Hybridogenetic Reproduction and Maternal Ancestry of Polyploid Iberian Fish: The *Tropidophoxinellus alburnoides* Complex

José A. Carmona,* Oris I. Sanjur,† Ignacio Doadrio,* Annie Machordom* and Robert C. Vrijenhoek†

**Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal, 2. 28006 Madrid, Spain and †Center for Theoretical and Applied Genetics, Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, New Jersey 08903-0231*

Manuscript received November 26, 1996
Accepted for publication April 1, 1997

ABSTRACT

Iberian minnows collectively known as the *Tropidophoxinellus alburnoides* STEINDACHNER complex comprise diploid and polyploid forms with highly female biased sex ratios. Previous investigators suggested that all-female clonal reproduction and interspecific hybridization may occur in this complex. We examined nuclear (allozymes) and cytoplasmic genes (mtDNA) to assess the evolutionary origins, relationships, and reproductive modes of *T. alburnoides* from western Spain. The multi-locus allozyme data clearly revealed the hybrid nature of all polyploid forms of this fish and some diploid forms as well. Diagnostic markers identified fish from the genus *Leuciscus* as the paternal ancestor of hybrids in the Duero and Guadiana River Basins. Additionally, analysis of nuclear markers revealed that hybridogenetic reproduction occurs in the diploid and triploid hybrids. The hybrids fully express the paternal Leuciscus genome and then discard it during oogenesis. Hybridogenetic ova contain only maternal nuclear genes and mtDNA from a non-hybrid *T. alburnoides* ancestor. Apparently diploid and triploid hybrids of *T. alburnoides* persist as sperm parasites on males of a sexually reproducing Leuciscus host species.

APPROXIMATELY 70 "unisexual" (*i.e.*, all-female) biotypes of fishes, amphibians, and reptiles are presently recognized (DAWLEY 1989; VRIJENHOEK *et al.* 1989). The majority (64%) of these biotypes are polyploid ($3n$ or $4n$), and essentially all appear to have arisen as interspecific or interracial hybrids. Sterility often associated with hybridization provides strong selection pressure for any oogenetic mechanism that rescues egg production and retains or restores diploidy (SCHULTZ 1969; WHITE 1978). Assuming such hybrids are viable and fertile, an oogenetic "rescue mechanism" will rapidly be fixed if demographic advantages also arise with all-female reproduction (MORITZ *et al.* 1989; VRIJENHOEK 1989). Thus, it is not surprising that several modes of clonal reproduction are found among unisexual vertebrates (parthenogenesis, gynogenesis, and hybridogenesis) and that the cytological mechanisms underlying these modes may be unrelated (reviewed by CUELLAR 1974; DAWLEY 1989). In some well-studied cases involving fish and lizards, polyploidization resulted from the addition of a third genome to a diploid asexual hybrid (DENSMORE *et al.* 1989; QUATTRO *et al.* 1992b). Additionally, several tri-hybrid unisexual triploids (*i.e.*, involving three species or races) are known (SCHULTZ 1977; TURNER *et al.* 1983; LOWCOCK *et al.* 1987; DESSAUER and COLE 1989). However, this scenario may not apply to the origin of many all-female insects for which spontaneous (*i.e.*, non-hybrid) parthenogenesis is documented (SUOMALAINEN *et al.* 1987).

Corresponding author: Robert C. Vrijenhoek, Center for Theoretical and Applied Genetics, Rutgers University, New Brunswick, NJ 08903-0231. E-mail: vrijen@ahab.rutgers.edu

Iberian minnows (Pisces: Cyprinidae) collectively known as the "*Tropidophoxinellus* (a.k.a. *Rutilus*) *alburnoides* (STEINDACHNER 1866) complex" comprise diploid ($2n = 50$) and triploid ($3n = 75$) forms (COLLARES-PEREIRA 1984; FERNÁNDEZ-DELGADO and HERRERA 1994; PERIS *et al.* 1994). As the triploids are essentially all female, a unisexual method of reproduction has been hypothesized but the mechanism is unknown (COLLARES-PEREIRA 1985). Members of the *T. alburnoides* complex hybridize with species in the genus Leuciscus (COLLARES-PEREIRA 1989) but the role of hybridization in the origin of triploid forms also remains unknown. If a non-hybrid origin is verifiable, the *T. alburnoides* complex would represent a highly unusual case for unisexual vertebrates.

The purpose of this study was to determine the evolutionary origins, relationships, and reproductive modes of diploid and polyploid forms of *T. alburnoides* from western Spain. We used multilocus allozymes and restriction fragment length polymorphism (RFLP) analysis of mitochondrial cytochrome *b* DNAs to examine diploid and polyploid members of the *T. alburnoides* complex. We also examined related Iberian species that might be involved in hybridization. The combination of allozymes and mtDNA provides a powerful tool for elucidating the origins and reproductive modes of unisexual organisms (AVISE *et al.* 1992). Allozymes allow detection of fixed heterozygosity at multiple loci, a condition that is typical of unisexual hybrids. Species-diagnostic markers typically are codominantly expressed in a unisexual hybrid and thereby help to identify the putative parental species involved in original hybridiza-

TABLE 1

Cyprinid samples from Spanish rivers

Basin, river[a]	Sample size[b]		
	Collected	Allo	RFLP
Duero Basin, Agueda River (Le Fregeneda, Salamanca)			
Tropidophoxinellus alburnoides[c]	233	132	15
Leuciscus carolitertii	31	31	5
Chondrostoma polylepis	31	31	5
Duero Basin, Turones River (Bouza, Salamanca)			
Rutilus lemmingii	20	20	5
Guadiana Basin, Estena River (Navas de Estena, Toledo)			
Tropidophoxinellus alburnoides[c]	41	31	15
Leuciscus cf. *pyrenaicus*	20	20	5
Rutilus lemmingii	18	18	5
Anaecypris hispanica	6	6	5

[a] Locality and district in parentheses.

[b] Numbers collected, used for allozyme studies, and for RFLP analysis.

[c] *T. alburnoides* complex (including putative hybrids and polyploids).

tion events. Diploid and triploid biotypes often can be distinguished by asymmetrical staining patterns of allozymes that reflect underlying dosages of the parental genomes (BALSANO *et al.* 1972; UZZELL *et al.* 1975; VRIJENHOEK 1975). mtDNA reveals the maternal ancestor of a hybrid lineage and becomes coupled to the nuclear genome with clonal inheritance. Together, allozymes and mtDNA are useful for estimating clonal diversity in unisexual populations that arise through multiple hybridization events or postformational mutations (BROWN and WRIGHT 1979; QUATTRO *et al.* 1991, 1992a).

MATERIALS AND METHODS

Specimens: We used electrofishing to collect minnows from several river systems in Spain (Table 1; Figure 1). The fish were transported alive to the laboratory (Madrid) and sex was determined upon dissection. We focused our sampling effort on fish from the Agueda River, a tributary of the Duero basin, located at the northern limit of the *T. alburnoides* complex. This river is characterized by relatively low diversity of freshwater fish species comprising several cyprinids (*T. alburnoides* complex, *Leuciscus carolitertii, Chondrostoma polylepis duriensis, Barbus bocagei,* and *Rutilus lemmingii*), a cobitid (*Cobitis paludica*) (DOADRIO *et al.* 1991; VELASCO 1994), and an introduced salmonid, *Salmo trutta.* Hybridization between members of the *T. alburnoides* complex and members of three other cyprinid genera (Leuciscus, Chondrostoma, and Rutilus) has been suspected (COLLARES-PEREIRA 1989). We examined genetic characters of representative Iberian species potentially involved in hybridization with members of the *T. alburnoides* complex.

Cytogenetics and cytometry: To determine ploidy, all *T. alburnoides* specimens were karyotyped. Four hours before sacrifice, each fish was injected intraperitoneally with 0.2 cc of 0.1% colchicine solution. Kidney and gill samples were removed, lightly minced, and placed for 30 min in 1 ml 0.075 M KCl. To isolate cells, the KCl suspension was spun for 20 min at 1400 rpm. The supernatant fluid was removed and the cells were fixed with Carnoy's ethanol-acetic mixture. The cells were carefully resuspended in fresh fixative and spun again, twice under the same conditions. The cell suspension was dropped from a height of 1 m onto ice-cold slides, air dried and stained with Giemsa 3%.

We used a Becton Dickinson FACS SORT flow cytometer to verify chromosome counts. DNA content of frozen heart cells was measured using the method of VINDELØV *et al.* (1982). We used human peripheral blood cells as a DNA standard and distinguished ploidy levels from histograms of fluorescence values. Discrete peaks of DNA content had coefficients of variation ranging from 1.5 to 5%.

Allozymes: We stored liver, skeletal muscle, and gonad tissues from each specimen at −70° before electrophoretic analyses. Individual tissues were homogenized in an equal volume of grinding buffer (0.01 M Tris, 0.0025 M EDTA, pH 7.0). Paper wicks dampened with tissue extracts were inserted in 12% starch gels (Sigma Chemical Co.). We used standard buffer recipes and histochemical staining protocols (PASTEUR *et al.* 1987) to screen 19 enzymatic systems encoded by 26 putative gene loci (Table 2). To identify species-diagnostic markers, we examined 66 individuals of the *T. alburnoides* complex from the Duero Basin and all other species for all 26 loci. We then examined 66 additional *T. alburnoides* from the Duero Basin for a subset of species-diagnostic markers (*sAAT-1*, PGDH** and *PGM**).

To test for hybridogenetic *vs.* gynogenetic modes of reproduction, we analyzed primary oocytes from three gravid diploid and 60 gravid triploid females for *sAAT-1*, PGDH** and *PGM**. Individual and grouped oocytes were crushed on paper wicks and loaded in starch gels.

Multiple loci encoding the same enzyme were designated as −1, −2, etc. following the recommendation of SHAKLEE *et al.* (1990). We used the BIOSYS-1 program (SWOFFORD and SELANDER 1981) to perform population genetic analyses and estimate diversity parameters with respect to allozymes.

RFLP analysis: A modified CTAB protocol (DOYLE *et al.* 1987) was used to extract whole genomic DNA from 1–3 cc of muscle tissue. A 1.2-kb fragment including the whole mitochondrial cytochrome *b* gene (cyt*b*) was generated by PCR. We used primers designed by SCHMIDT and GOLD (1993) for a North American cyprinid, *Lythrurus roseipinnis:* LA 5′-GTGACTTGAAAAACCACCGTTG-3′ and HA 5′-CAACGATCTCCGGTTTACAAGAC-3′. Cyt*b* was amplified in 50 μl reactions containing 2 μl of genomic DNA; 4 units of Taq Polymerase (Promega, Madison, WI); 5 μl of 10× buffer; 5 μl of $MgCl_2$ (0.025 mol L^{-1}, supplied with the polymerase); 5 μl of a C, T, A, G nucleotide mix (2 mmol L^{-1} for each nucleotide; Boehringer Mannheim, Indianapolis, IN); 28 μl sterile distilled water; and 2.5 μl each of the two primer stock solutions (10 μmol/liter). PCR involved three initial cycles at 95° for 60 sec (denaturation), 50° for 60 sec (annealing), and 72° for 90 sec (extension), followed by 25 cycles at 95° for 60 sec (denaturation), 55° for 60 sec (annealing), and 72° for 90 sec (extension). Ten microliters of the DNA product was used for restriction analysis with each of the following enzymes: *Ava*I, *Ava*II, *Bcl*I, *Bam*HI, *Bgl*II, *Bst*EII, *Bst*NI, *Eco*RI, *Eco*RV, *Hin*dIII, *Mlu*I, *Mse*I, *Nco*I, *Nde*I, *Nhe*I, *Pvu*II, *Sac*I, *Sac*II, and *Taq*I. Reactions were prepared following enzyme manufacturer's protocols.

Restriction fragment lengths were determined with GelReader v2.0 (NCSA Software Tools Group 1991). We used the program REAP v4.0 (MCELROY *et al.* 1992) to generate a

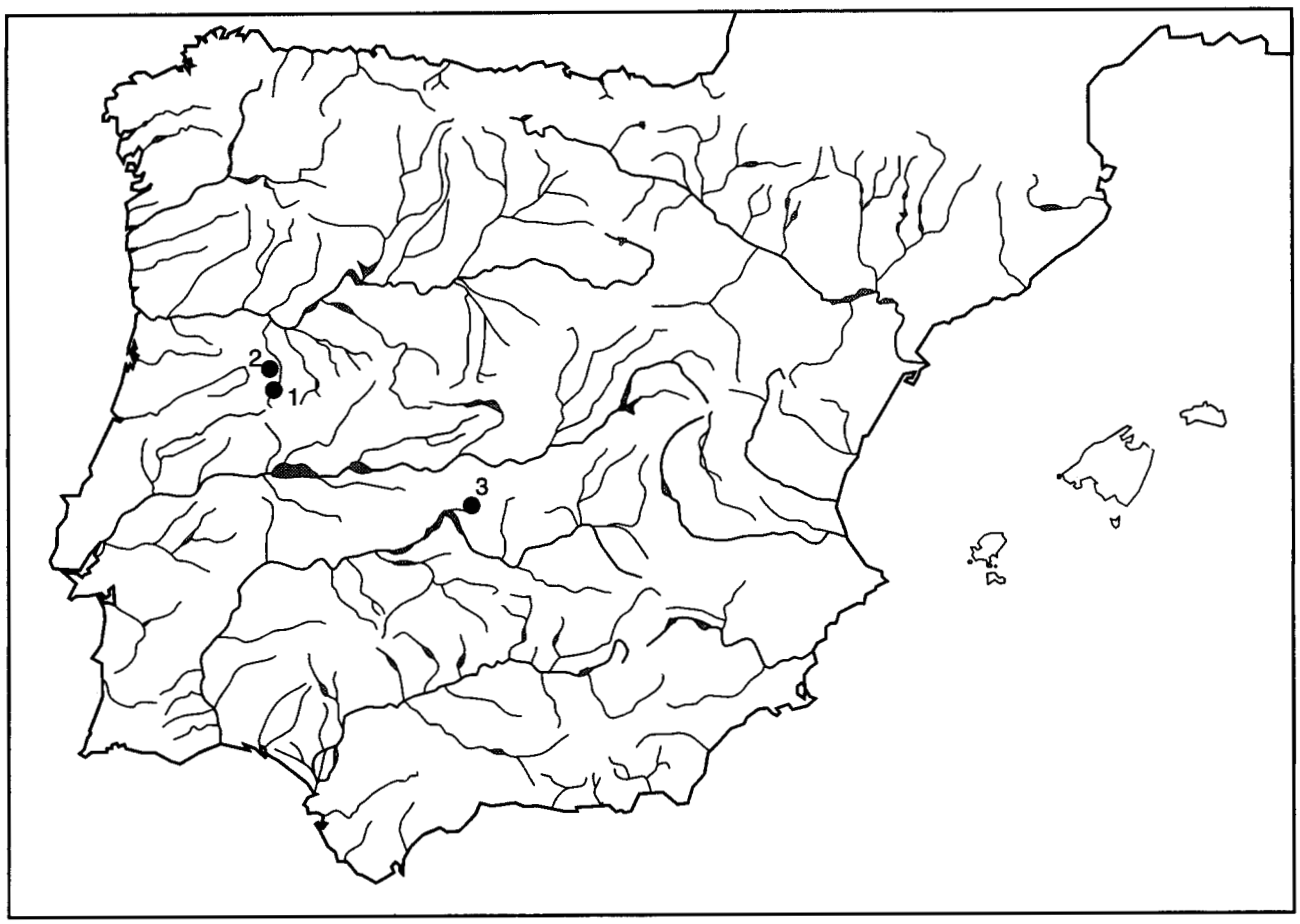

FIGURE 1.—Map of the Iberian Peninsula. Collection sites are as follows: (1) Agueda River (Duero basin), (2) Turones River (Duero basin) and (3) Estena River (Guadiana basin).

haplotype character-state matrix from the restriction fragment profiles, and then to generate a matrix of inferred nucleotide substitutions per site ($\hat{d}$) by the method of NEI and LI (1979). Cyt*b* genealogies were generated by two methods. We used MEGA v1.01 (KUMER *et al.* 1993) to generate a neighbor joining tree from the $\hat{d}$-value distance matrix, and PAUP v3.1 (SWOFFORD 1993) to generate a maximum parsimony tree from the original haplotype character-state matrix. *Chondrostoma polylepis* was used as an outgroup for these analyses.

RESULTS

***T. alburnoides* complex:** Morphological and cytogenetic criteria were used to sort members of the *T. alburnoides* complex by sex and ploidy level (Table 3). In the Duero Basin (Agueda River) sample, males were predominantly diploid, although some polyploids were found. Females were predominantly triploid. Three immature individuals also were triploid. Overall, triploids comprised 65.2% of the Duero Basin sample. Similarly, in the Guadiana Basin, triploids constituted 58.1% of the sample.

The percentage of *T. alburnoides* males from Duero Basin sample (18%) was higher than previously reported samples from the Agueda river (3.2%, PERIS *et al.* 1994). Males constituted 12% of the population in the South of the Iberian Peninsula (HERRERA 1991) and 24% males were found in Portugal (COLLARES-PEREIRA 1983). We also found 12% males in the Guadiana Basin sample. The low number of males found in some other studies may be affected by collection bias due to the smaller size of males. Unfortunately, the other studies did not determine whether *T. alburnoides* males were hybrids.

Multilocus (allozyme) heterozygosity was high in the sample of *T. alburnoides* from the Duero Basin (Table 4). Observed heterozygosity in a subsample of diploid fish (mostly males) greatly exceeded the heterozygosity expected under a Hardy-Weinberg model. This excess of heterozygotes resulted from fixed heterozygosity at three loci (*sAAT-1**, *PGDH**, and *PGM**). Triploid and tetraploid individuals from this river also were fixed heterozygotes at these loci (Table 5). Fixed multilocus heterozygosity is a characteristic of unisexual-hybrid vertebrates (VRIJENHOEK 1990). The present data clearly refute the hypothesis of Mendelian segregation and assortment in this population of *T. alburnoides*. With gene frequencies equal to $p = q = 0.5$ at each of the three fixed-heterozygous loci, the probability of drawing a single sexually produced individual heterozygous at all three loci is $(1/2)^3$, or 0.125, assuming the loci are unlinked. The probability of drawing 31 sexually pro-

TABLE 2

Enzymatic systems and electrophoretic conditions employed

Enzyme	EC no.	Locus	Tissue[a]	Buffer[b]
Aspartate aminotransferase	2.6.1.1	*sAAT-1**	M/L	A
		*mAAT-1**	M/L	A
Adenylate kinase	2.7.4.3	*AK**	M	A
Carbonate deshydratase	4.2.1.1	*CAH-1**	L	B
		*CAH-2**	L	B
Creatine kinase	2.7.3.2	*CK**	M	A
Esterase	3.1.1.-	*EST**	M	D
Fumarate hydratase	4.2.1.2	*FH**	M	D
Glyoxalasa-I	4.4.1.5	*GLO1**	M	B
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*GAPDH**	M	B
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-1**	M/L	C
		*GPI-2**	M	D
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.14	*IDHP-1**	M	A
		*IDHP-2**	L	A
		*IDHP-3**	L	A
Lactate dehydrogenase	1.1.1.27	*LDH-1**	M/L	C
		*LDH-2**	M/L	C
Malate dehydrogenase	1.1.1.37	*sMDH-1**	M	A
		*sMDH-2**	L	A
Malic enzyme (NAD^+)	1.1.1.38	*ME-1**	M	B
		*ME-2**	L	B
Manose-6-phosphate isomerase	5.3.1.8	*MPI**	L	A
Peptidase-B	3.4.11.4	*PEPB**	M	B
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH**	L	C
Phophoglucomutase	2.7.5.1	*PGM**	L	C
Superoxide dismutase	1.15.1.1	*SOD**	M/L	C

[a] M, skeletal muscle; L, liver.
[b] Buffers: A, Tris-citrate, pH 6.7; B, Tris-citrate, pH 8.0; C, Poulik, pH 8.6; D, Tris-malate-EDTA, pH 6.9.

duced, triple heterozygotes (out of a sample of 31 males) is vanishingly small, or $(0.125)^{31} \approx 10^{-28}$.

In contrast, the *T. alburnoides* sample from the Guadiana Basin included both hybrid and non-hybrid individuals. Twenty individuals were hybrids that all exhibited fixed heterozygous genotypes at three diagnostic loci (*sAAT-1**, *PGDH**, *PGM**). However, 11 non-hybrid individuals were homozygous at the same three loci and polymorphic at seven loci. Hardy-Weinberg analysis of the polymorphic loci revealed no significant deviations from random mating expectations (Fisher's exact test). Additionally, the mean heterozygosity observed in this subset of fish (Table 4, direct count) did not exceed that expected under random mating. Thus, a portion of this *T. alburnoides* complex comprises sexually reproducing diploid individuals.

TABLE 3

Sex and ploidy of *Tropidophoxinellus alburnoides* complex fish

Basin, river	Ploidy level[a] 2n	3n	4n	Total examined	Total collected
Duero Basin, Agueda River					
Males	31	3	3	37	42
Females	10	80	2	92	188
Juveniles	0	3	0	3	3
Total	41	86	5	132	233
Guadiana Basin, Estena River					
Males	4	1	0	5	5
Females	1	12	0	13	22
Juveniles	8	5	0	13	14
Total	13	18	0	31	41

[a] Numbers subjected to cytogenetic examination.

Hybrid origins of *T. alburnoides*: Allozyme variation was examined in five putative cyprinid species that might be considered candidates for hybridization with members of the *T. alburnoides* complex (Table 4). None of these samples deviated substantially from random mating expectations. In general, observed multilocus heterozygosity fit expected heterozygosity for each sample, and single locus genotypic proportions fit Hardy-Weinberg expectations. *Rutilus lemmingii* from the Guadiana Basin had higher genetic diversity than the sample of this species from the Duero Basin. Overall, Guadiana Basin populations appear to maintain higher levels of gene diversity than Duero Basin populations.

The 26 gene loci examined in this study were sorted into three categories (Table 5) according to their state in *T. alburnoides* hybrids from the Duero and Guadiana Basins: (1) nine loci were homozygous in all hybrids;

TABLE 4

Genetic variability expressed as the mean number of alleles per locus, polymorphism and heterozygosity of the taxa studied

Basin, river	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity	
				Direct count	Expected
Duero Basin, Agueda River					
T. alburnoides, diploids	25.0 ± 0.8	1.5 ± 0.1	26.9	0.151 ± 0.063	0.094 ± 0.034
L. carolitertii.	29.2 ± 1.1	1.1 ± 0.1	7.7	0.025 ± 0.015	0.026 ± 0.026
Ch. polylepis	25.7 ± 1.2	1.3 ± 0.1	11.5	0.010 ± 0.005	0.031 ± 0.012
Duero Basin, Turones River					
R. lemmingii	19.8 ± 0.1	1.0 ± 0.0	3.8	0.020 ± 0.020	0.015 ± 0.015
Guadiana Basin, Estena River					
T. alburnoides, nonhybrids	9.7 ± 0.3	1.3 ± 0.1	30.8	0.084 ± 0.032	0.092 ± 0.031
L. cf. *pyrenaicus*	19.3 ± 0.6	1.3 ± 0.1	15.4	0.042 ± 0.022	0.051 ± 0.022
R. lemmingii	16.2 ± 0.7	1.5 ± 0.1	26.9	0.067 ± 0.033	0.089 ± 0.032
A. hispanica	4.9 ± 0.1	1.4 ± 0.1	38.5	0.138 ± 0.046	0.137 ± 0.040

TABLE 5

Allozyme genotypes of *T. aburnoides* hybrids and alleles in possible progenitor species

	Duero Basin						Guadiana Basin					
	Genotypes of *T. alburnoides* hybrids			Alleles in non-hybrids[a]			Genotypes of *T. allb.* hybrids		Alleles in non-hybrids[a]			
Locus	2n	3n	4n	*L. car.*	*Ch. pol.*	*R. lem.*	2n	3n	*T. alb.*	*L.* cf. *pyr.*	*R. lem.*	*A. his.*
1. Homozygous loci in all *T. alburnoides* hybrids												
*CK**	aa	aaa	aaaa	a	a	B	aa	aaa	a	a	B	a
*GLO1**	aa	aaa	aaaa	a	a	a	aa	aaa	a	a	a	a
*IDHP-1**	aa	aaa	aaaa	a	a	a	aa	aaa	a	a	a	a
*LDH-1**	aa	aaa	aaaa	a	a	B	aa	aaa	a	a	a	a
*LDH-2**	aa	aaa	aaaa	a	B,C	B	aa	aaa	a	a	B	a
*sMDH-1**	aa	aaa	aaaa	a	a	a	aa	aaa	a	a	B	C
*ME-1**	aa	aaa	aaaa	a	a	a	aa	aaa	a	a	B	a
*PEPB**	aa	aaa	aaaa	a	E	E	aa	aaa	a	a	a,C	B,D
*SOD**	aa	aaa	aaaa	a	D	E	aa	aaa	a	a	E	B
2. Heterozygous loci in all *T. alburnoides* hybrids												
*sAAT-1**	ab	a-b[b]	abbb	a	b	b	ab	a-b[b]	b	a	b	b
*PGDH**	ac,ad	aac,acc,acd	accc,aadd	a	c	c	ac	aac,acc	c	a	c	b
*PGM**	ac	aac,acc	accc	a	a,c	c	ac	aac,acc	c	a,c	c,D	a,c
3. Polymorphic loci in *T. alburnoides* hybrids												
*mAAT-1**	aa	aaa	aaaa	a	a	a	ab	aaa	a	a	a	a
*AK**	aa,ac	aaa	aaaa	a	B	a	aa	aaa	a	a	a	a
*CAH-1**	aa,ad	aaa	aaaa	a	a	Null	aa,ae	aaa,aac,aae	a,e	a,c	e	a
*CAH-2**	aa,ac	aaa	aaaa	a,c	a	a	aa	aaa	a,D	a	a,B	a,D,E
*EST**	aa,ab	aaa	aaaa	a	C	a	aa	aaa,ddd,a-d[2]	a,d	a,b,d	a,d	a,d
*FH**	aa	aaa	aaaa	a	a,b	a	aa	aaa,aab	a	a	a	a
*GAPDH**	aa	aaa	aaaa	a	a	a	aa	aaa,aac	a	a,c	a,b,c	a
*GPI-1**	aa	aaa	aaaa	a	a	B,D	aa	aaa,aac	a	a	B,D	a,c
*GPI-2**	aa,ab,ac	aaa,aac	aaaa	a	a,c	a	aa	aaa,aab,abb	a,b	a	a	a,b
*IDHP-2**	aa,ac	aaa,aac	aaaa	a	D	B	aa	aaa,aae	a,e	a	a	a,c
*IDHP-3**	aa	aaa	aaaa	a	D	B	aa	aaa,aae	a,e	a	a	C
*sMDH-2**	aa,ab	aaa,aab,abb	aaaa	a	a,b	b	ab	aab,abb	b	a	a	a,b
*ME-2**	aa,ab	aaa,a-b[b]	aaaa,a-b[b]	a,b	a,b	a	aa,ab	abb,bbb	a,b	a,b	a,b	a,b
*MPI**	aa	aaa,aac	aaaa	a,c	a,b	a	aa	aaa,aac	a	a,c	a,b	a,c

[a] Capital letters represent species-diagnostic alleles.
[b] Symmetry of patterns did not allow inference of gene dosage.

(2) three loci were heterozygous in all hybrids; and (3) 14 loci were variable among the hybrids. The homozygous loci were used to exclude several species as possible progenitors of hybrid *T. alburnoides*. Species-diagnostic alleles (capital letters in Table 5) allowed us to exclude *Ch. polylepis, R. lemmingii,* and *A. hispanica* as possible progenitors of hybrids in both river basins. For example, alleles at three loci (*LDH-2*, PEPB*,* and *SOD**) in *Ch. polylepis* were not found in the hybrids. Similarly, diagnostic alleles at several loci allowed us to exclude *R. lemmingii* and *A. hispanica* as hybrid progenitors. However, *L. carolitertii* could not be excluded as a parent of Duero Basin hybrids, and similarly, non-hybrid *T. alburnoides* and *L.* cf. *pyrenaicus* could not be excluded as parents of the Guadiana Basin hybrids.

The three loci (*sAAT-1*, PGDH*,* and *PGM**) with fixed heterozygosity also were used to assess parentage of the hybrids. As before, the alleles found in non-hybrid *T. alburnoides* and *L.* cf. *pyrenaicus* were consistent with these species having been the parents of Guadiana Basin hybrids. Also, the alleles found at these three loci in *L. carolitertii* were consistent with it having been one of the parents of Duero Basin hybrids. Although the other parent was not identified in the present Duero Basin samples, these heterozygous genotypes also suggest that a non-hybrid lineage of *T. alburnoides,* similar to sexual lineage in the Guadiana Basin, participated in these hybridizations.

Genotypes at 14 loci were polymorphic among the hybrid forms of *T. alburnoides*. Polymorphic alleles found at six loci (*CAH-1*, EST*, GPI-2*, IDHP-2, IDHP-3*,* and *ME-2**) in non-hybrid *T. alburnoides* from the Guadiana Basin also occurred in different hybrids. Also, polymorphic alleles at six loci (*CAH-1*, EST*, GAPDH*, ME-2*, MPI*,* and *PGM**) in *L.* cf. *pyrenaicus* often differentiated diploid and triploid hybrid forms from this river. However, some hybrids carried alleles (*e.g., mAAT-1*b, FH*b,* and *GPI-1*c*) that were not observed in non-hybrid *T. alburnoides* or *L.* cf. *pyrenaicus*. Thus, our present identification of the putative progenitors of the Guadiana Basin hybrids should be considered tentative until larger sample sizes are obtained and additional populations are investigated.

Polymorphisms were found at nine loci in the Duero Basin hybrids. Alleles at three of these loci (*CAH-2*, ME-2*,* and *MPI**) also were polymorphic in the local *L. carolitertii* population. However, we could not identify sources of polymorphism for the other six loci. Also, some $3n$ individuals from this sample were heterozygous for three alleles at *PGDH** locus. Perhaps these polymorphic alleles reside in a non-hybrid *T. alburnoides* population that remains to be discovered in the Duero Basin or other rivers of the Iberian Peninsula.

Gene dosage patterns: Genotypes of $3n$ and $4n$ heterozygotes could be inferred from gene dosage patterns recognizable in the staining intensities of electromorphs. For example, *MDH-2** heterozygotes in triploids were inferred to represent the genotypes **aab* or **abb* based on dosage patterns (Figure 2). Two doses of the A subunit and one dose of the B subunit would randomly polymerize into functional dimers according to the following expectation: the binomial $(2A + B)^2$ expands to 4AA:4AB:1BB. Likewise, the **abb* genotype would produce a 1AA:4AB:4BB ratio of bands. Exceptions were *ME-2*, AAT-1*,* and *EST** for which resolution of zymograms did not permit inferences about the gene dosage (Table 5).

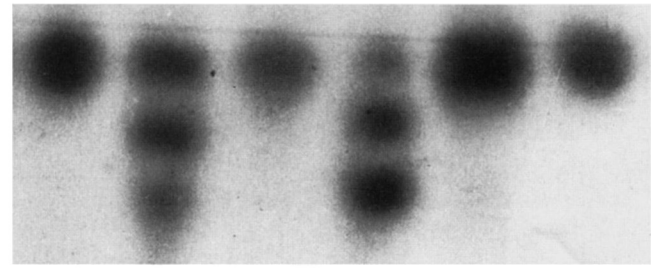

FIGURE 2.—Gene dosage patterns in triploids. Dimeric *MDH-2** genotypes of homozygous and heterozygous triploid hybrids of *T. alburnoides*.

Reproductive modes: Three unisexual modes of reproduction found among vertebrates were considered: parthenogenesis, gynogenesis, and hybridogenesis. Parthenogenesis and gynogenesis are strictly clonal modes of reproduction that faithfully replicate the maternal (diploid or triploid, etc.) genotype. However, gynogenetic females require sperm from males of a sexually reproducing host species to activate embryogenesis of their unreduced ova. Without laboratory rearing experiments, we could not discriminate between these two clonal modes of reproduction.

In contrast, hybridogenesis is a hemiclonal mode of reproduction that delivers only one genome of a hybrid to the functional oocytes (SCHULTZ 1969; CIMINO 1972). For example, an *MP* hybrid would produce haploid eggs containing only the maternal *M* genome. The *P* genome is replaced in each generation by mating with males of sexual species *P*. If loss of the *P* genome occurs before oogenesis, the *P* gene products should not be expressed in ova of a hybridogenetic female. If *P* genomes are lost during meiosis-I, however, *P* gene products may be expressed. Hybridogenetic females of the European waterfrog, *Rana esculenta,* express only maternal allozymes in the eggs, an indication of premeiotic exclusion of the paternal genome (GRAF and POLLS-PELAZ 1989). Premeiotic exclusion also occurs in hybridogenetic Poeciliopsis (CIMINO 1972). In contrast, clonal reproduction in parthenogens and gynogens is expected to result in diploid expression of both parental alleles.

Allozyme patterns of mature primary oocytes from *T. alburnoides* were examined to test these predictions. Of the three fixed heterozygous loci in Duero Basin hy-

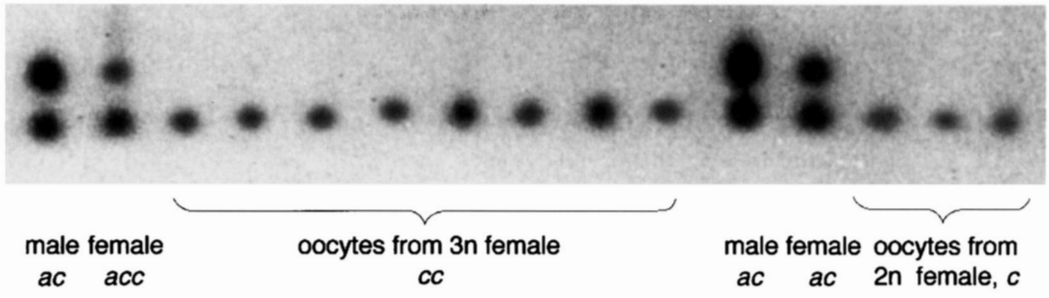

FIGURE 3.—Hybridogenesis demonstrated in *PGM** phenotypes of primary oocytes. Lanes 1 and 2, and 11 and 12 show heterozygous patterns expressed in adult liver tissues from diploid (genotype **ac*) and triploid (**acc*) hybrids. Oocytes from triploid (lanes 3–10) and diploid (lanes 13–15) *T. alburnoides* females expressed only the *T. alburnoides PGM*c* allele. The *PGM*a* allele (presumably from *L. carolitertii*) was not expressed.

brids, *sAAT-1** was not expressed in oocytes. Oocytes from diploid and triploid females expressed only the *PGM*c* allele, although liver tissues from these hybrids expressed heterozygous patterns, *ac* and *acc*, respectively (Figure 3). Similarly, although not illustrated, oocytes from these diploid and triploid females expressed only the *PGDH*c* allele, although muscle tissues from these hybrids were heterozygous, *ac* and *acc*, respectively. For both loci, the **a* allozyme was not expressed in the oocytes of hybrids, while the **c* allozyme was. Apparently, these hybrids transmitted only one of the parental genomes (lets call it the *M* genome) to mature ova, while the alternative genome (*P*) was excluded from oogenesis before meiosis-I. Diploid females produced reduced ova with one *M* genome, and triploid females produced reduced ova that probably had two *M* genomes. Based on the mitochondrial DNA information (below), the clonally transmitted *M* genome probably derived from a non-hybrid *T. alburnoides* lineage.

The substitutable *P* genomes of Duero Basin hybrids probably derived from local *L. carolitertii*. A characteristic of hybridogenesis that distinguishes this reproductive mode from gynogenesis is the expression of paternally derived allelic polymorphism in the hybrids (VRIJENHOEK *et al.* 1977). Although sample sizes were small in the present study, polymorphic alleles at three loci in *L. carolitertii* (*CAH-2**, *E-2**, and *MPI**) also appeared in the hybrids (Table 5). Evidence for paternal substitution was reinforced by inspection of *T. alburnoides* hybrids from the Guadiana basin. Polymorphisms at six loci in the local *L. cf. pyrenaicus* population also appeared in various diploid and triploid hybrids.

The Guadiana Basin hybrids also varied for alleles that segregated in the local population of non-hybrid *T. alburnoides* (Table 5). Multiple origins of these hybrid lineages may explain this variation if non-hybrid *T. alburnoides* were the maternal ancestors of clonally inherited *M* genomes. The presence of both *T. alburnoides* and *L. cf. pyrenaicus* in the Guadiana provides opportunities for recurrent synthesis of new hybridogenetic lineages.

Mitochondrial cyt*b* analysis: Because it is cytoplasmically inherited, mitochondrial DNA can be used to identify the maternal ancestor of non-recombinant hybrid lineages (reviewed in AVISE *et al.* 1992). Of the 19 restriction enzymes examined, 10 were polymorphic among the present samples and indicative of six distinct haplotypes (Table 6). All 2*n*, 3*n*, and 4*n* *T. alburnoides* from the Duero Basin had haplotype *I* (hereafter, hap-I). *Tropidophoxinellus alburnoides* from the Guadiana Basin had two related haplotypes (haps-II and -III) that differed respectively from hap-I for *Bst*NI (hap-II, Figure 4) and *Ava*II restriction patterns (hap-III). *Leuciscus* cf. *pyrenaicus* from the Guadiana Basin also had hap-II.

Distinct mitochondrial haplotypes occurred in *L. carolitertii* and *Ch. polylepis* from the Duero Basin. *Leuciscus carolitertii* had hap-IV, which differed from *T. alburnoides* for restriction pattern generated by six enzymes. *Chondrostoma polylepis* was polymorphic for hap-V and hap-VI, which differed from *T. alburnoides* for eight restriction enzymes. Percentage sequence divergence between these haplotypes (Table 7) was estimated by the fragment method of NEI and LI (1979). Members of the *T. alburnoides* complex and *L.* cf. *pyrenaicus* exhibited little sequence divergence (0.503–1.449%). However, *L. carolitertii* was divergent from *T. alburnoides* (3.145–3.682%), and *Ch. polylepis* haplotypes were most divergent (5.664–7.491%). This placement of *Ch. polylepis* is consistent with the treatment of HOWES (1991): Chondrostoma belongs to the Abramin lineage and Tropidophoxinellus and Leuciscus belong to the Leuciscus assemblage.

RFLP analysis of mitochondrial cyt*b* patterns in *R. lemmingii* and *A. hispanica* produced highly divergent patterns for most restriction enzymes. Preliminary sequence analysis of this gene confirms the high degree of divergence of the two species. Because estimates of sequence divergence from the RFLP fragments were likely to be unreliable, we did not include the two species in subsequent phylogenetic analyses.

Based on taxonomic and the present molecular criteria, we chose *Ch. polylepis* as an outgroup for phylogenetic analyses. Maximum parsimony (MP) and neighbor joining (NJ) trees (based on character-state and distance matrices, respectively) exhibited the same topology of cyt*b* haplotypes (Figure 5). All members (2*n*, 3*n*, and 4*n*) of the *T. alburnoides* complex and *L. cf.*

TABLE 6

Diagnostic restriction fragments expressed as binary characters (0 = absent; 1 = present)

Haplotype	*Ava*II abcde	*Bam*HI efg	*Bst*EII hij	*Bst*NI klmnopqr	*Eco*RI stu	*Eco*RV vwxyz	*Mse*I ABCDEF	*Nco*I HIJ	*Nhe*I KLM	*Taq*I NOPQRS
I	01001	110	001	00010010	001	00001	011100	110	001	011010
II	01001	110	001	10111000	001	00001	011100	001	001	011010
III	11010	110	001	10111000	001	00001	011100	001	001	011010
IV	11010	001	001	10010100	110	00001	110010	110	110	101001
V	00110	001	110	01000001	110	10010	000001	001	001	000101
VI	00110	001	110	01000001	110	11100	000001	001	001	000101

pyrenaicus clustered tightly (99% bootstrap value in MP analysis). *L. carolitertii* was basal to this group.

DISCUSSION

Our aim was to determine evolutionary origins, relationships, and reproductive modes of diploid and polyploid forms of *T. alburnoides* complex fish from the Iberian Peninsula. The genetic data clearly revealed the hybrid nature of most diploid and all polyploid *T. alburnoides* individuals in the present samples. Allozyme analyses revealed that diploid and polyploid *T. alburnoides* from the Duero Basin all were hybrids that contained a (non-hybrid) *T. alburnoides*-like genome plus a genome from *L. carolitertii*. mtDNA analysis also revealed that a *T. alburnoides*-like ancestor provided the mitochondrial genome of these hybrids. However, the putative *T. alburnoides*-like (non-hybrid) maternal ancestor was not identified in the present sample from the Duero Basin (Agueda River). In contrast, *T. alburnoides* hybrids from the Guadiana Basin probably involved local non-hybrid *T. alburnoides* as one of the parental forms and *L.* cf. *pyrenaicus* as the other.

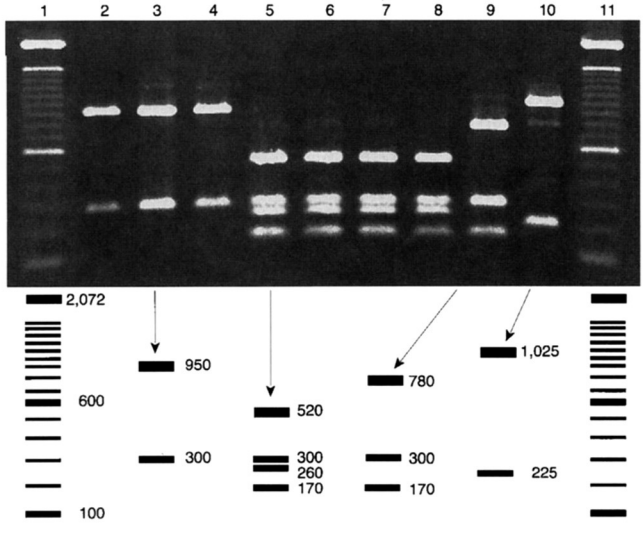

FIGURE 4.—RFLP analysis of mitochondrial cyt*b* with restriction enzyme *Bst*NI. (Top) Lanes 1 and 11 include a 100-bp ladder; lanes 2–4, hybrid *T. alburnoides* (2*n*, 3*n*, 4*n*, respectively from the Duero Basin); lane 5, a non-hybrid *T. alburnoides* (Guadiana Basin); lanes 6 and 7, hybrid *T. alburnoides* (Guadiana Basin); lane 8, *L. pyrenaicus* (Guadiana Basin); lane 9, *L. carolitertii* (Duero Basin); lane 10, *Ch. polylepis* (Duero Basin). (Bottom) Schematic of fragments sizes from top.

Unfortunately, fish of the *T. alburnoides* complex are not easy to breed under laboratory conditions, so direct tests of the breeding system have not been possible. Nevertheless, allozyme analysis of individuals from the Duero Basin sample suggests that hybridogenetic reproduction occurs in both the diploid and triploid hybrids. Hemizygous expression of allozymes in mature ova produced by the hybrid females suggested that the paternal *L. carolitertii* genome was excluded before meiosis. Premeiotic exclusion of the paternal genome precludes the expression of paternal genes in the maturing oocytes of other hybridogenetic systems, *e.g., Rana esculenta* (GRAF and POLLS-PELAZ 1989). Cytogenetic investigation of hybridogenetic Poeciliopsis directly demonstrated exclusion of one of the parental genomes before oogenesis and Meiosis-I (CIMINO 1972).

The hybridogenetic reproductive mode of Duero Basin *T. alburnoides* discards the paternal *L. carolitertii* genome and produces haploid or diploid *T. alburnoides*-like ova depending, respectively, on whether the female is diploid or triploid. Although most hybridogens are diploid (*e.g.,* in Poeciliopsis), precedents for triploid hybridogenesis occur in salamanders of the genus Ambystoma, fish of the genus Phoxinus, and the waterfrog *R. esculenta* (BERGER 1973; BOGART *et al.* 1985; GODDARD *et al.* 1989; GRAF and POLLS-PELAZ 1989).

Hybridogenetic reproduction in *T. alburnoides* females requires the availability of a genetically suitable paternal host from the genus Leuciscus. In the Duero Basin, *L. carolitertii* served as the paternal host, and in the Guadiana Basin, *L.* cf. *pyrenaicus* served as the paternal host. Evolutionary relationships between *L. carolitertii* and *L.* cf. *pyrenaicus* should be examined in more detail, however. A previous allozyme analysis of putative *L. pyrenaicus* from the Guadiana Basin suggested that this population should be considered conspecific with *L. carolitertii* (COELHO *et al.* 1995). Our present allozyme data are consistent with that interpretation, but the mtDNA data may not be. The mtDNA of Guadiana Basin *L.* cf. *pyrenaicus* did not differ from that of local *T. alburnoides*. The possibility exists that Guadiana Basin *L.*

TABLE 7

Percentage sequence divergence ($\hat{d} \times 100$) between *T. alburnoides* complex *cytb* haplotypes

	I	II	III	IV	V
II	0.953	—			
III	1.449	0.503	—		
IV	3.292	3.682	3.145	—	
V	7.454	7.334	5.664	4.314	—
VI	7.491	7.357	5.718	4.387	0.345

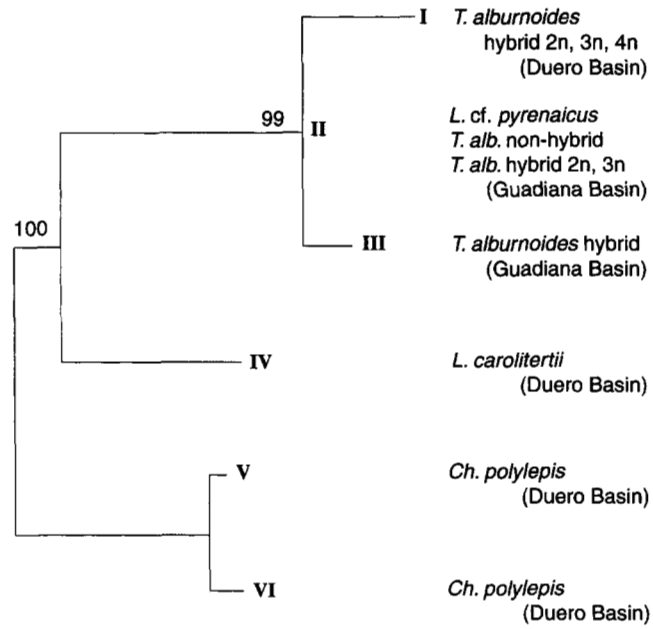

FIGURE 5.—Maximum parsimony tree of cyt*b* haplotypes. The numbers along the internodal branches are bootstrap values from 1000 replications of the MP analysis. The NJ tree showed the same topology.

cf. *pyrenaicus* are reticulate forms. Alternatively, lineage sorting of ancestral mtDNA polymorphisms (see AVISE *et al.* 1990) may explain these apparently contradictory results. Nevertheless, this problem warrants further investigation.

The incorporation and expression of paternal Leuciscus genes may benefit the hybrids in two ways. First, paternal expression may increase similarity of the hybrids to Leuciscus females and thereby increase the likelihood of hybrid matings (see LIMA *et al.* 1996). Second, paternal expression may help the hybrids adapt to local conditions for which the paternal species are already well adapted. For example, paternal variation contributes to thermal adaptation in hybridogenetic Poeciliopsis (BULGER and SCHULTZ 1982). Hybridogenetic forms of Poeciliopsis can use three different paternal hosts, *P. lucida, P. occidentalis,* and *P. latidens* depending on the river system (SCHULTZ 1977). It is likely that broader surveys of *T. alburnoides* complex fishes may reveal other paternal hosts, as well.

The high number of *T. alburnoides* males found in the Agueda population should not be considered indicative of sexual reproduction. All males found in the Agueda were identified as hybrids. Such males might result from *L. carolitertii* sperm that carried male-determining genes, as suggested for males of the gynogenetic cyprinid *Phoxinus eos-neogaeus* (GODDARD *et al.* 1989). Hybrid males found in the present study were mostly diploid. They may have been hybridogenetic, as males of hybridogenetic *R. esculenta* are fertile and not uncommon (GRAF and POLLS-PELAZ 1989). However, the presence of hybridogenetic males may have significant genetic consequences. Matings of hybridogenetic *M*P* females to *M*P* males (* marks the hemiclonal genome) results in production of *MM* progeny. Thus, non-hybrid, sexual diploids of *T. alburnoides* may be regenerated in this way. However, the *MM* progeny of hybridogenetic females may have low fitness due to the expression of deleterious mutations that accumulated in sheltered hemiclonal genomes. Considerable mutational load has been demonstrated in other hybridogenetic systems (LESLIE and VRIJENHOEK 1978; GRAF and POLLS-PELAZ 1989).

Relatively uncommon tetraploid hybrids of *T. alburnoides* may have resulted from triploid hybrid intermediates that produced unreduced triploid oocytes. However, this phenomenon does not appear to be common in the Duero Basin and it may not occur in the Guadiana Basin. We do not know if the tetraploid males and females were fertile.

As in Poeciliopsis, clonally reproducing forms of *T. alburnoides* may share a propensity for multiple hybrid origins. Allozyme and mtDNA analyses of Poeciliopsis hybridogens clearly identified discrete hemiclonal lineages that arose independently both within and among river systems (VRIJENHOEK *et al.* 1977, 1978; QUATTRO *et al.* 1991). Similarly, *T. alburnoides* hybrids from the Duero and Guadiana Basins differed with respect to mitochondrial genotypes. Diploid non-hybrid individuals of *T. alburnoides* with mtDNA haplotype II occurred in the Guadiana Basin; an endemic origin of the hybridogenetic lineage bearing this haplotype is likely. This hypothesis needs to be investigated with additional nuclear markers. In contrast, hybrids bearing haplotype I (Duero Basin) and haplotype III (Guadiana Basin) were not identified in corresponding sexual lineages. These haplotypes may represent "orphan" genotypes (TURNER *et al.* 1983) that once existed in local populations of the maternal ancestor but now are extinct. Alternatively, these haplotypes may still exist in sexual lineages that are rare or unsampled. Similarly, Guadiana Basin hybrids expressed several allozymes that were not found in either of the putative sexual progenitors, although samples were small. A thorough allozyme and mtDNA analysis of other rivers containing *T. alburnoides*-like fishes in the Iberian Peninsula and other locations in Europe is warranted.

A significant consequence of the present study was the recognition that the alpha-level taxonomy of the *T. alburnoides* complex and the larger Leuciscine assemblage needs thorough reevaluation. Morphology and cytology of this highly reticulate group has not identified clear species boundaries or higher level relationships (COLLARES-PEREIRA 1989; HOWES 1991). Multilocus allozymes provided some resolution in the present analysis, but overall these markers were not highly divergent. RFLP analysis of the cyt*b* gene provided clearer resolution of differences among *T. alburnoides, L. carolitertii,* and *Ch. polylepis.* Additional mitochondrial sequences may prove to be a powerful tool in categorizing this difficult group. Additionally, nuclear DNA markers that are not as conservative as allozymes would be useful for clearly identifying hybrids and their progenitors.

We thank JOHN GOLD for suggesting and sharing his cyt*b* primers with us. ROBERT DAWLEY kindly taught us flow cytometric methods. We thank JOHN AVISE and an anonymous reviewer for their helpful comments and criticisms. This is New Jersey Agricultural Experiment Station Publication No. D-32104-3-97, supported by state funds and National Science Foundation grants OCE-9302205 and OCE-9633131.

LITERATURE CITED

AVISE, J. C., and R. M. BALL, JR., 1990 Principles of genealogical concordance in species concepts and biological taxonomy. Oxford Surv. Evol. Biol. **7:** 45–67.

AVISE, J. C., J. M. QUATTRO and R. C. VRIJENHOEK, 1992 Molecular clones within organismal clones. Evol. Biol. **26:** 225–246.

BALSANO, J. S., R. M. DARNELL and P. ABRAMOFF, 1972 Electrophoretical evidence of triploidy associated with populations of the gynogenetic teleost *Poecilia formosa.* Copeia **2:** 292–297.

BERGER, L., 1973 Systematics and hybridization in European green frogs of *Rana esculenta* complex. J. Herpetol. **7:** 1–10.

BOGART, J. P., L. E. LICHT, M. J. OLDHAM and S. J. DARBYSHIRE, 1985 Electrophoretic identification of *Ambystoma laterale* and *Ambystoma texanum* as well as their diploid and triploid interspecific hybrid (*Amphibia: Caudata*) on Pelee Island, Ontario. Can. J. Zool. **63:** 340–347.

BROWN, W. M., and J. W. WRIGHT, 1979 Mitochondrial DNA analysis and the origin and relative age of parthenogenetic lizards (genus *Cnemidophorus*). Science **203:** 1247–1249.

BULGER, A. J., and R. J. SCHULTZ, 1982 Origins of thermal adaptation in northern vs southern populations of a unisexual hybrid fish. Evolution **36:** 1041–1050.

CIMINO, M. C., 1972 Egg production, polyploidization and evolution in a diploid all-female fish of the genus *Poeciliopsis.* Evolution **26:** 294–306.

COELHO, M. M., R. M. BRITO, T. R. PACHECO, D. FIGUEIREDO and A. M. PIRES, 1995 Genetic variation and divergence of *L. pyrenaicus* and *L. carolitertii* (Pisces, Cyprinidae). J. Fish Biol. **47:** 243–258.

COLLARES-PEREIRA, M. J., 1983 Estudo sistematico e citogenetico dos pequenos ciprinideos ibericos pertenecentes aos generos *Chondrostoma* (AGASSIZ 1835), *Rutilus* (RAFINESQUE 1820), and *Anaecypris* (COLLARES-PEREIRA 1983) Ph.D. Thesis, University of Lisbon, Lisbon.

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:** 69–90.

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. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany, NY.

CUELLAR, O., 1974 On the origin of parthenogenesis in vertebrates: the cytogenetic factors. Am. Nat. **108:** 625–648.

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. Bulletin 466. New York State Museum, Albany.

DENSMORE, L. D. III, C. C. MORITZ, J. W. WRIGHT and W. M. BROWN, 1989 Mitochondrial—DNA analyses and the origin and relative age of parthenogenetic lizards (genus *Cnemidophorus*). IV. Nine *sexlineatus*-group unisexuals. Evolution **43:** 969–983.

DESSAUER, H. C., and C. J. COLE, 1989 Diversity between and within nominal forms of unisexual teiid lizards, pp. 49–71 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

DOADRIO, I., B. ELVIRA and Y. BERNAT (Editors), 1991 *Peces Continentales Españoles. Inventario y Clasificación de Zonas Fluviales.* Colección Técnica, ICONA, Madrid.

DOYLE, J. J., and E. DICKSON, 1987 Preservation of plant samples for DNA restriction endonuclease analysis. Taxon **36:** 715–722.

FERNÁNDEZ-DELGADO, C., and M. HERRERA, 1994 Population structure, growth and reproduction of *Tropidophoxinellus alburnoides* (STEINDACHNER 1866) in an intermittent stream of the Guadalquivir River basin (southern Spain). Arch. Hydrobiol. **130:** 359–369.

GODDARD, K. A., R. M. DAWLEY and T. E. DOWLING, 1989 Origin and genetic relationships of diploid, triploid, and diploid-triploid mosaic biotypes in the *Phoxinus eos-neogaeus* unisexual complex, pp. 268–280 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

GRAF, J. D., and M. POLLS-PELAZ, 1989 Evolutionary genetics of the *Rana esculenta* hybrid complex, pp. 289–302 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

HERRERA, M., 1991 Estrategias en los ciclos de vida de una comunidad de ciprínidos en un arroyo de primer orden en la cuenca del Río Guadalquivir, Ph.D. Thesis, Universidad de Córdoba, Córdoba.

HOWES, G. J., 1991 Systematics and biogeography: an overview, pp. 1–33 in *Cyprinid Fishes: Sytematics, Biology, and Exploitation,* edited by I. J. WINFIELD and J. S. NELSON. Chapman & Hall, London.

KUMER, S., K. TAMURA and M. NEI, 1993 MEGA: molecular evolutionary genetics analysis, version 1.0. Distributed by The Pennsylvania State University, University Park.

LESLIE, J. F., and R. C. VRIJENHOEK, 1978 Genetic dissection of clonally inherited genomes of *Poeciliopsis.* I. Linkage analysis and preliminary assessment of deleterious gene loads. Genetics **90:** 801–811.

LIMA, N. R. W., C. J. KOBAK and R. C. VRIJENHOEK, 1996 Evolution of sexual mimicry in sperm-dependent clonal forms of *Poeciliopsis* (Pisces: Poeciliidae). J. Evol. Biol **9:** 185–203.

LOWCOCK, L. A., L. E. LICHT and J. P. BOGART, 1987 Nomenclature in hybrid complexes of *Ambystoma* (Urodela: Ambystomatidae): no case for the erection of hybrid "species." Syst. Zool. **36:** 328–336.

MCELROY, D., P. MORAN, E. BERMINGHAM and I. KORNFIELD, 1992 REAP: an integrated environment for the manipulation and phylogenetic analysis of restriction data. J. Hered. **83:** 157–158.

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* (Gekonidae), pp. 87–112 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

NCSA Software Tools Group, 1991 NCSA GelReader for the Macintosh. University of Illinois, Urbana-Champaign.

NEI, M., and W.-H. LI., 1979 Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76:** 5269–5273.

PASTEUR, N., G. PASTEUR, F. BONHOMME, J. CATALAN and J. BRITTON-DAVIDIAN, 1987 *Manuel Technique de Génétique par Électrophorèse des Protéines: Collection Technique et Documentation.* Lavoisier, Paris.

PERIS, S. J., A. MARTÍN and J. C. VELASCO, 1994 Age, growth and reproduction of the cyprinid *Tropidophoxinellus alburnoides* (STE-

PHANIDIS 1974) in River Agueda, West-Central Spain. Arch. Hydrobiol. **131**: 341–352.

QUATTRO, J. M., J. C. AVISE and R. C. VRIJENHOEK, 1991 Molecular evidence for multiple origins of hybridogenetic fish clones (Poeciliidae: *Poeciliopsis*). Genetics **127:** 391–398.

QUATTRO, J. M., J. C. AVISE and R. C. VRIJENHOEK, 1992a An ancient clonal lineage in the fish genus *Poeciliopsis* (Atheriniformes: Poeciliidae). Proc. Natl. Acad. Sci. USA **89:** 348–352.

QUATTRO, J. M., J. C. AVISE and R. C. VRIJENHOEK, 1992b Mode of origin and sources of genotypic diversity in triploid fish clones (*Poeciliopsis:* Poeciliidae). Genetics **130:** 621–628.

SCHMIDT, T. R., and J. R. GOLD, 1993 Complete sequence of the mitochondrial cytochrome *b* gene in the cherryfin shiner, *Lythrurus roseipinnis* (Teleostei: Cyprinidae). Copeia **3:** 880–883.

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 ecology of unisexual fishes. Evol. Biol. **10:** 277–331.

SHAKLEE, J. B., F. W. ALLENDORF, D. C. MORIZOT and G. S. WHITT, 1990 Gene nomenclature for protein-coding loci in fish. Trans. Amer. Fish. Soc. **119:** 2–15.

STEINDACHNER, F. H. (1866) Ichthyologischer Bericht über eine nach Spanien und Portugal unternommene Reise (Dritte Forsetzung). Sitzungsb. der Kais Akad. der Wissenschaften **54:** 261–272.

SUOMALAINEN, E., A. SAURA and J. LOKKI, 1987 *Cytology and Evolution in Parthenogenesis.* CRC Press, Boca Raton, FL.

SWOFFORD, D. L., 1993 *PAUP: Phylogenetic analysis using parsimony, version 3.1.* Formerly distributed by Illinois Natural History Survey, Champaign, IL.

SWOFFORD, D. L., and R. K. SELANDER, 1981 BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Hered. **72:** 281–283.

TURNER, B. J., J. S. BALSANO, P. J. MONACO and E. M. RASCH, 1983 Clonal diversity and evolutionary dynamics in a diploid-triploid breeding complex of unisexual fishes (*Poecilia*). Evolution **37:** 798–809.

UZZELL, T., L. BERGER and GÜNTHER, 1975 Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia, Salientia). Proc. Natl. Acad. Sci. USA **127:** 81–91.

VELASCO, J. C., 1994 Atlas de los peces de las provincias de Salamanca y Zamora. Valoración de especies y tramos fluviales. Ph.D. Thesis, University of Salamanca, Salamanca.

VINDELØV, L. V., I. J. CHRISTENSEN, N. KEIDING, M. SPANG-THOMSEN and N. I. NISSEN, 1982 Long term storage of samples for flow cytometric DNA analysis. Cytometry **3:** 317–322.

VRIJENHOEK, R. C., 1975 Gene dosage in diploid and triploid unisexual fishes, pp. 463–475 in *Isozymes IV. Genetics and Evolution,* edited by C. L. MARKERT. Academic Press, New York.

VRIJENHOEK, R. C., 1989 Genetic and ecological constraints on the origins and establishment of unisexual vertebrates, pp. 24–31 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

VRIJENHOEK, R. C., 1990 Genetic diversity and the ecology of asexual population, pp. 175–197 in *Population Biology and Evolution,* edited by K. WÖHRMANN and S. JAIN. Springer-Verlag, Berlin.

VRIJENHOEK, R. C., R. A. ANGUS and R. J. SCHULTZ, 1977 Variation and heterozygosity in sexually vs. clonally reproducing populations of *Poeciliopsis.* Evolution **31:** 767–781.

VRIJENHOEK, R. C., R. A. ANGUS and R. J. SCHULTZ, 1978 Variation and clonal structure in a unisexual fish. Am. Nat. **112:** 41–55.

VRIJENHOEK, R. C., R. M. DAWLEY, C. J. COLE and J. P. BOGART, 1989 A list of known unisexual vertebrates, pp. 19–23 in *Evolution and Ecology of Unisexual Vertebrates,* edited by R. DAWLEY and J. BOGART. Bulletin 466. New York State Museum, Albany.

WHITE, M. J. D., 1978 *Modes of Speciation.* Freeman, San Francisco.

Communicating editor: A. G. CLARK