Diversity of mycobiota isolated from tissues of four cultivars of *Pistacia vera*

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Accepted 30. May 2016

Key words: endophytes, communities, host, phylogeny, species diversity.

Abstract: The pistachio (*Pistacia vera* L.) belonging to Anacardiaceae family is a tree found in Iran and provides food that has economic values. Considering the importance of this plant, a study was conducted to determine the diversity and colonization frequency of fungi from *P. vera* L. A total of 614 fungal isolates were recovered from the fruits and leaves. Based on morphological, rDNA ITS region sequence and phylogenetic analysis, 41 fungal species were identified, representing 38 species of Ascomycota, two species of Basidiomycota and one species of Mucoromycotina. The most frequently isolated fungi were *Aspergillus flavus*, *A. niger*, *Byssochlamys spectabilis*, *A. tamarii*, *Alternaria malorum*, *Chaetomium globosum* and *Colletotrichum gloeosporioides*. The samples from fruits of the 'Kalle-Ghuchi' cultivar showed more species richness of fungi and higher Shannon`s diversity index than those in the other cultivars.

Zusammenfassung: Die Pistazie, *Pistacia vera*, gehört zur Familie *Anacardiaceae* und ist ein im Iran kultivierter vieljähriger Baum, der hochgeschätzte Lebensmittel liefert und beträchtlichen wirtschaftlichen Wert hat. In Anbetracht der Bedeutung dieser Pflanze wurde eine Untersuchung durchgeführt, um die Diversität und die Besiedlungshäufigkeit von Pilzen in *P. vera* zu bestimmen. Insgesamt wurden 614 Pilzisolate aus den Früchten und Blättern gewonnen. Basierend auf morphologischen Merkmalen, der rDNA-ITS-Sequenz und phylogenetischer Analyse wurden 41 Pilzarten identifiziert, nämlich 38 Arten der Ascomycota, zwei der Basidiomycota und eine Art der Mucoromycotina. Die am häufigsten isolierten Pilze waren *Aspergillus flavus, A. niger, Byssochlamys spectabilis, A. tamarii, Alternaria malorum, Chaetomium globosum* und *Colletotrichum gloeosporioides*. Die Proben von Früchten der Kultursorte 'Kalle-Ghuchi' zeigten einen höheren Artenreichtum und einen höheren Shannon-Diversitätsindex als die der anderen Kultivare.

Fungal endophytes are specified as microorganisms which reside in internal tissues of living plants without causing any immediate obvious detrimental effects (STONE & al. 2000). In the present paper, the terminus endophyte is used in the sense of Petrini (1991). Fungal endophytes appeared to have symbiotic relationship with the major taxonomic groups of living plants in natural ecosystems. This highly diverse group of fungi could have profound impacts on plant communities through inproving vitality in presence of abiotic and biotic stress, increasing biomass and reducing water consumption, or even decreasing fitness by altering resource allocation (SCHULZ & BOYLE

2005, ARNOLD 2007, RODRIGUEZ & al. 2009, ZHANG & al. 2014). On the other hand, novel antibiotics, antimycotics, immune suppressants, and anti-cancer compounds are only a few examples of compounds which are produced by endophytes (STROBEL & al. 2005, ALY & al. 2010, REFAEI & al. 2011). Some endophytic fungi are actually saprotrophs or latent pathogens and disease may be induced by time under specific environmental conditions that increase the stress on the plant, or plant senescence begins (ÁLVAREZ-LOAYZA & al. 2011).

The pistachio, *Pistacia vera* L., is recognized notably as a popular tree nut which is important all over the world because of its nutritional value and health attributes as well as economic importance. The distinguished characteristics of pistachio such as unsaturated fatty acids in high amount and the low proportion of saturated fatty acids, a rich source of proteins, dietary fibers, vitamins, minerals and antioxidants make it a noteworthy nut, that shows beneficial effects on human diet like other nuts (KASHANINEJAD & TABIL 2011).

Pistachio ranks second of non-oil and third among whole exported goods of Iran, because it's the most delicious and expensive nut compared to others (ARDAKANI 2006). The total pistachio-growing area in Iran is more than 350000 ha. Kerman Province with its 270000 ha of pistachio orchards is the most important and largest pistachio-growing area which has the most genetic diversity of pistachio compared to other regions in Iran. The main city of this province, Rafsanjan, is located at 30° 25' N and 55° 54' E which has an altitude of 1577 m above sea level. There are about 107000 ha of pistachio orchards (mainly bearing trees) in Rafsanjan that produce 1200 $kg.ha^{-1}$ of nut (on average). Production quota in Rafsanjan exceeded for 54 % of Kerman Province in total. More than 70 cultivars have been recorded for this province, among them cv. 'Ohadi', 'Fandoghi', 'Kalle-Ghuchi', 'Ahmad Aghaei', 'Badami', 'Rezaei', and 'Momtaz' as the major cultivars being grown (KASHANINEJAD & TABIL 2011). Those ecosystems with greatest biodiversity also seem to have endophytes in the greatest number and biodiversity (STROBEL & DAISY 2003). Hence, this study focused on endophytic fungi in leaves and fruits of *P. vera* in Rafsanjan, Kerman. This analysis included the identification and quantification of the species associated with four of the major growing cultivars in Kerman province.

Material and methods

Plant material: In each garden, five healthy trees in different parts of the garden were randomly selected and after collecting the fruits or leaves, they were combined and considered one sample. In total, eight fruit samples and eigth leaf samples from each major pistachio cultivar, cv.'Ohadi', 'Kalle-Ghuchi', 'Ahmad Aghaei' and 'Akbari', were collected during the spring of 2014 from different regions of Rafsanjan, Kerman, Iran (Fig. 1). They were placed in sterile plastic bags and stored in a refrigerator.

Isolation of endophytic fungi: Fungal isolation was conducted within 48 h of sample collection. Plant tissues were surface-sterilized using the procedure described by LI & al. (2015) with minor modification. All the samples were washed properly in running tap water. Then, they were sterilized in 75 % ethanol for 1 min, 3 % sodium hypochlorite for 3 min, 75 % ethanol for 0.5 min, and then rinsed tree times in sterile water and dried under aseptic conditions. 16 fruits and leaves from each sample were used. From each fruit and leaf, one piece approximately 5 × 5 mm was taken by using a sterile scalpel from the middle portion of tissue. The fragments were transferred aseptically to Petri dishes containing Potato Dextrose Agar (PDA) medium (potato 200 g/l, dextrose 20 g/l and agar 15 g/l) supplemented with streptomycin (100 mg/l) to inhibit the growth of bacteria and incubated at 25 °C for up

to 21 d. Emerged colonies were transferred into new Petri dishes and incubated under the same conditions at room temperature. The resulting fungal cultures were purified by single spore or hyphal tip isolation.

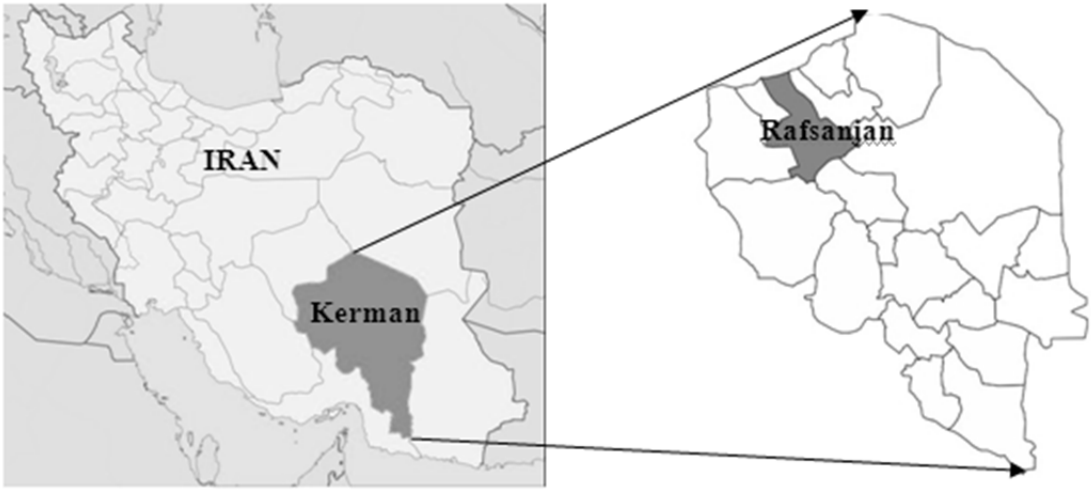

Fig. 1. Rafsanjan County in Kerman Province

Tab. 1. Identification of endophytes from the four cultivars 'Ohadi', 'Kalle-Ghuchi', 'Ahmad Aghaei' and 'Akbari' of *Pistacia vera* from different areas of Rafsanjan by morphological and ITS sequences (ITS1-5.8S rDNA-ITS2).

Endophytic fungi	Method of identification	GenBank accession No.	Identity (%)	Endophytic fungi	Method of identification	GenBank accession No.	Identity (%)
Acremonium sclerotigenum	M, I*	**KP641158**	99%	*Fomes fomentarius*	I	**KP641149**	99%
Alternaria brassicae	M, I	**KP641144**	100%	*Fusarium chlamydosporum*	M, I	**KP641161**	99%
Alternaria consortialis	M, I	**KP641143**	99%	*Fusarium* sp.	M, I	**KT351800**	99%
Alternaria infectoria	M, I	**KP641146**	100%	*Fusarium tricinctum*	M, I	**KP641142**	99%
Alternaria malorum	M			*Microsphaeropsis olivacea*	M, I	**KP641139**	99%
Ascorhizoctonia sp.	M, I	**KP641147**	99%	*Neoscytalidium dimidiatum*	M, I	**KP641157**	99%
Aspergillus flavus	M			*Nigrospora oryzae*	M, I	**KP641153**	99%
Aspergillus nidulans	M			*Penicillium chrysogenum*	M, I	**KP641164**	99%
Aspergillus niger	M			*Periconia byssoides*	M, I	**KP641155**	99%
Aspergillus tamarii	M, I	**KP641150**	100%	*Quambalaria cyanescens*	M, I	**KP641151**	99%
Biscogniauxia sp.	M, I	**KP641152**	99%	*Scopulariopsis brevicaulis*	M, I	**KP641165**	99%
Byssochlamys nivea	M, I	**KP641160**	99%	*Thielavia arenaria*	M, I	**KP641154**	99%
Byssochlamys spectabilis	M, I	**KP641135**	99%	*Thielaviopsis paradoxa*	M, I	**KP641163**	99%
Chaetomium elatum	M, I	**KP641140**	99%	*Trichoderma atroviride*	M, I	**KT351796**	99%
Chaetomium globosum	M, I	**KP641141**	99%	*Trichoderma harzianum*	M, I	**KT351798**	99%
Chaetomium interruptum	M, I	**KP641156**	99%	*Trichoderma longibrachiatum*	M, I	**KP641159**	100%
Clonostachys rosea	M, I	**KP641134**	99%	*Trichoderma reesei*	M, I	**KU756483**	99%
Colletotrichum gloeosporioides	M, I	**KP641148**	99%	*Ulocladium* sp.	M, I	**KP641145**	100%
Curvularia australiensis	M, I	**KP641162**	99%	*Umbelopsis isabellina*	M, I	**KP641166**	99%
Cytospora chrysosperma	M, I	**KP641137**	99%	*Valsa leucostoma*	M, I	**KP641136**	98%
Cytospora ribis	M, I	**KP641138**	99%				

*M: Morphological, I: ITS sequences

Identification of fungi: A total of 614 fungal samples were isolated. Morphological identification of endophytic fungi was obtained through study on colony appearance, culture morphology, mycelium structure and colour, type of asexual morph, conidiomata, conidia and conidiophore morphology (e.g. size, colour, shape, ornamentation), conidiogenous cells and the mechanism of spore production (ELLIS 1976, DOMSCH & GAMS 1980, ARX 1981, BARNETT & HUNTER 1998, SEIFERT & al. 2011). Each of the fungal isolates was separately subcultured onto different media; PDA, CYA (Czapek Yeast extract Agar), CMA (Corn Meal Agar), PCA (Potato Carrot Agar), MEA (Malt Extract Agar) and TWA (Tap Water Agar) + wheat straw for inducing sporulation. Microscopic slides were prepared, stained using lactophenol cotton blue and examined under a light microscope (Olympus, BX40) (RIDDELL 1950). All isolates according to their morphological appearances were classified into morphotypes, one isolate of each morphotype was kept for molecular identification.

DNA extraction: Colonies of each fungal isolate were grown in 250 ml Erlenmeyer flasks containing 100 ml Potato Dextrose Broth at 25 °C. After 15 days, genomic DNA of each isolate was extracted by using a modified CTAB method (DOYLE & DOYLE 1990).

The rDNA ITS region was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (WHITE & al. 1990). Each reaction mixture was carried out in volume of 25 µl, containing 50 ng of template DNA, 5 µl of 10 × PCR buffer, 3 µl of $MgCl_2$ (25 mM), 4 µl of dNTPs (2.5 mM), 1µl of each primer (10 pmol), and 2.5 units of Taq DNA polymerase. PCR amplification was carried in a Techne Genius Thermal Cycler (Canada). The amplification program was as follows: 1 cycle of 4 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C , and finally 1 cycle of 7 min at 72 °C. The ITS sequences were compared with that of the most similar fungal species using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) in consultation with observed colony and spore morphology to confirm the taxonomic status of the investigated fungal isolates. Sequence data were deposited in GenBank.

Data analysis: The Colonization Frequency (CF) percentage of an endophyte species was acquired by the ratio number of segments colonized versus total number of segments incubated × 100. The dominant percentage of the endophytic fungi was calculated according to division of the CF percentage of a given endophyte on the average of the CF percentages of all endophytes × 100 (HATA & FUTAI 1995, KUMAR & HYDE 2004). The diversity of endophytic communities was evaluated by use of the Shannon's Diversity Index (H') and Simpson's Diversity Index (1–D) (SHANNON & WEAVER 1949, SIMPSON 1949).

$$H' = -\sum_{i=1}^{s} P_i \times \ln P_i \, , P_i : \frac{n_i}{N}$$

$$D = 1 - \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N - 1)}$$

P is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), ln is the natural log, Σ is the sum of the calculations, and sis the number of species (ZHANG & al. 2014).

Results

Incubation of the different tissues (fruit and leaf) of the four major cultivars of *P. vera* has led to the isolation of different endophytic fungi. A total of 614 endophytic fungi were isolated from 1024 segments of *P. vera*, among these 191 isolates from cv. 'Kalle-Ghuchi', 159 isolates from cv. 'Ohadi', 149 isolates from cv. 'Ahmad Aghaei' and 115 isolates from cv. 'Akbari'. According to morphological characteristics and the ITS sequences of these isolates, 41 endophyte taxa were characterized and identified;

among these 37 taxa were identified to the species level and four of them to the genus level (Tab. 1).

Seven species were particularly common occurring in the four major cultivars grown in Kerman province: *Aspergillus flavus* (13.84 %), *A. niger* (13.68 %), *Byssochlamys spectabilis* (6.84 %), *Aspergillus tamarii* (5.54 %), *Alternaria malorum* (4.72 %), *Chaetomium globosum* (3.75 %) and *Colletotrichum gloeosporioides* (2.93 %) (Tab. 2).

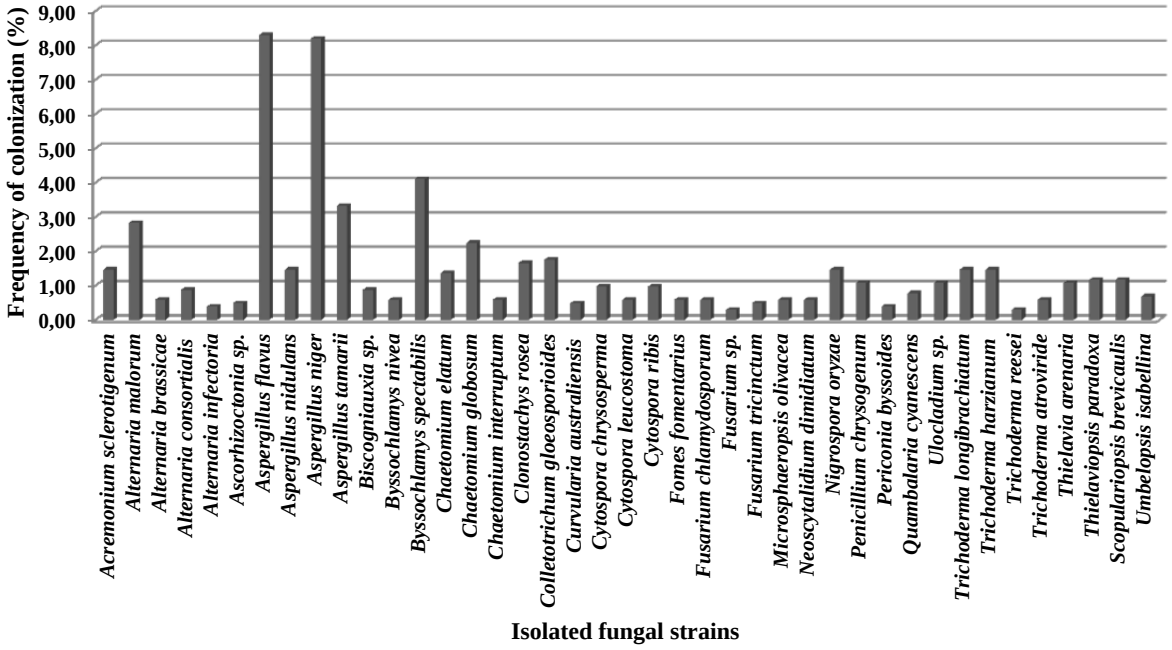

Fig. 2. Colonizing frequency (%) of endophytic fungi isolated from the four cultivars 'Ohadi', 'Kalle-Ghuchi', 'Ahmad Aghaei' and 'Akbari' of *Pistacia vera* from different areas of Rafsanjan, Kerman, Iran.

Biscogniauxia sp., *Chaetomium interruptum, Fusarium* sp., *Microsphaeropsis olivacea, Neoscytalidium dimidiatum* and *Trichoderma reesei* were only found on cv. 'Kalle-Ghuchi'. *Fomes fomentarius*, *Fusarium chlamydosporum* and *F. tricinctum* were seen on cv. 'Ohadi'. *Alternaria infectoria*, *Ascorhizoctonia* sp., *Curvularia australiensis*, *Cytospora ribis*, *Trichoderma atroviride* and *Umbelopsis isabellina* were isolated on cv. 'Ahmad Aghaei'. In addition, *Byssochlamys nivea*, *Periconia byssoides* and *Thielavia arenaria* were found on cv. 'Akbari' (Tab. 2). The samples from fruits of cv. 'Kalle-Ghuchi' showed more species richness of endophytes than the other cultivars. Different tissues (fruit and leaf) of *P. vera* exhibited different diversity levels and abundance of the endophytic fungi. Among the 614 endophytic fungal isolates, 340 and 274 isolates were obtained from fruits and leaves, respectively.

The Shannon's diversity index was 2.887 and 2.769 in fruits of cv.'Kalle-Ghuchi' and cv. 'Ohadi', higher than in their leaves (2.625 and 2.134, respectively). The Simpson's diversity index was 0.947 and 0.939 in fruits of cv. 'Kalle-Ghuchi' and cv. 'Ohadi', also higher than in their leaves (0.926 and 0.870, respectively). Shannon's and Simpson's diversity indices in leaves of 'Ahmad Aghayi' and 'Akbari' were higher than those in fruits (Tab. 2).

Tab. 2. Fungal diversity indices, species richness and fungal endophytes isolated from fruits and leaves of the four cultivars 'Ohadi', 'Kalle-Ghuchi', 'Ahmad Aghaei' and 'Akbari' of *Pistacia vera* from different areas of Rafsanjan.

Endophytic fungi	Kalle-Ghuchi		Ohadi		Ahmad Aghaei		Akbari			Dominance
	Leaf	Fruit	Leaf	Fruit	Leaf	Fruit	Leaf	Fruit	Total	
Acremonium sclerotigenum	3*			6	6				15	2.44
Alternaria brassicae		1		4				1	6	0.98
Alternaria consortialis		4	3			2			9	1.47
Alternaria infectoria						4			4	0.65
Alternaria malorum	9	4		8	2	4		2	29	4.72
Ascorhizoctonia sp.						5			5	0.81
Aspergillus flavus	16	8	12	14	8	7	12	8	85	13.84
Aspergillus nidulans	5	1		5			3	1	15	2.44
Aspergillus niger	8	10	13	12	11	18	8	4	84	13.68
Aspergillus tamarii	5	11	1	3	5	4	3	2	34	5.54
Biscogniauxia sp.		9							9	1.47
Byssochlamys nivea								6	6	0.98
Byssochlamys spectabilis	4	7		7	2	2	9	11	42	6.84
Chaetomium elatum		2		5	3	4			14	2.28
Chaetomium globosum	2	5	5		4		4	3	23	3.75
Chaetomium interruptum		6							6	0.98
Clonostachys rosea		6	3	6	2				17	2.77
Colletotrichum gloeosporioides	3		2		6	4	3		18	2.93
Curvularia australiensis					1	4			5	0.81
Cytospora chrysosperma		5		5					10	1.63
Cytospora ribis						10			10	1.63
Fomes fomentarius				6					6	0.98
Fusarium chlamydosporum				6					6	0.98
Fusarium sp.		3							3	0.49
Fusarium tricinctum				5					5	0.81
Microsphaeropsis olivacea	4	2							6	0.98
Neoscytalidium dimidiatum	6								6	0.98
Nigrospora oryzae		5					6	4	15	2.44
Penicillium chrysogenum	3		4	4					11	1.79
Periconia byssoides							4		4	0.65
Quambalaria cyanescens	5				3				8	1.30
Ulocladium sp.	3	4	2				2		11	1.79
Thielavia arenaria							6	5	11	1.79
Thielaviopsis paradoxa	7				5				12	1.95
Trichoderma atroviride						6			6	0.98
Trichoderma harzianum	5	4			4		2		15	2.44
Trichoderma reesei		3							3	0.49
Trichoderma longibrachiatum			3	5		4	3		15	2.44
Scopulariopsis brevicaulis			6	1	2		3		12	1.95
Umbelopsis isabellina						7			7	1.14
Valsa leucostoma		3		3					6	0.98
Total number of isolates (N)	88	103	54	105	64	85	68	47	614	
Species richness (S)	16	21	11	18	15	15	14	11		
Shannon's diversity index (H')	2.625	2.887	2.134	2.769	2.536	2.529	2.483	2.17		
Simpson's diversity index (1–D)	0.926	0.947	0.870	0.939	0.923	0.913	0.917	0.884		

***** The number of isolate

Overall frequency of colonization was 59.96 %. The highest frequency of colonization was seen by *Aspergillus flavus* (Fig. 2).

Most of the isolated fungi from *P. vera* were *Ascomycota* of subphylum *Pezizomycotina* (representing 96.58 % of the isolates) within 11 orders: *Hypocreales,*

Microascales, Glomerellales, Sordariales, Diaporthales, Xylariales, Trichosphaeriales, Pleosporales, Botryosphaeriales, Eurotiales and *Pezizales*. Few isolates belonging to *Agaricomycotina, Ustilaginomycotina* and *Mucoromycotina* were collected, representing only 0.98, 1.30 and 1.14 %, respectively.

Discussion

Various studies have been performed on the relationships between environmental conditions such as climate, quality of soil and water with the quality formation of pistachio nuts, however, these studies just were limited to the contribution of external environmental factors, but not to the internal environment of plants. Endophytes´ communities and distribution can affect plant growth and development, hence, the importance of investigating endophytes´ diversity and relationships between the endophytes and their hosts are considerable (JIANG & al. 2013). To the best of our knowledge, this study is the first report of distribution of endophytic fungi in major cultivars of *P. vera* in the world. The diversity of plants in tropical, subtropical, temperate and Arctic ecosystems was investigated for endophytic fungal communities in association which numbers of fungi observed (HIGGINS & al. 2007, ROSA & al. 2010).

To identify differences in fungal endophyte communities among cultivars of *P. vera*, we investigated and identified the fungal endophytes of fruits and leaves for *P. vera* in Kerman. According to morphological characteristics and the rDNA ITS region sequences, 37 taxa were identified to species level and four taxa to genus level.

Molecular techniques have been successfully employed by researchers for identification of different endophytic fungal communities (CHEN & al. 2010, ROSA & al. 2010, SETTE & al. 2006). In this study, we employed molecular techniques to identify these fungi using the rDNA ITS region. LI & al. (2008) isolated around 63 endophytic fungi from *Paris polyphylla*. The isolates were separated into the groups based on morphological characteristics and the presence of reproductive structures. Afterwards, molecular identification through analyzing the rDNA-ITS region was carried out. The isolates were identified as *Gliocladiopsis irregularis*, *Plectosphaerella cucumerina*, *Padospora* sp., *Gliomastix murorum* var. *murorum*, *Aspergillus fumigatus*, *Pichia guilliermondii*, *Neonectria radicicola*, *Fusarium redolens*, *F. oxysporum* and a mycorrhizal ascomycete.

As our results show, species of *Pezizomycotina* were predominantly isolated with 95.6 % frequency, *Agaricomycotina, Ustilaginomycotina* and *Mucoromycotina* which were isolated from leaves and fruits were present with very low frequency. The predominance of *Ascomycota* appears characteristic of endophytic mycota (HIGGINS & al. 2007, HOFFMAN & ARNOLD 2008). GAZIS & CHAVERRI (2010) reported that *Ascomycota* was dominant with representing almost 97 % of the isolates. In contrast, *Basidiomycota* and *Zygomycota* were represented by 1 % and 2 %, respectively. However, *Basidiomycota* occur as normal components of the endophytic mycota in diverse plant species, but in lower numbers (THOMAS & al. 2008). PAUL & al. (2012) reported that 21 fungal genera were characterized, for instance 16 *Ascomycota* and five *Basidiomycota* in *Capsicum annuum* L., based on rDNA ITS region and phylogenetic analysis.

Different parts of the tree were dominated by different endophytic species. *Aspergillus flavus* constituted the highest percent of isolates that came from leaf samples, and it was also present in fruit isolates, 48 and 37 %, respectively. *Aspergillus niger*

was the dominant species within the fruit isolates but was also an important component of the leaf isolates, 44 and 40 % respectively.

Many genera such as *Aspergillus*, *Alternaria*, *Fusarium* and *Colletotrichum* found in this study coincide with genera reported in other studies (KRISHNAMURTHY & al. 2009, PAUL & al. 2012, PATIL & al. 2015, ORLANDELLI & al. 2015). The present study has found other genera that are not commonly isolated such as *Umbelopsis isabellina*, *Quambalaria cyanescens*, *Thielavia arenaria* and *Microsphaeropsis olivacea*.

Some isolated fungi from the tissues of pistachios have the potential to be a pathogen in pistachio plants. *Aspergillus flavus* is an opportunistic pathogen of crops. It is important because toxigenic strains of this fungus can produce aflatoxin in the pistachio nuts (KLICH 2007). *Aspergillus niger* causes *Aspergillus* blights (MICHAILIDES & al. 1995). Some of these fungi cause major diseases in other plants. *Cytospora chrysosperma* causes canker in poplars (BLOOMBERG 1962). POLIZZI & al. (2009) reported that *Neoscytalidium dimidiatum* causes shoot blight, canker, and gummosis on citrus. Some of the isolated fungi are beneficial fungi. HARMAN & al. (2004) reported that *Trichoderma* species are opportunistic, avirulent plant symbionts, as well as parasites of other fungi.

The fruits of cv. 'Kalle-Ghuchi' and cv. 'Ohadi' had higher Shannon's and Simpson's diversity indices than their leaves. Vice versa, the leaves of cv.'Ahmad Aghayi' and cv. 'Akbari' had higher Shannon's and Simpson's diversity indices than their fruits. This is perhaps related to flowering and maturity time of cultivars. Cv. 'Kalle-Ghuchi' and cv. 'Ohadi' are almost early-flowering and maturing cultivars. Formation of flower and fruits in these cultivars starts sooner than in cv. 'Ahmad Aghayi' and cv. 'Akbari'; as a result, endophytic fungi have more time to colonize fruits and fungal diversity increases in fruits. Many factors such as environmental conditions, nutritional ingredients of hosts, tissue structures and interaction between host plants and endophytic fungi affect the endophytic fungal diversity (BANERJEE 2011, WU & al. 2014). The present study improves our understanding of fungal endophyte communities and can be a model for further study.

This work was funded by a University of Tehran grant to M. Javan-Nikkhah (Grant number 7110022/6/36).

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