博士（水産科学）　趙　岩

学位論文題名

Studies on spermatozoa of polyploid, hybrid and clonal *Misgurnus* loaches

（倍数体、雑種およびクローンドジョウの精子に関する研究）

学位論文内容の要旨

In teleost fishes, most species are sexually reproducing diploids with two sets of homologous chromosomes, but some have irregular chromosome constitutions including polyploids, hybrid-origin diploids, and aneuploids. These varieties have been found in nature or induced by experimental chromosome manipulation. Fishes with irregular chromosome constitutions raise many fundamental scientific questions as well as many ideas to meet human needs. Irregular chromosome constitutions could trigger a change in the meiotic and subsequent gametogenesis process, and thus various kinds of gametes with different chromosome constitutions occur. In this thesis, the author wants to know how irregular chromosome constitutions influence the spermatogenesis and its resultant products, i.e., spermatozoa in the loach *Misgurnus anguillicaudatus* (Teleostei : Cobitidae). Different kinds of spermatozoa derived from tetraploid, interspecific hybrid diploid, sex-reversed clonal diploid and hyper-triploid / tetraploid male loaches are analyzed from physiological and morphological aspects, and their spermatogenesis is discussed. Various polyploid and aneuploid spermatozoa or spermatozoon-like cells are very important germ resources for chromosome manipulation in aquaculture. The information gained from this study will increase the knowledge of fish spermatology, and also create new ideas for breeding technology in aquaculture.

The loach, a small freshwater fish, is being distributed in Japan, Korea, Taiwan and east coasts of Asian continent from Amur River to North Vietnam (Saitoh, 1989). Usually this species is sexually reproducing diploid fish with 2n=50, but polyploid loaches also exist in nature or can be obtained artificially (Arai, 2009). In chapter 1, two sources of tetraploid individuals were analyzed. The one source of tetraploid males was flow-cytometrically sorted from a total of 451 loach individuals, which were obtained from the wholesale market in Tokyo from March to April, 2011(Zhao et al., 2012). According to the fish dealers, these farmed animals were imported as food from Taiwan. The other source is neo-tetraploid males, which have been produced by fertilizing eggs from a normal diploid with diploid sperm of a natural tetraploid obtained in market samples, followed by inhibition of the second polar body release shortly after fertilization (Fujimoto et al., 2010). Normal sperm of wild

diploid loach males exhibited only haploid cell population, while the sperm of tetraploid and neo-tetraploid males showed diploid cell population. Haploid spermatozoa from normal diploids, diploid spermatozoa from tetraploids and those from neo-tetraploid exhibited vigorous total motility (92.3%, 94.0%, 90.0%). General ultrastructural architecture of spermatozoa produced by loach specimens with different ploidy status was similar to that of normal diploid except for the size. The average head size (length / width of head, means ± SD) of diploid spermatozoa (2.24 ± 0.13 / 2.23 ± 0.11 μm) from tetraploids and neo-tetraploid was approximately 1.24 times larger than that of haploid spermatozoa (1.81 ± 0.07 / 1.80 ± 0.07 μm) ($P < 0.05$) from normal diploids. This value may correspond to a doubled sperm volume under the assumption of a spherical configuration of the sperm head. Flagellum was longer in diploid spermatozoa (means ± SD, 30.23 ± 4.32 μm), when compared with haploid spermatozoa (24.20 ± 2.31 μm) ($P < 0.05$). However, the ratios of the spermatozoa head length to head width and those of the spermatozoa head length to flagellum length were not significantly different among all males ($P > 0.05$). The differences in cell size were also detected by flow cytometric analyses. Number of mitochondria per spermatozoon in normal diploids males ranged from 7 to 14, while the number of mitochondria per spermatozoon in tetraploid and neo-tetraploid males ranged from 14 to 22. Thus total volume of mitochondrial mass per spermatozoon of diploid spermatozoa from tetraploid and neo-tetraploid evaluated by MitoTracker Green FM was larger than that of haploid spermatozoa from normal diploid. ATP content of sperm from tetraploid males (362.63 ± 5.78 nmol/10^9 spermatozoa) and neo-tetraploid male (365.47 ± 5.76 nmol/10^9 spermatozoa) increased significantly than sperm from normal diploid males (81.32 ± 5.10 nmol/10^9 spermatozoa). In a word, tetraploid loaches generate reduced motile spermatozoa through meiotic process, not only morphological characteristics but also energy-related factors are changed to reach a new balance that sustains the motility of such diploid spermatozoa.

Mud loach *Misgurnus mizolepis*, a well-known exotic cobitid species (Teleostei: Cobitidae), is morphologically distinguishable from *M. anguillicaudatus* distributed in Japan (Fujita, 2007). *M. anguillicaudatus* has 2n=50 chromosomes (Ojima and Hitotsumachi, 1969), and its related species *M. mizolepis* has 2n=48 chromosomes including a pair of large metacentric chromosomes (Kim et al., 1995). Both species have the same arm number (NF) of 64, and karyotype differences can be well explained by Robertsonian translocation (Kim et al., 1995). Interspecific diploid hybrid between *M. anguillicaudatus* female and *M. mizolepis* male were obtained and previous studies indicated that diploid hybrid male exhibit semi-sterility (Fujimoto et al., 2008). Some hybrid individuals produce sperm with haploid, diploid and tetraploid cells, while the others produce sperm with large population of tetraploid cells. The tetraploid spermatozoon-like cells are studied in this chapter (2) by using the same approaches used in chapter 1. Poor total motility (<5%) and apparently lower average concentration of spermatozoa (32.4 x 10^6 cells/ml) were detected in sperm from the interspecific hybrid males. The ultrastructure of spermatozoa or spermatozoon-like cells from hybrid male loaches deviated considerably from the normal. Most of spermatozoa or spermatozoon-like cells from hybrid had one flagellum (46.7%), but those without flagellum (36.4%) or with two flagella (16.9%) were also found. The average length of the flagella in hybrid, with higher SD (12.47 ± 7.05 μm), was obviously shorter than that of the normal spermatozoa (23.85 ± 2.29 μm) (P<0.05). The average head size of tetraploid spermatozoa or spermatozoon-like cell from hybrid (2.83 ± 0.24 / 2.80 ± 0.24 μm) was approximately 1.6 times larger than that of normal spermatozoa from wild diploid male loaches (1.80 ± 0.08 / 1.80 ± 0.07 μm) (P < 0.05). The ratios of the tetraploid spermatozoa head length to flagellum length in hybrid (0.225 ± 0.213) were significantly different from that of normal spermatozoa (0.075 ± 0.09) (P < 0.05). Cells with less dense nuclear and flagellum were observed in sperm from hybrids. Besides normal 9+2 microtubule structure of the flagellum, abnormal 9+1 microtubule was also detected. Number of mitochondria per spermatozoon or spermatozoon-like cell in interspecific hybrid males ranged from 4 to 14. The tetraploid spermatozoon-like cell population was dominant in the studied hybrid loaches, and such 4n spermatozoon-like cell had large head and abnormal flagella. These results indicate that despite the absence of normal meiosis after replication of chromosomes (2n→4n) the spermiogenesis proceeds on schedule. The abnormal morphometric parameters of spermatozoa or spermatozoon-like cells from hybrid loaches are considered to be closely related to abnormal spermatogenesis. The reduced sperm motility may be explained by the malformations especially in flagellum which is the motor of the spermatozoon.

All-female diploid clonal lineages of the *Misgurnus* loach found in Japan are considered hybrid origin, and they spawn unreduced diploid eggs which develop without any genetic contribution of sperm donor (Morishima et al., 2002, 2008; Itono et al., 2006, 2007). When clonal diploid males are sex-reversed by administration with 17-α methyltestosterone, they produce unreduced diploid sperm with genetically identical genotypes (Yoshikawa et al., 2007). Such unreduced diploid spermatozoa exhibited a significant reduction in total motility (<5%) when compared with the spermatozoa from normal diploid and neo-tetraploid males. Electron microscopy revealed that diploid spermatozoa from sex-reversed clonal diploid and neo-tetraploid males exhibited normal shape similar to haploid spermatozoa from normal diploid males, except for larger sperm-head size and longer flagellum. Although the two kinds of diploid spermatozoa had the same volume of mitochondrial mass per spermatozoon, the number of mitochondria per spermatozoon did not increase in sex-reversed clonal diploid. This result suggests that mitochondria of spermatozoa from sex-reversed clone should become bigger in size. Diploid spermatozoa from sex-reversed clonal diploid males did not show increased ATP content. In neo-tetraploid male, diploid spermatozoa are apparently formed by meiosis and gametogenesis starting from tetraploid germ cells (Fujimoto et al., 2010). In contrast, diploid spermatozoa in sex-reversed clonal diploid males, which are considered to have a hybrid origin, are produced by chromosome doubling (premeiotic endomitosis) from diploid to tetraploid germ cells in early spermatogonial stages, followed by quasi-normal meiosis and spermatogenesis (Yoshikawa et al., 2009). Such difference may result in different motility of diploid spermatozoa between the two kinds of spermatozoa.

In chapter 4, hyper-triploid and hyper-tetraploid individuals were easily detected by flow cytometry using standard eudiploid, eutriploid, and eutetraploid controls. Hyper-triploid males were shown to be sterile, because they produced no fertile sperm comprising hexaploid range cell populations mostly. The results obtained from hyper-triploids were very similar to those from interspecific diploid hybrid which is discussed in chapter 2. It is probably that not due to the supernumerary microchromosome but the triploidy block the meiosis. On the contrary, hyper-tetraploids produced motile hyper-diploid spermatozoa; no differences were found between hyper-diploid spermatozoa and diploid spermatozoa in morphological characteristics and energy-related factors. These results strongly suggested that supernumerary microchromosomes did not affect the spermatogenesis. Then, the reproductive capacities of these hyperpolyploid males were examined. Viable progeny occurred in crosses between normal wild-type diploid females and hyper-tetraploid males, but androgenotes induced by the fertilization of UV-irradiated eggs with the sperm of hyper-tetraploid males were inviable hyper-diploids. Cytogenetic analyses of those androgenotes indicated that hyper-tetraploid males should produce hyper-diploid spermatozoa with $2n = 54$ (i.e., four supernumerary microchromosomes).

In this thesis, the author found that the irregular chromosome constitutions can change the morphological and physiological features of spermatozoa dramatically in loach. Similar results were also reported in other fishes with irregular chromosome constitutions. Thus, the author concludes that fish spermatogenesis is very sensitive to the chromosome constitutions of germ cell and there are some cellular and molecular mechanisms which check the chromosome constitutions of germ cell before meiosis. Atypical spermatogenesis may be triggered by irregular chromosome constitutions rather than expression of mutant gene(s) which is relevant to meiotic events. Further studies using cellular and molecular methods will provide a new insight into the mechanisms of gametogenesis.

学位論文審査の要旨

主　査	教　授	都　木　靖　彰	
副　査	教　授	荒　井　克　俊	
副　査	准教授	藤　本　貴　史	
副　査	教　授	山　羽　悦　郎	（北方生物圏フィールド科学センター）

学位論文題名

Studies on spermatozoa of polyploid, hybrid and clonal *Misgurnus* loaches

（倍数体、雑種およびクローンドジョウの精子に関する研究）

多くの魚類は二倍体で両性生殖により繁殖するが、倍数性、異数性、雑種、クローンなどの染色体構成に関する変異を持つ例も比較的多く見られる。これらの変異は天然集団のみならず、一部の種では人為的に誘起することもできる。この様な染色体変異は、減数分裂と配偶子形成過程に変化をもたらし、結果として、正常とは異なる多様な変異を持つ配偶子を形成することが予想される。本邦産ドジョウ *Misgurnus anguillicaudatus* には、両性生殖により繁殖する野生型二倍体(2n=50)のほか、非還元的に遺伝的に同一の二倍性卵を産し、これらの雌性発生により繁殖するクローン二倍体が一部地域に生息する。一方、中国には両性生殖により繁殖する二倍体(2n=50)に加えて、四倍体（4n=100）が同所的に生息し、これらは日本国内市場標本からも得られる。最近、異数体（高三倍体、高四倍体）ドジョウも市場標本から得られている。また、外来種カラドジョウ *M. mizolepis* の交雑を通じた国内在来種への影響が懸念されている。

倍数性、交雑、クローン性および異数性がどのような影響を精子形成に与えるかを検討するためには、以上のような変異体や近縁種を有するドジョウは好適なモデル生物である。本研究では、ドジョウの四倍体、ネオ四倍体、種間雑種、性転換クローン二倍体および異数体（高三倍体、高四倍体）雄を材料に、それらの産する精子（あるいは精子様細胞）について光学顕微鏡による運動性、濃度の観察、走査型、透過型電子顕微鏡による形態と微細構造の観察、フローサイトメトリーを用いた倍数性（細胞核DNA量）決定とミトコンドリア量測定、さらにATP含量測定を含む、一連の形態学的生理学的検討を行なった結果、以下の評価すべき成果を得た。

（1）四倍体の精子：市場標本（国外産起源不明）からフローサイトメトリーによる倍数性判定により選別した自然四倍体および二倍体野生型雌×自然四倍

体雄交配の後、第二極体放出阻止により作出したネオ四倍体を材料として、これらの精子を得たところ、いずれも正常二倍体の産する半数性精子の倍の DNA 量を持つ二倍性精子であった。二倍性精子は接水後 90%以上が活発な運動を示し、全体の構造に形態的異常は認められなかった。二倍性精子の平均の頭部サイズ（頭長・頭幅）は半数性精子の 1.24 倍であり、鞭毛（尾）も長かった。二倍性精子あたりのミトコンドリア数は半数性精子の 7-14 に対して 14-22 であり、Mito Tracker Green FM 染色後のフローサイトメトリーでも、二倍性精子のミトコンドリア量が多いことが判明した。二倍性精子の ATP 含量は半数性精子に比べ、著しく高かった。以上の結果より、四倍体、ネオ四倍体では減数分裂と精子形成は正常に進行し、機能的な精子が形成されると考えられた。また、二倍性精子が頭部サイズの肥大にもかかわらず、正常な運動能を示すことは、ミトコンドリア量と ATP 含量の増加に起因すると考えられた。

（2）雑種の精子：カラドジョウは日本国外に生息する外来種と考えられており、近年、その生息が国内で認められ、遺伝的な汚染が懸念される。人為的に作出したドジョウ雌×カラドジョウ雄の種間雑種雄の精液には、個体により半数体、二倍体、四倍体細胞集団が見られる場合とほぼ四倍体細胞集団のみが見られる場合がある。後者の四倍性精子様細胞では接水後の運動率は 5%以下であり、濃度も著しく低かった。また、約半数（47%）は鞭毛が 1 本であったが、無鞭毛（36%）や二鞭毛（17%）の細胞もみられ、鞭毛自体の平均長も正常半数体精子の約 23.9μm に比較して、12.5μm と短く、標準偏差も大きかった。精子様細胞の頭部サイズは正常半数性精子の約 1.6 倍であり、微細構造にも微小管の異常などが見られた。この様な異常な構造が雑種の精子（精子様細胞）の運動性を妨げているものと推察された。

（3）性転換クローンの精子：本邦には雌性発生により繁殖するクローン二倍体が生息するが、その子孫に雄性ホルモン処理を行うことにより、二倍性精子を産する性転換クローンを作出することができる。クローン雄の産生する二倍性クローンの運動性は著しく悪いが（5%以下）、頭部サイズが四倍体の産する二倍性精子同様に大きくなり、鞭毛が長くなっている点以外には、形態上の異常は見られなかった。クローン雄の二倍体精子のミトコンドリア量はネオ四倍体の作る二倍性精子と同様であったが、ミトコンドリア数は少ない傾向にあった。また、ATP 含量が正常な半数性精子よりは高いが、四倍体由来の二倍体に比べると著しく低いことが判明した。おそらく、これらのことが頭部の大きくなったクローン二倍性精子の貧弱な運動性に関連すると考えられた。

（4）異数体（高三倍体、高四倍体）の精子：高三倍体および高四倍体は四倍体と同様に、市場標本からフローサイトメトリーにより核 DNA 量を測定して選別した。これらは正三倍体、正四倍体個体と比べ過剰な DNA 量をもつ異数体である。高三倍体の産する精子（精子様細胞）は運動性をもたず、六倍体相当の DNA 量を有していた。高三倍体の精子（精子様細胞）は種間雑種の産する精子と同様の形態異常を示し、大型の頭部を有し、短い鞭毛を有した。また、鞭毛 1 本を持つ細胞のほか、無鞭毛と複数鞭毛の細胞が見られた。ミトコンドリア数は正常な半数性精子と同程度であったが、ATP 含量は高く四倍体、ネオ四倍体と同程度であった。

一方、高四倍体は接水後活発な運動性を示す高二倍性精子を産生した。これらの精子の形態や運動能力は四倍体あるいはネオ四倍体の形成する二倍性精子と同様であり、ミトコンドリア数、量、ATP 含量もそれらと同様であった。

そこで、これら精子を用いて、野生型正常二倍体の卵を授精したところ、子孫として正常な胚が生じ、その倍数性は三倍体（高三倍体を含む）の範囲にあった。しかし、紫外線照射により卵核不活性化を施した卵に授精した場合、正常な胚は発生せず、孵化に至らなかった。染色体観察からこれらの胚は 54 本の染色体をもつことが判明し、高二倍性精子は正二倍性精子に比較して 4 本過剰な微小染色体をもつことが分かった。

申請者による以上の研究成果は、染色体変異の精子形成への影響のみならず、育種素材としての倍数性、異数性、クローン精子等の形態と機能を明らかにしたもので、基礎生物科学および水産科学上重要な成果と評価でき、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。