Multiplex PCR for Identifying Common Dust Mites Species (*Dermatophagoides pteronyssinus, Dermatophagoides farinae* and *Blomia tropicalis*)

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Summary

Introduction: **Dust mites are known to be an important source of inhalant allergens causing allergic rhinitis and asthma worldwide. The sizes of dust mite populations in patients' houses are useful to monitor the risk of allergen exposure. However, mite identification using the conventional microscopic technique requires specific expertise and is time consuming; therefore a molecular technique has been developed in order to solve these drawbacks.**

Objective: **To develop a multiplex PCR assay for identifying the three common dust mite species in Thailand, namely *Dermatophagoides pteronyssinus* (Dp), *D. farinae* (Df) and *Blomia tropicalis* (Bt), and to evaluate the efficacy of the technique.**

Methods: **Pairs of primers were designed and tested in either singleplex PCR or multiplex PCR. The multiplex PCR technique was also optimized in order to obtain specific products. The reaction mixture contained 5 pmole of individual primers, 10 mM dNTP, 5 units Taq DNA polymerase and genomic DNA (gDNA). The reaction was run for 25 cycles at 94 °C for 20 seconds, 58 °C for 20 seconds and 72 °C for 30 seconds. The PCR products were analyzed by 1.5% agarose gel electrophoresis with GelRed™ fluorescence dye. The optimized multiplex technique was also tested with 30 house dust samples and dust samples spiked with DNA from other insect and mite species.**

Results: **Three PCR products were obtained with the relevant gDNA templates as expected; 143 bp for DF, 221 bp for DP and 318 bp for BT, respectively. The detection limit of the tests was found to be as low 1 ng of gDNA, whereas mixed gDNA species confirmed the 100% specificity of this assay. The total duration from the preparation of the PCR reaction mixture until the analysis by agarose gel electrophoresis was approximately 2 hours. No amplified product was obtained from mites and insects of other species.**

Conclusion: **The multiplex PCR was successfully developed for identifying 3 common dust mite species. This technique can be helpful, not only for non-acarologist personnel for dust mite identification, but also for patients who are allergic to dust mites.** ***(Asian Pac J Allergy Immunol 2012;30:224-30)***

Key words: Dust mite, Species identification, Multiplex PCR, Dust examination, Allergens

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Introduction

House dust mites are known to be the major source of indoor allergens worldwide. The mite species *Dermatophagoides pteronyssinus, Dermatophagoides farinae* and *Blomia tropicalis* have been reported to be predominantly found in many tropical countries.[1-5] Among the remedies for allergic treatment is allergen avoidance and information on dust mite species that patients are exposed to is important.[6] To date, mite identification is mostly based on the morphological appearance under the microscope which requires the well-trained and experienced personnel. In order to overcome this problem, assays using molecular-based techniques have been developed. These techniques, such as the barcode system, PCR-RFLP and AFLP have been widely used in the identification of insects and other arthropods including dust mites.[7] However, some require relatively large specimens to be effective. Multiplex PCR is a useful strategy by which

multiple-data can be obtained from one assay with small amount of specimens.

Molecular markers are an important key for identifying mites at taxonomic levels. The ideal properties of molecular markers have been previously described by several taxonomists.[8,9] Some markers are suitable for genus or species identification.[9-12] For example, genes within internal transcribe spacer 2 (ITS2), ribosomal DNA (rDNA), and mitochondrial genes have been used. ITS2, a DNA region between 5.8s rDNA and 28s rDNA is highly evolved in some species. The gene ITS2 has been used for species identification of spider mites[13] whereas 12S rRNA has been used for dust mite identification.[14]

Herein, the multiplex PCR for identifying three common dust mite species mostly found in Thai houses has been developed and its sensitivity and specificity have been determined. The assay is simple and practicable for any standard laboratory.

Methods

Dust Mites

Three common dust mite species; *Dermatophagoides pterronyssinus* (DP), *D. farinae* (DF), and *Blomia tropicalis* (BT) were obtained from laboratory cultures providing by Siriraj Dust Mite Center, Faculty of Medicine Siriraj Hospital. Pure mites were isolated and used for genomic DNA (gDNA) extraction.

Genomic DNA (gDNA) extraction

The gDNA of dust mites were obtained by using a commercially available extraction kit (NucleoSpin Tissue, MN) with some modifications. Briefly, a hundred mites isolated from the laboratory culture were homogenized in 100 ul of lysis buffer with a micropestle. The homogenate was centrifuged at 10,000 rpm for 5 minutes and the supernatant was mixed with the same volume of 95% ethanol and was loaded into a purifying column. After centrifugation, the column-bound gDNA was washed several times with the buffers providing in the kit. In the last step, the gDNA was eluted with Tris buffer. The amount and quality of gDNA were checked by spectrophotometer prior to being stored at -20 °C.

Primer design

A number of primers for detecting *D. pteronyssinus* and *D. farinae* mites were generated from the mites' internal transcribed spacer 2 (ITS2) gene collections,[15] whereas primers generated from cytochrome oxidase I (Cox I) gene were used for detecting *B. tropicalis*. The primers were tested in a polymerase chain reaction until species specific and optimum conditions were obtained.

Polymerase Chain Reaction (PCR)

Singleplex PCR was performed to select the species specific primers for detecting the 3 common dust mites (DP, DF, BT). Basically, each reaction was run in 20 ul containing 10x reaction buffer, 10 mM dNTP, 10 pmoles Forward primer, 10 pmoles Reverse primer, 5 units *Taq* DNA polymerase and variable quantities of gDNA template, respectively. The PCR products were run on 1.5% agarose gel electrophoresis and were visualized with UV light after being stained with fluorescense dye (GelRed^{TM}).

Thereafter, multiplex PCR was carried out by using the same amounts of reagents as in Singleplex PCR but only 5 pmoles of individual primers were used. The optimum condition of the reaction was: 94°C for 2 minutes, 25 cycles of 94°C 20 seconds, 58°C for 20 seconds, and 72°C for 30 seconds, respectively with the final step at 72°C for 2 minutes.

Efficacy of Multiplex PCR

The specificity and sensitivity of multiplex PCR were evaluated by both singleplex and multiplex PCR. The specificity was also tested with other mites and insects usually found in households: e.g., storage mite (*Tyrophagus* sp.), predator mite (*Cheyletus* sp.), American cockroach (*Periplaneta americana*), mosquito (*Culex* sp.), and booklice (*Liposcelis* sp.). The positive control reactions for mosquito and booklice were performed with their actin gene primers, whereas the ITS2 primers were used for the positive control of cockroaches and mites. The sensitivity, or detection limit, was investigated with two-fold serial dilutions from 0.00625 ng to 102.4 ng of gDNA template. The experiment was repeated ten times.

In addition, multiplex PCR was also performed with 30 house dust samples, of which, three (D001, D002, D003) were spiked with variable numbers of mites (50, 100, 200 mites of each species). 2.5 mg of these samples was extracted for genomic DNA (gDNA) using a similar method to that mentioned above. The resulting gDNA samples were then used as template in the multiplex assay.

Result

Primer selection

Using singleplex PCR, five primers generated from the genes ITS2 (ITS2-116_F, ITS2-199_F, ITS2_R2) and Cox I (Cox I_F3, Cox I_R2) were completely matched with three common dust mite species. As shown in Figure 1, the primers ITS2-116_F and ITS2_R2 amplified 195-bp product with *D. pteronyssinus* gDNA (Lane 1), whereas the primers ITS2-199_F and ITS2_R2 amplified 143-bp product with *D. farinae* gDNA (Lane 6). As for *B. tropicalis*, the primers Cox I_F3 and Cox I_R2 gave 318-bp product (Lane 11). In order to check the probability of non-specific products, the singleplex reactions with cross-matched pairs of these primers were also performed. No amplified products were obtained from these reactions as summarized in Table 1.

Efficacy of multiplex PCR

The multiplex PCR with mixed gDNA of *D. farinae*, *D. pteronyssinus* and *B. tropicalis* showed that non-specific amplification did not occur with non-relevant templates, even when large amounts were added (Figure 2). The highest sensitivity at a particular amount of template was for *B. tropicalis*, followed by *D. pteronyssinus* and *D. farinae*, respectively. In addition, the multiplex PCR with the gDNA of *Tyrophagus* sp., cockroach (*Periplaneta americana*), mosquito (*Culex* sp.), predator mite (*Chyletus* sp.), and booklice (*Liposcellis* sp) did not give any amplified DNA products, whereas the presence of gDNA template in the reaction was confirmed (data not shown).

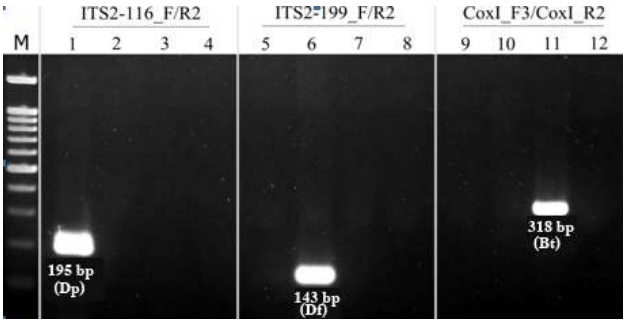

Figure 1. PCR products obtained from singleplex PCR with species-specific primers analyzed by agarose gel electrophoresis showing species specificity. The 20-μl reactions contained 10 mM dNTP, 10 pmoles forward primer, 10 pmoles reverse primer, 5 units Taq DNA polymerase and 50 ng gDNA of *D. pterronyssinus* (lane 1, 5, 9), *D. farinae* (lane 2, 6, 10) and *B. tropicalis* (lane 3, 7, 11), respectively. Negative controls without template were run in lane 4, 8 and 12.

Table 1. Summary of singleplex PCR with matched pairs of designed primers to examine the species specificity of the generated primers for 3 common dust mites; *D. pteronyssinus* (DP), *D. farinae* (DF) and *B. tropicalis* (BT).

Pair no.	Primer pairs		Mite species		
	Forward	Reverse	DP	DF	BT
1	ITS2-116_F	ITS2_R2	✓	--	--
2	ITS2-116_F	ITS2-119_F	--	--	--
3	ITS2-116_F	Cox I_F3	--	--	--
4	ITS2-116_F	Cox I_R2	--	--	--
5	ITS2-119_F	ITS2_R2	--	✓	--
6	ITS2-119_F	Cox I_F3	--	--	--
7	ITS2-119_F	Cox I_R2	--	--	--
8	Cox I_F3	Cox I_R2	--	--	✓
9	Cox I_F3	ITS2_R2	--	--	--
10	Cox I_R2	ITS2_R2	--	--	--

✓ = with PCR product, -- = no PCR product

With regard to sensitivity, the pre-tested singleplex PCR showed that the species specific PCR products were obtained from the lowest amounts of gDNA template were: 0.05 ng for *D. farinae*, and 0.00625 ng for both *D. pteronyssinus* and *B. tropicalis* (data not shown). However, it was slightly different in the multiplex PCR. The detection ability of the multiplex assay was 0.1 ng for DF, 0.025 ng for DP and 0.00625 ng for BT, respectively (Figure 3). Therefore, at least 0.1 ng of gDNA template should be used in multiplex PCR.

The multiplex PCR with 30 house dust samples showed that 12 of them contained *D. farinae*, whereas 16 samples contained *D. pteronyssinus* and 20 samples contained *B. tropicalis* mites. Some of them contained either single, double or triple mite species in the same samples (Figure 4). As summarized in Table 2, half of the samples gave 100% matchability between multiplex PCR and microscopic examination, especially for those spiked dust samples (D001 – D003), although less matchability could be seen in 5 samples (33.3%). Dust samples with either *D. pteronyssinus* or *D. farinae* mites showed higher frequencies of matchibility using both methods. Higher positivity was frequently found with samples containing *B. tropicalis*. There were only three house dust samples

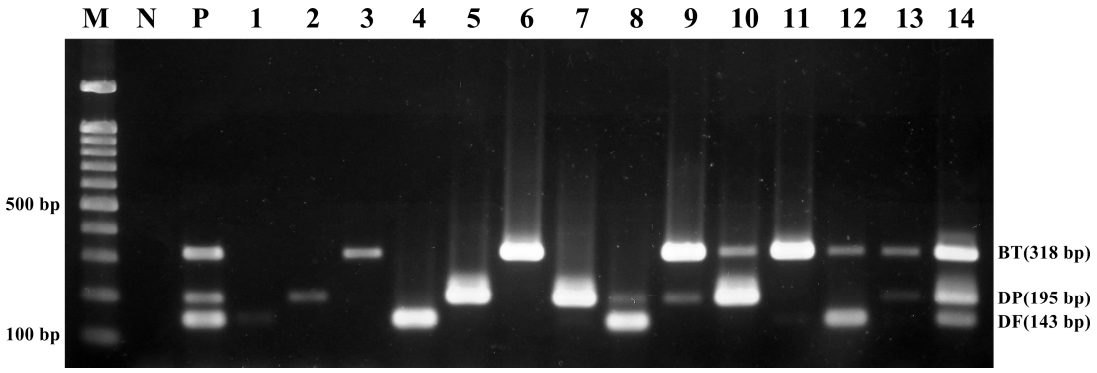

Figure 2. PCR products of multiplex PCR with varied amounts of gDNA template from *D. farinae* (DF), *D. pteronyssinus* (DP) and *B. tropicalis* (BT) were analyzed for the specificities and sensitivities. The 20-ul reaction contained 10 mM dNTP, 5 pmoles forward primer, 5 pmoles reverse primer and 5 unts Taq DNA polymerase. The reaction was run at 94 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 30 seconds for 25 cycles with the initial step at 94 °C for 2 minutes and the final step at 72 °C for 2 minutes.
Lane 1, 2, 3 : 0.1 ng gDNA of only DF, DP and BT respectively.
Lane 4, 5, 6 : 10 ng gDNA of only DF, DP and BT respectively.
Lane 7 : 0.1 ng gDNA of DF + 10 ng gDNA of DP
Lane 8: 0.1 ng gDNA of DP + 10 ng gDNA of DF
Lane 9: 0.1 ng gDNA of DP + 10 ng gDNA of BT
Lane 10: 0.1 ng gDNA of BT + 10 ng gDNA of DP
Lane 11: 0.1 ng gDNA of DF + 10 ng gDNA of BT
Lane 12: 0.1 ng gDNA of BT + 10 ng gDNA of DF
Lane 13 0.1 ng gDNA of DF, DP, BT
Lane 14, 10 ng gDNA of DF, DP, BT

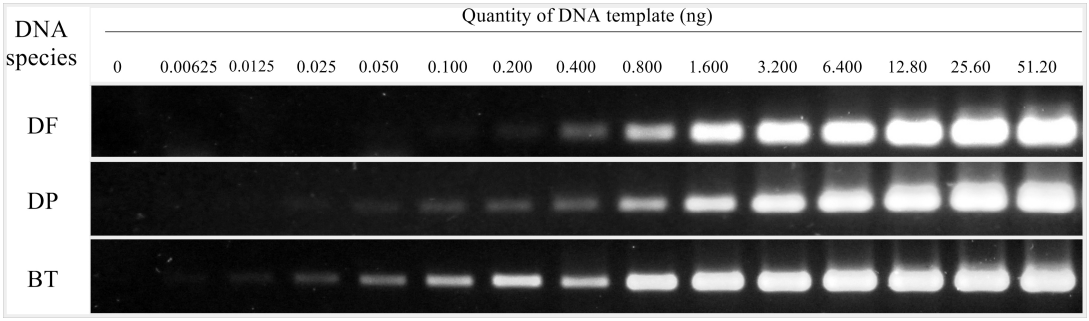

Figure 3. Results of multiplex PCRshowing the detection limit of the novel assay for three common dust mite species; *D. farinae* (DF), *D. pterronyssinuss* (DP), and *B. tropicalis* (BT). The amounts of gDNA are indicated above. The 20-ul reaction contained 10 mM dNTP, 5 pmoles forward primer, 5 pmoles reverse primer, 5 units Taq DNA polymerase and 10 ng of DNA template. The reactions were run at 94 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 30 seconds for 25 cycles with the initial step at 94 °C for 2 minutes and the final step at 72 °C for 2 minutes. As shown, the detection ability was 0.1 ng for DF, 0.025 ng for DP and 0.00625 ng for BT respectively.

that showed negative results due to the absence of dust mites.

Discussion

The main limitation of microscopic mite identification is the need for expert laboratory personnel, since it requires specialist expertise. Hence, entomological investigation is limited in some laboratories. Moreover, several factors such as dead bodies, damaged bodies and immature stages of mite development can also affect specific identification. Herein, the multiplex PCR assay has been established for identification of three common dust mite species causing allergic diseases in Thailand. The assay is simpler and less time consuming than the conventional technique. The duration of the assay is only 2 hours. Only a PCR machine and reagents are required and these are available in most standard laboratories. As confirmed in the experiment with dust samples spiked with 50 individual mite species, the results were quite consistent. Although the lowest number of mites (50 mites) that was spiked into dust samples was rather high compared to the assay's sensitivity, only 1 ng of DNA template is sufficient, which is a small amount compared to the approximately 160 ng of DNA available from one mite.[16] Therefore, the assay would be able to detect even one mite being present in the dust.

Internal transcribed spacer 2 (ITS2), a DNA region between 5.8s and 28s rDNA has been used as a molecular marker for insect and mite identification.[8,9,15,17] The nucleotide composition of this region is highly variable but similar in closely related species. It has been reported that the nucleotide variations of ITS2 are less than 4% in astigmatid mite.[15] The multiple copy number of this ITS2 region has also been previously reported.[18] Therefore, this DNA region is rather specific and suitable for use in dust mite identification. Moreover, the specificity of the assay tested using other common species of mites or insects has also been determined. The PCR-RFLP assay for mite identification has recently been reported, but requires 8 restriction enzymes to obtain the particular patterns for species identification.[7]

The two common dust mite species found in Thailand have been reported by Malainual et al.[2] to be *D. pteronyssinus* and *D. farinae* whereas *B. tropicalis* had been abundant during the period 1991-1994. However, studies in neighboring countries, such as Singapore[4] and Malaysia,[3] have found that it is abundant in houses. Therefore, the new multiplex PCR assay also included specific primers for identifying this storage mite species. It is possible that the difference in species diversities of mites in dust examination could be due to differences

Table 2. Summary of data obtained from thirty household dust examinations by the novel multiplex PCR in comparison with microscopic identification. Both assays were performed in triplicate, a positive result was considered to be present once any of them was positive. Sample D001 to D003 were the dust samples spiked with individual species of mites as indicated.

Sample ID.	Amount of gDNA (ng/ml)	Number of mites per sample			Multiplex PCR		
		DP	DF	BT	DP	DF	BT
D001	ND	50	50	50	✓	✓	✓
D002	ND	100	100	100	✓	✓	✓
D003	ND	200	200	200	✓	✓	✓
D004	1.1 - 4.0	2	--	1	✓	--	✓
D005	3.5 - 12.2	--	--	13	✓	--	✓
D006	3.1 - 14.0	--	--	2	✓	--	✓
D007	2.3 - 14.0	3	--	4	✓	--	✓
D008	11.8 - 19.2	1	--	3	✓	--	✓
D009	4.0 - 10.4	1	3	--	✓	✓	✓
D010	7.1 - 12,5	2	4	--	✓	✓	✓
D011	4.5 - 8.0	1	4	--	✓	✓	--
D012	20.5 - 34.5	--	4	--	--	✓	--
D013	29.6 - 42.0	--	2	1	--	--	--
D014	34.0 - 68.7	--	--	--	--	--	--
D015	12.2 - 30.4	2	--	5	✓	--	✓
D016	2.4 - 3.4	--	1	--	--	--	✓
D017	6.6- 37.1	--	--	--	✓	--	✓
D018	2.8 - 7.1	--	--	--	✓	--	--
D019	272.6 - 397.4	--	✓	--	--	✓	--
D020	43.1 - 184.3	--	✓	--	--	✓	✓
D021	16.6 - 19.1	6	--	--	✓	--	✓
D022	3.0 - 4.0	1	--	✓	✓	--	✓
D023	1.4 - 8.9	1	--	--	✓	✓	✓
D024	16.1 - 22.7	3	1	--	--	--	✓
D025	0.6 - 3.9	--	--	--	--	--	✓
D026	3.1 - 9.2	--	--	--	--	✓	✓
D027	1.0 - 3.0	--	--	1	--	--	✓
D028	4.0 - 9.4	--	--	--	--	--	--
D029	1.5 - 7.9	1	3	1	✓	--	✓
D030	2.6 - 3.5	--	--	--	--	--	--

✓ = found mite, -- = not found, ND = not done

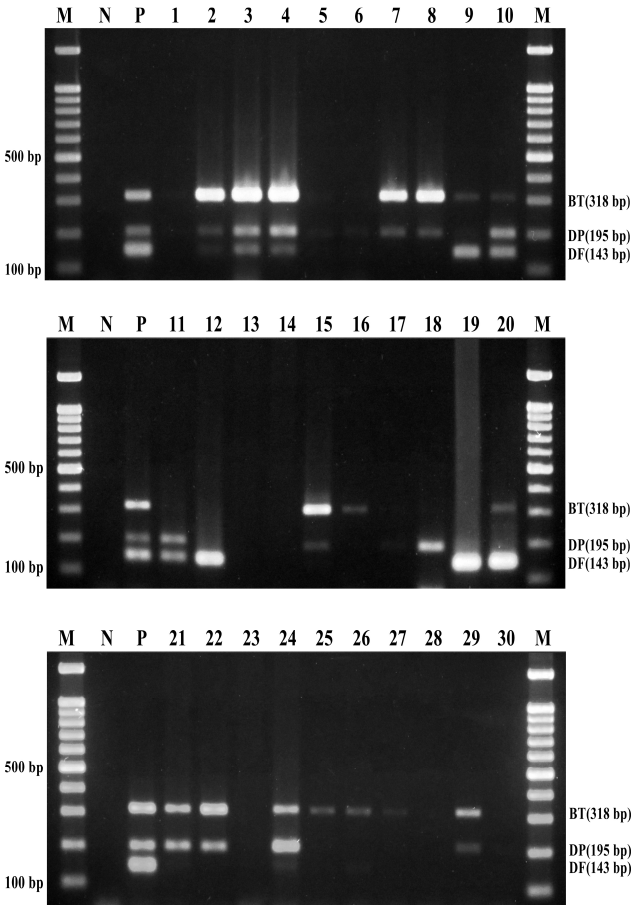

Figure 4. Results of multiplex PCR with DNA extracted from 30 house dust samples (Lane no.1-30). The 20-ul reaction contained 10 mM dNTP, 5 pmoles forward primer, 5 pmoles reverse primer, 5 units Taq DNA polymerase and 10 ng of DNA template. The reactions were run at 94 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 30 seconds for 25 cycles with the initial step at 94 °C for 2 minutes and the final step at 72 °C for 2 minutes. Lane N : negative controls (no template); Lane P : 1 ng gDNA of DF, DP, BT.

in mite recovery methods. Malainual et al. examined dust specimen with a flotation technique, whereas Chew et al. used the absolute number of mites in the whole sample. The flotation technique[19] requires only 100 mg of dust sample and it is less time consuming. However, the bodies of B. tropicalis are hairy and cannot be easily detached from the debris during recovery. Although the examination of the whole dust specimen under the microscope can give the absolute number of mite species present, it involves time consuming and laborious work. As shown in the results, the multiplex PCR could detect the presence of *B. tropicalis* in 22 house dust samples. We also demonstrated that this assay required only small amount of specimen, reagents and a short reaction time in order to achieve mite identification. Moreover, three mite species identification could be obtained from one reaction. This assay offers more advantages and is appropriate for using either in epidemiological studies or in service laboratories. In addition, the assay could be further developed with real-time quantitative multiplex PCR technique in order to examine levels of mites in dust samples.

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