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Pestalotioid fungi from *Restionaceae* in the Cape Floral Kingdom

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Abstract: Eight pestalotioid fungi were isolated from the *Restionaceae* growing in the Cape Floral Kingdom of South Africa. *Sarcostroma restionis*, *Truncatella megaspora*, *T. restionacearum* and *T. spadicea* are newly described. New records include *Pestalotiopsis matildae*, *Sarcostroma lomatiae*, *Truncatella betulae* and *T. hartigii*. To resolve generic affiliations, phylogenetic analyses were performed on ITS (ITS1, 5.8S, ITS2) and part of 28S rDNA. DNA data support the original generic concept of *Truncatella*, which encompasses *Pestalotiopsis* species having 3-septate conidia. The genus *Sarcostroma* is retained as separate from *Seimatosporium*.

Taxonomic novelties: *Pestalotiopsis matildae* (Richatt) S. Lee & Crous comb. nov., *Truncatella betulae* (Morochk.) S. Lee & Crous comb. nov., *Sarcostroma restionis* S. Lee & Crous sp. nov., *Truncatella megaspora* S. Lee & Crous sp. nov., *Truncatella restionacearum* S. Lee & Crous sp. nov., *Truncatella spadicea* S. Lee & Crous sp. nov.
Key words: Fungi imperfecti, fynbos, microfungi, South Africa, systematics.

INTRODUCTION

The *Restionaceae* (restios) is a monocotyledonous family distributed in the Southern Hemisphere, which includes more than 30 genera and about 400 species (Figs 1–6). In Africa approximately 330 species are found, mostly in the south-western tip of South Africa (Haaksma & Linder 2000). This area, comprising 90 000 km^2 and known as the Cape Floral Kingdom, is home to more than 8 500 plant species, of which 5 800 are endemic (Cowling & Richardson 1995). Fynbos is the dominant vegetation type of the Kingdom contributing 80 % of its species. Approximately 94 % of the restios growing in fynbos are indigenous. Locally, the stems of the plants are used for thatching, matting or brooms (Fig. 7). Research on the diversity of saprobic microfungi in fynbos was initiated in 2000 with an emphasis on two major plant groups: the dicotyledonous *Proteaceae* and the *Restionaceae*. About 500 fungal specimens have been collected from restios, of which 40 % represent coelomycetous anamorphs including the so-called pestalotioid fungi. Pestalotioid fungi are defined as those having multi-septate, more or less fusiform conidia with appendages at both or either ends, resembling those taxa accommodated in *Pestalotia* De Not. or *Pestalotiopsis* Steyaert, of which teleomorphic connections are found with the members of the *Amphisphaeriaceae*, *Broomella* Sacc., *Discostroma* Clem., and *Pestalosphaeria* M.E. Barr.

The aim of this study was to characterise pestalotioid fungi from restios growing in fynbos. Four new and four known species are treated. To clarify the phylogenetic relationships between these and other related pestalotioid fungi, DNA sequence data were generated for the partial 28S gene and ITS region (ITS1, 5.8S, ITS2) and phylogenetic analyses were applied.

MATERIALS AND METHODS

Isolates

Field collections were made in Western Cape Province nature reserves and in undisturbed areas of the fynbos during 2000–2002. Culm litter was collected in paper bags. Host identification was done either with the assistance of curators of the Kirstenbosch Botanical Garden or by using Intkey (Linder 2001).

Specimens were either studied immediately or air-dried for later use. Dried specimens were re-hydrated in damp chambers with wet filter paper. Single-conidium isolations were made from spore suspensions on 2 % malt extract agar (Merck, Gauteng, South Africa) supplemented with 0.04 g/L streptomycin sulfate, and incubated at room temperature. Reference cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands. Herbarium specimens have been deposited in the National Collection of Fungi, Pretoria (PREM), South Africa.

DNA amplification and phylogeny

Fungal isolates were grown in 1 mL 2 % malt extract broth in three 2 mL Eppendorf tubes for up to 7 d. Mycelium was collected and DNA was isolated following a modification of the method of Möller *et al*. (1992). The primers ITS1 and ITS4 (White *et al*. 1990) were used to amplify part of the nuclear rDNA spanning the 3'end of the 18S rDNA, the internal transcribed spacers, the 5.8S rDNA and a part of the 5' end of the 28S rDNA. The primers LR0R and LR7 were used to amplify part of the large subunit nuclear rDNA (Vilgalys & Hester 1990). Amplification reactions were started with 3 min denaturation in 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 1 min annealing at 55 °C and 1.5

min extension at 72 °C, and 10 min extension at 72 °C. For the amplification of partial 28S rDNA, the annealing temperature was adjusted to 50 °C. For specimens that could not be cultivated, direct PCR was performed from conidia with increased cycles (40 cycles). PCR products were separated by electrophoresis at 80–90 V for 15 min in 1 % (w/v) agarose gel in 1× TAE running buffer (0.1 mM Tris, 0.01 mM EDTA, 2 % SDS, pH 8.0) and visualised under UV light.

The amplification products were purified using a modified PEG method (Steenkamp *et al*. 2005). The purified products were sequenced in both directions using the same primers used in the amplification reactions except for the reverse primer of the partial 28S rDNA where LR5 was used (Vilgalys & Hester 1990). Sequencing reactions were performed using a PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, U.K.). Nucleotide sequence data were generated with an ABI Prism 3100™ automated DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). The raw sequence data were processed using the Sequence Navigator v. 1.0.1 software package (Perkin-Elmer Applied BioSystems, Foster City, California).

Sequences were assembled and aligned using ClustalW algorithm in MEGA v. 3.1 (Kumar *et al*. 2004) and finally optimised by eye. Phylogenetic analyses of sequence data were done in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). For parsimony analysis, alignment gaps were treated as fifth character and all characters were unordered and of equal weight. Maximum parsimony was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Neighbour-Joining (NJ) with the Tamura-Nei parameter model (Tamura & Nei 1993) was performed with adjusted settings: proportion of invariable sites (I) = 0.6169, gamma distribution (G) = 0.5970, base frequency equal, rate matrix 1.00, 2.3919, 1.00, 1.00, 5.5792 for partial 28S rDNA; I = 0, G = 0.3769, base frequency equal, substitution model (Ti/tv ratio) 1.6846 for ITS regions. These models were chosen as suggested by MODELTEST v. 3.5 (Posada & Crandall 1998). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Other measures calculated included tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC). GenBank accession numbers of sequences generated in this study are listed in Table 1. The DNA sequence alignment is deposited in TreeBASE (Study accession number S1442).

Taxonomy

A Zeiss Axioskop 2 Plus microscope was used with differential interference contrast to examine specimens. For some observations, phase contrast (PhC) or bright field (BF) was employed and indicated. Images were captured using a Canon digital camera equipped with a Canon Utilities Remote Capture v. 2.7.3.23. Measurements were done using Axiovision software (AxioVs 40 v. 4.3.0.101). Where possible, thirty measurements were made of all structures. Apical and/or basal appendages were excluded in measurements of conidial length, and were measured separately. For conidial dimensions the 95 % confidence levels were calculated, and extremes provided in parentheses.

To study the internal and peridial structures, vertical sections of conidiomata were made. Small pieces of plant tissue containing conidiomata were taken from dried herbarium material, placed on water agar with a drop of water, and incubated overnight. Tissues were mounted on a disc with Jung tissue freezing medium™. Sections were made (10–12 µm thick) using a Cryomicrotome (Leica CM1100). Sections were lifted onto a coverslip, mounted in lactic acid (85 %), and slides were placed on a heated plate to remove trapped air bubbles.

RESULTS

Phylogenetic analyses

ITS: Approximately 550 bases were determined for the isolates as indicated in Table 1. The manually adjusted alignment consisted of 29 taxa (including the two outgroups) and 612 characters including alignment gaps, of which 247 were parsimony-informative, 111 were variable and parsimony-uninformative, and 254 were constant. Parsimony analysis of the alignment yielded six most parsimonious trees, one of which is presented (Fig. 8). Ingroups consisted of four clades referred to as a *Truncatella* Steyaert clade, a *Pestalotiopsis*-A clade, a *Pestalotiopsis*-B clade and a *Sarcostroma* Cooke clade with 99 %, 100 %, 100 % and 100 % bootstrap support, respectively.

The *Truncatella* clade consisted of two sub-clades. The one sub-clade included five *Truncatella* species from our collections (100 % bootstrap support). And the other included *T. angustata* (Pers.) S. Hughes and species of *Bartalinia* Tassi with 96 % bootstrap support. The *Pestalotiopsis*-A clade included six *Pestalotiopsis* (*Ps.*) species having conidia with concolorous median cells, and *Ps. matildae* (Richatt) S. Lee & Crous having conidia with versicolorous median cells. The *Pestalotiopsis*-B clade included four *Pestalotiopsis* species having conidia with versicolorous median cells, and formed a sister clade to *Ps. theae* (Sawada) Steyaert, which had conidia with concolorous median cells and knobbed apical appendages (R. Jeewon, pers. comm.). The *Sarcostroma* (*Sa.*) clade included *Sa. restionis* S. Lee & Crous and *Seimatosporium* (*Se.*) *grevilleae* (Loos) Shoemaker which has a characteristic of *Sarcostroma*, centric apical and excentric basal appendages. The distance tree gave the same topology. Similar bootstrap values were obtained for both parsimony and distance analyses except for the branches supporting two *T. restionacearum* isolates and four *Truncatella* species within the *Truncatella* clade. These branches have higher support in distance analysis (95 % and 92 %, respectively) than in parsimony analysis (63 % and 58 %, respectively).