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Fungal endophytes in the needles of native and exotic pine species in a plantation in Northwestern Türkiye

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Abstract: The endophytic fungi present in needles of *Pinus sylvestris*, *Pinus pinaster*, *Pinus nigra*, *Pinus taeda* and *Pinus radiata* were investigated in Kerpe Research Forest, İzmit in 2016. Ten trees of each pine species were sampled systematically. Previous years green needles were sampled from the lower part of the canopy, from two equally spaced positions around the tree. Each needle was surface sterilized and cut into 0.5 cm sections then individually placed onto malt extract agar plates. DNA was extracted from reprensentative seven isolates and amplified using primers ITS1 and ITS4 targeting the nuclear 5.8S rDNA gene and the two ITS regions flanked between 18S and 28S rDNA genes. Amplicons sequenced in both directions using the universal fungal primers ITS1 and ITS4. Isolations from a total of 1000 needles (200 from each pine species) yielded 750 fungal isolates. *Pestalotiopsis funerea* (Desm.) Steyaert was isolated at the highest frequency followed by *Acremonium* sp., *Cladiosporum* sp. and *Cyclaneusma minus*.
Keywords: Fungal community, Marmara Region, ITS region

Türkiye'nin kuzeybatısındaki bir plantasyonda yerli ve egzotik çam türlerinin ibrelerinde görülen fungal endofitler

Özet: İzmit Kerpe Araştırma Ormanı'nda 2016 yılında *Pinus sylvestris*, *Pinus pinaster*, *Pinus nigra*, *Pinus taeda* ve *Pinus radiata* ibrelerinde bulunan endofitik funguslar araştırılmıştır. Her çam türünden on ağacın tepe tacının alt kısmından sistematik olarak önceki yılın ibreleri örneklenmiştir. Her bir ibre yüzeysel olarak steril edilmiş ve 0,5 cm büyüklüğünde parçalara ayrılmış daha sonra Malt Ekstrakt Agar besi ortamına yerleştirilmiştir. Gelişen izolatlardan seçilen temsili 7 izolattan DNA ekstraksiyonu yapılarak, 5.8S rDNA, 18S ve 28S rDNA genleri arasında yer alan iki ITS bölgesini hedef alan ITS1 ve ITS4 primerleri kullanılarak amplifiye edilmiştir. Amplikonlar, evrensel fungus primerleri kullanılarak her iki yönde dizilenmiştir. Toplam 1000 ibreden (her çam türünden 200 ibre) yapılan izolasyonda 750 fungus izolatı elde edilmiştir. En fazla oranda *Pestalotiopsis funerea* izole edilmiş, bunu *Acremonium* sp., *Cladiosporum* sp. ve *Cyclaneusma minus* takip etmiştir.
Anahtar kelimeler: Fungal topluluk, Marmara Bölgesi, ITS bölgesi

1. Introduction

Many species of fungi are specialized to infect conifers. Some of these fungi are pathogens that cause disease symptoms after relatively short incubation period. Others are endophytic causing quiescent infections (Sieber, 2007). Together, pathogens and endophytes from the microbial communities in conifer tissues. Fungal endophytes defined as organisms that inhabit plant organs or colonize internal plant tissues without causing apparent harm to the host (Petrini, 1991), can significantly affect plant responses to other abiotic and biotic agents or contribute to acquired resistance (Ganley et al., 2004; Rodriguez et al., 2009). Members of classes including Dothideomycetes, Sordariomycetes, Leotiomycetes, Eurotiomycetes and Pezizomycetes are the main classes with endophytic life strategies colonies in most plants (Jumpponen and Jones, 2009).

Most endophytes of conifer needles are filamentous Ascomycota (Petrini, 1991). Some endophytes in the natural flora of plants may become pathogenic due to stress factors that affect the host. Very few endophytes have been identified as weak pathogens, except where the host is affected by physiological stress (Brown et al., 1998). Changes in host or environment, however, may trigger pathogenicity in previously asymptomatic endophytes. Therefore, detection of the fungus is difficult and may take time. As the host or pathogen matures, signs or symptoms of disease appear under the influence of adverse environmental and/ or nutritional conditions (Agrios, 1988; Rojas et al., 2010). A single conifer can host hundreds of fungal species (Arnold et al., 2007). In conifer needles, fungal endophytes grow slowly, but remain viable in the tissues despite having limited host biomass for colonization and nutrition. Thus, restricted infections allow many different types of fungal endophytes to colonize the same needle. Arnold et al. (2007) tested detection of the endophytic fungi in needles of *Pinus taeda* (L.) both by culture and PCR cloning, demonstrated that and the molecular method was better. As the study indicated that many of

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the endophytic fungi could not be isolated from the host plant, it was suggested that greater effort was required to verify their presence with a taxonomic approach (Arnold et al., 2007). In other countries, *Picea abies* (L.) H. Karst, *Pinus mugo* Turra, Giorn., *Pinus sylvestris* L., *Pinus monticola* Douglas ex D. Don., *Pinus nigra* (J.F.) Arnold, *Pinus tabulaeformis* Carr., *Pinus taeda* Carr., *Pinus thunbergii* Parl., and *Pinus densiflora* Siebold & Zucc. have all been examined for the endophytes in a range of plant tissues, including roots, stems, and foliage (Hata et al., 1998; Müller and Hallaksela, 2000; Ganley et al., 2004; Giordano et al., 2009; Arnold et al., 2007; Guo et al., 2008).

The aim of this study was to determine the fungal communities present in needles of endemic and exotic pine species growing in a plantation forest in the Marmara Region of Türkiye.

2. Material and methods

2.1. Material

Needles of endemic *Pinus sylvestris*, *Pinus nigra* and exotic *Pinus pinaster* Ait., *Pinus taeda* and *Pinus radiata* D. Don were collected from twenty years old trees in in İzmit - Kerpe Research Forests (Figure 1). In İzmit-Kerpe, an industrial afforestation with fast growing species was established for research and development purposes within the framework of the project UNDP TUR-71/521 in cooperation with Research Institute for Poplar and Fast-Growing Forest Trees and FAO in 1976 (Ercan, 2002). Soil types in the stands were forest brown earth, light hydromorphic grey-brown podzolic brown earth, and andesite brownstone earth over the rock.

2.2. Methods

2.2.1. Collection of needles from native and exotic pine species

In 2016, twenty previous season green needles were collected from 10 trees of *P. sylvestris*, *P. nigra*, *P. taeda*, *P. radiata*, and *P. pinaster*. In total, 1000 needles (200 from each tree species) were analyzed. Needles were first placed in paper bags which were stored in bigger plastic bags for transfer to the laboratory in cold boxes at 4°C. Isolations were performed within 24 hours, after collection.

2.2.2. Isolation of fungi from needles and morphological identification

Each needle was soaked in 96% ethanol for 1 minute, 6% sodium hypochlorite for 5 minutes, washed with 96% ethanol for 30 seconds and dried on sterile blotting papers. Each needle was divided into pieces and, placed onto 2% (w / v) Malt extract agar (MEA) amended with 100 mg/L streptomycin to suppress bacterial growth and incubated at 20°C for two weeks. The fungal colonies that developed from each fragment were recorded, grouped according to their morphology and re-isolated onto fresh MEA media.

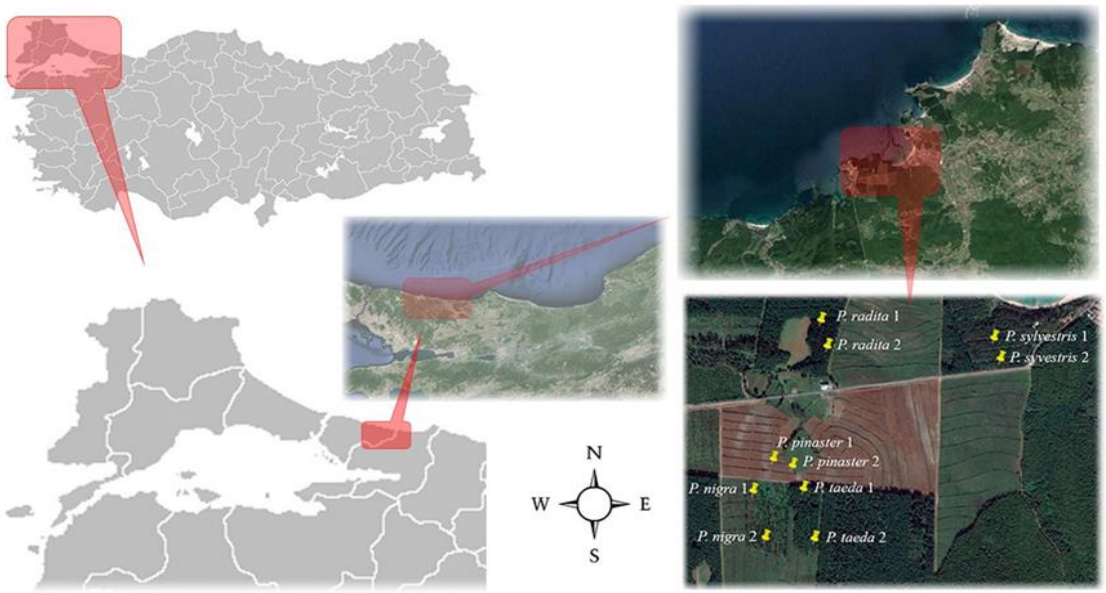

Figure 1. Map of the surveyed areas

2.2.3. Molecular identification

2.2.3.1. DNA extraction and ITS-PCR analysis

Isolates were subcultured to petri dishes containing fresh MEA covered with cellophane membrane and incubated at 21°C for 7 days. Mycelia were ground to fine powder in liquid nitrogen using a mortar and pestle and genomic DNA was extracted using Qiagen DNeasy Plant Mini kit.

PCR amplification of the internal transcribed spacer (ITS) region of the rDNA gene was performed using the primer set ITS1 (5′–TCCGTAGGTGAACCTGCGG–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′; (White et al., 1990) in 50 µl reactions containing 50 ng genomic DNA, 250 nM of each primer, 200 µM of each dNTP, 25 mM MgCl2, 1U Taq polymerase, 1 × Q solution and 1 × PCR buffer (Promega Corporation, Madison, WI, USA). PCR was conducted in a Bio-Rad MJ Mini Personal Thermal Cycler. Conditions were denaturing at 95°C for 1 min, followed by 30 cycles of amplification (20s denaturation at 94°C, 25s annealing at 55°C and 1 min extension at 72°C). The final extension was at 72°C for 10 min. Amplification products were separated by electrophoresis on gels containing 1% (w/v) of agarose (Biobasic Inc., Canada) and length of the products estimated using DNA molecular size markers with 100 bp repeats (Biobasic Inc., Canada). PCR products were sequenced in both directions by IonTek (Istanbul, Türkiye) using the primer set ITS1 and ITS4. Sequences were compared with those in the GenBank database (National Centre for Biotechnology Information, NCBI) using BLAST (Basic Local Alignment Search Tool).

3. Results

Isolations from a total of 1000 needles (200 from each pine species) yielded 750 fungal isolates. Details on fungal colonization are shown in Table 1. The highest numbers of isolates were obtained from the exotic species *P. pinaster* and *P. radiata*, 299 and 159, respectively. The number of fungal colonies obtained from the native *P. nigra* and *P. sylvestris* and the exotic *P. taeda* were 96, 102 and 94. The representative isolates from mainly obtained species sequences were submitted to the Gen bank with accession number (OR527451-OR527452-OR527453-OR527454-OR527455-OR527456-OR527457).

However, species richness (number of species) did not differ greatly among exotic and native trees. Additionally, Shannon and Simpson indices were not significantly different among host species as revealed by t –tests ($P<0.05$).

Pestalotiopsis funerea (Desm.) Steyaert was isolated at the highest frequency in both native and exotic pine species followed by *Cladosporium* sp. *Acremonium* sp. was isolated from native pine species. *Cyclaneusma minus* was present on all pine species except for *P. taeda*.

Lophodermium conigenum was isolated from only native pine species while *C. ferruginosum* was isolated from exotic pine needles except *P. pinaster*. *Eurotium repens* was infrequently isolated from four pine species, the highest percentage being on *P. taeda* (14%).

Frequently isolated Ascomycota were *Alternaria* sp. and *Aspergillus niger*. In addition to these species, other contaminants such as *Mucor* and *Rhizopus* were also abundant in needles of each pine species.

4. Discussion

The discovery of novel endophytic species colonizing various plant organs contributes to our expanding knowledge of these intricate inter-Kingdom relationships. The estimated number of fungal endophyes species ranges from 500,000 and 600,000 with approximately 465,000 of these species yet to be formally described (Botella and Diez, 2011). Despite ongoing research, the roles played by endophytic fungi remain largely enigmatic.

In our study, the diversity of fungal isolates was not pronounced in *Pinus pinaster* and *P. sylvestris* among the pine species examined. However, this richness was relatively modest, a finding consistent with the results of Tokumasu (1978). This phenomenon might be due to morphology and structure of pine needles. Needles of *Pineaceae* are characterized by an enveloping, robust, waxy cuticle, acting as a barrier to water loss and to microbial invasion. The penetration of this cuticl3e is a pivotal step for fungal colonization.

Table 1. Incidence of fungal species isolated from needles of different tree species

Fungal species	Incidence of detected endophyte fungi %				
	Native		Exotic		
	Pinus nigra	*Pinus sylvestris*	*Pinus pinaster*	*Pinus radiata*	*Pinus taeda*
Cenangium ferruginosum (Fr.)	0	5	0	8	9
Cyclaneusma minus (Butin) DiCosmo, Pered &Minter	14	10	3	3	0
Cladosporium sp.	13	12	13	14	19
Colletotrichum gloeosporioides (Penz.) Penz &Sacc	3	2	0	0	9
Acremonium sp.	23	20	20	0	5
Pestalotiopsis funerea (Desm.) Steyaert	16	13	25	34	32
Rhizosphaera	0	2	1	6	0
Lophodermium conigenum (Brunaud) Hilitzer	3	5	0	0	0
Fusarium oxysporum Schlecht Synder &Hansen	2	3	5	4	4
Eurotium repens de Bary Hedwigia	2	9	3	0	14
Aspergillus niger van Tieghem	6	4	10	9	4
Alternaria sp.	6	7	14	12	2
Contaminants	12	8	6	10	2

Some studies on endophytes have focused on host-specific fungal colonization (Petrini,, 1990; Lehtijärvi and Barklund 1999;; Collado et al., 2000; El-Morsy et al., 2006). In addition to these studies, changes in the number of endophyte species from a particular tree species or depending on environmental conditions in relation to the season are frequently discussed (Collado et al., 1999; Suryanarayanan et al., 2002; Martín et al., 2006). Kowalski and Zych (2002) reported that the species composition varied depending on the position of the needle in the crown, as the top of the tree is subject to sunshine and wind more than the lower part. Zamora et al. (2008) obtained 45 fungal species in isolations made from healthy and necrotic needles from 40-50 years old *P. nigra*, *P. pinaster*, *P. sylvestris* and *P. uncinata* trees. Fungal species did not differ based on plantation sites and pine species, but they were differences in needle sampling, in terms of the season in which they were taken and the isolation method. The number of taxa recorded in this study is similar to fungal communities reported from coniferous hosts growing in temperate climates elsewhere (Bills 1996; Collado et al., 1999; 2000; Danti et al., 2002; Santamaría and Diez, 2005; Göre and Bucak, 2007).

In the wok reported here, *Cyclaneusma minus*, *Cenangium ferruginosum* and *Clodiosporum* sp. appeared to be dominant fungi based on culturing in all pine species. *C. ferruginosum,* isolated from *P. radiata*, *P. taeda* and *P. sylvestris* needles, is known as a common opportunistic fungus that kills the cambium of the bark and branches and shoots of trees weakened as a result of pests or adverse environmental conditions (Sinclair et al., 1987; Jurc et al., 2000). However, Santamaria et al. (2006) found that *C. ferruginosum* does not cause damage on most pine tissues.

Many Leotiomycetes are considered important as endophytes within gymnosperms (Berbee and Taylor, 2001; Scheneider et al., 2004). In our study, *L. conigenum* was an endophyte in*Pinus* sp. and specifically of *Pinus nigra* and *P. sylvestris*. However, it is possible that this fungal species is restricted to native *Pinus* species in Türkiye as it was not isolated from the exotic species. *Lophodermium conigenum* was rare in needles of native pine species in the study area. While *L. conigenum* was a common fungus on senescent needles of *P. sylvestris* (e.g. Minter and Millar, 1980) it may not be so common as an endophyte, neither in symptomatic nor in healthy looking needles (e.g. Kowalski, 1982; 1993). *Lophodermium* Chevall., a genus in the Rhytismataceae, includes over 20 species commonly isolated from needles of coniferous trees and shrubs, the majority of which can survive endophytically in green needles without causing any damage to the hosts (Sinclair et al., 1987; Kirk et al., 2001). It is known that the pathogenic species of this genus on pines is *L. seditiosum* (Minter and Millar, 1980). In our country, the existence of 4 different species of *Lophodermium* has been reported which include *L. seditiosum* Minter, Staley & Millar, *L. nervisequium* (DC.) Chevall, *L. conigenum* (Brunaud) Hilitzer and *L. pinastri* (Schrad.) Chevall (Lehtijärvi et al., 2010).

In this study, *P. funerea* was isolated from green needles of all pine species. It is already known that several *Pestalotiopsis* species may have endoph ytic and pathogenic stages in their life cycle (Wei et al., 2007). *Rhizopus stolonifer*, *Mucor hiemalis*, *Trichothecium roseum*, and *Penicillium* spp. are saprophytes and rarely found as endophytes in healthy plant tissues; they have been isolated from green, healthy, and surface-sterilized needles, possibly as a form of contamination. No clear conclusions might be drawn regarding any of the fungi detected in this study and their relationship to tree health.

In conclusion, this is the first report of endophytic fungi in needles of native and exotic pine species planted in Türkiye. This will provide a comprehensive assessment of the endophytic mycobiota of *Pinus* sp. in this country. It contributes to comprehensive assessment of the endophytic mycobiota of *Pinus* sp. in this country. The evaluation and understanding of the ecology of endophytic fungal biodiversity in Türkiye is still in progress. This study was carried out to determine fungal endophyte diversity by isolation from needles. Further works needs to be planned to use high throughput sequencing methods to identify the diversity of fungal endophytes in needles of the same host species.

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