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Identification of *Glycycometus malaysiensis* (for the first time in Brazil), *Blomia tropicalis* and *Dermatophagoides pteronyssinus* through multiplex PCR

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Abstract
Blomia tropicalis and *Dermatophagoides pteronyssinus* play an important role in triggering allergy. *Glycycometus malaysiensis* causes IgE reaction in sensitive people, but is rarely reported in domestic dust, because it is morphologically similar to *B. tropicalis* making the identification of these species difficult. The identification of mites is mostly based on morphology, a time-consuming and ambiguous approach. Herein, we describe a multiplex polymerase chain reaction (mPCR) assay based on ribosomal DNA capable to identify mixed cultures of *B. tropicalis*, *D. pteronyssinus* and *G. malaysiensis*, and/or to identify these species from environmental dust. For this, the internal transcribed spacer 2 (ITS2) regions, flanked by partial sequences of the 5.8S and 28S genes, were PCR-amplified, cloned and 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 used to verify the frequency of different mites in house dust samples ($n = 20$) from homes of Salvador, Brazil. *Blomia tropicalis* was the most frequent, 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 in mixed cultures and could be applied in future epidemiological studies, and for quality control of mite extract production for general use.

Keywords Allergy · Multiplex PCR · Species identification · *Blomia tropicalis* · *Glycycometus malaysiensis* · *Dermatophagoides pteronyssinus*

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Introduction

The subclass Acari (Arthropoda: Chelicerata) includes ca. 54,617 described species (Zhang 2011), although the number of mite species is estimated at around 1 million worldwide (Walter and Proctor 2013). Based on their habits, some mites may be classified as storage mites and as house dust mites. *Lepidoglyphus destructor* (Schrank) and *Glycyphagus domesticus* (De Geer) (both Glycyphagidae) are considered as storage mites, as they are commonly found in wheat, corn and barley storage facilities (van Hage-Hamsten and Johansson 1992; Arlian and Morgan 2003; Palyvos et al. 2008; Ling et al. 2019). *Dermatophagoides farinae* (Hughes) and *Dermatophagoides pteronyssinus* (Trouessart) (Pyroglyphidae) are regarded as important house dust mites, commonly found in pillows, mattresses and house carpets worldwide (Nadchatram 2005). In tropical and subtropical countries (Chew et al. 1999b; Kuo et al. 1999; Yadav and Naidu 2015; Alimuddin et al. 2018), including Brazil (Baqueiro et al. 2006), *Blomia tropicalis* (Van Bronswijk, Cock & Oshima) (Echymyopodidae) along with *D. pteronyssinus* are the most medically important mites described so far (Hurtado and Parine 1987; Arlian et al. 1992; Mariotta et al. 1993; Caraballo et al. 1998).

Since the first descriptions of the allergenic roles of mites, in the mid-1960s, numerous mite species were described as being sources of allergens in house dust worldwide (Fernández-Caldas et al. 2014). House dust mites live in human habitats and may trigger allergic manifestations (e.g., allergic/atopic asthma, allergic rhinitis and atopic eczema) (Frankland 1971; Thomas et al. 2010). Mite antigens are able to bind immunoglobulin E (IgE) (Caraballo et al. 1994) that 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 and Joseph 1992).

Nevertheless, some species classified as storage mites are also capable of inducing sensitization and causing allergic respiratory symptoms (Solarz et al. 2004; Vidal et al. 2004; Ruoppi et al. 2005; Storaas et al. 2005; Koistinen et al. 2006; Jeebhay et al. 2007), causing occupational allergies. Some occupational and non-occupational allergy reports have been associated with the poorly known mite *Glycycometus malaysiensis* (Fain & Nadchatram) (=*Austroglycyphagus malaysiensis*) (Aeroglyphidae) (Baratawidjaja et al. 1999; Chew et al. 1999a; Tang et al. 2011; Chong et al. 2015). In addition, it has been suggested that *G. malaysiensis* allergens may cross-react with *B. tropicalis* allergens, as IgE levels for *B. tropicalis* and *G. malaysiensis* were highly correlated (Chew et al. 1999a).

Traditionally, the identification of mite species is done based on morphological characters provided by original description of taxa, taxonomic revisions and identification keys, sometimes aided by observed ecological and biological traits (Colloff 2009). However, taxonomic identification based on morphological characters may present several drawbacks, such as the need for certain developmental stages and preserved and well mounted individuals, for example. In addition, the use of morphological characters for identification of several mite groups is frequently hampered by factors such as cryptic and polytypic species, phenotypic plasticity and polymorphisms (Houck and OConnor 1991; Athias-Binche 1995; Radwan 2003; Gwiazdowicz 2004; Klimov et al. 2004; Skoracka et al. 2015; Solarz et al. 2016; Proctor and Knee 2018; Stefan et al. 2018). Besides, the presence of morphologically similar species may lead to misidentification (Colloff and Spieksma 1992; Colloff 1998, 2009).

Due to these drawbacks, molecular techniques based on ribosomal DNA (rDNA) and cytochrome oxydase subunit 1 (*coxI*) have been used as important tools on mite identification and phylogenetic analysis (Lava Kumar et al. 1999; Navajas and Fenton 2000; Vargas et al. 2005; Ben-David et al. 2007; De Rojas et al. 2007; Osakabe et al. 2008; Wong et al. 2011; Yang et al. 2011; Matsuda et al. 2012; Thet-Em et al. 2012; Arimoto et al. 2013; Roussel et al. 2013; Stephenson et al. 2013; Navia et al. 2014; Beroiz et al. 2014; Khaing et al. 2014; Lv et al. 2014; Juan et al. 2015; Li et al. 2015; Stalažs and Moročko-Bičevska 2016; Peltier et al. 2018; Syromyatnikov et al. 2018; Zélé et al. 2018; Zhou et al. 2020; Gómez-Martínez et al. 2020; Oliveira et al. 2021; Zhang et al. 2021).

In eukaryotes, the ribosomal region internal transcribed spacers 1 and 2 (ITS1 and ITS2) is located between the 28S, 5.8S and 18S rDNA subunit genes (Fig. 1). These three subunits are highly conserved, and can be applied in taxonomic group identification; on the other hand, the ITS regions diversify in sequence and can be used for species identification (Hillis and Dixon 1991; Navajas and Fenton 2000). ITS regions possess some characteristics, such as being (1) located between conserved genes, (2) short sequences, and (3) present in a huge number of copies in the genome (Long and Dawid 1980), which make them important and useful, even in poorly conserved environmental samples (Thet-Em et al. 2012; Beroiz et al. 2014).

Previous reports in tropical countries have identified *B. tropicalis* and *G. malaysiensis* in dust samples (Chew et al. 1999b; Mariana et al. 2000; Miranda et al. 2002). As differentiating these species morphologically can be challenging (Colloff 2009; Ling et al. 2019),

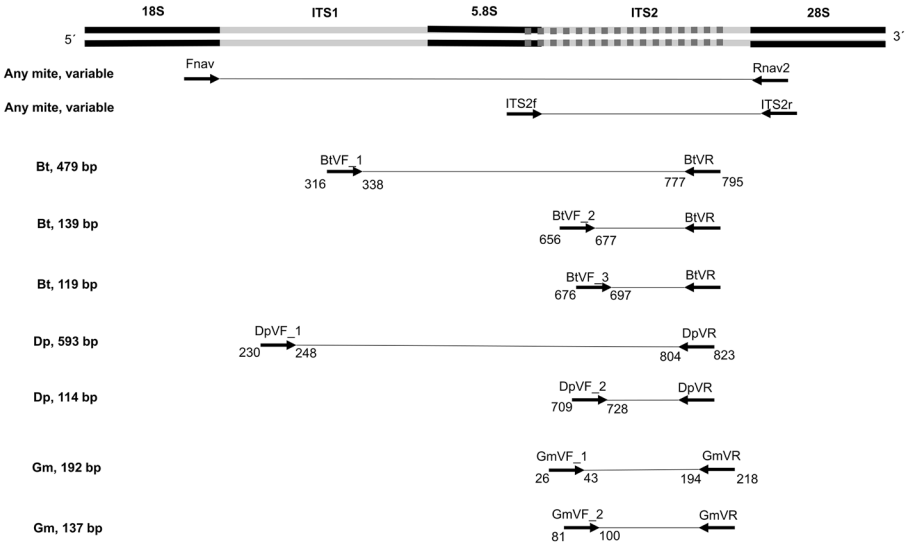

Fig. 1 Schematic overview of the position of the annealing sites of the primers at the rDNA and the corresponding PCR products used for identification of *Dermatophagoides pteronyssinus* (Dp), *Blomia tropicalis* (Bt) and *Glycycometus malaysiensis* (Gm). Mite-specific primers are shown, targeting the 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. The numbers near the arrows indicate the nucleotide positions of the designed primers, and are based on the GenBank sequences KC215364.1, KC215344.1 and MW763258.1 as template, respectively. The dotted region at the end of the 5.8S and part of the ITS2 regions shows the region used for the phylogenetic analyzes

mistaken cultivation can occur. Indeed, we present the first report of *G. malaysiensis* contamination of *B. tropicalis* samples in Brazil, identified in our laboratory by an expert in the field of entomology.

This study aimed to perform a phylogenetic analysis and to develop a multiplex PCR (mPCR), based on ITS rDNA, capable to identify *B. tropicalis* and *D. pteronyssinus*, major allergy-associated mites in tropical and subtropical countries, and to discriminate *B. tropicalis* from *G. malaysiensis*, whose role in allergy is still poorly known, from stock colonies or collected from residences. In addition, we intended to apply mPCR to evaluate the frequency of these species in house dust collected from 20 residences located in Salvador, northeast Brazil.

Materials and methods

Dust collection

Dust samples from mattresses ($n = 20$) were collected from homes in the city of Salvador, Bahia, northeastern Brazil (12.9777° S, 38.5016° W). The samples were collected in eight neighborhoods during a period spanning 11 months, from May 2019 to March 2020. Dust sampling was carried out using a residential vacuum cleaner (Electrolux Professional, 1220 W), containing a cellulose filter where the dust was trapped. Dust was collected for 5 min in an area of 1 m^2 at the head of the mattress. Part of the dust (4 mg) was stored at −20 °C until DNA extraction.

Obtaining mites from dust and mite cultivation

In order to isolate and rear mites for use in assays, dust samples were collected as described above and placed in a Tullgren funnel overnight for mite concentration. Then the mites were cultivated in small cell culture bottles, in a BOD incubator, at 25 °C and 70–80% RH, using spirulina and yeast as food. After 15 days, units of each mite were aliquoted for posterior PCR analysis. Clones of each species were carried out, placing pregnant females of the mites isolated, in 50 small plastic bottles, and cultivated as indicated above. After 2 months, the bottles positive for mite growth, had four mite specimens mounted per slide for morphological identification, and more individual mites aliquoted for PCR analysis. Subsequently the cloned mites were transferred to cell culture bottles for obtaining the various mites in large quantities for carrying out the PCR standardization. As we could not obtain *D. pteronyssinus* in large amounts from the mite cultures, for PCR standardization we obtained this mite from Stallergenes Greer (Lenir, NC, USA).

The mites were purified using a 5 M NaCl solution. Floating mites were sucked up with a vacuum pump and the mite mass was centrifuged with 11,000×*g* for 7 min to remove the saline solution, and washed once with distilled water by a second centrifugation. Afterwards, 20 mg of each mite species was stored in a −20 °C freezer for later use.

DNA extraction

DNA extraction was carried out from material from three sources: (1) dust (4 mg), from dust collection, (2) masses of frozen *B. tropicalis*, *G. malaysiensis* and *D. pteronyssinus* mites (20 mg per species), and (3) individual mites for each of the three species. 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 18S region (Navajas et al. 1999), and Rnav2, based on the 5′ end of the 28S region—Fig. 1 shows the position of the annealing sites of the primers at the rDNA (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, 1 × enzyme buffer supplied by the manufacturer (Sinapse, Sao Paulo, SP, Brazil), 200 µM dNTP mix, 0.2 µM of each primer, 1 U of Taq DNA polymerase (Sinapse). Primer sequences and 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 ITS2F and ITS2R primers (Noge et al. 2005) (Table 1, Fig. 1). The PCR products of Rnav2/Fnav (0.5 µl) were used with the concentration of reagents described above. 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, Waltham, MA, USA).

rDNA cloning and sequencing

For *G. malaysiensis*, the PCR products of the Fnav/Rnav2 primer pair and the nested PCR products with the ITS2F/ITS2R primer pair were purified (PCR DNA and Gel Band Purification; GFX) and cloned according to pGEM-T easy vector kit instructions (Promega, Madison, WI, USA). Four isolated colonies for both PCR products were selected for plasmid purification (NucleoSpin Plasmid QuickPure Kit; Macherey–Nagel, Düren, Germany) and insert sequencing. Purified nested PCR products from *B. tropicalis* were cloned according to pCR4-TOPO TA vector kit instructions (TOPO TA Cloning; Invitrogen, Waltham, MA, USA) and three colonies were selected for insert sequencing.

Sequencing was bi-directional for all clones and was outsourced at 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 (v.7.0.5.3, RRID:SCR_007361) (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 (RRID:SCR_001598) (Altschul et al. 1990) analysis against the nucleotide collection (nr/

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

Pair no.	Primer name	Sequence (5′→3′)	Target gene	Species	Approx. product size (bp)	Thermal conditions						References
						ID	D	A	E	FE	N	
1	Fnav	F-AGAGGAAGTAAAAGTCGTAACAAG	18S	All mites	Variable	95 °C,	92 °C,	48 °C,	72 °C,	72 °C,	45	Navajas et al. (1999)
	Rnav2	R-ATATGCTTAAATTCAGCGGG	28S			10 min	30 s	30 s	1 min	10 min		Navajas et al. (1998)
2	ITS2F	F-CGACTTTCGAACGCATATTGC	5.8S			94 °C,	94 °C,	52 °C,	72 °C,	72 °C,	30	Noge et al. (2005)
	ITS2R	R-GCTTAAATTCAGGGGGTAATCTCG	28S			1 min	30 s	30 s	1 min	2 min		
3	BtVF_1	F-AGTGATGGATGCAGGGTATCATT	ITS1	*B. tropicalis*	482	94 °C,	94 °C,	58 °C,	72 °C,	72 °C,	30	This study
	BtVR	R-GTCCGATCTGTAATGCACG	ITS2			5 min	30 s	1 min	30 s	5 min		
4	BtVF_2	F-ATGAAAGCCACAAACTATATGC	ITS2		147							
	BtVR	R-GTCCGATCTGTAATGCACG										
5	BtVF_3	F-GCAAGACTCGTTCATCGTGTTG	ITS2		120							
	BtVR	R-GTCCGATCTGTAATGCACG										

Table 1 (continued)

Pair no.	Primer name	Sequence (5′→3′)	Target gene	Species	Approx. product size (bp)	Thermal conditions						References
						ID	D	A	E	FE	N	
6	DpVF_1	F-GTTGGACCG AATCATGTCA	ITS1	*D. pteronyssinus*	593							
	DpVR	R-AGGTGTTTA GCTGGAAACGC	ITS2									
7	DpVF_2	F-CGTGAATAC GTCGTCAGGTC	ITS2		115							
	DpVR	R-AGGTGTTTA GCTGGAAACGC										
8	GmVF_1	F-TGGGGACAT CCTCAAGCT	ITS2	*G. malaysiensis*	193							
	GmVR	R-AGGTCGAAC ATTTACAAA CAAAGTC										
9	GmVF_2	F-TCGAAAGAC CTGTTGTCGTG	ITS2		138							
	GmVR	R-AGGTCGAAC ATTTACAAA CAAAGTC										

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

nt) was performed. Subsequently, the sequences obtained from the GenBank database were used for an alignment in ClustalW (BioEdit). To determine substitutions, insertions and deletions in these alignments, as there are no reference sequences in the GenBank for the ITS2 region of the studied mites, we used as a parameter the most frequently found sequences (the most conserved). For *G. malaysiensis*, as there were no record sequences in the GenBank prior to our study, no BLASTN analysis was performed, we just performed an alignment with all *G. malaysiensis* sequences obtained and took as reference the group of sequences that were more conserved. Thus, the events of substitutions, insertions and deletions were determined when they diverged from the majority group of sequences.

Phylogenetic relationships were constructed using the sequences obtained in this study and sequences deposited in GenBank for *D. pteronyssinus* and *B. tropicalis*. As no ITS2 sequence of mites from the Aeroglyphidae family or from the genus *Glycycometus* were available for comparison with the sequences obtained from *G. malaysiensis*, we also selected sequences for *G. domesticus* and *L. destructor*, both members of the Glycyphagidae, a family close to the Aeroglyphida and Echimyopodidae (*B. tropicalis*), all within the superfamily Glycyphagoidea (Colloff 2009). Table 2 shows the access numbers and country of each selected sequence for phylogenetic studies. The rDNA sequences were trimmed at the position of the ITS2F/ITS2R primer pair, comprising the complete ITS2 region flanked by partial sequences of 5.8S and 28S subunits (see Fig. 1). Molecular Evolutionary Genetics Analysis (MEGAX v.10.1.8, RRID:SCR_000667) was used to construct a maximum likelihood phylogenetic tree considering 1000 replicates of Bootstrap. The best-fit substitution model was T92 + G, as predicted by the Bayesian Information Criterion (BIC) of MEGAX.

In the design of primers, sequences obtained in the study were used, as well as sequences retrieved from GenBank for *D. pteronyssinus* (KC215344.1, KC215341.1, KC215342.1 and KC215340.1), *D. farinae* (KC215334.1), *D. microceras* (KC215315.1), *B. tropicalis* (KC215364.1) and *G. domesticus* (KC215365. 1, KC215374.1, KC215373.1 and KC215368.1).

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 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, RRID:SCR_003095) and OligoAnalyzer (Integrated DNA Technologies OligoAnalyzer, RRID:SCR_001363).

For *D. pteronyssinus* and *B. tropicalis*, forward primers for the ITS1 region were also designed, generating larger PCR products, consisting of an ITS1 partial sequence, 5.8S complete sequence and ITS2 partial sequence (Fig. 1). We designed a total of 10 primers: two forward and one reverse primers for *G. malaysiensis*, three forward and one reverse primers for *B. tropicalis* and two forward and one reverse primers for *D. pteronyssinus* (Table 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, 1 × enzyme buffer (Promega), 200 µM dNTP mix, 0.5 µM of each primer (Integrated DNA Technologies), 1 mM $MgCl_2$ and 0.8 U of

Taq DNA Polymerase (Promega). All singleplex PCR reactions included negative controls, using water as the template. The PCR cycle conditions were empirically adjusted until obtaining optimal results for all prime pairs (Table 1).

As larger PCR products were generated, primer pair 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 (water as the template). The mPCR and singleplex PCR products were visualized in 1% agarose gel stained with SYBR (Applied Biosystems).

Specificity and detection limit of mPCR

To evaluate the sensitivity of the assay, two tests were performed, using (1) dilutions of DNA of each species, separately, or (2) a mixture containing 100 ng of DNA of each species which was then diluted. 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. We also tested the mPCR against a sample containing DNA of only one mite of each species (*B. tropicalis*, *G. malaysiensis* and *D. pteronyssinus*) and two mites of each species.

The detection limit test was also used to assess the specificity of mPCR primer pairs in the presence of DNA from the studied mites. In addition, we tested the method against 50 ng of DNA from seven species of mites, including storage mites, predators and phytophagous mites: *Thyreophagus cracentiseta* (Astigmata: Acaridae), *Rhizoglyphus vicantus* (Astigmata: Acaridae), *Rhizoglyphus columbianus* (Astigmata: Acaridae), *Sancassania* sp. (Astigmata: Acaridae), *Suidasia pontifica* (Astigmata: Suidasiidae), *Raoiella indica* (Prostigmata: Tenuipalpidae) and *Euseius.* sp (Gamasina: Phytoseiidae).

Cloning and sequence analysis of mPCR products from dust samples

To further confirm the specificity of the mPCR assay, 11 of the 20 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*, three, six and eight colonies, respectively, were selected for insert sequencing (Supplementary Table 1).

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, the access numbers are shown in Table 2. BLASTN analysis against the nucleotide collection (nr/nt) was performed using the NCBI website. An alignment between the sequences of the dust clones and the sequences used in the phylogenetic tree generated previously was applied to construct a phylogenetic tree with the same configurations as the previous one.

Barcode gap analysis

All sequences presented in Table 2 were selected for the barcode gap analysis using two methods. The first, considered a traditional method, used the Geneious Prime software (v.2020, RRID:SCR_010519) and R program (RRID:SCR_001905). The sequences of the

Table 2 Species used in phylogenetic analyses, GenBank accession numbers, the country to which it is assigned and the literature sources

Species	GenBank acc. nr	Country	References
Lepidoglyphus destructor	MH794027.1, MH794029.1, MH794028.1, MH794059.1, MH794055.1, MH794058.1	China	Direct submission
	KX581028.1, KX121291.1, KX121295.1, KX121294.1		Niu et al. (2017)
	KY994139.1	Switzerland	Direct submission
	KC215377.1, KC215380.1, KC215384.1	Spain	Beroiz et al. (2014)
Glycyphagus domesticus	KC215374.1, KC215367.1, KC215371.1, KC215370.1, KC215369.1, KC215373.1, KC215366.1, KC215368.1, KC215372.1, KC215365.1	Spain	Beroiz et al. (2014)
Glycycometus malaysiensis	MW763261.1, MW763260.1, MW763259.1, MW763258.1, MW763254.1, MW763253.1, MW763252.1, MW763251.1, MW763250.1, OL331189.1, OL331190.1, OL331191.1	Brazil	This study
Blomia tropicalis	MW763268.1, MW763267.1, MW763266.1, MW763255.1, MW763256.1, MW763257.1, OL312657.1, OL312658.1, OL312659.1	Brazil	This study
	KC215364.1, KC215361.1, KC215363.1, KC215360.1, KC215359.1, KC215355.1, KC215362.1, KC215357.1, KC215356.1, KC215358.1	Spain	Beroiz et al. (2014)
	AB105003.1, AB105002.1	Japan	Noge et al. (2005)
	MG709495.1, MG709494.1, MG655150.1, MG709496.1	China	Direct submission
	MT782118.1	USA	Direct submission
	GQ205596.1, GQ205595.1, GQ205587.1, GQ205591.1, GQ205593.1, GQ205592.1, GQ205590.1, GQ205588.1, GQ205594.1	China	Yang et al. (2011)
Dermatophagoides pteronyssinus	MW763269.1, OL312660.1, OL312661.1	Brazil	This study
	KC215337.1, KC215339.1, KC215335.1, KC215344.1, KC215342.1, KC215338.1, KC215336.1, KC215341.1, KC215340.1	Spain	Beroiz et al. (2014)
	MK589426.1, MK589422.1, MK589425.1, MK589423.1, MK589429.1, MK589427.1, MK589428.1, MK589424.1	China	Direct submission
	MN906967.1	Portugal	Oliveira et al. (2021)

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 (RRID:SCR_014601) (Wickham 2016). To assess the reliability of the data, the Wilcoxon test was performed. The second method was the barcode gap analysis, using Automatic Barcode Gap Discovery (ABGD) software (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with default parameters. A species was considered identified if the interspecific distance was larger than the intraspecific distance (Schoch et al. 2012).

Results

rDNA amplification and phylogenetic analysis

The amplification with the primer combination Fnav and Rnav2 with DNA from *B. tropicalis* and *G. malaysiensis*, showed bands of approximately 854 and 890 bp, respectively (Supplementary Fig. 1). For *G. malaysiensis* four clones were sequenced (access numbers MW763262-MW763265 at NCBI).

The nested PCR for *B. tropicalis* and *G. malaysiensis* mites showed bands of approximately 314 and 250 bp, respectively, on agarose gel (Supplementary Fig. 1). The bands were cloned and four insert-containing clones for *G. malaysiensis* (access numbers MW763258-MW763261) and three 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 and 250–256 bp for *B. tropicalis* and *G. malaysiensis*, respectively. There were, for both mites, intraspecific polymorphisms in the sequences, which consisted of insertions, deletions and substitutions.

The phylogenetic tree (Fig. 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*, *B. tropicalis*, *G. domesticus* and *L. destructor* retrieved from the GenBank database (Table 2) and from the clones obtained in this study showed that the *B. tropicalis* clones clustered in the same branch as the *B. tropicalis* sequence, with a significant high bootstrap value (97%). The *G. malaysiensis* clones clustered in the same branch, with a robust bootstrap value (99%). It is also possible to infer that the mites *B. tropicalis*, *G. malaysiensis*, *G. domesticus* and *L. destructor* share the same common ancestor (99% bootstrap), as they belong to the superfamily Glycyphagoidea, and that the Aeroglyphidae (*G. malaysiensis*) and Glycyphagidae (*G. domesticus* and *L. destructor*) families are more closely related when compared to the Echimyopodidae (*B. tropicalis*) family (64% bootstrap).

Singleplex and multiplex PCR

The primer pairs used in the singleplex PCRs successfully amplified the DNA of *D. pteronyssinus*, *B. tropicalis* and *G. malaysiensis*, with fragments in the expected size (see Table 1). As shown in Supplementary Fig. 2, primer pairs 3–9 amplified PCR products of approximately 482, 147, 120, 593, 115, 193 and 138 bp, respectively.

The mPCR with primer pairs 3 (482 bp), 6 (593 bp) and 8 (193 bp) for *B. tropicalis*, *D. pteronyssinus* and *G. malaysiensis* mites, respectively, was successfully used to amplify the partial ITS1, 5.8S subunit and partial ITS2 regions, of *B. tropicalis* and *D. pteronyssinus*

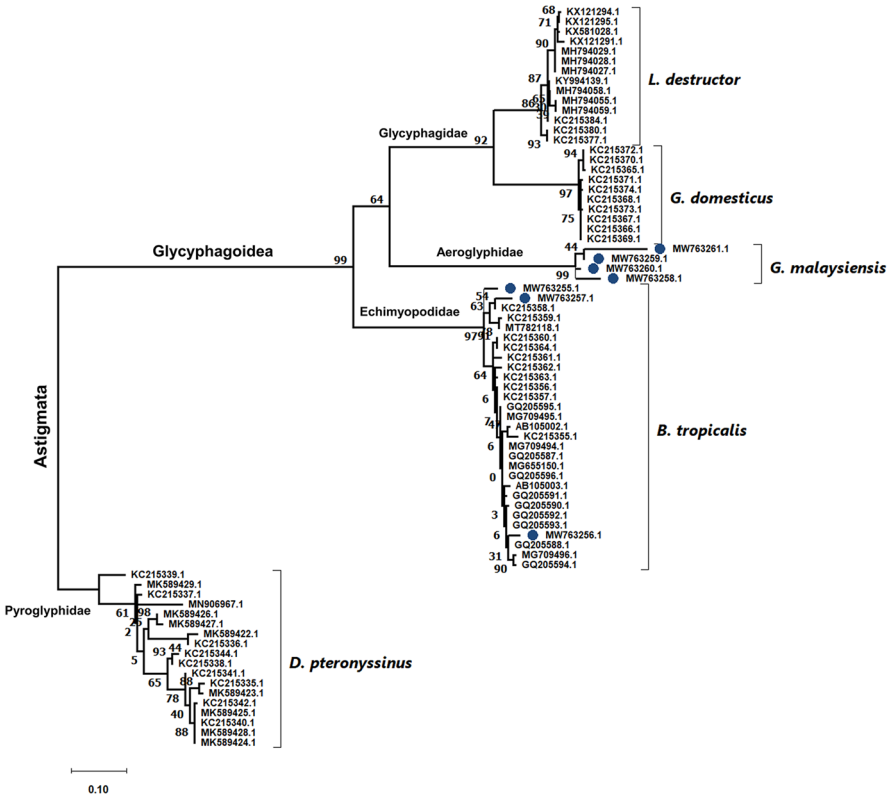

Fig. 2 Maximum likelihood phylogenetic tree considering 1000 replicates of Bootstrap and T92 + G model constructed using alignment of the sequences obtained in our study for *Blomia tropicalis* and *Glycycometus malaysiensis* (blue dots before the accession numbers) and representative sequences available in the GenBank database for *Dermatophagoides pteronyssinus*, *B. tropicalis*, *Glycyphagus domesticus* and *Lepidoglyphus destructor*, consisting of the ITS2 region flanked by partial sequences of 5.8S and 28S subunits. (Color figure online)

and partial ITS2 region for *G. malaysiensis*. 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. malaysiensis* (Supplementary Fig. 3, 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. malaysiensis* was 0.1 ng, increasing 10 × (Supplementary Fig. 3, panel D). Furthermore, the mPCR assay was able to detect even samples containing individual mites (Supplementary Fig. 4). In the specificity assay against DNA from seven mite species, only the sample containing the DNA of *R. indica* amplified (see Supplementary Fig. 5).

Supplementary Fig. 6 shows the mPCR agarose gels using DNA extracted from 20 dust samples. The Venn diagram in Fig. 3 indicates that *B. tropicalis* was the most found mite,

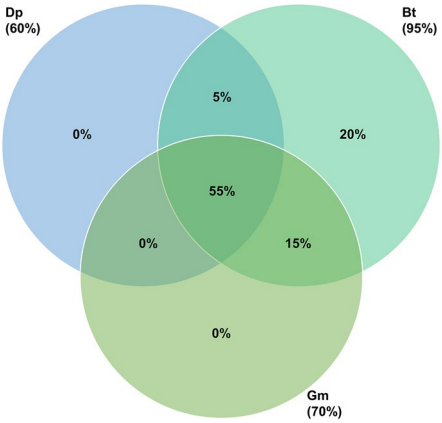

Fig. 3 Venn diagram showing the positivity of the 20 dust samples analyzed in the multiplex PCR reaction: 95% of the samples were positive for *Blomia tropicalis* (Bt), 70% for *Glycycometus malaysiensis* (Gm) and 60% for *Dermatophagoides pteronyssinus* (Dp). Twenty % of the samples were positive just for Bt, 5% only for Dp + Bt, and 15% for Bt + Gm; 55% were positive for all studied mites

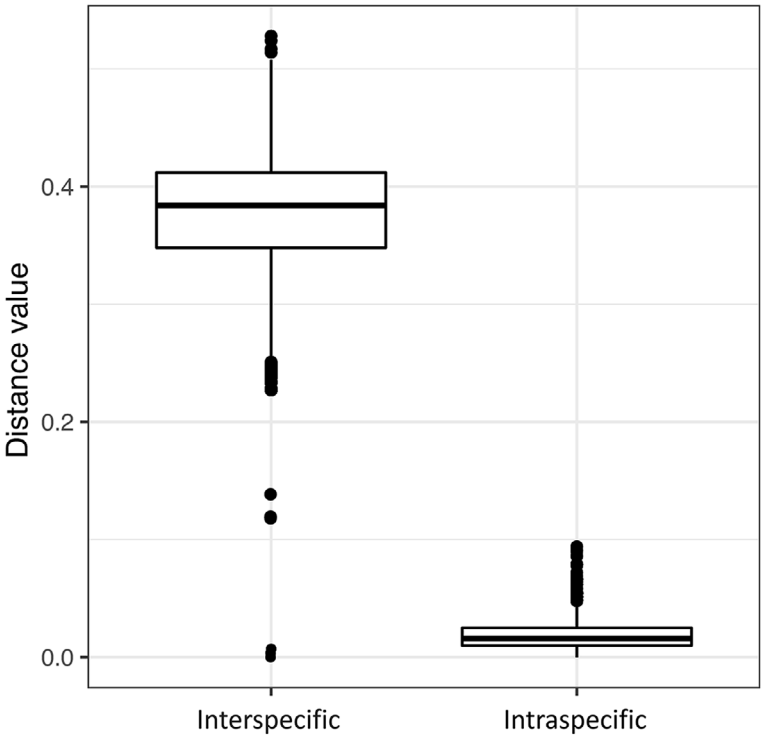

Fig. 4 Barcode gap analysis for the species *Blomia tropicalis*, *Dermatophagoides pteronyssinus*, *Glycycometus malaysiensis*, *Glycyphagus domesticus* and *Lepidoglyphus destructor* based on the ITS2 region and the intra- and interspecific distances. The top and bottom box represent the first and third quartiles of the data, the horizontal line in between indicates the median. The whiskers indicate 24.65% intervals, whereas the dots are outliers (remaining 0.35% of data)

present in 95% of the samples, except for sample 15, which was negative for all mites (Supplementary Fig. 6). *Glycycometus malaysiensis* proved to be the second most found mite, present in 70% of the samples, followed by *D. pteronyssinus* in 60% of the samples. *Blomia 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. malaysiensis*, and 5% harboring *B. tropicalis* and *D. pteronyssinus*. In addition, all three mite species were found in 55% of the samples. Finally, no house dust was found harboring only *D. pteronyssinus* and *G. malaysiensis*.

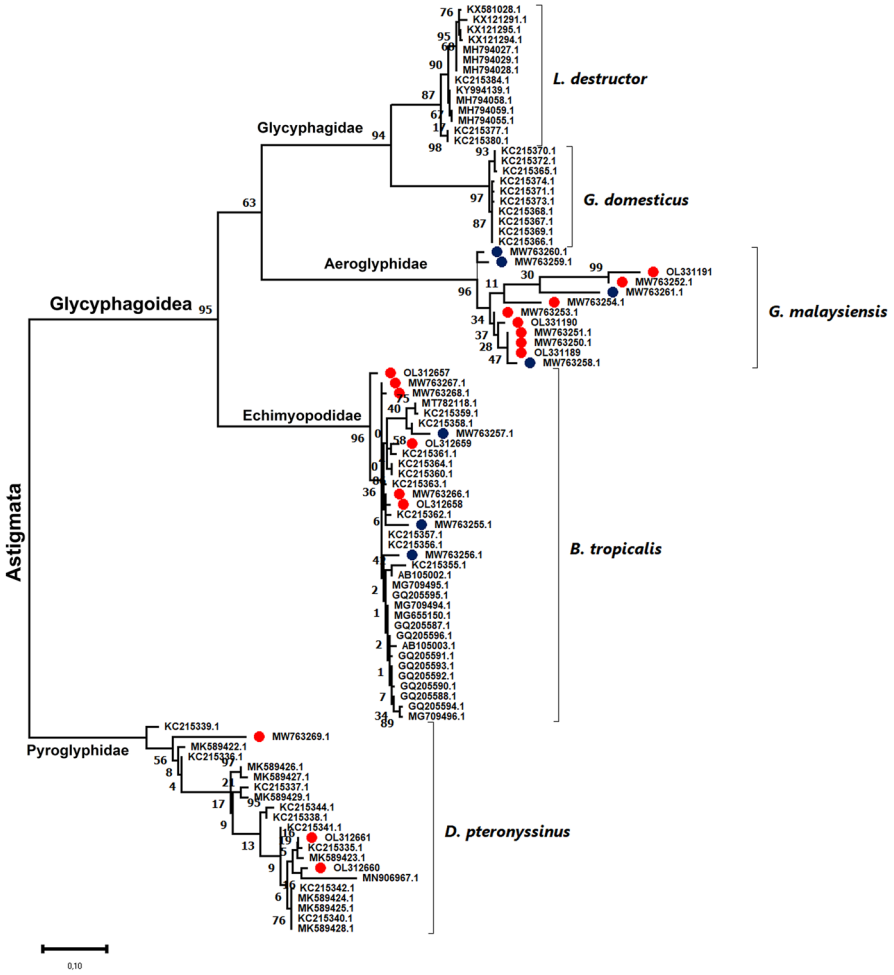

Fig. 5 Maximum likelihood phylogenetic tree considering 1000 replicates of Bootstrap and T92 + G model constructed using alignment containing all sequences obtained herein and representative sequences available in the GenBank database for *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Glycyphagus domesticus* and *Lepidoglyphus destructor*, consisting of the ITS2 region flanked by partial sequences of 5.8S and 28S subunits. The dust clone sequences obtained using primer pairs 3 and 6, for *B. tropicalis* and *D. pteronyssinus*, respectively, were trimmed, containing the end of the 5.8S region and partial ITS2 (see Fig. 1). Blue dots before the accession numbers indicate the sequences obtained for the ITS2 rDNA region flanked by partial sequences of the 5.8S and 28S subunits, the red dots indicate the sequences of the dust clones. (Color figure online)

Barcode gap analysis

The barcode gap analysis using all clones obtained in our study, including the dust ones, together with the sequences obtained in the GenBank database corresponding to *D. pteronyssinus*, *B. tropicalis*, *G. domesticus* and *L. destructor*, showed that there are five distinct groups and that all clones were grouped according to their species. As it can be seen in the boxplots in Fig. 4, 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.

The sequences of the dust clones for *B. tropicalis*, *G. malaysiensis* and *D. pteronyssinus* 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 all clones correspond to their respective species. As expected, the sequences of all clones showed an intraspecific variation. Polymorphisms consisted of substitutions, insertions and deletions. As shown in Fig. 5, 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 (Fig. 2), indicating that the sequences of the clones were grouped into clades corresponding to each studied species, and this result was supported by high bootstrap values (> 95%).

Discussion

Morphological identification of mites is a time-consuming and ambiguous technique and cannot be applied to purified mite fractions or poorly preserved environmental samples. Only four studies have shown up to now that the storage mite *G. malaysiensis* has a role in triggering allergy manifestations (Baratawidjaja et al. 1999; Chew et al. 1999a; Tang et al. 2011; Chong et al. 2015). Furthermore, because *G. malaysiensis* and *B. tropicalis* are morphologically similar (Colloff 2009; Ling et al. 2019), they can represent an additional challenge for morphological identification, which can lead to misidentification and mistaken cultivation of these species. To overcome these problems and verify the frequency of these species in 20 dust samples from the city of Salvador, Bahia, Brazil, we developed a molecular technique based on ITS rDNA for the identification of these species. The full-length ITS2 region flanked by partial sequences of 5.8S and 28S subunits were obtained for these studied mites and publicly deposited in the GenBank database, and this is the first description for *G. malaysiensis*. Furthermore, it was the first time that a mPCR was designed for simultaneous identification of *B. tropicalis*, *D. pteronyssinus* and *G. malaysiensis* and the first report of the presence of *G. malaysiensis* in Brazil.

Our first sequencing of the *G. malaysiensis* PCR products with the primer pair Fnav and Rnav2 proved to be fungal rDNA. As this combination of primers is not species-specific and the sequences of the ribosomal subunits are conserved among different taxonomic groups, the PCR product generated fungal rDNA, used as food for mites in culture, together with Astigmata rDNA. To overcome this problem, we performed a nested PCR with the primer pair targeting the ITS2 region. The sequencing of the nested PCR products showed that it has only Astigmata rDNA. Thus, these data indicate that a nested PCR using the ITS2F/ITS2R primers after amplification with the Fnav/Rnav2 primer pair can be considered a good option to amplify the ITS2 region from mites and increased both the detection resolution and the specificity of the technique for Astigmata ITS2 rDNA.

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 led to the increase of time to obtain the sequence and consequent identification of a given mite species of interest; however, after the design of the primers and standardization of the assay, it can be a valuable tool, reducing the need for experienced acarologists. Nonetheless, the polymorphisms did not interfere in the identification of the species, as validated by the barcoding gap analysis and by the phylogenetic trees, where the clones and co-specific sequences of each species clustered together, confirming the effectiveness of mPCR identification. These results 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.

Our trees followed the phylogenetic relationships of the Glycyphagoidea families, as presented by Colloff (2009). It is worth noting that the co-specific rDNA sequences obtained from the GenBank database of mites from other geographic regions (Table 2) also clustered with the sequences generated in our study. The present results support that the ITS2 region presents a good resolution for species discrimination and little intra-species variation that did not interfere 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, the variation between geographically isolated strains was very similar to that within strains, and that, therefore, the ITS2 region can be a reliably genomic source used for the identification of mites. In the case of *G. malaysiensis*, further analysis is necessary to verify whether paralogous copies of the ITS2 rDNA have been sufficiently homogenized by evolutionary processes (Rich et al. 1997).

Although 20 samples are insufficient to verify the frequency of these species in the city of Salvador, we used them as a proof of concept to validate the method developed. The most abundant species among the samples was *B. tropicalis*, a result consistent with previous works investigating the frequency of mites in tropical regions (Fernandes-Caldas et al. 1993; Chew et al. 1999b; Baqueiro et al. 2006). Previous studies, using morphological identification, also reported the presence of *G. malaysiensis* in domestic dust samples. This species was found in Singapore in approximately 35% of the samples (Chew et al. 1999b) and in 40% of the households in Klang Valley, Malaysia (Mariana et al. 2000). Miranda et al. (2002) found *G. malaysiensis* for the first time in Panama, in 6.37% of the samples collected in urban areas. As *G. malaysiensis* was found in a significant part of the samples (70%) in our study and it was not described by previous studies on house dust mite frequencies 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, *G. malaysiensis* being morphologically very similar to *B. tropicalis* (Chew et al. 1999b; Colloff 2009; Ling et al. 2019) can lead to its misidentification as *B. tropicalis*. Indeed, Chew et al. (1999a) showed that 78.2% of asthma and/or allergic rhinitis patients in Singapore were skin prick positive for *G. malaysiensis*. Moreover, *G. malaysiensis* and *B. tropicalis* share similar antigens, leading to the production of cross-reactive antibodies (Chew et al. 1999a; Tang et al. 2011), which can give a false impression that *B. tropicalis*, and not *G. malaysiensis*, is present in a given mite sample.

Some identification approaches based on the ITS and *coxI* regions have been carried out previously. Yang et al. (2011) sequenced the ITS2 and *coxI* 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 PCR–RFLP (restriction fragment length polymorphism) for the identification of mites. Beroiz et al. (2014), using PCR–RFLP, identified 13 mite species used for allergic extract production, in culture and environmental samples. The PCR–RFLP assay based on the ITS2 region was used by Wong et al. (2011) to identify six mite species, including the three evaluated in our study. Previous to enzymatic digestion, the ITS2 region of the mites was amplified with the primers ITS2F and ITS2R and the sizes of the PCR products for *B. tropicalis* and *G. malaysiensis* found here are in accordance with those found in Wong et al.'s study. However, the PCR–RFLP technique developed was used to identify cultured mites, and it is not possible to use this technique to identify mites from mixed cultures, or even from dust samples. Therefore, the novelty of our study is that for the first time we have developed a direct method of PCR to identify the three species (*B. tropicalis*, *G. malaysiensis* and *D. pteronyssinus*) in a single assay, from mixed cultures, or even from dust samples. 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, as the digestion step can take 2–16 h. On the other hand, the outputs are generated in < 3 h, which is an advantage over studies that used PCR–RFLP. Moreover, due to the fact that the primers used by these studies are not species-specific, there is a chance of ambiguity and errors in the interpretation of the digestion profiles. Therefore, more effective approaches should include the design of specific primers for identifying the species of interest.

Oliveira et al. (2021) designed an rDNA-based mPCR capable of distinguishing *D. pteronyssinus* and *D. farinae*. The method was tested on five dust samples and, to assess specificity, they sequenced only two mites, one per species. To date, Thet-Em et al. (2012) was the only study that used an mPCR to identify *B. tropicalis* and *D. pteronyssinus* in environmental samples. To assess the prevalence of these mites and *D. farinae* from house dust samples in Thailand, the mPCR assay used three primer pairs for amplification of the ITS2 region of *D. pteronyssinus* and *D. farinae*, and *coxI* for *B. tropicalis*. However, the primer sequence was not publicly available, contrary to our study, where the sequences of the 10 designed primers are made available. Moreover, Thet-Em et al. (2012) verified the frequency of the abovementioned 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 study, the sequencing of 17 bands was performed, and the results, both the barcode gap and the phylogenetic tree, support the species-specificity of the designed primers when using house dust samples.

Although a non-specific amplification occurred for *R. indica* (Supplementary Fig. 5), this mite is not found in domestic dust; it is a plant-feeding mite that infests economically important crops (Peña et al. 2012). The sequencing carried out in our study showed that the method was specific even against household dust samples, that 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, if known, may not have the genetic sequence available. Thus, our technique was reliable when applied to dust samples from Brazil; however, we recommend the sequencing of the mPCR products before implementing our technique to identify species in environmental samples worldwide.

The mPCR assay developed herein proved to be a reliable and fast technique for the identification of these mites in poorly preserved environmental samples, in culture or in individual mites. Furthermore, our mPCR test requires a small amount of DNA for identification (just one mite is enough) (Supplementary Fig. 4), which is important if few

specimens are available in the laboratory. We identified *G. malaysiensis* for the first time in Brazil, being found in most of the analyzed dust samples. Many of these samples also harbored *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 morphological certification of the studied species. Furthermore, this technique can be used as a quality control for the production of extracts to be used in clinical trials. Moreover, our mPCR assay and the other primers designed herein can be adapted for use in the quantitative multiplex real-time PCR technique, in order to assess the abundance of mites in environmental samples and promote new insights into the epidemiology and clinical implications of these mites.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10493-022-00694-y.

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Data availability Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) with the accession numbers MW763250-MW763269 (https://www.ncbi.nlm.nih.gov/nuccore/?term=MW763250:MW763269[accn]), OL331189:OL331191 (https://www.ncbi.nlm.nih.gov/nuccore/?term=OL331189:OL331191[accn]), and OL312657:OL312661 (https://www.ncbi.nlm.nih.gov/nuccore/?term=OL312657:OL312661[accn]). All other data are included in the article and its Supplementary Information.

Code availability Not applicable.

Declarations

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

Ethical approval This study was approved by the Research Ethics Committee (CEP) of the Institute of Health Sciences of the Federal University of Bahia (UFBA) (CAAE 22804719.0.0000.5662).

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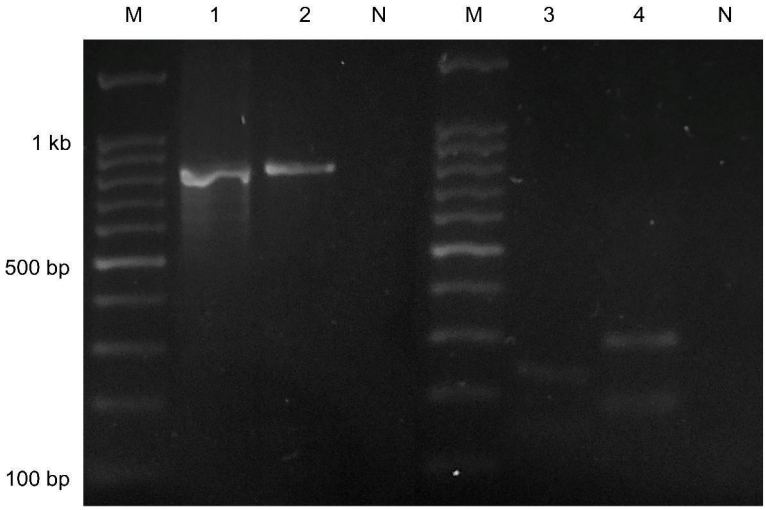
Supplementary Table 1: Dust samples used in the multiplex PCR assay selected for cloning and sequencing, and the number of clones sequenced for *D. pteronyssinus* (Dp), *B. tropicalis* (Bt) and *G. malaysiensis* (Gm) mite species. Dp, Bt and Gm mites had 3, 6 and 8 clones sequenced, respectively

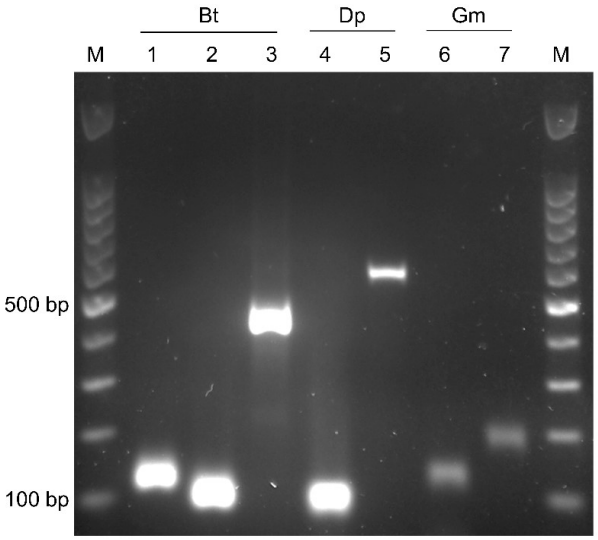
Species	Number of clones											Total
	D01	D02	D03	D04	D05	D06	D07	D14	D16	D17	D20	
Dp	0	1	1	1	0	0	0	0	0	0	0	3
Bt	0	0	0	1	1	1	0	1	1	1	0	6
Gm	1	0	1	1	1	0	1	1	0	1	1	8

D dust sample

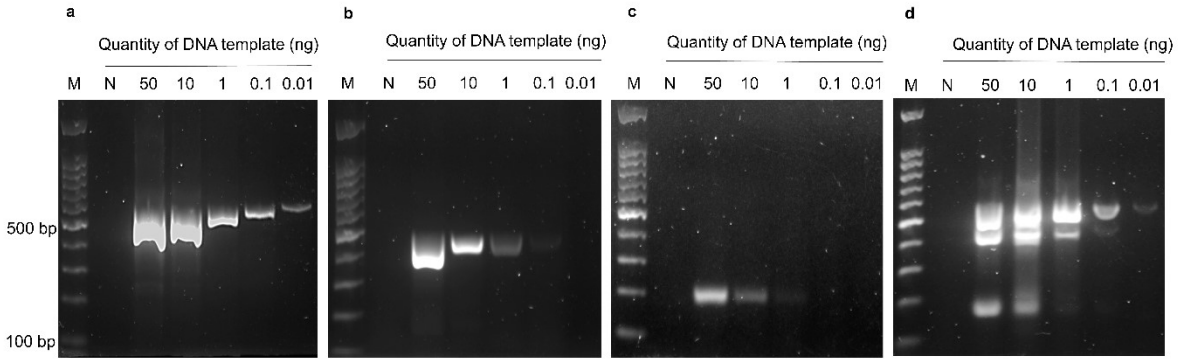
Supplementary Figure 1: The sizes of the amplified rDNA, with the combination of Fnav and Rnav2 primers, and ITS2 regions, with the combination of ITS2f and ITS2r primers, for *G. malaysiensis* (lane 1: 854 bp and lane 3: 250 bp, respectively) and *B. tropicalis* (lane 2: 890 bp and lane 4: 314 bp, respectively). Lane M 100 bp size marker, N negative control



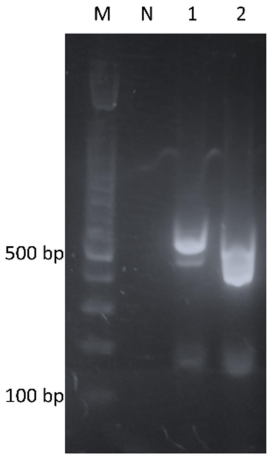
Supplementary Figure 2: Polymerase chain reaction (PCR) showing the band pattern generated by the designed mite specie-specifics primers. Lanes 1-3 correspond to the primer pairs designed for *B. tropicalis* (Bt): 4 (lane 1: 147 bp), 5 (2: 120 bp) and 3 (3: 482 bp), respectively. Lanes 4-5 correspond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



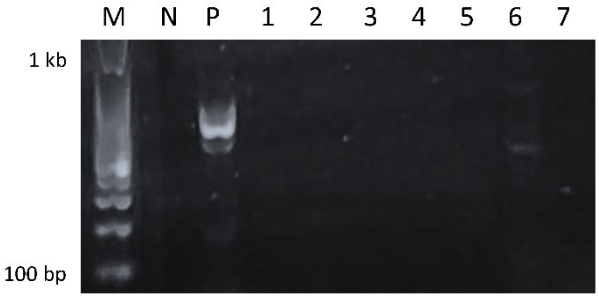
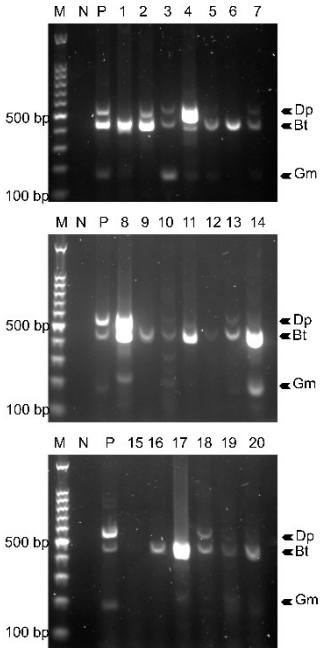
Supplementary Figure 3: Results showing the detection limits of multiplex PCR for *D. pteronyssinus* (Dp), *B. tropicalis* (Bt) and *G. malaysiensis* (Gm) mites. Panel A: when using Dp DNA, the detection limit was 0.01 ng. Panel B: when Bt DNA was used, the detection limit was 0.1 ng. Panel C: when Gm 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.



Supplementary Figure 4: Assay testing the method against a sample containing only 1 mite of each species (*B. tropicalis*, *G. malaysiensis* and *D. pteronyssinus*) (lane 1) and against a sample containing 2 mites of each species (lane 2). Lane M 100 bp size marker, lane N negative control



Supplementary Figure 5: Multiplex polymerase chain reactions (mPCR) specificity assay against 50 ng of DNA from the mites *Thyreophagus cracentiseta*, *Rhizoglyphus vicantus*, *Rhizoglyphus columbianus*, *Sancassania* sp., *Suidasia pontifica*, *Raoiella indica* and *Euseius*. sp, lanes 1-7, respectively. Lane M 100 bp size marker, lane N negative control, P positive control



Supplementary Figure 6: 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, lane P positive control