

# Molecular characterization and phylogeny of four new species of the genus *Trichonympha* (Parabasalia, Trichonymphea) from lower termite hindguts

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## Abstract

Members of the genus *Trichonympha* are among the most well-known, recognizable and widely distributed parabasalians symbionts of lower termites and the wood-eating cockroach species of the genus *Cryptocercus*. Nevertheless, the species diversity of this genus is largely unknown. Molecular data have shown that the superficial morphological similarities traditionally used to identify species are inadequate, and have challenged the view that the same species of the genus *Trichonympha* can occur in many different host species. Ambiguities in the literature, uncertainty in identification of both symbiont and host, and incomplete samplings are limiting our understanding of the systematics, ecology and evolution of this taxon. Here we describe four closely related novel species of the genus *Trichonympha* collected from South American and Australian lower termites: *Trichonympha hueyi* sp. nov. from *Rugitermes laticollis*, *Trichonympha deweyi* sp. nov. from *Glyptotermes brevicornis*, *Trichonympha louiei* sp. nov. from *Calcaritermes temnocephalus* and *Trichonympha webbyae* sp. nov. from *Rugitermes bicolor*. We provide molecular barcodes to identify both the symbionts and their hosts, and infer the phylogeny of the genus *Trichonympha* based on small subunit rRNA gene sequences. The analysis confirms the considerable divergence of symbionts of members of the genus *Cryptocercus*, and shows that the two clades of the genus *Trichonympha* harboured by termites reflect only in part the phylogeny of their hosts.

The genus *Trichonympha* includes large and morphologically complex eukaryotic flagellates found exclusively in the hindgut of some wood-eating insects [1–3]. The genus *Trichonympha* is a member of Parabasalia, a divergent clade of protists in the supergroup Excavata [4, 5]. It was previously classified among the hypermastigids, a non-monophyletic group including complex and ‘derived’ parabasalid taxa with a single nucleus and many flagella [6, 7]. Hypermastigids are now considered polyphyletic, and in the most recent classification, the genus *Trichonympha* is the type genus of the class Trichonymphea [4]. Members of this class are characterized by a bilaterally or tetradially symmetrical rostrum and hundreds or thousands of flagella arranged in parallel whorls of kinetosomes that are inherited by daughter cells during division [4]. Species of the genus

*Trichonympha* are easily identified by their relatively short rostral flagella, and constitute a common and eye-catching component of lignocellulose-digesting communities harboured by lower termites and the wood-eating cockroach species of the genus *Cryptocercus* [3, 6]. Despite being known for almost 150 years (*Trichonympha agilis* was described by Leidy in 1877 [8]), the diversity of the genus *Trichonympha* at the species level remains largely unexamined due to the paucity and unreliability of morphological characters. Historically, taxonomists have tended to assume that the same species of the genus *Trichonympha* could be harboured by many unrelated host species, but this assumption has not been supported by molecular data. Different termite species seem to harbour distinct symbiotic lineages, and several species of the genus *Trichonympha* have been

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**Abbreviations:** mtLSU, mitochondrial large subunit; SSU, small subunit.

The GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper are: MF062147–MF062150 (mtLSU rRNA gene of *Rugitermes laticollis*, *Glyptotermes brevicornis*, *Calcaritermes temnocephalus* and *Rugitermes bicolor*), MF062151–MF062154 (SSU rRNA gene of *Trichonympha hueyi*, *Trichonympha deweyi*, *Trichonympha louiei* and *Trichonympha webbyae*).

Supplementary data is available with the online Supplementary Material.

found even within the same host species [3, 9]. Specimens of members of the genus *Trichonympha* with high genetic similarity are consistently found only in one, or a few very closely related, termite species (e.g. *Zootermopsis angusticollis* and *Zootermopsis nevadensis* [10]).

Here, we describe four novel species of the genus *Trichonympha* from South American and Australian drywood termites (Kalotermitidae) with little or no previous record of flagellate diversity, and provide a molecular phylogeny of the genus based on small subunit (SSU) rRNA gene sequences.

*Rugitermes laticollis* was collected from the dead wood of a live tree in La Carolina park in Quito (Ecuador), close to the equator and at 2850 m above sea level, making this one of the highest elevations where termites have been sampled [11]. *Rugitermes bicolor* and *Calcaritermes temnocephalus* were collected in Peru, 15 km north of Tingo Maria and in the Campoverde District, respectively. Specimens for these three populations are deposited at the University of Florida termite collection. *Glyptotermes brevicornis* was collected at Mount Glorious in Australia, and inspected for symbionts with a portable microscope in the field.

All termite hosts were identified by morphological criteria and by DNA barcoding using the mitochondrial large subunit (mtLSU) rRNA gene, as previously described [12]. The most similar sequence to the barcodes of *R. laticollis* and *R. bicolor* belongs to *Rugitermes* sp. A TB-2014 (accession number: KP026284 [13]), with which they share 91.2 and 90.7 % similarity, respectively. Although the mtLSU rRNA gene has been the most common barcode marker for termites to date, there are currently no other available data for this genus. The similarity (non-corrected p-distance) between the two *Rugitermes* sequences obtained here is 90.2 %, also consistent with their heterospecific assignment. The barcode sequence of *C. temnocephalus* is 98.4 % identical to that of *C. temnocephalus* voucher BYU IGC IS19 (accession number: EU253743 [14]), confirming its morphological identification. There is no available sequence for *G. brevicornis*, and the barcode sequence most similar to that of the specimens reported here belongs to *Glyptotermes satsumensis*, distributed in Taiwan and China (accession number: KP026257 [13]). The low molecular similarity (90.9 %) confirmed that they are not the same species.

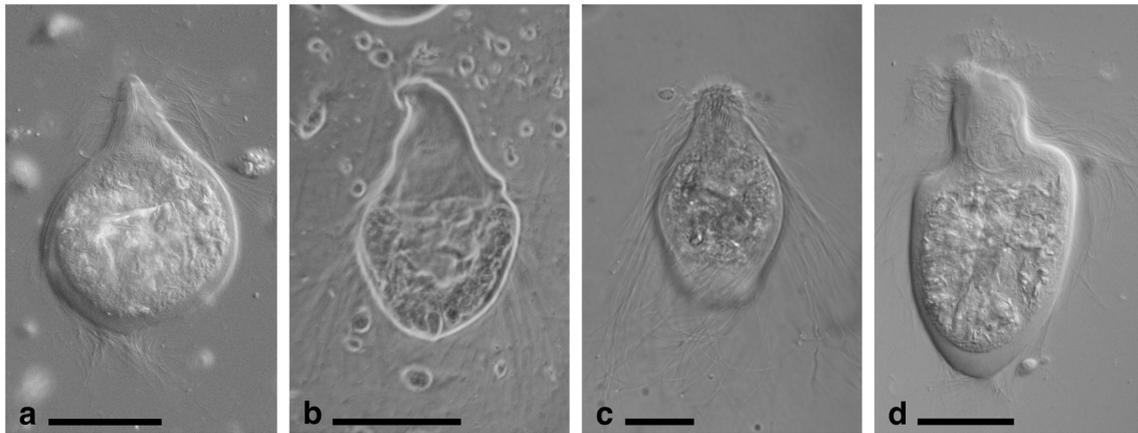
The microbial communities of the termite hindguts investigated were released by dissection in Trager's medium U [15] and observed with a differential interference contrast (DIC) microscope (Zeiss Axioplan 2). A single *Trichonympha* morphotype was identified for each host (Fig. 1). Specimens were collected individually with a glass micropipette, photographed and their SSU rRNA gene sequenced as previously described [12]. One clone per cell of *Trichonympha* was sequenced in *R. laticollis* (two single cells), *R. bicolor* (three single cells) and *C. temnocephalus* (five single cells). Two clones were obtained from a single isolated cell and a pool of about 50 cells collected from *G. brevicornis*.

An additional clone from whole-gut DNA extraction of *G. brevicornis* was also obtained, sharing high similarity with clones from isolated cells belonging to the genus *Trichonympha*. Mean sequence similarities were 98.9–99.4 % for specimens from the same host, and lower than 96.2–98.0 % when compared with specimens from a different host (including those investigated here). The variability among clones from the same hindgut community was 2.7 to 3.6 times lower than their mean divergence with the most similar clones representing the genus *Trichonympha* from a different termite.

A consensus sequence was inferred from the clones of each *Trichonympha* population using SeaView v4.6.1 [16]. One clone sequence was picked as a representative and used to infer phylogeny (choosing, whenever possible, the clone most similar to the consensus. All clone sequences are provided in Supplementary Data S1). Representative sequences were aligned using mafft v7.310 [17] (setting: -auto) with 153 available sequences from members of the genus *Trichonympha* harboured by termites and wood-eating cockroaches, plus the sequence of *Staurojoenina assimilis* as an outgroup (accession number: AB183882 [18]). The alignment matrix was trimmed using trimAl v1.4 [19] (settings: -gt 0.3 -st 0.001); columns with missing data at both ends were removed. A maximum-likelihood topology was calculated on the character matrix (1369 bp) using IQ-TREE v1.5.3 [20] (GTRGAMMA model as recommended by the BIC criterion; 1000 standard nonparametric bootstraps).

The phylogenetic tree is shown in Fig. 2. In accordance with the findings of other studies [21, 22], members of the genus *Trichonympha* isolated from species of the genus *Cryptocercus* formed a strongly supported (100 % bootstrap), fast-evolving clade. It has been suggested that the ancestor of lower termites and wood-eating cockroaches already harboured the ancestor of extant members of the genus *Trichonympha*, and that the parabasalian co-evolved with its host, being largely or exclusively vertically transmitted [2, 23, 24]. Species of the genus *Trichonympha* from termites were separated into two main subgroups, as previously noted [22, 24, 25]. The four novel species formed a weakly-supported (59 % bootstrap) clade within the subgroup dominated by symbionts of the class Kalotermitidae – the only exception being *Trichonympha magna*, isolated from the Australian *Porotermes adamsoni* (Stolotermitidae) [26]. The tree topology does not particularly support the strict co-speciation between the members of the genus *Trichonympha* and its termite hosts, since none of the host families, and few of the host genera, correspond to nodes in the symbiont tree.

No hindgut flagellate has been formally described or reported from *R. laticollis*, *R. bicolor* or *C. temnocephalus* [3], and so by extension there is no formally described species of the genus *Trichonympha* associated with any of these host species.



**Fig. 1.** Holotype specimens of the four novel species of the genus *Trichonympha* observed by differential interference contrast microscopy. (a) *Trichonympha hueyi* sp. nov. from *Rugitermes laticollis*. (b) *Trichonympha deweyi* sp. nov. from *Glyptotermes brevicornis*. (c) *Trichonympha louiei* sp. nov. from *Calcaritermes temnocephalus*. (d) *Trichonympha webbyae* sp. nov. from *Rugitermes bicolor*. Bars, 50  $\mu\text{m}$ .

The only species of the genus *Trichonympha* that has been described from any member of the genus *Glyptotermes* is *Trichonympha chattoni*, a taxon reported in the literature as also being found in *Rugitermes rugosus* and several species of the genus *Incisitermes* based on morphological similarities [3]. A sequence assigned to *T. chattoni* from *Incisitermes schwarzi* (accession number: AB434794 [22]) is quite distant from that of the *G. brevicornis* symbiont reported here (Fig. 2), suggesting that the classic morphospecies does not correspond to a single *Trichonympha* lineage. The name *T. chattoni* should probably be used only for flagellates compatible with the original morphological description and found in the type host, the Australian *Glyptotermes iridipennis* [2]. The symbiont of *G. brevicornis* possesses clear morphological differences from the original description of *T. chattoni*, most importantly a significantly larger size (about 200  $\mu\text{m}$  vs no more than 132  $\mu\text{m}$ ) and a conspicuous flagellated (rostral) area that extends to almost half the body length (vs about a third in *T. chattoni*).

Overall, the four taxa of the genus *Trichonympha* described here all appear to represent novel species, which are named as follows: *Trichonympha hueyi* sp. nov. for the symbiont of *R. laticollis*, *Trichonympha deweyi* sp. nov. for the symbiont of *G. brevicornis*, *Trichonympha louiei* sp. nov. for the symbiont of *C. temnocephalus* and *Trichonympha webbyae* sp. nov. for the symbiont of *R. bicolor*. Brief morphological descriptions and measurements are supplied for each taxon in the Taxonomic summary. However, due to the phenotypical plasticity within species of the genus *Trichonympha* and the degree of morphological overlap among species, the sequence of the SSU rRNA gene (along with host identity) should be treated as the most reliable diagnostic character. Since specimens are destroyed in order to obtain the sequence, photographic holotypes are provided in accordance with Article 73.1 of the International Code of Zoological Nomenclature and Declaration

45 of the International Commission on Zoological Nomenclature [27, 28].

## TAXONOMIC SUMMARY

***Trichonympha hueyi* sp. nov. Boscaro et al. 2017** (hu'ey.i. N.L. gen. n. *hueyi* referring to the Disney character Huey, the oldest of the three small and similar nephews of Donald Duck).

urn:lsid:zoobank.org:act: B4E7D4AA-73E6-422D-9DE1-B22C84905BD8

**Type host:** *Rugitermes laticollis* (Isoptera, Kalotermitidae; barcode MF062147).

**Type locality:** La Carolina park, Quito, Ecuador (0.1885 S 78.4860 W).

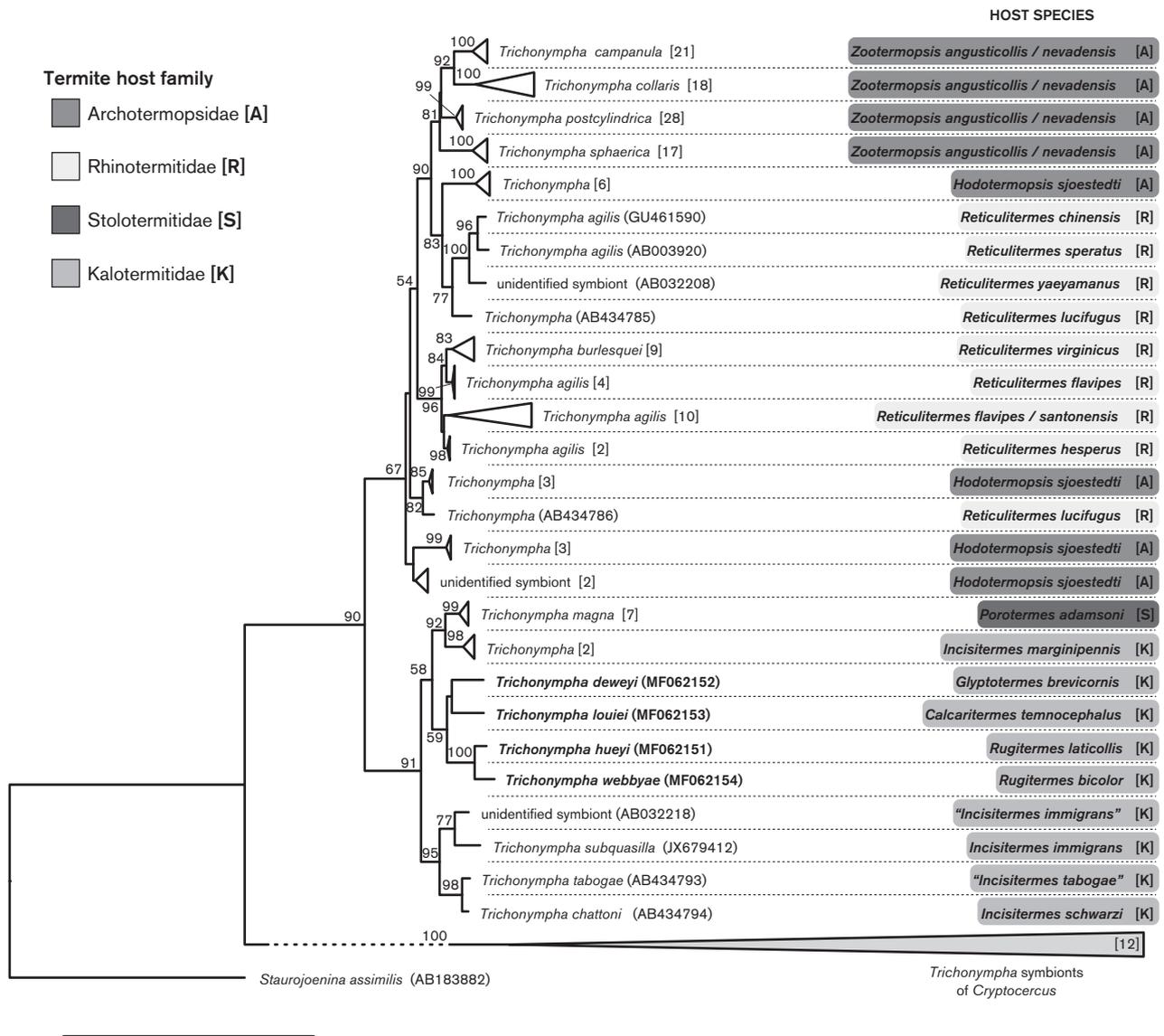
**Host collection:** University of Florida termite collection, accession number EC1465. Collectors: Mullins, Scheffrahn and Krecek.

**Description:** Parabasalian flagellate with morphological characteristics of the genus *Trichonympha*. Cells about 113  $\mu\text{m}$  in length and 79  $\mu\text{m}$  in width. Posterior (post-rostral) section of the body inflated, often spherical, entirely filled with wood particles, clearly differentiated from the short, tapered rostrum. Large, translucent operculum. Very thick ectoplasm throughout the cell body, with little or no difference in thickness between the rostral and post-rostral areas. Found in the hindgut of *Rugitermes laticollis*. Distinct SSU rRNA gene sequence.

**Holotype:** Specimen in Fig. 2a of the present publication.

**Gene sequence:** SSU rRNA gene GenBank accession number MF062151.

***Trichonympha deweyi* sp. nov. Boscaro et al. 2017** (dew'ey.i. N.L. gen. n. *deweyi* referring to the Disney



**Fig. 2.** Maximum-likelihood phylogenetic tree of the genus *Trichonympha* based on the SSU rRNA gene sequence (1369 bp). Sequences obtained in this study are in bold type. Clades of sequences attributed to the same species and collected from the same host species are collapsed; numbers in square brackets correspond to the number of sequences in the collapsed clade. Sequences attributed to *Trichonympha agilis* from different host species form a non-monophyletic assemblage. Termite species of uncertain status are in quotation marks: one population labelled '*Incisitermes immigrans*' is potentially a misidentified *Incisitermes schwarzi* [25], and '*Incisitermes tabogae*' is a junior synonym of *I. schwarzi* (unpublished data). Numbers at nodes correspond to nonparametric bootstrap values (percentages of 1000 replicates; values below 50 were omitted). Host organisms are listed on the right. Bar, estimated distance of 0.2. The branch associated with the symbionts of members for the genus *Cryptocercus* has been shortened for clarity.

character Dewey, the middle of the three small and similar nephews of Donald Duck).

urn:lsid:zoobank.org:act: F25F2F54-5B32-49C7-9564-50ED F46A332D

**Type host:** *Glyptotermes brevicornis* (Isoptera, Kalotermitidae: barcode MF062148).

**Type locality:** Mount Glorious, Australia (27.3377 S 152.7703 E).

**Host collection:** University of Florida termite collection, accession number AUS116. Collector: Keeling.

**Description:** Parabasalian flagellate with morphological characteristics of the genus *Trichonympha*. Cells about 207 µm in length and 125 µm in width. Large rostral section occupying almost half of the body. Tapered anterior end. Wood particles only observed in the post-nuclear endoplasm. Found in the hindgut of

*Glyptotermes brevicornis*. Distinct SSU rRNA gene sequence.

**Holotype:** Specimen in Fig. 2b of the present publication.

**Gene sequence:** SSU rRNA gene GenBank accession number MF062152.

***Trichonympha louiei* sp. nov. Boscaro et al. 2017** (lou'ie.i. N.L. gen. n. *louiei*, referring to the Disney character Louie, the youngest of the three small and similar nephews of Donald Duck).

urn:lsid:zoobank.org:act: 07EA5EBB-9984-4E61-86F1-8D3F9E1C3524

**Type host:** *Calcaritermes temnocephalus* (Isoptera, Kalotermitidae; barcode MF062149).

**Type locality:** Campoverde District, Peru (8.6085 S 74.9363 W).

**Host collection:** University of Florida termite collection, accession number PU512. Collector: Scheffrahn et al.

**Description:** Parabasalian flagellate with morphological characteristics of the genus *Trichonympha*. Cells about 116 µm in length and 65 µm in width. Short, non-tapered rostrum and rounded posterior end. Very long flagella, up to twice the cell length. Found in the hindgut of *Calcaritermes temnocephalus*. Distinct SSU rRNA gene sequence.

**Holotype:** Specimen in Fig. 2c of the present publication.

**Gene sequence:** SSU rRNA gene GenBank accession number MF062153.

***Trichonympha webbyae* sp. nov. Boscaro et al. 2017** (web'by.ae. N.L. gen. n. *webbyae* referring to the Disney character Webby, a small and adorable duckling unrelated to Donald Duck but unofficially referred to as the fourth nephew due to her similarity and friendship with the triplets).

urn:lsid:zoobank.org:act: 0920F31A-9148-4 DB2-AA64-3C033AC8BE2D

**Type host:** *Rugitermes bicolor* (Isoptera, Kalotermitidae; barcode MF062150).

**Type locality:** Tingo Maria, Peru (9.149 S 75.9923 W).

**Host collection:** University of Florida termite collection, accession number PU946. Collector: Scheffrahn et al.

**Description:** Parabasalian flagellate with morphological characteristics of the genus *Trichonympha*. Cells about 128 µm in length and 60 µm in width. Stout and large rostral section with a comparatively small operculum and rounded anterior end. A conspicuous enlargement separates the rostrum and post-rostral area in almost all specimens. The nucleus can be found in this region or in a slightly posterior position. The post-rostral, wood particles-filled area slightly tapers toward the posterior end. The ectoplasm is very thick throughout the cell, but usually slightly more in the rostral

area. Found in the hindgut of *Rugitermes bicolor*. Distinct SSU rRNA gene sequence.

**Holotype:** Specimen in Fig. 2d of the present publication.

**Gene sequence:** SSU rRNA gene GenBank accession number MF062154.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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