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SHORT COMMUNICATION

The Phylogenetic Position of *Kofoidia loriculata* (Parabasalia) and its Implications for the Evolution of the Cristamonadea

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ABSTRACT

Kofoidia loriculata is a parabasalid symbiont inhabiting the hindgut of the lower termite *Paraneotermes simplicicornis*. It was initially described as a lophomonad due to its apical tuft of multiple flagella that disintegrate during cell division, but its phylogenetic relationships have not been investigated using molecular evidence. From single cell isolations, we sequenced the small subunit rRNA gene and determined that *K. loriculata* falls within the Cristamonadea, but is unrelated to other lophomonads. This analysis further demonstrates the polyphyly of the lophomonads and the necessity to re-assess the morphological and cellular evolution of the Cristamonadea.

KOFOIDIA loriculata (Light), the only known species of its genus, is a parabasalid symbiont found exclusively in the hindgut of the lower termite *Paraneotermes* (formerly *Kalotermes*) *simplicicornis* (Banks). It is a visually striking protist: a large, spherical cell topped with several cylindrical bundles of flagella arranged in a short spiral. Its morphology is unique, but it was proposed that the closest known relative of *Kofoidia* was *Lophomonas* based on its overall large size, physical complexity, and more specifically due to the location and arrangement of the flagella as well as similarities with *Lophomonas* during cell division (Light 1927).

Initially, *Kofoidia* was placed in its own family, the Kofoidiidae, in the order Lophomonadida (Light 1927), which was later included in the class Hypermastigida together with the Trichonymphida and the Spirotrichonymphida (Brugerolle and Lee 2000). The Hypermastigida are characterized by having extensive multiplication of flagella and a single nucleus. Lophomonads include *Kofoidia*, *Lophomonas*, *Deltotrichonympha*, *Koruga*, *Joenia*, *Joenina*, and *Joenoides* among others, all of which share flagella located apically in a tuft and, during cell division, a nucleus that migrates away from the flagellar organelles and divides at the opposite end of the cell. The deserted flagellar organelles gradually degenerate except for four privileged basal bodies from which a new flagellar system is rebuilt *de novo* in the daughter cells (Light 1927).

The evolution of the parabasalids was assumed to have proceeded from simple to complex and the organisms were classified into two orders, the simpler Trichomonadida for organisms with up to six kinetosomes in the mastigont and the multiflagellated Hypermastigida. These orders are now considered polyphyletic and a revised classification has split the Parabasalia into six classes: the Cristamonadea, Hypotrichomonadea, Spirotrichonymphea, Trichonymphea, Trichomonadea, and Tritrichomonadea (Cepicka et al. 2010). The lophomonads are included in

the Cristamonadea, and not the Trichonymphea or Spirotrichonymphea implying that their flagellar multiplication evolved independently of these other groups (Noda et al. 2012). The Cristamonadea was established based on similarities in the organization of the mastigont and also includes species previously classified in the Trichomonadida such as the calonymphids and devescovinids (Brugerolle and Patterson 2001; Cepicka et al. 2010).

Molecular support for the class Cristamonadea is strong (Cepicka et al. 2010; Noda et al. 2009, 2012). The association of devescovinids and joeniids (subgroups of cristamonads) is also supported by the shared presence of a modified recurrent flagellum and similar ultrastructure of the axostyle (Brugerolle and Patterson 2001). Within the Cristamonadea, however, internal phylogenetic resolution is generally lacking. Many taxonomic groups are polyphyletic and their classification is in need of revision. Due to the poor phylogenetic resolution, all cristamonad genera were lumped into a single family named Lophomonadidae after *Lophomonas*, the first-described genus included (Cepicka et al. 2010). The lophomonads, however, have been shown to be completely polyphyletic within this family. Joeniids tend to branch at the base of the Cristamonadea, but this phylogenetic position lacks statistical support while *Deltotrichonympha* and *Koruga* form a strongly supported monophyletic group typically positioned sister to a calonymphid clade comprising *Calonympha*, *Snyderella*, and *Stephanonympha* (Gile and Slamovits 2012; Noda et al. 2009, 2012). Furthermore, *Lophomonas striata*, a close relative of the type species of this group *Lophomonas blattarum*, was recently found to be a basal taxon of the Trichonymphida and unrelated to the other lophomonads or any of the Cristamonadea (Gile and Slamovits 2012).

To determine the phylogenetic placement of *Kofoidia* and further investigate the evolution of the polyphyletic lophomonads, we sequenced the small subunit (SSU) rRNA gene from single cells morphologically consistent with *K. loriculata*. Using these data, we show that *Kofoidia* is a phylogenetically distinct taxon within the Cristamonadea, and not demonstrably related to other lophomonads from which molecular data have been characterized.

MATERIALS AND METHODS

Termite collection and molecular barcoding

Termites identified as *Paraneotermes simplicicornis* were collected west of Terlingua, Texas, USA (latitude 29.280, longitude −103.716) on October 30, 2008 and from Tucson, Arizona, USA (latitude 32.302, longitude −110.907) on July 25, 2012. To validate that the Arizona and Texas specimens were the same species and provide a measure of their genetic relatedness, a fragment of the mitochondrial 16S (large subunit) rRNA gene was sequenced from the termites collected from Arizona following methods previously described (Gile et al. 2011) and deposited in GenBank (accession KJ831626). The mitochondrial 16S rRNA sequence was previously obtained for the termites collected from Texas (GenBank accession KJ438371).

Single cell isolations and imaging of *K. loriculata*

The hindgut contents of *P. simplicicornis* were diluted in Trager's solution U (Trager 1934) and viewed using an Axiovert 2 inverted light microscope with differential interference contrast (DIC) optics (Zeiss, Jena, Germany). From Arizona termites, four single cells morphologically consistent with *K. loriculata* were individually collected and washed using micromanipulation. Two cells from a Texan *P. simplicicornis* were collected together. These cells were photographed with a MicroImager II camera (QImaging, Burnaby, Canada). Scanning electron microscopy was also used to obtain images of *K. loriculata* as described previously (Carpenter et al. 2013). Video of *K. loriculata* was obtained by viewing the cells with an Axioplan 2 upright light microscope with DIC optics (Zeiss) and a XL-M1S camera (Canon, Mississauga, Canada).

Small subunit rRNA gene sequencing and phylogenetic analysis

DNA extracted from single cells or the pool of two were used as templates to PCR amplify, clone, and sequence a ~1,530 bp fragment of the SSU rRNA gene following methods previously described (Tai et al. 2013). One to two clones were sequenced from each of the single cells and six clones were sequenced from the pool of two cells.

Two distinct clusters of *K. loriculata* SSU rRNA gene sequences were obtained, comprising six and seven sequences each. One representative sequence from each cluster was chosen for phylogenetic analysis and deposited in GenBank under accession numbers KJ831624 and KJ831625. These *K. loriculata* SSU rRNA gene sequences were aligned to representative parabasalid sequences obtained from GenBank using MAFFT L-INS-i (Katoh and Toh 2008). The ends were trimmed manually and Gblocks was used to remove highly variable and ambiguously aligned sites, but allowing gaps (Castresana 2000).

Maximum likelihood (ML) analysis using RAxML 7.0.4 (Stamatakis 2006) and Bayesian analysis using MrBayes 3.2 (Ronquist and Huelsenbeck 2003) were used to construct phylogenetic trees. The ML analysis implemented a general time reversible (GTR) model of nucleotide substitution with the gamma model of rate heterogeneity. Statistical support for the consensus tree was assessed from 1,000 bootstrap replicates. The Bayesian analysis also used a GTR + gamma model. Four chains were sampled every 100 generations from two runs for 1,000,000 generations. Diagnostics were run every 1,000 generations with a relative burnin of 25% of the tree samples. After 1,000,000 generations, the average standard deviation of the split frequencies from the two runs was 0.004.

RESULTS AND DISCUSSION

The single cells of *K. loriculata* collected for molecular analysis were morphologically consistent with their initial description (Light 1927) (Fig. 1A). Scanning electron micro-

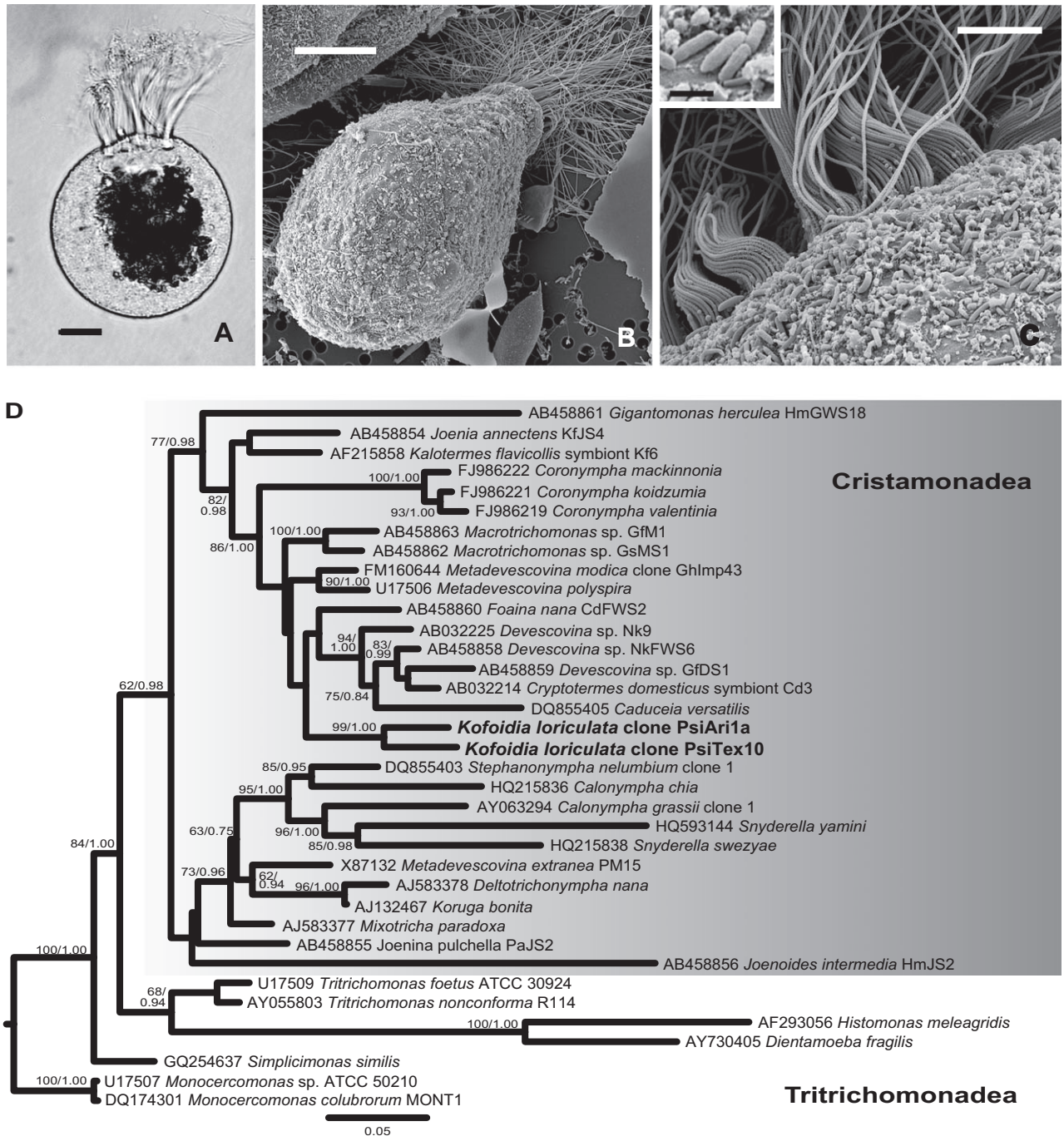

Figure 1 **A.** Differential interference contrast light micrograph of the *Kofoidia loriculata* used to obtain the PsiAri1a 18S rRNA sequence used for the phylogenetic analysis in D. Scale bar = 20 μm. **B.** Scanning electron micrograph (SEM) of *K. loriculata*. Scale bar = 20 μm. **C.** SEM of flagellar bundles at the apical end of a *K. loriculata* cell. Bacteria can be seen associated with the *K. loriculata* cell surface. Scale bar = 5 μm. *Inset*: higher magnification view of the surface bacteria. Scale bar = 1 μm. **D.** Maximum likelihood tree of small subunit (SSU) rRNA gene sequences showing the phylogenetic position of *K. loriculata* (in bold) relative to representative sequences of cristamonads and tritrichomonads. Statistical support is shown at nodes with > 60% maximum likelihood bootstrap support and > 0.70 Bayesian posterior probability.

graphs show additional details of *K. loriculata*, particularly the bundles of flagella and the association of straight and slightly curved rod-shaped bacteria with the cell surface (Fig. 1B, C). Each flagellar bundle consisted of at least 20 flagella. The bacteria were observed on most but not all *Kofoidia* cells. A video of live *K. loriculata* under light

microscopy is provided, highlighting the movement of the flagellar bundles (Movie S1).

Diversity of *K. loriculata*

The *K. loriculata* SSU rRNA gene sequences were more divergent than expected. Two clusters of *K. loriculata* were observed (Fig. S1) possibly representing cryptic species. The sequences within a cluster shared at least 98% similarity with each other, but on average only 94% similarity with sequences from the other cluster. From one cluster, the sequences were all from single cells collected from Arizonan *P. simplicicornis*. In the second cluster, one of the sequences was from a fourth cell from Arizona and the other six were from *Kofoidia* cells isolated from Texan *P. simplicicornis*. The sequences from this cluster contained many small insertions, ranging from 5 to 10 bp, relative to the *K. loriculata* sequences from the first group. Distinct species of *Kofoidia* may be represented by these two clusters, but under light microscopy no morphological distinctions were identified. The mitochondrial 16S rRNA barcode sequences of *P. simplicicornis* collected from Arizona and Texas were also divergent, sharing 97% similarity. Neither *Kofoidia* nor their host, *P. simplicicornis*, however, were sufficiently sampled to accurately estimate the genetic variation that exists in these organisms.

Kofoidia loriculata is a cristamonad

The SSU rRNA gene sequences from single cells of *K. loriculata* formed a distinct clade within the Cristamonadea (Fig. 1D). More specifically, *K. loriculata* branched distantly from other cristamonad taxa with a high level of structural complexity, including other lophomonads, and instead tended to branch most closely with genera such as *Devescovina*, *Foania*, *Metadevescovina*, and *Macrotrichomonas* which are the simplest in structure.

Taxonomy and evolution of the Cristamonadea

Many traditional families within the Cristamonadea have been found to be polyphyletic indicating that the morphological evolution of cristamonad taxa is more complex than previously assumed (Noda et al. 2009). The evolution of the Cristamonadea includes dynamic transitions of multiplication and reduction in flagellar, cytoskeletal, and nuclear elements (Noda et al. 2012). Phylogenetic evidence is mounting that several families should be redefined or newly established within the Cristamonadea. For example, molecular data now suggest that the Calonymphidae should include *Snyderella*, *Stephanonympha*, and *Calonympha*, but not *Coronympha* (Gile et al. 2011; Harper et al. 2009; Noël et al. 2007). *Coronympha* species form a distant but strongly supported monophyletic group, and are also morphologically distinguished by having separate rather than bundled axostyles (Harper et al. 2009; Noël et al. 2007 and references therein). As a result, in the absence of any clear association with other cristamonad subgroups, a distinct family for *Coronympha* is warranted. The Devescovinidae should include *Devescovina* and *Caduceia*, and likely *Foaina*, *Macrotrichomonas* and *Metadevescovina*, but not *Gigantomonas* (Noda et al. 2009). And, as shown in this study, *Kofoidia* is sufficiently distinct to reinstate the family Kofoidiidae for these organisms (Light 1927). Of the other described cristamonad subgroups, the monophyly of *Deltotrichonympha* and *Koruga* likely supports the family Deltotrichonymphidae, but the joeniids remain completely unresolved, molecular data are lacking from the rhizonymphids, and *Lophomonas*, the type genus of the Lophomonadidae, likely falls elsewhere entirely (Gile and Slamovits 2012).

These changes are interesting beyond simply classifying the Parabasalia, since they also have implications for character evolution (Hampl et al. 2004; Noda et al. 2012; Ohkuma et al. 2005). In the case of lophomonads, it seems most likely that the ancestor of cristamonads was a relatively simple trichomonad-like flagellate probably bearing four flagella, one of which was recurrent and likely attached to the cell by an undulating membrane underlain by the costa (Noda et al. 2012). This implies that "lophomonad" characters and the structural complexity that underlies them have evolved multiple times within the cristamonads, and even in parallel outside the group (in *Lophomonas*).

Cristamonads, with the notable exception of possibly basal taxa such joeniids and symbionts from *Mastotermes darwiniensis* (*Deltotrichonympha nana*, *Koruga bonita*, *Metadevescovina extranea*, and *Mixotricha paradoxa*), have diversified almost exclusively within the hindguts of termites from the family Kalotermitidae (Kirby 1937; Noda et al. 2009; Tai, unpubl. data; Yamin 1979), but the factors driving this diversification are not known. Presumably, differences in host behavior and diet have had a role. In this regard, *P. simplicicornis*, the host of *Kofoidia* as well as other cristamonads such as *Foaina*, *Hoplonympha*, and *Macrotrichomonas* (Yamin 1979), is unique among the kalotermitid termites. While the majority of kalotermitid termites live in and feed on damp wood, *P. simplicicornis*, the host of *Kofoidia*, is the only known member of the Kalotermitidae that evades the heat and dryness of its xeric habitats by foraging in the soil for wood away from its nest like true subterranean termites (Light 1937).

CONCLUSIONS

Molecular phylogenetic analyses demonstrate that *Kofoidia* is a phylogenetically distinct taxon of the Cristamonadea. Its phylogenetic placement provides further evidence for the polyphyly of lophomonads, even within the Cristamonadea. The molecular phylogeny questions the homology of "lophomonad" characters and provides a useful framework to recount the morphological and cellular evolution of *Kofoidia*, the cristamonads, and the Parabasalia.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maximum likelihood tree of *Kofoidia loriculata* SSU rRNA gene sequences. Two distinct clusters resulted. One cluster comprises only of sequences derived from *K. loriculata* cells isolated from *Paraneotermes simplicicornis* from Arizona (PsiAri). The second cluster comprises *K. loriculata* sequences obtained from cells isolated from *P. simplicicornis* from Texas (PsiTex) plus one from Arizona. Statistical support is shown at nodes with > 60% maximum likelihood bootstrap support and > 0.70 Bayesian posterior probability.

Movie S1. Video of live *Kofoidia loriculata* showing the cell body filled with wood particles and the movement of the flagellar bundles.