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Evolution

An ancient clonal lineage in the fish genus *Poeciliopsis* (Atheriniformes: Poeciliidae)

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ABSTRACT **Genetic diversity in mtDNA was assessed within the unisexual (all female) hybridogenetic fish *Poeciliopsis monacha–occidentalis* and the two sexual species from which it arose. Results confirm that *P. monacha* was the maternal ancestor and that paternal leakage of *P. occidentalis* mtDNA has not occurred. Of particular interest is the high level of *de novo* mutational divergence within one hybridogenetic lineage that on the basis of independent zoogeographic considerations, protein electrophoretic data, and tissue grafting analysis is of monophyletic (single hybridization) origin. Using a conventional mtDNA clock calibration, we estimate that this unisexual clade might be >100,000 generations old. Contrary to conventional belief, this result shows that some unisexual vertebrate lineages can achieve a substantial evolutionary age.**

Clonally reproducing organisms are believed to have short evolutionary life-spans due to a presumed lack of sufficient genotypic diversity for adaptive change to variable environments (1–3) or to an accumulation of deleterious mutations and gene combinations that cannot be purged in the absence of recombination (2, 4). However, many unisexual (all female) vertebrate populations are not devoid of genetic variation, and some clearly enjoy short-term ecological success relative to their sexual progenitors (5). Can such unisexual lineages also persist over long evolutionary time scales?

Genetic studies reveal that virtually all unisexual vertebrates arose recently from hybridization involving congeneric sexual species (for reviews, see ref. 6). Clonal diversity within a unisexual "biotype" (a particular combination of two or more heterospecific genomes) results primarily from multiple, independent, hybrid origins; however, additional variation can accrue within independent lineages after their inception via polyploidization and mutation (5).

Inferences concerning the evolutionary age of unisexual organisms must avoid confounding postformational processes that are indicative of an old lineage with genetic diversity arising from multiple hybrid origins. However, incomplete sampling of the sexual ancestors and incomplete assessment of diversity in the unisexual populations often defeat attempts to discriminate multiple origins from postformation processes. For example, unisexual lineages often are marked by unique alleles not observed in samples of their sexual relatives (7–11). Although unique alleles might represent postformational mutations, most authors interpret them as "orphan alleles," variants that exist in unsampled sexual populations or that might have existed in extinct sexual progenitors (12). With this conservative approach, postformational mutations could be overlooked and potential evidence for antiquity could be discounted.

The present study avoids this dilemma by focusing on postformational mtDNA and allozyme mutations within a monophyletic lineage of the unisexual fish *Poeciliopsis monacha–occidentalis* (hereafter *MO*). The *MO* biotype arose in northwestern Mexico (Fig. 1*A*) via crosses between two sexual species, *P. monacha* and *P. occidentalis* (14). Reproduction is by hybridogenesis, a "hemiclonal" mechanism whereby only the haploid maternal *monacha* genome (*M*) is transmitted without recombination to ova; the paternal *occidentalis* genome (*O*) is excluded during a premeiotic cell division, preventing synapsis and crossing-over (15, 16). Fertilization of *M* eggs by *O* sperm from *P. occidentalis* males restores diploidy and results in expression of maternal and paternal traits.

The monophyletic hemiclonal lineage on which we focus arose in the Río Mayo, where the southern limit of *P. occidentalis* and the northern limit of *P. monacha* overlap. Previous allozyme studies revealed that several distinct *MO* strains from Río Mayo were marked by local *P. monacha* alleles, verifying multiple, endemic, hybrid origins in this river (14). Apparently, a single unisexual lineage colonized, in a stepwise manner, the next four rivers to the north (Fig. 1*A*). In previous studies, the Ríos Yaqui, Matape, and Sonora were reported to contain a single "E-type," *MO/II* (the Roman numeral identifies the multilocus electrophoretic genotype, or E-type, of the *M* genome). The northernmost river, Río de la Concepción, harbors *MO/I*, an E-type characterized by two unique alleles, including a silencing mutation (17), not observed elsewhere in sexual or unisexual *Poeciliopsis*. In contrast with the diverse Río Mayo strains, tissue grafts among unisexual strains from the four northern rivers (Fig. 1*B*) corroborated that some strains were closely related antigenically and thus comprise a monophyletic lineage (13). Unfortunately, allozyme and histocompatibility data were not suitable for estimating the evolutionary age of this unique unisexual clade.

Divergence in mtDNA provides an ideal metric to gauge the relative age of an all-female lineage. mtDNA evolves rapidly in vertebrates (18) and is known to be maternally inherited in *Poeciliopsis* (19, 20). We examine mtDNA restriction site variation and allozyme diversity within *P. monacha–occidentalis* and its sexual relatives. Our results corroborate a monophyletic origin for the *MO/II* lineage and reveal a considerable accumulation of mutations within this unisexual clade.

MATERIALS AND METHODS

***Poeciliopsis* mtDNA.** We analyzed mtDNA restriction site variation among 15 *P. occidentalis* and 36 *P. monacha–occidentalis* specimens representing all five rivers within their range (Table 1; Fig. 1*A*). Also included are data gathered previously (20) from 50 specimens of *Poeciliopsis monacha–lucida, P. lucida*, and *P. monacha* (including three laboratory strains of *P. monacha* originally collected from the Río Mayo where this species might now be extinct). Wild-caught indi-

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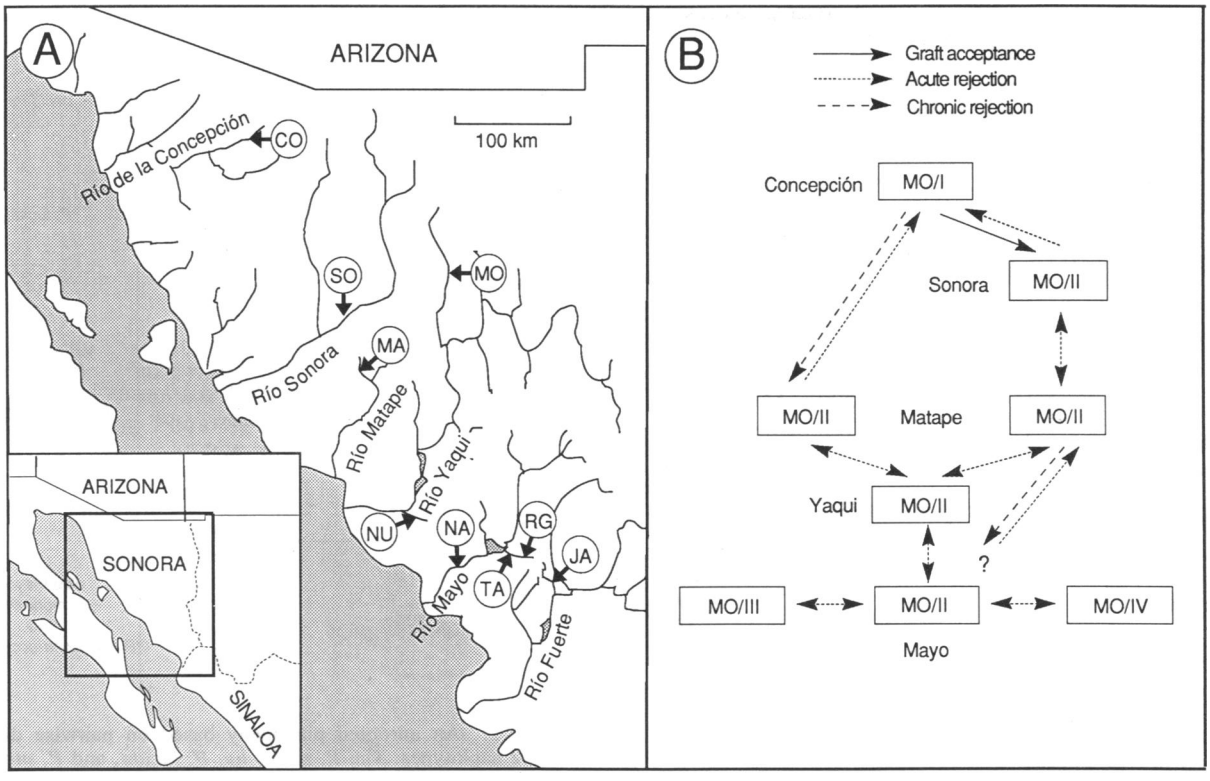

FIG. 1. (*A*) River drainages of Sonora, Mexico. Sampling localities are indicated by abbreviations (see Table 1). (*B*) Tissue graft relationships among E-type clones of *P. monacha–occidentalis*, adapted from Angus (13). E-type clones are arranged according to the river of collection. Dotted lines represent acute rejections (<20 days), dashed lines represent chronic rejections (20–30 days), and solid lines represent acceptances (no rejection after 30 days). Arrows indicate direction of response. For clarity, not all graft responses are shown but, when absent, represent acute rejections.

viduals were dissected in the field, and tissues (muscle, liver, gill, brain, ova, and kidney) were stored on ice in MSB/EDTA buffer (21) for up to 7 days. Specimens were returned to Rutgers University, where protein electrophoresis for 25 loci was used to distinguish among hemiclonal E-types by published methods (14). Purified mtDNAs (isolated following ref. 21) were digested according to the manufacturer's specifications with 16 restriction enzymes having 5-base (*Ava* I, *Ava* II, *Hin*cII) and 6-base (*Bam*HI, *Bcl* I, *Bgl* I, *Bgl* II, *Bst*EII, *Eco*RI, *Hin*dIII, *Nde* I, *Pst* I, *Pvu* II, *Spe* I, *Stu* I, *Xba* I) recognition sequences. DNA fragments were end-labeled with [^{35}S]dNTPs and separated in 0.8–1.2% agarose gels. After electrophoresis, gels were dried under vacuum and exposed to x-ray film at room temperature for 24–72 hrs.

Phylogenetic Analyses. Estimates of nucleotide sequence divergence between mtDNA haplotypes were calculated by the fragment (interspecific) and site (intraspecific) comparison methods (22). An unrooted phylogeny linking haplotypes was constructed by the neighbor-joining algorithm (23). A minimum-length network using information from both the allozyme and mtDNA mutations was constructed with the minimum spanning tree algorithm of the NTSYS statistical package (24).

RESULTS

Maternal Ancestry of *P. monacha–occidentalis*. A previous study revealed 12 mtDNA haplotypes among 50 specimens of *P. monacha*, *P. lucida*, and hybridogenetic *P. monacha–lucida* from the Río Fuerte (20). Fifteen new mtDNA haplotypes were identified among the 51 *P. occidentalis* and *P. monacha–occidentalis* specimens examined here. All 27 haplotypes were clustered in an unrooted phylogeny (Fig. 2) revealing two major clades: one composing the sibling species *P. lucida* and *P. occidentalis* and a second linking *P. monacha* with all *MO* and *ML* hybridogens. The *monacha* clade was subdivided further into two groups, one comprising Río Fuerte *P. monacha* and *P. monacha–lucida*, and a second including Río Mayo *P. monacha* and all *P. monacha–occidentalis*.

The average degree of sequence divergence between *P. monacha* and *P. occidentalis* mtDNAs is large (11.0%). Fragment profiles for all 16 restriction enzymes differentiate the species and allow an unequivocal assignment of *P. monacha* as the maternal parent of all surveyed *P. monacha–occidentalis* hybridogens.

mtDNA and Allozyme Diversity Within *P. monacha–occidentalis*. Five distinct mtDNA haplotypes were observed among the 38 *P. monacha–occidentalis* samples (Table 1; Fig. 2). Mutational divergence among the *MO* haplotypes could be attributed to base substitutions within four polymorphic restriction sites and an occurrence of a length polymorphism (Fig. 3). None of the *MO* haplotypes was identical to those in the available sample of Río Mayo *P. monacha* (*M.5* and *M.6*), but all the *MO* clones clustered with *P. monacha* haplotypes from the Río Mayo and not with those from the Río Fuerte (Fig. 2) (the latter drainage lying outside the range of *P. monacha–occidentalis*).

The protein electrophoretic survey identified two additional E-types within *P. monacha–occidentalis*, one each in the Río Yaqui (*MO/V*) and Río de la Concepción (*M/VI*) (Fig. 3). *MO/V* harbors an *Adh* null allele, and *MO/VI* carries a new allele at the creatine kinase locus *Ck-A* (referred to as *Mp-3* in ref. 14). Appending the mtDNA haplotypes to the allozyme genotypes allowed delineation of "composite" *MO* hemiclones, of which seven were observed in the four northern rivers. Their relationships in a minimum length network are presented in Fig. 3. Composite hemiclones

Table 1. Sources of fish specimens and observed multilocus electrophoretic (E-type) and mtDNA (mt-type) haplotype

River, locality, and biotype	Strain: Wild	Strain: Lab	E-type	mt-type
Rio Mayo				
NA (Navojoa)				
monacha–occidentalis		2	*III*	*M.16*
	1		*IV*	*M.16*
occidentalis	1		—	*O.3*
	1		—	*O.4*
TA (El Tabelo)				
monacha–occidentalis		5	*II*	*M.16*
	1		*III*	*M.16*
	2		*IV*	*M.16*
occidentalis	1		—	*O.3*
RG (Rancho Guamuchil)				
monacha		1	—	*M.5*
		2	—	*M.6*
Río Yaqui				
MO (Moctezuma)				
monacha–occidentalis		4	*II*	*M.17*
occidentalis	1		—	*O.1*
	2		—	*O.5*
NU (Nuri)				
monacha–occidentalis		1	*II*	*M.17*
monacha–occidentalis		3	*VI*	*M.17*
Río Matape				
SP (San José de Pimas)				
monacha–occidentalis		6	*II*	*M.18*
	1		II	*M.19*
occidentalis	1		—	*O.1*
	2		—	*O.2*
Río Sonora				
SO (Ures)				
monacha–occidentalis		5	*II*	*M.17*
occidentalis	1		—	*O.5*
	2		—	*O.6*
Río de la Concepción				
LP (La Providencia Cienega)				
monacha–occidentalis		5	*I*	*M.17*
	1		*II*	*M.20*
	1		*V*	*M.17*
occidentalis	1		—	*O.7*
	2		—	*O.8*

Sample sizes for each strain (wild caught or laboratory maintained) are given.

carrying the *MO/II* allozyme genotype but distinct mtDNA haplotypes were observed in the Río Matape (*MO/II.18* and *MO/II.19*), Río de la Concepción (*MO/II.20*), and Ríos Yaqui and Sonora (*MO/II.17*). Conversely, three composite hemiclones carried indistinguishable mtDNA haplotypes but differed in allozyme genotype: *MO/V.17*, Río Yaqui; *MO/I.17* and *MO/VI.17*, Río de la Concepción (Fig. 3).

DISCUSSION

Origin of *P. monacha–occidentalis*. The average degree of sequence divergence between *P. monacha* and *P. occidentalis* mtDNAs is large. Because all *MO* females carry *monacha*-type mtDNA, they must have arisen via crosses of *P. monacha* females with *P. occidentalis* males. As in previous studies of hemiclonal *P. monacha–lucida* (19, 20), sperm-mediated paternal leakage of mtDNA does not appear to occur in *MO*; no evidence for *occidentalis*-type mtDNA was found in the *MO* individuals surveyed. Since mtDNAs found in *MO* are most similar to those of *P. monacha* from the Río Mayo (Fig. 2), crosses giving rise to *MO* hybrids apparently

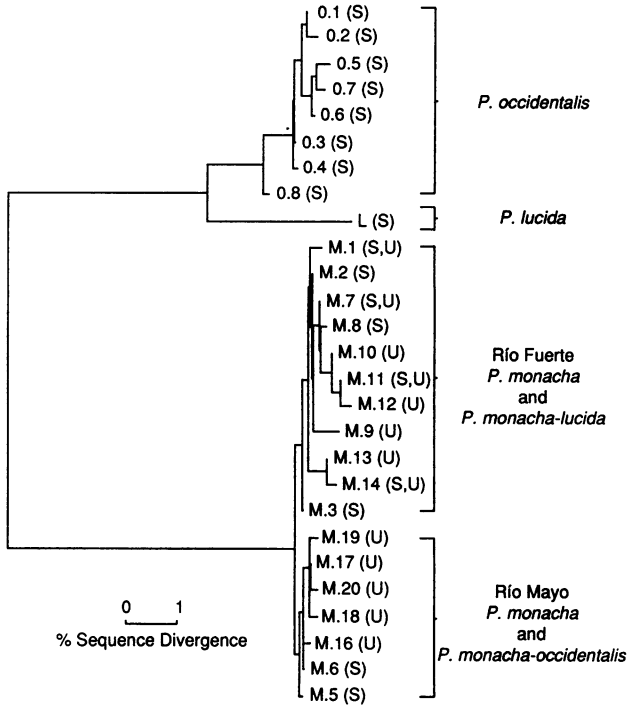

FIG. 2. An unrooted phylogeny based on pairwise distances among haplotypes. The *P. monacha*, *P. lucida*, and *P. monacha–lucida* data are from a previous publication (20). The presence of unisexual (U) and sexual (S) specimens associated with each haplotype is indicated.

were limited to this river, corroborating earlier allozyme studies.

Although mtDNA diversity exists in *P. monacha* from the Río Mayo, no mtDNA heterogeneity was observed in *MO* strains taken from upstream and downstream sites >100 km

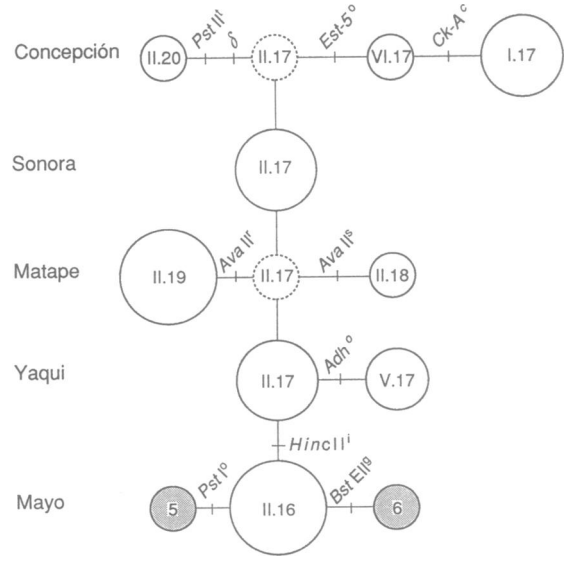

FIG. 3. An unrooted minimum mutation network summarizing relationships among composite haplotypes in sexual *P. monacha* (shaded circles) and *P. monacha–occidentalis* E-type *II* and derivatives (open circles). Haplotypes are arranged according to the river of collection. Sizes of circles are drawn in proportion to the observed frequency of individuals with that haplotype. Dashed circles are haplotypes not found in the respective rivers but represent hypothetical intermediate steps. Slash represents specific allozyme and mtDNA mutations as indicated. The symbol δ represents an ≈200-base-pair mtDNA length polymorphism.

apart in this river. The lack of mtDNA diversity in Río Mayo hemiclones suggests that the origin of successful hybridogens in this river has been constrained to a single maternal lineage of *P. monacha* defined by mtDNA type *M.16* (Fig. 2). Nevertheless, previously discovered allozyme diversity (14) indicates that independent hybridizations involving this *P. monacha* lineage have given rise to E-types *MO/II*, *MO/III*, and *MO/IV* (Fig. 1*B*). These E-types differ at two gene loci for common alleles that segregate in *P. monacha* populations in the upstream Río Mayo sites.

The mtDNA data are consistent with earlier histocompatibility and allozyme information in suggesting a monophyletic origin for the *MO* hemiclones north of the Río Mayo (Fig. 3). These northern hemiclones lack a *Hinc*II^{i} site (Fig. 3), which is otherwise present in all surveyed *P. monacha* and *P. monacha–occidentalis* from the Río Mayo. The absence of this *Hinc*II^{i} site was observed also in mt-type *M.3* in *P. monacha* from the Río Fuerte. However, we provisionally attribute the occurrence of *Hinc*II^{i} in *M.3* to a parallel site loss based on the drainage boundaries involved and the overall relationship among haplotypes in the unrooted phylogeny (Fig. 2). Furthermore, all mtDNA haplotypes found in Río Mayo *P. monacha* and *P. monacha–occidentalis* (except haplotype *M.5*) contain a *Pst* I^{o} site (Fig. 3) that has not been observed in a larger survey of Río Fuerte *P. monacha* or *P. monacha–lucida* (20).

Within the northern clade of *MO* hybridogens, the widespread hemiclone *MO/II.17* represents an intermediate step linking other composite types in the network (Fig. 3) and by this criterion might represent the ancestral genotype. Although the putative ancestor, *MO/II.17*, was not observed in the limited Río Mayo sample, earlier tissue grafting data (Fig. 1*B*) suggest that a genetically similar clone exists (or existed) in this river (13). In the next river to the north, the Río Yaqui, an alcohol dehydrogenase null allele (*Adh*^{o}) distinguishes *MO/V.17* from the putative ancestor. Although the ancestral lineage was not observed in the next river, Río Matape, mutational diversification apparently gave rise to *MO/II.18* and *MO/II.19*, which differ from *MO/II.17* for mtDNA restriction sites *Ava* II^{r} and *Ava* II^{s}, respectively. Haplotype *MO/II.17* appears again in the Río Sonora, and it probably occurred in the Río de la Concepción, where mutation gave rise to three new hemiclones. Concepción hemiclone *MO/II.20* differs from the ancestral lineage at one mtDNA restriction site (*Pst* I^{t}) and for an mtDNA length variant (δ). Río de la Concepción hemiclone *MO/VI.17* differs from the ancestral lineage by the presence of a silent esterase mutation (*Es-5*^{o}) and provides an intermediate link to *MO/I.17*, which also carries a creatine kinase mutation (*Ck-A*^{c}).

An alternative hypothesis for the origin of the northern *P. monacha–occidentalis* populations postulates that *P. monacha* was once present in the northern rivers, and that *M* genome diversity in *MO* represents remnant genetic variation captured during independent endemic hybridizations in isolated river systems. However, this hypothesis is strictly incompatible with the pattern of antigenic similarity between hemiclonal strains inhabiting different river systems (Fig. 1*B*). Furthermore, this hypothesis fails when examined with respect to patterns of *in situ* hybrid origins in neighboring rivers to the south. In different tributaries of the Ríos Fuerte and Sinaloa, multiple hybridizations between *P. monacha* and *P. lucida* produced diverse *P. monacha–lucida* assemblages marked by endemic allozymes and mtDNA haplotypes, specific to the local *P. monacha* population (11, 20). If *P. monacha* once existed in the four northern rivers, independent hybridizations should have produced a similar pattern of unrelated, endemic hemiclones in each river. Instead there is an absence of a highly subdivided pattern of genetic diversity in *P. monacha–occidentalis* inhabiting these river drainages. This pattern cannot likely be explained by a recent connection of drainage basins, since populations of *P. occidentalis* inhabiting these same rivers exhibit large genetic differences (25).

Evolutionary Age of a Unisexual Clade. The monophyletic status of the *MO/II* lineage and its mutational descendants in the northern rivers allows a unique opportunity to estimate the relative age of a unisexual clade, without the confounding effects of multiple hybrid origins. Mean sequence diversity, p (22), between mtDNA haplotypes of individuals in the *MO/II* lineage is 0.12%, approximately one-third the degree of matriarchal lineage diversification found in the sexual ancestor *P. monacha* across the Ríos Mayo and Fuerte (p = 0.38%) and about one-half that in *P. monacha* from the Río Fuerte alone (p = 0.28%). If we ignore haplotypic frequencies, the maximum interhaplotypic distance (p_{max}) between mtDNA of the two most divergent haplotypes (*MO/II.16* and *MO/II.20*) is 0.30%. Both estimates of sequence divergence within the *MO/II* lineage are far less than that found in comparisons between recognized sexual species of *Poeciliopsis: P. monacha* vs. *P. occidentalis* (p = 11.03%); *P. occidentalis* vs. *P. lucida* (p = 4.03%).

It has been estimated that the mean rate of evolution, averaged across the mtDNA molecule, is ≈1% sequence divergence per lineage per million years in a variety of vertebrate taxa (26, 27). If this calibration applies to *Poeciliopsis* mtDNA, then a p value of 0.12% suggests that mutational diversification within *MO/II* began ≈60,000 years ago. If we use the estimate for maximum mtDNA distance between any two haplotypes within this unisexual clade (p_{max} = 0.30%), the origination time becomes ≈150,000 years ago. Assuming a minimum of two generations per year in *Poeciliopsis*, these age estimates translate into 120,000 and 300,000 hemiclonal generations, respectively. A relatively ancient origin for this unisexual clade is supported by the observation of *de novo* mutations at three allozyme loci (*Adh*^{o}, *Est-5*^{o}, and *Ck-A*^{c}), substantial divergence in histocompatibility genotypes, and an mtDNA length variant (δ). Unfortunately, the latter types of variation cannot be converted easily into estimates of divergence time due to rate uncertainties and other caveats at least as great as those that apply to mtDNA sequence divergence calibrations (28).

The overall degree of mutational diversification displayed by this monophyletic lineage across its 550-km geographic range reflects considerable evolutionary longevity and ecological success. Perhaps a primary factor contributing to the persistence of this unisexual lineage is the absence of *P. monacha* from the four northern rivers. Vrijenhoek (29) proposed that the ecological success of a unisexual population depends on the opportunity for recurrent hybrid origins to create ecologically relevant hemiclonal diversity. Ensuing selection would act on this interclonal variability, producing an ecologically structured unisexual population that partially displaces the sexual progenitors from their ancestral niche. In rivers containing multiple hemiclones, unisexual fish comprise a majority of the total *Poeciliopsis* population and occupy a wider range of habitats compared to unisexual populations in monoclonal locales (29, 30). In the Ríos Mayo and Fuerte (where new hybrid origins are possible because *P. monacha* overlaps with *P. occidentalis* and *P. lucida*, respectively), high hemiclonal diversity is associated with high unisexual density. Yet, none of the numerous Río Mayo and Río Fuerte hemiclones have diverged significantly in allozymes or mtDNA from their local *P. monacha* ancestors (20). Apparently, unisexual biotypes in these rivers participate in a dynamic interplay, whereby newly synthesized hemiclones that comprise fortuitous combinations of parental genomes replace preexisting hemiclones that are more poorly adapted or genetically deteriorating (30). Over time, clones will blink in and out of existence, but a particular unisexual biotype (e.g., *P. monacha–occidentalis* or *P. monacha–lucida*) can

persist indefinitely as long as the sexual progenitors remain in sympatry and new clones can arise.

In contrast, *de novo* hybrid origins cannot occur in rivers where *P. monacha* is absent, and thus the scope for interclonal selection is limited. Monoclonal unisexual populations in the northern rivers are more limited ecologically, comprising a small fraction of the total *Poeciliopsis* population (29). For the northern *MO* populations, migration among drainages and mutation in preexisting clones are the only sources of unisexual variation. The absence of *P. monacha* prevents recurrent hybrid synthesis that would permit dynamic clonal turnover. Thus, individual hemiclones can persist in this river as long as the proper ecological factors permit.

Results of the present study indicate that monophyletic lineages within at least some unisexual vertebrates can reach considerable evolutionary age. In fact, several other unisexual vertebrates exhibit large mtDNA distances from their nearest potential sexual relatives. However, because of the opportunity for multiple hybrid origins of a particular biotype and the likelihood that the sexual progenitors are extinct or unsampled, the possibility that particular unisexual biotypes arose recently could not be eliminated. If evolutionary antiquity can be demonstrated in other clonal vertebrates, we must conclude that individual clones are not particularly prone to rapid extinction. Thus, the rarity of unisexual taxa might have as much to do with low origination rates as with high extinction probabilities (31, 32).

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