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Morphological and Phylogenetic Characterization of *Tyrophagus putrescentiae* Schrank (Sarcoptiformes: Acaridae) from *Hypsizygus marmoreus* in Korea

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The present study reports the morphological and phylogenetic characteristics of T*yrophagus putrescentiae* from *Hypsizygus marmoreus* in Korea. This species is morphologically characterized by two pigmented eyespots on the prodorsal shield, concave posterior margin of coxal plate Ⅱ, prominently and apically enlarged supracoxal seta shaft at bases of pectinations, and solenidion omega1 on tarsi I and Ⅱ, respectively and length of setae *d1* compared to setae *c1* and setae *d2*. The internal transcribed spacer 2 (ITS2) in the ribosomal DNA (rDNA), which is reliable for species identification, and the cytochrome oxidase subunit I (COI) in the mitochondrial DNA, which is a highly conserved region in eukaryotes, were sequenced. The resulting phylogenetic trees for COI and ITS2 apparently showed that the tested samples in this study were clustered with *T. putrescentiae* and separated from other species in the genus. This research would be useful for controlling mites from *H. marmoreus* in Korea and could help improve the quality and quantity of *H. marmoreus* production.

Key words: *Tyrophagus putrescentiae*, *Hypsizygus marmoreus*, COI, ITS2

INTRODUCTION

Mushrooms are one of the most cultivated crops in the world. Depending on the species and strain of mushroom, the environmental conditions and substrate resources required for cultivation differ (Dong and Wang, 2008; Qin and Qin, 2010). Some mite species prefer to live in wet environments and destroy stored foods such as cereals, grain, dog food, and flour, or organic matter debris (Rodriguez and Rodriguez, 1987). These mites can also survive in moist condition at 20–25°C for as long as 31 days without food (Sass *et al*., 2006). Since mushrooms are cultivated in warm and humid environments, control of pests, especially mites, is an economically important venture. Furthermore, domestic mites inhabit beds, carpets, and human living spaces (Spieksma, 1997). However, it is difficult to observe and control mites until the population density of the pests increases enough to cause noticeable damage (Wu and Lu, 1996; Lan *et al*., 2012).

The genus *Tyrophagus* is an economically important group of the superfamily Acaroidea. In particular, *Tyrophagus putrescentiae*, which is a non–predatory mite species is a common pest of stored foods and various fungi around the world (Chmielewski, 1978; Hubert *et al*., 2004, 2012; Nesvorná *et al*., 2012). This species has been reported from fungal and insect colonies in laboratories (Duek *et al*., 2001), indoor facilities, and the food industry (Solarz *et al*., 2007). It also lives in commercial bumblebee colonies (Rozej *et al*., 2012), agricultural soils (Smrz and Jungova, 1989) and the nests of small mammals and birds (O'Connor, 1979; O'Connor, 1982). In general, it feeds on protein and on fat–rich substances (Garcia, 2004; Palyvos *et al*., 2008; Erban *et al*., 2015; Erban *et al*., 2016). Infestation of this species on the mycelia and fruiting bodies of edible mushrooms decreases production and causes human allergic diseases (Li *et al*., 2003; Jeong *et al*., 2007). Previously *T. putrescentia*e has been found in *Agaricus bisporus*, *Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius* (Zou and Gao, 1987; Zhang *et al*., 1992).

Shimeji mushrooms, including *H. marmoreus*, is a mushroom group characterized by its bunched–together clusters with long stems and tight concave caps. Currently, production of shimeji mushrooms does not meet demand despite expansion of cultivation. Furthermore, farmers who cultivate *H. marmoreus* in Korea suffer economical losses from unidentified mites. Mite species have been identified generally using adult morphological characteristic keys, but their minuscule body sizes and the lack of morphological keys for egg and immature stages often make it difficult to identify these species (Vargas *et al*., 2005). Molecular diagnostic methods are useful for identifying acaroid mites at the species level, and the cytochrome oxidase subunit I (COI) in the mitochondrial DNA (mtDNA) and the internal transcribed spacer 2 (ITS2) in the ribosomal DNA (rDNA) have often been used for investigating relationships between and within species as well as for identifying mites to the species level (Ros and Breeuwer, 2007; Yang *et al*., 2011; Khaing *et al*., 2014). This study presents the results of studies on *T. putrescentiae* found on *H.*

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marmoreus in Korea using morphological characteristics and the phylogenetic trees of COI and ITS2.

MATERIALS AND METHODS

Sample collection and morphological identification

Specimens were collected for this study from pots used for *H. marmoreus* cultivation at a farm in Gaknam–myeon, Cheongdo–gun, Gyeongsangbuk–do, Korea in 2016. After collection, mites were immediately selected for genomic DNA extraction and morphological identification. The specimens were morphologically identified under a stereo microscope (Carl Zeiss Axioskop 2, Germany) using the characteristic keys described by Fan and Zhang (2007).

Molecular analysis

A total of seven samples were used for the molecular study. For partial sequences of COI and ITS2, genomic DNA was extracted from mites using genomic DNA extraction kits (Bioneer Corp., Daejeon, Korea). Because the samples were so small, the genomic DNA extraction process was modified from the original manufacturer's protocol. The specimens were combined with 1× PBS and 5 μL of Proteinase K in 1.5–mL tubes. After grinding, 50 μL of binding buffer was added and mixed immediately with a vortex mixer. The samples were incubated at 60°C for 10 min. After adding 25 μL of isopropanol, mites were washed with two types of washing buffers. Genomic DNA in the columns was eluted with an elution buffer. Polymerase chain reaction (PCR) was performed using forward (5'–GTTTTGGGATATCTCTCATAC–3') and reverse (5'–GAGCAACAACATAATAAGTATC–3') for COI and forward (5'–CGACTTTCGAACGCATATTGC–3') and reverse (5'–GCTTAAATTCAGGGGGTAA TCTCG–3') for ITS2 (Yang *et al*., 2011). A total volume of 25 μL for each PCR reaction mixture contained 0.5 μL of the genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer for COI and ITS2, and 1 unit of *Taq* DNA polymerase. PCR cycling conditions for both regions proceeded as follows: initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C 40 sec, 50°C (COI)/59°C (ITS2) for 40 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were electrophoresed through an ethidium bromide 1.5% agarose gel, and a gel imaging system with ultraviolet light illumination was used to check the amplicons. After PCR purification using ExoSAP–IT (USB, Cleveland, OH, USA), PCR products were sent to SolGent Co., Ltd. (Daejeon, Korea) for DNA sequencing.

The sequence alignments for COI and ITS2 were carried out using BioEdit 7.2.5 (Hall, 1999). All sequences were deposited in GenBank under accession no. LC190827–LC190833 for ITS region and LC190834–LC190839 for COI gene. Neighbor–joining (NJ) analysis was performed using MEGA 7.0 with 1000 bootstrap pseudo–replicates for node support (Kumar *et al*., 2016).

RESULTS

The morphological characteristics of the specimens for this study were examined using the key for *T. putrescentiae* (Fan and Zhang, 2007) (Fig. 1). In Figure 1, arrows and triangles indicate morphological fea-

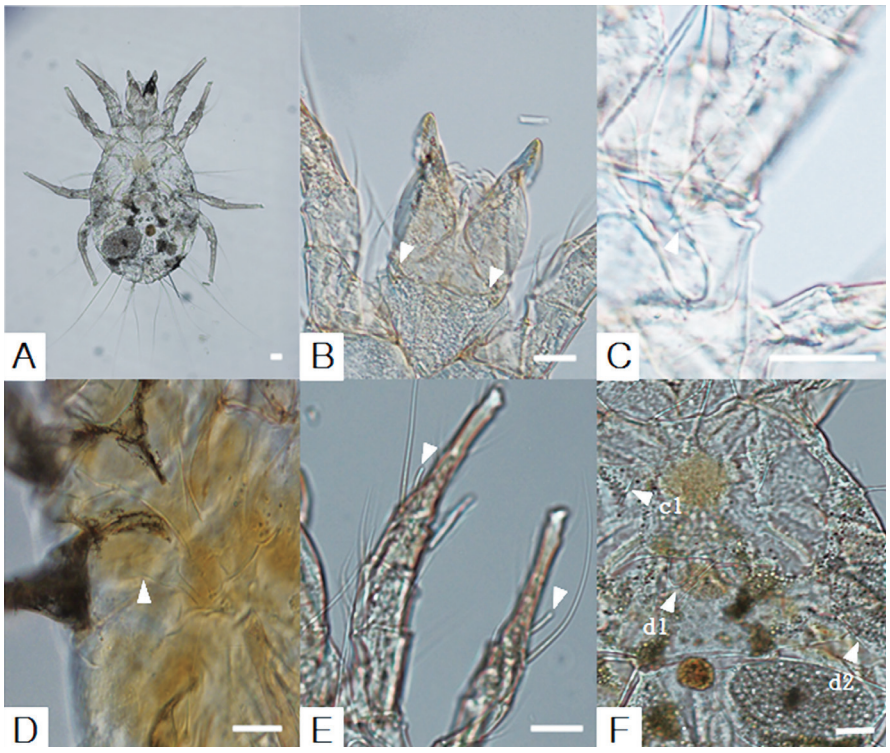

Fig. 1. Morphological characteristics of *T. putrescentiae*. A: Whole body of *T. putrescentiae*. B: Pigmented eyespots on prodorsal shield. C: Supracoxal setae at bases of pectinations. D: Posterior margin of coxal plate Ⅱ. E: Solenidion omega1 on tarsi I and Ⅱ. F: Setae *c1*, *d1*, and *d2*. Scale bars = 25 μm.

tures of *T. putrescentiae* for identification such as pigmented eyespots, shaft of supracoxal setae, coxal plate Ⅱ, solenidion omega1 on tarsi Ⅰ and Ⅱ, and setae *c1*, *d1*, and *d2*. The morphological key of *T. putrescentiae* is described as follows.

Key to *T. putrescentiae* (Fan and Zhang, 2007)
Two pigmented eyespots on prodorsal shield; prominently enlarged shaft of supracoxal seta at bases of pectinations; concave posterior margin of coxal plate II; apically enlarged solenidion omega1 on tarsi Ⅰ and Ⅱ; setae *d1* 2.5 times or more than length of setae c1 and setae *d2*..... *T. putrescentiae*

The taxonomic comparison based on neighbor–joining analyses of COI and ITS2, which are identified in the present study and the GenBank database, are shown in Fig. 2 and Fig. 3. In an analysis of COI, all seven sample sequences were identical. The sequence alignments of COI in this study were branched into four clusters. The samples in this study clustered together with the known COI sequence of *T. putrescentiae* from China (GenBank accession no. EU078968) although the known COI sequence of *T. putrescentiae* from Korea (GenBank accession no. KJ820777) was separated from the cluster. *Tyrophagus longior* (GenBank accession no. KR869095) and *Tyrophagus similis* (GenBank accession no. KM199641), which are the only available sequences in the genus from GenBank, were apparently separated from the clusters of *T. putrescentiae*. *Aleuroglyphus ovatus* (GenBank accession no. EU078964) was used as an outer group of the COI analysis. The analysis of COI in this study clearly showed distinct clusters between species.

The phylogenetic tree of ITS2 showed two main clusters. All samples in this study clustered together with the known ITS2 sequences of *T. putrescentiae* from Korea, Japan, and China from GenBank (accession no. KJ605165, AB104977, and GQ205623 respectively) and were clearly distinguished from the other species clusters. Two clusters for *Tyrophagus neiswanderi* and *Tyrophagus fanetzhangorum* (GenBank accession no. AB105036 and KC215303 respectively) were separated from the cluster of *T. putrescentiae*. Two clusters for *T. similis* and *T. longior* (GenBank accession no. AB104978 and AB104997 respectively) were apparently separated from the other species clusters. *Tyroborus lini* (GenBank accession no. AB105024) was used as an outer group of the ITS2 analysis. The analysis of ITS2 also prominently showed distinct clusters between species.

DISCUSSION

Tyrophagus putrescentiae found on *H. marmoreus* in Korea was morphologically and phylogenetically characterized in this study. There are at least 18 species in the genus *Tyrophagus*, which include *Tyrophagus curvipenis* Fain & Fauvel, *Tyrophagus debrivorus Chinniah* & Mohanasundaram, *Tyrophagus glossinarum* Fain, *Tyrophagus houstoni* Fain, *Tyrophagus jingdezhenensis* Jiang–Zhenta, *Tyrophagus lini* Oudemans, *T. longior* Gervais, *Tyrophagus mimlongior* Jiang, *T. neiswanderi* Johnson & Bruce, *Tyrophagus neotropicus* Oudemans, *Tyrophagus palmarum* Oudemans, *Tyrophagus perniciosus* Zakhvatkin, *T. putrescentiae* Schrank, *Tyrophagus robertsonae* Lynch, *Tyrophagus savasi* Lynch, *T. similis* Volgin, *Tyrophagus tropicus* Robertson, *T. fanetzhangorum* Klimov & O'Connor (O'Connor, 2008; Klimov and O'Connor, 2009). *Tyrophagus putrescentiae* was morphologically identified by means of a number of pigmented eyespots on the prodorsal shield, enlargements of supracoxal seta shaft at the bases of pectinations and solenidion omega1 on tarsi Ⅰ and Ⅱ, the posterior margin shape of coxal plates Ⅱ, and length comparison between setae *c1*, *d1*, and *d2*. However, species information is still needed for most of the species in this genus, including information on immature stages, in order to conduct a full investigation.

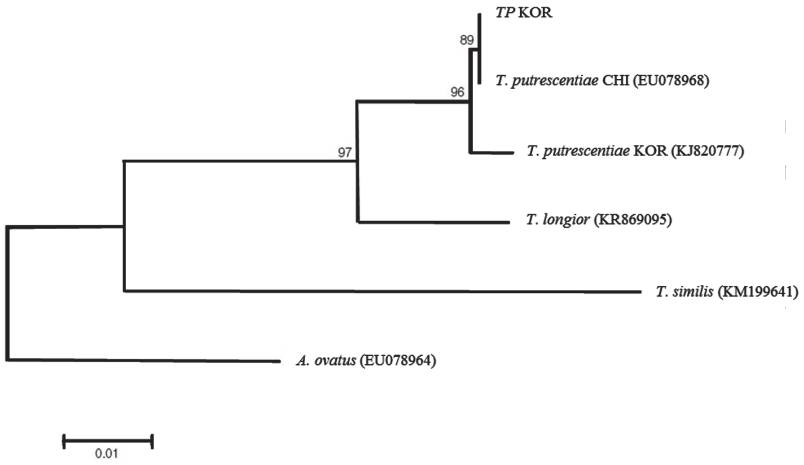

Fig. 2. Neighbor-joining tree for COI. Node support is given as bootstrap percentages for 1,000 replicates. One haplotype of *T. putrescentiae* for COI (*TP* KOR) in this study was produced. *Tyrophagus putrescentiae* KOR and CHI indicate strains of *T. putrescentiae* from Korea and China respectively. *Aleuroglyphus ovatus* was used as an outgroup for the analysis.

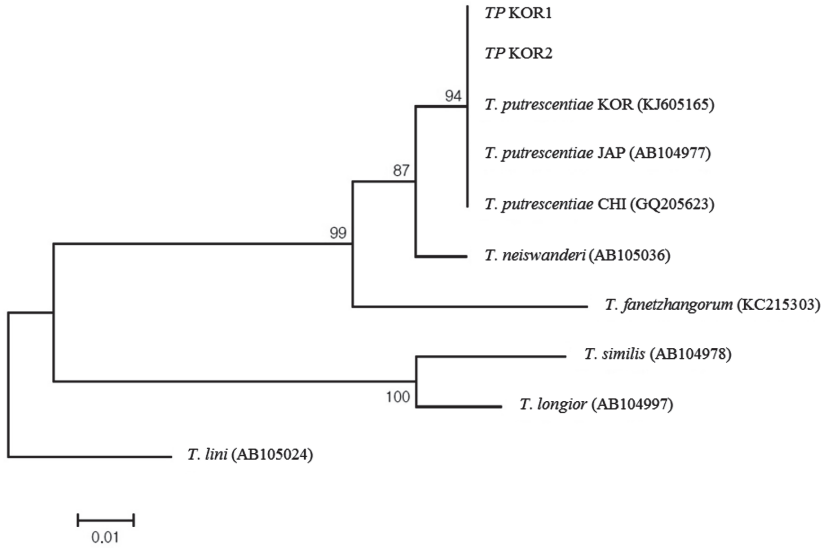

Fig. 3. Neighbor-joining tree for ITS2. Node support is given as bootstrap percentages for 1,000 replicates. Two haplotypes of *T. putrescentiae* for ITS2 (*TP* KOR1 and *TP* KOR2) were produced in this study. *Tyrophagus putrescentiae* KOR, JAP and CHI indicate strains of *T. putrescentiae* from Korea, Japan, and China respectively. *Tyroborus lini* is an outgroup for the analysis.

mtDNA is haploid and non–recombinant (Dowton and Campbell, 2001). Based on these features, phylogenetic studies often use regions of mtDNA as molecular markers, such as COI used for a DNA barcoding region and cytochrome oxidase subunit Ⅱ (COII), NADH dehydrogenase subunit 5 (ND5) and cytochrome b (CYTb) genes for species identification. In this study, there were four clusters in the phylogenetic tree of COI. Two species, *T. longior* and *T. similis*, were apparently separated from *T. putrescentiae*. *Tyrophagus putrescentiae* was branched into two clusters. One cluster grouped the samples from this study and the COI sequence of *T. putrescentiae* from China, and the other cluster comprised the COI sequence of *T. putrescentiae* from Korea. Yang *et al*. (2011) showed two clusters within *T. putrescentiae* from China in the analysis of COI. Khaing *et al*. (2014) reported that two strains of *T. putrescentiae* from Korea and China, which were the same as those used in this study, were separated in their analysis of COI. This separation might be the result of the influence of food and habitats found in different environments. However, the clusters of *T. putrescentiae* for COI were distinctly separated from the other mite species.

The analysis of ITS2 in this study showed that all clusters for each species were clearly separated in the phylogenetic tree, including *T. putrescentiae* from Korea, Japan, and China. Although sequence variation in most of the coding regions in the rDNA is limited by selection, the low level of evolutionary constraint in some of the spacers and even functional domains, combined with the presence of tandem repeats in rDNA and their subsequent homogenization (Dover 1982), can lead to the rapid spread of a new sequence variant. Genetically isolated populations thus may be rapidly homogenized for different variants. Therefore, sequence variation between closely related species in the internal transcribed spacer 1 (ITS1) or ITS2 regions of the rDNA is reliable for species identification, including for mite species (Hillis and Dixon, 1991; Navajas and Fenton, 2000; de Rojas *et al*., 2007; Beroiz *et al*., 2014). Yang *et al*. (2011) and Khaing *et al*. (2014) reported that the analysis of ITS2 clearly distinguished mite species with two haplotypes for *T. putrescentiae*. Beroiz *et al*. (2014) also reported that five species in the genus *Tyrophagus*—*T. putrescentiae*, *T. neiswanderi*, *T. fanetzhangorum*, *T. similis*, and *T. longior*—were apparently separated, with several haplotypes for each species. Although there were several haplotypes of ITS2 within species, the five species in the genus *Tyrophagus*—at least those which were analyzed in this study and others—were distinctly separated in the analysis of ITS2.

In conclusion, *T. putrescentiae* was represented using the characterization of its morphological features and by molecular analyses of COI and ITS2. This study can provide useful information for the control of this mite species on *H. marmoreus*, and may prove helpful in improving the profits of mushroom farmers.

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