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SHORT COMMUNICATION

Development and application of long-PCR for the assay of full-length animal mitochondrial DNA

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Native mitochondrial (mt) DNA in higher animals exists as a closed-circular molecule typically 16–20 kilobase pairs (kb) in length. For nearly two decades, this cytoplasmically housed genetic system has been employed to estimate matrilineal phylogeny within and among species (Avise 1994). Beginning in the late-1970s, a primary assay method involved restriction analyses of whole mtDNA molecules that had been physically isolated from nuclear DNA. In the late-1980s, many studies moved to PCR-based (Saiki *et al.* 1988) restriction-site or sequence assays of particular mtDNA regions typically a few hundred to 1000 bp in length.

Here we document successful 'long-PCR' amplification (Cohen 1994) of full-length animal mtDNA, using conserved 16S ribosomal gene sequences. Heretofore, PCR amplification of full-length mtDNA has been reported only for humans, and in a clinical context (Cheng *et al.* 1994b; Li *et al.* 1995).

Assays were focused primarily on armadillos (Mammalia, Edentata, Dasypodidae): *Dasypus novemcinctus* (*n* = 12 specimens from scattered sites in the south-eastern United States); and *Tolypeutes matacus* (*n* = 1), *Zoedyus pichiy* (*n* = 2), *Chaetophractus vellerosus* (*n* = 2), and *C. villosus* (*n* = 2) collected in northern Argentina. From the non-*Dasypus* species, total genomic DNA was prepared from blood by standard methods involving phenol chloroform extraction and ethanol precipitation. For the other specimens, mtDNA was purified from heart and liver by conventional CsCl gradient centrifugation (Lansman *et al.* 1981). Total genomic DNA also was prepared from 20 additional specimens of *Dasypus* according to Taggart *et al.* (1992), using clips of ear tissue preserved in 90% ethanol.

Sequences (*c.* 500 bp long) within the mtDNA 16S ribosomal RNA gene were PCR-amplified using the 'universal' primers 16sar-L and 16sbr-H (Palumbi *et al.* 1991). Templates for these amplifications were CsCl gradient-

purified mtDNA (*c.* 50–100 ng) from *Dasypus*, and total genomic DNA (0.5–1 µg) from the other armadillo species. The following cycling parameters were employed in 100 µL reaction volumes (1 x Promega *Taq* buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 25 pmol of each primer, and 2.5 units of *Taq* polymerase): initial denaturation at 94 °C for 2 min, followed by 35 cycles of 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplification products were purified from unincorporated primers using the 'Promega Wizard PCR Preps DNA Purification System'. Heavy and light strands of amplified fragments were cycle-sequenced from one specimen per species at the Molecular Genetics Facility, University of Georgia, using the fluorescent-dye chain-terminator method (Applied Biosystems automatic sequenator). Sequences were aligned with the computer program Sequence Navigator (Applied Biosystems) and five regions (ranging from 32 to 41 bp in length) of 100% sequence similarity across the armadillo species were noted. From the two longest of these regions (36 and 41 bp), 32-bp primers were designed with assistance from the program Oligo (National Biosciences, Inc.):

Dnov16S-for (5'-AATAGGGTTTACGACC-TCGATGTTGGATCAGG-3');

Dnov16S-rev (5'-TGATTATGCTACCTTTG-CACGGTCAGGGTACC-3').

These primers then were employed in a three-step amplification using a Hybaid Thermal Cycler and the Expand™ Long Template PCR system from Boehringer-Mannheim (B-M). This system jointly employs two polymerases: a nonproofreading *Taq* that is the main polymerase in the reaction, and a proofreading *Pwo* at lower concentration (see the B-M manual for further details). Optimized conditions, achieved according to the manufacturer's recommendations, were as follows, carried out in 50-µL reaction volumes: 1 x buffer 3 [whose 10 x stock contains 22.5 mM $MgCl_2$, 500 mM Tris-HCl pH 9.2, 160 mM $(NH_4)_2SO_4$, 20% v/v DMSO, 1% v/v Tween 20], 2.5 units enzyme mix, 100 ng of genomic DNA or 50–100 ng of CsCl gradient-purified mtDNA, 350 µM of each dNTP, and 25 pmol of each long-PCR primer. Cycling parameters consisted of initial denaturation at 94 °C for 2 min; 10 cycles at 92 °C for 30 s, annealing at 64 °C for 30 s, and

elongation at 68 °C for 12 min; another 25 such cycles with 20 additional seconds for the elongation step per cycle; and a final extension period of 7 min at 68 °C.

Each initial check for proper amplification of full-length (c. 16.7 kb) mtDNA involved electrophoresis of 3 μL of the reaction mixture along side a 1-kb ladder standard (Bethesda Research Laboratories) through a 1% agarose gel (1 x TBE), followed by EtBr visualization.

From the closed-circular mtDNAs (c. 500 ng), as well as from 3 μL of the linear full-length mtDNAs amplified by long-PCR, restriction digests were performed according to the manufacturer's recommendations using DraI, EcoRI, EcoRV, HindII, HindIII, HpaII, and TaqI. MtDNA fragments from the CsCl-based preparations were radioactively end-labelled with ^{35}S-tagged nucleotides before electrophoresis (Lansman et al. 1981). Restricted mtDNA fragments from the long-PCR were revealed by EtBr staining following electrophoresis. In all cases, mtDNA fragments were separated through 1.5% agarose gels and sized against 1-kb ladders.

The long-PCR primers successfully amplified full-length linear mtDNA from all armadillo species examined. The Dnov16S-for and Dnov16S-rev primers are separated by 366 bp in Dasypus mtDNA, so by 'full-length' amplification we mean that about 98% of the mtDNA molecule was copied. The initial gauge of success was the appearance of single DNA bands of appropriate size (c. 16–17 kb) in EtBr-stained agarose gels whose lanes had been loaded with reaction mixtures resulting from amplification by the long-PCR primers described above. Lanes with negative controls failed to show these bands. Successful amplification of full-length mtDNA was confirmed by agreement of restriction digestion profiles between the mtDNAs isolated from long-PCR and those from conventional CsCl gradient purifications (Figs 1, 2).

The right-hand lanes in Figs 1(b) and 2(b) indicate that the Dnov long-PCR primers also amplified full-length mtDNA suitable for restriction digestion from total genomic preparations from each of the three other armadillo genera examined. Furthermore, 75% of the 20 long-PCR attempts succeeded when the starting template involved genomic DNA preparations from the ethanol-preserved Dasypus ear clips.

Preliminary attempts to amplify full-length mtDNA from other vertebrates using the long-PCR armadillo primers met with mixed success. Species yielding an amplification product of expected full-length mtDNA size (c. 16–17 kb) included the fox squirrel Sciurus niger, snow goose Chen (or Anser) caerulescens, chuckwalla Sauromalus obesus, desert iguana Dipsosaurus dorsalis, western garter snake Thamnophis elegans, spring salamander Gyrinophilus porphyriticus, rainbow trout Oncorhynchus mykiss, and Atlantic salmon Salmo salar. However, each of these species also displayed smaller fragments in the gels per-
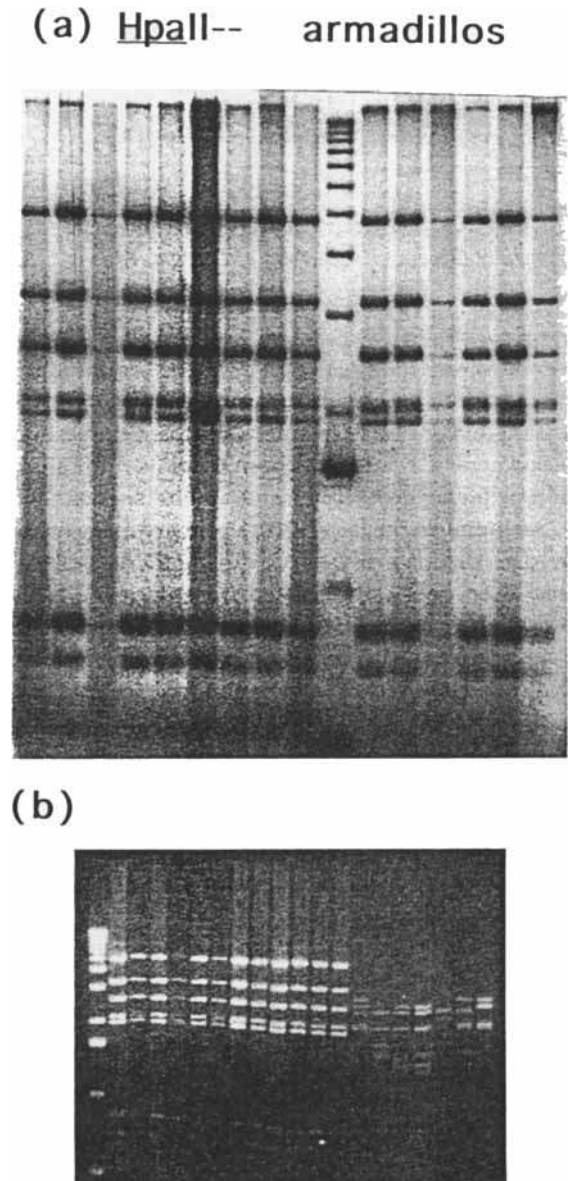

(a) HpaII-- armadillos

(b)

Fig. 1 HpaII digests of armadillo mtDNA. (a): autoradiograph based on end-labelling of closed-circular mtDNA as isolated by conventional CsCl gradient centrifugation from 15 specimens of Dasypus novemcinctus. The seventh lane from the right is a 1-kb ladder size standard. (b) EtBr-stained gel in which mtDNAs had been amplified by long-PCR. The leftmost lane is a 1-kb molecular size standard, lanes 2–13 are Dasypus novemcinctus, lanes 14–17 Chaetophractus sp., lane 18 Tolypeutes matacus, and lanes 19–20 Zoedyus pichiy. For Dasypus, note the appearance in Figs 1(a) and 1(b) of the same seven mtDNA fragments ranging in size from about 0.7 to 4.9 kb. This outcome also suggests that one of the HpaII restriction sites is close to the PCR priming sites, an inference confirmed by direct examination of the 16S rRNA gene sequence where a HpaII restriction site occurs within the 366 bp region excluded from long-PCR amplification. Digestion profiles produced by three other restriction enzymes (DraI, HindII and TaqI) similarly appeared identical in assays of both long-PCR and CsCl-isolated mtDNA.
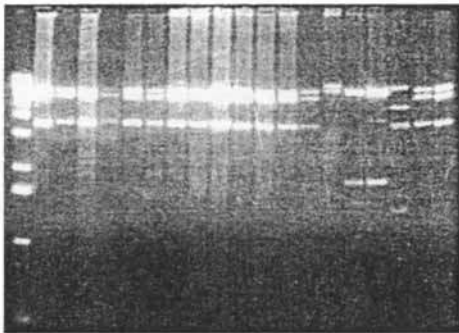
(a) EcoRI-- armadillos
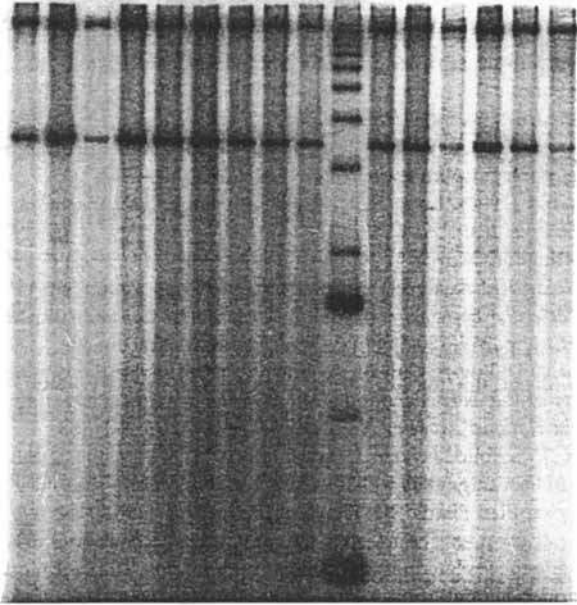
(b)

Fig. 2 *Eco*RI digests of armadillo mtDNA (see legend to Fig. 1 and the text). For this enzyme, as well as for *Eco*RV and *Hind*III, digestion profiles from the long-PCR products were different from those of conventional digests in ways reflective of the fact that long-PCR mtDNA products are linear as opposed to circular. For example, conventional *Eco*RI digestions of closed-circular mtDNA in *Dasypus novemcinctus* revealed two fragments of sizes 3.4 and c. 13 kb (Fig. 2a), whereas digestions of the linear long-PCR products revealed three fragments of sizes 3.4, and c. 6 and 7 kb (Fig. 2b). In this case, the priming sites for long-PCR occur near the middle of the 13-kb fragment obtained in the conventional digestion. Such differences in digestion profiles also further confirm that the latter reflect PCR-amplified mtDNA product as opposed to mere presence of CsCl gradient-purified mtDNA.

haps indicative of occasional mis-priming or lack of primer specificity for the 16S rRNA gene. Species failing to yield a full-size mtDNA amplification product in our preliminary assays were the sooty tern *Sterna fuscata*, sharp-tailed sparrow *Ammodramus caudacutus*, green turtle *Chelonia mydas*, and spotted bass *Micropterus punctulatus*.

Finally, a search of nucleotide sequences in GenBank was conducted to identify targets with 100% identity to the Dnov16S-for and Dnov16S-rev long-PCR primer sequences. Available 16S rRNA gene sequences matched both primers perfectly for the three-toed sloth *Bradypus variegatus*, two-toed sloth *Choloepus didactylus*, extinct ground sloth *Mylodon darwinii*, another armadillo species *Cabassous unicinctus*, and proboscis monkey *Nasalis larvatus*. Sloths (and anteaters) also belong to Edentata and are thought to be close relatives of armadillos (Höss et al. 1996). Another 105 species had sequences that matched either Dnov16S-for or Dnov16S-rev. These results, together with the empirical findings described above, suggest that the armadillo-derived long-PCR primers may amplify full-length mtDNA from some other vertebrates also. However, further evaluation will require species-specific experimentation with varied PCR conditions.

Following early attempts to amplify lengthy DNA fragments *in vitro* (e.g. Kainz et al. 1992; Ponce & Micol 1992), 'long-PCR' methods were refined by Barnes (1994) and Cheng et al. (1994a) who demonstrated high fidelity amplification of phage λ templates over 35 kb. Long-PCR relies on stringent annealing of long primers (permitting high annealing temperatures and thus a reduced risk of false priming), polymerase proofreading activity, and short high-temperature denaturations coupled with exacting temperatures of elongation.

In population and evolutionary genetics, several advantages can be envisioned for the assay of whole mtDNA by long-PCR: (a) laborious and time-consuming steps of physical mtDNA isolation by conventional methods are circumvented; (b) the isolations do not require large amounts of starting tissue, nor inevitable sacrifice of the organism; and (c) mtDNA amplification product should be unlimited, thus permitting fast gel screening by methods (e.g. EtBr staining) that avoid sensitive and expensive detection techniques. Additional advantages are that: (a) some population survey methods (e.g. by restriction enzymes that cut at five- and six-bp recognition sites) are better suited for full-length animal mtDNA than for short gene sequences within it (because of the scorable number of fragments typically produced); (b) extensive data from traditional whole-mtDNA surveys are available for many species for comparison; and (c) the priming site in long-PCR provides a consistent (across enzymes) anchor for restriction site mapping.

Potential disadvantages of long-PCR include those that apply to any PCR-based method: (a) the effort involved in

developing suitable primers and assay conditions for the species in question; and (b) the danger of amplification from non-target DNA. As applied to animal mtDNA, this latter concern is in one respect diminished in long-PCR as compared with regular PCR applications. One prominent source of nonspecific amplification occurs when PCR primers developed expressly for gene sequences in mito-chondria 'inadvertently' amplify paralogous sequences in the nucleus. Nuclear transfer of short mtDNA sequences is a rather common and ongoing phenomenon in animals and plants (Blanchard & Schmidt 1995, 1996), and the resulting mtDNA pseudogenes can create difficulties (as well as novel opportunities) in population and evolution-ary analysis (Smith et al. 1992; Arctander 1995; Dowling et al. 1996; Zhang & Hewitt 1996). Such complications are less likely to compromise mtDNA long-PCR because no transfers of full-length mtDNA to the nucleus have yet been reported.

We have demonstrated the technical feasibility of PCR amplification of full-length animal mtDNA. The current results involving armadillos suggest that with further refinement, the long-PCR approach for animal mtDNA may find a variety of applications in population genetic and evolutionary studies.

Acknowledgements

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