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Fine structure of micropylar regions of *Cobitis hankugensis*, *Iksookimia longicorpa* and their unisexual natural hybrids

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Research Article

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Summary

Iksookimia longicorpa and *Cobitis hankugensis* are two species of fish distributed only on the Korean Peninsula. They have a unique reproductive ecology that naturally hybridizes into three widely known unisexual types, maintaining populations of almost all females. In this study, the fine structure of the micropyles of *I. longicorpa*, *C. hankugensis* and their hybrids was analyzed to find out how egg–sperm interaction, a common interspecies isolation mechanism, is possible between heterogeneous species. Analysis of 30 eggs from five females of each species revealed that all had one funnel-shaped micropylar region and a manhole-shaped micropyle canal. With the exception of *C. hankugensis*, which had no spiral grooves or ridges, the rest had counter-clockwise spiral grooves and ridges on the micropylar region. All five species, however, showed identical groove patterns for the micropyle canal. The egg size was the largest in HL (one from the *C. hankugensis* locus with one from the *I. longicorpa* locus) and the smallest in *C. hankugensis*. In the hybrids, the HL type had the largest egg and HHL (two from the *C. hankugensis* locus with one from the *I. longicorpa* locus) type the smallest. For the diameter of the micropylar region and micropyle canal, the diploid *I. longicorpa*, *C. hankugensis* and HL were smaller than those of the triploid. In addition, as the ratio of the canal diameter to the eggs was lower in *I. longicorpa* than in *C. hankugensis*, it was confirmed that *I. longicorpa* has a relatively small micropyle canal compared with *C. hankugensis*.

Introduction

The micropyle is the entrance through which spermatozoa without acrosomes pass for fertilization. Although the numbers vary slightly depending on the taxa, teleost fishes generally have one micropyle in the region of the animal pole (Coward *et al.*, 2002). According to previous studies and despite few differences between closely related species, morphological characteristics of the micropyle can be used as an important indicator for taxonomic purposes (Riehl, 1980; Hirai and Yamamoto, 1986; Chen *et al.*, 1999) because they differ according to taxa. This fundamental difference can be described as the most basic of barriers to prevent cross-breeding. Nevertheless, there is a phenomenon in which this barrier collapses in the natural world, leading to hybridization.

Hybrids are individuals resulting from hybridization between different species, meaning by the combination of different genes. In some cases hybrids play an important role in the sympatric speciation process (Chevassus, 1983; Barton and Hewitt, 1985; Abbott *et al.*, 2013). Natural hybrids are often found to exist in limited populations in their native environments and generally are not fertile; hybrids alone cannot maintain a population and their traits cannot be passed on to the next generation. One of the results of common reproductive isolation, especially among species presenting with post-zygotic isolation, is hybrid sterility (Scopece *et al.*, 2008). Obviously, there is also a pre-zygotic mechanism that prevents hybrid formation.

The genus of *Cobitis* hybrids distributed in Korea is derived from two parental groups of *Cobitis* fishes, *Cobitis hankugensis* and *Iksookimia longicorpa* (first reported as *C. longicorpus* by Kim *et al.* (1976)). They maintain their lineage of almost all females with the extremely rare males (Park *et al.*, 2011). Three types of the hybrid are identified through microsatellite loci analysis [i.e. one diploid hybrid: one *C. hankugensis* locus with one *I. longicorpa* locus (HL), two triploid hybrids: one *C. hankugensis* locus with two *I. longicorpa* locus (HLL) and two *C. hankugensis* locus with one *I. longicorpa* locus (HHL)]. In the above unisexual fishes, the rare males that do occur have been considered sterile with low counts of sperm cells. Such an abnormal reproductive mode has attracted attention. According to previous research, the *C. hankugensis*–*I. longicorpa* hybrid forms through the normal hybridogenesis process and then combines with a haploid male through a premeiotic endomitosis mechanism to form a triploid. These triploids generate haploid eggs after heterogeneous allele removal and are fertilized with the male genome. For homozygous, intact *C. hankugensis* or *I. longicorpa*, when heterozygous, they form the HL-type diploid C. *hankugensis*–*I. longicorpa* complex again (Lee, 1992, 1995; Ko, 2009; Yun, 2017). To date, research on these unisexual fishes in the wild has tended to focus only on this process, while the structure of the micropyle remains poorly understood. Therefore, this study for Korean unisexual fishes sought to understand and compare the fine structure of the micropyle, given that it may be useful for identifying species and hybrids as a taxonomic characteristic.

Materials and methods

Ethics, sample collection and identification

All of the fish in this study were treated based on the 'Ethical review process by Chonbuk National University Institutional Animal Care and Use Committee (JBNUIACUC)'.

Specimens of *I. longicorpa*, *C. hankugensis* and their hybrids were collected during their spawning period in Ram Stream, Nakdong River basin, Namwon, Jeollabuk-do, April to June 2019 (35°27′26′′N, 127°36′26′′E) with a cast net (6 × 6 mm mesh). In total, 25 individuals were collected: *I. longicorpa* ($n = 5$), *C. hankugensis* ($n = 5$), HL type ($n = 5$), HLL ($n = 5$) and HHL type ($n = 5$). The identification of the hybrid type followed the allele dosage effects using microsatellite loci (Yun, 2017).

Egg collection and ultrastructural analysis

Under MS-222 (Sigma Aldrich Co., St. Louis, USA) anaesthesia conditions, the oocyte of each fish was collected under gentle pressure through the abdomen and water, blood and urine were carefully removed to avoid contamination. For scanning electron microscopy observation, the collected oocytes (30 eggs from five females of each species) were fixed in 2.5% glutaraldehyde solution with 0.1 M phosphate buffer at pH 7.4. Then the eggs were fixed in 1% osmium tetroxide solution with 0.1 M phosphate buffer for 6 h and dehydrated using a graded series of ethanol and tert-butyl alcohol and freeze dried using evaporation under vacuum (VFD-21S, Vacuum Device Co., Ltd, Ibaragi, Japan). The dried samples were coated with osmium tetroxide using ion sputtering (HPC-1SW, Vacuum Device Inc., Tokyo, Japan) and then observed under a field emission scanning electron microscope (Carl Zeiss, SUPRA40VP, Germany). The evaluation of the number of attachments over the egg surface was measured 10 times for each egg in the range 10 × 10 μm.

Statistical analysis

Egg diameter and morphometric values of micropylar components were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post hoc test in SPSS v.22. The level of significance was set at 5% ($P < 0.05$). Data are presented as mean and standard deviation.

Results

In a scanning electron microscopic observation, all unfertilized eggs had only a single micropyle complex composed of a funnel-shaped micropylar region and a manhole-shaped micropylar canal on its innermost. Repetitive rotational grooves and ridges were observed in the other four species of micropylar regions except for *C. hankugensis*. In all species, the micropylar canal has grooves and ridges down to the egg surface. The direction of the spirals in the micropylar region and the canal was counterclockwise from the outer boundary of the micropyle towards the centre (Figure 1).

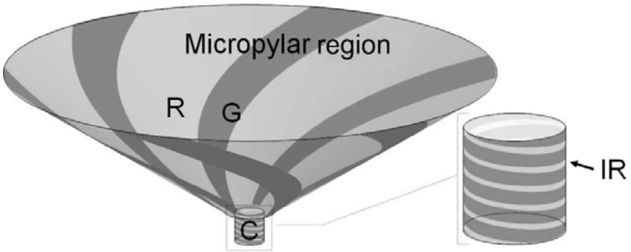

Figure 1. Schematic diagram of micropyle complex of *Iksookimia longicorpa*, *Cobitis hankugensis* (exceptionally no spiral pattern in the micropylar region), and their hybrids. C, micropyle canal; G, groove; IR, inner ridge; R, outer ridge.

However, their detailed morphological characteristics differed slightly by type. The unfertilized eggs of *I. longicorpa* were an average of 1.36 mm in diameter, similar in size to those of the HLL hybrid (1.35 mm). *C. hankugensis* with 1.11 mm was the smallest. The diameters of HL type and HHL type were 1.43 mm and 1.21 mm, respectively (Table 1).

The micropylar region was covered with numerous pore canals being surrounded by attachments that showed villous and granular forms (Figure 2 and Table 1). *I. longicorpa* and the HLL hybrid had a villus type and the number of villi was in the ranges 6–9 and 15–19, respectively. In addition, the other three species, *C. hankugensis*, HL hybrid and HHL hybrid, had a granular type and were 20–27, 13–19 and 20–27 in number, respectively. Unlike the other two types, in particular, the pore canals of *C. hankugensis* were barely exposed due to compacted arranged granules (Figure 2B).

Inside the micropylar region, counterclockwise spirals formed at least four to eight grooves in four species: *I. longicorpa*, HL type, HHL type and HLL type. Interestingly, however, for *C. hankugensis*, these spiral structures were not observed. Their absence may be due the many granules covering the entire egg surface as well as the micropylar region, but the surface that was covered with the granules was slightly curved and traces of grooves could be presumed. In addition, the multilayered grooves of the inner canal that were not fully connected were observed equally in all species (Figure 2A′–E′ and Table 1).

Micropylar regions were easily distinguished by the boundary where the chorion sank inward abruptly (Figure 2A). The HLL hybrid had the largest diameter of 49 μm and the *C. hankugensis* was the smallest at 35.36 μm. *I. longicorpa* and the HL hybrid were similar, 38.46 μm and 38.72 μm, respectively, while the diameter of the HHL hybrid was 44.93 μm (Table 1).

The circular opening of the canal was placed in the centre of the funnel. In contrast with the egg diameter, the micropylar canal diameter was the smallest in *I. longicorpa* (3.38 μm) and the *C. hankugensis* (3.56 μm) was larger than that of *I. longicorpa*. The largest species were the HHL hybrid (4.37 μm), followed by HL and HLL at 3.98 μm and 4.01 μm, respectively (Table 1).

Upon analysis of the ratio of the size of the micropylar canal to the egg diameter, *I. longicorpa*, HL hybrid and HLL hybrid were less than 0.3% and both *C. hankugensis* and HHL hybrid were greater than 0.3%, indicating that the size of the micropylar canal was relatively larger than that of the other three species (Table 1).

Discussion

In the evolution of vertebrates, fish have developed distinctive reproductive strategies such as the micropyle for optimization of

Table 1. Comparison of microstructural characters of the unfertilized eggs of in *Iksookimia longicorpa*, *Cobitis hankugensis* and their natural hybrids. Values within each column with different letters are significantly different ($P < 0.05$)

Genotype	*Iksookimia longicorpa*	*Cobitis hankugensis*	HL	HHL	HLL
Egg diameter, ED (mm)	1.36 ± 0.06^{a} (1.21–1.48)	1.11 ± 0.05^{b} (0.97–1.2)	1.43 ± 0.07^{c} (1.31–1.54)	1.21 ± 0.06^{d} (1.11–1.33)	1.35 ± 0.0^{a} (1.24–1.42)
Micropylar region diameter, RD (μm)	38.46 ± 3.52^{a} (32.59–41.94)	35.36 ± 3.6^{a} (29.9–41.14)	38.72 ± 3.04^{a} (35.2–47.55)	44.93 ± 4.74^{b} (37.24–57.51)	49 ± 4.65^{b} (38.27–57.92)
Micropyle canal diameter, CD (μm)	3.38 ± 0.15^{a} (3.17–3.52)	$3.56 \pm 0.17^{a,b}$ (3.42–3.77)	$3.98 \pm 0.11^{b,c}$ (3.86–4.09)	4.37 ± 0.3^{c} (3.86–4.75)	$4.01 \pm 0.3^{b,c}$ (3.59–4.29)
Ratio of CD/ED (%)	0.25 ± 0.01^{a} (0.23–0.26)	$0.32 \pm 0.02^{b,c}$ (0.3–0.35)	$0.28 \pm 0.02^{a,b}$ (0.26–0.31)	0.35 ± 0.03^{c} (0.32–0.4)	0.3 ± 0.02^{b} (0.27–0.31)
Number of spirals	4–8	None	5–6	6–8	7–8
Type of surface attachment	Villus	Granule	Granule	Granule	Villus
Number of surface attachment (10 × 10 μm)	7.2 ± 1.0^{a} (6~9)	23.4 ± 2.2^{b} (20~27)	15.9 ± 1.9^{c} (13~19)	22.5 ± 2.4^{b} (20~27)	17 ± 1.4^{c} (15~19)

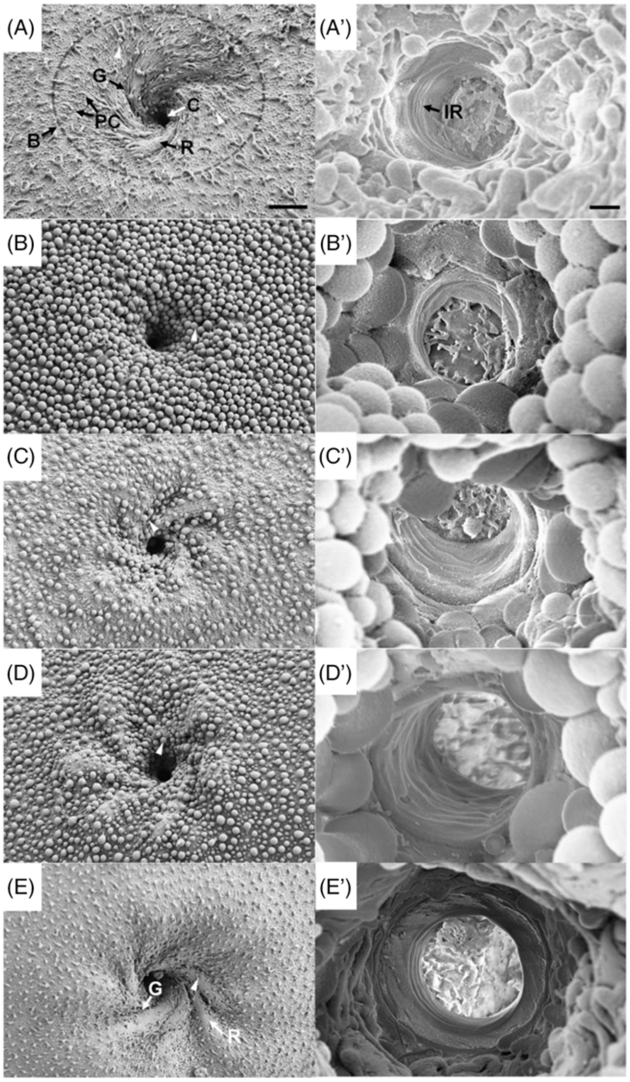

Figure 2. Scanning electron microscopy of micropylar region (A–E) and pore (A′–E′) in *Iksookimia longicorpa* (A, A′), *Cobitis hankugensis* (B, B′) and their natural hybrids, HL (C, C′), HHL (D, D′) and HLL (E, E′). The white triangle represents surface attachment, where (A) and (E) are villus type, and (B), (C) and (D) are granule type. B, micropylar region boundary; C, micropyle canal; G, groove; IR, inner ridge; PC, micropyle pore canal; R, outer ridge. Bars indicates 10 μm (A–E) and 1 μm (A′–E′).

mass spawning and oviparity. These processes can be found by backtracking from Teleostei to Agnatha. The hagfish, *Eptatretus burgeri*, has been reported to have a micropylar-like structure and spermatozoa containing acrosomes, which are predominantly observed in mammals (Morisawa, 1999). Acrosomes are also observed in sturgeon, a ray-finned fish with micropyles varying in number from 2 to 52 (Ginsburg, 1968; Debus *et al.*, 2008; Psenicka *et al.*, 2010). However, among teleosts, the largest group of fish comprising 96% of fish species (Nelson, 2006), a single spermatozoon without an acrosome passes through a single micropyle and fuses with the egg. This is supported by a series of excellent reviews published continuously since the 1950s (Yamamoto, 1952; Riehl and Schulte, 1977; Hart, 1990; Coward *et al.*, 2002). In scanning electron microscope observation, it was confirmed that not only do the relatively recently differentiated cobitid fish have one micropyle [30–35 million years ago (MYA); Slechtová *et al.*, 2008], but that *C. hankugensis* and *I. longicorpa* and their hybrids do as well, a characteristic typical of the teleost.

Studies on the morphological characteristics of micropyles have distinguished them into several types. Riehl and Kock (1989) focused on the size of the micropylar pit and canal: deep pit and a short canal (type I), flat pit and a longer canal (type II), only canal without a pit (type III) and two pits and a short canal (type IV). In a more recent study, Yanagimachi *et al.* (2017) classified these into three types based on the form in which the micropylar region is depressed: flat or slightly depressed micropylar region with manhole-like canal (type I), flat or slightly depressed micropylar region with funnel-like canal (type II) and a deeply depressed micropylar region such as a sinkhole or one with distinct grooves with a short canal (type III). In this study, we demonstrated the micropyle structure of all fishes with the same appearance with a shallow funnel-type micropylar region and a short manhole-type canal at the centre. Although our results did not correctly belong to the previous classification scheme, they were most similar to the type III suggested by Yanagimachi *et al.* (2017).

The grooves situated on the surface of the micropylar region guide sperm into the micropylar canal, this was mainly observed in species corresponding to the type III proposed by Yanagimachi *et al.* (2017) and their effectiveness has already been proven (Amanze and Iyengar, 1990). Although this varies depending on the species, *I. longicorpa* and three hybrids that received its genes had similar counterclockwise spiral structures, while in *C. hankugensis* spirals were absent. These spiral grooves are very distinctive even in the sister groups: *Misgurnus anguillicaudatus* has irregular grooves with sinkhole-shaped micropylar regions (Yanagimachi *et al.*, 2017) and *Kichulchoia multifasciata* has only funnel-shaped micropylar regions without grooves and ridges (Kim *et al.*, 2011). Only toadfish (*Thalassophryne amazonica*) and anabantoid fishes showed a spiral pattern around the micropyle, but the extent of the pattern was not limited to the micropylar region and appeared throughout the chorion (Riehl and Kokoscha, 1993; Britz *et al.*, 1995; Britz and Toledo-Piza, 2012). Consequently, the morphological characteristics of the micropylar region, such as the spiral of *I. longicorpa* or the non-spiral of *C. hankugensis* observed in this study seem to be species specific. It is unclear what advantage is offered by the linear structures appearing in the majority of grooves and equally unclear what the presence of the spiral structures or the absence of the grooves identified in this study means. More considerable research on the topic is required. In addition, the existence of the grooves was dependent on the L gene, which can be seen for the grooves of *I. longicorpa*; for HLL with two L gene this was clearer than on HL and HHL hybrids. The structure of the micropyle canal is a comparatively well defined component among the micropylar complex. Funnel-type structures observed in some fish such as *Oryzias* (Iwamatsu *et al.*, 1997) and Sparidae (Chen *et al.*, 1999) are intended to guide sperm to the centre of the canal. A similar manhole-type structure was also seen in this study.

On morphological analysis, the egg diameter of *I. longicorpa* was significantly similar to that of the HLL hybrid. There was no significant relationship, but the *C. hankugensis* and HHL hybrid egg diameters were smaller than in other examples, and the HL hybrid was the largest among them. This seems to be related to the reproductive mode of the *C. hankugensis* and *I. longicorpa* hybrids, in which the HL hybrid produces diploid eggs (HL) and HHL and HLL hybrids produce haploid eggs, H and L, respectively (Yun, 2017). As genome size affects egg diameter (Hardie and Hebert, 2004), the diploid egg, in the HL hybrid, had the largest diameter. The HLL hybrid with the haploid L gene had a size similar to that of the L and the HHL hybrids; the haploid H gene produced eggs of small size, as found for *C. hankugensis*. However, other metrics did not follow the 'genome-size relationship'. Namely, diploid eggs showed smaller diameters in micropylar region and canal than triploids, HHL and HLL hybrids, which are believed to be the result of 'cell size coordination by ploidy level' were identified in many organisms (Mendell *et al.*, 2008; Matsunaga *et al.*, 2013). Nevertheless, for canal diameter, the difference was not significant between species. As there has been no research on this so far, a new approach will therefore be needed to understand the relevance of the ploidy level and micropylar components in hybrids.

The attachment architecture of the egg membrane may play a role in adhesive or non-adhesive properties in demersal eggs (Mito, 1979). *Oryzias latipes* are among well reported examples of this type of adhesive envelope with long filaments on its entire egg surface, and which attach its fertilized eggs to substrates such as seaweed. Moreover, the number and length of the adhesive envelope have been used to identify between *O. latipes and O. sinensis* (Hart *et al.*, 1984, Kim and Park, 2021). In addition, villus or granular types have been already known for the family Cobitidae, as well as teleost fishes, as a tool for species identification (Mazzini *et al.*, 1984; Park, 1996; Park and Kim, 1997). We confirmed in this study that there have been two types, a villus and a granular form. Interestingly, the shape and number of the attachment structures in *Cobitis* hybrids were dependent on their parental species, not being fixed in a regular form. In case the L gene was dominant, the villus type with a small number was shown, whereas, in the dominant H gene, the granule type with a large number appeared. Exceptionally, for the HL hybrid, the attachment appeared to be affected by the H gene in shape and by the L gene in number.

The micropyle canal is the part through which the head of spermatozoa directly passes and has a very important role in taxonomic terms (Ginsburg, 1968; Kobayashi and Yamamoto, 1981). Interestingly, there are reviews about the relationship between the size of the micropylar canal and the head of spermatozoa. In *Pleuronectes obscurus*, Yanagimachi *et al.* (2013) reported that the size of the sperm head was ~87% of the micropyle canal. According to Solis Murgas *et al.* (2017), the corresponding ratios of the four neotropical fishes, *Prochilodus lineatus*, *Salminus brasiliensis*, *Piaractus mesopotamicus* and *Brycon orbignyanus*, were reported to be 31%, 19%, 23% and 43%, respectively. As such, the ratio varied depending on the species, but the important thing was, of course, that the size of the micropyle canal was larger than the head of the sperm and is a fundamental feature of teleost fishes (Psenicka *et al.*, 2010). In previous research, we could obtain only limited information about the morphology of *C. hankugensis* and *I. longicorpa* hybrid sperm (Park *et al.*, 2011). In that study, the size of the sperm head was 1.7 μm for *I. longicorpa*, 2.0 μm for *C. hankugensis* and 3.9 μm for the hybrid and when calculated in relation to the canal size of all species in this study, sperm of *I. longicorpa* was 39–50% and sperm of *C. hankugensis* was 46–59% and hybrid sperm were identified as 89–115%, respectively. Based on this, it was possible to carefully infer the answer as to why this hybrid population consisted mostly of females. Although that value was measured without the identification of three hybrids, in this population, male hybrids are basically unable to participate in the breeding competition as a result of their abnormally large diameter spermatozoa head.

Consequently, given that research on unisexual fishes showing polyploidy has focused mainly on genetic aspects, results based on micropyle may be used as another key for identifying and estimating hybrid populations from generation to generation.

In conclusion, the main finding of this study is the basic morphological characteristics on the micropyle of the *I. longicorpa*, *C. hankugensis* and their natural hybrids. They all have in common a single micropyle composed of a funnel-shaped micropylar region that can guide sperm to the centre of the micropyle with a manhole-type micropylar canal. The groove patterns with their counterclockwise spirals in the micropylar region were species specific and, moreover, *C. hankugensis* lacked the groove. These differences suggested a strong possibility for the participation of the L gene in the manifestation of the groove: *I. longicorpa* and HLL hybrid with two sets of L genes showed more distinct grooves than did HL and HHL hybrids with one set of L genes. The egg diameter, which is directly affected by the genome size, was the largest in the HL hybrid, which forms diploid eggs and in other somatic characteristics of the micropyle; the values were relatively high in triploid hybrids. Consequently, *I. longicorpa* had a large egg diameter with a small micropyle canal, whereas *C. hankugensis* had a small egg diameter with a relatively large micropyle canal. In addition, their hybrids showed the intermediate values of the parent species, being particularly dependent on the ploidy of the egg. Now, in addition to the many studies that have been performed on unisexual fishes, this type of approach may be helpful for obtaining insight into determining polyploidy or complicated taxa.

Data availability. Research data are not shared.

Competing interest. The authors declare no conflict of interest.

References

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J. W., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., Jones, J., Keller, B., Marczewski, T., Mallet, J., Martinez-Rodriguez, P., Möst, M., Mullen, S., Nichols, R., Nolte, A. W., Parisod, C., Pfennig, K., Rice, A. M., Ritchie, M. G., Seifert, B., Smadja, C. M., Stelkens, R., Szymura, J. M., Väinölä, R., Wolf, J. B., Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, **26**(2), 229–246. doi: 10.1111/j.1420-9101.2012.02599.x

Amanze, D. and Iyengar, A. (1990). The micropyle—A sperm guidance-system in teleost fertilization. *Development*, **109**(2), 495–500. doi: 10.1242/dev.109.2.495

Barton, N. H. and Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**(1), 113–148. doi: 10.1146/annurev.es.16.110185.000553

Britz, R. and Toledo-Piza, M. (2012). Egg surface structure of the freshwater toadfish *Thalassophryne amazonica* (Teleostei: Batrachoididae) with information on its distribution and natural habitat. *Neotropical Ichthyology*, **10**(3), 593–599. doi: 10.1590/S1679-62252012000300013

Britz, R., Kokoscha, M. and Riehl, R. (1995). The Anabantoid genera *Ctenops*, *Luciocephalus*, *Parasphaerichthys*, and *Sphaerichthys* (Teleostei, Perciformes) as a monophyletic group—evidence from egg surface-structure and reproductive-behavior. *Japanese Journal of Ichthyology*, **42**, 71–79.

Chen, K. C., Shao, K. T. and Yang, J. S. (1999). Using micropylar ultrastructure for species identification and phylogenetic inference among four species of Sparidae. *Journal of Fish Biology*, **55**(2), 288–300. doi: 10.1111/j.1095-8649.1999.tb00679.x

Chevassus, B. (1983). Hybridization in fish. *Aquaculture*, **33**, 245–262.

Coward, K., Bromage, N. R., Hibbitt, O. and Parrington, J. (2002). Gamete physiology, fertilization and egg activation in teleost fish. *Reviews in Fish Biology and Fisheries*, **12**(1), 33–58. doi: 10.1023/A:1022613404123

Debus, L., Winkler, M. and Billard, R. (2008). Ultrastructure of the oocyte envelopes of some Eurasian acipenserids. *Journal of Applied Ichthyology*, **24**(s1), 57–64. doi: 10.1111/j.1439-0426.2008.01093.x

Ginsburg, A. S. (1968). *Fertilization in Fishes and the Problem of Polyspermy*, p. 354. Izdatelnaya Nauka.

Hardie, D. C. and Hebert, P. D. N. (2004). Genome-size evolution in fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, **61**(9), 1636–1646. doi: 10.1139/f04-106

Hart, N. H. (1990). Fertilization in teleost fishes: Mechanisms of sperm–egg interactions. *International Review of Cytology*, **121**, 1–66. doi: 10.1016/s0074-7696(08)60658-0

Hart, N. H., Pietri, R. and Donovan, M. (1984). The structure of the chorion and associated surface filaments in *Oryzias*–evidence for the presence of extracellular tubules. *Journal of Experimental Zoology*, **230**(2), 273–296. doi: 10.1002/jez.1402300213

Hirai, A. and Yamamoto, T. S. (1986). Micropyle in the developing eggs of the anchovy, *Engraulis japonica*. *Japanese Journal of Ichthyology*, **33**(1), 62–66. doi: 10.1007/BF02905561

Iwamatsu, T., Onitake, K., Matsuyama, K., Satoh, M. and Yukawa, S. (1997). Effect of micropylar morphology and size on rapid sperm entry into the eggs of the medaka. *Zoological Science*, **14**(4), 623–628. doi: 10.2108/zsj.14.623

Kim, H. T. and Park, J. Y. (2021). Comparative morphology and morphometry of the micropyle of two Korean rice-fishes, *Oryzias latipes* and *Oryzias sinensis* (Pisces, Adrianichthyidae). *Journal of Vertebrate Biology*, **70**(1), 20130.1–8. doi: 10.25225/jvb.20130

Kim, C. H., Kim, J. G. and Park, J. Y. (2011). Structure of egg envelope and oogenesis of *Kichulchoia multifasciata*. *Korean Journal of Microscopy*, **41**, 189–196.

Kim, I. S., Choi, K. C. and Nalbant, T. (1976). *Cobitis longicorpus*, a new cobitid fish form Korea. *Korean Journal of Zoology*, **19**, 171–178.

Ko, M. H. (2009). Reproductive mechanisms of the unisexual diploid-tripoid hybrid complex of between the spined loach *Cobitis hankugensis* and *Iksookimia longicorpa* (Teleostei, Cobitidae) in Korea [Chonbuk National University Dotoral Thesis], 160 pp.

Kobayashi, W. and Yamamoto, T. S. (1981). Fine-structure of the micropylar apparatus of the chum salmon egg, with a discussion of the mechanism for blocking polyspermy. *Journal of Experimental Zoology*, **217**(2), 265–275. doi: 10.1002/jez.1402170213

Lee, E. H. (1995). A study of reproductive mode of the unisexual Cobitid fishes, *Cobitis sinensis-longicorpus* complex (Cobididae) by hybridization with its parental species [Chonbuk National University Dotoral Thesis], 92 pp.

Lee, J. H. (1992). A systematic study of the unisexual Cobitid fish, *Cobitis sinensis-longicorpus* complex in the Naktong River, Korea [Chonbuk National University Doctoral Thesis], 103 pp.

Matsunaga, S., Katagiri, Y., Nagashima, Y., Sugiyama, T., Hasegawa, J., Hayashi, K. and Sakamoto, T. (2013). New insights into the dynamics of plant cell nuclei and chromosomes. *International Review of Cell and Molecular Biology*, **305**, 253–301. doi: 10.1016/B978-0-12-407695-2.00006-8

Mazzini, M., Callaini, G. and Mencarelli, C. (1984). A comparative analysis of the evolution of the egg envelopes and the origin of the yolk. *Bolletino di Zoologia*, **51**(1–2), 35–101. doi: 10.1080/11250008409439457

Mendell, J. E., Clements, K. D., Choat, J. H. and Angert, E. R. (2008). Extreme polyploidy in a large bacterium. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(18), 6730–6734. doi: 10.1073/pnas.0707522105

Mito, S. (1979). Fish eggs. *Gekkan Kaiyo-Kagaku*, **11**, 126–130.

Morisawa, S. (1999). Fine structure of micropylar region during late oogenesis in eggs of the hagfish *Eptatretus burgeri* (Agnatha). *Development, Growth and Differentiation*, **41**(5), 611–618. doi: 10.1046/j.1440-169x.1999.00458.x

Nelson, J. S. (2006). *Fishes of the world* (4th edn) John Wiley and Sons, Inc., 601 pp.

Park, J. Y. (1996). *A morphological study on the gonad of the species in the family Cobitidae (Pisces: Cypriniformes) from Korea* [Dissertation]. Chonbuk National University (pp. 6–158).

Park, J. Y. and Kim, I. S. (1997). Egg membrane in five cobitid species of *Cobitis* (Pisces: Cobitidae). *Korean Journal of Ichthyology*, **9**, 121–129.

Park, J. Y., Kim, I. S. and Ko, M. H. (2011). Characteristics of rare males in the cobitid unisexual complex, *Cobitis hankugensis–Iksookimia longicorpa*. *Folia Zoologica*, **60**(4), 290–294. doi: 10.25225/fozo.v60.i4.a4.2011

Psenicka, M., Rodina, M. and Linhart, O. (2010). Ultrastructural study on the fertilisation process in sturgeon (*Acipenser*), function of acrosome and prevention of polyspermy. *Animal Reproduction Science*, **117**(1–2), 147–154. doi: 10.1016/j.anireprosci.2009.03.013

Riehl, R. (1980). Micropyle of some Salmonins and Coregonins. *Environmental Biology of Fishes*, **5**(1), 59–66. doi: 10.1007/BF00000950

Riehl, R. and Kock, K.-H. (1989). The surface-structure of Antarctic fish eggs and its use in identifying fish eggs from the Southern Ocean. *Polar Biology*, **9**(3), 197–203. doi: 10.1007/BF00297176

Riehl, R. and Kokoscha, M. (1993). A unique surface pattern and micropylar apparatus in the eggs of *Luciocephalus* sp. (Perciformes, Luciocephalidae). *Journal of Fish Biology*, **43**(4), 617–620. doi: 10.1111/j.1095-8649.1993.tb00444.x

Riehl, R. and Schulte, E. (1977). Scanning electron microscopical investigations of micropyles of selected freshwater teleost fishes. *Archiv für Fischereiwissenschaft*, **28**, 95–107.

Scopece, G., Widmer, A. and Cozzolino, S. (2008). Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. *American Naturalist*, **171**(3), 315–326. doi: 10.1086/527501

Slechtová, V., Bohlen, J. and Perdices, A. (2008). Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): Delimitation of genera, mitochondrial introgression and evolution of sexual dimorphism. *Molecular Phylogenetics and Evolution*, **47**(2), 812–831. doi: 10.1016/j.ympev.2007.12.018

Solis Murgas, L. D., Paulino, M. S., Palhares, P. C., Miliorini, A. B., Alves, E. and Oliveira Felizardo, Vd. (2017). Ultrastructural and morphometric analysis of gametes in Neotropical teleost fishes. *Journal of FisheriesSciences.com*, **11**(1), 56–61. doi: 10.21767/1307-234X.1000109

Yamamoto, K. (1952). Studies on the fertilization of the egg of the flounder. 2. The morphological structure of the micropyle and its behavior in response to sperm-entry. *Cytologia*, **16**(4), 302–306. doi: 10.1508/cytologia.16.302

Yanagimachi, R., Cherr, G., Matsubara, T., Andoh, T., Harumi, T., Vines, C., Pillai, M., Griffin, F., Matsubara, H., Weatherby, T. and Kaneshiro, K. (2013). Sperm attractant in the micropyle region of fish and insect eggs. *Biology of Reproduction*, **88**(2), 47. doi: 10.1095/biolreprod.112.105072

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T., Andoh, T., Vines, C. and Cherr, G. (2017). Chemical and physical guidance of fish spermatozoa into the egg through the micropyle. *Biology of Reproduction*, **96**(4), 780–799. doi: 10.1093/biolre/iox015

Yun, S. W. (2017). Molecular biological study of ploidy level determination and mode of hybridization in the loach *Cobitis hankugensis—Iksookimia longicorpa* complex [Chonbuk National University Doctoral Thesis], 115 pp.