



Development of a new multiplex PCR to detect prevalent species of house dust mites in house dust

Ana Sofia Oliveira, Carlos Gaspar, Joana Rolo, Cristiana Costa Pereira, Rita Palmeira-de-Oliveira, João Paulo Teixeira, José Martinez-de-Oliveira & Ana Palmeira-de-Oliveira

To cite this article: Ana Sofia Oliveira, Carlos Gaspar, Joana Rolo, Cristiana Costa Pereira, Rita Palmeira-de-Oliveira, João Paulo Teixeira, José Martinez-de-Oliveira & Ana Palmeira-de-Oliveira (2021): Development of a new multiplex PCR to detect prevalent species of house dust mites in house dust, International Journal of Environmental Health Research, DOI: [10.1080/09603123.2021.1893282](https://doi.org/10.1080/09603123.2021.1893282)

To link to this article: <https://doi.org/10.1080/09603123.2021.1893282>



View supplementary material [↗](#)



Published online: 08 Mar 2021.



Submit your article to this journal [↗](#)



Article views: 61



View related articles [↗](#)



View Crossmark data [↗](#)



Development of a new multiplex PCR to detect prevalent species of house dust mites in house dust

Ana Sofia Oliveira^{a,b}, Carlos Gaspar^{a,b,c}, Joana Rolo^a, Cristiana Costa Pereira^{d,e}, Rita Palmeira-de-Oliveira^{f,a,c}, João Paulo Teixeira^{d,e}, José Martinez-de-Oliveira^{a,b} and Ana Palmeira-de-Oliveira^{a,b,c}

^aHealth Sciences Research Centre (CICS-UBI), University of Beira Interior Av. Infante D. Henrique, Covilhã, Portugal; ^bFaculty of Health Sciences, University of Beira Interior, Av. Infante D. Henrique, Covilhã, Portugal; ^cLabfit – Health Products Research and Development Lda, UBLmedical, Estrada Nacional, Covilhã, Portugal; ^dNational Institute of Health, Environmental Health Department, Rua Alexandre Herculano, Porto, Portugal; ^eEPIUnit, EPIUnit - Instituto de Saúde Pública da Universidade do Porto, Rua das Taipas, Porto, Portugal; ^fCNC – Center for Neurosciences and Cell Biology, Center for Innovative Biomedicine and Biotechnology (CIBB), University of Coimbra, Faculty of Medicine (Polo 1), Rua Larga, Coimbra, Portugal

ABSTRACT

Dermatophagoides pteronyssinus and *Dermatophagoides farinae* are the most common House Dust Mite (HDM) species in home environments worldwide and responsible for HDM allergy. Since the prevalence of HDM-related clinical conditions is linked to exposure to the mite itself, the detection of HDM in the human households gains importance. We aimed to develop a fast and accessible multiplex PCR to detect and distinguish two relevant HDM species in house dust. New primers were designed, and sensitivity analysis was performed. Sequencing of PCR products was also performed to confirm the method's specificity. The limit of detection of the multiplex PCR for both species was as low as 30 pg μL^{-1} . The application of the multiplex PCR to dust samples also resulted in the identification of both species with high sensitivity. The protocol required small amount of template, reagents and a short reaction time thus presenting an alternative to classically used techniques for HDM identification.

ARTICLE HISTORY

Received 7 December 2020
Accepted 17 February 2021

KEYWORDS

Dust; *Dermatophagoides*;
Multiplex PCR

Introduction

Mite species that live in close proximity to humans are referred to as House Dust Mites (HDM). These mites are typically found in human households in places like carpets, fabrics, pillows, and mattresses and use the components present in house dust as food supplies (Nadchatram 2005; Gøtzsche and Johansen 2008; Hubert et al. 2019).

The most prevalent species globally found in house dust belong to the family Pyroglyphidae, being *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Euroglyphus maynei* the top three species in terms of global frequency and abundance (Colloff 2009). These species can also co-habit, since one particular household can host multiple species (Arlian et al. 2002). *D. pteronyssinus* and *D. farinae* are described to typically occur together and take special relevance due to their well-characterized allergenicity, as the prevalence of HDM allergy is intricately linked to exposure to the mite itself (Gøtzsche and Johansen 2008; Calderón et al. 2015; Insung et al. 2015; Miller 2018; Li

CONTACT Ana Palmeira-de-Oliveira ✉ apo@fcsaude.ubi.pt Faculty of Health Sciences, University of Beira Interior, Av. Infante D. Henrique, Covilhã 6200-506, Portugal

Supplemental data for this article can be accessed [here](#).

© 2021 Informa UK Limited, trading as Taylor & Francis Group

et al. 2018). In fact, one of the most clinically important sources of indoor allergens comes from the scales and faecal pellets of various mite species (Casley et al. 2018).

Since *Dermatophagoides* species are globally ubiquitous in human habitats and are mainly recovered from bedroom dust (particularly from mattresses), it makes them one of the most important etiological agents underlying allergic rhinitis and allergic asthma (Htut et al. 1996; Atambay et al. 2006; Colloff 2009; Calderón et al. 2015). In fact, allergy to HDM accounts for more than 10% of the general population, and 90% of individuals suffering from allergic asthma are sensitive to HDM (Cui 2014; Li et al. 2018). Despite HDM are widely recognized due to their allergenic potential, they are also responsible for other non-allergic condition called acariasis. Little is known about this condition, except that it is caused by parasitic mite infestation of the human host and that has been described in several tissues ranging from pulmonary, cutaneous, intestinal, oral and optic to urinary and vaginal systems (Li et al. 2003; Zhang et al. 2004; Cui 2014).

To address the exposure to HDM, three methods have been used in different studies, specifically mite counts by microscopic visualisation, detection of specific mite allergens by enzyme-linked immunosorbent assay (ELISA), and measurements of guanine using Acarex test® (Sander et al. 2012; Roussel et al. 2013; Cui et al. 2018; Casley et al. 2018).

From these classical methods, only mite counts by microscopic visualisation truly address the presence of HDM, as ELISA measures the level of mite allergens (that can be present in faeces, scales) and Acarex test® estimates, by a colorimetric test, the amount of guanine contained in the excrement of mites (Reboux et al. 2019). Therefore, most of the environmental studies rely on species identification through microscopic visualization to address distribution and diversity of HDM (Boquete et al. 2006; Sun et al. 2013; Solarz and Pająk 2019a, 2019b). Nonetheless, the availability of DNA sequence data of various mite species in international online available databases has opened the door for simpler molecular-based techniques, like polymerase chain reaction (PCR), to be used for the identification of various mites at the species level.

Despite the advantages presented by these molecular-based techniques, the majority of the available protocols are complex and require an increased number of steps and higher quantities of template amount. Multiplex PCR and qPCR techniques have also been developed, though without published primer sequences or PCR product sequencing to truly confirm the reaction specificity (Thet-Em et al. 2012; Roussel et al. 2013; Reboux et al. 2019).

Therefore, we propose the use of a new multiplex PCR assay to identify and distinguish the two most prevalent HDM species in different environments using a fast and accessible protocol. Primers were designed to specifically detect *D. pteronyssinus* and *D. farinae* in domestic dust. Analytical characteristics as 'in silico' specificity, limit of detection, sensitivity in complex dust matrices and PCR product sequencing were determined to validate the new method.

Material and methods

Samples

Dust samples were collected from either mattresses, living room carpets or textile sofas, using a vacuum cleaner with at least an 800 W engine effect and a flow rate of minimum 45 L per minute. The vacuum cleaners were equipped with a 40 micron nylon filter (DUSTREAM® Filters, Indoor Biotechnologies™, USA) attached to a Dustream® collector (Indoor Biotechnologies™, USA). Dust collections were carried out in four separate areas with approximately 0.06 m² for 30 seconds to achieve a total area of 0.25 m² and a harvest time of 2 minutes. After collection, the filter was visually inspected to confirm that at least one-fourth of the maximum capacity was occupied. The filter was then transferred to a zipped plastic bag and the samples were identified and stored at 4°C until processed.

D. pteronyssinus and *D. farinae* extracts were kindly provided by Diater Laboratories (Madrid, Spain), and consisted of adult mite bodies from each mite species.

DNA extraction

Genomic DNA extraction from mite extracts (separated and combined) and from house dust samples was performed using DNeasy® PowerSoil® Kit (QIAGEN, Hilden, Germany). Frozen mite extracts from each species were suspended in ultra-pure water (1 mg mL^{-1}) and used for DNA extraction. For dust DNA extraction, approximately 100 mg of each dust sample was used.

The extraction process was performed according to the manufacturer's instructions. In the final step, the purified genomic DNA was eluted with 50 μL of Qiagen elution Buffer and used directly in PCR.

Purity and concentration of DNA were determined using Thermo Scientific NanoDrop™ 1000 spectrophotometer (Wilmington, DE, USA).

Primer design

rDNA gene sequences of the full-length internal transcribed spacers (ITS1 and ITS2) of the two most prevalent HDM species isolated indoors, were retrieved from the GenBank™ database (www.ncbi.nlm.nih.gov/genbank) and aligned using EMBL-EBI Clustal Omega Multiple Sequence Alignment tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to choose the most appropriate areas for primer design.

Primers were designed with NCBI/Primer-BLAST using the following parameters: the minimum and maximum melting temperatures (T_m) permitted for primers were 55°C and 60°C , respectively. The maximum of CG content permitted by each primer was 60%. The optimal length for each primer was 20 bases (18 bases minimum and 25 bases maximum) and the amplified region length permitted ranged from 100 to 1000 bps. Among the primer candidates that were retrieved from Primer-Blast and further analysed with Oligo Analyser software 1.0.2, a selection was made based on similar melting temperatures and absence of cross reactivity between primers. The selected primers were synthesized by Stabvida (Caparica, Portugal).

To verify the specificity of the newly designed primers sets for the two target species, 80 mite DNA sequences of the full-length internal transcribed spacers present in GenBank™ (www.ncbi.nlm.nih.gov/genbank), representing both HDM species and storage mites were used, in order to perform 'in silico' primer hybridization analysis. Specifically *D. pteronyssinus*, *D. farinae*, *E. maynei*, *D. microceras*, *Blomia tropicalis* (HDM) and *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, *Acarus siro* (storage mite species) were included.

Each primer set was also checked for specificity using BLAST and global alignment algorithm [accessed at 12 March 2019] to screen new designed primers against Nucleotide collection (nt) database (Ye et al. 2012).

Validation of species-specific primers

All primers, corresponding to species-specific mite rDNA genomic sequences of the full-length internal ITS1 and ITS2, were first validated in a uniplex PCR reaction, using genomic DNA extracted from pure mite extracts of corresponding mite species, to attest primer specificity. In order to check for non-specific cross reactivity, each mite species was also amplified by the cross-matched primer pair.

One microliter of HDM DNA containing $3 \text{ ng } \mu\text{L}^{-1}$ was used in uniplex PCR amplifications in a $12.5 \mu\text{L}$ reaction mixture containing $6.25 \mu\text{L}$ of NZYTaQ 2x Green Master Mix (Nzytech, Lisboa, Portugal), $0.5 \mu\text{M}$ of each primer, and $5 \mu\text{L}$ nuclease-free water. The mixture was amplified in the T100™ Thermal Cycler (Biorad, Hercules, California, USA) with following optimum conditions:

initial denaturation at 95°C for 3 min followed by 28 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, elongation at 72°C for 10s, and a final elongation step at 72°C for 5 min.

Amplification products were loaded on a 1% agarose gel containing GreenSafe Premium (Nzytech, Lisboa, Portugal) at 0.02 $\mu\text{L mL}^{-1}$ and horizontal electrophoresis was performed in 1X TAE buffer at 120 V for 40 minutes. DNA fragments were then visualized on a UV transilluminator (UVITEC Cambridge, England).

Multiplex PCR protocol

Multiplex amplification of the two selected HDM species was performed using the newly designed species-specific primers. The reaction was first optimized for uniplex PCR using the above described cycling program and, afterwards, was adjusted for multiplex PCR. For the multiplex reaction, 1 μL of DNA template, 6.25 μL of NZYTaQ 2x Green Master Mix (Nzytech, Lisboa, Portugal), 0.125 μL of each forward primer (0.5 μM), 0.250 μL of common reverse primer (1 μM) and 4.75 μL of nuclease-free water, were used. The mixture was amplified using the same conditions described for uniplex PCR.

Efficacy of uniplex and multiplex PCR protocols

Both PCR protocols were evaluated regarding the method's limit of detection (LOD) using gDNA extracted from individual and combined mite extracts, in order to characterize and compare the efficacy of the two methods. The LOD was the lowest amount of DNA that was amplified by the PCR reaction and visualized in the agarose gels.

From a starting quantity of 3000 $\text{pg } \mu\text{L}^{-1}$, serial dilutions of extracted gDNA were performed (1:10 v/v) in ultrapure water. Both multiplex and uniplex PCR protocols were then tested with serial diluted gDNA in order to determine the LOD.

The sensitivity of each HDM species in complex samples was also determined for both protocols, using a pool of total DNA extracted from house dust samples, and expressed as total DNA concentration ($\text{pg } \mu\text{L}^{-1}$).

Detection of HDM in house dust samples

In order to determine the method's ability to identify both HDM species in a complex matrix, as genomic DNA extracted from house dust, both PCR protocols were applied to five house dust samples. Products from DNA extraction were used directly in the PCR reaction, without further dilution or purification steps.

For each PCR reaction, a negative control containing sterile double distilled water, and a positive control using 1 μL of DNA extracted from pure mite cultures were included.

Sequencing of PCR products

For sequencing of PCR products, PCR reactions with both primer sets were performed in 50 μL reaction mixtures, using total DNA extracted from house dust, as template. To check the success of the reaction, 5 μL of amplification products were loaded on a 1% agarose gel. The remaining 45 μL of each PCR product were purified using the NZYGelpure (Nzytech, Lisboa, Portugal), according to the manufacturer's protocol.

Premium Sanger DNA sequencing service from Stabvida (Stabvida, Caparica, Portugal) was used for direct sequencing of purified PCR products on both strands, using the same forward and reverse primers as for the PCR.

The resulting forward and reverse sequences were analysed using SnapGene software (GSL Biotech LLC), checked against the chromatograms and assembled into consensus sequences. Consensus gene sequences of internal transcribed spacer 1 (partial sequence), 5.8S ribosomal DNA gene (complete sequence), and internal transcribed spacer 2 (partial sequence) from *D. pteronyssinus* and *D. farinae* were submitted to GenBank™, and were assigned accession numbers **MN906967** and **MN906968**, respectively. The sequences were compared with previously published sequences in the Nucleotide collection (nr/nt) database in GenBank™ using the program Nucleotide BLAST of the National Center for Biotechnology Information (NCBI).

Results

Primer selection

The alignment of HDM sequences enabled us to choose areas for primer design using the ITS1, 5.8S and ITS2 rDNA regions, flanked by partial sequences corresponding to the 18S and 28S subunits.

The selected primer sequences and their analytical characteristics are present in Table 1. Reverse primer was the same for both species, corresponding to the flank 28S subunit. Primer length ranged between 20 and 21 bps and the highest and lowest CG contents were 60% for DP_Fwd and 50% for DF_Fwd, respectively.

The expected product sizes were 600 bps for *D. pteronyssinus* and 800 bps for *D. farinae*, which provided a convenient distance to product separation in multiplex PCR.

Primer specificity

The analysis performed with Primer-BLAST indicated that each primer set was species-specific, retrieving from the search just target template sequences of the desired species.

Regarding the ‘in silico’ primer hybridization analysis against the sequences deposited in GenBank™ for our two target HDM, three additional HDM species, and three storage mites (Supplementary Material – Table S1), the forward primer for *D. pteronyssinus* aligned with seven out of the ten *D. pteronyssinus* sequences (7/10). No nonspecific hybridization occurred against the second target species (*D. farinae*) sequences, neither with the additional six mite species tested, thus predicting a high degree of specificity. The same profile occurred with *D. farinae* forward primer, in this case with the specific hybridization occurring in all the *D. farinae* deposited sequences (10/10) and no unspecific hybridization with the additional six mite species tested. Regarding the common reverse primer, corresponding to the conserved flank 28S subunit, hybridization occurred in the majority of the deposited sequences, as expected. For one species, no hybridization of the common reverse primer occurred, as we considered only 100% homology as a positive result.

Table 1. Species-specific *Dermatophagoides* spp. forward (Fwd) and reverse (Rev) primer sets designed in this study and their analytical characteristics.

Species name	Target	Expected Amplicon Length (bp)	Primer	Sequence (5'-3')	Length	GC (%)	Tm (°C)
<i>D. pteronyssinus</i>	ITS1, 5.8S and ITS2 rDNA regions flanked by 18S and 28S	600	DP_Fwd	CCTCGGGACAGATGTGGATG	20	60	59.89
			DER_Rv	CTCGCTTGATCTGAGGTCGAA	21	52	59.87
<i>D. farinae</i>		800	DF_Fwd	TCGACTGACTTGTTGCCA	20	50	59.90
			DER_Rv	CTCGCTTGATCTGAGGTCGAA	21	52	59.87

Tm – Melting temperature.

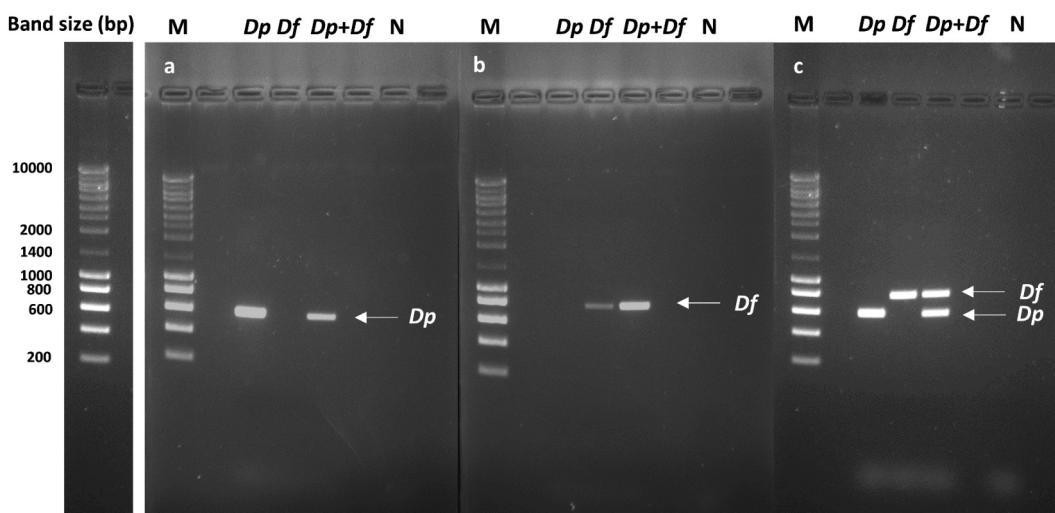


Figure 1. Agarose gel electrophoresis of amplified products from uniplex PCR reactions with *D. pteronyssinus* primer pair (a), *D. farinae* primer pair (b) and multiplex PCR with both primer sets (c). Reactions were performed using gDNA extracted from pure mite extracts, as template. M – 10,000 bp molecular size marker (Ladder III from Nzytech, Lisboa, Portugal); *Dp* – amplicon of *D. pteronyssinus*; *Df* – amplicon of *D. farinae*; *Dp+Df* – amplicons of both *Dermatophagoides* spp.; N – negative PCR control without DNA template (ultra-pure water).

Regarding experimental validation for primer specificity, agarose gel electrophoresis of PCR product from pure mite extracts from each species, both alone and combined, are present in [Figure 1](#). The expected amplicon size was observed for each HDM species.

In all the reactions, specific amplification of each mite species was achieved without observation of non-specific products regarding the second non-target species.

Limit of detection

DNA extraction from pure mite extracts using DNeasy® PowerSoil® Kit from QIAGEN (QIAGEN, Hilden, Germany) yielded 3 ng of gDNA per μL , that were serially diluted and used in LOD determinations. A uniplex PCR reaction for each species was primarily performed with a dilutions ranging from $3000 \text{ pg } \mu\text{L}^{-1}$ to $0.3 \text{ pg } \mu\text{L}^{-1}$. This protocol was able to identify both species with a detection limit of $30 \text{ pg } \mu\text{L}^{-1}$ (Supplementary Material – Figure S1a and S1b).

When applying the multiplex PCR protocol, the obtained results were not altered, maintaining the LOD of $30 \text{ pg } \mu\text{L}^{-1}$, when detecting the presence of only one or both species at the same time ([Figure 2](#)).

Applying the PCR protocol to dust samples

The DNA extraction process from house dust yielded 13–2.7 ng of gDNA per μL , depending on the sample. Samples 1 and 2 yielded the highest gDNA concentrations, with 13 ng and 5.5 ng per μL , respectively. The lowest amount of DNA extracted was obtained from Sample 3 ($2.7 \text{ ng } \mu\text{L}^{-1}$).

In all five samples, both protocols were able to identify the presence of HDM species. In the uniplex PCR ([Figure 3\(a,b\)](#)), the obtained bands corresponded to the expected amplicon size, with no unspecific amplification for the second target species.

Applying the multiplex PCR ([Figure 3\(c\)](#)), all samples were in accordance with the previously performed uniplex, with both amplicons with the expected sizes and good band separation. Higher

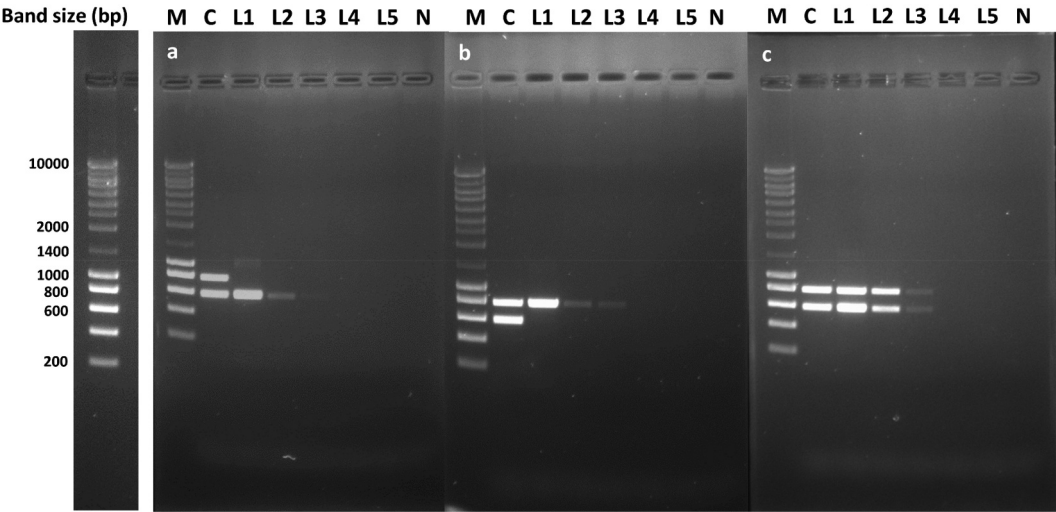


Figure 2. Agarose gel electrophoresis of amplified products from multiplex PCR using serial dilutions of gDNA extracted from pure mite extracts, as template. M – 10,000 bp molecular size marker (Ladder III from Nzytech, Lisboa, Portugal); C – Positive control; Lanes 1–5 represent ten-fold dilution series of gDNA from *D. pteronyssinus*(a), *D. farinae* (b), and from the two mite species (c) ranging from 3000 to 0.3 pg μL^{-1} . N - negative PCR control (ultra-pure water).

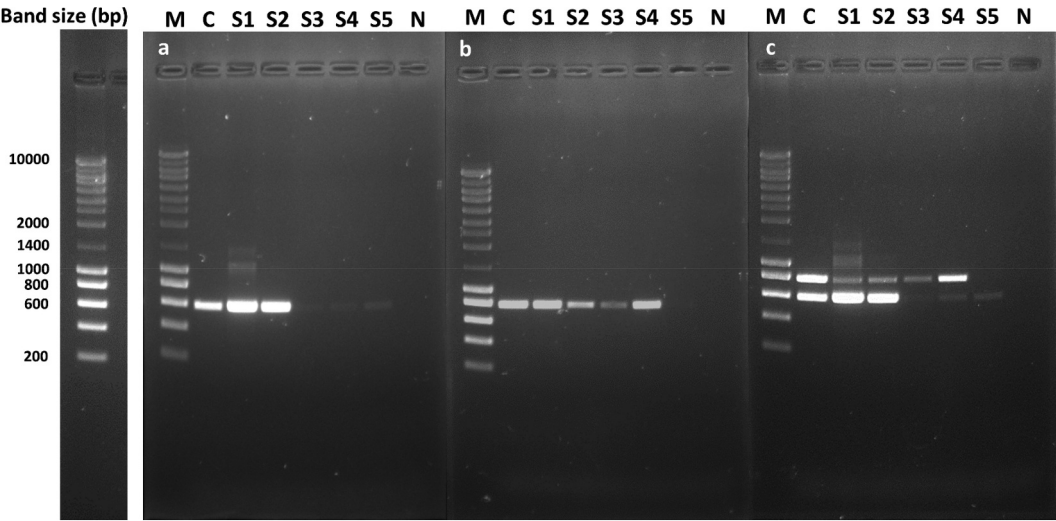


Figure 3. Agarose gel electrophoresis of amplified products from uniplex PCR reactions for *D. pteronyssinus* (a), *D. farinae* (b) and multiplex PCR (c) of DNA extracted from five house dust samples (S1-S5). C - Represents positive control using gDNA extracted from mite extract containing *D. pteronyssinus* and *D. farinae*. N - represents negative control (no DNA template). M - 10,000 bp molecular size marker (Ladder III from Nzytech, Lisboa, Portugal). The faint band at ≈ 1000 bps in sample S1, corresponds to a second product of DP_Fwd/DER_Rv primer pair hybridization, specific for *D. pteronyssinus* species.

band intensity was present in samples with higher DNA concentration (Sample 1 and Sample 2), as expected.

A loss of band intensity and definition regarding *D. farinae* was present in multiplex PCR. In addition, some loss of band intensity was also present in samples with faint bands in the uniplex protocol (Sample 3). Still, the relative intensity of one band towards the other was also in accordance with both methods.

The detection of HDM species was also in accordance with both protocols for all samples, where *D. pteronyssinus* was present in all the tested samples, while *D. farinae* was detected in four-fifths of the tested samples.

Sensitivity of PCR reaction in complex samples

Both species of HDM were identified with high sensitivity. Using the uniplex PCR protocol, sensitivity results were $30 \text{ pg } \mu\text{L}^{-1}$ and $300 \text{ pg } \mu\text{L}^{-1}$ of total gDNA, to detect *D. pteronyssinus* and *D. farinae*, respectively (Supplementary Material – Figure S1c and S1d).

When applying multiplex PCR protocol the results were similar, though a very faint band (almost imperceptible) was present for *D. pteronyssinus* at $30 \text{ pg } \mu\text{L}^{-1}$, decreasing sensitivity for this species for $300 \text{ pg } \mu\text{L}^{-1}$ (Figure 4).

Sequencing analysis

The results of the sequencing analyses for *D. pteronyssinus* (MN906967) and *D. farinae* (MN906968) were compared with previously published sequences in the Nucleotide collection (nr/nt) database in GenBankTM using the program Nucleotide BLAST, in order to conclude about the method specificity. The results from sequence comparisons are present in Table 2.

For sequenced PCR products, BLAST search retrieved a high correspondence with the desired species. For *D. pteronyssinus*, from the total of 80 aligned sequences, 70 corresponded to the target species. The obtained species-specific alignments had a wide query cover value ranging from 100%-

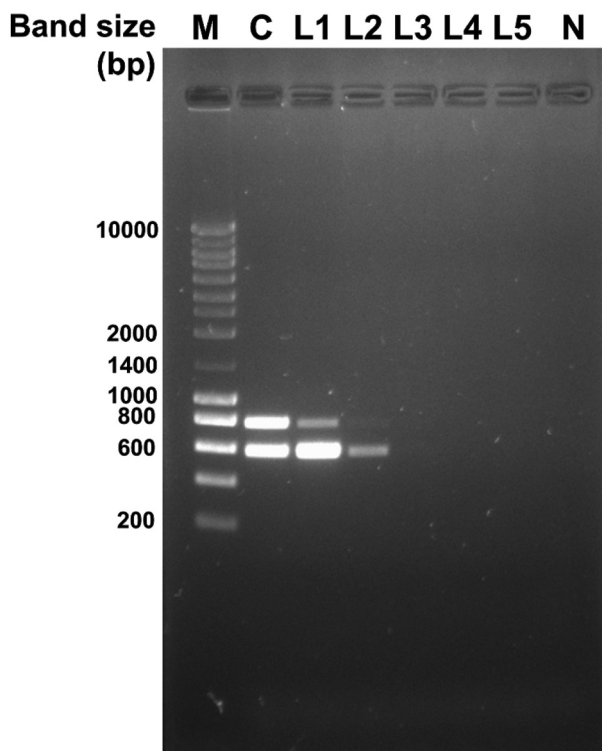


Figure 4. Agarose gel electrophoresis of amplified products from multiplex PCR assay using serial dilutions of a mixture of gDNA extracted from house dust. M – 10,000 bp molecular size marker (Ladder III from Nzytech, Lisboa, Portugal); C – Represents positive control using gDNA extracted from mite extract containing both HDM species; Lanes 1–5 represent ten-fold dilution series of gDNA from house dust ranging from 3000 to $0.3 \text{ pg } \mu\text{L}^{-1}$. N - negative PCR control (ultra-pure water).

Table 2. Summarized results from BLAST analysis of the sequenced PCR products from *D. pteronyssinus* and *D. farinae* species.

HDM species	Specific alignments ‡	Query Cover (%)	Identity (%)	Accession Number
<i>D. pteronyssinus</i>	70/80	100–92 52–27*	96.23–93.39 98.68–92.42*	MN906967
<i>D. farinae</i>	92/102	97–96 38*	95.83–92.97 96.45–96.13*	MN906968

‡Significant alignments with sequences corresponding to the target species previously published in Nucleotide collection (nt) database. *Alignment to second part of the rDNA gene cluster (corresponding to the 5.8S+ITS2 gene region) sequences.

27%, with lower values corresponded with alignments only to second part of the rDNA gene cluster. The highest value of percentage of identity was 98.68%.

For *D. farinae*, from the total of 102 aligned sequences, 92 corresponded to the target species. As for *D. pteronyssinus*, a wide range of query cover values were retrieved, with lower values corresponding to sequences covering only the second part of the rDNA gene cluster. The highest value of percentage of identity was 96.45%.

Regarding the remaining alignments obtained, for *D. pteronyssinus* and *D. farinae*, 10 alignment sequences referred to *E. maynei* and *D. microceras*, respectively, however with lower query covers and percentages of identity.

Discussion

With this study, we proposed a new multiplex PCR technique to detect the two most prevalent species of HDM, specifically *D. pteronyssinus* and *D. farinae*, in dust samples. With it, we intended to broad the range of available methods to address the presence HDM species in different environments, while simultaneously overcoming some of the limitations presented by the available molecular-based methods and some classical techniques, presenting an alternative or complement to their use.

In our study, we start by the design of two new specific primer sets, sharing a common reverse primer, using the full-length internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal gene cluster sequences of *D. farinae* and *D. pteronyssinus* mite species. This cluster consists of three genes (18S rDNA, 5.8S rDNA and 28S rDNA) separated by two non-coding regions (ITS1 and ITS2) and has already been used for the identification of different eukaryotic organisms with known success (Nilsson et al. 2012). In fact, Cruickshank (2002) elegantly reviewed the strategy of designing primers within the slowly evolving genes that flank the rapidly evolving spacers (ITS1 and ITS2) as good targets for distinguishing between closely related species. In addition, it was reported that more than 100 copies of this cluster may exist in a genome thus favouring its amplification of genomic DNA by PCR, and highlighting its potential as a possible target for primer design (Wen et al. 1974).

From our selected primer candidates, ‘*in silico*’ specificity analysis enables us to select primer sequences that hybridize with the highest number of sequences of each species. Six additional HDM or storage mite species, whose rDNA gene cluster sequences were deposited in GenBankTM, aside from the target species, were included in the ‘*in silico*’ analysis, thus confirming primer specificity. This analysis, when choosing from the primer candidates, in addition to allow us to predict the method specificity, enables us to assure that we overcome some of the variation that is typically present with rapid evolving sequences, as ITS1 and ITS2.

Beroiz et al. (2014) already used these target sequences to identify different species of HDM with success. However, the differentiation between species was not direct with PCR, and was only achieved with the addition of RFLP profile analysis. Additionally, a semi-nested re-amplification step had to be introduced before the RFLP, in order to apply the method to environmental samples (Beroiz et al. 2014). The same additional technique was also used by Wong et al. for the identification of mites at the species level (Wong et al. 2011).

The addition of further processing steps to the PCR, like nested PCR or RFLP profile analysis, or even additional DNA purification steps, although sometimes necessary, can lead to a higher demand of starter template quantities and to an increase of processing time and reagent consumption. Additionally, due to the higher manipulation required, ultimately, the risk of sample contamination is also elevated. Therefore, one-step protocols, as qPCR and conventional multiplex PCR assays, in which data from different species can be obtained from one assay with small amount of DNA template, are advantageous.

Also the use of commercial kits for DNA isolation, over classic extraction methods like phenol/chloroform extraction, also presents several advantages when applied to environmental samples, since DNA products of this origin usually contain organic contaminants or polysaccharides, proteins, and tannins that may inhibit the PCR amplification (Moreira 1998; González-Santos and Montealegre 2006).

As shown in the results section, our multiplex PCR was able to detect the presence of mite species in five house dust samples, with both mite species being simultaneously detected in four out of five house dust samples. The fact that *D. pteronyssinus* and *D. farinae* are described to typically occur together (Arlian et al. 2002) may lead us to hypothesise that the lack of *D. farinae* in one sample could be related to the method's sensitivity, as more sensitive methods have been described in the literature (Reboux et al. 2019). Still, different authors also found the presence of only one of our two target species in some environmental samples, thus aligning our results with previously published literature (Thet-Em et al. 2012; Beroiz et al. 2014; Schildt et al. 2017). Also, when applying the uniplex PCR protocol, the results were correspondent in all five samples, therefore supporting the results obtained with our multiplex protocol.

In order to determine the lowest amount of DNA extracted from house dust in which the presence of HDM could be detected, sensitivity analysis was performed. From uniplex PCR, 300 pg and 30 pg of total house dust DNA was sufficient to amplify *D. farinae* and *D. pteronyssinus*, respectively.

Since the starter DNA concentration of each HDM species was unknown, the higher sensitivity for *D. pteronyssinus* in the dust samples can be related to a possible higher presence of this species in the starter sample. When the multiplex PCR was applied, a very faint band was present for *D. pteronyssinus* at 30 ng μL^{-1} , however it was almost imperceptible, decreasing sensitivity value for this species by 10-fold. This loss of sensitivity between uniplex and multiplex PCR was already described in mite related and non-related protocols (Park et al. 2011; Thet-Em et al. 2012; Bilgiç et al. 2013).

Regarding the new multiplex PCR LOD, we used gDNA extracted from pure mite extracts, consisting of adult mite bodies, to overcome the limitation of the unknown relative quantity of each species in template DNA extracted from house dust. Therefore, starting with gDNA extracted from each species, both alone and combined, and serially diluting this DNA, we were able to effectively characterize both protocols.

Finally, in order to truly evaluate the specificity of the new protocol in highly complex samples such as house dust, the amplification products of PCR reactions for each species were sequenced using Sanger dideoxy sequencing method.

As presented in the results section, the large majority of the aligned sequences obtained after a BLAST database search corresponded with the desired species. No hits were obtained matching the second target species, thus corroborating the predicted specificity of the method. Despite the high diversity of genetic material that can be present in house dust (human epidermal scales, animal dander, fungi, bacteria, pollen, among others), the method was proven robust, as no hits besides house dust mites were retrieved from Blast search.

A similar one-step multiplex PCR strategy to search HDM in house dust samples, like the one here described, was already been developed and applied (Thet-Em et al. 2012; Schildt et al. 2017). However, no primer sequence was published, and no sequencing of the final PCR products was performed to confirm the specificity of the method. Also, the method here proposed, presented an overall higher sensitivity compared with the published protocol since it was able to detect both

species with 30 pg of DNA extracted from pure mite cultures against to 25–100 pg depending on the mite species (Thet-Em et al. 2012).

Quantitative PCRs (qPCRs) have also been developed, that provide results with great speed combined with higher sensitivity when compared with the available conventional PCR techniques (Roussel et al. 2013; Reboux et al. 2019). However, the high base cost of the qPCR supplies, combined with the need for different probes in addition to primer sets, ultimately increases the resource need for the overall process, not being accessible to some laboratories.

Regarding other methods used to identify HDM, three methods have been classically applied, specifically mite identification by microscopy, allergen quantification by ELISA and guanine measurements (Sander et al. 2012; Roussel et al. 2013; Cui et al. 2018; Casley et al. 2018). Mite quantification by microscopic analysis, also used for specifying the founding species using morphological characterization, has been conventionally used as gold-standard in mite identification and is the only method, apart for PCR, that truly detects the presence of the actual mite. However, microscopic mite identification has limitations not allowing the differentiation of some sibling species (Colloff 2009). Besides, it's a very time-consuming task and requires the presence of expert taxonomic personnel (Colloff 2009; Ernieenor et al. 2018).

When aiming to investigate specific allergens from mites in the environment, ELISA is the most commonly used method (Dunn et al. 2008; Reboux et al. 2019). However, ELISA kits search for only one specific antigen per kit, making the search for multiple allergens, over expensive, when trying to discriminate between allergens from different mite species. Also, cross-reactivity of allergens from close related species is described to occur. Still, since ELISA only detects the presence of mite allergens, it is not appropriate for studying species distribution, being mite microscopic identification often used for this purpose (Boquete et al. 2006; Sun et al. 2013; Solarz and Pająk 2019a, 2019b). There are also reports in the literature addressing the presence of positive samples using molecular-based techniques that were negative by ELISA, suggesting that these techniques could be more sensitive in detecting the presence of HDM in complex samples (Schildt et al. 2017). Therefore, molecular-based techniques may present some advantages to some classical methods in order to achieve simultaneously mite identification and classification.

The method here proposed is an accessible multiplex protocol designed to detect the two most prevalent species of HDM in total DNA extracted from house dust. We demonstrated that this protocol required only small amount of DNA template, reagents and a short reaction time to simultaneously detect both mite species with high specificity and sensitivity, using relatively accessible equipment as standard PCR machines and gel electrophoresis equipment.

In the future, we aim to validate this method for a broader application in other complex matrices besides domestic dust, in order to further investigate mite colonization in other environments and even human samples, thus deepening the knowledge of the poorly characterized acariasis condition.

Acknowledgments

The authors thank Diater Laboratories for kindly providing mite extracts used in this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Foundation for Science and Technology (FCT), through funds from the State Budget, and by the European Regional Development Fund (ERDF), under the Portugal 2020 Program, through the Regional Operational Program of the Center (Centro2020), through the Project with the Grant [UIDB/00709/2020]. Financial

support was also provided by the Foundation for Science and Technology (FCT) through a PhD fellowship to A.S.O under Grant [SFRH//BD/136192/2018].

References

- Arlian LG, Morgan MS, Neal JS. 2002. Dust mite allergens: ecology and distribution. *Curr Allergy Asthma Rep.* 2 (5):401–411. doi:10.1007/s11882-002-0074-2.
- Atambay M, Aycan OM, Daldal N. 2006. [House dust mite fauna in Malatya]. *Türkiye Parazitolo Derg.* 30(3):205–208.
- Beroiz B, Couso-Ferrer F, Ortego F, Chamorro MJ, Arteaga C, Lombardero M, Castañera P, Hernández-Crespo P. 2014. Mite species identification in the production of allergenic extracts for clinical use and in environmental samples by ribosomal DNA amplification. *Med Vet Entomol.* 28(3):287–296. doi:10.1111/mve.12052.
- Bilgiç HB, Karagenç T, Simuunza M, Shiels B, Tait A, Eren H, Weir W. 2013. Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. *Exp Parasitol.* 133(2):222–229. doi:10.1016/j.exppara.2012.11.005.
- Boquete M, Iraola V, Fernández-Caldas E, Arenas Villaroel L, Carballada FJ, González De La Cuesta C, López-Rico MR, Núñez Orjales R, Parra A, Soto-Mera MT, et al. 2006. House dust mite species and allergen levels in Galicia, Spain: A cross-sectional, multicenter, comparative study. *J Investig Allergol Clin Immunol.* 16(3):169–176.
- Calderón MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez De Rojas D, Virchow JC, Demoly P. 2015. Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol.* 136 (1):38–48. doi:10.1016/j.jaci.2014.10.012.
- Casley LS, Godec T, Logan JG, Pearce JC, Smith HMP, Stewart SA, Dewhirst SY. 2018. How clean is your house? A study of house dust mites, allergens and other contents of dust samples collected from households. *Int J Environ Health Res* [Internet]. [accessed 2020 Oct 15] 28(4):341–357. <https://www.tandfonline.com/doi/full/10.1080/09603123.2018.1457141>
- Colloff MJ. 2009. Dust mites. Dordrecht: Springer.
- Cruickshank RH. 2002. Molecular markers for the phylogenetics of mites and ticks. *Syst Appl Acarol.* 7(3):3.
- Cui Y. 2014. When mites attack: domestic mites are not just allergens. *Parasit Vectors.* 7(1):411. doi:10.1186/1756-3305-7-411.
- Cui Y, Wang Q, Jia H. 2018. Consideration of methods for identifying mite allergens. *Clin Transl Allergy.* 8(1):14. doi:10.1186/s13601-018-0200-4.
- Dunn JA, Thind BB, Danks C, Chambers J. 2008. Rapid method for the detection of storage mites in cereals: feasibility of an ELISA based approach. *Bull Entomol Res.* 98(2):207–213. doi:10.1017/S0007485308005634.
- Ernieenor FCL, Ernna G, Jafson AS, Mariana A. 2018. PCR identification and phylogenetic analysis of the medically important dust mite *Suidasia medanensis* (Acari: suidasiidae) in Malaysia. *Exp Appl Acarol.* 76(1):99–107. doi:10.1007/s10493-018-0285-4.
- González-Santos M, Montealegre F. 2006. Evaluation of extraction methods for the isolation of dust mites, bacterial, and fungal PCR-quality DNA from indoor environmental dust samples: A new scope for indoors research. *J Environ Heal Res.* 5(2):105–110.
- Gotzsche PC, Johansen HK. 2008. House dust mite control measures for asthma: systematic review. *Allergy Eur J Allergy Clin Immunol.* 63(6):646–659. doi:10.1111/j.1398-9995.2008.01690.x.
- Htut T, Houlbrook K, Lumley E, Maunder JW, Vickers L. 1996. A novel physical method to control mites and their allergens in bedding. *Int J Environ Health Res* [Internet]. [accessed 2020 Oct 16] 6(3):233–243. <http://www.tandfonline.com/doi/abs/10.1080/09603129609356894>
- Hubert J, Nesvorna M, Kopecky J, Erban T, Klimov P. 2019. Population and culture age influence the microbiome profiles of house dust mites. *Microb Ecol.* 77(4):1048–1066. doi:10.1007/s00248-018-1294-x.
- Insung A, Mongkut K, Lad T, Ruangsomboon S, Mongkut K, Lad T. 2015. Discrimination between house dust mite *Dermatophagoides pteronyssinus* and *D. farinae* by polymerase chain reaction-restriction fragment length polymorphism. *Chulalongkorn Med J.* 59(3):241–251.
- Li C, Cui Y, Wang J, Yang Q, Tian Y. 2003. Diarrhea and acaroid mites : a clinical study. *World J Gastroenterol.* 9 (7):1621–1624. doi:10.3748/wjg.v9.i7.1621.
- Li L, Qian J, Zhou Y, Cui Y. 2018. Domestic mite-induced allergy: causes, diagnosis, and future prospects. *Int J Immunopathol Pharmacol.* 32:205873841880409. doi:10.1177/2058738418804095.
- Miller JD. 2018. The role of dust mites in allergy. *Clin Rev Allergy Immunol.* 57:1–18.
- Moreira D. 1998. Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Res.* 26(13):3309–3310. doi:10.1093/nar/26.13.3309.
- Nadchatram M. 2005. House dust mites, our intimate associates. *Trop Biomed.* 22(1):23–37.
- Nilsson RH, Tedersoo L, Abarenkov K, Ryberg M, Kristiansson E, Hartmann M, Schoch CL, Nylander JAA, Bergsten J, Porter TM, et al. 2012. Five simple guidelines for establishing basic authenticity and reliability of newly generated fungal ITS sequences. *Mycoskeys.* 4:37–63. doi:10.3897/mycokeys.4.3606.

- Park SH, Hanning I, Jarquin R, Moore P, Donoghue DJ, Donoghue AM, Ricke SC. 2011. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157: H7, and *Salmonella* serotypes in water samples. *FEMS Microbiol Lett*. 316(1):7–15. doi:[10.1111/j.1574-6968.2010.02188.x](https://doi.org/10.1111/j.1574-6968.2010.02188.x).
- Reboux G, Valot B, Rocchi S, Scherer E, Roussel S, Millon L. 2019. Storage mite concentrations are underestimated compared to house dust mite concentrations. *Exp Appl Acarol*. 77(4):511–525. doi:[10.1007/s10493-019-00376-2](https://doi.org/10.1007/s10493-019-00376-2).
- Roussel S, Reboux G, Naegele A, Martinez J, Vacheyrou M, Scherer E, Millon L. 2013. Detecting and quantifying mites in domestic dust: A novel application for real-time PCR. *Environ Int*. 55:20–24. doi:[10.1016/j.envint.2013.02.002](https://doi.org/10.1016/j.envint.2013.02.002).
- Sander I, Zahradnik E, Kraus G, Mayer S, Neumann HD, Fleischer C, Brüning T, Raulf-Heimsoth M. 2012. Domestic mite antigens in floor and airborne dust at workplaces in comparison to living areas: a new immunoassay to assess personal airborne allergen exposure. *PLoS One*. 7(12):e52981. doi:[10.1371/journal.pone.0052981](https://doi.org/10.1371/journal.pone.0052981).
- Schildt KJM, Seppänen RTK, Hielm-Björkman AK, Saijonmaa-Koulumies LE, Belova S. 2017. Prevalence of house dust mites in the homes of atopic dogs in Finland. *Vet Dermatol*. 28(2):225–254. doi:[10.1111/vde.12421](https://doi.org/10.1111/vde.12421).
- Solarz K, Pająk C. 2019a. Risk of exposure of a selected rural population in South Poland to allergenic mites. Part I: indoor acarofauna of one-family houses. *Exp Appl Acarol* [Internet]. [accessed 2021 Jan 22] 77(3):375–386. <https://pubmed.ncbi.nlm.nih.gov/30820791/>
- Solarz K, Pająk C. 2019b. Risk of exposure of a selected rural population in South Poland to allergenic mites. Part II: acarofauna of farm buildings. *Exp Appl Acarol* [Internet]. [accessed 2021 Jan 22] 77(3):387–399. <https://doi.org/10.1007/s10493-019-00355-7>.
- Sun JL, Shen L, Chen J, Yu JM, Yin J. 2013. Species diversity of house dust mites in Beijing, China. *J Med Entomol* [Internet]. [accessed 2021 Jan 22] 50(1):31–36. <https://academic.oup.com/jme/article-lookup/doi/10.1603/ME12036>.
- Thet-Em T, Tungtrongchitr A, Tiewcharoen S, Malainual N. 2012. Multiplex PCR for identifying common dust mites species (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Blomia tropicalis*). *Asian Pacific J Allergy Immunol*. 30(3):224–230.
- Wen WN, León PE, Hague DR. 1974. Multiple gene sites for 5S And 18 + 28S RNA on chromosomes of *Glyptotendipes Barbipes* (staeger). *J Cell Biol*. 62(1):131–144. doi:[10.1083/jcb.62.1.132](https://doi.org/10.1083/jcb.62.1.132).
- Wong SF, Chong AL, Mak JW, Tan J, Ling SJ, Ho TM. 2011. Molecular identification of house dust mites and storage mites. *Exp Appl Acarol*. 55(2):123–133. doi:[10.1007/s10493-011-9460-6](https://doi.org/10.1007/s10493-011-9460-6).
- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13(1):134.
- Zhang R, Huang Y, Li C, Cui Y. 2004. Diagnosis of intestinal ascariasis with avidin-biotin system enzyme-linked immunosorbent assay. *World J Gastroenterol*. 10(9):1369–1371. doi:[10.3748/wjg.v10.i9.1369](https://doi.org/10.3748/wjg.v10.i9.1369).

Supplementary Material

Development of a New Multiplex PCR to Detect Prevalent Species of Domestic Mites in House Dust

Table of contents:

Table S1. Results of in silico primer hybridization testing (100% homology) of new primer sets for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* with full-length internal transcribed spacers (ITS1 and ITS2) sequences deposited in GenBank™, for our target species and **six** additional HDM species.

Figure S1. Agarose gel electrophoresis of amplified products from Uniplex PCR for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* using serial dilutions gDNA extracted from pure mite extracts (a and b) and from house dust (c and d).

Table S1. Results of *in silico* primer hybridization testing (100% homology) of new primer sets for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* with full-length internal transcribed spacers (ITS1 and ITS2) sequences deposited in GenBank™, for our target species and six additional HDM species.

Species	GenBank accession number™	DP_Fwd hybridization	DF_Fwd hybridization	Mite_Rv hybridization
<i>Dermatophagoides pteronyssinus</i>	<u>KC215334</u>	-	-	X
	<u>KC215333</u>	X	-	X
	<u>KC215332</u>	X	-	X
	<u>KC215331</u>	X	-	X
	<u>KC215330</u>	X	-	X
	<u>KC215329</u>	-	-	X
	<u>KC215328</u>	X	-	X
	<u>KC215327</u>	-	-	X
	<u>KC215326</u>	X	-	X
	<u>KC215325</u>	X	-	X
<i>Dermatophagoides farinae</i>	<u>KC215344</u>	-	X	X
	<u>KC215343</u>	-	X	X
	<u>KC215342</u>	-	X	X
	<u>KC215341</u>	-	X	X
	<u>KC215340</u>	-	X	-
	<u>KC215339</u>	-	X	X
	<u>KC215338</u>	-	X	X
	<u>KC215337</u>	-	X	X
	<u>KC215336</u>	-	X	X
	<u>KC215335</u>	-	X	X
<i>Euroglyphus maynei</i>	<u>KC215354</u>	-	-	X
	<u>KC215353</u>	-	-	X
	<u>KC215352</u>	-	-	X
	<u>KC215351</u>	-	-	X
	<u>KC215350</u>	-	-	X
	<u>KC215349</u>	-	-	X
	<u>KC215348</u>	-	-	X
	<u>KC215347</u>	-	-	X
	<u>KC215346</u>	-	-	X
	<u>KC215345</u>	-	-	X
<i>Dermatophagoides microceras</i>	<u>KC215324</u>	-	-	X
	<u>KC215323</u>	-	-	X
	<u>KC215322</u>	-	-	X
	<u>KC215321</u>	-	-	X
	<u>KC215320</u>	-	-	X
	<u>KC215319</u>	-	-	X
	<u>KC215318</u>	-	-	-
	<u>KC215317</u>	-	-	X
	<u>KC215316</u>	-	-	X
	<u>KC215315</u>	-	-	X
Acarus siro	<u>KC215264</u>	-	-	-
	<u>KC215263</u>	-	-	-
	<u>KC215262</u>	-	-	-
	<u>KC215261</u>	-	-	-
	<u>KC215260</u>	-	-	-
	<u>KC215259</u>	-	-	-

	KC215258	-	-	-
	KC215257	-	-	-
	KC215256	-	-	-
	KC215255	-	-	-
<i>Lepidoglyphus destructor</i>	MH794059	-	-	X
	MH794058	-	-	X
	MH794057	-	-	X
	MH794056	-	-	X
	MH794055	-	-	X
	MH794054	-	-	X
	MH794053	-	-	X
	MH794052	-	-	X
	MH794051	-	-	X
	MH794050	-	-	X
<i>Tyrophagus putrescentiae</i>	MF354011	-	-	X
	MF354010	-	-	X
	MF354009	-	-	X
	MF354008	-	-	X
	MF354007	-	-	X
	MF354006	-	-	X
	MF354005	-	-	X
	MF354004	-	-	X
	MF354003	-	-	X
	MF354002	-	-	X
<i>Blomia tropicalis</i>	KC215364	-	-	X
	KC215363	-	-	X
	KC215362	-	-	X
	KC215361	-	-	X
	KC215360	-	-	X
	KC215359	-	-	X
	KC215358	-	-	X
	KC215357	-	-	X
	KC215356	-	-	X
	KC215355	-	-	X

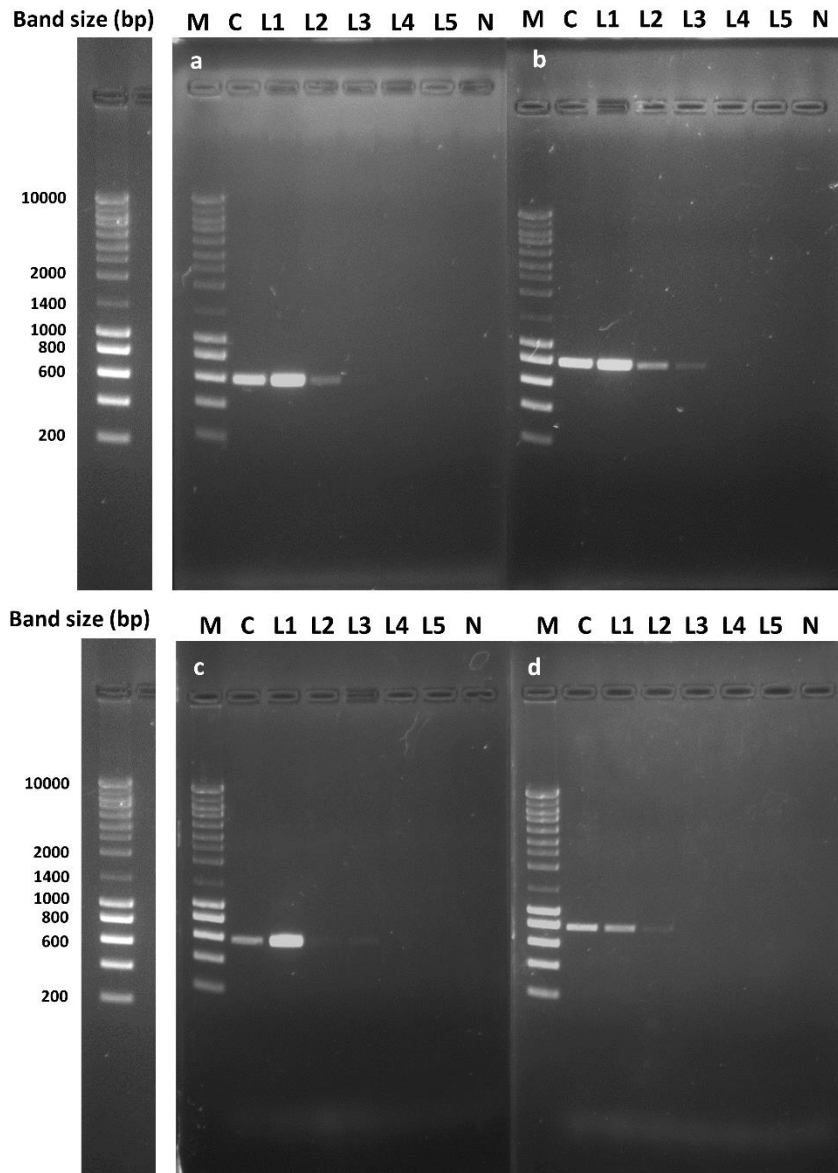


Figure S1. Agarose gel electrophoresis of amplified products from Uniplex PCR for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* using serial dilutions gDNA extracted from pure mite extracts (a and b) and from house dust (c and d). M -10,000 bp molecular size marker (Ladder III from Nzytech, Lisboa, Portugal); lanes 1–5, ten-fold dilution series of mixed DNA sample ranging from 3000 to 0.3 pg μL^{-1} ; N -negative PCR control (ultra-pure water). Faint product can be observed in Lane 3 (a) (30 pg μL^{-1}) for *Dermatophagoides pteronyssinus*.