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Source: Zoological Science, 21(7) : 795-798

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.795

ZOOLOGICAL SCIENCE **21**: 795–798 (2004)

Mitochondrial Gene Introgression between Spined Loaches via Hybridogenesis

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ABSTRACT—This report deals with an unusual mode of mitochondrial gene introgression between *Cobitis hankugensis* (*C. sinensis*) and *C. longicorpus* which is mediated by a unisexual hybridogenetic system of diploid-triploid *C. hankugensis-longicorpus* complex. Mitochondrial DNA sequences of 3329-3330bp encompassing from upstream ND6 to 12S rDNA indicated that mitochondrial genomes from the diploid hybrids, triploid hybrids, and their parental species are almost identical. Because triploid hybrids produce haploid ova with *C. hankugensis* chromosome set, normal diploid *C. hankugensis* regenerates upon insemination with *C. hankugensis* sperm. If the hybrid carries *C. longicorpus* mitochondrial genome, the regenerated *C. hankugensis* is a nucleo-cytoplasmic hybrid, thus accomplishing the unusual mode of mitochondrial gene introgression.

Key words: polyploidy, hybridization, unisexual reproduction, *Cobitis*

INTRODUCTION

Genetic introgression between closely related fish species is widely recognized (Smith, 1992; Mukai, 2001 and references therein). The process of gene introgression has been represented by production of fertile hybrid and backcross gradually incorporating genes into recipient populations upon genetic recombination. In this report we show an unusual, probably non-recombinant, and leaping mode of mitochondrial gene introgression which is mediated by a unisexual hybridogenetic system.

Some loaches (family Cobitidae, Osteichthyes) contain diploid-polyploid complexes (Kim and Lee 2000; Saitoh *et al*., 2000 and references therein; Zhang and Arai, 1999). Occurrence of unisexual (all-female) populations of hybrid origin in some of these complexes is emphasized to be a source of establishment of gonochoric tetraploid population (Vasil'ev *et al*., 1989), but no one except Kim and Lee (2000) recognized that normal diploid individual can be born from unisexual hybrids. Establishment of tetraploids via unisexual hybrids in loaches has been thought to be a one-way process.

A diploid-triploid hybrid complex occurs (*Cobitis hankugensis-longicorpus* [*Cobitis sinensis-longicorpus*] complex) in Nakdong River, Korea (Kim and Lee, 1990; Kim *et al*., 2003). The hybrid complex contains both diploid and triploid populations with few male occurrences. The diploid hybrid contains haploid genomes from *C. hankugensis* and *C. longicorpus* each, and the triploid does two haploid genomes from *C. hankugensis* and one from *C. longicorpus* (Kim and Lee, 1990). Artificial crossing experiment showed the diploid hybrid produces unreduced diploid hybrid ova, and the triploid does ova of *C. hankugensis* haploid genome eliminating *C. longicorpus* genome and reducing (Fig. 1) (Kim and Lee, 2000). Then, normal diploid *C. hankugensis* regenerates from the hybridogenetic triploid, crossing with male *C. hankugensis*. The process of establishment of polyploid populations thus may not be a one-way process. If so, this hybrid complex can mediate mitochondrial gene introgression from *C. longicorpus* to *C. hankugensis*.

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Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) AB120176-AB120177.

MATERIALS AND METHODS

We have sequenced a portion of mitochondrial genome (3329–3330 bp) from two female *C. hankugensis*, three diploid hybrids (one male and two females), three triploid hybrids (one male and two females), and two female *C. longicorpus* individuals. Species and ploidy diagnosis followed Kim and Lee (1990) employing morphological and chromosomal examination. These loaches came from Inwol-myon, Namwon-gun, Chollabuk-do, Korea (127°35'E, 35°27'N) except one *C. hankugensis*. The other *C. hankugensis* being examined as a comparative material was from Seangcho-myon, Sanchong-gun, Gyeongsangnam-do, Korea (127°50'E, 35°27'N). Both collecting sites are in the Nakdong River basin. The sequenced region encompasses from upstream NADH dehydroge-

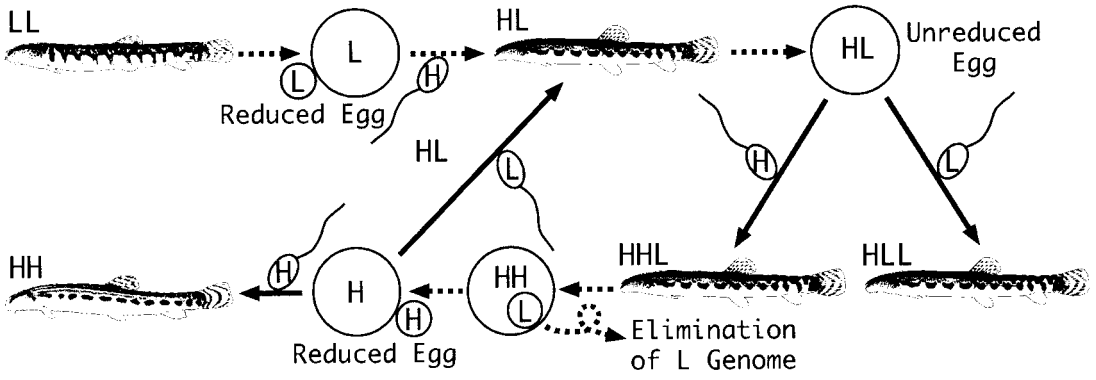

Fig. 1. Reproductive mode of *Cobitis hankugensis-longicorpus* complex. Single letters stand for *C. hankugensis* (H) or *C. longicorpus* (L) haploid genome. Solid arrows indicate experimental hybridization (Kim and Lee 2000), while dotted arrows denote presumed pathways. Large circles indicate eggs or oocytes. Haploid genomes being eliminated or released as polar bodies are set in small circles.

Table 1. PCR and sequencing primers used in this study

Name/ Position*	Sequence (5'–>3')
L12321**	GGT CTT AGG AAC CAA AAA CTC TTG GTG CAA
L14279	GAA TAC ATY ARA GCT ACC CCA C
L14504	GCC AAW GCT GCW GAA TAM GCA AA
L14547	AGG CGC CGG GTT AGA AGC AAC
L14760	AAC CTC TAA TGG CAA GCC TAC G
H14834	GAG CCA AAG TTT CAT CA
L15007	AAC ATA CAT GCC AAC GGA GC
H15149	GGT GGC KCC TCA GAA GGA CAT TTG KCC TCA
L15441	TCA TAT AAA GAC TTA TTA GGC TT
H15680	CGG AAT GTT AGT CCT CGT TG
L15926	TGA AAG CGC TGG TCT TGT AAT CC
L15936	GGT CTT GTA ATC CGA AGA TCG GAG GTT AAA
H15986	TAG TTT AGT TTA GAA TTC TGG CTT TGG GAG
L16019	GCT ACC AAA GCC AGA ATT CTA A
L16019a	GCT CCC AAA GCT AGT ATT CTA A
L220D***	AAT TAC TAA GGT GTG CAT AAG TC
L317D***	TCA TGC ATG ATA GAA CCA GGG AC
H342D***	AAC CAG ATG CCA GTA ATA GTT C
H594D***	ATA TGC AAT GCT TAA GTT ATG TC
H732D***	TTT MGG GGT TTG ACA AGG ATA
L620	AAA GCK TAG TAC TGA AGA TGT TA
H651	ATA AGG TCG GGA CCA TGC CT
H690	GCG GAG GCT TGC ATG TGT A
H721	CGG GCA GGG GAT TGA GGG CAT
H884	AAC CGC GGT GGC TGG CAC GAG
L1083	ACA AAC TGG GAT TAG ATA C
H1358	CGA CGG CGG TAT ATA GGC
H1631	ACA GGA TCC GGA TGT CTT CTC GGT GTA AG
H2990**	TGC ACC ATT RGG ATG TCC TGA TCC AAC ATC

* Primer names begin with strand name which they are designed on (L or H), followed by sequence positions of the 3' ends corresponding to positions on the human mt genome (Anderson *et al*., 1981) or to those on the carp mt genome (Huang *et al*., 1994) for primers on the D-loop containing region (***).

** Primers used for long PCR.

nase subunit-6 (ND6) to small subunit ribosomal DNA, corresponding to nucleotide positions from 14260 to 1017 of *C. striata* mitochondrial genome (Saitoh *et al*., 2003). We employed the two step PCR direct sequencing technique (Miya and Nishida, 1999; Kawaguchi *et al*., 2001). About 7 kb region was first amplified from genomic DNA with long-PCR primer pair. The long-PCR products then worked as templates for short PCRs with combination of 27 primers (Table 1) for direct sequencing using a commercial kit

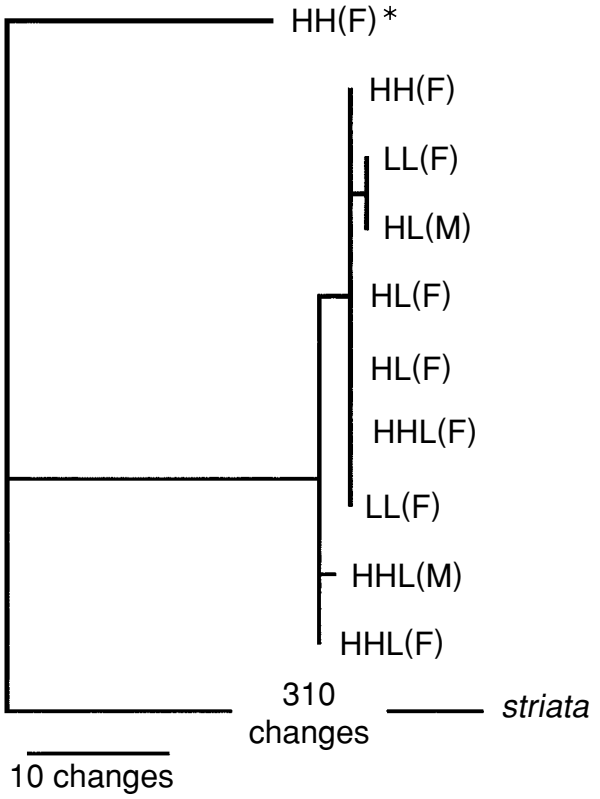

Fig. 2. A maximum parsimony tree of two equally parsimonious topologies obtained by an exhaustive search without character weighting on PAUP* ver. 4.0b (Swofford 1998). Gaps are treated as the fifth character. See Fig. 1 for genome composition of three biotypes. Letters in parentheses indicate male (M) or female (F). A *C. hankugensis* individual with an asterisk was collected at a different collecting site (Seangcho-myon) from other nine individuals.

Table 2. Sequence variable sites (corresponding to *C. striata* [Saitoh *et al*., 2003]) in *Cobitis hankugensis-longicorpus* complex

	1	1111111111111111	11111111111111	
Nucleotide	4	4444444455555555	55666666666666	
Position	2	67788999911223344	99000112234455	67788
of *C. striata*	5	72319667912893746	37277561327812	33302
	9	27894035662135811	46701880960669	15768
		*	*	
Gene	ND6	Cyt-*b*	CR	12S
HH(F)**,***	C	TGATAACCTTTGTTCAC	TGTGAAGTCTTTAA	ACACC
HH(F)	T	CACCGTTCCCCATCTGT	CACAGGT-TGTCGT	GTGTT
HL(M)	T	CACCGTTTCCCATCTGT	CACAGGT-TGTCGT	GTGTT
HL(F)	T	CACCGTTCCCCATCTGT	CACAGGT-TGTCGT	GTGTT
HL(F)	T	CACCGTTCCCCATCTGT	CACAGGT-TGTCGT	GTGTT
HHL(M)	T	CACCGTTCCCCACCTGT	CACAGGTTTGCCGT	GTGTT
HHL(F)	T	CACCGTTCCCCATCTGT	CACAGGT-TGTCGT	GTGTT
HHL(F)	T	CACCGTTCCCCATCTGT	CACAGGTTTGCCGT	GTGTT
LL(F)	T	CACCGTTTCCCATCTGT	CACAGGT-TGTCGT	GTGTT
LL(F)	T	CACCGTTCCCCATCTGT	CACAGGT-TGTCGT	GTGTT

* Non-synomymous substitution.
** See Fig. 1 for genome composition of three biotypes. Letters in parentheses indicate male (M) or female (F).
*** *C. hankugensis* from different locality from the others.

(Amersham, Bucks, UK) and an ABI373S automated DNA sequencer (ABI, Norwalk, USA).

RESULTS

Nine individuals of *C. hankugensis*, *C. longicorpus*, and hybrids from Inwol-myon turned out to be very close or identical (none to one nucleotide gap, none to three transitions, and no transversion) in their mitochondrial DNA sequences regardless of sex (Table 2). On the other hand one *C. hankugensis* individual from a different collecting site carried a heterogenic mitochondrial genome with 28–30 transitions, five transversions, and two amino-acid substitutions being observed between this individual and other nine individuals. Number of estimated nucleotide substitutions per site (Kimura, 1980) (transition/transversion ratio=5.71) between the two *C. hankugensis* individuals from different localities was 0.01. On the other hand, the values between individuals from the same locality were 0–0.0009 (average=0.0003) regardless of their biotypes.

We could not find any sequence differences between one *C. hankugensis*, one *C. longicorpus*, two diploid hybrids and one triploid hybrid individual. Similarly, one *C. longicorpus* and one diploid hybrid individual were identical in mitochondrial DNA sequences. A maximum parsimony tree with *C. striata* sequence as an outgroup showed a nested distribution of the three biotypes in the tree (Fig. 2).

DISCUSSION

Sequence divergence between *Cobitis* species is so far reported to range between 4.6 to 19.2% (Kim *et al*., 2000; Perdices and Doadrio, 2001; Kitagawa *et al*., 2001). Sequence divergence between *C. hankugensis* individuals from different localities actually was 1% indicating loach populations are localized and prone to diverge even within a single basin. From an empirical view taking these reports and our result into account, it is unusual that two morphologically and cytologically (Kim and Lee, 1990) distinct species carry mitochondrial genomes with little sequence divergence. Lineage sorting is unlikely over a geological timescale.

One possible explanation is the diploid-triploid hybrid complex as a vehicle of mitochondrial genome between two parental diploid species. If the mother of the initial diploid hybrid was *C. longicorpus*, it transferred the *C. longicorpus* mitochondrial genome to triploid hybrids of the next generation (Fig. 1) (Kim and Lee, 2000). The triploid hybrids produce ova with *C. longicorpus* mitochondrial genome and *C.* hankugensis haploid chromosome set which sometimes presumably accept *C. hankugensis* sperm in the natural habitat. Since hybridogenetic complexes show no genetic recombination between heterospecific genomes (Graf and Pelaz, 1989; Schmidt, 1996, but see Mateos and Vrijenhoek, 2002), next generation individuals would be nucleo-cytoplasmic hybrids between *C. hankugensis* and *C. longicorpus*. This pathway can accomplish hereby the unusual, probably non-recombinant, and leaping mode of mitochondrial gene introgression. Ecological study is necessary focusing on a mate recognition system between the hybrid complex and their parental diploid species.

Our study sheds light over unusual mitochondrial

grouping among some diploid fish species. Carmona *et al*. (1997) observed unusual mitochondrial clustering of minnows and postulated a hybrid origin or ancient lineage sorting. Kitagawa *et al*. (2001) postulated mitochondrial genome exchange between two *Cobitis* lineages. Introgression events at the diploid level (hybridization and backcrossing) may be responsible for such mitochondrial clustering or genome exchange, but also hybridogenesis can mediate gene introgression. We should especially consider the latter possibilities in minnows and loaches, because unisexual reproduction and polyploidy of hybrid origin occur frequently in these fish groups.

ACKNOWLEDGMENTS

We are grateful to Tadashi Inada for providing this opportunity for us to collaborate.

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(Received November 6, 2003 / Accepted May 13, 2004)