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A rapid identification method for common astigmatid species based on multiplex polymerase chain reaction

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Abstract

Astigmatid mites are economically significant pests of stored products and sources of inhalant allergens causing allergic rhinitis and asthma worldwide. The morphological identification of astigmatid mites at the species level is often a difficult task due to their small size, phenotypic similarity and lack of diagnostic characters. We used multiplex polymerase chain reaction (PCR) to identify astigmatid mite species, which could complement the morphological data for the species-specific identification of mites. Internal ribosomal transcribed spacer (ITS) sequences (i.e., partial 18S, the full length of ITS1-5.8S-ITS2 and partial 28S) from eight astigmatid species (*Acarus siro*, *Tyrophagus putrescentiae*, *Suidasia nesbitti*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Lepidoglyphus destructor*, *Chortoglyphus arcuatus* and *Gohieria fuscus*) were obtained by DNA extraction and then sequenced after PCR amplification. Specific primers were designed in the ITS2 region manually. Results revealed that an identification method for eight common astigmatid species was established based on multiplex PCR, which should be effective for the identification of other species of mites by redesigning species-specific primers in future experiments.

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Introduction

Astigmatid mites are frequent pests of animal fur, dried fruit, medicinal herbs and stored commodities.[1,2] They are also sources of indoor allergens due to their prevalence and close proximity to humans.[3,4] Astigmatid mite species are small and often co-occur in the same habitats.[5] Identification of these mites is mainly based on the morphological characters of different stages.[6] Mite identification using light microscope observation is time-consuming and requires expertise.[7]

Molecular markers have been used to resolve taxonomic and phylogenetic relationships as well as interspecific differentiation in mites.[8] The ribosomal internal transcribed spacer (ITS; 18S rDNA, 5.8S rDNA and 28S rDNA) and mitochondrial cytochrome oxidase subunit I (COI) regions are highly conserved in the eukaryotes.[9] There is a sufficient variation in these regions to allow their use as molecular barcodes for some taxonomic groups.[10]

The ITS2 region has been used for species identification.[11] For example, ITS2 sequences have been used as barcodes for identifying and analyzing spider mites.[12] The ITS2 sequences have also been used for six species of storage mites and analyses of their phylogenetic relationships.[13] However, all current methods are relatively time-consuming and expensive or require large specimens to be effective, due to the requirement of polymerase chain reaction (PCR) sequencing or using restricted fragment length polymorphisms.[9] Multiplex PCR has also been used to identify arthropod species,[14] including the Acari.[15]

In this study, a multiplex PCR method was developed for identifying eight common astigmatid mite species, and its specificity was determined.

Materials and Methods

Sample collection. A total of 80 astigmatid mite individuals, belonging to eight species, were used in this

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study. We randomly selected 10 astigmatid mite individuals from different populations, and their haplotype numbers are shown in Table 1. All of these samples were collected from stored commodities and indoor dust in China during 2018 (Table 1). Individual mites were separated under a microscope. Preliminary morphology identification was conducted using an optical microscope (BX51; Olympus, Tokyo, Japan) based on mite characteristics.[16] Isolated mites were placed in 0.20 mL centrifuge tubes containing 75.00% ethanol and kept at – 80.00 ˚C until genomic DNA (gDNA) extraction.

Genomic DNA extraction. The gDNAs of astigmatid mites were obtained through the protocol described by Jia *et al.*[17] The gDNA extraction of each mite was carried out separately. The gDNA template was prepared by homogenizing a single adult mite in a 25.00 µL mixture of sodium chloride-Tris-EDTA buffer (100 mM NaCl (Tiangen, Beijing, China), 10.00 mM Tris-HCl (Tiangen) and 1.00 mM EDTA (Tiangen); pH: 8.00 and 1.00 µL proteinase K (20.00 mg mL^{-1}) (Tiangen). This mixture was incubated at 37.00 ˚C for 30 min, and proteinase K was inactivated at 95.00 ˚C for 5 min. The extracted gDNAs were centrifuged and used immediately for PCR reaction or stored at – 20.00 ˚C for later use.

Polymerase chain reaction amplification and sequencing. Molecular identification of eight common astigmatid mites was performed using primers for the anterior region of COI gene, which forward and reverse sequences were 5'-CTTTTGGGATATCTCTCATAC-3' and 5'-GAGCAACAACATAATAACTATC-3', respectively.[18] Each PCR reaction was run in 25.00 µL containing 12.50 µL 2X PCR Master Mix (Tiangen), 1.00 µL (5.00 µmol L^{-1}) universal COI forward primer, 1.00 µL (5.00 µmol L^{-1}) universal COI reverse primer, 1.00 µL genomic DNA, and 9.50 µL double-distilled H_2O. The PCR reaction procedure was as follows: Initial denaturation at 94.00 ˚C for 5 min, 36 cycles at 94.00 ˚C for 30 s, 48.00 ˚C for 30 sec, 72.00 ˚C for 1 min, and a finial elongation step at 72.00 ˚C for 10 min. Afterwards, samples were stored at 4.00 ˚C for 59 min for heat preservation. The PCR amplification products were detected by 1.00% agarose gel electrophoresis and purified using a TIANgel Midi Purification Kit (Tiangen). All PCR products were commercially sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The obtained sequences were submitted and compared with known astigmatid mite sequences in the NCBI database. The GenBank accession numbers for astigmatid mite populations in the study are shown in Table 1.

Amplification of ITS gene region was performed using the species-specific primers following the protocol described by Beroiz *et al.*[19] The ITS2 forward and reverse sequences were 5'-AGAGGAAGTAAAAGTCGTAACAAG-3' and 5'-ATATGCTTAAATTCAGCGGG-3', respectively.[19] The PCR reaction and amplification procedures of ITS gene were the same as above. The obtained ITS sequences were used for subsequent primer design.

Primer design. Since 5.8S is a highly conserved region in ITS among different species, a universal forward primer was designed in the 5.8S region in this study. Due to the sequence length and composition polymorphism of ITS2, each of the eight reverse primers was designed to bind to one species only (using BLAST primer on the NCBI website; Table 2).

Table 1. Summary of astigmatid mite DNA samples included in this study.

Mite species	Accession No.	No.*	Location
Acarus siro	MH793874-MH793882	9	Bozhou
Tyrophagus putrescentiae	MH793968-MH793974	7	Wuhu
Suidasia nesbitti	MH794077-MH794086	10	Bozhou
Dermatophagoides pteronyssinus	MK589422-MK589429	8	Tongling
Dermatophagoides farinae	MH793948-MH793957	10	Wuhu
Lepidoglyphus destructor	MH794022-MH794031	10	Wuhu
Chortoglyphus arcuatus	MH794000-MH794008	9	Shaoxing
Gohieria fuscus	MH794087-MH794096	10	Wuhu

* The number of haplotype of each species.

Table 2. All multiplex polymerase chain reaction primers designed for eight species of astigmatid mites.

Mite species	Primers	Primer sequences
-	Asti-F*	5'-GTGGTGGATCACTCGGC - 3'
A. siro	Acas-R	5'-GGCAAACATACATTGGCTCCC-3'
T. putrescentiae	Tyrp-R	5'-AGCCCCCTACATTAGACTACCA-3'
S. nesbitti	Suin-R	5'-AGAGTCGACACGACCAACTG-3'
D. pteronyssinus	Derp-R	5'-TGGAAATGACCTGACGACGTT-3'
D. farinae	Derf-R	5'-GTGATGAATGTGCTACAAAGCCA-3'
L. destructor	Lepd-R	5'-ACCCGATCGATGCAATATGCT-3'
C. arcuatus	Choa-R	5'-CGTGTGTCCGCTAAAACAACTT-3'
G. fuscus	Gohf-R	5'-AATAGGCTTATGACATGTTGCC-3'

* The Asti-F primer represents a universal forward primer.

Amplification of multiplex PCR. Each multiplex PCR reaction was run in 25.00 µL containing 12.50 µL 2X PCR Master Mix (Tiangen), 1.00 µL (5.00 µmol L^{-1}) universal forward primer Asti-F, 1.00 µL (5.00 µmol L^{-1}) of each of the eight reverse primers, 1.00 µL gDNA and 2.50 µL double-distilled H_2O. Multiplex PCR was carried out using the following optimized reaction procedure: Initial denaturation at 94.00 ˚C for 3 min, 26 cycles at 94.00 ˚C for 30 s, 60.00 ˚C for 30 sec, 72.00 ˚C for 1 min, and a final elongation step at 72.00 ˚C for 10 min, afterward, samples were stored at 4.00 ˚C for 59 min for heat preservation. The multiplex PCR products were detected using 2.00% agarose gel electrophoresis for 60 min, and DNA ladder A Plus (Sangon Biotech Co., Ltd.) was used as a reference.

Results

Before DNA extraction, preliminary morphology identification was conducted under an optical microscope based on mite characteristics (Fig. 1). The obtained COI sequences were submitted and compared with known astigmatid mite sequences using BLAST searches of the nucleotide collection (nr/nt; nr: non-redundant protein sequence database, nt: nucleotide sequence database) database of the NCBI. The results showed that the identities of COI sequences were all above 98.00%. Therefore, the specimens collected in this study were identified as *A. siro*, *T. putrescentiae*, *S. nesbitti*, *D. pteronyssinus*, *D. farinae*, *L. destructor*, *C. arcuatus* and *G. fuscus*.

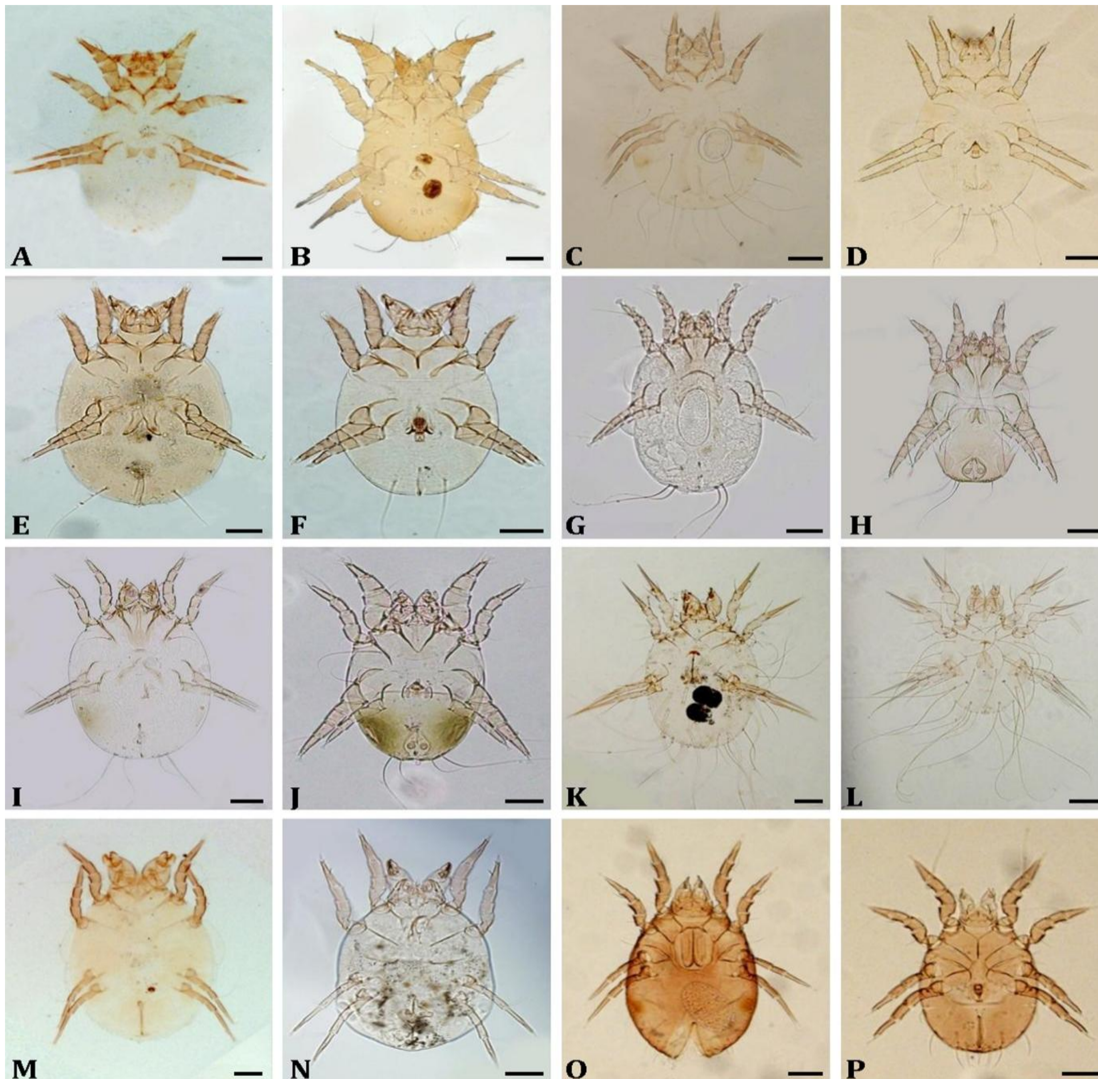

Fig. 1. Morphological images of eight common astigmatid mites. **A)** *Acarus siro* (female, scale 100 µm); **B)** *Acarus siro* (male, bar = 100 µm); **C)** *Tyrophagus putrescentiae* (female, bar = 100 µm); **D)** *Tyrophagus putrescentiae* (male, scale 100 µm); **E)** *Suidasia nesbitti* (female, bar = 100 µm); **F)** *Suidasia nesbitti* (male, bar = 100 µm); **G)** *Dermatophagoides pteronyssinus* (female, bar = 50.00 µm); **H)** *Dermatophagoides pteronyssinus* (male, bar = 50.00 µm); **I)** *Dermatophagoides farinae* (female, scale 50 µm); **J)** *Dermatophagoides farinae* (male, bar = 50.00 µm); **K)** *Lepidoglyphus destructor* (female, bar = 100 µm); **L)** *Lepidoglyphus destructor* (male, bar = 100 µm); **M)** *Chortoglyphus arcuatus* (female, bar = 50.00 µm); **N)** *Chortoglyphus arcuatus* (male, bar = 50.00 µm); **O)** *Gohieria fuscus* (female, bar = 50.00 µm); **P)** *Gohieria fuscus* (male, bar = 50.00 µm).

For the eight species of astigmatid mites, the total length of ITS2 ranged from 249 bp to 445 bp, and the base composition was similar when it was close to the flanking conservative area (5.8S rDNA or 28S rDNA). The mite-specific primers based on the used ITS2 region were shorter than expected. The primers designed in this region were kept at a sufficient distance as far as possible. This strategy achieved the diagnostic separation of the eight species.

From the ITS2 sequence data, each of the eight reverse primers was designed to bind to only one of the eight species. A universal forward primer was designed in the conservative 5.8S region. We were able to determine from the sequences that the lengths of the products were 429 bp for *A. siro*, 355 bp for *T. putrescentiae*, 192 bp for *S. nesbitti*, 215 bp for *D. pteronyssinus*, 288 bp for *D. farinae*, 306 bp for *L. destructor*, 409 bp for *C. arcuatus* and 382 bp for *G. fuscus* (Fig. 2).

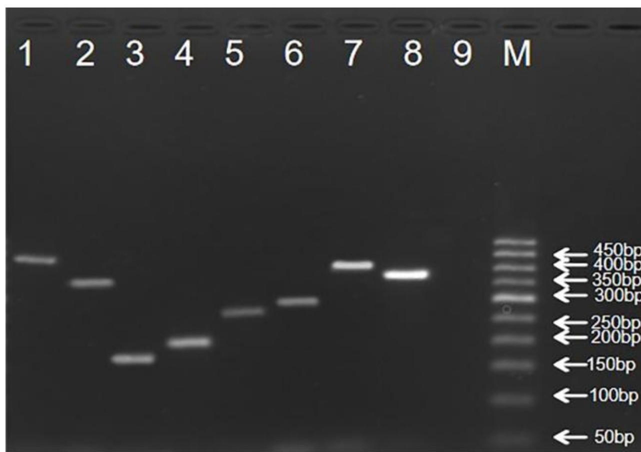

Fig. 2. Results of multiplex polymerase chain reaction with DNA extracted from 8 astigmatid mite species (Lanes 1-8). Lane 1: *Acarus siro*, 429 bp; Lane 2: *Tyrophagus putrescentiae*, 355 bp.; Lane 3: *Suidasia nesbitti*, 192 bp; Lane 4: *Dermatophagoides pteronyssinus*, 215 bp; Lane 5: *Dermatophagoides farinae*, 288 bp; Lane 6: *Lepidoglyphus destructor*, 306 bp; Lane 7: *Chortoglyphus arcuatus*, 409 bp; Lane 8: *Gohieria fuscus*, 382 bp; Lane 9: negative control (A universal forward primer and eight reverse primers were added without extracted gDNAs); Lane M: DNA ladder A Plus (50 bp DNA ladder marker).

Discussion

Previous mite identification studies have shown that multiplex PCR is an effective tool for distinguishing related species.[15,19,20] This study was the first to establish a multiplex PCR method for the identification of eight common astigmatid mites.

Identification of eight astigmatid mite species could be obtained from one reaction requiring only 3 hr from DNA extraction to species identification. The morphological characteristics used in the taxonomy of astigmatic mites make it very difficult to identify related species. Compared to the traditional morphological identification, the molecular identification methods are less affected by the growth stages or incomplete mite, which could provide a powerful supplement for traditional morphological identification. The method developed in this study tested different individual mites, and the results showed that it had better stability and reliability. However, due to the limited numbers of samples and species of mites in this study, further scientific researchers are needed to be tested on the reliability and extended applications of this method in the future. Additional species of mites could be detected by redesigning species-specific primers in future experiments.

This assay could be further developed with real-time quantitative multiplex PCR technique to examine levels of mites in samples. This simple molecular method can be used by plant quarantine offices to identify commonly occurring species of astigmatid mites infecting stored products. This method may be extended to other species in the future, which would lay a foundation for the future identification of species by multiplex PCR.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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