

Fungi associated with Aizoaceae seed in the Succulent Karoo

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Abstract

The Aizoaceae, commonly known as mesembs or “ice plants”, is a plant family endemic to Namaqualand, an area inside the Succulent Karoo biodiversity hotspot in South Africa. The more than 1800 unique mesembs are in part characterised by their hygrochastic seed capsules dispersing their seed by jet action. Six capsule types: *Mesembryanthemum*-type, *Delosperma*-type, *Drosanthemum*-type, *Lampranthus*-type, *Ruschia*-type and *Leipoldtia*-type are distinguished by differences in funicles, covering membranes and closing bodies. With the existence of microbial endemism now widely recognised, research into community ecology in the Succulent Karoo is needed to enable proper conservation. Sadly the microbial life associated with Aizoaceae has received little attention, *Alternaria*, *Colletotrichum* and *Fusarium*, have however been isolated from the halophyte *Sesuvium portulacastrum*, one of the few species from Aizoaceae but not endemic to South Africa. Fungi are known to aid in germination, seedling establishment, growth, water relations and nutrition in the Cactaceae (a family closely related to Aizoaceae). We believe that fungi may play a role in the ability of Aizoaceae to thrive in Namaqualand due to their specialization in the form of thick-walled spores that remain viable and have the ability to grow slow even at extreme aridity. We have isolated species of *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Fusarium* and *Talaromyces* from seeds of common mesemb species from Namaqualand. This is the first report of fungi associating with the specialised propagative material of Aizoaceae.

Keywords: arid, biodiversity, capsule, hotspot, hygrochastic, mesembs, seed-borne

INTRODUCTION

The Aizoaceae with more than 1800 unique species commonly known as mesembs or “ice plants” is a plant family endemic to Namaqualand, an area inside the Succulent Karoo biodiversity hotspot in South Africa and the most diverse arid ecoregion in the world (SANParks, 2013; Sloan et al., 2014). The area, included in the original 25 biodiversity hotspots accepted by Conservation International (CI) in 1989 (Myers et al., 2000), has exceptional local (alpha) diversity, extraordinary beta diversity along habitat gradients and gamma diversity along geographical gradients (Cowling et al., 1989). Currently 35 regions are recognized as hotspots, with only the Horn of Africa and the Succulent Karoo regarded as arid biodiversity hotspots (Sloan et al., 2014).

World renowned for its immaculate Spring flower displays, the Succulent Karoo is one of the best examples where biome specific factors drive germination success (Klak et al., 2015). Many fruit construction types occur within Aizoaceae, though complex, hygrochastic capsules with high structural diversity (including differences in funicles, covering membranes and closing bodies) are the norm (Chesselet et al., 2002). Falling raindrops enable capsules to expel seeds by jet-action dispersal via controlled trajectories depending on structural characteristics that have evolved in a recent speciation burst (Ihlenfeldt, 1994; Klak et al., 2004). More advanced capsule types have higher seed retention and dispersal ability over larger distances, while more primitive capsule types have less restriction in dispersal time (Parolin, 2006).

Fungi are increasingly being investigated for their role in scarification of seed coats, breaking seed dormancy and assisting with seedling establishment, especially in arid environments as larger areas of the world are becoming drier, due to climate change (Delgado-Sanchez et al., 2011; Sanchez-Coronado et al., 2011). Arid and semi-arid regions represent the extremes of potential habitats that life can occupy. While adaptations of plant and animal life in arid ecosystems have been well studied, the relationships of plants co-inhabiting with microbes that may confer thermotolerance, drought resistance and other important survival strategies has only recently received attention (Moncrieff et al., 2015).

We hypothesize that seed-borne fungi play a role in germination and establishment of Aizoaceae seedlings. However it is not known if fungi inhabit the capsules of Aizoaceae and whether the potential inhabitants are commonly isolated species. The present work represents a first contribution to the study of fungi associated with seeds of Aizoaceae in the Succulent Karoo biome. The influence of different capsule types is investigated.

MATERIALS AND METHODS

Study site and sampling

Plant material (including seed capsules) from ten common endemic Aizoaceae species was collected at the end of the dry season (May) and just after the highest rainfall period (which occurs from June to August) during September-October from a selected sampling area close to the Skilpad camp in Namaqua National Park (30°09'19"S; 17°43'55"E) situated in the Succulent Karoo biodiversity hotspot. This semi-desert region has a warm-temperate climate with mean annual temperature between 16.8 and 19.4°C (Mucina and Rutherford, 2006). Mean annual precipitation is relatively reliable, with an annual low of 140 mm in the Soebatsfontein area and a high of 340 mm in the Skilpad section (Desmet, 2007; SANParks, 2013). No endangered species were sampled or disturbed in this study.

Plant material was transported in brown paper bags at temperatures below 10°C prior to identification and fungal isolation. Plants were morphologically identified using a combination of capsule type morphology and leaf and flower characteristics according to the "Key to Mesemb Genera" from Smith et al. (1998). Plant identity was confirmed molecularly using *matK* and *trnL-F* primers (Taberlet et al., 1991). Total DNA was isolated from fresh leaf material using the Nucleospin Plant II Midi kit (Machery-Nagel, GmbH and Co., Düren, Germany) according to manufacturer's instructions. Polymerase chain reactions (PCR's) consisted of 12.5 µl DreamTaq reaction buffer, 2.5 mM MgCl₂, 4% dimethyl sulfoxide (DMSO) and 3% bovine serum albumin (BSA), dNTPs (2.5 mM each), primers (0.5 µM each), template DNA (25 ng), 1.0 U of DreamTaq DNA polymerase (Thermo Fisher Scientific). The amplification profile for *trnL-F* and *matK* consisted of initial denaturation samples at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min and a final extension phase of 7 min at 72°C. Cycle sequencing reactions were performed in a GeneAmp PCR system 9700 thermal cycler (ABI, Applied Biosystems, Warrington, Cheshire, UK) using the ABI BigDye Terminator v3.1. Cycle sequencing kit. BLASTn comparisons were done for sequences generated.

Isolation of seed-borne fungi

Seeds were grouped according to seed capsule type (Table 1). Isolations from seed were performed by grinding seed capsules to release individual seed, selecting seeds with forceps and surface sterilizing by immersion for 1 min inside a 1% sodium hypochloride (NaOCl) solution or 75% ethanol, followed by removal of excess NaOCl/EtOH by rinsing twice in double distilled (dd) H₂O and leaving to air dry inside a laminar flow cabinet. Seeds were plated on potato dextrose agar (PDA) (Biolab, Merck, Darmstadt, Germany), ½ PDA, malt extract agar (MEA), potato carrot agar (PCA), water agar (WA) or synthetic nutrient agar (SNA) in 90 mm diameter Petri dishes. Petri dishes were incubated at 10, 20, 25 and 30°C, under 12 h day/night light for up to 6 weeks. Petri dishes were examined every 48 h and emerging fungal colonies purified for identification. Isolates were maintained on PDA in 45 mm diameter Petri dishes, while long term storage cultures were stored in cryotubes containing 15% sterile glycerol at -80°C, and deposited in the South African National Collection of Fungi (PPRI collection) (Table 2).

DNA extraction and amplification

All filamentous fungi isolated were further examined by molecular methods. DNA was extracted using the DNAeasy® Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. Extracted DNA was used as a template in PCR's using the primer set ITS 1 and ITS 4 (White et al., 1990) in a total reaction volume of 25 µl, which included 2 µl of 10 x DreamTaq DNA buffer (Thermo Fisher Scientific, Waltham, MA), 2 µl of 25 µM dNTP's (Promega Corp., Madison, WI), 0.5 µl (0.2 µM) of each primer (Sigma-Aldrich, St. Louis), 0.2 µl (5 U/µl) DreamTaq DNA polymerase (Thermo Fisher Scientific), 2 µl template DNA and ddH₂O to a total volume of 25 µl. The PCR reaction conditions were initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and elongation at 72°C for 45 s, with final elongation at 72°C for 7 min. PCR products were visualized on a 1% agarose gel at 80 V for 30 min and stained with ethidium bromide at 1 µg/µl.

DNA sequencing and sequence comparisons

DNA sequences were determined from PCR amplicons using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq® DNA polymerase (Applied Biosystems, Paisley, UK). Sequences were manually edited (where necessary) in BioEdit and BLASTn comparisons performed on MycoBank (www.mycobank.org) and NCBI's GenBank sequence database (www.ncbi.nlm.nih.gov), where the top hits (lowest e-value) were used to assign identity. Newly generated ITS sequences were analysed with all available type-derived sequences. Phylogenetic analyses consisted of generating multiple sequence alignments with MAFFT v. 7.0 (Kato and Standley, 2013). Maximum likelihood (ML) analyses were performed after selecting the best-fit parameters in jModelTest v. 2.1.3 (Darriba et al., 2013) under the Akaike Information Criterion (AIC). Maximum likelihood (ML) trees were calculated, viewed and edited for aligned data sets using Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al., 2016) with the following options: initial tree with Bio-Neighbour-Joining (BioNJ), followed by Heuristic search with Nearest-Neighbour-Interchange (NNI). Support in nodes was calculated using a bootstrap analysis with 1000 replicates. Phylograms generated for all genera can be obtained from the authors.

RESULTS AND DISCUSSION

Sampled plants were identified as *Carpobrotus acinaciformis* L. Bolus, *Delosperma echinatum* Schwantes, *Drosanthemum diversifolium* L. Bolus, *Lampranthus bicolor* N. E. Br., *Leipoldtia schultzei* (Schltr. & Diels) Friedrich, *Mesembryanthemum barklyi* N. E. Br., *Mesembryanthemum crystallinum* L., *Ruschia diversifolia* L. Bolus, *Trichodiadema bulbosum* Schwantes and *Vanzijlia annulata* L. Bolus (Figure 2). In Table 1, these mesembs are grouped by fruit capsule type in order of ascending complexity, as described in *Mesembs of the World* (Smith et al., 1998).

Table 1. Grouping of mesembs from Namaqua National Park according to fruit capsule type.

| Plant name | Capsule type | Group |
|--------------------------------------|-----------------------|-------|
| <i>Mesembryanthemum crystallinum</i> | Mesembryanthemum-type | 1 |
| <i>Mesembryanthemum barklyi</i> | Mesembryanthemum-type | 1 |
| <i>Drosanthemum diversifolium</i> | Drosanthemum-type | 2 |
| <i>Delosperma echinatum</i> | Delosperma-type | 3 |
| <i>Trichodiadema bulbosum</i> | Delosperma-type | 3 |
| <i>Lampranthus bicolor</i> | Lampranthus-type | 4 |
| <i>Ruschia diversifolia</i> | Ruschia-type | 5 |
| <i>Leipoldtia schultzei</i> | Leipoldtia-type | 6 |
| <i>Vanzijlia annulata</i> | Leipoldtia-type | 6 |
| <i>Carpobrotus acinaciformis</i> | Fleshy-type | 7 |

In the “Illustrated Handbook of Succulent Plants” Hartmann (2002) noted that the black colour on the outer parts of some of the closing bodies from the plant family Aizoaceae may be due to the presence of a fungus. However no research has been done to investigate whether this statement holds truth. In our study seed-borne fungi were isolated from seed of all seven capsule types. Fungal isolates representing 16 different species from the orders Capnodiales (4 species), Eurotiales (3), Hypocreales (2) and Pleosporales (7) in the phylum Ascomycota (Table 2) were identified. Other arid-land studies have also indicated the prevalence of Dothideomycetes (orders Capnodiales and Pleosporales), Eurotiomycetes (order Eurotiales) and Sordariomycetes (order Hypocreales) in aboveground plant tissues (Suryanarayanan et al., 2005; Bezerra et al., 2013; Massimo et al., 2015).

Table 2. Fungi isolated from mesemb seed from Namaqua National Park

| PPRI# | Genus | specie | Phylum | Order |
|-------|---------------------|---------------------|------------|--------------|
| 13100 | <i>Alternaria</i> | <i>alternata</i> | Ascomycota | Pleosporales |
| 16053 | <i>Alternaria</i> | <i>citricancri</i> | Ascomycota | Pleosporales |
| 16047 | <i>Alternaria</i> | <i>palandui</i> | Ascomycota | Pleosporales |
| 14425 | <i>Alternaria</i> | <i>porri</i> | Ascomycota | Pleosporales |
| 14422 | <i>Alternaria</i> | sp. | Ascomycota | Pleosporales |
| 16051 | <i>Alternaria</i> | sp. | Ascomycota | Pleosporales |
| 14427 | <i>Aspergillus</i> | <i>toxicarius</i> | Ascomycota | Eurotiales |
| 13096 | <i>Aspergillus</i> | <i>niger</i> | Ascomycota | Eurotiales |
| 13102 | <i>Bipolaris</i> | sp. | Ascomycota | Pleosporales |
| 16046 | <i>Cladosporium</i> | <i>basiinflatum</i> | Ascomycota | Capnodiales |
| 16049 | <i>Cladosporium</i> | <i>tenuissimum</i> | Ascomycota | Capnodiales |
| 16052 | <i>Davidiella</i> | <i>allicina</i> | Ascomycota | Capnodiales |
| 16050 | <i>Davidiella</i> | sp. | Ascomycota | Capnodiales |
| 15942 | <i>Fusarium</i> | <i>oxysporum</i> | Ascomycota | Hypocreales |
| 13098 | <i>Fusarium</i> | <i>oxysporum</i> | Ascomycota | Hypocreales |
| 15944 | <i>Talaromyces</i> | <i>amestolkiae</i> | Ascomycota | Eurotiales |

Ruschia-type capsules (Figure 2h) harboured the greatest diversity of seed-borne fungi with all the isolated genera being present (Figure 1). In contrast the fleshy-type capsule of *Carpobrotus* (Figure 2a) was infected with only a single mycelial fungus, *Bipolaris* sp. (Figure 1). The much cultivated ice plant *Mesembryanthemum* was colonised mostly by *Alternaria* species. The shrubby mesembs with glittering leaves, *Drosanthemum* and *Delosperma*-type (Figure 2g), harboured similar fungal diversity (Figure 1). The structural characteristics of capsule types failed to provide sufficient evidence to determine whether a specific fungal species would be present or absent, with the exception of *Ruschia*-type capsules where the deep capsule base may play a role.

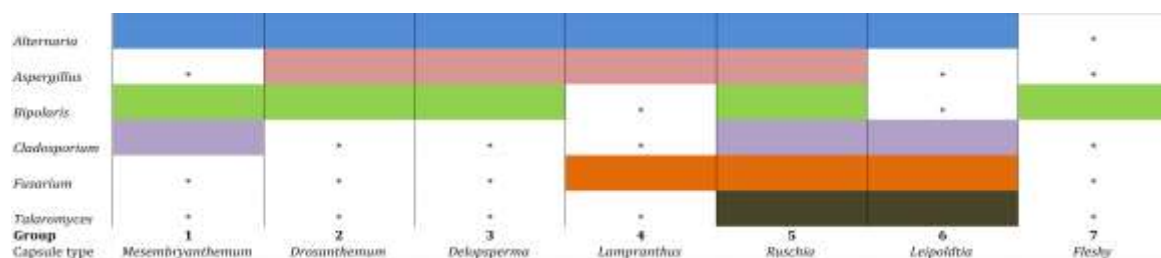


Figure 1. Fungal genera isolated from seed of Aizoaceae grouped according to capsule type, * indicates a particular genus was not found.

Species of genus *Alternaria* were most commonly isolated and were found to be seed-borne on mesemb seed from six of the seven seed capsule types investigated (Figure 1). ITS sequences delineated most of the species as belonging to *Alternaria alternata* (Figure 2j), the section with the lowest molecular variation. Isolate PPRI16051 clustered into sect. *gypsophilae*, commonly associated with carnation species from the plant family Caryophyllaceae, a family inside the order Caryophyllales (Woudenberg et al., 2013), the same order as Aizoaceae. Isolate PPRI14425 grouped most closely with species inside the largest section of *Alternaria*, sect. *porri* (Woudenberg et al., 2013). We also frequently isolated *A. alternata*, known to aid in dispersal of the fourwing saltbush, *Atriplex canescens* Nutt., a plant genus also characterized by the presence of encapsulated seed (Barrow and Havstad, 1997).

Aspergillus species were isolated from seed of capsule types 2 to 5. Most of the *Aspergillus* isolates morphologically resembled *Aspergillus niger* Tiegh. (Figure 2k). Barrow et al. (1998) reported that *Aspergillus* sp. enabled *A. canescens* to transport a previously insoluble phosphorous source, increase plant and root biomass and stabilize soil particles thereby improving nutrient and water retention as well as photosynthetic rates. *Aspergillus* species were not isolated from *Mesembryanthemum* or *Leipoldtia* plants. Both these genera prefer disturbed gravel habitats where soil stabilisation is not a priority (Smith et al., 1998).

It was not possible to distinguish morphologically between the five isolates of *Bipolaris* cultivated from seeds of capsule types 1, 2, 3, 5 and 7 and therefore molecular analysis was only done for one isolate. The *Carpobrotus* isolate was selected due to it being the only mycelial fungus isolated from this mesemb. This isolate returned a closest match for *Bipolaris* sp. in GenBank. However upon phylogenetic evaluation the species clustered more closely with *Curvularia spicifera* (Bainier) Boedijn. *Curvularia* is known to be somewhat host specific and to confer drought tolerance to rice, wheat and tomato plants (Redman et al., 2001; Rodriguez et al., 2001). Since Aizoaceae grow in water deficient habitats it could be expected to be a frequent inhabitant of the mesembs.

Genus *Cladosporium* was present in seed capsule types 1, 5 and 6. Four distinct species of *Cladosporium*, including *Davidiella allicina* anamorph to *Cladosporium allicinum* (Fr.) Bensch, U. Braun & Crous and an unidentified anamorph, clustering closely with this species according to ITS sequence, were isolated. *Cladosporium tenuissimum* Cooke and *C. basiinflatum* Bensch, Crous & U. Braun were also isolated. *Cladosporium* species have previously been reported seed-borne in Cactaceae (Fonseca-García et al., 2016).

The pigmented seed-borne fungi *Cladosporium* and *Curvularia* increase water absorption, protecting plants in arid environments (Bezerra et al., 2013), while *Alternaria* and *Cladosporium* are known to increase nutrient uptake and seedling vigour in *Atriplex canescens*. The common denominator in genera *Ruschia* and *Leipoldtia* is erect stems that may benefit from increased water absorption.

Limited by the shortcomings of the universal fungal barcode gene region (ITS), *Fusarium* species, isolated from seed capsule types 4, 5 and 6 could only be identified as belonging to the *Fusarium oxysporum* Schltdl. species complex (Figure 2l). *Alternaria*, *Bipolaris*, *Cladosporium* and *Fusarium* have previously been found to be endophytic in tissue of the mesemb, *Sesuvium portulacastrum* L. (Zhang and Li, 2009), but has only now been isolated from the seed of a species in the same plant family.

Seed-borne fungi are capable of eroding the seed funiculus and producing compounds that enhance seed germination and seedling growth. They provide an ecological advantage, and have the ability to positively impact both their hosts and progeny in challenging habitats (Delgado-Sanchez et al., 2011; Lucero et al., 2011). Although some seed-borne fungi may lead to reduced germination, seed deterioration, disease and production of toxins harmful to man and animal (Yassin et al., 2010), many have proved essential for germination of seed from capsules since 1934 (Pfeiffer, 1934). The seed fungal endophytes benefit from plant associations due to the provision of habitat, nutrition and means of dissemination, while plant fitness is enhanced (Card et al., 2016; Tahtamouni et al., 2016).



Figure 2. a) *Carpobrotus acinaciformis*; b) *Drosanthemum diversifolium*; c) *Mesembryanthemum barklyi*; d) Closed *Lampranthus* capsule; e) Opening *Lampranthus* capsule; f) Open *Lampranthus* capsule; g) *Drosanthemum* capsule; h) *Ruschia* capsule; i) *Leipoldtia* capsule; j) *Alternaria alternata*; k) *Aspergillus niger*; l) *Fusarium oxysporum*.

In this study, certain fungi were shown to have preference for specific mesemb species, however in depth investigation into host specificity of fungal communities associated with Aizoaceae is still required. Further investigation is also required to determine whether fungi associated with Aizoaceae confer drought tolerance and increase

survival in their harsh environment as is the case in Cactaceae and *Agave* (Coleman-Derr et al., 2016).

CONCLUSIONS

Species of *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Fusarium* and *Talaromyces* are seed-borne inhabitants of the specialised capsules of common Aizoaceae species from the Succulent Karoo biodiversity hotspot. Further investigation is needed to detect unculturable species, completing the microbiome and to elucidate the role of fungi in germination and establishment of Aizoaceae seedlings.

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