Comparative genomic analysis reveals contraction of gene families with putative roles in pathogenesis in the fungal boxwood pathogens Calonectria henricotiae and C. pseudonaviculata

Layne W. Rogers¹, Alyssa M. Koehler², Jo Anne Crouch³, Marc A. Cubeta¹ and Nicholas R. LeBlanc⁴*

Abstract

Background: Boxwood blight disease caused by Calonectria henricotiae and C. pseudonaviculata is of ecological and economic significance in cultivated and native ecosystems worldwide. Prior research has focused on understanding the population genetic and genomic diversity of C. henricotiae and C. pseudonaviculata, but gene family evolution in the context of host adaptation, plant pathogenesis, and trophic lifestyle is poorly understood. This study applied bioinformatic and phylogenetic methods to examine gene family evolution in C. henricotiae, C. pseudonaviculata and 22 related fungi in the Nectriaceae that vary in pathogenic and saprobic (apathogenic) lifestyles.

Results: A total of 19,750 gene families were identified in the 24 genomes, of which 422 were rapidly evolving. Among the six Calonectria species, C. henricotiae and C. pseudonaviculata were the only species to experience high levels of rapid contraction of pathogenesis-related gene families (89% and 78%, respectively). In contrast, saprobic species Calonectria multiphialidica and C. naviculata, two of the closest known relatives of C. henricotiae and C. pseudonaviculata, showed rapid expansion of pathogenesis-related gene families.

Conclusions: Our results provide novel insight into gene family evolution within C. henricotiae and C. pseudonaviculata and suggest gene family contraction may have contributed to limited host-range expansion of these pathogens within the plant family Buxaceae.

Keywords: Nectriaceae, Host range, Trophic lifestyle

Background

Boxwood blight is an emerging invasive disease of broadleaf evergreen shrubs and trees in the plant family Buxaceae [15]. Due to the widespread commercial production and negative impact of boxwood blight in native ecosystems, this disease poses a major threat to the worldwide ornamental horticulture industry and native Buxus populations in Asia and Europe [47]. Boxwood blight was first discovered in the United Kingdom in 1994 and subsequently identified in the United States in 2011, where it occurs in 30 states and the District of Columbia [27, 29, 32]. Symptoms of boxwood blight begin as dark lesions on leaves of infected plants that eventually lead to extreme leaf blighting, stem lesions,
and defoliation under conducive environmental conditions. Few effective boxwood disease management practices have been identified and current research efforts are focused on developing host–plant resistance strategies as multiple fungicide applications are costly and unsustainable for the year-round occurrence of this disease [15].

Boxwood blight is caused by the ascomycete fungi *Calonectria henricotiae* (*Che*) and *C. pseudonaviculata* (*Cps*), two closely related sister species in the family Nectriaceae. *Cps* also infects two additional genera in the plant family Buxaceae, *Pachysandra* and *Sarcococca* [7]. When boxwood blight disease was first discovered in the 1990s in Europe, *Cps* was the only known causal agent. However, in 2005, a second species—*Che* was identified from diseased boxwood in the UK and continental Europe [24]. Several studies have shown limited genetic diversity from natural populations of *Che* and *Cps*, consistent with the hypothesis of predominant asexual reproduction and introduced clonal lineages [8, 37, 38]. Malapi-Wight et al. (2019) determined that populations of *Cps* have a single mating type idiomorph (MAT1-1) compared to populations of *Che* which possess the MAT1-2 idiomorph [44]. Separate studies demonstrated that pathogen populations have low genetic diversity and no evidence of sexual recombination, suggesting limited opportunities for mating with predominately clonal asexual reproduction [8, 37]. Despite possessing opposite mating types and a sympatric geographic distribution in Europe and the UK, successful mating between *Che* and *Cps* has not been observed in nature or under laboratory conditions [7, 37, 44]. Additional population genomic analyses of *Che* and *Cps* have also shown limited gene flow between the two species and absence of shared genetic polymorphisms [38].

The genus *Calonectria* includes more than 160 described species that inhabit a broad range of ecological habitats and lifestyles globally [41]. In addition to *Che* and *Cps*, several other species of *Calonectria* are plant pathogens and the causal agents of diseases on approximately 335 plant species across 100 plant families [13, 42]. For example, *C. ilicicola* infects at least 70 plant species in multiple families while *C. gordoniae* is a pathogen of a single host plant species native to the southeastern US, loblolly bay (*Gordonia lasianthus*) [21]. Despite the incredible diversity of lifestyles employed by *Calonectria* species, little is known about the mechanisms that these fungi utilize to successfully infect their hosts and extract nutrients. Two comparative genomic and transcriptomic studies were recently conducted on the *Eucalyptus* pathogen *C. pseudoreteaudii* (*Cpr*) to elucidate pathogenesis mechanisms. In these studies, enzymes involved in secondary (specialized) metabolite biosynthesis were up-regulated in *Cpr* mycelia grown in *Eucalyptus* tissue culture medium [75]. These authors identified expanded gene families of Major Facilitator Superfamily (MFS) transporters that enhance pathogenicity suggesting that MFS proteins may provide an adaptive mechanism for degrading and transporting compounds produced by *Eucalyptus* that are toxic to the fungus. Ye et al. (2017) analyzed *Cpr* gene expression profiles at three temporal stages of *Eucalyptus* infection and disease symptom development. The authors identified differentially expressed genes involved in plant cell wall degradation, detoxification of phytoalexins, toxin synthesis, iron uptake, and reactive oxygen species scavenging. Genes encoding cutinase enzymes, which are crucial for plant pathogenic fungi that penetrate through the host cuticle, were also up-regulated during plant pathogenesis and expressed earlier than other cell wall degrading enzymes [76]. An additional report of secondary metabolites as virulence factors was observed in *C. ilicicola* and production of the PF1070A phytotoxin was correlated with increased disease symptom expression in 17 isolates examined [49]. The extracellular proteomes of *Che* and *Cps* were recently examined and revealed 124 putative effectors produced by both species which are hypothesized to be involved in plant pathogenesis [74]. However, to date, gene expression profiles of *Che* and *Cps* during boxwood blight disease development have not been the subject of comprehensive investigation in a gene family evolution framework.

During genome evolution, gene duplication and gene loss events can contribute to contraction and expansion of gene families [17]. Genome changes can be linked to evolutionary processes that result in environmental niche adaptation [17]. Studying changes in gene family contraction and expansion can provide useful insight into organismal, ecological, and lifestyle transitions of plant pathogenic fungi. In many disease-causing fungal species, changes in gene family size have been linked to observed variation in host adaptation, pathogenesis, and virulence [54, 61]. Rapid expansion of gene families in plant pathogenic fungi associated with host cell wall degradation, secondary, and carbohydrate metabolism is providing insight into pathogenesis and virulence processes [40, 48, 64]. Rapid contraction of gene families involved in similar processes have also been linked to biotrophy (obligate parasitism) and ecological lifestyle [65, 78]. For example, in insect and plant pathogenic fungi, contraction of gene families was associated with cuticle and cell wall degradation and limited (narrow) host range [3, 71]. In plant and insect systems, analysis of gene family evolution has elucidated different aspects of pathogen biology and ecology. In the Northern California black walnut (*Juglans hindsii*), a plant species native to the western US, contraction of gene families involved in abiotic stress and...
disease were associated with resistance to Armillaria root rot disease [69]. In another recent study, identification of rapidly evolving gene families led to the development of novel strategies for managing blood-feeding insects [23].

In this study, we deployed comparative phylogenomic tools to characterize and identify rapidly evolving gene families within the genomes of Che, Cps, and 22 additional fungal taxa. Using multiple analytical methods, we generated annotations for protein sequences within rapidly evolving gene families to determine putative functional classes. Further annotation of putative pathogenicity factors and secreted effectors within rapidly evolving gene families of Che, Cps, closely related plant pathogenic and saprobic (apathogenic) species of Calonectria, and less-aggressive pathogens of hosts Buxaceae Pseudonectria buxi (Pbu), P. foliicola (Pfo), and Coccinnectria pachysandricola (Cpa), were conducted to identify shared patterns in gene family evolution associated with fungal-host plant adaptation, pathogenesis, and virulence. We hypothesized that gene families important for host infection and pathogenesis have expanded in Che and Cps, relative to other pathogenic and saprobic species of Calonectria and closely related non-Calonectria Buxaceae pathogens. Here we report on (1) the quantity and predicted functional classes of rapidly contracting and expanding gene families in 24 fungal taxa in the Nectriaceae that vary in pathogenic and saprobic lifestyle; and (2) the predicted functional annotation and comparison of putative pathogenicity factors and secreted effectors within rapidly evolving gene families of Che, Cps, and closely and distantly related species of Calonectria and non-Calonectria pathogens of hosts in Buxaceae.

**Results**

Gene family identification based on time-calibrated phylogenetic analyses

Genome assemblies and predicted proteomes for each of the 24 fungal taxa showed high levels of completeness based on BUSCO scores of 95% or higher (Additional file 1). Overall, 95% of the predicted protein sequences across all the taxa were assigned to a gene family, with a total of 19,750 gene families identified. The average number of proteins in a gene family was 16.7 and 2154 gene families had single copy proteins found in all 24 taxa. Construction of a maximum likelihood phylogenetic tree using protein sequence data from 2154 single copy genes showed 100% confidence in tree topology (Additional file 2).

Identification of rapidly evolving gene families

Across the time-calibrated phylogeny of the 24 fungal taxa examined in this study, CAFE4 identified 422 gene families evolving at a non-random rate (rapidly evolving) (p ≤ 0.01; Additional file 3, Additional file 4). In total, 17,596 gene families experienced a change in size across the phylogeny, either through expansion or contraction. To provide a measure of rapid gene family evolution that each species experienced relative to total changing (both rapidly evolving and randomly evolving) gene families, calculations of the percent of rapidly evolving gene families per total changing gene families were performed (Fig. 1). For 17 species, rapidly expanding gene families accounted for ≥1% of total expanding gene families. Rapidly contracting gene families accounted for ≥1% of total contracting gene families in seven species. However, randomly contracting gene families were more numerous than randomly expanding gene families across the 24 taxa and may partially explain the generally lower observed percentages.

The percentage of rapidly evolving gene families showed variation among the 24 fungal taxa. For example, Cpa, which has the smallest assembled genome among the 24 taxa (26.4 Mb), contained the largest number of total contracting gene families (11,967 gene families) and mean gene losses (-8.4 genes) but was one of five species to have <10 total rapidly evolving gene families (six gene families) (Fig. 1, Additional file 1, and Additional file 5). Surprisingly, Che exhibited the highest and second highest percentages of rapidly contracting and expanding (respectively) gene families, despite having the second and first smallest totals (contracting and expanding, respectively) (Fig. 1). Similar to Che, Cps experienced comparable trends in both total changing and total rapidly evolving gene families (Fig. 1 and Additional file 5). The proportion of rapidly expanding gene families compared to rapidly contracting gene families for each species showed that each of the 24 taxa exhibited distinct patterns of gene family evolution directionality, with either more rapid gene family contraction or more rapid gene family expansion (Fig. 2).

Among species of Calonectria, Che and Cps were the only species to undergo more rapid gene family contractions than expansions and had considerably fewer total expanding and contracting gene families than Cmu, Cna, Cle, and Cpr (Figs. 1, 2, and Additional file 5). The saprobic species Cmu and Cna exhibited nearly exclusive rapid gene family expansion. For example, Cna had the greatest number of total expanding gene families across all 24 taxa (Figs. 1, 2, and Additional file 5). Compared to the non-Calonectria Buxaceae pathogens (Cpa, Pbu, and Pfo), Che and Cps experienced more rapid gene family expansions and contractions than Cpa, Pbu, and Pfo. However, Che and Cps had a similar number of total contracting and expanding gene families compared to the two species of Pseudonectria examined in this study.
For example, *Pseudonectria* species had ~11,000 fewer total contracting gene families than *Cpa*, which had the most total contracting gene families among the 24 taxa examined (Figs. 1, 2, and Additional file 5). The non-*Calonectria* pathogens of plants in the family Buxaceae consistently placed in the bottom five species with the fewest total rapidly evolving gene families (*Pbu*, *Pfo*, and *Cpa* had one, two and six total rapidly evolving gene families, respectively; Additional file 5).

To identify rapidly evolving gene families shared between *Che* and *Cps* and the other *Calonectria* species and non-*Calonectria* pathogens of species in Buxaceae (*Cpa*, *Pbu*, and *Pfo*), a series of UpSet plots were generated (Additional file 6). For rapidly contracting and expanding gene families, *Che* and *Cps* shared two or fewer gene families with saprobic *Calonectria* species, pathogenic *Calonectria* species, and non-*Calonectria* pathogens of plants in the family Buxaceae (Additional file 6). *Che* and *Cps* shared the most rapidly contracting and rapidly expanding gene families with the pathogenic *Calonectria* species (*Cle* and *Cp*) (Additional file 6). *Che* and *Cps* did not share any rapidly evolving gene families within the “rapidly expanding” or “rapidly contracting” categories but did share three gene families that were rapidly evolving in opposite directions in each species (OG0000649, OG0001150, and OG0007608). Individually, *Che* and *Cps* experienced rapid expansion and rapid contraction of three gene families (OG0000150, OG0000101, and OG0001150) and were not rapidly evolving in any of the other 22 additional fungal taxa.

**Annotation of rapidly evolving gene families**

While 422 gene families were identified by CAFE4 as rapidly evolving across the phylogeny, only those rapidly evolving at terminal taxa were characterized (403 gene families). Of the 7221 protein sequences grouped into the 403 extant gene families, 5912 received a COG annotation (sequences that received an ‘NA’ or not annotated designation by eggNOG were removed from subsequent analyses) (Table 1). The 5912 annotated sequences were used to classify 332 rapidly evolving gene families into a putative functional category which were annotated with between one and 11 (1.56 ± 1.09; Mean ± SD) COG categories based on the annotations of protein sequences within each gene family. Approximately 68% (225 gene families) of the COG-annotated gene families were annotated with a single COG category. For Pfam annotation,
5294 of the 7221 protein sequences received a Pfam hit with an e-value ≤ $1\times 10^{-5}$ and spanned 317 of the 403 rapidly evolving gene families with between one and 207 (16.700 ± 22.616) sequences per gene family. Protein sequences that received both a COG and Pfam annotation (4468 out of the 7221 protein sequences) spanned 304 of the 403 rapidly evolving gene families in extant species.

Among the 24 fungal taxa, 101 annotated gene families (~33%) were of the unknown function COG category (S category; Table 1). Of the 115 Pfam targets (13.6 ± 27.4 proteins per target) spanning the S-categorized gene families, heterokaryon incompatibility protein (HET; PF06985.12; 253 proteins) was the most frequently observed and was found in seven S-categorized gene families. The second most frequently observed Pfam target was NACHT domain (PF05729.13; 97 protein sequences), which is associated with programmed cell death and heterokaryon incompatibility (HET) loci. Together, HET and NACHT domain Pfam targets spanned 13 S-categorized gene families which were rapidly evolving in 14 of the 24 taxa (Che, Cmu, Cna, Cps, Cp, Dactyonectria macrodidiyma, Fusarium fujikuroi, F. oxysporum 4287, F. oxysporum Fo47, F. solani, Neonectria hederae, N. punicea, Stachybotrys chartarum, and S. chlorohalonata) (Fig. 3). Excluding F. oxysporum 4287 and Fo47, and S. chlorohalonata, each species experienced either exclusive rapid expansion or contraction of these gene families. After S-categorized gene families, Q-categorized gene families (secondary metabolism, biosynthesis, and catabolism) were the second most frequently observed COG category with 40 gene families (709 protein sequences). Pfam targets within Q-categorized gene families spanned a narrower range than in S-categorized gene families of 56 targets (12.7 ± 35.1 proteins per target) with the most frequently observed targets being cytochrome P450 (PF00067.23; 243 protein sequences), short-chain dehydrogenase (PF00106.26; 88 protein sequences), and enoyl-(acyl carrier protein) reductase (PF13561.7; 76 protein sequences). Combined, these Pfam-targets spanned

---

**Fig. 2** Time-calibrated maximum likelihood tree constructed from 2154 single copy orthologs that showed 100% confidence in tree topology for the boxwood blight pathogens *Calonectria hennicotaie* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae. The scale bar units are in millions of years. Percentages and frequencies of rapidly expanding and contracting gene families are plotted to the right of the phylogeny.
23 of 40 Q-categorized gene families which were rapidly evolving in 18 of the 24 taxa (Cp, Cmu, Cna, Cps, Corinectria fucelliana, D. macrodidyma, F. oxysporum 4287, F. fujikuroi, F. graminearum, F. oxysporum 47, F. solani, Ilyonectria destructans, N. ditissima, N. hederae, N. punicea, Pbu, S. chartarum, S. chlorohalonata) (Fig. 3). These gene families were exclusively expanding in half of the species and either both expanding and contracting or exclusively contracting in the remaining species. Combined with the S- and Q-categorized gene families, G-categorized gene families (carbohydrate transport and metabolism; 19 gene families; 240 protein sequences) represented more than 50% of the COG-annotated gene families. Pfam targets within G-categorized gene families spanned 33 Pfam targets (7.27 ± 8.71 proteins per target) with the most frequently observed being major facilitator family (PF07690.17; 39 protein sequences), tannase and feruloyl esterase (PF07519.12; 36 protein sequences), and glycoside hydrolase family 18 (PF00704.29; 15 protein sequences). These three Pfam targets spanned seven of the 19 G-annotated gene families which were rapidly evolving in eight of 24 taxa and exclusively expanding or contracting in each species (Cmu, Cna, Cps, F. fujikuroi, F. oxysporum Fo47, F. solani, N. hederae, S. chartarum). The remaining 27 COG categories contained ≤ 17 gene families per category and were represented by 304 annotated.

### Table 1

| COG abbreviation | COG category                        | Number of rapidly evolving gene families | Number of proteins |
|------------------|-------------------------------------|-----------------------------------------|--------------------|
| S                | Function unknown                     | 101                                     | 1566               |
| Q                | Secondary metabolite biosynthesis, transport, and catabolism | 40                          | 709                |
| G                | Carbohydrate transport and metabolism | 19                                      | 240                |
| E                | Amino acid transport and metabolism  | 17                                      | 228                |
| C                | Energy production and conversion     | 16                                      | 207                |
| K                | Transcription                        | 16                                      | 229                |
| L                | Replication, recombination, and repair | 12                                     | 114                |
| T                | Signal transduction mechanisms       | 12                                      | 118                |
| I                | Lipid transport and metabolism       | 8                                       | 118                |
| P                | Inorganic ion transport and metabolism | 8                                      | 99                 |
| U                | Intracellular trafficking, secretion, and vesicular transport | 8                                      | 105                |
| Z                | Cytoskeleton                         | 7                                       | 131                |
| M                | Cell wall/membrane/envelope biogenesis | 6                                      | 39                 |
| O                | Post-translational modification, protein turnover, chaperones | 6                                      | 252                |
| B                | Chromatin structure and dynamics     | 4                                       | 45                 |
| H                | Coenzyme transport and metabolism    | 3                                       | 9                  |
| J                | Translation, ribosomal structure, and biogenesis | 3                                      | 37                 |
| V                | Defense mechanisms                   | 3                                       | 48                 |
| A                | RNA processing and modification      | 2                                       | 26                 |
| D                | Cell cycle control, cell division, chromosome partitioning | 2                                      | 30                 |
| OT               | Post-translational modification, protein turnover, chaperones/signal transduction mechanisms | 2                                      | 24                 |
| AT               | RNA processing and modification/signal transduction mechanisms | 1                                      | 17                 |
| CG               | Energy production and conversion/carbohydrate transport and metabolism | 1                                      | 13                 |
| CH               | Energy production and conversion/coenzyme transport and metabolism | 1                                      | 8                  |
| DK               | Cell cycle control, cell division, chromosome partitioning/transcription | 1                                      | 15                 |
| FQ               | Nucleotide transport and metabolism/secondary metabolite biosynthesis, transport, and catabolism | 1                                      | 5                  |
| IQ               | Lipid transport and metabolism/secondary metabolite biosynthesis, transport, and catabolism | 1                                      | 10                 |
| KL               | Transcription/replication, recombination, and repair | 1                                      | 1                  |
| TZ               | Signal transduction mechanisms/cytoskeleton | 1                                      | 13                 |
| UY               | Intracellular trafficking, secretion, and vesicular transport/nuclear structure | 1                                      | 12                 |
gene families (Table 1). The comprehensive range of Pfam functional targets within each COG-annotated gene family are presented in Additional file 7.

Identification of putative pathogenicity factors and secreted effectors

To further screen rapidly evolving gene families in the genus Calonectria and non-Calonectria pathogens of hosts in Buxaceae for potential roles in plant pathogenesis, protein sequences in these gene families were compared to accessions in the Pathogen Host Interactions (PHI) database. The PHI database catalogues pathogenicity, virulence, and effector genes that have been experimentally tested in pathogen-host interactions of fungal, oomycete, and bacterial pathogens with animal, plant, fungal, and insect hosts [70]. In total, 2682 sequences were searched against the PHI database, and 1566 sequences spanning 112 rapidly evolving gene families received hits with e-values ≤ 1e−5. Protein sequences from all species except the non-Calonectria Buxaceae pathogen Pfo received PHI hits. To identify PHI-annotated sequences putatively involved in virulence and pathogenicity, sequences homologous to proteins with annotated mutant phenotypes of reduced virulence (RV), loss of pathogenicity (LOP), or effector (E) in other pathogens were identified from the dataset. Of 1566 sequences with similarity to sequences in the PHI database, 738 sequences matched these criteria and spanned 64 rapidly evolving gene families (Fig. 4 and Additional file 8). The same set of sequences used for PHI annotation were also classified into secreted and other (no signal peptide identified) protein categories using SignalP v5.0 [1]. Of 2682 sequences, 123 sequences were classified as secreted proteins and spanned 35 rapidly evolving gene families (Fig. 5 and Additional file 8). All species except for Pbu and Cpa had sequences classified as secreted proteins in rapidly evolving gene families. Sequences classified as secreted proteins were further classified into effector and non-effector categories using EffectorP v2.0 (Fig. 5) [66].

The predominant PHI phenotype for annotated sequences within rapidly evolving gene families was RV followed by LOP and E (Fig. 4 and Additional file 8). Several shared gene families identified with the UpSet plots between Che, Cps, saprobiic Calonectria species, non-Buxaceae Calonectria pathogens, and non-Calonectria pathogens of species in Buxaceae also contained PHI-annotated sequences (Fig. 4 and Additional file 6). Of 123 sequences classified as secreted proteins by SignalP, seven were classified as
effectors and spanned five gene families which were rapidly expanding in \textit{Cmu}, \textit{Cna}, \textit{Cle}, and \textit{Cpr} and rapidly contracting in \textit{Cps} and \textit{Cpr} (Fig. 5). Gene families containing secreted effectors were not rapidly evolving in \textit{Che}. Gene families rapidly evolving within saprobic \textit{Calonectria} species \textit{Cmu} and \textit{Cna}, pathogenic \textit{Calonectria} species, and non-\textit{Calonectria} \textit{Buxaceae} pathogens. A Percentage of annotated protein sequences from nine species searched against the PHI database from each rapidly evolving gene family with effector (E), loss of pathogenicity (LOP) and reduced virulence (RV) phenotypes. B Gene families containing PHI annotations and direction of rapid evolution (rapid expansion, rapid contraction, not rapidly evolving).

\textbf{Discussion}

Since the global emergence of boxwood blight disease in the 1990s, research on the evolution of \textit{Che} and \textit{Cps} has focused primarily on understanding factors influencing pathogen population genetic and genomic diversity. However, gene family evolution in \textit{Che} and \textit{Cps} and its putative role in plant pathogenesis has not been studied. For this study, we identified and annotated rapidly evolving gene families in the genomes of \textit{Che} and \textit{Cps} and its putative role in plant pathogenesis has not been studied.
Here, we tested the hypothesis that gene families important for plant host infection and pathogenesis have expanded in *Che* and *Cps*, relative to other pathogenic and saprobic species of *Calonectria* and distantly related non-*Calonectria* Buxaceae pathogens.

Among the pathogenic and saprobic species of *Calonectria* examined in this study, only *Che* and *Cps* exhibited predominant rapid contraction of gene families with putative involvement in host plant infection based on combined use of multiple annotation analyses. Gene families were assumed to play putative roles in plant pathogenesis based on examination of proteins that displayed similarity to proteins documented in the PHI database or contained predicted secreted effectors. While rapid expansion of gene families involved in pathogenesis has been reported for fungal plant pathogens, rapid contraction of pathogenesis-related gene families has been linked to biotrophy (obligate parasitism) and a restricted host range [3, 62, 65, 71, 78]. Given that infection by *Che* and *Cps* results in necrosis of diseased leaves and stems and because *Che* and *Cps* can be readily cultured on artificial nutrient medium, it is unlikely that they are obligate biotrophs. However, it remains unknown whether *Che* and *Cps* possess an initial biotrophic phase to obtain nutrients from living cells upon entry into plant tissues during infection, and whether these fungi exhibit hemibiotic or necrotrophic behaviors. Better studied fungal plant pathogens in the Nectriaceae including *Neonectria* spp. and *Fusarium* spp. have been characterized as hemibiotrophs [43, 57, 68]. Without knowledge of the trophic behavior and lifestyle of *Che* and *Cps*, it is challenging to interpret how the mechanism(s) of nutrient acquisition influence gene family evolution in these species. Future comprehensive investigation on the trophic behaviors and lifestyles of *Che* and *Cps* is warranted to provide additional insight into the relative contribution of
these behaviors on contraction of gene families involved in plant pathogenesis in these species.

To date, Cps has been isolated and identified from environmental samples of diseased leaves on plants in three genera in the family Buxaceae: Buxus, Pachysandra, and Sarcococca [21]. This restricted host range provides at least one plausible explanation for predominant rapid contraction of pathogenesis-related gene families. Che causes disease primarily on species of Buxus in nature, although artificial inoculation experiments in the laboratory indicate that Pachysandra terminalis ‘var. Compacta’ is a host [4]. Differences in Che and Cps host range may explain observed differences in gene family evolution between these closely related species. Interestingly, contraction of gene families involved in trophic behavior and plant/animal host recognition among 45 fungal genomes sampled in the order Hypocreales were associated with a restricted host range [78]. Comparative genomic analyses of fungal insect pathogens in the Ophiocordyceps unilateralis species complex also revealed contraction of gene families involved in cuticle degradation and other insect-host interactions and specificity [71]. The contraction of gene families involved in cell-wall degradation and secondary metabolite biosynthesis associated with plant pathogenesis in fungal plant pathogens is also well documented [65]. In other plant pathogens, the absence or presence of pathogenicity-related genes have been shown to be strong determinants of plant host range for lineages of Magnaporthe oryzae, Verticillium dahliae, Leptosphaeria maculans—Leptosphaeria biglobosa species complex, and closely related Zymoseptoria species [12, 16, 25, 26]. For example, effector genes were hypothesized to have emerged after speciation and contributed to differences in host specificity in the closely related sister species Zymoseptoria pseudotritici and Z. ardibiliae [25]. Perhaps this is the case for Che and Cps and explains the limited overlap in rapidly evolving, pathogenesis-related gene families between these species. Based on these observations, our hypothesis that gene families involved in plant pathogenesis are expanding in Che and Cps was rejected.

Three gene families were rapidly evolving in both Che and Cps, but none had putative roles in plant pathogenesis. Instead, these gene families received COG annotations of unknown function, coenzyme transport, and cytoskeleton and did not contain proteins that received PHI annotations or secreted effector classifications. Individually, Che and Cps contained three rapidly evolving gene families each that were not rapidly evolving in any of the additional 22 fungal taxa investigated. In Cps, one

| Gene family ID      | Rapidly evolving in Cps | Rapidly evolving in Che | COG category | Top Pfam target                          | Contains PHI annotated sequences | Contains putative secreted effectors |
|--------------------|-------------------------|-------------------------|--------------|------------------------------------------|---------------------------------|------------------------------------|
| OG00000000         | (C)                     | S                       | NACHT        | (PF05729.13)                             | Yes                             | No                                 |
| OG00000003         | (C)                     | O                       | CHAT         | (PF12770.8)                              | Yes                             | No                                 |
| OG00000008         | (C)                     | U                       | Sugar transporter | (PF00083.25)                          | Yes                             | No                                 |
| OG00000103         | (C)                     | Z                       | Phosphorylase superfamily | (PF01048.21)                          | No                              | Yes                                |
| OG00000018         | (C)                     | S                       | NACHT        | (PF05729.13)                             | Yes                             | No                                 |
| OG00000029         | (C)                     | Q                       | Short-chain dehydrogenase | (PF00106.26)                          | Yes                             | No                                 |
| OG00000036         | (C)                     | G                       | Glycoside hydrolase family 18 | (PF00704.29)                          | Yes                             | Yes                                |
| OG00000041         | (C)                     | G                       | Carboxylesterase family | (PF00135.29)                          | Yes                             | No                                 |
| OG00000049         | (C)                     | D                       | Protein kinase domain | (PF00069.26)                          | Yes                             | No                                 |
| OG00000069         | (C)                     | U                       | Trichothecene efflux pump | (PF06609.14)                          | Yes                             | No                                 |
| OG00000091         | (C)                     | G                       | LysM domain   | (PF01476.21)                            | Yes                             | Yes                                |
| OG00001157         | (C)                     | Q                       | Amine oxidase | (PF001179.21)                           | Yes                             | No                                 |
| OG00001187         | (C)                     | U                       | Sugar transporter | (PF00083.25)                          | Yes                             | No                                 |
| OG0000321          | (E)                     | Q                       | AMP-binding enzyme | (PF00501.29)                          | Yes                             | No                                 |
| OG0000702          | (C)                     | E                       | Aminotransferase class III | (PF00202.22)                          | Yes                             | No                                 |
| OG0000852          | (C)                     | S                       | Major facilitator family | (PF07690.17)                          | Yes                             | No                                 |
| OG00001175         | (E)                     | K                       | Fungal specific transcription factor domain | (PF04082.19)                  | Yes                             | No                                 |
| OG0001226          | (E)                     | IQ                      | AMP-binding enzyme | (PF00501.29)                          | Yes                             | No                                 |

(C) and (E) indicate rapid contraction and expansion of each gene family, respectively.
of three uniquely evolving and rapidly contracting gene families contained a top Pfam target of CFEM domain (PF05730.12) which has a proposed role in fungal pathogenesis and contained putative secreted proteins [34]. In Che, none of the three uniquely evolving gene families had putative roles in pathogenesis. Che and Cps are closely related sister species with minor genetic and genomic differences, but exhibit phenotypic differences in thermotolerance, fungicide sensitivity, and secreteme composition [24, 37, 74]. Additionally, the geographic distribution of Cps is more widespread than Che which has not expanded its range beyond the UK and continental Europe [38]. Differences in geographic range may contribute to the rapid evolution of different gene families in these species. However, the relationship between genetic diversity and biogeography are not well documented in fungal species except in certain pathogenic, mushroom-forming, and arbuscular mycorrhizal fungi [2, 18, 60, 79]. One isolate genome of Che and Cps was included for comparing rapidly evolving gene families between these species since pathogen populations of Che and Cps have been shown to be clonal in nature and display limited genetic diversity [37]. However, complete genome analyses of asexual pathogens like Verticillium dahliae have revealed that this plant pathogenic species can harbor substantial numbers of accessory genes, which can be enriched in candidate effectors not shared between strains [58]. Future studies should confirm similar trends in gene family evolution across genetically different isolates of Che and Cps.

Two of the closest known relatives of Che and Cps included in this study were the apathogenic soil saprob, Calonectria multiphialidica (Cmu) and C. naviculata (Cna), which exhibited nearly exclusive rapid gene family expansion and had the greatest number of rapidly evolving gene families among Calonectria species investigated. The greatest number of rapidly expanded gene families in Cmu and Cna were classified into the unknown function, secondary metabolite biosynthesis, and carbohydrate metabolism COG categories. Expanded gene families involved in plant cell wall degradation and secondary metabolite biosynthesis are commonly observed in saprobic fungal genomes due to their involvement in nutrient degradation and defense against competing microorganisms, respectively [36, 39]. Mutualistic ecto- and endomycorrhizal fungi are also known to produce a variety of secreted PCWDEs and effectors known as mycorrhiza-induced small secreted proteins (MiSSPs) that allow them to initiate their symbiotic association with plants [45]. Interestingly, many rapidly expanding gene families in Cmu and Cna contained proteins similar to those characterized in pathogen-host interactions and were the same gene families rapidly contracting in Che and Cps. While there are no published reports of plant diseases caused by Cmu and Cna, rapid expansion of pathogenesis-related gene families suggests that these species may be evolving in a similar manner to plant pathogenic fungi [40, 48, 64]. Saprobic fungi have been shown to produce and secrete large repertoires of effectors similar in sequence to those produced by plant pathogenic fungi. However, the function of these effectors in a saprobic lifestyle remains unclear [22]. Functional annotation of gene families involved in pathogenesis in plant pathogenic Calonectria species of non-Buxaceae hosts, Calonectria leucothoes (Cle) and Calonectria pseudoreteaudii (Cp), were also performed in this study. Cle and Cpr are well documented pathogens of Leucothoe spp. and Eucalyptus spp. in the Ericaceae and Myrtaceae plant families, respectively [21]. Based on these reports, Cle and Cpr have a similar size and restricted host range to Che and Cps. However, the same observation of predominant rapid contraction of gene families involved in plant pathogenesis was not observed. Compared to Che and Cps, Cle and Cpr had fewer total rapidly evolving gene families and experienced predominant expansion of rapidly evolving gene families including those involved in plant pathogenesis. Based on these observations, Cle and Cpr displayed the most typical trends in gene family evolution observed in other fungal plant pathogens compared to the other Calonectria species examined [40, 48, 64]. A valuable and future comparison of gene family evolution between the restricted plant host range Calonectria species examined in this study and a species of Calonectria with a broader host range is warranted. At the time of initiating our study, relatively few Calonectria genomes were publicly available for this comparison. Lastly, we compared rapidly evolving gene families in Che and Cps to non-Calonectria Buxaceae pathogens Pseudonectria buxi (Pbu), P. foliicola (Pfo), and Coccinonectria pachysandricola (Cpa). Pbu, Pfo, and Cpa are the causal agents of Volutella leaf blight on different hosts in the family Buxaceae and are considered non-aggressive pathogens that typically occur on plants experiencing abiotic stress compared to Che and Cps [56, 73]. Pbu causes disease on species of Buxus, while Pfo causes disease on both Buxus and Sarcococca spp., and Cpa causes disease on Sarcococca and Pachysandra spp. [21]. Pbu, Pfo, and Cpa clustered with Aquanectria peniciilliodes and Thelonectria rubi with the fewest total rapidly evolving gene families while Cpa shared two rapidly contracting gene families involved in pathogenesis with Che. Because of the relatively low quantity of rapidly evolving gene families in Pbu, Pfo, and Cpa comparisons with Che and Cps were limited. However, the relatively low number of rapidly
evolving gene families may suggest that these species are experiencing different selective pressures than Che and Cps [17].

In addition to the functional annotation analyses of pathogenesis-related gene families within Calonectria species and non-Calonectria Buxaceae pathogens, we performed broad functional annotation of gene families rapidly evolving across all 24 fungal taxa selected. Approximately, one third of rapidly evolving gene families across the 24 taxa were classified into the unknown function COG category. Within unknown function gene families, the predominant Pfam targets were heterokaryon incompatibility protein (HET; PF06985.12) and NACHT domain (PF05729.13). Both HET and NACHT domains are subunits of proteins commonly found in the HNWD gene family that allow fungi to recognize self from non-self for successful cell and cytoplasmic fusion [10]. Cell and cytoplasmic fusion are essential and fundamental processes in fungi that allow them to transition from unicellular to multicellular organisms and form hyphal networks for maximizing nutrient acquisition. HET genes have been shown to be highly polymorphic and contribute to the rapid evolution of members within HNWD gene families [10, 33, 53]. Constant rapid evolution of HET genes and their associated gene families allows fungi to maintain genome integrity and evade mycoparasitic exploitation or mycovirus infection, which is critical for fungal species success, irrespective of trophic behavior and lifestyle [52]. This would partially explain why the greatest number of rapidly evolving gene families contained proteins important for heterokaryon (vegetative) incompatibility across the 24 taxonomically diverse taxa examined in this study. Among species of Calonectria, heterokaryon incompatibility and HET loci have not been well studied. However, HET loci in Che and Cps likely have a similar function to other previously examined fungi in the Ascomycota where an incompatible (cell death) reaction is initiated when there are allelic differences at HET loci of two interacting fungal isolates of the same species.

Conclusions
In this study, we used comparative phylogenomic methods to identify and characterize gene families that are rapidly evolving in Che and Cps and other closely related fungi to better understand adaptation and pathogenesis mechanisms for infection of hosts in the plant family Buxaceae by these pathogens. Our work highlights and provides new information on the evolutionary trajectories of Che and Cps and their close relatives that suggest a restricted host range in Che and Cps and gene family evolution trends in saprobic species Cmu and Cna that are analogous to many plant pathogenic fungi. Our results serve as a framework for future studies examining Che and Cps during infection and pathogenesis on Buxaceae hosts that may be used to develop novel disease management strategies. This research also raises new questions about the complex involvement of gene family evolution in the trophic lifestyles of Calonectria species and provides further evidence for an evolutionarily relevant role of pathogenesis-related gene families in fungi with saprobic lifestyles.

Materials and methods
Genome selection and assembly quality assessment
Twenty-four fungal taxa representing 10 genera in the family Nectriaceae and two outgroup species of Stachybotrys (Stachybotryaceae) were selected for this study (Additional file 1). Genome assemblies were obtained from NCBI GenBank for all taxa except Calonectria multiphialidica. References and accession numbers for genome assemblies are shown in Additional file 1. Genome data for C. multiphialidica were generated using Illumina sequencing technology and assembled as previously described [38]. Predicted protein sequence data were also downloaded from NCBI GenBank where available; otherwise, proteins were predicted using the Funannotate v1.8.1 pipeline [51]. Completeness of all predicted proteomes and underlying genome assemblies were assessed using BUSCO v3.1.0 using the fungal-specific gene set ‘Fungi odb9’ [63]. No plant material was used in this study.

Estimation of gene families and construction of time-calibrated phylogeny
Clusters of orthologous genes were identified with OrthoFinder v2.2.7 using the “diamond” option for sequence alignment and “msa” option for gene-tree inference [20]. Single copy orthologs identified using OrthoFinder were concatenated into an alignment and poorly conserved regions were filtered with Gblocks v0.91 [6]. The best-fit model of protein evolution was identified using ProtTest v3.4.2 [14]. The protein sequence alignment was used to construct a maximum-likelihood phylogeny with RAxML v8.2.12 using the JTT model of protein evolution and 100 bootstrap replicates to assess confidence in tree topology [67]. The program r8s v1.81 was used to generate a time-calibrated ultrametric tree from the RAxML phylogeny using an estimated 244 MYA median divergence between Stachybotrys chartarum and Fusarium graminearum determined from the TimeTree database [35, 59]. The time-calibrated phylogeny and orthogroup data were used to measure changes in gene family size and identify rapidly evolving gene families.
Identification and annotation of rapidly evolving gene families

Rapidly evolving gene families were identified using CAFE v4.2.1 which models gene family evolution through time using a stochastic birth and death model and identifies gene families that have experienced a significant change in size [28]. Input data were represented by the orthogroups (gene families) identified with OrthoFinder and the time-calibrated phylogeny representing evolutionary relationships among the 24 fungal taxa. A birth–death parameter (lambda, -s option) of 0.00353252 was estimated for the phylogeny using an optimization algorithm that maximizes the log likelihood of the data for all gene families. A default significance level of 0.01 (-p option) was used to calculate Viterbi p-values to assess rapid (significant) contraction or expansion of gene families along each branch. A custom Python script was developed to extract gene families that were rapidly evolving in extant species to perform additional analyses (Additional file 5).

Protein sequences within rapidly evolving gene families for each species were annotated to determine putative functional classes of gene families. Broad functional annotation was performed using eggNOG-mapper v2 which identifies Clusters of Orthologous Groups (COG) functional categories for each sequence using an e-value threshold of 1e−3 [30, 31]. The most frequently observed COG category within rapidly evolving gene families was used to categorize the gene families for further analysis. Gene families with equivalent frequencies of more than one COG category were classified to the first of the tied categories, which were ordered alphabetically for a given gene family. Protein sequences from *Aquanectria penicillioides* (20 total protein sequences) and *Pfo* (eight total protein sequences) did not receive COG annotations and were not used in gene family characterization. Additional annotation of protein sequences within gene families was performed by searching sequences against the Pfam-A database v33.1 using HMMER 3 with an e-value threshold of 1e−5 [19, 46]. For each annotation procedure, hits with the lowest e-value were selected for annotation of protein sequences that matched multiple subject sequences. Interspecific gene family annotation summaries and analyses were conducted in R v4.0.3 [55] using the following packages: tidyverse v1.3.0, UpSetR v1.4.0, ggtern v2.4.1, seqinr v2.4.2–5, Biostrings v2.58.0 [9, 11, 50, 72, 77].

Identification of putative pathogenicity factors and secreted effectors

Protein sequences within rapidly evolving gene families from *Che, Cps*, two saprobic *Calonectria* species (*C. multiphialidica* [Cmu] and *C. naviculata* [Cna]), two *Calonectria* species pathogenic to non-boxwood hosts (*C. leucothoes* [Cle] and *C. pseudoreteaudii*, [Cpri]), and non-*Calonectria* pathogens of hosts in Buxaceae (*Pbu, Pfo*, and *Cpa*) were used to identify putative pathogenicity factors in the Pathogen Host Interactions (PHI) database v4.10 [70]. Searches were conducted using blastp v2.10.0 with an e-value threshold of 1e−5 [5]. Putative secreted proteins were identified (probability >0.5) from protein sequences described above using SignalP v5.0 [1]. Predicted secreted proteins were further classified (probability >0.5) as effectors using EffectorP v2.0 [66]. Unspecified parameters for all programs discussed were left as default values.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12862-022-02035-4.

**Additional file 1:** Genome metadata for the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 2:** Maximum likelihood phylogenetic tree constructed from 2154 single copy orthologs that showed 100% confidence in tree topology for the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 3:** Gene gains and losses identified by CAFE for rapidly evolving gene families in the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 4:** Time-calibrated maximum likelihood phylogenetic tree constructed from 2154 single copy orthologs that showed 100% confidence in tree topology for the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 5:** CAFE output statistics for the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 6:** UpSet plots generated for comparison of rapidly evolving gene families in *Calonectria, Coccinonectria* and *Pseudonectria* species.

**Additional file 7:** COG and Pfam annotations and e-values for each protein sequence within rapidly evolving gene families of the boxwood blight pathogens *Calonectria henricotiae* and *C. pseudonaviculata*, and 22 fungal taxa in the Nectriaceae.

**Additional file 8:** PHI, SignalP, and EffectorP annotations for each protein sequence within rapidly evolving gene families in *Calonectria, Coccinonectria* and *Pseudonectria* species.

**Acknowledgements**

The authors thank Ashley Yow for pre-submission review of this manuscript, Dr. H. David Shew for advice on organization and early review of this manuscript; and Ashton Larkin for assistance in developing the Python script used to extract rapidly evolving gene family information from CAFE output.

**Author contributions**

JC and NRL designed the research. LR and NRL generated and analyzed data. LR wrote the manuscript. All authors reviewed, edited, and approved the final manuscript.

**Funding**

This work is supported by the US Department of Agriculture (USDA) National Institute of Food and Agriculture Award No. 2019-67012-29631 to NL, USDA Agricultural Research Service (ARS) Floriculture and Nursery Research Initiative
References

1. Armenteros JJ, Tisogos KD, Sanderby CK, Petersen TN, Winther G, Brunak S, et al. SignaPL 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol. 2019;37:420–3.

2. Avio L, Cristani M, Stran P, Giovannetti M. Genetic and phenotypic diversity of geographically different isolates of Glomus mosseae. Can J Microbiol. 2009;55:242–53.

3. Barone P, Amby DB, Zapparana A, Sarracino S, Vannacci G, Le Floc’h G, et al. Gene family expansions and contractions are associated with host range in plant pathogens of the genus Colletotrichum. BMC Genomics. 2016;17:553.

4. Bartkóvá M, Brand T, Belz H, Safářková I. Host susceptibility and microclimatic conditions influencing the development of blight diseases caused by Colletotrichum hemicoccum. Eur J Plant Pathol. 2020;157:103–17.

5. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Baeuler K, et al. BLAST: architecture and applications. BMC Bioinform. 2009;10:1–9.

6. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol. 2000;17:540–2.

7. Castroagudín VL, Weiland JE, Baysal-Guler F, Cubeta MA, Daughtrey ML, Gauthier NW, et al. One clonal lineage of Colletotrichum pseudonaviculatum is primarily responsible for the boxwood blight epidemic in the United States. Phytopathology. 2020;110:1845–53.

8. Castroagudín VL, Yang X, Daughtrey ML, Luster DG, Pischekid JW, Weiland JE, et al. Boxwood blight disease: a diagnostic guide. Plant Health Progr. 2020;21:291–300.

9. Charif D, Lobry JR. SeqinR 1.0–2: a contributed package to the R project for statistical computing devoted to biological sequences retrieval and analysis. In: Gerstmann BS, editor. Structural approaches to sequence evolution. New York: Springer; 2007. p. 207–32.

10. Chevanne D, Bastiaens E, Debets A, Sauge SJ, Clavé C, Paolletti M. Identification of the het-r vegetative incompatibility gene of Podospora anserina as a member of the fast evolving HNWD gene family. Curr Genet. 2009;55:93–102.

11. Conway JR, Leav A, Gehlenborg N. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics. 2017;33:2938–40.

12. Couch BC, Fudal I, Lebrun M, Tharreau D, Van Kim P, et al. Origins of host-specific populations of the blast pathogen Magnaporthe oryzae in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. Genetics. 2005;170:613–30.

13. Crous PW. Taxonomy and pathology of Cylindrocladium (Calonectria) and allied genera. St. Paul: American Phytopathological Society Press; 2002.

14. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics. 2011;27:1164–5.

15. Daughtrey ML. Boxwood blight: threat to ornamentals. Annu Rev Phytopathol. 2019;57:189–209.

16. de Jonge R, van Esse HP, Maruthachalam K, Bolton MD, Santhanam P, Saber MK, et al. Tomato immune receptor Vte1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Proc Natl Acad Sci USA. 2012;109:5110–5.

17. Demuth JP, Hahn MW. The life and death of gene families. BioEssays. 2009;31:29–39.

18. Diao Y, Zhang C, Xu J, Lin D, Liu L, Mtung’E O, et al. Genetic differentiation and recombination among geographic populations of the fungal pathogen Colletotrichum truncatum from chili peppers in China. Evol Appl. 2015;8:108–18.

19. Eddy SR. Accelerated profile HMM searches. PLoS Comput Biol. 2011;7: e1002195.

20. Emms DM, Kelly S. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogoup inference accuracy. Genome Biol. 2015;16:1–14.

21. Fair DF, Rossman AM. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. https://nt.ars-grin.gov/fungaldatasees/ accessed 26 July 2021.

22. Feldman D, Yarden O, Hadar Y. Seeking the roles for fungal small-secreted proteins in affecting saprophytic lifestyles. Front Microbiol. 2020;11:455.

23. Freitas L, Nery MF. Expansions and contractions in gene families of independently-evolved blood-feeding insects. BMC Evol Biol. 2020;21:8–1.

24. Ghebresilassie B, Crouch JA, Marra RE, Van Poucke K, Rys F, Maes M, et al. Characterization and taxonomic reassessment of the box blight pathogen Colletotrichum pseudonaviculatum, introducing Colonectria hemicoccum sp. nov. Plant Pathol. 2016;65:57–52.

25. Grandaubert J, Bhattacharyya A, Stuckenbork EH. RNA-seq-based gene annotation and comparative genomics of four fungal grass pathogens in the genus Zymoseptoria identify novel orphan genes and species-specific inversions of transposable elements. G3-Genomes Genom Genet. 2015;5:1323–33.

26. Grandaubert J, Love RG, Soyer JL, Schoch CL, Angléla P, Fudal I, et al. Transposable element-assisted evolution and adaptation to host plant within the Leptosphaeria maculans-Leptosphaeria biglobosa species complex of fungal pathogens. BMC Genomics. 2014;15:1–27.

27. Hall CR, Hong C, Gouker FE, Daughtrey M. Analyzing the structural shifts in US boxwood production due to boxwood blight. J Environ Hortic. 2021;39:1–9.

28. Han MV, Thomas GWC, Lugo-Martinez J, Hahn MW. Estimating gene gain and loss rates in the presence of error in genome assembly and annotation using CAFE 3. Mol Biol Evol. 2013;30:1987–97.
29. Henricott B, Sierra AP, Prior C. A new blight disease on Buxus in the UK caused by the fungus *Cylindrocladium*. Plant Pathol. 2000;49:6.

30. Huerta-Cepas J, Szklarczyk D, Heller D, Hernandez-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5000 organisms and 2502 viruses. Nucleic Acids Res. 2019;47:D1039–D1048.

31. Huerta-Cepas J, Forslund K, Szklarczyk D, Jensen LJ, Von Mer ing C, et al. Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. Mol Biol Evol. 2017;34:2115–22.

32. Ivors KL, Lacey LW, Milks DC, Douglas SM, Inman MK, Marra RE, et al. First report of boxwood blight caused by *Cylindrocladium pseudonaviculatum* in the United States. Plant Dis. 2012;96:1070.

33. James TY. Ancient yet fast: rapid evolution of mating genes and mating systems in fungi. In: Singh RS, Xu J, Kulathinal RJ, editors. Rapidly evolving genes and genetic systems. Oxford: Oxford University Press; 2012. p. 187–200.

34. Kulkarni RD, Kelkar HS, Dean RA. An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. Trends Biochem Sci. 2003;28:118–19.

35. Kumar S, Stecher G, Sulejko M, Hedges SB. TimeTree: a resource for time-calibrating evolutionary histories. Version 2015;16:1–14. https://funanotate.readthedocs.io/en/latest/.

36. Martin F, Kohler A, Murat C, Veneault-Fourrey C, Hibbett DS. Unearthing species concepts in fungi: a new blight disease on *Buxus* in the UK caused by the fungus *Cylindrocladium pseudonaviculatum*. Plant Pathol. 2012;61:1070.

37. Liu QL, Li J, Wingfield MJ, Duong TA, Wingfield BD, Crous PW, et al. Comparative genomics trace clonal diversification and intercontinental migration of an emerging fungal pathogen of boxwood. Phytopathology. 2021;111:184–93.

38. Lebreton A, Zeng Q, Miyauchi S, Kohler A, Dai Y, Martin FM. Evolution of the mode of nutrition in symbiotic and saprotrophic fungi in forest ecosystems. Annu Rev Ecol Evol Syst. 2021;52:385–405.

39. Li Q, Wu Y, Dong Q, Li L, Rollins JA, Zhang R, et al. Pathogenic adaptations of *Colletotrichum* fungi revealed by genome-wide gene family evolutionary analyses. PLoS ONE. 2018;13: e0196303.

40. Liu Q, Li J, Wingfield MJ, Duong TA, Wingfield BD, Crous PW, et al. Reconsideration of species boundaries and proposed DNA barcodes for *Calonectria*. Stud Mycol. 2020;97:100095.

41. Lombard L, Crous PW, Wingfield BD, Wingfield MJ. Species concepts in *Calonectria*. Stud Mycol. 2010;66:113–131.

42. Lyons R, Stiller J, Powell J, Rusu A, Manners JM, Kazan K. *Fusarium oxysporum* triggers tissue-specific transcriptional reprogramming in *Arabidopsis thaliana*. PLoS ONE. 2015;10:e0192100.

43. Malapi-Wight M, Veltri D, Gehesquiere B, Heungens K, Crouch JA. Limited genetic diversity across pathogen populations responsible for the global emergence of boxwood blight identified using SSRs. Plant Pathol. 2019;68:861–8.

44. LeBlanc N, Cubeta MA, Crouch JA. Population genomics. 2017. https://funanotate.readthedocs.io/en/latest/.

45. LeBlanc N, Gehesquière B, Salgado-Salazar C, Heungens K, Crouch JA. Genome sequencing reveals cryptic speciation in the human pathogen *Histoplasma capsulatum*. MBio. 2018;7:1339.

46. Sharpton TJ, Stajich JE. Genomes with single–copy orthologs. Bioinformatics. 2013;31:3210–2.

47. Sipes C, Prianista AN, Walter MC, O’Connor E, Bäntin B, Krízsán K, et al. Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungus *Armillaria*. Nat Ecol Evol. 2017;1:1931–41.

48. Sippel LD, Stich RE, Sipe CM, Schmid B, Forslund SK, Smith HO, et al. BUSCO: assessing genome assembly and annotation completeness with single–copy orthologs. Bioinformatics. 2015;31:1543–6.

49. Speerschneider J, Dodds PN, Gardiner DM, Singh KB, Taylor JM. Improved prediction of fungal effector proteins from secretomes with EffectoRP 2.0. Mol Plant Pathol. 2018;19:2094–110.

50. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3.

51. Swett CL, Kirkpatrick SC, Gordon TR. Evidence for a hemibiotrophic association of the pathogen *Fusarium cincinatum* with *Pinus radiata*. Plant Dis. 2016;100:79–84.

52. Trouern-Trend AJ, Falk T, Zaman S, Caballero M, Neale DB, Langley CH, et al. Comparative genomics of six *Juglans* species reveals disease-associated gene family contractions. Plant J. 2020;102:410–23.

53. Urban M, Cuzick A, Seager J, Wood V, Rutherford K, Venkatesh SY, et al. PHI-base: the pathogen–host interactions database. Nucleic Acids Res. 2020;48:D613–20.

54. Widdowson DM, Messina H, Crous PW, et al. BUSCO: assessing genome assembly and annotation completeness with single–copy orthologs. Bioinformatics. 2015;31:3210–2.

55. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, et al. Welcome to the Tidyverse. J Open Source Softw. 2019;4:1686.

56. Wickham H, Doy Fortin D, Bache K,使用的文本内容是页面15的内容。
76. Ye X, Liu H, Jin Y, Guo M, Huang A, Chen Q, et al. Transcriptomic analysis of Calonectria pseudoreteaudi during various stages of Eucalyptus infection. PLoS ONE. 2017;12: e0169598.
77. Yu G, Smith DK, Zhu H, Guan Y, Lam TT. ggtrree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol Evol. 2017;8:28–36.
78. Zhang W, Zhang X, Li K, Wang C, Cai L, Zhuang W, et al. Introgression and gene family contraction drive the evolution of lifestyle and host shifts of hypocrealean fungi. Mycology. 2018;9:176–88.
79. Zhao M, Huang C, Chen Q, Wu X, Qu J, Zhang J. Genetic variability and population structure of the mushroom Pleurotus eryngii var. tuoliensis. PLoS ONE. 2013;8:e83253.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.