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SHORT COMMUNICATION

Molecular Characterization of Parabasalian Symbionts *Coronympha clevelandii* and *Trichonympha subquasilla* from the Hawaiian Lowland Tree Termite *Incisitermes immigrans*

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ABSTRACT

An important and undervalued challenge in characterizing symbiotic protists is the accurate identification of their host species. Here, we use DNA barcoding to resolve one confusing case involving parabasalian symbionts in the hindgut of the Hawaiian lowland tree termite, *Incisitermes immigrans*, which is host to several parabasalians, including the type species for the genus *Coronympha, C. clevelandii*. We collected *I. immigrans* from its type locality (Hawaii), confirmed its identity by DNA barcoding, and characterized the phylogenetic position of two symbionts, *C. clevelandii* and *Trichonympha subquasilla*. These data show that previous molecular surveys of "*I. immigrans*" are, in fact, mainly derived from the Caribbean termite *I. schwarzi*, and perhaps also another related species. These results emphasize the need for host barcoding, clarify the relationship between morphologically distinct *Coronympha* species, and also suggest some interesting distribution patterns of nonendemic termite species and their symbionts.

ESTABLISHING the correct identity of microbial symbionts is dependent on first correctly identifying their hosts, which can be problematic as taxonomic expertise is generally limited to one or the other. This problem has repeatedly occurred in the identification of parabasalian symbionts in the hindgut of lower termites. Lower termite species each contains a unique assemblage of protist symbionts, mostly parabasalians, and the composition of this community is stable over geography and time for that species. This gives the system some interesting advantages for the study of symbiont diversity; however, misidentification of termites or changes to their taxonomy have introduced a number of mistakes in our identification that can be misleading and confusing to later studies. One case involves *Incisitermes immigrans* (Snyder), which is the lowland tree termite native to Hawaii and the western neotropics, but which has also been putatively identified in Japan (Takematsu 1997). *Incisitermes immigrans* is the type-host for several parabasalian species, including *Trichonympha subquasilla* and *Coronympha clevelandii* (Kirby 1929, 1944), the latter being the type species of the genus *Coronympha*. *Coronympha clevelandii* is also of interest because it is morphologically distinct from all other described *Coronympha* species in possessing 16 karyomastigonts, compared with the eight found in all other species (Kirby 1929), and lacking a "metacoronympha" morphological stage, which seems to be common to other species (Dolan et al. 2000; Harper et al. 2009; Kirby 1929, 1939). A molecular survey of Japanese termites identified as *I. immigrans* revealed *Trichonympha*- and *Coronympha*-like sequences (Ohkuma et al. 2000), but subsequent sampling of sequences directly from manually isolated cells showed the *Coronympha* sequence to be nearly identical to an eight-karyomastigont *Coronympha* from a Caribbean Basin species, *I. schwarzi* (Harper et al. 2009), suggesting a misidentification of the host.

To resolve this conflict, we have collected *I. immigrans* from its type locality, the Hawaiian island of Oahu, and confirmed its identity by DNA barcoding. We then characterized the small subunit ribosomal RNA (SSU rRNA) from manually isolated cells matching the descriptions of *C. clevelandii* and *T. subquasilla*, and determined the phylogenetic positions of both species relative to "*I. immigrans*" environmental data.

MATERIALS AND METHODS

Collecting, barcoding, symbiont isolation

Incisitermes immigrans was collected on the Kamehameha Highway, Oahu (21°39′50″, 158°3′4″) on 21 July 2009 by PJK and deposited in the University of Florida termite collection (accession HI120). Collection of *I. schwarzi* (Banks) and mitochondrial large subunit ribosomal RNA (mtLSU rRNA) barcoding of both species were as described (Gile et al. 2011; Harper et al. 2009). Additional *I. immigrans* barcode sequences were generously provided by Allan Szalanski and their University of Florida termite collection accession numbers and country of collection are as follows: HN495_4383, Honduras; NI800_4363, Nicaragua; PN201_4718, Panama; EC137_4282, Ecuador; and lastly, HI98_4471, which was isolated on Oahu, a short distance from our above isolate, but was taken in 1996. *Coronympha clevelandii* and *Trichonympha subquasilla* were identified and documented by high-resolution video microscopy. A pool of 50 cells of each species was manually isolated and purified, and nuclear SSU rRNA amplified from both pools, as described (Harper et al. 2009). Five individual clones of each were completely sequenced. The diversity of *Trichonympha* was also assessed using *Trichonympha*-specific primers, Trich1F: 5′-GATATACAAATTCTATCTTGAAAT-3′ and Trich1R: 5′-TCAAATCCATCCTTAAAGCTCTCT-3′.

Molecular and phylogenetic analyses

All available *Incisitermes* mtLSU termite barcodes were downloaded from Genbank and aligned with our new sequences using MAFFT (Katoh and Toh 2010), and refined by eye using SeaView (Gouy et al. 2010). Poorly aligned regions were automatically removed with trimAl using a gap threshold of 0.9 (Capella-Gutierrez et al. 2009). Akaike's Information Criterion (AIC) weight as calculated with the perl script MrAIC.pl (Nylander 2004) was used to determine the evolutionary model that best fit the data, which corresponded to GTR + Γ + I in all cases. For the termite phylogeny, Maximum Likelihood (ML) estimation was carried out using RAxML 7.2.5 (Stamatakis 2006) with statistical support inferred from 1000 bootstrap replicates. Parabasalian SSU rRNA phylogenies were inferred using ML and Bayesian tree reconstruction methods, with PHyML v.3 (Guindon and Gascuel 2003) and MrBayes v.3.2 (Ronquist and Huelsenbeck 2003), respectively. For PHyML, eight rate categories were used with the gamma shape parameter and the proportion of invariable sites estimated from the data. The subtree prune and regraft (SPR) method of tree improvement was chosen, and 1000 bootstrap replicates performed for evaluating the support. For MrBayes, the inference used four Metropolis-coupled Markov Chain Monte Carlo consisting of 1,000,000 generations with sampling every 100 generations. The average standard deviation of split frequencies was used to assess the convergence of the two runs. Bayesian posterior probabilities were calculated after the initial burn-in period corresponding to 20% of the generations (200,000 generations). To better assess the phylogenetic position of *T. subquasilla,* a topology comparison using the approximately unbiased (AU) test was performed (Shimodaira 2002). For each tested tree, site likelihoods were calculated using RAxML and the AU test was performed using CONSEL (Shimodaira and Hasegawa 2001) with default scaling and replicate values.

RESULTS AND DISCUSSION

Host identification by DNA barcoding

The lower termite DNA barcode marker mitochondrial LSU rRNA was sequenced from workers of both *I. immigrans* and *I. schwarzi*, and in both cases, the barcode confirmed the identification. The *I. immigrans* barcode shared 97–100% identity to other *I. immigrans* barcodes (Fig. 1A), while the *I. schwarzi* barcode shared 94–98% identity to "*I. tabogae*" (Snyder) isolates (recognized as synonymous with *I. schwarzi*: Scheffrahn R. H., unpubl. data), and 95% identity to the problematic "*I. immigrans*" isolate from Japan (Fig. 1A). The nearest species in both cases shared only 83–87% identity.

Phylogentic position of *C. clevelandii*

The SSU rRNA from manually isolated *C. clevelandii* cells (Fig. 1B–D) was determined, and found to be branched with strong support with all other *Coronympha* sequences (Fig. 1G). It branched weakly as the sister to other *Coronympha* species, and showed no specific relationship to the "*I. immigrans*" environmental sequence, which is instead related to *C. mackinnonia* from *I. schwarzi* (Harper et al. 2009). This supports the interpretation that the Japanese "*I. immigrans*" was a misidentified *I. schwarzi*, and is also consistent with the monophyly of the eight-karyomastigont morphology common to most described species of *Coronympha* (although the polarity of evolutionary change between eight- and 16-karyomastigont states, or which came first, remains unclear).

Phylogenetic position of *T. subquasilla*

The SSU rRNA was also characterized from manually isolated *T. subquasilla* cells (Fig. 1E, F), but in this case, the results were different. Whereas the Japanese "*I. immigrans*" barcode and *Coronympha*-like sequence are both consistent with a misidentification of *I. schwarzi*, the *Trichonympha* phylogeny was not. Instead, *T. subquasilla* from *I. immigrans* was sister to the Japanese "*I. immigrans*" environmental sequence with moderate support, although the two sequences show a high level of divergence (Fig. 1H). The environmental sequence from "*I. immigrans*" was not specifically related to *T. chattoni*, as expected if "*I. immigrans*" is misidentified *I. schwarzi*, and this alternative topology with "*I. immigrans*" sister to *I. shwarzi* was rejected at the 5% level by the AU test.

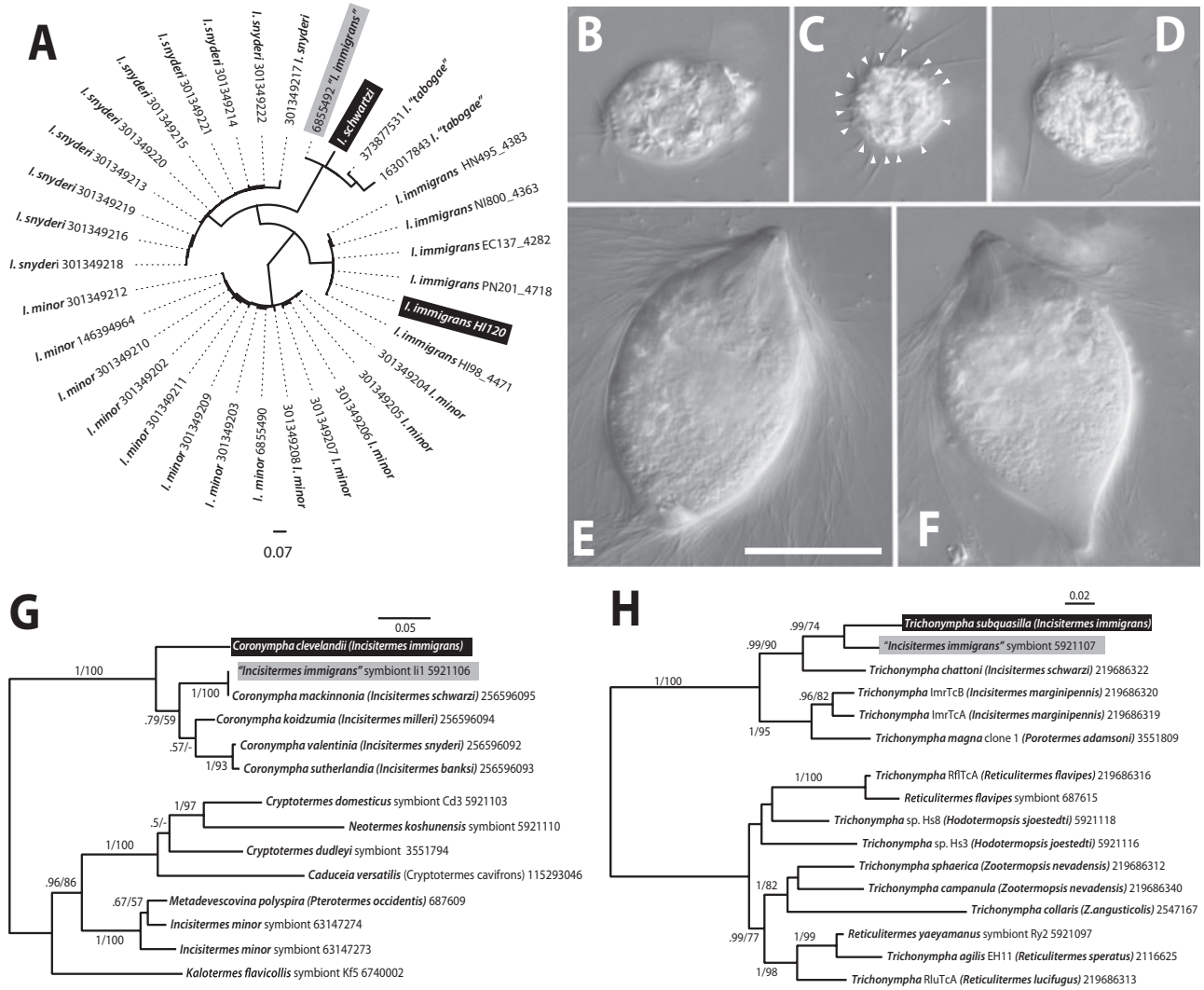

Fig 1. Characterizing I. immigrans and its parabasalian symbionts. (**A**) Mitochondrial LSU rRNA barcode for I. immigrans collected from its type locality on the Hawaiian Island of Oahu (white on black) shares 97–100% identity with other I. immigrans barcodes (for collection data on I. immigrans isolates, see methods). In contrast, the Japanese "I. immigrans" (black on gray) shares 94% identity with I. schwarzi from Florida (white on black) and 94–97% identity with synonymous I. tabogae isolates. (**B**–**D**) Differential interference contrast micrographs of C. clevelandii, showing the overall shape of the cell (B & D) and its 16-fold karyomastigont symmetry (C, triangles each corresponding to one of 16 robust recurrent flagella), matching previous descriptions. (**E** & **F**) Differential interference contrast micrographs of T. subquasilla showing the overall appearance of the cell. All micrographs are to the same scale and the scale bar is 50 μm. (**G** & **H**) Maximum likelihood SSU rRNA phylogeny showing the relationship of C. clevelandii and T. subquasilla to other members of their respective genera. (F) Coronympha clevelandii (white on black) is sister to all other sampled Coronympha species, and the Japanese "I. immigrans" environmental sequence (black on gray) is related to Coronympha from I. schwarzi. (G) Trichonympha subquasilla (white on black) is sister to T. chattoni from I. schwarzi, and the "I. immigrans" environmental sequence (black on gray) is distantly related to T. subquasilla.

There are several possible explanations for this incongruence. First, it is possible that *I. schwarzi* harbors additional cryptic *Trichonympha* species. We tested this by characterizing 10 sequences amplified from barcode-verified *I. schwarzi* hindguts using *Trichonympha*-specific primers, but only found sequences branching with *T. chattoni* (within 98%: not shown), and no sequence similar to *T. subquasilla*. It is also possible that the "*I. immigrans*" data from Japan come from more than one colony or a mixed colony, such that the termite and *Coronympha* data come from *I. schwarzi* and the *Trichonympha* data from another as yet unidentified termite (even though it branches with *T. subquasilla*, the two sequences are quite distant suggesting it did not come from *I. immigrans*). However, the most interesting possibility is that the symbiont community is mixed. *Incisitermes schwarzi* is a Caribbean Basin species that may have been introduced to Japan; if this nonendemic species has acquired symbionts

from closely related native termites, it would be an interesting case to explore further.

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