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Research article/Araştırma makalesi

Mycorrhizal diversity in some species of *Dactylorhiza* genus (Orchidaceae)

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Abstract

The research was designed to identify the mycorrhizal fungi of four of eleven species of *Dactylorhiza* in Turkey. To isolate and identify of mycorrhizal fungi of *Dactylorhiza euxina* var *euxina, D. osmanica* var. *osmanica* (endemic), *D. romana* subsp. *romana* and *D. urvilleana,* the roots of them were collected from three habitats of Black sea region. In additon to, to determine the relationship between color polymorphysim flowers of *D. romana* subsp. *romana* and mycorrhizal diversity*,* the isolations were done from the roots of the pink and yellow flowering individuals of *D. romana* subsp. *romana* separately. The isolates were characterized via morphological and molecular methods. For molecular identification, the fungal DNA were extracted from purified fungal culture and fungal ITS regions were PCR amplified using the primer pairs ITS1 and ITS4 and sequenced. Phylogenetic analysis revealed that the majority of *Dactylorhiza* mycorrhizal fungi are Tulasnellaceae and Ceratobasidiaceae belonging to Basidiomycota. Interestingly, *Verpa conica verpa*, a member of Morchellaceae, in individual pink flowering plants of *D. romana* subsp. *romana* was also found. *Tulasnella bifrons* were shared by plants of *D.euxina* var. *euxina, D. osmanica* var. *osmanica* and *D. urvilleana.* Fungal partners of *Dactylorhiza* species in wet meadows belong to Tulasnellaceae, those of under *Quercus* scrubs and on the wet forest margins are Ceratobasidiaceae, Morchellaceae and Tulasnellaceae, respectively. These results may indicate that fungal partner(s) of orchids may vary depending on habitat.

Key words: *Orchidaceae, Dactylorhiza, fungal diversity, mycorrhiza*

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***Dactylorhiza* cinsine(Orchidaceae) dahil bazı türlerin mikorizal çeşitliliği**

Özet

Bu araştırma, Türkiye'deki onbir *Dactylorhiza* türünden dördünün mikorizal çeşitliliğini belirlemek için yapılmıştır. *Dactylorhiza euxina* var *euxina, D. osmanica* var. *osmanica* (endemic), *D. romana* subsp. *romana* ve *D. urvilleana* türlerinin mikorizal funguslarını izole etmek ve tanımlamak için, bu türlerin kökleri Karadeniz Bölgesinde üç farklı habitatdan toplanmıştır. Buna ilave olarak, *D. romana* subsp. *romana*'nın çiçeklerindeki renk polimorfizmi ve fungal çeşitlilik arasındaki ilişkiyi belirlemek için sarı ve pembe çiçekli bireylerin köklerinden ayrı ayrı izolasyonlar yapılmıştır. İzolatlar morfolojik ve moleküler yöntemlerle tanımlanmıştır. Moleküler tanımlama için, saf fungus kültüründen DNA ekstrakte edildi ve fungal ITS bölgeleri, ITS1 ve ITS 4 primer zincirleri kullanılarak PCR ile çoğaltılıp sekansları yapılmıştır. Filogenetik analizler, *Dactylorhiza* türlerinin mikorizal funguslarının çoğunluğunun Basidiomycota'ya ait Tulasnellaceae ve Ceratobasidiaceae familyalarından olduğunu ortaya koydu. İlginç olarak, *D. romana* subsp. *romana*'nın pembe çiçekli bireylerinin köklerinde Morchellaceae familyasına ait *Verpa conica verpa* bulundu. *Tulasnella bifrons, D.euxina* var. *euxina, D. osmanica* var. *osmanica* ve *D. urvilleana* türlerinin köklerinde ortak olarak bulunmuştur. Bu araştırmanın sonuçlarına göre, nemli çayırlardaki *Dactylorhiza* türlerinin fungal partnerlerinin *Tulasnella*'ya, meşe ormanı altındaki ve nemli orman kenarlarındakilerin *Ceratobasidiaceae, Morchellaceae ve Tulasnellaceae* 'ye ait oldukları belirlendi. Bu sonuçlar, orkidelerin fungal partnerlerinin habitata bağlı olarak değişebileceğini işaret etmektedir.

Anahtar kelimeler: *: Orchidaceae, Dactylorhiza, fungus çeşitliliği, mikoriza*

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1. Introduction

The Orchidaceae is one of the largest flowering plant families and they have very different life strategies such as epiphytic, saprophytic, terrestrial (Dressler,1981; McCormick et al., 2004). It is known that mycorrhizal colonization is very important for both the life cycle of adult plants and understanding of the evolutionary history of orchids and seed germination (Rasmussen,1995; Rasmussen and Whigham, 2002; Rasmussen and Rasmussen 2009; Rasmussen and Rasmussen 2014). Little is known about what role fungal diversity plays in affecting an orchid's distribution, population size, and genetic diversity of the fungal association (McCormick et al.,2004). The fungi of orchid mycorrhiza have been largely included teleomorph genus such as *Ceratobasidium*, *Sebacina*, *Tulasnella* and *Thanatephorus* belonging to Basidiomycetes (Currah et al., 1997; Rasmussen,2002; Zettler et al., 2004; Dearnaley,2007). Mycorrhizal preference of orchids is variable (Xing et al.,2015); nonphotosynthetic orchids have very specific associations including saprophytic or ectomycorrhizal fungi (Dearnaley et al., 2012) while in photosynthetic orchids, the level of specificity varies considerably (McCormick et al., 2004; Waud et al., 2016). Therefore, the available of appropriate fungal partner (or partners) may determine habitat and geographic distribution of orchid plants (McCormick et al., 2009; Illyeset al., 2010; Jacquemyn et al.,2011; Jacqumyn et al., 2012b; Jacquemyn et al.,2014; Xing et al.,2015). The appropriate mycorrhizal fungus is a requirement for the orchid seed germination, as the seeds do not have the endosperm. The mycorrhizal fungi support nutrition for the seed germination and early development (Rasmussen,1995). More knowledge about orchid-fungal diversity in the same or different habitats will be very important and useful for orchid seed germination and propagation (Xing et al.,2015).

Turkey has a high species richness of Orchidaceae. There are about 200 species that belong to 24 genera in Turkey. According to the most recent data, there are 11 species of *Dactylorhiza* in Turkey (Guner, 2012). Their tubers are used for ice-cream and "salep" (as a Turkish drink) and as medicinal materials. Therefore, millions of tubers arecollected every year. All orchid species of Turkey are under serious threat of extinction due to over-collection and environmental destruction (Türkiş and Ertürk, 2015). To conserve surviving of wild orchid populations (*Dactylorhiza* and the others) and reintroduce the plants into their habitat require understanding mycorrhizal partner of each orchid species. But no research has been done related to the identification of orchid mycorrhizal fungi by using molecular techniques and diversity and distribution of the fungi in the same or different ecosystems of Turkey. We considered that the relationship of mycorrhizal diversity of orchids in the different habitats (wet meadow, *Quercus* shrubs or wet forest marjins) of Turkey should be well-known to produce the orchids in natural conditions from the seeds. For this reason, we designed this research. The orchids of the research are four *Dactylorhiza* species. Some of *Dactylorhiza* species and the others grow in forest and meadow ecosystems in Turkey, respectively. The roots of *Dactylorhiza romana* subsp. *romana* (habitat1) and *D. urvilleana* (habitat2) were collected at an altitude of 50m from Quercus scrub and at an altitude of 900m from wet forest margins, respectively. *D. euxina var euxina* and *D. osmanica var. osmanica* (habitat3) were collected at an altitude of 2350m from the meadow of the plateau of Black Sea Region of Turkey.

The distance between habitat 2 and 3 is 40km, habitat 1 is about 400-450km away from both of 2 and 3. With the completion of this research, the diversity of mycorrhizal fungi of some *Dactylorhiza* species in Turkey and the relationship between habitats of *Dactylorhiza* species and their mycorrhizal fungi will be well-known.

2. Materials and methods

1.1. Habitats and Plant Collection

The roots of *Dactylorhiza urvilleana (habitat2), D. euxina, D. osmanica* var. *osmanica* (endemic) *(habitat3)* were collected from East Black Sea Region in Turkey, the roots of *D. romana* subsp *romana* at flowering stage were from middle Black sea. *Dactylorhiza romana* subsp. *romana* have both purple and white flowers (habitat1). The roots of white and purple flowering plants were collected separately to determine the relationship between color polymorphism and mycorrhizal diversity. To avoid damaging the plants, we carefully hollowed around the individuals and one or two root parts collected from per individual plant.

Habitat1: Samsun/Turkey: ***D. romana* subsp. *romana***
Habitat2: Köprübaşı-Trabzon/Turkey: ***D. urvilleana*** (950m)
Habitat3: Ismail Aga Plato-Köprübaşı/Trabzon/Turkey: ***D. euxina* and *D. osmanica* var. *osmanica*** (2300m)

1.2. Microscopical observations for fungal pelotons, fungus isolation and morphological analysis

2.2.1 Microscopical observation

The roots were washed with tap water and then the cross sections were taken from the roots with a razor. The fungal colonizations were confirmed with a light microscope (Leica).

2.2.2 Fungus isolation

After microscopical observations, the roots parts containing fungal pelotons were surface sterilized with 1.5% NaOCl for 2-3 minutes and then washed with sterile distilled water for three times. The roots parts were sectioned in

aseptic conditions and the sections were placed in fungus isolation medium in the Petri dishes Clements et al., 1985). Fungus isolation medium was prepared according to Clements et al., 1985. The Petri dishes were covered with aluminum foil and incubated at 25 ºC (±2 ºC) in the dark in an incubator for 2 weeks.

2.2.3. Morphological analysi

The fungi growing on Potato Dextrose Agar (PDA, Merck) were examined for colony type and color, sclerotium color, nucleus number and hyphe diameter with Leica microscope. For preidentification, hyphal features and nuclear condition of the isolates were examined as explained in Karaca et al., 2002 after stained with Safranin O and 3% KOH (Bandoni, 1979). The colony and sclerotium colours were determined according to Royal Horticultural Society (RHS) Colour Chart (1995).

2.3. Molecular phylogeny

2.3.1 DNA extraction, amplification, sequencing

Genomic DNA (gDNA) was extracted from each piece of root according to Doyle (1991) protocol with some modifications. Firstly, 50 mg of fungal tissue was grounded to powder under liquid nitrogen using a mortar and pestle. The powder mycelium was transferred into a 1.5-µL centrifugation tube containing cetyltrimethyl ammonium bromide (CTAB) buffer (500 µL, 100 mM Tris-HCl, pH 8,0, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, and 0.1% b-mercaptoethanol). The lysis mixture was incubated at 65°C for 60 min and the content of the tubes was mixed by turning the tubes every 10 min. At room temperature, the solution was extracted twice with chloroform: isoamyl alcohol (24: 1) by centrifugation at 12.000rpm. The supernatant was precipitated with 0.6 volumes of ice–cold ethanol. The precipitate was then collected by centrifugation at 12000rpm. The pellet was washed with 70% ethanol and air dried. Finally, the DNA pellet was dissolved in 25 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at –20◦C prior to use. Amplification of the rDNA-ITS region was done with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR procedure of Pascual et al.,(2000) was used to amplify rDNA-ITS intergenic spacer loci with an MWG Primus thermal cycler. PCR amplifications were carried out in a total reaction volume of 50 µL, containing approx. 50 ng gDNA as the template, 50 pmol of each primer, 2.5 mM dNTP mix (Ambresco USA), 1U Tag polymerase (Promega, USA), 1.5 $MgCl_2$, 1X PCR buffer(Promega, USA) and dd H_2O. An MWG Primus thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C at 1mins, 2 min at 49°C, 72°C for 3 min and the final extension at 72°C for 7 min. The PCR product were electrophoresed on 1% agarose gel (Amresco, USA) prepared in 1X TBE (Tris-Borate- EDTA) buffer. And finally the PCR product was stained with Gel Red(Biotium), gels were visualized with the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK).

2.3.2. Phylogenetic analysis

The rDNA-ITS regions of the isolates were sequenced in both directions by Macrogen (Korea) with using ABI 3730 XL sequencer. The sequences were checked and edited using the program SeqMan II module of the LASERGENE 99 system (Applied Biosystem) and aligned with CLUSTAL X(Thomson etal.,1997) then optimized by hand. Firstly, the identity of isolates was determined by making BLAST search. Later, data sets were analyzed using the package program MrBayes version 3.2 (Ronquist et al.,2012). Bayesian analyses were run for 10 million generations, a sampling frequency of 1000 and a burn-in of 20%.

3. Results

1.1. Fungus isolation and morphological characterization

Mycorrhizal colonization was observed in cortical cells in the cross sections of the roots of all of *Dactylorhiza* species in the research (Figure 1).

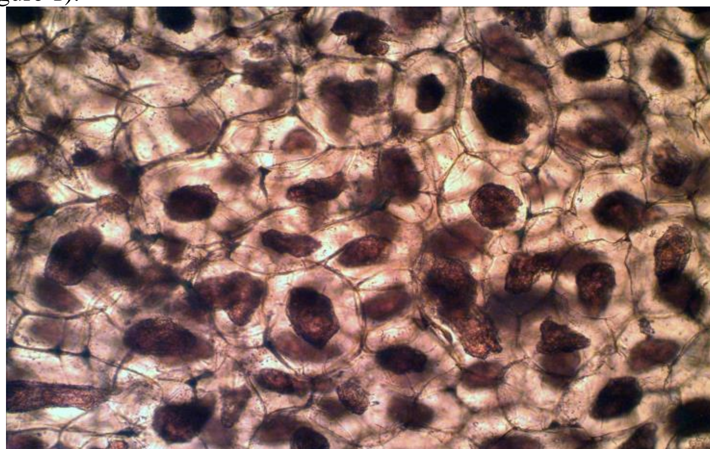

Figure 1: Mycorrhizal colonization in the cortical cells of *Dactylorhiza* roots

The fungal hyphae growing in the medium was purified by being transferred to the fresh fungus isolation medium. Then the purified fungi were grown on the PDA for morphological analysis (hyphae diameter, grow rate, colony features) and stocked on the PDA at 4ºC.

Eight fungi were isolated from the roots of three adult plants of each *Dactylorhiza* species, totally.

In all the *Dactylorhiza* species investigated, eight *Rhizoctonia*-like isolates and one *Verpa* spp. were obtained and morphological characteristics of them were given in Table 1 and Figure 2.

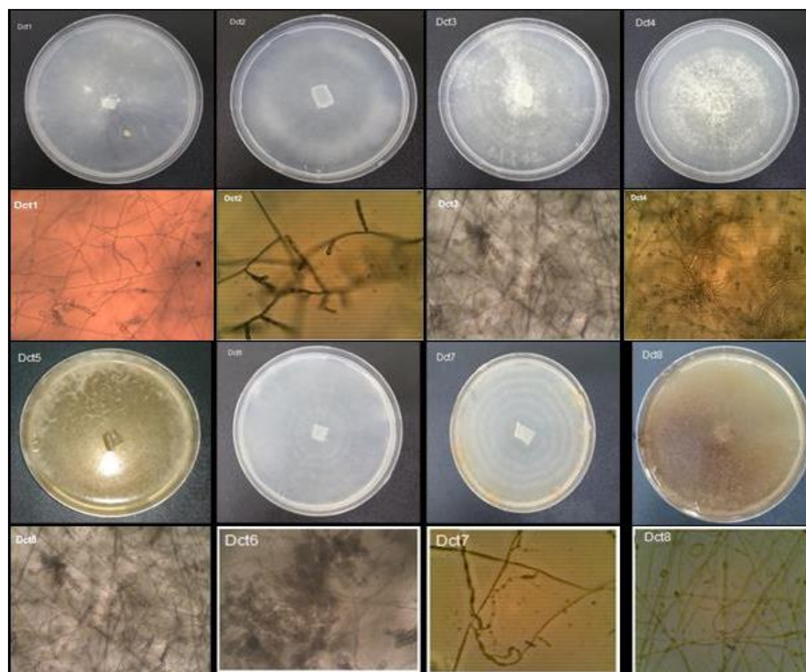

Figure 2: Colony and hyphal features of the isolated fungi

Dct1 isolate (from *D. osmanica* subsp. *osmanica*) formed the colony with aerial hyphae and colony white, hyphal diameters was 3.86±1,02µm. Colony colors of Dct2 (from *D.euxina* var. *euxina*) and Dct3 (*D. urvilleana*) isolates were white with submerged hyphae. The hyphal diameters of them were 4,3±1,26µm and 4,91±0,59µm, respectively. Dct4 formed the colony with aerial hyphae and sclerotium. Colony and sclerotium colors were gray-white, (colony and sclerotium colors were the same) and hyphae diameter 5,12±0,60 µm. Colony type, colony, and sclerotium colors, hyphae diameter of Dct5 (from *D. romana* subsp. *romana*, pink flowers) were with aerial hyphae, gray orange, brown and 4,68±0,47µm, respectively. Dct6 (from *D. romana* subsp. *romana* white flowers) was grey-white with submerged hyphae, no sclerotium, and 5,01±0,34µm in diameter. Dct7 (from *D. romana* subsp. *romana,* white flowers) formed grey-white colony and sclerotium, hyphal colony and hyphal diameter were aerial and 5,43±0,35µm, respectively. Finally, Dct8 was were isolated from *D. romana* subsp. *romana* (pink flower). The fungus had a gray-orange colony with aerial hyphae and sclerotium, hyphae diameter was 9,21± 2,24µm, nucleus number was 20.

1.2. Molecular characterization

DNA was extracted from fungal culturesand PCR amplification was carried out using the standard set of primers ITS1-ITS4. Amplification of the corresponding internal transcribed spacer region gave rise to amplicons whose electrophoretic profiles displayed bands from 500 to 800 bp. Representative cases were sequenced and the results were examined by BLAST against the GenBank database. Seven of the obtained sequences corresponded to Basidiomycetes and one of them Ascomycetes sequences. We found that three of the isolates, five isolates, and one isolate belonged to Tulasnellaceae, Ceratobasidiaceae, and Morchellaceae, respectively. Results of the BLAST analysis were shown in Table 2. The sequences were compared with known sequences in the Gen Bank using BLASTN. Based on a 95% cutt off value of similarity between sequences. Phylogenetic relationships based on the individual datasets were inferred from Bayesian inference (BI) analyses. BI analyses of individual marker and datasets were performed in MrBayes v.3.1.2.. The best fitting model of evolution was chosen for ITS marker using ModelTest v.3.7 The evolutionary models selected by the Akaike information criteria (AIC) estimator were GTR+I+G for ITS. On the tree, Tulasnellaceae family species grouped in three main clades. Tulasnellaceae members constituted the ITS-5.8S rDNA sequence similarities between 93% and 99%. Sequences of the isolates Dct1,Dct2, Dct3 shared high homology with those of uncultured Tulasnellaceae (Table 2). *Additionally,* data sets were formed for *Tulasnella* spp sequences using phylogenetic algorithms such as Mr. Bayesian. The ITS sequences of the several strains exhibited 100% identity. The group consists

of 36 sequences, shown by sequence similarity analysis to be related to the *Tulasnellaceae* family. In the Mr. Bayesian tree of *Tulasnellaceae*, there were three major lineages supported with a bootstrap value of 91 %. Dct 1, Dct 2, Dct 3 sequences placed in the clade with *Tulasnella bifrons.* Dct 1, Dct 2, Dct 3 do not associate closely with *Tulasnella bifrons* but they were closer to this species than the other species presented in the database (Figure 3).

The sequences of Dct4, Dct5 and Dct 6 showed high sequence identity (99, 100, 99%, respectively) to uncultured Ceratobasidiaceae. Sequences of the isolates Dct7 showed high sequence identity (100 %) uncultured Ceratobasidiaceae. The biggest group consists of 38 sequences, shown by sequence similarity analysis to be related to the *Ceratobasidiaceae* family. Mr. Bayesian analyses generated three lineages with a bootstrap value of 100 %. Uncultured Ceratobasidiaceae (Dct4, Dct5) were clustered in clade II, and also Dct6 were in clade III with *Ceratobasidium albasitensis* (Figure 4).

Finally, The sequences of Dct8 revealed high identity 100% of those of *Verpa conica verpa* (Morchellaceae-Ascomycota) in GenBank (accession numbers AF008230). The smallest group consists of 13 sequences, shown by sequence similarity analysis to be related to the *Morchellaceae* family. There were two major linages supported with a bootstrap value of 100%.The 712 bp Dct 8 sequence placed in a clade with *Verpa conica*. On the tree, Dct 8 from *Dactylorhiza romana* subsp. *romana* (pink flower) showed the close relation with *Verpa conica* (GenBank accession AF008230) with 100% nucleotide sequence similarity (Figure 5).

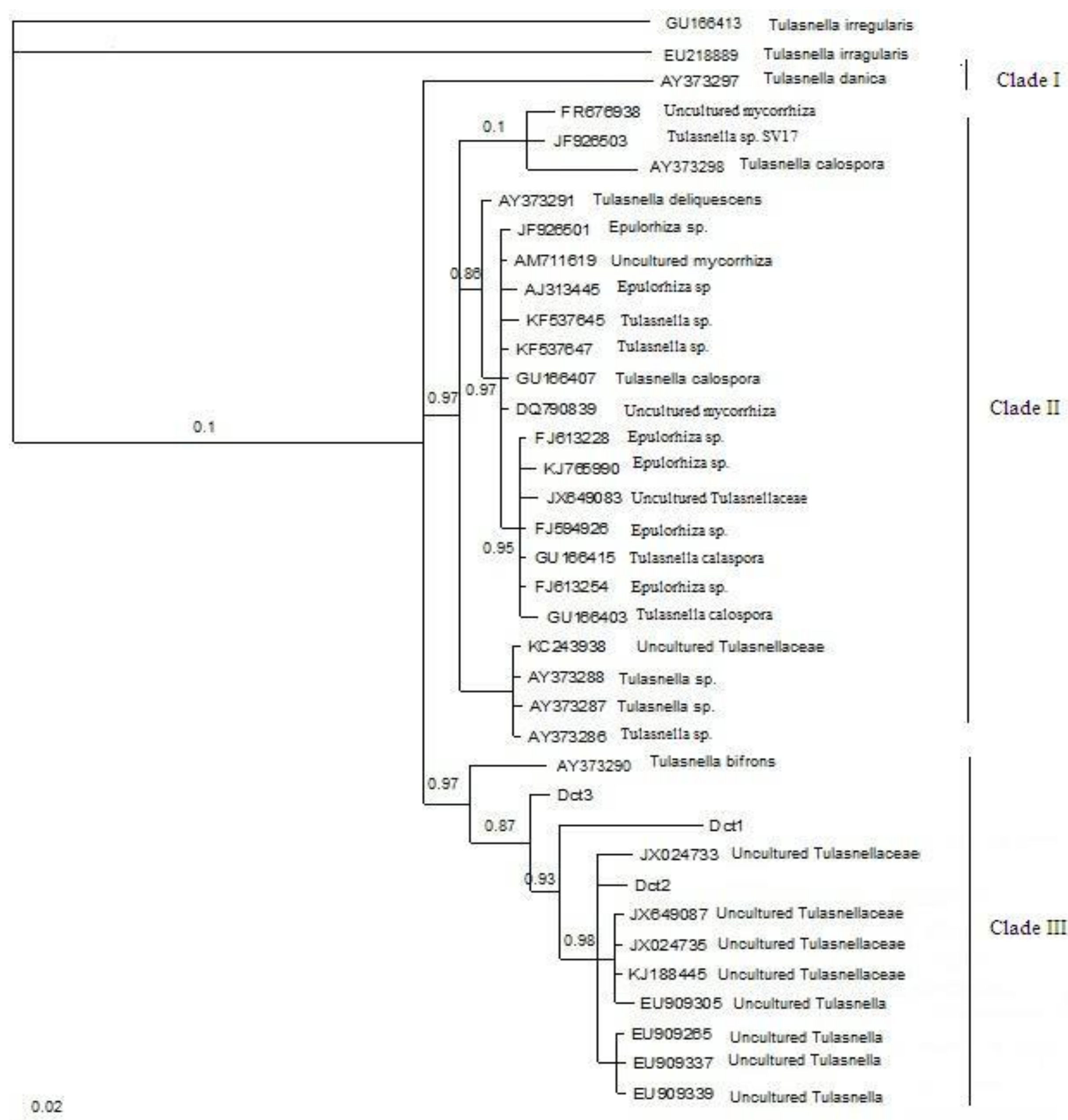

Figure 3: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Basidiomycota (Tulasnellaceae) ITS sequences

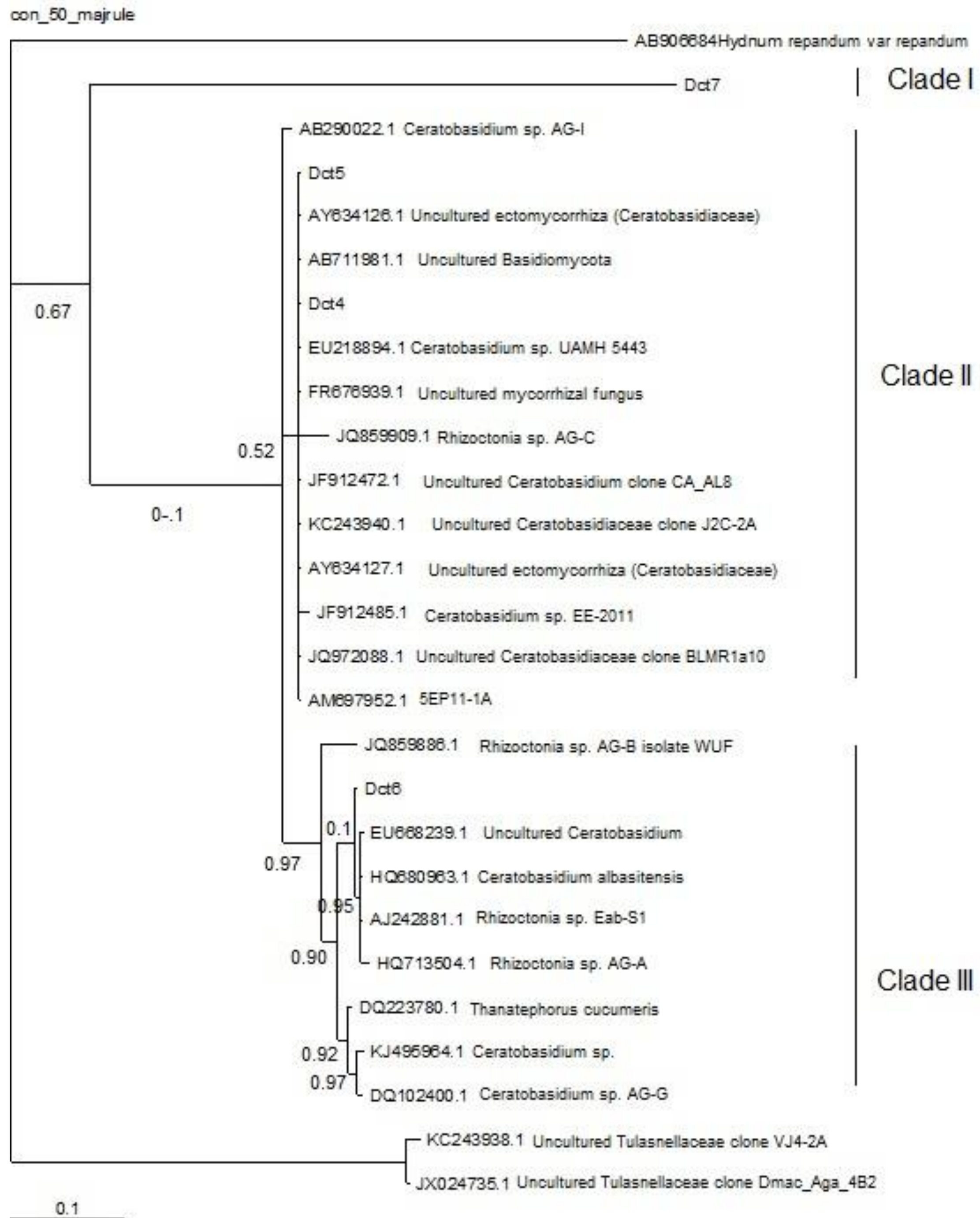

Figure 4: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Basidiomycota (Ceratobasidiaceae) ITS sequences

Table1: Summary of cultural, morphological and molecular characteristics of the fungal isolates

Isolate	**Host**	**Colony type**	**Hyphal diameter (µm)**	**Colony color**	**Sclerotium color**	**Number of nucleus**	**GenBank Accession code**	**Length(bp)**	**Sequence Identity**	**Phylogenetic relationship[a] Closest match in GenBank**
Dct 1	*D.osmanica* var. *osmanica*	Aerial	3,86±1,02	White	Absent	2	(JX024735)	543	93	Uncultured Tulasnellaceae clone Dmac_Aga_4B2 from *Dactylorhiza* sp(*D. fuchsii, D. incarnata, D. maculata, D. majalis and D. praetermissa*)
Dct 2	*D.euxina* var. *euxina*	Submerged	4,3±1,26	White	Absent	2	(KC243938)	640	99	Uncultured Tulasnellaceae clone VJ4-2A from Gymnadenia conopsea
Dct 3	*D. urvilleana*	Submerged	4,91±0,59	White	Absent	2	(EU909346)	644	99	Uncultured Tulasnella mycobiont of Aneura pinguis
Dct 4	*D.urvilleana*	Aerial	5,12±0,60	Gray-white	Gray-white	2	(KC243940)	661	99	Uncultured Ceratobasidiaceae from Gymnadenia conopsea
Dct5	*D.romana* subsp *romana*(pink flower)	Aerial	4,68±0,47	Gray-orange	Brown	2	(KC243940)	554	100	Uncultured Ceratobasidiaceae from Gymnadenia conopsea
Dct 6	*D.romana* subsp *romana* (white flower)	Submerged	5,01±0,34	Gray-white	Absent	2	(JX649076)	649	99	Uncultured Ceratobasidiaceae clone OTUC1_OgaAQ01) from *Anacamptis morio* and *Dactylorhiza fuchsii*
Dct 7	*D.romana* subsp *romana* (white flower)	Aerial	5,43±0,35	Gray-white	Gray-White	2	(KC243940)	663	100	Uncultured Ceratobasidiaceae
Dct 8	*D.romana* subsp *romana* (pink flower)	Aerial	9,21±2,24	Gray orange	Absent	20	(AF008230)	719	100	*Verpa conica verpa*

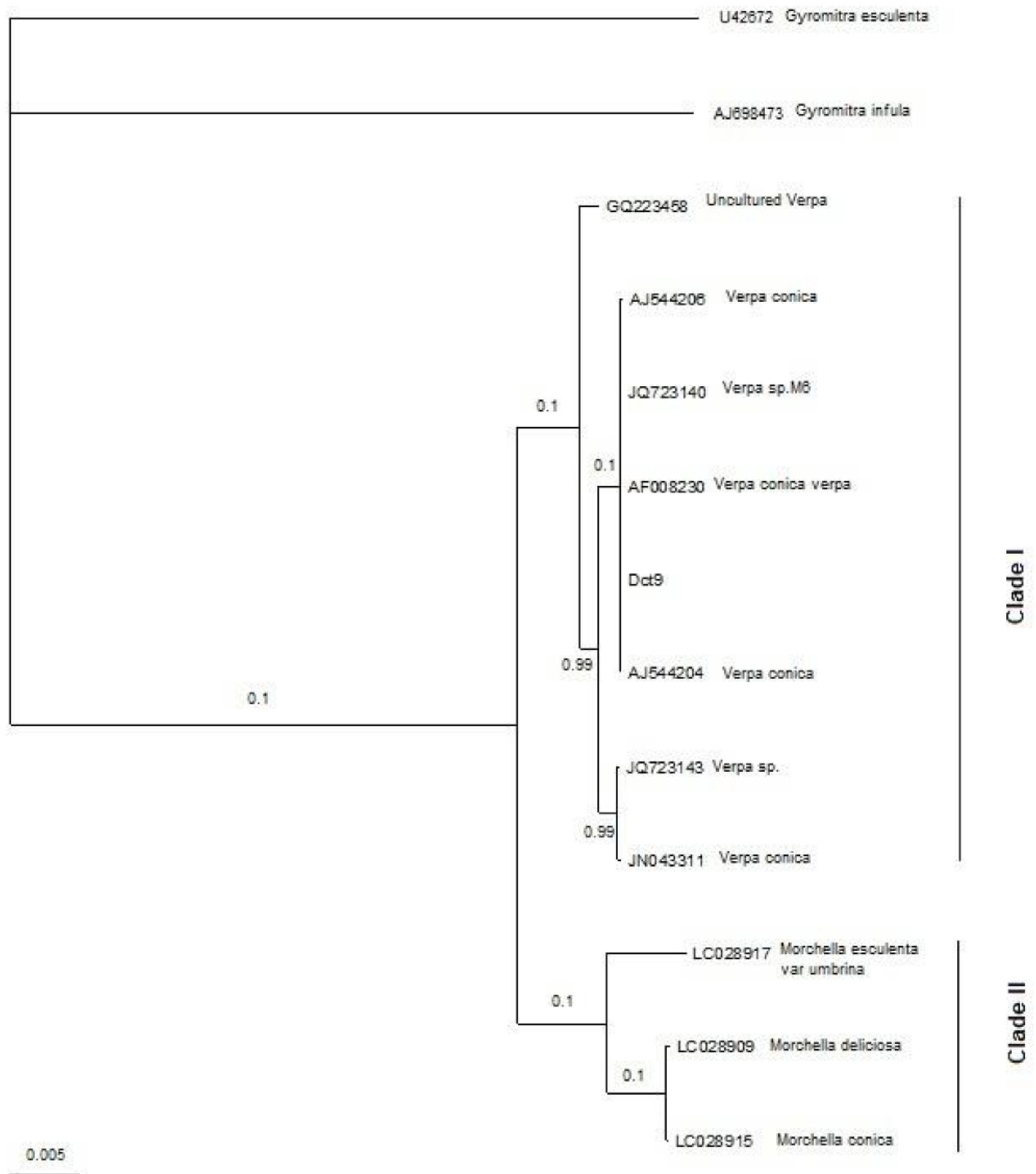

Figure 5: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Ascomycota (Morchellaceae) ITS sequences.

4. Conclusions and discussion

This study represents the first investigation of phylogenetic relationship mycorrhizal fungi associated with some *Dactylorhiza* species in Turkey. Researchers revealed that only hyphal characteristics for the identification of *Rhizoctonia*-like fungi may not be sufficient. Recently, a molecular analysis in addition to the morphological characterizations is used for identification of *Rhizoctonia*- like fungi and the other fungi (Rasmuusen, 2002; Taylor et al.,2008).We also used the both morphological and molecular analysis methods for identification of *Rhizoctonia*-like fungi and the others.

Fungi isolated from the roots of four *Dactylorhiza* species have been characterized using morphological and molecular analysis techniques. All of the isolated fungal partners from *Dactylorhiza* were identified as Tulasnellaceae

and Ceratobasidiaceae, which are the main mycorrhizal partners for terrestrial orchids (Rasmussen, 2002; Dearnaley,2007; Yukawa et al,2009) except Dct 8, the fungus was identified as Pezizalean Genus, *Verpa* (Morchellaceae). The few studies published so far also found that species of the genera *Dactylorhiza* commonly associate with fungal taxa related to *Tulasnella.* In *D. majalis*, ITS sequences of symbionts found in roots fell into two main clades: one of the genera *Tulasnella* and the second one of distantly related *Laccaria* (Shefferson et al.,2008). In the analysis of mycorrhizal associations in four *Dactylorhiza* species associated with a wide range of Tulasnella and Ceratobasidiaceae fungi. Phylogenetic analysis of fungal ITS sequences related that unique fungal partner of *D. euxina* var. *euxina*, *D. osmanica* var. *osmanic*a (endemic) and was *Tulasnella bifrons*, those of *D. urvilleana* were *T. bifrons* and *Tulasnella* spp. Fungal partners of *D. romana* subsp. *romana* (Pink and White flower) were different according to flower color polymorphism. Fungal partners of the pink flowering were identified as uncultured Ceratobasidiaceae (Dct5) and *Verpa conica verpa* (Dct8). Those of the white flowers were identified as uncultured Ceratobasidiaceae (Dct 6 and Dct 7).On the other hand, Dct6 associated exclusively with *Ceratobasidium albasitensis* from *Dactylorhiza purpurella* in Hungary. Color polymorphism of flowers of *Dactylorhiza sambucina* did not affect fungal diversity in the roots. Although little data are available on the relationship between the mycorrhizal partner and floral color variation, Taylor et al. (2003) showed that floral variation correlates with single *Sebacina* taxa and *Hexalectris spicat*a. In addition to, Pellegrino and Bellusci (2009) suggested that there is no relationship between mycorrhizal partner and flower colors of orchid. Additional researches must be done to reveal the relationship between the color polmorphism and mycorrhizal diversity of *D. romana* subsp. r*omana.* All the *Rhizoctonia*-like fungi, except *Verpa*, are well-known the partners of orchid mycorrhiza in all over the World (Kristiansen et al., 2001; Rasmussen, 2002; Pellegrino and Bellusci, 2009). Although fungal specificity is doubtful in photosynthetic orchids, fully mycoheterotrophy orchids have high fungal specificity (Girlanda et al., 2006). The results of the research revealed that there is no specific relation between four *Dactylorhiza* species and their mycorrhizal partners. *Verpa conica verpa* was isolated from orchid roots for the first time (Pezizales/Ascomycota) in Turkey. Similarly, the roots from *Gymnadenia conopsea* was isolated five Pezizalean genera (Stark et al., 2009) and they suggested that mycorrhizal taxa within Pezizales may be a potential fungal partner of orchids. Even so, most of the mycorrhizal fungi in the roots of orchids are related to Basidiomycetes fungi, some members of Ascomycetes have been isolated from the roots of some *Dactylorhiza* species and the other orchids (Bidartondo et al. 2004; Selosse et al., 2004). Mycorrhizal status of *Verpa conica verpa* isolated from the roots of *D. romana* subsp. *romana* should be investigated in detail in future researches.

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