An anaerobic mitochondrion that produces hydrogen
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subject to the constraints that no lichen covered the trunk and no young trees with a trunk circumference less than 0.9 m were used. Colour matches of treatments to natural bark were verified by spectrophotometry of stimuli and bark, followed by modelling of predicted photon catches\(^*\) of a typical passerine bird, the blue tit’s (Parus caeruleus) single cone photoreceptors\(^*\), with irradiance spectra from overcast skies in the study site. Our acceptance criterion was simply that cone captures for the experimental stimuli fell within the measured range of those for oak bark.

Experiment 1 used black patterns printed onto dark brown card. Patterns were samples of digital photos of the oak trees at 1:1 reproduction, converted using Image\(^*\) to greyscale and thresholded at 50% to binary (black/white) images to provide, when printed onto brown card, bark-like brown/black spatial variation (Fig. 1). Different samples, from different trees, were used for each replicate target.

Experiment 2 used bicoloured targets printed onto waterproof paper (Hewlett Packard Laserjet Tough Paper) with a Hewlett Packard Colour Laserjet 2500 (600 dots per inch) printer, with colour pairs chosen to have either high or low contrast. Colours were chosen from frequency distributions of the eight-bit RGB (red, green, blue) values from digital photographs of the oak trees in the study site, reduced to 16 bins in each colour channel. Photos (about 267 mm \(\times\) 200 mm; 2,560 pixels \(\times\) 1,920 pixels) were taken with a Nikon Coolpix 5700 camera, calibrated\(^*\) to linearize the relationship between radiance and the greyscale in each colour channel, and saved as uncompressed TIFF files. Digital photographs lack ultraviolet information that birds can see\(^*\), but lichen-free oak bark reflects negligible ultraviolet\(^*\). Even a properly calibrated RGB image does not precisely simulate the avian-perceived colour of many natural objects, owing to differences in the spectral sensitivity of bird long-wave, medium-wave and short-wave cones compared with human cones\(^*\). However, because our treatments varied only in relative colour contrast, any error introduced by this method was considered minor, an assumption verified retrospectively by spectrophotometry and colour-space modelling. We chose colour pairs from the eight most frequent RGB triplet in the bark photos as follows: a ‘background’ colour, then a triplet that was similar to the background (low contrast), and one that differed markedly (high contrast). The major difference between colours was in overall brightness not hue, but we could not systematically vary only one colour dimension within the available common bark colours. Sample numbers of background and contrasting colours were balanced for which was darker/lighter, and so there were no significant differences between bicoloured treatments in the brightest or darkest colour or average colour (analyses of variance on RGB sums and all possible ratios; \(P > 0.05\)). Monochrome treatments were also created as the means of the respective R, G and B values of the two colours in bicoloured high-contrast and low-contrast treatments. Different colour pairs and patterns, from different trees, were used for each replicate target.

Survival analysis was by Cox regression\(^*\) with the factors treatment and block. Cox regression assumes that all survival functions have the same shape; this proportional hazards assumption was checked by plotting partial residuals against ranked survival times\(^*\). There were significant block effects in both experiments (in experiment 1, \(\text{Wald} = 121.78, d.f. = 9, P < 0.001\); in experiment 2, \(\text{Wald} = 271.50, d.f. = 9, P < 0.001\)), reflecting differences in average predation rates in different parts of the woods on different dates, but this was not relevant to our hypotheses.

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letters to nature

An anaerobic mitochondrion that produces hydrogen

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Hydrogenosomes are organelles that produce ATP and hydrogen\(^*\), and are found in various unrelated eukaryotes, such as anaerobic flagellates, chytridiomycete fungi and ciliates\(^*\). Although all of these organelles generate hydrogen, the hydrogenosomes from these organisms are structurally and metabolically quite different, just like mitochondria where large differences also exist\(^*\). These differences have led to a continuing debate about the evolutionary origin of hydrogenosomes\(^*\). Here we show that the hydrogenosomes of the anaerobic ciliate Nyctotherus ovatus, which are found in the hindgut of cockroaches, have retained a rudimentary DNA encoding components of a mitochondrion electron transport chain. Phylogenetic analyses reveal that these proteins cluster with their homologues from aerobic ciliates. In addition, several nucleus-encoded components of the mitochondrial proteome, such as pyruvate dehydrogenase and complex II, were identified. The N. ovatus hydrogenosome is sensitive to inhibitors of mitochondrial complex I and produces succinate as a major metabolic end-product—biochemical traits typical of anaerobic mitochondria\(^*\). The production of hydrogen, together with the presence of a genome encoding respiratory chain components, and biochemical

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Figure 1 A 14,027-bp fragment (mtg 1) of the hydrogenosomal genome of *N. ovalis* var. *Blaberus* Amsterdam. Black boxes, RNA coding genes; shaded boxes, genes with significant similarity to mitochondrial genes; white boxes, unknown ORFs (named according to the number of codons); arrows, cDNAs identified so far. The numbers indicate the nucleotide positions on the 14-kb clone (mtg 1). The longest ORF (4,179–9,728) contains a stretch with significant similarity to *nad5*. A potential start codon for a putative *nad5* transcript is marked with an asterisk.

Figure 2 Phylogenetic analysis of hydrogenosomal genes. Both the organelar 12S (SSU) rRNA gene (b) and the nuclear hsp60 (c) reveal a ciliate ancestry for the hydrogenosome of *N. ovalis*. The same is true for the components of a ‘mitochondrial’ complex I, the *nad7* (49 kDa; organelar, a) and 51 kDa (nuclear, d) genes. The phylogenies were derived using MrBayes and neighbour joining: the topologies correspond to the maximum-likelihood (MrBayes) approach, and the values at the nodes indicate the posterior probability for the partition and its bootstrap value, respectively. Only values higher than 50% are indicated. See Supplementary Information. EB, Eubacteria.
features characteristic of anaerobic mitochondria, identify the *N. ovalis* organelle as a missing link between mitochondria and hydrogenosomes.

Hydrogenosomes and their highly reduced relatives, mitosomes, generally lack an organelle genome, hampering clarification of their origin. Two models for the origin of hydrogenosomes are currently debated. The first posits that the ancestral mitochondrial endosymbiont gave rise to aerobically functioning mitochondria, which subsequently evolved into hydrogenosomes by the acquisition of genes encoding enzymes essential for an anaerobic metabolism. The second hypothesis presumes that hydrogenosomes and mitochondria originated from one and the same ancestral—facultatively anaerobic—(endo)symbiont, followed by specialization to aerobic and anaerobic niches during eukaryotic evolution.

To address this issue we investigated DNA in hydrogenosomes of *N. ovalis*, which was previously identified by immunocytochemical methods. Intact *N. ovalis* hydrogenosomes isolated by cell fractionation contained DNA between 20 and 40 kilobases (kb) long. Long-range polymerase chain reaction (PCR) with this DNA with the use of specific primers for the hydrogenosomal small-subunit (SSU) ribosomal RNA and *nad7* (obtained earlier by PCR with degenerated primers) yielded a 12-kb fragment of the organelle genome. It encodes four genes of a mitochondrial complex I (*nad2, nad4L, nad5* and *nad7*), two genes encoding mitochondrial ribosomal proteins RPL 2 and RPL 14, and a 15S rRNA gene (Fig. 1). *nad2* and *nad4L*, which are generally poorly conserved among ciliates, could be identified by using multiple sequence alignments and an analysis of their membrane-spanning domains as described

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### Table 1: Nyctotherus ovalis genes encoding mitochondrial proteins and RNAs

<table>
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<tr>
<th>Type</th>
<th>Gene product</th>
<th>Localization of the gene</th>
<th>Codon use</th>
<th>cDNA Target</th>
<th>Accession no.</th>
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<td>Mt complex I</td>
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<tr>
<td>NAD2</td>
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<td>NAD4L</td>
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<td>NAD5</td>
<td>H Mt Yes*</td>
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<td>ND7</td>
<td>H Mt Yes†</td>
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<td>24 kDa</td>
<td>N Nuc Yes†</td>
<td>Yes</td>
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<td>51 kDa</td>
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<td>75 kDa</td>
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<td>Mt complex II</td>
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<td>SDH b</td>
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<tr>
<td>Mt protein synthesis</td>
<td>Putative rRNA methyltransferase 2</td>
<td>N Nuc</td>
<td>AY871313</td>
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<tr>
<td>Mt ribosomal proteins</td>
<td>RPL 2</td>
<td>H Mt</td>
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<tr>
<td></td>
<td>RPL 14</td>
<td>H Mt</td>
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<td></td>
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<td>tRNA tyrosine</td>
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<td>Mt tRNA (SSU)</td>
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<td>Mt catabolism/energy metabolism</td>
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<td>Acetyl-CoA synthase A synthase 2</td>
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<td>AY871315</td>
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<td></td>
<td>(EC 6.2.1.1)</td>
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<td></td>
<td>Adenylate kinase 2 (EC 2.7.4.3)</td>
<td>N Nuc</td>
<td>AY871316</td>
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<td></td>
<td>Adenylate kinase 2 isofrm c = HK2418</td>
<td>N Nuc</td>
<td>AY871317</td>
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<td></td>
<td>Probable α-lactate dehydrogenase</td>
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<td>AY871318</td>
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<tr>
<td></td>
<td>[cytochrome] (EC 1.1.1.4)</td>
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<td>Succinyl-CoA ligase</td>
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<td>AY871319</td>
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<td></td>
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<td>Succinyl-CoA-3-keto acid-cyclohydrolase</td>
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<td>AY871320</td>
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<td></td>
<td>A transaminase</td>
<td>N Nuc</td>
<td>?</td>
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<td>Mt carrier family</td>
<td>AAC</td>
<td>N Nuc</td>
<td>AF480921</td>
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<td></td>
<td>Putative mt carrier protein PET8</td>
<td>N Nuc</td>
<td>AY871322</td>
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<tr>
<td>Mt import/processing</td>
<td>Mt processing peptidase alpha subunit</td>
<td>N Nuc</td>
<td>?</td>
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<td></td>
<td>HSP 60</td>
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<td>Heat shock protein HSP82 YMR186W_Chr</td>
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<td></td>
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<tr>
<td>Mt protein import protein</td>
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<tr>
<td>Mt stress-70 protein, mt precursor</td>
<td>N Nuc</td>
<td>?</td>
<td>AY871329</td>
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</tbody>
</table>

For a complete table see Supplementary Information. N, nucleus; H, hydrogenosome; nuc, nuclear; Mt, mitochondrial; mtg1, 12-kb clone of hydrogenosomal genome (AJ871267); nd, not detectable. Accession numbers for cDNAs: *AJ871574 and AJ871575; †AJ871576; ‡AY608633 and AY608634; §AY616150 and AY616151; ¶AY619981; ‡‡AY623919; ^AY628684; ††AY623926; ‡‡‡AY608633 and AY608634.

### Table 2: Glucose metabolism of Nyctotherus ovalis

<table>
<thead>
<tr>
<th>Labeled end products</th>
<th>([U-14C]glucose) (µmol h⁻¹ per mg protein) (%)</th>
<th>([U-14C]glucose) (µmol h⁻¹ per mg protein) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>427</td>
<td>53</td>
</tr>
<tr>
<td>Lactate</td>
<td>220</td>
<td>27</td>
</tr>
<tr>
<td>Succinate</td>
<td>112</td>
<td>14</td>
</tr>
<tr>
<td>Ethanol</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>CO₂</td>
<td>205</td>
<td>-</td>
</tr>
<tr>
<td>Formate</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

Cells were incubated for 48 h at 25°C in micro-aerobic conditions in medium with either [U-14C]glucose or [6-14C]glucose. Excreted end products are shown as means ± s.d. of three independent experiments ([U-14C]glucose) or as the means of two independent experiments ([U-14C]glucose). Other excreted end products were less than 2% of the total excreted end products. ND, not detectable.

*Percentage of the total of acetate, lactate, succinate and ethanol.
Phylogenetic analysis revealed clustering of these genes with their homologues from the mitochondrial genomes of aerobic ciliates (Fig. 2, and Supplementary Information). All genes exhibit a characteristic mitochondrial codon-usage and lack amino-terminal extensions that could function as a mitochondrial targeting signal (Table 1). Complementary DNAs isolated for nad5 and nad7 show that they are transcribed. Translation with a nuclear genetic code from N. ovalis, rather than the ciliate mitochondrial code, leads to numerous stop codons (not shown). Five additional open reading frames (ORFs 236, 262, 71, 161 and 199) do not show significant sequence similarity to ORFs from the mitochondrial genomes accessible in the EMBL database. Two ORFs overlap with neighbouring ORFs as in other mitochondrial genomes.

Macronuclear gene-sized chromosomes encoding the 24-kDa, 51-kDa and 75-kDa subunits of mitochondrial complex I and the Fp and Ip subunits of mitochondrial complex II were cloned with a PCR-based approach. These have a nuclear codon usage, are transcribed (Table 1), encode a putative N-terminal mitochondrial targeting signal and branch with their mitochondrial homologues from aerobic ciliates in phylogenetic analyses (Fig. 2, Table 1 and Supplementary Information). They are similar to the two complex I-like Ndh51 and Ndh24 proteins discovered in Trichomonas vaginalis, because a phylogenetic analysis including the mitochondrial homologues from N. ovalis and certain aerobic ciliates reveals that all these proteins belong to a cluster of mitochondrial complex I homologues (see Supplementary Information). Thus, in N. ovalis, 7 of the 14 genes encoding core proteins of mitochondrial complex I, and two of the four proteins of mitochondrial complex II, have been identified so far. They are well conserved, are transcribed, and cluster with the mitochondrial homologues of their aerobic (ciliate) relatives, indicating that the hydrogenosomes of N. ovalis have retained parts of a functional mitochondrial electron-transport chain.

Hydrogenosomes of N. ovalis have typical mitochondrial cristae and contain cardiolipin. They are closely associated with endosymbiotic methanogens, which are biomarkers for hydrogen formation by the N. ovalis hydrogenosomes (Fig. 3a). The organelles stain with Mitotracker Green FM and fluoresce with rhodamine 123, indicating the presence of a membrane potential (Fig. 3). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (5 μM) prevented staining with rhodamine 123, indicating the possible presence of a proton gradient. Moreover, staining of the hydrogenosomes with rhodamine 123 was also prevented after incubation of the ciliates with rotenone, piericidin, fenazaquin and 1-methyl-4-phenylpyridinium (MPP⁺) (classical inhibitors of mitochondrial complex I (ref. 21)), but not with cyanide (1 mM) or antimycin A (inhibitors of mitochondrial complex III and IV; Fig. 3). Similarly, treatment with cyanide and salicylhydroxamic...
acid (SHAM), inhibitors of mitochondrial complex IV of the respiratory chain and the plant-like alternative oxidase known from certain mitochondria\(^3\), respectively, neither killed \textit{N. ovalis} nor interfered with its oxygen consumption under aerobic conditions (not shown). These observations not only indicate the absence of a functional complex III and IV and the absence of a terminal (plant-like) alternative oxidase, but also reveal the presence of a functional mitochondrial complex I as the source of the organellar proton gradient\(^7\). The oxygen consumption of \textit{N. ovalis} observed under aerobic conditions is most probably a detoxification mechanism, and longer exposure to atmospheric oxygen kills the ciliates effectively.

Metabolic experiments using tracer amounts of uniformly labelled (U-)\(^14\)C-glucose revealed that \textit{N. ovalis} catabolizes glucose predominantly into acetate, lactate, succinate and smaller amounts of ethanol, in addition to CO\(_2\) (Table 2). The presence of oxygen did not cause significant changes in the pattern of excreted end products (not shown). Notably, incubations in the presence of [\(^6\)\(^14\)C]glucose did not result in the formation of labelled CO\(_2\). Because \(^14\)C-labelled CO\(_2\) is released from [\(^6\)-\(^14\)C]glucose by successive decarboxylations, the absence of labelled CO\(_2\) after application of [\(^6\)-\(^14\)C]glucose indicates the absence of a complete Krebs cycle. The observed excretion of \(^14\)C-labelled CO\(_2\) after incubation with [\(^U\)-\(^14\)C]glucose could be the result of either pyruvate dehydrogenase (PDH) activity, as in typical aerobic mitochondria, or pyruvate:ferredoxin oxidoreductase (PFO) activity, as in the hydrogenosomes of \textit{T. vaginalis}. A third possibility for pyruvate catabolism, pyruvate formate lyase activity\(^22,23\), can be excluded because no detectable amounts of formate were produced from [\(^U\)-\(^14\)C]glucose (Table 2). We failed to identify genes for PFO but succeeded in isolating three of the four PDH genes, namely the E1\(\alpha\), E1\(\beta\) and E2 subunits, which are expressed as cDNA, indicating that \textit{N. ovalis} uses a mitochondrial PDH for oxidative decarboxylation. Significant amounts of \(^14\)C-labelled succinate from both [\(^U\)-\(^14\)C]glucose and [\(^6\)-\(^14\)C]glucose (Table 2) indicate that endogenously produced fumarate is used as a terminal electron acceptor, as in some anaerobic mitochondria\(^3\). Fumarate reduction in \textit{N. ovalis} (to account for the production of succinate) is most probably catalysed by a membrane-bound complex II (see above; Table 1, and Supplementary Information), which is coupled to complex I through electron transport mediated by quinones\(^7\). Mass spectrometry coupled to liquid chromatography of lipid extracts from \textit{N. ovalis} revealed the presence of small amounts of quinones (rhodoquinone 9 and menaquinone 8) at a concentration of about 2,000 six-frame-translated clones from our genomic DNA library of \textit{N. ovalis} (approx. 1 g of beef steak). In addition, the ciliates were fed with \textit{Esherichia coli}, which were added at weekly intervals. Ciliates were harvested by centrifugation.

### Methods

#### Strains

\textit{Nycotethus ovalis} ciliates were isolated from the hindgut of the cockroach \textit{Blaberus sp.} (strain Amsterdam), taking advantage of their unique (anodic) galvanotactic swimming behaviour.\(^8\) After the ciliate’s arrival at the anode, cells were picked up with a micropipette, inspected individually under a dissecting microscope at 40 magnification, and collected in an Eppendorf tube and washed three times with anaerobic electromigration buffer. Ciliates belonging to the \textit{Euplotes} genus were cultured in \textit{N. ovalis} (strain Amsterdam), taking advantage of their unique (anodic) galvanotactic swimming behaviour.\(^8\) The oxygen consumption of \textit{N. ovalis} has been acquired by lateral gene transfer.

#### Microscopy

Electron microscopy of \textit{N. ovalis} and \textit{Euplotes sp.} was performed as described previously\(^1,2\). Fluorescence microscopy was performed with a Nikon \textit{OZ} video-rate confocal microscope as described previously\(^3\). Inhibitors were used in concentrations of 1 mM. They were dissolved in \textit{N. ovalis} culture medium\(^4\). The rotenone solution contained 10% dimethyl sulphoxide, the fenaquazin solution 1% dimethyl sulphoxide.

#### Metabolite and quinone determinations

Micro-aerobic incubations with \textit{N. ovalis} were performed in rotating (2 r.p.m.) sealed incubation flasks containing 5 ml incubation medium (containing 10,000–15,000 cells). All incubations were performed for 48 h and contained either 10\(\mu\)g [\(^U\)-\(^14\)C]glucose or 10\(\mu\)g [\(^6\)-\(^14\)C]glucose (2.07 GBq mmol\(^{-1}\)), both from Amersham. Incubations were terminated by the addition of 300 \(\mu\)l 6 M HCl to lower the pH from 7.2 to 2.0. \textit{N. ovalis} cells were separated from the medium by centrifugation (5 min at 500 \(\times \) g and 4 \(^\circ\) C); excreted end products were analysed by anion-exchange chromatography on a Dowex 1X8 column. Quinones were separated by liquid chromatography and detected with a Sciex API 300 triple quadrupole mass spectrometer (see Supplementary Information).

#### Isolation of organellar DNA

\textit{N. ovalis} cells were washed once with isolation buffer (0.35 M sucrose, 10 mM Tris-HCl pH 7, 2 mM EDTA) and disrupted in a Dounce homogenizer. Nuclei were centrifuged at 3,000 \(\times\) g for 5 min, and organelles were pelleted from the supernatant at 10,000 \(\times\) g for 30 min. Genomic DNA was isolated by using standard procedures or after lysis of the cells in 8 M guanidinium chloride.

#### Genomic DNA library

Gene-sized chromosomes were randomly amplified by PCR with telomere-specific primers, size-fractionated in agarose gels, and cloned in \textit{pGEM-T easy} (Promega). Clones with sizes between 0.5 and 5 kb were end-sequenced and analysed manually by TBLASTX (http://www.ncbi.nlm.nih.gov/BLAST). Searches were conducted with BLASTN and FASTA.

#### c-DNA library

RNA was isolated with the \textit{RNeasy Plant mini-kit} (Qiagen). cDNA was prepared with the
Complete macronuclear gene-sized chromosomes

Telomere-specific primers in combination with internal gene sequences allow a straightforward recovery of the complete gene. The specific (internal) primers were based on the DNA sequences of internal fragments of the various genes, which were recovered previously by PCR with degenerated primers for conserved parts of the various genes.

Phylogenetic analysis

Protein sequences were aligned with ClustalW and Muscle; unequivocally aligned positions were selected with Gblocks or manually. Phylogenies were inferred with maximum likelihood by using a discrete gamma-distribution model with four rate categories plus invariant positions and the Poisson amino acid similarity matrix, and maximum likelihood by using a discrete gamma-distribution model with four rate positions were selected with Gblocks or manually. Phylogenies were inferred with neighbour joining as implemented in ClustalW, correcting for multiple substitutions with categories plus invariant positions and the Poisson amino acid similarity matrix, and maximum likelihood by using a discrete gamma-distribution model with four rate categories plus invariant positions and the Poisson amino acid similarity matrix, and neighbour joining as implemented in ClustalW, correcting for multiple substitutions with the Gonnet amino acid identity matrix, and bootstrapping with 100 samples.

OBW with a lower size limit of 100 nucleotides were identified with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). tRNAs were identified with tRNAscan-SE (http://www.genetics.wustl.edu/eddy/tnRnascan-SE). Potential mitochondrial import sequences were identified with BLASTX (http://www.ncbi.nlm.nih.gov/BLASTX), BLASTN and FASTA. For references on phylogenetic analysis see Supplementary Information.

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