A new blind species of the cave genus Oreonectes from Guizhou, China (Nemacheilinae)

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Abstract
This study aimed to describe a new specimen of cavefish collected from a karst cave in the Daqikong area of Libo County, Guizhou. Twenty-six cavefish specimens were collected and identified as a new species of Balitoridae: Nemacheilinae, and named Oreonectes daqikongensis sp. n. A genetic analysis was performed and showed that its genetic distances from Oreonectes shuilongensis and Oreonectes platycephalus are higher than intraspecific distances. Discovery of this species will be helpful to understand the distribution of Oreonectes.

Keywords
Cavefish, Libo, new species, Oreonectes daqikongensis sp. n.

Introduction
Nemacheilinae are common in tropical Asia. They occur in a great variety of habitats, particularly abundant in swiftly flowing hillside streams. The similar living environment may help explain why many cavefish of Asia belong to this subfamily (Kottelat 1990).

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There are 15 genera and more than 100 species found belonging to the Nemacheilinae subfamily in China so far (Zhu 1995). The *Oreoneutes* was first established by Günther (1868) with *O. platycephalus* as the type species. A total of 16 species of Oreoneutes are considered valid. Du et al. (2008) divide Oreoneutes into two groups that including furcocaudalis group and platycephalmus group. The platycephalmus group includes the *O. platycephalus*, *O. Polystigmus*, *O. guananensis* (Yang et al. 2011), *O. luochengensis* (Yang et al. 2011), and *O. anophthalmus* (Zhu 1989). The furcocaudalis group includes the *O. microphthalmus* (Du et al. 2008), *O. macrolepis*, *O. retrodorsalis* (Lan et al. 1995), *O. acidorsalis*, *O. barbatus*, *O. duanensis*, and *O. donglanensis* (Lan et al. 2013), *O. elongates* (Tang et al. 2012), *O. translucens* (Zhang et al. 2006), *O. furcocaudalis* (Zhu 1989), and *Oreoneutes* sp. n. (Chen et al. 2011). They all dwell in underground rivers of the karst environment (Yang et al. 2011; Lan et al. 2013). During a cave biodiversity survey on Libo County, Guizhou in 2011, we using seines nets and the bait collected 26 new cavefish specimens at Daqikong area. This study aimed to describe and identify the new specimen of cavefish.

**Materials and methods**

The holotype was fixed and preserved in 10% formalin, and the paratypes were preserved in anhydrous ethanol. The specimens were stored in the Animal Specimen Room of the School of Life Sciences, Guizhou Normal University (GNUG). All measurements are taken on the left side of the fish specimens. All measurements were taken point to point with digital calipers to 0.1 mm. The new species was identified according to the morphological features, molecular phylogenetic evidence, and distribution regions. Counts and proportional measurements follow Tang et al. (2012). The sources of material of other *Oreoneutes* species is in Appendix 1.

The tissue sample was extracted from the right side of the specimen no. 25, from which the genomic DNA was extracted using from muscle tissues by standard phenolchloroform methods (Sambrook and Russell 2001). Then the cytb gene segment was amplified using the Cyprinidae universal primers L14724 and H15915 (Rui et al. 2012). Both the amplification and sequencing were completed in Beijing Ruijie Gene Technology Co., Ltd. (Beijing, China).

The Sequence Alignment Editor (BioEdit) software was used to analyze sequencing peaks and delete carrier sequences, and then Seqman v5.51 (DNASTar) was used to perform the sequence assemble and alignment. Complete cytb sequences of other 29 species of Nemacheilinae were obtained from GenBank (Table 1). The cytb gene nucleic acid sequences of all the 30 species were compared with the ClustalW method of MEGA 6.0 software (Tamura et al. 2013), the terminal irregular regions were removed manually. Subsequently, the phylogenetic tree was established using the maximum likelihood (ML) method in MEGA 6.0, while reliability was tested using the Kimma2-Pamameter distance model and bootstrap method by repeating 1000 times.
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Table 1. GenBank accession numbers for the analyzed samples included in the phylogenetic analysis.

| Genus    | Species          | Accession |
|----------|------------------|-----------|
| Oreonectes | shuilongensis    | KF640641  |
|          | daqikongensis    | KU987436  |
|          | platycephalus    | DQ105197  |
|          | fasciolata       | HM010565  |
|          | caudofurca       | JN837651  |
|          | desmotes         | GQ174368  |
|          | callichroma      | JN837652  |
|          | latifasciata     | JN837653  |
|          | bucculenta       | JN837654  |
|          | macrotaenia      | JN837655  |
|          | amplizona        | JN837656  |
|          | cryptofasciata   | JF340401  |
|          | sikmaiensis      | JF340405  |
|          | pociuli          | JF340407  |
|          | longa            | JF340408  |
| Schistura | pycnolepis       | KF041000  |
|          | acuticephala     | HM010527  |
|          | longidorsalis    | HM010550  |
|          | potanini         | JF340388  |
| Homatula  | pulcher          | JF340402  |
| Traccatichthys | shuangjiangensis | JF340404 |
| Schistura | anguilliioides    | HM010582  |
| Paracobitis | xiangziensis    | JN96407   |
|          | stoliczki        | DQ105249  |
| Triphylphysa | siluroides      | EF212443  |
|          | bleeker          | FJ406605  |
|          | stenura          | JN837657  |
|          | orientalis       | DQ105251  |
| Nemacheilus | maysae          | GQ174377  |
|          | ornatus          | GQ174363  |
|          | pallidus         | GQ174370  |

Results

Oreonectes daqikongensis sp. n.
http://zoobank.org/598D9793-208A-45ED-BCA9-A1C9203003A3

Type materials. The 26 specimens were collected from Daqikong area of Libo County, Guizhou; the overall length of the specimen was 37.82–83.10 mm and the body length was 31.28–70.96 mm.

Holotype. (No. CNGZNU20110128001; Figure. 1a–b) The total length is 77.14 mm and the body length is 61.46 mm. Holotype was collected from a subterranean river of the Daqikong area (N 25°17’05.1", E 107°44’54.3"; H 488 m) in January 2011.
Figure 1. a Holotype of Oreonectes daqikongensis sp. n. NO.CNGZNU20110128002. b A living Oreonectes daqikongensis sp. n.

It was stored in the animal specimen room of the School of Life Sciences, Guizhou Normal University, Guiyang, China.

**Paratypes.** (25, No. CNGZNU20110128002–CNGZNU20110128026) Paratypes were collected and stored in the same places as the holotype.

**Habitat.** This species was found only in the Daqikong scenic area. The opening of the cave was halfway up the mountain, and the distance from the opening to the pool was about 15–20 m. The cave got no sunshine because of the twisty pathway. A large number of *Hipposideros armiger* lived in the cave and a thick layer of bat dung was found on the ground. Groundwater extended into the cave, and the water rushed outside the cave in the case of heavy rain. So far, no other fish, shrimps, or aquatic animals were found in the cave. The subterranean river belonged to the Dagou river system, and was the main river of the Libo County, which runs through the whole county, enters Guangxi from the Laocun Xiang, and was the major tributary of the Duliu River system (Figure 2).

**Diagnosis.** The species has a large head, and the width of the head is larger than its depth. The frontal torso is nearly cylindrical, the backend gradually compresses, and the head is slightly flattened. There is a short distance between the anterior and posterior nostrils, and the anterior nostril forms a short and tubular structure, which is truncated backward. The pectoral fin extends backward to or beyond the starting point of the pelvic fin. The body is naked. The eyes are completely degraded; and eye socket was filled in fat tissue and without any outside remnant indicating their presence. The superior and inferior caudal peduncles have well-developed soft finfolds. No carneous
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fin flaps are present in the pelvic fin axilla. The air bladder is wrapped in a bony capsule, and the posterior chamber of the air bladder is developed into a membranous chamber, which is separated from the abdominal cavity and connected to the anterior chamber by a short duct. The whole body is white and transparent, when they are alive, they look a little red because the blood inside, and is unlikely to become black when it is fed in sunlight for a long term.

Description. The species has a slightly elongated body, slightly ridged back, body slightly spindle, and compressed hindquarters, and its widest part is at the gill cover. The main measurable characters of O. daqikongensis are shown in Table 2. The head is slightly flattened, lips are rounded, eyes vestigial. Anterior and posterior nostrils well separated. Anterior nostril forms short tubular and posterior nostrils elliptical. The fish has an inferior mouth, and the upper and lower lips are connected at the corner of the mouth. The upper jaw is curved and the lower jaw is spoon shaped. The mouth aperture is U-shaped, and its rear end reaches the bottom part of the posterior edge of the naris. It has three pairs of slim barbels, and one pair each of inner rostral barbels, outer rostral barbels, and mouth corner barbels. The inner rostral barbels are shorter, and the outer rostral barbels extend backward to exceed the rear edge of the posterior naris, while the maxillary barbels extend backward and their ends are appropriate at the center of the rear edge of the opercular bone. The superior and inferior sides of the caudal peduncle have well-developed ridge-like fatty soft fin folds; especially, the soft

Figure 2. Collection localities of O. daqikongensis sp. n.
Table 2. Main morphometric characters of *O. daqikongensis* sp. n.

| Measurements                        | Holotype | Range          | Mean ± SD      |
|-------------------------------------|----------|----------------|----------------|
| Total length (mm)                   | 50.96    | 41.62–77.14    | 57.15 ± 10.36  |
| Standard length (mm)                | 41.68    | 34.22–61.46    | 46.49 ± 8.11   |
| Percentage (%) of SL                |          |                |                |
| Body height                         | 18.04    | 15.30–23.26    | 20.43 ± 1.92   |
| Body width (at dorsal fin origin)   | 13.72    | 12.42–19.74    | 15.86 ± 1.77   |
| Predorsal length                    | 50.82    | 50.79–56.32    | 53.06 ± 1.74   |
| Length of caudal peduncle           | 18.67    | 13.16–19.18    | 16.50 ± 1.95   |
| Depth of caudal peduncle            | 7.15     | 7.15–9.90      | 8.26 ± 0.82    |
| Percentage (%) of HL                |          |                |                |
| Body height                         | 60.74    | 56.94–82.90    | 70.48 ± 6.93   |
| Head height (at nape)               | 44.75    | 44.30–58.84    | 50.19 ± 4.38   |
| Head width                          | 49.11    | 49.11–61.73    | 55.47 ± 4.03   |
| Length of inner rostral barbel      | 17.12    | 12.36–17.75    | 15.03 ± 1.83   |
| Length of outer rostral barbel      | 24.39    | 18.42–33.12    | 25.86 ± 4.29   |
| Length of maxillary barbel          | 25.85    | 22.92–30.17    | 27.04 ± 1.68   |
| Percentage (%) of TL                |          |                |                |
| Body height                         | 14.76    | 12.69–18.77    | 16.43 ± 1.57   |
| Body width (at dorsal fin origin)   | 11.22    | 10.30–15.91    | 12.90 ± 1.36   |
| Head length                         | 24.29    | 22.29–25.93    | 23.62 ± 0.93   |

Fin folds between the superior caudal peduncle and the dorsal fin are more apparent than those in the inferior caudal peduncle, where its front end reaches the upper part of the anal fin. The superior soft fin folds originate from the rear edge of the dorsal fin base to one third of the front edge of the caudal fin base, where compressing the dorsal fin backward can reach the origin of the soft fin folds. The inferior soft fin folds originate from the rear edge of the anal fin base to one third of the front edge of the caudal fin base.

The distance from the dorsal fin origin to the rostral end is larger than that from the dorsal fin origin to the caudal fin base, and the outer edge is truncated or slightly concave. The rear end of the dorsal fin can be compressed to reach the soft fin fold origin. The fish has a long pectoral fin, which extends backward to or beyond the pelvic fin base. Also, the pectoral fin has a very special morphology, which does not have branched fin rays. The first and second fin rays are very long, forming a spoke-like shape. The ventral fin originates at a place opposite to the origin of the dorsal fin, and it extends backward to cover the anus and close to the origin of the anal fin. The distance from the anus to the anal fin origin is about 1 mm. The anal fin extends to its base to half of the caudal fin base. The posterior edge of the caudal fin is forked, and the upper lobe is slightly longer than the lower lobe.

The fish is naked, and the intact lateral line is superficially subcutaneously buried, which is flattened from the upper angle of the gill cover and extends backward to the center of the caudal fin base. Sensory tubes are present in the head connecting to the lateral
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line at the upper part of the posterior edge of the gill cover, and bifurcate into two lateral lines toward the head. These tubes travel from the supraorbital bone to the inner side of the anterior nostrils and from the infraorbital bone to the outer side of the nostrils, and connect to the two lateral lines via a transverse lateral line at the parietal bone. The whole body is colorless, and the living fish is translucent, where the internal organs are visible. The stomach is U-shaped, and the intestine is in its rear part, which is slightly curved and extends to the anus. The anterior bladder chamber is completely coated by the bony bladder sac, which has a bony posterior wall and no opening. However, the posterior bladder is a well-developed membranous chamber, which is separated from the abdominal cavity and connected to the anterior chamber by a short tube. An oval transparent area exists in the posterior branchial aperture, which is inset in both sides of the body.

Color. The whole body of the living species is pale pink and translucent, where the vertebra, body segment at caudal peduncle, cardinal gill, and internal organs are visible. Its body color is unlikely to change when it is fed in the laboratory under light for a long term.

Phylogenetic findings. In the 25 specimens of O. daqikongensis sp. n., the cyt b sequence was at 1140 bp, and the base did not show any difference among them, in which $T = 27.6\%$, $C = 28.7\%$, $A = 28.8\%$, and $G = 14.9\%$, and the overall transition/transversion rate was $R = 0.50$. The genetic distances between the new species and O. platycephalus and O. shuilongensis were 0.1802 and 0.1212, respectively, which were smaller than the genetic distance among species of other categories. The genetic distance between Oreonectes and the other categories of Nemacheilinae ranged from 0.1518 to 0.2546. The interspecific genetic distance of Nemacheilinae was 0.0009–0.2533 (Figure 3). The sequence divergence of Cyt b between this species and O. shuilongensis was 13.7, and that between this species and O. platycephalus was 19.4. Additionally, the divergence ranged from 19.8 to 27 between this species and species of other genus of the Nemacheilinae subfamily (Figure 4). The divergence of Oreonectes was smaller compared with the other genus of Nemacheilinae. Since the genetic distances between O. daqikongensis sp. n. and the other species of Oreonectes were greater than the interspecific distance of each category of Nemacheilinae, O. daqikongensis sp. n. was considered as a new species. In the phylogenetic tree (Figure 5), O. daqikongensis sp. n. was clustered with O. shuilongensis.

Figure 3. Genetic distance between O. daqikongensis sp. n. and species of Nemacheilinae.
Figure 4. The sequence divergence of Cyt b between *O. daqikongensis* sp. n. and species of Nemacheilinae.

Figure 5. Maximum likelihood phylogenetic trees of Nemacheilinae.
A new blind species of the cave genus Oreonectes from Guizhou, China (Nemacheilinae) (Bootstrap value (BP) = 99) and O. platycephalus (BP = 74). Oreonectes with Schistura, Homatula, and Nemacheilus genera was divided into two subsets (BP = 97). This species inhabits in the karst caves. Cave environments were easier to form geographical isolation for the independence of different caves. Therefore, according to the genetic distance, differences in sequences, and phylogenetic tree analysis, O. daqikongensis sp. n. belongs to a new species of Oreonectes.

**Discussion**

This species has nearly cylindrical forequarters, gradually compressed hindquarters, and a slightly flat head. The anterior and posterior nostrils are separated by a short distance, and the anterior nostrils form a short and tubular structure with their rear ends extending to become whisker or cusp. It has three pairs of barbels, and naked body. The anterior bladder chamber is completely coated by the bony bladder sac, and the posterior bladder is a well-developed membranous chamber, which is separated from the abdominal cavity and connected to the anterior chamber by a slender duct. Its stomach is U-shaped. All these features are consistent with the typical characteristics of Oreonectes described by Zhu (1989).

The new species has completely degraded eyes, without any outside remnant indicating their presence. This feature differed from the features of the following four species: O. donglanensis and T. xiangxiensis had degraded eyes, with only a small black spot visible, and the eye sockets are filled with loose fat globules; O. macrolepis had very small eyes, almost blind; and O. microphthalmus had highly degraded eyes, with only eyespots or eye sockets visible. This feature can also be differentiated from the features of the following five species that have normal eyes in appearance: O. platycephalus, O. polystigma, O. luochengensis, O. retrodorsalis, and O. elongates.

Forked caudal fins of the new species, so the new species belong to furcocaudalis type. Eyes completely degradation of this new species can be different from other species of furcocaudalis group with eyes nomal. Such as O. microphthalmus, O. macrolepis, O. retrodorsalis, O. duanensis, O. donglanensis and O. furcocaudalis. Lateral line complete of the new species, this characteristic make a distinction between O. barbatus, O. elongatus, O. translucens and O. acridorsalis which are lateral line incomplete or no lateral line. The comparison of main traits between the new species and the similarity species of Oreonectes (O. barbatus, O. elongatus, O. translucens and O. acridorsalis) is in Table 3.

Oreonectes daqikongensis sp. n. and O. shuilongensis are both distributed in Guizhou province, but in different county. The two species which have close genetic distance. There are different characteristics as following: a forked caudal fin (vs. truncated or slightly concave belong to platycephalus group), possessing adipose crests of the caudal peduncle (vs. no adipose crests), disappeared eye (vs. eye normal), lateral line is completely (vs. incomplete, 8–10 pores) and body translucence (vs. top of head and body gray and black). Oreonectes daqikongensis sp. n. and O. platycephalus which have close genetic distance with the new species. However, they can be differs by naked body (vs.
**Table 3.** Comparison of traits between *O. daqikongensis* sp. n. and the similarity species of *Oreonectes*.

| Trait                        | *O. daqikongensis* sp. n. | *O. acridorsalis* | *O. barbatus* | *O. elongatus* | *O. translucens* |
|------------------------------|----------------------------|-------------------|---------------|---------------|-----------------|
| No.                          | 15                         | 5                 | 8             | 3             | 3               |
| Locality of collection       | Daqikong area, Libo County, Guizhou | Tian'e County, Guangxi | Nandan County, Guangxi | Huangjiang County, Guangxi | Duan County Guangxi |
| Dorsal fin rays              | iii, 8–9                   | iii, 7            | iii, 8–10     | iii, 8–9      | iii, 8          |
| Anal fin rays                | iii, 6                     | iii, 5            | ii, 5–6       | ii, 6–7       | iii, 6          |
| Pectoral fin rays            | i, 11–12                   | i, 10–12          | i, 6          | i, 6          | i, 11           |
| Pelvic fin rays              | i, 6–7                     | ii, 5–6           | ii, 6         | i, 6          | i, 6            |
| Caudal fin branched rays     | 13–14                      | 13–14             | 14            | 14            | 16              |
| Pectoral fin rays            | Pectoral fin extend to or beyond the origin of the ventral fins | Pectoral fin Fan-shaped, extend to half of the distance between origins of pectoral fin and ventral fin | Pectoral fin extend to approx. two thirds of the distance between origins of pectoral fin and ventral fin | Pectoral fin long and narrow, greater than 1/2 the distance between origins of pectoral and pelvic fins | Pectoral fin long and narrow, almost reaching pelvic-fin origin |
| Pelvic fin rays              | The first and second branched rays of ventral fin are long, but do not form a spiny shape | Pelvic fin is shorter, origin is ahead of dorsal fin origin, extend, but cannot reach anus | Pelvic fin Origin is opposite to the dorsal fin origin, extend but cannot reach the anus | Pelvic fin relatively slender, extending slightly over anus | Pelvic fin extending slightly beyond anus |
| Anal fin rays                | Anal fin extend to reach half between the anal fin base and caudal fin base | Anal fin truncated outer edge | Anal fin truncated or slightly convex outer edge | Anal fin origin next to anus, tip nearly reaching middle of caudal peduncle | Anal-fin origin just posterior to anus |
| Lateral line                 | Lateral line is subcutaneously buried completely, which in the head has three branches | No lateral line in the body, lateral line in the head is not well-developed | No lateral line in the body, lateral line in the head is not well-developed | Lateral line incomplete, with 4 pores behind opercle, connecting to the cephalic lateral-line system. | Lateral line incomplete, with only 3 or 4 lateral pores behind head. |
| Eyes                         | Absent                     | Absent            | Absent        | Absent        | Absent          |
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Both morphological and molecular phylogenetic evidence revealed that *O. daqikongensis* sp. n. is a new species of *Oreonectes*. *Oreonectes* is distributed in the underground rivers in the karst region of Southwest China, of which *O. platycephalus* is most widely distributed. All these areas belong to the Pearl River and the Red River systems. Most of this species is distributed in the Karst regions of Guangxi. Currently, *O. shuilongensis* and *O. daqikongensis* sp. n. have been discovered in Guizhou, and most are distributed in the southern area of Guizhou. This work is the first time to describe the new species in detail. The discovery of this species will be conducive to comprehensively understand the distribution of *Oreonectes*.

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### Table 4. Comparison of traits between *O. daqikongensis* sp. n. and similar species of *Oreonectes*.

| Trait                     | *O. daqikongensis* | *O. shuilongensis* | *O. platycephalus* |
|---------------------------|--------------------|--------------------|--------------------|
| No. of specimens          | 15                 | 16                 | 4                  |
| Location                  | Libo, Guizhou      | Sandu, Guizhou     | Zhaoping, Guangxi  |
| Dorsal fin rays            | iii, 8–9           | iii, 7–8           | iii, 6–7           |
| Anal fin rays              | iii, 6             | iii, 6             | iii, 5             |
| Pectoral fin rays          | i, 11–12           | i, 11–12           | i, 10              |
| Pelvic fin rays            | i, 6–7             | i, 6               | i, 6–7             |
| Anterior nostril           | Anterior nostril in short tubular structure, which is obliquely cut tube | Anterior nostril in short tube extending into relatively long barbel, beyond posterior edge of eye | Anterior nostril in short tube extending into relatively long barbel, beyond edge of posterior nostril |
| Lateral line               | Lateral line is subcutaneously buried completely, which in the head has three branches | Lateral line incomplete, terminates above pectoral fin; 8–10 pores | Lateral line incomplete, terminates above pectoral fin |
| Caudal fin                 | caudal fin is forked | Truncated or slightly concave | Rounded |
| Body color                | The whole body of the living species is pale pink and translucent | ‘Top of head and body gray and black in fresh condition; grayish in dorsum and body light brown after preservation in alcohol; fins transparent | In formaldehyde, body light brown; dorsum and side of body with irregular dark gray; dark brown horizontal stripe at end of caudal fin; fins without stains |
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Appendix 1

The source of the material of other Oreonectes species.

| Species            | Number                      | Standard length (mm) | Distributions locations          | River system |
|--------------------|-----------------------------|----------------------|----------------------------------|--------------|
| O. platycephalus   | KIZ200304597–200304600      | 50.9–58.6            | Jinxiu county, Guangxi           | Zhujiang     |
| O. polystigmus     | KIZ2001060507 (holotype)    | 57.7                 | Guilin city, Guangxi             | Guijiang     |
| O. microphthalmus  | KIZ2004009395 (holotype)    | 39.0                 | Du’an county, Guangxi            | Red River    |
| O. macrolepis      | CLJH91065 (holotype)        | 54.8                 | Huanjiang county, Guangxi        | Liujiang     |
| O. retrodorsalis   | 9110001 (holotype)          | 37.00                | Nandan county, Guangxi           | Red River    |
| O. acridorsalis    | CLJH04100608 (holotype)     | 48.3                 | Tian’e county, Guangxi           | Red River    |
| O. barbatu         | CLJH2009080003 (holotype)   | 54.1                 | Nandan county, Guangxi           | Liujiang     |
| O. duanensis       | CLJH2011090302 (holotype)   | 56.1                 | Du’an county, Guangxi            | Red River    |
| O. donglanensis    | CLJH2010010051 (holotype)   | 45.2                 | Donglan county, Guangxi          | Red River    |
| O. elongatus       | ASIZB189288 (holotype)      | 78.2                 | Huanjiang county, Guangxi        | Zhujiang     |
| O. guananensis     | KIZ2010003067 (holotype)    | 77.0                 | Huanjiang county, Guangxi        | Xijiang      |
| O. luochengensis   | KIZ2010003073 (holotype)    | 74.9                 | Luocheng county, Guangxi         | Xijiang      |
| O. translucens     | ASIZB94785 (holotype)       | 45.8                 | Du’an county, Guangxi            | Red River    |
| O. furcocaudalis   | KIZ9309003, KIZ9309004      | 58.0–58.7            | Du’an county, Guangxi            | Red River    |
| O. anophthalmus    | KIZ1994001-005              | 25.3–36.9            | Wuming county, Guangxi           | Youjiang     |