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Taxonomical re-evaluation of *Phoma*-like soybean pathogenic fungi

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ABSTRACT

Coelomycetous fungi classified in *Ascochyta*, *Phoma*, and *Phyllosticta* have been recorded from spots on leaves and pods of soybeans. Based on the Genealogical Concordance Phylogenetic Species Concept, the authors suggest the re-evaluation of the taxonomic status of *Phoma sojicola* (syn. = *Ascochyta sojicola*) and *Phyllosticta sojicola*. In spite of the former delimitation of *Phoma sojicola* based on small differences in morphological features, it has proved to be identical to *Phoma pinodella*. Similarly, it was also confirmed that *Phyllosticta sojicola* was identical to *Phoma exigua* var. *exigua*. The authors supply tools for identification of *Phoma*-like fungi by combined conventional and molecular methods. Protein-encoding genes (*tef1* and *β-tubulin*) were successfully applied within the *Phoma* genus to infer phylogenetic relationships.

Introduction

Different species of hyaline-spored pycnidial fungi have been associated with leaf and pod spot diseases of soybean (*Glycine max*) from different parts of the world. These diseases might periodically occur causing yield losses under favourable environmental conditions. Comparison of herbarium materials and pathogenicity tests made with various isolates have demonstrated that *Phoma sojicola* was identified as one of the specific pathogens of soybean (Kövics *et al.* 1999). This fungus, which had been described earlier as *Ascochyta sojicola* (as '*sojaecola*') by Abramov (1931) resembles some plurivorous *Phoma* species, which may also be pathogenic on soybean, *viz.* *Phoma exigua* var. *exigua* (listed as '*A. phaseolorum*', Wallace & Wallace 1947, 1949) and *Phoma pinodella* (listed as '*A. pinodella*', Noll 1939). Finally *Phyllosticta sojicola* Massalongo (1900) has been reported causing similar disease symptoms (Böning 1939; Walters & Martin 1981) on soybean leaves and pods. The situation is further complicated in that other species of *Ascochyta* and *Phoma* have also been found on soybean; the occurrences of which were registered only sporadically and mainly as opportunistic parasites (Kövics *et al.* 1999). Because

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these fungi can only be differentiated with certainty *in vitro* (van der Aa *et al.* 1990), the early literature pertaining to them is very confusing. The present paper elucidates the phylogenetics of the specific *Phoma*-like species associated with soybean using molecular markers. Other hyaline-spored coelomycetes recorded on soybean in the genera *Phoma*, *Phyllosticta*, and *Ascochyta* are briefly discussed and their diagnostic characters are contrasted.

Up to now, molecular-based phylogenetic analyses within the *Phoma* genus have only been used for defining phylogenetic relationships among isolates within one or closely related species (Fatehi *et al.* 2003; Mendes-Pereira *et al.* 2003; Balmas *et al.* 2005; Voigt *et al.* 2005). In present paper the authors supply combined tools for the identification and delimitation of *Phoma*-like fungi of soybean, which could be applicable in *Phoma* taxonomy. In addition to conventional morphological criteria, there are some other supplementary methods that can contribute to the comparison and proper delimitation of *Phoma* and *Phoma*-like fungi. The complete sequence of the ITS region and fragments of the *tef1* and *β-tubulin* genes were obtained from representative isolates of *Phoma sojicola* (CBS 567.97) and *Phyllosticta sojicola* (CBS 301.39). Sequences from *Phoma pinodella*, *P. exigua* var. *exigua,* and *P. glomerata* isolated from soybean and other hosts, as well as non-soybean pathogenic *Phoma* and *Ascochyta* species were also used in the comparative analyses.

The primary objective of this research was to determine the evolutionary relationships among *Phoma*-like species occurring on soybean, especially *Phoma sojicola*, *P. exigua* var. *exigua*, *P. pinodella,* and *Phyllosticta sojicola*. As phylogenetic markers, we have analysed the sequences of ITS containing part of regions 1 and 2 and the 5.8S rDNA, the large intron of the translation elongation factor gene (*tef1*), and part of the *β-tubulin* gene (*tub1*).

Materials and methods

Strains and morphological analysis

Twenty-two isolates of nine different *Phoma* species were obtained from reference culture collections (Table 1). Seven of them were isolated from soybean; the others were collected from different hosts. All cultures were maintained on oatmeal agar (OA) at 5 °C during the study. The species were characterized morphologically according to Boerema *et al.* (2004).

The isolates were cultured on OA (20 g oat flakes, boiled in 0.5 l tap water, filtered through cheesecloth, and made up to 1 l with tap water) and malt agar (MA; 40 g malt extract Oxoid L39 (Hampshire, UK), 15 g Oxoid agar, 1 l tap water) incubated at 22 °C in dark, and examined 7 d later. Rayner's (1970) terminology was used for the description of colony colours. Petri dishes were then exposed to cycles of 13 h N_{UV} light/11 h darkness to stimulate the formation of pycnidia. Two weeks later colony descriptions were noted, and after three weeks the morphology of the pycnidia, conidia, and other structures, such as chlamydospores, were studied from the OA cultures. The sodium hydroxide (NaOH) spot test was performed on MA by the addition of a drop of 1 N NaOH on the colony margin, and a change in colour was noted. Conidia dimensions refer to 30 measurements with oil-immersion at ×1250.

DNA extraction

For PCR-based methods the studied cultures were grown in 100 ml malt broth (MB, containing 2 % malt extract) for 48 h at room temperature in the dark on a rotary shaker (125 rev min^{-1}). The mycelium was harvested by vacuum filtration. Total genomic DNA was extracted from freeze-dried mycelium and isolated using E.Z.N.A.® TM Fungal DNA Isolation Kit (Omega Bio-tek, Norcross, USA) according to the manufacturer's instructions. DNA concentrations were estimated in comparison to known standards in agarose gels stained with ethidium bromide (EtBr).

Nuclear ribosomal ITS, tef1, and β-tubulin sequencing

Amplifications of 50 μl PCR reaction mixture contained 25 μl 2 × PCR Master Mix (Fermentas, #K0171, Burlington, Canada), 40–40 pmol each primer, 20–40 ng DNA and nuclease free water were run out.

Primers used to amplify *ca* 520 bp of the ITS region containing the ITS regions 1 and 2, moreover the 5.8S rDNA are based on published composite sequences, SR6R and LR1 (White *et al.* 1990) with the following amplification protocol: 3 min initial denaturing at 95 °C, followed by five cycles of 1 min at 95 °C, 1 min annealing at 50 °C, 1 min at 72 °C, and 25 cycles of 1 min at 90 °C, 1 min annealing at 50 °C, 1 min at 72 °C, and 15 min final extension at 72 °C.

The large intron (*ca* 300 bp) of the *tef1* gene was amplified by the EF1-728F and EF1-986R primer pair (Druzhinina & Kubicek 2005) according to the previously described protocol with a temperature of 56 °C rather than 50 °C.

Primers Bt2a and Bt2b (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997) were used to amplify a fragment (*ca* 300 bp) of the *β-tubulin* gene, and PCR conditions were carried out as described above with an annealing temperature of 58 °C. PCR was performed in a Primus (MWG Biotech, Martinsried, Germany) thermocycler. Amplification products were subjected to electrophoresis in a 0.7 % agarose gel containing EtBr and visualized by UV illumination. The PCR products were purified by using YM-100 Microcon Centrifugal Filter Devices (Millipore, Billerica, USA). Purified amplification products were sequenced by MWG Biotech in Germany.

Data analysis

The obtained DNA sequences were aligned first with ClustalX (Thompson *et al.* 1997) and manually checked for ambiguities and adjusted, when necessary, using Genedoc (Nicholas *et al.* 1997). Single gaps were treated either as missing data or as the fifth base and multistate characters as uncertain.

In phylogenetic analyses of *tef1*, *β-tubulin* and ITS fragments, sequences of other *Phoma* and *Ascochyta* from GenBank maintained by NCBI were also included.

For the Bayesian analysis, models of sequence evolution were evaluated for each dataset, and model parameter estimates were obtained with Modeltest v.3.7 (Posada & Grandall 1998) using Bayesian Information Criterion (BIC). For the nuclear ribosomal ITS dataset, the TrNef + I model with equal base frequencies, six substitutions rate parameters (A–C = 1, A–G = 4.8974, A–T = 1, C–G = 1, C–T = 0.9815, G–T = 1), equal rates