Phylogenetics and Taxonomy of the Fungal Vascular Wilt Pathogen *Verticillium*, with the Descriptions of Five New Species

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Abstract

Knowledge of pathogen biology and genetic diversity is a cornerstone of effective disease management, and accurate identification of the pathogen is a foundation of pathogen biology. Species names provide an ideal framework for storage and retrieval of relevant information, a system that is contingent on a clear understanding of species boundaries and consistent species identification. *Verticillium*, a genus of ascomycete fungi, contains important plant pathogens whose species boundaries have been ill-defined. Using phylogenetic analyses, morphological investigations and comparisons to herbarium material and the literature, we established a taxonomic framework for *Verticillium* comprising ten species, five of which are new to science. We used a collection of 74 isolates representing much of the diversity of *Verticillium*, and phylogenetic analyses based on the ribosomal internal transcribed spacer region (ITS), partial sequences of the protein coding genes actin (ACT), elongation factor 1-alpha (EF), glyceraldehyde-3-phosphate dehydrogenase (GPD) and tryptophan synthase (TS). Combined analyses of the ACT, EF, GPD and TS datasets recognized two major groups within *Verticillium*, Clade Flavexudans and Clade Flavnonexudans, reflecting the respective production and absence of yellow hyphal pigments. Clade Flavexudans comprised *V. albo-atrum* and *V. tricorpus* as well as the new species *V. zaregamsianum*, *V. isaaeci* and *V. klebanhii*, of which the latter two were morphologically indistinguishable from *V. tricorpus* but may differ in pathogenicity. Clade Flavnonexudans comprised *V. nubilum*, *V. dahliae* and *V. longisporum*, as well as the two new species *V. alfalfae* and *V. nonalfalfae*, which resembled the distantly related *V. albo-atrum* in morphology. Apart from the diploid hybrid *V. longisporum*, each of the ten species corresponded to a single clade in the phylogenetic tree comprising just one ex-type strain, thereby establishing a direct link to a herbarium specimen. A morphology-based key is provided for identification to species or species groups.

Introduction

The genus *Verticillium* comprises a small group of plant-pathogenic fungi that cause billions of dollars of damage annually to a variety of agricultural crops in many parts of the world [1]. *Verticillium* species are soil-borne and cause *Verticillium* wilt, a plant disease that affects the vasculature of many different hosts [1], and can cause significant crop losses [2].

Control of *Verticillium* wilt is difficult and costly [3,4]. In the absence of a suitable plant host, *Verticillium* species can remain dormant in the soil for years by means of small, melanized resting structures that are extremely durable, and will only germinate in the proximity of a suitable host [5].

*Verticillium* has a long taxonomic history. The first species of *Verticillium* was first found in 1816 [6], and approximately 190 species have since been described [7]. The species share the characteristic *Verticillium* conidiophore that is comprised of spore-forming cells that are narrowly flask-shaped, and are assembled into whorls (verticils) and attached along a main axis. The advent of molecular systematics confirmed that *Verticillium* was composed of several distantly related and ecologically diverse groups which were subsequently removed from *Verticillium* [8,9] and placed in other genera. These include *Lecanicillium*, containing insect and fungus pathogens [10,11,12], *Pochonia* and *Haplocladium* comprising nematode parasites [13,14], and *Gibellulopsis* and *Musicillium* containing plant pathogens [15]. The reduced genus *Verticillium*, also referred to as *Verticillium* sensu stricto, thus consisted of only five species of plant associates and plant pathogens [13], and was reclassified with *V. dahliae* [16]. *Verticillium* is placed in the family Plectosphaerellaceae [15] that is closely related to *Colletotrichum* in the Glomerellaceae [17], another important group of plant pathogens. Both Plectosphaerellaceae and Glomerellaceae are families of uncertain phylogenetic position in the Hypocreomycetidae, a subclass within the fungal phylum Ascomycota [17,18]. *Gibellulopsis* and *Musicillium* are also part of the Plectosphaerellaceae [15], whereas *Lecanicillium*, *Pochonia* and *Haplocladium* are...
Verticillium diversity. We then studied evolutionary relationships using herbarium material and the literature, and described new species based on morphological investigations. Finally, we determined the correct species boundaries using multigene phylogenetic analyses and DNA sequence data.

This led to a significant improvement of our knowledge of Verticillium species, which will allow for a more reliable and consistent identification of species, and will provide a means for their identification. The new taxonomic system allows for pathogen exclusion and successful quarantine.

Verticillium morphology is a suitable character for species delimitation in Verticillium biology. Potential practical applications are many, and may include the confident relationship between Verticillium species and their hosts.

### Results

**DNA sequence data**

In order to investigate the phylogenetic relationships between Verticillium species, we generated DNA sequence data for 64 isolates, which were sequenced for the ITS, ACT, GPD, EF, and TS regions. We then analyzed the data using parsimony and maximum likelihood methods.

We found that the DNA sequence data from the five single-locus datasets (ITS, ACT, GPD, EF, TS) contained similar phylogenetic information.

### Single-locus analyses

To investigate whether the five single-locus datasets (ITS, ACT, GPD, EF, TS) contained similar phylogenetic information, we first analyzed each dataset individually using parsimony. For each single-locus analysis, we included only one representative of each species.

We did not detect any significant conflict between the most parsimonious trees from the five single-locus datasets on a 70% bootstrap support level.

### Table 1.

Statistics of the ITS, ACT, EF, GPD and TS single-locus datasets, the combined four-locus dataset and their respective most parsimonious trees.

| Locus | Haplotypes | Taxa | Characters | Variable characters | Parsimony informative characters | MPTs: number/ steps | CI/Ri | Clades >70% support |
|-------|------------|------|------------|--------------------|---------------------------------|----------------------|-------|---------------------|
| ITS   | 15         | 74   | 514        | 74 (14%)           | 62 (12%)                        | 1/94                 | 0.904/0.943 | 10 |
| ACT   | 17         | 77   | 638        | 283 (44%)          | 230 (36%)                       | 9/427                | 0.855/0.925 | 20 |
| EF    | 19         | 77   | 614        | 338 (55%)          | 234 (38%)                       | 12/599               | 0.825/0.896 | 20 |
| GPD   | 23         | 77   | 781        | 252 (32%)          | 209 (27%)                       | 2/430                | 0.802/0.917 | 27 |
| TS    | 26         | 77   | 625        | 298 (48%)          | 236 (38%)                       | 396/565              | 0.772/0.911 | 25 |
| ACT, EF, GPD, TS | 32 | 77 | 2658 | 1171 (44%) | 996 (27%) | 48/2041 | 0.805/0.944 | 35 |

*Percentages refer to the proportions of variable and parsimony informative characters in each dataset.

Ci: consistency index; Ri: retention index.

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Combined analyses

With the goal of improving the phylogenetic resolution, we combined the ACT, EF, GPD and TS datasets into a single alignment for combined analysis. We did not include the ITS dataset since for *V. longisporum*, the ITS phylogeny does not retrace the evolution of that species [27]. The resulting combined four-locus alignment comprised 77 taxa and 2658 characters, and was submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S11756). There were a total of 32 unique multilocus haplotypes (Table 1). The Bayesian consensus tree is illustrated in Figure 1, it was congruent with the most likely tree (ln likelihood = 12016.40) and with the 40 most parsimonious trees (Table 1) that differed at poorly supported branches within *V. dahliae* and the outgroup *Globodulafus nigrescens* (data not shown, but see support values in Figure 1).

We analyzed the four single-locus datasets jointly despite topological conflicts between them (Figures S2, S3, S4, S5). To evaluate whether single-locus datasets should be concatenated for combined analyses, a conditional combinability approach is often used which states that datasets should not be combined if there are significant differences between them [28,29]. There is no agreement how much the single-locus datasets are allowed to differ, but topological differences supported by 70-90% of the bootstrap replicates have been used as cutoffs [28,30]. In our case, there were topological differences supported by up to 100% of the parsimony bootstrap replicates between the single-locus datasets, involving the positions of *V. nubilum*, Species A1 and *V. zaregamsianum*. However, we found that the four-locus phylogeny comprised 35 groups with >70% support, higher than any of the single-locus trees (Table 1). Also, for *V. nubilum*, Species A1 and *V. zaregamsianum*, the combined analyses resulted for each species in the topology that had strongest overall support from the single-locus phylogenies. But the phylogenetic affinities of *V. nubilum* and Species A1 remain uncertain (Figure 1), and more data is needed to conclusively determine the closest relatives of these two species [27]. Species A1 and Species D1 were not linked to any type material and could not be officially described, since Species A1 and Species D1 have never been found [27].

The evolutionary relationships among the *Verticillium* species was overall well resolved, the species fell into two major clades reflecting morphological similarity. The major clades were Clade Flavexudans containing species producing yellow-pigmented hyphae including *V. albo-atrum*, *V. isacii*, *V. klebahnii*, *V. tricorpus* and *V. zaregamsianum*, and Clade Flavonexudans with species devoid of yellow-pigmented hyphae, including *V. alfalfa*, *V. dahliae*, *V. nonalfalfa*, and *V. longisporum* (Figure 1). The exception was *V. nubilum* whose placement in Clade Flavonexudans agreed with morphological data, but was only supported in the parsimony analyses (Figure 1). The other exception was the position of the *V. longisporum* ancestor Species A1 whose placement in the Bayesian consensus tree (Figure 1) contradicted phylogenetic analyses by Inderbitzin et al. [27] who used a different dataset.

Mating type distribution in *V. alfalfa* and *V. nonalfalfa*

All seven *V. alfalfa* and nine *V. nonalfalfa* isolates were screened for presence of MATI-1 and MATI-2 dimorphs which are the two mating compatibility alleles in ascomycetes [32]. All *V. alfalfa* isolates showed the MATI-1 specific PCR band whereas all *V. nonalfalfa* isolates lacked that band. All *V. alfalfa* isolates lacked the MATI-2 specific band, whereas the MATI-2 specific band was present in all *V. nonalfalfa* isolates (Figure 2). Thus, all *V. alfalfa* isolates likely have MATI-1 dimorphs whereas *V. nonalfalfa* isolates have MATI-2 dimorphs.

Taxonomy

The genus *Verticillium* sensu stricto corresponds to a monophyletic group of taxa comprising *V. dahliae* that has been conserved as the type of *Verticillium* [15,16]. We recognize ten species in *Verticillium* sensu stricto that are listed below in alphabetic order. The information provided for each species was obtained from morphological examination of cultures and herbarium specimens (Figure 3), literature surveys and phylogenetic analyses (Figure 1).

*Verticillium albo-atrum* Reinke & Berthold, Untersuchungen aus dem Botanischen Laboratorium der Universität Göttingen 1:75 (1879) **Figure 4**

MycoBank: MB199278 (as *V. alboatrum*)

**Description.** Colonies on PDA after two weeks 4.5–5.5 cm diam, white at first, later turning yellow to orange due to the formation of yellow-pigmented hyphae, then darkening due to formation of resting mycelium immersed in the agar medium (Figures 4a, 4b). Aerial mycelium generally abundant, floccose to pruinose, hyphae smooth-walled, (1–) 1.5–4 µm wide. Conidiophores erect or slanted, generally determinate (Figure 4c), branched or unbranched (Figure 4d), formed disjointedly throughout the colonies, hyaline, base brown-pigmented at times, (80–) 480 µm in length, 3–6 µm wide, narrowing towards the apex to 2–2.5 µm, transversely septate, septa spaced more narrowly towards the apex. Conidigenous cells are phialides, arranged in 1–4 (–6) whorls along conidiophores (Figures 4c, 4d), arising below transverse septum (Figure 4e). Whorls spaced 20–140 µm apart, closer towards the apex, consisting of (1–) 2–4 (–6) phialides (Figures 4c, 4d, 4e). Apical whorls consisting of one apical and one to several lateral phialides (Figures 4c, 4d, 4e). Phialides subulate, tapering from 1.5–3 µm at the base to 1–1.5 µm at the tip, terminal phialides 40–80 µm long, lateral phialides 20–50 µm long (Figure 4e). Conidia hyaline, smooth-walled, cylindrical with rounded apices to oval (Figure 4f), tapering at times, (3.0–) 6.0 µm × 2.5–5.5 µm.
Resting mycelium present, consisting of brown-pigmented hyphae, up to 7 μm wide, thick-walled, straight or curved, solitary or aggregated, up to 25 μm wide (Figures 4g, 4h). Microsclerotia present, composed of tightly intertwined, torulose brown-pigmented hyphae, rounded or variously shaped, up to 230 μm.
diam and consisting of rounded to elongate cells, up to 10 μm diam (Figures 4i, 4j, 4k). Yellow-pigmented hyphal cells present at times, up to 5 μm wide (Figure 4).

**Types.** Holotype: Missing, not found at GOET, B, M; Lectotype (designated herein): Illustrations from protolog: Figures of Plate (“Tafel’) 0 and Figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 of Plate 9 in Reinke and Berthold [21], available online at http://books.google.com/books?id=3WgVAAAAYAAJ&q=Die%20Kr%C3%A4uselkrankheit%20der%20Kartoffel&pg=PA107#v=onepage&q=Die%20Kr%C3%A4uselkrankheit%20der%20Kartoffel&f=false (accessed on October 5, 2011); Epitype (designated herein): Dried culture of *Verticillium albo-atrum* strain PD747 (Canada: Prince Edward Island; potato field soil) deposited at UC (UC 1953892), an ex-epitype culture at CBS (CBS 130340) and NRRL (NRRL 54797).

**Specimens examined.** The description was based on *Verticillium albo-atrum* strains PD670 (USA: WI; Irish potato), PD693 (UK; Irish potato), PD746 (Canada: New Brunswick; potato field soil), PD747 (Canada: Prince Edward Island; potato field soil) and PD748 (Canada: Prince Edward Island; potato field soil)(Table S1).

**Distribution and host range.** Currently known from Canada, Germany, UK and USA (WI). Substrates include Irish potato and soil collected from Irish potato fields.

**Commentary.** *Verticillium albo-atrum* was described by Reinke and Berthold in 1879 from diseased potato plants collected near Göttingen, Germany [21]. The protolog of *V. albo-atrum* contains detailed descriptions and drawings, but no reference is made to type material. We inquired at the herbaria of Göttingen (GOET),
Berlin (B) and München (M), none of which has any 
\textit{V. albo-atrum} type material or any other 
\textit{V. albo-atrum} material deposited by 
Reinke and Berthold. According to Klebahn [33], original cultures 
of \textit{V. albo-atrum} are no longer available. We did not find any 
\textit{V. albo-atrum} cultures by Reinke and Berthold in any of the major 
culture collections (CBS, IMI, DSMZ, ATCC). Thus, in absence 
of any original fungal material, we designated the illustrations from 
the \textit{V. albo-atrum} protolog in Plate 8 (Figures 1, 2, 3, 4, 5, 6, 7, 8) 
and Plate 9 (Figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11) in Reinke and 
Berthold [21] as the lectotype for \textit{V. albo-atrum}. According to The 
International Code of Botanical Nomenclature (ICBN) [34] this is 
permissible when the holotype, all cited or uncited original 
specimens and all original cultures are missing (Art. 8.4, Art. 9.2, 
Art. 9.10, Art. 37.4). To serve as an interpretive type, we 
designated a \textit{V. albo-atrum} epitype with an ex-epitype culture for 
molecular studies. Designation of an epitype is permissible 
according to ICBN when serving the precise application of a 
name (Article 9.7).

The original description of \textit{V. albo-atrum} by Reinke and Berthold 
[21] was based on observations from decaying potato stems, and is 
congruent with our observations from the \textit{V. albo-atrum} isolates 
examined in this study. The exception was the presence of yellow 
pigment associated with hyphal cells (Figure 4l) not seen by Reinke 
and Berthold [21]. However, Klebahn [35] reported that 
\textit{V. albo-atrum} mycelium on Salep Agar medium was white with a yellow tinge 
(p. 64), whereas \textit{V. dahliae} mycelium was described as white (p. 65).

In addition to resting mycelium, \textit{Verticillium albo-atrum} also forms 
microsclerotia (Figures 4i, 4j, 4k). Microsclerotia are very ‘small, 
firm, frequently rounded masses of hyphae with or without the 
addition of host tissue or soil.’ [36]. The \textit{V. albo-atrum} micro-
sclerotia were described and illustrated in the protolog on pages 74 
and 75, and in Figures 1 and 2 of Plate 9 [21], a translation from 
the German original is provided by Isaac [22]. The microsclerotia 
consist of aggregations of brown-pigmented, thick-walled hyphae, 
no lateral cell divisions are involved in their formation [21]. This is 
opposed to the microsclerotia of \textit{V. dahliae} where an increase in 
width is achieved by the lateral divisions of hyphal cells as 
described by Klebahn [35] on pages 56 and 57, and illustrated in 
Klebahn’s [35] Figure 8. Microsclerotia were only observed on 
WA-p and PLYA media, they were absent from strains cultured on

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**Figure 4.** Morphological features of \textit{Verticillium albo-atrum}. 4a. Colony of strain PD747 after 10 days on PDA, frontal view. 4b. Colony of strain 
PD747 after 10 days on PDA, reverse view. 4c. Conidiophore of strain PD748 after 29 days on WA-p. 4d. Branched conidiophore of strain PD670 after 
29 days on WA-p. 4e. Phialide of strain PD670 after 29 days on PDA. 4f. Conidia of strain PD670 after 29 days on PDA; Insets: Pigmented, septate and 
constricted conidium of strain PD670 after 29 days on PDA, budding conidium and conidium germinating by formation of a phialide, both of strain 
PD748 after 29 days on WA-p. 4g. Resting mycelium of strain PD747 after 33 days on WA-p. 4h. Aggregated hyphae of resting mycelium in strain 
PD670 after 28 on WA-p. 4i. Microsclerotium of strain PD670 after 47 days on PLYA. 4j. Microsclerotium of strain PD670 after 28 on WA-p. 4k. 
Microsclerotium of strain PD747 formed in the lumen of a thick-walled plant cell after 32 days on WA-p. 4l. Hypha of strain PD747 containing yellow 
pigment after 10 days on PDA. Scale bar: 4a, 4b = 2 cm; 4c, 4d = 50 \(\mu\)m; 4e–4h, 4i–4l = 20 \(\mu\)m; 4i = 100 \(\mu\)m. Imaging method: 4a, 4b = DS; 4c, 4d, 4g– 
4l = BF; 4e, 4f = DIC.

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PDA. *Verticillium albo-atrum* has frequently been confused with *V. alfalfae* and *V. nonalfalfae* that form resting mycelium but no microsclerotia.

The name ‘*V. albo-atrum*’ is correct with or without hyphen (Art. 23.1), the hyphenated form is more commonly encountered in the literature.

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**Figure 5. Morphological features of Verticillium alfalfae.** 5a. Colony of strain PD682 after 24 days on PDA, frontal view. 5b. Colony of strain PD682 after 24 days on PDA, reverse view. 5c. Conidiophore of strain PD682 after 31 days on WA-p. 5d. Phialide of strain PD489 after 30 days on WA-p. 5e. Conidia of strain PD682 after 30 days on WA-p. 5f. Resting mycelium of strain PD489 after 30 days on WA-p. 5g. Aggregated hyphae of resting mycelium in strain PD682 after 73 days on PDA. 5h. Resting mycelium of strain PD683 in the lumen of a thick-walled plant cell after 32 days on WA-p. Scale bar: 5a, 5b = 2 cm; 5c = 50 μm; 5d–5h = 20 μm. Imaging method: 5a, 5b = DS; 5c, 5f–5h = BF; 5d, 5e = DIC.

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**Figure 6. Morphological features of Verticillium dahliae strain PD322 (ex-epitype) unless otherwise noted.** 6a. Colony after 14 days on PDA, frontal view. 6b. Colony after 14 days on PDA, reverse view. 6c. Inflated cells present in mycelium after 28 days on PDA. 6d. Conidiophore after 15 days on WA-p. 6e. Branched conidiophore after 12 days on WA-p. 6f. Whorl phialide after 25 days on WA-p. 6g. Solitary phialide after 14 days on PDA. 6h. Conidia after 9 days on PDA. 6i. Microsclerotia after 12 days on WA-p. 6j. Microsclerotia of the *V. dahliae* holotype material from stem of *Dahlia* sp. 6k. Short brown-pigmented hypha composed of torulose cells attached to microsclerotium after 49 days on PDA. Scale bar: 6a, 6b = 2 cm; 6c, 6f–6k = 20 μm; 6d, 6e = 50 μm. Imaging method: 6a, 6b = DS; 6c, 6f–6h = DIC; 6d, 6e, 6i, 6k = BF; 6j = PC.

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Verticillium Systematics with Five New Species

Verticillium alfalfae Inderb., H. W. Platt, R. M. Bostock, R. M. Davis & K. V. Subbarao, sp. nov.

Figure 5
MycoBank: MB563552

Etymology: Medicago sativa (‘alfalfa’), the only currently known host of this species.

Latin diagnosis. Verticillio nonalfalae morphologia simile, sed characteribus sequentiarum nucleidearum distinguendum.
Actin positione 21 (T), 72 (G), 78 (T), 459 (A), 462 (A); Elongation factor 1-alpha positione 149 (G), 157 (G), 175 (G), 225 (A), 265 (T), 266 (A), 271 (A), 280 (C), 304 (T), 346 (C), 428 (T), 429 (T), 441 (G), 465 (T), 469 (C), 474 (T), 591 (C), 596 (T), 600 (C), 624 (T); Glyceraldehyde-3-phosphate dehydrogenase positione 173 (A), 324 (C); Tryptophan synthase positione 87 (A), 161 (T), 169 (C), 246 (T), 273 (T), 315 (T), 583 (T), 601 (C).

Description. Colonies on PDA after two weeks 3.5–4.5 cm diam, white at first (Figures 5a, 5b), later darkening due to the formation of resting mycelium immersed in the agar. Aerial...
The description was based on V. alfalfa strains PD330 (USA; alfalfa), PD353 (USA; alfalfa), PD489 (USA; alfalfa), PD620 (Canada; alfalfa), PD681 (USA; alfalfa), PD682 and PD683 (Japan: Hokkaido; alfalfa) (Table S1).

**Distribution and host range.** Currently known only from Canada, Japan and the USA (PA), only from alfalfa.

**Commentary.** Verticillium alfalfa is morphologically indistinguishable from V. nonalfalfae.

**Verticillium dahliae** Kleb., Mycologisches Centralblatt 3: 66 (1913) Figures 3a, 6

*Verticillium dahliae* is a well-known pathogen of various crops, including alfalfa, causing Verticillium wilt. It is characterized by the formation of microsclerotia, which are resistant to environmental stress and are a key feature for the survival of the fungus between host generations. The fungus is typically found in soil and can infect a variety of host plants, leading to significant economic losses in agriculture.

**Verticillium nubilum** (G. Zech) U.S. Domsch, Mycologia 64: 979 (1972) Figures 11. Morphological features of *Verticillium nubilum*. 11a. Colony of strain P742 after 13 days on PDA, frontal view. 11b. Colony of strain PD742 after 13 days on PDA, reverse view. 11c. Conidiophore of strain PD621 after 17 days on WA-p. 11d. Apical phialide of strain PD621 after 17 days on WA-p. 11e. Conidia of strain PD621 after 17 days on WA-p. 11f. Solitary chlamydospore of strain PD742 after 17 days on WA-p. 11g. Linear chain of chlamydospores of strain PD621 after 25 days on PDA. 11h. Angular chain of chlamydospores of strain PD621 after 25 days on PDA. 11i. Brown-pigmented hyphae of strain PD742 after 17 days on WA-p. Scale bar: 11a, 11b = 1 cm; 11c–11i = 20 μm; Imaging method: 11a, 11b, = DS; 11c = PC; 11d, 11e = DIC; 11f–11i = BF.

*Verticillium nubilum* is another member of the *Verticillium* genus, often associated with soilborne diseases. It is characterized by the formation of microsclerotia and its distinctive morphological features, which are crucial for its identification. This fungus can be found across various locations and is known for its ability to cause significant plant disease control issues in agriculture.
Types. Holotype: Specimen V. dahliae (Germany; Dahlia sp.) at HBG (Figures 3a, 6j) [16]; Epitope (designated herein): Dried culture of Verticillium dahliae strain PD322 (USA: CA; lettuce), PD327 (USA: CA; bell pepper) and PD502 (USA: WI; maple) (Table S1). The V. dahliae holotype specimen was also examined (Figures 3a, 6j).

Distribution and host range. Currently known from Brazil, Canada, Denmark, France, Germany, Iran, Israel, Italy, Japan, Netherlands, Russia, Spain, Sweden, UK, Ukraine, and USA (CA, ID, IL, OR, TX, WA, WI) [27]. Substrates include Anaheim pepper, annual sunflower, apricot, ashi, bell pepper, cabbage,celandine, chili pepper, common flax, eggplant, European smoke tree, garden tomato, globe artichoke, horseradish, hybrid strawberry, Icelandic poppy, Irish potato, jalapeno, Japanese maple, lettuce, maple, olive, opium poppy, paprika, pepper, peppermint, pistachio nut, purple coneflower, rape, sneezewort false mayweed, spinach, sweet almond, udo, upland cotton, and watermelon [27] that represent fourteen different plant families (Aceraceae, Amaranthaceae, Anacardiaceae, Araliaceae, Asterales, Brassicaceae, Cucurbitaceae, Fabaceae, Liliaceae, Malvaceae, Oleaceae, Papaveraceae, Rosaceae, Solanaceae).

Commentary. Verticillium dahliae is the type of Verticillium and was described by Klebahn [35] from Dahlia sp. cv. Geiselher in Germany (Figure 3a). Verticillium dahliae is not the oldest species of the genus, but it has the largest impact as a pathogen, is common and genetically relatively homogenous, and has thus been conserved as the type of the genus [16,34]. Since a viable ex-holotype culture is no longer available [33], and DNA extraction attempts from the holotype specimen failed, we designated a V. dahliae epitope with an ex-epitype culture that serves as an interpretive type for molecular studies.

The original description of V. dahliae by Klebahn [35] was based on material from Dahlia sp., and from cultures on Salep Agar medium which is a mixture of polysaccharides contained in orchid tubers [37]. The composition of Klebahn’s medium is unknown, but as a reference, Noéll [38] isolated fungal symbionts of orchids using a clear, weak decoction of salep containing 2% agar. We examined the V. dahliae holotype material which contains an ex-epitype culture at CBS (CBS 130341) and NRRL (NRRL 54783).

Specimens examined. The description was based on Verticillium dahliae strains PD322 (USA: CA; lettuce), PD327 (USA: CA; bell pepper) and PD502 (USA: WI; maple) (Table S1). The V. dahliae holotype specimen was also examined (Figures 3a, 6j).

Verticillium isaaei Inderb., R. M. Bostock, R. M. Davis & K. V. Subbarao, sp. nov. Figure 7

MycoBank: MB563553

Etymology. Named after Ivor Isaac (1914–1978), in recognition of significant contributions to Verticillium taxonomy.

Latin diagnosis. Verticillium tricorpus morphología simile, sed characteribus sequentiariun nucleidearum distingueat. Actin positione 79 (T), 115 (T), 292 (T), 390 (T), 410 (T), 432 (A), Elongation factor 1-alpha positione 142 (C), 162 (A), 166 (T), 105 (A), 190 (T), 230 (A), 235 (G), 240 (A), 260 (A), 351 (A), 363 (T), 366 (G); Glyceraldehyde-3-phosphate dehydrogenase positione 135 (C), 278 (G); Tryptophan synthase positione 135 (A), 145 (A), 383 (G).

Description. Colonies on PDA after two weeks 2.5–6 cm diam, white at first, later yellow, reverse orange to yellow, then darkening due to the formation of resting mycelium, chlamydospores and microsclerotia (Figures 7a, 7b). Aerial mycelium generally abundant, floccose, hyphae smooth-walled, 1–3.5 μm wide. Conidiophores erect or slanted (Figure 7c), generally determinate, branched or unbranched, formed disjointly throughout the colonies, hyaline, verruculose surface ornamentation present at times, 103–690 μm in length, 3–6 μm wide, narrowing towards the apex to 2–3.5 μm, transversely septate, septa spaced more narrowly towards the apex. Conidiogenous cells are phialides (Figure 7d), arranged in (1–) 2–4 (–6) whorls along conidiophores (Figure 7c), arising below transverse septum. Whorls spaced 25–60 μm apart, closer towards the apex, consisting of (1–) 3–5 (–6) phialides (Figure 7c). Apical whorls consisting of one apical and one to several lateral phialides (Figures 7c, 7d). Phialides subulate, tapering from 2.5–3.5 μm at the base to 1–1.5 μm at the tip, terminal phialides 30–65 μm long, lateral phialides 20–40 μm long (Figure 7d). Conidia hyaline, smooth-walled, cylindrical with rounded apices to oval (Figure 7e), tapering at times, (3.5–) 6.0 μm×1.5 μm (3.4–)×(1.5–) 3.0 μm±0.5 μm (–5.0) (V/W=(1.4–) 1.9±0.3 (–3.5), n=73), accumulating at the tip of the phialides (Figure 7c). Conidia rarely one- or two-septate, constricted at the septum at times (Figure 7c). Resting mycelium, chlamydospores and microsclerotia present. Resting mycelium consisting of brown-pigmented hyphae, up to 5 μm wide (Figure 7f), chlamydospores solitary or in chains, up to 12 μm wide (Figure 7g), microsclerotia rounded or variously shaped, up to 70 μm diam and consisting of rounded to elongate cells, up to 10 μm wide (Figure 7h). Yellow-pigmented hyphal cells present, up to 5.5 μm wide, containing globules of yellow pigment, at times pigment accumulating as crystals outside the cells, up to 21 μm diam (Figures 7i, 7j).

Types. Holotype: Dried culture of V. isaaei strain PD660 (USA: CA; lettuce) deposited at UC (UC 1953896), an ex-holotype culture at CBS (CBS 130343) and NRRL (NRRL 54792).

Specimens examined. The description was based on V. isaaei strains PD341, PD343, PD367, PD437, PD610, PD611, PD612, PD613, and PD660 (USA: CA; lettuce), PD618 (UK; garden tomato), PD619 (Canada; soil), PD661 (USA: WA; lettuce), PD752 and PD753 (USA; WA; spinach) (Table S1).

Distribution and host range. Currently known from Canada, UK and USA (CA, WA). Substrates include garden tomato, globe artichoke, hairy nightshade, lettuce, spinach and soil.

Commentary. Verticillium isaaei is morphologically indistinguishable from V. klebahnii and V. tricorpus.

Verticillium klebahnii Inderb., R. M. Bostock, R. M. Davis & K. V. Subbarao, sp. nov. Figure 8
Verticillium longisporum

**Etymology.** Named after Heinrich Klebahn (1859–1942), in recognition of significant contributions to *Verticillium* taxonomy.

**Latin diagnosis.** Verticillium tricorpus morphologia simile, sed characteribus sequentiarum nucleidearum distinguendum. Actin positione 92 (C), 92 (T), 256 (C); Elongation factor 1-alpha positione 160 (C), 166 (T), 191 (A), 195 (T), 196 (G), 203 (A), 215 (A), 220 (A), 264 (C), 312 (C), 352 (G), 355 (C), 363 (G), 304 (C); Tryptophan synthase positione 167 (T).

**Description.** Colonies on PDA after two weeks 4–6.5 cm diam, white at first, later yellow, reverse orange to yellow, then darkening due to the formation of brown-pigmented hyphae, chlamydospores and microsclerotia (Figures 8a, 8b). Aerial mycelium generally abundant, flocose, hyphae smooth-walled, 1–3.5 μm wide. Conidiophores erect or slanted (Figure 8c), generally determinate, branched or unbranched, formed disjointedly throughout the colonies, hyaline, verruculose surface ornamentation present at times, 130–700 μm in length, 3–5 μm wide, narrowing towards the apex to 2–3 μm, transversely septate, septa spaced more narrowly towards the apex. Conidiogenous cells are phialides, arranged in (1–) 2–7 (–8) whorls along conidiophores (Figure 8c). Whorls spaced 30–65 μm apart, closer to the apex, consisting of (1–) 2–5 (–7) phialides (Figure 8c), arising below transverse septum. Apical whors consisting of one apical and one to several lateral phialides (Figure 8c). Phialides subulate, tapering from 1.5–2.5 μm at the base to 1–1.5 μm at the tip, terminal phialides 30–60 μm long, lateral phialides 18–45 μm long (Figure 8c). Conidia hyaline, smooth-walled, cylindrical with rounded apices to oval (Figure 8d), tapering at times, (3.5–) 5.0 μm × (0.0)–1.5 μm × (0.0)–4.5 μm (n = 73), accumulating at the tip of the phialides (Figure 8c). Resting mycelium, chlamydospores and microsclerotia present. Resting mycelium consisting of brown-pigmented hyphae, up to 8 μm wide (Figure 8c), chlamydospores solitary or in chains, up to 13 μm wide (Figure 8d), microsclerotia rounded or variously shaped, up to 80 μm diam and consisting of rounded to elongate cells, up to 9 μm wide (Figure 8g). Yellow-pigmented hyphal cells present, up to 7.5 μm wide, containing globules of yellow pigment, at times pigment accumulating as crystals outside the cell, up to 14 μm diam (Figure 8h).

**Types.** Holotype: Dried culture of *V. klebahnii* strain PD401 (USA: CA; lettuce) deposited at UC (UC 1953897), an ex-holotype culture at CBS (CBS 130344) and NRRL (NRRL 54789).

**Specimens examined.** The description was based on *V. klebahnii* strains PD347, PD401, PD407 and PD458 (USA: CA; lettuce), PD657, PD658 and PD659 (USA: WA; lettuce) (Table S1).

**Distribution and host range.** Currently only known from the USA (CA, WA) from lettuce.

**Commentary.** Verticillium klebahnii is morphologically indistinguishable from *V. isaaci* and *V. tricopus*. Verticillium isaaci and *V. klebahnii* were described as new species because no synonyms of the morphologically similar *V. tricopus* were available (www.indexfungorum.org, accessed on September 30, 2011).

*Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale, Mycological Research 101(11): 1293 (1997) Figures 3b, 9

Basionym: *Verticillium dahiae* var. *longisporum* C. Stark, Gartenbauwissenschaft 26(8): 508 (1961)

Mycobank: MB413108

**Description.** *Verticillium longisporum* was described by Stark [42] and in more detail by Karapapa et al. [24]. We documented colony morphology (Figures 9a, 9b), conidia (Figure 9c) and microsclerotia (Figures 9d, 9e, 9f, 9g). We measured microsclerotia and conidia, and assessed the number of phialides per whorl. Microsclerotia were rounded to elongate, 37–240 × 25–52 μm (Figures 9d, 9e). Conidia were (3–) 6.5 × 2.5 μm (15.0) × (2.0)–3.5 μm (± 1.0 μm (–6.5)) (l/w = (1.6)–2.5 × 0.7 (–4.5), n = 29). Whorls consisted of (1–) 2–5 (–6) phialides.

**Types.** Holotype: Specimen CBS H-19247 at CBS (Germany: Niedersachsen; horseradish) (Figures 3b, 9g), an ex-holotype culture at CBS (CBS 124.64) included in this study as *V. longisporum* strain PD687 and submitted to NRRL (NRRL 54793).

Stark [42] (p. 509) submitted permanent slides of type material to the Herbarium des Staatsinstitutes für Allgemeine Botanik Hamburg, these slides are missing at HBG.

**Specimens examined.** *Verticillium longisporum* strains PD348 (USA: CA; cauliflower), PD356 (USA: IL; horseradish) and PD687 (Germany: Niedersachsen; horseradish) (Table S1), representing the three lineages of *V. longisporum* [27], and the holotype specimen CBS H-19247 (Germany: Niedersachsen; horseradish), a dried agar culture (Figures 3b, 9g), were examined in this study.

**Distribution and host range.** Currently known from France, Germany, Japan, Sweden and USA (CA, IL). Substrates include birdrape, cabbage, cauliflower, horseradish, radish, rape, sugar beet and wild radish [27].

**Commentary.** *Verticillium longisporum* is a diploid hybrid that originated at least three different times from four different parental lineages in three different species, including *V. dahiae*, Species A1 and Species D1 (Figure 1) [27]. *Verticillium dahiae* is the only known parent of *V. longisporum*, Species A1 and Species D1 have never been collected [27]. The holotype of *V. longisporum* represented by ex-holotype strain PD687 belongs to *V. longisporum* lineage A1/D3 that is one of the three lineages of *V. longisporum*, and *V. longisporum* is thus polyphyletic [27]. There is general agreement that fungal species should be monophyletic. However, we decided that *V. longisporum* should remain a polyphyletic species, because it seems impractical to name each lineage of *V. longisporum*. We currently know of three lineages of *V. longisporum* that represent three independent hybridization events, but there might be many more. Little is known about fungal hybrids, but in plants, hybrids can evolve frequently over short periods in small areas [43].

We included the ex-holotype isolate *V. longisporum* strain PD687 in our studies, strain PD687 did not form any microsclerotia. But microsclerotia were present in the holotype that is a dried culture of strain PD687 (CBS 124.64) (Figure 3b). The microsclerotia in the holotype documented in Figure 9g were similar to the ones described by Stark [42] on page 500 for ‘Typ X’ as *V. longisporum* was referred to prior to its description. Thus, *V. longisporum* strain PD687 likely lost its ability to produce microsclerotia due to prolonged culturing.

Karapapa et al. [24] compared *V. longisporum* to the morphologically similar *V. dahiae*, and found that *V. longisporum* microsclerotia and conidia were longer than the ones in *V. dahiae*, and that *V. longisporum* conidiophores had fewer phialides in each whorl than *V. dahiae*.

We evaluated those characters and found that for the isolates used in this study grown on PDA, microsclerotia and conidia size might be useful to distinguish *V. longisporum* from *V. dahiae*. In *Verticillium longisporum* strain PD356, the majority of microsclerotia were elongate (Figure 9d), but rounded microsclerotia were still present (Figure 9e), and in some sectors of the colony, rounded microsclerotia were in the majority (Figure 9e). In *V. longisporum* strain PD348, there were roughly as many elongate microsclerotia as there were rounded microsclerotia. *Verticillium dahiae* microsclerotia were mostly rounded, but in some areas elongate microsclerotia were prevalent. The short brown-pigmented...
hyphae that were frequently attached to microsclerotia (Figure 9f) are possibly immature microsclerotia as illustrated by Isaac [22]. Similar structures were seen in this study in *V. dahliae* (Figure 6k). The third strain of *V. longisporum* investigated here, the ex-holotype strain PD687 did not form any microsclerotia. Conidia of *V. longisporum* were on average 6.5 x 3.0 µm (Figure 6c) and conidia of *V. dahliae* 6.5 x 3.0 µm (Figure 6h). However, conidia lengths might also at times be misleading, as the size ranges overlap, standard errors were 2.5 and 1.5 µm, respectively. We found that both *V. longisporum* and *V. dahliae* had similar numbers of phialides in each whorl, 2–4 for *V. dahliae*, and 2–5 for *V. longisporum*, this is unlike that proposed by Karapapa et al. [24] who reported 4–5 in *V. dahliae* and mostly 3 in *V. longisporum*. In our hands, *Verticillium longisporum* strain PD348 very frequently had 5 phialides per whorl. Thus, a combination of conidia length and microsclerotia morphology might in many cases yield correct species identifications, but the two characters will also be misleading at times.

Another differentiating character was given by Stark [42]. He found that *V. longisporum* culture filtrate fluoresced, whereas fluorescence was absent in *V. dahliae*. We did not investigate fluorescence in the two species.

**Verticillium nonalfalfae** Inderb., H. W. Platt, R. M. Bostock, R. M. Davis & K. V. Subbarao, sp. nov.

**Figure 10**

MycoBank: MB563555

Etymology: Known to occur on a variety of hosts, but not *Medicago sativa* (‘alfalfa’).

**Latin diagnosis.** Verticillio alfalfae morphology simile, sed characteribus sequentiarum nucleiclearum distinguendium. Actin positione 16S, 18S (eta); Elongation factor 1-alpha: positione 148 (C), 179 (G), 190 (C), 248 (G), 316 (G), 332 (G), 342 (T), 414 (G), 470 (C), 473 (C), 494 (G), 513 (T), 541 (C), 580 (G), 595 (T), 597 (C), 639 (T); Glyceraldehyde-3-phosphate dehydrogenase positione 234 (T), 267 (T); Tryptophan synthase positione 471 (C), 534 (C).

**Description.** Colonies on PDA after two weeks 3.5–5.5 cm, white at first, later darkening due to the formation of resting mycelium immersed in the agar (Figures 10a, 10b). Aerial mycelium generally abundant, floccose to pruinose, hyphae smooth-walled, 1.5–3 µm wide. Conidiophores erect or slanted (Figures 10c, 10d), generally determinate, branched or unbranched (Figure 10c, 10d), formed disjointedly throughout the colonies, hyaline, 30–710 µm in length, 4.5–6 µm wide, narrowing towards the apex to 2–3 µm, transversely septate, septa spaced more narrowly towards the apex. Conidiogenous cells are phialides (Figure 10e), arranged in (1–) 2–6 whors along conidiophores (Figures 10d, 10e). Whors spaced 50–160 µm apart, closer towards the apex, consisting of (1–) 2–5 (–7) phialides (Figure 10e), arising below transverse septum. Apical whors consisting of one apical and one to several lateral phialides (Figures 10d, 10e). Phialides subulate, tapering from 2–3 µm at the base to 1–1.5 µm at the tip, terminal phialides 40–60 µm long, lateral phialides 30–45 µm long (Figure 10e). Conidia hyaline, smooth-walled, cylindrical with rounded apices to oval (Figure 10f), allantoid at times, (4.0–) 6.0 ± 1.0 µm (–10.5–) (25.3–) 3.0 µm ± 0.5 µm (–3.5) (l/w = (1.3–) 2.0 ± 0.2 (–2.7), n = 80), accumulating at the tip of the phialides (Figure 10d). Resting mycelium present (Figures 10g, 10h, 10i), consisting of brown-pigmented hyphae, up to 9 µm wide, thick-walled, straight or curved, solitary or aggregated (Figures 10g, 10h), turgidose at times (Figure 10i).

**Types.** Holotype: Dried culture of *V. nonalfalfae* strain PD592 (Japan: Hokkaidou; Irish potato) deposited at UC (UC 1953898), an ex-holotype culture at CBS (CBS 130339) and NRRL (NRRL 54791).

**Specimens examined.** The description was based on *V. nonalfalfae* strains PD592 (Japan: Hokkaidou; Irish potato), PD616 and PD626 (UK; common hop), PD74 (Cuba; potato field soil), PD743 (Canada: Manitoba; spinach); PD908, PD909 and PD011 (Slovenia; common hop) and PD010 (Slovenia; petunia) (Table 1).

**Distribution and host range.** Currently known from Canada, Cuba, Japan, Slovenia and UK. Substrates include common hop, Irish potato, petunia and spinach.

**Commentary.** *Verticillium nonalfalfae* is morphologically indistinguishable from *V. alfalfae*, but the two species differ in pathogenicity. *Verticillium nonalfalfae* causes disease on a variety of different hosts, whereas *V. alfalfae* causes disease mainly on lucerne [44]. Other differences include vegetative compatibility groups [45], mating types (Figure 2), as well as the DNA characters listed in the species descriptions. *Verticillium alfalfae* and *V. nonalfalfae* were described as new species because no synonyms of the morphologically similar *V. albo-atrum* were available (www.indexfungorum.org, accessed on September 30, 2011).

*Verticillium nonalfalfae* and *V. alfalfae* have long been recognized as two genetically distinct groups referred to as non-lucerne and lucerne pathotype, respectively [46,47,48].

Within *Verticillium*, *V. nonalfalfae* and *V. alfalfae* lack unique, diagnostic morphological characters and were frequently confused with the distantly related *V. albo-atrum*. The three fungi share an overall similar morphology, including the formation of resting mycelium (Figures 4g, 4h, 5f, 5g, 5h, 10g, 10h, 10i, 10j). *Verticillium albo-atrum* also forms microsclerotia (Figures 4i, 4j, 4k, one-septate, brown-pigmented conidia (Figure 4f), as well as phialides that originate directly from conidia (Figure 4f). However, microsclerotia were only observed on WA-p and on PLYA media, not on PDA medium, and one-septate, brown-pigmented conidia, and conidia germinating by phialide formation are relatively rare. Thus, based on our data, it is not possible to consistently differentiate *V. nonalfalfae* and *V. alfalfae* from *V. albo-atrum* using morphological characters. *Verticillium albo-atrum* may co-occur with *V. nonalfalfae* on some hosts, as Keyworth [49] isolated *Verticillium* strains forming resting mycelium, as well as *Verticillium* strains forming microsclerotia and resting mycelium simultaneously, from diseased potato plants in Connecticut.

**Verticillium nubilum** Pethybr., Transactions of the British Mycological Society 6: 117 (1919) **Figure 11**

MycoBank: MB225664

**Description.** Colonies on PDA after two weeks 2.5–6 cm diam, white at first, later darkening due to the chlamydospores immersed in the agar (Figures 11a, 11b). Aerial mycelium generally abundant, floccose to pruinose, hyphae smooth-walled, (1–) 2–4 µm wide. Conidiophores present (Figure 11c). Conidiogenous cells are phialides (Figure 11d) arranged in whors along conidiophores (Figure 11e), arising below transverse septum. Whors consisting of one or more phialides (Figures 11c, 11d). Phialides subulate (Figure 11d). Conidia hyaline, smooth-walled, cylindrical with rounded apices to oval (Figure 11e), allantoid at times, rarely with central septum, (4.5–) 7.5 µm ± 2.0–0.5 µm (–14.5) x (2.0–) 2.5 µm ± 0.5 µm (–3.5) (l/w = (2.0–) 3.0 ± 0.5 (–5.0), n = 50) (Figure 11f). Chlamydospores present, rounded to elongate, 6–14 µm diam, solitary or in chains of up to 6, straight or curved (Figures 11f, 11g, 11h). Brown-pigmented hyphae present at times (Figures 11h, 11i), generally attached to chlamydospores (Figure 11h).

**Types.** Holotype: Missing, not at DBN, IMI, K; Lectotype (designated herein): Illustration from protolog: Figure 5 on Plate 4
in Pethybridge [50], available online from Cyberliber, an Electronic Library for Mycology at http://www.cybertruffle.org.uk/cyberliber/59531/0006/002/p004b.jpg (accessed on October 5, 2011); Epitype (designated herein): Dried culture of *Verticillium nubilum* strain PD742 (obtained from CBS as CBS 457.51)(UK; soil) deposited at UC (UC 1953094) and NRRL (NRRL 54796).

**Specimens examined.** The description was based on *V. nubilum* strains PD621 (UK; mushroom compost), PD702 (UK; Irish potato), PD741 (UK; soil), PD742 (UK; soil) (Table 1).

**Distribution and host range.** Currently known from the UK. Substrates include mushroom compost, Irish potato and soil.

**Commentary.** *Verticillium nubilum* was described by Pethybridge [50] from the surface of a potato tuber attacked by *Phytophthora infestans*. The protolog of *V. nubilum* contains descriptions of the *V. nubilum* morphology and a photograph of chlamydospores, but no reference is made to type material. We inquired at Kew (K), CABI Bioscience (IMI) and Dublin (DBN), none of which has any *V. nubilum* type material in its possession. Isaac [23] who studied *V. nubilum* in detail did not mention any herbarium material. We did not find any *V. nubilum* cultures by Pethybridge in any of the major culture collections (CBS, IMI, DSMZ, ATCC). Thus, in absence of any original fungal material, we designated the illustration from the *V. nubilum* protolog, Figure 5 on Plate 4 in Pethybridge [50], as the lectotype for *V. nubilum*.

Isaac [23] studied *V. nubilum* in detail and submitted several strains to CBS, of which we selected a dried culture of strain PD742 (CBS 457.51) as epitype. Our observations of *V. nubilum* agreed with the accounts by Pethybridge [50] and Isaac [23]. Pethybridge [50] noted that *V. nubilum* conidia were larger than those of *V. albo-atrum*. We found that *V. nubilum* conidia were on average 7.5 × 2.5 μm (Figure 11c), the largest in *Verticillium*, with the exception of *V. longisporum* conidia that were on average 8.5 × 3.5 μm (Figure 9c). Differing from both Pethybridge [50] and Isaac [23], small numbers of brown-pigmented hyphae not directly associated with chlamydospores were sometimes present (Figure 11i), but these were lighter colored than the resting mycelium in other species (eg Figures 4g, 4h).

All the *V. nubilum* isolates that we examined formed very few conidia and conidiophores, which prevented us from conclusively assessing conidiophore morphology and dimensions. However, the few conidiophores and phialides that we saw were similar to other *Verticillium* species, in both morphology and dimensions (Figures 11e, 11d). *Verticillium nubilum* can be differentiated from other *Verticillium* species by the near exclusive formation of chlamydospores as resting structure (Figures 11f, 11g, 11h), in combination with the relatively large conidia (Figure 11c), but can be confused with *Gibellulopsis nigrescens* that forms distinctly smaller chlamydospores [15,23].

**Verticillium tricorpus** I. Isaac, Transactions of the British Mycological Society 36(3): 194 (1953) Figures 3c, 12

MycoBank: MB307745

**Description.** *Verticillium tricorpus* was described in detail by Isaac [23]. We provide illustrations of the culture morphology (Figures 12a, 12b), the conidia (Figure 12c), resting structures including resting mycelium, chlamydospores and microsclerotia (Figures 12d, 12e, 12f, 12g), and yellow-pigmented hyphae (Figure 12h).

**Types.** Holotype: Missing, not found at K, IMI, CBS, an ex-holotype culture (UK; garden tomato) available (IMI 51602, CBS 447.54), culture CBS 447.54 included in this study as *V. tricorpus* strain PD690 and submitted to NRRL (NRRL 54794); Lectotype (designated herein): Specimen K(M) 172015, originally IMI 51602 (England: Fareham, South Hampshire; wilted garden tomato), marked ‘isotype’? (Figures 3c, 12g).

**Specimens examined.** *Verticillium tricorpus* strains PD593 (Japan; Irish potato), PD594 (Japan: Chiba; garden tomato), PD685 (Japan; larkspur), PD690 (UK; garden tomato), and PD703...
(Netherlands; carnation) (Table S1), as well as *V. tricorpus* lectotype specimen IMI 51602 (UK; garden tomato) were included in this study (Figures 3c, 12g).

**Distribution and host range.** Currently known from Japan, Netherlands and UK. Substrates include carnation, garden tomato, Irish potato and larkspur.

**Commentary.** Isaac [23] (p. 194) deposited *V. tricorpus* type material at IMI, K and CBS. We were only able to locate specimen IMI 51602, a dried *V. tricorpus* culture on PDA medium labeled ‘isotype ?’ (Figure 3c). Specimen IMI 51602 is likely derived from ex-type strain IMI 51602 deposited at CBS by I. Isaac as strain CBS 447.54. Thus, since the holotype appeared to be missing, we designated specimen IMI 51602, a likely isotype, as the lectotype of *V. tricorpus* (Art. 9.2, Art. 9.9, Art. 9.10). Specimen IMI 51602 did not display typical *V. tricorpus* morphology. Whereas verticillate conidiophores and microsclerotia were present (Figure 12g) in agreement with the description provided by Isaac [23], yellow-pigmented hyphae, chlamydospores and resting mycelium were absent. However, according to the ICBN, lectotypes have to be chosen from among isotypes if they exist (Art. 9.10). *Verticillium tricorpus* specimen IMI 51602 is a likely isotype and was thus designated as lectotype. Upon initial culturing, *Verticillium tricorpus* colonies on agar medium are yellow to orange (Figures 12a, 12b) due to the presence of yellow-pigmented hyphae (Figure 12h). Resting mycelium, chlamydospores and microsclerotia are also formed simultaneously (Figures 12d, 12e, 12f, 12g). The yellow to orange coloration is typically less intense after prolonged culturing, or if obscured by resting structures. *Verticillium tricorpus* is morphologically indistinguishable from *V. isaacii* and *V. klebahnii*. All three species are characterized by the formation of resting mycelium (Figures 7f, 8e, 12d), chlamydospores (Figures 7g, 8f, 12e) and microsclerotia (Figures 7h, 8h, 12f, 12g), as well as yellow-pigmented hyphae (Figure 7i, 8i, 12h) that confer agar cultures yellow to orange coloration (Figure 7b, 8b, 12b).

There is evidence for differences in pathogenicity. Whereas *V. isaacii* strains PD343, PD610–PD613 were not pathogenic on lettuce or artichoke [41], *V. klebahnii* strain PD401 was pathogenic on lettuce [51]. *Verticillium tricorpus* is only pathogenic on tomato [23].

*Verticillium zaregamsianum* Inderb., T. Usami, Takeshi Kanto, R. M. Bostock, R. M. Davis & K. V. Subbarao, *sp. nov.* Figure 13

MycoBank: MB563556
Etymology: Named after Rasoul Zare and Walter Gams who collaboratively established the modern taxonomic framework this study is based on.

Latin diagnosis. Verticillio dahiae simile sed pigmentum croceum exsudans.

Description. Colonies on PDA after two weeks 3-6.5 cm, white at first, later yellow, reverse orange to yellow, then darkening due to the formation of microsclerotia (Figures 13a, 13b). Aerial mycelium generally abundant, floccose, hyphae smooth-walled, 1-4 μm wide. Conidiophores erect or slanted (Figures 13c, 13d), generally determinate, branched or unbranched, formed disjointedly throughout the colonies, halyine, 50-800 μm in length, 3-4 μm wide, narrowing towards the apex to 2-3 μm, transversely septate, septa spaced more narrowly towards the apex. Conidiogenous cells are phialides (Figure 13d), arranged in (1–) 3–7 (–11) whorls along conidiophores (Figures 13c, 13d). Whorls spaced 25-100 μm apart, closer towards the apex, consisting of (1–) 2–5 (–6) phialides, arising below transverse septum. Apical whors consisting of one apical and one to several lateral phialides (Figures 13c, 13d). Phialides subulate, tapering from 2–3 μm at the base to 1–1.5 μm at the tip, terminal phialides 25-60 μm long, lateral phialides 20-60 μm long (Figure 13d). Conidia halyine, smooth-walled (Figure 13e), cylindrical with rounded apices to ellipsoidal, (4.0–) 5.5 μm±1.0 μm (–12.5)×(2.0–) 3.0 μm±0.5 μm (–6.5) (l/w = (1.4–) 2.0±0.3 (–2.8), n = 88), accumulating at the tip of the phialides (Figure 13c), one-septate, constricted at septum, and brown-pigmented at times with age (Figure 13e). Microsclerotia regularly or irregularly distributed throughout the colony, rounded to variously shaped, up to 90 μm diam and consisting of rounded cells, up to 14 μm diam (Figures 13f, 13g, 13h, 13i). Structures resembling chlamydospores, possibly microsclerotia initial, present at times, up to 10 μm wide (Figures 13f, 13g). Scattered brown-pigmented hyphae present at times, thick-walled, up to 5 μm wide (Figure 13j). Yellow-pigmented hyphal cells present (Figures 13k, 13l), up to 6 μm wide, containing globules of yellow pigment (Figure 13k), at times yellow pigmented crystals present outside the cells (Figure 13l).

Types. Holotype: Dried culture of V. zaregamsianum strain PD736 (Japan: Chiba; lettuce) deposited at UC (UC 1953899), an ex-holotype culture at CBS (CBS 130342) and NRRL (NRRL 54795).

Specimens examined. The description was based on V. zaregamsianum strains PD586, PD739 and PD740 (Japan: Chiba; tenweeks stock), PD731, PD733 and PD734 (Japan: Hyogo; lettuce), PD735 (Japan: Kagawa; lettuce), PD736, PD737 and PD738 (Japan: Chiba; lettuce) (Table S1).

Distribution and host range. Currently only known from Japan. Substrates include lettuce and tenweeks stock.

Commentary. Verticillium zaregamsianum differs from all other Verticillium species by the formation of microsclerotia (Figures 13h, 13i) simultaneously with yellow-pigmented hyphae (Figures 13k, 13l). Only a few potential chlamydospores, possibly immature microsclerotia (Figure 13f, 13g), and sparse resting mycelium (Figure 13j) were observed. Verticillium tricorpus, V. isaacii and V. klebahnii differ by the formation of abundant chlamydospores (Figures 7g, 8f, 12e) and resting mycelium (Figures 7f, 8f, 12d). Verticillium zaregamsianum was described as a new species because according to Index Fungorum (www.indexfungorum, accessed on September 30, 2011), there were no synonyms available for V. tricorpus. None of the two synonyms of the morphologically similar V. dahiae listed in Index Fungorum (V. oawum G.H. Berkeley & A.B. Jackson, V. trachephiilum Curzi) matched the morphology of V. zaregamsianum in that no yellow-pigmented hyphae were mentioned [52,53].

Discussion

We have generated a solid taxonomic framework for Verticillium that recognizes ten species, five of which are new to science. Our results show that resting structure morphology, traditionally the most important morphological character to differentiate Verticillium species still plays a part in species identification, but the near-complete reliance on resting structure morphology to identify Verticillium species will have to be abandoned.

As other recent studies of fungal diversity [54,55,56,57,58,59,60], our approach combined phylogenetic analyses, literature research and morphological comparisons, and established that each Verticillium species, except the hybrid V. longisporum, corresponded to a single group in the phylogenetic tree. We included ex-type strains that are derived from herbarium type material to which fungal names are permanently linked according to the International Code of Botanical Nomenclature (ICBN). All species-level phylogenetic groups contained a single ex-type strain that thus conferred a species name to all current and future group members guaranteeing taxonomic stability.

This study recognized all previously known species of Verticillium [15]. These were V. albo-atrum [21], V. dahiae [35], V. longisporum [24], V. nubilum [50] and V. tricorpus [23]. In order to stabilize the application of names, we selected several new types. For V. albo-atrum and V. nubilum, we designated illustrations as lectotypes since no herbarium material was available, and for V. tricorpus an isotype was designated as lectotype. For V. dahiae and V. albo-atrum, epitypes were selected based on our morphological comparisons, and a V. nubilum epitype was chosen among strains deposited by Isaac [20], who studied V. nubilum in detail.

The five new Verticillium species

Five species-level phylogenetic groups did not contain any ex-type strains, and were thus described as new species (Figure 1). These were V. albo-atrum (Figure 5) and V. nonalfalfae (Figure 10) that are relatives of V. dahiae (Figure 6) and V. longisporum (Figure 9), as well as V. zaregamsianum (Figure 13), V. isaacii (Figure 7) and V. klebahnii (Figure 8), all related to V. tricorpus (Figure 12).

The sister species Verticillium albo-atrum and V. nonalfalfae (Figure 1) were previously referred to as the respective lucerne and non-lucernae pathotypes of ‘V. albo-atrum’ [46], and have long been recognized as two genetically distinct groups [47,48]. The two species are morphologically indistinguishable, but differ in pathogenicity. Verticillium nonalfalfae causes disease on a variety of hosts whereas V. albo-atrum causes disease on lucerne [44]. Other differences include vegetative compatibility groups [45], mating types (Figure 2), as well as the DNA characters listed in the species descriptions. Molecular data have previously been included in species descriptions [53,58,59,61,62]. We did not detect any genetic variation within V. albo-atrum and V. nonalfalfae (Figure 1). However, variation within V. nonalfalfae has been demonstrated using AFLP markers and a proteomics approach [63,64,65,66].

Verticillium albo-atrum and V. nonalfalfae are related to V. dahiae and V. longisporum (Figure 1), but differ morphologically by the formation of resting mycelium. However, resting mycelium is also present in the distantly related V. albo-atrum with which V. albo-atrum and V. nonalfalfae have frequently been confused [15].

The existence of two distantly related groups in Verticillium forming resting mycelium was established earlier. Verticillium albo-atrum and V. nonalfalfae have been referred to as ‘V. albo-atrum’ group 1, and V. albo-atrum as ‘V. albo-atrum’ group 2 [67,68]. Robb et al. [67] suggested that ‘V. albo-atrum’ group 2 was characterized by the formation of brown-pigmented hyphae aggregating in bundles, whereas brown-pigmented hyphae in ‘V. albo-atrum’ group 1 were
solitary. However, we found bundles of brown-pigmented hyphae in both *V. alfalfae* (Figure 3g), *V. nonalfalfae* and *V. albo-atrum* (Figure 4h), and thus, this character is not suitable for species differentiation.

It was earlier suggested that ‘*V. albo-atrum*’ groups 1 and 2 may constitute different species [68,69], but details have been unclear. Using comparisons to the *V. albo-atrum* type description and illustrations [21], it was apparent that *V. albo-atrum* corresponded morphologically to ‘*V. albo-atrum*’ group 2. *Verticillium albo-atrum* forms microsclerotia (Figures 4i, 4j, 4k) in addition to resting mycelium [69,70,71] (Figures 4g, 4h), as well as yellow-pigmented hyphae as observed by Klebahn [35] (Figures 4b, 4l). Recently, the name ‘*V. albo-atrum*’ has possibly been applied more frequently to ‘*V. albo-atrum*’ group 1 now comprising *V. alfalfae* and *V. nonalfalfae*, than to ‘*V. albo-atrum*’ group 2, now *V. albo-atrum*. Thus, in the absence of molecular data, detailed morphological descriptions or cultures, it is not possible to relate the previous literature on ‘*V. albo-atrum*’ with absolute certainty to the current species concepts of *V. albo-atrum*, *V. alfalfae* and *V. nonalfalfae*. Adding to the confusion, there might be additional groups with similar morphology, as in a study of ‘*V. albo-atrum*’ diversity isolates from pea formed a separate cluster [46,68].

The remaining three new species proposed here, *V. isaacii*, *V. klebahnii* and *V. zaregamsianum* are related to *V. tricorpus* (Figure 1). *Verticillium isaacii*, *V. klebahnii* and *V. tricorpus* are morphologically indistinguishable, they are characterized by the formation of resting mycelium (Figures 7f, 8e, 12f), chlamydospores (Figures 7g, 9f, 12e) and microsclerotia (Figures 7h, 8d, 12b), as well as the presence of yellow-pigmented hyphae (Figure 7i, 8h, 12h), providing the colonies on agar medium with a yellow or orange coloration (Figure 7b, 8b, 12b). The three species are a monophyletic group (Figure 1), and they could have been considered as three different lineages within just one species, *V. tricorpus*. However, compared with other *Verticillium* species, *V. tricorpus*, including what are now *V. isaacii* and *V. klebahnii*, was known to be very diverse, both in terms of ITS sequence data [48] and vegetative compatibility groups [72]. There is evidence for differences in pathogenicity. *Verticillium tricorpus* was only pathogenic on tomato [23], *V. klebahnii* was pathogenic on lettuce [51], and *V. isaacii* was not pathogenic on either lettuce or artichoke [41]. Further research is needed to determine the host ranges of these species.

*Verticillium zaregamsianum*, the third new species related to *V. tricorpus* (Figure 1), is morphologically distinct from all other *Verticillium* species, *V. zaregamsianum* forms predominantly microsclerotia (Figure 13b), as well as yellow-pigmented hyphae (Figures 13k, 13l). *Verticillium zaregamsianum* is a pathogen of lettuce in Japan [73].

Table 2 provides an overview of the taxonomic changes made in this paper and relates the new taxonomic system to previously described species.

### Phylogenetic relationships of *Verticillium* species

In agreement with previous studies [15,74], we identified two major groups in *Verticillium* that we named Clades Flavexudans and Flaxonexudans, respectively (Figure 1). Clade Flavexudans comprised all species that produced yellow-pigmented hyphae that were absent in all members of Clade Flaxonexudans. Whereas Clade Flavexudans was well supported by the phylogenetic analyses, Clade Flaxonexudans, in particular the monophyly of *V. nubilum* with the remaining members of Clade Flaxonexudans, only received support in the parsimony analyses (Figure 1). More research is needed to conclusively determine the phylogenetic placement of *V. nubilum* within *Verticillium*.

The phylogenetic relationships within the major clades were well resolved (Figure 1). Within Clade Flavexudans, the branching order of *V. albo-atrum*, *V. isaacii*, *V. klebahnii*, *V. tricorpus* and *V. zaregamsianum* had maximal support in all analyses. The topology of Clade Flaxonexudans was also well resolved, except for the placement of Species A1, an ancestor of the diploid hybrid *V. longisporum*. Species A1 that is unknown and has never been collected [27], is basal to the clade of *V. alfalfae*, *V. dahliae*, *V. nonalfalfae* as well as Species D1, another unknown species and second ancestor of *V. longisporum* [27], but only supported by the Bayesian analyses (Figure 1). Inderbitzin et al. [27] studied the evolutionary history of *V. longisporum* in detail, they found that *V. longisporum* evolved at least three different times from four different parental lineages representing three different species. The results of Inderbitzin et al. [27] differ from the current study with regard to the placement of Species A1 that formed a clade with Species D1 and *V. dahliae*, whereas in this study, Species A1 was a sister group to the clade of *V. alfalfae*, *V. dahliae*, *V. nonalfalfae* and Species D1. The topological divergence involving Species A1 might be due to differences in taxon sampling and the use of an additional locus for phylogenetic analyses in Inderbitzin et al. [27].

### Hosts and geographic distribution of *Verticillium* species

The isolates used in this study represent only a small fraction of the vast literature on *Verticillium* [1] and therefore do not paint a complete picture on geographic distribution and host range. However, the data provided here and in Inderbitzin et al. [27] are associated with correctly identified isolates and constitute an initial

**Table 2.** Correspondence of previous to current taxonomic system and summary of taxonomic changes enacted.

| Previous names | Taxonomic changes | Current names |
|----------------|------------------|---------------|
| *Verticillium albo-atrum* | Split into three species, designation of epitype for *V. albo-atrum* | *V. albo-atrum, V. alfalfae or V. nonalfalfae* |
| *Verticillium dahliae* | Epitype specimen designated | *V. dahliae* |
| *Verticillium longisporum* | None | *V. longisporum* |
| *Verticillium nubilum* | Epitype specimen designated | *V. nubilum* |
| *Verticillium tricorpus* | Split into three species, designation of lectotype for *V. tricorpus, V. isaacii* or *V. klebahnii* | Described as new species |

*V. albo-atrum* is more closely related to *V. tricorpus* than to *V. dahliae*, whereas *V. alfalfae* and *V. nonalfalfae* are closely related to *V. dahliae* (Figure 1).

*V. zaregamsianum* was referred to as *V. tricorpus* at least once [73], but differs morphologically.

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approximation of the distributions and host associations of *Verticillium*. For *V. dahliae*, *V. longisporum* and *V. nubilum* distribution and host range data are in general agreement with the literature [1,23,35,73]. *Verticillium dahliae* is known from four continents and fourteen host families, and is by far the most widespread *Verticillium* species. This contrasts with *V. nubilum* that is only known from Irish potato in the UK, and with *V. longisporum* that occurs in Europe, Japan and North America but is restricted mainly to hosts in the Brassicaceae. More work is needed to expand our knowledge on the distributions and host ranges of the remaining species, including the newly described *V. alfalfae*, *V. isaacii*, *V. klebahnii*, *V. nonalfalfae* and *V. zaregsianum*, as well as *V. albo-atrum* and *V. tricorpus* that are now more narrowly defined.

**Identification of *Verticillium* species**

Correct and consistent identification is crucial for effective and efficient disease control [76], but we found *Verticillium* species may frequently have been misidentified. Based on DNA sequencing and phylogenetic analyses, we determined that at least 34 of the 293 isolates used in this study and the study by Inderbitzin et al. [27], were not correctly identified (Table 3). Given that the majority of *Verticillium* strains were from *Verticillium* research labs, the error rate among non-specialists is likely to be higher.

*Verticillium* is difficult to separate from similar genera as it lacks morphological characters that are unique. The most conspicuous characters of *Verticillium*, the conidiophores bearing whorls of conidiogenous cells, as well as the resting structures, are also present in other genera including *Gibellulopsis* and *Musciillium*. This problem is illustrated by the fact that 26 of the 34 misidentified isolates belonged to genera other than *Verticillium* (Table 3).

Within *Verticillium*, resting structure morphology, conidia size, conidiophore size and pigmentation, the number of phialides per conidiophore, size and pigmentation, the number of phialides per conidiophore, and the formation of yellow-pigmented hyphae has been used to differentiate species [23,24,35,50]. It is known that resting structure morphology may vary depending on culture medium used to differentiate species [23,24,35,50]. We did not investigate the influence of environmental conditions on *Verticillium* morphology in detail, but found that differences in resting structures between *V. albo-atrum*, *V. alfalfae* and *V. nonalfalfae* were more readily observed on WA-p and PLYA media than on PDA medium. Thus, based on our results, we recommend the combined use of PDA and WA-p for species identification.

In Figure 14 we provide a key to *Verticillium* species based on morphological characters. However, given the morphological variability of *Verticillium* species as discussed above, the key is more intended as an overview of *Verticillium* morphology than as an authoritative means for species identification. All results obtained using the key should be confirmed by DNA sequencing and phylogenetic analyses with ex-type isolates.

**Conclusions**

The new taxonomic system presented here is based on a multifaceted approach that included phylogenetic and morphological investigations, herbarium and literature research, and allows for a more reliable and consistent identification of *Verticillium* species. We envision that over time, this taxonomic system will lead to a significant improvement of our knowledge of *Verticillium* biology. Potential practical applications are many, and may include more efficient and effective disease management strategies and quarantine regulations.

**Future Research**

Future research will focus on the determination of host ranges of some of the new species of *Verticillium* as well as *V. albo-atrum*. Also, inclusion of more isolates from non-agricultural systems in studies of *Verticillium* diversity would be desirable.

**Materials and Methods**

**Taxon selection, origin of fungal strains and DNA sequences retrieved from GenBank**

Taxes were selected to cover the known diversity of *Verticillium* [46], and included 74 strains representing *V. tricorpus*, *V. nubilum*, *V. albo-atrum*, *V. longisporum* and *V. dahliae* as well as the outgroup *Gibellulopsis nigrescens* based on results from Zare et al. [15]. We previously clarified the phylogenetic relationship of *V. dahliae* and *V. longisporum* [27], and for *V. dahliae* and *V. longisporum* only included six taxa representing the main lineages of the two species. The isolates were obtained from a variety of different sources (Table S1), and initially identified based on morphology. Common

### Table 3. Names of misidentified isolates received are given in top row, approximate correct names based on DNA sequencing and comparison to GenBank are in left column, numerals refer to numbers of isolates in each category.

| Incorrect name* | Correct name | *V. albo-atrum* | *V. dahliae* | *V. longisporum* | *V. nubilum* | *V. tricorpus* |
|----------------|--------------|----------------|-------------|----------------|-------------|----------------|
| *V. albo-atrum* |              | 1              |             |                |             |                |
| *V. dahliae*    |              | 1              |             |                |             |                |
| *V. tricorpus*  | 1            | 5              |             | 1              |             |
| *Gibellulopsis nigrescens* | 5 | 1 |             |             |             |
| *Leptodontium sp.* | 1 |             | 1            |             |             |
| *Lecanicillium sp.* | 2 |             | 1            |             |             |
| *Musciillium theobromae* | 3 |             | 1            |             |             |
| *Nectria sp.*   | 1            | 10             | 10           | 1             |             |
| *Neosartorya sp.* |             |              | 1            | 1             |             |
| *Plectosphaerella sp.* | 1 |             | 4            |             |             |

*A total of 34 incorrectly identified isolates were among the 293 isolates from this study and Inderbitzin et al. [27]. Origins of misidentified isolates available upon request.

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names used for hosts were obtained from www.ITIS.gov accessed on June 6, 2010.

For the following 13 isolates DNA sequence data by Inderbitzin et al. [27] was retrieved from GenBank. *V. dahliae* strains PD322 (HQ206718, HQ206921, HQ414624, HQ414719, HQ414909) PD-327 (HQ206723, HQ206925, HQ414628, HQ414723, HQ414913), PD502 (HQ206813, HQ206942, HQ414645, HQ414740, HQ414930); *V. alfalfae* strains PD338 (HQ206733), PD533 (HQ206742, HQ206933, HQ414636, HQ414731, HQ414921), PD620 (HQ206851, HQ206965, HQ414668, HQ414763, HQ414953), PD681 (HQ206891), PD692 (HQ206892); *V. nubilum* strain PD621 (HQ206552, HQ206966, HQ414669, HQ414764, HQ414954); *V. isaaci* strain PD660 (HQ206873, HQ206985, HQ414683, HQ414783, HQ414973); *V. longisporum* strains PD348 (HQ206738, HQ206930, HQ206931, HQ414633, HQ414634, HQ414729, HQ414792, HQ414918, HQ414919), PD356 (HQ206745, HQ206934, HQ414635, HQ414637, HQ414638, HQ414732, HQ414733, HQ414922, HQ414923), PD687 (HQ206893, HQ206993, HQ414696, HQ414697, HQ414791, HQ414792, HQ414981, HQ414982).

Stock culture maintenance and growth conditions
All strains were single-conidium purified and maintained as conidia suspensions at −80°C in glycerol diluted by half strength potato dextrose broth (25% glycerol vol/vol), and retrieved anew for each experiment. Cultures were grown on the following media. Potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD), supplemented with autoclaved stems of unidentified herbaceous plants in the Asteraceae and the Malvaceae (WA-p), and prune lactose yeast agar (PLYA) using food grade prune juice [79,80]. Plates were sealed with Parafilm, and left on a lab bench inside a plastic container (crisper) subject to natural and artificial light and darkness at night. To document culture morphology, plates were left unsealed.

Species recognition, description and naming
Species were defined as terminal or subterminal clades inferred from multigene phylogenetic analyses in accordance with the Genealogical Concordance Phylogenetic Species Recognition approach outlined by Taylor et al. [31] and named by the inclusion of ex-type strains. Except for the diploid hybrid *V. longisporum*, each species-level clade contained a single ex-type strain. New species were described for all species-level clades for which no existing names were available. Existing, readily available names include synonyms that are listed in Index Fungorum (www.indexfungorum.org). We were unable to search for additional synonyms among the 266 described *Verticillium* species listed in Index Fungorum (accessed September 30, 2011).

Morphological descriptions were based on cultures grown on PDA, WA-p and PLYA media. Microscopy was performed using a Leica DM5000 B microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany), with bright field (BF), differential interference contrast (DIC) and phase contrast (PC) illumination of specimens mounted in water. Photographs were taken with a Leica DFC310...
FX camera, using Leica Application Suite Version 3.6.0 software. Culture photographs were generated with a desktop document scanner (DS). The terminology used in the species diagnoses follows Kirk et al. [36]. For conidia dimensions, standard deviations are given. Nucleotide substitutions in the species diagnoses included all derived substitutions shared by all members of a species, except the substitutions that were in alignment regions of low complexity (single or multi-nucleotide repeats) or near gaps in regions of ambiguous alignment.

Nomenclature
The electronic version of this document in itself does not represent a published work according to ICBN, and hence the new names contained in the electronic version are not effectively published under that Code from the electronic edition alone. Therefore, a separate edition of this document was produced by a method that assures numerous identical printed copies, and those copies were simultaneously distributed on the publication date noted on the first page of this article for the purpose of providing a public and permanent scientific record, in accordance with Article 29 of the Code. Copies of the print-only edition of this article were distributed on the publication date to botanical or generally accessible libraries of the following institutions, BPI, CBS, CLUP, DAOM, HMAS, IMI, IRAN, NY, SFSU, TNS, UBC, and UC. The separate print-only edition is available on request from PLoS (Public Library of Science) by sending a request to PLoS ONE, Public Library of Science, 1160 Battery Street, Koshland Building East, Suite 100, San Francisco, CA 94111, USA along with a check for $10 (to cover printing and postage) payable to “Public Library of Science”. This article is digitally archived in PubMed Central and LOCKSS.

DNA extraction, PCR amplification for direct sequencing and DNA sequencing conditions
DNA was extracted according to Inderbitzin et al. [27]. For extraction of DNA from the V. dahliae type material, the same protocol as for extractions from mycelium recovered from agar plates was used.

Loci used for phylogenetic analyses and primer design
Five loci were used in this study, including actin (ACT), elongation factor 1-alpha (EF), glyceraldehyde-3-phosphate dehydrogenase (GPD), tryptophan synthase (TS) and the ribosomal internal transcribed spacer region ITS. Primers used to PCR amplify and sequence the ITS region were ITS1-F [81], ITS4 and ITS5 [82]. The TS region of Verticillium albo-atrum was at times PCR amplified and sequenced with primer pair VTs5f (5'-ACC TAT GTC ACT GCC GGC T-3') and VTs4r (5'-CAA TGA AGC CGT TGA CGC C-3'). For more details on TS as well as the remaining loci, PCR conditions and DNA sequencing, see Inderbitzin et al. [27].

MAT screening
Isolates in V. alfalfae and V. nonalfalfae were screened for presence of MAT1-1 and MAT1-2 idiomorphs according to Inderbitzin et al. [27].

Phylogenetic analyses
Besides the single-locus ACT, EF, GPD, TS and ITS datasets, a combined, four-locus dataset comprised of concatenated ACT, EF, GPD and TS datasets was analyzed.

The datasets were analyzed as outlined in Inderbitzin et al. [27] using three different algorithms. DNA sequences were assembled and aligned in Geneious v4.8.5 [83]. Single-locus datasets were analyzed under the maximum parsimony criterion using PAUP v.4.0b 10 [84]. The combined four-locus dataset was analyzed using parsimony, maximum likelihood as implemented in PAUP v.4.0b 10, as well as MrBayes v3.0b4 [85] implementing a Bayesian approach to inferring phylogenies.

Most parsimonious trees were inferred using 30 random addition replicates. Otherwise, default settings were used, including treating insertion/deletion gaps as missing data. Bootstrap support values were based on 500 replicates. Maximum likelihood analyses were done using default settings and 30 random addition replicates, bootstrap supports were based on 415 replicates. Bayesian analyses were performed with default settings, running four chains over 10 million generations and sampling each 100th tree. The first 1000 of the 10,000 saved trees were omitted and the consensus tree was based on the remaining 9,000 trees. Maximum likelihood and Bayesian analyses implemented an optimal model of DNA sequence evolution determined using Modeltest 3.7 [86]. All analyses were run with a single representative of each haplotype.

Supporting Information

Figure S1 Phylogenetic tree of Verticillium based on the ITS dataset comprising 74 taxa and 514 characters. Shown is one of the nine equally parsimonious trees, 427 steps in length. Isolates are represented by a strain identifier; species are delimited by a vertical bar followed by a name. Branches with 100% bootstrap support are in bold, other support values above 70% are given by the branches.

(TIF)

Figure S2 Phylogenetic tree of Verticillium based on the ACT dataset comprising 77 taxa and 638 characters. Shown is one of the 12 equally parsimonious trees, 427 steps in length. Isolates are represented by a strain identifier; species are delimited by a vertical bar followed by a name. Branches with 100% bootstrap support are in bold, other support values above 70% are given by the branches.

(TIF)

Figure S3 Phylogenetic tree of Verticillium based on the EF dataset comprising 77 taxa and 614 characters. Shown is one of the 2 equally parsimonious trees, 430 steps in length. Isolates are represented by a strain identifier; species are delimited by a vertical bar followed by a name. Branches with 100% bootstrap support are in bold, other support values above 70% are given by the branches.

(TIF)

Figure S4 Phylogenetic tree of Verticillium based on the GPD dataset comprising 77 taxa and 781 characters. Shown is one of the 2 equally parsimonious trees, 430 steps in length. Isolates are represented by a strain identifier; species are delimited by a vertical bar followed by a name. Branches with 100% bootstrap support are in bold, other support values above 70% are given by the branches.

(TIF)

Figure S5 Phylogenetic tree of Verticillium based on the TS dataset comprising 77 taxa and 625 characters. Shown is one of the 396 equally parsimonious trees, 365 steps in length. Isolates are represented by a strain identifier; species are delimited by a vertical bar followed by a name. Branches with 100% bootstrap support are in bold, other support values above 70% are given by the branches.

(TIF)
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Author Contributions

Conceived and designed the experiments: PI RMB RMD KVS. Performed the experiments: PI. Analyzed the data: PI. Contributed reagents/materials/analysis tools: PI RMB RMD TU HWP KVS. Wrote the paper: PI RMB RMD TU HWP KVS. Published a preliminary version of this paper: PI. RMB. RMD. KVS. Final version: PI. RMB. RMD. KVS. Wrote the paper: PI. RMB. RMD. KVS.

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