Trichonympha burlesquei n. sp. from *Reticulitermes virginicus* and evidence against a cosmopolitan distribution of *Trichonympha agilis* in many termite hosts

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Historically, symbiotic protists in termite hindguts have been considered to be the same species if they are morphologically similar, even if they are found in different host species. For example, the first-described hindgut and hypermastigote parabasalian, *Trichonympha agilis* (Leidy, 1877) has since been documented in six species of *Reticulitermes*, in addition to the original discovery in *Reticulitermes flavipes*. Here we revisit one of these, *Reticulitermes virginicus*, using molecular phylogenetic analysis from single-cell isolates and show that the *Trichonympha* in *R. virginicus* is distinct from isolates in the type host and describe this novel species as *Trichonympha burlesquei* n. sp. We also show the molecular diversity of *Trichonympha* from the type host *R. flavipes* is greater than supposed, itself probably representing more than one species. All of this is consistent with recent data suggesting a major underestimate of termite symbiont diversity.

Members of the genus *Trichonympha* are large and structurally complex parabasalians exclusively found in the symbiotic, lignocellulose-digesting hindgut community in numerous species of lower termites and wood-eating cockroaches (Kirby, 1932, Yamin, 1979). The type species, *Trichonympha agilis*, was the first of the now well-studied parabasalian symbionts of this community to be described. In 1877 Joseph Leidy observed *T. agilis* in *Reticulitermes flavipes* and, being reminded of '*the nymphs in a recent spectacular drama, in which they appeared with their nakedness barely concealed by long cords suspended from the shoulders*', coined the name *Trichonympha* (Leidy, 1877). Since then the genus *Trichonympha* has proved to be one of the more widespread genera of hindgut protists, and is even found in the hindgut of the sister lineage of lower termites, the wood-eating cockroach *Cryptocercus* (Carpenter *et al.*, 2009, Cleveland *et al.*, 1934, Ohkuma *et al.*, 2009, Yamin, 1979). While many different species of the genus *Trichonympha* have been described (Kirby, 1932, Yamin, 1979), one dominant trend in taxonomy of the genus *Trichonympha* has been to conclude that one species of the genus *Trichonympha* can be found in several different species of termite: in practice, similar-looking symbionts of the genus *Trichonympha* from different termite species are assumed to be the same species. As a result, since the late 1800s '*T. agilis*' has been reported to inhabit six additional species of the genus *Reticulitermes* (Yamin, 1979). This practice has clear effects on our overall view of symbiont biodiversity and evolution, but with few exceptions (notably by Kirby, who often discussed this issue), the assumptions underlying it are not questioned or even discussed. For example, in a study of wood preference of termites, Mannesmann defined the symbiont of the genus *Trichonympha* of *Reticulitermes virginicus* as *T. agilis* with no discussion as to the possibility that it might be a distinct species (Mannesmann, 1972), and this identification has now become part of the recorded host distribution of the species (Yamin, 1979). Molecular phylogenetic analyses of manually isolated cells has begun to call such assumptions into question for other species (e.g. (Gile *et al.*, 2011, Harper *et al.*, 2009, Saldarriaga *et al.*, 2011)), so here we analyse the molecular phylogeny of symbionts of the genus *Trichonympha* from *R. virginicus* and those from other species of the genus *Reticulitermes*, including the type host, *Reticulitermes flavipes*, to begin to determine whether *T. agilis* is really distributed widely within host species of the genus *Reticulitermes*, or if this practice is obscuring symbiont diversity.

Reticulitermes virginicus (collection details below) was identified by morphology and by DNA barcoding at the mitochondrial LSU locus [see James *et al.*, (2013)]. Hindgut contents were released by dissection in Trager's medium U

Abbreviation: SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the small subunit rRNA sequences of *Trichonympha burlesquei* and of novel *Trichonympha* strains isolated from *Reticulitermes flavipes* are KC494343–KC494351 and KC494352–KC494360 respectively.

A supplementary video is available with the online version of this paper.

and documented by video microscopy, as described previously (James *et al.*, 2013). The symbiont community matched previous descriptions (James *et al.*, 2013, Mannesmann, 1972), including a single discernible morphotype of as member of the genus *Trichonympha* (Fig. 1a). Small subunit rRNA (SSU rRNA) sequences were characterized from eight individual manually isolated cells (e.g. Fig. 1b–d) and a pool of 30 isolated cells, as described previously (James *et al.*, 2013). In total, sequences from 10 clones were characterized, resulting in an average pairwise variation of 1.3 %, which is consistent with the presence of a single species of the genus *Trichonympha* in *R. virginicus*.

SSU rRNA sequences attributed to *Trichonympha* from *R. flavipes* are publicly available both from a pool of 30 manually isolated *Trichonympha* cells (GenBank accession numbers KC285195 and KC285196) and from an unidentified environmental sequence (GenBank accession number U17512), however, in no case has the host termite identification been verified by barcoding. Accordingly, we barcode-identified an *R. flavipes* isolate (a lab stock generously provided by Barbara Stay, University of Iowa, in October 2009) and sequenced nine SSU rRNA clones of members of the genus *Trichonympha* from its hindgut community to confirm the identity of the existing sequences.

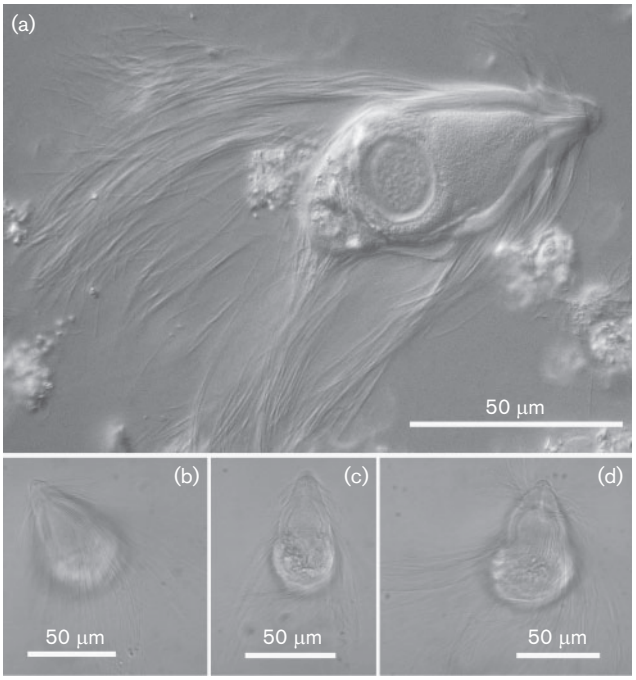

Fig. 1. Differential interference contrast light micrographs of *T. burlesqui* n. sp. from *R. virginicus*. (a) whole-cell image of *T. burlesquei* n. sp. showing the overall body shape and size, and in particular note the extensive number of long trailing flagella, which are not seen in *T. agilis* from *R. flavipes*. (b–d) three examples of isolated single cells of *T. burlesquei* n. sp. from which distinct SSU rRNA genes were sequenced. These cells correspond to GenBank accession numbers KC494348 (b), KC494349 (c) and KC494350 (d).

Phylogenetic analyses of all SSU rRNAs from members of the genus *Trichonympha* showed the sequences of members of the genus *Trichonympha* from *R. virginicus* and *R. flavipes* both fell into a well-defined subgroup of the genus *Trichonympha* (not shown), and a more detailed analysis focusing on all available sequences from this subgroup (Fig. 2) not only showed that the '*T. agilis*' isolated from *R. virginicus* is distinct from the '*T. agilis*' isolated from *R. flavipes*, but also showed that more than one species of the genus *Trichonympha* probably exists in *R. flavipes*. There is no universally accepted marker or level of sequence divergence applied to species level distinctions in protists, but by focussing on divergence levels within clades versus divergence between sister clades, we can draw some conclusions. The '*T. agilis*' clones isolated from *R. virginicus* formed a single well supported clade (100 %) with an average pairwise distance of 98.7 % similarity and an average distance to '*T. agilis*' clones from *R. flavipes* of 95.8 % similarity, all consistent with the simple conclusion that one, distinct species of *Trichonympha* exists in *R. virginicus*, which we here describe as *Trichonympha burlesquei* sp. nov.. There is one subclade (the lower part of *T. burlesqui* in Fig. 2) that could potentially represent a species-level difference, but the single isolated cells from which these sequences were derived (e.g. Fig. 1b) were not detectably different from cells with sequences that fell in the main part of the *T. burlesquei* clade (e.g. Fig. 1c,d), so we conservatively conclude they represent a single species. In contrast, the '*T. agilis*' clones isolated from *R. flavipes* formed three distinct groups: two groups represented by multiple clones from several different isolates that had average intra-clade pairwise distances of 1.2 % (A and B in Fig. 2) and a single clone from one isolate (C in Fig. 2) that was uniquely distinct. The '*T. agilis*' from *R. flavipes* clade B was most closely related to the *T. burlesquei* clade, but the inter-clade distance was substantially higher than the intra-clade distance.

Given the genetic distinction between symbionts of the genus *Trichonympha* from *R. virginicus* and *R. flavipes*, their apparent host-specificity and the fact that the hosts of the genus *Reticulitermes* are recognized as two species, we conclude that the symbionts of the genus *Trichonympha* also represent two distinct species and not a broad host range of *T. agilis*.

This work also uncovered some unexpected diversity in members of the genus *Trichonympha* from *R. flavipes*. This diversity could represent multiple paralogues of SSU rRNA within a single species of the genus *Trichonympha*, or (more probably) could represent multiple species of the genus *Trichonympha* within *R. flavipes*. A focused analysis of molecular data from many single cells will be required to make this determination, which is important because only this will allow the type species to be assigned to a molecular clade. Even without a molecular identification of the type species, it is clear that the morphology of strains of the genus *Trichonympha* found in *R. virginicus* is distinct from that of *T. agilis*: the flagella in members of the genus *Trichonympha* from *R. virginicus* are much longer than those described for *T. agilis* from *R. flavipes* and extend substantially beyond the posterior extreme of the body, sometimes by an entire body-length (Fig. 1A, Supplementary Video 1). Such obvious morphological differences may be absent and even when

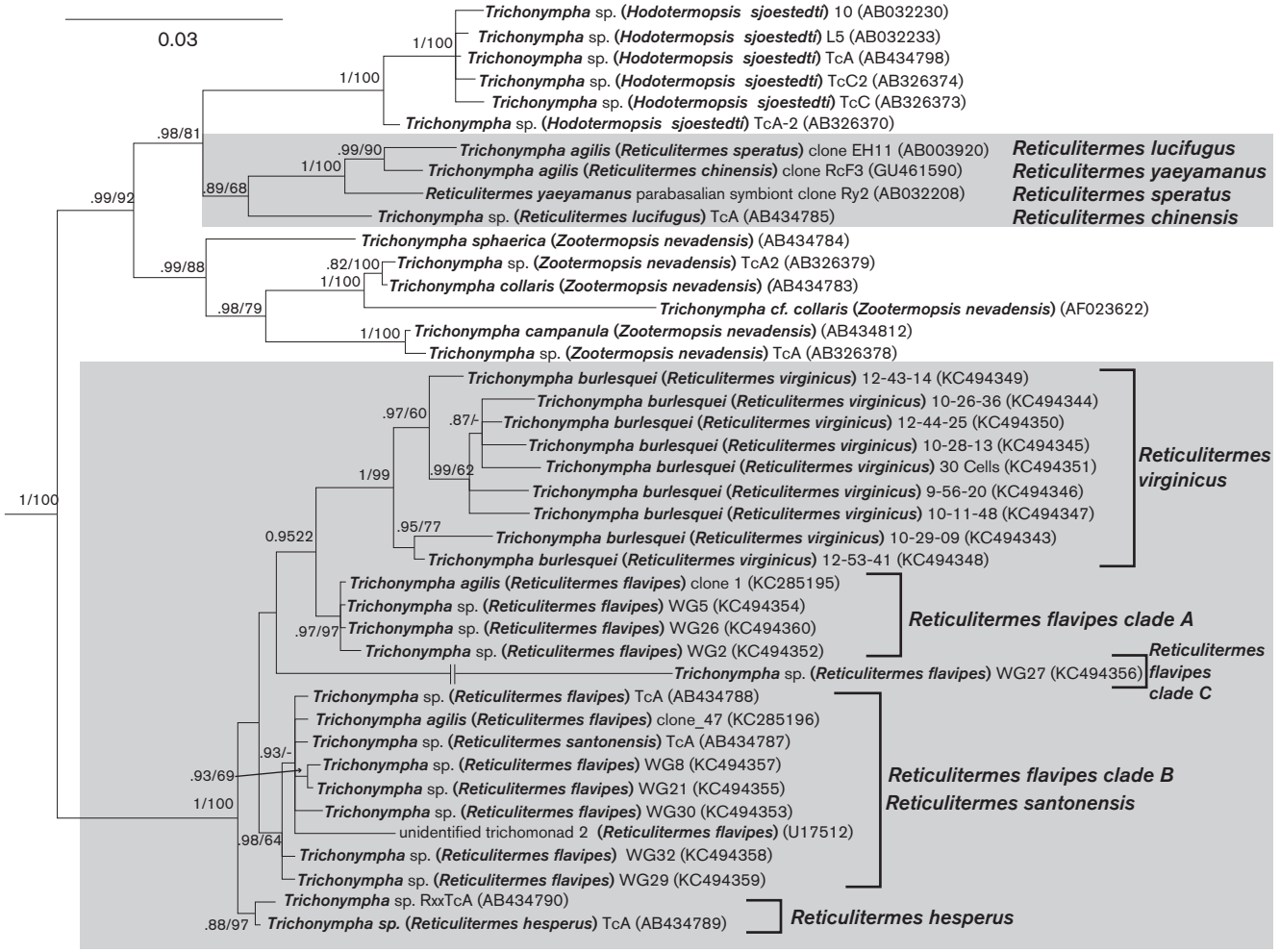

Fig. 2. SSU rRNA phylogeny of strains of species of the genus *Trichonympha*, including all sequences derived from hosts of the genus *Reticulitermes* and closely related hosts of the genera *Hodotermopsis* and *Zootermopsis*. Sequences from members of the genus *Trichonympha* derived from hosts of the genus *Reticulitermes* are indicated by grey boxes, and the host species for a clade are indicated in bold in parentheses. Symbionts formerly recognized as '*T. agilis*' form five distinct clades, suggesting that '*T. agilis*' is several species. All sequences from symbionts of *R. virginicus* fall in a single discrete clade, but sequences of symbionts from *R. flavipes* (the type host of *T. agilis*) fall into three discrete clades, labelled A–C.

present often go unnoticed because older descriptions sometimes lack detail or include errors [see Kirby, (1932) for a detailed history of early descriptions of members of the genus *Trichonympha*], so we feel that the molecular data and presence in distinct host species alone warrant a redescription of the *R. virginicus* symbiont.

Taxonomic Summary

***Trichonympha burlesquei* n. sp. James and Keeling, 2012**

urn:lsid:zoobank.org:act: E773C1FE-0474-4CDF-9A36-F4B6D359FC5E

Type host: *Reticulitermes virginicus(Isoptera, Rhinotermitidae: barcode JX975354)*

Type locality: Ft. Lauderdale, Secret Woods County Park, Florida, USA: 26° 5′ 8″ N 80° 10′ 48″ W.

Host collection: University of Florida termite collection, accession number FL2261. Collector R. H. Scheffrahn. Collected 21 February, 2005.

Description: Parabasalian flagellate with morphological characteristics of the genus *Trichonympha*. Cells are 77 μm in length and 40 μm in width (n=15). Found in the hindgut of *Reticulitermes virginicus*. Distinct SSU rRNA sequence (GenBank accession number JX975354).

Hapantotype: Microscope slide deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR201.

Gene sequence: SSU rRNA GenBank accession number KC494350.

Etymology: Species name refers to the original description of the genus *Trichonympha* by Leidy, which was based on the revealing nymph costumes in a 'spectacular drama' of his time.

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