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Phylogenetic analyses of *RPB1* and *RPB2* support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria

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ABSTRACT

Fusarium (Hypocreales, Nectriaceae) is one of the most economically important and systematically challenging groups of mycotoxigenic phytopathogens and emergent human pathogens. We conducted maximum likelihood (ML), maximum parsimony (MP) and Bayesian (B) analyses on partial DNA-directed RNA polymerase II largest (*RPB1*) and second largest subunit (*RPB2*) nucleotide sequences of 93 fusaria to infer the first comprehensive and well-supported phylogenetic hypothesis of evolutionary relationships within the genus and 20 of its near relatives. Our analyses revealed that *Cylindrocarpon* formed a basal monophyletic sister to a 'terminal *Fusarium* clade' (TFC) comprising 20 strongly supported species complexes and nine monotypic lineages, which we provisionally recognize as *Fusarium* (hypothesis F1). The basal-most divergences within the TFC were only significantly supported by Bayesian posterior probabilities (B-PP 0.99–1). An internode of the remaining TFC, however, was strongly supported by MP and ML bootstrapping and B-PP (hypothesis F2). Analysis of seven *Fusarium* genome sequences and Southern analysis of fusaria elucidated the distribution of genes required for synthesis of 26 families of secondary metabolites within the phylogenetic framework. Diversification time estimates date the origin of the TFC to the middle Cretaceous 91.3 million years ago. We also dated the origin of several agriculturally important secondary metabolites as well as the lineage responsible for Fusarium head blight of cereals. Dating of several plant-associated species complexes suggests their evolution may have been driven by angiosperm diversification during the Miocene. Our results support two competing hypotheses for the circumscription of *Fusarium* and provide a framework for future comparative phylogenetic and genomic analyses of this agronomically and medically important genus.

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1. Introduction

Fusarium species rank among the most economically destructive plant pathogens and mycotoxigenic fungi, posing a threat to plant and animal health and food safety. Notable plant diseases include Fusarium head blight (FHB) or scab of cereals (O'Donnell et al., 2000; Cuomo et al., 2007), sudden death syndrome (SDS) of soybeans (Aoki et al., 2005), ear rot of maize (Desjardins et al., 2002), root rot of pea (Coleman et al., 2009), and vascular wilts of scores of economically important crops (O'Donnell et al., 1998b; Skovgaard et al., 2001; van der Does et al., 2008). Fusarium-induced losses to crop yield and quality, as well as contamination with mycotoxins, are responsible for multi-billion US dollar losses to world agriculture annually (Wu, 2007). In addition, fusaria are responsible for keratitis (Chang et al., 2006) and finger and toenail infections in immunocompetent humans, as well as life-threatening infections in humans with chronically low levels of white blood cells (Sutton and Brandt, 2011).

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Most phylogenetic studies conducted within the genus have focused on resolving evolutionary relationships at the species level within clades of agriculturally and medically important fusaria (GCPSR; Taylor et al., 2000; O'Donnell et al., 2010 and references therein). In the most comprehensive phylogenetic assessment of the genus to date, Gräfenhan et al. (2011) analyzed a two-locus data set from 43 fusaria and 50 hypocrealean near relatives. They discovered that 17 of the fusaria were nested within basal lineages comprising non-fusaria, strongly indicating that *Fusarium*, as traditionally defined (Gerlach and Nirenberg, 1982), is polyphyletic. Although the remaining 26 fusaria included in their study formed eight strongly supported lineages, designated the 'terminal *Fusarium* clade' (TFC), support for this lineage was poor and evolutionary relationships within it were unresolved. At least seven teleomorph genera are connected taxonomically to the TFC (Geiser et al., 2013); however, these sexual states are rarely encountered by applied biologists working on fusarial diseases and toxins. In revising teleomorph genera within the TFC, and assigning the name *Fusarium* for unitary use to replace only one of them, Gräfenhan et al. (2011) and Schroers et al. (2011) set up an inevitable splitting of the TFC into at least nine genera, despite the fact that almost all of the species in the TFC produce *Fusarium* anamorphs, which historically are the principal form by which these organisms are recognized and reported.

Given this background, we conducted the most comprehensive phylogenetic assessment of *Fusarium* to date using portions of the DNA-directed RNA polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits, which are noted for their informativeness in analyses of diverse fungi (Schoch et al., 2009), including *Fusarium* (O'Donnell et al., 2010). Our goals were to (i) infer evolutionary relationships within the TFC to determine whether it is monophyletic, (ii) assess how well the traditional morphology-based subgeneric sectional classification corresponds to the molecular phylogeny, and (iii) construct the first time scale for the evolutionary origin and diversification of fusaria. Herein *Fusarium* is defined phylogenetically as a genealogically exclusive clade that is synonymous with the 'terminal *Fusarium* clade' (TFC sensu Gräfenhan et al., 2011). Thus, all of the species within the TFC are considered to be fusaria, irrespective of whether they produce a *Fusarium*-like anamorph. Given the economic importance of *Fusarium* and its toxins to world agriculture and food safety, the well-supported evolutionary framework developed in the present study should help guide future comparative phylogenetic and genomic studies on this genus.

2. Materials and methods

2.1. Taxon sampling and molecular phylogenetics

The 113 isolates included in this study (Supplementary Table S1) were chosen to represent the known morphological (Gerlach and Nirenberg, 1982) and phylogenetic diversity of *Fusarium* (O'Donnell et al., 2010; Gräfenhan et al., 2011). DNA extraction, PCR amplification and DNA sequencing followed published protocols (O'Donnell et al., 2010). Based on the results of model tests (Posada, 2008), the GTR + Γ + I default model of molecular evolution was selected for the ML-BS analyses, which were run with GARLI ver. 1.0 (Zwickl, 2006) on the CIPRES Science Gateway site (http://www.phylo.org/portal2/login). Clade support (Table 1) was assessed by: (i) nonparametric ML-BS using GARLI ver. 1.0 on CIPRES, (ii) maximum parsimony bootstrapping (MP-BS) in PAUP* ver. 4.0b10 (Swofford, 2003), employing 1000 pseudoreplicates of the data, 10 random taxon-addition sequences per replicate, TBR branch swapping, and MAXTREES set to automatically increase by 100, and (iii) Bayesian posterior probabilities using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on the University of Oslo Bioportal (https://www.bioportal.uio.no/appinfo/show.php?app=mrbayes). Two Bayesian analyses of four chains were run for 5×10^7 generations, sampling trees every 100 generations. Inspection of t files generated from the analyses indicated chains had reached stationarity within the first quarter of each run so 12,500 trees from each run were discarded as the burn-in sample. To obtain posterior probabilities (PPs), the 37,500 trees from each run were combined into a single tree file that was imported into PAUP to obtain 85% and 95% majority-rule consensuses.

DNA sequence data generated in this study have been deposited in GenBank (accession numbers JX171444–JX171669) and the concatenated two-locus alignment was deposited in TreeBASE (accession number S12813, Tree number Tr56612). To promote DNA sequence-based identification through web-based tools, all of the data reported in this study have been incorporated into *Fusarium*-ID (Geiser et al., 2004; Park et al., 2010) and *Fusarium MLST* (O'Donnell et al., 2010).

2.2. Secondary metabolites

The presence of secondary metabolite biosynthetic genes was assessed using three methods: (i) BLAST analysis of published genome sequences of *Fusarium graminearum* (Cuomo et al., 2007), *Fusarium pseudograminearum* (Gardiner et al., 2012), *Fusarium oxysporum*, *Fusarium verticillioides* (Ma et al., 2010) and '*Fusarium solani*' (Coleman et al., 2009) and unpublished genome sequences of *Fusarium avenaceum* and *Fusarium langsethiae* (Frandsen and Lysøe, unpubl. results) (Table 2); (ii) SOUTHERN blot analysis of selected polyketide synthase (PKS) genes of multiple isolates representing seven species complexes (Supplementary Table S3); and (iii) reports in the literature for which rigorous methods were used to determine species identities, e.g., comparisons of DNA sequences of unknown strains to those of previously validated strains. Because the correct species name for the isolate of '*F. solani*' ('*Nectria*' *haematococca* mating population VI) used for genome sequencing is unknown, it is listed with single quotation marks.

Table 1
Tree statistics and summary sequence for individual and combined partitions (see Figs. 1 and 2).

Locus	# Characters	# MPTs[a]	MPT length	CI[b]	RI[c]	UIC[d]	PIC[e]	PIC/bp[f]	# Nodes supported [g]		
									MP-BS	ML-BS	PP
RPB1	1606	8	8747	0.19	0.68	50	827	0.51	85	87	91
RPB2	1777	6	8913	0.18	0.67	44	802	0.45	78	80	89
Combined	3383	4	17,738	0.18	0.67	94	1629	0.48	94	99	104

[a] MPTs, most-parsimonious or shortest trees.
[b] CI, consistency index.
[c] RI, retention index.
[d] UIC, parsimony-uniformative, autapomorphic, or uniquely derived character.
[e] PIC, parsimony-informative, synapomorphic or shared derived character.
[f] PIC/bp, parsimony-informative characters/base pair.
[g] Number of nodes supported by maximum parsimony bootstrapping (MP-BS), maximum likelihood bootstrapping (ML-BS) and Bayesian posterior probability (PP).