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Research Article

Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*

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Abstract. Cellulase genes of *Pseudotrichonympha grassii* (Hypermastigida: Eucomonymphidae), the symbiotic flagellate in the hindgut of the wood-feeding termite *Coptotermes formosanus*, were isolated and characterized. The nucleotide sequences of the major cellulase component in the hindgut of *C. formosanus* were determined based on its N-terminal amino acid sequence. The five isolated nucleotide sequences (*PgCBH-homo*s) had an open reading frame of 1350 bp showing similarity to catalytic domains of glycoside hydrolase family (GHF) 7 members, and primary structure comparison with GHF7 members whose tertiary structures are well-characterized revealed the overall similarity between PgCBH-homo and the catalytic domain of a processive cellulase Cel7A (formerly CBHI) from the aerobic fungus *Trichoderma reesei*. Functional expression of *PgCBH-homo*s in *Escherichia coli*, using the carboxymethylcellulose-Congo red assay, demonstrated the actual cellulolytic activity of PgCBH-homo. RT-PCR showed that *PgCBH-homo*s were expressed, from the three flagellates in the hindgut, specifically in *P. grassii*.

Key words. Cellulase; *Pseudotrichonympha grassii*; parabasalia; symbiosis; termite; *Coptotermes formosanus*; glycoside hydrolase family 7.

Cellulose is the most abundant organic compound on earth and the major polysaccharide component of plant cell walls. Despite of its abundance in the biosphere, it had been believed until recently that, without help from microbes, animals could not utilize cellulose due to their inability to produce cellulases. Though some animals have recently been demonstrated to produce endogenous cellulase [1], others have achieved effective cellulose utilization by developing intimate relationships with microbes harbored in their gut as primary cellulolytic agents. One of the most prominent among such animals is, due to its ecological and economic importance, the termite.

As decomposers, termites are a keystone species forming a base to the grazing food chain in tropical terrestrial ecosystems [2]. They possess their own systems of nutritional mutualisms between various microorganisms, in which they overcome obstacles in utilizing a diet consisting of recalcitrant lignocellulosic plant material ranging from sound wood to soil organic matter [3]. Among these specialized assimilating functions, the cellulose utilization by some wood-feeding termites harboring dense populations of unique flagellates in their hindgut

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[4, 5] is well known and studied, possibly due to the large economic impact of these termites [6]. Since early demonstrations of the indispensability of these intestinal flagellates to the survival of the host termites on a diet of wood or pure cellulose [7, 8], cellulase production [9–11] and the anoxic fermentation of cellulose to acetate [12–15] by these flagellates have been established. In the present model of metabolic flow in the hindgut of wood-feeding termites, the major role of flagellates is the primary digestion of wood particles, and the acetate produced in the hindgut is subsequently absorbed through the hindgut walls and utilized by the host termites as an energy source and a precursor for biosynthesis [16–18].

Despite these findings on the biochemical aspects of the flagellates, the majority of investigations have been restricted primarily to their morphology, due to the difficulties involved in cultivating these organisms. Thanks to advances in molecular biological techniques such as PCR and in situ hybridization, studing these symbionts independent of culture has recently become possible. First, the phylogenetic positions of the organisms were determined. These flagellates, which belong to the protist groups Oxymonadida, Trichomonadida, and Hypermastigida (the latter two belong to the class Parabasalia), represent early-branching eukaryotes [19, 20]. Following these studies, the presence was demonstrated of diverse genes encoding putative cellulases homologous to members of glycoside hydrolase family (GHF; [21]) 45 in the hindgut of the wood-feeding termite *Reticulitermes speratus* (family Rhinotermitidae), and some of these genes were identified as originating from the hypermastigotes *Teranympha mirabilis* and *Trichonympha agilis* [22].

A wood-feeding termite *Coptotermes formosanus* (family Rhinotermitidae) is a cosmopolitan pest species, which harbors in its hindgut three species of symbiotic hypermastigotes: *Spirotrichonympha leidyi* (Spirotrichonymphidae), *Holomastigotoides mirabile* (Holomastigotidae), and *Pseudotrichonympha grassii* (Eucomonymphidae) (fig. 1) [4, 23, 24]. These flagellates have specific roles in decomposition of wood particle, and *P. grassii* is essential to utilize cellulose of high molecular weight [25–27]. Our previous report demonstrated the presence of two separate cellulolytic systems in *C. formosanus* – that of the host (the termite) itself in the midgut and that originating from its symbiotic fauna in the hindgut – but as a result of molecular analysis, in which we placed more weight on the host system [28]. Here we report the isolation and characterization of cDNAs encoding a major cellulase component in this symbiotic system. The isolated sequences were similar to fungal cellulases placed in GHF7 and originated from the major cellulolytic agent in the symbiotic cellulose digestion, *P. grassii*.

Materials and methods

Animals

C. formosanus workers were collected from a colony maintained in the laboratory under dark conditions at 26 °C with 65 % relative humidity with blocks of *Pinus densiflora* as food.

cDNA cloning

Degenerate oligonucleotide primers, CfF (5′-AAY CAR GCI GAR AAY CAY CC-3′) and F7R (5′-GCY TCC CAD ATR YCC ATY TC-3′), were designed, respectively, from the amino acid sequences of the N terminus of the major hindgut cellulase component of *C. formosanus* [28] and from the conserved region among most GHF7 members, corresponding to the catalytic center. First-strand cDNAs were synthesized from mRNAs purified from the salivary glands, foregut, midgut, and hindgut of *C. formosanus* as previously described [29] and used as templates in PCRs performed with the degenerate primers (CfF and F7R). Amplified fragments were ligated into pGEM-T plasmid vector (Promega) and transformed into JM109 bacterial host cells. The constructs were sequenced in both directions with an ABI3700 automated capillary DNA sequencer (Applied Biosystems) using the BigDye Terminator Sequencing Reaction Kit (Applied Biosystems) with SP6 or T7 primer. Both 5′ and 3′ flanking regions of the fragments were obtained using the SMART RACE cDNA amplification kit (Clontech) with gene-specific primers, and subsequently cloned and sequenced as described above. To confirm closely related sequences or Taq errors, at least five colonies were sequenced for each cloned product.

RT-PCR

Total RNA was isolated from single cells of flagellates as follows. The hindgut contents of the workers were suspended in a 90 % concentration of Solution-U [30], and

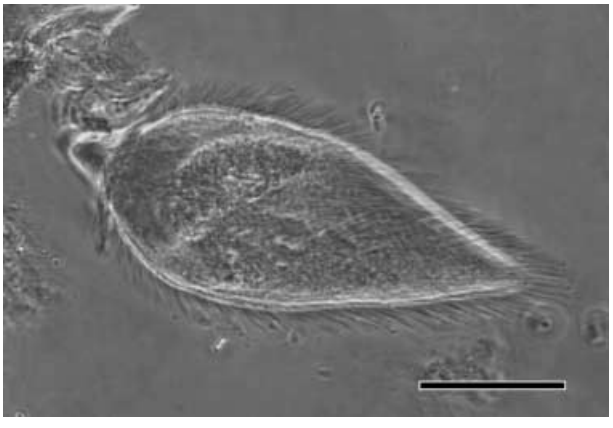

Figure 1. A phase contrast micrograph of *Pseudotrichonympha grassii* from the hindgut of *Coptotermes formosanus*. (bar, 50 μm).

single cells of flagellates were pipetted manually with Finpipette 10 (Labsystems) under a microscope. Each pipetted flagellate was ejected into 100 µl of acetone in separate PCR tubes (0.5 ml) to be dehydrated, and the acetone was dried up by heating the tubes at 94 °C for 1 min. From the dried specimens including isolated RNAs, first-strand cDNAs were synthesized using oligo-dT primer with Superscript II (Life Technologies) following the manufacturer's directions and used as templates in PCRs performed with gene-specific primers designed to amplify the entire open reading frame (ORF) of any cloned sequences: PgF (5′-CCG CTC GAG ATG TTT GYT WTT GTT TTA C-3′) for the sense strand and PgR (5′-GGC GGA TCC TTA GTA AGT TGA GTC AAT TG-3′) for the antisense strand.

Sequence analysis

Homology searches of the determined sequences were performed using the BLAST program [31] through the NCBI server at http://www.ncbi.nlm.nih.gov/BLAST/. Multiple alignments of amino acid sequences were performed using CLUSTAL_X software [32] and refined manually against the three-dimensional structure-based alignment of GHF7 members [33]. Based on alignments of nucleotide sequences using CLUSTAL_X, a phylogenetic tree was constructed by TREE-PUZZLE software [34] with quartet sampling and neighbor joining of 1000 puzzling steps, using the HKY substitution model.

Expression of cloned cDNAs in *Escherichia coli*

The entire ORFs of the cloned cDNAs (except for the putative signal peptide sequences) were subcloned into the *lacZ* frame of pBluescript II plasmid vector (Stratagene) and transformed into the JM109 bacterial host. The transformants were assayed for hydrolase activity on carboxymethylcellulose (CMC), employing a Congo red staining method as previously described [29].

Results

cDNA cloning

In the PCR experiments with the degenerate primers, amplification occurred in the hindgut sample, but not in any other tissue samples. The amplified fragment was cloned, and its flanking 5′ and 3′ regions were obtained by RACE amplification. As a result, five sequences (*PgCBH-homo1*, *PgCBH-homo2a*–*PgCBH-homo2d*) with ORFs of 450 amino acid residues were isolated. PgCBH-homo1 differed from PgCBH-homo2 (encoded in *PgCBH-homo2a*–*PgCBH-homo2d*) at four positions (Val3/Ala3, Phe4/Ile4, Trp8/Gly8, and Gln11/Arg11); however, these residues were located upstream of the experimentally determined N-terminal sequence (LGTGTNQAEN) [28] starting from Leu13, so PgCBH-homo1 and PgCBH-homo2 are identical in their matured forms (PgCBH-homo). The determined sequences will appear in the nucleotide sequence databases under accession numbers AB071864 to AB071868.

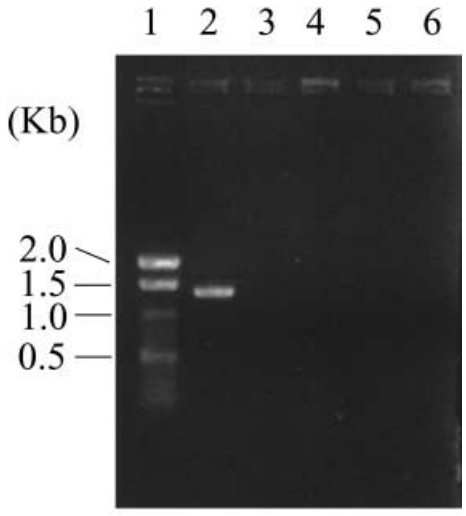

Figure 2. Expression of *PgCBH-homo*s in the hindgut of *C. formosanus*. PCR with primers specific to *PgCBH-homo*s was performed using a single cell of the hindgut flagellates as a template. Lane 1, marker; lane 2, *P. grassii*; lane 3, *H. mirabile*; lane 4, *S. leidyi*; lane 5, background (Solution-U); lane 6, negative control without template.

RT-PCR

To identify the origin of the obtained genes, RT-PCRs for the entire ORFs of the five cloned sequences were performed using a single cell of each flagellate species as templates. Among the templates (*P. grassii*, *H. mirabile*, *S. leydii*, and background Solution-U), only the *P. grassii* sample amplified to the expected size (1371 bp) (fig. 2). Subsequent sequencing of the amplified fragments confirmed the expression of the genes in *P. grassii*.

Structural and molecular-phylogenetic analyses

The homology search by BLAST-P revealed the similarity of PgCBH-homo to members of GHF7. To investigate the potential function of PgCBH-homo, multiple alignments were performed for PgCBH-homo and the structurally analyzed GHF7 members *Trichoderma reesei* Cel7A [35] and Cel7B [33], *Humicola insolens* Cel7B [36], and *Fusarium oxysporum* CelB [37]. A total of 96 out of 438 residues were conserved among all of the aligned sequences, including residues corresponding to the catalytic triad of Glu212 (nucleophile), Asp214, and Glu217 (general acid/base) [38] and to the 16 cysteines forming the disulfide bonds of *T. reesei* Cel7A [35] (fig. 3). Regions corresponding to the covering loops over the catalytic tunnel in *T. reesei* Cel7A [35] were conserved in PgCBH-homo (Asn191-Gly204, Val232-Gly254, Asn323-Met327, Asp335-Thr348, and Ile388-Val400; fig. 3).

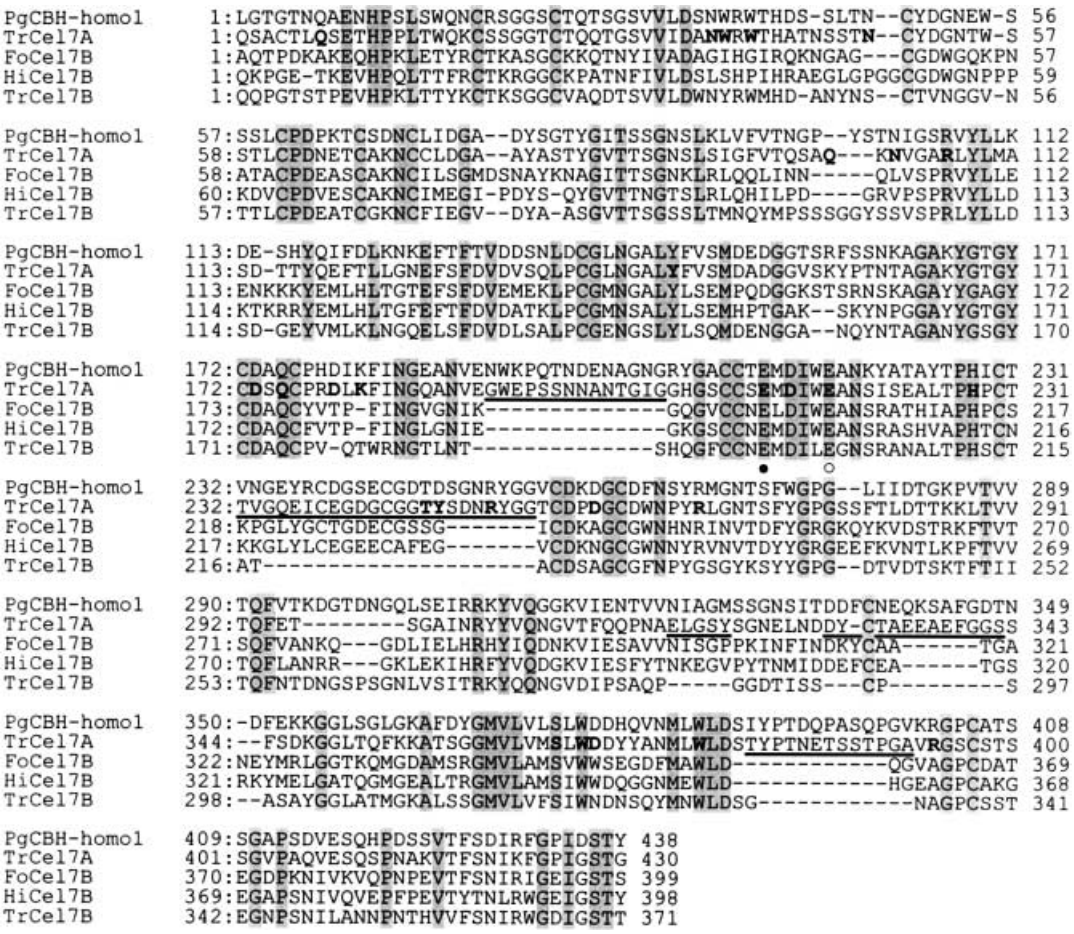

Figure 3. Multiple alignments of PgCBH-homo and the catalytic domains of glycoside hydrolase family 7 members. TrCel7A, a cellobiohydrolase component, *Trichoderma reesei* Cel7A [Swiss Prot. P00725]; FoCel7B, an endo-β-1,4-glucanase (EG) component, *Fusarium oxysporum* Cel7B [P46237]; HiCel7B, an EG component, *Humicola insolens* Cel7B [P56680]; TrCel7B, an EG component, *T. reesei* Cel7B [P07981]. The alignments were performed using CLUSTAL_X [32] and subsequent manual refinement based on the three-dimensional structures of reference sequences. Arabic numerals denote the number of residues from each N terminal in the mature form. Solid and open circles under the column indicate the sites of proton donors and general acids/bases, respectively. Shaded columns represent conserved positions within the sequences. Bold type denotes residues which interact with substrate cellulose chains in the catalytic tunnel of *T. reesei* Cel7A [39]. The underlined sequences in TrCel7A indicate loop-forming regions covering the catalytic tunnel [33, 35].

Most residues interacting with the substrate sugar chain in the catalytic tunnel of *T. reesei* Cel7A [39] were conserved in PgCBH-homo (fig. 3).

Phylogenetic analysis based on the nucleotide sequences showed that GHF7 members are mostly divided into two clusters: one made up of endo-β-1,4-glucanases (EGs) and the other of CBHs (fig. 4), and *PgCBH-homo1* is included in the CBH-cluster.

Expression of *PgCBH-homo1* in *E. coli*

The PgCBH-homo-coding region of *PgCBH-homo1* was subcloned into the *lacZ* frame of the plasmid and transformed into *E. coli* strain JM109, and the transformants formed clear halos on the CMC-LB plate after staining with Congo red and subsequent washings, whereas negative controls harboring the plasmid without inserts did not form halos (fig. 5).

Discussion

In the present study, novel GHF7 cellulases were isolated from the hindgut of the wood-feeding termite *C. formosanus*, which harbors dense populations of three species of symbiotic flagellates. In these flagellates, the isolated genes, *PgCBH-homo*s, were expressed specifically in *P. grassii* (fig. 2). Since the cDNAs were isolated by the poly-(A)-tail-based cloning method, possible prokaryotic origins of *PgCBH-homo*s in intracellular bacteria/archea or in externally attached spirochaeta on the body of *P. grassii* can most likely be excluded. Given that 15 genes encoding homologous GHF45 cellulases are expressed in the hindgut of another wood-feeding termite *R. speratus* [22], the present 5 genes encoding the same protein may represent a similar diversity of homologous cellulase genes in the hindgut fauna of *P. grassii*.

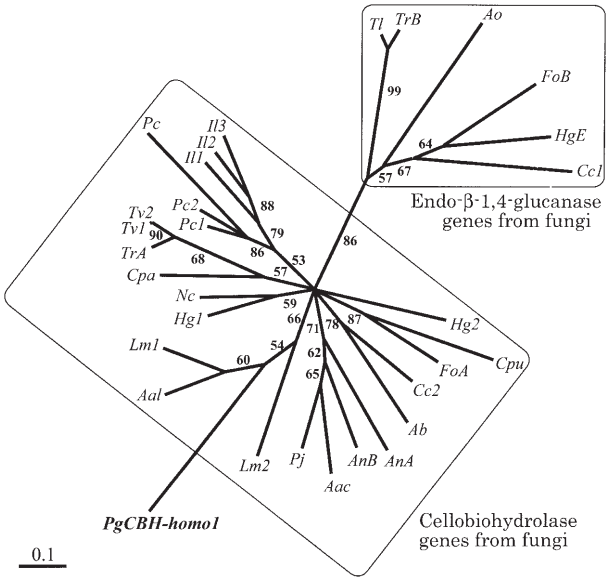

Figure 4. Phylogenetic relationships among GHF 7 members. This is an unrooted tree inferred from the maximum-likelihood method using 1382 aligned nucleotide positions, corresponding to the putative catalytic domains, of 30 fungal genes (6 endo-β-1,4-glucanase and 24 cellobiohydrolase genes) in GenBank and a parabasalian gene, *PgCBH-homo1*. Numbers at the internal branches indicate the percent occurrence of a group in 1000 quartet puzzling steps. The scale bar indicates the number of substitutions per position as given by TREE-PUZZLE [34]. The GenBank accession numbers together with the source organism of the reference sequences are: *Aac*, AB002821, from *Aspergillus aculeatus*; *Aal*, AF176571, from *Alternaria alternata*; *Ab*, Z50094, from *Agaricus bisporus*; *AnA*, AF156268, from *Aspergillus niger*; *AnB*, AF156269, from *A. niger*; *Ao*, D83732, from *Aspergillus oryzae*; *Cc1*, U25129, from *Cochliobolus carbonum*; *Cc2*, AF336799, from *C. carbonum*; *Cp*, L43048, from *Cryphonectria parasitica*; *Cpu*, Y07550, from *Claviceps purpurea*; *FoA*, L29379, from *Fusarium oxysporum*; *FoB*, L29378, from *F. oxysporum*; *Hg1*, D63515, from *Humicola grisea*; *Hg2*, AF123441, from *Humicola grisea* var. *thermoidea*; *HgE*, D63516, from *H. grisea*; *Il1*, AB019375, from *Ilpex lacteus*; *Il2*, AB019376, from *I. lacteus*; *Il3*, AB019377, from *I. lacteus*; *Lm1*, AF240000, from *Leptosphaeria maculans*; *Lm2*, AF240001, from *L. maculans*; *Nc*, X77778, from *Neurospora crassa*; *Pc*, S40817, from *Phanerochaete chrysosporium*; *Pc1*, M22220, from *P. chrysosporium*; *Pc2*, L22656, from *P. chrysosporium*; *Pj*, S56178, from *Penicillium janthinellum*; *Tl*, X60652, from *Trichoderma reesei*; *TrA*, X69976, from *T. reesei*; *TrB*, M15665, from *T. reesei*; *Tv1*, X53931, from *Trichoderma viride*; and *Tv2*, AB021656, from *T. viride*.

To our knowledge, all GHF7 members reported so far are from fungi; however, we found clones similar to GHF7 members in public databases of expressed sequence tag libraries of a social amoeba *Dictyostelium discoideum* (*Dictyostelium discoideum* cDNA Project at http://www. csm.boil.tsukuba.ac.jp/cDNAproject.html) and a cereal *sorghum bicolor* (Sorghum EST Project at http://www. botany.uga.edu/prattlab/Sorghum.htm), although they are not full-length protein-coding sequences. So the present dominance of fungal sequences in GHF7 seems to reflected of our unawareness, and new members will come to light outside mycelia.

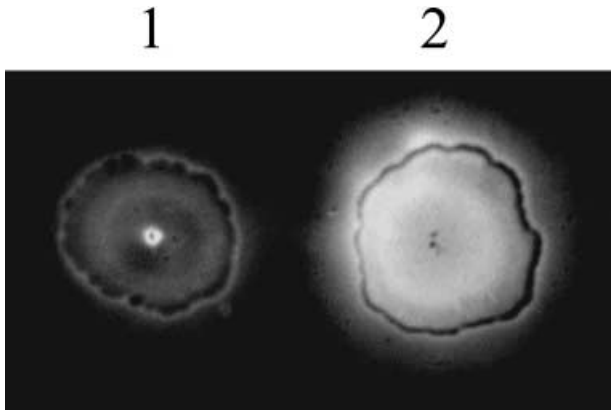

Figure 5. Heterologous expression of *PgCBH-homo1* in *Escherichia coli*. The *PgCBH-homo1* ORF (not including putative signal peptide sequences) was ligated in pBluescript II plasmid vector and expressed in *E. coli* strain JM109, on an LB plate containing 0.1% (w/v) CMC under IPTG induction. The transformant formed a translucent halo after staining with Congo red (2). No halos were formed by colonies lacking the *PgCBH-homo1* insert (1).

GHF7 is subdivided, according to the mode of catalytic action, into two groups: EG and CBH. EGs randomly cut internal linkages of a cellulose chain with a preference for amorphous regions of cellulose, and CBHs processively cleave cellobiosyl units from a chain end and can degrade crystalline regions of cellulose [40, 41]. Structural analyses revealed the general folding of GHF7 and the structural basis for the difference between EGs and CBHs [33, 35–37, 39]. GHF7 folds a β-sandwich structure of two β sheets, one concave and one convex. Structural differences between EGs and CBHs can be seen in active sites located in the clefts between the two β sheets. EG has an intact, cleft-shaped (U-shaped) active site that is open, allowing random access to the internal region of a cellulose chain. On the other hand, CBH has a tunnel-shaped (O-shaped) active site enclosed by loops that restrict the access of a cellulose chain to the active site, by only allowing it to be inserted from the chain ends. PgCBH-homo possesses regions corresponding to the tunnel-forming loops of the processive CBH component, Cel7A, from *T. reesei* [35] and preserves residues which interact with substrate sugars in the tunnel of *T. reesei* Cel7A [39], including the catalytic carboxylate triad of GHF7 (Glu212, Asp214 and Glu217; the residues of PgCBH-homo in fig. 3) [38]. Considering the conservation of all 16 cysteine residues forming disulfide bonds and the high overall similarity (61%) between *T. reesei* Cel7A and PgCBH-homo, we infer PgCBH-homo to have a folding similar to *T. reesei* Cel7A and, thus, to function as a processive enzyme, possibly active against crystalline cellulose, as formerly demonstrated in the crude hindgut extract of *C. formosanus* [28]. Molecular phylogenetic analysis of GHF7 members supported this idea; the GHF7 tree was divided into two clusters com-

prising fungal EGs and CBHs, respectively, and *PgCBH-homo1* was placed in the latter (fig. 4).

Our present results imply the presence of processive cellulases in *P. grassii*. The putative amino acid sequences of these enzymes indicated the presence of secreting signals leading the N terminals of the mature protein PgCBH-homo, so the enzymes could be secreted into food vacuoles containing endocytosed wood particles [42]. Since processivity is considered as a key character for effective degradation of crystalline cellulose [41], the presence of PgCBH-homo in *P. grassii* might explain its active endocytosis and degradation of native crystalline cellulose in the wood particles [27, 42]. Previously, we proposed a symbiotic cellulose-digesting system separate from the termite's own endogenous system in *C. formosanus* and demonstrated that each can digest crystalline cellulose, producing glucose, independently [28]. Our present report has suggested the possible major agents acting in this symbiotic system, which might have given this termite the competitive edge necessary for its survival.

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