Mitochondrial d-loop sequence variation and phylogeny of gobiobotine fishes*

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Abstract The mitochondrial DNA control region is amplified and sequenced from 8 genera and 10 species of gobiobotine fishes. The phylogenetic tree of Gobiobotinae and some representative species of other Cyprinid subfamilies obtained by the method of neighborhood joining, maximum likelihood and maximum parsimony with *Danio rerio* as an outgroup indicates that Gobiobotinae fishes are a monophyletic group which is close to Gobioninae subfamily. Gobiobotinae should be included into subfamily Gobioninae in terms of phylogenetic analysis. The research result supports that Gobiobotinae can be divided into genus *Xenophysogobio* and *Gobiobotia*. *Xenophysogobio* is the most primitive genera in the subfamily.

Keywords: **Gobiobotinae, Cyprinid mitochondrial d-loop, molecular systematics.**

Gobiobotine fishes are endemic to East Asia and classified as a subfamily in family Cyprinidae[1,2]. They are a group of small bottom-dwellers in running water and have some obvious monophyletic characters such as 3 pairs of chin barbels besides 1 pair of mouth barbels and the unique osteological air bladder capsule. Gobiobotine fishes are divided into two genera: *Xenophysogobio*, which contains two species only, and *Gobiobotia*, which contains 2 subgenera, *Progobiobotia* and *Gobiobotia*. There are 18 species in Gobiobotinae recorded at present time. Gobiobotine fishes are a typical East Asian group that mainly occurs in the mainstream of rivers' champaign areas. They are distributed in Taiwan & Hainan Island, Korea peninsula and in Amur River. However, we cannot find them beyond Yuanjiang River to the west, obviously because of the barrier of Qinghai-Tibet plateau. For these reasons, Gobiobotine fishes are considered ideal materials in the biogeographic analysis. As an obvious monophyletic group, they are also excellent objects in systematics research. The systematic position of gobiobotine fishes in Cyprinidae has not been affirmed. Many authors believed that they are a group between Cyprinidae and Cobitiade. Liu suggested that Gobioninae and Gobiobotinae should be grouped to Gobiobotidae[3]. Other authors thought they should be a genus in Gobioninae[4]. But gobiobotine fishes are popularly attributed to Cyprinidae for their large scale and pharyngeal teeth form, and they are always treated as a subfamily for the reason of taxonomy. A few studies have been conducted on gobiobotine fishes in China. Chen et al.[1] studied the gobiobotine fishes and divided them into three subgenera, and indicated that the osteological air bladder capsule has very important systematic significance. He et al.[2,5,6] grouped gobiobotine fishes in China into two genera. Former systematic work on gobiobotine fishes is mainly based on the morphological and osteological characters. Now mitochondrial DNA (mtDNA) has been widely used as a marker for evolutionary and population studies[7] because of its compact size, nearly complete maternal inheritance, and fast evolutionary rate. The mitochondrial DNA of fish is a small circular molecule of 16 kb ~ 21 kb nucleotides with a compact and conserved organization. The d-loop of mtDNA may reveal evolutionary relationships in terms of taxonomy[8~10]. And analysis of the d-loop region has been widely used in molecular systematics of fishes. In this study, we analyze the d-loop region to study the relationship among gobiobotine fishes.

1 Materials and methods

All the fishs for analysis are listed in Table 1. The study also included some representative species from other cyprinid subfamilies to testify the monophyletic status of Gobiobotinae.

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Table 1. The source of gobiobotine fishes in China

No.	Species	Source
1	*Xenophysogobio boulengeri*	Hejiang, Sichuan
2	*X. nudicorpa*	Yibin, Sichuan
3	*Gobiobotia. kolleri*	Shangrao, Jiangxi
4	*G. filfer*	Hejiang, Sichuan
5	*G. tungi*	Wuyuan, Jiangxi
6	*G. longibarba*	Jian'ou, Fujian
7	*G. cheni*	Taiwan
8	*G. longibarba*	Liuzhou, Guangxi
9	*G. kolleri*	Liuzhou, Guangxi
10	*Gobio gobio*	GG0388392[a)]
11	*Labeo labeo*	LBI388414[a)]
12	*Barbus barbus*	BME388416[a)]
13	*Acrossocheilus sp*	APA131833[a)]
14	*Danio rerio*	ACO24175[a)]
15	*Aphyocypris chinensis*	Liaoning
16	*A. kikuchii*	Taiwan

a) Sequences from GenBank.

Total genomic DNA of fishes was extracted by routine method. Target region of the mitochondrial DNA was amplified using the polymerase chain reaction (PCR) with the primers DL7 (5′-ACCCCTG-GCTCCCAAAGC-3′) and DH2 (5′-ATCTTAG-CATCTTCAGTG-3′)[10]. The PCR reaction contained approximately 100 ng of template DNA, 1 μL each primer, 5 μL 10 × buffer, 2 μL of each dNTPs, and 2.0 units Taq DNA polymerase in a total volume of 50 μL. The reactions were carried out by an initial 94 ℃ denaturation for 4 min, then 35 cycles at 94 ℃ for 45 s, 58～62 ℃ for 45 s, 72 ℃ for 1 min, and a final extension at 72 ℃ for 8 min. The amplified DNA fragments were electrophorezed, purified, and sequenced by the routine methods.

Multiple alignment of the sequences was performed using the CLUSTAL X software and reexamined by eye. The Kimura two-parameter distances were calculated using MEGA2.0 and PHYLIP3.25 softwares. The methods included the maximum parsimony (MP), maximum likelihood and the neighbor-joining (NJ) to reconstruct the molecular phylogenetic trees. Bootstrap analyses with 1000 replications were carried out using the MEGA software.

2 Result and discussion

The sequences of d-loop region obtained from 11 fish species collected in China (Table 1) with other 5 sequences downloaded from GenBank were read into CLUSTALX to align. After the alignment, the matrix was used to phylogenetic analysis. Low G content (14.6%) and almost equal A, T, and C content (32.5%, 32%, 20.9%, respectively) were found. The A + T% content (64.5%) was higher than that of G + C% (35.5%). The 982 analyzed sites included 443 conservative sites, 519 mutation sites, 250 informative sites for parsimony, and 266 dot mutation sites.

The systematic trees obtained from NJ, MP and ML analyses showed identical results. In the NJ tree with *Danio rerio* being the outgroup (Fig. 1), gobiobotine fishes form a monophyletic group, closing to Gobioninae. *Labeo labeo* in *Labeoninae* is relatively primitive and far from Gobiobotinae, next to it is *Barbus barbus* in Barbinae and then *Acrossocheilus* sp. Both *Aphyocypris chinensis* and *A. kikuchii* belong to *Aphyocypris* Gunther and form a sister group. *Xenophysogobio boulengeri* and *Xenophysogobio nudicorpa* form a sister group. This is accordant to morphological result which classified them into *Xenophysogobio* Chen et Tsao. While other five species belonging to *Gobiobotia* Kreyenberg in taxonomy form a monophyletic group and establish an sister group with *Xenophysogobio*. Among them, *G. longibarba* and *G. kolleri* form an sister group, while *G. cheni* and *G. filfer* form another one which combines the former to establish a new sister group. The *G. tungi* is relatively primitive. This result is basically similar to that based on morphological study, in which *G. longibarba*, *G. kolleri*, *G. cheni* and *G. filfer* all belong to the southern group. Southern group represents the more special grade and we think that it is the most special systematic grade among gobiobotine fishes.

He et al.[5] gave a detailed description of the air-bladder capsule and its adjacent structure in gobiobotine fishes. They considered that gobiobotine fishes originated from *Microphysogobio* in Gobioninae. *Gobiobotia filfer* has the most specialized air-bladder capsule and this means that it is the most specialized species in the Gobiobotinae group. The result we obtained from molecular analysis is identical to our morphological observation, in that gobiobotine fishes are a group close to the subfamily Gobioninae, and should be divided into two genera. The result supports the hypothesis of dispersal in gobiobotine fishes' biogeographic process, which indicates that all the gobiobotine fishes came from one origin and then dispersing in different directions.

Taiwan was a part of the protrusion in the Ningnan Mountains of Fujian and Zhejiang Provinces in

the middle and late periods of Yanshan Movement in Mesozoic. In the early Tertiary, the collision of Euroasian and Indian plate caused the Himalayan orogenic movement, the ancient plate separated and subsided. At that time the ancient central mountains of Taiwan constituted the island arches in margin of sea groove in early Tertiary. The Taiwan Movement in the late Himalayan Movement led to the formation of the Taiwan Island. In Cenozoic Taiwan Island had a similar environmental background to mainland of China. In Pleistocene, since the Quaternary Ice age, the tropical fauna had moved back to south. Taiwan and East Himalayas shared the same climate, plant cover and fauna. In Fig. 1, the *Gobiobotia longibarba* distributed in Jian'ou, Fujian Province, do not form a monophyletic group with the same species collected from Liuzhou, Guangxi Province, but is related to *Gobiobotia cheni* from Taiwan. We think that it might relate to the Ice Age and interval Ice Age of Quaternary. After its appearing, the Taiwan Island was connected with and separated from the continent many times. This change in sea/land is closely related to the Ice Age and interval Ice Age of Pleistocene. In the four Ice Ages, Guunz, Mindel, Riss and Wurm, the sea level degraded, the Taiwan Island was connected to the continent; in the interval Ice Age, the sea level upgraded, the Taiwan Island separated from the continent. So the main species of freshwater fish fauna in southeast seashore of China has been preserved in Taiwan until now.

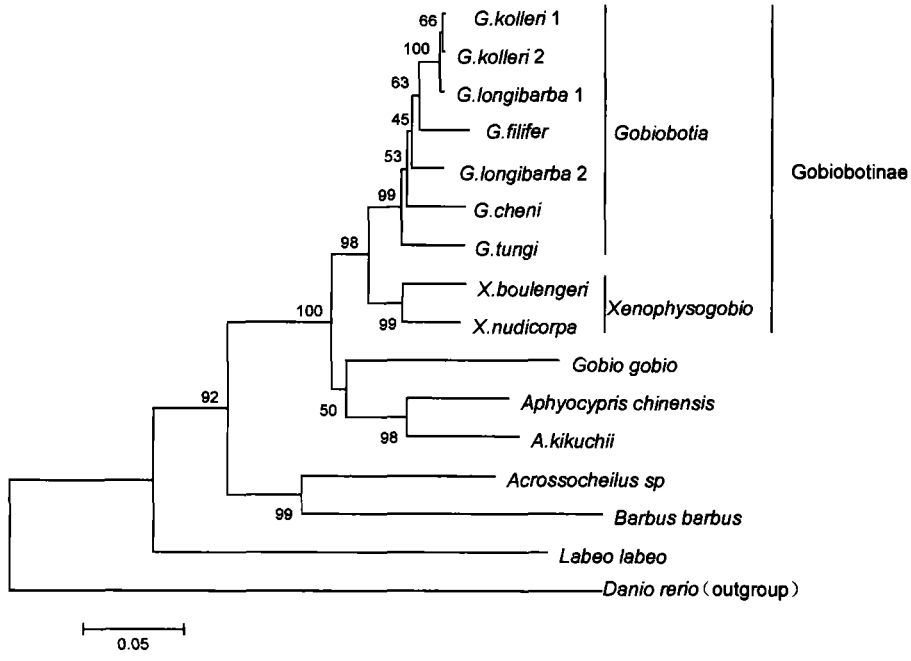

Fig. 1. Neighbor-joining tree using the Kimura two-parameter distance based on the control region sequence. Bootstrap analysis values (>50%) from 1000 replications are indicated at the nodes.

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