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Cloning and sequence analysis of chitin synthase gene fragments of *Demodex* mites*

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Abstract: To our knowledge, few reports on *Demodex* studied at the molecular level are available at present. In this study our group, for the first time, cloned, sequenced and analyzed the chitin synthase (*CHS*) gene fragments of *Demodex folliculorum*, *Demodex brevis*, and *Demodex canis* (three isolates from each species) from Xi'an China, by designing specific primers based on the only partial sequence of the *CHS* gene of *D. canis* from Japan, retrieved from GenBank. Results show that amplification was successful only in three *D. canis* isolates and one *D. brevis* isolate out of the nine *Demodex* isolates. The obtained fragments were sequenced to be 339 bp for *D. canis* and 338 bp for *D. brevis*. The *CHS* gene sequence similarities between the three Xi'an *D. canis* isolates and one Japanese *D. canis* isolate ranged from 99.7% to 100.0%, and those between four *D. canis* isolates and one *D. brevis* isolate were 99.1%–99.4%. Phylogenetic trees based on maximum parsimony (MP) and maximum likelihood (ML) methods shared the same clusters, according with the traditional classification. Two open reading frames (ORFs) were identified in each *CHS* gene sequenced, and their corresponding amino acid sequences were located at the catalytic domain. The relatively conserved sequences could be deduced to be a *CHS* class A gene, which is associated with chitin synthesis in the integument of *Demodex* mites.

Key words: *Demodex*, Chitin synthase genes, Cloning and sequencing, Phylogenetic study, Function analysis
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1 Introduction

Chitin, whose synthesis and degradation is one of the most important metabolic processes in insects, is an important component of exoskeleton and peritrophic membrane (PM), and the related metabolic enzymes and regulating factors are the potential targets of neotype insecticides. The literature on insect chitin synthases (CHSs) has just started in the recent decade, later than that on insect chitin catabolic enzymes. The genes or cDNAs of CHSs were isolated and identified from *Lucilia cuprina* (Tellam *et al*., 2000), *Drosophila metamorphosis* (Gagou *et al*., 2002), *Aedes aegypti* (Ibrahim *et al*., 2000), *Anopheles gambiae* (Hogenkamp *et al*., 2005), *Anopheles quadrimaculatus* (Zhang and Zhu, 2006), *Manduca sexta* (Zhu *et al*., 2002; Hogenkamp *et al*., 2005), *Spodoptera frugiperda* (Hogenkamp *et al*., 2005), *Spodoptera exigua* (Chen *et al*., 2007; Kumar *et al*., 2008), *Mamestra brassicae* (Piao and Fan, 2008), *Choristoneura fumiferana* (Ampasala *et al*., 2011), *Tribolium castaneum* (Arakane *et al*., 2004), and *Ostrinia furnacalis* (Qu *et al*., 2011) in Insecta, and *Boophilus microplus* (Tellam *et al*., 2000) in Arachnida, yet few *CHS* genes of Acariformes are available.

Demodex mites are tiny permanent parasites, belonging to Arachnida, Demodicidae. Up to now, 140 species or subspecies have been identified, infesting the hair follicles, sebaceous glands, meibomian glands, and internal organs of 11 orders of mammals such as human, dog, sheep, cat, pig, and

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mouse. Once excessive amount of mites parasitize the host, demodicosis might emerge, such as canine demodicosis (Izdebska, 2010; Mederle *et al*., 2010), goat demodicidosis (Smith, 1961; Xu *et al*., 2009), livestock demodicidosis (Chakrabarti and Pradhan, 1985), and human demodicidosis (Ayres and Anderson, 1932; Ayres and Ayres, 1961; Forton and Seys, 1993; Forton *et al*., 2005; Zhao *et al*., 2010; 2011a; 2011c; 2012a; 2012c). However, the pathogenesis of demodicidosis is still uncertain, and there are no effective prevention measures. A few reports on *Demodex* at molecular level can be obtained, as *Demodex* mite is tiny (about 0.2–0.4 mm long) and autolyze easily in vitro (Zhao *et al*., 2009a; 2011b), which makes them hard to study. There are no reports on successful culture in vitro or animal inoculated culture. Thus, it is difficult to get standard experimental mites. Moreover, the hard chitin of mites makes the homogenate difficult, so the quality of genomic DNA (gDNA) extracted by traditional methods can hardly meet the experimental requirements, making the study on *Demodex* at the gene level more difficult. In 2002, the first partial DNA sequence of *Demodex canis CHS* gene was submitted to GenBank (No. AB080667) by Japanese scholars, but DNA sequences of *Demodex* could not be retrieved in succession until 2008.

Until now, only a few reports on *Demodex* at the gene level exist in PubMed (Toops *et al*., 2010; Ravera *et al*., 2011; Rojas *et al*., 2012; Zhao and Wu, 2012a; 2012b; Zhao *et al*., 2012b; 2012d) and two reported recently by us in China National Knowledge Infrastructure (CNKI) (Zhao and Cheng, 2009; Zhao *et al*., 2009b). However, literature on chitin of *Demodex* could not be retrieved.

This study first cloned and sequenced the *CHS* gene fragments of *Demodex*, using the gDNA extracted from nine isolates of *Demodex folliculorum*, *Demodex brevis*, and *D. canis* (three isolates, respectively) as the template, and specific primers designed based on the single *CHS* gene partial sequence of *D. canis* from Japan retrieved in GenBank. The open reading frames (ORFs) of obtained *CHS* gene fragments of three *D. canis* isolates and one *D. brevis* isolate, out of the total nine isolates of three *Demodex* species, were found and aligned with those of *Mamestra brassicae* (Piao and Fan, 2008). The functions of corresponding amino acid sequences were deduced for further development of neotype gene-targeted acaricides.

2 Materials and methods

2.1 *Demodex* mite collection

The *D. folliculorum* and *D. brevis* mite isolates were sampled from sufferers' faces of Xi'an, China by cellophane tape method, and collected using a self-made needle after being identified morphologically under a 10×4 microscope (*D. folliculorum*, three isolates, 1500 adult mites/isolate; *D. brevis*, three isolates, 2000 adult mites/isolate). Three *D. canis* isolates (1000 mites/isolate) were sampled via scraping from infection focus of dogs. The mite isolates were preserved in Eppendorf tubes (EPs) at −20 °C, respectively. Ethical permission is not necessary in this study because the mite collection methods are non-invasive sampling techniques, doing no harm to the subjects.

2.2 DNA extraction

The mite samples were put into liquid nitrogen for 1 min freeze-thawing and then 10 min milling. This was repeated four times. gDNA was extracted using DNA Extraction Kit (OMEGA, Georgia, USA) following the slightly improved manufacturer's protocol and preserved in EPs at −20 °C.

2.3 *CHS* gene fragment amplification

The specific primers (sense: 5′-GACCCGGATT ATTATGAGT-3′; anti-sense: 5′-TTAGCTTAATCT TACACTAA-3′) were designed according to the *D. canis CHS* gene sequence in GenBank (No. AB080667) and synthesized in Beijing AuGCT biotechnology Co., Ltd., China. All polymerase chain reactions (PCRs) were conducted on AB Applied Biosystems 2720 Thermal Cycler, and each reaction volume was 25 μl. The PCR thermal profile was as follows: an initial degeneration for 5 min at 94 °C; 30 cycles of degeneration for 1 min at 94 °C, annealing for 1 min at 50 °C and extension for 2 min at 72 °C; and finally extension for 10 min at 72 °C. The PCR products were separated by electrophoresis in 2.0% agarose gels (0.02 g/ml) and visualized under UV light with 100 bp DNA ladder (TaKaRa, Japan) as marker.

2.4 PCR product cloning and sequencing

The PCR products were purified using DNA Gel Extraction Kit (Biodev-tech, Beijing, China), and

then recombined with pMD18-T vector (TaKaRa, Japan) and cloned into *Escherichia coli* (DH5α). Plasmids were isolated by alkaline lysis method and identified by PCR. The positive clones were screened for sequencing in Shanghai Sunny Biotechnology Co., Ltd., China.

2.5 *CHS* gene sequence analysis

The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was employed to identify ORFs in the *CHS* gene fragments. The amino acid sequences of ORFs were deduced, and then their molecular weights and structural domains were analyzed by associated analytical tool in proteomics on the ExPASy website (http://expasy.org/proteomics). The *CHS* gene fragments of the two *Demodex* species (five isolates) and the representative medical arthropods in GenBank (Table 1) were aligned using ClustalX software (Version 1.8) in the multiple alignment mode (Thompson *et al*., 1997). Molecular evolutionary genetics analysis (MEGA) software Version 4.0 (Tamura *et al*., 2007) was employed to analyze divergences of the *CHS* gene fragments for the five *Demodex* isolates. And then, the phylogenetic trees based on maximum parsimony (MP) and maximum likelihood (ML) methods were reconstructed in MEGA 4.0 (Tamura *et al*., 2007) and PhyML (Guindon *et al*., 2010), respectively.

Table 1 Information of *CHS* gene fragments in phylogenetic trees

Species	GenBank No.
Arachnida	
Demodex canis 0	AB080667
Demodex canis 1	GQ370811*
Demodex canis 2	JQ768842*
Demodex canis 3	JQ768843*
Demodex brevis	GU075871*
Boophilus microplus	AF227260
Ixodes scapularis 1	XM_002405187
Ixodes scapularis 2	XM_002405190
Insecta	
Lucilia cuprina	AF221067
Haematobia irritans	AF222067
Chrysomya bezziana	AF226626
Aedes aegypti	XM_001662150
Anopheles gambiae	XM_321336
Anopheles quadrimaculatus	DQ415985
Pediculus humanus corporis	XM_002423552

* Reported in this study

3 Results

3.1 PCR amplification, cloning, and sequencing of *CHS* genes

Fig. 1 shows clear amplification bands about 350 bp for the three *D. canis* isolates (Lanes 1–3) and one *D. brevis* isolate (Lane 5), while no bands for the other two *D. brevis* isolates (Lanes 4 and 6) and three *D. folliculorum* isolates (Lanes 7–9). After being purified, cloned, and sequenced, the DNA sequence lengths turned out to be 339 bp for *D. canis* and 338 bp for *D. brevis*.

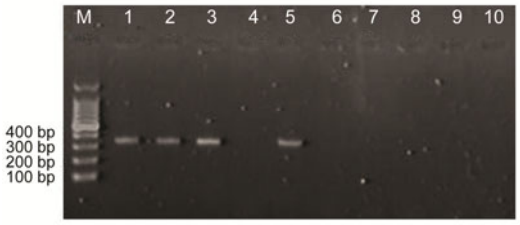

Fig. 1 Electrophoretogram of PCR amplification
Lane M: 100 bp DNA ladder marker; Lanes 1–3: three *Demodex canis* isolates; Lanes 4–6: three *Demodex brevis* isolates; Lanes 7–9: three *Demodex folliculorum* isolates; Lane 10: negative control

3.2 Sequence analysis

The deduction of ORF Finder and TMHMM2.0 on the ExPASy website revealed that the *CHS* gene segments of *D. canis* contained two ORFs at the extracellular domain: one was from Sites 79 to 338 (261 bp), encoding an 86-amino acid sequence; and the other was from Sites 1 to 147 (147 bp), encoding a 49-amino acid sequence. For *D. brevis*, there were also two ORFs located at the extracellular domain: one was from Sites 79 to 327 (249 bp), encoding an 82-amino acid sequence; and the other was from Sites 1 to 147 (147 bp), encoding a 49-amino acid sequence. According to the alignment with the related sequences from GenBank, the translated amino acid sequences were identified to be located at the catalytic domain of CHS. The alignment results (Fig. 2) also indicate that the sequence similarities of *D. canis* *CHS* genes between the three Xi'an (China) isolates and one Japanese isolate ranged from 99.7% to 100.0%, and those between the four *D. canis* isolates and one *D. brevis* isolate were 99.1%–99.4%.

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D. canis 0  GACCCGGATT ATTATGAGTT TGAGGTTAAT ATCTTTTTTG ATGATGCATT TGAGCTTTGT GATGAAAATG ATGAAGACAT GGTGGTGAAT AGATTTGTTA [100]
D. canis 1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [100]
D. canis 2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [100]
D. canis 3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [100]
D. brevis   .......... .......... .......... .......... .......... .......... .......... T......... .......... .......... [100]

D. canis 0  AACAGTTTGT TGAAGTAATT GATGAAGCGG CGAGTAATGT TCATCAGTGT AACATTAAAC TTAAGCCGCC AAAAAAGTAT CCAACACCAT ATGGTGGAAG [200]
D. canis 1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [200]
D. canis 2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [200]
D. canis 3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [200]
D. brevis   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [200]

D. canis 0  GTTAGAATGG ATTTTGCCCG GAGGCAACAA ATTGGTGGTT CATTTGAAAG ACAAAATTAA AATCAGACAT CGTAAAAGAT GGAGTCAGGT AATTGAGAGA [300]
D. canis 1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [300]
D. canis 2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [300]
D. canis 3  .......... .......... .......... G......... .......... .......... .......... .......... .......... .......... [300]
D. brevis   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [300]

D. canis 0  GTTGTAATTT TATTTACAAT TAGCTTAATC TTACACTAA [339]
D. canis 1  .......... .......... .......... ......... [339]
D. canis 2  .......... .......... .......... ......... [339]
D. canis 3  .......... .......... .......... ......... [339]
D. brevis   ....-..... .......... .......... ......... [339]
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Fig. 2 Alignment of *CHS* gene fragments of two *Demodex* species
D. canis 0–3: four *Demodex canis* isolates; *D. brevis*: one *Demodex brevis* isolate

3.3 Phylogenetic tree reconstruction

The phylogenetic analysis involved the *CHS* gene sequences of all medical Arachnida species in GenBank, and took seven representative species from medical insects as the outgroup. The MP and ML trees looked exactly the same. As seen in Fig. 3, the five isolates of two *Demodex* species made up a branch first, then formed the Arachnida cluster together with the three Ixodida species, and finally gathered with mosquitoes, flies and lice in Insecta. The phylogenetic relationship was in accordance with the morphological classification.

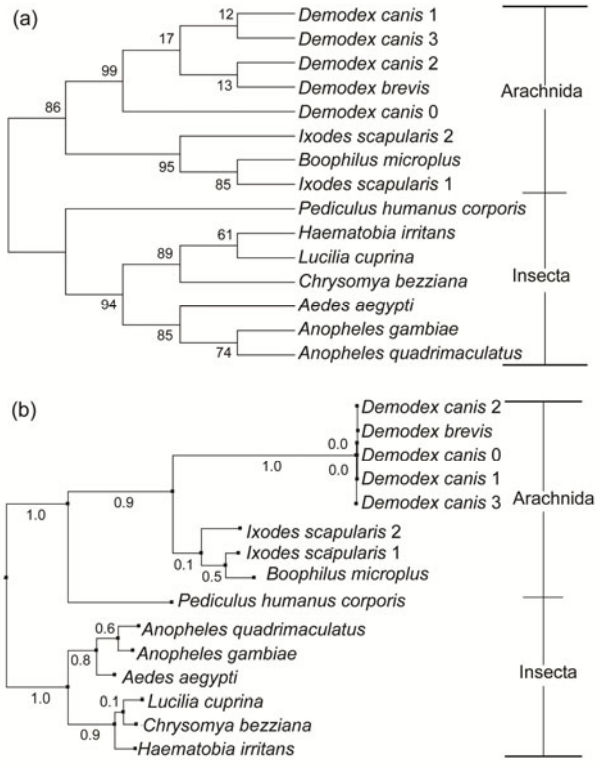

Fig. 3 Phylogenetic trees derived from *CHS* gene fragments of Arachnida and Insecta species
(a) MP tree; (b) ML tree

4 Discussion

The chitin synthesis is a highly complicated and various facets-associated biochemical and biophysical process only observed in invertebrates and fungi. Chitin, a linear polymer of β-(1,4)-linked *N*-acetyl-D-glucosamine, is an essential component of cuticle and PM in insects. The cuticle in insects (exoskeleton) is a rigid structure due to high levels of chitin and sclerotized proteins, and thus plays an important role in insect growth. During growth and development,

the insects have to periodically molt and form a new cuticle in order to adapt to the coming life stage. During ecdysis, the nascent and non-sclerotized epidermis is covered by the old one, and is gradually expanding when the old one is molting. Therefore, the insects synthesize and degrade chitin endlessly, with some regularity, to ensure the molting completion and PM regeneration (Merzendorfer and Zimoch, 2003).

Previous researches indicate there are two types of insect *CHS* genes, *CHS* gene A (*CHA*) and *CHS* gene B (*CHB*). The former is mainly responsible for the chitin synthesis of cuticle and trachea, and the latter for the formation of peritrophic matrix in midgut (Hogenkamp *et al*., 2005). In this study, the alignments demonstrated that the insect *CHS* genes with high identities were all *CHA*, and thus the obtained *Demodex CHS* gene fragments could be deduced to be *CHA*, related with chitin synthesis of body wall in *Demodex* mites.

The insect CHSs are transmembrane proteins and have varying numbers of transmembrane helices. In this study, the corresponding amino acid sequences of *CHS* genes from the two *Demodex* species were not located at the transmembrane regions, but at the catalytic domain. The sequence similarities among the three *D. canis* isolates were 100.0%, and those between the *D. canis* isolates and the *D. brevis* isolate were as high as 99.99%. The clusters of *Demodex* mites in phylogenic study showed no difference from the morphological relationship.

The quality of extracted gDNA has a direct impact on the amplification of target genes. The gDNA extracted from *Demodex* mites, which was restricted by the number, autolysis and difficult membrane rupture of mites, could hardly meet the experimental requirements in terms of purity and concentration. In this study, the *CHS* gene was amplified successfully only in four isolates out of the total nine isolates from three *Demodex* species, and we consider the insufficient quantity of extracted DNA to be responsible for the amplification failure in the other five isolates. Therefore, in order to study *Demodex* mites at the molecular level, one must solve the problem of increasing the DNA production and optimizing the conditions for DNA extraction.

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