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Intraspecific relationship of *Plasmopara halstedii* isolates differing in pathogenicity and geographic origin based on ITS sequence data

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Abstract

Sequence parts of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA were analysed to screen for the intraspecific variability of a non-coding genomic region in 15 *Plasmopara halstedii* populations of different pathotype and geographic origin. Samples revealed uniformity in a ca. 790 Bp fragment comprising of the ITS-1, 5.8S and front parts of the ITS-2. In contrast, clear differences were found in a ca. 810 Bp fragment of the ITS-2 thus allowing differentiation between populations of pathotype 100, 310 and 330 and a group of populations representing pathotypes 700, 701, 703, 710 and 730. Samples of pathotypes 700 to730 originated from Slovakia, France, and Germany, but were uniform in both ITS sequence parts, thus indicating very recent origin of these highly aggressive physiological races. The potential use of ITS sequences for pathotype differentiation and phylogenetic studies in *P. halstedii* is discussed.

Introduction

Plasmopara halstedii, the causal agent of sunflower downy mildew, is a pathogen of worldwide economic importance in sunflower crop production (Sackston, 1981; Gulya et al., 1997). The number of identified physiological races increased rapidly within the past three decades (Viranyi, 2002) raising questions on the origin and intraspecific relationships of the phenotypes. Molecular genetic investigations of the large subunit (LSU) of the nuclear ribosomal DNA revealed uniformity between field isolates of various pathotypes, but confirmed clear phylogenetic distinctiveness of isolates collected from *Helianthus* when compared with samples pathogenic to other Asteraceae genera (Riethmüller et al., 2002; Spring et al., 2003; Voglmayr et al., 2004). Except for PCR-based fingerprint techniques (Roeckel-Drevet et al., 1997; Intelmann and Spring, 2002) no suitable tools are so far available for molecular genetic investigations of *P. halstedii* on the intraspecific level.

Only recently, primers for the amplification of the ITS in Oomycetes were successfully employed for phylogenetic studies in different taxa of the Peronosporales (Cooke et al., 2000, 2002; Leclerc et al., 2000; Petersen and Rosendahl 2000; Constantinescu and Fatehi, 2002; Voglmayr, 2003; Göker et al., 2004; Constantinescu et al., 2005). Amplification of the ITS in *P. halstedii* was achieved after employment of modified primers (Bachofer, 2004) and finally resulted in a ca. 2600 bp product which exceeds the length of ITS in most other

Peronosporales by a factor of three. Meanwhile, the complete sequence of 2587 bp has been clarified (Thines et al., 2005) and revealed the presence of four large repetitive elements within the ITS-2 part of the *P. halstedii* genome. The extraordinary length and structure of the ITS in *P. halstedii* impedes its use for phylogenetic studies under routine PCR conditions. We have selected a ca. 790 bp fragment from the 5′ side (including the whole ITS-1, 5.8S and adjacent parts of ITS-2) and ca. 810 bp fragment from the 3′ end of ITS-2 for a comparative investigation of *P. halstedii* isolates differing in pathogenicity and geographic origin. The results of this study are reported here and its phylogenetic and epidemiologic implications are discussed.

Materials and methods

Sampling and origin of pathogen isolates

Pathogen isolates used in this study were selected from the oomycete collection of our laboratory. This collection consists of sporangia and sporangiophores harvested from the plant surface of infected plants and stored at −70 °C. In the case of *P. halstedii*, prior to freezing, vital cultures had been established by using fresh sporangia from field isolates as inoculum for the infection of general susceptible sunflower cultivars (e.g. HA-821), as previously described (Rozynek and Spring, 2000). In order to determine its pathotype each culture had been carefully studied for its pathogenicity on selected sunflower differential lines according to Gulya (1995). When possible, single spore strains or single sporangium strains had been generated as described by Spring et al. (1998).

The following samples were used in this study (origin and pathotype (PT) given in parenthesis): Ph19-01 (Hungary; PT 100); Ph1-97a (Germany, PT 310, single sporangium strain GG030797-A20); Ph1-97b (Germany, PT 330, single zoospore strain GG161097-A25); Ph9-98 (Germany, PT 330, single zoospore strain LE220499-A1), Ph5-99 (Slovakia, PT 700), Ph1-00a (France, PT 701, single zoospore strain IN190101-B7), Ph1-00b (France, PT 703, single zoospore strain IN190101-B9), Ph3-99 (Germany, PT 710), Ph5-96a (Germany, PT 710, single sporangium strain GE261196-B12), Ph5-96b (Germany, PT 730, single sporangium strain GE261196-A6), Ph5-96c (Germany, PT 730, single sporangium strain GE261196-C9), Ph1-94 (Germany, PT 730, single zoospore strain HA141097-C13), Ph8-99a (France, PT 730, single zoospore strain BL110602-A17), Ph8-99b (France, PT 730, single zoospore strain BL110602-A18), field isolate from *H.* × *laetiflorus* (Germany, PT undefined, voucher # OS 403, 07/2001, Herbarium of the University of Hohenheim (HUH)).

In addition, field isolates of *P. viticola* (voucher # OS 491, 08/2002, HUH), *P. nivea* (voucher # OS 410, 07/2001, HUH) and *P. pusilla* (voucher # OS 463, 06/2002, HUH) were used.

Isolation of total DNA from zoosporangia

Total DNA was extracted from approximately 5 mg of zoosporangia. Zoosporangia were disruptured dry at room temperature using a Mixer Mill (Retsch, Germany) at 10 Hz for 3 min. Samples were suspended in 400 μl AP1 buffer of the DNEasy Mini Kit (Qiagen, Germany) and treated according to the manufacturer's instruction. 200 μl DNA solution per sample was obtained and stored at −18 °C until further use. DNA concentration in samples was estimated by subjecting 1 μl of sample solution to agarose gel electrophoresis and computer-aided densitometry (E.A.S.Y. Win32 software; Herolab, Germany), through comparison with known quantities of λ-phage DNA applied on the same gel.

DNA amplification

The ITS-region was amplified using the oomycete specific primer ITS-O 5′-CGG AAG GAT CAT TAC CAC-3′ (Bachofer, 2004) and the primer LR-0 5′-GCT TAA GTT CAG CGG GT-3′ (reverse complement to LR-0R, Moncalvo et al., 1995). PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) using the following conditions: initial denaturation at 94 °C for 180 s, denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s, elongation for 1 min at 72 °C. Cycles were repeated 35 times and succeeded by a final elongation step of 10 min at 72 °C. Amplification products were analysed on 1.5% agarose gels (19.5 × 10 cm; 125 V for 90 min; ethidiumbromide staining; documentation under UV 302 nm using the Easy Store system of INTAS, Germany). ITS amplicons

from agarose gel separations were extracted by means of the QIAquick PCR purification Kit (Qiagen, Germany). Cycle sequencing was carried out on an automated DNA sequencer (ABI 373, Perkin Elmer) using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and ITS-O or LR-0 primers, respectively. Genbank accession numbers of sequences under study: Ph19-01 (DQ270143); Ph1-97a (DQ270144); Ph1-97b (DQ270145); Ph9-98 (DQ270146), Ph5-99 (DQ-270147), Ph1-00a (DQ270150), Ph1-00b (DQ 270149), Ph5-96a (DQ270151), Ph5-96b (DQ27-0155), Ph5-96c (DQ270153), Ph1-94 (DQ270154), Ph8-99a (DQ270148), Ph8-99b (DQ270152), field isolate from *H.* × *laetiflorus* (DQ270156), *P. viticola* (DQ270157).

Data analysis

For sequence comparison, the alignment was initially produced by means of the ClustalX software (Thompson et al., 1997) and visually checked and refined manually. For phylogenetic inference, sequences were aligned with MAFFT (Katoh et al., 2002). Manual refinement was limited to positions with leading and trailing gaps and based on background knowledge of primer location. Phylogenetic analysis based on the second part of the ITS (see below) was performed according to the NJ method (Saitou and Nei, 1987) in the BioNJ modification of Gascuel (1997) using PAUP* software (Swofford, 2002). Pairwise distances were estimated under maximum likelihood (Swofford et al., 1996) after determining the appropriate model of site substitution using the Akaike information criterion (AICc) as implemented in Modeltest 3.6 (Posada and Crandall, 1998; Posada and Buckley, 2004). The support for the internal nodes of the tree was assessed with bootstrap analysis (Felsenstein, 1985) using 1000 replicates.

Results

ITS amplicon length comparison

Amplification of ITS using the ITS-O and LR-0 primers resulted in clear, bright products of different size on agarose gels (Figure 1). All samples of *P. halstedii* from *Helianthus* showed ITS amplicons of ca. 2600 bp in length, while *Plasmopara* samples from other host genera gave significantly smaller products ranging from ca. 1100 bp in *P. pusilla* to ca. 2300 bp in *P. viticola*.

Sequence analyses

Cycle sequencing using the ITS-O and LR-0 primers provided sequence information alignable for the *Plasmopara* samples within two large parts of the ITS (Figure 2). Part 1 consisted of a ca. 790 bp fragment from the 5′ side which including the whole ITS-1, 5.8S and adjacent parts of ITS-2. The second part represented a ca. 810 bp fragment from the 3′ end of ITS-2 that was used for phylogenetic analysis.

Comparison of the sequences revealed complete identity for the *P. halstedii* samples in the first part of 790 bp, while a degree of variation up to 30% was found in *P. viticola*. Differences were found in the ITS-2 fragment, however. The AICc estimated K81 with unequal base frequencies (see Swofford et al. (1996) for a survey of these models) as best substitution model for phylogenetic analysis. The resulting BioNJ tree basally separated *P. halstedii* in two well-supported (bootstrap value 100%) groups (Figure 3). Two strains of pathotype 310 and 330 formed a first clade. The second, larger cluster was basically formed by strains representing the pathotypes 700 to 730 and included the sample isolated from the perennial sunflower hybrid *H.* × *laetiflorus*. At the base of this cluster a strain of pathotype 330 and one of pathotype 100 were associated, but separated from the core group with moderate to strong bootstrap support (76 and 85%, respectively). The isolates of pathotype 700–730 originated from Slovakia, France, and Germany, but were identical in ITS sequences.

Discussion

Until 1980 only two physiological races of *P. halstedii* were known to endanger sunflower cultivation, the European race 1 and the North American race 2 (Zimmer, 1974). Almost contemporarily with the development of highly resistant host cultivars new pathogen isolates of increased virulence were identified and rapidly replaced races of lower pathogenicity all over the world (for reviews see Gulya et al., 1997; Viranyi,

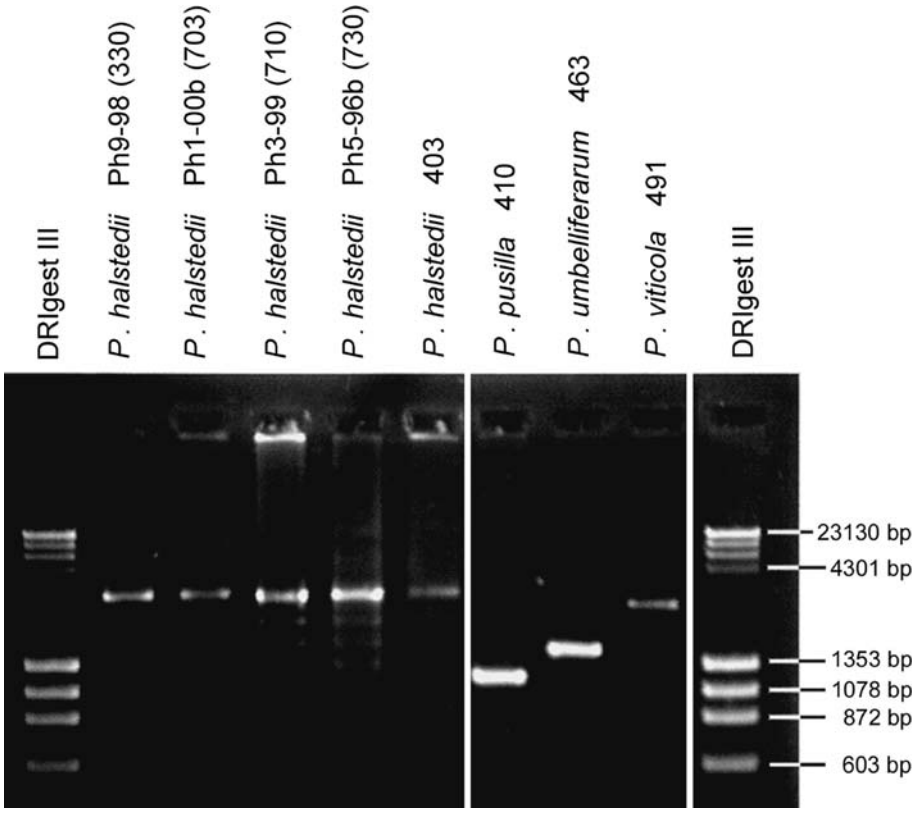

Figure 1. ITS amplicons of *Plasmopara halstedii* samples (representing different pathotypes 730 (Ph 4-93), 710 (Ph 5-95) and 330 (Ph 1-97)), *P. pusilla*, *P. umbelliferarum* and *P. viticola*. Lane 1, 9: size marker, lane 2–8: PCR products generated with the primers ITS-O and LR-0. Agarose gel (1,5%) stained with ethidiumbromide, photographed under ultraviolet light (302 nm).

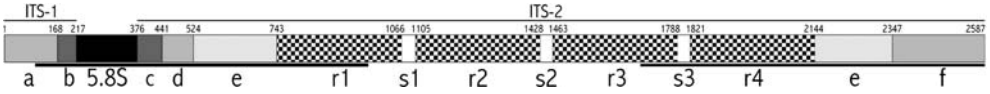

Figure 2. Structure of the ITS-region in *Plasmopara halstedii* (according to Thines et al. 2005): (a, b) ITS-1; 5.8S, region coding for the 5.8S ribosomal subunit; (c–f) ITS-2; (e) part of ITS-2, reported for neither *Phytophthora* (AF228084 and AF266794) nor *Peronospora* (AF465758), including the repetitions, r1 to r4, and the spacers in between the repetitive units, s1 to s3; 28S, part of the large ribosomal subunit (LSU). Figures above the drawing relate to base pairs, indicating the beginning of each of the parts described. Greyscales indicate the level of similarity to *Phytophthora* and *Peronospora*; black (5.8S, 28S): similarity higher than 90%, dark grey (b, c): similarity between 80 and 90%, grey (a, d, f): similarity less than 80%, other colours: regions reported for neither *Phytophthora* nor *Peronospora.* Black bars beneath the drawing indicate the parts of ITS used for phylogenetic analysis.

2002). Theories on the origin and spreading of the different genotypes remained speculative due to the lack of molecular genetic tools suitable for clarification.

The new ITS-O primer (Bachofer, 2004) provided access to the ITS of *P. halstedii* and for the first time enabled population studies and phylogenetic comparison based on a not fully conserved genomic region. The total length of 2600 bp shown in the current study for the ITS clearly separates *P. halstedii* from all other species of the genus investigated so far. This coincides with the results recently reported for the taxon based on LSU sequences which further separated isolates on *Helianthus* host plants from those of other Asteraceae genera (Spring et al., 2003). The ITS in *P. halstedii* exceeds the length of ITS in *P. pusilla* and *P. umbelliferarum* by a factor of two and those

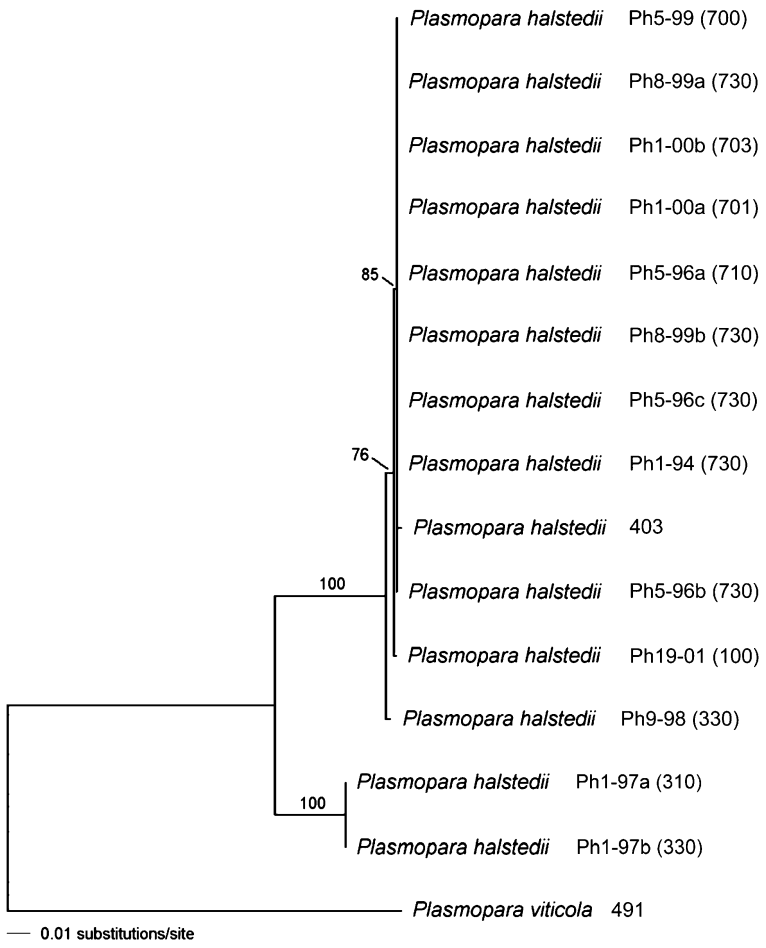

Figure 3. Phylogenetic tree according to Neighbour-joining analysis of an alignment of 810 bp of the ITS-region of the nuclear ribosomal DNA. Tree topology was rooted with the *Plasmopara viticola* (# OS 491) sequence. Figures on branches indicate bootstrap values (1000 replicates, values less than 50% not shown). Scale bar and branch lengths indicate the expected number of substitutions per site.

reported from most other Peronosporaceae by a factor of three (e.g. about 800 bp in most *Peronospora* and *Hyaloperonospora*, Voglmayr, 2003; Göker et al., 2004). Insertions in the ITS-2 are present in some species of *Hyaloperonospora*, resulting in a total sequence length of at most 1100 bp (Göker et al., 2004). Only *P. viticola* with ca. 2300 bp reached a similar size as in *P. halstedii* for this genomic region. A recent study (Thines et al., 2005) established the complete ITS sequence of *P. halstedii* and provided an explanation for the unusual size by the discovery of four large repetitive elements within the ITS-2 part. Whether parts of these elements occur in other *Plasmopara* species as well and if their number may account for the size differences, will have to be studied in the future.

Within the ITS of *Plasmopara*, conserved and variable regions were found. While all strains of *P. halstedii* were identical in ITS-1, 5.8S and first 420 bp of ITS-2, except for one single base substitution in *P. halstedii* on *Helianthus* × *laetiflorus*, variability in the 810 bp fragment at the 3′ end of ITS-2 separated the strains of lower virulence from pathotypes 700–730. Interestingly, the pathogen isolated from a naturalized population of the perennial sunflower hybrid *H.* × *laetiflorus* showed a nearly identical ITS-2 sequence. This does not support the separation of sunflower downy mildew

pathogens into specialized subgroups pathogenic to perennial or annual hosts as suggested by Novotelnova (1962). Within the group of pathotypes 700–730 no correlation to geographic origin of samples was found. Although the number of tested samples of pathotypes 100–330 is low (and augmentation for future studies will be difficult due to the current rarity of such phenotypes in sunflower fields; Rozynek and Spring, 2000), the results support the view of a very recent and common phylogeny of pathotypes 700–730. If all genotypes have coexisted over a long period, a higher degree of variation should be expected. To clear up the phylogeny of the whole *P. halstedii* complex, sequence analyses of pathogens isolated from different Asteraceae genera (as reviewed by Leppik, 1966) will have to be carried out. This will be particularly of interest for downy mildew isolates from *Eupatorium purpureum*, the assumed type host of *P. halstedii* and might help to clarify the nomenclatural dispute with respect to *P. helianthi* (for details see Gulya et al., 1997).

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