Morphological and molecular characterization of Taify rose endophytic fungi from Saudi Arabia

Alaa Baazeem
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, SAUDI ARABIA
aabaazeem@tu.edu.sa

Abstract
Endophytic fungi live inside plant tissues but do not cause any disease symptoms. They produce secondary metabolites that prevent herbivores from eating the plant by making them poisonous or tasteless and have other major roles in nutrient absorption, heat tolerance and biodiversity. The aim of current study was to isolate and identify endophytic fungi from leaves of Taify rose grown in Al-Hada region at Taif, Saudi Arabia. Isolation of endophytic fungi was carried out by microscopic examinations based on the colony's color, shape, strings, conidia, conidiophores and arrangements of spores. The molecular identification was conducted by ITS region sequencing and molecular characterization was achieved by ISSR-PCR. Nine endophytic fungi isolates were obtained from Taify rose leaves. Five of them were identified as Aspergillus niger, two isolates were identified as Alternaria alternata and Penicillium citrinum.

Finally, ISSR-PCR markers revealed a total of 77 bands in all isolates, with about 50.6% monomorphism and 49.4% polymorphism. The dendrogram drew based on genetic similarity and intraspecies variability grouped them into two different clusters with about 0.57 genetic similarity. Aspergillus sp. was the most abundant fungus isolated from Taify rose leaves, while Alternaria and Pencillium species were less prevalent. The ITS sequencing method is absolutely the best in identifying endophytic fungi for their ease and speed while the morphological method can be inaccurate and could consume more time.

Keywords: Endophytic fungi, Taify rose, ISSR-PCR, ITS region sequencing, Saudi Arabia.

Introduction

The rose is one of the most important crops in the flower growing industry[1]. Roses are used as cut flowers, preserved plant and ornamental garden plants[2]. Roses belong to the genus Rosa, which contains more than 100 species distributed in different regions including Europe, Asia, North America and the Middle East[3,4].

It has also been used in some food industries and the production of perfumes and cosmetics for a long time in Saudi Arabia[4-6]. A few of the genus Rosa have been used to produce essential oils, among which *R. damascena* is superior to producing high-quality essential oils[7]. The name (Damascene) species depends on Damascus, Syria, where it was originally found as a wild plant. However, it is now grown in different countries around the world[8,9].

Rosa damasina has a long history in Taif, the Western of Saudi Arabia, where essential oil is produced from high-quality roses and strong financial production. Endophytic fungi are the microorganisms found in healthy plant tissues during one or more stages during the life cycle of some plants, which do not cause problems or diseases for these plants[10-12]. Various previous studies regarding the role of endophytic fungi in host plants indicate that they can stimulate plant growth, improve plant's ability to withstand environmental stresses, increase disease resistance and recycle nutrients[11-13].

It has also been shown that some internal fungal cells can produce multiple biochemical and active chemicals and have potential applications in biocontrol of diseases and resistance to unfavorable conditions[14,15]. The appearance of the molecular classification of microorganisms was important to distinguish between species more precisely[16-18]. Internal transcribed spacer (ITS1 and ITS2) and 5.8S are the most widely used units of ribosomal nuclear identification of fungal endophytes and as a barcode marker in most fungi[19-22]. In this context, the main aim of current study was to morphologically and molecularly characterize the endophytic fungi associated with *R. damascene* from Taif region, Saudi Arabia.

Material and Methods

This investigation was carried in the Biology Department, College of Science, Taif University, Kingdom of Saudi Arabia.

Plant material: Leaves of *Rosa damascena* plant, Taify cultivar, were collected from Al-Hada region at Taif governorate and then stored at 4°C.

Isolation and identification of endophytic fungi: Leaves of Taify rose were cleaned under rinsed tap water and immersed in 70% ethanol for 60 sec., 5 % sodium hypochlorite for 5 min and finally in 70% ethanol for 30 sec. Leaves were then washed several times in sterile distilled water [23]. The sterilized leaves were shade dried under aseptic conditions. Leaves were divided into small pieces (1 × 1 cm^2), then placed on PDA growth medium and incubated at 28 ± 2°C for 2–3 weeks in an incubator until the appearance of the emerging hyphae and pure isolates.

Mycelial fragments of each isolate were removed with a sterile scalpel and transferred to a new PDA plate for purification[24]. Three replicates were used for each isolate. Morphological identification of the isolates was carried out microscopically at magnification of 40x according to Von-ARX et al[24]. Purified isolates were stored at 4 °C. Fresh fungal mycelium was transferred to a new plate every 2 weeks.

Molecular identification of endophytic fungi:

Total genomic DNA extraction: Endophytic fungi isolates were cultured on Czapek Dox broth at 28°C for five days, then the Norgen Plant/Fungi DNA isolation Kit (Sigma, Canada) was used to extract total genomic DNA of each endophytic fungus according to Hassan et al[20].

PCR amplification of 5.8S-ITS region: The ITS region of the 5.8S gene in rRNA was amplified using the site of primersITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as designed[26,27].PCR amplification was conducted in 25 ml reaction mixtures using 1X PCR buffer (DreamTaq™) and was carried out in the C1000™ Thermo Cycler Bio-Rad, Germany[21].

Sequence analysis of 5.8S-ITS region: The sequencing of the 5.8S region for all fungal isolates was done at Macrogen Co., Seoul, South Korea. The multiple nucleotide alignment of the ITS regions was analyzed using BioEdit version 7.2.5 software. The obtained sequences with about 600 bp were aligned with known sequences of the 5.8S-ITS region obtained from Genbank and subsequently used for the construction of a phylogenetic tree (MEGA 7.10) as described previously[28].

Inter simple sequence repeats (ISSR) analysis: For ISSR analysis, the total genomic DNA of the endophytic fungi isolates was subjected to PCR amplification as described previously[15]. ISSR primers names and sequences listed in table 1, were used in separate single PCR reactions according to Lakhani et al.[15] The similarity matrix was estimated based on a simple matching coefficient that was estimated by means of Jaccard's coefficient[29].

Table 1
ISSR Primers used in study of genetic variation among endophytic fungi isolates

Primers Name	Primers sequences 5'————3'
Asp-04	AGA GAG AGA GAG AGA GG
Box-A1	CTA CGG CAA GGC GAC GCT
GTG-5	GTG GTGGTGGTGGTG
M-13	GTT TTC CCA GTC ACG AC
Rep-29	ACA CAC ACA CAC ACA CT

Results

Morphological identification of isolated endophytic fungi: A total of nine endophytic fungal isolates belonging to three species were isolated from Taify rose in the Taif region of western Saudi Arabia. Five of them were classified as *Aspergillus niger*, their colonies grew rapidly on PDA and their initially white floccose mycelium spread rapidly, turning quickly into black-colored colonies that produced black spores. Two of them were recognized as *Penicillium citrinum*, their colonies were typically fast growing in shades of green, occasionally white and consisted of a dense felt of conidiophores. Finally, two isolates were identified as *Alternaria alternate*, their conidia were of muri-form shape and light brown color (Figure 1).

Molecular identification of endophytic fungi: The universal primers ITS 1 and ITS 2 were used to amplify the internal transcribed spacer regions of rDNA yielding products of approximately 600 bp as estimated by agarose gel electrophoresis (Figure 2). PCR amplified products were extracted and sequenced by an automatic sequencing device incorporating the 5.8S rDNA gene.

The obtained sequences were subjected to BLAST identification and confirmation and subsequently submitted to the National Center for Biotechnology Information (NCBI) GenBank. The accession number of sequences for ITS region form the nine isolates of endophytic fungi is represented in table 2.

Table 2
Comparison among endophytic fungi isolates and related strains in NCBI data base and accession numbers

Isolates code	Accession numbers	Closely related fungal sequence	Similarity %
TU-2	MT013014	*Aspergillus niger*MK713445	99
TU-3	MT013015	*Aspergillus niger* MK713445	100
TU-4	MT013016	*Alternaria alternata*MF102100	97
TU-5	MT013017	*Alternaria alternata* MF102100	99
TU-10	MT013018	*Penicillium citrinum*MN518391	99
TU-12	MT013019	*Penicillium citrinum* MN518391	97
TU-13	MT013020	*Aspergillus niger* MH237638	100
TU-15	MT013021	*Aspergillus niger* MH237638	99
TU-16	MT013022	*Aspergillus niger* MN513383	100

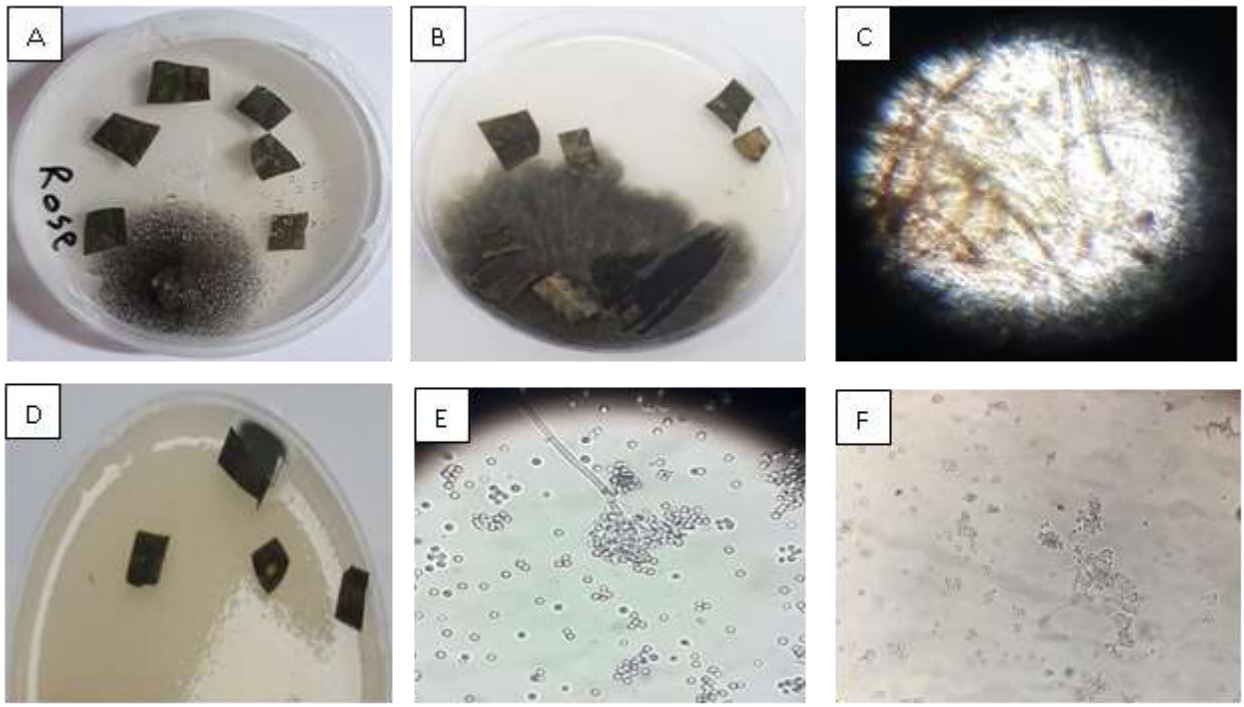

Figure 1: Endophytic fungi isolated from Taify rose leaves. A, B and C (*Aspergillus niger*); D, E and F (*Penicillium citrinum*)

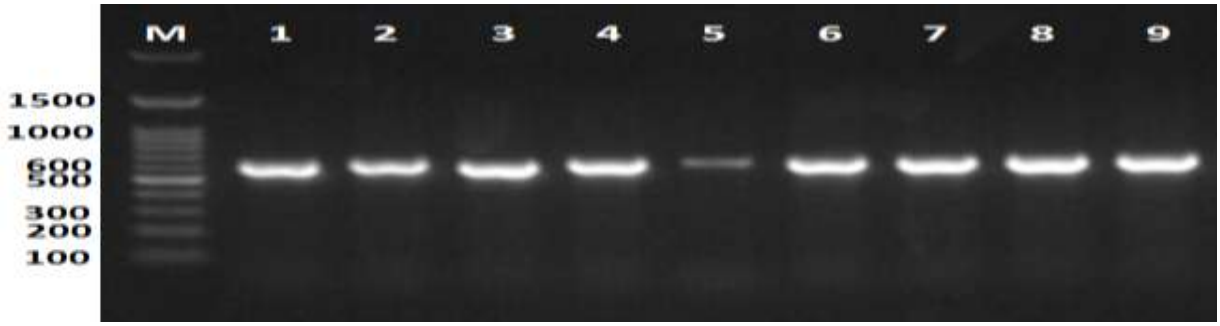

Figure 2: ITS region of the 5.8S gene amplification of nine endophytic fungi isolates

The multiple nucleotide alignment of ITS regions was analyzed using BioEdit program version 7.2.5. There was sub-stantial disparity in length of ITS sequences between endophytic fungi isolates (Figure 3). The isolates of endophytic fungi showed similarity value ranging from 97-100%. The endophytic isolates TU-2, TU-3, TU-13, TU-15 and TU-16 were identified as *Aspergillus niger* with similarity value ranging from 99 to 100%. TU-2 and TU-3 isolates were related to *Aspergillus niger* MK713445, TU-13 and TU-15 were similar to *Aspergillus niger* MH237638 and TU-16 was related to *Aspergillus niger* MN513383.

However, endophytic isolates TU-4 and TU-5 were identified as *Alternaria alternate* and they were related to *Alternaria alternate* MF102100 with similarity value ranging from 97 to 99% respectively. Moreover, isolates TU-10 and TU-12 were identified as *Penicillium citrinum* and they were related to *Penicillium citrinum* MN518391 with similarity value ranged from 97 to 99% (Table 2).

To elucidate the genetic closeness of the endophytic isolates, a phylogenetic tree was constructed based on sequence analysis of ITS regions using the neighbour-joining method in MEGA 7.1 for windows version on sequences aligned. A random sequence was used as an out-group to demonstrate the situation of the root. Bootstrap analysis of ITS region with 1000 bootstrap replication demonstrated two main branches (Figure 4).

All isolates of *Aspergillus niger* and *Penicillium citrinum* formed two subcluster into one group supported with a bootstrap value ranging from 97 to 99% among each species. While, *Alternaria alternata* formed other cluster consisting of endophytic isolates TU-4, TU-5 and *Alternaria alternate* MF102100.

Molecular characterization of endophytic fungi by ISSR-PCR: PCR based molecular markers can play an essential role in genetic diversity of endophytic fungi. Here, five ISSR primers were used and generated 77 bands ranging in length from 140 to 3000 bp (Table 3 and Figures 5-9). The total monomorphic pattern was 50.6 % whereas the total polymorphic pattern was with lower percentage of 49.4%. The maximum and minimum number of amplified bands

belonged to Asp-04 and M-13 that produced 11 and 18 bands respectively. The percentage of polymorphic bands was varied from 36.4% for Asp-04 primer to 56.3% for GTG-5 primer. The average band per primer was 15.4. PCR-based molecular markers can perform a significant part in the investigation of genetic diversity in such fungi. The use of molecular markers was pointing to display fast and consistent discrimination of genetic relatives of endophytic fungi isolated from *Rosa damascena* plants in Taif region, Saudi Arabia.

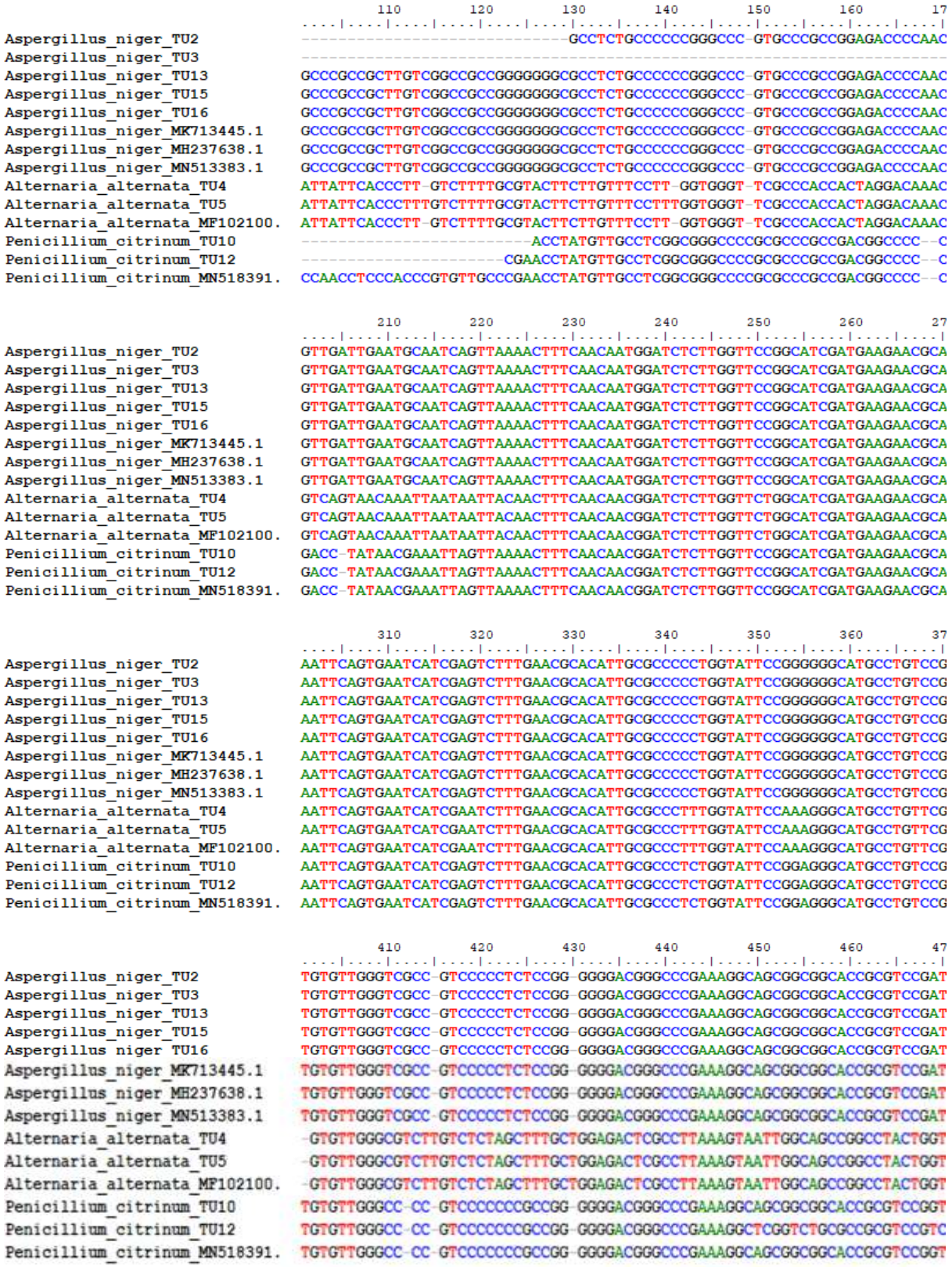

Figure 3: Alignment of nine endophytic fungi isolates compared with reference strains from NCBI database

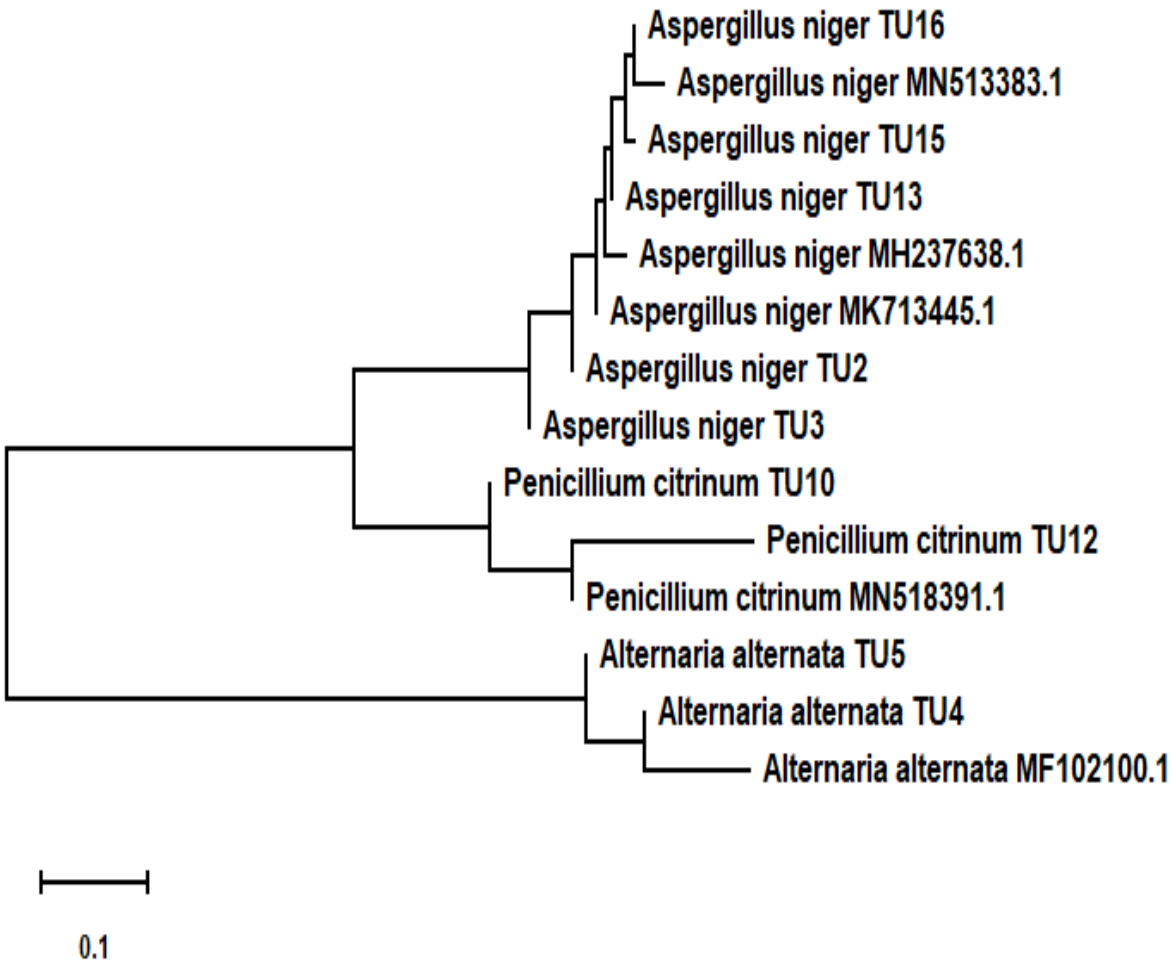

Figure 4: Phylogenetic tree and diversity of the 5.8S-ITS region in nine fungi isolates compared with reference strains from NCBI database. The phylogenetic tree was generated using parsimony neighbor-joining and maximum likelihood analysis

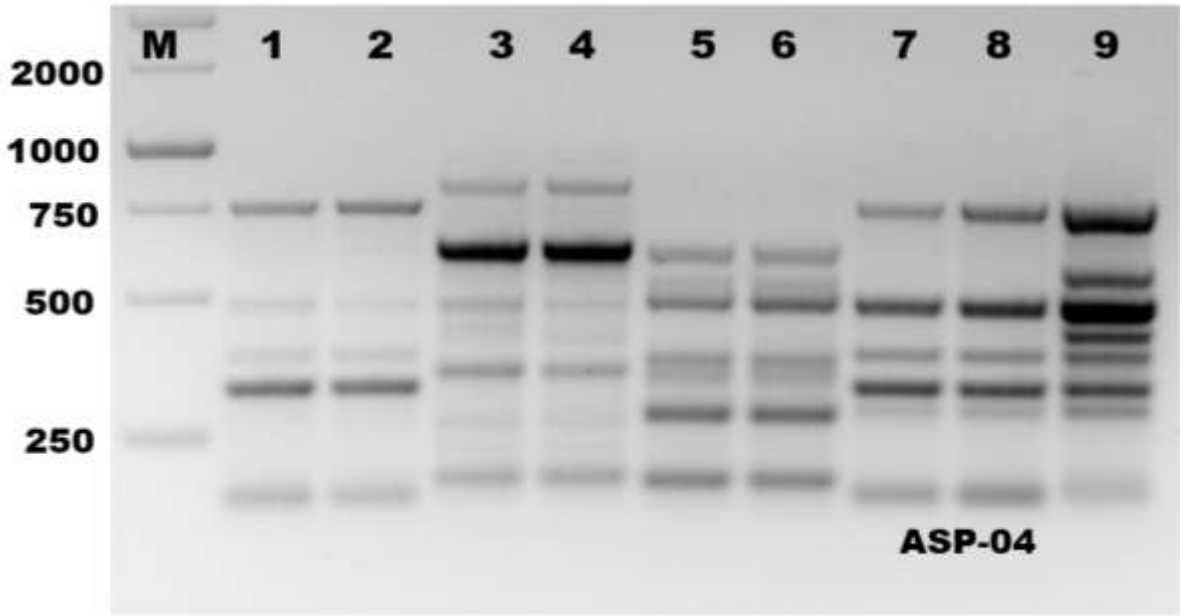

Figure 5: ISSR-PCR profile of nine endophytic fungi isolates generated with ISSR primer (ASP-04). First lane on each panel is 1kb molecular weight markers.

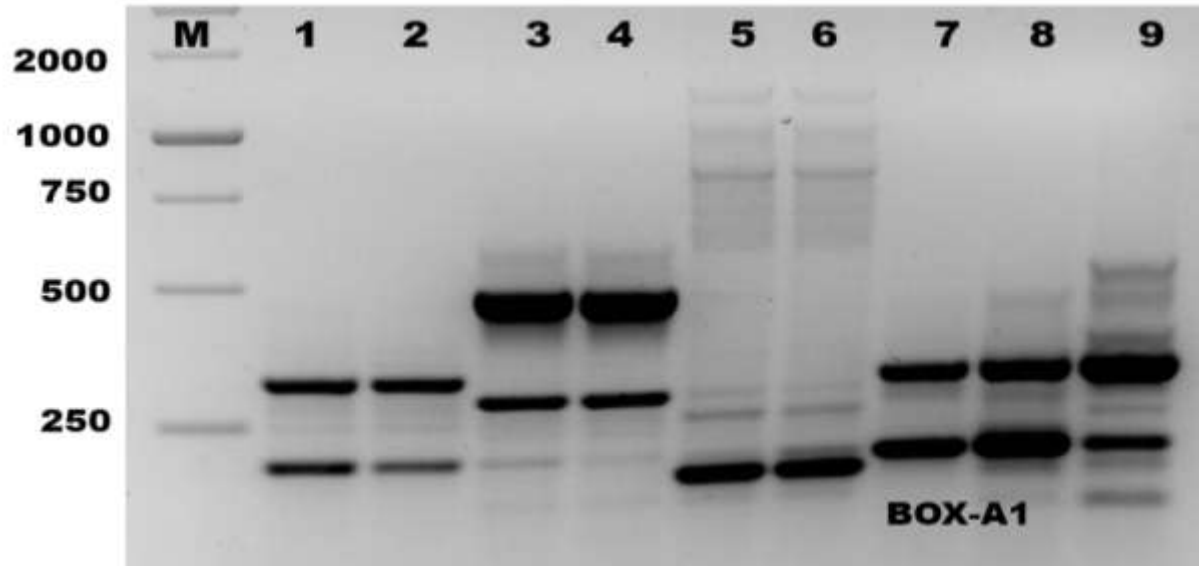

Figure 6: ISSR-PCR profile of nine endophytic fungi isolates generated with ISSR primer (BOX-A1). First lane on each panel is 1kb molecular weight markers.

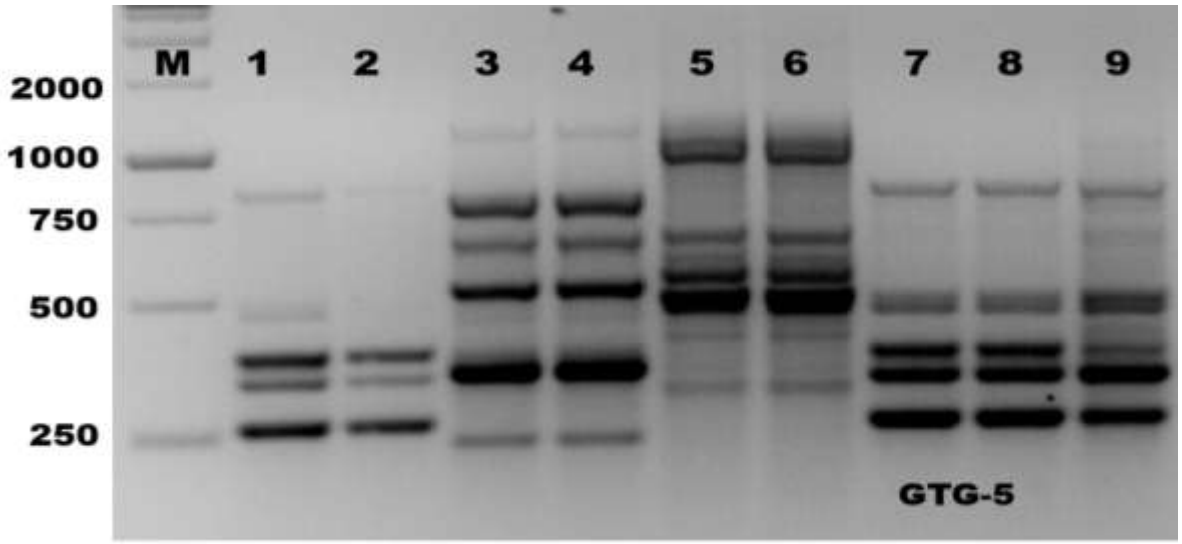

Figure 7: ISSR-PCR profile of nine endophytic fungi isolates generated with ISSR primer (GTG-5). First lane on each panel is 1kb molecular weight markers.

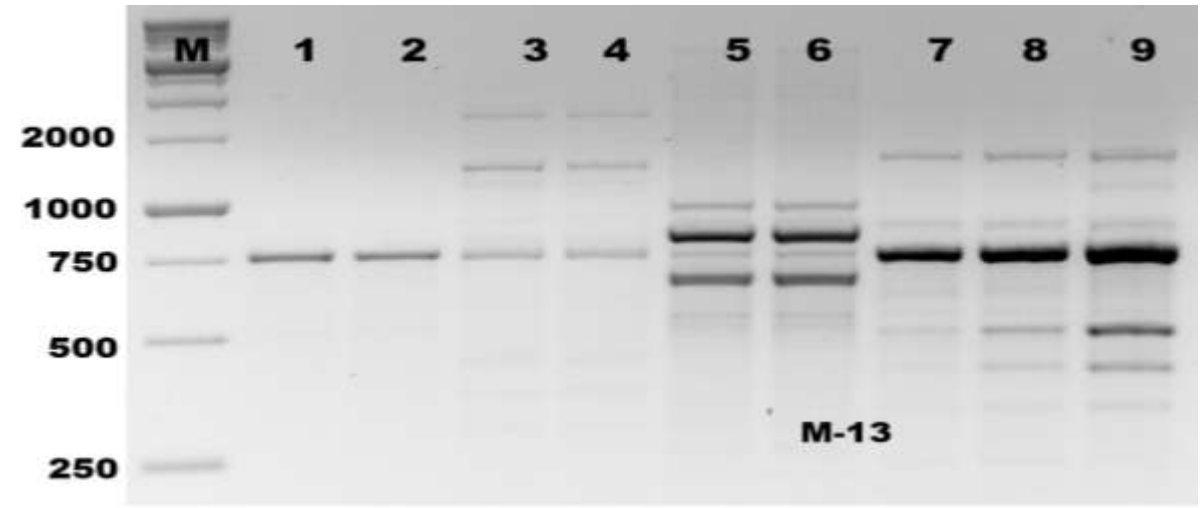

Figure 8: ISSR-PCR profile of nine endophytic fungi isolates generated with ISSR primer (M-13). First lane on each panel is 1kb molecular weight markers.

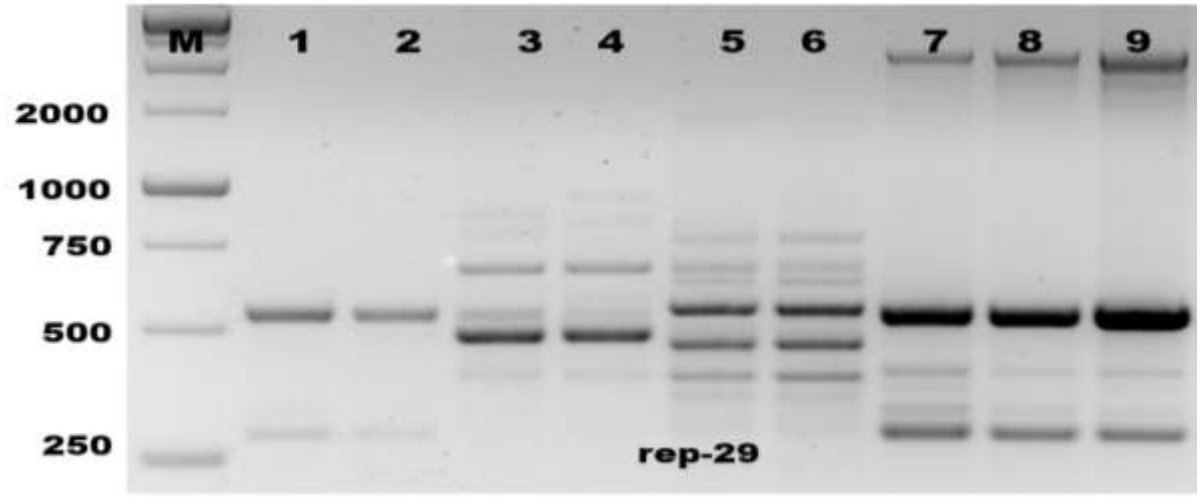

Figure 9: ISSR-PCR profile of nine endophytic fungi isolates generated with ISSR primer (rep-29). First lane on each panel is 1kb molecular weight markers.

Table 3
ISSR Profile of endophytic fungi isolates using five ISSR primers. Total bands (TB), polymorphic bands (PB), percentage of polymorphic bands (PPB) and number of specific bands (NSB).

Primers Name	TB	PB	PPB (%)	NSB	Bands range
Asp-04	11	4	36.4	2	140-800
Box-A1	15	8	53.3	3	160- 1400
GTG-5	16	9	56.3	2	240-1100
M-13	18	9	50.0	2	250-2100
Rep-29	17	8	47.1	2	260-3000
Total	77	38	49.4[m]		

*m = mean of percentage of polymorphic bands

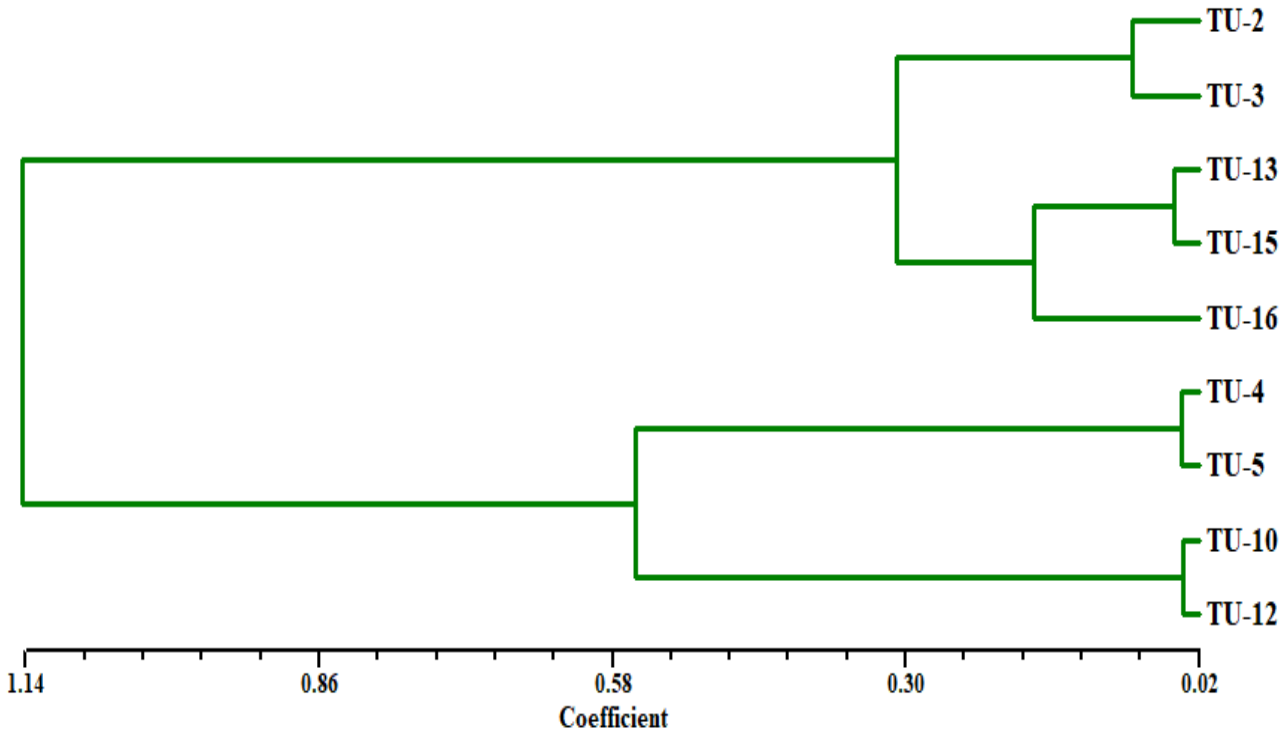

Figure 10: UPGMA dendrogram based on cluster analysis of ISSR data among some endophytic fungi isolates collected from rose plants leaves, Taif, Saudi Arabia

Scoring of banding batten of ISSR marker in endophytic fungi was used to draw the dendrogram based on the result of genetic similarity and intraspecific differentiation, the endophytic fungi isolates were assembled into two major clusters with about 0.57 genetic similarity (Figure 10).

Interestedly, the first main cluster contained five *Aspergillus niger* isolates and divided into two subcluster. The first subcluster contained *Aspergillus niger*TU-2 and *Aspergillus niger* TU-3. The second subcluster contained *Aspergillus niger* TU-13, *Aspergillus niger*TU-15and *Aspergillus niger* TU-16.

Meanwhile, the second main cluster contained two subclusters, the first one contained isolates TU-4 and TU-5 which were identified as *Alternaria alternata* whereas the second subcluster contained samples TU-10 and TU-12 which were identified as *Penicillium citrinum* (Figure 10).

Discussion

This study was conducted to use different morphological and molecular identification methods to characterize endophytic fungi isolated from Taiy rose plant leaves grown at Taif, Saudi Arabia. Three fungal species were isolated and identified at species level using rDNA ITS sequence as *Aspergillus niger, Alternaria alternate* and *Penicillium citrinum* after morphological identification to genus level. The obtained information from the morphological study alone is not sufficient to determine the types of endophytic fungi precisely because endophytic fungi have a relatively few characters at morphological level and the limited variety that may cause interference in determine the properties of them[22,30]. Besides that, morphological characteristics are influenced by culture conditions[31].

Therefore, there is a need to use molecular technology to compensate for the limitations of morphological characterization. Molecular identification is a fast and effective alternative to finding internal taxol-producing endophytic fungi in contrast to morphological identification method[32,33].

In current study, COI mtDNAsequencing and ISSR-PCR analysis were carried out. ITS rDNA were amplified and sequenced for identification endophytic fungi. Isolates (TU-2, TU-3, TU-13, TU-15 and TU-16) related to *Aspergillus* sp. based on the ITS sequence were classified as *A. niger* when the other partial ITS sequence was identified other isolates TU-4 and TU-5 as *Alternaria alternata*. Isolates TU-10 and TU-12 were closely related to *Penicillium citrinum*.

Many studies have traditionally used sequence data from the ITS region to identify sterile cultures and evaluate morphotaxon boundaries[21,34,35]. ITS data are considered useful for these purposes due to the rapid evolution of the ITS. However, most fungi are not represented in GenBank and some GenBank records are misidentified or lack taxonomic information[18,34,37].

Therefore, BLAST and phylogenetic analyses of other genomic regions should be combined with those of the ITS region to improve the accuracy of identification. In this study, we selected representative isolates from morphotypes and then conducted phylogenetic analysis based on ITS partial sequences[18,21,36]. ISSR-PCR analysis was carried out using five ISSR primers. The average band per primer was 15.4. fragments for each primer of ISSR.

Conclusion

Finally, the ISSR molecular marking method is a smooth and high-performance method and one will likely describe it rather in relation to the RAPD method and higher production potential due to high steel temperatures. In this case, a set of minimum number of primers are required to be selected for the varietal identification and to select a set with minimum number of primers, it is important to consider level of marker polymorphism[36]. In case of ISSR markers, total genetic polymorphism of 49.4% was observed, showing that ISSR markers have moderate potential polymorphisms compared to other marker in discriminating in some endophytic fungi species[18,36].

On other hand, Hassan et al[36] reported that the total polymorphism amongst some fungi by using twenty ISSR primers was 0.764%. It was interesting to know the correlation among percent polymorphism and attributes of the markers for sequences different[21].

References

1. Senapati S.K. and Rout G.R., Study of culture conditions for improved micropropagation of hybrid rose, *Horticul. Sci*., **35(1)**, 27–34 **(2008)**

2. Guterman I. et al, Rose scent: genomics approach to discovering novel floral fragrance-related genes, *Plant Cell*, doi:10.1105/tpc. 005207, **14**, 2325–2338 **(2002)**

3. Attia O.A., El-Dessoky S.D. and El-Tarras A.E., *In vitro* propagation of *Rosa hybrida*L. cv. Al-Taif Rose plant, *Afric. J. Biotechnol*., doi: 10.5897/AJB12.781, **11(48)**, 10888-10893 **(2012)**

4. Alotaibi S.S., Hassan M.M., Gaber A. and Aljuaid B.S., Genetic relationship and diversity of Taif-roses plant by using three different types of molecular markers, *Res. J. Biotechnol*., **14(6)**, 130-138 **(2019)**

5. Uggla M., Gustavsson K.E., Olsson M.E. and Nybom H., Changes in color and sugar content in rose hips (*Rosa dumalis*L. and *Rosa rubiginosa*L.) during ripening, *J. Horticul. Sci. Biotechnol.*, doi.org/10.1080/14620316.2005.11511918, **80**, 204–208 **(2005)**

6. Kaur N., Sharma R.K., Sharma M., Singh V. and Ahuja P.S., Molecular evaluation and micropropagation of field selected elites of *R. damascene*, *Gen. Appl. Plant Physiol*., **33**, 171–186 **(2007)**

7. Naquvi K.J., Ansari S.H., Ali M. and Najmi K., Volatile oil composition of *Rosa damascena*Mill (Rosaceae), *J. Pharm. Phytochem.*, **2**, 177–181 **(2014)**

8. Rusanov K., Kovacheva N., Stefanova K., Atanassov A. and Atanassov I., Rosa damascene genetic resources and capacity building for molecular breeding, *Biotechnol. Equip*., doi:10.2478/V10133-009-0009-3, **23**, 1436–1439 **(2009)**

9. Amer S., Salih A. and Basaid E.A., Molecular identification of *Rosa x damascene* growing in Taif region, Saudi Arabia, *Intern. J. Plant Biol*., doi:10.4081/pb.2016.6307, **7(1)**, 7-10 **(2016)**

10. Petrini O., Fisher P.J. and Petrini L.E., Fungal endophytes of bracken (*Pteridium aquilinum*), with some reflections on their use in biological control, *Sydowia*, **44**, 282–293 **(1992)**

11. U'Ren J.M., Lutzoni F., Miadlikowska J. and Arnold E., Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens, *Microbiol. Ecol*., doi: 10.1007/s00248-010-9698-2, **60**, 340–353 **(2010)**

12. Soca-Chafre G., Rivera-Ordun˜a F.N., Hidalgo-Lara M.E., Hernandez R.C., Marsch R. and Flores-Cotera L.B., Molecular phylogeny and paclitaxel screening of fungal endophytes from Taxus globose, *Fungal Biol*., doi: 10.1016/j.funbio.2010.11.004 **115**, 143–156 **(2011)**

13. Sturz A.V. and Nowak J., Endophytic communities of rhizobacteria and the strategies required creating yield-enhancing associations with crops, *Appl Soil Ecol*, doi.org/10.1016/S0929-1393(00)00094-9, **15**, 183–190 **(2000)**

14. Liu L. et al, Chloropestolide A, an antitumor metabolite with an unprecedented spiroketal skeleton from Pestalotiopsisfici, *Org. Lett.*, doi:10.1021/ol901039m, **11(13)**, 2836–2839 **(2009)**

15. Lakhani H.N., Vakharia D.N., Hassan M.M. and Eissa R.A., Fingerprinting and molecular comparison among two parental strains of *Trichoderma* spp. and their corresponding fusants produced by protoplast fusion, *Biotechnol. Equip.*, doi.org/10. 1080/13102818.2016.1230478, **30(6)**, 1065–1074 **(2016)**

16. Fierer N., Microbial biogeography: Patterns in microbial diversity across space and time, In Zengler K., eds., Accessing uncultivated microorganisms: From the environment to organisms and genomes and back, Washington, DC, ASM Press, 95–115 **(2008)**

18. Tibpromma S. et al, Identification of endophytic fungi from leaves of Pandanaceae based on their morphotypes and DNA sequence data from southern Thailand, *MycoKeys*, doi.org/10.1007/s13225-010-0034-4, **33**, 25–67 **(2018)**

19. Yuan Z.L., Chen Y.C. and Ma X.J., Symbiotic fungi in roots of Artemisia annual with special reference to endophytic colonizers, *Plant Biosys.*, doi.org/10.1080/11263504.2010.544863, **145**, 495–502 **(2011)**

20. Pecoraro L., Girland M., Kull T., Perini C. and Perotto S., Molecular identification of root fungal associates in Orchis paucifloraTenore, *Plant Biosys*., doi:10.1080/11263504.2011. 634447, **146**, 985–991 **(2012)**

21. Hassan M.M., Farid M.A. and Gaber A., Rapid identification of *Trichoderma koningiopsis* and *Trichoderma longibrachiatum* using sequence characterized amplified region markers, *Egyp. J. Biol. Pest Cont*., doi.org/10.1186/s41938-019-0113-0, **29**, 13 **(2019)**

22. Mazrou Y.S.A., Makhlouf A.H., Elseehy M.M., Awad M.F. and Hassan M.M., Antagonistic activity and molecular characterization of biological control agent *Trichoderma harzianum* from Saudi Arabia, *Egyp. J. Biol. Pest Cont.*, doi.org/10.1186/s41938-020-0207-8, **30**, 4 **(2020)**

23. Ananda K. and Sridhar K.R., Diversity of endophytic fungi in the roots of mangrove species on the West Coast of India, *Can. J. Microbiol.*, doi: 10.1139/w02-080, **48**, 871-878 **(2002)**

24. Huang X.L. and Xin M.X., Experiential Technique in Microbiology, Beijing, Higher Education Press, 55-79 **(2008)**

25. Von-Arx J.A., Guarro J. and Figueras M.J., The ascomycete genus Chaetomium, *Beih. Nova. Hedwig*., **84**, 1–162 **(1986)**

26. Hermosam M.R. et al, Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp., *Appl. Environ. Microbiol*., doi: 10.1128/aem.66.5.1890-1898.2000, **66**, 1890-1898 **(2000)**

27. Hassan M.M., Influence of protoplast fusion between two *Trichoderma* spp. on extracellular enzymes production and antagonistic activity, *Biotechnol. Equip.*, doi: 10.1080/13102818. 2014.978206, **28(6)**, 1014–1023 **(2014)**

28. Kumar S., Stecher G. and Tamura K., MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets, *Mol. Biol. Evol*., doi: 10.1093/molbev/msw054, **33**, 1870-1874 **(2016)**

29. Rohlf F.J., NTSYS-PC numerical taxonomy and multivariate analysis system, version 2.1, Exeter Software, Setauket, New York, doi: 10.12691/wjar-1-6-6 **(2000)**

30. Anees M. et al, Characterization of field strains of *Trichoderma* antagonistic against *Rhizoctonia solani*, *Fun. Biol*., doi: 10.1016/j.funbio.2010.05.007, **114**, 691-701 **(2010)**

31. Diguta C.F., Vincent B., Guilloux-Benatier M., Alexandre H. and Rousseaux S., PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes, *Food Microbiol*., doi: 10.1016/j.fm.2011.03.006, **28**, 1145-1154 **(2011)**

32. Mirjalili M.H., Farzaneh M., Bonfill M., Rezadoost H. and Ghassempour A., Isolation and characterization of *Stemphylium sedicola* SBU-16 as a new endophytic taxolproducing fungus from Taxus baccata grown in Iran, *FEMS Microbiol. Lett*., doi: 10.1111/j.1574-6968.2011.02488.x, **328**, 122–129 **(2012)**

33. Xiong Z., Yang Y., Zhao N. and Wang Y., Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglojap yew, Taxus x media, *BMC Microbiol*., doi: 10.1186/1471-2180-13-71, **13**, 71 **(2013)**

34. Arnold A.E., Henk D.A., Eells R.L., Lutzoni F. and Vilgalys R., Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR, *Mycologia*, doi.org/10.1080/15572536.2007. 11832578, **99**, 185–206 **(2007)**

35. Lacap D.C., Hyde K.D. and Liew E.C., An evaluation of the fungal morphotype concept based on ribosomal DNA sequences, *Fungal Divers*., **12**, 53–66 **(2003)**

36. Gherbawy Y.A. and Gashgaric R.M., Molecular characterization of fungal endophytes from *Calotropis procera* plants in Taif region (Saudi Arabia) and their antifungal activities, *Plant Biosys.*, doi.org/10.1080/11263504.2013.819043, **2013**, 1-8 **(2013)**

37. Hassan M.M., Gaber A. and El-Hallous E.I., Molecular and morphological characterization of *Trichoderma harzianum* from different Egyptian soils, *Wulfenia J*., **21**, 80–96 **(2014)**.

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