

学位論文題名

Genetic differentiation of Japanese *Misgurnus* loach inferred from microsatellite variation, marker-centromere map and reproduction of hybrids between two populations

（マイクロサテライト変異、マーカー-動原体地図および
集団間雑種の生殖特性から見た日本産ドジョウの遺伝的分化）

学位論文内容の要旨

The loach *Misgurnus anguillicaudatus* in Japan has been studied from the view points of taxonomy using morphological characters and population genetics using allozyme electrophoresis and RFLP analyses of mitochondrial DNA. These studies suggest genetic differentiation among geographically different populations. In Japan, the population in the Memanbetsu Town, northern area of Hokkaido Island was reported to be genetically different from populations in southern area of Hokkaido and other Islands (Khan and Arai, 2000; Khan *et al.*, 2005). In northern area of Hokkaido, clonal loaches generate diploid eggs genetically identical to the somatic cells of the mother and reproduce in nature by gynogenesis (Morishima *et al.*, 2002). The sympatric triploid loaches appear by accidental incorporation of sperm genome into unreduced diploid eggs (Morishima *et al.*, 2002). A possible origin of such clonal and polyploid vertebrates has been argued for long time and a tight linkage between unusual reproduction and hybridization has been speculated as a major cause (see review, Dawley, 1989).

Outline of the present thesis is as follow. In Chapter I, unisexual development, unreduced gametogenesis and spontaneous polyploidies were reviewed based on published literatures to show the background and the goal of the present thesis. To answer a hybrid origin theory of clonal and polyploid fishes, the author isolated and characterized newly developed microsatellite markers as genetic tools (Chapter II)

and then genetically compared two populations, Memanbetsu, northern area of Hokkaido and Kita, southern area of Hokkaido (Chapter III). In Chapter IV, recombination rates between microsatellite locus and centromere were estimated by half-tetrad analysis using induced gynogenetic diploids at newly and previously developed loci and different marker-centromere map distances were detected in three marker loci between the two populations. In Chapter V, inter-population hybrids were artificially produced and reproductive traits were examined in female (Chapter VI) and male hybrids (Chapter VII). All the results were summarized and the conclusion was addressed in the last chapter (Chapter VIII).

Forty five *Mado* primer sets were developed and 23 markers were informative. They are categorized to 4 kinds. 1) Some markers like *Mado1* are polymorphic in the Memanbetsu population, while they are fixed to specific one allele in the Kita population. 2) Some other markers such as *Mado5* showed polymorphisms in both populations and common alleles exist between the two populations. 3) Polymorphisms are observed only in the Kita population and no PCR products or null alleles are detected in the Memanbetsu population, as in *Mado14*, *Mado29* and *Mado31*. 4) Microsatellite markers are monomorphic at *Mado16*, *Mado42* and *Mado44*, but diagnostic allele is specific to the Memanbetsu or the Kita population at each locus. Substitution of alleles at several marker loci suggests the apparent genetic difference between the two populations.

Microsatellite-centromere map distances were assessed by half-tetrad analysis at a total 21 *Mado* and *Mac* marker loci using a total of seven gynogenetic diploid progeny from the Memanbetsu and the Kita population, which were induced by chromosome manipulation. In normal crosses, diploid progeny gave segregation of microsatellite genotypes according to the manner of Mendelian inheritance. Recombination rates between markers and centromere were estimated from $y=0$ (y : second division segregation frequency) to $y=1$. These results showed microsatellite loci distributed from centromeric to telomeric region on loach chromosome. Apparent differences were detected in marker-centromere map distances at the three loci between the Memanbetsu (0.2 centiMorgan (cM) at *Mado7*; 12.5 cM at *Mac3*;

15.2 at *Mac49*) and the Kita (18.6 centiMorgan (cM) at *Mado7*; 5.0 cM at *Mac3*; 2.6 at *Mac49*). In other microsatellite loci, no differences were recorded in marker-centromere map distances between the two populations. Map distances estimated in loaches collected from southern Hokkaido and Honshu (Morishima *et al.*, 2001) were similar to those estimated in the Kita population. Differences in map distances at some loci suggest the inter-population differences probably due to chromosome rearrangement during the genetic differentiation.

Inter-population hybrids were artificially produced to examine their reproductive traits and the hybrid nature was genetically verified by heterozygous genotypes at the diagnostic microsatellite loci. Survival rates of hybrids were slightly lower than those in intra-population control crosses.

Hybrid females spawned fertile eggs with various egg sizes from normal or small to large. Some hybrid females gave higher proportions of large eggs, while other hybrids laid large number of normal or small eggs. Larger egg sizes are related to the elevation of ploidy in eggs of hybrids, because large-sized eggs fertilized with normal haploid sperm gave triploid progeny, while most normal or small-sized eggs developed to diploid progeny after normal fertilization. The large eggs gave viable gynogenetic progeny with normal shape after fertilization with UV-irradiated sperm. Thus, most large eggs would be unreduced diploid eggs produced by the hybrids. Microsatellite genotypes of induced gynogenetic diploid and triploid progeny indicated that most unreduced eggs were genetically identical or very similar to hybrid female. However, unusual aneuploid eggs were also formed in the hybrids. These results were confirmed by ploidies of progeny after fertilization with goldfish sperm. These observations showed that some hybrid females had the reproductive capacity to generate fertile, unreduced diploid eggs. Unreduced egg formation is essentially similar to that reported in the clonal lineage of loaches in the Memanbetsu (Morishima *et al.* 2002) and other natural and artificial hybrid vertebrates (Dawley, 1989), but diploid eggs of hybrid females never develop by gynogenesis.

Physiological traits such as percentage of motile cells, grade of movement, time of motility, number of spermatic cells and hatching rates after fertilization were very poor in inter-population hybrid males, when compared with counterpart

control male. Scanning electron microscopy showed that spermatozoa from hybrid males exhibited larger head sizes as well as various abnormalities. On the contrary, normal shape and size were observed in spermatozoa from the counterpart control diploid. When normal eggs of wild diploid loaches were fertilized with a small quantity of sperm of hybrid males, very few survivors appeared and flow cytometry indicated that they were diploid and triploid progeny. Microsatellite genotyping of most triploid survivors indicated that two alleles were absolutely identical to the hybrid males and one allele was transmitted from the normal mother, suggesting the production of fertile unreduced spermatozoa in the hybrids. However, diploid survivors had heterozygous genotype comprising one allele from hybrid male and another allele from normal female. Thus, haploid spermatozoa can be produced in hybrid males by normal meiotic process.

In the present thesis, genetic differentiation in *Misgurnus* loach between the two populations was inferred from microsatellite variation, marker-centromere map distances and aberrant reproductive capacities observed in inter-population hybrids. These results concluded that unreduced gametogenesis was linked to hybridization between the two differentiated populations. Altered gametogenesis observed in the hybrids also reinforced the genetic differences between the two populations. However, inter-population hybrids are not the direct origin of the clonal lineages reported in the loach, because unreduced eggs of hybrids never developed gynogenetically. Further studies are required to disclose the mechanisms responsible for the occurrence of clonal and polyploid individuals in nature as well as to clarify genetic differentiation in the *Misgurnus* loach and related species.

学位論文審査の要旨

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日本産ドジョウ *Misgurnus anguillicaudatus* は一属一種とされてきたが、アロザイム等を遺伝マーカーとした遺伝学的研究から、大きく分化した集団の存在が示唆されてきた。しかも、ドジョウには自然雌性発生により繁殖するクローン二倍体系統が知られており、自然三倍体はクローンの産する非還元二倍体卵の偶発的精子取り込みにより生じる。これらの自然クローン・倍数体は遺伝的に異なる集団の境界付近に生じることから、それらの起源として、異なる集団間の交雑の関与が推定されてきたが、いまだ実験的手法により確認されていない。そこで、本研究では、マイクロサテライト DNA を用いたドジョウの 2 野生集団〔女満別、現大空町、北海道北部網走管内、北村、南部空知管内〕の集団解析およびマーカー-動原体地図距離の相違から、両集団の遺伝的分化を示し、さらに、集団間交雑の子孫雌雄における生殖能力の調査から、交雑が特殊な生殖の起源となるか否かを検討し、以下の評価すべき成果を得た。

1) ドジョウの DNA 断片を用いて DNA ライブラリーを作成し、スクリーニングを行なった。単離したマイクロサテライト配列を挟み込むように PCR 増幅用プライマーセットを設計し、それらを *Mado1-45* と命名した。これらのうち、安定した増幅を示し、何れかの集団で多型的な、あるいは両集団を区別可能な 23 座を解析に用いた。

2) マイクロサテライトの中には、女満別において高度に多型的であるが、北村で単型のもの、両集団で多型的で共通のアレルをもつもの、北村では多型を示すが、女満別では増幅が見られずヌルアレルに固定されたもの、両集団において単型であるが、集団特異的アレ

ルに固定されたものがあつた。これら複数のマイクロサテライト座におけるアレル頻度あるいは置換により、女満別と北村の集団は区別可能であつた。

3) 女満別および北村由来のドジョウから、雌性発生二倍体子孫を誘起し、これらを用いてマーカー—動原体間地図距離の推定を行なつたところ、多くのマイクロサテライト座には相違は見られなかつた。調査した座は、動原体側に位置するものから、染色体端部に位置するものまで、見られた。しかし、*Mado 7*、*Mac3*、および *Mac49*におけるマーカー—動原体間地図距離は2集団間で異なり、染色体の再配列を含む両者の遺伝的な相違を示唆した。

4) 女満別と北村の二つの集団間で人為交雑を行つたところ、雑種雌には、大型卵を多数産む個体と、通常サイズの卵を多数産む個体の両方が見られた。人工受精より生じる子孫のフローサイトメトリー分析の結果、大型の卵からは三倍体子孫が、通常の卵からは二倍体子孫が生じ、このほかに異数体も認められた。紫外線照射精子を用いた人為雌性発生を行つた場合、大型卵からは染色体倍加処理を行わなくても、正常稚魚が得られた。従つて、一部雑種個体は非還元二倍体卵を産むことが明らかになったが、これら卵の受精から生じる子孫は三倍体となり、自然雌性発生は生じなかつた。マイクロサテライト分析から、非還元卵の多くは雑種由来の二つのアレルを全ての座でもつクローンであることが示された。以上の結果は、遺伝的違いが示唆された集団間交雑に由来する雌のなかに、母親と遺伝的に同一なクローン卵子を産むものがあることを示す。従つて、交雑は非還元卵形成を引き起こした。しかし、今回見られた非還元卵はいずれも精子を取り込み、三倍体となることから、女満別集団で見られるクローン二倍体の直接の起源とは考え難い。

5) 雑種雄の精子の活性と運動持続時間で見た運動能力は劣り、細胞数も著しく少なかつた。精液のフローサイトメトリーは、半数体のほか二倍体および四倍体相当の DNA 量に大きなピークを示した。走査電子顕微鏡観察により、精子の多くは中片の欠落、二本あるいは三本の尾を示すなどの形態異常を示すことが分つた。雑種の作る精子の頭部のサイズは長径、短径ともに、明らかに正常の精子に比較して大きかつた。人工受精より生じた少数の子孫の倍数性はフローサイトメトリーの結果、二倍体と三倍体であり、前者は正常卵と正常精子、後者は正常卵と非還元二倍性精子の受精によるものと考えられた。マイクロサテライト分析は一部二倍体精子のクローン性と正常な減数分裂による半数体精子形成を示した。以上の結果は、集団間交雑は雄においても、異常な配偶子形成をもたらすことを示し、同種内交配とは著しく異なる結果となつた。

申請者による以上の研究成果は、魚類における自然クローン・倍数体の出現機構の解明に大きく寄与するものであり、審査員一同は、申請者が博士〔水産科学〕の学位を授与される資格のあるものと判定した。