Phylogenetic lineages in the *Botryosphaeriales*: a systematic and evolutionary framework

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Abstract: The order *Botryosphaeriales* represents several ecologically diverse fungal families that are commonly isolated as endophytes or pathogens from various woody hosts. The taxonomy of members of this order has been strongly influenced by sequence-based phylogenetics, and the abandonment of dual nomenclature. In this study, the phylogenetic relationships of the genera known from culture are evaluated based on DNA sequence data for six loci (SSU, LSU, ITS, EF1, BT, mtSSU). The results make it possible to recognise a total of six families. Other than the *Botryosphaeriaceae* (17 genera), *Phyllostictaceae* (*Phyllosticta*) and *Planistromellaceae* (*Kellermania*), newly introduced families include *Aplosporellaceae* (*Aplosporella* and *Bagnisiella*), *Melanopsaceae* (*Melanops*), and *Saccharataceae* (*Saccharata*). Furthermore, the evolution of morphological characters in the *Botryosphaeriaceae* were investigated via analysis of phylogeny-trait association. None of the traits presented a significant phylogenetic signal, suggesting that conidial and ascospore pigmentation, septation and appendages evolved more than once in the family. Molecular clock dating on radiations within the *Botryosphaeriales* based on estimated mutation rates of the rDNA SSU locus, suggests that the order originated in the Cretaceous period around 103 (45–188) mya, with most of the diversification in the Tertiary period. This coincides with important periods of radiation and spread of the main group of plants that these fungi infect, namely woody Angiosperms. The resulting host-associations and distribution could have influenced the diversification of these fungi.

Key words: *Aplosporellaceae*, *Melanopsaceae*, molecular dating, *Phyllostictaceae*, *Planistromellaceae*, *Saccharataceae*, systematics.
Taxonomic novelties: New families – *Aplosporellaceae* Slippers, Boissin & Crous, *Melanopsaceae* Phillips, Slippers, Boissin & Crous, *Saccharataceae* Slippers, Boissin & Crous.

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INTRODUCTION

DNA sequence-based phylogenetics has dramatically influenced both the taxonomy and systematics of the *Botryosphaeriaceae* during the course of the past decade (Crous *et al*. 2006), as it has done in most other groups of *Fungi* (James *et al*. 2006, Hibbett *et al.* 2007). At a higher taxonomic level, DNA sequence data have led to the recognition that the *Botryosphaeriaceae* represents a distinct order within the *Dothideomycetes*, leading Schoch *et al*. (2006) to introduce the *Botryosphaeriales*. The circumscription of the *Botryosphaeriales* has suffered from insufficient sampling and it was only recently that Minnis *et al*. (2012) provided molecular evidence to show that the *Planistromellaceae* resides in this order. In a subsequent study, Liu *et al*. (2012) provided a comprehensive phylogenetic analysis of genera in the *Botryosphaeriales* and they also concluded that, other than the *Botryosphaeriaceae* and *Planistromellaceae*, a number of clearly defined evolutionary lineages exist.

Apart from the *Planistromellaceae*, the genera traditionally associated with *Botryosphaeria* and *Phyllosticta* have sexual morphs that are clearly distinct phylogenetically, morphologically and ecologically. However, both are still grouped within the *Botryosphaeriaceae*. Members of the *Botryosphaeria* group are common endophytes of leaf and woody tissue of many woody plant species, have hyaline to dark ascospores, multilocular ascomata, and a wide range of asexual morphs that typically lack a mucoid sheath and apical appendage. Species in the *Guignardia* group (= *Phyllosticta*) typically infect leaves and fruit, less commonly wood, have unilocular ascomata with smaller ascospores that typically have mucoid appendages, and *Phyllosticta* asexual morphs. The *Phyllostictaceae* has been resurrected to accommodate this group of taxa (see Wikee *et al*. 2013b, this volume).

Substantial changes to the definition of sexual and asexual genera linked to the *Botryosphaeriaceae* have been made during the past decade (e.g. Crous *et al*. 2006, Phillips *et al*. 2008, Liu *et al.* 2012). Only a selection of the most common examples is discussed here. The first DNA sequence data for the *Botryosphaeriaceae* appeared to reveal a distinction between asexual morphs with hyaline fusicoccum-like conidia and those with pigmented diplodia-like conidia, termed sections *Hyala* and *Brunnea* (Jacobs & Rehner 1998, Denman *et al*. 2000, Zhou & Stanosz 2001). This distinction became increasingly less obvious as sampling increased and it was evident that conidial pigmentation is a feature that evolved more than once. It was, for example, shown that dark, septate and even muriformly septate dichomera-like conidia could be synasexual morphs of well-known genera such as *Fusicoccum*

and *Neofusicoccum* (Barber *et al*. 2005, Phillips *et al.* 2005). Furthermore, dark, septate ascospores were shown to be a polyphyletic character of several genera and more common than previously believed (Phillips *et al.* 2008). As the true phylogenetic diversity within the group emerged, a number of new genera were described (e.g. *Botryobambusa*, *Cophinforma*, *Neofusicoccum*, *Neoscytalydium*, *Pseudofusicoccum*, etc.) or older genera re-defined (e.g. *Auerswaldia*, *Barriopsis*, *Dothiorella*, etc.) (e.g. Crous *et al*. 2006, Phillips *et al*. 2008, Liu *et al.* 2012). The most recent work by Liu *et al*. (2012) reviewed these genera, and this study reflects a growing consensus regarding the circumscription of the majority of the genera (29 in total, of which sequence data are available for 20).

DNA-based sequence analyses have also resulted in significant changes to the nomenclature, identification and circumscription of species in the *Botryosphaeriaceae*. These changes have resulted in the implementation of a single nomenclature for all morphs of a species (Crous *et al.* 2006, Hawksworth *et al*. 2011, Wingfield *et al*. 2012). For the *Botryosphaeriaceae*, this has included the description of cryptic species based on DNA sequence data, where morphological characters were not variable enough for this purpose (Pavlic *et al*. 2009a, Sakalidis *et al.* 2011).

Insights gained from contemporary studies on the *Botryosphaeriaceae* have led to uncertainty regarding the application of names published in the older literature. The analyses show for example that morphological characters typically used for species identification (chiefly conidia or ascospore dimensions, shape, septation and pigmentation) are frequently unreliable. Even ecological and geographical data are difficult to interpret, with some species occurring on numerous hosts, and single locations or hosts often yielding numerous co-occurring species (Slippers & Wingfield 2007, Slippers *et al*. 2009). For this reason (together with the significant changes in generic descriptions mentioned above) many, if not most, of the taxa dealt with before the introduction of DNA sequence-based phylogenetic inference will need to be redefined (possibly neo- or epitypified), to allow meaningful comparisons with currently applied names (also see the discussion in Phillips *et al*. 2013, this volume). Where it is not possible to follow this approach, older names may have to be ignored and new species introduced that are supported by DNA data (see Slippers *et al.* 2014).

The *Botryosphaeriales* is an important group of fungi due to the ecological and economic significance of many of its species. All species are plant-associated, and many are classified as pathogens, known to cause disease on a wide range of ecologically and economically important plants (Mehl *et al.* 2012). Some species are also known to cause opportunistic infections in humans (de Hoog *et al.* 2000). Most species exist as endophytes living in healthy plant tissues for extended periods of time (Slippers & Wingfield 2007). Their roles as endophytes or pathogens often overlap, as is for example found in the case of *Diplodia sapinea*. This well-known pathogen of *Pinus* (Swart *et al*. 1991) is also a common endophyte in branches, the trunks and seed cones of these trees. In an extreme example, *D. sapinea* has been isolated from the wood of *Pinus* in South Africa, where it must have existed without causing disease subsequent to the tree being infected as long as a decade previously (Bihon *et al.* 2011).

Unlike the case for *D. sapinea*, the ecological roles for the majority of species of *Botryosphaeriaceae* are unknown. The changes to the taxonomy of the group are already strongly promoting an ability to characterise the diversity in this group. In turn, this is providing an evolutionary framework making it possible to study the ecological role that remains obscure for the majority of these fungi.

In this paper, the phylogenetic relationships of all the genera known from culture and considered to reside in the *Botryosphaeriales* and *Botryosphaeriaceae* are determined based on DNA sequence data for six loci. The *Planistromellaceae* is well defined within the *Botryosphaeriales*. As expected, *Phyllosticta* (= *Guignardia*) also forms a strongly supported monophyletic lineage, recognised as the *Phyllostictaceae* (see Wikee *et al*. 2013b, this volume). *Saccharata*, however, groups separately with respect to all other genera in the *Botryosphaeriales*, as does *Aplosporella, Bagnisiella* and *Melanops*. The nomenclatural changes necessary to reflect these distinctions are considered in this study. With the well-supported phylogeny provided by these analyses, we also test hypotheses regarding the evolution of major morphological features typically used in taxonomy of the *Botryosphaeriaceae*. Finally, we use the nuclear ribosomal subunit data to date the divergence in the major groups of the *Botryosphaeriales*.

MATERIALS AND METHODS

Isolates and DNA extractions

A total of 96 strains corresponding to 85 species were grown on 2 % potato dextrose agar (PDA) plates incubated at 25 ºC. Genomic DNA was extracted from mycelium using the PrepMan™ Ultra protocol (Applied Biosystems). Sequences from additional species were retrieved from GenBank. A total of 140 taxa were included in the ingroup and six taxa in the outgroup (see Table 1 for details).

PCR and sequencing

A total of six partial gene portions were used in this study: the nuclear ribosomal small subunit (SSU), the nuclear ribosomal large subunit (LSU), the intergenic spacer (ITS), the translation elongation factor 1-alpha (EF1), the β-tubulin gene (BT) and the mitochondrial ribosomal small subunit (mtSSU).

The primers used were NS1 and NS4 (White *et al*. 1990) for SSU, LROR and LR5 (Vilgalys Laboratory, Duke university, www.biology.duke.edu/fungi/mycolab/primers.htm) for LSU, ITS-1 and ITS-4 (White *et al*. 1990) for ITS, EF-AF and EF-BR (Sakalidis *et al*. 2011) for EF1, BT2A and BT2B (Glass & Donaldson 1995) for BT and mrSSU1 and mrSSU3R (Zoller *et al.* 1999) for mtSSU. All PCR reactions were conducted in 15 µL containing 1.5 mM of $MgCl_2$, 0.5 mM of dNTP, 1 × final concentration of buffer, 1 µM of each primer, 0.25 U of FastStart *Taq* Polymerase (Roche), 1.5 µL of DNA template and Sabax sterilised water (Adcock Ingram) to complete up to 15 µL. The cycling parameters were as follows: a first step of denaturation at 95 ºC for 5 min followed by 35 cycles of (i) denaturation at 95 ºC for 60 s, (ii) annealing at optimal temperature (55 ºC for ITS, EF1, LSU and 45 ºC for SSU, mtSSU, BT) for 80 s, (iii) elongation at 72 ºC for 90 s, and a final elongation step of 5 min was applied.

Sephadex columns (Sigma-Aldrich) were used to clean the samples both before and after the sequencing reactions. The sequencing PCRs were performed in 10 µL containing 1 µL of PCR product, 0.7 µL of Big Dye Terminator v. 3.1 (Applied Biosystems), 2.5 µL of sequencing buffer (provided with Big Dye), 1 µL of primer (10 µM) and 4.8 µL of Sabax sterilised water. Cycling parameters consisted of 25 cycles with three steps each: 15 s at 95 ºC, 15 s at 55 ºC (for ITS, EF1, LSU) or 45 °C (for SSU, mtSSU, BT) and 4 min at 60 ºC. The sequencing PCR products were sent to a