Seasonal variability in fungal endophytes from Aizoaceae plants in the Succulent Karoo biodiversity hotspot, South Africa

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ABSTRACT

All ten species of Aizoaceae screened in this study were populated with endophytes. Fifty nine fungal species from 25 genera, including fourteen rare species, were identified. Seasonal specificity was observed; with 32 and 21 fungal endophytes isolated only during the flowering and dry seasons, respectively. The genus *Fusarium* was the most abundant in samples from the flowering season, whilst *Alternaria* and *Cladosporium* were equally abundant in the dry season. Rarely isolated genera included *Neophaeosphaeria, Periconia, Preussia, Schizothecium* and *Truncatella*. *Fusarium oxysporum, Paecilomyces victoriae* and *Talaromyces pinophilus* were the largest contributors to the differences in community structure observed for fungal endophytes from the different seasons. Endophytic fungal richness was very high in comparison to other global arid regions. This is the first record of all these fungal species isolated from Aizoaceae plants in their endemic environment in the most biodiverse arid region in the world, the Succulent Karoo in South Africa.

KEYWORDS

Conservation; fungal diversity; mesembs; Namaqua National Park; rare species; seasonality

1. Introduction

Since the official establishment of Biodiversity Hotspots in 1989, the International Union for Conservation (IUCN) has recognised the Succulent Karoo in South Africa as the most biodiverse arid region in the world due to its incomparable alpha (local) diversity, astonishing beta diversity along habitat gradients and gamma diversity along geographical gradients (Myers, 2000; Desmet and Cowling, 2004; Sloan *et al.*, 2014). In spite of its ecological and socioeconomic importance the natural habitat of the Succulent Karoo has been subjected to intense destruction and little has been done to protect its natural resources (SANParks, 2014). Within this ecoregion a family of leaf succulents, the Aizoaceae (Caryophyllales, Plantae) represent the most remarkable radiation in the entire plant kingdom, with a single ancestor diverging into numerous species (Klak *et al.*, 2004). Commonly known as mesembs or "ice plants", or by their Afrikaans vernacular name of "vygies" in South Africa, these endemic plants thrive despite nutrient poor soils, limited water availability and large diurnal temperature fluctuations (Smith *et al.*, 1998).

Recent studies have highlighted the importance of the contribution of microbial life to plant thermotolerance, drought resistance and other important survival strategies by showing that diverse secondary metabolites are produced by the endophytic fungi harboured inside the plants rather than by the plants themselves (Moncrieff *et al.*, 2015; Mishra *et al.*, 2016). Fungal endophytes are phylogenetically diverse microscopic, eukaryotic organisms that colonise, either inter- or intra-cellularly, the healthy living, internal tissues of their host without causing any disease symptoms (Rodriguez *et al.*, 2009; Massimo *et al.*, 2015). Most studies exploring endophytes have been done in humid regions, with surprisingly few studies done in arid and semi-arid regions, where the focus has only been on cacti or grass species (Suryanarayanan *et al.*, 2005; Loro *et al.*, 2012; Bezerra *et al.*, 2012, 2013).

Seasonality has been shown to influence fungal endophytic communities associated with olive trees in the Mediterranean (Martins et al., 2016) as well as black plum in India (Yadav et al., 2016) while in other regions such as the Atlantic rain forest seasonality has been shown to be of minor importance (Bonfim et al., 2016). In the Namaqua National Park, sited in the South African Succulent Karoo biodiversity hotspot, the dynamics of plant communities change dramatically after the rainfall period, from May to September, when more than 60% of the annual precipitation occurs (South African Weather Service, www.weathersa.co.za).

This study is founded on the hypothesis that the fungal endophyte diversity associated with mesembs in the Succulent Karoo will be high and influenced by seasonality. The objectives of this study were: 1) to isolate and identify fungal endophytes from Aizoaceae species in the Namaqua National Park during the dry and flowering seasons; 2) to determine the influence of seasonality on community composition and 3) to explore fungal species richness and diversity in comparison with similar studies from other dry environments.

2. Materials and methods

2.1. Study site and sampling

Sampling occurred before and after the highest rainfall period (June-August) in the Succulent Karoo, Northern Cape Province, South Africa. In September 2013, during the flowering season Aizoaceae plants were collected from three selected sampling locations near the Skilpad camp (30°09'19"S; 17°43'55"E), in Namaqua National Park, a protected area inside the Succulent Karoo biodiversity hotspot (Fig. 1). Plots were $20m^2$ in size, divided into 10 blocks in three rows. Aizoaceae plants were also collected from the same three sampling locations during the flowering season (September-October) of 2014.

Fig. 1.a) Map of South Africa indicating the sampling site; b Map of the sampling site, close to Skilpad camp in the Namaqua National Park, in the Succulent Karoo biome in South Africa (Google maps, 2017); c) three species of mesemb plants, (i) *Drosanthemum diversifolium*, (ii) *Mesembryanthemum barklyi*, (iii) *Carpobrotus edulis*.

These sites were also re-sampled at the end of the dry season, in May of 2014 and 2015. As this area is a South African National Park, a permit to conduct this study was obtained from South African National Parks (SANParks: permit number CRC/2015/010--2014). No endangered species were sampled or disturbed during the sampling process. Common Aizoaceae plants were removed from each site on each of four collecting trips, for a total of 45 plants. Plant material was transported in brown paper bags at temperatures below 10°C in a cooler box, then refrigerated at 4°C and processing took place within 96h of collection. All samples collected during all sampling trips were treated in the same manner to minimize the possible influence of fungal opportunists that might have intruded during transportation.

2.2. Endophyte isolation

Aerial plant tissues were surface sterilized by immersion in 70% ethanol (EtOH) for 1min, followed by 1% aqueous sodium hypochlorite (NaClO) solution for 3min and 75% ethanol for 1min to remove epiphyllous fungi and contaminants. Samples were rinsed three times with double distilled (dd) H_2O and dried under sterile conditions inside a laminar flow cabinet. Small fragments (5 – 10mm) of leaf material were aseptically removed from each plant with forceps and plated onto potato dextrose agar (PDA) (Biolab, Merck, Darmstadt, Germany) in 90mm Petri dishes. Plates were sealed with Parafilm® and incubated at 10, 20 and 30°C, under 12h day/night light regime for a maximum period of six weeks. Plates were evaluated every 48h and hyphae from emerging fungal colonies were selected and transferred to fresh PDA in 45mm Petri dishes for purification and further morphological identification. All representative isolates were deposited into the South African National Collection of Fungi living fungal collection (PPRI), where cultures are maintained.

2.3 Endophyte identification

Fungal identity was determined by colony morphology and microscopic morphological characters and distinct isolates were subjected to further molecular identification. DNA was extracted from fungal mycelia using the DNAeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. Extracted DNA was used as a template to PCR amplify the ITS region using the primer set ITS 1 and ITS 4 (White *et al.*, 1990), performed in a total reaction volume of 25µl, which included 2µl of 10x DreamTaq DNA buffer (Thermo Fisher Scientific, Waltham, MA), 2µl of 25µM dNTPs (Promega Corp., Madison, WI), 0.5µl (0.2µM) of each primer (Sigma-Aldrich, St. Louis), 0.2µl (5U/µl) DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA), 2µl template DNA and ddH_2O to a total volume of 25µl. The PCR reaction conditions were an initial denaturation step at 94°C for 2min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 52°C for 30s and elongation at 72°C for 45s, with a final elongation step of 72°C for 7min. PCR products were visualized on 1% agarose gels at 80V for 30min and stained with ethidium bromide at a concentration of 1µg/µl. DNA sequences from PCR amplicons were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, Paisley, UK) using both forward and reverse primers for each gene region. Consensus sequences were manually edited (where necessary) in BioEdit version 7.2.5. (Hall, 1999) and compared to those published on NCBI's GenBank sequence database (http://www.ncbi.nlm.nih.gov) as well as MycoBank (www.mycobank.org) (Crous *et al.*, 2004) by means of BLASTn analyses. If the similarity based on the ITS region was >97%, the most similar reference sequence from these databases was used to assign identity. An identical name was assigned to sequences sharing less than 5% nucleotide difference. The global fungal nomenclature database www.indexfungorum.org was used to ensure that the most current name of fungal species was used and to eliminate synonymy.

2.4. Biodiversity measurements

The relative abundance of endophytic fungi was calculated by dividing the number of isolates of each species by the total number of isolates and multiplying by 100 (Table 1). Colonization frequency was calculated by dividing the number of colonized segments into the total number of segments incubated and reported as a percentage. Species diversity was estimated by various indices (Table 2) using PAST 3.14 (Hammer *et al.*, 2001) with a bootstrap value of 9999 and a 95% confidence interval. The species accumulation curve was constructed by implementing the analytical solution known as "Mao's tau", with standard deviation and standard error converted to 95% confidence intervals. Analysis of similarity (ANOSIM) was used to determine differences in relative abundance of fungal endophytes (Clarke, 1993). Differences in fungal endophyte communities isolated from Aizoaceae in the dry and flowering season were visualised by nonmetric multidimensional scaling (NMDS) ordination using the Bray-Curtis dissimilarity index for relative abundance data as well as the Jaccard dissimilarity index for binary (presence / absence) data (Taguchi and Oono, 2005). A similarity percentages analysis (SIMPER) was used to investigate whether certain species were chiefly responsible for differences observed with NMDS analysis (Clarke, 1993).

3. Results

All mesemb species (*Mesembryanthemum crystallinum*, *Mesembryanthemum barklyi*, *Drosanthemum diversifolium*, *Delosperma echinatum*, *Trichodiadema bulbosum*, *Lampranthus bicolor*, *Ruschia diversifolia*, *Leipoldtia schultzei*, *Vanzijlia annulata* and *Carpobrotus acinaciformis*) screened in this study were populated with endophytes on all occasions investigated. In total, 450 plant fragments were plated from which 253 mycelial fungal endophytes were isolated, yielding a colonization frequency of 56.2%. These isolates comprised

59 fungal species, from 25 genera (Table 1, Fig. 2). Fourteen of the isolated species (23.7%) can be considered as rare species associated with Aizoaceae in the region – with sequences corresponding with genera with only a single representative in the sample (singletons). *Alternaria alternata*, *Aspergillus parasiticus*, *Chaetosphaeronema hispidulum* and *Cladosporium cladosporioides* were among the only six species isolated from samples of both the dry and flowering seasons. Seasonal specificity was observed; with 32 and 21 fungal endophytes isolated only from samples collected during the flowering season and dry season, respectively.

Table 1 List of fungal endophytes isolated from Aizoaceae from the Namaqua National Park in the Succulent Karoo biodiversity hotspot, South Africa, during the dry and flowering seasons.

Endophytic fungi			**Season**			
	GenBank#	**PPRI#**	**Dry**	**Flr**	**Tot**	**RA**
Alternaria alternata	**AF347031**	16010	11	13	24	9.486
Alternaria eureka	**NR136016**	17624	0	5	5	1.976
Alternaria palandui	**DQ323682**	16053	6	0	6	2.372
Alternaria pellucida	**AF347031**	14425	0	1	1	0.395
Alternaria sp.	**AF347031**	14422	7	2	9	3.557
Alternaria tenuissima	**EF031053**	14429	0	4	4	1.581
Alternaria yali-inficiens	**AF347031**	16051	1	0	1	0.395
Aspergillus aureolus	**KY808743**	17735	0	6	6	2.372
Aspergillus fischeri	**EF669936**	17632	0	6	6	2.372
Aspergillus flavus	**AF027863**	14424	0	2	2	0.791
Aspergillus fumigatiaffinis	**EF669936**	13089	0	2	2	0.791
Aspergillus neoniveus	**NR137474**	13094	0	2	2	0.791
Aspergillus parasiticus	**JN942865**	14430	3	4	7	2.767
Aspergillus ustus	**EF652455**	17765	0	2	2	0.791

Endophytic fungi			Season			
	GenBank#	PPRI#	Dry	Flr	Tot	RA
Boeremia exigua	**KR653200**	16016	2	0	2	0.791
Chaetomium carinthiacum	**KT214565**	17666	0	1	1	0.395
Chaetomium funicola	**GU563369**	17640	0	2	2	0.791
Chaetosphaeronema hispidulum	**KF251148**	17732	4	4	8	3.162
Cladosporium allicinum	**EF679350**	16052	5	0	5	1.976
Cladosporium cladosporioides	**JN942904**	15983	8	6	14	5.534
Cladosporium licheniphilum	**HM148111**	16014	1	0	1	0.395
Cladosporium oxysporum	**HM148120**	16032	1	0	1	0.395
Cladosporium phyllophilum	**HM148154**	16013	2	0	2	0.791
Cladosporium sp.	**JN942904**	15990	5	0	5	1.976
Cladosporium tenuissimum	**HM148197**	16038	2	0	2	0.791
Cladosporium uredinicola	**AY362001**	16046	1	0	1	0.395
Curvularia borreriae	**HE861848**	14431	0	3	3	1.186
Curvularia spicifera	**KJ922377**	13102	0	2	2	0.791
Curvularia trifolii	**HG779023**	13091	0	6	6	2.372
Epicoccum nigrum	**FJ426996**	16007	5	0	5	1.976
Fusarium chlamydosporum	**GQ505439**	17664	0	4	4	1.581
Fusarium oxysporum	**U34566**	17777	0	24	24	9.486
Fusarium solani	**AF178409**	17659	0	1	1	0.395
Neophaeosphaeria sp.	**NR1378331**	16017	1	0	1	0.395
Paecilomyces victoriae	**JN899393**	17711	0	8	8	3.162
Penicillium abidjanum	**GU981618**	17635	0	1	1	0.395
Penicillium atrosanguineum	**GU944599**	17702	0	2	2	0.791
Penicillium canescens	**AF033493**	17642	0	1	1	0.395

Endophytic fungi			Season			
	GenBank#	PPRI#	Dry	Flr	Tot	RA
Penicillium chrysogenum	**AF033465**	13087	0	3	3	1.186
Penicillium corylophilum	**AF033450**	15997	3	0	3	1.186
Penicillium rubefaciens	**KC411677**	15986	3	0	3	1.186
Penicillium vanluykii	**JX997007**	13099	0	2	2	0.791
Periconia macrospinosa	**KP183999**	17627	0	1	1	0.395
Phoma herbarum	**JF810530**	16006	2	0	2	0.791
Phoma sp.	**KT389535**	13095	6	4	10	3.953
Phyllosticta capitalensis	**JF261459**	14433	0	3	3	1.186
Pleosporales sp.		16048	8	0	8	3.162
Preussia terricola	**GQ203765**	17623	0	1	1	0.395
Pseudodiplodia ruticola	**GQ203765**	16003	2	0	2	0.791
Pseudopithomyces chartarum	**DQ384571**	16033	2	0	2	0.791
Purpureocillium lilacinum	**FR734101**	13104	0	4	4	1.581
Rhizopus arrhizus	**AF543522**	13092	0	2	2	0.791
Schizothecium curvisporum	**AY999119**	17628	0	1	1	0.395
Talaromyces amestolkiae	**JX315678**	16043	6	0	6	2.372
Talaromyces pinophilus	**JN899382**	17634	0	10	10	3.953
Talaromyces purpureogenus	**JX315671**	16009	3	0	3	1.186
Trichoderma ghanese	**Z31015**	13101	0	2	2	0.791
Trichoderma spirale	**AF400262**	17661	0	5	5	1.976
Truncatella spadicea	**DQ278989**	16027	1	0	1	0.395
Total			101	152	253	100.00

* Abbreviations: *GenBank#* Closest match to GenBank accession, *PPRI#* Accession number in the South African National Collection of Fungi, *Dry* Number of dry season isolates, *Flr* Number of flowering season isolates, *Tot* Total number of isolates, *RA* Relative abundance of each fungal endophyte species

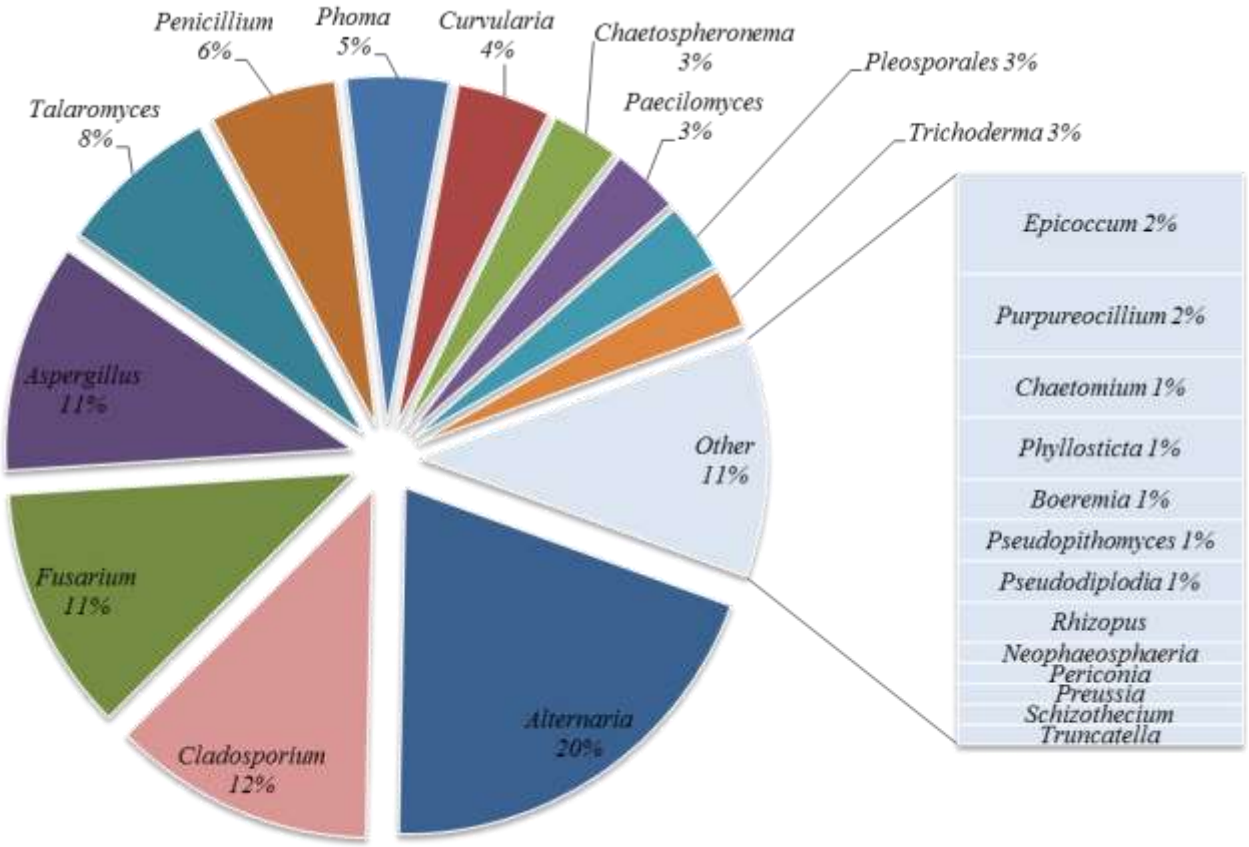

Fig. 2. Relative abundance of fungal genera isolated from Aizoaceae from the Namaqua National Park in the Succulent Karoo biodiversity hotspot, South Africa, during the dry and flowering seasons combined (total population).

Non-metric multidimensional scaling (NMDS) clearly separated samples from the two seasons (Fig. 3), illustrating highly significant seasonality in the culturable fungal endophyte communities. The different samples taken during the flowering season were more similar to each other than the different samples taken during the dry season, as was evident from the closer clustering of the former samples. ANOSIM was used to visualise differences in relative abundance of the fungal endophyte communities associated with seasonality (Fig. 4a) and presence / absence data (Fig. 4b). It is clear that differences between the dry and flowering season were significantly higher, compared to differences within each season, as evident from the higher ranked distances.

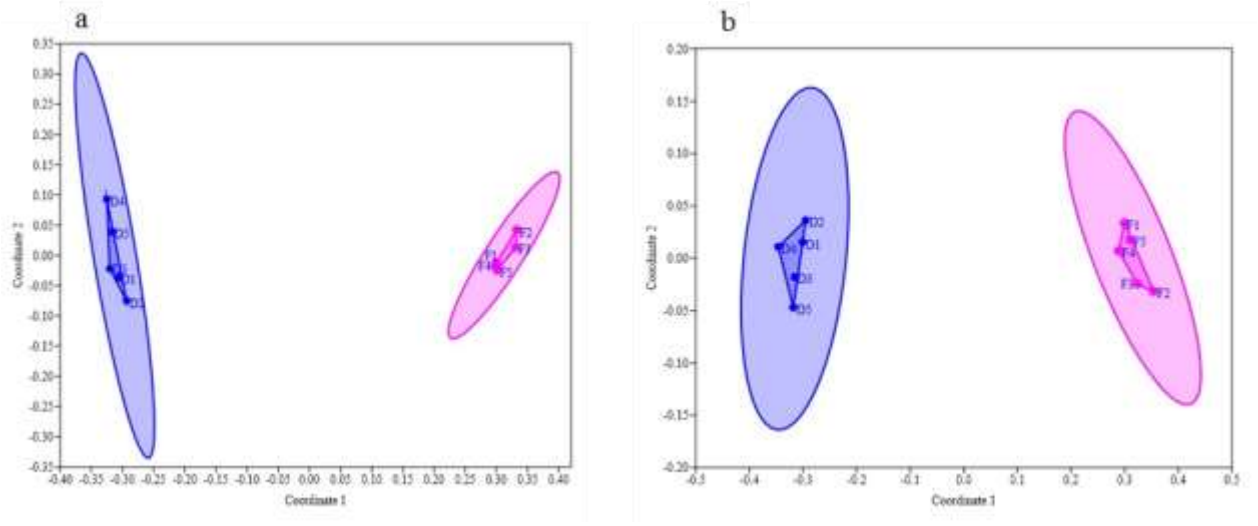

Fig. 3. Non-metric multidimensional scaling (NMDS) plots showing relationships between the fungal endophyte communities isolated from Aizoaceae sampled from the Namaqua National Park, during different sampling seasons (D = Dry season; F= Flowering season) using a) relative abundance as an indicator with the Bray-Curtis dissimilarity index and b) presence / absence data as an indicator with the Jaccard dissimilarity index. Convex hulls and 95% confidence ellipses are shown. (stress = 0.1689 and 0.127, respectively).

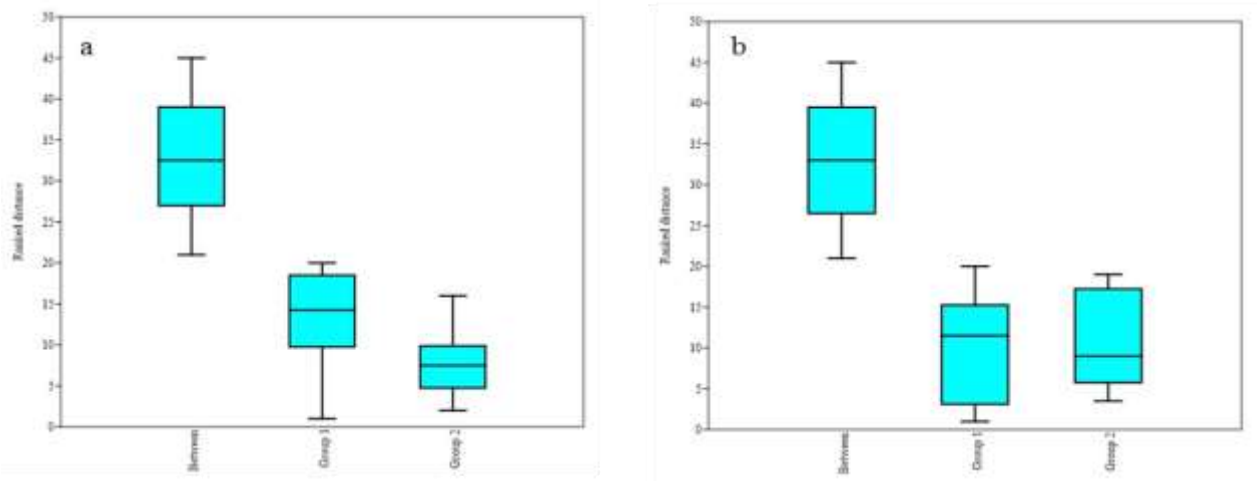

Fig. 4. One way analysis of similarity (ANOSIM) of fungal endophytes from Aizoaceae from the Namaqua National Park, comparing distances between and within fungal communities sampled during different seasons using the (a) Bray-Curtis dissimilarity index ($R = 1$, $p < 0.0073$) and (b) Jaccard dissimilarity index ($R = 1$, $p < 0.0083$) with 9999 permutations performed. Group 1 = Dry samples; Group 2 = Flowering samples.

The genus *Fusarium* (29 isolates) was the most abundant isolate from samples from the flowering season followed by *Alternaria* (25 isolates) and *Aspergillus* (24 isolates). In the dry season *Alternaria* and *Cladosporium* (25 isolates each) were most abundant followed by *Talaromyces* (9 isolates) (Table 1). The total fungal population was dominated by four genera: *Alternaria* (20%), *Cladosporium* (12%), *Fusarium* (11%) and *Aspergillus* (11%) (Fig. 2). The remaining 46% of fungi were represented in 21 genera. Genera rarely isolated included *Neophaeosphaeria, Periconia, Preussia, Schizothecium* and *Truncatella* (Fig. 2). SIMPER analysis (data not shown) confirmed that *Fusarium oxysporum*, *Paecilomyces victoriae* and *Talaromyces pinophilus* were the largest contributors to the differences in community structure observed for fungal endophytes from the different seasons.

Rarefaction curves did not show saturation, indicating that fungal endophyte diversity was underestimated and more sampling is advised (Fig. 5). Diversity indices (Table 2) indicate that a diverse community of endophytic fungi can be cultured from Aizoaceae plants. The diversity observed was higher during the flowering season compared to the dry season.

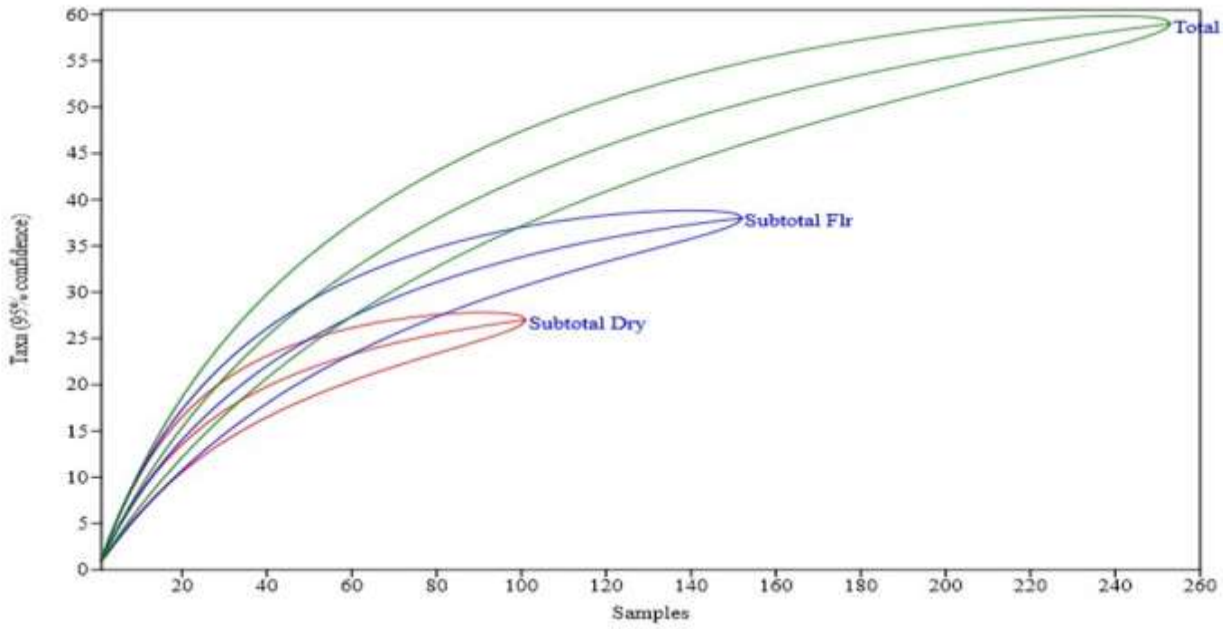

Fig. 5. Species accumulation curve of the fungal endophyte communities isolated from Aizoaceae in the Namaqua National Park, during the flowering (Subtotal Flr) season, dry (Subtotal Dry) season and both seasons combined (Total).

Table 2 Diversity indices of fungal endophytes isolated from Aizoaceae plants from the Namaqua National Park in the Succulent Karoo biodiversity hotspot, South Africa.

Indices	**Season**		**Total**
	Dry	Flowering	
Richness of species	27	38	59
Number of isolates	101	152	253
Margalef's (richness)	5.63	7.37	10.48
Fisher's α (diversity)	12.07	16.26	24.19
Shannon's (H') (entropy)	3.06	3.27	3.55
Simpson's (evenness)	0.94	0.94	0.96
Berger-Parker (dominance)	0.11	0.16	0.09

4. Discussion

Among the more exciting ecological discoveries of the past century is the realisation that all macro organisms are hosts to microorganisms. The significance of the diverse communities and indispensable functions performed by fungal endophytes has only recently been recognised, but with limited research focussing on plants inhabiting dry environments (Murali *et al.*, 2007; Bezerra *et al.*, 2012, 2013; Loro *et al.*, 2012). Low moisture ecosystems characterise almost 40% of land surface on Earth, with studies on global climate change predicting a continued increase in the percentage of arid land, highlighting the importance of arid region research and conservation (Collins *et al.*, 2008; Young *et al.*, 2016).

Estimation of fungal endophyte diversity is biased by many parameters including sampling strategy, method of isolation, initial incubation temperature, ability to grow in culture and technique of identification (Higgins et al., 2011; Suryanarayanan et al., 2011). Certain fungal groups like endophytic basidiomycetes (as was the case in this study) fail to be detected by culturing approaches alone (Suryanarayanan et al., 2011, Singh et al., 2017). Therefore it has recently become more commonplace to investigate the total fungal endophyte community from different environments, using a combination of culturing and culture-independent approaches,

such as next generation sequencing (NGS) (Hibbett et al., 2011; Singh et al., 2017). The culturing approach was used in this study firstly to enable depositing of fungal endophytes from Aizoaceae from this unique environment in the South African National Collection of Fungi living fungal collection (PPRI). Secondly, to highlight the significance of the area and contribute to the understanding of its importance with a view of creating a platform for future studies that will no doubt need to include NGS approaches.

In this study, the endophytic fungal community associated with Aizoaceae in the Namaqua National Park in the Succulent Karoo biodiversity hotspot was observed to be highly diverse. The fungal endophyte community associated with Aizoaceae shows some similarities with those of the closely related family Cactaceae, with respect to species richness and colonization frequency. Bezerra *et al.* (2012) isolated 44 fungal endophytes from *Opuntia ficus-indica*, or "prickly pear", grown in the semi-arid regions of Brazil. A similar number of endophytic fungi were isolated from forage cacti in Brazil (Bezerra *et al.*, 2013), while 23 and 22 fungal endophytes have been isolated from cacti in Australia and Arizona (USA), respectively (Fisher *et al.*, 1994; Suryanarayanan *et al.*, 2005). Most of these fungal endophytes belonged to the phylum Ascomycota, as is the case in the current study.

In our study *Alternaria* was the most frequently isolated genus: this genus has previously been reported as the most frequently observed in many arid regions (such as Arizona, Mexico and Australia) as well as in different plant species, including cacti (such as *Myrtillocactus geometrizans* and *Opuntia* spp.) and *Agave* (Suryanarayanan *et al.*, 2005; Bezerra *et al.*, 2012; Fonseca-García *et al.*, 2016). As for our study with Aizoaceae, *Alternaria* was found to be non-host specific in Cactaceae – a plant family also associated with dry environments (Bezerra *et al.*, 2013; Fonseca-García *et al.*, 2016). In this study, different species of *Alternaria* were isolated during different seasons, but the genus *Alternaria* did not contribute significantly to seasonality. The absence of seasonality was also observed by Yadav *et al*. (2016) in their study on endophytic fungi associated with *Eugenia jambolana* Lam.

The percentage of rarely isolated species identified from Aizoaceae in the Succulent Karoo biome (36.4%) was almost 10% higher than the figure reported by Loro *et al.* (2012) for the endophyte community associated with plants commonly found in semi-arid Northwest Venezuela (27%). The percentage of rarely isolated fungal endophyte species in this study was lower than found (58%: Bezerra *et al.,* 2013) in a study on *Cereus jamacaru*. However, these authors included 30% sterile mycelia in their count of rare species and only used morphological characteristics to determine species identity. Both the studies of Loro *et al.* (2012) and Bezerra *et al*. (2013) were also based on once-off sampling. In the current study three fungal genera; *Periconia*, *Preussia* and *Schizothecium* were isolated only from flowering season samples while *Neophaeosphaeria* and *Truncatella* were isolated only from the dry season samples. Results of once-off sampling studies are therefore highly unlikely to yield a complete picture of the culturable diversity of rare fungal endophytes present in arid regions. Interestingly, only a single species from genus *Preussia* was isolated from Aizoaceae in the Succulent Karoo biodiversity hotspot although this genus was the most frequently isolated fungal endophyte in a study of fungal endophytes from the Sonoran Desert (Massimo *et al*., 2015).

Results from this study were in stark contrast with those reported by Suryanarayanan *et al.* (2005) from their study of endophytic fungi associated with cacti in Arizona, where they reported low diversity in endophyte assemblages of arid zone plants due to water and nutrient limitation. Endophytic fungi isolated from cacti from Arizona were found to have a maximum Fisher's alpha value of 2.1 (Suryanarayanan *et al.,* 2005), which was significantly less than the lowest value of 12.07 calculated for dry season sampling of Aizoaceae from the Succulent Karoo. Results of the current study correlated better with fungal endophyte diversity in the cactus *C. jamacaru,* where culturable diversity was determined to have a Fisher's alpha value of 19.32 and a Margalef index of 9.722 (Bezerra *et al.,* 2013). These authors also relied only on morphological identification for their conclusion that endofungal diversity was high in *C. jamacaru*. We suggest that morphological identification methods may over-estimate species diversity and therefore result in

higher diversity index values. Loro *et al.* (2012) estimated endofungal diversity, using Fisher's alpha values, as 25.06 for semi-arid Northwest Venezuela, only slightly higher than the 24.19 calculated for the total community in the Succulent Karoo. The high endofungal diversity associated with Aizoaceae in the Succulent Karoo may result, in part, to the presence of micro-environmental niches, known to have an effect in other arid regions (Porras-Alfaro *et al.*, 2008).

Pawlowska *et al.* (2014) presented the concept that environmental factors associated with different seasons may influence species diversity. In the current study, seasonal specificity was clearly observed, with higher endofungal diversity estimates in the flowering season than in the dry season. In their study of seasonal influences on Indian endophytic mycobiota, Yadav *et al.* (2016) found species of genera *Aspergillus* and *Chaetomium* only present during the rainy season. Seasonal specificity was also observed for the genus *Phyllosticta* (Suryanarayanan *et al.*, 2005). Similarly, species of *Aspergillus*, *Chaetomium* and *Phyllosticta* isolated during this study showed seasonal specificity. These genera were only isolated after the highest rainfall period in the Namaqua National Park, during the flowering season, with the exception of *Aspergillus parasiticus* that was also present as an endophyte in the dry season.

Fusarium oxysporum, Talaromyces pinophilus and *Paecilomyces victoriae* were the greatest contributors to the seasonal differences observed in the fungal endophyte communities associated with Aizoaceae in this study. All three these species were absent in dry season samples, but 24, 10 and 8 isolates, respectively, were isolated from flowering season samples. A possible explanation for such differences is that fungal genera only isolated during the flowering (wet) season may require higher temperatures and humidity for growth. For example, *Fusarium* populations are known to increase after periods of rainfall (Bateman and Murray, 2001). Different selection pressures, as observed during the different seasons in this study, are known to play a role in endophyte species composition (Yadav *et al.*, 2016). Seasonality observed in fungal endophyte communities can result from the relationships these fungi have with plants during increased plant productivity in the environment (Giauque and Hawkes, 2016). Some endophytes

have the ability to establish themselves in the plant under active plant growth conditions, while the growth of other endophytes declines with an increase in plant metabolic activity (Martins *et al*., 2016).

Arid region plants may even be dependent on endofungal associations for production of secondary metabolites such as enzymes that increase plant fitness (Loro *et al.*, 2012). The diversity of secondary metabolites produced by fungal endophytes, including those produced by endophytes from arid regions, has recently been comprehensively reviewed (Aly *et al.*, 2010, 2011). Several of these secondary metabolite-producing fungal genera were isolated as endophytes associated with Aizoaceae in this study, including *Alternaria, Aspergillus, Curvularia, Fusarium, Penicillium, Phyllosticta* and *Periconia* (one of the rarely isolated species identified in this study). Redman *et al.* (2001) hypothesized that extreme environments may alter production of fungal secondary metabolites, favouring the conversion from a free-living or pathogenic to a mutualistic lifestyle. It is therefore possible that some fungal species, such as *Fusarium oxysporum*, typically known to be pathogenic, may in preference exhibit an endophytic lifestyle in a stressful environment (Leslie and Summerell, 2006) such as the arid environment of the Succulent Karoo biodiversity hotspot investigated in this study. *Fusarium oxysporum* has been isolated as an endophyte from succulent Cactaceae plants (Bezerra *et al.*, 2013) and was isolated as an endophyte from Aizoaceae in this study.

In conclusion, we have observed that seasonality plays a critical role in determining the culturable fungal endophyte communities associated with Aizoaceae in the Namaqua National Park in the Succulent Karoo biodiversity hotspot, South Africa. Our results agree with the recent findings of Bezerra *et al.* (2013) and Massimo *et al.* (2015) that fungal endophytes from arid environments are highly diverse and include species rarely isolated elsewhere. In addition, the results of this study highlight the importance of considering seasonality, including the influence of temperature, rainfall and plant growth stage, in fungal diversity studies. We argue that once-off fungal diversity studies cannot in any way represent of the dynamics in fungal populations.

Symbiotic relationships can have a direct or indirect impact on the structure, function and composition of plant communities (Loro *et al.*, 2012; Rudgers *et al.* 2015). Fungal endophytes (e.g. *Curvularia* and *Fusarium*) known to improve stress tolerance in plants from arid environments (Redman *et al*., 2001; Bezerra *et al*., 2013) have been isolated. Some fungal endophytes (e.g. *Penicillium, Phyllosticta* and *Periconia*) have also been shown to have the ability to transfer the 'plant fitness' benefits they bestow on native plants to plants of agricultural and horticultural importance (Aly *et al.*, 2010, 2011). Although research efforts in arid regions remain challenging, it can be justified by the countless insights that can be gained in informing global biodiversity studies (Loro *et al.*, 2012; Bezerra *et al.*, 2013). The value of researching and protecting the fungal biodiversity of the Succulent Karoo and documenting native fungi has been highlighted by the recent efforts to exploit the area for its shale gas. Improving human well-being by protecting biological richness and adopting green economies is increasingly becoming important in South Africa, as is the case worldwide. Further research into endophytes that improve plant fitness and health of Aizoaceae in the Succulent Karoo and how these endophytes can be protected and sustainably used is needed.

Declarations of interest: none

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