

تنوع و توزیع مکانی قارچ‌های اندوفیت فندق (*Corylus avellana*) در رویشگاه‌های مختلف آن در ایران

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چکیده

مقدمه: اندوفیت‌ها یا درون‌رست‌ها گروه بزرگ و متنوعی از ریزموجوداتی‌اند که بدون ایجاد علائم در بافت‌ها یا اندام‌های گیاهان زندگی می‌کنند. اندوفیت‌ها نقش مهمی در حفاظت از گیاه در برابر تنش‌های زیستی و غیرزیستی دارند. هدف مطالعه حاضر جداسازی و شناسایی قارچ‌های اندوفیت در *Corylus avellana* L. و ارزیابی اثرات احتمالی بافت‌های مختلف و شرایط جغرافیایی بر جوامع آنها بود.

مواد و روش‌ها: نمونه‌گیری از اندام‌های سالم (برگ، ساقه و ریشه) گیاه فندق در استان‌های گلستان، مازندران، گیلان، اردبیل، زنجان و قزوین در شمال و شمال غربی ایران انجام شد. به دنبال ضدعفونی سطحی، قارچ‌های اندوفیت با استفاده از تکنیک‌های استاندارد کشت، جداسازی و براساس مشخصات ریخت‌شناسی شناسایی شدند. نمایندگان هر گونه به روش مولکولی و توالی‌یابی نواحی ITS، rDNA، LSU، TEF1 و β -tubulin شناسایی شدند. داده‌ها با استفاده از شاخص‌های تنوع و تحلیل تناظری (CA) تجزیه و تحلیل شدند.

نتایج: در مجموع، ۷۹۱ جدایه قارچی اندوفیت از برگ‌های سالم (۳۹ درصد)، ساقه‌ها (۳۵/۶۵ درصد) و ریشه‌ها (۲۵/۱۶ درصد) به دست آمد که به ۲۴ گونه قارچی متعلق به هفت راسته در چهار رده قارچی طبقه‌بندی شدند که شامل Dothideomycetes (Capnodiales و Pleosporales)، Sordariomycetes (Diaporthales و Hypocreales)، Eurotiomycetes (Eurotiales و Chaetothyriales) و Agaricomycetes (Polyporales) بودند. Ascomycota و Basidiomycota به ترتیب ۹۶/۲ و ۳/۸ درصد از جدایه‌ها را تشکیل دادند. ساقه و ریشه به ترتیب بیشترین و کمترین میزان کل کلونیزاسیون و شاخص‌های تنوع قارچ‌های اندوفیت را داشتند. همچنین، شاخص‌های تنوع اندوفیت‌ها در گیلان از سایر استان‌های مورد مطالعه بیشتر بودند.

بحث و نتیجه‌گیری: فراوانی و تنوع اندوفیت‌های قارچی در گیاه *C. avellana* تأثیر گرفته از عواملی مانند شرایط جغرافیایی، تنوع زیستی و نوع بافت‌اند؛ بنابراین، آگاهی از ویژگی‌های بافت‌های مختلف گیاهی، مکان‌های متعدد نمونه‌برداری و شاخص‌های اکولوژیکی مربوط به آنها، به درک علت وجود تنوع زیستی بالا در جمعیت قارچ‌های اندوفیت مربوط به یک گونه گیاهی خاص کمک می‌کند.

واژه‌های کلیدی: تنوع زیستی، *Corylus avellana*، اندوفیت‌ها، توزیع قارچ، بافت‌های داخلی

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Investigating Diversity and Spatial Distribution of Endophytic Fungi in Hazelnut (*Corylus avellana*) in its Different Habitats of Iran

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Abstract

Introduction: Endophytes are a large and diverse group of microorganisms living in the plants' tissues and/or organs without causing any symptoms. Endophytes play an important role in plant protection against biotic and abiotic stress. The aim of the present study was to isolate and identify endophytic fungi in *Corylus avellana* L. and evaluate the possible effects of different tissues and geographical conditions on their communities.

Materials and Methods: Plant sampling of healthy tissues (leaf, stem, and root) of the hazelnut plants was carried out in Golestan, Mazandaran, Guilan, Ardabil, Zanzan, and Qazvin provinces in north and northwest of Iran. Following surface sterilization, endophytic fungi were isolated using standard culturing techniques and identified based on morphological characteristics. Representatives of each taxon were subjected to molecular identification based on ITS -rDNA, LSU, TEF1, and β -tubulin sequences. Data were analyzed using diversity indices and correspondence analysis (CA).

Results: In total, 791 endophytic fungal isolates were isolated from healthy leaves (39%), stems (35.65%), and roots (25.16%). The isolates were classified into 24 fungal species belonging to seven orders in four fungal classes, including Dothideomycetes (Capnodiales and Pleosporales), Sordariomycetes (Diaporthales and Hypocreales) Eurotiomycetes (Eurotiales and Chaetothyriales), and Agaricomycetes (Polyporales). Ascomycota and Basidiomycota represented 96.2% and 3.8% of the isolates, respectively. The stem and root showed the highest and lowest total colonization frequency and diversity indices of endophytic fungi respectively. Also, the diversity indices of endophytes in Guilan were higher than those in other studied provinces.

Discussion and conclusion: The frequency and diversity of fungal endophytes in *C. avellana* are under the influence of factors such as geographical conditions, biodiversity, and tissue type. Different plant tissues, multiple sites, and related ecological indicators have helped define how maximum biodiversity may be found in a fungal endophyte population in a given plant species.

Key words: Biodiversity, *Corylus avellana*, Endophytes, Fungal Distribution, Inner Tissues

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Introduction

Endophytes are microorganisms associated with living plant tissues with no obvious signs of being present and appear to cause no damage to their host plants (1, 2). The most common endophytes are fungi (usually Ascomycete and to a lesser extent Basidiomycete and Zygomycete) as well as bacteria and actinomycetes (3). They are found in almost all plant organs (roots, stems, leaves, flowers, fruits, and seeds) (4). After decades of research on fungal endophytes, it is now clear that they are diversely distributed throughout the plant taxonomic groups and in all ecological conditions (5).

In recent years, endophytic microorganisms have attracted much attention for a variety of reasons, including plant protection from pests, pathogens, and even herbivores, as well as their potential to produce bioactive compounds (6). Currently, endophytes are considered as a source of naturally occurring and biologically active compounds with great potential in the production of valuable medicinal compounds such as Taxol, which has been shown to possess antifungal and anti-cancer properties (7, 8, 9). Some studies have been carried out on endophytes biodiversity, classification, host environment, and their effects on the host well-being (8, 10, 11). Also, it has been shown that a variety of factors such as ecological and environmental conditions, sampling season, type, and age of infected tissues are effective in the colonization of a plant by endophytic fungi (12, 13). On the other hand, although a unique and diverse assemblage of biologically active secondary metabolites was produced by different endophytic species, their spectrum of metabolites changes with changes in environment, geography, host, and tissue type (14). Therefore, to obtain the maximum benefits from this excellent natural resource, it is necessary to study endophytic communities in different plants (5).

Hazelnut (*C. avellana*) used to be cultivated for its nutritional aspects. But, it is now being considered for its phytochemical content (15). Indeed, some valuable drugs such as taxol, have been reported from some endophytes isolated from hazelnut (16). This deciduous plant is native to Europe and western Asia (17). Iran is the seventh-largest producer of hazelnuts in the world (18) and its main cultivation area is in the northern and northwest provinces of Golestan, Mazandaran, Guilan, Ardabil, Zanjan, and Qazvin (19).

In the present study, our first aim is to isolate, identify, and classify endophytic fungi from leaf, stem, and root of hazelnuts from different geographical regions using morphological and molecular methods. The second goal is to investigate the possible effects of environmental and geographical changes as well as the type of plant organ on the rate of *C. avellana* colonization by the endophytes. Since the information available about the biodiversity of *C. avellana* endophytes is scarce, this study can provide valuable information about their biodiversity and shed light on important determining factors in their distribution under different geographical and environmental conditions.

Materials and Methods

Study Sites and Plant Sampling: *C. avellana* samples were collected from natural habitats and gardens in six north and northwest provinces of Iran (Golestan, Mazandaran, Guilan, Ardabil, Zanjan, and Qazvin) from April to June 2017 (Table 1; Figure 1). Similar sampling with a Z-shaped sampling pattern was performed to collect healthy leaves, stems, and roots randomly from 9 trees at each site (five samples of leaves, stems, and roots per tree). Samples were collected from 6- to 8-year-old trees with a height of 2 to 4 meters. In total, three regions represented each province, and 18 trees from each region were selected (a total of 324 trees). The samples were sealed in polyethylene bags and transferred to the

Central Laboratory of Gonbad Kavous University, and stored at 4 °C to be analyzed later.

Fungal Isolation and Morphological Identification: In order to isolate fungal endophytes from leaf, stem, and root tissues, 30 pieces (10 pieces from each organ) from each hazelnut tree were cultured according to Tan et al. (20). Before surface sterilization, the samples were washed thoroughly under running tap water to remove the remnant soil and debris. They were sequentially sterilized with 70% ethanol for 1 min followed by immersion in 3% Sodium hypochlorite (NaOCl) for 3-8 min (depending on the type of sample, 3 min for leaf, 5 min for the stem, and 8 min for root) and once again in 70% ethanol for 1 min. They were then rinsed several times in sterile distilled water and were dried on sterile filter paper (20). The sterilized plant

fragments were cut into about 0.5×1 cm using a sterile blade. Four segments of each sample were placed on Petri dishes containing potato dextrose agar (PDA; Merck, Darmstadt, Germany), malt extract agar (MEA; Merck, Darmstadt, Germany), and water agar. They were incubated at 25 °C for 2-4 weeks and were daily checked for any fungal growth. The number of individual fungal colonies emerged from different samples was recorded separately to calculate the fungal abundance and species diversity. The colonies were subcultured on fresh media to purify endophytic fungi and maintain them in PDA for further study. Morphological identification of isolates was performed according to the standard taxonomic keys based on the fungal colony morphology, characteristics of the hyphae, spores, and reproductive structures (21-24).

Table 1- Geographical Coordinates, Altitude, and Mean Annual Rainfall of the *C. avellana* Sampling Locations in Iran

Location			Geographic coordinates		Altitude (m)	Mean annual rainfall(mm)
			N	E		
Golestan	1	Minoudasht-Sasang	37°04'05.1 "	55°22'49.3 "	589	720
	2	Minoudasht-Takht	37°08'54.7"	55°26'42.6"	728	712
	3	Kalaleh-Maraveh Tapeh	37 38'41.24"	55 56'32.73"	1006	360
Mazandaran	1	Tonekabon-Dohezar	36° 37' 50.016"	50° 44'40.16 "	906	1500
	2	Royan- Lazir	36°24'22.3"	51°55'47.2"	930	655
	3	Behshahr-Hezar Jarib	36°35'58.9"	53°55'55.4"	1260	670
Guilan	1	Siahkal-Pirkooh	36°49'44.6 "	50°00'50.3 "	1256	1302
	2	Siahkal-Deylaman	36°53'19.2"	49°54'26.7"	1434	620
	3	Roudsar- Rahim Abad	36°45'02.4"	50°15'06.7"	1375	1508
Ardabil	1	Fandoqlou forest- 1	38°24'6.82 "	48°32'44.71 "	1460	290
	2	Fandoqlou forest- 2	38°24'7.79 "	48°33'47.13 "	1507	290
	3	Fandoqlou forest- 3	38°25'4.45 "	48°33'57.63 "	1589	290
Zanjan	1	Tarom- 1	36°39'13.4"	48°28'57.2"	1607	268
	2	Tarom- 2	36°39'09.8"	48°30'26.9 "	1618	268
	3	Tarom- 3	36° 39'08.2 "	48°29'31.0 "	1619	268
Qazvin	1	Almot- Ekojan	36°37'31.8 "	50°08'30.9 "	1520	530
	2	Almot- Hir	36°35'44.4 "	50°15'28.5 "	1700	515
	3	Almot- Via	36°37'20.3 "	50°16'36.4 "	1813	550

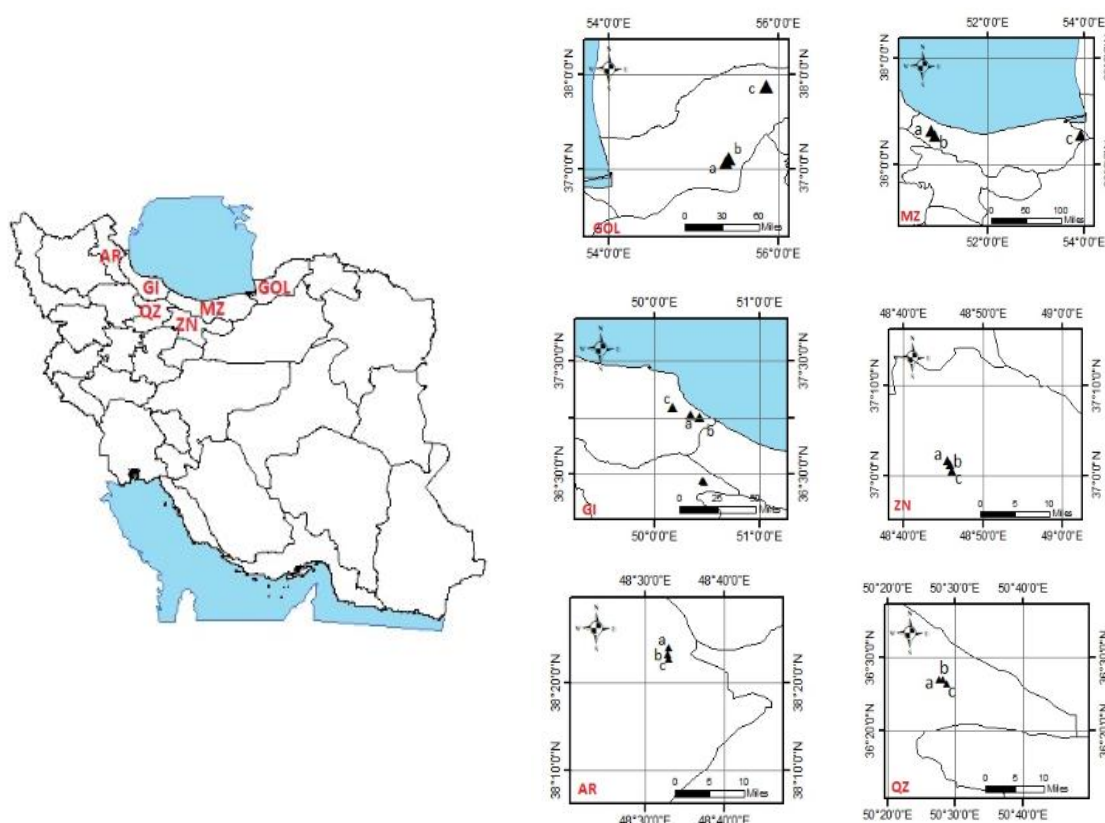


Fig. 1- Map of the six sampling site of *C. avellana* in Iran. GOL: Golestan. Mz: Mazandaran. Gi: Guilan. Zn: Zanjan. Ar: Ardabil. Qz: Qazvin.

DNA Extraction and Molecular Identification: For molecular identification, at least one isolate was selected as a representative of each morphotype and was grown in 200 mL potato dextrose broth (PDB) in a shaker incubator (WiseCube WIS-10R) at 110 rpm and 25 °C for 7 days. The mycelium was harvested and washed with distilled water, freeze-dried, and the genomic DNA was extracted by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Zhang et al. (25).

The extracted DNA was subjected to the Polymerase Chain Reaction (PCR) using four molecular markers: ITS (Internal transcribed spacer), LSU (partial large subunit nrDNA), TEF1 (Translation elongation factor), and TUB (β -tubulin) (Table 2). PCR was performed for PCR mixture (as shown in Table 3) using a T100™ thermal cycler (Bio-Rad, USA) with the initial denaturation at 94 °C for 5 min,

followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were analyzed electrophoretically in 1% (w/v) agarose gels stained with gel red (Biotium®) and visualized with a UV light (UVP MultiDoc-It™, Analytik Jena, Germany). The purified products were sent to the Microsynth AG genome center (Balgach, Swiss) for sequencing by the Sanger Sequencing Method.

Analysis of the Data: The resulting DNA sequences were trimmed and edited by MEGA 5.0 software. They were analyzed by the nBlast search tool on the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All the sequences were deposited in the NCBI's GenBank database, and an Accession Number was obtained for each sequence.

The Colonization Frequency (CF) was calculated as the number of fragments from

which one or more endophytic fungi were isolated, and divided by the total number of incubated fragments (30). The diversity of endophytic fungi isolated from six hazelnut growing provinces as well as three plant organs (leaf, stem, and root) were evaluated using the Shannon–Weiner Index [$H' = -\sum (P_i \times \ln P_i)$ ($P_i = n_i/N$)], Simpson's index [$D = 1 / \sum P_i^2$], Pielou's Evenness Index [$E = H' / \ln(S)$], Margalef richness index [$R = (S - 1) / \ln N$], and Jaccard similarity index [$J_c = C / (A + B - C)$]. Here, p_i is the proportion of individuals that species i contributes to the total; n_i represents the numbers of individuals; N represents the total number

of individuals; S indicates the total number of species (31); C is the number of species shared by these three tissues; A and B are the total number of species isolated from any of the three tissues (32). Also, the diversity index was calculated for each location and organ type using primer software

(<https://primer.software.informer.com/6.0/>). Correspondence Analysis (CA) was used to study the ecological interrelationships between the fungal species and different tissue types and also between the fungal species and Sampling areas (PAST software) (33).

Table 2- Primers Used for Generic Amplification and Sequencing

Locus	Primer	Primer sequence 5'-3'	Orientation	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	(26)
	ITS4	TCCTCCGCTTATTGATATGC	Reverse	(26)
LSU	LROR	CC CGC TGA ACT TAA GC	Forward	(27)
	LR5	TCCTGAGGGAAACTTCG	Reverse	(27)
TEF-1 α	EF1-983F	GCCYGGHCAYCGTGAYTTYAT	Forward	(28)
	Efgr	GCAATGTGGGCRGTRTGRCARTC	Reverse	(28)
β -tubulin	T1	AACATGCGTGAGATTGTAAGT	Forward	(29)
	β -Sandy-R	GCRCGNGGVACRTACTTGTT	Reverse	(29)

Table 3- Materials for DNA Amplification using PCR

Components	Concentration	Amount (μ l)
Master mix red (Ampliqon)	2X	12.5
Reverse primers	10 pmol/ μ l	1
Forward Primer	10 pmol/ μ l	1
DNA diluted	0.75-0.5 ng	2
Sterile distilled water		8.5
the Final content		25 μ l

Results

A total of 791 endophytic fungal isolates were recovered from 9720 leaf, stem, and root fragments. They were assigned to 24 species or morphological types. Their accession numbers and other complementary information are presented in Table 4. Most of the isolates were grouped in phylum Ascomycota, subphylum Pezizomycotina, in three classes of Dothideomycetes, Sordariomycetes, and Eurotiomycetes, and only one belongs to

subphylum Agaricomycotina of the phylum Basidiomycota (Agaricomycetes). Overall, the class Dothideomycetes represented by the orders of Pleosporales (8 species) and Capnodiales (4 species); the class Sordariomycetes represented by the orders of Hypocreales (5 species) and Diaporthales (2 species); class Eurotiomycetes represented by order Eurotiales (Three species), and the class Agaricomycetes represented by the order Polyporales (one species) (Table 4).

Table 4- Endophyte Isolate Accession Numbers and Other Supplementary Information of the Endophytic Fungi

Associated with *C. avellana* in Six Provinces of Iran

Isolate ID (Accession no.)	Endophytic Fungi	Tissue	Order /Phylum/Class	ITS identity (%)	Dominance of fungi
GOLB4 (MN963671)	<i>Epicoccum nigrum</i>	Leaf	Pleosporales/ Ascomycota/ Dothideomycetes	100	10.75
GOLB6 (MN963672)	<i>Nothophoma quercina</i>	Leaf	Pleosporales/ Ascomycota/ Dothideomycetes	100	2.66
GOLS20 (MN963673)	<i>Neocucurbitaria unguis</i>	Stem	Pleosporales/ Ascomycota/ Dothideomycetes	100	2.91
GOLR5 (MN963674)	<i>Alternaria alternata</i>	Root	Pleosporales/ Ascomycota/ Dothideomycetes	100	14.03
GOLR6 (MN963675)	<i>Paraphaeosphaeria sporulosa</i>	Root	Pleosporales/ Ascomycota/ Dothideomycetes	100	0.75
MZB3 (MN963676)	<i>Purpureocillium lilacinum</i>	Leaf	Hypocreales/ Ascomycota/ Sordariomycetes	100	2.79
MZB12 (MN963694)	<i>Fusarium sp.</i>	Leaf	Hypocreales/ Ascomycota/ Sordariomycetes	99	2.41
MZR1 (MN963677)	<i>Fusarium proliferatum</i>	Root	Hypocreales/ Ascomycota/ Sordariomycetes	100	4.56
GIB1 (MN963678)	<i>Diaporthe sp.</i>	Leaf	Diaporthales/ Ascomycota/ Sordariomycetes	100	2.53
GIS2 (MN963679)	<i>Cladosporium tenuissimum</i>	Stem	Capnodiales/ Ascomycota/ Dothideomycetes	100	3.29
GIS3 (MN963680)	<i>Pyrenochaetopsis sp.</i>	Stem	Pleosporales/ Ascomycota/ Dothideomycetes	100	2.53
GIR2 (MN963681)	<i>Exophiala sp.</i>	Root	Chaetothyriales/ Ascomycota/ Eurotiomycetes	100	3.92
ZNB7 (MN963682)	<i>Fusarium fujikuroi</i>	Leaf	Hypocreales/ Ascomycota/ Sordariomycetes	100	8.09
ZNB8 (MN963683)	<i>Fusarium equiseti</i>	Leaf	Hypocreales/ Ascomycota/ Sordariomycetes	100	8.09
ZNS2 (MN963684)	<i>Bjerkandera adusta</i>	Stem	Polyporales/ Basidiomycota/ Agaricomycetes	100	1.39
ZNR4 (MN963685)	<i>Penicillium chrysogenum</i>	Root	Eurotiales/ Ascomycota/ Eurotiomycetes	100	4.30
ARB5 (MN963689)	<i>Angustimassarina sp.</i>	Leaf	Pleosporales/ Ascomycota/ Dothideomycetes	100	2.41
ARB6 (MN963686)	<i>Gnomoniopsis idaeicola</i>	Leaf	Diaporthales/ Ascomycota/ Sordariomycetes	99	3.03
ARB21 (MN963687)	<i>Cercospora sp</i>	Leaf	Capnodiales/ Ascomycota/ Dothideomycetes	100	2.53
ARS4 (MN963690)	<i>Pleosporales sp.</i>	Stem	Pleosporales/ Ascomycota/ Dothideomycetes	99	2.80
ARR2 (MN963688)	<i>Cladosporium sphaerospermum</i>	Root	Capnodiales/ Ascomycota/ Dothideomycetes	98	3.42
QZS1 (MN963691)	<i>Cladosporium herbarum</i>	Stem	Capnodiales/ Ascomycota/ Dothideomycetes	100	2.15
QZR4 (MN963692)	<i>Penicillium citrinum</i>	Root	Eurotiales/ Ascomycota/ Eurotiomycetes	100	5.05
QZR10 (MN963693)	<i>Talaromyces amestolkiae</i>	Root	Eurotiales/ Ascomycota/ Eurotiomycetes	100	3.67

Distribution in Tissues: During individual leaf, stem, and root sample analysis, significant fluctuations were recorded in the recovery of endophytic fungi (Table 5). The leaf samples harbored 310 fungal isolates, followed by root (282 isolates), and stem (199 isolates). Among the organs examined in the current study, the CF of the leaf was higher than other organs (Table 5). Of all the identified taxa,

Fusarium, *Alternaria*, *Epicoccum*, *Penicillium*, and *Cladosporium* were the most dominant genera, and *Fusarium* had the highest CF, followed by *Alternaria*, *Epicoccum*, *Penicillium*, and *Cladosporium*, respectively (Figure 2). The data show that the diversity of endophytic fungi in *C. avellana* stem is higher than the leaf and root samples, and most of them belong to the phylum Ascomycota. The

fungal community of stem appeared to be dominated by *Epicoccum nigrum*, *Pyrenochaetopsis* sp, *Cladosporium tenuissimum*, *Alternaria alternate*, and *Pleosporales* sp (Table 5). Furthermore, the richness of endophytic fungi in the stem (4.14) was higher than in other organs (Table 5). Root samples were mainly by *A.*

alternate and *Fusarium equiseti*, also, the other dominant were *Fusarium fujikuroi*, *Fusarium proliferatum*, and *Penicillium citrinum* (Table 5). The endophytic population of leaf samples was dominated by *E. nigrum*, *A. alternate*, *F. fujikuroi*, and *F. equiseti* which occurred on more than 50% of the leaf segments.

Table 5- Number and Percentage of Colonized Segments of *C. avellana* according to Plant Tissues

Tissues	No. of samples	No. of endophytic fungi isolated	Endophyte species	CF%
Leaf	1620	310	21	9.41
Root	1620	282	23	8.61
Stem	1620	199	23	6.11
Total	4860	791	24	

CF: Colonization frequency

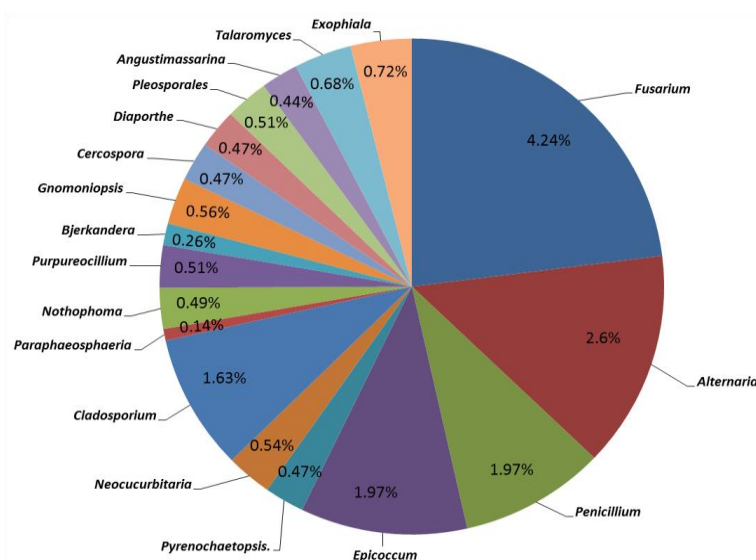


Fig. 2- The colonization frequency (CF %) of each endophytic fungus genera from all *C. avellana* tissues and sampling location in Iran

Spatial Distribution: In terms of the number of endophyte isolates, sampling locations showed different results (Figure 3). The maximum number of isolates was found in samples of Golestan (149) followed by Guilan (138), Mazandaran (136), Zanjan (126), Qazvin (124), and Ardabil (118) (Table 6). Also, Endophytes of the Golestan location had the highest colonization rate (9.19), followed by Mazandaran, Guilan, Zanjan, Qazvin, and

Ardabil, respectively (Table 6). Moreover, *Alternaria*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Cladosporium* were the dominant genera, and they were found in the majority of sampling sites as well (Figure 3). In general, leaf and root samples from the Guilan location and stem samples from the Mazandaran location had the most diverse endophytic assemblage (Figures 3 b and c).

Table 6- The Numbers and Percentages of Colonized Segments of *C. avellana* according to Six Sampling Locations in Iran

Sampling location	No. of samples	No. of fungi isolated	Endophyte species	CF%
Golestan	810	149	23	18.39
Mazandaran	810	136	22	16.79
Guilan	810	138	23	17.03
Zanjan	810	126	17	15.55
Ardabil	810	118	21	14.56
Qazvin	810	124	21	15.30
Total	4860	791	24	

Among all the sampling areas, endophytes from the Guilan location had the maximum Shannon–Wiener index (2.91) and Simpson’s index (0.95), while Zanjan had the least diversity indices (Table 7). In contrast, the stem had the highest diversity indices among the studied organs. The highest species richness and evenness of the endophytic fungi communities in tissues were observed in

the stem (4.14 and 0.95), while in sampling sites, Guilan had the highest level of species richness (4.45) and Ardabil showed the highest species evenness (0.94). Maximum Jaccard similarity coefficients (Jc) were found between Guilan and Mazandaran locations, whereas it was the lowest between Zanjan and Qazvin (Figure 4).

Table 7- The Results of Species Diversity Indices for Collected Data According to Tissues and Locations from *C. avellana* in Iran

index	Tissue type			Locations					
	Root	Stem	Leaf	GOL	GI	MZ	ZN	AR	QZ
Species richness-margalef	3.877798	4.144478	3.494313	4.391	4.458	4.275	3.298	4.142	4.163
Shannon–Wiener	2.755025	2.992317	2.665741	2.815	2.915	2.717	2.499	2.868	2.797
Simpson's	0.923569	0.947884	0.90749	0.9272	0.951	0.9172	0.904	0.9428	0.936
pielou's evenness	0.878657	0.954337	0.875586	0.8979	0.9297	0.879	0.882	0.9419	0.918

GOL: Golestan; MZ: Mazandaran; GI: Guilan; ZN: Zanjan; AR: Ardabil; QZ: Qazvin

Ecological Associations: Correspondence analysis revealed a clear distinction between endophytic fungal communities obtained from tissues and different sampling areas (Figures 5 and 6). The analysis showed that some isolates have an affinity for a specific tissue or area. Moreover, in correspondence analysis, the

isolates were categorized based on their abundance in each tissue or area. For example, *Paraphaeosphaeria sporulosa* was exclusively isolated from the root in the Golestan location (Figures 3, 5, and 6). *Cladosporium herbarum* was categorized more frequently from the Qazvin location (Figures 3 and 5).

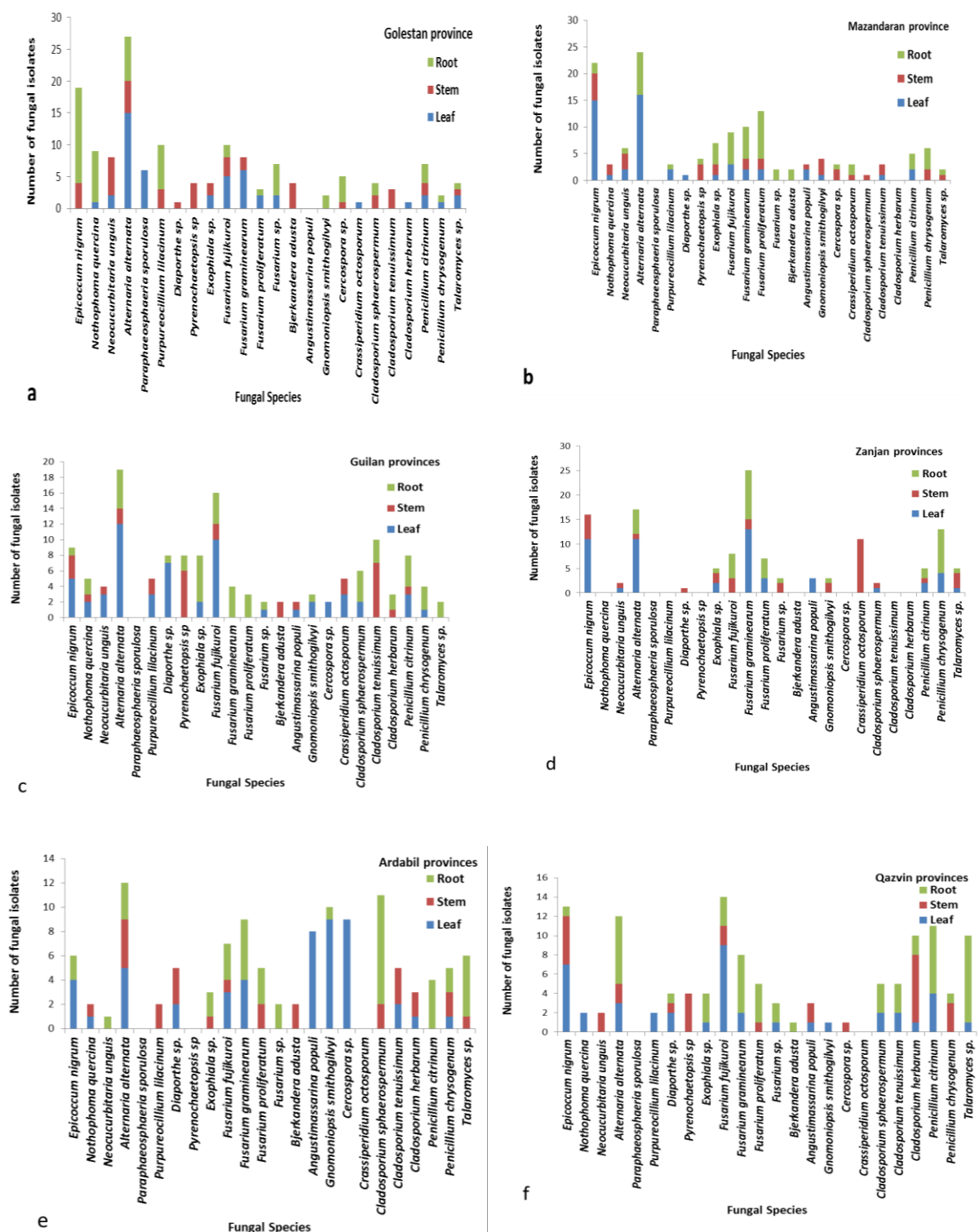


Fig. 3- Distribution of endophytic fungi in tissues according to sampling location and based on the species presence or absence data; a. GOL: Golestan; b. MZ: Mazandaran; c. GI: Guilan; d. ZN: Zanzan; e. AR: Ardabil; f. QZ: Qazvin

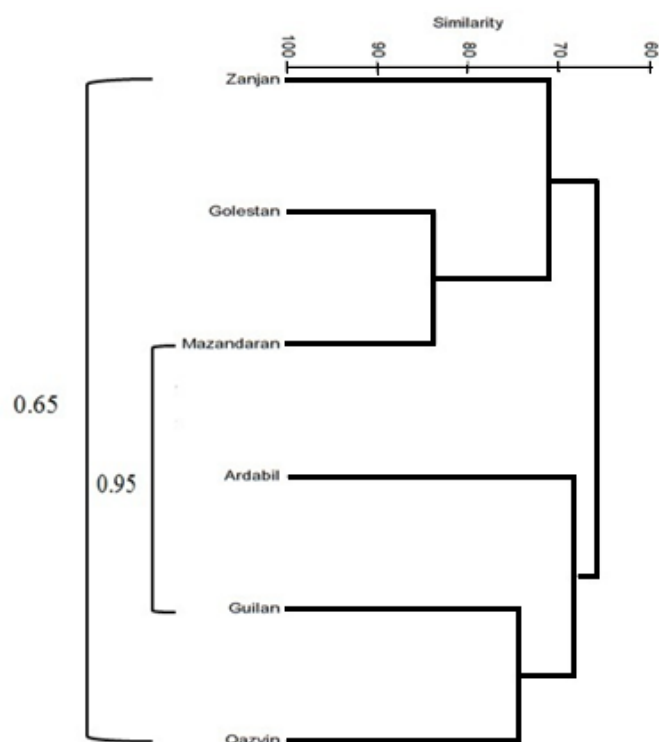


Fig. 4- Analysis of the similarity degree using Jaccard method (32) among six provinces based on endophytic communities isolated from *C. avellana* tissues

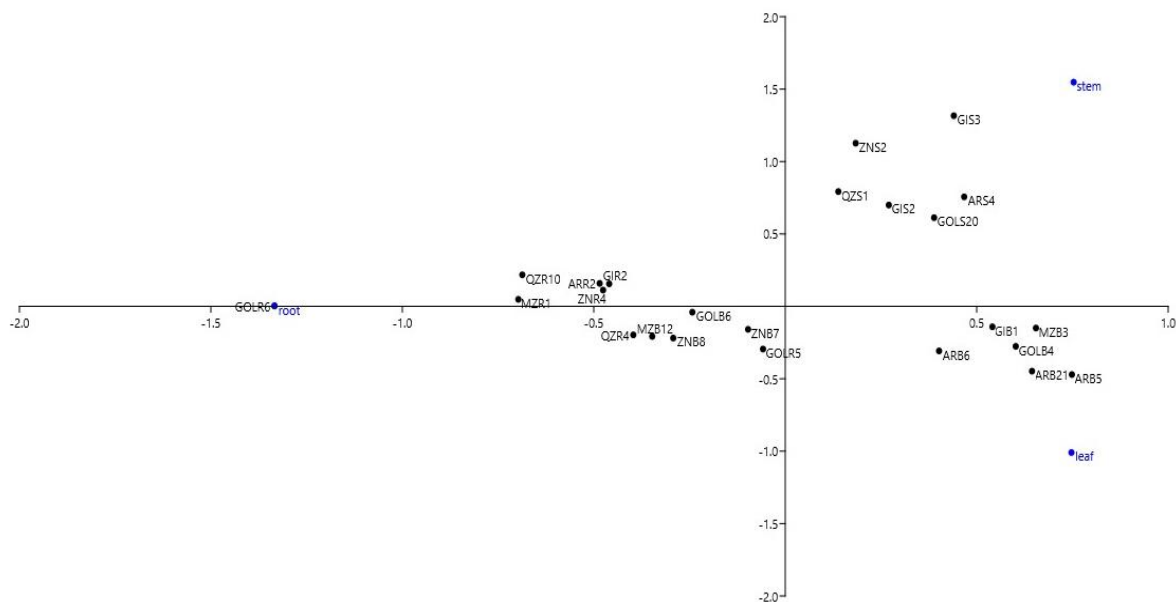
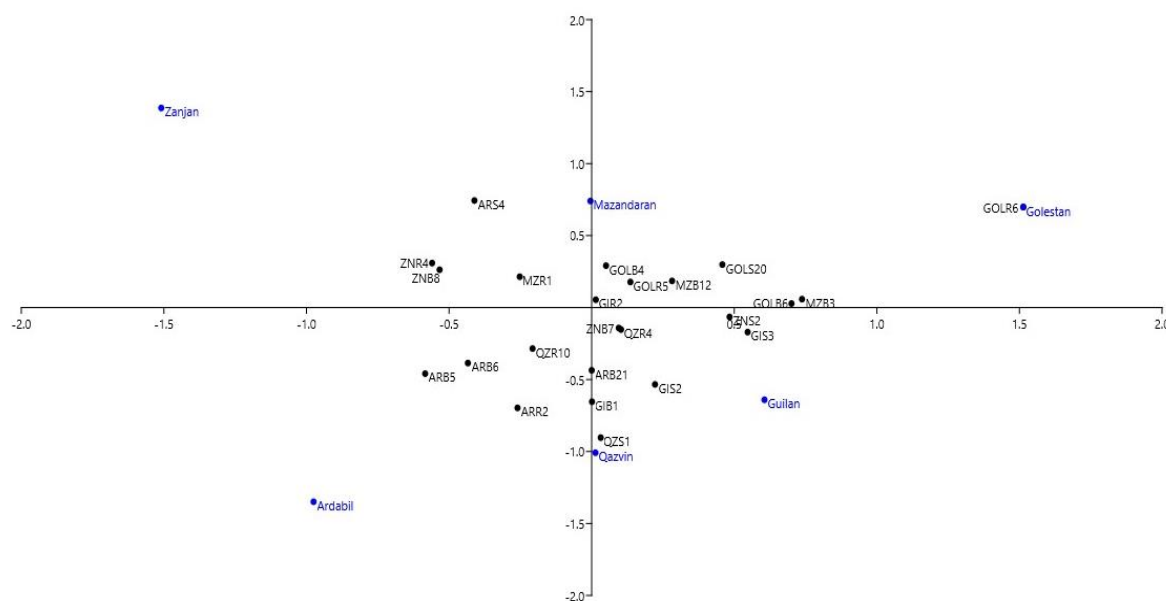


Fig. 5- Component analysis (CA) of fungal endophytes isolated from different tissues of *C. avellana*



avellana

Discussion

According to the results of the present study, a variety of endophytic fungi colonize various organs of the hazelnut trees. All of the 791 isolates were morphologically and molecularly classified into 24 species, most of the isolates belonged to the phylum Ascomycetes (Table 4). Indeed, in most studies, Ascomycetes are reported as endophytic fungi and also as endophytic mycobiota of other woody plants (34, 35). Basidiomycetes' endophytes have only been reported in a limited number of studies (36).

The results obtained for fungal frequency and diversity index in hazelnut tissues show that fungal frequency in leaves is higher than other tissues, which is consistent with Kharwar et al.'s study (37). They found that leaves harbored the maximum colonization of endophytic fungi greater than stem. Although the fungal frequency in the stem is lower, fungal diversity in the stem is higher than the leaf and root (Table 4). It appears that the fungal richness in stems of woody plants is higher than other organs. Tissues with a longer lifespan are more exposed to endophyte inoculation, and thus, have the greatest fungal richness in plant life history

(38). Of course, the stem and bark of woody plants have a longer lifespan and are exposed to air, rain, and dust throughout the year. As a result, stem structures and substrates can affect fungal infections and increase their colonization frequency and species richness (39, 40). Conversely, leaf lifespan (even in evergreen trees) is not so long and doesn't last as long as stem and root (41). Since *C. avellana* is a deciduous plant and its leaves have short durability, it can explain lower species diversity and species richness in leaf endophytes. In addition, the distribution and frequency of endophytic fungi may be affected by differences in the chemical content (terpenoids, phenols, and tannins) of tissues and organs (42). However, certain fungal taxa are able to utilize certain organs and survive in a particular tissue (43), for example, CA showed increased occurrence and frequency of certain species in specific tissues. Previous studies in other plants have also indicated the tissue-specificity of endophytes (36, 44, 45). In the present study, *Paraphaeosphaeria sporulosa* was isolated only from the root, whereas, some species colonized more than one tissue type

(i.e. *Pyrenochaetopsis* sp. and *Bjerkandera adusta* only in stems and roots). *Angustimassarina* sp. occurred only in leaves and stems (Figures 1a and 5). Other studies also have shown similar results about the distribution of endophytic fungi exclusively in one or more tissues and sites (46, 47, 48).

Changes in the assemblage of endophytes present in a single host plant at different study locations include a relatively persistent group of fungal species characterized by several dominant species (42, 49), which is also supported by this study. *Alternaria alternata*, *Epicoccum nigrum*, *Fusarium equiseti*, and *Fusarium fujikuroi* were recorded as the most dominant species with different frequencies in various hazelnut tissues in each sampling location (Figure 3), which is in agreement with another report on *Brassica napus* endophytes (50) and medicinal plants (51). For example, *A. alternata* was the predominant species in the leaves and roots at the Mazandaran location as well as the main species in the roots and stems at the Golestan site. *F. equiseti* was the predominant endophyte in leaves and roots at the Zanjan location. However, at the Qazvin, Golestan, and Mazandaran sites, it was the main species only in roots. *E. nigrum* was also the predominant species in leaves, stems, and roots at Mazandaran, and in leaves at the Golestan location. Such endophyte distribution indicates the influence of tissue type in harboring fungi (2, 52), as well as the ability of some endophytes to move from one part of the host to another (53). The presence or absence of some endophytic fungi in certain tree tissues may indicate the endophytes priority for the colonization of host tissues during the establishment of their symbiosis (54).

Different geographical and climatic conditions also affect the colonization of plant tissues by endophytic fungal communities (55, 56). The geographical

heterogeneity of fungal communities has been explained by dispersion limitations, host domain range, the evolution of regional adaptability, and environmental selection (56, 57). The findings of this study also indicate the influence of geographical factors on the distribution of endophytic fungi in hazelnut trees. It appears that the greater abundance and diversity of *C. avellana* endophytic fungi in the northern regions of Iran (Golestan, Mazandaran, and Guilan) is the result of their favorable geographical location and suitable climatic conditions. As reviewed by Vacher et al. (58), Fitt et al. (59), and Sadeghi et al. (60), rainfalls mainly contributed to shaping the endophytic community. Rainfalls might be important for fungal spore's dispersion and colonization of endophytes and also high humidity help fungal spore germination. In this regard, the results of the present study are consistent with the results of the above studies.

In addition, the moderate annual temperature may allow the fungi to survive longer and thus, increase their success rate in colonizing plant tissues (41). Therefore, the highest abundance and richness of fungi observed could be due to the high temperature or rainfall observed during these areas. In contrast, endophytes isolated from the northwestern regions of Iran (Zanjan, Ardabil, and Qazvin) have less species richness and diversity (Table 7). These areas are highly cold and icy during some seasons, which can affect the colonization rate of hazelnut tissues by endophytic fungi. Hence, the climate can affect the richness of endophytic species in different regions (61). Researchers believe that the widest biodiversity of endophytes occurs in tropical and temperate regions (62, 63, 64). Also, the results of the present study indicated that the microbial abundance decreased with the altitude (Tables 1 and 6) which is stated in previous studies on endophytes of other

psychrophilic (65, 66) and Mediterranean plants (67).

The effects of geographical factors also improved the composition of endophytes in plant tissues, provided that the sampling areas are distant or geographically different. This result was in agreement with previous studies in which climatic factors influence the endophytic community in organs (61, 68, 69). This result was predictable because fungi might be subjected to different selection pressures at different ecological sites, which can lead to the selection of a very specific fungal population in each location (12). The total isolates of hazelnuts at six sampling locations were compared by the Jaccard similarity index (Jc) (Figure 4). As seen, the highest observed overlap in *C. avellana* communities is in Golestan, Mazandaran, and Guilan sampling locations. This finding is logical because these locations share more similar ecological features than the rest of the sampling locations. It can be concluded that the environmental conditions and type of tissues (examined in this study) and other factors such as seasonality, sampled plant/tissue, age, and phenological stage examined in other studies (13, 69, 70) may affect these relationships.

However, in the present study, the role of endophytes in the production of secondary metabolites and increasing the resistance of hazelnuts to cold stress has not been investigated. But, it is suggested that endophytes can perform or modulate various essential functions in plants directly affecting growth, development, and tolerance to different biotic and/or abiotic conditions (71, 72). In extreme environments which impose severe limitations for growth, the establishment of functional symbioses with microorganisms can play a fundamental role in the adaptation of plants to environmental stress events due to a greater accumulation of solutes, reduced foliar conductance,

decreased transpiration, or the formation of thicker cuticles (73, 74, 75, 76). Many endophytes can also produce secondary metabolites, which offer protection against different phytopathogens and/or herbivores including insects (36). In summary, endophytes confer strong fitness to their host plants and this may affect the adaption of plants to different climates.

The overall findings of the present study showed that fungal endophytes densely inhabit *C. avellana* tissues and that spatial structure tends to influence the species composition. It was also shown that hazelnut endophytic communities include a diverse range of fungi. Since this is the first work on the diversity and distribution of hazelnut-related fungal endophytes under diverse environmental and geographical conditions, further research is needed to identify the functional and ecological significance of these endophytes. It is also essential to understand the role of endophytic fungi in a broader perspective about the production of secondary metabolites, biochemical control agents, and response to environmental conditions in the host micro-ecosystem.

Acknowledgement

The authors of the study are thankful to Mr. Hosseini, the expert of the Central Laboratory, Gonbad Kavous Agricultural University for all laboratory assistance. We are grateful to Golestan province Natural Resources Department, Maraveh Tappeh area Agricultural Service Center, Minoodasht Agricultural Jihad, Tonekabon Agricultural Jihad, Dohezar area Agricultural Service Center, Guilan province Agricultural Jihad, Qazvin Research Center, Ardabil Agricultural Jihad and Zanzan Research Center and Agricultural Jihad for their cooperation with sampling. This article is an excerpt from the PhD dissertation of Zabol University, Iran.

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