



HOKKAIDO UNIVERSITY

Title	The occurrence of hypertetraploid and other unusual polyploid loaches <i>Misgurnus anguillicaudatus</i> among market specimens in Japan
Author(s)	Zhao, Yan; Toda, Masakuni; Hou, Jilun et al.
Citation	Fisheries science, 78(6), 1219-1227 https://doi.org/10.1007/s12562-012-0556-7
Issue Date	2012-11
Doc URL	https://hdl.handle.net/2115/51004
Rights	© 2012 公益社団法人日本水産学会
Type	journal article
File Information	second revise for Fish. Sci Zhao Yan.pdf



Second Revised Version FISC-D-12-00149

The occurrence of hyper-tetraploid and other unusual polyploid loaches *Misgurnus anguillicaudatus*
among market specimens in Japan

Yan Zhao • Masakuni Toda • Jilun Hou • Mami Aso • Katsutoshi Arai

Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, 041-8611,
Japan

Katsutoshi Arai (Corresponding author) E-mail: araikt@fish.hokudai.ac.jp; FAX. +81-138-40-5537

Abstract

Exotic animals may cause a genetic contamination of indigenous species if they escape and reproduce in wild populations. Loach *Misgurnus anguillicaudatus* and its related species have been imported to Japan for commercial uses. We collected live loach specimens from central wholesale market in Tokyo. Among 451 specimens, ploidy status was examined by DNA content flow cytometry and polyploid loaches with triploid, tetraploid and other higher DNA content ranges were detected. Hyper-triploid and hyper-tetraploid individuals could be easily detected by flow cytometry using a standard euploid, eutriploid and eutetraploid controls and then reproductive capacity of these hyper-polyploid males was examined. Sperm of hyper-triploid males did not exhibit active progressive motility and major populations of spermatozoa or spermatozoon-like cells were detected in triploid and hexaploid ranges. Motile haploid spermatozoa were very few in sperm from hyper-triploid males. Therefore, hyper-triploid males were sterile, whereas hyper-tetraploid males produced fertile hyper-diploid spermatozoa with active progressive motility after contact with ambient water. Viable progeny occurred in the cross between normal wild-type diploid female and hyper-tetraploid males, but androgenotes induced by fertilization of UV-irradiated eggs with sperm of hyper-tetraploid males were inviable hyper-diploid. Cytogenetic analyses in such androgenotes indicated that hyper-tetraploid males should produce hyper-diploid spermatozoa with $2n=54$, i.e. presence of four supernumerary micro-chromosomes.

Keywords Androgenesis • Aneuploidy • Conservation • Euploidy • Exotic species

Introduction

Exotic live fishes, which are introduced for foods, live baits of fishing, aquarium animals and other purposes, often threaten biodiversity of indigenous ecosystems, when they escape into the wild. Genetic disturbances have been strongly suggested in Japanese wild populations of medaka [1] and bitterlings [2]. Most individuals of *M. anguillicaudatus* are sexually reproducing diploid organisms with 50 chromosomes ($2n=50$) in Japanese wild populations, but gynogenetically reproducing clonal diploid ($2n=50$) and clone-origin triploid ($3n=75$) individuals sympatrically occur in a few wild populations of northern Hokkaido and Noto Peninsula in Ishikawa Prefecture, Honsyu Island, Japan [3, 4]. In other localities, frequencies of natural triploid loach were generally low except for several places [5, 6]. Although tetraploid loaches with 100 chromosomes ($4n=100$) are distributed along Changjiang river system in central China together with diploid and triploid individuals [7-11], so far natural tetraploid individuals have not been found in Japanese wild populations [3-6]. Therefore, tetraploid individuals, which have been detected in Japanese market samples since 1970s [12, 13], are likely to be Chinese loaches imported to Japanese markets. Such an occurrence of tetraploid loaches in Japanese market samples suggests simultaneous import of sympatric diploid and triploid Chinese loaches to Japan.

Inferred from mitochondrial (mt) DNA sequences analyses, individuals of Japanese *M. anguillicaudatus* can be categorized into three different groups: A, B-1 and B-2 in Morishima et al. [4] or A, B and C in Koizumi et al. [14]. Arai [6] suggested that the group B-2 in [4] might be originated or strongly related to Chinese loach population. Koizumi [15] came to the same conclusion about the B group in [14] distributed in Japanese wild populations. Introduction of exotic individuals into natural waters in Ehime Prefecture, Shikoku Island was recently indicated by results of partial mtDNA analysis

[16]. These results suggest that genetic disturbance might occur by intra-specific hybrid event. Inter-population hybridization between genetically different groups in *M. anguillicaudatus* resulted in disruption of regular meiosis and normal gametogenesis, causing formation of unreduced diploid and other unusual aneuploid and polyploidy eggs in females [17] and sterility in males [18]. Molecular genetic relationship between Japanese and Chinese loach populations is still fragmentary and inconclusive, and it is impossible to distinguish them based on morphological characteristics. However, polyploidy and other unusual cytogenetic characteristics are indicators of the cryptic invasion of exotic loaches to find imported individuals among the market specimens.

In the present study, we obtained loach samples from the central wholesale market in Tokyo and examined ploidy status by DNA content flow cytometry in order to find exotic loach individuals. We found hyper-triploid, hyper-tetraploid and other unusual polyploids. Furthermore, the motility and ploidy status of sperm from hyper-triploid and hyper-tetraploid males were examined. Normally-fertilized and androgenetically induced progeny were then produced by using sperm of hyper-tetraploid males to investigate their reproductive capacity.

Materials and methods

Fish specimens

A total of 451 loach individuals were obtained from the wholesale market in Tokyo from March to April, 2011. According to the fish dealers, these farmed animals were imported as food from Taiwan, but they did not know their exact origin, and aquaculture conditions remain unknown. These specimens were identified as *M. anguillicaudatus* on the basis of external appearance and kept in the Aquarium room of

the Environment Control Experiment Building, the Faculty and Graduate School of Fisheries Sciences,
Hokkaido University.

Flow cytometry and ploidy determination

The ploidy status was determined as relative value in each individual by measuring DNA content using a flow-cytometer (Ploidy Analyzer, Partec GmbH, Münster, Germany) as described previously [3]. Briefly, a fin clip of each specimen was suspended in 200 µl of solution A (Cystain DNA 2step, Nuclei Extraction solution, Partec GmbH) and incubated at room temperature for 20 min. The mixture was filtered with a 50-µm mesh (Cell Trics, Partec GmbH) and stained for 5 min with Solution B (Cystain DNA 2step, DAPI (4'-6-diamidino-2-phenylindole) Staining solution, Partec GmbH). Ploidy status was determined by relative DNA content of the target samples with the standard DNA content of euploid, triploid and tetraploid specimens. The normal wild-type euploid loach was taken from Kitamura, Iwamizawa City, Hokkaido. The triploid sample was produced by fertilizing eggs of a normal wild-type female with diploid sperm of a sex-reversed clonal loach [19]. Diploid sperm was considered to be formed by premeiotic endomitosis (chromosome doubling in early spermatogonia and subsequent quasi-normal meiotic divisions) in sex-reversed clonal diploid loach [20]. As standard control of tetraploidy, neo-tetraploid loach was induced by fertilizing eggs of normal wild-type female with diploid sperm of a natural tetraploid male obtained in market samples, followed by inhibition of the second polar body release shortly after fertilization [21].

Motility and ploidy of sperm

Based on DNA content flow cytometry of samples, four hyper-triploid males (hyper-3n-#1, hyper-3n-#2, hyper-3n-#3 and hyper-3n-#4) and three hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and hyper-4n-#3) were sorted for further examination on reproductive capacity. Then, maturation was induced by injecting hCG (human Chorionic Gonadotropin, Aska Pharmaceuticals, Tokyo) [22]. Fish were maintained at 27°C for 12 h, and then sperm was collected in hematocrit glass tube by gently squeezing the abdomen. Ploidy status of sperm sample from each specimen was assayed by flow cytometry as described in the previous study [23]. The sperm motility was analyzed with a video recorder (Sharp VHS VC-HF920) according to procedures previously reported for loach [23].

Production of progeny

To verify the chromosome number of each specimen, we fertilized UV-inactivated eggs in order to produce androgenetic progeny. Androgenetic individuals do not survive for long beyond first feeding stage (3 to 4 days after hatching) due to so-called “haploid syndrome” although diploid individuals develop normally into adults. To induce androgenetic progeny, mature eggs from the normal wild-type diploid female were genetically inactivated by UV irradiation (150 mJ/cm²) according to the procedures previously optimized by Fujimoto et al. [24]. Irradiated eggs were then fertilized with the sperm from one normal wild-type diploid male (eudiploid control producing 1n sperm), one neo-tetraploid male (eutetraploid control producing 2n sperm), and three hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and hyper-4n-#3). At the same time, putative triploid progeny were produced by fertilizing non-inactivated eggs from the same female with sperm from the same neo-tetraploid and hyper-tetraploid males. Fertilized eggs were reared in dechlorinated tap water at 20°C. Fertilization rate was percentage of

cleaved eggs at 8 to 64-cell stage in total eggs used for fertilization. Cleaved eggs were clearly observable in 8 to 64-cell stages. Hatch rate was percentage of hatched larvae in fertilized eggs. Normal rate was percentages of hatched larvae with normal external appearance.

Ploidy and chromosomes of progeny

The relative DNA contents of hatched larvae were measured by flow cytometry as described previously [24]. Embryos at the 10-20 somite stage were randomly selected from different crosses for chromosome preparation. Yolk sacs were mechanically removed from the embryos using fine forceps and the embryos were then treated with 0.0025% colchicine in Ringer's solution for 30 min. The embryos were then placed in 0.075M KCL for 25 min and fixed with Carnoy's solution (1 acetic acid : 3 methanol). Chromosome preparations were made and chromosome numbers per metaphase plate were counted as described in Inokuchi et al. [25]. Chromosomes were classified according to Levan et al. [26].

Results

By flow cytometry, loach specimens (total $n=451$) were categorized into diploid range ($n=366$, 81.2%), triploid range ($n=52$, 11.5%), tetraploid range ($n=30$, 6.7%), pentaploid range ($n=1$, 0.2%), hexaploid range ($n=1$, 0.2%) and heptaploid range ($n=1$, 0.2%) (Fig. 1, Electronic Supplementary Material(ESM) Table S1). Although eudiploid, eutriploid and eutetraploid had just twice, three times and four times DNA content of normal haploid genome size, respectively, clear hyper-triploid (Fig. 2a) and hyper-tetraploid (Fig 2b) were recognized when compared with control triploid or tetraploid (neo-tetraploid) sample. Detection of hyper-diploid individuals and hypo-polyploid individuals has not been successfully

completed in the present study, because of the difficulty to distinguish them from variances of fluorescent peak(s) in flow cytometrical histograms.

Although normal sperm exhibited only haploid cell population (Fig. 3a), haploid, triploid and hexaploid-range cell populations were detected in sperm from hyper-triploid males (Fig. 3bc). Size of each cell population was different, but hexaploid-range cell population was always dominant in any hyper-triploid male (Fig. 3bc). Spermatozoa or spermatozoon-like cells with relatively bigger head sizes did not exhibit active progressive motility after contact with ambient water, but the spermatozoa with the smallest head sizes sometimes showed relatively active movement.

On the other hand, hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and hyper-4n-#3) demonstrated vigorous progressive motility of spermatozoa, as normally observed in haploid sperm of wild-type diploid males after activation. The relative DNA content of spermatozoa from these hyper-tetraploid males was half of their corresponding somatic cells and higher than that of normal diploid spermatozoa from neo-tetraploid loach (Fig. 4), thus hyper-tetraploid males were apparent to produce hyper-diploid spermatozoa.

When the eggs of a normal wild-type diploid female were fertilized with sperm from a normal wild-type diploid male, 98.4% eggs were successfully cleaved and 90.6% hatched (Table 1). DNA content flow cytometry indicated that the resultant progeny were diploids. In the cross between normal diploid female and neo-tetraploid male, viable triploid progeny appeared (Fertility, 94.3%; Hatch, 92.2%; Normal, 94.5%; Table 1, Fig. 5a). In the cross between normal diploid female and hyper-tetraploid males, normal larvae with triploid range DNA content appeared and viabilities were not different from the result used neo-tetraploid male (Fertility, 90.6–95.8%; Hatch, 85.0–90.3%; Normal, 84.3–92.8%; Table 1, Fig. 5b).

When UV-irradiated eggs of normal diploid female were fertilized with normal sperm of wild-type male, inviable haploid progeny appeared by induced androgenesis (Fertility, 90.3%; Hatch, 28.3%; Normal, 0%; Figure not shown). Androgenetic progeny developed from the cross between UV-irradiated eggs of normal diploid female and diploid sperm of neo-tetraploid males were viable diploid with high percentage of normally hatched larvae exhibiting normal external appearance (Fertility, 95.1%; Hatch, 35.6%; Normal, 90.4%; Table 1, Fig 5c). On the other hand, no viable progeny occurred in the crosses between UV-irradiated eggs of normal diploid female and sperm of hyper-tetraploid males (Fertility, 80.2–90.7%; Hatch, 25.2–34.7%; Normal, 0%; Table 1) and abnormal larvae exclusively occurred in androgenetically activated eggs (Fig. 5d).

DNA content of viable progeny between normal diploid female and neo-tetraploid male was just 1.5 times higher of that of somatic diploid cells and thus showed eutriploidy in these progeny (Table 1, Fig. 6a). Androgenetic progeny developing from sperm of neo-tetraploid male gave just euploid DNA content (Fig. 6b). Euploidy of these androgenetic progeny was also verified by cytogenetic analysis: modal chromosome number was $2n=50$ in the UV-irradiated eggs of normal diploid female $\times$ sperm of neo-tetraploid male cross (Table 2, Fig. 7ab). On the contrary, DNA content of viable progeny between normal diploid female and hyper-tetraploid males was slightly larger than that of euploid progeny (Fig 6c). Similarly, slightly larger DNA content was also detected in progeny from UV-irradiated eggs of normal diploid female $\times$ sperm of hyper-tetraploid males (Fig. 6d). These flow-cytometrical results indicated that hyper-tetraploid males generated hyper-diploid spermatozoa. Hyper-diploidy of spermatozoa of hyper-tetraploid males was also supported by modal chromosome number of $2n=54$ found in androgenetic progeny (Table 2). Most frequent diploid cell in the androgenetic progeny of

hyper-tetraploid males exhibited four supernumerary micro-chromosomes besides regular members of 50 chromosomes (Fig 7cd).

Discussion

In the present study, we detected various kinds of loaches with diploid, triploid, tetraploid, pentaploid, hexaploid and heptaploid-range DNA content among Japanese market samples. The occurrence of polyploid loaches is not surprising, because diploid, triploid and tetraploid loaches are sympatrically distributed in wild populations along Changjiang river system in central China [7-11], and natural triploid loaches have been detected among wild populations in Japan [3-6]. Recently, wild hexaploid loach with $6n=150$ chromosomes was also discovered in China [27]. Viable hexaploid loaches, which were experimentally induced from the cross-breeding between natural tetraploids followed by inhibition of the second polar release after fertilization, exhibited viability as well as fertility, and tetraploid, pentaploid and hexaploid progeny were produced by cross-breeding using fertile triploid gametes of the mature hexaploid loaches[28]. All these facts indicate that loach species can tolerate an elevation of genome size or ploidy status, in terms of surviving and reproductive capacity.

However, frequencies of euploid loaches were low in our present samples, and most individuals were considered aneuploids. Viable hyper-diploid loaches ($2.2n-2.5n$) were successfully produced by fertilization of eggs from wild-type diploids with aneuploid sperm from artificially induced triploids [29]. Adult-size hyper-diploid loaches with $2n=51, 52, 53$ and 58 , comprising additional telocentrics and supernumerary micro-chromosomes, were found in families produced by the cross-breeding of wild-type [30]. Hyper-triploid progeny ($3.1n-3.8n$) which were produced by cross-breeding between natural

tetraploid females and artificially induced triploid males were also viable [31]. These previous results suggest that loach species can also tolerate hyperploidy comprising extra or supernumerary chromosomes. The occurrence of such hyperploidy loaches in market samples also suggests that hyperploidy loaches should have survival capacity, but it was difficult to distinguish hypoploid individuals from artifact in the present study.

In our study, hyper-triploid males were shown to be sterile, because they produced no fertile spermatozoa except for few cases. On the contrary, hyper-tetraploids produced motile hyper-diploid spermatozoa, and crosses between diploid wild-type female and hyper-tetraploid males gave viable progeny. Thus, hyper-tetraploid males are considered fertile.

When UV-irradiated eggs of wild-type were fertilized with sperm of hyper-tetraploid males, androgenetic progeny were induced, and the resultant androgenetic hyper-diploid progeny exhibited four supernumerary micro-chromosomes ($2n=54$) when compared with wild-type eudiploid ($2n=50$). Although hyper-diploid loaches in previous studies were all viable [29, 30], these androgenetic hyper-diploid progeny exhibited abnormal appearance and could not survive beyond feeding stage. At present, however, we could not explain the genetic and cytogenetic reason why these androgenetic hyper-diploid progeny were inviable and externally abnormal. Simple explanation may be deleterious influences of supernumerary chromosomes on survival capacity of induced androgenetes.

In the present study, we reported sterility of hyper-triploid males and fertility of hyper-tetraploid males found in Japanese market samples. Although reproductive capacity of such unusual aneuploid females has not been investigated yet, it is noteworthy that fertile exotic hyper-tetraploid males may cause genetic disorders on the wild populations when they escape to invade Japanese waters. Very small number

of higher-polyploids exhibiting DNA content corresponding to pentaploid, hexaploid and heptaploid range also appeared in market samples, but reproductive capacity of these higher polyploids was not examined herein. Further investigation on genetic and reproductive capacity of these higher polyploid loaches is required in future to assess the risk of exotic species. The other important problem is the exact origin of market samples. To identify the origin, mitochondrial DNA haplotypes and nuclear DNA genotypes are also required to analyze in near future. The mechanism responsible for the occurrence of aneuploids and higher polyploids are also enigmatic. Random mating among different fertile (hyper- or hypo-) polyploid individuals should be imaginable.

Acknowledgments

This work was supported in part by the Grant-in-Aid for Scientific Research (B) (21380114) from Japan Society for the Promotion of Science (JSPS) to KA.

References

1. Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2004) Geographic variation and diversity of the cytochrome *b* gene in Japanese wild populations of Medaka, *Oryzias latipes*. Zool Sci 20: 1279–1291.
2. Hashiguchi Y, Kado T, Kimura S, Tachida H (2006) Comparative phylogeography of two bitterlings, *Tanakia lanceolata* and *T. limbata* (Teleostei, Cyprinidae), in Kyushu and adjacent districts of western Japan, based on mitochondrial DNA analysis. Zool Sci 23: 309–322.
3. Morishima K, Horie S, Yamaha E, Arai K (2002) A Cryptic clonal line of the loach *Misgurnus*

253 *anguillicaudatus* (Teleostei: Cobitidae) Evidenced by induced gynogenesis, interspecific
254 hybridization, microsatellite genotyping and multilocus DNA fingerprinting. Zool Sci 19: 565–575

255 4. Morishima K, Nakamura-Shiokawa Y, Bando E, Li YJ, Boroń A, Khan M M R, Arai K (2008)
256 Cryptic clonal lineages and genetic diversity in the loach *Misgurnus anguillicaudatus* (Teleostei:
257 Cobitidae) inferred from nuclear and mitochondrial DNA analyses. Genetica 132: 159–171

258 5. Zhang Q, Arai K (1999) Distribution and reproductive capacity of natural triploid individuals and
259 occurrence of unreduced eggs as a cause of polyploidization in the loach *Misgurnus anguillicaudatus*.
260 Ichthyol Res. 46:154–161

261 6. Arai k (2009) Polyploidy, clone and atypical reproduction in the loach. J Anim Genet 37: 59–80 (in
262 Japanese)

263 7. Li K, Li Y, Zhou D (1983) A comparative study of the karyotypes in two species of mud loaches.
264 Zool Res 4: 75–81(in Chinese)

265 8. Li YC, Li k (1987) Karyotypes of *Opsariichthys bidens* and *Misgurnus anguillicaudatus* (pisces),
266 with a consideration of the relationship between polymorphism of chromosome number and
267 taxonomy of fish. J Wuhan Univ 1: 107–112 (in Chinese)

268 9. Yin J, Zhao ZS, Chen XQ, Li YQ, Zhu LY (2005) Karyotype comparison of diploid and tetraploid
269 loach, *Misgurnus anguillicaudatus*. Acta Hydrobiologica Sinica 29: 469–472 (in Chinese)

270 10. Li YJ , Jie Y, Wang JB , Xu Y , Jie W, Sun XW, Arai K (2008) A study on the distribution of polyploid
271 loaches in China. Nippon Suisan Gakkaishi 74: 177–182 (in Japanese)

272 11. Yang CT, Cao L, Wang WM, Yang Y, Abbas K, Yan BL, Wang HP, Su L, Sun YH, Wang HL (2009)
273 Comparative and evolutionary analysis in natural diploid and tetraploid weather loach *Misgurnus*

- 274 *anguillicaudatus* based on cytochrome b sequence data in central China. *Env Biol Fish* 86: 145-153.
- 275 12. Ojima Y, Takai A (1979) The occurrence of spontaneous polyploid in the Japanese common loach,
- 276 *Misgurnus anguillicaudatus*. *Proc Japan Acad* 55B: 487–491
- 277 13. Arai K, Matsubara K, Suzuki R (1991) Karyotype and erythrocyte size of spontaneous tetraploidy and
- 278 triploidy in the loach *Misgurnus anguillicaudatus*. *Nippon Suisan Gakkashi* 57: 2157–2172
- 279 14. Koizumi N, Takemura T, Watabe K, Mori A (2008) Genetic variation and diversity of Japanese loach
- 280 inferred from mitochondrial DNA: phylogenetic analysis using the cytochrome b gene sequences. *T*
- 281 *Jpn Soc Irrig Drain Rural Eng* 77:7–16 (in Japanese with English abstract)
- 282 15. Koizumi N (2009) Conserving habitat of freshwater fishes inhabiting haruno-ogawa,
- 283 irrigation/drainage ditches, in rice paddies. The Gakuho press, Tokyo (in Japanese)
- 284 16. Shimizu T, Takagi M (2010) Genetic structure of natural populations of *Misgurnus anguillicaudatus*
- 285 in Ehime Prefecture impacted by recent exotic introductions, inferred from mitochondrial DNA
- 286 analysis. *Japan J Ichthyol* 57: 13–26 (in Japanese)
- 287 17. Arias-Rodriguez L, Yasui GS, Arai K (2009) Disruption of normal meiosis in artificial
- 288 inter-populational hybrid females of *Misgurnus* loach. *Genetica* 136: 49–56
- 289 18. Arias-Rodriguez L, Yasui GS, Kusuda S, Arai K (2010) Reproductive and genetic capacity of
- 290 spermatozoa of inter-populational hybrid males in the loach, *Misgurnus anguillicaudatus*. *J Appl*
- 291 *Ichthyol* 26: 653–658.
- 292 19. Yoshikawa H, Morishima K, Kusuda S, Yamaha E, Arai K (2007) Diploid sperm produced by
- 293 artificially sex-reversed clone loaches. *J Exp Zool* 307A:75-83.
- 294 20. Yoshikawa H, Morishima K, Fujimoto T, Saito T, Kobayashi T, Yamaha E, Arai K (2009)

- 295 Chromosome doubling in early spermatogonia produces diploid spermatozoa in a natural clonal fish.
296 Biol Reprod 80: 973–979.
- 297 21. Fujimoto T, Yasui GS, Hayakawa M, Sakao S, Yamaha E, Arai K (2010) Reproductive capacity of
298 neo-tetraploid loaches produced using diploid spermatozoa from a natural tetraploid male.
299 Aquaculture 308 (Suppl): S133–S139
- 300 22. Suzuki R, Yamaguchi M (1975) Influence of water temperature on inducing spawning by hormone
301 injection in the loach, cyprinid fish. Suisanzoshoku 22:135–139 (in Japanese)
- 302 23. Fujimoto T, Yasui GS, Yoshikawa H, Yamaha E, Arai K (2008) Genetic and reproductive potential of
303 spermatozoa of diploid and triploid males obtained from interspecific hybridization of *Misgurnus*
304 *anguillicaudatus* female with *M. mizolepis* male. J Appl Ichthyol 24: 430–437
- 305 24. Fujimoto T, Sakao S, Yamaha E (2007) Evaluation of different doses of UV irradiation to loach eggs
306 for genetic inactivation of the maternal genome. J Exp Zool 307A: 449–462
- 307 25. Inokuchi T, Abe S, Yamaha E, Yamazaki F, Yoshida MC (1994) BrdU replication banding studies on
308 the chromosomes in early embryos of salmonid fishes. Hereditas 121: 255–265
- 309 26. Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes.
310 Hereditas 52: 201–220
- 311 27. Abbas K, Li MY, Wang WM, Zhou XY (2009) First record of the natural occurrence of hexaploid
312 loach *Misgurnus anguillicaudatus* in Hubei Province, China. J Fish Biol 75: 435–441
- 313 28. Arai K, Taniura K, Zhang Q (1999) Production of second generation progeny of hexaploid loach.
314 Fish Sci. 65:186–192
- 315 29. Arai k, Inamori Y (1999) Viable hyperdiploid progeny between diploid female and induced triploid

male in the loach, *Misgurnus anguillicaudatus*. Suisanzoshoku, 47: 489–495

30. Zhang Q, Arai K (2003) Extensive karyotype variation in somatic and meiotic cells of the loach
Misgurnus anguillicaudatus (Pisces: Cobitidae). Folia Zool 52: 423–429

31. Zhang Q, Arai K (1999) Aberrant meioses and viable aneuploid progeny of induced triploid loach
(*Misgurnus anguillicaudatus*) when crossed to natural tetraploids. Aquaculture 175: 63–76

Figure captions

Fig. 1 Histograms showing the number of specimens with different ploidy status inferred from relative DNA content estimated by flow cytometry

Fig. 2 Comparison of flow-cytometrical histograms between control eudiploid and hyper-triploid/hyper-tetraploid in somatic cells. Fluorescent peak 1 of normal diploid loach indicates 2C DNA content; fluorescent peak 2 of control triploid loach indicates 3C DNA content; fluorescent peak 3 of hyper-triploid loaches indicates approximately 3.3C DNA content (a). Fluorescent peak 1' of normal diploid loach indicates 2C DNA content; fluorescent peak 2' of neo-tetraploid loach indicates 4C DNA content; fluorescent peak 3' of hyper-tetraploid loaches indicates approximately 4.3C DNA content (b)

Fig. 3 Flow-cytometrical histograms of normal diploid and hyper-triploid in sperm. Fluorescent peak 1 of haploid spermatozoa from normal wild-type diploid loach indicates 1C DNA content (a). Fluorescent peak 1', 2' and 3' of spermatozoa or spermatozoa-like cells from sperm of hyper-triploid loach indicate approximately 1.7C, 3.3C and 6.7C DNA content, respectively (b). Fluorescent peak 1'' and 2'' of spermatozoa or spermatozoa-like cells from sperm of hyper-triploid loach indicate approximately 3.3C and 6.6C DNA content, respectively (c)

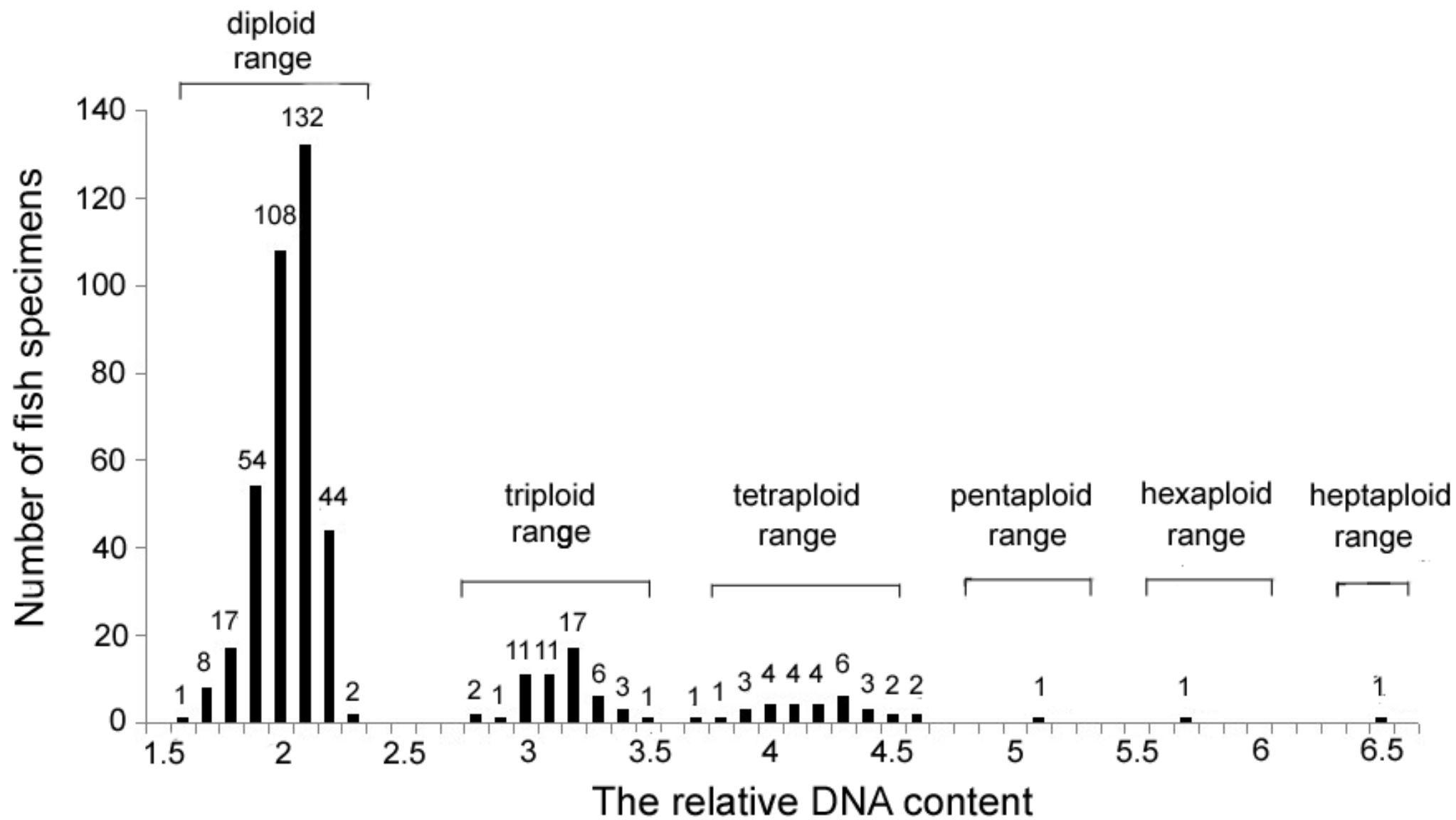
Fig. 4 Flow-cytometrical histograms of neo-tetraploid and hyper-tetraploid in sperm. Fluorescent peak 1 of haploid spermatozoa from normal diploid loach indicates 1C DNA content; fluorescent peak 2 of diploid spermatozoa from neo-tetraploid loach indicates 2C DNA content; fluorescent peak 3 of spermatozoa from hyper-tetraploid loaches indicates approximately 2.3C DNA content

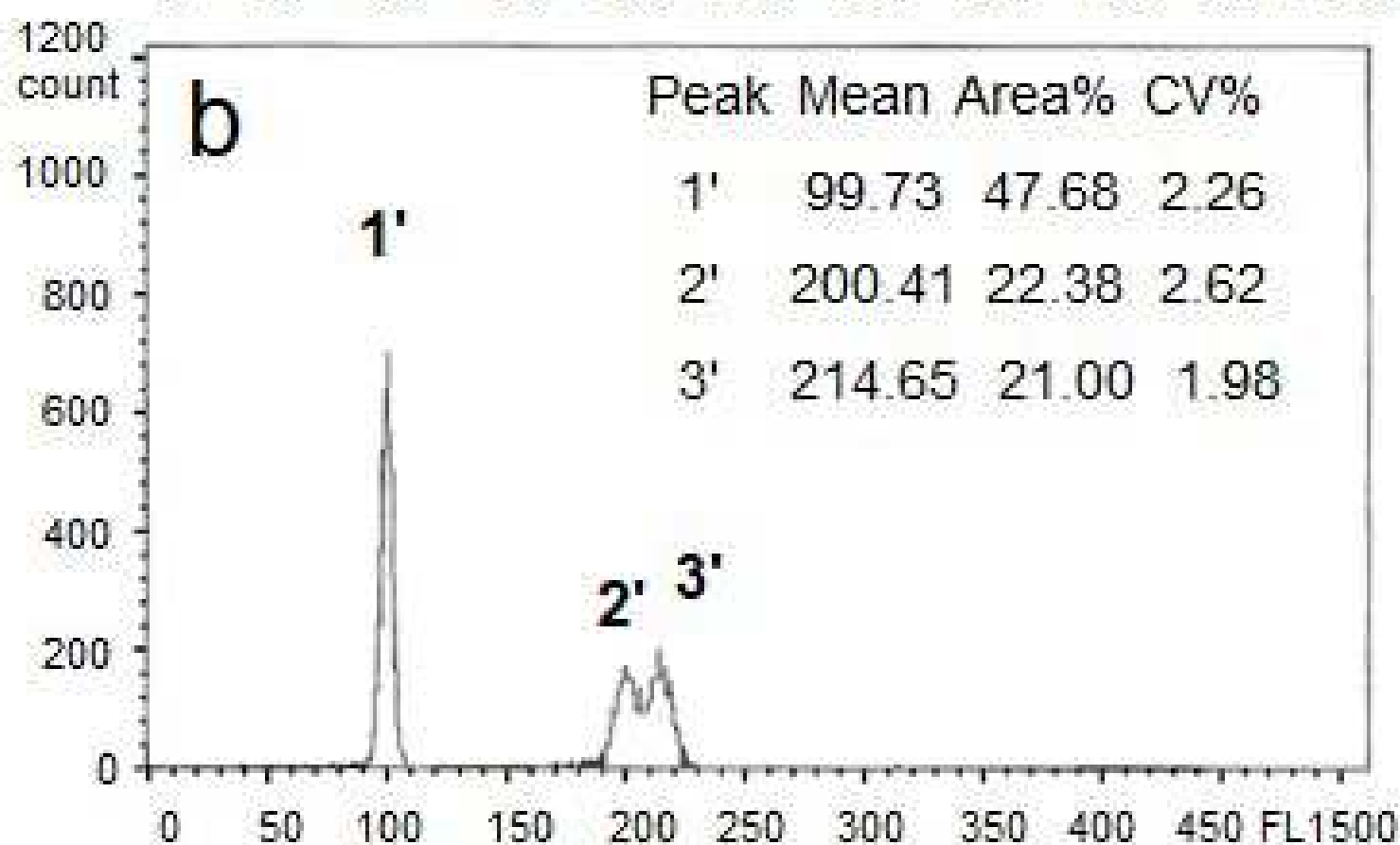
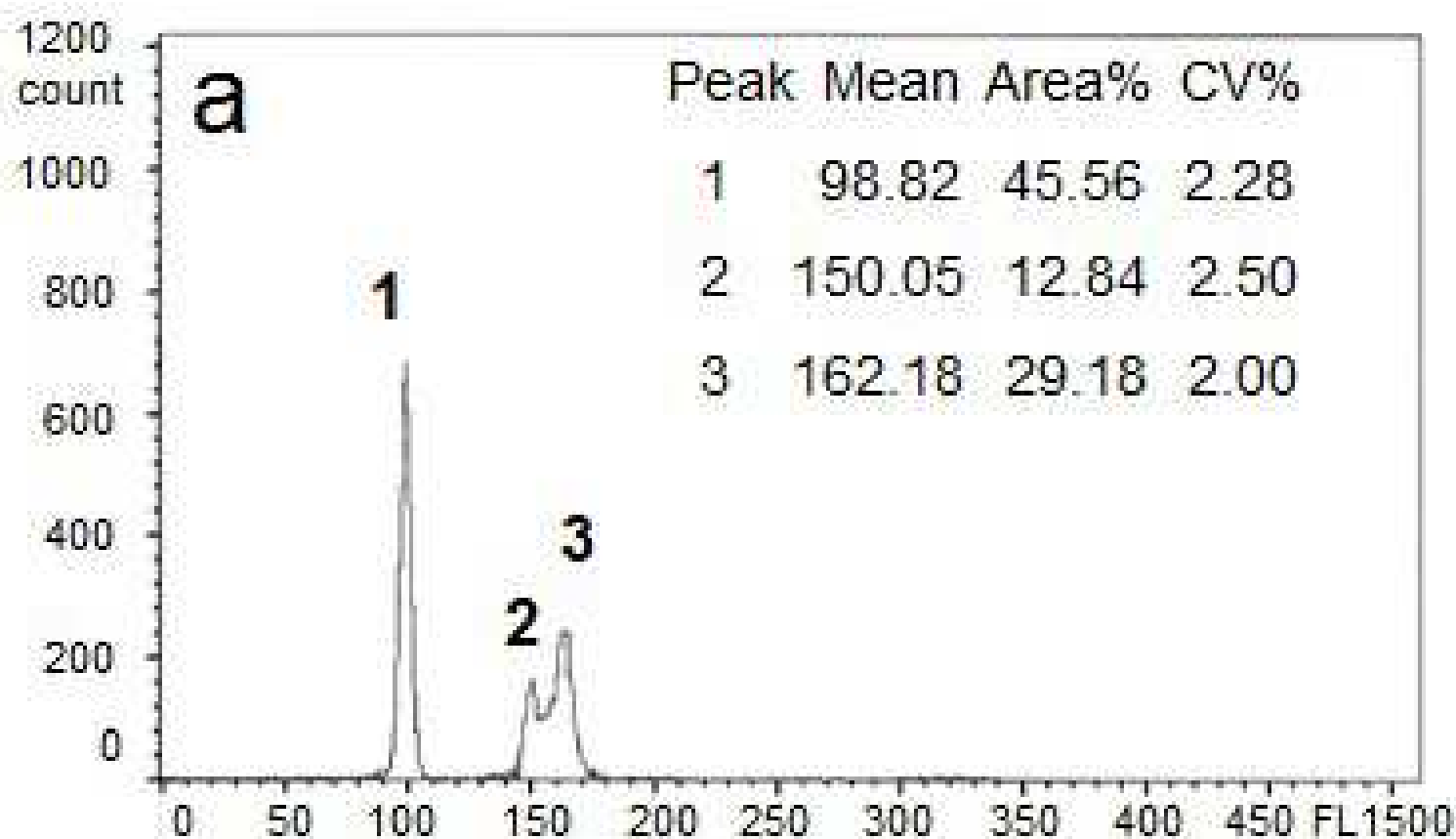
Fig. 5 External appearance of larvae. Normal larvae from diploid female $\times$ neo-tetraploid male (a).

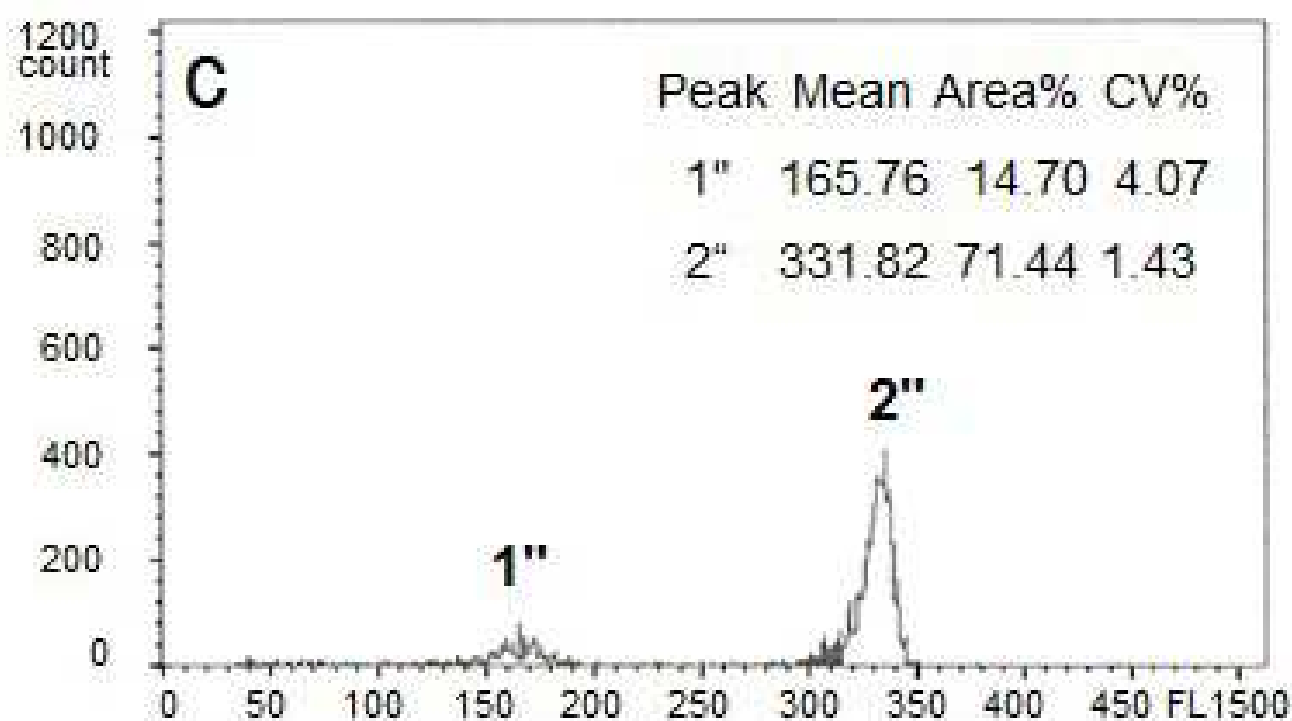
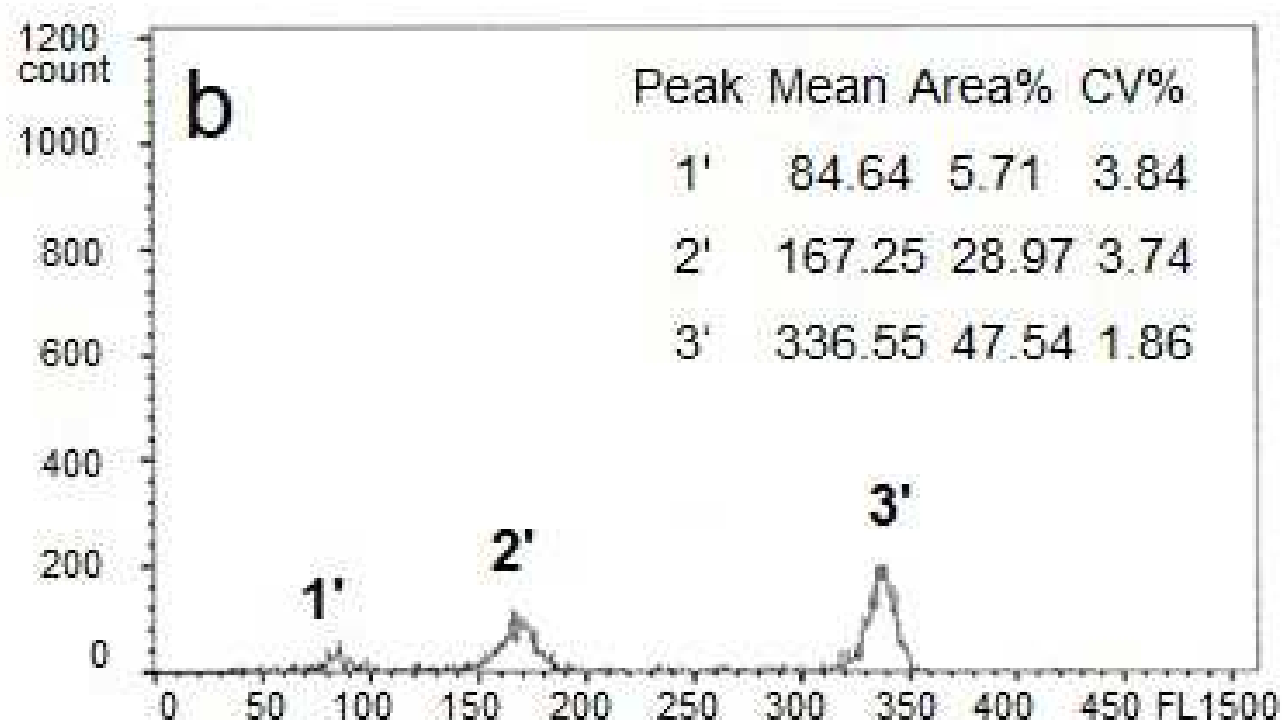
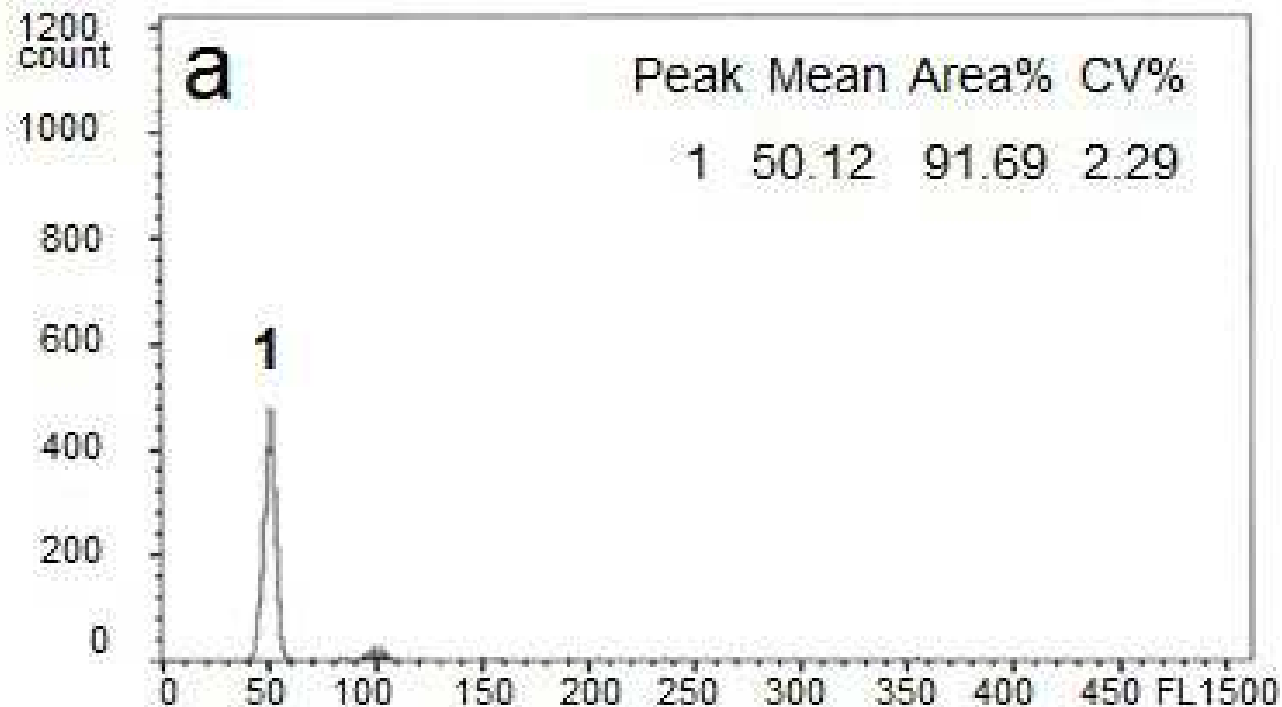
Normal larvae from diploid female $\times$ hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and hyper-4n-#3) (b). Normal larvae produced by induced androgenesis, diploid female (UV-irradiated eggs) $\times$ neo-tetraploid male (c). Abnormal larvae with a few or no melanophores produced by induced androgenesis, diploid female (UV-irradiated eggs) $\times$ hyper-tetraploid males (d). Arrows indicate melanophores. A scale bar denotes 1mm

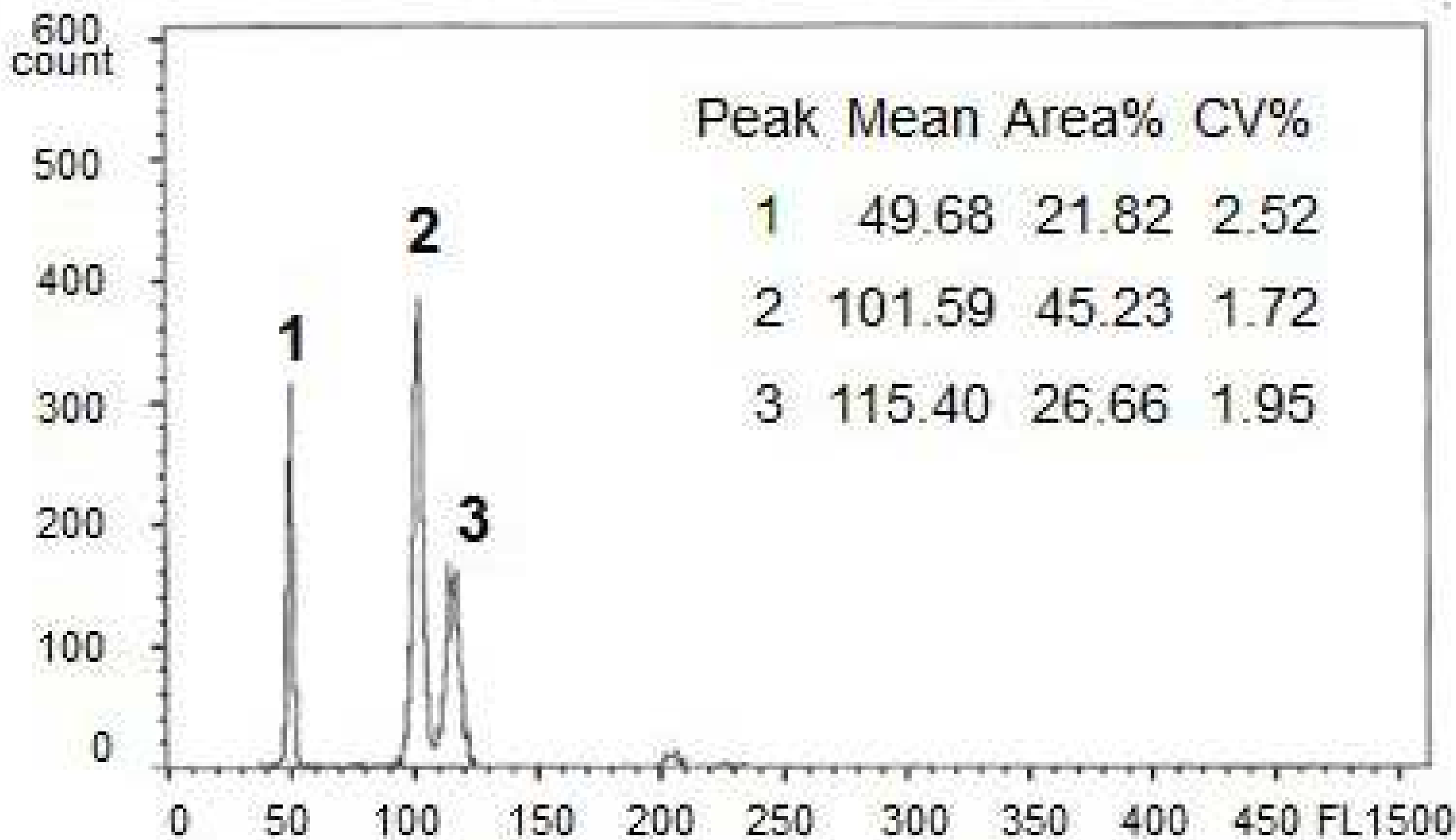
Fig. 6 Relative DNA contents of larvae from different crosses measured by flow cytometry. Fluorescent peak 1 of larvae from normal diploid female $\times$ normal diploid male cross indicates 2C DNA content. Fluorescent peak 2 of embryos from diploid female $\times$ neo-tetraploid male indicates 3C DNA content (a). Fluorescent peak 3 of larvae from diploid female (UV-irradiated eggs) $\times$ neo-tetraploid male cross indicates 2C DNA content (b). Fluorescent peak 1' of larvae from normal diploid female $\times$ normal diploid male cross indicates 2C DNA content. Fluorescent peak 2' of Embryos from diploid female $\times$ hyper-tetraploid males cross indicates approximately 3.3C DNA content (c). Fluorescent peak 3' of larvae from diploid female (UV-irradiated eggs) $\times$ hyper-tetraploid males cross indicates approximately 2.2C DNA content (d)

Fig. 7 Metaphase chromosomes (a, c) and corresponding karyotypes (b, d) of the loach *Misgurnus anguillicaudatus*. Diploid female (UV-irradiated eggs) $\times$ neo-tetraploid male, $2n=50$ (a, b). Diploid female (UV-irradiated eggs) $\times$ hyper-tetraploid males, $2n=54$ (c, d). Arrows indicate supernumerary chromosomes. Scale bars denote 10 μm

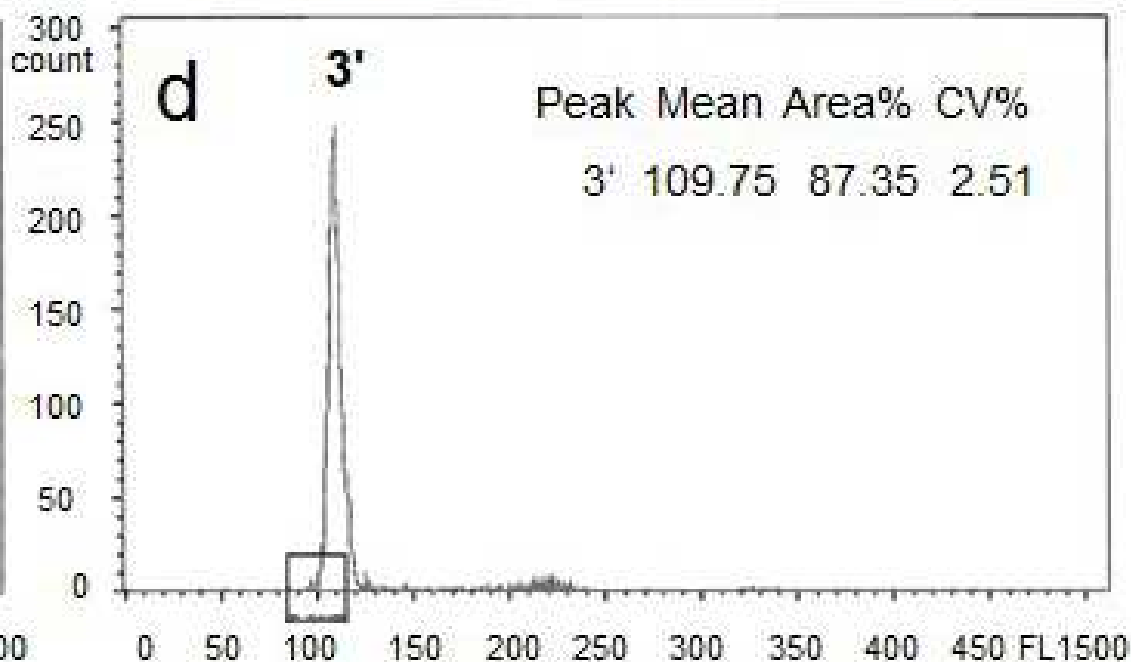
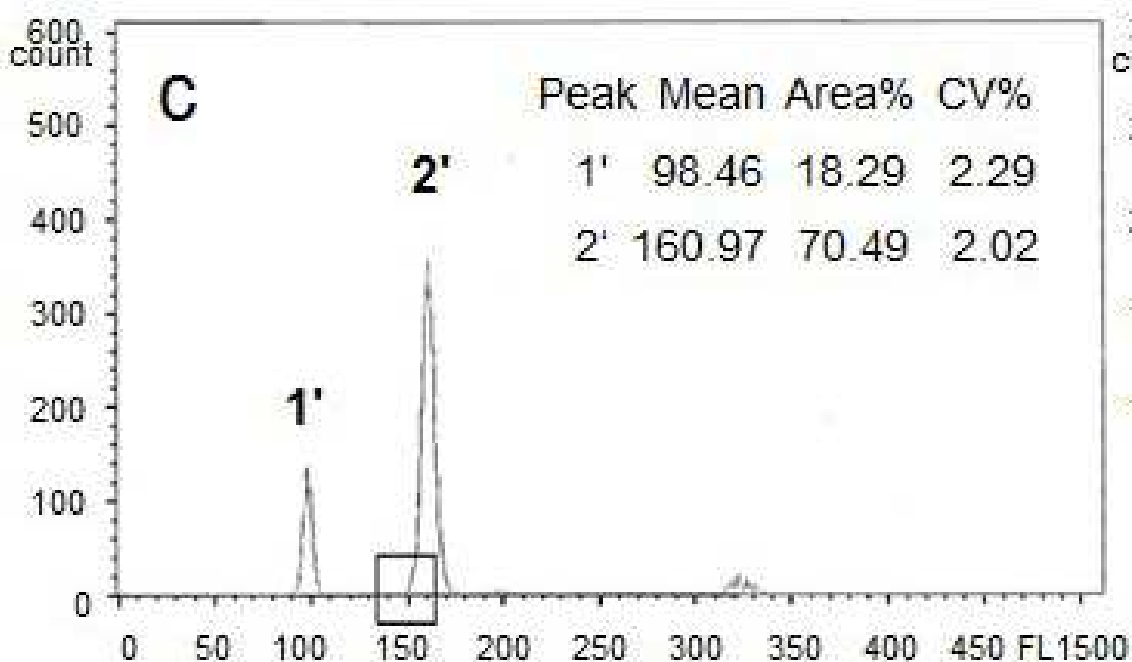
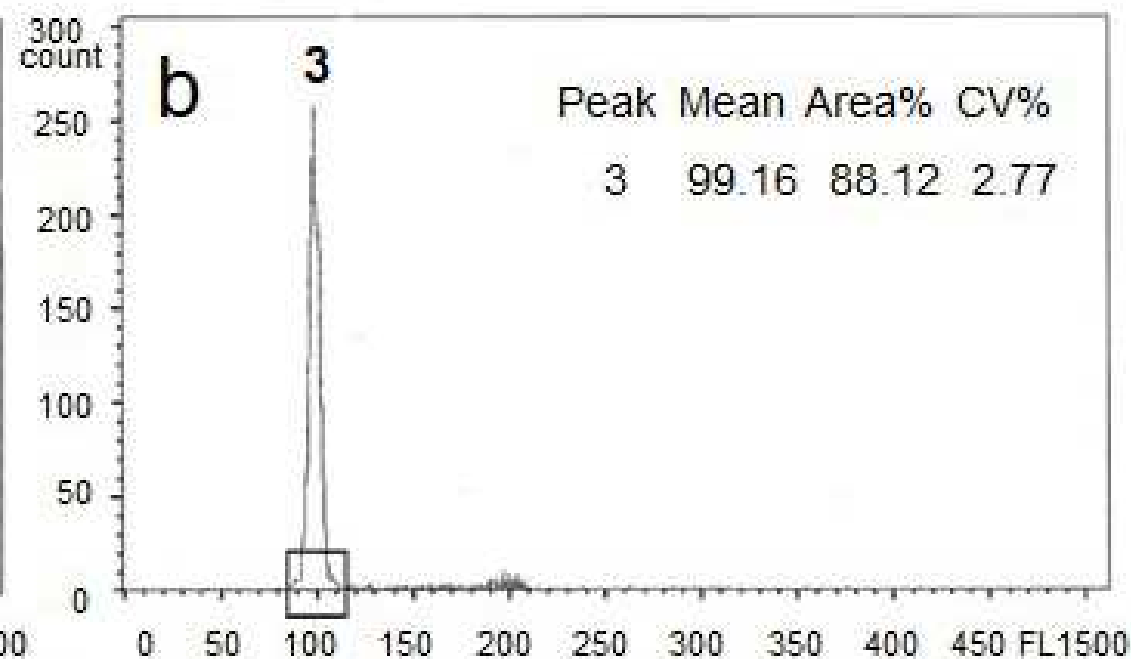
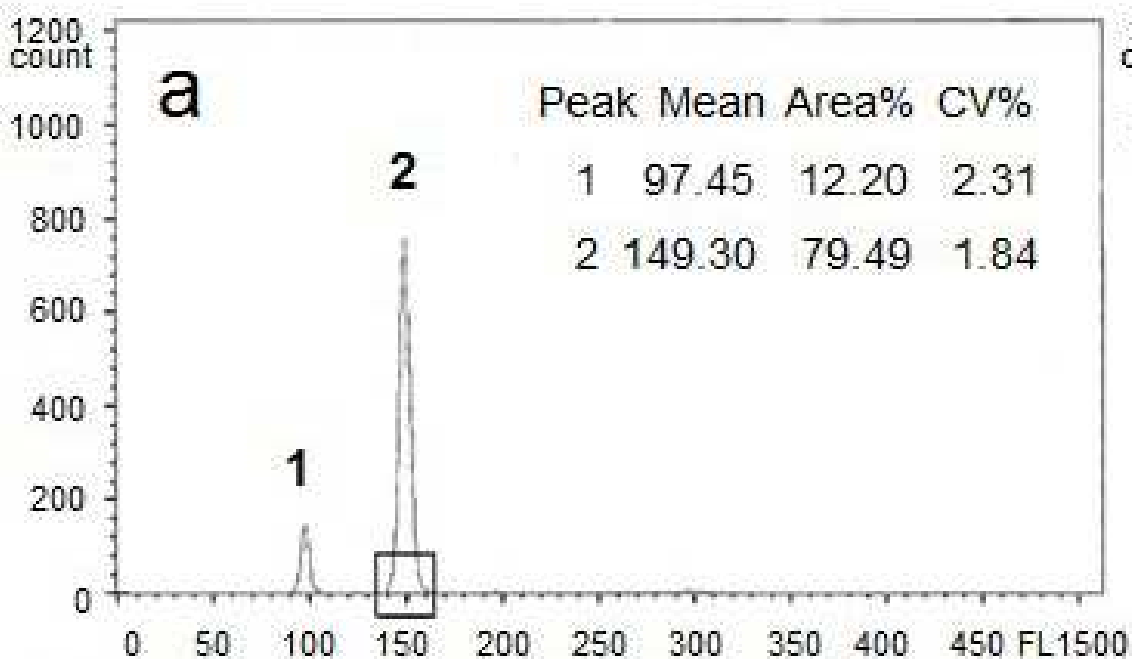












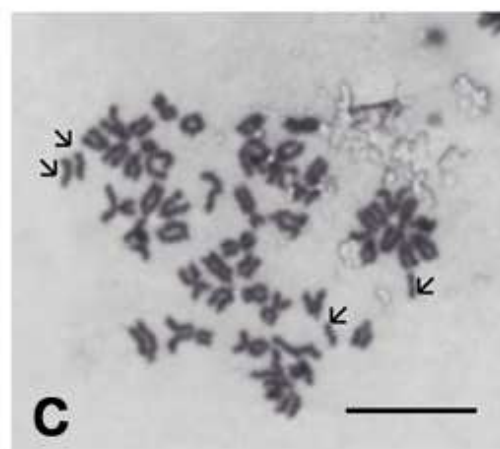
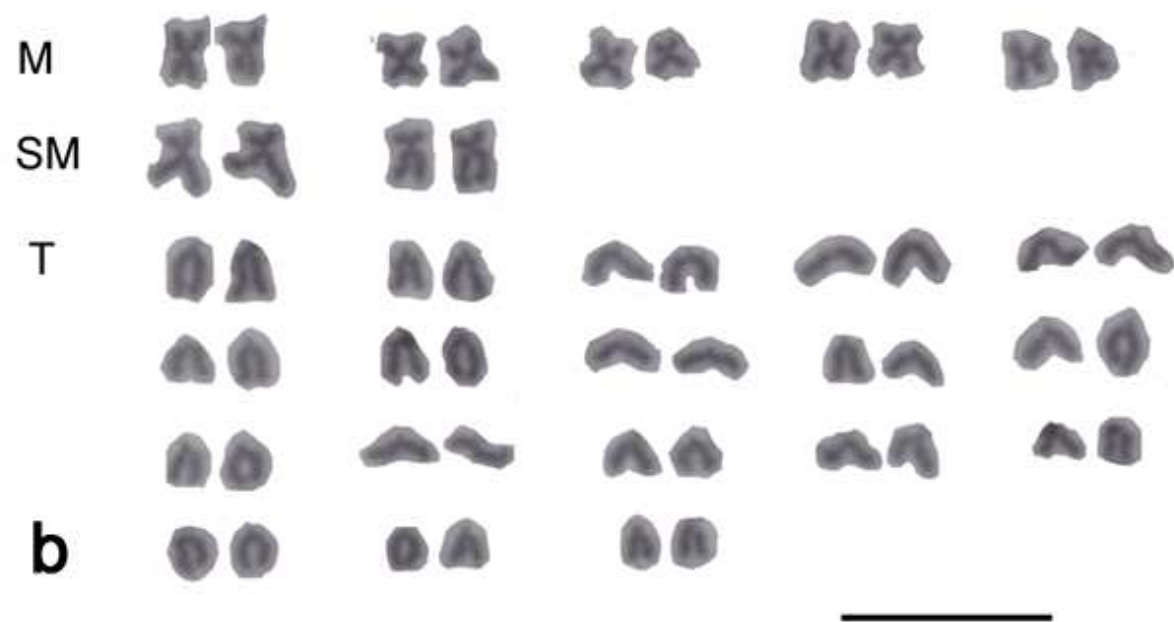
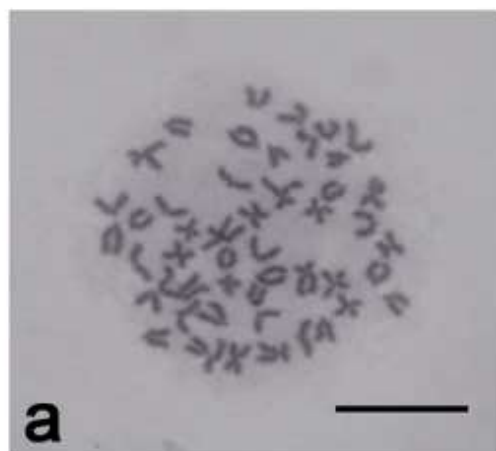


Table 1. Developmental capacity and ploidy status in progenies derived from sperm of normal 2N, neo-4N and hyper-4N males.

Putative progeny	Cross		Fertility (%)	Hatch (%)	Normal (%)	Sample no.	Ploidy range		
	Female*	Male					1n	2n	3n
Control-2n	2N	2N	98.4	90.6	97.5	10	0	10	0
3n	2N	neo-4N	94.3	92.2	94.5	10	0	0	10
3n	2N	hyper-4N-#1	90.6	85.0	84.3	10	0	0	10
3n	2N	hyper-4N-#2	95.8	86.5	92.4	10	0	0	10
3n	2N	hyper-4N-#3	95.5	90.3	92.8	10	0	0	10
Andro-1n	(2N)**	2N	90.3	28.3	0	10	10	0	0
Andro-2n	(2N)**	neo-4N	95.1	35.6	90.4	10	0	10	0
Andro-2n	(2N)**	hyper-4N-#1	90.7	30.4	0	10	0	10	0
Andro-2n	(2N)**	hyper-4N-#2	80.2	25.2	0	10	0	10	0
Andro-2n	(2N)**	hyper-4N-#3	90.5	34.7	0	10	0	10	0

* Intact or UV-inactivated eggs were obtained from the same diploid female.

** UV-irradiated

Table 2. Chromosome numbers in progenies derived from control cross (2N×2N) and androgenetes derived from sperm of neo-4N and hyper-4N males

Cross			Chromosome number												
Female	Male	n	45	46	47	48	49	50	51	52	53	54	55	56	
2N	2N	30		1	1	2	4	22							
(2N)*	neo-4N	41		1	1	1	8	27	2	1					
(2N)*	hyper-4N-#1	25							1	1	7	15	1		
(2N)*	hyper-4N-#2	9								1	4	4			
(2N)*	hyper-4N-#3	34								1	9	21	2	1	

* UV-irradiated