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REVIEW

Trail of decryption of molecular research on Botryosphaeriaceae in woody plants

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Summary. The family Botryosphaeriaceae is species rich and includes pathogens, saprobes and endophytes of economically important agricultural crops and plants. As pathogens, Botryosphaeriaceae species employ evolving defensive and counter-defensive strategies that enable them to infect their hosts. Molecular genetic results showed high genetic variability among Botryosphaeriaceae species, which could be a key determinant to their differing environmental roles on hosts and differences in their virulence. Improved technologies such as whole genome re-sequencing, integrated RNA-Seq, comparative and *ab initio* approaches for molecular genetics and mutation analysis have revealed possible virulence factors that might be involved in the pathogenicity of these fungi. Several genes involved in the pathogenicity processes of botryosphaeriaceous fungi have been identified and characterized. There are numerous reports on the involvement of phytotoxic metabolites in the pathogenicity of these taxa. Availability of whole genomes, genetic transformation and transcriptome analysis of some botryosphaeriaceous species have contributed towards identifying the biological functions of many genes in a fast and accurate way, enabling these fungi to be used as model organism for molecular research of plant opportunistic fungal pathogens in woody plants. The research on botryosphaeriaceous species will provide understanding of the infection mechanisms, and with designing control strategies against diseases caused by opportunistic fungal pathogens.

Key words: genetic diversity, genomics, molecular markers, pathogenicity genes, secondary metabolites.

Introduction

Species of Botryosphaeriaceae (Botryosphaeriales, Dothideomycetes) Theiss & Syd. are cosmopolitan and cause dieback, cankers, shoot blights, leaf spots, fruit and seed rots and witches' brooms, in a broad range of host plants of agricultural and economic importance (Denman *et al*., 2000; van Niekerk *et al*., 2004; Úrbez-Torres, 2011; Phillips *et al*., 2013). They are found in terrestrial habitats worldwide especially on woody branches, stems, herbaceous leaves, culms of grasses, on twigs and in thalli of lichens (Denman *et al*., 2000; Mohali *et al*., 2005; Liu *et al*., 2012). The family Botryosphaeriaceae has been well circumscribed in recent studies (Phillips *et al*., 2013; Hyde *et al*., 2014) and comprises many genera (Table 1).

Members of Botryosphaeriaceae have been described as endophytes and as latent pathogens causing serious diseases (Slippers and Wingfield, 2007; Sakalidis, 2011; Yan *et al*., 2013). Biochemical and genetic responses caused by external stimuli resulting from changing environmental conditions inside hosts (changes in host behaviour or microbial equilibrium) or outside hosts (changes in climate or extreme environmental events), triggers the fungi to change their lifestyles from endophytic to pathogenic. Because of this transition these fungi can be regarded as plant opportunistic fungal pathogens

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Table 1. Currently accepted genera of Botryosphaeriaceae.

Botryosphaeriaceae genera and authority	References
Alanphillipsia Crous & M.J. Wingf.	Crous *et al*., 2014
Barriopsis A.J.L. Phillips, A. Alves & Crous	Phillips *et al*., 2013; Liu *et al*., 2012; Hyde *et al*., 2013
Botryobambusa R. Phookamsak, J.K. Liu & K.D. Hyde	Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Botryosphaeria Ces. & De Not.	Denman *et al*., 2000; Slippers *et al*., 2004a; Crous *et al*., 2006; Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Cophinforma Doilom, J.K. Liu & K.D. Hyde	Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Diplodia Fr.	Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Dothiorella Sacc.	Phillips *et al*., 2013; Crous *et al*., 2006; Liu *et al*., 2012; Hyde *et al*., 2013
Endomelanconiopsis E.I. Rojas & Samuels	Rojas *et al*., 2008; Hyde *et al*., 2013; Phillips *et al*., 2013
Eutiarosporella Crous	Crous *et al*., 2015c
Lasiodiplodia Ellis & Everh.	Pavlic *et al*., 2004; Slippers *et al*., 2004a; Phillips *et al*., 2013; Liu *et al*., 2012; Hyde *et al*., 2013
Macrophomina Petr.	Crous *et al*., 2006; Hyde *et al*., 2013; Phillips *et al*., 2013
Marasasiomycyes Crous	Crous *et al*., 2015c
Mucoharknessia Crous, R.M. Sánchez & Bianchin.	Crous *et al*., 2015c
Neodeightonia C. Booth	Phillips *et al*., 2013; Liu *et al*., 2012; Hyde *et al*., 2013
Neofusicoccum Crous, Slippers & A.J.L. Phillips	Crous *et al*., 2006; Pavlic *et al*., 2009; Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Neoscytalidium Crous & Slippers	Crous *et al*., 2006; Pavlic *et al*., 2008; Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013
Phaeobotryon Theiss. & Syd.	Phillips *et al*., 2013; Liu *et al*., 2012; Hyde *et al*., 2013
Pseudofusicoccum Mohali, Slippers & M.J. Wingf.	Crous *et al*., 2006; Pavlic *et al*., 2008; Sakalidis *et al*., 2011; Liu *et al*., 2012; Phillips *et al*., 2013
Sakireeta Subram. & K. Ramakr.	Crous *et al*., 2015c
Spencermartinsia A.J.L. Phillips, A. Alves & Crous	Phillips *et al*., 2013; Inderbitzin *et al*., 2010; Liu *et al*., 2012; Hyde *et al*., 2013
Sphaeropsis Sacc.	van Niekerk *et al*., 2004; Phillips *et al*., 2013; Liu et al., 2012
Tiarosporella Höhn.	Crous *et al*., 2015c; Hyde *et al*., 2013; Phillips *et al*., 2013

(POFPs), since their pathogenic nature may appear when induced by environmental factors or when the vigour of plants becomes weak. Due to their ability to shift between endophytic and pathogenic phases, botryosphaeriaceous taxa have been subjected to various critical studies concerning pathogenicity (Beas-Fernandez *et al.*, 2006; Amponsah *et al*., 2011; Sakalidis, 2011; Baskarathevan *et al*., 2012a; Ramírez-Suero *et al*., 2012; Fernandes *et al*., 2014; Abou-Mansour *et al*., 2015). These POFPs attack perennial organs of many important plants including grape, apple, peach, pear, poplar and blackberry, ultimately leading to their death, with no obvious restriction to host colonization or habitat (Slippers and Wingfield, 2007; Sakalidis, 2011).

Morphological diversity and host association have been overruled as valid taxonomic characters for the identification of botryosphaeriaceous species

(Jacobs and Rehner, 1998). Studies after the 1990s have used molecular techniques to improve segregation of these fungi at species level (Crous *et al*., 2006; Phillips *et al*., 2013; Hyde *et al*., 2014). Molecular approaches provide improved accuracy, reliability and reproducibility in identifications, and eliminate the difficulty of naming sequence data from next generation sequencing techniques. They can be used in studies focusing on the diversity of botryosphaeriaceous taxa and to understand the genetic structure of pathogen populations, which is essential to predict disease epidemics (Begoude Boyogueno *et al*., 2012). A better understanding of the biology of these species is required to develop effective management strategies to control pathogenic species in the family (McDonald, 1997).

Despite the importance attributed to pathogenic botryosphaeriaceous taxa, few molecular studies have been conducted to improve the understanding of the biology and ecology of these species. This paper therefore reviews the understanding of molecular aspects underlying the pathogenesis and other metabolisms in Botryosphaeriaceae. The review focuses on (1) molecular markers and techniques used in the identification of pathogenic and quarantinable Botryosphaeriacae species, (2) genetic diversity and variability among the species, (3) genes and virulence factors affecting molecular pathogenesis, and (4) genomics in Botryosphaeriaceae. This review provides baseline knowledge on molecular data that answer some of the questions involved in the pathogenicity in the Botryosphaeriaceae. Species in this family include some of the most important pathogens of plants and agricultural crops, primarily perennial crops, worldwide. Hence, placing molecular knowledge in a review will contribute to future molecular research on Botryosphaeriaceae.

Molecular markers and techniques used for identification of Botryosphaeriacae species

Molecular markers used for identification

Traditionally, species recognition was mainly based on morphological characters in many different fungal taxa. While still useful for identification of species, this approach tends to underestimate the true diversity of botryosphaeriaceous species (Taylor *et al*., 2000). Compared to other groups of fungi, the narrow range of morphological and ecological characteristics available in Ascomycetes (Hawksworth, 2001) has partially contributed to this underestimation of species diversity. Existence of species complexes, specifically the cryptic species that could not be resolved using classical taxonomy methods, has questioned the reliability of using only the morphological species concept for differentiation of fungal species (Sakalidis, 2011). The ecological species concept, which is based on host specialization, has been used in the identification of many fungal species. However, the wide host ranges of botryosphaeriaceous species, and their coexistence with other species, does not allow the application of the ecological species concept in this family (Slippers and Wingfield, 2007). In the early 1990's, DNA based molecular techniques, in particular DNA sequence-based phylogenetic inference, have been used widely, allowing meaningful comparisons between old names and currently applied names (Slippers and Wingfield, 2007; Slippers *et al*., 2013, 2014; Hyde *et al*., 2014). This phylogenetic species concept allows the identification of morphologically similar new fungal species belonging to phylogenetically distinct lineages (Stewart *et al*., 2014).

Many molecular markers have been used for generating species-specific sequences for diagnostic and classification studies. They are also used in population genetics and pathogenicity studies, and have resolved identification of pathogens to species or strain level, which was not previously possible. Compared to traditional markers, such as morphological and biochemical markers (isozymes or allozymes), molecular markers, including those based on hybridization or the polymerase chain reaction (PCR), are highly reproducible, cost-effective and allow direct manifestations of genetic content without any influence from environmental factors (Kumar *et al*., 2009). Therefore, molecular markers serve as reliable indices of genetic or pathotypic variations. Many studies have used proteins, isozymes and nucleic acids (DNA, RNA) as potential markers (Babujee and Gnanamanickam, 2000).

The selection of a suitable target DNA region is the key factor in designing molecular markers for different species. Universal ribosomal DNA (rDNA) sequences contain conserved and variable regions that can differentiate taxa at various levels. Hence, rDNA sequences are commonly used as molecular markers. Rapidly evolving, highly variable regions

such as internal transcribed spacer (*ITS*), known as the primary fungal barcode (Schoch *et al*., 2012; Nilsson *et al*., 2014), are easy to access using universal fungal primers. These have been used extensively in resolving phylogenies of *Alternaria* Nees (Jasalavich *et al*., 1995), *Leptosphaeria* Ces. & De Not. (Morales *et al*., 1993), *Mycosphaerella* Johanson (Goodwin *et al*., 2001) and many other fungal groups (Zhang *et al*., 2012; Slippers *et al*., 2013, 2014; Phillips *et al*., 2013; Hyde *et al*., 2013, 2014; Ariyawansa *et al*., 2015; Crous *et al*., 2015a; Liu *et al*., 2015). Similarly, *ITS* data have also been used in Botryosphaeriaceae to identify and link asexual genera, leading to the clarification of the family and individual genera (Jacobs and Rehner, 1998; Denman *et al*., 2000; Pavlic *et al*., 2004, 2007; Phillips *et al*., 2007). When results of *ITS* phylogeny contradicts with morphological results (Denman *et al*., 2000; Crous *et al*., 2004; Phillips *et al*., 2007), other gene regions such as 28S rDNA (*LSU*), Translation Elongation Factor 1-*α* (*EF1-α)* and *β-tubulin* were applied successfully (Pavlic *et al*., 2004; Crous *et al*., 2006). Crous *et al*. (2006) used part of the *LSU* gene to resolve Botryosphaeriaceae species into ten lineages, representing ten distinct genera. This study resolved some of the confusions pointed out in the previous studies of Botryosphaeriaceae. Sakalidis (2011) used part of the *EF1-α* gene to resolve the *Lasiodiplodia* species complex. This study suggested that there can be four different taxa and two potential hybrids in the *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. species complex. The mitochondrial small subunit ribosomal gene region (mt *SSU*) as well as the small subunit ribosomal gene region (*SSU*) have been effectively used for the identification of *Diplodia sapinea* (Fr.) Fuckel, which surpasses the high degree of homology between *Diplodia* species (Zhou and Stanosz, 2001; Smith and Stanosz, 2006; Luchi *et al*., 2011).

Despite the high levels of species discrimination resulting from these single molecular markers, they underestimate the true diversity among cryptic or closely related species (Slippers and Wingfield, 2007). For example, Crous *et al.* (2006) was unable to resolve the clade consisting of *Diplodia*, *Lasiodiplodia* and other genera with pigmented conidia using *LSU* rDNA phylogeny alone. Similarly, Silva-Hanlin and Hanlin (1999) failed to determine the placement of *Botryosphaeria-Guignardia* clade using *SSU* rRNA phylogeny. The studies of Pavlic *et al*. (2004, 2007) on Botryosphaeriaceae showed that *ITS* phylogeny could not separate the cryptic species in the *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips/ *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips complex on native *Syzygium* R.Br. Gaertn. trees in South Africa.

Multigene phylogenies have therefore replaced the single genes or gene regions that were initially used to establish species boundaries. Recent phylogenetic studies often employed a combination of particular nuclear, ribosomal and protein-coding genes in different analysis approaches such as maximum parsimony, maximum likelihood and Bayesian inference (Crous *et al*., 2015b). Crous *et al*. (2015b) stated that the more genes that were used in phylogenetic analyses, the greater was the likelihood that results would reflect reality. This concept is known as genealogical concordance phylogenetic species recognition (GCPSR), a form of phylogenetic species concept (Taylor *et al*., 2000). GCPSR uses phylogenetic concordances of single gene genealogies to show evolutionary independence of the lineages and eliminate the limitations of single gene genealogies (Taylor *et al*., 2000). It allows the effective identification of cryptic species and species complexes. Multigene genealogies are commonly used in many phylogenetic studies involving important phytopathogenic genera such as *Alternaria* (Woudenberg *et al*., 2014), *Bipolaris* Shoemaker (Manamgoda *et al*., 2014), *Colletotrichum* Corda (Cannon *et al*., 2012) and many other genera (Zhang *et al*., 2012; Wikee *et al*., 2013; Ariyawansa *et al*., 2015; Liu *et al*., 2015). In these studies, various previously unidentified, cryptic phylogenetic species were revealed.

Due to the high genetic similarities between Botryosphaeriaceae species, existence of cryptic species and species complexes such as *N. parvum*/*N. ribis* and *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips/*Neofusicoccum australe* Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillip complex (Pavlic *et al*., 2008), the GCPSR approach has been used in recent studies (de Wet *et al*., 2003; Slippers *et al*., 2004a, 2013; Phillips *et al*., 2013; Hyde *et al*., 2014). For example, multiple gene genealogy was effectively used to distinguish *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. as a sister species of *D. sapinea* (de Wet *et al*., 2003). Multiple gene genealogy also helped in identifying *Neofusicoccum eucalypticola* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips as sister species of

Neofusicoccum eucalyptorum (Crous, H. Smith & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, and *N. australe* as a sister of *N. luteum* (Slippers *et al*., 2004b).

Sequence analyses of the *ITS* region in combination with protein coding genes such as *ß-tubulin* and *EF1-α* (Alves *et al*., 2006; Slippers *et al*., 2004a; van Niekerk *et al*., 2004; Phillips *et al*., 2013) have made major contributions in resolving genera in Botryosphaeriaceae. *ITS* together with *EF1-α* is a commonly used combination in most of the recent phylogenetic studies (Alves *et al*., 2008; Pavlic *et al*., 2008; Slippers *et al*., 2013; Phillips *et al*., 2013; Hyde *et al*., 2014, Ariyawansa *et al*., 2015; Liu *et al*., 2015). For example, this combination was used in the identification of *Dothiorella longicollis* Pavlic, T.I. Burgess & M.J. Wingf., *Fusicoccum ramosum* Pavlic, T.I. Burgess & M.J. Wingf., *Lasiodiplodia margaritacea* Pavlic, T.I. Burgess & M.J. Wingf., *Neoscytalidium novaehollandiae* Pavlic, T.I. Burgess & M.J. Wingf., *Pseudofusicoccum adansoniae* Pavlic, T.I. Burgess & M.J. Wingf., *Pseudofusicoccum ardesiacum* Pavlic, T.I. Burgess & M.J. Wingf. and *Pseudofusicoccum kimberleyense* Pavlic, T.I. Burgess & M.J. Wingf. from baobab and surrounding endemic trees in Australia (Pavlic *et al*., 2008). Gene combinations selected for phylogenetic analyses depend mainly on the species that are being analyzed. For example, in some genera *ITS* is identical for some of the species, hence alone it is uninfomative (Phillips *et al*., 2013). In contrast, *EF1-α* can distinguish cryptic species in genera such as *Lasiodiplodia* (Phillips *et al*., 2013). However, it is always more suitable to use other gene regions such as *ß-tubulin* for the combined gene analyses.

Adesemoye *et al*. (2011) used *ITS*, *ß-tubulin* and *EF1-α* to fully resolve the identity of *Botryosphaeria* sp., and this combination of loci has also resolved the confusion in the identification of the pathogen causing Diplodia canker of cypress (Alves *et al*., 2006). Using the same combination along with phenotypic characters, Slippers *et al*. (2004b) resolved *Botryosphaeria dothidea* (Moug.) Ces. & De Not. from *Neofusicoccum ribis*, as well as *N. parvum*. Pavlic *et al*. (2009), used GCPSR of five loci (*ITS*, *EF1-α*, *ß-tubulin*, RNA polymerase II subunit (*RPB2*) and locus BotF15 containing microsatellites) to resolve the cryptic speciation in the *Neofusicoccum parvum*/*N. ribis* complex. They showed that the *N. parvum*/*N. ribis* species complex consists of at least three cryptic species (*Neofusicoccum cordaticola* Pavlic, T.I. Burgess & M.J. Wingf., *Neofusicoccum kwambonambiense* Pavlic, T.I. Burgess & M.J. Wingf. and *Neofusicoccum umdonicola* Pavlic, T.I. Burgess & M.J. Wingf.). Furthermore, *RPB2* with its slow evolutionary rate (Liu *et al*., 1999), was shown to be the most informative gene region to distinguish the species in the *N. parvum*/*N. ribis* complex. This suggests that *RPB2* together with *ITS* sequences should be used for the delimitation of species in the *N. parvum*/*N. ribis* complex.

Multiple gene genealogies based on six loci (*ITS*, *EF1-α*, Glyceraldehydes-3-phosphate dehydrogenase (*GPD*) gene, Heat shock protein (*HSP*), Histone-3 (H_3) and *ß-tubulin*) has been used to show high species diversity in Botryosphaeriaceae (Inderbitzin *et al*., 2010). In this study, they compared phylogenetic resolutions of different dataset combinations to determine the best combination for the greatest resolution. Acccording to them, six locus combination resulted in the greatest phylogenetic resolution and resolved 37 nodes. Even the diverse *Neofusicoccum* clade, which could not be resolved by any other combination, was resolved successfully. However, combined analyses of *ITS*, *EF1-α* and *ß-tubulin* resolved 34 nodes, whereas five locus combination (*EF1-α*, *GPD*, *HSP*, H_3 and *ß-tubulin*) resolved 36 nodes in Botryosphaeriaceae. Although greatest resolution was achieved using six loci, there are doubts whether the resolution has yet been maximized (Inderbitzin *et al*., 2010).

The importance of using multigene phylogeny for fungal identification was evident from a series of revisions of the placement of *Phyllosticta* Pers. Initially the genus was placed in the family Phyllostictaceae, of the order Phyllostictales by Seaver (1922). Then Barr (1970) placed *Phyllosticta* in Botryosphaeriaceae together with its sexual state *Guignardia* Viala & Ravaz, which was later replaced by the name *Phyllosticta* due to one name-one fungus approach (Wijayawardene *et al*., 2014). This placement was further confirmed by Schoch *et al*. (2006), Crous *et al*. (2006) and Liu *et al*. (2012). However, Crous *et al*. (2006) and Liu *et al*. (2012) mentioned the possibility that *Phyllosticta* could be placed elsewhere. Wikee *et al*. (2013) used a multi gene analysis based on *ITS*, Actin (*ACT*), *EF1-α*, *LSU* and *GPD* and proposed that *Phyllosticta* should reside in its original family Phyllostictaceae as a sister family to Botryosphaeriaceae, but in the order Botryosphaeriales. Slippers *et al*. (2013) confirmed this using *LSU*, *SSU*, *ITS*, *EF1-α*, *ß-tubulin* and mt *SSU* genes. The robustness of multigene inference was evident from the greater species

resolution resulting in these studies. All the studies reviewed above reflect the critical importance of using multiple gene genealogies to identify cryptic species and to characterize the true diversity within the Botryosphaeriaceae.

Molecular techniques used for identification

Apart from molecular markers, a broad range of molecular techniques and approaches has been successfully applied for identifying fungal pathogens (Crous *et al*., 2015b). In 1990's, initial attempts at fungal identification using DNA data were reported, where tools such as randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) were used (Hyakumachi *et al*., 2005). These techniques have been successful in differentiating phytopathogens such as species of *Fusarium* Link (Lievens *et al*., 2007) and *Elsinoë* Racib. (Hyun *et al*., 2009), and many other pathogenic fungi (Daval *et al*., 2010; Pan *et al*., 2010). Similarly, RAPD and RFLP analyses have been used as valuable tools to characterize Botryosphaeriaceae populations (Babujee and Gnanamanickam, 2000). RAPD and RFLP molecular markers have been developed and used to differentiate the four morphotypes of *Diplodia sapinea,* which were later defined as separate species (Smith and Stanosz, 1995; Burgess *et al*., 2001; Burgess *et al*., 2004a).

Chen *et al*. (2011) used RFLP markers to identify Botryosphaeriaceae species in *Eucalyptus* L'Hér. plantations in China, and used the locus BotF15 as a molecular marker to successfully distinguish isolates in the *Neofusicoccum parvum*/*N. ribis* complex. Pavlic *et al*. (2007) identified eight Botryosphaeriaceae species based on anamorph morphology, together with *ITS* data and RFLP analysis. RFLP analysis distinguished *N. parvum* isolates from *N. ribis* isolates using BotF15 and BotF16 primers. However, *N. luteum* and *N. australe* isolates could not be resolved using RFLP. Furthermore, many studies confirmed that the *ITS* region in RFLP-PCR is not suitable for assessing genetic variability in *Macrophomina phaseolina* (Tassi) Gold., since the *ITS1* region has a high mutation rate with single nucleotide polymorphisms compared to *ITS2* and 5.8S rDNA, which have no polymorphism in *Macrophomina* (Su *et al*., 2001; Almeida *et al*., 2003).

The use of *ITS* and *LSU* as molecular markers in Amplified ribosomal DNA restriction analysis (ARDRA) allowed the identification of many Botryosphaeriaceae species. ARDRA has been shown to be an outstanding tool for the identification of cryptic species such as *N. ribis, N. parvum* and *Dothiorella sarmentorum* (Fr.) A.J.L. Phillips, J. Luque & A. Alves and *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves (Alves *et al*., 2005).

PCR-based genomic fingerprinting techniques have been useful in identifying unique molecular markers to distinguish individuals within a population by analyzing the whole genome using specifically targeted primers. Microsatellites/simple sequence repeats (SSR) and minisatellites are frequent components in the eukaryotic genomes that are used as highly versatile markers for DNA fingerprinting (Capote *et al*., 2012). Due to high polymorphism, and the multiallelic and co-dominant nature of these markers (Capote *et al*., 2012), they were widely used to study many plant pathogenic fungi, including *Ceratocystis fimbriata* Ellis & Halst. (Rizatto *et al*., 2010), *Puccinia graminis* Pers. (Szabo, 2007) and *Magnaporthe oryzae* B.C. Couch (Zheng *et al*., 2008).

In Botryosphaeriaceae, microsatellite-primed polymerase chain reaction (MSP-PCR) was used to differentiate species such as *Botryosphaeria dothidea* (Ma *et al*., 2004), and repetitive-sequence-based polymerase chain reaction (rep-PCR) was used for molecular typing (Alves *et al*., 2007). Molecular markers designed by Alves *et al*. (2007) for MSP-PCR and rep-PCR have been successful in producing complex species-specific genomic fingerprints to differentiate 27 species in Botryosphaeriaceae, with high interspecies variation and high reproducibility. This study also allowed the clear identification of cryptic species such as *Neofusicoccum parvum*/*N. ribis* or *N. luteum*/*N. australe*. These markers, derived from repetitive sequences of prokaryotes, were shown to be excellent in amplifying regions in eukaryotic fungal genomes (Alves *et al*., 2007). Baskarathevan *et al*. (2012b) also developed markers for isolate-specific PCR assays for *N. parvum* and *N. luteum*, using universal primed PCR (UP-PCR) based on the polymorphism found in the marker band using the *TaqI* restriction endonucleases. Specificity of these markers has been tested for six Botryosphaeriaceae species resulting in unique bands specific to their species and isolates.

Several studies have shown the suitability of microsatellite markers for the differentiation of *M. phaseolina* (Su *et al*., 2001; Arias *et al*., 2011; Mahdizadeh

et al., 2012). The SSR markers developed for *Diplodia sapinea* by Burgess *et al.* (2004a) have demonstrated the allelic polymorphism in Brazilian isolates of *Lasiodiplodia theobromae* (Cardoso and Wilkinson, 2008). In addition to these markers, new markers have been designed using inter-short sequence repeats (ISSR-PCR) and by genome walking for *L. theobromae* (Burgess *et al*., 2003). Slippers *et al*. (2004b) has developed eight sets of microsatellite markers for *Neofusicoccum parvum*, which were also applied successfully for *N. ribis*, *Fusicoccum* sp., *Neofusicoccum mangiferae* (Syd. & P. Syd.) Crous, Slippers & A.J.L. Phillips, *N. eucalyptorum*, *N. luteum* and *B. dothidea*. The same microsatellite markers have been successfully applied with *N. parvum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* to study their species and population diversity (Pavlic-Zupanc *et al*., 2015). These studies confirmed that MSP-PCR and rep-PCR are better methods than RFLP and RAPD, for differentiating botryosphaeriaceous taxa.

With the introduction of next generation sequencing techniques, whole genome sequencing has become an increasingly feasible approach in fungal identifications (Ohm *et al*., 2012). The availability of fungal genomes provides an opportunity to reconstruct evolutionary events through phylogenomics, which will expose fungal relationships more clearly. The phylogenomic approach overcomes the limitations associated with single and multiple gene phylogenies and provides strong and stable markers with maximum amount of discrete DNA or amino acid characters for use in large scale phylogenetics (Fitzpatrick *et al*., 2006). Phylogenomic data has resulted in consistent tree topology with well-supported nodes across a suite of different algorithms (Fitzpatrick *et al*., 2006; Wang *et al*., 2009). In addition to contributing towards phylogenomics, whole genome sequences enable the development of species-specific microsatellite markers (Ropars *et al*., 2014). They screen the random regions of the fungal genomes producing species-specific sequences, an advantage over conserved genes whose variations sometimes become insufficient for successful identification of the species.

Although many molecular techniques have been introduced, the advantages of using phylogenetic inferences based on multiple gene genealogies have changed the direction of fungal taxonomy. This has proved to be the most powerful tool in species recognition and is expected to be used in future for the discovery of new species and species complexes. The application of molecular techniques such as microsatellite markers will help in understanding the diversity, evolution and the process of speciation in Botryosphaeriacae. These techniques are the latest tools in identification, but the best is yet to be discovered.

Genetic diversity in populations of Botryosphaeriaceae

Genetic diversity is the number of genetic characteristics in the genetic makeup of a species, which are reflected in morphological, physiological and behavioural differences between individuals and populations (Frankham *et al*., 2002). This plays a vital role in the survival and adaptability of the fungi. The diversity available in the population's gene pool, allows variable traits, which will allow the species to survive or thrive in different environments (Toro and Caballero, 2005). Genetic variability is the tendency of genetic characteristics to vary (Schlegel, 2003) and lays the foundation for organisms to have genetic diversity. Genetic diversity and variability are essential for the evolution of a species (Falk *et al*., 2001). Variability affects their responses to environmental stresses and leads to differential survival modes within a population (Dobzhansky, 1970). Diversity provides information on distribution and virulence of fungal genotypes in different localities (Reeleder, 2003). This is influenced by the reproductive nature, with sexually reproducing taxa having more genetically diverse populations than asexually reproducing species (Cortesi and Milgroom, 2001). Population genetics is the study of the frequency and interaction of alleles and genes (Postlethwalt and John, 2009), which provides understanding of adaptation and speciation under evolutionary processes such as natural selection, generic drift, mutation and gene flow (Hartl and Clark, 2007).

Many studies have been conducted on genetic diversity of different plant pathogenic fungi using different molecular approaches. Examples are with *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara (Padder *et al*., 2007), *Fusarium graminearum*, *Magnaporthe oryzae* (Babu *et al*., 2013), and other pathogenic fungi (Overmeyer *et al*., 1999). Several studies have been conducted on genetic variability, genetic diversity and population structure of taxa in Botryosphaeriaceae (Burgess *et al*., 2004b, 2006).

Table 2. Common techniques that have been used in studies of genetic diversity.

Technique	Reference
Amplified fragment length polymorphism (AFLP)	Reyes-Franco *et al*., 2006; Piškur *et al*., 2010
Amplified ribosomal DNA restriction analysis (ARDRA)	Machouart *et al*., 2004; Alves *et al*., 2005; Madrid *et al*., 2008
Diversity array technology (DarT)	Kilian *et al*., 2005
ISSR	Zietkiewicz *et al*., 1994
microsatellite-primed PCR (MSP-PCR)	Alves *et al*., 2007; Bahkali *et al*., 2012; Mahdizadeh *et al*., 2012
Randomly amplified polymorphic DNA (RAPD)	Fuhlbohm, 1997; Su et al., 2001; Omar *et al*., 2007; Almeida *et al*., 2008
Restriction fragment length polymorphism (RFLP)	Almeida *et al*., 2003; Purkayastha *et al*., 2006
Single nucleotide polymorphism (SNPs)	Chen and Sullivan, 2003
Vegetative compatibility grouping (VCGs)	Ma *et al*., 2001, 2003, 2004; Dakin *et al*., 2010; Baskarathevan *et al*., 2012a
Universal primed PCR (UP-PCR)	Bulat *et al*., 1998; Abdollahzadeh *et al*., 2013

Similar to species identification, genetic diversity can also be determined using morphological, biochemical and molecular approaches. However, molecular approaches provide rapidly obtainable results without any interference from environmental factors (Gonçalves *et al*., 2009). Many techniques have been used in genetic diversity studies (see Table 2).

Vegetative compatibility grouping (VCG) is a method traditionally used for evaluating population structure and genetic diversity of asexually reproducing fungi, which provides information on genetic diversity and the potential for genetic exchange in populations (Dakin *et al*., 2010; Baskarathevan *et al*., 2012a). Botryosphaeriaceae species are thought to reproduce asexually in the field (Phillips, 2002), with rare sexual reproduction (van Niekerk *et al*., 2006). Thus, surprisingly high genetic diversity has been observed in studies for these apparently asexual taxa (Dakin *et al*., 2010; Baskarathevan *et al*., 2012a; Bihon *et al*., 2012a).

The genetic diversity of Californian isolates of *B. dothidea* have been assessed using three complementary approaches; microsatellite-primed PCR, phylogenetic analysis on *RPB2* gene and VCG analysis. Ma *et al.* (2003) identified 20 VCGs from 360 isolates of *B. dothidea* from various hosts excluding grapevine, and 94% of them were assigned to one of two VCGs. This suggests that they were genetically uniform, yet were a highly pathogenic population with little variation. Similarly, MSP-PCR and RPB2 gene analyses have shown high genetic similarity (>98%) between *B. dothidea* isolates suggesting that they are primarily asexual on Californian trees and plants. This low genetic diversity suggests that there is a high probability of successfully controlling these populations. However, *B. dothidea* isolates from outside California were genetically diverse (Ma *et al*., 2001, 2004).

In South Africa, 62 VCGs were identified from 107 isolates of *D. sapinea* from exotic *Pinus patula* Schiede ex. Schltdl. & Cham., and four VCGs were identified among 83 isolates from Northern Sumatra, indicating high genetic diversity (Smith *et al*., 2000). Similar results were observed using microsatellites (Burgess *et al*., 2004b; Bihon *et al*., 2011, 2012a). Genotypic diversities observed for *Diplodia* populations in Australia and New Zealand were greater when compared to native Californian populations, with South African populations being of even greater diversity (Burgess *et al*., 2001). This high genetic diversity of *D. sapinea* could have resulted from either the multiple introductions which were believed to have occurred in South Africa or from cryptic sexual reproduction, genetic recombination or mutations (Burgess *et al*., 2004b; Bihon *et al*., 2012b). Similarly, the low genotypic diversities observed in Australia and California could result from very few introductions into those regions (Smith *et al*., 2000; Burgess *et al*., 2004a; Bihon *et al*., 2012a).

Genetic diversity of *D. sapinea* changes with different morphotypes, and taxonomy, biology and virulence also differ accordingly (Burgess *et al*., 2001). An endophytic population of *D. sapinea* showed greater genetic variation, compared to pathogenic populations obtained from dieback symptoms of *P. patula* after hail damage (Bihon *et al*., 2012a). Increased genetic variation in endophytic populations can result from numerous individual infections over time. Endophytic communities cause diseases under host stress conditions in absence of wounding. When wounding occurs, other infections from asexual conidia lead to disease resulting in uniform pathogenic populations (Bihon *et al*., 2012a). Smith *et al.* (2000) explained this scenario as a result of competition and selection among endophytic strains.

Ability of UP-PCR to amplify DNA from any organism is a good option for investigating genetic diversity, as it produces fungal band profiles according to their genetic relatedness (Bulat *et al*., 1998). UP-PCR provides greater resolution of population structure than VCGs, allowing identification of clonal isolates from different regions (Baskarathevan *et al*., 2012a). *Neofusicoccum parvum* isolates were distinguished into seven main groups with 97 % genetic similarity between them, and high inter- and intra-vineyard variation was demonstrated among 56 genotypes of New Zealand *N. parvum* population with low clonality. This indicated the occurrence of anastomosis to increase the genetic diversity of the species. Similar results were obtained for the *N. parvum* population in Iran by Abdollahzadeh *et al*. (2013). They suggested that *Neofusicoccum mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips is a complex of species due to the significant differences in *ITS* and *EF1-α* sequences. Populations of *N. australe* in Australia showed high diversity, with no host or habitat specificity (Sakalidis *et al*., 2011).

Genetic divergence of *Botryosphaeria* species assessed using ARDRA fingerprinting showed very low intraspecific variability among species and intraspecific polymorphism was detected only in *N. parvum* and *B. dothidea* (Alves *et al*., 2005). Genetic diversity of *Neoscytalidium hyalinum* (C.K. Campb. & J.L. Mulder) A.J.L. Phillips, M. Groenew. & Crous has been evaluated with respect to geographical origins. Genotyping conducted on *ITS*, *LSU*, *β-tubulin*, and the chitin synthase genes revealed low genetic variability (Madrid *et al*., 2008), similar to that reported by Machouart *et al*., (2004).

Amplified fragment length polymorphism (AFLP) is another highly sensitive, reproducible tool used widely to detect polymorphisms simultaneously in different genomic regions at several taxonomic levels (Meudt and Clarke, 2007). This approach has detected polymorphisms in Botryosphaeriaceae (Reyes-Franco *et al*., 2006; Piškur *et al*., 2010). Piškur *et al*. (2010) revealed diversity between *B. dothidea* isolates based on AFLP markers and stated that their diversity did not link with their geographical origin but with their hosts.

The RAPD technique has been used extensively in studying genetic diversity. It has been used for studying genetic variation and genetic relatedness within and between populations of *Macrophomina phaseolina* (Fuhlbohm, 1997; Omar *et al*., 2007). Almeida *et al*. (2008), using RAPD analysis, showed that *M. phaseolina* is a highly diverse species, and that isolates were separated into clusters at 85% similarity. Analysis of molecular variance revealed that 21% of the genetic variability was due to the differences between populations, and 79% was due to the differences within populations. The mean coefficient of gene differentiation indicated 27% between populations, and explained the existence of restricted gene flow among populations. This may have been due to the variable resistance of different hosts, which applies considerable selection pressure on the pathogens leading to increases in pathotypes. Based on RAPD analysis, Su *et al*. (2001) observed DNA polymorphism among the *M. phaseolina* isolates from soybean, sorghum, corn and cotton in the USA, and showed that this genetic variation was restricted to populations bounded by host species. Similar results were obtained from studies of genetic variation in *M. phaseolina* in Australia (Fuhlbohm, 1997), Brazil (Almeida *et al*., 2003) and Mexico (Mayek-Perez *et al*., 2001). Significant correlation has been shown between geographical data, host plants and genetic diversity of fungal isolates (Mayek-Perez *et al*., 2001; Su *et al.*, 2001). These studies suggest that host selection is the evolutionary force in genetic variation among *M. phaseolina* isolates.

In contrast to Almeida *et al*. (2003), who used RFLP techniques to assess genetic diversity, Purkayastha *et al*. (2006) showed that RFLP is not appropriate for assessing genetic diversity of *M. phaseolina*. However, MSP-PCR has been successful for assessing genetic diversity (Alves *et al*., 2007; Bahkali *et al*., 2012; Mahdizadeh *et al*., 2012; Begoude Boyogueno *et al*., 2012)

and evolution (Wostemeyer *et al*., 2002) of many fungal pathogens. This technology has also produced a single marker (Bahkali *et al*., 2012) for *M. phaseolina* for assessment of intra-specific polymorphism. Similar studies have been conducted on *M. phaseolina* from soybean (Baird *et al*., 2010) and other plant hosts (Baird *et al*., 2010; Mahdizadeh *et al*., 2012). These PCR-based fingerprinting techniques have been effective for studying intraspecific variability.

Begoude Boyogueno *et al.* (2012) investigated the genetic diversity and population structure of *L. theobromae* and *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous in Cameroon, using microsatellites. Bayesian inference suggested that the population structure of these species was not influenced by hosts or the geographic location. A high level of gene diversity and gene flow between isolates from *Theobroma cacao* L. and *Terminalia* sp., and moderate levels of genotypic diversity, were apparent. Random association of the SSR alleles of both species suggested that out-crossing had occurred. This is because both species occur in tropical and subtropical regions without any host specialization (Alves *et al*., 2008; Mohali *et al*., 2005). These fungi are considered as high-risk pathogens in Cameroon, due to their high diversity, out-crossing, highly pathogenic nature, and gene flows between geographically different populations. Alves *et al*. (2007) used microsatellite markers to demonstrate high genetic diversity in *L. theobromae* isolates. Using the same technique, Palvic-zupanc *et al*. (2015) found that *N. parvum* populations from urban areas are more genetically diverse than populations from disturbed plantations. They suggested that biological invasions with multiple introductions were possible causes. Asexual reproduction or homothallic sexual cycles were suggested as the reasons for the existence of identical halotypes between the populations.

From these studies, we conclude that there is considerable genetic diversity among many botryosphaeriaceous taxa. This high diversity may be the reason for their success in adapting to many life styles under different environmental conditions. Gene pools of these taxa must be rich, since the fungi occur in many environments. Although RFLP, RAPD, ISSR and AFLP markers have been commonly used in genetic diversity studies, reproducibility has been a problem. Therefore, microsatellite markers have become the markers of choice. Discovery of single nucleotide polymorphism, microarray-based techniques and whole genome analyses have been generally useful for studying genetic diversity and variability. However, they have rarely been used in genetic diversity studies of Botryosphaeriaceae.

Studies focused on genetic diversity and pathogenicity of Botryosphaeriaceae species (Úrbez-Torres and Gubler, 2009; Amponsah *et al*., 2011; Baskarathevan *et al*., 2012a) have demonstrated variable levels of virulence between isolates. These observations have led to the initiation of further molecular studies to demonstrate correlations between genetic diversity and virulence. Although botryosphaeriaceous taxa have high genetic diversity, low genetic variability is reported from many geographical locations for some of the species such as *Botryosphaeria*, *Diplodia*, *Neoscytalidium* and *Lasiodiplodia*. This low genetic variability will allow researchers to design strategies for management of the diseases they cause. Hence, understanding genetic diversity and variability is important.

Genes and virulence factors affecting molecular pathogenesis

Pathogenicity is the ability of a pathogen to infect a host (Baarlen *et al*., 2007). Pathogens produce toxins or other virulence factors which may directly or indirectly damage their hosts or promote host immune responses (Casadevall and Pirofski, 1999). Most fungal plant pathogens enter their hosts through openings such as stomata or wounds, and extremely specific pathogens forcibly enter the host by piercing the leaf with an infection peg, arising from an appressorium that helps to exert the pressure (Bechinger *et al*., 1999). The evolution of pathogenicity does not occur as an independent event for each host. The genetic structure of both the host and pathogen facilitate these compatible molecular interactions (Lynch and Conery, 2003) with some influence from the environmental conditions (Baarlen *et al*., 2007). All pathogens produce molecular components that play important roles in nutrient release or uptake or in the evasion or suppression of host immune systems (Read, 1994). Pathogenicity also involves cell wall degrading enzymes and phytotoxins which are involved in the breakdown of plant tissues and the induction of cell death (Saldanha *et al*., 2007).

Recent studies have shown an increasing number of Botryosphaeriaceae species associated with many hosts. In grapevine, their effect is significant and caus-

es many diseases (Yan *et al*., 2013; Hyde *et al*., 2014). As the first attempt towards understanding the pathogenicity of these species associated with grapevine, van Niekerk *et al*. (2004) conducted *in vitro* and *in vivo* assays to test the pathogenicity of eleven Botryosphaeriaceae species. In subsequent studies, pathogenicity assays have been conducted using many approaches (Baskarathevan *et al*., 2012c; Billones-Baaijens *et al*., 2012, 2013). Most of these studies showed *N. parvum*, *N. luteum, L. theobromae*, *N. australe, Diplodia mutila* (Fr.) Mont. and *Diplodia seriata* De Not. to be highly pathogenic, and the most prevalent Botryosphaeriaceae species. They are also highly variable in their pathogenicity. Studies have also revealed the effects of host and environmental factors on the pathogenicity of these taxa. These observations have led to initiation of research which explains the molecular basis of the results. Molecular genetic analyses have helped to provide understanding of the strategies that fungal pathogens employ to infect their hosts.

Recent studies, using tagged mutagenesis techniques, have identified many fungal genes which are believed to have roles in the formation of infection structures, cell wall degradation, overcoming or avoiding plant defenses, responding to the host environment, production of toxins and in signal cascades (Balhadère and Talbot, 2001; Shah *et al*., 2009; Cobos *et al*., 2010; Jami *et al*., 2010; Lambert *et al*., 2012, Zhao *et al*., 2013). These were named as pathogenicity genes. They are defined as genes necessary for disease development, but not essential for a pathogen to complete its life cycle (Idnurm and Howlett, 2001). In addition to pathogenicity genes, avirulence genes also have a role in pathogenicity (Idnurm and Howlett, 2001). Involvement of these genes in pathogenesis depends on the infection mechanism of the pathogen.

Most popular techniques used to study pathogenicity genes are targeted and random gene disruption techniques. These include restriction enzyme mediated integration, *Agrobacterium tumafaciens*-mediated transformation, and transposon tagging. These techniques have been applied successfully to many plant pathogenic fungal taxa such as *Magnaporthe oryzae* (Villalba *et al*., 2001), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Yokoby *et al*., 2001), *Fusarium solani* (Mart.) Sacc. (Rogers *et al*., 1994), *Botrytis cinerea* Pers. (ten Have *et al*., 1998) and many others (Tonukari *et al*., 2000; Balhadère and Talbot, 2001). In recent years, several studies have been conducted on Botryosphaeriaceae by employing these techniques. Zhang *et al*. (2014) produced pathogenicity mutants of *L. theobromae* using restriction enzyme-mediated integration (REMI). This was applied with *Hind III* and *KpnI* restriction enzymes to produce 1369 transformants, as the first successful genetic transformation for *L. theobromae*. Similarly Lopez-Zambrano *et al*. (2012) used suppression subtractive hybridization, and identified a 125 bp gene responsible for hydrolytic enzymes, arabinases, in the genome of *L. theobromae* that might be involve in the cell wall degradation of the hosts.

In order to identify possible pathogenicity genes and to expand understanding of the biology of *D. seriata*, a proteome-level study was conducted by Cobos *et al*. (2010). This approach has been successful for many fungal strains (Shah *et al*., 2009; Jami *et al*., 2010). Several known virulence factors have been identified during the proteome and secretome analysis of *D. seriata* by peptide mass fingerprinting and tandem mass spectrometry. According to the functional classification of the cytoplasmic proteins, most (68%) were metabolic enzymes, and 88% were implicated in cell rescue, defense and virulence, such as Mitochondrial peroxiredoxin-1, Heat shock protein and a Flavohemoprotein. The secretome analysis suggested that the majority of proteins identified, such as glucosidases and peptidases, have significant roles in the pathogenicity process. These are responsible for the degradation of polysaccharides and cell wall proteins, allowing the pathogens to penetrate into plant cells. Peptidases are also believed to be involved in inactivation or inhibition of plant defense response proteins, allowing the pathogen to successfully establish infection (Yajima and Kav, 2006). Necrosis and induced proteins (*Nep*) identified in the secretome represent a new class of necrotic elicitors. These *Nep* proteins were reported to cause different symptoms such as necrosis (García *et al*., 2007), leading to severe injuries and cell death in susceptible plants. They are capable of inducing responses in a variety of plants, including induction of pathogen related (*PR*) genes, callose apposition, altered cell respiration, induction of hypersensitive responses and localized cell death (Bae *et al*., 2006). Detection of large amounts of putative secreted *PhiA* proteins may ensure fungal survival by producing large numbers of conidia in response to the presence of toxic metabolites.

In the Cobos *et al*. (2010) study, the medium was supplemented with carboxymethyl-cellulose (CMC) to simulate the host plant environment. In the pres-

ence of CMC, three proteins were up-regulated, including Alcohol dehydrogenase (*ADH*) and Mitochondrial peroxyrredoxin-1. Among the 30 down-regulated proteins, nine were identified with few of them responsible for carbohydrate metabolism (Cobos *et al*., 2010). Previous studies confirmed that increased peroxiredoxin levels in phytopathogenic fungi contribute in the development of pathological responses (Choi *et al*., 2003; Quan *et al*., 2008). Since several of the glycolytic enzymes are down-regulated, up-regulated *ADH* activity could produce energy (Mukherjee *et al*., 2006). However, the limited number of up- and down-regulated proteins reveals that this strategy is not effective in identifying pathogenicity factors in pathogenic fungi.

Fernandes *et al*. (2014) conducted a secretome analysis for the phytopathogen *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque, to elucidate the molecular mechanisms of pathogenesis. The analysis identified 16 proteins including 12 that were extracellular (carbohydrate degrading enzymes, proteases, putative glucan-β-glucosidses, neuraminidases and ferulic acid esterases relate to pathogenicity), showing homology to *M. phaseolina* and *N. parvum* (Islam *et al*., 2012; Blanco-Ulate *et al*., 2013). The importance of this study is that the genomes of both *M. phaseolina* and *N. parvum* have been sequenced whereas *D. corticola* has not. Lack of genomic data constricts the number of proteins identified, which in turn reflects the low homology with other fungi (Fernandes *et al*., 2014). Proteins identified in this study differed from those of *D. seriata* (Cobos *et al*., 2010), suggesting differences in the infection strategies of these fungi.

There have been several studies of *M. phaseolina* to understand pathogenicity, unlike other botryosphaeriaceous taxa. Two aspects help *M. phaseolina* to successfully produce disease. This pathogen has high physiological variabilities (Manici *et al*., 1995; Mihail and Taylor, 1995), morphological differences (Mihail and Taylor, 1995; Mayek-Perez, 1999), pathogenicity variations (Manici *et al*., 1995; Mihail and Taylor, 1995; Su *et al*., 2001) and genetic diversity (Mihail and Taylor, 1995; Jones *et al*., 1998; Su *et al*., 2001), which helps it to adapt to diverse environments. The other aspect that helps the pathogen is the existence of two lifestyles in its life cycle, one saprobic (*Rhizo-ctonia bataticola* (Taubenh.) E.J. Butler) and the other pathogenic (*M. phaseolina*). The pathogenic stage produces pycnidia and is non-specific, hence capable of infecting a wide range of crops (Dhingra and Sinclair, 1978).

Macrophomina phaseolina produces two types of cellulases, endoglucanases and cellobiohydrolases, which play major roles in pathogenicity. Two β-*1*,4 endoglucanase genes (*egl1*, *egl2*), which are also common in other pathogens, have been found from *M. phaseolina*. The *egl1* gene is responsible for penetration of hyphae into the host in addition to cellulolytic activity, and the *egl2* gene has properties similar to *egl3* gene of *Trichoderma reesei* E.G. Simmons (Wang and Jones, 1995a, b). Beas-Fernandez *et al*. (2006) suggested that the pathogenic capacity of *Macrophomina* may be based on the production of small but abundant microsclerotia. They also suggested a relationship between pathogenicity and geographic origin of isolates. Conidiation is another factor that affects fungal survival and infection, and hypo-osmotic stress triggers conidiation (Duran *et al*., 2010). Islam *et al*. (2012) found the *OSM1* gene in *M. phaseolina* which regulates the osmotic stress response, and is involved in pathogenicity. This was shown in *Magnaporthe oryzae* when gene deletion reduces pathogenicity (Dixon *et al*., 1999). Adhesion to the plant surface is essential for the establishment of the disease. Homologs of the glycoprotein Cellulase-Binding Elicitor Lectin (CBEL) and Class II hydrophobic proteins have been identified from the *M. phaseolina* genome. These are known to have roles, respectively, in adhesion to host cell walls (Gaulin *et al*., 2002) and communication between the fungus and the environment (Whiteford and Spanu, 2001).

Pathogens use virulence factors to overcome plant defenses and establish successful infection (Sacristán and García-Arenal, 2008). Previous studies have shown that the virulence factors contribute in the initial stages of infection by establishing adhesion between pathogen and host (Tan and Liang, 2013). Environmental factors have significant effects on the virulence of botryosphaeriaceous species, indirectly affecting pathogenicity. This was shown by Swart and Wingfield (1991), who found that environmental extremes such as water stress and other factors such as pruning and wounding through hail favour *D. sapinea* infections. Differences in host susceptibility, wounding, virulence of the pathogen, adverse environmental conditions such as drought further amplify pathogenicity of Botryosphaeriaceae taxa (Zwolinski *et al*., 1990). The effects of drought in enhancing fungal diseases of plants has been studied extensively (Schoeneweiss, 1975). It has been reported that pathogens *B. dothidea* (Ma *et al*., 2001),

D. sapinea (Johnson *et al*., 1997) and *D. mutila* (Ragazzi *et al*., 1999) were able to develop their infections only after the water stress of the hosts reached high levels. These observations were further confirmed, when Botryosphaeriaceae pathogens (*N. australe*, *N. parvum*, *L. theobromae* and *D. seriata*) showed severe symptoms after exposure to water stress (van Niekerk *et al*., 2011). This was further demonstrated when water stress greatly increased sycamore canker development by a mildly virulent strain of *L. theobromae* (Lewis and van Arsdel, 1978). However, *D. sapinea* infections on red pine showed significant effects of drought in increasing disease development only for aggressive morphotypes (Blodgett *et al*., 1997).

Effects of secondary metabolites on pathogenesis

There is considerable information available on the effects of secondary metabolites on pathogenicity of Botryosphaeriaceae. Several pathogens produce phytotoxic metabolites whose synergic activities could lead to symptoms observed on hosts. However, the cause of these symptoms and role of these metabolites in disease development remain unclear. These toxins have been chemically characterized and tested for their toxicity, to establish their roles in virulence and their modes of action (Andolfi *et al*., 2011, 2014; Buckel *et al*., 2013; Abou-Mansour *et al*., 2015).

Martos *et al*. (2008) isolated *B. dothidea*, *D. seriata*, *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous, *N. luteum* and *N. parvum* from grapevines in Spain, and tested the fungi for production of high molecular weight hydrophilic toxins. *Neofusicoccum parvum* and *N. luteum* produce lipophilic phytotoxins which are likely to be involved in the virulence of these taxa. Gas chromatography-mass spectrometry (GC-MS) analysis on exopolysaccharides (EPSs), dihydrotoluquinones, pyranones and dihydroisocoumarins secreted from *N. parvum* (Barbosa *et al*., 2003; Abou-Mansour *et al*., 2012, 2015) revealed that they are *O*-methyl glycosides. They differ from botryosphaerans produced by *L. theobromae* (Corradi da Silva *et al*., 2005). Evidente *et al*. (2010) also isolated four toxic metabolites from *N. parvum*, including (3R,4R)-(-)-4-hydroxy- and (3R,4S)-(-)-4-hydroxy-mellein, isosclerone and tyrosol. Leaf assays showed that these toxins caused slight to severe leaf wilting. The toxins have also been detected in *D. seriata* (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al*., 1991), *D. sapinea* and another Botryosphaeriaceae species which cause decline of *Pinus radiata* D. Don (Cabras *et al*., 2006). *Dothiorella* sp. produce the cytotoxic compounds cytosporone B, dothiorelone A, B, C and D (Xu *et al*., 2004, 2005). Andolfi *et al*. (2014) has shown that *Lasiodiplodia* species produce jasmonic acid esters, which are signaling molecules in plant defence, activating responses to wounding and pathogens.

Diplodia seriata produces several phenolic dihydroisocoumarins, including mellein, cis-(3R,4R)-4-hydroxymellin, 5-hydroxymellin, (3R,4R)-4,7-dihydroxymellin on grapevine (Castillo-Pando *et al*., 2001; Larignon *et al*., 2001) and on apple (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al*., 1991). Djoukeng *et al*. (2009) has shown that mellein occurred in vine wood infected by *D. seriata.* This enables mellein to be used as a diagnostic molecular marker for *D. seriata*, which can be used to differentiate between the two grapevine diseases, esca and Black dead arm. These melleins and their derivatives are restricted to botryosphaeriaceous fungi (Cabras *et al*., 2006; Djoukeng *et al*., 2009).

Lignin was initially fragmented by laccases, composed of one of three polyphenol oxidase groups (Eriksson *et al*., 1990). Being a member of the multicopper family of oxidases, this enzyme is typically extracellular (Messerschmitt, 1997) and has a key role in delignification in fungi (Eriksson *et al*., 1990). Laccase genes belonging to different organisms differ slightly and regulated by factors such as pH, substrate condition and fungal metabolism (Mansur *et al*., 1997). *Lasiodiplodia theobromae*, which has been screened for its ligninolytic activity (Barbosa *et al*., 1996), has been studied for the production of laccases (Dekker *et al*., 2007). The functional diversity observed for fungal laccases could be related to their genetic and phylogenetic differentiation (Castilho *et al*., 2009).

Studies have shown that *Botryosphaeria* species not only produce laccases and cellulases, but also produce pectinases and xylanases (Alves *et al*., 2003), confirming the pectinolytic nature of the genus. Laccase production was subjected to complex regulation by many regulators, including nutrients, and they affect the transcription levels of laccase genes. In the presence of certain aromatic inducers, laccase encoding genes are differentially expressed (Terron *et al*., 2004).

Ramírez-Suero *et al*. (2014) evaluated the effects of fungal extracellular compounds on expression of defense genes. Different toxicities of the extracellular compounds of *N. parvum* and *D. seriata* were

observed. Total extracellular compounds from *N. parvum* and *D. seriata* induced different levels of necrosis and different gene expression profiles. Extracellular compounds from *N. parvum* and *D. seriata* induced genes participating in cellular detoxification (*VvGST1*), pathogenesis-related proteins (*VvPR10*, *VvCHIT4c*), jasmonic acid pathway (*VvLOX*), synthesis of secondary metabolites of the phenylpropanoid pathway (*VvPAL*), phytoalexin production (*VvSTS1*) and the hyposensitive response gene (*VvHSR*). Phytotoxins produced by *N. parvum* were more toxic than toxins from *D. seriata*. Molecular analyses confirmed that when mellein concentration reached its greatest amount, induction of defense gene expression became significant. This gene expression was strongly induced with two specific pathways in the presence of mellein: cellular detoxification (*VvGST1*) and pathogenesis-related proteins (*VvPR6*, *VvGLU*, *VvCHIT4c* and *VvPR10.1*). Different patterns of defense gene expression between two varieties of *Vitis vinifera* showed that response is induced in the presence of extracellular compounds and toxins (Ramírez –Suero *et al*., 2012).

Abou-Mansour *et al*. (2015) have provided evidence for the highly conserved genes in *N. parvum* in grapevine that are responsible for the synthesis of secondary metabolites. Secondary metabolites belonging to dihydrotoluquinones, epoxylactone, dihydroisocoumarins and hydroxybenzoic acid chemical families have been isolated using High performance liquid chromatography with Diode-Array Detection (HPLC-DAD) analysis. Despite the known phytotoxicity shown previously by Djoukeng *et al*. (2009) and Evidente *et al*. (2010), compounds isolated for the first time in this study ((-)-R-3-hydroxymellin and (-)-terremutin) induced highest level of necrosis. Magnin-Robert *et al*. (2011) showed that defense related gene expression is affected and detected at initial stages of infection, and is amplified during symptom expression in *Vitis*. Abou-Mansour *et al*. (2015) elaborated phytotoxic effects of the newly found (-)-terremutin on gene expression. They showed that expression patterns of several defense related genes were involved in cellular detoxification, including the jasmonic acid pathway, synthesis of secondary metabolites of phenylpropanoid pathway, phytoalexin production, flavonoid synthesis and pathogenesis related proteins, similar to the results of Ramírez-Suero *et al*. (2014). The phytotoxic effects were time-dependent. This provided evidence for the presence of toxins in grapevine wood from plants showing *Botryosphaeria* dieback symptoms. Further studies are needed for better understanding of the roles of extra cellular compounds in disease symptoms expression, their toxicity against other life forms and their biological functions.

Although these phytotoxic metabolites have been chemically characterized, their modes of action and their molecular targets remain mostly to be elucidated. However, Andolfi *et al*. (2011) hypothesized that these metabolites initially act on the wood where they are produced, causing tissue darkening, necrosis of parenchyma cells surrounding the vessels, excretion of dark gum into the vessels and the blockage of the xylem vessels, since the symptoms they produced are localized and mostly on leaves. It has also been suggested that the main effect of EPSs is to plug the xylem vessels, but also to have direct effects on chloroplasts. Andolfi *et al*. (2011) also stated that toxic levels and rate of increment of these toxin levels in leaves was responsible for variation of leaf symptoms over time and space.

Morales-Cruz *et al*. (2015) annotated a predicted proteome of *N. parvum* and *D. seriata* using integrated RNA-Seq, comparative and *ab initio* approaches, with the focus on key functions associated with pathogenesis and virulence such as wood degradation, nutrient uptake and toxin production. This study demonstrated the effectiveness of integrating transcriptome sequencing with comparative and *ab initio* approaches for gene prediction. Carbohydrate active enzymes (CAZymes) are proteins involved in the degradation of plant cell walls during colonization by pathogens. Their annotation and prediction of protein secretion, has been used extensively to identify and classify cell wall degrading enzymes of plant pathogens (Suzuki *et al*., 2012; Blanco-Ulate *et al*., 2014; Jones *et al*., 2014).

Morales-Cruz *et al*. (2015) showed that *D. seriata* and *N. parvum* (*GH5*, *GH3*, and *GH43*) carry the greatest number of Glycoside hydrolases (GHs) in their genomes. These genes produce enzymes such as xyloglucan transglucosylase/hydrolases (*GH16*), endo-ß-1,4-cellulases (*GH5*) and ß-xylosidases (*GH3*, and *GH43*), which are responsible for the degradation of cellulose and hemicellulose in plant cell walls (Floudas *et al*., 2012; Blanco-Ulate *et al*., 2014). In order to deconstruct lignocellulosic material, auxiliary activity (AA) CAZymes are required in conjugation with other enzymes with redox activity. The *N. par-*

vum genome was enriched with *AA3* genes, and *AA1* genes were also abundant in *N. parvum* (12 genes) and *D. seriata* (9 genes) genomes. These AA genes produce enzymes that catalyze oxidative processes involved in degradation of plant cell wall components. *AA3*s are mainly involved in the degradation of cellulose, hemicellulose and lignin (Kremer and Wood, 1992). CAZyme family genes were enriched in *N. parvum* genomes. As pathogens of woody plants, presence of these genes in their genomes is important. The genome of *N. parvum* consists of highly variable genes. This explains the capability of the fungus to more rapidly colonize grapevine wood than other pathogens (Travadon *et al*., 2013).

According to the results of Morales-Cruz *et al*. (2015) genomes of *D. seriata* and *N. parvum* also have a range of genes involved in the secondary metabolites. P450s is a family of genes responsible for variety of functions such as synthesis of essential membrane lipids and secondary metabolites. Morales-Cruz *et al*. (2015) revealed different classes of P450 genes (*CYP65*s, *CYP531*s and *CYP58*s) abundant in Ascomycete trunk pathogens including *N. parvum* and *D. seriata*. In addition to these genes, cellular transporters are responsible for transport of compounds involved in pathogenesis, and in protection against these secondary metabolites during pathogenesis (Choquer *et al*., 2007). Genomes of all the trunk pathogens were abundant with the greatest number of genes involved in primary active transporters such as MgtE (TCDB code 1.A.26.1.1) involved in the transport of Mg^{2+} and Co^{2+} and Major Facilitator Super family (MFS, TCBD code 2.A.1.14.11). Fungal chromosomes often have physical clusters of genes involved in the same secondary metabolic pathways (Brakhage, 2012). These gene clusters each consist of a central biosynthetic gene together with genes encoding transporters, and are involved in post-synthesis modification of metabolites such as cytochrome P450s and dehydrogenases (Brakhage, 2012). According to Morales-Cruz *et al*. (2015), the *D. seriata* genome consists of 142 genes related to secondary metabolism. However, *N. parvum* together with *Eutypa lata* (Pers.) Tul. & C. Tul. and *Diaporthe ampelina* (Berk. & M.A. Curtis) R.R. Gomes, Glienke & Crous have the greatest number of genes related to secondary metabolism. The study of Morales-Cruz *et al*. (2015) suggests that the differences in gene counts of these secondary metabolite clusters between *N. parvum* and *D. seriata* reveal their difference of colonization rates of wood (Amponsah *et al*., 2011), and the highest rate of wood necrosis caused by *N. parvum* (Bénard-Gellon *et al*., 2014).

Morales-Cruz *et al*. (2015) also revealed 13 functional gene categories over-represented in the genomes of trunk pathogens. These categories include genes involved with cell wall degradation (CAZymes), secondary metabolism, protein catabolism, oxidative processes and cellular defense (transporters and P450s). According to principal component analyses conducted in this study, *N. parvum* showed a distinct expansion of AA3 CAZymes and 49 genes encoding secondary metabolic functions.

The protein coding genes presented in the studies discussed above provide basic resources for molecular studies involved in *in planta* expression profiling, targeted knock-out mutations, over expression and gene tagging. Whole genome sequencing and reverse genetic approaches will provide information to understand pathogenicity mechanisms underlying the different diseases. This will enable the development of more accurate diagnostic tools and novel effective control strategies.

Genomics

Understanding the mechanisms related to the pathogenicity of Botryosphaeriaceae fungi have been prevented by the lack of genome sequence information in addition to variability in virulence among the isolates (van Niekerk *et al*., 2004; Úrbez-Torres and Gubler, 2009). Deciphering these genomes allows greater understanding of pathogenesis and their pathogen survival.

Blanco-Ulate *et al.* (2013) presented the draft genome of *N. parvum*, providing the first insight in to the complex set of putative virulence factors using the Core Eukaryotic Genes Mapping Approach (CEGMA). As indicated in Table 3, the draft genome of 42.5Mb consists of 63 million paired reads which were assembled into 1877 contigs. Among validated protein coding genes, 1,097 were identified as potentially secreted, including several enzymes responsible for the colonization of host tissues which includes cutinases, polysaccharide lyases and glycoside hydrolases. In addition, lignin peroxidases and cytochrowme P450 monooxygenases have been annotated, with their functions related to lignin degradation (Hammel and Cullen, 2008).

In an effort to gain a glimpse into the molecular basis of pathogenesis, the genome of highly destruc-

Table 3. Features of assembled genomes in Botryosphariaceae pathogens.

Species name	Assembly size (Mb)	Gene space completeness	Scaffolds	Validated protein coding genes	Reference
M. phaseolina	49.29	92.83 %	94	9934	Islam *et al*., 2012
N. parvum	42.5	97.20 %	1287	10470	Blanco-Ulate *et al.* 2013
B. dothidea	43.1	-	-	13571	Zhang *et al*., 2013
L. theobromae	43.1	-	-	13013	Zhang *et al*., 2013
N. parvum	41.7	-	-	13339	Zhang *et al*., 2013
D. seriata	37.1	96.0 %	695	9398	Morales-Cruz *et al*. 2015

tive *M. phaseolina* has been sequenced using a shot gun approach. Raw sequences of size 6.92 Gb were assembled and mapped to the reference sequence, resulting in 15 super-scaffolds with significantly distinct genome coverage (Table 3), compared to other Ascomycete fungi. Protein coding genes validated by transcriptome analysis include secreted peroxidases, hydrolytic enzymes, oxidases, polysaccharides, lignocelluloses, several glycosidases, membrane transporters, transposases and several secondary metabolites involved in cell wall degradation and host tissue penetration. The genome of *M. phaseolina* was recorded to have fewest proteases and the greatest number of backbone genes. In addition the genome encodes the greatest number of distinct carbohydrate esterases that have been recorded in any fungal genome. Microarray analysis revealed the adaptability of *M. phaseolina* to a wide range of osmotic and pH environments, specifically the robustness of its pH sensing system shows its extraordinary capacity to live in adverse conditions. As a plurivorous pathogen, *M. phaseolina* encodes many pathogen-host interaction genes for cell wall breakdown, adhesion, purine biosynthesis, potent mycotoxin patulin and signal transduction, which together account for its pathogenic life style. The genome of *M. phaseolina* consists of a diverse set of enzymes and toxins that are required for infection and to overcome host plant defence. Further understanding of these plant-pathogen interactions will be influential in designing strategies for disease control and to ensure crop production and security globally (Islam *et al*., 2012).

Our research group has sequenced the genomes of *L. theobromae*, *B. dothidea* and *N. parvum* using next-generation sequencing approaches. Details of those assemblies are given in Table 3 (Zhang *et al*., 2013). The genome size of *N. parvum* was similar to that shown in the parallel study conducted by Blanco-Ulate *et al.* (2013), although more of the genes were predicted in Zhang *et al*. (2013).

Morales-Cruz *et al*. (2015) presented whole genomes of three grapevine trunk pathogens using shotgun sequencing approach and CEGMA technique similar to Blanco-Ulate *et al.* (2013). Genome assembly statistics are given in Table 3. Compared to the estimated genome based on DNA k-mer distributions, shotgun sequencing resulted in a genome with 96% gene space completeness. CEGMA and tRNA analyses confirmed this higher degree of completeness. According to the *ab initio* gene discovery approach, validated protein coding genes includes 910 secreted proteins. All of the predicted genes were evenly distributed among the scaffolds without forming any gene-rich islands on the genome. Among the identified proteins, there were proteins associated with plant cell wall degradation and wood colonization (335 annotated genes for carbohydrate-active enzymes, 53 annotated genes for peroxidases, 122 annotated genes for cytochrome 450s), cellular transporters (1345 annotated genes), and proteins associated with secondary metabolism including toxin production (142 annotated genes).

Next generation sequencing techniques can be used to further improve the assembled genomes and draft genomes of species in Botryosphaeriaceae. Lack of whole genome data of botryosphaeriaceous fungi affects other molecular studies such as identification of the variety of molecules, mainly proteins, responsible for fungal-plant interaction. Identification of these molecules is important to provide un-

derstanding of host-pathogen interactions and to design strategies to alter the balance of these interactions to decrease pathogenicity. Hence much effort must go into whole genome sequencing of botryosphaeriaceous fungi which will enhance the fungal annotated databases important for many studies.

Conclusions

Over the last decade (to 2016) there have been a significant number of publications reporting molecular studies on species in Botryosphaeriaceae, focusing particularly on identification and diversity. However, there have been fewer publications related to molecular aspects such as pathogenicity. With the development of next generation sequencing techniques and the availability of whole genome sequences, many molecular studies have now been focusing on pathogenicity. Significant progress towards a unified phylogenetic system has been achieved, for which further advances were obtained by multi-gene phylogenies that use protein coding, non-coding single-copy and multi-copy gene regions and phylogenomic analyses. Development of genetic markers provides a means to create genetic maps, and also provides tools to assess population diversity and the evolution of lineages.

This review has indicated that analyses based on microsatellites are a popular tool for the identification of poly- and mono-morphic loci that can be used to monitor the genetic diversity in botryosphaeriaceous taxa. Use of next generation sequencing techniques in whole genome sequencing and annotation of predicted genes will provide necessary molecular resources for genetic diversity and association studies. Diversity studies provide information on distribution, virulence, and genetic structure of pathogen populations. The elucidation of pathogen life cycles and their genome sequences are significant steps towards understanding virulence.

Recent advances exploring the genetic basis of pathogenicity have been achieved through molecular approaches, including whole genome re-sequencing, integrated RNA-Seq, genome-wide insertional mutagenesis, development of expressed sequence tags, transposon tagging and DNA microarray techniques, amongst others. These have led to the discovery of pathogenicity genes. Identification of these genes has attracted a large amount of research interest in recent years. Several newly identified genes related to pathogenicity with no homologous genes available in the existing gene databases reveal new aspects of the plant-pathogen interactions. Genomic analyses have been shown to be a promising approach to explore the mechanisms underlying the host-pathogen interactions.

The introduction of next generation sequencing approaches will enhance the results by enabling new strategies to discover regulatory and evolutionary processes. Techniques such as RNA-seq, shotgun sequencing and mapping of core eukaryotic genes provide unlimited access to the transcriptomes of pathogens, thus enhancing genome annotations and identification of new genes. Since very few studies have applied these techniques to botryosphaeriaceous fungi, new studies can be designed to analyze pathogens under different environmental conditions and to discover new virulence genes and the roles they play in pathogenicity. Dual RNA-Seq approaches have been applied successfully in other fungi enabling the simultaneous analysis of the host and pathogen transcriptomes providing further insights, which cannot be obtained from individual sequencing. These techniques can be applied in future research on the Botryosphaeriaceae.

Continued research on pathogen surveillance coupled with knowledge of pathogen biology, genetic variability, adaptability, their host associations and pathogen genomics is important for the development of novel and durable strategies to manage these pathogens and the devastating diseases they cause. Genetic diversity/variability allows pathogens to tolerate environmental stresses and leads to different survival modes within a population by adapting to many lifestyles. These factors help to determine what type of control strategies are suitable for a particular pathogen and the suitable period for the application of the treatment. Botryosphaeriaceous species infect a wide range of hosts with various levels of virulence. Diversity observed in the pathogenicity of these species, their ability to survive in different environmental conditions and their amenability to in-depth genome-wide analyses, suggest the suitability of these species to be used as model organisms to analyze genetic diversity and virulence in pathogenic fungi.

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