Production of Fertile Unreduced Sperm by Hybrid Males of the *Rutilus alburnoides* Complex (Teleostei, Cyprinidae): An Alternative Route to Genome Tetraploidization in Unisexuals

M. Judite Alves, M. Manuela Coelho, M. Isabel Próspero and M. João Collares-Pereira

Centro de Biologia Ambiental, Departamento de Zoologia e Antropologia, Faculdade de Ciências, Universidade de Lisboa, 1700 Lisboa, Portugal

Manuscript received April 9, 1998
Accepted for publication September 18, 1998

ABSTRACT

The hybrid minnow *Rutilus alburnoides* comprises diploid and polyploid females and males. Previous studies revealed that diploid and triploid females exhibit altered oogenesis that does not involve random segregation and recombination of the genomes of the two ancestors, constituting unisexual lineages. In the present study, we investigated the reproductive mode of hybrid males from the Tejo basin, using experimental crosses and flow cytometric analysis of blood and sperm. The results suggest that diploid hybrids produced fertile unreduced sperm, transmitting their hybrid genome intact to offspring. Triploid hybrids also produced unreduced sperm, but it was not possible to obtain data concerning their fertility. Finally, tetraploid hybrids produced fertile diploid sperm, which exhibited Mendelian segregation. Tetraploid *R. alburnoides* may reestablish biparental reproduction, as individuals of both sexes with the appropriate constitution for normal meiosis (two haploid genomes from each parental species) are likely to occur in natural populations. Tetraploids probably have arisen from syngamy of diploid eggs and diploid sperm produced by diploid hybrid males. Diploid hybrid males may therefore play a significant role in the dynamics of the complex, starting the evolutionary process that may ultimately lead to a new sexually reproducing species.

INTERSPECIFIC hybridization generally leads either to sterile F_1's or to hybrids with some measure of fertility that exhibit normal meiosis and act, through backcrossing, as a bridge for the transfer of genetic material between the parental species (introgression; reviewed in Arnold 1997). Occasionally, however, hybridization disrupts oogenesis so that hybrids produce viable eggs without recombination and often without a reduction in ploidy, founding unisexual lineages (reviewed in Dawley 1989). Among vertebrates, this phenomenon is apparently uncommon, with ~70 unisexual taxa described (Vrijenhoek *et al.* 1989). The genetic basis of the origin of unisexuality via hybridization remains uncertain. It has been suggested that hybridization brings together specific sets of alleles that, in combination, cause changes in the regulation of oogenesis, allowing for unisexual reproduction ("balance hypothesis"; Moritz *et al.* 1989). Genes seem to be differentially expressed in oocytes *vs.* spermatocytes, as rare males of unisexual lineages are apparently sterile (Cimino and Schultz 1970; Darevsky *et al.* 1978; Rasch *et al.* 1982; Goddard and Dawley 1990). *Rana esculenta* is an exception, because both females and males are hybridogenetic (during gametogenesis, one of the parental genomes is eliminated without recombination, and only the other parental genome is retained in mature gametes; reviewed in Graf and Polls Pelaz 1989).

The Iberian minnow *Rutilus* (a.k.a. *Tropidophoxinellus*) *alburnoides* (Steindachner 1866) comprises diploid and polyploid forms and is of hybrid origin, incorporating genomes from *Leuciscus carolitertii* or *L. pyrenaicus* and that from an undescribed species (Alves *et al.* 1997a,b; Carmona *et al.* 1997). Hybrid males are usually rare, but in some populations they represent about 30% of total specimens collected (Collares-Pereira 1984, 1985, 1989; Alves *et al.* 1997a; Carmona *et al.* 1997). Previous studies have focused on the reproductive modes of diploid and triploid females, revealing altered oogenesis that does not involve random segregation and recombination between the genomes of the ancestors (Carmona *et al.* 1997; Alves *et al.* 1998). *R. alburnoides* females seem to exhibit distinct reproductive modes according to their geographic origin. Diploid and triploid females from the northern Douro basin reproduce by hybridogenesis, discarding the *L. carolitertii* genome during oogenesis (Carmona *et al.* 1997). Diploid females from the southern Tejo basin transmit their hybrid genome clonally to the eggs, which upon fertilization yield triploid progeny, whereas triploid females from the Tejo and Guadiana basins present a modified hybridogenesis in which the *L. pyrenaicus* genome is discarded in each generation, but the remaining genomes are not transmitted clonally to the eggs as the

Corresponding author: M. João Collares-Pereira, Centro de Biologia Ambiental, Departamento de Zoologia e Antropologia, Faculdade de Ciências, Universidade de Lisboa, Campo Grande C2 - Piso 3, 1700 Lisboa, Portugal. E-mail: mcolares@fc.ul.pt

elimination of the unmatched genome permits ready synapsis of the homospecific genomes ("meiotic hybridogenesis"; Alves *et al.* 1998). Whether hybrid males are fertile and participate in the reproduction within the complex remained unknown.

As a key to exploring the population dynamics and the evolutionary history of the *R. alburnoides* complex, we have investigated the reproductive mode(s) of hybrid diploid, triploid, and tetraploid males from the Tejo basin. The present study is based on experimental crosses and flow cytometric analysis of blood and sperm and revealed a greater role for hybrid males than initially suspected.

MATERIALS AND METHODS

Specimens used in this study were collected in 1996 from the Sorraia River of the Tejo basin (detailed locality data are available from M.J.C.P.), during the reproductive season (April–May).

DNA content of erythrocytes and spermatozoa from 31 *R. alburnoides*-like males was determined by flow cytometry, using an EPICS Profile II cytometer. Blood samples were drawn from the caudal vein, and milt samples were collected by applying light pressure to the abdomen. Both samples were stabilized in buffer (40 mm citric acid trisodium salt, 0.25 m sucrose, and 5% dimethyl sulfoxide) and immediately frozen at −80°. Prior to analysis, samples were diluted at approximately the same concentration (5×10^6 cells/ml) with the help of a hemocytometer and stained as described in Dawley and Goddard (1988) using propidium iodide as fluorochrome. Chicken erythrocytes were used both as an internal and external standard. The same sample of chicken erythrocytes was used throughout the study, and the flow cytometer was calibrated daily with fluorescent microspheres (DNA-Check, Coulter, Hialeah, FL). For each sample, two measurements were made at a flow rate of less than 300 events/sec, using an excitation emission of 488 nm. Approximately 6×10^4 cells were examined per measurement, and only peaks of the fluorescence histograms with a coefficient of variation lower than 4% were scored (Dressler and Seamer 1994). DNA content of erythrocytes and spermatozoa in individual fish was estimated by calculating the ratio of mean fluorescence of fish cells to mean fluorescence of chicken cells and multiplying by 2.5 pg (the standard DNA content value for chicken erythrocytes; Tiersh *et al.* 1989). The data were converted and analyzed using the software Pro2FCS (version 3.2) and Win MDI (version 1.3.5).

Additional specimens were used in crossing experiments. Crosses were done blindly without knowledge of the ploidy level of the mates. For the crosses that involved hybrid males, progeny were produced by stripping eggs and milt from the parents simultaneously into a bowl containing aquarium water. The young hatched in <1 wk and were reared to the age of 9 mo (2–4 cm). High mortality occurred in all broods due to fungal contamination. Parents and offspring were killed with an overdose of MS222 and frozen at −80°. Offspring were sexed by dissection and inspection of gonads. Ploidy of parents and offspring was determined by flow cytometric measurement of erythrocyte DNA content as described above. Parents and a subsample of offspring were analyzed by DNA fingerprinting using the human minisatellite probes 33.6 and 33.15 (Jeffreys *et al.* 1985), as described in Alves *et al.* (1998). The number of fragments detected by both probes out of *n* scored that were transmitted to a number *r* of offspring in a sibship of *N* was compared with the expected number given by the binomial distribution $({}^{N}Cr/2^{N})n$ assuming 50% transmission (Jeffreys *et al.* 1986), using the *G*-test for goodness of fit (Sokal and Rohlf 1981).

The hybrid nature of specimens analyzed in this study was investigated by allozyme electrophoresis at the *sAAT** (aspartate aminotransferase, EC 2.6.1.1) and *PGDH** (phosphogluconate dehydrogenase, EC 1.1.1.44) loci. These loci have been found to be diagnostic for the *R. alburnoides* complex; virtually all specimens are heterozygous, exhibiting one set of alleles associated with members of the genus Leuciscus and a second set that could not be attributed to any described species (Alves *et al.* 1997a; Carmona *et al.* 1997).

RESULTS

Flow cytometric analysis of blood and sperm: Flow cytometric measurement of DNA content in erythrocytes revealed that the 31 *R. alburnoides*-like males sampled in the Sorraia River comprised 21 diploids (mean DNA content of 2.43 ± 0.07 pg/cell), 2 triploids (mean DNA content of 3.64 ± 0.16 pg/cell), and 8 tetraploids (mean DNA content of 4.83 ± 0.08 pg/cell; Table 1). Ten diploid males produced sperm that showed DNA values ranging from 1.11 to 1.21 pg/cell, with a mean of 1.16 ± 0.03 pg/cell. The ratio of erythrocyte to spermatozoon DNA content per male varied from 1:0.44 to 1:0.50, averaging 1:0.48 ± 0.02. The remaining 11 diploid males produced sperm with DNA content ranging from 2.25 to 2.43 pg/cell, and the mean was 2.34 ± 0.06 pg/cell. Erythrocyte and spermatozoon DNA content ratio varied from 1:0.88 to 1:1, with a mean of 1:0.96 ± 0.03. Sperm produced by triploid males also exhibited DNA values similar to those of the erythrocytes (mean ratio 1:0.95 ± 0.03), averaging 3.44 ± 0.04 pg/cell. The DNA content of sperm produced by tetraploid males was from 2.19 to 2.57 pg/cell, with a mean of 2.34 ± 0.13 pg/cell. The ratio of the erythrocyte and spermatozoon DNA values ranged from 1:0.45 to 1:0.54, averaging 1: 0.48 ± 0.03.

Allozyme electrophoresis revealed that the diploid males with ratios of somatic cell and gamete DNA content of about 1:1 were all heterozygous at the diagnostic *sAAT** and *PGDH** loci, whereas the remaining diploids with ratios of about 1:0.5 were homozygous for alleles presumably attributed to the undescribed parental species (Alves *et al.* 1997a; Carmona *et al.* 1997). All triploid and tetraploid males showed heterozygous patterns at the diagnostic loci.

The additional specimens used in the crossing experiments reported here included two diploid and four tetraploid males and one diploid and seven triploid females. All specimens were heterozygous at the diagnostic loci.

Crosses involving diploid hybrid males: Diploid ♂121 crossed to diploid ♀122 yielded apparently all male tetraploid progeny (four fish were not sexed), whereas diploid ♂117 and ♂121 mated to triploid females produced triploid progeny of both sexes (Table 2).

TABLE 1

DNA content of erythrocytes and spermatozoa of *R. alburnoides*-like males collected from the Tejo basin

	No. of individuals	Erythrocytes (pg/cell)		Spermatozoa (pg/cell)	
		Mean ± SD	CV range	Mean ± SD	CV range
2*n*	10	2.43 ± 0.06	1.64–2.27	1.16 ± 0.03	2.45–3.61
	11	2.44 ± 0.08	1.57–2.63	2.34 ± 0.06	1.64–2.55
3*n*	2	3.64 ± 0.16	3.56–3.78	3.44 ± 0.04	2.56–2.96
4*n*	8	4.83 ± 0.08	1.30–1.59	2.33 ± 0.14	1.73–2.73

SD, standard deviation per group of males; CV, within-sample coefficients of variation.

DNA fingerprinting analysis of cross ♀122 × ♂121 (Figure 1a, Table 3) revealed that all offspring exhibited identical patterns, having coinherited all scorable maternal DNA fragments and almost all scorable paternal fragments. Probe 33.15 detected polymorphisms in the region around 9.4 kb, as one paternal fragment was transmitted to no offspring, and one fragment observed in four young could not be traced back to either parent.

Crosses involving tetraploid hybrid males: Tetraploid ♂130 and diploid ♀122 produced one brood of 24 tetraploid and 1 diploid young. Tetraploid offspring included both females and males; the single diploid was a female (Table 2). DNA fingerprinting analysis revealed that all tetraploid offspring exhibited bands associated with the male parent, but the diploid offspring displayed only bands that could be traced back to the mother (Figure 1b, Table 3). All scorable maternal DNA fragments were coinherited by all tetraploid and diploid progeny, indicating that the female hybrid genome was transmitted intact to the eggs. The observed distribution of the paternal bands in the tetraploid offspring followed the expected binomial distribution for alleles showing Mendelian inheritance ($0.25 < P < 0.50$). Probe 33.15 detected in four tetraploid progeny a fragment with length >9.4 kb that could not be attributed to either parent (Figure 1b).

Tetraploid ♂112, ♂115, and ♂134 crossed to triploid females resulted in only triploid progeny. Crosses ♀110 × ♂112, ♀111 × ♂112, and ♀133 × ♂134 produced offspring of both sexes, whereas cross ♀114 × ♂115 yielded apparently all female progeny (two fish were not sexed; Table 2). Families fathered by ♂115 and ♂134 were analyzed by DNA fingerprinting (Table 3). In each cross, sets of five and six maternal minisatellite bands, respectively, were transmitted to no offspring. The remaining maternal fragments and the paternal

TABLE 2

Laboratory crosses involving diploid and tetraploid hybrid males and diploid and triploid females of *R. alburnoides* collected from the Tejo basin

Crosses	Ploidy	Analyzed progeny		
		Sex		
		♀	♂	?
2*n* ♀ × 2*n* ♂				
#122 #121	4*n*	—	9	4
3*n* ♀ × 2*n* ♂				
#116 #117	3*n*	1	—	1
#132 #121	3*n*	4	3	—
#136 #121	3*n*	—	4	—
2*n* ♀ × 4*n* ♂				
#122 #130	2*n*	1	—	—
	4*n*	3	6	15
3*n* ♀ × 4*n* ♂				
#110 #112	3*n*	2	1	—
#111 #112	3*n*	2	4	1
#114 #115	3*n*	10	—	2
#133 #134	3*n*	1	4	—

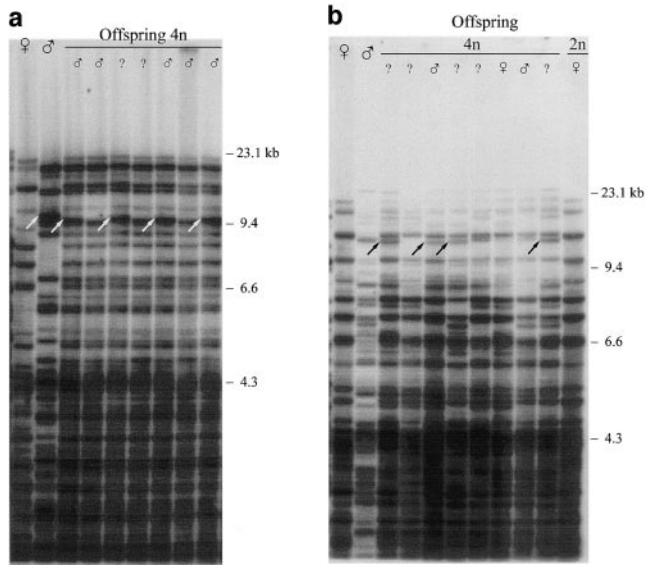

Figure 1.—DNA fingerprints of parental fish and offspring of crosses ♀122 × ♂121 (a), and ♀122 × ♂130 (b), as obtained with probe 33.15 after *Mbo*I digestion. The arrowed fragments in offspring are present in neither parent and are probably new mutants; the arrowed fragment in female of cross #121 is present in no offspring and probably it was lost due to mutation.

TABLE 3

Segregation of hypervariable single fragments produced by the minisatellite probes 33.6 and 33.15 (Jeffreys *et al.* 1985) in the experimental families

	Observed single fragments transmitted to *r* offspring							
	♀ 122 × ♂ 121 ($N = 7$)		♀ 122 × ♂ 130 ($N = 8$)		♀ 114 × ♂ 115 ($N = 8$)		♀ 133 × ♂ 134 ($N = 5$)	
No. of offspring (*r*)	Maternal	Paternal	Maternal	Paternal	Maternal	Paternal	Maternal	Paternal
0	0	1	0	0	5	0	6	1
1	0	0	0	0	1	1	3	0
2	0	0	0	1	1	4	2	5
3	0	0	0	3	3	2	4	5
4	0	0	0	9	2	5	2	2
5	0	0	0	2	6	2	0	0
6	0	0	0	3	2	6		
7	19	18	0	2	1	0		
8			16	0	0	0		
Total no. of bands (*n*)	19	18	16	20	21	20	17	13

Fragments transmitted to all offspring may be from homozygous loci and were ignored in all but the diploid males and females. All but one of each set of linked or pair of apparently allelic fragments were excluded. Linkage may result from the cutting of a single minisatellite allele at internal recognition sites, generating two or more fragments which are always coinherited; alternatively, two distinct minisatellite regions may be situated close together on a chromosome so that recombination between them occurs infrequently (Bruford *et al.* 1992). Bands codetected by both probes were not observed. *N*, number of offspring analyzed in each brood.

fragments were inherited by the triploid offspring following the binomial distribution for alleles segregating in Mendelian fashion ($P > 0.1$).

DISCUSSION

The measurement of DNA content in erythrocytes and spermatozoa demonstrated that some diploid *R. alburnoides*-like males produced haploid sperm (ratios of somatic cell and gamete DNA content of about 1:0.5), whereas others produced unreduced sperm (ratios of about 1:1). Allozyme analysis suggested that the former males possessed nonhybrid nuclear DNA, whereas the latter exhibited hybrid nuclear DNA. The origin of the nonhybrid diploid males lacks consensus among authors. Taking into account that the Hardy-Weinberg analysis of polymorphic loci revealed no significant deviations from random expectations, Carmona *et al.* (1997) suggested that nonhybrid specimens correspond to a sexually reproducing diploid form, which most likely was the maternal ancestor of the complex. Alves *et al.* (1998; M. J. Alves, M. J. Collares-Pereira, T. E. Dowling and M. M. Coelho, unpublished data), considering that nonhybrid specimens exhibit *L. pyrenaicus*-like mitochondrial DNA and show highly male-biased sex ratios, suggested that they probably were reconstituted from *R. alburnoides* hybrids. Previous studies revealed that nonhybrid males exhibit normal Mendelian meiosis (Alves *et al.* 1998), which is consistent with either of the above hypotheses.

DNA fingerprinting of progeny fathered by the diploid hybrid revealed that each inherited the almost intact genome of the male. These data together with the flow cytometry data clearly show that, like the diploid hybrid females from the Tejo basin (Alves *et al.* 1998), diploid hybrid males produced unreduced clonal gametes. Although it has been hypothesized for *Rana esculenta* (Günther 1970; Uzzell *et al.* 1977), production of fertile unreduced sperm by natural diploid hybrid vertebrates has never before been confirmed.

Triploid *R. alburnoides* males produced unreduced sperm (ratios of somatic cell and gamete DNA content of about 1:1). However, due to their low number in natural populations, they were not used in the crossing experiments, and no data concerning their fertility was obtained. There are reports of fertile triploid hybrid males in amphibians, but they all produced reduced sperm (Heppich *et al.* 1982; Nishioka and Ohtani 1984; Shen *et al.* 1984; Vinogradov *et al.* 1990; Berger and Günther 1991–1992; Sumida and Nishioka 1993).

Tetraploid *R. alburnoides* males produced reduced sperm (ratios of somatic cell and gamete DNA content of about 1:0.5) that exhibited Mendelian segregation at the minisatellite markers. Therefore, these males seem to have undergone normal meiosis. Theoretically, *R. alburnoides* tetraploids have three possible combinations of parental genomes: PPAA, PAAA, and PPPA, where P is the *L. pyrenaicus* genome and A is the genome of the other ancestor. Only in the first of these is normal meiosis likely to occur, as the presence of two haploid

genomes from each parental species permits ready synapsis (Vasil'ev *et al.* 1989). We may, therefore, expect that the tetraploid males presently analyzed had a PPAA constitution.

The inheritance patterns of the maternal bands of both the diploid and triploid females used in the present study followed the patterns described in Alves *et al.* (1998). Diploid females from the Tejo basin transmit clonally their hybrid genome to the eggs, whereas triploid females present a modified hybridogenesis in which the *L. pyrenaicus* genome is discarded in each generation, but the remaining genomes undergo meiosis (meiotic hybridogenesis). According to Alves *et al.* (1998), eggs produced by both diploid and triploid females needed syngamy to initiate development. However, the present study revealed that a small proportion (3% in the present example) of the diploid eggs produced by diploid females may develop directly without syngamy into diploid clones. In fact, diploid ♀122 produced one diploid female young that inherited the intact hybrid genome of the female parent but exhibited no DNA fingerprinting bands that could be traced back to the father. Such young seems to have originated by gynogenesis, where sperm only stimulates development of the egg. This is the first direct evidence for gynogenesis in the *R. alburnoides* complex, constituting the third mode of reproduction described for diploid females (Carmona *et al.* 1997; Alves *et al.* 1998).

Probe 33.15 detected polymorphisms at crosses ♀122 × ♂121 and ♀122 × ♂130 that are probably accounted for by germ line mutation. Mutations in minisatellites can involve gain or loss of numbers of repeat units, originating new length alleles (Jeffreys *et al.* 1988). In cross ♀122 × ♂121, one paternal fragment was transmitted to no offspring, while one fragment observed in some young could not be traced back to either parent. In cross ♀122 × ♂130, a new fragment was also observed in some offspring. According to Bruford *et al.* (1992), typical apparent mutation rates in fingerprints are of the order of 10^{-3} per fragment per gamete. Evidence for genomic variability in as short a time as one generation has also been found in one diploid *R. alburnoides* female (Alves *et al.* 1998).

The mechanism of sex determination in the *R. alburnoides* complex is not well understood. Although cytological data indicated that the parental species *L. pyrenaicus* has a *ZW* female/*ZZ* male sex chromosome heteromorphism (Collares-Pereira *et al.* 1998), the sex ratio of offspring mothered by triploid *R. alburnoides* females (Alves *et al.* 1998) has suggested that non-*W*-linked genes, expressed differently depending on the species and the population to which parents belong, are involved in sex determination. The present data are also not consistent with a simple *ZW*/*ZZ* sex-determining mechanism. According to this model, all progeny produced in cross ♀122 × ♂121 should have been female and not male, because they have received both a *Z* and *W* chromosome from their diploid mother. In fact, the occurrence of apparently all male progeny suggests an *XX* female/*XY* male sex chromosome heteromorphism. Male heterogamety would also explain the production of female and male offspring in the cross ♀122 × ♂130: because tetraploid males seem to undergo normal meiosis, we may expect that some spermatozoa carried two *X* chromosomes producing females, whereas others carried *X* and *Y* chromosomes producing males. However, this mechanism of sex determination does not explain the sex ratio of progeny of crosses ♀116 × ♂117 and ♀132 × ♂121, which involved diploid hybrid males and triploid females: according to this model, having received both an *X* and *Y* chromosome from their father, no female young should have been observed. Therefore, sex determination in *R. alburnoides* can be fully explained neither by a *ZW* female/*ZZ* male nor by an *XX* female/*XY* male sex-determining system.

Schultz (1969) postulated that hybridization and unisexuality provide a path to evolution by polyploidy. Restoration of an even ploidy value in tetraploids would allow a return to normal meiosis, serving as a stepping-stone to biparental reproduction (Schultz 1977, 1980, 1989). The typical route to tetraploidy in clonal vertebrates seems to be the syngamy of unreduced triploid eggs and haploid sperm produced by a male of a related bisexual species (Dawley 1989). This pathway could yield tetraploids with several possible combinations of parental genomes, though only one (two haploid genomes from each bisexual parental species) is optimal for meiotic reproduction (Vasil'ev *et al.* 1989). The formation of such tetraploids is, however, apparently not possible in many clonal vertebrates, as only one parental species is present (*e.g.*, Bogart *et al.* 1987). In fact, although tetraploids are abundant in some unisexual complexes, namely in Ambystoma (Bogart 1989), *Carassius auratus langsdorfii* (Kobayasi and Kawasima 1972) and Cobitis (Vasil'ev *et al.* 1989), all females seem to exhibit unisexual reproduction and the males, when they exist, are sterile. The *R. alburnoides* complex presents an alternative route to tetraploidy, where tetraploids may have originally arisen from syngamy of diploid eggs and diploid clonal sperm produced by a diploid hybrid male. At least two types of diploid eggs occur in the *R. alburnoides* complex, depending on their origin (Alves *et al.* 1998): clonal eggs with PA constitution, if they are originated by diploid female hybrids; or recombined eggs with AA constitution, if they are produced by PAA triploid females. Fertilization of the former by clonal sperm yields isogenic tetraploids with PPAA constitution, whereas fertilization of the latter leads to genetically diverse tetraploids with PAAA constitution. The occurrence of matings between female and male diploid hybrids is restricted by the low frequency of these forms in natural populations, and we may speculate that PPAA tetraploids were initially infrequent. However, once they arose, they also produced fertile diploid gametes, yield-

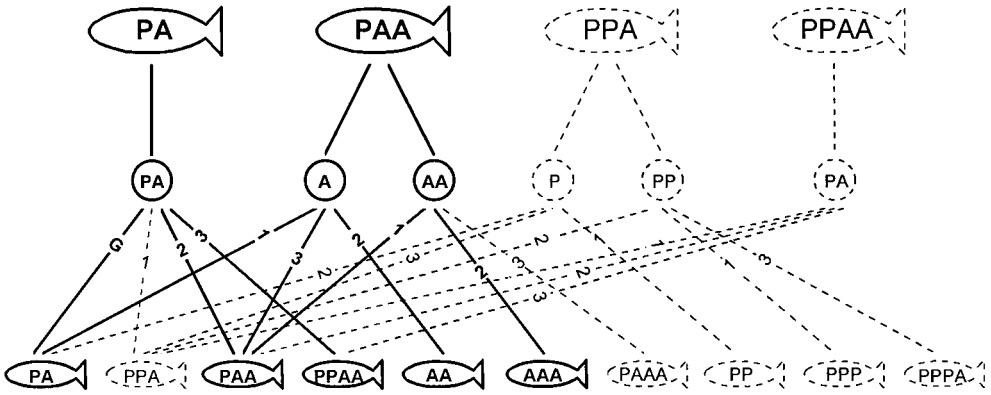

Figure 2.—Summary of the putative reproductive modes and interrelationships between the different forms of the *R. alburnoides* complex in the Tejo basin (Alves *et al.* 1998; present study). (—) Forms and pathways confirmed experimentally; (- - -) hypothetical forms and pathways. P is the genome of *L. pyrenaicus*, and A is the genome of the other ancestor. (1) Fertilization by P sperm, produced by normal meiosis by *L. pyrenaicus*; (2) fertilization by A sperm, produced by normal meiosis by nonhybrid males; and (3) fertilization by PA sperm, produced either clonally by diploid hybrid males or by normal meiosis by tetraploid males. (G) Occurrence of gynogenesis.

ing more tetraploids with the appropriate constitution for normal meiosis, either through backcrossing to diploid hybrids or through mating to other symmetric tetraploids. Symmetric tetraploids produced in both of these ways are genetically diverse, in contrast with clonal tetraploids whose parents are both diploid hybrids. Occurrence of PPAA tetraploids of both sexes in natural populations is the necessary condition for achieving biparental reproduction. The experimental hybridization between diploid hybrids reported here (cross ♀122 × ♂121, Table 2) produced apparently all male PPAA progeny. However, the cross involving a diploid female and a tetraploid male produced tetraploid females (cross ♀122 × ♂130) that should also present PPAA constitution, because they received a clonal PA genome from their mother and a recombined PA genome from their father. Therefore, we may expect that PPAA tetraploids of both sexes occur in nature, and that they may occasionally mate, leading to biparental reproduction.

Tetraploids seem to occur only in the central and northern part of the distribution area of the *R. alburnoides* complex, apparently being absent in the Guadiana and Sado basins (Alves *et al.* 1997a,b; Carmona *et al.* 1997; Martins *et al.* 1998). One explanation is the apparent absence of diploid hybrid males in the latter basins (Alves *et al.* 1997a; Carmona *et al.* 1997; M. J. Alves, M. M. Coelho and M. J. Collares-Pereira, unpublished data). Therefore, in these basins, the only possible route to tetraploidy would involve the fertilization of triploid eggs. However, if such a phenomenon occurs, it seems to be very rare or localized.

The present study and the previous studies of Carmona *et al.* (1997) and Alves *et al.* (1998) suggest that hybridization in *R. alburnoides* has apparently "opened" many novel reproductive pathways, altering both oogenesis and spermatogenesis (see Figure 2 for summary of the putative reproductive modes of the different forms of the *R. alburnoides* complex in the Tejo basin). Contrasting with most unisexual vertebrates, hybrid *R. alburnoides* males are fertile and participate in the perpetuation of the Iberian complex. Among the known hybrid complexes, only in *R. esculenta* are hybrid males fertile (reviewed in Graf and Polls Pelaz 1989). Diploid hybrid males seem to have played a particularly important role in the dynamics and evolution of the *R. alburnoides* complex, as they might have initiated tetraploidization, starting the evolutionary process that may ultimately lead to a new sexually reproducing polyploid species.

We are especially grateful to Toomas Saat for performing the breeding experiments. We thank Dominic Poccia for receiving M.J.C.P. and M.I.P. in his laboratory to improve technical aspects of flow cytometry, Terry Burke for receiving M.J.A. to learn DNA fingerprinting methods, and Diogo Thomaz and Olivier Hanotte for technical assistance during that time. We acknowledge Alec Jeffreys and Robert Dawley, who kindly provided the human minisatellite probes and the protocols for preservation and staining of erythrocyte samples for flow cytometry, respectively. We also thank Carlos Almaça and Eduardo Crespo for comments on the manuscript, Ricardo Pires and Luís Miguel Vieira for caring for the progeny on many occasions, Maria Graça Vieira for use of her laboratory facilities, and Líbia Zé-Zé and Maria do Céu Sampaio for technical assistance. We acknowledge Direcção Geral das Florestas for permission to collect specimens. This work was supported by Centro de Biologia Ambiental, by the Junta Nacional de Investigação Científica e Tecnológica (JNICT) project Programa Específico para o Ambiente (PEAM)/C/GAG/227/93, and by grants CIÊNCIA/BD/2185/92-RN and PRAXIS XXI/BD/5735/95 to M.J.A.

LITERATURE CITED

Alves, M. J., M. M. Coelho and M. J. Collares-Pereira, 1997a The *Rutilus alburnoides* complex (Cyprinidae): evidence for a hybrid origin. J. Zool. Syst. Evol. Res. **35:** 1–10.

Alves, M. J., M. M. Coelho, M. J. Collares-Pereira and T. E. Dowling, 1997b Maternal ancestry of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae) as determined by analysis of cytochrome *b* sequences. Evolution **51:** 1584–1592.

Alves, M. J., M. M. Coelho and M. J. Collares-Pereira, 1998 Diversity in the reproductive modes of females of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): a way to avoid the genetic constraints of uniparentalism. Mol. Biol. Evol. **15:** 1233–1242.

Arnold, M. L., 1997 *Natural Hybridization and Evolution*. Oxford University Press, Oxford.

Berger, L., and R. Günther, 1991–1992 Inheritance patterns of water frog males from the environments of Nature Reserve Steckby, Germany. Zool. Pol. **37:** 87–100.

Bogart, J. P., 1989 A mechanism for interspecific gene exchange

via all-female salamander hybrids, pp. 170–179 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Bogart, J. P., L. A. Lowcock, C. W. Zeyl and B. K. Mable, 1987 Genome constitution and reproductive biology of hybrid salamanders, genus *Ambystoma*, on Kelleys Island in Lake Erie. Can. J. Zool. **65:** 2188–2201.

Bruford, M. W., O. Hanotte, J. F. Y. Brookfield and T. Burke, 1992 Single-locus and multilocus DNA fingerprinting, pp. 225–269 in *Molecular Genetic Analysis of Populations: A Practical Approach*, edited by A. R. Hoelzel. Oxford University Press, New York.

Carmona, J. A., O. I. Sanjur, I. Doadrio, A. Machordom and R. C. Vrijenhoek, 1997 Hybridogenetic reproduction and maternal ancestry of polyploid Iberian fish: the *Tropidophoxinellus alburnoides* complex. Genetics **146:** 983–993.

Cimino, M. C., and R. J. Schultz, 1970 Production of a diploid male offspring by a gynogenetic triploid fish of the genus *Poeciliopsis*. Copeia **1970:** 760–763.

Collares-Pereira, M. J., 1984 The "*Rutilus alburnoides* (Steindachner, 1866) complex" (Pisces, Cyprinidae). I. Biometrical analysis of some Portuguese populations. Arq. Mus. Bocage Ser. A **2:** 111–143.

Collares-Pereira, M. J., 1985 The "*Rutilus alburnoides* (Steindachner, 1866) complex" (Pisces, Cyprinidae). II. First data on the karyology of a well-established diploid-triploid group. Arq. Mus. Bocage Ser. A **3:** 69–89.

Collares-Pereira, M. J., 1989 Hybridization in European cyprinids: evolutionary potential of unisexual populations, pp. 281–288 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Collares-Pereira, M. J., M. I. Próspero, R. I. Biléu and E. M. Rodrigues, 1998 *Leuciscus* (Pisces, Cyprinidae) karyotypes: transect of Portuguese populations. Gen. Mol. Biol. **21:** 63–69.

Darevsky, I. S., L. A. Kupriyanova and M. A. Bakradze, 1978 Occasional males and intersexes in parthenogenetic species of Caucasian rock lizards (genus *Lacerta*). Copeia **1978:** 201–207.

Dawley, R. M., 1989 An introduction to unisexual vertebrates, pp. 1–18 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Dawley, R. M., and K. A. Goddard, 1988 Diploid-triploid mosaics among unisexual hybrids of the minnows *Phoxinus neogaeus*. Evolution **42:** 649–659.

Dressler, L. G., and L. C. Seamer, 1994 Controls, standards, and histogram interpretation in DNA flow cytometry, pp. 241–262 in *Flow Cytometry*, edited by Z. Darzynkiewicz, J. P. Robinson and H. A. Crissman. Academic Press, San Diego.

Goddard, K. A., and R. M. Dawley, 1990 Clonal inheritance of a diploid nuclear genome by a hybrid freshwater minnow (*Phoxinus eos-neogaeus*, Pisces: Cyprinidae). Evolution **44:** 1052–1065.

Graf, J.-D., and M. Polls Pelaz, 1989 Evolutionary genetics in the *Rana esculenta* complex, pp. 289–301 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Günther, R., 1970 Der Karyotyp von *Rana ridibunda* Pall. und das Vorkommen von Triploidie bei *Rana esculenta* L. (Anura, Amphibia). Biol. Zbl. **89:** 327–342.

Heppich, S., H. G. Tunner, and J. Greilhuber, 1982 Premeiotic chromosome doubling after genome elimination during spermatogenesis of the species hybrid *Rana esculenta.* Theor. Appl. Genet. **61:** 101–104.

Jeffreys, A. J., V. Wilson and S. L. Thein, 1985 Hypervariable "minisatellite" regions in human DNA. Nature **314:** 67–73.

Jeffreys, A. J., V. Wilson, S. L. Thein, D. J. Weatherall and B. A. J. Ponder, 1986 DNA "Fingerprints" and segregation analysis of multiple markers in human pedigrees. Am. J. Hum. Genet. **39:** 11–24.

Jeffreys, A. J., N. J. Royle, V. Wilson and Z. Wong, 1988 Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature **332:** 278–281.

Kobayasi, H., and Y. Kawasima, 1972 On the chromosomes of an all-female population in the ginbuna, *Carassius auratus langsdorfii*. Jpn. Wom. Univ. J. **17:** 259–263.

Martins, M. J., M. J. Collares-Pereira, I. G. Cowx and M. M. Coelho, 1998 Diploid *vs.* triploid *R. alburnoides*: spatial segregation and morphological differences. J. Fish Biol. **52:** 817–828.

Moritz, C., W. M. Brown, L. D. Densmore, J. W. Wright, D. Vyas *et al.*, 1989 Genetic diversity and the dynamics of hybrid parthenogenesis in *Cnemidophorus* (Teiidae) and *Heteronotia* (Gekkonidae), pp. 87–112 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Nishioka, M., and H. Ohtani, 1984 Hybridogenetic reproduction of allotriploids between Japanese and European pond frogs. Zool. Sci. **1:** 291–326.

Rasch, E. M., P. J. Monaco and J. S. Balsano, 1982 Cytophotometric and autoradiography evidence for functional apomixis in a gynogenetic fish, *Poecilia formosa*, and in its related triploid unisexuals. Histochemistry **73:** 515–533.

Schultz, R. J., 1969 Hybridization, unisexuality and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. Am. Nat. **103:** 605–619.

Schultz, R. J., 1977 Evolution and the ecology of unisexual fishes. Evol. Biol. **10:** 277–331.

Schultz, R. J., 1980 The role of polyploidy in the evolution of fishes, pp. 313–339 in *Polyploidy: Biological Relevance*, edited by W. H. Lewis. Plenum Press, New York.

Schultz, R. J., 1989 Origins and relationships of unisexuals poeciliids, pp. 69–87 in *Ecology and Evolution of Livebearing Fishes (Poeciliidae)*, edited by G. F. Meffe and F. F. Snelson, Jr. Prentice-Hall, Englewood Cliffs, NJ.

Shen, J., Z. Fan, S. Li, R. Cheng and S. Xue, 1984 Comparative studies of the somatic cell and spermatozoon DNA contents and ploidy of Fangzheng crucian carp and Zhalong lake goldfish. Acta Zool. Sin. **30:** 7–13.

Sokal, R. R., and F. Rohlf, 1981 *Biometry*. Freeman, San Francisco.

Sumida, M., and M. Nishioka, 1993 Reproductive capacity of allotriploids between *Rana tsushimensis* from Tsushima and *Rana japonica* from Ichinoseki and Hiroshima. Sci. Rep. Lab. Amphib. Biol. Hiroshima Univ. **12:** 133–175.

Tiersch, T. R., R. W. Chandler, S. S. Wachtel and S. Elias, 1989 Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry **10:** 706–710.

Uzzell, T., R. Günther and L. Berger, 1977 *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). Proc. Acad. Nat. Sci. Phila. **12:** 147–171.

Vasil'ev, V. P., K. D. Vasil'eva and A. G. Osinov, 1989 Evolution of a diploid-triploid-tetraploid complex in fishes of the genus *Cobitis* (Pisces, Cobitidae), pp. 153–169 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Vinogradov, A. E., L. J. Borkin, R. Günther and J. M. Rosanov, 1990 Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. Genome **33:** 619–627.

Vrijenhoek, R. C., R. M. Dawley, C. J. Cole and J. P. Bogart, 1989 A list of the known unisexual vertebrates, pp. 19–23 in *Evolution and Ecology of Unisexual Vertebrates*, edited by R. M. Dawley and J. P. Bogart. New York State Museum, Albany, NY.

Communicating editor: S. Yokoyama