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POECILIA MEXICANA IS THE RECENT FEMALE PARENT OF THE UNISEXUAL FISH *P. FORMOSA*

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Poecilia formosa, a small live-bearing fish native to northeastern Mexico, was the first recognized vertebrate with unisexual reproduction (Hubbs and Hubbs, 1932). This all-female "species" produces diploid apomictic eggs (Rasch et al., 1982; Monaco et al., 1984), and embryogenesis is subsequently activated by sperm from a related bisexual species, normally either *P. latipinna* or *P. mexicana* (Turner et al., 1980*a*, 1980*b*). The process apparently takes place without syngamy, such that genetic transmission is predominantly or exclusively clonal (Balsano et al., 1989).

The gynogen *P. formosa* almost certainly arose via hybridization of *P. latipinna* and *P. mexicana* (Hubbs and Hubbs, 1932; Turner, 1982). It is sympatric with these species (Darnell and Abramoff, 1968), possesses an intermediate morphology (Hubbs and Hubbs, 1932; Meyer, 1938), and exhibits nearly fixed heterozygosity at numerous protein and allozyme loci that distinguish or are polymorphic in *P. latipinna* and *P. mexicana* (Abramoff et al., 1968; Balsano et al., 1972; Simanek, 1978; Turner, 1982). There is a relative paucity of allozyme variation among the unisexuals, and the best fit to the particular *P. formosa* alleles occurs in populations of *P. mexicana* in the Rio Tigre drainage near Tampico, Mexico. This led Turner (1982) to hypothesize that the gynogen may have arisen recently in this area (perhaps from a single hybridization event), and that its current geographic distribution reflects a rapid northward expansion via efficient colonizing ability. A relatively recent origin for *P. formosa* is also consistent with the finding that the species retains a capacity to express male-specific genes and to undergo normal spermatogenesis upon experimental induction with exogenous androgens (Turner and Steeves, 1989).

One open evolutionary question concerns the direction of the cross that produced *P. formosa*. Unlike the situation in hybridogenetic *Poeciliopsis* fishes from western Mexico, where results of laboratory hybridization experiments have been critical in deciphering the male and female parents of the unisexuals (Schultz, 1973, 1989; Wetherington et al., 1989), attempts to synthesize unisexual *P. formosa* through laboratory crosses thus far have failed (Turner, 1982). Balsano et al. (1989) suspect that *P. mexicana* was the female parent, but note that direct evidence from maternally transmitted mitochondrial DNA (mtDNA) might settle the issue. Here we survey mtDNA genotypes in these *Poecilia* fishes, and show unequivocally that *P. mexicana* was indeed a recent female parent of *P. formosa*.

MATERIALS AND METHODS

Fish were collected from the following locales (Table 1): (1) Live Oak Island, Wakulla Co., FL; (2) Escondido Creek, Kleberg Co., TX; (3) Resaca del Rancho Viejo, Cameron Co., TX (Rio Grande drainage); (4) south of San Benito, Cameron Co., TX (Rio Grande drainage); (5) Rio Tigre, Tamaulipas, Mexico; (6) coastal lagoon drainage north of Altamira, Tamaulipas, Mexico. All assayed specimens of *P. formosa* were diploid. Although triploid *P. formosa* also occur in some drainages (Rasch and Balsano, 1989; Balsano et al., 1989), most notably the Rio Sota la Marina, none was represented in our collections.

To enhance mtDNA yields, most work was conducted with large, gravid females. Embryos, liver, and muscle from individual fish provided the tissue source for isolation of mtDNA by either of two methods: (a) ultracentrifugation in cesium chloride gradients (Lansman et al., 1981); or (b) an "alkaline lysis" procedure (Tamura and Aotsuka, 1988). Mitochondrial DNA was then digested with the following restriction enzymes that proved to produce multiple cuts in most samples: (1) *Ava*I; (2) *Ava*II; (3) *Bam*HI; (4) *Bcl*I; (5) *Bgl*I; (6) *Bst*EII; (7) *Eco*RI; (8) *Hin*cII; (9) *Hin*dIII; (10) *Pvu*II; (11) *Stu*I; and (12) *Xba*I. The fragments were end-labeled with ^{35}S nucleotides, separated by molecular weight through 1% agarose gels, and revealed by autoradiography, all according to standard procedures (Brown, 1980; Lansman et al., 1981; Maniatis et al., 1982). No attempt was made to score fragments less than about 0.6 kilobases in length.

For unknown reasons, *Poecilia* proved rather refractory to the mtDNA isolation procedures that we have employed successfully with numerous other vertebrates (Avise et al., 1987). The mtDNA yields were highly variable, often heavily contaminated with nuclear DNA, and many isolations were unsuccessful entirely. Altogether, among 187 individuals attempted, only 44 (24%) provided adequate mtDNA for complete scoring by all (or all except one) of the 12 informative endonucleases. These form the heart of our analysis. An additional 71 individuals (38%) were scored for a

TABLE 1. MtDNA haplotype descriptions in *Poecilia* fishes. Upper-case letters from left to right refer to the multi-fragment mtDNA digestion profiles for the 12 endonucleases listed in same order as in Materials and Methods. Locales are also described in Materials and Methods.

MtDNA clone	No. of individuals	Collection locale	Genotype description											
P. latipinna														
a	6	1	C	C	C	C	C	C	C	C	C	*D*	C	C
b	2	1	C	*D*	C	C	C	C	*D*	C	C	C	C	C
c	8	2, 3	C	*G*	C	*D*	C	C	*D*	C	C	C	*D*	*D*
P. mexicana														
d	9	6	X	X	D	X	X	D	X	X	D	B	X	X
e	2	6	X	X	D	X	X	D	X	X	*E*	B	X	X
f	1	5	X	X	D	*Y*	X	D	X	X	D	B	X	X
g	1	5	X	X	D	X	X	D	X	*Y*	D	B	X	X
P. formosa														
d	15	2–4	X	X	D	X	X	D	X	X	D	B	X	X

small subset of enzymes (usually 1–7), and hence provided ancillary information only.

For most endonucleases, the mtDNA digestion profiles for *P. mexicana* and *P. latipinna* were sufficiently different to preclude simple site interpretations. Therefore, overall estimates of sequence divergence were based on the "fragment" comparison approach of Nei and Li (1979). A matrix of genetic distances between mtDNA "clones" was clustered phenetically using the unweighted pair-group method with arithmetic means (Sneath and Sokal, 1973).

RESULTS

Composite mtDNA designations for the 44 individuals fully assayed are listed in Table 1. An average of 50 mtDNA fragments was monitored per individual. This represents approximately 283 base pairs (bp) of recognition sequence (or 1.7% of the *Poecilia* mtDNA genome, which we estimate to be about 16.5 kb. in length).

Three mtDNA haplotypes were observed among the 16 *P. latipinna* that were fully scored. Two of these characterized the Florida sample, while the third was confined to the collection sites in Texas. The Texas and Florida genotypes differed in at least four digestion profiles, with mean estimated sequence divergence $p = 0.012$. In *P. mexicana*, four mtDNA haplotypes were distinguished among the 13 specimens from two surveyed locales in Mexico. All differences among these *P. mexicana* genotypes were minor, attributable to one or two restriction site changes. An additional 40 specimens of this species from these same Mexican locales were scored for only one to six enzyme patterns, but in all cases they too exhibited the "*P. mexicana*" mtDNA profile.

In comparison to the moderate mtDNA differences observed within either species, the distinction between the *P. latipinna* and *P. mexicana* was dramatic: digestion patterns for all endonucleases were distinct, and mean mtDNA sequence divergence was $p = 0.070$. After correction for within-species polymorphism (Nei, 1987), the net nucleotide divergence between *P. latipinna* and *P. mexicana* remained $p = 0.066$. If mtDNA in *Poecilia* evolves at a rate considered "conventional" in several other vertebrate groups (about 2% sequence divergence per million years—Brown et al., 1979; Shields and Wilson, 1987; Wilson et al., 1985), then these species may last have shared a common ancestor roughly three to four million years ago.

All fully scored specimens of *P. formosa* were identical in assayed genotype to the most common mtDNA clone in *P. mexicana*. This finding is particularly impressive because these *P. formosa* were from three locales in Texas near the northern distributional limit of the species, whereas the *P. mexicana* were from the southern portion of *P. formosa* range near Tampico, Mexico (close to the putative site of origin of *P. formosa*—Turner, 1982). In Texas, *P. formosa* uses *P. latipinna* as sexual host exclusively (Turner, 1982). Thus, beyond reasonable doubt, *P. mexicana* rather than *P. latipinna* contributed the mtDNA now present in northern populations of *P. formosa*, and hence was the female parent in the original cross or crosses producing these unisexuals. A phenogram summarizing relationships among the fully assayed *Poecilia* forms is presented in Figure 1.

Although we had great difficulty purifying mtDNA from other *P. formosa*, we provisionally typed an additional 18 individuals from the Mexican locales. In a single scorable digest (*Hin*cII), all of these specimens exhibited the diagnostic, high-molecular weight mtDNA bands characteristic of *P. mexicana*. Thus, they too probably had a *P. mexicana* (rather than *P. latipinna*) matriarchal ancestry, although we cannot speculate further as to their clonal diversity or age.

DISCUSSION

As recently as 1978, in referring to the hybrid-derived parthenogenetic grasshopper *Warramaba virgo*, the late M. J. D. White lamented that "we are never likely to know which species was the female parent." Within a year, the female parent of two parthenogenetic lizards (*Cnemidophorus tesselatus* and *C. neomexicanus*) was determined unambiguously using recently introduced mtDNA methods (Brown and Wright, 1979). Since then, studies utilizing the female-transmitted mtDNA molecule have helped resolve questions con-

cerning the maternal origins and ages of several unisexual or clonally reproducing vertebrates, including additional *Cnemidophorus* and *Heteronotia* lizards, *Ambystoma* salamanders, and *Menidia, Poeciliopsis,* and *Phoxinus* fishes [see Dawley and Bogart (1989) and references therein, and also several papers in Evolution 43(5) (1989)]. An emerging generalization from these studies is that most vertebrate parthenoforms are unidirectional in maternal source. Furthermore, most unisexuals exhibit only a limited subset of the mtDNA diversity present in their female sexual progenitors (an exception involves *Poeciliopsis*—Quattro et al., 1991), suggesting relatively recent origins through a small number of successful hybridization events.

Ironically, the first unisexual vertebrate discovered (Hubbs and Hubbs, 1932) has not previously been the subject of mtDNA analysis. Here we have employed mtDNA assays to determine that the hybridization(s) producing the gynogenetic fish *Poecilia formosa* involved *P. mexicana* as the female parent. Furthermore, the close genetic similarity of the *P. formosa* mtDNA genome to the most common mtDNA haplotype observed in *P. mexicana* indicates that the assayed unisexuals arose recently through one or a few hybridization events involving closely related females.

How recent may these hybridization(s) have been? Our assays with 12 restriction endonucleases produced some 50 mtDNA fragments per individual. A change in one such fragment translates into an estimate of sequence divergence of about 0.2%, or about 100,000 years of lineage separation under the "conventional" mtDNA clock calibration mentioned above. Since no fragment changes distinguished the mtDNA in *P. formosa* from the common haplotype in *P. mexicana,* a literal interpretation indicates a time of *P. formosa* origin less than 100,000 years ago.

Of course, more intensive molecular assays and broader geographic sampling (particularly within the southern range) might likely reveal additional mtDNA haplotypes in *Poecilia formosa,* and perhaps lead to more refined estimates of clonal ages and the possibility of multiple origins. Genetic variation within *P. formosa* clearly does exist, as judged by differences in allozymes (Turner, 1982), nuclear DNA fingerprints (Turner et al., 1990), ribosomal DNA (Monaco et al., 1988), and histocompatibility response (Kallman, 1962). However, a difficulty in interpreting genetic variation within *P. formosa* or any other parthenogen involves determining whether the differences accumulated within a monophyletic lineage after separation from a perhaps distant bisexual progenitor, or alternatively whether they reflect multiple hybridization events and retentions of polymorphisms from more recent ancestors that may or may not still exhibit these genotypes today (Turner, 1982).

Most of the known allozyme alleles within *P. formosa* have also been observed in *P. mexicana* or *P. latipinna* (Turner, 1982); and in terms of nuclear DNA fingerprints, the *maximum* genetic distance (proportional band dissimilarity) within *P. formosa* appears to be much less than the *average* distance within bisexual fish species (Turner et al., 1990). Such observations led these authors to conclude that unisexual *P. formosa* clones "are ultimately descended from hybridization events involving the same or very closely related individuals" (Turner et al., 1989). Similarly, we have found mtDNA haplotype heterogeneity within *P. formosa* to be less than that within *P. mexicana* (and *P. latipinna*).

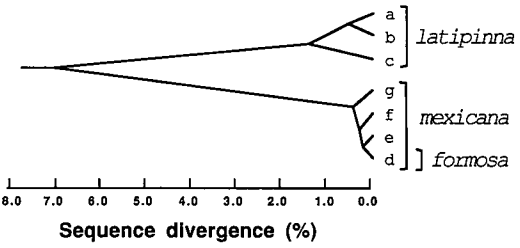

FIG. 1. UPGMA phenogram summarizing distances among the *Poecilia* mtDNA clones.

This study contributes to the growing catalogue of unisexual vertebrates for which the bisexual female ancestor has now been determined. It also contributes to the emerging view that most unisexual vertebrate species are evolutionarily young, and in terms of matriarchal phylogeny are embedded within the broader matriarchal diversity of their female sexual progenitors.

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