Title
Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates.

Journal
Journal of Heredity, 77(4)

ISSN
0022-1503

Authors
Bermingham, Eldredge
Lamb, Trip
Avise, John C

Publication Date
1986-07-01

DOI
10.1093/oxfordjournals.jhered.a110230

The Journal of Heredity 77:249–252. 1986.

Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates

ABSTRACT: The mitochondrial DNA of the bowfin fish and each of two species of treefrogs displays large-scale size variation. Within each species, mitochondrial genomes span more than a 700 base pair range, and the size polymorphism is localized to one portion of the genome. In addition, about 5 percent of the total 357 individuals surveyed were observed to carry two size classes of mtDNA. These findings are among the few documented instances of extensive within-species mtDNA size polymorphism and individual heteroplasmy, and constitute exceptions to previously reached generalizations about the molecular basis of mtDNA variation.

Eldredge Bermingham

Trip Lamb

John C. Avise

THE MOLECULAR BASIS of mitochondrial DNA (mtDNA) polymorphism has been studied in numerous mammalian and other higher animal species by restriction mapping and nucleotide sequencing techniques (reviews in Avise and Lansman[3] and Brown[7]). Two general conclusions from these earlier works are relevant to this report. First, most intraspecific mtDNA variation is due to base substitutions or to very small (a few base pair) addition/deletions[8]. Although some examples of large-scale mtDNA size differences among related vertebrates are known, mtDNA genome size within most assayed species has proved to be very stable[7]. Second, individual organisms usually appear homoplasmic; that is, they contain predominantly if not exclusively a single mtDNA genotype[3].

In this study, we report exceptions to both of these generalizations of mtDNA polymorphism. During the course of population genetic surveys of mtDNA in various species of frogs and fishes, we have uncovered several examples of: 1) macrovariation in the size of the mtDNA molecule (i.e., on the order of several hundred base pairs) among conspecifics, including members of local demes; and 2) heteroplasmy, in which at least two mtDNA genomes differing greatly in size coexist within an individual. Here we document these observations, comment on their frequencies of occurrence, and consider whether large-scale mtDNA size variation and heteroplasmy may be more prevalent in some groups of animals than in others.

Materials and Methods

Data will be presented for three species: the fish *Amia calva* (bowfin; Amiiformes, Amiidae) and the frogs *Hyla cinerea* (green treefrog; Anura, Hylidae), and *Hyla gratiosa* (barking treefrog). The bowfin were collected from 13 drainages in the southeastern United States, extending from the Santee-Cooper River in South Carolina to the Mississippi River. The treefrogs were collected from a series of adjacent catfish ponds near Auburn, Alabama. Totals of 52 bowfin and 305 treefrogs were assayed for mtDNA.

For each individual, mtDNA was isolated from fresh tissue (bowfin: heart; treefrog: heart, liver, muscle) by CsCl gradient centrifugation as described by Lansman et al.[18]. Restriction endonuclease digestions of purified mtDNA were carried out generally under conditions recommended by the vendor (New England Biolabs). Restriction fragments were end-labeled using Klenow and ^{32}P-αdNTP[5] and, following electrophoresis through 0.6 percent agarose gels, revealed by autoradiography[19]. Fragment sizes were compared against the 1-kilobase ladder standard available from Bethesda Research Labs.

The authors were affiliated with the Department of Genetics, University of Georgia, Athens, GA 30602. Dr. Bermingham's present address is: NMFS/CZES, Genetics, 2725 Montlake Blvd. E., Seattle, WA 98112. Dr. Lamb's present address is: Savannah River Ecology Laboratory, Drawer E, Aiken, South Carolina 29801. This work was supported by NSF grants BSR-8217291, BSR-8603775, and by an NIH predoctoral fellowship to E. B. Publication costs were supported by DE-AC09-76SROO-819 between the U.S. Department of Energy and the University of Georgia's Institute of Ecology. Please address reprint requests to Dr. John C. Arise, Department of Genetics, University of Georgia, Athens, GA 30602.

Results

Amia calva (*bowfin*)

In our surveys, mtDNA size variation and heteroplasmy were evidenced by concordant patterns of differences in digestion profiles produced by separate restriction endonucleases. For example, the mtDNA of bowfin "a" in Figure 1 exhibited an *Xba*I fragment that was about 500 base pairs (bp) smaller than its homologue in individual "b" (other fragments in their *Xba*I digestions appeared identical in size). In digestions with other enzymes (such as *Ava*I and *Pvu*II, Figure 1), the mtDNA of "a" consistently showed a digestion profile of same magnitude smaller size. Similarly, individual "c" was detectably heteroplasmic for two size classes of mtDNA, one of which was approximately 450 bp larger than the other. Because of the effective redundancy of information presented by separate restriction enzymes, conclusions about size differences and size heteroplasmy are not here confused with technical artifacts such as might result from incomplete restriction digestion.

Additional examples of size polymorphism and heteroplasmy in bowfin mtDNA are shown in Figure 2. Overall, bowfin mtDNA genomes ranged in size from about 16000–16900 bp, a difference of 900 nucleotide pairs. Maximum mtDNA size difference observed within a river was about 700 bp, and all sampled populations exhibited some large-scale mtDNA size variation. Among the 52 assayed fish, 4 (8 percent) were detectably heteroplasmic under our assay conditions. Each heteroplasmic fish exhibited two mtDNA size classes.

Hyla (*treefrogs*)

Patterns of size variation and heteroplasmy in the mtDNAs of *Hyla cinerea* and *H. gratiosa* are shown in Figure 3. Again, the documentation consists of concordant patterns of genome size change across digestion profiles of separate endonucleases. Additional examples of mtDNA size polymorphism for *H. gratiosa* are pictured in Figure 4.

The mtDNA genomes of *Hyla cinerea* and *H. gratiosa* are easily distinguished, exhibiting an estimated sequence divergence of more than 18 percent[17]. At the Auburn site, these species engage in extensive introgressive hybridization[14,20]. Nonetheless, any frog (whether hybrid or nonhybrid) exhibited either "*cinerea*-type" or "*gratiosa*-type" mtDNA, with no evidence of intra-individual mixtures of the two genome patterns[17A]. Thus we believe the examples of size polymorphism and heteroplasmy reported here are unrelated to the phenomenon of interspecific hybridization for these treefrogs.

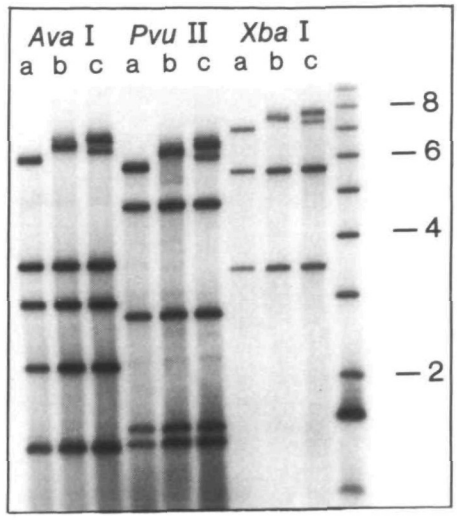

FIGURE 1 *Ava*I, *Pvu*II, and *Xba*I restriction fragment patterns for mtDNA from three specimens of *Amia calva*. Individuals "a" and "b" appear homoplasmic for mtDNAs differing in size; individual "c" is heteroplasmic for two mtDNA size classes. In the lane to the far right, approximate molecular weights in the standard (which is a 1 kilobase ladder available from Bethesda Research Labs) are indicated.

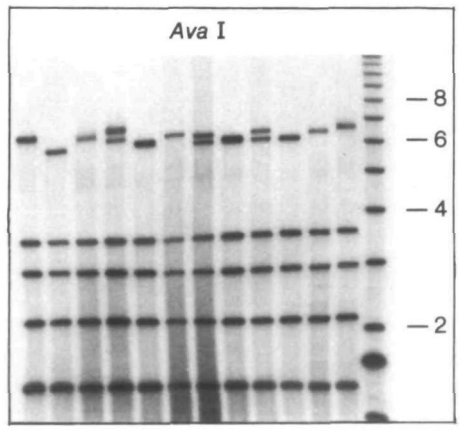

FIGURE 2 *Ava*I digests of mtDNA from 12 *Amia calva*. Individuals in lanes 4, 7, and 9 (from the left) appear heteroplasmic, and the mtDNAs of several other fish differ in size. Molecular weight standard is labeled as in Figure 1. Individuals were collected from the following rivers: lane 1, Santee-Cooper, South Carolina; lanes 2–4, Savannah, Georgia; lanes 5–7, Satilla, Georgia; lanes 8 and 9, St. Johns, Florida; lanes 10–12, Apalachicola, Florida. In this gel, as in the entire study, proportions of individuals heteroplasmic should be considered *minimal* estimates for two reasons: 1) differences among fragments similar in molecular weight may have remained undetected under our assay conditions; and 2) low frequency (<10 percent) size variants within the mtDNA pool of an individual would likely have gone undetected. There are obvious stoichiometric differences among mtDNA size classes within some individuals (see lane 4 in this figure).

Among frogs with *H. cinerea*-type mtDNA, genome size estimates ranged from 17500–18400 bp; among *H. gratiosa*-types, from 18000–18900 bp. Two of the 142 frogs (1.4 percent) carrying *cinerea* mtDNA were detectably heteroplasmic as were 13 of the 163 frogs (8 percent) carrying *gratiosa* mtDNA. No heteroplasmic frog exhibited more than two mtDNA types.

Characterization of the size variation

For both the bowfin and the treefrogs, it is tempting to speculate that the mtDNA size variation is localized in the D-loop (heavy strand origin of replication) and adjacent nontranscribed regions, areas that are known to harbor between-species macrovariation in mtDNA genome size in other vertebrates[6,7]. We have no direct evidence on the absolute position of the additions/deletions in *Amia* and *Hyla*, but in both groups the size differences are indeed localized (rather that scattered throughout the genome). Evidence for this conclusion stems from the apparent confinement of size heterogeneity of particular restriction fragments in various digestion profiles (examples in Figures 1–4). In the bowfin, among digestion profiles for 17 enzymes, the tightest bracketings of the variable-size region are accomplished by two *Hin*dIII sites and two *Hin*cII sites, both located about 3000 bp apart. Furthermore, no restriction sites were observed within the variable-size area. For example, the digestion profile for *Mbo*I (recognition site GATC) consists of numerous (>11) fragments smaller than 1500 bp, plus one larger fragment (~3000 bp) that exhibits the variation in size.

In *Hyla*, the size variation and heteroplasmy also appear localized to one region of the mtDNA genome. Among assays with five endonucleases, the tightest observed bracketing of the variable-size region was accomplished by two *Stu*I sites (in *H. cinerea*) located about 4900 bp apart.

It seems likely (though not proven) that the mtDNA size differences in *Amia* and *Hyla* represent products of individually large addition/deletion events. However, with our data we cannot exclude the possibility that independent (but localized) small-size variations arise and accumulate very rapidly, and that the larger differences in mtDNA genome size observed (e.g., within heteroplasmic individuals) are due to loss (extinction) of molecules intermediate in size.

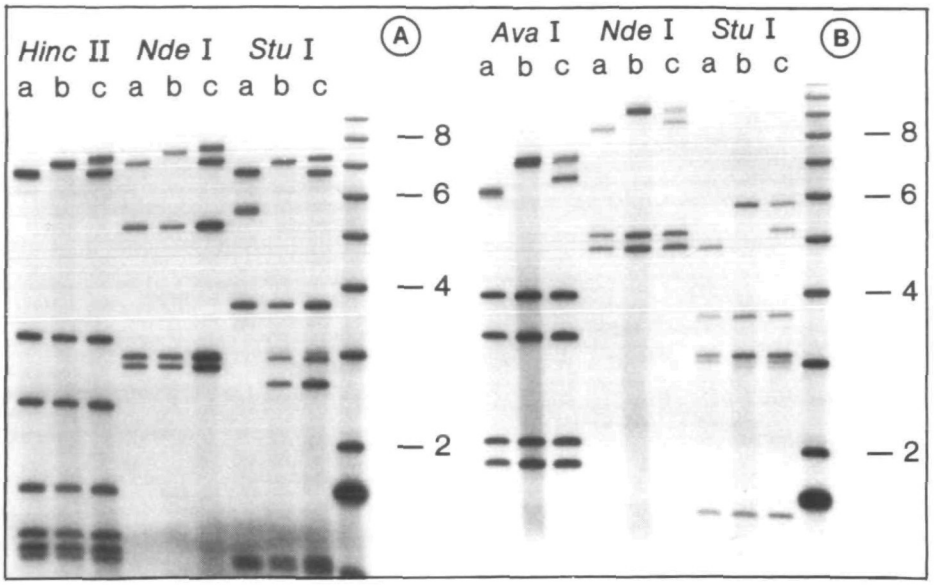

FIGURE 3 Selected restriction fragment profiles for mtDNA of *Hyla gratiosa* (*A*) and *H. cinerea* (*B*). In each species, individuals "a" and "b" appear homoplasmic for mtDNAs differing in size, and individual "c" is heteroplasmic for two mtDNA size classes. In the *Stu*I digests for *H. gratiosa* (*A*), frogs "b" and "c" also differ from frog "a" by addition of a restriction site that cleaves the 5.7 kilobase fragment into fragments of length 3.0 and 2.7 kilobases. Molecular weight standards are labeled as in Figure 1.

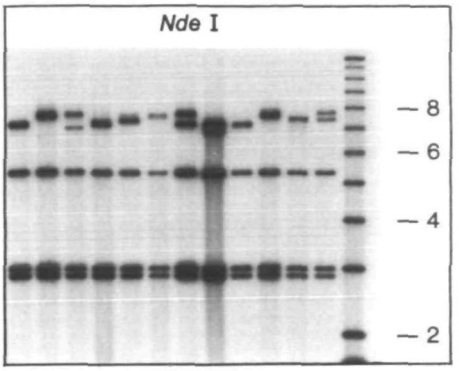

FIGURE 4 *Nde*I digests of mtDNA from 12 *Hyla gratiosa*. Individuals in lanes 3, 7, 8, and 12 (from the left) appear heteroplasmic, and the mtDNAs of several other individuals differ in size. Selected molecular weights are indicated to the right (see legend to Figure 1).

Discussion

Prerequisite to an understanding of mtDNA evolution is an adequate description of the kinds of genetic differences that exist and their relative frequencies of occurrence. Here we have documented examples of two molecular aspects of mtDNA polymorphism for which there is limited empirical precedent in higher animals: large-scale intraspecific size variation, and individual heteroplasmy.

Intraspecific macrovariation in mtDNA size

In higher animals as diverse as mammals, frogs, sea urchins, and insects, mtDNA genome size ranges from 15700 to about 20000 bp[7]. It is generally unknown whether these size differences represent long-term accumulated effects of numerous small (a few bp) addition/deletions, or alternatively whether they can arise through larger, discontinuous changes in genome size. From direct sequencing and fine-scale mapping techniques, microvariations in mtDNA size are known. Perhaps the best example involves human mtDNA, where 14 observed length variants in 112 individuals were due to additions or deletions each about 6–14 bp in length[8]. Size changes of 1 and 2 bp also have been reported in human mtDNA[1].

On the other hand, large-scale differences in mtDNA size (e.g., hundreds of bp) are known to occur between representatives of some closely related species. Such interspecific macrovariations in mtDNA size have been noted among *Drosophila* fruitflies[11], *Cnemidophorus* lizards[25], and *Hyla* treefrogs[17]. Gorilla mtDNA differs from that of other higher primates by a 95 bp deletion[13]. Intraspecific macrovariations in mtDNA size have been observed in several insect species[12,15,23,24], and in a few vertebrates (*Cnemidophorus* lizards[7,10] and *Rana* frogs[21]).

The finding of large-scale size variation within *Amia calva*, *Hyla cinerea*, and *H. gratiosa* is thus noteworthy. While our observations do not allow final conclusions about the mechanistic basis of changes in mtDNA genome size (e.g., rapid accumulation of individually small addition/deletions *and* saltational larger-size changes are both consistent with our data; sequencing or fine-scale mapping will be required to decide between these competing hypotheses), they nonetheless illustrate that large differences in mtDNA genome size can arise over a relatively short evolutionary time.

Individual heteroplasmy

There have been fewer well-documented reports of mtDNA heteroplasmy in higher animals. Small-size heteroplasmy (1–10 bp) has been reported in cattle[16,22]. Large-scale heteroplasmies (involving up to several hundred bp) have been found in the mtDNAs of *Drosophila mauritiana*[24], in two species of *Gryllus* crickets[15], in two species of *Cnemidophorus* lizards[10], and in *Rana esculenta* frogs[21]. Our findings for *Amia calva*, *Hyla cinerea*, and *H. gratiosa* thus provide some of the few direct documentations of mtDNA heteroplasmy in higher animals.

Given the great preponderance of mtDNA base substitution over addition/deletion changes in mtDNA evolution, it is surprising that for most of the species in which mtDNA heteroplasmy has been observed, large-scale *size* differences were involved. One possible explanation for this bias is the kind of technical information required to document heteroplasmy. Large-scale size polymorphisms alter simultaneously the digestion profiles for all endonucleases (indeed, such concordant alterations immediately signal an alert to size differences). In contrast, base substitutions affect only particular restriction sites. In routine population surveys, since the gel patterns for restriction site heteroplasmy are often difficult to distinguish from those expected for incomplete digests (see discussions in Avise et al.[2] and Avise and Lansman[3]), many true cases of restriction-site heteroplasmy may have remained unreported. If this suggestion is correct, more detailed molecular characterizations of mtDNA should eventually result in the documentation of a somewhat higher frequency of restriction-site heteroplasmy than is currently recognized. This in turn would be of significance to the development of evolutionary models for mtDNA, a prime concern of which has been

to understand the transitory heteroplasmic phase whereby intraindividual mtDNA polymorphisms are converted to interindividual differences[4,9].

We have some evidence against the possibility that the intraindividual mtDNA size polymorphisms in *Amia* and *Hyla* represent long-term evolutionary retentions of heteroplasmic states within particular lineages. If the mtDNA size classes are ancient *and have evolved independently after separation* (i.e., no gene conversion, recombination), they should have accumulated considerable sequence differences. Yet the various size classes within each species often exhibit identical restriction-site patterns (Figures 1–4).

Phylogenetic distribution of mtDNA size macrovariation

Despite the greater volume of data available for mammalian mtDNA, most of the known examples of large-scale, within-species size polymorphism and heteroplasmy of mtDNA occur elsewhere—in insects, fishes, frogs, lizards. These also are the animal groups for which larger between-species differences in mtDNA genome size are known. Indeed, the range of mtDNA size observed here within *Amia calva*, *Hyla cinerea*, and *H. gratiosa* is about as large as the maximum mtDNA size difference between any surveyed mammalian species (including rabbits, rodents, and primates[7]), or between any surveyed birds (including waterfowl, sparrows, and warblers[17]). These results suggest that mtDNA size macrovariation and heteroplasmy may be more prevalent in the lower vertebrates (and invertebrates) than in mammals and birds. Much additional data will be needed to verify this possibility.

Although we have emphasized examples of mtDNA macrosize polymorphism in this paper, it would be misleading to imply that this phenomenon is ubiquitous in species of lower vertebrates. For example, using similar restriction enzyme techniques, we have surveyed several species of teleost fish without noting any comparable instances of intraspecific mtDNA size variation[2, 3A]. Even within *Amia* and *Hyla*, most (~90 percent) of the individuals appear to be homoplasmic. Thus our findings should not be interpreted as a complete overthrow of previous generalizations about the major features of mtDNA evolution.

References

1. AQUADRO, C. F. and B. D. GREENBERG. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* 103:287–312. 1983.
2. AVISE, J. C., E. BERMINGHAM, L. G. KESSLER, and N. C. SAUNDERS. Characterization of mitochondrial DNA variability in a hybrid swarm between subspecies of bluegill sunfish (*Lepomis macrochirus*). *Evolution* 38:931–941. 1984.
3. —— and R. L. LANSMAN. Polymorphism of mitochondrial DNA in populations of higher animals. *In* Evolution of Genes and Proteins. M. Nei and R. K. Koehn, Eds. Sinauer, Sunderland, Mass. p. 147–164. 1983.

3A. BERMINGHAM, E. and J. C. AVISE. Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* In press 1986.

4. BIRKY, C. W., Jr., A. R. ACTON, R. DIETRICH, and M. CARVER. Mitochondrial transmission genetics: replication, recombination, and segregation of mitochondrial DNA and its inheritance in crosses. *In* Mitochondrial Genes. P. Slonimski, P. Borst, and G. Attardi, Eds. Cold Spring Harbor Lab., New York. p. 333–348. 1982.
5. BROWN, W. M. Polymorphism in mitochondrial DNA of humans as revealed by restriction endonuclease analysis. *PNAS* 77:3605–3609. 1980.
6. ——. Mechanisms of evolution of animal mitochondrial DNA. *Ann. N. Y. Acad. Sci.* 361:119–134. 1981.
7. ——. Evolution of animal mitochondrial DNA. *In* Evolution of Genes and Proteins. M. Nei and R. K. Koehn, Eds. Sinauer, Sunderland, Mass., p. 62–88. 1983.
8. CANN, R. L. and A. C. WILSON. Length mutations in human mitochondrial DNA. *Genetics* 104:699–711. 1983.
9. CHAPMAN, R. W., J. C. STEPHENS, R. A. LANSMAN, and J. C. AVISE. Models of mitochondrial DNA transmission genetics and evolution in higher eucaryotes. *Genet. Res.* 40:41–57. 1982.
10. DENSMORE, L. D., J. W. WRIGHT, and W. M. BROWN. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (genus *Cnemidophorus*). *Genetics* 110:689–707. 1985.
11. FAURON, C. M.-R. and D. R. WOLSTENHOLME. Structural heterogeneity of mitochondrial DNA molecules within the genus *Drosophila*. *PNAS* 73:3623–3627. 1976.
12. —— and ——. Intraspecific diversity of nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules of *Drosophila mauritiana*, *Drosophila melanogaster*, and *Drosophila simulans*. *Nucleic Acids Res.* 8:5391–5410. 1980.
13. FERRIS, S. D., A. C. WILSON, and W. M. BROWN. Evolutionary tree for apes and humans based on cleavage maps of mitochondrial DNA. *PNAS* 78:2431–2436. 1981.
14. GERHARDT, H. C., S. I. GUTTMAN, and A. A. KARLIN. Natural hybrids between *Hyla cinerea* and *Hyla gratiosa*: morphology, vocalization, and electrophoretic analysis. *Copeia* 1980:577–584. 1980.
15. HARRISON, R. G., D. M. RAND, and W. C. WHEELER. Mitochondrial DNA size variation within individual crickets. *Science* 228:1446–1448. 1985.
16. HAUSWIRTH, W. W., M. J. VAN DE WALLE, P. J. LAIPIS, and P. D. OLIVO. Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue. *Cell* 37:1001–1007. 1984.
17. KESSLER, L. G. and J. C. AVISE. A comparative description of mitochondrial DNA differentiation in selected avian and other vertebrate genera. *Mol. Biol. Evol.* 2:109–125. 1985.

17A. LAMB, T. and J. C. AVISE. Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: the influence of mating behavior. *PNAS* 83:2526–2530. 1986.

18. LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA, and J. C. AVISE. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. *J. Mol. Evol.* 17:214–226. 1981.
19. MANIATIS, T., E. F. FRITSCH, and J. SAMBROOK. Molecular Cloning. Cold Spring Harbor Lab., New York. 1982.
20. MECHAM, J. S. Introgressive hybridization between two southeastern tree frogs. *Evolution* 14:445–457. 1960.
21. MONNEROT, M., J.-C. MOUNOLOU, and M. SOLIGNAC. Intra-individual length heterogeneity of *Rana esculenta* mitochondrial DNA. *Biol. Cell* 52:213–218. 1984.
22. OLIVO, P. D., M. J. VAN DE WALLE, P. J. LAIPIS, and W. W. HAUSWIRTH. Nucleotide sequence evidence for rapid genotypic shifts in the bovine mitochondrial D-loop. *Nature* 306:400–402. 1983.
23. REILLY, J. G. and C. A. THOMAS, JR. Length polymorphisms, restriction site variation, and maternal inheritance of mitochondrial DNA of *Drosophila melanogaster*. *Plasmid* 3:109–115. 1980.
24. SOLIGNAC, M., M. MONNEROT, and J.-C. MOUNOLOU. Mitochondrial DNA heteroplasmy in *Drosophila mauritiana*. *PNAS* 80:6942–6946. 1983.
25. WRIGHT, J. W., C. SPOLSKY, and W. M. BROWN. The origin of the parthenogenetic lizard *Cnemidophorus laredoenis* inferred from mitochondrial DNA analysis. *Herpetologica* 39:410–416. 1983.