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Molecular identification of pathogenic house dust mites using 12S rRNA sequences

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House dust mites are microarthropods implicated in the cause of allergic diseases. Currently, there is no phylogenetic analysis of dust mites based on genomic or mitochondrial DNA (mtDNA) evidence. For the first time, we report evolutionary relationships based on partial mtDNA 12S rRNA sequences among the four dust mite families Pyroglyphidae (*Dermatophagoides pteronyssinus*), Glycyphagoidea (*Glycyphagus privatus*), Acaridae (*Aleuroglyphus ovatus*), and Echimyopodidae (*Blomia tropicalis*). Thirteen sequence variants were obtained and phylogenetic analysis showed two monophyletic clades composed of two species each. Contrary to current taxonomic classification, the Acaridae clustered in a monophyletic group with the Pyroglyphidae. Considering the current difficulties in identifying these medically important species for the purpose of eradication and treatment, it is significant that sequence data are capable of discriminating between species belonging to different families of dust mites.

Keywords: Dust mites / Phylogeny / 12S rRNA gene

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1 Introduction

Dust mites are chelicerate microarthropods (250–300 µm in size), currently classified in the Acariformes superorder, one of the three major divisions of the subclass Acari. Dust mites are included within the suborder Astigmata, fossils of which have been found in amber, dating back 28 million years [1]. Dust mites are octopods in the adult stage, lack eyes, and have respiratory structures that are not well developed. These mites feed on dander, shed skin particles (containing collagen and keratin), other human excretions and secretions, as well as grains, fungi, and some bacteria [1]. Geographically, these chelicerate microarthropods are distributed worldwide. Four families of mites have been consistently identified as a common component of house dust: Pyroglyphidae, Glycyphagoidea, Acaridae, and Echimyopodidae [1–4]. Commonly referred to as “domestic mites”, they have been extensively studied as one of the major causes of allergic diseases, such as asthma, rhinitis and atopic dermatitis [1, 5–8].

Current identification of house dust mites is based mainly on their morphological and developmental characteristics. However, these methods often yield ambiguous results since some species are difficult to identify in this

manner. For example, only three members of the Pyroglyphidae family (*Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae*, and *Euroglyphus maynei*) have been precisely differentiated at larval and nymphal stages [9]. The remaining domestic mite species have no reliable differentiation at these life cycle stages. Similarly, two genera of the Acaridae family (*Acarus* and *Tyrophagus*), which are taxonomically complex and morphologically very similar in the adult stage, are very difficult to differentiate with a high degree of certainty [4].

Phylogenetic relationships between domestic mite families have been proposed based on previously described evolutionary ecology hypotheses [1, 2]. Criteria for these relationships include morphological and allergenic evidence [10, 11], physiological responses to the environment, mites associations with birds and small mammals' nests [12–16] as well as geographic distribution [1]. Based on this type of evidence, for example, it has been postulated that the Astigmata suborder evolved from a common soil-dwelling ancestor (the Oribatida) [1].

Current scientific literature contains no reported mitochondrial DNA (mtDNA) sequences for any domestic mite species. Consequently, there are, so far, no dust mites phylogenetic analyses based on genomic or mtDNA data. Only a few mitochondrial genomes of other chelicerate species have been completely sequenced: *Rhipicephalus sanguineus*, *Ixodes hexagonus*, and *Limulus polyphemus* [17]. In contrast, partial mtDNA sequencing of other eukaryotes has been extensively employed to ascertain the location of genes as well as to determine phylogenies at different taxa levels, including families and orders [18–23].

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Abbreviations: HP, heuristic parsimony; mtRNA, mitochondrial RNA; TBR, tree bisection and reconnection branch swapping

Several mitochondrial regions have been identified as highly conserved in arthropods [24]. These areas enabled the design of several primer sets considered to be “universal” in order to target arthropod mitochondrial genomes [24]. One of these universal primer sequence sets is included within a region of the 12S rRNA. This region has been highly informative in the examination of phylogenetic relationships [25–29]. In this study, dust mite 12S rRNA mtDNA sequences are reported for the first time and used to establish evolutionary relationships between the four domestic mite families Pyroglyphidae, Glycyphagoidea, Acaridae, and Echiomyopodidae represented by *Dp*, *Glycyphagus privatus* (*Gp*), *Aleuroglyphus ovatus* (*Ao*), and *Blomia tropicalis* (*Bt*), respectively. Our findings support a monophyletic origin of these four families which segregate into two sister clades in the phylogenetic tree. The data provided by this molecular approach allow a clearer understanding of the phylogenetic relationships between these domestic mite families and of the nature of their respective allergens as proposed by Colloff *et al.* [1, 4]. Considering the difficulties in identifying these medically important species for the purpose of eradication and treatment, it is significant that sequence data are capable of discriminating between species belonging to different families of dust mites. With additional sequence data from nuclear and mitochondrial genomes this approach could be employed to delineate further species or even geographical groups in an effort to identify allergen based causes.

2 Materials and methods

2.1 Culture and harvesting of dust mites

Dp, *Gp*, *Ao*, and *Bt* were selected as representatives of the Pyroglyphidae, Glycyphagoidea, Acaridae, and Echiomyopodidae families, respectively. Cultures were started by adding 8.6 g of live ready cultures to 30 g fish food flakes (Tetra) previously weighted and treated with UV (three rounds of 90 s) in a crystal 9” round plate. Dust mites were grown for 20 days and then processed in a 300 μ m sieve with exposure to the heat generated by a 40 W bulb. The live mites migrated from the sieve located on the top of a funnel to a 50 mL sterile tube located at the bottom (heat-escaped bodies) [30]. The collected bodies were immediately frozen in liquid nitrogen and stored at -80°C prior to DNA extraction procedures.

2.2 DNA isolation

A total of 200 mg of frozen dust mite bodies from each species were ground into a fine particulate matter using a previously cooled (-80°C) mortar and pestle. Total DNA

extraction was performed using the RNA STAT-60™ procedure (RNA/DNA/Protein Isolation Kit, Leedo Medical Laboratories) [31]. DNA pellets were dissolved in 200 μ L of 1 mM Tris-EDTA (TE) buffer. Subsequently, the 200 μ L DNA solutions of *Dp*, *Gp*, *Ao*, and *Bt* were purified using the QIAamp DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications.

2.3 Mitochondrial 12S DNA amplification

Amplification of a 391–396 bp portion of the mitochondrial 12S rRNA gene was performed using the following primer set: 12S forward primer 5'-TACTATGTTAGCACTTA-3' and a 12S reverse primer 5'-AAACTAGGATTAGATACCA-3'. The amplification reaction contained 200 ng DNA from *Dp*, *Gp*, *Ao*, or *Bt*, 22.5 pmol of each of the primers, 8 μ L of 10 mM dNTPs, 10 μ L 10 $\times$ *Taq* buffer, and 2.5 U *Taq* polymerase in a total volume of 100 μ L. Positive and negative control reactions were performed using *Bombyx mori* silk gland DNA and sterile dH_2O , respectively. The PCR parameters were the following: initial denaturation for 10 min at 95°C and 40 cycles of amplification using a step program of 1 min at 95°C , 1 min at 38°C , and 1 min at 72°C . This was followed by a final extension for 10 min at 72°C and held at 4°C . If not immediately electrophoresed, samples were stored at -20°C [32].

2.4 12S rRNA libraries

The 12S rRNA gene libraries were created using the Original TA Cloning kit (Invitrogen, Carlsbad, CA, USA) including the pCR2.1 linearized plasmid vector. The kit provides a one-step cloning strategy for the insertion of a PCR product into the plasmid. *Taq* polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3'-ends of PCR products. Having a single deoxythymidine (T) residue on both ends of the linearized pCR2.1 vector, the freshly manufactured PCR insert is efficiently ligated into the plasmid. The 10 μ L ligation reactions contained 2.0 μ L PCR product, 1.0 μ L 10 $\times$ ligation buffer, 2.0 μ L pCR2.1 vector (25 ng/ μ L), and 1.0 μ L T4 DNA ligase (4 U/ μ L). The ligation reactions were incubated overnight at 14°C . INV α F' competent cells were transformed according to Invitrogen specifications and plated on LB-amp plates containing 100 μ g/mL ampicillin. Ampicillin-resistance and β -galactosidase blue-white screening was used to identify white, recombinant colonies. The colonies were individually picked and suspended in 1 mL SOC media supplemented with ampicillin (100 μ g/mL) in 1.5 mL Eppendorf tubes and grown overnight. The species-specific 12S rRNA PCR libraries were stored at -80°C and regrown for sub-

sequent screening and sequencing purposes. Experimental and control groups were processed under the same conditions [32].

2.5 Sequencing 12S rRNA variants

Recombinant clones were grown overnight at 37°C in 5 mL SOC media containing ampicillin (100 µg/mL) with constant shaking. Using the Wizard 373 DNA purification system kit from Promega (Madison, WI, USA), the plasmids were isolated and purified as described by the manufacturer. Isolated plasmids were linearized by overnight *Hind* III digestion at 37°C. Agarose gel electrophoresis with dilutions of mass standards was employed to estimate the DNA concentration of the restricted plasmids. Sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Kit. The 10 µL sequencing reactions contained 4.0 µL terminator ready reaction premix (ABI Prism), 1.6 pmol M13 forward or reverse primers (Promega), and approximately 600 ng purified plasmid. The pGEM (–3Zf(+)) (200 ng/µL) control included in the kit was sequenced using 400 ng of DNA. The sequencing reactions were performed in a 9600 Perkin-Elmer thermocycler (Norwalk, CT, USA) for 30 cycles as specified by the ABI Prism Protocol. The cycle sequencing products were ethanol precipitated, vacuum-dried, and stored at –20°C until needed. Sequences were obtained using the ABI Prism 377 Sequencer from Perkin-Elmer. The resultant sequences were edited and analyzed using the ABI Autoassembler Program. A BLAST data bank search confirmed the obtained sequencing product. All generated sequences were submitted to GenBank and are now registered under the following accession numbers: AF529916: *Bt* variant A; AF529917: *Bt* variant B; AF529918: *Bt* variant C; AF529919: *Bt* variant D; AF529920: *Bt* variant E; AF529921: *Bt* variant F; AF529911: *Dp* variant A; AF529912: *Dp* variant B; AF529913: *Dp* variant C; AF529914: *Dp* variant D; AF529915: *Dp* variant E; AF529908: *Gp* variant A; AF529909: *Ao* variant A; AF529910: *Ao* variant B.

2.6 Sequence alignment and phylogenetic analyses

Alignment of the dust mite's 12S rDNA sequences was performed using the Clustal Ver 1.7 program [33]. *I. hexagonus* (hedgehog tick) sequence retrieved from GenBank (accession number: NC02010) was included as a reference sequence. *I. hexagonus* belongs to the same Acari subclass as the domestic mites and is currently the closest related sequence in the GenBank database. Once aligned, the 12S sequences were examined for nucleotide similarities and differences (GeneDoc/Multiple Sequence Alignment Editor & Shading Utility, Ver 2.6.001, 1997) [34].

The Neighbor-Joining (NJ) [37] PHYLIP Phylogeny Inference Package Version 3.5c [35] method was employed to analyze the evolutionary relationships among the 12S rRNA variants with *I. hexagonus* included as an out-group. Heuristic parsimony (HP) was performed using PAUP 4.0 [36], using the following parameters: simple stepwise sequence addition, tree bisection and reconnection branch swapping (TBR), all characters equally weighted, uninformative characters were excluded, one tree held at each step, MulTrees option on and steepest descent option not in effect. Tree robustness for both methods was tested by bootstrapping (1000 replicates). The species sequences included in the HP analysis were retrieved from GenBank (*A. mellifera*: NC 001566; *S. scabiei*: AF372989; *R. sanguineus*: NC00074; *I. hexagonus*: NC02010; *D. pulex*: NC000844; *P. monodon*: NC002184; *P. longicarpus*: NC003058; *L. bleekeri*: NC 002507; *A. franciscana*: NC 001620; *P. strigosa*: NC002176; *L. terrestris*: NC001673; *K. tunicata*: NC001636; *R. nigromaculata*: NC002805; *P. marinus*: NC001626; *F. peregrinus*: NC0000878; *G. gallus*: NC001323; *I. iguana*: NC002793; *R. radiata*: NC000893; *A. japonica*: NC002707; *L. africana*: NC000934; *F. catus*: NC01700; *C. domesticus*: NC002008; *R. norvegicus*: NC001665; *P. pygmaeus*: NC001646; *H. sapiens*: NC001807; *G. gorilla*: NC001645; *C. hominivorax*: NC002660; and *D. melanogaster*: NC001709). Base composition statistics and nucleotide *p* distances were obtained using MEGA 2.1 [37].

3 Results

3.1 DNA sequence analysis and variants

A 391–396 bp long fragment of the 12S rRNA mitochondrial gene from four mite species (*Dp*, *Ao*, *Bt*, and *Gp*) was successfully amplified by PCR, cloned, and subsequently sequenced. Alignment was performed using *I. hexagonus* as a reference sequence (Fig. 1). Variants were identified as *bona fide* 12S rRNA sequences only after being detected more than once from independent PCR reactions within the same species. The sequences obtained from only one clone were excluded from the analyses. Variants for three of the four species were detected: six from *Bt* (AF529916–AF529921), five from *Dp* (AF529911–AF529915), and two from *Ao* (AF529909, AF529910). Only one 12S rRNA form was observed in *Gp* (AF529908) (Table 1). The differences between the *Dp* and *Ao* sequence variants consist only of point mutations, including an insertion at position 358 and four transitional substitutions (215, 220, 242, and 351 nt) (Fig. 1). In contrast, the sequences obtained from *Bt* and *Gp*

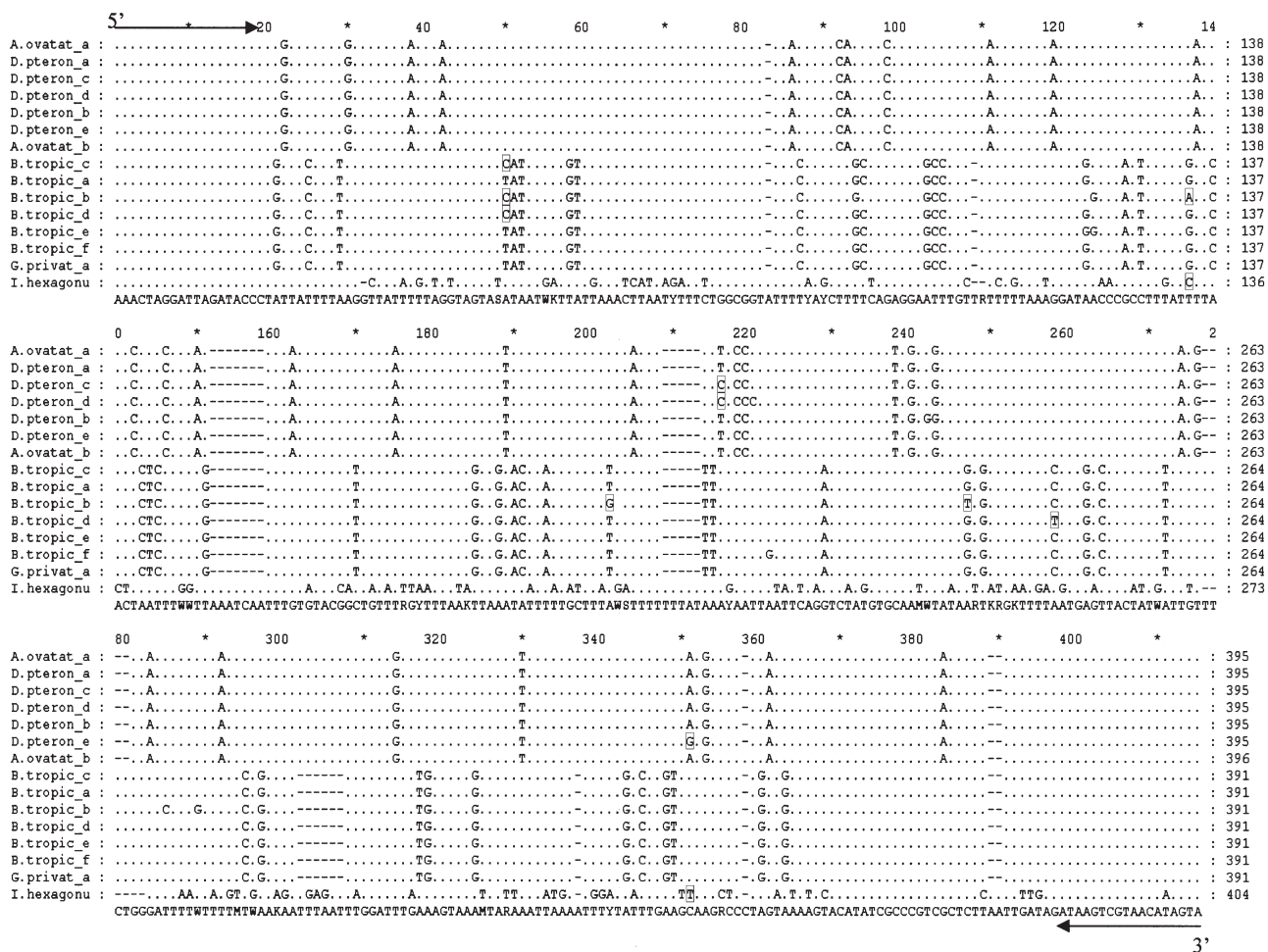


Figure 1. Sequence alignment of a fragment (390–396-bp) of the mitochondrial 12S rRNA gene from four domestic mite species: *Dp*, *Gp*, *Ao*, and *Bt* representing the Pyroglyphidae, Glycyphagoidea, Acaridae, and Echimyopodidae families, respectively. *I. hexagonus* was used as the comparison sequence and used in combination with the 12S rRNA house dust mite variants to generate the consensus sequence. A dot indicates same as consensus nucleotide. Denoted nucleotides represent base differences from the consensus sequence. 5' and 3' primer areas are indicated by arrows in their respective directions.

show more variability ranging from 1–6 nucleotide differences within their variants, 11 in total: nine transitions located at 50, 95, 123, 124, 136, 222, 258, 285, and 289 and two transversions at positions 202 and 247 (Fig. 1). Fifty-one nucleotide differences were identified among all the variants from the four dust mite species, representing 12.9% of the analyzed sequences. All the variants detected (independently of the species) exhibit a base composition bias toward A and T, ranging from a mean of 65.5 for *Bt* and *Gp* to 73.1 for *Dp* and *Ao* (Table 2). In the *Dp* and *Ao* species, the transition *versus* transversion ratio (*Ts/Tv*) was not determined (only four transitions were reported). On the other hand, the *Ts/Tv* ratios for the *Bt* and *Gp* variants range from 2 to 4.7, with a mean of 4.3 (Table 2). When the four species were analyzed together,

the average overall *Ts/Tv* ratio was 0.9. The mean *p* distance within *Dp* and *Ao* was 0.004 and within *Bt* and *Gp* was 0.008. When all four species were included an overall mean *p*-value of 0.159 was obtained (Table 2).

3.2 Molecular phylogeny

The 12S rRNA gene variants from the four house dust mite species representing the four family taxa clustered together and away from the reference arthropod *I. hexagonus* in the NJ tree (Fig. 2). Generally, the variants segregated along species lines. When other species were included in an HP analysis to ascertain the strength of the proposed clustering, the HP tree presented a topology for

Table 1. House dust mite 12S rRNA variants *per* family and species

Family	Species	Total number of clone sequences <i>per</i> variants	Variants	Total number of variants <i>per</i> species	GenBank accession number
Glycyphagidae	<i>G. privatus</i>	36	GprivatA	1	AF529908
Acaridae	<i>A. ovatus</i>	22	AovatusA	2	AF529909
		3	AovatusB		AF529910
Pyroglyphidae	<i>D. pteronyssinus</i>	9	DpteronA	5	AF529911
		6	DpteronB		AF529912
		3	DpteronC		AF529913
		2	DpteronD		AF529914
		2	DpteronE		AF529915
Echimyopodidae	<i>B. tropicalis</i>	11	BtropicA	6	AF529916
		3	BtropicB		AF529917
		3	BtropicC		AF529918
		2	BtropicD		AF529919
		2	BtropicE		AF529920
		2	BtropicF		AF529921

Table 2. Base composition, A–T bias, *Ts/Tv* ratios, and *p* distance among house dust mite 12S rRNA isoforms

Clade	A ^{a)}	T ^{a)}	C ^{a)}	G ^{a)}	A–T bias ^{a)}	<i>Ts/Tv</i> ^{b)}	Distance ^{c)}
<i>Dp/Ao</i>	34.0	39.1	11.6	15.1	73.1	–	0.004
<i>Bt/Gp</i>	23.6	41.9	14.4	20.2	65.5	4.3	0.008
<i>Dp/Ao</i> + <i>Bt/Gp</i>	28.8	40.5	13.0	17.6	69.3	0.9	0.159

a) All values of base composition are presented in percentages (%).

b) Mean values among pairwise comparison

c) *p* distance values (transitions + transversions)

A hyphen indicates that no value was obtained.

the dust mite variants similar to that obtained in the NJ tree (Fig. 3). Two sister clades for the four house dust mites were consistently differentiated in both the HP and the NJ analyses (Figs. 2, 3). One of the clades encompasses the variants belonging to *Dp* and *Ao*, while the other includes the variants belonging to *Bt* and *Gp*. Bootstrap values above 500, corresponding to the appropriate nodes in both the NJ and HP trees, are indicated in both dendrograms (represented as percentages).

4 Discussion

4.1 Base composition analyses

Base composition analyses of the 12S rRNA variants uncovered a bias toward A and T in the two clades formed (Table 2). This tendency is usually observed in metazoans mitochondrial sequences [19, 38, 39]. The nucleo-

tide differences observed among the four species studied represent 12.9% of the 12S rRNA sequences examined. Comparison of the *p* distances (transition + transversion) from the variant sequences shows that the isoforms in the *Bt/Gp* clade (0.008) have twice the amount of variation than the variants in the *Dp/Ao* cluster (0.004) (Table 2). Not surprisingly, the bifurcations in the *Bt/Gp* clade were supported by higher bootstrap values (Figs. 2, 3). These results suggest that the genetic distances are not equal between the species of the two clades representing the four dust mite families. In addition, it suggests that *Dp* and *Ao* are phylogenetically closer than *Bt* and *Gp*. The analysis of the *Ts/Tv* ratios indicated a transitional bias that has been frequently reported either among recently evolved groups or in slowly evolving genes [26, 40]. Given that the *Ts/Tv* ratio between recently diverging DNA sequences is usually 2.0 or more [19], the results (4.3) suggest a non-ancient divergence within the *Bt/Gp* clade (Table 2). The *Ts/Tv* ratio of the *Dp/Ao* clade was not determined due to

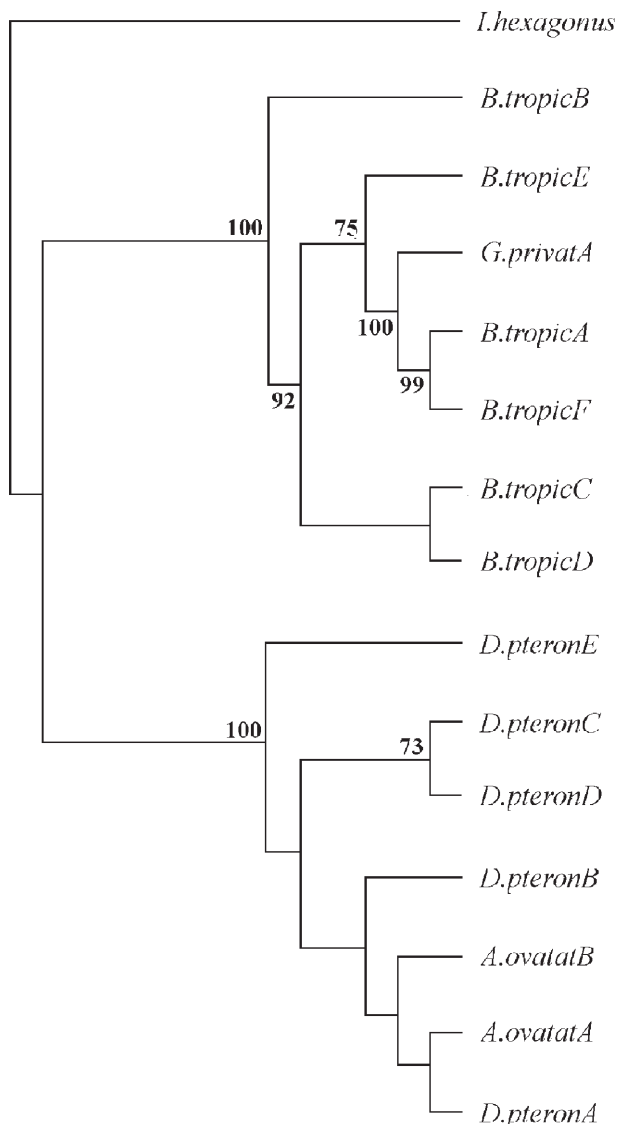


Figure 2. NJ tree using the mitochondrial 12S rRNA gene sequence variants of domestic mites *Dp*, *Gp*, *Ao*, and *Bt*. Bootstrapped 1000 × (represented as percentages) and rooted with the *I. hexagonus* sequence. Values <50% are not shown.

the lack of transversions on the reported sequences. However, when the Ts/Tv ratio analysis of the four species was performed, a more ancient divergence within them is indicated ($Ts/Tv = 0.9$) (Table 2).

4.2 Phylogenetic relationships

Previous studies suggest that the Astigmata suborder evolved from the ancient Oribatida, a group of soil-dwelling mites [1]. Fossils of the Oribatida have been recovered from Devonian mudstones dating back 376–379 million

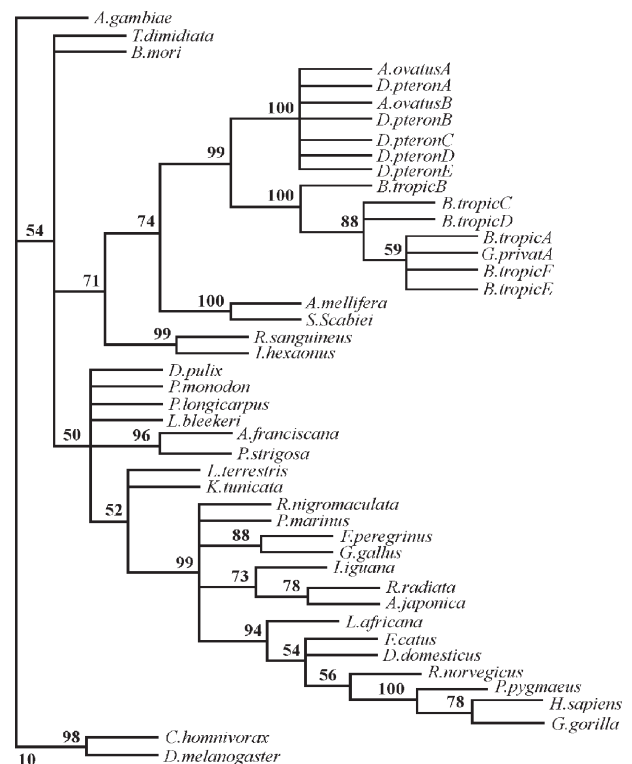


Figure 3. HP based on tree of the 12S rRNA sequences from various species along with the isoform sequences of domestic mites *Dp*, *Gp*, *Ao*, and *Bt*. This dendrogram is the most parsimonious tree generated using heuristic search with simple stepwise sequence addition, TBR, and bootstrapping of 1000 replicates. Bootstrap values <50% are not shown.

years [41]. The possible relationships that have been proposed for the Pyroglyphidae, Glycyphagidae, Echi-myopodidae, and Acaridae families suggest that the latter three evolved separately from the Pyroglyphidae [1, 12–15]. This hypothesis was partially supported by our 12S rRNA sequence analyses. According to the results of the present study, the Glycyphagidae and Echi-myopodidae families (*Bt/Gp* clade) (both belonging to the Glycyphagoidea superfamily) appear to be closely related (Figs. 2, 3). This finding was expected based on current taxonomy and the hypothesis that both migrated from terrestrial settings (e.g., small mammal ground nests to houses) [16]. However, our phylogenetic analyses suggest a closer relationship than expected between the Pyroglyphidae and Acaridae families (*Do/Ao* clade) (Figs. 2, 3). Furthermore, these latter families share common variant sequences (*DpteronA* and *AovatusA*). This was a surprising finding due to the fact that these two species differ not only in their proposed migration patterns (*Ao* from terrestrial mammalian nests and *Dp* from ground bird-nests dwellers) [14, 16], but also in their morphological characters (pictorial keys) (Collof, 1998b).

4.3 Conclusions

Variants of the mtDNA 12S rRNA sequence from four domestic mite species were obtained and analyzed. These sequences may serve as templates for designing specific primers to differentiate between members of the Glycyphagoidea superfamily (*Bt* and *Gp*) and the Pyroglyphidae (*Dp*) and Acaridae (*Ao*) families. Identification of domestic mites from environmental samples or cultures using PCR and DNA sequencing will be highly advantageous. It is significant that the 12S rRNA sequences examined are capable of discriminating between these economically and medically important species of dust mites. If required, additional sequence data from nuclear and mitochondrial genomes could be used to discriminate further among species or even geographical populations in an effort to identify allergen based causes.

Examining the phylogenetic relatedness among domestic mite families (all considered sources of allergens) may help to understand the nature of their allergenic proteins, as well as their adaptability to different environments during their evolutionary process. As observed in the phylogenetic analyses of the Pyroglyphidae (*Dp*) and Acaridae (*Ao*) families, the close evolutionary relationship of the Pyroglyphidae and Acaridae families may explain similarities in protein expression and cross-reactivity, which had not been explained by more traditional methods based on the physiochemical and biochemical properties of the allergens [1, 3].

The results observed in the NJ and HP trees support the close relationship and current classification of the Echi-myopodidae and Glycyphagidae families in the Glycyphagoidea superfamily. However, our phylogenetic analyses identified an unexpected closer relationship between the Pyroglyphidae and Acaridae families. This data provide evidence to suggest further consideration to reclassify the taxonomically controversial *Ao* species or to consider the creation of a superfamily containing these two families. Analyses of other sequences from the remaining mitochondrial genome as well as other domestic mite species, may provide information on the proposed relationships.

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