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Research Article

Delastria, A NEW GENUS OF HYPOGEOUS FUNGI RECORD FOR THE TURKISH MYCOBIOTA

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Abstract: In the present study, fungal samples collected from Enez and Süloğlu districts of Edirne Province, in Türkiye with the help of truffle-detecting dogs were analyzed. The macroscopic features and environmental details of the collection sites were documented in their natural habitats. The samples were investigated with light- and scanning electron microscopy (SEM) and were also used in ITS rDNA-based molecular phylogenetic analysis, which revealed that they belong to *Delastria rosea* Tul. & C. Tul. The identification was further supported by morphological data. This is the first record of *D. rosea* in Türkiye at the genus and species level. A brief description of the newly reported species is provided. Macro- and microphotographs of the spores taken by both ligth and electron microscop (SEM). With this study, the number of genera and species of Turkish truffles and truffle-like fungi has increased to 36 and 105, respectively.

Özet: Bu çalışmada 2022 yılında, Edirne'nin Enez ve Süloğlu ilçerinden trüf dedektör köpeklerin yardımıyla toplanan hipogean fungal örnekler analiz edilmiştir. Örneklerin makroskobik özellikleri ve araştırma alanının ekolojik detayları doğal yaşam alanlarında belgelenmiştir. Örnekler ışık ve taramalı elektron mikroskobuyla araştırılmış, ve aynı zamanda ITS rDNA temelli moleküler filogenetik analiz edilerek *Delastria rosea* Tul. & C. Tul.'a ait oldukları tespit edilmiştir. Bu tanı, morfolojik verilerle de desteklenmiştir. Bu kayıt Türkiye'den *D. rosea*'nın cins ve tür düzeyinde ilk kaydıdır. Yeni kaydedilen türün kısa bir tanımı verilmiştir. Sporların ışık ve taramalı elektron mikroskobu (SEM) ile çekilen fotoğrafları dahil edilmiştir. Bu çalışma ile Türkiye trüf ve tüf benzeri mantarların cins ve tür sayısı sırasıyla 36 ve 105'e yükselmiştir.

Introduction

Delastria Tul., & C. Tul. is a genus of hypogeous fungi belonging to the order *Pezizales* (*Ascomycota*, *Pezizomycetes*). The genus was first described by the Tulasne brothers to honor the French botanist Charles Jean Louis Delastre (Tulasne & Tulasne 1843) and was initially classified under the family *Terfeziaceae* based on morphology, and then in the family *Tuberaceae* by Castellano *et al.* (2004). *Delastria* was represented for almost 200 years with only one single species, *D. rosea*. Paz & Lavoise (2013) described *D. supernova* A. Paz & Lavoise as the second species of the genus. In 2018, Paz *et al.* (2018) published a comprehensive study on the genus, in which they described four additional species, *D. evae* M. Romero, A. Paz & Lavoise, *D. faustiniana* A. Paz, Lavoise & P. Juste, *D. javieri* A. Paz, Lavoise & R. Molina and *D. liebanensis* A. Paz, F. Rodríguez & Lavoise. Early molecular studies on *Delastria rosea* Tul. & C. Tul. demonstrated its connection to both pezizoid and tuberoid lineages within the Pezizaceae family, highlighting the distinctiveness of the *Delastria* genus within this family (Alvarado *et al.* 2011, Paz *et al.* 2018).

Delastria rosea, the type species of the genus, is mainly distinguished by its ascomata encased in an extremely delicate white down, which gradually diminishes to unveil the outer surface of the gleba, displaying a strikingly pink color and spino-reticulate spores (Alvarado *et al.* 2011). The species has been

reported to associate with various host plants, including members of the genera *Pinus* L., *Quercus* L., *Cistus* L., *Helianthemum* Mill., *Tuberaria* L. and *Carpinus* L. Even though the exact distribution of the species remains uncertain, it was predominantly reported in the Mediterranean region (North Africa to Southern Europe) and North America (Alvarado *et al.* 2011, Gómez-Reyes *et al.* 2017, Paz *et al.* 2018).

In the existing literature on Turkish mycobiota (Sesli *et al*. 2020, Akata *et al.* 2022), no records have been reported associated with *Delastria*. In the present study, we report the presence of *D. rosea* in Türkiye for the first time based on the material obtained from Edirne Province.

Materials and Methods

The material included in the study was obtained in March and December 2022 with the help of truffle dogs in field studies in Enez and Süloğlu districts of Edirne Province in northeast Türkiye. The fruiting bodies of *Delastria* specimens found in the field were collected for further analysis. The macroscopic features of the specimens and the environmental details of the collection sites were recorded on-site. The collected material was brought to the laboratory for evaluation. The microscopic structures of the specimens were analyzed using a light microscope (LM) (Euromex Oxion Trinocular) and a scanning electron microscope (SEM) (SEM-Zeiss EVO 40XVP). Thirty measurements were taken for each microscopic structure under the LM at 100× magnification, and the measurement data were statistically analyzed. Melzer's reagent, 5% KOH, Congo red and distilled water were used for analysis. To visualize the specimens under the SEM, pieces of hymenium were embedded in carbon paste and coated with gold particles with an accelerating voltage of 15 kV. The specimens were identified as AKATA&SEN TT 001, AKATA TT 335, and AKATA TT 403 in accordance with Alvarado *et al.* (2011), Gómez-Reyes *et al.* (2017), and Paz *et al.* (2018) and deposited in the Ankara University Herbarium (ANK).

Molecular Phylogeny

The genomic DNA of the samples was extracted using the CTAB DNA extraction method as described in Rogers & Bendich (1994). After the spectrophotometric (Nanodrop Lite Thermo Scientific) assessment of the quality and quantity of the isolated genomic DNAs, they were used as a template for PCR (AppliedBioSystems/MiniAmpPlus) to amplify the nuclear Internal Transcribed Spacer (nrITS) rDNA regions. In these particular PCR amplifications, the ITS1 forward and ITS4 reverse universal oligonucleotide primers were used as previously described (Stielow *et al.* 2015). After the electrophoretic validations of the PCR amplicons as single and sharp bands on an agarose gel, they were purified with the DNA Clean & Concentrator-25 (DCC-25) kit (Zymo Research) and sequenced with Sanger dideoxy sequencing method at Macrogen (Spain).

In molecular phylogenetic analyses, the sequences obtained from the Sanger chromatograms were assembled using The Clustal Omega online alignment tool (EMBL's European Bioinformatics Institute), and NCBI BLASTn was used for determining the similarity indexes of the assembled sequences. Using this search tool, the in-group and the out-group members were determined, and their sequence data were obtained from the NCBI GenBank for the phylogenetic analyses. The assembled sequences and the ITS rDNA sequences of the defined in-group and out-group members were aligned using the ClustalW algorithm of the MEGAX software (Kumar *et al.* 2018). The evolutionary relatedness of the samples with the selected members was predicted from a phylogenetic tree built using the Maximum Likelihood method and Tamura 3 + G + I nucleotide substitution model (Tamura 1992). For the accuracy prediction of the tree branches, the bootstrap method was used by applying 1000 bootstrap replicates (Felsenstein 1985).

Results

The investigated material was presented below with the dates and places of collections, habitat observations, geographic coordinates, and herbarium reference numbers. The morphological characteristics of the species, both macroscopic and microscopic, were detailed. SEM images of the spores were also included to help understanding of the distinct traits of the species,

Macroscopic and microscopic features

Delastria rosea Tul. & C. Tul. (Figs 1, 2).

Ascomata 20-40 mm in diameter, globose to subglobose, lobulate or slightly irregular. **Peridium** whitish to pale yellowish, pubescent surface soil particles and organic materials at first, more or less strong pinkish hue, eventually transitioning to a brownish color upon reaching full maturity. **Gleba** solid, white at first, with rounded fertile areas of bright pinkish spots with numerous white veins joining the peridium at maturity. **Odor** and **taste** not distinctive. **Peridium** extremely thin and easily separable, exhibiting a whitish hue, composed of aseptate thin-walled, branched hyphae up to 11 µm, pigmented near the surface and hyaline, with a slight enlargement towards the gleba. **Gleba's sterile veins** consist of gelatinized hyphae, hyaline, and hooked. **Asci** (90) 110-150 (160) × (35) 4050 (55) µm, hyaline, clavate to reniform, thin-walled, with a short peduncle, 3(-4) spored. **Ascospores** (28) 30-34 (35) µm (without ornamentation), (37) 38-42 (44) µm (with ornamentation), globose, greenish-yellow, with a reticulate pattern, and dimples, upon maturation, dimples develop spines (up to 5 µm) only at their peaks, creating a conical shape with a sharp tip.

Material examined: Türkiye-Edirne, Süloğlu, pine forest, under pine (*Pinus* sp.), 08.03.2022, AKATA & ŞEN TT 001; Türkiye-Edirne, Enez, pine forest, under pine (*Pinus* sp.), 40°38′58.89″K, 26°04′39.00″D, 20 m, 06.12.2022, AKATA TT 335; pine forest, under pine (*Pinus* sp.), 40°39′01.33″K, 26°04′41.32″D, 15 m,10.12.2022, AKATA TT 403.

Distribution: Europe (France and Spain), North Africa (Morocco), and North America (Mexico and The USA) (Tulasne & Tulasne 1843, Harkness 1899, Parks 1921, Gilkey 1954, Gómez-Reyes *et al.* 2017, Alvarado *et al.* 2011, Paz *et al.* 2018, Henkrar *et al.* 2022).

Molecular Phylogeny of Samples

The nrITS rDNA sequences of AKATA&SEN TT 001, AKATA TT 335, and AKATA TT 403 were deposited into the NCBI GenBank under the accession numbers OR223353, OQ955742, and OQ955743, respectively. In regards to the molecular phylogeny analyses of these sequences, by taking the results of the BLASTn searches into account, some nrITS rDNA sequences from members of the genus *Delastria* were selected to represent ingroup members and the nrITS rDNA sequence of *Picoa juniperi* Vittad. (ANK Akata 6991) was chosen to represent the outgroup member. As a result of the molecular phylogenetic analysis, two separate clades emerged apart from the outgroup (Fig. 3). While clade 2 included some distinct isolates of *Delastria rosea* and the specimens AKATA&SEN TT 001, AKATA TT 335, and AKATA TT 403, the clade 1 comprised other species of the genus *Delastria* including *D. evae*, *D. liebanensis*, *D. faustiniana*, *D. supernova*, and *D. javieri*. On the other hand, *P. juniperi* branched separately from the ingroup clades and formed an outgroup as predicted. The BLAST analyses performed with the nuclear ITS rDNA sequences of the samples showed similarity rates above 99% with different isolates of *D. rosea*. The phylogenetic analyses performed further verified the close relatedness of these specimens with *D. rosea* with a high branch bootstrap rate.

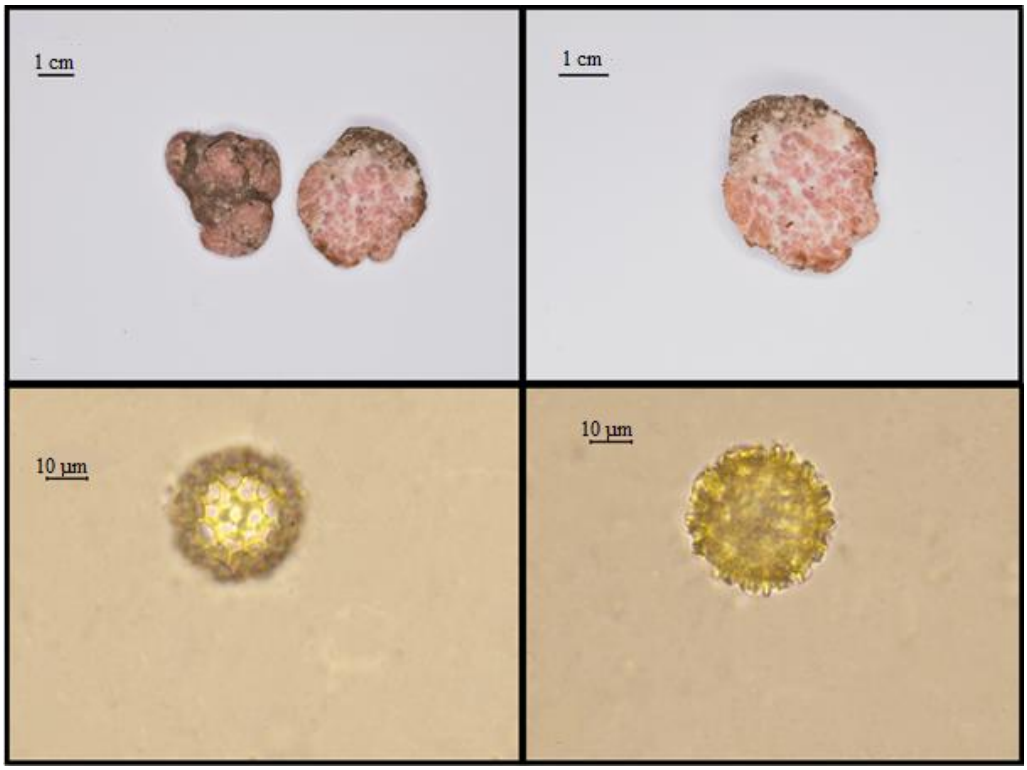

Fig. 1. *Delastria rosea*: **a, b**. ascomata, **c, d.** spores (LM).

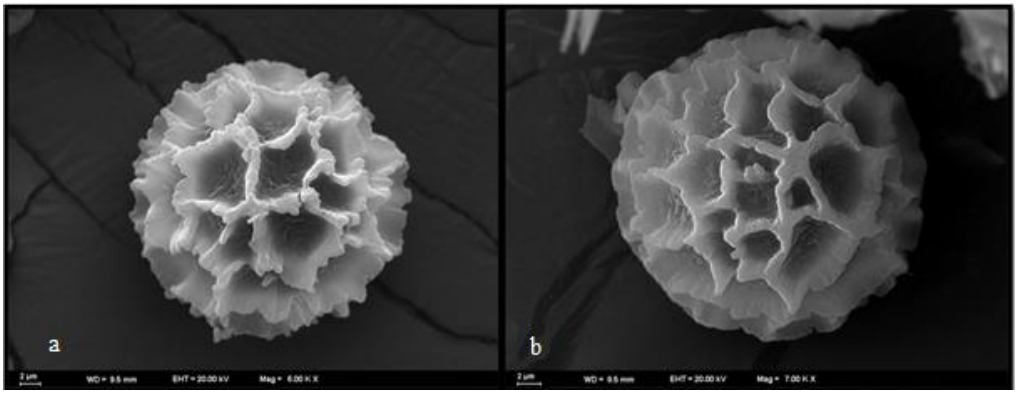

Fig. 2. *Delastria rosea*: **a, b.** spores (SEM).

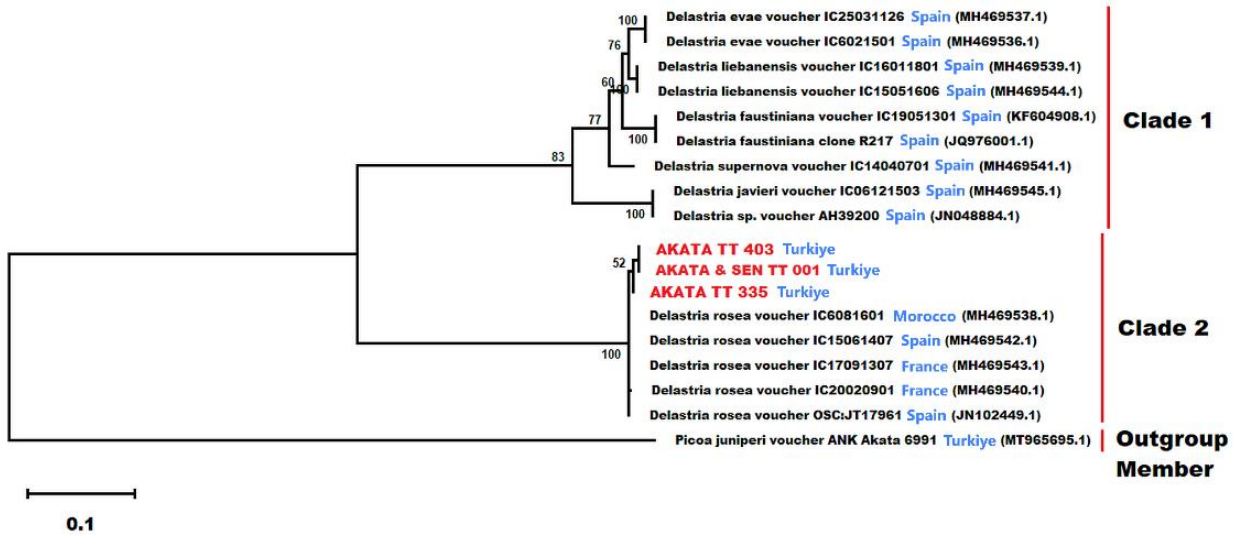

Fig. 3. The ML phylogenetic tree demonstrating the evolutionary relationships of 18 fungal specimens deduced from the nrITS rDNA region. The highest log likelihood value of the tree is -2699.36. Bootstrap rates (≥50) were given for each branch. All of the sequences used to build the tree were retrieved from NCBI GenBank except for AKATA&SEN TT 001, AKATA TT 335, and AKATA TT 403, shown in red. *Picoa juniperi* was used as the outgroup member in the phylogenetic tree. The countries of the specimens, the sequences used to construct the tree were stated in blue color. GenBank accession numbers are provided in parenthesis for each sequence. The scale bar (lower left) represents a genetic distance of 0.1.

Discussion

Delastria rosea is primarily distinguished by its whitish pubescent peridium, vivid pink gleba featuring wide spots between slim white sterile veins, and spherical ascospores ornamented by spines originating from the wall sides of indentations. The collection sites of the known records of the species are connected to sandy and siliceous soils in forests that include various plant species like *Pinus pinaster* Aiton, *P. pinea* L., *P. halepensis* Mill., *Cistus ladanifer* L., *C. monspeliensis* L., *Tuberaria guttata* (L.) Fourr., and *Carpinus caroliniana* Walter (Alvarado *et al.* 2011, Paz & Lavoise 2013, Gómez-Reyes *et al.* 2017, Paz *et al.* 2018, Henkrar & Khabar 2022). There are also a few instances of the species occurrence under *Quercus* and *Helianthemum* (Paz *et al.* 2018).

Delastria supernova, *D. evae*, *D. faustiniana*, *D. javieri* and *D. liebanensis* typically differ from *D. rosea* due to their persistent peridium, which features an encrusted cream-cinnamon pubescence and marbled gleba in compact patches (Paz *et al.* 2018). Additionally, other micromorphological characteristics like the form and dimensions of the asci, ascospores, and the length of the exosporium spines are crucial for differentiating *Delastria* species (Gómez-Reyes *et al.* 2017, Paz & Lavoise 2018). A comparison of ascomata, asci, and ascospore measurements, as well as habitat characteristics for *Delastria* species, can be found in Fig. 1.

In conclusion, we report here *Delastria rosea* as a new generic record for Turkish mycobiota. The genus *Delastria* is the 35th truffle genus reported from Türkiye. Numerous analyses of the growing sequence data related to fungal taxa revealed that the genetic diversity of fungal species far exceeds their morphological diversity. Thus for accurate identifications and phylogenetic inferences of fungal species, it is compulsory to use genetic information along with the morphological data. There are various helpful genetic markers including rRNA gene regions such as nrITS, nrSSU, and nrLSU as well as sequences of protein-coding genes such as beta tubulin (BT2) and translation elongation factor 1-alpha (TEF-1α) are employed for molecular systematics for the last several decades (Raja *et al.* 2017). Among them, nrITS is the most commonly utilized genetic marker for fungal taxa. A considerable amount of nrITS sequence data related to fungal taxa has been accumulated in NCBI GenBank since 1994 (Clark *et al.* 2016). Therefore, this database provides useful guidance for molecular taxonomic studies. Thus, we consulted nrITS rDNA sequence based phylogenetic analyses for the molecular identification of the samples. As a result of the nrITS rDNA-based molecular identifications, more than 99% similarity rates were observed between *D. rosea* and the three specimens (GenBank IDs: OR223353, OQ955742, and OQ955743) (Fig. 3).

Paz *et al.* (2018) postulate that the global presence of the genus *Delastria* is primarily concentrated within a relatively narrow geographical region, with Spain being its epicenter. It highlights the interesting fact that despite the global recognition of the genus, its representation seems to be focused in this area. A limited number of published reports suggest the occurrence of *D. rosea* in the United States (specifically California) and Mexico (Harkness 1899, Gilkey 1954, Gómez-Reyes et al. 2017). However, it's important to note that until the time of these reports, there were no published sequences that belonged to this particular lineage which originated from locations outside of Spain, Portugal, Morocco, Italy, or France.

In the study of Paz *et al.* (2018), a proposed hypothesis suggests that "The current distribution of *Delastria* in the

Western Mediterranean is likely, not artificial and might indicate true regional endemism". The present paper documents instances of *D. rosea* collected in distinct regions within Edirne, located in the European part of Turkey. These findings lead us to reevaluate the notion that *Delastria*'s current distribution in the Western Mediterranean is a reflection of genuine regional endemism. This suggests that the genetic information of this species, as it's known so far, is largely based on specimens from these Mediterranean countries. The lack of published sequences from other geographical areas either indicates the limited spread of this species or a lack of comprehensive studies in those areas. To obtain a more global perspective on the distribution and diversity of *Delastria*, more research and sequencing from other potential habitats, such as those in North America, would be essential.

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Data Sharing Statement: All data are available within the study.

The authors confirm that the data supporting the findings of this study are available within the supplementary material of the article.

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