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Hybrid male sterility between the fresh- and brackish-water types of ninespine stickleback *Pungitius pungitius* (Pisces, Gasterosteidae)

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Abbreviated title: Hybrid male sterility in sticklebacks

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ABSTRACT—Two ecologically distinct forms, fresh- and brackish-water types, of ninespine stickleback co-exist in several freshwater systems on the coast of eastern Hokkaido. Recent genetic analyses of 13 allozyme loci revealed genetic separation between the two types even though their spawning grounds were in close proximity. On the other hand, there is only a small difference in mitochondrial DNA (mtDNA) sequence between the two types suggesting that they diverged quite recently or that mtDNA introgression occurred between them. To test for postzygotic reproductive isolating mechanisms and hybrid mediated gene flow, we examined the viability and reproductive performance of reciprocal F_1 hybrids. The hybrids grew to the adult size normally and both sexes expressed secondary sexual characters in the reciprocal crosses. The female hybrids were reciprocally fertile, while the male hybrids were reciprocally sterile. Histological and flow-cytometric analyses of the hybrid testis revealed that the sterility pattern was classified as 'gametic sterility,' with gonads of normal size but abnormal spermatogenesis. To our knowledge, the present finding is a novel example of one sex hybrid sterility in the stickleback family (Gasterosteidae).

Key words: sterility pattern, speciation, flow-cytometry, Haldane's rule

INTRODUCTION

The ninespine stickleback *Pungitius pungitius* is a small euryhaline fish belonging to the family Gasterosteidae (Pisces) known as sticklebacks, an important model system in evolutionary biology (Mattern, 2004). This species has a nearly continuous circumpolar distribution, occurring in fresh and coastal waters of northern Eurasia and North America (Münzing, 1969; Wootton, 1976). Although its widespread distribution and morphological variability are comparable to those of the threespine stickleback *Gasterosteus aculeatus*, the ninespine sticklebacks has received less attention from biologists than the latter species (Wootton, 1976).

Takata *et al*. (1987) revealed that there are two ecologically and morphologically distinct forms, which co-occur abundantly in several freshwater systems on the coast of eastern Hokkaido, Japan. The two forms are identified as “freshwater type” and “brackish-water type,” though taxonomically undefined, based on the spawning habitat (Takata *et al*., 1987). They differ from one another in three meristic characters; the freshwater type has a high number of dorsal spines and gill-rakers, and a low number of vertebrae, when compared with the brackish-water type (Takata *et al*., 1987). The body color of the brackish-water type is typically silvery, and the freshwater type is usually yellowish or greenish brown. Although their habitats frequently overlap in lower reaches, discrete habitat preferences are generally maintained throughout the year. The freshwater type exclusively occupies freshwater areas within river systems, whereas the brackish-water type occupies brackish-water areas, such as estuaries and lagoons. Since these two forms are reciprocally monophyletic (Takahashi *et al*., 2003), the evolutionary background is differ from that of the anadromous–freshwater system in the threespine stickleback of which freshwater forms were considered to have multiple, independent

origins (McKinnon and Rundle, 2002).

Takata *et al*. (1987) examined allozyme variations between the two types in the Biwase River, eastern Hokkaido, and revealed complete allelic displacement at three of 13 loci examined. They claimed that the two types should be regarded as independent species, according to the biological species concept (Mayr, 1963). On the other hand, Takahashi and Goto (2001) suggested that the fresh- and brackish-water types had diverged quite recently or otherwise exchanged mitochondrial DNA (mtDNA) through introgressive hybridization, on the grounds that there was no obvious difference in their mtDNA control region sequences. Similar examples of discordant patterns of nuclear and mtDNA are abundant in sticklebacks (e.g., Taylor and McPhail, 1999, 2000; Takahashi and Takata, 2000). These studies suggested that mtDNA introgression has erased mtDNA history of the recipient population. Although information about the postzygotic reproductive isolating mechanisms will provide insight into the discrepancy between the allozyme and mtDNA data (e.g., Takahashi and Takata, 2000), little is known about such mechanisms between the two types.

As a first step to examine postzygotic reproductive isolating mechanisms between the two stickleback types, we examined the viability, growth potential, and reproductive performance of their artificial hybrids. It should be noted that these fitness components are part of postzygotic reproductive isolating mechanisms. Although, postzygotic reproductive isolating mechanisms are classified into extrinsic and intrinsic barriers (Coyne and Orr, 2004), the former such as ecological inviability, behavioral sterility (e.g., Rundle and Whitlock, 2001; Vamosi and Schluter, 1999) were not tested in the present study. To elucidate the cause of hybrid sterility found in the present study, we also made histological observation of gonads and comparison of the DNA contents

between the gonad and somatic cells.

MATERIALS AND METHODS

Rearing of hybrid stocks

Mature fishes of the fresh- and brackish water types of *Pungitius pungitius* were collected from the Bekanbeushi River, eastern Hokkaido, Japan, in June 2000. Three females and six males of each type were used as parents of artificial hybrids and of controls. We used the semi-dry method for artificial insemination, because of limited amounts of sperm in sticklebacks. The eggs were pressed out with fingers from a single mature female and halved into two Petri dishes. The testes were surgically removed from a single male and cut with scissors in a drop of normal saline. The halves of the eggs were fertilized with sperm suspensions obtained from a single different type male and with a single same type male as a control. The fertilization rate was estimated by the frequency of eggs that had undergone cleavage at 3 h after fertilization (2- or 4-cell stages). The fertilized eggs gathering in a cluster were detached from each other to avoid suffocation, and then maintained in a Petri dish at 15°C until they hatched. The hatching rate was calculated as the relative percentage of the initial eggs incubated.

Each full sib stock was kept in a separate aquarium (15 × 10 cm and 10 cm high) for about two months, and then 20 fishes were chosen randomly and transferred to a larger aquarium (60 × 30 cm and 30 cm high). They were maintained at 15°C with a 14L:10D cycle and initially fed one to two times a day with freshly hatched *Artemia* (brine shrimp) until they were large enough to accept frozen bloodworms (larval chironomids). Thereafter fishes were fed with frozen bloodworms and live tubifexes once a day. After about six months, the fish rearing condition was switched to a lower temperature 10°C with a 12L:12D cycle to simulate winter. The condition was shifted

once again to warmer temperature 15°C with a 14L: 10D cycle in April 2001 to facilitate the final maturation of the fish. Water salinity was maintained at 1-3 ppt throughout the experiment.

To test the reproductive performance of the F_1 hybrids, the fertilization and hatching rates for those gametes were examined by artificial fertilization with the normal control gametes. Three mature individuals were randomly chosen for both sexes in each full sib stock of F_1 hybrids, and backcrossed to the controls. The fertilization and hatching rates were estimated in a similar manner as above. The experiments were finished when all of the eggs hatched or deceased.

Gonad histology

Histological observation of gonads was made on F_1 hybrid that expressed a reduced survival rate in the backcross experiment. Additional three mature individuals were used for each full sib stock of F_1 hybrids and of both types of controls in gonad histology and flow-cytometric analysis. A small part of each gonad sample was used in flow-cytometric analysis (see below), the remainder being fixed overnight with Bouin's fixative and dehydrated in a butyl alcohol series for gonad histology. After embedding in paraffin, the entire gonad was sectioned transversally at 8 μm thickness and stained with Delafield's hematoxylin and eosin according to the standard procedures. Histological nomenclatures followed Ruby and McMillan (1970).

Flow-cytometric analysis

Flow cytometry to detect the DNA content of testis cells was used to elucidate the stage of spermatogenesis in the hybrids and controls using the Partec PA flow cytometer (Partec GmbH, Münster, Germany). Approximately 10 mg of testis were incubated for 5 min. in 100 ml of Partec Cystain solution A (Partec) at room temperature. After

filtration of the cell suspension, the nuclei were stained by adding Partec Cystain solution B (Partec) according to the manufacture's instructions. Relative DNA content of testis cells was measured as fluorescence intensity with respect to reference diploid cells (fin).

RESULTS

Growth and maturation of hybrids

The fertilization and hatching rates were more than 95% in all of the F_1 hybrids and controls, and these rates were not significantly different between the hybrids and controls (Wilcoxon signed-ranks test with exact probability, $Z = -1.00$, $P = 1.00$; $Z = -0.37$, $P = 0.86$). The fish grew normally to the adult size (approx. 4-6 cm, SL), and all of them expressed secondary sexual characters such as a head-up display in female and a nest construction behavior in male with nuptial coloration at the next year breeding season (May to July). No mortality was observed in larger aquariums for each full sib stock, with the exception of a single dead fish observed in two of six control stocks.

In the backcross experiments, the fertilization and hatching rates in some crosses were lowered (Table 1) as compared to those in controls (more than 95%). The rates for the F_1 hybrid females were as high as those in controls (more than 90%) except for one of six cases (56.3% in hatching rate, Table 1). Extremely low hatching rates (0%–3.7%) were observed for the progeny of F_1 hybrid male crossed with both pure types although fertilization rates were high (68.3%-100%). All of these embryos exhibited the typical haploid syndrome (arrested development, distorted body axis, small eyes) (Onozato and Yamaha, 1982), and died at hatching with the exception of one backcross progeny, of which four out of 107 eggs were successfully hatched. These four had a curved body axis, small eyes, and died within two days of hatching.

Histological observation of hybrid testes

Since reduced reproductive performance was observed only in F_1 hybrid males, histological observations were made only in the males. No significant difference was observed between the samples in outside appearances of testis such as shape and size except for the color of epiorchium. Melanophores on the epiorchium were highly developed in the freshwater type, rendering the testes dark gray, while those were reduced in the brackish-water type testes, rendering it white. Both of the reciprocal F_1 hybrids represented intermediate testes with grayish color.

Microscopically, the majority of seminiferous tubules in testes of both controls were occupied by mature sperm and by spermatids with condensed nuclei that stained strongly with hematoxylin (Fig. 1a, b). Mature sperm also occupied the interior of the seminal ducts. A regular array of phagocytes containing sperm nuclei was observed on the interior wall of some tubules. A relatively small number of cysts involving primary or secondary spermatocytes were observed in putative secondary seminiferous tubules located near the outside of testis. No obvious difference was detected in the histological features of the fresh- and brackish-water type controls.

The testes of reciprocal F_1 hybrids consisted of seminiferous tubules without mature sperm (Fig. 1c, d). Many vacant spaces were observed in the interior of tubules. A relatively large number of tubules contained cysts with spermatocytes and/or a small number of spermatids. The nuclei of such spermatids were stained in various strengths with hematoxlin and these cells had an irregular shape. Phagocytes containing spermatid nuclei were observed in several tubules. The configuration of such phagocytes was irregular, and these cells were liberated from the interior wall of the tubules.

Flow-cytometric analysis for hybrid testes

A single sharp peak in fluorescence intensity was present in the flow-cytometric histogram for each fin sample. This fluorescence intensity was regarded as an indication of 2n DNA content (Fig. 2c). In the testis, three distinct peaks are expected: spermatozoa (1n), static and replicated phases of spermatocytes (2n or 4n), and somatic cells (2n). The flow-cytometric analyses revealed that the testes of both controls consisted of a prominent 1n-cell population (spermatozoa) and a lower 2n-cell population (Fig. 2a). On the other hand, the testis cells of reciprocal hybrids showed only a prominent 2n-cell population with no 1n spermatozoa (Fig. 2b). In addition, a small 4n-cell population was detected in some fish.

DISCUSSION

Three points will be helpful in discussing the characteristics of fish hybrids: viability, growth potential, and reproductive performance (Chevassus, 1983). In the present study, no significant difference in the former two was observed between reciprocal F_1 hybrids and controls. In the hybrid females, the last characteristic (reproductive performance) was also normal, although the viability of the backcrosses has been examined only through the hatching period. On the other hand, extremely low hatching rates were observed in the backcrosses of male hybrids, indicating that the reciprocal hybrid males were sterile. Relatively high fertilization rates were expressed in the hybrid male experiments, but all backcross embryos exhibited typical haploid syndrome. Furthermore, eggs laid by control females under natural mating with hybrid males showed no fertilization in an aquarium experiment (Takahashi, unpublished data). For these reasons, the relatively high fertilization rates were probably due to the artificial insemination method by which the parthenogenetic activation of the eggs was

induced.

In fish hybrids, various sterility patterns have been observed, but these patterns fall roughly into three types: zygotic sterility, gametic sterility and gonadic sterility (Chevassus, 1983). “Zygotic sterility” is that the conditions where the gametes are viable (normal in size and structure) and fertilization occurs but results embryos fail to develop. In the present study, as noted above, estimates of fertilization rate were uncertain, because of possible parthenogenetic activation of eggs. However, no mature sperm was observed in seminiferous tubules of the testes in reciprocal F_1 hybrids and flow-cytometric analyses revealed the absence of 1n. These observations indicate the sterility pattern of the hybrid males was more serious one than the zygotic sterility.

In “gametic sterility” the gonads are normal in size but abnormal in gametogenesis. Histology indicated that a large number of tubules contained cysts with spermatocytes in the hybrid males, but only a small number of such tubules located near the outside of testis in the controls. In a closely related gasterosteid fish, brook stickleback (*Culaea inconstans*), spermatocyte formation completed in the first autumn of life, and most of the cysts filled with spermatocytes broke down prior to the winter season for spermiogenesis (Ruby and McMillan, 1970). Taking into account the similarity in the life cycles between the brook and ninespine sticklebacks (Ruby and McMillan, 1970; Goto *et al*., 1979), the present result for hybrid males suggested delay or arrest of spermatogenesis reducing the number of spermatids and distorting their shape.

“Gonadic sterility” is characterized by reduced gonad size. Since the testes of hybrid males were normal in size, the sterility pattern of the hybrids was determined to be the gametic sterility characterized by abnormal spermatogenesis. The male hybrids exhibited the secondary sexual characteristics similar to the controls, suggesting that the

testis retained endocrine functions (i.e., producing androgens) essential for the development of secondary characteristics (Wootton, 1976). Similar examples are abundant: Suzuki and Fukuda (1973), for example, reported that the hybrids of salmonid fishes with zygotic or gametic sterility generally exhibit secondary sexual characteristics.

In the present study, hybrid sterility was observed only in the male hybrids. To our knowledge, the present finding is a novel example of one sex (male) hybrid sterility in gasterosteid fishes. Few different patterns of intrinsic postzygotic isolation have been observed. Honma and Tamura (1984), for example, reported that F_1 hybrids between female marine and male landlocked forms of threespine stickleback (*Gasterosteus aculeatus*) were sterile in both sexes, while hybrids of the opposite direction were fertile in both sexes. These marine and landlocked forms correspond to the Japan Sea and Pacific Ocean groups, respectively, suggested by Higuchi and Goto (1996). A different pattern, breakdown in backcrosses, was observed in the well-studied limnetic and benthic forms of threespine stickleback. Hatfield and Schluter (1999) revealed that hatching success of the benthic backcrosses was significantly lower than that of the limnetic, F_1 and F_2 crosses in a laboratory cross experiment between the two forms.

Similar observations to that found in the present study are abundant in a broad array of animal taxa, a general rule known as Haldane's rule (Haldane, 1922). This rule states that when in the F_1 offspring of two species or populations, one sex is inviable or sterile, that sex is usually the heterogametic sex. Taking the remarkable consistency of this rule among taxa into consideration (Coyne, 1992), the present sterility pattern suggests that ninespine sticklebacks have XY heterogametic males, even with homo-morphic sex chromosomes (e.g., Klinkhardt and Buuk, 1990). Indeed,

male-specific DNA sequence was identified by the amplified fragment length polymorphism (AFLP) method in the close relative, the threespine stickleback (Griffiths *et al*., 2000). As outlined by Orr (1997), the faster evolution of hybrid male sterility (faster-male theory) likely plays an important role in Haldane's rule for hybrid male 'sterility' in addition to the fundamental dominance theory. The faster-male theory explains the Haldane's rule on the basis of two following reasons: (*i*) spermatogenesis is particularly sensitive to perturbation in gene expression, perhaps due to lack of postmeiotic transcription regulation; and (*ii*) sexual selection might cause faster evolution of male- than female-expressed genes (Wu and Davis, 1993). The current results would seem to be consistent with the former, because of hybridization defects were observed only in the spermatogenesis but not in the other aspects of male reproduction. Further studies on genes associated with hybrid male sterility can provide many insights into the genetic bases of the reproductive isolating mechanisms, though such genes have never been identified in the ninespine sticklebacks.

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Table 1. Fertilization and hatching rates for gametes of the F_1 hybrids between the fresh- and brackish-water types of ninespine stickleback, *Pungitius pungitius*.

Parents*		Egg number	Fertilization rate	Hatching rate
Female	Male			
BF-a	BB	102	102 (100%)	99 (97.1%)
BF-b	BB	37	37 (100%)	37 (100%)
BF-c	FF	64	59 (92.2%)	36 (56.3%)
FB-a	BB	126	126 (100%)	115 (91.3%)
FB-b	FF	61	60 (98.4%)	59 (96.7%)
FB-c	FF	46	46 (100%)	46 (100%)
BB	BF-a	117	115 (98.3%)	0
BB	BF-b	32	32 (100%)	0
FF	BF-c	145	99 (68.3%)	0
BB	FB-a	115	89 (77.4%)	0
FF	FB-b	107	107 (100%)	4 (3.7%)
FF	FB-c	138	138 (100%)	0

* BF: hybrids F_1 of brackish-water type female and freshwater type male parents, FB: of freshwater type female and brackish-water type male parents, and BB and FF: brackish-water and freshwater types controls, respectively. Three individuals (a-c) of F_1 hybrids were used for each cross.

Figure legends:

Fig. 1. Transverse sections through seminiferous tubules of the testes in both controls (a: the freshwater type, b: the brackish-water type) and hybrids (c: the freahwater type female and the brackish-water type male; d: the brackish-water type female and the freshwater type male), showing spermatids (S), phagocytes (arrow head), and cysts with spermatocytes (SC). Both hybrids testes consisted of seminiferous tubules without mature sperm, many vacant spaces being observed. Scale bars indicate 0.05 mm.

Fig. 2. Examples for the Partec PA flow cytometer output showing flow-cytometric histogram in testis of the freshwater type control (top) and the F_1 hybrid between the freshwater type female and the brackish-water type male (middle). Relative DNA contents of testis cells were measured as fluorescence intensity (x-axis) with respect to the reference of fin as diploid cells (bottom).

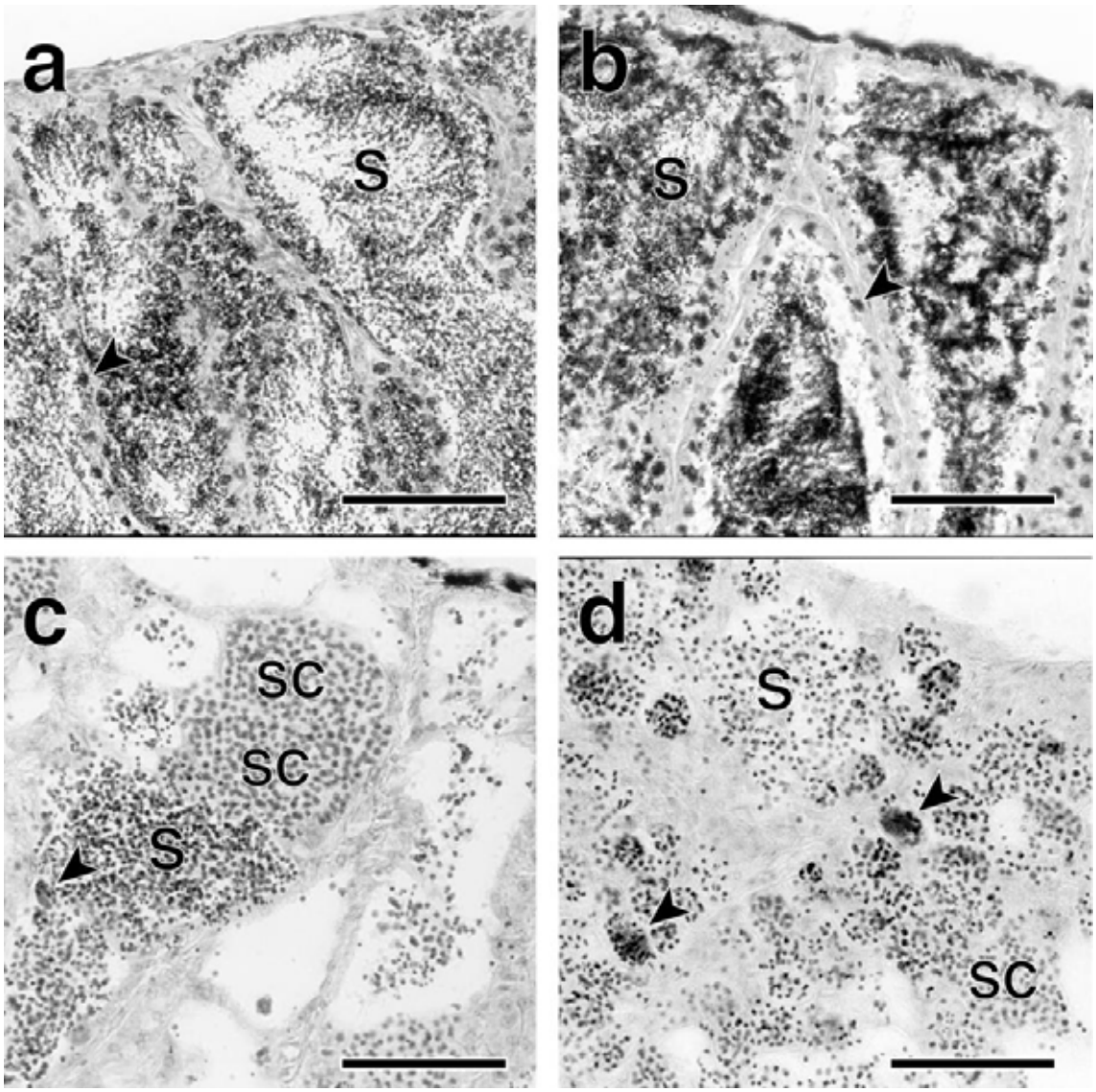

Figure 1.

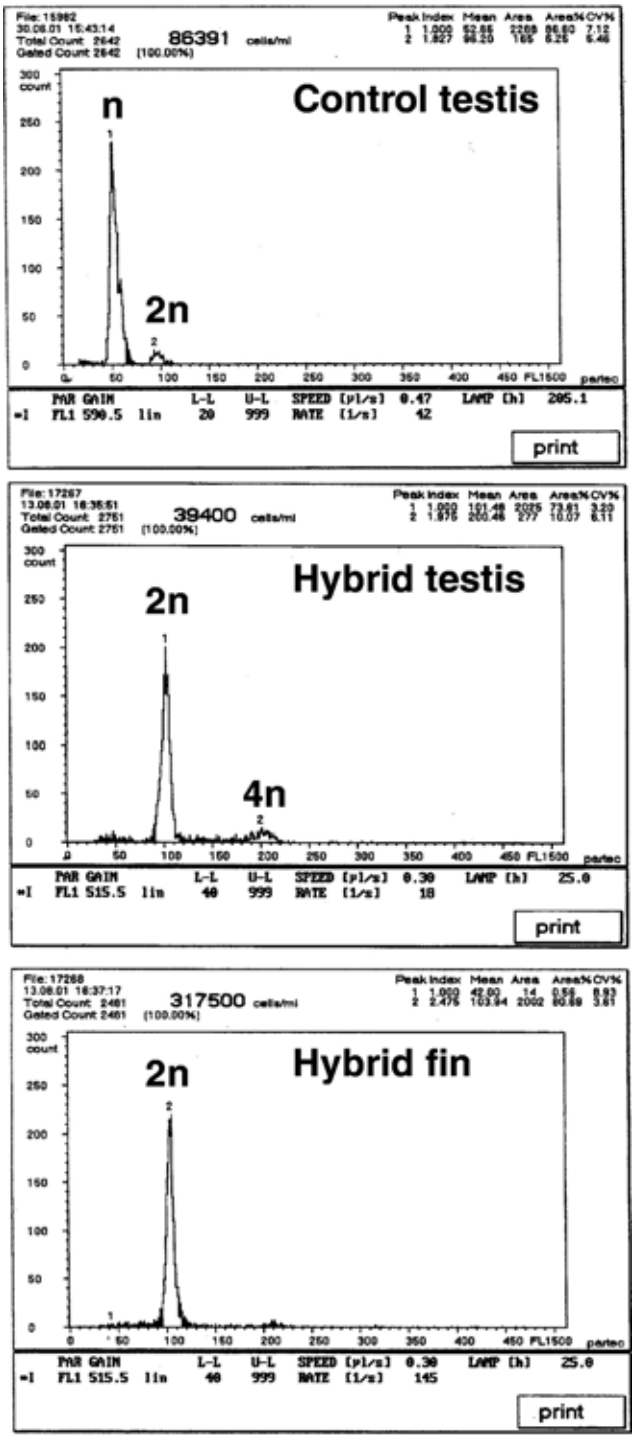
86391 cells/ml
n
Control testis
2n
print
39400 cells/ml
Hybrid testis
2n
4n
print
317500 cells/ml
2n
Hybrid fin
print

Figure 2.