A Large-Subunit Mitochondrial Ribosomal DNA Sequence Translocated to the Nuclear Genome of Two Stone Crabs (*Menippe*)

Robin Schneider-Broussard† and Joseph E. Neigel**

*Department of Biology, University of Southwestern Louisiana; and †University of Texas, M. D. Anderson Cancer Center

Two DNA sequences that appear to be homologous to large-subunit mitochondrial ribosomal RNA genes have been identified in the stone crabs *Menippe mercenaria* and *M. adina.* Amplification from whole genomic DNA by polymerase chain reaction (PCR) with oligonucleotide primers based on conserved portions of large-subunit mitochondrial rRNA genes consistently amplified two products of similar length (565 and 567 bp). These products differed at 3% of their nucleotide bases, and could be distinguished by a *Hin*dIII site. Only one of these sequences (designated the A sequence) was detected by PCR in purified mitochondrial DNA. The other (designated the B sequence) hybridized to total genomic DNA at a level consistent with a nuclear genome location. It is unlikely that the type B product would have been recognized as a nuclear copy by examination of its sequence alone. This is the first report of a mitochondrial gene sequence translocated into the nuclear genome of a crustacean.

Introduction

Animal mitochondrial genomes have been regarded as effectively haploid because they are generally free of gene duplications and sequence variants within an individual. However exceptions to both of these generalizations have been recognized following the isolation of multiple forms of a mitochondrial sequence from single individuals. Duplicated mitochondrial genes have been found both within the mitochondrial genome (Moritz and Brown 1986, 1987; Heinonen, Schnare, and Gray 1990) and as sequences that have been translocated into the nuclear genome (van den Boogaart, Samallo, and Agsteribbe 1982; Farrelly and Butow 1983; Jacobs et al. 1983; Wright and Cummings 1983; Grohmann, Brennicke, and Schuster 1992; Smith, Kelley, and Patton 1992). Multiple forms of the mitochondrial genome within an individual (heteroplasmy) have been documented in insects, lizards, mice, cattle, seals, frogs, fish, and mussels (Solignac et al. 1984; Bentzen, Leggett, and Brown 1988; see references in Clark 1988; Boyce, Zwick, and Aquadro 1989; Hoeh, Blakley, and Brown 1991; Hoelzel, Hancock, and Dover 1993).

Duplications of mitochondrial genes are of significance in at least two respects. First, they provide an opportunity to study the evolution of a particular sequence under different conditions. For example, one copy may be constrained to perform its original function, while another copy is not. Thus translocations of mitochondrial genes into the nuclear genome can reveal the effects of genomic location on sequence evolution. However, these duplications are also significant because they have the potential to confound systematic and population genetic studies. In studies that utilize mitochondrial DNA (mtDNA) sequence variants as genetic markers, it is generally assumed that sequences compared between individuals or taxa are orthologous and are components of haploid, nonrecombining maternally transmitted genomes (Avise et al. 1987). With the now widespread use of the polymerase chain reaction (PCR) to amplify mitochondrial sequences from total genomic DNA, sequences that have been translocated to the nuclear genome may be mistaken for those expected from mitochondria unless they are clearly pseudogenes. For example, Quinn and White (1987) identified a translocated mitochondrial DNA sequence from inconsistencies in the Southern hybridization patterns of DNA isolated from different tissues. The source of the tissue determined which of the two sequences was obtained. The mitochondrial form was predominant in DNA from liver tissue, while a duplicated nuclear form was predominant in DNA from blood. Smith, Kelley, and Patton (1992) realized that a cytochrome *b* sequence was probably not of mitochondrial origin when sequence analysis revealed three stop codons that would prevent the translation of a functional protein product. Further work with purified mtDNA yielded a second, functional sequence. In each of these cases, the misidentification of the duplicated and translocated nuclear sequence was discovered because it happened to produce a striking anomaly. This suggests that there may be other, more subtle, cases in which similar occurrences have gone unnoticed. Here we report the occurrence of two mitochondrial large-subunit ribosomal DNA (LSrDNA) sequences in the stone crabs *Menippe adina* and *M. mercenaria* and provide evidence that only one sequence is located within the mitochondrial genome. We also examine the pattern of nucleotide substitutions in these sequences, and consider whether the putative nuclear copy would have been recognized as such by its sequence characteristics alone.

Materials and Methods

Specimens Examined

Stone crabs (genus *Menippe*) were collected in wire crab pots by local fishermen or by Dr. D. Felder from three locales. *Menippe adina* was collected from upper Terrebonne Bay near Cocoderie, La. *Menippe mercenaria* was collected from Rookery Bay, north of Marco Island near Naples, Fl. The third locality, Saint Simon Sound near Brunswick, Ga., is within the broader range of *M. mercenaria,* but may represent a hybrid zone be-

Key words: mitochondrial DNA, large-subunit ribosomal DNA, translocation, *Menippe.*

Address for correspondence and reprints: Joseph E. Neigel, Department of Biology, University of Southwestern Louisiana, Lafayette, Louisiana 70504. E-mail: jneigel@usl.edu.

Mol. Biol. Evol. 14(2):156–165. 1997
© 1997 by the Society for Molecular Biology and Evolution. ISSN: 0737-4038

tween *M. adina* and *M. mercenaria* (Bert 1986; Bert and Harrison 1988). Species determinations were based on locality as previously determined by Williams and Felder (1986). Crabs were transported to our laboratory alive in buckets of aerated seawater.

Sample Preparation

Within 24 hours of arrival, male crabs were placed on ice until cessation of movement and then dissected. Total genomic DNA (including nuclear and mitochondrial DNA) was isolated from testes by the procedure described by Neigel et al. (1991). Purified mitochondrial DNA (mtDNA) was isolated from individuals collected in Terrebonne Bay and Calcasieu Lake, Cameron Parish by the method of Thorpe, McGregor, and Cumming (1993). Briefly, mitochondria were pelleted by centrifugation, resuspended, and centrifuged through a 1.5 M/1.0 M sucrose step gradient. The mitochondria were then collected from the gradient interface, washed, and lysed with SDS and Proteinase K. The lysate was phenol/chloroform extracted, and DNA was precipitated by the addition of ethanol. Mitochondrial DNA was further purified by agarose gel electrophoresis. Distinct bands with mobilities corresponding to 15-kb supercoiled DNA molecules were excised from 0.7% agarose gels stained with 500 ng per ml ethidium bromide. DNA was recovered from gel slices with Prep-A-Gene (Biorad) as per the manufacturer's specifications.

Mitochondrial Genome Size

The size of the mitochondrial genome was determined by restriction endonuclease digestion and agarose gel electrophoresis. Purified mitochondrial DNA was digested with *Hin*dIII and *Ava* I as per supplier's instructions. The digested DNA was end-labeled with [α-^{32}P]dCTP and electrophoresed on 0.6% agarose gels. Fragment sizes were estimated by the method of Schaffer and Sederoff (1981). The total size of the mitochondrial genome was calculated by summing the individual fragment sizes.

Primer Design and Synthesis

Oligonucleotide primers for both PCR and DNA sequencing were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. The sequences of the amplification primers matched a region known to be highly conserved within the 3′ portion of the mitochondrial large-subunit ribosomal RNA (LSrRNA) gene and have been designated 16sar-L and 16sbr-H (Palumbi et al. 1991). M13 forward and reverse primers were used for initial sequencing of cloned PCR amplification products. For more sequence information, two additional primers that match *Menippe* LSrRNA sequences were designed with the NAR program (Rychlik and Rhoads 1989). We have designated these: 16sar-M (ATAAGACCCTATAAAGC) and 16sbr-I (CCGCCCCAGCAAAATAAA).

Amplification and Cloning of PCR Products

PCR amplifications were performed using one cycle of 95°C for 5 min, followed by 35 cycles, each of 94°C for 1 min; 53°C for 1.5 min; ramp for 2 min to 72°C and hold for 2.5 min, followed by one cycle of 72°C for 5 min. Each reaction mixture included 20 ng of template DNA; 0.4 μM of each primer; 0.2 mM of each dNTP; 10 mM Tris, pH 8.3; 50 mM KCl; 4.5 mM $MgCl_2$; 0.01% gelatin; and 0.6 U *Taq* DNA polymerase (Boehringer Mannheim).

Amplification products were electrophoresed along with size standards on 1.5% agarose gels in 1 × TBE stained with ethidium bromide. Products of amplification reactions that produced a single strongly staining band of the expected size were directly cloned into the pCRII vector (Invitrogen Corporation) and transformed into One-Shot competent cells using the TA cloning system according to the supplier's specifications. Clones were screened and characterized by restriction profiles for *Alu* I and *Hin*dIII (New England Biolabs).

Sequence Determination and Analysis

For sequencing, plasmid DNA was prepared on a large scale for selected clones (Birnboim and Doly, 1979; Maniatis, Fritsch, and Sambrook 1982). Sequences were determined using dideoxy termination (Sanger, Nicklen, and Coulson 1977; Chen and Seeburg 1985). Sequence alignments were performed manually and with CLUSTAL (Higgins and Sharp 1988, 1989). A maximum-parsimony cladogram was generated by MEGA (Kumar, Tamura, and Nei 1993) using the Branch-and-Bound Search option to examine the relationships of these sequences to homologous sequences from other animal taxa. Sequences from the liver fluke, *Fasciola hepatica* (GenBank accession X07364), and the purple sea urchin, *Strongylocentrotus purpuratus* (X12631), were used as outgroups. The remaining sequences were from arthropods. The marsh crab *Sesarma reticulatum* (unpublished data) is classified in the same section (Brachyrhyncha) as *Menippe.* The hermit crabs *Clibanarius vittatus* and *Coenobita sp.* (Cunningham, Blackstone, and Buss 1992) are in the same order (Decapoda). The brine shrimp, *Artemia sp.* (M21833) is in the same subphylum (Crustacea), and the mosquito *Aedes albopictus* (X01078) is in the same phylum (Arthropoda).

A hypothetical secondary structure for the *Menippe* mitochondrial large-subunit ribosomal RNA sequence was developed by comparison of an *M. adina* type A sequence with sequences for which secondary structures have been determined: from a mosquito, *Aedes* (Hsu-Chen, Kotin, and Dubin 1984), and from a branchiopod crustacean, *Artemia* (Palmero, Renart, and Sastre 1988). A diagram of this structure was generated using the CARD program (Winnepenninckx et al. 1995).

Southern Blot Analyses

Southern hybridization (Southern, 1975) was used to map restriction sites within and adjacent to the amplified portion of the LSrRNA sequence in mtDNA. Single and double digests of 10-μg purified mtDNA samples using *Alu* I, *Hae* III, *Hin*fI, *Hin*dIII, and *Rsa* I (New England Biolabs) were electrophoresed through a 1.5% agarose gel. The gel was blotted onto a MagnaGraph nylon membrane (Micron Separations Inc.) and hybridized at 67°C overnight in 5 × SSC with a hybridization

probe of digoxigenin-labeled type B *Menippe* large-subunit rDNA (see Results for definition of a type B sequence). The labeled probe was generated by PCR amplification from a cloned type B sequence that had originally been amplified from *M. adina.* Immunological detection of the hybridized DNA fragments was performed according to manufacturer's specifications for the Genius Nonradioactive DNA Detection Kit (Boehringer Mannheim).

Genomic copy numbers were estimated by Southern hybridization of a ^{32}P-labeled cloned type B probe to genomic DNA digested with *Hin*dIII. Digests were electrophoresed through a 0.6% agarose gel, which was blotted and hybridized as described above. Serially adiluted samples of a cloned type B sequence corresponding to molar equivalents of from 1 to 2,000 copies per genome were probed on the same blot as internal standards. Autoradiographs were exposed for up to 2 weeks, after which hybridization was quantified by scanning the autoradiographs with a Zeineh SLR Scanning Laser Densitometer.

Results

PCR Products

Electrophoresis of PCR amplification products from *Menippe* genomic DNA with primers 16sar-L and 16sbr-H revealed a single intensely staining band. The mobility of this band corresponded to a molecule with the expected length of approximately 580 bp. However, restriction endonuclease digestion of the PCR product with either *Hin*dIII or *Alu* I produced fragments with estimated sizes that summed to over 580 bp (fig. 1). Repeated amplifications from genomic DNA samples isolated from nine different individuals, followed by restriction endonuclease digestions under different conditions, failed to eliminate any of the fragments. Because every *Hin*dIII site contains an *Alu* I site, these results could be explained by amplification of two products of equal length that differed in the presence of one *Hin*dIII/*Alu* I site. The product with two *Hin*dIII sites (three *Alu* I sites) was designated type A, the other, with a single *Hin*dIII site (two *Alu* I sites) was designated type B. In general, the quantity of the type B product that was amplified was less than that of the type A product (on both a molar and mass basis), as judged by ethidium staining intensity.

PCR products from one individual from each locale (*M. adina* from Louisiana, *M. mercenaria* from Florida and Georgia) were cloned, and analyzed by restriction digests with *Hin*dIII and *Alu* I. For the PCR products from the *M. adina* individual and the *M. mercenaria* from Georgia, eight of the nine clones produced the fragments predicted for the type A sequence; the remaining clone produced the fragments predicted for the type B sequence. For the PCR product from the *M. mercenaria* individual from Florida, 19 of the 20 clones produced the fragments predicted for the type A sequence; the remaining clone produced the fragments predicted for the type B sequence. This procedure was repeated for genomic DNA samples from a total of nine individuals, with three individuals sampled from each collection locale. Clones from all except two of these individuals included both type A and type B sequences as assessed by *Alu* I digestion profiles. Although restriction analysis indicated that both sequences were present in the PCR products of the other two individuals, only the more abundant type A sequence was found among the clones that were examined.

Sequence Analysis

Nucleotide sequences were determined for both strands of the nine type A and seven type B PCR products that were amplified and cloned as described above. These sequences have been deposited in GenBank; accession numbers are given in table 1. Restriction maps based on these sequences (fig. 2) verified our interpretation of the alternate restriction fragment profiles. All of the sequences appeared to be homologous to the 3′ portion of known crustacean mitochondrial LSrRNA genes.

The cladogram (fig. 3) illustrates the relationships of the *M. adina* and *M. mercenaria* type A and B sequences to each other and to other invertebrate LSrDNA sequences. It appears that both sequences are of crustacean origin and are more similar to each other than to other crustacean sequences. Furthermore, although both type A and type B sequences varied among individuals, they are clearly resolved as distinct clusters. The marsh crab, *Sesarma reticulatum,* is also under investigation in our laboratory, and so was considered a possible source of contamination. However, the sequence obtained by PCR amplification from this species is clearly different from any sequence that we have obtained from *Menippe.*

The type A and B sequences differed by at least 18 base substitutions, with transitions outnumbering transversions. The most common type A sequence (sequence 1) was compared with the most common type B sequence (sequence 7). These two sequences were also the most similar among comparisons of type A with type B sequences. The location of each substitution was examined with respect to the hypothetical RNA secondary structure shown in figure 4. In addition to the designated stem and loop regions, there were several regions of undetermined structure that would be expected to base-pair with nucleotides outside the known sequence. Substitutions were classified as transitions or transversions, and by their locations within the hypothetical RNA secondary structure. The lowest proportion of base substitutions occurred within the stem regions (table 2). Alignment of type A and B sequences also indicated a two-base deletion in the type A sequence (after position 253) in a presumed loop region. All of the substitutions between these sequences that were in presumed stem regions preserved base-pairing, with uracil pairing with either adenine or guanine.

Polymorphisms were also found among sequences of the same type from different individuals (table 3). Both in terms of number of polymorphic sites and numbers of base substitutions in pairwise comparisons, type A sequences were more variable than type B sequences. However, the number of sequences examined is not suf-

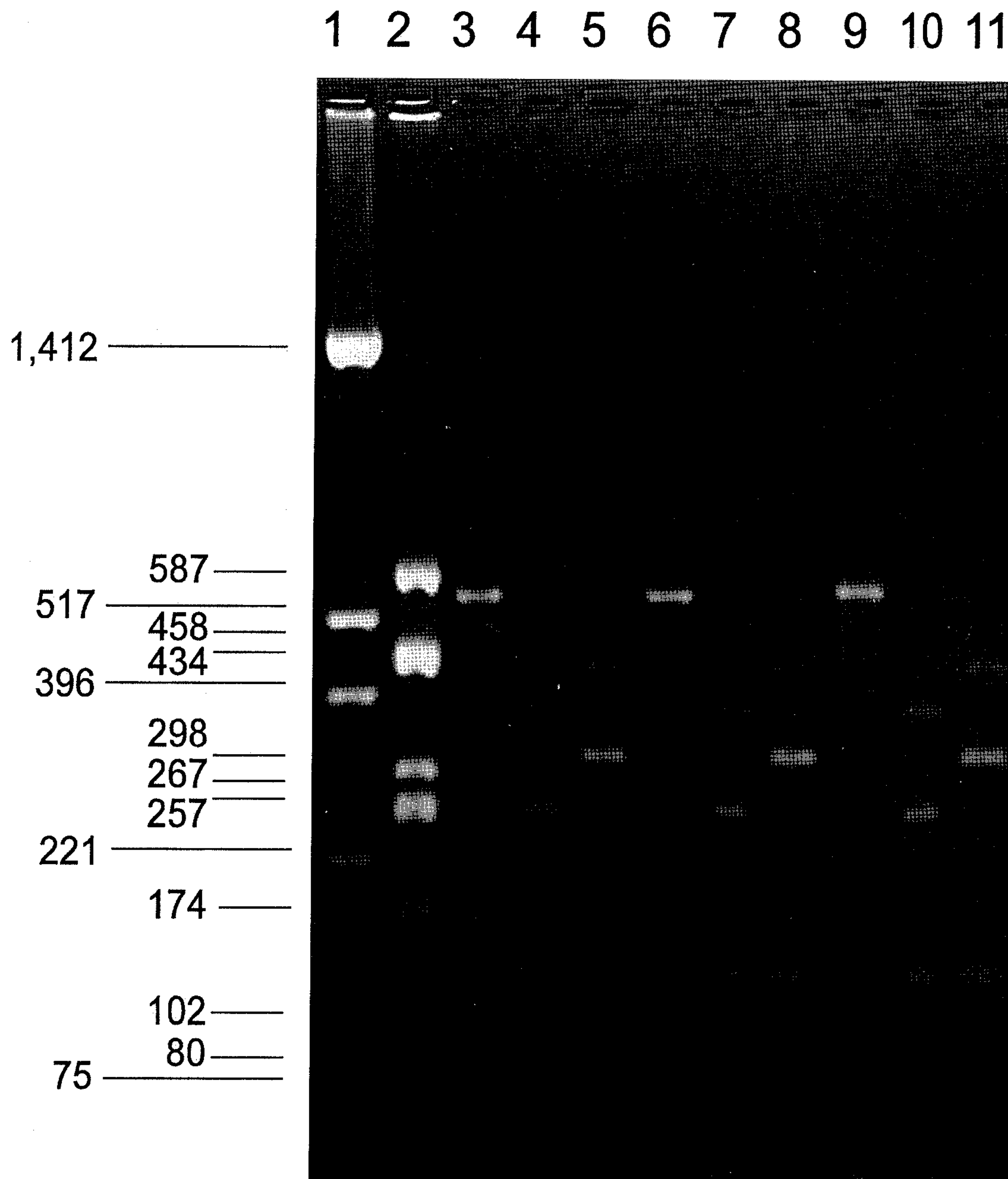

FIG. 1.—Agarose gel electrophoresis of PCR amplification products. The amplification products in lanes 3, 6, and 9 are from three individuals: *M. adina* (LA), *M. mercenaria* (FL), and *M. mercenaria* (GA), respectively. The PCR amplification products were restriction digested with *Alu* I, (lanes 4, 7, and 10), and *Hind*III, (lanes 5, 8, and 11). Lengths in base pairs are indicated for size standard fragments from pUC 18 digested with *Hin*fI (lane1) and *Hae* III (lane 2).

ficient to demonstrate statistically significant differences in these parameters. Only one exception to conservation of base-pairing in a stem region was found. This occurred in sequence 4, a type A sequence, at position 504 (see fig. 4). This uracil-to-cytosine transition would prevent base-pairing with the adenine at position 511 in our hypothetical secondary structure. However, these positions are flanked by G-C pairs, which may maintain overall stability of the structure. Among the type A sequences, a polymorphism consisting of a pair of substitutions that would preserve complementary base-pairing was found at positions 478 and 493.

Genomic Locations of Sequences

Several lines of evidence suggest that only the type A sequence is present within the *Menippe* mitochondrial genome. First, PCR products amplified from purified mtDNA and digested with *Hind*III did not produce the visible 434-bp fragment expected for type B sequences on ethidium-stained gels, but did produce the 321-bp and 111-bp fragments expected only from type A sequences, as well as the 132-bp fragment expected from both sequence types (fig. 5*A*). In contrast, *Hind*III fragments expected from both type A and type B sequences were produced by digestion of the products amplified

Table 1
Sequences and GenBank Accession Numbers

Sequence	Accession	Individual	Source
Type A sequences			
1 . . .	U20744	56	*M. adina*
1 . . .	U20744	72	*M. adina*
1 . . .	U20744	42	*M. mercenaria*—Florida
1 . . .	U20744	54	*M. mercenaria*—Georgia
1 . . .	U20744	101	*M. mercenaria*—Georgia
2 . . .	U20746	66	*M. adina*
3 . . .	U20739	38	*M. mercenaria*—Florida
4 . . .	U20742	95	*M. mercenaria*—Florida
5 . . .	U20743	94	*M. mercenaria*—Georgia
Type B sequences			
6 . . .	U20745	56	*M. adina*
7 . . .	U20748	72	*M. adina*
7 . . .	U20748	95	*M. mercenaria*—Florida
7 . . .	U20748	101	*M. mercenaria*—Georgia
8 . . .	U20740	38	*M. mercenaria*—Florida
9 . . .	U20742	42	*M. mercenaria*—Florida
10 . . .	U20750	94	*M. mercenaria*—Georgia

from total genomic DNA, a mixture of organellar and nuclear DNA (figs. 1 and 5*A*). This comparison was repeated for three other mtDNA isolations from different individuals, with similar results. The *Hin*dIII-digested PCR products from an additional individual were probed on a Southern blot with a cloned type B sequence. Southern hybridization can detect quantities of DNA below 100 pg, while ethidium staining can detect quantities of DNA only as low as about 25 ng. Again, the 321-bp *Hin*dIII fragment expected from a type A sequence was detected in the products amplified from purified mtDNA, but the 434-bp fragment expected from the type B sequence was not detected. Fragments expected for both sequence types were detected in the product amplified from total genomic DNA (data not shown).

The actual sizes of the putative nuclear sequence restriction fragments could not be predicted, because each of the fragments would extend to restriction sites outside of the region that we have characterized. However, a Southern blot of total genomic (mitochondrial and nuclear) DNA, digested with *Hin*dIII and probed with a ^{32}P-labeled cloned type B mitochondrial LSrDNA produced two strongly hybridizing bands with mobilities corresponding to fragments expected from the type A sequence (8.2 and 3.7 kb, see below), and two weakly hybridizing bands (1.8 and 1.4 kb) that were above the minimum sizes predicted for the type B sequence (fig. 5B). These results can be most simply explained if the weakly hybridizing bands correspond to the type B sequence located within the nuclear genome. Because there are generally more copies of the mitochondrial genome per cell than copies of the nuclear genome, differences in hybridization intensity are expected. Estimates of copy number from laser densitometer scans of Southern blot autoradiographs compared with internal standards indicated that about one copy of the type B sequence is present per haploid nuclear genome, while there are about 600 copies of the type A sequence (data not shown).

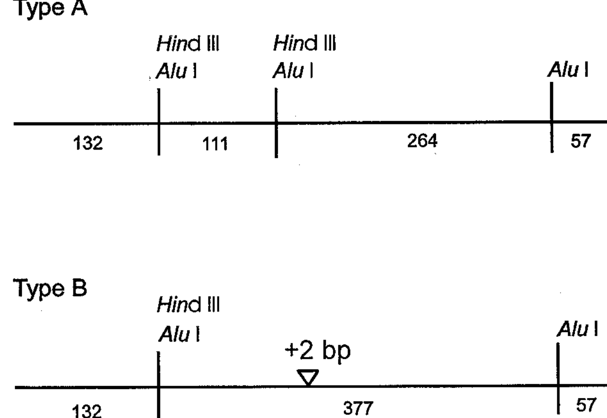

FIG. 2.—Restriction site maps of the amplified region of the *Menippe* mitochondrial large-subunit rRNA based on sequence data. Fragment sizes in base pairs are those expected for *Alu* I digests of PCR products, and include primer sequences. The 2-bp insertion in the type B sequence is indicated.

We used restriction mapping to address the possibility of a duplication of the LSrDNA sequence within the mitochondrial genome. We estimated the size of the *Menippe* mitochondrial genome from restriction fragment analysis of DNA that was extracted from mitochondria isolated on sucrose gradients. The estimated size, 16.5 kb, is typical for metazoa, and not indicative of a large duplication within the genome. Southern hybridization analysis was used to develop a restriction site map for the region of the mitochondrial genome that hybridized to the amplified portion of the LSrDNA sequence. Only one of the two sequences will fit within this region, and the locations of the *Alu* I sites are diagnostic for the type A sequence (fig. 6).

Discussion

Animal mitochondrial genomes are generally haploid (Brown 1985), and analysis of mitochondrial nucleotide sequence data usually assumes this condition. However, multiple sequences of mitochondrial DNA have been isolated from single individuals. These exceptions were due to either heteroplasmy or duplication

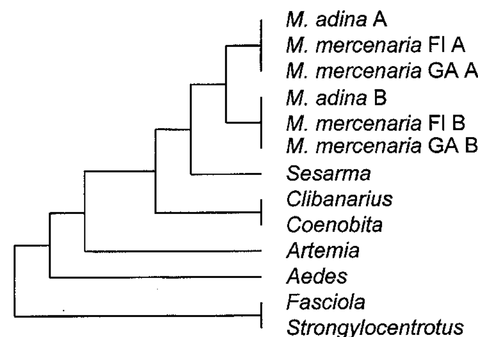

FIG. 3.—Maximum-parsimony cladogram of *Menippe* and other invertebrate large-subunit rRNA sequences. Representative type A (A) and type B (B) sequences are shown for *M. adina* and for *M. mercenaria* from Florida (FL) and Georgia (GA). GenBank accession numbers or literature citations for all sequences are provided in the text.

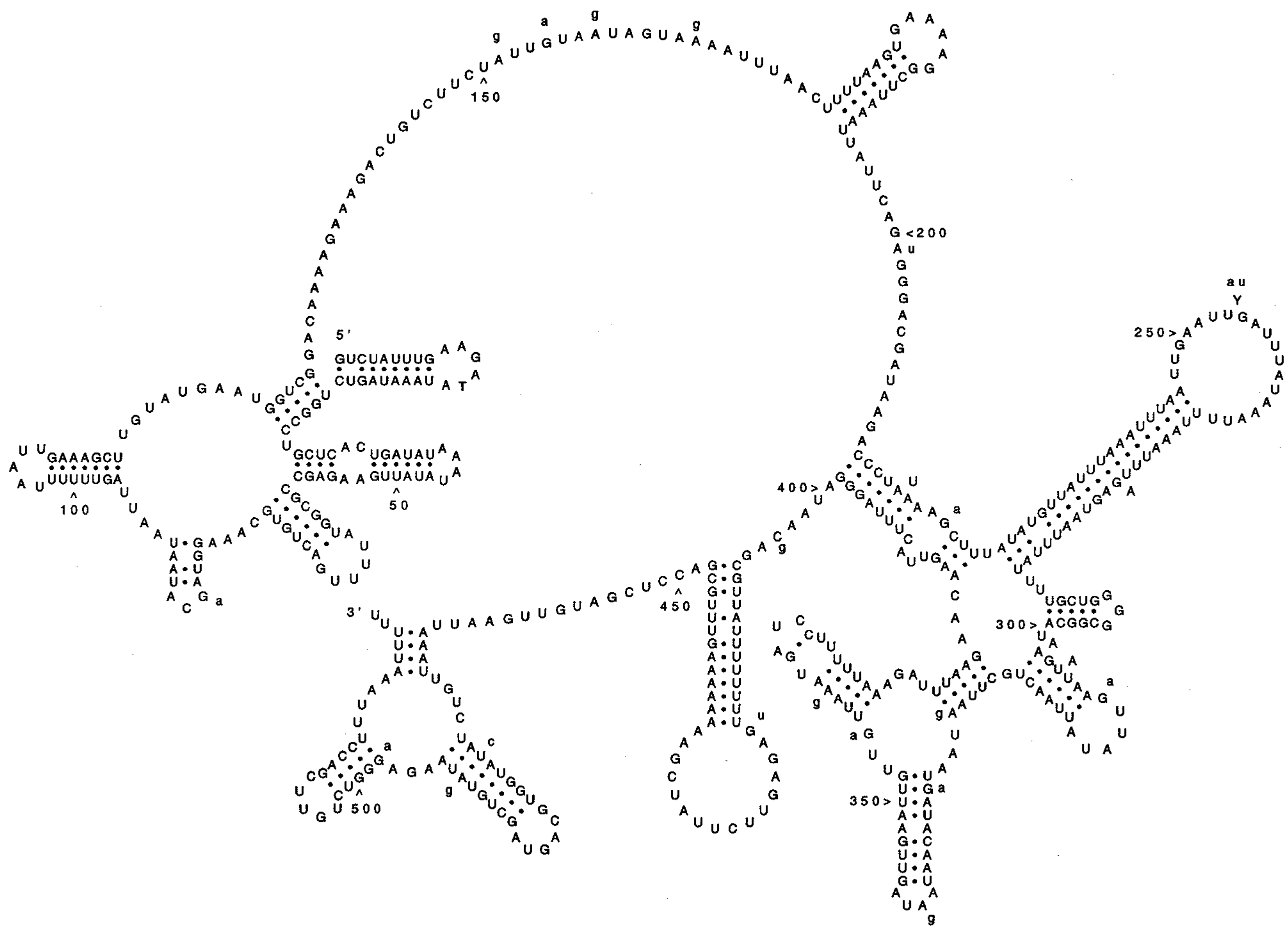

FIG. 4.—Hypothetical secondary structure of the amplified portion of the large-subunit rRNA sequence. This structure was based on manual comparisons of the type A sequence from *Menippe adina* with those from a mosquito (*Aedes*) and a branchiopod crustacean (*Artemia*). The base substitutions found in the type B sequence are shown adjacent to the type A sequence base.

of a mitochondrial sequence, the latter either within the mitochondrial genome or at a nuclear location. A third possible explanation for the detection of multiple sequences is contamination, either by other organisms (e.g., parasites) or by exogenous DNA in the laboratory.

It is unlikely that the presence of two sequences in the PCR product can be explained by contamination. Cladistic analysis of the two LSrDNA sequences indicates that they are both of crustacean origin, and closely related (fig. 3). No parasites were observed in the tissues used as sources of DNA, and undetected parasites would be not be expected to carry *Menippe*-like sequences.

Our results can be explained by a nuclear genome location for the type B *Menippe* LSrDNA sequence. Only the type A sequence was detected in LSrDNA PCR products amplified from purified mtDNA, while both type A and type B sequences could be detected in products amplified from total (nuclear plus mitochondrial) DNA. On Southern blots of genomic DNA from both *Menippe adina* and *M. mercenaria* that were probed with type B LSrDNA, faintly hybridizing bands were observed that were of sufficient length to include the PCR amplified portion of the type B sequence. The intensity of hybridization to these fragments was at a level

Table 2
Base Substitutions Between Type A and Type B Sequences

	Bases Examined	Substitutions	Percent Substitutions	Number of Transitions	Number of Transversions	Transition : Transversion Ratio
Stems.	272	5	1.8	5	0	3
Loops	170	8	4.7	6	2	3
Undetermined.	83	5	6.0	4	1	4
Total	525	18	3.5	15	3	5

Table 3
Variation Within Each Type of Sequence

	Number of Comparisons	Polymorphic Sites (total)	Polymorphic Stem Sites	Polymorphic Loop Sites	Percent Stem Sites	Substitutions		
						Minimum	Mean	Maximum
Type A.........	36	11	4	6	36	0	3.4	9
Type B.........	21	8	2	5	25	0	2.3	6

Note.—All pairwise comparisons were made for nine type A sequences and seven type B sequences.

consistent with a single-copy nuclear sequence, and was much lower than intensity of hybridization to the type A sequence. This is consistent with a nuclear location for the type B sequence and a lower number of copies of the nuclear genome per cell relative to the mitochondrial genome.

Our results cannot be readily explained by either heteroplasmy or a duplication of the LSrDNA sequence within the mitochondrial genome. In either case, both type A and type B sequences would be expected in products amplified from purified mtDNA. In the case of heteroplasmy, other regions of the genome would also be

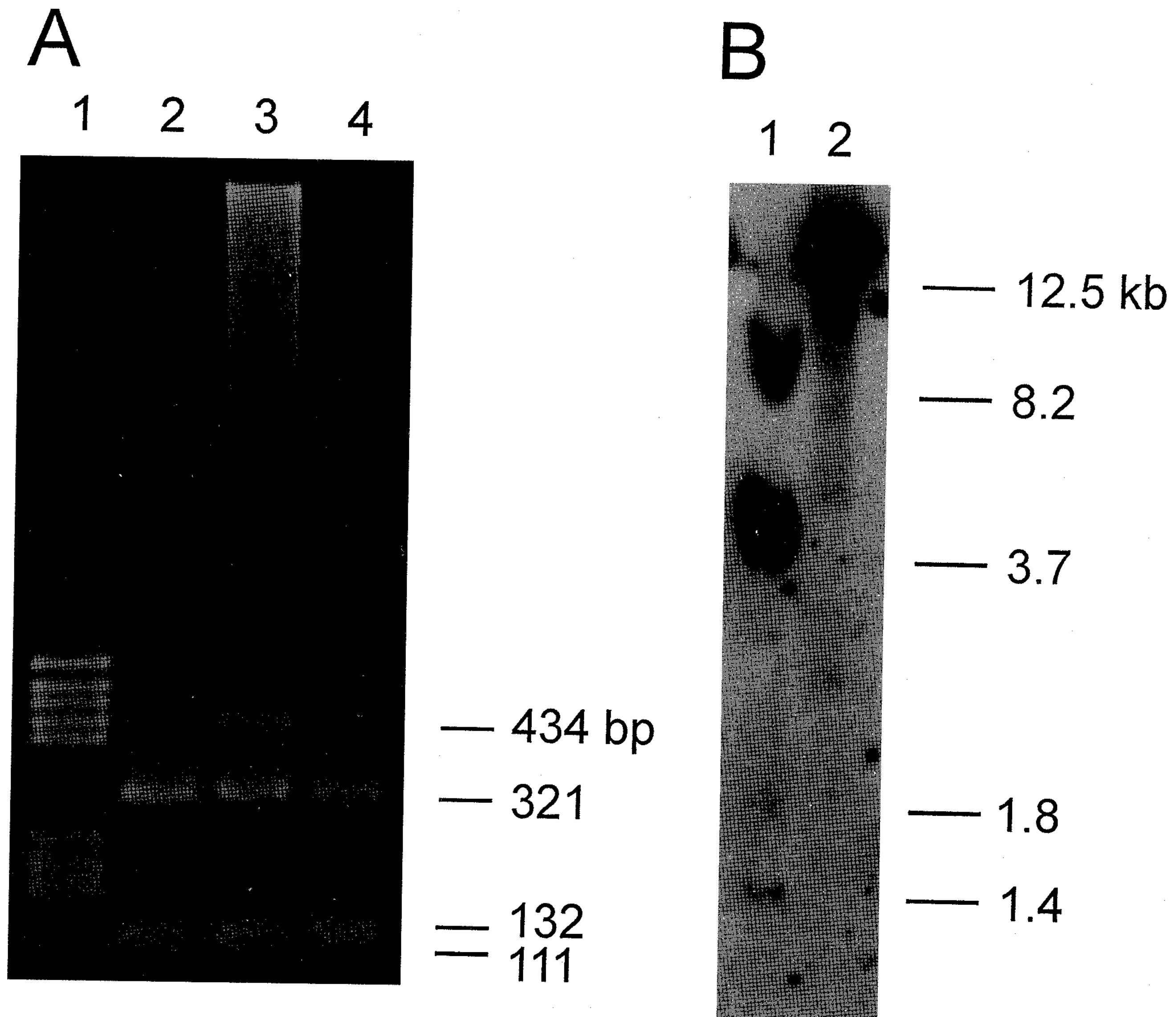

Fig. 5.—*A*, *Hin*dIII digests of PCR products. Lane 1 is pBR322 digested with *Hae III* as a size marker. Lanes 2 and 4 are digests of products amplified from purified mtDNA isolated from two different individuals. Lane 3 is a digest of the products amplified from total genomic DNA isolated from the same individual as in lane 4. The 434-bp fragment seen in lane 3 is characteristic of type B sequences. *B*, Southern blot of total genomic DNA digested with *Hin*dIII and probed with a cloned *Menippe* type B LSrDNA. Lane 1 is a *Hin*dIII digest of genomic DNA. In addition to the strongly hybridizing fragments corresponding to a type A sequence, the two weakly hybridizing bands are of sufficient size (1.8 and 1.4 kb) to correspond to a type B sequence. The strongly hybridizing band in lane 2 is an *Ava* I fragment.

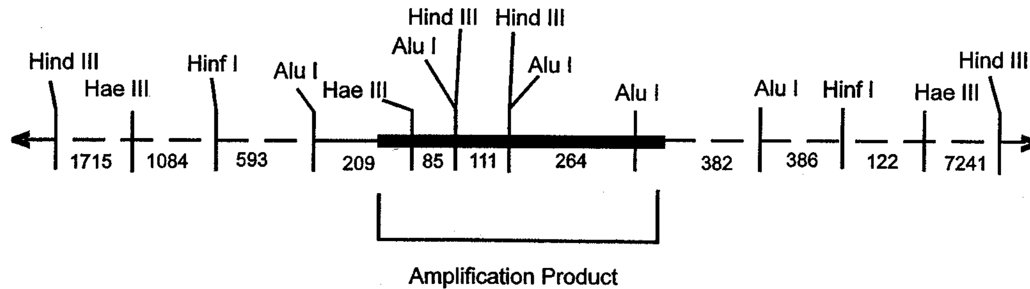

FIG. 6.—Restriction site map of the large-subunit rDNA region of the mitochondrial genome based on Southern hybridization analysis. A bracket indicates the region of the mitochondrial genome that includes the PCR-amplified portion of the large-subunit ribosomal DNA. Fragment sizes are in base pairs. Dashed lines indicate areas where the drawing is not to scale.

likely to exhibit multiple sequences from single individuals. We examined 370 bp of cytochrome oxidase I sequence from two individuals (one *M. adina* and one *M. mercenaria*), and found no sequence variation within individuals (data not shown). In the case of a duplication within the mitochondrial genome, the duplicated region should be detectable by restriction mapping. Our analysis of the restriction sites in the region of the mitochondrial genome that hybridizes to an LSrDNA probe excludes the presence of either a tandem or inverted duplication (fig. 6). The absence of the type B sequence from at least the majority of isolated mtDNA molecules is demonstrated by the absence of the characteristic 375-bp *Alu* I fragment on Southern blots of mitochondrial DNA probed with a homologous sequence.

Other examples of sequences translocated from the mitochondrion to the nucleus have been reported, although this is the first case within the Crustacea, and of a translocation shared by two species within same genus. Aside from the gene translocations that are believed to have occurred shortly after the original endosymbiotic events that gave rise to mitochondria, more recent translocations of mtDNA sequences to the nuclear genome have been reported for fungi (van den Boogaart, Samallo, and Agsteribbe 1982; Farrelly and Butow 1983; Wright and Cummings 1983), and plants (Kemble et al. 1983; Grohmann, Brennicke, and Schuster 1992), and for ribosomal RNA sequences in other metazoa (Gellison et al. 1983; Jacobs et al. 1983; Lopez et al. 1994).

The amounts of type A and type B products amplified from total genomic DNA appeared more similar than the 600:1 ratio estimated for their relative abundances in total genomic DNA. Based on relative intensity of PCR products on ethidium-stained gels, and on the proportions of the sequences in clones generated from PCR products, it appears that the type A sequence is about 10 times more abundant as a PCR product than the type B sequence. It is possible that the 60-fold difference in these ratios is the result of differential amplification of the two products. Differential PCR amplification of sequences can occur when primers mismatch with the template DNA (Mullis and Faloona 1987). Under moderately stringent annealing conditions, a low percentage of primers may bind with partially mismatched template sequences, so that these sequences are amplified along with more precisely matched template sequences. Thus, while there may be a 600:1 excess of type A sequences in total genomic DNA, this may be partially offset by preferential amplification of the type B sequence.

The consistent amplification of two distinct sequences homologous to mitochondrial LSrDNA from *Menippe* suggests that caution should be used in interpreting PCR products as mitochondrial sequences simply because they appear homologous to other mitochondrial sequences. We can use our results to evaluate some presumed safeguards against confusing a translocated nuclear duplication with a mitochondrial sequence. First, it might be assumed that by using partially purified mitochondrial DNA for PCR, only mitochondrial sequences will be represented in significant amounts as products. However, we found that although the type B sequence was present as only a minor contaminant in our total DNA isolations (<0.2%), it was preferentially amplified to the extent that it represented a significant fraction of the final PCR product. Second, it is often expected that pseudogene sequences should exhibit an unusually high degree of polymorphism, which would be likely to include base substitutions that obviously disrupt function. These "telltales" did not appear in our sample of seven type B LSrDNA sequences. They were actually less polymorphic than the mitochondrial type A sequences, the proportion of substitutions in stems was no higher, and neither the single indel nor any of the base substitutions between type A and B sequences disrupted the presumed rRNA secondary structure. Thus, while PCR provides many advantages over other methods of mtDNA sequence isolation, it may be prudent to verify at least a subset of results obtained with PCR using mtDNA isolated by other methods.

Acknowledgments

This research was supported by grants from the NSF doctoral dissertation improvement grant program to R.S.-B., from NSF/EPSCoR (1992–1996)-ADP-02 and the Louisiana LEQSF Fund to J.E.N., and by the Graduate Student Organization at USL.

LITERATURE CITED

AVISE, J. C., J. ARNOLD, R. M. BALL, E. BERMINGHAM, T. LAMB, J. E. NEIGEL, C. A. REEB, and N. C. SAUNDERS.

1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. **18**:489–522.

BENTZEN, P., W. C. LEGGETT, and G. G. BROWN. 1988. Length and restriction site heteroplasmy in the mitochondrial DNA of American shad (*Alosa sapidissima*). Genetics **118**:509–518.

BERT, T. M. 1986. Speciation in western Atlantic stone crabs (genus *Menippe*): the role of geological processes and climatic events in the formation and distribution of species. Mar. Biol. **93**:157–170.

BERT, T. M., and R. G. HARRISON. 1988. Hybridization in western Atlantic stone crabs (genus *Menippe*): evolutionary history and ecological context influence species interactions. Evolution **42**:528–544.

BIRNBOIM, H. C., and J. DOLY. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.

BOYCE, T. M., M. E. ZWICK, and C. F. AQUADRO. 1989. Mitochondrial DNA in the bark weevils: size, structure and heteroplasmy. Genetics **123**:825–836.

BROWN, W. M. 1985. The mitochondrial genome of animals. Pp. 95–130 *in* R. J. MACINTYRE, ed. Molecular evolutionary genetics. Plenum Press, New York.

CHEN, E. Y., and P. H. SEEBURG. 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.

CLARK, A. G. 1988. Deterministic theory of heteroplasmy. Evolution **42**:621–626.

CUNNINGHAM, C. W., N. W. BLACKSTONE, and L. W. BUSS. 1992. Evolution of king crabs from hermit crab ancestors. Nature **355**:539–542.

FARRELLY, F., and R. A. BUTOW. 1983. Rearranged mitochondrial genes in the yeast nuclear genome. Nature **310**:296–301.

GELLISON, G., J. Y. BRADFIELD, B. N. WHITE, and G. R. WYATT. 1983. Mitochondrial DNA sequences in the nuclear genome of a locust. Nature **310**:631–634.

GROHMANN, L., A. BRENNICKE, and W. SCHUSTER. 1992. The mitochondrial gene encoding ribosomal protein S12 has been translocated to the nuclear genome in *Oenothera*. Nucleic Acids Res. **20**:5641–5646.

HEINONEN, T. Y. K., M. N. SCHNARE, and M. W. GRAY. 1990. Sequence heterogeneity in the duplicate large subunit ribosomal RNA genes of *Tetrahymena* pyriformis mitochondrial DNA. J. Biol. Chem. **265**:22336–22341.

HIGGINS, D. G., and P. M. SHARP. 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene **73**:237–244.

———. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. CABIOS **5**:151–153.

HOEH, W. R., K. H. BLAKLEY, and W. M. BROWN. 1991. Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. Science **251**:1488–1490.

HOELZEL, A. R., J. M. HANCOCK, and G. A. DOVER. 1993. Generation of VNTR's and heteroplasmy by sequence turnover in the mitochondrial control region of two elephant seal species. J. Mol. Evol. **37**:190–197.

HSUCHEN, C.-C., R. M. KOTIN, and D. T. DUBIN. 1984. Sequences of the coding and flanking regions of the large ribosomal subunit RNA gene of mosquito mitochondria. Nucleic Acids Res. **20**:7771–7785.

JACOBS, H. T., J. W. POSAKONY, J. W. GRULA, J. W. ROBERTS, J.-H. XIN, R. J. BRITTEN, and E. H. DAVIDSON. 1983. Mitochondrial DNA sequences in the nuclear genome of *Strongylocentrotus purpuratus*. J. Mol. Biol. **165**:609–632.

KEMBLE, R. J., R. J. MANS, S. GABAY-LAUGHNAN, and J. R. LAUGHNAN. 1983. Sequences homologous to episomal mitochondrial DNAs in the maize nuclear genome. Nature **304**:744–747.

KUMAR, S., K. TAMURA, and M. NEI. 1993. MEGA: molecular evolutionary genetics analysis. Version 1.0. The Pennsylvania State University, University Park, Pa.

LOPEZ, J., N. YUHKI, R. MASUDA, W. MODI, and S. O'BRIEN. 1994. A recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol. **39**:174–190.

MANIATIS, T., E. F. FRITSCH, and J. SAMBROOK. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MORITZ, C., and W. M. BROWN. 1986. Tandem duplication of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science **233**:1425–1427.

———. 1987. Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. Proc. Natl. Acad. Sci. USA **84**:7183–7187.

MULLIS, K. B., and F. A. FALOONA. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **155**:335–350.

NEIGEL, J. E., D. L. FELDER, C. A. CHLAN, and R. LA PORTE. 1991. Cloning and screening of DNA probes for genetic studies in stone crabs (Decapoda: Xanthidae: *Menippe*). J. Crust. Biol. **11**:496–505.

PALMERO, I., J. RENART, and L. SASTRE. 1988. Isolation of cDNA clones coding for mitochondrial 16s ribosomal RNA from the crustacean *Artemia*. Gene **68**:239–248.

PALUMBI, S., A. MARTIN, S. ROMANO, W. O. MCMILLAN, L. STICE, and G. GRABOWSKI. 1991. The simple fool's guide to PCR. Version 2.0. University of Hawaii, Honolulu, HI.

QUINN, T. W., and B. N. WHITE. 1987. Analysis of DNA sequence variation. Pp. 163–198 *in* F. COOKE and P. A. BUCKLEY, eds. Avian genetics. Academic Press, London.

RYCHLIK, W., and R. E. RHOADS. 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucleic Acids Res. **17**:8543–8551.

SANGER, F., S. NICKLEN, and A. R. COULSON. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

SCHAFFER, H. E., and R. R. SEDEROFF. 1981. Improved estimation of DNA fragment lengths from agarose gels. Anal. Biochem. **115**:113–122.

SMITH, M. F., W. KELLEY, and J. L. PATTON. 1992. Mitochondrial DNA-like sequence in the nuclear genome of an akodontine rodent. Mol. Biol. Evol. **9**:204–215.

SOLIGNAC, M., J. GENENERMONT, M. MONNEROT, and J.-C. MOUNOLOU. 1984. Genetics of mitochondria in Drosophila: mtDNA inheritance in heteroplasmic strains of *D. mauritiana*. Mol. Gen. Genet. **197**: 183–188

SOUTHERN, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

THORPE, R. S., D. MCGREGOR, and A. M. CUMMING. 1993. Population evolution of western Canary Island lizards (*Gallotia galloti*): 4-base endonuclease restriction fragment length polymorphisms of mitochondrial DNA. Biol. J. Linn. Soc. **49**:219–227.

VAN DEN BOOGAART, P., J. SAMALLO, and E. AGSTERIBBE. 1982. Similar genes for a mitochondrial ATPase subunit in the nuclear and mitochondrial genomes of *Neurospora crassa*. Nature **298**:187–189.

WILLIAMS, A. B., and D. L. FELDER. 1986. Analysis of stone crabs: Menippe mercenaria (Say), restricted, and a previ-

ously unrecognized species described (Decapoda: Xanthidae). Proc. Biol. Soc. Wash. **99**:517–543.

WINNEPENNINCKX, B., Y. VAN DE PEER, T. BACKELJAU, and R. DE WACHTER. 1995. CARD: a drawing tool for RNA secondary structure models. BioTechniques **18**:1060–1063.

WRIGHT, R. M., and D. J. CUMMINGS. 1983. Integration of mitochondrial gene sequences within the nuclear genome during senescence in a fungus. Nature **302**:86–88.

SIMON EASTEAL, reviewing editor

Accepted October 31, 1996