A Nondichotomous Key to Protist Species Identification of *Reticulitermes* (Isoptera: Rhinotermitidae)

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ABSTRACT A key was developed using morphological and behavioral characters to identify nine genera and 13 species of protists found in the hindgut of three *Reticulitermes* species— *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), and *Reticulitermes hageni* Banks—by using the online IDnature guides by Discover Life. There are seven characters and 13 taxa, each attached to species descriptions, digital stills, or movies to aid in protist species identification. We chose characters for protist species identification that were easy to observe with live samples and a light microscope at 400× magnification. All 11 protists from *R. flavipes* and nine each in *R. virginicus* and *R. hageni* were recognized using original and revised species descriptions. This was the first report of the protist genera *Trichomitus* from both *R. virginicus* and *R. hageni*.

KEY WORDS symbiotic protists, termite identification, anaerobic protists identification, Parabasalia, Oxymonadida

The anaerobic symbiotic protist orders found in the hindgut of lower termites (Isoptera) include Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà (Yamin 1979). None of these protist species are found outside of the insect host (Kirby 1941, Margulis et al. 1986), and their identification has relied on two established techniques, high-definition microscopy of fixed cells and light microscopy of living cells (Inoue et al. 2000). Although molecular techniques can identify protist species, verification still requires correct morphological identification (Kudo et al. 1998; Ohkuma et al. 1999, 2000). Examining fixed cells by using high-definition microscopy is time-consuming and often requires a specialist to identify distinguishing characteristics. In contrast, observing live cells simplifies species identification and study of the protist community (Kirby 1932, Lewis and Forschler 2004a).

Three *Reticulitermes* Holmgren (Rhinotermitidae) termite species have been described in the southeastern United States: *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), and *Reticulitermes hageni* Banks (Weesner 1970, Nutting 1990). Eleven protists are recognized from *R. flavipes*, eight in *R. virginicus*, and eight in *R. hageni* (Yamin 1979, Lewis and Forschler 2004b). It was proposed, although not widely accepted, that the termite protist community can substitute for termite species identification (Brown 1930a, Kirby 1937, Dropkin 1944, Cook 1996, Lewis and Forschler 2004b). The presence of *Dinenympha gracilis* Leidy (1877) distinguishes *R. flavipes* (workers have ≈57 ± 11%), because it is not found in *R. virginicus* and *R. hageni* (Lewis and Forschler 2004b). *Trichonympha agilis* Leidy (1877) is ≈19 ± 5% of the protist population in *R. virginicus* compared with 4 ± 3% in *R. flavipes* and 7 ± 5% in *R. hageni* (Lewis and Forschler 2004b). *Dinenympha fimbriata* Kirby (1924) is ≈27 ± 9% of the protist population in *R. hageni* compared with 11 ± 7% in *R. flavipes* and 2 ± 2% in *R. virginicus* (Lewis and Forschler 2004b).

There are generic keys to termite protists (Calkins 1926, Lee et al. 1985), but they do not provide species-level determinations. Here, we describe an approach to identify termite hindgut protists from *R. flavipes*, *R. virginicus*, and *R. hageni* by using a nondichotomous key with morphological and behavioral characters easily observed with a light microscope by using the online IDnature guides by Discover Life (Lewis and Forschler 2006) and a revision to and consolidation of Koidzumi (1921) and Kudo (1966).

Materials and Methods

We referred to all original and revised protist species descriptions (Leidy 1877, 1881; Grassi 1879, 1892, 1917; Koidzumi 1916, 1917; Dubosq and Grassé 1924, 1928; Kirby 1924, Powell 1928, Brown 1930a,b, 1931; Boykin et al. 1986; Lewis and Forschler 2004b) following the terminology of Koidzumi (1921) and Kudo (1966) for distinguishing characters (Figs. 1–3; *Appendix* 1). These characters include termite host, protist cell size and shape, number and placement of flagella, axostyle, and indicator protist species used in termite identification.

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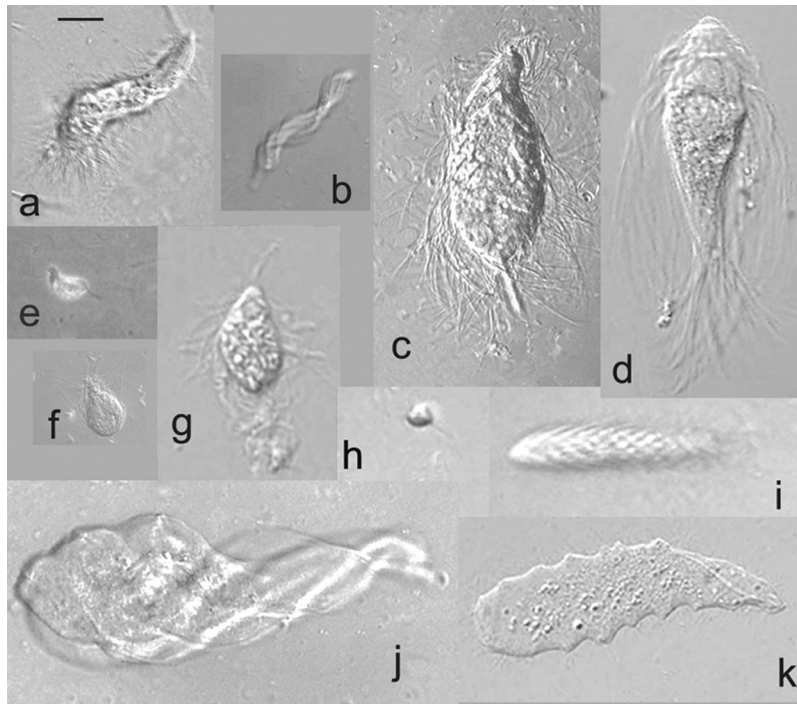

Fig. 1. Protist species in *R. flavipes*. (a) *Dinenympha fimbriata* Kirby 1924. (b) *Dinenympha gracilis* Leidy 1877. (c) *Spirotrichonympha flagellata* (Grassi 1892). (d) *Trichonympha agilis* Leidy 1877. (e) *Trichomitus trypanoides* (Dubosq and Grassé 1924). (f) *Microjoenia fallax* (Dubosq and Grassé 1928). (g) *Spironympha kofoidi* Koidzumi 1917. (h) *Monocercomonas* sp. Grassi 1879. (i) *Holomastigotes elongatum* Grassi 1892. (j) *Pyrsonympha vertens* Leidy 1877. (k) *Pyrsonympha major* Powell 1928. Scale bar = 10 μm (photos taken using a Leica microscope at 400× magnification and images acquired using an AxioCam digital camera).

Termites were collected from different established field sites and laboratory colonies from Georgia, USA. Collections were made in Clarke, Lamar, McIntosh, Spalding, and Union counties as presented by Lewis and Forschler (2004b). Termite species were identified using dichotomous keys to both the soldier and alate castes (Scheffrahn and Su 1994). Termites collected from field sites were sampled for protists within 72 h. Laboratory cultures were sampled at various times after collection and maintained as described previously (Grube and Forschler 2004). Voucher specimens from each termite collection were preserved in 100% ethanol and deposited at the Household and Structural Entomology Laboratory at the University of Georgia (Athens, GA).

The termite hindgut was removed using forceps to pull the last two abdominal segments away from the rest of the termite and placed in a saline solution as described by Lewis and Forschler (2004a). The worker caste was chosen because this caste has all representatives of the protist community for each termite species (Dropkin 1944, Mannesmann 1972, Lewis and Forschler 2004b). Protists were observed with a Leica compound microscope at 400× magnification. Digital stills were acquired with an AxioCam digital camera, with all scale bars equal to 10 μm. All movies were taken at 400× magnification with a Nikon CoolPix 995 digital camera equipped with 4× digital zoom.

Results

We identified nine genera and 13 species of protists found in the hindgut of *R. flavipes*, *R. virginicus*, and *R. hageni* (Figs. 1–3; *Appendix* 1; Lewis and Forschler 2006). All 11 protist species previously described from *R. flavipes* were observed (Yamin 1979, Lewis and Forschler 2004b). These species included *Dinenympha fimbriata*, *D. gracilis*, *Holomastigotes elongatum* Grassi (1892), *Microjoenia fallax* (Dubosq and Grassé 1928), *Monocercomonas* sp. Grassi (1879), *Pyrsonympha major* Powell (1928), *P. vertens* Leidy (1877), *Spironympha kofoidi* Koidzumi (1917), *Spirotrichonympha flagellata* (Grassi 1892), *Trichomitus trypanoides* (Dubosq and Grassé 1924), and *Trichonympha agilis* (Fig. 1; Lewis and Forschler 2006). Other than being in a separate termite species, we could not distinguish *P. major* from *P. minor*.

We identified nine protist species from *R. virginicus: D. fimbriata*, *H. elongatum*, *M. fallax*, *Monocercomonas* sp., *P. minor* Powell (1928), *S. kofoidi*, *S. flagellata*, *T. trypanoides*, and *T. agilis* (Fig. 2; Lewis and Forschler 2006). Nine protists were identified from *R. hageni*, including: *D. fimbriata*, *H. elongatum*, *Microjoenia*

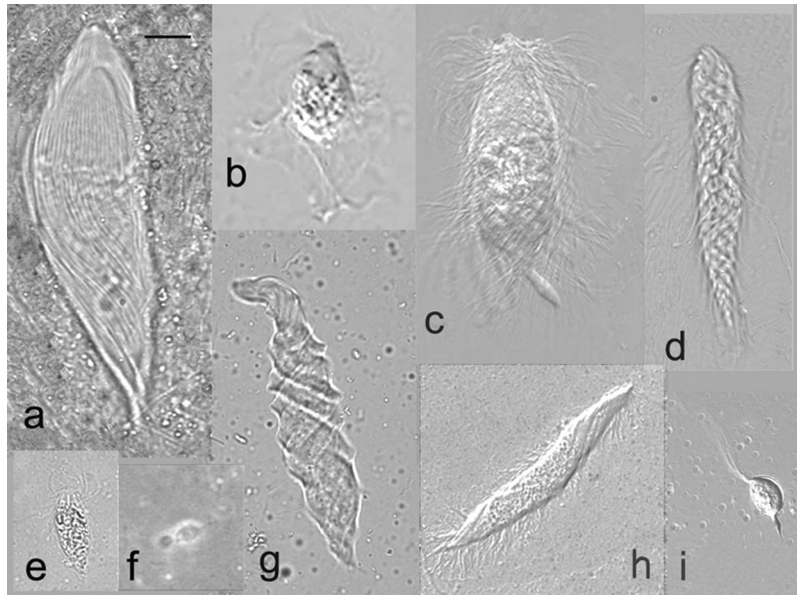

Fig. 2. Protist species in *R. virginicus*. (a) *Trichonympha agilis* Leidy 1877. (b) *Spironympha kofoidi* Koidzumi 1917. (c) *Spirotrichonympha flagellata* (Grassi 1892). (d) *Holomastigotes elongatum* Grassi 1892. (e) *Microjoenia fallax* (Dubosq and Grassé 1928). (f) *Trichomitus trypanoides* (Dubosq and Grassé 1924). (g) *Pyrsonympha minor* Powell 1928. (h) *Dinenympha fimbriata* Kirby 1924. (i) *Monocercomonas sp.* Grassi 1879. Scale bar = 10 μm (photos taken using a Leica microscope at 400× magnification and images acquired using an AxioCam digital camera).

pyriformis Brown (1930), *Monocercomonas* sp., *P. minor*, *S. kofoidi*, *S. flagellata*, *T. trypanoides*, and *T. agilis* (Fig. 3; Lewis and Forschler 2006). This is the first report of *T. trypanoides* in *R. hageni* and *R. virginicus*.

We were able to identify termite protists from *R. flavipes*, *R. virginicus*, and *R. hageni* by using the following characters: termite host, cell size and shape, number and placement of flagella, and axostyle (Figs. 1–3; *Appendix* 1; Lewis and Forschler 2006).

Discussion

We were able to distinguish between morphologically similar protist species from *R. flavipes*, *R. virginicus*, and *R. hageni* by using key characteristics and movement patterns with the online IDnature guides by Discover Life (Lewis and Forschler 2006). The key characters include presence of axostyle and placement of flagella, as easily seen in live specimens.

Termite protist identification has relied on original species descriptions since the 1800s (Leidy 1877). Although there have been several revisions (Grassi 1879, 1892, 1917; Koidzumi 1916, 1917; Dubosq and Grassé 1924; Kirby 1924; Powell 1928; Brown 1930a,b; Brown 1931; Boykin et al. 1986), there is not a single, comprehensive morphological key.

Protist species identification is based on cell morphology (Kirby 1937), including cell size and shape, number of flagella, site of flagellar attachment, and the presence of an axostyle (Koidzumi 1921, Honigberg 1963, Kudo 1966). Preserving protist cells is difficult because different stains are needed for some distinctive organelles; thus, several slide preparations are often needed, and finding the same protist species on each slide is time-consuming. Preparing a fresh slide mount allows for correct species identification by observing protist movement patterns, which simplifies distinguishing similar species, especially hard-to-observe organelles, such as axostyle, flagellum number, and placement of flagella.

This is the first termite protist key to consolidate the literature and provide descriptions, digital stills, and movies to aide in protist identification (Figs. 1–3; *Appendix* 1; Lewis and Forschler 2006). We hope that this key will stimulate further study of termite protist speciation. Termite hindgut protists were first described >100 yr ago. Many of the described species were revised because of variable cell morphology that lead to designations as separate species (Brown 1930b, 1931), only to be collapsed into original species (Dubosq and Grassé 1928, Brown 1930a, Cook 1996). In addition, some taxa, such as *Microjoenia* spp. and *Pyrsonympha* spp., were designated simply because they were recovered from different host termites (Kirby 1937). Further study with both morphological and molecular techniques should verify species level distinctions (Lewis and Forschler 2004b).

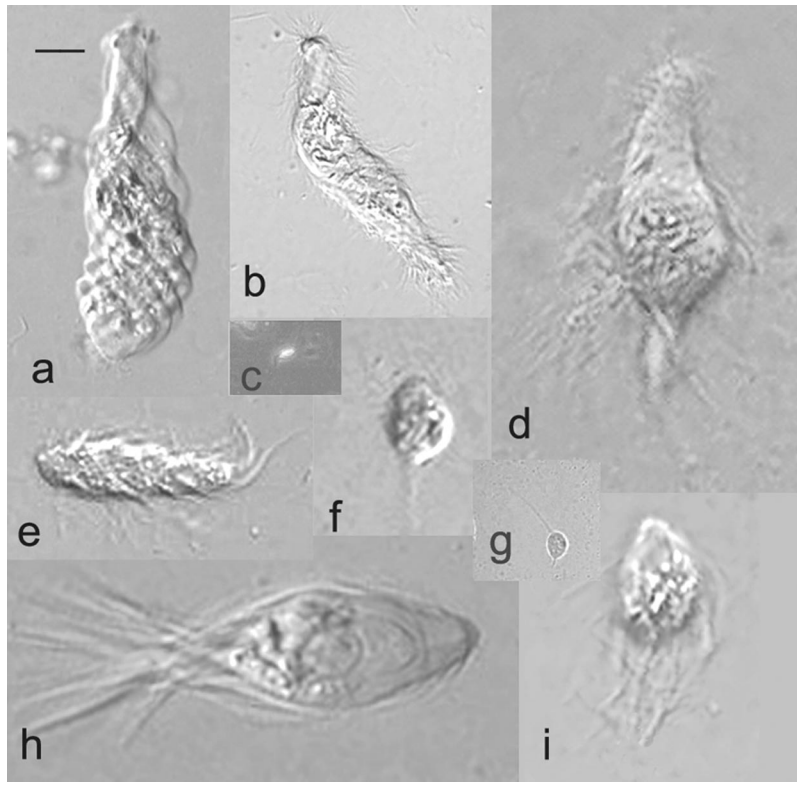

Fig. 3. Protist species in *R. hageni*. (a) *Pyrsonympha minor* Powell 1928. (b) *Dinenympha fimbriata* Kirby 1924. (c) *Trichomitus trypanoides* (Dubosq and Grassé 1924). (d) *Spirotrichonympha flagellata* (Grassi 1892). (e) *Holomastigotes elongatum* Grassi 1892. (f) *Microjoenia pyriformis* Brown 1930b. (g) *Monocercomonas* sp. Grassi 1879. (h) *Trichonympha agilis* Leidy 1877. (i) *Spironympha kofoidi* Koidzumi 1917. Scale bar = 10 μm (photos taken using a Leica microscope at 400× magnification and images acquired using an AxioCam digital camera).

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Appendix 1

Host Species Protist Community

Reticulitermes flavipes (Fig. 1).
Reticulitermes virginicus (Fig. 2).
Reticulitermes hageni (Fig. 3).

Cell Size

4.5–30 μm (Fig. 1e and f, Fig. 2e and f, Fig. 3c and f).
20–80 μm (Fig. 1a, b, g, and i; Fig. 2b, d, and h; Fig. 3b, e, and i).
65–150 μm (Fig. 1c, d, and k; Fig. 2a, c, and g; Fig. 3a, d, and h).
100–275 μm (Fig. 1j).

Cell Shape

Anterior rostrum (Fig. 1c and d; Fig. 2a and c; Fig. 3d and h).
Fusiform (Fig. 1c, d, and i; Fig. 2a, c, and d; Fig. 3d, e, and h).
Lanceolate (Fig. 1a, b, d, j, and k; Fig. 2a, g, and h; Fig. 3a, b, and h).

Oval (Fig. 1e, f, and h; Fig. 2e, f, and i; Fig. 3c, f, and g).
Pyriform (Fig. 1a, c, g, j, and k; Fig. 2b, c, g, and h; Fig. 3a, b, d, and i).

Number of Flagella

8 or Less (Fig. 1a, b, h, i, k, and e; Fig. 2f–i; Fig. 3a–c and g).
More than eight (Fig. 1c, d, f, g, and i; Fig. 2b–e; Fig. 3d–f, h, and i).

Placement of Flagella

Anterior region only (Fig. 1d, f, and h; Fig. 2a, e, and i; Fig. 3f and g).
Anterior and posterior region, including recurrent flagella (Fig. 1h, Fig. 2i, Fig. 3g).
Flagellar cords (Fig. 1a, b, and j; Fig. 2g and h; Fig. 3a and b).
Posterior region only (Fig. 1a, b, j, and k; Fig. 2g and h; Fig. 3a and b).
Spiraling flagellar rows extend almost to the posterior end (Fig. 1c and g; Fig. 2b and c; Fig. 3d and i).
Spiraling flagellar rows extend to posterior end (Fig. 1i, Fig. 2d, Fig. 3e).
Undulating membrane (Fig. 1e, Fig. 2f, Fig. 3c).

Axostyle

Articulated (Fig. 1c, e, f, and h; Fig. 2c, e, f, and i; Fig. 3c, d, f, and g).
Extend from posterior end (Fig. 1a, b, j, k, and e; Fig. 2f–h; Fig. 3a–c).
Nonarticulated (Fig. 1c, d, and f–i; Fig. 2a–e, and i; Fig. 3d–j).

Indicator Protist Species Used in Termite Identification

Reticulitermes flavipes (Fig. 1b).
Reticulitermes virginicus (Fig. 2a).
Reticulitermes hageni (Fig. 3b).

Original and revised protist species descriptions (Leidy 1877, 1881; Grassi 1879, 1892, 1917; Koidzumi 1916, 1917; Dubosq and Grassé 1924, 1928; Kirby 1924, Powell 1928, Brown 1930a,b, 1931; Boykin et al. 1986; Lewis and Forschler 2004b) following the terminology of Koidzumi (1921) and Kudo (1966)