1. INTRODUCTION

Wood-feeding cockroaches in the genus Cryptocercus and phylogenetically basal lower termites depend on gut symbiotic flagellates for cellulose digestion (Inoue et al. 2000). This symbiotic relationship is considered a key element in the evolution of social behaviour in their hosts. However, there has been controversy concerning the origin of these symbiotic flagellates. Here, molecular sequences encoding small subunit rRNA and glyceraldehyde-3-phosphate dehydrogenase were identified in the symbiotic flagellates of the order Trichonymphida (phylum Parabasalia) in the gut of Cryptocercus punctulatus and compared phylogenetically to the corresponding species in termites. In each of the monophyletic lineages that represent family-level groups in Trichonymphida, the symbionts of Cryptocercus were robustly sister to those of termites. Together with the recent evidence for the sister-group relationship of the host insects, this first comprehensive study comparing symbiont molecular phylogeny strongly suggests that a set of symbiotic flagellates representative of extant diversity was already established in an ancestor common to Cryptocercus and termites, was vertically transmitted to their offspring, and subsequently became diversified to distinct levels, depending on both the host and the symbiotic lineages.

Keywords: Cryptocercus; termite; symbiosis; Parabasalia; symbiont diversification; evolution of social behaviour

Cryptocercus cockroaches and lower termites harbour obligate, diverse and unique symbiotic cellulolytic flagellates in their hindgut that are considered critical in the development of social behaviour in their hosts. Among them, Trichonymphida members (commonly trichonymphids) are abundant in the gut of Cryptocercus as well as many termite species.

Since the elaborative description of the flagellates in the gut of Cryptocercus cockroaches (Cleveland et al. 1934), there has been controversy over the evolutionary origin of the symbionts. Most notably, in Proc. R. Soc. B during the early in 1990s, Thorne (1990, 1991) and Nalepa (1991) debated the relative merits of two hypotheses: that these symbionts had been inherited from a common ancestor of Cryptocercus and lower termites or had been secondarily transferred from one taxon to the other. Phylogenetic analyses encompassing both host insects and symbiotic flagellates were necessary to resolve the controversy. Recent rigorous phylogenetic studies on the hosts have confirmed the sister-group relationship between Cryptocercus and termites, with this clade embedded within cockroach lineages (Lo et al. 2000; Inward et al. 2007; Ware et al. 2008); this is also supported by the parallel phylogeny of intracellular bacteria (Blattabacterium spp.) vertically transmitted via the eggs (Lo et al. 2003). These observations imply the inheritance of symbiotic flagellates in their common ancestor. However, no comparative phylogenetic study on the gut flagellates in these hosts has been reported, due to the absence of a comprehensive investigation on Cryptocercus symbionts. Although recently the phylogenetic positions of some Cryptocercus symbionts have been investigated using molecular data (Heiss & Keeling 2006; Carpenter & Keeling 2007), the evolutionary relationship of these protists with their host insects has not been addressed. Since some trichonymphid genera such as Trichonympha and Eucomonympha are shared in

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the extant hosts, a possibility of lateral transfer of some restricted symbiont taxa remained. Furthermore, there is significant diversity amounting to at least 16 species in 7 hypermastigid genera in the Cryptocercus gut. Termites also harbour hypermastigid diversity comprising nearly 25 genera (Yamin 1979). Table 1 lists representative flagellate genera in Cryptocercus and termites. Many of these symbiont genera are specific to either host group, implying their parallel diversification. Nevertheless, the origin of this diversity is crucial in understanding their evolutionary history. The reconstruction of a reliable phylogeny of the symbionts is necessary to address these questions.

In this study, molecular sequences of the parabasalid symbionts, particularly those in Cryptochnida, were investigated in Cryptocercus punctulatus, and compared with those from termites, in order to test whether the symbionts have been inherited from an ancestor common to the hosts, and to address the evolutionary origin of the extant diversity of trichonymphids.

2. MATERIAL AND METHODS

The collection of Appalachian C. punctulatus and the Japanese termites Hodotermopsis spoestdi and Reticulitermes speratus, and their maintenance in the laboratory, were described previously (Noda et al. 2006). DNA in the gut microbial community of C. punctulatus was extracted, purified and used for polymerase chain reaction (PCR) amplification of small subunit (SSU) rRNA gene with primers for eukaryotes as described previously (Ohkuma et al. 2000). The amplification products of expected size for parabasalids (approx. 1.5 kbp), but not for oxymonads (above 2.0 kbp), were gel fractionated, purified and cloned into pCR2.1-TOPO vector (Invitrogen). Sixty-three clones were sequenced and sorted as described previously (Ohkuma et al. 2000), and only representative sequences were used for phylogenetic inference. The flagellate species in C. punctulatus were identified by their morphological characters. The cells of trichonymphid species of typical morphology (Trichonympha acuta, Eucomonympha imla, Urynympha talea and Barbulanympha sp.) were manually isolated under a microscope equipped with a micromanipulator (CellTram and Eppendorf) and used for subsequent PCR and reverse transcription-PCR (RT-PCR) of SSU rRNA gene. The PCR using 5–20 manually isolated cells was performed as described previously (Ohkuma et al. 2005). A single cell was usually used for RT-PCR (otherwise 20 cells), with reverse transcription using primer Euk1772H and subsequent PCR with primers Euk18 and Euk1627 (Ohkuma et al. 1998, 2000). The gene sequences encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by RT-PCR from a single isolated cell in each of the total seven flagellate species and analysed as described previously (Ohkuma et al. 2007a); these species were T. acuta and E. imla in C. punctulatus, two Trichonympha spp. and two Eucomonympha spp. in H. spoestdi, and Teranympha mirabilis in R. speratus. The database accession numbers of the DNA sequences determined in this study are AB443588–AB443609.

Fluorescence in situ hybridization (FISH) for the identification of trichonymphid species was performed according to Noda et al. (2006). The sequence specific probes used in this study were: 5′-TGCCGCTCCATGGAATCCTG-3′ for Cp20, 5′-TGCTTAGTTGCATAAGCGATTTC-3′ for Cp38, 5′-ATCCAAATCAGGATTGC-3′ for Cp07, 5′-GCTAGTTGCGTTGATACGACAT-3′ for Cp13, 5′-GCTAGTACCGCTAAATT3′ for Cp26, and 5′-TGCTAGTTTGTGTAGAAATT-3′ for Cp49. Each of these probes was 5′-labelled with 6-carboxyfluorescein and used for FISH simultaneously with a probe for all eukaryotes (Ohkuma et al. 1998) 5′-labelled with Texas-Red.

The new sequences were added to the pre-existing alignments (Ohkuma et al. 2005, 2007a,b) and manually refined by juxtaposing conserved secondary structure in the case of SSU rRNA gene. For the SSU rRNA gene sequences of Pseudotrichonympha species, only three among 16 sequences from different termite species were used in the analyses; these 16 sequences were closely related and formed a robust monophyletic group (Noda et al. 2007). The maximum-likelihood (ML) tree was inferred with PHYML2.4.4 (http://atgc.lirmm.fr/phyml/) using the general-time reversible model with gamma-distributed rate variation and a proportion of invariant sites. Gamma shape parameter and fraction of invariant sites were estimated from the data. Bootstrap values were obtained from 100 replicates. Bayesian analysis was performed with MrBayes v. 3.1.2 (http://mrbayes.ebc.uu.se/mrbayes/) using the same model as described above. Three hot and one cold Markov chains were run in duplicate, each from a random starting tree for 2 000 000 generations sampled every 1000 generations with burn-in values set at 200 000 generations. GAPDH amino acid sequences were also analysed with PHYML and MrBayes as described above using the JTT substitution model with gamma distributed rate variation and a proportion of invariant sites. Differences in alternative tree topology were compared by the approximately unbiased (AU) test implemented in CONSEL (http://www.is.titech.ac.jp/~shimo/prog/consel/). The alternative tree topologies were obtained by Bayesian inference under constraint of considered taxa. The sets of GAPDH sequences examined for the monophyletic constraints were as follows: (i) CpT20 and CpT23 of T. acuta, Trichonympha agilis and Trichonympha p. HrT29; (ii) CpT20 and CpT23 of T. acuta and Trichonympha sp. HrT36; (iii) all the four in the genus Eucomonympha; (iv) all the four in the genus Eucomonympha
and T. mirabilis; and (v) CpT20 and CpT23 of T. acuta, and CpE21 and CpE23 of E. imla (all the Cryptocercus symbionts). The constraints of the sets (i) and (ii) were used for evaluating the nesting of cockroach Trichonympha within termite Trichonympha. The inference under the set (iv) constraint resulted in the basal position of P. grassii in the Eucomonymphidae plus Teranymphidae group.

3. RESULTS

Thirteen representative sequences of parabasalian SSU rRNA gene were obtained from C. punctulatus. The FISH experiments using sequence-specific probes (figure 1), and/or the clonal analyses of PCR or RT-PCR products of manually isolated trichonymphid cells, identified the species origins of these sequences; these were Trichonympha acuta, Trichonympha sp., Urinympha talea, Barbulanympha uvalula, two Barbulanympha spp. and Eucomonympha imla. Although the sequence of E. imla was identified only from the manually isolated cells, the sequence showed 96 per cent identity to that recently reported for this species (Carpenter & Keeling 2007). These sequences were phylogenetically analysed along with published sequences for trichonymphids in diverse termites (Ohkuma et al. 2005 and references therein; Noda et al. 2007; see also figure 2 for termite taxa sampled).

In the phylogenetic tree inferred from the SSU rRNA gene sequences (figure 2), four sequences from Cryptocercus, including two identified Trichonympha sequences, formed a robust monophyletic lineage. This Trichonympha lineage was a sister to Trichonympha sequences from five termite genera in evolutionarily diverse positions, although the monophyly of all the Trichonympha sequences was only weakly supported (ML bootstrap value/Bayesian posterior probability is 74/46). The sequences of Barbulanympha and Urinympha (both belong to Hoplonymphidae) formed a robust monophyletic lineage that was sister to Hoplonymphida, the only Hoplonymphidae genus known in termites. Eucomonympha, Pseudotrichonympha (both belong to Eucomonymphidae) and Teranymphia (Teranymphidae) formed a monophyletic group; however, the nested position of E. imla was dubious. The grouping of Trichonympha was robustly supported (100/100) in a large phylogenetic tree using a broad sampling of parabasalian taxa (data not shown), in which the root of Trichonympha located at the node dividing (Trichonymphidae + Staurojenina) and (Hoplonymphidae + Eucomonymphidae + Teranymphia) when the other parabasalian taxa were treated as out-groups and the monophyletic groups described above were confirmed. In this large tree, the other four sequences from Cryptocercus were found to be close relatives of Hexamastix spp. from reptiles (Hampl et al. 2004) and three unidentified sequences from termites (clones Cbre1, Gf8 and Cd5) (Keeling et al. 1998; Ohkuma et al. 2000).

The sequences of Trichonympha and Eucomonympha from Cryptocercus were extremely divergent and showed very long branches in the tree, which was in clear contrast to Trichonympha in termites and to Barbulanympha and Urinympha in Cryptocercus. These divergent sequences were considered to encode authentic rRNA because near-identical sequences were obtained from RNA by RT-PCR. Base frequency among the trichonymphid sequences was homogeneous (χ²-test; p = 0.996, χ² = 46.33). Artificial inference known as long-branch attraction (Felsenstein 1978) was seemingly not the case because step-wise exclusions of these taxa resulted in no substantial change of tree topology with slight increase in support values for some nodes (data not shown).

Since some phylogenetic relationships involving Trichonympha and Eucomonympha in Cryptocercus were poorly resolved in the SSU rRNA gene tree, the relationships of these symbionts between Cryptocercus and termites were investigated in the more robust analysis using another molecular sequence, GAPDH. The GAPDH sequence has been shown to be useful as a phylogenetic marker in Parabasalia despite the presence of multiple sequences in the genome (Gerbod et al. 2004; Ohkuma et al. 2007a). The GAPDH gene sequences were identified from
Figure 2. Unrooted ML tree inferred from nuclear SSU rRNA gene sequences of trichonymphids, using 1142 unambiguously aligned positions. The sequences in bold were obtained from trichonymphids in the gut of Cryptocercus. The name of host termite species is shown in parentheses after each flagellate taxon. The family-level groups and sequences of termite Trichonympha were indicated with vertical bars. The accession numbers of the sequences of the symbionts in termites have been reported in Ohkuma et al. (2001). It is becoming increasingly

4. DISCUSSION

This is the first molecular phylogenetic study comprehensively comparing trichonymphid flagellates between Cryptocercus and termites. The results indicate that Cryptocercus symbionts probably have a sister-group relationship to the corresponding symbionts of termites in each group of Trichonympha. The nesting of Cryptocercus symbionts within clades of corresponding termite symbionts was rejected. Since the host Cryptocercus is phylogenetically sister to termites, the results strongly suggest that a set of trichonymphid flagellates that correspond to ancestors of extant Trichonymphidae, Hoplonymphidae and Eucomonymphidae plus Teranymphidae was established in the common ancestor of the hosts and vertically transmitted to offspring. One line of evidence supporting this hypothesis is that species in the genus Trichonympha, one of the few genera common to Cryptocercus and termites, are clearly differentiated. Trichonympha cells in Cryptocercus all possess a nuclear sleeve that is absent in termite Trichonympha species; they also differ in their capacity for forming cysts (Kirby 1947).

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recognized that complexity of social behaviour can be associated with the mode of acquisition of symbionts (Lombardo 2008).

The strict vertical transmission of trichonymphids suggested in this study implies that the set of flagellate species in the common host ancestor represents the origins of present-day diversity of symbionts; the inherited symbionts subsequently diversified within each host lineage. The extent of the species diversification, however, depends on the group of symbiotic flagellates. Species of Trichonympha are rich in both Cryptocercus and termites. In the case of Hoplonymphidae, Trichonympha stably harbours Cryptocercus species. Among molecularly yet-uncharacterized genera in Cryptocercus and Idionympha (Staurojoeninidae) is probably sister to Staurojoenina in termites. Leptospironympha and Macrospironympha were originally described as belonging to Spirotrichonymphidae (Spirotrichonympha; Cleveland et al. 1934); however, they are now classified into Spirotrichosomidae (Spirotrichonympha), none of which have been investigated by their molecular sequences. Prolophomonas was formerly classified as Lophomonadida (included in the previous Hypermastigida), but now Lophomonadida is reclassified into Cristamonadida (Brugerolle & Patterson 2001). Because molecular phylogeny and traditional classification is often incongruent in Parasalalia (Hampi et al. 2004; Ohkuma et al. 2005; Noël et al. 2007), some uncertainties as to their evolutionary positions remain. Since Spirotrichonympha and Cristamonadida exclusively comprise symbionts of termites, the yet-uncharacterized genera in Cryptocercus discussed above are possible candidates for sister taxa to these orders found in termites. Of course, this possibility is somewhat speculative and unfortunately we failed to detect sequences likely to have derived from these genera in C. punctulatus. Future molecular identifications of these are of significant importance for understanding the origin and evolution of these orders. The situation of the oxymonads, which are also unique to Cryptocercus and

Figure 3. ML tree of GAPDH sequences showing sister relationships of trichonymphid genera between Cryptocercus and termites. The tree was inferred from 324 amino acid positions aligned unambiguously. The parabasalid sequences outside Trichonymphidae were used as out-groups owing to the clear dichotomy between Trichoephyllum and the other parabasalids as well as the most likely root position of parabasalids at the node dividing these groups (Ohkuma et al. 2007a,b). The sequences of trichonymphids in C. punctulatus are shown in bold. The family-level groups (T: Trichonymphidae, E: Eucomonymphidae plus Teranymphidae) and the orders Spirotrichonymphida (S) and Cristamonadida (C) are indicated with vertical bars. The accession numbers of the other sequences have been reported in Gerbod et al. (2004) and Ohkuma et al. (2007a,b). Bayesian posterior probability and PHYML bootstrap value (divided by slash) are indicated in each node. Asterisks at nodes indicate full support (100/100). Scale bars indicate 0.10 substitutions per position.

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Carpenter & Keeling 2007) and confirmed here, Eucomonymphus spp. in termites are distantly related to E. imla in Cryptocercus, and rather closely related to Teranympha. Indeed, the monophyly of Eucomonymphus was rejected by the AU tests with the GAPDH data as well as SSU rRNA gene data (each p<0.01). This indicates that the acquisition of Eucomonymphus by a lateral transfer between Cryptocercus and termite lineages is unlikely. The results also suggest that the morphological characteristics that differentiate Teranympha (and probably Pseudotrichonympha) from Eucomonymphus are autapomorphies.

Associations of bacteria with the symbiotic flagellates are a prominent feature in the gut microbial community of termites and play important roles in the efficient usage of cellulose for both host flagellates and termites (Hongo et al. 2008; Inoue et al. 2008; Ohkuma 2008). Members of the Holoannelidae possess ectosymbiotic bacteria attached to the surface of the flagellate cells and, as previously reported (Noda et al. 2006), these ectosymbionts form a monophyletic lineage in the order Bacteroidales. Many species of Trichonympha in termites harbour endosymbiotic bacteria belonging to the candidate phylum ‘Termite group 1’ (Ikeda-Ohitsu 2007; Ohkuma et al. 2007b; Hongo et al. 2008) and Pseudotrichonympha species examined so far harbour endosymbiotic Bacteroidales bacteria (Noda et al. 2005, 2007). However, no abundant association of endosymbiotic bacteria was observed in Trichonympha spp. and E. imla in Cryptocercus when they were examined by microscopic observation after DNA staining, although some ectosymbiotic bacteria were present as described previously (Carpenter & Keeling 2007). The endosymbionts were probably acquired only in the flagellate lineages in termite guts after the divergence of the host insects. These observations also support the host lineage-dependent evolution of flagellate symbionts.

It is remarkable that Cryptocercus cockroaches retain more diverse flagellate species than any extant termite species. Among molecularly yet-uncharacterized genera in Cryptocercus, Idionympha (Staurojoeninidae) is probably sister to Staurojoenina in termites. Lepotospironympha and Macropymnomena phyla were originally described as belonging to Spirotrichonymphidae (Spirotrichonympha; Cleveland et al. 1934); however, they are now classified into Spirotrichosomidae (Spirotrichonympha), none of which have been investigated by their molecular sequences. Prolophomonas was formerly classified as Lophomonadida (included in the previous Hypermastigida), but now Lophomonadida is reclassified into Cristamonadida (Brugerolle & Patterson 2001). Because molecular phylogeny and traditional classification is often incongruent in Parasalalia (Hampi et al. 2004; Ohkuma et al. 2005; Noël et al. 2007), some uncertainties as to their evolutionary positions remain. Since Spirotrichonympha and Cristamonadida exclusively comprise symbionts of termites, the yet-uncharacterized genera in Cryptocercus discussed above are possible candidates for sister taxa to these orders found in termites. Of course, this possibility is somewhat speculative and unfortunately we failed to detect sequences likely to have derived from these genera in C. punctulatus. Future molecular identifications of these are of significant importance for understanding the origin and evolution of these orders. The situation of the oxymonads, which are also unique to Cryptocercus and

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termites, seems to be similar. The reported SSU rRNA gene sequences of the oxymonads in Cryptocerus (Sacchnobaculidae) showed a sister-group relationship to Pyrsonymphidae and Oxymonadidae species in termites; however, the resolution of phylogenetic relationships of these three families was poor (Heiss & Keeling 2006). The analysis of protein-encoding genes has already started in oxymonads of Cryptocerus (de Koning et al. 2008), but studies of those from termites are still limited. Considering that Cryptocerus probably harbours the descendants of the original set of symbiotic flagellates that represent their extant diversity, Cryptocerus is an important ‘model taxon’ not only for termite evolution (Nalepa 1988, 1994; Klass et al. 2008) but also for the evolution of Parabasalia and Oxymonadida as well.

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