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AN INTEGRATIVE TAXONOMY OF MOLECULAR AND TRADITIONAL APPROACHES FOR IDENTIFICATION OF A STORAGE MITE, ALEUROGLYPHUS OVATUS (ACARI: ASTIGMATA: ACARIDAE) IN MALAYSIA

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ABSTRACT

A reliable and rapid taxonomic identification of a mite is the basis for a correct diagnosis of important mite associated allergies as they produce species-specific allergens. A double approach (molecular and morphological) to the taxonomic identification of *Aleuroglyphus ovatus* was presented. Molecular identification was performed with amplification of the internal transcribed spacer region (ITS2), whilst morphological characters were examined under light microscope. The BLAST results obtained from molecular analysis of *A. ovatus* was shown to be in concordance with the morphological identification with 97% genetic similarity. Thus, the molecular identification based on the ITS2 region can be applied as a reliable and efficient tool for species identification of *Aleuroglyphus* and probably any other astigmatid mites. Our findings suggest the need for a broad taxonomic sampling especially from closely related species for an accurate identification of local mites using both DNA sequences and morphology.

Keywords

Aleuroglyphus ovatus, taxonomy, ITS2, storage mite

Academic Discipline And Sub-Disciplines

Molecular biology

SUBJECT CLASSIFICATION

Genetic

TYPE (METHOD/APPROACH)

Experimental

INTRODUCTION

Aleuroglyphus ovatus Troupeau, 1879 (Astigmata: Acaridae) is a storage mite that can be found in stored bran, dried fish product, medicinal herbs and grain products worldwide (Palyvos *et al*, 2008; Sun *et al*, 2014). The mite was first reported in Peninsular Malaysia in 1980 (Yunus and Ho, 1980). Since then, there was no other report of the presence of this mite until the species was found to be the most common mite isolated from the Animal Holding Facility of International Medical University, Kuala Lumpur, Malaysia in 2012 (Tan, 2012). Since then, several studies have reported *A. ovatus* as a source of sensitizing allergens and have been confirmed in patients with allergic asthma by skin test, Radio-AllergoSorbent Test (RAST) and nasal challenge (Silton *et al*, 1991; Geary *et al*, 2000; Valdivieso *et al*, 2006).

Current species identification of dust mites is based mainly on their morphological and developmental characteristics of adult stage (Colloff and Spieksma, 1992; Khaing *et al*, 2014). Taxonomic recognition of mites using morphological features alone can be difficult due to their microscopically small body size, few distinct characteristics among sibling species specifically in immature stages and when specimens are not intact (Suarez-Martinez *et al*, 2005; Colloff, 2009). Expertise and experienced personnel in taxonomy therefore are required for specimen mounting, scanning electron microscope (SEM) examination and identification. Taxonomically complex and similar morphological characteristics in the adult stage between the two genera of the family Acaridae, *Tyrophagus* and *Aleuroglyphus* make them difficult to be differentiated with a high degree of certainty (Hughes, 1976).

The difficulties in identifying these mites at the species level have led to the search for a simpler and more rapid method using the molecular-based techniques. Online sequences databases such as GenBank are used commonly in molecular systematics to identify a variety of medically important species of mites that causes

allergic diseases, such as asthma, rhinitis and atopic dermatitis (Silton et al. 1991). Species identification in mite cultures is a key factor in standardization of allergen production especially when the colonies established are from field-derived mites (Beroiz *et al*, 2014). Proper species identification may contribute to improved management of mite associated allergies as they produce species-specific allergens besides being an essential first step of acarological evidence for a legal investigation (de Pancorbo *et al*, 2004; Yang *et al*, 2011).

Utilization of DNA as a molecular marker for analysis allows identification from any developmental stage or from non-intact adult mite specimens (Navajas and Fenton, 2000). Some markers have been widely tested and their use has been proven valid for genus and species identification of arthropods. For example, nucleotide composition of ribosomal second internal transcribed spacer (ITS2) region is highly variable but similar in closely related species (Thet-Em *et al*, 2012).

In order to provide a complementary tool for morphological identification of *A. ovatus* mite, this study investigated the use of ITS2 gene as a molecular marker in an integrative approach.

MATERIALS AND METHODS

Culture of mites

Aleuroglyphus ovatus colonies maintained at the Acarology Unit, Institute for Medical Research, Malaysia were used. Colonies consist of mixed generations of mites maintained at 25°C ± 2°C and relative humidity of 75% ± 2%. Purity of the mites was checked by microscopy examination of a few randomly mounted specimens.

Molecular analysis

Harvesting of mite and DNA extraction

The mite cultures were harvested with heat escape method in which the live mites migrated from the sieve located on top of a funnel to a 1 ml sterile collection Eppendorf tube at its bottom. The collected bodies were immediately resuspended in 25 µl of phosphate buffered saline (PBS) and 75 µl of ATL buffer. The mites were then crushed using sterile plastic tips for 5 mins prior to adding 20 µl of Proteinase K. The following steps were performed using the manufacturer's supplementary protocol for Qiagen DNeasy® Kit for animal tissues (Qiagen, Germany). Purity and concentration of the DNA was determined using Thermo Scientific NanoDrop™ 1000 spectrophotometer. The extracted DNA was either directly used as a template for PCR amplification or stored at -20°C until further usage.

Amplification of ITS2 gene and gel electrophoresis

Amplification of ITS2 gene region was performed using the following universal primer set: ITS2-forward primer 5'-CGACTTTCGAACGCATATTGC-3' and ITS2-reverse primer 5'-GCTTAAATTCAGGGGGTAATCTCG-3'. The primers will span a portion of the 5.8S rDNA, the full length ITS2 region and a part of the 28S rDNA of astigmatid mite (Noge *et al*, 2005). The amplification reaction contained 10 µl of DNA, 25 µl of 2X Taq PCR Master Mix, 2.5 µl of each primer (10 µM), and 10 µl nuclease free water in a total volume of 50 µl. Sterile double distilled water was used to substitute DNA sample for the negative control. Thermocycling was carried out in an Eppendorf Master Cycler Personal (Germany) with the following parameters: initial denaturation at 94°C for 2 min, followed by 25 cycles, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 50 s and final extension at 72°C for 7 min. Amplicons were analyzed by 1.5% agarose gels electrophoresis and ethidium bromide stained bands were viewed under an ultraviolet trans-illuminator.

Sensitivity of PCR

Ten serial dilutions (1 µl to 10 µl) of DNA template were used to detect threshold for determination of sensitivity for the PCR amplification of the ITS2 gene. The PCR temperature profiles were set as previously described. The highest dilutions of the DNA showing a visible band was confirmed by analyzing 6 µl of PCR products on 1.5% agarose gel. The experiment was repeated five times.

DNA purification and DNA sequencing

The DNA fragment was excised from the gel using sterile, sharp gel cutter and purified using 5 Prime PCR Agarose Gel Extract Mini Kit (Hamburg, Germany) according to the manufacturer's protocol. All PCR products were then sent to First Base Laboratory, Malaysia for DNA sequencing. The sequencing was bi-directional for all specimens and the primer combination for this step was the same as that used in the PCR amplification. Multiple sequence alignment was carried out using Clustal-W programme of BioEdit (Hall, 2005).

Gene sequence analysis

The obtained sequences were then compared with those available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/nucleotide) for the identification of species and detection of sequence contamination. The approach enabled the similarity of sequences to be measured depending on several criteria's such as expected value, maximum identical, query coverage and maximum score.

Morphological analysis

Mites were placed in cavity block containing lactic acid and heated on a hot plate at 200°C for 5 minutes before being mounted in Hoyer's medium. The preparation slides were covered with a small cover slip. Mounted slides were incubated at 40°C for a week prior to examination under a light microscope (20x and 40x magnification).

RESULTS

Molecular analysis

ITS2 DNA region of *A. ovatus* was successfully amplified using a combination of ITS2-forward and ITS2-reverse primers. The PCR products (including the primers) ranged from 450 – 490 bp in length (Figure 1). Amplified DNA fragment was sent for sequencing and the results from BLAST analysis showed that the local *A. ovatus* ITS2 region had 97% sequence homology with *A. ovatus* sequence in the GenBank database (Figure 2). Small differences in the ITS2 region were mainly found in terms of its length because of the short deletions or insertions of single nucleotides at several sites. With regards to sensitivity, the PCR products were obtained from the lowest volume of 4 µl DNA template onwards (Figure 3).

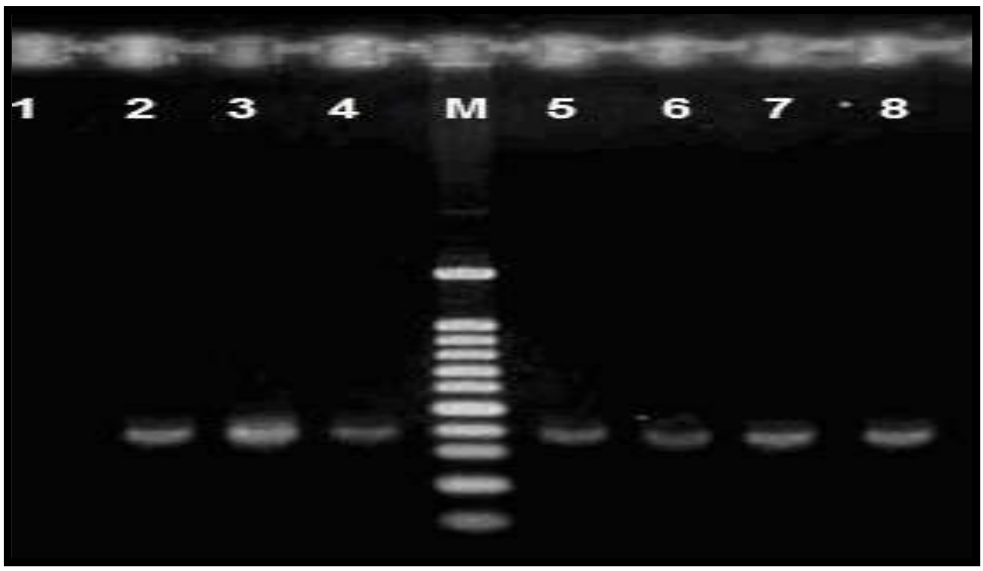

Fig 1-Gel photograph showing 489 bp of ITS2 rDNA gene by PCR amplification with specific primers. Lane 2-8: DNA of *A. ovatus*. M is molecular weight marker (100-bp DNA ladder).

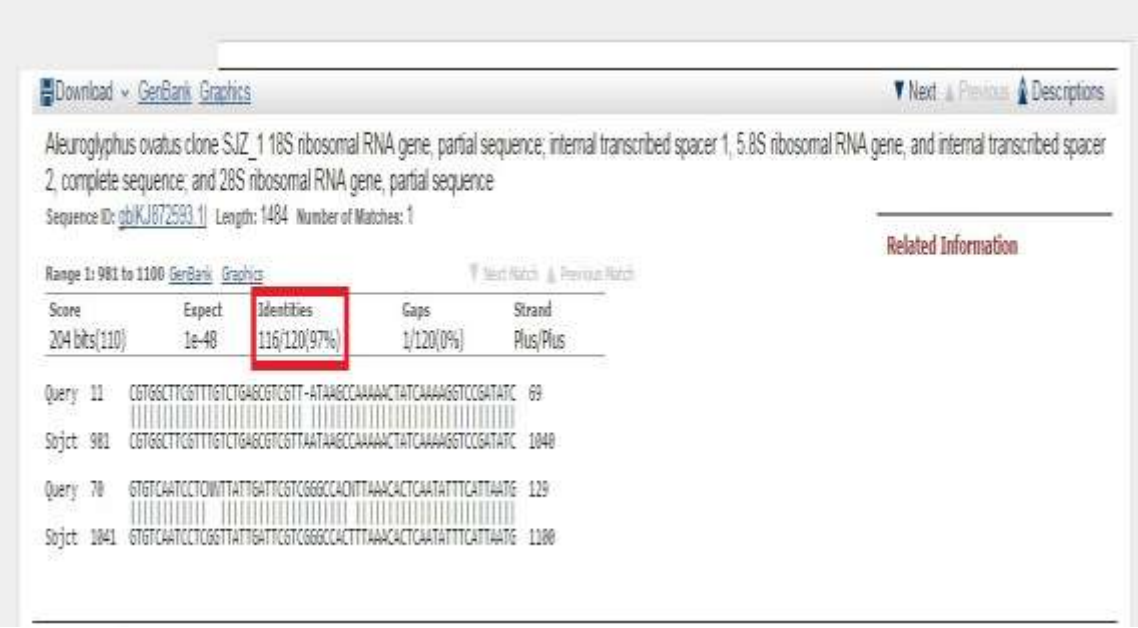

Fig 2-The similarity of local *A. ovatus* sequence with available sequence in Genbank database.

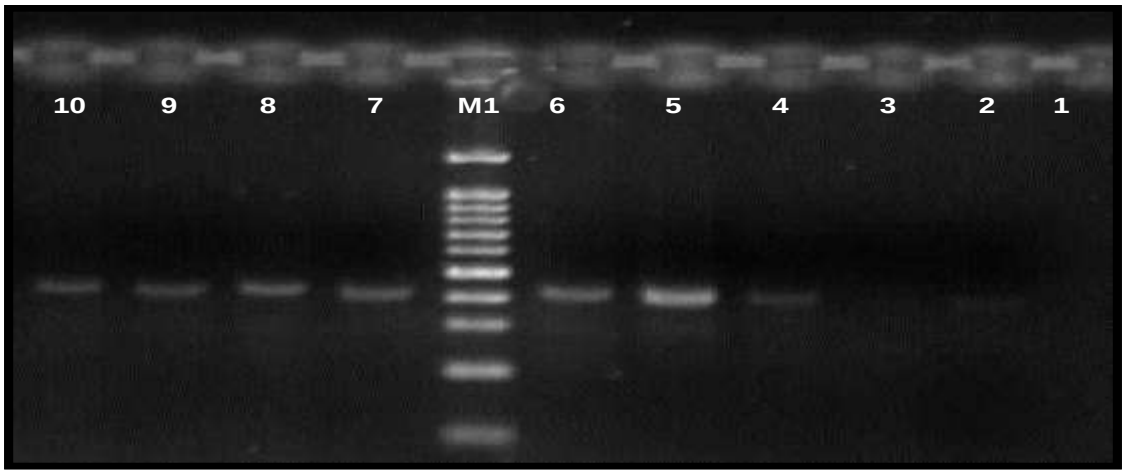

Fig 3-Gel photograph showing 489 bp of ITS2 rDNA gene by PCR amplification of 10 serial dilutions tested where each well contains different volume of extracted DNA (1 – 10 µl). M is molecular weight marker (100-bp DNA ladder).

Morphological analysis

General morphology

A. ovatus can be easily distinguished from other mite species as it has an intense tanned (dark reddish to brown) chelicerae and legs compared to other parts of its body (Figure 4). The mite is composed of an anterior gnathosoma and a posterior idiosoma. The gnathosoma consists of a pair of chelicerae i.e a fixed digit blade and a mobile digit. The propodosomal shield is oblong, with slightly concave lateral margins and a pitted surface. A pair of external vertical setae (*ve*) is longer than the internal vertical setae (*vi*). Both vertical setae rise to the same level. Internal scapular setae, *sci* are shorter than the external scapular setae, *sce* (Figure 4). A pair of long post-anal setae is also present.

There is a dorsal transverse groove dividing propodosoma from hysterosoma (Figure 4). The dorsal of idiosoma has four pairs of setae; the last pair of dorsal setae at the posterior end of the idiosoma being longer than the other three anterior dorsal setae. The dorsal hysterosomal setae (*d1* to *d3*) are short. These setae are about the same length as *sci*, whilst *d4* is slightly shorter. Attached to the body are four pairs of legs with only three pairs present in larval and protonymph stages. Their legs are short and stout.

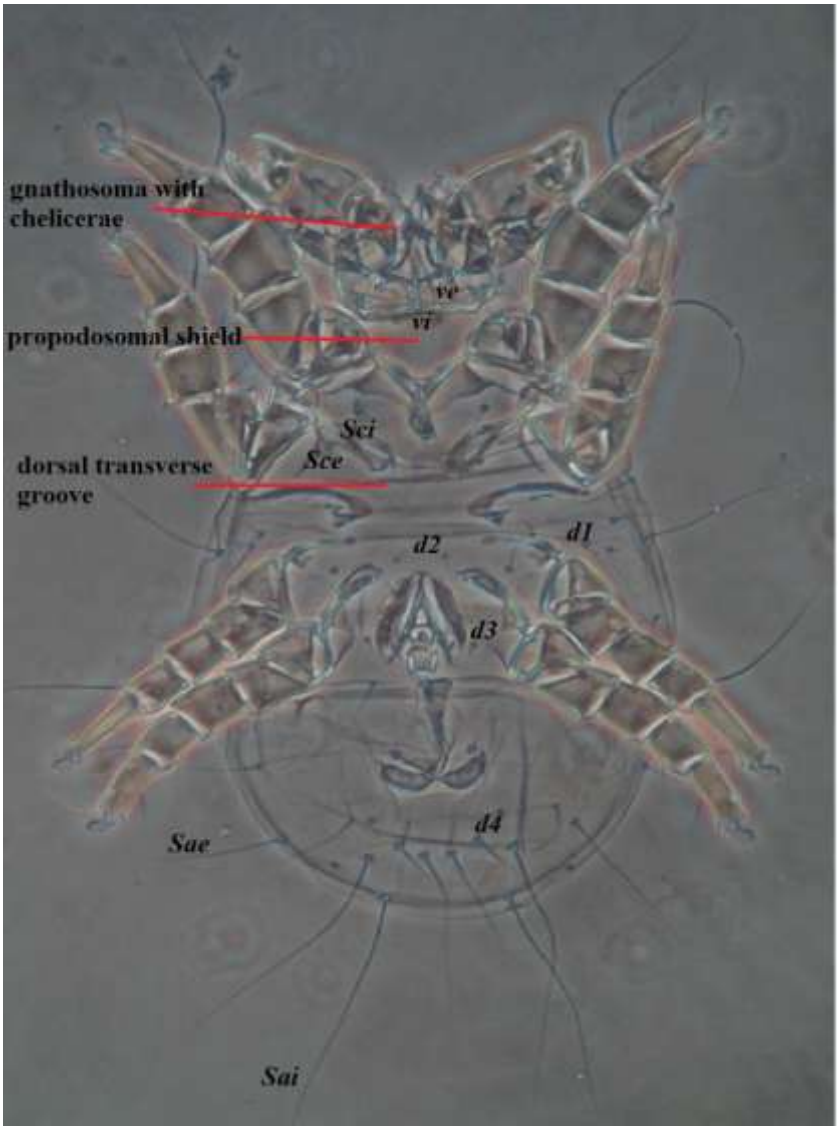

Figure 4-Dorsal view of *A. ovatus* (40x magnifications) showed intense tanned chelicerae and legs. (*vi*: internal vertical setae; *ve*: external vertical setae; *sci*: internal scapular setae; *sce*: external scapular setae; *d1-d4*: dorsal setae; *Sai*: internal posterior setae; *Sae*: external posterior setae).

Sexual dimorphism

The female *A. ovatus* mite closely resembles the male except for the anal opening. Female mite has broader anal opening with four pairs of setae (Figure 5). The *a2* setae are long and extend beyond the posterior edge of the body. The other two pairs of post anal setae (*pa1* and *pa2*) also are long and almost in line with one another (Figure 5). The male mite has a pair of anal sucker and three pairs of post-anal setae that are located almost in the same line (Figure 6).

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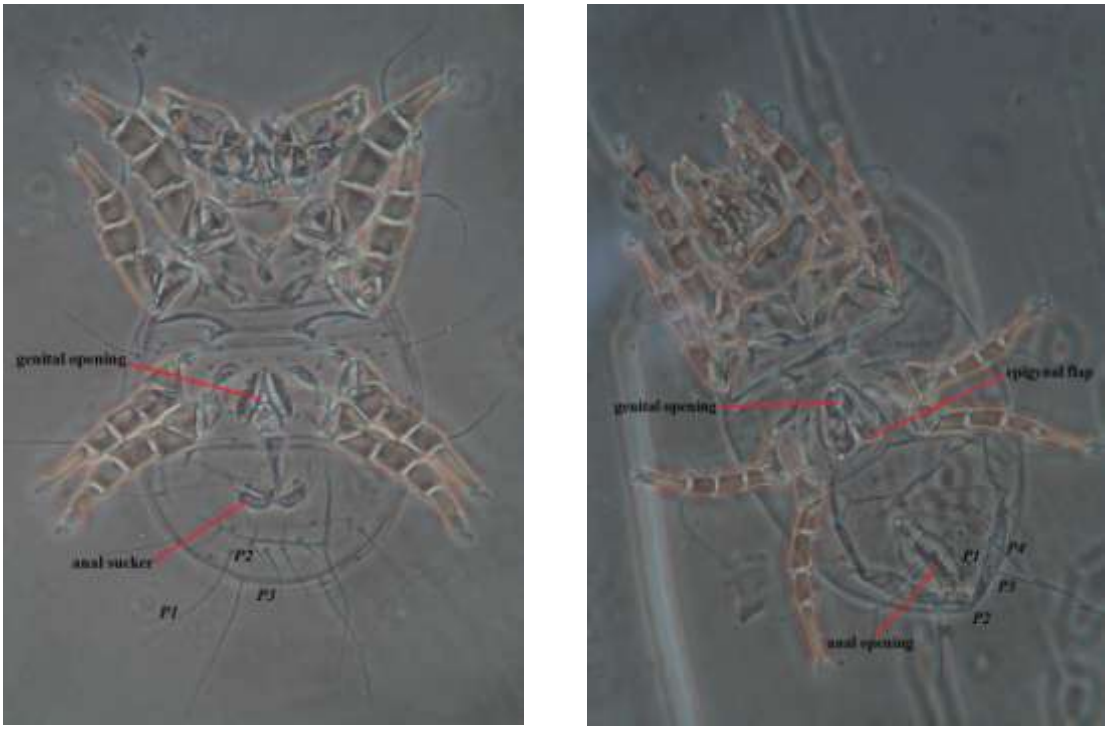

Fig 5-Ventral view of a male (left) and female (right) *A. ovatus* (40x magnifications).

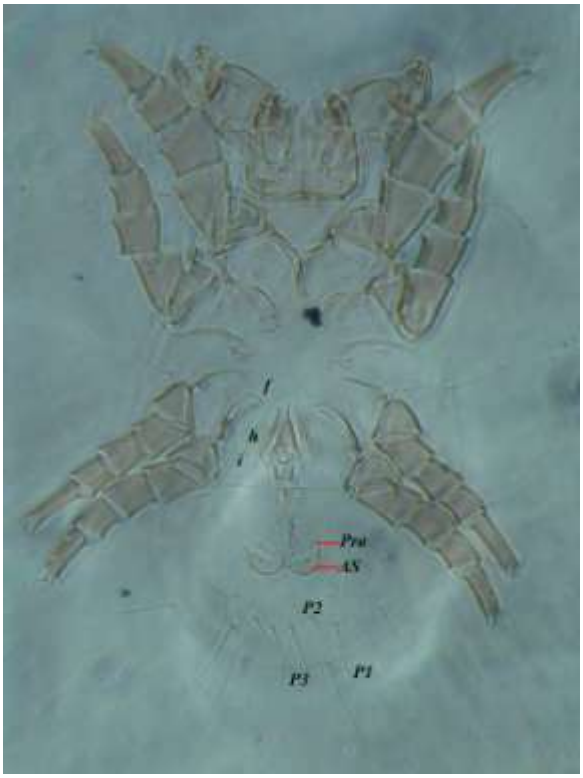

Fig 6-Ventral view of *A. ovatus* under 40x magnifications of light microscope. (*i*: posterior paragenital bristle; *h*: middle paragenital bristle; *f*: anterior paragenital bristle; *Pra*: pre-anal setae; *AS*: anal sucker; *P1-P3*: post anal setae).

DISCUSSION

Molecular markers are an important key for identifying mites at taxonomic levels (Hinomoto *et al*, 2007; Ros and Breeuwer, 2007). The ideal properties of molecular markers have been previously described by several taxonomists. Not all markers serve as suitable candidate for molecular identification purpose. One of the most useful markers was the ITS2 rDNA gene where it is highly conserved in eukaryotes and, hence, is used to identify closely related species. The multiple copy number of this region has also been previously reported, which favors its amplification by polymerase chain reaction (PCR) from genomic DNA of dust mites (Noge *et al*, 2005; Wong *et al*, 2011; Thet-Em *et al*, 2012). In the present study of *A. ovatus*, the length of the ITS2 sequences varied considerably (450 – 490 bp). The results were consistent with studies reported by Noge *et al.* (2005), in which a slightly higher intraspecific variation among the ITS2 sequences of *A. ovatus* was observed as compared to other astigmatid mites. Another reason was probably due to the ITS2 region of *A. ovatus* that was not homogenized completely within and/ or between individuals as been observed by Yang *et al.* (2011).

The most significant aspect of the PCR method is its sensitivity to detect minuscule amount of DNA. The sensitivity usually depends on both the quantity of target DNA and the progressive degradation of DNA (Li *et al*, 2010; Lah *et al*, 2012). The established PCR technique is sensitive in detecting diminutive volume of DNA template as confirmed in this study by using serial dilutions. Thus it can therefore be implied to detect even a smaller number of dust mites. Our finding coincides with the study by Thet-Em *et al*. (2012) that reported the detection limit of identifying some common dust mite species (*Dermatophagoides farinae, D. pteronyssinus* and *Blomia tropicalis*) as low 1 ng of genomic DNA. This method allows identification for immature of the target species and broken mites (Wong *et al*, 2011), which cannot be identified morphologically. Furthermore, 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 (Beroiz *et al*, 2014). One possible reason for the faint bands and decline of visualization of PCR products signal below 4 µl may be due to the increasing insensitivity of ITS2 gene amplification for detecting a smaller amount of mite's DNA in a less concentrated dilution. Successful detection of the ITS2 gene fragment using large amounts of DNA template does not guarantee high amplification of target DNA by PCR. This might be probably due to preservation of specimens and the methods of DNA extraction which may affect the quality of DNA and therefore the PCR amplification (Jeyaprakash and Hoy, 2010).

Identification of astigmatid mites by their morphological characteristics alone is difficult. The need for an expert taxonomist that requires skills and experience is a major drawback of this conventional method (Vargas *et al*, 2005). Moreover, several factors such as broken bodies, immature stages and closely morphological characters can also affect specific morphological identification (Colloff and Spieksma, 1992; Noge *et al*, 2005). The present study showed that the *A. ovatus* mite has its own morphological characteristics. It has been well-known as brown-legged grain mite (Sun *et al*, 2014) due to its intensely tanned chelicerae and legs which are near to dark reddish-brown in color, in contrast with whitish appearance of the body. These morphological observations were supported and in close accordance with the molecular analysis using the ITS2 region. Moreover, the combined approach used in this study could reveal further discrepancies in the actual classification and identification of *A. ovatus*.

CONCLUSION

In conclusion, this study showed an integrative approach, combining morphology and molecular data that is useful for species identification of astigmatid mites. The molecular analysis of ITS2 region of *A. ovatus* allows a clearer understanding to complement the current morphological identification of this mite to the species level. Another advantage is that the molecular method also allows the identification of mite species even when the mites' bodies are broken which eventually limit the morphological identification. In addition, the molecular identification technique is simpler, less time consuming and appropriate for epidemiological studies or in diagnostic laboratories of allergy-causing mites.

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