

A New Putative Chitinase from *Reticulitermes speratus* KMT001¹

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ABSTRACT

Termites are pests that cause serious economic and cultural damage by digesting wood cellulose. Termites are arthropods and have an epidermis surrounded by a chitin layer. To maintain a healthy epidermis, termites have chitinase (β -1,4-poly-N-acetyl glucosaminidase, EC 3.2.1.14), an enzyme that hydrolyzes the β -1,4 bond of chitin. In this study, the amino acid sequence of the gene, which is presumed to be termite chitinolytic enzyme (NCBI accession no. KC477099), was obtained from a transcriptomic analysis of *Reticulitermes speratus* KMT001 in Bukhan Mountain, Korea. An NCBI protein BLAST search confirmed that the protein is a glycoside hydrolase family 18 (GH18). The highest homology value found was 47%, with a chitinase from *Araneus ventricosus*. Phylogenetic analysis indicated that the KC477099 protein has the same origins as those of arthropods but has a very low similarity with other arthropod chitinases, resulting in separation at an early stage of evolution. The KC477099 protein contains two conserved motifs, which encode the general enzymatic characteristics of the GH18 group. The amino acid sequences Asp¹⁵⁶-Trp¹⁵⁷-Glu¹⁵⁸, which play an important role in the enzymatic activity of the GH18 group, were also present. This study suggests that the termite KC477099 protein is a new type of chitinase, which is evolutionarily distant from other insect chitinases.

Keywords: termite, *Reticulitermes speratus* KMT001, phylogenetic analysis, chitinase

1. INTRODUCTION

Chitin is an amino sugar in which N-acetyl-D-glucose-2-amine is polymerized by β -1,4 bonds (Aronson *et al.*, 1997; Bussink *et al.*, 2007; Han *et al.*, 2005). It is found in the eggshells of nematodes (Brydon *et al.*, 1987) and cell walls of fungi (Bartnicki-Garcia, 1968) and comprises the skeleton of the shells of arthropods (Kramer and Koga, 1986). Chitinase (β -1,4-poly-N-acetyl glucosaminidase, EC 3.2.1.14) is an enzyme that hydrolyzes the β -1,4 bonds

of chitin (Henrissat and Davies, 1997; Kramer and Koga, 1986) and belongs to the glycoside hydrolase family 18 (GH18) (Reynolds and Samuels, 1996). Chitinase has a wide range of functions in organisms as diverse as insects, bacteria, fungi, plants, and animals. Insects use chitinase to decompose old cuticle layers for the synthesis and reconstitution of new cuticle layers during ecdysis in the growth process (Henrissat and Davies, 1997; Merzendorfer and Zimoch, 2003). Chitinases are also found in organisms that do not have chitin. These enzymes protect plants from pathogenic fungi

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(Badariotti *et al.*, 2007; Taira *et al.*, 2002). Chitinases in some bacteria and animals decompose chitin in food to produce nutrients, or they may be used to defend against external chitin toxicity (Guan *et al.*, 2011; Kawada *et al.*, 2007; Renkema *et al.*, 1998; van Eijk *et al.*, 2005).

GH18 has eight similar α/β -barrel structures and two conserved regions, which play an important role in enzymatic activity. All structure and regions are located on the $\beta 3$ and $\beta 4$ strands (Cho *et al.*, 2010; Fukamizo, 2000; Henrissat and Bairoch, 1993; Korb *et al.*, 2012; Kramer and Muthukrishnan, 1997; Sharma *et al.*, 2011). Of the two conserved domains, domain II, which is located on the $\beta 4$ strand and includes acidic amino acids, such as aspartic and glutamic acids, is known to play an important role in enzymatic activity (Lu *et al.*, 2002; Terwisscha van Scheltinga *et al.*, 1996; Thomas *et al.*, 2000).

Termites are insects belonging to the order Blattodea and are known as pests that damage wooden buildings by degrading cellulose (Matsui *et al.*, 2009). Termites may ingest chitin while eating wood infested with fungi (Mishra and Sensarma, 1981). The amount and activity of chitinases in the digestive tract of insects were observed to be increased when chitinous foods were ingested (Fukamizo *et al.*, 1985; Liu *et al.*, 2013; Merzendorfer and Zimoch, 2003). Efforts to combat termites using chemicals have been made (Hadi *et al.*, 2018; Kim and Chung, 2017; Mun and Nicholas, 2017). Previous studies suggest the use of chemicals that inhibit chitin synthesis as insecticides (Sandoval-Mojica and Scharf, 2016; Zhu *et al.*, 2016). Pentoxifylline, an inhibitor of chitinases, was shown to kill termites (Husen and Kamble, 2013; Husen *et al.*, 2015). According to our research results, no reports on the chitinases of termites are available until now. In this study, a gene that encodes a putative chitinase was obtained from a transcriptomic analysis of *Reticulitermes speratus* in Bukhan Mountain, Seoul, Korea. By using the amino

acid sequence, its taxonomic position was identified through phylogenetic analysis, and the amino acid sequences, which are an important motif in GH18, were compared using multiple sequence alignment.

2. MATERIALS and METHODS

2.1. Termites

The termites used were *R. speratus* KMT001 (Cho *et al.*, 2010), which were collected in the Bukhan Mountain, Seoul, Korea (latitude: 37.614009, longitude: 126.990545). The termites were grown at 26°C and 70% humidity without light.

2.2. Chitinase genes of *R. speratus* KMT001

A putative chitinase gene was selected using transcriptomic analysis from previous studies (Park *et al.*, 2014) aimed at elucidating its biological function. The amino acid sequence, including the open reading frame (ORF), was inferred from the selected gene sequence. The chitinase gene identified from *R. speratus* KMT001 was registered in the NCBI database (accession number: KC477099).

2.3. Phylogenetic analysis of the putative chitinase

A protein BLAST search (<http://blast.ncbi.nlm.nih.gov/>) was performed on the NCBI website using the amino acid sequence of the putative chitinase to identify homologous genes. For the multiple sequence alignment and phylogenetic analysis, we selected the gene with the highest homology score from each genus among 100 homologous sequences. The putative chitinase sequence in this study and 19 homologous sequences were aligned using ClustalW in the MEGA4 program (<https://www.megasoftware.net/mega4/>). For the phylo-

genetic analysis of the aligned amino acid sequences, a neighbor-joining method was used to assess the evolutionary distance using bootstrap and cross-validation methods with 1,000 replications to estimate the reliability of the results (Nei and Saitou, 1987; Tamura *et al.*, 2007).

3. RESULTS and DISCUSSION

3.1. Putative chitinases of *R. speratus* KMT001

A previous transcriptomic analysis of *R. speratus* KMT001 using the GS FLX System (Park *et al.*, 2014) provided sequence information for the expressed mRNA.

Among the genes whose biological functions were suggested, two contigs (contig00176 and contig 03679) and 17 singletons, which appear to be chitinases, were selected (Table 1). The biological function analysis of contig00176 (NCBI accession number: KC477099) indicated that the closest gene in the NCBI database was a chitinase of *Araneus ventricosus*, a spider. The gene was 1,300 bp in length, and the complete ORF of the putative chitinase was 1,199 bp. The most similar gene to contig03679 in the NCBI database was a chitinase from *Ixodes scapularis*, a mite subspecies. In contig03679, only an ORF with a short base sequence, 178 bp, could be identified. The other 17 singletons did not have an identifiable ORF and were excluded from this study.

Table 1. Putative chitinase genes from transcriptomic analysis of *R. speratus* KMT001.

Identification name	Strains that have the closest gene	Length of sequence (base pair)
(Assembled sequences)		
Contig00176	<i>Araneus ventricosus</i>	1300
Contig03679	<i>Ixodes scapularis</i>	178
(Singleton sequences)		
GEKBKKN03C2OJA	<i>Beta vulgaris</i> subsp. <i>Vulgaris</i>	456
GEKBKKN03DGRNH	<i>Culex quinquefasciatus</i>	137
GEKBKKN04EANL5	<i>Clostridium phytofermentans</i>	504
GEKBKKN04EBH72	<i>Nasonia vitripennis</i>	391
GEKBKKN04EC1T3	<i>Clostridium</i> sp.	489
GEKBKKN04EGXG0	<i>Listeria seeligeri</i>	394
GEKBKKN04EH9RU	<i>Anopheles gambiae</i>	389
GEKBKKN04EMBY Y	<i>Clostridium botulinum</i>	438
GEKBKKN04ENHY3	<i>Listeria welshimeri</i>	390
GEKBKKN04ENU5M	<i>Clostridium</i> sp.	457
GEKBKKN04EPUMP	<i>Ostrinia furnacalis</i>	462
GEKBKKN04EQ9DU	<i>Clostridium botulinum</i>	488
GEKBKKN04ER1D0	<i>Clostridium paraputrificum</i>	411
GEKBKKN04ETLU4	<i>Clostridium botulinum</i>	354
GEKBKKN04EUAMJ	<i>Clostridium paraputrificum</i>	362
GEKBKKN04EULGE	<i>Clostridium</i> sp.	358
GEKBKKN04EZ9ET	<i>Nasonia vitripennis</i>	487

3.2. Identification of homologous genes using protein BLAST

We searched for genes homologous to the ORF KC477099 in the NCBI database using a protein BLAST search at the National Center for Biotechnology Information (Table 2). The 100 most similar genes were from GH18, distributed over in 19 orders. Among the search results, none were found in the order Blattodea, to which *R. speratus* belongs. The highest homology with KC477099 was AAN39100 from *A. ventricosus* with an e-value of 7E-112. In addition to chitinase, chitotriosidase and acidic mammalian chitinase (AMCase), which belong to the same GH18 group, were found to have high homology scores with KC477099.

The orders Decapoda, Diptera, and Mesostigmata in

the phylum Arthropoda have homologous chitinase genes in the GH18 group. Members of the class Enteropneusta in the phylum Hemichordata, order Ostreoida in the phyla Mollusca, Rodentia, Perissodactyla, Carnivora, Primates, Squamata, Dasyuromorphia, and Diptera have genes for chitotriosidase and AMCase in GH18 (Bussink *et al.*, 2007; Reardon and Farber, 1995). Homologous chitinase genes have been identified in various animals. Previous studies suggest that AMCase and chitotriosidase in insects, bacteria, and plants are highly homologous to chitinase (Arakane and Muthukrishnan, 2010; Henrissat, 1999). The role of chitinase in non-insect animals and plants is different from that in insects that have a chitin exoskeleton. Chitinase in the former is used for the digestion of chitin for nutrients or as a defense mechanism against insects,

Table 2. Results of TBLASTN searches using the amino acid sequence of KC477099 (contig00176) ORF from *R. speratus* KMT001.

Accession no. in NCBI	Species	Common name	Query coverage (%) / Identities (%)	E-value
AAN39100	<i>Araneus ventricosus</i>	Spider	91 / 47	7E-112
EFN88161	<i>Harpegnathos saltator</i>	Ant	90 / 47	6E-110
XP_003739697	<i>Galendromus occidentalis</i>	Mite	99 / 44	9E-109
ACR23315	<i>Penaeus vannamei</i>	Shrimp	90 / 47	1E-106
XP_002413492	<i>Ixodes scapularis</i>	Tick	97 / 43	2E-102
XP_002597592	<i>Branchiostoma floridae</i>	Lancelet	97 / 43	2E-99
EHJ70785	<i>Danaus plexippus</i>	Butterfly	97 / 41	2E-97
XP_001959669	<i>Drosophila. ananassae</i>	Fly	96 / 42	5E-97
EFX90412	<i>Daphnia pulex</i>	Water flea	94 / 42	4E-96
XP_970191	<i>Tribolium castaneum</i>	Beetle	92 / 42	3E-95
EKC38802	<i>Crassostrea gigas</i>	Oyster	90 / 44	5E-94
XP_002740150	<i>Saccoglossus kowalevskii</i>	Acorn worm	93 / 41	4E-94
NP_997469	<i>Rattus norvegicus</i>	Rat	90 / 40	6E-93
NP_001137269	<i>Equus caballus</i>	Horse	88 / 42	1E-92
XP_003999521	<i>Felis catus</i>	Cat	88 / 42	1E-91
XP_514112	<i>Pan troglodytes</i>	Chimpanzee	90 / 41	2E-91
XP_003220370	<i>Anolis carolinensis</i>	Lizard	90 / 39	8E-91
XP_003769823	<i>Sarcophilus harrisi</i>	Tasmanian devil	90 / 39	1E-89
XP_001372881	<i>Monodelphis domestica</i>	Opossum	90 / 39	2E-89

but the latter is mainly used for the maintenance of the chitin exoskeleton (Rathore and Gupta, 2015). The results of this study confirm that AMCase and chitotriosidase have high homology scores with chitinases found in insects, bacteria, and plants (Rathore and Gupta, 2015). Hypothetical proteins from Amphoxiformes, Cladocera, Diptera, and Coleoptera have a conserved region, but the active sites of GH18 and their activities remain to be confirmed.

3.3. Multiple sequence alignment of KC477099

To compare the amino acid sequences of KC477099, one amino acid sequence with the highest homology score in each of the 19 orders among the 100 homologous sequences was selected, and multiple sequence alignments were performed using ClustalW in the MEGA4 program (Fig. 1). KC477099 shared two conserved regions (motifs I and II in Fig. 1) and an

	$\beta 1$	$\beta 2$	Motif I $\beta 3$	Motif II $\beta 4$
KC477099	36 VVCY YGSWATYR	63 CTH IVYSFMGLE	109 KAILAIGG WNEGS	150 FDGLD LDWEYP
XP_003220370	24 LSCYFTN WQQR	51 CTH LIYAFAGMT	91 KTL LAI GGW NFGT	132 FDGLD FDWEYP
XP_001372881	24 L TCY FTN WQQR	51 CTH LIYAFAGMS	91 KTL LAI GGW NFGT	132 FDGLD FDWEYP
XP_003769823	24 L TCY FTN WQQR	51 CTH LIYAFAGMR	91 KTL LAI GGW NFGT	132 FDGLD FDWEYP
NP_997469	24 L VCY FTN WQQR	51 CTH LIYAFAGMQ	91 KTL LAI GGW NFGT	132 FDGLD LDWEYP
NP_001137269	24 L VCY FTN WQQR	51 CTH LIYAFAGMN	91 KTL LAI GGW NFGT	132 FDGLD LDWEYP
XP_003999521	24 L VCY FTN WQQR	51 CTH LIYAFAGMT	91 KTL LSI GGW NFGT	132 FDGLD LDWEYP
XP_5144112	34 L VCY FTN WQQR	61 CTH LIYAFAGMT	101 KTL LAI GGW NFGT	142 FDGLD LDWEYP
XP_002740150	27 R VCY YSN WQQR	54 CTH IVYAFANMN	101 R TLIA LGGW NFGT	142 FDG FDVDWEYP
XP_002597592	29 R VCY H TNWSQQR	56 CTH LIY SFAKMT	101 KTL LG VGGW NFGS	142 FDGLD LDWEYP
EKC38802	23 R VCY Y TNWSQQR	50 CTH VIYAFAKMN	96 KTL LAV GGW NMGS	137 FDGLD MDWEYP
XP_001959669	32 V VCY Q GTWSTYR	57 CTH LIYAF LGIE	105 KTL VAV GGW NEGS	146 FDGLD LDWEYP
EHJ70785	27 V ICY H GTWATYR	51 CTH IVY GFMGIN	99 K ALLAV GGW NEGS	140 FDGLD LDWEYP
XP_002413492	68 F ICY W GSWSHYR	95 CTH LVY TFAKLE	142 K TIIA I GGW NEGS	173 FDGLD MDWEYP
XP_970191	34 V VCY L GTWSVYR	61 CTH IVY SFAGLD	110 K VTLA I GGW NEGS	151 FDGLD LDWEYP
EFX90412	33 M VCY Y GSWAVYR	60 CTH IIY GFTGLG	108 K ALIA I GGW NEGS	149 FDGLD FDWEYP
ACR23315	1 M VYI Y GFAGLK	28 CTH LIY GFAGLK	77 KTL LAI I GGW NEGS	118 FDGLD LDWEYP
AAAN39100	31 V VCY L GSWAVYR	58 CTH VIY GFAKLS	105 S TLLA I GGW NEGS	146 FDGLD MDWEYP
XP_003739697	34 V VCY Y GSWAVYR	61 CTH LIY GFAGLG	110 K TIIA I GGW NEGS	151 FDGLD MDWEYP
EFN88161	32 I VCY Y GSWAVYR	59 CTH LIY TFVGIS	107 KTL VAI I GGW NEGS	148 FDG FDLDWEYP
	* *		**	** *
	$\beta 5$	$\beta 6$	$\beta 7$	$\beta 8$
KC477099	190 M LTV AVCAD	221 M SFD YHTATS	271 K LIM GIPL YGR	365 G TM M VA LESE DF
XP_003220370	179 M VTA AVAAG	210 M TYD FHG-SW	259 K LIV GF PTYGH	356 G AMV W SLD LD DF
XP_001372881	179 M VTA AVAAG	210 M TYD LHG-SW	259 K LIV GF PSYGH	356 G AMV W AI DL DDF
XP_003769823	179 M VTA AVAAG	210 M TYD LHG-SW	259 K LIV GF PSYGH	356 G AMV W AI DL DDF
NP_997469	179 M VTA AVAAG	210 M TYD LHG-SW	259 K LIV GF PPEYGH	356 G AMI W AI DL DDF
NP_001137269	179 L LSA AVPAG	210 M AYD FHG-SW	259 K LIL GM PTYGR	354 G AMV W AL DM DDF
XP_003999521	179 L LSA AVPAG	210 M AYD FHG-SY	259 K LIL GM PAYGR	354 G AMV W AL DM DDF
XP_5144112	189 L LSA AVPAG	220 M AYD FHG-SW	269 K LIL GM PTYGR	364 G AMV W AL DL DDF
XP_002740150	189 L LTA AVAAG	220 M SYD LNG-AW	269 K LIV GM PTYGR	365 G TMV W AM DL DDF
XP_002597592	189 L LTA AI PAG	220 M AYD LHG-QW	269 K INL GM LYGR	364 G AMV W AL DT DDF
EKC38802	177 M LSA AVPAG	208 M TYD FHGGSF	258 K INL GM LYGR	356 G IMV W AL DL DDF
XP_001959669	188 I LTA AVGSA	219 M AYD LHG P -W	272 K LVL GV PTYGR	370 G IMI W S LE SDDF
EHJ70785	182 I LSA AAVAV	213 M AYD IN P -T	267 K LVL GL PTYGH	355 G AMI W S IE TDDF
XP_002413492	223 L LTA AVSAG	254 M GYD FFGA-W	303 K LVL GL PLYGR	401 G IMV W S IE TDDF
XP_970191	191 L LTA AFGAG	222 M CYD YHG-AW	264 K LVL GV PLYGR	370 G IMV W S IE TDDF
EFX90412	189 L LTA AVSPG	220 M NYD YHGS-W	272 K IIM GM LYGR	370 G ALT W S IE TDDF
ACR23315	158 M LTA AVSAG	189 M AYD LHG T -W	239 K LVL GI LYGR	336 G AMV W S IE TDDF
AAAN39100	186 L LSA AVSAG	217 M AYD LHGS-W	266 K VIL GM GTYGR	361 G GM IW S LE TDDF
XP_003739697	191 L LTA AVSAG	224 M AYD FHG G -W	271 K IIL GM LYGR	369 G GMV W S IE TDDF
EFN88161	188 I LSA AVGAA	219 M SYD LHGS-W	268 K IVV GV PTYGR	374 G VM LW S VE TDDF
	**	* ** *	**	*

Fig. 1. Multiple amino acid sequence alignment of KC477099 from *R. speratus* KMT001 and chitinase from 19 strains obtained from a protein BLAST search of the National Center for Biotechnology Information. Motifs I and II are indicated on the top of the alignments. The bold characteristics indicate the consensus sequences in the active sites of GH18. The star marks on the bottom of the alignments indicate the active sites of GH18 chitolectin chitotriosidase.

active site (shown in bold in Fig. 1) involved in the enzymatic activity of chitinase belonging to the GH18 group with other sequences (Henrissat and Bairoch, 1993; Korb *et al.*, 2012; Lu *et al.*, 2002; Sharma *et al.*, 2011; Terwisscha van Scheltinga *et al.*, 1996; Thomas *et al.*, 2000). KC477099 have 17 active amino acid sites in the GH18 chitolectin chitotriosidase (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?hsI=1&uid=119351&#seqhrch>), which is the family with the most specific hits to KC477099 (Fig. 1). Among the active sites, 16 sites are located in the β strands (Fig. 1).

The amino acid sequence of motif II is FDGLDIDWEYP, which is part of the amino acid sequence of KC477099. The Asp-Trp-Glu site in motif II has a significant role in the enzymatic activity of GH18 (Henrissat and Bairoch, 1993; Huang *et al.*, 2010; Synstad *et al.*, 2004; Zhang *et al.*, 2002). Glu¹⁵⁸ in KC477099 may be involved in the cleavage of glycosidic bonds through protonation as a proton donor in the GH18 enzymatic activity (Sinnott, 1990). The switching of Glu¹⁵⁸ to another amino acid significantly reduces its enzymatic activity (Henrissat and Bairoch, 1993). Asp¹⁵⁶ has been suggested as an electrostatic

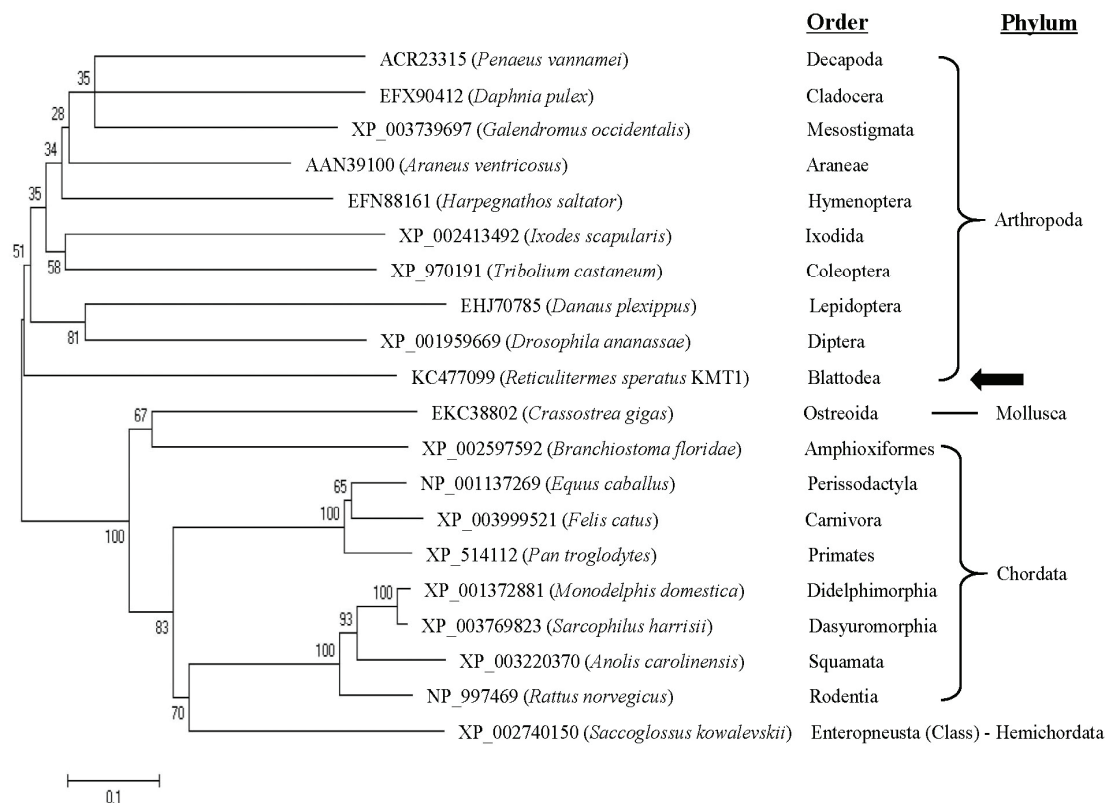


Fig. 2. Phylogenetic analysis of KC477099 from *R. speratus* KMT001 and chitinase from 19 strains from a protein BLAST search at the National Center for Biotechnology Information. A neighbor-joining method with a bootstrap of 1,000 replications was used. The order and phylum of each strain are listed on the right side of the figure. KC477099 is indicated using a black arrow. *Saccoglossus kowalevskii* class is listed because it has not been assigned to an order.

stabilizer in the transition state and is less involved in the enzymatic activity of GH18 than Glu¹⁵⁸ (Huang *et al.*, 2010; Synstad *et al.*, 2004). Glu¹⁵⁴ is involved in the ionization state of Glu¹⁵⁸ and affects the *pK* values of Glu¹⁵⁸ and Asp¹⁵⁶ (Henrissat and Bairoch, 1993). An additional amino acid in motif II that affects the enzymatic activity of GH18 is Trp¹⁵⁷. Trp¹⁵⁷ maintains the abnormal *pK* values of other amino acids during enzymatic activity. It has an important role in the extension of chitinase activity within an alkaline pH range (Zhang *et al.*, 2002).

3.4. Phylogenetic analysis

To investigate the evolutionary relationships between genes, we analyzed the phylogeny of the 20 genes shown in Fig. 1 (Fig. 2). KC477099 was grouped in the phylum Arthropoda with the Decapoda, Mesostigmata, Araneae, Hymenoptera, Lepidoptera, and Ixodidae. The biological function of chitinase in the phylum Arthropoda is the digestion of food, decomposition of the exoskeleton in ecdysis, and defense of the body against toxicity (Henrissat and Davies, 1997; Merzendorfer and Zimoch, 2003).

The phylum Mollusca, order Ostreoida, phylum Hemichordata class Enteropneusta, and phylum Chordata, including orders Perissodactyla, Carnivora, Primates, Rodentia, Squamata, Didelphimorphia, and Dasyuromorphia, were grouped in one of the main branches. All of them were identified as chitotriosidase and AMCase in GH18. Chitotriosidase was the first chitinase found in humans, and AMCase is chitotriosidase expressed in an acidic condition (Reardon and Farber, 1995). In the group of the phylum Arthropoda, the KC477099 branch is separated at the earliest stage. This phylogenetic analysis confirms that the putative chitinase KC477099 of *R. speratus* KMT001 is unique from other reported chitinases in the phylum Arthropoda and has low homology in the BLAST search.

4. CONCLUSION

The transcriptomic analysis of *R. speratus* KMT001 identified a putative chitinase gene, KC477099, which includes the complete ORF of the chitinase gene with the consensus amino acids of GH18. The homology between KC477099 and the sequence with the highest homology score was *A. ventricosus* at 47%. Multiple sequence alignment identified two conserved motifs in GH18, and the active amino acid sites Asp¹⁵⁶-Trp¹⁵⁷-Glu¹⁵⁸. Phylogenetic analysis showed that KC477099 is grouped with other chitinases of the phylum Arthropoda, but its branch separates at the earliest stage. All of the analyses suggest that KC477099 is a new termite-derived chitinase gene of *R. speratus* KMT001. This new chitinase can provide an important biological target for the control of termites.

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