Mitochondrial DNA in the Bark Weevils: Size, Structure and Heteroplasmy

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ABSTRACT

Mitochondrial DNA of higher animals has been described as an example of extreme efficiency in genome structure and function. Where exceptionally large size molecules have been found (>20 kb), most have occurred as rare variants within a species, suggesting that these variants arise infrequently and do not persist for long periods in evolutionary time. In contrast, all individuals of at least three species of bark weevil (Curculionidae: Pissodes) possess a mitochondrial genome of unusually large size (30–36 kb). The molecule owes its large size to a dramatically enlarged A+T-rich region (9–13 kb). Gene content and order outside of this region appear to be identical to that found in Drosophila. A series of 0.8–2.0-kb repeated sequences occur adjacent to the large A+T-rich region and have perhaps played a role in the generation of the large size as well as an unprecedented frequency of size variant heteroplasmy. Every weevil sampled in all three species (n = 219) exhibits anywhere from two to five distinct size classes of mtDNA. The persistence of this large amount of size polymorphism through two speciation events combined with the abundant size variation within individuals suggests that these molecules may not be subject to strong selection for small overall size and efficiency of replication. This pattern of variation contrasts strongly with the conservation of gene content and arrangement in the coding region of the molecule.

Animal mitochondrial DNA is generally known for its conservation of gene content and size across a variety of taxa (Moritz, Dowling and Brown 1987; Sederoﬀ 1984). The majority of animal mtDNAs encode 13 protein genes, 22 transfer RNAs, and two ribosomal RNAs. Most taxa studied have mtDNA molecules of similar size ranging from 15 to 20 kb. Coding sequences are tightly abutting if not overlapping, and lack introns. Thus, it has been thought that mtDNA size has been constrained in the evolutionary history of animals. Recently, however, mitochondrial genomes of relatively large size (greater than ca. 20 kb) have been found in several vertebrate taxa (Moritz and Brown 1987), in the deep sea scallop, Placopecten magellanicus (Snyder et al. 1987) and in the nematode Romanomermis culicivorax (Powers, Platzer and Hyman 1986). These molecules have gained their large size in a variety of ways. Tandem duplications of control region sequences are responsible for size increases in newts and fishes, whereas coding sequences have been duplicated in lizards (Moritz and Brown 1986; Moritz, Dowling and Brown 1987). The large size of the scallop mtDNA molecule is in part the result of the addition of ca. 11 kb of unknown sequence, over and above the approximately 14 to 16 kb present in typical mitochondrial genomes. The mtDNA of the nematode, R. culicivorax, owes its large size to many dispersed repeats (Powers, Platzer and Hyman 1986).

In addition to these large sizes, heteroplasm for size variants may be relatively common in many organisms other than mammals (Bermingham, Lamb and Avise 1986; Harrison 1989). Minor size variation, ca. 500 base pairs (bp) or less and typically localized to the control region, is common within and among lower vertebrates and insects studied to date (Moritz, Dowling and Brown 1987). Tandem repetition of a 1.2-kb element of unknown sequence is responsible for large size variants in the sea scallop (Snyder et al. 1987) and a similar pattern with a 1.5-kb repeat is found in the american shad (Bentzen, Legget and Brown 1988).

Nevertheless, in most taxa, large size mtDNA molecules appear only as rare variants within species, suggesting that these variants do not persist for long periods in evolutionary time. This observation is consistent with the view that mtDNA size is somewhat constrained in animals (Hale and Singh 1986; Sederoﬀ 1984; Moritz, Dowling and Brown 1987). Experiments tracing size variant frequency changes in isofemale lines of Drosophila mauritiana (Sollignac et al. 1984) and heteroplasmic female crickets and their progeny (Rand and Harrison 1986), while not conclusive, have suggested that smaller sized molecules are more frequently transmitted. A simple model for such an advantage stems from the potential kinetic advantage a smaller molecule may have during repli-
cation (RAND and HARRISON 1986, 1989; WALLACE 1989). Larger size molecules are viewed to be at a selective disadvantage simply because they take longer to replicate, leaving fewer copies to be transmitted. Under this hypothesis, the largest molecules should be under the most intense selection and have the shortest residency time in a population if the transmission rates of different size variants are equal.

We have been using mtDNA polymorphisms in three species of bark weevil (Curculionidae: Pissodes) to assess the degree and nature of genetic differentiation of various pest populations of these insects. As groundwork for this study, we have characterized the gene content, order and size variation of Pissodes mitochondrial genomes and found them to be uniformly large (30–36 kb) in three species. Furthermore, across the three species all individuals sampled (n = 219) are heteroplasmic for up to five major size classes. These size variants appear to be the result of different copy numbers of a tandemly repeated region, itself varying in size from 0.8 to 2.0 kb. Through the use of restriction site mapping, transfer-hybridization, and cloning of Pissodes mtDNA, we determined the nature and extent of the size increase and heteroplasmacy. For comparison, we have characterized the mitochondrial genomes of two other species of weevil, the boll weevil (Anthonomus grandis) and the pales weevil (Hylobius pales). Furthermore, we have aligned the complete Drosophila genetic map to maintain relative gene order and compact arrangement of various pest populations of these insects. As groundwork for this study, we have characterized the gene content, order and size variation of Pissodes mitochondrial genomes and found them to be uniformly large (30–36 kb) in three species. Furthermore, across the three species all individuals sampled (n = 219) are heteroplasmic for up to five major size classes. These size variants appear to be the result of different copy numbers of a tandemly repeated region, itself varying in size from 0.8 to 2.0 kb. Through the use of restriction site mapping, transfer-hybridization, and cloning of Pissodes mtDNA, we determined the nature and extent of the size increase and heteroplasmacy. For comparison, we have characterized the mitochondrial genomes of two other species of weevil, the boll weevil (Anthonomus grandis) and the pales weevil (Hylobius pales). Furthermore, we have aligned the complete Drosophila genetic map of mtDNA with the Pissodes restriction site map. Our findings imply that while selection appears to be acting to maintain relative gene order and compact arrangement of the major coding regions, the large size of the Pissodes mtDNA molecule and extensive heteroplasmacy suggest that there must not be strong constraints on total size of the mtDNA molecule in weevils, in contrast to patterns found in most animal taxa studied to date.

MATERIALS AND METHODS

Isolation of mtDNA: For preparation of purified mtDNA, live Pissodes nemorensis were obtained from a culture founded with collections from Gainesville, Florida, and Tully, New York. Live P. strobi were excavated from white pine shoots from Ithaca, New York, and live Anthonomus grandis were provided from a collection at the University of Mississippi. mtDNA was isolated using the protocol of LANS-MAN et al. (1981) with the following modifications. Homogenization was carried out in 0.21 M mannitol/0.07 M sucrose/0.05 M Tris (pH 7.5)/0.003 M CaCl2/0.01 M EDTA using a Tekmar Tissumizer. Nuclei and debris were pelleted and the supernatant resuspended in fresh homogenization buffer. The sample was rehomogenized and debris removed again. Samples were pooled and mitochondria were pelleted, resuspended in 0.21 M mannitol/0.07 M sucrose/0.05 M Tris (pH 7.5)/0.01 M EDTA and repelleted. The mitochondria were resuspended in 100 mM NaCl/50 mM Tris-HCl/10 mM EDTA and lysed with SDS. The SDS was precipitated from solution with CsCl salt and the supernatant made 0.6 mM in propidium iodide. Covalently closed circular DNA was isolated through CsCl gradients (density 1.578 g/ml, refractive index of 1.878). The propidium iodide was extracted with saturated CsCl/isoamyl alcohol (1:1, v:v), the volume adjusted with TE. The sample was rehomogenized and the supernatant resuspended in fresh homogenization buffer. The sample was rehomogenized and debris removed again. Samples were pooled and mitochondria were pelleted, resuspended in 0.21 M mannitol/0.07 M sucrose/0.05 M Tris (pH 7.5)/0.01 M EDTA and repelleted. The mitochondria were resuspended in 100 mM NaCl/50 mM Tris-HCl/10 mM EDTA and lysed with SDS. The SDS was precipitated from solution with CsCl salt and the supernatant made 0.6

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of P. strobi mtDNA that extend in either direction from the BamHI site (clones pPSH.6.1, pPSBX.28, and pPSBX.2).

Gene content and order: Mitochondrial gene content and order were determined by hybridization to probes representing specific genes or sets of genes in Drosophila mtDNA prepared from Drosophila yakuba mtDNA cloned in pBR322 (CLARY and WOLSTENHOLME 1985); D. melanogaster mtDNA cloned into pBR322 (GARESSE 1988; DE BRUIJN 1983), or from purified mtDNA prepared from D. melanogaster (Table 2). For fragments from purified D. melanogaster mtDNA, the desired fragments were separated via electrophoresis in low melting point agarose, excised and labeled as described above. Additionally, a 382-bp XbaI restriction fragment of D. melanogaster mtDNA within the 12S rRNA gene (SOLIGNAC, MONNEROT, and MOUNOLOU 1986) was cloned into appropriately digested pBS vector DNA (Stratagene, Inc.) and recombinant plasmid DNA prepared as above. Hybridization of Drosophila mtDNA to weevil DNA was carried out as described above but under reduced stringency (hybridization and washes at 42°C).

RESULTS

Restriction enzyme site maps and size: MtDNA restriction site maps of Pissodes nemorensis, P. strobi, P. terminalis, Hylobius pales (the pales weevil) and Anthonomus grandis (the boll weevil) were constructed for several enzymes (Figure 1). Consistent restriction site maps for Pissodes species were obtainable only through a combination of techniques. Single and double restriction enzyme digests provided relative positions of sites in most cases. However, several fragments 0.8–2.0 kb long were evidently repeated as judged by darker intensity on autoradiographs, leading to some ambiguity in site positions. Digestions with BamHI revealed that it consistently cleaves the mtDNA of all Pissodes species at only one conserved site. Likewise, PstI cleaves the molecules at two invariant sites on either side of the BamHI site (Figure 1). Fragment sizes and their relative placements for particular enzymes were obtained via indirect end-labeling of partial digests of DNA previously cut with BamHI or PstI. This technique revealed the order in which fragments are arranged extending from the BamHI site and confirmed that all bands arise from a closed, circular molecule (Figure 2).

The restriction site maps for the three Pissodes species show a ca. 16-kb region where most mapped enzymes cleave, a 9–13-kb fragment that is not cleaved by most enzymes used, and a series of repeated fragments (Figure 1). Cleavage patterns for 219 individual weevils were examined and all revealed sizes for the three species of pine weevil in the range of 30 kb to 36 kb, although P. strobi typically has the largest genome and P. terminalis the smallest of the three species. Percent nucleotide sequence divergence (π; NEI and TAJIMA 1981) among single representative mtDNA types of three species of Pissodes was estimated using the presence or absence of mapped restriction sites in the nonduplicated regions of each species. The estimated divergence between P. strobi and P. nemorensis is 2.87%; between P. strobi and P. terminalis, 3.66%; and between P. nemorensis and P. terminalis, 1.83%. Hylobius and Anthonomus restriction enzyme maps are too divergent to align to those of Pissodes with confidence and hence values of divergence were not estimated.

Remarkably, every weevil examined in all three species was heteroplasmic for several distinct size classes of mtDNA (Figure 3). These size classes most often differed by multiples of the average repeat unit size (ca. 1.5 kb). Particular digestion patterns of repeated fragments could not be consistently correlated with particular heteroplasmic profiles, suggesting that any particular size class may be arrived at through a number of different combinations of repeated fragments.

Verification of mtDNA as weevil DNA: The unusual size and configuration of the mtDNA raised the concern that the DNA analyzed may not have been of weevil origin. In particular, the possibility that the DNA being analyzed was from gut endosymbionts needed to be ruled out. Fungi, yeast, and various other organisms are known to be endosymbionts of wood feeding insects, including Pissodes (BUCHNER 1965) and may have large mtDNA genomes (SEDER-OFF 1984). To that end, several experiments were performed.
TABLE 2
Restriction fragments of D. melanogaster and D. yakuba used in determining gene order in Pissodes mtDNA

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<thead>
<tr>
<th>Fragment</th>
<th>Fragment/clone description</th>
<th>Reference</th>
<th>Coding regions in fragment</th>
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<tr>
<td>A</td>
<td>382-bp XbaI fragment of D. melanogaster cloned into pBS, clone pDMX.20</td>
<td>Solignac, Monnerot and Mounolou (1986), and this paper</td>
<td>125 rRNA</td>
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<td>B</td>
<td>896-bp EcoRI fragment of D. melanogaster cloned into pBR322 (7245-8141)</td>
<td>Garesse (1988)</td>
<td>16S rRNA, tRNA&lt;sup&gt;7&lt;/sup&gt;, ND-1</td>
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<td>C</td>
<td>2198-bp Smal fragment of purified mtDNA of D. melanogaster (5374-7572)</td>
<td>Garesse (1988)</td>
<td>Cyt b, ND-6, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;8&lt;/sup&gt;, ND-4, ND-5</td>
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<td>D</td>
<td>3970-bp SstI fragment of purified mtDNA of D. melanogaster (2285-6253)</td>
<td>Garesse (1988)</td>
<td>ND-5, tRNA&lt;sup&gt;8&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;8&lt;/sup&gt;, tRNA&lt;sup&gt;8&lt;/sup&gt;, ND-3, tRNA&lt;sup&gt;8&lt;/sup&gt;, COII</td>
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<td>E</td>
<td>3719-bp MspI fragment of purified mtDNA of D. melanogaster (1654-5573)</td>
<td>Garesse (1988)</td>
<td>tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, ATPase 6, ATPase 8</td>
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<td>F</td>
<td>1772-bp EcoRI fragment of D. melanogaster cloned into pBR322 (86-1858)</td>
<td>Garesse (1988)</td>
<td>COI</td>
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<td>G</td>
<td>970-bp XbaI fragment of D. yakuba, clone pDYHB (5753-4723)</td>
<td>Clary and Wolstenholme (1985)</td>
<td>tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, ATPase 6, ATPase 8</td>
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<td>H</td>
<td>600-bp SstI fragment of D. yakuba, clone pDYHB of Clary and Wolstenholme (1735-2334)</td>
<td>Clary and Wolstenholme (1985)</td>
<td>COII, ATPase 6, ATPase 8, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, COII, tRNA&lt;sup&gt;6&lt;/sup&gt;, COI, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, ND-2</td>
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<td>I</td>
<td>4868-bp HindIII fragment of D. melanogaster cloned into pBR322 (1-4869)</td>
<td>De Bruijn (1983)</td>
<td>COII, ATPase 6, ATPase 8, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, COII, tRNA&lt;sup&gt;6&lt;/sup&gt;, COI, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, tRNA&lt;sup&gt;6&lt;/sup&gt;, ND-2</td>
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* Letters refer to fragments diagrammed in Figure 1.

* The numbers in parentheses refer to the base pairs contained in the fragment as determined from DNA sequences published in the appropriate reference.

First, the purified Pissodes mtDNA was digested to completion with HindIII, electrophoresed in 0.8% agarose, stained with ethidium bromide, and the banding pattern compared to that obtained through probing of HindIII digested total DNA with Drosophila and presumed Pissodes mtDNA under normal stringency (55° incubation and washes). The band patterns were identical, indicating no contamination by more than one species in the DNA being analyzed and also that the DNA is likely to be insect mtDNA.

Second, to test for gut endosymbiont contamination, thoracic muscle and complete gut tissues were dissected from live individual weevils of P. strobi and P. nemorensis and their separate DNAs prepared. Endosymbionts of Pissodes species are known to inhabit the mycetomes of the anterior mid-gut (Buchner 1965) and the likelihood of endosymbionts occurring in thoracic muscle is remote. Gut tissue DNAs were prepared without evacuating the gut and included as much of the gut as could be removed intact (the anterior esophagus to the anus). Thoracic tissues were removed before dissection of the gut. HindIII banding patterns of these DNAs were then compared through probing with purified mtDNA. Species specific band patterns were observed with no loss or gain of bands between DNAs from thoracic muscle and gut tissue, indicating that there was little likelihood of endosymbiont contamination.

Insect pathogens may also contain large mtDNA genomes displaying size variation (Powers, Platzer and Hyman 1986). Dissected weevils displayed no obvious pathology (e.g., fungal or nematode infection) that might have led to identical band patterns in the two DNAs. Furthermore, the probability is remote that all individuals across the wide seasonal and geographic ranges of all three species would contain levels of fungal or nematode infection sufficient to mimic mtDNA of the host.

Third, total genomic DNA of D. melanogaster was digested and probed with purified Pissodes mtDNA at normal stringency (55° incubation and washes) and the restriction fragment patterns compared to published data. The purified mtDNA hybridized to Drosophila mtDNA under the conditions used, except for A+T-rich regions; again suggesting that the DNA is insect mtDNA. Taken together, the results of these experiments sufficiently rule out the possibility that the DNA being analyzed is of endosymbiont or pathological origin and strongly indicate that it is mtDNA of Pissodes.

**Gene order and genome organization:** The restriction enzyme map of Pissodes species obtained through double digestion profiles and indirect end labeling was wholly consistent with the map obtained through...
probing with specific fragments of Drosophila mtDNA and through probings of Drosophila melanogaster mtDNA with Pissodes mtDNA clones. In no case did a Drosophila fragment probe two discontinuous Pissodes fragments, indicating that there has not been any major duplication or extensive rearrangement of gene sequences. Drosophila genes all map along a single, contiguous segment of Pissodes mtDNA; gene order appears identical in Pissodes and Drosophila (Figure 1). The region of Pissodes mtDNA containing the coding sequences is the region cut most often by restriction enzymes, while the large, typically uncut region and the repeated sequences in Pissodes mtDNA map to the A+T-rich region of Drosophila mtDNA.

Probing of mapping digests for the three Pissodes species with particular fragments of P. nemorensis mtDNA allowed the alignment of homologous restriction sites. Probing of mapping digests of H. pales and A. grandis with the 4.8-kb HindIII fragment of D. yakuba and the Pissodes clone pPSBX.28 aligned the restriction site maps of these weevils with that of Pissodes and Drosophila. The mtDNA of A. grandis is of a more common size, 18.2 kb. The restriction site map of H. pales reveals that it is slightly larger (ca. 26 kb). No obvious repeated sequence motifs are observed in the restriction site maps of these two species.

**Characterization and mapping of repeated sequences:** The presence of variable numbers and sizes of repeated sequences in Pissodes mtDNA can be seen in restriction enzyme digestion patterns for HindIII, XbaI, and HaeIII. Mapping digests probed with a clone of the repeat unit (pPSH.2) show that several fragments in the 0.8–2.0-kb size range as well as the very large, uncut fragment contain these repeated sequences (Figure 4). Indirect end-labeling of partial digestions with these enzymes (as described above) reveal that the repeat units are arranged tandemly (Figure 2). None of the other three clones of Pissodes mtDNA contain repeated sequences. MtDNA from H. pales and A. grandis, and D. melanogaster do not
contain sequences similar enough to the repeated region in Pissodes to be probed under the conditions of reduced stringency used (42°).

DISCUSSION

The mtDNA of these species of Pissodes is unusual in the following respects: (1) it is uniformly nearly twice as large as most metazoan mtDNAs, (2) the large size is accompanied by extensive size variation and heteroplasmy, and (3) these characteristics are shared by all observed individuals in the three species examined. These three species of Pissodes are members of the Pissodes strobi species group, and are considered to be closely related (PHILLIPS 1984). Yet, the mtDNA

FIGURE 2.—Fragment size and order via indirect end-labeling of Pissodes mtDNA. Total genomic DNA from an individual with *P. nemorensis* mtDNA was digested to completion with *PstI* then partially digested with *HindIII*, electrophoresed and transferred to a nylon filter. The filter was probed in succession with pPSBX.28 (lane A) and pPSH.6.1 (lane B). Thus, the two band patterns represent the opposite orders of *HindIII* restriction sites. Note that this map corresponds with that of *P. nemorensis* diagrammed in Figure 1. Distances between bands correspond to fragment lengths except for *PstI/HindIII* double digestion products as indicated at the bottom of each lane. The 1.8-kb *PstI* fragment appears in both lanes A and B, and is proportionately darker since it is a complete digestion product. Size standard is Bethesda Research Laboratories 1-kb ladder in middle lane (std). Standard band sizes are (from top to bottom in kb) 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0, 0.5, 0.4. The 1.6, 0.5, and 0.4 kb standard fragments share sequence with the vector used (pBS) and thus appear exceptionally dark, partially masking the 2.0-kb standard fragment just above it.
mtDNA in Bark Weevils

Figure 3.—Heteroplasmy in Pissodes nemorensis (lanes 2–9), P. strobi (lanes 10–16 and 18), and P. terminalis (lanes 19–22 and 24–27). Individual weevils typically display several size classes of the largest BglI digestion product indicated by arrow. BglI restriction sites lie outside the size variable region for all three species (Figure 1). Close examination of the autoradiographs and digestion patterns with other enzymes confirm that weevils appearing homoplasmic in this figure, e.g., lanes 19 and 27, are, in fact, heteroplasmic. Size standards (lanes 1, 17, and 23) are Bethesda Research Laboratories 1-kb ladder.

Figure 4.—Size variation and distribution of repeated sequences. A and B are the same mapping digests of an individual P. nemorensis probed with (A) clone pPSH.2 containing repeated sequences and (B) with complete Pissodes mtDNA. Digestion patterns are from digests with: lane 1, HindIII; lane 2, HindIII and HaellI; lane 3, HaellI; lane 4, HindIII; lane 5, HindIII and HinfI; lane 6, HinfI. The cloned fragment corresponds to the 1.6-kb HindIII fragment identified by the arrow. Note that several bands (400 bp to ca. 2 kb) and the largest band (ca. 16+ kb) are probed by the clone. Fragments not probed by the cloned repeated sequences map to the Pissodes coding region.

P. nemorensis

P. strobi

P. terminalis

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The very large size mtDNA of these weevils is not apparent in the other weevil taxa studied (Figure 5). A. grandis displays a typical size of 18.2 kb. The mtDNA of H. pales, while not as large as that of Pissodes, is ca. 6 kb larger than typical. Whether a tendency for large mtDNA size (and/or heteroplasmy) is a characteristic of the clade to which Pissodes is a member will require more extensive characterization of several genomes in the clade and the rest of Pissodes as well.

Gene content and order: Through the use of genespecific clones and restriction enzyme fragments from Drosophila mtDNA, we were able to determine that the mtDNA of Pissodes is similar to that of Drosophila in that it is a closed, circular molecule and contains the same set of gene sequences. No duplications of coding sequence were detected. Furthermore, the major coding regions of Pissodes mtDNA occur in the same order as in Drosophila and display the same contiguous arrangement. As the Diptera and Coleoptera lineages diverged at least 250 million years ago (RIEK 1970), this observation further reinforces other work suggesting that major gene rearrangements may occur only rarely, with relatively few (two to six) rearrangements accounting for different gene orders among major phyla (JACOBS et al. 1988; but see YANG and ZHOU 1988).

Transfer-RNA genes, however, appear to be more labile to rearrangement than the major protein coding genes in some taxa (JACOBS et al. 1988; CANTATORE et al. 1987; MORITZ, DOWLING and BROWN 1987). In insects, HAUCKE and GELLISSEND (1988) have found the order of the tRNA genes coding for lysine and aspartate to be reversed in Locusta migratoria (Orthoptera) as compared to Drosophila yakuba (Diptera). Within the Diptera, the tRNA genes for arginine and alanine in the mosquito Aedes albopictus are reversed relative to D. yakuba, and the direction of transcription has been reversed for the serine tRNA (DUBIN, HSUCHEN and TILLOTSON 1986). Because tRNA coding sequences are short, they may not have been detectable with the probes and hybridization conditions employed and we cannot completely rule out
such rearrangements in Pissodes mtDNA. Similarly, while the mtDNA of Pissodes appears to contain all of the major coding regions of Drosophila mtDNA, we cannot completely rule out the possibility that additional novel coding sequences exist that result in the large size of the molecule. For reasons cited below, however, this possibility seems unlikely.

The large size of Pissodes mtDNA results from the addition to the molecule of a large sequence (9-13 kb) and many tandemly repeated sequences (0.8-2.0 kb in length), over and above sequences required for mtDNA coding functions. This pattern is similar to that found in the sea scallop where ca. 11 kb has been added to the 14 to 16 kb necessary for the coding sequences of a typical mitochondrial genome. In Pissodes, we have found that these two additional regions map genetically to the presumed A+T-rich region of the molecule. The majority of the added sequence has characteristics expected of an A+T-rich region, including a rarity of sites for the restriction enzymes used. The repeated sequences in Pissodes all occur at one end of the presumed A+T-rich region, between the 12S rRNA gene and the large sequence addition. Repeated sequences in Cnemidophorus lizards also occur at one end of the control region of the molecule, adjacent to the rRNA genes, and in fact contain partial coding sequences of the rRNA genes (MORITZ and BROWN 1986). However, as judged by lack of cross hybridization to Drosophila mtDNA and their location in the Pissodes restriction site map, the repeated sequences in Pissodes do not show any sequence similarity to either of the rRNA genes, nor to any other known mtDNA coding region.

Size variation and repeated sequences: The magnitude of size variant differences, the number of size classes possible within single individuals, and the abundance of mtDNA heteroplasmy in all individuals of three species of Pissodes is unprecedented. Most taxa exhibit size class heteroplasmy at population frequencies much lower than observed in Pissodes, although heteroplasmy involving small scale size differences (0.2 kb or less) may be very frequent (e.g., *Rana esculenta*; MONNEROT, MOUNOLOU and SOLIGNAC 1984). Pissodes mtDNA is exceptional in that 100% of the 219 individuals surveyed are heteroplasmic for size variants differing by at least 0.8 kb, and may contain as many as five distinct size classes differing by as much as 7.5 kb (Figure 3). These size classes apparently correspond to the presence of different copy numbers of the repeated sequences in different sized molecules.

The repeated sequences in Pissodes themselves vary in size (0.8-2.0 kb), most often by discrete units of ca. 200 bp, indicating that duplication of the sequence is not always complete, or that smaller insertions/deletions are common within the larger repeating sequence. Insertion and deletion of both large and small fragments may be the result of intermolecular recombination among different mtDNA molecules of the same individual, or the result of intramolecular recombination (RAND and HARRISON 1989).

Slipped mispairing (either within or between molecules) during replication has been implicated as a significant force in DNA sequence evolution (LEVenson and GUTMAN 1987) and could play a role in the generation of size variation in Pissodes. Slipped mis-
pairing involves the generation of insertions and deletions through the mispairing of complementary sequences of tandem repeats. Small size variants in mtDNA may be the result of this mechanism operating on homopolymer tracts (Hauswirth et al. 1984) and larger variants may be the result of similar mechanisms operating on repeat sequences separated by several hundred base pairs. Repeats of a symmetric dyad sequence have been found in cricket mtDNA at the bounds of a repeated 206-bp sequence responsible for size heteroplasmy (Rand and Harrison 1989). Insight into the mechanism by which so many repeated sequences of Pissodes have been generated may be gained through a similar analysis of the repeat (clone pPSH.2).

That the cloned repeat sequence hybridizes to the large, typically uncut fragment indicates sequence similarity between the two regions. The repeated sequences may be implicated in the generation of the overall large size of the molecule via additions to the A+T-rich region through mechanisms of duplication/deletion coupled with high mutation rates in A+T-rich sequences. If the mechanism of duplication/deletion is dependent on the presence of highly similar sequences from repeat to repeat, mutation in those sequences could protect a region from further duplication and deletion. Repeat units changed in this way would presumably not be able to form secondary structures necessary for excision of the unit, and would remain as extra sequence in the A+T-rich region. Extra sequence would continue to accumulate as repeats adjacent to the region lose the ability to excise. The amount of divergence necessary to prevent deletion of a member of the repeated family may not be much. The frequency of deletion of a 1.5-kb repeat in bacteriophage f1 was significantly reduced by the change of only a single base pair in a 14-bp inverted repeat sequence (Albertini et al. 1982). Thus, the large, typically uncut A+T-rich region in Pissodes mtDNA may consist of older repeat units no longer able to be excised. That the extra sequences have accumulated only on one side of the repeated regions may be related to the direction of replication; presumably the repeated regions are the last to be replicated (see below). If this mechanism is responsible for the size increase, sequence similarity between repeat units and the large fragment should decrease as distance from the 12S rRNA gene region increases.

The high degree of insertion/deletion polymorphism may also be related to the location of the repeated sequences relative to the sites of initiation and termination of replication. Rand and Harrison (1986) have suggested that the presence of multiple initiation sites for replication could be a mechanism generating significant frequencies of large size variants. The repeated region of Pissodes mtDNA lies adjacent to the 12S rRNA gene, near or at the presumed origin of replication (Figure 1). If the repeated region contains the initiation site for replication, multiple copies of the region may allow for multiple replication forks, each producing a potentially different sized molecule. This mechanism would still require several duplication events to initially generate multiple sites for initiation of replication since the products of multiple replication forks could only be as large or smaller than the original template molecule.

Alternatively, if the origin of replication is not repeated, the formation of secondary structures involved in slipped mispairing, and thus the generation of larger size variants, could be facilitated by the delay in light strand replication. Drosophila mtDNA initiates heavy strand replication just prior to the 12S rRNA gene and the initiation of light strand replication is typically delayed until heavy strand replication is nearly complete (Goddard and Wolstenholme 1978). If the location of the repeating units lies just prior to the initiation site for heavy strand replication, secondary structures important for duplication and deletion (Rand and Harrison 1989) may be able to form during the delay between heavy and light strand synthesis, promoting the rapid formation of different size classes.

Population genetics of heteroplasmy and mtDNA size: There are a variety of mechanisms that could maintain the high degree of heteroplasmy observed in the three species depending on the degree of selection acting on size variants and the demographics of Pissodes species. These mechanisms can be classified as to whether stochastic (drift of neutral variants) or deterministic forces (selection) are expected to dominate the fate of size variants.

Mitochondrial DNA size variants have usually not been viewed as strictly neutral (Hale and Singh 1986; MacRae and Anderson 1988; Rand and Harrison 1986; Sederoff 1984; Wallace 1989). It is often argued that mtDNA is under selection that acts to keep the molecule compact, perhaps because smaller molecules are more efficiently replicated. Evidence for the selective advantage of smaller genomes has been reported in D. mauritiana (Solignac et al. 1984) and in the cricket Gryllus firmus (Rand and Harrison 1986). This poses a dilemma in explaining the patterns of size variation in Pissodes since there appears to be ample size variation and no clear indication that smaller genomes are selectively favored. If smaller molecules have a significant selective advantage, one would expect the smallest size class to be most frequent, if not fixed in most individuals as is seen in a world-wide survey of D. melanogaster mtDNA size classes (Hale and Singh 1986). In the representatives of Pissodes examined to date, the smallest size classes
are not the most frequent (Figure 3 and T. Boyce, unpublished data). Clark (1988) has shown that a balance of mutation, sampling, and selection can produce globally stable equilibrium frequencies of heteroplasmy under conditions that are not particularly stringent. In particular, where mutation is bidirectional, as among size classes generated via duplication and deletion of repeated elements, mutation and sampling are sufficient for the maintenance of heteroplasmy. If smaller mtDNA molecules in Pissodes are in fact at a selective advantage, then the mutation rate to larger size classes must be high enough to effectively counter the effects of such an advantage.

An additional factor that may act to help maintain significant frequencies of heteroplasmy and large size variants in Pissodes may be related to maternal age effects. Solignac et al. (1987) have shown that the frequency of long mtDNA size variants in progeny increased with female age at oviposition in D. melanogaster. Pissodes adults may live in nature for up to 4 years and reproduce in each of those seasons (McMullen and Condrashoff 1973). If a similar age-dependent bias in size class transmission occurs in Pissodes as in D. melanogaster, population processes that effect time to reproduction or age-dependent fecundity could significantly contribute to the retention of larger mtDNA size variants.

The persistence of the large size through the divergence of the three species suggests that the large size is not at a strong disadvantage. If the size variants observed in Pissodes are strictly neutral, then the level of heteroplasmy becomes a function of the effective population size (which removes variants due to genetic drift) and the mutation rate to new size classes (which generates new variants). Rand and Harrison (1989) used theory developed by Birky, Maruyama and Fuertz (1983) in a hierarchical analysis of size class variation in two species of crickets and found that the balance of mutation rate and drift was such that size class frequency changes were significant from individual to individual due to the effects of drift within cell lineages, but that the mutation rate was high enough to maintain a substantial frequency of heteroplasmy and little differentiation among populations.

Qualitatively, the distribution of size class variation among individuals of Pissodes is similar to that described for crickets in that individuals usually contain unique profiles of size classes, indicating some influence of drift within lineages. However, the overall incidence of heteroplasmy within populations is substantially higher in Pissodes than in crickets (100% vs. 61.5%). While not conclusive, these observations suggest that mutation rates to different size classes and/or mitochondrial effective population sizes may be significantly larger, or rates of vegetative segregation lower in Pissodes relative to most other taxa displaying mtDNA heteroplasmy for size variants (Birky, Fuertz and Maruyama 1989). These results may be particularly relevant to the use of mtDNA markers in studying population structure in Pissodes since high levels of heteroplasmy may lead to significant errors in estimations of population subdivision and gene diversity (Birky, Fuertz and Maruyama 1989). A larger analysis of heteroplasmy in Pissodes, partitioning size variation among individuals, populations and species will be necessary to more completely understand the population genetics of size variation in these species.

An additional, and not necessarily exclusive explanation for the high level of heteroplasmy in Pissodes is paternal leakage of mtDNA. It is commonly assumed that mtDNA is maternally inherited, either because there is no parental contribution at fertilization, or because such contributions would necessarily be a small fraction of mtDNA in the developing embryo and would most likely not become fixed in the germ line (Avise and Vrijenhoek 1987; Lansman, Avise and Huettel 1983; Gyllensten, Wharton and Wilson 1985). However, recent studies have suggested paternal contributions of mtDNA in Drosophila and marine mussels (Satta et al. 1988; W. Brown, personal communication). Significant paternal leakage or outright equal contribution to mtDNA polymorphism would increase the mitochondrial effective population size, and if mtDNA length variants are strictly neutral, larger effective population size would allow the accumulation of a greater number of mtDNA size variants. Furthermore, a significant paternal contribution would allow new combinations of different size classes in progeny. Following the frequencies of size variants from parents through progeny of specific crosses among lines with distinct mtDNA types will be necessary to determine if significant paternal leakage is occurring in these species.

Whatever balance of selection, mutation and drift that may be responsible, the ubiquitous heteroplasmy for size variants differing by as much as 7.5 kb, and the large overall size of mtDNA in all three species of Pissodes is unique among animals studied so far. If, as has been suggested from other studies, smaller sized mtDNA molecules are at some replicative or selective advantage, some mechanism is present in Pissodes that has generated an unusually large size molecule in the face of whatever selective disadvantages might be associated with large size. The large mtDNA of Pissodes has existed since before the divergence of the three species examined, and not as a rare variant within a species. Likewise, heteroplasmy involving an apparently high mutation rate for mtDNA variants of substantially different size has probably persisted over the same period of time. The high frequency of substantial size differences within and among species sug-
gests that, at least in Pissodes, smaller size molecules may not be at any strong selective advantage. Moreover, the extensive size variation contrasts strongly with the conservation of order and structure within the coding region of the molecule, suggesting that the two regions of the molecule experience significantly different selective regimens.

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