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Analyses of mitochondrial and satellite DNAs on spined loaches of the genus *Cobitis* from Japan have revealed relationships among populations of three diploid-tetraploid complexes

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Abstract. Restriction analysis of mitochondrial DNA (mtDNA) revealed considerable genetic diversification among local populations of diploid-tetraploid complexes of spined loaches in Japan. Branching of some lineages can be explained by Tertiary and Quaternary geological events. Regardless of their morphology, all tetraploids had mtDNAs similar to some of the diploid *biwae* complex. Those results, combined with the presence of *striata* complex-specific tandem repeat, indicate that all tetraploids but one are of hybrid origin.

Key words: teleost fish, speciation, tandem repeat, polyploid, hybridization, genetic differentiation

Introduction

Many ichthyologists and herpetologists have concentrated on the evolutionary biology of polyploids. This study field is attractive, not only because of the particular mode of speciation, but also it provides insights into the evolution of higher vertebrates by gene duplication. Polyploidy occurs in some lower teleostean groups including cobitids.

Three Japanese *Cobitis* nominal species, *C. taenia*, *C. striata* and *C. biwae*, contain diploid-tetraploid complexes (Ueno & Ojima 1976). Here we call these groups "yamato", *striata* and *biwae* complex respectively. The nominal *C. taenia* is clearly not conspecific with European populations and is named according to the Japanese common name, "yamato-shimadojo". These complexes may provide good material to examine the establishment of polyploid populations. In spite of morphological similarity however, interrelationships between diploid and tetraploid in each nominal group are not simple. Several authors have reported morphological, ecological, physiological and chromosomal differentiation within each group, even at the diploid level (Minamori 1951, 1956, Ueno 1981, Aizawa & Hibiya 1982, Saitoh & Aizawa 1987). Such variants have their specific geographic range and they constitute local races. It is then necessary to investigate from which local race each of the polyploid races originated. In addition, recent studies have revealed that several polyploid loaches are of hybrid origin (Vasiľev & Vasiľeva 1982, Kim & Lee 1990, Suzawa & Taniguchi 1990, Boroń 1992, Sezaki et al. 1994, Ráb & Slavík 1996).

In this study we analyzed mitochondrial (mt) and satellite DNAs of the diploid-tetraploid complexes from Japan to clarify interrelationships between diploid and tetraploid races. Genealogy and homology of these elements have revealed both maternal and paternal lineages of some polyploid races.

Materials and Methods

Specimens and localities

We used 38 individuals of spined loaches, including 18 populations from 19 localities, and also two individuals of mud loach (*Misgurnus anguillicaudatus*) as comparative material (Fig.1, Table 1). Identification of local races is based mainly on morphology. Because of possibilities for coexistence of a number of tetraploid races of the yamato complex with different chromosome number (Kimizuka et al. 1979), we observed chromosome spreads for every individual of this complex following Ojima & Kurishita (1980).

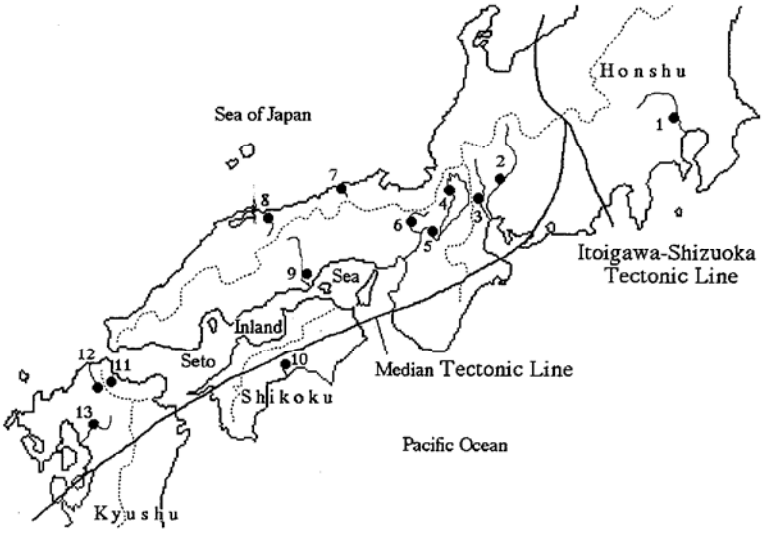

Fig. 1. Sampling localities (closed circles) and geography of central to western Japan. 1) Ara River; 2) Nagara River; 3) Ibi River; 4) Lake Biwa; 5) Uji River; 6) Ooi River; 7) Kishida River; 8) Hino River; 9) Asahi River; 10) Kagami River; 11) Ima River; 12) Onga River; 13) Kikuchi River. Dotted lines indicate major watersheds.

DNA analyses

Routinely extracted total genomic DNA received enzyme digestion, agarose gel electrophoresis, Southern blotting, and hybridization with total, cloned or PCR-amplified mtDNA probes. Probes and conditions of blotting, hybridization and detection were described in Saitoh et al. (1995). Restriction endonucleases used here were *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Kpn*I, *Pst*I, *Xba*I and *Xho*I (Toyobo, Japan). Estimation of the number of nucleotide substitutions followed Nei & Tajima (1983). We employed the neighbor-joining method (Saitou & Nei 1987) to infer interrelationships among mtDNA haplotypes.

We have cloned a restriction satellite DNA specific to the *striata* complex. A 940bp prominent band by *Hind*III-digestion of genomic DNA from the Biwa small race extracted from low melt agarose (FMC, USA) was ligated into pUC19 compatible site and cloned into DH5α (BRL, USA). The cloned insert purified from low melt agarose and labeled with digoxigenin (Boehringer Mannheim, Germany) was the probe for the detection of the satellite by Southern hybridization. Post hybridization washing condition was 1×SSC (0.15M NaCl, 0.015M 3Na-citrate), 0.1%SDS for 5min at room temperature and then 0.1×SSC, 0.1%SDS at 65°C for 5 and 10min.

Table 1. Specimens and their collecting localities in this study.

Nominal species	Local race/ form	Abbreviation	Chromosome number	Locality	Number of individuals
"Yamato"		Y86	86*	Kikuchi R.	3
		Y90	90*	Onga R.	3
		Y94	94*	Ima R.	2
striata	large race	Sl	98**	L. Biwa	3
	Biwa small race	SB	50**	L. Biwa	3
	Yodo small race	SY	50**	Uji R.	2
	small race	Ss	49–50****	Asahi R.	2
	Tokai small race	ST	50**	Ibi R.	1
				Nagara R.	1
	Spotted small race	Ssp	50***	Hino R.	2
	Kyushu form	SK	50**	Kikuchi R.	2
	middle race	Sm	50**	Ooi R.	2
biwae	tetraploid race	B4n	96**	Ooi R.	1
	diploid form	B2nB	48**	L. Biwa	2
		B2nN	48**	Nagara R.	2
		B2nKs	48**	Kishida R.	2
		B2nA	48**	Ara R.	3
		B2nKg	48**	Kagami R.	2
Misgurnus anguillicaudatus		Ma		Ooi R.	2

* Present study. ** Ueno & Ojima (1976) and Ueno (1981). *** Kimizuka (1987). **** Saitoh (1989).

Results

Chromosomal characteristics

Samples of the yamato complex from Kikuchi River basin were the 86 chromosome race, while those from Onga River basin were 90 and from Ima River basin 94 (Fig. 2). The 90 chromosome race is a novel form of the yamato complex. Chromosomal characteristics of other local races or forms have been reported previously (Table 1).

Restriction analysis of mtDNAs

Digestion by nine restriction endonucleases of 40 loach individual mtDNA revealed a total of 86 cleavage sites (Table 2). The homology test with cloned or PCR amplified mtDNA fragments of known position determined orders of *Xba*I-digested fragments of several mtDNA haplotypes. This procedure and routine double digestion technique mapped cleavage sites unambiguously.

We found 26 mtDNA haplotypes. Most individuals carry specific haplotypes, while only two cases were found where a number of races carried a common haplotype. In all cases of polymorphisms within a spined loach population, there are only one cleavage site differences. There were only two cleavage sites common to all haplotypes analyzed. These conservative sites were both of *Xba*I and were around the control region. Partial sequencing indicated one is in the 12SrRNA gene at the position corresponding to nucleotide 1208–1213 in humans (Saitoh unpublished).

Phylogenetic analysis revealed substantial deep branching among several haplotypes (Fig. 3). the order of deeper branches may not be clear because of limited number of restriction sites compared, but some clusters based on the approximate results were recognizable. The diploid *striata* complex contained four major mitochondrial clusters:

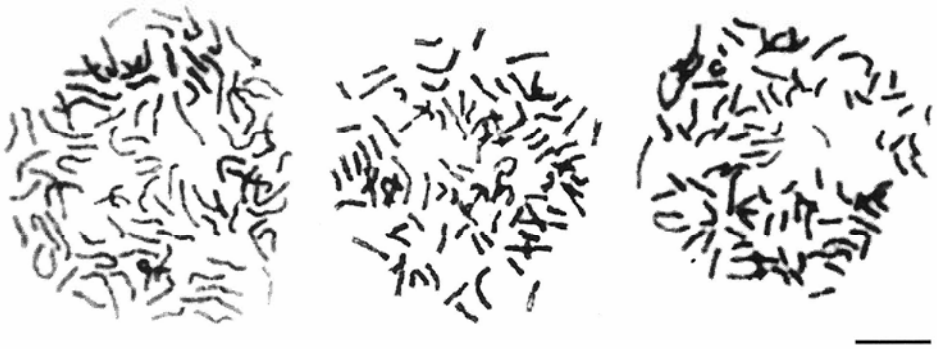

Fig. 2. Chromosome spreads of yamato complex with 86 (left), 90 (center) and 94 (right) chromosomes. Scale bar indicates 10 μm.

Kyushu form, middle race, spotted small race and other smaller races. The *biwae* complex consisted of three major mitochondrial clusters: populations from western part of Honshu Island, eastern part, and Pacific slope of Shikoku Island.

All of the tetraploid races carried mtDNA haplotypes within the clusters of the *biwae* complex. Tetraploid *biwae* had a haplotype close to those of a diploid from geographic proximity. In spite of karyological difference, all haplotypes of the yamato complex were close to each other. The closest diploid *biwae* to the yamato complex in terms of mtDNA haplotype was from Shikoku Island. Regardless of morphological resemblance, the large race of the *striata* complex did not carry haplotype close to sympatric diploid *striata* (Biwa small race), nor a haplotype similar to diploid *biwae* of sympatry. The closest diploid form to large race in terms of mtDNA haplotype was *biwae* from Shikoku Island.

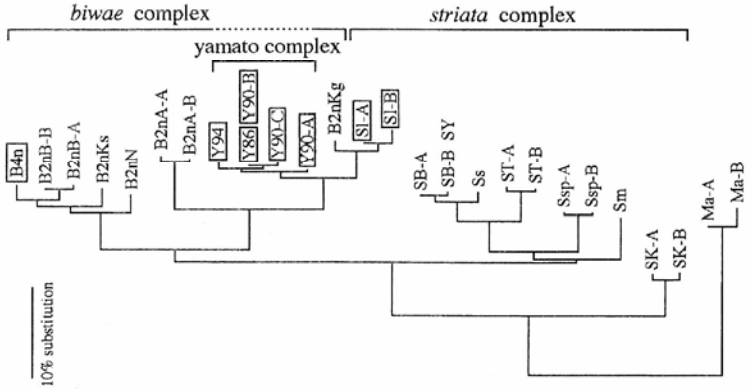

Fig. 3. Neighbour-joining tree showing mitochondrial lineages of Japanese spined loaches. See Table 1 for abbreviations.

Characterization of satellite DNA

Southern hybridization of satellite DNA cloned from Biwa small race of the *striata* complex detected distribution of homologous elements among spined loaches (Fig. 4). Diploid *striata* had some homologous elements with the cloned fragment. Tetraploid *striata* (large race) and all tetraploid yamato complex also showed the presence of homologous elements in their genomes. On the other hand, *biwae* from Shikoku Island showed no homology with that element. Local

Table 2. Distribution of restriction sites in Japanese spined loach and mud loach showing presence (1) or absence (0) state at each site.

Type/Enzyme	*Bam*HI	*Bgl*I	*Bgl*II	*Eco*RI	*Eco*RV	*Kpn*I	*Pst*I	*Xba*I	*Xho*I
B4n	00011010	100010100011	0000000	000100000010	000000000	0010100	0001010	11001000000	01000100000011
B2nB-A	00011010	100110100011	0000000	001101000010	001000000	0010101	0001011	11001000000	11000000000011
B2nB-B	00011010	100110100011	0000000	001101000010	001000000	0010101	0001010	11001000000	11000000000011
B2nN	00000110	100110101011	0000000	000100010010	001000000	0000101	1001010	11001000000	11000100000011
B2nKs	00010010	100110111011	0001000	000101000010	000000001	0010000	0001010	11001000000	11000000000011
B2nA-A	00100000	100010001001	1000001	001100000000	000000000	0000000	1100000	11000000100	00100001000000
B2nA-B	00100000	100010001001	1000001	001100000000	000000000	0000000	1100000	11000000000	00100001000000
B2nKg	00100000	100010001000	0000000	000100001010	000001000	0100010	0001001	11000010000	00000000001000
Y86&Y90-B	00100000	100010001001	1000001	000100001010	001100000	0000010	0001101	11000010000	00000000001100
Y90-A	00100000	100010001001	1000001	000100001010	001100000	0000010	0001001	11000010000	00000000001100
Y90-C	00100000	100010001001	1000001	000100000010	001100000	0000010	0001101	11000010000	00000000001100
Y94	00100000	100010001001	1100001	000100001010	001000000	0000010	0001101	11000010000	00000000001100
Sl-A	00100000	100010001000	0000000	000100001010	000001000	0000000	0001001	11001010000	00000000001000
Sl-B	00100000	100000001000	0000000	000100001010	000001000	0000000	0001001	11001010000	00000000001000
SB-A	00000001	000010000011	0010010	000100010000	001000000	0000100	0000010	11000010001	00000000000100
SB-B&SY	00000001	000010000011	0010010	000100010000	001000100	0000100	0000010	11000010001	00000000000100
Ss	00000001	000010000011	0010010	000100010100	001000000	0000100	0000110	11000010011	00000000000100
ST-A	00000000	000010000011	0000110	000100010100	001001000	1000100	1000010	11000000001	01000000000001
ST-B	00000000	000010000011	0000110	000100010100	001000000	1000100	1000010	11000000001	01000000000001
Ssp-A	00000000	100010100011	0001100	010100000000	000100000	0000000	1000010	11000100001	01000001001000
Ssp-B	00000000	100010000011	0001100	010100000000	000100000	0000000	1000010	11000100001	01000001001000
Sm	00010010	100010000001	0001101	000100010010	001010000	0001100	0000000	11010100101	11000001001000
SK-A	00010000	100000010000	0001000	000100000000	011000000	0001100	0000010	11010101011	00001000000000
SK-B	00010000	100000010000	0001000	000100000000	011000000	0000000	0000010	11010101011	00001000000000
Ma-A	11000000	011001010100	0000000	100011100001	100000010	0000000	0111000	11100100000	00010000100000
Ma-B	11000000	010000010100	0000000	100011100001	100000010	0000000	0110000	11100100000	00010000100000

races other than Biwa small race and Kyushu form of the *striata* complex also showed the same hybridization pattern, while no *biwae* complex indicated hybridization with the element (data not shown). The cloned 940bp fragment is a member of a *striata* genome-specific satellite family.

Restriction pattern outlined some characteristics of the satellite family. In *Hin*dIII-digested genomic DNAs it appeared as the 940bp main fragment and a 1.9kb sub-band. Sometimes a weak 2.8kb band was detectable. The same pattern also appeared in *Eco*RI-digested DNAs. Digestion of genomic DNAs by both *Hin*dIII and *Eco*RI made the main band pale. Instead, two bands at 610bp and 330bp appeared. These characteristics indicate that this satellite family is a tandem repeat in which *Hin*dIII and *Eco*RI cleavage sites appear in turn almost every 940bp with 610/330bp shift. Some variation among paralogous elements eliminate cleavage sites resulting in doublets or triplets etc.

While most local races of the *striata* complex showed a strong hybridization signal with the cloned fragment, hybridization signals of Kyushu form were considerably weaker. Similarly, the yamato complex showed weak signals of hybridization. Instead, the Kyushu form and the yamato complex commonly exhibited a 1.4kb prominent band in their DNA smears, though we have not yet cloned it. The 1.4kb band did not hybridize with the 940bp fragment at all.

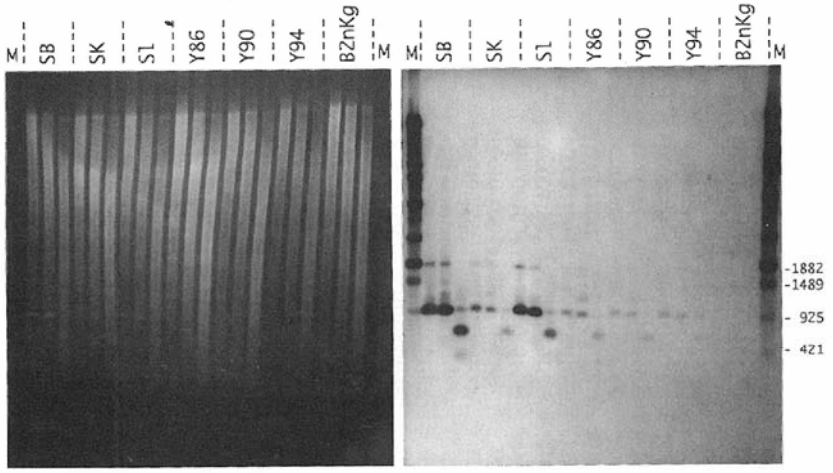

Fig. 4. Southern hybridization analysis of *striata* complex-specific restriction satellite showing ethidium bromide stained gel (left panel) and hybridization pattern (right panel). For each individual DNA sample, lanes from left to right show patterns of *Eco*RI, *Hin*dIII and double digestion by *Eco*RI-*Hin*dIII. M, molecular weight marker (*Eco*T14I-digested λ-DNA). See Table 1 for other abbreviations.

Discussion

The present study indicates substantial local differentiation among Japanese spined loaches, especially among diploids in terms of mtDNA analysis. Small mitochondrial differentiation within each diploid population indicates that mitochondrial branching pattern is close to the branching pattern among populations, though the possibility of mitochondrial gene introgression between diploid populations cannot be ruled out. Each tetraploid carries mtDNA closely related to some diploids. Therefore we should first overview differentiation among diploids in order to discuss the maternal origin of tetraploids.

Distinction among three major mitochondrial groups in *biwae* complex is coincident with the major land mass borders called Itoigawa-Shizuoka and the Median tectonic lines which broke out in the Tertiary period (F u j i t a 1973). Two morphological units have already been

recognized across the Itoigawa-Shizuoka line (Aizawa & Hibiya 1982). The present study indicated the existence of a third cryptic unit. If separation of these lineages occurred before the establishment of this geological structure, the history of the *biwae* complex can be traced back to the Tertiary period.

Discrimination among four major mitochondrial groups in the diploid *striata* complex coincides with some recent geological events. In the early Quaternary period, the ancient Lake Biwa overflowed east (Kawabe 1994). Separation among four mitochondrial lineages of diploid *striata* thus established by possible watersheds between the early Quaternary Lake Biwa and the westward areas. Later an orogenic activity at the east of the lake switched its effluent into the Seto Inland Sea slope. Separation between the Tokai small race and smaller races in Seto Inland Sea slope, including Lake Biwa, possibly occurred in that period. Distribution of the small race may be due to recent dispersal from Lake Biwa through oceanic recession during ice-ages.

The branching order of the *biwae* complex from western Honshu Island is somewhat different from that of *striata* complex. This may be explained by habitat preference of the *biwae* complex to upper reaches. Watersheds might not be strict barriers against their dispersal because of possible river capture in headwaters. Further study, however, is necessary to confirm the order of close branching points in this group.

Depths of mitochondrial branches between tetraploids and their closest diploid relatives are usually much smaller than branches among diploids, indicating recent establishment of tetraploids. The tetraploid *biwae* is close to the diploid *biwae* from Lake Biwa basin. Allozyme data and experimental gynogenesis indicated tetrasomy of tetraploid *biwae* (Suzawa & Taniguchi 1990, Kusunoki et al. 1994). Their results and mitochondrial similarity suggest autotetraploidy, though the possibility of allotetraploidy by interratial hybridization in the *biwae* complex cannot be ruled out.

On the other hand, the yamato complex and the large race of the *striata* complex carry genetic elements similar to both the *biwae* and *striata* complexes, indicating their hybrid origin between *biwae* and *striata* complexes. Their mitochondrial haplotypes showing affinity with diploid *biwae* and presence of the *striata*-specific satellite clearly indicate directional hybridization. The putative mother of those tetraploids, however, has not been found in sympatry. In the Lake Biwa basin another form of diploid *biwae* appears. There is no record of the *biwae* complex from the sampling localities in Kyushu Island. Remnants of the group of diploid *biwae* from Shikoku Island, which appear in tetraploid genomes, suggest existence of this group in the western Japan in recent geological time.

In contrast to maternity, candidates for the father of those tetraploids may coexist sympatrically. Though the homology test of the *striata* genome-specific satellite cannot distinguish among middle and smaller races, morphological similarity may be an evidence of paternity of the Biwa small race for the large race. Faint hybridization signals and the presence of another satellite common to both Kyushu form of the *striata* complex and the yamato complex suggest that the father of the yamato complex is Kyushu form. Further study, however, is necessary to test the homology and presence-absence state of the new satellite.

Recent studies on Cobitis hybrid complexes indicate gynogenetic or hybridogenetic propagation of triploid hybrids (Vasiľev & Vasiľeva 1982, Kim & Lee 1990, Boroń 1992, Ráb & Slavík 1996). If triploid eggs accept haploid sperm, they may become tetraploids. Thus a gynogenetic triploid intermediate can be postulated for the establishment of bisexual tetraploid of hybrid origin. If so, however, lack of both unisexual triploids and maternal diploids from Japan indicates that the present tetraploids drove away their mother. This poses some ecological or physiological problems for further study.

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