Phytochemistry Letters 27 (2018) 36–43

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Phylogenetic analysis and diversity of novel endophytic fungi isolated from medicinal plant *Sceletium tortuosum*

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ARTICLE INFO

Keywords:
Diversity
Endophytic fungi
Novel
Elongation factor
Phylogenetic

ABSTRACT

Throughout history, mankind has used plants as their primary source of sustainability, in agricultural commodities, clothing, fragrances, fertilizers, flavours, and providing shelter. There is a strong symbiotic relationship between the plant and its endophytes. Endophytes are harboured within the living plant tissues without causing neither diseases nor symptoms. They produce bioactive compounds that protect the host plants against attack of insects, pathogens and herbivores. The bioactive compounds might be utilized for pharmaceutical, agricultural, or biotechnological applications.This paper reported on the various endophytic fungi strains that were isolated from isolated from a medicinal plant, *Sceletium tortuosum*. Fifty *Sceletium tortuosum* plants were collected from three different provinces in South Africa and leaves and roots used to isolate culturable endophytes. Morphological characteristics and a genus specific PCR designed to amplify fungal internal transcribe spacer (ITS) region (ITS1 and ITS4) and elongation factor (EF 1 and 2) was used for identification. A total of 60 fungal isolates belonging to 16 genera were identified and classified. Isolates were identified to species level based on similarities with known sequences in GenBank and a large proportion of the fungi were *Fusarium* species (37%) followed *Aspergillus* (25%) and *Penicillium* (7%) species. Phylogenetic analysis was performed using nuclear ribosomal DNA sequences and three potentially new isolates (DR 019 *Fusarium penzigii*, DR 010 *Phomopsis columnaris*, DR 007 *Fusarium oxysporum* f. sp. *lycopersici*) were identified in the phylogenic tree that was constructed. Our results offers basic data on the symbiotic/or mutualistic relationship between the medicinal plant *Sceletium tortuosum* and its endophytic fungi, as well as novel species.

1. Introduction

Sceletium tortuosum is a small succulent plant that is well-known for its medicinal properties and *Sceletium* species are widely distributed within South Africa, especially in the south-western area that is predominantly dry (Gericke and Viljoen, 2008). This dicotyledonous flowering slow-growing plant is endemic to the Cape Region of South Africa and belongs to the family Aizoaceae (Smith et al., 1996). This herbal plant that has been used as a mood-altering drug (Gericke and Viljoen, 2008) has several common names that range from Kanna to Channa, and Kougoed, meaning something to chew or chewable and is known as a psychoactive plant. A traditional concoction called "Kougoed" is prepared from the plant and used to treat cases of intoxication. Although the concoction is not known to be hallucinogenic nor habit forming, it is taken prior to stressing events such as hunting due to its cognitive effects. Numerous studies (Shikanga et al., 2011; Setshedi, 2012) involving the plant have focused on phytochemical analysis and structural elucidation of crude extracts.

Endophytic fungi are known to live and spend either all or part of their life cycle by colonizing the inter-and/or intra-cellular tissues of healthy host plants (Namasivayam et al., 2014). The presence of these fungi provides several benefits to the plant host such as drought tolerance, protection against pathogens, enhanced growth and prevention from destruction by herbivores (Higginbotham et al., 2013). It has also been reported that endophytic fungi play a very important role in affecting the quality and quantity of the crude extracts produced by host plants through a particular fungus-host interaction and this indicates the need to understand the occurence of these fungi in medicinal plants that are used traditionally for the treatment of infections (Faeth and Fagan, 2002). This paper focuses on assessing the diversity of novel

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https://doi.org/10.1016/j.phytol.2018.06.004
Received 19 February 2018; Received in revised form 4 May 2018; Accepted 11 June 2018
Available online 21 June 2018

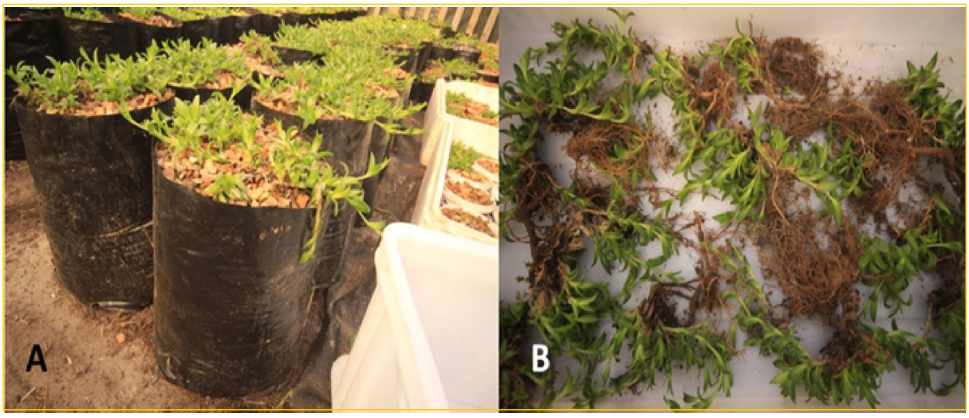

Fig. 1. *Sceletium tortuosum* medicinal plant (A) *Sceletium tortuosum* plantation (B) Individual *Sceletium tortuosum* with fine roots.

Table 1
Oligonucleotide primer sequences used to amplify ITS and EF target regions in endophytic fungi.

Primer	Sequence (5'-3')	Target species	PCR cycling conditions
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	All fungal isolates except *Fusarium* species	94 °C for 5 min; 94 °C for 30 secs 50 °C for 45 secs 30 Cycles 72 °C for 45 secs 72 °C for 7 mins
EF 1	CGAATCTTTGAACGCACATTG	*Fusarium* species	94 °C for 5 min 94 °C for 30 secs 54 °C for 45 secs 30 Cycles 72 °C for 45 secs 72 °C for 7 mins

endophytic fungi from a medicinal plant *Sceletium tortuosum* based on their phylogenic relationships.

Identification of endophytic fungi was previously based on morphological characters cultured on artificial media (Hyde and Soytong, 2007, 2008). Morphological structures such as the type of conidia and colony description have been used for identification of fungi. It is also known that secondary metabolites produced by plants are obstacles to the colonization of endophytic fungi and therefore these organisms must secrete the matching detoxification enzymes, such as cellulases, lactase, xylanase, and protease, in order to decompose the secondary metabolites such that they can penetrate through the defense systems of the resided host-plants.

A number of secondary metabolites, such as saponin as well as some essential oils are produced as a resistance mechanism against pathogens including endophytic fungi by medicinal plants when they cohabit (Sieber, 2007). To overcome this, endophytic fungi usually assume a latent state once inside the tissues of a host-plant either for the whole lifetime of the host plant (neutralism) or for an extended period of time (mutualism or antagonism) until environmental conditions are favorable for the fungi to its metabolites (Sieber, 2007). Against this background, bioactive compounds that are produced by endophytic fungi, with the exception of those produced by their host plants may enhance the tolerance of both the fungi and the plants to abiotic and biotic stress. Moreover, these compounds produced by endophytic fungi can in turn induce the production of a variety of novel bioactive secondary metabilites that may serve as important medicinal resoureces for humans (Zhang et al., 2006; Firáková et al., 2007; Rodriguez et al., 2009).

Despite the fact that endophytic fungi usually possess paired conidiophores with whorls of 2–3 phialides that produce one-celled, smooth surface and mostly globose green conidia that are ovoidal in shaped as well as thick and rough-walled, globose to subglobose chlamydospores that serve as specific morphological identification targets (Majid et al., 2015), modern techniques that incorporate DNA specific assays provide more reliable identification schemes that also reduce misclassification. DNA sequencing of the internal transcribed region (ITS) of fungal genomes is considered the goal standard technique for identification and determination of genetic relatedness. (Abd-Elsalam et al., 2003; de Beeck et al., 2014; Kozel and Wickes, 2014; Xu, 2016)

To the best of our knowledge, there is currently no study that documents the diversity of endophytic fungi isolated from *Sceletium* plants in South Africa since previous reports focused on phytochemical analysis of crude extracts (Patnala and Kanfer, 2009; Shikanga et al., 2011). The present study is therefore designed to investigate, identify and establish the genetic relationship of the endophytic fungi isolated from *Sceletium tortuosum* plants. This baseline data may provide valuable options for the identififfcation of novel antimicrobial agents for pharmaceutical and agricultural industrial applications.

2. Materials and methods

2.1. Collection of samples

A total of 50 *Sceletium tortuosum* plants that appear in Fig. 1 were collected from three different locations in South Africa and these comprised Roodepoort in Johannesburg, Gauteng Province; Sunndale and Klien Karoo in Cape Town, Western Cape Province with co-ordinates 26.1201 °S, 27.9015 °E, 34.1241 °S, 18.3875 °E, 25.6444 °S, 27.7773 °E, respectively. Fresh plant materials were wrapped in newspapers to reduce excessive moisture prior to transportation. Upon arrival in the laboratory, samples were temporally stored at 4 °C in a cold room and were processed within 48 h.

2.1.1. Isolation of endphytic fungi (surface sterilization and calculation of colonizing frequency)

Mature healthy *Sceletium tortuosum* plants with no visual symptoms of disease were selected and used for isolation of endophytic fungi. The plant samples were thoroughly washed with running water to remove dust and debris, and their surfaces were disinfected using a standard method (Araújo et al., 2001). Leaf, stem and root samples of plants were excised, cut into small portions and used for isolation of fungi. Each sample was rinsed with 70% (v/v) ethanol for 1 min and their surfaces were disinfected with 2% (v/v) sodium hypochlorite solution for 2 min. The samples were rinsed again 70% (v/v) ethanol for 20 s and latter twice with sterile distilled water based on a previous protocol (Araújo et al., 2001). Small portions of these plant material were placed on Nutrient-poor media that comprised Selective Fusarium Agar and Potato Carrot Agar supplemented with antibiotics, WA). Each plate was inoculated with 2–5 pieces of plant material and incubated for 7–10

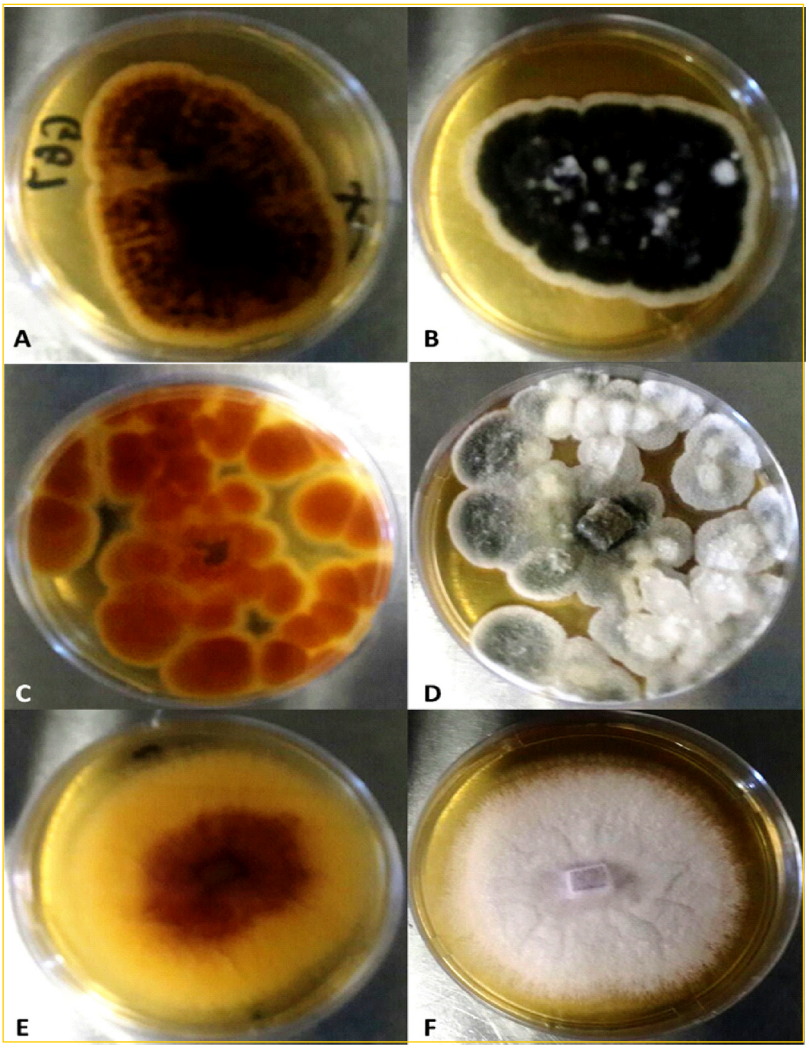

Fig. 2. Macroscopical characters of endophytic fungi on PDA (A) Front view GG 7 (B) Reverse view GG 7 (C) Reverse view GG 1 (D) Front view GG 1 (E) Reverse view GG 8 (F) Front view GG 8.

days at 25 °C. Each sample was analysed in duplicates of five.

Colonization Frequency (CF) was calculated as described by Suryanarayanan et al., 2003.

$$\text{Colonization frequency of endophyte} = \left\{\frac{\text{Number of segments colonized by fungi}}{\text{Total number of segment observed}}\right\} \times 100$$

2.2. Morphological identification

All isolates identified were purified using single spore technique, preserved, maintained and stored in the Plant Protection Research Institute (PPRI) collection of the National Collections of Fungi, Biosystematics Programme, Agricultural Research Council, South Africa. Each isolate was assigned a unique accession PPRI number. Hyphal morphology, mycelia and spore characteristics were observed using an EVOS FL microscope (AMEP4708) obtained from Life Technologies and fungal structures were captured (Carmichael et al., 1980; Barnett and Hunter, 1998; Carris et al., 2012). Colours were named using 'A Mycological Colour Chart'.

2.3. Molecular identification endophytic fungi

To determine the identity and phylogentic relationship among the endophytic fungi isolated in this study the Internal Transcribed Spacer (ITS) and Elongation Factor (EF) regions were used as targets for all other fungal species and *Fusarium* species, respectively (Khorasani, 2013). The Qiagen DNeasy Mini Plant Kit (Hilden, Germany) was used to extract genomic DNA from fungal mycelium based on the manufacturer's instructions. The DNA was quantified using a Nanodrop (Thermo Scientific, NanodropLite, USA) and stored at −20 °C for PCR analysis. PCR amplifications were performed using ITS and EF specific oligonucleotide primer sequences that presented in Table 1 to target all fungal isolates except *Fusarium* and *Fusarium* species respectively (Khorasani, 2013). Amplifications were performed in a Thermal Cycler (C1000 Touch, Bio-Rad, Johannesburg, South Africa) using conditions outlined in Table 1. PCR products were resolved by electrophoresis on a 1.7% (w/v) agarose gel containing 0.1 µg/mL ethidium bromide. Amplicons were visualized under UV light. The PCR products were purified and sequenced using an Applied Biosystems ABI 3500XL Genetic Analyser (Model 4,401,689) at Inqaba Biotechnologies (Pretoria, South Africa).

2.4. Phylogenetic analysis

Consensus sequences were constructed for all isolates and were subjected to the Basic Local Alignment Search Tool (BLAST) on http://wwwncbinlmgov/BLAST in NCBI database. Prior to this manual editing was conducted and then pairwise alignment was accomplished by

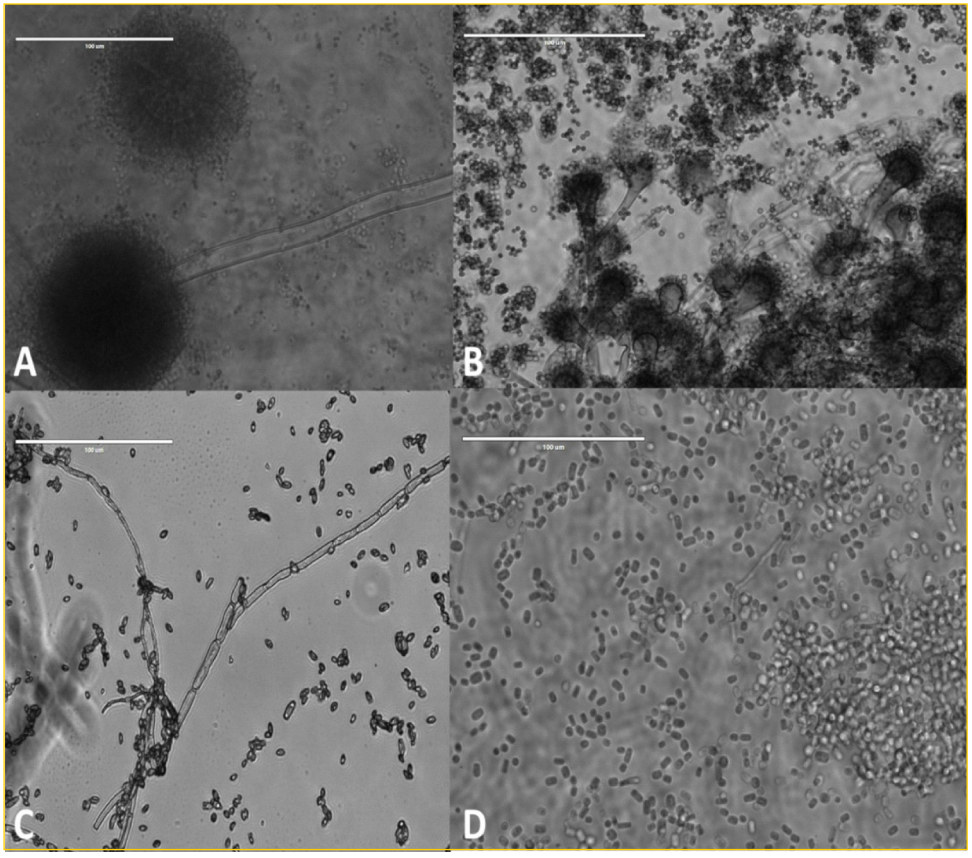

Fig. 3. Morphology characters used for identification (A) GG 14, (B) ND 20, (C) DR 16(D) and ND 17.

Fig. 4. Colonization rate of endophytic fungi isolated in leaves and roots at GP (Gauteng), WC1 (Western Cape site 1), WC2 (Western Cape site 2).

BioEdit software. Fungal identities were generated by comparing investigative sequences with those previously submitted in Genbank. The genetic relationships between the isolates was established with the MEGA 52 software using Neighbor joining method. The consensus sequences were contracted from the Internal Transcribed Spacer (ITS) and Elongation Factor (EF) regions to construct phylogenetic tree with 1000 replications bootstrap.

3. Results and discussion

In the current study, sixty endophytic fungi were successfully isolated from 50 *Sceletium* plants. All 60 isolates were subjected to morphological characterisation. The microphotographs of morphological structures and taxonomic parameters that were used for classification of the species are shown in Figs. 2 and 3. Cultures on PDA produced moderately fast growing colonies that were transparent, but with colours comprising black, pink and white (Fig. 2). Isolates grew well at 25 °C and plates were usually covered within 7–10 days. Mycelia were usually simple but with branched hyphae in some situations and also possessed conidiophores (Fig. 3). The reverse view of colonies were either black, pink or white (Fig. 2B, C and E). Iolates were successfully identified to Genus level using morphological characteristics and they belonged to 16 genera. The dominant endophytic fungi belong to genera *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* and this was in

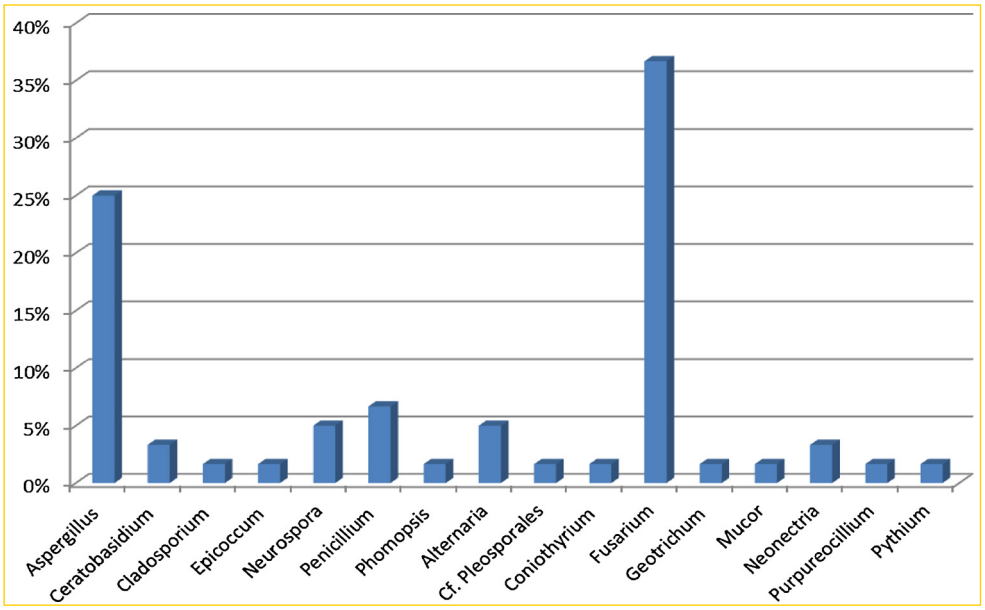

Fig. 5. Distribution and diversity of Sceletium endophytic fungi at genus levels.

agreement with the results of Qi et al. (2009). In addition, our results revealed that only 40% (24/60) of the fungi analysed were identified to genus level.

Colonization rate (CR) was defined as the number of fragments from which one or more endophytic fungi (EF) was isolated, divided by the total number of incubated fragments (Petrini et al., 1982). There was significant difference in the CR between leaves and roots since the CR in leaves were higher than roots. The colonization rates for the leaves of plants obtained from the Western Cape site 2 (WC2) was higher (22%) than those of the roots (16%) of the same plants. In addition, the fungal CR for plants obtained from WC2 was higher than those from Western Cape site 1 (WC1) as well those from Gauteng province (GP). Similarly, the colonization rate was significantly higher in leaves (20%) compared to roots (13%) of plants obtained from Gauteng (Fig. 4). Generally endophytic fungi were more prevalent on leaf tissue (> 50%) than roots. On the contrary previous findings by Naik et al. (2009), revealed an overall fungal colonization rate of *Oryza sativa* was higher in the roots than leaves.

In order to determine the phylogenetic relationship among the endophytic fungi from *Sceletium tortuosum* plants and related species, the ITS and EF genes amplified from isolates and sequenced were compared. The identities of the isolates based on BLAST search as well as the percentage similarities to previously deposited sequences are shown in Table 1. The results revealed that 56 (93%) of the isolates had 97%–100% sequence similarities with relevant sequences in GenBank. However, isolates GG012, GG016, ND5, DR019 respectively, had 87%, 95%, 92% and 83% sequence similarities with previous sequences in GenBank and were identified as *Fusarium oxysporum* (JX840353.1), *Aspergillus niger* (KM460938.1), *Neurospora* (KT844664.1) and *Fusarium penzigii* (NR_137707.1) respectively. The proportion of isolates belonging to the genus *Fusarium* (37%; 22/60) was higher than those identified as *Aspergillus* (25%; 15/60) and other related species (*Penicillium*, *Altenaria*, *Phompsis*, *Neurospora*) that were detected among less than 10% (Fig. 5). *Neurospora* species have enormous potential to adapt to changing environments, and are therefore capable of switching their disease-causing capabilities from endophytic to pathogenic or saprotrophic fungi (Hsiao-Che et al., 2014). In addition, a large proportion of these fungi belong to genera that have been reported to be pathogenic to both humans and animals (Perez-Nadales et al., 2014). Our findings are similar to those previously reported in which *Fusarium* and *Aspergillus* species were dominant among endophytic fungi isolated (Zakaria et al., 2010; Musavi and Balakrishnan, 2013; Srivastava, 2017). Isolates DR 13, GG 7, GG 8, ND 10, DR 1, DR 8, DR 13 revealed 100% sequence similarities with *Fusarium equiseti*, *Alternaria* sp. *Fusarium oxysporum, Alternaria* sp. *Aspergillus niger, Fusarium* sp. *Fusarium equiseti* repectively. The consensus sequences were submitted in NCBI database, GenBank bankit and accession numbers were issued (Table 2).

The phylogenetic relationships of the isolates was determined through the Bootstrap analysis using 1000 replications in order to assess the relative of the branches of the tree that was constructed. The sequences generated from the isolates were compared to reference fungal taxa in the database. The sum of branch length was calculated as 156.35442900 and the final dataset total was 226 positions. This was extracted from 60 nucleotide sequences.

All the consensus sequences were aligned using MAFF (Multiple Alignment using Fast Fourier Transform) program and construction of the phylogenetic tree was based on the neighbor joining method consisting of 1000 bootstrap replications associated taxa clustered (Fig. 6). Phylogenetic analysis using ITS region of endophytic fungi is distributed into three Clusters which is illustrated in Fig. 6. Cluster 2 subdivision shows a significant weakness in the bootstrap support which was less than 50%.

Further interpretation reveals that all clusters (Cl 1–3) have strains belonging to Division ascomycota while the Division basidiomycota is located in cluster 1 and 2. Nevertheless, the genera Ceratobasidium (GG 6, DR 11) are clustered under different taxa groups 1 and 3. Thought-provoking strains are situated in cluster 1 but in different sub-clusters (DR 12, GG2) belonging to the division Oomycota and Zygomycota. Ascomycota dominates the scientific classification with an average of 81% in the phylogenetic tree. Strong phylogenetic relationships/homology (95–100%) was shown with symbols. On the other hand 50% and less were not displayed in the phylogenetic tree. This data correspond with Miguel et al., (2017), Basidiomycota and Ascomycota were the dominate phyla isolated from eucalyptus leaves. Based on the allocation of stains in the phylogenetic tree, there are three potential novel endophytic fungi (DR 7, DR 10, DR 19) situated in all the clusters. Furthermore, the strains were isolated from the same location. All strains were belonging to the genus *Fusarium* with different species and subspecies.

Table 2
Sequence identities of endophytic fungi isolated from *S. tortuosum* based on BLAST analysis.

Sample ID	Closest related species		GenBank Best BLAST Match	
			Accession No.	No. coverage
GG 001	*Aspergillus*	sp.	KR154911.1	100%
GG 002	*Mucor*	*Circinelloides*	DQ118990.1	99%
GG 003	*Fusarium*	*Phaseoli*	KF717534.1	99%
GG 004	*Penicillium*	*Janthinellum*	KM268704.1	99%
GG 005	*Fusarium*	*Solani*	KP784419.1	100%
GG 006	*Ceratobasidium*	sp.	KT265713.1	100%
GG 007	*Alternaria*	sp.	KX270745.1	99%
GG 008	*Fusarium*	*oxysporum*	KJ774041.1	100%
GG 009	*Neurospora*		KT844666.1	100%
GG 010	*Aspergillus*	*terreus*	KJ685810.1	100%
GG 011	*Fusarium*	*solani*	KX349467.1	100%
GG 012	*Fusarium*	*oxysporum*	JX840353.1	87%
GG 013	*Aspergillus*	*fumigatus*	HE864321.1	100%
GG 014	*Aspergillus*	*niger*	KP172477.1	98%
GG 015.1	*Aspergillus*	sp.	KP881423.1	98%
GG 015.2	*Aspergillus*	sp.	KM924435.1	99%
GG 016	*Aspergillus*	*niger*	KM460938.1	95%
ND 1	*Aspergillus*	sp.	KM924435.1	100%
ND 2	*Aspergillus*	*fumigatus*	KF305755.1	98%
ND 3	*Penicillium*	*brevicompactum*	KR912330.1	98%
ND 4	*Penicillium*	*echinulatum*	KP411588.1	100%
ND 5	*Neurospora*		KT844664.1	92%
ND 6	*Penicillium*	sp.	KT192314.1	100%
ND 7	*Geotrichum*	sp.	KU571521.1	97%
ND 8	*Alternaria*	sp.	KP027305.1	98%
ND 9	*Aspergillus*	sp.	KP881423.1	98%
ND 10	*Alternaria*	sp.	KR094438.1	96%
ND 12	*Epicoccum*	*nigrum*	KP721576.1	99%
ND 13	*Fusarium*	*oxysporum* f. sp. *ciceris*	JN400682.1	100%
ND 14	*Aspergillus*	*fumigatus*	JQ776545.1	99%
ND 15	*Aspergillus*	*niger*	KJ881376.1	99%
ND 16	*Aspergillus*	*niger*	JQ929761.1	99%
ND 17	*Aspergillus*	sp.	KF305740.1	99%
ND 18	*Neurospora*		KT844666.1	99%
ND 19	*Fusarium*	*oxysporum f.* sp. *lycopersici*	KC478629.1	99%
DR 001	*Aspergillus*	*niger*	KP748369.1	100%
DR 002	*Fusarium*	*oxysporum*	EF495235.1	100%
DR 003	*Coniothyrium*	*aleuritis*	KP749188.1	98%
DR 004	*Fusarium*	*oxysporum* f. sp. *ciceris*	KU097318.1	98%
DR 005	*Cf. Pleosporales*	sp.	HM596868.1	98%
DR 006	*Fusarium*	*equiseti*	KU715166.1	97%
DR 007	*Fusarium*	*oxysporum* f. sp. *lycopersici*	KC478629.1	97%
DR 008	*Fusarium*	sp.	DQ446211.2	99%
DR 009	*Fusarium*	*equiseti*	KU715166.1	99%
DR 010	*Phomopsis*	*columnaris*	GU934561.1	99%
DR 011	*Ceratobasidium*	sp.	KT428729.1	99%
DR 012	*Pythium*	*heterothallicum*	KU210972.1	99%
DR 013	*Fusarium*	*equiseti*	JQ412109.1	99%
DR 014.1	*Neonectria*	sp.	HQ731630.1	99%
DR 014.2	*Neonectria*	sp.	HQ731630.1	86%
DR 015	*Purpureocillium*	sp.	KJ935014.1	100%
DR 016	*Cladosporium*	sp.	KF976501.1	99%
DR 017	*Fusarium*	*solani*	KP784419.1	99%
DR 018	*Fusarium*	*dimerum*	JQ434586.1	98%
DR 019	*Fusarium*	*penzigii*	NR_137707.1	83%
DR 020	*Fusarium*	*subglutinans*	KU715164.1	97%
DR 021	*Fusarium*	*equiseti*	JQ412109.1	99%
DR 022	*Fusarium*	sp.	DQ446211.2	100%
DR 023	*Fusarium*	*oxysporum*	KU746660.1	100%
DR 024	*Fusarium*	sp.	DQ446211.2	99%

To the best of our knowledge, there is no study that investigated the diversity of endophytic fungi isolates from indigenous *S. tortuosum*. The current study demonstrates the relatively high variety of fungal communities from the biodiversity of three locations in South Africa. The extensive richness of endophytes in the roots and leaves is directly proportional to physiological and/or ecological importance of medicinal plants. In the current study, the CR value is higher in leaves, hence more endophyic fungi habour the leaves than the roots. Three novel isolates were identified in the phylogenetic tree. Our findings lay a critical foundation in a deeper understanding of *S. tortuosum*. This baseline data provides valid directions for future research particularly analysis of the bioactive compounds produced by these fungi and their therapeutic applications as well as extracellular enzymes that they produce.

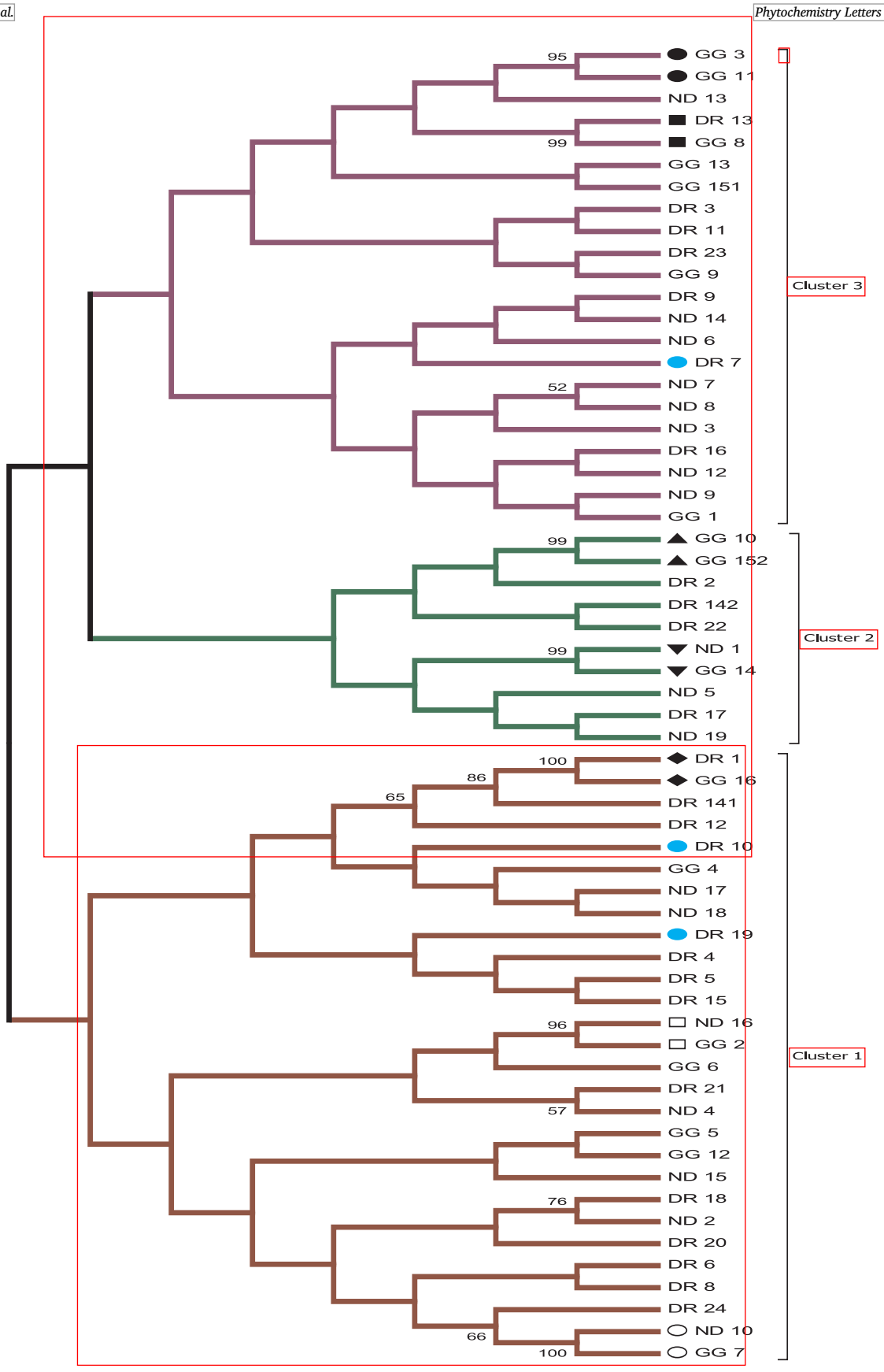

Fig. 6. Phylogenetic tree constructed by neighbor joining method using ITS sequences of 60 fungal strains. Bootstrap values is based on 1000 replicates while above 50% are indicated on the branches.

Acknowledgements

This work was supported by the National Research Fund (NRF) and Health and Welfare Sector Education and Training Authority (HWSETA) of South Africa.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2018.06.004.

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