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Discrimination between house dust mite *Dermatophagoides pteronyssinus* and *D. farinae* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

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Problem/Background : *House dust mites, Dermatophagoides pteronyssinus and D. farinae are the most common among all mite species distributed worldwide. They are also the most common sources of allergens causing asthma and other allergic diseases. Epidemiological studies of these house dust mites are based on morphological identification. This procedure faces some difficulties, including requirements of expertise to identify the mites and inability to discriminate immature stages. Therefore, we applied the molecular method for discriminating between the two most common house dust mites, D. pteronyssinus and D. farinae.*

Objective : *To demonstrate the usage of the cytochrome oxidase I (COI) gene for discriminating between D. pteronyssinus and D. farinae by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).*

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Design : *Descriptive study.*

Setting : *Department of Parasitology, Faculty of Medicine, Chulalongkorn University*

Methods : *The house dust mites; D. pteronyssinus and D. farinae, were maintained at Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The DNA extraction was amplified for COI gene by PCR technique. The DNA sequences were compared with GenBank database. As for PCR-RFLP analysis, the PCR products were digested in separate reaction with AluI.*

Results : *PCR yielded 1,559 and 1,562 bps for the COI gene of D. pteronyssinus and D. farinae respectively. AluI restriction digestions of the PCR amplicons produce different RFLP patterns between these house dust mite species.*

Conclusions : *This technique provides accurate identification of the house dust mites which can be used for epidemiological surveys, and therefore to perform diagnostic testing and for prescribing immunotherapy more efficiency.*

Keywords : *House dust mite, mitochondrial DNA, cytochrome oxidase, PCR-RFLP.*

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บทนำ : *ไรฝุ่นสายพันธุ์ Dermatophagoides pteronyssinus และสายพันธุ์ D. farinae เป็นสายพันธุ์ที่พบกระจายทั่วโลก ไรฝุ่นเป็นสาเหตุอย่างมากที่ทำให้เกิดอาการแพ้ซึ่งเป็นสาเหตุทำให้เกิดเป็นโรคหอบหืดและอาการอื่น ๆ ที่เกี่ยวข้องกับโรคภูมิแพ้ การศึกษาทางด้านระบาดวิทยาของไรฝุ่นมักศึกษาเกี่ยวกับลักษณะรูปร่างของไรฝุ่น ซึ่งค่อนข้างยากและไม่สามารถแยกความแตกต่าง ในระยะตัวอ่อนได้ ดังนั้นงานวิจัยนี้จึงนำเทคนิคทางอณูชีววิทยามาประยุกต์ ใช้เพื่อจำแนกสายพันธุ์ของไรฝุ่น*

วัตถุประสงค์ : *เพื่อศึกษาการจำแนกสายพันธุ์ของไรฝุ่นสายพันธุ์ D. pteronyssinus และสายพันธุ์ D. farinae ที่ตำแหน่งยีนไซโตโครม ออ็กซิเดซ วัน (COI) โดยใช้เทคนิคพีซีอาร์อาร์เอฟแอลพี (PCR-RFLP).*

รูปแบบการศึกษา : *การศึกษาเชิงพรรณนา*

สถานที่ทำการศึกษา : *ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

วิธีการศึกษา : *ไรฝุ่นสายพันธุ์ D. pteronyssinus และ สายพันธุ์ D. farinae ทำการเพาะเลี้ยงที่คณะเทคโนโลยีการเกษตร สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง ทำการเพิ่มจำนวนดีเอ็นเอบริเวณ COI โดยเทคนิคพีซีอาร์ ลำดับนิวคลีโอไทด์ที่ได้จะถูกเปรียบเทียบกับฐานข้อมูล GenBank และทำการศึกษาด้วยเทคนิค PCR-RFLP โดยใช้เอนไซม์ตัดจำเพาะ AluI*

ผลการวิจัย : *ผลการหาลำดับนิวคลีโอไทด์พบว่าบริเวณ COI ของไรฝุ่นสายพันธุ์ D. pteronyssinus และสายพันธุ์ D. farinae มีขนาดประมาณ 1,559 และ 1,562 bp ผลการวิเคราะห์ PCR-RFLP สามารถจำแนกความแตกต่างของไรฝุ่นทั้งสองสายพันธุ์นี้ได้โดยใช้เอนไซม์ตัดจำเพาะ AluI*

สรุป : *เทคนิคพีซีอาร์อาร์เอฟแอลพีสามารถนำมาใช้เป็นเครื่องมือในการจำแนกชนิดของไรฝุ่นได้อย่างถูกต้องแม่นยำ สามารถใช้ในการศึกษาทางด้านระบาดวิทยา และการรักษาทางด้านภูมิคุ้มกันให้มีประสิทธิภาพมากขึ้น*

คำสำคัญ : *ไรฝุ่น, mitochondrial DNA, cytochrome oxidase, PCR-RFLP.*

House dust mites or domestic mites are classified as members of Arachanida, related to ticks, spiders and scorpions. Studies on house dust mites and allergies have grown dramatically since 1964, and they have been known to be the most common household allergens causing asthma and other allergic diseases. [1] The most common allergy-causing mites found in houses worldwide including, *Dermatophagoides farinae* (American house dust mite), *D. pteronyssinus* (European house dust mite), *Euroglyphus maynei*, and the storage mite *Blomia tropicalis*.[2,3]

D. farinae and *D. pteronyssinus* are the most prevalent house dust mites species found worldwide. Surveys of house dust mites conducted around the world reported that these two species usually occur together. Although *D. pteronyssinus* is usually more prevalent than *D. farinae*[4]; however, some reports state that *D. farinae* has more prevalence than *D. pteronyssinus*.[5]*D. farinae* and *D. pteronyssinus* are always found in houses; however, they are rarely found or absent in arid climates unless indoor relative humidity in the house is artificially raised. [6] Reports of the prevalence of the house dust mites are usually based on identification of mites using morphological characteristics. Morphology of mites in the genus *Dermatophagoides* is very similar. However, some morphological characteristics such as in the male ventral posterior idiosoma and the aedeagus, and in the female genital opening and bursa copulatrix can be used to identify the mite species. Morphological differences between *D. pteronyssinus* and *D. farinae*, in the male show the pair of the first legs are extraordinary larger in *D. pteronyssinus*, whereas in *D. farinae* are not larger than the other three pairs. In the female, however, a shape of bursa copulatrix in *D. pteronyssinus* showes a flower-shape, but *D. farinae* showed a cup-shape. [7, 8] This procedure faces some difficulties because of the similarities of the mites' organs, especially in the immature stages [9, 10]; therefore, it needs persons of high expertise to identify the mites.

Identification of the arthropods using molecular biology techniques has been developed to overcome the difficulties associated to taxonomic identification previously described. The mitochondrial DNA (mtDNA) is one of the sources of DNA suitable for molecular identification because of the high abundance of the mtDNA in tissues when compared to the nuclear DNA (nuDNA) therefore, it is suitable for DNA extraction although in spite of the small amount of samples.[11] Furthermore, mtDNA is maternal inheritance and lack of genetic recombination, it is therefore a good candidate for evolutionary and genetics population studies.[12] The cytochrome oxidase gene is a part of mtDNA and was chosen in this study because it has relative high degree of variations. We, hereby, demonstrate the application of partial mitochondrial cytochrome oxidase I (COI) sequences for discriminating between the two house dust mite species, *D. pteronyssinus* and *D. farinae* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The benefits of this study include further epidemiological surveys, and therefore for performing diagnostic testing to house dust mite allergy as well as prescribing more efficient immunotherapy.

Materials and Methods

House dust mite culture and isolation of adult mites

House dust mite; *D. pteronyssinus* and *D. farinae,* were maintained at the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The mites were reared in 75 cm^3 flasks and incubated at a controlled temperature and humidity incubator. These mites were raised on a large-scale basis at 25 ± 1 °C and 75 ± 5 % relative humidity. A culture medium consisting of yeast, wheat flour and rice was used for the large-scale cultivation. The adult mites were removed from the cultures using a writing brush under a stereomicroscope. One thousand live adults of *D. pteronyssinus* and *D. farinae* from the cultures were collected in 1.5 ml microcentrifuge tubes for further DNA extraction steps.

DNA extraction

The house dust mite samples were placed in liquid nitrogen for 1 minute. Having recovered from liquid nitrogen, 200 µl of lysis buffer (provided with the DNA extraction kit) was added into to each sample. The house dust mite samples were then ground with a sterile plastic pestle. Genomic DNA was isolated using DNA extraction kits: Invisorb ® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 100 µl of elution buffer; the fraction of extracted DNA was spectrophotometrically quantitated using a Nanodrop 2000c (Thermo Scientific, Singapore). The extracted DNA samples were kept at -80 ^{0}C for long-term storage.

PCR amplification

Sequences of the COI gene of the *D. pteronyssinus* and *D. farinae* were obtained from GenBank with accession numbers; EU884425 [13] GQ465336 [14], respectively. The sequences were aligned using the multiple alignment programs ClustalX version 1.81. [15] The degenerate oligonucleotide primers were designed as forward primer (5′ TAATGCRAAGAGTTTAAGCC 3′) and reverse primer (5′ CAAGCYTCMACATTAGTGTG 3′). Primers were synthesized by 1st BASE Oligonucleotide (Oligo) Synthesis services company (1st BASE Laboratories, Malaysia). The amplification reaction was set up in the final volume of 25 µl, containing approximately 200 ng of extracted DNA, 10 µM of each primer, 2.5 mM of $MgCl_2$, 2 mM of dNTPs and 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad CA, USA). The PCR reactions were performed in a GeneAmp PCR system 2400; Applied Biosystems®, USA. The reaction conditions were as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 47.8 °C for 1 min, and 72°C for 1 min and the final extension at 72°C for 10 min. Aliquots of the amplicons were analyzed on an 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

To evaluate the sensitivity of the PCR for amplifying the COI gene from the extracted DNA of the house dust mites, PCR was performed with serial dilutions of 1 µl of extracted DNA (1×), 1 µl of 1×10^{-1}, 1 µl of 1×10^{-2} and 1 µl of 1×10^{-3}.

DNA sequencing and RFLP patterns prediction

After PCR amplifications, the PCR products were recovered from the gel and purified by using Agarose Gel DNA Purification Kit: Invisorb® Fragment CleanUp (STRATEC Molecular GmbH, Germany) following the manufacturer's instructions. The purified DNA was sent for direct DNA sequencing which was performed by 1st BASE DNA Sequencing Service Company (1st Base Laboratories, Malaysia). PCR was performed 5 times on each mite species; 5 PCR samples were sequenced for each species. Determination of the nucleotide sequences was performed in both directions using the same forward and reverse primers used in PCR steps. Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor Version 7.0.9.0 [16] and the consensus sequences were BLAST search (available at http://www.ncbi.nlm.gov/ BLAST) for species identification. The nucleotide sequences of COI gene obtained from this study were submitted to the GenBank sequence database. The resulting sequences were used for prediction of species-specific restriction sites by using the NEBcutter V2.0 web-based program (available at http://tools.neb.com/NEBcutter2/index.php). From restriction prediction data, *Alu*I restriction endonucleases were chosen for RFLP.

PCR-RFLP

The PCR products were digested in separate reactions with *Alu*I (New England Biolabs, Ipswich, USA). The reaction mixture was composed of approximate 200 μg of PCR product, 1 μl of 10× fastdigest buffer, 0.5 unit of restriction enzyme and DNase-free water to the final volume of 10 μl. The mixture was incubated at 37°C for 15 min followed by heat inactivation at 65 °C for 5 min. The restriction products were electrophoresed through 8% native polyacrylamide gel electrophoresis run at 100 V for 70 min (MiniProtein 3 cell; Bio-Rad®, USA), followed by ethidium bromide staining and visualized on a Quantity One Quantification Analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

Results

DNA extraction, PCR amplification and sensitivity of PCR

The extracted DNA yielded 193 ng/μl and 214 ng/μl for *D. pteronyssinus* and *D. farinae*, respectively. PCR was able to amplify the COI gene from both house dust mite species. The expected PCR products of approximately 1.5 kb were demonstrated on 1.5% agarose gel (data not shown). The sensitivity of PCR was determined by using serial DNA dilutions as DNA template for PCR amplifications. PCR was able to amplify 1 μl of diluted DNA to 1×10^{-3} dilutions for *D. pteronyssinus* (Fig. 1).

Sequence analysis

Consensus COI gene sequences of *D. pteronyssinus* and *D. farinae* were submitted to GenBank, with assigned accession numbers as HQ823623 and HQ823622 for *D. pteronyssinus* and *D. farinae*, respectively. Sequence comparisons between *D. pteronyssinus* and *D. farinae* of this study were compared with sequence data from GenBank for *D. pteronyssinus* accession number EU884425 [13] and for *D. farinae* accession number GQ465336 [14] showed the percentage of sequence similarity of 98% and 99%, respectively (data not shown).

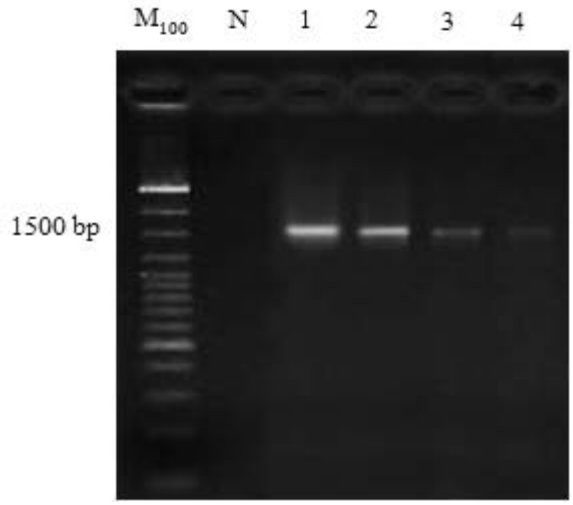

Figure 1. The PCR-amplified product of approximately 1.5 kb in length for *D. pteronyssinus*. PCR was able to amplify extracted DNA from 1 μl to the serial dilution of 1×10^{-3}. N: Negative, Lane 1-4: PCR amplifications using, 1 μl of extracted DNA (1×), 1 μl of 1×10^{-1}, 1 μl of 1×10^{-2} and 1 μl of 1×10^{-3} dilutions, as DNA template respectively. M_{100}: 100 bp, DNA standard marker (Invitrogen®).

PCR-RFLP

PCR-RFLP prediction was performed with sequences of the house dust mites obtained from this study by using the NEBcutter V2.0 web-based program (available at http://tools. neb.com/ NEBcutter2/index.php). *Alu*I restriction endonuclease was allowed the differentiation of *D. pteronyssinus* and *D. farinae* as summarized in the Table 1. PCR products of each species were digested with the *Alu*I restriction enzyme and the fragments separated by 8% native polyacrylamide gel electrophoresis. Fig.1 demonstrate the PCR-amplified product of approximate 1.5 kb in length for both species, the PCR-RFLP patterns for each species and mixed DNA of both species (Fig. 2).

Discussion

Mitochondrial DNA sequences provide essential data for population genetics, phylogenetic studies, species identification and the analysis of molecular evolution studies. The mitochondrial DNA sequences of the arthropod have been used as efficient molecular markers in *Aleuroglyphus ovatus*, *Blomia tropicalis*, *D. farinae*, *D. pteronyssinus* and *Tyrophagus putrescentiae*.[17] Many mitochondrial genes are conserved in arthropods and specific of species such as cytochrome oxidase subunits I-II (COI-II), small ribosomal subunit RNA (12S-rRNA and 16S-rRNA) and cytochrome b (Cytb). [13] In this study, the 1.5 kb region coding for the cytochrome oxidase subunits I (COI) of the mitochondrial DNA was

Table 1. Comparative RFLP patterns between *D. pteronyssinus* and *D. farinae*.

Species	COI digested with *Alu*I
D. pteronyssinus	618, 327, 313, 249, 24, 24 and 4 bp
D. farinae	664, 272, 252, 211 and 163 bp

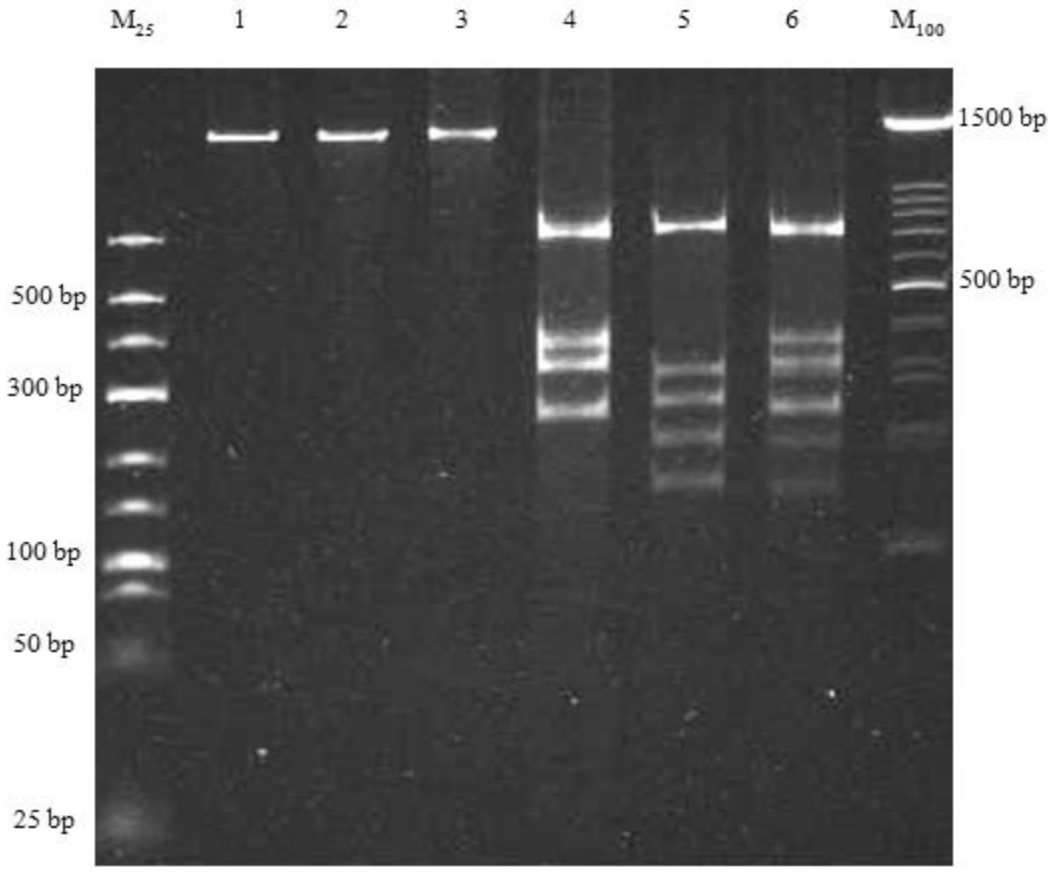

Figure 2. Eight percent native polyacrylamide gel electrophoresis shows PCR-RFLP patterns of COI amplicons digested with *Alu*I restriction enzyme. From left to right as follows: undigested PCR products amplified from *D. pteronyssinus, D. farinae* and mixed of *D. pteronyssinus* and *D. farinae* DNA respectively (lanes 1-3); RFLP patterns of *D. pteronyssinus* (lane 4); *D. farinae* (lane 5) and mixed of *D. pteronyssinus* and *D. farinae* (lane 6) from *Alu*I digestion. Lane M_{25} and M_{100} are 25 bp and 100 bp, DNA standard marker (Invitrogen®), respectively.

selected for discriminating between *D. pteronyssinus* and *D. farinae*. The extracted DNA from the house dust mites using method in this study was suitable for PCR amplification. The sensitivity of PCR was validated by using 1 μl of serial dilution of the extracted DNA to 1×10^{-3} as DNA template, PCR was able to amplify to 1 μl of 1×10^{-3} dilutions. The results revealed that PCR used in this study was able to amplify the house dust mite COI gene even at a very small amount of DNA; therefore, it can be implied to detect event a small number of the house dust mite. Comparison of the COI gene sequences obtained from this study and the sequences reported in the GenBank revealed that 98% and 99% similarity to sequences reported for *D. pteronyssinus*[13] and *D. farinae*[14] respectively.

The PCR-RFLP analysis has been used to identify and differentiate between closely related species such as spider mites.[18] In this study we demonstrated the PCR-RFLP technique in order to discriminate between the closely related house dust mite species *D. pteronyssinus* and *D. farinae*. RFLP

profiles of both species when digested with *Alu*I restriction endonuclease were demonstrated on an 8% native polyacrylamide gel electrophoresis stained with ethidium bromide (Fig. 2.). Native polyacrylamide gel has high resolution for discriminating the between very close fragments such as 327, 313 bp fragments of *D. pteronyssinus* and 272, 252, 211 bp fragments of *D. farina*e. The expected RFLP fragments of *D. farinae* were demonstrated on the gel; however, the small fragments (below 50 bps) of *D. pteronyssinus* disappeared because they were run out of the gel. In case of mixed DNA of both house dust mite species, the RFLP showed combination patterns between *D. pteronyssinus* and *D. farinae*.

In conclusion, we demonstrated the PCR-RFLP of the COI region to discriminate between house dust mite species, *D. pteronyssinus* and *D. farinae*. This technique is quick, easy and low cost-effective compare to other molecular techniques. The primers used in this study and reaction conditions were able to amplify the COI gene of both *D. pteronyssinus* and *D. farinae*. The amplified and restricted sequences of the COI regions were separated by agarose gel electrophoresis and the PCR-RFLP profiles between the arthropods is based on differences in the sizes of the restriction fragments obtained from the amplified DNA region generated by a specific endonuclease. The results of this study PCR-RFLP markers successfully differentiated adults of *D. pteronyssinus* and *D. farinae* and confirmed the usefulness of molecular markers in differentiating and identifying mites causing allergic because house dust mites have species-specific allergens and their distribution and biology are different; moreover, the mites produce over 30 different proteins; which can sensitize and induce immunoglobulin E (Ig E)-mediated allergic reactions in humans. [19] Techniques demonstrated in this study would be a useful tool for further investigation of house dust mites survey and therefore for more effective controlling the house dust mites and for diagnosis and treatments of house dust mite allergy.

Acknowledgements

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