The Genome of the Saprophytic Fungus *Verticillium tricorpus* Reveals a Complex Effector Repertoire Resembling That of Its Pathogenic Relatives

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Vascular wilts caused by *Verticillium* spp. are destructive plant diseases affecting hundreds of hosts. Only a few *Verticillium* spp. are causal agents of vascular wilt diseases, of which *V. dahliae* is the most notorious pathogen, and several *V. dahliae* genomes are available. In contrast, *V. tricorpus* is mainly known as a saprophyte and causal agent of opportunistic infections. Based on a hybrid approach that combines second and third generation sequencing, a near-gapless *V. tricorpus* genome assembly was obtained. With comparative genomics, we sought to identify genomic features in *V. dahliae* that confer the ability to cause vascular wilt disease. Unexpectedly, both species encode similar effector repertoires and share a genomic structure with genes encoding secreted proteins clustered in genomic islands. Intriguingly, *V. tricorpus* contains significantly fewer repetitive elements and an extended spectrum of secreted carbohydrate-active enzymes when compared with *V. dahliae*. In conclusion, we highlight the technical advances of a hybrid sequencing and assembly approach and show that the saprophyte *V. tricorpus* shares many hallmark features with the pathogen *V. dahliae*.

Vascular wilt diseases caused by members of the fungal genus *Verticillium* collectively affect hundreds of plant hosts (Fradin and Thomma 2006; Klosterman et al. 2009). Genus *Verticillium* was recently revised to encompass only ten species of soil-borne fungi that differ in morphological features, such as their resting structures, as well as in their ability to cause plant diseases (Inderbitzin et al. 2011). Within this genus, *V. dahliae* is the most notorious plant pathogen, due to the ability to cause vascular wilt on hundreds of dicotyledonous plant species, including ecologically important plant hosts and many high-value crops (Fradin and Thomma 2006; Klosterman et al. 2009). Furthermore, *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae*,

and *V. longisporum* are also plant pathogens, albeit with narrower host ranges and more restricted distribution than *V. dahliae* (Inderbitzin and Subbarao 2014). However, not all *Verticillium* species are genuine plant pathogens (Inderbitzin and Subbarao 2014). For instance, although occasionally reported as an opportunistic pathogen, *V. tricorpus* is mainly known as a soil-borne saprophyte that thrives on decaying organic matter (Isaac 1967; Klosterman et al. 2009; Powell et al. 2013; Qin et al. 2008). Comparative genomics between pathogenic members of the *Verticillium* genus, in particular strains of *V. dahliae*, recently facilitated the identification of key factors that mediate successful establishment of host infection and mechanisms that foster adaptation in the evolutionary arms race with plant hosts (de Jonge et al. 2012; 2013; Klosterman et al. 2011; Seidl and Thomma 2014). To further identify genomic differences within the *Verticillium* genus and to identify genetic components that enable particular *Verticillium* species to successfully infect plant hosts, determination of the genome sequence of nonpathogenic members of the *Verticillium* genus, such as *V. tricorpus*, is instrumental.

In the past, de novo sequencing of complex eukaryotic genomes was expensive and time-consuming. However, recent advances in next-generation sequencing technologies, which can now be divided into second and third generation, facilitate affordable, rapid, and high-quality genome sequencing (Faino and Thomma 2014; Metzker 2010). Second generation sequencing generates billions of short strings of nucleotides (called reads) of high quality and can be used for genome resequencing, in which genome assemblies are based on mapping of reads to a reference genome, as well as for de novo genome sequencing. However, de novo assemblies that are based on short reads are obstructed by repetitive elements, GC- or AT-rich stretches, and homo-nucleotide stretches. Consequently, genome assemblies based only on second generation sequencing generally contain many gaps, leading to fragmented draft genomes (Faino and Thomma 2014). Third generation technology, such as single molecule real-time (SMRT) sequencing, generates long continuous sequences (up to 35 kb) that overcome assembly problems by increasing the probability of having unique overlaps between reads (Faino and Thomma 2014; Schadt et al. 2010). However, SMRT sequencing has a high per-base error rate that can only be corrected by large sequence depths, which results in considerable sequencing costs. To overcome the specific limitations of second and third generation sequencing platforms, hybrid approaches have been developed (Faino and Thomma 2014; Gnerre et al. 2011; Koren et al. 2012). Such

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approaches combine reads generated by second and third generation sequencing platforms to maximize continuity and minimize the per-base error rate. So far, however, hybrid approaches have only been used for relatively small microbial (bacterial) genomes (Koren et al. 2012).

Here, we report on the genome of the saprophyte *V. tricolorpus*. We applied a recently described hybrid pipeline (Faino and Thomma 2014) to establish a draft genome of *V. tricolorpus*. This 36-Mb genome assembly encompasses 58 nearly gapless scaffolds, of which four represent complete chromosomes. Comparative genomics between *V. tricolorpus* and *V. dahliae* was performed in an attempt to identify genomic features that foster pathogenicity within the *Verticillium* genus.

RESULTS AND DISCUSSION

V. tricolorpus MUCL9792

does not cause wilt disease on tomato.

In order to confirm the inability of *V. tricolorpus* to cause Verticillium wilt disease on tomato (*Solanum lycopersicum*), inoculations were carried out with *V. tricolorpus* MUCL9792 on 'MoneyMaker' tomato plants that lack any characterized source of Verticillium wilt resistance (Fradin et al. 2009). As controls, inoculations were performed with two *V. dahliae* strains, VdLs17 and JR2, that have previously been characterized as relatively weak and strong tomato pathogens, respectively (de Jonge et al. 2013; Fradin et al. 2009; Klosterman et al. 2009). As expected, inoculation of tomato plants with *V. dahliae* JR2 resulted in clear stunting of the inoculated plants as a typical symptom of Verticillium wilt disease, whereas inoculation with strain VdLs17 resulted in considerably fewer disease symptoms, as only mild stunting was observed (Fig. 1A). In contrast, inoculation of tomato plants with *V. tricolorpus* did not lead to visible stunting of tomato plants (Fig. 1A). This was

quantitatively corroborated as plants inoculated with *V. dahliae* strains JR2 or VdLs17 show considerably reduced foliage area when compared with mock-inoculated plants, while plants inoculated with *V. tricolorpus* do not show any reduction (Fig. 1B). Moreover, fungal recovery assays using stem sections of the inoculated plants showed that all sections of *V. dahliae*-inoculated plants were colonized by fungus, while no fungal biomass was recovered from stem sections of *V. tricolorpus*-inoculated plants (Fig. 1C). Finally, real-time polymerase chain reaction (PCR) quantification of fungal biomass revealed that no *V. tricolorpus* biomass could be recorded in inoculated tomato plants, while ample *V. dahliae* biomass could be detected in VdLs17- and JR2-inoculated plants (Fig. 1D). These data confirm previous observations that *V. tricolorpus* is not a genuine tomato pathogen (Isaac 1967). *V. tricolorpus* has only incidentally been reported as a plant pathogen on other hosts (Powell et al. 2013; Qin et al. 2008). For instance, inoculation of lettuce with *V. tricolorpus* resulted in mild disease symptoms on only two out of 400 assessed lettuce plants (Qin et al. 2008). Thus, *V. tricolorpus* should be considered an opportunistic pathogen that causes occasional infections on weakened plant hosts. Indeed, *V. tricolorpus* is able to induce wilt symptoms when plants have been challenged with additional abiotic stresses, such as high soil nitrogen levels or waterlogging conditions (Isaac 1956).

A high-quality *V. tricolorpus* genome assembly.

The genome sequence of *V. tricolorpus* MUCL9792 was determined using sequencing-by-synthesis on the Illumina HiSeq-2000 and SMRT sequencing on the PacBio platform. To this end, 1.2 Gb of paired-end (PE) library-derived reads (500-bp insert size; 100-bp read length) was produced in combination with 1 Gb of mate-pair (MP) library-derived reads (5-kb insert size; 50-bp read length), together with approximately 200 Mb

Fig. 1. *Verticillium tricolorpus* does not cause wilt disease on tomato. Pathogenicity of two *V. dahliae* strains (highly pathogenic JR2, mildly pathogenic VdLs17) and of *V. tricolorpus* was assessed on tomato. **A**, Side and top view of tomato plants at 13 days after mock inoculation (control) or inoculation with *V. dahliae* JR2, *V. dahliae* VdLs17, and *V. tricolorpus* MUCL9792. **B**, Average canopy area of six tomato plants at 13 days after mock inoculation or inoculation with *V. dahliae* JR2, *V. dahliae* VdLs17, and *V. tricolorpus* MUCL9792. An independently performed inoculation resulted in highly similar results (squared Pearson correlation coefficient = 0.99). **C**, Fungal outgrowth at 10 days after plating tomato stem sections harvested at 15 days postinoculation with *V. dahliae* JR2, *V. dahliae* VdLs17, and *V. tricolorpus* MUCL9792. Quantification shows the percentage of stem section slices that showed fungal outgrowth. **D**, Real-time polymerase chain reaction quantification of fungal biomass based on tomato plants harvested at 15 days postinoculation with *V. dahliae* JR2, *V. dahliae* VdLs17, and *V. tricolorpus* MUCL9792.

of long reads produced with PacBio sequencing (10-kb insert size) (Supplementary Table S1). In order to assemble the Illumina-derived short reads and the PacBio-derived long reads, we applied a recently described hybrid assembly approach (Fig. 2A) (Faino and Thomma 2014) that increases contiguity of the assembly in several steps (Fig. 2B). In the first step, the PE and MP sequences were assembled using the A5 pipeline (Tritt et al. 2012) (Fig. 2A). This initial assembly resulted in 195 scaffolds with an N50 of approximately 4 Mb, with the longest contig of 240 kb and the longest scaffold of 5.3 Mb

Fig. 2. A hybrid sequencing and assembly approach yields a nearly gapless draft genome of *Verticillium tricornus*. **A**, A five-step hybrid assembly approach applied to assemble the 36-Mb draft genome of *V. tricornus*. **B**, Sequence length accumulation curve showing the successive reduction in the number of contigs and scaffolds during the different steps of the hybrid assembly. The curve displays the number of bases in the assembly as a function of the (sorted) number of sequences (contigs or scaffolds). **C**, Sequence length accumulation curve showing the comparison between the draft genome assembly of *V. tricornus* and the previously assembled genomes of *V. dahliae* strains JR2 and VdLs17 as well as *V. alfalfae*.

(Table 1). This assembly has approximately 2,500 gaps and approximately 600 Ns (unknown nucleotides) per 100 kb. In the second step, gaps were filled using three types of sequences: i) corrected long reads, ii) uncorrected long reads, and iii) sequences generated upon local reassembly of short reads at gap positions (Fig. 2A). This step improved the assembly statistics considerably, reducing the number of contigs from approximately 2,700 to approximately 750, with the longest contig of 535 kb, and reducing the number of gaps from approximately 2,500 to approximately 550. At this stage, gaps that are not closed may actually represent wrongly scaffolded contigs. Therefore, uncorrected long reads were used to scaffold contigs in the third step. Subsequently, the obtained scaffolds were subjected to another scaffolding step, using MP reads in the fourth step, resulting in 604 contigs and 166 scaffolds (Table 1). More importantly, the total number of gaps was reduced to 438, while the number of Ns per 100 kb dropped to approximately 240. Manual curation included the removal of about 100 extremely short contigs (<500 bp) and further scaffolding. The A5 software will not merge contigs into scaffolds in case of insufficient support by paired Illumina reads. However, in about 10 cases in which MP reads could unambiguously be mapped at the edges of two contigs, these were joined manually. Subsequently, we globally evaluated the scaffolding by remapping the MP reads to the scaffolds. As this did not lead to the identification of any discordantly mapped MP reads that would suggest erroneous scaffolding, this assembly was considered final (Supplementary Fig. S1). This manual curation yielded a final assembly of 399 contigs that composed 61 scaffolds (58 genomic and three mitochondrial) ranging in size between 9 Mb and 500 bp (scaffold N50, 4.7 Mb; contig N50, 250 kb) with 338 gaps and 229 Ns per 100 kb, on average (Table 1). The 10 largest scaffolds encompass 95% of the genome (Figs. 2C and 3A).

To determine its quality, the genome assembly was compared with that of previously sequenced *Verticillium* genomes. The genome of *V. dahliae* VdLs17 as well as that of *V. alfalfae* (formerly known as *V. albo-atrum*) VaMs102 were sequenced using whole-genome shotgun Sanger sequencing (7.5× and 4× coverage, respectively) (Klosterman et al. 2011), while *V. dahliae* JR2 was sequenced with the Illumina HiSeq2000 (30× coverage) (de Jonge et al. 2012). All these assemblies yielded a higher number of contigs (1,562 for *V. dahliae* VdLs17, 4,098 for *V. alfalfae*, and 4,515 for *V. dahliae* JR2) when compared with the *V. tricornus* assembly (Fig. 2C), leading to a considerably higher number of Ns per 100 kb. Consequently, the scaffolded genome assembly of *V. alfalfae* and *V. dahliae* VdLs17 contained 2.5 and 0.9 Mb of Ns, respectively. Although optical mapping further refined the *V. dahliae* assemblies by combining individually assembled scaffolds into eight chromosomes (de Jonge et al. 2013; Klosterman et al. 2011), this process introduced a considerable number of additional gaps.

To assess whether any of the 58 scaffolds of the *V. tricornus* assembly represent full-length chromosomes, we queried the scaffolds for characteristic fungal telomeric sequence repeats (Fulnecková et al. 2013). We identified 14 such repeats, all of which were located at the ends of scaffolds (Fig. 3A). Moreover, eight telomeric repeats were found on the ends of four of the 10 largest scaffolds that are, thus, likely to represent full-length chromosomes (Fig. 3A). Pulse-field gel electrophoresis was performed to determine the karyotype of *V. tricornus* MUC9297. This analysis revealed the presence of seven chromosomes, suggesting that we sequenced and assembled all telomeres present in the genome (Fig. 3B). Combining the information from the karyotyping and the assembly, we estimate the sizes of the chromosomes to be 9 Mb (Scaffold 1; not entered the gel), 7.5 Mb (Scaffold 2), 5.7 Mb (not completely

assembled), 4.8 Mb (not completely assembled), 4.1 Mb (Scaffold 4), 3.3 Mb (Scaffold 5), and 1.8 Mb (not completely assembled) in size (Fig. 3B), summing up to 36.2 Mb and corresponding to the calculated genome size. Thus, whereas previously sequenced *V. dahliae* strains carry eight chromosomes, this *V. tricornis* strain only carries seven.

To further assess the completeness of the assembled gene space, we queried for orthologs of 248 core eukaryotic gene families, using the CEGMA pipeline (Parra et al. 2007, 2009). The assembled *V. tricornis* genome assembly was found to contain 237 members (95.56%) of these eukaryotic core families, comparable to previously assembled *V. dahliae* draft genomes

and higher when compared with *V. alfalfae* (81%) (de Jonge et al. 2013; Klosterman et al. 2011), indicative of a highly complete gene space in the *V. tricornis* genome assembly.

V. tricornis genome annotation.

We classified different types of repetitive elements in the genome of *V. tricornis* with Repeatmasker, utilizing a combination of known repetitive elements and de novo repeat identification (Jurka et al. 2005; Smit et al. 1996). In total, 2.5% (800 kb) of the assembled *V. tricornis* genome accounts for repetitive DNA and included traces of long terminal repeats (110 kb, 0.3%), long interspersed nuclear elements (35 kb,

Table 1. Genome assembly statistics for the A5 assembly, the hybrid assembly, and the final assembly of the draft genome of *Verticillium tricornis*

Statistics	A5	Hybrid assembly	Final assembly
Final statistics			
Read depth (fold)	62.1X	67.5X	67.5X
Estimate genome size (Mb)	36.16	36.10	36.09
Number of Ns (unknown nucleotides)	217,012	87,959	82,887
Number of Ns per 100 kb	600.20	242.65	229.6
Contig statistics			
Number	2,789	604	399
Total length (Mb)	35.94	36.04	36.01
Largest (kb)	241.5	677.2	677.2
N ₅₀ (kb)	35.9	180.7	249.2
Number of genes (partial)	10,417 (1,032)	11,291 (167)	11,338 (120)
Scaffold statistics			
Number	195	166	61
Largest (kb)	5,284	6,960	9,084
N ₅₀ (kb)	4,123	4,117	4,670
Number of Ns per 100 kb	600.71	243.65	229.64
Number of genes (partial)	11,107 (345)	11,398 (60)	11,406 (52)

Fig. 3. Overview of the draft genome assembly of *Verticillium tricornis*. **A**, Schematic representation of the 10 largest scaffolds in the genome assembly of *V. tricornis*. Characteristic fungal telomeric repeats are displayed on the ends of the scaffolds (indicated by shading; an additional single small scaffold with distal telomeric repeat is displayed as well), and assembly gaps are displayed on each scaffold. Gene (gray) and repeat (black) density as well as GC content is displayed in a sliding window of 10 kb with a slide of 2 kb. **B**, Chromosome karyotype by pulsed-field gel electrophoresis of the sequenced *V. tricornis* MUCL9792. Chromosomal DNA of *Schizosaccharomyces pombe* (M1) and *Hansenula wingei* (M2) were loaded as size markers.

0.1%), and short interspersed nuclear elements (1 kb, 0.002%) that are not uniformly distributed along the scaffolds (Fig. 3A). Surprisingly, their quantity and relative contribution is considerably smaller compared with that of previously sequenced *Verticillium* genomes, in which 4.2% of the assembled genome account for repetitive elements (4.05% for *V. dahliae* VdLs17 and 4.33% for *V. dahliae* JR2) (de Jonge et al. 2013; Klosterman et al. 2011). Repetitive elements are strong drivers of genome evolution, since they promote large-scale genomic variations (Seidl and Thomma 2014). In *V. dahliae*, repetitive elements have been shown to foster genomic rearrangements that drive the evolution of pathogenicity (de Jonge et al. 2013; Seidl and Thomma 2014). It is, thus, tempting to speculate that the increase of repetitive elements in the genome of *V. dahliae* is associated with its pathogenic capacity.

We inferred a reference gene annotation by integrating de novo, homology-based, and transcription data using the Maker2 pipeline (Holt and Yandell 2011). To this end, we generated 1-Gb RNA-Seq data on *V. tricorpus* growing in a suspension of tomato cells, to mimic a plant-associated environment, and about 97% of the reads could be aligned to the genome assembly using TopHat2 (Kim et al. 2013). To further improve gene structure annotation by Maker2, we assembled the RNA-Seq reads into transcripts, using Oases (Schulz et al. 2012) and Trinity (Grabherr et al. 2011), which were subsequently aligned to the genome assembly using PASA (Haas et

al. 2003). Additionally, we provided Maker2 (Holt and Yandell 2011) with 35 predicted fungal proteomes that represent a broad phylogenetic diversity (Supplementary Table S2) to guide gene structure annotation and applied three independent de novo gene predictors: SNAP (Korf 2004), GeneMark-HMM (Lukashin and Borodovsky 1998), and Augustus (Stanke et al. 2004). Maker2 combined these data and produced a consolidated set of gene models. Subsequently, we refined these gene models by manually performing RNA-Seq- and homology-guided gene structure annotation. If necessary, gene models were corrected, leading to the identification of 418 previously undetected genes, mainly due to erroneous gene fusions, and the correction of approximately 4,000 gene models, thus yielding a total of 11,454 protein-coding genes. Of these, 89% have RNA-Seq support (≥ 1 read mapped, on average 464 reads per gene model) and 91% have homology-based support in other fungal species. The 11,454 protein-coding genes were functionally annotated using InterProScan (Zdobnov and Apweiler 2001) and Blast2GO (Conesa et al. 2005), yielding approximately 65% of the predicted genes with a predicted functional annotation (Supplementary Fig. S2; Supplementary Table S3).

The secretome of *V. tricorpus*.

The vast majority of fungal species comprise saprophytes that thrive on decaying organic matter. These species deploy a plethora of secreted proteins to decompose complex organic material in their proximity to acquire nutrients and to establish their niche. Independently emerging from saprophytic ancestors, particular fungal species evolved to establish symbioses with various hosts, including plants, requiring them to successfully overcome host immunity. Irrespective of whether the symbiosis involves parasitism, commensalism, or mutualism, secreted fungal proteins play an important role, as they comprise so-called effectors that are required for establishment of the interaction (de Jonge et al. 2011; Rovenich et al. 2014; Thomma et al. 2011). Therefore, pathogenic species often encode larger secretomes (i.e., the total set of secreted proteins) as compared with their saprophytic relatives (Choi et al. 2010), potentially reflecting the acquisition of additional factors, e.g., effector genes, that facilitate their pathogenic lifestyle. To predict the secretome of *V. tricorpus*, we queried the predicted proteome for secretion signals, leading to the identification of 952 secreted proteins, which accounts for approximately 8.3% of its proteome. Using the same approach, the genomes of the pathogenic *V. dahliae* strains JR2 and VdLs17 encode 926 and 907 secreted proteins accounting for approximately 8.5% of their predicted proteomes, respectively. The genome of *V. alfalfae* encodes fewer (870) secreted proteins, which may be the consequence of the relatively low sequence coverage that hampered whole-genome assembly and annotation (Klosterman et al. 2011) and, thus, should be taken with caution.

By tracing the gain and losses of gene families encoding secreted proteins in the *Verticillium* genus using Dollo parsimony, we observed comparable absolute numbers of these gene families in *V. dahliae*, *V. alfalfae*, and *V. tricorpus* ranging from 715 to 801, corresponding to 779 to 877 proteins (Fig. 4A). Since pathogenic *Verticillium* species likely evolved from a nonpathogenic saprophytic ancestor, the 667 families (2,691 proteins) that are shared between *V. tricorpus*, *V. dahliae*, and *V. alfalfae* could represent a 'core' saprophytic secretome. In *V. dahliae* JR2, only 56 of the 668 genes belonging to these families are in planta induced (fold change $> 1.5 \log_{10}$), and thus, the majority have other functions that are likely not associated with its pathogenic lifestyle. Only a few gene families (99) encompassing 254 genes encoding secreted proteins have been specifically gained in the genomes of *V. dahliae* and *V. alfalfae*, of which the vast majority encode proteins of unknown function. Moreover, a consider-

Fig. 4. The secretome of *Verticillium tricorpus* encodes a secretome of similar size as pathogenic *Verticillium* spp. **A**, Evolutionary history of the gene families constituting the predicted secretome of the analyzed *Verticillium* spp. according to DOLLOP. At each branch, the number of gene family gains and losses is displayed and the inferred ancestral gene family set is shown. **B**, Distribution of nonsynonymous substitutions per nonsynonymous site (K_A) to synonymous substitutions per synonymous site (K_S) ratios for *V. tricorpus* genes encoding secreted proteins with orthologs in any of the analyzed *Verticillium* secretomes and for all other *V. tricorpus* genes with orthologs in any of the analyzed *Verticillium* species. The difference between the distributions was assessed using the nonparametric Wilcoxon rank sum test and significance is highlighted. Whiskers indicate the 1.5 interquartile range and outlier were not displayed for clarity. The total number of considered gene pairs (n) is indicated. **C**, Distribution of K_A/K_S ratios for *V. tricorpus* genes encoding secreted proteins with orthologs in the other analyzed *Verticillium* secretomes. Genes with K_A/K_S ratios > 1 , indicative of positive selection, are highlighted.

able amount of secreted proteins do not display similarity to any other sequence and are thus considered lineage-specific. For example, *V. dahliae* JR2 contains 49 lineage-specific genes encoding secreted proteins, including the experimentally verified virulence effector *XLOC009059* and the in planta-induced candidate effector *XLOC008951* (de Jonge et al. 2013). Similarly, *V. tricornis* encodes a considerable amount of lineage-specific genes, mainly encoding secreted proteins with unknown function. The vast majority (70%) of these genes are expressed during in vitro growth.

Many genes encoding secreted proteins, but particularly pathogen effectors, are expected to rapidly diversify due to selection pressure imposed by the host during the evolutionary arms race between pathogens and their hosts (de Jonge et al. 2013; Guyon et al. 2014; Klosterman et al. 2011; Raffaele et al. 2010; Wicker et al. 2013). To assess the speed of gene evolution and positive selection within the secretome, we used the ratio of nonsynonymous substitutions per nonsynonymous site (K_A) to synonymous substitutions per synonymous site (K_S) for *V. tricornis* genes with orthologs in the other analyzed *Verticillium* spp. as a proxy (Fig. 4B). While the vast majority of *V. tricornis* genes were found to be under purifying selection (i.e., $K_A/K_S < 1$), genes encoding secreted proteins were found to display significantly higher K_A/K_S ratios when compared with pairs of genes derived from the full set of protein-coding genes (Fig. 4B and C), thus suggesting accelerated evolution within the secretome. However, only a single highly expressed gene, encoding a secreted protein with similarity to chitinases, displays signs of positive selection, i.e., $K_A/K_S > 1$ (Fig. 4C). In contrast, *V. dahliae* JR2 contains 17 genes encoding secreted proteins with signs of positive selection (Supplementary Fig. S3), of which the majority are of unknown function, but that also includes genes encoding secreted proteins with predicted functions in cellulose binding or cutinase activity. In many pathogenic microbes, an extended repertoire of genes that are under positive selection and encode secreted proteins has been described (Raffaele et al. 2010; Wicker et al. 2013). However, the small number of genes under positive selection corroborates previous analysis that suggested that diversification at nucleotide levels does not play a major role in *V. dahliae* evolution (de Jonge et al. 2013). Notably, the majority of effector genes in *V. dahliae* with roles in virulence are lineage-specific, hampering the assessment of positive selection, and suggesting that presence or absence polymorphisms are crucial to drive the evolution of pathogenicity in *V. dahliae*.

We observed a distinct quantitative difference in repeat content between *V. dahliae* JR2 and *V. tricornis*. To further assess the hypothesis that the expansion of repeats in *V. dahliae* JR2 is associated with its pathogenicity (de Jonge et al. 2013; Seidl and Thomma 2014), we analyzed whether genes, particularly those encoding secreted proteins, are directly flanked by repetitive elements. In *V. dahliae* JR2, 25% of all genes and 27% of those encoding secreted proteins are directly flanked by at least a single repetitive element (excluding simple nucleotide repeats). In contrast, only 15% of all predicted genes and 18% of those encoding secreted proteins are flanked by repeats in *V. tricornis*. Interestingly, when considering in planta-expressed genes ($>1.5 \log_{10}$ fold change), 20 of 79 genes that encode secreted proteins are directly flanked by repeats in *V. dahliae* JR2, including two previously functionally characterized JR2-specific effector proteins (de Jonge et al. 2013). In contrast, only three highly expressed *V. tricornis* genes ($>2 \log_{10}$ fold change) when compared with the average genome-wide expression) that encode secreted proteins are flanked by repeats. Thus, our data suggest that the presence and expansion of repetitive elements is associated with pathogenicity in the *Verticillium* genus.

Genes encoding secreted proteins are clustered in *Verticillium* spp.

The 10 largest scaffolds encode, on average, 9.5% secreted proteins. On some of the smaller scaffolds, considerably more genes encode secreted proteins, e.g., up to 18% of the genes on scaffold 10, thus indicating that many of these genes localize in close physical proximity to each other. Moreover, these small scaffolds contained one of the characteristic telomeric repeats and, thus, many proteins encoding secreted protein cluster in proximity of the chromosomal ends. Therefore, we analyzed the global position of genes encoding secreted proteins on the complete chromosomes (scaffolds 1, 2, 4, and 5) (Fig. 5A). Interestingly, genes encoding secreted proteins are not randomly distributed along the chromosomes of *V. tricornis* but, rather, cluster locally, both in areas within the chromosomes and also, in some cases, close to telomeres (Fig. 5A). Also, genes encoding predicted membrane- or nuclear-localized proteins cluster (Fig. 5A). However, compared with genes encoding secreted proteins, where approximately 45% of the genes are clustered, only about 27% of the genes encoding proteins predicted to localize in membranes or in the nucleus are located within clusters (Fig. 5B). Similarly, this local clustering, even though less pronounced, is also observed in both *V. dahliae* strains, e.g., on chromosome 1 for strain JR2 and chromosome 2 for VdLs17 (Fig. 5C and D). Clustering of genes encoding secreted proteins has been reported previously in different strains of *V. dahliae*, in which in planta-expressed effector genes are enriched in highly dynamic lineage-specific regions (de Jonge et al. 2013). Similarly, many filamentous pathogens contain a genome architecture that is often characterized by the local co-occurrence of effector genes (Raffaele and Kamoun 2012). For example, genes encoding secreted proteins in *Fusarium graminearum* are clustered preferentially in regions close to the telomeres or in regions with increased recombination frequency (Brown et al. 2012). Clustering of genes, particularly genes encoding secreted proteins, in or in the proximity of unstable genomic regions can foster the swift alterations of these genes, thus enabling the rapid adaptation to changing environments (Seidl and Thomma 2014).

The genome of *V. tricornis* encodes an expanded repertoire of CAZymes.

Saprophytes and plant pathogens secrete a plethora of enzymes that are involved in the breakdown of complex polysaccharides that compose plant cell walls. Whereas saprophytes degrade these complex polysaccharides solely for nutritional purposes, pathogens need to also overcome these physical barriers to establish infection. Therefore, plant pathogens often encode larger repertoires of carbohydrate-active enzymes (CAZymes) when compared with saprophytes (Zhao et al. 2013). To explore the diversity of CAZymes in *Verticillium* species, we queried the predicted proteomes of *V. tricornis*, *V. alfalfae*, and the two *V. dahliae* strains (JR2 and VdLs17) for CAZyme signature domains (Yin et al. 2012) that belong to the four catalytic classes of CAZymes—glycoside hydrolases (GH), polysaccharide lyases, carbohydrate esterases, glycosyltransferases—and carbohydrate-binding modules (CBM), leading to the identification of 646, 508, 531, and 532 CAZymes (approximately 5% of their proteomes), respectively. When compared with six filamentous pathogens (Supplementary Fig. S4) and three saprophytes, *Verticillium* spp., together with the vascular wilt pathogen *Fusarium oxysporum*, encode the highest absolute number of CAZymes in their genomes (Supplementary Fig. S5; Supplementary Table S4) (Klosterman et al. 2011). Moreover, *Verticillium* spp. devote the largest proportion of their proteome to CAZymes (Fig. 6A). Notably, the saprophytic dung-decomposing fungus *Podospira anserina*, the white rot fungus

Phanerochaete chrysosporium, and *Neurospora crassa* encode considerably lower CAZyme numbers. Moreover, *Verticillium* spp. devote a large proportion (about 25%) of their secretome to CAZymes (Fig. 6B), which is greater than most other fungi, with the exception of the saprophyte *Phanerochaete chrysosporium* (Fig. 6B). Contrary to previous observations that saprophytes encode fewer CAZymes than encoded by pathogens (Zhao et al. 2013), *V. tricornis* contains more CAZymes than the two pathogenic *V. dahliae* strains. The high number of CAZymes in *V. tricornis* is mainly due to a higher abundance of GH families (in particular families GH3, GH43, and GH78) and secreted CBM1-containing proteins. CBM1 is generally considered as a fungal cellulose-binding domain that is often identified in proteins containing GH (Klosterman et al. 2011). Saprophytic fungi, such as *Podospira anserina* and *Phanerochaete chrysosporium*, also encode an expanded repertoire of CBM1 containing genes. The expanded CAZyme repertoire in *V. tricornis*, therefore, likely reflects its saprophytic lifestyle. In contrast, only six CAZyme families are expanded in the pathogenic *Verticillium* strains when compared with *V. tricornis*. Moreover, a single member belonging to CAZyme family GH54 is present in all pathogenic *Verticillium* strains but is absent from *V. tri-*

cornis. This gene is predicted to encode a secreted arabinofuranosidase with C-terminal CBM42. Interestingly, the gene that encodes this member is highly induced during in planta infection in *V. dahliae* JR2 (up to 3 log₁₀ (fold change) at 12 days postinoculation), suggesting a possible role of this gene during pathogenicity. However, only two other genes that belong to the six expanded CAZyme families show induced in planta expression (>1.5 log₁₀ fold change), suggesting that expansion or the presence or absence of particular CAZymes is not associated with pathogenicity.

The *V. tricornis* effector repertoire largely resembles that of other *Verticillium* spp.

The surprising finding that the secretome of the saprophyte *V. tricornis* is even larger than the secretome of the *V. dahliae* strains spurred us to further explore the presence and abundance of genes that have been implicated in fungal pathogenicity, and could, thus, reflect the adaptation of pathogenic *Verticillium* spp. to their particular lifestyle. We queried the genomes for genes encoding small secreted cysteine-rich proteins (SSCP) that are often characterized as lineage-specific pathogen effector molecules (de Jonge et al. 2011; Thomma et al. 2011). When

Fig. 5. Genes encoding proteins belonging to distinct functional classes cluster in the genome of *Verticillium tricornis*. **A**, Genomic position of genes encoding secreted proteins (i), genes encoding transmembrane localized protein (iii) and genes encoding nuclear localized proteins (v) are indicated along the four scaffolds that represent complete *V. tricornis* chromosomes. Clusters of genes for each of the functional classes are shown (ii, iv, and vi; window of 20 genes, minimum of five genes, consecutive windows were merged). **B**, Fraction of genes in each of the functional classes that is clustered on the four complete chromosomes. Total number of genes per class (*n*) is indicated. **C**, Clustering of genes for a chromosome of *V. dahliae* JR2 and **D**, VdLs17 is shown for comparison.

querying secreted proteins for size and cysteine content (≤ 150 aa and containing $\geq 3\%$ cysteine-residues [Saunders et al. 2012]), we identified 46 SSCP in *V. tricorpus*, of which more than 75% do not have any functional annotation. Similarly, 67 SSCP were identified in *V. dahliae* JR2, 43 in VdLs17, and 36 in *V. alfalfae*, of which more than 85, 70, and 70% do not have any functional annotation, respectively (Supplementary Table S5). *V. tricorpus* and *V. dahliae* considerably overlap in the functionally annotated proteins present in their respective SSCP catalogue, suggesting that *V. tricorpus* shares effectors with *V. dahliae*. However, the *V. tricorpus* catalogue of SSCP contains proteins that cannot unambiguously be implicated in pathogenicity, such as hydrophobins. Even though particular hydrophobins play roles in pathogenicity (Talbot et al. 1993), they are also present in nonpathogenic fungi and act in the formation of resting structures in *Verticillium* spp. (Klimes et al. 2008). Similarly, several proteins with a cerato-platanin domain were identified, of which homologs occur in filamentous fungi with all types of lifestyles and that may act in fungal growth and development (Gaderer et al. 2014).

Fig. 6. The genomes of *Verticillium* spp. encode a large repertoire of carbohydrate active enzymes (CAZymes). **A**, Boxplots display the relative number of CAZymes in the full proteomes and **B**, secretome of *Verticillium* spp. and related fungi. The relative contribution belonging to the five CAZyme classes—glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), and glycosyltransferases (GT), and carbohydrate-binding modules (CBM)—are shown as boxplots; whiskers indicate the 1.5 interquartile range, and outliers are shown as open circles. The position of each of the *Verticillium* spp. is indicated.

We further mined the secretomes for the presence of LysM effectors, a class of secreted proteins that do not contain any other recognizable features except LysM domains (Bolton et al. 2008; de Jonge and Thomma 2009; de Jonge et al. 2010; Kombrink and Thomma 2013). Even though several LysM effectors have been implicated in fungal pathogenicity (de Jonge et al. 2010; Marshall et al. 2011; Mentlak et al. 2012; Sánchez-Vallet et al. 2013), they are commonly observed in saprophytes as well and may, thus, play alternative roles in fungal biology besides promoting pathogenicity (Kombrink and Thomma 2013; Rovenich et al. 2014). The genome of *V. tricorpus* encodes five LysM effectors, containing three to five LysM domains, of which one, Vt01g11890, is expressed when *V. tricorpus* is grown in a tomato cell suspension (Fig. 7A). Initially, seven LysM effector candidates were identified in the genome of *V. dahliae* VdLs17 (Klosterman et al. 2011), of which only five qualify as bona fide LysM effectors (Kombrink 2014). Notably, one of these five is a strain-specific LysM effector that is

Fig. 7. The genome of *Verticillium tricorpus* encodes effector candidates similar to those encoded by pathogenic *Verticillium* spp. **A**, RNA-Seq expression of a gene encoding the LysM effector Vt01g11890. **B**, Phylogenetic relationship of necrosis and ethylene inducing-like (NLP) in related filamentous fungi. Orthologous groups of previously described NLP classes in *Verticillium* are highlighted in gray (Santhanam et al. 2013) and the single highly expressed NLP (Vt02g01010.1) in *V. tricorpus* is indicated by an arrow.

expressed during infection and acts as a virulence factor on tomato (de Jonge et al. 2013). So far, the role of the remaining four core LysM effectors during plant infection could not be demonstrated and their function remains enigmatic (Kombrink and Thomma 2013). Rather than a role in pathogenicity, *Verticillium* LysM effectors could be involved in protection from competing microbes in the soil (de Jonge and Thomma 2009; Kombrink and Thomma 2013; Rovenich et al. 2014).

Similar to LysM effectors, necrosis and ethylene inducing-like proteins (NLP) also are widespread and occur in organisms with diverse life styles (Gijzen and Nürnberger 2006). Even though they have been observed in both pathogenic and saprophytic species, e.g., *Neurospora crassa* (Fig. 7B), they have gained considerable attention in pathogenic species (Gijzen and Nürnberger 2006). Many fungi contain few (one or two) NLP, while the NLP family is expanded in several pathogenic oomycetes and particular fungi (Gijzen and Nürnberger 2006; Santhanam et al. 2013; Seidl et al. 2011), including *V. dahliae* and *V. alfalfae*, that, depending on the strain, contain either seven or eight members (Santhanam et al. 2013). Recent analyses revealed that only two of the *V. dahliae* NLP, NLP1 and NLP2, induce cell death and are required for virulence on tomato and *Arabidopsis* (Santhanam et al. 2013) while the role of the remaining, noncytolytic NLP remains unclear. Interestingly, VdNLP1 also plays a role in vegetative growth and asexual production, hinting to alternative functions of NLP besides their necrotic activity (Santhanam et al. 2013). An initial survey of *V. tricorpus* prior to the completion of the genome assembly identified only four NLP homologs (Santhanam et al. 2013). However, the presently assembled genome of *V. tricorpus* contains seven NLP genes, of which six are clear orthologs of previously described *V. dahliae* NLP (Fig. 7B). Interestingly, *V. tricorpus* encodes orthologs of NLP1 and NLP2. Both of these orthologs were expressed at low levels (7.8 and 15 read counts per 1 kb, respectively), in contrast to a *V. tricorpus*-specific NLP homolog (Vt02g01010) that was highly expressed in the tomato cell suspension (368.7 read counts per 1 kb). Thus, presently the function of *V. tricorpus* NLP remains enigmatic.

In summary, we revealed an effector repertoire in *V. tricorpus* that is surprisingly similarly composed when compared with its pathogenic relatives. Members of some of the identified effector families are commonly observed in nonpathogenic species such as saprophytes and, thus, do not necessarily represent genuine pathogenicity factors. Moreover, homologs of *V. dahliae* effector genes, such as NLP2, are not always identical in *V. tricorpus* and may, thus, have diverged to different functions. Furthermore, a considerable number of SSCP do not display significant similarity to any known (effector) family, and several of these uncharacterized proteins might play important roles in niche establishment and competition with other microbes. For example, *V. tricorpus* has recently been shown to be an antagonist of *V. dahliae* on lettuce and artichoke (Qin et al. 2008) and SSCP of unknown function might be involved in this interaction. Thus, the genome assembly of *V. tricorpus* presents the foundation for the identification and initial characterization of effector families. Realistically, however, the functions of most effector families and their members remain unknown and need experimental characterization.

Conclusions.

In the last decade, rapid technological advances made genomic sequencing a commodity that provides a plethora of genomic sequences. Many of these genomes are from closely related species that differ in their ecological niches. We anticipated that a high-quality genomic sequence of the saprophyte *V. tricorpus* would shed light on the origin of pathogenicity within the genus *Verticillium*. However, contrary to our expecta-

tations, the genome of *V. tricorpus* contains many of the hallmarks that also characterize pathogenic *Verticillium* spp. In particular, *V. tricorpus* encodes an effector repertoire that largely resembles that of its pathogenic relatives. Assuming that pathogenicity in fungi evolved from saprophytes, alternative roles for effectors, e.g., during interactions with other microorganisms that are encountered in the same niche, seem likely (Rovenich et al. 2014). During the evolution of pathogenicity, these proteins might have acquired altered functionalities that facilitate the pathogenic lifestyle. However, a distinct difference in the repetitive element content between *V. tricorpus* and its pathogenic relatives may suggest that repeats are involved in the evolution of pathogenicity in the *Verticillium* genus. We anticipate that further comparative genomics together with functional characterization of effector proteins in species with different lifestyles will enhance our understanding of their role in facilitating a pathogenic lifestyle.

MATERIALS AND METHODS

Plant inoculations.

All plant inoculations were performed on 10-day-old tomato seedlings (cv. MoneyMaker) using root-dip inoculation as described previously (Fradin et al. 2009). Briefly, seedlings were uprooted and inoculated by dipping the roots for 2 min in a conidial suspension (10^6 conidia per milliliter) in water. After replanting in soil, plants were incubated at standard greenhouse conditions of a 16-h-light and 8-h-dark regime and 60% relative humidity. Disease progression was monitored for 15 days after inoculation. To determine in planta colonization, stem sections at the height of the first internode were taken, surface-sterilized, and sliced. The slices were placed on potato dextrose agar supplemented with chloramphenicol at 50 μ g/ml and were incubated at 22°C for 10 days. For in planta biomass quantification, stems of three inoculated plants were harvested at 15 days after inoculation. The samples were ground to powder and genomic DNA was isolated. Real-time PCR on genomic DNA was carried out and was analyzed using the 7300 SDS software (Applied Biosystems). A *Verticillium*-specific primer pair was used to quantify fungal colonization (5'-CATTGCCC AAGTTTACCTCC-3' combined with 5'-GCCAGCGTGTCTA TCTTCTC-3').

DNA extraction.

DNA was isolated from conidia and mycelium fragments that were harvested from 10-day-old cultures grown in liquid potato dextrose broth at 28°C. DNA isolation was performed according to the following protocol. First, mycelium was scraped directly from the surface of an agar plate, was suspended in water, and was transferred to a 50-ml tube. The tube was vortexed briefly and was centrifuged at maximum speed for 10 min. After centrifugation, 0.5 ml of 0.1 M Tris HCl (pH 8) was added to the pellet, which was mixed and subsequently transferred to a 2-ml screw-cap tube. Three metal beads were added and the tube was shaken in a bead beater at the highest speed for 45 s. After shaking, 1 ml of phenol/chloroform/isoamylalcohol (24:24:1) was added. The tube was mixed and centrifuged at maximum speed for 7 min, and then, the water phase (400 μ l) was transferred to a new tube. In the new tube, 800 μ l of isopropanol was added and the tube was centrifuged, after mixing at maximum speed for 20 min in the cold room. After centrifugation, the isopropanol was removed and the DNA pellet was air-dried. Subsequently, the pellet was dissolved in 50 μ l of deionized H₂O.

Genome sequencing and assembly.

Two sequence libraries (500-bp and 5-kb insert size) of *V. tricorpus* were prepared and paired ends were sequenced at the

Beijing Genome Institute (BGI), using the Illumina high-throughput sequencing platform. In addition, a long-insert library (10 kb) was generated and sequenced with SMRT sequencing technology on a PacBio platform at BaseClear (Leiden, The Netherlands). Genome assembly was performed as described previously (Faino and Thomma 2014). Contiguity of scaffolds and the correct placement of contigs were manually assessed. To this end, we manually inspected paired-end reads for concordant mapping and coverage of gapped regions between contigs. Genome statistics were calculated using QUAST (Gurevich et al. 2013). Repeats were identified and characterized using the de novo repeat identification with RepeatModeler (Smit and Hubley 2008) (1.0.7; default setting). Subsequently, de novo-identified repeats were combined with the repeat library from RepBase (Jurka et al. 2005) (release 31-04-2014) to identify and annotate families of repetitive elements, using RepeatMasker (Smit et al. 1996) (open-4.0.5) in sensitive mode.

Karyotyping.

Karyotyping was performed as outlined by de Jonge and associates (2013). Briefly, *V. tricornis* protoplasts were prepared as described by Mehrabi and colleagues (2012). Karyotyping was performed using a CHEF Mapper XA pulsed field electrophoresis system (Bio-Rad), using the auto algorithm function with low- and high-molecular weight setting at 2 and 6 Mb, respectively. Chromosomes were separated in 0.8% low EEO agarose gel (US Biologicals). Two chromosome size markers (Bio-Rad) were loaded as a reference marker. After electrophoresis, the gel was stained with ethidium bromide (1 µg/ml) in water for 1 h and was de-stained in water for 2 h.

Gene prediction and annotation.

For RNA sequencing, *V. tricornis* was cultured in a 10-day-old tomato cell suspension for 2 days. RNA was isolated based on Trizol RNA extraction (Simms et al., 1993). An RNA-Seq library (180 bp insert size) was prepared and sequenced (100-bp reads) at BGI. The RNA-Seq reads were mapped onto the draft *V. tricornis* genome using Tophat (Trapnell et al. 2009) and PASA (Haas et al. 2003). Oases (Schulz et al. 2012) and Trinity (Grabherr et al. 2011) were used to assemble the RNA-Seq data independently from the genome assembly. The assembled transcripts derived from Oases, Trinity, and PASA were combined and used as cDNA expressed sequence tag evidence in Maker2 (Holt and Yandell 2011). Moreover, assembled RNA-Seq reads from *V. dahliae* were used as evidence for gene prediction as well as protein sequences from 35 different fungal proteomes. The annotation of the predicted genes was done by BLASTp (Altschul et al. 1990) searches to the National Center for Biotechnology Information (NCBI) nonredundant database and by InterProScan (Jones et al. 2014). The BLASTp analysis and the InterProScan were merged together using Blast2GO (Conesa et al. 2005).

To estimate gene expression levels in *V. tricornis*, RNA-Seq reads were mapped to the draft genome of *V. tricornis* using TopHat (Trapnell et al. 2009). Subsequently, the number of reads that were mapped to each gene were determined and normalized for the length of the transcript (number of reads per 1 kb of transcript). Similarly, gene expression in *V. dahliae* JR2 was estimated using previously generated RNA-Seq data (de Jonge et al. 2012). For each condition, the reads were mapped to the draft genome assembly of *V. dahliae* JR2 using TopHat (Trapnell et al. 2009) and were summarized as reads per kilobase of transcript per million of mapped reads.

Secreted proteins were predicted using a combination of SignalP3 (Dyrlov Bendtsen et al. 2004), SignalP4 (Petersen et al. 2011), TargetP (Emanuelsson et al. 2000), and TMHMM

(Krogh et al. 2001). Proteins showing positive predictions from i) both predictors (HMM and neural network, default settings) of SignalP3, ii) SignalP4 (default settings), and iii) TargetP (predicted localization “secreted”; default settings) were considered as candidate effectors. Moreover, we filtered this list by removing proteins that contained i) more than a single TMHMM predicted transmembrane domain or ii) proteins with a single TMHMM predicted transmembrane domain, if this transmembrane domain starts outside of the first 35 amino acids or overlaps less than 10 amino acids with the predicted signal peptide. Protein domains observed in candidate effectors proteins, e.g., NLP and LysMs, were identified by mining the InterProScan annotations.

Comparative genomics.

The species phylogeny was based on the amino acid sequences of 130 single-copy gene families that were identified in the predicted proteomes of *V. tricornis* and nine related fungi using the CEGMA pipeline (Parra et al. 2007). Individual families were aligned using mafft (LINSi; v7.047b) (Katoh et al. 2002) and were subsequently concatenated. Maximum likelihood phylogeny was inferred using RAXML (v7.6.3) (Stamatakis 2006; Stamatakis et al. 2008) with the GAMMA model of rate heterogeneity and the Whelan and Goldman (WAG) model of amino acid substitutions. The robustness of the inferred phylogeny was assessed by 1,000 rapid bootstrap approximations.

The phylogeny of the NLP was based on the identified NLP domain (PF05630, excluding short fragments) using InterProScan (Jones et al. 2014) of 10 filamentous fungi. Subsequently, the NLP domain was excised, aligned with mafft (LINSi; v7.047b) (Katoh et al. 2002). Maximum likelihood phylogeny was inferred using RAXML (v7.6.3) (Stamatakis 2006; Stamatakis et al. 2008) with the GAMMA model of rate heterogeneity and the WAG model of amino acid substitutions. The robustness of the inferred phylogeny was assessed by 1,000 rapid bootstrap approximations.

We built families of gene-encoding secreted proteins in the analyzed *Verticillium* spp. and seven related fungi using OrthoMCL (default setting) (Li et al. 2003). Beforehand, similarity between proteins was established by an all-vs.-all analyses using BLASTp (E-value cutoff 1e-5, soft filtering) (Altschul et al. 1990, 1997). The ancestral set of families and the gains and losses were inferred by reconciliation of the binary presence or absence profiles of members constituting an orthologous group with the species phylogeny using DOLLOP (Felsenstein 2002), assuming the irreversibility of the loss an orthologous group, i.e., an orthologous group can only be gained, however individual members can be lost multiple times independently. To calculate the value of K_S , K_A , and the K_A/K_S ratio, we extracted sequences of the four analyzed *Verticillium* strains from the previously determined OrthoMCL families. Subsequently, we aligned the coding sequences per gene family guided by protein alignment and determined the pairwise K_S , K_A , and K_A/K_S ratios using the ‘kaks’ function of the ‘seqinr’ package. For further analysis, only genes with K_A or K_S value ≤ 5 were considered, to avoid saturation effects. Similarly, we built OrthoMCL families for all genes of the four *Verticillium* spp. and determined K_A , K_S , and K_A/K_S ratios as outlined above.

To identify clustering of genes that encode secreted proteins, we scanned each scaffold with CROC (Pignatelli et al. 2009). Briefly, CROC scans each scaffold, represented as a string of genes, with a sliding window (number of genes per window = 20; slide per window = 1; minimum of five genes of interest per window). For each window, the probability to observe that many (or more) genes encoding secreted genes by chance is assessed using a hypergeometric distribution test (Pignatelli et

al. 2009). Subsequently, multiple-testing correction using Benjamini-Hochberg was applied. Transmembrane localized proteins were predicted using TMHMM (Krogh et al. 2001), and nuclear localized proteins were predicted, using a combination of WoLF PSORT (Horton et al. 2007) and NLStradamus (Nguyen Ba et al. 2009), and clustering was a subsequently assessed as described above.

Data access.

The genome assembly has been associated with the NCBI GenBank BioProject PRJNA229139 and the BioSample SAMN02415140. The assembly is deposited in the DDBJ/EMBL/GenBank database under the accession JPET00000000. The raw RNA-Seq sequence reads have been associated with the NCBI Sequence Read Archive under the same BioProject and BioSample numbers and is deposited under the accession SRX658508.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The R project seqinr package:
cran.r-project.org/web/packages/seqinr/index.html