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ORIGINAL ARTICLE

Non-motile tetraploid spermatozoa of *Misgurnus* loach hybrids

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Abstract We have compared various properties of spermatozoa from the wild diploid male pond loach *Misgurnus anguillicaudatus* to those from the interspecific male hybrid of the cross between a female *M. anguillicaudatus* and a male mud loach *M. mizolepis*. Our results show that spermatozoa from this interspecific hybrid had poor motility, low viability, abnormal morphology, a larger volume of mitochondrial mass per cell and higher ATP content of spermatozoa with tetraploid DNA content, and they were present at a low concentration. The interspecific hybrid males produced spermatozoa with a larger head, with either no flagellum (36.4 %), one flagellum (46.7 %) or two flagella (16.9 %). These flagella were shorter than those of the normal wild-type male *M. anguillicaudatus* and often presented with abnormalities in microtubule structure. An abnormally shorter flagellum has difficulty in propelling tetraploid spermatozoa with an increased head size in normal progressive motility, although they had higher energy, as shown by their larger volume of mitochondrial mass and higher ATP content. These tetraploid spermatozoa are likely produced by the arrest of the regular meiotic division after chromosomal replication, followed by abnormal spermiogenesis.

Introduction

Hybrid female fishes between genetically closed species are often fertile and able to produce fertile eggs that can develop normally after fertilization with sperm of the parental species [1–5]. To the contrary, hybridizations between remotely related species often give rise to sterile progeny even if the progeny are able to survive to the adult stage, while hybrids from several combinations of different species produce fertile eggs and then generate viable progeny by means of alternative atypical reproductive processes, such as unreduced oogenesis, clonal gynogenesis or semi-clonal hybridogenesis [6–9]. Hybrid male fishes are often sterile even when they are the offspring of combinations between relatively closed species, such as Japanese char *Salvelinus leucomaenis* × brook trout *S. fontinalis* [2], among others [5]. However, there are a few exceptions; for example, fertile unreduced diploid spermatozoa were reported in Iberian minnow with a natural hybrid origin [10], common carp × crucian carp hybrids [11–13] and sex-reversed clonal loach which is considered to have a hybrid origin [14, 15].

In Japan, the mud loach, *Misgurnus mizolepis*, is a well-known exotic cobitid species (Teleostei: Cobitidae) which has been observed and recorded in several areas [16]. *M. mizolepis* is morphologically distinguishable from the pond

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loach, *M. anguillicaudatus*, which is widely distributed throughout Japan [17], and is considered to be a synonym of *Paramisgurnus dabryanus* [16, 18]. *M. anguillicaudatus* has $2n = 50$ chromosomes—ten of which are metacentric (m), four are submetacentric chromosomes (sm) and 36 are acrocentric (a) [19], while *M. mizolepis* has $2n = 48$ with a karyotype of 12 m + 4 sm + 32 a [20]. Since both species have the same arm number (NF) of 64, the interspecific karyotype difference can be well explained by Robertsonian translocation, i.e., centric fusion or fission [20]. Reproductive performance has been examined in interspecific hybrids between *M. anguillicaudatus* and *M. mizolepis* to assess the genetic influence of exotic species on indigenous species. Park et al. [21] reported fertility of hybrid males based on the histologically proven presence of spermatozoa. Fujimoto et al. [22] artificially induced *M. anguillicaudatus* female × *M. mizolepis* male hybrids and then observed that a number of the hybrid males had testes, including haploid, diploid and tetraploid cell populations, while others had mainly tetraploid cells. These authors also reported that of the spermatozoa from hybrid males, only haploid spermatozoa were fertile, and they generated the next generation of the progeny by backcrossing [22]. In the case of haploid spermatozoa, spermatogenesis should successfully proceed due to regular meiotic division between balanced chromosomes in the hybrid because there are little or no differences in chromosomal dosage between *M. anguillicaudatus* and *M. mizolepis*. On the other hand, tetraploid spermatozoa from the hybrid males were not fertility, although they might have been matured and spermiated without the completion of meiosis [22].

Little is known about the physiological and morphological characteristics of tetraploid spermatozoa in hybrid males. Here, we investigated motility-related parameters, such as total motility, progressive motility, duration of motility, concentration and viability, in spermatozoa collected from hybrid males. We then observed the ultrastructure of the tetraploid spermatozoa by electron microscopy and measured the head length, head width, flagellum length and number of mitochondria in these spermatozoa. Finally, the volume of mitochondrial mass per cell and ATP content were estimated.

Materials and methods

Ethics statement and fish used

This study was performed in accordance with the "Guide for the care and use of laboratory animals in Hokkaido University." The fishes were kept in the aquarium of the Environment Control Experiment Building, Faculty and Graduate School of Fisheries Sciences, Hokkaido University.

Three adult normal wild-type diploid *M. anguillicaudatus* males [range of standard length (SL) 75–80 mm] and three adult interspecific diploid hybrid males (range of SL 78–83 mm) were used for this study. Normal wild-type diploid *M. anguillicaudatus* loaches were obtained from Kitamura, Iwamizawa City, Hokkaido. Interspecific diploid hybrid males were produced by fertilizing eggs of *M. anguillicaudatus* with sperm of *M. mizolepis* [22]. Pure mud loach *M. mizolepis* males were not available for this study.

Collection of spermatozoa

Sperm collection was performed according to Fujimoto et al. [22]. The ploidy status of the sperm sample from each individual was assessed by flow cytometry as described previously [22–25]. For this evaluation, the collected samples were immediately placed in 1.5-ml microtubes containing 1 ml immobilizing solution (IS) (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl$_2$, 2.4 mM NaHCO$_3$; Kurokura et al. [26]), followed by vortexing. The diluted sperm samples were subsequently stored at 4 °C prior to analysis. For the electron microscopy study, the collected samples were immediately mixed with 2.5 % glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2).

Evaluation of motility, concentration and viability of spermatozoa

Motility was assessed using our previously described procedures for loach [23, 24, 27]. Total motility (%), progressive motility (%) and the duration of motility (s) were determined from the analysis of video sequences recorded on a video system (model VHS VC-HF920; Sharp Corp., Osaka, Japan) that included subjective visualization of sperm movement based on Iwamatsu et al. [28]. The proportion of total motility and progressive motility and the duration of motility were measured in triplicate for each sample evaluated. The spermatozoa diluted in IS were fixed by the fixative solution (1 % formalin, 5 % NaHCO$_3$) and cell numbers were counted three times using Thoma's counting chamber in each sample after sedimentation of spermatozoa for 5 min. The average concentration value was also calculated. The viability of spermatozoa was assessed by the DUAL-staining [SYBR-14 and propidium iodide (PI)] procedure using the LIVE/DEAD Sperm Viability kit (Molecular Probes, Inc. Eugene, OR) following the instructions of the manufacturer. The evaluation was carried out under a fluorescence microscope (model ECLIPSE E800; Nikon Corp., Tokyo, Japan). One hundred spermatozoa from each sample were counted to determine the percentage of PI-negative (live) and positive (damaged or dead) spermatozoa.

Electron microscopy

The ultrastructure of the spermatozoa was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), according to previously described procedures for loach sperm [23, 24]. The morphological characteristics of spermatozoa were evaluated by SEM. The length of the head of spermatozoa was measured along the head–tail axis from the anterior tip to the posterior tip of the head. Width was determined as the largest transverse distance of the head of a spermatozoon. For the measurement of head size of spermatozoa without flagellum, major and minor diameters were used, i.e., the length and width of the head of spermatozoa, respectively. For flagellum length, the length of the tail part without the mid-piece was measured. Number of mitochondria per spermatozoon was counted in TEM images from 180 different cells of wild-type diploid males and hybrid males.

Estimation of volume of mitochondrial mass per spermatozoon

Spermatozoa were stained with MitoTracker Green FM (MTGFM: Molecular Probes, Inc.) according to Zhao et al. [23, 24]. MTGFM is a mitochondrion-specific probe that becomes fluorescent in the lipid environment of mitochondria. This probe contains a thiol-reactive chloromethyl moiety, resulting in stable peptide and protein conjugates following its accumulation in mitochondria. MTGFM appears to preferentially accumulate in mitochondria regardless of the mitochondrial membrane potential ($\Delta\Psi$m), making it an important tool for determining mitochondrial mass [29, 30]. Spermatozoa were collected from each of the three normal wild-type diploid males and then mixed together, following which the sample was diluted to obtain a final concentration of 10^6 cells ml^{-1} prior to analysis. Samples from the three hybrid males were prepared in the same way. The spermatozoa were assessed using a flow cytometer (model EPICS ALTRA Flow Cytometer Cell Sorter; Beckman-Coulter, Brea, CA) equipped with an argon laser at 488 nm and a 525-nM filter to detect fluorescence. Forward and side scatter (hereafter abbreviated FS and SS, respectively) from the cells were used to observe the cell distribution profile. Flow-check™ Fluorescent beads (10 μm; Beckman-Coulter) were used to optimize the analyzer. The data generated by the flow cytometer were plotted in a single dimension to produce a histogram. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates."

Measurement of ATP content

The sperm samples collected from each of the three wild-type males and each of the three hybrid males were diluted 100-fold in IS prior to measure the ATP content. ATP content was measured using Bioluminescence Assay kit HS II (Roche Diagnostics GmbH, Hoffmann-La Roche, Basel, Switzerland), following the instructions of the manufacturer [23, 24]. Luminescence was read with a Luminescencer-JNRII AB-2300 (ATTO Co. Ltd., Tokyo Japan). The ATP content of each sample was expressed as nmol ATP/10^9 spermatozoa. The ATP content of each sample was measured six times.

Statistical analysis

The data on the morphological parameters and ATP content were tested for statistical significance using one-way analysis of variance with the Least-Significant Difference post hoc test in SASS version 11.0 (IBM Corp., Armonk, NY). Statistical significance was set at $p < 0.05$.

Results

Ploidy status of sperm

The major proportion of sperm cells from the normal wild-type diploid males ($n = 3$) had 1C DNA content (Fig. 1a), while that of sperm cells from the interspecific hybrid males ($n = 3$) had a DNA content corresponding to 4C (Fig. 1b). These results show that the hybrid males produced tetraploid spermatozoa, and the control diploid males produced haploid spermatozoa.

Viability, concentration and motility of spermatozoa

Various parameters of the spermatozoa from normal diploid and interspecific hybrid are shown in Table 1. Haploid spermatozoa from normal diploid exhibited vigorous total motility (91.7 %), active progressive motility (87.3 %) and a long motility duration (175.0 s). In contrast, poor total motility (<5 %), no progressive motility and a short motility duration (96.7 s) were detected in sperm from the interspecific hybrid males. On average, the concentration of spermatozoa in the hybrid males was distinctly lower than that in the normal diploid males (32.4×10^6 vs. 3090.0×10^6 cells/ml, respectively).

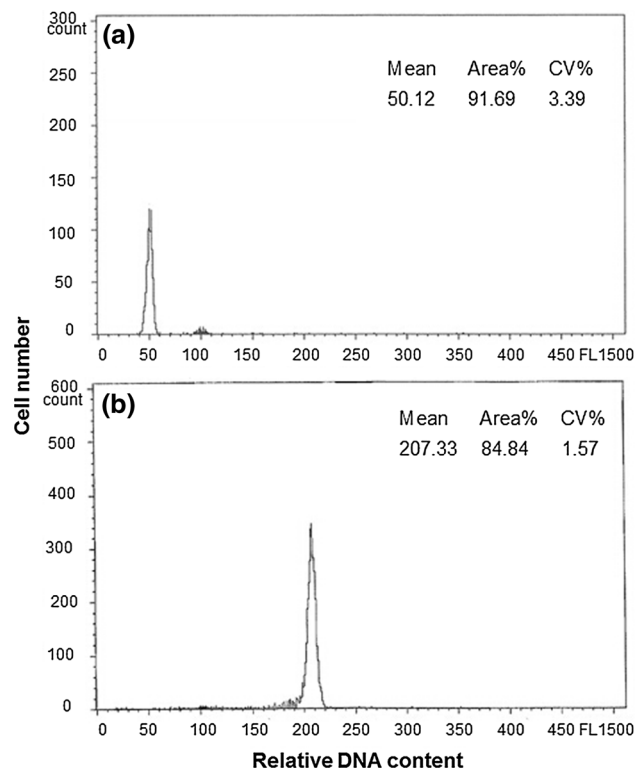**Fig. 1** Flow cytometry histograms of sperm samples from normal diploid wild-type pond loach *Misgurnus anguillicaudatus* (**a**) and from hybrids between a *M. anguillicaudatus* female and a mud loach *M. mizolepis* male (**b**). Fluorescence peak of haploid spermatozoa from normal wild-type diploid loaches indicates a DNA content of 1C (**a**), and that of tetraploid spermatozoa from interspecific hybrid loaches indicates a DNA content of 4C (**b**). *Mean* Average relative DNA content detected automatically by the flow cytometer, *Area %* proportion of cells contained in the highest peak of the histogram relative the total number of cells analyzed, *CV %* coefficient of variation of the histogram × 100 (i.e. CV % = standard deviation/average × 100)

Microscopic evaluations of spermatozoa

About one-half of the spermatozoa from the hybrids had one flagellum (46.7 %), but spermatozoa without a flagellum (36.4 %) or with two flagella (16.9 %) were also found (Fig. 2; Table 2). Average flagellum length (± standard deviation) of hybrid spermatozoa (cells without flagellum were not measured) was 12.47 ± 7.05 µm and clearly shorter than that of the normal spermatozoa (23.85 ± 2.29 µm) ($p < 0.05$) (Fig. 2; Table 3). The head length of spermatozoa from the hybrid males ranged from 2.11 to 3.49 µm (Figs. 2, 3), and spermatozoa from these hybrid males without a flagellum had a slightly longer head than those with one flagellum or two flagella (2.99 ± 0.36 vs. 2.75 ± 0.21 µm, respectively; $p < 0.05$) (Table 3). The average head size (length/width of head) of tetraploid spermatozoa from the hybrid males ($2.83 \pm 0.24/2.80 \pm 0.24$ µm) was approximately 1.6-fold larger than that of normal spermatozoa from the wild-type diploid male loaches ($1.80 \pm 0.08/1.80 \pm 0.07$ µm) ($p < 0.05$) (Table 3). The ratios of head length to head width were approximately 1.0, i.e., sphere like, in all spermatozoa observed and were not significantly different between wild-type diploid and hybrid males (Table 3). The head length to flagellum length ratio in tetraploid spermatozoa from hybrid males (0.225 ± 0.213) was significantly different from that of normal spermatozoa (0.075 ± 0.09) ($p < 0.05$) (Fig. 2; Table 3). The forward light-scattering linear scale (FS Lin) from the flow cytometry analysis revealed that spermatozoa from hybrid males had larger cell volumes than those of wild-type diploid males, but significant differences between these two groups in inner morphological complexity were not detected by the side light-scattering linear scale (SS Lin) between (Fig. 4).

Table 1 Average viability, concentration, total motility, progressive motility and duration of motility of spermatozoa from the normal diploid wild-type pond loach (*Misgurnus anguillicaudatus*) and from hybrids between a *M. anguillicaudatus* female and a mud loach *M. mizolepis* male

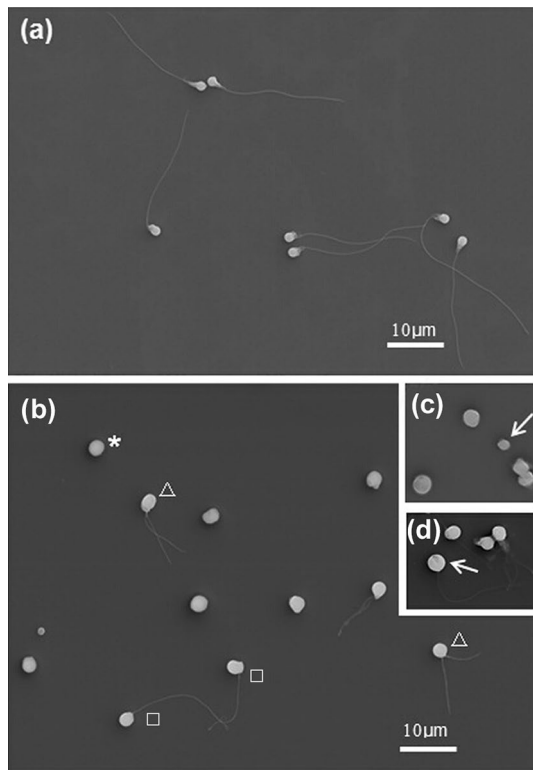| Biotype	Fish no.	Viability (%)	Concentration (×10⁶cells/ml)	Total motility (%)	Progressive motility (%)	Duration of motility (s)
Normal wild-type diploid male	1	90	2950	90	85	170
	2	95	3200	95	91	180
	3	90	3120	90	86	175
	Mean of 1–3	91.7	3090.0	91.7	87.3	175.0
Interspecific hybrid male	1	75	54.3	<5	0	95
	2	75	19.2	<5	0	105
	3	80	23.8	<5	0	90
	Mean of 1–3	76.7	32.4	<5	0.0	96.7

Fig. 2 Scanning electron microscopy study of spermatozoa from normal diploid (**a**) and from interspecific hybrid (**b**, **c**, **d**) male loaches. **b** *Asterisk* Cell without flagellum, *triangle* cell with two flagella, *square* cell with one flagellum, **c** *white arrow* cell with a relatively small size, **d** cell with a relatively large size

Table 2 Percentage of abnormal spermatozoa in spermatozoa of normal diploid wild-type and interspecific hybrid male loaches

Flagellum characteristics	Normal diploid wild-type pond loach	Hybrid loach
N	90	165
No-flagellar spermatozoa	0	60 (36.4 %)
Mono-flagellar spermatozoa	90 (100 %)	77 (46.7 %)
Bi-flagellar spermatozoa	0	28 (16.9 %)

Although patch- or spot-like vesicles or vacuoles were seldom detected in the condensed nucleus of spermatozoa from normal diploid wild-type males (Fig. 5a), such structures were detected in the condensed nucleus of spermatozoa from hybrid males (Fig. 5b). Some spermatozoa with two flagella (Fig. 5b, c) exhibited separate cytoplasmic channels (Fig. 5b, d), while others with two flagella had a communal cytoplasmic channel (Fig. 5c, e). Only the normal (i.e., 9 + 2) microtubule structure was found in spermatozoa from wild-type diploid males (Fig. 5f), but both abnormal (i.e., 9 + 1) and normal (i.e., 9 + 2) microtubule structures were found in spermatozoa from hybrid males (Fig. 5g).

The mean number of mitochondria counted in spermatozoa ($n = 180$) was similar between the control diploid wild-type and interspecific hybrid males, but spermatozoa with smaller numbers (4–6) of mitochondria were present in hybrids (Fig. 6).

Volume of mitochondrial mass per spermatozoon

Total volume of mitochondrial mass per spermatozoon from the interspecific hybrid male loaches was larger than that from the normal diploid wild-type loaches (Fig. 7).

ATP content of spermatozoa

The ATP content of sperm from interspecific hybrid males had a higher ATP content (257.37 ± 8.30 nmol/10^9 spermatozoa) than those from normal diploid wild-type males (80.06 ± 5.16 nmol/10^9 spermatozoa) ($P < 0.05$) (Fig. 8). The inter-male variability for ATP content was low in all spermatozoa.

Discussion

In the present study, we found that interspecific hybrid *M. anguillicaudatus* female × *M. mizolepis* males predominantly produced non-motile tetraploid spermatozoa which had approximately 1.6-fold larger head sizes than those of normal haploid spermatozoa from normal wild-type diploid males. In addition, about 47 % of spermatozoa from the interspecific hybrid male loaches had one abnormally short flagellum, 17 % had two flagella and 36 % had no flagellum. These results demonstrate that the diploid hybrid male loaches generated abnormal spermatozoa (or spermatozoon-like cells without flagellum) with replicated chromosomes equivalent to a DNA content of 4C, which underwent the process of spermiogenesis. Our observations of the production of spermatozoa with an unusually large head size, a nucleus with 4C DNA content and differentiated flagellum(a) were quite similar to those reported by Shimizu et al. in the interspecific hybrid males between *Oryzias latipes* and *O. curvinotus* [31]. In these medaka hybrids, the authors concluded that there was arrest of the meiotic cell cycle based on cytological observation, absence of the expression of protamine mRNA and a cell culture of primary spermatocytes, in which production of one spermatozoon-like cell from one spermatocyte isolated from the hybrid was observed [31]. We suggest that abnormal spermatozoa in the interspecific hybrid male loach were also likely produced by meiotic arrest after the replication of chromosomes, similar to that observed in the medaka hybrids, resulting in an elevation of chromosomal content from 2C (diploidy) to 4C (tetraploidy). This meiotic

Table 3 Morphometric characteristics of spermatozoa of normal diploid wild-type and interspecific hybrid male loaches

Biotype	Fish no.	Type of flagella	N	Head size of spermatozoa (μm)		Length of flagellum (μm)	Head length/head width	Head length/flagellum length
				Length of head	Width of head			
Normal diploid	1	MonoF	30	1.80 ± 0.07 a	1.80 ± 0.07 a	23.89 ± 2.35 a	1.00 ± 0.052 a	0.075 ± 0.010 a
	2	MonoF	30	1.81 ± 0.09 a	1.79 ± 0.06 a	23.42 ± 2.26 a	1.01 ± 0.062 a	0.077 ± 0.008 a
	3	MonoF	30	1.80 ± 0.08 a	1.80 ± 0.07 a	24.25 ± 2.25 a	1.00 ± 0.049 a	0.074 ± 0.008 a
	Mean of 1–3		90	1.80 ± 0.08*	1.80 ± 0.07*	23.85 ± 2.29 a	1.00 ± 0.054 a	0.075 ± 0.009 a
Hybrid	1	NoF	19	3.00 ± 0.38 b	2.97 ± 0.35 b	–	1.01 ± 0.078 a	–
		MonoF	24	2.76 ± 0.19 c	2.76 ± 0.20 c	11.69 ± 7.21 b	1.00 ± 0.071 a	0.240 ± 0.250 b
		BiF	9	2.74 ± 0.24 c	2.71 ± 0.22 c	13.55 ± 6.92 b	1.01 ± 0.076 a	0.209 ± 0.186 b
	2	NoF	19	2.99 ± 0.35 b	2.96 ± 0.33 b	–	1.01 ± 0.077 a	–
		MonoF	26	2.74 ± 0.19 c	2.71 ± 0.19 c	12.45 ± 7.11 b	1.01 ± 0.072 a	0.224 ± 0.235 b
		BiF	10	2.74 ± 0.23 c	2.71 ± 0.21 c	11.91 ± 7.09 b	1.01 ± 0.075 a	0.232 ± 0.217 b
	3	NoF	22	2.98 ± 0.35 b	2.98 ± 0.33 b	–	1.00 ± 0.076 a	–
		MonoF	27	2.77 ± 0.18 c	2.74 ± 0.20 c	13.21 ± 7.01 b	1.01 ± 0.074 a	0.211 ± 0.201 b
		BiF	9	2.76 ± 0.21 c	2.73 ± 0.19 c	12.04 ± 6.98 b	1.01 ± 0.076 a	0.238 ± 0.194 b
	Mean of NoF		60	2.99 ± 0.36 b	2.97 ± 0.34 b	–	1.01 ± 0.077 a	–
	Mean of MonoF and BiF		105	2.75 ± 0.21 c	2.72 ± 0.20 c	12.47 ± 7.05 b	1.01 ± 0.074 a	0.225 ± 0.213 b
	Mean of 1–3		165	2.83 ± 0.24*	2.80 ± 0.24*	12.47 ± 7.05 b	1.01 ± 0.075 a	0.225 ± 0.213 b

Data for head length, head width, length of flagellum and head length:head width ratio are presented as the mean ± standard deviation (SD) Values within a column followed by different lowercase letters or values marked with an * are significantly different at $p < 0.05$

N number of cells measured, *NoF* no-flagellar spermatozoa, *MonoF* monoflagellar spermatozoa, *BiF* biflagellar spermatozoa
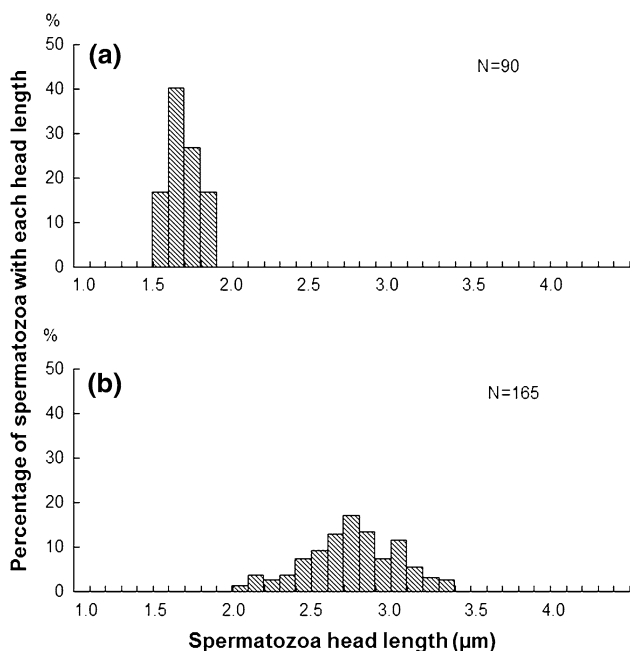

Fig. 3 Head length of spermatozoa from normal diploid wild-type (**a**) and interspecific hybrid (**b**) male loaches. *Columns* represent the percentages of spermatozoa with each head length. N number of the cells measured

arrest is presumably due to the failure of pairing between homologous chromosomes derived from the different species. However, as in medaka hybrids, loach hybrids also produced unusual spermatozoa with flagellum(a) by the process of spermiogenesis.

Fujimoto et al. [22] studied hybrids from the same interspecific cross as those in our study and reported 13.2 $\times 10^6$ cells/ml concentration, 4.1 % (range 0–8 %) total motility, 21.9 % progressive motility (range 0–39 %) and 102.9 s duration of motility (range 63–131 s) for spermatozoa. These parameters are almost the same as ours with the exception of the 0 % progressive motility our study. Since Fujimoto et al. [22] reported the occurrence of a very small number of viable diploid progeny after backcrossing a hybrid male to an *M. anguillicaudatus* female, the difference in progressive motility between the two studies could be explained by the proportion of motile haploid spermatozoa. Also, previous genetic studies using microsatellite DNA markers revealed that the above-mentioned viable diploid progeny possessed alleles derived from two species, and thus fertile haploid spermatozoa were formed by meiotic segregation even in the inter-specific hybrid male [22]. At the present time, however, it is not known why some hybrid males generate motile haploid spermatozoa

Fig. 4 Volume and morphological complexity of spermatozoa assessed by flow cytometry from normal diploid wild-type (**a**) and interspecific hybrid (**b**) male loaches. *FS Lin* Forward light-scattering linear scale, indicates the volume of cells; *SS Lin* side light-scattering linear scale, indicates complexity of inner structure of cells
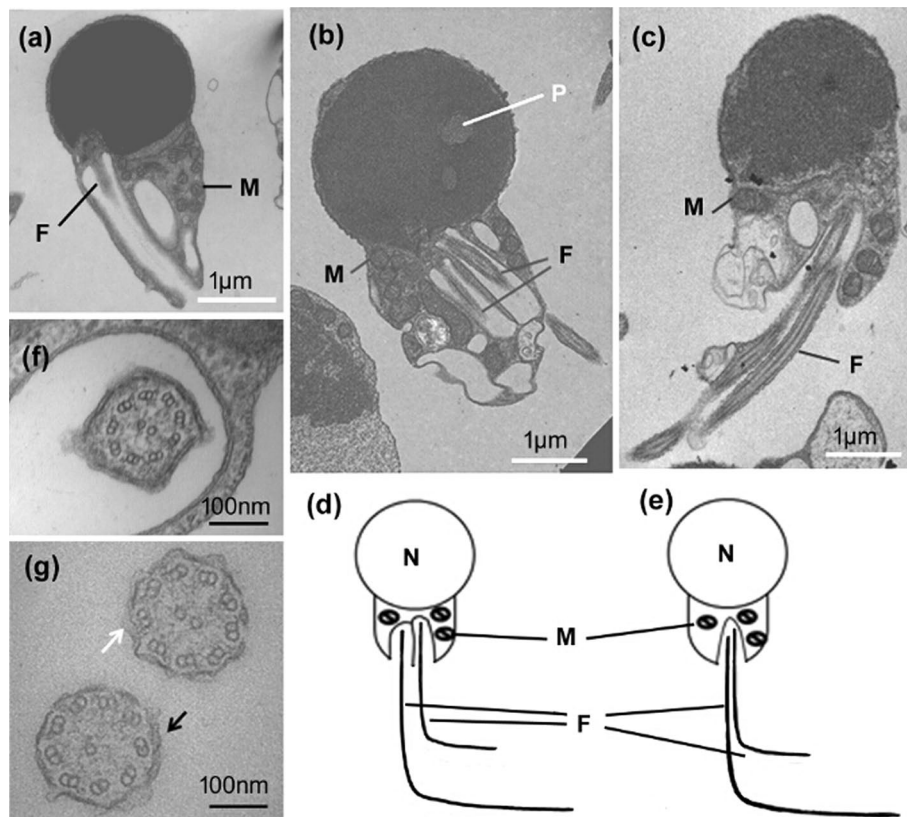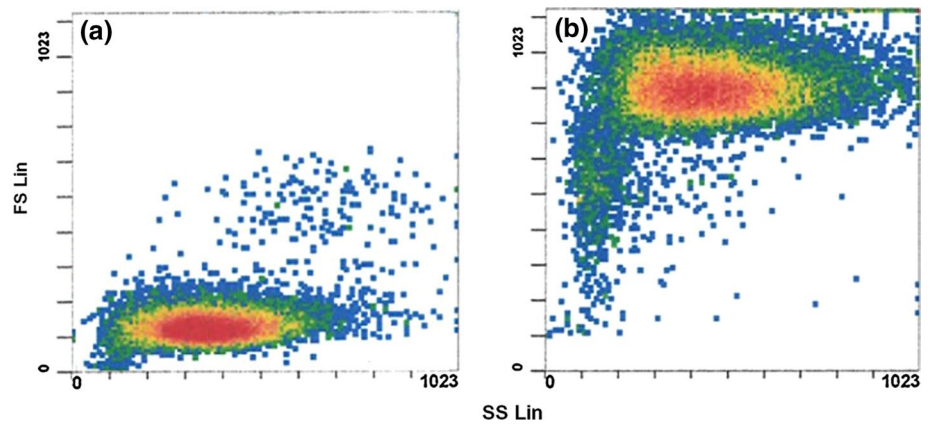

Fig. 5 **a–c**, **f**, **g** Transmission electron microscopic (TEM) images of a spermatozoon from a normal diploid wild-type male loach (**a**), bi-flagellar spermatozoon with separate cytoplasmic channels from an interspecific hybrid male loach (**b**), bi-flagellar spermatozoon with a communal cytoplasmic channel from an interspecific hybrid male loach (**c**), typical 9 + 2 microtubule structure of flagellum of spermatozoon from a normal diploid wild-type male loach (**f**) and typical 9 +2 (*white arrow*) and abnormal 9 + 1 (*black arrow*) microtubule structure of flagellum from an interspecific hybrid male loach (**g**). **d**, **e** Schematic representation of bi-flagellar spermatozoon with separate cytoplasmic channels (**d**) and bi-flagellar spermatozoon with a communal cytoplasmic channel (**e**). **d**, **e** are schematic representations of **b**, **c**, respectively. *F* Flagellum, *M* mitochondrion, *N* nucleus, *P* patch- or spot-like vesicles or vacuoles (**a–e**).

by meiosis and that other males do not due to the arrest of meiosis after the replication step. A question which remains to be answered is: What is the difference between the two cases in terms of spermatogenesis of *Misgurnus* hybrids? Further cytogenetic and molecular studies are required to answer this question.

Total volume of mitochondrial mass per spermatozoon and ATP content of sperm apparently increased in the interspecific hybrid male loaches, possibly compensating for the reduced motility due to the increased sperm head sizes due to the elevation of ploidy status or the increase in genetic material content in the cell nucleus. However,
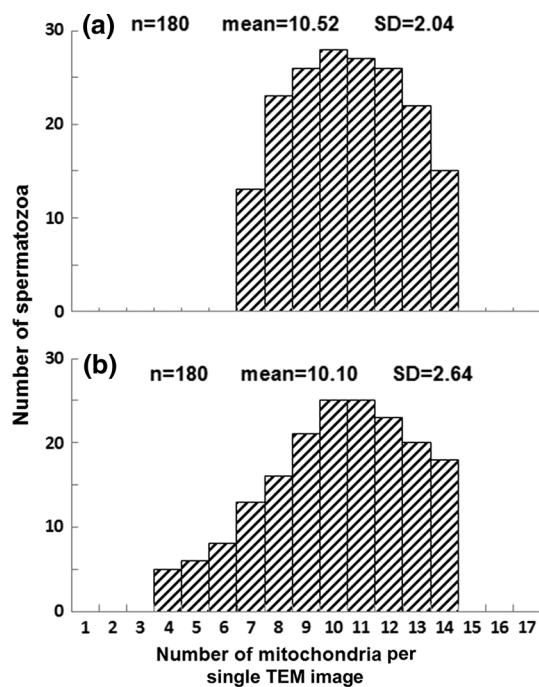
Fig. 6 Number of mitochondria (mean ± standard deviation) counted in TEM sections from normal diploid wild-type (**a**) and interspecific hybrid (**b**) male loaches
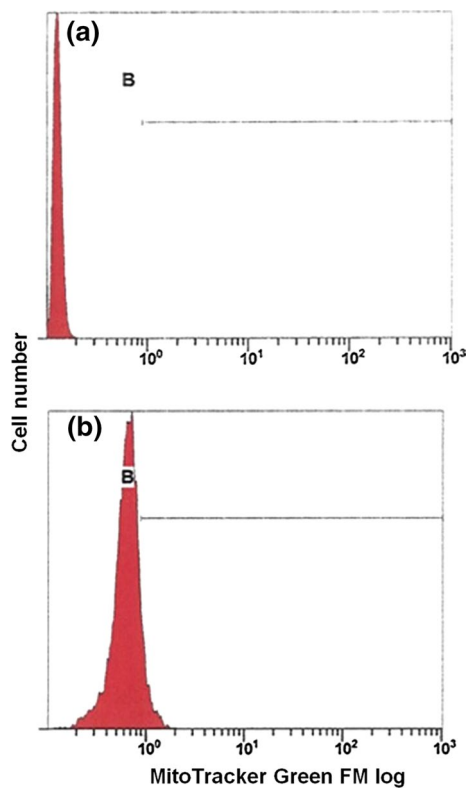

Fig. 7 Total volume of mitochondrial mass per spermatozoon assessed by flow cytometry from normal diploid wild-type (**a**) and interspecific hybrid (**b**) male loaches. *X* axis MitoTracker Green FM intensity (logarithmic scale), *Y* axis cell counts (linear scale)
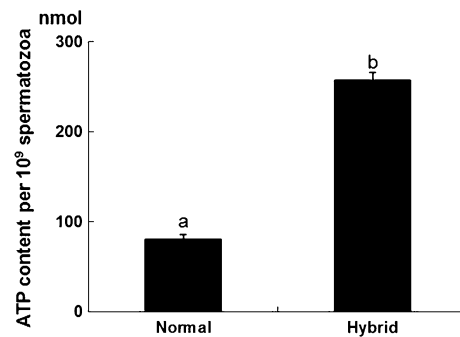

Fig. 8 ATP content of spermatozoa from loach males. Significant differences in ATP content were recorded between haploid spermatozoa from normal diploid wild-type male loaches (*Normal*) and tetraploid spermatozoa from interspecific hybrid males (*Hybrid*). *Different lowercase letters at top of column* indicate significant differences at $p < 0.05$

the spermatozoa of these hybrids did not exhibit active progressive motility. We found poor motility in tetraploid spermatozoa, possibly due to malformations, especially in the flagellum which is the motor of the spermatozoon. The absence of a flagellum or the presence two short flagella apparently inhibited the propulsion movement of spermatozoa with increased head sizes. In addition, in spermatozoa with one flagellum, the length of the flagellum was significantly shorter than that of wild-type diploid male loaches. Similar situations have been reported in abnormal hexaploid spermatozoa formed in hyper-triploid loaches [24].

Another factor related to the reduced motility of the spermatozoa from the interspecific hybrid male loaches may be the abnormal microtubule structure of the axoneme. Although the axoneme of a motile flagellum has two central microtubule singlets in addition to the nine outer doublets (called a 9 + 2 axoneme), tetraploid spermatozoa often show a 9 + 1 structure. Such a deviation from the typical 9 + 2 microtubule structure has been reported to be linked to the formation of non-motile flagellum [32]. Abnormalities in flagellar number (no-flagellar or bi-flagellar) and structure (9 + 1 axonema and others) have also been found in hexaploid spermatozoa of the hyper-triploid loach [24]. The number of mitochondria in tetraploid spermatozoa from the interspecific hybrid male loaches in our study was similar to that observed in hexaploid spermatozoa from the hyper-triploid loach, with almost the same mean and standard deviation, in spite of the large difference in ploidy status [24]. These common morphological features are likely caused by the spermiogenesis of unusual spermatids which are formed without two successive meiotic divisions. Therefore, abnormalities of spermatozoa of interspecific hybrid male loaches may be closely linked to the unusual process of spermiogenesis without the completion of the meiotic divisions.

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