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Differences in sexual morphological dimorphisms between two loach species of the genus *Misgurnus* (Cypriniformes: Cobitidae) in the River Shono system, Fukui Prefecture, Japan

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Abstract. A cryptic loach species of the genus *Misgurnus* (Cypriniformes: Cobitidae) has been reported as *Misgurnus* sp. Type I *sensu* Okada et al. (2017) (hereafter called Type I loach) from the Japanese Archipelago, in addition to the common nominal species, *M. anguillicaudatus* (hereafter called Type II loach). Although both species co-exist in the Naka-ikemi Wetland, Fukui Prefecture, their mechanism of reproductive isolation is unknown. Differences in sexual dimorphism might reflect differences in reproductive behaviour that contribute to reproductive isolation between the two species. We investigated the morphological characteristics of the two loach species at two sympatric sites and observed several morphological differences. Type I differ from Type II loaches in the following morphological traits and sexual dimorphisms: in males, generally less developed lamina circularis, shorter anal fins, and lack of a pair of raised ridges in the anterior portion of the body side; in females, lack of spawning scars on the body side. These morphological differences between the two sympatric loaches may reflect differences in their spawning behaviour. Furthermore, it may represent one of the factors responsible for reproductive isolation between the two species.

Key words: cryptic species, reproductive isolation, reproductive behaviour

Introduction

The weather loach, genus *Misgurnus* Lacepède, 1803 (Cypriniformes: Cobitidae), inhabits muddy bottoms of creeks, ponds, wetlands, and paddy fields across Europe and East Asia, including the Japanese Archipelago. The Japanese *Misgurnus* was historically considered a single species, *M. anguillicaudatus* (Cantor, 1842), called the dojo loach (Saitoh 1989). A rare mitochondrial DNA (mtDNA) type (hereafter

called Type I after Kitagawa et al. (2011)) has been reported in some populations in certain parts of north-eastern Japan, along with a common mtDNA type (hereafter called Type II after Kitagawa et al. (2011)) that has been detected throughout Japan (Morishima et al. 2008, Koizumi et al. 2009, Shimizu & Takagi 2010, Kitagawa et al. 2011, Fujimoto et al. 2017, Okada et al. 2017). The highly divergent mtDNA polymorphisms have been attributed to past mtDNA introgression from another genus, *Cobitis* (Type I original *vs.* Type

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II introgressed) (Šlechtová et al. 2008, Kitagawa et al. 2011, Nakajima & Uchiyama 2017). Both mtDNA types are often sympatrically detected in populations containing triploid or clonal lineages originating from hybrids of the two mtDNA type populations (Morishima et al. 2008, Yamada et al. 2015, Fujimoto et al. 2017). The coexistence of the two bisexual populations with their respective mtDNA types and the reproductive isolation between them was confirmed in the Naka-ikemi Wetland, Tsuruga City, Fukui Prefecture, Japan (Okada et al. 2017) (Type I as a cryptic species *Misgurnus* sp. Type I *sensu* Okada et al. (2017) (hereafter called Type I loach) and Type II as a common species *M. anguillicaudatus* (hereafter called Type II loach)). No distinct habitat segregation has been observed between the two loach species in recent distribution surveys using direct fish capture and environmental DNA methods in wetlands (Okada & Kitagawa 2018, Okada et al. 2020b), and their detailed reproductive isolation mechanisms are unknown. Okada et al. (2017) reported differences in vertebral numbers between the two species, but no differences in external morphological characteristics were found. The Japanese *Misgurnus* loach has been reported to exhibit several sexually dimorphic morphological traits associated with its reproductive behaviour (Okamoto 1921, Ikeda 1936, Tsukahara

1948, Kobayashi 1951, Kubota & Matsui 1955, Kubota 1961). Differences in sexual dimorphism (especially reproductive traits in morphological characteristics) might reflect differences in reproductive behaviour, contributing to the reproductive isolation between the two species.

In this study, we compared the morphological characteristics of the two loach species from two sympatric sites to investigate the sexual dimorphism and morphological differences between them.

Material and Methods

Sample collection

A total of 36 loach specimens were collected from the Naka-ikemi Wetland (35°39'34.8" N, 136°05'18.6" E, altitude 50 m) in the River Shono system (Fig. 1, Table 1). An additional 18 specimens from the Ikenokochi Wetland (35°39'11.5" N, 136°08'00.6" E, altitude 300 m) located on the upper part of the same river system were collected to eliminate effects of phenotypic plasticity caused by environmental differences. Our preliminary genetic analysis using mtDNA cytochrome *b* (*cytb*) sequencing revealed that both populations belonged to the same local genetic group (R. Okada et al., unpublished data). Only specimens

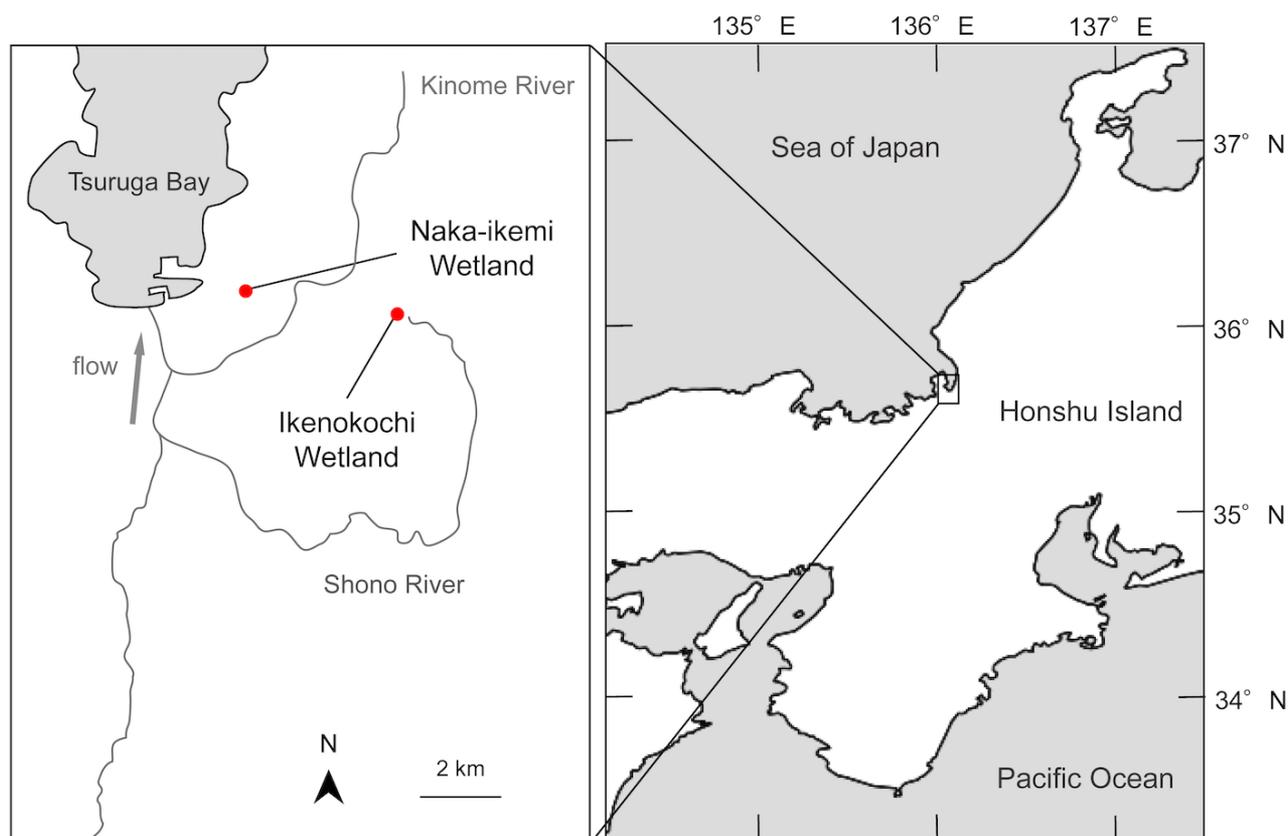


Fig. 1. Location of the Naka-ikemi and Ikenokochi wetlands.

Table 1. continued

No. of specimens*	Type I Loach		Type II Loach	
	Male 13 (12)	Female 16 (11)	Male 11 (7)	Female 14 (6)
Pelvic-fin length	6.5-8.9	3.9-6.3	8.8-11.8	6.1-8.6
Caudal-fin length	15.7-20.0	14.0-17.5	15.4-18.0	14.2-18.4
Snout length	5.1-7.1	5.2-7.1	5.9-7.3**	6.0-7.3
PRR	10.7-13.6	-	6.5-9.2	-
As % HL				
Orbit diameter	10.7-13.7	7.3-14.1**	10.5-16.5**	8.6-14.7**
Interorbital width	14.8-22.7	11.6-20.3	14.6-23.0	15.8-21.1
Rostral barbel length	29.7-44.2	24.1-37.0	24.4-41.8	19.9-28.0
Maxillary barbel length	25.0-37.1	26.4-38.7	24.3-38.9	23.4-31.5
Mandibular barbel length	26.4-36.6	24.0-34.3	31.7-49.8	24.4-37.9

* Numbers in parentheses indicate individuals from Naka-ikemi Wetland. **The characteristics show significant differences between the two wetlands.

with a standard length (SL) of 70 mm and over were used to account for secondary sexual development. Fish were euthanised using benzoic acid, following which the right pelvic fin was cut and preserved in 99.5% ethanol for genetic species identification (Type I or Type II loach). The rest of the body was fixed in 10% formalin and preserved in 70% ethanol.

Genetic species identification

Total DNA was extracted from ethanol-preserved fin tissues following the procedure described by Asahida et al. (1996). Species identification for all specimens was conducted via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses of the *cytb* region in the mtDNA and the ribonuclease subunit H2 gene (*RNaseh2*) region in the nuclear gene according to the method previously reported by Okada et al. (2017). Additionally, RFLP analysis of nuclear recombination activating gene 1 (*RAG1*) was conducted for all specimens to identify particular species. PCR amplification was performed using the primer pair RAG-1F (5'-AGC TGT AGT CAG TAY CAC AAR ATG-3') and RAG-9R (5'-GTG TAG AGC CAG TGR TGY TT-3') (Quenouille et al. 2004). PCR conditions were 2 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 54 °C, 1 min at 72 °C and 5 min at 72 °C. The PCR products were then digested with the restriction enzyme *Pst*I (TaKaRa, Kyoto, Japan) selected based on sequence information reported by Šlechtová et al. (2008). Digestion was performed following the manufacturer's instructions. The expected RFLP fragments for the *Pst*I digestion in each species were ca. 511 and 474 bp for the Type I loach and 474, 316, and 195 bp for the Type II loach. The restriction fragments were subsequently separated by horizontal electrophoresis on 3% agarose gel.

Morphological analysis

Meristic methods followed Kottelat & Freyhof (2007), and morphometric methods generally followed Kottelat (1984), except for the following characteristics: dorsal-fin base and anal-fin base length were measured according to Kottelat & Freyhof (2007); the anal-fin length was measured as the distance between the base and the tip of the unbranched ray, even if any branched ray was longer; the pectoral-fin length was measured both as the length of the longest unbranched ray and as the 1st branched ray; the caudal peduncle depth was measured both at the narrowest part of the caudal peduncle and the caudal-fin base. Vertebrae, including the four components associated with the Weberian apparatus, were counted from soft x-ray photos. The sex of each specimen was determined by

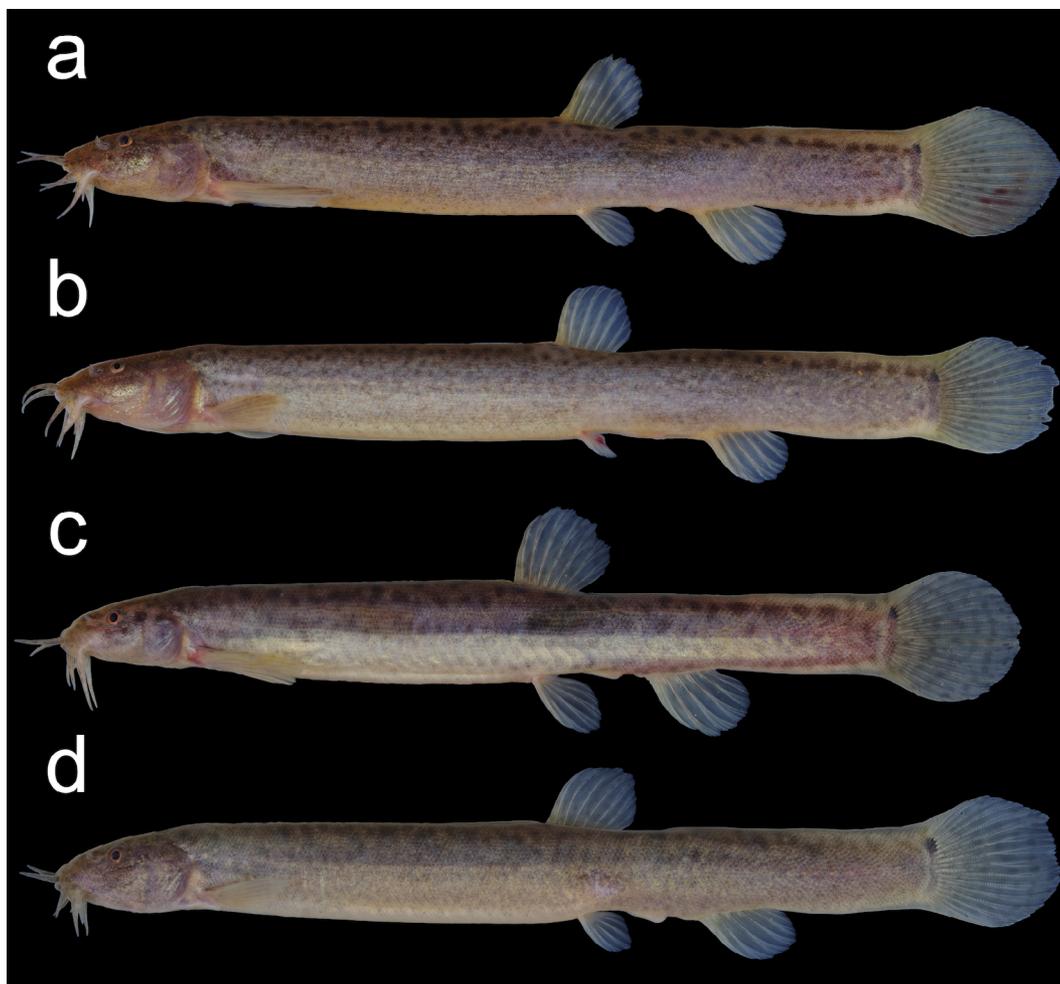


Fig. 2. Fresh specimens of Type I and Type II loaches from Naka-ikemi Wetland. (a) Type I loach, KUN-P 59954, 92.7 mm standard length (SL), male; (b) Type I loach, KUN-P 59917, 104.6 mm SL, female; (c) Type II loach, KUN-P 59924, 96.3 mm SL, male; (d) Type II loach, KUN-P 59928, 86.8 mm SL, female.

the presence of the lamina circularis at the base of the 1st unbranched ray of the pectoral fin that only male has. To observe the features of the lamina circularis, the right pectoral fin was placed in 4% KOH for 24 h to make it transparent and then stained with Alizarin Red S + 1% KOH for 24 h, according to the method described by Nakajima (2012). The description of the lamina circularis follows Nakajima & Uchiyama (2017) and Hata et al. (2020). In male specimens, the distance between the dorsal fin origin and the top of the raised ridge located posterior to the dorsal fin was measured as the position of the raised ridge (PRR). All measurements were made using a digital calliper and recorded to 0.1 mm. Differences in meristic and morphometric characteristics between the two species or the sexes were tested using Welch's t-test. Differences in characteristics between the same species and sex specimens from two different sites were also tested using Welch's t-test, except for Type I males, where only one individual was included from the Ikenokochi Wetland. Statistical analyses were conducted using R v. 3.5.2 (R Core Team 2018).

Materials examined

The information on each specimen listed below is shown in Table S1.

Male Type I loach (n = 13). KUN-P59948-59950, 59952-59954, 64124, 64128-64130, 64826, 64827, 78.0-109.2 mm in standard length (SL), Naka-ikemi Wetland; KUN-P60028, 104.4 mm SL, Ikenokochi Wetland.

Female Type I loach (n = 16). KUN-P59917, 59947, 59951, 59955, 64126, 64127, 64131-64133, 64828, 64829, 78.7-130.3 mm SL, Naka-ikemi Wetland; KUN-P60023-60027, 118.7-126.8 mm SL, Ikenokochi Wetland.

Male Type II loach (n = 11). KUN-P59918, 59924-59927, 59929, 59930, 73.9-99.9 mm SL, Naka-ikemi Wetland; KUN-P60018-60021, 80.2-90.6 mm SL, Ikenokochi Wetland.

Female Type II loach (n = 14). KUN-P59919-59923, 59928, 86.8-113.0 mm SL, Naka-ikemi Wetland;



Table 2. Results of analysis of Welch's t-test for meristic and morphometric characters of mature two loach species.

	Sexual dimorphisms of two loach species				Morphological difference between two loach species					
	Type I		Type II		Male		Female			
	t-value	df	P	t-value	df	t-value	df	t-value	P	
Dorsal-fin rays	-	-	-	-1.000	10.000	0.341	10.000	0.341	-	-
Pectoral-fin rays	0.501	22.178	0.621	1.240	21.466	0.229	21.957	0.000**	5.146	26.840
Anal-fin rays	-	-	-	1.000	10.000	0.341	10.000	0.149	-	-
Pelvic-fin rays	0.601	26.930	0.553	0.125	22.178	0.902	-	-	-	-
Total vertebrae	-1.799	16.722	0.090	-1.771	23.698	0.089	4.333	0.000**	5.895	24.181
Head length	0.244	22.207	0.810	0.630	18.816	0.536	-0.424	0.477	-2.094	27.999
Head depth	0.398	24.203	0.694	0.481	14.696	0.638	-1.367	0.188	-2.498	27.909
Body width (at dorsal origin)	-1.210	26.928	0.237	2.235	23.000	0.035*	2.669	0.016*	-1.035	23.617
Body width (at anal origin)	-4.088	24.013	0.000**	-1.030	22.916	0.314	2.147	0.043*	-0.248	21.933
Body depth	-3.272	26.857	0.002**	-1.026	22.873	0.316	-2.209	0.039*	-3.845	27.459
Caudal-peduncle length	-0.829	25.315	0.415	-2.429	18.013	0.026*	-1.241	0.229	0.388	27.993
Caudal-peduncle depth (at the narrowest part)	-1.844	26.728	0.076	-1.290	21.734	0.211	-1.201	0.245	-1.326	25.185
Caudal-peduncle depth (at caudal-fin base)	-2.502	26.249	0.019*	-2.286	20.488	0.033*	-4.123	0.001**	-4.176	25.159
Pre-dorsal-fin length	0.758	25.228	0.456	2.490	21.496	0.021*	0.787	0.440	-0.797	27.998
Pre-pelvic-fin length	0.769	26.586	0.449	1.719	22.341	0.100	-0.219	0.829	-1.629	23.916
Pre-anal-fin length	1.943	25.157	0.063	2.279	21.343	0.033*	1.032	0.314	0.721	27.538
Pre-anus length	2.078	25.904	0.048*	-0.041	17.521	0.968	0.140	0.890	0.159	23.721
Dorsal-fin length	-4.526	26.333	0.000**	-6.338	20.487	0.000**	-5.369	0.000**	-3.449	22.371
Dorsal-fin base length	-3.242	22.161	0.004**	-0.972	19.338	0.343	-4.497	0.000**	-9.681	27.672
Pectoral-fin length (at unbranched ray)	-10.468	21.001	0.000**	-14.386	22.688	0.000**	-2.919	0.009**	-2.544	26.872
Pectoral-fin length (at 1 st branched ray)	-14.608	14.302	0.000**	-12.190	13.381	0.000**	-1.486	0.152	-5.998	23.809



Table 2. continued.

	Sexual dimorphisms of two loach species				Morphological difference between two loach species							
	Type I		Type II		Male		Female					
	t-value	df	P	t-value	df	P	t-value	df	P			
Anal-fin length	-7.134	25.915	0.000**	-4.661	21.476	0.000**	-3.165	17.208	0.006**	-2.444	21.825	0.023*
Anal-fin base length	-3.692	21.352	0.001**	-2.902	22.012	0.008**	-1.135	18.779	0.271	-4.095	27.493	0.000**
Pelvic-fin length	-11.140	21.364	0.000**	-8.552	20.538	0.000**	-6.731	19.875	0.000**	-8.436	22.558	0.000**
Caudal-fin length	-4.470	11.999	0.000**	-3.267	17.276	0.004**	1.110	11.477	0.290	-0.612	19.582	0.548
Snout length	0.294	23.502	0.772	0.402	20.610	0.692	-1.139	21.829	0.267	-1.525	27.955	0.139
PRR	-	-	-	-	-	-	11.237	21.328	0.000**	-	-	-
Orbit diameter	-1.567	23.085	0.131	-1.752	21.940	0.094	-1.140	14.935	0.272	-0.401	27.770	0.691
Interorbital width	-2.036	24.813	0.053	-0.972	19.155	0.343	0.299	21.876	0.768	-1.724	27.812	0.096
Rostral barbel length	-2.796	25.935	0.010**	-5.675	12.369	0.000**	0.537	16.485	0.598	6.884	24.951	0.000**
Maxillary barbel length	0.401	26.149	0.692	-3.985	15.809	0.001**	-0.152	20.276	0.881	4.151	26.368	0.000**
Mandibular barbel length	-0.657	23.034	0.518	-3.691	17.790	0.001**	-3.369	17.153	0.004**	-0.100	23.986	0.921

*P < 0.05, **P < 0.01.

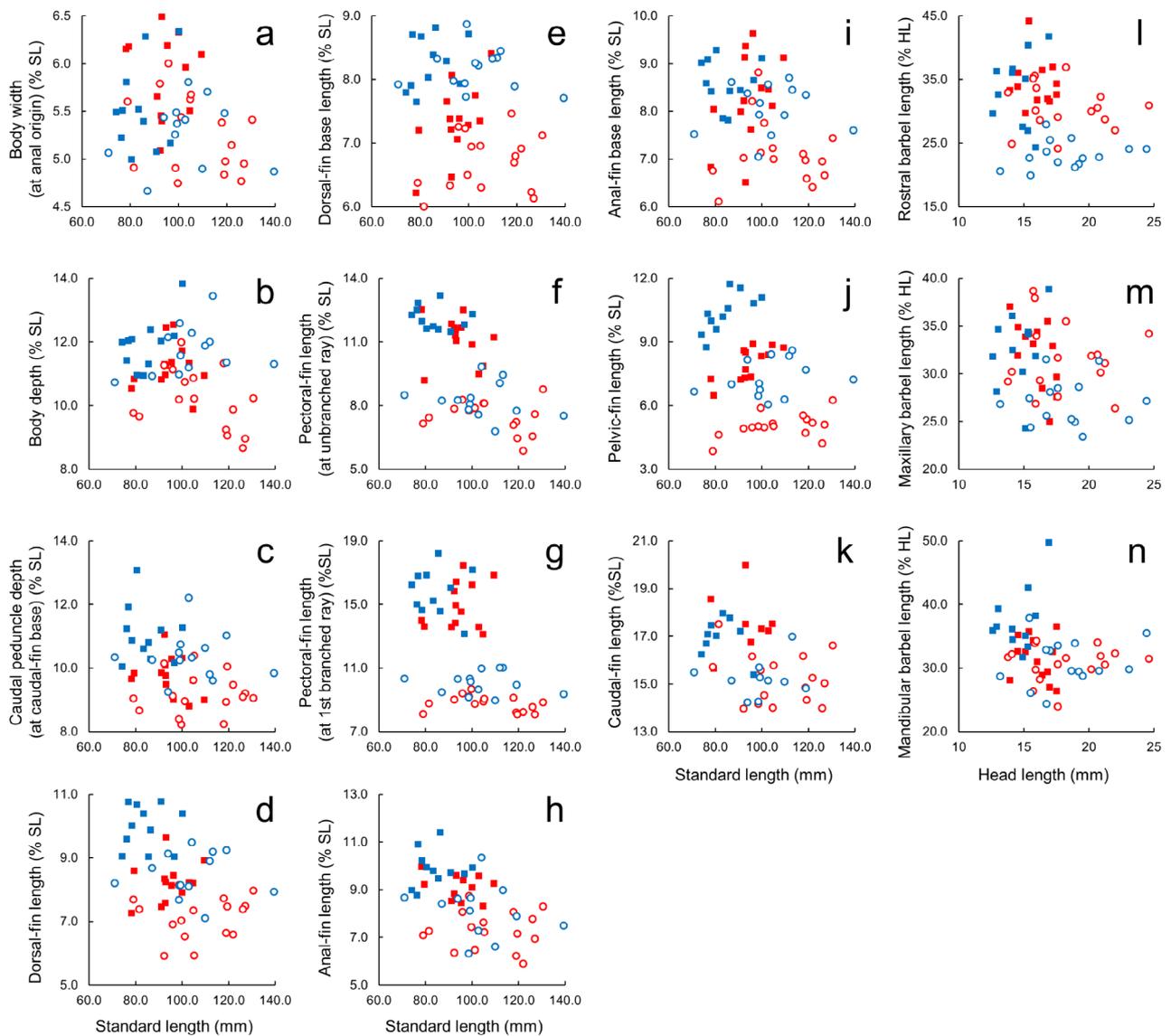


Fig. 3. Relationships between (a) body width (at anal origin), (b) body depth, (c) caudal-peduncle depth (at caudal-fin base), (d) dorsal-fin length, (e) dorsal-fin base length, (f) pectoral-fin length (at unbranched ray), (g) pectoral-fin length (at 1st branched ray), (h) anal-fin length, (i) anal-fin base length, (j) pelvic-fin length, (k) caudal-fin length (as % of standard length), (l) rostral barbel length, (m) maxillary barbel length and (n) mandibular barbel length (as % of head length) to standard length and head length in Type I loach (male: red squares, female: red circles) and Type II loach (male: blue squares, female: blue circles).

KUN-P60010-60012, 60014-60017, 70.7-139.1 mm SL, Ikenokochi Wetland.

Results

Genetic species identification

All RFLP banding patterns of the mtDNA (*cytb*) and two nuclear gene regions (*RNaseh2* and *RAG1*) concord with the expected results. All examined specimens were successively identified as Type I loaches (13 males and 16 females) and Type II loaches (11 males and 14 females) (Table 1). Only one Type I male was found among the specimens collected from the Ikenokochi Wetland. Hybrids of the two species were not detected.

Sexual dimorphisms of two loach species

Meristic and morphometric data of Type I and Type II loaches and the results of the statistical analyses are shown in Table 1. Of the 32 characters, significant ($P < 0.05$) differences were observed for 13 and 15 characters between the males and females in Type I and Type II loaches, respectively (Welch's t-test, Table 2). In both species, the dorsal and pectoral fin length (at the unbranched and 1st branched rays), anal-fin length, anal-fin base length, pelvic- and caudal-fin length, and the rostral barbel length were longer in males than in females and commonly showed significant ($P < 0.01$) differences (Fig. 2). The caudal peduncle depth of the males (at the caudal-fin base) was significantly ($P < 0.05$) greater than that of females.

Among these characteristics, significant ($P < 0.05$) differences in the dorsal- and pectoral-fin length (at the unbranched and 1st branched rays) and anal-fin base length were observed between specimens of the same species from the two sites (Welch's t-test, Table 1, Table S1). In Type I loaches, the body width (at the anal origin), body depth, and dorsal-fin base length differed significantly ($P < 0.01$) between the sexes. In the Type II loaches, the maxillary and mandibular barbel length differed significantly ($P < 0.01$) between the sexes. Males of both species have raised ridges on both sides of the body, posterior to the dorsal fin. Among these characteristics, body width (at the anal origin) and depth showed significant ($P < 0.05$) differences between Type I loach specimens from two sites (Welch's t-test; Table 1, Table S1).

Morphological differences between two loach species

A variety of body colours and spots were observed among individuals of both species, and both Type I and Type II loaches commonly have a black spot on the caudal peduncle base (Fig. 3). Significant ($P < 0.05$)

Table 3. Frequency of total vertebrae counts of two loach species.

	Total vertebrae							
	44	45	46	47	48	49	50	51
Male Type I loach				1	3	5	4	
Female Type I loach				1	4	7	4	
Male Type II loach			1	6	3	1		
Female Type II loach	1	3	6	4				

differences for 13 characters in male loaches and 15 characters in female loaches were observed between the two loach species (Welch's t-test, Table 2). Type I loaches have more pectoral-fin branched rays and total vertebrae than Type II loaches in both sexes (Tables 2-3). The caudal peduncle depth (at the caudal fin base), dorsal-fin length, dorsal-fin base length, pectoral-fin length (at the unbranched ray), and pelvic-fin length were significantly ($P < 0.01$) higher in Type II loaches (Fig. 2); body depth and anal-fin length were also significantly ($P < 0.05$) higher (Table 2). Among these characteristics, the dorsal-



Fig. 4. Dorsal view of preserved specimens of male (a, b) Type I and (c, d) Type II loaches from Naka-ikemi Wetland. Arrows indicate raised ridges. (a) KUN-P 59954, 92.7 mm standard length (SL); (b) KUN-P 59952, 99.7 mm SL; (c) KUN-P 59918, 99.9mm SL; (d) KUN-P 59924, 96.3 mm SL.

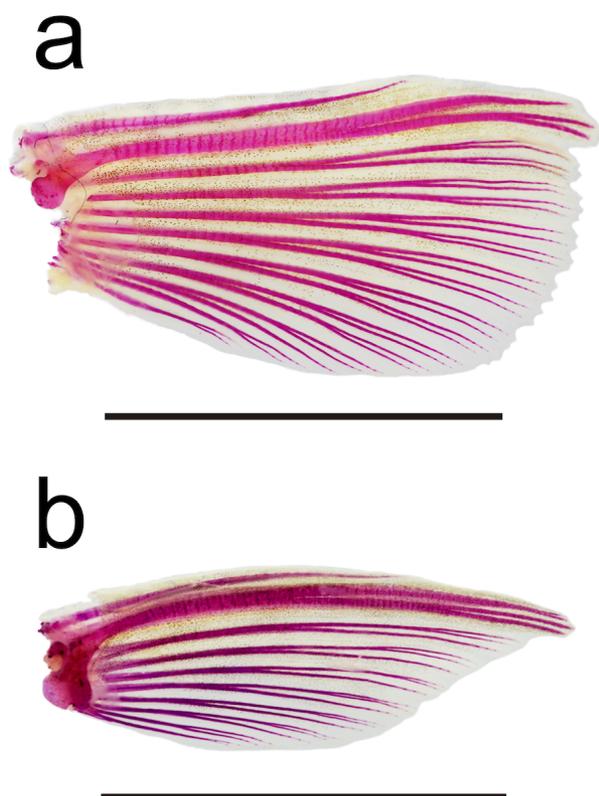


Fig. 5. Dorsal views of pectoral-fin of mature males stained with Alizarin Red S. (a) Type I loach, KUN-P 60028, 104.4 mm standard length (SL), Ikenokochi Wetland; (b) Type II loach, KUN-P 60020, 83.1 mm SL, Ikenokochi Wetland.

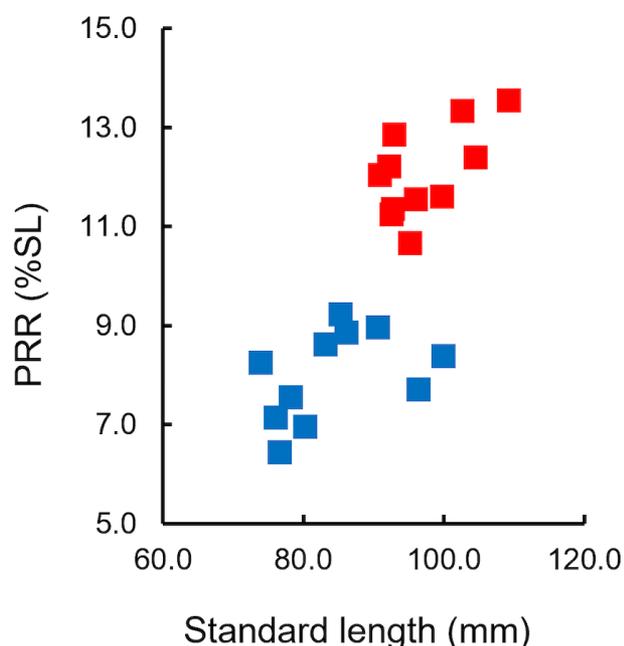


Fig. 6. Relationships between the top of the raised ridge below the dorsal fin when the body side is the base and the dorsal fin origin (as % of standard length) to standard length in male Type I loach (red squares) and male Type II loach (blue squares).

fin length, body depth, and pectoral fin length (at the unbranched ray) differed significantly ($P < 0.05$) between specimens of the same species from the two sites (Welch's t-test, Tables 1, Table S1). In contrast, the head length and depth, body width (at both the dorsal and anal origin), pectoral-fin length (at the 1st branched ray), and rostral, maxillary, and mandibular barbel length differed significantly ($P < 0.05$) in only one sex. Among these characteristics, body width (at the dorsal and anal origin) in males and pectoral-fin length (at the 1st branched ray) in females differed significantly ($P < 0.05$) between specimens of the same species from the two sites (Welch's t-test, Table 1, Table S1). The lamina circularis varies considerably in males, with intermediate shapes and sizes. It tends to be rice-scoop shaped in Type I loaches and pole-axe shaped in Type II loaches and is smaller in Type I than in Type II loaches (Fig. 4). All male Type I loaches had one pair of raised ridges on both body sides posterior to the dorsal fin (Fig. 5a). All mature Type II loaches had an additional keel-like ridge that extended along the body axis on both sides anterior to the dorsal fin (Fig. 5b). PRR was larger in Type I than in Type II loaches (Table 2, Fig. 6). Most adult Type II loach females (91.7%) had spawning scars above the pelvic fin (Fig. 3d), irrespective of the population. These scars were absent in Type I loach females (Fig. 3b).

Discussion

Sexual dimorphism and morphological differences between the two loach species

Sexual dimorphism in the Japanese loach has been reported in previous studies before the two biological species (Type I and Type II loaches) were recognised (Okamoto 1921, Ikeda 1936, Tsukahara 1948, Kobayashi 1951, Kubota & Matsui 1955, Kubota 1961). This observation suggests that the sexual dimorphisms of the two species were recognised equivocally. Our comparison of the two genetically distinct species in a single river system revealed morphological differences and sexual dimorphisms between the two loach species.

Kubota & Matsui (1955) reported the sexually dimorphic morphological traits of the loach *M. anguillicaudatus* from Shimonoseki, Yamaguchi Prefecture, Japan. This population was identified as Type II loaches based on the distribution area (Morishima et al. 2008, Koizumi et al. 2009, Fujimoto et al. 2017). The males have longer pectoral and dorsal fins, dorsal fin bases, pelvic, anal and caudal fins, barbels (rostral, maxillary, and mandibular barbel), and greater caudal peduncle depth than females.



Females have a wider body width than males, and the dorsal fin of males is located more anteriorly than females. In this study, Type II loaches showed sexual dimorphisms similar to those previously reported. Furthermore, the following characteristics were observed: Type II male loaches have a wider body width at the dorsal fin origin, longer caudal peduncle, and anterior anal fin; however, no sexual dimorphism in the dorsal-fin base length was observed in this study, consistent with the report by Kubota & Matsui (1955). These differences may be attributed to genetic, geographical, or environmental differences among the local populations of Type II loaches.

The elongation of the male pectoral fin is one of the most common sexual dimorphisms in Cobitidae (e.g. Vladykov 1935, Šlechtová et al. 2008), and noticeable sexual dimorphism was observed in this species, even when environmental factors were considered. Kubota & Matsui (1955) also reported that males of this species have elongated pectoral fins. The male pectoral-fin length (at the unbranched and 1st branched rays) differed significantly among Type II loach specimens from the two sites despite genetic homogeneity and geographic proximity (Table 1) and without range overlap between males and females (% SL 11.5-13.2 in males and 6.8-9.8 in females), and is considered a sexually dimorphic trait in this species.

In Type I loaches, males showed longer pectoral, dorsal, pelvic, anal, and caudal fins and rostral barbel than females, similar to the Type II loaches in this study. Additionally, males had a longer dorsal fin base, greater body depth, and wider body width at the anal fin origin than females, and the male anus was located more posteriorly than females. Although only one individual was investigated from the Ikenokochi population, the measurement of these characteristics was within the range of the Naka-ikemi population. Among the sexually dimorphic characters in Type I loaches, body width (at the anal origin), body depth, pectoral-fin length (at the unbranched and 1st branched rays), and anal-fin base length differed significantly in the female specimens from the two sites (Table 1). Of these, differences in body width and depth should not be regarded as sexual dimorphisms, as they generally vary depending on environmental factors (e.g. food availability, water temperature, and collection season). Pectoral fin length (at the unbranched and 1st branched rays) showed significant differences without range overlap between males and females (% SL 9.2-12.5 in males and 5.9-8.8 in females), which is also thought to be a sexual dimorphism of this species.

Based on an examination of a population from Kyushu Island (Fukuoka Prefecture), Tsukahara (1948) reported that males had a well-developed lamina circularis at the base of the 1st branched ray of the pectoral fin and two pairs of raised ridges on the body side and similar depressions. The specimen was identified as a Type II loach. In this study, although all Type II loach males had two pairs of raised ridges on the body side, all Type I loaches lacked anterior keel-like ridges and only had one raised ridge on the posterior side of the dorsal fin. The PRR was also distinctly different between the two loach species. In this study, the lamina circularis was more developed in Type II than in Type I loaches. Tsukahara (1948) also reported that most adult females have a small depression on the body of the upper part of the pelvic fin (referred to as spawning scars). Spawning scars were observed in the wild captured Type II loach females regardless of the season but not in the female Type I loach (Fig. S1). As the spawning scars remained in captivity for more than one year (Y. Yashima, unpublished data), the presence of spawning scars may be regarded as a lifelong mark in female Type II loaches that had spawned previously.

Tsukahara (1948) reported that Type II loach males squeeze eggs from the female body by winding their body around that of the female during spawning. The developed lamina circularis on the pectoral fin and two ridges on the body side fix and squeeze the female's body during spawning. The lamina circularis of the male creates spawning scars on the female's body during this process. The smaller size and degenerated shape of the lamina circularis, lack of anterior keel-like ridges, longer PRR in males, and absence of spawning scars in females suggest that the spawning behaviour of the Type I loach may differ from that of the Type II loach, and may be among the factors responsible for the reproductive isolation between the two species in Naka-ikemi Wetland. Direct observation of Type I loach spawning behaviour is important to examine whether the sexual dimorphisms observed in the two loach species act as a reproductive barrier between them.

Morphological identification and taxonomic problems of the two loach species

Although a difference in the total number of vertebrae was reported between the two loach species in the Naka-ikemi Wetland ($P < 0.01$, Welch's *t*-test; Okada et al. 2017), no useful external characteristics were known for distinguishing them. In our observation, distinguishing the two species based on colouration was difficult because of the considerable variation in



colour in both species. Other external morphological characteristics, regardless of site, were observed between the two species. In the morphological analysis, Type I loaches could be distinguished from Type II loaches by pectoral fin branched rays (8-10 (mode 9) vs. 7-9 (mode 8)), caudal-peduncle depth, and dorsal-fin, dorsal-fin base, pectoral-fin (at the unbranched rays), and pelvic-fin lengths, which were shorter than those of Type II loaches of the same sex. Some characteristics showed some overlap between Type I and Type II loaches. Therefore, identification of the sex of each individual is necessary for morphological species identification. Male Type II loaches have a keel-like ridge on both body sides anterior to the dorsal fin in addition to the ridge on the posterior side, and most female Type II loaches have spawning scars, which are absent in Type I loaches. Type II loaches have been reported to have hybridised and genetically introgressed with an unknown member of the genus *Cobitis* in the past (7-10 million years ago; Kitagawa et al. 2011, Okada et al. 2020a). The contribution of genomic introgression from hybrid species to these morphological differences must be addressed. Further comparisons of reproductive dimorphism with a wider range of species, including the genus *Cobitis*, are required.

In this study, common differences between the two species were observed between the specimens from the two sites at different altitudes, indicating that the traits are stable in this river system (Table 1). However, the morphological differences observed in this study may only be common to some populations of the two loach species across Japan. Nakajima & Uchiyama (2017) described the morphological characteristics of populations from Lake Tofutsu in Hokkaido with Type I mtDNA as *Misgurnus* sp. (Clade A) *sensu* Nakajima & Uchiyama (2017) and reported that the shape of the lamina circularis could be a diagnostic characteristic of *Misgurnus* sp. (Clade A) and Type II loaches. However, morphological differences in the lamina circularis between the two species were not as apparent as previously reported, and considerable variation was observed between the two species. Recently, Shedko & Vasil'eva (2022) described a new species of the genus *Misgurnus* loach with Type I

mtDNA as *M. chipisaniensis* from Sakhalin Island without examining specimens from Japan, including *Misgurnus* sp. Type I *sensu* Okada et al. (2017) and *Misgurnus* sp. (Clade A) *sensu* Nakajima & Uchiyama (2017). Recent genetic studies have revealed that highly genetically diverged local populations are included within Type I loaches (R. Okada et al., unpublished data). Taxonomic examination of Type I loaches from a wider area, including *M. chipisaniensis*, *Misgurnus* sp. (Clade A) *sensu* Nakajima & Uchiyama (2017), is therefore necessary.

Type II loaches are also distributed in East Asia. It has been reported that Japanese Type II loach populations are monophyletic and morphologically distinguishable from the continental populations (Matsui & Nakajima 2020). Zhang et al. (2021) assigned scientific names to each of these two populations, but the taxonomic identity of the genus *Misgurnus*, including the type specimens from Japan, needs to be re-examined.

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Author Contributions

All authors participated in sampling. Y. Yashima and T. Kitagawa designed the study and wrote the manuscript. Y. Yashima undertook data collection and analyses. R. Okada developed the nuclear species identification DNA marker. All authors have accepted responsibility for the entire manuscript content and approved its submission.



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Supplementary online material

Table S1. A list of morphological data of all *Misgurnus* specimens examined in this study (<https://www.ivb.cz/wp-content/uploads/JVB-vol.-72-2023-Yashima-Y.-et-al.-Table-S1.xlsx>).

Fig. S1. Lateral view photos of the female individuals captured from the wild population in Naka-ikemi Wetland; Type II loaches captured on (a) April 3, 2021, and (b) November 2, 2021; Type I loaches captured on (c) April 3, 2021, and (d) November 2, 2021. Red arrowheads point spawning scars (<https://www.ivb.cz/wp-content/uploads/JVB-vol.-72-2023-Yashima-Y.-et-al.-Fig.-S1.pdf>).