Delineation of species within the *Trichoderma viride/atroviride/koningii* complex by UP-PCR cross-blot hybridization

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Abstract

Sequences from the ribosomal DNA ITS regions have shown that there is limited variation among the species in the *Trichoderma viride/atroviride/koningii* species complex also known as *Hypocrea rufa* complex, and that infraspecies variation sometimes is larger than interspecies variation. Strains belonging to *T. viride*, *T. atroviride*, *T. koningii*, *T. asperellum*, and respective teleomorphs were analyzed using cross-blot hybridization of PCR products generated by the universally primed PCR (UP-PCR) technique. The hybridization results showed that the morphologically defined species could be delineated molecularly, suggesting that the species are more separated than indicated by ITS sequence phylogeny. However, cross-hybridization signals at infra- and interspecies level within this species complex was overlapping, again raising the question of how many species there are. Due to the heterogeneity of *T. viride* revealed, a further revision of this species is needed. In addition, this study shows that a macroarray (DNA chip) containing membrane-bound UP-PCR products for reference strains can be developed for routine identification of *Trichoderma* strains.

Keywords: *Trichoderma*; UP-PCR; PCR fingerprinting; Species complex

1. Introduction

Since Rifai’s [1] revision of *Trichoderma*, in which he operated with species aggregates, considerable effort has been devoted to define species in this genus [2–4]. The combination of molecular data (in particular rDNA sequences) and morphology has proven useful for reevaluation (neotypification) of known and widely accepted species [5–8], for recognition of new species [9] and for linking of anamorphs with their respectively teleomorphs [6,10]. The rDNA sequences have also been used to phylogenetically evaluate the sections of *Trichoderma* proposed by Bissett [2,11–14].

Species now accepted to belong to section *Trichoderma* (*T. viride*, *T. asperellum*, *T. atroviride*, *T. koningii*, *T. hamatum* neotype and related teleomorphs *Hypocrea rufa*, *H. atroviridis*, *H. koningii*, *H. vinosa*) have very limited ITS variation, and sometimes the interspecies variation can be less than the infraspecies variation of the morphologically defined species [6,8]. Thus the ITS region is not universally applicable as a species-level marker, and defining species solely on basis of ITS identity may be prone to error, unless a 100% sequence identity is found between a strain and the extype strain as proposed by [15].

Universally primed PCR (UP-PCR) technique has been applied to *Trichoderma* strains in several recent
studies [16–20] and has proven useful for strain and species identification and for testing their genetic relatedness. Visual inspection of aligned PCR banding profiles often may be used as a criterion for species identification [15,19,21]. In cases where the profiles are too different from relevant reference or type strains, UP-PCR products can be tested in hybridization experiments [22]. Cross-hybridization of UP-PCR products resembles traditional DNA/DNA hybridization by facilitating investigation of sequence complementarity. The hybridization signals of UP-PCR products indicate the genetic relatedness of the isolates to the reference isolate (labeled probe) [16,17,23,24]. DNA/DNA hybridization between even closely related species has been found to be low for fungi [25,26] and can thus be used as an aid in species designation and studies of genetic relationship.

The aim of this study was to use UP-PCR cross-hybridization as a tool for testing whether genotypic groups revealed by cross-hybridization correlate with morphospecies belonging to section Trichoderma of the genus Trichoderma.

2. Materials and methods

2.1. Fungal strains and DNA extraction

Table 1 lists sixteen Trichoderma strains, which were selected on the basis of previous characterization. References are given in Table 1. The strains were maintained on 2% cornmeal agar in Petri dishes (CMA, Sigma, Detsenhofen, Germany). For DNA extraction, the strains were grown in liquid medium [27] for 48 h at room temperature and 120 rpm. DNA extraction was performed as described in [28] and the concentration was adjusted to 10 ng μl⁻¹.

2.2. UP-PCR conditions

UP-PCR was performed essentially as described in Lübeck et al. [18]. Three different UP primers were used: L45 (5′-GTTAACACAGCGCGCGT-3′) [16], L15/AS19 (5′-GAGGGTTGCGCGCTAG-3′) [18] and AA2M2 (5′-CTGCGACCCAGAGCGG-3′) [29]. Two microlitre UP-PCR products (1/10th part of the total reaction) were electrophoresed in 1.7% 7 cm long agarose gels at 300 V with TBE buffer for 40 min. The gels were stained with ethidium bromide, and scanned using the Image-Master VDS (Pharmacia Biotech, Uppsala, Sweden).

2.3. Cross-dot blot hybridization

Cross-dot blot hybridization of UP-PCR products for selected strains was performed according to Bulat et al. [16] with slight modifications. All UP-PCR products generated with one primer (L15/AS19 or AA2M2) for the isolates was dotted on a filter in a systematic scheme and all UP-PCR products from each isolate using the same primer (L15/AS19 or AA2M2) have been labeled and used as probe in separate experiments. Labeling was conducted as follows: A small aliquot (about 0.5 μl of 20 μl amplification) of total UP-PCR products generated from one strain with the respective UP primer was re-amplified in the same conditions as

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no.</th>
<th>UP-PCR HG⁶</th>
<th>Taxon label in CA⁷</th>
<th>Geographic origin</th>
<th>References⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. asperellum</td>
<td>CBS 361.97=TR48</td>
<td>I</td>
<td>Na</td>
<td>USA/lab</td>
<td>[7,8]</td>
</tr>
<tr>
<td></td>
<td>GJS 90–14</td>
<td></td>
<td>Na</td>
<td>Vietnam</td>
<td>[7,8]</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Nb</td>
<td>USA/Maryland</td>
<td>[6–10]</td>
</tr>
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<td>GJS 72–16</td>
<td>II</td>
<td>At</td>
<td>Germany</td>
<td>[7]</td>
</tr>
<tr>
<td>H. atroviridis²</td>
<td>CTR 81–50=Hy5</td>
<td></td>
<td>At</td>
<td>USA/Indiana</td>
<td>[7,10]</td>
</tr>
<tr>
<td>T. viride</td>
<td>CBS 994.97=TR8</td>
<td>III</td>
<td>Vb</td>
<td>USA/Wisconsin</td>
<td>[6,7,9]</td>
</tr>
<tr>
<td></td>
<td>GJS 91–62</td>
<td></td>
<td>Vb</td>
<td>USA/Virginia</td>
<td>[7]</td>
</tr>
<tr>
<td>H. vinosa</td>
<td>GJS 96–163</td>
<td>IV</td>
<td>Ve</td>
<td>Taiwan</td>
<td>[7]</td>
</tr>
<tr>
<td>T. hamatum</td>
<td>DAOAM167057</td>
<td>V</td>
<td>Ha</td>
<td>Canada</td>
<td>[6,7,12]</td>
</tr>
<tr>
<td>T. koningii</td>
<td>CBS 457.96</td>
<td>VI</td>
<td>Ko</td>
<td>Netherlands</td>
<td>[6,7,10]</td>
</tr>
<tr>
<td>H. cf. rufa</td>
<td>GJS 97–243</td>
<td>VII</td>
<td>Vd</td>
<td>USA/Georgia</td>
<td>[7]</td>
</tr>
<tr>
<td>H. rufa</td>
<td>GJS 94–118</td>
<td></td>
<td>Vd</td>
<td>France</td>
<td>[7]</td>
</tr>
<tr>
<td>T. viride</td>
<td>ATCC32630=TR26</td>
<td>VIII</td>
<td>Vd</td>
<td>Sweden</td>
<td>[7,8]</td>
</tr>
<tr>
<td>T. viride</td>
<td>GJS 90–20</td>
<td></td>
<td>Ve</td>
<td>USA/Wisconsin</td>
<td>[7,8]</td>
</tr>
</tbody>
</table>

⁶ UP-PCR Hybridization Group revealed in this study.
⁷ Taxon label in CA revealed by Lieckfeldt et al. [7].
⁸ References where the strains have been described previously.
⁹ Strain kindly provided by Dr. H. Nirenberg.
¹⁰ Strain named as H. rufa in [7] but later as H. atroviridis in [10].
described in Lübeck et al. [19] except dCTP was replaced with (α-32P) dCTP (5 μCi) (Amersham Pharmacia, Uppsala, Sweden). Furthermore PCR was run for three cycles with the primer extension step for 10 min instead of 3 min. The reaction was terminated at 95 °C for 3 min, and subsequently the labeled products were added to the hybridization solution. Hybridization signals were revealed by exposing the filters to autoradiographic films for (a) approximately 30 min and (b) overnight, and then scored for intensity of signals.

3. Results

3.1. UP-PCR analysis of Trichoderma strains

Banding profiles generated with each of the UP primers were analyzed separately and aligned so that similar strains were placed together (Fig. 1). Only few of the 16 strains had similar banding profiles and a rather large variation was detected. The resolution of the fragment patterns obtained with three UP primers was comparably similar among the strains investigated, so that we
could easily reveal differences and similarities of the strains (Fig. 1).

3.2. Cross-dot blot hybridization of Trichoderma strains

Cross-dot blot hybridization experiments were carried out using UP-PCR products generated with UP primer L15/AS19 and AA2M2, respectively. The radioactive signals were scored in four categories of which (1) strong signal means same signal as the probe (labeled UP-PCR products from each isolate) to itself (indicated by S in Table 2), (2) significant signal but with less intensity than (1) (S* in Table 2), (3) weak signal (W in Table 2), and (4) weak or no signal after overnight exposure (− in Table 2). The hybridization results generated with L15/AS19 are shown in Table 2. Based on strong radioactive hybridization signals the strains were distributed into eight hybridization groups. Both primers revealed the same groups. Some strains from different groups cross-hybridized weakly with strains from other groups indicating that they had some sequence similarity. Only minor differences of generation of weak signals of the two primers were observed.

The correlation of the eight hybridization groups with the grouping of the strains based on correspondence analysis (CA) of a large number of molecular and morphological characters obtained by Lieckfeldt et al. [7] are shown in Table 1.

4. Discussion

By UP-PCR cross-hybridization 16 Trichoderma strains were distributed into eight hybridization groups which correspond perfectly with previous characterization of the strains using CA [7]. CA was carried out on a mixed-type data set containing information from morphological, physiological and molecular studies, 55 characters in total. The UP-PCR cross-hybridization experiments were carried out in a double blind experiment. The strains were selected and numbered with a code. Then the DNA from each strain was extracted and sent for analysis to the Danish lab. Thus their identity was unknown until the analysis was finished.

The radioactive signals from the cross-hybridization experiments can be related to DNA/DNA hybridization values using the scale in Lu¨beck and Poulsen [23]. Roughly strong hybridization signals (S) correspond to a DNA hybridization value of more than 75%, significant signals (S*) indicate a DNA hybridization value of approximately 60–75%, weak signals (W) indicate a DNA hybridization value of approximately 40–60%, while no signal is obtained with a DNA hybridization value less than 40%. Accordingly, only strong and significant hybridization signals (>60% hybridization values) will be found between strains of the same genetic entity.

According to the hybridization data, revealed in this study, the Trichoderma strains of section Trichoderma are grouped into the different morphologically defined species: T. asperellum, T. hamatum, T. koningii, T. atroviride, and several groups of T. viride. The weak signals corresponding to DNA hybridization values between 40% and 60% between some strains of the eight hybridization groups can be used as an estimate of how closely related the species are. The recently discovered species T. asperellum seems thus to be well separated as first found by Lieckfeldt et al. [7] and Samuels et al. [9].

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**Table 2**

UP-PCR cross-hybridization of Trichoderma strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no.</th>
<th>I*</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
</tr>
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<tbody>
<tr>
<td><strong>T. asperellum</strong></td>
<td>CBS 361.97</td>
<td>S*</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>GJS 90–14</td>
<td>S*</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>CBS 433–97</td>
<td>S</td>
<td>−</td>
<td>−</td>
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<td></td>
<td>GJS 94–81</td>
<td>S</td>
<td>−</td>
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<tr>
<td></td>
<td>CBS 362.97</td>
<td>S</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><strong>T. atroviride</strong></td>
<td>BBA68768</td>
<td>−</td>
<td>S</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td><strong>H. atroclavis</strong></td>
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<td>−</td>
<td>S</td>
<td>W</td>
<td>−</td>
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<tr>
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<td>−</td>
<td>W</td>
<td>S</td>
<td>W</td>
<td>−</td>
<td>−</td>
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<td>W</td>
<td>S</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><strong>H. vinosa</strong></td>
<td>GJS 96–163</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><strong>T. hamatum</strong></td>
<td>DAOM 167057</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>S</td>
<td>−</td>
</tr>
<tr>
<td><strong>T. koningii</strong></td>
<td>CBS 457.96</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>S</td>
<td>−</td>
</tr>
<tr>
<td><strong>H. cf. rufa</strong></td>
<td>GJS 97–243</td>
<td>−</td>
<td>W</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td><strong>H. rufa</strong></td>
<td>GJS 94–118</td>
<td>−</td>
<td>W</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>W</td>
<td>S</td>
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<tr>
<td><strong>T. viride</strong></td>
<td>ATCC32630</td>
<td>−</td>
<td>W</td>
<td>W</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>W</td>
<td>S</td>
</tr>
<tr>
<td><strong>T. viride</strong></td>
<td>GJS 90–20</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>S</td>
</tr>
</tbody>
</table>

S strong hybridization signal.
S* strong hybridization signal (but with slightly lower intensity than S).
W weak hybridization signal.
− Complete absence of signal or very weak signal after overnight exposure.

*UP-PCR hybridization groups revealed by the L15/AS19 primer.*
addition, data of the extype strain of *T. hamatum* indicate that this species is well separated from others within section *Trichoderma*. *T. koningii*, *T. atroviride* and the four *T. viride* groups seem to be less well separated from each other as indicated by weak hybridization between some of the strains. However, using the scale from Lübeck and Poulsen [23] in which weak hybridization signals correspond to a DNA hybridization value of less than 60%, the existence of the different morphospecies seems justified. There is evidence from other fungal species that infraspecific DNA hybridization values are most often higher than 70% and values lower than 60% are in the order of interspecies relationship [26].

*T. viride* Pers. is the type species of the genus and characterized by having warted conidia. Rifai [1] proposed the *T. viride* aggregate comprising of strains with warted conidia despite a considerable variation was recognized among the strains. The distribution of *T. viride* strains into four groups also indicates that more species could perhaps be defined as is suggested from morphological observations and preliminary EF-1α sequence data by Dodd et al. [10]. The heterogeneity of “true” *T. viride* revealed here and by Lieckfeldt et al. [7] suggests that a further revision of this species is needed.

Although it is widely accepted that a multifactorial approach is needed for the analysis of phylogenetic relationship of strains in *Trichoderma* [7,11], we in this study have shown that UP-PCR cross-hybridization has a great capacity to reliable classify and identify strains. For routine identification a macroarray (DNA chip) containing membrane bound UP-PCR products for all reference strains as proposed by Bulat et al. [16] could be developed.

References


