Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helicis*

Julius Lukes1,2, Milan Jirků, Nuraly Avliyakulov and Oldřich Benada3

Institute of Parasitology, Czech Academy of Sciences and 1Faculty of Biology, University of South Bohemia, Branišovská 31, 37005 České Budějovice and 3Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 110 00 Prague 10, Czech Republic

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Introduction

Evidence gathered so far from various mitochondrial genomes favors the theory that all present day mitochondria are descendants of a single early endosymbiotic event (Gray and Spencer, 1996), although a polyphyletic origin of the mitochondrion cannot be excluded (Simpson and Thiemann, 1995). In all organisms studied, circular or linear mitochondrial DNA contains varying numbers of genes with a slow but steady tendency of mitochondrial genomes to be transferred into the nucleus over the course of evolution (Palmer, 1997). For unknown reasons, how-evver, mitochondrial (mtDNA) kDNA of kinetoplastid protozoa retained the most complicated DNA structure known in nature. In an insect trypanosomatid *Crithidia fasciculata*, kDNA represents ~15% of the total cellular DNA and is composed of ~5000 minicircles and 25 maxicircles, topologically interlocked in a single network which is located in the mitochondrial matrix close to the kinetosome of a single flagellum (Robinson and Gull, 1994; Shapiro and Englund, 1995). The kDNA network forms a disc of 1 μm in diameter and 0.3 μm thick (Ferguson et al., 1992) with an extremely high DNA concentration (Rauch et al., 1993). Recently it has been shown that each minicircle is connected to three neighbors by a single interlock (Chen et al., 1995a), the number of interlocks increasing during the kinetoplast division up to six per minicircle (Chen et al., 1995b). Furthermore, minicircles are relaxed rather than negatively supercoiled because the supercoiling would probably be incompatible with the sophisticated structure of the network (Rauch et al., 1993).

Maxicircles are homologs of the mitochondrial DNA of other eukaryotes. They bear mitochondrial genes coding for ribosomal 9S and 12S RNAs and subunits of the respiratory chain complexes. Several transcripts undergo unusual processing by uridilate insertions and deletions, called RNA editing. This is mediated by guide RNAs homologous to the minicircles to plasmids, we present a theory on the formation of the kDNA network.

Keywords: catenation/kDNA/kinetoplastida/
mitochondrial DNA/supercoiling

The mitochondrial DNA (mtDNA) of a primitive kinetoplastid flagellate *Cryptobia helicis* is composed of 4.2 kb minicircles and 43 kb maxicircles. 85% and 6% of the minicircles are in the form of supercoiled (SC) and relaxed (OC) monomers, respectively. The remaining minicircles (9%) constitute catenated oligomers composed of both the SC and OC molecules. Minicircles contain bent helix and sequences homologous to the minicircle conserved sequence blocks. Maxicircles encode typical mitochondrial genes and are not catenated. The mtDNA, which we describe with the term ‘pankinetoplast DNA’, is spread throughout the mitochondrial lumen, where it is associated with multiple electron-lucent loci. There are ~8400 minicircles per pankinetoplast-mitochondrion, with the pan-kDNA representing ~36% of the total cellular DNA. Based on the similarity of the *C. helicis* minicircles to plasmids, we present a theory on the formation of the kDNA network.

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and from the classical kDNA network, that no feasible evolutionary theory can be drawn.

During the ultrastructural study of Cryptobia vaginalis from leeches, Vickerman (1997) noted that its kDNA seemed to be dispersed throughout the mitochondrial matrix and coined for such an arrangement the term ‘pankinetoplast’. Further ultrastructural analyses of selected bodonids revealed that the pankinetoplast morphology is widespread among members of the suborder Bodonina (Brugerolle et al., 1979). However, any information about this enigmatic structure has been missing until now. In this work, we show that the pankinetoplast of Cryptobia helicis contains a uniquely organized mitochondrial DNA, with both relaxed and supercoiled minicircles, which remain largely noncatenated. We term this kind of DNA ‘pan-kinetoplast DNA’ (pan-kDNA). We also present a theory that attempts to explain the origin of catenated kDNA networks.

Results

Topology of the pan-kDNA

Because the standard protocols failed to isolate kDNA from C. helicis, we isolated total cellular DNA and subjected it to agarose gel electrophoresis (Figure 1A). Unexpectedly, a large amount of undigested DNA entered the gel; the two most prominent bands migrated at 2.6 and 4.4 kb, or 4.2 and 7.0 kb when compared with linear or supercoiled markers, respectively. Most DNA migrated in the compression zone and only a small amount of DNA remained in the slot. When total DNA of another bodonid, Thorrelia, was analyzed under the same conditions, bands of this type were not observed. Furthermore, in the case of catenated kDNA of C. fasciculata, only a low intensity band of linear 2.4 kb minicircles could be detected (Figure 1A, lanes 3 and 4).

To further investigate the topology of the dominant bands visible in C. helicis total DNA, it was treated with topoisomerase II and subjected to agarose gel electrophoresis, followed by Southern blotting using a cloned C. helicis minicircle Z4 (see below) (Figure 1B). Relaxation of the fast migrating band (2.4 kb) into a population of species coiled. Treatment with topoisomerase I, DNase I or Fe2+/H2O2 at 25°C resulted in the conversion of the fast migrating band into open circles migrating at 4.4 kb. Treatment with Fe2+/H2O2 produced a band at 4.2 kb which represents linear molecules. Significant mobility changes also occurred in several higher molecular weight bands which are present in the untreated sample. We assume that the weak band visible only in undigested DNA, slightly above the most prominent open circle band, is a dimer composed of two singly interlocked supercoiled minicircles (SC–SC) (Figure 1B). It is the first species to disappear after any treatment. The dimer composed of open and supercoiled rings (OC–SC) is only slightly more stable and withstands short incubations with topoisomerases. On the contrary, the dimer containing two interlocked open circles (OC–OC) becomes the most abundant species after the treatment with topoisomerase I, since it transforms the available dimers into this species by nicking (Figure 1B). In the agarose gels, it is difficult to distinguish between individual types of catenanes composed of three or more interlocked circles, and in the pan-kDNA there are not enough of them for the analysis of a gel-isolated DNA by electron microscopy. However, since topoisomerase I nicks all the supercoils, we assume that the prominent band migrating at 15.0 kb is an open circle trimer (OC–OC–OC) (Figure 1B). Although >95% of the Z4 minicircle signal hybridized with DNA in the gel, the possibility that some larger catenanes remained in the slot cannot be excluded. In lanes 8 and 9 (Figure 1B), the restriction digestion of total DNA by BglII, which linearized most of the minicircles and MvaII, which cuts minicircles into small fragments, is visible.

The BglII-linearized minicircles were isolated from the gel, several 4.2 kb inserts were cloned, and the clone Z4 was analyzed further. To investigate a possible homology of the Z4 circle with minicircles, the bent helix region of
C. fasciculata minicircle (pPK201/CAT) was used as a probe to screen the whole 4.2 kb Z4 insert. We subcloned and sequenced a 1.44 kb long fragment which contained sequence motifs characteristic of the trypanosomatid minicircles. In this sequence we identified two regions showing significant similarity to the invariant 12 bp universal minicircle sequence (CSB3) and an 8 bp conserved sequence (CSB2). Between CSB2 and CSB3, a region with high sequence similarity to the C. fasciculata minicircle bent helix was present. Also, two other bent helix-like regions that contained irregularly spaced A-tracks were identified (Figure 1C). Total C. helicis DNA digested with \( {\text{H}}_{11022} \) restriction enzymes was hybridized separately with the Z4 probe and a probe prepared from the gel-isolated minicircle band. The hybridization patterns and the intensity of signal revealed high sequence homogeneity of the minicircle population and showed that the Z4 clone is a representative of a major minicircle class (data not shown).

The sedimentation properties of the C. helicis lysate centrifuged in a continuous sucrose gradient are presented in Figure 2. The obtained fractions were hybridized with the Z4 minicircle probe, a maxicircle probe [300 bp fragment of the cytochrome oxidase subunit 1 gene (cox1)] and a nuclear probe [2kb small subunit rRNA gene (SSU rRNA)]. The cell lysate of C. fasciculata hybridized with the pDP312 minicircle probe was used as a control (Figure 2A). Both the mitochondrial and nuclear probes proved the absence of a large network in the C. helicis genome (Figure 2B–D). Poor sedimentation, and thus retention, of most of the signal in the upper fractions corresponds with the expected individual status of the majority of mini- and maxicircles. It should be noted that minor peaks in the central part of the gradient of Figure 2B were indicative of possible small minicircle catenanes. A count retention experiment was performed by comparison of the hybridization signal between serially diluted total DNA and the sum of the signals of individual fractions. For both C. helicis and C. fasciculata, it ranged from 70–80%. These relatively low values are most likely due to a partial loss of DNA during precipitation of fractions prior to their loading onto membrane.

Together, the analysis of DNA by agarose gel electrophoresis (Figure 1A and B), the use of cell lysates in the propidium iodide gradient (data not shown), and the results from sucrose gradient (Figure 2) strongly indicate the absence of large networks in C. helicis. However, because the kDNA differs among species with respect to its fragility (Shapiro and Englund, 1995), and because a C. helicis network might be extremely sensitive to pipetting and shearing, the above methods might fail to prove its existence and only detect its breakdown products. To avoid any manipulation with the lysate or the nucleic acid, cells embedded in the low-melting agarose were subjected to pulse-field gel electrophoresis (PFGE). As shown in Figure 3B, lane 1, the large majority (~90% in different experiments) of the minicircles entered the gel, individual bands representing different single and catenated species. The small amount (<10%) of the minicircles that stayed in the slot most likely represent those minicircles that could not leave the block due to the interference of poorly lysed membranes and cell debris etc. Under the same conditions, a large majority of minicircles of stationary phase C. fasciculata cells stayed in the slot (Figure 3B, lane 3).

**Electron microscopy of the pan-kDNA**

In order to verify the existence of various forms of minicircles that we detected in agarose gels and hybridization studies, we resorted to electron microscopical analysis of C. helicis DNA. Only carefully isolated DNA that did not come into contact with ethidium bromide or other intercalating agents was studied. By the contour measure-
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Fig. 3. PFGE of agarose-embedded cells was carried out as described in Materials and methods. (A) Ethidium bromide-stained agarose gel. CHEF size standard (lane 1); T. borreli (lane 2); C. helicis (lane 3); (B) Southern blot. C. helicis DNA was hybridized with Z4 minicircle probe (lane 1) and cox2 maxicircle probe (lane 2); C. fasciculata DNA was hybridized with pDP312 minicircle probe (lane 3).

From the representative number of circular molecules we confirmed that the minicircle population consisted of 4.2 kb molecules only (Figures 1 and 4A–L). To perform accurate measurements, most of the measured molecules were OC forms.

Among the 398 analyzed pan-kDNA components on the grids, we were able to find all the predicted minicircle forms, e.g. the SC (n=328) and OC monomers (n=19) (Figure 4A), the SC–SC (n=18), OC–SC (n=6) and OC–OC (n=7) dimers (Figure 4B–D), all the possible combinations of SC and OC molecules catenated into trimers (n=12) (Figure 4E–G), as well as rare larger catenanes (n=8). As shown in Figure 4I–L, the latter included catenanes up to the size of octamers. They were either composed solely of OC or SC forms, or both forms catenated at random (Figures 4I–L). In most cases, the neighbors were connected by a single interlock, as in the trypanosomatid kDNA networks. Two unusual structures that would not fit into the predicted C. fasciculata network model (Chen et al., 1995a,b) were detected only once: a trimer with minicircles mutually interlocked into a triangle (Figure 4H) and a minicircle connected with four OC neighbours by single interlocks (Figure 4K). Although in these cases the localization of circles in different planes was not rigorously excluded, this possibility can be considered unlikely due to the low concentration of DNA, and especially the physical repulsion of DNA on grids (Fergusson and Davis, 1978). To exclude the possible disruption of larger catenanes during the preparation of DNA samples, the kDNA networks of Trypanosoma carassii were used as a control (Figure 4M).

In a group of 398 pan-kDNA components counted on randomly chosen sections of the grid, 82% and 5% were the SC and OC monomers, respectively; the SC–SC, SC–OC and OC–OC dimers together comprised 8%, and oligomers represented only about 5% of the analyzed molecules. In the agarose gels (Figure 1, lane 2), supercoiled minicircles also were the dominating species in untreated samples.

Fig. 4. Electron microscopy of the pan-kDNA. (A) OC and SC monomers. (B) SC dimer. (C) OC dimer. (D) OC–SC dimer. (E) OC–OC–SC trimer. (F) OC–SC–OC trimer. (G) OC trimer. (H) OC trimer with neighbor minicircles catenated as a triangle. (I) OC tetramer. (J) pentamer composed of three SC and two OC minicircles. (K) OC heptamer with a central minicircle (arrow) joint with four neighbors. (L) octamer composed of seven OC and one SC minicircles (arrow). (M) kDNA network of T. carassii. The magnification of all panels is equal except panel M; bars = 0.5 nm (~1.6 kb).
Localization of the pan-kDNA in the mitochondrion

When trypanosomatid cells are stained for DNA, the kDNA appears as a small dot positioned adjacent to the flagellar kinetosome (Robinson and Gull, 1994). In contrast, when the C.helicis cell was stained with DAPI or propidium iodide, the pan-kDNA appeared as a prominent rod-shape structure, staining as intensely as the nucleus (Figure 5A and B). This is a strong indication that the pan-kDNA is distributed throughout the mitochondrial matrix. To exclude the possibility that bands considered as the pan-kDNA minicircles were in fact plasmids of cytoplasmic origin, we performed the in situ hybridization with the Z4 minicircle probe. As shown in Figure 5C, the probe hybridized with the mitochondrion only, thus confirming the organellar localization of the circles. As a control, the in situ hybridized cells were co-stained with propidium iodide (Figure 5D).

To investigate further the distribution of pan-kDNA in the mitochondrion, we studied the fine structure of the organelle in osmium-fixed and Epon-Araldite-embedded cells, this procedure enabling optimal preservation of the ultrastructure. In all the analyzed cells, a single elongated mitochondrion contained two prominent structures: short tubular-shaped cristae that stretched from the periphery into the matrix and multiple electron-lucent loci distributed evenly in the matrix (Figure 6A–D). These structures were not analyzed in detail, but the negative results of the polysaccharide-specific Thiery-staining indicated their protein composition (data not shown). In order to localize the pan-kDNA at the ultrastructural level, we fixed the cells with paraformaldehyde and embedded them in LR White resin. Mildly deproteinized ultrathin sections were treated with the α-DNA antibodies and secondary antibodies coupled with colloidal gold. As can be seen in Figure 6B–D, the colloidal gold particles were spread in the mitochondrial matrix. However, they were bound only to the electron-lucent loci, while no signal was associated with the tubular cristae or the periphery of the organelle.

Maxicircles and the abundance of pan-kDNA components

For the detection of maxicircles in the pan-kDNA, we PCR-amplified and cloned a fragment of the C.helicis cox2 gene and used it as a probe. To avoid hydrodynamic shearing of large maxicircles, we embedded cells in agarose blocks and used PFGE (see Materials and methods). Most of the maxicircle signal migrated at approximately 43 kb, indicating that, similar to the minicircles, there is no maxicircle network (Figure 3B, lane 2). The topology of maxicircles (circularity or linearity) has not been investigated. We also cloned and sequenced internal part of the cox1 gene which was, according to the hybridization studies (data not shown), situated adjacent to the cox2 gene.

Quantitative dot blot analysis was performed to determine the relative abundance of the maxi- and minicircles (Figure 7). As a reference, we used a plasmid construct, in which ~300 bp fragments of the nuclear LSU rRNA gene, the cox 1 gene and the conserved bent helix region of Z4 minicircle (Y6) were cloned in tandem. Because a single-copy gene of C.helicis is currently not available, we used as a copy number reference the nuclear LSU rRNA gene, which is known to be present in 100–200 copies in related flagellates (Leon et al., 1978; Castro et al., 1981; L.Floeter-Winter, personal communication).

Assuming that there were 150 copies of the LSU rRNA gene in the C.helicis genome, we calculated that there were 14 cox1 genes (and thus 14 maxicircles) and 8400 minicircles (considering that, as in trypanosomatids, each minicircle contains a single conserved bent helix region). The ratio between maxi- and minicircles hence was 1:600, and the total amount of mitochondrial DNA was ~35 Mbp.

In order to determine the relative amount of the pan-kDNA, non-dividing cells were stained by Feulgen reaction and the intensity of the signal was quantified by confocal microscopy as a sum of measurements in five arbitrarily chosen planes. By measuring the ratio between the mitochondrial and nuclear DNAs, we found that on average ~36% (33–41%) of the total cellular DNA was localized in the mitochondrion. Based on these values, the total genome size of C.helicis appeared to be about ~35 Mbp, this size being in good agreement with that obtained for related T.borrelia (Yasuhiira and Simpson, 1996).

Discussion

Cryptobia helicis was chosen as an organism useful for evolutionary studies because it fulfils the following criteria:
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Fig. 6. Transmission electron microscopy and immunogold labelling. (A) The elongate mitochondrion in Epon-Araldite-embedded cells with peripheral cristae and multiple electron-lucent loci in its lumen. Longitudinal (B and D) and cross (C) sections of a mitochondrion in LR-White-embedded cells treated with the α-DNA antibodies and secondary antibodies coupled with colloidal gold. All the colloidal gold granules are associated with the electron-lucent loci while no DNA is detected at the peripheral cristae. Bars - 250 nm (A) and 100 nm (B–D) (arrows: mitochondrial cristae, arrowhead: cellular membrane).

(i) it represents the earliest known evolutionary branch of the kinetoplastid lineage as deduced from the SSU and LSU rRNA sequences (D.Doležel, M.Jirků, D.A.Maslov, J.Lukeš, unpublished data); (ii) although with difficulties, it can be obtained pure in sufficient amounts, while all the other bodonids can only be cultivated with feeder bacteria or not at all (T.borrelli being a single exception); and (iii) it contains the pan-kDNA.

Presence of the two prominent and several weak bands in the undigested samples of C.helicis DNA is indicative of either an extremely fragile network or free minicircles. However, the results obtained with the agarose-embedded cells run in a pulse field gel excluded the former possibility. In comparison, when DNA of the stationary phase C.fasciculata cells was analyzed under the same conditions, only ~1% of the minicircles entered the gel compared with ~90% of the C.helicis minicircles. Further support for the absence of a large network in C.helicis was provided by the centrifugation of various DNA components in sucrose gradient; in contrast to C.fasciculata, the minicircles did not reach the lower part of the gradient. On the contrary, the minicircle signal showed several minor peaks in the middle of the gradient, and increased to a major peak in the uppermost fraction. This distribution of minicircles in the gradient, when compared with the steep increase of the maxicircle and nuclear signals in the upper fractions, was indicative of small catenanes and free circles.

The kDNA minicircles of trypanosomatids represent the only nonsupercoiled circular DNA in nature (Rauch et al., 1993). Unexpectedly, in C.helicis, most of the minicircle monomers and a significant part of dimers and oligomers are supercoiled. Supercoiled monomers represented the most abundant species followed by open circles. We do not know if these circles are covalently closed or they contain nicks or gaps, because the division of minicircles could not be studied due to apparent absence of the replication intermediates in the samples analyzed, which were prepared from the stationary phase C.helicis cells. We have also found catenanes composed of 2–8 minicircles, although catenanes larger than trimers were rare. With two exceptions, minicircles were always joined by a single interlock and members of the catenane were linked with up to four neighbours.

Replication of decatenated Trypanosoma equiperdum minicircles occurs via θ-intermediates, the daughter circles being segregated from one another by topoisomerase II (Ryan and Englund, 1989). Inhibition of this activity results in the appearance of oligomers, up to pentamers (Shapiro, 1994). We cannot exclude that the oligomers are products of an unusual minicircle replication or arise as a consequence of a local absence of topoisomerase II. However, only very speculative interpretations would explain the existence of some catenanes (Figures 4H–L) as replication intermediates.

Using the C.fasciculata minicircle probe, we subeloned
a 1.44 kb part of the C.helicis Z4 minicircle, in which regions with high homology to typical minicircle motifs were found. The putative CSB2 and CSB3 blocks differed by one and three mismatches, respectively, from the universally conserved CSB2 and CSB3 blocks of trypanosomatids (Ray, 1989; Simpson, 1997). A short but significant homology with the F.asciulata bent helix was localized in the region between CSB2 and CSB3. In trypanosomatid minicircles, however, this characteristic sequence motif (Kitchin et al., 1986) is invariably situated in the region outside the CSB2–CSB3 conserved region (Shapiro and Englund, 1995; Simpson, 1997). Furthermore, two other regions with irregularly spaced A-tracts were reminiscent of a bent helix sequence. The identified regions of similarity, although not very strong, indicate that the 4.2 kb circles already contain typical minicircle sequences and can therefore be considered to be equivalents to minicircles of modern flagellates.

We have also demonstrated that minicircles are distributed in the mitochondrial lumen, and not in a kDNA disc close to the flagellar kinetosome, as is the case in most kinetoplastids studied thus far. We were able to localize the DNA to the multiple electron-lucent structures in the elongate mitochondrial. The highest percentage of the mtDNA when compared to the total cellular DNA is known from trypanosomatids (15%) (Shapiro and Englund, 1995) and Acanthamoeba castellani (up to 20%) (Burger et al., 1995). In the mitochondrion of C.helicis, the pan-kDNA represents >36% of the cell genome and thus might be the largest mitochondrial genome known.

The existence of a huge network of interlocked circles in the trypanosomatid mitochondrial puzzled investigators until the discovery of guide RNA (gRNA) genes coded by minicircles (Pollard et al., 1990; Sturm and Simpson, 1990) and their function in editing the maxicircle transcripts (reviewed by Simpson and Thiemann, 1995; Arts and Benne, 1996). Obviously, catenation and a sophisticated mechanism of the kDNA division might limit the potential loss of the minicircle-encoded gRNA genes during the mitochondrial division (Borst, 1991). Trypanoplasma borrelii, in which minicircles are absent, solved the problem of losing the gRNA genes by placing them in tandem in a 180 kb circular molecule (Yasuhiro and Simpson, 1996; Simpson, 1997).

The description of the pan-kDNA structure in C.helicis, which differs from the kDNA of morphologically closely related Tborreli, has a taxonomic consequence in final justification, after a three decade long taxonomic dispute (reviewed by Lom and Dyková, 1992), of the separate generic status of these flagellates.

In contrast to the kDNA minicircles, the pan-kDNA minicircles resemble plasmids by their monomeric status, supercoiling and loose distribution in the mitochondrial matrix. Based on this resemblance, an intriguing scenario can be drawn, in which minicircles might be descendants of a plasmid that invaded the mitochondrion of an ancient free-living bodonid. This mitochondrion was already equipped with its DNA, preserved until now in the form of a maxicircle. Multiple interactions of mitochondrial plasmids with each other and with mtDNA have been observed in the fungus Neurospora crassa, including the formation of transcript hybrids (reviewed by Griffith and Yang, 1995). After RNA editing arose by an as yet unknown mechanism (Simpson and Thiemann, 1995; Arts and Benne, 1996; Cavalier-Smith, 1997), mitochondrial plasmids possibly became involved, functioning as vehicles for the gRNA genes. A process of interlocking individual minicircles into a network proved to be an efficient way to prevent their loss during the mitochondrial division. RNA editing has not yet been demonstrated in the C.helicis mitochondrial. Although the cox2 gene and the internal part of the cox1 gene are not edited similar to the related Tborreli (Lukes et al., 1994; D.Blim, A.de Haan, M.Van den Berg, P.Sloof, M.Jirků, J.Lukes, R.Benne, in preparation), and although homology search failed to find the gRNA genes in available minicircle sequences, the presence of RNA editing in this primitive flagellate can be expected. In that case, we can only speculate about the precise segregation mechanism of the dispersed minicircle population. Based on the estimation of various C.helicis DNA components mentioned above, the pan-kDNA contains about three times as much DNA as the kDNA of F.asciulata, with minicircles accounting for this difference. Therefore, a redundancy of minicircles might be an alternative solution to the network structure. A challenge is to confirm, by a comparative analysis of the kDNA of primitive flagellates, that in Cryptobia such an ancient kDNA structure remained preserved.

### Materials and methods

#### Organisms and DNA isolation

Cells of C.helicis were obtained from the pin-head sized receptaculum seminis dissected from garden snails, Helis pomatia, captured in southern and central Bohemia, Czech Republic, and northern Italy. After opening the wall of the receptaculum, flagellates (about 2×10^7 cells/receptaculum) were carefully washed out by micromanipulation from the space between the receptaculum wall and the spermatophorous matrix. In most cases the flagellate population was in the stationary phase. F.asciulata (Steinert strain), Tcarassii (CC-Nem strain), and Tborreli (T-JH strain) were cultivated as described elsewhere (Kleisen et al., 1975; Lukes et al., 1994; Jirků et al., 1995).

Total DNA was isolated from cells after repeated washes in NET–50 (50 mM EDTA, 100 mM NaCl, 10 mM Tris, pH 8.0), lysed by N-lauroylsarcosine (Fluka) and pronase E (Merck) at the final concentration of 3% (v/w) and 1 mg/ml, respectively, for 1 h at 4°C. The lysate was phenol-chloroform extracted and the DNA was ethanol-precipitated, air dried and resuspended in TE buffer. During the isolation, any vortexing and shearing was avoided.

#### Electrophoresis, blotting, hybridization and PCR

Agarose gel electrophoresis and blotting were performed according to standard protocols (Sambrook et al., 1989). Low-melting agarose blocks with cells embedded at a concentration of either 4×10^7 (minicircle probe) or 4×10^9 (maxicircle probe) cells/ml were prepared as described elsewhere (Rovai et al., 1992). PFGE was performed in a FIGE Mapper apparatus (Bio-Rad) in 1% agarose gel and 0.5× TBE buffer at forward and reverse voltages of 180V and 120V, respectively, on a linear switch ramp (0.1–8 s) at 10°C for 11 h. Gels obtained from PFGE or regular agarose gel electrophoresis were blotted following treatment of the gel either with 0.5N HCl for 15 min or at 600 kJ at 254 nm, prior to denaturation and neutralization. With all the probes used, hybridization was performed at 65°C overnight, and the membranes were washed three times for 20 min in 3×SSC, 0.1% SDS at 65°C.

The cytochrome oxidase subunit (cox1) (410 bp) and cox2 (330 bp) maxicircle probes were generated by PCR from genomic DNA using C112 and C115 oligonucleotides (Lukes et al., 1994) and oligonucleotides G566 (AAAGTATAGGGTTT[C][C][A][G][T][G]) and G567 (GGTTTCTAGG[A][G][A][T][C][T][G][A][G][G][T][T][A]), respectively. The fragments of small rRNAs (5S and 18S) nuclear genes were amplified using oligonucleotides S762 and S1842, and S1842 and S1843 of Maslov et al. (1996), respectively. All PCR amplifications were performed as described elsewhere (Lukes et al., 1996).
In 1994; Maslov et al., 1996). The conserved region minicircle probe pDP312 (Ferguson et al., 1992) and the bent helix minicircle probe pPK201CAT (Kitchin et al., 1986) from C.fasciculata were kindly provided by L. Guibride and PT.Englund.

**Restriction analysis and topology**

Treatments with restriction endonucleases (Fermentas, Promega), DNAse I (Boehringer Mannheim), topoisomerases I (Fermentas) and II (Amersham) were performed according to manufacturers’ instructions. Treatment with Fe²⁺ ions reduced by hydrogen peroxide into OH-radicals, which degrade the deoxyribose and thus break the DNA backbone, was performed according to Foja and Paleček (1997).

Conditions of a representative experiment are described in Figure 1B.

**Sucrose gradients**

Approximately 6×10⁶ cells of H. helicis (or 2×10⁶ cells of C. fasciculata) were lysed as described above. The sample was diluted to 5 ml with water and loaded onto a 30 ml sucrose gradient prepared according to Englund (1979) and centrifuged at 7000 r.p.m. for 45 min at 4°C in a Sorvall HB-6 rotor. 1.4 ml fractions were collected from the bottom of the gradient using a Minipuls 2 gradient fractionator (Gilson). The fractions were diluted with 1.4 ml water, ethanol-precipitated, air-dried, redissolved in 100 μl water, spotted on Hybond-N (Amersham), and hybridized with the appropriate probes. The amount of signal was measured by phosphorimager Storm S60. Count retention was established by comparing the signal from all fractions and was compared with serially diluted total DNA hybridized with the same probe.

**Cloning and sequencing of cox1 fragment and Z4 minicircle**

A 410 bp PCR-amplified fragment of the cox1 gene was cloned into the EcoRV site of pBluestick vector (Novagen), and the BglII-linearized minicircle (Z4) was cloned into the BamHI site of pBluestick SK(-) (Strategene) and transformed in XL-1 competent cells (Strategene). The cox1 fragment and the 1.44 kb long subclone of Z4 were sequenced using the Sequenase 2.0 kit (Amersham). The Z4 sequence has been deposited in GenBank under accession number AF034623.

**In situ hybridization, confocal microscopy and DNA electron microscopy**

For in situ hybridization, cells were attached to poly-L-lysine pretreated slides and fixed (4% formaldehyde; 5% acetic acid; 0.9% NaCl) for 5 min at room temperature. Z4 digested with HhaI was digoxigenin-labelled with the Dig-DNA labeling kit (Boehringer Mannheim) according to the manufacturer’s instructions, following the technique of Pearce et al. (1996). The detection of hybridized probes was performed with the Fab fragments of the sheep anti-digoxigenin antibody directly conjugated to the alcaline phosphatase (1:500 dilution in PBS) and its conjugate to the alcaline phosphatase (1:500 dilution in PBS). The sections were picked up on 200 mesh copper grids with a carbon coated formvar membrane. The grids were placed in a moist chamber for the following incubations: 20 min in 2% proteinase K in phosphate buffered saline (PBS); 10 min in 0.5% fatty acid free bovine serum albumin; 1 h in 0.1M-DAI antibodies at room temperature; 20 min wash in PBS; 1 h in secondary antibodies (anti-mouse IgG) coupled with 10nm colloidal gold; 20 min wash in PBS; 30 min postfixation in 2% glutaradehyde; five minutes 5 min in deionized water. The grids were poststained with 5% uranyl acetate and lead citrate, and examined in a JEOL JEM 1010 electron microscope.

**Quantitation of DNA components**

A 410 bp fragment of the cox1 gene, 290 bp of the Z4 minicircle fragment containing the conserved bent helix (Y6), and the 320 bp Drel and HireII fragment of the LSU rRNA gene were cloned in tandem into appropriate restriction sites of the same plasmid, termed X. In this plasmid, which was used as reference for quantitative dot blot analysis, the maxi- and minicircular and nuclear DNA fragments were present in equimolar amounts. HhaI-digested genomic DNA from C.helicis and EcoRl-linearized X3 DNA were serially diluted and spotted on Hybond-N membrane (Amersham). With each of the three DNA fragments that were labeled by nick translation, one of three identical blots was hybridized at 65°C overnight and washed three times 20 min in 3× SSC and 0.1% SDS. The experiment was performed four times.

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