Evolution of Anaerobic Ciliates from the Gastrointestinal Tract: Phylogenetic Analysis of the Ribosomal Repeat from Nyctotherus ovalis and its Relatives

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The 18S and 5.8S rDNA genes and the internal transcribed spacers ITS-1 and ITS-2 of ciliates living in the hindgut of frogs, millipedes, and cockroaches were analyzed in order to study the evolution of intestinal protists. All ciliates studied here belong to the genus Nyctotherus. Phylogenetic analysis revealed that these ciliates form a monophyletic group that includes the distantly related anaerobic free-living heterotrichous ciliates Metopus palaeformis and Metopus contortus. The intestinal ciliates from the different vertebrate and invertebrate hosts are clearly divergent at the level of their rDNA repeats. This argues for the antiquity of the associations and a predominantly vertical transmission. This mode of transmission seems to be controlled primarily by the behavior of the host. The different degrees of divergence between ciliates living in different strains of one and the same cockroach species most likely reflect the different geographical origins of the hosts. In addition, host switches must have occurred during the evolution of cockroaches, since identical ciliates were found only in distantly related hosts. These phenomena prevent the reconstruction of potential cospeciation events.

Introduction

About 7,500 species of ciliated protozoa have already been described, and extrapolations suggest that there might be more than 10,000 species of free-living ciliates thriving in the most divergent ecological niches (Grell 1973; Hawksworth and Kalin-Arroyo 1995; Finlay et al. 1996; Hausmann and Hülsmann 1996). Ciliates even live in the intestinal tracts of animals: they represent a substantial fraction of the complex microbiota that populate the rumina and the hindguts of many herbivorous mammals (Hungate 1966; Williams 1986; Williams and Coleman 1991). Potentially, the digestive tracts of hundreds of herbivorous mammalian species provide a wealth of anaerobic ecological niches. Such environments should favor the coevolution of the intestinal microbiota with their hosts, and one might expect that the intestinal ciliates which have been described to date represent only a minor fraction of a hitherto unknown number of anaerobic species (Hackstein 1997).

Anaerobic ciliated protozoa are also found in the guts of many arthropods (Lucas 1927; Kudo 1931; Williams and Roth 1960; Hoyte 1961a; Breznak 1982; Cruden and Markovetz 1987; Gijzen et al. 1991). A recent survey of 20 higher taxa of arthropods revealed that permanent associations between ciliates and their arthropod hosts were nonrandom: only millipedes and cockroaches were found to host substantial numbers of ciliated protozoa (Hackstein and Stumm 1994). Systematic screens among 40 species of cockroaches belonging to various genera confirmed that at least 17 cockroach species host intestinal ciliates (Hackstein 1997). The majority of these ciliates seem to belong to the genus Nyctotherus (Leidy 1849, 1853). Ciliates belonging to this taxon are also known to occur in the intestinal tract of frogs and reptiles (Bhatia and Gulati 1927; Lucas 1927; Wichterman 1937; McKeen 1972). However, the taxonomic relationships between the intestinal ciliates of the various hosts and their phylogenetic positions with respect to their potential free-living relatives and ancestors has remained largely unclear until now (Schlegel 1991; Embley et al. 1995; Hirt et al. 1995; Hammerschmidt et al. 1996).

Here, we describe the phylogenetic analysis of the 5.8S and 18S rDNA genes and the internal transcribed spacers (ITS-1 and ITS-2) of Nyctotherus ovalis ciliates from the hindgut of the cockroaches Periplaneta americana and Blaberus sp. In addition, we show that these ciliates are closely related to Nyctotherus cordiformis and similar ciliates from the intestinal tracts of frogs and millipedes. They form a monophyletic clade that also includes the anaerobic free-living heterotrichous ciliates Metopus palaeformis and Metopus contortus. Lastly, we present evidence for substantial DNA sequence divergence not only between ciliates living in different host species, but also between ciliates that live in different, isolated strains of one and the same cockroach species. We show that horizontal transfers between host species did occur, and we discuss the significance of the DNA sequence divergence for the antiquity of the associations between intestinal ciliates and their hosts.

Materials and Methods

Sources

The cockroach strains Periplaneta americana var. Amsterdam, P. americana var. Dar es Salaam, and P. americana var. Nijmegen were isolated from free-living populations. The cockroach strains P. americana var.
Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Positions on Eukaryotic rDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euk-forward</td>
<td>ATCTGTTGATCCTGCCAGT</td>
<td>18S</td>
</tr>
<tr>
<td>Euk300F</td>
<td>CGGGATATCCGAGCTCC</td>
<td>18S</td>
</tr>
<tr>
<td>Euk300R</td>
<td>CTCGGAATCGAAACCTT</td>
<td>360–376</td>
</tr>
<tr>
<td>Euk528F</td>
<td>GGGAATCCGATGCTCC</td>
<td>360–376</td>
</tr>
<tr>
<td>Euk528R</td>
<td>CTCCGGAATCGAACCCT</td>
<td>376–360</td>
</tr>
<tr>
<td>Euk690F</td>
<td>AGAATTTGACCTTCTTG</td>
<td>568–583</td>
</tr>
<tr>
<td>Euk690R</td>
<td>GGTGGTGCATGGCCGG</td>
<td>568–583</td>
</tr>
<tr>
<td>Euk1055F</td>
<td>GGTGGTGCATGGCCGG</td>
<td>1238–1252</td>
</tr>
<tr>
<td>Euk1055R</td>
<td>GGGCAGTCAGCAACC</td>
<td>1238–1252</td>
</tr>
<tr>
<td>Euk-reverse</td>
<td>GTATCCCTCTCGAGGCTACCTAC</td>
<td>1737–1726</td>
</tr>
<tr>
<td>M13-forward</td>
<td>GCCCGAAGCCGAGCTCC</td>
<td>18S</td>
</tr>
<tr>
<td>M13-reverse</td>
<td>CAGGACACACTATGAC</td>
<td>18S</td>
</tr>
<tr>
<td>5.8S</td>
<td>TCAAAGATCTGATGACTCGC</td>
<td>5.8S</td>
</tr>
<tr>
<td>5.8SR</td>
<td>TCAAAAGATCTGATGACTGCC</td>
<td>5.8S</td>
</tr>
<tr>
<td>28S</td>
<td>AATATGCTTAAGTTCAGCGG</td>
<td>28S</td>
</tr>
</tbody>
</table>

Note: Positions are based on the rDNA genes of Tetrahymena thermophila (X54512).

Bayer, Blaberus sp. var. Nijmegen, Blaberus sp. var. Amsterdam, and Blaberus sp. var. Düsseldorf originated from laboratory cultures. Blaberus sp. var. Nijmegen is identical to Blaberus sp. var. Amsterdam, but has been kept for 6 years in Nijmegen as a separate line. The cockroaches were cultured at a temperature of 21°C. They were fed apple, potato, commercial pelleted food for rabbits, and water ad libitum.

The julid millipede, listed as “Unidentified A” in Hackstein and Stumm (1994) was cultured in the laboratory at 21°C and fed potato.

Frogs and tadpoles were obtained from Dr. H. Strijbosch, Department of Environmental Biology, University of Nijmegen.

Isolation of Ciliates

Cockroaches were selected for high methane production (>90 nmol CH₄/g cockroach/h, as described earlier [Hackstein and Stumm 1994]), since such cockroaches were likely to host large ciliate populations. Cockroaches were anesthetized with CO₂ and dissected under a dissecting microscope. The hindgut was removed and placed in an electromigration device. After application of an electric field (20 V), the ciliates moved toward the anode, where they were picked up with a Pasteur pipette (Wagener, Stumm, and Vogels 1986). They were centrifuged for 5 min at 2,000 rpm and washed three times in sterile electromigration buffer (2.7 mM K₂HPO₄, 1.8 mM KH₂PO₄, 21.5 mM KCl, 20 mM NaCl, 6.1 mM MgSO₄·7H₂O, 0.5 mM L-cysteine, 0.5 mM CaCl₂·2H₂O, 0.5 mM titanium citrate [Zehnder and Wuhrmann 1976], and 1 mM NaHCO₃ [pH 7.5]). A solution of 100 µl 5% Chelex-100 (Walsh, Metzger, and Higuchi 1991) was added to the pellet. The sample was frozen in liquid nitrogen and stored at −20°C.

Alternatively, single ciliate cells were picked up with a drawn out Pasteur pipette and washed and centrifuged three times in a fresh droplet of sterile electromigration buffer. Individual cells were transferred into Eppendorf tubes. After addition of 50 µl 5% Chelex-100 the cells were frozen and stored at −20°C.

The galvanotactic behavior of the ciliates was also used to isolate the organisms from the intestinal tract of the julid millipede (“Unidentified A,” described in Hackstein and Stumm 1994), from the hindguts of tadpoles of Rana ridibunda, and from young adults of Rana temporaria.
Evolution of the Intestinal Ciliates 1197

FIG. 2.—Histogram of the size distribution of *Nyctotherus ovalis* from the different cockroach hosts. Samples of 250 ciliates from a single hindgut were analyzed. The few individuals of a size above 160 μm most likely represent zygotes.

FIG. 3.—Riboprints of the 18S rDNA genes of single ciliates of two different cockroach hosts after digestion with the enzyme *DdeI*. Lanes 1–6: *Nyctotherus ovalis* from *Periplaneta americana* var. Bayer. Lane 7: Biozym low ladder (1 kb–100 bp). Lanes 8–9: *N. ovalis* from *P. americana* var. Nijmegen.

Length Measurements

All ciliates from the hindgut of one specimen of each of the seven different cockroach strains were isolated by electromigration and transferred to a Sedgewick rafter (Graticules LTD, Tonbridge Kent, England). Swimming, untreated ciliates were photographed at 40× magnification with a Leitz photomicroscope. The sizes of 250 ciliates from each of the seven strains were measured on enlarged photographic prints.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy of the hindgut of *P. americana* var. Amsterdam was performed as described in Cazemier et al. (1997).

DNA Isolation, PCR Amplification, and Sequencing

Four or eight microliters of proteinase K (10 mg/ml) was added to the frozen single ciliates and pellets, respectively. Pellets were homogenized with a sealed Pasteur pipette. Single-cell samples were vortexed for half a minute. After incubation for 3–4 h at 56°C, the homogenates were heated at 95°C for 10 min in order to inactivate the proteinase K, chilled on ice, and centrifuged at 13,000 rpm for 10 min. Aliquots of the supernatants were used for the amplification of the 18S and 5.8S rDNA genes and the internal transcribed spacers (ITS-1 and ITS-2) of the ciliates.

PCR amplification of the total 18S rDNA genes of the ciliates from cockroaches was performed using the
primers **euk-forward** and **euk-reverse** in 25-μl reaction volumes (table 1). The 5.8S rDNA and the ITS regions were amplified with the primers **18SF** and **28SR** in 25-μl reaction volumes. PCR amplification of the 18S rDNA genes of the ciliates from a millepede and the anurans was performed using the **euk300F** and **euk-reverse** primers in 25-μl reaction volumes. DNA concentrations were estimated after electrophoresis on a 6% PAGE gel and staining with silver nitrate (Bassam, Cae

The cloning of the 18S rDNA gene of *N. ovalis* from *P. americana* var. Amsterdam was performed with a blunt-end ligation of the gene using the SureClone Ligation Kit (Pharmacia Biotech) and *Escherichia coli* JM 109 competent cells. The FlexiPrep Kit (Pharmacia Biotech) was used to harvest and purify the plasmid DNA from recombinant clones for sequencing. Two clones with the 18S rDNA gene were sequenced with five forward and five reverse eukaryotic SSU rDNA primers (table 1, cf. Elwood, Olsen, and Sogin 1985) and the M13-forward and -reverse primers.

PCR products of the 18S rDNA, ITS-1, 5.8S rDNA, and ITS-2 of one individual ciliate from each of the various hosts were sequenced directly. The PCR products were separated on a 1% agarose gel, and the desired band was electroeluted. DNA was precipitated with 0.1 volumes 7.5 M ammonium acetate (pH 5.2) and 2.5 volumes 96% ethanol for 1 h at −20°C. Finally, the PCR fragments were purified with the FlexiPrep Kit (Pharmacia Biotech) and used for sequencing with the
Table 2
Restriction Fragment Analysis (ARDRA) of the 18S rDNA Genes of the Intestinal Ciliates of the Different Cockroaches with the Restriction Enzymes DdeI, HaeIII, Hinfl, and SspI

<table>
<thead>
<tr>
<th>Species</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N. ovalis from Periplaneta americana var. Amsterdam</td>
<td>9</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>9</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>2. N. ovalis from P. americana var. Bayer</td>
<td>13</td>
<td>10</td>
<td>19</td>
<td>9</td>
<td>19</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>3. N. ovalis from P. americana var. Dar es Salaam</td>
<td>10</td>
<td>13</td>
<td>9</td>
<td>13</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. N. ovalis from P. americana var. Nijmegen</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. N. ovalis from Blaberus sp. var. Amsterdam</td>
<td>9</td>
<td>19</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. N. ovalis from Blaberus sp. var. Düsseldorf</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>7. N. ovalis from Blaberus sp. var. Nijmegen</td>
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<td></td>
<td></td>
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<td></td>
<td>19</td>
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Distance matrix

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N. ovalis from P. americana var. Amsterdam</td>
<td>0.135</td>
<td>0.112</td>
<td>0.135</td>
<td>0.139</td>
<td>0.026</td>
<td>0.139</td>
<td></td>
</tr>
<tr>
<td>2. N. ovalis from P. americana var. Bayer</td>
<td>0.071</td>
<td>0.123</td>
<td>0.139</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. N. ovalis from P. americana var. Dar es Salaam</td>
<td>0.155</td>
<td>0.071</td>
<td>0.135</td>
<td>0.071</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. N. ovalis from P. americana var. Nijmegen</td>
<td>0.123</td>
<td>0.155</td>
<td>0.123</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td></td>
</tr>
<tr>
<td>5. N. ovalis from Blaberus sp. var. Amsterdam</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td></td>
</tr>
<tr>
<td>6. N. ovalis from Blaberus sp. var. Düsseldorf</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td></td>
</tr>
<tr>
<td>7. N. ovalis from Blaberus sp. var. Nijmegen</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
<td></td>
</tr>
</tbody>
</table>

NOTE.—Distance matrix was calculated according to Swofford and Olsen (1990).
This matrix was used to construct a tree with the neighbor-joining method (Saitou and Nei 1987).

Phylogenetic Analysis

18S rDNA sequences from 41 ciliates, 2 apicomplexa, 2 dinoflagellates, and 1 stramenopile were obtained from the GenBank and EMBL databases. These sequences, together with the 18S rDNA sequences of 9 ciliates, 2 apicomplexan, and 2 dinoflagellates, and 1 stramenopile were obtained from the GenBank and EMBL databases. These sequences, together with the 18S rDNA sequences of Nyctotherus ovalis from P. americana var. Amsterdam, N. cordiformis from R. ridibunda and R. temporaria, and N. velox from the julid millipede “Unidentified A” were aligned using the PileUp program of the Wisconsin package, version 9.1. Hypervariable regions were removed from the alignment. For distance and parsimony analyses, the alignment was reduced to approximately 1,100 positions.

EDNADIST (modified PHYLIP version 3.572c of DNADIST by Felsenstein [1993]) was used to calculate the sequence similarity and evolutionary distances using the Jukes and Cantor (1969) and Kimura (1980) nucleotide substitution models. A distance matrix tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The distance data were bootstrap resampled 100 times (Felsenstein 1985).

EDNAPARS (modified PHYLIP version 3.572c of DNADIST by Felsenstein [1993]) was used to perform a parsimony analysis.

Results

Ciliates from the Hindguts of Cockroaches

In the hindguts of full-grown cockroaches, hundreds to thousands of Nyctotherus ciliates were found (Fig. 1). A conspicuous size polymorphism was characteristic for these ciliates. Ciliates of largely varying sizes were present in a single hindgut. In addition, ciliates from the hindguts of the different Blaberus spp. appeared to be smaller than those from the P. americana strains. However, a biometric analysis showed that ciliate sizes were normally distributed (Fig. 2). Since the ciliates were also morphologically similar, it is likely that all ciliates in the hindgut of a single cockroach belong to one population. The modes of the length distributions of the ciliates were host-specific, and they confirm our observation that the smallest ciliates live in the largest host (Blaberus sp.; Fig. 2). Only rarely (<1%) were double-sized ciliates observed in all hosts; these morphs had been interpreted as macrogamonts or zygotes by McKean (1972).

Restriction analysis with 28 different enzymes of the amplified 18S rDNA genes was used to confirm that there is only one ribotype of ciliates present in the hindgut of an individual cockroach. The restriction patterns obtained from 10–20 individual ciliates isolated from at least 4 different cockroaches from each of the 7 host strains were analyzed. Consistently, the restriction patterns revealed that only one ciliate ribotype was present in all of the cockroaches belonging to one and the same strain (Fig. 3).

Phylogenetic Analysis of the Ribosomal Small Subunit rDNA

The complete 18S rDNA gene of Nyctotherus ovalis from the hindgut of the cockroach P. americana var. Amsterdam has a length of 1,707 nucleotides (accession number AJ222678). There were no indications of structural peculiarities or fragmentation of the coding region. The GC content of the coding region of the small subunit is 43.8%. Also, partial sequences (±1,340 nt) of the 18S rDNA of Nyctotherus ciliates from a millipede and two frog species were obtained. Phylogenetic analysis of the small subunit rDNA genes was performed by distance matrix analysis using the Jukes and Cantor (1969) algorithm for 45 ciliate, 2 apicomplexan, and 2 dinoflagellate species, with an alga as outgroup. The results strongly suggested that the intestinal Nyctotherus ciliates from the various hosts form a monophyletic group together with the free-living anaerobic heterotrichous ciliates M. palaiformis and M. contortus. High bootstrap
values for the *Nyctotherus-Metopus* cluster strongly support the close relationship and confirm the postulated branching order (fig. 4).

DNA Sequence Divergence Among Ciliates from Different Cockroaches

Restriction analysis of the 18S rDNA of *Nyctotherus* ciliates revealed that 4 (*DdeI, HaeIII, HinII, and SspI*) of the 28 restriction enzymes generated different restriction patterns for 5 of the 7 ciliate samples from the different cockroach strains (fig. 5). Only ciliates from the cockroach species *Blaberus* sp. var. Nijmegen, *Blaberus* sp. var. Amsterdam, and *P. americana* var. Bayer exhibited identical restriction patterns (table 2). The informative patterns generated by these four enzymes were used to construct a distance matrix tree (see below). It became evident that the five different ciliate samples were closely related but occupied clearly different positions in the phenogram. This divergence was confirmed by DNA sequence analysis of the 5.8S rDNA genes and the adjacent internal transcribed spacers (ITS-1 and ITS-2). The ITS-1 and ITS-2 regions are remarkably short (93 nt and 136–137 nt, respectively). The G/C contents of these regions are 34.6 ± 1.4% and 43.3 ± 1.7%, respectively (table 3). Alignment of the ITS regions and the 5.8S rDNA gene revealed no obvious gaps or length differences within the *Nyctotherus* group (fig. 6). The pairwise comparison of the 18S, 5.8S and ITS sequences from ciliates living in the various cockroach strains revealed a divergence of up to 5% (table 5). Phylogenetic analysis of the 5.8S rDNA genes and the ITS-1 and ITS-2 sequences from the ribosomal repeats allowed the construction of a distance matrix tree (fig. 7) that is similar to the tree obtained by ARDRA of the amplified 18S rDNA genes (not shown).

**Discussion**

**Phylogenetic Aspects**

Ciliates represent a large group of morphologically very diverse protists that are characterized by a nuclear dimorphism, sexual reproduction in form of conjugation, and a complex infraciliature (Small and Lynn 1981; Foissner and Foissner 1988; Finlay et al. 1996; Hausmann and Hülsmann 1996). DNA sequencing data have confirmed that ciliates form a monophyletic taxon which radiated early after their common ancestor separated from the other eukaryotes (Sogin and Elwood 1986; Schlegel 1991; Embley et al. 1995; Hirt et al. 1995; Hammerschmidt et al. 1996).

The phylogenetic analysis of 18S rDNA sequencing data of *N. ovalis* from the hindgut of the cockroach *P. americana* var. Amsterdam, *N. velox* from the hindgut of the julid millipede “Unidentified A,” and *N. cordiformis* from the intestinal tract of two anurans places them with high bootstrap values in a clade with the distinctly related free-living anaerobic heterotrichs *M. palaeformis* and *M. contortus* (fig. 4). This position is not subject to change when different distance and tree-building algorithms (i.e., Jukes and Cantor 1969; Kimura 1980; parsimony) are used. Therefore, the phylogenetic analysis of the 18S rDNA genes of the various *Nyctotherus* species confirms that anaerobic heterotrichous ciliates cluster clearly distinctly from the aerobic heterotrichs (De Puytorac, Grain, and Legendre 1994; Hirt et al. 1995).

**Sequence Divergence**

The DNA sequence divergence between the ribosomal genes of the different *Nyctotherus* species is substantial (e.g., approximately 5% between *N. ovalis* and *N. cordiformis*; table 4). However, a divergence up to 5% can also be observed between *N. ovalis* from the various cockroach strains (table 5). The divergence with respect to their closest free-living relatives, i.e., *Metopus* species, exceeds 10%. Unexpectedly, the divergence for the (noncoding) ITS regions is not significantly higher than that for the coding parts of the 18S rDNA genes. However, the ITS-1 and ITS-2 of the ribosomal repeats are short (93 and 136–137 nt, respectively; see table 3); they are the smallest among eukaryotes (cf. Odorico and Miller 1997). Dot-plot analysis did not provide any evidence for the presence of repeated DNA sequences (not shown). It seems reasonable to assume that only those parts of the ITS that are under functional constraints have been retained in all the ciliates of the *Nyctotherus* cluster.

**Mode of Transmission**

Phylogenetic analysis of the 18S rDNA genes of the different *Nyctotherus* species from frogs, millipedes, and cockroaches reveals that these ciliates represent well-separated evolutionary lines, although the branching order of the millipedes and the frog symbionts cannot be resolved (fig. 4). The remarkable divergence is rather unexpected since the gastrointestinal tract in animals is an open ecosystem. This raises the question of how, under these conditions, a long-lasting genetic isolation is achieved that is rigid enough to allow the evolution of such a divergence. The intestinal tracts of animals must be colonized by a complex microbiota, including *Nyctotherus*, after hatching from the eggs, molting, and metamorphosis. Cockroaches, for example, molt several times before reaching the adult stage. *Periplaneta americana*, for instance, molts approximately 12 times over a period of 1 year (Guthrie and Tindall 1968). During every molt, the intestinal biota of the hindgut are shed, together with the cuticle covering this part of the intestinal tract. Freshly molted cockroaches eat their own meconium or those from members of their local population. This behavior allows the recolonization of the intestinal tract with a microbial biota that is characteristic for the host population (cf. Nalepa and Bell 1997). Consequently, the behavior of the hosts, at least in cockroaches, might favor a vertical transfer. However, ciliates of the genus *Nyctotherus* form cysts as resting and propagation stages. Therefore, a horizontal transfer to other host species (or strains) should be possible during the (re-)infection phases. The presence of ciliates with identical 18S rDNA in two only distantly related cockroach species, i.e., *P. americana* (*Blattellidae*) and *Blaberus* sp. (*Blaberidae*), proves that host switches can occur under...
1. Nystotherus ovalis from P. americana var. Amsterdam
   GCGG-AGGATCATATAAATCGAATTCAAAACATTCTAACCATTACCTTGACAGCC-T
2. N. ovalis from P. americana var. Bayer
   A..................A..................---A..................---A..................---A
3. N. ovalis from P. americana var. Dar es Salaam
   A..................A..................---A..................---A..................---A
4. N. ovalis from P. americana var. Nijmegen
   A..................A..................---A..................---A..................---A
5. N. ovalis from Blaberus sp. var. Amsterdam
   A..................A..................---A..................---A..................---A
6. N. ovalis from Blaberus sp. var. Düsseldorf
   A..................A..................---A..................---A..................---A
7. N. ovalis from Blaberus sp. var. Nijmegen
   A..................A..................---A..................---A..................---A
8. Cryptocaryon irritans (#)
   A..................---A..................---A..................---A..................---A
9. Ichthyophthirius multifiliis (#)
   A. AT..................---A..................---A..................---A
10. Tetrahymena thermophila (*)
    A. AT..................---A..................---A..................---A

1. TCTCCAC--TGGAGATA-GTTA-TACCTACCT--AAAA--C-AATTGCGAATG--AAA--T--AAAA--AAAA
2. ---------------GACCATTTCACGATGATATCTAGGTCACATAGAAGAGAC
3. ---------TATACATA---------TACAT--------AG.AA--------G.A--------T.C--------A
4. ATGCTCAAGATCTCGATA-TATCATCACAAGAAAATTAAG.AA--------G.A
5. ATGCTCAAGATCTCGATA-A-CATCCAAGACGAAG---AG.AA.A.A.GA.C.A.C.G.GG

1. GAGCAAAATGTCGATAGCAGTGCAGAAAACCCCGGATACTGAAATGAGAACCCCGGACCTGGGACCAACC
2. G..................A..................T..................T..................A
3. G..................A..................T..................T..................A
4. G..................A..................T..................T..................A
5. G..................A..................T..................T..................A
6. G..................A..................T..................T..................A
7. G..................A..................T..................T..................A
8. G..................A..................T..................T..................A
9. C..................A..................---C..................---C..................---C
10. GGAAGGGG.---C.G.---C...G.T......GAT......A.CCGTCAC.G.A......TC......AC...

1. TTCCGCATGTTTGTTCATGGACCTACTAATTTAAGAATTTTGATGGCAAGCTGCAAGCTGCAAGCTGCAAG
2. GT..............A......T..............-TA..............-TA..............---A
3. GT..............A......T..............-TA..............-TA..............---A
4. GT..............A......T..............-TA..............-TA..............---A
5. GT..............A......T..............-TA..............-TA..............---A
6. GT..............A......T..............-TA..............-TA..............---A
7. GT..............A......T..............-TA..............-TA..............---A
8. GT..............A......T..............-TA..............-TA..............---A
9. G..................---C..................---C..................---C
10. GGAGG.---GG.---C...G.T......GAT......A.CCGTCAC.G.A......TC......AC...

1. TTATGCATGTTTGTTCATGGACCTACTAATTTAAGAATTTTGATGGCAAGCTGCAAGCTGCAAGCTGCAAG
2. GT..............A......T..............-TA..............-TA..............---A
3. GT..............A......T..............-TA..............-TA..............---A
4. GT..............A......T..............-TA..............-TA..............---A
5. GT..............A......T..............-TA..............-TA..............---A
6. GT..............A......T..............-TA..............-TA..............---A
7. GT..............A......T..............-TA..............-TA..............---A
8. GT..............A......T..............-TA..............-TA..............---A
9. C..................---C..................---C..................---C
10. C..................---C..................---C..................---C

1. TCTCCAC--TGGAGATA-GTTA-TACCTACCT--AAAA--C-AATTGCGAATG--AAA--T--AAAA--AAAA
2. ---------------GACCATTTCACGATGATATCTAGGTCACATAGAAGAGAC
3. ---------TATACATA---------TACAT--------AG.AA--------G.A--------T.C--------A
4. ATGCTCAAGATCTCGATA-TATCATCACAAGAAAATTAAG.AA--------G.A
5. ATGCTCAAGATCTCGATA-A-CATCCAAGACGAAG---AG.AA.A.A.GA.C.A.C.G.GG

1. GAGCAAAATGTCGATAGCAGTGCAGAAAACCCCGGATACTGAAATGAGAACCCCGGACCTGGGACCAACC
2. G..................A..................T..................T..................A
3. G..................A..................T..................T..................A
4. G..................A..................T..................T..................A
5. G..................A..................T..................T..................A
6. G..................A..................T..................T..................A
7. G..................A..................T..................T..................A
8. C..................A..................---C..................---C..................---C
9. C..................A..................---C..................---C..................---C
10. C..................A..................---C..................---C..................---C

1. TCTCCAC--TGGAGATA-GTTA-TACCTACCT--AAAA--C-AATTGCGAATG--AAA--T--AAAA--AAAA
2. ---------------GACCATTTCACGATGATATCTAGGTCACATAGAAGAGAC
3. ---------TATACATA---------TACAT--------AG.AA--------G.A--------T.C--------A
4. ATGCTCAAGATCTCGATA-TATCATCACAAGAAAATTAAG.AA--------G.A
5. ATGCTCAAGATCTCGATA-A-CATCCAAGACGAAG---AG.AA.A.A.GA.C.A.C.G.GG

1. TCTCCAC--TGGAGATA-GTTA-TACCTACCT--AAAA--C-AATTGCGAATG--AAA--T--AAAA--AAAA
2. ---------------GACCATTTCACGATGATATCTAGGTCACATAGAAGAGAC
3. ---------TATACATA---------TACAT--------AG.AA--------G.A--------T.C--------A
4. ATGCTCAAGATCTCGATA-TATCATCACAAGAAAATTAAG.AA--------G.A
5. ATGCTCAAGATCTCGATA-A-CATCCAAGACGAAG---AG.AA.A.A.GA.C.A.C.G.GG
natural conditions (fig. 7). However, such switches seem to occur only rarely. Also in zoological gardens, where large populations of free-living Blattela germanica, Pycnocelus surinamensis, P. australasia, and P. americana coexist with Blaberus sp. cultures, we did not observe any transfers of intestinal ciliates (Hackstein 1997). We also did not find any evidence for an acquisition of “foreign” ciliates by Blaberus sp. var. Amsterdam over a period of 4 years.

Experimental studies revealed that transfers between different species are hampered by several constraints. For example, transfers are only possible between certain host species, and they can occur only if the recipient is freshly molted (Hoyte 1961a, 1961b; unpublished data). DNA sequence data also do not provide any evidence that cockroaches acquired intestinal ciliates from millipedes or anurans. Moreover, the ciliates that live in the intestinal tracts of the various vertebrates and arthropods exhibit distinct morphological traits, and, consequently, they have been described as different species. Our study confirms the assumption that the ciliates living in the intestinal tracts of animals exhibit a remarkable host- and strain-specificity (Bhatia and Gulati 1927; Dolein and Reichenow 1953; Hoyte 1961a, 1961b). Spatial and behavioral isolation of their hosts might be responsible for a predominantly vertical transmission of the ciliates described in this study. But in addition, a horizontal spread of intestinal ciliates might also be hampered by a genetically controlled character of the host, since intestinal protists are distributed in a nonrandom manner (Hackstein and Stumm 1994; Hackstein 1997). Such a phenomenon has also been observed for other arthropods and their intestinal protists, and it has been speculated as to whether host taxonomy is of significance for the presence of intestinal symbionts (Hennig 1981; Nalepa 1991; Thorne 1991; Hackstein and Stumm 1994; Grancolas and Deleporte 1996). Consequently, one of the main criteria for differentiation of the various Nyctotherus species has been their association with particular hosts (Hoyte 1961a). Since we have demonstrated that host switches can occur, this concept, at least for cockroaches, is of limited value. Moreover, the substantial DNA sequence divergence (up to 5%; see tables 4 and 5) between ciliates living in different strains of one and the same host species might be used as an argument to assign these ciliates to different species (Vandamme et al. 1996). However, despite the substantial DNA sequence divergence, ciliates from cockroaches look rather similar. Nyctotherus species from millipedes and frogs, on the other hand, exhibit rather characteristic traits that allow an easy discrimination from the ciliates from cockroaches. Therefore, we suggest the

Fig. 6.—Alignment of the 5.8S rDNA, ITS-1, and ITS-2 sequences, including parts of the 18S and 28S rDNA genes of Nyctotherus ovalis from the different cockroach hosts. The alignment includes sequences from two parasitic ciliates (*Diggles and Adlard 1997) and a free-living ciliate (*Engberg and Nielsen 1990). Points indicate identical nucleotides. Dashes mark gaps in the alignment.

Fig. 7.—Unrooted neighbor-joining tree (Saitou and Nei 1987) based on 5.8S rDNA, ITS-1, and ITS-2 sequences (fig. 6) of the intestinal ciliates from the different cockroach hosts using the Jukes and Cantor (1969) algorithm. Distance data were bootstrap resampled 100 times (Felsenstein 1985). The scale bar indicates the distances.
maintenance of the arbitrary description of *N. ovalis* for ciliates living in cockroaches, *N. velox* for ciliates from millipedes, and *N. cordiformis* for ciliates living in anuran hosts until additional morphological, physiological, and genetic data support a more differentiated classification. The additional indication of the particular host strain might be sufficient to identify the various ciliates and to avoid discussions about their classification as separate species or subspecies.

Antiquity of the Symbiotic Associations

The phylogenies of the intestinal ciliates and the significant degree of DNA sequence divergence between the different *Nyctotherus* species and variants argue for the antiquity of the associations between ciliates and their arthropod and vertebrate hosts. Notwithstanding, highly divergent ciliates are also found in different strains of one and the same host species, and because of host switches, the phylogenies of intestinal ciliates and their cockroach hosts do not match. Such a situation is clearly different from the coevolution of the mycetome-inhabiting flavobacteria and their cockroaches hosts, for which a complete match between the phylogenies of the symbionts and their hosts has been demonstrated (Bandi et al. 1995). A comparable coevolution has also been described for *Buchnera* and their aphid hosts (Moran and Baumann 1994; Baumann et al. 1995). Such a coevolution is the result of a strictly vertical transmission of the symbionts that is facilitated by an egg-mediated transfer. Also, *Wolbachia*, a rickettsia-like germ line parasite of many arthropods (Werren 1997), is transmitted by eggs from the mother to the filial generation. However, switches of *Wolbachia* between hosts that belong to different higher taxa have occurred (Schluhuizen and Stouthamer 1997) similar to the situation observed for the intestinal ciliates of cockroaches. Such host switches hamper the reconstruction of potential cospeciation events, but fossilized insects might provide clues for a calibration of the molecular clock. Fossils that exhibit very suggestive cockroach traits are as old as 250–300 Myr (Hennig 1981). The morphology of the chewing appendages of many of these insects did not change substantially over at least 250 Myr, suggesting that the feeding habits of fossil cockroach-like insects were similar to those of extant cockroaches. Therefore, one might speculate as to whether the ancestors of cockroaches hosted ciliates similar to those of their present-day relatives. A more detailed analysis of the intestinal tract of fossilized cockroaches for the presence of ciliate cysts should allow the identification of potential *Nyctotherus* species and thereby provide direct evidence for the antiquity of these symbiotic associations.

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Sequence Availability

The sequence data from the *Nyctotherus* species described here have been deposited in the EMBL database: 18S rDNA gene of *N. ovalis* from *P. americana* var. Amsterdam (AJ222678), 18S rDNA gene (partial) of *N. cordiformis* from *R. ribibunda* (AJ006711), 18S rDNA gene (partial) of *N. cordiformis* from *R. temporalia* (AJ006712), 18S rDNA gene (partial) of *N. velox* from the julid millipede “Unidentified A” (AJ006713), 5.8S rDNA gene and flanking ITS sequences of *N. ovalis* from *P. americana* var. Amsterdam (AJ006714), 5.8S rDNA gene and flanking ITS sequences of *N. ovalis* from *P. americana* var. Dar es Salaam (AJ006715), 5.8S rDNA gene and flanking ITS sequences of *N. ovalis* from *P. americana* var. Nijmegen (AJ006716), 5.8S rDNA gene and flanking ITS sequences of *N. ovalis* from *P. americana* sp. var. Düsseldorf (AJ006720).
Table 5
Pairwise Comparison of the 18S a and 5.8S rDNA, and the ITS-1 and ITS-2 Sequences of *Nyctotherus ovalis* from the Different Cockroach Hosts

<table>
<thead>
<tr>
<th>Length (nt)</th>
<th>ITS-1</th>
<th>ITS-2</th>
<th>18S</th>
<th>5.8S</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. var. Amsterdam</td>
<td>95.7</td>
<td>100.0</td>
<td>97.5</td>
<td>98.9</td>
</tr>
<tr>
<td>5. var. DuÈsseldorf</td>
<td>95.7</td>
<td>100.0</td>
<td>97.5</td>
<td>98.9</td>
</tr>
<tr>
<td>5. var. Nijmegen</td>
<td>95.7</td>
<td>100.0</td>
<td>97.5</td>
<td>98.9</td>
</tr>
</tbody>
</table>

NOTE – Numbers represent percentage identity calculated with the *Best*®t program of the Wisconsin package, version 9.1.

a Partial sequence (65%). For lengths of 5.8S rDNA, ITS-1, and ITS-2, see table 3.

**LITERATURE CITED**


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