Letter to the Editor

A Mitochondrial Ancestry of the Hydrogenosomes of *Nyctotherus ovalis*

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Hydrogenosomes are membrane-bounded organelles about 1 μm in size that, like mitochondria, produce ATP (Müller 1993). They compartmentalize terminal steps of anaerobic energy metabolism but, unlike mitochondria, hydrogenosomes cannot use oxygen as an electron acceptor; they reduce protons to molecular hydrogen (Embley and Martin 1998; Martin and Müller 1998; Müller 1998). Hydrogenosomes have been found only in anaerobic protists and fungi, for example, the parabasalian flagellate *Trichomonas vaginalis*, the amoeboflagellate *Psalteriomonas lanterna*, the anaerobic ciliate *Nyctotherus ovalis*, and the anaerobic chytridiomycete fungi *Neocallimastix frontalis* and *Piromyces* sp. (Müller 1993).

A wealth of biochemical and molecular genetic evidence argues that hydrogenosomes share a common ancestor with mitochondria (Biagini, Finlay, and Lloyd 1997; Embley, Horner, and Hirt 1997; Sogin 1997; Martin and Müller 1998). However, since—with one notable exception—all hydrogenosomes studied so far lack a genome, a direct proof remained difficult. Recently, we provided evidence for the presence of a genome in the hydrogenosomes of the gut-dwelling anaerobic ciliate *N. ovalis* (Akhmanova et al. 1998). Immunogold labeling allowed colocalization of hydrogenase and double-stranded DNA in the hydrogenosomes of the ciliate. These organelles look like mitochondria, but they are surrounded by endosymbiotic methanogenic archaea that indicate (1) anoxic conditions in the immediate vicinity of the mitochondria-like organelles and (2) the presence of intracellular hydrogen (cf. Fenchel and Finlay 1995). Using reverse transcription–polymerase chain reaction (RT-PCR) and PCR approaches, we succeeded in isolating and sequencing a complete SSU rRNA that is abundantly expressed in the ciliates (Akhmanova et al. 1998). This putative hydrogenosomal SSU rRNA (accession number Y16670) proved to be greatly different with regard to nucleotide sequence and the lengths of variable regions from the respective SSU rRNA genes of the ciliate and its methanogenic endosymbionts (van Hoek et al. 1998, 1999, 2000). Notwithstanding a high degree of sequence divergence, secondary-structure analysis clearly revealed that all stem/loop structures characteristic of the ''structural cores'' of the SSU rRNAs (Stiegler et al. 1981; Gray, Sankoff, and Cedergren 1984) were conserved. Phylogenetic analysis based on evolutionary conserved rDNA sequence blocks (universally conserved regions U1—U8; Gray, Sankoff, and Cedergren 1984) revealed that the isolated sequence clustered together with mitochondrial SSU rRNA genes of aerobic ciliates (Akhmanova et al. 1998).

We showed earlier that the ciliates hosted by different cockroach strains exhibit substantial sequence divergence of their nuclear ribosomal genes. The degree of this sequence divergence (up to 9%), the presence of host-specific methanogenic endosymbionts, and differences in morphology and swimming behavior suggest that different cockroach strains host different (sub)species of *N. ovalis* (van Hoek et al. 1998, 1999, 2000). Here, we report the isolation of additional hydrogenosomal SSU rRNA genes of different *N. ovalis* isolates from several cockroach strains. In addition to the previously described complete hydrogenosomal SSU rRNA sequence of *N. ovalis* from *Periplaneta americana* var. Nijmegen, we isolated partial SSU rRNA sequences from ciliates hosted by the strains *P. americana* var. Bayer and *P. americana* var. Amsterdam. These sequences display substantial DNA sequence divergence, and ciliates from the host strain *P. americana* var. Amsterdam exhibit heteroplasmy (cf. Lightowlers et al. 1997). The SSU rRNA sequences Amsterdam 1 and Amsterdam 2 differ in the nonconserved regions of the fragment for several nucleotides. However, an alignment of conserved blocks of hydrogenosomal, mitochondrial, and bacterial SSU rRNAs is possible (fig. 1). The alignment allows a phylogenetic reconstruction that is supported by several methods of data analysis. Notably, phylogenetic analysis reveals monophyly of the SSU rRNAs from hydrogenosomes and mitochondria from ciliates (fig. 2*a*). This would be expected if hydrogenosomes of anaerobic ciliates evolved from the mitochondria of their aerobic relatives.

Northern blotting has revealed that the hydrogenosomal SSU rRNA genes are abundantly expressed, excluding the possibility that the isolated genes are inactive or a PCR artifact (Akhmanova et al. 1998). In addition, Southern blotting strongly suggests that the SSU rRNA genes described here are regular constituents of an organelle genome: SSU rDNA probes hybridize to a band of an approximate size of 11 kb of undigested genomic DNA from the various ciliate isolates (fig. 2*b*). This DNA fragment is substantially larger than the rRNA gene itself (and also a putative association of a SSU and LSU gene). Since there is also no indication

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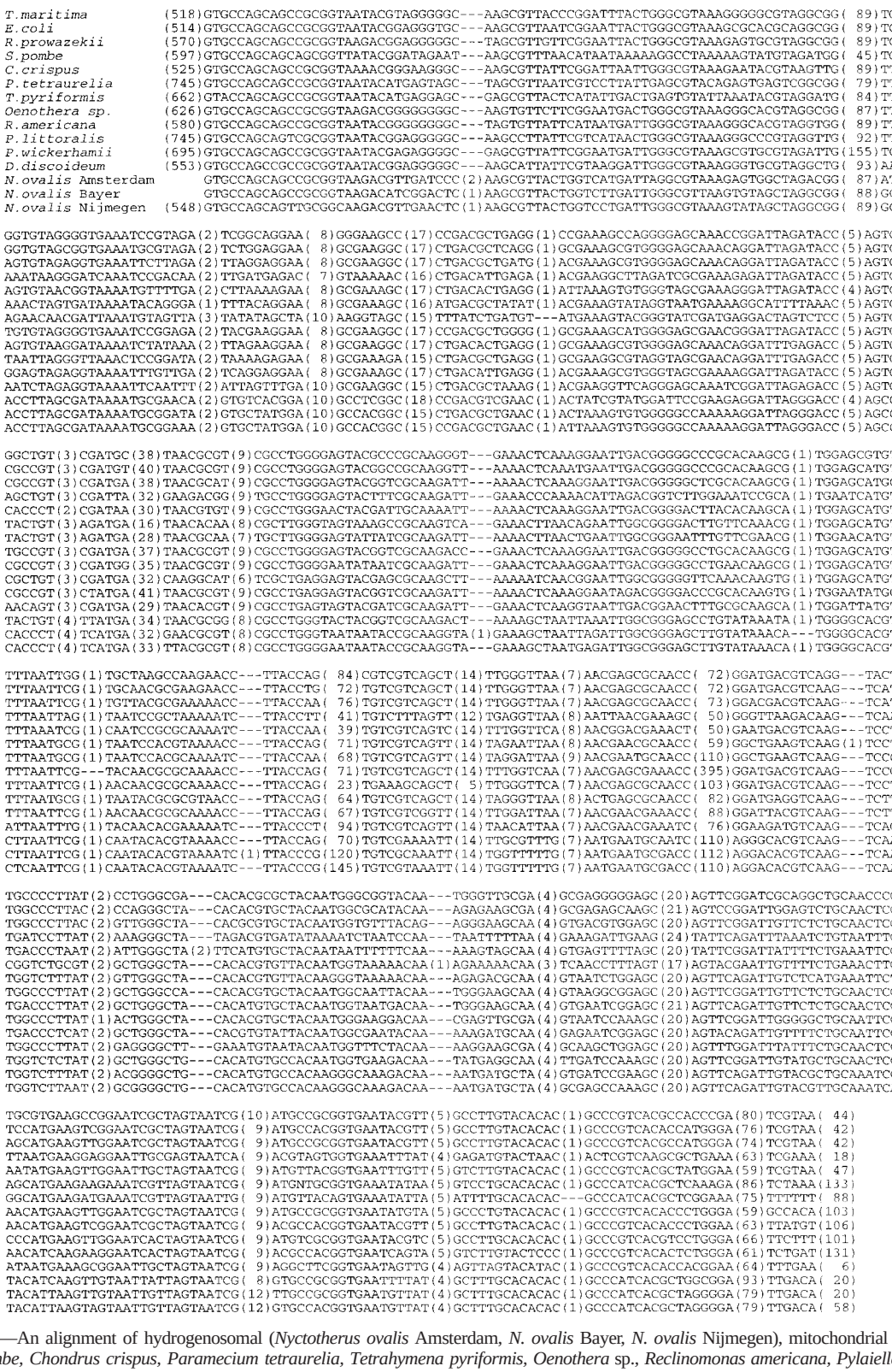

FIG. 1.—An alignment of hydrogenosomal (*Nyctotherus ovalis* Amsterdam, *N. ovalis* Bayer, *N. ovalis* Nijmegen), mitochondrial (*Schizosaccharomyces pombe*, *Chondrus crispus*, *Paramecium tetraurelia*, *Tetrahymena pyriformis*, *Oenothera* sp., *Reclinomonas americana*, *Pylaiella littoralis*, *Prototheca wickerhamii*, *Dictyostelium discoideum*), and bacterial (*Thermotoga maritima*, *Escherichia coli*, *Rickettsia prowazekii)* SSU rRNAs. The alignment was created using the CLUSTAL X package (Jeanmougin et al. 1998). Only blocks that are well conserved across all sequences (J. Castresana, personal communication) are indicated and used for phylogenetic reconstruction. The partial sequences of the hydrogenosomal SSU rRNAs from *N. ovalis* from *Periplaneta americana* var. Amsterdam (accession numbers AJ237908 and AJ237909) and from *P. americana* var. Bayer (accession number AJ237907) were obtained by PCR using primers derived from conserved regions of the mitochondrial SSU rRNA of *Paramecium tetraurelia* (accession number K01751): 5′-TGTGCCAGCAGCCGCGGTAA-3′ (positions 625–644) and 5′-CCC(AC)TACC(AG)GTACCTTGTGT-3′ (positions 1637–1657). The sequence from *N. ovalis* from *P. americana* var. Amsterdam 2 is for the positions used in the alignment and phylogenetic analysis identical to that for *N. ovalis* var. Amsterdam 1. The numbers in parentheses are the numbers of nucleotides that have been omitted from a particular sequence.

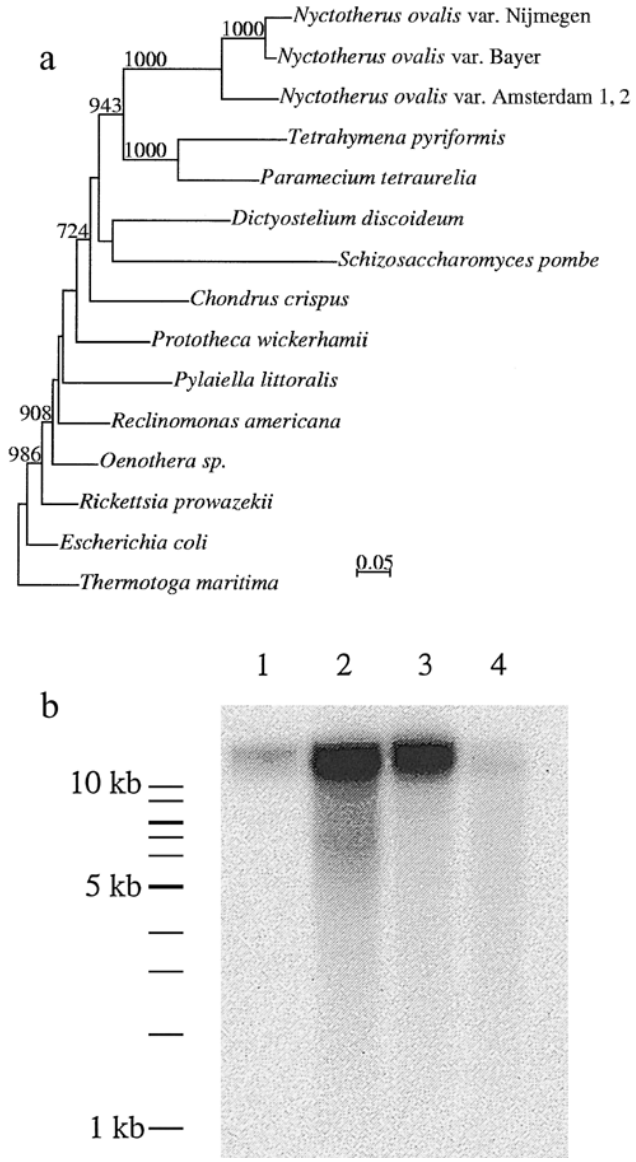

FIG. 2.—*a,* A neighbor-joining tree based on the alignment displayed in figure 1. Bootstrap values larger than 700 are indicated. The tree was created using CLUSTAL X (Jeanmougin et al. 1998), which uses the Kimura (1980) model to estimate evolutionary distances. The monophyly of the hydrogenosomal SSU rRNAs with the mitochondrial SSU rRNAs of aerobic ciliates was supported by a maximum-likelihood analysis with MOLPHY (Adachi and Hasegawa 1996). With approximate likelihood, the 1,000 best trees were calculated using a semiconstrained tree in which the *Nyctotherus ovalis* cluster, the *Tetrahymena pyriformis* + *Paramecium tetraurelia* cluster, and the *Schizosaccharomyces pombe* + *Dictyostelium discoideum* cluster were fixed, and using the HKY85 model (Hasegawa, Kishino, and Yano 1985) to account for biases in substitution rates and nucleotide composition. Among the 1,000 trees, one in which the ciliate mitochondria and hydrogenosomes were monophyletic had the highest probability in a full likelihood analysis, although the probability was not significantly higher than that of the best tree that did not support this specific monophyly. Furthermore, quartet puzzling (Strimmer and von Haeseler 1996) using the HKY85 model and a uniform model of rate heterogeneity, or, alternatively, eight gamma-distributed rate categories, supports the monophyly of the ciliate mitochondria and hydrogenosomes with reliability values of 99 and 95, respectively. Finally, a neighbor-joining tree in which variation among nucleotide frequencies of the various sequences was taken into account using log-determinant distances (Steel 1994) supported the monophyly of the ciliate mitochondria and hydrogenosomes with a bootstrap value of 96. Increasing the number of positions included from the alignment did not alter the clustering of the hydrogenosomal SSU rRNAs with the mitochondrial SSU rRNAs of aerobic ciliates in the neighbor-joining tree. *b,* Southern blot of undigested genomic DNA from *N. ovalis* from the various cockroach strains (from the left: *Periplaneta americana* var. Amsterdam, *P. americana* var. Bayer, *P. americana* var. Nijmegen, *Blaberus* sp. var. Amsterdam) probed with ^{32}P-labeled DNA from the hydrogenosomal SSU rRNA gene of *N. ovalis* from *P. americana* var. Nijmegen. The probe labels a fragment with an apparent size of approximately 11 kb. The wash consisted of 70 mM Na_2PO_4, pH 7.3, at 65°C; exposure occurred overnight. The marker was 1–10 kb. Under these conditions, the probe does not hybridize with the 18S rDNA from *N. ovalis* or the SSU rDNAs from the anerobic chytrids *Piromyces* sp. L2 and *Neocallimastix* sp. E2, the methanogenic archaeon *Methanomicrococcus blatticolus,* or the α proteobacterium *Echerichia coli. Nyctotherus ovalis* cells were isolated from dissected cockroach hindguts using their characteristic galvanotactic swimming toward the anode over a distance of about 5 cm (van Hoek et al. 1999). Since all other ciliates besides the cockroach-dwelling *N. ovalis* species swim to the cathode under the influence of a constant DC-field (Machemer and de Peyer 1977; Wagener, Stumm, and Vogels 1986; Machemer-Röhnisch, Machemer, and Bräucker 1996; van Hoek et al. 1999), a contamination by other ciliates—whether aerobic or anaerobic—can be excluded. Moreover, it has been shown by both DNA sequencing and ribotyping (ARDRA analysis) of the SSU rDNA genes of individual ciliates that the hindgut of a given cockroach strain hosts only one host-specific *Nyctotherus* (sub)species (van Hoek et al. 1998).

for a cross-hybridization with both the ciliate's and the endosymbiont's SSU rDNAs (cf. also Akhmanova et al. 1998), it is likely that the DNA fragment with an apparent size of approximately 11 kb represents the hydrogenosomal genome.

We do not yet know whether the putative genome is circular or linear. Assuming a genome of 11 kb or larger (if circular), then the hydrogenosomal genome might be well within the size range of mitochondrial genomes (Gray, Burger, and Lang 1999). The most related (linear) mitochondrial genomes of the aerobic ciliates *Paramecium* and *Tetrahymena* measure approximately 40–50 kb (Nosek et al. 1998; Gray, Burger, and Lang 1999). We have not yet proven that the putative hydrogenosomal SSU rRNA genes are transcribed inside the hydrogenosomes or that the corresponding rRNAs are actually used for hydrogenosomal protein synthesis. However, to our knowledge, there is not a single report on the presence of (transcribed) mitochondrial ribosomal genes outside a mitochondrion in any eukaryote whatsoever. Moreover, immunogold labeling using antibodies against double-stranded DNA has revealed the presence of DNA in the macronucleus and the micronucleus of the ciliate, the methanogenic endosymbionts, and the mitochondria-like hydrogenosomes (Akhmanova et al. 1998; unpublished data). Since the cytoplasm is virtually free of label, it is reasonable to assume that the ''mitochondrial'' SSU rDNA is located in the mitochondria-like hydrogenosomes. Therefore, we have to conclude that the hydrogenosomes of *N. ovalis* evolved from mitochondria—most likely in a process that involved adaptation of the ciliates to anaerobic environments (Embley et al. 1995; Hirt, Wilkinson, and Embley 1998).

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