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Molecular characterization of phytoseiid mites in Turkey based on the internal transcribed spacer (ITS) region, with a new record for the country

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Abstract
The family Phytoseiidae contains many predatory mite species and some are used in biological control programs worldwide. The identification of phytoseiid mites is based on tiny morphological structures and sometimes species diagnosis is not easy especially for non-taxonomists. DNA-based approaches may offer a fast and accurate diagnosis to overcome these difficulties, nevertheless more DNA sequences are needed to determine intra- and inter-specific variations and to provide accurate decision rules based on genetic distances between the taxa considered. In this study, we provide the molecular characterization of seven phytoseiid species based on the internal transcribed spacer (ITS) region. Several populations of these species collected in Turkey were considered. A phylogenetic tree was also constructed. Finally, we record the presence of *Neoseiulus reductus* (Wainstein) in Turkey.

Keywords Phytoseiidae · Internal transcribed spacer · *Euseius finlandicus* · *Neoseiulus reductus*

Introduction

The family Phytoseiidae includes many predatory mite species, some being commonly used in biological control programs (McMurtry et al. 2013; Tixier 2018). Predatory mites can control pest mites as well as small insects such as whiteflies and thrips (van Lenteren 2012). Phytoseiid mites are reported from all continents except Antarctica (Tixier et al. 2010a; McMurtry et al. 2015) and more than 2500 species have been described so far (Chant and McMurty 2007; Demite et al. 2020).

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Morphological identification of phytoseiid mites is based on tiny morphological structures and seta measurements (Chant and McMurtry 2007). The observation of these structures is sometimes difficult. Futhermore, diagnosis decisions are sometimes difficult to take because of lack of knowledge concerning intraspecific variation for continuous characters (e.g., seta length) (Tixier 2012, 2013). As a consequence, a high level of expertise is often required (Döker et al. 2018) and the existence of cryptic species is difficult to assess (Tixier et al. 2006; Kanouh et al. 2010a; Skoracka et al. 2015). Besides, keys for morphological identifications are only available for adult females that prevent the correct identification of other stages (immatures and males) (Okassa et al. 2012). Diagnosis improvement is thus required especially because the basis of success of biological control is the correct identification of the natural enemies (Rosen 1986).

Along with progress in molecular techniques, DNA-based identification methods have been used to overcome morphological diagnosis difficulties (Hebert et al. 2003; Navajas and Fenton 2000; de Mendonça et al. 2011). However, using molecular analysis in phytoseiid mites is not so easy (Jeyaprakash and Hoy 2002) and there are also some critical views to public GenBank database due to misidentification, contamination and nomenclatural errors/updates (Tixier et al. 2011). To overcome this problem, the combination of molecular and morphological approaches (by DNA extraction of single mites and recovering the carcass of mites after DNA extraction) can be used (Jeyaprakash and Hoy 2010; Tixier et al. 2010b). Another key point for molecular identification is to characterise intra- and inter-specific variation to propose decision rules (Tixier et al. 2017, 2019). The challenge is thus to obtain more sequences for more species and populations collected in different localities. Only two DNA sequences (ITS) of a species of Phytoseiidae from Turkey are present in GenBank (*Kampimodromus ragusai* Swirskii & Amitai; Döker et al. 2018). The objective of this study is to fill this gap, obtaining and analysing the DNA sequences of seven Phytoseiidae species collected in Turkey.

Material and methods

Mites

The specimens were collected from various locations of Turkey in 2019 (Fig. 1). They were placed in plastic vials containing 70 and 100% alcohol for morphological and molecular identification, respectively. Detailed information on collection sites is presented in Table 1. Hoyer's medium was used for the preparation of permanent slides and morphological identification (Zhang 2003). The generic classification of Chant and McMurtry (2007) was used in this paper. The specimen identified as *Neoseiulus reductus* (Wainstein) was measured, and terminology for seta notation follows that of Lindquist and Evans (1965) as adapted by Rowell et al. (1978) for Phytoseiidae. The permanent slides are deposited at Ankara University, Department of Plant Protection, in case of future verification.

DNA extraction

Genomic DNA was extracted from individual female mites using Qiagen DNeasy Blood & Tissue Kits as described by Kanouh et al. (2010b). As the mites were crushed during the DNA extraction process, only samples with more than one mite specimen were herein

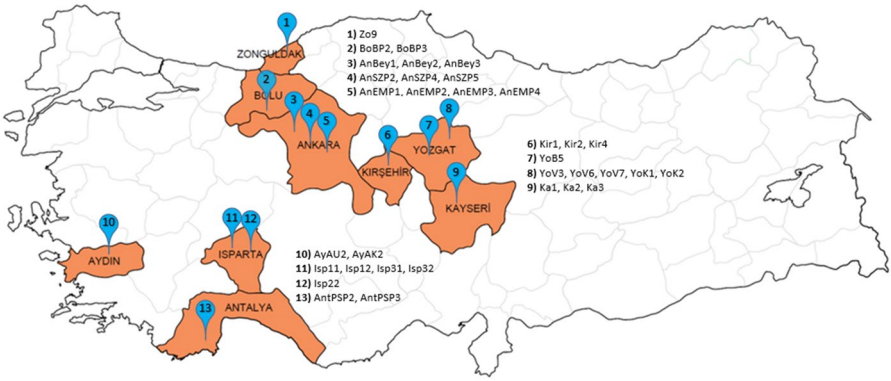

Fig. 1 Map of sampling areas of phytoseiidae species. Numbers indicate the location areas in Table 1

considered (one part being used for morphological identification and the other part for DNA analysis).

DNA fragment considered

Various molecular markers have been used in mite identification so far (e.g., Navajas and Fenton, 2000; Cruickshank 2002; Dabert 2006; Ros and Breeuwer 2007; dos Santos and Tixier 2016). The internal transcribed spacers (ITS1 and ITS2) of the non-coding region of ribosomal DNA (rDNA) have variable sequences and are among the earliest markers used in Acari (Navajas et al. 1992). They have been reported to usually allow the separation of species (Cruikshank 2002; Tixier et al. 2006). In addition, the ITS region has been reported as a reliable marker to investigate Phytoseiidae phylogeny at species and genus level (Navajas et al. 1999; dos Santos and Tixier 2016).

PCR conditions

The primers used to amplify the ribosomal internal transcribed spacer (ITS) region including ITS1, 5.8S and ITS2 regions, were as follows: 5′-3′ AGAGGAAGTAAAAGTCGT AACAAG and 3′-5′ ATATGCTTAAATTCAGGGGG (Navajas et al. 1999). The PCR reaction was performed in a total volume of 30 µl, containing 5 µl of mite DNA, 0.5 µl of both forward and reverse primer, 18 µl of ultrapure nuclease-free water and 6 µl of FIREPol Master Mix (containing reaction buffer, $MgCl_2$ and dNTPs) (Solis Biodyne). DNA amplification was performed with a thermal cycler (BioRad T100) under the following conditions: 4 min at 94 °C, 40 cycles of 60 s at 92 °C, 60 s at 50 °C and 90 s at 72 °C, followed by a final extension for 5 min at 72 °C. Electrophoresis was carried out on a 2% agarose gel in 0.5X TAE buffer during 35 min at 100 V.

Cytochrome oxidase I (COI) gene was also amplified only for *N. reductus* specimens. Primers used for COI amplification were as follows: 5′- GGAGGATTTGGAAATTGATTA GTTCC -3′ and 5′- TACAGCTCCTATAGATAAAAC -3′ (Navajas et al. 1994; Navajas and Boursot 2003). PCR mix and conditions were the same as those mentioned above.

Table 1 Location, host plants and accession numbers of sampled phytoseiid mites

Species—code	Location (number on map, Fig. 1)	Host plant	Accession number
Neoseiulus californicus Isp11–Isp12	Isparta/Senirce (11)	*Malus communis* L	MT436725-26
N. californicus Isp22	Isparta/Eğirdir (12)	*M. communis*	MT436728
N. californicus Isp31–Isp32	Isparta/Çünür (11)	*M. communis*	MT436727, MT436729
N. californicus AyAU2	Aydın/Umurlu (10)	*Phaseolus vulgaris* L.	MT436724
Amblyseius andersoni Zo9	Zonguldak/Centrum (1)	Unknown	MT436719
Amblyseius swirskii AntPSP2–AntPSP3	Antalya/Kumluca (13)	*Citrus sinensis* (L.)	MT436720-21
Kampimodromus aberrans AnSZP2–AnSZP4–AnSZP5	Ankara/Sincan (4)	Unknown	MT436730-32
Phytoseiulus persimilis AyAK2	Aydın/Efeler (10)	*P. vulgaris*	MT436752
Neoseiulus reductus BoBP2–BoBP3	Bolu/Mengen (2)	Unknown	MT436722-23
Euseius finlandicus AnEMP1–AnEMP2–AnEMP3–AnEMP4	Ankara/Eymir (5)	Unknown	MT436742-45
E. finlandicus YoV3–YoV6–YoV7	Yozgat/Aydıncık (8)	*Prunus cerasus* L.	MT436733-35
E. finlandicus YoK1–YoK2	Yozgat/Aydıncık (8)	*Prunus avium* L.	MT436737-38
E. finlandicus YoB5	Yozgat/Centrum (7)	Unknown	MT436736
E. finlandicus Kir1–Kir2–Kir4	Kırşehir/Akçakent (6)	*Juglans regia* L.	MT436746-48
E. finlandicus Ka1–Ka2–Ka3	Kayseri/Talas (9)	*Prunus armeniaca* L.	MT436749-51
E. finlandicus AnBey1–AnBey2–AnBey3	Ankara/Beypazarı (3)	*Solanum lycopersicum* L.	MT436739-41

Data and phylogenetic analysis

The sequences obtained were submitted to the Basic Local Alignment Search Tool (BLAST) in GenBank to check for potential contaminations (other mite families, insects, Human DNA …). Because of high identity level, all specimens herein considered belong to the family Phytoseiidae. Furthermore, as DNA sequences of the seven species are included in GenBank (and mainly deposited by one of the authors of this article), the blast process allowed to assign without any doubts the present DNA sequences to the correct species name.

To build the phylogenetic tree, the sequences of the species herein obtained and some retrieved in the public GenBank database were also used. The GenBank sequences considered were mostly deposited by M-S Tixier, co-author of this paper. The sequences obtained were cleaned using BioEdit v.7.0.5 (Hall 1999) and all sequences aligned using MAFFT v.7 with default settings (Katoh et al. 2019). A Maximum likelihood (ML) phylogenetic analysis was performed with Molecular Evolutionary Genetics Analysis (MEGA v.7) (Kumar et al. 2016) using the Tamura 3 + G model (identified to be the best-fit model in MEGA) (Tamura 1992) with 1,000 bootstraps. The outgroup species is *Galendromus occidentalis* (Nesbitt) a species of the sub-family Typhlodrominae as all the species herein considered belong to the sub-family Amblyseiinae. Genetic distances between the specimens considered were calculated using the K2Parameter model in MEGA7 (Kumar et al. 2016).

Results and discussion

Identification and phylogenetic tree

After alignment, the sequences had a total of 610 nucleotides (including gaps), used for phylogenetic tree construction, and 263 out of the 610 nucleotides were conserved. The aligned sequences had 42.8% GC-content (57.2% AT), on average. 34 ITS sequences were obtained: *Amblyseius andersoni* (Chant) (one specimen), *Amblyseius swirskii* Athias-Henriot (2 specimens), *Euseius finlandicus* (Oudemans) (19 specimens), *Kampimodromus aberrans* (Oudemans) (3 specimens), *Neoseiulus californicus* (McGregor) (6 specimens), *N. reductus* (2 specimens) and *Phytoseiulus persimilis* Athias-Henriot (one specimen). Accession numbers of sequences in GenBank database are included in Table 1.

The phylogenetic tree based on ITS-region is presented in Fig. 2. The phylogenetic tree structure is similar to the ones obtained by Tsolakis et al. (2012) and dos Santos and Tixier (2016, 2018), as almost all sequences are issued from these works. The genus *Euseius* seems to be paraphyletic as it should include the genus *Iphiseius*. The genera *Neoseiulus* and *Amblyseius* seem to be polyphyletic.

All the specimens of the seven species herein considered are included in clades containing specimens of these species, showing a correct assignation and identification using this molecular marker. The genetic distances between the DNA sequences herein obtained and those of specimens of the same species retrieved from GenBank are very low (0–0.77%; Table 2). It is interesting to note that some interspecific genetic distances are sometimes very low especially between *N. fallacis* and *N. californicus* (0.9%) and between *P. persimilis* and *P. macropilis* (0.45%). These values are lower than the maximal intraspecific distance reported in Tixier et al. (2017). However, these

Fig. 2 Phylogenetic tree of phytoseiid mites based on ITS region. Only bootstrap values higher than or equal to 70% are shown. Sequences of the species obtained in this study are indicated with red dots. (color figure online)

Table 2 Mean genetic distances (%) (min–max) between the specimens of a single species and between specimens of different species of a single genus

	No. specimens	Genetic distances between specimens herein considered; mean (min–max)	Genetic distances between specimens herein considered and the ones retrieved in GenBank; mean (min–max)	Interspecific genetic distances between the species herein considered and other species in the same genus; mean (min–max)
Amblyseius andersoni	1	–	0.45 (0.45–0.45)	12.10 (11.05–13.16)
Amblyseius swirskii	2	0	0	15.82 (11.05–20.60)
Euseius finlandicus	19	0.44 (0–0.77)	0.30 (0–0.58)	2.42 (2.14–2.81)
Kampimodromus aberrans	3	0.14 (0–0.22)	0.30 (0.22–0.45)	5.80 (5.25–6.67)
Neoseiulus californicus	6	0	0.05 (0–0.22)	6.61 (1.38–12.76)
Neoseiulus reductus	2	0	0.45 (0.45–0.45)	11.26 (6.32–12.76)
Phytoseiulus persimilis	1	–	0	1.32 (0.62–2.03)

interspecific distances are much higher than the intraspecific distances and no overlap between intra- and interspecific distances is observed, for the four species here mentioned. This may be due to the fact that *N. californicus* and *N. fallacis* on the one hand, and *P. persimilis* and *P. macropilis* on the other hand, are morphologically very close and probably recently divergent species (Kanouh et al. 2010b; Okassa et al. 2010). This result clearly shows the difficulty to use only the marker ITS for molecular identification, and also the difficulty to use only one decision rule for the whole Phytoseiidae family.

Genetic distance between the 19 specimens of *E. finlandicus* herein considered range between 0 to 0.77% (0.44% on average). No structuring of these specimens is observed in the phylogenetic tree because of the very small distances. The pairwise distances within the specimens of the populations from Ankara, Yozgat, Kayseri and Kirsehir were 0.16, 0.42, 0.00 and 0.13%, respectively. The average pairwise distances between specimens from Kirsehir and Yozgat populations were the lowest (0.29%) and those between specimens from Kirsehir and Kayseri were the highest (0.71%). No correlation between genetic distance values and geographic distances is observed (Table 3).

First report of *Neoseiulus reductus* in Turkey

Neoseiulus reductus has been identified morphologically and molecularly for the first time in Turkey. BLAST results of the two *N. reductus* sequences herein obtained (ITS) showed 99.3 and 99.6%, similar to the reference sequence in GenBank (GU966582 posted by Pham and Van der Linden in 2010), confirming the identity of the two specimens collected. A COI sequence (859 bp) has also been deposited in GenBank (accession nr. MT439329). There is no COI sequence in this database, and the Blast analysis only showed that the nearest COI fragment belongs to *P. persimilis* (accession nr. GQ222414).

In addition, re-description of *N. reductus* is provided based on one female (Fig. 3). All measurements are provided in micrometers. Length and width of dorsal shield is 338 and 142, respectively. Measurements of dorsal setae as follows: *j1* 18, *j2* 13, *j4* 13, *j5* 13, *j6* 16, *J2* 20, *J5* 10, *z2* 24, *z4* 24, *z5* 13, *Z1* 19, *Z4* 41, *Z5* 65, *s4* 41, *S2* 41, *S4* 34, *S5* 31, *r3* 29, and *R1* 24. Measurements of ventral setae as follows: *ST1* 10, *ST2* 10, *ST3* 10, *ST4* 10, *ST5* 11, *JV1* 15, *JV1* 16, *JV2* 18, *JV4* 15, *JV5* 40, *ZV1* 13, *ZV2* 18, *ZV3* 10. Length of ventrianal shield is 101; width at level of *ZV2* and anus is 77 and 61, respectively. Length of macrosetae on leg IV basitarsus is 36. Although minor morphological differences are present, the lengths of dorsal setae of the Turkish specimens are quite similar with those of Dutch specimens reported by Miedema (1987).

Table 3 Mean genetic distances (%) (min–max) between the specimens of *Euseius finlandicus* collected in different locations in Turkey

	No. specimens	Ankara	Yozgat	Kırşehir	Kayseri
Ankara	7	–			
Yozgat	6	0.43 (0–0.58)	–		
Kırşehir	3	0.49 (0.19–0.58)	0.29 (0–0.58)	–	
Kayseri	3	0.61 (0.58–0.77)	0.68 (0.58–0.77)	0.71 (0.58–0.77)	–

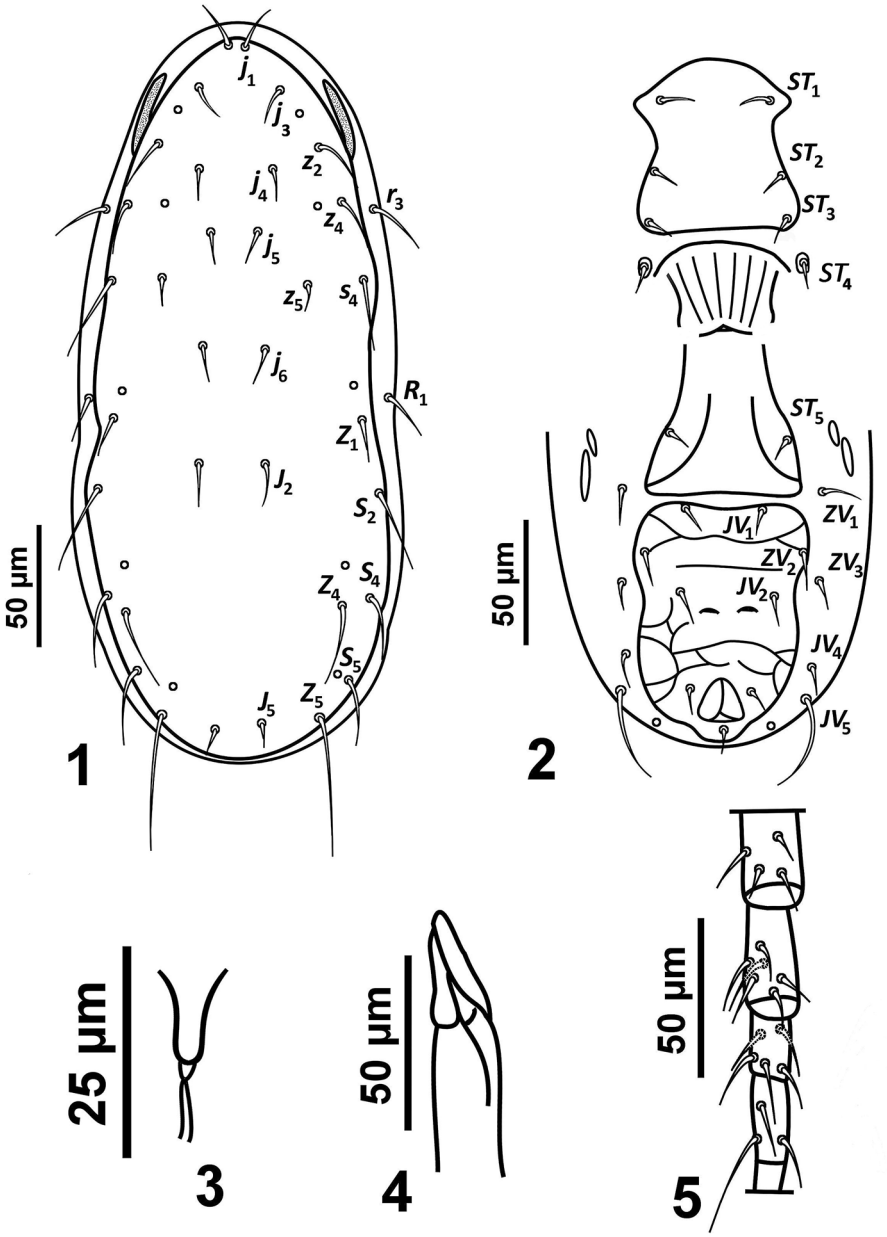

Fig. 3 Female of *Neoseiulus reductus*: 1. dorsal view, 2. ventral view, 3. spermatheca, 4. chelicera, 5. leg IV

Neoseiulus reductus is morphologically very close to *Neoseiulus cucumeris* (Oudemans). Ventrianal shield with one pair of distinct eye-shaped pores in *N. reductus* and punctiform circular pores in *N. cucumeris*. In addition, *J2* and *Zl* are shorter than *S2* in *N. reductus*, whereas both setae are about equal length to *S2* in *N. cucumeris* (Tuovinen 1993).

Conclusion

Accurate identification of phytoseiid mites is essential to design proper pest control programs for agriculture. DNA-based approaches may allow mite identification even by non-experts under two conditions (i) the existence of a complete reference database, and (ii) clear rules for molecular identification. For this, determination of intra- and interspecific variation is essential to reveal the gaps for molecular identification. The present study correctly assigned the specimens to the species they belong to in the phylogenetic tree. However, we confirm here that ITS sequence is not very variable in phytoseiid mites and intra- and interspecific distances are sometimes very close if not overlapping to those of species of different genera (Navajas et al. 1999; dos Santos and Tixier 2016). This clearly shows that a simple and universal decision rule based on distance overlap cannot be applied at family level. More probably, decision rules proposed would have to be different for some genus with more rapid DNA rate evolution (as *Phytoseiulus*) (Kanouh et al. 2010b; Okassa et al. 2010). Mitochondrial DNA fragments seem more appropriate for separating morphologically close species and the study of intraspecific variation (Okassa et al. 2010; dos Santos and Tixier 2016) because of higher genetic distances within and between species.

In the public GenBank database, only one ITS sequence of *N. reductus* was available so far. Now, we provided the sequences of ITS and COI of two specimens that will help species identification. One important issue is the correct morphological identification. It is worth to consider that different species might co-occur in the same plant samples and it should be prefered to use the non-destructive extraction techniques proposed by Jeyaprakash and Hoy (2010) and Tixier et al. (2010b) to check for the identity of specimens after DNA extraction, especially in case of mismatching. In our study, we did not reveal such problems and only used specimens for DNA analyses, when several specimens were present in a same sample. However, in the future, non-destructive DNA extraction techniques should clearly be favored.

The sequences of ITS regions of seven phytoseiid mite species from Turkey were obtained for the first time. Although about 100 phytoseiid species have been recorded for Turkey (Faraji et al. 2011; Döker et al. 2016), we only have the ITS sequences of eight species (Döker et al. 2018; present study) and only one COI sequence in the GenBank database with a limited number of individuals. Therefore, additional studies on DNA-based identification should be performed to both increase our knowledge on molecular variation within and between species for more precise diagnosis and to elucidate the evolutionary history of phytoseiid mites.

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