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Molecular identification of eriophyoid mites in Thrace using the 28S and COI genes

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Abstract: Eriophyoid mites are recognized as the second most economically important group among the Acari following spider mites. The identification of eriopyhoid mites traditionally based on morphological characters. However, the size of these diagnostic structures is very small and this often causes misidentification of species. In this study, we used DNA-based identification for five eriophyoid species collected from the Thrace region of Turkey (*Aceria erinea, Rhyncaphytoptus ficifoliae*, *Eriophyes pyri*, *Aceria massalongoi*, *Colomerus vitis* collected from *Juglans regia* L., *Ficus carica* L., *Pyrus communis* L., *Vitex-agnus castus* L., *Vitis vinifera* L., respectively) using both cytochrome oxidase subunit I (MW396744-MW396748) and 28S rRNA (MW396565-MW396571) in combination with morphological taxonomy. A phylogenetic tree has also been constructed for each gene to get a deeper understanding of the evolutionary history of Eriophyoidea. Besides all the molecular data herein obtained are the first eriophyoidea sequences for the country, some of the sequences have been submitted to the public GenBank database for the first time. Further studies are urgently needed to reveal genetic variation within and between eriophyoid species to make accurate species identification using molecular technics.

Key words: Eriophyoidea, cytochrome c oxidase subunit I, 28S rRNA, DNA taxonomy

1. Introduction

Eriophyoid mites (Acari: Eriophyoidea) are tiny, obligatory phytophagous and mainly host-specific invertebrates (Lindquist et al., 1996). These mites are recognized as the second most economically important group among the Acari following spider mites (Acari: Tetranychidae) (Van Leeuwen et al., 2010). Their economic importance is related to (i) direct feeding damage to their host plant (ii) their vector feature that allows transmitting plant pathogens, especially viruses (Van Leeuwen et al., 2010; Stenger et al., 2016). Although more than 5000 species have been recorded so far (Zhang, 2017), it is estimated that the total number of eriophyoid species may amount to more than 50,000 (Amrine, 1996; Lindquist et al., 1996). Although a significant eriophyoid biodiversity may be expected considering the geographical position and botanical background of Turkey (Ekim and Güner, 2000), only around 130 species have been recorded so far indicating the lack of studies to reveal eriophyoid fauna (Denizhan et al., 2006, 2008; Denizhan and Çobanoğlu, 2010, Denizhan et al., 2015).

The identification of eriopyhoid mites traditionally based on morphological characters such as genital area, prodorsal shield, empodium and opisthosomal setae length (de Lillo et al., 2009). However, the size of these diagnostic structures is very small and this often causes misidentification of species (de Lillo et al., 2009). In addition, identification keys are only available for adult females which limit species identification using immature stages. Considering all these difficulties and the importance of accurate species identification in pest management, DNA-based approaches may offer a solution to overcome these problems (Navajas and Navia, 2010).

Species identification using a specific gene sequence was first proposed by Hebert et al. (2003) and called as DNA barcoding. Similar to many biological organisms, this approach has been applied to Acari (Navajas and Fenton, 2000; Cruickshank, 2002; Dabert, 2006) including eriophyoid mites (Navajas and Navia, 2010). However, the molecular diagnosis of eriophyoid mites has still not reached the desired level and reference sequences for many species are still lacking in the public GenBank. Moreover, delimitation of species boundaries (intra- and interspecific variation), which is crucial for accurate diagnosis, is not known for most of the eriophyoid mites.

In this study, we obtained the first DNA-barcoding sequences based on cytochrome c oxidase subunit I (COI) and D1-D2 region of the large subunit ribosomal gene (28S rRNA) of five eriophyoid species collected from Turkey. In addition, genetic distances between certain species/genera from Turkey and other countries of the world were analyzed. Last, phylogenetic trees were constructed to reveal the evolutionary relationships among the Eriophyoidea.

2. Materials and methods

2.1. Sampling of eriophyoid mites

A total of 7 mite populations belonging to 4 different genera were sampled from herbaceous plants and fruit trees showing visible symptoms of injuries caused by eriophyoid mites in the Thrace region of Turkey (Edirne, Çanakkale, Kırklareli) in 2020 (Figure 1; Table 1). The eriophyoid mites collected from the host plants were directly examined under a dissecting stereomicroscope (Leica ES2) and subsequently mounted on

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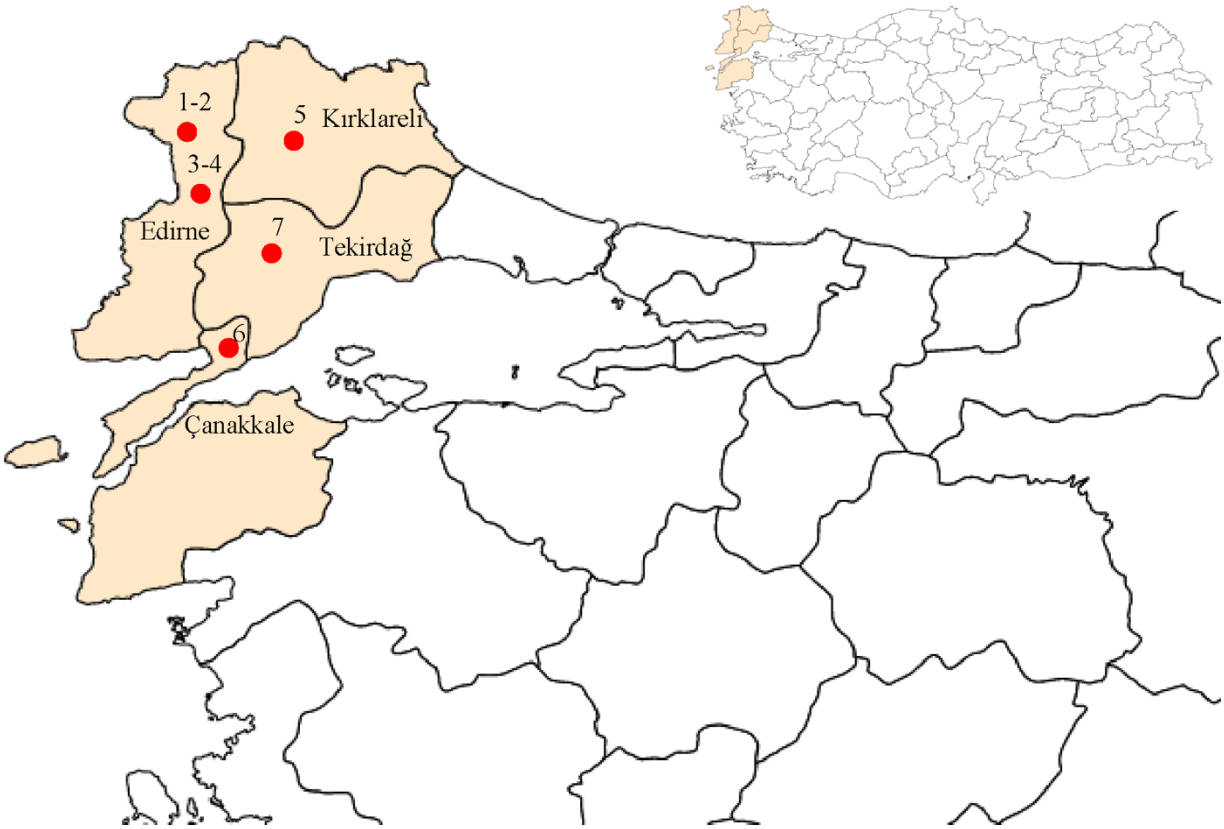

Figure 1. Map of the study area showing the sampling fields.

Table 1. Location, date, host plant of sampled eriophyoid mites from Turkey.

Species	Location - date	Host plant	Host (family)	Accession number	
				28S	COI
1 *Aceria erinea* Nalepa, 1891	Edirne – 15.07.2020	*Juglans regia* L.	Juglandaceae	MW396567	MW396748
2 *Rhyncaphytoptus ficifoliae* Keifer, 1939	Edirne – 20.07.2020	*Ficus carica* L.	Moraceae	MW396571	MW396747
3 *Eriophyes pyri* Pagenstecher, 1857	Edirne – 05.08.2020	*Pyrus communis* L.	Rosaceae	MW396566	-
4 *Aceria massalongoi* Canestrini,1890	Edirne – 07.08.2020	*Vitex-agnus castus* L.	Verbenaceae	MW396565	MW396746
5 *Aceria erinea* Nalepa, 1891	Kırklareli – 21.08.2020	*Juglans regia* L.	Juglandaceae	MW396569	MW396745
6 *Aceria erinea* Nalepa, 1891	Çanakkale – 03.08.2020	*Juglans regia* L	Juglandaceae	MW396568	MW396744
7 *Colomerus vitis* Pagenstecher, 1857	Tekirdağ – 12.08.2020	*Vitis vinifera* L.	Vitaceae	MW396570	-

slides according to Keifer (1975). Morphological identification of eriophyoid species was performed by Evsel Denizhan under a phase-contrast microscope (Leica DM 1000). The morphological nomenclature and systematic classification follow Lindquist (1996) and Amrine et al. (2003), respectively.

Permanent slides were deposited at Trakya University, Department of Biology in case of further verification. Mites were transferred to ATL buffer (Qiagen DNeasy Blood & Tissue Kit) for molecular purposes.

2.2. DNA isolation and PCR analysis

Total DNA was extracted from pools of 10 adult female mites per sample using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. After the final wash, genomic DNA was eluted with 50 µL of elution buffer. The concentration and quality of the isolated DNA were checked by agarose gel electrophoresis (1.5%) and UV spectrophotometer (Thermo Scientific NanoDrop 2000). All DNA extracts were stored at –20 °C until the PCR process.

An approximately 650 basepairs (bp) fragment of cytochrome oxidase I (COI) was amplified using the universal primer pairs LCO1490 (forward) and HCO2198 (reverse) (Folmer, 1994). PCR temperature cycling conditions were as follows: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 48 °C and 60 s at 72 °C, and a final extension of 7 min at 72 °C.

The D1-D2 region of the large subunit ribosomal gene (28S rRNA) was multiplied using a set of primers 5'-ACAAGTACCDTRAGGGAAAGTTG-3' and 5'-GCATAGTTCACCATCTTTCG-3' according to the PCR conditions reported by Sonnenberg et al. (2007).

All PCR reactions were performed in a total volume of 30 µL containing 3 µL of mite DNA, 0.5 µL of each primer, 18 µL of ultrapure water and 6 µL of FIREPol Master Mix (Solis Biodyne). The resulting PCR products were purified using HighPrep PCR clean-up system (MagBio Genomics Inc.) and subsequent sequencing was performed at Macrogen Inc. (Seoul, Korea).

2.3. Phylogenetic and genetic analysis

The phylogenetic trees based on 28S and COI genes have been built using the sequences herein obtained and the ones retrieved from the public GenBank database. All sequences were aligned and then trimmed to obtain equal size sequences using Clustal X v2.0 (Larkin et al., 2007) and using BioEdit v.7.0.5 (Hall, 1999), respectively.

A maximum likelihood (ML) phylogenetic tree has been constructed using MEGA X (Molecular Evolutionary Genetics Analysis) with 1000 bootstraps (Kumar et al., 2018). According to Bayesian information criterion (BIC) scores, TN93+G and GTR+G+I were identified to be the best-fit substitution model by MEGA X for 28S and COI, respectively. Intra- and interspecific genetic distance analyses were performed by selecting different group/groups and subsequently computing within or between groups mean distance using MEGA X (Kumar et al., 2018).

Mite species belonging to the family Phytoptidae were used as an outgroup in both phylogenetic trees, since all the mites obtained in the present study were belong to Eriophyidae and Diptilomiopidae.

3. Results and discussion

After alignment, final fragments of 28S rRNA and mtCOI sequences including around 800 bp and 560 bp, respectively, were used for further data analysis. A total of 12 sequences belonging to five eriophyoid species were obtained; *Aceria erinea*, *Eriophyes pyri*, *Rhyncaphytoptus ficifoliae*, *Colomerus vitis*, *Aceria massalongoi* (MW396744-MW396748 for COI; MW396565-MW396571 for 28S rRNA). Unfortunately, COI gene amplification of *C. vitis* and *E. pyri* species have been failed despite using both the Folmer primer pair and HCOd_R, an alternative reverse primer for the Folmer fragment (Chetverikov et al., 2015). AT content of COI and 28S genes were 69.4% and 52.5%, respectively.

The phylogenetic trees based on 28S and COI are presented in Figures 2 and 3. COI-based tree showed better clustering pattern according to genera than 28S-based tree, however, many genera belonging to the Eriophyoidea do not seem to be monophyletic. The conflict between morphological- and molecular-based taxonomy caused by the homoplasmic evolution of eriophyoid mites, have been known (Li et al., 2014). The complexity in the phylogenetic trees could be explained by this misclassification based on morphological characters used in traditional taxonomy. Integrated taxonomy combining multiple data should be favoured in future studies.

Our results showed that the families Eriophyidae and Diptilomiopidae have been clustered together, in line with Li et al. (2014) and Chetverikov et al. (2015). Even full mitochondrial sequences were not able to separate these closely related families (Arribas et al., 2020), probably caused by homoplasmic evolution within Eriophyoidea.

Aceria is known to be the largest genus of the Eriophyoidea comprising more than 1000 species (Amrine et al., 2003; de Lillo et al., 2020). In addition, the presence of cryptic species that create challenges in species identification within this genus has been known (Skoracka et al, 2012; Skoracka et al., 2015). Although not always supported by high bootstrap values, the results indicated that the genus *Aceria* seems to be polyphyletic (Figures 2 and 3), in line with Li et al. (2014) and Chetverikov et al. (2015). The genetic differences among *Aceria* spp. might be caused by the monophagous feature of most eriophyiod mites allowing high-level of genetic differentiation (Skoracka et al., 2009). Although initial studies showed that the evolution of speciation in eriophyoid mites is not correlated with host evolution (Fenton et al., 2000), host-plant specificity may be associated with speciation (Magalhães et al., 2007; Li et al., 2014).

In previous studies, genetic distances based on COI sequences were higher than 28S sequences between the Eriophyoidea species (Lewandowski et al., 2014; Chetherikov et al., 2015; Szydło et al., 2015). Zivkovic et al. (2017) reported that the average genetic distances based on COI between *Aceria* spp. collected from six Brassicaceae hosts was 18.3%, on the other hand, variation between clades of *Aceria tosichella* s.l. was determined to be 13.9% (Skoracka et al. 2012). In contrast to the previous studies, genetic variation of 28S (30.3%) was higher than COI (27%) between *Aceria* and other genera, as well as within the genus *Aceria* (27.7% and 25.4% for 28S and COI, respectively). This might be caused by the limited number of individuals and partial sequences of the genes used in this study, therefore, a larger dataset should be used to reach a more general conclusion about the Eriophyoidea. In addition, the amplified region (D1-D2 regions of 28S) in the present study contains length variable parts resulting in high variation (Sonnenberg et al., 2007). Although this unequal sequence size of indels might pose a problem for multiple sequence alignments, these variable fragments should be considered an advantage for DNA-based identification, because they provide more character for comparison, as stated in Sonnenberg et al. (2007). Since the minimum genetic distance between some species (i.e. 3.4% distance between *A. kunminensis* and *A. abalis*) was quite low, using 28S sequences alone without matching morphological diagnosis may lead to misidentification.

Since a universal similarity cut-off is not available, those assessments should be performed for each certain group (DeSalle et al., 2005), including the Eriophyoidea. In this study, the genetic variations based on a mitochondrial and a nuclear gene sequences of two *Aceria* species (*A. erinea* and *A. massalongoi*) were compared with the other species

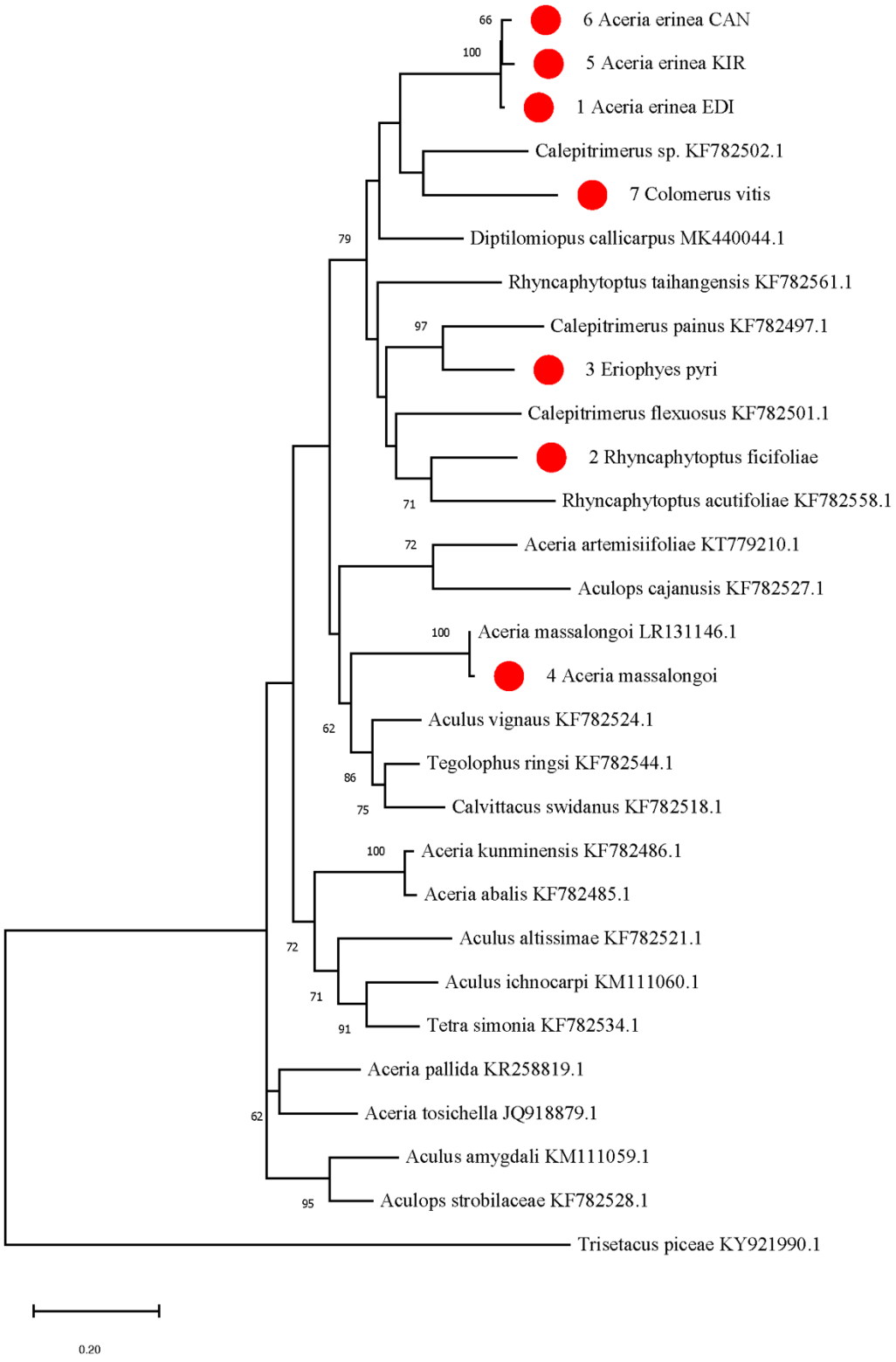

Figure 2. Phylogenetic tree of the family Eriophyoidea based on 28S rRNA. Bootstrap values lower than 50% are not shown.

belonging to the same genus (Table 2). The outgroup taxon was excluded in genetic distance analyses. The results showed that the intra- and interspecific genetic distances for both genes did not overlap allowing species delimitation for each species.

4. Conclusion

Application of DNA-based solutions for accurate species identification has a great potential in pest management, especially for small-sized pests such as Eriophyoidea. In this context, determination of intra- and interspecific variation among the specimens from different geographical

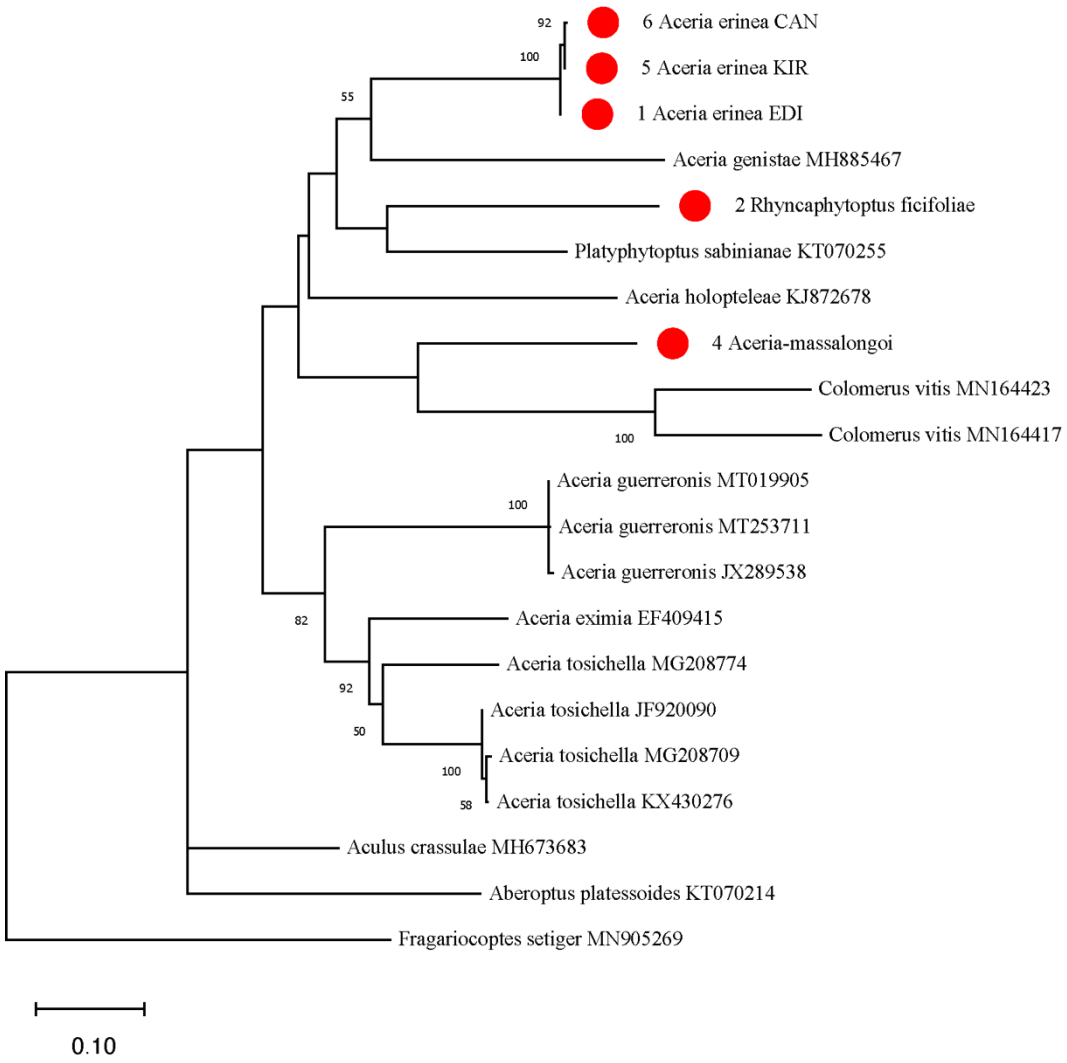

Figure 3. Phylogenetic tree of the family Eriophyoidea based on COI. Bootstrap values lower than 50% are not shown.

Table 2. Intra- and interspecific genetic distances of two *Aceria* species obtained in this study, and mean genetic diversity within the genus *Aceria*.

	No. specimens	Intraspecific genetic distances; mean (min-max)	Genetic distances between the species herein considered and other species in the same genus; mean (min-max)	Genetic distance within the genus *Aceria*; mean (min-max)	Genetic distances between the genus *Aceria* and other genus herein considered; mean (min-max)
28S					
Aceria erinea	3	0	29.0 (25.8–31.1)	27.7 (3.4–31.7)	30.3 (22.0–47.2)
Aceria massalongoi	2	0	31.0 (30.1–31.7)		
COI					
Aceria erinea	3	0.4 (0.3–0.5)	24.7 (20.7–28.7)	25.4 (15.6–29.2)	27.0 (20.2–31.3)
Aceria massalongoi	1	-	28.0 (27.2–29.2)		

backgrounds is of vital importance. Here we obtained the sequence data of eriophyoid mites from Turkey based on a mitochondrial (COI) and a nuclear gene (28S rRNA).

Although species identification seems to be possible (with some exceptions) using DNA-based methods, more and more sequences are required to resolve the evolutionary relationship of eriophyoid mites. Our study showed that constructing a phylogenetic tree using a single gene to resolve the evolutionary relationship of eriophyoid mites does not seem to be possible. In addition, more comprehensive studies revealed the conflict between morphology- and molecular-based taxonomy indicating the need for further studies using integrative taxonomy (Li et al., 2014).

In this study, specimens of eriophyoids were separately collected for morphological and molecular purposes. Although the extremely monophagous nature of eriophyoid

mites has relatively minimized the risk of coexistence on the same host plant, nondestructive DNA extraction methods should still be preferred to prepare voucher specimens (Castalanelli et al., 2010).

The sequences obtained in the present study are the first molecular data for five eriophyoid mite species, moreover, there were no sequence data of *A. erinea*, *R. ficifoliae*, and *E. pyri* (for 28S) in the public GenBank so far. However, more sequences of specimens belonging to the same or different species are needed to deeply elucidate the genetic variation among eriophyoid mites.

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