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Original Article

A New Putative Chitinase from *Reticulitermes speratus* KMT001[1]

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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 glucosamidinase, 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 Asp^{156}-Trp^{157}-Glu^{158}, 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 β3 and β4 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 β4 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	*Araneus ventricosus*	1300
Contig03679	*Ixodes scapularis*	178
(Singleton sequences)		
GEKBKKN03C2OJA	*Beta vulgaris* subsp. *Vulgaris*	456
GEKBKKN03DGRNH	*Culex quinquefasciatus*	137
GEKBKKN04EANL5	*Clostridium phytofermentans*	504
GEKBKKN04EBH72	*Nasonia vitripennis*	391
GEKBKKN04EC1T3	*Clostridium* sp.	489
GEKBKKN04EGXG0	*Listeria seeligeri*	394
GEKBKKN04EH9RU	*Anopheles gambiae*	389
GEKBKKN04EMBYY	*Clostridium botulinum*	438
GEKBKKN04ENHY3	*Listeria welshimeri*	390
GEKBKKN04ENU5M	*Clostridium* sp.	457
GEKBKKN04EPUMP	*Ostrinia furnacalis*	462
GEKBKKN04EQ9DU	*Clostridium botulinum*	488
GEKBKKN04ER1D0	*Clostridium paraputrificum*	411
GEKBKKN04ETLU4	*Clostridium botulinum*	354
GEKBKKN04EUAMJ	*Clostridium paraputrificum*	362
GEKBKKN04EULGE	*Clostridium* sp.	358
GEKBKKN04EZ9ET	*Nasonia vitripennis*	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	*Araneus ventricosus*	Spider	91 / 47	7E-112
EFN88161	*Harpegnathos saltator*	Ant	90 / 47	6E-110
XP_003739697	*Galendromus occidentalis*	Mite	99 / 44	9E-109
ACR23315	*Penaeus vannamei*	Shrimp	90 / 47	1E-106
XP_002413492	*Ixodes scapularis*	Tick	97 / 43	2E-102
XP_002597592	*Branchiostoma floridae*	Lancelet	97 / 43	2E-99
EHJ70785	*Danaus plexippus*	Butterfly	97 / 41	2E-97
XP_001959669	*Drosophila. ananassae*	Fly	96 / 42	5E-97
EFX90412	*Daphnia pulex*	Water flea	94 / 42	4E-96
XP_970191	*Tribolium castaneum*	Beetle	92 / 42	3E-95
EKC38802	*Crassostrea gigas*	Oyster	90 / 44	5E-94
XP_002740150	*Saccoglossus kowalevskii*	Acorn worm	93 / 41	4E-94
NP_997469	*Rattus norvegicus*	Rat	90 / 40	6E-93
NP_001137269	*Equus caballus*	Horse	88 / 42	1E-92
XP_003999521	*Felis catus*	Cat	88 / 42	1E-91
XP_514112	*Pan troglodytes*	Chimpanzee	90 / 41	2E-91
XP_003220370	*Anolis carolinensis*	Lizard	90 / 39	8E-91
XP_003769823	*Sarcophilus harrisii*	Tasmanian devil	90 / 39	1E-89
XP_001372881	*Monodelphis domestica*	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

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                                                                Motif I          Motif II
                        β1                 β2                  β3                β4
KC477099        36 VVCYYGSWATYR   63 CTHIVYSFMGLE   109 KAILAIGGWNEGS   150 FDGLDIDWEYP
XP_003220370    24 LSCYFTNWGQYR   51 CTHLIYAFAGMT    91 KTLLAIGGWNFGT   132 FDGLDFDWEYP
XP_001372881    24 LTCYFTNWAQYR   51 CTHLIYAFAGMS    91 KTLLAIGGWNFGT   132 FDGLDFDWEYP
XP_003769823    24 LTCYFTNWAQYR   51 CTHLIYAFAGMR    91 KTLLAIGGWNFGT   132 FDGLDFDWEYP
NP_997469       24 LVCYFTNWAQYR   51 CTHLIYAFAGMQ    91 KTLLAIGGWNFGT   132 FDGLDLDWEYP
NP_001137269    24 LVCYFTNWAQYR   51 CTHLIYAFAGMN    91 KTLLAIGGWNFGT   132 FDGLDLDWEYP
XP_003999521    24 LVCYFTNWAQYR   51 CTHLIYAFAGMT    91 KTLLSIGGWNFGT   132 FDGLDLDWEYP
XP_5144112      34 LVCYFTNWAQYR   61 CTHLIYAFAGMT   101 KTLLAIGGWNFGT   142 FDGLDLDWEYP
XP_002740150    27 RVCYYSNWAQYR   54 CTHIVYAFANMN   101 RTLIALGGWNFGT   142 FDGFDVDWEYP
XP_002597592    29 RVCYHTNWSQYR   56 CTHIIYSFAKMT   101 KTLLGVGGWNFGS   142 FDGLDLDWEYP
EKC38802        23 RVCYYTNWSQYR   50 CTHVIYAFAKMN    96 KTLLAVGGWNMGS   137 FDGLDMDWEYP
XP_001959669    32 VVCYQGTWSTYR   57 CTHLIYAFLGIE   105 KTLVAVGGWNEGS   146 FDGLDLDWEYP
EHJ70785        27 VICYHGTWATYR   51 CTHIVYGFMGIN    99 KALLAVGGWNEGS   140 FDGLDIDWEYP
XP_002413492    68 FICYWGSWSHYR   95 CTHLVYTFAKLE   142 KTIIAIGGWNEGS   173 FDGLDMDWEYP
XP_970191       34 VVCYLGTWSVYR   61 CTHIVYSFAGLD   110 KVTLAIGGWNEGS   151 FDGLDLDWEFP
EFX90412        33 MVCYYGSWAVYR   60 CTHIIYGFTGLG   108 KALIAIGGWNEGS   149 FDGLDFDWEYP
ACR23315         1 MVYYYGSWAVYR   28 CTHLIYGFAGLK    77 KTLLAIGGWNEGS   118 FDGLDLDWEYP
AAN39100        31 VVCYLGSWANYR   58 CTHVIYGFAKLS   105 STLIAIGGWNEGS   146 FDGLDMDWEYP
XP_003739697    34 VVCYYGSWAVYR   61 CTHLIYGFAGLG   110 KTIIAIGGWNEGS   151 FDGLDMDWEYP
EFN88161        32 IVCYYGSWAVYR   59 CTHLIYTFVGIS   107 KTLVAIGGWNEGS   148 FDGFDLDWEYP
                       *    *                                   **                  * **

                        β5                 β6                  β7                β8
KC477099       190 MLTVAVCAD     221 MSFDYHTATS     271 KLIMGIPLYGR     365 GTMMWALESEDF
XP_003220370   179 MVTAAVAAG     210 MTYDFHG-SW     259 KLIVGFPTYGH     356 GAMVWSLDLDDF
XP_001372881   179 MVTAAVAAG     210 MTYDLHG-SW     259 KLIVGFPSYGH     356 GAMVWAIDLDDF
XP_003769823   179 MVTAAVAAG     210 MTYDLHG-SW     259 KLIVGFPSYGH     356 GAMVWAIDLDDF
NP_997469      179 MVTAAVAAG     210 MTYDLHG-SW     259 KLIVGFPEYGH     356 GAMIWAIDLDDF
NP_001137269   179 LLSAAVPAG     210 MAYDFHG-SW     259 KLILGMPTYGR     354 GAMVWALDMDDF
XP_003999521   179 LLSAAVPAG     210 MAYDFHG-SY     259 KLILGMPAYGR     354 GAMVWALDMDDF
XP_5144112     189 LLSAAVPAG     220 MAYDFHG-SW     269 KLILGMPTYGR     364 GAMVWALDLDDF
XP_002740150   189 LLTAAVAAG     220 MSYDLNG-AW     269 KLIVGMPTYGR     365 GTMVWAMDLDDF
XP_002597592   189 LLTAAIPAG     220 MAYDLHG-QW     269 KINLGMGLYGR     364 GAMVWALDTDDF
EKC38802       177 MLSAAVPAG     208 MTYDFHGGSF     258 KINLGMPLYGR     356 GIMVWALDLDDF
XP_001959669   188 ILTAAVGSA     219 MAYDLHGP-W     272 KLVLGVPFYGR     370 GIMIWSLESDDF
EHJ70785       182 ILSAAVAAV     213 MAYDINNP-T     267 KLVLGLPFYGH     355 GAMIWSIETDDF
XP_002413492   223 LLTAAVSAG     254 MGYDFFGA-W     303 KLVLGLPLYGR     401 GIMVWSIETDDF
XP_970191      191 LLTAAFGAG     222 MCYDYHG-AW     264 KLVLGVPLYGR     370 GIMVWSIDTDDF
EFX90412       189 LLTAAVSPG     220 MNYDYHGS-W     272 KIIMGMPLYGR     370 GALTWSIETDDF
ACR23315       158 MLTAAVSAG     189 MAYDLHGT-W     239 KLVLGIGLYGR     336 GAMVWSIETDDF
AAN39100       186 LLSAAVSAG     217 MAYDLHGS-W     266 KVILGMGTYGR     361 GGMIWSLETDDF
XP_003739697   191 LLTAAVSAG     224 MAYDFHGG-W     271 KIILGMGLYGR     369 GGMVWSIETDDF
EFN88161       188 ILSAAVGAA     219 MSYDLHGS-W     268 KIVVGVPFYGR     374 GVMLWSVETDDF
                        **           * **    *              * *              *
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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?hslf=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 FDGLDI**DWE**YP, 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). Glu^{158} 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 Glu^{158} to another amino acid significantly reduces its enzymatic activity (Henrissat and Bairoch, 1993). Asp^{156} has been suggested as an electrostatic

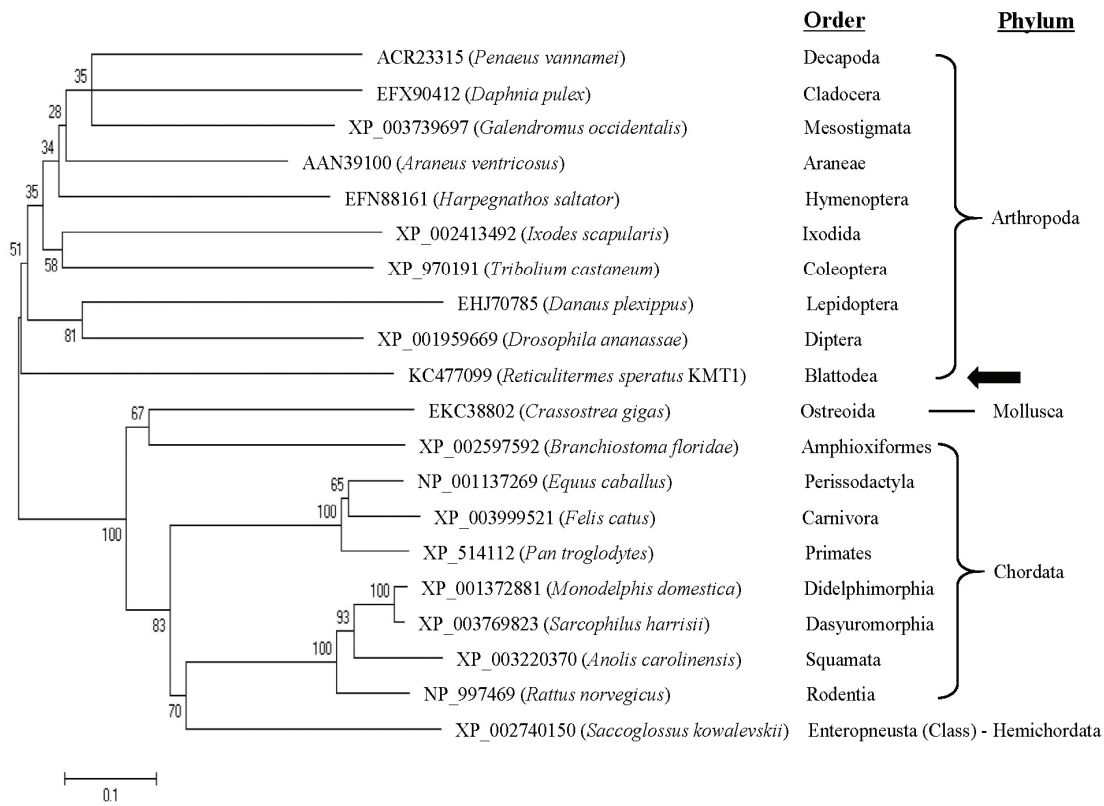

Fig. 2. Phylogenetic analysis of KC477099 from *R. speratus* KMT001 and chitinase from 19 strains from a protein BLAST search at the National Center for Biotechnology Information. A neighbor-joining method with a bootstrap of 1,000 replications was used. The order and phylum of each strain are listed 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 Glu^{158} (Huang *et al.*, 2010; Synstad *et al.*, 2004). Glu^{154} is involved in the ionization state of Glu^{158} and affects the *pK* values of Glu^{158} and Asp^{156} (Henrissat and Bairoch, 1993). An additional amino acid in motif II that affects the enzymatic activity of GH18 is Trp^{157}. Trp^{157} 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 Ixodide. 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 Asp^{156}-Trp^{157}-Glu^{158}. 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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