SHORT COMMUNICATION

Symbiotic nitrogen fixation in the New Zealand dampwood termite (*Stolotermes ruficeps*)

Nicola M. Reid* and Gareth Lloyd-Jones
Scion, Private Bag 3020, Rotorua Mail Centre, Rotorua 3046, New Zealand
*Author for correspondence (Email: nicki.reid@scionresearch.com)

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Abstract: This study investigates symbiotic microorganisms in the New Zealand dampwood termite *Stolotermes ruficeps* using culture-independent techniques to describe the diversity of nitrogen-fixing organisms within this termite. Phylogenetic analysis of a portion of the *nifH* gene (encoding dinitrogenase reductase) revealed 19 phylotypes (>98% sequence identity) with 77–86% similarity to published nucleotide sequences from uncultured microorganisms described from termite guts. The majority of sequences obtained in this study were most closely related to sequences obtained from basal families Kalotermitidae, Termopsidae and the closely related wood-feeding cockroach species *Cryptocercus*. This adds to the growing amount of evidence suggesting that the composition of *nifH* sequences is characteristic of a termite family. This study also identifies wood-dwelling termites as a potentially important source of nitrogen input into temperate forests, something previously neglected and warranting further investigation.

Keywords: gut community; *NifH*; Termopsidae; wood decay; woody debris

Introduction

Termites thrive in tropical terrestrial ecosystems and play an important part in the bio-recycling of lignocellulose as the major decomposers of woody material. Due to the low nitrogen content of wood (approximately 0.03–0.15%) a supply of useable nitrogen is essential for wood decomposition (Potrikus & Breznak 1981). A group of microorganisms living within the gut of termites fix atmospheric nitrogen and thus supply the nitrogen required by the termite and its symbionts to consume wood material. Nitrogen has also been shown to be extensively recycled through proctodeal trophallaxis by dampwood termites (Machida et al. 2001).

More than 2600 species of termites are recognised (Inward et al. 2007) and their distribution is predominantly tropical (Eggleton 2000). New Zealand with its temperate climate has three endemic termite species: *Stolotermes ruficeps, S. inopinus* and *Kalotermes brouni*. The role of these New Zealand termites in wood decomposition and the nitrogen-fixing microorganisms associated with them have not been well documented, most likely due to their low impact (neither mound builders nor house-demolishers) and minor economic importance to the timber industry (J. Bain, Scion, Rotorua New Zealand pers. comm.). *Stolotermes ruficeps*, the most common of the New Zealand termites, is widespread in forests, inhabiting only dead and decaying standing trees, branch stubs, logs and stumps of a wide variety of native hosts, and decaying wood of introduced *Pinus* species (Bain & Jenkin 1983; Milligan 1984). *S. ruficeps* is a wood feeder only and exhibits a 'one-piece' life type, nesting and feeding in the same piece of decayed wood (Milligan 1984).

Stolotermes is a relict genus of modern termites within the basal clade of the Termopsidae. Termopsidae constitute a small family of termites containing 4–5 extant genera with 13–20 living species. The placement of *Stolotermes* in Termopsidae has been disputed recently on both genetic and morphological grounds (Klass et al. 2000; Inward et al. 2007; Sillam-Dussès et al. 2007). *Stolotermes* have retained many plesiomorphic characters such as a well-developed, pigmented, compound eye (Thorne & Lenz 2001) and very primitive worker/imago dentition (Emerson 1942; Ahmad 1950). In comparison with other families, the Stolotermitinae have not been well examined, and taxonomic relationships are unclear.

Gut microorganisms are specific symbionts that have co-evolved with termites. By examining the phylogeny of gut microorganisms in different termites the co-evolution of termites and their gut microorganisms can be determined (Hongoh et al. 2005). Nitrogen-fixing symbionts represent a physiologically distinct group of bacteria in the termite

gut. The amount of nitrogen supplied by the diet of solely wood-feeding termites such as *Stolotermes* is very low and for this reason nitrogen fixation should be more important to achieve a physiological C:N balance than for other life types that have a soil, plant litter or mixed-wood diet (Tayasu et al. 1997).

The microbial population in the termite gut is not easily cultured and so we used a culture-independent approach to phylogenetically identify the nitrogen-fixing microorganisms in *S. ruficeps* by analysing the *nifH* gene. The *nifH* gene encodes an essential iron-containing component of nitrogenase, and it is widely used as the basis for investigating the diversity of nitrogen-fixing organisms in a variety of natural samples (reviewed in Zehr et al. 2003). The nitrogenase enzyme complex catalyses the fixation of atmospheric dinitrogen (N_2), which is converted to ammonia. This approach has been used to examine *nifH* diversity in the gut of approximately 20 species of termites isolated mostly from one biogeographical region (Asia) (Ohkuma et al. 1996; 1999; Noda 1999; Yamada et al. 2007); in these studies, two species in the Termopsidae were examined, neither belonging to the genus *Stolotermes*. The NifH sequences were found to cluster in groups that corresponded to those from the guts of other closely related termites indicating a correlation between both host taxonomic position and lifestyle and NifH community phylogeny (Yamada et al. 2007).

This study describes the phylogenetic diversity of the *nifH* genes in the gut microbial community of *S. ruficeps* revealing the diversity of its nitrogen-fixing microorganisms. This study also highlights a valuable source of nitrogen input into temperate forests by nitrogen-fixing symbionts hosted by non-tropical, wood-degrading termites and representing a basal lineage amongst extant termites.

Materials and methods

Termite nymphs were collected from a colony in a decaying *Pinus radiata* log from Whakarewarewa Forest at latitude 38.5°13′ S and longitude 176°00′ E, in the Bay of Plenty region, North Island, New Zealand.

Five whole nymphs were thoroughly washed to remove surface cells and homogenised whole in 200 µl of phosphate buffer (100 mM), 200 µl of SDS lysis buffer (3% SDS, 0.5-M Tris pH 8, 0.1-M NaCl), 200 µl of chloroform-isoamyl alcohol (24:1; vol/vol), and a mixture of silica–zirconium beads (0.5 g of 0.1-mm beads and 0.5 g of 3.0-mm beads) and shaken in a FastPrep machine (Bio 101, Vista, CA) at 4.5 m s^{-1} for 40 s. To this homogenate 200 µl of TE buffer (10-mM TrisCl, 1-mM EDTA, pH 8), 100 µl of 5-M NaCl, and 80 µl of CTAB/NaCl (10% CTAB, 0.7-M NaCl) were added and mixed by vortexing, followed by incubation at 65°C for 60 min. The mixture was then extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1; vol/vol/vol) and then with chloroform–isoamyl alcohol (24:1). Bulk nucleic acids were precipitated from solution with twice the volume of ethanol and collected by centrifugation, rinsed with 70% ethanol, and resuspended in 30 µl of sterile water. DNA was purified using the Wizard® DNA Clean-up System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The *nifH* gene was amplified from the extracted DNA using the universal *nifH* PolF and PolR PCR primers, cloned, sequenced and analysed as described previously (Bowers et al. 2008) except the PCR products were ligated into pCR-TOPO and transformed into *Escherichia coli* One Shot TOP10 competent cells using the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmids were prepared using the PureLink Hipure Plasmid Filter Midiprep Kit (Invitrogen). The sequences obtained in this study have been deposited in GenBank as accession numbers EU791899 to EU791917.

Results

A total of 29 *nifH* nucleotide sequences were obtained and compared with other previously described *nifH* sequences. Comparison with the GenBank database showed that the closest matches for the majority of clones (26/29) were with *nifH* gene sequences derived from other uncultured termite gut symbionts. In general the similarity of matches on the basis of nucleotide sequences was low (77–86%).

The *nifH* sequences were translated to the amino acid sequence and aligned using CLUSTAL_X (version 1.81; Thompson et al. 1997) and analysed using PHYLIP (version 3.65; Felsenstein 1989) and the furthest neighbour method in DOTUR (Schloss & Handelsman 2005). A phylogenetic tree was inferred from the translated nucleotide sequences, with 19 phylotypes (defined with 98% sequence identity) (Table 1). Rarefaction analysis revealed that diversity at phylotype definitions < D = 0.57 sequence difference was undersampled. Rarefaction curves showed no tendency for the rate of accumulation of new phylotypes to level off until the 57% level (Fig. 1). This implies that the sequencing of additional *nifH* amplicons would lead to the capture of additional NifH phylotypes from other *S. ruficeps* gut symbionts. This is consistent with a Chao1 richness estimator of 34 (95% CI = 25–77) for unique sequences and 31 (95% CI = 20–72) for D = 0.08.

Discussion

The presence of nitrogen-fixation genes is accepted as a marker for nitrogen fixation, therefore it is likely that nitrogen fixation is occurring in the gut of *S. ruficeps*.

Table 1. Phylogenetic assignation of *NifH* clones found in the gut of New Zealand *Stolotermes ruficeps*.

Clone[a]	GenBank accession no.	*NifH* phylogenetic group	Closest match		
			AA Id (%)	Host	Host family
c134, c4	EU791903	II	96[e]	*Zootermopsis nevadensis*	Termopsidae
c12	EU791904	III-3	98[c]	*Zootermopsis angusticollis*	Termopsidae
c131	EU791905	III-3	100[d]	*Zootermopsis angusticollis*	Termopsidae
c31, c17, c151	EU791906	III-3	99[c]	*Zootermopsis angusticollis*	Termopsidae
c11, c23	EU791910	III-3	99[b]	*Cryptotermes punctulatus*	Cryptocercidae
c10	EU791909	III-3	97[b]	*Cryptotermes punctulatus*	Cryptocercidae
c9	EU791913	III-3	96[b]	*Cryptotermes punctulatus*	Cryptocercidae
c98, c111	EU791914	III-3	96[b]	*Cryptotermes punctulatus*	Cryptocercidae
c20	EU791912	III-3	97[b]	*Cryptotermes punctulatus*	Cryptocercidae
c152	EU791911	III-3	97[b]	*Cryptotermes punctulatus*	Cryptocercidae
c15	EU791907	II	97[b]	*Cryptotermes punctulatus*	Cryptocercidae
c3	EU791908	II	96[b]	*Cryptotermes domesticus*	Kalotermitidae
c1, c2	EU791902	II	94[b]	*Glyptotermes fuscus*	Kalotermitidae
c133	EU791901	II	99[e]	*Zootermopsis angusticollis*	Termopsidae
c16	EU791915	III-2	90[b]	*Cryptotermes punctulatus*	Cryptocercidae
c32	EU791916	III-2	90[b]	*Nasutitermes dimorphus*	Nasutitermitinae
c65	EU791917		92[b]	Mine spoilings	-
c14, c27, c24	EU791899	I	94[b]	*Neotermes koshunensis*	Kalotermitidae
c21, c8, c28	EU791900	I	93[b]	*Neotermes koshunensis*	Kalotermitidae

[a] Clonal groupings based on ≥ 98% match
[b] Closest match was dinitrogenase reductase (uncultured nitrogen-fixing bacterium)
[c] Closest match was dinitrogenase reductase (*Treponema primitia* ZAS-2)
[d] Closest match was dinitrogenase reductase (*Treponema azotonutricium* ZAS-9)
[e] Closest match was dinitrogenase reductase (*Treponema denticola* ZAS-1)

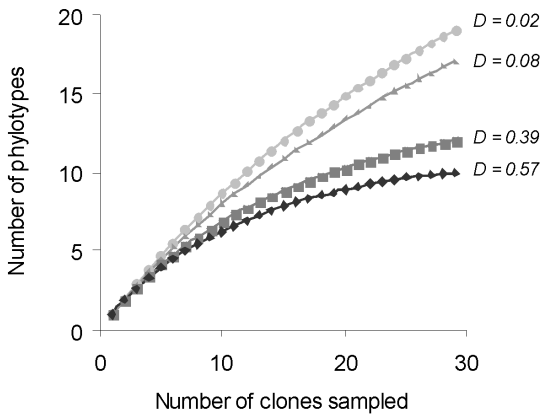

Figure 1. Rarefaction curves of the observed number of NifH phylotypes in New Zealand *Stolotermes ruficeps* at various evolutionary distances. Rarefaction curves were constructed based on analyses performed in DOTUR (Schloss & Handelsman 2005).

The majority of clones obtained (24/29) were related to the 'KTC' group, a group defined by Yamada et al. (2007) as containing microorganisms from the guts of termites belonging to the basal families (Kalotermitidae, Termopsidae as well as *Cryptocercus*). Termites and *Cryptocercus* species evolved from a common wood-feeding cockroach ancestor (Inward et al. 2007). The placement of the New Zealand termite in this grouping is in agreement with the taxonomy and lifestyle of *S. ruficeps*. Recent research demonstrates a correlation between NifH phylogeny and termite taxonomic grouping suggesting co-evolution (Ohkuma et al. 1999; Hongoh et al. 2005; Yamada et al. 2007). Hongoh et al. (2005) states that 'congeneric termites harbor very similar bacterial gut microbiota, irrespective of the individual, colony, location and host species' and the symbiotic relationship between gut bacteria and their host termites is very stable and strong. In termites, proctodeal trophallaxis (shared feeding) allows for the transmission of gut microbiota between individual members. Gut symbionts are carried over from the mother nest to a newly founded termite colony by the alates (king and queen), ensuring the inheritance of a conserved gut microbial population (Machida et al. 2001).

Only three of the four widely accepted NifH clusters were represented: the proteo-cyano cluster (I), the alternative NifH (anf-methano) cluster (II), and the anaerobe cluster (III) (Chien & Zinder 1994; Ohkuma et al. 1999; Zehr et al. 2003). The anaerobe cluster (III) was divided into sub-clusters according to the classification of Ohkuma et al. (1999). No clones grouped within Cluster IV (consisting of 'pseudo NifH' sequences). The majority of clones (15/29), representing 11 phylotypes, were identified as belonging to Cluster III, the anaerobe NifH group. NifH sequences from the so called 'lower termites' have been shown to group mostly in Clusters II and III (Ohkuma et al. 1999). Six families (including the Termopsidae), comprising 40% of known termite species, are classified as 'lower termites'.

Only the termites of 'one-piece' life type harbour Fe-only alternative nitrogenases (Cluster II) that, unlike ordinary nitrogenases, do not use a molybdenum metal cofactor (Yamada et al. 2007). Molybdenum concentrations are low in wood debris and the seven clones (five phylotypes) of Fe-only nitrogenases (encoded by the *anf* gene) found in this study may reflect the wood diet of *S. ruficeps*. EU791901 closely matched (99%) the dinitrogenase reductase of *Treponema* ZAS-1 within Cluster II. It is generally thought that Fe-nitrogenase is only expressed under molybdenum and vanadium starvation conditions; however, Noda et al. (1999) demonstrated that a termite Fe-nitrogenase was preferentially transcribed and was not regulated by molybdenum. The Fe-nitrogenases were critical for nitrogen fixation in the termite *Neotermes koshunensis* as they were expressed more often than Mo-containing nitrogenases (Noda et al. 1999). Fe-only nitrogenase sequences may be more ubiquitous than previously thought (Zehr et al. 2003) as *anf* genes have been recently isolated from soil, lignocellulosic wastewater treatment plants and wood chips (Betancourt et al. 2008; Bowers et al. 2008) as well as from estuary and rice root samples (reviewed in Zehr et al. 2003).

Eight clones, representing five phylotypes, had close homology with NifH amino acid sequences obtained from ZAS-1, ZAS-2 and ZAS-9 (Lilburn et al. 2001). The amino acid sequence from EU791905 matched 100% with the nitrogenase of the strict anaerobe *Treponema azotonutricium* ZAS-9. *Treponema* strains ZAS-1, ZAS-2 and ZAS-9 were isolated from the dampwood termite *Zootermopsis angusticollis* (Family Termopsidae) (Leadbetter et al. 1999). *Treponema* species have been shown to exhibit nitrogen-fixing activity, with ZAS-9 exhibiting the greatest specific activity, about 100-fold greater than that of ZAS-1 and ZAS-2 (Lilburn et al. 2001). In one case their contribution to nitrogen fixation was calculated to be as much as 5 ng of N_2 per hour (Lilburn et al. 2001).

Apart from EU791905, none of the amino acid sequences for the NifH protein identified in this study were identical to published sequences and 14 phylotypes had an uncultured nitrogen-fixing bacterium as their closest phylogenetic relative, emphasising the uniqueness of this environment and lack of cultured representatives.

This is the first time the NifH gut community of a termite belonging to this genus has been investigated (Nurse 1945) and the third species amongst the Termopsidae (Yamada et al. 2007). Tighter phylogenetic groupings may be revealed as more species are examined. The rarefaction results indicate that only a portion of the

richness in the *nifH* community (at the ≥ 98% sequence similarity level) was surveyed within the clone libraries. As with other studies of termite guts, the *nifH* sequences that were retrieved showed a low level of homology to published *nifH* sequences. This initial characterisation of the gut microbial community of *S. ruficeps* provides a basis for addressing further questions related to the translocation of nitrogen into decomposing wood, as well as host phylogeny, biogeography and co-evolution of symbionts.

The termite contribution to biologically fixed nitrogen can be estimated by comparing the difference between the nitrogen content of the wood and the nitrogen content of the termite. *S. ruficeps* has an average weight of 2.7 mg dry weight and inhabits colonies of c. 365 ± 175 members (Thorne & Lenz 2001). With body nitrogen contents of 8–13% dry weight (Higashi et al. 1992) a colony of 365 termites must obtain 80–130 mg of nitrogen to support its own biomass in addition to that lost through excretion. A colony of *S. ruficeps* inhabiting *Pinus radiata* wood (which has a nitrogen content of 0.08% and a carbon content of 48.5%) would need to consume over 100 times its body mass of wood to obtain the necessary nitrogen. This is clearly improbable and a symbiotic relationship with nitrogen-fixing bacteria is required to overcome this shortfall. It is believed that 60% of termite biomass nitrogen is obtained from nitrogen fixation (Lilburn et al. 2001).

Extrapolation to ecosystem scales is possible if we are able to estimate the density of *S. ruficeps* colonies within the forest ecosystem. Colonies of *S. ruficeps* are widespread and are found in a range of host wood species (Bain & Jenkin 1983); 50 colonies of *S. ruficeps* were detected at 0.5-km intervals along a 3.5-km transect near Muriwai Beach (Thorne & Lenz 2001). Based on these reports, and our field observations of the ease of discovery of colonies, it is not unreasonable to assume the presence of c. 50 colonies per hectare of *S. ruficeps* in forest ecosystems. By using a range of 1–100 colonies per hectare, we estimate that New Zealand's forest resource of 6.4 million hectares of indigenous forest and 1.8 million hectares of planted forest potentially receives 0.7–110 Mg of nitrogen from *S. ruficeps*. This estimate pays no account of the loss of nitrogen from the termite by excretion. Excreted nitrogen incorporated via the termite frass to the decaying wood may well contribute an additional nitrogen input such as that observed with decay in a New Zealand *Pinus radiata* forest (Garrett et al. 2008). Accordingly it is likely that the recycling of nitrogen through proctodeal trophallaxis in *S. ruficeps* colonies is inefficient. The input of nitrogen by *S. ruficeps* to the nutrient budget of the forest ecosystem probably contributes part of the 'missing nitrogen' input attributed to nitrogen fixation (Chestnut et al. 1999). Since both managed and indigenous forests in New Zealand contain large quantities of standing dead or fallen logs (wood debris), the retention of wood debris may be an important source of nitrogen for sustaining long-term soil productivity.

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