

## Phylogenetic position of the genus *Bibarba* as revealed from molecular genetic data (Teleostei: Cobitidae)

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Phylogenetically, the family Cobitidae consists of an assemblage of lineages that are referred to as ‘southern lineages’, out of which stems a monophyletic bunch of lineages that is referred to as ‘Northern clade’. Up to now, 17 of the 21 valid genera have been included into genetic phylogenies. The present phylogenetic study analyses the only two known species of *Bibarba* using nuclear and mitochondrial DNA sequences. Both species together formed a monophyletic lineage that is sister to the Northern clade of Cobitidae, but well-separated from the four other major lineages within the Northern clade. The results support the validity of the genus and show it to represent a major lineage on its own. The morphological synapomorphy of the northern clade is in the sexual dimorphism, with males bearing an ossified structure (lamina circularis or scale of Canestrini) on the second branched pectoral-fin ray in males. *Bibarba* was reported to have such structure on the third instead of second fin ray, but our observations reveal the presence of two lamina circularis, one on the second and one on the third fin ray (character doubling). Due to the presence of this synapomorphy in *Bibarba* and its distribution in the area of the Northern clade we consider *Bibarba* as member of the Northern clade. This clade is enlarged by *Bibarba* forming a fifth major lineage.

### Introduction

The family Cobitidae (spined loaches) includes small benthic freshwater fishes that are found in nearly every river in Eurasia. It is presently composed of 21 genera and 171 species (Kottelat, 2012). According to recent studies, all species from the northern part of the distribution area including Europe, Near East and Asia north of the Himalayas Mountains and Mekong River basin together form a monophyletic group that was referred to as ‘Northern clade’ (Šlechtová

et al., 2008). The Northern clade stems out of an assemblage of genera from the southern part of the distribution area, meaning Asia south of the Himalayas, reaching from Pakistan to Vietnam and south to the Sunda Islands, that have been referred to as ‘Southern lineages’. The distribution range of the Northern clade does not overlap with the range of the southern lineages; the only exception is the occurrence of an undescribed species of *Microcobitis*, a genus belonging to the Northern clade, in the Mekong basin in Laos (Šlechtová et al., 2008, Bohlen & Harant, 2010, Kottelat, 2012).

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**Fig. 1.** **a**, *Bibarba bibarba*, not preserved, male, about 50 mm SL; China: Guangxi: Du'An county: stream Cheng Jiang; right side, reversed; **b**, *Bibarba pavoculum*, ZRC 54454, male, 51.9 mm SL; China: Guangxi: cave in Liuzhou county; right side, reversed; both shortly after capture.

The main synapomorphy of the Northern clade is the presence of a 'lamina circularis' or 'scale of Canestrini' in adult males; this is an ossified postero-dorsal extension of the upper hemitrichium of the first branched pectoral-fin ray (Kottelat, 2017). The lamina circularis is embedded into a skin sack, so that details of the ossified elements become visible only after removal of the skin sack. Size and shape of the lamina circularis are variable: in *Microcobitis* and most species of *Misgurnus* and *Cobitis* it is dorso-ventrally flat, not thicker than the bearing fin ray and triangular, axe-shaped, semi-circular or distally elongated in dorsal view (Chen & Chen, 2005, Chen et al., 2018, Economidis & Nalnant, 1996, Kim et al., 1999). In some *Cobitis* (e.g. *C. brachysoma*, *C. choii* and *C. microcephala*) the distally elongated lamina is serrated on the posterior margin (Chen & Chen, 2011, Chen et al., 2018, Kim et al., 1999). In *Paramisgurnus dabryanus* it is thicker than the fin ray and finger-shaped into posterior direction (Kottelat & Freyhof, 2007); in *Cobitis bilseli* it is thick, knob-shaped and the skin above the lamina bears tubercles (Erkakan et al., 1999). The complete lamina circularis is secondarily reduced in *Sabanejewia*, *Kichulchoia* and *Niwaella*, in a few species of *Cobitis* and one species of *Misgurnus* (Šlechtová et al., 2008). In several species of *Cobitis* on the Balkan Peninsula and the Near East two lamina circularis are present: one on the single unbranched ray and one on the first branched ray;

these species have been pooled in the subgenus *Bicanestrinia* (Băcescu, 1962, Erkakan et al., 1998). The presence of a second lamina circularis on a different fin ray is best interpreted as character doubling rather than as independent evolution of a new morphological character. The precise function of the lamina circularis is unknown, but it most likely plays a role in mating behaviour (Bohlen, 2008).

Genetic studies have consistently identified four well-supported lineages within the Northern clade, two of them containing a single genus (*Microcobitis* and *Sabanejewia*, respectively), one lineage ('Misgurnini') includes the genera *Misgurnus*, *Paramisgurnus* and *Koreocobitis*, while the last and largest lineage ('*Cobitis* s.l.') includes all species of *Cobitis*, *Iksookimia*, *Kichulchoia* and *Niwaella* (Šlechtová et al., 2008, Perdices et al., 2016).

Up to now, 17 of the 21 valid genera of Cobitidae (Kottelat, 2012) have been subject to a genetic analysis. The remaining four genera (*Bibarba*, *Paralepidocephalichthys*, *Protocobitis*, *Theriodes*) together contain eight species, of which five are cave species that are known from only a single locality in China (*Bibarba parvoculus*, *Paralepidocephalus translucens*, *Protocobitis anteroventris*, *P. polylepis*, *P. typhlops*) and also the three epiterranean species (*Bibarba bibarba*, *Paralepidocephalus yui*, *Theriodes sandakanensis*) have very restricted distribution ranges (Liu et al., 2016).



Fig. 2. Comparison of *Bibarba bibarba* and the co-occurring *Cobitis* sp. A: top, *Bibarba bibarba*, IAPG A8786, 44.6 mm SL; bottom, *Cobitis* sp. A, IAPG A8734, 41.2 mm SL; both China: Guangxi: Du'an county: stream Cheng Jiang.

The genus *Bibarba* is known only by its type species, *B. bibarba*, from karstic waters in Du'an county and *B. parvoculus* from a cave in Liuzhou county, both in the Pearl River basin in Guangxi province, China (Chen & Chen, 2007, Lan et al., 2013, Wu et al., 2015) (Fig. 1). According to the available literature, *Bibarba bibarba* (Fig. 1a) is morphologically very similar to *Cobitis* (Fig. 2), with two major differences: *Bibarba* has only a pair of rostral and a pair of mandibular barbels while *Cobitis* additionally has a pair of maxillo-mandibular barbels. The barbels are very short and the lamina circularis in males is located on the second branched pectoral-fin ray (Chen & Chen, 2007) instead of the first branched (or unbranched plus first branched in Bicanestrinia) in *Cobitis* (Nalbant, 1963, Šlechtová et al., 2008). Chen & Chen (2007) give as additional characters for the separation of *Bibarba* from *Cobitis* the usually smaller size of *Bibarba*, the absence of a stripe from eye to occiput and the thicker medio-lateral process of the suborbital spine; but these character states are also found in some species of *Cobitis*, were not used by Chen & Chen for comparison (JB, pers. observ., Erkakan et al., 1998). *Bibarba parvoculus* (Fig. 1b) is a cave species with reduced eyes and pigmentation, but with the same arrangement of barbels and sexual dimorphism as in *B. bibarba* (Wu et al., 2015). However, the morphological differences between *Bibarba* and *Cobitis* do not seem to be very large, especially when considering the vast variation of morphology within the genus *Cobitis*. The validity of *Bibarba* therefore is in need of another validation on the base of characters not previously studied.

We present here the mitochondrial and nuclear sequence data for the two species of the genus *Bibarba* as well as for *Cobitis* from geographically close localities and include them into the dataset published by Šlechtová et al. (2008) in order to test if the distinctness of *Bibarba* is supported by genetic data.

### Material and methods

Table 1 provides an overview about the newly analysed specimens; the complete dataset of Šlechtová et al. (2008) is used as comparative material to find the phylogenetic position of *Bibarba* within Cobitidae. Altogether we have analysed 104 specimens (53 species) of Cobitidae plus 3 outgroup taxa from the sister family Nemacheilidae, including 32 novel sequences of the genera *Bibarba*, *Cobitis* and *Niwaella*. Among the newly analysed species is *Niwaella* sp. from the Yangtze basin in Anhui province as well as five species of *Cobitis* (*C. brachysoma*, *C. cf. oxycephala* and the three undescribed species: *Cobitis* sp. A, *Cobitis* sp. B, and *Cobitis* sp. C) from the Pearl River basin in Guangxi and Guangdong provinces. The concatenated dataset consisted of 2045 bp (922 bp of RAG-1 and 1123 bp of *cyt b*).

Specimens were obtained from local markets or collected by hand nets; tissue samples were preserved in 96 % ethanol, voucher specimens were fixed in 4 % formaldehyde and preserved in 70 % ethanol. Voucher specimens are stored in the collections of IAPG, Liběchov (IAPG) and Lee Kong Chian Natural History Museum, Singapore

(ZRC). Morphological observations were carried out under an Olympus SZX7 dissecting scope equipped with a uEye camera system.

Genomic DNA was extracted from white muscle tissue or from fin clips using Dneasy Tissue Kit (Qiagen). An approximately 900 bp long portion of RAG-1 was amplified using the primers RAG-1F (5'-AGCTGTAGTCAGTAYCACAAATG-3'; Perdices et al., 2005) and RAG-RV1 (TCCTGRAAGATYTTGTAGAA-3', Šlechtová et al., 2007). The PCR conditions followed Šlechtová et al. (2007). The complete mitochondrial cytochrome *b* (*cyt b*) was amplified and sequenced using the primers Glu-L.Ca14337-14359: 5'-GAA GAA CCA CCG TTGTTATTCAA-3' and Thr-H.Ca15568-15548: 5'-ACC TCC RATCTYCGG ATT ACA-3' as described in Šlechtová et al. (2006). For comparison, the whole dataset from Šlechtová et al. (2008) was used to compute representative phylogenetic trees of Cobitidae including all formerly analysed genera. The Genbank accession numbers and geographic origin of the newly sequenced specimens are in Table 1; the data for the other Cobitidae are in Šlechtová et al. (2008).

The combined (*cyt b* and RAG-1) molecular dataset was analysed using the Bayesian inference in MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003).

The analyses were performed in two independent runs of 10 million generations, each using six Markov Chains, starting with random trees and sampling frequency set to 100 generations. The dataset was partitioned by genes and by codon positions, ending up with six partitions. Prior to the analyses we have run the model estimation for each of the partitions in the MEGA 5.10 software (Tamura et al. 2011), which evaluates different models according to the Bayesian information criterion (BIC) and selects the model with best BIC score. During the analyses a best fitting model was specified for each partition. The log-likelihood score distribution was examined in order to assess if stationarity was reached. The first 5000 saved trees were discarded as the burn-in and a 50 % majority rule consensus of the remaining trees was computed. Statistical support of clades was assessed by posterior probabilities.

## Results

The Yesmodels selected for each partition according to BIC were the following: K2+G+I, HKY+I and YESGTR+G+I for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> codon positions of *cyt b*, respectively, and K2+G, JC+G

**Table 1.** Specimens analysed for the genetic markers, identification numbers, geographic origin and Gene Bank accession numbers.

Species	ID	Origin	Gene Bank	Gene Bank
			Acc. No. Cyt b	Acc. No. RAG
<i>Bibarba bibarba</i>	IAPG A 8724	China, Guangxi, Du'An county, market in Dong	KT585690	KT585706
	IAPG A 8725	Miao	KT585689	KT585705
<i>Bibarba bibarba</i>	IAPG A 8728	China, Guangxi, Du'An county, carstic crater	KT585688	KT585704
	IAPG A 8729	24°00'N 107°59' E	KT585687	KT585703
<i>Bibarba parvoculus</i>	ZRC 54454_1	China, Guangxi, Liuzhou county, carstic cave	KT585682	KT585698
	ZRC 54454_2	24°47'N 108°44' E	KT585683	KT585699
<i>Cobitis</i> sp. A	IAPG A 8734	China, Guangxi, Du'An county, Chen Jiang	KT585686	KT585702
	IAPG A 8735	24°06'N 107°59' E	KT585685	KT585701
<i>Cobitis</i> sp. B	IAPG A 8835	China, Guangxi, Lingchuan county, Li Jiang 25°11'N 110°25' E	KT585684	KT585700
<i>Cobitis</i> sp. C	IAPG A 8589	China, Guangxi, Lingchuan county, Li Jiang 25°11'N 110°25' E	KT585693	KT585709
<i>Cobitis</i> cf. <i>oxycephala</i>	IAPG A 9154	China, Guangdong, Longchuan county, Dong Jiang	KT585681	KT585697
	IAPG A 9155	23°31'N 114°41' E	KT585680	KT585696
<i>Cobitis brachysoma</i>	IAPG A 9162	China, Guangdong, Longchuan county, Dong Jiang	KT585679	KT585695
	IAPG A 9163	23°31'N 114°41' E	KT585678	KT585694
<i>Niwaella</i> sp.	IAPG A 8656	China, Anhui, Shitai county, Qiupu River	KT585692	KT585708
	IAPG A 8657	30°11'N 117°31' E	KT585691	KT585707

and K2+G for the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> codon positions of RAG-1, respectively. Those were taken into account for the subsequent Bayesian analyses.

In the reconstructed consensus tree, the analysed samples of *Bibarba* together formed a monophyletic lineage ('*Bibarba* lineage'), with the two samples of *B. parvoculus* together representing the sister lineage of the samples of *B. bibarba* (Fig. 3). The *Bibarba* lineage was assigned with high statistical support as sister lineage to the Northern clade. All newly sequenced co-occurring samples of *Cobitis* and *Nivaeella* from the Pearl and Yangtze River basins turned out to be closely related to other samples of the genus *Cobitis*, but only distantly related to the *Bibarba* lineage. The phylogenetic relationships of all other Cobitidae was as discussed in Šlechtová et al. (2008).

In both species of *Bibarba* only two pairs of barbels are present: one rostral between snout and upper lip and one mandibular at the corner of the mouth (Fig. 4). The second pectoral-fin ray (i. e. first branched ray) of males is prolonged (ca. 140 % of length of second branched ray), strongly broadened (width at least 5 times of width of second branched ray) and branched only at the tip (Fig. 5). Two dissected males (ZRC 54453, IAPG A9085) exhibit an ossified postero-dorsal projection (lamina circularis) on the base of the upper hemitrichium of first and second branched pectoral-fin rays. The projection on the second branched ray is several times larger than the one on the first branched ray. Both projections are surrounded by thick tissue. The upper and the lower hemitrichia were in direct contact along their whole length.

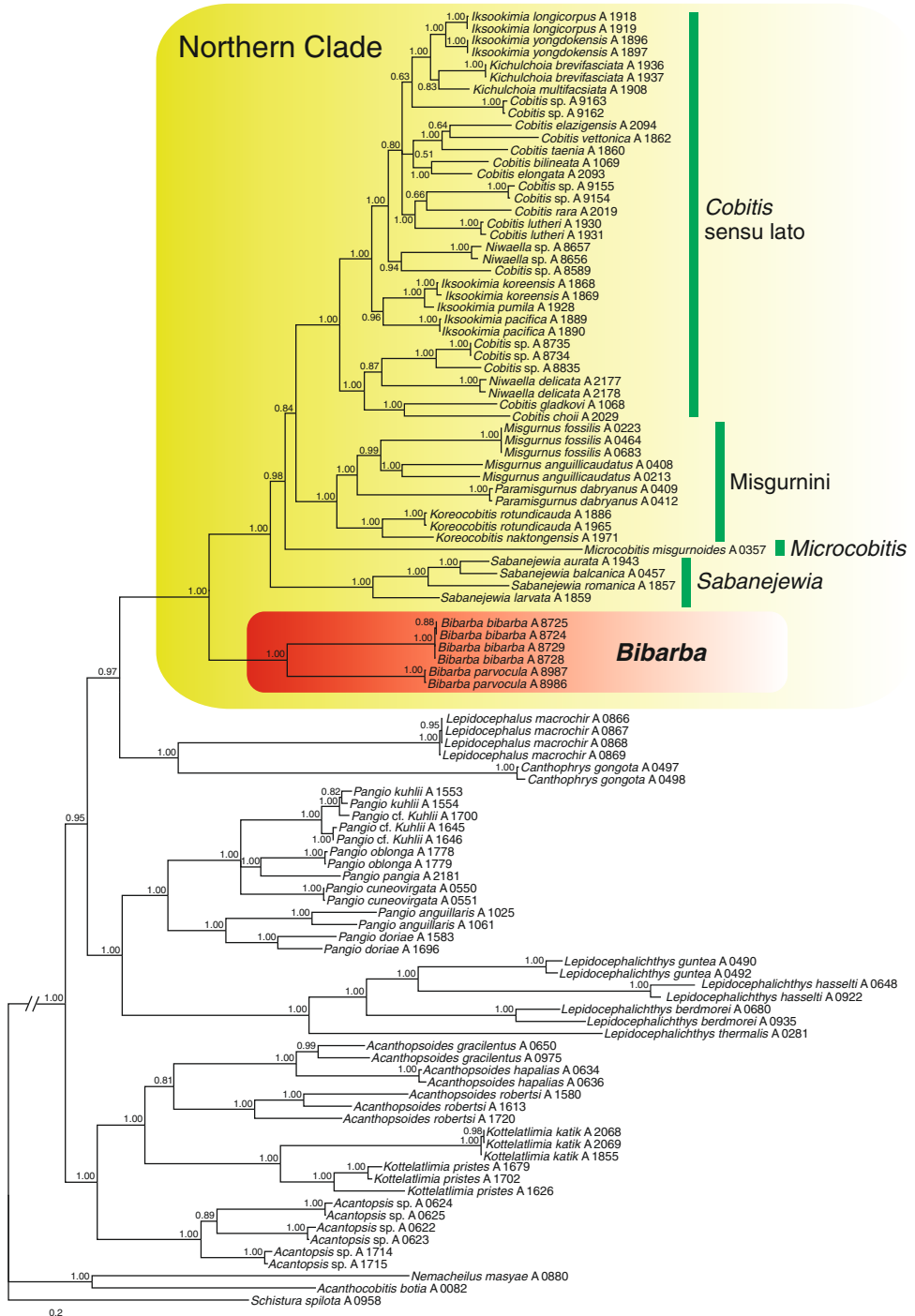
## Discussion

In our genetic analyses, all samples of *Bibarba* together formed a monophyletic, well-supported lineage, supporting the morphology-based hypothesis that it represents a distinct genus. The combined dataset of nuclear and mitochondrial DNA clearly demonstrated a sister relationship between *Bibarba* and the Northern clade; the presence of a lamina circularis in males of both species of *Bibarba* is evidence enough to support *Bibarba* as a member of the Northern clade. The scenario is additionally supported by biogeographic data, since the distributions of the Northern clade and the southern lineages are nearly not overlapping and both species of *Bibarba* occur within the geo-

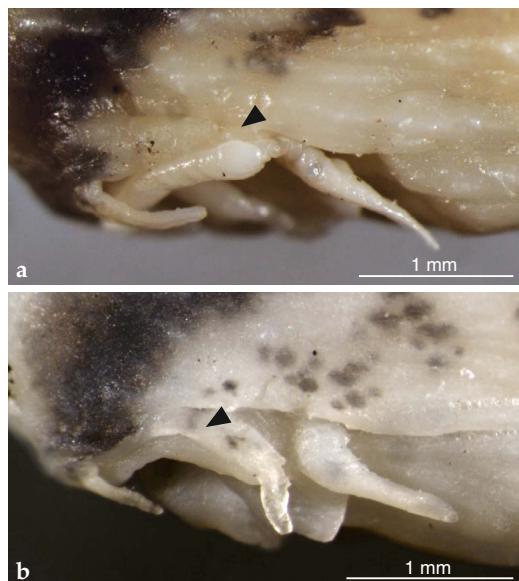
graphic range of the Northern clade. Therefore, morphological, biogeographic and genetic data support each other and substantiate the conclusion that *Bibarba* is a distinct valid genus, a member of the Northern clade and a fifth distinct lineage within the Northern clade. Since *Bibarba* is sister to the other lineages of the Northern clade it enlarges our concept of the phylogenetic structure of the Northern clade.

The most important characters that have been used to diagnose the genus *Bibarba* were the absence of the maxillo-mandibular barbel and the position of the lamina circularis on the third pectoral-fin ray (Chen & Chen, 2007). Otherwise all but one species of Cobitidae have three barbels, one rostral, one maxillo-mandibular and one mandibular (Nalbant, 1963). The only exception is *Neoeocirrichthys maydelli* among the Southern lineages which lacks barbels completely (Bănărescu & Nalbant, 1968). However, *Bibarba* has buds at the place where the maxillo-mandibular barbels in other Cobitidae are located, indicating that they have been secondarily reduced. Nevertheless, the reduction of the maxillo-mandibular barbels in *Bibarba* can be considered an autapomorphy and a diagnostic character. The fact that *Bibarba* has the maxillo-mandibular barbels completely reduced and the other two pairs have less than 1/3 of the length of most *Cobitis* species points to a decreased importance of barbels for *Bibarba*, most likely by a different ecology when compared to *Cobitis*. Most species of *Cobitis* are specialised for a life in and on fine sand, into which they burrow for hiding and from which they filter fine organic material for food (Robotham, 1978; Bohlen, 2002). In contrast, both species of *Bibarba* live in carstic area and were in several localities observed to live on bare rock surface (pers. obs.). In the Chen River where *B. bibarba* co-occurs with *Cobitis* sp., a microhabitat separation was visible – while *Bibarba* was observed on rocks, *Cobitis* was present only on sand (pers. obs.). Most likely, the reduction of barbel length and number is an adaptation for living on hard substrates.

In most species of the Northern clade adult males bear a lamina circularis; most often a single one on the base of the first branched pectoral-fin ray. This structure is a postero-dorsal projection of the upper hemitrichium; the lower hemitrichium is not involved in the formation of the lamina circularis (Rendahl, 1930, 1933; Bohlen & Harant, 2010). However, in some species of *Cobitis*, the character is doubled and a second lamina is pre-

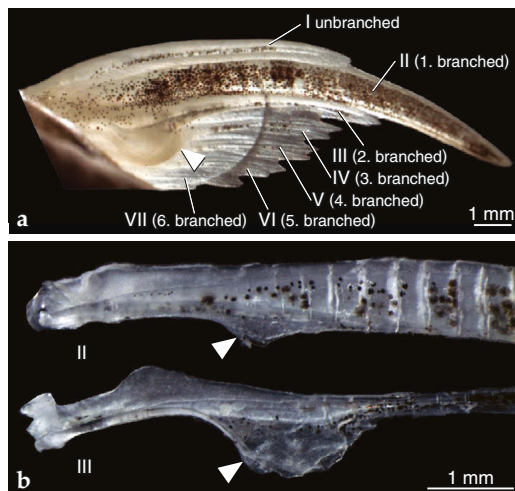


**Fig. 3.** Phylogenetic relationships among Cobitidae and position of genus *Bibarba* as revealed by Bayesian analysis of 2045 bp alignment consisting of the nuclear RAG-1 gene and the mitochondrial cytochrome *b* gene. The Northern Clade and its subclades are indicated. The values at the nodes correspond to Bayesian posterior probabilities.



**Fig. 4.** Comparison of mouth region of *Bibarba* and *Cobitis*: **a**, *Bibarba bibarba*, ZRC 54453, male, 45.9 mm SL. **b**, *Cobitis* sp., IAPG A8734, juvenile, 41.2 mm SL; both collected together in China: Guangxi: Cheng Jiang. Both in lateral view from the left side. Arrowheads indicate the base of the second barbel in *Cobitis* and a conspicuous bud in *Bibarba* at the same place. The bud might represent the rest of a reduced barbel.

sent on the unbranched pectoral-fin ray (Băcescu, 1962; Bohlen et al., 2006, Kottelat, 2017). Among the Southern lineages, an ossified postero-dorsal projection of the upper hemitrichium of the first branched pectoral-fin ray is present in *Kottelatlimia* (Kottelat & Tan, 2008). Chen & Chen (2007) described the first branched pectoral-fin ray in males of *Bibarba* as ‘thickened and elongated’ and the lamina circularis as present on the second branched ray. Our results confirm these observations, but add the presence of a second, smaller lamina circularis on the first branched pectoral-fin ray. As mentioned before, the presence of a lamina circularis on the first branched pectoral-fin ray is a diagnostic character of the Northern clade. It appears as doubled in *Bibarba* in a similar way like in *Bicanestrinia*, with the difference that the two laminae are located on the first and second branched rays (vs. on the unbranched and first branched ray in *Cobitis*). Like in other species of the Northern clade, both lamina circularis of *Bibarba* are derivate of the upper hemitrichium only.



**Fig. 5.** *Bibarba bibarba*, ZRC 54453, male, 45.9 mm SL; China: Guangxi: Cheng Jiang: **a**, right pectoral fin in dorsal view, arrowhead indicates skin sack covering lamina circularis; **b**, base of dissected first branched (top) and second branched (below) ray of right pectoral fin in dorsal view; arrowheads indicate laminae circularis.

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