



Endophytic fungus, *Radulidium subulatum* from *Phragmites australis* in Iran

F. Salimi

Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

A. Alizadeh

Department of Plant Protection, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran

A. Mirzadi Gohari

M. Javan-Nikkhah ✉

Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

Abstract: In order to isolate and identify endophytic fungi of *Phragmites australis*, numerous samplings were conducted from the plants grown in the southern areas of the Caspian Sea in Guilan, Mazandaran and Golestan provinces and saline soils around the Lake Urmia at province of East Azarbaijan. Twenty-one isolates of the genus *Radulidium* were obtained from healthy tissues of *P. australis* plants. Morphological studies and molecular analysis based on ITS-rDNA sequence revealed that the isolates belonged to the *R. subulatum*. To the best of our knowledge, this is the first report of *R. subulatum* from *P. australis* to the mycobiota of Iran.

Key words: Endophyte, mycoflora, ITS-rDNA taxonomy, symbiosis

INTRODUCTION

Endophytic fungi are diverse group of plant symbionts that live locally and sometimes systematically within plant tissues without causing symptoms of disease (Porrás-Alfaro & Bayman 2011). There are several studies reporting the beneficial features of the fungal endophytic groups (González-Teuber 2016). The important ecological roles of endophytes in the issue of environmental adaptation of their hosts can result in increasing resistance against pathogens and herbivores (Clay &

Schardl 2002), assisting the plants for mineral nutrition uptake (Kipfer et al. 2011), mitigating the effects of water stress (Atala et al. 2012) and affecting plant responses towards other biotic and abiotic stresses (Clay & Schardl 2002, Rodríguez et al. 2008, Cheplick & Faeth 2009, Soares et al. 2015). Symbiotic relationships can have a direct or indirect impact on the structure, function and composition of plant communities, the expansion of host niches (Rudgers et al. 2015) and food webs and ecosystem processes (Aschehoug et al. 2014).

Common reed (*Phragmites australis* (Cav.) Trin. Ex Steud.) is a perennial grass found in most habitats throughout the world. The microbial endophytic communities of *P. australis* have been examined in several previous studies (Wirsel et al. 2001, Li et al. 2012, Angelini et al. 2012, Fischer & Rodríguez 2013, Kim et al. 2014, Hipol & Cuevas 2014, Sim et al. 2015, Sauvêtre & Schröder 2015, Soares et al. 2015) and thought to play a key role in aiding plant establishment in marginal and high stress habitats. It has been suggested that success in invasion of this plant is related to some factors including plant ecological capability to tolerate and grow in a wide range of soil salinity and fertility levels, its wide dispersion efficiency (Meyerson et al. 2012), high genetic diversity (Fer & Hroudova 2009), and microbial symbiosis (Kowalski et al. 2015). These plants have also been shown to tolerate heavy metals as well as other stresses (Bonanno & Giudice 2010). The ability of *P. australis* to cope with salt stress plays a crucial role in colonization of coastal habitats (Achenbach & Brix 2014), although it is unclear how much the plant's microbiome can influence the resistance to salt stress. According to our knowledge, there is no comprehensive study of endophytic fungi from common reed plants in Iran.

Radulidium is an ascomycetous fungal genus that was described by Arzanlou et al. (2007) as a new taxon. *Radulidium* has been isolated and identified from a limited but diverse types of the substrates/hosts including *Epichloë typhina*, an unknown Poaceae plant, *Puccinia allii*, *Phragmites australis*, incubator for cell cultures and *Lasioptera arundinis* (Arzanlou et al. 2007), so far.

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✉ Corresponding Author E-mail: jnikkhah@ut.ac.ir

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The aim of this study was morphological and molecular characterization of *Radulidium* isolates which were isolated previously in a study on endophytic fungi from *P. australis* plants in different natural habitats of the north and west provinces of Iran.

MATERIALS AND METHODS

Sample collection

Numerous samplings were conducted from healthy *Phragmites australis* plants (without disease symptoms) grown in some areas in the southern parts of the Caspian Sea and saline soils around the Lake Urmia, during the summer and autumn of 2017 and 2018. At each sampling site, whole plants (1.5–3.0 m height) with undamaged leaves, roots and rhizomes were randomly collected and transferred to the laboratory in polythene bags and processed for the isolation of endophytic fungi in the same day or within a maximum of 48 hours using standard techniques (Rodriguez et al., 2008). Briefly, samples were washed in running tap water to remove dust and debris for 15 min, dried in the air and then cut into 1 cm segments. For surface sterilization, the plant slices were soaked in 70% ethanol for 1 min, then in sodium hypochlorite (3% available chlorine) for 2 min for leaf and stem and 3 min for root segments. Disinfection processes continued in 70% ethanol for 1 min and subsequently, the samples rinsed 5 times with autoclaved distilled water. The plant tissues were placed on the petri dishes containing potato dextrose agar (PDA, Merck, Darmstadt, Germany) and 2% water agar (WA, 2 %) media supplemented with chloramphenicol (20 µg/ml) and streptomycin (10 µg/ml). Petri dishes were kept at 20–25 °C in the darkness for 7–21 days. WA plates supplemented with chloramphenicol (20 µg/L) were inoculated with fungal tissues that had formed on WA and PDA

plates. Single spore or hyphal tips isolates were prepared on PDA. Pure cultures were deposited at the Mycology Laboratory (UTFC ...) of College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran (Table 1).

Morphological characterization

Morphological characterization was performed according to Arzanlou et al. (2007). Cultural growth rates and morphology were recorded from colonies grown on malt extract agar (MEA) for 14 days at 24 °C in the darkness and colony colors were determined according to Rayner (1970). Microscopic observations were conducted from colonies cultivated on MEA and OA (oatmeal agar, Gams et al., 2007). Structures were mounted in lacto-phenol cotton blue, and 30 measurements ($\times 1000$ magnification) determined wherever possible.

DNA extraction and PCR amplification

Genomic DNA was extracted from colonies grown on PDA (25 °C for 7–10 days) using a standard phenol-chloroform extraction protocol (Sambrook & Russell, 2001). Amplification of the internal transcribed spacer (ITS) region of rDNA was performed using the ITS1 and ITS4 primers (White et al., 1990). Polymerase chain reaction was performed in a Corbett Thermocycler (Corbett Research, Australia) in a total volume of 25 µL. The PCR mixture contained 10–15 ng genomic DNA, 0.2 µM of each primer, 1 \times Taq PCR buffer, 2 mM MgCl₂, 20 µM of each dNTP, 0.75 µl DMSO and 0.25 U Smart Taq DNA Polymerase (CinnaGen Co, Iran). The PCR program was as follows: an initial step of 5 min at 98 °C, 35 cycles of 10 s at 98 °C, 30 s at 60 °C and 30 s at 72 °C, followed by 10 min at 72 °C. The PCR products were sequenced by Bioneer Co. (South Korea) with the amplifying primers.

Table 1. *Radulidium* isolates obtained from *Phragmites australis*, and characterized in this study.

Isolate	Collection Code	Host Tissue	Locality		Date
			Region/Province	GPS data	
S77	UTFC-S77	Stem	Gorgan/Golestan	36.841468, 54.359102	July-2017
S150	UTFC-S150	Leaf	Sangar/Guilan	37.206559, 49.703512	July-2017
187	UTFC-187	Stem	Lasht-e Neshan/Guilan	37.297241, 49.779597	July-2017
S21-1	UTFC-S21-1	Stem	Behshahr/Mazandaran	36.712068, 53.536062	July-2017
S21-2	UTFC-S21-2	Stem	Behshahr/Mazandaran	36.712068, 53.536062	July-2017
S21-3	UTFC-S21-3	Stem	Behshahr/Mazandaran	36.712068, 53.536062	July-2017
N1P8R6	UTFC-N1P8R6	Root	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P4S6	UTFC-N1P4S6	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P4S5	UTFC-N1P4S5	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P3S1	UTFC-N1P3S1	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P2S2	UTFC-N1P2S2	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P9S4	UTFC-N1P9S4	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N1P10S3	UTFC-N1P10S3	Stem	Sharafkhane/East Azerbaijan	38.11361, 45.28190	July-2018
N2P8R1	UTFC-N2P8R1	Root	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P8L1	UTFC-N2P8L1	Leaf	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P7L3	UTFC-N2P7L3	Leaf	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P6R1	UTFC-N2P6R1	Root	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P5S1	UTFC-N2P5S1	Stem	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P4S4	UTFC-N2P4S4	Stem	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P1S4	UTFC-N2P1S4	Stem	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018
N2P6R4	UTFC-N2P6R4	Root	Sharafkhane/East Azerbaijan	38.10205, 45.29125	July-2018

Phylogenetic analysis

The programs ChromasPro 1.34 and EditSeq, parts of the DNA Lasergene (DNASTAR, Madison, WI) software package, were used for viewing, editing and assembling the sequences.

The sequences were aligned using MUSCLE, a multiple sequence alignment method (Edgar 2004). The sequences of the two Iranian *Radulidium* isolates were compared with other fungal DNA sequences from NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank/) using BLAST algorithms. Six sequences with high similarity were added to the alignment as reference strains (Fig. 1).

Analysis were performed using the software MEGA v.6 (Tamura et al. 2013). Distance matrices of the aligned sequences were calculated using the Kimura 2-parameter model (Kimura 1980), and analyzed with the neighbor-joining (NJ) algorithm (Felsenstein 1981). The reliability of the inferred trees was estimated by bootstrap analyses with 1000 replicates.

In the maximum parsimony (MP) analysis, the evolutionary distances were computed using the maximum composite likelihood method (Varin 2008). Gaps were treated as missing data in the pairwise sequence comparisons (Pairwise deletion option). The MP tree was obtained using the close-neighbor-interchange algorithm of Nei & Kumar (2000) with search level 1 (Felsenstein 1985, Nei & Kumar 2000) in which the initial trees were obtained by random addition of sequences (100 replicates). Clade stability was assessed in a bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa. The tree branches were drawn to scale, with lengths calculated using the average pathway method (Nei & Kumar 2000), as the units of the number of changes over the whole sequence. Sequence data were deposited in GenBank and assigned accession numbers (UTFC-N1P10S3: MK968565; UTFC-N1P10S4: MK968567).

Characterization of the species

A total of 21 isolates of *Radulidium* were isolated as endophytes from leaves, stems and roots of healthy *P. australis* plants showing no disease symptoms from six different geographical areas at four Northern provinces of Iran: East Azerbaijan, Guilan, Mazandaran and Golestan (Table 1). Investigating colonies and morphological characteristics of the isolates showed that all isolates belonged to only one morphotype with similar or identical characteristics. Based on the detailed morphological studies, it became clear that all isolates belonged to the *R. subulatum* species.

To confirm morphological identification, two isolates were selected for molecular identification by sequencing of ITS-rDNA genomic region. A BLAST search of the resulting sequences of N1P10S3 and N1P10S4 isolates showed 99.77% similarity to *R. subulatum* CBS 405.76 ex-type strain (EU041845;

Arzanlou et al., 2007). Analysis of ITS-rDNA sequences of two selected isolates confirmed morphological identification and could resolve the identity of the *R. subulatum* isolates, totally in concordance of morphological characters. To the best of our knowledge, this is the first report of *R. subulatum* from *P. australis* to the mycobiota of Iran.

Phylogeny

The ITS sequences of N1P10S3 and N1P10S4 isolates were combined and aligned with the eight reference sequences of three taxa from GenBank. The alignment consisted of 542 characters including alignment gaps, of which 360 were constant, 185 were variable and 108 were parsimony-uninformative. Maximum parsimony analysis of the parsimony-informative characters resulted in three most parsimonious trees with a consistency index of 0.91, the retention index of 0.91 and composite index of 0.85 (Fig. 1). NJ analysis resulted in the trees with the same topology as the MP trees. The MP tree is shown with bootstrap values of both analyses at the nodes (Fig. 1). Phylogenetic analyses based on ITS-rDNA sequence (Fig. 1) placed the examined isolates N1P10S3 and N1P10S4 with reference strains of *R. subulatum* (including ex-type strain CBS405.76) in a strongly supported monophyletic clade with 74% bootstrap support. Furthermore, all isolates of the *R. subulatum* were well separated from the ex-type strain of the close species *R. epichloes*. Therefore, the molecular analysis based on ITS-rDNA sequence confirmed that the isolates belong to the *R. subulatum*.

Radulidium subulatum (de Hoog) Arzanlou, W. Gams & Crous, *Studies in Mycology* 58: 89 (2007)

Basionym: *Ramichloridium subulatum* de Hoog, *Studies in Mycology* 15: 83. 1977.

Cultural characteristics: Colonies on MEA rather fast growing, reaching 50 mm diam after 14 d at 24 °C, with entire but vague margin, velvety, floccose near the margin, centrally with fertile hyphal bundles up to 10 mm high, about 2 mm diam; mycelium whitish, later becoming greyish brown; reverse grey, zonate.

Morphology on MEA: Submerged hyphae hyaline, thin-walled, 1–2.5 µm wide; aerial hyphae brownish. Conidiogenous cells arising laterally from vegetative hyphae, pale brown, smooth, thick-walled, sometimes without a basal septum, cylindrical to aculeate, tapering gradually towards the apex, widest at the base, 25–40 × 2–3 µm; proliferating sympodially, forming a pale brown rachis, with densely crowded, prominent, blunt conidium-bearing denticles, with pale brown apex. Conidia solitary, sub-hyaline, thin-walled, smooth, ellipsoidal to almost clavate, 5–6.5 × 1.5–2 µm, with a slightly pigmented, non-refractive hilum, about 1 µm diam (Fig. 2). These morphological characters were completely accorded to the characteristics that

examined and established by Arzanlou et al. (2007) in specimens examined: Czech Republic, on *Phragmites*

australis, ex-type culture CBS 405.76; Czech Republic, from *Lasioptera arundinis*, CBS 101010.

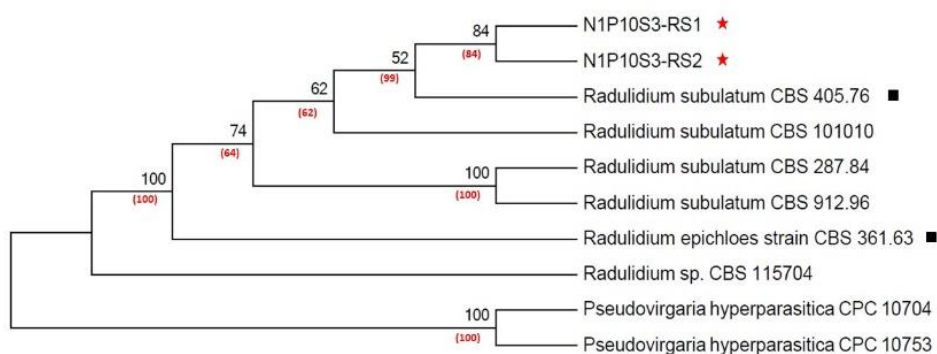


Fig. 1. The phylogenetic tree constructed from a maximum parsimony analysis for *Radulidium subulatum* based on the ITS-rDNA sequence data. Bootstrap values > 50% (1000 replicates) of MP analysis is exhibited above the branches and those of neighbor-joining in brackets. Obtained isolates N1P10S3 and N1P10S4 in this study are marked with red stars. Black squares indicate the ex-type strains. *Pseudovirgaria hyperparasitica* CPC10704 and *P. hyperparasitica* CPC10753 are used as out-group taxa.

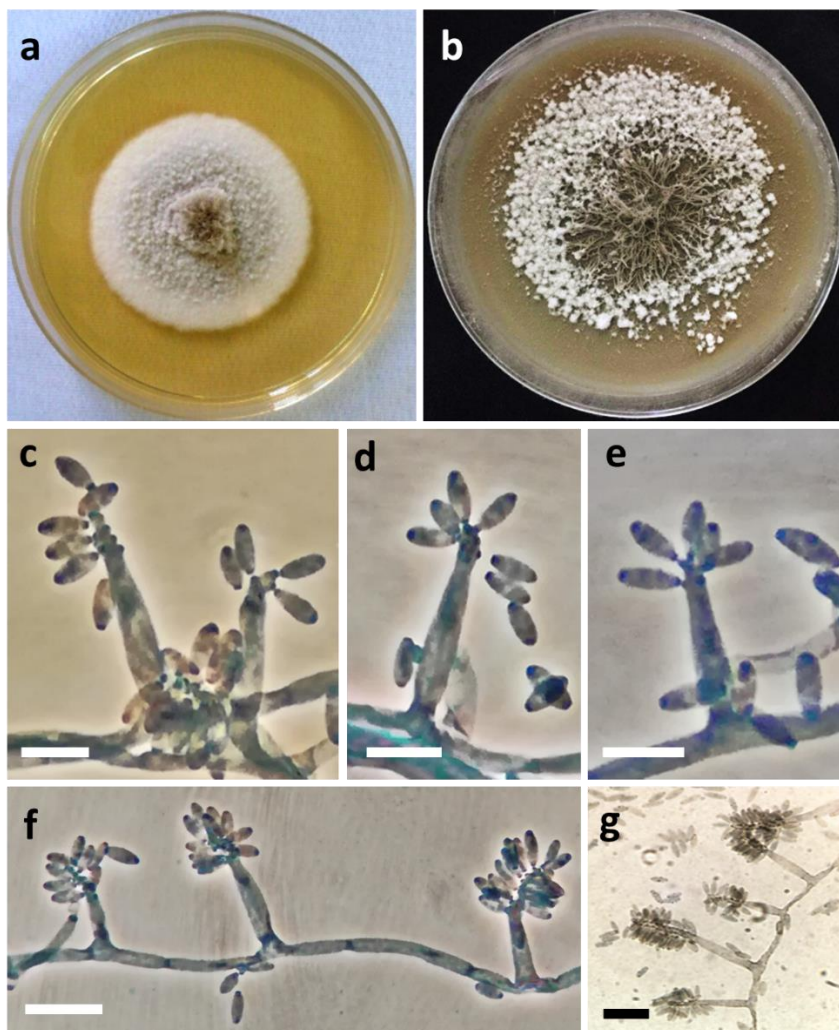


Fig. 2. *Radulidium subulatum* (N1P10S3). a. Colony on MEA, 7 d growth; b. Colony on PDA, 14 d growth; c-g. Macronematous conidiophores with sympodially proliferating conidiogenous cells, resulting in a conidium-bearing rachis. — Scale bars: c-e = 10 µm; f-g = 50 µm.

Specimen examined. UTFC-S77, UTFC-S150, UTFC-187, UTFC-S21-1, UTFC-S21-2, UTFC-S21-3, UTFC-N1P8R6, UTFC-N1P4S6, UTFC-N1P4S5, UTFC-N1P3S1, UTFC-N1P2S2, UTFC-N1P9S4, UTFC-N1P10S3, UTFC-N2P8R1, UTFC-N2P8L1, UTFC-N2P7L3, UTFC-N2P6R1, UTFC-N2P5S1, UTFC-N2P4S4, UTFC-N2P1S4 and UTFC-N2P6R4.

Note: The genus *Radulidium* was first described and reported by Arzanlou et al. (2007) as a new taxon. So far, four species of *Radulidium* including *R. subulatum*, *R. epichloe*, *R. xigazense* and *R. guttiforme* have been described and reported (Arzanlou et al. 2007, Wu & Zhang 2013). According to the Arzanlou et al. (2007), the ITS-rDNA is an efficient genomic region for phylogenetic studies of *Radulidium* species and can well distinguish *Radulidium* species from each other and other close taxa. Therefore, to clarify the relationship and accurate identification of *Radulidium* isolates, we conducted phylogenetic analyses for sequence of ITS-rDNA combined with morphological characters. The sequences of the ITS-rDNA gene could resolve the relationship of the examined *R. subulatum* isolates, totally in concordance of morphological characters. Thus far, no genetic data has been provided for both *R. xigazense* and *R. guttiforme* species in the literatures and their descriptions were based only on morphological data. However, morphological characteristics are sufficiently effective for the discriminate of the *Radulidium* species and it is possible to accurately identify species through morphological characteristics (Arzanlou et al., 2007, Wu & Zhang 2013, Eliss & Dearn 2016). Morphologically, *R. subulatum* differing from other three species by the significant differences in conidia features; *R. subulatum* conidia contain no oil droplets and were ellipsoidal to almost clavate $5-7 \times 1.5-2 \mu\text{m}$, with a slightly pigmented, nonrefractive hilum. Whereas, *R. epichloes* produced larger ($5-11 \times 2-3 \mu\text{m}$) verruculose conidia, and conidia of *R. xigazense* contained several oil droplets. Similarly, *R. guttiforme* (Wu & Zhang, 2013) differing from *R. subulatum* by its shorter and wider conidia ($3.5-6 \times 2.5-4 \mu\text{m}$) which were papillate or slightly truncate at the base.

In conclusion, morphological studies and molecular characterization based on rDNA-ITS sequence revealed the that endophytic *Radulidium* isolates obtaining from healthy tissues of *P. australis* plants, belonged to *R. subulatum*. To the best of our knowledge, this is the first report of *R. subulatum* from *P. australis* to the mycobiota of Iran.

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قارچ اندوفیت *Radulidium subulatum* به دست آمده از گیاه نی (*Phragmites australis*) در ایران

فاطمه سلیمی^۱، علیرضا علیزاده^۲، امیر میرزادی گوهری^۱، محمد جوان نیکخواه[✉]

۱- گروه گیاهپزشکی، دانشکده علوم و مهندسی کشاورزی، پردیس کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران

۲- گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه شهید مدنی آذربایجان، تبریز، ایران

چکیده: برای جداسازی و شناسایی قارچ‌های اندوفیت گیاه نی (*Phragmites australis*)، نمونه‌برداری‌های متعددی از گیاهان نی روئیده در برخی مناطق جنوبی دریای خزر در استان‌های گیلان، مازندران و گلستان و خاک‌های شور اطراف دریاچه ارومیه در استان آذربایجان شرقی انجام شد. بیست و یک جدایه از جنس *Radulidium* از بافت‌های سالم گیاهان *P. australis* به دست آمد. مطالعات ریخت‌شناختی و تجزیه و تحلیل فیلوژنتیکی بر اساس توالی ناحیه ITS-rDNA نشان داد که جدایه‌ها متعلق به *R. subulatum* هستند. بر اساس اطلاعات موجود، این اولین گزارش از *R. subulatum* از گیاه نی برای میکوبیوتای ایران می‌باشد.

واژه‌های کلیدی: اندوفیت، فلور قارچی، ITS-rDNA، طبقه بندی، همزیستی