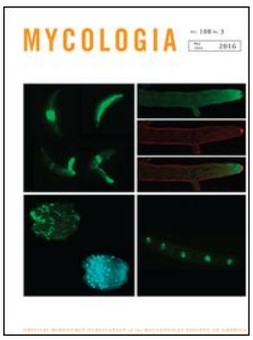

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Epichloë hybrida, sp. nov., an emerging model system for investigating fungal allopolyploidy

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ABSTRACT

Endophytes of the genus *Epichloë* (Clavicipitaceae, Ascomycota) frequently occur within cool-season grasses and form interactions with their hosts that range from mutualistic to antagonistic. Many *Epichloë* species have arisen via interspecific hybridization, resulting in species with two or three subgenomes that retain all or nearly all of their original parental genomes, a process termed allopolyploidization. Here, we characterize *Epichloë hybrida*, sp. nov., a mutualistic species that has increasingly become a model system for investigating allopolyploidy in fungi. The *Epichloë* species so far identified as the closest known relatives of the two progenitors of *E. hybrida* are *E. festucae* var. *lolii* and *E. typhina*. We confirm that the nuclear genome of *E. hybrida* contains two homeologs of most protein-coding genes from *E. festucae* and *E. typhina*, with genome-wide gene expression analysis indicating a slight bias in overall gene expression from the *E. typhina* subgenome. Mitochondrial DNA is detectable only from *E. festucae*, whereas ribosomal DNA is detectable only from *E. typhina*. Inheriting ribosomal DNA from just one parent might be expected to preferentially favor interactions with ribosomal proteins from the same parent, but we find that ribosomal protein genes from both parental subgenomes are nearly all expressed equally in *E. hybrida*. Finally, we provide a comprehensive set of resources for this model system that are intended to facilitate further study of fungal hybridization by other researchers.

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KEYWORDS
Endophyte; hybridization; mtDNA; rDNA; transcriptome analysis

INTRODUCTION

Filamentous fungi of the genus *Epichloë*, many formerly classified in the anamorph genus *Neotyphodium* (Leuchtmann et al. 2014), are widespread and largely asymptomatic symbionts of cool-season grasses (Pooideae) (Clay 1990; Schardl 2010). *Epichloë* species form extensive hyphal networks throughout the intercellular spaces of the aerial tissues of their grass hosts, with dispersal via the seed being the dominant mode of transmission. For some species, horizontal transmission can also occur via a sexual stromatal stage, causing a phenomenon known as 'choke.' Choked and asymptomatic tillers can be produced on the same host plant, thus enabling both vertical and horizontal modes of transmission (Leuchtmann et al. 1994; Leuchtmann and Schardl 1998; Schardl and Leuchtmann 1999). This ability to have pathogenic and asymptomatic growth means that the interactions of *Epichloë* species with their hosts can be mutualistic or antagonistic, with species falling on a continuum between these two extremes (Schardl et al. 2014). The mutualistic interactions stem from benefits that *Epichloë* species provide to their hosts, which at least in part derive from the fungus producing a variety of secondary metabolites, including compounds toxic to insects or grazing herbivores (Johnson et al. 2013).

A key feature of the genus *Epichloë* is the frequent formation of interspecific, typically asexual, allopolyploid species that are often referred to as hybrids (Tsai et al. 1994; Schardl and Wilkinson 2000; Moon et al. 2004, 2007). Allopolyploidy, the process of forming new species by the merging of different parent species, is widespread across the eukaryotic tree of life (e.g., Otto and Whitton 2000; Wood et al. 2009; Albertin and Marullo 2012; Wendel 2015) but has not been widely described in fungi

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(Campbell et al. 2016). To date, *Epichloë* has the most known interspecific hybrids of any fungal genus (Schardl and Craven 2003; Moon et al. 2004, 2007; Gentile et al. 2005; Schardl 2010; Leutchmann et al. 2014; Campbell et al. 2016; Shymanovich et al. 2017). Haploid, sexual *Epichloë* species typically carry only one copy of a gene at a given locus, but interspecific hybrids were originally identified by the presence of two or three homeologs, thus indicating their origin from two or more parent species (Schardl et al. 1994; Tsai et al. 1994; Moon et al. 2004). The genome sizes of these interspecific hybrids are also typically increased, often being double or triple those of haploid lineages (Kuldau et al. 1999). The prevalence of allopolyploidy in this endophyte group may be a way to alleviate the deleterious consequences of Muller's ratchet in asexual species and may be facilitated by the absence of a vegetative incompatibility system (Chung and Schardl 1997; Selosse and Schardl 2007). Furthermore, asexual propagation (vertical transmission) is theorized to be selected for in mutualistic relationships (Ewald 1987). Two or more subgenomes can often produce a more diverse range of alkaloids than one haploid genome as well, thus potentially increasing host fitness (Schardl et al. 2013b).

One of the first interspecific hybrids identified in the genus *Epichloë* is a strain commonly known in the literature as Lp1 (Schardl et al. 1994). Lp1 is an allodiploid originating from the hybridization of two haploid parent species, *E. festucae* var. *lolii* and *E. typhina*, and full genomes appear to be retained from both parent species (Cox et al. 2014). Although the date when the hybridization event occurred is unknown, genetic work has put an upper bound of approximately 300 000 years (Cox et al. 2014). On the basis of isozyme analysis, Lp1 was assigned early on to a larger taxonomic group called LpTG-2 (*Lolium perenne* taxonomic group 2) (Christensen et al. 1993), and the new species definition provided in this report likely applies to all other LpTG-2 strains, such as those listed by Van Zijll de Jong et al. (2008). Although Lp1 has been used as an agricultural strain, commercial releases have been limited (Barker et al. 1997; Kerr et al. 2012). It has, however, become a model system for exploring the outcomes of interspecific hybridization in filamentous fungi, including genomic (Ganley and Scott 1998) and transcriptomic (Cox et al. 2014) responses.

Despite these agricultural and scientific applications, no taxonomic description of Lp1 has been published. Here, we formally describe Lp1 as *Epichloë hybrida*, and we outline the key morphological and molecular resources that make this organism an ideal model system for studying interspecific fungal hybridization.

HISTORY OF *EPICHLOË HYBRIDA*

E. hybrida Lp1 was isolated from perennial ryegrass (*L. perenne* L.) seeds collected as part of a national breeding program funded by the French Ministry of Agriculture in the early 1980s. Run by Gilles Charmet and François Balfourier at the National Institute of Agricultural Research (INRA) in Clermont-Ferrand, 547 natural ryegrass populations were sampled across France by INRA researchers and a national team of 10 private breeders. A core collection of 110 accessions, including the *E. hybrida* source population, was transferred to INRA Lusignan in 2000, where it now forms the basis of the modern French perennial ryegrass breeding program.

Accession F187 was collected in the summer of 1983 by Joël Meunier, an employee of the French breeding company Maïsadour, and at present is entered into the INRA collection under accession number 010204. The collection site was a natural meadow near the village of Bidache in the French administrative department of Pyrénées Atlantiques in southwestern France (SUPPLEMENTARY FIG. 1). Less than 30 km from the Atlantic coast and under 100 m in altitude, this area has hot summers and mild winters and is very wet with substantial rainfall throughout the year.

In the late 1980s, the INRA collection was shared with Sydney Easton, a plant breeder in what was then the New Zealand Department of Scientific and Industrial Research (DSIR), and this New Zealand branch of the collection is now housed in the Margot Forde Germplasm Centre at the AgResearch Crown Research Institute. Accession F187 was recoded as accession A6056 at that time.

An endophyte program aimed at systematically isolating agriculturally promising *Epichloë* strains from this and other ryegrass seed collections was undertaken by Garrick Latch in the late 1980s/early 1990s. A ryegrass seedling from the INRA seed lot F187 accession was recognized as having a desirable fungal chemotype, in which the animal neurotoxin lolitrem B was not produced, but the desirable insect feeding deterrent peramine was present. An *Epichloë* strain isolated from this accession was used to inoculate cultivars of perennial and hybrid ryegrasses and was recorded as 187A, later known as AR5 (AR = "AgResearch").

Later examination of additional seedlings from the same F187 seed collection identified another *Epichloë* strain that was easier to culture and inoculate, but with apparently the same chemotype. Originally called 187BB, *E. hybrida* Lp1 was, in 1989, the 28th isolate obtained (under the labeling

system: A, B, . . ., Y, Z, AA, BB, . . .). Although not formally named, the strain was placed within a group of *Epichloë* strains known as *Lolium perenne* Taxonomic Group 2, LpTG-2 (Christensen et al. 1993), all of which are expected to fall under this new species definition. The identifier "Lp1" was assigned by Michael Christensen (Lp = "*Lolium perenne*") and is synonymous with AR6, an alternative identifier assigned later by Garrick Latch (pers. comm.; see also Christensen et al. 2012). The strain has also been referenced under the identifier e144 (Schardl et al. 2013b). Because *E. hybrida* makes several classes of secondary metabolites that deter insect feeding (Christensen et al. 1993), perennial ryegrass cultivars carrying *E. hybrida* were advanced for commercialization in 1990.

Schardl et al. (1994) identified *E. hybrida* as an interspecies hybrid between *E. festucae* var. *lolii* and *E. typhina*, observing that two genes (*tub2, pyr4*) and seven allozymes examined were duplicated in *E. hybrida*, whereas the rRNA gene repeats (rDNA) only appeared as a single sequence type. The duplicated genes were shown to have sequences most similar to *E. festucae* var. *lolii* and *E. typhina*. Additional evidence indicated that *E. hybrida* possesses only one mitochondrial sequence type that is similar to *E. festucae* var. *lolii*, in contrast to the single rDNA sequence type that is similar to *E. typhina*. Because of its similarity to the *E. festucae*–like genome in *E. hybrida*, together with its discovery in the same sample collection, the nonhybrid haploid strain AR5 has often been used as a proxy for the *E. festucae* parent (Cox et al. 2014). For the other homeologous genome within *E. hybrida*, only two strains of *E. typhina* are known to have been isolated from perennial ryegrass: the first from a golf course in Massachusetts, USA (strain E8), and the second from a batch of the perennial ryegrass cultivar Callan in France (strain E432) (Leuchtmann and Schardl 1998). Strain E8 has often been used as a proxy for the *E. typhina* parent, although E8 is genetically more distant from the true *E. typhina* parent than strain AR5 is from the true *E. festucae* var. *lolii* parent (Cox et al. 2014). *Epichloë hybrida* Lp1, together with *E. festucae* var. *lolii* AR5 and *E. typhina* E8, now constitute a key model experimental system for studying the genomic outcomes of fungal hybridization. Although likely not the immediate parents of Lp1, we refer to *E. festucae* var. *lolii* AR5 and *E. typhina* E8 as the "parental species" here, given that they are the closest known extant relatives to the true parents.

MATERIALS AND METHODS

Strain and data access.—Isolates of *E. hybrida* Lp1 (PN2197), *E. festucae* var. *lolii* AR5 (PN2225), and *E. typhina* E8 (PN2065) were provided by Garrick Latch and Michael Christensen at AgResearch, New Zealand. The type specimen of *E. hybrida* has been deposited in the American Type Culture Collection (ATCC; https://www.atcc.org; accession TSD-66). All genomic and transcriptomic data described below have been deposited in publicly accessible databases (see SUPPLEMENTARY TABLE 1 for full access details). A fragmentary genome assembly was reported by Schardl et al. (2013b).

Morphological examination.—Colony morphology was evaluated by subculturing strains onto potato dextrose (PD) agar and growing at 20 C in darkness. Colonies were photographed and submitted for scanning electron microscopy analysis after 21 d growth.

For electron microscopy, cultures were excised from agar plates and fixed in 10% (*v*/*v*) ethanol before being dehydrated stepwise in graded ethanol series up to 100%. The cultures were critical point dried using liquid CO_2 as the critical point fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus; Quorum Technologies, Laughton, UK), before being mounted onto aluminum stubs and sputter coated with approximately 100 nm of gold (BAL-TEC SCD 005 sputter coater; Leica Biosystems, Eisfeld, Germany). The mounts were viewed in a FEI Quanta 200 scanning electron microscope (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at an accelerating voltage of 20 kV.

Conidiospore staining.—*Epichloë hybrida* was subcultured and grown for 10 d on five potato dextrose (PD) agar plates. Pure sterile water (2 mL) was flooded on to each plate, and the colonies gently scrubbed with a glass spreader to collect conidiospores. The final spore suspension from all plates was filtered through a 1-mL pipette tip filled with sterile glass wool. A 5-µL aliquot of the spore suspension was placed onto a glass slide with 5 µL of fresh fluorescent probe 4′,6-diamidino-2-phenylindole (DAPI; Sigma Corporation, Kawasaki, Japan) at a final concentration of 0.025 mg/mL.

Imaging of single nuclei was carried out using a Leica DM6000B SP5 confocal laser scanning microscope system running LAS AF 2.7.3.9723 software (Leica Microsystems, Wetzlar, Germany). Images were acquired with a HCX PL APO 40× (numerical aperture [N.A.] = 1.25) lens with an optical zoom of 2.3×. The fluorescent probe DAPI was imaged through excitation at 405 nm and emission collection at 415–500 nm. Differential interference contrast (DIC) was imaged

simultaneously at 488 nm. Z-series images were collected with a step size of 1.60 µm. Image analysis was undertaken using ImageJ 1.47 with the LOCI Bio-Formats Importer plugin.

DNA isolation, sequencing, and assembly.—DNA isolation was performed using a phenol-chloroform DNA extraction, as described previously (Byrd et al. 1990), with strain-specific barcodes added for sequencing. Whole-genome shotgun sequences were generated from a library pooled in a 2:1:1 ratio to evenly represent the *E. hybrida* Lp1:*E. festucae* var. *lolii* AR5:*E. typhina* E8 genome sizes. Sequencing was performed on a single Illumina MiSeq lane (Illumina, San Diego, California, USA), to minimize technical variance, with 300 nucleotide paired-end reads obtained using version 3 chemistry. The quality of demultiplexed reads was assessed and low-quality bases removed with SolexaQA++ 3.1.3 (Cox et al. 2010). Subsequently, adapter contamination was removed with Trimmomatic 0.33 (Bolger et al. 2014). To increase assembly success of high-copy-number rDNA and mitochondrial DNA (mtDNA) sequences, cleaned DNA sequence data were normalized with BBNorm, part of the BBMap 34 bioinformatic tools (Bushnell 2015). To obtain complete rDNA and mitochondrial genome sequences, assembly was performed for each organism separately across a range of *k*mer values with Velvet 1.2.10 (Zerbino and Birney 2008).

DNA sequence analysis.—The mitochondrial and rDNA contigs were identified from the assemblies with BLASTn 2.3.0 (Altschul et al. 1990) using a complete *Metarhizium anisopliae* mitochondrial genome sequence (GenBank accession AY884128) and a partial *E. typhina* rDNA sequence (accession AB105953) as references.

Mitochondrial assemblies were checked for circularity by identifying an overlapping segment at the start and end of the contigs that matched the *k*mer length of the assembly. The mitochondrial genomes were annotated with the MFannot Web server (http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl; accessed September 2015). Open reading frames detected but not annotated by MFannot were identified with Blast2GO 3.0.10 (Conesa and Götz 2008) using a comparison with the National Center for Biotechnology Information (NCBI) protein reference database and an *E* value $<1 \times 10^{-3}$ threshold. The mitochondrial genomes were aligned with Mauve 2.4.0 (snapshot 2015-02-25) (Darling et al. 2004) and the list of single-nucleotide polymorphisms (SNPs) was exported from the alignment.

The nuclear rDNA sequences were identified by BLASTn alignment to the *E. typhina* reference, and the sequences from *E. hybrida* Lp1, *E. festucae* var. *lolii* AR5, and *E. typhina* E8 were extracted from the contigs for alignment. These sequences were aligned using the Geneious 9 (Kearse et al. 2012) multiple sequence aligner with default settings followed by minor manual editing.

To obtain a comparative data set of housekeeping genes, sequences of the genes encoding γ-actin (actG), calmodulin (calM), translation elongation factor 1-α (tefA), and β-tubulin (tubB) were extracted from the University of Kentucky endophyte database (Schardl et al. 2013a; http://www.endophyte.uky.edu; accessed September 2017) by blasting the EfM3 gene model for each gene against the genomes of *Epichloë* spp. present in the database using BLASTn (Altschul et al. 1990). The sequences retrieved were then aligned with MAFFT 7.273 (Katoh and Standley 2013) using the set of parameters "linsi," and the multiple sequence alignments of each gene were manually verified to confirm the presence of the correct homolog in the BLASTn matches.

For the parental strains *E. festucae* var. *lolii* AR5 and *E. typhina* E8, sequences of the genes *actG, calM, tefA*, and *tubB* were assembled from genome sequence reads (SUPPLEMENTARY TABLE 1) using GRAbB (Brankovics et al. 2016) and dipSPAdes 1.0 (Safonova et al. 2015). These sequences were then aligned and curated manually.

For *E. hybrida*, Illumina reads from the genome sequencing (SUPPLEMENTARY TABLE 1) were aligned to the *actG, calM, tefA*, and *tubB* gene sequences from *E. festucae* var. *lolii* AR5 and *E. typhina* E8 with HISAT 2.1.0 (Kim et al. 2015). Read mapping files were analyzed manually using IGV 2.3 (Robinson et al. 2011), and for each gene, the two homeolog sequences were rebuilt manually by identifying SNPs (both presence and absence) and the linkage between them. The resulting sequences were then aligned, and all SNPs were verified to be present on the correct copy of each reconstructed gene homeolog.

The alignments of individual genes were automatically trimmed and cleaned using trimAl 1.4.rev6 (Capella-Gutiérrez et al. 2009) with the parameter "gappyout." A maximum likelihood phylogeny was reconstructed using PhyML 20160115 (Guindon et al. 2010), with the substitution model chosen using pModeltest 1.4 from ETE 3 (Huerta-Cepas et al. 2016). Approximate likelihood-ratio tests were computed as branch supports, and the tree was formatted on the interactive Tree Of Life (iTOL) Web site (Letunic and Bork 2007, 2016; https://itol.embl.de; accessed September 2017). The trees were rooted on the *E. inebrians* branch, as suggested by Chen et al. (2015). All sequences

have been deposited in GenBank: *actG* (MF928038–MF928054), *calM* (MF928055–MF928071), *tefA* (MF928021–MF928037); and *tubB* (MF928004–MF928020) (also see SUPPLEMENTARY TABLE 1); alignments are available on TreeBASE (TB2:S21646); and Newick records for each gene phylogeny are given in SUPPLEMENTARY DATA SET 1.

RNA sequencing and genome-wide gene expression analysis.—Transcriptomic sequence data were generated from RNA extractions made from endophyte-inoculated *Lolium perenne* plants for all three species (*E. hybrida* Lp1, *E. festucae* var. *lolii* AR5, and *E. typhina* E8), as described in SUPPLEMENTARY DOCUMENT 1. Reads were cleaned with SolexaQA++ 3.0 (Cox et al. 2010) and mapped to the only *Epichloë* species reference gene models available at that time, namely, *E. festucae* E2368 (http://epichloe.massey.ac.nz; accessed March 2015; Schardl et al. 2013a) with Bowtie 2 2.02 (Langmead et al. 2009; Langmead and Salzberg 2012). Resulting alignments were analyzed in the Hybrid Lineage Transcriptome Explorer (HyLiTE) 1.6.2 (Duchemin et al. 2015). The genomic DNA sequence data described above were included in the SNP calling part of this analysis, as they increase the effectiveness of later assigning RNA-seq reads to a subgenome origin. The resulting read count matrix, containing *E. festucae* and *E. typhina* orthologs from the parent species, and *E. festucae*– and *E. typhina*–derived homeologs within *E. hybrida*, was analyzed in DESeq2 1.6.3 (Love et al. 2014). Differentially expressed (DE) genes detected by DESeq2 were additionally filtered for $\log_2$ fold change with an absolute value >1 and an adjusted *P* value <0.05. To obtain ribosomal protein gene models from the *E. festucae* E2368 database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) identifier "KO0310 Ribosome" was used.

A Pearson's χ^2 test was performed in R 3.2.0 (Pearson 1900; R Core Team 2015) to test for statistical significance between biased/unbiased gene sets from the parental species (between orthologs), and from *E. hybrida* (between homeologs) for all gene models, and for the ribosomal protein gene set.

Secondary metabolite genes and alkaloid production.—Genes involved in the peramine, indole-diterpene, and ergot alkaloid pathways (Tanaka et al. 2005; Young et al. 2006; Fleetwood et al. 2007) were identified in *E. hybrida* Lp1 using the BLASTn function of Geneious 8.1.5 (Kearse et al. 2012). Alkaloid production by *E. hybrida* Lp1, *E. festucae* var. *lolii* AR5, and *E. typhina* E8 within the same perennial ryegrass background was assessed by tandem mass spectrometry (MSMS) using the methods of Rasmussen et al. (2012), in samples taken at the same time as those for the transcriptome analysis.

RESULTS

Morphological characteristics.—The colony morphology of *E. hybrida* Lp1 appears as a compact undulate form with wavy edges, differing from the smooth-edged morphology of *E. festucae* var. *lolii* AR5 and in striking contrast to the loose spiral mycelial form of *E. typhina* E8 (FIG. 1A–C). Scanning electron microscopy examination of Lp1 cultures revealed that *E. hybrida* forms a simple conidiophore structure that releases a single elliptical-shaped conidium (FIG. 1D). To confirm that *E. hybrida* is a true allopolyploid (Schardl et al. 1994), conidia were isolated and stained with DAPI (FIG. 1E). A single fluorescent nucleus was observed in all *E. hybrida* conidia examined, confirming that Lp1 is mononucleate and not simply an interspecies dikaryon.

Growth in planta is almost entirely asymptomatic. The only known exception is transitory white epiphytic growth that sometimes occurs on the lower portion of the spike during seed set and development (Christensen et al. 2012). This epiphytic growth depends on growth conditions and plant genotype, lasts no longer than a few days, and appears morphologically dissimilar from stromata ("choke"). As it only rarely affects seed development in some spikelets, it is not considered a significant issue for seed production.

***Molecular evidence confirms the allodiploid nature of* E. hybrida.**—Previous work indicated that *E. hybrida* Lp1 has two homeologs for several genes, in each case with one related to *E. typhina* and one related to *E. festucae* (Schardl et al. 1994; Collett et al. 1995). To check this attribution of *E. hybrida* origin, we performed a phylogenetic sequence analysis of four housekeeping genes, *actG, calM, tefA*, and *tubB*, in a comparative set of *Epichloë* species (Schardl et al. 2013a; http://www.endophyte.uky.edu; accessed September 2017). In all cases, the two *E. hybrida* homeologs fall separately within the *E. festucae* and *E. typhina* clades (FIG. 2). Support values for the placement of these homeologs on the gene tree are high, further suggesting correct parental attribution of *E. hybrida* homeologs to *E. festucae* and *E. typhina*.

A previous investigation of the in culture transcriptome of *E. hybrida* Lp1 found that almost all gene models that were able to be mapped from the parental species are retained as two homeologs (Cox et al. 2014). This suggests that *E. hybrida* is a true allodiploid without any

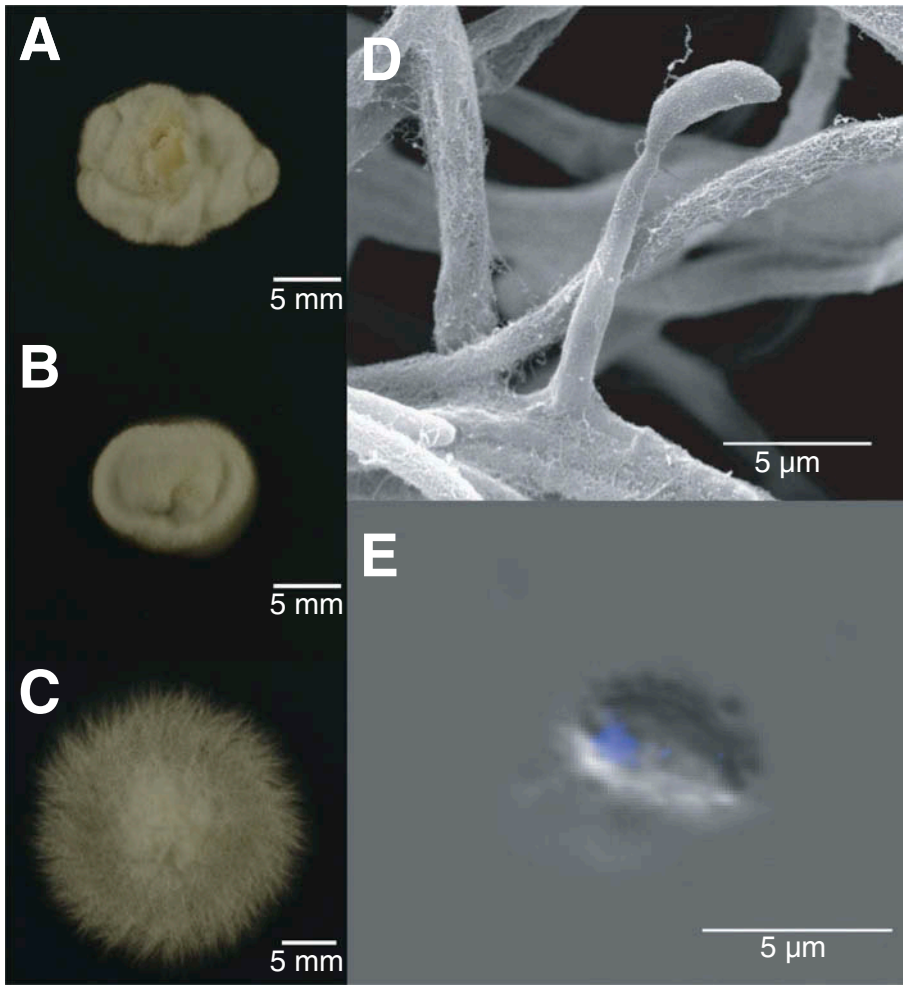

Figure 1. Colony morphology and conidial nuclear status of *Epichloë hybrida*. A–C. Plate colony morphologies. A. *E. hybrida* Lp1. B. *E. festucae* var. *lolii* AR5. C. *E. typhina* E8. D–E. Microscopy of *E. hybrida* Lp1. D. A conidiogenous cell. E. A DAPI (4′,6-diamidino-2-phenylindole)–stained conidium.

aneuploidy, and with almost no loss of homeologs. To confirm these results and to provide a resource that reflects the natural transcriptional status, we sequenced the *E. hybrida* Lp1 in planta transcriptome, alongside that of each parental species. Of the 8369 *E. festucae* E2368 gene models in our reference set, 8172 genes were detectably expressed in both *E. festucae* var. *lolii* AR5 and *E. typhina* E8. The remaining 207 gene models showed no expression in one or both of the parental species. To determine whether expression of homeologs has changed in *E. hybrida* Lp1, we performed a differential expression analysis. A HyLiTE analysis indicated that 90.5% and 88.0% are expressed by either the *E. festucae* or *E. typhina* parental subgenomes, respectively, within *E. hybrida* Lp1. We found 8108 genes (99.2%) that had detectable expression in *E. hybrida* Lp1, although only 7679 genes were included in the following differential expression analysis, as some reads lacked sufficient information to assign to parent homeologs. More gene models (n = 312) were expressed only by the *E. festucae* subgenome than only by the *E. typhina* subgenome (n = 105), potentially suggesting a suppression bias.

In *E. hybrida* Lp1, the majority of homeolog pairs (5946, 77.4%) show unbiased expression, greater than the 67.4% observed in the in culture transcriptome study (Cox et al. 2014). More genes in *E. hybrida* Lp1 showed a bias in favor of expression towards the *E. typhina*–derived homeolog (13.4%) than a bias towards the *E. festucae*–derived homeolog (9.1%) (FIG. 3). A similar bias as that observed with the parental species was also observed for *E. hybrida* Lp1 when grown in planta, with more genes showing expression bias towards *E. typhina* (16.2%) than towards *E. festucae* (11.3%) (FIG. 3). This is in contrast with the previous in culture transcriptome study, which found no bias in the parental species (Cox et al. 2014). The development of HyLiTE has provided greater statistical power to detect this bias towards *E. typhina* than earlier manual mapping methods, and this bias can now be detected in

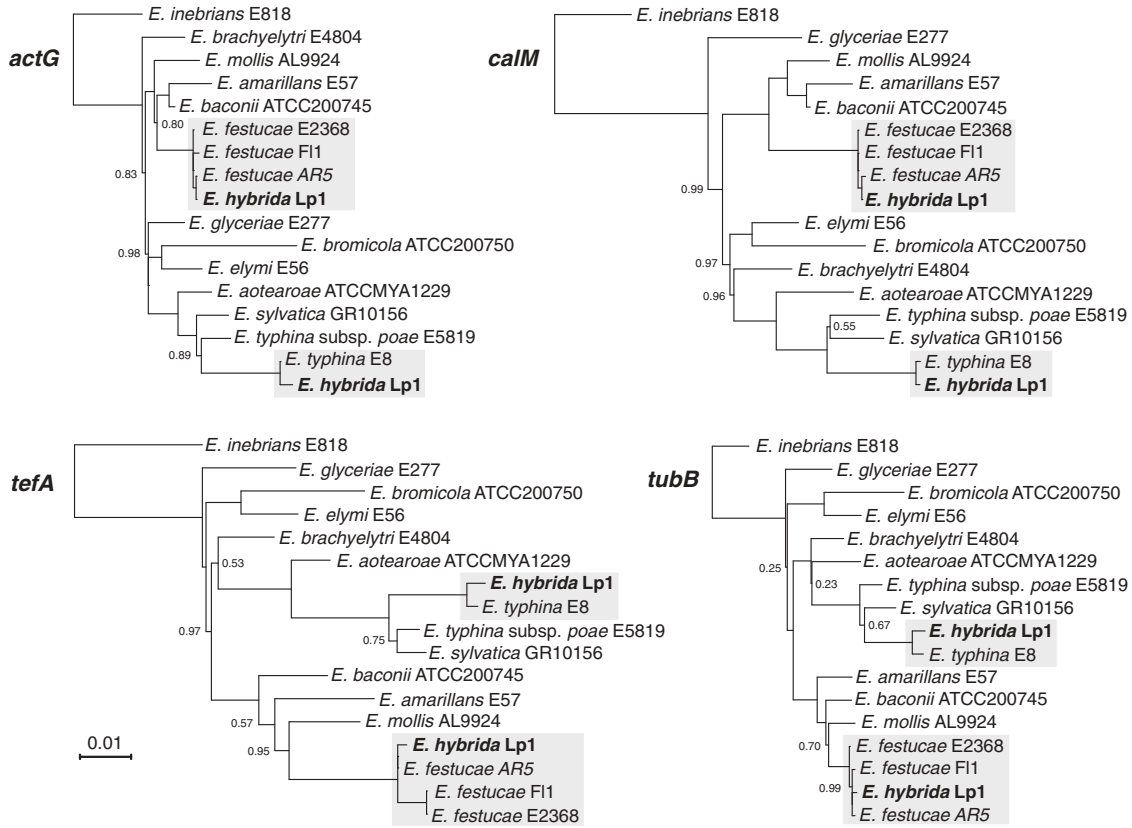

Figure 2. Phylogenetic placement of *E. hybrida* Lp1 and proxy parental species *E. festucae* var. *lolii* AR5 and *E. typhina* E8 in the *Epichloë* genus using *actG, calM, tefA*, and *tubB* gene sequences. Support values <1 are indicated at nodes, and sequence divergence is indicated by branch lengths according to the scale bar. Placements of the *E. festucae*–like and *E. typhina*–like homeologs from *E. hybrida* Lp1 are indicated in bold.

the original in culture data set as well (24.3% versus 17.3%; see Cox et al. 2014 for comparison).

Uniparental inheritance of mitochondrial and ribosomal DNA.—In contrast to most genes, the *E. hybrida* mtDNA (Schardl et al. 1994) and rDNA (Schardl et al. 1994; Ganley and Scott 1998) appear to derive from just one of the parent species. Assembly of whole-genome sequence data produced mitochondrial genomes with lengths of 88 749 bp for *E. hybrida* Lp1, 88 744 bp for *E. festucae* var. *lolii* AR5, and 84 630 bp for *E. typhina* E8. Consistent with expectations of homoplasy and previous results, the *E. hybrida* Lp1 and *E. festucae* var. *lolii* AR5 mitochondrial genomes are extremely similar, differing at just 21 of 88 752 sites (including 10 single-base gaps; FIG. 4). In contrast, the *E. hybrida* and *E. typhina* mitochondrial genomes differ at 437 nucleotide sites in the alignment, indicating that the mtDNA derives solely from the *E. festucae* parent. Annotation by MFannot predicts 83 features (protein-coding genes, tRNAs, and open reading frames) in the *E. hybrida* Lp1 and *E. festucae* var. *lolii* AR5 mitochondrial genomes, whereas *E. typhina* E8 has 79. General gene composition and order is the same in all three species, but the *E. typhina* mitochondrial genome differs from that in *E. hybrida* and *E. festucae* var. *lolii* in the number of introns in five protein-coding genes (FIG. 4).

In the case of the rDNA, it has been proposed that in *E. hybrida* Lp1 all the *E. festucae* repeats have been converted to *E. typhina* rDNA sequence (Ganley and Scott 2002). To investigate the inheritance of the rDNA further, we identified and analyzed the rDNA locus from the whole-genome assembly. Only one rDNA sequence type was obtained from *E. hybrida* Lp1, as expected (Ganley and Scott 2002). Consistent with previous reports of rDNA repeat assembly with Illumina data (Agrawal and Ganley 2016), complete assemblies of the entire rDNA repeat matching previously published lengths (Ganley and Scott 1998) were not possible. Therefore, we focused on the rRNA coding regions. The assembly produced rDNA unit sequences of 18S-ITS1-5.8S-ITS2-28S of 6014 bp from *E. hybrida* Lp1 and *E. typhina* E8, and 6493 bp from *E. festucae* var. *lolii* AR5 (FIG. 5). Most of this length polymorphism results from a putative group I intron inserted in the *E.*

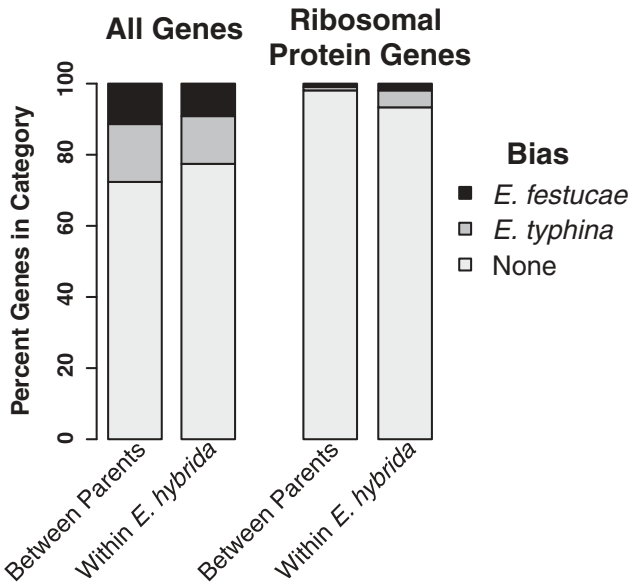

Figure 3. In planta differential expression bias. Gene expression differences between all orthologs (n = 8172 gene models), all homeologs (n = 7679), and ribosomal protein orthologs/homeologs (n = 105; KEGG KO03010). Genes were characterized as biased towards either *E. festucae* or *E. typhina* orthologs (parental species comparisons) or homeologs (*E. hybrida* comparisons), or not differentially expressed, and are plotted as the percent of all expressed genes (leftmost two columns). The same analysis for the ribosomal protein gene models is shown in the rightmost two columns.

festucae var. *lolii* AR5 rDNA homeolog (near another group I intron that is shared between all three species), which is absent from the *E. hybrida* Lp1 and *E. typhina* E8 rDNA sequences. The *E. hybrida* Lp1 rDNA shares greater sequence identity with *E. typhina* E8 than *E. festucae* var. *lolii* AR5, with 56 of the 58 polymorphisms in the three-way alignment occurring between *E. hybrida* and *E. festucae*, the majority of which are present in the noncoding internal transcribed spacer (ITS) and group I intron regions (FIG. 5). These results are consistent with the proposed conversion of *E. festucae* rDNA to *E. typhina* sequence (Ganley and Scott 2002), although they do not rule out other explanations. Whatever the mechanism, the data show that *E. festucae* rDNA has been purged from the *E. hybrida* genome, at least to the level of detection obtainable here.

Gene expression of ribosomal protein homeologs.—The presence of both parental homeologs of the protein-coding genes but uniparental inheritance of the rDNA creates a situation where *E. hybrida* ribosomes may be hybrids of *E. typhina* rRNA and *E. festucae* ribosomal proteins. To determine whether this situation is avoided by the silencing of *E. festucae*–derived ribosomal protein homeologs, gene expression was determined for 105 ribosomal protein genes in *E. hybrida*. We found that ribosomal protein gene expression is largely unbiased, with 98 genes showing equal expression between the *E. festucae* and *E. typhina* homeologs (FIG. 3), and indeed gene expression is less biased among ribosomal protein genes than among protein-coding genes as a whole, both between orthologs in the progenitor species ($P = 3.54 \times 10^{-8}$), and between homeologs in *E. hybrida* ($P = 7.93 \times 10^{-4}$). These results suggest that ribosomal protein divergence is not sufficient to produce deleterious interspecies interactions within ribosomes in *E. hybrida* Lp1.

Mating type genes.—*E. hybrida* Lp1 contains two mating type genes (SUPPLEMENTARY TABLE 1), both belonging to the MTB idiomorph, suggesting that both the *E. festucae* and *E. typhina* progenitors were the same mating type. One mating type homeolog (Lp1_mtBA-f) has an identical sequence to *E. festucae* var. *lolii* AR5, whereas the other mating type homeolog (Lp1_mtBA-t) differs and therefore likely derives from the *E. typhina* progenitor. However, *E. typhina* E8 has the MTA idiomorph (Hettiarachchige et al. 2015), again suggesting that the *E. typhina* progenitor of *E. hybrida* Lp1 comes from a lineage that is similar to, but distinct from, *E. typhina* E8.

Secondary metabolite genes and alkaloid production.—To profile the secondary metabolites of *E. hybrida* Lp1, genomic reads were mapped against genes previously characterized as being responsible for peramine, indole-diterpene, and ergovaline biosyntheses (Tanaka et al. 2005; Young et al. 2006; Fleetwood et al. 2007; Schardl et al. 2013a). Assembled genes have been deposited in GenBank (SUPPLEMENTARY TABLE 1; see also Schardl et al. 2013b for additional accessions of these genes).

Two homeologs of the peramine gene *perA* were identified from *E. hybrida* Lp1 genomic DNA. The *E. festucae*–like homeolog appears to be intact, whereas the *E. typhina* homeolog carries a small deletion in the 5′ terminus that produces a frameshift and premature termination (SUPPLEMENTARY FIG. 2). This deletion was detected in independent sequence reads from multiple biological replicates from both genomic DNA and RNA sequencing. Expression analysis at the deletion site indicates that 84% (n = 21) of the transcribed homeologs of *perA* in *E. hybrida* Lp1 derive from the *E. festucae*

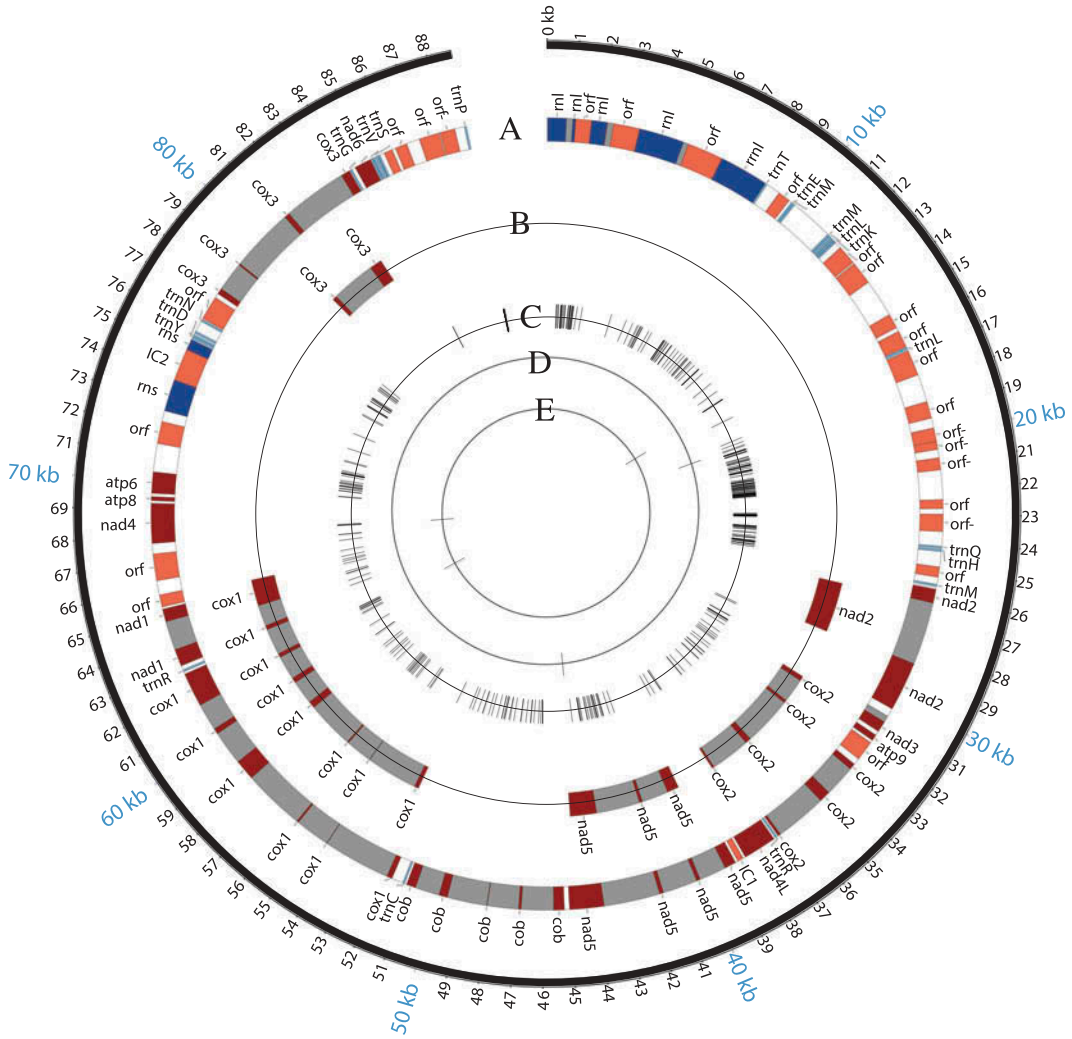

Figure 4. Comparison of mitochondrial genomes from *E. hybrida* Lp1, *E. typhina* E8, and *E. festucae* var. *lolii* AR5. The *E. hybrida* Lp1 mitochondrial genome is shown with major features in track A. Features are color coded by RNA (blue), open reading frames and other nonintron features (orange), protein-coding gene regions (red), introns (gray), and intergenic regions (white). Features on the minus strand are indicated with "-" beside the feature name, e.g., "orf-." The five protein-coding genes that differ in intron composition between *E. hybrida* Lp1 and *E. typhina* E8 are shown on track B. *E. festucae* var. *lolii* AR5 has exactly the same structure as *E. hybrida* Lp1 and so is not shown separately. Tracks C, D, and E show SNPs with a shared state in two of the species but distinct from the third. Track C, *E. hybrida/E. festucae* different in state from *E. typhina*; Track D, *E. hybrida/E. typhina* different in state from *E. festucae*; and Track E, *E. festucae/E. typhina* different in state from *E. hybrida*.

homeolog. MSMS analysis confirms that at least the *E. festucae*–derived *E. hybrida* Lp1 homeolog is functional, with high levels of peramine produced (TABLE 1).

For the indole-diterpene pathway, all genes except *idtE* and *idtJ* (formerly known as *ltmE* and *ltmJ*) are present as single homeologs derived from the *E. festucae* parent. (This pathway is not present in *E. typhina* E8.) The absence of *idtE* and *idtJ* is consistent with previous results (Young et al. 2009; Schardl et al. 2013b) and precludes the production of lolitrem B, as confirmed here by MSMS analysis (TABLE 1). No compounds of the lolitrem class of indole-diterpenes were detected, but earlier pathway intermediates were identified. These earlier intermediates comprise paspalanes from the early stages of the pathway (paspaline and 13-desoxypaxilline), the epoxy-bridged terpendole I, through to more decorated terpendoles (terpendoles C, K, M, and N) (Schardl et al. 2013b).

Consistent with earlier MSMS analyses demonstrating that the ergovaline pathway is fully functional within *E. hybrida* (Panaccione et al. 2003), all genes required for ergovaline production were identified in *E. hybrida* Lp1 from genomic reads and appear to have been inherited as single homeologs derived from the *E. festucae* progenitor. (This pathway is also absent in *E. typhina* E8.) All known intermediates (chanoclavine, agroclavine, elymoclavine,

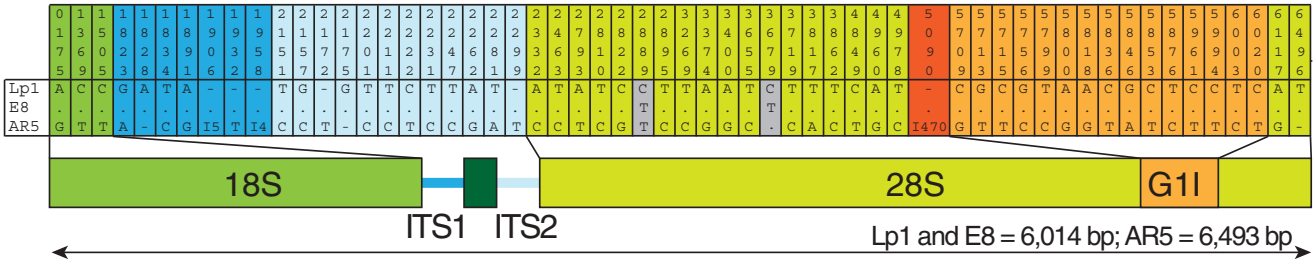

Figure 5. *Epichloë hybrida* Lp1 rDNA sequence derives from *E. typhina*. The 56 positions in the rDNA in *E. festucae* AR5/*E. typhina* E8 that differ from the *E. hybrida* sequence are indicated. Shading corresponds to the rDNA coding region schematic shown underneath (to scale). Numbers above represent positions in the three-way alignment. Polymorphisms between *E. hybrida* Lp1 and *E. typhina* E8 are shown in gray; one (position 2889) is likely a mutation in the *E. hybrida* lineage and the other (position 3679) is likely a mutation in the *E. typhina* lineage. Three polymorphisms, indicated by "I" and the number of nucleotides involved, are multinucleotide indels. The 470-bp indel starting at position 5090 (red) is a group I intron that has inserted in the *E. festucae* var. *lolii* AR5 28S rRNA gene near another group I intron (G1I, orange) that is shared between all three species. The 5.8S rRNA gene (dark green box in the schema) has no polymorphisms. The majority of the polymorphisms (35 of 58) are found in the noncoding regions (ITS1, ITS2 and group I intron). The lengths of the rRNA coding regions from the three species are shown at the bottom.

and lysergic acid) are present at detectable levels, and there are substantial levels of pathway end products (ergovaline, ergine, and lysergyl alanine) (TABLE 1).

TAXONOMY

Epichloë hybrida M.P. Cox et M.A. Campbell, FIG. 1A, D, E

MycoBank MB815747

Typification: FRANCE. PYRÉNÉES ATLANTIQUES: Bidache, from mature seeds of *Lolium perenne*, 1983, leg. J. Meunier. Isolated by G. Latch (187BB, Lp1, AR6) (**holotype**, ATCC-TSD-66).

Etymology: Latin *hybrida*, referring to the origin from distinct progenitor lineages.

Colonies on potato dextrose agar white, slightly undulate, margin broadly wavy. Reverse of colony creamy white. Growth moderate, reaching approximately 15 mm diameter in 21 d at 20 C. Conidiogenous cells arise perpendicularly from hyphal cells, hyaline, phialidic, 10.5–12.5 µm long, and 1.3–1.7 µm wide at base. Conidiogenous cells gradually taper to approximately 0.7 µm at the tip, always with a basal

Table 1. Secondary metabolite chemical levels.

Chemical class	Alkaloid[a]	*E. hybrida* Lp1	*E. festucae* var. *lolii* AR5	*E. typhina* E8	MS1 precursor ion (*m/z*)	MS2 filter ion (*m/z*)
Peramine	Peramine	+++[b]	+++	+++	248	206
Indole-diterpenes[c]	Paspaline	+++	+++	–	422	406
	Paspaline B	+	+	–	436	420
	13-Desoxypaxilline	++	++	–	420	402
	Paxilline	+	+	–	436	420
	Terpendole E	++	++	–	438	422
	Terpendole I	++	++	–	454	436
	Terpendole J	+	+	–	522	506
	Terpendole C	++	++	–	520	504
	Terpendole K	++	++	–	518	502
	Terpendole N	+++	+++	–	534	516
	Terpendole M	++	++	–	536	518
	Terpendole A	++	++	–	536	518
	Lolitriol	–	–	–	620	562
	Lolitrem B	–	–	–	686	628
Ergot alkaloids	Chanoclavine	++	++	–	257	226
	Agroclavine	+	+	–	239	208
	Elymoclavine	+	+	–	255	224
	Lysergic acid	+	+	–	269	223
	Lysergic acid amide (Ergine)	++	+++	–	268	223
	Lysergyl alanine	++	+++	–	340	223
	Ergovaline	++	+++	–	534	223

[a]All metabolites were screened in the same samples used for transcriptome analysis and in the same perennial ryegrass background.
[b]Relative intensity of compound peaks: +++ strong signal; ++ medium signal; + weak signal (towards limit of detection); – no peak observed.
[c]Indole-diterpenes: assignment of terpendoles E, I, J, K, N, M, and A are tentative, as authentic standards are unavailable.

septum. Conidia lunate with a basal apiculum-like bulge, hyaline, aseptate, 4.2–5.5 × 1.5–1.7 µm. Moderate sporulation in culture. Teleomorph not observed. Hybrid with genetic relationships to *E. typhina* (Pers.) Tul. & C. Tul. and *E. festucae* var. *lolii* (Latch, M.J. Chr. & Samuels).

Known host range: Lolium perenne L.

Habitat and distribution: Pyrénées Atlantiques, France. Introduced for agricultural purposes outside native range throughout New Zealand and other temperate countries.

DISCUSSION

Here, we provide a formal taxonomic description for a hybrid fungus that likely arose sometime in the last 300 000 years, *E. hybrida*. Although first collected in 1983 and since used extensively for both applied and basic research purposes, this organism has not been formally described. We remedy this here and simultaneously describe the broad range of molecular resources that are now available to study fungal hybridization using this model organism.

It became clear in the early 1990s that the genome size of *E. hybrida* is doubled relative to several other *Epichloë* species, and that it contains genes from two different species (Schardl et al. 1994; Kuldau et al. 1999). The parental genomes that formed *E. hybrida* are very distinct (~5% divergent), a trait that is not uncommon among *Epichloë* hybrids (Schardl and Craven 2003), and several molecular studies have shown that it derives from a fusion of *E. festucae* var. *lolii* and *E. typhina* (Schardl et al. 1994; Collett et al. 1995; Cox et al. 2014). We confirm these findings, showing that *E. festucae* var. *lolii* and *E. typhina* from *L. perenne* are close relatives to the parental strains, and that almost all genes still have both parental homeologs present. The mechanism behind the allopolyploidy event that produced *E. hybrida* remains unknown. It has been proposed that asexual allopolyploid *Epichloë* are generated by somatic hybridization of two *Epichloë* species that co-occur in a new host species, followed by selection for vertical transmission (Sellosse and Schardl 2007), but this remains to be verified.

Hybrid fungi are not widely known or studied outside the yeasts (Campbell et al. 2016). Therefore, *E. hybrida* is a valuable model system to study the consequences of hybridization in filamentous fungi. This value is enhanced by the identification of close relatives to the parental species, thus allowing comparative analyses before and after hybridization. To facilitate the study of allopolyploidy, we previously developed new software (Duchemin et al. 2015) that allows the identification of a large number of parentally informative markers in hybrids, and most sequencing reads can now be unambiguously assigned to the two proxy progenitors. From this, we were able to allocate RNA sequence reads to parental homeologs for 7679 gene models in *E. hybrida*, with comparable levels of gene coverage in the parental transcriptomic data sets. These data sets comprise a more comprehensive and biologically relevant picture of transcription in *E. hybrida* than that available previously and form a valuable set of resources for the community.

We confirm earlier reports that, in contrast to most genes in the genome, the mtDNA and rDNA sequences are each derived from only one of the parent species (Schardl et al. 1994; Ganley and Scott 2002), and we provide sequences of both loci from *E. hybrida* and its parents. In the case of mtDNA, uniparental inheritance is not surprising, as even in fungi that have biparental mitochondrial inheritance, mitochondria quickly become homoplasmic (Basse 2010). However, it is more challenging to explain the single-parent nature of the multicopy, chromosomally encoded rDNA. One simple explanation, that one rDNA-containing chromosome has been lost in *E. hybrida*, is rendered unlikely by data showing that both homeologs of almost all genes are retained. It is possible, however, that the *E. festucae*–derived rDNA repeat array has suffered a relatively clean deletion. An alternative explanation, backed by previous evidence (Ganley and Scott 2002), is that all of the *E. festucae* rDNA sequence has been converted to *E. typhina* rDNA sequence by a process known as interlocus homogenization. This is believed to involve recombination-based turnover of rDNA repeats (Ganley and Kobayashi 2011) that may be augmented by selfish or adaptive selective pressure (Dover 1982) and has previously been observed in lizard and cotton hybrids (Hillis and Dixon 1991; Wendel et al. 1995). A chromosome-level assembly of *E. hybrida* would allow this question to be definitively answered. Regardless, our genome sequence data indicate that the purging of *E. festucae* rDNA has been complete or nearly complete, and they also reveal a polymorphism in group I intron presence in the 28S rRNA gene. That this group I intron did not insert into the *E. typhina* E8–derived rDNA array upon the *E. hybrida* hybridization event suggests that it does not contain a homing endonuclease, and, indeed, although open reading frames are present, they show no evidence for homology with any proteins in GenBank (protein BLAST expect value <1).

One consequence of biparental inheritance of most protein-coding genes but only a single type of rDNA is that ribosomes consisting of *E. typhina* rRNA and some *E. festucae* ribosomal proteins are likely to form in *E. hybrida*. Divergence of the ribosomal proteins may result in deleterious incompatibilities. The in planta transcriptome data show that both homeologs of all annotated ribosomal protein genes are retained, and that most *E. festucae* and *E. typhina* homeologs within *E. hybrida* (98 of 105 gene models) have similar expression. Therefore, there has been no suppression of *E. festucae* ribosomal protein gene expression despite the potential for ribosomal incompatibilities. Little literature is available on this phenomenon in other species, but homeolog-specific expression of a gene encoding one ribosomal protein has been observed in hybrid maize (Springer and Stupar 2007). In addition, ribosomal protein gene expression occurs nearly exclusively from a single parental homeolog for each ribosomal protein in hybrid catfish (Chen et al. 2016), although which parental homeolog is expressed differs for each ribosomal protein gene, meaning that hybrid ribosomes will still be produced. The silencing observed in these other systems may reflect a response independent of ribosome function. Given that the date of formation of *E. hybrida* is not known, there may not have been sufficient time to silence homeologs. Collectively, these observations suggest that the presence of ribosomal protein homeologs is not strongly deleterious to ribosome function.

The secondary metabolite genes required for indole-diterpene and ergot alkaloid synthesis have been inherited from the *E. festucae* parent, as the *E. typhina* parent does not possess these genes. Unsurprisingly, the production of indole-diterpene and ergot alkaloids by *E. hybrida* Lp1 matches that seen for *E. festucae* var. *lolii* AR5. One homeolog of the *perA* gene for peramine synthesis has been inherited from each progenitor, but the homeolog originating from the *E. typhina* parent appears to be nonfunctional. Since *perA* is fully functional in both *E. festucae* var. *lolii* AR5 and *E. typhina* E8, the mutated homeolog could either have arisen after hybridization or have been inherited from an *E. typhina* strain with a *perA* gene containing a frameshift. This latter explanation is possible, as mating type genes show that *E. typhina* E8 is not the direct progenitor of *E. hybrida* Lp1.

In summary, we have provided a formal description for *E. hybrida*, a model system for the study of fungal hybridization responses. We also provide new data sets that contribute to a comprehensive set of genomic resources for this model system and are intended to extend its use to a wider sphere of researchers to facilitate further study of fungal hybridization in this fascinating group of organisms. Together, our work illustrates the utility of the *E. hybrida* system for studying hybridization.

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LITERATURE CITED

Agrawal S, Ganley AR. 2016. Complete sequence construction of the highly repetitive ribosomal RNA gene repeats in eukaryotes using whole genome sequence data. In: Németh A, ed. The nucleolus: methods and protocols. Methods in Molecular Biology 1455:161–181.

Albertin W, Marullo P. 2012. Polyploidy in fungi: evolution after whole-genome duplication. Proceedings of the Royal Society Series B: Biological Sciences 279:2497–2509.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Barker DJ, Christensen MJ, Wever C, Bacon CW, Hill NS. 1997. Application of endophyte allozyme analysis to *Lolium perenne* population ecology in hill country pasture. In: Bacon CW, Hill NS, eds. *Neotyphodium*/grass interactions. New York: Springer. p. 41–43.

Basse CW. 2010. Mitochondrial inheritance in fungi. Current Opinions in Microbiology 13:712–719.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

Brankovics B, Zhang H, van Diepeningen AD, van der Lee TAJ, Waalwijk C, de Hoog GS. 2016. GRAbB: selective assembly of genomic regions, a new niche for genomic research. PLoS Computational Biology 12:e1004753.

Bushnell B. 2015. BBMap: BBMap short read aligner, and other bioinformatic tools. [accessed 2016 Mar 01]. Available from: http://jgi.doe.gov/data-and-tools/bbtools/

Byrd AD, Schardl CL, Songlin PJ, Mogen KL, Siegel MR. 1990. The β-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). Current Genetics 18:347–354.

Campbell MA, Ganley ARD, Gabaldón T, Cox MP. 2016. The case of the missing ancient fungal polyploids. The American Naturalist 188:602–614.

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. TrimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25:1972–1973.

Chen A, Wang R, Liu S, Peatman E, Sun L, Bao L, Jiang C, Li C, Li Y, Zeng Q, Liu Z. 2016. Ribosomal protein genes are highly enriched among genes with allele-specific expression in the interspecific F1 hybrid catfish. Molecular Genetics and Genomics 291:1083–1093.

Chen L, Li XZ, Li CJ, Swoboda GA, Young CA, Sugawara K, Leuchtmann A, Schardl CL. 2015. Two distinct *Epichloë* species symbiotic with *Achnatherum inebrians*, drunken horse grass. Mycologia 107:863–873.

Christensen MJ, Leuchtmann A, Rowan DD, Tapper BA. 1993. Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea)*, meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). Mycological Research 97:1083–1092.

Christensen MJ, Saulsbury K, Simpson WR. 2012. Conspicuous epiphytic growth of an interspecific hybrid *Neotyphodium* sp. endophyte on distorted host inflorescences. Fungal Biology 116:42–48.

Chung K-R, Schardl CL. 1997. Vegetative compatibility between and within *Epichloë* species. Mycologia 89:558–565.

Clay K. 1990. Fungal endophytes of grasses. Annual Review of Ecology and Systematics 21:275–297.

Collett MA, Bradshaw RE, Scott DB. 1995. A mutualistic fungal symbiont of perennial ryegrass contains two different pyr4 genes, both expressing orotidine-5′-monophosphate decarboxylase. Gene 158:31–39.

Conesa A, Götz S. 2008. Blast2GO: a comprehensive suite for functional analysis in plant genomics. International Journal of Plant Genomics 2008:619832.

Cox MP, Peterson D, Biggs P. 2010. SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics 11:485.

Cox MP, Dong T, Shen G, Dalvi Y, Scott DB, Ganley ARD. 2014. An interspecific fungal hybrid reveals cross-kingdom rules for allopolyploid gene expression patterns. PLoS Genetics 10:e1004180.

Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Research 14:1394–1403.

Dover G. 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Duchemin W, Dupont P-Y, Campbell MA, Ganley AR, Cox MP. 2015. HyLiTE: accurate and flexible analysis of gene expression in allopolyploid species. BMC Bioinformatics 16:8.

Ewald PW. 1987. Transmission modes and evolution of the parasitism-mutualism continuum. Annals of the New York Academy of Sciences 503:295–306.

Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD. 2007. A complex ergovaline gene cluster in *Epichloë* endophytes of grasses. Applied and Environmental Microbiology 73:2571–2579.

Ganley AR, Kobayashi T. 2011. Monitoring the rate and dynamics of concerted evolution in the ribosomal DNA repeats of *Saccharomyces cerevisiae* using experimental evolution. Molecular Biology and Evolution 28:2883–2891.

Ganley AR, Scott B. 1998. Extraordinary ribosomal spacer length heterogeneity in a *Neotyphodium* endophyte hybrid: implications for concerted evolution. Genetics 150:1625–1637.

Ganley AR, Scott B. 2002. Concerted evolution in the ribosomal RNA genes of an *Epichloë* endophyte hybrid: comparison between tandemly arranged rDNA and dispersed 5S *rrn* genes. Fungal Genetics and Biology 35:39–51.

Gentile A, Rossi MS, Cabral D, Craven KD, Schardl CL. 2005. Origin, divergence, and phylogeny of *Epichloë* endophytes of native Argentine grasses. Molecular Phylogenetics and Evolution 35:196–208.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59:307–321.

Hettiarachchige IK, Ekanayake PN, Mann RC, Guthridge KM, Sawbridge TI, Spangenberg GC, Forster JW. 2015. Phylogenomics of asexual *Epichloë* fungal endophytes forming associations with perennial ryegrass. BMC Evolutionary Biology 15:72.

Hillis DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. The Quarterly Review of Biology 66:411–453.

Huerta-Cepas J, Serra F, Bork P. 2016. ETE 3: reconstruction, analysis and visualization of phylogenomic data. Molecular Biology and Evolution 33:1635–1638.

Johnson LJ, Bonth ACM, Briggs LR, Caradus JR, Finch SC, Fleetwood DJ, Fletcher LR, Hume DE, Johnson RD, Popay AJ, Tapper BA, Simpson WR, Voisey CR, Card SD. 2013. The exploitation of epichloae endophytes for agricultural benefit. Fungal Diversity 60:171–188.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30:772–780.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

Kerr G, Chapman DF, Thom ER, Matthew C, Van der Linden A, Baird DB, Johnston E, Corkran J. 2012. Evaluating perennial ryegrass cultivars: improving testing. Proceedings of the New Zealand Grassland Association 74:127–136.

Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. Nature Methods 12:357–360.

Kuldau GA, Tsai H-F, Schardl CL. 1999. Genome sizes of *Epichloë* species and anamorphic hybrids. Mycologia 91:776–782.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9:357–359.

Langmead B, Trapnell C, Pop M, Salzberg S. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10:R25.

Letunic I, Bork P. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23:127–128.

Letunic I, Bork P. 2016. Interactive Tree Of Life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Research 44:W242–W245.

Leuchtmann A, Bacon CW, Schardl CL, White JF, Tadych M. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106:202–215.

Leuchtmann A, Schardl CL. 1998. Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congeneric European species. Mycological Research 102:1169–1182.

Leuchtmann A, Schardl CL, Siegel MR. 1994. Sexual compatibility and taxonomy of a new species of *Epichloë* symbiotic with fine fescue grasses. Mycologia 86:802–812.

Love M, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550.

Moon CD, Craven KD, Leuchtmann A, Clement SL, Schardl CL. 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Molecular Ecology 13:1455–1467.

Moon CD, Guillaumin J-J, Ravel C, Li C, Craven KD, Schardl CL. 2007. New *Neotyphodium* endophyte species from the grass tribes Stipeae and Meliceae. Mycologia 99:895–905.

Otto SP, Whitton J. 2000. Polyploid incidence and evolution. Annual Review of Genetics 34:401–437.

Panaccione DG, Tapper BA, Lane GA, Davies E, Fraser K. 2003. Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. Journal of Agricultural and Food Chemistry 51:6429–6437.

Pearson K. 1900. X. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. London, Edinburgh and Dublin Philosophical Magazine and Journal of Science 50:157–175.

R Core Team. 2015. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. [accessed 2016 Feb 15]. Available from: https://www.r-project.org

Rasmussen S, Lane GA, Mace W, Parsons AJ, Fraser K, Xue H. 2012. The use of genomics and metabolomics methods to quantify fungal endosymbionts and alkaloids in grasses. Methods in Molecular Biology 860:213–226.

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011. Integrative Genomics Viewer. Nature Biotechnology 29: 24–26.

Safonova Y, Bankevich A, Pevzner PA. 2015. dipSPAdes: assembler for highly polymorphic diploid genomes. Journal of Computational Biology 22:528–545.

Schardl CL. 2010. The Epichloae, symbionts of the grass family Poöidae. Annals of the Missouri Botanical Garden 97:646–665.

Schardl CL, Chen L, Young CA. 2014. Fungal endophytes of grasses and morning glories, and their bioprotective alkaloids. In: Osbourn A, Goss RJ, Carter GT, eds. Natural products: discourse, diversity, and design. New York: John Wiley & Sons. p. 125–145.

Schardl CL, Craven KD. 2003. Interspecific hybridization in plant-associated fungi and oomycetes: a review. Molecular Ecology 12:2861–2873.

Schardl CL, Leuchtmann A. 1999. Three new species of *Epichloë* symbiotic with North American grasses. Mycologia 91:95–107.

Schardl CL, Leuchtmann A, Tsai HF, Collett MA, Watt DM, Scott DB. 1994. Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. Genetics 136:1307–1317.

Schardl CL, Wilkinson HH. 2000. Hybridization and cospeciation hypotheses for the evolution of grass endophytes. In: Bacon CW, White JF, eds. Microbial endophytes. New York: Marcel Dekker. p. 63–83.

Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ, Haws DC, Moore N, Oeser B, Panaccione DG, Schweri KK, Voisey CR, Farman ML, Jaromczyk JW, Roe BA, O'Sullivan DM, Scott B, Tudzynski P, An Z, Arnaoudova EG, Bullock CT, Charlton ND, Chen L, Cox MP, Dinkins RD, Florea S, Glenn AE, Gordon A, Güldener U, Harris DR, Hollin W, Jaromczyk J, Johnson RD, Khan AK, Leistner E, Leuchtmann A, Li C, Liu J, Liu J, Liu M, Mace W, Machado C, Nagabhyru P, Pan J, Schmid J, Sugawara K, Steiner U, Takach JE, Tanaka E, Webb JS, Wilson EV, Wiseman JL, Yoshida R, Zeng Z. 2013a. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genetics 9:e1003323.

Schardl CL, Young CA, Pan J, Florea S, Takach JE, Panaccione DG, Farman ML, Webb JS, Jaromczyk J, Charlton ND, Nagabhyru P, Shi C, Leuchtmann A. 2013b. Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. Toxins 5:1064–1088.

Selosse M-A, Schardl CL. 2007. Fungal endophytes of grasses: hybrids rescued by vertical transmission? An evolutionary perspective. New Phytologist 173:452–458.

Shymanovich T, Charlton ND, Musso AM, Scheerer J, Cech NB, Faeth SH, Young CA. 2017. Interspecific and intraspecific hybrid *Epichloë* species symbiotic with the North American native grass *Poa alsodes*. Mycologia 109:459–474.

Springer NM, Stupar RM. 2007. Homeolog-specific expression patterns reveal biases and embryo-specific parent-of-origin effects in hybrid maize. Plant Cell 19:2391–2402.

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B. 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Molecular Microbiology 57:1036–1050.

Tsai H-F, Liu J-S, Staben C, Christensen MJ, Latch GC, Siegel MR, Schardl CL. 1994. Evolutionary diversification

of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. Proceedings of the National Academy of Sciences of the United States of America 91:2542–2546.

Van Zijll de Jong E, Dobrowolski MP, Bannan NR, Stewart AV, Smith KF, Spangenberg GC, Forster JW. 2008. Global genetic diversity of the perennial ryegrass fungal endophyte *Neotyphodium lolii*. Crop Science 48:1487–1501.

Wendel JF. 2015. The wondrous cycles of polyploidy in plants. American Journal of Botany 102:1753–1756.

Wendel JF, Schnabel A, Seelanan T. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proceedings of the National Academy of Sciences of the United States of America 92:280–284.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. Proceedings of the National Academy of Sciences of the United States of America 106:13875–13879.

Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B. 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genetics and Biology 43:679–693.

Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B. 2009. Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltm* gene analysis. Applied and Environmental Microbiology 75:2200–2211.

Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Research 18:821–829.