Zootaxa 5162 (5): 525–540
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5162.5.4
http://zoobank.org/urn:lsid:zoobank.org:pub:02E91F18-BF2D-4A6C-859F-2D29D9F9DE94

A new species of the genus *Misgurnus* (Cypriniformes, Cobitidae) from Ryukyu Islands, Japan

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Abstract

A new loach species, *Misgurnus amamianus*, is described based on 27 type specimens sampled from Ryukyu Islands, Japan. This new species can be distinguished from its congeners by the following combination of characteristics. 1) In adult males, a lamina circularis at the base of the pectoral fin is poleaxe-shaped and rounded at the back. 2) The last ray of dorsal fin is not branched, and its length is less than half the length of the second-branched soft ray. 3) The projected length from the posterior edge of the anal-fin base to the caudal-fin base is longer than the length from the insertion point of the pelvic fin to the anterior edge of the anal-fin base. 4) The ridges on the caudal peduncle at the upper and lower edges are weakly developed. The validity of this new species is also supported by the phylogenetic analysis of the mitochondrial DNA control region.

Key words: Cobitoidei, weather loach, freshwater fish, DNA barcoding

Introduction

Weather loaches of the genus *Misgurnus* La Cepède, 1803 (Cypriniformes: Cobitidae) are slender freshwater fishes distributed in Europe and East Asia (Kottelat & Freyhof 2007; Kottelat 2012). This genus is characterised by the following features: suborbital spine hidden under muscles; lower lip with four long, barbel-like mental lobes; caudal fin edge rounded; and no regular rows of spots (Kottelat & Freyhof 2007; Nakajima & Uchiyama 2017). At present, it is generally agreed that this genus consists of eight species, of which five are distributed in Northeast Asia: *M. anguillicaudatus* (Cantor, 1842), *M. buphoensis* Kim & Pak, 1995, *M. dabryanus* (Dabry de Thiersant, 1872), *M. mohoity* (Dybowski, 1869), and *M. nikolskyi* Vasil'eva, 2001 (Kottelat 2012; Zhang et al. 2021). Previous genetic and morphological studies have shown that there are other species with undetermined scientific names in Japan (Morishima et al. 2008; Koizumi et al. 2009; Kitagawa et al. 2011; Shimizu et al. 2011; Kano et al. 2012; Fujimoto et al. 2017; Nakajima & Uchiyama 2017; Okada et al. 2017; Oka et al. 2021; Yashima et al. 2021).

Misgurnus sp. IR was first reported from the Iriomote-jima Island, Yaeyama Islands, Okinawa Prefecture, Japan, by Shimizu et al. (2011) as a species with an undetermined scientific name distinct from *M. anguillicaudatus*. Kano et al. (2012) pointed out that the population on Iriomote-jima Island is most likely attributed to anthropogenic transfer. Thereafter, Nakajima & Uchiyama (2017) reported *M.* sp. IR from Amami Islands, which includes Amami-oshima, Kikai-jima, Tokuno-shima and Okinoerabu-jima islands, Kagoshima Prefecture, central part of Ryukyu Islands, Japan, suggesting that they should be treated as an independent species endemic to these islands. However, no taxonomic study has been conducted on this species. Therefore, we describe *M.* sp. IR as a new species based on the 27 type specimens in this study.

Materials and Methods

We examined 27 specimens collected from the Amami-oshima Island, Kikai-jima Island, Tokuno-shima Island,

Accepted by Y.-H. Zhao: 26 Jun. 2022; published: 11 Jul. 2022

Okinoerabu-jima Island, and Iriomote-jima Island, Ryukyu Islands, Japan (Fig. 1). All specimens were fixed in 10% neutral formalin and preserved in 70% ethanol. We used the counting and measurement methods described by Kottelat & Freyhof (2007). The ratio of LPC (= the projected length from the posterior edge of the anal-fin base to the base of the caudal fin) / DPA (= the projected length from the insertion point of the pelvic fin to the anterior edge of the anal-fin base), and L (= the length of the last ray of the dorsal fin) / S (= the length of the second branched soft ray of the dorsal fin) were also examined in this study (Fig. 2). Counts and measurements were obtained on the left side. All measurements were obtained using a digital caliper and recorded to the nearest 0.1 mm. The lamina circularis of the right pectoral fin was examined in males. The right pectoral fin was resected and made transparent by placing it in 4% KOH for 24 h. After staining with Alizarin Red S + 1% KOH for 24 h, the fin was examined and sketched using a stereomicroscope. Vertebral counts were performed using soft X-ray photographs, confirming the composition of the abdominal and caudal vertebrae in the holotype and in some paratypes. The counting method of Sawada (1982) followed. Measurements were obtained based on color and X-ray photographs for nine types of specimens deposited British Museum of Natural History, London (BMNH), Naturalis Biodiversity Center, Leiden (RMNH), and Swedish Museum of Natural History, Stockholm (NRM). Measurements from these images were performed using ClickMeasure (https://onochi-lab.com/page-832/).

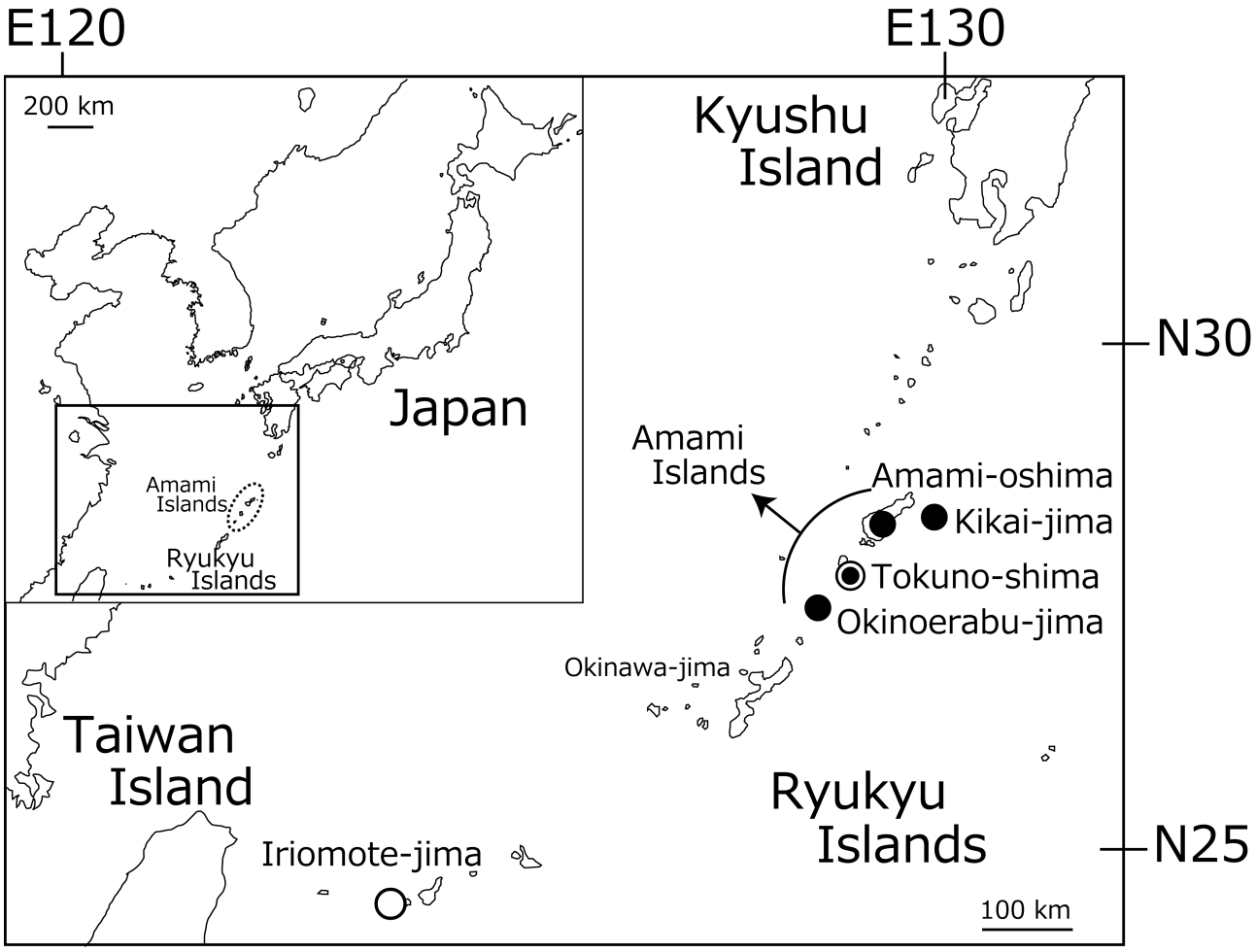

FIGURE 1. Map showing the collection sites of the type series of *Misgurnus amamianus* sp. nov. A double circle indicates the locality of holotype. Double and closed circles indicate native populations, and open circle indicate an introduced population.

Other abbreviations used are JNC, collection of Jun Nakajima, Dazaifu; KMNH, Kitakyushu Museum of Natural History, Kikakyushu; KPM, Kanagawa Prefectural Museum of Natural History, Odawara; NSMT, National Museum of Nature and Science, Tsukuba; OMNH, Osaka Museum of Natural History, Osaka; TKPM, Tokushima Prefectural Museum, Tokushima; WMNH, Wakayama Prefectural Museum of Natural History, Kainan; YCM, Yokosuka City Museum, Yokosuka.

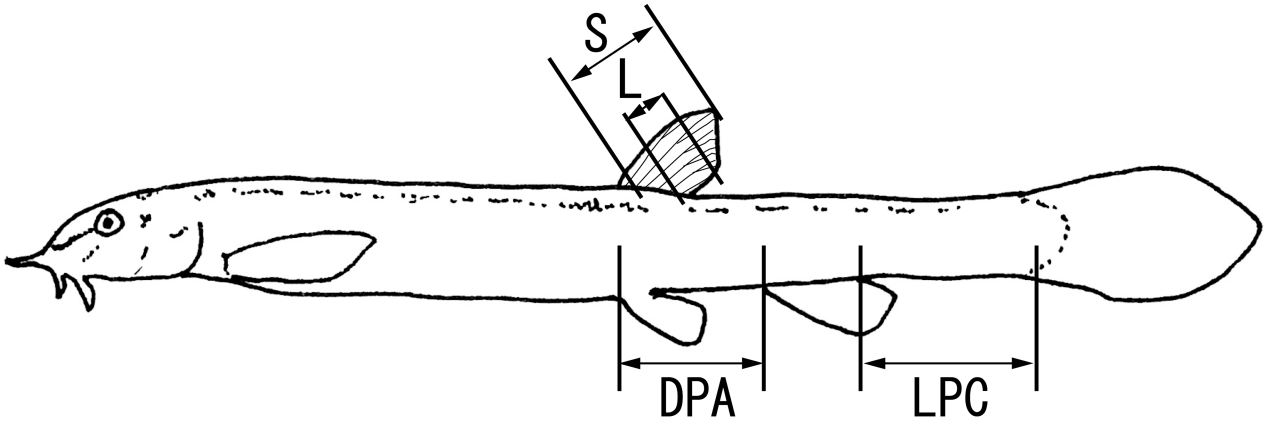

FIGURE 2. Measurement methods of DPA (= the projected length from the insertion point of the pelvic fin to the anterior edge of the anal-fin base), LPC (= the projected length from the posterior edge of the anal-fin base to the base of the caudal fin), S (= the length of the second branched soft ray of dorsal fin), and L (= the length of last ray of dorsal fin).

Comparative materials examined in this study are as follows— *Misgurnus anguillicaudatus* (Cantor, 1842) (China clade): BMNH 1843.7.21.15-18 (syntypes), tow males & two females, 87.0–114.9 mm SL, China: Chusan Island; KMNH VR 100185–100187, three males, 126.3–136.4 mm SL, China: East Tiaoxi River, Zhejiang Prov.; *Misgurnus anguillicaudatus* (Cantor, 1842) (Japan clade): KYUM-PI 4520, male, 93.4 mm SL, Japan: Fukutsu, Fukuoka Pref.; KYUM-PI 4522, male, 101.3 mm SL, Japan: Hitoyoshi, Kumamoto Pref.; KYUM-PI 4523, male, 88.4 mmSL, Japan: Hasami, Nagasaki Pref.; KMNH VR 100171, male, 72.9 mm SL, Japan: Kitatsugaru, Aomori Pref.; *Misgurnus anguillicaudatus formosanus* Rendahl, 1936: NRM 10354 (holotype), female, 105.1 mm SL, Taiwan: Lake Candidius; *Misgurnus buphoensis* Kim & Pak, 1995: Uchida Collection, Kyushu University, Japan, five males & three females, 67.9–87.6 mm SL, North Korea: Beonpo, Hamgyeongbuk-do; *Misgurnus dabryanus* (Dabry de Thiersant, 1872): JNC130, male, 115.5 mm SL, Japan: Otou, Fukuoka Pref.; *Misgurnus dichachrous* (Bleeker, 1860): BMNH1866.5.2.96 (lectotype), male, 113.4 mm SL, Japan: Jedo [Tokyo], Tokyo Met.; *Misgurnus enalios* (Bleeker, 1860): BMNH1866.5.2.26 (lectotype), female, 64.2 mm SL, Japan: Kaminoseki, Yamaguchi Pref.; *Misgurnus mohoity* (Dybowski, 1869): JNC563 & 564, male & female, 71.7–75.7 mm SL, Japan: Ibaraki Pref.; *Misgurnus nikolskyi* Vasil'eva, 2001: JNC300 & 301, two males, 121.3–139.8 mm SL, Japan: Fish market, Tokyo; *Misgurnus polynema* (Bleeker, 1860): BMNH 1866.5.2.24 (lectotype), female, 125.7 mm SL, Japan: Jedo [Tokyo], Tokyo Met.; *Misgurnus rubripinnis* (Temminck & Schlegel, 1846): RMNH.PISC.2705 (lectotype), female, 147.5 mm SL, Japan: Surroundings of Nagasaki, Nagasaki Pref.; *Misgurnus* sp. (Clade A) *(sensu* Nakajima & Uchiyama, 2017): KYUM-PI 4524–4526, two males & female , 86.8–115.8 mm SL, Japan: Memanbetsu, Hokkaido Pref.: JNC345, male, 110.5 mm SL, Japan: Osorezan, Aomori Pref.; *Misgurnus* sp. OK (*sensu* Shimizu et al., 2011): KMNH VR 100189, male, 77.1 mm SL, Japan: Yonaguni-jima Is., Okinawa Pref.; OMNH-P 37928–37934, two males & five females , 57.5–75.5 mm SL, Japan: Okinawa-jima Is., Okinawa Pref.

To infer the phylogenetic position of this new species in relation to the Japanese and some Northeast Asian species of *Misgurnus*, a phylogenetic tree was constructed using partial sequences of the mitochondrial DNA control region (CR). First, genomic DNA of the seven individuals of the new species and four individuals of *M. anguillicaudatus* were extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), following the manufacturer's protocol. Second, the mitochondrial CR sequence (around 1,085 bp) was amplified using primers designed by Morishima et al. (2008) (231F: 5'-TTGTAATCCGAAGATCGGAG-3'; 1366R: 5'-CATGCAAGTCTCCGCAAA-3'). The PCR amplification method involved an initial denaturation at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 52–55 ºC for 30 sec, and extension at 72 ºC for 1 min, using MightyAmp™ DNA Polymerase (Takara Bio). Third, PCR products were purified using Agencourt AMPure XP (Beckman Coulter) and sequenced on an automated DNA sequencer ABI 3130 Genetic Analyzer (Thermo Fisher Scientific) using amplification primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). The nucleotide sequences obtained in this study were submitted to the DNA Data Bank of Japan (Accession Nos. LC672439–LC672449).

TABLE 1. Localities and accession numbers for the material analysed in the present study

Species	Locality	Deposited no.	Accession No.	Reference
Misgurnus amamianus sp. nov.	Japan: Tokuno-shima Is., Amami Isls., Ryukyu	KPM-NI 68404	LC672439	present study
	Japan: Tokuno-shima Is., Amami Isls., Ryukyu	TKPM-P 26178	LC672440	present study
	Japan: Tokuno-shima Is., Amami Isls., Ryukyu	JNC554	LC672441	present study
	Japan: Tokuno-shima Is., Amami Isls., Ryukyu	KMNH VR 100463	LC672442	present study
	Japan: Okinoerabu-jima Is., Amami Isls., Ryukyu	KMNH VR 100188	LC415023	Hashiguchi & Nakajima (unpubl.)
	Japan: Okinoerabu-jima Is., Amami Isls., Ryukyu	JNC140	LC672443	present study
	Japan: Okinoerabu-jima Is., Amami Isls., Ryukyu	JNC141	LC672444	present study
	Japan: Iriomote-jima Is., Yaeyama Isls., Ryukyu	WMNH-PIS 0311	AB645738	Kano et al. (2012)
	Japan: Iriomote-jima Is., Yaeyama Isls., Ryukyu	JNC128	LC672445	present study
Misgurnus anguillicaudatus (Japan)	Japan: Fukutsu, Fukuoka, Kyushu	KYUM-PI 4520	LC415010	Hashiguchi & Nakajima (unpubl.)
	Japan: Hitoyoshi, Kumamoto, Kyushu	KYUM-PI 4522	LC672446	present study
	Japan: Hasami, Nagasaki, Kyushu	KYUM-PI 4523	LC672447	present study
	Japan: Kitatsugaru, Aomori, Honshu	KMNH VR 100171	LC672448	present study
	Japan: Tsushima Is., Nagasaki, Kyushu	KMNH VR 100178	LC672449	present study
	Japan: Nakano-shima Is., Tokara Isls, Ryukyu	KMNH VR 100307	LC600318	Nakajima et al. (2021)
Misgurnus anguillicaudatus (China)	China: East Tiaoxi R., Zheijiang	KMNH VR 100185	AB645739	Kano et al. (2012)
Misgurnus dabryanus	China: Huangshan, Anhui		DQ105316	Tang et al. (2006)
Misgurnus fossilis	Croatia: unknown		GU583685	Jakovlic et al. (unpupl.)
Misgurnus mohoity	China: Haerbin, Heilongjiang		DQ105309	Tang et al. (2006)
Misgurnus nikolskyi	China: Amur R., Neha, Nenjiang		AB242171	Saitoh et al. (2006)
Misgurnus sp. (Clade A)	Japan: Mutsu, Aomori, Honshu		LC494591	Hata et al. (2020)
Misgurnus sp. OK	Japan: Yonaguni-jima Is., Yaeyama Isls., Ryukyu	KMNH VR 100189	LC415024	Hashiguchi & Nakajima (unpubl.)
Leptobotia elongata	China: Luzhou, Sichuan, China		AY600875	Tang et al. (2006)
Barbatula barbatula	Europe: Lake Constance		AY833831	Barluenga and Meyer (2005)

Definitions of *M.* sp. (Clade A) and *M.* sp. OK are according to Nakajima & Uchiyama (2017).

Species, localities, and accession numbers of the nucleotide sequences used for the phylogenetic analysis are shown in Table 1. The CR sequences of known *Misgurnus* species as well as the outgroup species were retrieved from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/). Nucleotide sequences were aligned using MAFFT v.7.392 (Katoh & Standley 2013). A phylogenetic tree was constructed using the maximum likelihood (ML) method with the T92 + G model in the MEGAX software package (Kumar et al. 2018). The best-fit substitution model was selected based on the Bayesian information criterion (BIC) using MEGAX. The reliability of each tree node was assessed by the bootstrap method with 1,000 replicates.

Results

Misgurnus amamianus sp. nov.

(Figs. 3, 4, 5, 6, 7a)

Misgurnus anguillicaudatus (Cantor, 1842): Ogawa 1937: 18, Fig. 15 (Japan: Tokuno-shima Is., Amami Isls., Kagoshima Pref.).

Misgurnus sp. IR (*sensu* Shmizu et al. 2011): Shimizu et al. 2011: 144, Fig. 1 (OMNH-P20968, Japan: Iriomote-jima Is., Yaeyama Isls., Okinawa Pref.); Nakajima & Uchiyama 2017: 72, 74 (Japan: Amami Isls., Kagoshima Pref.), 75 (Japan: Iriomote-jima Is., Yaeyama Isls., Okinawa Pref.); Nakajima 2018: 111 (no data); Hosoya 2019: 180 (Japan: Kagoshima Pref.).

Holotype. KPM-NI 68404, male, 83.8 mm SL, Japan: Isen, Tokuno-shima Is., Amami Isls., Kagoshima Pref., 12. XII. 2015., T. Kitano leg.

FIGURE 3. *Misgurnus amamianus* sp. nov. a, holotype (male, KPM-NI 68404); b, paratype (female, TKPM-P 26178).

Paratypes. 16 males and 10 females, 55.1–126.3 mm SL, all from the Ryukyu Islands, Japan. TKPM-P 26178, 26179, male & female, same data as the holotype; JNC353, 354, male & female, same locality as the holotype, 29. VII. 29. 2016., J. Nakajima leg.; KMNH VR 100463, male, Noda, Isen, Tokuno-shima Is., Amami Isls., Kagoshima Pref., 20. VIII. 2015., Y. Kano leg.; JNC554, female, same locality, 10. XI. 2019., J. Nakajima leg.; NSMT-P 34733, male, Otsukan, China, Okinoerabu-jima Is., Amami Isls., Kagoshima Pref., 3. VIII. 1958., S. Ueno & Y. Morimoto leg.; OMNH-P 1145, 1146, male & female, Kamihirakawa, China, Okinoerabu-jima Is., Amami Isls., Kagoshima Pref., 29. VIII. 1964., Y. Shibata leg.; KPM-NI 68405, 68406, KMNH VR 100188, TKPM-P 26180, 26181, JNC140, 141, four males & three females, Kamihirakawa, China, Okinoerabu-jima Is., Amami Isls., Kagoshima Pref., 17. XI. 2013., J. Nakajima leg.; YCM-P 31002, male, Suitengu-no-ike, Kikai-jima Is., Amami Isls., Kagoshima Pref., data unknown (1930s)., H. Ikeda leg.; NSMT-P 14268, male & female, Amuro, Uken, Amami-oshima Is., Amami

Isls., Kagoshima Pref., 7. VIII. 1970., collector unknown; NSMT-P 28933, two males & two females, Nishi-nakama, Amami-oshima Is., Amami Isls., Kagoshima Pref., 4. IV. 1975., H. Sakai & M. Sato leg.; JNC128, 1 male, Urauchi, Iriomote-jima Is., Yaeyama Isls., Okinawa Pref., 26. X. 2007., H. Mizutani leg.; OMNH-P 37946, male, same locality, 24. X. 2008., T. Suzuki, M. Hosokawa & M. Baba leg.; WMNH-PI 0311, male, Kumotta, Urauchi, Iriomote-jima Is., Yaeyama Isls., Okinawa Pref., 8. IX. 2011., J. Nakajima leg.

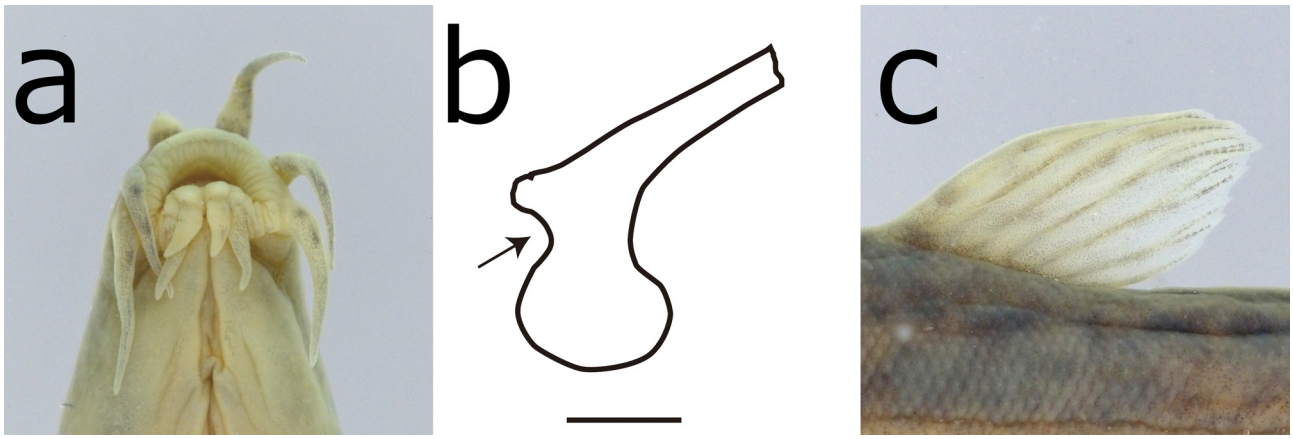

FIGURE 4. *Misgurnus amamianus* sp. nov., KPM-NI 68404, holotype. a, mouth; b, lamina circularis (allow indicating the neckline at the base of lamina circularis), bar 1 mm; c, dorsal fin.

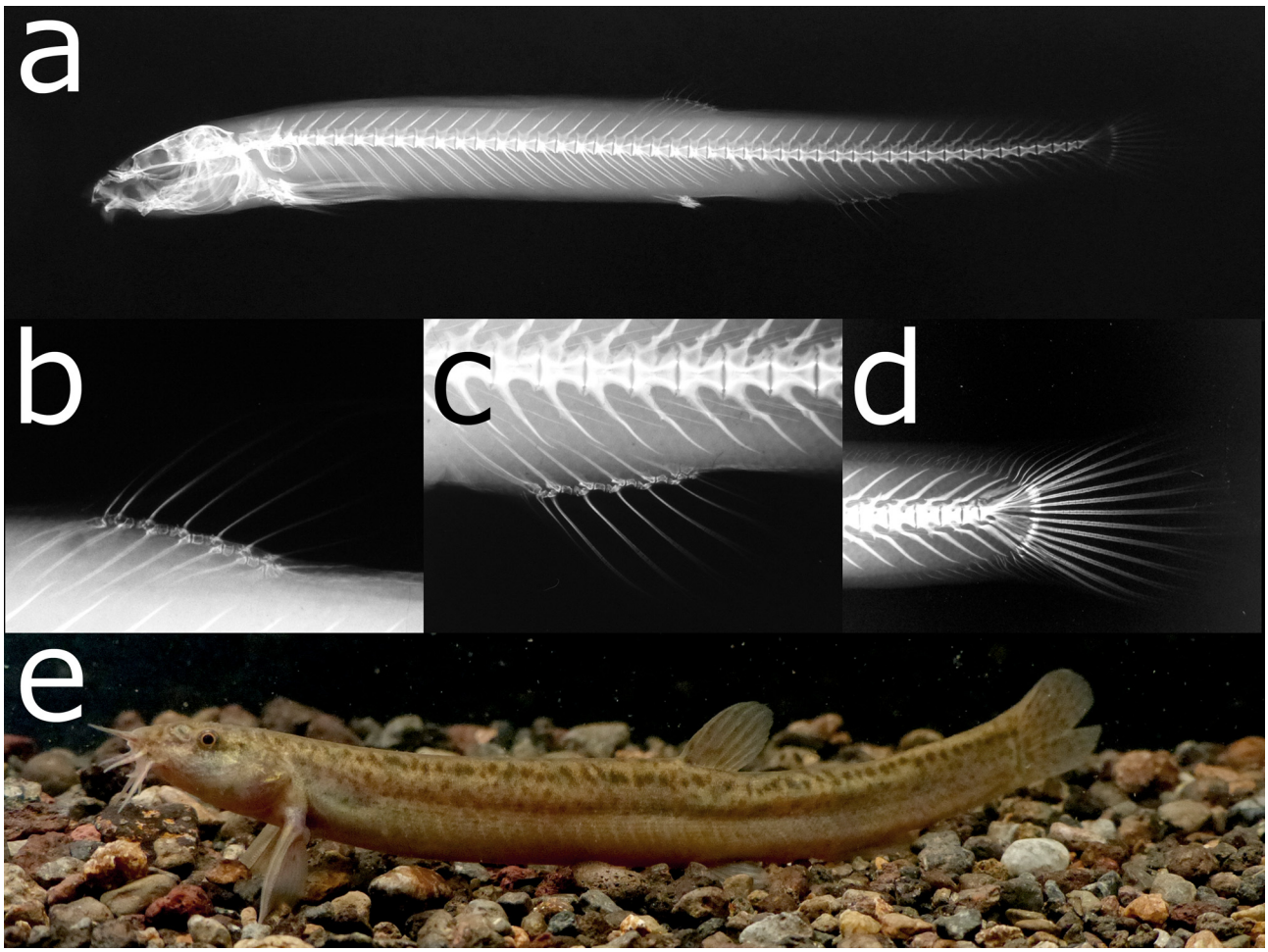

FIGURE 5. *Misgurnus amamianus* sp. nov., KPM-NI 68404, holotype. a, radiograph; b, radiograph of dorsal fin; c, radiograph of anal fin; d, radiograph of caudal fin; e, living body.

Non-type specimens. OMNH-P 37941-37945, 37947, two males & four females, Japan: Urauchi, Iriomote-jima Is., Yaeyama Isls., Okinawa Pref. 24. X. 2008., T. Suzuki, M. Hosokawa & M. Baba leg.

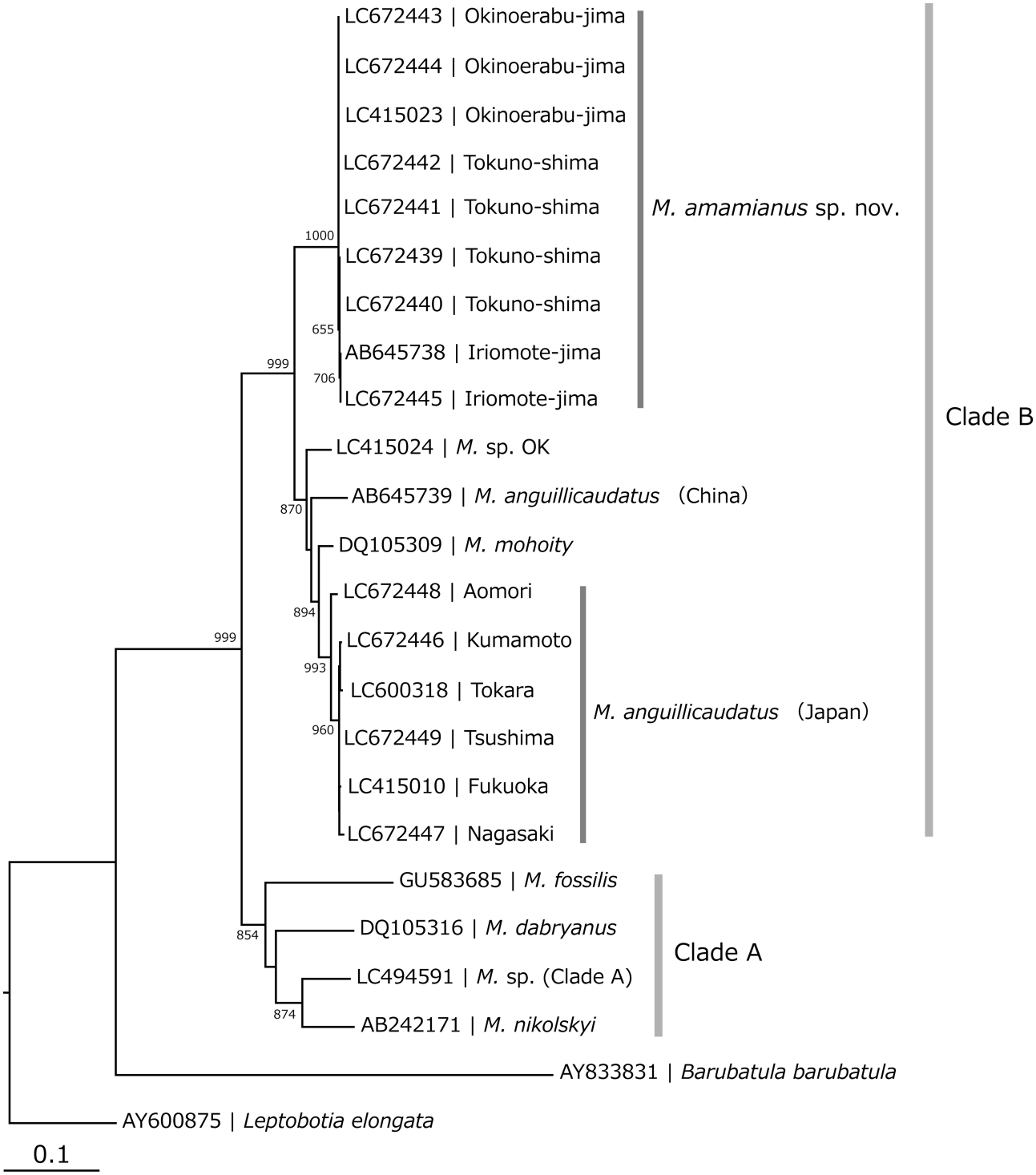

FIGURE 6. Maximum likelihood tree of the partial sequences of mitochondrial DNA control region (CR) in *Misgurnus amamianus* sp. nov. and Japanese and Northeast Asian species of *Misgurnus*. CR sequences of *Barbatula barbatula* and *Leptobotia elongata* are used as outgroups. Numbers on each branch indicate bootstrap values with 1,000 replications.

Diagnosis. The new species is distinguished from other species of *Misgurnus* by the combination of the following characteristics (Table 3): lamina circularis at base of pectoral fin in adult male poleaxe-shape (vs. spatula-shape in *M. buphoensis* and *M. nikolskyi*; elongated horizontally in *M. dabryanus*; absent in *M. fossilis*); neckline at base of lamina circularis shallow (vs. deep in *M. anguillicaudatus*); dorsal fin ray iii+6 (vs. commonly iii+7–8 in *M. anguillicaudatus*); last ray of dorsal fin commonly not branched (vs. commonly branched in *M. anguillicaudatus*, *M. buohoensis*, *M. dabryanus*, *M. fossilis*, *M. mohoity* and *M. nikolskyi*); L/S less than 0.5 (vs. over 0.5 in *M. anguil-*

licaudatus, *M. buphoensis*, *M. dabryanus*, *M. mohoity* and *M. nikolskyi*); LPC/DPA over 1.1 (vs. less than 1.0 in *M. anguillicaudatus* (China clade), *M. dabryanus* and *M. tonkinensis*); barbel length general (vs. long in *M. dabryanus* and *M. tonkinensis*); caudal-peduncle depth shallow (vs. deep in *M. dabryanus, M. multimaculatus* and *M. tonkinensis)*; and total vertebrae number 44–48 (vs. over 49 in *M. buphoensis*, *M. fossilis* and *M. nikolskyi*).

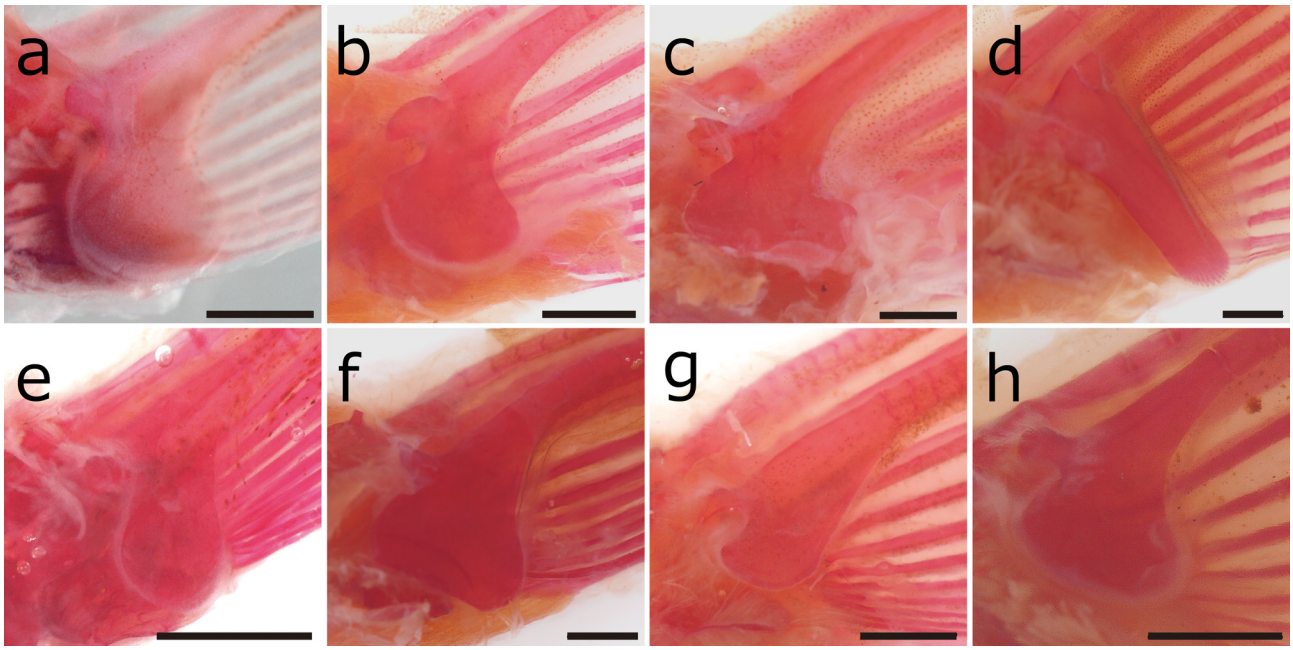

FIGURE 7. Lamina circularis of Northeast Asian species of *Misgurnus*. a, *M. amamianus* sp. nov. (KPM-NI 68404, holotype, Tokuno-shima Is., Japan, 83.8 mm SL); b, *M. anguillicaudatus* (Japan clade) (KYUM-PI 4520, Fukuoka, Japan, 93.4 mm SL); c, *M. anguillicaudatus* (China clade) (KMNH VR 100186, Zheijiang, China, 128.9 mm SL); d, *M. dabryanus* (JNC130, Fukuoka, Japan, 115.2 mm SL); e, *M. mohoity* (JNC563, Ibaraki, Japan, 71.7 mm SL); f, *M. nikolskyi* (JNC301, Tokyo, Japan, 121.3 mm SL); g, *M.* sp. (Clade A) (JNC345, Aomori, Japan, 110.5 mm SL); e, *M.* sp. OK (KMNH VR100189, Okinawa, Japan, 77.1 mm SL). Bar 1mm.

Description. The morphometric and meristic data for the type specimens (17 males and 10 females) are summarised in Table 2. Data for the holotype are presented first, followed by those for the paratypes in parentheses, if different.

Body elongated, slightly compressed. Head elongated, snout rather short. Interorbital space convex. Eye moderately small, positioned upper and middle on head. Caudal peduncle compressed; ridges on the caudal peduncle at upper and lower edge weakly developed. Mouth small, inferior arched with fleshy lips; lower lip with 4 long, barbel-like mental lobes; upper lip with transverse wrinkles on surface (Fig. 4a). Barbels 3 pairs, first on rostrum, second on maxilla, and third on mandible; each barbels well developed, approximately 30% of head length. No lateral line. Ridges on caudal peduncle at upper and lower edge weakly developed. Small cycloid scales on trunk; head without scale. Lamina circularis at base of pectoral fin in adult males, poleaxe shape, backend round; neckline at base of lamina circularis shallow (Figs. 4b, 7a). First branched soft ray of pectoral fin longer than others, broad and slightly extended; pectoral fin in males longer than that in females. Dorsal-fin ray thin and weak; last ray unbranched, its length less than half of second branched soft ray (Figs. 4c, 5b). Margin of dorsal fin rounded, slightly pointed at center tip. Dorsal-fin base behind middle of body. Pelvic-fin origin below third or fourth branched dorsal-fin ray. Anal fin not reaching caudal-fin base. Margin of caudal fin rounded, slightly pointed at center tip. Abdominal vertebrae 30 (27–30); caudal vertebrae 18 (15–19); total vertebrae 48 (44–48) (n = 20) (Fig 5a).

Colouration of fresh specimens. Head and back blackish brown with irregular and indistinct spots; slightly purplish on cheeks and body; abdomen yellowish brown (Figs. 3, 5e). Unclear streak running from tip of snout to occiput, crossing to eye. Dorsal and caudal fins with irregular and indistinct spots. Upper spot at caudal-fin base ambiguous, size smaller than eye diameter. Ridges on caudal peduncle at upper and lower edge tinged with yellowish.

Sexual dimorphism. A lamina circularis of pectoral fin only in males; pectoral fin in males longer than that in females. Generally, body size of females larger than that of males.

TABLE 2. Counts and morphometric measurements of *Misgurnus amamianus* sp. nov.

	Holotype	Paratypes	
		males (n=16)	females (n=10)
Standard length (mm); mean (range)	83.8	75.2 (63.3–89.4)	92.1 (55.1–126.3)
Counts			
Dorsal fin rays	iii+6	iii+6	iii+6
Anal fin rays	ii+5	ii+4–5	ii+4–6
Pectoral fin rays	i+9	i+8-9	i+8–9
Pelvic fin rays	i+5	i+4–5	i+3–5
Caudal fin rays*	5+5	6–8+7	7–8+6–7
In % standard length; mean (range)			
Head length	16.8	17.0 (15.4–18.7)	17.7 (16.1–18.7)
Body depth	10.9	12.4 (10.1–15.6)	12.1 (10.5–15.3)
Depth of caudal peduncle	8.8	10.0 (8.4–11.4)	9.9 (8.0–11.0)
Predorsal length	56.3	56.2 (54.2–58.9)	57.1 (56.0–59.2)
Prepelvic length	57.5	58.1 (56.0–60.2)	59.2 (57.3–60.6)
Preanal length	69.1	68.9 (66.8–71.8)	69.9 (68.4–72.0)
In % head length; mean (range)			
Snout length	38.3	38.0 (31.2–42.5)	38.8 (36.9–42.3)
Orbit diameter	12.8	14.8 (12.3–16.8)	12.8 (10.1–14.7)
Interorbital width	23.4	22.9 (16.0–26.8)	23.2 (16.9–28.5)
Rostral barbel	31.9	30.1 (21.7–36.2)	25.1 (13.7–31.1)
Maxillary barbel	31.2	32.4 (28.5–37.7)	27.4 (16.5–32.2)
Mandibular barbel	33.3	36.1 (30.7–45.1)	32.1 (19.4–40.8)
LPC/DPA	1.5	1.3 (1.1–1.6)	1.2 (1.1–1.4)
L/S	0.4	0.4 (0.4–0.5)	0.4 (0.3–0.5)

DPA, distance between the pelvic and anal fin; LPC, length of caudal peduncle. L, the length of last soft ray of dorsal fin; S, the length of the second soft ray of dorsal fin. See also Figure 2. *, Counted only branched rays.

Mitochondrial DNA sequence. Control region: LC672439 (Holotype); 12SrRNA: LC278291.

Distribution. Japan: Amami Islands. (Kikai-jima Island, Amami-oshima Isand, Tokuno-shima Island and Okinoerabu-jima Islamd), Kagoshima Prefecture, introduced to Iriomote-jima Island, Okinawa Prefecture, Japan (Fig. 1).

Habitat and biology. *Misgurnus amamianus* inhabits the muddy bottoms of small streams, paddy fields, and ponds with rich vegetation. The life cycle is unknown, but it breeds from May to August in captivity. The loach has a strong tendency to hide in vegetation.

Etymology. The specific name was derived from the Amami Islands, which is the primary distribution area of this species. Japanese name is suggested as Shinobi-dojyô.

Remarks. There are many synonyms for *Misgurnus anguillicaudatus*, but no species or subspecies have been described from the Ryukyu Islands, including the Amami Islands (Kottelat 2012). This species is morphologically and genetically distinguished from the known species and populations distributed in Northeast Asia (Figs. 6, 7; Table 3). In this study, we also compared the type specimens of *Misgurnus anguillicaudatus* as well as the five nominal species described from Japan and Taiwan Island. We confirmed that the combination of the ray number in the dorsal fin, the features of the last ray of the dorsal fin, the LPC/DPA ratio, the L/S ratio, and total vertebrae number morphologically can aid in the distinguishing of this species from *M. amamianus* (Fig. 8; Table 4).

There are two different mitochondrial DNA lineages in the genus *Misgurnus*: clades A and B (or Type I and Type II) (Morishima et al. 2008; Koizumi et al. 2009; Kitagawa et al. 2011; Fujimoto et al. 2017; Okada et al. 2017). These two lineages were confirmed by phylogenetic analysis with high bootstrap values (Fig. 6). Phylogenetic analysis revealed that all *M. amamianus* individuals belonged to clade B and clearly diverged from other species

TABLE 3. Comparison of morphological features between *Misgurnus*

Scientific name	Shape of lamina circularis	Ray number in dorsal fin	Last ray of dorsal fin	L/S ratio	LPC/DPA ratio	Barbel length	Caudal peduncle depth	Body pattern	Total vertebrae number	Abdominal vertebrae number	Caudal vertebrae number	Reference
M. amamianus sp. nov.	poleaxe, neckline at the base of lamina circularis shallow	iii+6	not branched	less 0.5	over 1.1	general	shallow	ambiguous	44–48	27–30	15–19	present study
M. anguillicaudatus (Japan clade)	poleaxe, neckline at the base of lamina circularis deep	iii+6–7	branched	over 0.5	over 1.1	general	shallow	ambiguous	46–47	28–30	17–18	Matsui & Nakajima (2020); present study
M. anguillicaudatus (China clade)	poleaxe, neckline at the base of lamina circularis deep	iii-iv+7–8	branched	over 0.5	less 1.0	long	moderately deep	ambiguous	45–46	28–29	17	Matsui & Nakajima (2020); Zhang et al. (2021); present study
M. buphoensis	spatula	iii+6–7	branched	over 0.5	over 1.1	general	shallow	ambiguous	50–53	33	17–20	Uchida (1939); Kim & Pak (1995); present study
M. dabryanus	elongated horizontally	iii-iv+6–8	branched	over 0.5	less 1.0	long	deep	ambiguous	46–53	24–27	17–19	Vasil'eva (2001); Takahashi & Shimizu (2016); Hosoya (2019); Zhang et al. (2021)

.....*Continued on the next page*

TABLE 3. (continued)

Scientific name	Shape of lamina circularis	Ray number in dorsal fin	Last ray of dorsal fin	L/S ratio	LPC/DPA ratio	Barbel length	Caudal peduncle depth	Body pattern	Total vertebrae number	Abdominal vertebrae number	Caudal vertebrae number	Reference
M. mohoity[*1]	poleaxe, neckline at the base of lamina circularis shallow	iii-iv+6	branched	over 0.5	over 1.1	general	shallow	ambiguous	51	31	20	Vasil'eva (2001); Zhang et al. (2021); present study
M multimaculatus .	-	iii+6	-	-	over 1.1	general	deep	spotted	-	-	-	Rendahl (1944)
M. nikolskyi[*2]	spatula or elongated poleaxe	iii-iv+6	branched	over 0.5	over 1.1	general	shallow	ambiguous	49–53	33–34	16–18	Vasil'eva (2001); Zhang et al. (2021); present study
M. tonkinensis	-	-	-	-	less 1.0	long	deep	spotted	-	-	-	Rendahl (1937)
M. sp. (Clade A)[*3]	spatula	iii+6–7	branched	over 0.5	over 1.1	general	shallow	ambiguous	48–49	29–30	17–19	Nakajima & Uchiyama (2017); present study
M. sp. OK[*3]	poleaxe, neckline at the base of lamina circularis deep	iii+7–8	branched	over 0.5	less 1.1	long	moderately deep	spotted	43–45	27–29	16–17	Nakajima & Uchiyama (2017); present study

*1, Treated as *M. bipartitus* in Zhang et al. (2021); *2, treated as *M. mohoity* in Zhang et al. (2021); *3, definitions are according to Nakajima & Uchiyama (2017).

TABLE 4. Comparison of morphological features between type speciemns of *Misgurnus* described from Japan and Taiwan, and syntypes of *Misgurnus anguillicaudatus*.

Scientific name	Type	Type locality	Deposited no.	Ray number in dorsal fin	Last ray of dorsal fin	LPC/DPA ratio	L / S ratio	Total vertebrae number
M. amamianus sp. nov.	Holotype	Tokuno-shima Island, Amami Islands, Kagoshima Prefecuture, Japan	KPM-NI 68404	iii+6	not branched	1.5	0.4	48
M. anguillicaudatus (Cantor, 1842)	Syntypes	Chusan Island [Zhoushan Island, Zhejiang Province, China]	BMNH 1843.7.21.15-18a	iii+7	branched	0.6	0.5	46
			BMNH 1843.7.21.15-18b	iii+7	branched	0.9	0.5	48
			BMNH 1843.7.21.15-18c	iii+7	branched	0.6	0.7	46
			BMNH 1843.7.21.15-18d	iii+7	branched	0.6	0.7	45
M. rubripinnis (Temminck & Schlegel, 1846)	Lectotype	Surroundings of Nagasaki [Nagasaki Prefecture, Kyushu, Japan]	RMNH.PISC.2705	iii+6	branched	1.1	0.6	47
M. enalios (Bleeker, 1860)	Lectotype	Kaminoseki [Yamaguchi Prefecture., Honshu, Japan]	BMNH 1866.5.2.26	iii+6	branched	0.4	0.5	37
M. dichachrous (Bleeker, 1860)	Lectotype	Jedo [Tokyo Metropolis, Honshu, Japan]	BMNH 1866.5.2.96	iii+6	branched	1.1	0.6	48
M. polynema (Bleeker, 1860)	Lectotype	Jedo [Tokyo Metropolis, Honshu, Japan]	BMNH 1866.5.2.24	iii+6	branched	1.2	-	48
M. anguillicaudatus formosanus Rendahl, 1936	Holotype	Lake Candidius [Nantou County, Taiwan]	NRM 10354	iii+7	branched	0.9	0.5	47

within this clade (Fig. 6). The monophyly of *M. amamianus* is supported by a 100% bootstrap value (Fig. 6). Pairwise differences (i.e., p-distances) of the CR nucleotide sequences between *M. amamianus* and other *Misgurnus* species in clade B ranged from 6.3% to 7.8% (mean: 7.2%). These results support the validity of a distinct species, *M. amamianus*.

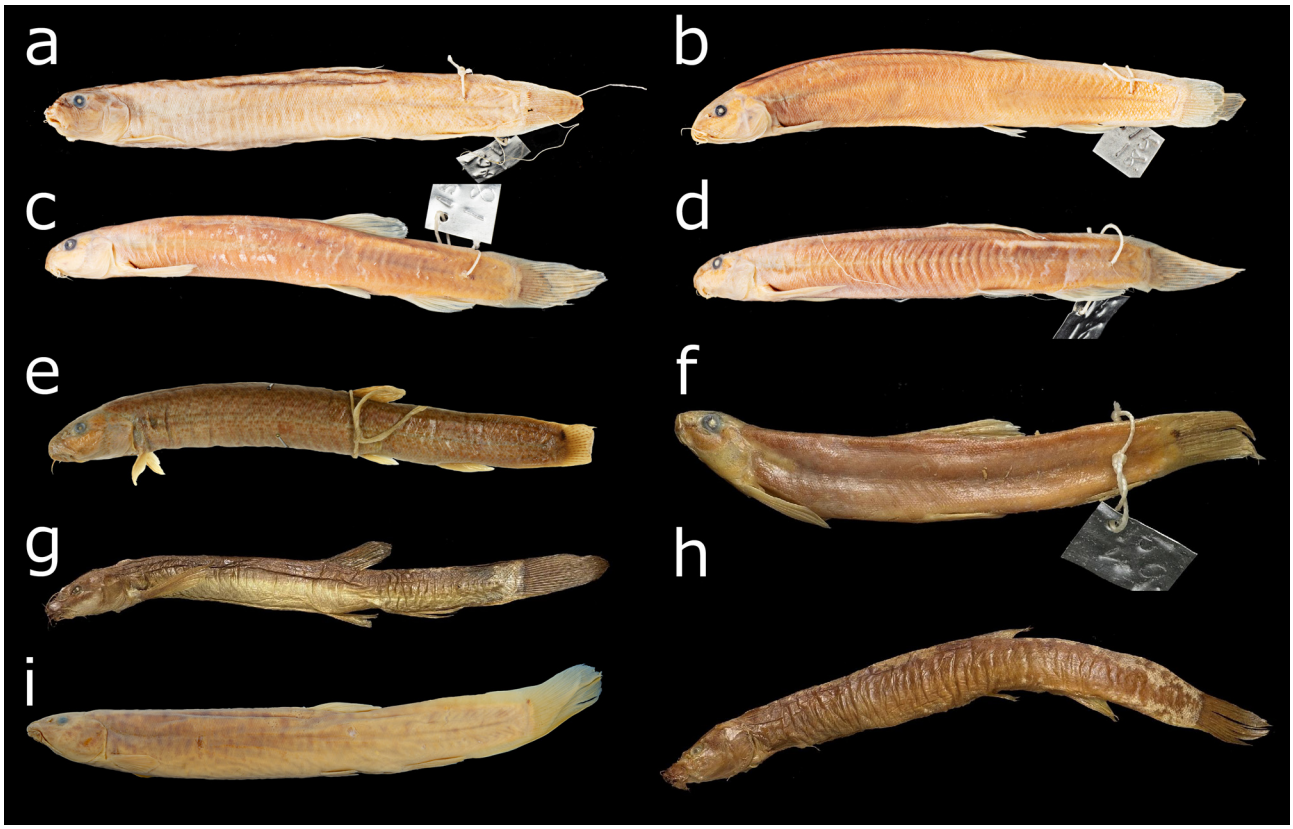

FIGURE 8. Type specimens of *Misgurnus anguillicaudatus* and five *Misgurnus* loaches described from Japan and Taiwan. a, BMNH 1843.7.21.15-18a (syntype of *Cobitis anguillicaudata* Cantor, 1842, Chusan Island [Zhoushan Island], China, 103.9 mm SL); b, BMNH 1843.7.21.15-18b (syntype of *Cobitis anguillicaudata* Cantor, 1842, 114.9 mm SL); c, BMNH 1843.7.21.15-18c (syntype of *Cobitis anguillicaudata* Cantor, 1842, 87.0 mm SL); d, BMNH 1843.7.21.15-18d (syntype of *Cobitis anguillicaudata* Cantor, 1842, 91.5 mm SL); e, RMNH.PISC.2705 (lectotype of *Cobitis rubripinnis* Temminck & Schlegel, 1846, Nagasaki, Japan, 147.5 mm SL); f, BMNH1866.5.2.26 (lectotype of *Cobitichthys enalios* Bleeker, 1860, Kaminoseki, Japan, 64.6 mm SL); g, BMNH1866.5.2.96 (lectotype of *Cobitichthys dichachrous* Bleeker, 1860, Jedo [Tokyo], Japan, 102.8 mm SL); h, BMNH1866.5.2.24 (lectotype of *Cobitichthys polynema* Bleeker, 1860, Jedo [Tokyo], Japan, 123.6 mm SL); i, NRM 10354 (holotype of *Misgurnus anguillicaudatus formosanus* Rendahl, 1936, Lake Candidius, Taiwan, 105.1 mm SL). Photos by James Maclaine & Lucie Goodayle (BMNH) (a, b, c, d, f, g, h), Esther Dondorp (RMNH) (e), and Andrea Hennyey (NRM) (i).

Many amphibian and reptile species are known to have evolved in this way in the central part of the Ryukyu Islands, including the Amami Islands (e.g., Lin et al. 2002; Matsui et al. 2005; Tominaga et al. 2013), and the same has been reported for freshwater fishes such as the Asian swamp eel, *Monopterus* (Matsumoto et al. 2010). Our phylogenetic analysis suggests that *M. amamianus* is also an endemic species that was isolated when the Amani Islands were separated from the Asian continent. Further detailed molecular phylogenetic analyses will help to clarify the evolutionary origins of this species.

Misgurnus amamianus is listed as Data Deficient (DD) in the Japanese Red List under the name *Misgurnus* sp. IR (Ministry of the Environment 2020). Our survey showed that the last collection on Kikai-jima Island was in the 1930s, and the last collection on Amami-oshima Island was in the 1970s; the species has not been rediscovered since then. In addition, a pond that was the only habitat in Okinoerabu-jima Island (the collection site of the specimen used as the paratypes in this study) underwent renovation in the spring of 2021; the species may already be extinct. Therefore, there are only three definite habitats for this species: two ponds on Tokuno-shima Island and one paddy field on Iriomote-jima Island. In the Amami Islands, paddy fields which are the main habitat of this species, have been largely lost because of the rapid conversion from the once-common paddy rice cultivation to sugarcane

cultivation (Hagihara 1992). We believe that *M. amamianus* is on the verge of extinction, and urgent conservation measures are needed.

Acknowledgements

We thank T. Kiatno, Y. Kano, M. Minobe, N. Inahata, H. Mizutani and the Office of Tokuno-shima, Ministry of Environment for their support in sample collection and Y. Suzawa and S. Hirakawa for their invaluable information. We are also grateful to Y. Hibino (KMNH), H. Senou (KPM), T. Ito (TKPM), G. Shinohara (NSMT), S. Matsui (OMNH), Y. Kaji (WMNH) and K. Hagiwara (YCM) for the donation of specimens, A. Tawa and H. Senou for obtaining the X-ray photographs, and J. Maclaine (BMNH), L. Goodayle (BMNH), E. Dondorp (RMNH), M. Oijen (RMNH), A. Hennyey (NRM) and B. Delling (NRM) for obtaining the photographs of the old type specimens. This study was partly supported by JSPS KAKENHI Grant Number 19K04682 and Research and Development of River Erosion Control Technology, Ministry of Land, Infrastructure, Transport and Tourism, Japan. We would like to thank Editage (www.editage.com) for English language editing.

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