Species-Specific Primers for *Eutypella parasitica*, the Causal Agent of Eutypella Canker of Maple

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ABSTRACT

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Eutypella parasitica was recently reported in Europe for the first time, and this study reports the molecular evaluation of the internal transcribed spacer (ITS)1/5.8S/ITS2 regions of 68 isolates of the fungus obtained in pure culture with polymerase chain reaction restriction fragment length polymorphism (RFLP). The RFLP patterns of all isolates proved identical and the restriction profiles served to differentiate *E. parasitica* from *Eutypa lata*, another pathogenic member of the family Diatrypaceae. Low intraspecific variability was detected in the sequenced ITS1/5.8S/ITS2 regions of eight *Eutypella parasitica* isolates originating from different hosts and geographical locations. Based on this ITS region, EpR/F primers specific to *E. parasitica* were constructed and tested with a wide range of fungal isolates. The EpR/F primer pair successfully amplified the expected fragment size of 341 bp from isolates of *E. parasitica* and also directly from infected maple wood shavings. The RFLP patterns and species-specific primers represent a step toward routine, large-scale, and rapid molecular diagnostics and identification of *E. parasitica*.

The family Diatrypaceae (Ascomycota) includes several genera that typically occur on a broad range of declining or dead woody angiosperms; most are considered saprobes, except *Eutypa lata* and *Eutypella parasitica*, which are pathogens (1,4). Molecular phylogenetic studies of Diatrypaceae (1) showed little correlation with the current morphology-based classical taxonomic scheme; however, there are no reports of phylogenetic placing of *E. parasitica* within the family.

E. parasitica causes the Eutypella canker of maple (*Acer* spp.), a destructive disease originally found in North America (5,19,39). In Europe, the disease was reported recently from several locations in Slovenia (17). Moreover, field maple (*Acer campestre*) was reported for the first time as a host of *E. parasitica* (30). In Europe, extensive areas of maple stands are predicted to be at considerable risk from *E. parasitica* infection (31). Although the potential economic loss due to the Eutypella canker is yet to be estimated for Europe, it is desirable to track the disease

and to initiate measures to avoid its spread, which could threaten *Acer* spp. growing in forests, urban environments, or nurseries (31).

The first visual sign of Eutypella canker is dieback of the bark and, due to the slow progression of the fungus, the site of initial infection often escapes notice. The necrosis slowly advances with growth of the tree and, 5 to 8 years after the dieback of the bark, numerous perithecial necks surrounded by poorly developed stromata are seen on the surface of the canker. A characteristic sign of the canker is mycelial fans in the infected bark (17,23,30). The fungus overgrows the wood underneath the wound, causing a brown discoloration and slow degradation of wood (19).

Eutypa lata has been reported on a broad range of tree species, but it is not yet clear whether all isolates are pathogenic (4,28). According to the Centraalbureau voor Schimmelcultures (CBS) Filamentous Fungi Database, *E. lata* var. *aceris* Rappaz can appear on *A. campestre* and *A. pseudo-platanus*. Carter et al. (4) confirmed the pathogenicity of one strain of *E. lata*, isolated from *A. campestre*, on apricot and grapevine. Like *Eutypella parasitica* on maple, *Eutypa lata* causes cankers on grapevines and apricot, and typically is observed as perithecia embedded in the stromata composed of fungal and host tissues, with numerous spindle-shaped, eight-spored asci with long stipes (28). The described symptoms and signs of *E. lata* are similar to those of *Eutypella parasitica*, and could be the reason for misidentification of the fungus, although

we have not found any report of *Eutypa lata* being pathogenic to the maple.

Eutypella parasitica was placed on the alert list of the European and Mediterranean Plant Protection Organization in 2005 (29) and a quick and reliable method for its detection and identification would be required to implement the quarantine measures. Conventional methods of identifying *E. parasitica* and other plant-pathogenic fungi include observations of visible symptoms on the host and isolation and culturing of the pathogen, followed by a macro- and microscopic examination of the mycelium and fruiting structures in the culture. Inoculation of the host with the isolated fungus and reisolation from the infected host usually confirms pathogenicity. These procedures are time consuming, and successful identification requires experience, knowledge, and skill (26). Identification based on the comparison of molecular markers is much faster, does not require fully developed morphological features, and can be used with any infected material by workers with limited experience in plant pathology. Methods such as restriction fragment length polymorphism (RFLP) of the polymerase chain reaction (PCR) products or a PCR with species-specific primers also can provide confirmation of the presence of the pathogen and are potential candidates for automation. A number of species-specific primers have been developed for identifying plant pathogens and the first online database of primers for plant pathogenic fungi has been released (8).

The objectives of this work were to (i) confirm the identification of *E. parasitica*, obtained by using morphology of the isolates in pure culture with that obtained by using PCR-RFLP; (ii) conduct a preliminary screening of the variability of internal transcribed spacer (ITS) region among isolates of *E. parasitica* using PCR-RFLP and to sequence this region; (iii) develop primers specific to *E. parasitica* based on the rDNA ITS region for rapid and large-scale identification of the pathogenic fungus from various sources, including infected plant material; and (iv) extract DNA directly from diseased wood for developing a method for direct detection of *E. parasitica*.

MATERIALS AND METHODS

Fungal isolates. We used 68 isolates of *E. parasitica* obtained from different loca-

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tions in Slovenia from which outbreak of the disease had been reported (Table 1), isolated either from infected wood or directly from perithecia embedded in the bark. We also included *E. parasitica* ex-type isolate CBS 210.39 as a reference strain. The isolates were grown at 24°C on malt extract agar (20.0 g of malt extract, 15.0 g of agar, and 1 liter of distilled water) and then stored at 4°C until needed. Thirty-three diverse fungal isolates (Table 2) were used for testing the specificity of the constructed species-specific primers. Additionally, fresh plant material comprising infected and uninfected wood and leaves was used for testing the sensitivity of the method with primers specific to *E. parasitica* or as a negative control.

DNA extraction. DNA was extracted from approximately 1 cm² of the mycelium scraped from the surface of the agar plate or, in the case of field samples, from either approximately 4-mm² pieces of wood shavings from infected wood tissue at the margins of the discoloration or young leaves. The plant material was handled aseptically throughout the process. DNA was extracted using the 2% (wt/vol) hexadecyltrimethylammonium bromide (CTAB) protocol (6,35). To the initial DNA from the plant material extracted in a buffer, 1% (wt/vol) polyvinylpyrrolidone (PVPP) was added (13) and the extracted DNA was resuspended in 50 µl of sterile distilled water. DNA concentrations in DNA samples extracted from pure *E. parasitica* cultures and infected wood tissue were measured spectrophotometrically (Hewlett Packard 8453) by using the molar extinction coefficient expressed in molar concentration of base pairs (calf thymus and

salmon testes DNA; $\epsilon_{259} = 12,800 \text{ M}^{-1} \text{ cm}^{-1}$; 34) and pure distilled water as a reference solution. In addition to the 2% CTAB protocol, DNA also was extracted from the wood shavings by using the method described by Lecomte et al. (24). Serial dilutions of the DNA samples from pure *E. parasitica* cultures and wood shavings were prepared. Extracted uninfected maple genomic DNA was spiked with serial dilutions of extracted *E. parasitica* genomic DNA to check the influence of possible inhibitors on PCR reaction. Original solutions and dilutions were used for PCR reactions.

PCR amplification, restriction, and sequencing. Amplifications were performed with universal fungal primers ITS1f (7) and ITS4 (40) in 10- or 40-µl reaction volumes, depending on the method for which it was to be used next. The 10-µl reaction mix comprised 6.35 µl of sterile double-deionized H₂O, 1 µl of 10× PCR buffer, 1 µl of 25 mM MgCl₂, 1 µl of 2 mM dNTPs, 0.2 µl each of 10 µM ITS1 and 10 µM ITS4 primers, 0.05 µl of Taq DNA polymerase (5 U/µl; Eppendorf), and 0.2 µl of DNA extract per reaction. The 40-µl reaction mix comprised the same ingredients in the same proportions. The cycling parameters followed the procedure described by Kraigher et al. (21). Initial DNA denaturation was at 94°C for 85 s; followed by 35 cycles of denaturation (95°C for 35 s), primer annealing (55°C for 55 s), and DNA extension at 72°C (for 45 s for the first 13 cycles, 120 s for the next 13 cycles, and for 180 s for the final 9 cycles); and final DNA extension at 72°C for 10 min. All PCRs, including negative and positive controls, were checked for

positive amplification on a 0.5× Tris-borate EDTA (TBE) (Sigma) 2% (wt/vol) agarose gel (LE; Promega) stained with ethidium bromide. Only those reactions that yielded single and strong bands on the gel were used for subsequent analysis.

For restriction analyses, 5-µl aliquots of the PCR products were digested in separate reactions with *Hinf*I, *Mbo*I, and *Taq*I endonucleases (18) according to the manufacturer's instructions (Promega). The gels were read and the data on fragment size from Polaroid or GelDoc photos were processed using Adobe Photoshop and analyzed in the Taxotron software system, which is designed especially for processing RFLP data (14), as described in Grebenc et al. (12), and subsequently compared with the PCR-RFLP database at the Slovenian Forestry Institute (SFI) (9,11,12). Fragments shorter than 100 bp were not included in the restriction analysis.

Amplified DNA from the isolates intended for sequencing (Table 1) was cleaned using Wizard SV Gel and PCR Clean-Up System (Promega) kit. Both strands of the amplicons were sequenced on ABI Prism 310 using BigDye Terminator v3.1 Matrix Standard Kit (Applied Biosystems). The sequences were aligned with the corresponding sequences of *Eutypella* spp. accessed from the GenBank database (Table 3) using DIALIGN2 (27).

The constructed primers specific to *E. parasitica* were compared against the GenBank "nr" database by searching for short and nearly exact matches using BLASTN. The primers were checked for specificity against the isolates of *E. parasitica* (Table 1) and other fungal or plant material (Table 2). In the specific PCR, cycling parameters were the same as those used in the amplification with primers ITS1f and ITS4, but with different annealing temperatures (55, 60, or 63°C).

RESULTS

The PCR-RFLP with *Mbo*I, *Hinf*I, and *Taq*I restriction enzymes (Figs. 1, 2, and 3) of the 650-bp long ITS region, amplified with the ITS1f and ITS4 primers, showed a uniform restriction pattern for all isolates of *E. parasitica* (Table 4). The restriction profile of *E. parasitica* obtained with *Hinf*I was clearly different from that of *E. prunastri* (CBS. 277.87), *Eutypa lata* (CBS. 208.87), and *E. lata* var. *aceris* (CBS. 290.87, CBS 217.87) obtained with the same restriction enzyme (Table 4; Fig. 2). Restriction enzymes *Mbo*I and *Taq*I revealed either no differences or only minor differences in RFLP patterns among isolates of *Eutypella parasitica*, *E. prunastri*, *Eutypa lata*, and *E. lata* var. *aceris* included in this analysis (Table 4; Figs. 1 and 3).

The sequences of the ITS1/5.8S/ITS2 region of selected isolates of *Eutypella parasitica* (Table 1) from different locations and host species were identical to *E. parasitica* sequences from GenBank (Ta-

Table 1. Isolates of *Eutypella parasitica* from the wood of infected trees or directly from perithecia

Designation ^a	Acer spp. host	Geographic origin
D1/1	<i>Acer pseudoplatanus</i>	Ljubljana, SI
D4/1, 2, 3, 4	<i>A. pseudoplatanus</i>	Ljubljana, SI
D5/1	<i>A. pseudoplatanus</i>	Ljubljana, SI
D6/1	<i>A. pseudoplatanus</i>	Ljubljana, SI
D7/1, 2, 3, 4	<i>A. pseudoplatanus</i>	Ljubljana, SI
D11/1, 2, 3	<i>A. pseudoplatanus</i>	Ljubljana, SI
D14/1, 2, 3, 4	<i>A. pseudoplatanus</i>	Ljubljana, SI
D15/1	<i>A. pseudoplatanus</i>	Ljubljana, SI
D24/1, 2, 3, 4, 5, 6	<i>A. pseudoplatanus</i>	Ljubljana, SI
D26/1*, 2, 3, 4, 5	<i>A. pseudoplatanus</i>	Ljubljana, SI
G7/2	<i>A. pseudoplatanus</i>	Ljubljana, SI
IZ1/1, 2, 3	<i>A. pseudoplatanus</i>	Sevnica, SI
IZ2/1, 2, 3	<i>A. pseudoplatanus</i>	Sevnica, SI
K1/1*, 2, 3	<i>A. pseudoplatanus</i>	Kozje, SI
M1/1*, 2, 3	<i>A. campestre</i>	Ljubljana, SI
M2/1, 2, 3, 4, 5, 6	<i>A. campestre</i>	Ljubljana, SI
M3/1, 2, 3, 4, 5	<i>A. campestre</i>	Ljubljana, SI
OJ1/1*, 2	<i>A. platanoides</i>	Ljubljana, SI
RS1/1*, 2, 3	<i>A. campestre</i>	Rogaška Slatina, SI
SE1/1	<i>A. pseudoplatanus</i>	Sevnica, SI
SE2/1*, 2, 3	<i>A. pseudoplatanus</i>	Sevnica, SI
TO1/1*, 2, 3	<i>A. pseudoplatanus</i>	Topol, SI
ZN1/1*, 2	<i>A. campestre</i>	Znojile pri Krki, SI

^a Each number after the slash represents a separately cultured isolate and the entire series after the slash indicates isolates obtained from the same canker. All isolates were analyzed with polymerase chain reaction restriction fragment length polymorphism; asterisks indicate the isolates sequenced and used for constructing species-specific primers.

ble 3). An exception was isolate TO1/1, which differed from the rest in its cytosine-to-thymine transition at site 14 in the ITS2 sequence region, confirmed by both sequenced amplicon strands. The point mutation did not affect the RFLP pattern (Table 4). The sequences were deposited in the EMBL Nucleotide Sequence Database under accession numbers AM295770–AM295777.

Based on the sequence alignment of *E. parasitica* with other *Eutypella* spp. sequences in Table 3, the forward primer EpF 5'-CGCCGAAGGACTGTAAACT-3' and the reverse primer EpR 5'-CTGACACCACTGAAGCGAGA-3' were constructed. Amplification with these specific primers yielded the expected fragment of 341 bp for all isolates of *E. parasitica* (Fig. 4). The other fungi included in the analysis (Table 2) and DNA extracts from host species yielded no amplification products, except for *E. prunastri*, which targeted an amplicon at an annealing tem-

perature of 55°C but not at 60 or 63°C (Fig. 4). Amplification with primers specific to *E. parasitica* using DNA extracts made directly from infected wood using the 2% CTAB extraction protocol required up to 100× serial dilution for some extracts to yield the expected fragment. The DNA concentrations (in base pairs) were around 160 µM_{bp} for DNA extracts made from pure cultures of *E. parasitica* and 45 µM_{bp} for DNA samples extracted from wood shavings infected with *E. parasitica*. Diluting of DNA samples indicated the detection limit for the PCR assay with EpR/F to be around 0.04 µM_{bp} (Fig. 5). DNA extracts from infected wood diluted up to 100× following the method of Lecomte et al. (24) revealed no products after PCR assays with either specific or universal primers.

DISCUSSION

Our aim to construct primers specific to *E. parasitica*, the causal agent of Eutypella canker of the maple, and to select a proto-

col suitable for DNA extracts from wood shavings was achieved. We amplified DNA fragments of the expected size from extracts made directly from wood shavings (from tissues taken from the margin of the brown discolorations typical of Eutypella canker). The BLAST search showed high specificity of the designed primer pair EpF/R among the sequences deposited in the GenBank database. The specificity of primer pair EpF/R also was confirmed with tested fungal isolates used in this study. Clear amplification was achieved

Table 2. Species or isolates of fungi comprising some members of Diatrypaceae and other ubiquitous fungi or those associated with wood used for confirming specificity of primers specific to *Eutypella parasitica*^a

Species or isolate	Origin ^b	Comments ^c
<i>Acremonium</i> sp.	SFI TBR1	Co-isolated with <i>Eutypella</i>
<i>Alternaria alternata</i>	ZIM 101	Kept at 4°C on PDA
<i>Antredia vaillantii</i>	ZIM 80	Kept at 4°C on PDA
<i>Armillaria mellea</i>	ZIM 1	Kept at 4°C on PDA
<i>Aureobasidium pullulans</i>	ZIM 103	Kept at 4°C on PDA
<i>Botryosphaeria dothidea</i>	SFI B1/4	Isolated with <i>Ostrya carpinifolia</i>
<i>Chaetomium globosum</i>	SFI C/1	Co-isolated from <i>Eutypella</i>
<i>Eutypa lata</i>	CBS 208.87	...
<i>E. lata</i> var. <i>aceris</i>	CBS 217.87	Isolated with <i>Acer campestre</i>
<i>E. lata</i> var. <i>aceris</i>	CBS 290.87	Isolated with <i>A. pseudoplatanus</i>
<i>Eutypella prunastri</i>	CBS 277.87	...
<i>Exophiala</i> sp.	SFI PBR/1	Co-isolated from <i>Eutypella</i>
<i>Exophiala</i> sp.	SFI E/2	Co-isolated from <i>Eutypella</i>
<i>Fomes fomentarius</i>	ZIM 59	Kept at 4°C on PDA
<i>Ganoderma applanatum</i>	ZIM 15	Kept at 4°C on PDA
<i>Heterobasidium annosum</i>	ZIM 19	Kept at 4°C on PDA
<i>Hypoxylon fragiforme</i>	ZIM 508	Kept at 4°C on PDA
<i>Nectria galligena</i>	SFI Nectria/1	Co-isolated with <i>Eutypella</i>
<i>N. galligena</i>	SFI Nectria/2	Co-isolated with <i>Eutypella</i>
<i>Penicillium janthinellum</i>	ZIM 410	Kept at 4°C on PDA
<i>Phanerochaete chrysosporium</i>	ZIM 1015	Kept at 4°C on PDA
<i>Pleurotus ostreatus</i>	AG PLA B	Kept at 4°C on PDA
<i>P. ostreatus</i>	AG Plo S2	Kept at 4°C on PDA
<i>P. ostreatus</i>	AG Plo 3	Kept at 4°C on PDA
<i>P. ostreatus</i>	ZIM 1002	Kept at 4°C on PDA
<i>P. ostreatus</i>	ZIM 1006	Kept at 4°C on PDA
<i>P. ostreatus</i>	ZIM 76	Kept at 4°C on PDA
<i>Saccharomyces cerevisiae</i>	ZIM 0074	Kept at 4°C on YM
<i>Schizophyllum commune</i>	SFI E/1	Co-isolated with <i>Eutypella</i>
<i>S. commune</i>	ZIM 41	Kept at 4°C on PDA
<i>Stropharia rugosoannulata</i>	AG STRA 1	Kept at 4°C on PDA
<i>Trametes versicolor</i>	ZIM 97	Kept at 4°C on PDA
<i>Trichoderma longibrachiatum</i>	ZIM 413	Kept at 4°C on PDA

^a The isolates were grown at 24°C on malt extract agar and then stored at 4°C until further use or as specified.

^b Abbreviations of culture collections: AG, collection at the Institute of Natural Sciences, Ljubljana, Slovenia; CBS, collection at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; SFI, collection of plant-pathogenic fungi at the Slovenian Forestry Institute, Ljubljana, Slovenia; ZIM, collection at the Department of Wood Science and Technology and at the Department of Food Science and Technology, Biotechnical Faculty, Ljubljana, Slovenia (33).

^c Abbreviations of culture media: PDA = potato dextrose agar (Difco); YM = yeast maltose agar (3.0 g of yeast extract, 3.0 g of malt extract, 5.0 g of peptone, 10.0 g of glucose, 20.0 g of agar, and 1 liter of distilled water); and co-isolated with *Eutypella* = co-isolated from discolored wood from a *Eutypella* canker.

Table 3. Internal transcribed spacer (ITS)1/5.8S/ITS2 sequences and accession numbers in the GenBank nucleotide database of *Eutypella* spp. used for multiple sequence alignment and search of regions specific to *Eutypella parasitica*

Fungal species	GenBank accession number
<i>Eutypella vitis</i>	AY462578
<i>E. vitis</i>	AY462577
<i>E. scoparia</i>	AF373064
<i>E. scoparia</i>	AJ302465
<i>E. quaternata</i>	AJ302469
<i>E. prunastri</i>	AJ302464
<i>E. leprosa</i>	AJ302463
<i>E. kochiana</i>	AJ302462
<i>E. cerviculata</i>	AJ302461
<i>E. cerviculata</i>	AJ302468
<i>E. caricae</i>	AJ302460
<i>E. alsophila</i>	AJ302467
<i>E. parasitica</i>	DQ118964
<i>E. parasitica</i>	DQ118965
<i>E. parasitica</i>	DQ118966

Fig. 1. *Mbo*I restriction fragment length polymorphism patterns of polymerase chain reaction-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 regions using primer pair ITS1f/ITS4 from DNA isolated from pure cultures. Digests were run on a 2% agarose gel in 0.5× Tris-borate EDTA buffer. Lane M, molecular ladder, Kbp units (Gene Ruler 100-bp DNA Ladder; Fermentas); lane 1, *Eutypella prunastri* (CBS 277.87); lane 2, *Eutypella lata* (CBS 208.87); lane 3, *E. lata* var. *aceris* (CBS 290.87); lane 4, *E. lata* var. *aceris* (CBS 217.87); and lane 5, *Eutypella parasitica* (SFI D26/1).

with all *E. parasitica* isolates and no reaction was observed with 33 isolates of other fungi. Methods based on PCR using species-specific primer pairs for fungi enabled rapid detection and identification of fungal pathogens (25) even before the infected plants show significant symptoms (15,37).

Fig. 2. *HinfI* restriction fragment length polymorphism patterns of polymerase chain reaction-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 regions using primer pair ITS1f/ITS4 from DNA isolated from pure cultures. Digests were run on a 2% agarose gel in 0.5× Tris-borate EDTA buffer. Lane M, molecular ladder, Kbp units (Gene Ruler 100-bp DNA Ladder; Fermentas); lane K, negative control (no DNA); lane 1, *Eutypella prunastri* (CBS. 277.87); lane 2, *Eutypa lata* (CBS. 208.87); lane 3, *E. lata* var. *aceris* (CBS 290.87); lane 4, *E. lata* var. *aceris* (CBS 217.87); and lane 5, *Eutypella parasitica* (SFI D26/1).

Fig. 3. *TaqI* restriction fragment length polymorphism patterns of polymerase chain reaction-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 region using primer pair ITS1f/ITS4 from DNA isolated from pure cultures. Digests were run on a 2% agarose gel in 0.5× Tris-borate EDTA buffer. Lane M, molecular ladder, Kbp units (Gene Ruler 100-bp DNA Ladder; Fermentas); lane K, negative control (no DNA); lane 1, *Eutypella prunastri* (CBS. 277.87); lane 2, *Eutypa lata* (CBS. 208.87); lane 3, *E. lata* var. *aceris* (CBS 290.87); lane 4, *E. lata* var. *aceris* (CBS 217.87); and lane 5, *Eutypella parasitica* (SFI D26/1).

Several undiluted samples of DNA extracted directly from infected wood with the 2% CTAB extraction protocol revealed no amplification or only weak amplification with the species-specific primers (EpR and EpF); however, after the original DNA extract was diluted, the amplification was successful. An important step in molecular diagnostics is the isolation of DNA free of any DNA amplification inhibitors (25). PCR inhibitors in extracts made from maple tissue were confirmed by PCR reaction with EpR/F using uninfected maple DNA extracts, spiked with serial dilutions of DNA from pure *E. parasitica* culture. The presence of such compounds probably affected the PCR amplification of DNA extracted directly from infected wood.

Another reason for the weak amplification or no amplification at all could be a low concentration of fungal DNA due to the particular location of tissues taken for the DNA extraction, such as the early-infection area or the edge of an infected lesion. PCR detection with *E. parasitica*-specific primers in extracts made directly from infected maple wood tissue shows rather inconsequential results, as explained previously. These results indicate the need for diluting the original extracts before performing PCR reaction to reduce possible PCR inhibitors present in the sample. Lecomte et al. (24) reported a simple DNA extraction protocol that they used successfully for in situ detection of *Eutypa lata* in a diseased grapevine. Lecomte's protocol

Table 4. Restriction fragment length polymorphism patterns of polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS) regions (650 bp long) obtained with restriction enzymes *HinfI*, *MboI*, and *TaqI* using primer pair ITS1f/ITS4 from DNA of fungal species or genotypes included in the analysis

Species	Length of restriction fragments of PCR products (bp)		
	<i>HinfI</i>	<i>MboI</i>	<i>TaqI</i>
<i>Eutypella parasitica</i> (all isolates from Table 1)	231, 156, 140, 121	319, 214	289, 233
<i>E. parasitica</i> (isolate SFI TO1/1)	231, 156, 140, 121	319, 214	289, 233
<i>E. prunastri</i> (CBS 277.87)	345, 286	319, 214	289, 233
<i>Eutypa lata</i> (CBS 208.87)	345, 151, 135	319, 214	289, 217
<i>E. lata</i> var. <i>aceris</i> (CBS 290.87)	345, 151, 135	319, 214	289, 233
<i>E. lata</i> var. <i>aceris</i> (CBS 217.87)	345, 151, 135	319, 214	289, 233

Fig. 4. Products of polymerase chain reaction (PCR) amplification at annealing temperature of 63°C with primers EpR/F from DNA isolated from pure fungal cultures of some members of Diatrypeaceae and isolates co-isolated from wood beneath *Eutypella* canker. PCR products were run on a 2% agarose gel in 0.5× Tris-borate EDTA buffer. Lane M, molecular ladder, Kbp units (Gene Ruler 100-bp DNA Ladder; Fermentas); lane K, negative control (no DNA); lane 1, *Acremonium* sp. (SFI TBR1); lane 2, *Chaetomium globosum* (SFI C/1); lane 3, *Nectria galligena* (SFI Nectria/1); lane 4, *N. galligena* (SFI Nectria/2); lane 5, *Exophiala* sp. (SFI PBR/1); lane 6, *Schizophyllum commune* (SFI E/1); lane 7, *Exophiala* sp. (SFI E/2); lane 8, *Eutypella prunastri* (CBS. 277.87); lane 9, *Eutypa lata* (CBS. 208.87); lane 10, *E. lata* var. *aceris* (CBS 290.87); lane 11, *E. lata* var. *aceris* (CBS 217.87); lane 12, *Eutypella parasitica* (SFI D26/1); lane 13, *E. parasitica* (SFI TO1/1); and lane 14, *E. parasitica* (CBS 210.39).

(24) also was tested in our study as the method for direct extraction of DNA from infected maple wood. This method did not work in the subsequent PCR with the wood shavings included in our experiment—either with universal fungal primers or with those specific to *Eutypella parasitica*. A possible explanation for this outcome is that our samples contained larger amounts of PCR inhibitors or smaller amounts of DNA than those used by Le-compte et al. (24).

The species-specific EpR/F primers constructed during this work proved adequate to distinguish *E. parasitica* from *Eutypa lata* and *E. lata* var. *aceris*, which is difficult to accomplish using only morphological features of their mitospore stage. As reported by the CBS database and Carter et al. (4), such fungi can be present in wood of *Acer* spp. Applied techniques successfully differentiate between *Eutypella prunastri* and *E. parasitica*, which are both representatives of the same taxonomic unit based on morphological features. This is another confirmation of successful application of the EpR/F primer pair in identification of fungi. The biology of Diatrypaceae species is not well known, the taxonomy not settled, and phylogenetical position of *E. parasitica* not clear (36). These reasons obstruct easy identification. The proposed method will facilitate proper identification of the causal agent of the cankers of maple, although *Eutypa lata* or any other Diatrypaceae species have not been reported so far as being pathogenic to maple.

The cankers caused by *Eutypella parasitica* are morphologically similar to those caused by *Botryosphaeria dothidea*

(mostly on *A. platanoides*) and *Nectria galligena* (mostly on *A. pseudoplatanus*) found in Slovenia (*unpublished observations*). However, the tested primers specific to *E. parasitica* successfully distinguished between the different causal agents of the canker. Robustness of EpR/F primers also was confirmed by their ability to distinguish isolates of *E. parasitica* from those of *Acremonium* spp., *Chaetomium globosum*, *Exophiala* spp., and *N. galligena*, obtained from cankers on maple, and other ubiquitous ascomycetes and basidiomycetes or those associated with woody tissues.

PCR-RFLP represents a relatively rapid and simple method that has been used successfully for species identification and separation in many plant groups (3,32), identification of ectomycorrhizae (2,9–11,18,20–22), and identification of fungi that cause wood decay (15). It also has been used to distinguish among species, as in the case of *Armillaria* spp. by Schulze et al. (38), to study intraspecific variation in *A. ostoyae* (37), and also for identification of *Eutypa lata*, another member of Diatrypaceae (36). The results obtained through the sequencing and RFLP profiling show highly conserved ITS regions of the collected *Eutypella parasitica* isolates and indicate low intraspecific variability in the ITS1/5.8S/ITS2 region. Intraspecific polymorphism was not detected among *Eutypa lata* isolates when examined with PCR-RFLP of the ITS region (36). The RFLP patterns in *Eutypella parasitica* point to an autonomous cluster in the SFI PCR-RFLP database in comparison with the previously deposited RFLP patterns. The PCR-RFLP method used in this study was specific enough to distinguish *E. parasitica* from *E. prunastri*, *Eutypa lata*, another pathogenic member of Diatrypaceae, and from two isolates of *E. lata* var. *aceris*.

Thus, molecular methods are feasible as additional tests to supplement the classical identification protocols or as independent identification methods for forest pathogens in Slovenia, as was indicated also by Jurc et al. (16) and reinforced in this study.

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LITERATURE CITED

1. Acero, F. J., González, V., Sánchez-Ballesteros, J., Rubio, V., Checa, J., Bills, G. F., Salazar, O., Platas, G., and Peláez, F. 2004. Molecular phylogenetic studies on the Diatrypaceae based on rDNA-ITS sequences. *Mycologia* 96:249-259.
2. Agerer, R., Kraigher, H., and Javornik, B. 1996. Identification of ectomycorrhizae of *Hydnum rufescens* on Norway spruce and the variability of the ITS region of *H. rufescens* and *H. repandum* (Basidiomycetes). *Nova Hedwigia* 63:183-194.
3. Ballian, D., Grebenc, T., Božič, G., Melnik, V., Wraber, T., and Kraigher, H. 2006. History, genetic differentiation and conservation strategies for disjunct populations of *Sibiraea* species from Southeastern Europe and Asia. *Conserv. Genet.* 7:895-907.
4. Carter, M. V., Bolay, A., and Rappaz, F. 1983. An annotated host list and bibliography of *Eutypa armeniaca*. *Rev. Plant Pathol.* 62:251-258.
5. Davidson, R. W., and Lorenz, R. C. 1938. Species of *Eutypella* and *Schizoxylon* associated with cankers of maple. *Phytopathology* 28:733-745.
6. Doyle, J. J., and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19:11-15.
7. Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2:113-118.
8. Ghignone, S., and Migheli, Q. 2005. The database of PCR primers for phytopathogenic fungi. *Eur. J. Plant Pathol.* 113:107-109.
9. Grebenc, T. 2005. Types of ectomycorrhizae on beech (*Fagus sylvatica* L.) in natural and managed forest. Ph.D. thesis, University of Ljubljana, Ljubljana, Slovenia.
10. Grebenc, T., and Kraigher, H. 2000. Establishment of the PCR-ITS-RFLP database for mycorrhizal fungi and types of ectomycorrhizae on Norway spruce from the permanent forest research plot of the Slovenian Forestry Institute on Pokljuka. Pages 179-190 in: *The Rhizosphere: Studies of Forest Soils and the Rhizosphere and Their Influences on Chosen Physiological Parameters of Forest Trees in Selected Forest Ecosystems, Forest Types and Developmental Phases of the Forest*. H. Kraigher and I. Smolej, eds. Slovenian Forestry Institute, Ljubljana, Slovenia.
11. Grebenc, T., and Kraigher, H. 2007. Changes in the community of ectomycorrhizal fungi and increased fine root number under adult beech trees chronically fumigated with double ambient ozone concentration. *Plant Biol. (Stuttg.)* 9:279-287.
12. Grebenc, T., Piltaver, A., and Kraigher, H. 2000. Establishment of a PCR-RFLP library for Basidiomycetes, Ascomycetes and their ectomycorrhizae in *Picea abies* (L.) Karst. *Phyton (Horn)* 40:79-82.
13. Grebenc, T., Rupnik, M., Hacin, J. I., Janc, M., and Kraigher, H. 1999. A contribution to the studies of actinorrhizal symbioses of *Alnus* spp. in Slovenia. *Phyton (Horn)* 39:207-211.
14. Grimont, P. A. D. 1998. *Taxotron User's Manual*. Institute Pasteur, Paris.
15. Jellison, J., and Jasalavich, C. 2000. A review of selected methods for the detection of degradative fungi. *Int. Biodeterior. Biodegrad.* 46:241-244.
16. Jurc, D., Ogris, N., Grebenc, T., and Kraigher, H. 2006. First reported *Botryosphaeria dothidea* causing bark dieback of European hop hornbeam in Slovenia. *Plant Pathol.* 55:299.
17. Jurc, D., Ogris, N., Slippers, B., and Stenlid, J. 2006. First report of *Eutypella* canker of *Acer pseudoplatanus* in Europe. *Plant Pathol.* 55:577.
18. Kären, O., Hogberg, N., Dahlberg, A., Jonsson,

Fig. 5. Polymerase chain reaction (PCR) detection of *Eutypella parasitica* in infected wood tissue at the margins of the discolorations with primers EpR/F at annealing temperature of 63°C. PCR products were run on a 2% agarose gel in 0.5× Tris-borate EDTA buffer. Lane M, molecular ladder, Kbp units (Gene Ruler 100-bp DNA Ladder; Fermentas); lane 1, undiluted DNA extract; lane 2, 100-fold diluted DNA extract; lane 3, 1,000-fold diluted DNA extract; and lane 4, 10,000-fold diluted DNA extract.

- L., and Nylund, J. E. 1997. Inter- and intra-specific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytol.* 136:313-325.
19. Kliejunas, J. T., and Kuntz, J. E. 1974. Eutypella canker, characteristics and control. *For. Chron.* 50:106-108.
20. Kraigher, H. 1994. Molecular ecology in forestry: taxonomy of ectomycorrhizae by anatomical characteristics and molecular markers. Pages 189-196 in: *Proc. Int. Colloquium Impact Plant Biotechnol. Agric. Rogla, Slovenia*. B. Javornik, B. Bohanec, and I. Kreft, eds. Biotechnical Faculty, Agronomy Department, Centre for Plant Biotechnology and Breeding, Ljubljana, Slovenia.
21. Kraigher, H., Agerer, R., and Javornik, B. 1995. Ectomycorrhiza of *Lactarius lignyotus* on Norway spruce, characterized by anatomical and molecular tools. *Mycorrhiza* 5:175-180.
22. Kraigher, H., Batič, F., and Agerer, R. 1996. Types of ectomycorrhizae and mycobioindication of forest site pollution. *Phyton (Horn)* 36:115-120.
23. Lachance, D., and Kuntz, J. E. 1966. Eutypella canker of sugar maple. *Phytopathology* 56:885-886.
24. Lecomte, P., Péros, J. P., Blancard, D., Bastien, N., and Délye, C. 2000. PCR assays that identify the grapevine dieback fungus *Eutypa lata*. *Appl. Environ. Microbiol.* 66:4475-4480.
25. Malvick, D. K., and Grunden, E. 2005. Isolation of fungal DNA from plant tissues and removal of DNA amplification inhibitors. *Mol. Ecol. Notes* 5:958-960.
26. McCartney, H. A., Foster, J. S., Fraaije, B. A., and Ward E. 2003. Molecular diagnostics for fungal plant pathogens. *Pest Manage. Sci.* 59:129-142.
27. Morgenstern, B. 1999. DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. *Bioinformatics* 15:211-218.
28. Munkvold, G. P. 2001. Eutypa dieback of grapevine and apricot. *Plant Health Progress Online*, doi: 10.1094/PHP-2001-0219-01-DG.
29. OEPP/EPPPO. EPPPO Alert List. Entry date 2005-11. Online publication, 2005.
30. Ogris, N., Jurc, D., and Jurc, M. 2005. Eutypella canker (*Eutypella parasitica*: *Ascomycota: Fungi*) on sycamore maple and field maple: characteristics and differences. *Gozd. Vestn.* 63:411-418.
31. Ogris, N., Jurc, D., and Jurc, M. 2006. Spread risk of Eutypella canker of maple in Europe. *Bull. OEPP* 36:475-485.
32. Pučko, M., Grebenc, T., Božič, G., Brus, R., and Kraigher, H. 2005. Identification of types of ectomycorrhizae on seedlings in a beech provenance trial. *Zb. Gozd. Lesar.* 75:87-104.
33. Raspor, P., Smole-Možina, S., Podjavoršek, J., Pohleven, F., Gogala, N., Nekrep, F. V., Rogelj, I., and Hacin, J. 1995. ZIM, the Collection of the Industrial Microorganisms. Catalog of biocultures. Biotechnical Faculty, Ljubljana, Slovenia.
34. Riley, M., Maling, B., and Chamberlin, M. J. 1966. Physical and chemical characterization of two- and three-stranded adenine-thymine and adenine-uracil homopolymer complexes. *J. Mol. Biol.* 20:118-124.
35. Rogers, S. O., and Bendich, A. J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* 5:69-76.
36. Rolshausen, P. E., Trouillas, F., and Gubler, W. D. 2004. Identification of *Eutypa lata* by PCR-RFLP. *Plant Dis.* 88:925-929.
37. Schulze, S., Bahnweg, G., Möller, E. M., and Sandermann, Jr. H. 1997. Identification of the genus *Armillaria* by specific amplification of an rDNA-ITS fragment and evaluation of genetic variation within *A. ostoyae* by rDNA-RFLP and RAPD analysis. *Eur. J. For. Pathol.* 27:225-239.
38. Schulze, S., Bahnweg, G., Tesche, M., and Sandermann, Jr. H. 1995. Identification of European *Armillaria* species by restriction-fragment-length polymorphisms of ribosomal DNA. *Eur. J. For. Pathol.* 25:214-223.
39. Sinclair, W. A., Lyon, H. H., and Johnson, W. T. 1989. *Diseases of Trees and Shrubs*. Cornell University Press, New York.
40. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols. A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.