



Geometric Morphometric Analysis of the Morphological Variation among Three Lenoks of Genus *Brachymystax* in China

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ABSTRACT

The genus *Brachymystax* mainly distributes in the Amur River and streams of the Qinling Mountains of northern China. There is a debate on the validation of subspecies *B. lenok tsinlingensis* Li for a long time. Some ichthyologists thought that there should be two species (*B. lenok* and *B. tumensis*) in Amur River and a subspecies (*B. lenok tsinlingensis*) in the Qinling Mountains, while others believed no division of the subspecies. Thus, 169 specimens of *Brachymystax* spp. were collected from three locations (Heihe River, Amur and Ussuri River) to identify the taxonomic status in terms of morphological variation among these species or subspecies. Results of geometric morphometric analysis indicated significantly morphological variation in body shape among three morphotypes based on 18 landmarks. Principal component analysis (PCA) showed that the cumulative contribution rate of the first five principal components were 72.99%. CV1 (65.77%) and CV2 (34.23%) were well explained 100% of the observed variation among three morphotypes by Canonical variate analyses (CVA). The morphological variation was well defined by PCA and CVA: *B. lenok tsinlingensis* had wider and elongate head, the longest eye diameter and the widest dorsoventral orientation; *B. lenok* had tapered and narrow head, sharp snout and medium diameter of eye; *B. tumensis* had short head, blunt snout and shortest eye diameter and narrow dorsoventral orientation. Furthermore, discriminant function analysis (DFA) showed that all samples (except six) were correctly reclassified. Our morphological analysis supported the validity of taxonomic status of *B. lenok* and *B. tumensis* as two species, and *B. lenok tsinlingensis* could be considered as an independent species.

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Authors' Contribution

YM and DX designed the study. YM and GW collected morphological data. HL, LW and JZ collected samples. YM analyzed the data. YM and DX wrote the manuscript. DX and XL supplied financial support.

Key words

Brachymystax, Geometric morphometrics, Shape variation.

INTRODUCTION

The genus *Brachymystax* Günther, 1866, belonging to Salmonidae, Salmoniformes, widely distributes in the rivers of Siberia, Mongolia, the Korea Peninsula and north of China (Li, 1966; Song and Fang, 1984; Qin and Wang, 1989; Froufe *et al.*, 2008). In China, the genus *Brachymystax* distributes in Amur River drainage, Tumen River, Yalu River, Luanhe River, Irtysh River, Burqin River, Ulungur River and the southern and northern streams of Qinling Mountains (Huang *et al.*, 1964; Li, 1984; Song and Fang, 1984; Qin and Wang, 1989; Liu, 1992). Based on comparing some characters of specimens from two geographical populations (the Amur River and the streams of Qinling Mountains), such as number of gill rakers, lateral-line scales and pyloric caeca, Li (1966) considered

that the *Brachymystax* fish distributed in the Qinling differ from that of Amur River, and described it as an endemic subspecies, and named as *Brachymystax lenok tsinlingensis* Li. *B. lenok tsinlingensis* is thought to be a glacial relict, as one of the southernmost Salmonidae species (the other one is *Hucho bleekeri*) (Li, 1984; Yue and Chen, 1998). In recent years, overexploitation, environmental pollution, dam constructions, and other reasons have caused a rapid reduction of *B. lenok tsinlingensis* populations in the wild (Ren and Liang, 2004), and rare fish can be found in the brook of the Qinling Mountains where far away from human activities, only in the region with altitude range from 1100 m to 2300 m (Yue and Chen, 1998; Ren and Liang, 2004; Gong *et al.*, 2009). Therefore, the *B. lenok tsinlingensis* has been listed as a class II state protected wild animal in China Red Data Book of Endangered Animals since 1998, because of highly sensitive to ecology environment and limited numbers in the wild (Yue and Chen, 1998; Zhao and Zhang, 2009).

Up to present, there have been some studies

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referring to genus *Brachymystax*, most of them focused on their early development, age and growth, and artificial propagation (Lee *et al.*, 2001; Xu *et al.*, 2010, 2015; Shi *et al.*, 2012; Guo *et al.*, 2016; Lee and Yoshizaki, 2016). Whereas little attention is paid to explore the taxonomic problem of genus *Brachymystax* fish. Hence, the problem of species or subspecies differentiation belonging to genus *Brachymystax* is still controversial. On the one hand, *B. lenok tsinlingensis* Li, 1966 (Qinling lenok) was described as a subspecies differ from *B. lenok lenok* (Pallas, 1773) (Li, 1966; Yue and Chen, 1998; Du *et al.*, 2016), nevertheless, other ichthyologists considered that *B. lenok tsinlingensis* couldn't be treated as a subspecies, just as the synonym of *B. lenok lenok* (Song and Fang, 1984; Wang, 1988; Huang *et al.*, 1989; Qin and Wang, 1989; Zhang, 1995). On the other hand, *B. lenok* (Pallas, 1773) (sharp-snouted lenok) and *B. tumensis* Mori, 1930 (blunt-snouted lenok) distributed in Amur River and its tributary were revised as two species of *Brachymystax*, because they were different in the shape and length of their snouts as well as a number of biological characters and karyotypes (Ma *et al.*, 2005, 2009; Mou *et al.*, 2006; Ma and Jiang, 2007; Froufe *et al.*, 2008; Wang *et al.*, 2010; Frolov *et al.*, 2015). Thereafter, the taxonomic relationship among *B. lenok*, *B. tumensis* and *B. lenok tsinlingensis* is even more vague. For example, Wang (2011) thought *B. lenok tsinlingensis* was different from both *B. lenok* and *B. tumensis*. Ma *et al.* (2005) identified the morphological traits of *B. lenok tsinlingensis* were more similar to *B. tumensis*. Xing *et al.* (2015) even redefined and revalidated *B. lenok tsinlingensis* Li, 1966 as an independent species and named as *Brachymystax tsinlingensis* Li, 1966. Some non-Chinese scholars held that the *Brachymystax* distributed in Korea was also *B. lenok tsinlingensis* (Kim and Park, 2002; Jang *et al.*, 2003).

However, rarely published studies have examined the morphological divergence or genetic diversity among the three of *B. lenok tsinlingensis*, *B. lenok* and *B. tumensis* (Xing *et al.*, 2015; Du *et al.*, 2016). The taxonomic boundary of them has been still vague, especially the taxonomic status of *B. lenok tsinlingensis*. It will affect the conservation of germplasm resources of this endangered wild fish and its further biological research.

Fish morphological variation is the most intuitive adaptability change to specific habitat conditions. The morphological characteristics of fish are affected by genetic and environmental factors, which are important basis for species identification and species classification (Kinsey *et al.*, 1994). Morphometrics is a good research method that specialized in the shape variation and its covariation with other variables (Bookstein, 1991). As the revolution of morphometrics, geometric morphometric method combined with multivariate statistical analysis could capture the overall morphological changes of shape, avoid the loss of information of specimens structure and

consider the global anatomic context (Rohlf and Marcus, 1993; Adams *et al.*, 2004; Slice, 2007), which could express accurately the characteristics of biological form and provide the complete information for shape of the individuals, comparing with traditional morphometric analysis based on the relation between linear dimensions taken from two anatomical landmarks (Rezić *et al.*, 2017; Strauss and Bookstein, 1982). Due to its obvious advantages, geometric morphometrics is widely applied on hydrocole to analysis the relationship between morphology and habitat (Zimmermann *et al.*, 2012; Idaszkin *et al.*, 2013; Foster *et al.*, 2015), growth stages and shape (Frédéric and Vandewalle, 2011), morphological differences among geographic populations (Fruciano *et al.*, 2011; Braga *et al.*, 2017), as well as between species or subspecies (Tofilski, 2008; Addis *et al.*, 2010; Stange *et al.*, 2016).

In the present paper, the landmark-based geometric morphometrics was used to investigate the morphometric variation of the genus *Brachymystax* in China, which had been recognized as different species (*B. lenok* and *B. tumensis*) or subspecies (*B. lenok tsinlingensis*) in previous studies. The objective of the present study was to evaluate the taxonomic status of three morphotypes of genus *Brachymystax*, especially the subspecies validation of *B. lenok tsinlingensis* in Qinling, based on shape data.

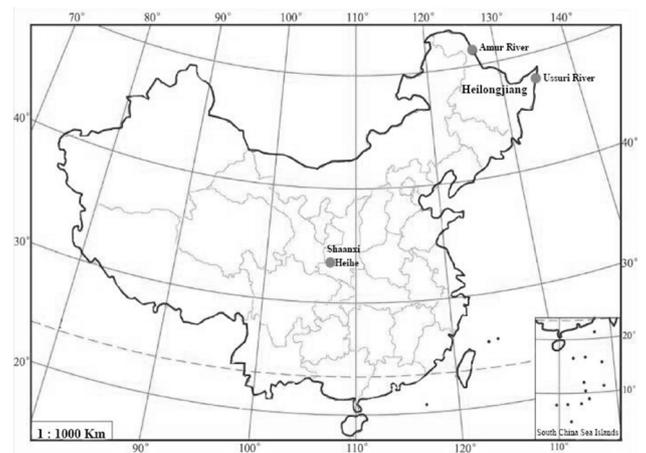


Fig. 1. Geographical locations of the samples used in the present study.

MATERIALS AND METHODS

Sampling

All specimens (n=169) of genus *Brachymystax* were collected with a gill net from August 2014 to June 2017 in three different locations from Shaanxi and Heilongjiang Province of China. Sampling sites located at Heihe River (more than 1100 m altitude), Amur River (about 170 m altitude) and Ussuri River (a tributary of Amur

River; about 83 m altitude) (Fig. 1). All samples were stored initially on wet ice and frozen immediately after catching, and preserved in the laboratory of Northwest A&F University, Yangling, China, after transportation. Taxonomic identification of all specimens were on the basis of characteristics of morphological traits (*i.e.*, snout shape, body color and colour spots) and geographical distribution following Ma *et al.* (2005), Wang (2008) and Gong *et al.* (2009). These specimens recognized as three morphotypes (*B. lenok*, *B. tumensis* and *B. lenok tsinlingensis*). The average body length and weight of *B. lenok* was 21.75 ± 3.87 cm and 188.12 ± 99.11 g; the average body length and weight of individuals for *B. tumensis* was 34.43 ± 2.69 cm and 550.17 ± 132.83 g; the average body length and weight of examined samples of *B. lenok tsinlingensis* was 15.44 ± 2.83 cm and 49.58 ± 28.98 g. More detailed sampling information was showed in Table I.

Geometric morphometric data collection

The absolutely thawed fish were laid in a straight horizontal position on a polystyrene board and photographed the left side of each fish with a Nikon 60D digital camera (Nikon Ltd., Japan), while using

a straightedge as scale. The fins were stretched out and fixed with pins. All raw images were further processed using the tpsUtil v.1.70 (Rohlf, 2016). Eighteen landmarks were placed and computed scale factors in each image of sample to describe the body shape changes (Fig. 2). These unambiguously identified landmarks (*e.g.* where the fins join the body) were predominantly type 1 to 2 landmarks as defined by Bookstein (1990), which represented significant skeletal or structural features (Helland *et al.*, 2009; Arbour *et al.*, 2010). To remove the bending effects of samples owing to preservation, the ‘unbending landmarks’ procedure was applied in tpsUtil, and three additional unbending landmarks (landmark 19, 20 and 21; removed before analysis) were digitized for that reason, which were in the middle of the line, respectively (Fig. 2). The coordinates of 21 landmarks were digitized using tpsDig2.0 (Rohlf, 2016) for each specimen. The new coordinates (X, Y) were calculated after removing the ‘unbending landmarks’ by tpsUtil. Finally, new coordinates data was tested and confirmed the suitability for further analysis, using tpsSmall v.1.33 (Ristovska *et al.*, 2008; Rohlf, 2015).

Table I.- Locations and number of samples of each morphotype.

| Morphotypes | Locations | | | Sample size | Body length (cm) | Weight (g) |
|-------------------------------|--------------|------------|-------------|-------------|------------------|---------------|
| | Ussuri River | Amur River | Heihe River | | Mean±SD | Mean±SD |
| <i>B. lenok</i> | 8 | 32 | - | 40 | 21.75±3.87 | 188.12±99.11 |
| <i>B. tumensis</i> | 71 | - | - | 71 | 34.43±2.69 | 550.17±132.83 |
| <i>B. lenok tsinlingensis</i> | - | - | 58 | 58 | 15.44±2.83 | 49.58±28.98 |

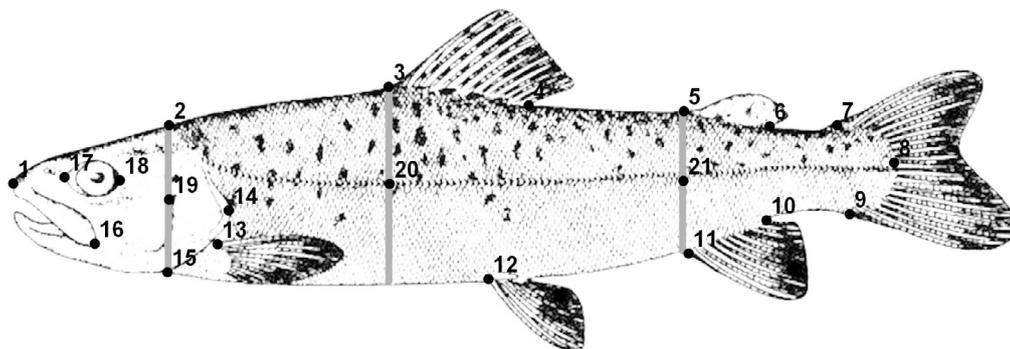


Fig. 2. Position of the 21 landmarks used for body shape analyses of the *Brachymystax*: 1, anterior tip of the snout at the level of the upper jaw; 2, posterior margin of the neurocranium; 3, dorsal fin origin; 4, posterior insertion of the dorsal fin base; 5, origin of adipose fin; 6, terminal of adipose fin base; 7, dorsal origin of caudal fin; 8, middle of caudal base; 9, ventral origin of caudal fin; 10, posterior end of anal fin base; 11, origin of anal fin; 12, origin of pelvic fin; 13, origin of pectoral fin; 14, trailing edge of the gill cover; 15, indentation where the opercular cover and sternohyoideus muscle cross; 16, upper jaw tip (17 and 18, diameter of the eye); 19, the middle point of the line going from the caudal part of the head to the bottom of the head; 20, the middle point of the line going from the rostral point of the dorsal fin base to the bottom of the body; 21, the middle point of the line going from the rostral point of the adipose fin base to the bottom of the body (19, 20 and 21 were unbending landmarks). (Picture is cited from Yue and Chen, 1998).

Geometric morphometric analysis

All non-shape related variation in scale effects, orientation, and translation were removed from the dataset by means of a Generalised Procrustes analysis (GPA) (Adams *et al.*, 2004; Slice, 2007) until its position minimized the shape difference between specimens based on unbending energy (Haas, 2011). In this method, landmark configurations are superimposed by least squares optimisation and the process is iterated to compute the mean shape (Braga *et al.*, 2017). After Procrustes superimposition, shape differences can be analyzed by the differences between Procrustes coordinates. Centroid size (CS), which is calculated as the square root of the sum of the squared deviations of landmarks from a centroid for each specimen, was used as a size proxy (Zelditch *et al.*, 2012). The main tendencies in shape variation between samples within species were summarized through a principal component analysis (PCA) of the variance-covariance matrix of the Procrustes coordinates (Slice, 2007). Canonical variate analysis (CVA) was also used to visualize body shape changes that discriminated among groups (Klingenberg and Monteiro, 2005). CVA computes

axes of variance in a way that minimized within-group differences and maximized between-group differences. Discriminant function analysis (DFA) is to determine classification functions by Fisher's classification rule, followed by canonical analysis. Cross validation test was used to verify the accuracy of DFA method. The significance of differences among group means was tested through permutation tests with 10,000 permutations rounds, and meanwhile appeared the Procrustes distances and Mahalanobis distances among groups. All subsequent morphometric analyses were performed in the MorphoJ 1.06d (Klingenberg, 2011).

RESULTS

Morphological variation of average shape of each morphotype

The least-squares criterion regression analyses showed that the regression coefficient of the Tangent distance (y-axis) and the Procrustes distance (x-axis) was 0.99, indicating that the selected eighteen landmarks were valid and could be used for further analysis.

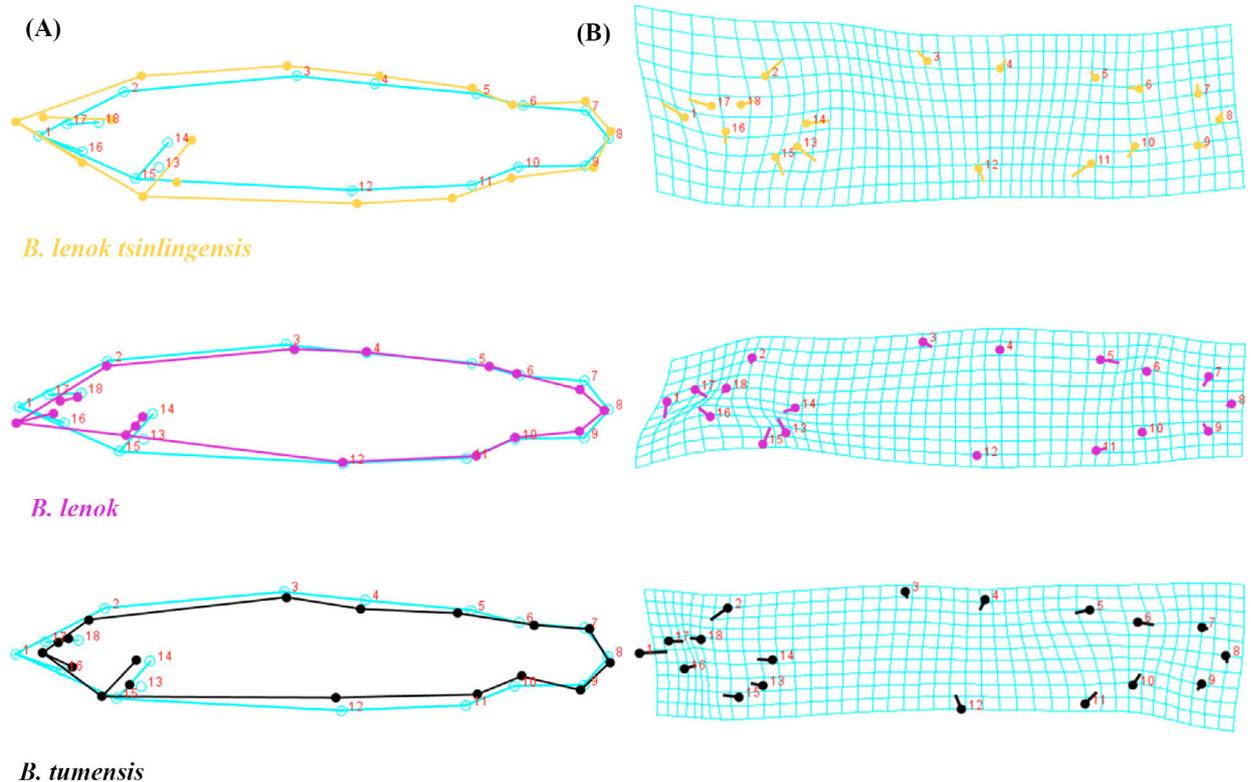


Fig. 3. Variation visualization of three morphotypes of *Brachymystax*. A, the wireframes graphs of three morphotypes (scale factor = 4, bluish green line; bluish green points show average position of landmarks of all individuals); B, the transformation grids of three morphotypes (scale factor = 4).

Four types of graphs were provided to visualize the morphological changes associated with the statistical results by MorphoJ. In the present study, wireframes graphs and transformation grids displayed that the morphological differences among three morphotypes of *Brachymystax* were mainly reflected in the changes of the head shape, the snout shape and the width of dorsal-ventral orientation (Fig. 3A, B). *B. lenok* and *B. lenok tsinlingensis* were more stretch on dorsal-ventral orientation than *B. tumensis*. All samples of *B. lenok* showed the sharpest snout, tapered and narrow head. *B. lenok tsinlingensis* had wider and elongate head and the longest eye diameter. *B. tumensis* had blunt and round and short head, blunt snout and shortest diameter of the eye.

Morphological variation among three *Brachymystax* morphotypes

PCA of 18 landmarks morphometric variables for 169 samples with a priori classification (Table I) displayed

overlap among three morphotypes. The first five PCs accounted for 72.99% of the body shape changes (PC1 31.52%, PC2 17.75%, PC3 9.66%, PC4 7.81% and PC5 6.25%) (Fig. 4A). The PCA results indicated that most individuals of *B. tumensis* and *B. lenok tsinlingensis* took negative values and positive values along PC1 in the morphospace plot, respectively. Samples of *B. lenok* were distributed randomly and showed a large overlap with the other two morphotypes. PC2 did not distinguish any of the morphotypes in the scatter plot. The least overlap between *B. tumensis* and *B. lenok tsinlingensis* was evident in the scatter plot of PC1 versus PC2 (Fig. 4B). The uppermost body shape changed along PC1 followed a dorsal-ventral orientation compression and stretching, as well as head length in the lateral view (Fig. 4C). The body height of the *B. lenok tsinlingensis* in Qinling was higher than that of *B. lenok* in Heilongjiang, and the head of the *B. lenok* morphotype was characterized more sharper than the other two.

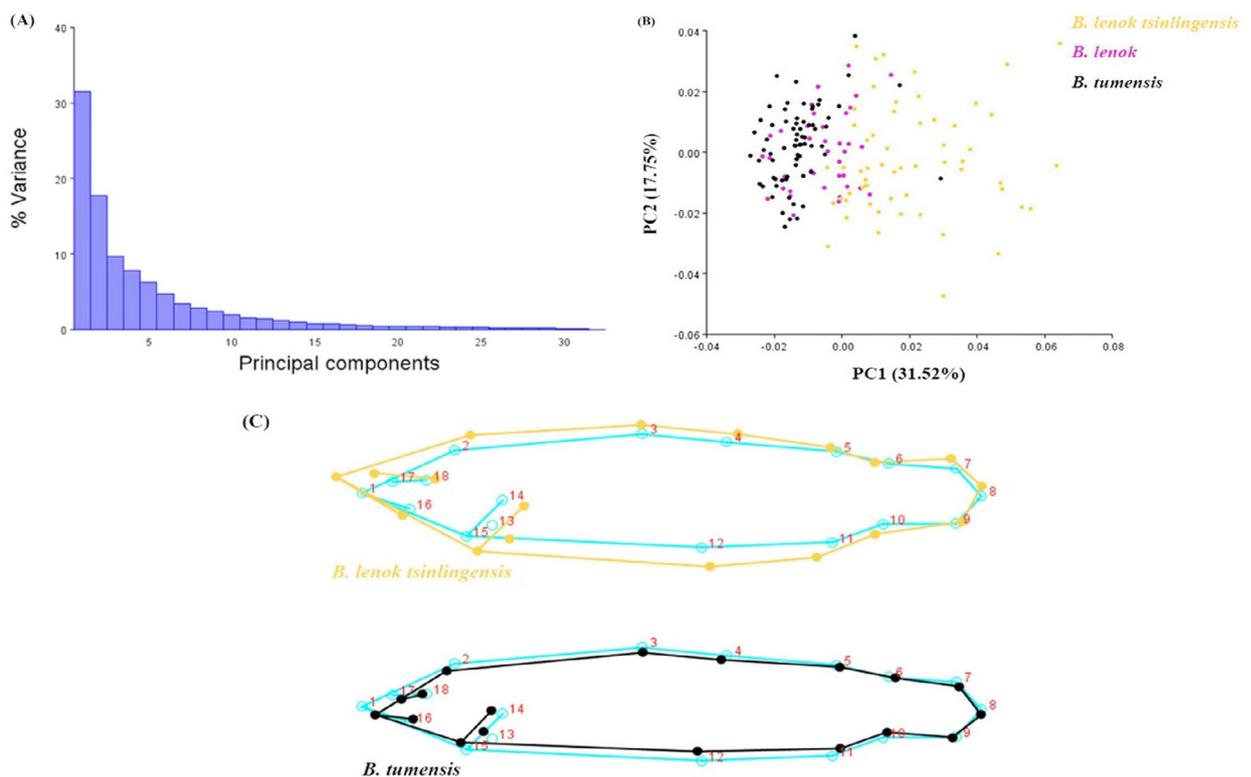


Fig. 4. The results of principal component analysis for three morphotypes (*B. lenok tsinlingensis*, *B. lenok* and *B. tumensis*). A, Bar chart of percent variance explained by 32 principal components of geometric morphometric analysis, the first five PCs together account for 72.99% of the total variation; B, Scatterplot of principal components 1 and 2 from principal component analysis of Cartesian coordinates of 18 landmarks for all individuals ($n = 169$) among the three morphotypes (*B. lenok tsinlingensis*, *B. lenok* and *B. tumensis*); C, wireframes showing shape changes related to extremes of variation along the PC1 axes, the upper wireframes represented the shape variation of *B. lenok tsinlingensis* (scale factor = 0.08), the below wireframes represented the shape variation of *B. tumensis* (scale factor = -0.04).

Results of CVA revealed two canonical correlations and separated all the samples of each morphotype into three non-overlapping clouds of points. The two canonical correlations explained 100% of the observed variation among *B. lenok*, *B. tumensis* and *B. lenok tsinlingensis* (Fig. 5). The first canonical variable (CV1, 65.77%) mainly discriminated from *B. lenok tsinlingensis* and the other two morphotypes, which mainly manifested as *B. lenok tsinlingensis* exhibiting the most negative values and the other two morphotypes having positive values. The variables that contributed most to the CV1 were coordinates 5Y, 17X and 18X. The second canonical variable (CV2, 34.23%) mainly separated the groups of *B. lenok* and *B. tumensis*, the former group took values smaller than zero and the latter one took values larger than zero. The variables that contributed most to the CV2 were coordinates 1X, 1Y and 16X, 16Y. The most pronounced differences were presented in the results of CVA by comparison of shape data. The shape change in CV1 from one morphotype to another was reflected in a dorsal-ventral orientation compression and stretching. The mainly shape change in CV2 among different species was reflected in head shape.

DFA was also used to judge individuals classification. After the 10,00 permutation test using the T-square ($P < 0.0001$), the discrimination function correctly reclassified all except six individuals in a cross-validation

test, the proportions of correct reclassification were 90% to 98.6% (Table II). Additionally, the Procrustes distance and Mahalanobis distance between *B. lenok tsinlingensis* and *B. tumensis* were farthest (the former distance was 0.0351 and the latter was 7.3094), the distances between *B. lenok tsinlingensis* and *B. lenok* were in the middle (the former was 0.0308 and the latter was 6.8108), the distances between *B. lenok* and *B. tumensis* were the nearest (the former was 0.0199 and the latter was 5.9646).

Table II.- Discriminant function analysis of three *Brachymystax* morphotypes.

| Predicted morphotypes | Discriminated morphotypes | | |
|---|---------------------------|--------------------|-------------------------------|
| | <i>B. lenok</i> | <i>B. tumensis</i> | <i>B. lenok tsinlingensis</i> |
| Results of discriminant function | | | |
| <i>B. lenok</i> | 40(100%) | 0 | 0 |
| <i>B. tumensis</i> | 0 | 71(100%) | 0 |
| <i>B. lenok tsinlingensis</i> | 0 | 0 | 58(100%) |
| Results of cross-validation | | | |
| <i>B. lenok</i> | 36(90%) | 4 | 0 |
| <i>B. tumensis</i> | 1 | 70(98.6%) | 0 |
| <i>B. lenok tsinlingensis</i> | 1 | 0 | 57(98.3%) |

The number outside of the parentheses represents discriminated samples, and the inside number means the discriminated proportions of samples that were correctly classified.

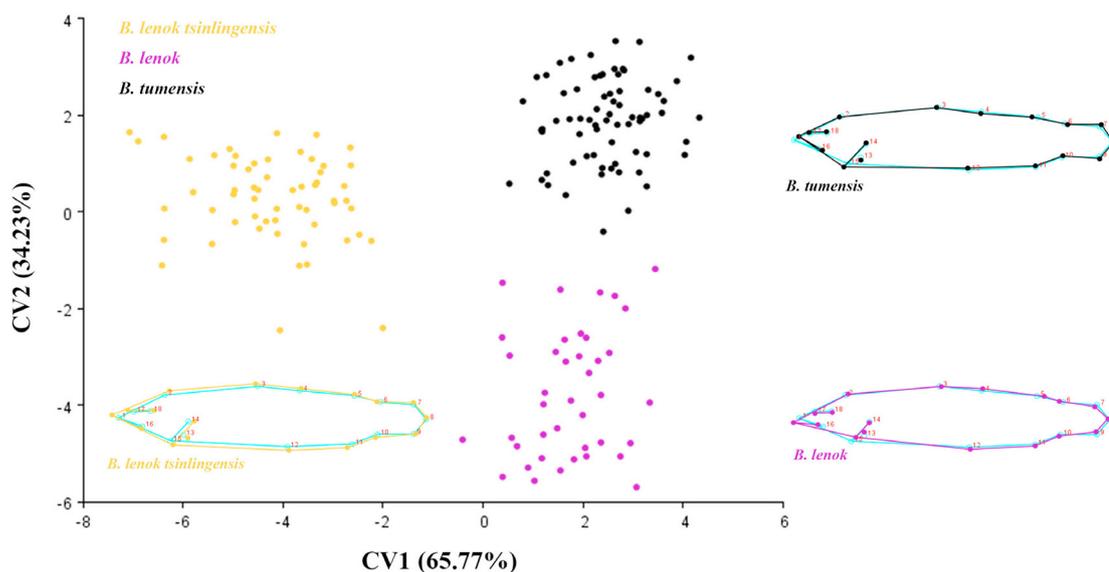


Fig. 5. The body shape variation in three morphotypes of genus *Brachymystax*. Scatterplot of individual values from the canonical variate analysis for the shape variation of three different morphotypes of *Brachymystax*. The first two canonical variates (CVs) capture the entire variance (100%) observed among the three morphotypes. Lower left wireframes showing shape changes of *B. lenok tsinlingensis* (yellow wireframes) along the CV1 axes, (scale factor = -8, bluish green line; bluish green points show average position of landmarks of all individuals). For CV2, (Top right) black wireframes represent the shape changes of *B. tumensis* (scale factor = 10); (Bottom right) purple wireframes represent the shape variation of *B. lenok* (scale factor = -10).

DISCUSSION

Three morphotypes of *Brachymystax* had been collected from Heihe, Amur River and Ussuri River, and these morphotypes had different biological characteristics and life-history (Mou *et al.*, 2006; Wang, 2008). The present study exhibited that three morphological types of *B. lenok*, *B. tumensis* and *B. lenok tsinlingensis*, whose shape was coherent with morphological description in previous research (Li, 1966; Ma *et al.*, 2005; Gong *et al.*, 2009; Xing *et al.*, 2015), differed significantly in body shape along the dorsal-ventral orientation, head morphology and snout shape, and eye diameter. Morphologically, all results of geometric morphometric analysis have shown that they belong to three different lineages.

Relationship of morphological variation and elevation habitats

Compared with other vertebrates, fish morphological characteristics are more diverse within or among populations, and more susceptible to the environmental influences (Wimberger, 1992). Salmonidae fishes originated from the north frigid zone of Eurasia, and all Salmonidae species distributed in China are thought to be the residual fishes after the glacial epoch (Li, 1984). *B. lenok tsinlingensis*, a landlocked salmon, is sealed off and stagnated in mountain streams with deglaciation (Song and Fang, 1984). Therefore, the *Brachymystax* fish from Qinling and from Amur River have been separated into two geographical populations approximately 200 million years ago and guaranteed reproductive isolation (Du, 2012; Du *et al.*, 2016). Obviously, the morphological variation of genus *Brachymystax* that distributed in Shaanxi and Heilongjiang Province are closely related to geographic isolation.

According to our results and the different altitude habitats of *Brachymystax*, we can divide all samples into two geographically isolated groups. One group is the high-altitude habitat (*B. lenok tsinlingensis*, more than 1100 meters) and another is the low-altitude habitat (*B. lenok* and *B. tumensis*, below 200 meters). PCA has shown the overlap in varying degree among three morphotypes, but the minimum overlap appeared somewhere in *B. lenok tsinlingensis* and *B. tumensis*. The similar findings of PCA have also been occurred in the morphometric variation analysis of other vertebrates, such as *Trinomys* and ariia catfish (Dalapicolla and Leite, 2015; Stange *et al.*, 2016). The results of CVA had showed that the specimens of high-altitude group were completely divided from the low-altitude group at CV1 axis without overlap. The DFA also got the same results, the Procrustes distance and Mahalanobis distance between *B. lenok tsinlingensis*

and *B. tumensis* were farther than the distances between *B. tumensis* and *B. lenok*, and the Procrustes distance and Mahalanobis distance between *B. lenok tsinlingensis* and *B. lenok* were also bigger than the distances between *B. tumensis* and *B. lenok*. In other words, the shape variation of specimens between high- and low-altitude habitat is significant difference ($P < 0.0001$). Maximal differences were observed in dorsaventral orientation and the head form in which *B. lenok tsinlingensis* had a wider dorsaventral orientation and the eye diameter longer than those from the group at low-altitude habitat, and the posterior end of the upper jaw (the 16th landmark) is below the center of the eye. These differences demonstrated that the body shape of *B. lenok tsinlingensis* was significantly more different comparing with *B. lenok* and *B. tumensis* which from Heilongjiang Province and reinforced the results of a recent multivariate morphometric study of *Brachymystax* in China that suggested the *B. lenok tsinlingensis* were neither the synonym of *B. lenok* nor the synonym of *B. tumensis* (Xing *et al.*, 2015).

Previous studies explored the validity of *B. lenok tsinlingensis* was based on comparing the isozymes or some meristic characters, such as, the number of gill rakers, the lateral-line scales and the pyloric caeca. The comparison results showed there was a narrow overlap, but clearly existed differences in these characters among *B. lenok tsinlingensis* and lenoks without specifying the sharp or blunt snout (Song and Fang, 1984; Wang, 1988; Qin and Wang, 1989). The number of gill rakers was considered to be a highly heritable trait (Svårdson, 1979) and the number of the lateral-line scales which was genetically regulated was also effective for the differentiation of the populations (Bochkarev *et al.*, 2017). Genetic research results using populations markers (*e.g.*, mitochondrial control region and cytochrome *b*) also supported there were significant differentiation of lenoks between geographical populations from Qinling and Amur River (Xia *et al.*, 2005, 2006). Our study of the body shape variation in *Brachymystax* belonging to different geographical populations in China allowed us to identify a different pattern of shape evolution.

Taxonomic status among three morphotypes of genus Brachymystax

Many early studies on the classification of genus *Brachymystax* in China included two fields, one is to explore whether the subspecies is exist or the validity of subspecies (without considering snout shape within populations) and another is to analysis two new sympatric species, sharp-snouted lenok (*B. lenok*) and blunt-snouted lenok (*B. tumensis*), from Amur River basin. However, few studies paid attention to the relationship of three distinct lineages in genus *Brachymystax* in China (Xing

et al., 2015; Du *et al.*, 2016). Morphological variation for three morphological types have been quantified in China only utilizing traditional morphometric analysis (Xing *et al.*, 2015). In the present study, analyses of morphological differences using landmark-based geometric morphometric analysis indicated that *B. lenok tsinlingensis*, *B. lenok* and *B. tumensis* were differentiated by particular body shape. The relatively distinct morphological characters of sharp-snouted and blunt-snouted lenok in our study is consistent with previous reports of morphological characters of lenoks distributing in Amur River basin (Ma *et al.*, 2005; Froufe *et al.*, 2008). These two also differ significantly in their biological characters and independent spawning sites, which may guarantee reproductive isolation in sympatry (Mou *et al.*, 2006; Froufe *et al.*, 2008).

Lenoks distributing in the Qinling Mountains has been considered as blunt-snouted lenok based on external morphology (Ma *et al.*, 2005), and *B. lenok tsinlingensis* was placed together with *B. tumensis* (Shed'ko and Shed'ko, 2003). Moreover, *B. lenok tsinlingensis* is considered as a synonym of *B. lenok* (Pallas, 1773), and the genus *Brachymystax* included only three currently recognized valid species: *B. lenok* (Pallas, 1773), *B. tumensis* Mori, 1930, and *B. savinovi* Mitrofanov, 1959 (Froese and Pauly, 2014). The results of the above studies were contrary to the results of our study about *B. lenok tsinlingensis*. Our results demonstrated that *B. lenok tsinlingensis* had more significant shape difference from both *B. lenok* and *B. tumensis* than the shape difference between *B. lenok* and *B. tumensis* in head shape, the eye diameter and the position of posterior end of the upper jaw.

Ma *et al.* (2009) had even synonymized *B. tumensis* in Tumen River with *B. lenok* based on mitochondrial control region sequence which indicated only slightly more interspecific genetic divergence (2.2%) than the intra-specific variation recorded for *B. tumensis* (1.2%) in China, and the blunt-snouted lenok was considered as *Brachymystax* sp. *B. tumensis* was still temporarily adopted in the present study. The complete mitochondrial genomes were sequenced and the level of divergence inferred from 12 protein-coding genes showed close proximity between sharp-snouted lenok and *B. lenok tsinlingensis*, but clear species boundaries between the blunt-snouted lenok and both sharp-snouted lenok and Qinling lenok (Si *et al.*, 2012; Balakirev *et al.*, 2016). In addition, karyotypes analysis showed these two forms have different chromosome number, and *B. lenok* karyotype were $2n=90$ with two cytotypes (I: $NF=110$; III: $NF=106\sim 136$), *B. tumensis* karyotype has $2n=92$ and $NF=116$ (Kartavtseva *et al.*, 2013), and the silver-staining exhibited a certain difference between *B. lenok* and *B. tumensis* karyotypes (Frolov *et al.*, 2015). The obtained karyotypic difference also strongly

supported *B. lenok* and *B. tumensis* from Amur River basin were different species. The results of morphological analyses in our study, as well as the results based on molecular analysis (*i.e.* mitochondrial control region and microsatellites) and chromosomal study of the lenoks in previous research supported the validity of the species status of sharp-snouted and blunt-snouted lenok. Though, the lenok from Yellow Sea basin (the Luan He River in China) examined herein also has the same karyotype as *B. tumensis* (Kartavtseva *et al.*, 2013). However, there are no karyotype data of *B. lenok tsinlingensis* in Qinling with aid to judge taxonomic status.

In other respects, researchers have proved a differentiation among these three lenoks, the subspecies validity of *B. lenok tsinlingensis* was determined by the partial sequence of the mitochondrial control region fragment (Du *et al.*, 2016). Based on cytochrome *b* gene analyses, *B. lenok tsinlingensis* was thought as an independent species, renamed as *B. tsinlingensis*, and *B. tsinlingensis* has a significant genetic divergence from *B. lenok* (0.020~0.022) and *B. tumensis* (0.034), respectively, and the above values were larger than the interspecific genetic divergence (ranged from 0.008 to 0.011) among *Hucho taimen*, *H. hucho* and *H. bleekeri* (Xing *et al.*, 2015). The clustering results of molecular phylogenetic trees from Xing *et al.* (2015) were consistent with our morphological results and conclusions. Therefore, it was speculated that *B. lenok tsinlingensis* was an independent species.

CONCLUSION

The present study provided morphological data that will help in the correct identification of *Brachymystax* in China. The landmark-based morphological analysis showed that *B. lenok tsinlingensis*, *B. lenok* and *B. tumensis* are characterized by significant differences from each other, which differ mainly in the head form, snout shape and the diameter of eye and the height of the dorsiventral orientation and the location of the posterior end of the upper jaw. Moreover, we speculated that the *B. lenok tsinlingensis* may be an independent species, but this required further evidences of molecular analysis, such as genetic diversity of mitochondrial DNA sequences and microsatellites to improve our understanding of the taxonomic status of genus *Brachymystax* fish.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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