

Reproductive Isolation between *Moroco oxycephalus* and *M. lagowskii* (Pisces; Cyprinidae) in Korea

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Sibling species

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Haldane's rule

To clarify taxonomic status of the two sibling species, *Moroco oxycephalus* and *M. lagowskii* reproductive isolation mechanisms were investigated at sympatric area located in Kansung-up, Kosung-gun, Kangwon-do, Korea. Genetic analysis was performed to reveal mating system and intensity of hybridization between the two species. The frequencies of hybrids were increased since 1989, and then the observed hybrid frequencies (H_o) did not significantly differ from the expected hybrid (H_E) in 1998 and 1999. However, based on histological analysis of two parents' and their hybrid's gonads, the hybridizations between *M. oxycephalus* and *M. lagowskii* produced mostly fertile females but sterile males in accordance with Haldane's rule. Although it was suspected that pre- and postmating isolation mechanisms were affected between the two species, *M. oxycephalus* and *M. lagowskii* seemed to be strongly isolated with microhabitat at sympatry until 1997. Since 1998, hybrid frequencies were increased by habitat disturbance. However, their hybrid frequencies would be reduced by postmating isolation mechanisms. Therefore, the two species are considered to be distinct species recently diverged.

Many species are divided into a mosaic of genetically distinct populations, separated by narrow zones of hybridization where these populations meet, mate and produce hybrids. Hybrid zones are natural laboratories that can tell us about the nature and effects of differences among incipient species, and about how selection at different levels affects the spread of alternative adaptations (Barton and Hewitt, 1989). For this reason, hybrid zones have long been the subject of evolutionary research as such study would elucidate mechanisms of speciation (Kocher and Sage, 1986). Speciation is the evolution of premating or postmating isolation preventing genetic exchange between populations (Dobzhansky, 1940), and the mechanisms that isolate one species reproductively from others are the most important set of attributes a species has (Mayr, 1963). As a biological species is defined as an "actually or potentially interbreeding group of populations" (Mayr, 1963), any taxa that can produce viable and fertile F_2 generations or backcrosses are regarded as being of the same species.

The Chinese minnow, *Moroco oxycephalus*, and Amur minnow, *M. lagowskii* (Cyprinidae: Pisces) are freshwater fishes which are distributed in Korea, Japan and China (Qingtai and Baoshan, 1987; Kawanabe and

Mizuno, 1989; Kim, 1997). Not only are these two species morphologically very similar, but also each of them exhibits a wide range of variation in meristics (Min and Yang, 1986; Jeon, 1989). However, the meristic character of the number of scale above lateral line (SAL) was the best single criterion for species identification (Min and Yang, 1986), and the two species were discriminated with genetic markers by the two species isozyme electrophoretic analysis (Yang and Min, 1988; Min and Yang, 1992). They differ in geographical distributions (Min and Yang, 1986), and the sympatric area of *M. oxycephalus* and *M. lagowskii* was discovered in Kosung-gun, Kangwon-do, Korea (Kang, 1987). Because isozymes are highly useful in the identification of hybrids as clearly as their parental species especially at sympatric area, the genetic markers were used at sympatry to discern *M. oxycephalus* from *M. lagowskii*, and to detect their hybrids (Yang and Min, 1988; Min and Yang, 1992). There were no hybrids at sympatric area in 1987 (Yang and Min, 1988), and 11.8% hybrids in 1989 (Min and Yang, 1992). However, the death rate of young hybrids was higher than that of young parental species and the frequencies of the after generations of F_2 backcrosses were very low (Min and Yang, 1992). Furthermore, the diagnostic character, SAL counts, between the two species were distinctly discriminated at sympatry in comparison with other allopatric area, and this phenomenon was regarded as a character displacement (Yang and Min, 1988; Min and Yang, 1992). Therefore, it was assumed

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Table 1. Four collection sites, dates and sample sizes of specimens for hybrid analysis at Kansung-up, Kosong-gun, Kangwon-do, Korea

Collection site	Collection date	No. of specimens
Bridge of Chinbu (Chinbu-ri)	Apr. 7, 1998	28
	June 15, 1998	98
Bridge of Chechu (Chinbu-ri)	July 28, 1997	329
	June 15, 1998	225
	Apr. 19, 1999	56
Changshin Resort (Changshin-ri)	May 1, 1998	24
	May 18, 1998	15
	May 31, 1998	18
	June 15, 1998	16
	July 8, 1998	28
Bridge of Changshin-5 (Changshin-ri)	May 1, 1998	29
	June 15, 1998	14
	July 8, 1998	18
	July 30, 1998	19
	Sept. 4, 1998	19

that *M. oxycephalus* and *M. lagowskii* were reproductively isolated by a premating isolation mechanism as well as a postmating isolation mechanism (Yang and Min, 1988; Min and Yang, 1992).

This study was designed to determine sterilities of the hybrids based on histological examinations of the testes and the ovaries of randomly selected hybrids. Besides, the extent of hybrid distributions, and the frequency changes of hybrids as well as parental species were surveyed from 1997 to 1999 using isozyme electrophoretic analyses. Finally, the speciation modes of *M. oxycephalus* and *M. lagowskii* were suggested.

Materials and Methods

Study specimens were collected using fish-pots and fish traps at 4 sites on sympatric area, Kusong-gun, Kangwon-do, Korea from 1997 to 1999 (Table 1). All fish specimens were transported on dry ice (-70°C) to the lab. To discern *M. oxycephalus*, *M. lagowskii*, and their hybrids, isozyme electrophoresis was performed. Genetic markers such as enzyme-X (on esterase), general protein (*Gp*) quoted from Min and Yang (1992), and creatine kinase (*Ck*) were used (Table 2). Voucher samples were fixed in 10% formalin, and preserved in 70% ethanol. They were deposited at the Lab. of Evolutionary Genetics, Dept. of Biology, Inha University.

Randomly selected ovaries and testes of *M. oxycephalus*, *M. lagowskii*, and hybrids (Table 3) were used in histological analysis. The gonads collected in April 1999 especially, were in fully mature condition (Kang and Min, 1999). For light microscopic observations, the

Table 2. Buffer systems and stains for electrophoresis

Buffer system	Enzyme	Volts & Time
Continuous Tris citrate II (T.C. II), pH 8.0	Creatine kinase (<i>Ck</i>)	100V 3½ hrs
Lithium hydroxide (LiOH), pH 8.1	Esterase (<i>E-X</i>) General protein (<i>Gp</i>)	250V 3½ hrs

Table 3. Number of *Moroco* specimens used for histological analysis collected at bridge of Chechu, Kansung-up, Kosong-gun, Kangwon-do

Collection date	Sex	<i>M. oxycephalus</i>	Hybrid	<i>M. lagowskii</i>
July 28, 1997	Female	3	6	5
	Male	2	11	2
June 15, 1998	Female	2	14	3
	Male	3	6	2
Apr. 19, 1999	Female	2	7	2
	Male	2	6	2

gonads were preserved in Bouin's fixative (Bouin, 1897), embedded in paraffin after dehydration through an ethanol series, sectioned and stained with Harris' hematoxylin and eosin.

Results

Range of hybrid distribution and frequency changes of hybrids

M. oxycephalus and *M. lagowskii* meet in a narrow zone expanded about 20-25 km along the mountain stream at Kansung-up, Kosong-gun, Kangwon-do and hybridize. According to the isozyme analysis on specimens collected from 1997 to 1999 (Table 4), the frequencies of hybrids were the highest at Br. Chechu (33.3%) (Fig. 1). At Br. Chinbu in the direction of Inje-gun about 6.8 km away from the southern upstream region, there were 14.3% hybrids. Moreover, 9.9% hybrids were found at the stream of Changshin resort about 10 km away from Br. Chechu, and 2.0% at Br. Changshin-5. However, *M. lagowskii* was hardly found at Br. Chinbu, and *M. oxycephalus* was rare at the regions of Changshin resort and Br. Changshin-5. *M. lagowskii* was 0.8% at Br. Chinbu and *M. oxycephalus* 1.0% at Changshin resort and 0.0% at Br. Changshin-5 (Fig. 1).

At the most flourishing hybrid zone, Br. Chechu, the frequencies were gradually increased since 1989. There

Table 4. Number of specimens of two parental species of *Moroco* and their hybrids at sympatry from 1997 to 1999

Collection site	Collection date	<i>M. oxycephalus</i>	Hybrid	<i>M. lagowskii</i>	Total
Br. Chinbu	Apr. 7, 1998	16	12	-	28
	June 15, 1998	91	6	1	98
Br. Chechu	July 28, 1997	121	82	126	329
	June 15, 1998	45	99	81	225
	Apr. 19, 1999	21	22	13	56
Changshin Res.	May 1, 1998	-	4	20	24
	May 18, 1998	-	2	13	15
	May 31, 1998	1	3	14	18
	June 15, 1998	-	-	16	16
	July 8, 1998	-	1	27	28
Br. Changshin-5	May 1, 1998	-	1	28	29
	June 15, 1998	-	1	13	14
	July 8, 1998	-	-	18	18
	July 30, 1998	-	-	19	19
	Sept. 4, 1998	-	-	19	19

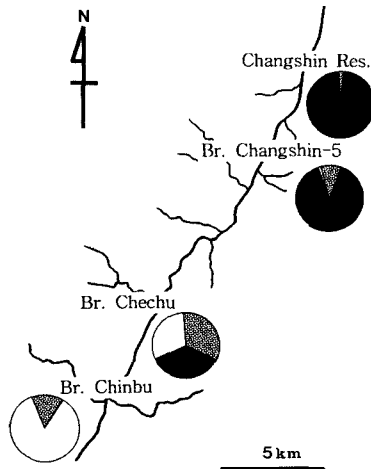


Fig. 1. Schematic view of hybrid zone. Pie diagrams illustrate the relative proportion of *M. oxycephalus* (□), *M. lagowskii* (■), and their hybrids (▨) at four sites from 1997 to 1999.

There were 11.7% hybrids in 1989 (Min and Yang, 1992), and 24.9%, 44.0% and 39.3% in 1997, 1998 and 1999, respectively (Table 4, Fig. 2). Collected sample sizes were yearly diminished at sympatric area. A total of 883 individuals was collected in 1989 (Min and Yang, 1992), but 56 individuals in 1999 (Table 4). On the other hand, the frequency of *M. lagowskii* was increased from 13.3% in 1989 to 23.2% in 1999. The frequency of *M. oxycephalus* was decreased from 75.0% in 1989 to 37.5% in 1999 (Fig. 2).

Hybrid abnormalities in histological analysis

The architecture of the hybrid gonads was almost normal. They had cone-shaped genital pores and normal gonad features like their parents'. During their ripe phase in mid April, the ovaries of the hybrids were expanded and filled with yellowish ripe eggs, and the testes of the hybrids were milky white and extended in size.

In histological analysis, the testes of the parental species had testicular germ cells and the cysts at testicular lobules were filled with spermatids and spermatozoa in April 1999 (Fig. 3A). However, the hybrids

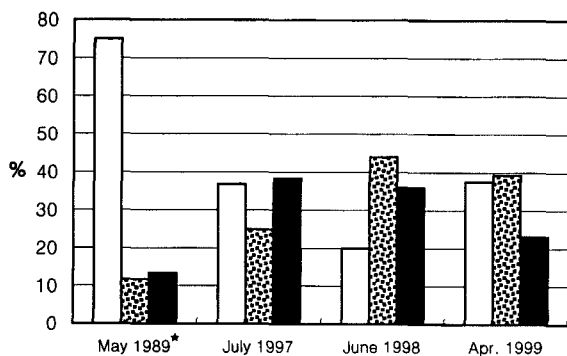


Fig. 2. Temporal changes of hybrid frequencies at Br. Chechu from 1989 to 1999. □: *M. oxycephalus*, ▨: hybrid, ■: *M. lagowskii*.

had dysgenic testes in histological observations. Some hybrid males had few or no germ cells, and instead, only filled with interstitial concretions and connective tissues (Fig. 3H). Few of these concretion tissues were found in the testes of *M. oxycephalus* and *M. lagowskii*. These were observed as a reddish round crystals stained with eosin. Another hybrid testes had spermatid germ cells, but no typical spermatids or spermatozoa were present in these sections (Figs. 3B, C). Most of the spermatid cells had hardly developed, or they had developed until the cell levels of secondary spermatocytes or spermatids (Figs. 3B, C). However, these cells were aggregated into an irregular mass (Fig. 3D), then the nucleus became vacuolated and degenerated (Figs. 3E, F, G). These degenerated nuclei were dark-violet in color when stained with hematoxylin.

On the contrary, most of the female hybrids seemed to be normal in ovary structures and histological observations of oogenesis (Figs. 4C, D, E, F). Hybrid ovaries were in the process of oogenesis like parental species. When fully matured, the hybrid females had ripe eggs (Fig. 4F). However, one out of the detected hybrid ovaries showed abnormal oogenesis. This ovary had a few oocyte and was relatively empty compared to the other hybrid female individuals (Figs. 4G, H).

Discussion

The interbreeding of closely related sympatric species of animals is usually prevented by a whole series of seasonal and habitat isolation, ethological isolation, and mechanical isolation (Mayr, 1963). These are evolved in response to selection against gametic wastage (Dobzhansky, 1940), and these efforts are classified as a premating isolation mechanism. However, when reproductive isolating mechanisms are destroyed by ecological habitat disturbance, hybridizations occur. In this investigation, the frequencies of *M. lagowskii* were increased, and *M. oxycephalus* were relatively decreased since 1989, and the hybrid frequencies were increased steadily (Fig. 2). Although there can be many factors for the increased hybridization, the main reason was suspected to be habitat disturbances involved at the sympatric area. Environmental disruption caused by rockslide restoration works, and various kinds of constructions around the sympatric area in Kosung-gun could induce habitat changes and provide unstable environmental pressures. The bridge of Chechu was under construction in 1997. This environmental transformation and destruction seemed to disturb their micro-habitat segregations between the two species. In fact, from the results of χ^2 test, the observed hybrid frequencies (H_o) did not significantly differ from the expected hybrid frequencies (H_E) in accordance with Hardy-Weinberg's rule (Table 5). *M. oxycephalus* and *M. lagowskii* segregated their own habitat at sympatric regions, and *M. oxycephalus* dominated at more upstream, Br. Chinbu, and *M. lagowskii* at more down-

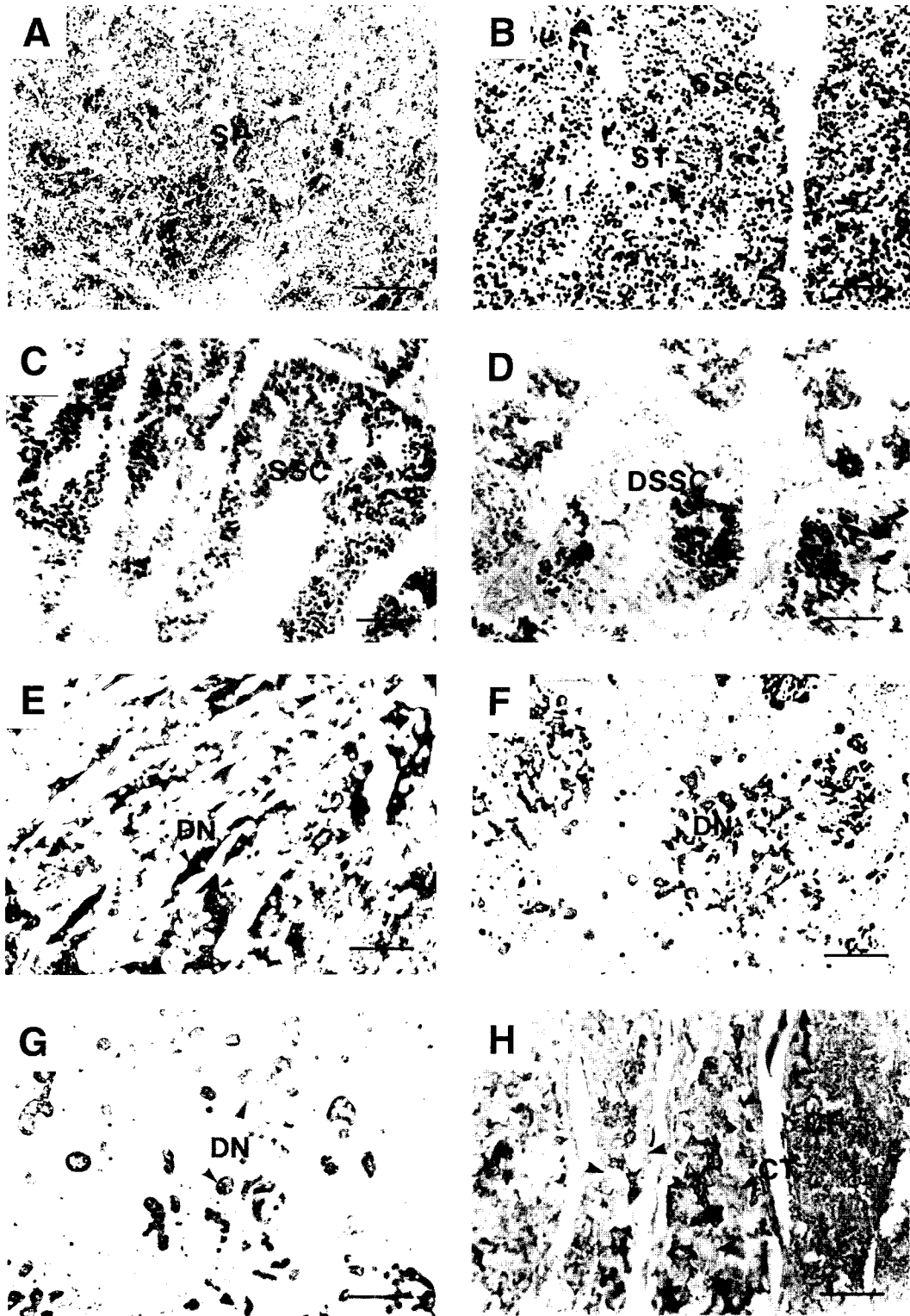


Fig. 3. Photomicrographs of testes of *Moroco oxycephalus*, *M. lagowskii* and their hybrids. A, Cross section of testicular lobules of *M. oxycephalus* and *M. lagowskii* filled with sperms (SP) at fully matured condition in April. B, C, D, E, F, G, H, Sections of testes of hybrids. B, A number of secondary spermatocytes (SSC) and a few spermatids (ST: arrowhead) within lobules. C, Section of hybrid testis filled with secondary spermatocytes (SSC). Note this testis was seen in April. D, Degenerating of secondary spermatocytes (DSSC) within a testicular lobe (arrowhead). E, F, G, Degenerated nucleus (DN) within hybrid testes. H, Testicular lumen filled with only connective fluid (CF) and concretion tissues (CT). Note the round-shaped concretion tissues (arrowheads). Scale bars=25 μ m.

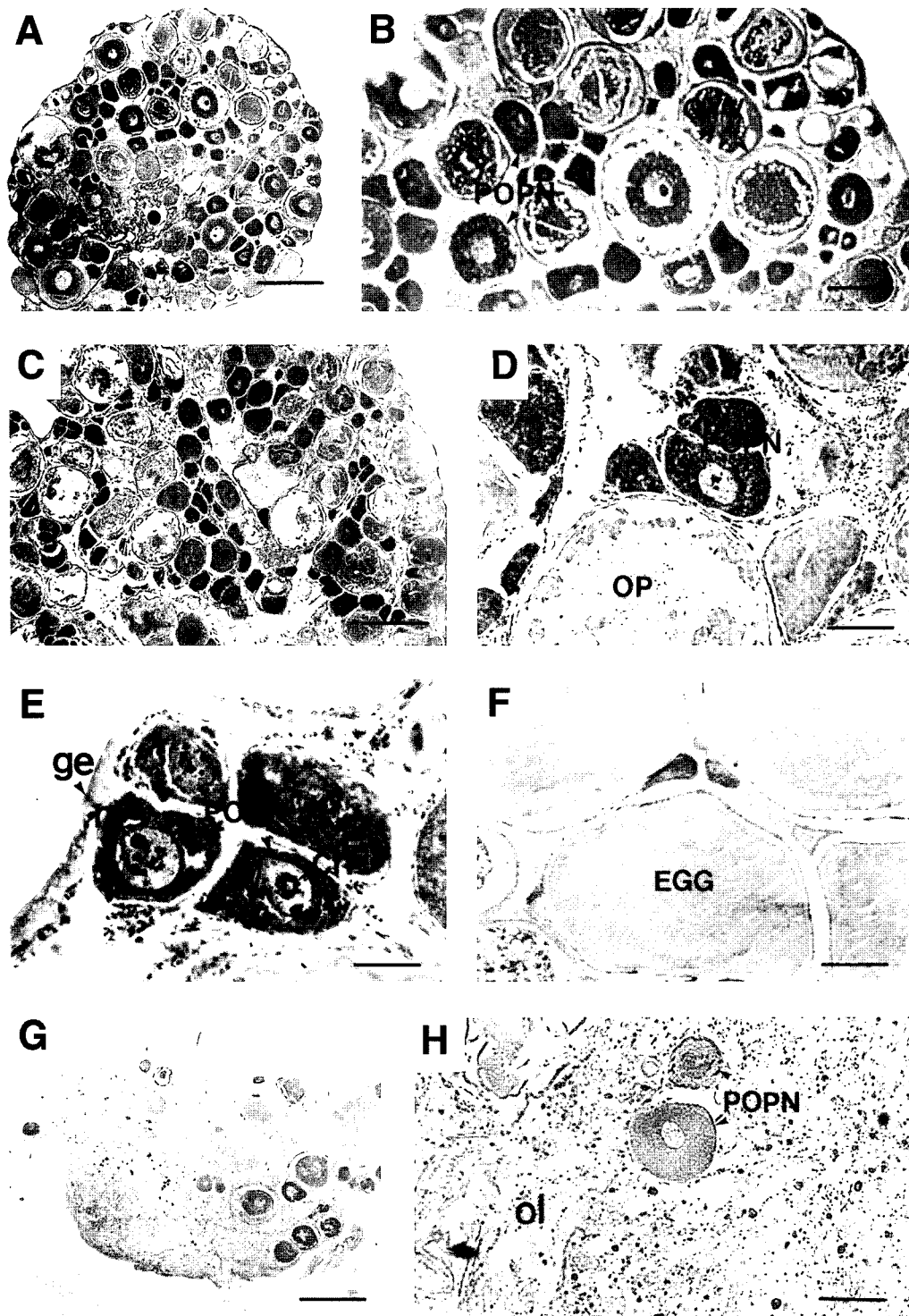


Fig. 4. Photomicrographs of ovaries of *Moroco oxycephalus*, *M. lagowskii* and their hybrids. A, Ovarian cross section of *M. oxycephalus* and *M. lagowskii* filled with oocytes in various developing stages. B, Enlarged view of A, having oocytes in yolk vesicle stage (QV) and primary oocytes in peri-nucleolus stage (POP). C, D, E, Sections of hybrid ovaries. Note these hybrid ovaries are normally developed like their parents'. C, Hybrid ovary filled with various oocytes. D, Same view of C, showing primary oocytes in peri-nucleolus stage (POP) and oocytes in pre-maturation stage (OP). E, Hybrid ovary showing primary oocytes in chromatin-nucleolus stage (POCN). Note the cells located nearby germinal epithelium (ge). Each has a nucleolus (n: arrowhead) and thread-like chromatin (ch: arrowhead). F, Eggs at fully matured condition of *M. oxycephalus*, *M. lagowskii* and their hybrids. G, H, A hybrid ovary showing abnormality. G, Cross section of abnormal ovary. H, Enlarged view of G. Note the poorly developed oocytes and empty ovarian lumen (ol). Scale bars=17 μ m (E), 25 μ m (D), 50 μ m (H), 100 μ m (B, F), and 200 μ m (A, C, G).

Table 5. Chi-square test for the population at sympatric area from 1989 to 1999

Year	H _E	H _O	χ^2
1989	0.30	0.12	341***
1997	0.50	0.25	82.7***
1998	0.49	0.44	2.11 ^{NS}
1999	0.49	0.39	2.20 ^{NS}

Significance levels for chi-square tests, H₀=*M. oxycephalus* : Hybrid : *M. lagowskii*=1:2:1; ***=P < 0.001, ^{NS}=non-significance, $\chi^2 = \sum [(observed - expected)^2 / expected]$, df=k-1, k is the number of parental species.

stream, around Br. Changshin-5 and Changshin resort. These habitat preferences are concordant with the reports of Itai (1976, 1977) that both of the two species are distributed from mid to upstream regions. However, *M. oxycephalus* dominated at upstream and *M. lagowskii* is at more downstream. Furthermore, the spawning periods of the two species are between mid April and mid May (Kang and Min, 1999) and the non-seasonal isolation of spawning would be contributed as a synergy effect of hybridization with other factors as well. In addition, interspecific competitions seemed to be involved in the frequency changes of two species. In the past, *M. lagowskii* population dominated at Changshin-ri according to the personal observation of the last author (S. Y. Yang). However, lately, dark chub (*Zacco temminckii* B type: Yang and Min, 1987) population was more prevalent there. These phenomena were considered to be a result of interspecific competition on habitat, and the defeated *M. lagowskii* population migrated into the sympatric area. Such forced crowding of fish on a limited spawning ground must have been one of the causes yielding many natural hybrids (Hubbs, 1955).

However, if this ecological or ethological set of barriers fail or become weak, a second set of barriers may prevent successful hybridization, at the cost of gametic wastage. The potential mates complete copulation but no offspring are produced, or the offspring have reduced viability or fertility (Mayr, 1963). Because ecological and ethological inferiority in hybrids reduces their chances of leaving offspring, they play a role as a postmating isolating mechanism. Haldane (1922) produced the empirical declaration that "when in the F₁ offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex." In birds the female is the heterozygous or digametic sex, and consequently the male hybrid is more likely to be fertile (Fitzpatrick, 1951; Swan, 1985). However, hybrid males of silver fox and blue fox had meiotic arrest in their spermatogenesis (Gustavsson et al., 1988). Exceptions to the fertility aspect of Haldane's rule have indeed been rare (Kulathinal and Singh, 1998). However, in *Oryzias latipes* × *O. curvinotus*, both male and female hybrids are sterile (Hamaguchi and Sakaizumi, 1992). In histological analysis of hybrids between *M. lagowskii* and *M. oxycephalus*, all of hybrid males were sterile. However, whereas the hybrid females

were mostly fertile. From the histologically, some of hybrid testes showed pre-spermatogenetic abnormalities. Testicular lobules of these hybrids had few or no germ cells only filled with concretions and connective tissues. The others had post-spermatogenetic abnormalities, and their testicular germ cells have developed into spermatids, but degenerated during spermiogenesis after the meiotic processes. Hybrid sterility is a part of syndrome termed hybrid dysgenesis, the underlying cause of which is unknown, although many possible mechanisms have been proposed (Swan, 1985). Such sterility seems to be found more frequently in males than in females (Gray, 1971). This difference has been attributed to chromosomal constellations formed during the first meiotic division, the male being the heterozygous sex (Haldane, 1922). Differences between stages at which abnormalities appear probably result from differences in the causes of sterility. Swan and Cristidis (1987) defined three forms of hybrid sterility according to the possible causes of breakdown of spermatogenesis. The first, meiotic arrest at the prophase stage of first meiosis (abnormal chromosomal behavior: Gustavsson et al., 1988); the second, degeneration of spermatogenic cells mediated by a defective blood-testis barrier (auto immune process: Berg, 1984; Swan, 1985), and the third, differences between genes of parental species (Liming and Pathak, 1981). On the other hand, the abnormal spermiogenesis on male hybrids of *Oryzias latipes* × *O. curvinotus* seemed to be caused by irregular cytoskeletal systems (Hamaguchi and Sakaizumi, 1992). Though the causes of dysgenesis on hybrid males between *M. oxycephalus* and *M. lagowskii* were suspected to be similar with those examples, more cytological investigations are required.

In cases of fertile hybrids, they may be less well adjusted to the existing ecological niches, and, where definite behavior patterns and species-specific stimuli play an important role, the hybrids are usually less successful in courtship than individuals of pure species (Mayr, 1963). For example, stickleback (*Gasterosteus aculeatus* complex) male hybrids were sexually selected against when they competed for the nests with parental males (Vamosi and Schluter, 1999), and fitness of the young sticklebacks hybrids was very low because of ecological selection pressure (Hatfield and Schluter, 1999), i.e., hybrids may be at disadvantage not only because they are sterile or inviable, but because they fall between the niches of two ecologically isolated taxa or cannot mate properly with either of their parental species (Coyne and Orr, 1989a).

The isolating mechanisms are arranged like a series of hurdles: if one breaks down, another must be overcome (Mayr, 1963). In cases of these two species, geographical isolation was broken down and then reproductive isolation was reinforced by postmating isolation mechanism. The consistent appearance of hybrid sterility early in the evolution of two genetically diverging species has marked it as the first step in the

process of speciation (Coyne and Orr, 1989b). Although it was suspected that pre- and postmating isolation mechanisms were affected between two species, *M. oxycephalus* and *M. lagowskii* seemed to be strongly isolated with microhabitat at sympatry until 1997. Subsequently they became reproductively isolated mostly by the postmating isolation mechanism after their habitat disturbances. Therefore, the two species are distinct species in accordance with biological species concept.

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