Cryphonectria naterciae: A new species in the Cryphonectria–Endothia complex and diagnostic molecular markers based on microsatellite-primed PCR

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Abstract
In a recent study intended to assess the distribution of Cryphonectria parasitica in Portugal, 22 morphologically atypical orange isolates were collected in the Midwestern regions. Eleven isolates were recovered from Castanea sativa, in areas severely affected by chestnut blight and eleven isolates from Quercus suber in areas with cork oak decline. These isolates were compared with known C. parasitica and Cryphonectria radicalis isolates using an integrated approach comprising morphological and molecular methods. Morphologically the atypical isolates were more similar to C. radicalis than to C. parasitica. Phylogenetic analyses based on internal transcribed spacer (ITS) and β-tubulin sequence data grouped the isolates in a well-supported clade separate from C. radicalis. Combining morphological, cultural, and molecular data Cryphonectria naterciae is newly described in the Cryphonectria–Endothia complex. Microsatellite-primed PCR fingerprinting with (GACA)4 primer discriminated between C. naterciae, C. radicalis, and C. parasitica.

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Introduction
The Cryphonectriaceae was introduced by Gryzenhout et al. (2006a) to accommodate genera from the Cryphonectria–Endothia complex (Castlebury et al. 2002). Genera in the Cryphonectriaceae can be distinguished from those in other families, or undescribed groups in the Diaporthales, by the development of orange stromatic tissue at some stage of their life cycle, a purple reaction in KOH and a yellow reaction in lactic acid associated with pigments in the stromatic tissue or in culture. There is also strong phylogenetic support in phylogenies based on ribosomal and β-tubulin gene sequences (Castlebury et al. 2002).
Cryphonectria was considered to be a synonym of *Endothia* until Barr (1978) separated the two genera based on the configuration and texture of the stromata and the septation and shape of the ascospores (Roane et al. 1986; Myburg et al. 2004b). Nevertheless, species of *Endothia* continue to be mistaken with Cryphonectria spp. due to their similar orange fruiting structures and a shared anamorph genus, Endothiella (Barr 1978; Gryzenhout et al. 2006b). Moreover, these species share the same hosts (Castanea spp. and Quercus spp) and geographical distributions (Gryzenhout et al. 2006b).

Recent taxonomic revisions restrict the name Cryphonectria (sensu stricto) to *Cryphonectria macrospora* (Tak. Kobay. & Kaz. Ito) M.E. Barr, *Cryphonectria nitschkei* (G.H. Otth) M.E. Barr *Cryphonectria parasitica* and *Cryphonectria radialis* (Schwein.: Fr.) M. E. Barr (Gryzenhout et al. 2006a, b, c). Of these *C. parasitica* is the most studied species since it is the causal agent of chestnut blight disease worldwide, and the only species of this genus that is considered to be a primary plant pathogen. This fungus has destroyed nearly all native stands of American chestnuts (*Castanea dentata* (Marsh.) Borkh.). In Europe, many European chestnuts areas (*Castanea sativa* Mill.) have been similarly affected by the blight since the start of the 1930s. However, impact and disease severity has been stalled due to natural higher resistance (*Castanea* spp. due to their similar orange fruiting structures and a shared anamorph genus, *Endothiella*) (Hoegger et al. 1987; Heining & Rigling 1994; Bissegger et al. 1997; Milgroom & Cortesi 2004). In the United States and in some European countries, *C. parasitica* has also been found on some species of *Quercus* (Torsello et al. 1994; Radócz & Tarcali 2005), although blight symptoms are not as severe as in Castanea (Radócz & Tarcali 2005).

The saprophytic *C. radialis* occurs in Europe, North America, and Japan, being closely related to *C. parasitica* (Hoegger et al. 2002; Venter et al. 2002; Myburg et al. 2004a, b). Isolates of *C. radialis* are difficult to detect in nature because they are possibly almost displaced or their occurrence is cryptically masked by *C. parasitica* (Hoegger et al. 2002).

Gryzenhout et al. (2006b) proposed two different phylogenetic groups of *C. radialis*. The first one (*C. radialis* sensu stricto) defined by the North America type specimen and corresponding morphologically to a group containing isolates from Switzerland, Greece, Italy, and Japan. The second group consisted of two isolates, one from Italy and another from Portugal (Myburg et al. 2004a, b). This Portuguese isolate was formerly described as *Endothiella gyrosa* and was collected from *Quercus suber* L. Nevertheless, the taxonomy of *C. radialis* remains unclear despite all the knowledge that have been gathered in recent years, mainly because the teleomorph (required for accurate morphological species differentiation) is not commonly found in nature and is not easily produced in vitro (Myburg et al. 2004b; Gryzenhout et al. 2006b).

In a recent study on the distribution of *C. parasitica* in Portugal (Bragança et al. 2007), morphologically atypical orange isolates were collected from chestnut stands that were severely affected by the blight in the Midwest of the country. The different morphology of these isolates was noticed on potato dextrose agar (PDA) among *C. parasitica* cultures. A close relationship was established between these and other isolates collected previously from declining *Q. suber*, also in the Midwest of Portugal (Santos et al. 2005). Cultural morphology of these Midwest isolates showed a strong similarity with an isolate of *E. gyrosa* Sacc. in the Instituto Nacional de Recursos Biológicos (INRB) working culture collection that was collected from *Q. suber* in 1960. This fungus was first reported in Portugal by Câmara (1929) causing the ‘ferrugem alaranjada’ (‘orange rust’) disease on cork oak stems.

The aim of this work was to use morphological and molecular techniques to determine the identity of the isolates from Portugal. In addition, MSP-PCR fingerprinting was used to provide a fast and reliable methodology to discriminate the species within the *Cryphonectria—Endothia* complexes.

### Material and methods

#### Fungal isolates

In this study, 22 orange *Cryphonectria* isolates from *Castanea sativa* and *Quercus suber* collected in the Midwest of Portugal (Beira Interior, Ribatejo, and Alentejo) were characterized (Table 1). All isolates were maintained in a culture collection at the Unidade de Silvicultura e Recursos Florestais, INRB, I.P., Oeiras, Portugal and in the Swiss Federal Research Institute (WSL). For comparison, three *Cryphonectria radialis* isolates from Switzerland (culture collection of the WSL) and four *Cryphonectria parasitica* isolates, three from the Portuguese collection, and one from USA (WSL collection), were used. Reference isolates were deposited in the public culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands and the INRB culture collection (MEAN).

#### Culture morphology and growth

Isolates were grown on PDA (Difco 39 g L$^{-1}$) in 90 mm diam. Petri dishes are incubated at 25 °C in the dark for 1 week. The cultures were then exposed to diffuse daylight at room temperature on the laboratory bench, and culture morphology was recorded once a week for a period of 4 weeks. All cultures were checked for typical *C. parasitica* characteristics, i.e., purple droplets (as seen with the dissecting microscope) and a ‘flat’ central area (Hoegger et al. 2002). Agar pieces (5 × 5 mm) taken from the edge of actively growing cultures were immersed in parallel into 0.2 ml of 3 % of KOH and into 0.2 ml of lactic acid to check for purple and yellow discolouration of the mycelium as described by Castlebury et al. (2002). In addition, all isolates were grown on corn meal medium (10 g of corn meal autoclaved for 20 min at 120 °C with 20 ml of distilled water in 100 ml Erlenmeyer flasks) to test for the purple discolouration that is characteristic of *C. radialis* in this medium (Hoegger et al. 2002). Two cultures per isolate were incubated at 25 °C in the dark and assessed for discolouration of the medium after 7 weeks.

Observations of micromorphological features and measurement from three isolates of each group of *Cryphonectria* (20-d-old PDA cultures) were made as described by Santos & Phillips (2009). Mean, standard deviation (SD) and 95 % confidence intervals were calculated from measurements of 150 conidia of each group of *Cryphonectria*. Minimum and maximum dimensions are given in parentheses. Differences for the conidia measurements among the three species were assessed by analysis of variance (ANOVA), followed by LSD post-hoc means test.

Colony growth rates for all 22 *Cryphonectria* isolates, together with three *Cryphonectria parasitica* and three *C. radialis*
reference isolates (Table 1), were assessed by the methods described by Hoegger et al. (2002) and Gryzenhout et al. (2004). Cultures were grown on PDA in 90 mm diam. Petri dishes in the dark at temperatures ranging from 10°C to 35°C at 5°C intervals. Five replicate plates per isolate and temperature were inoculated at the centre with 5 mm diam. agar discs taken from the edge of actively growing cultures. After 4 d, two perpendicular diameters of each culture, minus the 5 mm diam. of the agar disk, were measured and the average taken. The Newman–Keuls Test of multiple mean comparison for non-independent samples and uneven number of observations (Newman 1939; Keuls 1952) was used to test for significant differences \( p < 0.05 \) in growth between species for each temperature. Statistical analyses were made using Statistica 6.1 software (StatSoft, Tulsa, OK, USA).

**DNA extractions**

Mycelium was grown on PDA overlayed with cellophane for 7 d at 25°C in the dark. A square of fresh cultures (2 cm \times 2 cm) was stripped from the cellophane overlays, transferred to a 2 ml Eppendorf tube and DNA extracted following the procedures of Raeder & Broda (1985) with modifications. First, the mycelium was suspended in 500 \mu l lysing buffer (300 mM Tris–HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA, 0.5 % w v\(^{-1}\) SDS) and the equivalent of 200 \mu l of 450–600 \mu m glass beads (Sigma) was added. After vortexing for 2 min, the tubes were centrifuged (max V.) for 10 min at 4°C (adapted from Sampaio et al. 2001). The supernatant was mixed with 350 \mu l of phenol and 150 \mu l chloroform and centrifuged for 15 min (max V.). Subsequent steps were done exactly as described by Raeder & Broda (1985). The DNA was resuspended in 50 \mu l of TE (10 mM Tris–HCl, 2 mM EDTA, pH 8.0) and stored at 4°C until use.

**Amplification of the internal transcribed spacer (ITS) region and RFLP analysis**

The ITS region, which includes ITS1, 5.8S rRNA gene and ITS2, was amplified by PCR using forward primer IT5S and reverse

<table>
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<th>Cryptonectria species</th>
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<th>Origin</th>
<th>Collector</th>
<th>Year</th>
<th>GenBank accession no.</th>
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<td>C 0084</td>
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<td>D. Rigling</td>
<td>2005</td>
<td></td>
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a C – collections of the INRB, I.P., Oeiras (isolates under investigation are listed in bold), CBS – culture collection of the CBS, Utrecht, The Netherlands; MEAN – culture collection of INRB (Ex Estação Agronômica Nacional), M – culture collection of the Swiss Federal Research Institute (WSL), Switzerland.
c Ex-type culture.
primer ITS4 described by White et al. (1990). The PCR reaction mixture (50 µL) included 400 ng of template DNA, 2 U of Taq DNA polymerase (Life Technologies, England), 50 pmol of each primer (Life Technologies, England), 1× PCR buffer supplied with the enzyme, 4 mM MgCl₂ and 200 µM of each dNTP (Life Technologies, England). To each PCR tube 50 µL of mineral oil were added before amplification in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following program: 4 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 56 °C and 1 min at 72 °C; 5 min at 72 °C.

Based on Myburg et al. (1999) a restriction analysis of the ITS region was performed. From each PCR product 5 µL samples were digested with 3 U of restriction endonuclease AluI (New England Biolabs, Beverly, MA, USA), in a final volume of 10 µL, according to manufacturer’s instructions. The digested PCR products were separated on 2 % w/v agarose gel (Invitrogen) in 0.5× TBE (50 mM Tris, 45 mM boric acid, 1 mM EDTA) at 90 V for 3 h, using 100 bp DNA Ladder (Gibco-BRL) as a molecular size marker. After ethidium bromide staining, the gels were analysed with KODAK 1D 3.5.2 software.

β-tubulin gene amplification

Two regions within the β-tubulin gene were amplified using primer pairs Bt1a, Bt1b (amplifying β-tubulin region 1) and Bt2a, Bt2b (amplifying β-tubulin region 2) (Glass & Donaldson 1995). The amplification reaction mixture and reaction conditions were as described by Myburg et al. (2002).

Sequence analysis

All sequences were obtained in an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter, USA, in ICAT-Lisbon Faculty of Sciences Sequencing Services) by a dye-labelled dideoxy termination method (Dye Terminator Cycle sequencer (DTCS) start kit, Beckman Coulter). For sequencing, the PCR products were purified using Jet Quick-PCR Purification Kit (Genomed) as described by the manufacturer. Two sequencing reactions, one in each direction, were performed for each PCR product using the same primers as for PCR amplification. The two sequences were assembled using the CEQ Investigator program (software CEQ 8000, Beckman Coulter).

Sequences were edited and used in a phylogenetic analysis following the protocol of Santos & Phillips (2009) with minor changes. Phylogenetic tree were inferred in Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Swofford 2002) by Maximum Parsimony (MP) and gaps were regarded as missing data. Additional sequence data used in the phylogenetic analyses were obtained from Myburg et al. (2004a, b) and Gryzenhout et al. (2006a) and listed by their taxon name and accession number in the tree. Sequences from Diaporthe ambigua were used as outgroup to root the phylogenetic trees. Newly generated

Fig 1 – Culture morphology on PDA (top row), and colouration of corn meal medium (bottom row): (A) and (D) – Cryphonectria naterciae; (B) and (E) – C. radicalis; (C) and (F) – C. parasitica.
sequences have been deposited in GenBank (Table 1) and the alignment and phylogeny in TreeBASE (TB2:S11667).

**MSP-PCR fingerprinting**

Three different primers were tested: a core sequence of the phage M13, and two synthetic oligonucleotides, (GACA)$_4$ and (GTG)$_5$ (Meyer et al. 1993). All PCR reactions were performed in 25 μL Eppendorf tubes containing approximately 200 ng of template DNA, 1 U of Taq DNA polymerase (Life Technologies, England), 50 pmol of each primer (Life Technologies, England), 1× PCR buffer supplied with the enzyme, 2.5 mM MgCl$_2$ and 0.1 mM of each dNTP (Life Technologies, England). To each PCR tube 50 μL of mineral oil was added and amplification was performed in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following amplification program: 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C; with a final step of 5 min at 72 °C. Amplified DNA fragments were separated by electrophoresis in 1% (w v$^{-1}$) agarose gels (Invitrogen) at 90 V for 3 h using a 1 kb plus DNA Ladder (Invitrogen) as molecular size marker. After ethidium bromide staining, DNA banding patterns were visualized under UV transillumination and images were acquired using KODAK 1D 3.5.2 software.

### Results

**Culture morphology and growth**

On PDA after 1 week, the pinkish colouration due to the development of small droplets of purple exudates on the hyphae, visible under the dissecting microscope, was observed in only the three Cryphonectria radicalis isolates from Switzerland. Cryphonectria naterciae sp. nov showed, with some variation, an orange pigmentation in the centre of the culture, whereas the three C. radicalis remained white. After 4 weeks under artificial light, three different types of culture morphologies were associated with C. naterciae, C. radicalis, and Cryphonectria parasitica. As shown in Fig 1, C. radicalis and C. naterciae cultures had similar morphologies. However, the flat centre area was present only in C. radicalis isolates (Fig 1B).

![Fig 2 – Colony growth of Cryphonectria naterciae (22 isolates), C. radicalis (three isolates) and C. parasitica (three isolates) on PDA medium (mean colony growth after 4 d ± SD, 5 replications per isolate and temperature).](image-url)

### Table 2 – Dimension of conidia produced by C. naterciae, C. radicalis, and C. parasitica. Mean ± SD and range for each species (listed in bold) and isolate are given with 95 % confidence intervals (minimum and maximum dimensions are given in parenthesis).

<table>
<thead>
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<th>Isolate no.</th>
<th>n</th>
<th>Mean ± SD (μm)</th>
<th>Range (μm)</th>
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<td>C. naterciae</td>
<td></td>
<td>(2.9−3.6−3.8 (−4.9) × (1.0−1.2−1.3 (−1.6)</td>
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<tr>
<td>C 0612</td>
<td>150</td>
<td>3.7 ± 0.4 × 1.3 ± 0.1</td>
<td>(3.0−3.6−3.8 (−4.3) × (1.0−1.3−1.5)</td>
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<td>50</td>
<td>3.4 ± 0.2 × 1.2 ± 0.1</td>
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<tr>
<td>C 0705</td>
<td>50</td>
<td>4.0 ± 0.5 × 1.3 ± 0.1</td>
<td>(3.0−3.6−3.8 (−4.3) × (1.0−1.3−1.5)</td>
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<tr>
<td>C. radicalis</td>
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<td>(2.7−3.4−3.5 (−4.1) × (1.2−1.4−1.5 (−1.8)</td>
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<tr>
<td>M2269</td>
<td>150</td>
<td>3.4 ± 0.3 × 1.4 ± 0.1</td>
<td>(2.7−3.1−3.3 (−3.7) × (1.2−1.3−1.5)</td>
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</tr>
<tr>
<td>EP155</td>
<td>50</td>
<td>3.7 ± 0.4 × 1.4 ± 0.1</td>
<td>(2.9−3.6−3.6 (−4.3) × (1.2−1.4−1.5 (−1.7)</td>
</tr>
</tbody>
</table>
The mean colony diameters of the three taxonomic groups after 4 d of growth in relation to temperature are showed in Fig 2. All isolates grew best at 25°C. Isolates of C. radicalis showed the fastest growth at all temperatures tested, with the exception of 30°C where C. parasitica grew the fastest. However, despite the separation of the three taxonomic groups at 30°C, no statistical significant association could be found between growth and taxonomic groups when all data were analysed.

**ITS and β-tubulin gene amplification and RFLP analysis**

Amplification of the ITS (ITS1-5.8S-ITS2) and β-tubulin gene regions resulted in single fragments of approximately 700 and 600 bp, respectively. Digestion of the ITS region with the enzyme Alu I produced the same RFLP profile for all isolates (two fragments with approximately 475 and 240 bp), which is in accordance with the Cryphonectria parasitica profile presented by Myburg et al. (1999).

**Phylogeny**

The phylogenetic tree obtained from the ribosomal ITS sequence data is shown in Fig 3. The dataset consisted of 28 ingroup taxa and two outgroups and the alignment contained 594 characters including alignment gaps. Of the 594 characters, 167 were parsimony informative and

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**Fig 3** — The most parsimonious tree resulting from the alignment of 576 characters of ITS rDNA region. Length = 359; consistency index (CI) = 0.850; retention index (RI) = 0.944; homoplasy index (HI) = 0.150. Bootstrap values with 1000 replications for MP are shown above the branches. Bar represents 10 changes. *Diaporthe ambigua* (AF543817 and AF543818) were included as outgroups. Phylogeny deposited in TreeBASE (TB2:S11667). Newly generated sequences are listed in bold together with their isolate number. Isolate followed by § is ex-type isolate and additional sequence data used in the phylogenetic analyses were obtained from Myburg et al. (2004a,b) and Gryzenhout et al. (2006a).
included in the analysis resulting in one most parsimonious tree.

Since not all isolates used for the ITS region could be sequenced for ß-tubulin gene (Table 1), only the tree obtained from ribosomal ITS sequence data are presented taking into account that ITS and ß-tubulin phylogenies are congruent (data not shown).

The phylogenetic relations found amongst Cryphonectria spp. and Endothia spp. are compatible with the results presented by Myburg et al. (2004a, b) and Gryzenhout et al. (2006a, b). These data confirm the existence of two separate clades within Cryphonectria radicalis isolates considered by Gryzenhout et al. (2006b) as C. radicalis sensu stricto that includes two isolates from Switzerland and two from Italy. The new species, Cryphonectria naterciae groups our Portuguese isolates with another Portuguese isolate and an Italian isolate (100 % bootstrap support). As expected, Portuguese Cryphonectria parasitica isolates are in the same clade with the other C. parasitica (99 % bootstrap support). The final identification of isolates is presented in Table 1.

**MSP-PCR fingerprinting**

Distinct electrophoretic band patterns were obtained with M13, (GTG)₅, and (GACA)₄ primers, with each primer enabling differentiation of all three species, (Fig 4). Electrophoretic banding patterns obtained by (GACA)₄ were more evident than M13 and (GTG)₅ patterns. The (GACA)₄ PCR fingerprints showed well-separated main DNA fragments (bp ± SD), four associated with Cryphonectria naterciae isolates; (971 ± 10 bp, 744 ± 7 bp, 669 ± 4 bp, and 457 ± 4 bp); three with Cryphonectria radicalis isolates (974 ± 16 bp, 783 ± 7 bp, 673 ± 2 bp) and three with Cryphonectria parasitica isolates (762 ± 14 bp, 638 ± 8 bp, and 389 ± 2 bp). Since the Portuguese isolates had the same banding patterns, this method confirmed the C. naterciae identification for the nonsequenced isolates.

**Taxonomy**

*Cryphonectria naterciae* M.H. Braganc¸a, E. Diogo, & A.J.L. Phillips sp. nov. Fig 5.

Cryphonectria radicalis affinis sed coloniis in agaro solano tuberoso (PDA) uniformibus, laevibus, aurantiacis-ferrugineis non luteis-aurantiacis granulosibus, circulo centrali albido translucido abest et guttae parvae purpureae hyphae abest. A C. radicalis differt coloniis in farina frumento luteo-purpurascentibus non purpureis. Conidia (2.9–3.6–3.8(–4.9) × (1.0–)1.2–1.3(–1.6) μm.

Conidiomata pseudostromatic, globose, pulvinate, occurring separately, superficial to slightly immersed, erumpent through the host epidermis, yellow to orange, obpyriform, multilocular, variable in size up to 390 μm wide and 420 μm high, with a single ostiole (Fig 5(A–B)), basal tissues pseudoparenchymatous (Fig 5D). Conidiophores cylindrical, septate, branched, hyaline, smooth, up to 33 μm long, 1.9 μm wide (Fig 5(E–G)). Conidiogenous cells phialidic, apical or lateral on branches beneath the septum, cylindrical tapering towards the end, (6.9–)7.2–9.8(–10.9) long and (1.3–)1.5–2.0(–2.3) μm wide, collaretare and pericinal thickening inconspicuous (Fig 5(E–G)). Conidia (2.9–)3.6–3.8(–4.9) × (1.0–)1.2–1.3(–1.6) μm, hyaline, one celled (or aseptate), egutulate, cylindrical to

![Fig 4 – MSP-PCR profiles of Cryphonectria naterciae, C. radicalis, and C. parasitica isolates using (GACA)₄, M13, and (GTG)₅ primers: 1 – C 0084, 2 – C 0605, 3 – C 0607, 4 – C 0608, 5 – C 0611, 6 – C 0612, 7 – C 0614, 8 – C 0679, 9 – C 0685, 10 – C 0691, 11 – C 0705, 12 – CM2269, 13 – CM2270, 14 – CM4733, 15 – CEP155, 16 – C 0720, 17 – C 0721, 18 – C 0722. M – molecular size marker (1 kb plus DNA Ladder). Diagnostic bands are indicated by arrows: 457 bp for C. naterciae and 389 bp for C. parasitica.](image-url)
fusoid, seldom slightly allantoid (Fig 5F), exuded in the form of yellow to orange twisted cirri (Fig 5C).

Cultural characteristics: on PDA, colonies flat, felty, orange to ferruginous colour, uniform, margins even, with scarce sporulation (Fig 1A). Optimum temperature for growth 25 °C (Fig 2). On corn meal producing a reddish purple pigment (Fig 1D).

Etymology: After the Portuguese phytopathologist Maria Natércia Santos (b. 1940).

Teleomorph: Not seen.

Habitat: On Quercus suber and Castanea sativa.

Known distribution: Italy (Myburg et al. 2004a), Portugal.

Specimens examined: Portugal, Alentejo, Pegões, on bark of Q. suber, 2001, H. Bragança & P. Piloto, holotype CBS H-20572, culture ex-type CBS 129351 (C 0612, MEAN 950) See Table 1 for other isolates studied.

Notes: This species can be distinguished from C. radicalis by the absence of small droplets of purple exudates on the hyphae growing on PDA (visible under the dissecting microscope) and by the absence of a 'flat' centre area in PDA plates. It can be distinguished from Cryphonectria parasitica by causing a reddish purple colour change in corn meal (Fig 1D).

Fig 5 — Fruiting structures of C. naterciae — (A) and (B) pycnidia on Quercus suber and Castanea sativa stems respectively. (C) — tendrils of conidia on C. sativa stem in vitro; (D) — pycnidium; (E) — conidiogenous cells; (F) — conidia; (G) — conidiophores. Bars: (A–C) — 1 mm; (D) — 200 μm; (E–G) — 5 μm.
Discussion

Eleven isolates from Castanea sativa and eleven from Quercus suber were studied. All isolates from both hosts formed a clear and well-supported clade sister to Cryphonectria radicalis, and on account of the morphological and phylogenetic distinctions they were considered to represent a separate species described here as Cryphonectria naterciae. Thus far only two other isolates of this species are known, one collected in Italy and another in Portugal (Myburg et al. 2004a).

Morphologically all the Portuguese isolates belonged to the Cryphonectriaceae, which was recently described by Gryzenhout et al. (2006a). Furthermore, culture morphology on PDA as well as the reddish purple pigmentation in corn meal medium suggested a close relationship with C. radicalis. However, some of these characters, such as the absence of small purple droplets in the Portuguese cultures, did not allow us to definitely assign the Portuguese isolates to C. radicalis (Hoeger et al. 2002). Conidial dimensions of the three species scarcely differed but C. naterciae conidia were slightly narrower than in the other species. In a previous study that included two of these isolates (Gryzenhout et al. 2006a) it was suggested that they may represent a distinct species. However, Gryzenhout et al. 2006b did not introduce a new species name for these isolates because none of the sequences that they studied could be linked to cultures or herbarium specimens.

The polymorphisms found for each MSP-PCR primer, i.e., M13, (GTG)6, and (GACA)4, clearly distinguished three different patterns among the analysed isolates, and these groups corresponded to the three species under study. Of these primers, (GACA)4 produced the most distinctive banding patterns for each group of isolates. A single and well-separated PCR band of 457 bp was found to be specific for C. naterciae isolates and between the two specific bands for Cryphonectria parasitica (389 bp and 638 bp) the smaller band can be unequivocally diagnostic for this species. Cryphonectria radicalis could be identified due to the lack of the bands diagnostic for C. naterciae. Moreover, in C. parasitica the band around 970 bp is absent (this band is present in both C. naterciae and C. radicalis). These findings indicate that these bands are reliable markers to differentiate rapidly and easily C. radicalis, C. naterciae, and C. parasitica (diagnostic bands are indicated by arrows in Fig 4). This type of technique has previously been reported to give good differentiation of related species in other genera (Sampaio et al. 2001; Gadano et al. 2003; Roque et al. 2006).

ITS PCR-RFLP with Alu I revealed the existence of a common profile for each of the three species analysed, which is clearly distinct from the profile for Endothia gyrosa (Myburg et al. 1999). Although this method did not discern the three groups in study, it allowed us to reject the hypothesis that Portuguese isolates are E. gyrosa.

Cryphonectria radicalis was not found in Portugal during this study. To date, the known distribution of C. radicalis, in Europe is restricted to the central and south-eastern part of the continent including Switzerland, Italy, and Greece (Myburg et al. 2004a). Cryphonectria radicalis has been reported from Europe before the introduction of C. parasitica and was thought to have evolved with European chestnut (Elliston 1982). Whether both C. radicalis and C. naterciae found in Europe are indigenous fungi, however, remain to be shown. The new species described here, C. naterciae, was particularly common in the province of Alentejo on both Q. suber and C. sativa. In the main chestnut growing area of Portugal (Trás-os-Montes), however C. naterciae isolates were neither reported in the past nor detected during the recent national survey on chestnut blight, which yielded the isolates from chestnut in Alentejo (Bragança et al. 2007).

Preliminary results from an experiment with C. sativa and Q. suber showed that C. naterciae is not pathogenic on C. sativa, but no conclusive results were achieved with Q. suber (data not shown). More intensive studies are needed to evaluate the pathogenicity of C. naterciae and to determine its main host in Portugal and also in other areas in Europe. The combination of the morphological and molecular method based on MSP-PCR with (GACA)4 primer, as well as the phylogenetic approach used in this study, could contribute towards the development of diagnostic molecular markers to identify species in Cryphonectria.

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