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Bioinformatic Studies on the Group 2 Allergens of *Dermatophagoides farinae* from China

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SUMMARY The present study aimed to characterize the group 2 allergens of the house dust mite *Dermatophagoides farinae* (Der f 2) from Hainan Island, a tropical region in Southeastern China. We cloned and sequenced cDNA coding for Der f 2 and found an additional region of 87 base pairs (bp) (from +77 to +163 bp) in our strain that was absent in the reference sequence (GenBank AB195580) used for primer design. However, the BLAST analysis identified the same sequence in strains reported from Reinbek, Germany, and Guangzhou, China. A phylogenetic tree was constructed using the Der f 2 nucleotide sequences from different regions or countries and showed that the Hainan sequence clustered with the strains from Reinbek and Guangzhou. Analysis of the translated amino acid sequence suggests that the encoded peptide is hydrophobic and extracellular with a cleavage site between the 17th and 18th amino acid residues and contains a strong trans-membrane helix from the 6th amino acid to the 24th amino acid, indicating a MD-2-related lipid recognition domain in this protein. Furthermore, the secondary structure of the pro-protein consists of 16.57% alpha helix, 32.57% extended strand and 50.86% random coil. In brief, we obtained a gene coding for Der f 2 and predicted the molecular characteristics of this protein using bioinformatics tools. Our analysis identified that this gene showed several significant differences to those reported previously.

House dust mites are among the most common sources of indoor allergens worldwide. They have been found to produce over 30 types of allergens associated with asthma, atopic dermatitis, rhinitis and other allergic diseases. Specific immunotherapy with mite extracts has been considered by some to be the only effective approach to provide long-lasting relief of allergic symptoms to house dust mites. Unfortunately, these extracts contain non-allergenic proteins and lack effective concentration of important allergens, both of which may be associated with clinical side effects of mite extracts.[1] In contrast, recombinant allergens and their mixtures have shown to be effective and will likely be important for immunotherapy for dust mite-specific IgE-mediated hypersensitivity.[2]

Of the allergens produced by house dust mites, i.e. groups 1 and 2, were considered to be responsible for the high levels of IgE and Th2 cytokines in 80% of the allergic patients.[3] However, there is increasing evidence showing that both groups of allergens were highly polymorphic.[4-7] Therefore, it is very important to understand the molecular, physical

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and chemical properties of the diverse forms of the allergens before they can be effectively used in clinical diagnosis and immunotherapy. To characterize house dust mites' allergens from Hainan Island, a tropical region in Southeastern China, we cloned and sequenced the cDNA coding for Der f 2 in the house dust mite *Dermatophagoides farinae*. Surprisingly, the sequenced results showed an insertion of 87 bp that was not present in the reference sequence (GenBank AB195580). Here, we report our experimental results and the bioinformatics analysis of this sequence.

MATERIALS AND METHODS

D. farinae culture and isolation of adult mites

To isolate house dust mites, we first obtained house dust from the floors of rice and flour shops in Haikou City, Hainan Island and isolated house dust mites under the stereomicroscope. All of the mites potentially belonging to *D. farinae* were put into a culture chamber. After about two months, the whole culture in the chamber was taken to select for *D. farinae* by stereomicroscope. Subsequently, mites presumptively identified as *D. farinae* were purified by cultivating them in small culture chambers. Two months later, representative mites were taken from the chambers and the species were identified. When the mites found to be *D. farinae*, all of the mites in the chamber were treated to be *D. farinae*. These mites were then raised at a relative humidity of 75% and temperature of 25°C on a large scale. A culture medium comprising of yeast, wheat flour and rice was used for the large-scale cultivation. Following our previous protocols, the adults were isolated as follows.[8] The whole culture was taken and placed on glass plates, and after thirty minutes, the culture medium was removed manually. The adult mites, larval mites and some aliquots of the culture medium were collected in small ceramic cups using a small writing brush for the mite isolation. Under a lamp, the adults moved rapidly to the basal part of the ceramic cup and away from the larvae. The adults were removed with a writing brush under a microscope, and about 600 mites were collected the for total RNA isolation.

Total RNA isolation

Total RNA of the mites was isolated using an RNA isolator (TaKaRa Biotech Co. Led, Code No.D312) according to the manufacturer's instructions. Briefly, about 600 alive adult mites were rapidly frozen with liquid nitrogen and 1 ml of RNA isolator (RNAiso) was added. The mites in RNAiso were homogenized using a PowerGen 125 Tissue Homogenizer (Fisher Scientific) starting at 1,800 x *g* and gradually going up to approximately 7,200 x *g* for 30-60 seconds at room temperature. After homogenization, all contents in the tissue homogenizer were removed to an Eppendorf tube and 0.2 ml chloroform was added. After precipitation and air-drying, the RNA pellet was dissolved in RNase-free water and stored at - 80°C.

Preparation of cDNA and polymerase chain reaction (PCR)

For PCR amplification of the cDNA coding for Der f 2, two primers were designed and synthesized based on the GenBank sequence AB195580. The forward primer sequence was (5'GGATCCATGATTTCCAAAATCTTGTGCCTTT-C3') and the reverse was (5'CTCGAGTTAATCACGGATTTTACCATGGG3'). These two primers contained a *BamH*I site and an *Xho*I site at their 5' end (underlined), respectively. Firstly, reverse transcription (RT) was performed using the total RNA isolated from the mites with a 3'-Full RACE Core Set Ver.2.0 (TaKaRa Biotech Co. Led, Code No.D314) in the PCR Thermal Cycler Dice (TaKaRa Biotech Co. Ltd., Code TP600) for 30 minutes. Each 10 µl of reaction mixture for RT contained 3 µl of total RNA, 1x RNA PCR Buffer, 1 mM of dNTP mixture, 5 mM of $MgCl_2$, 1.25 U of M-MLV Reverse Transcriptase XL, 10 U of RNase inhibitor, 10 pmol each of the forward and reverse primers. Secondly, the RT product was used as template for PCR in the same Thermal Cycler Dice with an initial incubation at 94°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, at 55°C for 30 seconds and 72°C for 1 minute. The reaction mixture contained RT products (2 µl), 2x GC Buffer (5 mM Mg^{2+} Plus, 25 µl), 5 U/µl of LA *Taq* (0.5 µl), 1x cDNA dilution buffer (8 µl), 20 pmol of the primers F and R (1 µl each), and 12.5 µl of dH_2O. The PCR amplification products were ligated with the plasmid pMD19-T (TaKaRa Biotech Co. Led, Code No. D104) and then transformed into *E. coli* competent cells JM109 (TaKaRa Biotech Co. Ltd., Code No.D9052). Positive clones were selected by the blue/white coloration on Luria-Bertani (LB)

plates containing 100 μg/ml ampicillin. The presence of the inserted DNA was verified by restriction analysis and DNA sequencing with the *Bca*BEST M13-47 and *Bca*BEST RV-M primers on a MegaBACE 1000 DNA sequencer. Digested by *BamH* I and *Xho* I, the cloned DNA fragments were electrophoretically analyzed on an agarose gel.

Nucleotide sequence analysis, alignment and phylogeny

The obtained sequence was edited to remove vector and restriction sites, and a complete open reading frame (ORF) was obtained using the ORF finder in the NCBI (National Center for Biotechnology Information) Website. By BLASTn searches on NCBI, sequences coding for Der f 2 deposited from other geographic regions were aligned using the VECTOR NTI 9.0 software (IBI, New Haven, CT, USA), and a phylogenetic tree was constructed using MEGA3.0 software. The Der f 2 cDNA sequence and the reference sequence (GenBank AB195580) were aligned by ClustalW 1.83 (http://www.ebi.ac.uk/clustalw/index.html).

Deduced amino acid sequence and the bioinformatics analysis of the pro-protein

The amino acid sequence of Der f 2 was deduced by Translate Tools (http://www.expasy.org). Its potential subcellular localization and hydrophobicity were analyzed using the TopPred tools. The SignalP-NN was predicted with the EUK networks method by the SignalP 3.0 software in the SMART Server (http://www.cbs.dtu.dk/services/SignalP/).

The transmembrane region was predicted by the TMpred in ISREC Server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html). Its function was predicted using the Superfamily 1.69 software in the HMM library, and its secondary structure was generated by GOR4.

RESULTS

Cloning and sequence of Der f 2 cDNA from Hainan, China

Based on the nucleotide sequence of Der f 2 in the GenBank (AB195580), primers were designed to amplify the cDNA fragments by PCR using total RNA isolated from adult mites. By agarose electrophoresis, a DNA band was observed. The recovered PCR products were linked to the plasmid pMD19-T and transformed into the JM109 *E. coli* competent cells. Positive colonies were identified by the blue/white screening and the recombinant plasmids were extracted and analyzed by restriction enzyme digests using *BamH*I and *Xho*I. Two plasmids containing the targeted cDNA fragment were identified and confirmed by DNA sequencing with the primers of BcaBEST Primer M13-47 and BcaBEST Primer RV-M. According to the ORF Finder in the NCBI Web server, an ORF was found from the start codon

Fig. 1 Comparison of the Hainan Der f 2 cDNA sequence with the reference sequence (AB195580).

ATG to the stop codon TGA with a length of 528 bp. Unexpectedly, there was an additional 87 bp (position 77-163) in our cloned fragment that were absent in the reference sequence (AB195580) (Fig. 1). These two sequences shared 83% nucleotide identity.

Nucleotide sequence alignment and phylogeny for group 2 allergens reported from different regions or countries

The sequencing results were queried by BLAST in the NCBI web server and homologous sequences coding for the group 2 allergens from the same species but reported from different geographic regions were chosen and analyzed. Among the analyzed sequences, two GenBank accessions AF346905 and Emb AJ862838 reported from China and Germany, respectively, contained the same 87 base pair insertion (Fig. 2). Tables 1 and 2 show the sequence similarity for Der f 2 sequences reported from different regions. After alignment by VECTOR NTI 9.0 software and manual adjusts, a phylogenetic tree was constructed using the MEGA 3.0 software (Fig. 3).

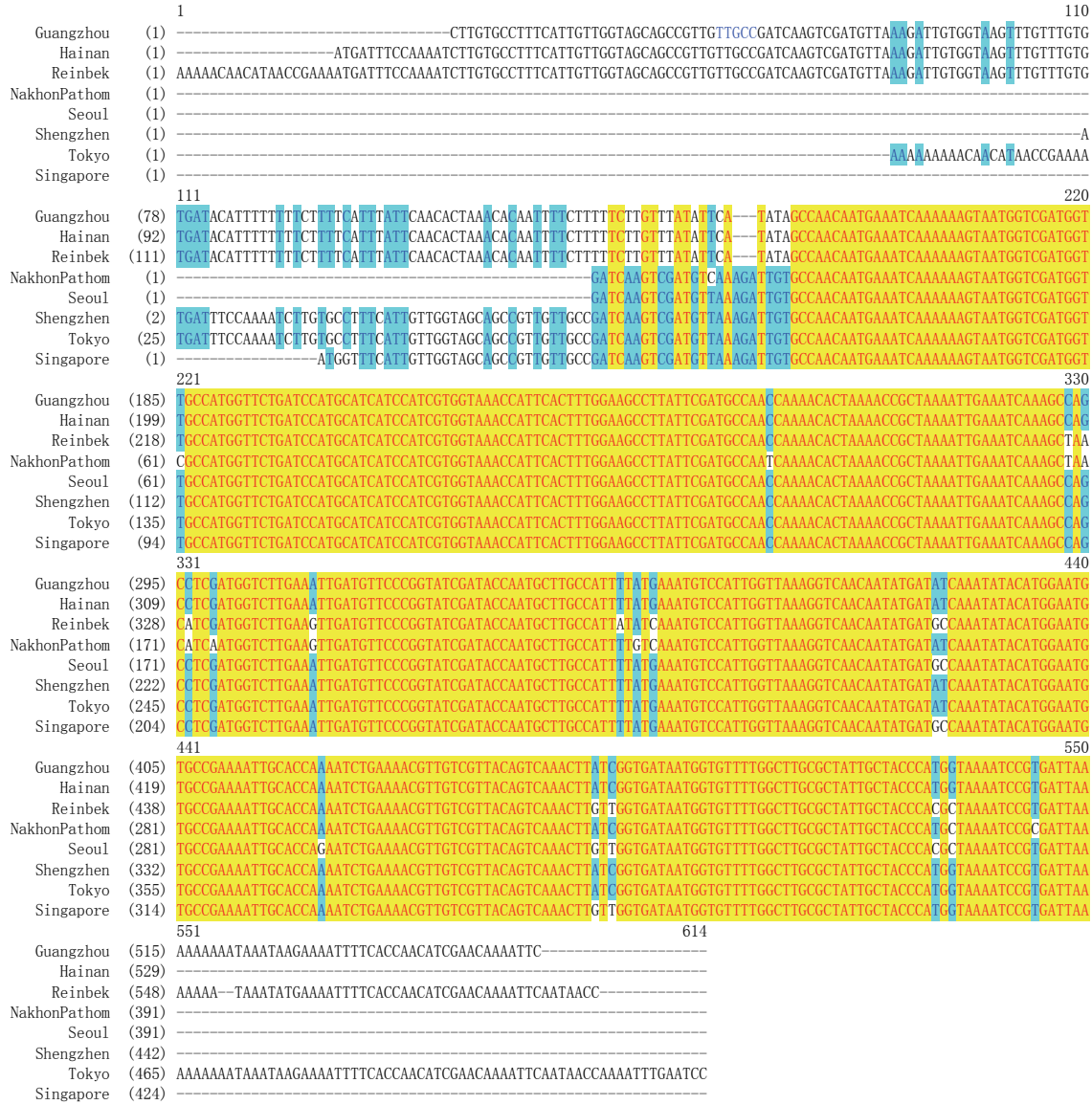

Fig. 2 Homology and polymorphism of group 2 allergen cDNA sequences reported from different regions or countries.

Table 1 Nucleotide sequence similarity among Der f 2 reported from different regions

District and countries	Accession code	Identity	E value (10,000)	References
Guangzhou, China	AF346905	100%	2.3e-206	Hao *et al.* (2003)[9]
Nakhon Pathom, Thailand	DQ185511	96%	5e-180	Piboonpocanun *et al.* (2006)[4]
Reinbek, Germany	AJ862838	97.7%	3.6e-203	Nandy *et al*. (2003)[10]
Seoul Korea	AY066008	98.1%	1.4e-139	Jin *et al.* (2003)[5]
Singapore	AY283288	98.9%	8.7e-142	Augus *et al.* (2003)[11]
Tokyo, Japan	D10448	83.5%	4.2e-146	Yuuki *et al*. (1991)[12]
Shenzhen, China	EF139432	83.5%	3.5e-146	Liu *et al.* (2006)[13]

By BLASTn searches on NCBI, sequences coding for Der f 2 deposited from other geographic regions were chosen. The identity and E value between our Der f 2 sequence and each of those sequences were computed by LALIGN. (http://www.ch.embnet.org/software/LALIGN_form.html)

Table 2 Similarity for Der f 2 reported from different districts or countries by the VECTOR NTI 9.0 software

	Guangzhou	Hainan	Reinbek	Nakhon Pathom	Seoul	Shengzhen	Tokyo	Singapore
Guangzhou	100	90	91	68	69	73	78	71
Hainan		100	87	73	74	78	68	76
Reinbek			100	62	62	65	71	64
Nakhon Pathom				100	97	90	76	92
Seoul					100	90	76	94
Shengzhen						100	86	96
Tokyo							100	82
Singapore								100

Deduced amino acid sequence and bioinformatics studies

Fig. 4 shows the deduced amino acid sequence of 175 residues. The protein encoded a hydrophobic and signature extracellular region with a loop length of 127 residues and 15 Lys and Arg according to the TopPred results (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html). The most likely cleavage site of the pro-protein was between positions 17 and 18 by the SignalP 3.0 software in SMART Server (http://www.cbs.dtu.dk/services/SignalP/). One strong transmembrane helix was found from position 6 to position 24 with a total score of 1300 as predicted by the TMpred in ISREC Server, suggesting this protein probably anchors on a cytoplasmic membrane and may function as an adaptor molecule that may play a role in cell signaling. InterProscan results (http://www.ebi.ac.uk/InterProScan) revealed that there were two possible domains in this protein: one homologous to the E1 protein, an epididymal secretory protein (from pos. 55 to 172) and the other homologous to the E set superfamily (from pos. 22 to 170). Both of these two domains were indexed in the ML family (MD-2-related lipid recognition domain). With regard to its secondary structure, GOR4 predicted that 16.57% (29) of the protein was in alpha helices, 32.57% (57) as extended strands, and 50.86% (89) as random coils (Fig. 5).

DISCUSSION

It has long been recognized that recombinant allergens are essential for clinical diagnosis of house dust allergies and hold a great potential for

therapy against house dust mite allergy. Previous reports were largely restricted to the screening of cDNA libraries for a gene fragment coding for an active protein. As a result, such obtained proteins would not contain the pro-peptide sequence. Subsequent experiments showed that the purified allergens expressed in *E. coli* had only 50% of the IgE-binding activity of the natural molecule.[9,10] In contrast, Best *et al*.[11] reported that recombinant forms of Der f 1 (rDer f 1) containing the pro-peptide sequence expressed in *Pichia pastoris* had a similar IgE binding activity as native Der f 1 isolated directly from mites. This result suggested that the pre-pro sequence was essential for the secretion of the mature form of the protein and both the alpha factor signal peptide and the pro-enzyme region were efficiently processed during secretion. Therefore, the production of recombinant allergens should include the pro-protein sequence.

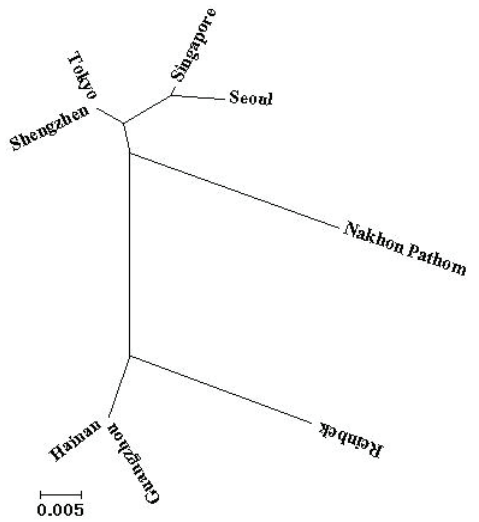

Fig. 3 A phylogenetic tree for group 2 house dust mite allergens reported from different regions or countries.

In this study, a complete CDS coding for

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1     ATG ATT TCC AAA ATC TTG TGC CTT TCA TTG TTG GTA GCA GCC GTT   45
1     Met Ile Ser Lys Ile Leu Cys Leu Ser Leu Leu Val Ala Ala Val   15

46    GTT GCC GAT CAA GTC GAT GTT AAA GAT TGT GGT AAG TTT GTT TGT   90
16    Val Ala Asp Gln Val Asp Val Lys Asp Cys Gly Lys Phe Val Cys   30

91    GTG ATA CAT TTT TTT TCT TTT CAT TTA TTC AAC ACT AAA CAC AAT   135
31    Val Ile His Phe Phe Ser Phe His Leu Phe Asn Thr Lys His Asn   45

136   TTT CTT TTT CTT GTT TAT ATT CAT ATA GCC AAC AAT GAA ATC AAA   180
46    Phe Leu Phe Leu Val Tyr Ile His Ile Ala Asn Asn Glu Ile Lys   60

181   AAA GTA ATG GTC GAT GGT TGC CAT GGT TCT GAT CCA TGC ATC ATC   225
61    Lys Val Met Val Asp Gly Cys His Gly Ser Asp Pro Cys Ile Ile   75

226   CAT CGT GGT AAA CCA TTC ACT TTG GAA GCC TTA TTC GAT GCC AAC   270
76    His Arg Gly Lys Pro Phe Thr Leu Glu Ala Leu Phe Asp Ala Asn   90

271   CAA AAC ACT AAA ACC GCT AAA ATT GAA ATC AAA GCC AGC CTC GAT   315
91    Gln Asn Thr Lys Thr Ala Lys Ile Glu Ile Lys Ala Ser Leu Asp   105

316   GGT CTT GAA ATT GAT GTT CCC GGT ATC GAT ACC AAT GCT TGC CAT   360
106   Gly Leu Glu Ile Asp Val Pro Gly Ile Asp Thr Asn Ala Cys His   120

361   TTT ATG AAA TGT CCA TTG GTT AAA GGT CAA CAA TAT GAT ATC AAA   405
121   Phe Met Lys Cys Pro Leu Val Lys Gly Gln Gln Tyr Asp Ile Lys   135

406   TAT ACA TGG AAT GTG CCG AAA ATT GCA CCA AAA TCT GAA AAC GTT   450
136   Tyr Thr Trp Asn Val Pro Lys Ile Ala Pro Lys Ser Glu Asn Val   150

451   GTC GTT ACA GTC AAA CTT ATC GGT GAT AAT GGT GTT TTG GCT TGC   495
151   Val Val Thr Val Lys Leu Ile Gly Asp Asn Gly Val Leu Ala Cys   165

496   GCT ATT GCT ACC CAT GGT AAA ATC CGT GAT TAA   528
166   Ala Ile Ala Thr His Gly Lys Ile Arg Asp End
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Fig. 4 The deduced amino acid sequence of the Hainan Der f 2 allergen.

Der f 2 was selected as the reference sequence for the design of primers to amply house dust mite allergen from adult *D. farinae*. Unexpectedly, an additional sequence was found in our results. After BLAST analysis against the GenBank database in the NCBI, only four sequences, i.e. GenBank accession numbers AJ862838, AJ862837, AJ862836 and AF346905, contained the extra 87 base pairs, and the other cDNA coding for Der f 2 did not contain this extra region. Our analysis identified sequence polymorphisms among the group 2 allergens. The polymorphisms were found corresponding to nucleotide positions 76, 88, 111 and 125 as reported by Yuuki *et al*.[12] When the cDNA sequences coding for Der f 2 from different geographic regions were analyzed, the Hainan strain was clustered with two sequences, one from Reinbek, Germany and the other from Guangzhou, China. Hainan Island is a tropical region in Southeastern China. If not by airplanes, everyone coming to Hainan Island from mainland China has to go through Guangzhou province first. Therefore, the close genetic relationship of *D. farinae* between Hainan and Guangzhou may have originated from the migrations brought about by hosts' migration and traveling. However, at present, the closeness of the relationship between mites from Hainan and that from Reinbek, Germany remains unknown. We suppose that mites from Hainan, China and Reinbek, Germany, belong to the same sub-species and the mites from Nakhon Pathom, Seoul, Tokyo and Singapore probably belong to another sub-species. The different sub-species of mites may travel from one country to another with international trading, consequently resulting in their particular geographic distribution. Furthermore, a highly conserved region was found between +240 and +555 bp among the Der f 2 sequences from all the indicated regions or countries via sequence comparison (Fig. 3), indicating that this region may be critical for their functions. Moreover, the polymorphisms among Der f 2 cDNA sequences, especially the deletion mutation from +77 to 163 bp, may reflect their functional difference in cell biology.

So far, the biochemical function of the group 2 allergens remains largely unknown and is still debated. Some scholars insisted that the group 2 allergens could be the products of epididymal secretions.[13] Other scholars showed that the tertiary structure of the group 2 allergens was similar to the third and fourth domains of the transglutaminase coagulation factor VIII.[14,15] However, our analysis suggested that group 2 allergens might have a transmembrane domain and a MD-2-related lipid recognition domain.

In summary, we cloned and sequenced the group 2 allergen from *Dermatophagoides farinae* isolated in Hainan, China. We found that this gene contained an insertion of 87 base pairs not seen in the reference sequence. Our analysis showed that the strain from Hainan was more similar to those from Reinbek, Germany and Guangzhou, China than to strains from other parts of East Asia.

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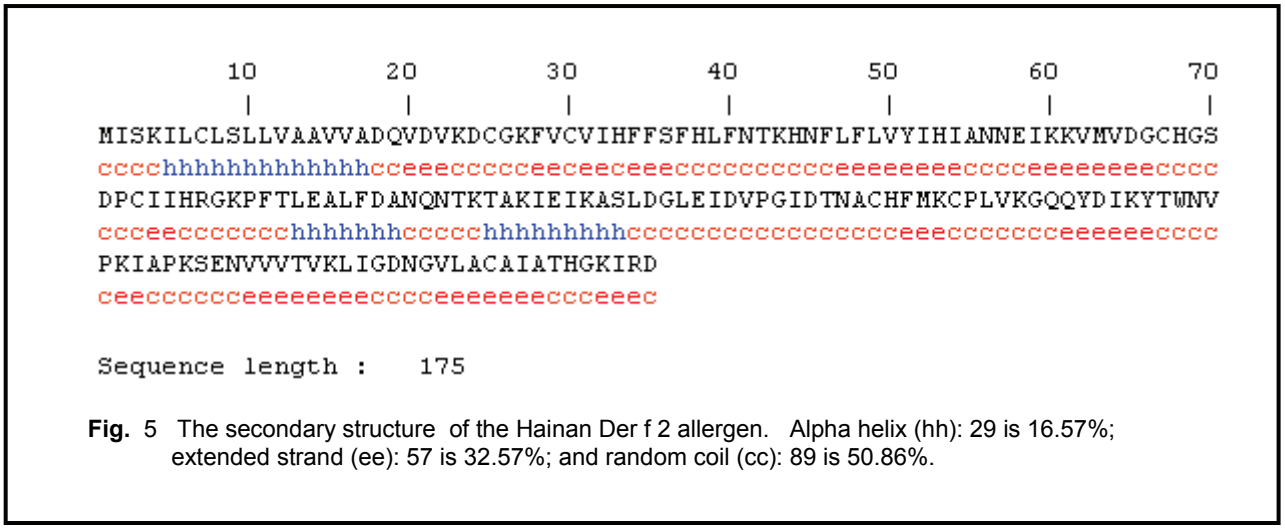

Fig. 5 The secondary structure of the Hainan Der f 2 allergen. Alpha helix (hh): 29 is 16.57%; extended strand (ee): 57 is 32.57%; and random coil (cc): 89 is 50.86%.

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