Mode of Inheritance and Variation of Mitochondrial DNA in Hybridogenetic Fishes of the Genus *Poeciliopsis*[1]

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A genetic survey of mitochondrial DNA (mtDNA) in a hybridogenetic complex of fishes (genus *Poeciliopsis*) was conducted to assess the possibility of low-level paternal transmission of mtDNA to progeny. In this reproductive system, females of the unisexual hybrid biotype between *P. monacha* and *P. lucida* effectively participate in a perpetual backcross to males of a sexual species, *P. lucida*. As judged on the basis of numerous restriction digests, the mtDNAs of the bisexual parental species (*P. monacha* and *P. lucida*) were highly distinct, yet the mtDNA of the natural hybridogens was not different from that of *P. monacha* from the same river system. Since the hybridogens are probably thousands of generations old, the present results demonstrate that paternal leakage of mtDNA must be extremely low or absent in these fishes. MtDNA genotypic differences among hybridogenetic strains were also present and corresponded to geographic locale. These differences provide a foundation for estimation of both origins and phylogeny of the unisexual forms.

Introduction

Poeciliopsis are small viviparous fishes that live in the rivers of northwestern Mexico. Some sexually reproducing species have hybridized in nature, giving rise to a series of diploid and triploid all-female biotypes (Schultz 1969; Moore 1984; Vrijenhoek 1984*a*). The unisexual diploid biotype *P. monacha-lucida,* the subject of this report, originated from hybridizations between *P. monacha* (♀) and *P. lucida* (♂) and reproduces by means of a semisexual process termed hybridogenesis (Schultz 1969). During hybridogenetic reproduction in *Poeciliopsis* (fig. 1), the haploid maternal set of chromosomes (M) is transmitted to the ova, while the paternally derived chromosomes (L) are discarded. This is accomplished in premeiotic oogonia through a unipolar spindle apparatus that attaches only to the M, leaving the L to degenerate in the apolar cytoplasm (Cimino 1972). In each generation, the diploid condition is reestablished through matings of hybridogenetic females with males of a sexual species. Protein-electrophoretic and tissue-grafting studies have corroborated the clonal pattern of inheritance of the M (Vrijenhoek 1972; Vrijenhoek et al. 1977, 1978; Angus and Schultz 1979; Angus 1980). Thus "leakage of paternal . . . genes into the maternal genome has not been observed in *Poeciliopsis*" (Moore 1984).

Whereas the above statements apply to the nuclear genome, in the present paper we examine the inheritance of a cytoplasmic genome, mitochondrial DNA (mtDNA), in hybridogenetic *Poeciliopsis*. The transmission of mtDNA is known to be predominantly maternal in higher animals (Dawid and Blackler 1972; Hutchison et al. 1974;

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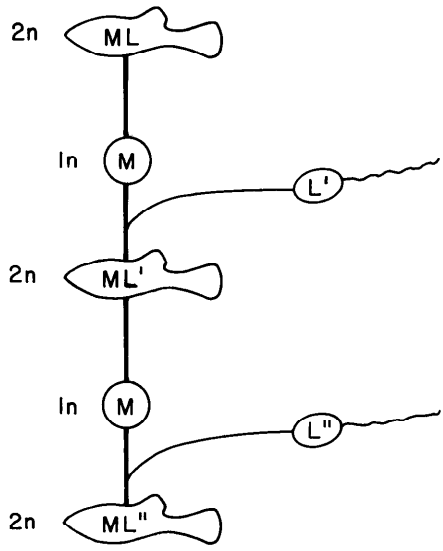

FIG. 1.—The hybridogenetic mode of reproduction in *Poeciliopsis*. The letters M and L refer to maternal and paternal nuclear genomes, respectively.

Hayashi et al. 1978; Avise et al. 1979; Francisco et al. 1979; Giles et al. 1980), but the possibility of a low-level paternal contribution of mtDNA has not been eliminated. Unisexual *P. monacha-lucida* provide an ideal natural situation in which to test for paternal leakage of mtDNA. Once formed, hybridogenetic females essentially participate in an endless backcross to males of *P. lucida*. Some naturally occurring hybridogenetic strains have been engaging in this backcross for thousands of generations (Leslie and Vrijenhoek 1980). Even a small amount of per-generation leakage of paternal mtDNA into the hybridogens should have resulted in the partial or complete replacement of *P. monacha* mtDNA with mtDNA of the sexual host, *P. lucida*.

The purposes of the present study are to (1) begin a characterization of restriction-site variation in mtDNA of *Poeciliopsis* and (2) capitalize on the unique advantages afforded by the hybridogenetic biotypes to critically assess the possibility of low-level paternal transmission of mtDNA.

Material and Methods

Seven strains of *Poeciliopsis* were assayed (table 1). The M61-31 strain of *P. lucida* (collected in the Rio del Fuerte in Sonora, Mexico, by R. R. Miller in 1961) was the paternal parent for each of the six hybridogenetic strains (fig. 1). Two hybridogenetic strains, ML/VII and ML/VIII, occur naturally in the Arroyo de Jaguari tributary of the Rio del Fuerte. The wild ancestors of these strains were captured near the Sonoran village of Agua Caliente (AC) and were given the collection numbers S68-4PCw (R. J. Schultz, 1968) and T70-3PCw (R. E. Thibault, 1970), respectively. Previous genetic studies based on allozymes revealed that ML/VII and ML/VIII had endemic origins at this locality (Vrijenhoek et al. 1978).

Wild *P. monacha* females used in the laboratory synthesis of new hybridogenetic strains were collected from two geographically separated river systems. The M genomes in synthetic hemiclones ML/H and ML/K derived from separate *P. monacha* females collected from the same locality (AC) that contained natural hemiclones ML/VII and

Table 1
Strains of *Poeciliopsis* Assayed for mtDNA Genotype

Biotype and Strain Designation	Strain (River, Site)	Mode of Reproduction	No. of Samples (No. of Fish Pooled in sample[s])
P. monacha-lucida:			
ML/VII	Natural[a] (Rio Fuerte, AC)	Hybridogenetic	2 (7,4)
ML/VIII	Natural[a] (Rio Fuerte, AC)	Hybridogenetic	1 (7)
ML/H	Synthetic (Rio Fuerte, AC)	Hybridogenetic	2 (3,6)
ML/K	Synthetic (Rio Fuerte, AC)	Hybridogenetic	1 (4)
ML/FF	Synthetic (Rio Mayo, TA)	Hybridogenetic	1 (3)
ML/S	Synthetic (Rio Mayo, TA)	Hybridogenetic	1 (4)
P. lucida:			
M61-31	Natural (Rio Fuerte, San Pedro)	Sexual	1 (6)
Total			9 (44)

[a] Maintained in the laboratory for 25–30 generations.

ML/VIII. Synthetic hemiclones ML/S and ML/FF derived from *P. monacha* females collected from a headwater tributary of the Rio Mayo (near El Tabelo, Sonora [TA]). The synthetic hybrid strains were produced by means of laboratory hybridizations within the past 5 years (Wetherington et al. 1987). Two of the synthetic ML strains (ML/H and ML/K) were in their fourth generation of existence as hybridogens, and two (ML/S and ML/FF) were in the third such generation. Because we had no wild *P. monacha* strains for comparative studies, we proceeded under the reasonable assumption that the M genomes of these synthetic ML strains exhibit the mtDNA of their recent *P. monacha* female parents.

MtDNA was purified in closed-circular form by means of cesium chloride/ethidium bromide gradient centrifugation (Lansman et al. 1981). In each workup, either fresh muscle tissue or eggs plus livers of three to seven pooled individuals was used (table 1). Both of these tissue sources provided more than adequate yields of mtDNA for successful assay by each of 15 informative restriction endonucleases: *Ava*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hin*cII, *Hin*dIII, *Msp*I, *Nde*I, *Pst*I, *Pvu*II, *Stu*I, and *Xba*I.

Restriction digestions of purified mtDNA were accomplished under conditions recommended by New England Biolabs. mtDNA fragments were end labeled with (α^{32}P)dNTP in the presence of the large fragment of *E. coli* DNA polymerase I and separated by means of 1.0% (1.8% for *Msp*I) horizontal agarose gels (Brown 1980). Fragments were visualized by means of autoradiography of vacuum-dried gels and sized by being compared with standards in a 1-kb ladder purchased from Bethesda Research Labs. For the 1% gels, no attempt was made to score fragments <500 bp in length. Estimates of nucleotide sequence divergence between mtDNA genotypes were computed by means of both the fragment and the site methods of Nei and Li (1979).

Results

mtDNA Characterizations

The 15 endonucleases utilized produced a total of 66–69 scored fragments in each *Poeciliopsis* strain (table 2). On the basis of the single-enzyme digestion profiles, the mtDNA genomes appear to be ~16.5–17.1 kb long. Uncertainties in exact size stem from the usual difficulties in (1) observing fragments < ~0.5 kb and (2) accurately

Table 2
MtDNA Genotypes Observed in Strains of *Poeciliopsis*

Genotype	Composite Designation[a]	No. of Fragments Scored				Strain
		Six-Base Enzymes[b]	Five-Base Enzymes[b]	Four-Base Enzymes[b]	Total	
1	MMMMMMMMMMMMMM	44	12	13	69	ML/VII; ML/VIII; ML/H; ML/K
2	MMMMM*NNN*M*N*M*N*MMM	43	11	12	66	ML/FF; ML/S
3	LLLLLLLLLLLLLL	37	12	17	66	M61-31

[a] Letters refer to the multifragment digestion profiles for enzymes in the following order: *Ava*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hin*cII, *Hin*dIII, *Msp*I, *Nde*I, *Pst*I, *Pvu*II, *Stu*I, and *Xba*I. M fragment patterns (or N patterns, which were one site gain or one site loss from M) were characteristic of *P. monacha;* the L fragment patterns were characteristic of *P. lucida.*

[b] The four-base enzyme (i.e., that having four bases in its recognition site) employed was *Msp*I; effective five-base cutters were *Ava*I and *Hin*cII; and all other enzymes had six-base recognition sequences.

sizing fragments > ~7.0 kb. Such size estimates are typical for mtDNA in many vertebrates, including fishes (Brown 1983; Bermingham et al. 1986).

Three different mtDNA genotypes were observed in the present study, two in the hybridogenetic forms and a third in *P. lucida* (table 2). Relationships among these genotypes are summarized in figure 2. In gel profiles for five restriction enzymes—*Bst*EII, *Eco*RI, *Hin*cII, *Msp*I, and *Pst*I—one hybridogenetic genotype, observed in Rio del Fuerte natural strains (ML/VII, ML/VIII) and synthetic strains (ML/H, ML/K), differs from the other, observed in Rio Mayo synthetic strains (ML/FF, ML/S). Each of these five profile changes can provisionally be accounted for in terms of a single restriction-site gain or loss. Overall, nearly 92% of the restriction fragments (and 98% of the restriction sites) are shared between the Rio del Fuerte and Rio Mayo *P. monacha-lucida* mtDNAs, and the nucleotide sequence divergence estimate is 0.5%.

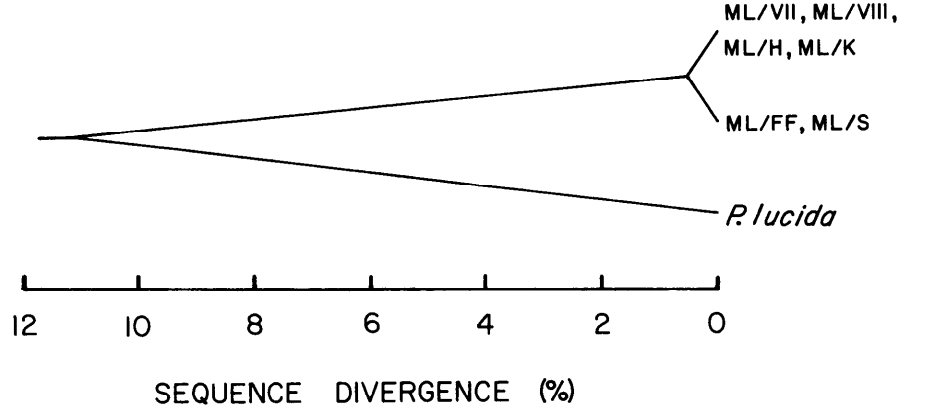

FIG. 2.—Phenogram (constructed by means of UPGMA clustering; Sneath and Sokal 1973, p. 230) summarizing relationships among the observed mtDNA genotypes in *Poeciliopsis.*

In contrast, *P. lucida* mtDNA differs dramatically from that of the *P. monacha-lucida* hybridogens (examples in fig. 3). For all 15 endonucleases, digestion profiles are distinct, and in no case can multifragment profile differences be attributed to single site gains or losses. The overall proportion of mtDNA fragments shared by *P. lucida* and either mtDNA genotype in *P. monacha-lucida* is 0.21, which translates into a nucleotide sequence divergence estimate of 11.5% (this assumes that all changes are due to base substitutions, a proposition that would ultimately need confirmation by means of direct nucleotide sequencing).

Tests of Paternal Leakage

The large number of mtDNA restriction-site differences between the *monacha* and *lucida* genomes provided many (redundant) opportunities to detect paternal leakage of mtDNA into the naturally occurring hybridogenetic strains, if leakage had in fact occurred. Nonetheless, we found no evidence for such paternal transmission of mtDNA. Altogether, 47 mtDNA fragments observed in *P. lucida* were absent in the mtDNA digests of all synthetic and natural hybridogenetic strains assayed (examples in figs. 3, 4).

In principle, paternal leakage of mtDNA could be evidenced in either of two ways, depending on the effective size (n) of the mtDNA population in germ-line cells (Gyllensten et al. 1985). First, in what we will refer to as the "sporadic input" scenario, if n is small (in the extreme case, only one or a few molecules per cell), a paternally derived mtDNA might rarely but quickly colonize a maternal line. With a neutral model, the per-generation probability (p) of this occurrence in any line is simply the proportion of mtDNA molecules contributed to a zygote by the sperm. In most species, mature oocytes carry huge numbers of mtDNA whereas sperm carry only a few (Dawid and Blackler 1972; Michaels et al. 1982), so p is often on the order of ≤ 0.0005 (Gyllensten et al. 1985). The detection of paternal leakage would then necessitate assay either of very large numbers of individuals or of progeny derived from a very large number of unidirectional backcross generations (g) to a paternal line. In the present study, the numbers of individuals (38) and hybridogenetic strains (6) assayed was only moderate (table 1), but the number of backcross generations involved in the evolutionary histories of the natural *P. monacha-lucida* biotypes is probably large (Leslie and Vrijenhoek 1980; Schenck and Vrijenhoek 1986). For the sake of argument, assume that each of the two natural hybridogenetic biotypes assayed is 1,000 generations old. In either strain, there would then have been 1,000 opportunities for paternal leakage of mtDNA, and, with $p \cong 0.0005$, the per-strain probability of detection of paternal leakage would be $\sim 1 - e^{-pg}$ or 0.39. Of course, the natural hybridogens may be much older than this, in which case the probability of detection of paternal leakage would be higher.

A second theoretical scenario for paternal leakage involves the gradual accumulation hypothesis. Here the effective size of the mtDNA population in germ cells is assumed to be large (n being perhaps several thousand or more). Sperm-mediated transfer of mtDNA into female lineages would then occur gradually and cumulatively, such that all progeny after any given g would possess some fraction of paternally derived mtDNA. When paternal leakage occurs at rate p, the total accumulated fraction of paternal mtDNA in g is $A_g = 1 - (1 - p)^{g+1}$ (Lansman et al. 1983), which, for small p, $\cong 1 - e^{-pg}$. If we again assume that the natural hybridogenetic biotypes of *Poeciliopsis* are $\sim$1,000 generations old and that $p = 0.0005$, then $A_g \cong 0.39$, a level that would have been detected in our gel autoradiographs (figs. 3, 4). Even if the

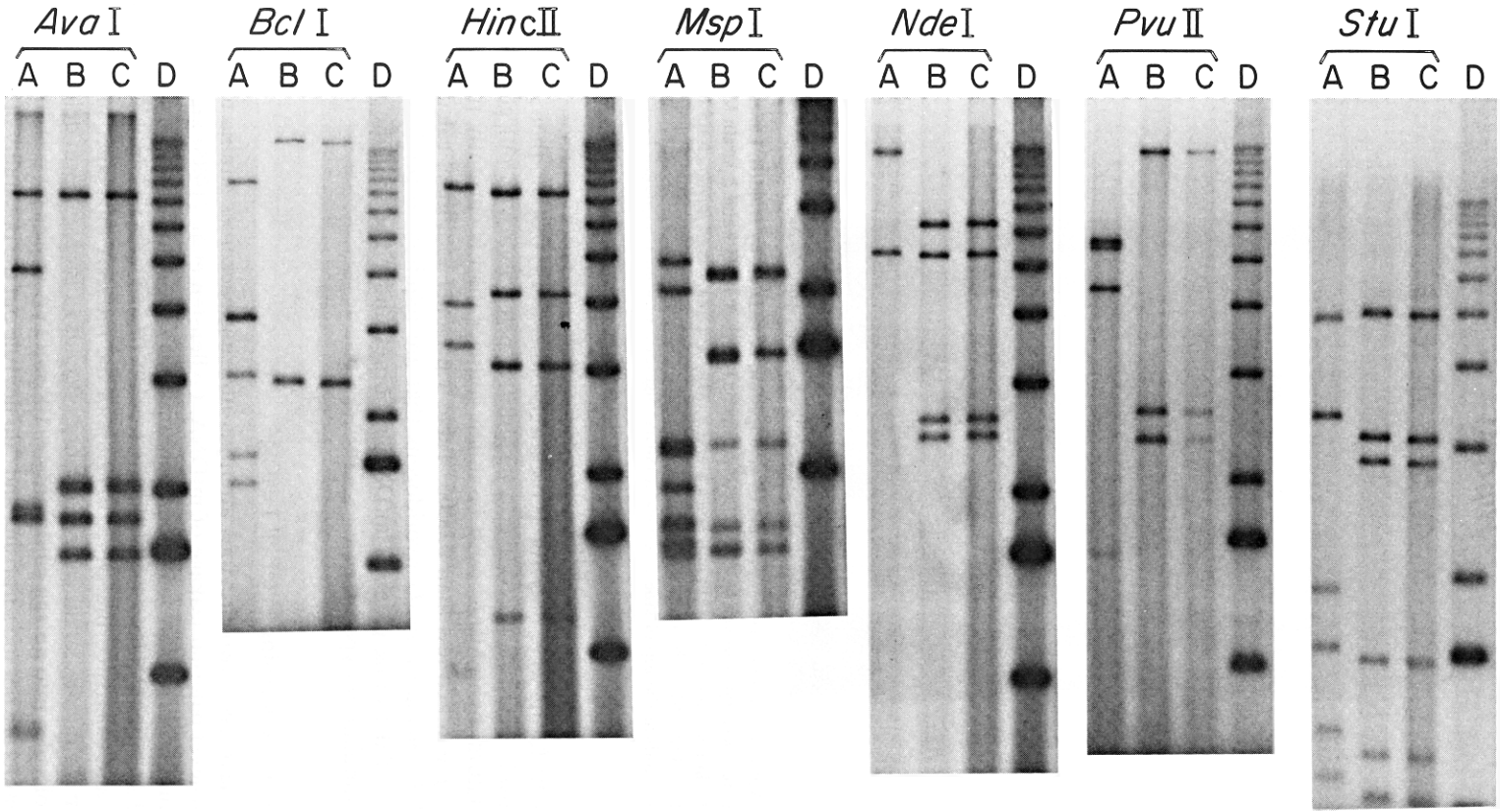

FIG. 3.—Selected autoradiographic patterns for mtDNA in strains M61-31 of *Poeciliopsis lucida* (lanes A) and ML/H of *P. monacha-lucida* (lanes B and C). In each lane D is a molecular-weight standard with fragment sizes (in kb), sequentially from bottom to top of gel, as follows: 1.0, 1.6, 2.0, 3.0, 4.1, 5.1, 6.1, 7.1, 8.1, 9.2, 10.2, 11.2, and 12.2.

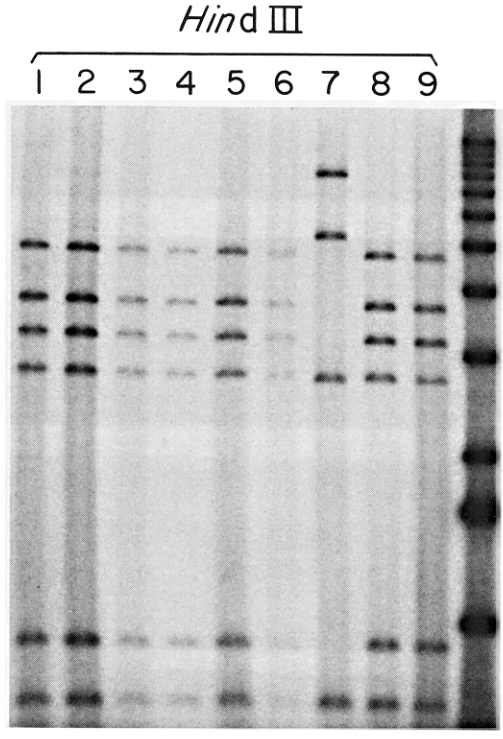

FIG. 4.—Autoradiographic patterns produced via digestion of mtDNA by *Hind*III. Lanes 1–6 and 8–9 from the left are *Poeciliopsis monacha-lucida* hybridogenetic biotypes: lanes 1 and 2, strain ML/VII; lanes 3 and 4, ML/K; lanes 5 and 6, ML/FF; and lanes 8 and 9, ML/H. Lane 7 is *P. lucida* strain M61-31. The molecular-weight standard in the rightmost lane is a 1-kb ladder described in the legend to fig. 3. The four extremely faint bands at the top of lane 2 represent partial digestion products, as judged by results of other gels.

hybridogenetic forms were only 100 generations old and $p = 0.0005$, then $A_g \cong 0.05$, a level probably near a conservative lower limit of detectability on our gels. Again, either the natural hybridogens must be much younger than is currently thought or, more likely, p is much less than 0.0005. Table 3 presents additional calculations of the ages of *P. monacha-lucida* hybridogens that are required to produce specified probabilities of detecting paternal mtDNA genomes under the sporadic input and gradual accumulation hypotheses.

Discussion

Considerations Concerning Paternal Leakage

There have been two previous attempts utilizing backcross strains to detect a possible low-level paternal leakage of mtDNA. Gyllensten et al. (1985) analyzed the 6–8-generation-backcross progeny of matings originally between *Mus musculus* (house mouse) and *M. spretus*. They observed no paternally derived mtDNA in the backcross individuals and calculated that *at least* 99.8% of the mtDNA molecules must be inherited from the female parent each generation. Because a mouse sperm delivers ~50 mtDNA molecules into an egg containing $\sim 10^5$ mtDNA copies ($p = 0.0005$), these backcross results are not too surprising and "do not rule out the possibility of a paternal

Table 3
Examples of g Required for *Poeciliopsis monacha-lucida* hybridogens to produce specified probabilities of detecting paternal mtDNA genomes

p	g BY PATERNAL LEAKAGE SCENARIO[a]	
	Sporadic Input[b]	Gradual Accumulation[c]
10^{-2} ...	70	5
10^{-3} ...	700	50
10^{-4} ...	7,000	500
10^{-5} ...	70,000	5,000
10^{-6} ...	700,000	50,000

[a] Calculated as $1 - e^{-pg}$.
[b] No. of g required for 50% of lineages to be fixed for paternal mtDNA.
[c] No. of g required for 5% (an amount that would have been scorable in our radiographs) of mtDNA in each strain to be of paternal origin.

contribution to mtDNA inheritance" (Gyllensten et al. 1985). Lansman et al. (1983) analyzed the 45- and 91-generation-backcross progeny of matings of male *Heliothis virescens* (tobacco budworm) to hybrid *H. virescens* × *H. subflexa* females. The backcross progeny exhibited *H. subflexa* mtDNA exclusively. The conditions of the experiment were such that a gradual paternal leakage of as little as 1 mtDNA molecule/25,000/generation backcross ($p = 0.00004$) would have been detected.

Results of the present study thus provide the third example of a failure to detect low-level paternal leakage of mtDNA in what are, in effect, advanced-generation backcross progeny. Since we do not know the exact evolutionary ages of the natural hybridogenetic strains of *Poeciliopsis* assayed, we cannot set a precise upper limit on magnitude of paternal leakage in this system. If, however, the natural hybridogens are even a few hundred or thousand generations old, as seems likely, any effective paternal leakage must be very low indeed (table 3), below the sperm:egg mtDNA ratios for at least some vertebrates for which this ratio is known (Michaels et al. 1982; Hecht et al. 1984; Gyllensten et al. 1985).

The reasons for failure to detect paternal leakage of mtDNA in all of the above-mentioned studies remain unclear. Particularly for the *Heliothis* and *Poeciliopsis* experiments, simple dilution effects attributable to the sheer preponderance of egg mtDNA in zygotes may not, when taken alone, be an adequate explanation, because the cumulative effects of low levels of paternal leakage under the gradual accumulation hypothesis should ensure that paternal mtDNA proportions would be elevated to detectable levels.

We suspect, however, that some version of the sporadic input hypothesis is a more likely mode by which any real paternal leakage of mtDNA might occur. In restriction-site surveys of numerous higher animals, the usual observation is of extensive mtDNA sequence heterogeneity among conspecific individuals—but of homoplasmy (the occurrence of predominantly a single mtDNA genotype) within each individual (Avise and Lansman 1983). Instances of heteroplasmy are known (Bermingham et al. 1986, and references therein), but they are sufficiently unusual to suggest that the heteroplasmic phase is quite transitory (Rand and Harrison 1986). Neutral models that might account for the joint observations of within-individual mtDNA homogeneity but between-individual mtDNA differences require small effective population sizes of

mtDNA molecules in intermediate germ-cell lineages, such that chance sampling drift leads to rapid mtDNA fixation (Chapman et al. 1982; Birky et al. 1983). In such neutral models, the probability that paternal mtDNA becomes fixed in a germ-line lineage is simply its frequency in the mtDNA pool of the zygote. If such a model is valid, then searches for rare and sporadic paternal leakage events would pose many of the same problems as would searches for newly arisen mutations. In the *Poeciliopsis* experiments, perhaps we simply failed to "capture" any of these rare instances of paternal takeover, despite the reasonable prospect of such detection if g for the hybridogenetic strains is large (table 3). Thus, searches for paternal leakage of mtDNA in other *Poeciliopsis* strains (and other species) should continue. In hybrid fishes of the genus *Morone,* one such putative instance of paternal leakage has been noted (Robert W. Chapman, personal communication).

Other hypotheses that might account for strict maternal inheritance of mtDNA involve more active mechanisms, such as directed degradation of sperm mtDNA in zygotes (Vaughn et al. 1980), a replication advantage for maternal mtDNA in the fertilized egg, or other epistatic mechanisms involving interactions between the nucleus and cytoplasm (Gillham 1978, p. 328; Gyllensten et al. 1985). However, it is apparent that at least in some cases the mtDNA from one species can survive, function, and replicate in the nuclear background of another species. In both the *Heliothis* and *Mus* experiments, for example, mtDNA genomes were placed through backcrossing into an almost totally heterologous nuclear background (Lansman et al. 1983; Gyllensten et al. 1985). In this particular respect, our current data with *Poeciliopsis* are probably less informative than those of earlier studies. In hybridogenetic "backcrossing," the nuclear genome is not progressively enriched with paternal nuclear DNA. Rather, the maternal mtDNA in each generation remains in a nuclear background that is 50% *monacha* and 50% *lucida* (fig. 1).

One previous study has also examined mtDNA evolution in a hybridogenetic complex (Spolsky and Uzzell 1986). The edible frog of Europe, *Rana esculenta,* is thought to have arisen by means of crosses between *R. lessonae* (L) males and *R. ridibunda* (R) females. *Rana esculenta* (RL) is maintained by means of hybridogenetic reproduction primarily involving crosses of RL females with L males. Surprisingly, most *R. esculenta* in central Europe now exhibit an L-type mtDNA, which is easily distinguishable from the R type (Spolsky and Uzzell 1986). However, unlike *P. monacha-lucida, R. esculenta* has males and females, and rare hybridogenetic crosses may involve RL males with L females. To account for the unexpected distribution of mtDNA, Spolsky and Uzzell (1986) raise two possibilities: "either (1) the original hybridizations were, contrary to expectations, mainly between *R. lessonae* females and *R. ridibunda* males or (2) each *R. esculenta* lineage has gone through at least one mating between an *R. esculenta* male and an *R. lessonae* female." The authors did not consider a third hypothesis—that of occasional paternal leakage of *R. lessonae* mtDNA into *R. esculenta* lineages during hybridogenetic crosses in the usual direction. In the present study, we have shown that such paternal leakage of mtDNA has not occurred in the hybridogenetic strains of *Poeciliopsis* assayed. Provided such results also apply to the *Rana* hybridogenetic complex, the original conclusions of Spolsky and Uzzell (1986) would then be bolstered.

mtDNA Divergence in *Poeciliopsis*

On the basis of data from the genetic strains employed in this survey, *P. lucida* and the *P. monacha* ancestors of our synthetic ML strains are highly divergent (11.5%)

in terms of mtDNA sequence. Brown et al. (1979) observed that mtDNA in mammals evolves at a rate of ~1%/lineage/Myr. If this rate applies to *Poeciliopsis,* the separation of *P. lucida* and *P. monacha* may have occurred ~5–6 Myr before the present.

Among the assayed hybridogenetic forms of *P. monacha-lucida,* the two observed mtDNA genotypes (which differ by ~0.5% sequence divergence) distinguish *monacha*-genome strains stemming from separate river drainages in Mexico (table 1). On the other hand, we could not distinguish on the basis of mtDNA genotype any of the assayed *monacha* genomes within a drainage. In particular, the naturally occurring hybridogenetic strains ML/VII and ML/VIII were identical at all 69 mtDNA restriction sites, despite being distinguishable in allozyme composition (Vrijenhoek et al. 1977, 1978), histocompatibility genotype (Angus and Schultz 1979), and a suite of ecological characteristics, such as food preference, predatory efficiency, and microspatial distribution (Vrijenhoek 1984*b;* Schenck and Vrijenhoek 1986). Such differences, however, are probably no greater than those that would be expected to exist between random draws of *monacha* genomes from the *P. monacha* gene pool in the Rio del Fuerte. Each successful hybridization between *P. monacha* and *P. lucida* isolates another *P. monacha* genome from the sexual gene pool, "freezing" whatever characteristics the *P. monacha* genomes encode (Vrijenhoek 1984*b;* Vrijenhoek and Wetherington, accepted). In laboratory experiments, synthetic hybridogenetic biotypes involving Rio del Fuerte *P. monacha* genomes combined with a common *P. lucida* background exhibit life-history differences that greatly exceed those between ML/VII and ML/VIII (Vrijenhoek and Wetherington, accepted). Thus, although the allozyme data strongly argue that ML/VII and ML/VIII arose from separate hybridization events in nature, the similarity in mtDNA genotype suggests that the two female *P. monacha* involved were closely related in a matriarchal pedigree. The mtDNA data presented in the present paper provide a solid foundation for further studies of maternal phylogeny (and of possible paternal leakage of mtDNA) in the *P. monacha-lucida* complex.

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