

Molecular and morphological analysis of the family Calonymphidae with a description of *Calonympha chia* sp. nov., *Snyderella kirbyi* sp. nov., *Snyderella swezyae* sp. nov. and *Snyderella yamini* sp. nov.

Gillian H. Gile,^{1†} Erick R. James,¹ Rudolf H. Scheffrahn,²
Kevin J. Carpenter,^{1‡} James T. Harper^{1,3} and Patrick J. Keeling¹

Correspondence
Patrick J. Keeling
pkeeling@interchange.ubc.ca

¹Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada

²University of Florida Research & Education Center, 3205 College Avenue, Davie, FL 33314, USA

³Department of Biology, Douglas College, 700 Royal Avenue, New Westminster, BC V3M 5Z5, Canada

Calonymphids are a group of multinucleate, multiflagellate protists belonging to the order Cristamonadida (Parabasalia) that are found exclusively in the hindgut of termites from the family Kalotermitidae. Despite their impressive morphological complexity and diversity, few species have been formally described and fewer still have been characterized at the molecular level. In this study, four novel species of calonymphids were isolated and characterized: *Calonympha chia* and *Snyderella yamini* spp. nov., from *Neotermes castaneus* and *Calcaritermes nearcticus* from Florida, USA, and *Snyderella kirbyi* and *Snyderella swezyae*, spp. nov., from *Calcaritermes nigriceps* and *Cryptotermes cylindroceps* from Colombia. Each of these species was distinguished from its congeners by residing in a distinct host and by differences at the molecular level. Phylogenetic analyses of small subunit (SSU) rDNA indicated that the genera *Calonympha* and *Stephanonympha* were probably not monophyletic, though the genus *Snyderella*, previously only represented by one sequence in molecular analyses, appeared with these new data to be monophyletic. This was in keeping with the traditional evolutionary view of the group in which the morphology of the genus *Snyderella* is considered to be derived, while that of the genus *Stephanonympha* is ancestral and therefore probably plesiomorphic.

INTRODUCTION

Calonymphids are multinucleate, multiflagellate, anaerobic or microaerophilic protists that live in the hindgut of drywood or dampwood termites (family Kalotermitidae). They belong to the Parabasalia, a group of symbiotic (mutualistic and parasitic) protists that includes the human pathogen *Trichomonas vaginalis*. In parabasalids,

the basic mastigont system consists of three anteriorly directed flagella and one recurrent flagellum (Brugerolle, 1991), connected to the Golgi apparatus by parabasal fibres and typically associated with an axostyle (Brugerolle, 1991; Cepicka *et al.*, 2010). Certain parabasalid lineages, found exclusively in the hindguts of lower termites and wood-eating cockroaches of the genus *Cryptocercus*, have elaborated on this basic mastigont organization and bear hundreds or thousands of flagella (Brugerolle & Lee, 2000). In the family Calonymphidae, nuclei and their associated axostyles have also multiplied, a characteristic that distinguishes them from the independently elaborated hypermastigote lineages (Kirby, 1947; Ohkuma *et al.*, 2005; Cepicka *et al.*, 2010). Calonymphid nuclei are either each associated with a mastigont system, as a karyomastigont, or are free in the cytoplasm, leaving akaryomastigonts at the cell's periphery. The presence and relative numbers of karyo- and akaryomastigonts have been used to define genera in this group (Brugerolle & Lee, 2000).

†Present address: Department of Biochemistry and Molecular Biology, Dalhousie University, 5850 College Street, Room 9-G1, Halifax, NS B3H 1X5, Canada.

‡Present address: Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, L-231, 7000 East Ave, Livermore, CA 94551, USA.

Abbreviations: ML, maximum-likelihood; SEM, scanning electron microscopy; SSU, small subunit; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the nuclear SSU and mitochondrial SSU and COII sequences determined in this study are HQ215836–HQ215844 and HQ593144–HQ593145.

Since the family Calonymphidae was proposed (Grassi & Foà, 1911) to include the genera *Calonympha* and *Microrhopalodina* (now known to be an oxymonad genus), nine additional calonymphid genera have been described and molecular phylogenetic analyses have shown that the group consists of two independent lineages (termed the *Calonympha*-group and *Coronympha*-group, Cepicka *et al.*, 2010). The genera *Coronympha* and *Metacoronympha* were previously included in the family Calonymphidae due to their multiple nuclei and flagella (Kirby, 1939, 1947). However, the axostyles in these genera do not form a bundle in the centre of the cell and molecular phylogenetic analyses have shown they branch independently from the *Calonympha*-group (Kirby, 1929, 1939; Gerbod *et al.*, 2002; Noël *et al.*, 2007; Harper *et al.*, 2009), so they should no longer be considered to be calonymphids. In addition, the genus *Metacoronympha* was recently determined to be a life cycle stage of the genus *Coronympha* (Harper *et al.*, 2009) and was therefore dissolved.

Calonymphid genera for which molecular data are available are *Stephanonympha*, *Calonympha* and *Snyderella*. These are also the best-studied morphologically, with several described species in each genus. In the genus *Stephanonympha*, all mastigont systems are karyomastigonts and they are arranged in a loose spiral or spirals extending from the cell apex (Janicki, 1911). The genus *Calonympha* also has karyomastigonts, in one or two turns of a loose spiral, but these are posterior to a larger number of akaryomastigonts (Foà, 1905). The genus *Snyderella* exhibits only akaryomastigonts and the nuclei are randomly distributed in the cytoplasm (Kirby, 1929). In each of these genera, the axostyle associated with each mastigont extends towards the centre axis of the cell where the strands form a bundle that proceeds towards or through the cell's posterior. Molecular data from six species and unnamed strains of these three genera so far do not address whether the genus *Snyderella* is monophyletic, but they do suggest that the genera *Calonympha* and *Stephanonympha* may not be. Together these three calonymphid genera form a clade in small subunit (SSU) rRNA trees (Gerbod *et al.*, 2002; Noël *et al.*, 2007; Noda *et al.*, 2009).

Currently, the kalotermitid species whose symbionts have not been studied significantly outnumber those whose have (Yamin, 1979), suggesting that the bulk of calonymphid diversity has yet to be observed and many more species await description. Here, we have contributed to addressing this deficiency by describing and providing SSU rRNA sequences for four novel species of calonymphids from four different termite hosts, as well as scanning electron microscopy (SEM) analysis of one of the novel species.

METHODS

Cell isolation, DNA extraction, PCR and sequencing. Several individuals of *Neotermes castaneus* and *Calcaritermes nearcticus* from Florida, USA, and *Calcaritermes nigriceps* and *Cryptotermes cylindroceps* from Colombia were collected and maintained at room

temperature in the laboratory. Termites were dissected and the hindgut contents were suspended in Trager medium U (Trager, 1934). Individual calonymphid cells were isolated by micropipette and extracted singly or pooled in samples of 4–75 cells for DNA extraction using the Masterpure Complete DNA and RNA Purification kit (Epicentre). DNA was also extracted from the whole gut contents of *N. castaneus* for environmental PCR. All PCRs used EconoTaq PLUS GREEN (Lucigen).

Parabasolid SSU rRNA gene sequences were amplified using the universal eukaryotic primers PF1 5'-TGCGCTACCTGGTTGATC-CTGCC-3' (Keeling, 2002) and FAD4 5'-TGATCCTTCTGCAGG-TTCACCTAC-3' (Medlin *et al.*, 1988; Deane *et al.*, 1998). This primer pair amplifies nearly the entire SSU: against the human 18S rRNA reference sequence NR_003286.2, PF1 binds to the first 18 nt and includes 5 nt upstream, while the FAD4 binding region ends just 3 nt before the 3' end of the gene. The SSU from *Snyderella yamini* sp. nov. was amplified using a nested reaction with primers PF1 and FAD4 followed by GGF 5'-CTTCGGTCTAGATTAAGCCATGC-3' and GGR 5'-CCTTGTTACGACTTCTCCTTCCTC-3' (this study), which were designed for nested amplification of parasasolid SSU. PCR conditions included a 3 min denaturation at 95 °C followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, then an additional 7 min at 72 °C. To confirm the identity of the host, termite mitochondrial SSU rRNA sequences were amplified with the primers LR-N-13398 5'-CGCCTGTTTATCAAAAACAT-3' (Simon *et al.*, 1994) and LR-J-13007 5'-TTACGCTGTATC-CCTAA-3' (Kambhampati & Smith, 1995) and the COII sequence from *N. castaneus* was amplified using A-tLeu 5'-ATGGCAGATTAG-TGCAATGG-3' and B-tLys 5'-GTTAAGAGACCAGTACTTG-3' (Wirth *et al.*, 1999). Cycle conditions were the same as for parasasolid SSU, except that the annealing temperature was lowered to 50 °C and the extension time was shortened to 1 min.

PCR products were purified using a QIAquick PCR purification kit (Qiagen). Parasasolid SSU sequences were cloned using a Strataclone PCR cloning kit (Agilent Technologies) and sequenced on both strands with a BigDye Terminator v. 3.1 kit (Applied Biosystems). Several clones were sequenced from each parasasolid and the single clone that most closely matched the consensus sequence was chosen for phylogenetic analyses and to submit to GenBank. Mitochondrial SSU and COII were sequenced directly from the purified PCR product, also with BigDye Terminator v. 3.1. All sequences were deposited in GenBank under accession numbers HQ215836–HQ215844 and HQ593144–HQ593145 (Table 1).

Phylogenetic analyses. New SSU sequences were added to an existing alignment in MacClade 4.08 (Maddison & Maddison, 2003). Preliminary phylogenetic analyses (not shown) including representatives of all parasasolid groups confirmed a cristamonad affinity for the novel calonymphids described here. For the final analysis (presented here), trichonymphid and spirotrichonymphid sequences were removed to increase the number of alignable characters, leaving only cristamonad sequences and close tritrichomonad relatives as an outgroup. Sequences were realigned using MAFFT (Kato *et al.*, 2002). Highly variable and ambiguously aligned regions were removed manually for a final alignment of 48 taxa and 1412 positions (83% of the raw 1692-site alignment). Maximum-likelihood (ML) and Bayesian phylogenetic analyses were performed with RAxML 7.0.4 (Stamatakis, 2006) and MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) respectively, on the freely available Bioportal (<http://www.bioportal.uio.no>), using the GTR+ Γ +I model (with the Γ distribution approximated by four rate categories) as specified by Modeltest v3.7 (Posada & Crandall, 1998) under the Akaike Information Criterion, implemented in PAUP* 4.0b10 (Swofford, 2002). For the ML analysis, support was assessed from 1000 bootstrap replicates. For the Bayesian analysis, two independent chains, sampled

Table 1. New sequences determined in this study

Organism	Gene	Length (bp)*	GenBank accession no.
<i>Calonympha chia</i> sp. nov.	SSU rDNA	1528	HQ215836
<i>Snyderella kirbyi</i> sp. nov.	SSU rDNA	1590	HQ215837
<i>Snyderella swezyae</i> sp. nov.	SSU rDNA	1541	HQ215838
<i>Snyderella yamini</i> sp. nov.	SSU rDNA	1482†	HQ593144
<i>Devescovina</i> sp.	SSU rDNA	1534	HQ215839
<i>Neotermes castaneus</i> symbiont	SSU rDNA	1523	HQ215840
<i>Calcaritermes nigriceps</i>	mitochondrial SSU rDNA	376	HQ215841
<i>Calcaritermes nearcticus</i>	mitochondrial SSU rDNA	375	HQ593145
<i>Cryptotermes cylindroceps</i>	mitochondrial SSU rDNA	373	HQ215842
<i>Neotermes castaneus</i>	mitochondrial SSU rDNA	379	HQ215843
<i>Neotermes castaneus</i>	mitochondrial COII	714	HQ215844

*Product length does not include primer binding regions.

†Amplified with nested primers.

each 100 generations, were run until they converged (the mean standard deviation of partition frequency values between the chains dropped below 0.01) with 25 % of the trees discarded as burn-in. Convergence was reached after 590 000 generations and a consensus tree was computed from the 8850 saved trees. This analysis was repeated using identical parameters except that the runs were allowed to continue for 5 000 000 generations rather than being stopped automatically upon reaching convergence. The consensus tree, built from the 75 000 saved trees, had nearly identical topology and posterior probabilities to the consensus of the shorter two runs.

Light and electron microscopy. Hindgut contents suspended in Trager medium U were viewed on a compound microscope (Axioplan 2; Zeiss) using Nomarski interference optics and photographed with a QImaging MicroImager II. Cell isolations were performed on an inverted microscope (Axiovert 200; Zeiss). For SEM, a few drops of hindgut contents in Trager medium U were pipetted onto a Millipore Isopore membrane filter (pore size of 3 or 5 µm) held in a plastic Millipore Swinnex cartridge affixed to a 10 ml syringe. Five millilitres of 2.5 % glutaraldehyde in Trager Medium U buffer were then poured into the syringe and the material was allowed to fix for 30 min. The syringe barrel was then inserted and used to force the glutaraldehyde out of the cartridge. Immediately after, the cartridge was unscrewed from the syringe and 10 ml buffer was pulled into the syringe. The cartridge was then screwed back on and buffer was pushed through for rinsing. This was repeated once or twice before a few millilitres of 1 % OsO₄ was pulled into the syringe (with the Swinnex cartridge off) and then placed into contact with the material, which was allowed to fix for 30 min. Following fixation, filters were rinsed with buffer, dehydrated in an ethanol series (50 %, 70 %, 90 %, 2 × 100 %) and critical-point-dried in a CO₂ critical-point dryer (Autosamdri 815B; Tousimis). Dried filters were affixed to aluminium SEM stubs with double-sided carbon sticky tape and coated with 5 nm Au or Au/Pd in a sputter coater (208 HR; Cressington). Samples were examined and photographed in a Hitachi 4700 FESEM at 3–5 kV.

RESULTS AND DISCUSSION

Calonympha chia sp. nov.

We observed a multinucleate, multiflagellate protist in the hindgut of *N. castaneus* from Dagny Johnson Park, Key

Largo, Florida, USA (N 25.17608 W 80.36945). The mastigont systems are organized in multiple helical rows from the cell apex. The foremost mastigont systems lack nuclei while the rearmost row or rows of mastigonts are associated with nuclei. An axostylar strand descends from each mastigont to a bundle in the centre of the cell, which reaches or protrudes from the posterior of the cell. In certain SEM images, the axostylar bundle can be seen protruding from the cell's posterior (Fig. 1b, c, f). The observed combination of karyo- and akaryomastigonts (which has been confirmed by transmission electron microscopy for a different isolate of the same organism, see below) places this protist in the genus *Calonympha* (Foà, 1905).

The cell is covered with a glycocalyx (Figs 1b, 2b, c), which can be seen in high magnification to be made up of a dense, fibrous network (Fig. 2d, e). The surface of the posterior half of the cell, which lacks flagella, is associated with a variety of ectobionts (Fig. 2b–e). Some of these appear to be spirochaetes or possibly bristle-shaped members of the order *Bacteroidales* (Hongoh *et al.*, 2007), while others are coccoid or rod-shaped cells (Fig. 2b, c). At least three distinct morphotypes of rod-shaped cells can be seen, including long rods with tapered ends (Fig. 2c), long rods with blunt ends (Fig. 2e) and smaller rods that can be as little as 300 nm in length (Fig. 1b–d).

We amplified the SSU of this organism from DNA extracted from pools of 20–50 cells isolated by micropipette, as well as from environmental PCR of DNA extracted from the entire gut contents. A total of five clones was sequenced on both strands and two independent PCR products were sequenced directly on both strands. All sequences were nearly identical, differing at a total of only seven positions collectively.

The consensus of the five clone sequences was 99 % identical in BLAST alignments to GenBank sequences AM747388 and X97976, both *Calonympha* sp. from *N. castaneus*, from a culture maintained at the Bundesanstalt

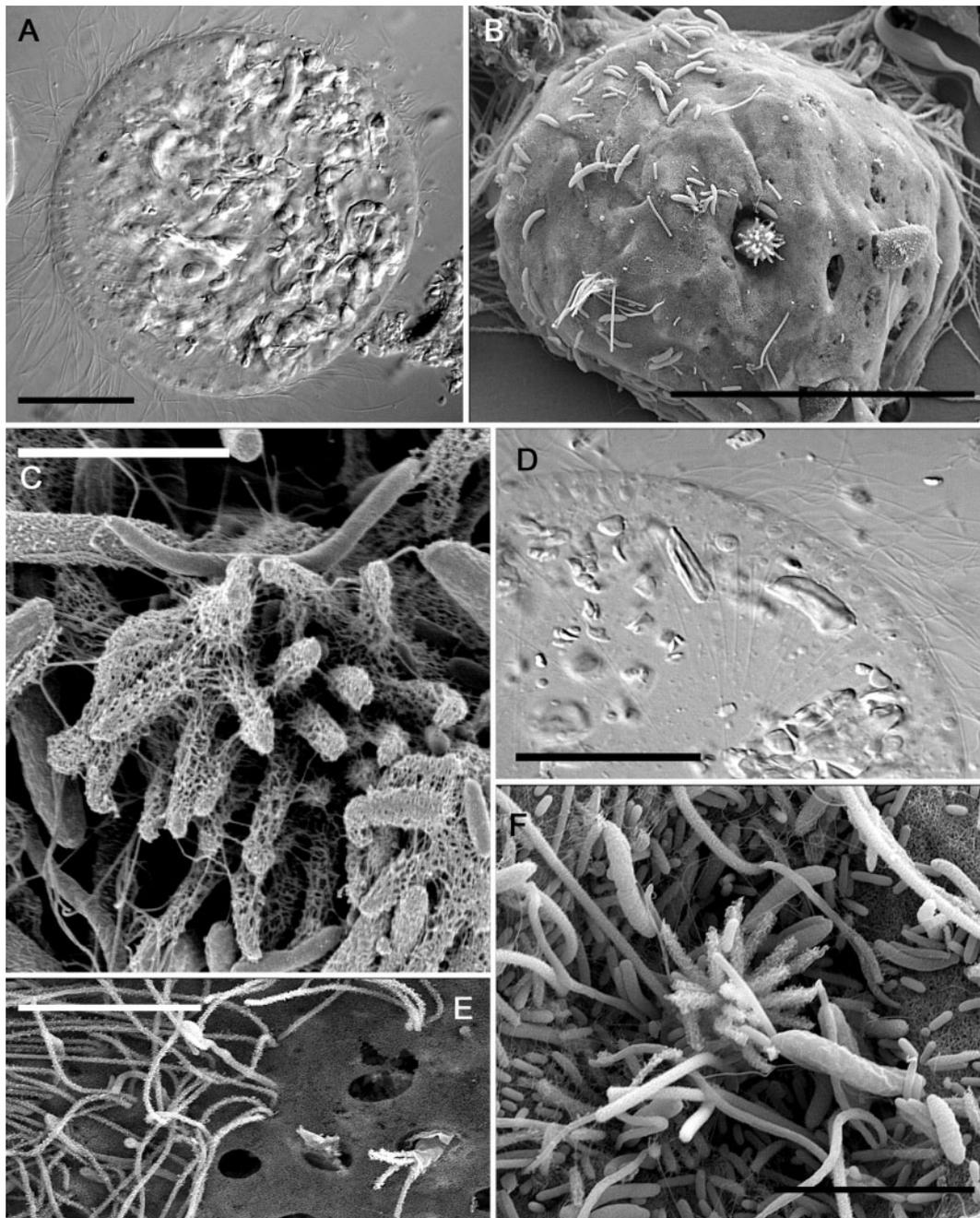


Fig. 1. Overview of *Calonympha chia* sp. nov. (a, d) Differential interference contrast light micrographs from the posterior of the cell showing ingested wood particles filling the cell and karyomastigonts arranged along the periphery. Axostylar strands are visible in (d). (b, c, e, f) SEM of the cell surface. (b, c, f) Posterior view of the cell with protruding axostylar bundle. (e) Lateral view of the cell. Flagella emerge in groups of four. Bars, a, b, d, 30 µm; c, 2.5 µm; e, 20 µm; f, 5 µm.

für Materialforschung und -prüfung (BAM), Germany (Branke, 1997; Ikeda-Ohtsubo *et al.*, 2007), which we therefore consider to derive from the same *Calonympha* species as investigated here. Gut symbionts from this termite strain were investigated by J. Branke before the termite was reliably identified as *N. castaneus* and the record for GenBank accession no. X97976 erroneously specifies

Neotermes jouteli as the host species. There is no doubt, however, that the BAM strain (collected in Cuba) and our *N. castaneus* from Florida, USA, are the same species, based on three lines of evidence. First, the symbiont community of our *N. castaneus* isolate is identical to that described for the BAM isolate (Branke, 1997; Ikeda-Ohtsubo *et al.*, 2007), while true members of *N. jouteli* have a very different gut

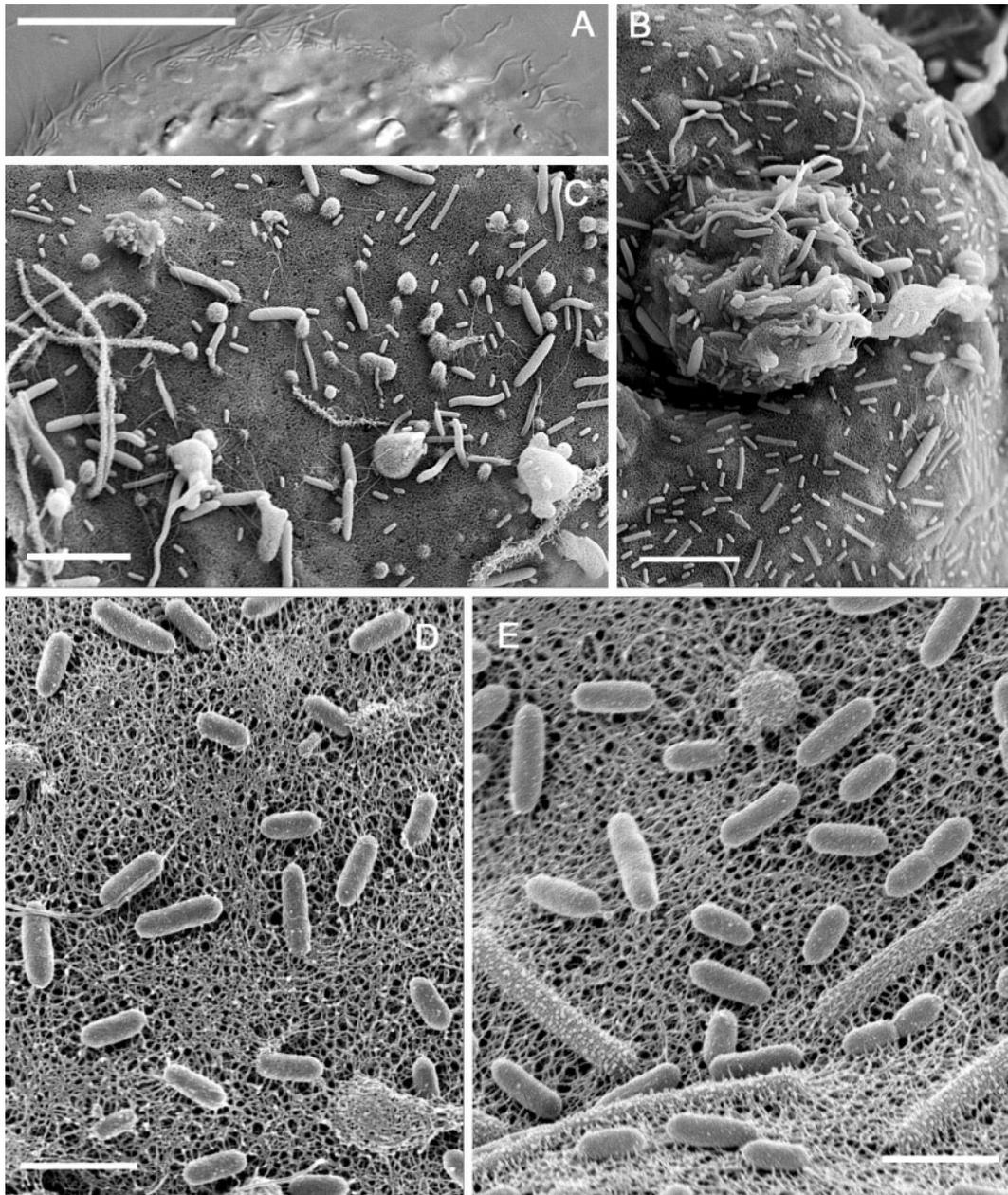


Fig. 2. Surface details of *Calonympha chia* sp. nov. (a) Differential interference contrast light micrograph with glancing optical section of cell surface showing rod-shaped bacteria. (b, c) SEM of the posterior of the cell showing considerable diversity of adherent bacterial morphotypes. (b) At least three morphotypes of rod-shaped bacteria are scattered at an even spacing across the cell surface. The protruding axostylar bundle and three spirochaete-like bacterial cells can be seen. (c) The diversity of bacterial ectosymbionts includes coccoid forms. Four emergent flagella can be seen to the left. (d, e) Ultra-high magnification micrographs showing the fibrous appearance of the fixed glycocalyx with adherent bacteria. (e) Coccoid and longer rod-shaped ectosymbionts appear to be embedded within the glycocalyx. Bars, a, 30 µm; b, c, 5 µm; d, e, 1 µm.

symbiont community that does not include a member of the genus *Calonympha* (our observations; Yamin, 1979). Second, we characterized two additional sequences in our environmental PCR from a *Devescovina* sp. and an unidentified tritrichomonad that were nearly identical to sequences from Branke's surveys (GenBank accession nos

X97974 and Y08538, Fig. 3). Finally, we determined the molecular barcode of the host using the mitochondrial COII sequence and found the two termites' sequences were identical (this study; Desai *et al.*, 2009). Altogether, these lines of evidence demonstrate that the BAM strain is *N. castaneus* and that the Branke (1997) study and the current

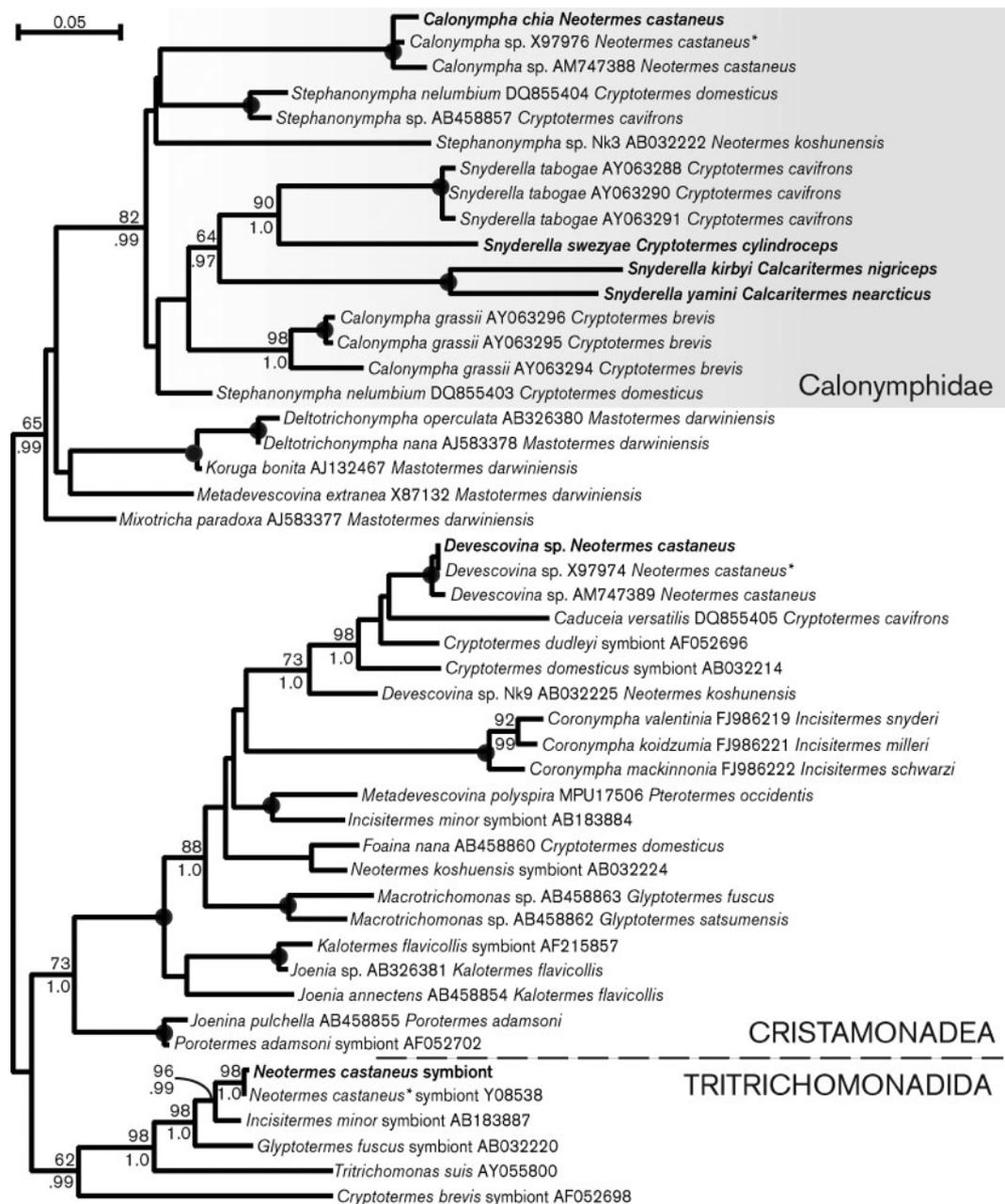


Fig. 3. ML phylogeny of SSU rRNA sequences from calonymphids, other cristamonads and close outgroup tritrichomonads. Numbers at nodes indicate ML bootstrap support as a percentage of the total in agreement out of 1000 replicates (above) and Bayesian posterior probability values (below). Filled circles indicate 100 % bootstrap support and Bayesian posterior probability of 1. Only bootstrap values >65 and posterior probabilities >0.95 are shown. New sequences determined in this study are indicated by bold type. Asterisks indicate sequences from *N. castaneus* from the Bundesanstalt für Materialforschung und -prüfung (BAM) erroneously attributed to *N. jouteli*, see text for details. Sequences AB032222 and AB032225, labelled '*Neotermes koshuensis* parasitoid symbiont clone' in GenBank, were determined by FISH to be species of the genera *Stephanonympha* and *Devescovina* (Noël *et al.*, 2007; Gerbod *et al.*, 2002). Bar, 0.05 substitutions per site.

study document the same calonymphid species. Previous transmission electron microscopy (TEM) of symbionts from the BAM strain showed akaryomastigonts at the apex of the *Calonympha* sp. found in that gut (G. Brugerolle, personal communication), further supporting our observations and

our conclusion that this novel species fits the morphological criteria for inclusion in the genus *Calonympha*.

The genus *Calonympha* has a somewhat turbulent history. A total of eight species names have been proposed since the

genus was erected in 1905, but five of these were synonymized with the type species, *Calonympha grassi*, as their host termites were identified as *Cryptotermes brevis*, and two more underwent later name changes (Foà, 1905; Calkins, 1936; Brown, 1941; Kirby & Margulis, 1994). *Calonympha grassi* is defined by the identity of its host, *Cryptotermes brevis*. Conversely, *Calonympha angusta* (Kirby 1994 ex. *Calonympha grandis* Brown 1941) is said to inhabit 16 termite species from five genera of termites from South and Central America (its type host is *Rugitermes panamae*), but it is morphologically indistinguishable from *Calonympha grassi*. In describing *Calonympha angusta*, Kirby noted that the continuum of variability observed in cell size, number, placement, size and shape of nuclei, and shape and size of the parabasal bodies, made it impossible to define the boundaries of populations, though he felt it likely that *Calonympha angusta* encompassed multiple species (Kirby & Margulis, 1994). The third species of the genus *Calonympha*, *Calonympha umbella* (Kirby 1994 ex. *Calonympha patella* Brown 1941) from *Neotermes tectonae*, is only known from Indonesia. It is distinguished by an irregular arrangement of karyomastigonts and a more gradual branching of axostyles from the central bundle to the individual mastigonts (Kirby & Margulis, 1994). The *Calonympha* species investigated here was isolated from *N. castaneus*, which is not the host of *Calonympha umbella*, *Calonympha grassi* or any of the hosts listed for *Calonympha angusta*. Furthermore, its SSU sequence was clearly distinct from that of *Calonympha grassi*, though no molecular data are yet available from representatives of *Calonympha angusta*. Accordingly, we propose a novel species, *Calonympha chia* sp. nov., because the flagella from karyo- and akaryomastigonts covering the apical half of the cell give it the appearance of a Chia Pet [an animal or human-shaped terracotta planter used to sprout chia (*Salvia hispanica*) in the USA; the chia sprouts and resembles the figure's fur or hair]. While it is clear that *Calonympha chia* sp. nov. and *Calonympha grassi* are distinct on the tree, it is impossible to conclude whether the genus *Calonympha* is monophyletic or not from this tree, since they are not separated by any strongly supported nodes (Fig. 3). If future molecular analyses confirm that the currently sampled *Calonympha* species are not monophyletic (see below), then the members of the *Calonympha grassi* clade will have priority and *Calonympha chia* sp. nov. will be transferred to a new genus. However, given the presence of *Calonympha*-defining features in the *N. castaneus* symbiont, the lack of support for their separation in phylogenetic trees and the poor sampling from this genus to date, we have conservatively retained this genus for the present.

***Snyderella kirbyi*, *Snyderella swezyae* and *Snyderella yamini* spp. nov.**

We observed multinucleate, multiflagellate protists in the hindguts of two termites from Colombia, *Calcaritermes nigriceps* and *Cryptotermes cylindroceps*, and one termite from Florida, *Calcaritermes nearcticus*. In all three flagellates, the nuclei are randomly arranged, free in the

cytoplasm and the anterior of the cell is covered with spiral rows of akaryomastigonts (Fig. 4). There is a distinct axostylar bundle that does not reach the cell's posterior. Several individuals of the symbiont from *Calcaritermes nigriceps* were seen in a stage of division (Fig. 4c, d). The internally scattered nuclei and lack of karyomastigonts place these flagellates in the genus *Snyderella* (Kirby, 1929).

The SSU sequences of all three symbionts were determined from isolated single cells or isolated pools of cells. In the case of the *Calcaritermes nigriceps* symbiont, ten pools of between one and 50 cells were isolated and the SSU was amplified in ten independent PCRs. A total of 15 clones were sequenced in both directions and differed at only 33 positions collectively. For the *Cryptotermes cylindroceps* symbiont, seven pools of 4–25 cells were isolated and a total of ten clones were sequenced from the seven independent PCRs. Again, the SSU sequences were found to differ at only 25 positions collectively. For the *Calcaritermes nearcticus* symbiont, 20 cells were isolated and five identical clones were sequenced from the single PCR product. In each case, a single clone sequence identical or nearly identical to the consensus was included in phylogenetic analyses and submitted to GenBank. In the phylogenetic analysis (Fig. 3), all three sequences were distinct from each other and from any known sequence, but they grouped closely with the existing *Snyderella tabogae* sequence, consistent with our identification. Furthermore, the *Snyderella* species isolated in this study from *Cryptotermes cylindroceps* branched with *Snyderella tabogae* from *Cryptotermes brevis*, while the two *Snyderella* species isolated from members of the genus *Calcaritermes* branched together, suggesting a certain amount of co-evolution between termites and their calonymphid symbionts.

Only four species of the genus *Snyderella* have been described previously. The type species, *Snyderella tabogae* (Kirby, 1929) was discovered in the hindgut of *Cryptotermes longicollis* from Taboga Island, Panama. *Snyderella bandeirantium* (de Mello, 1954b) from *Cryptotermes brevis* from an unspecified collection location in Brazil, differs from *Snyderella tabogae* by having only two basal bodies and two flagella per akaryomastigont and de Mello observed the nuclei to be placed somewhat more superficially. In *Snyderella ypiranga* (de Mello, 1954a) from *Rugitermes rugosus*, from the experimental station of Mount Alegre do Sul, Brazil, each axostylar filament branching off the central bundle leads to several akaryomastigonts rather than just one; there is no capitulum. Finally, *Snyderella froilanoi* from *Neotermes arthurimulleri* collected in Curitiba, Brazil (Kattar & de Oliveira, 1973), bears two flagella per akaryomastigont, each axostyle joins only one akaryomastigont with a well-developed capitulum and the parabasal bodies are elongated.

For our newly discovered symbionts, morphological and molecular data indicate that all three belong to the genus *Snyderella*. All three are from different host termite species collected in different locations to any of the other four

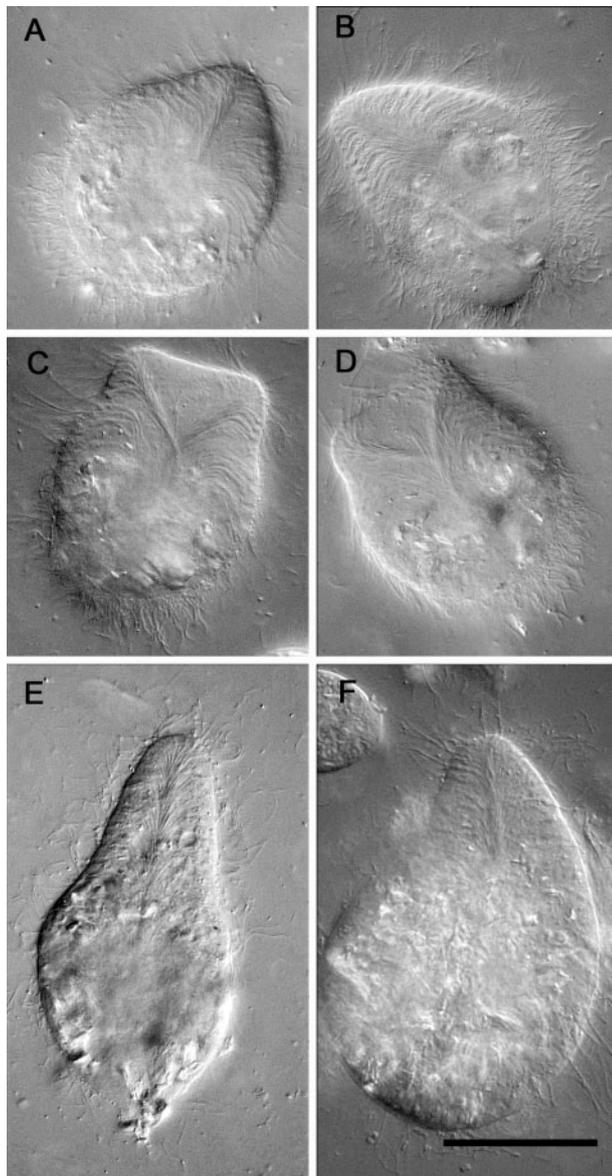


Fig. 4. Differential interference contrast light micrographs of species of the genus *Snyderella* described in this study. (a–d) *Snyderella kirbyi* sp. nov. Side views of cell showing surface akaryomastigonts and ingested wood particles. The central axostylar bundle appears helically twisted. Spirochaetes or other bristle-shaped bacteria adhere to the posterior of the cell. (c, d) Dividing cells. (e) *Snyderella swezyae* sp. nov. This cell is larger and more elongate than *S. kirbyi* sp. nov. (f) *Snyderella yamini* sp. nov., also a large elongate cell. The anterior portion of the axostylar bundle can be seen, though the posterior portion is obscured by wood particles that fill the expanded posterior of the cell. Bar, 50 μ m.

described species. The SSU sequences of the novel species are considerably divergent from each other and from that of *Snyderella tabogae*, though this sequence derives from a *Snyderella* sp. in *Cryptotermes cavifrons*, which is not the type host of *Snyderella tabogae* and therefore may itself represent a distinct species of the genus *Snyderella*. Furthermore, we

did not observe only two flagella per mastigont or axostylar filaments leading to multiple akaryomastigonts in either species, further ruling out their identity as *Snyderella bandeirantium*, *Snyderella arthurimulleri* or *Snyderella ypiranga*. The *Snyderella* species from *Cryptotermes cylindroceps* and *Calcaritermes nearcticus* were markedly larger and more elongate than the *Snyderella* sp. from *Calcaritermes nigriceps* (Fig. 4). Consequently, we propose *Snyderella kirbyi* sp. nov., from *Calcaritermes nigriceps*, *Snyderella swezyae* sp. nov., from *Cryptotermes cylindroceps* and *Snyderella yamini* sp. nov. from *Calcaritermes nearcticus*.

Phylogeny of the family Calonymphidae

In our molecular phylogeny of SSU rRNA sequences, as in others, the family Calonymphidae (here considered not to include the genus *Coronympha*) was monophyletic (Fig. 3). Previously published SSU and multigene phylogenies agree that this group is a part of the order Cristamonadida, along with the devescovinids, lophomonads and members of the genus *Coronympha*, and branches sister to a clade of members of the genera *Koruga* and *Deltotrichonympha* from the deepest-branching termite, *Mastotermes darwiniensis* (Gerbod *et al.*, 2002; Noël *et al.*, 2007; Noda *et al.*, 2009; Cepicka *et al.*, 2010). While the monophyly of the cristamonads in general and calonymphids specifically are both well established, relationships among and within calonymphid genera are less certain and in some cases the monophyly of genera is untested or questionable.

The genus *Stephanonympha* is the best studied in terms of molecular data (four sequences from three different termites) and number of species described (approx. 20). The two sequences from *Stephanonympha nelumbium* from *Cryptotermes domesticus*, its type host, group together with only a few polymorphisms. However, the other two species of this genus, from *Neotermes koshuensis* and *Cryptotermes cavifrons*, branch separately in our analyses and in previous analyses (Gerbod *et al.*, 2002; Noël *et al.*, 2007; Noda *et al.*, 2009; Cepicka *et al.*, 2010). This separation is not supported, so it remains possible that with deeper taxon sampling, the genus *Stephanonympha* may yet resolve itself as monophyletic (Lecointre *et al.*, 1993; Heath *et al.*, 2008), although there is no indication of this from current data. Moreover, the absence of akaryomastigonts is considered an ancestral state among calonymphids (Kirby, 1929), so the paraphyly of this state is perhaps unsurprising. Should this be borne out by additional data, the genus *Stephanonympha* would require a new, stricter definition, which will require more thorough study of the genus. In addition to the 17 recognized species for which no molecular data are available, many more species of the genus *Stephanonympha* probably inhabit the great number of kalotermitid species that have yet to be investigated, so this is clearly a genus where taxonomic changes are likely to be needed.

Molecular data from the genus *Snyderella* were previously available only from *Snyderella tabogae* from *Cryptotermes cavifrons* (the type species for the genus is *Snyderella tabogae* from the type host *Cryptotermes longicollis*), so the monophyly of this genus has been untested by molecular data, a concern given the lack of evidence for monophyly of the genera *Stephanonympha* and *Calonympha*. Here we describe three novel species of *Snyderella*: *Snyderella kirbyi* sp. nov. from *Calcaritermes nigriceps*, *Snyderella swezyae* sp. nov. from *Cryptotermes cylindroceps* and *Snyderella yamini* sp. nov. from *Calcaritermes nearcticus*, and provide SSU sequences for all three. All three sequences branch with the *Snyderella tabogae* sequence with 64 % bootstrap support and Bayesian posterior probability of 0.97 (Fig. 3), overall suggesting that the *Snyderella* species sampled here are monophyletic. The strong support for sisterhood of *Snyderella* species from the same host termite genus further suggests coevolution between *Snyderella* species and their kalotermitid hosts. In the converse situation to the genus *Stephanonympha*, the presence of only akaryomastigonts is considered to be a derived character, and therefore may be a good synapomorphy for the genus (Kirby, 1929).

Molecular data exist for two species of the genus *Calonympha*: *Calonympha grassi*, the type species from the type host *Cryptotermes brevis*, where some sequence diversity exists (Fig. 3), and a *Calonympha* sp. reported to be from *N. jouteli*, but which observations here and from other groups have shown is actually *N. castaneus*. The *Calonympha* sequences from *N. castaneus*, which we have here described as *Calonympha chia* sp. nov., form a distinct group branching distantly from those of *Calonympha grassi*, although once again this separation is not strongly supported (Fig. 3). The presence of both karyomastigonts and akaryomastigonts, which are defining characteristics of this genus, in both *Calonympha* phylogenotypes may be an indication that these characteristics have arisen independently from a *Stephanonympha*-like ancestor with only karyomastigonts, in keeping with Kirby's interpretation of the genus *Calonympha* as an intermediate stage in a phyletic progression beginning with all karyomastigonts in the genus *Stephanonympha* and culminating with complete nuclear dissociation in the genus *Snyderella* (Kirby, 1929).

Because molecular data are lacking for the majority of described calonymphid species, and many kalotermitid host species remain uninvestigated, it is clear that an understanding of the diversity and evolution of this group awaits further sampling. In particular, the five recognized genera for which no molecular data are available should be sampled to assess their validity, monophyly and placement within the group. The genus *Diplonympha* (Grassi, 1917) was reported to have two nuclei per karyomastigont, but doubts about this observation and the validity of this genus have been expressed in the literature (Kirby, 1947; Brugerolle & Lee, 2000). The genus *Metastephanonympha*

(de Mello & de Brito, 1929) was reported to carry only two flagella per karyomastigont, but may in fact be a synonym of the genus *Stephanonympha* (Kirby, 1947). The genus *Prosnyderella* is said to show the same axostylar branching pattern as the genus *Snyderella*, but has karyomastigonts interspersed with the akaryomastigonts (Dolan & Kirby, 2002). The genera *Criconympha* and *Gyronympha* were both described from *Rugitermes panamae* and are distinguished by an empty apex with no akaryomastigonts (*Criconympha*) and by a tightly ordered ring of karyomastigonts at the apex in a tuft (*Gyronympha*) (Dolan & Kirby, 2002). Ultimately the stability of genus-defining characters and the patterns of character evolution in the group, in particular whether the *Calonympha*-type morphology has arisen multiple times from a *Stephanonympha*-like ancestor, await molecular and morphological characterization of many more calonymphids.

TAXONOMIC SYNOPSIS

Calonympha chia Gile and Keeling sp. nov.

Type host. *Neotermes castaneus*.

Type locality. N 25.17608 W 80.36945, Dagny Johnson Park, Key Largo, FL, USA.

Host collection. University of Florida termite collection, accession number FL3191. Collector R. H. Scheffrahn. Collected 9 April 2008.

Diagnosis. Multinucleate flagellates from the hindgut of *N. castaneus*. Cells with apical spirals of mastigonts and nuclei associated with the hindmost ring or rings of mastigonts (karyomastigonts). Distinct from other species of the genus *Calonympha* by type host and locality and distinct SSU sequence.

Hapantotype. SEM stub deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada, under accession number MI-PR104.

Gene sequence. SSU rRNA GenBank accession number HQ215836.

Etymology. The many clustered flagella covering the anterior half of the cell and the usual absence of spirochaetes on the cell's posterior, give this species the appearance of a Chia Pet.

Snyderella kirbyi Gile and Keeling, sp. nov.

Type host. *Calcaritermes nigriceps*.

Type locality. N 11.1256 W 74.11972, above Minca, Colombia.

Host collection. University of Florida termite collection, accession number CO548. Collector R. H. Scheffrahn. Collected 5 June 2009.

Diagnosis. Multinucleate flagellates from the hindgut of *Calcaritermes nigriceps*. Cells with characteristics of the genus *Snyderella*, including spirals of akaryomastigonts covering the anterior half to two-thirds of the cell, nuclei randomly dispersed within the endoplasm and axostyles gathered in a bundle in the central axis of the cell. Distinct from other species of the genus *Snyderella* by type host and distinct SSU sequence.

Hapantotype. Unstained prepared slide deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada, under accession number MI-PR105.

Gene sequence. SSU rRNA GenBank accession number HQ215837.

Etymology. This species is named after the distinguished protistologist, Harold Kirby, who made significant contributions to the study of parabasalid symbionts of termites, including the establishment of the genus *Snyderella* in 1929.

***Snyderella swezyae* Gile and Keeling, sp. nov.**

Type host. *Cryptotermes cylindroceps*.

Type locality. N 9.86610 W 75.15836, NW San Jacinto, Colombia.

Host collection. University of Florida termite collection, accession number CO373. Collector R. H. Scheffrahn. Collected 3 June 2009.

Diagnosis. Multinucleate flagellates from the hindgut of *Cryptotermes cylindroceps*. Cells with characteristics of the genus *Snyderella*, including spirals of akaryomastigonts covering the anterior half to two-thirds of the cell, nuclei randomly dispersed within the endoplasm, and axostyles gathered in a bundle in the central axis of the cell. Distinct from other species of the genus *Snyderella* by type host and distinct SSU sequence.

Hapantotype. Unstained prepared slide deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada, under accession number MI-PR106.

Gene sequence. SSU rRNA GenBank accession number HQ215838.

Etymology. This species is named after the distinguished protistologist, Olive Swezy, whose contributions to the study of flagellate symbionts of termite guts included the recognition that trichomonads and hypermastigotes belong to the same clade.

***Snyderella yamini* Gile and Keeling, sp. nov.**

Type host. *Calcaritermes nearcticus*.

Type locality. N 27.99531 W 82.11748, Plant City, FL, USA.

Host collection. University of Florida termite collection, accession number FL3527. Collector R. H. Scheffrahn. Collected 26 August 2010.

Diagnosis. Multinucleate flagellates from the hindgut of *Calcaritermes nearcticus*. Cells with characteristics of the genus *Snyderella*, including spirals of akaryomastigonts covering the anterior half to two-thirds of the cell, nuclei randomly dispersed within the endoplasm and axostyles gathered in a bundle in the central axis of the cell. Distinct from other species of the genus *Snyderella* by type host and distinct SSU sequence.

Hapantotype. Unstained prepared slide deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada, under accession number MI-PR107.

Gene sequence. SSU rRNA GenBank accession number HQ593144.

Etymology. This species is named after the protistologist, Michael Yamin, whose compiled tables of parabasalid species and their hosts continue to be of great reference value for research into the diversity and evolution of symbiotic parabasalids.

ACKNOWLEDGEMENTS

We would like to thank Harris Falconer for technical assistance. All termite specimens from Colombia were collected under Permiso No. 21 del 26 de Noviembre de 2009, Ministerio de Ambiente, Vivienda y Desarrollo Territorial, Dirección de Licencias, Permisos y Trámites Ambientales, Expediente: IDB0053, Elabor: Yeison F. Rodriguez V. This work was supported by the Natural Science and Engineering Research Council of Canada (NSERC) (grant 227301) to P. J. K., and an NSERC postgraduate doctoral fellowship to G. H. G. P. J. K. is a Fellow of the Canadian Institute for Advanced Research and a Senior Scholar of the Michael Smith Foundation for Health Research.

REFERENCES

- Branke, J. T. (1997). *Phylogenetische und morphologische Charakterisierung pro- und eukaryontischer Mikroorganismen aus dem Mäuse- und Termitendarm*. PhD thesis, Universität Ulm, Germany.
- Brown, H. E. (1941). *Flagellates of the genus Calonympha from termites*. PhD thesis, University of Georgia, GA, USA.
- Brugerolle, G. (1991). Flagellar and cytoskeletal systems in amitochondrial flagellates: Archamoeba, Metamonada and Parabasala. *Protoplasma* **164**, 70–90.
- Brugerolle, G. & Lee, J. J. (2000). Phylum Parabasalia. In *An Illustrated Guide to the Protozoa*, pp. 1196–1250. Edited by J. J. Lee, G. Leedale & P. Bradbury. Lawrence, KS: Allen Press Inc.

- Calkins, G. N. (1936).** Some polymastigote and hypermastigote flagellates from Puerto Rican termites. *PR J Public Health Trop Med* **12**, 169–187.
- Cepicka, I., Hampl, V. & Kulda, J. (2010).** Critical taxonomic revision of Parabasalids with description of one new genus and three new species. *Protist* **161**, 400–433.
- Deane, J. A., Hill, D. R. A., Brett, S. J. & McFadden, G. I. (1998).** *Hanusia phi* gen. et. sp. nov. (Cryptophyceae): Characterization of ‘*Cryptomonas* sp. Φ ’. *Eur J Phycol* **33**, 149–154.
- de Mello, I. F. (1954a).** Contribution à l’étude des microparasites de termites brésiliens. II. Un nouveau Calonymphide, “*Snyderella ypiranga*” sp. n., de “*Rugitermes rugosus*” (Hagen 1858). *Rev Bras Biol* **14**, 71–78 (in French).
- de Mello, I. F. (1954b).** Sur un nouvelle espèce de Calonymphide du termite brésilien *Cryptotermes* sp. recolté dans la ville de Campinas. *An Inst Med Trop (Lisb)* **11**, 339–360 (in French).
- de Mello, I. F. & de Brito, J. (1929).** Sur trois espèces de *Devescovina* parasites de l’intestin d’un termite récolté à Damaun (Inde Portugaise) et de la complexité de leur appareil kinétoplastique. *Arq Esc Méd Cirurg Nova Goa ser. A* **5**, 693–706 (in French).
- Desai, M. S., Strassert, J. F. H., Meuser, K., Hertel, H., Ikeda-Ohtsubo, W., Radek, R. & Brune, A. (2009).** Strict cospeciation of devescovinid flagellates and Bacteroidales ectosymbionts in the gut of dry-wood termites (*Kalotermitidae*). *Environ Microbiol*.
- Dolan, M. F. & Kirby, H. (2002).** *Gyronympha*, *Prosnnyderella* and *Criconympha*, three new genera of calonymphids (Parabasalia: Trichomonadida) from wood-eating termites. *Eur J Protistol* **38**, 73–81.
- Foà, A. (1905).** Due nuovi flagellati parassiti. *Rend Reale Accad Lincei* **14**, 542–546.
- Gerbod, D., Noël, C., Dolan, M. F., Edgcomb, V. P., Kitade, O., Noda, S., Dufernez, F., Ohkuma, M., Kudo, T. & other authors (2002).** Molecular phylogeny of parabasalids inferred from small subunit rRNA sequences, with emphasis on the Devescovinidae and Calonymphidae (Trichomonadea). *Mol Phylogenet Evol* **25**, 545–556.
- Grassi, B. (1917).** Flagellati viventi nei termiti. *Mem Reale Accad Lincei* **12**, 331–394.
- Grassi, B. & Foà, A. (1911).** Intorno ai protozoi dei termitidi. *Rend Reale Accad Lincei* **20**, 725–741.
- Harper, J. T., Gile, G. H., James, E. R., Carpenter, K. J. & Keeling, P. J. (2009).** The inadequacy of morphology for species and genus delineation in microbial eukaryotes: an example from the parabasalid termite symbiont *coronympha*. *PLoS ONE* **4**, e6577.
- Heath, T. A., Hedtke, S. M. & Hillis, D. M. (2008).** Taxon sampling and the accuracy of phylogenetic analyses. *J Syst Evol* **46**, 239–257.
- Hongoh, Y., Sato, T., Noda, S., Ui, S., Kudo, T. & Ohkuma, M. (2007).** *Candidatus Symbiothrix dinenymphae*: bristle-like Bacteroidales ectosymbionts of termite gut protists. *Environ Microbiol* **9**, 2631–2635.
- Ikeda-Ohtsubo, W., Desai, M., Stingl, U. & Brune, A. (2007).** Phylogenetic diversity of ‘Endomicrobia’ and their specific affiliation with termite gut flagellates. *Microbiology* **153**, 3458–3465.
- Janicki, C. (1911).** Zur Kenntnis des Parabasalapparat bei parasitischen Flagellaten. *Biol Zentbl* **31**, 321–330 (in German).
- Kambhampati, S. & Smith, P. T. (1995).** PCR primers for the amplification of four insect mitochondrial gene fragments. *Insect Mol Biol* **4**, 233–236.
- Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002).** MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059–3066.
- Kattar, M. R. & de Oliveira, G. M. F. (1973).** Duas novas espécies de protozoários flagelados simbioses de térmita. *Acta Biol Paranense* **2**, 113–119 (in Portuguese).
- Keeling, P. J. (2002).** Molecular phylogenetic position of *Trichomitopsis termopsidis* (Parabasalia) and evidence for the Trichomito-psiinae. *Eur J Protistol* **38**, 279–286.
- Kirby, H. (1929).** *Snyderella* and *Coronympha* two new genera of multinucleate flagellates from termites. *Univ Calif Publ Zool* **31**, 417–432.
- Kirby, H. (1939).** Two new flagellates from termites in the genera *Coronympha* Kirby, and *Metacoronympha* Kirby, new genus. *Proc Calif Acad Sci* **22**, 207–220.
- Kirby, H. (1947).** Flagellate and host relationships of trichomonad flagellates. *J Parasitol* **33**, 214–228.
- Kirby, H. & Margulis, L. (1994).** Harold Kirby’s symbionts of termites: Karyomastigot reproduction and calonymphid taxonomy. *Symbiosis* **16**, 7–63.
- Lecointre, G., Philippe, H., V&acaron; Lé, H. L. & Le Guyader, H. (1993).** Species sampling has a major impact on phylogenetic inference. *Mol Phylogenet Evol* **2**, 205–224.
- Maddison, D. R. & Maddison, W. P. (2003).** *MacClade 4: Analysis of phylogeny and character evolution*, version 4.08. Sunderland, MA: Sinauer Associates, Inc.
- Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. (1988).** The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71**, 491–499.
- Noda, S., Mantini, C., Bordereau, C., Kitade, O., Dolan, M. F., Viscogliosi, E. & Ohkuma, M. (2009).** Molecular phylogeny of parabasalids with emphasis on the order Cristamonadida and its complex morphological evolution. *Mol Phylogenet Evol* **52**, 217–224.
- Noél, C., Noda, S., Mantini, C., Dolan, M. F., Moriya, S., Delgado-Viscogliosi, P., Kudo, T., Capron, M., Pierce, R. J. & other authors (2007).** Molecular phylogenetic position of the genera *Stephanonympha* and *Caduceia* (Parabasalia) inferred from nuclear small subunit rRNA gene sequences. *J Eukaryot Microbiol* **54**, 93–99.
- Ohkuma, M., Iida, T., Ohtoko, K., Yuzawa, H., Noda, S., Viscogliosi, E. & Kudo, T. (2005).** Molecular phylogeny of parabasalids inferred from small subunit rRNA sequences, with emphasis on the Hypermastigea. *Mol Phylogenet Evol* **35**, 646–655.
- Posada, D. & Crandall, K. A. (1998).** MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Ronquist, F. & Huelsenbeck, J. P. (2003).** MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994).** Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am* **87**, 651–701.
- Stamatakis, A. (2006).** RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690.
- Swofford, D. L. (2002).** PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, MA: Sinauer Associates.
- Trager, W. (1934).** The cultivation of a cellulose-digesting flagellate, *Trichomonas termopsidis*, and of certain other termite protozoa. *Biol Bull* **66**, 182–190.
- Wirth, T., Le Guellec, R. & Veuille, M. (1999).** Directional substitution and evolution of nucleotide content in the cytochrome oxidase II

gene in earwigs (dermapteran insects). *Mol Biol Evol* **16**, 1645–1653.

Yamin, M. A. (1979). Flagellates of the orders Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà

reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach *Cryptocercus* (Dictyoptera: Cryptocercidae). *Sociobiology* **4**, 5–119.