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Characteristics of rare males in the cobitid unisexual complex, *Cobitis hankugensis-Iksookimia longicorpa*

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Abstract. The *Cobitis hankugensis-Iksookimia longicorpa* (formerly *C. sinensis-C. longicorpus*) complex in South Korea includes diploid and triploid forms of hybrid origin that are most often females. However, there are a few males that show no differences from the hybrid females in their general morphology except the presence of a *lamina circularis* on the pectoral fin. An examination of 354 adult hybrid specimens revealed that 3 % of the complex are male specimens. The testes of the rare males are normal in shape and external morphology. The gonadosomatic index of hybrid males is similar to that observed in males of the parental species and changes during the year in a similar pattern. However, the testicular cells differ from those of the parental species in their morphology. Especially sperm cells are very rare and show abnormalities in their morphology, therefore we conclude that the rare males are sterile. This result suggests that although males are present with a low frequency in the complex, this complex functionally is unisexual and depends on the males of the parental species for perpetuation.

Key words: Cobitidae, reproduction, gynogenesis, cell morphology

Introduction

Natural populations of unisexual species (all or almost all female) are rare in vertebrate species; their modes of reproduction are gynogenesis and hybridogenesis (Cimino 1971, Dawley 1989, Goddard & Schultz 1993). Such unisexual populations have been reported in about 90 biotypes; in most cases they are of hybrid origin and polyploid (Dawley 1989, Vrijenhoek et al. 1989, Vasil'ev et al. 2005, Janko et al. 2007). Males in unisexual fish species are usually rare and sterile (Dawley 1989, Bohlen & Ritterbusch 2000, Lamatsch et al. 2002, Juchno & Boroń 2006), but there are some unisexual fish with fertile testes in the *Cobitis* complex described by Vasil'ev et al. (1989) and in the *Iberocypris alburnoides* complex (Alves et al. 1999, 2001).

The *Cobitis hankugensis-Iksookimia longicorpa* (formerly reported as *C. sinensis-C. longicorpus*) complex in South Korea has been shown to be a unisexual lineage originating from a hybridization event between *C. hankugensis* and *I. longicorpa* (Kim

& Lee 1990, 2000). They are diploid or triploid and the females reproduce by gynogenesis and spawn with males of both bisexual parental species, *C. hankugensis* and *I. longicorpa* (Kim & Lee 1990, 2000; Kenji et al. 2004). Although natural males occur rarely in wild populations of the complex, there is little information about their testes. Therefore, this study reviewed the morphology of the testes of naturally occurring males within the *C. hankugensis-I. longicorpa* complex.

Material and Methods

Monthly field observations of the *C. hankugensis-I. longicorpa* complex and two bisexual species, *C. hankugensis* and *I. longicorpa*, were performed from 1994 to 1995 and 2005 to 2007 at the Stream Ram of the River Nakdong, South Korea, using a cast net (5 × 5 mm) and seine net (5 × 5 mm). To investigate sex ratios, the males were identified by the presence of a *lamina circularis* on the base of the pectoral fin (Kim & Lee 1990, 2000). To evaluate the

sexual maturity of testes, the gonadosomatic index, $GSI = [\text{total weight of the gonad} / \text{total weight of the intact fish}] \times 100$, was used.

For light microscope examination (LM), the testes of males were fixed in 10 % neutral buffered formaldehyde followed by embedding in paraffin. For scanning electron microscope examination (SEM), the samples were fixed in 2.5 % glutaraldehyde in

in the same buffer. After dehydration in a graded alcohol series, fragments were embedded in Epon 812. The testes were observed with a JEOL JSM-T330A scanning electron microscope and a JEOL-1200EX transmission electron microscope. For photographs and evaluations of spermatogenic cells, Carl Zeiss vision was used (LE REL. 4.4, Germany).

Results and Discussion

Sexual dimorphism and sex ratio

In general, the hybrid fishes have an appearance intermediate between their parental species (Fig. 1). The hybrid males are essentially similar to their female counterparts in colour patterns and grow to a similar size (approximately 7-10 cm SL). Their pectoral fin bases have a well-developed *lamina circularis*, which occurs as a secondary sexual characteristic in both *C. hankugensis* and *I. longicorpa* males (Fig. 2). Monthly observations from 1994 to 1995 and 2005 to 2006 in fields revealed 136 males and 117 females of *C. hankugensis*, 102 males and 136 females of *I. longicorpa* and 10 males and 344 females of the *C. hankugensis*-*I. longicorpa* complex. While the sex ratio of the two species is within the normal range for

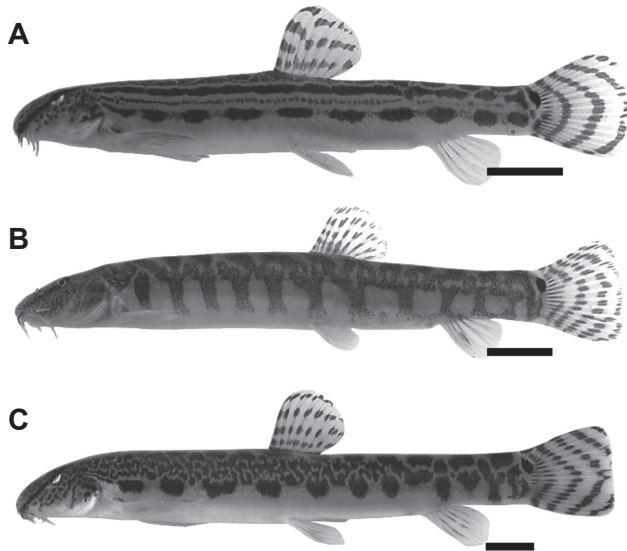


Fig. 1. The lateral color pattern of cobitid fish in the River Nakdong, Korea. A: *Cobitis hankugensis*; B: *Iksookimia longicorpa*; C: *C. hankugensis*-*I. longicorpa* complex. Bars indicate 10 mm.

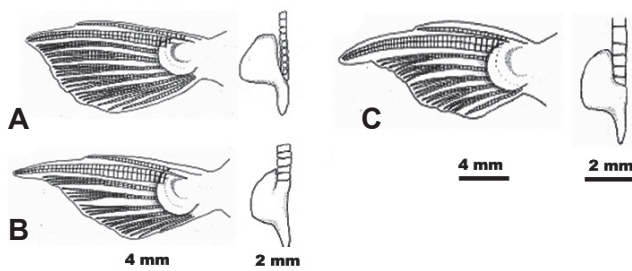


Fig. 2. Diagram showing lamina circularis formed on the base of a male's pectoral fin. A: *Cobitis hankugensis*; B: *Iksookimia longicorpa*; C: *C. hankugensis*-*I. longicorpa* complex.

0.1 m phosphate buffer at pH 7.2 and dehydrated in a graded alcohol series and critical point dried in CO_2 . The dried samples were coated with gold-palladium. For transmission electron microscope examination (TEM), the fragments were fixed in 2.5 % glutaraldehyde in a 0.1 m phosphate buffer at pH 7.2. Postfixation was performed in 1 % osmium tetroxide

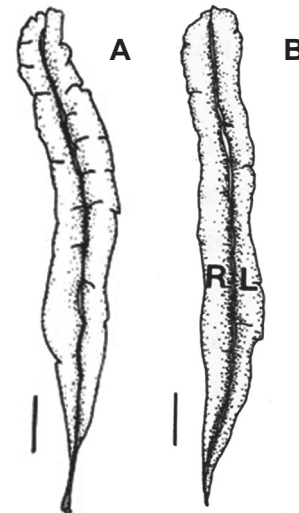


Fig. 3. Diagram showing a pair of testes in the spawning season: A: *Cobitis* and *Iksookimia*; B: *C. hankugensis*-*I. longicorpa* complex. R, right lobe; L, left lobe. Bars indicate 3 mm.

cobitid loaches (Bohlen & Ritterbusch 2000, Bohlen et al. 2008), it appears that the complex has an extremely female-biased sex ratio, with the ratio of females to males ranging between 1 : 0.03-1 : 0.04. In other cobitid diploid-polyploid complexes, gynogenetic forms are virtually all female or include females and males in a 2.3 : 1 ratio (Vasil'ev et al. 1989).

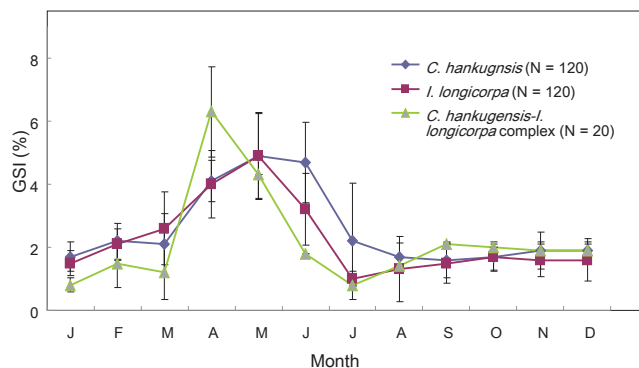


Fig. 4. Comparison of gonadosomatic indices of males of the *C. hankugensis*-*I. longicarpa* complex and those of the two parental bisexual species in 2005.

Testes morphology and gonadosomatic index (I_G)

The gonads of the complex males are a pair of elongated structures and are milky white in colour (Fig. 3), being morphologically very similar to the testes of both parental species, as reported by Park & Kim (1996). The gonadosomatic index (I_G) of the complex male testes was higher from April to May with a value that ranged from 1.8-6.3; this time period is thought to correspond to the spawning season (Fig. 4). Fish of both male parental types caught at the same time showed similarly high I_G values: 3.2-4.2 for *I. longicarpa* males and 4.1-4.9 for *C. hankugensis* males (April-June). During the non-spawning period, the I_G values were lower in the complex males ($I_G = 0.8$ -2.0), in the *I. longicarpa* males ($I_G = 1.0$ -2.6) and *C. hankugensis* males ($I_G = 1.5$ -2.2). These results indicate that the testes of the complex males are comparable to those of both parental male species in terms of shape, size and I_G values.

Testes cell morphology

Microscopic examination of the testes of the complex males, however, indicates that they are sterile. Although normal spermatogenesis is likely to occur, the architecture of the testes is abnormal (Figs. 5A, 5B). The seminiferous tubules are not located centrally and are scattered, and a relatively large number of tubules contain cysts with a large number of spermatocytes and/or a small number of spermatids (Figs. 5A, 5B). Many vacant spaces are present in the interior of the seminiferous tubules. The spermatocytes and spermatids are deformed, variously size and some cells appear to have collapsed. The nuclei of the cells showed a range of over staining with hematoxylin in an irregular shape. However, unlike these abnormal testes, those of the bisexual species showed normal structures, with a typical restricted lobular structure

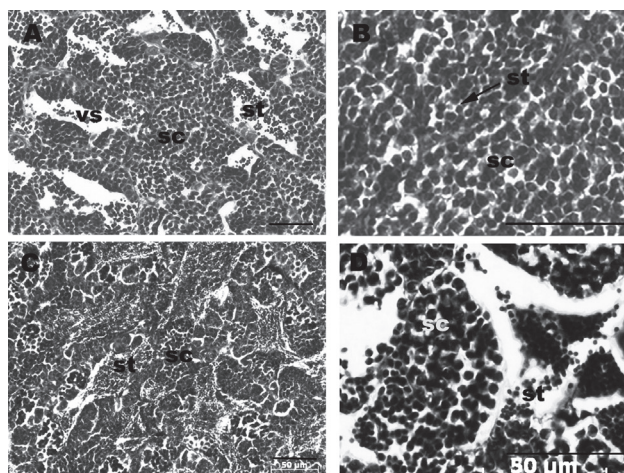


Fig. 5. Cross section of spermatogenic cells for abnormal (A-B) and normal testes (C-D) during spawning season stained with Ehrlich hematoxylin and eosin. A-B: *C. hankugensis*-*I. longicarpa* complex. Numerous vacant spaces (vs) are seen (A), and the deformed spermatocytes (sc) and spermatids (st) have nuclei with a range of hematoxylin staining intensity and irregular shape (B). Bars indicate 20 μ m (A) and 50 μ m (B). C-D: *C. hankugensis*. The testis is showing regular and well-developed lobular structures having normal spermatocytes (sc) and spermatids (st). Bars indicate 50 μ m.

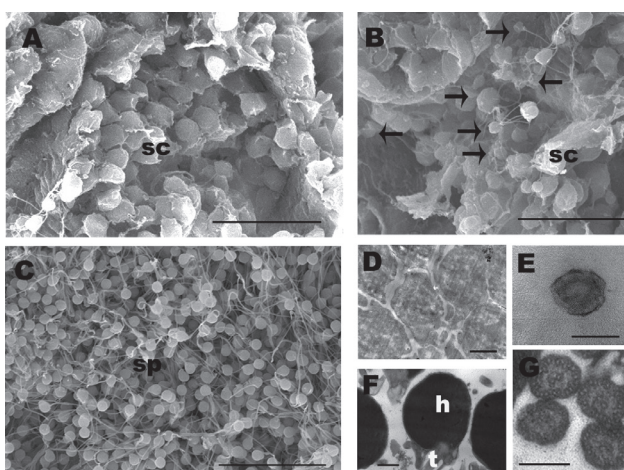


Fig. 6. Scanning and transmission electron microscopy during spawning season. *C. hankugensis*-*I. longicarpa* complex showed abnormal testicular cells (A, B, D and E): swollen spermatocytes (sc) (A), a few deformed sperm cells (arrows) (B), the deviations from the normal 9 + 2 structure in the axonemes (D), and malformation or excessive formation of the sperm head (E). Whereas in *C. hankugensis*, the testes are fully filled with sperm (sp) (C) with normal heads (h), tails (F), and a 9 + 2 structure (G). Bars indicate 20 μ m (A-C), 2 μ m (D), 0.5 μ m (F) and 200 nm (E and G).

Table 1. Comparison of spermatogenic cells in the testes of *Cobitis hagukensis*-*Iksookimia longicorpa* complex and the testes of its two parental bisexual species.

Developmental stage of testis Species	Diameter of spermatogenic cell Average (μm) ± SD		
	Spermatocytes	Spermatids	Head of sperm
<i>C. hagukensis</i> - <i>I. longicorpa</i> complex	4.8 ± 0.9	2.8 ± 0.6	3.9 ± 1.1
<i>C. hangukensis</i>	3.4 ± 0.3	1.5 ± 0.2	2.0 ± 0.2
<i>I. longicorpa</i>	2.6 ± 0.3	1.6 ± 0.2	1.7 ± 0.1

carrying out normal development (Figs. 5C, 5D), as suggested by Parenti & Grier (2004).

The diameters of the spermatocytes and the spermatids were about 1.4-1.8 times and 1.8-2.2 times larger than those of the parental species, respectively (Table 1). The complex male testes contained only a very small number of spermatozoa. Even in metamorphosed spermatozoa, morphological abnormalities, such as malformation or excessive formation of the head, were commonly seen (Figs. 6A, 6B and 6D). The diameters of the complex sperm nuclei were about 1.9 to 2.3 times larger than those of the parental bisexual species. Although many sperm cells appeared to have normal flagella, various deviations from the normal 9 + 2 structure were observed, as were abnormalities

in the arrangement of the outer double microtubules of axonemes (Figs. 6D, 6E). In contrast, the testes of their parental bisexual species during spawning season were fully filled with normally metamorphosed sperm with normal heads and tails with a 9 + 2 structure (Figs. 6C, 6F and 6G).

These abnormal structures of the testes suggest that the males are sterile, as is the case with abnormal testes of other sterile fishes (Kawamura et al. 1999, Tiwary et al. 2000). Also in other unisexual complexes, the rare males have been found to be sterile (Cimino 1971, Vasil'ev et al. 1989, Goddard & Schultz 1993). Our results confirm that the *C. hankugensis*-*I. longicorpa* complex is functionally a unisexual population.

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