Independent Origins of Allotriploidy in the Fish Genus Poeciliopsis

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Abstract

We examined mitochondrial DNA (mtDNA) sequences and allozymes to assess possible modes of origin, clonal diversity, and evolutionary age in a triploid all-female fish of the genus Poeciliopsis from the state of Sinaloa, Mexico. Analysis of multilocus allozymes revealed that the Rio Mocorito biotype (Poeciliopsis monacha-lucida-viriosa) is trihybrid, carrying haploid genomes from three sexually reproducing species, Poeciliopsis monacha, Poeciliopsis lucida, and Poeciliopsis viriosa. Composite allozyme and mtDNA genotypes identified four clones, all bearing closely related mitochondrial haplotypes originally derived from P. monacha. Apparently these trihybrids arose endemically by addition of a haploid genome from P. viriosa, a local sexual species, to an allodiploid biotype, P. monacha-lucida, also found in the Rio Mocorito. The present analysis clearly revealed that P. monacha-lucida-viriosa arose independently of the two allotriploid biotypes that live in a river to the north (Rio Fuerte). Although the origins of allotriploidy in Poeciliopsis are less constrained phylogenetically and geographically than previously thought, known triploid biotypes all had relatively recent origins, which supports the notion that most asexual lineages are evolutionarily short-lived.

Unisexual, or all-female reproduction, is rare among vertebrates. Approximately 80 distinct biotypes (or taxa) of clonally reproducing vertebrates are known (reviewed in Vrijenhoek et al. 1989; see Alves et al. 2001 for recent references). The majority (>60%) of these biotypes are polyploid, and essentially all of them originated by hybridization between sexually reproducing progenitors that are recognized as distinct species or as genetically discrete populations (e.g., chromosomal races). Each biotype comprises a particular combination of parental (A and B) chromosome sets—for example, AB (2n), AAB (3n), etc.—in various allotriploid or allopolyploid combinations. Unisexual vertebrates are not only rare, but most have had recent origins from extinct sexual ancestors. Most unisexual biotypes exhibit low mitochondrial DNA (mtDNA) diversity and little sequence divergence from their closest sexual relatives (reviewed by Avise et al. 1992), although some notable exceptions have been found (e.g., Goddard et al. 1989; Hedges et al. 1992; Moritz and Heideman 1993; Quattro et al. 1991; Spolsky et al. 1992). Nevertheless, compared to their sexual relatives, unisexual vertebrates and the vast majority of other asexual animals appear to be evolutionarily short-lived (Maynard Smith 1992; but see Judson and Normark 1996; Schón et al. 1998).

Allotriploid biotypes of fish in the genus Poeciliopsis follow the typical pattern for unisexual vertebrates. They exhibit limited mtDNA and allozyme divergence from extant sexual relatives, suggesting recent origins (Quattro et al. 1992b). Triploid forms of Poeciliopsis reproduce gynogenetically (Schultz 1967), a clonal form of reproduction that requires sperm to activate embryogenesis (Figure 1A). The P. 2 monacha-lucida (MML) and P. monacha-2 lucida (MLL) biotypes (the numbers in each biotype name represent genomic dosage) are comprised of genomes ultimately derived from P. monacha (M) and P. lucida (L), sexual species whose males are required as sources of sperm. Allozyme and mtDNA studies (Quattro et al. 1992b) revealed that the MML and MLL biotypes arose exogenously by “genome addition” from related allodiploid lineages of P. monacha-lucida (i.e., M1L2 + L → M1L2L3 and M1L2 + M → M1L2M2) and not endogenously via “genome duplication” (i.e., M1L2 → M1M2L and M1L2L3). The allodiploid biotype, P. monacha-lucida, reproduces hybridogenetically (Figure 1B), a uniallelic form of reproduction in which the haploid M genome is transmitted (cloned) to eggs, whereas the paternal (L) genome is replaced each generation by insemination from P. lucida males (Cimino 1972; Schultz 1969; Vrijenhoek et al. 1977). We know of three hybridogenetic biotypes—P. monacha-lucida (ML), P. monacha-occidentalis (MO), and P. monacha-latidens (Mlat)—each of which transmits the clonal M genome to its eggs (Schultz 1977). The geographic distributions of hybridogenetic biotypes are broad and
The origins of the gynogenetic triploid biotypes of *Poeciliopsis* were investigated. Among river systems (Quattro et al. 1991; Vrijenhoek et al. 1992b), these biotypes arose via multiple independent origins within and between species. Studies revealed that diverse hemiclones of ML and MO biotypes can use sperm from a related sexual species (e.g., *P. lucida*) to obtain inseminations (Figure 2). Allozyme and mtDNA markers reveal low levels of genetic diversity within and between biotypes. Their origins trace to one, or at most a few, closely related ML ancestors (i.e., a single matriline). Factors that might limit the origins of allodiploid gynogens and facilitate multiple independent origins of allodiploid hybrids are not known. To date, no evidence has been found among unisexual vertebrates to discriminate between hypotheses that unisexuality is an accidental outcome of dysgenic interactions between disparate genomes manifested only during gametogenesis in hybrids (Moritz et al. 1989; Vrijenhoek 1989; Wetherington et al. 1987) or it is due to interactions between a few genes that control the processing of chromosomes (e.g., DNA packing, attachment to spindle fibers, synopsis, etc.) prior to or during meiosis (Turner 1982).

This study investigates the origin of a third gynogenetic biotype of *Poeciliopsis* which, based on morphological and geographical considerations, was hypothesized to have had a trihybrid origin. Designated *Poeciliopsis monacha-lucida-viriosa* (Schultz 1971; Vrijenhoek and Schultz 1974), this biotype (MLV) is endemic to the Río Mocorito (Figure 2), where it relies on males of *P. viriosa* for insemination and co-occurs with the hybridogenetic *P. monacha-lucida* (Mateos and Vrijenhoek 2002). Our goals were twofold: (1) to examine, with genotypic data, the trihybrid origin hypothesis for this fish; and (2) to assess whether it arose independently of the MML and MLL biotypes found in rivers to the north. We used a combination of allozyme and mtDNA markers to accomplish these goals.

**Figure 1.** Asexual reproductive mechanisms in *Poeciliopsis*. (A) Gynogenesis in *Poeciliopsis*: a triploid gynogenetic female *Poeciliopsis monacha-lucida* (MLL) produces clonal triploid eggs (MLL). Sperm from a related sexual species (e.g., *P. lucida*) is required to activate embryogenesis, but its genetic material is not incorporated. (B) Hybridogenesis: a diploid hybridogenetic female *Poeciliopsis monacha-lucida* (ML) produces a hemiclonal (haploid) egg (M) with only maternal genes. Haploid sperm from *P. lucida* (different superscripts represent different males) fertilizes the M egg producing an ML female with the same maternal genome as her mother, but different paternal genome.

**Figure 2.** Collection localities and distribution of asexual hybrid biotypes of *Poeciliopsis* and the sexual species involved in these asexual hybrids. (A) Collection localities are indicated by abbreviations: CU, Cuchujaqui (26°56.4′N, 108°53.4′W); CA, El Cajón (26°51.8′N, 108°41.6′W); JA, Jaguari (26°54.1′N, 108°40.5′W); SP, San Pedro (26°32.5′N, 108°21.0′W); PA, El Paso (25°48.65′N, 107°49.9′W); SB, San Benito (25°32.7′N, 107°46.1′W); and LH, La Huerta (25°30.7′N, 107°45.8′W). (B) ML = *P. monacha-lucida* depends on sperm from *P. lucida*, MO = *P. monacha-occidentalis* depends on sperm from *P. occidentalis*, Mlat = *P. monacha-latidens* depends on sperm from *P. latidens*, MML = *P. monacha-lucida-mo*; MLL = *P. monacha-lucida-lucida*, and MLV = *P. monacha-lucida-viriosa*. Although in nature MML and MLL appear to use sperm from *P. monacha*, *P. lucida*, and *P. viriosa*, respectively, these triploids can use sperm from different species of *Poeciliopsis* to activate development of the clonal egg (Schultz 1967).
Materials and Methods

Specimen Collection

Collection localities are shown in Figure 2. Rio Mocorito samples were collected in 1978, 1999, and 2000 from two localities (LH and SB). In the field we examined several thousand specimens of *Poeciliopsis* from LH and SB to roughly sort hybrid biotypes from coexisting sexual species (i.e., *P. lucida*, *P. viriosa*, *P. latidens*, *P. prolifica*, and *P. presidionis*). Although found in the Rio Mocorito, neither *P. prolifica* nor *P. presidionis* appear to be involved in any asexual hybrids. Approximately 200 potential hybrids were retained for subsequent genetic screening. Unused fish were returned to their stream with minimal harm. Specimens retained for genetic analyses were euthanized in ice water (4°C) in the field and frozen immediately on dry ice.

Allozymes

We used cellulose acetate gel electrophoresis (CAGE) to screen specimens for multilocus allozymes (29 putative loci) that had previously been used to identify different sexual and asexual lineages of *Poeciliopsis* (Mateos and Vrijenhoek 2002; Morizot et al. 1990; Vrijenhoek et al. 1978). Allozyme loci, tissue sources and preparation, electrophoretic conditions, buffers, and stains have been described previously (Mateos and Vrijenhoek 2002). Storage of intact specimens at −80°C since 1978 had no appreciable effect on allozyme patterns. For *P. monacha*, which does not occur in the Mocorito, we relied on published data (Morizot et al. 1990; Vrijenhoek 1979) and analyses of laboratory strains from the Rio Fuerte. Allelic identifications were cross-referenced (side by side) on CAGE membranes with genetically defined laboratory strains of *P. monacha-lucida* (S68-4 and T70-3), of *P. 2 monacha-lucida* (S68-4 and S68-5), and of *P. monacha-lucida* (M61-31) from the Rio Fuerte, collected in 1968 (R. J. Schultz), 1970 (R. E. Thibault), and 1961 (R. R. Miller), respectively.

We distinguished *P. monacha-lucida-viriosa* (MLV) from sympatric asexual (i.e., *P. monacha-lucida* and *P. monacha-latidens*) and sexual diploids (i.e., *P. lucida*, *P. viriosa*, *P. prolifica*, *P. presidionis*, and *P. latidens*) based on the presence of fixed heterozygosity and allelic dosage at species-diagnostic loci. Fifteen of the examined loci are diagnostic to at least one of the three species presumably involved in MLV—that is, *P. monacha*, *P. lucida*, and *P. viriosa* (Mateos and Vrijenhoek 2002). Of these 15 loci, 6 are diagnostic between *P. monacha* and *P. viriosa* (i.e., *Pgld*, *Glo-I*, *Ck-C*, *Gda*, *Gpi-1*, and *Adh-2*), including 2 that are diagnostic for all three species (i.e., *Gpi-1* and *Adh-2*).

mtDNA

To infer maternal ancestry of MLV, we examined DNA sequences of mitochondrial ND2 (1047 bp) and cyt b (1140 bp) genes in MLV specimens previously identified by allozyme analyses. They were compared with new sequences of laboratory strains of the gynogenetic triploid MML and MLL (including the same strains examined for restriction fragment length polymorphisms [RFLPs] by Quattro et al. 1992b) and published sequences of the hybridogenetic diploid *P. monacha-lucida* (Mateos and Vrijenhoek 2002) and sexual lineages of *P. monacha*, *P. viriosa*, and *P. lucida* (Mateos et al. 2002). DNA extraction, amplification, and sequencing methods are described in Mateos et al. (2002). To exclude polymerase error as a source of observed haplotypic variation, we repeated the polymerase chain reaction (PCR) and sequencing of all individuals bearing unique haplotypes.

Results

Allozymes

We screened approximately 200 putative hybrids from the SB and LH localities of the Rio Mocorito for multilocus allozyme genotypes. Fixed heterozygosity at 15 species-diagnostic allozyme loci was used to distinguish hybrids from nonhybrids (Table 1). Diploid and triploid hybrids were distinguished by stain intensities of diagnostic parental allozymes at seven heterozygous loci. Altogether we identified 78 MLV triploids, all of which were heterozygous at 17 loci and homozygous at 12 monomorphic loci. All of the 78 triploids exhibited a triallelic genotype at *Adh-2*, and 74 of these also exhibited a triallelic genotype at *Gpi-1*; alleles at both loci distinguish *P. monacha*, *P. lucida*, and *P. viriosa* (Table 1). These triallelic genotypes confirm the trihybrid origin hypothesis for MLV. Although not triallelic, genotypes at the remaining heterozygous loci (except *Gpi-1*) were also consistent with a trihybrid origin based on allelic dosages, as inferred from staining intensities (Table 1).

Based on allozymes, clonal diversity of MLV was low. Only two loci were variable (i.e., *Gpi-1* and *Pep-lgg*), resulting in three different allozyme clones (distinguished by Roman numerals; Table 1). The most common genotype was found in 73 individuals referred to as MLV/I. The other two allozyme clones were less common; MLV/II was found in four individuals and MLV/III was found in a single individual. The *Gpi-1* phenotype of MLV/I and MLV/III was consistent with the triallelic genotype “abc” (denoted with uppercase letters in Table 1). On the other hand, MLV/II had a diallelic “ac” phenotype, because it was missing the diagnostic *viriosa* “b” allele. Nevertheless, asymmetrical staining intensities suggested that the allozyme patterns resulted from the expression of three genes rather than two (i.e., “aac” instead of “ac”). MLV/III, in turn, had a distinct phenotype from MLV/I and MLV/II at the *Pep-lgg* locus. Due to poor electrophoretic resolution and overlap with a distinct peptidase-coding locus, it was difficult to identify the allelic
variant that produced this unique phenotype. Nevertheless, the monacha and lucida allozymes (a and b) appeared to be the same as those in MLV/I and MLV/II, and thus the variant allozyme (b) may correspond to a novel P. viriosa allele (Table 1). We dismissed contamination from parasites and other artifacts as explanations for this phenotype because we consistently observed this pattern, regardless of the type of tissue (i.e., muscle or eye) and despite multiple electrophoretic runs.

**mtDNA**

We examined mtDNA sequences (cyt b, 1140 bp; ND2, 1047 bp) in 13 of the 78 MLV individuals. These included eight representatives of MLV/I, all four representatives of MLV/II, and the single representative of MLV/III. New sequences were deposited in GenBank (accession nos. AY093934–AY093947). Among the 13 sequences examined, we found three haplotypes (i.e., based on the combined cyt b and ND2 sequences), but divergence among them was low (one to two differences). The three haplotypes (D, E, and F; Figure 3) were closely related to haplotypes from other asexual Poeciliopsis biotypes (i.e., less than 0.78% divergent; uncorrected p). The most common haplotype (D) was found in seven of the eight MLV/I representatives examined, and in all four MLV/II representatives. Haplotype E differed by one mutation from haplotype D and was found in one of the eight MLV/I individuals examined. Similarly haplotype F

Table 1. Allozyme markers at 29 loci in the putative parental species and in the three allozyme-defined clones of Rio Mocorito gynogenetic triploids

<table>
<thead>
<tr>
<th>Locus</th>
<th>Putative parental alleles</th>
<th>MLV genotypes</th>
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<tbody>
<tr>
<td></td>
<td>monacha&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>viriosa&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>1. Heterozygous in MLV; dosage determined</strong></td>
<td></td>
<td></td>
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<tr>
<td>Aat-1</td>
<td>a</td>
<td>a</td>
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<td>Aat-2</td>
<td>a</td>
<td>a</td>
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<td>Adh-2</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Glo-1</td>
<td>A</td>
<td>b</td>
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<tr>
<td>Gpi-1</td>
<td>A</td>
<td>B</td>
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<tr>
<td>Mdh-2</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Pep-lgg</td>
<td>A, b</td>
<td>b</td>
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<td><strong>2. Heterozygous in MLV; dosage not determined</strong></td>
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<tr>
<td>Est-4</td>
<td>b, e</td>
<td>b</td>
</tr>
<tr>
<td>Est-5</td>
<td>c, d, e, f</td>
<td>e</td>
</tr>
<tr>
<td>Gpi-2</td>
<td>b</td>
<td>A</td>
</tr>
<tr>
<td>Idh-1</td>
<td>a, b</td>
<td>a</td>
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<tr>
<td>Idh-2</td>
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<td>Mp-1</td>
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<tr>
<td>Mp-2</td>
<td>a</td>
<td>a</td>
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<tr>
<td>Ck-C</td>
<td>b</td>
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<tr>
<td>Gda</td>
<td>b</td>
<td>A</td>
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<tr>
<td>Pgd</td>
<td>a, c</td>
<td>B</td>
</tr>
<tr>
<td><strong>3. Homozygous in MLV; dosage not determined</strong></td>
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<tr>
<td>Aat-3</td>
<td>b</td>
<td>b</td>
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<tr>
<td>Ak</td>
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<tr>
<td>Chc-A</td>
<td>a, b</td>
<td>a</td>
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<tr>
<td>Ldh-2</td>
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<td>Mp-4</td>
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</tr>
<tr>
<td>Pgmu</td>
<td>d, e</td>
<td>d, f</td>
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<tr>
<td>Ldh-1</td>
<td>a, b</td>
<td>b</td>
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<sup>a</sup> From monacha genomes found in sexual P. monacha populations and hemiclonal genomes in P. monacha-lucida strains from the Ríos Fuerte and Sinaloa (from Montzot et al. 1990; Vrijenhoek 1979; 1984).

<sup>b</sup> Boldface capital letter alleles are diagnostic to that species.

<sup>c</sup> From Río Mocorito P. viriosa and P. lucida populations.

<sup>d</sup> Although the Pep-lgg phenotype of MLV/III was clearly distinct from the others, we were uncertain of its exact genotype.

<sup>e</sup> Monomeric enzymes or dimeric enzymes for which electrophoretic separation was good enough to distinguish different alleles, but not good enough to infer allelic dosage accurately.
differed from haplotype D by one substitution and was found in the single MLV/III individual. Haplotypes E and F differed from each other by two substitutions. All three MLV haplotypes (D, E, and F) clearly derived from the *P. monacha* lineage, as they grouped with the mtDNA haplotype of sexual *P. monacha* (100% bootstrap support) and were very divergent from *P. viriosa* (mean uncorrected $p = 10.32\%$), the closest sexual relative of *P. monacha* (Mateos et al. 2002), and from the outgroup *P. lucida* (mean uncorrected $p = 16.46\%$). The MLV haplotypes were most closely related to the clade formed by haplotypes A, B, and C found in Río Mocorito and Río Fuerte ML hemi-clones. These haplotypes were not observed among the three Río Sinaloa ML individuals sampled in this study (Figure 3), which is surprising because Río Sinaloa is between the Fuerte and Mocorito (Figure 2).

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The mtDNA results clearly reveal that the Río Mocorito MLV biotype arose independently of the Río Fuerte MML and MLL triploid biotypes. Mocorito MLV haplotypes were very divergent (i.e., 13–18 steps, or 0.6–0.8%) from the four haplotypes (2, 2*, 2**, and 2***0) found in Fuerte MML and MLL individuals. In contrast, divergence among these Fuerte haplotypes was only two to five steps (0.09–0.23%).

**Clonal Origins and Diversity**

It is difficult to distinguish whether the presently defined Mocorito MLV clones had a single origin or multiple origins.
from a single matriline (i.e., a few closely related maternal lineages). Haplotypes D, E, and F formed a monophyletic clade, separate from their closest relatives (haplotypes A, B, and C, found in Mocorito and Fuerte ML diploids). On the other hand, haplotypes found in Fuerte triploids (MML and MLL) did not comprise a monophyletic clade relative to ML diploids. Within the MLV clade, haplotype D represented the most recent common ancestor of haplotypes E and F. Mutations that led to the divergence of haplotypes E and F from D could have occurred before (i.e., multiple origins) or after (i.e., single origin) the origin of MLV triploids. Clonal diversity within MLV was low in terms of number of clones and divergence among them. The combined analysis of mtDNA and allozyme results yielded four distinct “cytonuclear” clones in the Río Mocorito: MLV/1D, MLV/1LD, MLV/1E, and MLV/1IL. This first, MLV/1D, was most common.

### Discussion

The present allozyme analysis corroborates earlier hypotheses that the Río Mocorito *P. monacha-lucida-viriosa* triploid is a trihybrid comprised of genomes originally derived from *P. monacha*, *P. lucida*, and *P. viriosa* (Schultz 1971; Vrijenhoek and Schultz 1974). Furthermore, mtDNA haplotypes clearly identified *P. monacha* as its closest maternal relative, which is not surprising, because mitochondrial evidence has revealed that *P. monacha* is the maternal relative of all known unisexual biotypes of *Poeciliopsis* examined to date (Mateos and Vrijenhoek 2002; Quattro et al. 1991, 1992a,b). An unknown feature of the *P. monacha* genome causes it to become clonal when combined with *P. lucida*, *P. occidentalis*, or *P. latidens*, but not when paired with *P. viriosa* (Leslie 1982; Morizot et al. 1990; Vrijenhoek and Schultz 1974). Although inferences about the order in which *lucida* and *viriosa* nuclear genomes were added to the original monacha genome of *P. monacha-lucida-viriosa* are not straightforward, we distinguish among three hypotheses.

#### Hypothesis 1: ML + V → MLV

Schultz (1971) first suggested that a diploid *P. monacha-lucida* could have produced an unreduced diploid ML egg that was fertilized by a *viriosa* sperm, resulting in the Mocorito MLV triploid. This hypothesis seems most likely, because it follows the same genome addition pathway that gave rise to Río Fuerte MML and MLL triploids (Quattro et al. 1992b). Also, we know that Mocorito *P. monacha-lucida* hybridogens occasionally mate with males of *P. viriosa* (ML × V) because rare MV hybrids are found in this river (Mateos and Vrijenhoek 2002).

#### Hypothesis 2: ML × V → MV + L → MVL

A diploid MV hybrid generated by a cross between a *P. monacha-lucida* female and a *P. viriosa* male might have produced an unreduced diploid egg (MV) that was fertilized by *lucida* sperm (MV + L). However, this scenario is unlikely because MV hybrids have normal meiosis and produce haploid recombinant eggs (Leslie 1982; Morizot et al. 1990; Vrijenhoek and Schultz 1974). Indeed, this recombinant scenario was used to explain the origin of the Río Mocorito diploid hybridogens, which is essentially a *P. monacha-lucida* hybrid with a limited number of *viriosa* genes in its hemiclonal M genome (Mateos and Vrijenhoek 2002; Vrijenhoek and Schultz 1974). To illustrate this point, which is the basis for our third hypothesis, we temporarily label the Mocorito hybridogen as (M/v)-L (instead of ML), where M/v represents a haploid genome composed primarily of *monacha* (M) genes and a few introgressed *viriosa* (v) genes.

#### Hypothesis 3: (M/v)-L → (M/v)-(M/v)-L

Schultz (1971, 1977) also suggested that the Mocorito triploid might have arisen by retention of the *lucida* genome and duplication of the recombinant (M/v) hemiclonal genome in an (M/v)-L hybrid. Although (M/v)-L hybridogens express some *viriosa* morphological traits (Vrijenhoek and Schultz 1974), they express no unique *viriosa* allozymes (Mateos and Vrijenhoek 2002), thus this scenario cannot explain the trihybrid allozyme patterns expressed by the *Adh-2* and *Gpi-1* loci. Furthermore, the other *Poeciliopsis* allotriploids (MML and MLL) arose by genome addition, not genome duplication (Quattro et al. 1992b).

An endemic (Mocorito) origin of MLV appears most likely because two out of three of its most closely related mtDNA haplotypes (A and B; Figure 3) are found in this river. Its ancestor appears to have been a *P. monacha-lucida* hybridogen that migrated from the Río Fuerte or Río Sinaloa, because *P. monacha* does not occur in the Río Mocorito. Furthermore, the mtDNA haplotypes found in Mocorito MLV and ML biotypes are closely related to haplotypes found in Río Fuerte (Mateos and Vrijenhoek 2002). The mtDNA haplotypes found in Sinaloa ML hybridogens (PA locality) were more distantly related to Mocorito ML and MLV strains. Perhaps ancestors of the Mocorito ML and MLV biotypes once existed, or still exist, in the Río Sinaloa, because major portions of this river are poorly sampled and unexplored. It is presently unsafe to conduct fieldwork in remote areas of the Sinaloa drainage. Furthermore, this river has been severely altered during the past 30 years by the construction of large and small impoundments and the introduction of *Tilapia*. Though we presently have no evidence that Río Sinaloa ML strains were involved in the origin of Río Mocorito ML or MLV biotypes, we suspect that the ancestors of the Mocorito biotypes might have passed through this river.

The present mtDNA results clearly show that allotriploidy has arisen independently at least twice in *Poeciliopsis*. The three MLV haplotypes are most closely related to extant Río Mocorito and Río Fuerte ML haplotypes, and they are more distantly related to the ML haplotypes that coalesce with MML and MLL (Figure 3). Nevertheless, independent origins of triploid gyngenetic biotypes still appear to be rare, an observation consistent with the hypothesis that allotriploidy has arisen only a few times in *Poeciliopsis* from a limited number of ancestral matrilines (Quattro et al. 1992b). The
three triploid biotypes (MML, MLL, and MLV) coalesce with two distinct monacha matrilines, which stands in strong contrast to the pattern of multiple independent origins of ML hybridogens in the Rio Fuerte (Quattro et al. 1991).

The low levels of clonal diversity observed in Mocorito allotriploids may just be a consequence of the low diversity present in the presumed ancestor(s). ML hybridogens, their closest relatives in the Mocorito, also exhibit low clonal diversity, with one allozyme clone and two closely related mtDNA haplotypes (i.e., haplotypes A and B; Figure 3), representing a single matriline (Mateos and Vrijenhoek 2002). Therefore the opportunity for multiple origins from diverse and divergent ML matrilines probably was not present in the ancestor. In contrast, the low levels of clonal diversity reported in Fuerte allotriploids are only expected if allotriploid origins are rare. The Rio Fuerte houses a diverse assemblage of ML matrilines that exceeds the diversity seen in P. monacha; nevertheless all MML and MLL lineages coalesce to a single matriline (Figure 3) (Quattro et al. 1991).

Sources of clonal diversity in asexual lineages can be preformational (due to multiple origins from genetically diverse ancestors) or postformational (due to mutations or recombination events within asexual lineages). Without more extensive examinations of genetic diversity in the sexual relatives, we cannot distinguish among these hypotheses. Nevertheless, failure to find the Pep-lgg variant of MLV/III in the presently sampled sexual ancestors suggests a postformational mutation. On the other hand, the Gpi-1*aac genotype of MLV/II might have resulted from recombination—that is, replacement of the viriosa *b allele by the monacha *a allele (see Asher and Nace 1971). Alternatively, the Gpi-1*aac genotype might have resulted from a convergent charge-state mutation of the viriosa *b allele. Recombination versus mutation could be distinguished by examination of Gpi-I DNA sequences.

The low levels of clonal diversity in allotriploid Poeciliopsis suggest a relatively recent origin. Low mtDNA divergence between the trihybrid MLV haplotypes and their closest ML relatives (i.e., 0.14–0.27%, uncorrected \( p \)) is comparable to the divergence observed between the dihybrid triploids MML and MLL, and their closest ML relatives (i.e., 0–0.18%). These levels of divergence are much smaller than the levels of divergence observed among sexual species of Poeciliopsis. For example, P. monacha is at least 10% divergent (uncorrected \( p \)) from P. viriosa, its closest sexual relative. Similarly, most named species within the genus Poeciliopsis differ by at least 3% mtDNA sequence divergence (from Mateos et al. 2002). Thus, assuming a nucleotide substitution rate of 1–2% per million years for mitochondrial genes (from Mateos et al. 2002), most sister species of Poeciliopsis appear to be at least 1.5–3.0 million years old. Given the same substitution rate, the Mocorito MLV lineage could be as old as 135,000–270,000 years, approximately 11 times younger than most sexual species. However, this lineage could be younger if a closer maternal relative exists or existed among hybridogenetic ML or sexual P. monacha from unsampled localities in the Rios Fuerte, Sinaloa, and Mocorito.

Our results demonstrate two independent origins of allotriploidy in Poeciliopsis, but are still consistent with the hypothesis that allotriploidy origins in Poeciliopsis are extremely rare compared to allopolyploid origins. However, several questions remain regarding the origin of allotriploids: (1) What causes production of an unreduced egg? (2) Why are allotriploid origins so rare in Poeciliopsis? (3) Why do all allotriploids in Poeciliopsis carry at least one genome from P. monacha and one from P. lucida? For example, despite the existence of the hybridogenetic diploids P. monacha-occidentalis and P. monacha-latidens, allotriploids involving the genomes of P. occidentalis and P. latidens do not exist. This suggests that the monacha genomes involved in P. monacha-occidentalis and P. monacha-latidens are not capable of producing unreduced diploid eggs, or that only the specific combination of monacha and lucida genomes is capable of producing unreduced eggs. An alternative explanation is that allotriploids composed of other genomes are formed, but do not succeed ecologically. It is intriguing that despite the presence of P. lucida in the Rio Mocorito and our present evidence for an endemic origin of allotriploids (i.e., MLV), no allotriploids of the MLL biotype have been observed in this river. In general, our observation of low clonal diversity and relatively young evolutionary age of allotriploids is consistent with the notion that most asexual lineages are evolutionarily short-lived.

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