Androgenesis and conservation of fishes*

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Due to non-visibility of egg nucleus, the established scheme of nuclear manipulation to clone fish may prove a difficult task. However, fishes are amenable for interspecific androgenetic cloning. A recent discovery of using cadaveric sperm to successfully generate progenies has opened the possibility of adopting a simple, widely practicable method of post-mortem preserved (at – 20°C) sperm to induce androgenesis. Inactivation of maternal genome by UV-irradiation and activation of genome-inactivated homologous or heterologous egg by a single diploid or two haploid fresh or preserved sperms are some landmark events, which have not only accelerated research activity but also focused on the importance of androgenesis in aquaculture and conservation of fish germplasm. With the absence of acrosome in the teleostean sperm, fertilization in fish is not a species-specific event. Eggs of many teleosts are amenable for heterospecific insemination. Successful heterospecific insemination results in activation or fertilization of an ovum of an alien species and is the most important strategic step for induction of interspecific androgenetic cloning. Polyspermy, especially dispermy occurs in nature and can be experimentally achieved after incubation of the milt in calcium chloride or polyethylene glycol. The paternal origin of androgenotes is verified using selected phenotypic, protein and/or molecular markers as well as karyotyping and progeny testing. Recently, reporter genes, the green fluorescent protein gene and the Tc1 transposan-specific marker have also been used. While confirming the paternity of androgenotes, progeny testing has also indicated the unexpected occurrence of females, which are, however, shown to carry XY genotype. Survival of androgenotes can be improved using a single diploid, rather than two haploid sperms for activation. About 84% androgenotes succumb during embryonic development. Haploid genome regulates the time scale of developmental sequence in both homologous and heterologous eggs of *Puntius* spp., as effectively as that of diploid. A couple of research groups have restored a fish species using its preserved sperm and genome-inactivated eggs of another species. A comparison on the source, technique and genomes used for generation of clones of mammals and androgenetic clones of fishes indicates that from the point of conservation and aquaculture, interspecific androgenetic cloning in fishes has an edge over that of mammals.

IN biology, clones denote genetically identical progenies produced by a single parent. Clones are easily obtained in asexually-reproducing, simple organisms, plants and fungi. However, cloning does not occur in sexually-reproducing higher organisms, whose progenies are drawn from equal genomic contributions from both the parents. Consequently, their progenies need not necessarily be genetically identical copies of either of the parents. In almost all higher animals, only the egg/zygote is totipotent, i.e. the gametes have the ability to develop into a complete individual. Briefly, the unique molecular organization of egg cytoplasm alone provides the signals to the nucleus, drawn from the egg/sperm or from a differentiated cell, to execute the programme of embryonic development. With an orderly series of divisions of the egg/zygote, the original totipotency of the daughter cells is progressively reduced to pluripotency and finally to unipotency. The molecular organization of cytoplasm of these daughter and grand-daughter cells is not capable of providing appropriate signals to their respective nuclei to execute the orchestrated programme that regulates the development of a single cell into an organized multicellular entity, i.e. while the cell nucleus has the entire genetic information, its expression is controlled by signals being received from the surrounding cytoplasm (see also Lakhotia[1]).

Cloning was actually first achieved nearly four decades ago, when experimental embryologists generated a large number of clones of a frog. The basic scheme of manipulative events that were followed for cloning the frogs are the same for mammals too, but with the introduction of a surrogate mother to solve problems arising from viviparity, and super-ovulation to increase the number of progenies at a given time. The basic scheme includes: removal of haploid nucleus from the egg by microsurgery and implanting a diploid nucleus derived from a somatic cell of the animal to be cloned; the nucleus is microinjected into an enucleated egg and allowed to fuse with the egg. 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 due to the non-visibility of egg nucleus. Secondly, the nuclei drawn from somatic cells of fish appear less totipotent than those of frog or mammal. Even Lee *et al.*[2], who have enhanced the visibility of the nuclei by infecting the donor cells of zebrafish with green fluorescent protein (*GFP*) gene, could transfer the nuclei only from blastula or embryonic fibroblasts and not from adult cells (see also Pandian[3]). However, fishes are known for their amazing ability to tolerate genomes from haploid to heptaploid, genomic contribution from male or female parent alone and unequal contributions from parents belonging to the same or different species[4]. The

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ease with which gynogenetic clones can be generated has virtually resulted in the 'downpour' of publications, which have been reviewed by Cherfas[5] and Chourrout[6]. Based on our publications[4,7–11], this article comprehensively reviews available literature on androgenesis in fishes. Androgenesis is a developmental process facilitating the inheritance of exclusively paternal genome. In fishes, it obligately involves two or three steps: (i) elimination or inactivation of the egg genome, (ii) dispermic[10] or monospermic activation of embryonic development by haploid[12] or diploid[13] gamete and/or (iii) restoration of diploidy by suppression of the first mitotic cleavage, when embryonic development is activated by a haploid sperm (Figure 1). Table 1 summarizes the landmark events in induction of androgenesis in fishes.

Androgenesis may prove useful for the production of (i) viable Y^2Y^2 supermale in male-heterogametic species and Z^1Z^1 superfemale in female-heterogametic species, (ii) inbred isogenic lines, and (iii) intraspecific and interspecific androgenetic clones for conservation germplasm. Of course, YY male is known not to survive in some species, e.g. *Betta splendens*[14]; however, androgenetic clones have been successfully generated and viable Y^2Y^2 clones have been obtained in a few species of cyprinids, cichlids and salmonids (Table 2). Considering the immense potential for the production of new strains in commercially important fish and loss of available strains/species due to anthropogenic activity, there is a need to develop new techniques for conserving them.

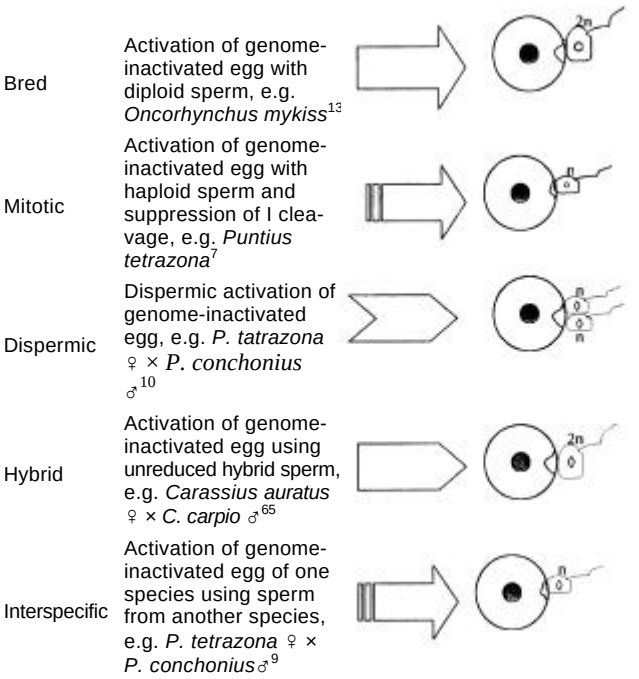

Figure 1. Established protocols for induction of androgenesis in fishes ⟹, Normal activation; ▮⟹, Activation followed by thermal shock; ≥⟹, Dispermic activation; ⟹, Activation by unreduced diploid sperm.

Gamete preparation

Recipient egg

As already indicated, the non-visibility of egg nucleus of many fishes renders them not amenable for enucleation and elimination of maternal genome of the egg. Consequently, the induction of androgenesis obligately involves irradiation of the fish egg (Table 2). Initially, the irradiation was limited to the use of *g*-rays at a dose ranging from 36 (ref. 12) to 88 kR (ref. 15); Russian scientists like Grunina *et al.*[15,16] used X-ray at the doses 25–30 kR. However, the irradiation may completely[17] or partially[18] destroy the chromosomes. Carter *et al.*[19] doubted the total elimination of the egg genome, since mtDNA and mRNA are present in large quantities in the egg[20]. Owing to protection by mitochondrial membrane, mtDNA in the eggs of *Oreochromis niloticus* suffered no damage from UV-irradiation[21]. Consequently, the treated eggs may still transfer some genetic material to F_1 progenies. This sort of accidental transfer of chromosomal fragments results in the undesired 'genomic impurity' of the androgenotes[22]. Because of their high penetrance, irradiation by *g*- or X-rays is also shown to destroy 'the maternal products' like proteins (e.g. enzymes), RNA (mainly mRNA) and mtDNA[23,24], obligately required for earlier development[25]. For instance, Stroband *et al.*[26] demonstrated that these maternal products control development in common carp zygotes until the stage of epiboly, which occurs 5–6 h after fertilization. Despite the cost and skill required for X-ray and *g*-irradiation, one of them was used to eliminate the maternal genome of the eggs, especially in salmonids until 1991.

Bongers *et al.*[27] were the first to claim 100% inactivation of the genome from *Cyprinus carpio* eggs by UV irradiation alone (Table 1). The carp eggs were immersed in synthetic ovarian fluid and exposed to UV radiation at the dose of 250 mJ/cm^2. Manual rotation of the eggs to expose the animal pole to radiation yielded better results than mechanical rotation. To focus the irradiation on animal pole, Arai *et al.*[22] exposed *Misgurnus anguillicaudatus* eggs to a UV source from the upper and lower sides. Owing to the pear-like shape of eggs of the bitterlings *Rhodeus ocellatus ocellatus*, the animal pole of the egg is always oriented upwards ensuring complete inactivation of the egg genome, even when the UV-irradiation source is limited to the upper side only[28].

UV-irradiation causes several types of damage, including pyrimidine-dimers, DNA–DNA cross-links, pyrimidine adducts in many species. However, pyrimidine-dimer formation (T–T, C–T, C–C) in adjacent DNA bases is the most common type of UV-damage[29]. Similar damage occurs in RNA with regard to the pyrimidines uracil and cytosin. In teleosts, the pyrimidine-dimer formation is repaired by the enzyme DNA-photolyase under the influence of visible light, specifically at 300–600 nm. This enzyme also repairs damage in RNA[29]. To prevent photo-

Table 1. Landmark events in induction of androgenesis in fish

Reference	Achievement	Limitation
Romashov and Belyaeva[67]	First to claim androgenesis in loach	No evidence for total elimination of maternal genome
Stanley *et al.*[52]	Recorded incidental occurrence of androgenetic grass carp while hybridizing with common carp	No evidence for purity of androgenotes
Arai *et al.*[68]	First to use *g*-rays to eliminate maternal genome in salmon	No evidence for total elimination of maternal genome
Thorgaard *et al.*[13]	First to show higher survival of androgenotes generated using tetraploid rainbow trout	No family lines established
Scheerer *et al.*[31]	First to use cryopreserved milt to generate androgenotes of rainbow trout	Observation contradictory to that of Bercsenyi *et al.*[22]
Bongers *et al.*[27]	First to use UV-irradiation to eliminate maternal genome in common carp	Other than colour, no marker used to confirm inactivation of maternal genome
Arai *et al.*[22]	Generated androgenetic loach using natural tetraploid	No family lines established
Corley-Smith *et al.*[56]	First to generate fertile androgenetic male zebrafish; confirmed its purity by RAPD, SSR and MHC analyses	No information on clonal XX androgenetic siblings
Bercsenyi *et al.*[39]	First to generate interspecific androgenetic goldfish	No information on maturity and reproduction of the androgenote
Nam *et al.*[69]	First to generate transgenic androgenetic mud loach	
Kirankumar and Pandian[9]	First to use cadaveric sperm to generate interspecific androgenetic rosy barb	–
Araki *et al.*[51]	First to generate dispermic, intraspecific androgenotes of rainbow trout	0.1% survival; no information on reproduction
Kirankumar and Pandian[10]	First to generate dispermic interspecific androgenetic rosy barb	–

Table 2. Protocols used to eliminate female genome and restore diploidy for induction of androgenesis in fishes (from ref. 4, modified and added)

Species	Inactivation of female genome	Genetic marker	Survival (%)	Sperm source/ remarks
Salmonidae				
Oncorhynchus mykiss	^{60}Co; 36 kR	Isozymes	Hatching: 7 Feeding: 5	Inbred
			Hatching: 9 Feeding: 7	Outbred
O. mykiss	^{60}Co; 40 kR	–	Hatching: 1 Feeding: < 1	Diploid
	^{60}Co; 40 kR	–	Hatching: 12 Feeding: 10	Tetraploid
O. mykiss	^{60}Co; 36 kR	Isozymes, colour	Hatching: 1.3 Feeding: 1.0	Cryopreserved
Salvelinus fontinalis	^{60}Co; 88 kR	Allozymes	? : 38	–
Cyprinidae				
Cyprinus carpio	X-ray; 25–30 kR	Colour	Hatching: 9	Inadequate genome elimination
C. carpio	UV; 100–250 mJ/cm^2	Colour	Hatching: 15 24 days: 10	Irradiation of eggs in ovarian fluid
C. carpio	X-ray	–	Hatching: ?	*C. auratus gibileo* sperm
C. carpio	UV-?	–	–	Gold fish sperm; hybrid eggs
Danio rerio	X-ray; 10000 R	RAPD, SSR, MHC	24 h after fertilization: 22	*Danio rerio*
C. carpio	^{60}Co *g*-rays; 25 kR	Colour, barbel, tail morphology	Hatching: 37.2	Gold fish sperm
Puntius tetrozona	UV	Fin morphology, PCR analysis, colour	Hatching: 15 Maturity: 7	*P. conchonius*; revived from preserved sperm (– 20°C)
P. conchonius	UV	Colour	Hatching: 7	100% elimination of egg genome
Cobitidae				
Misgurnus anguillicaudatus	UV; 7500 ergs/mm^2	Allozyme	Hatching: 8	100% elimination of egg genome; 2 *n* sperm
Cichlidae				
Oreochromis niloticus	UV	Colour	Hatching: 3	Fresh or cryopreserved sperm
Characidae				
Hemigrammus caudovittatus	UV	Colour	Hatching: 7	–
Others				
Acipenser ruthenus	UV	–	Hatching: ?	Sperm of *A. baeri*

reactivation of the inactivated chromosomes of gametes, the entire procedure of irradiation and diploidization is usually completed under total darkness. Myers *et al.*[21] performed Southern analyses of mtDNA from control and UV-irradiateded eggs of *O. niloticus* to assess the extent of damage, and found no difference between their autoradiograms. However, the positive controls (purified mtDNA irradiated directly with a 254-nm lamp) revealed extensive damage to the mtDNA. Due to the relative position of the egg pronucleus and the scattered distribution of mitochondria throughout the egg, the pronucleus perhaps suffers greater damage and even total inactivation, while a large number of mitochondria remain intact, partially or totally.

Hitherto used intensity of irradiation ranges from 100 to 7500 ergs/m^2 (ref. 23). Unfortunately, many authors have not even indicated the intensity (for example, see ref. 21) and the duration of UV-irradiation. In general, the eggs of *Puntius tetrazona*[7], *P. conchonius*[8], *Hemigrammus caudovittatus* and *Gymnocorymbus ternetzi*[30] are spherical and measure 0.8–1.5 mm in diameter. An intensity of 4.2 W/m^2 and a duration of 3.5 min were found adequate to inactivate the maternal genome in the eggs of these species. However, it is likely that the optimal duration required for inactivation of maternal genome may vary from species to species, depending upon size and cytoplasmic content of the egg as well as egg shape and position of the animal pole when the eggs are arranged in a single layer.

Ever since Bongers *et al.*[27] demonstrated the effective inactivation of maternal genome by UV-irradiation, it has been the choice for inactivation of the maternal genome in fishes belonging to Cyprinidae, Cobitidae and Characidae. However, the adequacy and effectiveness of UV-irradiation remains to be tested in many other groups of teleosts (Table 2). When donor sperm of the same species/strain is used for activation, the determination of optimal dose and duration of UV-irradiation at which 100% haploids are generated from the genome-inactivated eggs, is the procedure used to confirm the inactivation of maternal genome. The determination of haploidy requires karyotyping even at the embryonic stage, as these haploids/aneuploids suffer heavy mortality owing to haploid syndrome. For instance, following this procedure, Kirankumar and Pandian[7] determined that at the intensity of 4.2 W/m^2 UV-irradiation for 3.5 min duration was optimum for inactivation of the genome of *P. tetrazona* egg. Essentially, the procedure may prove tedious, but it is still followed by a number of researchers (for example, see ref. 31). However, the most commonly used procedure is to select the donor sperm of a different strain/species characterized by recessive colour and to determine the dose and duration of UV-irradiation required to inactivate the maternal genome of eggs, as evidenced by the recessive-coloured progenies in a cross in which the female was characterized by dominant colour (Table 3).

Sperm as genome donor

In fishes, the milt can be obtained by stripping. However, many silurids are known not amenable for stripping[32]. Hence milting these silurids has to be obligately invasive. Cryopreservation of fish sperm is possible[33] and a large number of publications are available on choice of extenders[34], cryoprotectants[35] and other parameters for long-term sperm preservation of fish (Cichlidae[36]; Salmonidae[37]; Cyprinidae[38]).

Sperm preservation: Scheerer *et al.*[31] were perhaps the first to show that the cryopreserved sperm can be used to induce androgenesis (Table 1). An important objective of androgenesis is to use the technique for conservation of fish genome by preserving the milt of the desired species/strain, and restoring it using genome-inactivated eggs of a suitable fish species. A couple of available publications on cryopreservation of sperm for induction of androgenesis report fragmentary and contradictory observations. Scheerer *et al.*[31] indicated 1.3 and 3.8% survival of the androgenetic clones of *Oncorhynchus mykiss* generated using cryopreserved and fresh sperm, respectively. Conversely, Bercsenyi *et al.*[39] indicated that the survival of interspecific androgenetic clones of *Carassius auratus* was higher (23%) while using cryopreserved sperm than using fresh sperm (19%). A reason for paucity of information in this area may be traced to the non-availability of liquid nitrogen facility, even in fairly big cities of developing countries. In fact, the need for costly equipment, including the liquid nitrogen facility has been the bottleneck in cryopreservation of fish sperm. For instance, India has a wealth of 2118 fish species and a large number of strains in 50 commercially important fish species. However, for want of a simpler and more widely practicable protocol for preservation of fish sperm, the National Bureau of Fish Genetic Resources, Lucknow has a sperm-bank facility to hold only a few fish species like *Catla catla*, *Labeo rohita*, *L. dussumieri*, *Cirrhina mrigala*, *C. carpio*, *O. mykiss*, *Salmo trutta*, *Tor putitora*, *T. khudree*,

Table 3. Colour as marker in induction of androgenesis (from ref. 4, modified and added)

Species	Female	Male	Progeny
Puntius conchonius	Gray (D)	Gold (R)	Gold
P. tetrazona	Gray (D)	Blond (R)	Blond
Cyprinus carpio	Black (D)	Blond (R)	Blond
C. carpio	Black (D)	Orange (R)	Orange
C. carpio	Normal (D)	Yellow (R)	Mostly yellow
Misgurnus anguillicaudatus	Black (D)	Orange (R)	Mostly orange; few black fry (0–10.6%)
Oncorhynchus mykiss	Black (D)	Albino (R)	Spectrum of colours
Hemigrammus caudovittatus	Black (D)	Albino (R)	Albino

D, Dominant colour; R, Recessive colour.

Tenualosa ilisha and *Harabagus brachysoma*. Using the preserved sperm, 65–100% hatching success has been achieved[40].

At Madurai Kamaraj University, progenies of the Indian catfish *Heteropneustes fossilis* were generated using live, fertile sperm drawn from specimens that were post-mortem preserved at – 20°C for more than 240 days[41]. A taxonomic survey indicated the successful use of 'cadaveric sperm' to fertilize or activate development in eggs of many freshwater and marine fishes. However, it was not clear whether these cadaveric sperms induced gynogenesis rather than syngamy of pronuclei of egg and sperm. Hence, Kirankumar and Pandian[9] made a comparative study using fresh and cadaveric sperm drawn from two different strains of *P. conchonius* characterized by a combination of two recessive traits, namely golden colour and unspotted tail, and dominant grey colour and spotted tail. Although post-mortem preserved cadaveric sperms suffered significant losses in count, motility and fertilizability (ref. 41), the milt of *P. conchonius* still ensured fertilization and hatchability, which were lower (16–21%) than those generated using fresh sperm (76–88%). Irrespective of whether fresh or cadaveric sperms were used, the sex ratio of the progenies of normal sires, and sires resulting from cadaveric sperm remained 1♂ : 1♀ (Table 4). The sex ratio of the F_1 progenies confirmed no selective damage or mortality to X- or Y-carrying sperm. Hence, the discovery of post-mortem preservation of sperm at – 20°C has opened the possibility of using a simple, widely practicable method of sperm preservation. This technique has special implication to us in India, where the liquid nitrogen facility is not available in every town, especially in the northeast and the Western ghats, where the need for sperm preservation of rare endemic fishes is urgently required. Also, a study is yet to be undertaken to compare the fertilizability of sperm drawn from fresh milt, cryopreserved milt and post-mortem preserved (– 20°C) specimens for different durations.

Heterospecific insemination: Typically, the spermatozoa of teleostean fishes do not have acrosome[42]. However, the absence of acrosome coincides with the presence of micropyle in the eggs[43]. Since entry of the sperm is made possible through micropyle during fertilization (Figure 2), fertilization in fishes is not a species-specific event. For instance, tilapia eggs can be activated by carp (Cyprinidae) sperm[44]; eggs of *Betta splendens* (Anabantidae) can be activated by milt of *O. mossambicus* (Cichlidae)[45]. Thus, eggs of a number of species are amenable for heterospecific insemination. Successful heterospecific insemination results in activation or fertilization of an ovum of an alien species and is the most important strategic step for induction of hybridization, hybridogenesis, gynogenesis, androgenesis and interspecific cloning, which may be defined as below: (i) hybridization, in which heterospecific insemination results in fertilization of an ovum of a fish species by the sperm of another fish species, and production of viable hybrid progenies; (ii) hybridogenesis, in which heterospecific insemination results in hybridization, but with almost total elimination of paternal chromosomes of the previous generation; (iii) gynogenesis, in which heterospecific insemination results in activation of development in haploid or diploid ovum, and (iv) interspecific androgenesis, in which heterospecific insemi-

Table 4. Sex distribution among F_1 progenies of the rosy barb generated from rosy barb eggs activated by sperms from randomly selected normal males or 30-day post-mortem preserved specimens of rosy barb (from ref. 9)

Sperm source (strain/no.)	No. of dams used for crossing	No. of hatchlings	Sex distribution ♀ (X^1X^2)	Sex distribution ♂ (X^1Y^2)
Normal sires				
Strain 4 ♂ 3	4	83	41	42
	9	79	38	41
	3	76	38	38
		Mean	39	40
Strain 2 ♂ 7	1	88	45	43
	2	81	41	40
	4	86	38	48
		Mean	41	44
Post-mortem preservation				
Strain 4 ♂ 2	9	18	9	9
	6	16	10	6
	5	17	8	9
		Mean	9	8
Strain 2 ♂ 14	8	19	11	8
	10	17	9	8
	1	21	12	9
		Mean	11	8

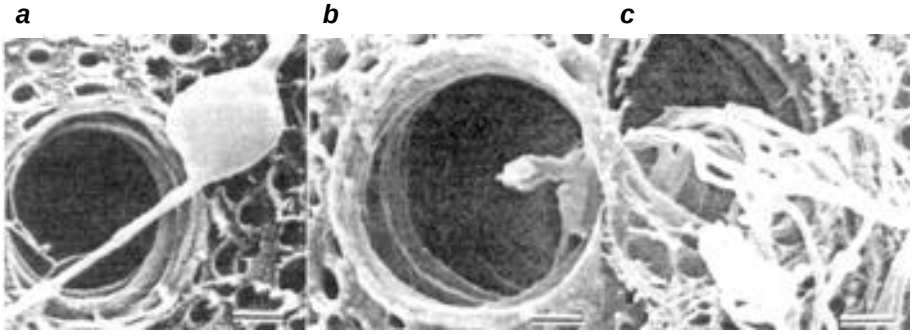

Figure 2. Scanning electron micrograph of (***a***) unfertilized egg (30 s after contact with sperm suspension in sea water, (***b***) fertilized egg (20 min after contact with sperm suspension; note sperm tail in micropyle) and (***c***) fertilized egg (note the presence of many sperms in the micropylar canal) of the Atlantic herring *Clupia harengus* (from ref. 66).

nation results in activation of the genome-inactivated ovum.

Table 5 presents selected examples of fishes in which heterospecific insemination results in the production of viable progenies. Hitherto, experiments on heterospecific insemination have mostly been limited to commercially important food- and ornamental fishes. From available information, *C. carpio* appears to be a universal donor whose sperm is accepted by a dozen species belonging to Cyprinidae, Cichlidae, etc. Among salmonids, *O. mykiss* is perhaps a universal recipient. Reciprocal heterospecific inseminations have been a success between the following pairs: *C. carpio* and *Ctenopharyngodon idella*, *C. carpio* and *Hypophthalmichthys molitrix*. Not surprisingly, hybridization among the Indian major carps is prevalent and has led to genetic retrogression[40]. Such reciprocal, heterospecific insemination does not necessarily result in hybridization but may induce gynogenesis, as in the case of *P. conchonius* ♀ × *P. tetrazona* ♂, or may also induce paternal (*C. idella* ♀ × *Hypophthalmichthys nobilis* ♂[46]), or maternal triploidy as in many cyprinids and salmonids. *Pangasius sutchi* successfully donates sperm to *Clarias macrocephalus*, but does not serve as recipient to the sperm of *C. macrocephalus*[32]. Likewise, *P. conchonius* can be a sperm donor to *P. tetrazona*, whose sperm is, however, not acceptable to *P. conchonius*[7].

Polyspermy: Fish eggs are also amenable to polyspermy. Mantelman[47] observed that 5% of just-fertilized eggs of *C. idella* contained three or more pronuclei and centromeres. When female *Fundulus heteroclitus* was crossed with male *Menidia notata*, more than 50% of the hybrid eggs was dispermic[48]. Similar records on induced dispermy in eggs of triploid rainbow trout[49] and triploid carp[50] were published. Chemicals like polyethylene glycol (PEG) and calcium chloride[51] are known to facilitate the entry of two or more sperms into an egg. Grunina *et al.*[15] explored the possibility of dispermic activation to reduce the homozygosity and generation of diploid androgenotes. They claimed to have achieved the desired dispermic (*C. carpio*) activation of genome-inactivated eggs of the hybrid *C. carpio* ♀ × *C. auratus* ♂. The objective of dispermic activation of genome-inactivated eggs to generate androgenetic clones is to improve hatchability and survival. However, survival of such dispermic androgenotes is too low (e.g. 0.1%; see ref. 51). Figure 3 presents a protocol adopted by Kirankumar and Pandian[10] for successful dispermic activation of genome-inactivated eggs of *Puntius* sp.

Markers

Although androgenetic clones of fish have been claimed to occur in nature[52] and also artificially generated[53], they have failed to confirm the integrity of androgenotes. The paternal origin of androgenotes is usually verified by inspecting the progenies for selected phenotypic, protein and/or molecular markers as well as karyotyping and progeny testing. By and large, most investigators have stuck to phenotypic markers like the colour (see Table 3). The design is to choose a recessive colour for male and dominant one for female, so that even a tint of colour present in the progeny can be easily detected. The total absence and presence of even a tint of the dominant colour in the progeny may indicate the elimination/inactivation of maternal genome and the occurrence of the undesired fractions of maternal genome, respectively. For instance, Disney *et al.*[54] observed that a spectrum of colours is inherited by the F_1 progeny of *O. mykiss*, clearly indicating the incomplete elimination of maternal genome. Few workers like Bercsenyi *et al.*[39] and Kirankumar and Pandian[9] have chosen more than one phenotypic markers to confirm the paternity of the respective androgenotes (Table 6). A few others have also chosen protein markers like the isozyme[12] or allozyme[22,55]. Scheerer *et al.*[31] have chosen both colour and isozymes to confirm the paternity of *O. mykiss* androgenotes.

More recently, molecular markers have been used to irrefutably confirm the paternity of androgenotes. Kiran-

kumar and Pandian[7] used green fluorescent protein gene, a reporter gene, as a marker to confirm the exclusive paternal origin of the haploid. It has a couple of advantages: (i) the destiny and distribution of the paternal genome can be traced from early embryonic stages, as early as 16-h-old embryo in *P. tetrazona* (Figure 4), and (ii) it can be used to confirm the paternal origin of haploid androgenotes which succumbed even at the embryonic stage.

Table 5. Heterospecific insemination in fishes (from ref. 4; modified and added)

Sperm donor	Sperm recipient
Cyprinus carpio	*Ctenopharyngodon idella*
	Carassius auratus
	Hypophthalmichthys molitrix
	Cirrhinus mrigala
	Misgurnus anguillicaudatus
	Cobitis biwae
	Tinca tinca
	Oreochromis niloticus
	O. mossambicus
C. auratus	*C. idella*
	M. anguillicaudatus
	O. niloticus
C. idella	*C. carpio*
H. nobilis	*C. idella*
Puntius conchonius	*P. tetrazona*
P. gonionotus	*C. carpio*
C. biwae	*M. anguillicaudatus*
M. anguillicaudatus	*C. biwae*
M. mizolepis	*Paralichthys olivaceus*
Barbus barbus	*C. carpio*
T. tinca	*O. niloticus*
Pangasius schwanenfeldii	*P. gonionotus*
P. sutchi	*Clarius macrocephalus*
Ictalurus furcatus	*I. punctatus*
Gnathopogan elongatus elongatus	*M. anguillicaudatus*
Herichthys cyanoguttatus	*O. mossambicus*
Osteochilus hosselti	*C. carpio*
Acanthopagrus schlegeli	*P. olivaceus*
Pagrus major	*Sparus aurata*
Menida notata	*Fundulus heteroclitus*
Acipenser ruthensis	*Huso huso*
A. baeri	*A. ruthensis*
Salmo salar	*O. mykiss*
Salmo trutta	
Salvelinus fontinalis	
O. kisutsch	
O. tshwystcha	
O. masou	
S. trutta	*S. salar*
Thymallus thymallus	*O. mykiss*
Abramis brami	*C. carpio*
S. fontinalis	*S. trutta*
Poecilia velifera	*P. sphenops*
P. sphenops	*P. velifera*
Oreochromis aureus	*O. niloticus*
O. hornorum	*O. niloticus*
O. hornorum	*O. mossambicus*
O. macrochir	*O. niloticus*
O. variabilis	*O. niloticus*
O. hornorum	*O. aureus*
O. vulcani	*O. aureus*
O. niloticus	*O. leucostictus*
O. niloticus	*O. spilurus niger*
O. niloticus	*O. mossambicus*
*O. aureus hornorum**	*O. niloticus*
O. mossambicus	*O. spilurus niger*
Prinotus paralatus	*P. alatus*
Semotilus atromaculatus	*Phoximus oreas*
Gila eremica	*G. ditaenia*
Micropterus dolomieui	*M. salmoides*

*, Hybrid resulting from the cross between *O. aureus* and *O. hornorum*.

While phenotypic markers, isozymes and allozymes are limited to one or few genes and/or alleles, RAPD analysis provides a more comprehensive picture of the genome. Therefore, it may be a good idea to go for RAPD analysis[56], besides one or more phenotypic markers[39]. Kirankumar and Pandian[9,11] generated monospermic and dispermic androgenetic clones of the rosy barb using genome-inactivated eggs of the tiger barb. For the first time in fishes, Tc1-transposon-specific primers were used to confirm the total inactivation of maternal genome, especially when interspecific androgenotes are induced. Transposons are mobile DNA elements that are widespread components of the genomes of most organisms. Tc1-like transposons belonging to class II occur widely in the genome of fishes[57]. When PCR analyses were made using Tcl-transposon-specific primers, the rosy barb genomic DNA produced an intense 800 bp product and the tiger barb genomic DNA, an intense 300 bp product. Hybrids between these two barbs (tiger barb ♀ × rosy barb ♂) produced both these products. Expectedly, the genomic DNA of the interspecific androgenetic clones of the rosy barb, *P. conchonius* resulting from monospermic or dispermic activation of the genome-inactivated eggs of the tiger barb produced an intense 800 bp product only, confirming the expected paternal inheritance (Figure 5). Therefore, this PCR analysis confirmed the purity of paternal genome inheritance by *P. conchonius* through the surrogate eggs of *P. tetrazona*.

Progeny testing

In male heterogametic species, androgenesis results in the production of supermales (Y^2Y^2) which are academically and economically important animals. They are useful to understand the sex-determining mechanism in the tested species and to sire all-male progenies. Monitoring sex ratios of progenies sired by supermales is a method to confirm the paternal integrity of the androgenotes. In male heterogametic species, the supermales are expected to sire all-male progenies[58]. Few authors have extended their investigation to rearing the androgenotes to sexual maturity and assessing the sex ratio of their progenies. Available publications clearly show the unexpected occurrence of 3–24% of F_1 female progenies sired by supermales. Kirankumar and Pandian[8] have induced successive generations of androgenotes (Figure 6) and recorded the unexpected occurrence of females up to F_3 progenies, more or less in the same ratio as it was in the F_1 progenies. This investigation clearly indicates the need to identify the genotype of such unexpected female progenies.

Information on the sex-linked DNA markers in fishes is fragmentary and diverse. In a few fish species, different types of markers have been characterized; for instance, the Y-chromosome-specific probe in the chinook salmon, *O. tshawytscha*[59], the sex chromosome-specific repetitive sequences in the Poeciliids[60], sex-specific quantitative DNA markers in *O. tshawytscha*[61] and the male-specific growth hormone pseudogene (GH-*j*) in the masu salmon, *O. masou masou*[62]. These studies are related to members of Salmonidae and Poeciliidae. Kirankumar *et al.*[11] have identified, isolated and characterized a Y-chromosome-specific molecular marker for the cyprinid *P. conchonius*.

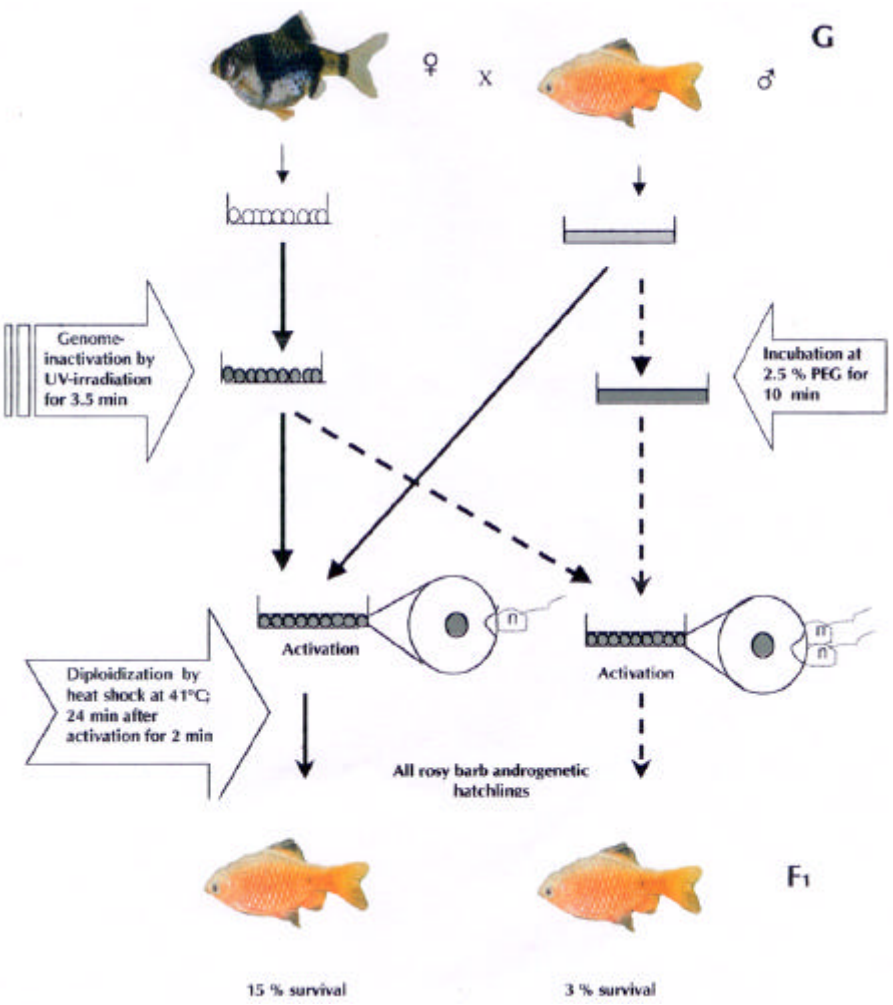

Figure 3. Dispermic activation of genome-inactivated eggs of the grey tiger barb *P. tetrazona* for interspecific androgenetic cloning of the golden rosy barb *P. conchonius* using its sperm incubated at 2.5% PEG for 10 min (from ref. 10).

Table 6. Homozygous dominant and recessive traits used as phenotypic markers to confirm the paternity of androgenotes of *Puntius conchonius* strains[9] and *Carassius auratus* strains[22].

Puntius conchonius Phenotypic marker	Strain 1	Strain 2	Strain 3	Strain 4
Tail morphology	Veil tail – dominant	Normal tail – recessive	Normal tail – recessive	Normal tail – recessive
Body colour	Gray – dominant	Gray – dominant	Gold – recessive	Gold – recessive
Spot near caudal peduncle	Absent – recessive	Present – dominant	Present – dominant	Absent – recessive
Carassius auratus				
	Strain 1	Strain 2	Strain 3	
Head morphology	Red cap – recessive	Bubble eyes – recessive	Telescope eyes – recessive	
Body colour	White – recessive	Red – recessive	Black – recessive	

Using SRY-specific primers, they have performed PCR analysis of the genomic DNA of male golden rosy barb, which has yielded three amplicons of 588 bp, 333 bp and 200 bp length (Figure 7). They have found that only the 200 bp product is amplified in the female genome. Hence the first two products may serve as molecular markers to rapidly identify a cyprinid fish possessing Y-chromosome. The consistent presence of the upper 333 and 588 bp fragments in normal (X^1Y^2), hormonally induced (Y^1Y^2) and androgenetic (Y^2Y^2) males and the absence of any relationship between the 200 bp fragment and the X-chromosome clearly indicates that the male-specific markers are specific to the Y-chrmosome only.

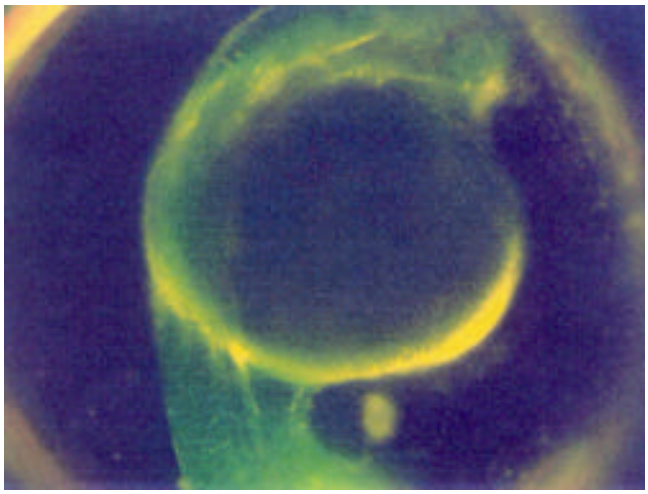

Figure 4. EGFP expression in the 16-h-old haploid androgenetic blond. *Puntius tetrazona* embryo (from ref. 7).

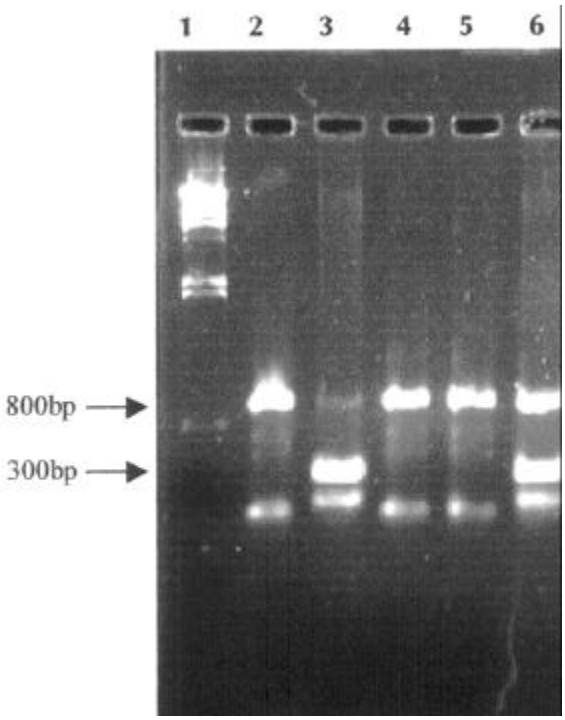

Figure 5. Agarose gel electrophorogram showing species-specific Tc1 amplification (size variants) in the rosy barb *P. conchonius*, the tiger barb *P. tetrazona*, the hybrid barb and the androgenetic clones Lane 1, *l Hin*d III marker; lane 2, Rosy barb; lane 3, Tiger barb; lanes 4 and 5, Androgenetic clones; lane 6, Hybrid barb (from ref. 9).

Incidentally, Kirankumar and Pandian[7–9] have reported that the reproductive performance of the androgenetic male is superior and that of the female inferior when compared to normal male and female, respectively (Table 7). If homozygosity is a cause for all the negative features recorded for the inferior reproductive performance of androgenetic female (X^2X^2), then it is difficult to comprehend the superior reproductive performance of androgenetic males (Y^2Y^2), which also possess an equal level of homozygosity.

Survival of androgenetic clones

In general, survival of androgenetic fishes is low. The two important causes for this are (i) injury and stress involved in genome inactivation of egg by irradiation, and stress imposed by thermal/pressure shock for diploidization, and (ii) homozygosity. *g*- and X-rays have high penetrance; hence they may cause damage not only to the genome but also to other important components of the eggs. Expectedly, the survival values reported for androgenotes generated using *g*- or X-rays for elimination of egg genome range from 0.7 to 12%. Conversely, the UV-irradiation, known for its relatively lower penetrance, seems to cause less damage to other important components like mRNA, mtDNA and proteins; expectedly, the survival of these androgenotes, generated using eggs in which the genome was inactivated by UV-irradiation, is higher and ranges around 15% (Table 2). Clearly, the UV-irradiation protocol, first developed by Bongers *et al.*[27], has not only removed the bottleneck of using costly and skilled technique of *g*- and X-rays, but also improved the survival of the androgenotes by threefold.

Two different approaches have been made to eliminate the stress involved in diploidization; the first one is to use a relatively more homozygous diploid sperm and the second one involves the dispermic activation of eggs. In *O. mykiss*, Thorgaard *et al.*[13] produced androgenotes using haploid and diploid sperms. Expectedly, viability of the androgenotes resulting from diploid sperm of a tetraploid male was significantly higher (43%) than those produced using haploid sperm (0.8%). Using diploid sperm, Arai *et al.*[22] have also improved the survival of androgenotes of *M. anguillicaudatus* by almost ten times. Clearly, elimination of the diploidization step in the protocol significantly increases the yield of androgenotes. Conversely, the survival of androgenotes arising from dispermic activation has not significantly improved; for instance, survival of such androgenotes is reported as low as 0.1% in *O. mykiss*[51] and 3% in *P. conchonius*[10]. It is not clear why the androgenotes generated by dispermic

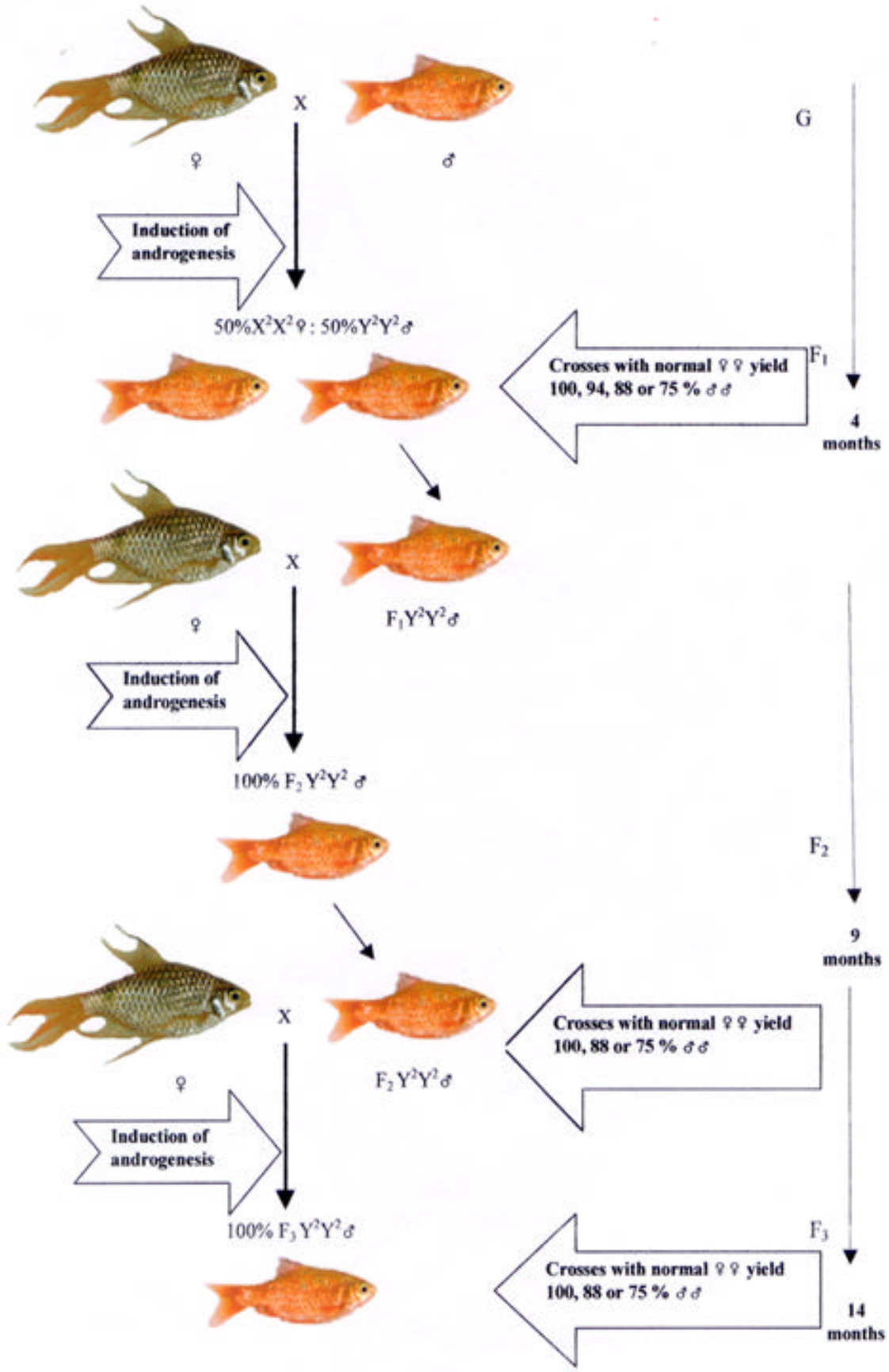

Figure 6. Protocol for production and progeny testing in successive generations of androgenetic rosy barb, *P. conchonius* (from ref. 8).

activation result in such low survival, despite the fact that the entry of two different sperms should have considerably increased the heterozygosity.

At hatching, the survival is relatively higher and ranges from 0.7% in *O. mykiss* to 15% in *C. carpio* (Table 2). As they attain feeding stage, it decreases to 0.3% in *O. mykiss*[13] and to 10% in *C. carpio*[27]. Thus the survival of androgenotes is known to decrease with advancing age; for instance, it decreases from 15% at hatching to 7% at sexual maturity for *P. tetrazona*[7] and *P. conchonius*[8]. Apparently, about 84% of the mortality of the androgenotes occurs during embryonic development[8]. Kirankumar and Pandian[8] described the stage-specific embryonic mortality and found that the stages between activation and the 18th somite stage, and those before and after hatching are critical. Interestingly, they also showed that (i) despite activation by sperm belonging to another species, the development proceeded almost precisely in the same time scale as that of the normal egg fertilized by sperm belonging to same species (Figure 8), and (ii) despite suffering severe mortality, a certain percentage of eggs, activated by sperms having the genome of related strain, regulated the normal development until hatching in the haploids, but for lifetime in the diploids.

In the context of heterospecific insemination and intergeneric androgenetic cloning, it is interesting to note the attempt made by Kirankumar and Pandian[9] to understand the role played by haploid, diploid or hybrid genome in regulation of time sequence of development and embryonic mortality in *P. conchonius* (egg size 882 μm) and *P. tetrazona* (egg size 1230 μm), characterized by differences in egg size, and pre- and post-embryonic durations (Table 8). They made the following conclusions: (i) the haploid genome regulates the developmental sequence as effectively as that of the diploid genome, (ii) the haploid or diploid rosy barb genome, drawn from one or two fresh

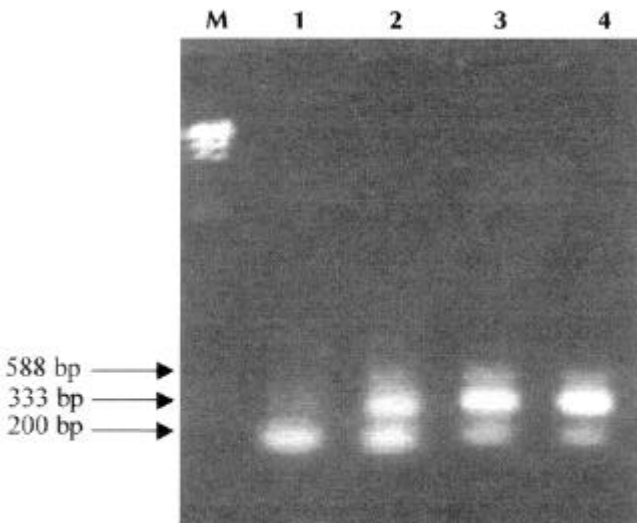

Figure 7. PCR products amplified by SRY primers in the genomic DNA of golden rosy barb, *P. conchonius* carrying different sex genotypes. Lane M, *l Hin*d III marker; lane 1, Normal female (X^1X^2); lane 2, Normal male (X^1Y^2), lane 3, Hormonally induced supermale (Y^1Y^2); lane 4, Androgenetic supermale (Y^2Y^2) (from refs 9 and 11).

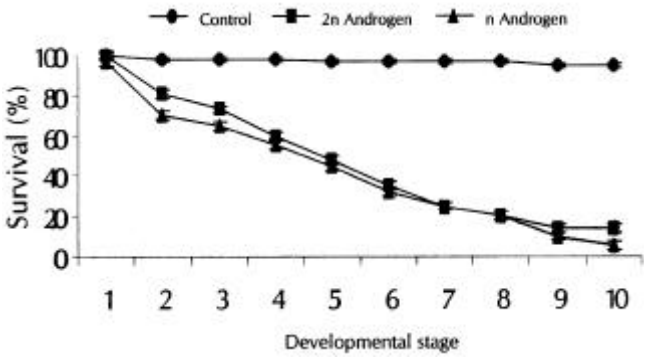

Figure 8. Survival of androgenetic clone of golden rosy barb, *P. conchonius* as a function of developmental stage. Numbers in the *X*-axis indicate selected embryonic stages (from ref. 8).

Table 7. Reproductive performance of androgenetic males and females of tiger barb, *P. tetrazona* and the rosy barb, *P. conchonius* (from refs 7, 8)

	Genotype of ♂ tiger barb		Genotype of ♂ rosy barb	
Parameter	X^1Y^2	Y^2Y^2	X^1Y^2	Y^2Y^2
Sexual maturity (days)	110	120	90	85
GSI	0.48	0.52	0.38	0.55
Sperm count (no./ml)	8.1×10^5	8.8×10^5	7.2×10^5	7.5×10^6
Motility duration (s)	–	–	95	87
Fertilizability (%)	97	95	–	–
	Genotype of ♀ tiger barb		Genotype of ♀ rosy barb	
	X^1X^2	X^2X^2	X^1X^2	X^2X^2
Sexual maturity (days)	115	140	90	130
Inter-spawning period (days)	18	30	15	26
GSI	0.52	0.38	0.42	0.28
Fecundity (no./spawn)	105	84	91	61
Hatchability (%)	98	85	95	70

sperms or one cadaveric sperm, regulates the developmental sequence, even in alien surrogate egg of tiger barb, as good as that of the diploid genome of the tiger barb, (iii) the time sequence characteristic of the rosy barb is maintained even when about 25% excess yolk was available in eggs of the tiger barb and (iv) hybrid eggs and haploids suffer heavy mortality.

Interspecific cloning

An alternate approach to increase heterozygosity is to generate interspecific androgenotes. Although interspecific androgenesis has been attempted in many species, for example, Salmonidae[63] and Cyprinidae[15], Bercknyski *et al.*[39] were the first to produce viable androgenetic goldfishes, *C. auratus* using its fresh or frozen sperms to activate genome-inactivated eggs of the common carp, *C. carpio* (Table 1). Our specific understanding of nucleo-cytoplasmic relation in fish is fragmentary. Chinese scientists presumed the enucleation of eggs of crucian carp, *C. auratus*, by pricking a glass microneedle at a point immediately below the small polar body. Subsequently, the nucleus obtained from the blastula cells of common carp was introduced into the presumed enucleated eggs of crucian carp. This kind of transplantation helped them to generate hybrids between these cyprinids. Briefly, their studies indicate that both nucleus and cytoplasm influence the expression of genetic information in the hybrid. Barring these Chinese publications, there is no readily available publication on nucleo-cytoplasmic relationship in eggs and hybrid eggs of fishes[3]. Until adequate information is made available on hybrid eggs, protocols for generation of interspecific androgenotes will have to be made on a trial and error basis. To make interspecific androgenetic

Table 8. Duration required for completion of pre- and post-embryonic development and survival of the tiger and rosy barb, their hybrids and rosy barb androgenotes (from ref. 9)

Cross	Duration from fertilization to hatching (h)	Duration from hatching to feeding (h)	Survival (%) at	
			hatching	maturity
Tiger barb control (TB)	26	42	98	94
Rosy barb control (RB)	24	36	98	95
RB ♀ × TB ♂ – hybrid	24	36	72	0
TB ♀ × RB ♂ – hybrid	24	40	80	75
2*n* interspecific androgenote (fresh RB sperm)	24	42	14	7
2*n* interspecific androgenote (cadaveric RB sperm)	24	42	7	3
2*n* interspecific androgenote (fresh RB *n* + *n* sperm)	24	42	3	2
n interspecific androgenote (fresh sperm source)	24	0	9	0
n interspecific androgenote (cadaveric sperm source)	24	0	3	0

Table 9. Source, technique and genome of the clones generated in mammals and fishes

	Mammal	Fish
Source	Enucleated ovum of recipient and nucleus of a highly differentiated cell of donor	Due to non-visiblity of nucleus, genome-inactivated (by UV-irradiation) egg as recipient and sperm as donor
Requirement	Viviparity requires a surrogate mother	With oviparity, the genome-inactivated egg replaces the requirement of surrogation
Techniques	Highly skilled technique of enucleation of recipient egg and donor cell	Not so skilled a technique of UV-irradiation to inactivate the egg genome
	Cell fusion technique to transfer the donor genome	Natural sperm-mediated transfer of the donor genome
	Surgical introduction of ‘fused’ egg in surrogate mother	Interspecific androgenetic cloning requires dispermy or diploid sperm, or monospermy or haploid sperm with restoration of diploidy by thermal/pressure shock
Genome of clone	Originating from a highly differentiated cell, clone develops in the egg cytoplasm, i.e. ‘old wine in a new bottle’.	Originating from the donor sperm, the clone develops in the egg cytoplasm, i.e. ‘new wine in a new bottle’.
	Nuclei of different cells of the same tissue may generate clones like ‘xerox copies’ of the donor, i.e. clones may be identical and may not differ genetically from each other.	Nuclei of different sperms may generate clones like ‘photos’ of the donor, i.e. clones may be identical but may genetically slightly differ from each other.
Sex of clone	Since the clones originate from the same nuclei, say, udder cells of a donor, they are all expected to be of the same sex, i.e. females only	Since the clones originate from nuclei of either X- or Y-carrying sperm, expected sex ratio of clones is 50% females and 50% males
Advantage/limitation	Requires live donor and recipient	Requires live recipient, but the donor can be from the sperm of either live or post-mortem preserved donor

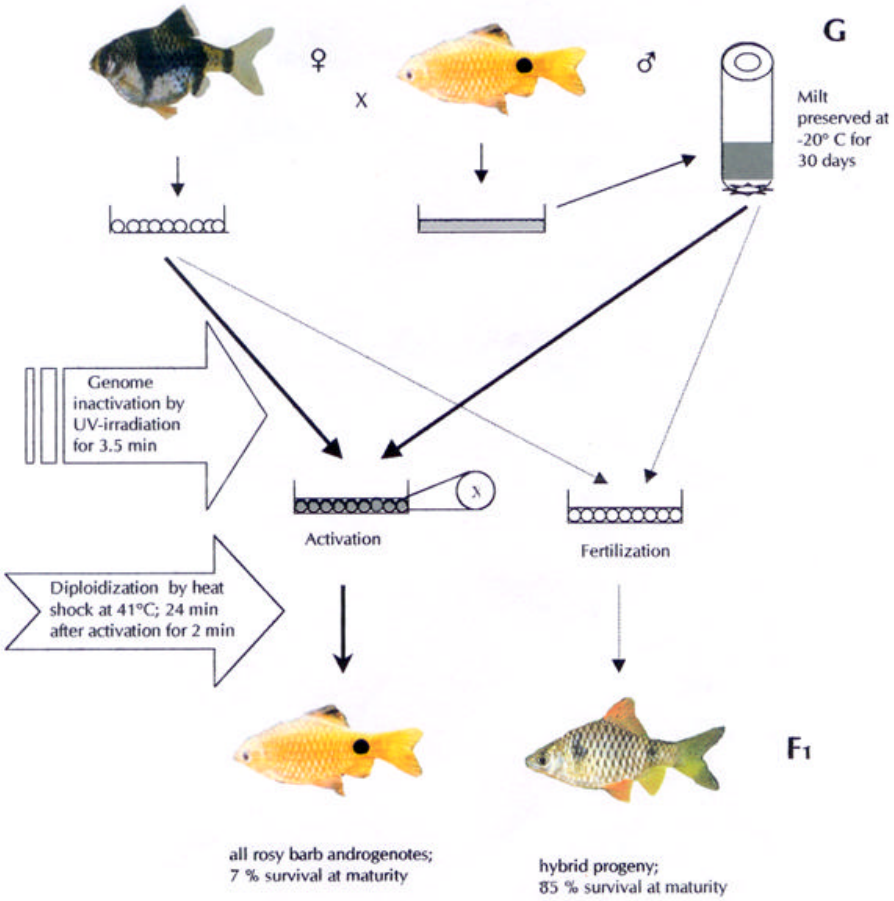

Figure 9. Induction of interspecific androgenetic cloning of *P. conchonius* using its preserved sperms for activation of genome-inactivated (surrogate) eggs of *P. tetrazona* (from ref. 9).

cloning a success, the following points are taken into consideration when selection for the two species is made: (i) compatability between the yolk volume and incubation period, (ii) compatibility of the head of the donor sperm and the micropyle of the host egg, (iii) availability of the phenotypic and protein markers as well as species-specific primers for PCR analysis and (iv) affording protection to 'the maternal produts' of the eggs, namely enzyme, mRNA and mtDNA, while the eggs are being irradiated to inactivate their genome. These technical difficulties may prove difficult, but are not totally unsurmountable.

Not surprisingly, interspecific androgenetic clones have been generated only in a couple of species, namely *C. auratus* using *C. carpio* eggs[39], and *P. conchonius* using *P. tetrazona* eggs[9] (Figure 9). Kirankumar and Pandian[9] have successfully reared the interspecific androgenetic clones to sexual maturity and found that the reproductive performance of these androgenetic males was superior and that of the females inferior compared to the respective controls (Table 8). Recently, intergeneric androgenetic clones of *Hemigrammus caudovittatus* using its preserved sperm and genome-inactivated eggs of *Gymnocorymbus ternetzi* have successfully been generated[30].

Among vertebrate groups, fishes are uniquely amenable for induction of interspecific androgenotes. Indeed techniques and protocols for induction of interspecific and intergeneric androgenotes will lay the foundation (i) for restoration of endangered fish using preserved/cadaveric sperm and surrogate egg, (ii) to produce seedlings almost throughout the year in annual spawners like the carps using surrogate eggs of undesired fish like tilapia which breed throughout the year, and (iii) for generation of seedlings in migratory species like eel, using their cryopreserved sperm and suitable surrogate eggs.

Despite its comparability, androgenetic cloning is not identical to the mammalian cloning achieved in recent years. Table 9 presents a comparative account on the source, techniques and genomes used for the generation of clones of mammals and androgenetic clones of fish. From the points of conservation and aquaculture, androgenetic cloning certainly has an edge over the technique of mammalian cloning[64].

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