Interspecific androgenetic restoration of rosy barb using cadaveric sperm

S. Kirankumar and T.J. Pandian

Abstract: Interspecific androgenetic rosy barb (*Puntius conchonius*) was generated using its cadaveric (–20 °C) or fresh sperm to activate nuclear genome inactivated oocytes of gray tiger barb (*Puntius tetrazona*). UV irradiation was used to inactivate nuclear genome of tiger barb oocytes. Thermal shock restored diploidy of rosy barb in the oocytes of tiger barb. Survival of androgenotes was 14% or 7% when fresh or cadaveric sperm was used. The diploid or haploid nuclear genome of rosy barb, individually or jointly with that of tiger barb, regulated the time sequence of embryonic development in an alien cytoplasm of tiger barb oocytes. Androgenetic males (Y^2Y^2) attained sexual maturity earlier and had significantly higher gonadosomatic index and sperm concentration, albeit suffering a slight decrease in fertilizing ability. Conversely, androgenetic females (X^2X^2) suffered extended interspawning period, reduced fecundity, and poor hatchability of their progenies. These results are discussed with respect to their significance for conservation biology.

Key words: nuclear genome inactivation, tiger barb, cadaveric sperm, rosy barb, interspecific androgenotes, Tc1 transposon.

Résumé : Des barbus rosés, *Puntius conchonius*, interspécifiques androgénétiques ont été générés en employant du sperme entreposé (-20 °C) ou frais pour activer des oocytes de barbus tigre (*P. tetrazona*) dont le génome avait été inactivé. L'irradiation aux UV a été employée pour inactiver le génome nucléaire des oocytes des barbus tigre. Un choc thermique a rétabli la diploïdie du génome du barbus rosé dans l'oocyte du barbus tigre. La survie des progénitures androgénétiques était de 14 ou de 7 % selon que le sperme employé était frais ou entreposé. Le génome nucléaire du barbus rosé, diploïde ou haploïde, régulait, seul ou conjointement avec le génome du barbus tigre, la séquence de développement de l'embryon dans le cytoplasme étranger du barbus tigre. Les mâles androgénétiques (Y^2Y^2) ont atteint la maturité sexuelle plus tôt et présentaient une concentration significativement plus élevée de sperme ainsi qu'un index gonadosomatique plus élevé. Malgré cela, ils souffraient d'une légère diminution de leur aptitude à la fécondation. Inversement, les femelles androgénétiques (X^2X^2) souffraient d'une période plus longue entre les fraies, d'une réduction de la fécondité et d'une moindre éclosion de leur progéniture. La signification de ces résultats est discutée en lien avec la conservation des ressources biologiques.

Mots clés : inactivation du génome nucléaire, barbus tigre, sperme entreposé, barbus rosé, progéniture androgénétique interspécifique, transposon Tc1.

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Introduction

Ex situ strategies like cloning may become important for conservation of valuable germplasm. In fishes, the established scheme of nuclear manipulation may prove a difficult task because of the nonvisibility of the egg nucleus. However, fishes are known to tolerate genome from haploidy to heptaploidy, genomic contribution from male or female parent alone, and unequal contributions from parents belonging to the same or different species (Pandian and Koteeswaran 1998). Androgenesis, a developmental process facilitating the presumable inheritance of exclusively paternal nuclear genome, involves three steps: (*i*) destruction of oocyte nuclear genome by irradiation with X or γ rays or inactivation of oocyte nuclear genome by UV irradiation, (*ii*) activation with a single spermatozoan, and (*iii*) restoration of diploidy by arresting the first mitotic cleavage in the zygote (Pandian and Koteeswaran 1998). Of more than 20 publications available on androgenesis in fishes, earlier researchers have concentrated on developing species-specific protocols for (*i*) irradiation with X or γ rays (Arai et al. 1979) or UV rays (Bongers et al. 1994) and (*ii*) confirmation of inactivation and (or) destruction of maternal nuclear genome. Most authors have used phenotypic markers, especially coloration (e.g., Bongers et al. 1994) or protein markers like isozymes (e.g., Scheerer et al. 1991), to confirm the inactivation and (or) destruction of maternal genome in oocytes. Whereas phenotypic and protein markers are limited to one or few genes and (or) alleles, molecular markers may provide a

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more comprehensive analysis of the genome. In recent years, many authors have used different molecular markers, e.g., SSR and MHC (Corley-Smith et al. 1996), RAPD (Berscenyi et al. 1998), microsatellites (Babiak et al. 2002*a*), and AFLP (Brown and Thorgaard 2002).

In fishes in which androgenesis has been achieved, most authors have generated intraspecific androgenotes using milt and nuclear genome inactivated/destroyed oocytes of the same species. Although more or less the same procedure may ensure interspecific androgenesis, a few have reported failure for one or more reasons (e.g., May and Grewe 1993; Babiak et al. 2002*a*). Bercsenyi et al. (1998) were the first to succeed in induction of interspecific androgenetic goldfish (*Carassius auratus*) using oocytes of common carp (*Cyprinus carpio*) (also see Recoubratsky and Grunina 2001). They hatched out goldfish from carp oocytes and reared the hatchlings for 3–6 months. More recently, Nam et al. (2002) have successfully induced interspecific androgenotes of the cyprinid mud loach (*Misgurnus mizolepis*) using oocytes of *C. carpio*. Brown and Thorgaard (2002) have induced interspecific androgenetic salmonid *Oncorhynchus mykiss* using sex-reversed male and normal females of *Oncorhyncus clarki* subsp. *bouveri*.

Scheerer et al. (1991) showed that cryopreserved sperm can be used to induce androgenesis in salmonids. Through an information-rich publication, Babiak et al. (2002*b*) confirmed that cryopreservation of sperm does not significantly decrease the efficiency of androgenesis. However, the need for costly equipment including a liquid nitrogen container has been the bottleneck in the development of protocols for cryopreservation of fish sperm, especially in developing countries like India. Recently, Koteeswaran and Pandian (2002) successfully generated progenies of catfish *Heteropneustes fossilis* using live fertile sperm collected from a specimen that was postmortem frozen at –20 °C for 240 days. This finding has made it possible to use cadaveric sperm to induce androgenesis. This paper reports on the production and life history traits (survival and reproduction) of interspecific androgenetic rosy barb (*Puntius conchonius*) (Hamilton 1822) using cadaveric sperm and genome-inactivated oocytes of tiger barb (*Puntius tetrazona*) (Bleeker 1855).

Materials and methods

Induction of androgenesis

Two closely related species, rosy barb and tiger barb, were used to produce androgenotes; the rosy barb was restored using tiger barb as the recipient (Fig. 1). Fresh or cadaveric sperm was drawn from four different strains, which were distinguishable with respect to three phenotypic markers: tail morphology (veil or normal), body colour (gray or gold), and a spot near the caudal peduncle (present or absent). The four respective genotypes were VVGGss, vvGGSS, vvggSS, and vvggss (see Table 4). Eggs were drawn from the dominant "gray" tiger barb. The barbs were purchased from a local dealer and their F_2 progenies were reared at 28 °C for experimentation. The barbs were cared for in accordance with the Canadian Council on Animal Care.

Kirankumar (2002) has shown that sperm collected from cadavers of rosy barb frozen at –20 °C up to 30 d successfully activated oocytes of tiger barb. Healthy, mature males were immersed in a plastic trough containing 1 L of water with 0.2% clove oil for 5 min and anaesthetized. When they failed to make spontaneous opercular movement, they were presumed to be dead and were frozen at –20 °C for up to 30 d. Subsequently, the frozen males were thawed and dissected to remove the testes, and each lobe of the testes was squashed in 5 mL of teleost Ringer's solution (Ogawa et al. 1973).

For each treatment, about 140 oocytes from two ripe females of the same age and strain were stripped in a thin plastic petri dish (1 mm thick) and spread in a single layer in teleost Ringer's solution. They were irradiated using a UV-G lamp (254 nm, 40 W). The distance between the lamp and the oocytes in the petri dish was 25 cm and exposure duration varied from 1.5 to 4.0 min with an increment of 0.5 min. Intensity of the irradiation (watts per square metre) was estimated using a RMX-3W-CX-254 radiometer (Vilber Lourmat, France), which was positioned near the petri dish containing the oocytes.

To activate the irradiated oocytes, one unit of fresh sperm or two units of cadaveric sperm were added, i.e., the oocytes were mixed with either 0.75 mL ($7.1 \pm 0.6 \times 10^5$ cells/mL) of fresh sperm or 1.5 mL ($8.2 \pm 0.7 \times 10^3$ cells/mL) of milt collected from postmortem preserved specimens of the chosen strain and diluted in teleost Ringer's solution. Subsequently, addition of 3 mL of water into the petri dish and gentle swirling for 45 s ensured activation of the oocytes. Following this, 2 mL of degumming solution containing the Ringer's solution and milk at a ratio of 4:1 was added and the petri dish was gently swirled for 45 s until the stickiness was completely removed. The whole procedure was completed in darkness.

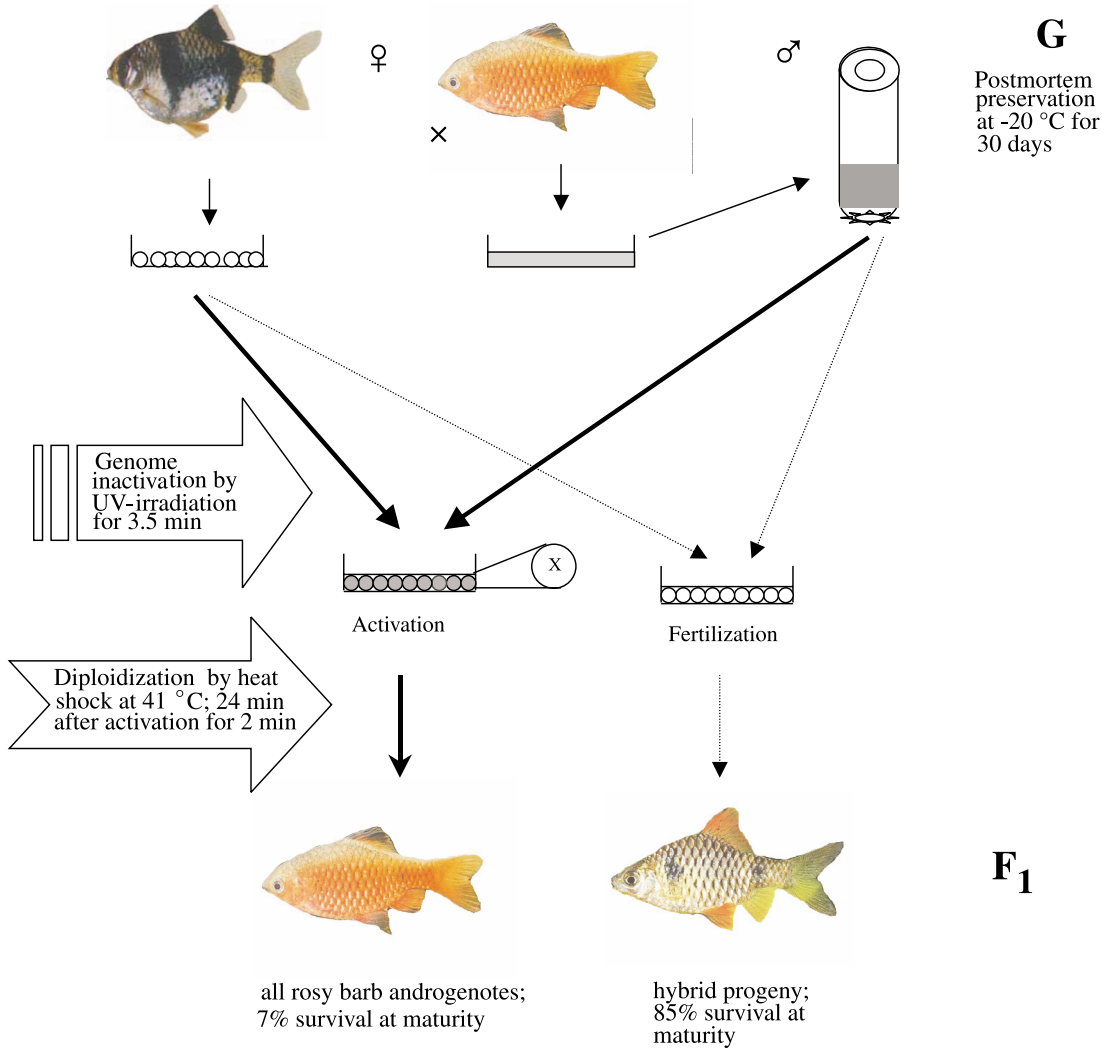
The activated eggs were kept at 28 °C. Thermal shock at 40, 41, and 42 °C was applied for 2.0 or 2.5 min at each temperature by suspending embryos in a plastic container (5 cm in diameter and 3 cm deep with a screen of 1-mm mesh at the bottom) in a water bath. After shock treatment, the embryos were transferred to a plastic tray filled with sterilized water to restore them to the preshock temperature.

From the respective treatment groups, randomly selected just-hatched fry were immersed in 0.01% colchicine solution for 6 h. They were then fixed in Carnoy's fixative (3 parts methanol to 1 part glacial acetic acid) for 30 min. Subsequently, the fixative was removed and the whole fry, one after the other, was dissociated by gentle aspiration in 200 μL of 45% acetic acid. The dissociated tissue with suspending cells was dropped from a height of 20 cm over a clean slide (Kligerman and Bloom 1977). The slide was air dried and stained in 5% Giemsa for 30 min. Determination of ploidy of each individual was based on 25 ± 5 metaphase spreads; for each treatment group, a minimum of seven individuals were analyzed.

Genomic purity of androgenotes

Tissue samples (fin clips, 10–50 mg each) were collected from the parents and randomly selected androgenetic progenies of the same strain at the age of 3 months for PCR analysis. Genomic DNA was isolated from tissues (300 mg)

Fig. 1. Protocol for interspecific androgenesis of rosy barb using nuclear genome-inactivated oocytes of tiger barb and fresh or cadaveric sperm of rosy barb; diploidy is restored by a thermal shock.



by proteinase-K digestion and phenol extraction (Sambrook et al. 1989). PCR analysis was done to compare the nuclear genome of the androgenotes with those of their parents using Tcl transposon specific primers, namely, TclF1 (CAGTTGAAGTCGGAAGTTTACA-22 mer) and TclR1 (AGTCGGAAGTTTACATACACC-21 mer). TclFl and TclR1 primers are expected to bind at the 5′ and 3′ ends to cover the full length of the Tcl transposon consensus region (1778 bp). The PCR protocol applied was as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and primer extension at 72 °C for 3 min. Thirty cycles of reactions were performed with a final extension of 5 min at 72 °C and an initial denaturation at 94 °C for 3 min. The resulting PCR amplicons were analyzed on 2% agarose gel. Thirty randomly selected androgenetic progenies belonging to the four strains were screened by this analysis.

Genomic DNA of the unexpected female progenies, sired by a cross between an androgenetic male (Y^2Y^2) and a normal female (X^1X^2), were isolated and subjected to PCR analysis using the primers developed by Kirankumar et al. (2003) to identify their genotype. Of eight such unexpected female progenies, six were screened for this analysis.

Embryonic mortality was estimated for individuals containing haploid, diploid, or hybrid genome to know the extent of the *P. conchonius* genome (in haploid or diploid status) regulated embryonic development in an “alien” egg in terms of time scale and survival.

Reproductive performance

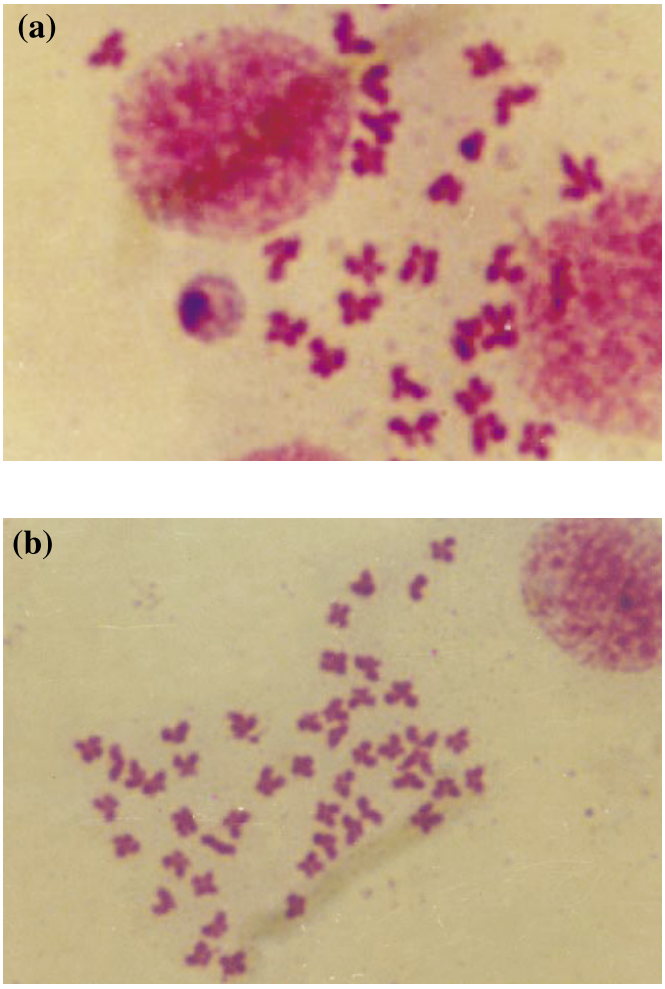
The interspecific androgenetic rosy barb were reared to sexual maturity and a long-term study was undertaken for progeny testing and to assess the breeding performance of these androgenetic males (Y^2Y^2) and females (X^2X^2). Gonadosomatic index was calculated by expressing gonad mass as a percentage of body mass. A mature male was stripped in 1 mL of Ringer’s solution and sperm concentration was estimated using a haemocytometer (Neu-Baur, Germany) by the method described in Linhart (1991). In a microscopic concave slide, 300 µL of tap water was added to

Table 1. Effect of different durations of UV irradiation on hatching of haploid eggs of tiger barb, which were activated after irradiation by fresh sperm of rosy barb (strain IV).

Duration of UV exposure (min)	No. of tiger barb oocytes	Hatchability of rosy barb fry (%)	Genotype of hatched fry (%)		
			Haploid (*n*)	Aneuploid	Diploid (2*n*)
1.5	1430	30±2.23	0	10±5.81	90±5.45
2.0	1460	21±2.16	0	15±4.93	85±4.73
2.5	1360	9±1.32	20±2.26	80±1.75	0
3.0	1480	16±3.24	95±1.38	5±2.48	0
3.5	1440	9±1.18	100	0	0
4.0	1360	12±3.72	9±2.64	91±2.52	0

Note: Each value is the mean (±SD) of a minimum of 10 replicates.

Fig. 2. Metaphase spread of (*a*) haploid (*n* = 24) and (*b*) diploid (*n* = 48) interspecific androgenetic rosy barb. Scale bar = 5 µm in Fig. 2*a* and 10 µm in Fig. 2*b*.



100 µL of Ringer's solution containing milt and sperm and motility duration was estimated using a stopclock (Shinco, India).

Two sexually mature interspecific androgenetic males, expected to sire 100% male progenies, were randomly selected from each of the four strains. Each male was crossed with three randomly selected females (X^1X^2) from a pool of 10 females belonging to each of the selected four strains. Their respective progenies were reared for 90 days or longer to morphologically identify sex and to estimate sex ratio.

Statistical analysis

All data are presented as mean ± SD. The level of significance among the corresponding data was calculated using ANOVA, followed by Duncan's multiple range test (Zar 1984).

Results

Interspecific androgenesis

When oocytes of tiger barb were subjected to UV irradiation for 3.5 min and activated by fresh or cadaveric sperm of rosy barb, 100% haploids were generated (Table 1). The hatchability of these haploids was 9%. In these fry, haploidy was confirmed by karyotyping (Fig. 2*a*). The treatment of 24-min-old (after activation) haploid rosy barb embryos at 41 °C for 2.0 min diploidized all of the treated embryos (Table 2).

To confirm restoration of rosy barb using its fresh and cadaveric sperm and genome-inactivated oocytes of tiger barb, karyotyping of the 2-day-old androgenetic fry was done (Fig. 2*b*). The veil tail became apparent on the fifth day in fry belonging to strain I. On the seventh day, the gray (strains I and II) or gold (strains III and IV) body colour, characteristic of rosy barb, became apparent; however, the black vertical bands, characteristic of tiger barb, were totally absent in all strains characterized by gray and gold colour. On the 15th day, the black spot on the caudal peduncle, characteristic of rosy barb, became visible in progenies belonging to strains II and III. These phenotypic markers and karyotype showed that rosy barb was restored from tiger barb oocytes and the desired interspecific androgenesis was achieved.

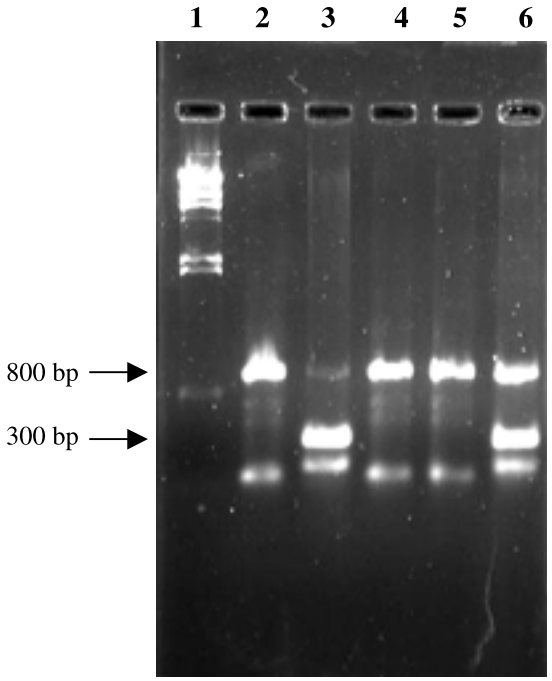
PCR analyses showed that the rosy barb genomic DNA produced an intense 800-bp amplicon and the tiger barb genomic DNA an intense 300-bp amplicon. The hybrids between these two barbs (tiger barb ♀ × rosy barb ♂) produced both of these amplicons (Fig. 3). Expectedly, the F_1 androgenotes produced the rosy barb specific 800-bp amplicon only. Therefore, PCR analysis confirmed that no tiger barb specific Tc1 marker sequence was present in the androgenetic rosy barb hatched from tiger barb oocytes.

Table 2. Estimation of optimum temperature and heat shock duration required to restore diploid rosy barb (strain IV) from the nuclear genome inactivated oocytes of tiger barb.

Treatment protocol					Genotype of rosy barb fry (%)		
Temperature (°C)	Commencement time (min after activation)	Duration (min)	No. of UV-irradiated tiger barb oocytes	Hatchability of rosy barb fry (%)	Haploid (*n*)	Aneuploid	Diploid (2*n*)
40	22	2.0	1340	7±1.25	9±1.22	91±1.27	0
		2.5	1390	8±2.63	10±1.39	90±1.43	0
	24	2.0	1430	6±1.74	0	22±2.37	78±2.49
		2.5	1470	6±4.46	0	16±2.82	84±2.38
41	22	2.0	1350	13±1.35	0	6±1.94	94±1.74
		2.5	1430	11±3.43	0	5±2.74	95±3.17
	24	2.0	1400	14±1.73	0	0	100
		2.5	1360	10±2.83	0	3±1.99	97±2.87
42	22	2.0	1380	8±2.49	0	82±3.32	18±3.47
		2.5	1370	9±1.64	0	92±3.79	8±3.83
	24	2.0	1420	6±1.48	0	87±1.65	13±2.26
		2.5	1350	5±2.73	0	77±2.32	23±1.95

Note: Each value is the mean (±SD) of a minimum of 10 replicates.

Fig. 3. Agarose gel electrophorogram showing species-specific Tc1 amplifications in rosy barb, tiger barb, the hybrid barb, and the androgenotes. Lane 1, λ *Hin*dIII marker; lane 2, rosy barb strain I; lane 3, gray tiger barb; lane 4, androgenote strain I; lane 5, androgenote strain I; lane 6, hybrid barb.



Survival of androgenotes

Survival of tiger and rosy barbs resulting from homologous fertilization was 98% and ~95% at hatching and sexual maturity, respectively (Table 3). It was reduced to <80% in hybrids, to <14% in interspecific androgenetic rosy barb, and to <7% or 3% when a single cadaveric sperm or double fresh was used to generate the androgenotes. Haploid androgenotes successfully hatched but hatching success was 9% or 3% when fresh or cadaveric sperm was used. A strain-specific analysis of survival of androgenetic rosy barb showed that at all of the selected developmental stages, the triple recessive strain suffered a significantly ($P < 0.05$) higher percentage of mortality than the double and single recessive strains (Table 4). Trends obtained for survival of diploid and haploid androgenetic rosy barb ran parallel, close to each other, with both suffering progressive mortality throughout embryonic development (Fig. 4), although haploids suffered more, especially during the early embryonic stages (activation to 18th somite stage) and immediately following hatching.

In reciprocal crosses, the hybrid genome of the barbs regulated the time sequence of embryonic development within 24 h (Table 3); likewise, the haploid and diploid genome of rosy barb also regulated the time sequence of embryonic development within 24 h (Table 3) ($P < 0.05$) in the alien cytoplasm of tiger barb oocytes (diameter 1230 ± 20 μm) (Fig. 4).

Reproduction in androgenetic clones

Selected reproductive parameters of normal heterozygous (X^1Y^2) male and homozygous (Y^2Y^2) androgenetic males of all four strains (pooled values) of the rosy barb indicated that androgenotes attained sexual maturity earlier and had significantly ($P < 0.05$) higher gonadosomatic index and sperm concentration (Table 5). However, the fertilizing capacity of their sperm was low; a strain-specific analysis clearly indicated that this was due to the significantly ($P < 0.05$) low value obtained for the triple recessive strain IV. Conversely, androgenetic females (X^2X^2) suffered significantly ($P < 0.05$) from extended interspawning period, reduced gonadosomatic index and fecundity, and poor hatchability of their progenies (Table 5).

Randomly selected androgenetic supermales (Y^2Y^2) belonging to strains I, II, and III always sired 100% male progenies when crossed with any randomly selected females from the pool. However, an unexpected 12.5% frequency of female progenies was recorded when sires 7 and 8 of the tri-

Table 3. Survival and duration required for completion of embryonic and postembryonic development of tiger and rosy barbs, their hybrids, and androgenotes of rosy barb.

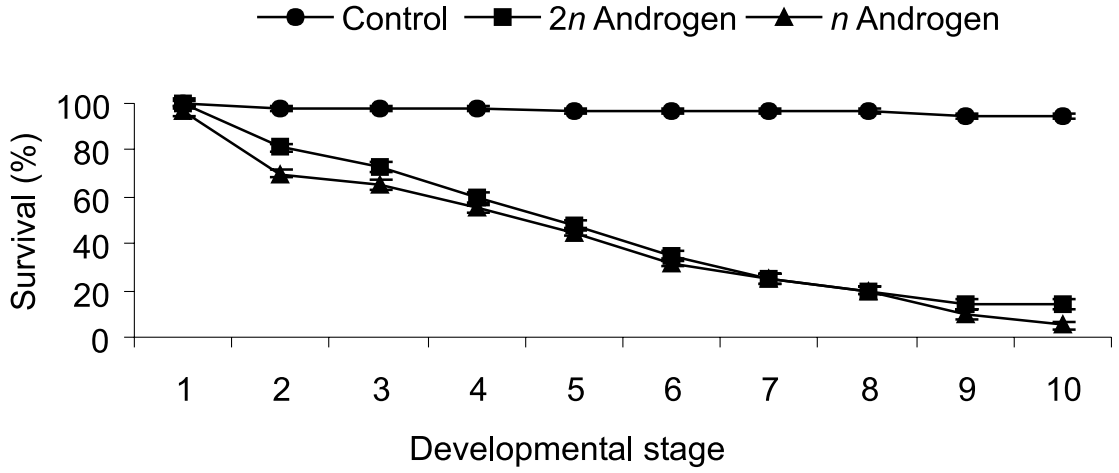
Cross	Time after fertilization (h): hatching	Time after hatching (h): feeding	Survival rate (%) at: hatching	Survival rate (%) at: maturity
Tiger barb control (TB)	26±1.35	42±1.32	98±0.12	94±0.32
Rosy barb control (RB)	24±1.15	36±1.40	98±0.15	95±0.46
RB ♀ × TB ♂ hybrid	24±0.75x	36±1.10	72±3.10	0
TB ♀ × RB ♂ hybrid	24±1.18	40±1.20	80±2.0	75±1.30
2*n* interspecific androgenote (fresh RB sperm)	24±1.25	42±1.30	14±1.50	7±1.10
2*n* interspecific androgenote (cadaveric RB sperm)	24±1.10	42±1.20	7±1.10	3±0.90
2*n* interspecific androgenote (fresh RB *n* + *n* sperm)*	24±1.10	42±1.20	3±1.62	2±1.17
n interspecific androgenote (fresh sperm source)	24±1.20	0	9±1.20	0
n interspecific androgenote (cadaveric sperm source)	24±1.37	0	3±1.30	0

Note: Each value is the mean (±SD) of 20 replicates.
*From Kirankumar and Pandian (2004*b*).

Table 4. Survival of the androgenetic clones of the rosy barb belonging to the selected four strains at different developmental stages.

Developmental stage	Strain I (VVGGss)	Strain II (vvGGSS)	Strain III (vvggSS)	Strain IV (vvggss)	Hybrid control
6±1 somite	51±4.3	49±3.3	55±5.4	38±3.4	92±1.7
Hatching	14±1.7	13±2.8	14±2.6	9±1.7	80±2.3
Maturity	5±2.5	7±2.3	7±1.5	3±1.6	75±1.4

Fig. 4. Survival of androgenote of gray rosy barb (strain I) as a function of embryonic stage. Arabic numbers represent the successive embryonic and postembryonic stages: 1, just activated/fertilized egg; 2, oblong; 3, epiboly; 4, bud; 5, sixth somite; 6, 12th somite; 7, 18th somite; 8, 24th somite; 9, hatching; 10, 1.5- to 2-day-old feeding stage.



ple recessive strain IV were crossed with dams 8 and 2, respectively (also see Kirankumar and Pandian 2004*a*).

PCR analysis revealed that these unexpected females possessed the Y-chromosome-specific molecular markers of 588- and 333-bp amplicons and thereby characterized that these females carried the XY genotype.

Discussion

This study reports on the successful induction of interspecific androgenesis and restoration of *P. conchonius* using its cadaveric sperm to activate the nuclear genome inactivated oocytes of *P. tetrazona*. For the first time, the interspecific androgenetic males and females have been reared to sexual maturity and their reproductive performance has been tested. However, Bercsenyi et al. (1998) were the first to cryopreserve sperm to successfully hatch out interspecific androgenotes of *C. auratus*. Babiak et al. (2002*a*) have shown that cryopreserved sperm does not generally decrease the efficiency of interspecific androgenesis. The present study on the use of cadaveric sperm of *P. conchonius* has confirmed the earlier observation of Koteeswaran and Pandian (2002) and has shown that this simple, practicle method of using cadaveric sperm from

Table 5. Reproductive performance of androgenetic male (Y^2Y^2) and female (X^2X^2) rosy barb.

Parameter	Genotype of ♂	
	X^1Y^2	Y^2Y^2
Sexual maturity (day)	90±3	85±3
Gonadosomatic index	0.38±0.03	0.55±0.02
Sperm concentration (no./mL)	$7.2±0.9 \times 10^5$	$7.5±0.7 \times 10^6$
Motility duration (s)	95±8.0	87±5.0
Fertilizing capacity (%)	95±3.2	88±10.1
	Genotype of ♀	
	X^1X^2	X^2X^2
Sexual maturity (day)	90±3	130±4
Interspawning period (days)	15±2	26±5
Gonadosomatic index	0.42±0.02	0.28±0.04
Fecundity (no./spawn)	91±3.5	60±5.2
Hatchability (%)	95±2.4	70±2.5

Note: Each value is the mean (±SD) of a minimum of 30 estimates using 20 sires from four strains.

postmortem frozen (–20 °C) specimens is capable of producing viable hatchlings.

Most authors used phenotypic markers such as colour (Bongers et al. 1994) and protein markers such as isozymes (Scheerer et al. 1991) to confirm complete inactivation and (or) destruction of maternal genome in oocytes. Only a few authors, such as Corley-Smith et al. (1996) and Bercsenyi et al. (1998), used a more comprehensive molecular analysis, namely random amplified polymorphic DNA, to confirm the destruction of the maternal genome. In the interspecific androgenotes of *O. mykiss*, Brown and Thorgaard (2002) traced mitochondrial and nuclear inheritance using amplified fragment length polymorphism analysis. Babiak et al. (2002*b*) used a single microsatellite locus to confirm homozygosity and paternal origin of androgenotes. In the present study, three contrasting morphological traits, namely body colour, tail feature, and peduncle spot, were used. The molecular marker Tcl transposon was used to confirm the inactivation of maternal genome in oocytes of fish. The simultaneous use of more "points of control" including microsatellites, random amplified polymorphic DNA, and restriction fragment length polymorphism may increase the probability for detection of maternal contamination; the persisting maternal nuclear fragments were assumed to cause poor viability of androgenotes (Babiak et al. 2002*a*). That survival at hatching of interspecific cyprinid androgenotes ranged from 14% in rosy barb to 19% in mud loach (Nam et al. 2002) to 17%–28% in goldfish (Bercsenyi et al. 1998) may indicate that the probability for maternal genome contamination is minimal in cyprinid interspecific androgenotes.

UV irradiation ensured a higher percentage of survival of androgenotes at hatching than that obtained with γ or X rays (Pandian and Kirankumar 2003). Corley-Smith et al. (1996) successfully induced intraspecifc androgenesis in zebrafish (*Danio rerio*) and obtained only a single functional male (also see Devlin and Nagahama 2002). Nam et al. (2002) established three clonal lines of interspecific androgenetically derived transgenic mud loach and followed their growth trends for a period of 5 months. In rosy barb, androgenetic clonal lines for four strains were established with a breeding population consisting of more than 50 males and 40 females. Scheerer et al. (1991) monitored the sex ratio of F_1 in intraspecific androgenetic *O. mykiss*; however, they limited their observations to the sex ratio of F_1 alone. The present study is the first to report on reproductive performance of an interspecific androgenetic fish.

Haploids of interspecific androgenetic salmonids do not hatch (Babiak et al. 2002*a*). However, successful hatching of haploids of interspecific androgenotes is reported; their hatching success ranges from 9% in rosy barb to 57% in mud loach (Nam et al. 2002). The trends obtained for embryonic mortality for intraspecific androgenetic rainbow trout (Babiak et al. 2002*b*) and interspcific androgenetic rosy barb are comparable and show progressive mortality of these androgenotes throughout embryonic development. Besides, this investigation has shown that the haploid nuclear genome of the rosy barb regulated the time sequence of embryonic development in tiger barb oocytes as precisely as that of the diploid.

In recent years, cloning has become possible and its usefulness for conservation of endangered species has been realized. The basic scheme includes removal of the haploid nucleus from an oocyte by microsurgery and implantation of a diploid nucleus derived from a somatic cell of the animal to be cloned. Like a typical totipotent zygote, the chimeric egg develops into an individual. In fishes, this established scheme of nuclear manipulation may prove a difficult task because of the nonvisibility of the egg nucleus. Furthermore, the nuclei drawn from somatic cells of fish appear to be less totipotent than those of frogs or mammals, as Lee et al. (2002) could transfer only the nuclei from blastula or embryonic fibroblasts and not from adult cells (also see Pandian 2002). Alternatively, induction of interspecific androgenesis in fishes may open a new avenue for restoration of endangered fish species to mass produce seedlings of less fecund and (or) periodic spawning (e.g., common carp) and migratory (e.g., eel, salmon) species using eggs of suitable donor fish species.

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References

Arai, K., Onozato, H., and Yamazaki, F. 1979. Artificial androgenesis induced with gamma irradiation in masu salmon, *Oncorhynchus masou*. Bull. Fac. Fish. Hokkaido Univ. **30**: 181–186.

Babiak, I., Dobosz, S., Goryczko, K., Kuzminski, H., Goryczko, K., Ciesielski, S., Brzuzan, P., Urbanyi, B., Horvarth, A., Lahnsteiner, F., and Piironen, J. 2002*a*. Failure of interspecies androgenesis in salmonids. J. Fish Biol. **61**: 432–447.

Babiak, I., Dobosz, S., Goryczko, K., Kuzminski, H., Brzuzan, P., and Ciesielski, S. 2002*b*. Androgenesis in rainbow trout using cryopreserved spermatozoa: the effect of processing and biological factors. Theriogenology, **57**: 1229–1249.

Bercsenyi, M., Magyary, I., Urbanyi, B., Orban, L., and Horvath, L. 1998. Hatching out goldfish from common carp eggs: interspecific androgenesis between two cyprinid species. Genome, **41**: 573–579.

Bleeker, P. 1855. Nalezingen op de vischfauna van Sumatra. Visschen van Lahat en Sibogha. Natuurkd. Tijdschr. Neder. Indië, **9**: 257–280.

Bongers, A.B.J., In't Veld, E.P.C., Abo-Hashema, K., Bremmer, I.M., Eding, E.H., Komen, J., and Richter, C.J.J. 1994. Androgenesis in common carp (*Cyprinus carpio* L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture, **122**: 119–132.

Brown, K.H., and Thorgaard, G.H. 2002. Mitochondrial and nuclear inheritance in an androgenetic line of rainbow trout, *Oncorhynchus mykiss*. Aquaculture, **204**: 323–335.

Corley-Smith, G.E., Lim, C.J., and Brandhorst, B.P. 1996. Production of androgenetic zebrafish (*Danio rerio*). Genetics, **142**: 1265–1276.

Devlin, R.H., and Nagahama, Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture, **208**: 191–364.

Hamilton, F. 1822. An account of the fishes found in the river Ganges and its branches. Archibald Constable and Co., Edinburgh, U.K.

Kirankumar, S. 2002. Induction of polyploidy in fish. Ph.D. dissertation, Madurai Kamaraj University, India.

Kirankumar, S., and Pandian, T.J. 2004*a*. Production and progeny testing of androgenetic rosy barb, *Puntius conchonius*. J. Exp. Zool. In press.

Kirankumar, S., and Pandian, T.J. 2004*b*. Use of heterologous sperm for the dispermic induction of androgenesis in barb. J. Fish Biol. In press.

Kirankumar, S., Anathy, V., and Pandian, T.J. 2003. Hormonal induction of supermale rosy barb and isolation of Y-chromosome specific molecular markers. Gen. Comp. Endocrinol. **134**: 62–71.

Kligerman, A.D., and Bloom, S.E. 1977. Rapid chromosome preparations from solid tissues of fishes. J. Fish. Res. Board Can. **34**: 266–269.

Koteeswaran, R., and Pandian, T.J. 2002. Live sperm from post-mortem preserved Indian catfish. Curr. Sci. **82**: 447–450.

Lee, K., Huang, H., Ju, B., Yang, Z., and Lin, S. 2002. Cloned zebrafish by nuclear transfer from long-term-cultured cells. Nat. Biotechnol. **20**: 795–799.

Linhart, O. 1991. Evaluation of the sperm and the activation and fecundation of eggs. Methodology of R.I.F.C.H. University of South Bohemia ceske Budejovice, Vodnany, Czech Republic.

May, B., and Grewe, P.M. 1993. Fate of maternal mtDNA following ^{60}Co inactivation of maternal nuclear DNA in unfertilized salmonid eggs. Genome, **36**: 725–730.

Nam, Y.K., Cho, Y.S., Cho, H.J., and Kim, D.S. 2002. Accelerated growth performance and stable germ-line transmission in androgenetically derived homozygous transgenic mud loach, *Misgurnus mizolepis*. Aquaculture, **209**: 257–270.

Ogawa, M., Yagasaki, M., and Yamazaki, F. 1973. The effect of prolactin on water influx in isolated gills of the goldfish, *Carassius auratus* L. Comp. Biochem. Physiol. A Comp. Physiol. **44**: 1177–1183.

Pandian, T.J. 2002. Cloning the fish. Curr. Sci. **83**: 1063–1064.

Pandian, T.J., and Kirankumar, S. 2003. Androgenesis and conservation of fishes. Curr. Sci. **85**: 917–931.

Pandian, T.J., and Koteeswaran, R. 1998. Ploidy induction and sex control in fish. Hydrobiologia, **384**: 167–243.

Recoubratsky, A.B., and Grunina, A.S. 2001. Nucleocytoplasmic incompatibility in androgenetic fish hybrids can be overcome. Ontogenez, **32**: 360–366. (In Russian)

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

Scheerer, P.D., Thorgaard, G.H., and Allendorf, F.W. 1991. Genetic analysis of androgenetic rainbow trout. J. Exp. Zool. **260**: 382–390.

Zar, H.J. 1984. Biostatistical analysis. Prentice-Hall International, Englewood Cliffs, N.J.