

Mite species identification in the production of allergenic extracts for clinical use and in environmental samples by ribosomal DNA amplification

B. BEROIZ¹, F. COUSO-FERRER¹, F. ORTEGO¹,
M. J. CHAMORRO^{2,3}, C. ARTEAGA^{2,3}, M. LOMBARDEO^{2,3},
P. CASTAÑERA¹ and P. HERNÁNDEZ-CRESPO¹

¹Department of Environmental Biology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, ²Department of Research and Development, ALK-Abelló SA, Madrid, Spain and ³Department of Research and Development, ALK-Abelló AS, Hørsholm, Denmark

Abstract. The identification of allergy-causing mites is conventionally based on morphological characters. However, molecular taxonomy using ribosomal DNA (rDNA) may be particularly useful in the analysis of mite cultures and purified mite fractions in the production of allergenic extracts. Full-length internal transcribed spacers (ITS1 and ITS2) were obtained from *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Dermatophagoides microceras* and *Euroglyphus maynei* (Astigmata: Pyroglyphidae), *Glycyphagus domesticus* and *Lepidoglyphus destructor* (Astigmata: Glycyphagidae), *Tyrophagus fanetzhangorum*, *Tyrophagus putrescentiae*, *Tyrophagus longior*, *Tyrophagus neiswanderi*, *Acarus farris* and *Acarus siro* (Astigmata: Acaridae), and *Blomia tropicalis* (Astigmata: Echymopodidae), using mite-specific primers. Polymerase chain reaction (PCR) products were digested with *Hpa*II and *Rsa*I restriction enzymes in order to produce species-specific PCR restricted fragment length polymorphism (RFLP) profiles. A semi-nested re-amplification step was introduced before the RFLP in order to apply the method to environmental samples. Results demonstrate that rDNA sequences can be used for the unambiguous identification of mite species. The PCR–RFLP system allows the identification of species in purified mite fractions when the availability of intact adult mite bodies for morphological identification is limited. This reliable and straightforward PCR–RFLP system and the rDNA sequences obtained can be of use in the identification of allergy-causing mite species.

Key words. *Acarus*, *Blomia*, *Dermatophagoides*, *Euroglyphus*, *Glycyphagus*, *Lepidoglyphus*, *Tyrophagus*, allergy, molecular taxonomy.

Introduction

Stored product and house dust mites of the order Astigmata are recognized as important respiratory allergy-causing agents (Thomas *et al.*, 2010). The most relevant species belong to the families Pyroglyphidae (*Dermatophagoides* spp. and *Euroglyphus* spp.), Acaridae (*Acarus* spp. and *Tyrophagus* spp.), Echymopodidae (*Blomia* spp.) and Glycyphagidae (*Glycyphagus* spp.

and *Lepidoglyphus* spp.). Allergen avoidance, drug therapy and immunotherapy are the main strategies currently employed to reduce disease caused by mites; the last of these represents the only disease-modifying approach and the most promising method of counteracting allergy. The treatment consists of the administration to the patient of gradually increasing quantities of an allergenic extract which, in the case of mites, is derived from mass-rearing cultures. The quality of an allergen extract

Correspondence: Pedro Hernández-Crespo, Centro de Investigaciones Biológicas, CSIC, Laboratorio Interacciones Planta-Insecto (Lab 240), Ramiro de Maeztu 9, 28040 Madrid, Spain. Tel.: + 34 91 8373 112 (ext. 4393); Fax: + 34 91 5360 432; E-mail: pedro@cib.csic.es

is related to the adequacy of the original material (Fрати *et al.*, 2012) because species identification in mite cultures is a key factor in the standardization of allergen production, especially when mite colonies are renewed with specimens sourced from other laboratories or field-derived mites.

Mite identification has been conventionally based on morphological characterization (Hughes, 1976; Colloff & Spieksma, 1992) and requires, in many cases, a specialized knowledge and analysis of adult stages. Thus, species certification by morphology in the production of mite allergenic extracts is time-consuming, represents an increased cost to the industry, and cannot be applied using purified mite fractions downstream in the production process. By contrast, a straightforward method based on molecular markers (Navajas & Fenton, 2000) could be designed to facilitate mite species identification or validation. The use of DNA markers is advantageous in that it does not require a given developmental stage or a source of living individuals for analysis. Once the correspondence between morphological and molecular identification has been correctly assessed (for a critical review, see de Mendonça *et al.*, 2011), a large number of samples can be routinely analysed, reducing both time requirements and subjectivity in comparison with morphological identification processes. Ideally, a DNA marker appropriate for species certification in the production process of allergenic extracts should identify mite species in purified fractions of bodies or faeces.

The nuclear ribosomal DNA region (rDNA) has been used previously for the identification of different eukaryote organisms with known success (Schoch *et al.*, 2012). The rDNA cluster consists of three conserved ribosomal subunits, 18S, 5.8S and 28S, linked by the two internal transcribed spacers, ITS1 and ITS2. More than 100 copies of this cluster may exist in a genome, which favours its amplification by polymerase chain reaction (PCR) from genomic DNA. The three subunits are highly conserved and are useful in discriminating taxonomic groups, whereas the ITS1 and ITS2 regions can be used for species identification (Hillis & Dixon, 1991; Navajas & Fenton, 2000; de Rojas *et al.*, 2007). Variation in ITS1 or ITS2 sequences has been successfully used to identify species of mites, insects and ticks (Gotoh *et al.*, 1998; Essig *et al.*, 1999; Navajas *et al.*, 1999, 2001; Knapp *et al.*, 2003; Ben-David *et al.*, 2007; Wei *et al.*, 2011). Combined PCR and restriction fragment length polymorphism (PCR–RFLP) analysis has been applied in the identification of ticks (Poucher *et al.*, 1999), mites (Osakabe *et al.*, 2002) and insect species (Toma *et al.*, 2000; Kampen *et al.*, 2003). The molecular identification of mites using 12S rRNA mitochondrial sequences has been proposed (Suarez-Martinez *et al.*, 2005), but the marker used is limited by the conservation of sequences comparing species of different genera. By contrast, the ITS2 sequence of rDNA was demonstrated to be useful in the differentiation of Astigmata species (Webster *et al.*, 2004; Noge *et al.*, 2005; Yang *et al.*, 2011). More recently, a PCR–RFLP method based on the amplification of the ITS2 region of rDNA and the restriction patterns generated by six to eight enzymes has been applied for the differentiation of six species (Wong *et al.*, 2011).

The current study reports the full-length ITS1, 5.8S subunit and ITS2 sequences of rDNA from 13 Astigmata species belonging to the genera *Dermatophagoides*, *Euroglyphus*,

Acarus, *Tyrophagus*, *Glycyphagus*, *Lepidoglyphus* and *Blomia*. Based on the sequences obtained, we developed a PCR–RFLP system that can be implemented for species certification in mite cultures and purified fractions (bodies and faeces) used for the industrial production of allergenic extracts. Finally, the system was assayed for the detection of Astigmata mites in environmental samples by introducing a second PCR amplification before the RFLP reaction.

Materials and methods

Mites

Thirteen Astigmata species belonging to seven different genera and four different families were studied. Ten species are regularly maintained at ALK-Abelló facilities: *Blomia tropicalis* Van Bronswijk, de Cock & Oshima (Bt); *Acarus siro* Linnaeus (As); *Dermatophagoides microceras* Griffiths & Cunningham (Dm); *Dermatophagoides farinae* Hughes (Df); *Dermatophagoides pteronyssinus* (Trouessart) (Dp); *Euroglyphus maynei* (Cooreman) (Em); *Glycyphagus domesticus* (de Geer) (Gd); *Lepidoglyphus destructor* (Schränk) (Ld); *Tyrophagus fanetzhangorum* Klimov & O'Connor (Tf), and *Tyrophagus putrescentiae* (Schränk) (Tp). Three species came from laboratory colonies maintained at the Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CIB–CSIC). These were: *Tyrophagus longior* (Gervais) (Tl), cultures of which were established from *cecina* (air-dried meat) from León (Spain); *Tyrophagus neiswanderi* Johnson & Bruce (Tn), and *Acarus farris* (Oudemans) (Af), which were collected on mature Cabrales cheese at Carreña de Cabrales (Spain) (Sanchez-Ramos *et al.*, 2007). Mite species were identified based on taxonomic keys (Hughes, 1976; Colloff & Spieksma, 1992). The mite sample to be identified was carefully added to a glass slide containing a drop of lactic acid. The preparation was covered with a coverslip. When the cuticle was clear enough, the sample was analysed under the microscope.

Environmental samples

House dust samples were collected using a standard vacuum cleaner fitted with a new dust bag as instructed (DEA-test system; ALK-Abelló, SA, Madrid, Spain). The dust samples were sieved (mesh size: 850 µm) and stored at –60 °C until they were required for DNA/allergen extraction. Der p 1 and Der f 1 allergen content was determined by enzyme-linked immunosorbent assay (ELISA), based on allergen-specific mAb, after extraction into phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) (Barber *et al.*, 2012). The detection limit of the ELISA was 0.05 µg allergen/g dust. Five samples were collected from houses in northwest Spain (A Coruña, Galicia: CH2, CH4, CH7; Infiesto, Asturias: CH3, CH5), and four were collected in the Canary Islands (Gran Canaria: 717, 718, 719, 720).

DNA extraction

Genomic DNA was purified from: (a) individual mites collected with the aid of a clean entomological needle; (b) mite

cultures in exponential growth containing mites and culture media (semisynthetic media containing among other components yeast, hydrolysed fish flour proteins, starch and vitamins); (c) purified fractions consisting of bodies and faeces obtained by serial sieving from whole mite culture, and (d) environmental house dust samples (described above). Genomic DNA from mite individuals and mite cultures was purified following a standard phenol-chloroform method previously described by Díaz-Mendoza *et al.* (2005). Briefly, samples were homogenized in a saline buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA; pH 8), prior to the addition of sodium dodecyl sulphate (SDS) (1%) and proteinase K (1 mg/mL). After overnight incubation at 37 °C, NaCl and cetyltrimethylammonium bromide (CTAB) were added to a final concentration of 0.7 M and 1%, respectively, and incubated at 62 °C for 10 min. DNA was purified through consecutive phases of phenol and chloroform/isoamyl alcohol. Then, the NaCl concentration was lowered to 0.5 M and DNA was precipitated by centrifugation after adding one volume of isopropanol. Finally, DNA pellets were washed with 70% ethanol, dried, re-suspended in TE 0.1× buffer (1 mM Tris, 0.1 mM EDTA; pH 8) with RNAase (0.1 µg/µL), and stored at 20 °C until use. DNA extraction from purified fractions (bodies or faeces, 20 mg) and environmental samples (50 mg) was performed using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany).

rDNA amplification

Amplification by PCR was performed using the mite-specific primers FNav (5'-AGAGGAAGTAAAAGTCGTAACAAG-3'), based on the 3' end of the rDNA 18S sequence (Navajas *et al.*, 1999), and RNav2 (5'-ATATGCTTAAATTCAGCGGG-3'), based on the 5' end of the rDNA 28S sequence (Navajas *et al.*, 1998). The PCR product contained the 3' end of the 18S subunit, the full-length sequences of ITS1, the 5.8S subunit and ITS2, and the 5' end of the 28S rDNA subunit. Polymerase chain reaction was performed in a total volume of 25 µL containing 100 ng of DNA template, 1× PCR Buffer II, 200 µM dNTP mix, 0.4 µM of each primer, 1.5 mM MgCl₂ and 1.5 units of Ampli Taq Gold DNA Polymerase (Applied Biosystems, Inc., Foster City, CA, U.S.A.). The PCR cycle consisted of a 95 °C hold for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C and 2 min at 72 °C, ending with an extension hold of 10 min at 72 °C. All PCR reactions included negative controls using water as the template. All PCR products were visualized after agarose gel electrophoresis using TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8), and staining with ethidium bromide.

Cloning and sequencing of PCR products

Purified PCR products (PCR Purification Kit; Qiagen GmbH) were ligated to the pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) and cloned into *Escherichia coli* (Enterobacteriales: Enterobacteriaceae) DH5α strain. Ten positive colonies were randomly selected for plasmid purification, using the High Pure Plasmid Isolation Kit (Roche

AG, Basel, Switzerland), and insert sequencing. Sequences were initially obtained using the vector primers SP6 (5'-AT TTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACT ATAGGG-3'). Later, two internal primers were designed according to a conserved region (for all species analysed) at the 5.8S subunit: Fast5.8 (5'-ACTCGACWTTCTGAACGCA-3'), and Rast5.8 (5'-TGCGTTTCGAAGWTCGAGT-3'). These primers were used for both clone sequencing and direct sequencing of PCR products (see above). Direct sequencing of PCR products obtained from DNA extracted from single individuals of three of the 10 species, *D. farinae*, *D. pteronyssinus* and *B. tropicalis*, was performed using primers FNav, RNav2, Fast5.8 or Rast5.8 on a 3730 DNA Analyzer (Applied Biosystems, Inc.).

Sequence analysis

In all cases, sense and antisense strand sequences were edited using 4Peaks software (Version 1.7.2) to obtain full-length readings. Clustal 2.1 multiple sequence analyses were performed at the European Molecular Biology (EMBL) European Bioinformatics Institute (EBI) ClustalW2 server (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Bootstrap neighbour-joining trees (1000 replicates) were obtained using ClustalQt 1.5a. The tree was visualized with Dendroscope software (Huson *et al.*, 2007). BLAST analysis against the nucleotide collection (nr/nt) was performed at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Sequences reported in this paper were deposited in the GenBank database (GenBank accession nos: KC215255–KC215386). The following sequences available in the GenBank database were also used in the ITS2 alignment: *A. farris* (AY525591); *Acarus gracilis* (AY525592); *Acarus immobilis* (AB105007); *A. siro* (AY525589); *B. tropicalis* (AB105002, GQ205587); *D. farinae* (AB105001, GQ205603); *D. pteronyssinus* (AB105008, GQ205614); *E. maynei* (GQ205616); *T. longior* (AB104997); *T. neiswanderi* (AB105036); *T. putrescentiae* (AB105000, GQ205623), and *Tyrophagus similis* (AB104978) (Webster *et al.*, 2004; Noge *et al.*, 2005; Yang *et al.*, 2011). The boundaries of ITS1 and ITS2 for the analysis performed in this study were assigned by conserved sequences at the 18S (5'-AGGATCATT-3'), 5.8S (5'-CTGYYAGTGG-3' and 5'-TGAGCGTCGT-3') and 28S (5'-CGACCTCAG-3') subunits.

Analysis by PCR-RFLP

Amplification by PCR was performed using the primers FNav and RNav2 as described above. Non-purified PCR products were digested using *Hpa*II or *Rsa*I restriction enzymes (Roche AG). The restriction enzyme reaction was performed at 37 °C for 2 h in a hot-lid thermal cycler to minimize evaporation and avoid partial digestion, in a final volume of 20 µL containing 8–11 µL of the PCR product, 2 µL of Sure/Cut Buffer L and four units of the corresponding enzyme. The resulting digested fragments were separated in 2% agarose gels.

Analysis by PCR–RFLP in environmental samples

No PCR products were visualized after the amplification of DNA purified from environmental samples by the standard protocol. Thus, the PCR–RFLP system was adapted to these samples using the DNeasy Blood and Tissue Kit (Qiagen GmbH) for DNA extraction, and by introducing a re-amplification step before the RFLP. The re-amplification consisted of a semi-nested reaction using the primers FNav and RAs5.8, amplifying the full-length ITS1 region flanked by partial sequences of the 18S and 5.8S subunits. The amplification cycle and the RFLP procedure did not differ from those described above. The resulting digested fragments were separated in 3% agarose gels (using conventional and low-melting agarose at a 1 : 1 ratio).

Results

The primer combination FNav and RNav2 was successfully used for PCR amplification of rDNA in DNA extracted from cultures of 13 Astigmata species (Fig. 1). Products of PCR were purified and cloned and 10 insert-containing clones for each reaction were randomly selected for sequencing (GenBank accession nos: KC215255–KC215384). In all cases, sequence analyses confirmed the amplification of the complete ITS1, 5.8S and ITS2 rDNA regions flanked by partial sequences corresponding to the 18S and 28S subunits. The similarity among the sequences obtained and their comparison with sequences available in the GenBank database identified all sequences as Astigmata rDNA. The minimum similarity at the 5.8S subunit (151–152 bp) among species of different families was 85%. Similarity among species of the same family at the same region ranged between 97% and 100%. Clones obtained for all species were polymorphic, but the polymorphism detected did not affect species identification. Thus, clones obtained from each species significantly clustered together in the phylogenetic tree generated by the alignment of the sequences (Figure S1).

The complete ITS2 region flanked by the 5.8S and 28S partial sequences from the 10 clones obtained for each species were aligned to other Astigmata rDNA sequences available in GenBank belonging to *Dermatophagoides* spp., *Tyrophagus* spp., *Acarus* spp., *B. tropicalis* and *E. maynei* (Webster *et al.*, 2004; Noge *et al.*, 2005; Yang *et al.*, 2011). As the phylogenetic tree generated from this alignment shows (Fig. 2), rDNA sequences form distinct species that are always clustered in the same branch with a highly significant bootstrap value (99–100%). In addition, sequences obtained in this study from *D. farinae*, *D. pteronyssinus*, *A. siro*, *A. farris*, *T. longior*, *T. putrescentiae*, *T. neiswanderi* and *B. tropicalis* unambiguously clustered to sequences assigned to these species in the GenBank database, with the notable exception of sequences reported by Yang *et al.* (2011) as *E. maynei* rDNA.

The estimated sizes for ITS1, 5.8S and ITS2 varied among species (Table 1). The ITS regions of closely related species belonging to the same family showed small differences in size. The 5.8S subunit was highly conserved, as stated earlier, but varied in the length of one nucleotide among different Astigmata families: it was 151 bp in the seven species



Fig. 1. Polymerase chain reaction (PCR) amplification of rDNA from 13 astigmatid mite species. PCR performed on DNA extracted from laboratory cultures of *Acarus siro* (As), *Acarus farris* (Af), *Tyrophagus neiswanderi* (Tn), *Tyrophagus putrescentiae* (Tp), *Tyrophagus fanetzhangorum* (Tf), *Tyrophagus longior* (Tl), *Dermatophagoides microceras* (Dm), *Dermatophagoides farinae* (Df), *Dermatophagoides pteronyssinus* (Dp), *Euroglyphus maynei* (Em), *Blomia tropicalis* (Bt), *Glycyphagus domesticus* (Gd) and *Lepidoglyphus destructor* (Ld). M, 100-bp ladder (Fermentas).

of the families Acaridae and Echymopodidae, and 152 bp in the six species of the families Pyroglyphidae and Glycyphagidae. Intraspecific polymorphism was observed in all species without exception, mainly consisting of a low number of discontinuous substitutions together with short insertions or deletions. The higher variation in length at the ITS1 and ITS2 regions was found in *A. farris* (ITS1, 773–789 bp) and *B. tropicalis* (ITS2, 214–227 bp). Direct sequencing of the PCR amplification of rDNA using DNA extracted from single individuals or from cultures failed as an identification method in *D. farinae*, *D. pteronyssinus* and *B. tropicalis*. All primers tested for sequencing (FNav, Fast5.8, Rast5.8 and Rnav2) produced chromatograms with very short clear sequences followed by double reading compatible with the existence of insertions or deletions in rDNA sequences. The partial sequences obtained were not of a length adequate for species identification. Direct sequencing in single individuals of other species was not assayed.

The PCR–RFLP method

A PCR–RFLP method has been designed for species identification. Products of PCR obtained using the primer combination FNav and RNav2 were digested with *HpaII* or *RsaI* restriction enzymes. The band patterns generated by each enzyme (Fig. 3) were in agreement with those predicted for each species by the analysis of the sequences of the 10 clones obtained and were compatible with the polymorphism detected (Table S1). Patterns were species-specific, although the digestion with both enzymes was useful for resolving ambiguities caused by the presence of bands of similar size. Thus, the restriction fragments generated by the enzymes could be used to unambiguously identify each species.

The PCR–RFLP was performed using both DNA extracted from single individuals of *D. farinae*, *D. pteronyssinus* and *B. tropicalis* and DNA extracted from mite cultures of the three species. No differences were observed in comparisons of profiles obtained from single individuals or mite cultures of the same species (Fig. S2, online). Finally, the method was

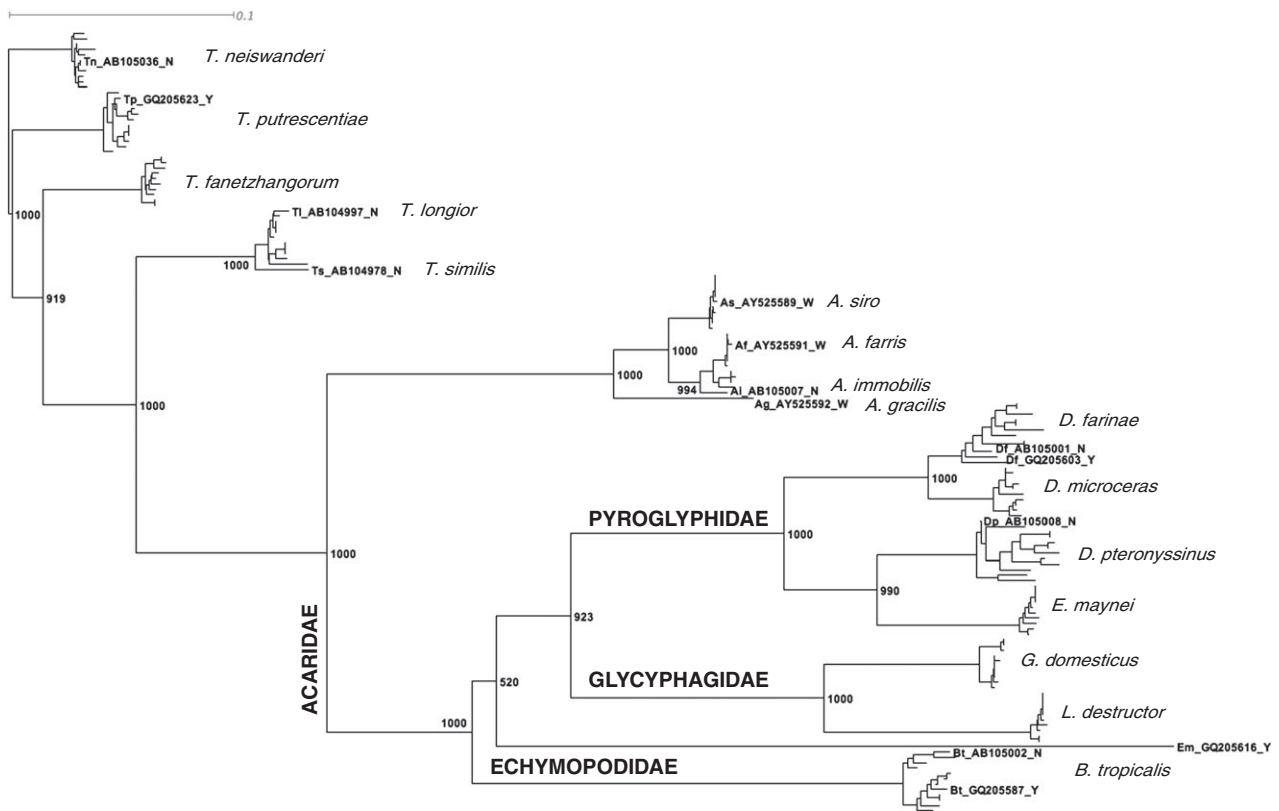


Fig. 2. Phylogenetic tree generated from the alignment of ITS2 rDNA region flanked by partial sequences of 5.8S and 28S subunits. Sequences obtained in this paper were aligned to representative sequences available in GenBank. The first and second letters indicate genus and species, respectively; the letter after the GenBank accession number indicates the bibliographic reference [(W) Webster *et al.*, 2004; (N) Noge *et al.*, 2005; (Y) Yang *et al.*, 2011]. All clones obtained from one mite species in this paper clustered in the group indicated by the scientific name. Bootstrap values correspond to 1000 replicates.

Table 1. Total size (base pairs) of cloned and sequenced polymerase chain reaction (PCR) products and estimated full-length size for ITS1, ITS2 and the 5.8S subunit (GenBank accession nos: KC215255–KC215384). The range observed corresponds to the polymorphism found in 10 clones analysed per species. Partial sequences for the 18S and 28S subunits were 57 bp and 43 bp, respectively, in length in all cases.

	PCR product	ITS1	5.8s	ITS2
<i>Acarus siro</i>	1438–1449	763–774	151	422–424
<i>Acarus farris</i>	1441–1458	773–789	151	416–420
<i>Tyrophagus neiswanderi</i>	1335–1354	698–713	151	384–390
<i>Tyrophagus putrescentiae</i>	1303–1310	668–671	151	384–388
<i>Tyrophagus fanetzhangorum</i>	1373–1376	725–726	151	396–399
<i>Tyrophagus longior</i>	1291–1295	684–685	151	355–359
<i>Dermatophagoides microceras</i>	951–960	466–478	152	228–233
<i>Dermatophagoides farinae</i>	953–959	468–474	152	233–235
<i>Dermatophagoides pteronyssinus</i>	950–954	477–481	152	220–223
<i>Euroglyphus maynei</i>	939–948	465–470	152	222–227
<i>Blomia tropicalis</i>	927–943	461–468	151	214–227
<i>Glycyphagus domesticus</i>	1258–1267	639–643	152	367–373
<i>Lepidoglyphus destructor</i>	1341–1347	719–721	152	370–374

successfully used for *D. farinae* and *D. pteronyssinus* species identification in purified fractions (bodies and faeces) of mite cultures used for allergenic extracts production. Results obtained in the analysis of three independent purified fractions of bodies and two of faeces from *D. farinae* cultures are shown in Fig. S3 (online).

Use of RFLP to detect mites in environmental samples

The RFLP method described herein was assayed for the detection of the dominant species in environmental samples consisting of dust samples from houses. The PCR reaction using the primers FNav and RNav2 was assayed on DNA extracted

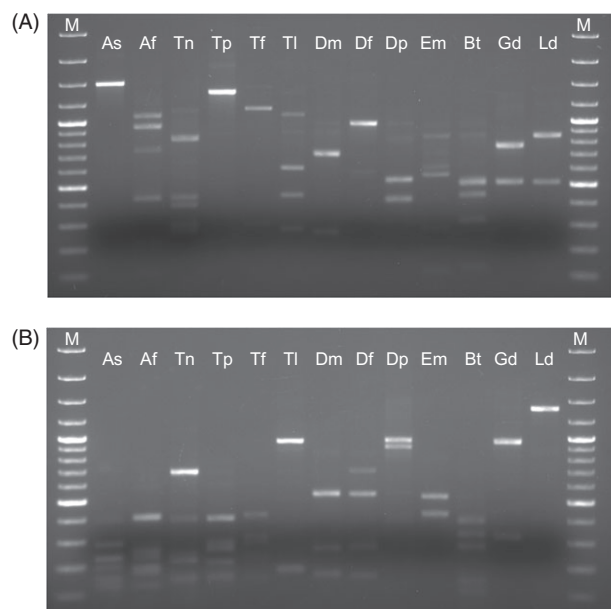


Fig. 3. Polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) profiles obtained from rDNA amplified from cultures of 13 Astigmata species. (A) *HpaII* digestion. (B) *RsaI* digestion. Species names are indicated by the first letters of the genus and species (see Fig. 1). M, 100-bp ladder (Fermentas).

from 0.1–0.2 g of sieved powder, but no PCR products were visualized in agarose gels. As the proportion of mite material in relation to the total weight of the sample may influence the efficiency of the PCR, several nested and semi-nested re-amplifications were performed without success. Then, different DNA extraction methods were assayed in order to avoid PCR inhibitors that may remain in the sample after DNA precipitation. Finally, the PCR–RFLP method was improved by using a specific DNA extraction protocol (see Materials and methods) and by performing a semi-nested re-amplification of the first PCR (FNav and RNav2 primers) to amplify the ITS1 region, using the primer FNav in combination with the primer Rast5.8 (based on the 5.8S subunit).

Figure 4(A) shows the semi-nested PCR products obtained from DNA from mite cultures (right), and from DNA extracted from environmental samples collected in houses in north-west Spain (A Coruña, Galicia: CH2, CH4, CH7; Infiesto, Asturias: CH3, CH5), and in the Canary Islands (Gran Canaria: 717, 718, 719, 720). The PCR products obtained from mite cultures were in agreement with those predicted for each species by the analysis of the sequences previously obtained (see Table S2). The size of the PCR products visualized (600–700 bp) in all samples, with the exception of CH5, was compatible with the detection of Pyroglyphidae mites (*Dermatophagoides* spp. and/or *E. maynei*) and/or *B. tropicalis*, discarding the presence of other species producing PCR products of higher molecular weight (see Fig. 4A). By contrast, the single band of < 500 bp amplified in sample CH5 and secondary bands of similar size observed in samples 717 and 718 did not match with any of the mite species studied in this work.

The unexpected PCR products of < 500 bp observed in samples CH5 and 717 were cloned and sequenced. The sequence data obtained (GenBank accession nos: KC215385 and KC215386) were compatible with mite rDNA based on their similarity with the sequences obtained in this study and sequences available in GenBank, although species identification was not possible.

In order to further identify mite species in the environmental samples, PCR–RFLP patterns using *HpaII* or *RsaI* restriction enzymes were generated after ITS1 re-amplification of mite cultures. The patterns obtained from all species in cultures are shown in Fig. S4 (online). Again, PCR products obtained from mite cultures were in agreement with those predicted for each species according to the analysis of the sequences previously obtained (Table S2, online). The patterns obtained from environmental samples, together with samples of compatible species based on the size of the PCR product amplified (see above), are shown in Fig. 4(B, C). As the PCR product amplified on sample CH5 did not correspond to a known species, no RFLP was performed for this sample. The interpretation of the patterns generated was complex as a result of the presence of more than one species in the samples. As Fig. 4(B, C) shows, bands compatible with *D. farinae* were observed in the RFLP profiles generated by the two enzymes in samples from Galicia (CH7) and Asturias (CH3), and less clearly (not all bands visualized) in samples from the Canary Islands (719 and 720); bands compatible with the presence of *D. pteronyssinus* were observed in samples collected in Galicia (CH4) and Gran Canaria (719 and 720); finally, bands compatible with the presence of *B. tropicalis* were observed in one sample collected in the Canary Islands (718).

The results of PCR–RFLP with the Der p 1 and Der f 1 content determined by ELISA in the same environmental samples are compared in Table 2. Complementary and non-contradictory results were observed using the two detection systems. Nonetheless, PCR–RFLP results were influenced by the presence of more than one mite species in the samples and by the dominance of one of them. Thus, the two detection systems identified *D. pteronyssinus* as the dominant species in samples CH4 and 720, and *D. farinae* as the dominant species in samples CH7 and CH3. However, the PCR–RFLP detection of the two species in samples 719 and 720 was ambiguous, whereas ELISA detected the presence of Der p 1/Der f 1 antigens in most of the samples. The detection of *D. pteronyssinus* or *D. farinae* in samples CH2, CH5, 717 and 718 by PCR–RFLP may have been influenced by the dominance or presence of other mite species, as seen in Table 2.

Discussion

Full-length ITS1 and 5.8S subunit sequences for the 13 species studied are reported for the first time in this paper. In addition, ITS2 sequences for *D. microceras*, *T. fanetzhangorum*, *G. domesticus* and *L. destructor* are reported for the first time. In previous works, ITS2 sequences have been used for the molecular identification of Astigmata mites and in phylogenetic studies (Webster *et al.*, 2004; Noge *et al.*, 2005; Yang *et al.*,

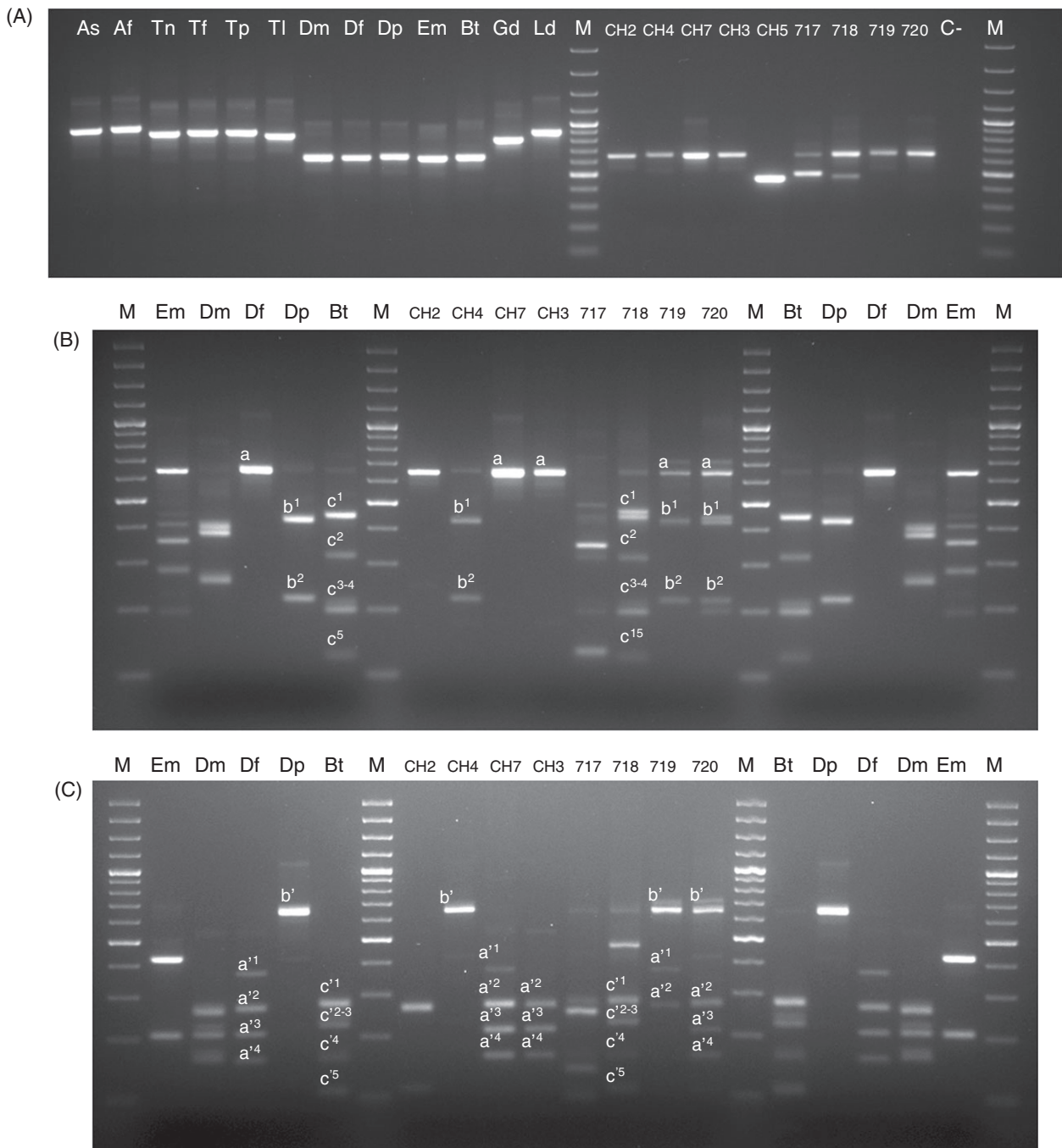


Fig. 4. Polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) system for the identification of astigmatid mites in environmental samples (house dust). (A) Re-amplification by PCR of ITS1 region. (B) *Hpa*II digestion. (C) *Rsa*I digestion. Em, Dm, Df, Dp and Bt: DNA extracted from mite cultures (species codes as in Fig. 1); CH2, CH4, CH7, CH3, 717, 718, 719 and 720: DNA extracted from environmental samples; C- negative control of the PCR using no DNA template. (3% agarose gel. Equal letters in different lanes are interpreted as the same RFLP band based on the profile visualized after digestion with the two restriction enzymes.

2011). The sequences obtained in this work have been used for species identification. Identification by sequence may be simpler than identification using taxonomic keys. However, identification by sequence requires cloning, because intraspecific and/or intra-individual polymorphism may complicate the readings of

direct sequencing, as we observed in three of the species analysed in this paper.

It is worth noting that the intraspecific or intra-individual polymorphism detected did not affect the species-specificity of the sequences. Polymorphic clones obtained from each species

unambiguously clustered together in the phylogenetic tree constructed after the alignment. However, the ITS2 sequences obtained in this paper clustered together with conspecific sequences obtained from individuals of different geographic origins reported in works by other authors (Webster *et al.*, 2004; Noge *et al.*, 2005; Yang *et al.*, 2011). The only discrepancy between the data reported in the current study and those cited in other reports corresponds to the sequence associated with *E. maynei* by Yang *et al.* (2011). However, as Fig. 2 shows, the sequences reported in this paper for this species are more consistent with a mite species belonging to the family Pyroglyphidae.

The PCR–RFLP system described in this paper avoids the sequencing handicaps derived from polymorphism and identifies species by comparing restriction profiles after gel electrophoresis. The sequence polymorphism is reflected in the PCR–RFLP profiles, but does not affect species identification in mite cultures. In fact, DNA extracted from single individuals produces the same profiles as DNA extracted from cultures. Thus, the system would be useful for guaranteeing the source species in cultures maintained for the industrial production of allergenic extracts for clinical use, and would fulfil the requirements expressed in the guideline for the production and quality of allergen products elaborated by the European Medicines Agency (Committee for Medicinal Products for Human Use, 2008). In addition, this method can be applied to DNA extracted from purified fractions of bodies and faeces which can be used as source materials in the production of allergenic extracts. Obviously, the mite species representing the source of these fractions can never be efficiently determined by morphology. The specificity of the primer combination of FNav and RNav2 allows the amplification of mite rDNA from a starting medium in which DNA from other eukaryotic and prokaryotic organisms (yeast, bacteria, etc.) may be present. The method has been tested on cultures of up to 13 species of stored product and house dust mites belonging to four families of Astigmata, which suggests that it may be broadly applicable in other astigmatid mites. The amplification of the ITS2 region followed by RFLP was previously proposed for the identification of *D. pteronyssinus*, *D. farinae*, *B. tropicalis*, *Glycyphagus malaysiensis*, *T. putrescentiae* and *Acarus ovatus* (Wong *et al.*, 2011), but the method proposed required the use

of up eight restriction enzymes, and no data on primer specificity were given. In comparison, our PCR–RFLP method is based on the amplification of both ITS1 and ITS2, and uses only two restriction enzymes to identify up to 13 species. Remarkably, the PCR–RFLP method successfully differentiates phylogenetically close species, such as the sibling species *D. farinae* and *D. microceras* (Colloff, 1998), and the recently described *T. fanetzhangorum* previously misidentified as *T. putrescentiae* (Klimov & O'Connor, 2009).

Thus, a PCR–RFLP method amplifying the ITS2 region has been adapted for the identification of astigmatid mites in house dust samples. The detection of mite species in environmental samples is currently performed in epidemiological studies using specific antibodies (Zock *et al.*, 2006; Barber *et al.*, 2012) or morphological identification (Boquete *et al.*, 2006; Soltani *et al.*, 2011). Immunodetection is generally restricted to a few species, mainly because it requires the demonstration of specific antibodies that do not show cross-reactivity between species. However, morphological identification is tedious and requires the provision of well-conserved samples, the presence of adult stages and the correct use of morphological keys. As far as we know, this is the first molecular method to be developed for the detection of allergy-causing mite species in environmental samples. The method requires the use of a DNA extraction procedure that presumably avoids the co-purification of mite DNA with unknown PCR inhibitors, and the introduction of a re-amplification step before RFLP that increases the detection threshold. Thus, PCR re-amplification products can be visualized in a gel, giving a first indication of the species found in a sample. Non-expected bands may be cloned and sequenced in order to provide reference material for future samples of unknown species. Finally, the species found in the samples can be identified after RFLP by comparing the patterns generated from individual species with those obtained from the house dust sample. The analysis by ELISA based on Der p 1 and Der f 1 specific monoclonal antibodies in the same samples analysed by PCR–RFLP (Fig. 4, Table 2) reveals that the method allows for the discrimination of at least the dominant species. One drawback of the PCR–RFLP method is the amplification of unknown mite species and the complex patterns visualized when rDNA of more than one species is amplified. However, the PCR products can be cloned in order to further elucidate the species amplified. A dominant species

Table 2. Comparison of the analysis of environmental samples by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and by enzyme-linked immunosorbent assay (ELISA).

Sample	Galicia			Asturias		Canary Islands			
	CH2	CH4	CH7	CH3	CH5	717	718	719	720
Dp	– (<0.05)	+ (2.00)	– (6.20)	– (3.06)	– (1.80)	– (19.08)	– (41.72)	+ (1.63)	+ (5.01)
Df	– (0.93)	– (0.09)	+ (92.61)	+ (24.20)	– (37.01)	– (<0.05)	– (2.36)	* (1.68)	* (1.79)
Bt	–	–	–	–	–	–	+	–	–
Unknown species	+	–	–	–	+	+	+	–	–

Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; Bt, *Blomia tropicalis*; +, indicates detection of an species by PCR–RFLP in patterns generated by both *Hpa*II and *Rsa*I restriction enzymes; *, indicates detection not confirmed by the presence of all diagnostic bands in both restriction enzyme patterns; –, indicates no detection. In parenthesis, µg/g of Der p 1 or Der f 1 estimated by ELISA. Cloning and sequencing of PCR products confirmed the presence of unknown mite species in samples CH5 and 717.

may mask the detection of others in the PCR, as seen in sample CH5, in which no bands compatible with *Dermatophagoides* spp. were observed in the PCR (Fig. 4A), despite the detection of the Der f 1 allergen. It is noteworthy that the presence of a dominant species does not always prevent the detection of others in the PCR or RFLP because more than one species were detected in some of the samples analysed (samples 717–720; Fig. 4, Table 2). In any case, specific primers for the detection of a given species could be designed according to the rDNA sequences obtained in this study.

Both the rDNA sequences and the PCR–RFLP system developed in this study will be of use in the identification and certification of mite species sources in industrial allergenic extract production and will provide an advantageous alternative to morphological analysis. Among other advantages, the method allows the identification of mite species in purified mite fractions when the availability of intact adult mite bodies suitable for morphological identification is limited. Additionally, we have demonstrated that rDNA-based methods can be implemented for the detection of allergy-causing mite species in environmental samples. The complexity of interpreting PCR–RFLP profiles when more than one species is present or when a dominant species masks the presence of others could be overcome by using the rDNA sequences obtained in this study to design specific primers for species identification. Thus, rDNA-based methods may significantly contribute to diagnostic or epidemiological studies of allergy-causing mites.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12052

Figure S1. Phylogenetic tree generated from the alignment of complete ITS1, 5.8S and ITS2 rDNA regions flanked by partial sequences of the 18S and 28S subunits obtained from DNA of 13 Astigmata species (10 clones per species). All clones obtained from one species clustered in the group indicated by the scientific name. Bootstrap values correspond to 1000 replicates.

Figure S2. Polymerase chain reaction (PCR) restricted fragment length polymorphism performed using DNA extracted from single individuals (I) and mite cultures (C) of *Dermatophagoides farinae* (Df), *Dermatophagoides pteronyssinus* (Dp) and *Blomia tropicalis* (Bt). Left, PCR using primers FNav and RNav2. Middle, *Hpa*II digestion of the PCR product. Right, *Rsa*I digestion. M, 100-bp ladder (Fermentas).

Figure S3. Species certification by polymerase chain reaction restricted fragment length polymorphism in purified fractions from *Dermatophagoides farinae* (Df) cultures. Above, *Hpa*II digestion. Below, *Rsa*I digestion. B1, B2 and B3, DNA extracted from purified fractions of bodies; F1 and F2, DNA extracted from purified fractions of faeces; 1, 2, 3, independent DNA extractions; M, 100-bp ladder (Fermentas).

Figure S4. Polymerase chain reaction restricted fragment length polymorphism profiles obtained from the ITS1 region of rDNA

amplified from cultures of 13 Astigmata species using primers FNav and RAst5.8. Above, *Hpa*II digestion. Below, *Rsa*I digestion. Species names are indicated by the first letter of the genus and species (see Fig. 1). 3% agarose gels; M, 100-bp ladder (Fermentas).

Table S1. Polymerase chain reaction product size (in parentheses) obtained using primers FNav and RNav2 and restriction fragments generated by *Hpa*II and *Rsa*I digestion, estimated based on the 10 clones per species sequenced in this study.

Table S2. Polymerase chain reaction product size (in parentheses) corresponding to the ITS1 region and obtained using primers FNav and RAst5.8, and restriction fragments generated by *Hpa*II and *Rsa*I digestion, estimated based on the 10 clones per species sequenced in this study.

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Conflicts of interest: CA, MJC and ML are employees of ALK-Abelló, SA. All authors declare no conflict of interest in connection with the present study.

References

- Barber, D., Arias, J., Boquete, M. *et al.* (2012) Analysis of mite allergic patients in a diverse territory by improved diagnostic tools. *Clinical and Experimental Allergy*, **42**, 1129–1138.
- Ben-David, T., Melamed, S., Gerson, U. & Morin, S. (2007) ITS2 sequences as barcodes for identifying and analysing spider mites (Acari: Tetranychidae). *Experimental and Applied Acarology*, **41**, 169–181.
- Boquete, M., Iraola, V., Fernandez-Caldas, E. *et al.* (2006) House dust mite species and allergen levels in Galicia, Spain: a cross-sectional, multicentre, comparative study. *Journal of Investigational Allergology and Clinical Immunology*, **16**, 169–176.
- Colloff, M.J. (1998) Taxonomy and identification of dust mites. *Allergy*, **53**, 7–12.
- Colloff, M.J. & Spieksma, F.T.M. (1992) Pictorial keys for the identification of domestic mites. *Clinical and Experimental Allergy*, **22**, 823–830.
- Committee for Medicinal Products for Human Use (2008) *Guideline on Allergen Products: Production and Quality Issues*. European Medicines Agency, London.
- Díaz-Mendoza, M., Ortego, F., de Lacoba, M.G. *et al.* (2005) Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides*

- (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. *Insect Biochemistry and Molecular Biology*, **35**, 1005–1020.
- Essig, A., Rinder, H., Gothe, R. & Zahler, M. (1999) Genetic differentiation of mites of the genus *Chorioptes* (Acari: Psoroptidae). *Experimental and Applied Acarology*, **23**, 309–318.
- Frati, F., Incorvaia, C., David, M. *et al.* (2012) Requirements for acquiring a high-quality house dust mite extract for allergen immunotherapy. *Drug Design, Development and Therapy*, **6**, 117–123.
- Gotoh, T., Gutierrez, J. & Navajas, M. (1998) Molecular comparison of the sibling species *Tetranychus pueraricola* Ehara et Gotoh and *T. urticae* Koch (Acari: Tetranychidae). *Entomological Science*, **1**, 55–57.
- Hillis, D.M. & Dixon, M.T. (1991) Ribosomal DNA – molecular evolution and phylogenetic inference. *Quarterly Review of Biology*, **66**, 411–453.
- Hughes, A.M. (1976) *The Mites of Stored Food and Houses* Technical Bulletin 9. Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationery Office, London.
- Huson, D.H., Richter, D.C., Rausch, C., DeZulian, T., Franz, M. & Rupp, R. (2007) Dendroscope: an interactive viewer for large phylogenetic trees. *BMC Bioinformatics*, **8**, 460.
- Kampen, H., Sternberg, A., Proft, J. *et al.* (2003) Polymerase chain reaction-based differentiation of the mosquito sibling species *Anopheles claviger* s.s. and *Anopheles petragani* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene*, **69**, 195–199.
- Klimov, P.B. & O'Connor, B.M. (2009) Conservation of the name *Tyrophagus putrescentiae*, a medically and economically important mite species (Acari: Acaridae). *International Journal of Acarology*, **35**, 95–114.
- Knapp, M., Wagener, B. & Navajas, M. (2003) Molecular discrimination between the spider mite *Tetranychus evansi* Baker & Pritchard, an important pest of tomatoes in southern Africa, and the closely related species *T. urticae* Koch (Acarina: Tetranychidae). *African Entomology*, **11**, 300–304.
- de Mendonça, R.S., Navia, D., Diniz, I.R., Auger, P. & Navajas, M. (2011) A critical review on some closely related species of *Tetranychus sensu stricto* (Acari: Tetranychidae) in the public DNA sequences databases. *Experimental and Applied Acarology*, **55**, 1–23.
- Navajas, M. & Fenton, B. (2000) The application of molecular markers in the study of diversity in acarology: a review. *Experimental and Applied Acarology*, **24**, 751–774.
- Navajas, M., Lagnel, J., Gutierrez, J. & Boursot, P. (1998) Species-wide homogeneity of nuclear ribosomal ITS2 sequences in the spider mite *Tetranychus urticae* contrasts with extensive mitochondrial COI polymorphism. *Heredity*, **80**, 742–752.
- Navajas, M., Lagnel, J., Fauvel, G. & De Moraes, G. (1999) Sequence variation of ribosomal internal transcribed spacers (ITS) in commercially important Phytoseiidae mites. *Experimental and Applied Acarology*, **23**, 851–859.
- Navajas, M., Gutierrez, J., Williams, M. & Gotoh, T. (2001) Synonymy between two spider mite species, *Tetranychus kanzawai* and *T. hydrangeae* (Acari: Tetranychidae), shown by ribosomal ITS2 sequences and cross-breeding experiments. *Bulletin of Entomological Research*, **91**, 117–123.
- Noge, K., Mori, N., Tanaka, C., Nishida, R., Tsuda, M. & Kuwahara, Y. (2005) Identification of astigmatid mites using the second internal transcribed spacer (ITS2) region and its application for phylogenetic study. *Experimental and Applied Acarology*, **35**, 29–46.
- Osakabe, M., Hirose, T. & Sato, M. (2002) Discrimination of four Japanese *Tetranychus* species (Acari: Tetranychidae) using PCR–RFLP of the internal transcribed spacer region of nuclear ribosomal DNA. *Applied Entomology and Zoology*, **37**, 399–407.
- Poucher, K.L., Hutcheson, H.J., Keirans, J.E., Durden, L.A. & Black, W.C. (1999) Molecular genetic key for the identification of 17 *Ixodes* species of the United States (Acari: Ixodidae): a methods model. *Journal of Parasitology*, **85**, 623–629.
- de Rojas, M., Ubeda, J.M., Cutillas, C., Mora, M.D., Ariza, C. & Guevara, D. (2007) Utility of ITS1, 5.8S, ITS2 and 16S mitochondrial DNA sequences for species identification and phylogenetic inference within the *Rhinonyssus coniventris* species complex (Acari: Rhinonyssidae). *Parasitology Research*, **100**, 1041–1046.
- Sanchez-Ramos, I., Alvarez-Alfageme, F. & Castanera, P. (2007) Development and survival of the cheese mites, *Acarus farris* and *Tyrophagus neiswanderi* (Acari: Acaridae), at constant temperatures and 90% relative humidity. *Journal of Stored Products Research*, **43**, 64–72.
- Schoch, C.L., Seifert, K.A., Huhndorf, S. *et al.* (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6241–6246.
- Soltani, A., Azizi, K., Saleh, V. & Dabaghmanesh, T. (2011) The fauna and distribution of house dust mites in residential homes of Bandar Abbas District, Southern Iran. *Experimental and Applied Acarology*, **54**, 269–276.
- Suarez-Martinez, E.B., Montealegre, F., Sierra-Montes, J.M. & Herrera, R.J. (2005) Molecular identification of pathogenic house dust mites using 12S rRNA sequences. *Electrophoresis*, **26**, 2927–2934.
- Thomas, W.R., Hales, B.J. & Smith, W.A. (2010) House dust mite allergens in asthma and allergy. *Trends in Molecular Medicine*, **16**, 321–328.
- Toma, T., Miyagi, I., Crabtree, M.B. & Miller, B.R. (2000) Identification of *Culex vishnui* subgroup (Diptera: Culicidae) mosquitoes from the Ryukyu Archipelago, Japan: development of a species-diagnostic polymerase chain reaction assay based on sequence variation in ribosomal DNA spacers. *Journal of Medical Entomology*, **37**, 554–558.
- Webster, L.M.I., Thomas, R.H. & McCormack, G.P. (2004) Molecular systematics of *Acarus siro* s. lat., a complex of stored food pests. *Molecular Phylogenetics and Evolution*, **32**, 817–822.
- Wei, D.D., Yuan, M.L., Wang, Z.Y. *et al.* (2011) Sequence analysis of the ribosomal internal transcribed spacers region in psocids (Psocoptera: Liposcelididae) for phylogenetic inference and species discrimination. *Journal of Economic Entomology*, **104**, 1720–1729.
- Wong, S.F., Chong, A.L., Mak, J.W., Tan, J., Ling, S.J. & Ho, T.M. (2011) Molecular identification of house dust mites and storage mites. *Experimental and Applied Acarology*, **55**, 123–133.
- Yang, B., Cai, J.L. & Cheng, X.J. (2011) Identification of astigmatid mites using ITS2 and COI regions. *Parasitology Research*, **108**, 497–503.
- Zock, J.P., Heinrich, J., Jarvis, D. *et al.* (2006) Distribution and determinants of house dust mite allergens in Europe: the European Community Respiratory Health Survey II. *Journal of Allergy and Clinical Immunology*, **118**, 682–690.

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Figure S1

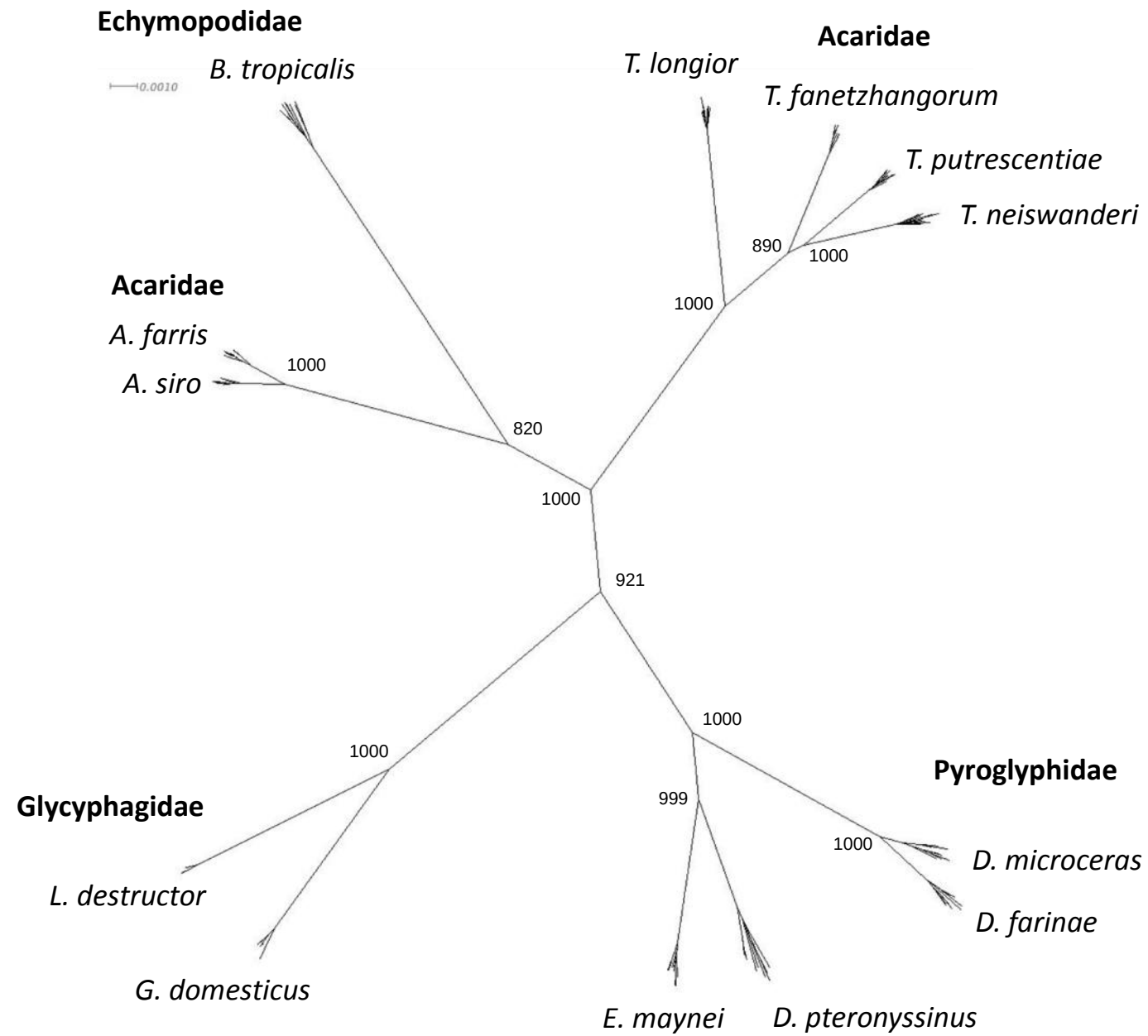


Figure S2

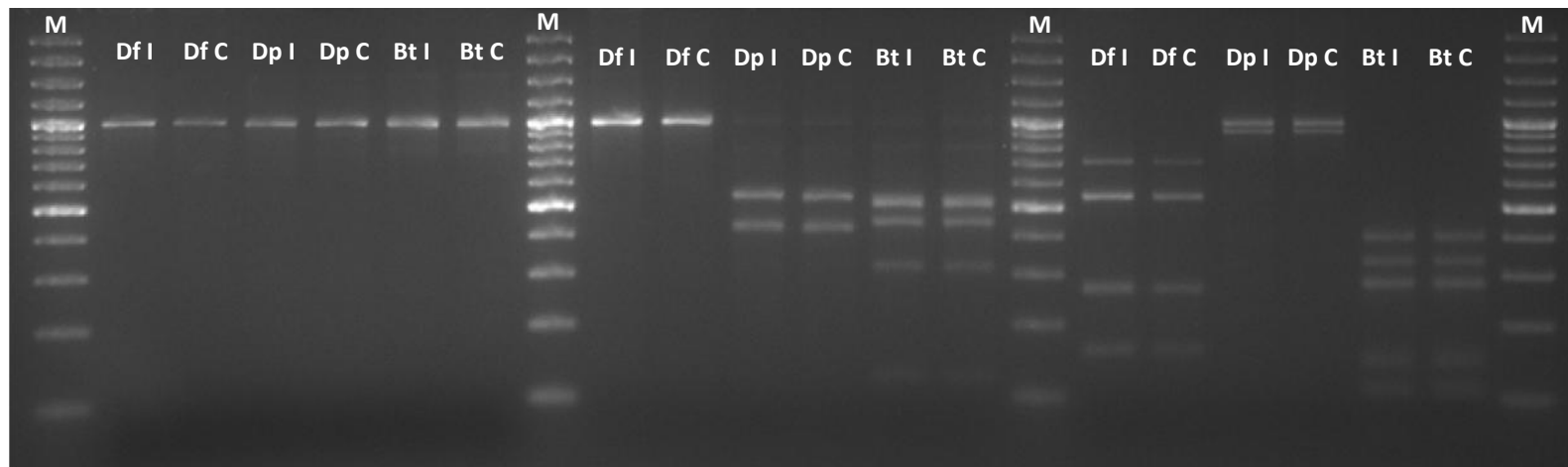


Figure S3

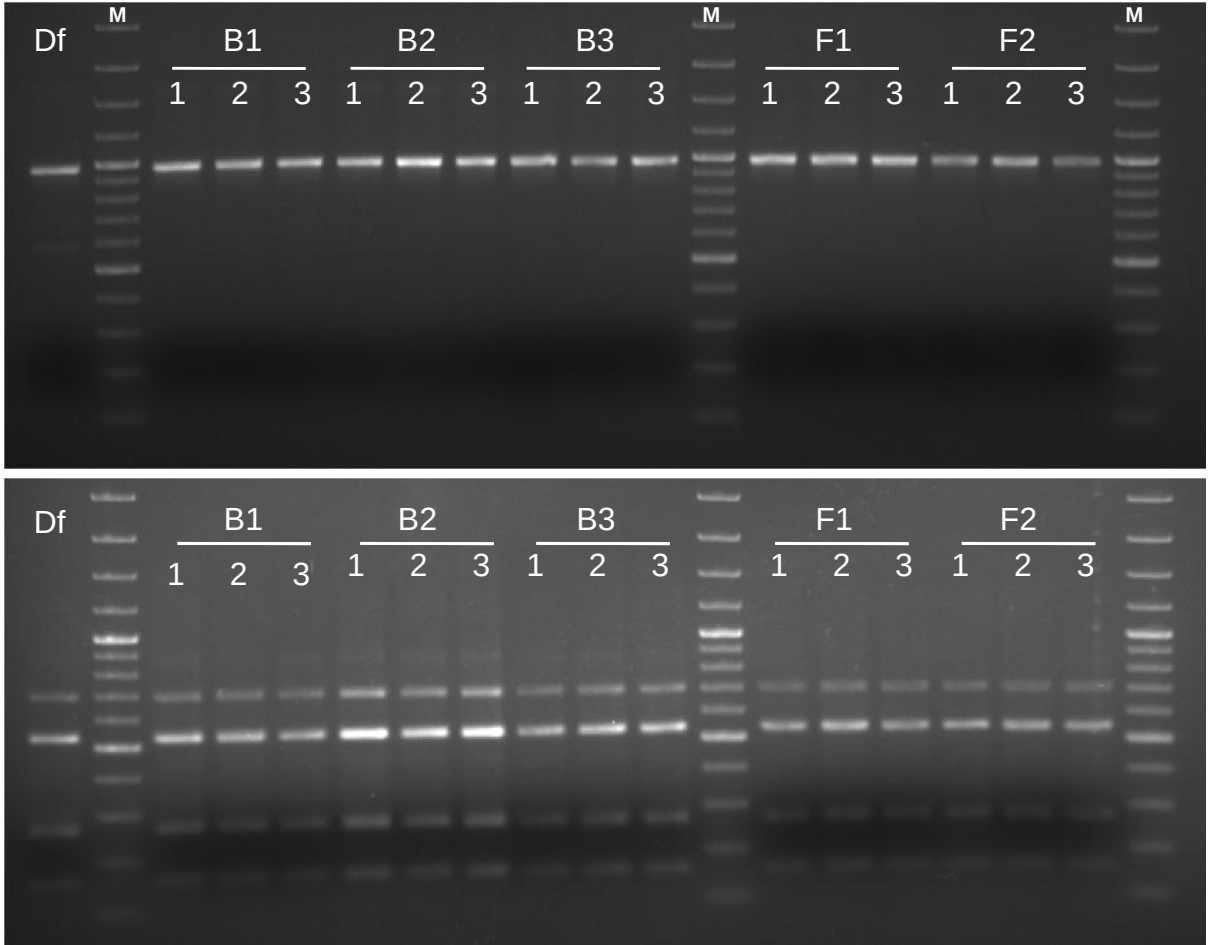


Figure S4

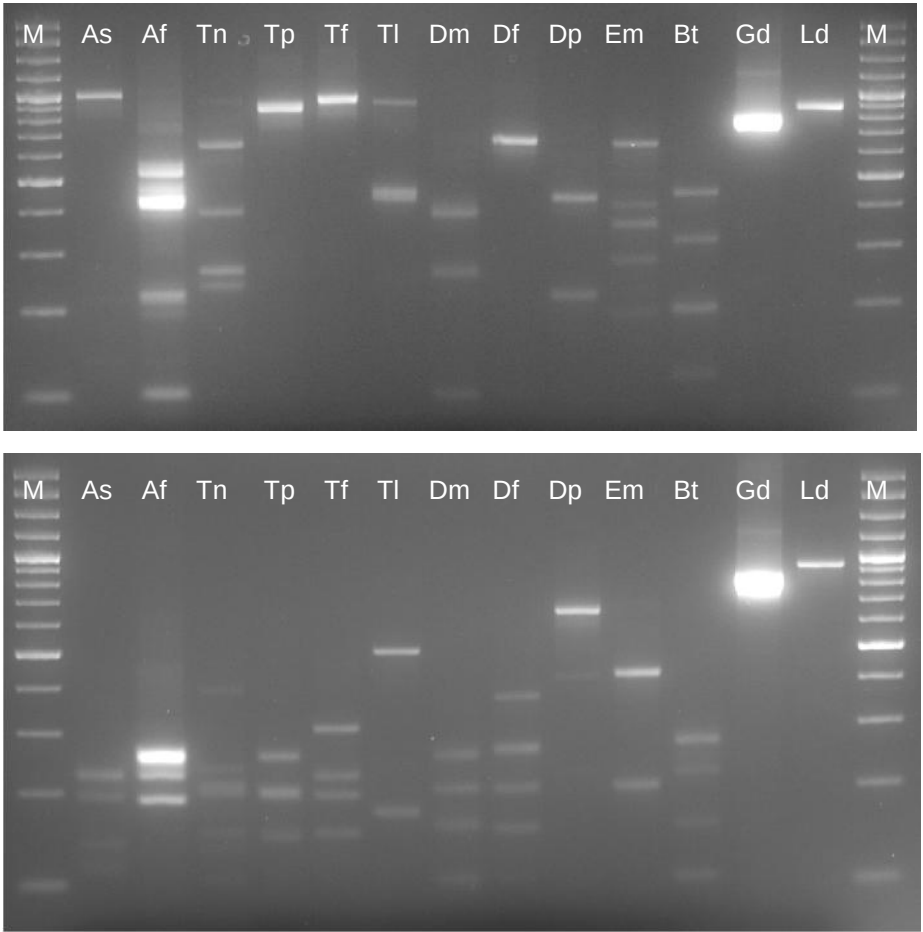


Table S1. PCR product size (in parenthesis) obtained using primers FNav-RNav2 and restriction fragments generated by *HpaII* and *RsaI* digestion, estimated in base to the ten clones per species sequenced in this paper.

Species (PCR product size, bp)	<i>HpaII</i> (bp)	<i>RsaI</i> (bp)
<i>A. siro</i> (1438-1449)	1442, 1384, 56	419, 286, 222-233, 194-199, 156-157, 131, 115-118, 79-80, 21-26
<i>A. farris</i> (1441-1458)	1024-1026, 918-920, 728-731, 423-427, 222-225, 211-212, 193, 179, 100-101, 10	403-407, 256, 246-256, 227-234, 193-194, 173-174, 123-127, 65, 13, 7
<i>T. neiswanderii</i> (1335-1354)	1065, 807-808, 734, 425, 38-0-382, 282-288, 250-259, 229, 67	675-682, 389, 286, 235-237, 205-213, 152, 134, 103, 97, 70
<i>T. putrescentiae</i> (1303-1310)	1303-1310	395, 278-282, 258, 204-207, 162, 147-150, 118, 18
<i>T. fanetzhangorum</i> (1373-1376)	1091, 282-285	407, 307, 278-281, 229, 152
<i>T. longior</i> (1291-1295)	1029, 861, 595-596, 434, 261-265	934-938, 180, 177
<i>D. microceras</i> (951-960)	697-708, 250-255	531-536, 256-261, 160-166
<i>D. farinae</i> (953-959)	953-959	689, 531-533, 264-269, 158
<i>D. pteronyssinus</i> (950-954)	530-533, 419-423	950-953, 890-891, 540, 479, 414, 64, 59
<i>E. maynei</i> (939-948)	839, 707-711, 612, 560-562, 274-278, 232, 193, 125, 107-108, 34	519-524, 420-424
<i>B. tropicalis</i> (927-943)	497-511, 427-433, 309-311, 120-122	385-394, 327-332, 267-271, 136-142, 104-106, 63, 30
<i>G. domesticus</i> (1258-1267)	756-760, 502-508	946-950, 312-318
<i>L. destructor</i> (1341-1347)	836-839, 505-509, 352, 155	1341-1347

Table S2. PCR product size (in parenthesis) corresponding to ITS1 region and obtained using primers FNav-RAst5.8, and restriction fragments generated by *HpaII* and *RsaI* digestion, estimated in base to the ten clones per species sequenced in this paper.

Species (PCR product size, bp)	<i>HpaII</i> (bp)	<i>RsaI</i> (bp)
<i>A. siro</i> (924-936)	924-936	419, 254, 228-233, 222-226, 194-199, 141, 115-118, 21-26
<i>A. farris</i> (935-951)	517, 423-427, 412-414, 222-225, 211-212, 193, 179, 100-101, 10	260-263, 246-256, 227-234, 193-194, 13, 7
<i>T. neiswanderii</i> (860-875)	874, 616-617, 543, 380-382, 250-259, 67	389, 286, 235-237, 202-214, 152, 134, 103, 95, 70
<i>T. putrescentiae</i> (830-833)	830-833	258, 206-207, 200, 147-150, 18
<i>T. fanetzhangorum</i> (888)	888	307, 229, 200, 152
<i>T. longior</i> (846-847)	846, 434, 412-413	489-490, 180, 177
<i>D. microceras</i> (627-639)	373-384, 250-255	256-261, 207-212, 160-166
<i>D. farinae</i> (629-635)	629-635	365, 264-269, 207-208, 158
<i>D. pteronyssinus</i> (638-642)	641, 419-423, 218-219	638-642, 414, 226-227
<i>E. maynei</i> (626-631)	626-631, 402, 352, 274-278, 193, 34	420-424, 205-207
<i>B. tropicalis</i> (622-629)	427-433, 309-311, 191-196, 120-122	326, 267-271, 239, 217-222, 157-159, 104-106, 63
<i>G. domesticus</i> (800-804)	800-804	800-804
<i>L. destructor</i> (880-883)	836-839, 44	880-883