A PHYLOGENETIC AND EVOLUTIONARY STUDY OF ENDOGENOUS CELLULOSE DIGESTION IN HIGHER TERMITES (ISOPTERA:TERMITIDAE)

By

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To my family, for their endless love,
even when I'm at my most unlovable

Family is a haven in a heartless world.

—Christopher Lasch

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Cellulose is the most abundant biopolymer in the world whereas termites are the most important metazoan cellulose processors. Termites are divided into lower and higher termites, with the latter being the most derived and most specious. Although termites are known for their ability to digest wood, members of the family Termitidae (higher termites) are nutritionally diverse in their use of cellulose. There have been numerous studies on the evolution of termites, but the evolution of endogenous cellulose digestion in termites, especially in higher termites, is poorly understood. Endogenously-produced termite cellulases consist of endo-β-1,4-glucanases and β-glucosidases only. Hence, using phylogenetic inferences from mitochondrial (16S) ribosomal RNA, nuclear (28S), endo-β-1,4-glucanase and β-glucosidase sequences, I attempt to explain the evolution of endogenous cellulose digestion in higher termites.

The translated endo-β-1,4-glucanase amino acid sequences obtained during this study showed high similarity to endo-β-1,4-glucanases in the glycosyl hydrolase family 9 (GHF9). The inferred endo-β-1,4-glucanase phylogenetic tree showed congruency with the mitochondrial/nuclear tree, with the fungus-growers being the most basal group and

the soil/litter- and wood/lichen/grass/litter-feeders being the most distal diphyletic feeding groups. The phylogenetic placement of the bacterial comb-grower was determined as the “missing link” between the fungus-growers and the soil/litter- and wood/lichen/grass/litter-feeders. There was also a strong diphyletic relationship between endo-β-1,4-glucanases of upper layer soil-feeders and the other soil-feeders. Within the monophyletic wood/lichen/grass/litter-feeding termites’ clade, the nasutitermitines were polyphyletic and a strong diphyletic relationship was also observed in the most distal groups, the lichen- and the grass/litter-feeders. In some species, I was able to obtain up to four paralogous copies with high degrees of substitutions among them, suggesting different alleles, and subsequently resulting in different gene function.

For β-glucosidase, the deduced amino acid sequences showed that they were similar to β-glucosidases in the glycosyl hydrolase family 1 (GHF1). However, phylogenetic incongruity was observed between the mitochondrial/nuclear and β-glucosidase trees. Instead of being the most basal feeding group, the fungus-growers formed a strong diphyletic relationship with the wood/grass- and soil/litter-feeders. Furthermore, instead of being an intermediary between the basal and derived groups as initially hypothesized, data suggested that bacterial comb-grower β-glucosidases were probably derived from fungus-growers β-glucosidases. Two different sequences from the bacterial comb-feeder suggest the involvement of two different alleles, resulting in different gene functionality. Lastly, while there is a high level of evidence to support the vertical gene transfer hypothesis in GHF9, the evolutionary origin of GHF1 could not be deduced at present.

CHAPTER 1
TERMITE ENDOGENOUS CELLULASES

General Introduction

Termites are eusocial insects comprising over 2,600 described species (Kambhampati and Eggleton, 2000). They are phylogenetically classified into lower (families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, and Rhinotermitidae) and higher termites (family Termitidae). The family Termitidae is recognized as being the most recently evolved and derived family (Miura et al., 1994). While it was suggested that primitive termites originated from the Upper Jurassic period (~161 Ma), the earliest recorded termitid fossil was from the Eocene period (~55.8 Ma) (Thorne et al., 2000). However, Engel et al. (2009) estimated that the family Termitidae arose from the family Rhinotermitidae in Early Paleogene and began radiating in the Late Eocene period (~40 Ma).

The family Termitidae comprises 84% of all termite species and is divided into seven subfamilies: Macrotermitinae, Sphaerotermitinae, Foraminitermitinae, Syntermitinae, Nasutitermitinae, Termitinae and Apicotermitinae (Engel et al., 2009). Although termites are known for their ability to digest wood, members of this family actually exploit a wide variety of feeding substrates, ranging from fungi, bacterial comb, wood, lichen, grass and soil/litter.

Cellulose

The three major components of lignocellulose are cellulose (28-50%), hemicelluloses (20-30%) and lignin (18-30%) (Thompson, 1983). Cellulose is composed of unbranched anhydro-β-1,4-glucose chains linked together by a β-1,4-$_D$-glycosidic bond, which is cleaved by cellulases during cellulose degradation (Han et al., 1995).

According to Brune (2006), the pathway for cellulose digestion is: wood polysaccharides → mono-, di- and oligosaccharides → lactate (ethanol) → acetate/formate → CO_2/CH_4. The penultimate end products of these reactions are acetate (which is used by termites as their energy source) and formate (H_2 and CO_2). Termites mainly utilize the glucose from the breakdown of cellulose and the acetate from the fermentation in the hindgut (Slaytor, 2000). Both H_2 and CO_2 are utilized by endosymbiotic: i) spirochetes to produce acetate; ii) acetogenic bacteria to produce acetate; and iii) methanogenic bacteria to produce methane (Adams and Boopathy, 2005).

Cellulose is the most abundant biopolymer in the biosphere and one of the cheapest resources to solve the problem of chemical and energy production (Sakka et al., 2000). Hence, cellulase can be utilized to manage cellulosic industrial and municipal waste by converting them into useful substances such as ethanol, or acetic acid (Sukhumavasi et al., 1989). Lately, there has been an effort to use termites and/or their endosymbionts as a potential resource of functional genes for industrial applications, where useful cellulolytic, lignolytic and aromatic hydrocarbon degradation genes are proposed for use in environmental solutions, biomass utilization and fine chemicals production (Matsui et al., 2009).

Cellulase and its Functions

There are three types of cellulases: i) endo-β-1,4-glucanases (EG) (1,4-$_D$-glucan 4-glucohydrolase, EC 3.2.1.4); ii) exoglucanase (β-1,4-$_D$-glucan cellobiohydrolase, EC 3.2.1.91) and finally iii) β-glucosidases (BG) (β-$_D$-glucoside glucohydrolase, EC 3.2.1.21) (Han et al., 1995). However, endogenous termite cellulases only consist of endo-β-1,4-glucanases and β-glucosidases. According to Robson and Chambliss (1989), endo-β-1,4-glucanase works by cleaving the internal β-1,4-$_D$-glycosidic bonds at

random. Exoglucanases remove the cellobiose unit from the non-reducing end, while β-glucosidases acts by cutting cellobiose and cello-oligosaccharides to convert them into glucose. Cellulases are members of the glycosyl hydrolase superfamily (GHF), which comprise 125 classified families based on amino acid sequence comparison (CAZy, Carbohydrate-Active enZYmes Database, website: http://www.cazy.org).

Cellulose Digestion in Lower Termites

In lower termites, endosymbiotic protozoans play an important role in the digestion of cellulose (Cleveland, 1923; 1924; 1925). According to Konig et al. (2006), the intestinal microbiota of lower termites consists of a mixture of protozoans, fungi, archaea and bacteria. Termites have a long evolved symbiotic relationship with these gut microbiota, which play important roles in the degradation of cellulose, hemicellulose, and aromatic compounds, as well as in nitrogen fixation (Breznak and Brune, 1994; Brune and Ohkuma, 2011).

Using *Coptotermes formosanus* Shiraki as an example, its symbiotic protozoan *Pseudotrichonympha grassii* Koidzumi decomposes highly polymerized cellulose while *Holomastigotoides* sp. and *Spirotrichonympha leidyi* Koidzumi utilizes low molecular weight cellulose only (Brugerolle and Radek, 2006). Watanabe et al. (2002) proved that protozoan symbionts of *C. formosanus* produced an endo-β-1,4-glucanase homologous to GHF7. Nakashima et al. (2002a) found that crude extracts from both the midgut and hindgut produced sugar and reducing sugar from crystalline cellulose and that GHF7 in the hindgut must have originated from the protozoans because secreting cells were absent there. Nakashima et al. (2002b) also isolated and characterized cellulase genes from *P. grassii* in *C. formosanus* and found that the nucleotide sequences (*PgCBH-homos*) showed similarity to GHF7 and the primary structure was similar to that of

cellulase Cel7A from the aerobic fungus *Trichoderma reesei* Simmons. Later, Inoue et al. (2005) found that *S. leidyi* in *C. formosanus* produced endo-β-1,4-glucanase similar to GHF5.

The site of cellulase secretion for lower termites is the salivary glands (Slaytor, 2000). About 80% of endo-β-1,4-glucanase activity is found in the salivary gland of *C. formosanus*, with its N-terminal amino acid sequence similar to fungal endo-β-1,4-glucanase and cellobiohydrolases from the GHF7, but not from GHF9 (Nakashima and Azuma, 2000). Nakashima et al. (2002a) reported that endo-β-1,4-glucanase much like GHF9 occured in the salivary gland, foregut and midgut of *C. formosanus* which transformed cellulose into cellobiose. Mo et al. (2004) also found high β-glucosidase activity in the *C. formosanus* midgut, which transformed cellobiose into glucose. According to Nakashima et al. (2002a), an independent dual cellulose-digesting system (endogenous and exogenous) occurs in *C. formosanus*. This was supported by Tokuda et al. (2002) in a drywood termite, *Neotermes koshunensis* (Shiraki) and Scharf et al. (2010) in the Eastern subterranean termite, *Reticulitermes flavipes* (Kollar). Nakashima et al. (2002a) also proposed that cellulose was partly ingested through the termite-derived system (endogenous) first, and then the remaining undigested cellulose moved to the hindgut to be further digested by the protozoans (exogenous). According to Nakashima and Azuma (2000), the total localized cellulase activity in the digestive system of *C. formosanus* are 80.8%, 2.4%, 8.9% and 7.9% in the salivary glands, foregut, midgut, and hindgut, respectively. This two-step cellulose degradation method is highly efficient and these termites were able to assimilate >90% of the wood (Ohkuma, 2003).

Cellulose Digestion in Higher Termites

According to Breznak (1984), the intestinal microbiota of higher termites consists of prokaryotes alone. Warnecke et al. (2007) who conducted a major metagenomic study of the microflora in the hindgut paunch of *Nasutitermes* sp. and found 1,750 bacterial 16S rRNA gene sequences that represent 12 phyla and 216 phylotypes supported this. Heterogenous bacterial populations reside within the hindgut of wood-eating higher termites (Anklin-Muhlemann et al., 1995). According to Slaytor (1992), because higher termites do not harbor protozoans, the role of bacteria in cellulose digestion was unclear as both the lower and higher termites produce endogenous cellulases. However, Lenoir-Labe and Rouland (1993) proved the presence of cellulolytic activity of bacteria in *Cephalotermes rectangularis* (Sjoestedt). Tokuda and Watanabe (2007) who showed the presence of endocellulases from symbiotic bacteria in the hindgut of *Nasutitermes takasagoensis* (Shiraki) and *Nasutitermes walkeri* (Hill) confirmed this. Warnecke et al. (2007) reported the presence of multiple sets of bacterial genes responsible for cellulose digestion in the hindgut of *Nasutitermes* sp.. Nevertheless, according to Bignell (2000), it is unproven if symbionts are exclusively responsible for cellulose digestion in termites. Furthermore, Slaytor (1992) found no evidence that exocellulases are necessary for cellulose digestion in termites.

Earlier, Rouland et al. (1988a) purified cellulase I_T, II, 1,4-β-glucan glucanohydrolase and 1,4-β-glucan cellobiohydrolase from *Macrotermes muelleri* (Sjostedt) and its symbiotic fungus *Termitomyces* sp.. According to Rouland et al. (1990), the subfamily Macrotermitinae degrade plant material using double symbiosis with the basidiomycete of the genus *Termitomyces* sp.; exosymbiosis when the termite workers consume pre-digested inferior fungus comb and endosymbiosis when the

Termitomyces sp. within the termite gut further digests cellulose together with termite-derived cellulase and endosymbiont-derived cellulase. Hyodo et al. (2000) confirmed that the role of mutualistic fungus *Termitomyces sp.* in the fungus-growing termite *Macrotermes gilvus* (Hagen) was to degrade lignin while increasing cellulose digestibility for the host. Kouame et al. (2005) purified β-fucosidases (β-glycosidase A and B) from *Macrotermes subhyalinus* (Rambur) and proposed that these degrade di- and oligosaccharides from hemicelluloses and celluloses.

According to Brune and Ohkuma (2011), a dual cellulose digestion system also occurs in higher termites, with both the host's endogenous cellulase and hindgut bacteria engaging in cellulose degradation. The site of cellulase secretion for higher termites is the midgut epithelium (Slaytor, 2000), although evidence from later studies by Tokuda et al. (2004; 2009) provided some variations to the initial finding. More recent developments in the higher termites' endogenous cellulases are discussed in detail in Chapters 3 and 4.

Problem Statement

A major occurrence in termite evolution is a single event loss of flagellates from the hindgut of higher termites (Breznak, 2000; Inoue et al., 2000; Tokuda et al., 2004), in parallel with significant changes in gut structure and nutrition (Bignell, 1994; Donovan et al., 2000). While there have been numerous studies on the evolution of termites, the evolution of cellulose digestion in termites, especially in higher termites, is still poorly understood. Inward et al. (2007a) found that it was impossible to elucidate the evolution of feeding group within the higher termites using nuclear and mitochondrial markers.

To date, endogenous cellulase sequences have been available only from four termitid species; *Odontotermes formosanus* (Shiraki), *Na. takasagoensis*, *Na. walkeri*

and *Sinocapritermes mushae* (Oshima and Maki); in the GenBank database. Due to the large number of termitid species and their diverse feeding habits, there is a substantial gap in our knowledge on endogenous cellulases in the molecular database to help us understand more about the evolution of endogenous cellulose digestion in higher termites.

Hence, the targeted enzymes for this study are the endogenous cellulases (endo-β-1,4-glucanase and β-glucosidase) because they are endogenously produced by the termites to digest cellulose. The termitid's diverse diets drive selective pressure, which cause changes in the coding sequence of these endogenous cellulases to encode for the appropriate enzyme to match a particular type of food. By examining the sequences that encode for these enzymes, I might begin to understand how termite endogenous digestion evolves at the molecular level.

Objectives

The main question I wished to answer was "How did endogenous cellulose digestion evolve in higher termites?" Therefore, my specific objectives were:

I. to elucidate the phylogeny of a representative selection of higher termites in this study with mitochondrial (16S) and nuclear (28S) markers,

II. to purify, clone, and sequence endo-β-1,4-glucanases, and to determine its evolution across higher termites of different feeding guilds,

III. to purify, clone, and sequence β-glucosidases, and to elucidate its evolution across nutritionally diverse Termitidae, and finally,

IV. to determine the phylogenetic placement of the bacterial comb-grower in the evolution of endogenous cellulose digestion in termitids and to posit how feeding behavior evolved in higher termites

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CHAPTER 2
THE PHYLOGENY OF HIGHER TERMITES BASED ON MITOCHONDRIAL AND NUCLEAR MARKERS

Termites have been closely linked with cockroaches through various morphological structures (Walker, 1922; McKittrick, 1965; Thorne and Carpenter, 1992; Klass, 1995), as well as the presence of endosymbionts (Cleveland et al., 1934; Koch, 1938). According to Kambhampati (1995; 1996), termites are sister-groups with the cockroach-mantid clade. However, through 18S ribosomal RNA, mitochondrial cytochrome oxidase subunit II (COII) and endo-β-1,4-glucanase gene sequences analyses, Lo et al. (2000) showed that eusocial termites probably shared a stem ancestor with the cockroach, *Cryptocercus*. More recently, Inward et al. (2007b) showed that termites form a clade within the cockroaches with *Cryptocercus* as their sister group, thus corroborating the hypothesis that termites are actually eusocial cockroaches.

While these previous studies have dealt with a broader question, the phylogeny within the family Termitidae has received little consensus, especially due to poor taxon sampling (Eggleton, 2001) and an inadequate number of genetic loci (Inward et al., 2007a). Similar problems exist not only in molecular-based studies, but also in gut anatomy-based studies (Lo and Eggleton, 2011). More comprehensive molecular studies were done by Inward et al. (2007a) and Legendre et al. (2008) using various molecular markers. Later, Engel et al. (2009) published the first morphology-based termite phylogeny that combined fossil and recent data and this has provided insight into the evolution of the family Termitidae.

In phylogenetic determinations, mitochondrial DNA (mtDNA) is widely used because it exhibits various genotypic characters and evolves rapidly (Moore, 1995).

However, because the gene is inherited as a single linkage group (haplotype), it does not provide independent estimates of the species tree (Moore, 1995). Nuclear genes, on the other hand, provide an independent estimate of the species tree because they can be selected from distinct chromosomes (Moore, 1995). Consequently, Pamilo and Nei (1988) proposed that one should use sequences of many different and independently-evolving (unlinked) loci to construct a species tree and Wu (1991) suggested the use of more than five non-orthologous loci to determine species phylogeny.

In this study, my goal was to elucidate the phylogeny of a selection of 25 species of higher termites with mitochondrial (16S) ribosomal RNA and nuclear (28S) gene sequences. Both gene markers have been used with success to infer the phylogeny of different species within the family Termitidae (Inward et al., 2007a, Legendre et al., 2008). I hypothesized that these gene segments will provide a clear separation among the 25 termitid species (Table 2-1), and show that fungus-growers are the most phylogenetically basal, while wood- and soil/litter-feeders are the most derived groups. I predict that phylogenetic trees inferred from these sequences will provide a clear delineation among different feeding groups of the higher termites and show congruency with the results from endogenous cellulase analyses.

Materials and Methods

Termite Species

The 25 higher termite species used in this study and their feeding habits are listed in Table 2-1. These termitids were selected because of their differing nutritional biology. *Nasutitermes corniger* (Motschulsky) was the only specimen obtained fresh from a laboratory population. Others were preserved in ethanol or on Whatman FTA® Plantsaver cards.

DNA Extraction

DNA from fresh and ethanol-preserved specimens were extracted using Qiagen® DNeasy Blood & Tissue Kit. Those preserved on Whatman FTA® Plantsaver cards were processed according to the methodology of Bujang et al. (2011). Only the termite heads were used to prevent contamination from the hindgut microbiota.

Polymerase Chain Reaction

PCR was performed with 16S mitochondrial and 28S (D4 expansion segment) nuclear markers as listed in Table 2-2. Amplifications were conducted in 50 µL final reaction volumes, each containing 2 µL DNA template, 33.8 µL dH_2O, 5 µL PCR Buffer (1.675 µL dH_2O, 1.25 µL 1 M KCl, 1 µL 1 M Tris, 0.5 µL 5% Tween 20, 0.075 µL 1 M $MgCl_2$ and 0.5 µL 1% gelatin), 0.04 mM of each dNTP, 50 ng of each primer and 1 U Econo*Taq* DNA polymerase (Lucigen Corp., Middleton, WI). Depending on the DNA extraction method, either water or a strip of unused FTA® card was incorporated into PCR reaction mixtures to serve as negative controls.

For the 16S gene, after initial denaturation at 94°C for 45 s, the thermocycling profile for 40 cycles was 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by final extension at 72°C for 10 min (Ye et al., 2004) before cooling to 4°C. For the 28S gene segment, after precycle denaturation at 94°C for 2 min, the thermocycler profile for 40 cycles was 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a postcycle extension at 72°C for 10 min before cooling to 4°C.

Visualization and Sequencing

The amplified PCR products were electrophoresed through 1% Agarose Low EEO electrophoresis grade agar (Fisher Scientific, Pittsburg, PA) using TAE (40 mM Tris-acetate and 1 mM EDTA) as running buffer and stained with ethidium bromide (EtBr)

before they were visualized by UV transillumination. Successful amplicons were purified using Wizard® PCR Preps DNA Purification System (Promega Corp., Madison, WI) and quantified by comparison with serial dilutions of uncut lambda DNA (Promega Corp., Madison, WI) by 1% Agarose (Low EEO) (Fisher Scientific, Pittsburg, PA) electrophoresis using TAE. Finally, purified PCR products were sent for sequencing using their respective primer pairs to the BioAnalytical Services Laboratory (BASLab), University of Maryland, Baltimore, MD.

Sequence Analysis

Consensus sequences for each PCR product were obtained using DNA Baser Sequence Assembler (Heracle BioSoft , Pitesti, Romania) and Lasergene® SeqMan Pro v7.2.0 (DNASTAR, Inc, Madison, WI) software. Consensus sequences were then aligned using Mega 4.1 software (Tamura et al., 2007). Sequence similarity or putative sister groups of nucleotide sequences were searched using BLAST (Basic Local Alignment Search Tool) in National Center for Biotechnology Information, USA (website: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence variation analysis was performed with Mega 4.1 software (Tamura et al., 2007). The homogeneity test of base frequencies was conducted using PAUP* 4.0 (Swofford, 2002, Sinauer Associates, Inc. Publishers, Sunderland, MA).

Phylogenetic analyses were done under Bayesian criteria. The appropriate model of DNA substitution for each of the 16S, 28S, and their combined dataset was chosen using MODELTEST 3.0 (Posada and Crandall, 1998). The parameters obtained under Akaike's Information Criterion (AIC) were subsequently used in PAUP* 4.0 (Swofford, 2002, Sinauer Associates, Inc. Publishers, Sunderland, MA). Tree inferences and posterior probabilities estimation with Markov Chain Monte Carlo sampling were carried

out using MrBayes v3.1.2 software (Ronquist and Huelsenbeck, 2003) for 1,000,000 generations and burnin setting at 1,000. All sites with missing data were regarded as missing characters.

Results and Discussion

I was able to sequence partial fragments of about ~600 bp and ~700 bp for the 16S (GenBank Accession No. xxxxxxx to xxxxxxx) and 28S (GenBank Accession No. xxxxxxx to xxxxxxx) genes, respectively, which provided a total of ~1,300 bp characters (Fig. 2-1) (GenBank Accession No. xxxxxxx to xxxxxxx). The results from the inferred tree supported my earlier hypothesis that fungus-growers are the most phylogenetically basal group, while wood- and soil/litter-feeders are the most derived groups, with the bacterial comb-feeder intermediate between the other subgroups (Fig. 2-2).

Results also supported earlier findings by Inward et al. (2007a), Legendre et al. (2008) and Engel et al. (2009), which posited that the family Termitidae is monophyletic. Macrotermitines formed a separate basal clade with strong posterior probability, distal from the other subgroups. The next major branch is the subfamily Sphaerotermitinae, which corroborated the molecular findings by Inward et al. (2007a). However, based on the morphological data of Engel and Krishna (2004), they placed Sphaerotermitinae as a sister group to the Macrotermitinae because of a combination of plesiomorphic and apomorphic traits.

Moving apically, the monophyly of subfamily Apicotermitinae is consistent with the findings of Inward et al. (2007a). The inferred tree also showed that the apicotermitines form a separate clade away from other soil/litter feeders. A paraphyletic relationship between the soil/litter feeders and the wood/grass/lichen/litter-feeders was also revealed. This outcome supported the study of Donovan et al. (2001), who outlined the

evolution of feeding groups based on gut content analysis and morphological characters of worker termites. They suggested that the ancestor of apicotermitines, termitines and nasutitermitines might have been the soil-feeders, which acquired the hindgut bacterial community from ingested soil. Hence, the presence of wood-feeding termitines in the same clade as soil interface-feeding termitines agreed with the trend of feeding group evolution proposed by Donovan et al. (2001). As it is more conceivable to think that the soil/litter-feeders are the most phylogenetically apical group, the presence of wood-feeding termitids amongst them shows the independent progression of this group (Donovan et al., 2001).

The termitines were paraphyletic with the syntermitines and nasutitermitines, consistent with the findings of Donovan et al. (2001) and Inward et al. (2007a). Instead of nesting within the nasutitermitines as initially expected, the syntermitines were found nesting within the termitines, as was shown by Inward et al. (2007a). Finally, my findings departed in some respects from Engel et al. (2009) and Legendre et al. (2008) regarding the nasutitermitines. Data from this study showed that Nasutitermitinae is polyphyletic, with *Subulitermes baileyi* (Emerson) falling outside of the otherwise monophyletic Nasutitermitinae group. Thus, this outcome is in agreement with Inward et al. (2007a).

Table 2-1. List of 25 termitid species used in this study

Species	Subfamily	Feeding habit	Locality
Macrotermes carbonarius (Hagen)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Malaysia, Pulau Pinang
Macrotermes gilvus (Hagen)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Malaysia, Pulau Pinang
Macrotermes subhyalinus (Rambur)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Tanzania, -4.67710/29.62260
Microtermes pallidus (Haviland)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Malaysia, Pulau Pinang
Odontotermes formosanus (Shiraki)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Taiwan, Pingtung County
Odontotermes hainanensis (Light)	Macrotermitinae	Wood/Grass/Litter, Fungus-grower	Malaysia, Pulau Pinang
Sphaerotermes sphaerothorax (Sjoestedt)	Sphaerotermitinae	Wood, Bacterial comb-grower	Congo, Pointe Noire
Syntermes grandis (Rambur)	Syntermitinae	Grass/Litter	French Guyana, 5.67540/-53.59198
Rhynchotermes bulbinasus Scheffrahn	Syntermitinae	Grass/Litter	Colombia, 9.31634/-74.90097
Amitermes dentatus (Haviland)	Termitinae	Wood	Malaysia, Pulau Pinang
Amitermes foreli Wasmann	Termitinae	Grass	Colombia, 8.92399/-75.8381
Microcerotermes crassus Snyder	Termitinae	Wood	Malaysia, Pulau Pinang
Globitermes sulphureus (Haviland)	Termitinae	Wood/Litter	Malaysia, Pulau Pinang
Hospitalitermes bicolor (Haviland)	Nasutitermitinae	Lichen	Malaysia, Pulau Pinang
Constrictotermes cavifrons (Holmgren),	Nasutitermitinae	Lichen	French Guyana, 5.02389/-53.0249
Constrictotermes guantanamensis Krecek, Scheffrahn and Roisin	Nasutitermitinae	Lichen	Cuba, 19.934/-75.098
Nasutitermes corniger (Motschulsky)	Nasutitermitinae	Wood	USA, Dania Beach

Table 2-1. Continued

Species	Subfamily	Feeding habit	Locality
Nasutitermes takasagoensis (Shiraki)	Nasutitermitinae	Wood	Japan, Iriomate Island
Nasutitermes sp.	Nasutitermitinae	Wood	Malaysia, Pulau Pinang
Subulitermes baileyi (Emerson)	Nasutitermitinae	Soil/Litter	Venezuela, 10.18533/-65.82158
Pericapritermes nitobei (Shiraki)	Termitinae	Upper soil/Litter	Taiwan, Taitung County
Pericapritermes sp.	Termitinae	Upper soil/Litter	Malaysia, Pulau Pinang
Sinocapritermes mushae (Oshima and Maki)	Termitinae	Upper soilLitter	Taiwan, I-Lan County
Grigiotermes metoecus Mathews	Apicotermitinae	Soil/Litter	Venezuela, 10.40245/-68.00039
Anoplotermes schwarzi Banks	Apicotermitinae	Soil/Litter	Guatemala, 14.69649/-89.62552

Table 2-2. List of nuclear and mitochondrial marker primers used in this study

Name	Gene	Orientation	Sequence (5' to 3')	References
Mitochondrial				
16Sar	16S	Forward	CCGGTCTGAACTCAGATCACGT	Simon et al., 1994, Marini and Mantovani, 2002
16Sbr	16S	Reverse	CGCCTGTTTAACAAAAACAT	Simon et al., 1994, Marini and Mantovani, 2002
Nuclear				
Hux	28S	Forward	ACACGGACCAAGGAGTCTAAC	Inward et al., 2007a
Win	28S	Reverse	GTCCTGCTGTCTTAAGCAACC	Inward et al., 2007a

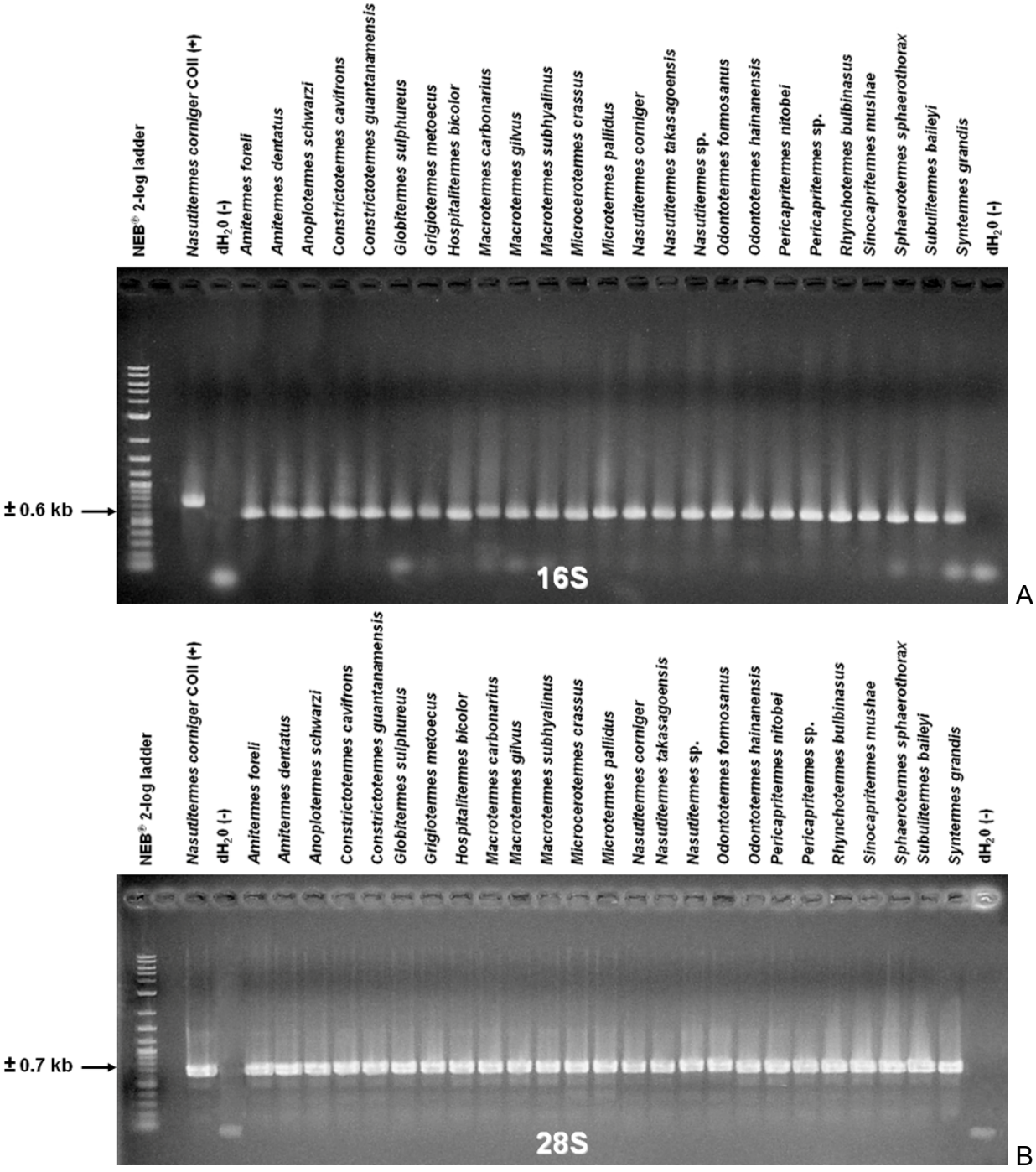

Figure 2-1. Agarose gel showing PCR amplification from 25 species of higher termites used in this study with positive and negative (water) control. A) PCR amplification of 16S mitochondrial (16S) ribosomal RNA gene and B) PCR amplification of nuclear (28S) gene.

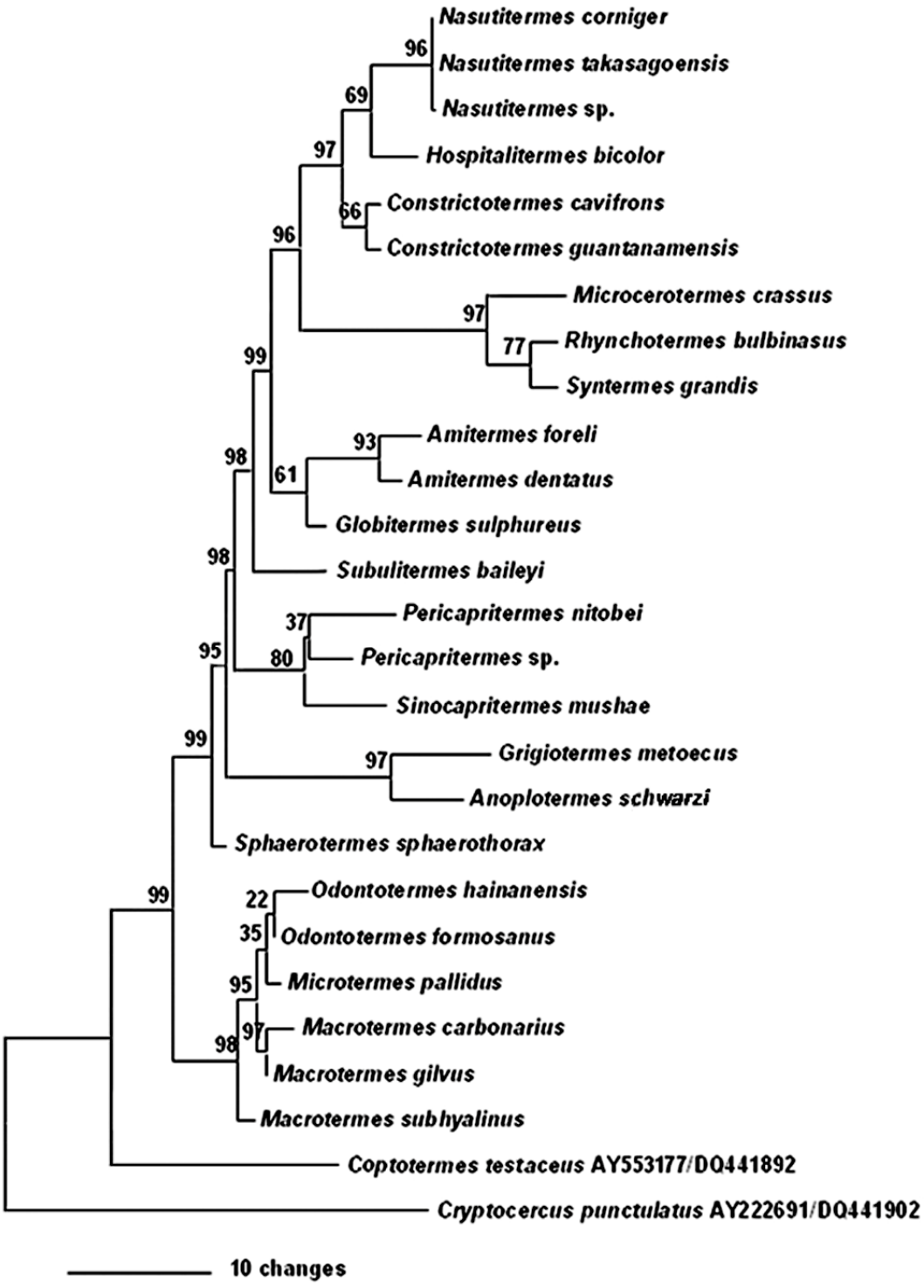

Figure 2-2. Consensus Bayesian tree inferred from combined 16s and 28s gene sequences (total ~1300 bp). Numbers above branch nodes indicate posterior probabilities recovered by the Bayesian analysis. Branch lengths are proportional to the number of changes.

CHAPTER 3
A PHYLOGENETIC AND EVOLUTIONARY STUDY OF ENDO-BETA-1,4-GLUCANASE IN HIGHER TERMITES

Endo-β-1,4-glucanases are members of GHF9, which comprise four known representatives and 732 components, as listed in CAZy (Carbohydrate-Active enZYmes Database, website: http://www.cazy.org). They occur in various insects, such as beetles, flies, cockroaches, and termites (Willis et al., 2010) and are an important component of termite digestion because they randomly hydrolyze the internal β-1,4-$_D$-glycosidic bonds on cellulose and convert it into cellobiose and cello-oligosaccharides (Robson and Chambliss, 1989). Slaytor (1992) reported that endo-β-1,4-glucanase is active against both crystalline cellulose and carboxymethylcellulose, hence proving that exocellulases are not crucial for cellulose digestion in termites.

In lower termites, Watanabe et al. (1998) were the first to sequence an endogenous cellulase, RsEG (GenBank Accession No. AB008778) from a termite, *Reticulitermes speratus* (Kolbe), after which endo-β-1,4-glucanases from other lower termite species were also sequenced (Tokuda et al., 2004; Zhou et al. 2007; Zhang et al. 2011). Zhang et al. (2009) cloned and overexpressed CfEG3a from C*. formosanus* in *Escherichia coli* and found that the hydrolytic activity of the recombinant native form (nCfEG) was higher than that of the C-terminal His-tagged form (tCfEG). Zhang et al. (2010) later conducted a functional analysis on recombinants of endo-β-1,4-glucanase and β-glucosidase obtained from the cDNA library of *C. formosanus* and found successful conversion from cellulose to glucose. Fujita et al. (2008) reported that in *Hodotermopsis sjoestedti* Holmgren, endo-β-1,4-glucanase activity was highest in the salivary gland, and was significantly higher in termite workers than soldiers.

Due to the loss of flagellates from their hindgut, higher temites depend heavily on endogenous cellulases for cellulose digestion (Slaytor et al., 1997; Ohkuma, 2003; Tokuda et al., 2004). Kovoor (1970) was the first to suggest that *Microcerotermes edentatus* Wasmann produce their own cellulase. According to Lo et al. (2011), endogenously-produced cellulases play a major role in termitid metabolism. Endo-β-1,4-glucanase activity has been quantified in numerous species such as *Trinervitermes trinervoides* (Sjostedt) (Potts and Hewitt, 1973), *Macrotermes natalensis* (Haviland) (Martin and Martin, 1978), *Speculitermes cyclops* Wasmann (Mishra and Sen-Sarma, 1985a), *Na. walkeri*, *Nasutitermes extiosus* (Hill) (Hogan et al., 1988), *Nasutitermes lujae* (Wasmann) (Chararas and Noirot 1988), *Crenetermes albotarsalis* (Sjostedt) (Rouland et al., 1989a), *M. subhyalinus*, *Macrotermes michaelseni* (Sjostedt) (Veivers et al., 1991), *Na. takasagoensis* (Tokuda et al., 1997; 2005; Tokuda and Watanabe, 2007; Fujita et al., 2008) and *O. formosanus* (Yang et al., 2004; Tokuda et al., 2005). Slaytor (1992) reported that the cellulolytic activity in the hindgut of higher termites was either undetectable or very low. Later, Slaytor (2000) reported that in areas where the gut microflora was absent, or present in trace amounts, such as the salivary glands and midgut, cellulolytic activity was found to be high.

The entire sequence of the coding region of *Na. takasagoensis* NtEG (GenBank Accession No. AB019146) was first determined by Tokuda et al. (1999) using a PCR-based strategy and found to consist of 10 exons and interrupted by nine introns. Khademi et al. (2002) later reported the structure of an endo-β-1,4-glucanase from *Na. takasagoensis*. Based on endo-β-1,4-glucanase sequences from *Na. takasagoensis*, a

sea squirt, *Ciona intestinalis* (Linnaeus) and an abalone, *Haliotis discus hannai* Ino, Lo et al. (2003) suggested that GHF9 was present in the ancestor of all bilaterian animals.

From endo-β-1,4-glucanase sequences of only three species of higher termites (*O. formosanus*, *Na. takasagoensis* and *S. mushae*), Tokuda et al. (2004) concluded that the more phylogenetically basal group is Macrotermitinae (fungus-growers), while the more apical groups are Termitinae (soil-feeders) and Nasutitermitinae (wood-feeders). They also found that the expression sites of endogenous cellulases in lower termites and *O. formosanus* were in the salivary glands while in those more distal expression sites occured in the midgut. Termitids have a wide range of feeding substrates, but there is a paucity of molecular information on endo-β-1,4-glucanases to help us understand the evolution of endogenous cellulose digestion in higher termites. Furthermore, Inward et al. (2007a) found that it was impossible to elucidate the evolution of feeding groups within the higher termites using nuclear and mitochondrial markers only.

In this chapter, my goal was to purify, clone, and sequence endo-β-1,4-glucanases, and to compare its evolution across higher termites of different feeding guilds. I hypothesized that the evolution of endo-β-1,4-glucanase should be congruent with the evolution of termites according to the mitochondrial and nuclear markers. I predict that the endo-β-1,4-glucanase phylogenetic tree will provide clues to understand the evolution of feeding groups within the higher termites. The bacterial comb-grower, *S. sphaerothorax* is of particular interest because it is the only species of higher termite known to cultivate bacterial combs and consume the bacterial pellets following bacterial action. Despite their unique feeding habit, how their feeding behavior was derived and

where this feeding group falls along the evolutionary line has yet to be investigated. Hence, the phylogenetic placement of the bacterial comb-grower in the evolution of endo-β-1,4-glucanase was also determined.

Materials and Methods

mRNA Extraction and cDNA Synthesis

Only the termite heads and salivary glands were used to prevent contamination from the hindgut microbiota. Messenger RNA was extracted using Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA). cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Polymerase Chain Reaction

PCR was performed with specifically-designed endo-β-1,4-glucanase primers (Clone Manager 9 Professional Edition, Scientific & Educational Software, Cary, NC) as listed in Table 3-1. The procedure used was the same as described in Chapter 2, but used a different thermal cycling program. The temperature profile for the first cycle was 94°C for 2 min, 52°C for 2 min, and 72°C for 3 min. For the remaining 44 cycles, the temperature profile was 94 °C for 1 min, 52°C for 2 min, and 72°C for 3 min before cooling to 4°C.

Cloning

After electrophoresis of PCR products through a 1% Agarose Low EEO gel (Fisher Scientific, Pittsburg, PA) followed by EtBr staining and visualization by UV transillumination, amplified DNA products were gel-purified with Lonza SeaPlaque® GTG® Agarose (Lonza Rockland, Inc., Rockland, ME) and excised bands were purified using Wizard® PCR Preps DNA Purification System (Promega Corp., Madison, WI). The

purified fragments were ligated overnight with pGEM®-T Vector System I (Promega Corp., Madison, WI) at 4°C and then used to transform into One Shot® TOP10 Chemically-competent *E. coli* (Invitrogen Corp., Carlsbad, CA). The transformed bacterial cultures were grown overnight at 37°C on Luria-Bertani (LB) medium (15g Bacto™ Agar, 10g Bacto™ Tryptone, 5g Bacto™ Yeast Extract, 10g NaCl in 1L volume) containing 100 µg/ml ampicillin (A100) with isopropyl beta-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal) as an overlay on the agar to enable blue/white colony screening. Fifty isolated white colonies were carefully selected and grown overnight at 37°C on LB_{A100} media patch plate.

PCR was carried out again on each transformed bacterial colony as described earlier, but using dH_2O-suspended bacterial cells of each clone as the DNA template. Restriction Fragment Length Polymorphism (RFLP) with restriction enzymes *Hinf*1 and *Mse*1 (New England BioLabs, Waverley, MA, USA) was conducted on each amplified product from successfully-transformed products to assess polymorphisms. These restriction enzymes were selected after virtual electrophoresis screening of endo-β-1,4-glucanase sequences from *N. takasagoensis* (GenBank Accession No. AB013272) and *N. walkeri* (GenBank Accession No. AB013273) with pDRAW32 1.0 (AcaClone software, http://www.acaclone.com). Digested products were electrophoresed through an 8% nondenaturing polyacrylamide gel using TBE (90mM Tris-borate, 2mM EDTA) as running buffer, stained with EtBr and then visualized by UV transillumination.

Forty-one clones were selected and the recombinant plasmids were grown overnight at 37°C in LB broth (10g Bacto™ Tryptone, 5g Bacto™ Yeast Extract, 10g NaCl in 1L volume). After purification with Wizard® *Plus* Minipreps DNA Purification

Systems (Promega Corp., Madison, WI), the plasmids were each quantified by comparison with serial dilutions of uncut lambda DNA (Promega Corp., Madison, WI) in 1% Agarose Low EEO electrophoresis grade agar (Fisher Scientific, Pittsburg, PA). Finally, they were sent for insert sequencing with the M13F and M13R primer pair at the Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Laboratory, University of Florida, Gainesville, FL.

Sequence Analysis

Consensus sequences were assembled and aligned as described in Chapter 2. Sequence similarity or putative sister groups of nucleotide and amino acid sequences were searched using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information, USA (website: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide and amino acid sequence variation analysis was performed with Mega 4.1 software (Tamura et al., 2007). The homogeneity test of base frequencies was conducted using PAUP* 4.0 (Swofford, 2002, Sinauer Associates, Inc. Publishers, Sunderland, MA). Phylogenetic analyses followed the outline described in Chapter 2. Amino acid sequences were subsequently aligned with Mega 4.1 (Tamura et al., 2007). Catalysis and substrate-binding sites were inferred from Sakon et al. (1997). Finally, N-glycosylation site search was performed with N-GlycoSite HCV sequence database (http://hcv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html) (Zhang et al., 2004).

Results and Discussion

Single PCR fragments of about 1.35 kb in length from 23 species of higher termites were amplified (Fig. 3-1). Based on RFLP profiles of the clones, 41 different clones from 23 termitid species were obtained (Fig. 3-2) (GenBank Accession No.

xxxxxxx to xxxxxxx). Collectively, the sequences showed at least 70% amino acid similarity with *Mastotermes darwiniensis* Froggatt endo-β-1,4-glucanase and at least 61% identity with *Panesthia cribrata* Saussure endo-β-1,4-glucanase. Protein BLAST searches also showed similarity to members of GHF9 family, which contain endo-β-1,4-glucanases from plants, bacteria and slime molds. However, plants and bacteria GHF9 lacks the linker and cellulose-binding domains sometimes found in members of this family (Tomme et al., 1995). Each of the sequences contain the proton donor glutamate and the nucleophile aspartate, which are typical characteristics of GHF9 (Watanabe and Tokuda, 2001; Zhou et al. 2007). These findings were consistent with endo-β-1,4-glucanases from other termitids (Tokuda et al., 2004) and lower termites (Watanabe and Tokuda, 2001).

The sequences obtained were consistent with the findings of Nakashima et al. (2002a) and Zhou et al. (2007) in as much as each sequence possesses conserved motifs involved in substrate binding and catalysis as described by Khademi et al. (2002). These motifs are “NEVA”, with “E” (Glu, glutamic acid) as the proton donor and “DAGD” with both “D”s (Asp, aspartic acid) as the nucleophiles (Fig. 3-3). The substrate-binding cleft structure allows random binding on the cellulose chain (Khademi et al. 2002; Zhou et al. 2007). The N-linked glycosylation, putative catalytic glutamic acid and aspartic acid residues (proton donor and nucleophile, respectively) and stop codon positions are shown in Fig. 3-3.

Only a few insertion/deletion events were evident among all the sequences obtained. The inferred endo-β-1,4-glucanase phylogenetic tree (Fig. 3-4) showed congruency with the mitochondrial/nuclear tree (Fig. 2-2). Generally, the fungus-growers

were the most basal group and the most distal diphyletic feeding groups were the soil/litter- and wood/lichen/grass/litter-feeders. Results of endo-β-1,4-glucanase sequences suggest that the bacterial comb-grower is phylogenetically placed as the "missing link" between the fungus-growers and the soil/litter- and wood/lichen/grass/litter-feeders.

Up to four paralogous copies from each of the 23 termitid species were obtained, with high degrees of substitutions among some paralogs. The high divergence among these paralogs suggests the involvement of different alleles. As predicted, endo-β-1,4-glucanase paralogs from the same species clustered with one another except in the case of *O. formosanus*. Although Lo et al. (2011) stated that the roles of multiple endo-β-1,4-glucanase gene copies remained unclear, I support the "neofunctionalization" hypothesis by Ohno (1970), Force et al. (1999) and Hahn (2009), which suggests a different function from the original gene.

In terms of endo-β-1,4-glucanase evolution, fungus-growers were the most phylogenetically basal group, concordant with the mitochondrial/nuclear data. This was also in agreement with earlier studies by Tokuda et al. (2004), who further showed that in *O. formosanus*, endo-β-1,4-glucanase is mostly expressed in the salivary glands, as observed in lower termites. There was a strong posterior probability for the monophyly of macrotermitines, although there was poor phylogenetic resolution for endo-β-1,4-glucanase sequences between species, except in the case of *M. carbonarius* and *M. gilvus*. According to Lo et al. (2011), although endogenously-produced cellulases are less important in fungus-growers, they are still preserved in the genome.

The low divergence of endo-β-1,4-glucanases from this feeding group was due to their high dependency on fungi to digest their food. With the loss of flagellates in the ancestors of the family Termitidae [through abrasion, as proposed by Rouland-Lefevre and Bignell (2001)], the macrotermitines' close association with cellulolytic fungi has allowed them to continue to use wood and litter as a raw product in their food production. By relying on fungal symbionts to process their food (Darlington, 1994), the fungus-growers have some form of an 'external gut' that partially digests cellulose for them. The fungus grows on fungus combs, which were constructed from primary feces (Grasse, 1978). According to Sands (1969), this method of fecal reuse substituted the role of proctodeal feeding. From a nutrition point of view, Sands (1956) found that the exclusion of fungi from *Odontotermes badius* (Haviland) diet caused an effect similar to that when starved. Because both the comb and fungi are major sources of food for the fungus-growers (Arshad et al., 1987), I suspect that the types of fungi with which the termites are associated drives selective pressure to code for the different substitution patterns to match a particular type of fungus.

In the monophyletic sphaerotermitine, molecular evidence suggests that bacterial comb-feeding termites bridge the transition from being fungus-growers to soil/litter- and wood-feeders. Donovan et al. (2001) suggested that the soil-feeding ancestor of Apicotermitinae, Termitines and Nasutitermitinae acquired the bacterial community from ingested soil. However, I propose that their hindgut bacterial community was acquired through bacterial comb-feeding. I was able to obtain four paralogous copies from *S. sphaerothorax*. The high divergence among these four paralogs suggests the involvement of different alleles. In fungus-growing termites, the fungal symbiont,

basidiomycete *Termitomyces* spp. grows on termite-constructed fungus-combs (made from mylosphere or primary faeces) as mycelium or seasonal basidiocarps (Heim, 1977). According to Garnier-Sillam (1989), Sphaerotermitinae builds two types of bacterial combs within its nest; the first occurs as a result of primary feces accumulation, while the second is by final feces accumulation (Garnier-Sillam, 1989). Both types of combs contain nitrogen-fixing bacteria, with the older (lighter) comb containing a significantly higher number of bacteria than the younger (darker) comb (Garnier-Sillam, 1989). Just as fungus-growers consume the comb following fungal action, bacterial comb-feeder eats up the light-colored pellets formed by bacterial action. Garnier-Sillam (1989) also reported that one bacterial type is more abundant than the others within the sphaerotermitine nest. I suspect that different bacterial strains present within the nest might produce bacterial pellets of different chemical/nutritional properties. In support of the idea of different functionality, I propose that the various bacterial strains present in bacterial combs might have caused different endo-β-1,4-glucanase paralogs to occur. I suspect that different paralogs may be used to degrade pellets of different physical and chemical properties that resulted from different bacterial strains.

A shift has occured from having an 'external gut' in the fungus- and bacterial comb-grower to having an 'internal gut' in the wood/lichen/grass/litter- and soil/litter-feeders. The inferred tree suggested a diphyletic relationship between the soil/litter-feeders and the wood/lichen/grass/litter-feeders. According to Donovan et al. (2001), the ancestor of wood- and soil/litter-feeders may have been the soil-feeders, where the acquisition of bacterial community within their hindgut was presumably achieved from

ingested soil. Earlier, Noirot (1992) suggested that the major source of nutrient for soil-feeding termites is the bacterial-fermented aromatic humus compound, but later, Ji and Brune (2001) provided evidence that soil-feeding termites, *Cubitermes orthognathus* (Emerson) utilize plant and bacterial polysaccharides as well as microbial biomass as their nutrient source.

Although Tokuda et al. (2004) predicted the next major branch to be the apicotermitines, sequences from this study showed a strong separation between *Pericapritermes* and the other soil-feeders, indicating high nucleotide substitutions between these groups. According to Brauman et al. (2000), “genuine” soil-feeders feed widely in the soil profile. Eggleton et al. (1995) and Eggleton and Bignell (1995) classified the “wood/soil interface feeders” as those that feed on highly-humified but still recognizable organic matter. Donovan et al. (2001) have categorized *Pericapritermes* under group III feeders that feed on the organic rich upper layers of the soil, while *Grigiotermes* was placed under group IV feeders (true soil feeders) that ingest mineral soil. Tokuda et al. (2004) showed that the majority of endo-β-1,4-glucanase activity in *S. mushae* occurs in the midgut, although its overall endo-β-1,4-glucanase activity was almost imperceptible when compared with that of lower termites. Regardless, it is retained in the genome despite its insignificance for soil/litter-feeders (Lo et al., 2011).

Within the wood/lichen/grass/litter-feeding clade, it was interesting to note the polyphyly of the nasutitermitines and the strong diphyletic relationship between the most distal groups, the lichen-feeders and the grass/litter feeders. The high divergence among *Globitermes* and *Amitermes* paralogs also suggests the involvement of different alleles. I speculate that this high level of substitution was because the selection

pressure on wood-feeders was more relaxed, thus allowing for the occurrence of multiple endo-β-1,4-glucanase paralogs to achieve increased resource utilization efficiency. Nonetheless, Tokuda et al. (2004) reported that the overall endo-β-1,4-glucanase activity in *Na. takasagoensis* was only 10% than that of lower termites.

The evolutionary origin of the endo-β-1,4-glucanase obtained here remains uncertain because of the unavailability of complete coding sequences. However, Zhou et al. (2007) found evidence to support the idea of vertical transfer of GHF9 from a cockroach ancestor in *R. flavipes*. Also, in *Na. takasagoensis* endo-β-1,4-glucanase NtEG (GenBank Accession No. AB019146), identical intron positions between GHF9 genes from *Na. takasagoensis* with those from two marine organisms suggested vertical transfer of this gene from a common ancestor. Nevertheless, the gene transfer status prior to that common ancestor remains unanswered.

Table 3-1. List of endo-β-1,4-glucanase primers used in this study

Name	Gene	Orientation	Sequence (5' to 3')
EG1f	EG	Forward	GCGGACCTGAAGGTAACTTG
EG1r	EG	Reverse	AGTACGCGCTGAGTTCCATC
EG2f	EG	Forward	CGCTTTGCCAAGGTGCTTAC
EG2r	EG	Reverse	GGCGAGAGCTGATTGGAAAC
EG3f	EG	Forward	CATGCTGCTTGCGACTAC
EG3r	EG	Reverse	AGCGACGAGAGCTGATTG
EG4f	EG	Forward	ATGATAGCGGCCAGAACG
EG4r	EG	Reverse	TAACCCAGCGCTACGAGAAC
EG5f	EG	Forward	GCTGCCGACTACAAGAAAG
EG5r	EG	Reverse	GGCGGATCAATGACCCAAC
EG6f	EG	Forward	CTTGGCGGAAAGATTCAG
EG6r	EG	Reverse	GTTGAGTGCCATCAAGAG
EG7f	EG	Forward	TTTGCCAAGCTGCGTATG
EG7r	EG	Reverse	ATAATCGCAGGCCACTTC
EG8f	EG	Forward	AAGAACGGACTGGACCTTAC
EG8r	EG	Reverse	TGGGCCACTAATAGCCTAAC
EG9f	EG	Forward	AAGGACTCCGCCTTAAACG
EG9r	EG	Reverse	ATACGAAACGGCAGGACAG
EG10f	EG	Forward	TTTGCCAAACTGCTTACC
EG10r	EG	Reverse	AGCCTGCGTTATAATCTG
EG11f	EG	Forward	GGAAAGATTCAGCCCTGAAC
EG11r	EG	Reverse	CCATCAAGTGGGCATGAAC
EG12f	EG	Forward	AAGGATTCCGCCCTCAATG
EG12r	EG	Reverse	CGTTACGAGAACGGAGATAG
EG13f	EG	Forward	AGCTGCTTACGACTATAACC
EG13r	EG	Reverse	TGGAAGCCTGCGTTATAATC

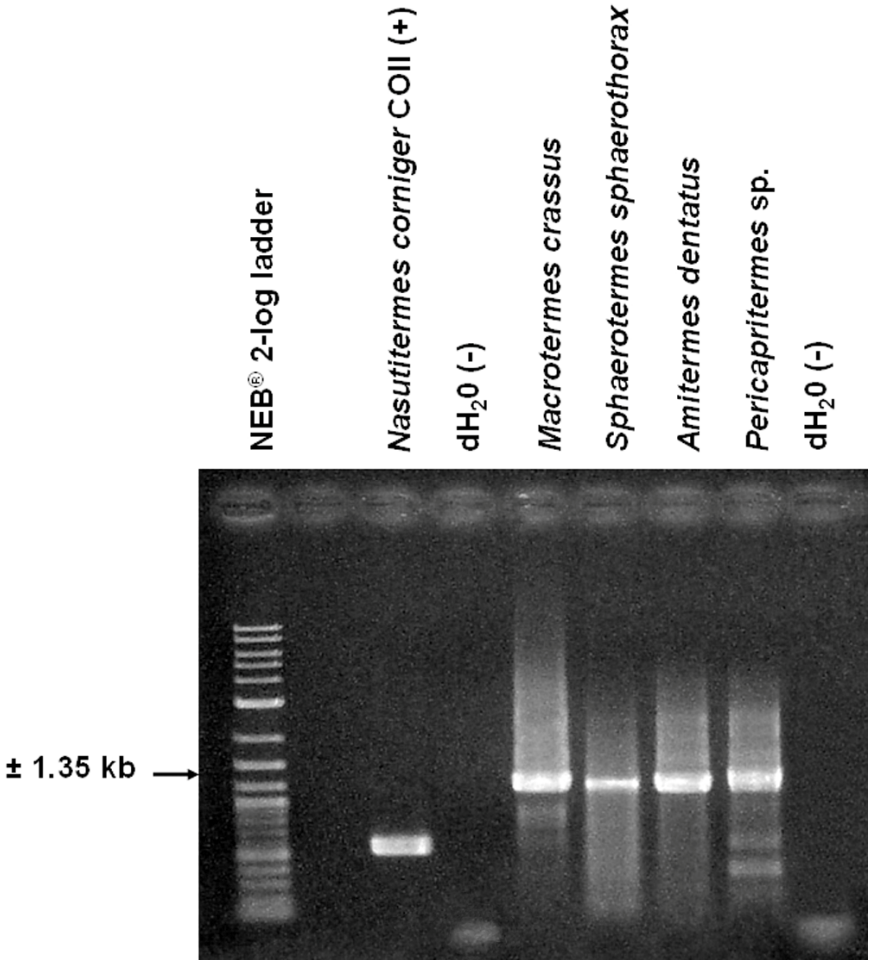

Figure 3-1. Agarose gel showing PCR amplification of endo-β-1,4-glucanase from four representative species of higher termites used in this study with positive and negative (water) control.

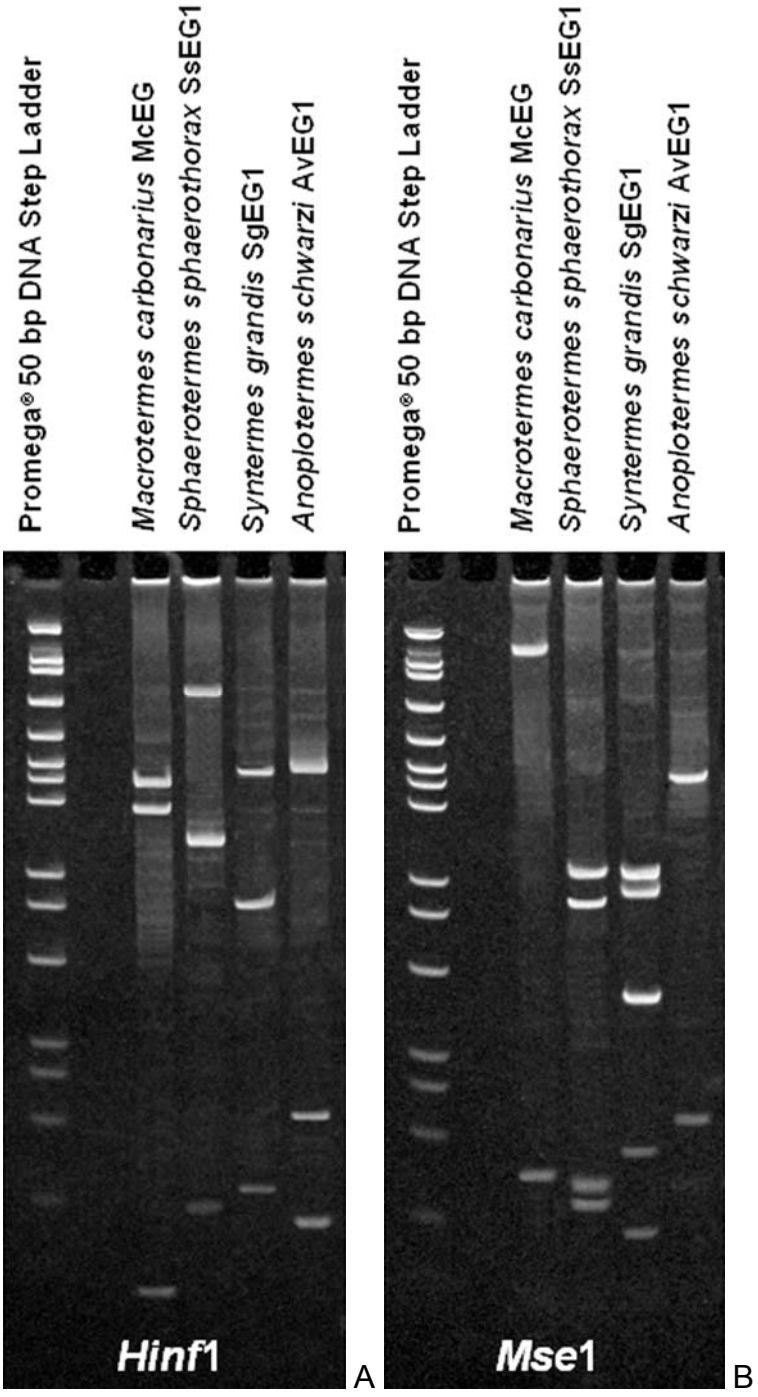

Figure 3-2. Restriction fragment length profiles of four representative endo-β-1,4-glucanase clones (ca. 1.35 kb) from four species of higher termites used in this study. A) Digestion with *Hinf*1 and B) Digestion with *Mse*1.

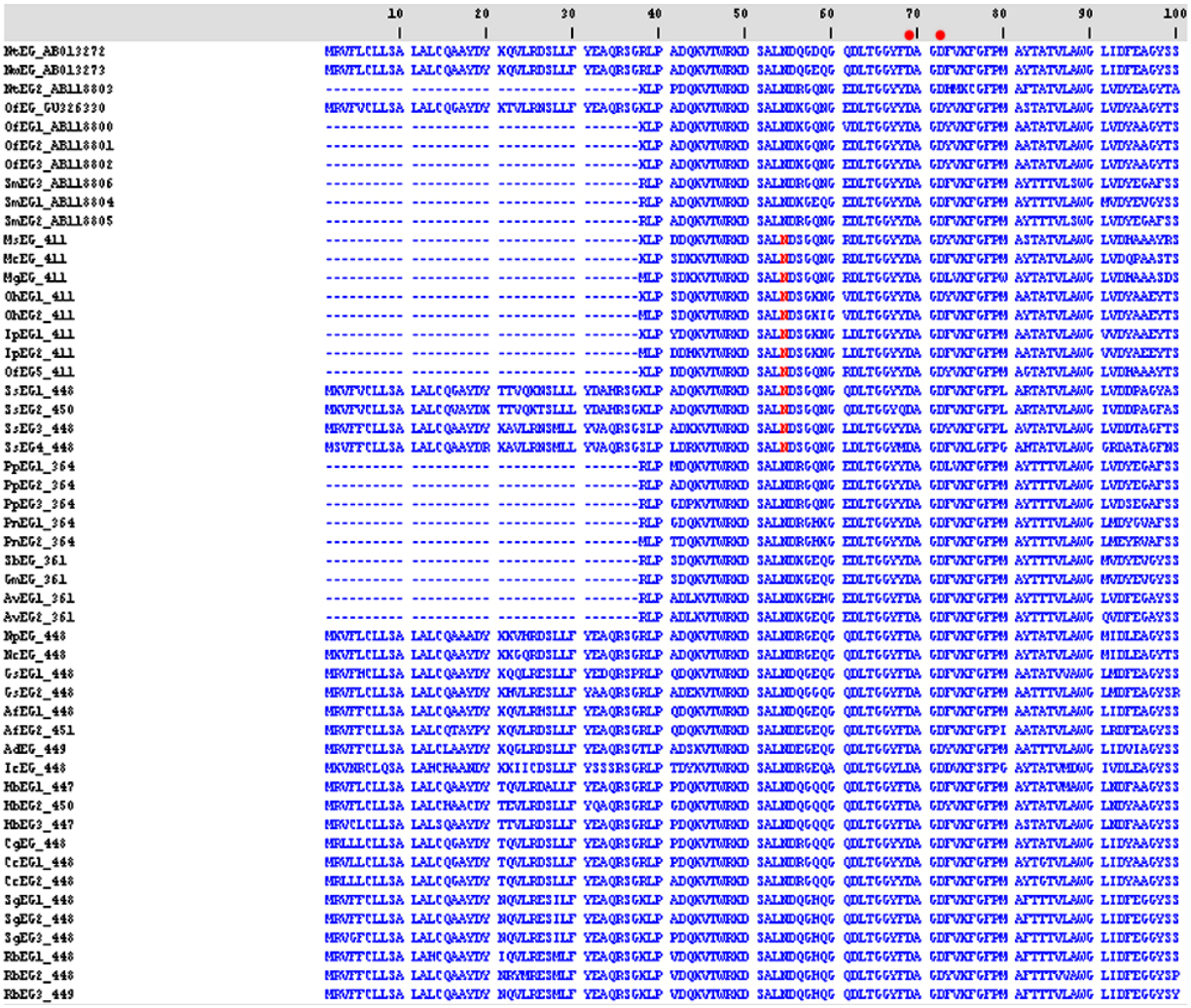

Figure 3-3. Multiple alignments of endo-β-1,4-glucanase amino acid sequences from higher termites. The number of encoded amino acids was listed next to the sequence names. N-linked glycosylation sites are highlighted in red. Blue dot indicates putative proton donor. Red dot indicates putative nucleophile. Black dot indicates the position of stop codon.

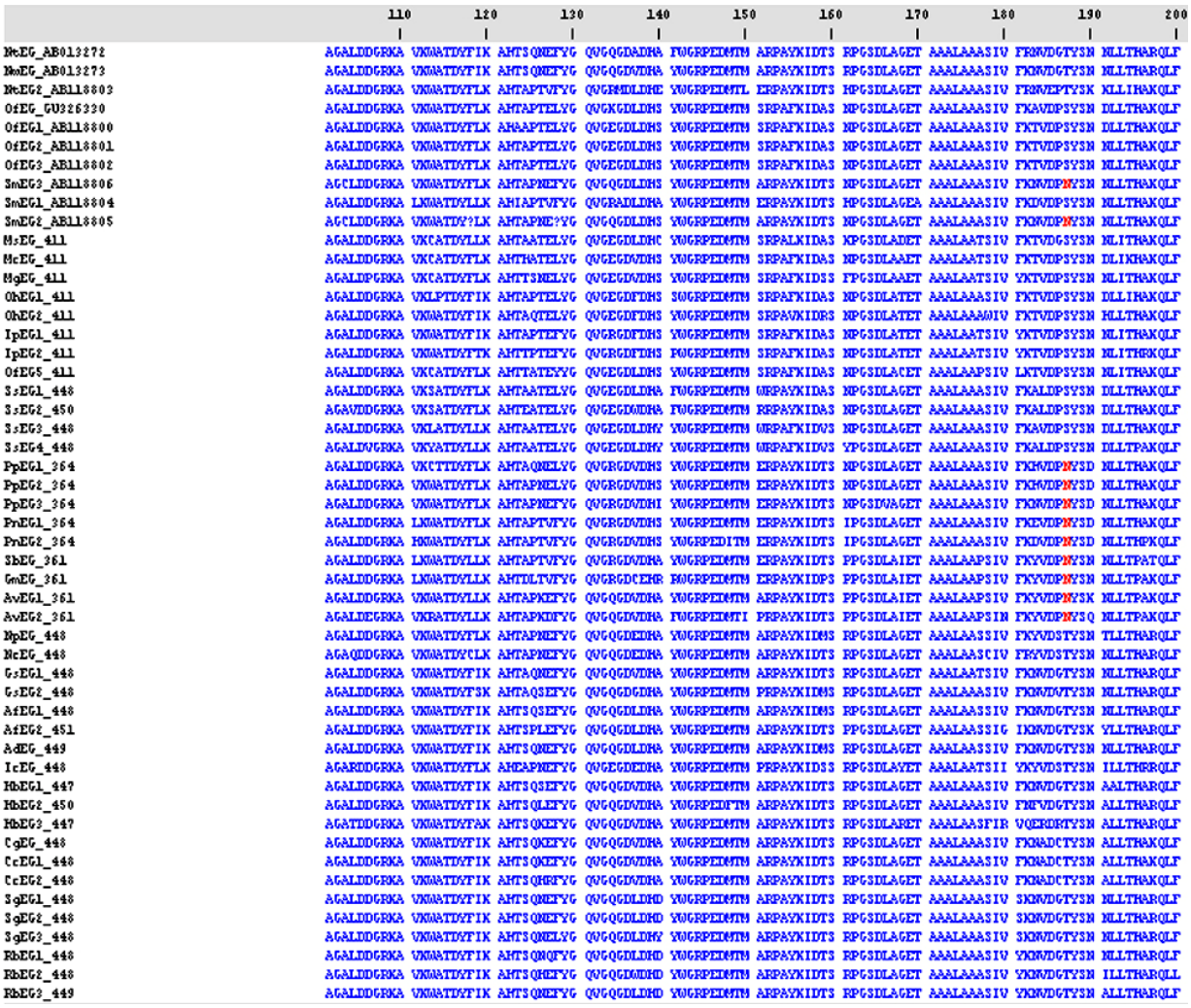

Figure 3-3. Continued.

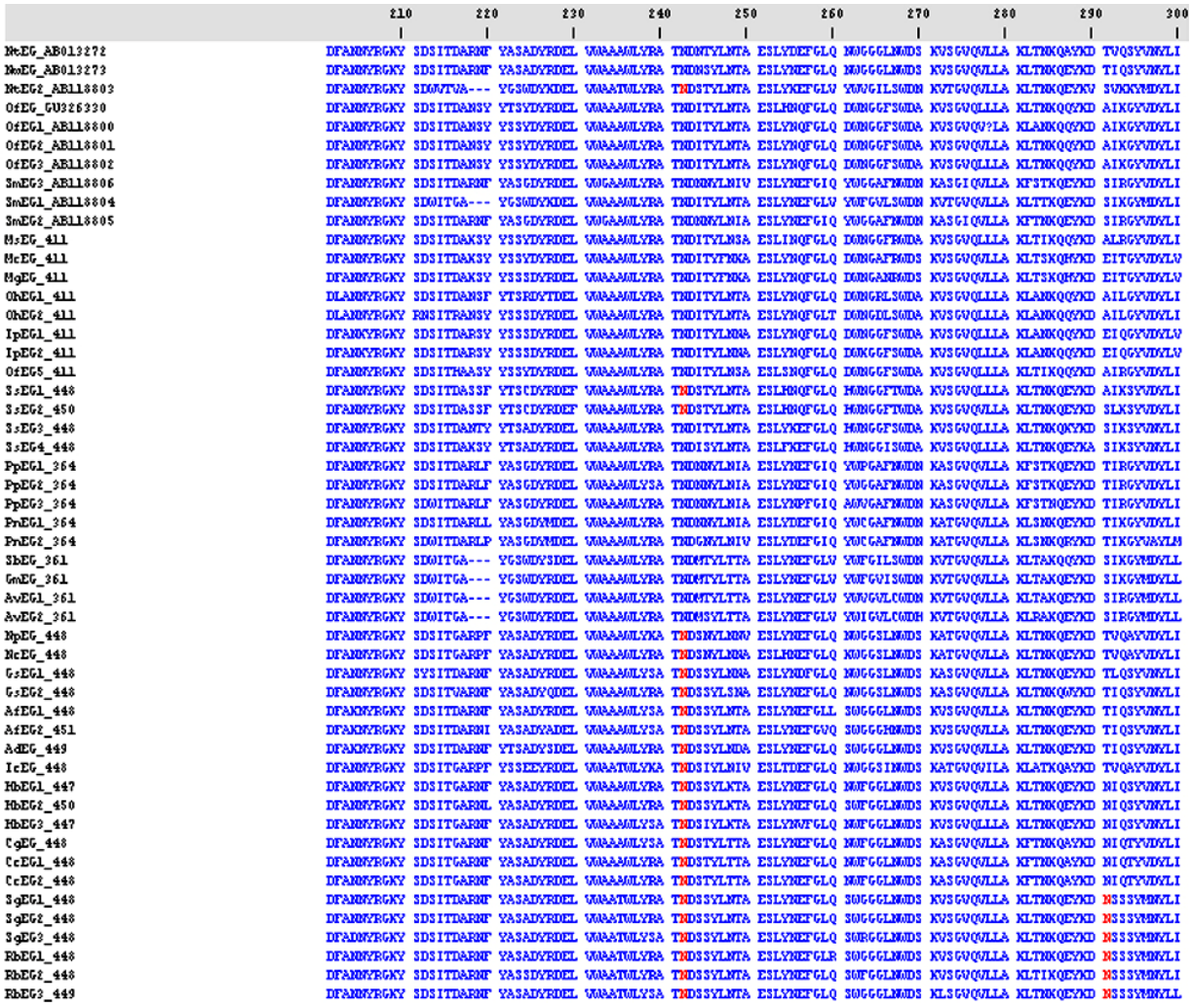

Figure 3-3. Continued.

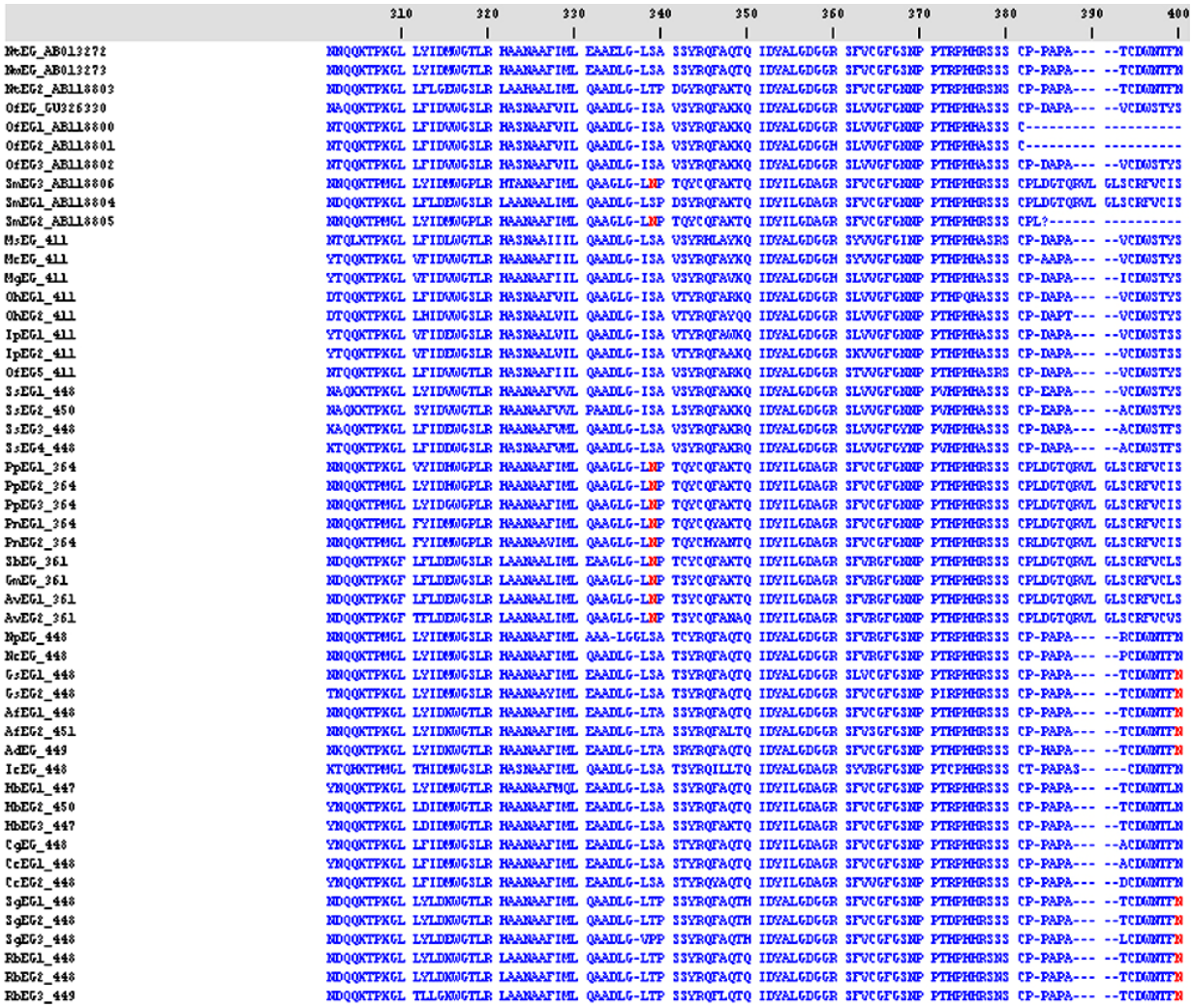

Figure 3-3. Continued.

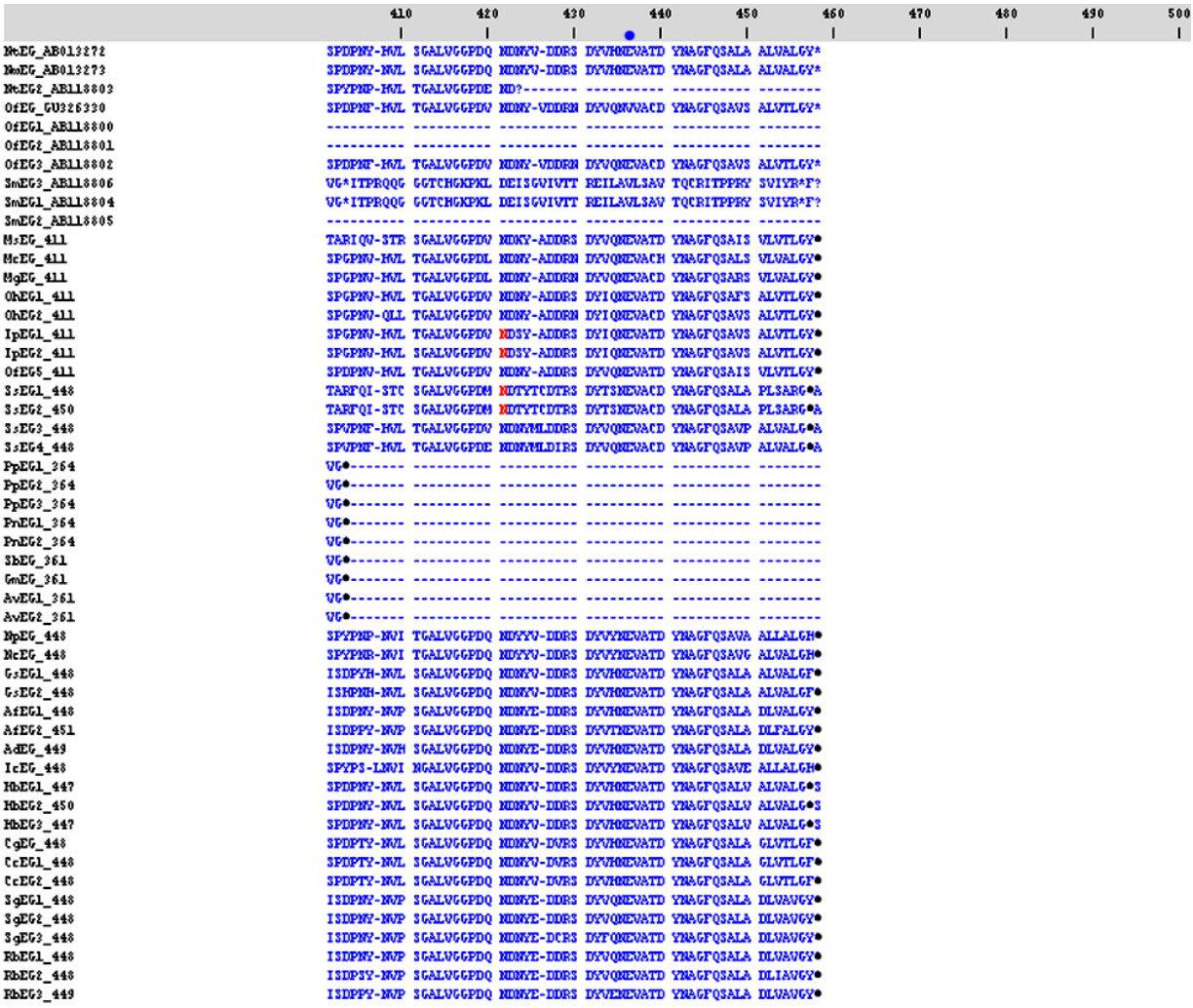

Figure 3-3. Continued.

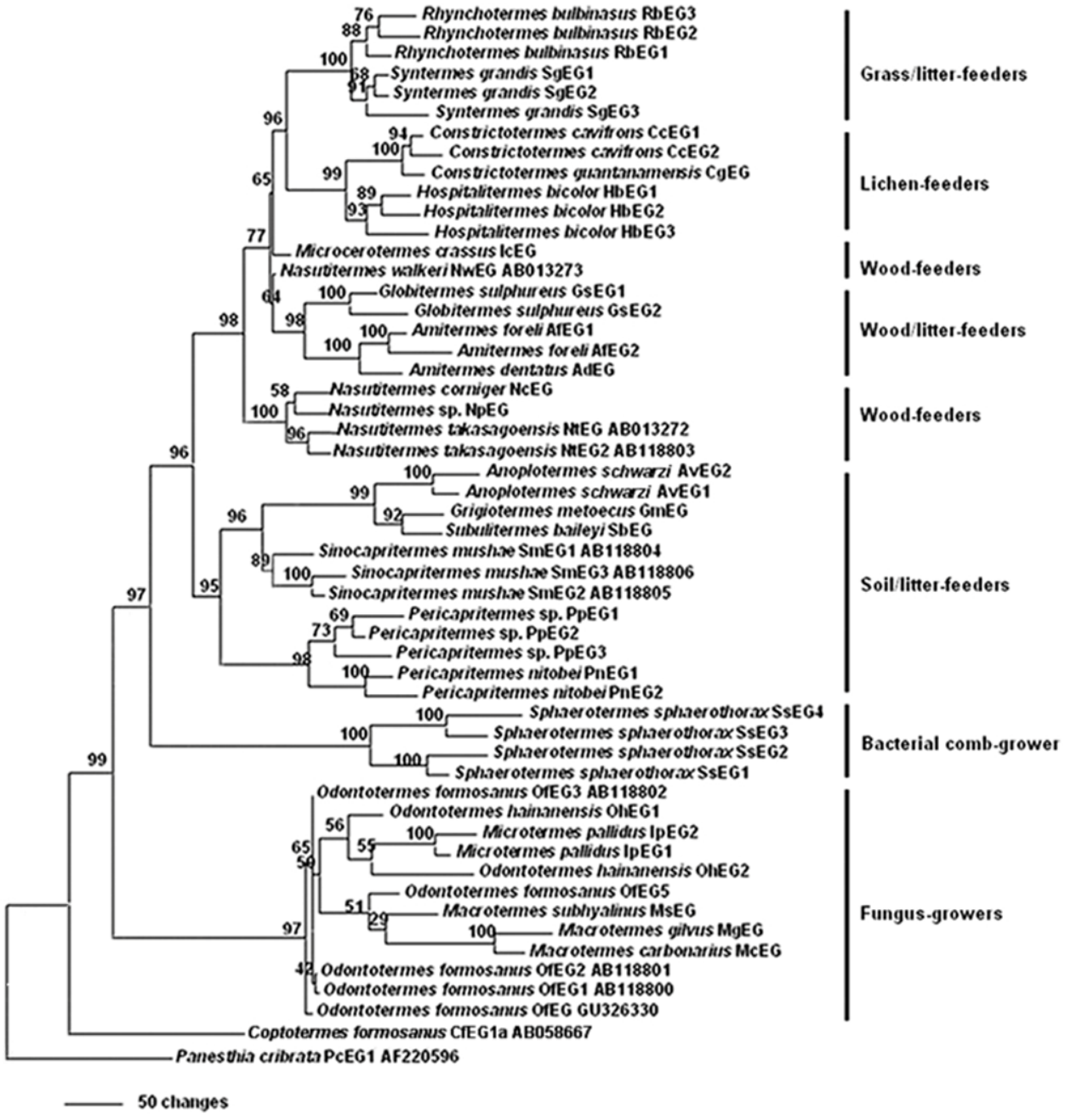

Figure 3-4. Consensus Bayesian tree inferred from endo-β-1,4-glucanase sequences. Numbers above branch nodes indicate posterior probabilities recovered by the Bayesian analysis. Branch lengths are proportional to the number of changes.

CHAPTER 4
A PHYLOGENETIC AND EVOLUTIONARY STUDY OF BETA-GLUCOSIDASE IN HIGHER TERMITES

β-glucosidases (or β-D-glucoside glucohydrolase) are Class 1 and 2 β-glycosidases which degrade cellobiose and other disaccharides (Marana et al., 2001). β-glucosidases are members of GHF1, which comprise 19 known representatives and 2,889 components, as listed in CAZy (Carbohydrate-Active enZYmes Database, website: http://www.cazy.org). β-glucosidase occurs in various insects, including termites (reviewed by Willis et al., 2010). It is a crucial component of termite digestion because it completes cellulose digestion by cleaving cellobiose and cello-oligosaccharides converting them into glucose (Robson and Chambliss, 1989). According to Lo et al. (2011), endogenously-produced cellulolytic enzymes are especially important in higher termites due to the lack of flagellates in their hindgut.

In higher termites, Potts and Hewitt (1972) reported β-glucosidase activity in the head and gut of *T. trinervoides*. However, at the time, its function was unknown because their findings showed that the β-glucosidase was incapable of hydrolyzing cellobiose. β-glucosidase activity was later reported in the foregut and midgut of *C. albotarsalis* (Rouland et al., 1989a) and shown as being most active in the salivary glands of *M. muelleri* (Rouland et al., 1989b). More recently, Binate et al. (2008) purified and characterized two β-glucosidases (β-Glc A and B) from *Macrotermes bellicosus* (Smeathman) intended for glycobiotechnology.

Tokuda et al. (2002) were the first to molecularly characterize β-glucosidase from termites, which was obtained from *N. koshunensis* (NkBG) (GenBank Accession No. AB073638). Following that, Ni et al. (2007) successfully overexpressed this NkBG cDNA in *E. coli*, which showed a 3-fold increase in the recombinant enzyme's specific

activity. Later, Tokuda et al. (2009) successfully sequenced β-glucosidases from the salivary gland and midgut of a higher termite, *Na. takasagoensis*. Scharf et al. (2010) later sequenced two β-glucosidases, RfBGluc-1 and RfBGluc-2, which were expressed in the salivary glands and foregut of *R. flavipes*. Zhang et al. (2010) recovered and cloned an endo-β-1,4-glucanase and a β-glucosidase from the cDNA library of *C. formosanus* and found successful cellulose to glucose conversion using the recombinant enzymes.

From an earlier study, Tokuda et al. (1997) found that the majority of β-glucosidase activity occurs in the salivary glands of *Na. takasagoensis* although they were uncertain of the function at the time (Tokuda et al., 2009), The recent discovery of egg-mimicry by the cuckoo fungus, Matsuura et al. (2009) demonstrated that β-glucosidase and lysozyme constitute the termite-egg recognition pheromone in *Reticulitermes* termites. Termite eggs were 'administered' with β-glucosidase during transportation into the nursery chamber and egg-grooming. Later, Matsuura and Yashiro (2010) reported a similar type of termite egg-mimicry by a different fungus with *Na. takasagoensis*. This is not surprising, because according to Ketudat Cairns and Esen (2010), β-glucosidases occur universally and serve many functions, including defense and plant-insect interactions.

Among different termitid castes, Deng et al. (2008) showed that β-glucosidase activity was highest in *O. formosanus* workers but that the level was not significantly different between soldiers and the *T. albuminosus* fungus with which the termites were associated. Fujita et al. (2008) reported that β-glucosidase activity was highest in the midgut of all castes of *Na. takasagoensis*. Low titers of β-glucosidase were also found in

the salivary glands of major and minor workers of *Na. takasagoensis* (Fujita et al., 2008), suggesting a similar egg-marking function to what was observed in *R. speratus*.

As mentioned previously, it is impossible to elucidate the evolution of feeding group within the higher termites using nuclear and mitochondrial markers alone (Inward et al., 2007a). To date, there have only been eight β-glucosidase sequence accessions from two species of higher termites [*Na. takasagoensis*, a wood-feeder (GenBank Accession No. AB508954-AB508960) and *O. formosanus*, a fungus-grower (GenBank Accession No. GU591172)] deposited in the GenBank database. The family Termitidae constitutes the majority of all termite species and the scarcity of molecular information on β-glucosidase makes it impossible to currently understand how termite endogenous digestion evolves at the molecular level.

Hence, in this study, my goal was to purify, clone, and sequence β-glucosidases, and elucidate its evolution across nutritionally diverse Termitidae. I hypothesized that the evolution of β-glucosidase will be the same as endo-β-1,4-glucanase, and congruent with the results from mitochondrial and nuclear markers. In addition, I aimed to determine the phylogenetic placement of *S. sphaerothorax* in the evolution of β-glucosidases among the higher termites.

Materials and Methods

Polymerase Chain Reaction

PCR was performed with specifically-designed β-glucosidase primers (Clone Manager 9 Professional Edition, Scientific & Educational Software, Cary, NC) as listed in Table 4-1. Amplifications were conducted using Terra™ PCR Direct Polymerase Mix (Clontech Laboratories, Inc., CA) in 50 µL final reaction volumes, each containing 12 µL dH_2O, 25 µL 2X Terra™ PCR Direct Buffer (with Mg^{2+} and dNTP), 2 µL cDNA template

(as was used in Chapter 3), 100 ng of each primer and 1.25 U Terra™ PCR Direct Polymerase Mix. After the initial denaturation at 98°C for 2 min, the temperature profile for 35 cycles was 98°C for 10 s, 60°C for 15 s, and 68°C for 2 min before cooling to 4°C.

Cloning and Sequence Analysis

The cloning procedures followed the method described in Chapter 3. However, the plasmids were sent for insert sequencing with M13F and M13R primer pairs to BioAnalytical Services Laboratory (BASLab), University of Maryland, Baltimore, MD. Sequence assembly, alignment and analyses of nucleotide and amino acid sequences also followed the procedures outlined in Chapter 3.

Results and Discussion

Of 25 termitid species, I was only able to amplify single PCR fragments of about 1.6 kb in length from four species of higher termites (Fig. 4-1). Based on RFLP profiles of the clones, I was able to obtain five different clones from *M. carbonarius*, *S. sphaerothorax*, *Anoplotermes schwarzi* Banks and *R. bulbinasus* (Fig. 4-2) (GenBank Accession No. xxxxxxx to xxxxxxx). Sequences from this study showed at least 63% amino acid similarity with *N. koshunensis* β-glucosidase and at least 52% identity with *Tenebrio molitor* Linnaeus β-glucosidase (GenBank Accession No. AAG26008). Protein BLAST search also showed that the amino acid sequences were similar to β-glucosidases in GHF1. This was consistent with β-glucosidases from *Na. takasagoensis* (Tokuda et al., 2009), although a β-glucosidase from GHF3 has been found in the salivary glands of a lower termite, *H. sjoestedti* (Yuki et al. 2008).

The sequences obtained also showed consistency with the findings of Tokuda et al. (2002; 2009) and Scharf et al. (2010), in that they possess conserved motifs involved

in substrate binding and catalysis, which are “NEPL”, with “E” (Glu, glutamic acid) as the proton donor and “TENG” with “E” (Glu, glutamic acid) as the nucleophile. The N-linked glycosylation, putative catalytic glutamic acid residues (proton donor and nucleophile) and stop codon positions are shown in Fig. 4-3.

The inferred β-glucosidase phylogenetic tree (Fig. 4-4) showed discrepancy with the mitochondrial/nuclear tree (Fig. 2-2). Instead of being the most basal group, the fungus-growers formed a strong diphyletic relationship with the wood- and soil/litter-feeders. The result further suggests that bacterial comb-grower β-glucosidases were derived from fungus-growers β-glucosidases.

As predicted, β-glucosidase paralogs from the same species clustered with one another, as seen with *Na. takasagoensis* and *S. sphaerothorax*. The β-glucosidase sequence from *R. bulbinasus*, a grass-/litter-feeder clustered within the wood-feeders. Both wood- and grass-feeders formed a paraphyletic group from the soil/litter-feeders.

In the fungus-growers/bacterial comb-grower clade, I suspect that the β-glucosidases of fungus-growers are least divergent because of the high dependency on fungi to digest their food. High levels of β-glucosidase are present in the fungal nodules with which *M. bellicosus*, *Odontotermes pauperans* (Silvestri), *Ancistrotermes cavithorax* (Sjoestedt) and *Pseudocanthotermes militaris* (Hagen) are associated (Sengupta and Sengupta, 1990). As stated by Darlington (1994) and Rouland-Lefèvre (2000), fungus-growers depend on fungal symbionts to process their food. When their ancestors lost their flagellates, these termites were still able to feed on wood and litter because of their close association with cellulolytic fungi, which function as an ‘external gut’ by partially digesting the wood for termites. According to Mishra and Sen-Sarma

(1985b), *T*. *albuminosus* contain glucosidases as well as laccase, chitinase and esterase, which are all essential in lignocellulose degradation. Rouland et al. (1988b) later reported that fungus-derived cellulases, β-glucosidase and another termite-derived cellulase work in synergy to digest cellulose. Earlier on, Abo-Khatwa (1978) showed that β-glucosidase activities in the *Termitomyces* conidiophores and midgut and hindgut of *M. subhyalinus* are almost equal, thus proving that the fungal nodules were able to replace the role of the missing flagellates.

Moreover, in macrotermitines, Martin and Martin (1978; 1979) suggested that *Termitomyces*-acquired digestive enzymes are required for digestion by *M. natalensis*. This was contested by Bignell et al. (1994), who suggested that the termite's endogenous cellulase activity alone is sufficient for resource utilization. According to a study by Hyodo et al. (2000), fungus-feeders consume the mature portion of the comb because the cellulose degradation in the old comb is three times higher than that of the fresh comb. Rouland et al. (1991) suggested that some fungus species produce fungal cellulases to match a substrate while others did not. Rouland-Lefèvre et al. (2006) later divided *Termitomyces* into a relatively generalist fungal genus (which contained various degradation enzymes depending on the substrate and grown by several termite species) and relatively specialist fungal species (which produced degradation enzymes for specific substrates and only associated with a single termite species). In the case of endogenous β-glucosidase evolution, I speculate that because the fungus itself is a major carbon source for these termites (Arshad et al., 1987), the types of fungi with which the termites are associated might have driven the selective pressure to code for the most adaptive β-glucosidase to occur.

In sphaerotermitines, I provide molecular evidence to suggest that bacterial comb-grower β-glucosidases probably evolved from fungus-growers β-glucosidases. Two β-glucosidase sequences, SSBG1 and SSBG2 were obtained from the bacterial comb-grower, suggesting two different alleles. Although Lo et al. (2011) stated that the role of different gene copies was unclear, in the case of *S. sphaerotermes*, the occurrence of two distinctly different β-glucosidases probably supports the "neofunctionalization" hypothesis by Ohno (1970), Force et al. (1999) and Hahn (2009), which resulted in a different function from the original gene. I suspect that different paralogs may be used to degrade cellobiose and cello-oligosaccharides of different physical and chemical properties, hence increasing cellulose digestion efficiency. Because of the presence of different bacterial strains within the *S. sphaerothorax* nest, (Garnier-Sillam, 1989), I speculate that this may have been the factor that drove the selection for different β-glucosidases to occur.

The nutritional dependency on fungus and bacteria has been lost in wood- and soil/litter-feeders. Henceforth, a shift has occured from having an 'external gut' in fungus- and bacterial comb-grower to having an 'internal gut' in wood- and soil/litter-feeding termites. Rouland et al. (1986; 1989a) reported a low endogenous digestive enzyme activity in the midgut and hindgut of soil/litter-feeding termites. Even though endogenously-produced cellulases are less important in the digestion of soil/litter-feeders, however, the gene is still maintained in the genome (Lo et al., 2011). I suspect that soil/litter-feeders developed an increased dependency on their hindgut microbiota to digest food with low cellulose content because of their highly humified nutritional requirements. Brauman et al. (2000) proposed that some compounds such as

polysaccharides are digested to a certain extent by a generalist gut flora after alkaline pretreatment and selected reduced substrates such as polyaromatic compounds are digested by a specialized symbiont population. There were some speculation that cellulolytic amoebae could play a role in cellulose utilization in some termitids (cited by Brauman, 2000; Slaytor, 2000; Eggleton, 2006).

The inferred tree shows that *R. bulbinasus* β-glucosidase, RbBG, falls within the wood-feeders clade. In wood-feeding termitids, Hogan et al. (1988) showed that β-glucosidase is secreted in the midgut of *Na. walkeri*. While the expression site has shifted exclusively to the midgut for endo-β-1,4-glucanase, β-glucosidase was still secreted in the salivary glands, as well as the midgut of *Na. takasagoensis* (Tokuda et al., 2009). This explains the considerable differences between salivary and midgut β-glucosidase paralogs, as observed in the inferred tree. According to Tokuda et al. (1999) and Slaytor (2000), the shift of expression sites from salivary glands in lower termites to the midgut in higher termites (which lack hindgut flagellates) enhanced cellulose digestion ability. Because I obtained only five different sequences from four termitid species, I could not support the idea of Tokuda et al. (2009) and Lo et al. (2011) that β-glucosidase gene copy numbers increased with more derived species. Nevertheless, I speculate that their high divergence was because selection pressure on wood-feeders was more relaxed, thus selecting for multiple types of β-glucosidases to occur, resulting in an increased efficiency in resource utilization.

The evolutionary origin of termitid β-glucosidases remains to be answered because there has yet to be a complete coding sequence of this gene available in the molecular database. While there is a high level of evidence to reject the horizontal gene

hypothesis in GHF9 (Lo et al., 2003; Davison and Blaxter, 2005), the evolutionary origin of GHF1, whether it was from a metazoan ancestor or horizontally-acquired more recently, is still debatable. Tokuda et al. (2002) supported vertical gene transfer because the *N. koshunensis* β-glucosidase (NkBG) that they obtained was closely related to those from various insects in the GenBank. However, in contrast, Ketudat Cairns and Esen (2010) have suggested that insect β-glycosidases have diverged from plants because some insects have adapted glycosides and glycoside hydrolases from the plants that they consumed. Unfortunately, such speculation could not be confirmed at present with endogenous termite β-glucosidases because of the very limited amount of available information.

Table 4-1. List of β-glucosidase primers used in this study

Name	Gene	Orientation	Sequence (5' to 3')
BG1f	BG	Forward	GGCAGAGCAACGAAATG
BG1r	BG	Reverse	AAGCGCCAGGGATATG
BG2f	BG	Forward	TTCCCGATGGATTTCTG
BG2r	BG	Reverse	ACAAACCGCTAGATGAAG
BG3f	BG	Forward	GACAGTTTGCTTCGTTATC
BG3r	BG	Reverse	ACTCGTAATCAGCAGTATG
BG4f	BG	Forward	ATTCCCCGATGGATTTC
BG4r	BG	Reverse	AACAAACCGCGTTTTC

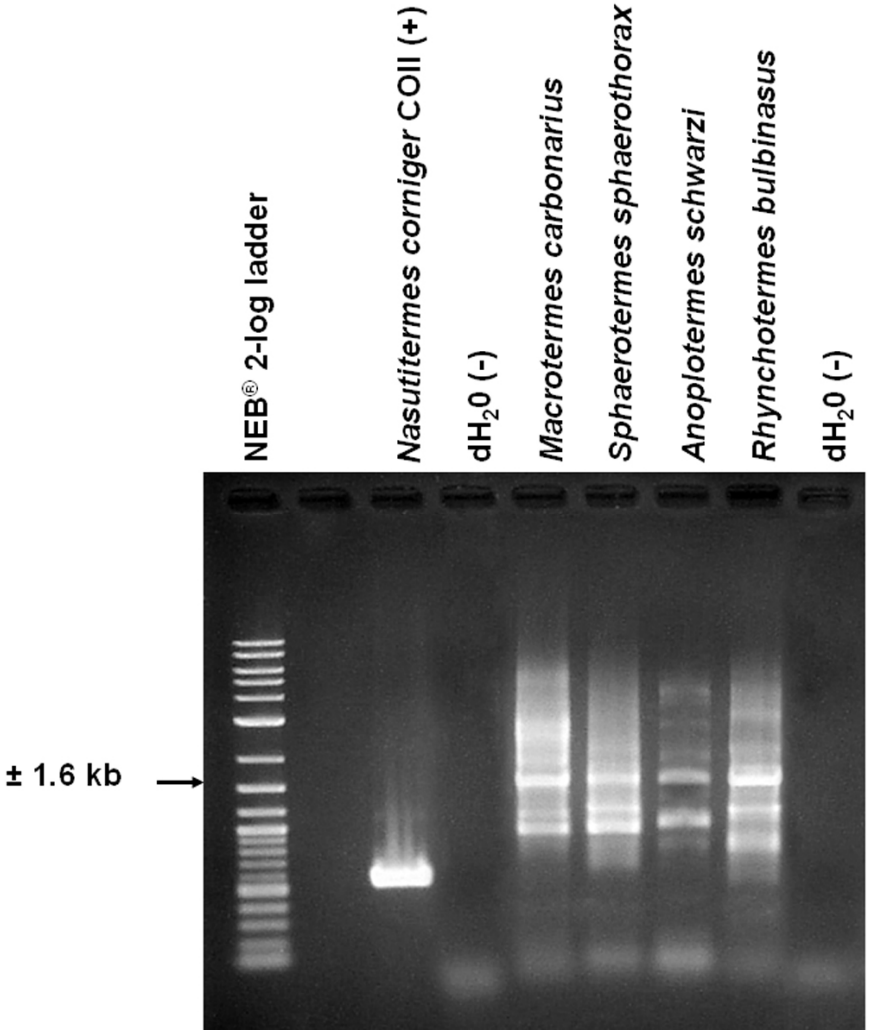

Figure 4-1. Agarose gel showing PCR amplification of β-glucosidase from four species of higher termites used in this study with positive and negative (water) control.

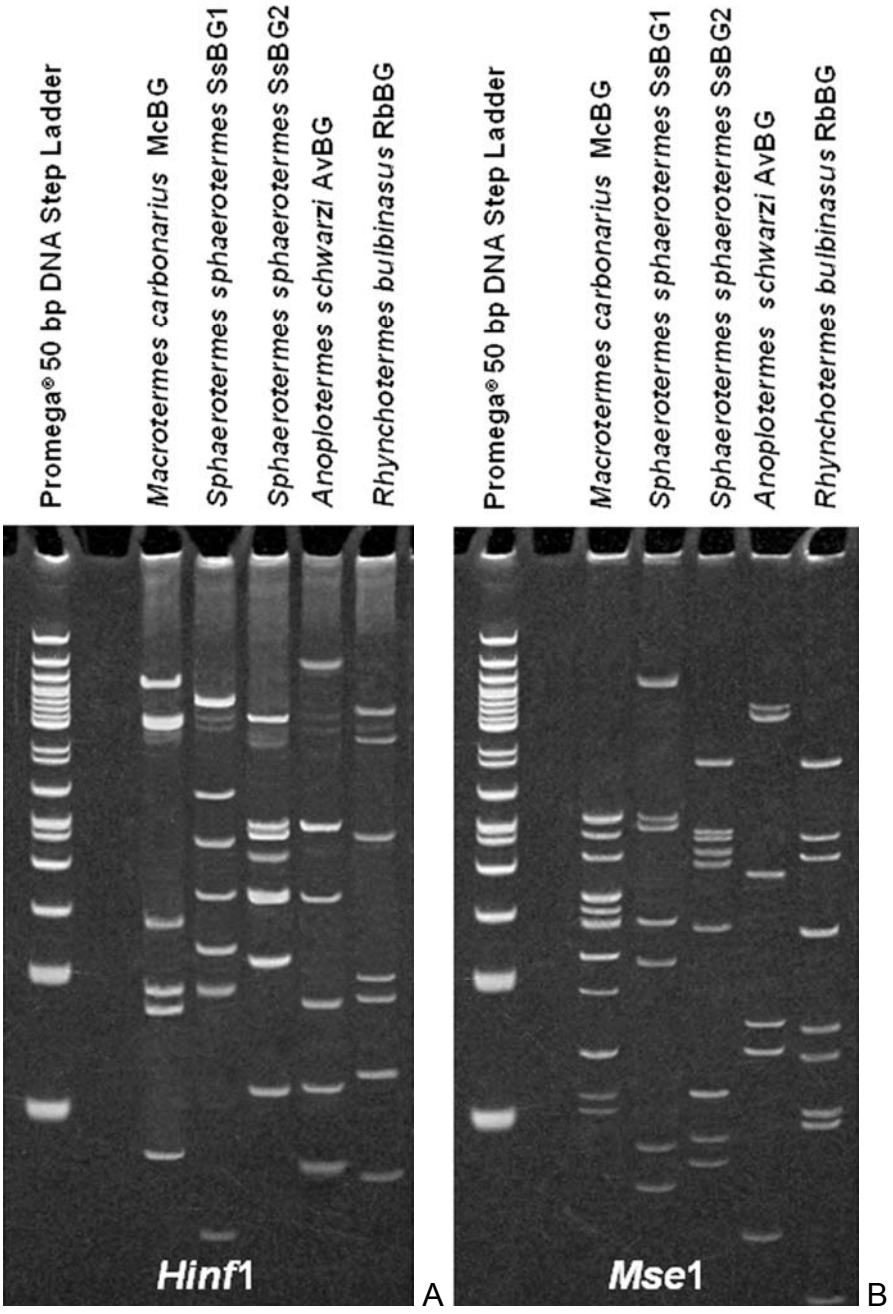

Figure 4-2. Restriction fragment length profiles of five β-glucosidase clones (ca. 1.6 kb) from four species of higher termites used in this study. A) Digestion with *Hinf*1 and B) Digestion with *Mse*1.

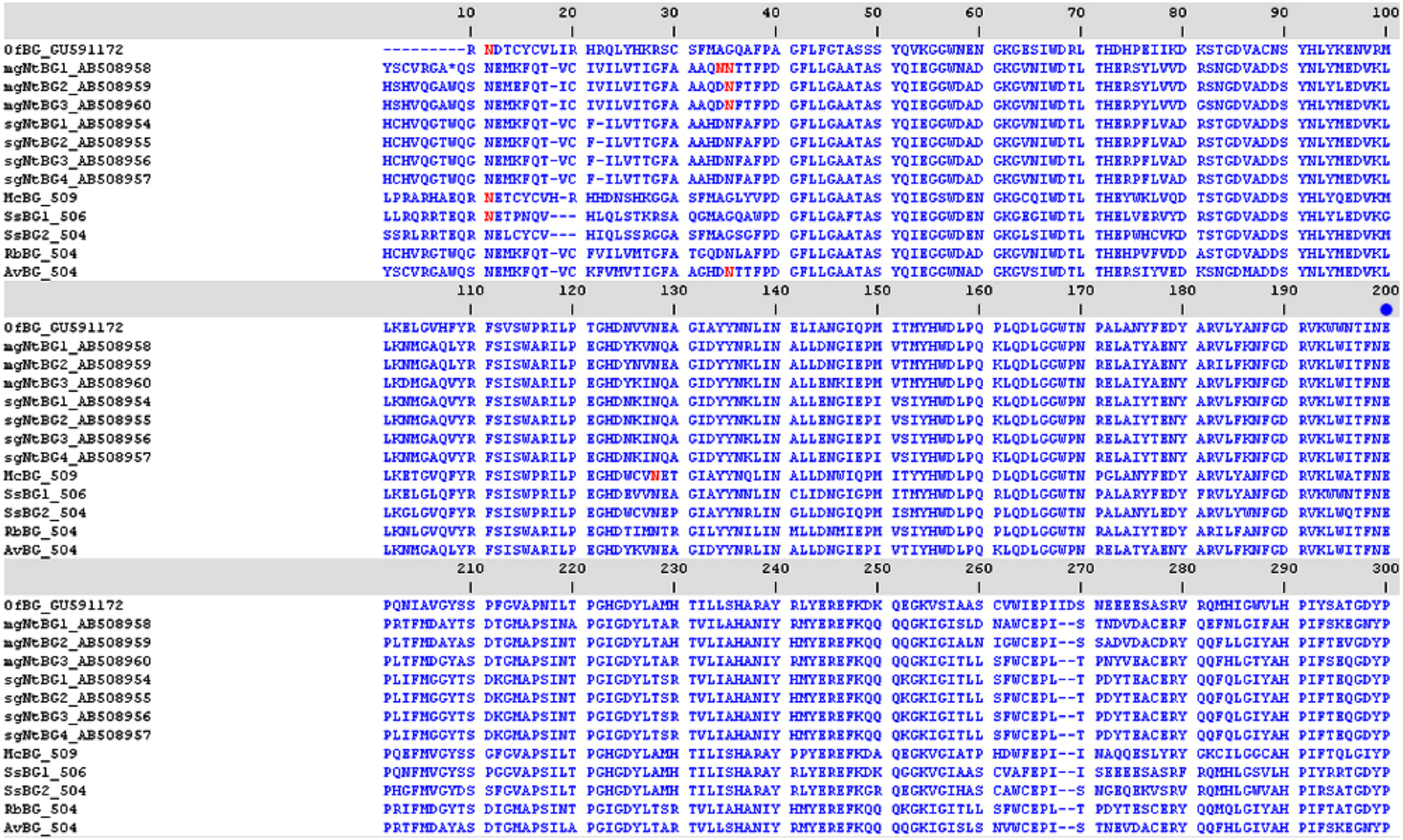

Figure 4-3. Multiple alignments of β-glucosidase amino acid sequences from higher termites. The number of encoded amino acids was listed next to the sequence names. N-linked glycosylation sites are highlighted in red. Blue dot indicates putative proton donor. Red dot indicates putative nucleophile. Black dot indicates the position of stop codon.

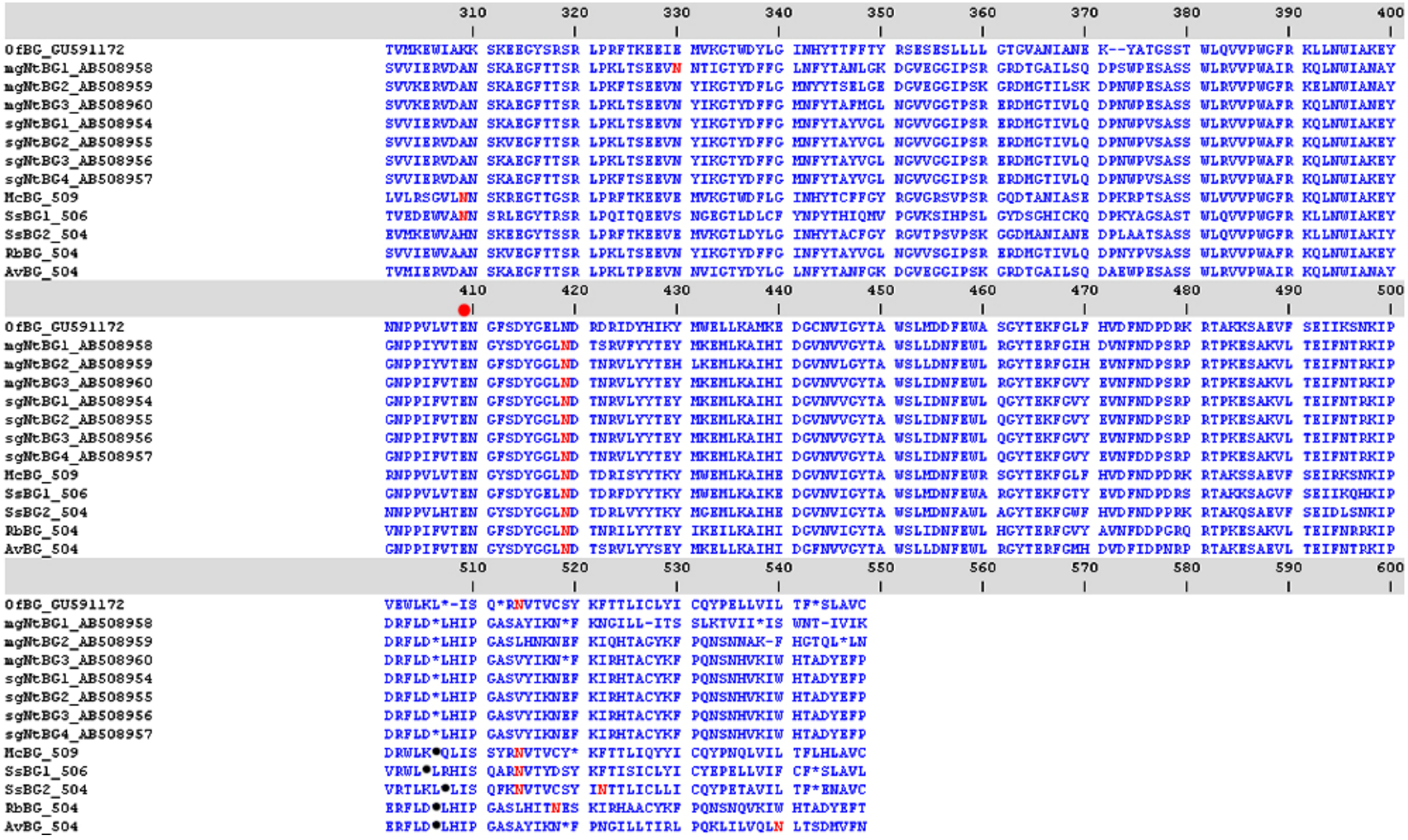

Figure 4-3. Continued.

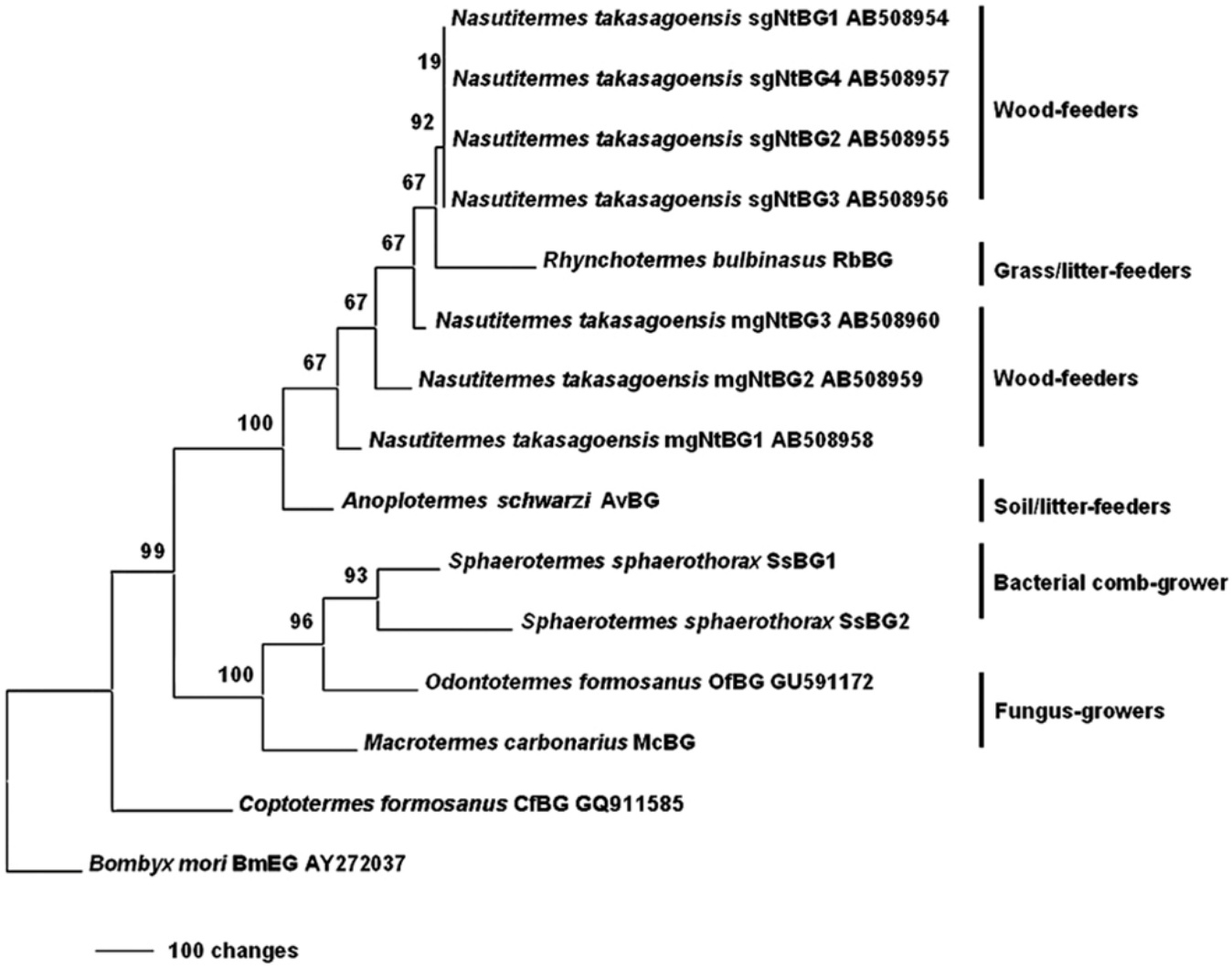

Figure 4-4. Consensus Bayesian tree inferred from β-glucosidase sequences. Numbers above branch nodes indicate posterior probabilities recovered by the Bayesian analysis. Branch lengths are proportional to the number of changes.

CHAPTER 5
CONCLUDING REMARKS AND FUTURE DIRECTIONS

The family Termitidae comprise a majority of all termite species and boasts an ability to exploit a wide range of feeding substrates, ranging from fungi, bacterial comb, plant materials of various stages of humification and soil/litter. In this study, I attempted to explain how endogenous cellulase digestion evolved in higher termites, with respect to the evolution of endo-β-1,4-glucanases and β-glucosidases.

Firstly, however, the phylogeny of a selection of 25 species of higher termites was delineated using mitochondrial (16S) ribosomal RNA and nuclear (28S) gene markers. Inferences from the sequences of fragments of the 16S and 28S genes corroborated with the outcome of studies by Inward et al. (2007a) and Legendre et al. (2008) regarding the monophyletic status of the family Termitidae relative to the lower termites. The fungus-growing termites were the most phylogenetically basal group, while the wood/lichen/grass/litter- and soil/litter-feeding termites were the most derived groups. The bacterial comb-growing termites were phylogenetically placed between fungus-growing and wood/lichen/grass/litter- and soil/litter-feeding termitids. The macrotermitines and apicotermitines were monophyletic, the termitines were paraphyletic and the nasutitermitines were polyphyletic. Finally, there was a paraphyletic relationship between the soil/litter feeders and the wood/grass/lichen/litter-feeders.

For endo-β-1,4-glucanase, I hypothesized that the evolution of endo-β-1,4-glucanase will be congruent with the evolution of termites according to the mitochondrial and nuclear markers. Forty-one endo-β-1,4-glucanase sequences were obtained from 23 species of higher termites. The deduced amino acid sequences showed that they

were similar to endo-β-1,4-glucanase in GHF9. The inferred tree suggested that the fungus-growing termites were the most phylogenetically basal group, while the wood- and soil/litter-feeding termites were the most distal groups. The bacterial comb-grower was phylogenetically placed as the intermediary that bridged the transition from being a fungus-growing termite to being a wood- and soil/litter-feeding termite. There were strong diphyletic relationships between endo-β-1,4-glucanases of upper layer soil-feeders and the other soil-feeders, and between the lichen- and the grass/litter-feeders within the wood/lichen/grass/litter-feeding termites clade. Sequences from the bacterial comb-feeder also showed that they were significantly different from each other, thus suggesting different alleles, which subsequently resulted in different functions from the original gene.

As with endo-β-1,4-glucanase, I hypothesized that the evolution of β-glucosidase would also be congruent with the evolution of termites according to the mitochondrial and nuclear markers. Five β-glucosidase sequences were obtained from four species of higher termites. The deduced amino acid sequences show that they are similar to β-glucosidases in GHF1. The inferred β-glucosidase phylogenetic tree conflicts with the mitochondrial and nuclear data, with the fungus-growers forming a strong diphyletic relationship with the wood- and soil/litter-feeders. Furthermore, it suggests that bacterial comb-grower β-glucosidases were probably derived from fungus-growers β-glucosidases. Two different sequences obtained from the bacterial comb-feeder suggest the involvement of two different alleles, resulting in different gene functionality.

While there was a high level of evidence to support vertical gene transfer hypothesis in GHF9, the evolutionary origin of GHF1 remains unanswered because of

the limited amount of available information. Further research should focus on providing the complete coding sequences to understand the evolutionary origin of β-glucosidases.

Molecular characterization of termite endogenous cellulases serves as a critical initial step towards using termites as bioresources for industrial applications. While this study has focused solely on termite endogenous cellulases, termites are also a rich source of xylanases, amylase, pectinase, lignin peroxidases, manganese peroxidases and laccases, regardless of whether they are termite-derived or of microbiotic origin. These recombinant enzymes or genes can be mass-produced for use in industrial application and biofuel production.

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BIOGRAPHICAL SKETCH

Nurmastini Sufina binti Bujang was born in Kuching, Sarawak in 1977. Growing up as a child in Borneo, she had often embarked on her own “exploration of nature”, taking nature walks, climbing trees, catching or simply observing small mammals, reptiles, amphibians, fish and insects. To her, nature is indeed, the best teacher.

After obtaining a Bachelor of Applied Science in parasitology from Universiti Sains Malaysia, Pulau Pinang in 2000, she developed a much deeper interest in insects. Under the guidance of Professor Lee Chow Yang, she studied the biological parameters and control of the “smooth” cockroach, *Symploce pallens* (Stephens). Nurmastini graduated with a Master of Science in entomology from Universiti Sains Malaysia in 2005.

In the Fall of 2006, she enrolled in a Doctor of Philosophy program at the University of Florida under Professor Nan-Yao Su. After working closely with Dr Nigel Harrison, she has now developed a deep interest in molecular biology. Her ultimate dream one day is to return home to set up her own independent molecular laboratory to study termites from the deep rainforest of Borneo. Nurmastini graduated with a Doctor of Philosophy in entomology from the University of Florida in the Summer of 2011.

In youth we learn; in age we understand.

—Marie von Ebner-Eschenbach