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# Structure of *Leishmania* Minicircle Kinetoplast DNA Classes

In a recent paper (5), Noyes et al. discussed a nested-PCR-based approach for identification and analysis of *Leishmania* kinetoplast DNA (kDNA) minicircle classes using the analysis of minicircle variable-region (VR) restriction profiles.

The kDNA of trypanosomatids consists of catenated minicircle (approximately $5 \times 10^3$ to $5 \times 10^4$ per associate) and maxicircle (20 to 50 per associate) molecules (8). It was shown that the minicircles encode guide RNA (gRNA) molecules involved in the RNA editing of maxicircle cryptogenes (for a review, see reference 9). To date, only this one important cellular function has been demonstrated for minicircles but the enigma of their redundancy remains topical. The prognosticated number of minicircle classes in *Leishmania* is more than 60 (4). This number comes from the hypothetical number of maxicircle cryptogenes requiring editing of pre-mRNA transcripts. Recently the nonrandom distribution of minicircle classes has been demonstrated for the following set of *Leishmania tropica* isolates: MHOM/SU/74/SAF/K-27, MHOM/TU/85/TAT3, MHOM/KE/84/NLB299, MRAT/IQ/72/MRCB-JBF, and MHOM/NA/76/ROSSI-II (3). Moreover, it has been proposed that some minicircle classes might be preferentially amplified for expression of required gRNA. The results obtained by Noyes et al. lead to the same conclusion. We think that analysis of the sequence (5) (GenBank accession no. AF032997) (which is in fact a minicircle VR) for the presence of specific gRNA genes (2a) or regulatory sequences (2) may shed more light on the functional role of these molecules.

We have analyzed the presented sequence (5) (GenBank accession no. AF032997) using the Parasite Genome Blast Server (5a) computational tool. The results of our search have revealed a rather high level of homology of the *L. infantum* MHOM/TN/80/IPT1 minicircle (5) with *L. donovani* MHOM/IQ/88/RTC6 (clone 11; GenBank accession no. AJ010074), *L. donovani* MHOM/SD/85/FORSTER (clone 7; GenBank accession no. AJ010080), and *L. infantum* (clone 10543; GenBank accession no. Z32846) minicircle DNAs (up to 90, 68, and 68%, respectively). We speculate that these minicircles might present one interspecies class for expression of the one certain gRNA type.

All Trypanosomatidae kDNA minicircles are organized by a uniform scheme and contain one or several highly conserved regions (CR). There are three highly conserved blocks (CSB) within CRs: CSB1 (GGGCGT), CSB2 (CCCCGTTC), and CSB3 (GGGGTTGGTGTA) (with interspecies homology of ca. 90 to 100%). CSB1 and CSB3 are almost identical in all species investigated to date, whereas CSB2 is less universal (6, 7). Such an organization model provides us with at least two different strategies for PCR analysis of minicircles with the outward-oriented PCR primers specific to CSB1 and CSB3 regions (for amplification of VRs adjacent to the neighboring CRs) (1) or to the CSB3 region (for amplification of CRs and VRs simultaneously) (3).

Noyes et al. (5) used another two-step-PCR approach. The first step was amplification of the minicircle template using the nested CSB2 primers. The second round was performed by using CSB1-CSB3 primers. We think it would be of interest to discuss the possibility of nonrandom amplification of a single minicircle class during the nested (CSB2) PCR. Our experience shows that using outward-oriented CSB1 and CSB3 primers leads to the detectable amplification of all minicircular templates.

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## Author's Reply

My colleagues and I thank Yurchenko et al. for their constructive and useful letter and in particular for showing that the minicircle sequence that we discovered is similar to a number of other minicircle sequences from *L. donovani* and *L. infantum*. This confirms that the sequence that we amplified was indeed of minicircle origin and not an artefact.

Yurchenko et al. ask whether the ability to amplify a single minicircle class is due to the nonrandom distribution of minicircles into the different classes or alternatively to nonrandom amplification of minicircle classes. In principle it seems likely that both these factors are involved; however, we did not have time to investigate these points since our primary objective was to demonstrate the sensitivity of the nested PCR and not to investigate minicircle organization.

Nonrandom distribution of minicircles into the different classes has been demonstrated, as Yurchenko et al. point out.

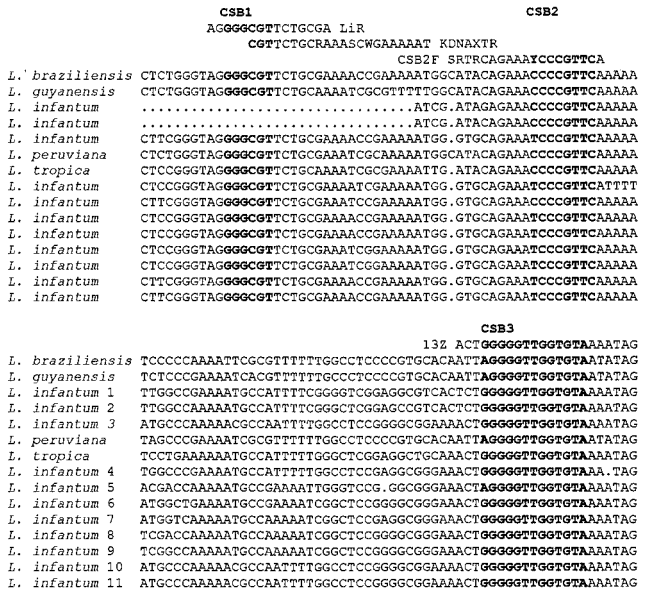


FIG. 1. Alignment of the conserved region of *Leishmania* kDNA minicircles showing the highly conserved sequence blocks (bold). The primers used in the nested PCR are shown. The reverse primers have been reversed and complemented. The alignment shows that the regions selected for the primers are highly conserved across the wide range of species used. It is therefore assumed that they will also be conserved across most minicircle classes in a given strain. The Genbank accession numbers of the strains are as follows: *L. braziliensis*, M87315; *L. guyanensis*, M87316; *L. infantum* 1, Z32847; *L. infantum* 2, Z32846; *L. infantum* 3, Z35501; *L. peruviana*, M87317; *L. tropica*, Z32843; *L. infantum* 4, Z35269; *L. infantum* 5, Z35270; *L. infantum* 6, Z35272; *L. infantum* 7, Z35273; *L. infantum* 8, Z35274; *L. infantum* 9, Z35292; *L. infantum* 10, Z35501-2; *L. infantum* 11, Z35501.

Nonrandom amplification is also likely for two reasons. First, although the amount of variability in the conserved region is small, it is possible that there are sufficient differences between minicircle classes to affect amplification efficiency of some of them. The greater effective primer length in the nested PCR will increase the risk of not amplifying some minicircle classes. Secondly, it seems likely that some minicircle classes will be thermodynamically or kinetically favored in the PCR, particularly given the extensive secondary structure of minicircle DNA. These minicircles will be more efficiently amplified and will be the major products of any PCR; they may be the only product of the nested PCR given the very small quantities of template.

We attempted to minimize the risk of the first problem by designing the primers from an alignment of a wide range of *Leishmania* species. Our alignment (Fig. 1) shows that we used regions that are conserved across the main *Leishmania* species. This strategy was based on the assumption that regions that were conserved across many species would also be conserved between classes in the same species. The inclusion of multiple sequences from *L. infantum* suggests that this is often the case. The complex fingerprints produced by the digestion of the PCR products from amounts of template greater than 1 fg showed that the nested PCR did indeed amplify multiple minicircle classes. Furthermore, it has been shown that the six 3′ bases of a primer are the most important for specificity and that some mismatches in the 5′ region are tolerated by the *Taq* enzyme (1). Our primers were anchored in regions in which the six 3′ bases were invariable. The alignment also shows that we designed the first set of primers against both CSB2 and CSB3 and not against CSB2 alone, as Yurchenko et al. seem to suggest.

The different efficiencies of amplification of different minicircle classes were largely beyond our control; however, it may be interesting to investigate the effect of different solvent systems, particularly dimethyl sulfoxide, on the amplification.

Consequently, we think it is likely that our primers will not amplify all minicircle classes and will not amplify any minicircle class in proportion to its original abundance. This is a point that merits further attention for the light it might shed on minicircle organization. Nevertheless, we would like to emphasize that our experiments showed that the nested PCR is the most sensitive method available for detecting and identifying kDNA without the use of isotopically labelled probes. This has recently been confirmed by trials in Guatemala, where both *L. braziliensis* and *L. mexicana* are endemic. Furthermore, our data showed that the nested PCR reproducibly amplified the same minicircle classes from different members of a presumed clonal population. Consequently, although some selection may be involved it is reproducible and can be managed by using suitable controls.

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