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Comparative Morphology and Morphometry of the Olfactory Organ of the Six Korean Spined Loaches, Genus *Iksookimia*, as a New Taxonomic Key

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Olfactory organ, Nostril, Rosette, Lamellar number, *Iksookimia*, Taxonomy

ABSTRACT

The olfactory structure of Korean spined loaches, *Iksookimia koreensis, I. pumila, I. pacifica, I. longicorpus, I. hugowolfeldi, I. yongdokensis*, were investigated by anatomical observation and statistical analysis. They share the same olfactory morphology characterized by tubular anterior and flat posterior nostrils and a rosette showing a radial arrangement of finger-like curved lamellae. However, the number of olfactory lamellae (LN) differs by species: 19-25 in *I. longicorpus* (with standard length, SL, 113.2±11.5 mm, mean±SD), 17-20 in *I. yongdokensis* (88.1±8.8 mm), 14-20 in *I. hugowolfeldi* (77.4±8.1 mm), 16-19 in *I. koreensis* (76.1±6.5 mm), 15-20 in *I. pacifica* (100.9±17.3mm), 15-19 in *I. pumila* (77.8±5.5 mm). In particular, *I. koreensis* (23.0±1.4%) and *I. hugowolfeldi* (22.4±2.4%) shows higher LN/SL ratios than do *I. longicorpus* (19.4±1.8%) and *I. pacifica* (17.5±2.0%). This comparison of LN and LN/SL ratio among species could be used as new taxonomic key of *Iksookimia*.

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INTRODUCTION

In addition to vision, hearing, current and acoustic pressure detection, electroreception, and magnetoreception, most teleost species rely on olfaction for detecting foods, hazardous materials, and pheromones; discriminating same from foreign species; and avoiding predators, migrating, and homing (Hara, 1986; Zielinski and Hara, 2006; Chung-Davidson *et al.*, 2010). Because such chemical reception is conducted by sensory cells on the lamellae of the rosette inside the olfactory chamber, teleost species shows species-specific structure in olfactory rosette based on their habitat environment and ecology (Cox, 2008). Teleosts show considerable morphological variability regarding the shape and location of the olfactory organ, number and arrangement of the olfactory lamellae, the distribution of sensory and non-sensory epithelium in relation to the diverse environments that the fish inhabit (Hara, 1986; Zeiske *et al.*, 1992; Kasumyan, 2004; Ghosh and Chakrabarti, 2016; Ghosh, 2019; Triana-Garcia *et al.*, 2021). In particular, although the number of olfactory lamellae (LN) tends to increase with growth and age, it remains constant upon teleost maturity (Hara, 1994). Accordingly, the number of olfactory lamellae has been used as a species-specific trait for taxonomy (LN 0 in genera *Periophthalmus*, *Boleophthalmus*, *Oryzias*, and *Rudarius*; LN 1 in *Navodon*, *Hemiramphus*, *Cheilopogon*, and *Cololabis*; LN > 100 in *Angulla* and *Conger*, and up to 200 in *Pseudoplatystoma corruscans*) among taxa (Matsuura, 1982; Matsuura and Tyler 1997; Tyler, 1997; Kim and Park, 2020), as well as between distant teleost species (Zeiske *et al.*, 1992; Kasumyan, 2004; Kuciel *et al.*, 2013).

The multiple ranges of mountains in Korea have resulted in numerous freshwater streams flowing independently to the sea (Kim *et al.*, 2005). This isolation of Korean water systems has resulted in evolution of *Iksookimia* into six endemic species: *Iksookimia*

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koreensis, Kim (1975); *Iksookimia pumila,* Kim and Lee (1987); *Iksookimia pacifica,* Kim *et al*. (1999); *Iksookimia longicorpus,* Kim *et al*. (1976); *Iksookimia hugowolfeldi,* Nalbant (1993); *Iksookimia yongdokensis,* Kim and Park (1997), based on habitat preference and morphology (Ko, 2015; Park *et al*., 2016; Ko *et al*., 2020). Recently, during Korean fish research to prove allopatric speciation, we have recognized that the olfactory organ of *Liobagrus* shows morphometrical differences in relation to its microhabitat preference (Kim and Park, 2020). So, we assumed that the olfactory organ of *Iksookimia* as an example of representative geographical speciation in South Korea also is likely to exhibit different adaptations. The present study focused on comparative morphology and morphometry of the olfactory organ of six Korean loaches based on taxonomy.

MATERIALS AND METHODS

Sample preparation

The six Korean spined loaches (a total of 120 specimens, 20 per species) of *Iksookimia* (*I. koreensis*, *I. pumila*, *I. pacifica, I. longicorpus, I. hugowolfeldi, I. yongdokensis*) were caught from numerous waterways from May to June 2022 (Table I). The endangered species *I. pumila* was caught with written permission (Number 2021-18) from the Ministry of Environment. After capture, two specimens per species were fixed with 2.5% glutaraldehyde solution (pH 7.4) for scanning electron microscopy (SEM), and the rest were preserved in 10% neutral formalin solution (pH 7.4) to measure the standard length (SL) and count the LN of the olfactory organ. We used full-grown adults demonstrating sexual dimorphism (Buj *et al*., 2015).

Anatomical structure

The olfactory organs were carefully dissected from the olfactory chamber on the dorsal side of the head under a stereo microscope (Stemi DV4, Carl Zeiss, Germany); counted and averaged the lamellae numbers; nostril and rosette images of olfactory organ were collected for morphological analysis with a digital camera (TG-3, Olympus, Tokyo, Japan). For SEM, dissected olfactory rosettes of each specimen were postfixed in 1.0% osmium tetroxide-phosphate buffer (pH 7.4) solution for six hours at room temperature, dehydrated with an ascending alcohol series (60% to 100%) for 20 minutes at each concentration, transferred to tert-butyl alcohol, freeze-dried (Vfd-21S, Vacuum Device Co., Ltd., Ibaragi, Japan), coated with white 1 µm thick gold by ion-sputter (HPC-1SW, Vacuum Device Inc., Tokyo, Japan), and filmed with a scanning electron microscope (S-3000N, Hitachi, Tokyo, Japan).

Statistical analysis

SPSS software (IBM® *SPSS*® Statistics, SPSS version 18.0, USA) was used for interspecies numerical analysis of the SL and LN of *Iksookimia*. For a parametric test based on the results of Kolmogorov-Smirnov or Shapiro-Wilk test ($P > 0.05$), one-way ANOVA was followed by Duncan's multiple range test and used to compare mean SL, LN, and LN/SL ratio between species. ANCOVA was used to analyze the LN difference based on the covariate SL. Pearson's correlation coefficient was used to identify a possible allometric association between SL and LN.

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Table I. Measurements of Korean loaches (genus *Iksookimia*) and collection localities.

	SL (mm)	Weight (g)	Collection locality (GPS)
I. koreensis (n=20)	76.1±6.5 (67.4~91.0)	4.8±1.4 (3~9)	Andeog-ri, Gui-myeon, Wanju-gun, Jeollabuk-do (35°44'34"N, 127°07'15"E)
I. longicorpa (n=20)	113.2±11.5 (100.6~141.8)	9.2±2.0 (6~13)	Dongsan-ri, Bokheung-myeon, Sunchang-gun, Jeollabuk-do (35°26'52"N, 126°55'09"E)
I. hugowolfeldi (n=20)	77.4±8.1 (61.8~93.8)	7.4±1.9 (5~12)	Eojeon-ri, Geumsan-myeon, Goheung-gun, Jeollanam-do (34°26'20"N, 127°09'42"E)
I. pumila (n=20)	77.8±5.5 (70.3~91.0)	5.0±1.2 (3~7)	Cheongnim-ri, Sangseo-myeon, Buan-gun, Jeollabuk-do (35°39'17"N, 126°36'35"E)
I. pacifica (n=20)	100.9±17.3 (75.3~128.6)	8.6±4.2 (4~17)	Sampo-ri, Jugwang-myeon, Goseong-gun, Gangwon-do (38°18'27"N, 128°31'38"E)
I. yongdokensis (n=20)	88.1±8.8 (75.7~105.0)	6.9±1.7 (5~11)	Sangwon-gil, Chuksan-myeon, Yeongdeok-gun, Gyeongsangbuk-do (36°30'23"N, 129°24'35"E)

Mean±SD (Min~Max); SL, standard length; n, number.

RESULTS

Anatomical structure of the olfactory organ

The *Iksookimia* species share same structure of anterior and posterior nostrils, rosette and lamellae of the olfactory organ, a paired sensory system located on both sides of the snout, with anterior and posterior nostrils present on each side (Fig. 1). The anterior nostril is a vertical wind-curve entrance tube protruding at differing heights from the skin surface (Fig. 1A). The posterior nostril is in the same level with skin surface and appears as an elliptical vent with a swollen skin edge (Fig. 1B). The two nostrils are separated by a nasal bridge on the skin and open to the outside. The olfactory rosette of the Korean spined loaches is comprised of several lamella originating from the ventral and lateral walls of the olfactory chamber and arranged based on the medium raphe (Fig. 2A-F). Each lamella is a center-bent finger-like structure, and they gradually increase in size from the anterior toward the posterior region. They are positioned parallelly at the front and middle part of the rosette and arranged radially at the posterior part. Each lamella also is thicker in the base and become thinner as getting upward (Fig. 2A-F). The center of the medium raphe is thicker and wider, and it narrows as going up and down (Fig. 2A-F).

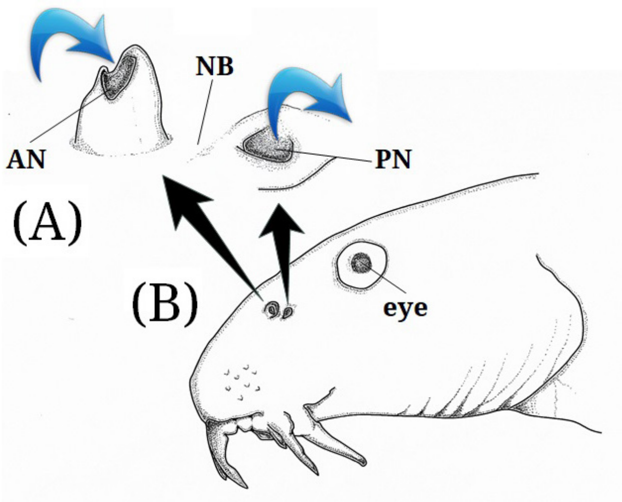

Fig. 1. The external anatomy of the olfactory organ and the head of genus *Iksookimia* showing two holes, anterior nostril (A) and posterior (B) nostril. The blue curved arrows indicate the direction of water flow. AN, anterior nostril; NB, nasal bridge; PN, posterior nostril.

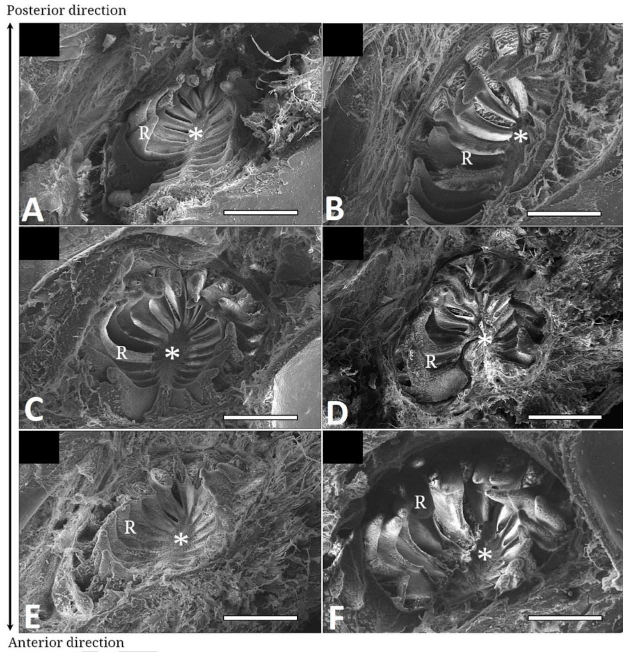

Fig. 2. Scanning electron micrographs of the olfactory rosette structure of the genus *Iksookimia* including *I. koreensis* (A), *I. longicorpus* (B)*, I. hugowolfeldi* (C)*, I. pumila* (D), *I. pacifica* (E)*, I. yongdokensis* (F). Asterisk, raphe; R, lamellae. All scale bars indicate 500 µm.

Statistical analysis of the olfactory lamellae

The SL showed different measurements: *I. longicorpus* was the longest (113.2±11.5, 100.6-141.8; Mean±SD, range); followed by *I. pacifica*, 100.9±17.3, 75.3-128.6; *I. yongdokensis*, 88.1±8.8, 75.7-105.0; *I. pumila*, 77.8±5.5, 70.3-91.0; *I. hugowolfeldi*, 77.4±8.1, 61.8-93.8; and *I. koreensis* was the shortest (76.1±6.5, 67.4-91.0). There was a significant difference in SL among the species (one-way ANOVA, $F_{5,\ 114}$ = 42.8, P < 0.001; Fig. 3A). The LNs also were different among the six Korean spined loaches: *I. longicorpus* had the highest number (21.9±1.7, 19-25; mean±SD, range), followed by *I. yongdokensis*, 18.0±0.9, 17-20; *I. hugowolfeldi*, 17.2±1.8, 14-20; *I. koreensis*, 17.4±1.0, 16-19; *I. pacifica*, 17.4±1.5, 15-20; and *I. pumila* with the smallest number (16.4±1.4, 15-19).There was a significant difference in LN among species (one-way ANOVA, $F_{5,\ 114}$ = 37.0, P < 0.001; Fig. 3B). The LN/SL ratio showed meaningful differences: *I. koreensis* had the highest ratio (23.0±1.4%, 19.5-24.9); followed by *I. hugowolfeldi*, 22.4±2.4%, 17.9-26.0; *I. pumila*, 21.1±1.1%, 19.1-23.8; *I. yongdokensis*, 20.5±1.4%, 18.4-22.9; *I. longicorpus*, 19.4±1.8%, 16.6-23.1; and then *I. pacifica* (17.5±2.0%, 14.8-21.4). The difference among them was significant (one-way ANOVA, $F_{5,\ 114}$ = 26.0, P < 0.001; Fig. 3C). The LN difference between species was affected strongly by SL as a covariate (ANCOVA, $F_{5,\ 114}$ = 19.5, P < 0.001). The SL and LN had a significant correlation in *I. koreensis* (Pearson's correlation coefficient, r = 0.650,

$P < 0.05$), *I. longicorpus* ($r = 0.481$, $P < 0.05$), *I. pumila* ($r = 0.779$, $P < 0.001$), *I. pacifica* ($r = 0.820$, $P < 0.001$), and *I. yongdokensis* ($r = 0.762$, $P < 0.001$) but not in *I. hugowolfeldi* ($r = 0.424$, $P = 0.063$) (Fig. 4).

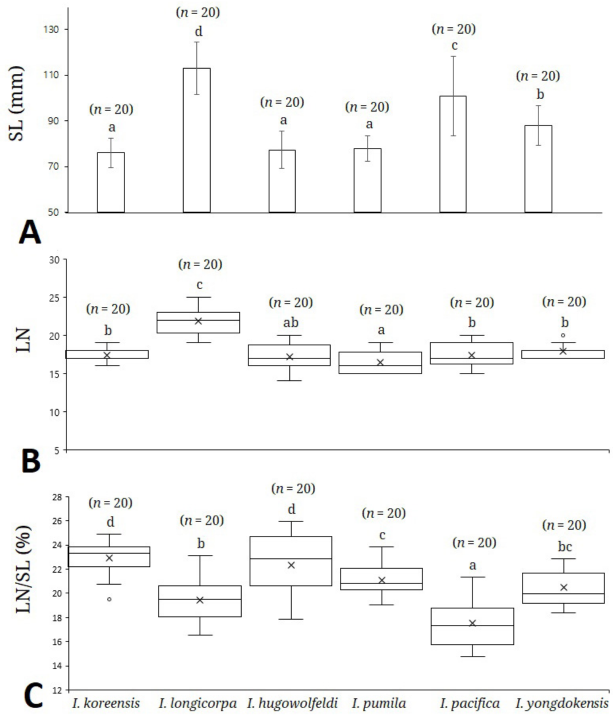

Fig. 3. Interspecific differences of genus *Iksookimia* in standard length (A), the number of olfactory lamellae (B), and the number of olfactory lamellae/standard length ratio (C). Letters above bars and box denote significantly different groups as determined by Duncan's multiple range test ($P < 0.05$). LN, the number of olfactory lamellae; SL, standard length (mm).

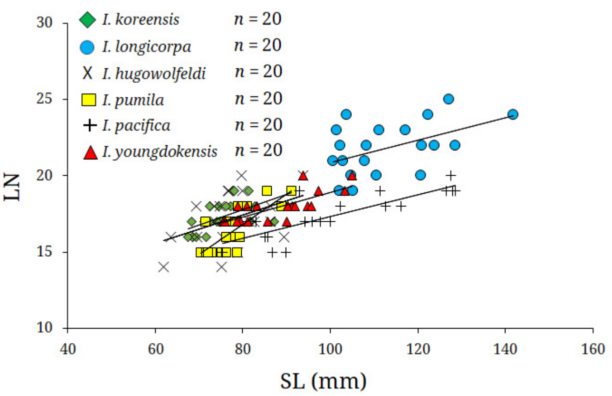

Fig. 4. Scatter plot of the dispersion between standard length (x-axis) and the number of olfactory lamellae (y-axis) of genus *Iksookimia*.

DISCUSSION

Distinct from the related genus *Cobitis* with Gambetta's zones as a pigmentary zone on lateral body but sharing similar habitat (the middle to upper streams with gravels and sandy bottom), genera *Iksookimia* is currently classified into six endemic Korean species based on (i) geological distribution, and (ii) morphology (the lamina circularis as bony process or plate at the base of the first and second rays of the pectoral-fin and the body pigmentation) (Kim and Park, 1997, 2002; Kim, 2009; Ko, 2015). The organisms in question also demonstrate different reproductive strategies in egg envelope structure (papilla, villi, grapevine, hillock, granule, unadorned form, and sawtooth-like form) in relation to the river bottom structure (Ekmekci and Erk'akan, 2003; Ko and Park, 2010), spawning character (Ko and Won, 2016), and early life history (Ko *et al*., 2017). These different adaptations of *Iksookima* are well-correlated with their geographical distribution and are a good example of allopatric speciation (Kim, 1997). The morphological variations, frequent interspecific hybridization, and similar genetic sequences of Korean spined loaches (Family Cobitidae) have interested many ichthyologists (Kim, 2009). However, these overlapping features have been the source of taxonomic debate in recent days. *I. pacifica* was reclassified from the genus *Cobitis* into the genus *Iksookimia* based on rippled pigmentation of its dorso-lateral surface of body (Kim, 2009). *Cobitis choii* was first described as a new species of *Cobitis* by Kim and Son (1984), but was reclassified into the genus *Iksookimia* by Nalbant (1993) before it finally was returned to the genus *Cobitis*. To clarify the taxonomy of *Iksookimia*, we described the LN and LN/SL ratio as a suggested new taxonomic key for morphometrical analysis.

All Korean spined loaches share the same olfactory morphology with two openings (tubular anterior nostril and posterior nostril flat against the skin surface) on each side of the snout and the olfactory rosette composed of several finger-like lamellae. The tubular nostril typically appears in bottom-dwelling teleosts is a functional boundary layer between inflow of chemical odors to the olfactory organ (Belanger, 2003; Sarkar *et al*., 2014; Kim and Park, 2020). Such an olfactory entrance may be related to the benthic habitat of Korean spined loaches, which spends their lifetime on the river bottom (Kim and Park, 2002; Park *et al*., 2018).

In contrast to this morphological similarity, the olfactory morphometry of *Iksookimia* shows interspecies variation in that the LN and LN/SL ratio differ by species size. Kim and Park (2020) demonstrated that, although the LN ranges overlap, *Liobagrus* fish showed significant

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mean difference: 16-19 in *L. andersoni*, 14-16 in *L. obesus*, 22-27 in *L. mediadiposalis*, 19-24 in *L. somjinensis*, 14-18 in *L. hyeongsanensis*, which could be useful to identify species based on microhabitat adaptation. Recently, Silva-Junior and Zanata (2022) reported variability among six geologically diverse species of the genus *Parotocinclus* based on LN to SL ration and sexual dimorphism that could be used as a new species indicator. Therefore, difference in LN and LN/SL ratio among species of *Iksookimia* might be a useful taxonomic key to reflect their different geographical distribution.

Regarding LN, *I. longicorpus* was highest (21.9±1.7), followed by *I. yongdokensis*, 18.0±0.9; *I. hugowolfeldi*, 17.2±1.8; *I. koreensis*, 17.4±1.0; *I. pacifica*, 17.4±1.5; and then *I. pumila* (16.4±1.4). However, based on LN/SL ratio, *I. koreensis* was highest (23.0±1.4%), followed by *I. hugowolfeldi*, 22.4±2.4%; *I. pumila*, 21.1±1.1%; *I. yongdokensis*, 20.5±1.4%; *I. longicorpus*, 19.4±1.8%; and then *I. pacifica*, 17.5±2.0%. Considering the correlation between LN and olfactory function, Fishelson *et al*. (2010) opined that the rosette of abundant lamellae in synodontids (active predator) indicates a more important role of olfaction in their life history compared to benny fishes with a small-sized rosette. Based on this collective finding, larger LN/SL ratio suggest that olfaction of *I. koreensis* and *I. hugowolfeldi* may be more important in their life history despite a smaller LN than that of *I. longicorpus* and *I. pacifica*. Analysis of correlation between olfactory lamellae and body size has focused on the LN increase with body size (Kudo *et al*., 2009; Atta, 2013). Herein, we confirmed that the LN/SL ratio among related species could distinguish species, highlight relative importance of olfaction, and is suggested as newly taxonomic key.

CONCLUSION

As a good example of allopatric speciation, the genus *Iksookima* shows different adaptations well-correlated with geographical distribution among them. They also reveal morphological variations, frequent interspecific hybridization, and similar genetic sequences. However, these overlapping features have been the source of taxonomic debate in recent days. Therefore, we aim to report new morphological taxonomic key using the features of the olfactory rosette morphology and morphometry for species identification. Our findings demonstrated that difference in LN and LN/SL ratio among species of *Iksookimia* might be a useful taxonomic key to reflect their different geographical distribution. So, LN and LN/SL ratio are recommended as a new and meaningful taxonomic character that distinguishes sympatric species at least in *Iksookimia*.

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IRB approval

This study was approved by the Institutional Review Board at Jeonbuk National University.

Ethical statement

All procedures of animal experiment strictly obeyed the rules of the Jeonbuk National University Institutional Animal Care and Use Committee (Licence Number: CBNU-2022-00060).

Statement of conflict of interest

The authors have declared no conflict of interest.

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