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Comparative Genomics of Conifer-Associated *Fusarium* spp.

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ABSTRACT

Recent studies have shown numerous *Fusarium* spp. are associated with symptomatic conifer seedlings in both bareroot and container nursery systems. Some of these species have been found pathogenic to conifer seedlings (e.g., *F. avenaceum*, *F. commune*, *F. oxysporum*, *F. solani,* and *F. verticillioides*), but the mechanisms and shared evolutionary history of these conifer pathogenic species have not been well studied in these pathosystems. We compared whole genomes of 17 *Fusarium* spp. associated with conifer seedlings to elucidate putative shared pathogenicity/virulence gene profiles presumably expressed for roles in causing damping-off and/or root disease of conifer seedlings. In addition, this work provides draft genomes of conifer-associated *Fusarium* spp. and genomes not previously referenced in public databases (e.g., *F. lactis*, *F. fredkrugeri*, *F. ipomoeae,* and *F. flocciferum*). We identified pathogenicity/virulence genes associated with *Fusarium* spp. pathogens of conifers including effectors, the secreted in xylem (SIX) genes 2, 4, 9 and 14 and secondary metabolites, and the mycotoxins fumonisin and deoxynivalenol. We conclude that gene profiles are shared within *Fusarium* species complexes and among closely related *Fusarium* species complexes; however, these shared profiles are widely distributed across all *Fusarium* pathogens. These findings highlight potential targets for detecting and/or identifying *Fusarium* pathogens of conifers, but multiple methods and/or targets will be required depending on the species complexes and clades. More research is needed to determine the roles of expressed pathogenicity/virulence genes and the downstream metabolic products that result in pathogenesis to conifers.

1 | Introduction

Fusarium comprises cosmopolitan fungi that infect many agriculturally important plant species. Due to the ubiquitous nature of many *Fusarium* spp., global interest has arisen to better understand diverse species and strains of pathogenic *Fusarium*. Currently, the host ranges of many *Fusarium* species are not well documented, and it remains unknown whether certain *Fusarium* species are limited to pathogenic, saprophytic and/or endophytic lifestyles. As climate change and extreme weather events (e.g., drought) result in stressed plant hosts and conditions favouring plant disease, native and seemingly innocuous saprophytic fungi can emerge as 'new' plant pathogens, which elevates the need to better understand the pathogenic potential of host-associated fungal species and strains (Herron et al. 2015). Of particular note, some *Fusarium* spp. were previously thought to only cause localised disease due to their soil-borne habits; however, recently these species have been reported as causing atypical disease on aboveground plant parts (Li et al. 2023). For example, one apparent shift in pathogenic behaviour from soil to foliage environments is perhaps attributable to changes in climatic conditions that increase plant susceptibility to the pathogen or favour pathogen infection of foliage (e.g., high humidity facilitating infection via airborne conidia) (Rekah, Shtienberg, and Katan 2000). Currently, the molecular mechanisms that drive such lifestyle changes are not well understood.

Conifer nurseries are vital for forest restoration, reforestation and timber production. However, nursery production can be impeded by conifer seedling-infecting *Fusarium* pathogens that cause pre- and postemergent damping-off and root diseases (Dobbs et al. 2023), a major problem for nursery managers. In addition, as changing climatic conditions occur, diseases caused by *Fusarium* pathogens may increase because of lifestyles shifting from saprophytic or opportunistic/low-virulence pathogens to high-virulence pathogens under the new climatic conditions (Quesada et al. 2019). Reforestation of areas impacted by fire is an ever-increasing need that translates to a growing requirement for nursery-grown seedlings. Heightened nursery production can contribute to inadvertent introductions of new pathogens to a broader suite of host species that can lead to the development of pathogens with broader host ranges (Fargione et al. 2021). Since the geographical region has a significant effect on *Fusarium* spp. community structure, the introduction of novel pathogenic genotypes of these pathogens across regions into nurseries or field sites could result in devastating seedling losses (Dobbs et al. 2023). Furthermore, multiple *Fusarium* spp. have been documented to cause damping-off and root disease in the same host (Gordon, Swett, and Wingfield 2015). If multiple conifer-pathogenic *Fusarium* species can co-infect a susceptible host utilising multiple virulence metabolic pathways to overcome host defences, the likely result is a devastating disease (Abdullah et al. 2017). Multiple *Fusarium* pathogens can each utilise unique virulence mechanisms to overwhelm their hosts' defences. It has been posited that multiple pathogens, even weakly virulent pathogens, can work in concert to cause disease on a given host, and repeated introductions of a pathogen on a host can facilitate the development of host-specific, virulence mechanisms in the pathogen (Abdullah et al. 2017; Newman and Derbyshire 2020).

The host-range of many *Fusarium* spp. has not been well documented, especially in recently described or understudied species, such as *F. commune* and *F. annulatum* (Skovgaard et al. 2003; Yilmaz et al. 2021). For example, *F. commune* has been reported as a pathogen of both conifer and nonconifer hosts; however, it has not been characterised as a generalist pathogen and it remains unknown if pathogenic strains display host specificity (Stewart et al. 2012; Hamini-Kadar et al. 2010; Kim et al. 2012). Species like *F. oxysporum* have been separated into strains by *formae speciales* designations that reflect host specificity (Czislowski, Zeil-Rolfe, and Aitken 2021). Generalist and host-specific pathogens have different management implications. For example, treatments are typically required once a generalist pathogen is identified in a nursery, whereas further investigation may be required before a treatment is deemed necessary for host-specific pathogens.

The current reclassification of *Fusarium* spp. coupled with the potential of multiple species causing a threat to conifer seedling production have generated considerable uncertainty about the appropriate disease management practices for nurseries and other agricultural systems. DNA sequence-based techniques, such as Sanger or whole-genome sequencing, are integral to pathogen identification, but these methods still rely on confirmation of species identification through databases (Torres-Cruz et al. 2022), and the main databases for *Fusarium* species identification do not always agree (Geiser et al. 2021; Crous et al. 2021). For this reason, an alternative method for identifying *Fusarium* pathogens is needed that does not rely on the conventional conserved-locus BLAST search that is currently used by the main databases. For example, one alternative method could utilise genetic markers that target pathogenicity genes expressed by the pathogens during disease development. Such pathogenicity-based identification methods would be more useful for managers in determining the presence of pathogens and informing when pathogen management is necessary.

Fusarium contains species complexes that comprise members that share hosts and virulence mechanisms—features that would be useful for rapid identification (Ma et al. 2013). Furthermore, such similarities among species complexes may provide insights for developing robust disease-management methods. However, the evolutionary history and genetic similarities have not been characterised for multiple *Fusarium* spp. that cause root rot and damping-off diseases on conifer seedlings. Identification of shared genes among diverse *Fusarium* pathogens of conifers will facilitate the development of affordable and robust management strategies. Pathogenomics is the first step in developing genomic and putative virulence gene profiles that can be used for developing these management strategies (Rampersad 2020).

It has been hypothesised that *Fusarium* pathogens of conifers may share similarities in virulence factors, possibly due to horizontal gene or chromosome transfer (Stewart et al. 2012). To further investigate this hypothesis, our goal was to determine if predicted virulence genes are shared among conifer-pathogenic *Fusarium* strains. Towards this goal, our research was directed towards the following objectives: (1) investigate genomic similarities among *Fusarium* species collected from conifer seedlings; (2) identify putative conifer-specific genes based on host-origins of a single species, *F. commune* and (3) identify the presence of putative conifer-specific genes among conifer collected *Fusarium* spp.

2 | Materials and Methods

2.1 | *Fusarium* spp. Isolates

Total DNA was extracted from 21 *Fusarium* spp. isolates collected from symptomatic conifer seedlings from the southwestern, Pacific northwestern and southeastern USA, which were previously described in Dobbs et al. (2023) (Table 1). In preparation for DNA extraction, the isolates were grown on ¼-strength potato dextrose agar (PDA) at 25°C for 3 days. These *Fusarium* isolates represent 17 *Fusarium* spp.[1–17] that are contained within six *Fusarium* species complexes [*F. fujikuroi* species complex (FFSC) including *F. annulatum*[1], *F. fredkrugeri*[2], *F. fujikuroi*[3], *F. lactis*[4] and *F. verticillioides*[5]; *F. nisikadoi* species complex (FNSC) including *F. commune*[6]; *F. oxysporum* species complex (FOSC) including *F. inflexum*[7] and *F. oxysporum*[8]; *F. incarnatum-equiseti* species complex (FIESC) including *F. clavum*[9], *F. ipomoeae*[10] and *F. luffae*[11]; *F. tricinctum* species complex (FTSC) including *F. acuminatum*[12], *F. avenaceum*[13], *F. flocciferum*[14] and *F. torulosum*[15] and *F. solani* species complex (FSSC) including *F. falciforme*[16] and *F. solani*[17]] (Table 1).

TABLE 1 | List of reference genomes available in GenBank and the isolates used in this study, including those without an available reference genome.

Fusarium species	Isolate	Accession number	Species complex[a]	Host: common name	Host: scientific name	Reference
F. tricinctum	MES5	JAVTNN000000000	*tricinctum*	Ponderosa pine	*Pinus ponderosa*	This study
F. annulatum	NASWWP3	JAVTNU000000000	*fujikuroi*	Southwest white pine	*Pinus strobiformis*	This study
F. avenaceum	NC74	JAVTNV000000000	*tricinctum*	Loblolly pine	*Pinus taeda*	This study
F. clavum	NAGrPIST2	JAVTNS000000000	*incarnatum-equiseti*	Southwest white pine	*Pinus strobiformis*	This study
F. commune	B62	JAVTNG000000000	*nisikadoi*	Rice	*Oryza sativa*	This study
F. commune	F10.2.2	JAVTNJ000000000	*nisikadoi*	Douglas-fir	*Pseudotsuga menziesii*	This study
F. commune	F13.4.1	JAVTNK000000000	*nisikadoi*	Douglas-fir	*Pseudotsuga menziesii*	This study
F. commune	JICPIPO1	JAVTNL000000000	*nisikadoi*	Ponderosa pine	*Pinus ponderosa*	This study
F. commune	MiAE120	JAVTNQ000000000	*nisikadoi*	Tomato	*Solanum lycopersicum*	This study
F. falciforme	SC35	JAVTNX000000000	*solani*	Loblolly pine	*Pinus taeda*	This study
F. flocciferum	31MAPSF17A	JAVTNF000000000	*tricinctum*	Ponderosa pine	*Pinus ponderosa*	This study
F. fredkrugeri	SC7	JAVTNZ000000000	*fujikuroi*	Loblolly pine	*Pinus taeda*	This study
F. fujikuroi	SC5	JAVTNY000000000	*fujikuroi*	Loblolly pine	*Pinus taeda*	This study
F. inflexum	JTHABCO3.1	JAVTNM000000000	*oxysporum*	White fir	*Abies concolor*	This study
F. ipomoeae	NC8	JAVTNW000000000	*incarnatum-equiseti*	Loblolly pine	*Pinus taeda*	This study
F. lactis	MES8	JAVTNP000000000	*fujikuroi*	Ponderosa pine	*Pinus ponderosa*	This study
F. luffae	CC	JAVTNH000000000	*incarnatum-equiseti*	Douglas-fir	*Pseudotsuga menziesii*	This study
F. oxysporum	MES6	JAVTNO000000000	*oxysporum*	Ponderosa pine	*Pinus ponderosa*	This study
F. solani	NAPSME6.1	JAVTNT000000000	*solani*	Douglas-fir	*Pseudotsuga menziesii*	This study
F. torulosum	DCN062.2.3H	JAVTNI000000000	*tricinctum*	Douglas-fir	*Pseudotsuga menziesii*	This study
F. verticillioides	NAGrPIPO5	JAVTNR000000000	*fujikuroi*	Ponderosa pine	*Pinus ponderosa*	This study
F. ambrosium	NRRL62606	NKCL00000000	*solani*	Beetles		Geiser et al. (2021)
F. avenaceum	NRRL54939	JPYM00000000	*tricinctum*	Barley	*Hordeum vulgare*	Geiser et al. (2021)
F. circinatum	NRRL25331	JAAQPE000000000	*fujikuroi*	Monterey pine	*Pinus radiata*	Geiser et al. (2021)
F. commune	NRRL28387	JABFES000000000	*nisikadoi*	Tomato	*Lycopersicon esculentum*	Geiser et al. (2021)
F. equiseti	NRRL66338	QGEB00000000	*incarnatum-equiseti*	Oats	*Avena sativa*	Geiser et al. (2021)
F. falciforme	NRRL43529	JABEEK000000000	*solani*	Human	*Homo sapiens*	Geiser et al. (2021)

(Continues)

TABLE 1 | (Continued)

Fusarium species	Isolate	Accession number	Species complex[a]	Host: common name	Host: scientific name	Reference
F. foetens	NRRL38302	JABFMM000000000	*oxysporum*	Monterey pine	*Pinus radiata*	Geiser et al. (2021)
F. fujikuroi	NRRL5538	GCF_900079805.1	*fujikuroi*	Rice	*Oryza sativa*	Geiser et al. (2021)
F. gaditjirrii	NRRL45417	JABFAI000000000	*nisikadoi*			Geiser et al. (2021)
F. guttiforme	NRRL22945	JAAQRL000000000	*fujikuroi*	Pineapple	*Ananas comosus*	Geiser et al. (2021)
F. hainanense	NRRL66475	JABFEW000000000	*incarnatum-equiseti*			Geiser et al. (2021)
F. hostae	NRRL29888	JABCJX000000000	*redolens*	Hostas	*Hosta* sp.	Geiser et al. (2021)
F. illudens	NRRL22090	JABFEX000000000	*solani*	Tawa	*Beilschmiedia tawa*	Geiser et al. (2021)
F. irregulare	NRRL31160	QGEA00000000	*incarnatum-equiseti*	Human	*Homo sapiens*	Geiser et al. (2021)
F. lyarnte	NRRL54252	JAAVUB000000000	*nisikadoi*			Geiser et al. (2021)
F. mangiferae	NRRL25226	FCQH00000000	*fujikuroi*	Mango	*Mangifera indica*	Geiser et al. (2021)
F. miscanthi	NRRL26231	JAAVUA000000000	*nisikadoi*	Silver grass	*Miscanthus* sp.	Geiser et al. (2021)
F. neocosporiellum	NRRL22166	SSHR00000000	*solani*		*Heterodera glycines*	Geiser et al. (2021)
F. nisikadoi	NRRL25179	JABFFB000000000	*nisikadoi*		*Phyllostachys nigra* var. *henonis*	Geiser et al. (2021)
F. oxysporum	NRRL34936	AAXH00000000	*oxysporum*	Tomato	*Solanum lycopersicum*	Geiser et al. (2021)
F. oxysporum	NRRL32931	AFML00000000	*oxysporum*	Human	*Homo sapiens*	Geiser et al. (2021)
F. redolens	NRRL22901	JAAVUJ000000000	*redolens*	Douglas-fir	*Pseudotsuga menziesii*	Geiser et al. (2021)
F. sacchari	NRRL66326	JABSTH000000000	*fujikuroi*			Gesier et al. (2021)
F. scirpi	NRRL66328	QHHJ00000000	*incarnatum-equiseti*	Debris from soil		Geiser et al. (2021)
F. subglutinans	NRRL66333	JAAOAV000000000	*fujikuroi*	Acoma blue maize	*Zea mays*	Geiser et al. (2021)
F. thapsinum	NRRL22049	JAAOAX000000000	*fujikuroi*	Sorghum	*Sorghum bicolor*	Geiser et al. (2021)
F. torulosum	NRRL22747	JABFMN000000000	*tricinctum*			Geiser et al. (2021)
F. tricinctum	NRRL25481	JAALXJ000000000	*tricinctum*	Winter wheat	*Triticum aestivum*	Geiser et al. (2021)
F. vanettenii	NRRL45880	ACJF00000000	*solani*			Geiser et al. (2021)
F. verticillioides	NRRL20956	AAIM00000000	*fujikuroi*	Maize	*Zea mays*	Geiser et al. (2021)
F. virguliforme	NRRL31041	JABEEP000000000	*solani*	Soybean	*Glycine max*	Geiser et al. (2021)
F. xylarioides	NRRL25486	JABFFK000000000	*fujikuroi*	Coffee	*Coffea* sp.	Geiser et al. (2021)

(Continues)

TABLE 1 | (Continued)

Fusarium species	Isolate	Accession number	Species complex[a]	Host: common name	Host: scientific name	Reference
F. commune	JCM11502	BCHB00000000	*nisikadoi*	Soil		
F. acuminatum	CHS3	JAPTGE000000000	*tricinctum*		*Bupleurum scorzonerifolium*	
F. annulatum	F8_4S_2B	JAHAPR000000000	*fujikuroi*	ISS environmental surface		
F. brachygibbosum	Fb-HN-1	JAEKIX000000000	*sambucinum*	Maize	*Zea mays*	
F. clavum	NRRL66337	QGEC00000000	*incarnatum-equiseti*	Oats	*Avena sativa*	
F. incarnatum	NRRL66325	GCA_004367075.1	*incarnatum-equiseti*	Pointed gourd	*Trichosanthe dioica*	
F. luffae	NRRL66473	GCA_013184325.1	*incarnatum-equiseti*			
F. metavorans	FSSC_6	LWBZ00000000	*solani*	Asian longhorned beetle	*Anoplophora glabripennis*	
F. proliferatum	ET1	FJOF00000000	*fujikuroi*	Orchid		

[a]*F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC) and *F. solani* (FSSC) species complexes.

2.2 | Whole Genome Sequencing and Assembly

To examine host-specificity in an understudied species reported as pathogenic to both conifer and nonconifer hosts, DNA was also extracted from five *F. commune* isolates [Representative isolates were collected from symptomatic hosts including rice (*Oryza sativa*), tomato 'Montfavet' (*Solanum lycopersicum*), ponderosa pine (*Pinus ponderosa*) and Douglas-fir (*Pseudotsuga menziesii*)] (Table 1). Hyphal tips were transferred to 200 mL of potato dextrose broth and shaken at 90 rpm for 7 days at room temperature. Mycelium was collected using a Buchner funnel and filtered through a Whatman No. 1 filter paper under vacuum. Mycelium was placed in 2-mL cryotubes and frozen at −75°C for 24 h prior to extraction. Total genomic DNA was extracted using a cetyl trimethyl ammonium bromide (CTAB) extraction method similar to Dobbs et al. (2020). Tissue preparation was modified in that frozen samples were ground to a fine powder using liquid N_2 in a flame-sterilised mortar and pestle. Tissue was homogenised after adding 750 μL of CTAB extraction buffer (1% w/v CTAB; 1 M NaC1; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinylpolypyrrolidone) to each sample using a FastPrep-24 (M.P. Biomedicals LLC, Santa Ana, CA, USA) at 5.5× speed for two 60-s runs. Samples were incubated for 30 min at 70°C in a heat block. One volume of chloroform:isoamyl alcohol (24:1) was added to each sample and mixed on a shaker for 20 min then centrifuged at 10,000 × g for 5 min. The upper, aqueous phase (600 μL) was transferred to a new 2-mL tube. Two volumes (1200 μL) of precipitation buffer were added to each sample, mixed in a shaker for 5 min, and then centrifuged at 13,000 × g for 15 min. The supernatant was removed and re-suspended in 350 μL 1.5 M NaCl. To each sample, 20 μL of 10 mg/mL RNase was added, followed by incubation at 37°C for 30 min. One volume of chloroform:isoamyl alcohol (24:1) was added to each sample and mixed on a shaker for 20 min, then centrifuged at 10,000 × g for 5 min. The upper phase was transferred to a new 1.5-mL tube and 0.6 volume of ice-cold isopropanol was added to each sample. Samples were mixed by inversion and incubated at 20°C for 1 h. After incubation, samples were centrifuged at 14,000 × g for 5 min at room temperature. The supernatant was discarded and 1 mL of 70% ethanol was added to each sample. After 1-min incubation at room temperature, samples were centrifuged at 14,000 × g for 3 min at room temperature. Each pellet was dried and resuspended in 80 μL TE buffer (10 mM TrisHCl, 1 mM EDTA). Extracted DNA was assessed for quality using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and gel electrophoresed in a 0.8% agarose gel. DNA was quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Extracted DNA was sent to Novogene (U.C. Davis DNA Sequencing Center, Davis, CA, USA) for TruSeq Illumina shotgun sequencing with paired-end reads of 2 × 151 bp. Paired-end reads were trimmed using BBDuk (Sourceforge.net/projects/bbmap/) in Geneious Prime v2022.0.1 (https://www.geneious.com/).

2.3 | Whole Genome Assembly

The trimmed reads were used in *de novo* genome assemblies that were constructed using SPAdes v3.15.4 (Bankevich et al. 2012). The SPAdes genome assemblies were assessed for quality using QUAST (Gurevich et al. 2013). Contigs were checked for

similarity with other *Fusarium* spp. sequences using a local BLAST of the National Center for Biotechnology Information (NCBI) nucleotide (nt) database displaying the top five results per hit (query sequence with similarity to the nucleotide database). Contigs were screened for the presence of bacterial DNA, and sequences that had three or more results containing similarity to bacterial DNA were removed.

2.4 | Comparative Genome Analysis

Fusarium spp. genomes previously used to phylogenetically place isolates (reference genomes; Geiser et al. 2021) and the genome of a closely related sister species (*Neonectria ditissima*), for use as an outgroup, were retrieved from GenBank (Table 1). Genomes of conifer-associated *Fusarium* spp. isolates were compared for size and quality assessment using QUAST (Gurevich et al. 2013) and for quantitative assessment of genome completeness using BUSCO v5.4.4 against the ascomycota_od10 database (Manni et al. 2021). A maximum likelihood (ML) phylogeny was inferred from 16 concatenated loci (*acl1*, *cal1*, *dpa1*, *dpe1*, *fas1*, *fas2*, *lcb2*, *mcm7*, *pgk1*, *rpb1*, *rpb2*, *tef1*, *top1*, *tsr1*, *tub1* and *tub2*) previously used to speciate *Fusaria* (Geiser et al. 2021) with 1000 pseudoreplicates using IQtree2 and Modelfinder to determine substitution model for the phylogeny (Minh et al. 2020). Abbreviations for loci names are described in Table 2. Loci substitution models were determined independently from one another through the partitioning of each locus. Beauti2 was used to format the aligned sequences for subsequent use in BEAST2 to generate the Bayesian phylogeny (Bouckaert et al. 2019). Bayesian posterior probability branch support and the maximum likelihood bootstrap values were added to the ML phylogeny. DNA sequences were aligned with the MUSCLE sequence aligner using default settings (Edgar 2004).

2.5 | Genome Annotation

Genomes were annotated in a manner similar to that of Dobbs et al. (2020). The *de novo* assembled genomes were annotated using MAKER v.3.01.03 annotation pipeline (Cantarel et al. 2007) with RepeatMasker v.4.1.2 (Smit, Hubley, and Green 2013–2015). A genome-specific repeat library was constructed using RepeatModeler v.2.0.3 (Flynn et al. 2020) to mask interspersed repeats and low-complexity DNA sequences. Three ab initio gene predictors, GeneMark-ES (Ter-Hovhannisyan et al. 2008), SNAP (Zaharia et al. 2011) and AUGUSTUS (Keller et al. 2011), were used in the pipeline, with *F. graminearum* serving as the species model for AUGUSTUS. To identify tRNA genes, tRNAscan-SE v2.0.9 (Lowe and Eddy 1997) was used with default settings. Genome annotations were also assessed for completeness using BUSCO v5.4.4 against the ascomycota_od10 database. Predicted transcripts (≥150 bp) and proteins (≥50 amino acids) were analysed for putative function. Putative functions of genes (i.e., putative virulence genes, secreted proteins, effectors, carbohydrate degrading enzymes (CAZymes), secondary metabolites and orthologous gene clusters) were predicted using the following databases: PHI-base 4.14 (Urban et al. 2017), deeploc2 (Thumuluri et al. 2022), EffectorP 3.0 (Sperschneider and Dodds 2022), dbCAN2 (Zhang et al. 2018), antiSMASH (Blin et al. 2023) and OrthoFinder (Emms and Kelly 2019) respectively. Online servers antiSMASH and dbCAN2 were used to analyse the predicted genes at default settings. Only proteins with dbCAN2 hits in at least two of the

TABLE 2 | Phylogenetic data summary of 16 genes analysed in this study.

Locus[a]	Identity	Model
acl1	ATP citrate lyase large subunit	TN+F+I+G4
cal1	Calmodulin	TIM2+F+I+G4
dpa1	DNA polymerase alpha subunit	TNe+I+G4
dpe1	DNA polymerase epsilon subunit	TN+F+I+G4
fas1	Fatty acid synthase alpha subunit	TIM2+F+I+G4
fas2	Fatty acid synthase beta subunit	TN+F+I+G4
lcb2	Sphinganine palmitoyl transferase subunit	TIM+F+I+G4
mcm7	DNA replication licensing factor	TIM2+F+I+G4
pgk1	Phosphoglycerate kinase	TN+F+I+G4
rpb1	RNA polymerase largest subunit	TN+F+I+G4
rpb2	RNA polymerase second largest subunit	TIM2+F+I+G4
tef1	Translation elongation factor 1-alpha	GTR+F+I+G4
top1	Topoisomerase	TIM2+F+I+G4
tsr1	Ribosomal biogenesis protein	TN+F+I+G4
tub1	Tubulin alpha subunit	TIM2e+I+G4
tub2	Tubulin beta subunit	SYM+I+G4

[a]Selected based on Geiser et al. (2021).

three databases (HMMER, DIAMOND and e-CAMI) were considered. OrthoFinder with multiple sequence alignment, deeploc2 and EffectorP were run locally with default settings and a local BLAST was used for the PHIbase database. Selection of putative proteins from BLASTx results was based on criteria of *e*-value < 1e-5, percent identity ≥ 65% and a coverage of ≥ 50%. Additionally, a local BLAST of Secreted in Xylem (SIX) genes collected from GenBank was used to predict the presence of SIX genes in the genomes used in this study (Table S1). Comparisons of gene contents were analysed for each database using R studio.

To further investigate the similarity among *Fusarium* taxa and the *F. commune* isolates from conifer and nonconifer hosts, including two reference genomes of *F. commune* [GCA 001599515.1 collected from soil; NRRL28387 collected from tomato (*Lycopersicon esculentum*)], principal coordinates analyses (PCoA) were conducted using Bray–Curtis dissimilarities based on abundance of predicted genes and contrasted by species complexes, species complex clades (e.g., SC1, SC2 and SC3) and host using Bray–Curtis distances with a PERMANOVA using the 'vegan' package (Oksanen et al. 2020). An UpSet plot was generated based on orthologous proteins among *Fusarium commune* isolates collected from conifer and nonconifer hosts using ComplexUpset (Lex et al. 2014) in R studio. Conifer-unique orthogroups were identified based on orthogroups only present among *F. commune* isolates collected from conifers. These orthogroups were analysed based on putative function including putative virulence proteins, secreted proteins and effectors and CAZymes. These orthogroups were also searched for presence among the other conifer collected *Fusarium* spp. using OrthoFinder.

3 | Results

3.1 | Whole Genome Assembly and Annotation

Assembled genomes in this study were deposited into the NCBI GenBank databank (BioProjectID: PRJNA1020449; Table 1 and Table S2). Genomes ranged from ca. 39–56 Mb with *F. ipomoeae* having the smallest genome and *F. falciforme* having the largest, both collected from loblolly pine (*P. taeda*) (Table 3 and Table S2). All assembled genomes had a completeness of > 97% based on 1706 genes of the ascomycota_db10, similar to the GenBank collected genomes that had a completeness of > 95% (Table 3 and Table S2). GC content averaged 48.38%, which is expected for *Fusarium* spp. (Table 3 and Table S2). Genome size was incongruent with the number of predicted genes (Table 4 and Table S2). For example, *F. facliforme* has the largest genome size (56.13 Mb) in this study with a modest number of predicted genes (17,518), whereas *F. lactis* (genome size 54.97 Mb) has the highest (22,049) number of predicted genes (Table 4 and Table S2). Annotations had similar completeness of > 97% based on 1706 genes of the ascomycota_db10 (Table 4 and Table S2).

3.2 | Phylogenetics and Phylogenomics of *Fusarium* spp.

The maximum likelihood phylogeny using 16 loci resolved isolates into six *Fusarium* species complexes (FFSC, FNSC, FOSC, FIESC, FTSC and FSSC) (Figure 1). Because reference genomes were not available for five *Fusarium* spp. (*F. flocciferum*, *F. fredkrugeri*, *F. inflexum*, *F. ipomoeae* and *F. lactis*), the identity of these *Fusarium* spp. and phylogenetic relationships of their 16 conserved loci with closely related species was confirmed using the BLAST similarity algorithm to match the translation elongation factor 1-alpha (*tef1*) and the RNA polymerase II second largest subunit (*rpb2*) loci to the GenBank (https://www.ncbi.nlm.nih.gov/) and Fusarioid-ID databases (Crous et al. 2021). These five genomes without associated reference genomes were placed within the expected species complex clades (SC1–SC3, please see details below) based on their respective BLAST sequence similarities (Figure 1). Clades of closely related species complexes were shown to be well supported among the FFSC, FNSC and FOSC hereafter referred to as species complex clade 1 (SC1); FIESC and FTSC were found to be similar (SC2) and the FSSC was unique (SC3).

3.3 | Comparative Gene Profiles

To compare gene profiles among species and species complexes, PCoA was generated based on total orthologous proteins (Figure 2) as well as putative pathogenicity/virulence genes, secondary metabolites, and CAZymes (Figure 3). Species complexes were significantly different based on total orthologous proteins ($p = 0.001$ and $R^2 = 0.692$; Figure 2), putative pathogenicity/virulence genes ($p = 0.001$ and $R^2 = 0.860$; Figure 3A), secondary metabolite genes ($p = 0.001$ and $R^2 = 0.662$; Figure 3B), and CAZymes ($p = 0.001$ and $R^2 = 0.410$; Figure 3C). Species complex clades (e.g., SC1, SC2, and SC3) were significantly different based on total orthologous proteins ($p = 0.001$ and $R^2 = 0.454$), putative pathogenicity/virulence genes ($p = 0.001$ and $R^2 = 0.618$), and secondary metabolite genes ($p = 0.001$ and $R^2 = 0.381$). However, when comparing CAZyme families, only glycosyl transferases were not significantly different among species complex clades ($p = 0.055$ and $R^2 = 0.193$; Figure S1). Species within the SC1 (e.g., FNSC, FFSC, and FOSC) ordinated most closely together, suggesting they share similar gene profiles likely involved in pathogenicity and secondary metabolite production (Figure 3A,B). The SC2 (e.g., FTSC and FIESC) also ordinated closely together when comparing putative virulence genes (Figure 3A) but are less similar when comparing secondary metabolite genes (Figure 3B). The SC3 and the species comprised within were the most dissimilar suggesting their gene profiles were uniquely distinct from the other *Fusarium* spp. This pattern was corroborated when comparing the profiles of putative virulence genes and secondary metabolite genes (Figure 3A,B). However, the gene profiles were more similar when comparing CAZymes (Figure 3C). Putative virulence genes unique to species complexes (FFSC, FNSC, FOSC, FIESC, FTSC, and FSSC) were identified (Table S3). Interestingly, the histone deacetylase inhibitor (DEP1-5) mitogen-activated protein kinase (MAPK) signalling pathway was shared between the FFSC, FNSC, and FOSC, which are clustered within SC1 (Table S3). The BLAST search of SIX genes that was conducted across genomes found that SIX2, SIX4, SIX9, and SIX14 were present in species collected from conifer seedlings in the FFSC and FNSC species complexes (Table S4). These species complexes also possessed some unique predicted secondary metabolites including some well-characterised species complex-specific

TABLE 3 | Comparison of genome size, completeness and GC content of the conifer collected *Fusarium* spp. genomes used in this study.

Isolate	*Fusarium* species	Host collected from	Genome size (Mb)	# Contigs	BUSCO completeness (%)	GC%
NASWWP3	*F. annulatum*	*Pinus strobiformis*	45.96	400	98.6	48.3
NC74	*F. avenaceum*	*P. taeda*	42.5	275	98.7	47.99
NAGrPIST2	*F. clavum*	*Pinus strobiformis*	47.15	2456	98.8	47.93
B62	*F. commune*	*Oryza sativa*	45.96	410	98.6	47.63
F10.2.2	*F. commune*	*Pseudotsuga menziesii*	48.85	648	98.7	47.74
F13.4.1	*F. commune*	*Pseudotsuga menziesii*	49.96	1441	98.7	47.68
JCM11502	*F. commune*	Soil	46.18	19	99.1	47.62
JICPIPO1	*F. commune*	*P. ponderosa*	49.43	1555	98.4	48.62
MiAE120	*F. commune*	*Solanum lycopersicum*	45.71	640	98.9	47.8
NRRL28387	*F. commune*	*Solanum lycopersicum*	48.32	1926	98.5	48.11
SC35	*F. falciforme*	*P. taeda*	56.13	1247	98.9	49.49
31MAPSF17A	*F. flocciferum*	*P. ponderosa*	40.77	197	99	47.57
SC7	*F. fredkrugeri*	*P. taeda*	46.23	334	98.6	47.24
SC5	*F. fujikuroi*	*P. taeda*	43.8	171	98.4	48.23
JTHABCO3.1	*F. inflexum*	*Abies concolor*	52.61	1637	98.8	47.74
NC8	*F. ipomoeae*	*P. teada*	38.79	56	98.6	48.09
MES8	*F. lactis*	*P. ponderosa*	54.97	1164	98.5	50.58
CC	*F. luffae*	*Pseudotsuga menziesii*	50.75	217	97.8	52.45
MES6	*F. oxysporum*	*P. ponderosa*	49.71	805	98.6	47.6
NAPSME6.1	*F. solani*	*Pseudotsuga menziesii*	51.79	601	98.7	50.82
DCN062.2H	*F. torulosum*	*Pseudotsuga menziesii*	43.12	499	98.9	47.55
MES5	*F. tricinctum*	*P. ponderosa*	44.45	253	99	47.71
NAGrPIPO5	*F. verticillioides*	*P. ponderosa*	42.84	467	98.4	48.25

toxins, such as fujikurin and fumonisin, that are only found in the FFSC (Table S5).

3.4 | Similarity Among Conifer-Associated *Fusarium* spp.

Analyses of total genes and their pathogenicity gene profiles for *F. commune* isolates collected conifer hosts (e.g., Douglas-fir, ponderosa pine), nonconifer hosts (e.g., rice, tomato), and soil revealed that the orthologous protein profiles were dissimilar among isolates originating from a conifer host, nonconifer host, and soil ($p = 0.024$ and $R^2 = 0.315$; Figure 4A). In contrast, comparisons of putative pathogenicity/virulence genes ($p = 0.399$ and $R^2 = 0.289$; Figure S2A), secondary metabolite ($p = 0.245$ and $R^2 = 0.282$; Figure S2B), and CAZyme ($p = 0.464$ and $R^2 = 0.174$; Figure S2C) gene profiles did not reveal significant separation among isolates derived from conifer hosts, nonconifer hosts, and soil. For this reason, genes conveying host specificity could not be clearly attributed to specific functions. However, some putative virulence genes including VFGLU1 (PHI:323 Beta-1,6-glucanase), Mocapn9 (PHI:6612 Calpain), HOG1 (PHI:149 MAP kinase), and BcSAK1 (PHI:1031 stress-activated MAPK) were found only in conifer-associated isolates F13.4.1 (collected from Douglas-fir) and JICPIPO1 (collected from ponderosa pine). In contrast, PCK1 (PHI:424 Phosphoenolpyruvate carboxykinase), Mopsr1 (PHI:11582 Serine/threonine-protein phosphatase dullard), and Annexin_A7 (PHI:2115 Unknown) were only found in nonconifer host-associated isolates (Table S6A).

Although significant genomic separation was not found among *F. commune* isolates based on their hosts or substrate, origin-associated variation was found for some aspects of the predicted metabolome. For example, differences were observed in secondary metabolite gene clusters and predicted metabolite-encoding genes, including the following: (1) the RiPP-like genes, lucilactaene, ilicolin H, and butyrolactone were uniquely identified in the ponderosa pine-associated isolate; (2) lijiquinone, fusatrixin, fungal RiPP-like genes,

TABLE 4 | Comparison of gene content within the *Fusarium* genomes used in this study.

Isolate	*Fusarium* species	Predicted genes	BUSCO completeness (%)	Secretome	Effectors	PHIbase	CAZymes	Secondary metabolite genes	Predicted metabolites
NASWWP3	*F. annulatum*	15,320	98.7	1680	583	552	675	65	20
NC74	*F. avenaceum*	13,485	98.7	1555	563	540	699	63	20
NAGrPIST2	*F. clavum*	17,440	98.8	2207	897	545	1024	45	11
B62	*F. commune*	14,958	98.6	1682	621	557	697	60	17
F10.2.2	*F. commune*	15,624	98.7	1727	614	573	705	58	19
F13.4.1	*F. commune*	15,969	98.8	1795	657	598	770	61	18
JCM11502	*F. commune*	14,567	99.1	1632	589	559	704	59	18
JICPIPO1	*F. commune*	17,105	98.4	2029	742	567	740	60	20
MiAE120	*F. commune*	14,573	98.9	1522	569	560	629	53	18
NRRL28387	*F. commune*	14,960	98.5	1689	600	577	701	60	19
SC35	*F. falciforme*	17,518	99.0	1921	596	561	826	50	13
31MAPSF17A	*F. flocciferum*	12,561	99.0	1429	540	532	640	56	16
SC7	*F. fredkrugeri*	14,850	98.7	1540	541	553	704	58	19
SC5	*F. fujikuroi*	14,692	98.5	1621	566	550	746	60	18
JTHABCO3.1	*F. inflexum*	16,624	98.8	1894	674	583	853	55	15
NC8	*F. ipomoeae*	12,721	98.6	1431	511	535	636	49	10
MES8	*F. lactis*	22,049	98.5	3136	1110	547	923	65	20
CC	*F. luffae*	21,632	97.9	2969	1097	536	803	57	12
MES6	*F. oxysporum*	16,150	98.7	1767	618	566	834	56	13
NAPSME6.1	*F. solani*	16,955	98.8	1817	571	560	776	45	11
DCN062.2H	*F. torulosum*	13,374	98.9	1478	557	541	657	58	21
MES5	*F. tricinctum*	14,046	99.0	4623	604	549	706	62	21
NAGrPIPO5	*F. verticillioides*	14,078	98.5	1552	522	540	701	57	16

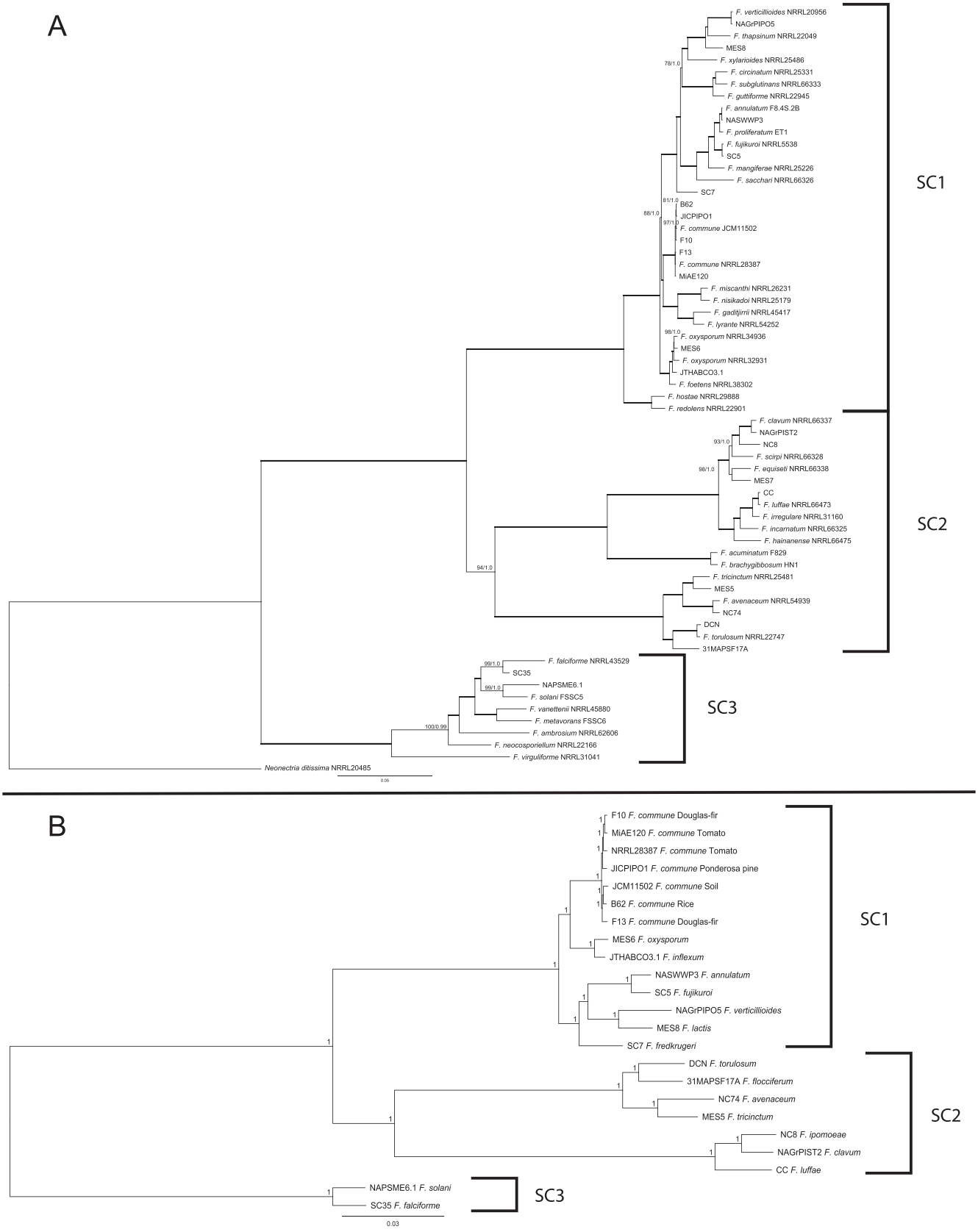

FIGURE 1 | Legend on next page

FIGURE 1 | A) Maximum likelihood phylogeny inferred from 16 loci (*acl1*, *cal1*, *dpa1*, *dpe1*, *fas1*, *fas2*, *lcb2*, *mcm7*, *pgk1*, *rpb1*, *rpb2*, *tef1*, *top1*, *tsr1*, *tub1* and *tub2*) with bootstrap (bs) values > 78 and posterior probability (pp) values > 0.99 (nodes with thicker lines indicate of bs = 100 and pp. = 1.0) showing good support for *Fusarium* species complex clades (SC1: Species complex clade 1 including *F. fujikuroi*, *F. nisikadoi* and *F. oxysporum* species complexes; SC2: Species complex clade 2 including *F. incarnatum-equiseti* and *F. tricinctum* species complexes; SC3: Species complex clade 3 including *F. solani* species complex). The phylogeny resolved isolates analysed in this study to species that had available reference genomes. Those isolates (*F. flocciferum*, *F. fredkrugeri*, *F. inflexum*, *F. ipomoeae* and *F. lactis*) without available reference genomes were confirmed based on species complex and BLAST analysis. Abbreviations for loci names are described in Table 2. B) Species tree inferred from 5,677 orthologous proteins also shows good support for *Fusarium* species complex clades.

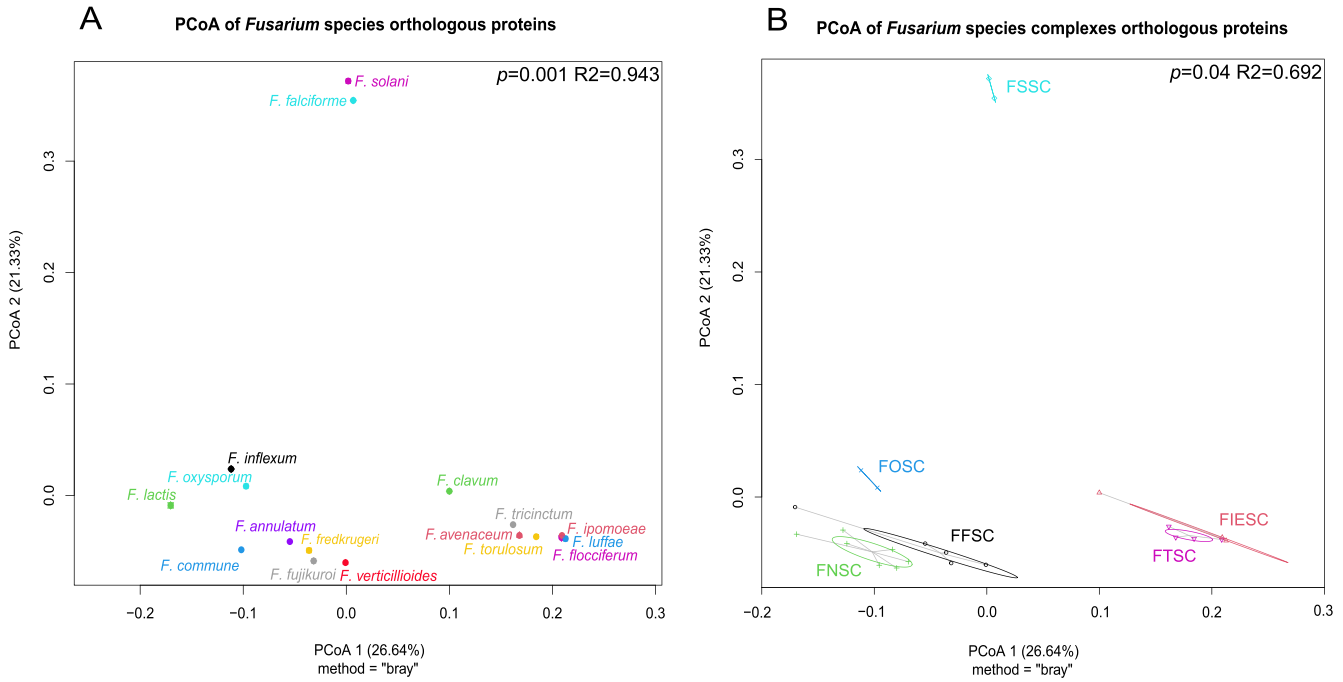

FIGURE 2 | (A) A principal coordinates analysis (PCoA) of orthologous proteins shared among and between 17 *Fusarium* species. (B) Of the six identified *Fusarium* species complexes, the *F. solani* species complex (FSSC) was the most dissimilar. The *F. fujikuroi* (FFSC), *F. oxysporum* (FOSC), and *F. nisikadoi* (FNSC) species complexes ordinated closely, and likewise, the *F. tricinctum* (FTSC) and *F. incarnatum-equiseti* (FIESC) species complexes ordinated close together.

fosfonochlorin, enniatin, choline, and AbT1 were identified in both the conifer- and nonconifer-associated isolates, but were not found in the soil-derived isolate; (3) more copies of fungal RiPP-like genes were found in conifer-associated isolates, with higher numbers in isolates JICPIPO1 and F13.4.1; (4) fusatrixin was found in both conifer- and rice-associated isolates; (5) more copies of T1PKs and terpenes were found in nonconifer-associated and soil-derived isolates, and (6) subglutinol A and B were only found in the rice-associated isolate (Table S6B).

When comparing orthologs of total proteins encoded in *F. commune* isolates based on hosts, 32 orthogroups were identified as unique to the three conifer-collected isolates (Figure 4B). When comparing these *F. commune* conifer-unique orthogroups with the other conifer-associated *Fusarium* spp., 10 orthogroups were identified as shared among all conifer isolates (Figure 5 and Table S7). However, these genes did not have significant similarity to proteins currently in the databases used in this study.

4 | Discussion

In this work, we used whole-genome sequencing and annotation to compare the genomes of 17 *Fusarium* spp. collected from symptomatic conifer seedlings, of which seven (*F. lactis*, *F. torulosum*, *F. flocciferum*, *F. ipomoeae*, *F. clavum*, *F. luffae*, and *F. falciforme*) were only recently reported on conifers (Dobbs et al. 2023). We found orthologous proteins that were shared among *Fusarium* species complexes, which suggests a shared evolutionary history among putative virulence proteins and secondary metabolite production within six *Fusarium* species complexes (FFSC, FNSC, FOSC, FIESC, FTSC, and FSSC) and larger species complex clades (SC1 including FFSC, FNSC, and FOSC; SC2 including FIESC and FTSC, and SC3 including FSSC). We identified unique genes shared among putative *Fusarium* pathogens of conifers, suggesting that conifer-associated isolates may contain host-specific genes involved in host colonisation, pathogenicity, or virulence. These genes could serve as targets for identification/detection of conifer pathogens. Although we identified genes that were

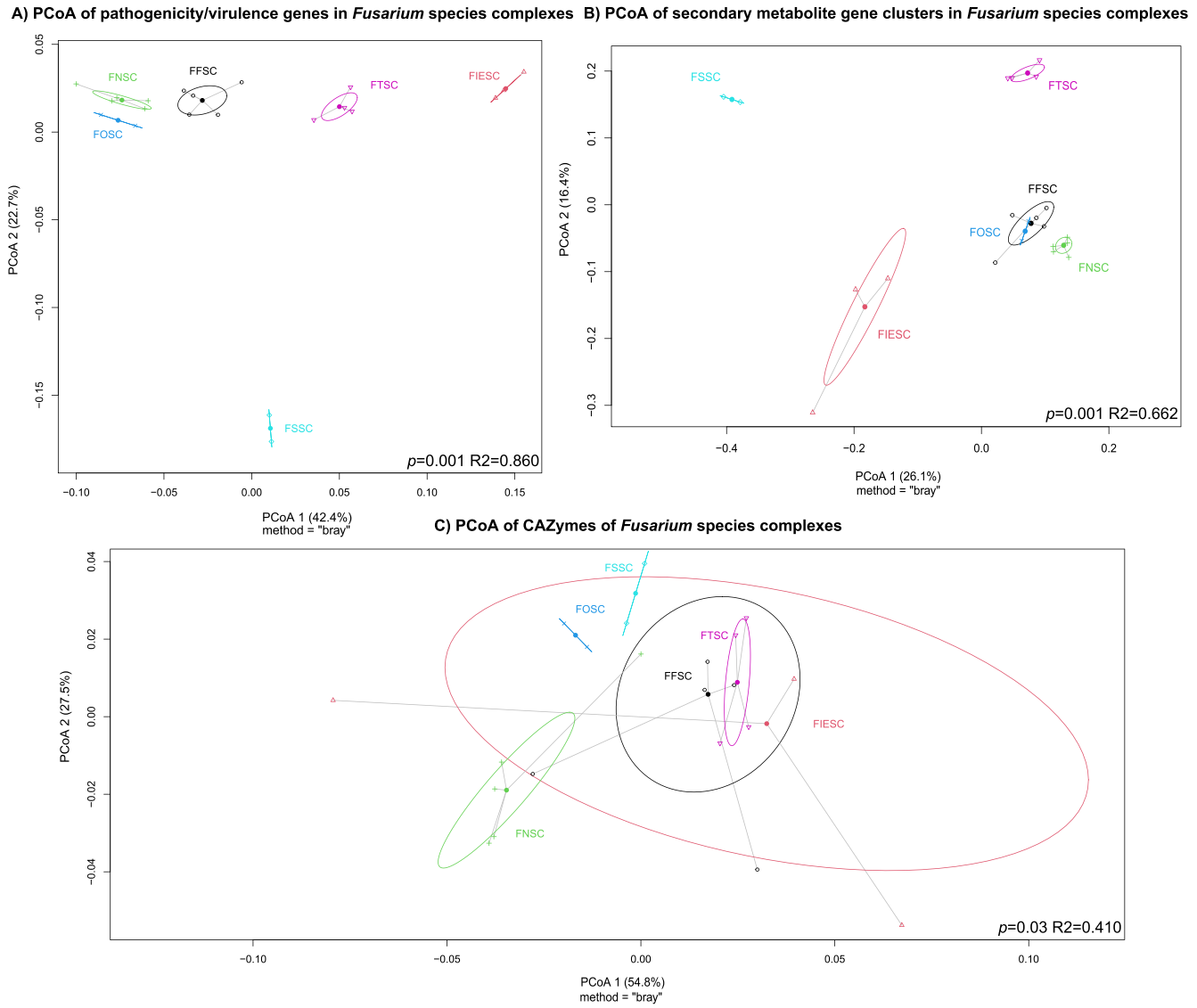

FIGURE 3 | Principal coordinates analysis (PCoA) of putative gene functions shows clustering of *Fusarium* species complexes when comparing putative pathogenicity/virulence genes (A) and secondary metabolites (B). *F. nisikadoi* (FNSC), *F. fujikuroi* (FFSC), and *F. oxysporum* (FOSC) ordinated closely together and may indicate that they contain similar virulence pathways and secondary metabolites. Analysis of CAZymes (C) did not show the same clustering of *Fusarium* species complexes *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes.

unique to conifer-associated isolates, we further showed that it may not be possible to identify a single gene across *Fusarium* species and species complexes that determines pathogenicity to conifer seedlings. In contrast, we provide evidence that it may be reasonable and necessary to identify multiple genes or downstream products that play roles in multiple pathways used by *Fusarium* lineages for pathogenicity to conifer hosts. For this reason, further study is necessary to better understand the roles of virulence- and secondary metabolite-associated gene expression in the pathogenicity and host-specificity of *Fusarium* species and lineages.

In this study, we investigated conifer-associated *Fusarium* species contained within six *Fusarium* species complexes: *Fusarium fujikuroi* (FFSC; SC1), *F. nisikadoi* (FNSC; SC1), *F. oxysporum* (FOSC; SC1), *F. incarnatum-equiseti* (FIESC; SC2), *F. tricinctum* (FTSC; SC2), and *F. solani* (FSSC; SC3) species complexes. Based on the phylogenetic and orthologous putative protein analyses, we observed a correlation between species complex clades (i.e., SC1, SC2, and SC3) and similarities among the gene profiles of closely related species complexes (Figures 1 and 2), which parallels similar results that have been found in previous phylogenetic and phylogenomic studies where multigene phylogenies were constructed for *Fusarium* (Geiser et al. 2021; Torbati et al. 2019; Crous et al. 2021).

Since most of the *Fusarium* species that were examined in this study have been reported as plant pathogens, we expected to find both unique and shared putative virulence genes among these species/species complexes and their respective species complex clades (SC1–SC3). For example, the SGE1 gene, which has been identified as important for virulence as a regulator of effectors and secondary metabolite production (Brown, Busman, and Proctor 2014), was found in the SC1 and SC2, but interestingly, was lacking in the SC3. Many of the unique putative virulence proteins in this study were described as associated with secondary

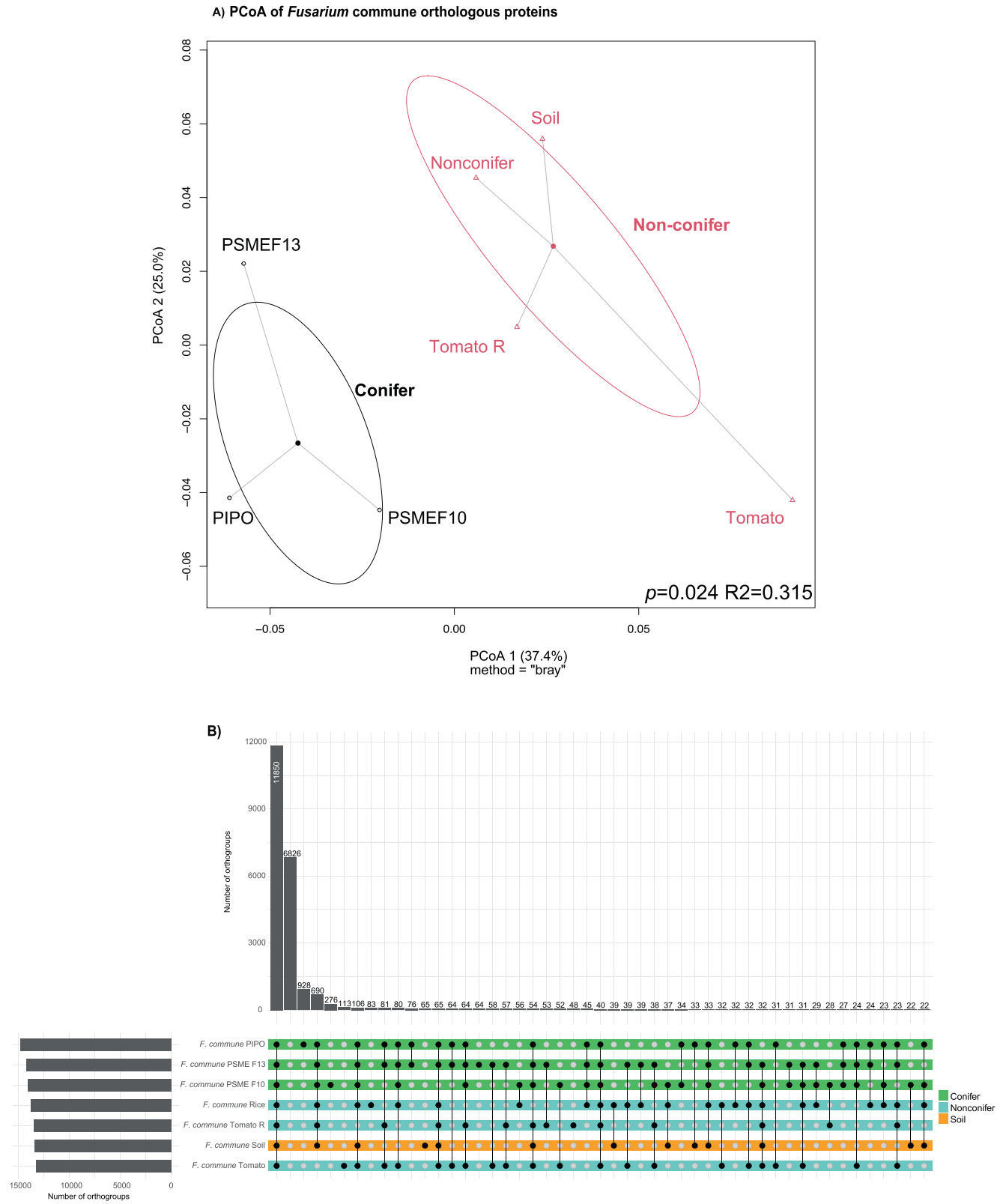

FIGURE 4 | (A) A principal coordinates analysis (PCoA) of *F. commune* isolates collected from conifer, nonconifer, and soil showed clustering based on host origins. (B) An UpSet plot of orthologous proteins from *Fusarium commune* isolates collected from conifer [in green: Ponderosa pine (*Pinus ponderosa*; PIPO), and Douglas-fir (*Pseudotsuga menziesii*; PSME)], nonconifer hosts [in sky blue: Rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*)], and soil (in orange). Thirty orthogroups were found exclusive to conifer-associated isolates.

metabolite production. In *Fusarium* spp., secondary metabolites are compounds produced by the fungus that are nonessential, except for roles in pathogenesis and competition with other microbes (Avalos and Limón 2021). In genomics analyses, gene clusters have been identified in the production of these secondary metabolites (Blin et al. 2023) and combinations of these secondary metabolites

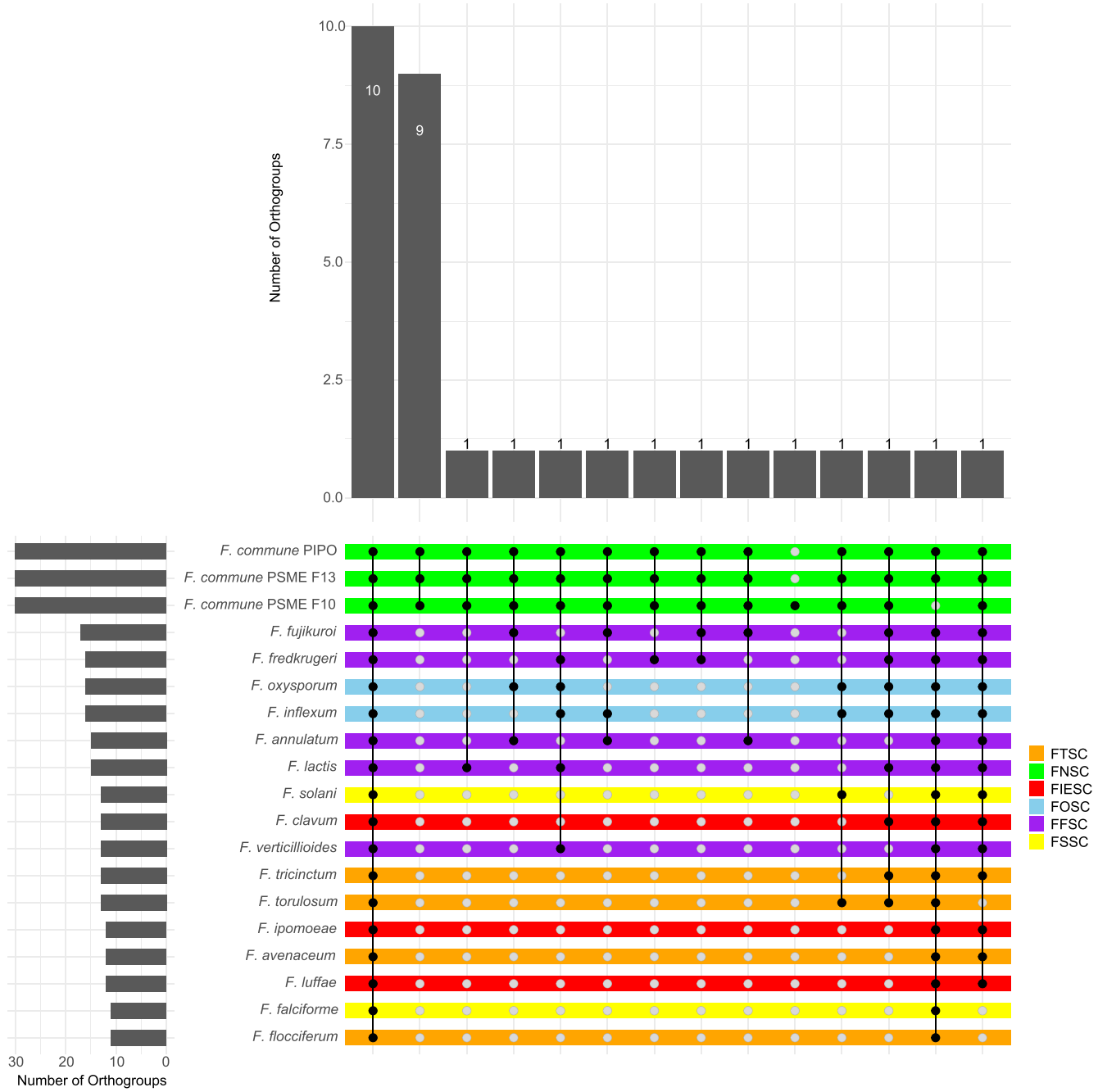

FIGURE 5 | An UpSet plot of *Fusarium commune* conifer-unique orthogroups compared to orthologous proteins from 23 *Fusarium* spp. isolates (17 *Fusarium* spp.) collected from conifer hosts. Orthogroups shared among species complexes were highlighted with 10 orthogroups identified as shared among all conifer-associated *Fusarium* spp.

have been linked to host-specificity (Niehaus et al. 2016; Witte, Harris, et al. 2021; Witte, Villeneuve, et al. 2021). Interestingly, in the *Fusarium* genomes of the three species complex clades (SC1–SC3) analysed in this study, SC1 uniquely possessed the depudecin gene cluster (dep1-5), which was previously identified in the putative virulence genes and secondary metabolite genes through comparative genomics (Wight et al. 2009).

Depudecin is a polyketide mycotoxin that acts as a histone deacetylase inhibitor (Wight et al. 2009). Fusaric acid was also identified as unique to SC1, as was expected since it has been previously reported in species within the FFSC and FOSC (Liu et al. 2020). Enniatin, which is an important mycotoxin, was shared among FNSC and FOSC, and it has been previously found in the FFSC and FOSC (Gautier, Pinson-Gadais, and Richard-Forget 2020). A primary finding of our study is that combinations of secondary metabolite genes, unique and common across species, may be necessary for conifer pathogenesis, but further research is needed to better understand the pathogenic roles of secondary metabolite gene combinations.

4.1 | Similarities Among Hosts and Virulence Pathways Within *Fusarium* Species Complex Clades (SC1–SC3)

4.1.1 | *Fusarium* Species Complex Clade 1 (SC1 Including FFSC, FNSC and FOSC Species Complexes)

Many agriculturally important *Fusarium* species have been reported as belonging to the *F. fujikuroi* species complex (FFSC). In this study, we analysed the genomes of five conifer-associated *Fusarium* spp. in the FFSC, including *F. annulatum*, *F. fujikuroi*, *F. fredkrugeri*, *F. lactis*, and *F. verticillioides*. *Fusarium annulatum*, *F. fujikuroi*, and *F. lactis* have all been previously reported as pathogens to nonconifer hosts including grapevine (*Vitis* sp.), millet (*Panicum* sp.), melon (*Cucumis* sp.), wheat (*Triticum aestivum*), corn (*Zea mays*), rice (*Oryza sativa*) and sweet pepper (*Capsicum annuum*) (Sekiguchi et al. 2021; Choi et al. 2021; Wiemann et al. 2013; Medeiros Araújo et al. 2021; Niehaus et al. 2016; Brown, Busman, and Proctor 2014). *Fusarium verticillioides* has been reported as pathogenic to conifer hosts (Maciel et al. 2017); however, *F. fredkrugeri* has not been reported as a pathogen (Sandoval-Denis, Swart, and Crous 2018). In the FFSC, putative virulence genes, such as SGE1, have been found to be vital to virulence as regulators of the production of many effectors and the production of secondary metabolites (e.g., fumonisins and fujikurins) (Brown, Busman, and Proctor 2014; Atanasoff-Kardjalieff and Studt 2022). In this study, the SGE1 gene was found in the isolates of *F. avenaceum*, *F. commune*, *F. inflexum*, *F. oxysporum*, *F. torulosum*, and *F. verticillioides*, suggesting that these isolates are likely pathogenic. The SGE1 gene was not found in the isolate of *F. fredkrugeri*, a potential indication that this isolate is not a plant pathogen.

Fusarium inflexum is a member of the *F. oxysporum* species complex (FOSC) that is not well studied or well characterised. It was originally described as a pathogen on beans (*Vicia faba*) (Schneider and Dalchow 1975), but recent investigations of this species have focused on its potential use as a biocontrol agent against nematodes (Lira et al. 2020). In contrast, *F. oxysporum* is a very well-characterised species with high-quality genomes available and descriptions of its various formae speciales (Edel-Hermann and Lecomte 2019). Effector profiles have been well characterised for several *Fusarium* species, including *F. oxysporum*. In particular, the SIX effectors have been used to determine host-specificity and a single gene has been identified as necessary for host specialisation (Czislowski, Zeil-Rolfe, and Aitken 2021; Li, Fokkens, and Rep 2020). Based on a BLAST search of SIX genes present in the *Fusarium* spp. genomes studied here, SIX2, SIX4, SIX9, and SIX14 genes were identified in conifer-associated *Fusarium* spp. SIX2 was found in *F. annulatum* and *F. circinatum*, which was expected since it has previously been found to be shared among other members of the FFSC—a potential example of horizontal gene transfer of SIX genes from *F. oxysporum* to other species (van Dam et al. 2017). SIX4 has been identified as necessary for high virulence on plant hosts due to its role in repressing host defence response (Jangir et al. 2021; Jenkins et al. 2021). SIX4 was found in *F. commune* and *F. verticillioides*, which have both been described as pathogens of conifer seedlings, suggesting a potential role in of SIX4 in virulence on conifers. SIX9, found in *F. redolens*, and SIX14, found in *F. lactis* and *F. verticillioides*, have both been described as unnecessary for pathogenicity; however, these genes could be related to virulence in specific hosts (Jenkins et al. 2021).

Although SIX genes are important for pathogenicity and/or virulence, their expression and that of SGE1 are regulated by two transcription factors, *Fusarium* transcription factors (FTF) 1 and 2 (Jangir et al. 2021). FTF1 appears to be lineage-specific and housed on accessory chromosomes (Jangir et al. 2021). Because FTF1 was only found in isolates of two species in this study, *F. commune* and *F. oxysporum*, it could be inferred that these isolates can express these SIX genes or they may share a lineage-specific chromosome necessary for SIX gene expression (Jangir et al. 2021). However, FTF1 and the SIX4 gene were found in two separate isolates of *F. commune*, which could be interpreted as SIX genes are not necessary for *Fusarium* pathogenicity and/or virulence on conifers. Alternatively, if these two isolates interacted, they could potentially transfer the FTF1-containing accessory chromosome to the SIX gene-containing strain or interact in other manners that could potentially affect pathogenicity/virulence on conifer seedling hosts. SIX genes were not identified in the *F. oxysporum* isolate in this study, which may indicate that it is a nonpathogenic strain or that SIX genes are not necessary for pathogenicity to conifer seedlings. However, FOW2 (PHI:734), a Zn finger transcription factor that has also been described as important for virulence in FOSC members, was found in all the *Fusarium* isolates surveyed in this study. FOW2 was identified within the *F. oxysporum* genome as being necessary for invasion and colonisation of plant hosts (Jangir et al. 2021). This perhaps indicates that all the studied isolates are potential plant pathogens, but it likely does not indicate that all of these isolates are conifer pathogens.

Fusarium commune is the only member of the *F. nisikadoi* species complex (FNSC) that we investigated since it was the only species in this complex that we recovered from conifer seedlings in our previous survey (Dobbs et al. 2023). Recently, *F. commune* has been described as a pathogen on both coniferous and nonconiferous hosts including rice, tomato, lotus (*Nelumbo nucifera*), and Douglas-fir (Stewart et al. 2012; Hamini-Kadar et al. 2010; Kuang et al. 2022; Husna, Zakaria, and Mohamed Nor 2021). A transcriptome analysis of *F. commune* pathogen of lotus root showed that genes involved in carbon metabolism and effectors were important for pathogenesis (Kuang et al. 2022). In this study, we found that the isolates collected from symptomatic ponderosa pine and Douglas-fir seedlings (JICPIPO1 and F13, respectively) had the most predicted effectors and CAZymes compared to the other *F. commune* isolates, which may indicate that these isolates are highly virulent isolates to conifer seedlings. However, virulence assays are necessary to confirm the connections among effectors and CAZymes with pathogenicity/virulence to coniferous hosts. Based on their genome content, all studied isolates have the potential to produce secondary metabolites, such as enniatin, depudecin, gibberellin, fusaric acid, alterpyranone, and subglutinol A/B, while infecting their hosts. Metabolomic and/or transcriptomic analyses would allow for the identification of pathogenicity/virulence genes involved in metabolism and effector production that are expressed during pathogenesis on conifer seedlings.

4.1.2 | *Fusarium* Species Complex Clade 2 (SC2 Including FIESC and FTSC Species Complexes)

We analysed three members of the *F. incarnatum-equiseti* species complex (FIESC), *F. ipomoeae*, *F. luffae*, and *F. clavum*, all of which have been described as pathogens of nonconiferous hosts including peanut (*Arachis hypogaea*), maize and tomato (Gilardi et al. 2021; Parime et al. 2022; Xu et al. 2021). None of these species have been tested for pathogenicity on conifers. Secondary metabolite gene clusters and products, such as enniatin, fusarin, and zearalone, have been found to vary among members of the FIESC, likely due to horizontal gene transfers that effect the functionality of the metabolic pathways (Villani et al. 2019). Zearalone was identified in *F. clavum* and *F. luffae*, while deoxynivalenol (DON) was identified in *F. ipomoeae* and *F. luffae*. However, enniatin and fusarin were not identified in any of these three species (*F. clavum*, *F. luffae*, and *F. luffae*), suggesting isolates studied herein may be pathogens but the lack of some mycotoxin-associated genes that may affect pathogenicity or virulence on conifer hosts.

The *F. tricinctum* species complex (FTSC) members are well-known cereal pathogens that cause head and seedling blight on wheat and barley (*Hordeum* sp.) and crown rot on wheat, but FTSC members have also been described as pathogens of pea (*Pisum sativum*) and faba bean (*Vicia faba*) (Šišić et al. 2020; Inbaia, Farooqi, and Ray 2023; Karlsson, Persson, and Friberg 2021; Wang et al. 2022). In addition, FTSC members are known for producing mycotoxins, such as enniatins and moniliformin, that can contaminate grain (Wang et al. 2022). The genomes of *F. tricinctum*, *F. avenaceum*, *F. flocciferum*, and *F. torulosum* were examined in this study, of which, only *F. avenaceum* has been previously reported as a pathogen of conifer seedlings (Morgan 1983; James et al. 1989). It has been posited that *F. tricinctum* is not a pathogen of conifer seedlings, even though it is commonly isolated from conifer seedlings (James 2004). In this study, isolates of *F. tricinctum* and *F. torulosum* did not share secondary metabolites that were identified in *F. avenaceum*, further suggesting these two species likely are not conifer pathogens (Table S5). The *F. avenaceum* isolate had unique predicted secondary metabolites and shared secondary metabolites with SC2. Enniatin, which has been found to be important for pathogenicity on plant hosts (Inbaia, Farooqi, and Ray 2023), was not found in the *F. avenaceum* isolate; however, apicidin gene clusters were identified in the *F. avenaceum* isolate. The apicidin gene cluster was recently found on accessory chromosomes in a closely related species, *F. poae* (Witte, Harris, et al. 2021), suggesting that this secondary metabolite may be important for conifer pathogenesis and that an accessory chromosome may be present in this *F. avenaceum* isolate.

4.1.3 | *Fusarium* Species Complex Clade 3 (SC3 Including FSSC Species Complex)

Taxonomists are currently disputing the taxonomic placement of the *F. solani* species complex (FSSC), and changing the genus from *Fusarium* to *Neocosmospora* has been recently proposed (Crous et al. 2021). A recently described species, *F. falciforme* (syn. *N. falciforme*) (Herron et al. 2015) has been reported as a pathogen of melons (Medeiros Araújo et al. 2021). Another member of FSSC, *F. solani* (syn. *N. solani*), has been described as a pathogen of both conifer and nonconifer hosts (James and Perez 2000; Šišić et al. 2018). Pectase lyase genes, which were identified in the FSSC species, have been described as important for the pathogenicity of *F. solani* (Covey et al. 2014).

4.2 | Unique Genes for Conifer Pathogens

In our study, we identified 12 conifer-exclusive genes. Though these genes did not have described functions based on BLAST similarity to proteins in databases searched in this study, they provide targets for further analyses including validation of expression through transcriptomic or functional analyses, such as proteomics or metabolomics during infection within a host. Though we did not conduct virulence assays on isolates in this study, comparing isolates based on their pathogenicity/virulence on conifer hosts may also provide gene targets to develop molecular tools for rapid identification of conifer pathogen.

4.3 | Detection Techniques

Methods to rapidly identify pathogenic members within the *Fusarium* species complexes through genetic, genomic, proteomic, and metabolomic analyses are necessary to develop robust management strategies to detect conifer pathogens, mitigate pathogen spread, and manage disease outbreaks. Conserved gene loci and comparative genomics have been previously used to generate PCR-based detection methods for *Fusarium* spp. (Dobbs et al. 2020; Pramunadipta et al. 2022). Soil and plant material samples can serve as substrates for detecting/identifying *Fusarium* spp. pathogens within a growing system by metabarcoding of environmental DNA (eDNA) (Cobo-Díaz et al. 2019). Effector gene profiles and protein mass spectral fingerprinting have also been used to identify pathogenic *Fusarium* spp. (Wigmann et al. 2019; Czislowski, Zeil-Rolfe, and Aitken 2021). However, secondary metabolites and the genes associated with their production can also be used to effectively and robustly differentiate pathogenic isolates of *Fusarium* spp. (Niehaus et al. 2016; Wiemann et al. 2013). The gene profiles and the putatively produced secondary metabolites identified in this study among *Fusarium* species complexes and species complex clades (e.g., SC1, SC2 and SC3) should prove useful for the development of rapid detection tools to identify conifer pathogens within *Fusarium*.

5 | Conclusions

In this study, we used whole-genome sequencing and predicted genes to analyse the relationships among *Fusarium* isolates and found clustering among closely related species complexes. This work provides draft genomes for conifer-associated *Fusarium* spp. and other genomes that were not previously referenced in public databases (e.g., *F. lactis*, *F. fredkrugeri*, *F. ipomoeae*, and *F. flocciferum*). We also took a closer look at a single species, *F. commune*, to determine which genes associated with host specialisation were present among isolates derived from conifer and nonconifer hosts. Also in this study, we predicted *Fusarium* putative pathogenicity/virulence genes

for conifers through genome comparisons; however, phenotypic validation through pathogenicity/virulence assays, transcriptomic, and metabolomic analyses are necessary to confirm the pathogenicity/virulence of the 17 *Fusarium* spp. isolates used in this study, confirm the expression of putative pathogenicity/virulence genes for conifers, and confirm the presence of secondary metabolites that contribute to pathogenicity and/or virulence. Based on our previous survey, we found associations of *Fusarium* species complexes with multiple conifer hosts including *Picea* spp. (FFSC, FOSC, FNSC, FIESC, FTSC, and FSSC), *Pinus* spp. (FFSC, FOSC, FNSC, FIESC, FTSC, and FSSC), *Pseudotsuga menziesii* (FFSC, FOSC, FNSC, FTSC, and FSSC), *Larix* spp. (FFSC), *Cupressus* spp. (FFSC, FOSC, FNSC, and FTSC), *Juniperus* spp. (FFSC, FOSC, and FSSC), *Sequoia* spp. (FOSC), and *Abies* spp. (FFSC, FOSC, and FNSC). These wide host associations suggest that directed pathogenicity assays of *Fusarium* spp. on these host genera would prove useful to determine conifer-host specialists and generalists.

Mycotoxins were shown to be specific to species complexes of the conifer-associated *Fusarium* spp. isolates used in this study. This finding is noteworthy as it determines that potential targets for detecting/identifying conifer pathogens within *Fusarium* will likely require multiple target-specific methods, depending on the species complex clades. Our results also support the importance of mass spectrometry as an alternative method for pathogen detection method. In addition, host responses to these mycotoxins is perhaps another useful approach that warrants further investigation for rapid detection of infected hosts through air sampling within nurseries to detect pathogens and disease.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in the NCBI GenBank databank at https://www.ncbi.nlm.nih.gov/, reference number PRJNA1020449.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.