Copyright © 2000 by the Genetics Society of America

A Multipartite Mitochondrial Genome in the Potato Cyst Nematode *Globodera pallida*

Miles R. Armstrong, Vivian C. Blok and Mark S. Phillips

Department of Nematology, Scottish Crop Research Institute, Dundee, Scotland DD2 5DA

Manuscript received June 1, 1998
Accepted for publication September 17, 1999

ABSTRACT

The mitochondrial genome (mtDNA) of the plant parasitic nematode *Globodera pallida* exists as a population of small, circular DNAs that, taken individually, are of insufficient length to encode the typical metazoan mitochondrial gene complement. As far as we are aware, this unusual structural organization is unique among higher metazoans, although interesting comparisons can be made with the multipartite mitochondrial genome organizations of plants and fungi. The variation in frequency between populations displayed by some components of the mtDNA is likely to have major implications for the way in which mtDNA can be used in population and evolutionary genetic studies of *G. pallida.*

THE increasing number of species for which the mitochondrial genome (mtDNA) has been completely sequenced has revealed that mtDNAs vary considerably in size, structure, and gene content. For example, the 367-kb mtDNA of the vascular plant *Arabidopsis thaliana* is thought to be composed of three configurations of circular molecules (and two additional subgenomic molecules) and to encode 57 known genes (Klein *et al.* 1994; Unseld *et al.* 1997). In contrast, the mtDNA of the protozoan malarial parasite *Plasmodium falciparum* consists of tandem arrays of a 6-kb sequence that encode only 5 genes (Feagin 1994). Complete mtDNA nucleotide sequences are available for a total of 76 species, of which 58 are metazoan. Within the metazoa, gene content and structure are remarkably stable. The mtDNA of all species characterized encodes the structural RNAs of the mitochondrion's own protein synthesizing machinery and the 12 or 13 proteins involved in electron transport and oxidative phosphorylation (Pont-Kingdon *et al.* 1995). Compared to the mtDNAs of plants and fungi, metazoan mtDNAs exhibit little size variation, ranging from 14 to 39 kb (Moritz *et al.* 1987). Repetitive sequences and the length of the control region generally account for whatever variation there is. Except for some lower metazoan Cnidarian species, where the mtDNA occurs either as a linear chromosome or as two unique 8-kb linear molecules (Bridge *et al.* 1992), metazoan mtDNAs have been found to be single circular molecules.

In this article, we present information regarding the organization of the mtDNA of the potato cyst nematode *Globodera pallida*, a sedentary endoparasite of major economic importance. In particular, we provide evidence that *G. pallida* is unusual among the metazoa in that its mtDNA has a multipartite structure. Six circular DNAs, ranging from ~6.3 to 9.5 kb, have been amplified from a British population of *G. pallida* by PCR, and additional components of the *G. pallida* mtDNA remain uncharacterized. Taken individually, these molecules are considerably smaller than the 13.7-kb mtDNA of the nematode *Onchocerca volvulus*, the smallest completely sequenced metazoan mtDNA (Keddie *et al.* 1998). All of these potential *G. pallida* mtDNAs contain sequences similar to known mitochondrial genes, with most containing sequences that show highest sequence similarity to previously described nematode mitochondrial genes. The complete sequence of the largest putative mtDNA reveals that it contains seven full-length open reading frames similar in sequence to nematode mitochondrial genes. Each mtDNA also shares a common noncoding sequence that may contain the origin of replication. Our data suggest that these small, circular mitochondrial DNAs (scmtDNAs) are present together in populations of *G. pallida*, although their relative frequencies vary considerably between populations. This interpretation was supported by electron microscopic examination of cesium chloride (CsCl)-enriched mtDNA preparations that consist of a population of circular DNAs of variable sizes. No evidence of a molecule >9.5 kb that could define a typical metazoan mtDNA has been observed. Possible mechanisms of mtDNA fragmentation are discussed in the context of animal, plant, and fungal mitochondrial genome organization.

MATERIALS AND METHODS

Cultivation of *G. pallida* populations: The three main *G. pallida* populations used as a source of DNA for PCR amplifications and Southern blotting (Lindley, Friskney, and Gourdie)

Corresponding author: Miles Armstrong, Nematology, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland DD2 5DA.
E-mail: marmst@scri.sari.ac.uk

TABLE 1

Primer sequences

Primer name	Clone	Gene	Primer sequence 5′-3′
p129f	s129a	ND3	CTTCTCTAGGTTTTTTTTGGAGTGG
p129r	s129a	CB	TCACGTAACCTAAAAAAGAGATAAGCA
p116f	s116	COII	CTATCAGTGGTATTGGAGCTATGA
p116r	s116	COII	TCATAGCTCCAATACCACTGATG
p91f	s91	COI	CCTTTTTTTTGGGGGTAAATTTAAC
p91r	s91	COI	CCCCTTCCAAAACTAAAACTACAAA
p226f	x226	ND2	TGGTAGTGGTCCTTTTCTTTTAACTTG
p226r	x226	ND2	TTTAAAAAGGGAAAGAACAACCCTC
p86f	s86	lrRNA	AAAGTTATGAAAATTGACTAAAATTTTGCC
p86r	s86	lrRNA	TGGGGCAAAATTTTAGTCAATTTTC
p222f	x222	—	AAGTTAGAATCGGTTATTCTCTCCACC
p222r	x222	—	CTAGAATGAGTTTTAAGAGTGTGTGAAAGG

Primer sequences used in the amplification of the six circular DNAs described in the text.

were obtained from the potato cyst nematode collection at the Scottish Crop Research Institute that had been collected previously from a variety of field sites in the United Kingdom. Potato cyst nematode populations are named after the location where they were originally collected. Extensive characterization of their mtDNA had failed to identify any RFLP variation between them (M. R. Armstrong, unpublished results). A fourth population, Luffness, which had been shown previously to exhibit mtDNA RFLP variation, provided additional CsCl-enriched mtDNA for electron microscopy analysis. All populations were multiplied as greenhouse cultures in 10-cm pots on the susceptible potato cultivar Désirée.

Plasmids and bacterial strains: The bacterial plasmids pUC19 and pBluescript II SK+ were used as vectors for molecular cloning and DNA sequencing. Competent XL1-Blue *Escherichia coli* strains (Stratagene, La Jolla, CA) were used as recipients in molecular cloning experiments (Sambrook *et al.* 1989).

Nucleic acid isolation, restriction enzyme digestion, and gel electrophoresis: *G. pallida* total genomic DNA was extracted from 50–100 cysts from each of three populations, Lindley, Friskney and Gourdie, using the procedure of Pastrik *et al.* (1995), except a phenol-chloroform (1:1) extraction was added before the isopropanol precipitation. Restriction enzyme digestion and gel electrophoresis were carried out using standard procedures.

mtDNA isolation and library construction: An ~0.5-ml packed volume of eggs from *G. Pallida* population Lindley was homogenized and mitochondria were isolated as described by Powers *et al.* (1986). mtDNA was purified on the basis of circularity by ethidium-cesium chloride (EtBr-CsCl) isopycnic centrifugation from a lysate of these mitochondria. Two libraries were constructed as follows: one with *Sau*3AI-digested mtDNA ligated into dephosphorylated pUC19 linearized with *Bam*HI, and a second with *Xba*I-digested mtDNA ligated into pUC19 linearized with *Xba*I. A second sample of mtDNA was purified, the same way as described previously, from population Luffness. Both the Luffness and Lindley samples were examined by electron microscopy (see below).

DNA sequencing and sequence analysis: DNA sequences of *Sau*3AI and *Xba*I mtDNA library clones were obtained by the dideoxy-chain termination procedure (Sanger *et al.* 1977), initially using the Sequenase sequencing kit (United States Biochemical, Cleveland) and later the ABI prism cycle sequencing kit (Perkin Elmer, Norwalk, CT). Nucleotide sequences were analyzed using the GCG software package, which includes FASTA (Pearson and Lipman 1988) for sequence similarity database searches. Modifications to the universal genetic code used in computer-assisted translation of the mt-nucleotide sequences were based on the putative nematode mitochondrial genetic code proposed for *Ascaris suum* and *Caenorhabditis elegans* by Okimoto *et al.* (1992) and applied successfully in other nematode species (Azevedo and Hyman 1993). These alternative codon assignments are as follows: AGA and AGG, serine; TGA, tryptophan; ATA, methionine.

Primer design and PCR: Primers were designed using the computer program PRIMER (Lincoln *et al.* 1991). In the case of the four primer pairs designed from mt-protein-coding sequences (Table 1), the forward primer was designed 5′-3′ in the direction of transcription and the reverse primer 5′-3′ from the opposite strand. Amplification was achieved using the total genomic DNA extracted from *G. pallida* population Gourdie as template and the GeneAmp XL PCR kit (Perkin Elmer) with the following reaction components: 1× buffer, 0.4 μm each primer, 1 mm $Mg(OAc)_2$, 0.8 mm dNTPs, 3 units of r*Tth* DNA polymerase, and 20–100 ng template DNA. Cycling parameters initially consisted of denaturation at 94° for 1 min; 20 cycles at 94° for 30 sec, annealing at 55° for 30 sec, and extension at 68° for 15 min; another 20 such cycles with an additional 15 sec for the extension step per cycle; and a final extension period of 7 min at 68°. After the size of amplification products had been determined, the extension time was reduced to 1 min/kb.

Determining the complete sequence of scmtDNA I: The PCR products generated with primers p129f-p116r and p129r-p116f were cloned into pBluescript II SK+. The sequence of each PCR product was determined for both strands using primer walking. Two clones of each PCR product were sequenced. The nucleotide sequence of scmtDNA I has been submitted to the EMBL Data Library under accession no. AJ249395.

DNA transfer-hybridization: DNAs were ^{32}P-labeled *in vitro* by random priming (Feinberg and Vogelstein 1983) and were annealed to electrophoretically fractionated DNA preparations that had been alkali transferred (Sambrook *et al.* 1989) to Hybond N^+ nylon filters (Amersham, Arlington Heights, IL). Approximately 1 μg of genomic DNA was transferred from each sample. Molecular weight markers were coelectrophoresed with the DNA preparations. After transfer and hybridization, the 1-kb marker (Promega, Madison, WI) hybridized to cloned probes, enabling direct size estimation of hybridizing mtDNA restriction fragments. Filters were washed under stringent conditions in 0.1× SSPE, 0.1% SDS at 65° for

TABLE 2

Group C clones from the mtDNA library

Clone name	Length (bp)	*A. suum* Similarity	*A. suum* % Identity	*C. elegans* Similarity	*C. elegans* % Identity	Gene
s91	184	80	52	82	53	COI
s116	210	70	60	70	60	COII
s129b	308	67	40	69	69	COII
s34	250	77	42	77	49	ND5
s36	290	66	25	64	16	ND1
x226	1318	54	20	57	21	ND2
s129a	582	75	34	75	30	ND3
		74	53	71	49	CB
s22	324	64	32	64	27	ATPase6
s86	204		62		60	lrRNA
s18	168		72		73	lrRNA

Percentage identity and similarity, with the mitochondrial genomes of *A. suum* and *C. elegans*, of the deduced amino acid sequences of eight translatable group C sequences and percentage identity of the two ribosomal sequences isolated from the mtDNA library.

at least 15 min. Bound probes were removed by incubating filters in 0.4 m NaOH for 30 min at 45°, followed by incubation in 0.2 m Tris-HCl, pH 8.0, 0.1× SSC for 15 min at 45°.

Electron microscopy: mtDNAs from populations Lindley and Luffness were spread for electron microscopy using the cytochrome-C-hypophase technique (Davis *et al.* 1971). Contour lengths were measured with a CalComp graphics tablet interfaced to an Elonex PC-425X computer. Double-stranded DNA plasmids M13mp-18RFI (Pharmacia, Piscataway, NJ) and Bluebac3 (Invitrogen, San Diego) were measured separately as size reference standards.

RESULTS

Sequences from the mtDNA libraries: Sequencing clones from the *Sau*3AI mtDNA library generated 53 sequences. In addition, two large clones of 1318 and 2201 bp, designated x226 and x222, from the *Xba*I library were sequenced completely. These 55 sequences can be divided into three groups on the basis of nucleotide sequence similarity to known mitochondrial sequences and degree of repetition within the library. Group A sequences (11 sequences) were represented once in the library and did not show significant similarity with any mitochondrial sequences on the EMBL database. Group B sequences (34 sequences) were repeated at least once within the library and did not show significant similarity with any mitochondrial sequences on the EMBL database. Of the group B sequences, 18 constitute various internal regions of the 2201-bp *Xba*I clone x222. Group C sequences (10 sequences) showed a high percentage identity with a total of nine mitochondrial genes. These clones are listed in Table 2 with the percentage identity and similarity of their deduced amino acid sequences with *C. elegans* and *A. suum* mt-proteins. With the exception of the putative NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), and subunit 6 of the F_o ATPase complex (ATPase6) putative homologues, which showed sequence similarities with a variety of invertebrate mitochondrial sequences, the best matches were with the mtDNAs of the nematodes *C. elegans* and *A. suum.*

Group B and C sequences hybridize with the same restriction fragments that hybridize with CsCl-enriched mtDNA: A series of autoradiographs, obtained after hybridization of a single Southern blot of digested *G. pallida* genomic DNA from population Friskney with three probes, is presented in Figure 1. After each round of hybridization, the Southern blot was stripped and was then reprobed. In Figure 1A, the probe is a circular DNA preparation (obtained using EtBr-CsCl isopycnic

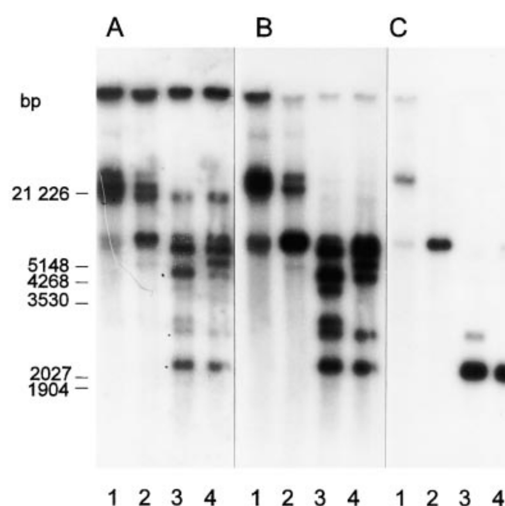

Figure 1.—Hybridization of a single Southern blot of restriction-digested *G. pallida* genomic DNA from population Friskney. Undigested (lane 1), *Sac*I (lane 2), *Sac*I/*Hin*dIII (lane 3), and *Hin*dIII (lane 4). (A) Probed with CsCl-enriched mtDNA. (B) Probed with clone x222. (C) Probed with clone s86.

centrifugation) prepared from a mitochondrially enriched fraction and likely to be enriched in mtDNA. In Figure 1, B and C, the probes are the group B clone x222 and a group C clone s86, similar in sequence to the *C. elegans* mitochondrial large ribosomal RNA (lrRNA) gene. In both cases, the hybridizing restriction fragments correspond to those in Figure 1A, suggesting that both clones are similar in sequence to *G. pallida* mtDNA. Although s86 hybridized to a limited subset of the restriction fragments observed in Figure 1A, as would be expected if s86 formed part of the mtDNA, x222 hybridized with many of the restriction fragments observed in Figure 1A. Given that clone x222 is not similar in sequence with known mitochondrial genes and is apparently noncoding, this may suggest that the *G. pallida* mtDNA contains a noncoding region. The physical relationships between the group C sequences, as well as those between the group C sequences and the group B sequence x222, were investigated by PCR.

PCR analysis: In an attempt to amplify the entire *G. pallida* mtDNA by PCR, five pairs of primers specific to group C clones from the mtDNA library were tested in various combinations (Table 1). The template for these amplifications was total genomic DNA extracted from population Gourdie. The majority of primer combinations were unsuccessful. Only 2 of the 27 primer combinations tested amplified a product. These two products were generated by combining a cytochrome c oxidase subunit II (COII) forward primer specific to s116 sense strand (p116f) with a cytochrome b (CB) antisense primer specific to s129a (p129r) and vice versa. However, by combining these five pairs of primers with a single pair of primers specific to clone x222, five pairs of PCR products were amplified. Each of the six pairs of PCR products represents a complete circular molecule with overlapping regions (shown diagrammatically in Figure 2). Accounting for overlaps, the sizes of these scmtDNAs were estimated to range from ~6.3 to ~9.5 kb. In the case of the five scmtDNAs amplified with primers derived from the sequence of clone x222, primers designed 5′-3′ in the direction of transcription of the sense strand generated a product when combined with primer p222f, and primers designed from the opposite strand generated a product in combination with p222r, suggesting that the direction of transcription of these mitochondrial genes, relative to the x222 sequence, is the same on all five scmtDNAs.

Associating clones from the mtDNA library with the amplified circular sequences: The results of probing Southern blots of the library clones listed in Table 2 with the 12 sequences that comprise the six scmtDNA molecules described previously are presented diagrammatically in Figure 2. In each case, the inner ring of the diagram represents a hypothetical gene content for the scmtDNA based on hybridization of available probes. We assume that any clone found to hybridize was derived from a full-length coding sequence and that *G. pallida* mt genes are approximately of the same length as those of *C. elegans.* These assumptions are supported by the results of sequencing scmtDNA I (Table 3).

From these data, it is evident that scmtDNA I has a sequence similar to the group B clone x222 downstream of p129f and upstream of p116r. The 10 PCR products constituting the five scmtDNAs generated using primers derived from x222 (scmtDNAs II–VI) also hybridized to clone x222. A number of group C clones were also found to be present on more than one of the scmtDNAs. Clone s129a (ND3 and CB) hybridized to PCR products that comprise both scmtDNAs I and III; clones s36 (ND1) and s129b (COII) hybridized to both scmtDNAs I and II. Some group C clones were found to be uniquely associated with certain scmtDNAs. For example, clones s18, s86, and s22 (lrRNA and ATPase6) hybridized exclusively to scmtDNA IV, clone s91 (COI) hybridized to scmtDNA V, and clone x226 (ND2) hybridized to scmtDNA VI.

Restriction mapping: Each scmtDNA was mapped with six restriction enzymes. Restriction maps of the six scmtDNAs are presented in Figure 2. In the case of scmtDNA IV, an additional enzyme (*Sac*I) was mapped to provide support for our interpretation of the hybridization pattern observed in Figure 1C. scmtDNAs IV and V share a *Pst*I site that mapped to within 500 bp of the p222f primer. Direct sequencing of these PCR products with primer p222f had predicted a site at 440 bp for these two sequences. The remaining four scmtDNAs share a common *Ava*I site ~500 bp from the same primer, and this observation was also confirmed by direct sequencing. scmtDNAs I and II have a similar order of restriction sites downstream of the p116f primer site. To determine if this conserved order of restriction sites was coincidental, the PCR products generated with p116f and p222f (scmtDNA II) and p116f and p129r (scmtDNA I) were digested with *Xba*I, *Eco*RI, and *Bam*HI, and the samples were coelectrophoresed. The *Xba*I, *Eco*RI, and *Bam*HI fragments immediately downstream of the p116f primer site from both these PCR products were shown to be indistinguishable in size (data not shown). This suggests that these two scmtDNAs are similar in sequence in the region extending between the shared COII sequence and *Bam*HI sites and presumably divergent thereafter, as demonstrated by the absence of a ND3 or CB sequence on scmtDNA II. Other than these similarities, it is hard to discern any direct relationships between the circular sequences on the basis of restriction mapping.

Given that clone x222 was isolated from an *Xba*I library, it would be expected that *Xba*I sites would map to the ends of the x222 sequence. Only scmtDNA III has *Xba*I sites in the expected positions. It is presumed that clone x222 was originally derived from scmtDNA III, with the other scmtDNAs containing similar but nonidentical sequences. This is further demonstrated

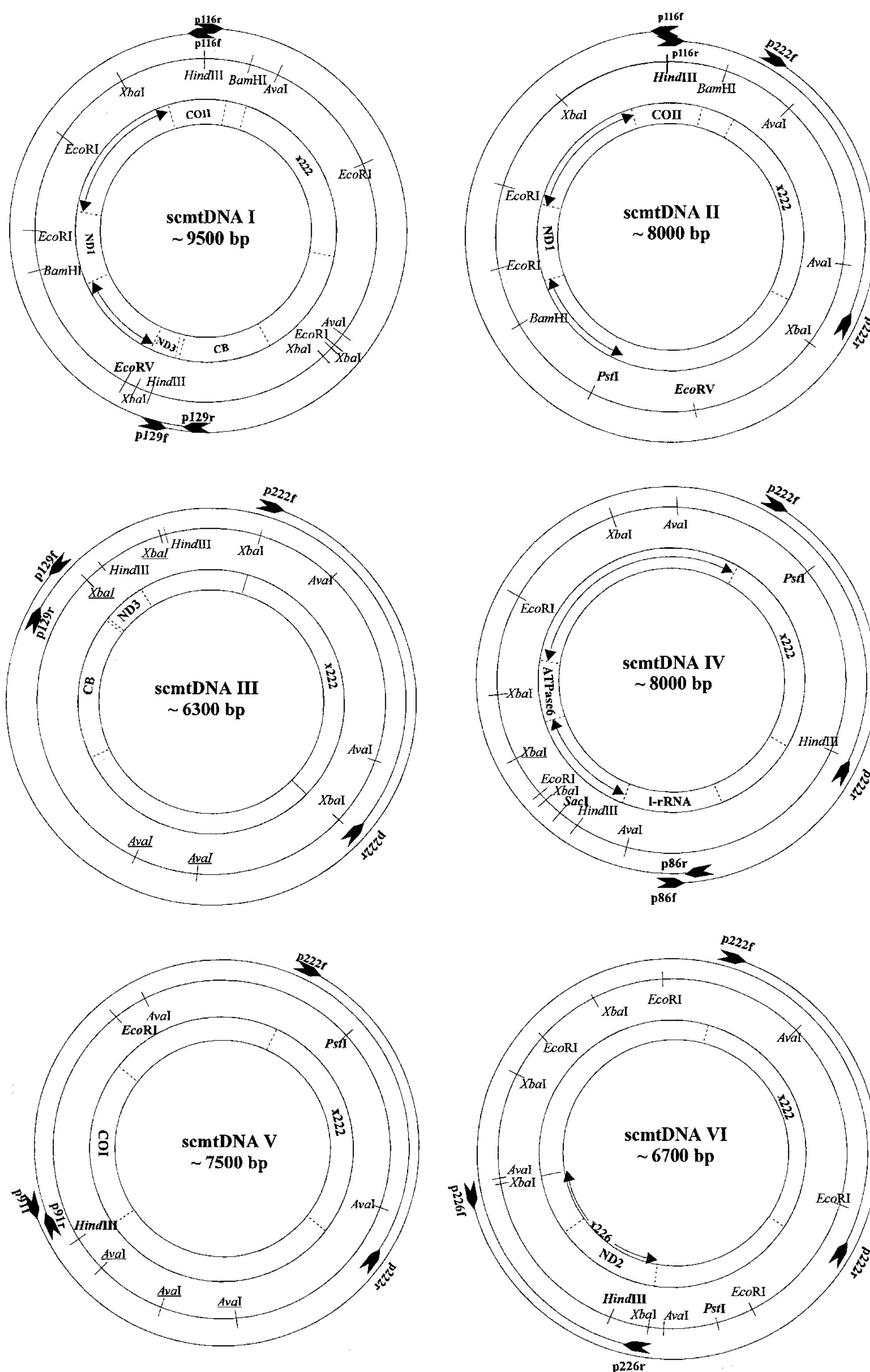

Figure 2.—Restriction maps of the six scmtDNAs amplified by PCR from population Gourdie. The outer rings show the orientation and position of the primers used to generate each scmtDNA. Inside these rings, a restriction map is presented, unordered restriction sites are underlined, and enzymes that have a single restriction site on any particular scmtDNA are in boldface type. The innermost ring depicts the presumed gene content on the basis of the primer sequences used to amplify the scmtDNA and hybridization to mtDNA library clones. Where a gene cannot be located precisely, the possible range of locations is indicated by arrows.

by the variation in restriction sites for various enzymes that map within the sequence of x222.

The complete sequence of scmtDNA I: The complete 9428-bp sequence of scmtDNA I was determined. For each of the seven protein-coding genes, the most likely translation initiation codon, based on alignments with other nematode mitochondrial genes, was assigned. Four of the proteins are proposed to begin with ATA and the remaining three with ATT; both of these codons have been proposed to commonly be used in nematode mtDNA translation initiation (Okimoto *et al.* 1992). Each gene has a complete TAA or TAG translation ter-

TABLE 3

Comparison of the seven *G. pallida* mt proteins found on scmtDNA I with those of *C. elegans* and *A. suum*

	Length (aa)			% Identity	
	G. pallida	*C. elegans*	*A. suum*	*A. suum*	*C. elegans*
COII	237	231	232	51.9 (233)	50.7 (229)
ND4	402	409	409	37 (400)	37.4 (401)
COIII	257	255	255	48 (254)	46.9 (254)
ND6	147	144	144	32.8 (122)	36.9 (122)
ND1	287	291	290	33.4 (287)	26.5 (291)
ND3	111	111	111	36.8 (106)	37 (92)
CB	360	370	365	50.6 (346)	46.7 (351)

Comparison of the length and percentage amino acid identity of the deduced amino acid sequences of the seven ORFs found on scmtDNA I with the corresponding mt proteins from *A. suum* and *C. elegans.* The length of the overlap from which the percentage amino acid identity was calculated is indicated in parentheses.

mination codon and is separated by a short intergenic region of between 25 and 173 bp. The percentage identity of these seven deduced amino acid sequences with the corresponding *C. elegans* and *A. suum* sequences is presented in Table 3 with a comparison of the lengths of *G. pallida* genes with the other nematode species. In general, *G. pallida* mt-protein genes are of similar length to those of *C. elegans* and *A. suum.* The distribution of these seven ORFs within the sequence of scmtDNA I is shown in Figure 3 with the precise order of restriction sites for the six enzymes used to map this molecule. A good correspondence between the order of restriction sites suggested by restriction mapping (Figure 2) and this order is evident. Other than the seven ORFs mentioned previously, only one ORF >300 nucleotides was identified, and this sequence was not similar to any known mitochondrial gene. In addition, no region of scmtDNA I similar in sequence to rRNA genes was identified.

As predicted from Southern hybridization analysis, scmtDNA I was demonstrated to contain a region similar in sequence with clone x222 downstream of the translation termination codon of the CB gene and upstream of the COII gene. Comparison of the 2201 bp of the x222 sequence with scmtDNA I revealed that they are 91.7% identical over a 2229-bp overlap. Given that clone x222 is presumed to have been derived from scmtDNA III, this is a measure of the sequence divergence between scmtDNAs I and III within this region. Further evidence of sequence dissimilarity between scmtDNAs was provided by the comparison of the protein-coding sequences present on scmtDNA I and those obtained from the mtDNA library described in Table 2. The sequence of clone s129a was found to be 99% identical to the scmtDNA I ND3 and CB genes over 581 bp of comparable sequence, clone s129b was found to be 98% identical with the scmtDNA I COII gene over 308 bp, and clone s36 was found to be 99.5% identical with the scmtDNA I ND1 sequence.

Verification of the restriction maps: The relationship between the six restriction-mapped scmtDNAs amplified by PCR from population Gourdie and related sequences in genomic DNA was examined by probing a series of Southern blots of restriction-digested DNA from population Gourdie with probes specific to the

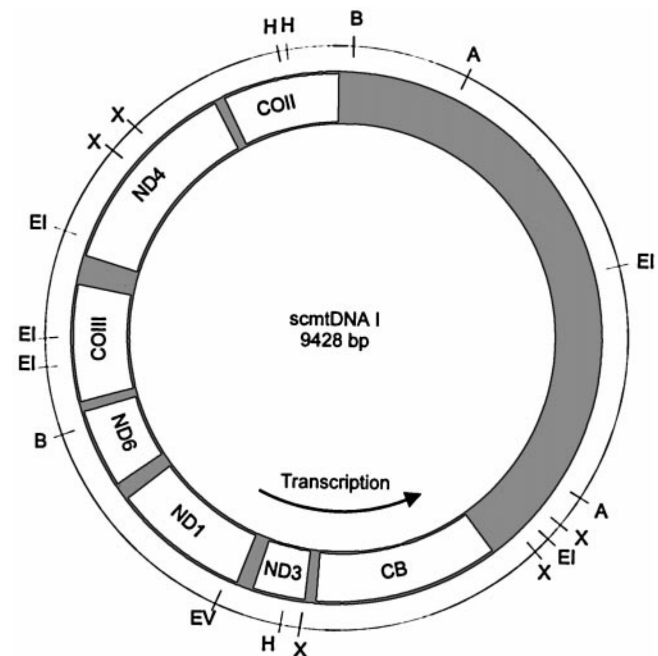

Figure 3.—The gene content of scmtDNA I. The direction of transcription of the seven mt-protein-coding genes is indicated with an arrow. These proteins are all components of the oxidative phosphorylation system: cytochrome b (CB), subunits of cytochrome c oxidase (COII and III), and subunits of the respiratory chain NADH dehydrogenase (ND1, 3, 4, and 6). The precise order of restriction sites for the six restriction enzymes mapped on scmtDNA I in Figure 2 is indicated. Abbreviations used for restriction enzymes are as follows: A, *Ava*I; B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII: X, *Xba*I. The noncoding region, which contains a sequence similar to clone x222, is hatched, as are the short intergenic sequences.

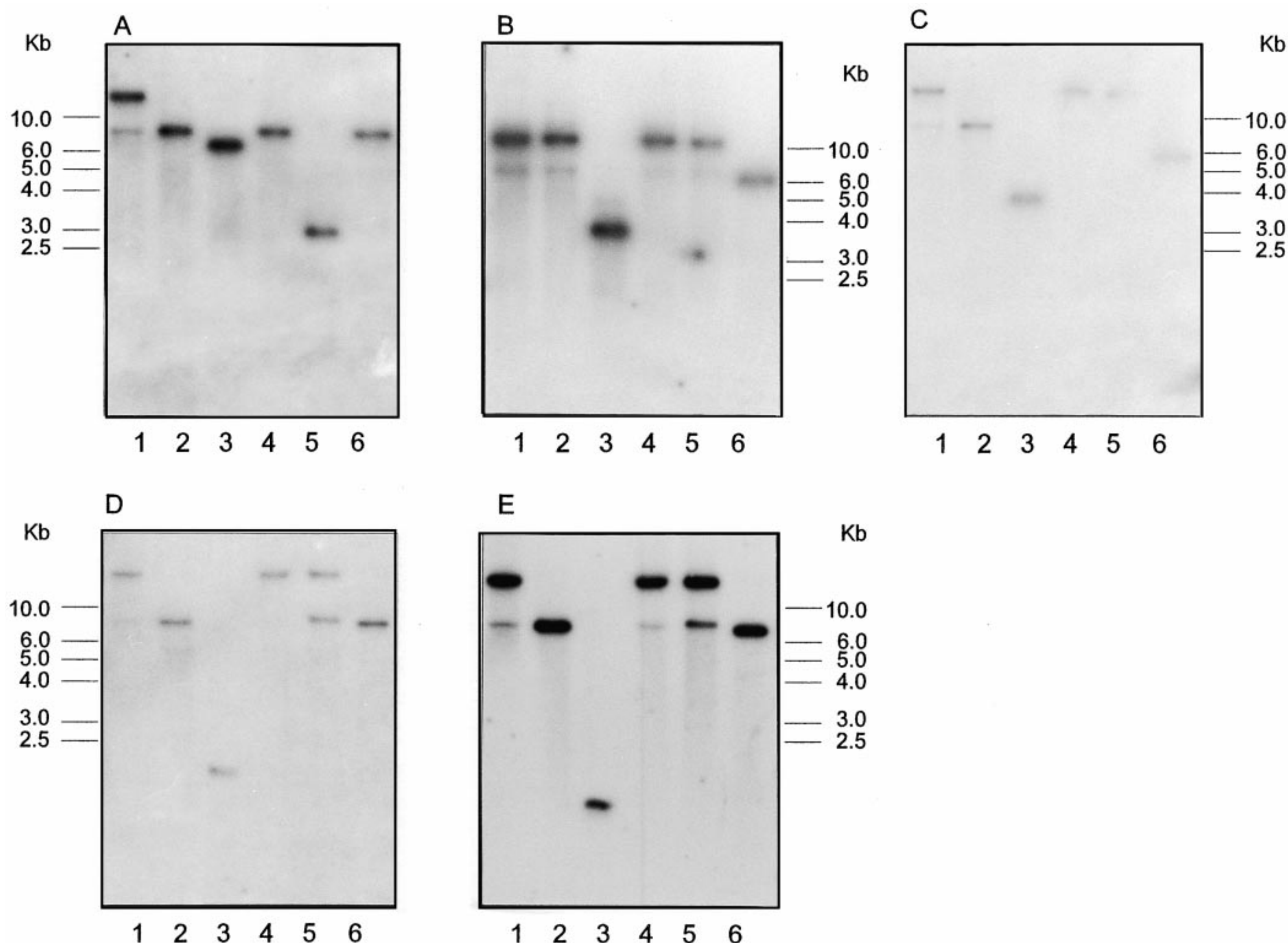

Figure 4.—Hybridization of two Southern blots of restriction-digested *G. pallida* genomic DNA from population Gourdie. (A–C) Blot 1. (D and E) Blot 2. Undigested (lane 1), *Pst*I (lane 2), *Ava*I (lane 3), *Eco*RV (lane 4), *Bam*HI (lane 5), and *Hin*dIII (lane 6). (A) Probed with s129b (scmtDNAs I and II). (B) Probed with s129a (scmtDNAs I and III). (C) Probed with s22 (scmtDNA IV). (D) Probed with s91 (scmtDNA V). (E) Probed with x226 (scmtDNA VI).

various scmtDNAs. The results of five such experiments are presented in Figure 4. In Figure 4A, the probe is s129b (COII). This clone had previously been found to hybridize with scmtDNAs I and II; consequently, for each restriction enzyme, two hybridizing restriction fragments would be expected. In each case, only one restriction fragment is observed; however, the pattern of hybridization is in complete agreement with the predicted pattern based on the restriction map of scmtDNA II. The samples digested with the three enzymes predicted to cut this molecule once (*Pst*I, *Eco*RV, and *Hin*dIII) contain a single restriction fragment estimated as being 7.6 kb in length. This is consistent with the previously predicted size of 8 kb for scmtDNA II. It is highly unlikely that these three restriction fragments could have been generated by digestion of a molecule larger than scmtDNA II, as the sizes of these restriction fragments would be expected to vary. Furthermore, the sample digested with *Ava*I contains a restriction fragment estimated as 6.4 kb in length. Restriction mapping predicted this fragment would be 6.7 kb. The observed *Bam*HI fragment was estimated to be 2.9 kb, which again is similar to the predicted size of 3.1 kb. After a 4-day exposure, no evidence of the restriction fragments predicted from the map of scmtDNA I, or any additional restriction fragments, was detected.

The undigested sample in Figure 4A contains at least two hybridizing DNA species. Circular DNA molecules can exist in a number of forms: supercoiled, nicked open circular, and linear. Supercoiled DNA would be expected to have migrated farthest after agarose gel electrophoresis, followed by linear DNA and finally by open circular DNA. The observation that the single ~7.6-kb restriction fragments observed in samples two, four, and six comigrate with the farthest migrating species in the undigested sample demonstrates that this species is likely to be linear rather than supercoiled DNA. The position of the more slowly migrating species suggests it is open circular DNA. The apparent absence of supercoiled mtDNA in the undigested sample is consistent with the observations of Poulton *et al.* (1993) and Ballinger *et al.* (1994), where human mtDNA,

after agarose gel electrophoresis and Southern blotting of total genomic DNA, was found to exist primarily as open circular and linear forms. Supercoiled mtDNA was not detected.

The result of reprobing this Southern blot with clone s129a is shown in Figure 4B. Clone s129a had previously been found to hybridize with scmtDNAs I and III. The restriction fragments detected in Figure 4B are entirely consistent with having originated from scmtDNA III, and no evidence of scmtDNA I was detected in population Gourdie. However, when both these probes were hybridized with genomic DNA samples from Luffness, a British population thought to be distinct from the majority of British populations (Phillips *et al.* 1992), the restriction fragments predicted from the map of scmtDNA I were observed in the absence of restriction fragments derived from scmtDNAs II and III (data not shown).

Figure 4C shows the result of reprobing this Southern blot with clone s22 (ATPase6). This clone had been found to hybridize with scmtDNA IV. Again, the pattern of hybridization is in agreement with the restriction map of scmtDNA IV. The putative linear species in the undigested sample comigrates with the single restriction fragment in the *Pst*I-digested sample, confirming that a single *Pst*I site is present on this scmtDNA and that the length of scmtDNA IV is ~8 kb.

Figure 4, D and E, are autoradiographs obtained after two rounds of hybridization using a duplicate Southern blot to that presented in Figure 4, A–C. In Figure 4D, the probe is s91 (COI). Primers derived from this clone generated scmtDNA V, which had been predicted to contain single recognition sequences for *Pst*I and *Hin*dIII and to lack recognition sequences for *Bam*HI and *Eco*RV. The observed pattern of hybridization is consistent with these expectaions. In Figure 4E, the probe was x226 (ND2), a clone uniquely associated with scmtDNA VI. The pattern of hybridization is entirely consistent with the restriction map of scmtDNA VI. Note the single restriction fragments in the *Pst*I- and *Hin*dIII-digested sample (enzymes predicted to cut this scmtDNA once) comigrating with the proposed linear species in the undigested sample. Also note the apparent lack of digestion in the samples treated with *Eco*RV and *Bam*HI, both of which were predicted not to digest scmtDNA VI.

Reinterpretation of Figure 1: The electrophoretic properties of scmtDNAs in total genomic DNA extractions observed in Figure 4 enabled the pattern of hybridization in Figure 1 to be interpreted more precisely. As supercoiled forms of the scmtDNAs were apparently not detected in the genomic DNA extractions in Figure 4, it seems reasonable to assume that the species migrating at ~8 kb and below in the undigested samples in Figure 1 are linear. The species migrating behind the 21-kb marker can be interpreted in a number of ways. They may represent circular concatemers or nuclear sequences with mitochondrial homology. However, the data in Figure 4 indicate that they might also be a collection of open circular scmtDNAs. This interpretation is supported by the observation that the undigested sample of Figure 1C (obtained by hybridization with clone s86 from scmtDNA IV) apparently contains a single putative linear species of ~8 kb and a single putative open circular species migrating behind the 21-kb marker. The *Sac*I-digested sample (Figure 1C, sample 2) contains a single restriction fragment that comigrates with the putative linear species in the undigested sample. This would be expected, as scmtDNA IV contains a single *Sac*I recognition sequence.

If the restriction fragments in Figure 1A are derived from a collection of scmtDNAs, this would explain to some extent why clone x222 (a sequence common to all scmtDNAs) hybridized with so many restriction fragments in Figure 1B. Indeed, the pattern of hybridization in the *Hin*dIII-digested sample (sample 4) of Figure 1, A and B, can be partially predicted from the restriction maps in Figure 2. However, the presence of scmtDNAs that are at present only partially characterized prohibits a complete explanation.

Electron microscopic examination of EtBr-CsCl-enriched mtDNA: In total, 56 measurements of the 7.2-kb double-stranded circular DNA plasmid M13mp18-RFI (Pharmacia) and 30 measurements of the 10.2-kb double-stranded circular DNA plasmid Bluebac3 (Invitrogen) were obtained. The mean contour lengths and sample standard deviations for M13 and Bluebac3 were 2287 ± 112 and 3184 ± 98 nm, respectively, having removed from the data two apparent Bluebac3 dimers and three M13 molecules of <3 kb. This suggests an average conversion factor of 313 nm/kb, a figure consistent with previous investigations (Bendich 1993). This figure was used to convert subsequent contour measurements from nanometers to kilobases. Figure 5 presents

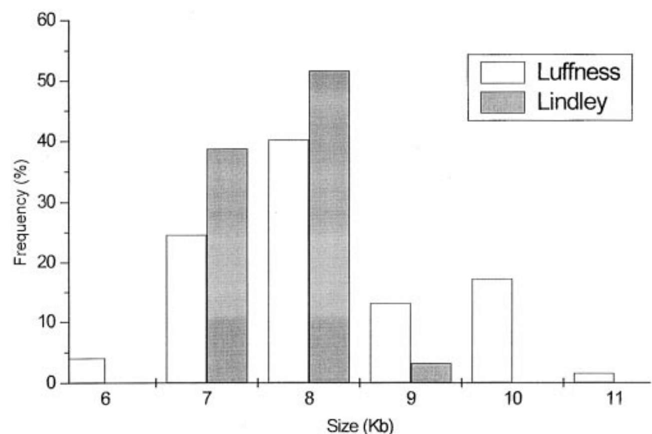

Figure 5.—Size estimation of *G. pallida* mtDNA. mtDNA from *G. pallida* populations Luffness and Lindley was prepared by CsCl-EtBr isopycnic centrifugation. mtDNA and double-stranded DNA plasmids were spread separately for electron microscopy. The histogram shows the size distribution of 122 Luffness mtDNA molecules and 31 Lindley mtDNAs.

a histogram showing the frequency distribution of 31 measurements taken from a series of electron micrographs of EtBr-CsCl-enriched mtDNA spreads from the British population Lindley alongside 122 measurements from the British population Luffness. The preparations contain populations of circular, double-stranded DNA molecules of variable lengths. The observed size range falls between 5.1 and 10.35 kb, with one observation of 0.9 kb from Luffness and one of 3.1 kb and another of 3.2 kb from Lindley. Because this size range overlaps with the sizes of the control DNAs, the plasmids were spread and measured separately from the mtDNA preparations. These results are entirely consistent with the previous PCR amplification, restriction mapping, and Southern blotting and support the interpretation that the mtDNA of *G. pallida* populations exists as a heterogeneous population of subgenomic-sized circular chromosomes.

DISCUSSION

Sequencing the mtDNA libraries identified 10 clones similar in sequence to mitochondrial genes. The 8 sequences similar to mitochondrial-protein-coding sequences were translated according to the modifications to the universal genetic code proposed for the mtDNAs of the nematodes *C. elegans* and *A. suum* (Okimoto *et al.* 1992). Clones x226 and s129a contained internal TGA codons and, as a result, could not be translated using the standard genetic code. In general, these 10 sequences were most similar to the mitochondrial genomes of *C. elegans* and *A. suum*, although 3 clones (x226, s22, and s36) were found to be similar to a variety of invertebrate ND2, ATPase6, and ND1 sequences. All 10 clones were found to hybridize to *G. pallida* genomic DNA and to cross-hybridize with restriction fragments detected when CsCl-enriched mtDNA was used as a probe. A sequence was also identified that was repeated within the library (x222). Southern hybridization suggested that this sequence was similar in sequence with CsCl-enriched mtDNA. PCR analysis further suggested that the x222 sequence was present on at least six scmtDNAs. These scmtDNAs have all been detected in Southern blots of *G. pallida* total genomic DNA, although scmtDNA I is not present at a detectable frequency in total genomic DNA from population Gourdie. Sequence analysis of the repetitive sequence x222 has failed to detect any regions of similarity with mitochondrial sequences at either the nucleic acid or amino acid level. The presence of this sequence on all the scmtDNAs identified thus far may suggest a role in replication and/or transcriptional control.

The complete sequence of scmtDNA I reveals that this molecule encodes seven full-length mitochondrial ORFs with no evidence of the frame-shift mutations that typify mitochondrial sequences introgressed into the nucleus. This sequence also confirms that scmtDNA I lacks rRNA genes. These observation provide support for the notion that scmtDNAs are generally functional, while also confirming that scmtDNA I in particular would not be functional in isolation. The gene content of scmtDNA I also explains why so few combinations of the primers listed in Table 1 were successful. Of the five pairs of primers designed from mt-protein-coding sequences, only the ND3/CB and COII primers are found together on scmtDNA I.

Possible sources of artifacts: There are numerous reports describing rearrangements of animal mtDNA. Duplications have been described in humans (reviewed by Poulton and Holt 1994), Cnemidophorus lizards (Moritz and Brown 1987) newts (Wallis 1987), scallops (Snyder *et al.* 1987), and nematodes (Okimoto *et al.* 1991; Azevedo and Hyman 1993). These reports describe within-genome repetition, whereas the repetition described here appears to be among the components of the mtDNA. Nonetheless, mtDNA duplications could be a source of artifacts if PCR analysis were exclusively relied upon. Similarly, the extensive literature concerning the introgression of mitochondrial sequences into the nuclear DNA (reviewed by Zhang and Hewitt 1996), especially examples such as the domestic cat, where a 7.9-kb mitochondrial sequence is tandemly repeated 38–76 times on chromosome 2D (Lopez *et al.* 1994), indicates that reliance on PCR and the expectation of circularity might prove misleading. However, the Southern data presented here provide compelling evidence that neither of these possibilities is relevant. For each of the scmtDNAs examined, the undigested genomic DNA sample has been shown to contain a single hybridizing linear species of the size predicted from PCR amplification and an associated putative open circular species. When the sample is digested with a restriction enzyme predicted to cut the particular scmtDNA once, the single restriction fragment detected comigrates with the linear species from the undigested sample, and the signal from the putative open circular species is removed. We would not anticipate that introgressed nuclear or duplicated mitochondrial sequences would repeatedly behave in this way, regardless of the restriction enzyme used.

Possible sources of subgenomic-sized mtDNAs: A number of recent reports have demonstrated the existence of deleted, subgenomic-sized mitochondrial species accumulating with age in postmitotic tissues from sources as diverse as man (Melov *et al.* 1995a), mouse (Melov *et al.* 1997), and nematode (Melov *et al.* 1995b). These reports are characterized by the low abundance of the deleted forms relative to the full-length "wild-type" sequence. This is in direct contrast to the situation described here, where any proposed "wild-type" sequence or sequences would have to be present at a much reduced frequency relative to their derivatives. None of the probes isolated from either mtDNA library has been found to hybridize to restriction fragments

suggestive of a mtDNA > ~9.5 kb. Electron microscopic examination of the closed circular fraction of DNA isolated from Lindley, a population previously shown to be indistinguishable from Gourdie in terms of mtDNA analysis, reveals a population of circular molecules in a size range consistent with that predicted by PCR and Southern blotting. An additional 122 measurements of mtDNA isolated from a fourth population also failed to identify a molecule of sufficient length to contain the full metazoan gene complement. As a result, we conclude that the mitochondrial genome of *G. pallida* is multipartite in structure, which implies that mitochondrial function at the level of the individual is encoded by as-yet-unknown combinations of the various scmtDNAs described here. A recent report that the primitive mesozoan animal Dicyema may encode each of its mitochondrial genes on separate, autonomously replicating mtDNA "minicircles" (Watanabe *et al.* 1999), as well as evidence for a similar structural organization of the chloroplast genome of the dinoflagellate *Heterocapsa triquetra* (Zhang and Cavalier-Smith 1999), suggest that the expected incompatibility between a multipartite organelle genome and the stochastic nature of organelle DNA replication has been overcome in some species. In addition, it is interesting to note that in Southern blots of genomic DNA from population Gourdie, scmtDNAs II and III were detected by Southern hybridization, while scmtDNA I could only be detected by PCR. In total genomic DNA from population Luffness, only scmtDNA I was present at a sufficiently high frequency to enable its detection by Southern hybridization. As scmtDNA I contains sequences duplicated on scmtDNAs II and III, this might suggest a degree of flexibility in mitochondrial genome organization.

Analogy with plant and fungal mtDNA organization: Any full-length circular sequence from which the observed diversity might have been derived would have to be present at a low frequency. This situation would be reminiscent of proposed models of plant mitochondrial genome structure, where a putative circular master chromosome gives rise to a population of smaller circular derivatives by recombination events between repeat regions (Fauron *et al.* 1995). The master chromosome is proposed to exist at low levels or in rare cell types, as its existence has rarely been demonstrated experimentally (Backert *et al.* 1997). However, the similarities with plant mtDNA organization end there. Plant mtDNA is typified by the presence of a low percentage of circular molecules with a continuous size distribution, as well as heterogeneous populations of linear molecules, often longer than the proposed circular mtDNA map. Indeed, some authors suggest that the rarely observed circular molecules may be the products of incidental recombination events rather than have any functional relevance (Bendich 1993). The Southern data presented here indicate that the majority of *G. pallida* mtDNA exists as discrete sized classes of circular molecules, with only a subpopulation of genome-length linear molecules, presumably generated by shearing during extraction. Analogy with fungal mitochondrial genomes is perhaps more robust than with plants. For example, the mitochondrial rearrangements associated with the senescence phenotype of *Podospora anserina* involve the complete replacement of wild-type mtDNA molecules with rearranged molecules (Griffiths 1992), a process that is under the control of nuclear genes (Jamet-Vierney *et al.* 1997). In *Neurospora crassa*, intramolecular recombination generates a range of usually smaller circular derivatives of the 62-kb mtDNA. These make up a small proportion of the total DNA of normal mitochondria. However, in some mutant strains, severe reversible fluctuations in the frequency of different classes of recombinant molecules are associated with the characteristic stop-start mode of growth (Gross *et al.* 1984), and selection for continuously growing derivatives of these mutants has led to the isolation of a strain with a mtDNA fragmented into two autonomously replicating subgenomic circular chromosomes. The stability of this novel mtDNA structure appeared to be associated with the loss of two extended sequences of the single chromosome of normal mitochondria (Gross *et al.* 1989). Thus, at least in laboratory strains, stable mtDNA fragmentation has been observed and depends on the presence of multiple origins of replication on the original mtDNA.

Possible mechanisms of mtDNA fragmentation in *G. pallida*: The similarity in gene order and conservation in distribution of restriction sites exhibited by scmtDNAs I and II over the region downstream of the COII gene and upstream of the shared *Bam*HI site is the best evidence for a direct relationship between any of the circular mtDNAs described. The presence of CB and ND3 sequences on scmtDNAs I and III also suggests that at least some of the scmtDNAs may have been derived from each other, or from a common ancestral sequence, by an unknown mechanism. One such mechanism by which subgenomic circular molecules might be derived from a larger molecule would be intramolecular recombination between direct repeats, such as that described in chloroplast mtDNAs (Palmer 1985) and proposed for plant and fungal mtDNAs (Gross *et al.* 1984; Fauron *et al.* 1995). However, no single model is likely to explain all the variation described here. In these models, nucleotide pairings between direct-repeat copies followed by intramolecular homologous recombination result in two daughter molecules, each containing one repeat copy. A second mechanism implicated in the generation of deleted mtDNAs in *C. elegans* (Melov *et al.* 1995b), *Homo sapiens* (Shoffner *et al.* 1989), and *Romanomermis culicivorax* (Hyman and Slater 1990) is slipped-strand mispairing. In this model, directly repeated sequences mispair, resulting in an unpaired single-stranded loop containing one repeat copy that would not participate in subsequent mtDNA replication. This latter model is generally regarded as the more probable mechanism

responsible for the generation of animal mtDNA deletions due to the accepted belief that recombination is not active in animal mitochondria. However, it has been demonstrated recently that mammalian mitochondria contain enzymes capable of catalyzing homologous recombination of DNA plasmids (Thyagarajan *et al.* 1996), and the products of intramolecular recombination have been detected at low levels in the mtDNA of the nematode *Meloidogyne javanica* (Lunt and Hyman 1997). In chloroplast DNA, copy correction between inverted repeats is thought to be a consequence of frequent recombination (Palmer 1985). The extremely low levels of sequence divergence found between repeats in the mtDNA of the nematode *R. culicivorax* have also been proposed to have resulted from "an active homogenizing mechanism," such as recombination-mediated gene conversion (Azevedo and Hyman 1993). However, even with the modest range of restriction enzymes used in this study, sequence divergence is evident between copies of the x222 sequence on different scmtDNAs, and there is also evidence of variation in coding sequences among different scmtDNA lineages, suggesting that such mechanisms are not active in *G. pallida* mitochondria or that there may be a physical barrier to their operation (Lightowlers *et al.* 1997). Evolutionary independence of repeated mtDNA sequences such as this is also observed in Cnemidophorus lizards and Heteronotia geckos, and is taken to accord with the apparent lack of intermolecular recombination in animal mtDNA (Moritz and Brown 1987; Moritz 1991). As a result, if scmtDNAs I and II were derived from a common ancestral sequence, the apparent divergence between the copies of the x222-like sequence from these molecules does not support a recent separation. Further analysis will be necessary to determine the mechanism responsible for the fragmentation of the *G. pallida* mtDNA and how widespread the phenomenon is among the cyst nematodes. Restriction enzyme analysis of the mtDNAs of the soyabean cyst nematode *Heterodera glycines* and sugar beet cyst nematode *H. schachtii* indicated that these species do not share the unusual mtDNA structure reported here (Radice *et al.* 1988).

What is not in doubt is that the complexity of mtDNA organization in populations of *G. pallida* dramatically reduces its utility as a population genetic marker. What might superficially appear to be RFLP variation detected by Southern hybridization in reality reflects variation in the frequency that the various scmtDNAs are found between populations. Also, the presence of sequences duplicated on different scmtDNAs presents a considerable practical obstruction to the reasonable identification of synapomorphic character states, especially when PCR is relied upon.

We thank W. G. F. Whitfield, B. C. Hyman, D. L. Trudgill and J. T. Jones for comments on the manuscript. Thanks also to G. Duncan for assistance with the electron microscopy. This work was supported by grants from the European Union (AIR3 CT-92-0062).

LITERATURE CITED

Azevedo, J. L., and B. C. Hyman, 1993 Molecular characterization of lengthy mitochondrial DNA duplications from the parasitic nematode *Romanomermis culicivorax.* Genetics **133:** 933–942.

Backert, S., B. L. Nielson and T. Borner, 1997 The mystery of the rings: structure and replication of mitochondrial genomes from higher plants. Trends Plant Sci. **2:** 477–483.

Ballinger, S. W., J. M. Shoffner, S. M. Gebhart, D. A. Koontz and D. C. Wallace, 1994 Mitochondrial diabetes revisited. Nat. Genet. **7:** 458–459.

Bendich, A. J., 1993 Reaching for the ring: the study of mitochondrial genome structure. Curr. Genet. **24:** 279–290.

Bridge, D., C. W. Cunningham, B. Schierwater, R. DeSalle and L. W. Buss, 1992 Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure. Proc. Natl. Acad. Sci. USA **89:** 8750–8753.

Davis, R. W., M. Simon and N. Davidson, 1971 Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21D:** 413–428.

Fauron, C., M. Casper, Y. Gao and B. Moore, 1995 The maize mitochondrial genome: dynamic, yet functional. Trends Genet. **11:** 228–235.

Feagin, J. E., 1994 The extrachromosomal DNAs of apicomplexan parasites. Annu. Rev. Microbiol. **48:** 81–104.

Feinberg, A. P., and B. Vogelstein, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

Griffiths, A. J. F., 1992 Fungal senescence. Annu. Rev. Genet. **26:** 351–372.

Gross, S. R., T. Hsieh and P. H. Levine, 1984 Intramolecular recombination as a source of mitochondrial chromosome heteromorphism in *Neurospora.* Cell **38:** 233–239.

Gross, S. R., A. Mary and P. H. Levine, 1989 Change in chromosome number associated with a double deletion in the *Neurospora crassa* mitochondrial chromosome. Genetics **121:** 685–691.

Hyman, B. C., and T. M. Slater, 1990 Recent appearance and molecular characterization of mitochondrial DNA deletions within a defined nematode pedigree. Genetics **124:** 845–853.

Jamet-Vierny, C., V. Contamine, J. Boulay, D. Zickler and M. Picard, 1997 Mutations in genes encoding the mitochondrial outer membrane proteins Tom70 and Mdm10 of *Podospora anserina* modify the spectrum of mitochondrial DNA rearrangements associated with cellular death. Mol. Cell. Biol. **17:** 6359–6366.

Keddie, E. M., T. Higazi and T. R. Unnasch, 1998 The mitochondrial genome of *Onchocerca volvulus*: sequence, structure and phylogenetic analysis. Mol. Biochem. Parasitol. **95:** 111–127.

Klein, M., U. Eckertossenkopp, I. Schmiedeberg, P. Brandt, M. Unseld *et al.*, 1994 Physical mapping of the mitochondrial genome of *Arabidopsis thaliana* by cosmid and YAC clones. Plant J. **6:** 447–455.

Lightowlers, R. N., P. F. Chinnery, D. M. Turnbull and N. Howell, 1997 Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. Trends Genet. **13:** 450–455.

Lincoln, S. E., M. J. Daly and E. S. Lander, 1991 Primer: a computer program for automatically selecting PCR primers. MIT Centre for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA.

Lopez, J. V., N. Yuhki, R. Masuda, W. Modi and S. J. O'Brien, 1994 Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol. **39:** 174–190.

Lunt, D. H., and B. C. Hyman, 1997 Animal mitochondrial DNA recombination. Nature **387:** 247.

Melov, S., J. M. Shoffner, A. Kaufman and D. C. Wallace, 1995a Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. Nucleic Acids Res. **23:** 4122–4126.

Melov, S., G. L. Lithgow, P. M. Fischer, P. M. Tedesco and T. E. Johnson, 1995b Increased frequency of deletions in the mitochondrial genome with age of *Caenorhabditis elegans.* Nucleic Acids Res. **23:** 1419–1425.

Melov, S., D. Hinerfeld, L. Esposito and D. C. Wallace, 1997 Multi-organ characterization of mitochondrial genomic rearrangements in *ad libitum* and caloric restricted mice show strik-

ing somatic mitochondrial DNA rearrangements with age. Nucleic Acids Res. **25:** 974–982.

Moritz, C., 1991 Evolutionary dynamics of mitochondrial DNA duplications in parthenogenetic geckos, *Heteronotia binoei.* Genetics **129:** 221–230.

Moritz, C., and W. M. Brown, 1987 Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. Proc. Natl. Acad. Sci. USA **84:** 7183–7187.

Moritz, C., T. E. Dowling and W. M. Brown, 1987 Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu. Rev. Ecol. Syst. **18:** 269–292.

Okimoto, R., H. M. Chamberlin, J. L. MacFarlane and D. R. Wolstenholme, 1991 Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): nucleotide sequences, genome location and potential for host-race identification. Nucleic Acids Res. **19:** 1619–1626.

Okimoto, R., J. L. MacFarlane, D. O. Clary and D. R. Wolstenholme, 1992 The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum.* Genetics **130:** 471–498.

Palmer, J. D., 1985 Comparative organization of chloroplast genomes. Annu. Rev. Genet. **19:** 325–354.

Pastrik, H. K., H. J. Rumpenhorst and W. Burgermeister, 1995 Random amplified polymorphic DNA analysis of a *Globodera pallida* population selected for virulence. Fundam. Appl. Nematol. **18:** 109–114.

Pearson, W. R., and D. J. Lipman, 1988 Improved tools for biological sequence comparisons. Proc. Natl. Acad. Sci. USA **85:** 2444–2448.

Phillips, M. S., B. E. Harrower, D. L. Trudgill, M. A. Catley and R. Waugh, 1992 Genetic variation in populations of *Globodera pallida* as revealed by isozyme and DNA analysis. Nematologica **38:** 304–319.

Pont-Kingdon, G. A., N. A. Okada, J. L. MacFarlane, C. T. Beagley and D. R. Wolstenholme, 1995 A coral mitochondrial *mut*S gene. Nat. **375:** 109–111.

Poulton, J., and I. J. Holt, 1994 Mitochondrial DNA: does more lead to less? Nat. Genet. **8:** 313–315.

Poulton, J., M. E. Deadman, L. Bindoff, K. Morten, J. Land *et al.*, 1993 Families of mtDNA rearrangements can be detected in patients with mtDNA deletions: duplications may be a transient intermediate form. Hum. Mol. Genet. **2:** 23–30.

Powers, T. O., E. G. Platzer and B. C. Hyman, 1986 Species specific restriction site polymorphism in root-knot nematode mitochondrial DNA. J. Nematol. **18:** 288–293.

Radice, A. D., T. O. Powers, L. J. Sandall and R. D. Riggs, 1988 Comparison of the mtDNA from the sibling species *Heterodera glycines* and *Heterodera schachtii.* J. Nematol. **20:** 443–450.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F. S., S. Nicklen and A. R. Coulson, 1977 DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

Shoffner, J. M., M. T. Lott, A. S. Vojavec, S. A. Soueidan, D. A. Costigan *et al.*, 1989 Spontaneous Kearns-Sayre/chronic external opthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc. Natl. Acad. Sci. USA **86:** 7952–7956.

Snyder, M., A. R. Fraser, J. Laroche, K. E. Gartner-Kepkay and E. Zouros, 1987 Atypical mitochondrial DNA from the deep-sea scallop *Placopecten magellanicus.* Proc. Natl. Acad. Sci. USA **84:** 7595–7599.

Thyagarajan, B., R. A. Padua and C. Campbell, 1996 Mammalian mitochondria possess homologous DNA recombination activity. J. Biol. Chem. **271:** 27536–27543.

Unseld, M., J. R. Marienfeld, P. Brandt and A. Brennicke, 1997 The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nat. Genet. **15:** 57–61.

Wallis, G. P., 1987 Mitochondrial DNA insertion polymorphism and germ line heteroplasmy in the *Triturus cristatus* complex. Heredity **58:** 229–238.

Watanabe, K. I., Y. Bessho, M. Kawasaki and H. Hori, 1999 Mitochondrial genes are found on minicircle DNA molecules in the mesozoan animal *Dicyema.* J. Mol. Biol. **286:** 645–650.

Zhang, D. X., and G. Hewitt, 1996 Nuclear integrations: challenges for mitochondrial DNA markers. TREE **11:** 247–251.

Zhang, Z., and T. Cavalier-Smith, 1999 Single gene circles in dinoflagellate chloroplast genomes. Nature **400:** 155–159.

Communicating editor: I. Greenwald