**Phytophthora ramorum** sp. nov., a new pathogen on *Rhododendron* and *Viburnum*

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Since 1993, a hitherto unidentified *Phytophthora* species has been found associated with twig blight disease in *Rhododendron* and, sporadically, *Viburnum*. The morphology and growth characteristics of fourteen isolates from Germany and the Netherlands were investigated, together with their breeding system, the internal transcribed spacer (ITS) regions of the ribosomal DNA, amplified fragment length polymorphism (AFLP) fingerprints, and isozyme profiles, which were compared to those of a number of outgroup species. Morphologically the isolates are characterized by abundant production of chlamydospores and elongate, ellipsoid, deciduous sporangia with a short pedicel, in which they resemble *P. palmivora*. However, sporangia were semi-papillate, chlamydospores were much larger and cardinal temperatures much lower than those of *P. palmivora*. Oogonia with amphigynous antheridia and plerotic oospores were produced in dual cultures with an A2 mating type strain of *P. cryptogea*. ITS1 and ITS2 sequences of the unidentified species were closest to those of *P. lateralis*, but differed in three and eight nucleotides respectively from the latter species. AFLP fingerprints and isozyme patterns of malate dehydrogenase (MDH-2) and malic enzyme (MDHP) showed that the isolates formed a homogeneous group, distinct from all examined outgroup species, including *P. lateralis*. It was concluded that they represent a new *Phytophthora* species, described here as *P. ramorum* sp. nov. In pathogenicity tests all isolates of *P. ramorum* were pathogenic to *Rhododendron*.

INTRODUCTION


Since 1993, a new twig blight of rhododendron has been observed in Germany and The Netherlands. It kills both nursery plants of rhododendron and mature bushes in gardens. *Phytophthora* isolates obtained from such plants did not fit the morphological description of any known *Phytophthora* species, though they showed some resemblance to *P. palmivora*. Since 1998 similar isolates have been found on diseased *Viburnum* sp., and obtained from water in recirculation systems of nurseries where *Rhododendron* or *Viburnum* plants are grown. Infection trials with the very first isolates showed that they were highly aggressive to rhododendron twigs (Werres & Marwitz 1997).

Identification of *Phytophthora* species on morphology is notoriously difficult due to intraspecific variation and to
Phytophthora ramorum, a new pathogen

overlap of morphological characters between species (Brazier
1991, Erwin & Ribeiro 1996). Therefore molecular and
biochemical techniques have been increasingly applied in the
identification process. Sequence analysis of the internal
transcribed spacer (ITS) region of the ribosomal DNA (rDNA)
will discriminate between species of Phytophthora (Crawford
et al. 1996, Cooke & Duncan 1997, Cooke et al. 2000, Förster,
Cummings & Coffey 2000). Most species have unique ITS
sequences, although some closely related species may have
identical sequences, e.g. P. infestans and P. mirabilis (Cooke
et al. 2000). Complementary tools for discriminating species are
amplified fragment length polymorphism (AFLP) finger-
printing (Vos et al. 1995, Mayer et al. 1997) and isozyme
analysis (e.g. Mills, Förster & Coffey 1991, Oudemans &
Coffey 1991a, b, Oudemans, Förster & Coffey 1994, Ilieva
et al. 1998, Man in ’t Veld et al. 1998, Bonants et al. 2000). The
aim of the present study was to characterise the morphology,
breeding system, pathogenicity, and biochemical and mol-
elar characteristics of the unassigned Phytophthora isolates
from Rhododendron and Viburnum in order to evaluate whether
they represent a new Phytophthora species, and to relate them
to other Phytophthora species using ITS, AFLP and isozyme-
based methods.

MATERIAL AND METHODS

Isolates

Fourteen Phytophthora ramorum isolates were examined, along
with reference isolates of eight other Phytophthora species for
comparison (Table 1). Of these 14 isolates, 10 were from
diseased Rhododendron twigs, 2 from diseased Viburnum sp.,
and 2 from recycling water from woody ornamental nurseries.
All of them were isolated between 1993 and 1999. The
isolates have been deposited with the Centraalbureau voor
Schimmelcultures (CBS) and with the Federal Biological
Research Centre for Agriculture and Forestry (BBA).

Media

Carrot piece agar (CPA) was prepared from 50 g carrot pieces
and 22 g agar per 1000 ml distilled water (Werres et al. 2001).
Cornmeal agar (CMA), V8 agar (V8), oatmeal agar (OA) and
cherry decoction agar (CHA) were prepared according to
Gams, Hoenstra & Aptroot (1998). Liquid V8 medium was
prepared as follows: 125 ml of V8 juice (Campbell Soup) and
11.5 g of oatmeal were added to 500 ml demineralised water,
boiled for 5 min and clarified by centrifugation (15 min at
1600 rev min−1). To the V8 medium 0.94 g CaCO3 was
added, and the solution was filled up with demineralised water
to 625 ml and autoclaved for 30 min at 110 °C. Soil extract
was prepared by shaking 400 g of sandy soil with 11 l of
distilled water and then filtering and autoclaving the extract.

Colony patterns and growth characteristics

Colony patterns were examined on CHA, CMA, CPA, OA
and V8. Vegetative growth was determined by culturing the
isolates on CPA at 11 different temperatures between 2 ° and
37 ° in the dark (Kröber 1985, Werres et al. 2001). Starter
CPA cultures were incubated at 20 °C. From these, pieces were
cut out with a cork borer at 10 mm from the edge of the
growing colony and placed onto fresh CPA plates. Plates
were incubated for 24 h at 25 °C, and subsequently incubated
at the series of temperatures in the dark. The colony radius
was measured at three different points of the colony edge
before the colony had reached the edge of the Petri dish. All
measurements were performed in duplicate using two agar
plates per temperature tested.

Production of sporangia and chlamydospores

Sporangial sizes were measured from pieces of CPA colonies
flooded with aqua bidest. or with Petri solution followed by
aqua bidest. (Werres et al. 2001) and incubated for 1–2 d at
room temperature (20–22 °C). Per isolate, 200 sporangia
were studied and measured. Sporangium formation was also induced
by flooding with azalea soil extract and by placing autoclaved
hemp seeds overnight on agar cultures, and transferring them
to Petri dishes with soil extract on the next day. Sporangia are
then formed within a few hours on the hemp seed surface.
Zoospore production was observed in cultures on autoclaved
hemp seeds in soil extract. Chlamydospores were examined
on thin layer cultures or from CPA cultures on Petri dishes
(Werres et al. 2001).

Sexual reproduction

Oogonia, antheridia and oospores were examined on CPA.
Oogonia and antheridia were not produced in single cultures,
suggesting that the isolates are self-sterile and heterothallic.
Attempts were made to induce the formation of sexual
structures by pairing the isolates with A1 and A2 tester strains
of P. cambivora, P. cinnamomi, P. cryptogea, P. drechsleri, and P.
palmivora (Table 1) on CPA during up to four weeks at room
temperature (20–22 °C) in the dark. Sexual structures were only
induced by P. cryptogea A2 tester BBA 63651. To establish
which of the strains produced the oogonia, experiments were
performed in which the mating partners were separated by a
sterile cellophane membrane.

Infection trials

To study the pathogenicity of the 14 isolates, two inoculation
trials were carried out. Cuttings of about 20 cm in length of
Rhododendron cv. ‘Catawbiense Grandiflorum’ were placed in
300 ml Erlenmeyer flasks filled with 200 ml tap water. Two
Erlenmeyer flasks each with three cuttings were used for each
experiment and isolate. In the first trial, three 1 cm diam pieces
from the edge of a growing CPA culture were added to each
Erlenmeyer flask. For negative controls, pieces of sterile CPA
agar were used. Flasks were sealed with parafilm to prevent
evaporation. In the second trial, the tips of the cuttings were
cut off and a 7 mm diam mycelium disc was placed onto the
fresh wound. In the controls, sterile agar disks were taken.
Each twig tip with agar piece was subsequently wrapped in
wet tissue and incubated under a small plastic bag. Three days
Table 1. Isolates of *Phytophthora* Species used in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Other code</th>
<th>Source</th>
<th>Country, year</th>
<th>Experiments</th>
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<td></td>
<td></td>
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<td>ramorum</td>
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<td><em>Rhododendron</em> sp.</td>
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<td>All experiments</td>
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<td>The Netherlands, 1998</td>
<td>All experiments</td>
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<td>All experiments</td>
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<td>Germany, 1995</td>
<td>All experiments</td>
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<td>All experiments</td>
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<td>All experiments</td>
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<td>All experiments</td>
</tr>
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<td><em>Viburnum bodnantense</em></td>
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<td>All experiments</td>
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<td>All experiments</td>
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<td><em>P. cactorum</em></td>
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<td><em>Rubus idaeus</em></td>
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<td>AFLP</td>
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<td>P6187</td>
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<td><em>Chamaecyparis lawsoniana</em></td>
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</tr>
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<td>Scotland</td>
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<td><em>Rhododendron simsii</em></td>
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<td></td>
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<td><em>Calluna sp.</em></td>
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<td>AFLP</td>
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<td>PD 92/25</td>
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<td>ITS, AFLP</td>
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<td>PD 94/1345</td>
<td><em>Cichorium intybus</em></td>
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<td>ITS, AFLP</td>
</tr>
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<td><em>P. drechsleri</em></td>
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<td></td>
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<td><em>P. lateralis</em></td>
<td>CBS 168.42 (ex-type)</td>
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<td>Isozymes, ITS, AFLP</td>
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<td></td>
<td>CBS 102608 E. Hansen 368</td>
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<tr>
<td></td>
<td>CBS 310.62</td>
<td><em>Nicotiana tabacum</em></td>
<td>North Carolina, USA</td>
<td>Isozymes</td>
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<td></td>
<td>CBS 311.62</td>
<td><em>Nicotiana tabacum</em></td>
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<td>AFLP</td>
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<td><em>P. palmivora</em></td>
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<td></td>
<td>P446</td>
<td>Fruit</td>
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</tbody>
</table>

after incubation, the plastic bag and the tissue were removed. In both trials, the cuttings were incubated at 23 °C and 20 °C, with a day length of 16 hours in the glasshouse. The *Mdh-2*¹⁰⁰ allele and the *Mdhp*¹⁰⁰ allele of *P. nicotianae* (CBS 310.62) were arbitrarily chosen as references.

### Isozyme analysis

Isozyme genotyping, employing malate dehydrogenase (MDH, EC 1.1.1.37) and malic enzyme (MDHP, EC 1.1.1.40) was performed as described before (Man in’t Veld et al. 1998).

### Isolation of DNA

Isolates were grown in 50 ml of liquid V8 medium. After incubation at 22 °C for 15–30 d, mycelium was harvested by filtration, washed with demineralised water, freeze-dried and stored at −20 °C until isolation of DNA. Freeze-dried mycelium was harvested.
Colony pattern of *Phytophthora ramorum* isolate CBS 101553 (ex-type strain) on CPA (left), V8 (middle) and CHA (right). Upper part, top of cultures; lower part, reverse side.

(10–50 mg) was ground in microcentrifuge tubes with sterile sand and a pestle. DNA was isolated with the Puregene kit (Gentra/Biozym, Landgraaf, The Netherlands) according to the instructions of the manufacturer.

**ITS sequence analysis**

ITS–PCR was performed with primers ITS1 and ITS4 (White *et al*. 1990) as previously described (Bonants *et al*. 1997). Products were separated on 1.0% agarose gels in 0.5 × TBE buffer (10 × TBE = 9 m Tris–HCl, pH 8.0, 0.9 m boric acid, 10 mm EDTA) and bands were visualized by ethidium bromide staining and UV illumination. ITS–PCR products were sequenced with ITS1 and ITS4 as sequencing primers on an ABI3700 automatic sequencer (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). Sequences from other *Phytophthora* species (Cooke *et al*. 2000, Crawford *et al*. 1996) were obtained from Genbank. ITS-1 and ITS-2 sequences were clustered with the MEGALIGN module of the DNASTAR software (DNASTAR, Madison, WIS).

**AFLP analysis**

Protocols used for fluorescent AFLP analysis were previously described (Baayen *et al*. 2000). AFLP patterns were analyzed with ImageMaster software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Bands were scored as present or absent and the results converted into binary data, and a similarity matrix was constructed using the method of Nei & Li (1979). UPGMA cluster analysis based on this matrix was performed with Treecon software (van de Peer & de Wachter, 1994), and dendrograms were constructed with a distance scale.

**RESULTS**

**Disease symptoms**

Characteristic symptoms on *Rhododendron* plants caused by the *Phytophthora* isolates were brownish to black discoloration and dieback of shoot tips, and formation of brown spots on leaves (particularly of the tips of the leaves). On *Viburnum*, however, the disease spread from the stem base upwards. The cambial and phloem tissue was totally discoloured and the discolouration extended into the wood. No twig blight was observed in *Viburnum*, and the roots remained healthy (Werres 2001).

**Morphology**

The morphology of the isolates from all sources was
Sporangia (Fig. 2, Table 3)

On agar, all the isolates, except CBS 109278 and CBS 109279, produced sporangia singly or in clusters of 2–12 (rarely 16), arranged sympodially on long sporangiophores. Older cultures often required flooding with water to induce sporangium formation. All isolates developed sporangia on CPA in azalea soil extract, in aqua bidest., in Petri solution followed by aqua bidest. and on hemp seed cultures in soil extract. At 17 °, zoospores were developed inside the sporangia and were also released. Sporangia were mostly ellipsoid, spindle-shaped or elongated-ovoid, with a rounded base (occasionally with a tapering base), and caducous either with a short pedicel (to 5 µm), or often without a pedicel. Sporangia were semi-papillate with one narrow papilla (mostly 5–8 µm diam), which was less pronounced in young sporangia; older sporangia sometimes had a shallow apical thickening. Lengths × widths ranged from 25–97 × 14–34 µm; averages were 45.6–65 x 21.2–28.3 µm, and the average length: width ratio was ca 1.8–2.4. For Phytophthora lateralis a length: width ratio of 1.7–2 was found, and for P. palmivora a ratio of 1.4–1.5. Other authors have reported a ratio of 1.2–1.8 for P. palmivora (Brasier & Griffin 1979, Mchau & Coffey 1994, Erwin & Ribeiro 1996). The sporangia thus were distinctly more elongate than those of P. lateralis or P. palmivora. In P. lateralis CBS 102608 a small but significant number of deciduous, non-papillate sporangia with short pedicels was observed. The sporangial opening in both isolates of this species was mostly 8–10 µm wide, which may be compared to the papilla diameter in the unassigned isolates (5–8 µm).

Chlamydospores (Fig. 3, Table 3)

The unassigned isolates produced numerous chlamydospores on all media. The globose, mostly thin-walled chlamydospores were formed intercalarily and terminally, occasionally laterally;
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Fig. 2. Sporangiophore and sporangia of *Phytophthora ramorum* (different isolates). (Bar = 20 µm).

they were sized 20–91 µm (averages 46.4–60.1 µm) diam and thus were significantly larger than those of *Phytophthora lateralis*. In the latter species chlamydospores were mostly lateral and intercalary. On CPA the chlamydospores of the unassigned isolates were colourless, but chlamydospores that were in contact with living plant tissue from *Rhododendron* were dark brown. Chlamydospores often germinated with a sporangiophore carrying a few sporangia, rather than with regular hyphae.

Oogonia, antheridia, oospores (Fig. 4, Table 3)

None of the *Phytophthora ramorum* isolates was self-fertile. All the isolates produced gametangia with the A2 tester strain BBA 63651 of *P. cryptogea* (but not with any of the other tester strains), suggesting that they are heterothallic and all of mating type A1. The within-species control pairings of all tester strains were successful. Sexual structures were also produced by the unassigned isolates when separated from A2 tester strain BBA 63651 by a sterile cellophane membrane. In contrast, the isolates did not induce the formation of gametangia in tester strain BBA 63651 under these conditions. Oogonia were terminal, often also laterally sessile, smooth, nearly spherical, in the range of 24–40 µm diam, with a mean range of 29.8–33 µm. Oospores were plerotic, 20–36 µm diam, with a mean of 27.2 × 31.4 µm. Antheridia were amphigynous and mainly rounded to barrel-shaped, approx. 12–22 × 15–18 µm. With isolate PD98/8/6743 a two-celled
Table 3. Morphological characteristics of *Phytophthora ramorum* in comparison with *P. lateralis* and *P. palmivora*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sporangia Length range</th>
<th>Sporangia Width range</th>
<th>Sporangia Average Length</th>
<th>Sporangia Average Width</th>
<th>Ratio</th>
<th>Chlamydospores Chlamydospores Range</th>
<th>Chlamydospores Average</th>
<th>Oogonia Range</th>
<th>Oogonia Average</th>
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<td></td>
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<tr>
<td>CBS 101327</td>
<td>25–79</td>
<td>14–32</td>
<td>45.6 × 25.7</td>
<td>1.77</td>
<td>22–85</td>
<td>57.6</td>
<td>28–34</td>
<td>31.6 × 32.0</td>
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<td>46.4</td>
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<td>2.04</td>
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<td>2.00</td>
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<td>1.72</td>
<td>26–52</td>
<td>39.4</td>
<td>33–50*</td>
<td>NA</td>
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<td><em>P. palmivora</em></td>
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<td>BBA 64972</td>
<td>36–56</td>
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<td>45.4 × 31.2</td>
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<td>33.0*</td>
<td>32–42*</td>
<td>29.4 × 30.0</td>
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</table>

* Data taken from Erwin & Ribeiro (1996).

Dimensions are given in µm (sporangia and chlamydospores, n = 200; oogonia, n = 30). NA: not available.

![Fig. 3. Chlamydospores of *Phytophthora ramorum* (a, b = CBS 101553; c, d = CBS 109278). Bar = 20 µm.](image)
Phytophthora ramorum, a new pathogen

Fig. 4. Amphigynous antheridia, oogonia and oospores of Phytophthora ramorum (a, b = CBS 101552 × P. cryptogea BBA 63651; c, d = CBS 101549 × P. cryptogea BBA 63651. (Bar = 20 µm).

Antheridium was observed on one occasion only. Production of sex organs was confined to the carrot pieces.

Pathogenicity test

All Phytophthora ramorum isolates were pathogenic on Rhododendron. The symptoms produced were identical to those of the Rhododendron twig blight observed in nurseries. Initial discoloration of the twigs became apparent three to seven days after inoculation. Reisolation was successful for all isolates. No symptoms were observed on the uninoculated twigs.

Molecular analysis

ITS sequence analysis (Fig. 5a, b)

In an ITS sequence analysis (ITS-1 + ITS-2) performed on all 14 Phytophthora ramorum isolates, all the isolates exhibited identical ITS sequences. Their common ITS sequence was then compared to the ITS sequences of several Phytophthora species available from GenBank. Phylogenetic trees were then constructed for the ITS-1 (Fig. 5a) and ITS-2 regions (Fig. 5b) separately. The P. ramorum isolates were shown to be most closely related to P. lateralis, from which they differed in the ITS-1 and ITS-2 regions by three and eight nucleotides, respectively. They were unrelated to P. palmivora and to the other species included as outgroups.

AFLP analysis (Fig. 6)

In an AFLP analysis the 14 P. ramorum isolates formed a distinct cluster, representing a different lineage from those of the eight species used for comparison. The variability among the 14 isolates was in the same range as that in other species.

Isozyme analysis (Fig. 7, Table 2)

Isozymes of malate dehydrogenase (MDH-2) and malic enzyme (MDHP) were investigated. All 14 P. ramorum isolates were identical and contained the Mdhh-2 and the Mdhp[102] alleles in the homozygous state, as indicated by the
Fig. 5. ITS phylogram of isolates of Phytophthora ramorum, relative to \( P. \) lateralis and other Phytophthora species: (a) Phylogram based on ITS-1 region; (b) Phylogram based on ITS-2 region. Isolate numbers are indicated as such; accession numbers of sequence data obtained from GenBank are indicated in parentheses. Sequences were clustered with the MEGALIGN module of DNASTAR software. The numbers above the branches indicate bootstrap percentages (1000 replicates).

DISCUSSION

The fourteen unassigned Phytophthora isolates from Rho
dendron, Viburnum and recirculating water were shown here
to be a relatively homogeneous group in regard to their
colony characteristics, temperature-growth relationships, spor-
angial and gametangial structure, chlamydospore production
and sexual behaviour. Their identical ITS and isozyme

Fig. 7. Isozyme patterns generated in Phytophthora ramorum (lanes
2–5) and \( P. \) lateralis (lanes 6 and 7) by malate dehydrogenase (MDH).
Lanes 1 and 8 (references: allele numbers indicated at the site); 2, CBS
101551; 3, CBS 101550; 4, CBS 101553 (ex-type); 5, CBS 101548;
6, CBS 102608; 7, CBS 168.42.

Fig. 6. AFLP similarity dendrogram of isolates of Phytophthora
ramorum and other Phytophthora species. UPGMA cluster analysis
performed with Treecon software.

presence of a single band for both isozyme loci. \( P. \) lateralis
possessed two different isozyme alleles (Mdhi-2\textsuperscript{108} and Mdhi\textsuperscript{98})
at these two loci. The alleles of \( P. \) palmivora (Mdhi-2\textsuperscript{107}, Mdhi\textsuperscript{98})
also differed.
Phytophthora ramorum, a new pathogen

Werres, De Cock & Man in’t Veld,

sp. nov.

Etym.: ramus, branch: referring to the pathogenicity to twigs and branches.

Coloniae in agaris fragmentis Dauci, V8 et farina maydis concocitis submersae, zonatae, in agaro cerasorum mycelio aerio compacto, lobis radiantisibus. Hyphae primariae ad 8 μm latae. Chlamydosporae copiosae in agar et in seminibus cannabis, intercalares vel terminales, raro laterales, (sub)globosae, 10–40(–5) μm diam. Sporangia copiosa in agar et in seminibus cannabis, singula vel prolificatione sympodiali aggregata, terminalia vel post prolificationem lateralia, elliptoidea, fusiformia vel elongata, ad basim rotundata vel raro angustata, indistincte papillata, 20–32 μm diam.

Zoosporae sub 20 μm diam. Species heterothallica. Oogonia subglobosa, 28–38 μm diam, in medio 31 ± 1.5 μm; paries laevis, hyalina, ad 2(–4) μm crassa. Antheridia singula, terminalia, diclinum, amphigynum, globosa vel cupata, 12–25 ± 15–18 μm. Oospores pleroticae. Temperatures crescentiae: minima 2 °C, optimum 20 °C, maximum 26 °C. Daily radial growth on CPA at 20 °C 2.8 mm.

Phytophthora ramorum can easily be identified morphologically by its unique combination of semipapillate, deciduous sporangia with short pedicels and high length:width ratio, large chlamydosporae, relatively slow growth and low cardinal temperatures for growth. P. palmivora, which it resembles superficially, has smaller chlamydosporae, papillate sporangia with a lower length:width ratio and higher cardinal temperatures. The closest known relative, P. lateralis, has mainly non-papillate sporangia, with a wider pore, and chlamydosporae which are mainly lateral, whereas in P. ramorum sporangia are semi-papillate with a narrow pore and chlamydosporae are mainly terminal and intercalary. Very few Phytophthora species have sporangia that are both semipapillate and deciduous. Of these species, only P. colocasiae produces chlamydosporae, however, its sporangia are ovoid and have a much lower length:width ratio than those of P. ramorum.

Phytophthora ramorum was isolated from Rhododendron and Viburnum. Artificial inoculation experiments with isolates from both host plants confirmed that this species causes twig blight in Rhododendron, identical to the symptoms with which the isolates were initially associated. Reisolation of the fungus confirmed the identity of the pathogen morphologically, thus fulfilling Koch’s postulates. On old Rhododendron plants, sometimes young shoots sprout from bases of branches attacked by dieback (J. de Gruyter, pers. comm.). Apparently the plant is sometimes able to arrest the infection or it dies out naturally.

The fact that fourteen isolates, isolated in different years and localities in Germany and The Netherlands only represent the A1 mating type is striking. This suggests that the investigated isolates may constitute a clonal lineage, introduced into western Europe in recent times. However, the inraspecific variation as reflected by the differences in AFLP fingerprints and lactate dehydrogenase profiles (W. A. Man in’t Veld, unpubl.) suggests that the population is more variable and therefore more ancient. Alternatively, P. ramorum may have been introduced on several occasions, or a single introduction occurred of an already variable population. A wider population-genetic study will be necessary to elucidate this.
ACKNOWLEDGEMENTS

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REFERENCES


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