UNIVERSIDADE FEDERAL DA BAHIA

INSTITUTO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

VÍTOR DOS SANTOS ALVES

ENSAIO DE MULTIPLEX PCR PARA IDENTIFICAÇÃO DE ÁCAROS EM CULTURA E AMOSTRAS AMBIENTAIS

Salvador

2021

VÍTOR DOS SANTOS ALVES

ENSAIO DE MULTIPLEX PCR PARA IDENTIFICAÇÃO DE ÁCAROS EM CULTURA E AMOSTRAS AMBIENTAIS

Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia do Instituto de Ciências da Saúde da Universidade Federal da Bahia como requisito parcial para obtenção do título de mestre em biotecnologia.

Orientadora: Drª Carina da Silva Pinheiro
Co-orientadora: Drª Neuza Maria Alcantara Neves

Salvador

2021

Ficha catalográfica elaborada pelo Sistema Universitário de Bibliotecas (SIBI/UFBA), com os dados fornecidos pelo(a) autor(a).

Alves, Vítor dos Santos
Ensaio de multiplex PCR para identificação de ácaros em cultura e amostras ambientais / Vítor dos Santos Alves. -- Salvador, 2021.
54 f. : il

Orientadora: Carina da Silva Pinheiro.
Coorientador: Neuza Maria Alcantara Neves.
Dissertação (Mestrado - Programa de Pós-Graduação em Biotecnologia) -- Universidade Federal da Bahia, Instituto de Ciências da Saúde (ICS), 2021.

1. Multiplex PCR. 2. Identificação de espécies . 3. Blomia. 4. Glycycometus. 5. Dermatophagoides. I. Pinheiro, Carina da Silva. II. Neves, Neuza Maria Alcantara. III. Título.

VÍTOR DOS SANTOS ALVES

ENSAIO DE MULTIPLEX PCR PARA IDENTIFICAÇÃO DE ÁCAROS EM CULTURA E AMOSTRAS AMBIENTAIS

Dissertação apresentada como requisito para obtenção do grau de mestre em Biotecnologia, Instituto de Ciências da Saúde da Universidade Federal da Bahia.

Aprovado em 22 de abril 2021

BANCA EXAMINADORA:

Carina da Silva Pinheiro – Orientadora_____
Doutora em Biomedicina pelo Instituto de Ensino e Pesquisa da Santa Casa de Belo Horizonte, IEP-SCBH, Brasil
Universidade Federal da Bahia.

Ricardo Wagner Dias Portela _____
Doutor em Bioquímica e Imunologia pela Universidade Federal de Minas Gerais, UFMG, Brasil.
Universidade Federal da Bahia.

Barbara de Castro Pimentel Figueiredo _____
Doutora em Bioquímica e Imunologia pela Universidade Federal de Minas Gerais, UFMG, Brasil.
Universidade do Estado da Bahia.

A

Agenor Conceição dos Santos, *in memoriam*

AGRADECIMENTOS

Dedico esse trabalho aos meus pais, os quais agradeço grandemente pelo amor e apoio diário.

Aos amigos do Laboratório de Alergia e Acarologia pela ajuda, ensinamentos e companhia.

A professora Carina Pinheiro pela orientação nesses quatro anos, ensinamentos e confiança. Certamente contribuiu grandemente para a minha formação.

Aos professores Luis Pacheco e Neuza Alcântara pela ajuda nos experimentos e dicas valiosas durante esses anos.

Ao professor Eric Aguiar e a Paula Fonseca pela revisão do manuscrito e pela ajuda nas análises filogenéticas e depósito das sequências.

A FAPESB pelo financiamento da pesquisa (APP0004/2016).

A CAPES pelo financiamento da minha bolsa de mestrado, permitindo a minha permanência na pesquisa.

Alves, Vítor dos Santos. Ensaio de multiplex PCR para identificação de ácaros em cultura e amostras ambientais. 54 f.: il. 2021. Dissertação (mestrado) – Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, 2021.

RESUMO

Os ácaros *Blomia tropicalis* e *Dermatophagoides pteronyssinus* desempenham um papel importante no desencadeamento de manifestações alérgicas. A identificação de ácaros é principalmente baseada na morfologia, uma técnica demorada e ambígua. O ácaro *Glycycometus malaysiensis* é alergênico e morfologicamente semelhante a *B. tropicalis*, o que torna crucial a identificação precisa dessas espécies. Neste estudo, projetamos um ensaio de reação em cadeia da polimerase multiplex (mPCR) baseado em DNA ribossomal (rDNA) para identificar os ácaros *B. tropicalis*, *D. pteronyssinus* e *G. malaysiensis*. Para isso, a região do espaçador transcrito interno 2 (ITS2), flanqueada por sequências parciais dos genes 5.8S e 28S, foi amplificada e sequenciada. As sequências obtidas foram alinhadas com sequências co-específicas disponíveis na base de dados Genbank visando o desenho de primers e estudos filogenéticos. Os resultados obtidos suportam que a região ITS2 apresenta uma boa resolução para identificação de espécies. Três pares de primers foram escolhidos para compor o mPCR, que foi utilizado para verificar a frequência dos ácaros estudados em vinte amostras de poeira domiciliar de residências na cidade de Salvador, nordeste do Brasil. Os dados mostraram que *B. tropicalis* foi o ácaro mais prevalente, encontrado em 95% das amostras, seguido por *G. malaysiensis* (70%) e *D. pteronyssinus* (60%), sendo que 55% das amostras abrigaram os três ácaros. Além disso, foi a primeira vez que *G. malaysiensis* foi identificado no Brasil e sua sequência ITS2 foi disponibilizada. O ensaio mPCR provou ser uma ferramenta rápida, confiável e simples para identificar esses ácaros e poderá ser aplicado em futuros estudos epidemiológicos ou diagnósticos, bem como para o controle de qualidade de produção de extratos de ácaros para serem usados em ensaios clínicos.

Palavras-chave: Alergia; Multiplex PCR; Identificação de espécies; Blomia; Glycycometus; Dermatophagoides

Alves, Vítor dos Santos. Multiplex PCR assay for identification of mites in culture and environmental samples. 54 f.: il. 2021. Master dissertation - Institute of Health Sciences, Federal University of Bahia, Salvador, 2021.

ABSTRACT

The *Blomia tropicalis* and *Dermatophagoides pteronyssinus* mites play an important role in triggering allergic manifestations. The identification of mites is mostly based on morphology, a time-consuming and ambiguous technique. The mite *Glycycometus malaysiensis* is allergenic and morphologically similar to *B. tropicalis* which makes the precise identification of these species crucial. In this study, we designed a multiplex polymerase chain reaction (mPCR) assay based on ribosomal DNA (rDNA) to identify the mites *B. tropicalis*, *D. pteronyssinus* and *G. malaysiensis*. For this, the internal transcribed spacer 2 (ITS2) region, flanked by partial sequences of the 5.8S and 28S genes, were amplified and sequenced. The sequences obtained were aligned with co-specific sequences available in Genbank database aiming at the design of primers and phylogenetic studies. The results support that the ITS2 region presents a good resolution for species identification. Three pairs of primers were chosen to compose the mPCR, which was used to verify the frequency of the mites studied in twenty house dust samples from homes in the city of Salvador, northeast Brazil. The data showed that the *B. tropicalis* was the most prevalent mite, found in 95% of the samples, followed by *G. malaysiensis* (70%) and *D. pteronyssinus* (60%), and that 55% of the samples harbored the three mites. Furthermore, it was the first time that *G. malaysiensis* was identified in Brazil and its ITS2 sequence was made available. The mPCR assay proved to be a fast, reliable and straightforward tool for identifying these mites and could be applied in future epidemiological or diagnostic studies, as well as for quality control of the production of mite extracts to be used in clinical trials.

Keywords: Allergy; Multiplex PCR; Species identification; *Blomia*; *Glycycometus*; *Dermatophagoides*

SUMÁRIO

1. INTRODUÇÃO E REVISÃO DA LITERATURA

A subclasse Acari possui cerca de 50.000 espécies descritas e, por ser cosmopolita, é estimado existir entre 540.000 e 1.133.000 espécies de ácaros no mundo, o que os torna, após os insetos, o grupo mais popular de artrópodes (WALTER; PROCTOR, 2003). Para lidar com a grande diversidade dos ácaros, os acarologistas os agrupam de acordo com o local em que são encontrados no espaço urbano. Sendo assim classificados em dois grupos: ácaros de poeira (*House Dust Mites* - HDM) e ácaros de armazenamento (*Storage Mites* - SM). A classificação dos ácaros em SM e HDM não é duradoura porque os ácaros podem migrar de SM para HDM, como aconteceu com *B. tropicalis*, originalmente identificado e caracterizado como SM (VAN BRONSWIJK; DE COCK, 1974) e que agora é aceito como um HDM.

Ácaros de poeira doméstica, como *Dermatophagoides pteronyssinus* (Pyroglyphidae: Dermatophagoidinae) e *Blomia tropicalis* (Glycyphagoidea: Echymyopodidae), consomem epitélio humano, resquícios de nutrientes, fungos, bactéria e pólen. Os HDM, por estarem próximos a humanos, tem uma importância médica, sendo responsáveis por crises de asma, rinite alérgica e eczema (FRANKLAND, 1971; THOMAS; HALES; SMITH, 2010). Alguns ácaros possuem diversos antígenos que são capazes de se ligar a imunoglubulina E (IgE) (CARABALLO et al., 1994) – para *B. tropicalis*, por exemplo, 25 proteínas alergênicas foram identificadas, 13 sendo reconhecidas pelo International Union of Immunological Societies (IUIS) (SANTOS DA SILVA et al., 2017) –, essa ligação promove a liberação de mediadores inflamatórios pelos mastócitos, tais como histamina, fatores quimiotáticos, citocinas e metabólicos do ácido araquidônico, os quais irão agir na musculatura lisa, tecido conjuntivo, glândulas de mucosa e células inflamatórias, levando a quadros de alergia (BORISH; JOSEPH, 1992).

Em países de clima tropical, como o Brasil, os HDM mais prevalentes e importantes do ponto de vista médico são *B. tropicalis* e *D. pteronyssinus* (PUERTA et al., 1993; CHEW et al., 1999a; BINOTTI et al., 2001). BAQUEIRO et al., (2006), utilizando identificação morfológica, avaliou a frequência de oito espécies de ácaros em amostras de poeira de 101 camas de dois grupos socioeconômicos da cidade de Salvador, Brasil. Os ácaros mais frequentemente encontrados foram *B. tropicalis* (71.8%) e *D. pteronyssinus* (39.9%).

Ácaros como *Tyrophagus putrescentiae*, *Acarus siro* e *Glycycometus malaysiensis* (Glycyphagoidea: Aeroglyphidae) são chamados de ácaros de armazenamento, pois são encontrados em instalações de armazenamento de grãos, como trigo, milho e cevada (VAN HAGE-HAMSTEN; JOHANSSON, 1992; PALYVOS; EMMANOUEL; SAITANIS, 2008).

Estes ácaros podem contaminar processos industriais que envolvem grãos, visto que encontram um ambiente ótimo para reprodução; além disso, podem infestar alimentos, um problema para indivíduos sensíveis (THIND; CLARKE, 2001; VOGEL; DAL BOSCO; FERLA, 2015).

O SM *G. malaysiensis* é morfologicamente parecido com o HDM *B. tropicalis* (COLLOFF, 2009b; LING et al., 2019), o que pode levar ao cultivo incorreto dessas espécies. Além disso, estudos mostraram que *G. malaysiensis* tem um papel no desencadeamento de manifestações alérgicas (CHEW et al., 1999b; TANG et al., 2011). CHEW et al., (1999b), mostrou que 78.2% dos pacientes que tinham asma e/ou rinite alérgica apresentavam teste de reação cutânea (*Skin prick test*) positivo para *G. malaysiensis.* Outro fato importante mostrado por esse estudo é que *G. malaysiensis* e *B. tropicalis* compartilham antígenos similares, os quais levam à produção de anticorpos que apresentam reação cruzada, com níveis de IgE altamente correlacionados.

G. malaysiensis nunca foi identificado nos estudos de prevalência de ácaros no Brasil, mas a sua prevalência já foi verificada em outros países através de identificação morfológica. Esse ácaro foi encontrado em 35% das amostras em Singapura (CHEW et al., 1999a), em 40% das casas em Klang Valley, Malaysia (MARIANA et al., 2000), e em 6.37% das amostras coletadas em áreas urbanas no Panamá (MIRANDA; QUINTERO; ALMANZA, 2002).

De acordo com a organização mundial de saúde (OMS) a estratégia de tratamento ideal para a rinite alérgica consiste em evitar o alérgeno, farmacoterapia e imunoterapia (BOUSQUET; LOCKEY; MALLING, 2005). A imunoterapia utiliza pequenas doses de extratos alergênicos para aumentar a tolerância clínica e diminuir a sensibilidade dos pacientes aos alérgenos (YANG; ZHU, 2017), sendo uma técnica valiosa para tratamento de rinite alérgica (KOUZEGARAN et al., 2018). Além disso, os extratos alergênicos são utilizados no diagnóstico, seja por meio de testes *in vitro* como o ELISA (*Enzyme-Linked Immunosorbent Assay*), ou por meio de testes cutâneos, como o *Skin Prick Test.*

Sendo assim, devido ao uso de extratos alérgicos na pesquisa científica, no diagnóstico e tratamento de alergias, os ácaros devem ser cultivados em culturas espécie-específicas, o que faz a identificação da espécie crucial na produção de extratos alergênicos de qualidade. A cultura dos ácaros pode começar através da obtenção de ácaros coletados em amostras de poeira domésticas, estes, posteriormente, são separados individualmente utilizando microscópio, e cultivados em garrafas de cultura celular suplementadas com alimento (spirulina e levedura), mantidas em estufas com controle da temperatura e umidade (COLLOFF, 2009a).

Os ácaros são geralmente identificados com base em chaves morfológicas, dados ecológicos, biológicos e comportamentais (COLLOFF, 2009b). No entanto, há problemas em

se utilizar apenas chaves morfológicas na identificação de ácaros. Além de ser uma técnica laboriosa, que requer amostras bem conservadas e presença de estágios adultos, espécies morfologicamente parecidas, como *Dermatophagoides farinae* e *Dermatophagoides microceras* e *G. malaysiensis* e *B. tropicalis* podem levar a incertezas na identificação até mesmo por acarologistas experientes (COLLOFF; SPIEKSMA, 1992; COLLOFF, 1998; LING et al., 2019). Além disso, os ácaros nem sempre são encontrados na fase adulta ou intactos (quando na forma de extratos alergênicos), dificultando ainda mais a certificação da espécie (COLLOFF; SPIEKSMA, 1992). Devido a estas dificuldades, técnicas biotecnológicas vêm sendo utilizadas como importante ferramenta na identificação de ácaros.

O DNA ribossômico (rDNA) é amplamente utilizado como marcador em estudos filogenéticos e identificação de espécies (HILLIS; DIXON, 1991). Em eucariotos, as regiões ribossomais espaçadoras transcritas internas (*internal transcribed spacer* - ITS), como a ITS1 e ITS2, são espaços situados entre os genes das subunidades 28S, 5.8S e 18S do rDNA; esse arranjo forma um *cluster* que se repete em tandem mais de 100 vezes no genoma, e pode até ser encontrado em mais de um cromossomo (WEN; LEÓN; HAGUE, 1974). As três subunidades são altamente conservadas e podem ser aplicadas em identificação de grupos taxonômicos, já as regiões ITS evoluem rapidamente e são bastante variadas em sequência, podendo ser utilizadas na identificação de espécies. Além disso, como as regiões ITS´s estão situadas entre genes conservados, a criação de iniciadores específicos para amplificar essas regiões é facilitada.

A região ITS2 e a subunidade 1 do citocromo oxidase (*cytochrome oxidase subunit* I – Cox I) já foram amplificadas e sequenciadas visando a identificação de seis espécies de ácaros (YANG; CAI; CHENG, 2011). As regiões ITS1, subunidade 5.8S e ITS2 também foram sequenciadas para estudo filogenético de 6 espécies de ácaros comercialmente importantes da família Phytoseiidae, aplicados como agentes no controle biológico de pragas (NAVAJAS et al., 1999).

Além do sequenciamento de DNA, outro exemplo de técnica molecular que pode ser aplicada na identificação de ácaros é a amplificação através da Reação em Cadeia da Polimerase (*Polymerase Chain Reaction* – PCR) de uma dada região do genoma seguida da digestão com enzimas de restrição e análise dos fragmentos gerados da digestão das bandas em gel, denominada polimorfismo no comprimento do fragmento de restrição (*Restriction Fragment Length Polymorphism* – RFLP). A PCR-RFLP baseia-se na capacidade das enzimas de restrição em clivar o DNA em sequências específicas de 4 a 8 nucleotídeos ou próximas a essas sequências. Quando a técnica é aplicada às regiões polimórficas do genoma, o padrão de

digestão gerado pelas enzimas de restrição será diferente de espécie para espécie. O polimorfismo pode ser constatado pela presença ou ausência de sequências específicas, o que altera o tamanho dos fragmentos digeridos ou ocasiona na ausência de clivagem pelas enzimas de restrição escolhidas para o ensaio.

A técnica de PCR-RFLP aplicada às regiões ITS foi utilizada na identificação de 3 espécies de ácaros comerciais (*Neoseiulus cucumeris*, *Amblyseius swirskii*, e *Neoseiulus barkeri*) sem a necessidade de sequenciamento (SYROMYATNIKOV et al., 2018). OSAKABE et al., (2008), buscando a padronização e simples identificação de ácaros do gênero *Tetranychus* (spider mites), considerados pragas agrícolas, aplicaram a PCR-RFLP a regiões ITS desses ácaros para criação de um catálogo que simplificava a visualização dos padrões de digestão gerado por 5 enzimas de restrição, o qual tem potencial aplicação na verificação da biossegurança de plantas em quarentena no Japão.

A região ITS2 de HDM também já foi utilizada na identificação dos ácaros *G. malaysiensis*, *B. tropicalis*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *T. putrescentiae* e *Acarus ovatus* com a utilização de 8 enzimas de restrição (WONG et al., 2011). Mais recentemente, BEROIZ et al., (2014) amplificou a região do rDNA de 13 ácaros que contemplava as regiões ITS1, 5.8S e ITS2. Os fragmentos foram sequenciados para análise dos sítios de restrição com finalidade de submeter esses fragmentos a PCR-RFLP com uso de apenas duas enzimas de restrição, permitindo a identificação de treze espécies de ácaros utilizados como extratos alérgicos em estudos clínicos. O estudo foi aplicado a ácaros em cultura, com crescimento exponencial, e a ácaros em amostras ambientais. Para ácaros em amostras ambientais, foi necessário realizar uma nested PCR para amplificar unicamente as regiões ITS1, que posteriormente eram digeridas, permitindo a discriminação das espécies.

As desvantagens em utilizar PCR-RFLP para identificação de espécies deriva do fato de demandar mais etapas, pois, além de extrair o DNA dos ácaros e amplificar por PCR a região de interesse, é necessária, ainda, a digestão enzimática, que pode levar de 2 a 16 horas. Além disso, devido ao grande número de bandas geradas da digestão e do fato da PCR amplificar o rDNA de qualquer ácaro presente (não sendo espécie-específica), há maiores problemas na interpretação dos resultados, podendo gerar erros e ambiguidades na interpretação dos perfis gerados.

Uma abordagem tecnicamente mais simples e eficaz seria o design de iniciadores de PCR específicos para os ácaros de interesse. Com isso, a banda só apareceria no gel de eletroforese se a amostra for positiva para um dado ácaro, diferentemente dos iniciadores utilizados na maioria dos trabalhos, que amplificam o DNA de qualquer ácaro. A multiplex

PCR (mPCR) é um ensaio de PCR que utiliza mais de um par de iniciadores, permitindo a amplificação de mais de um fragmento gênico. Após devida padronização, ela é útil em estudos de diagnóstico, pois reduz o tempo de análise por amostra e o gasto com reagentes.

Até o momento, poucos trabalhos fizeram o uso da mPCR para identificar ácaros. Alguns focaram na identificação de ácaros considerados pestes agrícolas (SINAIE; NAMAGHI; FEKRAT, 2018; ZÉLÉ; WEILL; MAGALHÃES, 2018; GÓMEZ-MARTÍNEZ et al., 2020) e outros na identificação de HDM. THET-EM et al., (2012) desenvolveu um ensaio de mPCR espécie-específico para avaliar a prevalência dos ácaros *D. pteronysinus*, *D. farinae* e *B. tropicalis* em 30 amostras de poeira doméstica da Tailândia. O ensaio utilizava 3 pares de iniciadores para amplificar a região ITS2 de *D. pteronysinus* e *D. farinae*, e Cox I para *B. tropicalis*. No entanto, as sequências dos iniciadores não foram disponibilizadas nos trabalhos publicados. Além disso, não houve sequenciamento de nenhuma das bandas das mPCR das 30 amostras de poeira, o que poderia dar uma segurança quanto a especificidade do ensaio para os ácaros estudados. Poeira doméstica pode conter DNA de diversas espécies e essa diversidade de espécies pode variar entre regiões geográficas (BARBERÁN et al., 2015). Algumas dessas espécies podem ser desconhecidas ou não ter a sequência nucleotídica disponível em bancos de dados. Com isso, o sequenciamento das bandas se torna importante em estudos de identificação molecular de espécies. Mais recentemente, OLIVEIRA et al., (2021) desenhou um ensaio de mPCR específico para os HDM *D. pteronysinus* e *D. farinae*, capaz de identificar os ácaros citados em amostras ambientais. O sequenciamento das amostras foi utilizado para confirmar a especificidade do método.

Há uma tendência na utilização de abordagens moleculares para identificação de espécies, e na acarologia não é diferente. As abordagens moleculares são mais precisas e rápidas em relação às análises morfológicas, permitindo análise quando não há possibilidade de verificação morfológica – quando há somente o extrato do ácaro ou em amostras ambientais pouco conservadas, por exemplo. Essas técnicas podem, portanto, ser utilizadas em estudos epidemiológicos e diagnósticos de ácaros de interesse.

Com isso, o objetivo do presente trabalho é desenvolver um ensaio de multiplex PCR espécie-específica, baseado em ITS-rDNA, capaz de identificar as espécies de ácaros *D. pteronyssinus*, *B. tropicalis* e *G. malaysiensis* em cultura e amostras ambientais. Além disso, avaliar a frequência desses ácaros na poeira de residências da cidade de Salvador (BA), nordeste do Brasil.

2. OBJETIVO

2.1. Geral

Desenvolver um ensaio de Reação em Cadeia da Polimerase multiplex (mPCR) espécie-específico capaz de identificar as espécies de ácaros *Blomia tropicalis*, *Glycycometus malaysiensis* e *Dermatophagoides pteronyssinus* a partir de DNA extraído de i) amostras ambientais, ii) massa de cultura de ácaros e iii) ácaros individuais. E testar esse ensaio verificando a prevalência desses ácaros em amostras de poeira doméstica da cidade de Salvador, nordeste do Brasil.

2.1. Específicos

1. Identificar sequências genômicas desses ácaros, para isso: amplificar, clonar e sequenciar o rDNA de *B. tropicalis* e *G. malaysiensis* e obter as sequências de *D. pteronyssinus* do banco de dados Genbank.
2. Desenhar iniciadores espécie-específicos baseados na região ITS-rDNA para *B. tropicalis*, *G. malaysiensis* e *D. pteronyssinus*.
3. Padronizar as extrações de DNA para i) amostras ambientais, ii) massa purificados de cultura de ácaros e iii) ácaros individuais.
4. Testar os iniciadores desenhados para o DNA dos ácaros estudados e selecionar os pares de iniciadores que serão utilizados no ensaio de mPCR.
5. Testar a mPCR quanto a sua especificidade e sensibilidade.
6. Coletar amostras de poeira do colchão de 20 residências da cidade de Salvador, Bahia e identificar os ácaros estudados nessas amostras através da mPCR.
7. Clonar e sequenciar as bandas dos três ácaros estudados das amostras de poeira para confirmação da especificidade da mPCR.
8. Realizar estudos de filogenia através de análises de barcode gap e árvores filogenéticas.

3. RESULTADOS E PRODUTO GERADO

3.1. Multiplex PCR assay for identification of mites in culture and environmental samples

Artigo a ser publicado na revista "*Experimental and Applied Acarology*" elaborado com os resultados de pesquisas realizadas no Laboratório de Alergia e Acarologia (LAA) da Universidade Federal da Bahia (UFBA), com colaboração do Laboratório de Bioinformática de Vírus da Universidade Estadual de Santa Cruz (UESC) e do Laboratório de Acarologia Agrícola da Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo (USP). O projeto foi financiado pela FAPESB e aprovado pelo Comitê de Ética em Pesquisa (CEP) da UFBA (CAAE 22804719.0.0000.5662).

MULTIPLEX PCR ASSAY FOR IDENTIFICATION OF MITES IN CULTURE AND ENVIRONMENTAL SAMPLES

Authors: Vítor dos Santos Alves[1], Fabián Salazar Garcés[1], Leonardo Freire Santiago[1], Paula Luize Camargos Fonseca[2], Antônio Márcio Santana Fernandes[1], Raphael Chagas Silva[1], Lorena Miranda de Souza[3], Pedro Paulo Ribeiro Silva Cunha[1], Marina Ferraz de Camargo Barbosa[4], Eric Roberto Guimarães Rocha Aguiar[5], Luis Gustavo Carvalho Pacheco[1], Neuza Maria Alcantara-Neves[1], Carina da Silva Pinheiro*[1]

[1]Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Brazil

[2]Depto. of Microbiology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 30270-901, Brazil

[3]Universidade Salvador, Salvador, Brazil

[4]Depto. Entomologia e Acarologia, ESALQ-USP, Piracicaba, São Paulo 13418-900, Brazil

[5]Depto. of Biological Science (DCB), Center of Biotechnology and Genetics (CBG), State University of Santa Cruz (UESC), Rodovia Ilhéus-Itabuna km 16, Ilhéus 45652-900, Brazil

* Corresponding author's address: Laboratory of Allergy and Acarology, Institute of Health Sciences, Federal University of Bahia. Avenida Reitor Miguel Calmon, S/n, Vale do Canela, CEP: 40110-100. Salvador, Bahia, Brazil. https://orcid.org/0000-0001-5623-1308. E-mail: carina.pinheiro@ufba.br

Abbreviation list

B. tropicalis	*Blomia tropicalis*
Bt	*Blomia tropicalis*
CAAE	Certificate of Presentation of Ethical Appreciation
CAPES	Coordination for the Improvement of Higher Education Personnel
CEP	Research Ethics Committee
Cox I	Cytochrome oxydase subunit I
DNA	Deoxyribonucleic Acid
Dp	*Dermatophagoides pteronyssinus*
D. pteronyssinus	*Dermatophagoides pteronyssinus*
G. domesticus	*Glycyphagus domesticus*
Gm	*Glycycometus malaysiensis*
G. malaysiensis	*Glycycometus malaysiensis*
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2
mPCR	Multiplex Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction - Restriction Fragment Length Polymorphism
rDNA	Ribosomal Deoxyribonucleic Acid
UFBA	Federal University of Bahia

Abstract

The *Blomia tropicalis* and *Dermatophagoides pteronyssinus* mites play an important role in triggering allergic manifestations. The identification of mites is mostly based on morphology, a time-consuming and ambiguous approach. The mite *Glycycometus malaysiensis* is allergenic and morphologically similar to *B. tropicalis* which makes the precise identification of these species crucial. Herein, we describe a multiplex polymerase chain reaction (mPCR) assay based on ribosomal DNA (rDNA) to differentiate the mites *B. tropicalis*, *D. pteronyssinus* and *G. malaysiensis*. For this, the internal transcribed spacer 2 (ITS2) regions, flanked by partial sequences of the 5.8S and 28S genes, were PCR-amplified and *de novo* sequenced. The sequences obtained were aligned with co-specific sequences available in the Genbank database for primer design and phylogenetic studies. Three pairs of primers were chosen to compose the mPCR assay, which was then used to verify the frequency of the different mites in house dust samples (n = 20) from homes in the city of Salvador, Brazil. *B. tropicalis* was the most frequent mite, found in 95% of the samples, followed by *G. malaysiensis* (70%) and *D. pteronyssinus* (60%). Besides reporting for the first time the occurrence of *G. malaysiensis* in Brazil, our results confirm the good resolution of the ITS2 region for mite identification. Furthermore, the mPCR assay proved to be a fast and reliable tool for identifying these mites and could be applied in future epidemiological or diagnostic studies, as well as for quality control of the production of mite extracts to be used in clinical trials.

Keywords: Allergy, Multiplex PCR; Species identification; *Blomia tropicalis*; *Glycycometus malaysiensis*; *Dermatophagoides pteronyssinus*

Introduction

The subclass Acari (Arthropoda: Chelicerata) includes *ca*. 54.617 described species, although it is estimated that there exist nearly 1 million mite species, making it the second biggest group of arthropods, after insects (BARNES, 1989; GROOMBRIDGE, 1993; WALTER; PROCTOR, 2003). Mites species are the mainly source of allergen in house dust in tropical countries and, since the first discovery of the allergenic role of mites in the mid-1960s, numerous species were described as source of allergens (FERNÁNDEZ-CALDAS; PUERTA; CARABALLO, 2014). According to their living sites, mites can be clustered and classified into two different groups: storage mite (SM) and house dust mite (HDM). SM mites comprise species such as *Glycyphagus domesticus* (De Geer) (Gd) (Glycyphagidae) and *Glycycometus malaysiensis* (Fain & Nadchatram) (Gm) (=*Austroglycyphagus malaysiensis*) (Aeroglyphidae) since they are commonly found in wheat, corn and barley storage places (VAN HAGE-HAMSTEN; JOHANSSON, 1992; WALTER; PROCTOR, 2003; PALYVOS; EMMANOUEL; SAITANIS, 2008). The mite specie *Blomia tropicalis* (Van Bronswijk, Cock & Oshima) (Bt) (Echymyopodidae) was first characterized as a SM (VAN BRONSWIJK; DE COCK, 1974). However, it is currently accepted that *B. tropicalis* is a very important HDM, along with *Dermatophagoides pteronyssinus* (Trouessart) (Dp) (Pyroglyphidae), being found in pillows, mattresses and house carpets (NADCHATRAM, 2005).

HDM has a medical importance since this group of mites lives close to humans and triggers allergic manifestations (e.g., allergic/atopic asthma, allergic rhinitis and atopic eczema) (FRANKLAND, 1971; THOMAS; HALES; SMITH, 2010). However, some species classified as SM are also capable of inducing sensitization and triggering allergic respiratory symptoms. Mite antigens are able to bind immunoglobulin E (IgE) (CARABALLO et al., 1994) and promote the release of inflammatory mediators such as: histamine, chemo-attractant factors, acid arachidonic metabolites and cytokines, which act directly on smooth musculature, conjunctive tissue, mucous glands and inflammatory cells, triggering the allergic symptoms (BORISH; JOSEPH, 1992).

In tropical and subtropical countries (CHEW et al., 1999a; KUO et al., 1999; YADAV; NAIDU, 2015; ALIMUDDIN et al., 2018), including Brazil (BAQUEIRO et al., 2006), *B. tropicalis* and *D. pteronyssinus* are currently considered the most common allergy-associated mites. Furthermore, these and some other SM are capable to cause occupational allergies (SOLARZ; SZILMAN; SZILMAN, 2004; VIDAL et al., 2004; RUOPPI; KOISTINEN; PENNANEN, 2005; STORAAS et al., 2005; KOISTINEN et al., 2006; SZILMAN et al., 2006; JEEBHAY et al., 2007; STEJSKAL; HUBERT, 2008). Although *G. malaysiensis* is a neglected

species of mite, its medical importance has already been demonstrated, since it promotes occupational and non-occupational allergies (CHEW et al., 1999b; COLLOFF, 2009b; TANG et al., 2011; LING et al., 2019). In addition, it has been suggested that *G. malaysiensis* allergens may cross-react with *B. tropicalis* allergens, since IgE levels for *B. tropicalis* and *G. malaysiensis* were highly correlated ($r = 0.60$, $P < 0.001$) (CHEW et al., 1999b).

Therefore, due to the importance of these mites in triggering allergies, the correct identification of each species is crucial for the quality and the production of specific extracts that can be used in diagnostics and/or allergy treatments. Traditionally, mites are identified based on morphological keys, ecological and biological data and behavior (COLLOFF, 2009b). However, there are problems in the identification of these mites based on morphology, since this technique requires preserved samples, and the presence of adult stages, a situation that is not often possible. Besides, the presence of morphologically similar species (e.g., *Dermatophagoides farina*, *Dermatophagoides microceras*, *G. malaysiensis* and *B. tropicalis*) may lead, even experient mite morphologists, to misidentification (COLLOFF; SPIEKSMA, 1992; COLLOFF, 1998, 2009b; LING et al., 2019). Due to these drawbacks, molecular techniques based on ribosomal DNA (rDNA) and Cytochrome Oxydase Subunit 1 (Cox 1) have been used as important tools on mite identification (HILLIS; DIXON, 1991; NAVAJAS; FENTON, 2000; WONG et al., 2011; YANG; CAI; CHENG, 2011; THET-EM et al., 2012; BEROIZ et al., 2014).

Moreover, the rDNA is widely used in phylogenetical studies and species identification (HILLIS; DIXON, 1991). In eukaryotes, the ribosomal regions called internal transcribed spacers 1 and 2 (ITS1 and ITS2) are regions located between the 28S, 5.8S and 18S rDNA subunit genes (Figure 1); this arrangement creates a in tandem cluster which is repeated over 100 times, and can even be found in more than one chromosome (WEN; LEÓN; HAGUE, 1974). These three subunits are highly conserved, and can be applied on taxonomic group identification; on the other hand, the ITS regions diversify in sequence and can be used for species identification. ITS regions possess some characteristics, such as i) being located between conserved genes; ii) being short sequences; and iii) being present in a huge number of copies in the genome, make them important and useful, even in poorly conserved environmental samples (THET-EM et al., 2012; BEROIZ et al., 2014).

Therefore, we aimed to perform phylogenic studies and develop a species-specific multiplex PCR (mPCR), based on ITS-rDNA, capable to identify and discriminate *D. pteronyssinus*, *B. tropicalis* and *G. malaysiensis* mites in culture and in environmental samples.

In addition, we intend to apply mPCR for evaluate the frequency of these species in house dust collected from twenty residences located in Salvador, Northeast Brazil.

Materials and methods

Dust collection

Dust samples were collected in mattresses from twenty residences in the city of Salvador, Bahia, northeastern Brazil. The samples were collected in eight neighborhoods during an 11-month interval, from May 2019 to March 2020. A map of the continental part of the city of Salvador was generated in the QGIS program (version 3.14.16) (QGIS Development Team 2020) to signal the neighborhoods where the collections were carried out (Figure S1). Dust collection was carried out as described by Baqueiro (2006), using a residential vacuum cleaner (Eletrolux Professional, 1220watts; Electrolux do Brasil, São Paulo, Brazil) for 5 minutes in an area of 1m² at the head of the mattress. The dust was weighed and stored at -20 °C until DNA extraction. This study was approved by the Research Ethics Committee/CEP of the Institute of Health Sciens of the Federal University of Bahia/UFBA (CAAE 22804719.0.0000.5662).

Mites culture

B. tropicalis and *G. malaysiensi* mites were obtained from domestic dust samples, and *D. pteronyssinus* was purchased from the company Indoor Biotechnologies (USA) since it was not possible to culture these mites in the laboratory. All dust samples collected were cultured in laboratory in a BOD incubator at 25 °C and 70-80% RH. After 7 days, all cultures were analyzed at stereoscopic magnifying glass (ZEISS, 475052 – 9901, West Germany) and the mites were collected individually and transferred to cell culture bottles supplemented with spirulina and yeast and cultured in the same conditions. After three weeks, a sample containing mites was taken for morphological identification by a specialist. After the mite growing, the *B. tropicalis* and *G. malaysiensi* cultures were placed in saline solution (5 mol/L) to allow the decantation of food and dirt. The mites on the surface were sucked with a vacuum pump and the mass of mites obtained was centrifuged for 11,000 G for 7 minutes to remove the saline solution. Afterwards, the mite masses were stored in a -20 ° C freezer for later use.

DNA extraction

DNA extraction was carried out from material from three different sources i) domestic dust (4 mg); ii) masses of frozen *B. tropicalis, G. malaysiensis* and *D. pteronyssinus* mites (20 mg) and iii) individual mites. All extractions were performed according to the recommendations

of the NucleoSpin DNA Insect kit (Macherey Nagel, Düren, Germany). To assess purity and concentration, the extracted DNA was analyzed using the µDrop plate in MultiSkan GO DNA quantification system (Thermo Scientific, Waltham, MA, USA), and subsequently stored at -20 °C.

Amplification of rDNA

PCR amplification was performed for *B. tropicalis* and *G. malaysiensis* mites, using the primer combination Fnav, based on the 3 'end of the rDNA 18S region (NAVAJAS et al., 1999), and Rnav2, based on the 5´ end of the 28S region (NAVAJAS et al., 1998). The PCR products generated consisted of a partial sequence of the 18S region, the complete sequence of the ITS1, 5.8S and ITS2 regions and the 5' end of the 28S subunit. The total reaction volume was 25 µl, containing 100-270 ng of DNA extracted from a mass of mites corresponding to the respective species, 1x enzyme buffer supplied by the manufacturer (Sinapse Inc), 200 µM dNTP mix, 0.2 µM of each primer, 1 IU of Taq DNA polymerase (Sinapse Inc). The PCR cycle conditions can be found in Table 1.

The PCR products were used as a template for the nested PCR of the ITS2 rDNA region flanked by partial sequences of the 5.8S and 28S subunits using the ITS2 f and ITS2 r primers (NOGE et al., 2005). The PCR products of Rnav2/Fnav (0.5 µl) were used with the concentration of reagents described above (see Table 1 for cycle conditions). All PCR reactions included negative controls using water as the template. The PCR products were visualized after 1% agarose gel electrophoresis and stained with SYBR (Applied Biosystems).

rDNA cloning and sequencing

For *G. malaysiensis*, the PCR products of the Fnav/Rnav2 primer pair and the nested PCR products obtained with the ITS2 f/ITS2 r primer pair were purified (PCR DNA and Gel Band Purification; GFX), ligated to the pGEM-T easy vector (Promega Corp., Madison, WI, USA) and transformed into *Escherichia coli* XL1-Blue strain. Four isolated colonies for both PCR products were selected for plasmid purification (NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel)) and insert sequencing. Purified nested PCR products from *B. tropicalis* (PCR DNA and Gel Band Purification; GFX) were ligated to the pCR™4-TOPO® TA vector (TOPO® TA Cloning®; Invitrogen) and cloned as described above. Three colonies were selected for insert sequencing.

Sequencing was bi-directional for *G. malaysiensis* and *B. tropicalis* clones and was carrying out by the company MYLEUS FACILITY (http://facility.myleus.com/) using the M13

forward and M13 reverse primers and the equipment ABI 3730 (Life Technologies/Thermo Fisher Scientific).

Sequence analysis and primer design

All sequences obtained from the sequencing were edited using the BioEdit program (version 7.0.5.3) (HALL, 1999) to obtain full-length readings and deposited in the Genbank database under the following accession numbers: MW763262-MW763265 for PCR with Fnav/Rnav2 primers, and MW763255-MW763257 and MW763258-MW763261 for the nested PCR for *B. tropicalis* and *G. malaysiensis*, respectively. The BLASTN (ALTSCHUL et al., 1990) analysis against the nucleotide collection (nr/nt) was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). For *D. pteronyssinus*, it were used the sequences available in the GenBank database (KC215344.1, KC215341.1, KC215342.1 and KC215340.1). Also, the *D. farinae* (KC215334.1), *D. microceras* (KC215315.1), *B. tropicalis* (KC215364.1) and *Glycyphagus domesticus* (KC215365.1, KC215374.1, KC215373.1 and KC215368.1) sequences available in the GenBank database were used for primer design. The phylogenetic relationships were constructed using the sequences obtained in this study and reference sequences deposited in GenBank for *D. pteronyssinus*, *B. tropicalis* and *G. domesticus*, as mentioned above. The rDNA sequences were trimmed at the position of the ITS2 f/ITS2 r primer pair, comprising the complete ITS2 region flanked by partial sequences of 5.8S and 28S subunits (see Figure 1). To infer the evolutionary history, a Neighbor-joining tree were generated using the Maximum Composite Likelihood method to compute the evolutionary distances and considering 1000 replicates of Bootstrap using the program MEGAX (version 10.1.8).

In order to observe intraspecific variation for each species studied, the ITS2 regions obtained in the sequencing and the sequences obtained in the GenBank database were aligned using the Clustal-W program of BioEdit (version 7.0.5.3) (HALL, 1999) and the conserved regions were selected. To evaluate interspecific variation and species specificity of the previously selected conserved regions, a new alignment was made using all sequences. Then, species-specific sequences were used for manual design of primers. The primers were evaluated using Primer-BLAST (NCBI) and OligoAnalyzer (Integrated DNA Technologies, Inc).

Table 1 lists the generated primer sequences. For *D. pteronyssinus* and *B. tropicalis*, forward primers for the ITS1 region were also designed, generating larger PCR products, consisting of a ITS1 partial sequence, 5.8S complete sequence and ITS2 partial sequence (Figure 1).

Singleplex and Multiplex Amplification

All designed primer pairs were tested with DNA from individual mites and mass of mites. The PCR reagent concentrations were the same for all singleplex PCR tested, with a final volume of 10 µl containing 10 ng of DNA, 1x enzyme buffer (Promega, Madison, WI, USA), 200 µM dNTP mix, 0.5 µM of each primer (Integrated DNA Technologies, Inc), 1 mM MgCl2 and 0.8 IU of Taq DNA Polymerase (Promega, Madison, WI, USA). All singleplex PCR reactions included negative controls, using water as the template. The PCR cycle conditions were empirically adjusted until obtention of optimal results for all prime pairs (Table 1).

As larger PCR products were generated, primer pairs numbers 3, 6 and 8, for *B. tropicalis*, *D. pteronyssinus*, and *G. malaysiensis* respectively (Table 1), were selected to compose the mPCR. The reaction was carried out as in singleplex PCR, using 100 ng of DNA. All mPCR reactions included positive (DNA extracted from individual mites) and negative controls. The mPCR and singleplex PCR products were visualized in 1% agarose gel stained with SYBR (Applied Biosystems).

Sensitivity, Specificity and detection limit of mPCR

To evaluate the sensitivity of the assay, two tests were performed, i) using dilutions of DNA of each species, separately, ii) using dilutions of a mixture containing proportional amounts of DNA from each species. For both tests, DNA extracted from individual mites was used, and were diluted for a final amount of 50, 10, 1, 0.1 and 0.01 ng of DNA. The detection limit test was also used to assess the specificity of mPCR primer pairs in the presence of DNA from the studied mites.

Cloning and sequence analysis of mPCR products from dust samples

To further confirmation of the specificity of the mPCR assay, seven of the twenty dust samples were selected for cloning, as previously described for the *G. malaysiensis* rDNA fragments, and sequencing. For the fragments of *D. pteronyssinus*, *B. tropicalis* and *G. malaysiensis*, one, three and five colonies, respectively, were selected for insert sequencing (Table 2).

Sequencing was bi-directional for all clones and was carried out by the company ACTGene Análises Moleculares Ltda (https://actgene.com.br/) using primers M13 forward (-40) and M13 reverse and the equipment AB 3.500.

All sequences obtained were deposited in the Genbank database under the following accession numbers: MW763266-MW763268, for *B. tropicalis*, MW763250-MW763254 for *G. malaysiensis* and MW763269 for *D. pteronyssinus*. BLASTN analysis against the nucleotide collection (nr/nt) was performed using the NCBI website. A bootstrap Neighbor Joining tree (1000 replicates), using the Maximum Composite Likelihood method in the MEGAX (version 10.1.8) program, was built by performing an alignment between the sequences of the dust clones and the sequences used in the phylogenetic tree generated previously.

Barcode gap analyses

All the sequences generated in this work and the sequences obtained in the GenBank database for *D. pteronyssinus, B. tropicalis* and *G. malaysiensis* were used for the barcode gap analysis using two different methods. The first, considered as traditional method, using the Geneious Prime software (V. 2020) and R program (R Core Team 2013). The sequences of the ITS2 region were used to generate a distance matrix (using P distance) with default parameters. The matrix was exported to an excel table and the intra- and interspecific distances were clustered. The distance data set was used to build boxplot and jitter plots using the R ggplot2 package (WICKHAM, 2016). To assess the reliability of the data, the *Wilcoxon test* was performed. The second method used to the barcode gap analysis was the Automatic Barcode Gap Discovery (ABGD) software (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using default parameters. A species was considered identified when the interspecific distance was larger than the intraspecific distance (SCHOCH et al., 2012).

Results

rDNA amplification and phylogenetic analysis

The primer combination Fnav and Rnav2, for the amplification of the with the 3′ end of the 18S subunit, the full-length sequences of ITS1, 5.8S subunit, ITS2 and the 5′ end of the 28S rDNA subunit of *B. tropicalis* and *G. malaysensis* mites only generated products visible on agarose gel when using DNA extracted from *G. malaysensis*. The PCR product was approximately 820 bp (Figure S2) and four clones were sequenced (under access numbers MW763262-MW763265 at NCBI). However, the analysis of similarity among the sequences obtained with the sequences from the GenBank database did not identify the sequences as Astigmata rDNA, but as fungal rDNA.

The nested PCR for *B. tropicalis* and *G. malaysensis* mites showed bands of approximately 350 bp and 260 bp, respectively, on agarose gel (Figure S2). The bands were

cloned and 4 insert-containing clones for *G. malaysensis* (under access numbers MW763258-MW763261) and 3 for *B. tropicalis* (under access numbers MW763255-MW763257) were selected for sequencing. For both mites, the analysis among the obtained sequences and the GenBank database confirmed the sequences as Astigmata rDNA. The sequences ranged from 314-323 bp and 250-256 bp for *B. tropicalis* and *G. malaysensis* respectively. There were, for both mites, intraspecific polymorphisms in the sequences, which consisted of insertions, deletions and substitutions.

As shown by the phylogenetic tree (Figure 2) constructed with the alignment of sequences referring to the complete ITS2 region flanked by partial sequences of 5.8S and 28S subunits of the *D. pteronyssinus* and *B. tropicalis* clones and the sequences obtained in the GenBank database, the *B. tropicalis* clones (*Bt1-3*) clustered in the same branch as the *B. tropicalis* sequence, with a significant high bootstrap value (100%). The *G. malaysensis* clones (*Gm1-4*) clustered in the same branch, with a robust bootstrap value (93%). It is also possible to infer that the mites *B. tropicalis, G. malaysensis* and *G. domesticus* share the same common ancestor (100% bootstrap), as they belong to the superfamily Glycyphagoidea, and that *G. malaysensis* and *G. domesticus* are closely related species, sharing the same clade (90% bootstrap).

Singleplex PCR

We designed a total of 10 primers: two forward and one reverse primers for *G. malaysenis*, three forward and one reverse primers for *B. tropicalis* and two forward and one reverse primers for *D. pteronyssinus*. The designed primers were used in pairs and are described in Table 1. The primer pairs used in the singleplex PCRs successfully amplified the DNA of *D. pteronyssinus, B. tropicalis* and *G. malaysensis*, with fragments in the expected size (see Table 1). As shown in Figure 3, primer pairs 3 to 9 amplified PCR products of approximately 482 bp, 147 bp, 120 bp, 593 bp, 115 bp, 193 bp and 138 bp, respectively.

Multiplex PCR

The mPCR was successfully used to amplify the partial ITS1, 5.8S subunit and partial ITS2 regions, of *D. pteronyssinus and B. tropicalis* and partial ITS2 region for *G. malaysensis*. The mPCR limit detection was assessed with DNA dilutions of individual mites and dilutions with a DNA mixture of the mites used in this study. When using individual mite DNA, the detection limit was possible starting from 0.01 ng for *D. pteronyssinus,* 0.1 ng for *B. tropicalis* and 1 ng for G. *malaysensis* (see Figure 4, panel A-C); furthermore, the results showed that

primer pairs used in the mPCR assay are species-specific when using the DNA of the mites studied. When using the mixture containing proportional amounts of DNA from the mites studied, the detection limit for *D. pteronyssinus* and *B. tropicalis* remained the same of using individual mite DNA as a template, however the detection limit for *G. malaysensis* was 0.1 ng, increasing 10 times. (Figure 4, panel D).

Figure 5 shows the mPCR agarose gels using DNA extracted from 20 dust samples. Looking at the venn diagram in Figure 6, it is possible to notice that *B. tropicalis* was the most prevalent mite, being present in 95% of the samples, except for sample 15, which was negative for all mites (Figure 5). *G. malaysensis* proved to be the second most prevalent mite, present in 70% of the samples, followed by *D. pteronyssinus*, found in 60% of the samples. *B. tropicalis* was the only mite found alone, in 20% of the samples, however it was also found associated with other mites, with 15% of the samples harboring *B. tropicalis* and *G. malaysensis* and 5% of them contained *B. tropicalis* and *D. pteronyssinus*. In addition, the 3 mites were found in 55% of the samples. Finally, no house dust was found harboring only *D. pteronyssinus* and *G. malaysensis*.

The sequences of the dust clones for *B. tropicalis* (*DBt1-3*) (access numbers MW763266-MW763268), *G. malaysensis* (*DGm1-5*) (access numbers MW763250-MW763254) and *D. pteronyssinus* (*DDp*) (access number MW763269) mites presented a size of 478-483, 193-214 and 595 bp, respectively. The BLASTN search against the Genbank database identified all sequences as Astigmata rDNA and the *DBt* and *DDp* clones as belonging to *B. tropicalis* and *D. pteronyssinus*, species, respectively. As expected, the sequences of all clones – including the *DDp* clone, when compared to the *D. pteronyssinus* sequences obtained from the Genbank database, showed an intraspecific variation. Polymorphisms consisted of substitutions, insertions and deletions. As shown in Figure 7, the phylogenetic tree built with the alignment of the sequences of the dust clones together with the sequences used in the design of the previous phylogenetic tree (Figure 2), showed that the sequences of the clones were grouped into clades corresponding to each studied species, and this results was supported by high bootstrap values (100%).

Barcode gap analysis

The barcode gap analyzes of the sequences of the clones *Bt1-3*, *DBt1-3*, *Gm1-4*, *DGm1-5* and *DDp*, together with the sequences obtained in the Genbank database corresponding to the mites *D. pteronyssinus*, *B. tropicalis* and *G. domesticus*, showed that there are four distinct groups and that all clones were grouped according to their species. As it can be seen in the

boxplot showed at Figure 8, the interspecific variation is greater than the intraspecific variation, allowing us to infer that each group is a species and the amplified regions can be considered robust enough to identify and separate mites from different species.

Discussion

Mites play a role in triggering allergic reactions worldwide, being *B. tropicalis* and *D. pteronyssinus* the mites of greatest medical importance (CHEW et al., 1999b; KUO et al., 1999; YADAV; NAIDU, 2015; ALIMUDDIN et al., 2018). Morphological identification is a time-consuming and ambiguous technique, and cannot be applied to mite extracts or poorly preserved environmental samples. Some studies have shown that the SM *G. malaysiensis* has a role in triggering allergy manifestations (CHEW et al., 1999b; COLLOFF, 2009b; TANG et al., 2011). Furthermore, because they are morphologically similar (COLLOFF, 2009b; LING et al., 2019), *G. malaysiensis* and *B. tropicalis*, can represent an additional challenge for morphological identification, which can lead to misidentifications and mistaken cultivation of these species. To overcome these problems and check the frequency of the mites studied in the city of Salvador, northeastern Brazil, we developed a molecular technique based on rDNA-ITS for the identification of these species.

The full-length ITS2 region flanked by partial sequences of 5.8S and 28S subunits were obtained for the studied mites and publicly deposited in the GenBank database, and this is the the first description for *G. malaysensis*. Furthermore, it was the first time that a mPCR was designed to identify the *B. tropicalis, D. pteronyssinus* and *G. malaysensis* and to demonstrate their frequencies; it was also the first time that *G. malaysensis* was identified in Salvador, northeast Brazil.

In this study, with the objective to amplify the ITS regions, PCRs with the combination of primers Fnav and Rnav2 were performed. However, we were not able to amplify the rDNA of *B. tropicalis* – contrary to Beroiz et al. (2014), that amplified the rDNA of this mite – and *G. malaysensis*. For *B. tropicalis*, there was no band on the gel after PCR and for *G. malaysensis* there was a band, which sequencing proved to be fungal rDNA. As this combination of primers is not species-specific and the sequences of the ribosomal subunits share several similarities between different taxonomic groups, the PCR product generated fungal rDNA, used as food for mites in culture, together with Astigmata rDNA. For overcoming this problem, we performed a nested PCR with the primer pair ITS2 f and ITS2 r. The sequencing of the nested PCR products showed that it was Astigmata rDNA. Thus, these data indicate that a nested PCR using the ITS2 f/ITS2 r primers after amplification with the Fnav/Rnav2 primer pair may be

interesting to amplify the ITS2 region and increased both the detection power and the specificity of the technique.

The sequences obtained in this study were used in phylogeny and species identification. All of them had intraspecific polymorphisms and, therefore, required a cloning step for its obtaining. This can increase the time to obtain the sequence and consequent identification of a given mite of interest, however, after the design of the primers and standardization of the assay, the identification by molecular techniques is more practical than the morphological identification. Nonetheless, the polymorphisms did not interfere in the identification of the species, as validated by the barcoding gap analysis and by the phylogenetic tree, where the clones and co-specific sequences of each species clustered together, confirming the effectiveness of mPCR identification. These result are supported by the findings of Beroiz et al. (2014), in which the sequences of the clones obtained for 13 species of mites, including *B. tropicalis* and *D. pteronyssinus*, clustered together in the phylogenetic tree, despite being polymorphic.

It is worth noting that the co-specific rDNA sequences obtained from the Genbank database (BEROIZ et al., 2014) of mites from other geographic regions also clustered with the sequences generated herein. The present results support that the ITS2 region presents a good resolution for species discrimination and a little intra species variation that did not interfered with the identification at the species level. These results corroborate the data published by Noge et al. (2005), who concludes that, for some species of Astigmata mites, including *B. tropicalis* and *D. pteronyssinus*, the variation between geographically isolated strains was very similar to that within strains, and that, therefore, the ITS2 region can be reliably used for the identification of these mites. The ITS2 region has been widely used for the identification of *B. tropicalis* and *D. pteronyssinus* by different techniques (WONG et al., 2011; THET-EM et al., 2012; BEROIZ et al., 2014). In the case of *G. malaysensis*, further analysis are necessary to verify whether paralogous copies of the rDNA-ITS2 have been sufficiently homogenized by evolutionary processes (RICH et al., 1997). Furthermore, it is necessary to evaluate more sequences from different populations of this mite to see if the variations between geographically isolated populations interferes with molecular identification, which could generate inaccurate phylogenetic relationships.

In the present study, we describe an mPCR assay capable of identifying *B. tropicalis* and *D. pteronyssinus*, mites, of known medical importance, and *G. malaysensis* mite, whose role in allergy is currently poor explored, in environmental samples and in culture. The evaluation of the frequency of these mites in twenty samples of domestic dust revealed that the

most abundant one was *B. tropicalis* (see Figures 5 and 6), a result consistent with previous works investigating the frequency of mites in tropical regions (PUERTA et al., 1993; CHEW et al., 1999a; BAQUEIRO et al., 2006). Due to the high prevalence of *B. tropicalis* over other mites, favored by the tropical climate of Brazil that provides the ideal conditions for this mite survival, all samples that harbored *G. malaysensis* and/or *D. pteronyssinus* also contained *B. tropicalis* (Figure 6). Baqueiro et al. (2006) morphologically identified mites at 101 residences in the city of Salvador, Brazil, finding that the most frequent mite was *B. tropicalis* (71.8%) followed by *D. pteronyssinus* (39.9%). Despite the difference in the number of samples analyzed between the studies, we attribute the increase in the frequency of *B. tropicalis* and *D. pteronyssinus* found in the present study to the sensitivity of the mPCR assay. Although the detection limit of the assay (Figure 4) for *D. pteronyssinus* was 10 times greater than for *B. tropicalis* and *G. malaysensis*, *B. tropicalis* and *G. malaysensis*, in that order, were the most found mites. This indicates that the mPCR assay reflected the actual frequency of these mites in environmental samples from this city. We assume that the increase in the detection limit for *G. malaysensis* when using the DNA mixture of the mites in comparison with dilutions only with *G. malaysensis* template, probably is a consequence of primer dimer formation. PCR reactions that contain the DNA mixture consume more primers, making them less available for dimer formation, which must have increased the detection limit for this mite.

Previous studies, using morphological identification, also reported the presence of the *G. malaysensis* mites in domestic dust samples. *G. malaysensis* was found in Singapore in approximately 35% of the samples (CHEW et al., 1999a) and in 40% of the households in Klang Valley, Malaysia (MARIANA et al., 2000). Miranda et al. (2002) found *G. malaysensis* for the first time in Panama, in 6.37% of the samples collected in urban areas. As *G. malaysensis* was found in a significant part of the samples (70%) in our study, a frequency even higher than that found for *D. pteronyssinus*, and it was not described by previous studies on the prevalence of HDM in Brazil (BINOTTI et al., 2001; BAQUEIRO et al., 2006), we infer that the ambiguity arising from the morphological identification used by these works to identify mite species may explain this apparent contradiction. In addition to *G. malaysensis* being morphologically very similar to *B. tropicalis* (CHEW et al., 1999a; COLLOFF, 2009b; LING et al., 2019), which can lead to its misidentification as *B. tropicalis*, is also allergenic. Indeed, Chew et al. (1999a) showed that 78.2% of asthma and/or allergic rhinitis patients in Singapore were skin prick positive for *G. malaysensis*. Moreover, *G. malaysensis* and *B. tropicalis* share similar antigens, leading to the production of cross-reactive antibodies (CHEW et al., 1999b; TANG et al., 2011).

Thus, further studies in Brazil must include *G. malaysensis* as a possible inductor or allergic reactions.

Some identification approaches based the ITS and Cox I regions have been carried out previously. Yang et al. (2011) sequenced the ITS2 and Cox I regions aiming the identification of six species of astigmatid mites, among them *D. pteronyssinus* and *B. tropicalis*. Further, Navajas et al. (1999) sequenced the ITS1 and ITS2 regions to explore the variation of these sequences, showing their reliability in the phylogenetic identification of six mites species belonging to the Phytoseiidae family with commercial importance. Some studies used the Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) for the identification of mites. The PCR-RFLP assay based on the ITS2 region was used to identify 6 species of mites, including *D. pteronyssinus*, *B. tropicalis* and *G. malaysiensis* (Wong et al. 2011). Previously to enzymatic digestion, the ITS2 region of the mites was amplified with the primers ITS2 f and ITS2 r; the sizes of the PCR products for *B. tropicalis* and *G. malaysensis* found here are in accordance with those found in this study. Beroiz et al. (2014), also using PCR-RFLP, identified, in culture and environmental samples, 13 species of mites used for allergic extract production. Nevertheless, there are some disadvantages of using PCR-RFLP for species identification. One of them derives from the fact that it demands the use of restriction enzymes, which can make the process more expensive and time consuming, since the digestion step can take two to sixteen hours. Moreover, due to the fact that the primers used by these studies are not species-specific – especially the combination of primers Fnav and Rnav2, which, as shown here, can amplify fungal rDNA –, there is a chance of ambiguity and errors in the interpretation of the digestion profiles. In this way, more effective approaches should include the design of specific primers for identify the species of interest.

To date, Thet-Em et al. (2012) was the only study that used a mPCR to identify *B. tropicalis* and *D. pteronysinus* in environmental samples. To assess the prevalence of the aforementioned mites and *D. farinae* from house dust samples at Thailand, the mPCR assay used three primer pairs for species-specific amplification of the ITS2 region of *D. pteronyssinus* and *D. farinae*, and Cox I for *B. tropicalis*. However, the primer sequence was not public available, contrary to our study, where the sequences of the 10 designed primers were made available. Moreover, Thet-Em et al. (2012) verified the frequency of the above mentioned mites using 30 dust samples. Despite testing the specificity of primers against DNA of several species present in the dust, no PCR product was sequenced to attest the specificity of this technique. In our work, the sequencing of nine bands was performed, and the results, both the barcode gap (Figure 8) and the phylogenetic tree (Figure 7), support the species-specificity of the designed

primers. Household dust samples can contain DNA from several species, which can vary among geographic regions (BARBERÁN et al., 2015). Some of these species may even be unknown or, when known, may not have the genetic sequence available, and in this case we recommend the sequencing of the mPCR products. Besides enabling the identification of three species of mites in the same assay, another advantage of mPCR is practicality, where the outputs are generated in less than three hours, which is an advantage over studies that used PCR-RFLP. Furthermore, our mPCR test requires a small amount of DNA for identification (just one mite is enough), which is important if few specimens are available in the laboratory.

The mPCR assay developed herein proved to be a reliable, fast and sensitive technique for the identification of these mites in poorly preserved environmental samples, in culture or individual mites. We identified the *G. malaysensis* mite for the first time in Brazil, a mite that has already been identified as a cause of allergies in other regions of the world, and sequenced its ITS2 region. This mite was found in most of the analyzed dust samples, many of them also harboring *B. tropicalis*, which can lead to morphological misidentification. The mPCR assay developed here can be used to solve this problem, being useful as a practical alternative to the identification and morphological certification of the studied species. Thus, this technique can be used as a quality control for the production of extracts to be used in clinical. Moreover, it may be useful in future epidemiological or diagnostic studies to assess the prevalence and frequency of these mite species.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by grant from FAPESB APP0004/2016, VSA was recipient of a fellowship from the Coordination for the Improvement of Higher Education Personnel (CAPES).

References

Alimuddin S, Rengganis I, Rumende CM, Setiati S (2018) Comparison of Specific Immunoglobulin E with the Skin Prick Test in the Diagnosis of House Dust Mites and Cockroach Sensitization in Patients with Asthma and/or Allergic Rhinitis. Acta Med Indones 50:125–131

562 Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol
563 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

564 Baqueiro T, Carvalho FM, Rios CF, et al (2006) Dust mite species and allergen concentrations
565 in beds of individuals belonging to different urban socioeconomic groups in Brazil. J Asthma
566 43:101–105. https://doi.org/10.1080/02770900500497958

567 Barberán A, Dunn RR, Reich BJ, et al (2015) The ecology of microscopic life in household
568 dust. Proc R Soc B Biol Sci 282:. https://doi.org/10.1098/rspb.2015.1139

569 Barnes RD (1989) Diversity of organisms: How much do we know? Integr Comp Biol 29:1075–
570 1084. https://doi.org/10.1093/icb/29.3.1075

571 Beroiz B, Couso-Ferrer F, Ortego F, et al (2014) Mite species identification in the production
572 of allergenic extracts for clinical use and in environmental samples by ribosomal DNA
573 amplification. Med Vet Entomol 28:287–296. https://doi.org/10.1111/mve.12052

574 Binotti RS, Muniz JR, Paschoal IA, et al (2001) House dust mites in Brazil--an annotated
575 bibliography. Mem Inst Oswaldo Cruz 96:1177–1184. https://doi.org/10.1590/S0074-
576 02762001000800027

577 Borish L, Joseph BZ (1992) Inflammation and the allergic response. Med Clin North Am
578 76:765–787. https://doi.org/10.1016/S0025-7125(16)30325-X

579 Caraballo L, Puerta L, Martinez B, Moreno L (1994) Identification of-allergens from the mite
580 Blomia tropicalis. Clin Exp Allergy 24:1056–1060. https://doi.org/10.1111/j.1365-
581 2222.1994.tb02743.x

582 Chew FT, Lim SH, Goh DYT, Lee BW (1999a) Sensitization to local dust-mite fauna in
583 Singapore. Allergy Eur J Allergy Clin Immunol 54:1150–1159. https://doi.org/10.1034/j.1398-
584 9995.1999.00050.x

585 Chew FT, Zhang L, Ho TM, Lee BW (1999b) House dust mite fauna of tropical Singapore.
586 Clin Exp Allergy 29:201–206. https://doi.org/10.1046/j.1365-2222.1999.00493.x

587 Colloff MJ (2009) Identification and taxonomy, classification and phylogeny. Springer,
588 Dordrecht

589 Colloff MJ (1998) Taxonomy and identification of dust mites. Allergy 53:7–12.
590 https://doi.org/10.1111/j.1398-9995.1998.tb04989.x

591 Colloff MJ, Spieksma FTM (1992) Pictorial keys for the identification of domestic mites. Clin
592 Exp Allergy 22:823–830. https://doi.org/10.1111/j.1365-2222.1992.tb02826.x

593 Fernández-Caldas E, Puerta L, Caraballo L (2014) Mites and allergy. Chem Immunol Allergy
594 100:234–242. https://doi.org/10.1159/000358860

595 Frankland AW (1971) Asthma and mites. Postgrad Med J 47:178–180.
596 https://doi.org/10.1136/pgmj.47.545.178

597 Groombridge B (1993) Global biodiversity: Status of the earth's living resources

598 Hall T. (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis
599 Program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98.
600 https://doi.org/10.14601/Phytopathol_Mediterr-14998u1.29

601 Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic
602 inference. Q Rev Biol 66:411–53. https://doi.org/10.1086/417338

603 Jeebhay MF, Baatjies R, Chang YS, et al (2007) Risk factors for allergy due to the two-spotted
604 spider mite (Tetranychus urticae) among table grape farm workers. Int Arch Allergy Immunol
605 144:143–149. https://doi.org/10.1159/000103226

606 Koistinen T, Ruoppi P, Putus T, et al (2006) Occupational sensitization to storage mites in the
607 personnel of a water-damaged grocery store. Int Arch Occup Environ Health 79:602–606.
608 https://doi.org/10.1007/s00420-006-0097-4

609 Kuo IC, Yi FC, Cheong N, et al (1999) Sensitization to Blomia tropicalis and
610 Dermatophagoides pteronyssinus - A comparative study between Singapore and Taiwan. Asian
611 Pacific J Allergy Immunol 17:179–188

612 Ling SJ, Wong SF, Mak JW, Ho TM (2019) Morphology of glycycometus malaysiensis, a
613 domestic mite in malaysia. Trop Biomed 36:263–273

614 Mariana A, Ho TM, Sofian-Azirun M, Wong AL (2000) House dust mite fauna in the Klang
615 Valley, Malaysia. Southeast Asian J Trop Med Public Health 31:712–721

616 Miranda JR, QUINTERO A. D, ALMANZA A (2002) House dust mites from urban and rural
617 houses on the lowland Pacific slopes of Panama. Syst Appl Acarol 7:23.
618 https://doi.org/10.11158/saa.7.1.3

619 Nadchatram M (2005) House dust mites, our intimate associates. Trop Biomed 22:23–37

620 Navajas M, Fenton B (2000) The application of molecular markers in the study of diversity in
621 acarology: A review. Exp Appl Acarol 24:751–774. https://doi.org/10.1023/A:1006497906793

622 Navajas M, Lagnel J, Fauvel G, De Moraes G (1999) Sequence variation of ribosomal Internal
623 Transcribed Spacers (ITS) in commercially important Phytoseiidae mites. Exp Appl Acarol
624 23:851–859. https://doi.org/10.1023/A:1006251220052

625 Navajas M, Lagnel J, Gutierrez J, Boursot P (1998) Species-wide homogeneity of nuclear
626 ribosomal ITS2 sequences in the spider mite Tetranychus urticae contrasts with extensive
627 mitochondrial COI polymorphism. Heredity (Edinb) 80:742–752.
628 https://doi.org/10.1046/j.1365-2540.1998.00349.x

629 Noge K, Mori N, Tanaka C, et al (2005) Identification of astigmatid mites using the second
630 internal transcribed spacer (ITS2) region and its application for phylogenetic study. Exp Appl
631 Acarol 35:29–46. https://doi.org/10.1007/s10493-004-1953-0

632 Palyvos NE, Emmanouel NG, Saitanis CJ (2008) Mites associated with stored products in
633 Greece. Exp Appl Acarol 44:213–226. https://doi.org/10.1007/s10493-008-9145-y

634 Puerta L, Mercado D, Lockey RF, Caraballo LR (1993) Mite fauna, Der p I, Der f I and Blornia
635 tropicalis allergen levels in a tropical environment. Clin Exp AlIergy 23:292–297

636 Rich SM, Rosenthal BM, Telford SR, et al (1997) Heterogeneity of the internal transcribed
637 spacer (ITS-2) region within individual deer ticks. Insect Mol Biol 6:123–129.
638 https://doi.org/10.1111/j.1365-2583.1997.tb00080.x

639 Ruoppi P, Koistinen T, Pennanen S (2005) Sensitisation to mites in laboratory animal workers
640 with rhinitis. Occup Environ Med 62:612–615. https://doi.org/10.1136/oem.2004.015685

641 Schoch CL, Seifert KA, Huhndorf S, et al (2012) Nuclear ribosomal internal transcribed spacer
642 (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A
643 109:6241–6246. https://doi.org/10.1073/pnas.1117018109

644 Solarz K, Szilman P, Szilman E (2004) OCCUPATIONAL EXPOSURE TO ALLERGENIC
645 MITES IN A POLISH ZOO. Ann Agric Env Med 11:27–33

646 Stejskal V, Hubert J (2008) Risk of Occupational Allergy To Stored Grain Arthropods. 29–35

647 Storaas T, Steinsvåg SK, Florvaag E, et al (2005) Occupational rhinitis: Diagnostic criteria,
648 relation to lower airway symptoms and IgE sensitization in bakery workers. Acta Otolaryngol
649 125:1211–1217. https://doi.org/10.1080/00016480510044205

650 Szilman P, Szilman E, Szilman M, et al (2006) Occupational exposure to allergenic mites
651 among workers of the Silesian Zoo. Biol Lett 43:375–380

652 Tang JC, Wong SF, Mak JW, Ho TM (2011) Antigenic profile of Blomia tropicalis,
653 Aleuroglyphus ovatus and Glycycometus malaysiensis. Trop Biomed 28:223–236

654 QGIS Development Team, 2020. QGIS Geographic Information System. Open Source
655 Geospatial Foundation. URL http://qgis.org

656 R Core Team (2013) R: A language and environment for statistical computing. R Foundation
657 for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

658 Thet-Em T, Tungtrongchitr A, Tiewcharoen S, Malainual N (2012) Multiplex PCR for
659 identifying common dust mites species (Dermatophagoides pteronyssinus, Dermatophagoides
660 farinae and Blomia tropicalis). Asian Pacific J allergy Immunol 30:224–30

661 Thomas WR, Hales BJ, Smith WA (2010) House dust mite allergens in asthma and allergy.
662 Trends Mol Med 16:321–328. https://doi.org/10.1016/j.molmed.2010.04.008

663 van Bronswijk JE, de Cock AW (1974) The genus Blomia Oudemans (Acari : Glycyphagidae).
664 II. Comparison of its species. Acarologia 15:490–505

665 van Hage-Hamsten M, Johansson SGO (1992) Storage mites. Exp Appl Acarol 16:117–128.
666 https://doi.org/10.1007/BF01201495

667 Vidal C, Boquete O, Gude F, et al (2004) High prevalence of storage mite sensitization in a
668 general adult population. Allergy Eur J Allergy Clin Immunol 59:401–405.
669 https://doi.org/10.1111/j.1398-9995.2003.00430.x

670 Walter DE, Proctor HC (2003) Biology, ecology, and prevalence of dust mites

671 Wen WN, León PE, Hague DR (1974) Multiple gene sites for 5S And 18 + 28S RNA on
672 chromosomes of Glyptotendipes Barbipes (staeger). J Cell Biol 62:131–144.
673 https://doi.org/10.1083/jcb.62.1.132

674 Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Use R! Springer International
675 Publishing : Imprint: Springer, Cham.

676 Wong SF, Chong AL, Mak JW, et al (2011) Molecular identification of house dust mites and
677 storage mites. Exp Appl Acarol 55:123–133. https://doi.org/10.1007/s10493-011-9460-6

678 Yadav A, Naidu R (2015) Clinical manifestation and sensitization of allergic children from
679 Malaysia. Asia Pac Allergy 5:78. https://doi.org/10.5415/apallergy.2015.5.2.78

680 Yang B, Cai J, Cheng X (2011) Identification of astigmatid mites using ITS2 and COI regions.
681 Parasitol Res 108:497–503. https://doi.org/10.1007/s00436-010-2153-y

Pair. no	Primer name	Sequence (5′→ 3′)	Target gene	Specie	~ Product size (bp)	Thermal conditions						References
						ID	D	A	E	FE	N	
1	Fnav Rnav2	F- AGAGGAAGTAAAAGTCGTAACAAG R- ATATGCTTAAATTCAGCGGG	18S 28S	Any mite	Variable	95 °C 10 min	92 °C 30 s	48 °C 30 s	72 °C 1 min	72 °C 10 min	45	Navajas et al. (1999) Navajas et al. (1998)
2	ITS2F ITS2R	F- CGACTTTCGAACGCATATTGC R- GCTTAAATTCAGGGGGTAATCTCG	5.8S 28S			94 °C 1 min	94°C 30 s	52 °C 30 s	72 °C 1 min	72 °C 2 min	30	Noge et al. (2005)
3	BtVF_1 BtVR	F- AGTGATGGATGCAGGGTATCATT R- GTCCGATCTGTAATGCACG	ITS1 ITS2	*B. tropicalis*	482	94 °C 5 min	94 °C 30 s	58 °C 1 min	72 °C 30 s	72 °C 5 min	30	This study
4	BtVF_2 BtVR	F- ATGAAAGCCACAAACTATATGC R- GTCCGATCTGTAATGCACG	ITS2		147							
5	BtVF_3 BtVR	F- GCAAGACTCGTTCATCGTGTTG R- GTCCGATCTGTAATGCACG	ITS2		120							
6	DpVF_1 DpVR	F- GTTGGACCGAATCATGTCA R- AGGTGTTTAGCTGGAAACGC	ITS1 ITS2	*D. pteronyssinus*	593							
7	DpVF_2 DpVR	F- CGTGAATACGTCGTCAGGTC R- AGGTGTTTAGCTGGAAACGC	ITS2		115							
8	GmVF_1 GmVR	F- TGGGGACATCCTCAAGCT R- AGGTCGAACATTTACAAACAAAGTC	ITS2	*G. malaysiensis*	193							
9	GmVF_2 GmVR	F- TCGAAAGACCTGTTGTCGTG R- AGGTCGAACATTTACAAACAAAGTC	ITS2		138							

ID initial denaturation, D denaturation, A annealing, E extension, FE final extension, N number of cycles, ~ Approximate

682 **Table 1** List of the primer pairs, their sequence, target gene, target specie, size of the generated polymerase chain reaction (PCR) products, thermal
683 conditions, references.

Specie	Number of clones							Total
	D03	D04	D05	D07	D14	D17	D20	
Dp	0	1	0	0	0	0	0	1
Bt	0	1	1	0	0	1	0	3
Gm	1	1	0	1	1	0	1	5

D dust sample

Table 2 Dust samples used in the multiplex PCR assay selected for cloning and sequencing, and the number of clones sequenced for *D. pteronyssinus* (Dp), *B. tropicalis* (Bt) and *G. malaysiensis* (Gm) mite species. Dp, Bt and Gm mites had 1, 3 and 5 clones sequenced, respectively.

Figures

Fig. 1 Schematic overview of the position of the annealing sites of the primers at the rDNA and the corresponding PCR products used for identification of *D. pteronyssinus* (Dp), *B. tropicalis* (Bt) and *G. malaysiensis* (Gm) mites. The mite-specific primers targeting the end of the 18S (Fnav) and 5.8S sequences (ITS2f) and the start of the 28S region (Rnav and ITS2r) and the designed primers targeting ITS1 (for Dp and Bt) and ITS2 sequences of rDNA are shown. The dotted space at the end of the 5.8S and part of the ITS2 regions shows the region used for the phylogenetic analyzes.

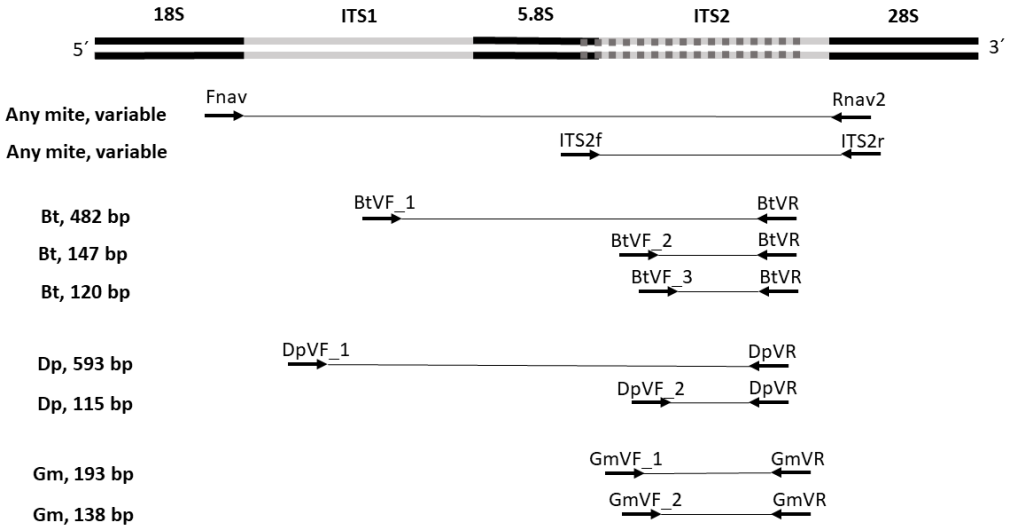
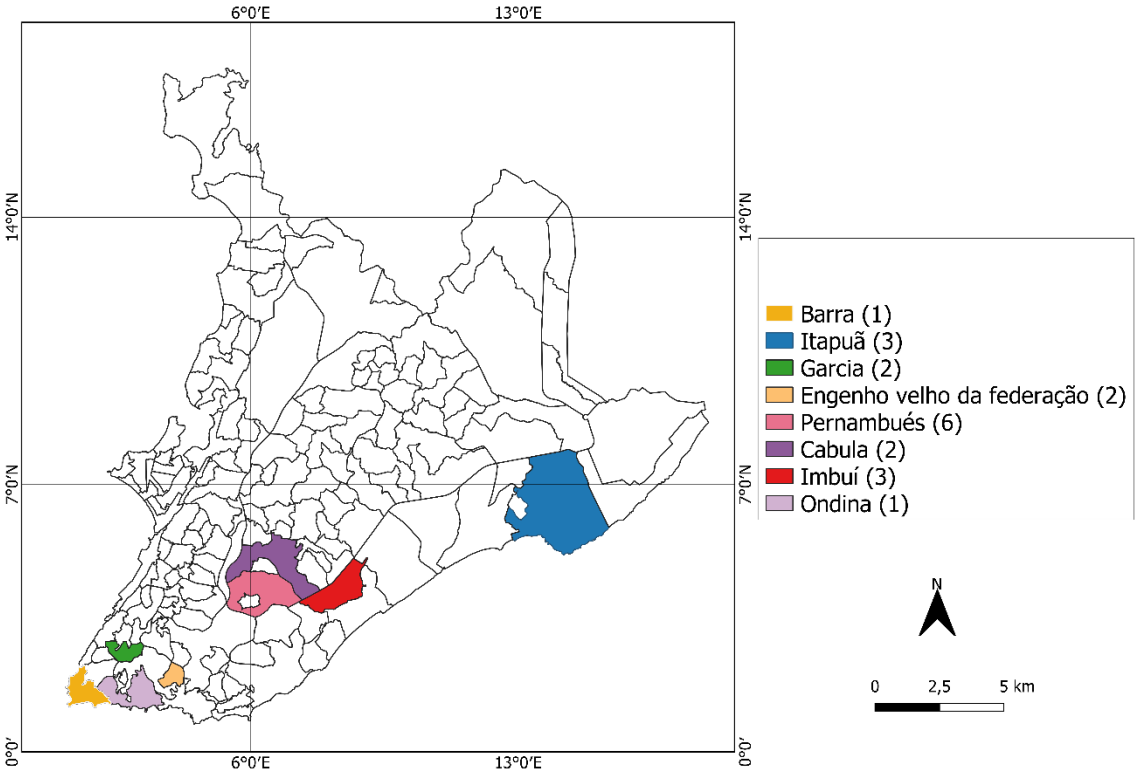


Figure 1

718 **Fig. 1S** Map of the city of Salvador, BA, showing the neighborhoods where dust samples were
719 collected, and the number of samples collected by neighborhood.



720

721 **Figure 1S**

722

723

724

725

726

727

728

729

730

Fig. 2S Panel A: electrophoresis agarose gel of the PCR products obtained with the combination of Fnav and Rnav2 primers. Lane 1 shows the 820 bp band generated for DNA extracted from the mass of *G. malaysiensis* mites. M-1000 bp size marker. Panel B: fragment pattern generated by the nested polymerase chain reaction (nested PCR) with the ITS2 f/ITS2 r primer pair. Lane 1 shows the bands generated for *G. malaysiensis*, with approximately 260 bp and lane 2 shows the bands generated for *B. tropicalis*, with approximately 350 bp. M-100 bp size marker, N negative control.

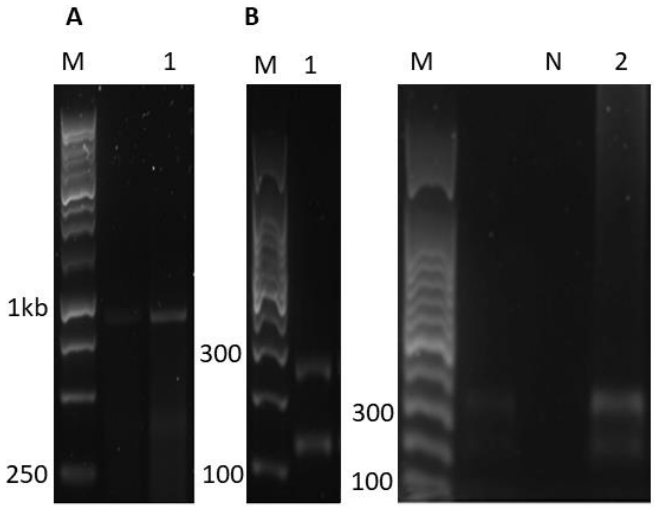


Figure 2S

Fig. 2 Phylogenetic tree generated by the Neighborhood Joining method using the alignment of the sequences of *Bt1-3* and *Gm1-4* clones, for Bt and Gm mites, respectively, and representative sequences available in GenBank database (Beroiz et al. 2014) for Bt, Dp and *G. domesticus* mites. The sequences consist of the ITS2 region flanked by partial sequences of 5.8S and 28S subunits. Bootstrap values correspond to 1000 replicates.

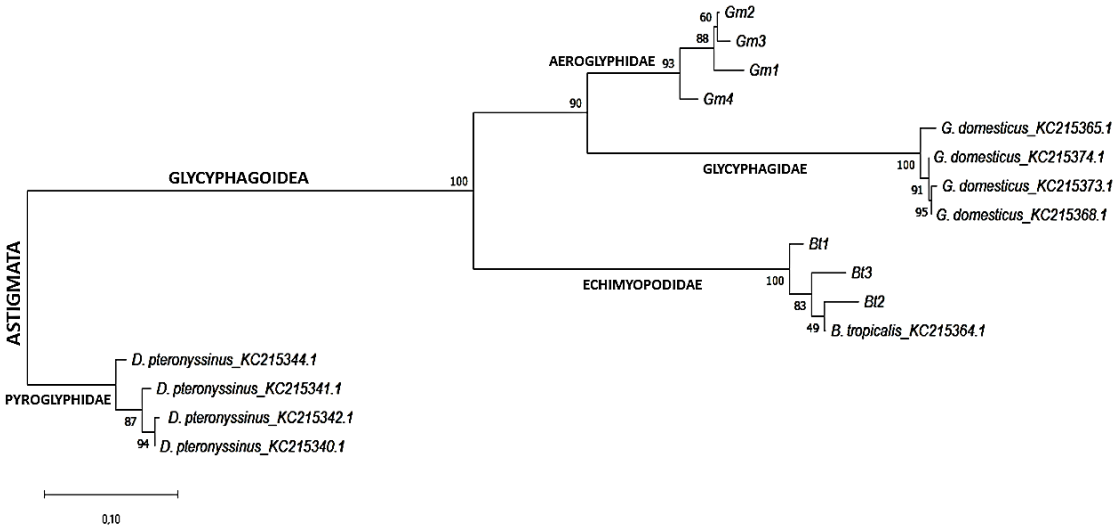
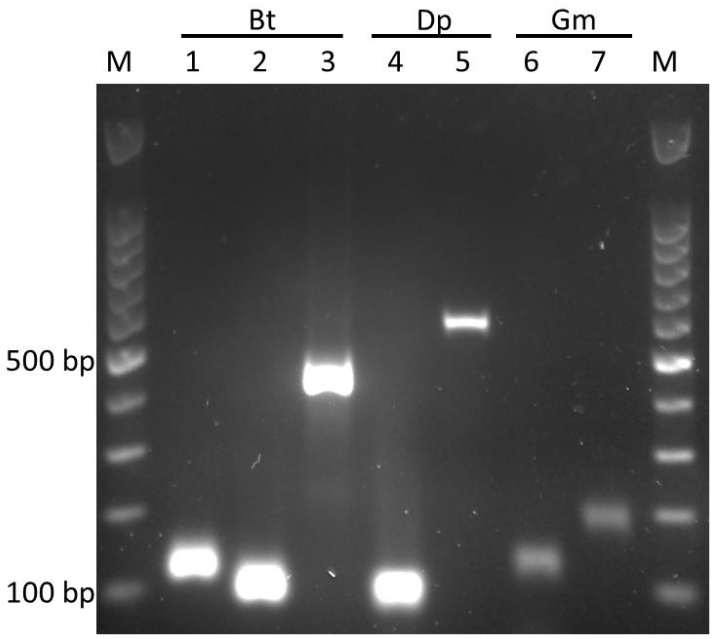


Figure 2

766 **Fig 3** Polymerase chain reaction (PCR) showing the band pattern generated by the designed
767 mite specie-specifics primers. Lanes 1-3 correspond to the primer pairs designed for *B.*
768 *tropicalis* (Bt): 4 (lane 1, 147 base pairs), 5 (2, 120) and 3 (3, 482), respectively. Lanes 4-5
769 correspond to the primer pairs designed for *D. pteronyssinus* (Dp): 7 (4, 115) and 6 (5, 593),
770 respectively. While lanes 6-7 correspond to the primer pairs designed for *G. malaysiensis* (Gm):
771 9 (6, 138) and 8 (7, 193), respectively. M-100 bp size marker.



772

773 **Figure 3**

774

775

776

777

778

779

780

781

Fig. 4 Results showing the detection limits of multiplex PCR for *D. pteronyssinus* (Dp), *B. tropicalis* (Bt) and *G. malaysiensis* (Gm) mites. Panel A: when using Dp DNA, the detection limit was 0.01 ng. Panel B: when Bt DNA was used, the detection limit was 0.1 ng. Panel C: when Gm DNA was used, the detection limit was 1 ng. Panel D shows the detection limits when a mixture containing the same amount of DNA of Dp, Bt and Gm was used. The detection limits were 0.01 ng for Dp and 0.1 ng for Bt and Gm. Lane M-100 bp size marker, lane N negative control.

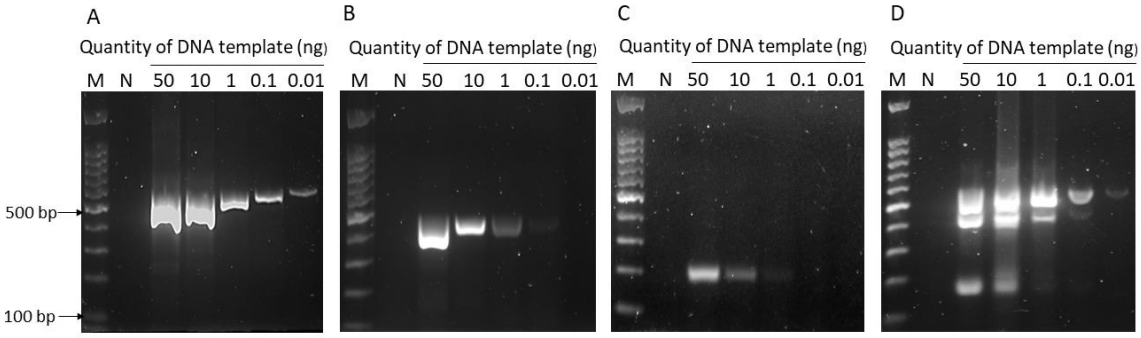


Figure 4

Fig 5 Multiplex polymerase chain reactions (mPCR) using 100 ng of DNA extracted from 20 dust samples (Lane 1-20) showing the positivity of the samples for the presence of *D. pteronyssinus*, *B. tropicalis* and *G. malaysiensis* mites. Lane M 100 bp size marker, lane N negative control.

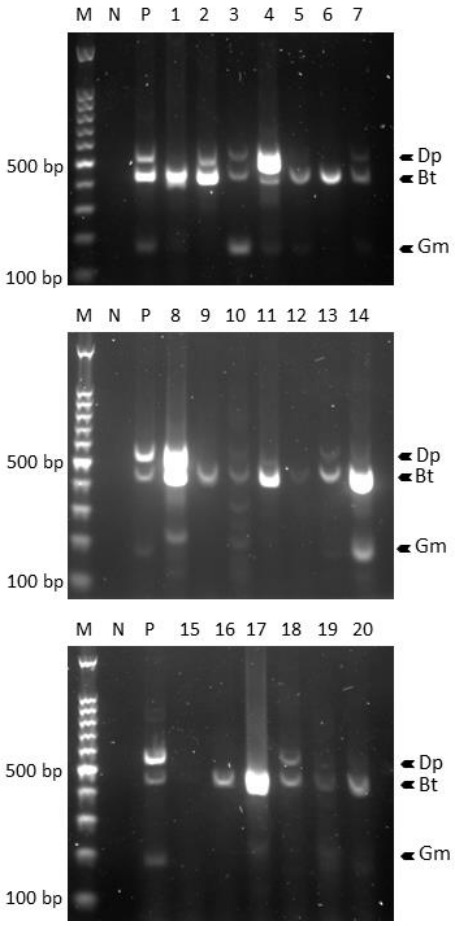


Figure 5

Fig. 6 Venn diagram showing the positivity of the 20 dust samples analyzed in the multiplex polymerase chain reaction (mPCR). 95% of the samples were positive for Bt, 70% for Gm and 60% for Dp. 20% of the samples were positive just for Bt, 5% of the samples were positive only for Dp and Bt and 15% for Bt and Gm. 55% were positive for all studied mites.

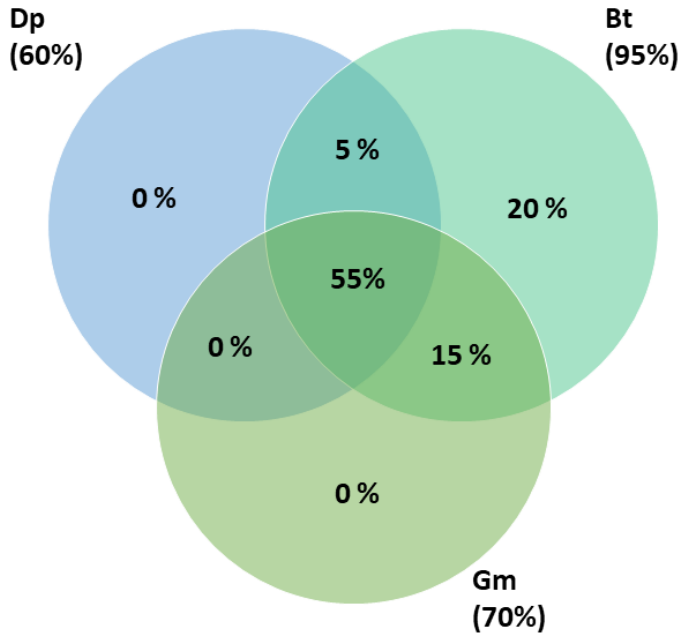


Figure 6

Fig. 7 Phylogenetic tree generated by the Neighborhood Joining method from the alignment of ITS2 rDNA region flanked by partial sequences of 5.8S and 28S subunits, containing sequences obtained herein (*Bt1-3* and *Gm1-4*), representative sequences available in the GenBank database (BEROIZ et al., 2014) and sequences obtained from dust clones for Bt (*DBt1-3*), Gm (*DGm1-5*) and Dp (*DDp*). The sequences obtained for the *DDp* and *DBt1-3* dust clones were trimmed, containing the end of the 5.8S region and partial ITS2 (see Figure 1). Bootstrap values correspond to 1000 replicates.

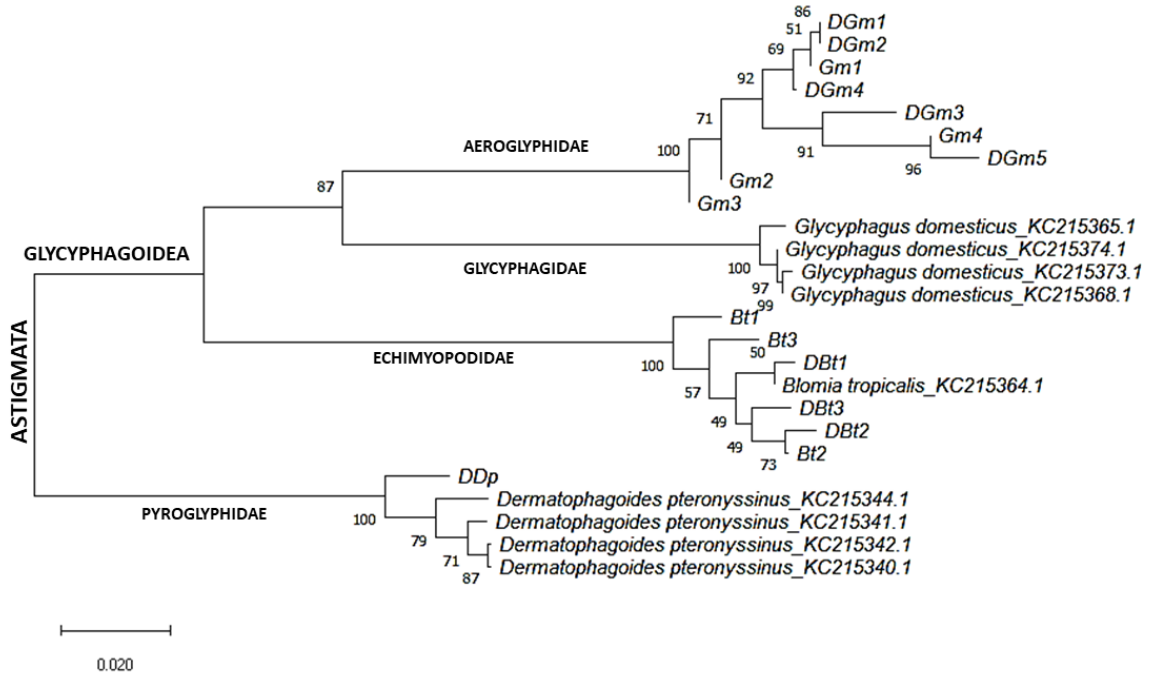


Figure 7

Fig. 8 Barcode gap analysis for the species Bt, Dp, Gm and *G. domesticus*, based on the ITS2 region and the intra and interspecific distances. Genetic distances are plotted in the axis Y. To assess the reliability of the data, the *Wilcoxon test* was performed.

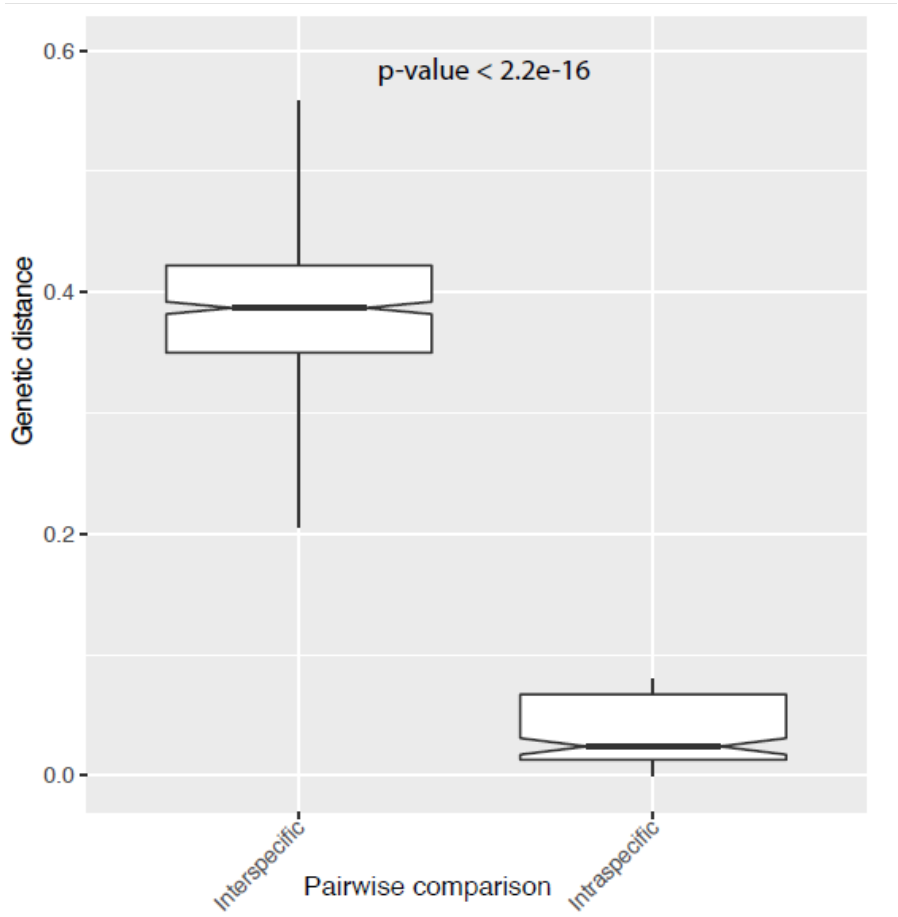


Figure 8

4. CONSIDERAÇÕES FINAIS

A identificação morfológica requer o espécime intacto, de preferência adulto, o que nem sempre é possível. Quando obtidos de amostras ambientais os ácaros são muitas vezes encontrados na forma de ovo, larva e ninfa, sendo um desafio para esse tipo de abordagem, o que é agravado quando os ácaros são morfologicamente parecidos, como é o caso das espécies *B. tropicalis* e *G. malaysiensis*, levando ao cultivo incorreto dessas espécies. Além desses desafios, o tempo gasto com a identificação morfológica é de até três semanas – compreendendo o tempo para a expansão do espécime encontrado, permitindo a obtenção de mais exemplares, e o tempo de preparar a lâmina e deixar agir os reagentes (meio de montagem de Hoyer, agente de Nesbitt ou lactofenol e 95-100% etanol), para que a análise das chaves morfológicas seja possível. Em contraste, o ensaio de multiplex PCR desenhado, é capaz de dar o resultado em até 3 horas – compreendendo a extração do DNA (35 min), PCR (1 hora e 20 min) e análise do resultado em gel de agarose (1 hora).

O ensaio de multiplex PCR se mostrou eficaz quando utilizado i) DNA extraído de ácaros individuais, ii) de massa obtida de cultura de ácaros em crescimento exponencial e iii) poeira. A especificidade da identificação pelo ensaio de multiplex PCR para as 3 espécies de ácaros estudados foi atestada pelos sequenciamentos de amostras de poeira positivas. Além disso, os resultados do sequenciamento foram utilizados para estudos filogenéticos, que mostraram que a região ITS2 apresenta uma boa resolução para identificação dessas espécies.

Depositamos as sequências parciais das subunidades 5.8S, 28S e a região completa da região ITS2 para os três ácaros estudados, sendo que esses resultados foram gerados pela primeira vez para o ácaro *G. malaysiensis*. Adicionalmente, o fragmento que compreende a sequência parcial da região ITS1 e ITS2 e a subunidade 5.8S completa foi obtido para os ácaros *D. pteronyssinus* e *B. tropicalis*. Por se tratar de sequências Brasileiras, isso pode auxiliar em futuros estudos filogenéticos para os ácaros estudados, com foco na evolução do DNA ribossomal e na identificação de polimorfismos.

Em conclusão, nosso estudo enfatizou a importância da identificação de ácaros por métodos moleculares devido à sua sensibilidade, especificidade e rapidez, como por exemplo, a técnica de mPCR aqui padronizada. Além disso, o uso desta técnica nos permitiu avaliar a frequência dessas espécies de ácaros na cidade de Salvador, Bahia, e assim demonstrar pela primeira vez a alta frequência do ácaro *G. malaysiensis* no Brasil, que já foi identificado como causador de alergias em outras regiões do mundo.

5. REFERÊNCIAS

BAQUEIRO, T. et al. Dust mite species and allergen concentrations in beds of individuals belonging to different urban socioeconomic groups in Brazil. **Journal of Asthma**, v. 43, n. 2, p. 101–105, 2006.

BARBERÁN, A. et al. The ecology of microscopic life in household dust. **Proceedings of the Royal Society B: Biological Sciences**, v. 282, n. 1814, 2015.

BEROIZ, B. et al. Mite species identification in the production of allergenic extracts for clinical use and in environmental samples by ribosomal DNA amplification. **Medical and Veterinary Entomology**, v. 28, n. 3, p. 287–296, 2014.

BINOTTI, R. S. et al. House dust mites in Brazil--an annotated bibliography. **Memórias do Instituto Oswaldo Cruz**, v. 96, n. 8, p. 1177–1184, 2001.

BORISH, L.; JOSEPH, B. Z. Inflammation and the allergic response. **Medical Clinics of North America**, v. 76, n. 4, p. 765–787, 1992. Disponível em: <http://dx.doi.org/10.1016/S0025-7125(16)30325-X>.

BOUSQUET, J.; LOCKEY, R.; MALLING, H. Allergen immunotherapy: Therapeutic vaccines for allergic diseases. A WHO position paper. **Journal of Allergy and Clinical Immunology**, v. 102, n. 4, p. 558–562, 2005.

CARABALLO, L. et al. Identification of-allergens from the mite Blomia tropicalis. **Clinical and Experimental Allergy**, v. 24, n. 11, p. 1056–1060, 1994.

CHEW, F. T. et al. House dust mite fauna of tropical Singapore. **Clinical and Experimental Allergy**, v. 29, n. 2, p. 201–206, 1999a.

CHEW, F. T. et al. Sensitization to local dust-mite fauna in Singapore. **Allergy: European Journal of Allergy and Clinical Immunology**, v. 54, n. 11, p. 1150–1159, 1999b.

COLLOFF, M. J. Taxonomy and identification of dust mites. **Allergy**, v. 53, p. 7–12, 1998.

COLLOFF, M. J. **Identification and taxonomy, classification and phylogeny**. [s.l.] Springer, Dordrecht, 2009. v. 5

COLLOFF, M. J.; SPIEKSMA, F. T. M. Pictorial keys for the identification of domestic mites.

Clinical and Experimental Allergy, v. 22, n. 9, p. 823–830, 1992.

FRANKLAND, A. W. Asthma and mites. **Postgraduate Medical Journal**, v. 47, n. 545, p. 178–180, 1971.

GÓMEZ-MARTÍNEZ, M. A. et al. Tracking mite trophic interactions by multiplex PCR. **Pest Management Science**, v. 76, n. 2, p. 597–608, 2020.

HILLIS, D. M.; DIXON, M. T. Ribosomal DNA: molecular evolution and phylogenetic inference. **The Quarterly review of biology**, v. 66, n. 4, p. 411–53, 1991. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/1784710>.

KOUZEGARAN, S. et al. Immunotherapy in allergic rhinitis: It's effect on the immune system and clinical symptoms. **Open Access Macedonian Journal of Medical Sciences**, v. 6, n. 7, p. 1248–1252, 2018.

LING, S. J. et al. Morphology of glycycometus malaysiensis, a domestic mite in malaysia. **Tropical Biomedicine**, v. 36, n. 1, p. 263–273, 2019.

MARIANA, A. et al. House dust mite fauna in the Klang Valley, Malaysia. **Southeast Asian Journal of Tropical Medicine and Public Health**, v. 31, n. 4, p. 712–721, 2000.

MIRANDA JR, QUINTERO A. D, ALMANZA A (2002) House dust mites from urban and rural houses on the lowland Pacific slopes of Panama. **Syst Appl Acarol** 7:23. https://doi.org/10.11158/saa.7.1.3.

NAVAJAS, M. et al. Sequence variation of ribosomal Internal Transcribed Spacers (ITS) in commercially important Phytoseiidae mites. **Experimental and Applied Acarology**, v. 23, n. 11, p. 851–859, 1999.

OLIVEIRA, A. S. et al. Development of a new multiplex PCR to detect prevalent species of house dust mites in house dust. **International Journal of Environmental Health Research**, p. 1–13, 2021.

OSAKABE, M. et al. Restriction fragment length polymorphism catalog for molecular identification of Japanese Tetranychus spider mites (Acari: Tetranychidae). **Journal of Economic Entomology**, v. 101, n. 4, p. 1167–1175, 2008.

PALYVOS, N. E.; EMMANOUEL, N. G.; SAITANIS, C. J. Mites associated with stored

products in Greece. **Experimental and Applied Acarology**, v. 44, n. 3, p. 213–226, 2008.

PUERTA, L. et al. Mite fauna, Der p I, Der f I and Blornia tropicalis allergen levels in a tropical environment. **Clinical and Experimental Allergy**, v. 23, p. 292–297, 1993.

SANTOS DA SILVA, E. et al. Allergens of Blomia tropicalis: An Overview of Recombinant Molecules. **International Archives of Allergy and Immunology**, v. 172, n. 4, p. 203–214, 2017.

SINAIE, S.; NAMAGHI, H. S.; FEKRAT, L. A multiplex pcr assay for simultaneous discrimination of two predominant spider mites of the genus tetranychus (Acari: Tetranychidae) in greenhouses of Iran. **Journal of Agricultural Science and Technology**, v. 20, n. 4, p. 733–744, 2018.

SYROMYATNIKOV, M. Y. et al. A simple molecular method for rapid identification of commercially used Amblyseius and Neoseiulus species (Acari: Phytoseiidae). **Zootaxa**, v. 4394, n. 2, p. 270, 2018.

TANG, J. C. et al. Antigenic profile of Blomia tropicalis, Aleuroglyphus ovatus and Glycycometus malaysiensis. **Tropical Biomedicine**, v. 28, n. 2, p. 223–236, 2011.

THET-EM, T. et al. Multiplex PCR for identifying common dust mites species (Dermatophagoides pteronyssinus, Dermatophagoides farinae and Blomia tropicalis). **Asian Pacific journal of allergy and immunology**, v. 30, n. 3, p. 224–30, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23156853>.

THIND, B. B.; CLARKE, P. G. The occurrence of mites in cereal-based foods destined for human consumption and possible consequences of infestation. **Experimental and Applied Acarology**, v. 25, n. 3, p. 203–215, 2001.

THOMAS, W. R.; HALES, B. J.; SMITH, W. A. House dust mite allergens in asthma and allergy. **Trends in Molecular Medicine**, v. 16, n. 7, p. 321–328, 2010. Disponível em: <http://dx.doi.org/10.1016/j.molmed.2010.04.008>.

VAN BRONSWIJK, J. E.; DE COCK, A. W. The genus Blomia Oudemans (Acari : Glycyphagidae). II. Comparison of its species. **Acarologia**, v. 15, n. 3, p. 490–505, 1974.

VAN HAGE-HAMSTEN, M.; JOHANSSON, S. G. O. Storage mites. **Experimental & Applied Acarology**, v. 16, n. 1–2, p. 117–128, 1992.

VOGEL, P.; DAL BOSCO, S. M.; FERLA, N. J. Ácaros Y Las Consecuencias Sobre La Salud Humana. **Nutricion Hospitalaria**, v. 31, n. 2, p. 944–951, 2015.

WALTER, D. E.; PROCTOR, H. C. **Biology, ecology, and prevalence of dust mites**. [s.l: s.n.]v. 23

WEN, W. N.; LEÓN, P. E.; HAGUE, D. R. Multiple gene sites for 5S And 18 + 28S RNA on chromosomes of Glyptotendipes Barbipes (staeger). **Journal of Cell Biology**, v. 62, n. 1, p. 131–144, 1974.

WONG, S. F. et al. Molecular identification of house dust mites and storage mites. **Experimental and Applied Acarology**, v. 55, n. 2, p. 123–133, 2011.

YANG, B.; CAI, J.; CHENG, X. Identification of astigmatid mites using ITS2 and COI regions. **Parasitology Research**, v. 108, n. 2, p. 497–503, 2011.

YANG, L.; ZHU, R. Immunotherapy of house dust mite allergy. v. 13, n. 10, p. 2390–2396, 2017.

ZÉLÉ, F.; WEILL, M.; MAGALHÃES, S. Identification of spider-mite species and their endosymbionts using multiplex PCR. **Experimental and Applied Acarology**, v. 74, n. 2, p. 123–138, 2018. Disponível em: <https://doi.org/10.1007/s10493-018-0224-4>.