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PCR-based identification of *Erysiphe pulchra* and *Phyllactinia guttata* from *Cornus florida* using ITS-specific primers

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Summary

The internal transcribed spacer (ITS) regions of rDNA and the intervening 5.8S rRNA gene for the powdery mildew fungi *Erysiphe* (sect. *Microsphaera*) *pulchra* and *Phyllactinia guttata* were amplified using standard polymerase chain reaction (PCR) protocols and the universal primer pairs, ITS_1 and ITS_4. PCR products for ITS were analysed by electrophoresis in a 1.5% agarose gel and sequenced. The size of the amplified ITS products (approximately 650 bp) were not sufficiently different to allow reliable differentiation of *E. pulchra* and *P. guttata*; however, their sequences were distinct. Specific primers for *E. pulchra* and *P. guttata* were developed and evaluated for use as diagnostic tools. The diagnostic band size from *E. pulchra*-specific primer pair was 568 bp while the *P. guttata* band was 597 bp; the two primer pairs were highly specific to *E. pulchra* and *P. guttata*. Comparison of ITS sequences with information in the GenBank showed a very close similarity between sequences of *E. pulchra* isolates from *Cornus florida* in the USA and isolates collected on *Cornus kousa* in Japan. BLAST analysis of the sequence of the 650-bp band from *P. guttata* revealed a close alignment with sequences of *P. moricola* (92%), *P. kakicola* (94%), and *P. fraxini* (92%). The sequence of *P. guttata* in *C. florida* also had a 98% identity with *P. guttata* in *Calycanthus occidentalis* and 94% identity with *P. guttata* in *Corylus cornuta*.

1 Introduction

Powdery mildew is one of the most problematic diseases encountered during dogwood production (McRitchie 1994; Ranney et al. 1994; Hagan et al. 1995; Hagan and Mullen 1995; Hartman 1998; Mmbaga 1998; Daughtrey and Hagan 2001). The sudden emergence of the disease in the southeastern USA and the swift spread of the disease further northward is a cause of growing concern because of economic impacts on dogwood as a landscape plant and ecological impacts on dogwood as a food source for wildlife. Although powdery mildew was previously believed to cause only superficial damage (Hagan et al. 1997) with no lasting growth effects, recent studies demonstrated that this powdery mildew significantly reduces plant growth (Mmbaga 1998). A study by Hartman (1998) showed that infected flowering dogwoods showed reduced flower bud set and diminished blooms during the season following heavy infection. Only two fungi, *Phyllactinia guttata* (Wallr.) Lev. [syn. *Phyllactinia corylea* (Pers.) P. Karst.] and *Erysiphe* (sect. *Microsphaera*) *pulchra* (Cook & Peck, Braun & Takamatsu) have been associated with this disease (Leigh et al. 1998; Braun and Takamatsu 2000). Both pathogens produce similar symptoms, and signs of the disease during the growing season are produced by the anamorph (asexual) stage (*Oidium* sp. for *E. pulchra* and *Ovulariopsis* sp. for *P. guttata*). Because the anamorph stages are similar for these pathogens, they are not easily distinguished during the time of plant infection and associated damage (Barnet and Hunter 1998). However, *P. guttata* and *E. pulchra* can be readily identified at the teliomorph stage because each produces very distinct ascocarps. Thus, the ascocarp is the

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primary diagnostic feature used for identification of these pathogens (Leigh et al. 1998). Unfortunately, ascocarps are formed late in the growing season (September–November), and are not found during the growing season when disease symptoms are most prominent (Williamson and Blake 1999; Mmbaga 2002).

The distribution of these two pathogens in dogwood growing areas and their associated virulence are not well known. While *E. pulchra* and *P. guttata* have been observed in North Carolina and Kentucky (Hartman 1998), the occurrence of *P. guttata* is relatively rare in Tennessee where *E. pulchra* is the predominant powdery mildew pathogen (Mmbaga 2000, 2002).

To understand the relative roles of each fungus in disease severity or determine their prevalence in production areas, reliable techniques are needed to correctly identify these fungi at the conidial stage. The objectives of this study were to apply DNA molecular diagnostic techniques based on the internal transcribed spacer (ITS) regions of rDNA to: (1) distinguish *P. guttata* and *E. pulchra*, (2) compare DNA sequences of *E. pulchra* and *P. guttata* with other data in the GenBank, and (3) develop polymerase chain reaction (PCR)-based specific primers for diagnosis of *P. guttata* and *E. pulchra* during the anamorph stage.

2 Materials and methods

2.1 Powdery mildew sample collection and single conidiospore isolation

Dogwood material infected with powdery mildew consisted of (a) samples from Tennessee and Kentucky containing conidiospores, (b) ascocarps of *E. pulchra* from Tennessee and Kentucky, and (c) ascocarps of *P. guttata* from Kentucky. Although, ascocarps of *P. guttata* and *E. pulchra* are very distinct (Fig. 1), conidiospores are similar morphologically. For this reason, single conidiospores were isolated from the field and multiplied on detached leaves *in vitro* before DNA analysis. Ten field isolates were collected from each of five counties (Cannon, Coffee, Franklin, Rutherford, and Warren) in Tennessee and one collection of mixed field isolates was collected from Kentucky by Dr J. R. Hartman (Department of Plant Pathology Extension Service, University of Kentucky, Lexington, KY, USA).

Single conidial chains were selected for single-spore isolations under a dissecting microscope. Single conidiospore lines were established for each field isolate collected, for a total of 50 single-spore isolates from Tennessee and 10 single-spore isolates from Kentucky. Each isolate was cultured on the surface of dogwood leaves that were surface sterilized with 10% Clorox™ (sodium hypochlorite, Clorox company, Oakland, CA, USA) before placement in Petri dishes containing 1% water agar (WA). Cultures were maintained under fluorescent lighting. Leaves used for cultures were collected from disease-free dogwoods (cv. 'Cherokee Princess') that were maintained in a greenhouse. After 2–3 weeks, samples of mycelia were harvested from each culture using disposable micropipette tips, and placed in 0.5-ml microcentrifuge tubes. Samples were stored at −20°C until DNA extraction.

Leaf samples were collected from powdery mildew-infected trees during December and observed for the presence of *P. guttata* and *E. pulchra* ascocarps. On approximately 100 leaves collected in Tennessee, only *E. pulchra* was observed. However, leaf samples collected in Kentucky contained ascocarps of both fungi. After collection, all leaf samples were stored at 4°C until ascocarps were harvested.

2.2 Preparation, amplification, and analysis of DNA from fungal mycelia and ascocarps

Cell lysis and DNA preparation from mycelial samples were performed using Lyse-N-Go™ reagents (Pierce, Rockford, IL, USA) following the manufacturer's protocol. When

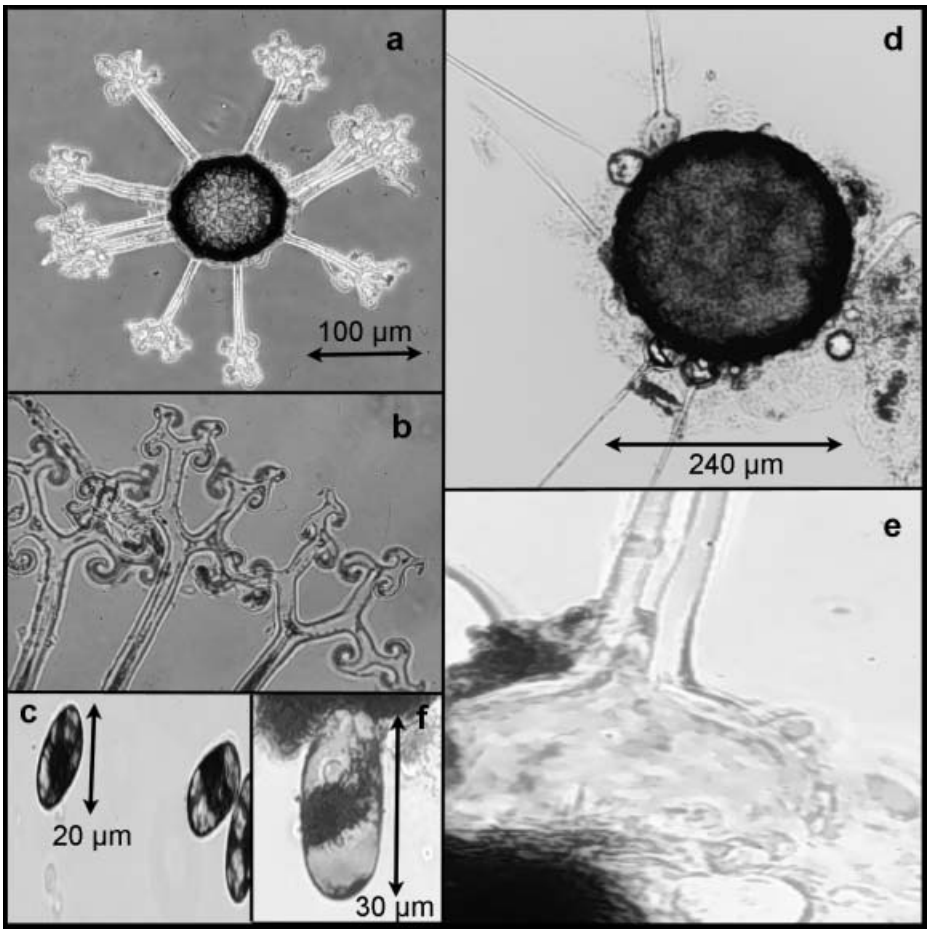

Fig. 1. Morphology of *Erysiphe pulchra* and *Phyllactinia guttata*: (a) Ascocarp of *E.* (sect. *Microsphaera*) *pulchra*; (b) dichotomously branched appendages of *E. pulchra* ascocarps; (c) ascospores of *E. pulchra* (approximately 20 μm in size); (d) larger ascocarp of *P. guttata*; (e) bulbous appendages of *P. guttata* ascocarps; (f) and *P. guttata* ascospore (approximately 30 μm in size)

DNA samples were used directly for PCR reactions, they were held at −80°C until the PCR reagents were added and PCR cycles initiated. DNA samples not used immediately were stored at −20°C. The Lyse-N-Go^{TM} reagent was also used to prepare DNA from samples containing 25–100 ascocarps; however, ascocarps were manually crushed and homogenized prior to each Lyse-N-Go^{TM} procedure.

The universal primer pairs, ITS_1 and ITS_4, were used to amplify the internal transcribed spacer (ITS) region, including the intervening 5.8S rRNA gene (White et al. 1990). Each 50-μl reaction mixture contained the DNA template prepared by Lyse-N-Go^{TM} reagent (or no DNA template for a negative control), 1 unit *Taq* polymerase (Applied Biosystems, Foster City, CA, USA), 4 mM $MgCl_2$, 200 μM dNTPs, and 0.5 μM of each primer (ITS_1 and ITS_4). A Techne Progene^{TM} (Techne Incorporated, Princeton, NJ, USA) thermal cycler was used. The cycling parameters were as follows: 94°C initial denaturation (5 min),

followed by 48 cycles of denaturation at 94°C (35 s), annealing at 46°C (1 min 10 s), extension at 72°C (1 min 40 s), and one cycle of final extension at 72°C (7 min) (Pimentel et al. 1998).

Polymerase chain reaction products were analysed by electrophoresis in a 1.5% agarose gel in 0.5X TBE [Tris–borate–ethylenediaminetetraacetic acid (EDTA)] buffer. Gels were stained with ethidium bromide (0.5 μg/ml) and DNA was visualized using UV light. PCR products for ITS region (approximately 650 bp) were purified using ExoSAP-IT^{TM} (USB Corporation, Cleveland, OH, USA) and sequenced using an ABI 377XL PRISM automatic sequencer (Applied Biosystems, Foster City, CA, USA). ITS sequences of *E. pulchra* and *P. guttata* were edited with BioEdit software (Hall 1999) and then compared with GenBank information using a BLAST search (Altschul et al. 1997).

Sequences of *E. pulchra* and *P. guttata* were used to develop specific primers for differentiating *E. pulchra* from *P. guttata*. The two sets of specific primers (forward and reverse: EP_1, and EP_2 for *E. pulchra*, and PG_1 and PG_2 for *P. guttata*) were prepared by the Iowa State University Biotechnology Services (Ames, IA, USA).

2.3 Evaluation of the specific primers designed for *E. pulchra* and *P. guttata*

Specific primers for *E. pulchra* (EP_1 and EP_2) were evaluated for rDNA amplification of *E. pulchra* and *P. guttata* DNA from dogwood. Mycelia and ascocarps were evaluated for *E. pulchra*, whereas only ascocarps for *P. guttata* were evaluated. All mycelial samples were collected from leaves of field-grown trees. To test whether the primers will cross-amplify DNA from the two fungi, specific primers designed for *P guttata* (PG_1 and PG_2) were evaluated for amplification of *P. guttata* and *E. pulchra* DNA. DNA extraction and amplification protocols described above were used to evaluate specific primers. Because ITS_1 and ITS_4 successfully amplified both *P. guttata* and *E. pulchra* (650 bp), they were included as the positive control in the evaluation of *P. guttata*- and *E. pulchra*-specific primers. A negative control consisted of tissue from the leaf surface of dogwood, where powdery mildew signs were not present.

3 Results

Cleistothecia of *P. guttata* and *E. pulchra* are very distinct (Fig. 1), and samples used in this study were from dry leaves collected during the previous year. Because powdery mildew spores are highly air-borne, single-spore isolation using detached leaves avoided contamination. Lyse-N-Go^{TM} DNA preparation reagents and associated protocol produced suitable template DNA from very small amounts of mycelia or ascocarps (approximately 25–100) of *E. pulchra* or *P. guttata*. The use of very small amounts of mycelia directly from leaf tissue is of particular significance in studies of obligate parasites, because obligate parasites cannot be grown in artificial media to produce sufficient fungal material for DNA extraction.

Polymerase chain reaction products from the rDNA ITS region of *E. pulchra* and *P. guttata* were about 650 bp (Fig. 2). However, the DNA sequence of *E. pulchra* was distinctly different from that of *P. guttata*, with sequence differing by more than 50% (GenBank accession no.: AY224136 for *E. pulchra* and AY224137 for *P. guttata*). All single-spore isolates exhibited a similar-sized PCR product as that of *E. pulchra* ascocarps, and their ITS sequences were identical (data not shown). Thus, these mycelial samples were confirmed to be *E. pulchra*. The ITS region sequence did not show any sequence variation between samples.

Because the sequence of *P. guttata* was distinctly different from that of *E. pulchra*, specific PCR primers could be designed for *E. pulchra* and *P. guttata.* Specific primers

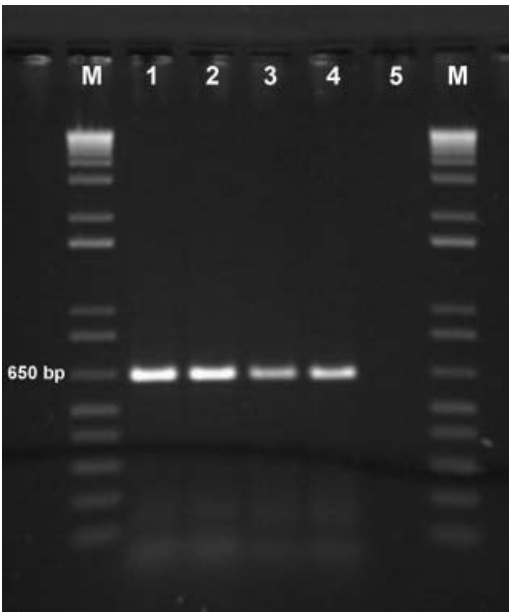

Fig. 2. PCR-amplified products using ITS_1 and ITS_4 primers on *Erysiphe* (Sect. *Microsphaera*) *pulchra* and *Phyllactinia guttata* from *Cornus florida*. Lane M: Molecular size standards (bp; 1 kb plus DNA ladder), lanes 1 and 2: *E. pulchra*, lanes 3 and 4: *P. guttata*, lane 5: negative control (no DNA)

designed for *E. pulchra* contained 18 forward bases 5′-GTGAACCTGCGGAAGATC-3′ (EP_1) and 20 complementary bases (reverse) 5′-CATGTGACTGGAACAAAAAG-3′ (EP_2). Specific primers for *P. guttata* had 18 forward bases 5′-CTGAGCGTGAA GACTCTC-3′ (PG_1), and 18 complementary (reverse) bases 5′-GGTATCCCTACCT GATTC-3′ (PG_2). These primers differentiated the two pathogens. The specific primers for *P. guttata* (PG_1 and PG_2) amplified only *P. guttata* DNA, and failed to amplify *E. pulchra* DNA. In addition, specific primers for *E. pulchra* (EP_1 and EP_2) amplified only *E. pulchra* DNA, and not *P. guttata* DNA. The diagnostic band size from *E. pulchra*-specific primer pair was 568 bp, while the *P. guttata* band was 597 bp. Thus, the two primer pairs (EP_1–EP_2 and PG_1–PG_2) were highly specific to *E. pulchra* and *P. guttata*, respectively (Fig. 3). Specific primers for *E. pulchra* and *P. guttata* were highly selective and consistently differentiated the two fungi associated with dogwood powdery mildew.

A comparison of ITS DNA sequences along with information from the GenBank for *E. pulchra* isolates collected on *C. florida* showed a 99% match with an *E. pulchra* var. *pulchra* isolate (GenBank accession no.: AB015935) on *C. kousa* in Japan (Takamatsu et al. 1999). BLAST analysis of the 650-bp band (DNA sequence) of *P. guttata* revealed a close alignment with *P. moricola* (92%), *P. kakicola* (94%), and *P. fraxini* (92%). The sequence of *P. guttata* in *C. florida* also had a 98% identity with *P. guttata* in *Calycanthus occidentalis* and 94% identity with *P. guttata* in *Corylus cornuta*. The primers were not tested against other powdery mildew fungi; however, other closely related powdery mildew species, which exhibit DNA sequence similarity to *E. pulchra* or *P. guttata*, cannot infect dogwood.

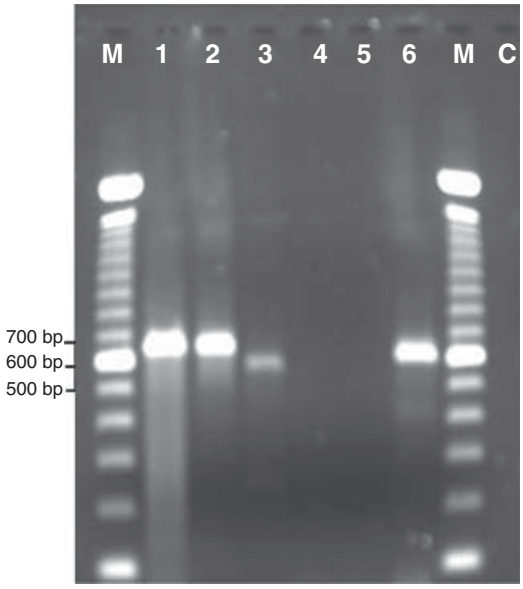

Fig. 3. PCR-amplified products using species-specific primers for *Erysiphe* (sect. *Microsphaera*) *pulchra* (EP_1 and EP_2) and *Phyllactinia guttata* (PG_1 and PG_2) for diagnosis of the powdery mildew pathogens in *Cornus florida.* Lane M: molecular size standard (bp; 100-bp DNA ladder), lane 1: *E. pulchra* using universal primers ITS_1 and ITS_4; lane 2: *P. guttata* using primers ITS_1 and ITS_4; lane 3: *E. pulchra* using species-specific primers EP_1 and EP_2; lane 4: *E. pulchra* using primers PG_1 and PG_2; lane 5: *P. guttata* using primers EP_1 and EP_2, lane 6: *P. guttata* using species-specific primers PG_1 and PG_2; and lane C: negative control using ITS_1 and ITS_4 on tissue from non-infected leaf surface

4 Discussion

Although, conidiospores from field isolates of *E. pulchra* and *P. guttata* were similar morphologically, their teliomorph stages were very distinct (Fig. 1). The isolation of *E. pulchra* in all single-spore cultures suggests that *E. pulchra* may be more widespread than *P. guttata* in the locations where collections were made. Because most single-spore isolates were collected in Tennessee, our results support other reports indicating that *E. pulchra* is the predominant powdery mildew pathogen on dogwood in Tennessee (Leigh et al. 1998; Mmbaga 1998). Dry leaves collected in Kentucky often contained both ascocarps of *E. pulchra* and *P. guttata*; however, some leaves only contained ascocarps of either *E. pulchra* or *P. guttata*. Leaves containing both fungi or *E. pulchra* alone, were covered with dry mycelia. However, leaves that contained only *P. guttata* did not have noticeable amounts of mycelia. These observations suggest that signs of powdery mildew in dogwood may be primarily from *E. pulchra* and that *P. guttata* may play only a minor role in disease damage. More studies are needed to confirm the relative damage caused by these pathogens.

The DNA sequence of the ITS region did not show sequence variation between isolates of *E. pulchra*; however, distinct sequence differences were observed between *E. pulchra* and *P. guttata*. Thus, the ITS region is likely of limited value for studying genetic variation within either species. Because ascocarps of *E. pulchra* are the main source of primary inoculum in Tennessee (Mmbaga 2000), genetic variation in *E. pulchra* is likely to occur. Identical ITS sequences from all *E. pulchra* isolates may be a reflection of a recent introduction of a pathogen (Pimentel et al. 1998), but

further studies are in progress to better understand the recent occurrence of the *E. pulchra* disease problem in dogwood.

The virtually identical sequences of the ITS region from Tennessee isolates of *E. pulchra* (GenBank accession no.: AY224136) and the Japanese *E. pulchra* isolate (GenBank accession no.: AB015935), suggest a possible explanation for the sudden emergence of powdery mildew in dogwood. The sudden appearance of the powdery mildew in the southeastern USA occurred at a time (1990s) when research efforts were focused on dogwood anthracnose, caused by *Discula destructiva*. At that time, efforts were underway to identify anthracnose-resistant sources from diverse geographic areas (RANNEY et al. 1994). Ascocarps of *E. pulchra* from Japan may have been introduced into Kentucky, Tennessee, or a neighbouring state on *C. kousa* imported as breeding material; if so, *E. pulchra* may have quickly spread on to susceptible native flowering dogwoods. Such implications warrant further studies.

The development of specific primers for *E. pulchra* and *P. guttata* can serve as an accurate diagnostic tool for distinguishing the two powdery mildew pathogens at the conidial stage. This tool will be useful in assessing the relative prevalence of each pathogen and their roles in disease severity. Such information will benefit disease management and the development of host resistance.

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Résumé

Identification par PCR de Erysiphe pulchra et Phyllactinia guttata sur Cornus florida par utilisation d'amorces spécifiques des ITS

Les régions ITS et le gène 5.8S de l'ADN ribosomal des champignons agents d'oïdium *Erysiphe* (Sect. *Microsphaera*) *pulchra* et *Phyllactinia guttata* ont été amplifiés en utilisant un protocole de PCR standard et les paires d'amorces universelles ITS_1 et ITS_4. Les produits PCR pour les ITS ont été analysés par électrophorèse sur gel d'agarose à 1.5% et séquencés. La taille des produits amplifiés pour les ITS (environ 650 pb) n'est pas suffisamment discriminante pour permettre une différenciation fiable de *E. pulchra* et *P. guttata*, mais les séquences sont distinctes. Des paires d'amorces spécifiques ont été développées pour *E. pulchra* et *P. guttata* et évaluées pour leur utilisation en diagnostic. La taille de la bande diagnostique obtenue avec la paire d'amorces spécifiques de *E. pulchra* est de 568 pb et de 597 pb pour *P. guttata* . Les deux paires d'amorces sont hautement spécifiques de *E. pulchra* et de *P. guttata* . La comparaison des séquences des ITS avec les informations contenues dans GenBank montre une très forte similarité entre les séquences des isolats de *E. pulchra* provenant de *Cornus florida* aux USA et les isolats récoltés sur *Cornus kousa* au Japon. L'analyse par BLAST de la séquence de la bande de 650 pb de *P. guttata* a montré un très proche alignement avec les séquences de *P. moricola* (92%), *P. kakicola* (94%), et *P. fraxini* (92%). La séquence de *P. guttata* sur *C. florida* présente aussi une identité à 98% avec *P. guttata* sur *Calycanthus occidentalis* et une identité à 94% avec *P. guttata* sur *Corylus cornuta*.

Zusammenfassung

PCR-basierte Identifizierung von Erysiphe pulchra und Phyllactinia guttata von Cornus florida mit Hilfe von ITS-spezifischen Primern

Die ITS-Region der rDNA inklusive des 5,8 rRNA-Gens der Echten Mehltaupilze *Erysiphe pulchra* und *Phyllactinia guttata* wurden mit PCR-Standardmethoden und den universalen Primern ITS_1 und ITS_4 amplifiziert. Die Produkte wurden auf Agarosegelen (1,5%) elektrophoretisch analysiert und sequenziert. *E. pulchra* und *P. guttata* konnten anhand der Grösse ihrer ITS-Banden (ca. 650 bp) nicht voneinander getrennt werden, sie hatten aber unterschiedliche Sequenzen. Es wurden spezifische Primer für die beiden Arten entwickelt und auf ihre diagnostische Eignung geprüft. Von dem spezifischen Primerpaar für *E. pulchra* amplifizierte Banden hatten eine Grösse von 568 bp, diejenigen

von *P. guttata* waren 597 bp gross. Beide Primerpaare waren in hohem Grade artspezifisch. Ein Vergleich der ITS-Sequenzen mit den in der Genbank vorhandenen Daten ergab eine sehr hohe Ähnlichkeit zwischen den *E . pulchra* – Isolaten von *Cornus florida* in den USA und Kollektionen von *C. kousa* aus Japan. Die 650 bp-Sequenz von *P. guttata* zeigte eine hohe Ähnlichkeit mit den entsprechenden Sequenzen von *P. moricola* (92%), *P. kakicola* (94%) und *P. fraxini* (92%). Die ITS-Sequenz von *P. guttata* von *C. florida* stimmte zu 98% mit der von *P. guttata* von *Calycanthus occidentalis* und zu 94% mit der von *P. guttata* auf *Corylus cornuta* überein.

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