both of which have been proposed to have a major influences on the modern landform formations and ecological and environment changes in the Three Parallel Rivers region. This phenomenon is similar to that hypothesized to have occurred in the highly specialized schizothoracine fishes (He and Chen 2007).

Morphological variation

Examination and comparisons of specimens from different localities revealed several descriptive characters (visible to the eye) in *T. stenura* with significant variation (Table 2). These features include body size, body color, mouth structure and intestine shape. We assume that the body shape differences are the result of a series of factors relating to population genetic differentiation, food abundance and habitat environment. Color variation might result from environmental conditions, and factors to protect fish from

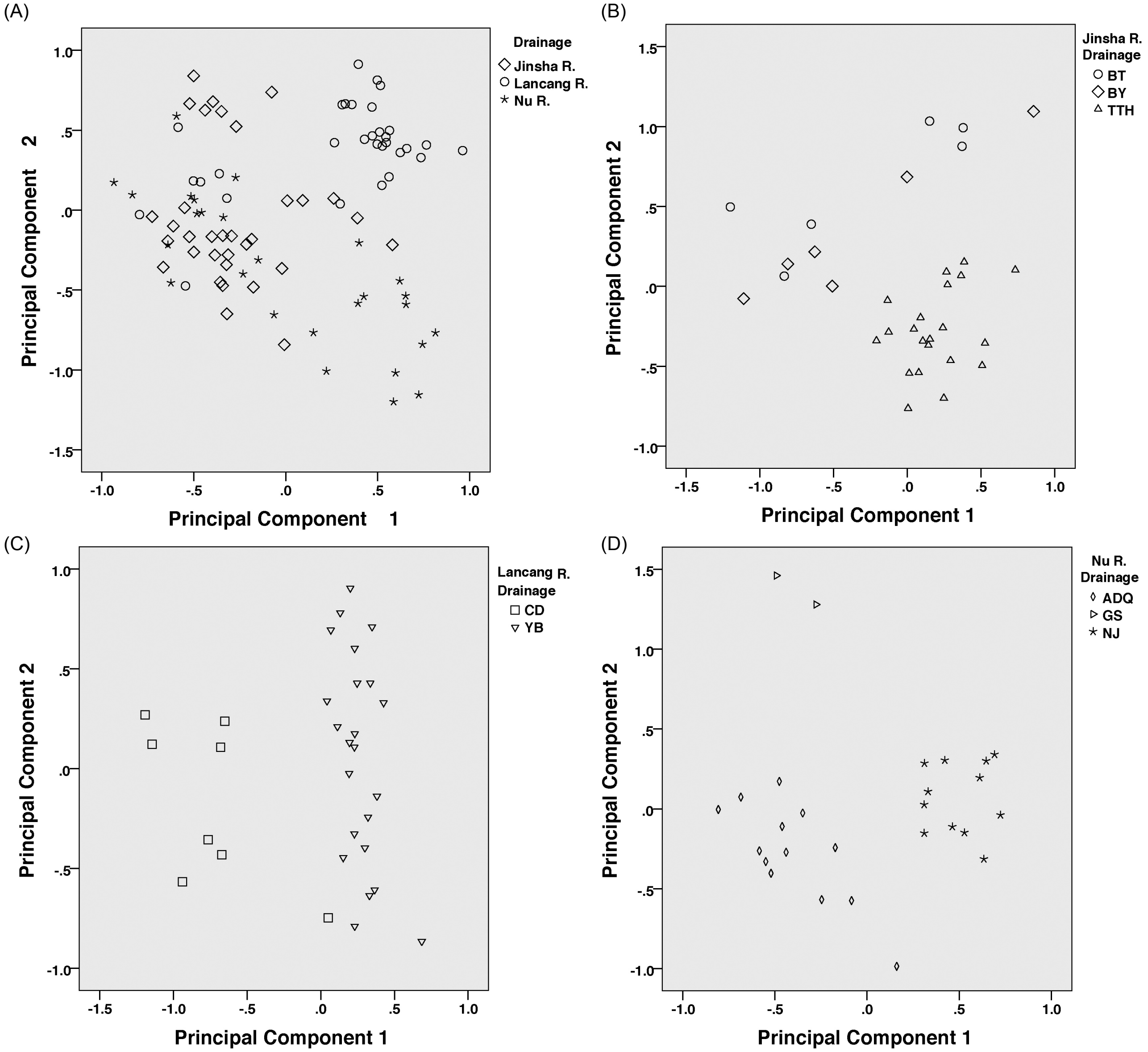

Fig. 5. Scatter plots of the first two principal components for *Triplophysa stenura* individuals from three drainages (A) and populations within three drainages respectively (B: Jinsha R. Drainage; C: Lancang R. Drainage; D: Nu R. Drainage). The locality abbreviations are shown in table 1.

predation. The ability to adjust color patterns of *Triplophysa* is hypothesized to produce adaptive changes to adjust to the surrounding area in the stream and remain cryptic. Intestinal variability in *T. stenura* may also be the result of feeding habits and the abundance of different food resources in their environmental settings. If the environmental situation in one population consists of more animal than plant food, then the loach is likely to feed mainly by predation and its intestine would tend to be short; if the habitat is dominated by algae and aquatic plants, then its intestine would tend to be long and form spirals (Zhu 1981).

T. stenura is widely distributed and exists in different environmental conditions, so some of the characters traditionally used to identify the species may display ecophenotypic variation. Thus, we hypothesize that adaptive intraspecific variation exists in *T. stenura*, and these characters are too unstable to diagnose and differentiate different species of *Triplophysa*.

For the morphometric data, ANOVA recognized 16 characters that are significantly different among drainages and 23 characters that are significantly different among populations (Table S1). This is consistent with the AMOVA results of moderate genetic variation among populations of *T. stenura*. PCA analyses (Fig. 5) demonstrated that individuals from different drainages are not clearly distinguishable, but individuals from different populations within drainages can be distinguishable, suggesting that there is some character differentiation among populations within drainages. We hypothesize that characters varying between populations are best explained as the result of genetic and environment influences.

Stable diagnostic characters of *Triplophysa*

In addition to the general characters mutually shared between species of *Triplophysa*, this study identified some stable and easily identifiable characters shared among different populations of *T. stenura*. These include being scaleless and having smooth skin; caudal peduncle nearly round, depth reduced sharply towards caudal-fin base; caudal peduncle length greater than head length; posterior chamber of air bladder degenerate; intestine bending in one to three coils behind stomach; tip of depressed pelvic-fin extending beyond anus; caudal fin slightly concave and the upper lobe equal to lower lobe. Through careful examination and comparisons of specimens of 45 *Triplophysa* species kept in KIZ, we conclude that some characters that are stable within species and relatively variable between species can be used to identify and distinguish species of *Triplophysa*. These include whether the skin smooth or not; whether skin is scaled or scaleless; size and position of eyes; shape of cross-section of base of caudal-peduncle; shape of caudal fin; whether or not tip of depressed pelvic fin reaches anus; possessing or lacking a posterior air bladder and the shape of an existing air bladder; and the shape of intestine.

With the uplift of the Qinghai-Tibet Plateau, the transformation of landforms and water systems, species of *Triplophysa* gradually dispersed to their present accumulative distributional range and pattern (Zhu 1989). During the long period of geographic isolation and evolution, and the influence of a series of factors such as different climate, elevation, hydrologic conditions, feeding conditions, intraspecific and interspecific competition, different populations of the same species can have degrees of morphological variation. Therefore, most differentiation should be viewed as intraspecific rather than interspecific variation (He 2008). We suggest that the classification and identification of species in *Triplophysa* should be based on multiple characters, and should not overemphasize some characters with ecophenotypic variation, such as body color, color patterns, beard length and mouth structure.

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Table S1. ANOVA depicting the morphometric characters that are significantly different among eight populations and three drainages. Only drainages or populations significantly different from one another are shown. Number of “*” indicates *P* value[1]. (download)