IDENTIFICATION AND DIFERENTIATION OF *Verticillium* SPECIES WITH PCR MARKERS AND SEQUENCING OF ITS REGION

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Introduction

The genus *Verticillium* is a group of ascomycete fungi, including plant-pathogenic species capable of infecting the vasculature of many agricultural crops, and therefore causes major economic losses worldwide (Figure 5). In 2011, a new taxonomic classification of the genus was proposed, which is now referred to as *Verticillium* sensu stricto, comprising ten species: *V. dahliae V. albo-atrum, V. alfalfae, V. longisporum, V. nonalfalfae, V. tricorpus, V. zaregamsianum, V. nubilum, V. isaacii and V. klebahnii*. Our *Verticillium* spp. collection was screened with the new taxonomic PCR-markers using simplex and multiplex PCR assays. In addition, we also evaluated an alternative ITS-sequence based approach for the identification purposes.

Material and methods

Fungal genomic DNA of 105 *Verticillium* isolates was extracted by the modified CTAB method (Kump and Javornik, 1996). For species identification, 18 new primers, combined to amplify 11 *Verticillium* sensu stricto species specific DNA fragments were used in simplex and multiplex PCR assays, developed by Inderbitzin et al. (2013). Products were analyzed with gel electrophoresis with 1.3 % wt/vol agarose. Primers ITS1 and ITS4, designed by White et al. (1990), were used to amplify and sequence fungal ITS region. Phylogenetic analysis was performed using CLC Genomic Workbench software. ITS sequences were aligned using Muscle algorithm. Maximum likelihood phylogenetic tree was obtained with implementing Neighbor joining/Kimura80 model and rooted to *Gibellulopsis nigrescens* ITS region sequence. Simultaneously, sequences were compared to NCBI data base using BLAST tool. PCR-based and ITS analysis-based data were collected and merged in comprehensive table for identification purposes.

Phylogenetic analisys of ITS region

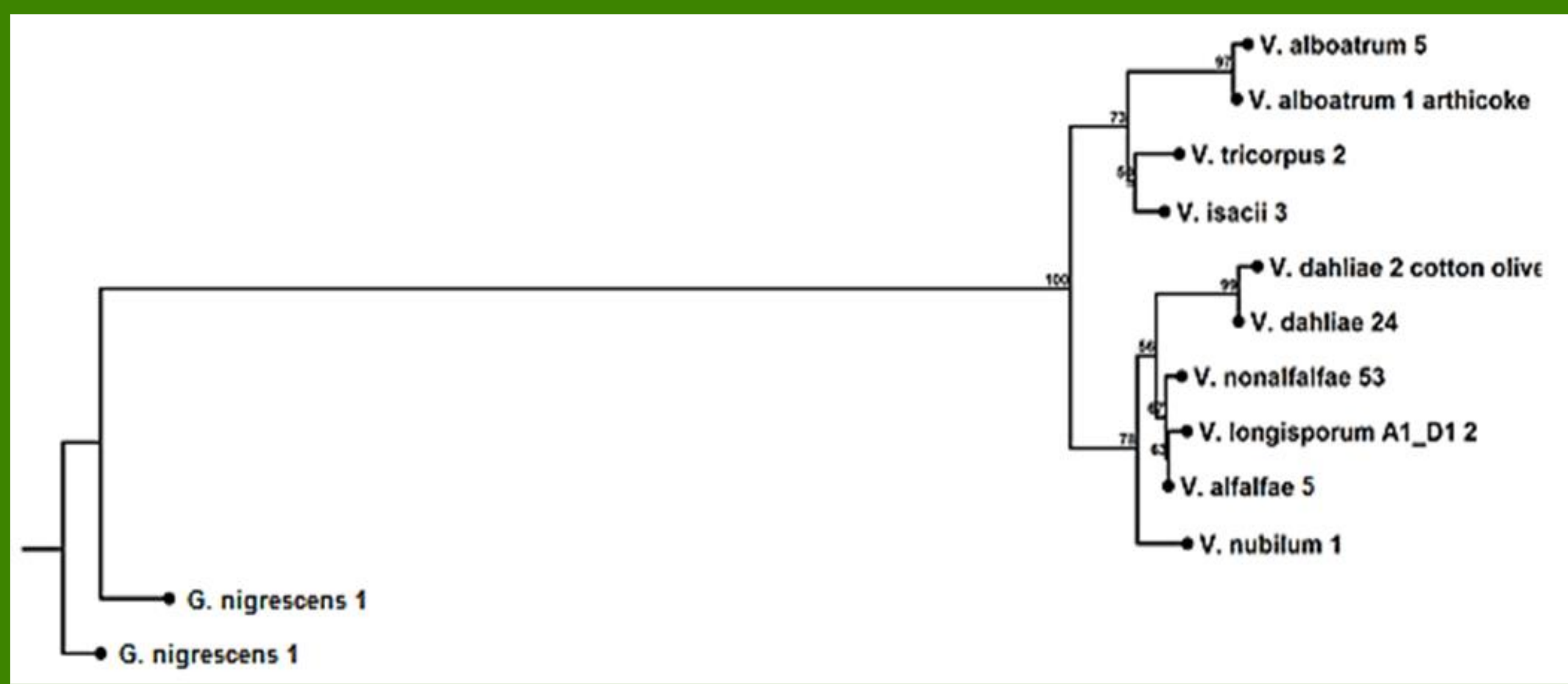


Figure 1: Maximum likelihood phylogenetic tree; branches represent representative ITS sequences of *Verticillium* sensu stricto species, subgroups of certain species and two *Gibellulopsis nigrescens* isolates; numbers beside species name identicate the number of isolates with identical ITS sequence.

Results

Among 105 *Verticillium* isolates included in the identification analysis, total of 88 isolates were successfully amplified by specific band length for each simplex PCR assay. The identified isolates represent eight out of ten species of the genus *Verticillium* sensu stricto. Two of them (*V. klebahnii* and *V. zaregamsianum*) were not presented in our collection. The remaining 17 isolates were not amplified due to the low quality of DNA or due to the no specificity of developed PCR primers. Thirty five isolates that couldn't be differentiated with simplex PCR assay have additionally undergone multiplex PCR assay. Of those, 2 isolates were identified as *V. longisporum* A1/D1, by two species specific bands amplified in multiplex PCR assays with primer pairs specific to Species A1 and Species D1. For other isolates, previous simplex PCR identification of *V. dahliae* species was confirmed.

Based on sequence data for ITS region, total of 100 isolates were included in the phylogenetic analysis. Two isolates were excluded, since no quality ITS sequences were obtained, along with three isolates representing *V. fungicola* and *V. leicanii* species, identified as such in NCBI data base. Maximum likelihood phylogenetic tree, using Neighbor joining/Kimura80 model, was obtained, dividing isolates into 12 groups of branches, representing eight species of *Verticillium* sensu stricto, subgroups of certain species and *Gibellulopsis nigrescens* species (Figure 1). Representative sequence for each group was used in additional aligning and tree design. Using phylogenetic analysis, 98 isolates were identified as *Verticillium* sensu stricto species and two as species *Gibellulopsis nigrescens*.

Validation of newly proposed PCR markers for identification of *Verticillium* sensu stricto species failed in differentiation of *V. dahliae* and *G. nigrescens* species, but on the other hand alternative ITS sequences based approach resulted in successful differentiation of these two different species.

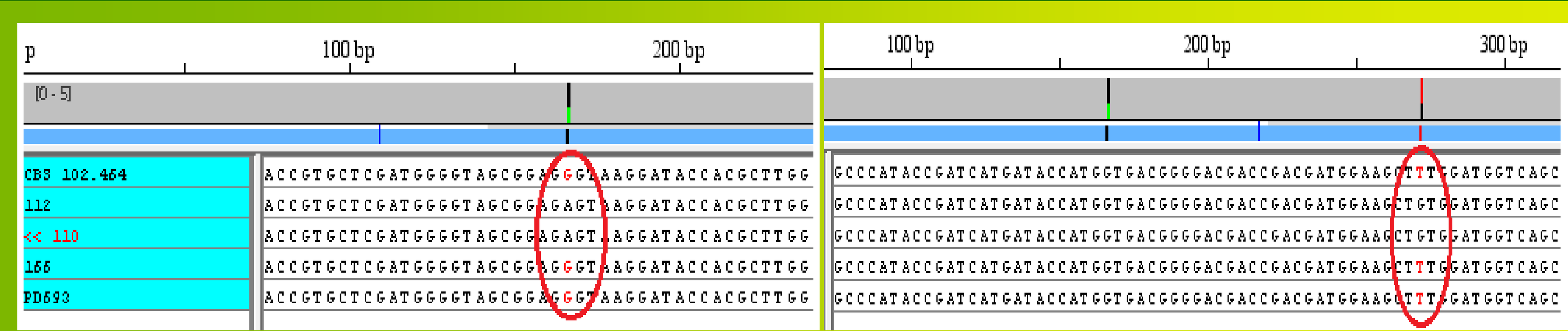
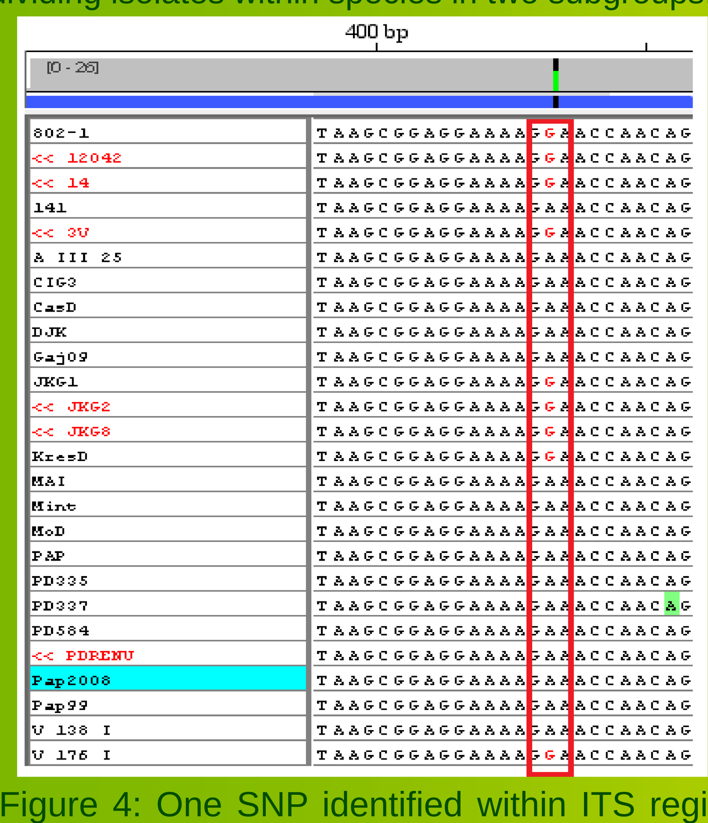


Figure 2: Two SNPs identified within region of actin, specific for *V. albo-atrum* species, dividing isolates within species in two subgroups; both isolates with nucleotide A in SNP region originate from Prince Edward Islands.

Figure 3: Six SNPs identified within region of elongation factor 1-alpha, specific for *V. isaacii* species, dividing isolates within species in two subgroups; isolate JKG20 was isolated from *Tilia* sp. (Netherlands).



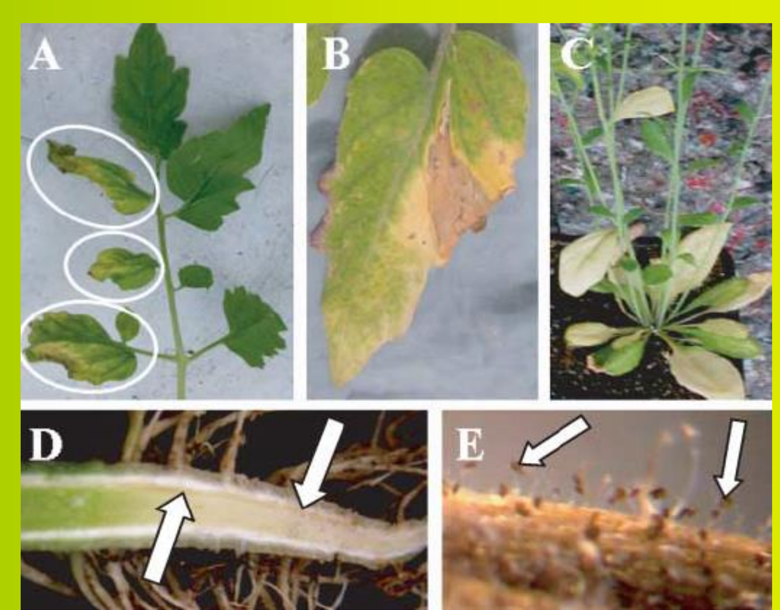


Figure 5: Symptoms of fungi (*Verticillium* sensu stricto) infection

Figure 4: One SNP identified within ITS region, specific for *V. dahliae* species, dividing isolates within species in two subgroups; isolates within the two subgroups have no characteristic common features.

Conclusions

The new PCR-marker analysis enabled us to confirm the identity of 88 isolates from various geographic locations in Europe, North America and Japan and from various host plants, including hop, potato, tomato, cotton, olive and alfalfa. According to the new taxonomic classification, 41 isolates were identified as *V. nonalfalfae*, 28 as *V. dahliae*, 6 as *V. albo-atrum*, 5 as *V. alfalfa*, 3 as *V. isaacii*, 2 as *V. tricorpus*, 2 as *V. longisporum* lineage A1/D1 and 1 as *V. nubilum*. Identification and differentiation of *V. longisporum* linegaes was performed by multiplex PCR assay. We were also able to determine all isolates by means of sequence ITS analysis, reflecting two main groups of isolates, Flavexudans and Flavnonexudans. Moreover, based on ITS sequencing analysis, we were able to obtain additional information on the subgroups within the species *V. albo-atrum* and *V. dahliae* that were not revealed by PCR-marker analysis (Figure 2,3,4).