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

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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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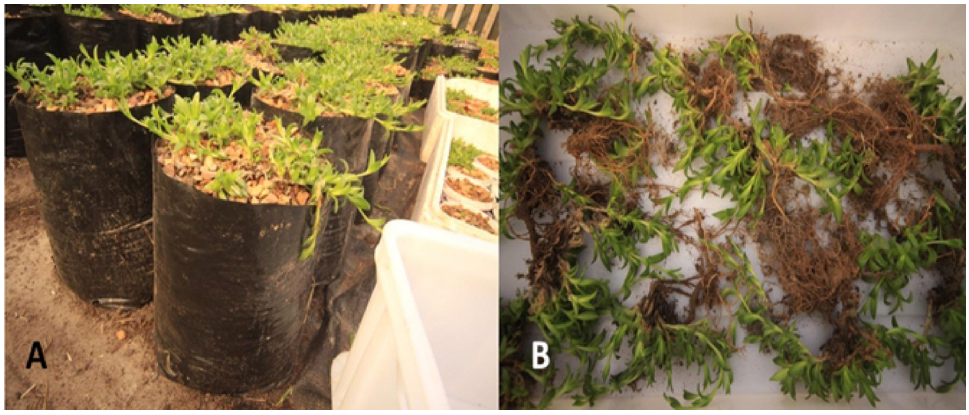

Fig. 1. *Sceletium tortuosum* medicinal plant (A) *Sceletium tortuosum* plantation (B) Individual *Sceletium tortuosum* with fine roots.

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 identifiifcation 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