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 eudiploid, 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

64 [16]. These results suggest that genetic disturbance might occur by intra-specific hybrid event.
65 Inter-populational hybridization between genetically different groups in *M. anguillicaudatus* resulted in
66 disruption of regular meiosis and normal gametogenesis, causing formation of unreduced diploid and
67 other unusual aneuploid and polyploidy eggs in females [17] and sterility in males [18]. Molecular
68 genetic relationship between Japanese and Chinese loach populations is still fragmentary and
69 inconclusive, and it is impossible to distinguish them based on morphological characteristics. However,
70 polyploidy and other unusual cytogenetic characteristics are indicators of the cryptic invasion of exotic
71 loaches to find imported individuals among the market specimens.

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

78

79 **Materials and methods**

80 Fish specimens

81 A total of 451 loach individuals were obtained from the wholesale market in Tokyo from March to April,
82 2011. According to the fish dealers, these farmed animals were imported as food from Taiwan, but they
83 did not know their exact origin, and aquaculture conditions remain unknown. These specimens were
84 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, Nulei 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 eudiploid, eutriploid and eutetraploid specimens. The normal wild-type eudiploid loach was taken from Kitamura, Iwamizawa City, Hokkaido. The eutriploid 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 eutetraploidy, 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

106 Based on DNA content flow cytometry of samples, four hyper-triploid males (hyper-3n-#1, hyper-3n-#2,
107 hyper-3n-#3 and hyper-3n-#4) and three hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and
108 hyper-4n-#3) were sorted for further examination on reproductive capacity. Then, maturation was induced
109 by injecting hCG (human Chorionic Gonadotropin, Aska Pharmaceuticals, Tokyo) [22]. Fish were
110 maintained at 27℃ for 12 h, and then sperm was collected in hematocrit glass tube by gently squeezing
111 the abdomen. Ploidy status of sperm sample from each specimen was assayed by flow cytometry as
112 described in the previous study [23]. The sperm motility was analyzed with a video recorder (Sharp VHS
113 VC-HF920) according to procedures previously reported for loach [23].
114
115 Production of progeny
116 To verify the chromosome number of each specimen, we fertilized UV-inactivated eggs in order to
117 produce androgenetic progeny. Androgenetic individuals do not survive for long beyond first feeding
118 stage (3 to 4 days after hatching) due to so-called “haploid syndrome” although diploid individuals
119 develop normally into adults. To induce androgenetic progeny, mature eggs from the normal wild-type
120 diploid female were genetically inactivated by UV irradiation (150 mJ/cm^2) according to the procedures
121 previously optimized by Fujimoto et al. [24]. Irradiated eggs were then fertilized with the sperm from one
122 normal wild-type diploid male (eudiploid control producing 1n sperm), one neo-tetraploid male
123 (eutetraploid control producing 2n sperm), and three hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2
124 and hyper-4n-#3). At the same time, putative triploid progeny were produced by fertilizing
125 non-inactivated eggs from the same female with sperm from the same neo-tetraploid and hyper-tetraploid
126 males. Fertilized eggs were reared in dechlorinated tap water at 20℃. Fertilization rate was percentage of

127 cleaved eggs at 8 to 64-cell stage in total eggs used for fertilization. Cleaved eggs were clearly observable
128 in 8 to 64-cell stages. Hatch rate was percentage of hatched larvae in fertilized eggs. Normal rate was
129 percentages of hatched larvae with normal external appearance.
130
131 Ploidy and chromosomes of progeny
132 The relative DNA contents of hatched larvae were measured by flow cytometry as described previously
133 [24]. Embryos at the 10-20 somite stage were randomly selected from different crosses for chromosome
134 preparation. Yolk sacs were mechanically removed from the embryos using fine forceps and the embryos
135 were then treated with 0.0025% colchicine in Ringer's solution for 30 min. The embryos were then placed
136 in 0.075M KCL for 25 min and fixed with Carnoy's solution (1 acetic acid : 3 methanol). Chromosome
137 preparations were made and chromosome numbers per metaphase plate were counted as described in
138 Inokuchi et al. [25]. Chromosomes were classified according to Levan et al. [26].
139
140 **Results**
141 By flow cytometry, loach specimens (total *n*=451) were categorized into diploid range (*n*=366, 81.2%),
142 triploid range (*n*=52, 11.5%), tetraploid range (*n*=30, 6.7%), pentaploid range (*n*=1, 0.2%), hexaploid
143 range (*n*=1, 0.2%) and heptaploid range (*n*=1, 0.2%) (Fig. 1, Electronic Supplementary Material(ESM)
144 Table S1). Although eudiploid, eutriploid and eutetraploid had just twice, three times and four times DNA
145 content of normal haploid genome size, respectively, clear hyper-triploid (Fig. 2a) and hyper-tetraploid
146 (Fig 2b) were recognized when compared with control triploid or tetraploid (neo-tetraploid) sample.
147 Detection of hyper-diploid individuals and hypo-polyploid individuals has not been successfully

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

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

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

162 When the eggs of a normal wild-type diploid female were fertilized with sperm from a normal
163 wild-type diploid male, 98.4% eggs were successfully cleaved and 90.6% hatched (Table 1). DNA content
164 flow cytometry indicated that the resultant progeny were diploids. In the cross between normal diploid
165 female and neo-tetraploid male, viable triploid progeny appeared (Fertility, 94.3%; Hatch, 92.2%; Normal,
166 94.5%; Table 1, Fig. 5a). In the cross between normal diploid female and hyper-tetraploid males, normal
167 larvae with triploid range DNA content appeared and viabilities were not different from the result used
168 neo-tetraploid male (Fertility, 90.6–95.8%; Hatch, 85.0–90.3%; Normal, 84.3–92.8%; Table 1, Fig. 5b).

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

178 DNA content of viable progeny between normal diploid female and neo-tetraploid male was just
179 1.5 times higher of that of somatic diploid cells and thus showed eutriploidy in these progeny (Table 1,
180 Fig. 6a). Androgenetic progeny developing from sperm of neo-tetraploid male gave just eudiploid DNA
181 content (Fig. 6b). Eudiploidy of these androgenetic progeny was also verified by cytogenetic analysis:
182 modal chromosome number was 2n=50 in the UV-irradiated eggs of normal diploid female × sperm of
183 neo-tetraploid male cross (Table 2, Fig. 7ab). On the contrary, DNA content of viable progeny between
184 normal diploid female and hyper-tetraploid males was slightly larger than that of eudiploid progeny (Fig
185 6c). Similarly, slightly larger DNA content was also detected in progeny from UV-irradiated eggs of
186 normal diploid female × sperm of hyper-tetraploid males (Fig. 6d). These flow-cytometrical results
187 indicated that hyper-tetraploid males generated hyper-diploid spermatozoa. Hyper-diploidy of
188 spermatozoa of hyper-tetraploid males was also supported by modal chromosome number of 2n=54 found
189 in androgenetic progeny (Table 2). Most frequent diploid cell in the androgenetic progeny of

190 hyper-tetraploid males exhibited four supernumerary micro-chromosomes besides regular members of 50
191 chromosomes (Fig 7cd).
192
193 **Discussion**
194 In the present study, we detected various kinds of loaches with diploid, triploid, tetraploid, pentaploid,
195 hexaploid and heptaploid-range DNA content among Japanese market samples. The occurrence of
196 polyploid loaches is not surprising, because diploid, triploid and tetraploid loaches are sympatrically
197 distributed in wild populations along Changjiang river system in central China [7-11], and natural triploid
198 loaches have been detected among wild populations in Japan [3-6]. Recently, wild hexaploid loach with
199 6n=150 chromosomes was also discovered in China [27]. Viable hexaploid loaches, which were
200 experimentally induced from the cross-breeding between natural tetraploids followed by inhibition of the
201 second polar release after fertilization, exhibited viability as well as fertility, and tetraploid, pentaploid
202 and hexaploid progeny were produced by cross-breedings using fertile triploid gametes of the mature
203 hexaploid loaches[28]. All these facts indicate that loach species can tolerate an elevation of genome size
204 or ploidy status, in terms of surviving and reproductive capacity.
205 However, frequencies of euploid loaches were low in our present samples, and most individuals
206 were considered aneuploids. Viable hyper-diploid loaches (2.2n-2.5n) were successfully produced by
207 fertilization of eggs from wild-type diploids with aneuploid sperm from artificially induced triploids [29].
208 Adult-size hyper-diploid loaches with 2n=51, 52, 53 and 58, comprising additional telocentrics and
209 supernumerary micro-chromosomes, were found in families produced by the cross-breeding of wild-type
210 [30]. Hyper-triploid progeny (3.1n-3.8n) which were produced by cross-breeding between natural

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

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

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

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

232 of higher-polyploids exhibiting DNA content corresponding to pentaploid, hexaploid and heptaploid
233 range also appeared in market samples, but reproductive capacity of these higher polyploids was not
234 examined herein. Further investigation on genetic and reproductive capacity of these higher polyploid
235 loaches is required in future to assess the risk of exotic species. The other important problem is the exact
236 origin of market samples. To identify the origin, mitochondrial DNA haplotypes and nuclear DNA
237 genotypes are also required to analyze in near future. The mechanism responsible for the occurrence of
238 aneuploids and higher polyploids are also enigmatic. Random mating among different fertile (hyper- or
239 hypo-) polyploid individuals should be imaginable.
240

241 **Acknowledgments**

242 This work was supported in part by the Grant-in-Aid for Scientific Research (B) (21380114) from Japan
243 Society for the Promotion of Science (JSPS) to KA.
244

245 **References**

246 1. Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2004) Geographic variation and
247 diversity of the cytochrome *b* gene in Jpanese wild populations of Medaka, *Oryzias latipes* . Zool Sci
248 20: 1279–1291.
249 2. Hashiguchi Y, Kado T, Kimura S, Tachida H (2006) Comparative phylogeography of two bitterlings,
250 *Tanakia lanceolata* and *T limbata* (Teleostei, Cyprinidae), in Kyushu and adjacent districts of western
251 Japan, based on mitochondrial DNA analysis. Zool Sci 23: 309–322.
252 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 anguillicanudatus*. 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 polypoid 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 tetrapoidy 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 meoiotic 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

337 **Figure captions**

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

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

347 **Fig. 3** Flow-cytometrical histograms of normal diploid and hyper-triploid in sperm. Fluorescent peak 1 of
348 haploid spermatozoa from normal wild-type diploid loach indicates 1C DNA content (a). Fluorescent
349 peak 1', 2' and 3' of spermatozoa or spermatozoa-like cells from sperm of hyper-triploid loach
350 indicate approximately 1.7C, 3.3C and 6.7C DNA content, respectively (b). Fluorescent peak 1" and
351 2" of spermatozoa or spermatozoa-like cells from sperm of hyper-triploid loach indicate
352 approximately 3.3C and 6.6C DNA content, respectively (c)

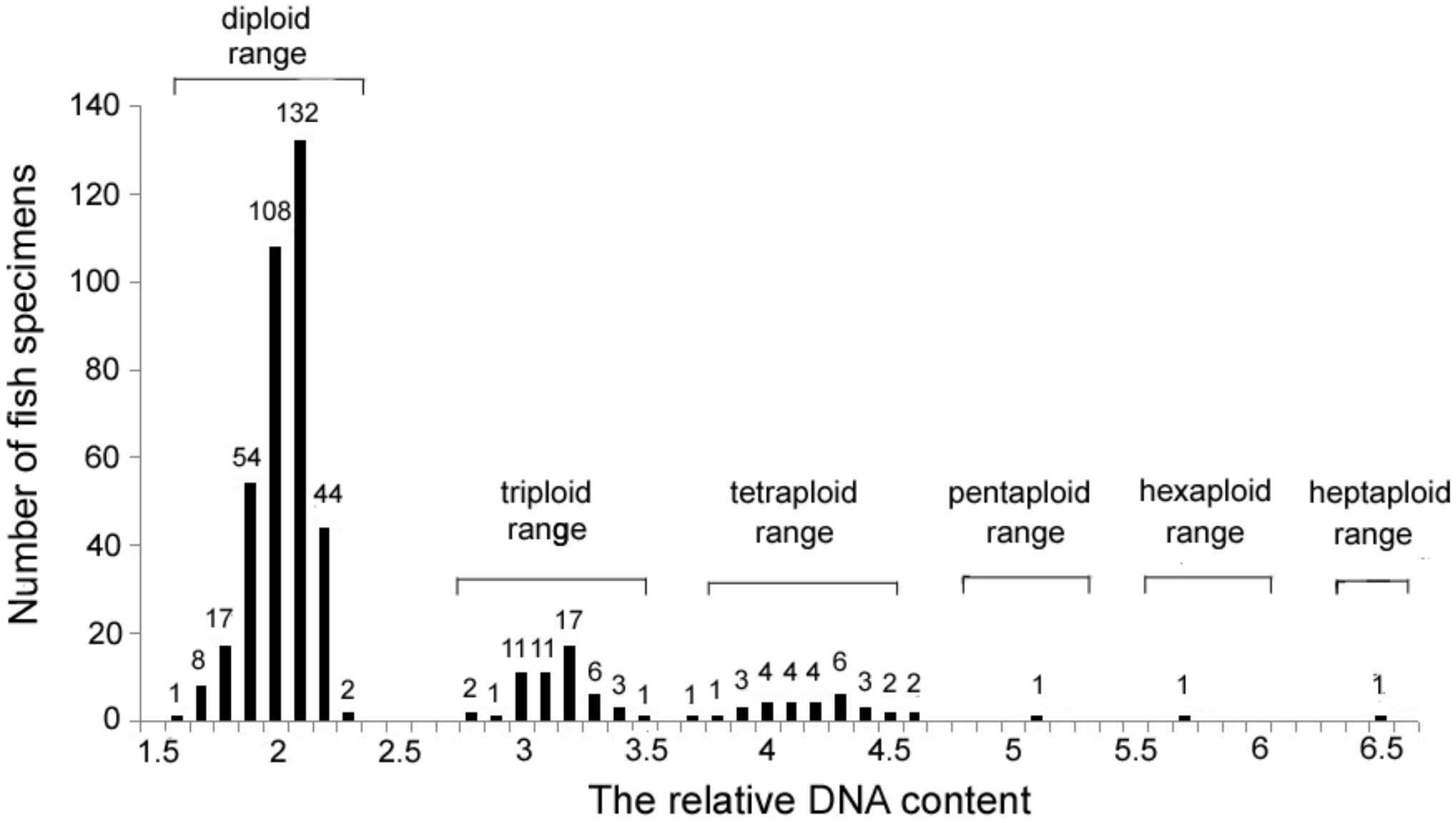
353 **Fig. 4** Flow-cytometrical histograms of neo-tetraploid and hyper-tetraploid in sperm. Fluorescent peak 1
354 of haploid spermatozoa from normal diploid loach indicates 1C DNA content; fluorescent peak 2 of
355 diploid spermatozoa from neo-tetraploid loach indicates 2C DNA content; fluorescent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

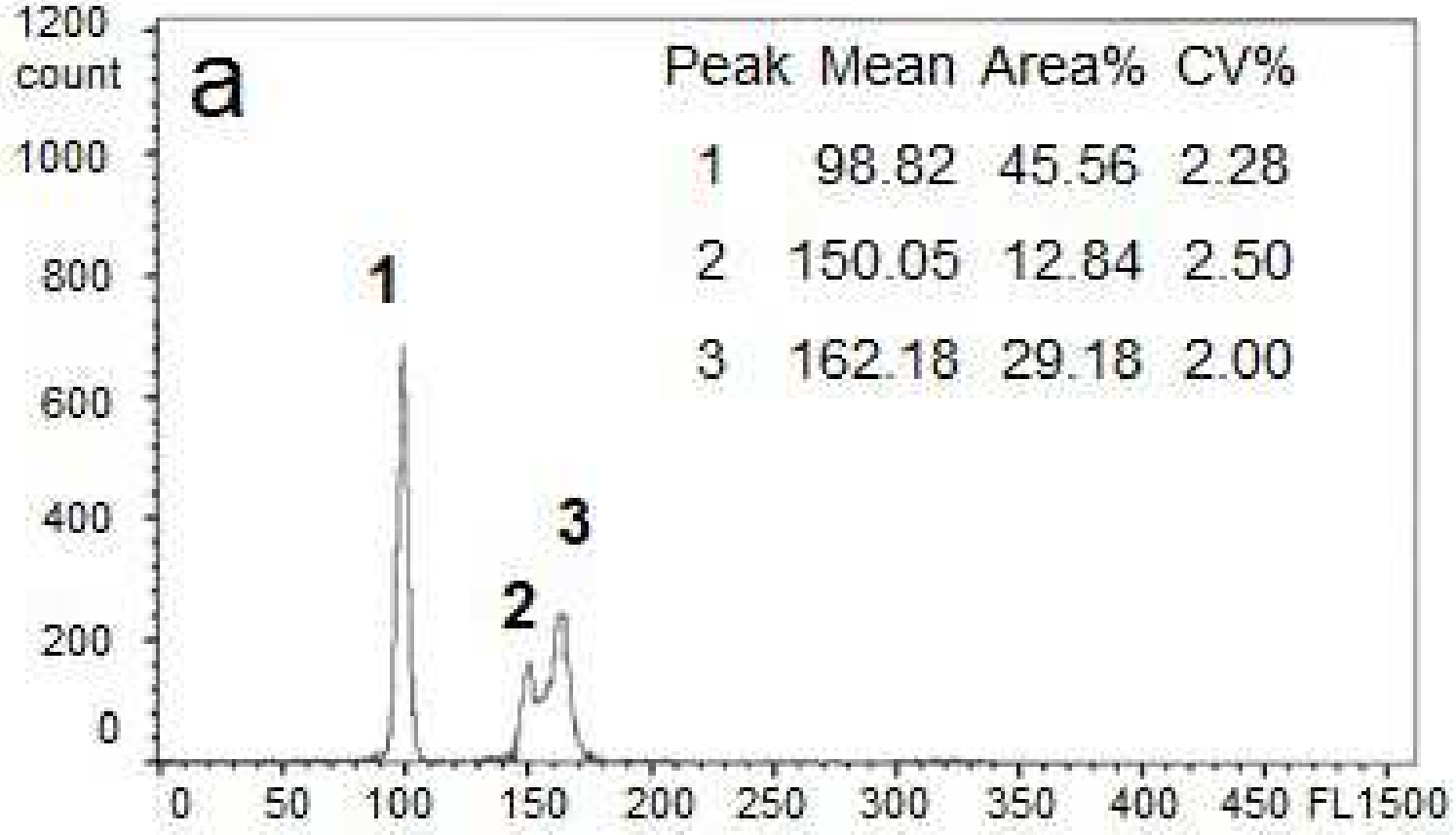
357 **Fig. 5** External appearance of larvae. Normal larvae from diploid female × neo-tetraploid male (a).

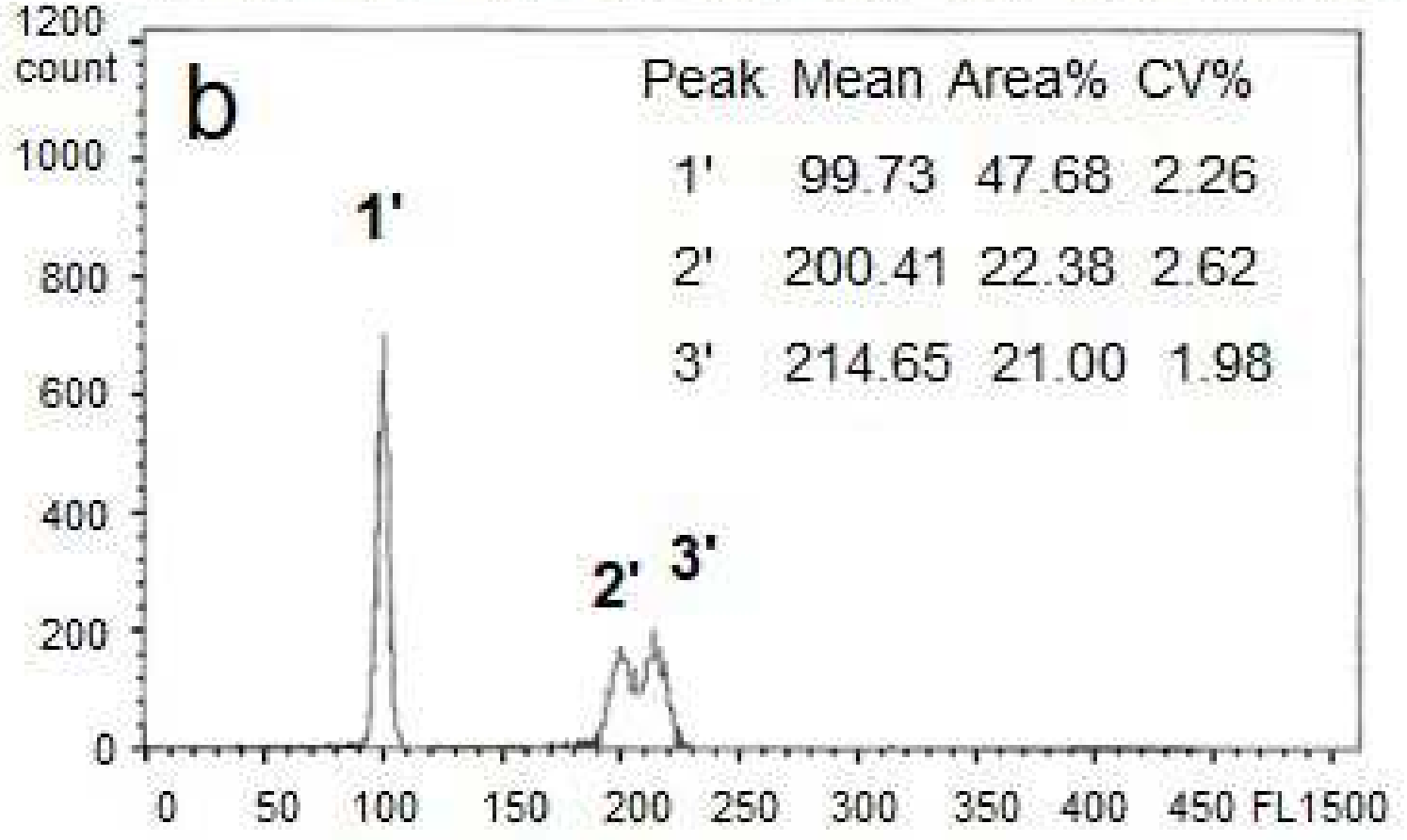
Normal larvae from diploid female × hyper-tetraploid males (hyper-4n-#1, hyper-4n-#2 and hyper-4n-#3) (b). Normal larvae produced by induced androgenesis, diploid female (UV-irradiated eggs) × neo-tetraploid male (c). Abnormal larvae with a few or no melanophores produced by induced androgenesis, diploid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

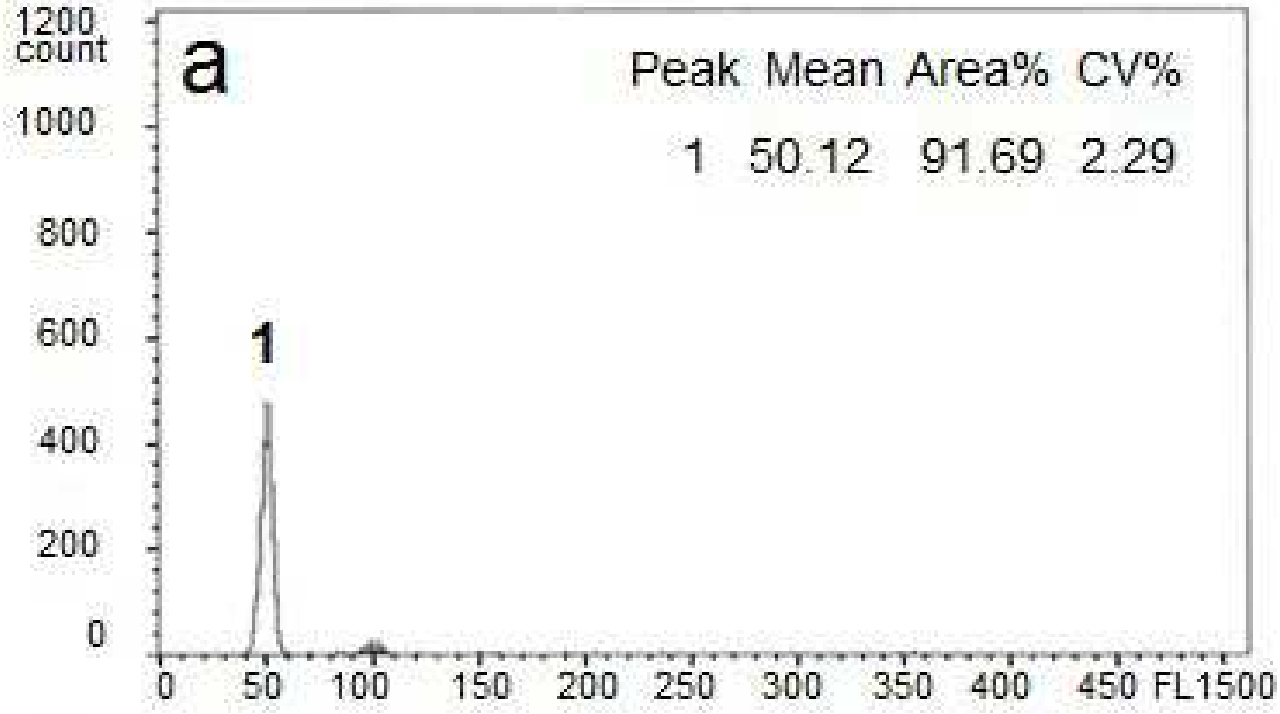
Fig. 6 Relative DNA contents of larvae from different crosses measured by flow cytometry. Fluorescent peak 1 of larvae from normal diploid female × normal diploid male cross indicates 2C DNA content. Fluorescent peak 2 of embryos from diploid female × neo-tetraploid male indicates 3C DNA content (a).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

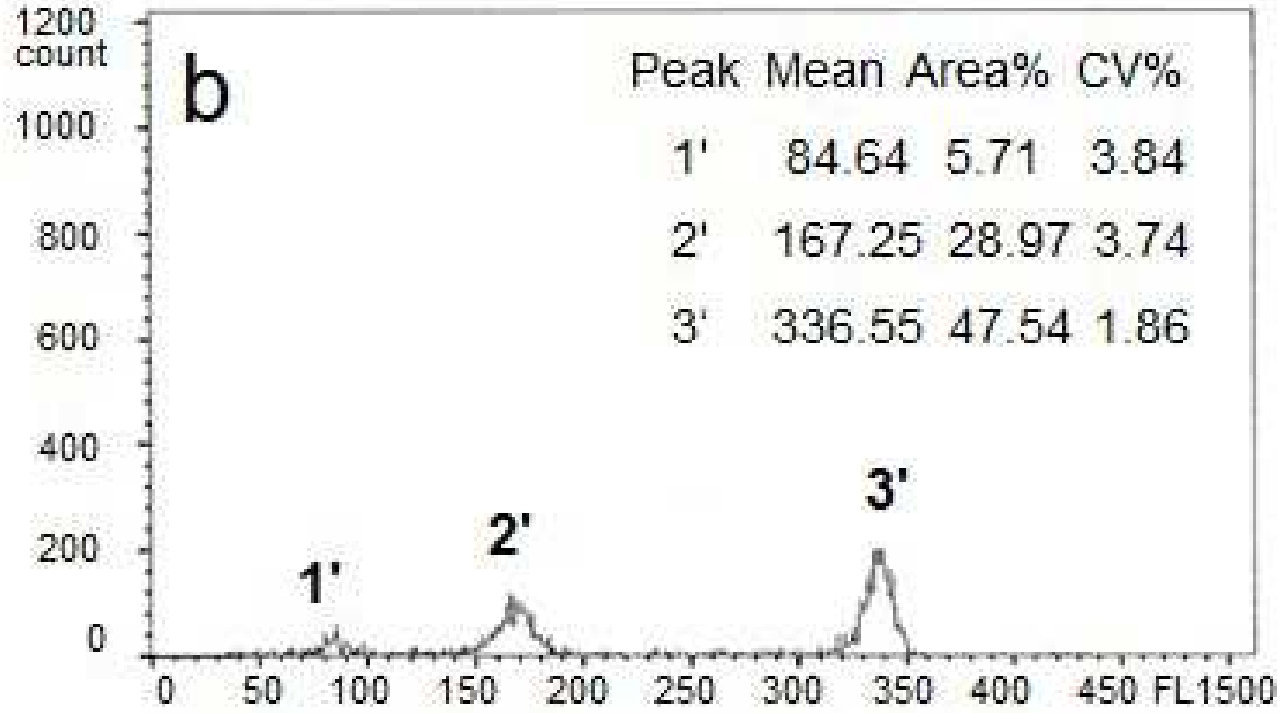
Fig. 7 Metaphase chromosomes (a, c) and corresponding karyotypes (b, d) of the loach *Misgurnus anguillicaudatus*. Diploid female (UV-irradiated eggs) × neo-tetraploid male, 2n=50 (a, b). Diploid female (UV-irradiated eggs) × hyper-tetraploid males, 2n=54 (c, d). Arrows indicate supernumerary chromosomes.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

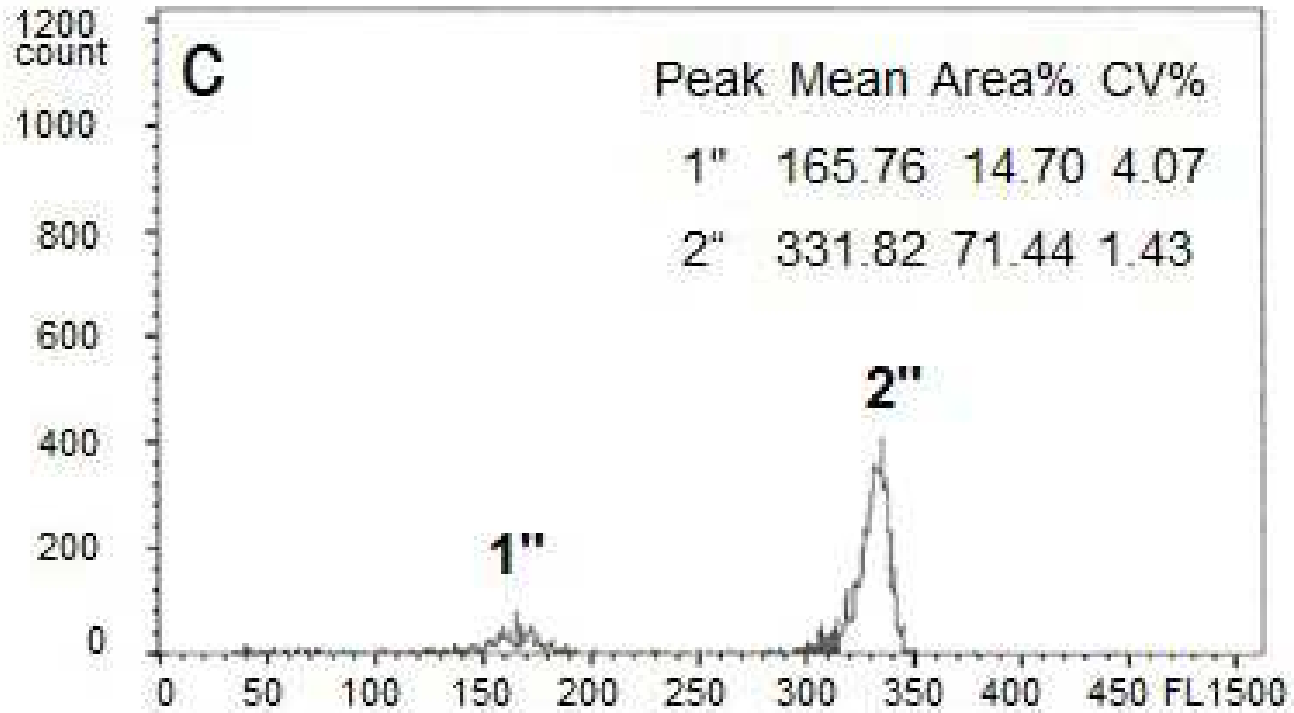

diploid range
triploid range
tetraploid range
pentaploid range
hexaploid range
heptaploid range
Number of fish specimens
The relative DNA content
0
20
40
60
80
100
120
140
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
1
8
17
54
108
132
44
2
2
1
11
11
17
6
3
1
1
1
3
4
4
4
6
3
2
2
1
1
1

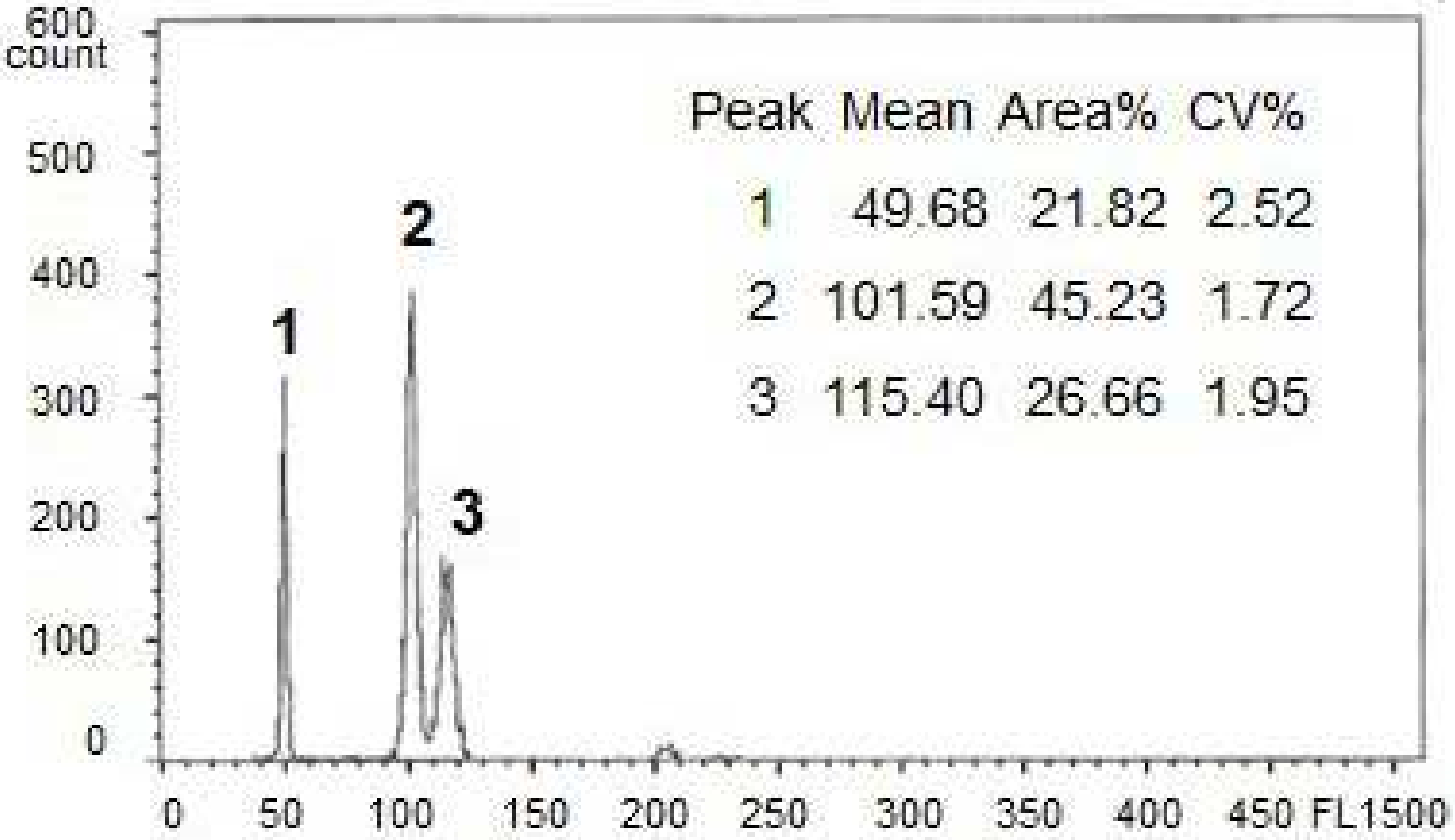

a
Peak Mean Area% CV%
1 98.82 45.56 2.28
2 150.05 12.84 2.50
3 162.18 29.18 2.00
1200
count
1000
800
600
400
200
0
1
2
3
0 50 100 150 200 250 300 350 400 450 FL1500

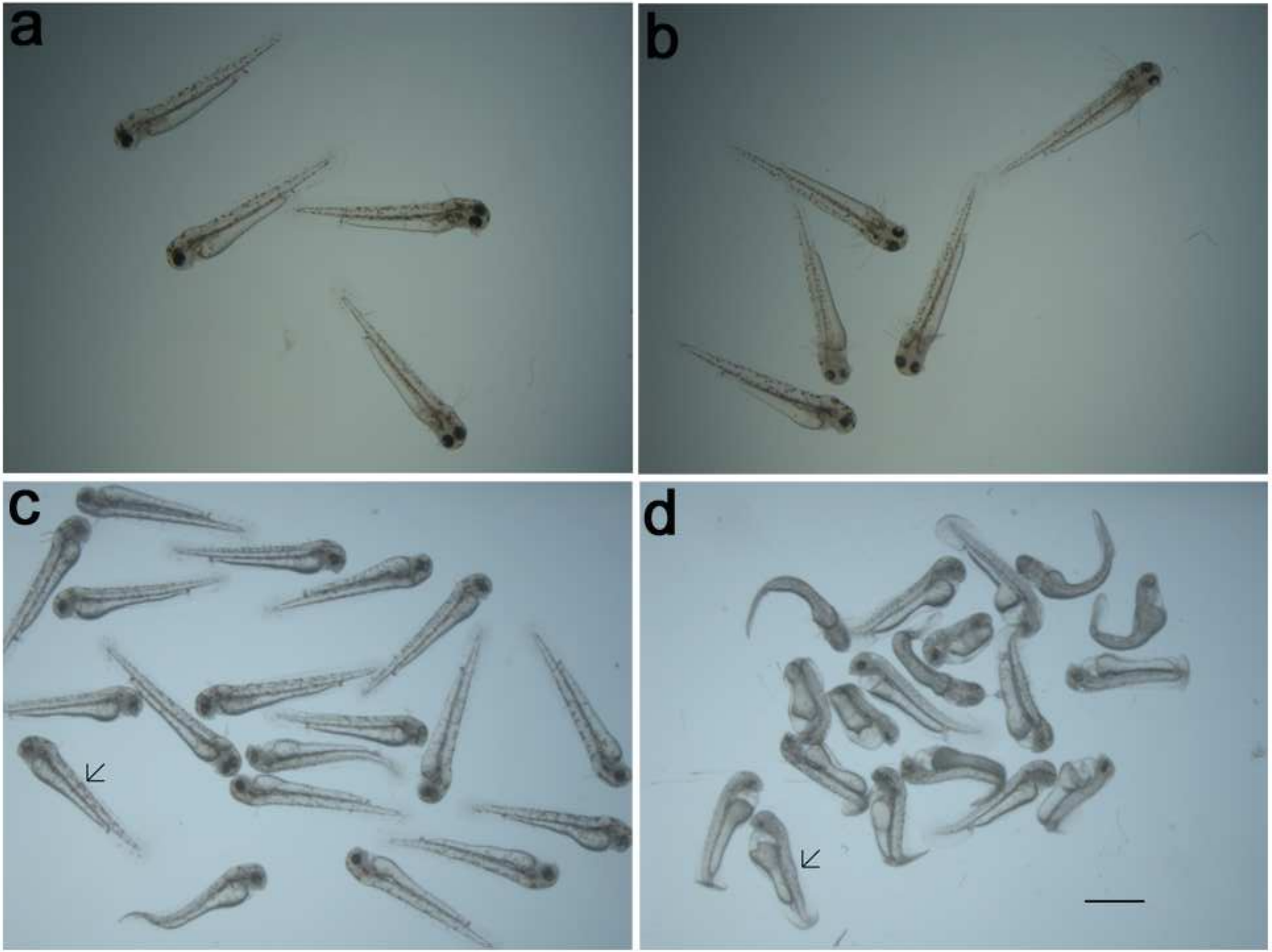

b
Peak Mean Area% CV%
1' 99.73 47.68 2.26
2' 200.41 22.38 2.62
3' 214.65 21.00 1.98
1200
count
1000
800
600
400
200
0
1'
2'
3'
0 50 100 150 200 250 300 350 400 450 FL1500

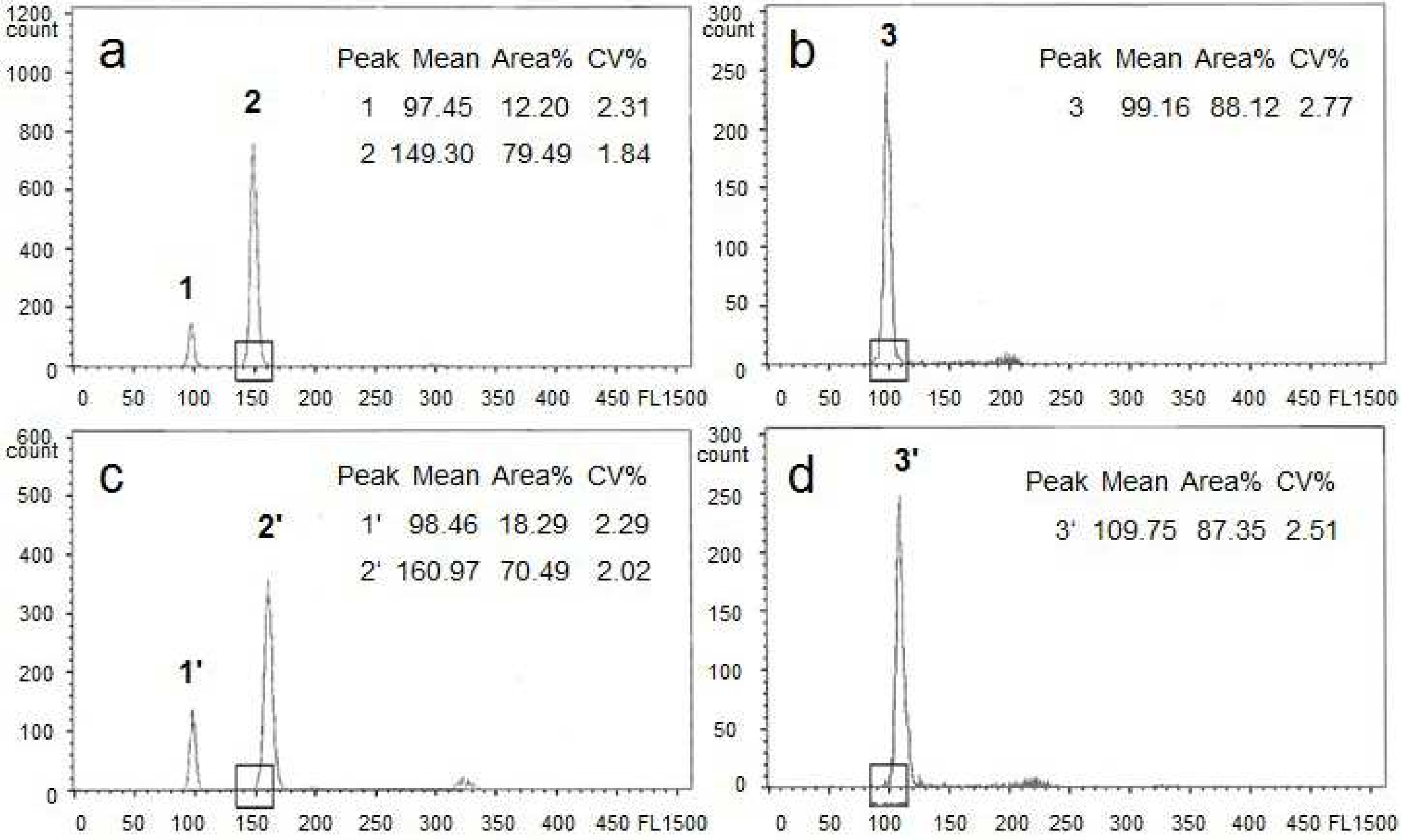

a
Peak Mean Area% CV%
1 50.12 91.69 2.29
1
1200 count
1000
800
600
400
200
0
0 50 100 150 200 250 300 350 400 450 FL1500


b
Peak Mean Area% CV%
1' 84.64 5.71 3.84
2' 167.25 28.97 3.74
3' 336.55 47.54 1.86
1'
2'
3'
1200 count
1000
800
600
400
200
0
0 50 100 150 200 250 300 350 400 450 FL1500


c
Peak Mean Area% CV%
1" 165.76 14.70 4.07
2" 331.82 71.44 1.43
1"
2"
1200 count
1000
800
600
400
200
0
0 50 100 150 200 250 300 350 400 450 FL1500


600
count
500
400
300
200
100
0
1
2
3
Peak Mean Area% CV%
1 49.68 21.82 2.52
2 101.59 45.23 1.72
3 115.40 26.66 1.95
0 50 100 150 200 250 300 350 400 450 FL1500


a
b
c
d


a
Peak Mean Area% CV%
1 97.45 12.20 2.31
2 149.30 79.49 1.84
1
2
count
FL1500
b
Peak Mean Area% CV%
3 99.16 88.12 2.77
3
count
FL1500
c
Peak Mean Area% CV%
1' 98.46 18.29 2.29
2' 160.97 70.49 2.02
1'
2'
count
FL1500
d
Peak Mean Area% CV%
3' 109.75 87.35 2.51
3'
count
FL1500

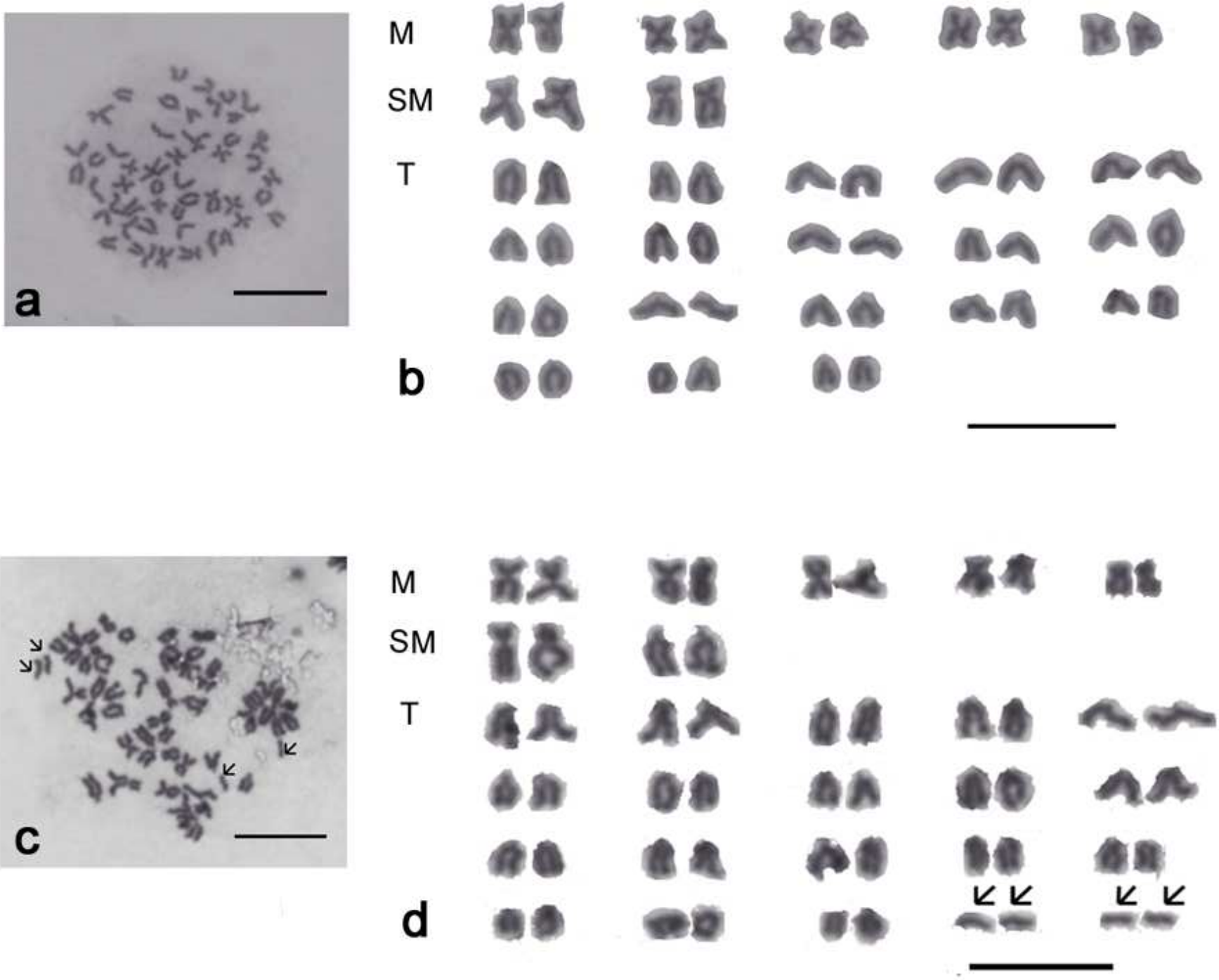

a
M
SM
T
b
c
d

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