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RESEARCH PAPER

Comparative morphology and morphometry of the micropyle of two Korean rice-fishes, *Oryzias latipes* and *Oryzias sinensis* (Pisces, Adrianichthyidae)

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Abstract. The morphology and morphometry of the micropyle of two Korean rice fishes, *Oryzias latipes* and *O. sinensis*, were investigated by light microscopy and scanning electron microscopy. Obtained from adult gravid females during the spawning season, the full-grown eggs of each species have a single micropyle at the animal pole. For the two species, the micropyles are similar in appearance, but there exist some differences in detail. The egg is larger in *O. latipes* than in *O. sinensis* (1,123.3-1,465.5 µm *vs.* 823.1-1,152.1 µm in diameter), and the outer diameter of a single micropyle for *O. latipes* is nearly two times larger than that of *O. sinensis* (18.5-22.4 µm *vs.* 10.0-12.5 µm). The micropylar diameter to egg diameter ratio (1.6 ± 0.1% *vs.* 1.0 ± 0.1%) is also larger in *O. latipes* than *O. sinensis*. In regard to micropyle structure, the two showed distinguishing characteristics: *O. latipes* has a funnel-shaped micropyle consisting of two regions, an outer gradual pit and an inner narrow canal, while *O. sinensis* has a conical-shaped micropyle having only a deep narrow canal. Consequently, these differences in micropylar structure between the two Korean rice-fishes may be considered as a useful taxonomic characteristic in closely related taxa and seem to be structural adaptations to shape and control entry velocity of spermatozoa into the micropyle.

Key words: micropylar diameter, egg diameter, ratio, structure, adaptation to spermatozoa, taxonomic character

Introduction

The micropyle of teleost fishes is a functional aperture through which spermatozoa enter the egg. It originates from the zona pellucida between late vitellogenic stage I and stage III before hatching (Hart 1990). For successful fertilization, it plays significant roles in: i) passage for sperm penetration to the oocyte, ii) attraction of spermatozoa by pheromones, iii) prevention of polyspermy, and iv) influx of water during formation of the perivitelline space (Riehl 1999). However, the number, diameter, and surface structure of the micropyle are species-specific (Chen et al. 1999, Debus et al. 2002). In number, almost all teleost fishes possess only a single micropyle on the egg envelope, although some species have two or several units (Yao et al. 1995, Lahnsteiner 2003). Iwamatsu et al. (1997) reported that the micropylar diameter (MD) in the Japanese medaka *Oryzias latipes* and the marine medaka *Oryzias melastigma* differ from each other. In particular, Riehl (1980) and Riehl & Kock (1989) classified micropyle structure into four types by surface and proximal

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morphology. Moreover, based on such variation, the micropyle possesses strong criteria that makes it useful for taxonomic classification (Riehl 1993, Breining & Britz 2000).

Belonging to the family Adrianichthyidae, the genus *Oryzias* currently consists of two species, *Oryzias latipes* and the Chinese medaka *O. sinensis*, from South Korea (Kim & Park 2002). These fish commonly show large prominent eyes and very small body size (not exceeding 5 cm in standard length) and prefer the habitat of ponds, marshes, paddy fields, and the near stagnant flow of floodplains (Kim & Park 2002). Due to this similarity, the genus *Oryzias* in South Korea was originally considered a single species, *O. latipes*. With additional evidence, such as distribution, morphology, embryology, and DNA analysis, it was subsequently confirmed that two species coexisted (Kim & Kim 1993, Park et al. 2006). Nevertheless, the identification of the two species has remained unclear due to their very similar morphological characteristics. The structure of the micropyles and the surface pattern of fish eggs play important roles for specific criteria in salmonids and four related species of coregonids (Riehl 1980), as well as other teleosts (Riehl & Schulte 1978, Riehl 1979, 1991). We already investigated the egg surface of two rice fishes and found unique structures to distinguish them (Kim & Park 2014). Therefore, as another method for applying taxonomic classification to the two Korean rice fishes, a study on the micropyle of the egg was performed. We describe and compare the micropyles of two rice fishes, *O. latipes* and *O. sinensis*, using microscopy and statistical analysis.

Material and Methods

Specimen preparation

During the spawning season (July to September 2019), ten adult gravid females each of *O. latipes* and *O. sinensis* (Fig. 1A) were caught using a scoop net (3 × 3 mm in mesh) in a slow-flowing stream of Sodong-ri, Ilun-myeon, Geoje-si, Gyeongsangnam-do, South Korea, 34°49′38″ N, 128°42′36″ E, and in a farming area of Songcheon-dong, Jeonju-si, Jeollabuk-do, South Korea, 35°53′29″ N, 127°6′56″ E, respectively (Fig. 1B). The identification of sexes based on dimorphism followed the research of Kim & Park (2002). Among the fishes collected, some females with spawned egg bundles hanging down just beneath the urogenital pore were immediately anesthetized with MS-222 (Sigma Aldrich Co., St. Louis, USA) and fixed in 2.5% glutaraldehyde solution (GA solution) with 0.1 M phosphate buffer at pH 7.4. The rest were taken to the laboratory and kept in two aerated glass tanks (60 × 30 × 30 cm, 27 ± 0.5 °C, measured three times daily) illuminated with a 20-watt incandescent light bulb. A 15-h

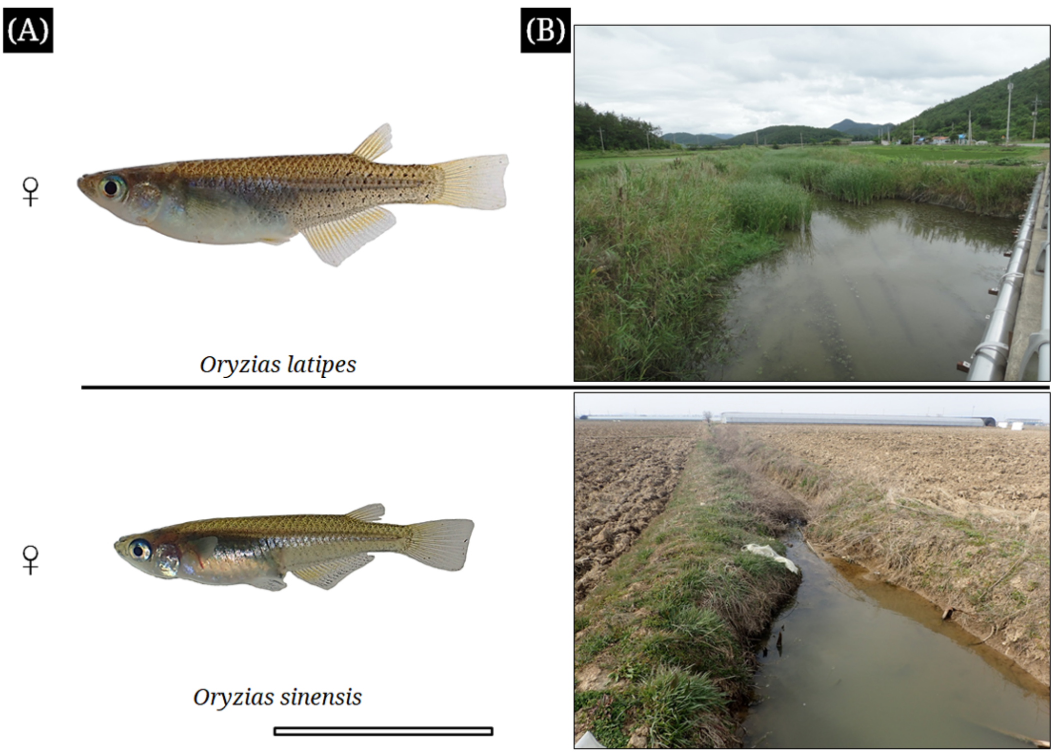

Fig. 1. Photographs of *Oryzias latipes* and *O. sinensis* (A), and their habitats (B). The bar indicates 2 cm.

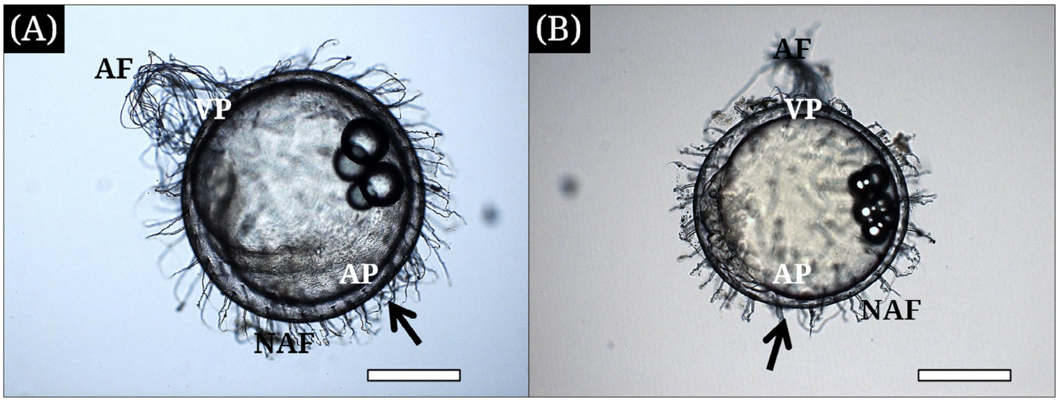

Fig. 2. Photographs of fully developed eggs with numerous filaments in *Oryzias latipes* (A) and *O. sinensis* (B). Arrows indicate position of the micropyle. AF – attaching filaments, AP – animal pole, NAF – non-attaching filaments, VP – vegetal pole. Bars indicate 500 μm.

light/9-h dark cycle was maintained to provide optimum conditions for reproduction. Hormone application was not used to induce sexual maturity of the fishes. Frozen bloodworm (Hikari Sales USA Inc.) were provided as a prey twice each day. All animal procedures strictly obeyed the rules of the Jeonbuk National University Institutional Animal Care and Use Committee.

Microscopic investigation

For scanning electron microscopy (SEM) of the micropyle of both species, 5-10 fully-grown eggs per female fish of *O. latipes* (n = 10) and *O. sinensis* (n = 10) over a month of breeding were collected in the laboratory. To obtain unfertilized eggs, before the eggs dropped to the bottom or attach to submerged vegetation, egg bundles were collected directly from females after release. The eggs obtained from the egg bundles were fixed in 2.5% GA solution for 24 hours, placed in buffered solution (pH 7.4) two times for 30 minutes, fixed in 1% osmium tetroxide solution with 0.1 M phosphate buffer (pH 7.4) for six hours, and placed in buffered solution (pH 7.4). They were dehydrated with a graded series of ethanol treatments (50%, 60%, 70%, 80%, 90%, 95%, 100%) and stored in pure tert-butyl alcohol. They were subsequently freeze-dried by evaporation under vacuum (VFD-21S, Vacuum Device Co., Ltd., Ibaragi, Japan). The dried samples were coated with osmium tetroxide by ion sputtering (HPC-1SW, Vacuum Device Inc., Tokyo, Japan) and observed under a scanning electron microscope (Carl Zeiss, SUPRA40VP, Germany).

Statistical analysis

Using SPSS statistics (SPSS version 18.0, IBM, USA), statistical analysis was conducted for interspecific comparison of egg diameter (ED), MD, and the ratio between the two. Independent two-sample t-test was applied for ED, MD, and MD:ED ratio. A simple-linear regression model was used for intraspecific linear regression between ED and MD.

Results

Morphology

The fully developed eggs from the two rice fishes were spherical in shape, with numerous filaments over the entire surface: egg diameter was 1,123.3-1,465.5 μm in *O. latipes* and 823.1-1,152.1 μm in *O. sinensis* (Fig. 4A). The number of lipid droplets was between four to seven with a diameter of 193.9 ± 36.6 μm (121.5-263.9, n = 20) in *O. latipes* and 140.1 ± 18.8 μm (111.6-178.4, n = 20) in *O. sinensis* (Fig. 2A, B). The eggs of both species have a single micropyle, circular in form from an upper view (Fig. 3A, B). The micropyle is located at the animal pole, covered with only non-attaching filaments, as opposed to the vegetal pole with long attaching filaments (Fig. 2A, B). Like relative egg size, the outer diameter of the micropyle is larger in *O. latipes* than in *O. sinensis* (18.5-22.4 μm *vs.* 10.0-12.5 μm in *O. sinensis*, Fig. 4B). Under SEM, the micropyle of *O. latipes* consists of two regions: an outer pit and an inner canal, whereas *O. sinensis* has only a deep canal without an outer pit (Fig. 3C). The micropyle in *O. latipes* is funnel-shaped and composed of flat or gradual pits with five-six spiral layers and a narrow canal at the proximal centre. Its boundary region is somewhat flat on the surface (Fig. 3A). Meanwhile, *O. sinensis* shows a conical-shaped micropyle, which comprises a deep inner canal with numerous spiral patterns over ten layers. Its boundary region has numerous pore canals and protrudes upward from the surface, higher than that of *O. latipes* (Fig. 3B).

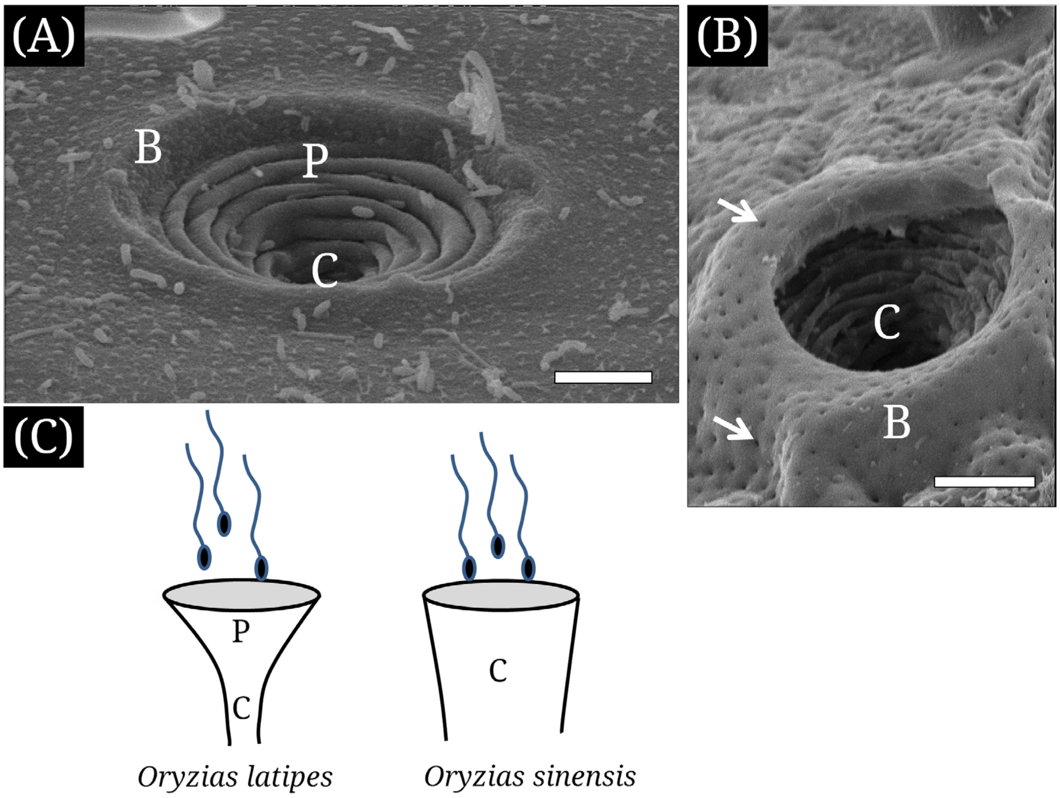

Fig. 3. Scanning electron micrographs showing types of micropyle in *Oryzias latipes* (funnel shape with a pit – A) and *O. sinensis* (conical shape without a pit – B). C is a schematic diagram of different micropyles. Arrow – pore canal, B – boundary part, C – micropyle canal, P – micropyle pit. All bars indicate 5 μm.

Morphometry

The ED (n = 86, mean ± SD: 1,293.5 ± 53.5 μm) of *O. latipes* was larger than that of *O. sinensis* (n = 86, 1,074.3 ± 42.2), and was significantly different (independent two-sample t-test, ED, t = 29.84, df = 170, $P < 0.001$; Fig. 4A). The MD (n = 40, 20.6 ± 0.9 μm) of *O. latipes* was also significantly larger (n = 40, 11.1 ± 0.8 μm) than that of *O. sinensis* (independent two-sample t-test, MD, t = 20.78, df = 78, $P < 0.001$; Fig. 4A). The MD to ED ratio showed a significant difference between *O. latipes* (n = 40, 1.6 ± 0.1%) and *O. sinensis* (n = 40, 1.0 ± 0.1%; independent two-sample t-test, MD, t = 36.44, df = 78, $P < 0.001$; Fig. 4C). The regression coefficient between ED and MD differed significantly from zero in both species: in *O. latipes* (linear regression, df = 1, F = 11.96, $P < 0.05$; Fig. 5A) and *O. sinensis* (df = 1, F = 9.02, $P < 0.05$; Fig. 5B).

Discussion

In almost all teleost fishes the micropyle number is typically one, located at the animal pole of the unfertilized egg. However, there are exceptions with some species possessing multiple micropyles: 1-13 units in *Acipenser stellatus*, more than 33 units in *Huso huso*, 55 units in *Acipenser gueldenstaedti*, 3-15 units in *Acipenser transmontanus* of the family Acipenseridae (Ginsburg & Dettlaff 1991), and 4-12 units in *Polyodon spathul*a of the family Polyodontidae (Linhart & Kudo 1997). The structure of the micropyle is also variable among species: i) a short canal with deep pit, ii) a long canal with flat pit, iii) only a micropyle canal without a pit, and iv) a short canal with two pits, as reported by Riehl & Kock (1989). In our results, the micropyles of *O. latipes* and *O. sinensis* were present on the animal pole of an unfertilized egg and commonly comprised a single circular opening. However, they presented different structures (a flat pit and a long canal in *O. latipes vs.* a deep canal without a pit in *O. sinensis*). These characteristics correspond with type II and type III micropylar categories of Riehl & Kock (1989), respectively. The overall form could be described as a funnel-shaped micropyle in *O. latipes*, as seen in *Oncorhynchus gorbuscha* (Stehr & Hawkes 1979), *Epinephelus malabaricus* (Li et al. 2000), *Dicentrarchus labrax* (Fausto et al. 1994), and *Bostrichthys sinensis* (He et al. 2009). Conversely, a conical-shaped micropyle is evident in *O. sinensis*, as reported in *Siganus rivulatus* and *Mugil cephalus* (Mikodina 1987). In particular, the conical-shaped micropyle of *O. sinensis* is a hole without a pit, which has not been reported previously for the

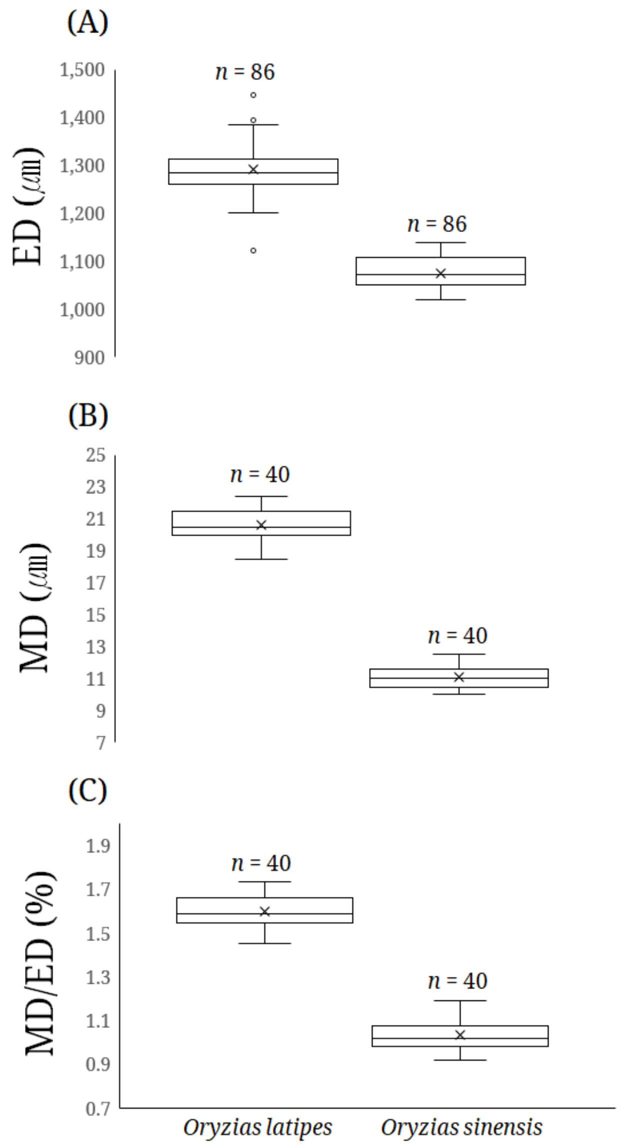

Fig. 4. Interspecific differences of *Oryzias latipes* and *O. sinensis* in egg diameter (A, μm), micropylar diameter (B, μm), micropylar diameter to egg diameter ratio (C, %). ED – egg diameter, MD – micropylar diameter, n – number.

genus *Oryzias*. In contrast, the funnel-shaped micropyle has been confirmed in the Japanese medaka *O. latipes* (Nakashima & Iwamatsu 1989, Iwamatsu 2004). Chen et al. (1999) reported that variable structures of the micropyle can act as an important indicator for identification of the egg as well as species for the taxonomic study of fishes. Riehl (1999) mentioned that the morphology and morphometry of the micropyle are affected by interaction with the spermatozoa. So, the morphological difference in micropyle of two rice-fishes may be considered a structural adaptation to spermatozoa shape or entry style and are a useful taxonomic characteristic. In addition, small pore canals in *O. sinensis* are notable on the surface near the micropyle. No anatomical or histological report of pore canals in the genus *Oryzias* has previously been published. However, it is estimated that the pore canal of teleost fish eggs functions to absorb water into the eggs to decrease the physical impact from the environment (Hosokawa et al. 1981, Groot & Alderdice 1985).

In morphometry, both the ED (1,293.5 ± 53.5 μm *vs.* 1,074.3 ± 42.2 μm) and the MD (20.6 ± 0.9 μm *vs.* 11.1 ± 0.8 μm) of *O. latipes* were larger than those of *O. sinensis*. We additionally compared the MD:ED ratio between the two species, which also proved (1.6 ± 0.1% *vs.* 1.0 ± 0.1%) larger in *O. latipes* than *O. sinensis*. The MD of teleost fishes has been measured at variable sizes: 3.3-4.3 μm in *Oncorhynchus mykiss*, about 17 μm in *Salmo salar*, 14-16 μm in *Salvelinus alpinus*, 2-2.5 μm in *Salvelinus fontinalis*, 15-18 μm in *Coregonus lavaretus*, and 14 μm in *Coregonus nasus* (Riehl 1980). In particular, for the genus *Oryzias*, micropylar size was compared between Japanese medaka (*O. latipes*) and Indian medaka (*O. melastigma*) to confirm that the MD

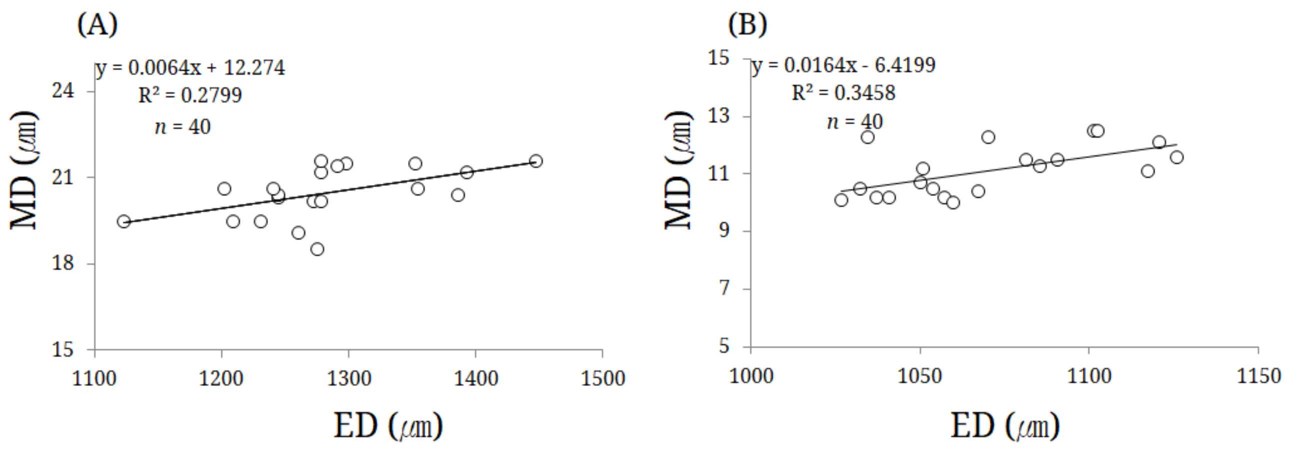

Fig. 5. Dispersion diagrams showing the relationship between egg diameter (x-axis) and micropylar diameter (y-axis) of *Oryzias latipes* (A) and *O. sinensis* (B). n – number.

(20.0 ± 0.6 μm) of *O. latipes* is larger than that of *O. melastigma* (9.8 ± 0.4 μm; Iwamatsu et al. 1997). Iwamatsu et al. (1997) demonstrated that the larger diameter of the micropyle of the Japanese medaka *O. latipes* facilitates a greater swimming velocity and a higher entry velocity of spermatozoa into the micropyle. Therefore, we consider that the wider and larger funnel-shaped MD of *O. latipes* is a morphological adaptation for higher entry velocity of spermatozoa into the micropyle than that of *O. sinensis*. This disparity may reflect an interspecific difference in the fertilization strategies of *O. latipes* and *O. sinensis*.

We used statistical analysis to compare the MD and MD:ED ratio of the two species. This statistical analysis was helpful in assessing interspecific morphological difference among species or individuals that had different body or egg size. We also attempted to observe 5-10 eggs per one female fish of *O. latipes* (n = 10) and *O. sinensis* (n = 10) under SEM. Although there was a total of 86 eggs per species, we measured only 40 eggs each for the two species as a result of failing to find the micropyle at the animal pole in all eggs and by restricting analysis to the best quality samples.

In conclusion, the micropyle of two Korean rice fishes, *Oryzias latipes* and *O. sinensis*, showed clear morphological differences: a funnel-shaped micropyle with a gradual pit and a narrow canal (*O. latipes*) *vs.* a conical-shaped micropyle having only a deep, narrow canal (*O. sinensis*). The egg diameter (ED) and micropylar diameter (MD) in *O. latipes* were larger than those of *O. sinensis*: 1,293.5 ± 53.5 μm *vs.* 1,074.3 ± 42.2 μm in ED; 20.6 ± 0.9 μm *vs.* 11.1 ± 0.8 μm in MD. The MD:ED ratio also was larger in *O. latipes* than in *O. sinensis,* 1.6 ± 0.1% *vs.* 1.0 ± 0.1%. These micropylar differences may be considered useful taxonomic characters to identify closely related species and may represent a functional morphological adaptation relating to the shape and entry velocity of spermatozoa into the micropyle.

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