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ORIGINAL ARTICLE

Diversity of endophytic fungi of common yew (*Taxus baccata* L.) in Iran

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Abstract To characterize the endophytic fungi of common yew, 125 fungal strains were isolated from 80 healthy twig and bark samples. Of them, 41 (32.8%) isolates were identified on the basis of morphological features and 84 (67.2%) isolates were sterile. Of the 41 morphotypes, 40 isolates belonged to 21 genera and 26 species, with an isolate as anamorphic *Xylaria*. Most of the identified endophytic fungi belonged to mycelia sterilia (67.2%) and Ascomycota (32%) and the others belonged to Zygomycota (0.8%). *Cladosporium* and *Alternaria* were the dominant genera. Most of the identified species are new endophytes for common yew and *Pseudodictyosporium elegans*, *Cladosporium basiinflatum*, *C. perangustum*, *C. subtilissimum*, *Geniculosporium serpens*, *Phoma pratorum*, *Paraphoma fimeti*, *Phomopsis archeri*, *Sclerostagonospora cycadis*, and *Seimatosporium* cf. *pezizoides* are reported as new taxa for the mycoflora of Iran. ITS rDNA phylogeny of 26 selected isolates revealed four clades, 1, 2, 3, and 4, corresponding to Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Dothideomycetes, respectively. Clade 1 included four groups, A, B, C, and D, representing, respectively, the Xylariales, Trichosphaeriales, Hypocreales, and Diaporthales identified. The results have shown that *T. baccata* in Iran harbors a wide and significant diversity of fungal endophytes.

Keywords Fungus · Taxon · Morphology · Molecular phylogeny and biodiversity

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Introduction

Common yew (*Taxus baccata* L.) is an evergreen dioecious gymnosperm tree belonging to the family Taxaceae, which grows naturally in the vast areas from the Astara to Ali Abad regions in Caspian Sea coastal provinces, including Gilan, Mazandaran, and Golestan in the north of Iran. Rare plants growing in remote locations represent rich potential sources of novel microorganisms, which may have the ability to produce secondary metabolites of medical and industrial interest. The term "endophyte" was first introduced by de Bary in 1866 and initially applied to microorganisms that reside in living plant tissue, persisting for the whole or part of the life cycle of the plant without causing negative effects (Wang et al. 2008). Endophytic fungi are to be found in almost all plants. These include trees, grass, algae, and herbaceous plants (Huang et al. 2001; Hyde and Soytong 2008). The plant-associated habitat is a dynamic environment in which many factors affect the structure and composition of species that colonize different tissues. It has been previously shown that endophytic communities may vary spatially in many kinds of plants (Rivera-Orduña et al. 2011). Caruso et al. (2000) recovered 150 fungal strains from woody and herbaceous tissues, mostly from *Taxus baccata* and less from *Taxus brevifolia*. *Alternaria*, *Fusarium*, and *Mucor* were the most dominant genera (Table 3). Wang et al. (2008) obtained 40 endophytic fungal isolates from healthy leaves of nine *Taxus mairei* trees on Fushan, Taiwan. Based on morphological and molecular characters, *Colletotrichum* and *Fusarium* were estimated as the most frequently isolated endophytic fungi from leaves of *T. mairei* (Table 3). Liu et al. (2009) obtained 115 endophytic fungal isolates from bark pieces of *Taxus chinensis*. Isolates were grouped into 23 genera based on the morphological features and sequence analysis of the ITS1-5.8S-ITS2 region. Among them, *Diaporthe*, *Phomopsis* (anamorph of *Diaporthe*), *Acremonium*, and *Pezicula* were the dominant genera (Table 3). Fungal

endophytes form a very various polyphyletic group, composed frequently of species of the phylum Ascomycota (Arnold and Lutzoni 2007; Huang et al. 2001), although there are reports on species from other fungal phyla, such as Basidiomycota (Rungjindamai et al. 2008). Rivera-Orduña et al. (2011) recovered 116 fungal isolates from bark, branches, leaves, and roots of healthy yew trees (*T. globosa* Schltdl.).

Based on morphological characteristics, 57 isolates were selected for taxonomic characterization by phylogenetic analysis of their 28S rDNA sequences (Table 3). The fungal isolates belonged to Ascomycota (77.2%) and Basidiomycota (22.8%). Some endophytic fungi have been found to produce chemical compounds similar to those produced by their host plants. Others have been shown to be possible sources of new natural products effective in medicine, agriculture, and industry (Liu et al. 2009). Many endophytic fungi have the potential to synthesize various bioactive metabolites such as Taxol, which may be directly or indirectly used as therapeutic agents against numerous diseases (Kusari and Spiteller 2012; Kusari et al. 2012; Strobel et al. 1996). In addition, the effect of endophytes has been shown in mutualism, decreasing the herbivory by the production of toxins and increasing the tolerance of host plants to biotic and abiotic stress factors (Hyde and Soytong 2008; Kusari et al. 2012; Sieber 2007). In that regard, they colonize ecological niches where the plant pathogens exist and fungal endophytes often increase the resistance against the pathogen and improve the growth of their host plants (Hassan 2007).

In Iran, no comprehensive study for the identification of common yew endophytic fungi has been done, although several efforts to identify some Taxol-producing fungal endophytes of common yew have been performed. In a survey, Nasiri Madiseh et al. (2010) recovered 80 fungal isolates with unknown species attribution as endophytes of common yew and investigated their ability to produce Taxol. Based on their results, only five isolates were able to produce Taxol up to 21.74 μg/L. Also, Mirjalili et al. (2012) isolated 25 endophytic fungal strains from the bark of common yew (*T. baccata*) in Iran and studied their potential to produce Taxol. Among them, only *Stemphylium sedicola* SBU-16 was proved to produce 6.9 μg/L Taxol and 2.2 μg/L its intermediate compound, 10-deacetylbaccatin III.

The very important step in the biological production of medically and industrially important substances from endophytic fungi is to identify the endophytic diversity of certain host plants in the defined regions. Thus, the main objectives of the present study were the isolation and morphological identification of some endophytic fungi from common yew and, also, the investigation of their diversity and phylogeny using the sequences of the ITS1-5.8S-ITS2 rDNA region in Iran.

Materials and methods

Plant materials and fungal isolates

Healthy and symptomless barks and 1- or 2-year-old twigs of common yew (*Taxus baccata* L.) from the Zarin Gol region of Ali Abad city in Golestan province as the main habitat of the plant in the north of Iran, and also from Karaj, in Alborz province, in the central part of Iran, were collected by the first author during the summer and autumn seasons of 2011. The samples were immediately transferred to a mycology laboratory and stored at 4 °C for future use. The method modified by Strobel and Daisy (2003) was used for surface sterilization of plant samples. Plant materials were thoroughly washed in running tap water for 10 min before disinfection. The plant samples were surface disinfected with 70% (v/v) ethanol for 1 min and subsequently rinsed with sterile water and the outer tissue of the plant materials were removed with a sterile scalpel. Disinfected twigs and barks were cut in small pieces (0.5 × 0.5 cm) and then placed in Petri dishes containing water agar (WA). Petri dishes were kept in continuous dark conditions at 25 °C for several days. After growth of the fungal colonies around the plant tissues, the fungal pure cultures were obtained by the hyphal tip method. Several hyphal tips were taken from the margin of colonies growing around the same plant tissues and transferred to potato dextrose agar (PDA) culture medium. Inoculated Petri dishes were kept at 25 °C for 7–10 days until the pure colonies of the fungal isolates appeared. For long-term storage, fungal isolates were grown on sterile filter papers placed on PDA and colonized filter papers were taken from the surface of culture medium and dried at room temperature and finally stored at −20 °C for future use.

Morphological characterization of fungal endophytes

Fungal isolates were first identified as morphospecies. For morphological identification, the fungal isolates were grown on different culture media depending on their type and ability to sporulate. First, young colonies of the fungal isolates were obtained by transferring one of the stored filter papers belonging to each isolate to PDA culture medium and incubation of inoculated Petri dishes at 25 °C in continuous dark conditions for several days. In cases where the endophytic fungus did not sporulate on PDA, it was grown on oat meal agar (OA), malt extract agar (MEA), or corn meal agar (CMA) to promote sporulation. Otherwise, to induce sporulation, leaf and twig extracts of common yew were added to the mentioned culture media or surface-sterilized leaves of the plant were put on the culture media beside the fungal colonies. For the latter, the cultures were transferred to a 12-h fluorescent light (around 400 nm) and 12-h continuous dark condition (Gazis and Chaverri 2010). According to the type of the fungal isolates, morphological studies were done on different culture media, temperature,

and light conditions, as provided by Chesters and Greenhalgh (1964), Booth (1971), Boerema et al. (2004), Ho et al. (2004), Simmons (2007), Bensch et al. (2010), Kirschner et al. (2013), Woudenberg et al. (2013), and others. Colony features, mycelium color and structure, type of conidioma, conidiophores, conidiogenous cells, and conidial features (size, color, shape, septation, ornamentation, etc.) were studied for the characterization and identification of morphospecies in microscopic slide preparations using lactophenol or lactophenol cotton blue under an Olympus, BH2 light microscope.

Phylogenetic analyses

For molecular identification and investigation of phylogeny, several mycelial plugs from the margins of pure cultures of the fungal isolates were transferred to 100-mL Erlenmeyer flasks containing 50 mL potato dextrose broth (PDB). Inoculated flasks were kept at 25 °C for 7–10 days on a shaker at 120 rpm, depending on the isolate's growth rate. Mycelia were harvested and washed by vacuum filtration on sterile filter paper and then lyophilized and stored at −20 °C. Genomic DNA was extracted from lyophilized mycelia by the method of Liu et al. (2000) or Zhong and Steffenson (2001). Polymerase chain reaction (PCR) amplification of the ITS1-5.8S-ITS2 region of ribosomal DNA was performed using the universal ITS1 and ITS4 primers (White et al. 1990) in a Palm-Cycler device (Corbett Research, Australia). The concentrations of PCR components in 25-μL PCR reactions were as follows: 50–100 ng/μL of template DNA, 0.4 μM of each primer, and 10 μL of 2× Master Mix (Ampliqon, China). The PCR amplification conditions were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 50 °C for 70 s, 72 °C for 90 s, and a final extension at 72 °C for 7 min. To ensure amplification of the ITS1-5.8S-ITS2 region in the investigated fungal isolates, PCR amplification products were separated by electrophoresis in 1% (w/v) agarose gels and stained with ethidium bromide for visual examination. Then, the PCR products were sent to Bioneer Corporation (Daejeon, South Korea) for purification and sequencing. The obtained ITS1-5.8S-ITS2 sequences of rDNA were viewed and edited using the Chromas software ver. 2.1 (Technelysium, Australia) and the edited sequences were compared with deposited sequences in the GenBank (NCBI) by the BLAST search tool (Altschul et al. 1997). All edited ITS1-5.8S-ITS2 sequences of the newly identified fungal isolates, along with 28 obtained sequences from GenBank (Table 1) and *Peziza vesiculosa* Bull. (AF491625) as the outgroup taxon, were aligned using ClustalW with default settings as available in the MEGA software ver. 5.2 (Tamura et al. 2011). The phylogenetic trees were obtained by the maximum likelihood (ML), maximum parsimony (MP) (Felsenstein 1981), and neighbor-joining (NJ) (Saitou and Nei 1987) methods using the MEGA software ver. 5.2. The confidence of individual clades was assessed by bootstrap analyses (Felsenstein 1985) with 1000 heuristic replicates (Hedges 1992), and values above 50% are shown on the branches. The newly obtained sequences of the ITS1-5.8S-ITS2 rDNA region in this study were deposited in the GenBank database using the Sequin software (NCBI, Bethesda, MD, USA).

Results

Morphological characterization of fungal endophytes

Among 125 isolates of endophytic fungi that were recovered from 80 twig and bark samples of *T. baccata* in Iran, 41 isolates (32.8%) were identified on the basis of morphological features of asexual or very rarely sexual reproduction stages and 84 isolates (67.2%) were mycelia sterilia with no asexual or sexual reproduction, could not be identified, were omitted from phylogenetic investigation, and, therefore, their cultures were discarded. Of the 125 recovered isolates, 44 isolates were obtained from healthy twigs and 81 isolates were recovered from bark samples. Only one isolate had sexual reproduction as well as asexual reproduction and was identified as *Absidia spinosa*. Of the 41 morphotypes, 37 isolates belonged to 21 genera and 26 species, three morphotypes considered as unknown species of the three respective genera (*Coniothyrium*, *Cyclothyrium*, and *Seimatosporium*), and, finally, an isolate was recognized as anamorphic *Xylaria* in the true fungi (Table 2). *Cladosporium* with nine isolates was the dominant genus, followed by *Alternaria* with four isolates, belonging to Davidiellaceae and Pleosporaceae, respectively (Schoch et al. 2009; Hyde et al. 2013; Ariyawansa et al. 2015). Among the 26 identified morphospecies, *Aureobasidium pullulans*, *Pseudodictyosporium elegans*, *Cladosporium herbarum*, *Cladosporium perangustum*, and *Lecanicillium lecanii* were the dominant species. *Pseudodictyosporium elegans*, *Cladosporium basi-inflatum*, *Cladosporium perangustum*, *Cladosporium subtilissimum*, *Geniculosporium serpens*, *Paraphoma fimeti*, *Phoma pratorum*, *Phomopsis archeri*, *Sclerostagonospora cycadis*, and *Seimatosporium* cf. *pezizoides* are new taxa for the mycoflora of Iran. Also, *Absidia spinosa*, *Alternaria alternata*, *Alternaria atra*, *Alternaria longipes*, *Aspergillus niger*, *Aureobasidium pullulans*, *Pseudodictyosporium elegans*, *Cladosporium basi-inflatum*, *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Cladosporium perangustum*, *Cladosporium subtilissimum*, *Cytospora leucostoma* (current name *Leucostoma persoonii*), *Epicoccum nigrum*, *Fusarium lateritium* (current name *Gibberella baccata*), *Fusarium tricinctum* (current name *Gibberella tricincta*), *Geniculosporium serpens* (current name *Nemania serpens*), *Lecanicillium lecanii*, *Nigrospora oryzae*, *Paraphoma fimeti*, *Phoma pratorum*, *Phomopsis archeri*, *Sclerostagonospora cycadis*, *Seimatosporium* cf. *pezizoides*, *Stachybotrys chartarum*, *Truncatella angustata*,

Table 1 ITS1-5.8S-ITS2 sequences used in this study from GenBank (NCBI)

Taxa	Isolate	GenBank no.	Reference
Nemania serpens	BF330	EF155504.1	–
Xylaria hypoxylon	152	GU300096	Hsieh et al. (2010)
Xylaria bambusicola	162	GU300088	Hsieh et al. (2010)
Seimatosporium walkeri	CPC 17644	JN871207	Barber et al. (2011)
Truncatella angustata	BPF5	JN038391	–
Nigrospora oryzae	Y2-1	FJ827037	–
Leucostoma persoonii	wxm92	HM061320	–
Fusarium lateritium	wxm147	HM061323	–
Fusarium tricinctum	T06	FJ459974	–
Verticillium cf. *lecanii*	RCEF104	AF368809	–
Stachybotrys chartarum	UAMH 6417	AF206273	Haugland and Heckman (1998)
Aspergillus niger	A-3204	JQ316522	Alborch et al. (2012)
Cladosporium subtilissimum	UFMGCB 3843	JQ346203	–
Cladosporium basi-inflatum	CBS 822.84	HM148000	Bensch et al. (2010)
Cladosporium cladosporioides	M61	JQ936096	–
Cladosporium perangustum	CPC 15192	HM148149	Bensch et al. (2010)
Davidiella tassiana	ATCC 6506	AY361974	Park et al. (2004)
Cyclothyrium sp.	B14-3242	FJ025227	–
Massarina corticola	–	AF383957	Liew et al. (2002)
Alternaria longipes	EGS30-033	AY751457	Xue et al. (2005)
Alternaria alternata	786949	GU594741	Alhanout et al. (2010)
Alternaria atra	UAMH 7840	AY625072	–
Epicoccum nigrum	PC4-3	JX914480	–
Leptosphaeria sp.	E-000535652	JN545781	Cueva et al. (2011)
Coniothyrium cereale	1401	AM262407	–
Phoma fimeti	M 4937	JN003240	–
Paraphoma fimeti	–	AB488489	–
Peziza vesiculosa	NSW 6124	AF491625	Hansen et al. (2002)

Coniothyrium sp., *Cyclothyrium* sp., and *Seimatosporium* sp. are reported as new endophytic fungi of common yew throughout the world. The identified isolates were deposited in the Iranian Fungal Culture Collection (IRAN…C) at the Iranian Research Institute of Plant Protection, Tehran, Iran and in the University of Tehran fungal culture collection (UTFC) at the Department of Plant Protection, Faculty of Agricultural Science and Engineering, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran (Table 2). All 41 recovered endophytic fungal isolates placed in nine different groups correspond to their taxonomy at the order level. Eight groups including Xylariales, Trichosphaeriales, Hypocreales, Diaporthales, Capnodiales, Dothideales, Eurotiales, and Pleosporales in the phylum Ascomycota and one group including Mucorales in the phylum Zygomycota were identified. All identified endophytic fungi were deposited in the herbarium of Iran.

Phylogenetic analyses

The total number of 26 newly recovered isolates representing 17 genera, 22 species, and one as anamorphic *Xylaria* were selected for phylogenetic reconstruction using the ITS1-5.8S-ITS2 rDNA sequences. The newly obtained sequences were deposited in the GenBank (NCBI) database with accession numbers from KF573970 to KF573995 (Table 2). In the studied fungal isolates, length of the ITS1-5.8S-ITS2 sequences after editing varied considerably from 476 in *Cladosporium basi-inflatum* 11TG to 739 in *Stachybotrys chartarum* 114 TB. Alignment of 26 sequences along with 28 obtained sequences from GenBank (Table 1) increased the characters of the dataset to 896. None of the characters were eliminated during the phylogenetic analyses. The phylogenetic trees obtained with the ML, MP, and NJ methods were considerably similar in topology, so only the

Table 2 Identified endophytic fungi

Taxa	Isolate	NCBI accession no.	Locality	IRAN accession no.	Plant tissue
Absidia spinosa[a]	22TG	–	Ali Abad, Golestan	IRAN 2167C	Bark
Alternaria alternata	2TG	KF573970	Ali Abad, Golestan	UTFC-TB1	Bark
	13 TB	–	Karaj, Alborz	UTFC-TB2	Twig
Alternaria atra[a]	125 TB	KF573995	Karaj, Alborz	UTFC-TB3	Bark
Alternaria longipes[a]	16TG	KF573971	Ali Abad, Golestan	UTFC-TB4	Bark
Aspergillus niger	117 TB	KF573973	Karaj, Alborz	IRAN 2168C	Bark
Aureobasidium pullulans[a]	119 TB	–	Karaj, Alborz	UTFC-TB5	Bark
	112 TB	–	Karaj, Alborz	UTFC-TB6	Twig
	113 TB	–	Karaj, Alborz	UTFC-TB7	Twig
Pseudodictyosporium elegans[b]	36TG	KF573974	Ali Abad, Golestan	UTFC-TB8	Bark
	48TG	–	Ali Abad, Golestan	UTFC-TB9	Bark
	49TG	–	Ali Abad, Golestan	UTFC-TB10	Bark
Cladosporium basi-inflatum[ab]	11TG	KF573975	Ali Abad, Golestan	IRAN 2159C	Twig
Cladosporium cladosporioides	35TG	KF573976	Ali Abad, Golestan	IRAN 2169C	Twig
Cladosporium herbarum	32TG	KF573977	Ali Abad, Golestan	IRAN 2170C	Bark
	96 TB	–	Karaj, Alborz	UTFC-TB11	Bark
	69TG	–	Ali Abad, Golestan	UTFC-TB12	Bark
Cladosporium perangustum[ab]	34TG	KF573978	Ali Abad, Golestan	IRAN 2160C	Twig
	17TG	–	Ali Abad, Golestan	UTFC-TB13	Bark
	47TG	–	Ali Abad, Golestan	UTFC-TB14	Twig
Cladosporium subtilissimum[ab]	85 TB	KF573979	Karaj, Alborz	IRAN 2161C	Bark
Coniothyrium sp.	97 TB	KF573980	Karaj, Alborz	UTFC-TB15	Bark
Cyclothyrium sp.	84 TB	KF573981	Karaj, Alborz	IRAN 2171C	Bark
Cytospora leucostoma[a]	88 TB	KF573982	Karaj, Alborz	IRAN 2172C	Twig
Epicoccum nigrum	54TG	KF573983	Ali Abad, Golestan	IRAN 2173C	Bark
Fusarium lateritium	56TG	KF573984	Ali Abad, Golestan	UTFC-TB16	Bark
Fusarium tricinctum[a]	1TG	KF573985	Ali Abad, Golestan	UTFC-TB17	Bark
Geniculosporium serpens[ab]	11(2)TG	KF573986	Ali Abad, Golestan	IRAN 2174C	Twig
Lecanicillium lecanii[a]	20TG	KF573987	Ali Abad, Golestan	UTFC-TB18	Bark
	23TG	–	Ali Abad, Golestan	UTFC-TB19	Bark
	12TG	–	Ali Abad, Golestan	UTFC-TB20	Twig
Nigrospora oryzae	15TG	KF573988	Ali Abad, Golestan	UTFC-TB21	Bark
Paraphoma fimeti[ab]	79 TB	KF573989	Karaj, Alborz	IRAN 2175C	Bark
Phoma pratorum[ab]	101 TB	–	Karaj, Alborz	IRAN 2176C	Bark
Phomopsis archeri[ab]	73 TB	–	Karaj, Alborz	IRAN 2177C	Bark
Sclerostagonospora cycadis[ab]	26TG	KF573990	Ali Abad, Golestan	UTFC-TB22	Twig
Seimatosporium cf. *pezizoides*[ab]	71 TB	KF573991	Karaj, Alborz	IRAN 2178C	Bark
Seimatosporium sp.	77 TB	KF573992	Karaj, Alborz	IRAN 2179C	Bark
Stachybotrys chartarum[a]	114 TB	KF573993	Karaj, Alborz	IRAN 2180C	Twig
Truncatella angustata[a]	81 TB	KF573994	Karaj, Alborz	UTFC-TB23	Bark
Anamorphic *Xylaria*	4TG	KF573972	Ali Abad, Golestan	UTFC-TB24	Bark

[a] New endophytic fungi for *Taxus baccata* throughout the world

[b] New taxon for the mycoflora of Iran

ML tree is presented (Fig. 1). The ML and MP trees were similar to each other from top to bottom and consisted of four main clades, 1, 2, 3, and 4, and within clade 1, four groups, A, B, C, and D, were detected. Members of clades 1–4 belonged to taxonomic classes of fungi including Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Dothideomycetes, and members of groups A–D belonged to taxonomic orders of fungi including Xylariales, Trichosphaeriales, Hypocreales,

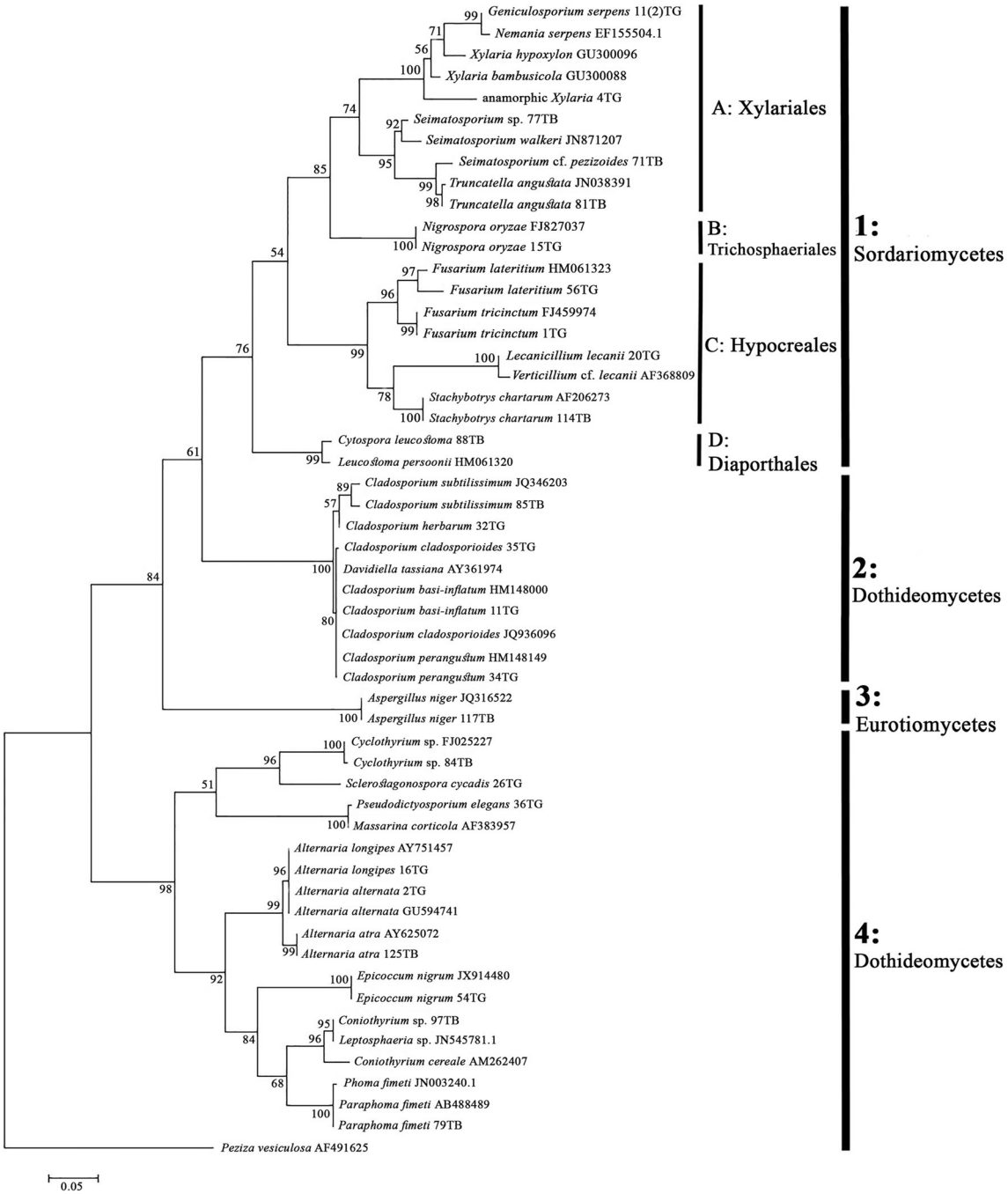

Fig. 1 Maximum likelihood tree of the ITS1-5.8S-ITS2 sequences of the endophytic fungi associated with *Taxus baccata* constructed by MEGA software ver. 5.2. *Peziza vesiculosa* was used as the outgroup. The numbers on branches are bootstrap scores (above 50%) obtained from 1000 replications. The *bars* indicate the nucleotide substitutions per site

and Diaporthales, respectively. In the ML tree, the bootstrap support for clades 1, 2, 3, and 4 were 76, 100, 100, and 98%, respectively. Also, in the ML tree, the bootstrap support for groups A, B, C, and D were 74, 100, 99, and 99%, respectively. In the NJ tree, the order of the clades was 1, 3, 2, and 4 and the order of the groups in clade 1 was A, B, D, and C as compared to the ML and MP trees. The composition of taxa in the identified clades and groups of the ML, MP, and NJ trees was the same. Clade 1 with 22 isolates (41.5%) was the biggest clade in the phylogenetic trees, followed by clade 4 with 19 isolates (35.8%), clade 2 with 10 isolates (18.9%), and clade 3 with 2 isolates (3.8%). In clade 1, group A with 10 isolates (18.9%) was the biggest group, followed by group C with 8 isolates (15%) and groups B and D each with 2 isolates (3.8%). Clade 1 consisted of 22 isolates belonging to 17 different taxa, including *Geniculosporium*, *Nemania*, *Xylaria*,

Seimatosporium, and *Truncatella* of the Xylariales; *Nigrospora* of the Trichosphaeriales; *Fusarium*, *Lecanicillium*, *Verticillium*, and *Stachybotrys* of the Hypocreales; and *Cytospora* and *Leucostoma* of the Diaporthales. Clade 2 was composed of ten isolates belonging to *Cladosporium* and *Davidiella* in Davidiellaceae of the Capnodiales. Clade 3 consisted of only two isolates belonging to *Aspergillus* in Trichocomaceae of the Eurotiales. Clade 4 was composed of 19 isolates belonging to 14 different taxa, including *Cyclothyrium*, *Sclerostagonospora*, *Pseudodictyosporium*, *Massarina*, *Alternaria*, *Ulocladium*, *Epicoccum*, *Coniothyrium*, *Leptosphaeria*, *Coniothyrium*, *Phoma*, and *Paraphoma*.

Discussion

A common method for the identification of plant endophytic fungi is the microscopic analysis of morphological features of fruiting bodies. However, a significant proportion of endophytic fungi such as mycelia sterilia never produce fruiting bodies in culture, because of adaptation to the host plants and the nature of their growth, and also the obligate endophytic fungi cannot be identified by this method, since they cannot be grown on cultures. Phylogenetic analysis of rDNA sequences has been successfully employed for the identification of different fungal morphospecies (Wang et al. 2008; Liu et al. 2009). In the present study, each isolate having fruiting structures was recovered and cultured on suitable culture media and then identified by morphology and studied by phylogenetic analysis of ITS rDNA sequences. Also, recovered mycelia sterilia without fructification were omitted from morphological and molecular investigations. According to the isolation and identification methods, we limited the present investigation to culturable fungi having fruiting structures. In other words, we neglected the obligate biotrophs that might be the important portion of the endophytic fungi of *T. baccata*. However, other molecular techniques based on DNA can be used with advantage to identify more rapidly and comprehensively the fungal diversity within *T. baccata* tissues.

Among the 125 isolates of endophytic fungi that were recovered from twigs and barks of common yew in Iran, 41 identified morphotypes belonged to 21 genera and 26 species of the true fungi. The isolates of *Cladosporium*, *Alternaria*, *Aureobasidium*, *Pseudodictyosporium*, and *Lecanicillium* were common among the recovered endophytic fungi. A number of fungi have been reported as endophytes in different *Taxus* species. Caruso et al. (2000) recovered 150 isolates from *T. baccata* tissues in Italy and identified 25 different genera, with *Alternaria* and *Fusarium* as the dominant genera. In *T. baccata*, endophytes were more frequent in woody tissues than herbaceous ones. Wang et al. (2008) found five genera and three unidentified endophytic fungi in 13 symptomless leaf samples of nine *T. mairei* trees in Taiwan. *Colletotrichum gloeosporioides* and *Fusarium solani* were the dominant species among 40 recovered endophytic fungal isolates. Liu et al. (2009) recovered 115 endophytic fungi isolates from bark segments of *T. chinensis* in China and grouped them into 23 genera, with *Diaporthe*, *Phomopsis*, *Acremonium*, and *Pezicula* as the dominant genera. Also, Rivera-Orduña et al. (2011) isolated 116 endophytic fungi from the bark, branches, leaves, and roots of healthy *Taxus globosa*. Of the 26 identified genera, the taxa *Alternaria*, *Aspergillus*, *Cochliobolus*, *Coprinellus*, *Hypoxylon*, *Polyporus*, and *Xylaria* were the most frequently isolated. It should be noted that the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Phoma*, and *Xylaria* are common to all four *Taxus* species (Table 3).

In the present study, the species including *Absidia spinosa*, *Alternaria atra*, *Alternaria longipes*, *Aureobasidium pullulans*, *Pseudodictyosporium elegans*, *Cladosporium basi-inflatum*, *Cladosporium perangustum*, *Cladosporium subtilissimum*, *Cytospora leucostoma*, *Fusarium tricinctum*, *Geniculosporium serpens*, *Lecanicillium lecanii*, *Paraphoma fimeti*, *Phoma pratorum*, *Phomopsis archeri*, *Sclerostagonospora cycadis*, *Seimatosporium* cf. *pezizoides*, *Stachybotrys chartarum*, and *Truncatella angustata* are new endophytic fungi for common yew trees throughout the world. *Pseudodictyosporium elegans*, *Cladosporium basi-inflatum*, *C. perangustum*, *C. subtilissimum*, *Geniculosporium serpens*, *Phoma pratorum*, *Paraphoma fimeti*, *Phomopsis archeri*, *Sclerostagonospora cycadis*, and *Seimatosporium* cf. *pezizoides* are new taxa for the mycoflora of Iran. Also, all identified species and strains in the present study are reported for the first time from *Taxus baccata* in Iran.

Most fungi reported as endophytes to date have been identified as ascomycetes. Basidiomycetous endophytes have only been reported in a limited number of studies (Sridhar and Raviraja 1995; Wang et al. 2005), with grasses (Sánchez Márquez et al. 2007, 2010), various liverworts (Duckett and Ligrone 2008), cocoa trees (Crozier et al. 2006; Thomas et al. 2008), and palms (Rungjindamai et al. 2008). In this study, most of the identified endophytic fungi belonged to mycelia sterilia (67.2%) and Ascomycota (32%), and the others belonged to Zygomycota (0.8%).

Endophytic fungi obtained from barks and twigs of *T. baccata* in Iran represented a phylogenetically diverse array of fungal taxa, including eight frequent species and 18 rare or not previously observed taxa (Table 2). The species composition of identified endophytic fungi in *T. baccata* between two studied geographic regions, Zarin Gol region of Ali Abad city in Golestan province in the north of Iran with temperate climate and forests, and Karaj city in Alborz province in the central part of Iran with semi-arid to arid climate, had significant differences. These two areas are separated by Alborz mountain chains. Only the genera *Alternaria* and *Cladosporium* were recovered from both areas, but with different species. The genera *Aspergillus*, *Aureobasidium*, *Coniothyrium*, *Cyclothyrium*,

Table 3 Comparison of identified endophytic fungi in three *Taxus* species

Taxus baccata in Iran, Jam Ashkezari and Fotouhifar	*Taxus baccata* in Italy, Caruso et al. (2000)	*Taxus mairei* in Taiwan, Wang et al. (2008)	*Taxus chinensis* in China, Liu et al. (2009)	*Taxus globosa* in Mexico, Rivera-Orduña et al. (2011)
Absidia spinosa	*Acremoniula*	*Colletotrichum* sp.	*Diaporthe eres*	*Lecythophora* sp.
Alternaria alternata	*Alternaria*	*C. gloeosporioides*	*Alternaria* sp.	*Aspergillus* sp.
Alternaria atra	*Aspergillus*	*Fusarium solani*	*Fusarium solani*	*Penicillium* sp.
Alternaria longipes	*Aureobasidium*	*Aspergillus nidulans*	*Rhizopus oryzae*	*Hypocrea jecorina*
Aspergillus niger	*Beauveria*	*Phanerochaete* sp.	*Paraconiothyrium brasiliense*	*Trichophaea abundans*
Aureobasidium pullulans	*Botryosphaeria*	*Rhizoctonia solani*	*Trichoderma* sp.	*Conoplea fusca*
Pseudodictyosporium elegans	*Cladosporium*		*Phomopsis* sp.	*Colletotrichum* sp.
Cladosporium basi-inflatum	*Cladotrichum*		*Aspergillus* sp.	*Cochliobolus* sp.
Cladosporium cladosporioides	*Drechslera*		*Acremonium alternatum*	*Letendraea helminthicola*
Cladosporium herbarum	*Epicoccum*		*Xylaria* sp.	*Phialophorophoma litoralis*
Cladosporium perangustum	*Fusarium*		Mycorrhizal basidiomycete	*Sporormia lignicola*
Cladosporium subtilissimum	*Gelasinospora*		*Cladosporium tenuissimum*	*Alternaria* sp.
Coniothyrium sp.	*Geotrichum*		*Sordaria* sp.	Pleosporomycetidae
Cyclothyrium sp.	*Gliocladium*		*Neonectria radicicola*	*Phoma medicaginis*
Cytospora leucostoma	*Humicola*		*Pezicula* sp.	*Massarina igniaria*
Epicoccum nigrum	*Monodictys*		*Epacris* sp.	*Xylomelasma sordida*
Fusarium lateritium	*Mucor*		*Metarhizium anisopliae*	*Cercophora* aff. *mirabilis*
Fusarium tricinctum	*Penicillium*		*Cryptococcus flavescens*	*Cercophora mirabilis*
Geniculosporium serpens	*Pestalotia*		*Hypocrea lixii*	*Nigrospora* sp.
Lecanicillium lecanii	*Phoma*		*Fusarium* sp.	*Xylaria juruensis*
Nigrospora oryzae	*Phomopsis*		*Diaporthe* sp.	*Xylaria cubensis*
Paraphoma fimeti	*Rhinocladiella*		*Botryosphaeria obtuse*	*Xylariaceae*
Phoma pratorum	*Rhizopus*		*Coniothyrium diplodiella*	*Daldinia eschscholzii*
Phomopsis archeri	*Trichoderma*			*Daldinia* sp.
Sclerostagonospora cycadis	*Trimmatostroma*			*Annulohypoxylon* sp.
Seimatosporium cf. *pezizoides*	Mycelia sterilia			*Hypoxylon* sp.
Seimatosporium sp.	Unidentified			*Coprinellus domesticus*
Stachybotrys chartarum				*Polyporus arcularius*
Truncatella angustata				*Trametes elegans*
Anamorphic *Xylaria*				
Mycelia sterilia				

Cytospora, *Paraphoma*, *Phoma*, *Phomopsis*, *Seimatosporium*, *Stachybotrys*, and *Truncatella* and their identified species were only recovered from plant samples of Golestan province and *Absidia*, *Pseudodictyosporium*, *Epicoccum*, *Fusarium*, *Geniculosporium*, *Lecanicillium*, *Nigrospora*, *Sclerostagonospora*, and anamorphic *Xylaria* and their identified species were only isolated from plant samples of Alborz province, reflecting the effect of geographic isolation and ecosystem condition in the host plant and endophytic fungi coevolution, as mentioned by Rodriguez et al. (2009), Liu et al. (2009), and Zhao et al. (2010).

Geniculosporium serpens was originally considered as *Hypoxylon serpens* and was transferred to the genus *Geniculosporium* by Chesters and Greenhalgh (1964). The conidiophores of *Geniculosporium serpens* are indistinctive in arrangement and conidia are not borne on distinct denticles. In the resulting ML tree, *Geniculosporium serpens* and *Nemania serpens* were clustered together in group A with 99% bootstrap support. The placement of *Geniculosporium* and *Seimatosporium* species in the same group may reflect the same type of conidioma (acervulus) and conidiogenous cells (annellidic). *Fusarium lateritium*, *Fusarium tricinctum*, *Lecanicillium lecanii*, and *Stachybotrys chartarum* were clustered together in group C with 99% bootstrap support, with similar conidiogenous cells (phialide). *Cytospora leucostoma* (current name *Leucostoma persoonii*) and *Leucostoma persoonii* were clustered together in group D with 99% bootstrap support. *Sclerostagonospora cycadis*, *Pseudodictyosporium elegans*, *Massarina corticola* (anamorph

Pseudodictyosporium elegans), *Alternaria longipes*, *Alternaria alternata*, *Alternaria atra*, *Epicoccum nigrum*, *Coniothyrium cereale*, *Phoma fimeti* (current name *Paraphoma fimeti*), *Paraphoma fimeti*, and a strain of *Cyclothyrium* sp., *Coniothyrium* sp., and *Leptosphaeria* sp. (anamorph *Coniothyrium*) were clustered together in Clade 4 with 98% bootstrap support. *Alternaria atra* was previously considered as *Ulocladium atrum* and recently transferred to the genus *Alternaria* by Woudenberg et al. (2013). *Cyclothyrium* sp., *Sclerostagonospora cycadis*, *Coniothyrium* sp., and *Paraphoma fimeti* have similar conidiomata (pycnidia). According to the morphological characteristics and results of phylogenetic analyses using nucleotide sequences of the ITS1-5.8S-ITS2 region, fungal isolates were mostly well resolved at the genus level. Also, in order to complete a phylogenetic study of this group of fungi and to achieve reliable taxonomic results, especially for species of *Cladosporium* and *Alternaria*, other sequences such as beta-tubulin, actin, calmodulin, EF1-α, and histone H3 should be used.

The ITS contains hypervariable regions, so it can be ideal for resolving closely related species (Schubert et al. 2009; Bensch et al. 2010). In this study, the sequence of ITS regions resolved most of the taxa as distinct species. But some isolates belonging to the class Dothideomycetes, such as *Cladosporium* (35TG, 11TG, and 34 TG) and *Alternaria* (16TG and 2TG), require the use of other gene sequences to provide better taxonomic resolution.

This study is the first comprehensive survey on fungal endophytes of *T. baccata* in Iran and the results showed that *T. baccata* harbors a wide and significant diversity of fungal endophytes that provides a baseline for the search of Taxol and other novel secondary metabolites which can be screened against different druggable targets.

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