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Identification and differentiation of Verticillium species and V. longisporum lineages by singleplex and multiplex PCR assays

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Abstract:	Accurate species identification is essential for effective disease management, but is challenging in microfungi including Verticillium sensu stricto (Ascomycota, Sordariomycetes, Plectosphaerellaceae), a small genus of ten species that includes important plant pathogens. Here we present fifteen PCR assays for the identification of all Verticillium species and the three lineages of the diploid hybrid V. longisporum. The assays were based on the ribosomal internal transcribed spacer region, actin, elongation factor 1-alpha, glyceraldehyde-3-phosphate dehydrogenase and tryptophan synthase gene sequences. The eleven single target (singleplex) PCR assays resulted in amplicons of diagnostic size for V. alfalfae, V. albo-atrum, V. dahliae including V. longisporum lineage A1/D3, V. isaacii, V. klebahnii, V. nonalfalfae, V. nubilum, V. tricorpus, V. zaregamsianum, and Species A1 and Species D1, the two undescribed ancestors of V. longisporum. The four multiple target (multiplex) PCR assays simultaneously differentiated the species or lineages within the following four groups: Verticillium albo-atrum, V. alfalfae and V. nonalfalfae; Verticillium dahliae and V. longisporum lineages A1/D1, A1/D2 and A1/D3; Verticillium dahliae including V. longisporum lineage A1/D3, V. isaacii, V. klebahnii and V. tricorpus; Verticillium isaacii, V. klebahnii and V. tricorpus. Since V. dahliae is a parent of two of the three lineages of the diploid hybrid V. longisporum, only a multiplex PCR assay is able to differentiate V. dahliae from all V. longisporum lineages. PCR assays were tested with fungal DNA extracts from pure cultures, and were not evaluated for detection and quantification of Verticillium species from plant or soil samples. The DNA sequence alignments used in this study encompass at each locus the total, currently known Verticillium diversity, and are provided for the design of additional primers.
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Editor, *PLoS One*

Davis, November 20, 2012

Dear Editor:

Please find attached our manuscript '**Identification and differentiation of *Verticillium* species and *V. longisporum* lineages by singleplex and multiplex PCR assays**', for consideration of publication in *PLoS One* as a research article.

The manuscript contains the description of 15 singleplex and multiplex PCR assays to identify and differentiate all currently known species of the ascomycete fungus and important plant pathogen *Verticillium.* We previously published in PLoS One on the evolution, phylogenetics and taxonomy of *Verticillium* including the description of several new species [1,2]. The PCR assays in the current manuscript will allow anyone to accurately identify all *Verticillium* species which is extremely difficult based on morphology. The primers were designed and tested using our extensive collection of *Verticillium* strains and molecular datasets from the two previous studies that included several isolates of each species and five different loci. It is by far the most comprehensive study of its kind in *Verticillium.*

This work was initiated following the suggestion of our last PLoS One editor, Dr. Alex Idnurm who suggested we publish species-specific primer sets.

Our findings are relevant to diagnostics labs, for plant breading and for other research projects that demand accurate identification of *Verticillium* species.

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Thank you very much for your time and consideration.

Sincerely,

Patrik Inderbitzin, pre-publication corresponding author, prin@ucdavis.edu

References:

1. Inderbitzin P, Bostock RM, Davis RM, Usami T, Platt HW, et al. (2011) Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS ONE 6: e28341.
2. Inderbitzin P, Davis RM, Bostock RM, Subbarao KV (2011) The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS ONE 6: e18260.

Identification and differentiation of *Verticillium* species and *V. longisporum* lineages by singleplex and multiplex PCR assays

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Abstract

Accurate species identification is essential for effective disease management, but is challenging in microfungi including *Verticillium* sensu stricto (Ascomycota, Sordariomycetes, Plectosphaerellaceae), a small genus of ten species that includes important plant pathogens. Here we present fifteen PCR assays for the identification of all *Verticillium* species and the three lineages of the diploid hybrid *V. longisporum.* The assays were based on the ribosomal internal transcribed spacer region, *actin*, *elongation factor 1-alpha*, *glyceraldehyde-3-phosphate dehydrogenase* and *tryptophan synthase* gene sequences. The eleven single target (singleplex) PCR assays resulted in amplicons of diagnostic size for *V. alfalfae, V. albo-atrum, V. dahliae* including *V. longisporum* lineage A1/D3, *V. isaacii, V. klebahnii, V. nonalfalfae, V. nubilum, V. tricorpus, V. zaregamsianum,* and Species A1 and Species D1, the two undescribed ancestors of *V. longisporum*. The four multiple target (multiplex) PCR assays simultaneously differentiated the species or lineages within the following four groups: *Verticillium albo-atrum, V. alfalfae* and *V. nonalfalfae*; *Verticillium dahliae* and *V. longisporum* lineages A1/D1, A1/D2 and A1/D3; *Verticillium dahliae* including *V. longisporum* lineage A1/D3, *V. isaacii, V. klebahnii* and *V. tricorpus*; *Verticillium isaacii, V. klebahnii* and *V. tricorpus*. Since *V. dahliae* is a parent of two of the three lineages of the diploid hybrid *V. longisporum*, only a multiplex PCR assay is able to differentiate *V. dahliae* from all *V. longisporum* lineages. PCR assays were tested with fungal DNA extracts from pure cultures, and were not evaluated for detection and quantification of *Verticillium* species from plant

or soil samples. The DNA sequence alignments used in this study encompass at each locus the total, currently known *Verticillium* diversity, and are provided for the design of additional primers.

Introduction

Verticillium sensu stricto is a small group of agriculturally important, plant associated fungi that cause Verticillium wilt, a type of vascular wilt affecting many different crops and resulting in significant losses in many parts of the world [1,2,3]. Among the ten species currently recognized in *Verticillium* sensu stricto, in the following referred to as *Verticillium* [4], *V. dahliae* is most widespread and most economically important [1,5,6], but *V. albo-atrum* [7], *V. alfalfae* [8,9], *V. longisporum* [10,11], *V. nonalfalfae* [12,13], *V. tricorpus* [7,14] and *V. zaregamsianum* [15] also cause significant losses, *V. nubilum* causes disease in pathogenicity tests [14], and both *V. isaacii* and *V. klebahnii* have been recovered from lettuce and artichoke, respectively [4,16]. One of the characteristic features of *Verticillium* species is the formation of resting structures that are highly melanized, thick-walled cells of different shapes and sizes [4]. The resting structures of *V. dahliae* are known as microsclerotia and can survive in the soil for years [17]. As few as two microsclerotia per gram of soil can result in plant infection and yield losses [18], and knowledge about the abundance of microsclerotia and other resting structures in the soil is an important factor to consider for disease management. *Verticillium* species also differ in host range and pathogenicity [8,14,19,20,21], and thus, expedient detection, quantification and identification of *Verticillium* species has

been the focus of extensive research efforts. These included the design of numerous PCR-based assays, targeting *V. albo-atrum* [22,23,24,25,26,27,28,29], *V. dahliae* [23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41], *V. longisporum* [29,37,42,43], and *V. tricorpus* [26,27,28,37,44], in a variety of substrates including alfalfa [22], oilseed rape [43], olive [40], pepper [30], potato [27,28,33,44,45], strawberry [31], soil [26,29,31,34,35,36,37,38,39], spinach seed [41], tomato [30], and herbaceous hosts in general [23,24,25,26,32].

PCR assays provide quantitative and qualitative information about a species or a lineage, and replace other techniques that are less desirable or are not applicable to a particular situation. However, incomplete knowledge of the genetic diversity underlying a PCR assay may result in misleading results, including false positives and false negatives, and potentially significant economic losses. Thus, a thorough understanding of the genetic diversity, beyond the target species or lineage and including relatives, is a prerequisite for the design of reliable PCR assays.

Significant advances have recently been made in our understanding of the genetic diversity in *Verticillium.* Five new *Verticillium* species were described [4], and the evolutionary history of *V. longisporum,* a diploid hybrid with four different parents, was elucidated [46]. *Verticillium longisporum* presents particular difficulties for the design of species-specific PCR assays. This is because *V. longisporum* consists of three different lineages that resulted by hybridization of Species A1, with Species D1, *V. dahliae* lineage D2 and *V. dahliae* lineage D3 (Figure 1). Species A1 and Species D1 have not formally been named, as they have never been found and are only known as parents of *V. longisporum. Verticillium dahliae* lineages D2 and D3

are two different lineages of *V. dahliae.* Thus, PCR assays for the identification of *V. longisporum* and *V. dahliae* have to account for the fact that *V. longisporum* contains *V. dahliae* alleles.

In this study, we took advantage of the data from recent studies on *Verticillium* diversity and evolution [4,46], and assembled DNA sequence datasets that represent our current knowledge of the intraspecific and interspecific genetic diversity of *Verticillium.* We used the comprehensive datasets to design eleven primer pairs to selectively amplify all *Verticillium* species and *V. longisporum* lineages, in eleven singleplex and four multiplex PCR assays. Our PCR assays are useful for confirmation of morphological species identification in diagnostics labs, for genotyping of isolates prior to pathogenicity tests, and for other research projects. The DNA sequence alignments are provided for design of additional PCR primers and probes.

Results

Primers designed

Eighteen primers were designed and combined into eleven lineage-specific primer pairs (Figure 2, Table S1). Five different datasets were used for primer design. Five primers were based on the *elongation factor 1-alpha* (*EF*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*) datasets, respectively, three primers were based on the *actin* (*ACT*) and *tryptophan synthase* (*TS*) datasets, respectively, and two primers were based on the nuclear ribosomal internal transcribed spacer region (ITS) dataset (Table 1). DNA sequence alignments for each dataset are available as text files in

FASTA format (Alignments S1, S2, S3, S4 and S5). Specificity of each primer pair was achieved by designing the primers in such a way that the binding site of at least one of the primers in each primer pair differed from homologous sites in non-target lineages by at least one substitution. Each primer pair amplified a region of different length, thereby enabling the identification of each *Verticillium* species and *V. longisporum* lineage based on the size of the PCR amplicon, or in the case of *V. longisporum* based on PCR banding pattern. The numbers of substitutions between primer sequences and primer sites in the *Verticillium* strains used for primer design are listed in Table S2. Primer annealing temperatures were between 53°C and 58°C as determined by the Nearest Neighbor Method [47] (Table S1). Primer names were chosen to reflect primer specificity, for instance, forward primer 'If', named after *V. isaacii,* was used only for amplification of *V. isaacii,* whereas reverse primer 'IKr' that was named after *V. isaacii* and *V. klebahnii,* was part of both *V. isaacii* and *V. klebahnii*-specific primer pairs (Figure 2).

Singleplex PCR assays

All eleven singleplex (=single target) PCR assays were specific and amplification of each primer pair was restricted to target isolates. Non-target isolates did not result in bright PCR bands of expected size as illustrated in Figure 3. The targets of the eleven singleplex PCR assays were *V. albo-atrum, V. alfalfae, V. dahliae* including *V. longisporum* lineage A1/D3, *V. isaacii, V. klebahnii, V. nonalfalfae, V. nubilum, V. tricorpus, V. zaregamsianum,* and Species A1 and Species D1, the two *V. longisporum* ancestors (Figure 3). Specificity testing for each PCR

assay was done with a target isolate as a positive control, and representatives of all non-target lineages where the more divergent of the two primer binding sites differed by four or fewer substitutions from the homologous target site (Table S2, Figure 2). *Verticillium nubilum* and *V. zaregamsianum* singleplex PCR assay specificity was also confirmed with the 26 *Verticillium* isolates and outgroups that were used to evaluate the multiplex assays as reported below (Figure S1, Table 2), since *V. nubilum* and *V. zaregamsianum* were not part of any multiplex PCR assays. PCR conditions, expected band sizes and suggested agarose gel concentrations for optimal PCR product separation are given in Table 3. Each PCR primer pair was tested in at least three different PCR runs, except for the Species A1, Species D1 and *V. dahliae* primer pairs.

Multiplex PCR assays

Primer pairs from the singleplex PCR assays were combined into four multiplex PCR assays to allow for simultaneous identification of the following morphologically or ecologically similar groups of *Verticillium* species or lineages: *Verticillium albo-atrum, V. alfalfae* and *V. nonalfalfae*; *V. dahliae* including *V. longisporum* lineage A1/D3*, V. isaacii, V. klebahnii* and *V. tricorpus; V. dahliae* and *V. longisporum* lineages A1/D1, A1/D2 and A1/D3*; V. isaacii, V. klebahnii* and *V. tricorpus*. Specificity testing for the multiplex PCR assays was more extensive than for the singleplex PCR assays, and involved all 24 *Verticillium* isolates that, with the exception of *V. dahliae*, represented the total genetic diversity in *Verticillium*, as well as two relatives of *Verticillium*, *Gibellulopsis nigrescens* strain PD595 and

Musicillium theobromae strain PD686 (Table 2). The four multiplex PCR assays contained between five and seven primers, detailed PCR conditions are given in Tables 4, 5, 6, 7 and 8. In all four multiplex PCR assays, only target isolates resulted in bright PCR bands of expected size (Figures 4, S2). All multiplex PCR assays were repeated at least three times on three different PCR machines.

Additional validation of PCR assays

Additional isolates were used for confirmation of the singleplex and multiplex PCR assays and, in all cases, the results were as expected (Figure S3). Most notably, eleven previously uncharacterized *Verticillium* isolates from lettuce in California, were identified using the multiplex PCR assay that targets *V. dahliae* including *V. longisporum* lineage A1/D3, *V. isaacii*, *V. klebahnii* and *V. tricorpus,* in the following referred to as the *V. dahliae* – *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay, and the PCR results were confirmed by DNA sequencing of the PCR bands followed by phylogenetic analyses (Figure S4).

For the other multiplex and singleplex PCR assays, previously genetically characterized isolates from Inderbitzin et al. [4,46] were used, since alternative isolates were unavailable. With the exception of *V. dahliae,* the new isolates were genetically identical at the loci available to isolates used for validation of the singleplex and multiplex PCR assays. The additional *V. dahliae* isolates were strains PD323, PD328, PD331, PD615, PD656 and PD718 that represented the majority of genotypes in the main group of *V. dahliae* [46] that was represented in the PCR assay validation by *V. dahliae* strain PD322. However, since all primer sites were

conserved within *V. dahliae*, the amplification of the *V. dahliae* diagnostic PCR band for each isolate was as expected (Figure S3b). Not tested with additional isolates were the *V. albo-atrum – V. alfalfae – V. nonalfalfae* multiplex assay, the *V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay, and the *V. tricorpus* singleplex PCR assay.

Evaluation of a *V. longisporum* PCR assay from the literature

Karapapa and Typas [42] proposed to use a 839-bp intron in the nuclear SSU rRNA gene as a marker for *V. longisporum*. Our results show that *V. longisporum* lineages A1/D1 and A1/D2 have the 839-bp intron, whereas *V. longisporum* lineage A1/D3 and *V. dahliae* lack the intron (Figure 5). This is in agreement with the fact that *V. longisporum* lineage A1/D3 has a *V. dahliae* ITS region, whereas the other two lineages have the ITS region of a different parent, Species A1 [46].

Discussion

We present eleven singleplex and four multiplex PCR assays for the identification of all species in *Verticillium* including the different lineages of the diploid hybrid *V. longisporum*. The assays were validated through rigorous testing to ascertain assay specificity and reliability, involving multiple isolates from each *Verticillium* species, as well as two outgroups, *Gibellulopsis nigrescens* and the more distantly related *Musicillium theobromae*. This is the most diverse sample of *Verticillium* genetic diversity considered to date for design of diagnostic PCR assays

and should provide a consistent and accurate means for identification of species and lineages of *Verticillium.*

Fifteen singleplex and multiplex PCR assays facilitate the identification of all *Verticillium* species

The goal of this study was to provide PCR assays for the identification of *Verticillium* species, and in particular, for the *Verticillium* species and lineages that are morphologically indistinguishable or challenging to differentiate [4,46], including the following three groups of species: *Verticillium albo-atrum, V. alfalfae, V. nonalfalfae; V. dahliae* and the three different lineages of the diploid hybrid *V. longisporum;* and *V. isaacii, V. klebahnii* and *V. tricorpus.* We designed a fourth multiplex PCR assay to simultaneously identify *V. dahliae, V. isaacii, V. klebahnii* and *V. tricorpus,* since *V. dahliae, V. isaacii* and *V. klebahnii* co-occur on California lettuce, and *V. tricorpus* is morphologically indistinguishable from *V. isaacii* and *V. klebahnii. Verticillium longisporum* lineage A1/D3 which could be confused with *V. dahliae* in the *V. dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay, has not been reported from California [46]. All the species- and lineage-specific PCR primer pairs in the multiplex assays were also tested and validated in singleplex PCR assays, as were the *V. nubilum* and *V. zaregamsianum* singleplex assays that were not multiplexed with any other primers. Thus, all ten *Verticillium* species and the three *V. longisporum* lineages can be identified using eleven singleplex and four multiplex PCR assays (Figures 2, 3, 4, S1, S2).

We also tested additional multiplex PCR combinations that were not discussed in this paper, including an assay that involved addition of *V. zaregamsianum*-specific primers to the *V. isaacii, V. klebahnii* and *V. tricorpus* multiplex assay, or a multiplex assay targeting all *Verticillium* species. However, in both cases there appeared to be primer interference, as the *V. zaregamsianum* and *V. nubilum* PCR bands tended to be inconsistent. This problem may be avoided by designing new primers, the DNA sequence alignments are provided (Alignments S1, S2, S3, S4, S5).

How to differentiate *V. dahliae* and *V. longisporum*

Verticillium dahliae is the most economically important pathogen in *Verticillium*, but it is the most difficult *Verticillium* species to identify by PCR assay. This is because *V. dahliae* is the parent of two of the three *V. longisporum* lineages, *V. longisporum* lineage A1/D2 and *V. longisporum* lineage A1/D3 (Figure 1). Due to the high genetic similarity between *V. longisporum* and *V. dahliae*, PCR primers specific to *V. dahliae* protein-coding genes will in most cases amplify the orthologs in *V. longisporum* lineages A1/D2 and A1/D3. The situation for the ITS region is different, as due to concerted evolution, each *V. longisporum* lineage only has a single ITS allele [46]. This is the *V. dahliae* ITS allele in *V. longisporum* lineage A1/D3, and the Species A1 ITS allele in *V. longisporum* lineages A1/D1 and A1/D2. The multiplex PCR assay differentiates *V. longisporum* from *V. dahliae* based on the Species A1 *EF* allele that is common to all *V. longisporum* lineages. The *V. longisporum* lineage A1/D1 is characterized by a Species D1 *GPD* allele that is unique to *V. longisporum* lineage A1/D1, and *V. longisporum* lineage A1/D3 has a *V.*

dahliae ITS allele that is absent in other *V. longisporum* lineages. Thus, the *V. longisporum* lineage A1/D1 PCR banding pattern consists of the 310-bp Species A1 *EF* and the 1020-bp Species D1 *GPD* amplicons, the *V. longisporum* lineage A1/D2 banding pattern consists of the 310-bp Species A1 *EF* amplicon, and the *V. longisporum* lineage A1/D3 pattern consists of the 310-bp Species A1 *EF* and the 490-bp ITS *V. dahliae* amplicons (Figures 2, 4). The *V. longisporum* lineage A1/D2 banding pattern is identical to the pattern expected for Species A1. However, Species A1 has never been found and is only known as one of the parents of *V. longisporum* [46].

Previously published PCR-based assays for identification of *V. dahliae* and *V. longisporum* may have to be re-evaluated. For instance, before the discovery of a *V. dahliae* ITS region in *V. longisporum* [46], Karapapa and Typas [42] proposed to use the presence of a 839-bp intron in the nuclear SSU rRNA gene as a marker for *V. longisporum.* However, in agreement with the ITS data, *V. longisporum* lineage A1/D3 also lacks the SSU intron (Figure 5), and based on Karapapa and Typas' assay, *V. longisporum* lineage A1/D3 would thus be identified as *V. dahliae*. A similar problem exists for *V. dahliae* diagnostic assays that use nuclear ribosomal markers [39], or markers based on *V. dahliae* protein coding genes which might falsely identify *V. longisporum* lineages A1/D2 and A1/D3 as *V. dahliae* [41]. However, *V. dahliae* and *V. longisporum* tend to have different host ranges, and in many cases, a *V. dahliae* assay that excludes all *V. longisporum* lineages may not be necessary.

Screening for the unknown *V. longisporum* parents Species A1 and Species D1

Two of the *V. longisporum* parents, the informally named Species A1 and Species D1 [46], have never been found and are only known as parents of *V. longisporum* (Figure 1). Since neither morphology nor ecology of Species A1 and Species D1 are known, and the two species may resemble *V. longisporum* and *V. dahliae* morphologically, PCR assays based on *V. longisporum* alleles provide an opportunity to screen existing or new collections for isolates of Species A1 and Species D1. Using the *V. dahliae* – *V. longisporum* multiplex PCR assay, and assuming that Species A1 and Species D1 are haploid as are the majority of *Verticillium* species and ascomycetes in general, the Species D1-diagnostic PCR banding pattern is expected to consist of only the 1020-bp Species D1 band, but the Species A1 banding pattern would be indistinguishable from the banding pattern of *V. longisporum* lineage A1/D2 that comprises one 310-bp Species A1 band (Figures 2, 4). To differentiate Species A1 from *Verticillium longisporum* lineage A1/D2, PCRs targeting protein coding genes, for instance using primer pairs VActF / VActR for *ACT*, VEFf / VEFr for *EF*, VGPDf2 / VGPDr for *GPD* [46], would result in amplicons that could only be sequenced without cloning in Species A1, and in phylogenetic analyses would cluster with Species A1. Alternatively, primer pairs specific to allele D2 of *V. longisporum* lineage A1/D2, could be used to confirm the absence of allele D2, either in singleplex PCRs, or as multiplex PCR assay with the *V. longisporum* multiplex primers. Suitable primer pairs targeting allele D2 that theoretically are compatible with the current *V. longisporum* multiplex primers,

include ActF2d2 / VActR targeting 503 bp of *ACT*, MATdf / MATdr targeting 419 bp of *MAT*, OxFd2 / VOxR targeting 505 bp of *OX*, TsFd2 / VTs2R targeting 511 bp of *TS* [46].

Identifying *Verticillium*

Identification of *Verticillium* to the level of genus, a prerequisite to using *Verticillium* PCR assays to species identification, is not trivial. If a fungus isolated from an agricultural substrate has thick-walled, dark-pigmented resting structures, and long, narrow conidiogenous cells arranged in whorls along the main axis of the conidiophore, chances are high that it belongs to *Verticillium* [4]. However, there are exceptions. *Gibellulopsis nigrescens* and *Musicillium theobromae* are associated with plants, resemble *Verticillium* species in terms of conidiophore and resting structure morphology, but are phylogenetically distinct and belong to different genera [48]. Also, numerous other unrelated fungi have conidiophores suggestive of *Verticillium* [49,50,51]. Confirmation of genus identification can be performed by sequencing the ITS region and undertaking a nucleotide BLAST search at GenBank, or preferably, phylogenetic analyses with a *Verticillium* ITS dataset that contains ex-type sequences [4], available from TreeBASE at www.treebase.org [52].

Identifying *Verticillium* species and setting up PCR assays

Details of all fifteen PCR assays designed in this study for identification and differentiation of *Verticillium* species, including primers and other PCR conditions, are listed in Tables 3, 4, 5, 6, 7 and 8.

As a starting point for PCR assay selection, morphological identification may be used, and a short morphological key is available in Inderbitzin et al. [4]. It is important to realize that *V. dahliae* can only be accurately identified with the *V. dahliae* – *V. longisporum* multiplex PCR assay which is the only assay that is able to distinguish *V. dahliae* from *V. longisporum*. The *V. dahliae* singleplex assay in Table 3 also amplifies isolates of the *V. longisporum* lineage A1/D3 (Figure 3), and can thus lead to false positive results.

Materials and Methods

DNA sequence data

A total of 104 DNA sequences from ten *Verticillium* species and *Gibellulopsis nigrescens* were retrieved from GenBank (Table S3). The sequences were chosen to reflect the genetic diversity at five loci in the ten *Verticillium* species in Inderbitzin et al. [4,46], or were used for primer design outside the regions sequenced by Inderbitzin et al. [4,46], and were from Klosterman et al. [53] and Pramateftaki et al. [54]. *Gibellulopsis nigrescens* sequences, used as outgroup by Inderbitzin et al. [4], were retrieved for *G. nigrescens* strain PD595. Sequences were retrieved from five different loci, including the ribosomal internal transcribed spacer (ITS) region, *actin* (*ACT*), *elongation factor 1-alpha* (*EF*), *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*), and *tryptophan synthase* (*TS*). Sixty-one of the retrieved sequences were from Inderbitzin et al. [4], 39 sequences were from Inderbitzin et al. [46], three sequences were from Klosterman et al. [53] and one sequence was from Pramateftaki et al. [54].

The ITS sequence of *Musicillium theobromae* strain PD686 (CBS 110322), an additional outgroup [48], was generated using primers ITS1-F [55] and ITS5 [56] with settings described in Inderbitzin et al. [46]. The ITS sequence was submitted to GenBank as JQ621980. The species identification of *M. theobromae* strain PD686 was based on GenBank ITS BLAST hits [57], and is thus tentative.

DNA sequence alignments

The DNA sequences of *Verticillium* and *Gibellulopsis* retrieved from GenBank were aligned separately for each of the five loci using CLUSTAL X version 2.0 [58,59], and alignments were manually optimized in Geneious Pro version 4.8.5 [60].

Primer design

Primer specificity was achieved by maximizing the number of mismatches between a primer's 3'-end and homologous sites in non-target lineages [61]. Primers were designed manually in Geneious Pro version 4.8.5 [60] based on the *ACT, EF, GPD,* ITS or *TS* alignments, and primer annealing temperatures and primer self-complementarity were determined using OligoCalc [47], available at http://basic.northwestern.edu/biotools/OligoCalc.html (last accessed February 3, 2012) using default settings.

Fungal culturing, DNA extraction, PCR conditions and gel electrophoresis

Fungal isolates were grown and DNA was extracted as described in Inderbitzin et al. [46]. PCRs were performed using GoTaq Colorless Master Mix (Promega Corp., Madison, WI, USA) in GeneMate 0.2 ml 8-strip PCR tubes (BioExpress, Kaysville, UT). Each PCR reaction comprised 10 µl template dilution containing 1, 10, or 100 ng DNA, 2.5 µl primer mixture (0.5 µM for each primer, except primers D3f and D3r that were 0.25 µM each when multiplexed) and 12.5 µl master mix, for a total volume of 25 µl. The PCR program consisted of a 2 min initial denaturation step at 94°C, 32 or 35 cycles of 10 sec at 94°C, 20 sec at the PCR assay-dependent annealing temperature, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. PCR reactions were set up at room temperature under sterile conditions in a laminar flow hood wearing gloves and using plugged pipet tips. The reactions were run immediately, or were stored in a freezer. PCR machines used were a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA), a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA) and a PTC-200 DNA Engine (BioRad Laboratories, Inc., Hercules, CA).

Agarose gel electrophoresis was performed in a RAGE RGX-60 gel box with 20-sample comb (Cascade Biologics, Inc., Portland, Oregon) or a larger Bio-Rad Wide Mini Sub Cell gel box (Bio-Rad Life Science, Hercules, CA) with a 30-sample box. Gels were run between 30 to 70 minutes at 70-90 V, using various agarose concentrations (Tables 3, 4). PCR product, 4-6 µl was loaded per well. A 2-log DNA ladder, 0.75 µg (New England Biolabs, Inc., Ipswich, MA) was loaded per well. Loading buffer contained xylene cyanol or bromophenol blue for small and large amplicons, respectively [62].

Confirmation of PCR assay specificity

PCR assays were validated with 24 *Verticillium* isolates representing the allelic diversity at *ACT, EF, GPD,* ITS and *TS* as determined by Inderbitzin et al. [4,46], with the exception of *V. dahliae* where only three strains were used to represent *V. dahliae* lineages D2, D3 and the main group of *V. dahliae* [46] (Table 2). Also included were two outgroups, *Gibellulopsis nigrescens* strain PD595 [46], and the more distantly related *Musicillium theobromae* strain PD686 [48].

The specificity of each of the eleven primer pairs was initially assessed in PCR assays comprising one representative of each target lineage as a positive control, and representatives of non-target lineages as negative controls. In every PCR assay, negative controls were the non-target lineages that differed by four or fewer substitutions at the more variable primer site. When the most variable non-target primer site differed by more than four substitutions, a random non-target isolate was selected as negative control. The numbers of substitutions between primer sites across lineages are given in Table S2.

The four multiplex PCR assays, the *V. albo-atrum – V. alfalfae – V. nonalfalfae* multiplex PCR assay, the *V. dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay, the *V. dahliae – V. longisporum* multiplex PCR assay, and the *V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay, and the *V. nubilum* and *V. zaregamsianum* singleplex PCR assays whose primers were not part of any multiplex PCR assay, were tested against all 24 *Verticillium* isolates, one *G.*

nigrescens isolate and one *M. theobromae* isolate (Table 2). Each multiplex PCR assay was run on all three different PCR machines.

Validation of PCR assays using additional isolates

The *V. dahliae* – *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay was used to identify eleven genetically uncharacterized isolates from lettuce in California. These were *V. dahliae* strains Ls.1867, Ls.1870, Ls.1871, Ls.1875, Ls.1877, Ls.1878, *V. isaacii* strains Ls.1864, Ls.1868, Ls.1869, and *V. klebahnii* strains Ls.1865 and Ls.1886 obtained from the Subbarao lab collection. The PCR results were confirmed by DNA sequencing with the respective species-specific primers (Table S1) followed by phylogenetic analyses using PAUP v.4.0b 10 [63] (Figure S4). The methods used were as in Inderbitzin et al. [4,46].

The remaining PCR assays were tested with additional isolates that, except for *V. dahliae,* were all genetically identical at the loci examined to the isolates in Table 2 used for assay validation [4,46]. For the *V. dahliae* – *V. longisporum* multiplex PCR assay, the isolates used were *V. longisporum* strains PD640, PD676, PD725 (lineage A1/D1), strains PD402, PD629, PD730 (lineage A1/D2), strains PD589, PD687 and PD715 (lineage A1/D3), for the *V. albo-atrum* singleplex assay the isolates used were *V. albo-atrum* strains PD746, PD747, PD748, for the *V. alfalfae* singleplex assay the isolates used were *V. alfalfae* strains PD353, PD489, PD681, PD620, PD682, PD683, for the *V. dahliae* singleplex assay the isolates used were *V. dahliae* strains PD323, PD328, PD331, PD615, PD656, PD718, for the *V. nonalfalfae* singleplex assay the isolates used were *V. nonalfalfae* strains PD616, PD626, PD744, PD745, PD808,

P809, PD811, PD810, for the *V. nubilum* singleplex assay the isolates used were *V. nubilum* strains PD702, PD741, PD742, and for the *V. zaregamsianum* singleplex assay the isolates used were *V. zaregamsianum* strains D733, PD736, PD737, PD738 and PD740. Not tested with additional isolates were the *V. albo-atrum* – *V. alfalfae* – *V. nonalfalfae* multiplex assay, the *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay, and the *V. tricorpus* singleplex PCR assay.

Evaluation of a *V. longisporum* PCR assay from the literature

The presence of the 839-bp intron [42] in *V. longisporum* and *V. dahliae* was assessed with primer pair Bas3 [64] and NS6 [56], with the PCR conditions described above, with an annealing temperature of 48°C and an extension time of 2 min. Isolates screened for the presence of the intron were *V. longisporum* strains PD590 (lineage A1/D1), PD730 (lineage A1/D2), PD614 and PD715 (lineage A1/D3), as well as *V. dahliae* strains Ls.1875 (retrieved as DNA from the Subbarao lab collection) and PD362 [46].

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References

1. Pegg GF, Brady BL (2002) Verticillium wilts. Wallingford, Oxon, UK: CABI Publishing. 552 p.

2. Subbarao KV, Hubbard JC, Greathead AS, Spencer GA (1997) Verticillium wilt. In: Davis RM, Subbarao KV, Raid RN, Kurtz EA, editors. Compendium of Lettuce Diseases. St. Paul, MN: The American Phytopathological Society. pp. 26-27.

3. Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV (2009) Diversity, pathogenicity, and management of *Verticillium* species. Annu Rev Phytopathol 47: 39-62.

4. Inderbitzin P, Bostock RM, Davis RM, Usami T, Platt HW, et al. (2011) Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS ONE 6: e28341.

5. Gams W, Zare R, Summerbell RC (2005) Proposal to conserve the generic name *Verticillium* (anamorphic Ascomycetes) with a conserved type. Taxon 54: 179.

6. Hawksworth DL, Talboys PW (1970) *Verticillium dahliae*. CMI Descriptions of Pathogenic Fungi and Bacteria 256.

7. Platt HW, MacLean V, Mahuku G, Maxwell P (2000) Verticillium wilt of potatoes caused by three *Verticillium* species. In: Tjamos EC, Rowe RC, Heale JB, Fravel DR, editors. Advances in *Verticillium*: Research and disease management. St. Paul, MN: APS Press. pp. 59-62.

8. Heale JB, Isaac I (1963) Wilt of lucerne caused by species of *Verticillium*. IV. Pathogenicity of *V. albo-atrum* and *V. dahliae* to lucerne and other crops; spread and survival of *V. albo-atrum* in soil and weeds; effect upon lucerne production. Ann Appl Biol 52: 439–451.

9. Hawksworth DL, Talboys PW (1970) *Verticillium albo-atrum*. CMI Descriptions of Pathogenic Fungi and Bacteria 255.

10. Dunker S, Keunecke H, Steinbach P, von Tiedemann A (2008) Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. J Phytopathol 156: 698-707.

11. Babadoost M, Chen W, Bratsch AD, Eastman CE (2004) *Verticillium longisporum* and *Fusarium solani*: two new species in the complex of internal discoloration of horseradish roots. Plant Pathol 53: 669-676

12. Down G, Barbara D, Radišek S (2007) *Verticillium albo-atrum* and *V. dahliae* on hop. Bull OEPP 37: 528-535.

13. Radišek S, Jakše J, Javornik B (2006) Genetic variability and virulence among *Verticillium albo-atrum* isolates from hop. Eur J Plant Pathol 116: 301-314.

14. Isaac I (1953) A further comparative study of pathogenic isolates of *Verticillium*: *V. nubilum* Pethybr. and *V. tricorpus* sp. nov. Trans Br mycol Soc 36: 180-195.

15. Usami T, Kanto T, Inderbitzin P, Itoh M, Kisaki G, et al. (2010) *Verticillium tricorpus* causing lettuce wilt in Japan differs genetically from California lettuce isolates. J Gen Plant Pathol 77: 17-23.

16. Qin Q-M, Vallad GE, Subbarao KV (2008) Characterization of *Verticillium dahliae* and *V. tricorpus* isolates from lettuce and artichoke. Plant Disease 92: 69-77.

17. Wilhelm S (1955) Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology 45: 180-181.

18. Harris DC, Yang JR (1996) The relationship between the amount of *Verticillium dahliae* in soil and the incidence of strawberry wilt as a basis for disease risk prediction. Plant Pathol 45: 106-114.

19. Isaac I (1949) A comparative study of pathogenic isolates of *Verticillium*. Trans Br mycol Soc 32: 137-157.

20. Zeise K, von Tiedemann A (2002) Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. J Phytopathol 150: 112-119

21. Eynck C, Koopmann B, Grunewaldt-Stoecker G, Karlovsky P, von Tiedemann A (2007) Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. Eur J Plant Pathol 118: 259-274.

22. Larsen RC, Vandemark GJ, Hughes TJ, Grau CR (2007) Development of a real-time polymerase chain reaction assay for quantifying *Verticillium albo-atrum* DNA in resistant and susceptible alfalfa. Phytopathology 97: 1519-1525.

23. Nazar RN, Hu X, Schmidt J, Culham D, Robb J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in detection and differentiation of Verticillium wilt pathogens. Physiol Mol Plant Pathol 39: 1-11.

24. Carder JH, Morton A, Tabrett AM, Barbara DJ (1994) Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. In: Schots A, Dewey FM, Oliver RP, editors. Modern assays for plant pathogenic fungi: Identification, detection and quantification. Wallingford, Oxford: CAB International. pp. 91-97.

25. Koike M, Itaya Y, Hoshino K, Nagao H, Ohshima S (1997) PCR detection of Japanese isolates of *Verticillium dahliae* and *V. albo-atrum* using European subgroup-specific primers. Microbes Environ 12: 15-18.

26. Lievens B, Brouwer M, Vanachter ACRC, Cammue BPA, Thomma BPHJ (2006) Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Science 171: 155-165.

27. Dan H, Ali-Khan ST, Robb J (2001) Use of quantitative PCR diagnostics to identify tolerance and resistance to *Verticillium dahliae* in potato. Plant Dis 85: 700-705.

28. Platt HW, Mahuku GS, Maxwell P, MacLean V (2000) Detection techniques for research on *Verticillium* species in potato soils. In: Tjamos EC, Rowe RC, Heale JB, Fravel DR, editors. Advances in Verticillium: Research and disease management. St. Paul, MN: APS Press. pp. 140-143.

29. Banno S, Saito H, Sakai H, Urushibara T, Ikeda K, et al. (2011) Quantitative nested real-time PCR detection of *Verticillium longisporum* and *V. dahliae* in the soil of cabbage fields. J Gen Plant Pathol 77: 282-291.

30. Gayoso C, Martínez de Ilárduya O, Pomar F, Merino de Cáceres F (2007) Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different *Solanaceae* cultivars. Eur J Plant Pathol 118: 199-209.

31. Kuchta P, Jecz T, Korbin M (2008) The suitability of PCR-based techniques for detecting *Verticillium dahliae* in strawberry plants and soil. Journal of Fruit and Ornamental Plant Research 16: 295-304.

32. Li K-N, Rouse DI, Eyestone EJ, German TL (1999) The generation of specific DNA primers using random amplified polymorphic DNA and its application to *Verticillium dahliae*. Mycol Res 103: 1361-1368.

33. Atallah ZK, Bae J, Jansky SH, Rouse DI, Stevenson WR (2007) Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97: 865-872.

34. Volossiouk T, Robb EJ, Nazar RN (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. Appl Environ Microbiol 61: 3972-3976.

35. Mahuku GS, Platt HW (2002) Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. Am J Potato Res 79: 107-117.

36. Kageyama K, Komatsu T, Suga H (2003) Refined PCR protocol for detection of plant pathogens in soil. J Gen Plant Pathol 69: 153-160.

37. Debode J, Van Poucke K, França SC, Maes M, Höfte M, et al. (2011) Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. Plant Dis 95: 1571-1580.

38. Schena L, Nigro F, Ippolito A (2004) Real-time PCR detection and quantification of soilborne fungal pathogens: the case of *Rosellinia necatrix*, *Phytophthora nicotianae*, *P. citrophthora*, and *Verticillium dahliae*. Phytopathol Mediterr 43: 273–280.

39. Bilodeau GJ, Koike ST, Uribe P, Martin FN (2012) Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology 102: 331-343.

40. Karajeh MR, Masoud SA (2006) Molecular detection of *Verticillium dahliae* Kleb. in asymptomatic olive trees. J Phytopathol 154: 496-499.

41. Duressa D, Rauscher G, Koike ST, Mou B, Hayes RJ, et al. (2012) A real-time PCR assay for detection and quantification of *Verticillium dahliae* in spinach seed. Phytopathology 102: 443-451.

42. Karapapa VK, Typas MA (2001) Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a group-I intron found in the nuclear SSU-rRNA gene. Curr Microbiol 42: 217-224.

43. Steventon LA, Fahleson J, Hu Q, Dixelius C (2002) Identification of the causal agent of Verticillium wilt of winter oilseed rape in Sweden, *V. longisporum*. Mycol Res 106: 570-578.

44. Moukhamedov R, Hu X, Nazar RN, Robb J (1994) Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84: 256-259.

45. Robb J, Hu X, Platt H, Nazar R (1994) PCR-based assays for the detection and quantification of *Verticillium* species in potato. In: Schots A, Dewey FM, Oliver R, editors. Modern assays for plant pathogenic fungi: Identification, detection and quantification. Wallingford, Oxford: CAB International. pp. 83-90.

46. Inderbitzin P, Davis RM, Bostock RM, Subbarao KV (2011) The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS ONE 6: e18260.

47. Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res 35: W43-W46.

48. Zare R, Gams W, Starink-Willemse M, Summerbell RC (2007) *Gibellulopsis*, a suitable genus for *Verticillium nigrescens*, and *Musicillium*, a new genus for *V. theobromae*. Nova Hedwigia 85: 463-489.

49. Gra□fenhan T, Schroers H-J, Nirenberg HI, Seifert KA (2011) An overview of the taxonomy, phylogeny, and typificiation of nectriaceous fungi in *Cosmospora*, *Acremonium*, *Fusarium*, *Stilbella*, and *Volutella*. Stud Mycol 68: 79–113.

50. Gams W, Zare R (2001) A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. Nova Hedwigia 72: 329–337.

51. Sung G-H, Hywel-Jones NL, Sung J-M, Luangsa-ard JJ, Shrestha B, et al. (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Stud Mycol 57: 5-59.

52. Piel WH, Donoghue MJ, Sanderson MJ (2002) TreeBASE: a database of phylogenetic knowledge. In: Shimura J, Wilson KL, Gordon D, editors. To the interoperable "Catalog of Life" — with partners Species 2000 Asia Oceanea. Tsukuba, Japan: Research Report from the National Institute for Environmental Studies No. 171. pp. 41-47.

53. Klosterman SJ, Subbarao KV, Kang S, Veronese P, Gold SE, et al. (2011) Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathog 7: e1002137.

54. Pramateftaki PV, Antoniou PP, Typas MA (2000) The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: Intraspecific heterogeneity within the intergenic spacer region. Fungal Genet Biol 29: 19-27.

55. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2: 113-118.

56. White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols. San Diego: Academic Press, Inc. pp. 315-322.

57. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

58. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

59. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882.

60. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2010) Geneious v4.8.5, Available from http://www.geneious.com.

61. Kwok S, Chang SY, Sninsky JJ, Wang A (1994) A guide to the design and use of mismatched and degenerate primers. Genome Res 3: S39-S47.

62. Sambrook J, Russell DW (2001) Molecular Cloning. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

63. Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

64. Inderbitzin P, Landvik S, Abdel-Wahab MA, Berbee ML (2001) *Aliquandostipitaceae,* a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. Am J Bot 88: 52-61.

Figure Legends

Figure 1. Evolutionary history of *Verticillium longisporum* illustrated by a cartoon phylogenetic tree based on Inderbitzin et al. [46]. *Verticillium longisporum* evolved at least three different times by hybridization of Species A1, to Species D1, *V. dahliae* lineage D2 and *V. dahliae* lineage D3, resulting in *V. longisporum* lineages A1/D1, A1/D2 and A1/D3, respectively. *Verticillium dahliae* isolates are in blue, Species D1 in green, Species A1 in orange and *V. alfalfae* in gray. Red arrows indicate parents of *V. longisporum,* connecting lines represent the three *V. longisporum* lineages. The *Verticillium dahliae* lineage D2 is marked as *'V. dahliae* D2' and comprises both *V. dahliae* isolates and D2-alleles of *V. longisporum* lineage A1/D2. The *Verticillium dahliae* lineage D3 ('*V. dahliae* D3') comprises only D3-alleles of *V. longisporum* lineage A1/D3. Most of the *V. dahliae* isolates in Inderbitzin et al. [46] belonged to the clade marked '*V. dahliae* main group'. Tree is not to scale.

Figure 2. Specificity, expected band sizes and target loci of the eleven PCR primer pairs designed in this study. Phylogenetic tree on the left summarizes relationships of *Verticillium* species from Inderbitzin et al. [4,46], branch lengths are not to scale. The asterisks indicates that primer pair Df / Dr only amplifies the *V.*

dahliae strains of *V. dahliae* lineage D2, but not the strains of *V. longisporum* lineage A1/D2 (Figure 1). For details see text.

Figure 3. Singleplex PCR assays are species-specific except for the *V. dahliae* singleplex PCR assay that also amplifies *V. longisporum* lineage A1/D3. Agarose gels demonstrating selective amplification of all eleven species-specific singleplex PCR assays. Each gel is delimited by 2-log ladders, penultimate lanes are negative controls except in Figure 3h, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given by the lanes for every fifth lane. The PCR assay target species are indicated at the bottom of gels. For explanation of isolates selected as negative controls see text.

3a. *Verticillium albo-atrum* PCR assay. Lanes 2, 3: *V. nubilum* strain PD621, 10 and 100 ng of DNA, respectively. Lanes 4, 5: *V. albo-atrum* strain PD693, 10 and 100 ng of DNA, respectively. Size marker = 700 bp.

3b. *Verticillium alfalfae* PCR assay. Lanes 2, 3: *V. nonalfalfae* strain PD592, 10 and 100 ng DNA. Lanes 4, 5: *V. alfalfae* strain PD683, 10 and 100 ng DNA. Size marker = 1000 bp.

3c. *Verticillium dahliae* PCR assay. Lanes 2, 3: *V. albo-atrum* strain PD670, 10 and 100 ng DNA. Lanes 4, 5: *V. alfalfae* strain PD338, 10 and 100 ng DNA. Lanes 6, 7: *V. klebahnii* strain PD347, 10 and 100 ng DNA. Lanes 8, 9: *V. nonalfalfae* strain PD592, 10 and 100 ng DNA. Lanes 10, 11: *V. nubilum* strain PD621, 10 and 100 ng DNA. Lanes 12, 13: *V. tricorpus* strain PD593, 10 and 100 ng DNA. Lanes 14, 15: *V. zaregamsianum* strain PD586, 10 and 100 ng DNA. Lanes 16, 17: *V. isaacii* strain

PD341, 10 and 100 ng DNA. Lanes 18, 19: *V. dahliae* strain PD323, 10 and 100 ng DNA. Lanes 20, 21: *V. longisporum* lineage A1/D3 strain PD589, 10 and 100 ng DNA. Size marker = 500 bp. Note that the *V. dahliae* assay also amplifies *V. longisporum* lineage A1/D3, see lanes 20 and 21.

3d. *Verticillium isaacii* PCR assay. Lanes 2, 3: *V. klebahnii* strain PD347, 10 and 100 ng DNA. Lanes 4, 5: *V. klebahnii* strain PD407, 10 and 100 ng DNA. Lanes 6, 7: *V. tricorpus* strain PD593, 10 and 100 ng DNA. Lanes 8, 9: *V. isaacii* strain PD341, 10 and 100 ng DNA. Size marker = 200 bp.

3e. *Verticillium klebahnii* PCR assay. Lanes 2, 3: *V. isaacii* strain PD341, 10 and 100 ng DNA. Lanes 4, 5: *V. klebahnii* strain PD347, 10 and 100 ng DNA. Size marker = 200 bp.

3f. *Verticillium nonalfalfae* PCR assay. Lanes 2, 3: *V. alfalfae* strain PD683, 10 and 100 ng DNA. Lanes 4, 5: *V. nonalfalfae* strain PD592, 10 and 100 ng DNA. Size marker = 1200 bp.

3g. *Verticillium nubilum* PCR assay. Lanes 2, 3: *V. nonalfalfae* strain PD592, 10 and 100 ng DNA. Lanes 4, 5: *V. nubilum* strain PD741, 10 and 100 ng DNA. Size marker = 1200 bp.

3h, 3i. *Verticillium tricorpus* PCR assay. Lanes 2, 3: *V. dahliae* strain PD322, 10 and 100 ng DNA. Lanes 4, 5: *V. longisporum* lineage A1/D1 strain PD591, 10 and 100 ng DNA. Lanes 6, 7: *V. longisporum* lineage A1/D2 strain PD356, 10 and 100 ng DNA. Lanes 8, 9: *V. alfalfae* strain PD338, 10 and 100 ng DNA. Lanes 10, 11: *V. nonalfalfae* strain PD592, 10 and 100 ng DNA. Lanes 12, 13: *V. nubilum* strain PD621, 10 and 100 ng DNA. Lanes 14, 15: *V. albo-atrum* strain PD670, 10 and 100

ng DNA. Lanes 18, 19: *V. albo-atrum* strain PD693, 10 and 100 ng DNA. Lanes 20, 21: *V. zaregamsianum* strain PD586, 10 and 100 ng DNA. Lanes 22, 23: *V. zaregamsianum* strain PD739, 10 and 100 ng DNA. Lanes 24, 25: *V. isaacii* strain PD341, 10 and 100 ng DNA. Lanes 26, 27: *V. klebahnii* strain PD347, 10 and 100 ng DNA. Lanes 28, 29: *Gibellulopsis nigrescens* strain PD595, 10 and 100 ng DNA. Lanes 30, 31: *V. tricorpus* strain PD685, 10 and 100 ng DNA. Size marker = 400 bp.

3j. *Verticillium zaregamsianum* PCR assay. Lanes 2, 3: *V. tricorpus* strain PD685, 10 and 100 ng DNA. Lanes 4, 5: *V. tricorpus* strain PD703, 10 and 100 ng DNA. Lanes 6, 7: *V. zaregamsianum* strain PD586, 10 and 100 ng DNA. Size marker = 400 bp.

3k. Species A1 PCR assay. Lanes 2, 3: *V. dahliae* strain PD323, 10 and 100 ng DNA. Lanes 4, 5: *V. dahliae* strain PD327, 10 and 100 ng DNA. Lanes 6, 7: *V. dahliae* strain PD332, 10 and 100 ng DNA. Lane 8: *V. longisporum* lineage A1/D1 strain PD720, 10 ng DNA. Size marker = 300 bp.

3l. Species D1 PCR assay. Lanes 2, 3: *V. dahliae* strain PD328, 10 and 100 ng DNA. Lanes 4, 5: *V. longisporum* lineage A1/D2 strain PD402, 10 and 100 ng DNA. Lanes 6, 7: *V. longisporum* lineage A1/D3 strain PD687, 10 and 100 ng DNA. Lanes 8, 9: *V. longisporum* lineage A1/D1 strain PD640, 10 and 100 ng DNA. Size marker = 1000 bp.

Figure 4. Multiplex PCR assays identify genetically diverse target isolates. Each agarose gel displays the results of one of the four multiplex PCR assays, controls with none-target isolates are shown in Figure S2. Gels are delimited

by 2-log ladders, penultimate wells are negative controls, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given for every fifth lane. Abbreviations below bands indicate species and *V. longisporum* lineages. All lanes contain 100 ng template DNA. For an explanation of isolates included see text.

4a. *Verticillium albo-atrum – V. alfalfae – V. nonalfalfae* multiplex PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lane 5: *V. nonalfalfae* strain PD592. Size markers = 700 bp, 1000 bp, 1200 bp.

4b. *Verticillium dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2-4: *V. dahliae* strains PD322, PD327, PD502. Lanes 5-8: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 9, 10: *V. klebahnii* strains PD347, PD407. Lanes 11-13: *V. tricorpus* strains PD593, PD685, PD703. Size markers = 200 bp, 400 bp, 500 bp.

4c. *Verticillium dahliae – V. longisporum* PCR multiplex PCR assay. Lanes 2-4: *V. dahliae* strains PD322, PD327, PD502. Lane 5: *V. longisporum* lineage A1/D1 strain PD348. Lane 6: *V. longisporum* lineage A1/D2 strain PD356. Lane 7: *V. longisporum* lineage A1/D3 strain PD589. Size marker = 300 bp, 500 bp, 1000 bp.

4d. *Verticillium isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2-5: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 6, 7: *V. klebahnii* strains PD347, PD407. Lanes 8-10: *V. tricorpus* strains PD593, PD685, PD703. Size markers = 200 bp, 400 bp.

Figure 5. *Verticillium longisporum* lineage A1/D3 shares a 839-bp SSU rRNA intron with *V. dahliae* that is absent in the other *V. longisporum* lineages. Agarose gel showing Bas3 / NS6 amplicons. Gel is delimited by 2-log ladders, penultimate lane is negative control, and relevant size markers are indicated by '<' and correspond to 500 bp and 1500 bp, respectively. Lanes are numbered from left to right, fifth lane is numbered. Abbreviations below bands refer to *V. longisporum* lineages and *V. dahliae*. For information on isolates selected see text. Lane 2: *V. longisporum* lineage A1/D1 strain PD590. Lane 3: *V. longisporum* lineage A1/D2 strain PD730. Lane 4: *V. longisporum* lineage A1/D3 strain PD614. Lane 5: *V. longisporum* lineage A1/D3 strain PD715. Lane 6: *V. dahliae* strain Ls.1875. Lane 7: *V. dahliae* strain PD362.

Figure S1. *Verticillium nubilum* and *V. zaregamsianum* PCR assays are species-specific as illustrated by agarose gels of multiplex PCR assays with additional non-target isolates. Each gel is delimited by 2-log ladders, penultimate wells are negative controls, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given for every fifth lane. Specificities of PCR assays are given at bottom of gels. For explanation of isolates included see text.

S1a. *Verticillium nubilum* PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lanes 5-7: *V. dahliae* strains PD322, PD327, PD502. Lanes 8-11: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 12, 13: *V. klebahnii* strain PD347, PD407. Lane 14: *V. longisporum* lineage A1/D1 strain PD348. Lane 15: *V. longisporum* lineage A1/D2 strain PD356.

Lane 16: *V. longisporum* lineage A1/D3 strain PD589. Lane 17: *V. nonalfalfae* strain PD592. Lanes 18-20: *V. tricorpus* strains PD593, PD685, PD703. Lanes 21-24: *V. zaregamsianum* strains PD740, PD731, PD735, PD739. Lane 25: *Gibellulopsis nigrescens* strain PD710. Lane 26: *Musicillium theobromae* strain PD686. Lane 27: *V. nubilum* strain PD621. Size marker = 500 bp.

S1b. *Verticillium zaregamsianum* PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lanes 5-7: *V. dahliae* strains PD322, PD327, PD502. Lanes 8-11: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 12, 13: *V. klebahnii* strain PD347, PD407. Lane 14: *V. longisporum* lineage A1/D1 strain PD348. Lane 15: *V. longisporum* lineage A1/D2 strain PD356. Lane 16: *V. longisporum* lineage A1/D3 strain PD589. Lane 17: *V. nonalfalfae* strain PD592. Lane 18: *V. nubilum* strain PD621. Lanes 19-21: *V. tricorpus* strains PD593, PD685, PD703. Lane 22: *Gibellulopsis nigrescens* strain PD710. Lane 23: *Musicillium theobromae* strain PD686. Lane 24: *V. zaregamsianum* strain PD586. Size marker = 500 bp.

Figure S2. Multiplex PCR assays are species-specific as illustrated by agarose gels of multiplex PCR assays with non-target isolates. Each gel is delimited by 2-log ladders, penultimate wells are negative controls, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given for every fifth lane. Specificities of PCR assays are given at bottom of gels. For explanation of isolates included see text.

S2a. *Verticillium albo-atrum – V. alfalfae – V. nonalfalfae* multiplex PCR assay. Lanes 2-4: *V. dahliae* strains PD322, PD327, PD502, respectively. Lanes 5-8: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 9, 10: *V. klebahnii* strain PD347, PD407. Lane 11: *V. longisporum* lineage A1/D1 strain PD348. Lane 12: *V. longisporum* lineage A1/D2 strain PD356. Lane 13: *V. longisporum* lineage A1/D3 strain PD589. Lanes 14: *V. nubilum* strain PD621. Lanes 15-17: *V. tricorpus* strains PD593, PD685, PD703. Lanes 18-21: *V. zaregamsianum* strains PD586, PD731, PD735, PD739. Lane 22: *Gibellulopsis nigrescens* strain PD710. Lane 23: *Musicillium theobromae* strain PD686. Lane 24: *V. nonalfalfae* strain PD592. Size marker = 1200 bp.

S2b. *Verticillium dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lane 5: *V. longisporum* lineage A1/D1 strain PD348. Lane 6: *V. longisporum* lineage A1/D2 strain PD356. Lane 7: *V. longisporum* lineage A1/D3 strain PD589. Lane 8: *V. nonalfalfae* strain PD592. Lane 9: *V. nubilum* strain PD621. Lanes 10-13: *V. zaregamsianum* strains PD586, PD731, PD735, PD739. Lane 14: *Gibellulopsis nigrescens* strain PD710. Lane 15: *Musicillium theobromae* strain PD686. Lane 16: *V. dahliae* strain PD363. Size markers = 500 bp. Note that *V. longisporum* lineage A1/D3 has an identical amplicon to *V. dahliae.*

S2c. *Verticillium dahliae – V. longisporum* PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lanes 5-8: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 9, 10: *V. klebahnii* strain PD347, PD407. Lane 11: *V. nonalfalfae* strain PD592. Lane 12: *V. nubilum* strain PD621.

Lanes 13-15: *V. tricorpus* strains PD593, PD685, PD703. Lanes 16-19: *V. zaregamsianum* strains PD586, PD731, PD735, PD739. Lane 20: *Gibellulopsis nigrescens* strain PD710. Lane 21: *Musicillium theobromae* strain PD686. Lane 22: *V. dahliae* strain PD678. Size marker = 500 bp.

S2d. *Verticillium isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lanes 5-7: *V. dahliae* strains PD322, PD327, PD502. Lane 8: *V. longisporum* lineage A1/D1 strain PD348. Lane 9: *V. longisporum* lineage A1/D2 strain PD356. Lane 10: *V. longisporum* lineage A1/D3 strain PD589. Lane 11: *V. nonalfalfae* strain PD592. Lane 12: *V. nubilum* strain PD621. Lanes 13-16: *V. zaregamsianum* strains PD586, PD731, PD735, PD739. Lane 17: *Gibellulopsis nigrescens* strain PD710. Lane 18: *Musicillium theobromae* strain PD686. Lane 19: *V. isaacii* strain PD341. Size marker = 200 bp.

Figure S3. PCR assays correctly identify additional isolates. Each gel is delimited by 2-log ladders, penultimate wells are negative controls except for Figure S3f, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given for every fifth lane. Specificities of PCR assays are given at bottom of gels. For explanation of isolates included see text.

S3a. *Verticillium dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2, 3: *V. isaacii* strains Ls.1868, Ls.1869. Lanes 4-7: *V. dahliae* strains Ls.1871, Ls.1870, Ls.1875, Ls.1878. Lane 8: *V. klebahnii* strain Ls.1886.

Lanes 9, 10: *V. dahliae* strains Ls.1877, Ls.1867; Lane 11: *V. klebahnii* strain Ls.1865. Lane 12: *V. isaacii* strain Ls.1864. Size markers = 200, 500 bp.

S3b. *Verticillium albo-atrum* PCR assay. Lanes 2-7: *Verticillium albo-atrum* strains PD746, PD747 and PD748, each strain 10 and 100 ng DNA, respectively. Size marker = 700 bp.

S3c. *Verticillium alfalfae* PCR assay. Lanes 2-15: *Verticillium alfalfae* strains PD353, PD489, PD681, PD620, PD682, PD683 and PD338, each strain 10 and 100 ng DNA, respectively. Size marker = 1000 bp.

S3d. *Verticillium dahliae* PCR assay. Lanes 2-13. *Verticillium dahliae* strains PD323, PD328, PD331, PD615, PD656 and PD718, each strain 10 and 100 ng DNA, respectively. Size marker = 500 bp.

S3e. *Verticillium longisporum* PCR assay. Lanes 2-19. *Verticillium longisporum* strains PD640, PD676, PD725, PD402, PD629, PD730, PD589, PD687 and PD715, each strain 10 and 100 ng DNA, respectively. Size markers = 300, 500, 1000 bp.

S3f. *Verticillium nonalfalfae* PCR assay. Lanes 2-11. *Verticillium nonalfalfae* strains PD616, PD626, PD744, PD745 and PD808, each strain 10 and 100 ng DNA, respectively. Size marker = 1200 bp.

S3g. *Verticillium nonalfalfae* PCR assay. Lanes 2-9. *Verticillium nonalfalfae* strains P809, PD811, PD810 and PD592, each strain 10 and 100 ng DNA, respectively. Size marker = 1200 bp.

S3h. *Verticillium nubilum* PCR assay. Lanes 2-9. *Verticillium nubilum* strains PD702, PD741, PD742 and PD621, each strain 10 and 100 ng DNA, respectively. Size marker = 1200 bp.

S3i. *Verticillium zaregamsianum* PCR assay. Lanes 2-11. *Verticillium zaregamsianum* strains PD733, PD736, PD737, PD738 and PD740, each strain 10 and 100 ng DNA, respectively. Size marker = 300 bp.

Figure S4. Phylogenetic trees confirming the identification of previously genetically uncharacterized strains using the *V. dahliae* – *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay. Shown are most parsimonious trees obtained using representative taxon samples from Inderbitzin et al. [4] for the *EF* tree on the left, and from Inderbitzin et al. [46] for the ITS tree on the right. See those publications for GenBank accession numbers. Previously unknown strains are in bold and clustered within the species expected based on the multiplex PCR results (Figure S3a).

Tables

Table 1. Details of DNA sequence alignments used for primer design, including the numbers of taxa, alignment lengths and the numbers of primers designed at each locus.

Alignment locus	**Number of taxa**	**Alignment length, characters**	**Number of primers designed**
ACT	17	1203	3
EF	22	610	5
GPD	23	1221	5
ITS	17	619	2
TS	26	1464	3

Table 2. *Verticillium*, *Gibellulopsis* and *Musicillium* strains used to validate PCR assays.

Species	**Strain identifier** [A]
G. nigrescens	PD710
M. theobromae	PD686
V. albo-atrum	PD670
V. albo-atrum	PD693
V. alfalfae	PD338[B]
V. dahliae	PD322[C]
V. dahliae	PD327
V. dahliae	PD502
V. isaacii	PD341
V. isaacii	PD343
V. isaacii	PD618
V. isaacii	PD752
V. klebahnii	PD347
V. klebahnii	PD407
V. longisporum lineage A1/D1	PD348[D]
V. longisporum lineage A1/D2	PD356
V. longisporum lineage A1/D3	PD589
V. nonalfalfae	PD592
V. nubilum	PD621[E]

V. tricorpus	PD593
V. tricorpus	PD685
V. tricorpus	PD703
V. zaregamsianum	PD586
V. zaregamsianum	PD731
V. zaregamsianum	PD735
V. zaregamsianum	PD739

[A] See Inderbitzin et al. [4,46] for *Verticillium* and *Gibellulopsis* strain information, for information on the *M. theobromae* strain see text.

[B] Replaced at times by genetically equivalent *V. alfalfae* strain PD683 [4].

[C] Replaced at times by genetically equivalent *V. dahliae* strains PD328, PD323 and PD332 [46].

[D] Replaced at times by genetically equivalent *V. longisporum* lineage A1/D1 strains PD591, PD640, PD720 [46].

[E] Replaced at times by genetically equivalent *V. nubilum* strain PD741 [4].

Table 3. Details of *Verticillium* singleplex PCR assays, including target loci, primer pairs, DNA template concentrations, PCR annealing temperatures, numbers of PCR cycles, PCR product sizes, and agarose gel concentrations for gel electrophoresis.

Target species	**Target locus**	**Primer pair[A]**	**DNA template / reaction[A], ng**	**Annealing temperature[B], °C**	**PCR cycles[B]**	**PCR amplicon, bp**	**Agarose in TAE buffer, % (w/v)**
V. albo-atrum	*ACT*	AaF / AaTr	10 or 100	62	35	725	1.5
V. alfalfae	*GPD*	AlfF / AlfD1r	10 or 100	62	35	1060	1.5
V. dahliae[C]	*ITS*	Df / Dr	1 or 10	67	32	490	1.5 or 2
V. isaacii	*EF*	If / IKr	10 or 100	64	35	195	2
V. klebahnii	*EF*	Kf / IKr	10 or 100	62	35	230	2
V. nonalfalfae	*TS*	NoF / NoNuR	10 or 100	64	35	1310	1.5
V. nubilum	*TS*	Nuf / NoNuR	10 or 100	62	35	1150	1.5
V. tricorpus	*ACT*	Tf / AaTr	10 or 100	64	35	415	1.5 or 2
V. zaregamsianum	*GPD*	Zf / Zr	10 or 100	64	35	360	2
Species A1	*EF*	A1f / A1r	10 or 100	64	35	310	2

Species D1	*GPD*	D1f / AlfD1r	10 or 100	70	35	1020	1.5

[A] Each 25 µl PCR reaction contained the following: 1.25 µl of each primer from 10 µM stocks, 12.5 µl Promega master mix, and 10 µl template containing 10 or 100 ng DNA.

[B] The PCR program consisted of a 2 min initial denaturation step at 94°C, 32 or 35 cycles of 10 sec at 94°C, 20 sec at the PCR assay-dependent annealing temperature, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. PCR reactions were set up at room temperature under sterile conditions and run immediately, or were stored in a freezer.

[C] Assay does not differentiate *V. dahliae* from *V. longisporum* lineage A1/D3.

Table 4. Details of *Verticillium* multiplex PCR assays, including target loci, primer pairs, DNA template concentrations, PCR annealing temperatures, numbers of PCR cycles, PCR product sizes, and agarose gel concentrations for gel electrophoresis.

Target species	**Target loci**	**Primer pairs[A]**	**DNA template / reaction[A], ng**	**Annealing temperature[B], °C**	**PCR cycles[B]**	**PCR amplicon, bp**	**Agarose in TAE buffer, % (w/v)**
V. albo-atrum – V. alfalfae – V. nonalfalfae	*ACT, GPD, TS*	AlfF / AlfD1r, NoF / NoNuR, AaF / AaTr	10 or 100	64	35	725, 1060, 1310	1.5
V. dahliae – V. longisporum lineages	*GPD, EF, ITS*	D1f / AlfD1r, A1f / A1r, Df / Dr	10 or 100	64	35	490, 310, 1020	1.5
V. dahliae[C] *– V. isaacii – V. klebahnii – V. tricorpus*	*ITS, EF, ACT*	Df / Dr, If / IKr, Kf / IKr, Tf / AaTr	10 or 100	64	35	490, 195, 230, 415	2
V. isaacii – V.	*EF, ACT*	If / IKr, Kf / IKr,	10 or 100	62	35	195, 230,	2

klebahnii – V. tricorpus		Tf / AaTr				415	

[A] Each 25 µl PCR reaction contained the following: 2.5 µl primer mixture (see Table XX), 12.5 µl Promega master mix, and 10 µl template containing 10 or 100 ng DNA.

[B] The PCR program consisted of a 2 min initial denaturation step at 94°C, 32 or 35 cycles of 10 sec at 94°C, 20 sec at the PCR assay-dependent annealing temperature, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. PCR reactions were set up at room temperature under sterile conditions and run immediately, or were stored in a freezer.

[C] Assay does not differentiate *V. dahliae* from *V. longisporum* lineage A1/D3.

1

Table 5. Preparation of 125 µl primer stock for *V. albo-atrum* – *V. alfalfae* – *V. nonalfalfae* multiplex PCR assay sufficient for 50 25-µl PCR reactions

Component	**Volume, µl**[A]
AlfF	6.25
NoF	6.25
NoNuR	6.25
AaF	6.25
AaTr	6.25
AlfD1r	6.25
diH20	87.5

[A] Primer initial concentrations = 100 µM each.

Table 6. Preparation of 125 µl primer stock for *V. dahliae* – *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay sufficient for 50 25-µl PCR reactions.

Component	**Volume, µl**[A]
AaTr	6.25
If	6.25
Kf	6.25
Tf	6.25
IKr	6.25
Df	3.125
Dr	3.125
diH20	87.5

[A] Primer initial concentrations = 100 µM each.

Table 7. Preparation of 125 µl primer stock for *V. dahliae* – *V. longisporum* lineages multiplex PCR assay sufficient for 50 25-µl PCR reactions.

Component	**Volume, µl**[A]
D1f	6.25
AlfD1r	6.25
A1f	6.25
A1r	6.25
Df	3.125
Dr	3.125
diH20	93.75

[A] Primer initial concentrations = 100 µM each.

Table 8. Preparation of 125 µl primer stock for *V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay sufficient for 50 25-µl PCR reactions.

Component	**Volume, µl**[A]
AaTr	6.25
If	6.25
Kf	6.25
Tf	6.25
IKr	6.25
diH20	93.75

[A] Primer initial concentrations = 100 µM each.

Table S1. Primers designed in this study, primer names reflect deployment in PCR assays: 'Aa' = *V. albo-atrum*, 'D' = *V. dahliae* except *V. dahliae* lineage D2, 'T' = *V. tricorpus*, 'A1' = Species A1, 'I' = *V. isaacii*, 'K' = *V. klebahnii*, 'Z' = *V. zaregamsianum*, 'Alf' = *V. alfalfae*, 'D1' = Species D1, 'D3' = *V. dahliae* lineage D3, 'No' = *V. nonalfalfae*, 'Nu' = *V. nubilum*; 'f' and 'r' refer to primer orientation, forward and reverse, respectively.

Table S2. Numbers of substitutions at primer sites among a representative sample of *Verticillium* strains and a *Gibellulopsis nigrescens* outgroup [4,46]. Substitution numbers marked by an asterisk are inferred from DNA sequence alignments (Alignments S1, S2, S3, S4, S5), see Table S3 for accession numbers. The remaining substitution numbers are derived from single-locus phylogenetic trees in Inderbitzin et al. [4,46].

Table S3. GenBank and other accession numbers of DNA sequences used for primer design.

Alignments

Alignment S1. FASTA text file with *ACT* alignment used for primer design, primer sites are indicated. Sequence accession numbers are given as part of sequence names for sequences in public databases.

Alignment S2. FASTA text file with *EF* alignment used for primer design, primer sites are indicated. Sequence accession numbers are given as part of sequence names for sequences in public databases.

Alignment S3. FASTA text file with *GPD* alignment used for primer design, primer sites are indicated. Sequence accession numbers are given as part of sequence names for sequences in public databases.

Alignment S4. FASTA text file with ITS alignment used for primer design, primer sites are indicated. Sequence accession numbers are given as part of sequence names for sequences in public databases.

Alignment S5. FASTA text file with *TS* alignment used for primer design, primer sites are indicated. Sequence accession numbers are given as part of sequence names for sequences in public databases.

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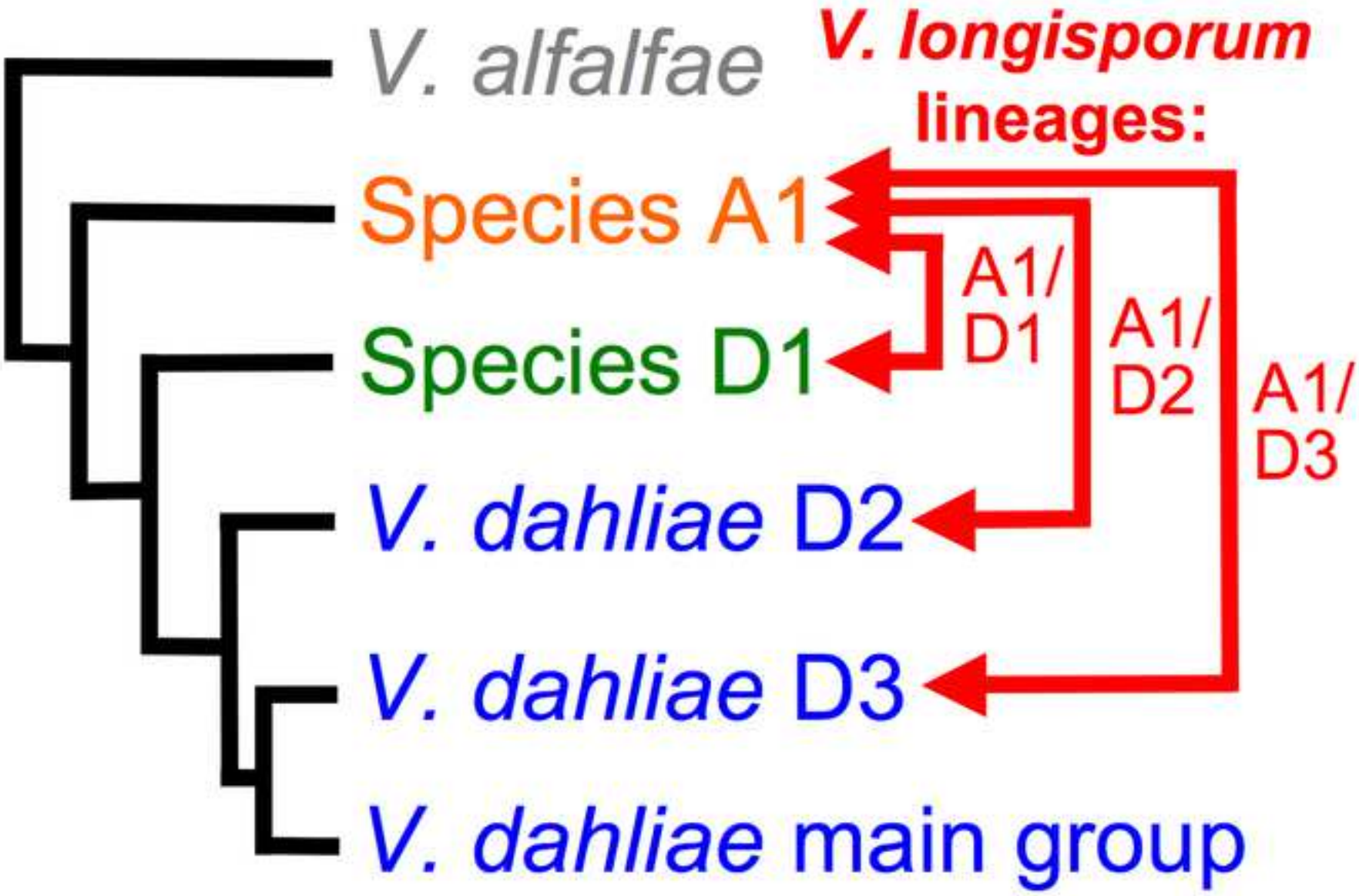

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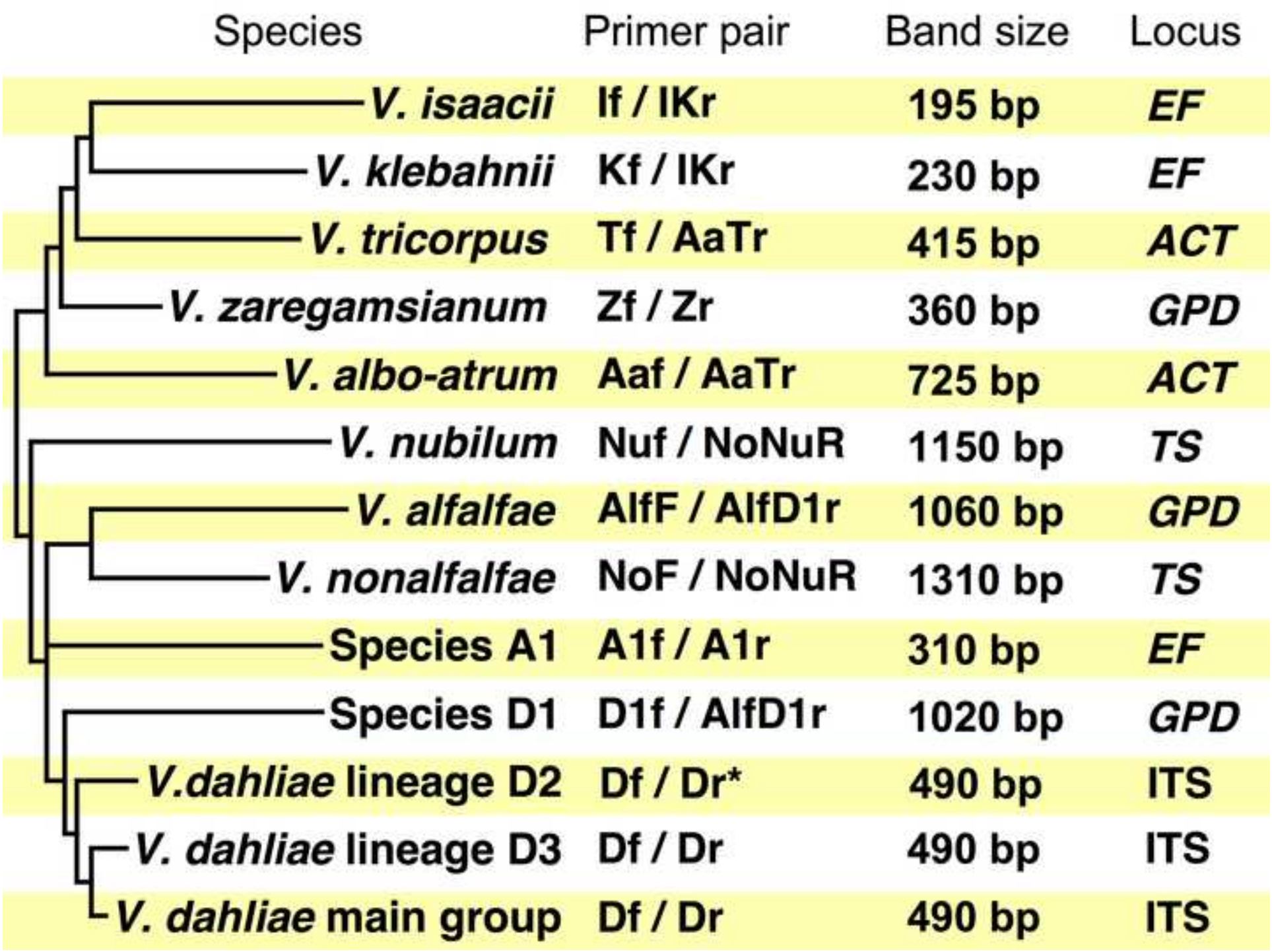

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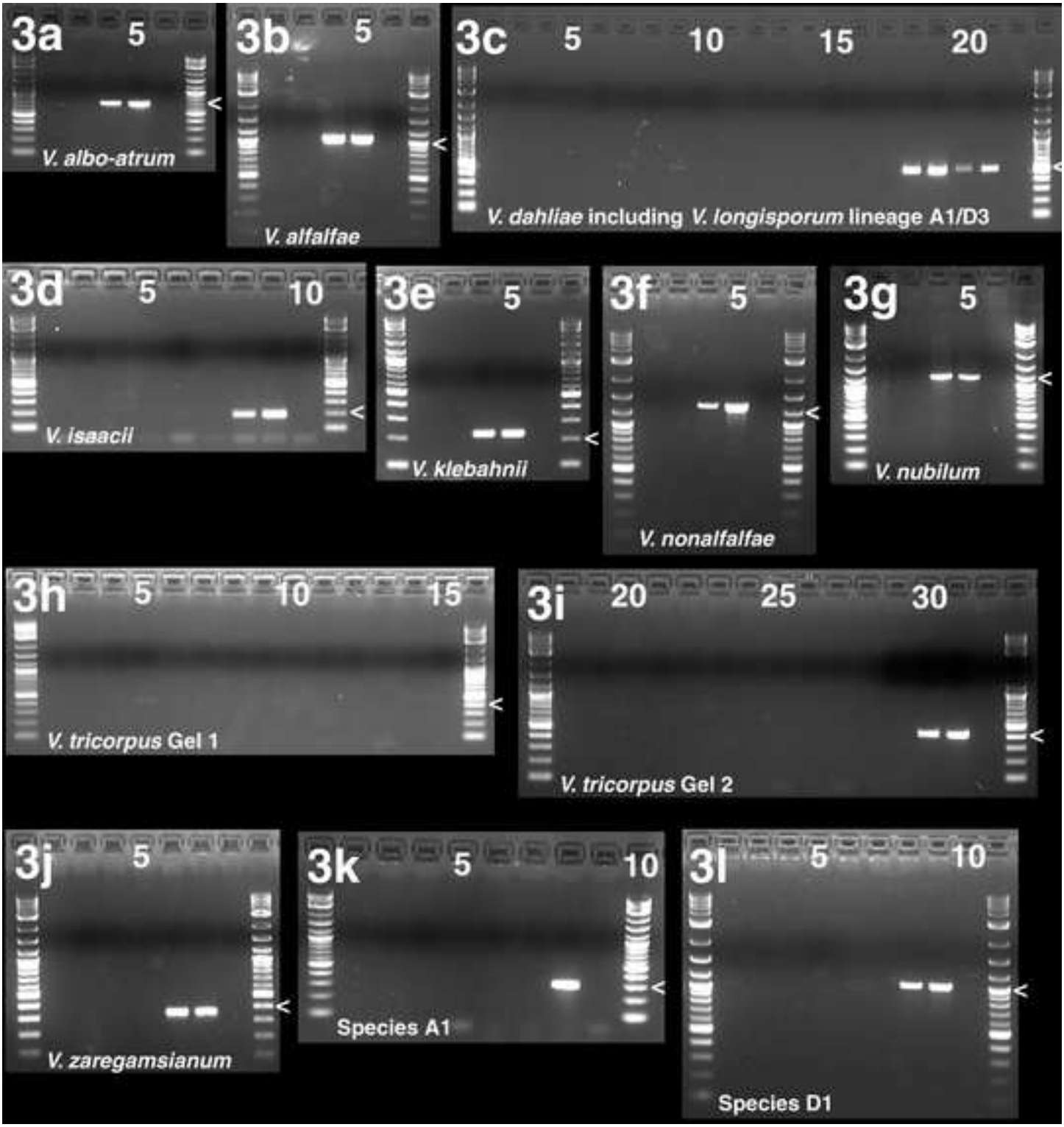

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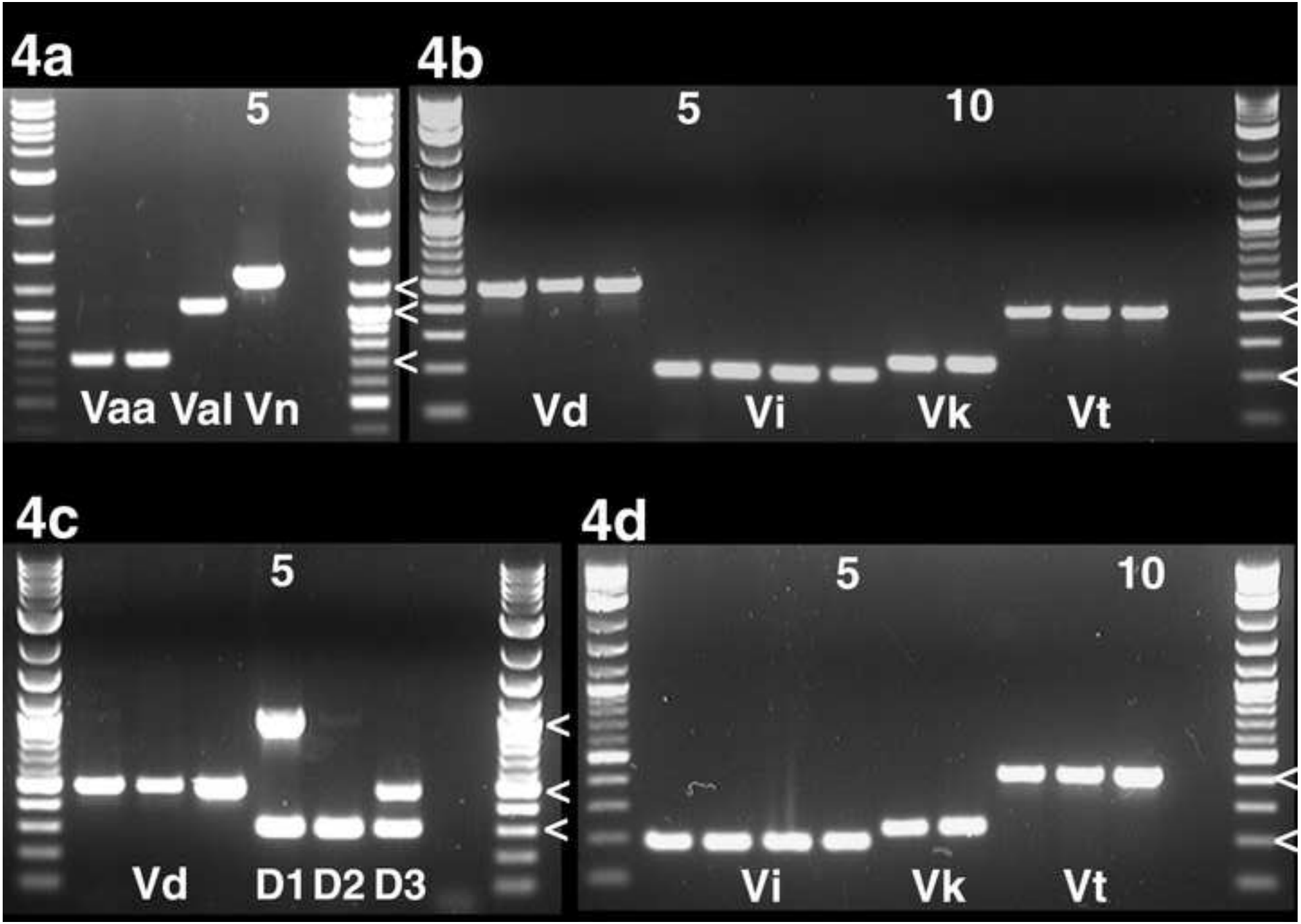

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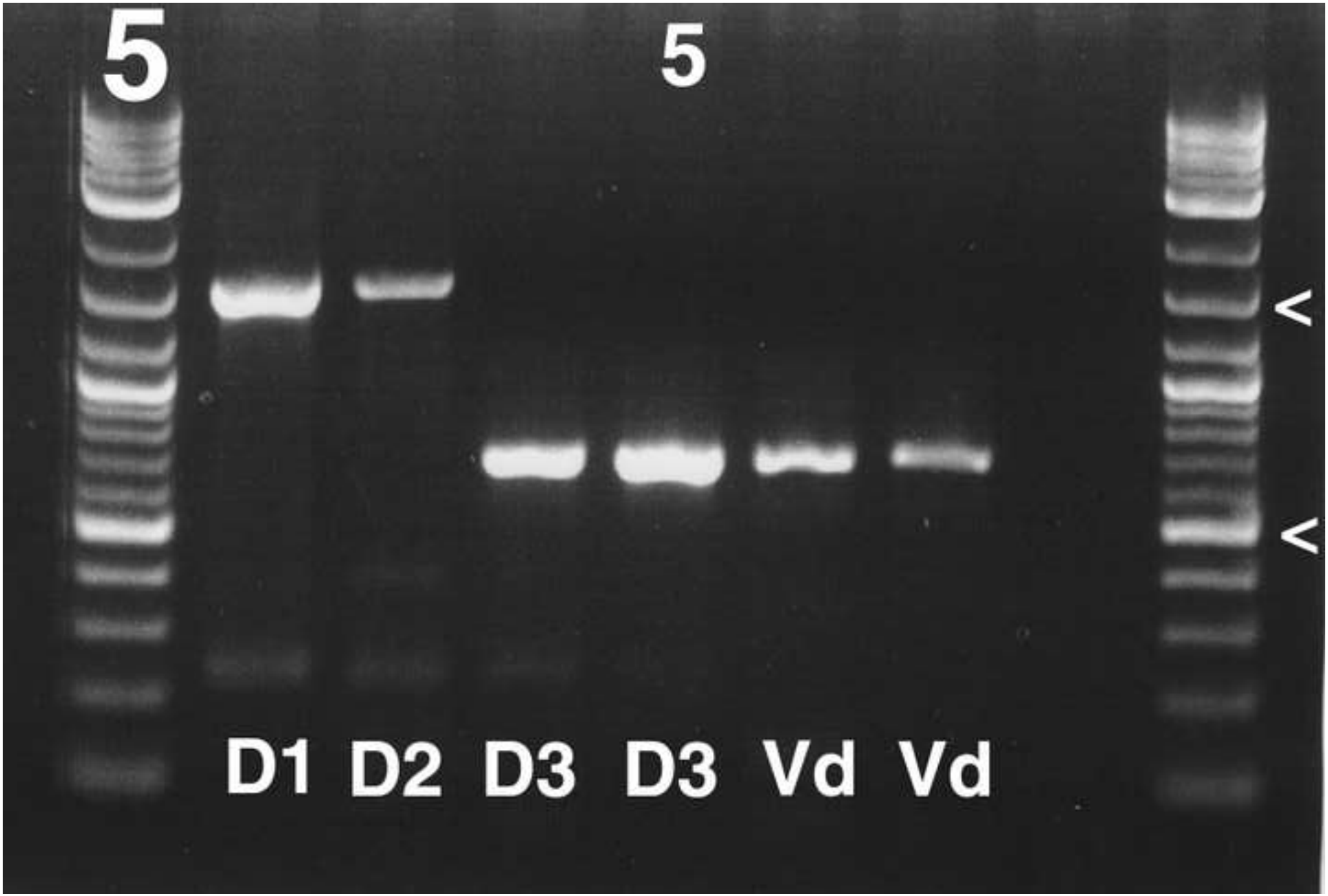

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