Characterization of border species among Pythiaceae: several *Pythium* isolates produce elicitins, typical proteins from *Phytophthora* spp.

FRANCK PANABIÈRES*, MICHEL PONCHET, VALÉRIE ALLASIA, LOÏC CARDIN AND PIERRE RICCI

Institut National de la Recherche Agronomique, station de Botanique et de Pathologie Végétale, BP 2078, F-06606 Antibes Cedex, France

Elicitins, holoproteins which act as inducers of hypersensitivity on tobacco, were considered as a characteristic of *Phytophthora*. They are also produced, along with glycosylated isoforms, by three species belonging to the related genus *Pythium*, *Py. vexans*, *Py. oedochilum* and *Py. marsipium*, while other *Pythium* species do not possess such proteins. Various elicitin-like sequences were determined, bringing novel features to the elicitin family, such as an histidine residue and C-terminal extensions on the deduced peptide sequences. As the unique elicitin content of these species supports a distinct location among Pythiaceae, we suggest the separation of *vexans*, *Py. oedochilum* and *Py. marsipium* from *Pythium* and consider them as linking species between *Phytophthora* and *Pythium*.

Efficient strategies for crop protection depend upon a precise knowledge of the identity of the pathogen. Unfortunately the systematics of phytopathogenic fungi such as Oomycetes (Peronosporomycetes, Dick, 1995) is imprecise and sometimes controversial. This class includes *Phytophthora* and *Pythium*, forming part of the Pythiaceae, which encompasses the most destructive fungal plant pathogens observed to date, causing a large array of diseases, on plant crops as well as in natural plant communities. Their taxonomy is still based on morphological characteristics of sexual reproductive structures (Waterhouse, 1963; Van der Plaats-Niterink, 1981; Dick, 1990), that are variable and may be influenced by environmental conditions (Hendrix & Papa, 1974). Moreover some species exhibit a wide intraspecific morphological variation, or appear to consist of several distinct taxa (Hansen & Maxwell, 1991; Förster, Learn & Coffey, 1995). Despite the development of molecular tools (for an overview, see Erwin & Ribeiro, 1996), and the emergence of new systematic approaches and evolutionary concepts (Hansen *et al.*, 1986; Brasier, 1991), the old nomenclature is still used as a reference, regardless of unresolved problems (Erwin, 1983; Brasier & Hansen, 1992), and redefinitions of species (Oudemans & Coffey, 1991; Mchau & Coffey, 1995).

Generally, *Pythium* isolates are easily distinguishable from *Phytophthora*, based on morphological (Erwin & Ribeiro, 1996), physiological (Masago *et al.*, 1977; Kato, New & Dick, 1990), and/or various molecular features (Klassen, McNabb & Dick, 1987; Martin & Kistler, 1990; Förster *et al.*, 1990; Lee & Taylor, 1992; Levesque, Vrain & DeBeer, 1994; Briard *et al.*, 1995). These criteria provide a classification which generally fits the traditional taxonomy, but some species do not exhibit all the characteristics of any of these genera, and subgeneric classifications have been proposed among *Pythium* (Belkhiri & Dick, 1988; Grosjean, 1992), if not the emergence of new genera (Ko *et al.*, 1978; Ho & Jong, 1990; Briard *et al.*, 1995). Thus new specific criteria or molecules would be of prime importance for taxonomic purposes.

A characteristic feature of *Phytophthora* species is the secretion of small holoproteins of 98 amino acids called elicitins (for reviews, see Ricci *et al.*, 1993; Ricci, 1997). These proteins, produced in high amounts by all *Phytophthora* species, except some isolates of *P. parasitica* pathogenic to tobacco, are highly conserved among the genus, but are unrelated to any other proteinaceous elicitors isolated from bacteria or fungi (Beer *et al.*, 1993; De Wit *et al.*, 1993). They act as inducers of tobacco defence responses. However, quantitative differences in activities seem to be correlated with differences in their physico-chemical properties (Ricci *et al.*, 1989; Le Berre *et al.*, 1994), which allow the definition of two classes of elicitins, acidic and basic elicitins. Both classes may be found concomitantly within a single isolate (Le Berre *et al.*, 1994).

Recently a gene encoding an elicitin from *P. parasitica* (Kamoun *et al.*, 1993) and a set of four clustered elicitin genes from *P. cryptogea* (Panabières *et al.*, 1995) were characterized. A comparison of the deduced aminoacid sequences shows a high conservation among the coding regions, including potential signal peptides which are identical between acidic elicitin genes from both species (Panabières *et al.*, 1995).

Beyond the dual distribution of elicitins among *Phytophthora*, the aminoacid sequence of an elicitin may be considered as a signature at the species level. The aminoacid sequence of two purified elicitins from distinct isolates from *P.*

* Corresponding author.

parasitica (Mouton-Perronnet *et al*., 1995) were shown to be strictly identical to the sequence deduced from a genomic clone of a third isolate (Kamoun *et al*., 1993). In contrast, both acidic and basic elicitins from two closely related, sometimes considered identical species, *P. cryptogea* and *P. drechsleri* (Förster, *et al*., 1995), exhibit 11 and 14 differences respectively (Huet, Nespoulous & Pernollet, 1992; Panabières *et al*., 1995). Therefore, elicitins may represent the new tools that would be of great value for taxonomic purposes. In this context, the purification of two elicitin-like proteins in the culture filtrate from an isolate of *Py. vexans* (Huet *et al*., 1995) would have excluded elicitins from the 'taxonomic toolbox', as they may occur in at least another genus. However, the taxonomic status of this species is unclear as it lacks some characteristics of *Pythium* (Belkhiri & Dick, 1988; Kato *et al*., 1990; Grosjean, 1992), though apparently lacking morphological characters that would enable it to be transferred to *Phytophthora*, as has been done for *P. undulata* (Dick, 1989).

We show in this report that elicitin production is not restricted to *Phytophthora*, but is shared by at least three *Pythium* species. These data suggest that a redefinition of *Pythium-Phytophthora* boundaries will eventually be necessary.

MATERIALS AND METHODS

Fungal isolates and culture

Phytophthora and *Pythium* isolates are listed in Table 1. *Phytophthora* cultures were from the INRA Antibes culture collection, and *Pythium* isolates were kindly provided by J. Mugnier (Rhône Poulenc Agrochimie, France). All strains were grown in slightly modified liquid glucose asparagine medium (Le Berre *et al*., 1994) for 3 or 10 days in the dark at 24 °C, unless otherwise indicated in the text.

Protein analysis

SDS-PAGE electrophoresis, western immunodetection, reversed phase hplc analysis and elicitin purification were performed essentially as previously described (Le Berre *et al*., 1994). The presence of sugars in glycosylated elicitins was determined according to Weiss, Postel & Gorg (1991) except that dansylhydrazine was dissolved in methanol prior to use.

Table 1. Isolates used

Isolate	Species	Origin
3	*Pythium arrhenomanes* Drechsler	RP 18.4
5	*Py. coloratum* Vaartaja	CBS 649.79
13	*Py. irregulare* Buisman	CBS 265.38
14	*Py. irregulare*	RP 97.10
17	*Py. marsipium* Drechsler	RP 108.2
20	*Py. oedochilum* Drechsler	CBS 292.37
31	*Py. ultimum* Trow	RP 176.1
34	*Py. vexans* de Bary	RP 183.4
36	*Py. vexans*	RP183.7
37	*Py. sylvaticum* W. A. Campb. & J. W. Hendrix	INRA, antibes
38	*Py. irregulare*	INRA, antibes
39	*Py. sylvaticum*	INRA, antibes
52	*Phytophthora cryptogea* Pethybr. & Laff.	INRA, antibes
149	*P. parasitica* Dastur	INRA, antibes
447	*P. undulata* (H. E. Petersen) M. W. Dick	RP 51.1

Nucleic acid manipulations

Total genomic DNA was extracted from frozen mycelium according to Panabières *et al*. (1989). Poly $(A)^+$ RNA was extracted from 3-day old frozen mycelia using the Quickprep micro mRNA purification kit (Pharmacia, Inc.). Digestion by restriction enzymes, electrophoresis, transfers and hybridizations were performed as described (Sambrook, Fritsch & Maniatis, 1989). Final washings following hybridizations were performed twice with 0·2X SSC and 0·1% SDS (w/v) for 15 min at 65°.

Cloning of amplified sequences, sequencing and data analysis

Two sets of primers were designed from conserved sequences of elicitins and called oligonucleotide 1 and oligonucleotide 2, respectively. 200 ng each of oligonucleotide 1 (5′ ATGAA-CTTCCGCGCTCTSYTYGC 3′) and oligonucleotide 2 (5′ CGAGAAGCCGTTCGCGTAC 3′) were used as primers in PCR amplification experiments, using 500 ng of genomic DNA as a template, in a 50 µl reaction mixture containing 200 µM each of dATP, dCTP, dGTP and dTTP, and 0·2 units of *Taq* DNA polymerase (Appligene, Inc.) in the amplification buffer supplied with the enzyme by the manufacturer. The cycling parameters were 35 cycles of 95° for 5 s/58° for 15s/72° for 30 s. Fragments of interest (a single fragment of *ca* 340 bp was generated in each distinct reaction) were purified and subcloned as described (Panabières *et al*., 1995).

cDNA was obtained starting from 200 ng of poly (A)+ RNA and 200 ng oligo (d)T-Not I primer using the First-strand synthesis kit (Pharmacia, Inc.) in a total volume of 25 µl. After incubation (60 min at 37°), the mixture was heated for 3 min at 90°. Then 200 ng oligonucleotide 1 were added with 0·4 unit *Taq* polymerase (Appligene, Inc.) in a final volume of 50 µl and PCR as well as purification of fragments and cloning were performed as described above. Recombinant colonies were grown overnight in LB medium and aliquots (20 µl) were centrifuged for 1 min. Subsequent pellets were dissolved in 7 µl of sterile water and used as template in PCR amplifications using oligonucleotide 1 and oligonucleotide 2 as primers. Subsequent selected clones were sequenced by the dideoxynucleotide chain termination method (Sanger, Nicklen & Coulson, 1977) using the Sequence Version 2·0 kit (U.S. Biochemical).

Sequences were aligned using Clustal W (Thompson, Higgins & Gibson, 1994). Phylogenetic analyses were carried out from the alignments, and were evaluated using the Neighbour-joining and parsimony methods included in the PHYLIP package v. 3. 572 (Felsenstein, 1993). Bootstrap confidence values (Felsenstein, 1985) were estimated from 10000 resamplings.

RESULTS

Characterization of elicitin-related peptides in some Pythium *species*

Culture filtrates from various *Phytophthora* and *Pythium* species were analysed by SDS-PAGE electrophoresis for the presence of elicitins. Results are shown in Fig. 1. A single, major peptide

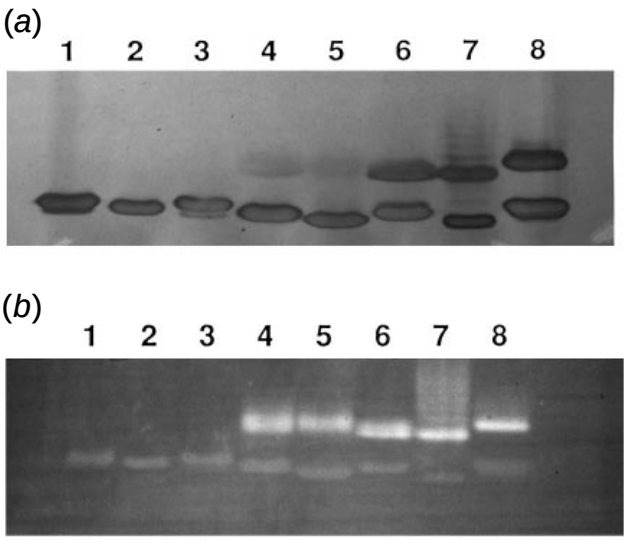

Fig. 1. Characterization of elicitin-like peptides in *Pythium* species. Culture filtrates from *P. cryptogea* (1), *P. parasitica* (2), *P. undulata* (3) *Py. vexans* isolate 34 (4), *Py. oedochilum* (5), *Py. marsipium* (6), *Py. sylvaticum* isolate 37 (7) and *Py. ultimum* (8), were analyzed by 15% polyacrylamide SDS-PAGE electrophoresis. Peptides were revealed by silver staining (*a*), while sugar moiety was characterized by dansylhydrazine (*b*) (see Materials and Methods).

of around 10000 Da, corresponding to a typical elicitin pattern, was detected in the *Phytophthora* filtrates, including *P. undulata*, whereas two main bands of around 10000 Da and 12500 Da, respectively, were detected in culture filtrates from all *Pythium* strains (Fig. 1*a*), as previously observed in a *Py. vexans* isolate (Huet *et al.*, 1995). Therefore these peptides were examined for their relationships with elicitins, and for their glycosylation status. Antibodies raised against cryptogein reacted with the elicitins of *Phytophthora* isolates as expected, and with both the 10 and 12·5 kDa bands of *Py. vexans*, *Py. marsipium* and *Py. oedochilum*, whereas no reaction was observed with bands of *Py. sylvaticum* and *Py. ultimum*, indicating the distinctive nature of these proteins (not shown). Dansylhydrazine reacted with the 12·5 kDa band of each *Pythium* species, indicating that these proteins are glycosylated (Fig. 1*b*). So it appeared that not only *Py. vexans*, but at least two additional *Pythium* species produce elicitin-related peptides.

After purification of these proteins, reverse phase-high performance liquid chromatography (RP-hplc) analysis allowed their identification as acidic elicitins, as demonstrated by their ion exchange behaviour which was similar to other elicitins such as capsicein or acidic elicitins from *P. cryptogea* (Le Berre *et al.*, 1994).

Cloning and sequence analysis

Two sets of degenerate primers (see Materials and Methods for the nomenclature of oligonucleotides) were designed from conserved sequences of elicitins, and amplified a single product from the genomic DNA of *Py. vexans*, *Py. oedochilum* and *Py. marsipium*, which was purified and subcloned into *E. coli*. Several recombinant clones were selected and sequenced, then compared with *Phytophthora* elicitin sequences (Kamoun *et al.*, 1993; Panabières *et al.*, 1995), as shown in Fig. 2. Homology level varied in a 79–98% range (Fig. 2*a*), and reflects the overall conservation of this protein family. The 60 bp 5′ terminal region, corresponding to the signal peptide, was highly conserved among all sequences, as already mentioned for elicitin genes from *P. cryptogea* (Panabières *et al.*, 1995), suggesting a common secretion pathway for all these proteins among Pythiaceae. The deduced ORFs, which span 92 amino acids, excluding the signal peptide, were aligned with both basic (cryptogein) and acidic (capsicein) elicitins, along with vex1 and vex2, recently characterized (Huet *et al.*, 1995), as shown in Fig. 2*b*. Three classes were identified. The first class comprises one sequence from *Py. vexans* (dV2), a sequence obtained from *Py. marsipium* (dM1) and capsicein. dM1, which could be called marsipin, is strictly identical to capsicein, while dV2 diverges at only one position from capsicein. The second class comprises the sequence from *Py. oedochilum* (dO1) and the cryptogein. When compared with dV2, they share several conservative replacements, such as Ala 2, Thr 14, Ser 28, Thr 65, Asn 70, and Asp 72. The third class includes three sequences from *Py. vexans*, dV1, vex1 and vex2. Surprisingly the sequences from this class are more distant from dV2 than elicitin sequences characterized in any other species from *Pythium* or *Phytophthora*. They exhibit from 18 to 21 substitutions when compared to dV2, with conserved typical features such as Asn 23, Thr 48, or Gln 57. It has to be noted that compared to this latter class, dV2 does not possess any *Py. vexans* features previously mentioned, and exhibits *Phytophthora* features, such as Leu 49 or Asn 58. This classification was strongly supported by a subsequent phylogenetic reconstruction (Fig. 3). Neighbour-joining method (Saitou & Nei, 1987), as well as parsimony analysis gave similar results. Bootstrap resampling (Felsenstein, 1985) fully confirmed the tree, and showed that the nodes separating the three groups were particularly strong (from 85 to 100%).

In order to get information about the entire coding regions of elicitins from *Pythium*, mRNA was extracted from 3 day-old cultures of *Py. vexans* and *Py. oedochilum*, and transformed into cDNA which was used as a template for RT-PCR, using primer 1 and oligo d(T). This strategy led to the characterization of three distinct sequences from *Py. oedochilum* and a single type of sequence from *Py. vexans* which were called rO1, rO2, rO3 and rV1 respectively (Fig. 4). The coding regions encompassed 360 bp, including the signal peptide, and encoded mature peptides of 100 amino acids, instead of 98 amino acids as observed in *Phytophthora* spp. rO3 exhibited several unusual features. Although 360 bp long, it corresponds to a mature peptide of 101 amino acids, as the signal peptide comprises 19 amino acids, unlike elicitins described so far (Terce-Laforgue Huet & Pernollet, 1992; Kamoun *et al.*, 1993; Panabières *et al.*, 1995). It possesses a glycosylation site at Asn 92. This location was already mentioned as a potential glycosylation site in vex1 (Huet *et al.*, 1995). Another notable feature is the presence of an His residue, never observed in any elicitin sequence to date, at position 89.

As previously observed (Panabières *et al.*, 1995) in elicitin genes from *P. cryptogea* or other *Phytophthora* species (Panabières *et al.*, unpublished results), sequence homology is mainly restricted to the coding region (Fig. 4). Thus the 3′

(*a*)

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dV2   ATGAACTTCCGCGCTCTGCTCGCCGCTACCGTCGCTGCCCTCGTCGGCTCCACCTCCGCC GCCACGTGCACGACCACCCAGCAGACCGCCGCGTACGTCG
dM1   ..................CT.......................................... .....................................T.
b14   ..................T.......C........C........G.............. A..........C..G...........G..T.........
ParA1 ..................T.......C........C..................... A..........C.....G.....A..T..G........G.
X24   .........AC............T..CGT..C...C...T.G.....A..TG..AA.... A..G.......CG...............T......AAGA
dO1   ..................T....T..CG...C...C......A.....A..G..AA.... ...G.C.....CT..................C.....TA
dV1   ..................T..T..C....C...C.............TGT.G...G. A..GGC.....C..G..G........G.............
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dV2   CCCTGGTGAGCATCCTCTCGGACTCGTCGTTCAACCAGTGCGCCACGGACTCTGGCTACTCGATGCTGACGGCTACGGCACTGCCCACGACGGCCCAGTA
dM1   ..........................................................................C.....G...................
b14   .T..C..........G.....T.....................................C.....C...T.G..C...........A......
ParA1 .G..C..A..............A.................T.G.................A...........C..CT.GT............AG.....
X24   .G..C.........T.G.....CG.................T.......T..G........C...........C.A...C..C.....C.....G.....
dO1   .G..C..C.A.................C...TCG......T.G..C.....G.................C..AA.G..T..G...GAC..G.....
dV1   .G...A.C...C.G..GAGC......AAC............T.G........G..T.................G.....T..C..G.......AG.....
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dV2   CAAGCTCATGTGCGCGTCGACGGCGTGCAACACGATGATCACCAAGATCGTGTCGCTGAACCCCCCTGACTGCGAGCTGATGGTGCCCACGAGCGGTCTG
dM1   ........................A.....................................................C.........A.........
b14   ..................C............T.C......G........A.TAGC......G.G..C............AC..............C...
ParA1 ..............................G..........A..........A..........G..C.........T...C......T........C...
X24   ..................C.....A.......C.......AG.........A..........G..CA.......C....C..............C...
dO1   ....AAG........C..................C.......A..........A..........G..CA.T.....C....C......G........C...
dV1   ..CCAAG.................T...C.GGGC.......AG.CC...A.CG.CGCC.....G...........C....C...C..G........C...
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dV2   GTGCTCAACGTTTACTCGTACGCGAACGGCTTCTCG----------------------
dM1   ..................................----------------------
b14   ...........G.......................GCCACGTGCGCTTCGCTGTAA
ParA1 ..A........G.T.A.............G......TCTACGTGCGCGTCACTGTAA
X24   ..A........G.......................AACAAGTGCTCGTCGCTGTAA
dO1   ...........G.......................----------------------
dV1   ...........G...GA..................----------------------
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(*b*)

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                10        20        30        40        50        60        70        80        90
dV2         ATCTTTQQTAAYVALVSILSDSSFNQCATDSGYSMLTATALPTTAQYKLMCASTACNTMITKIVSLNPPDCELMVPTSGLVLNVYSYANGFS--------
dM1         ..................................................................T.................--------
capsicein   ..................................................................T.................ATCASL--
cryptogein  TA..A.......KT.......A.....S..........K.....................K...T....N.D.T.................NKCSSL--
dO1         .A..S........T..N.......S..S...........T...D....K...........N...T....N.D.T.................--------
vex1        TA...................DN.S..S................E..TK.......QA..AN.IT........T...........E.....BATCASL--
vex2        TA.........F.....V..TDN....S................E..TK.......QG..QT.IAA.A.....T...........E......AKCSSLSS
dV1         TG.............I.L....N....S................E..TK.......QG..KT.IAA.....D.T...........E.....--------
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Fig. 2. Characterization of elicitin-like sequences from *Pythium* species. (*a*) Comparison of amplified sequences from *Py. vexans* (dV1 and dV2), *Py. marsipium* (dM1), and *Py. oedochilum* (dO1) with coding sequences of parasiticein (parA1) or acidic (B14) and basic (X24) elicitins from *P. cryptogea*. Bold nucleotides correspond to the positions of the primers locations. Underlined nucleotides correspond to putative signal peptides. (*b*) Alignment of the deduced peptides with elicitins.

untranslated regions (UTR), although similar in size (125–142 bp) and in their GC content (51–57%), largely diverged and could not be aligned properly. However, an exception presented here is the extreme homology observed between rO1 and rV1, which is close to 99%, including the 3′UTRs.

Comparison of cDNA and genomic sequences led to a refined nomenclature of *Pythium* elicitins. Thus rV1 and dV1 diverged only by three nucleotide substitutions, leading to two aminoacid changes, and were considered as vexin Ia and vexin Ib respectively, while dV2, which belongs to a distinct set of genes, was assessed as representative of vexin II. The elicitins already isolated from *Py. vexans* (Huet *et al*., 1995) may represent additional classes. rO1 was designed as oedochilin I. rO2 is identical to dO1 and probably represents its transcript. rO2 is quite identical to rO3, except the ten C-terminal aminoacids, where numerous non-conservative substitutions are observed. Thus rO2 and rO3 were designed as oedochilin II and oedochilin III, respectively.

An alignment of all elicitin sequences available was performed (Fig. 5), including those of *hae* genes, called B20 and B26 from *P. cryptogea*, that encode elicitin-like ORFs and from which no transcript has been observed (Panabières *et al*., 1995). A primary observation is that mutations are located on various regions of the sequence, alternating with more conserved positions. A finer analysis, based on crystallographic data recently published concerning the three-dimensional structure of cryptogein (Boissy *et al*., 1996), emphasizes some major points. Most of the mutations are located mainly in regions corresponding to α-helices. If we assume that all elicitins have a similar overall tertiary structure, we can propose that, except for the Thr58Gly mutation located in helix α4, most other mutations would not affect the tertiary structure. However, no mutations occur in the region of Ω-loop from Tyr33 to Pro42, as well as in the antiparallel two-stranded β-sheet which is also conserved among elicitins. As these two structures represent a beak-like motif which could be a major site for recognition of a receptor and/or ligand (Boissy *et al*., 1996), the conservation of this structure would imply that *Phytophthora* and *Pythium* elicitins may have similar biological properties. In addition all aminoacids which form

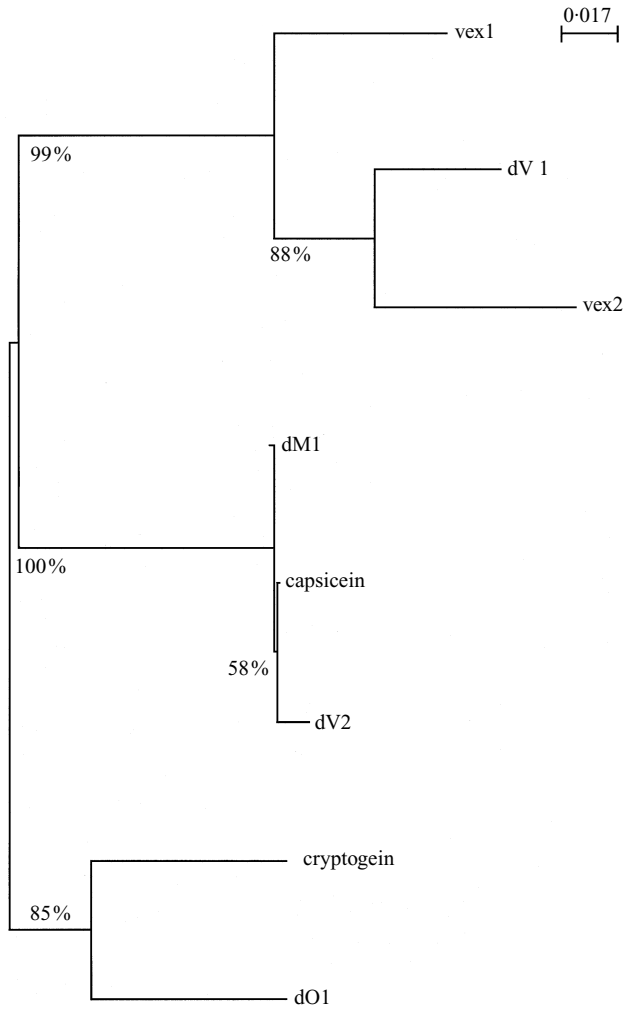

Fig. 3. Neighbour-joining phylogenetic tree of sequences aligned in Fig. 2*b*. Confidence limits of branches (indicated in % along the branches) were created in a bootstrap analysis using 10000 trials. The scale bar represents 1·7% sequence divergence.

the hydrophobic core of cryptogein represent invariant positions, except Phe91. In contrast a salt bridge (Asp21-Lys62) would be lacking in typical *Pythium* elicitins, due to the mutation at Lys62.

Phylogenetic reconstructions were performed as above and the resulting tree allowed the grouping of sequences in four clusters (Fig. 6). The first cluster (I), represented by the *hae* genes, is strongly supported by bootstrap analysis. A second cluster (II) is exclusively composed by *Pythium* elicitins. Here again, the nodes separating the different sequences are strongly supported. Other *Pythium* elicitins belong to different clusters, interspersed with *Phytophthora* elicitins. Oedochilin I and III belong to the cluster which encompasses the basic elicitins (cluster III), as marsipin and vexin II are clustered with most of the acidic elicitins (cluster IV). Despite the relevance of a distinction between cluster III and cluster IV (confidence limit of *ca* 75%), it has to be noted that the internal organization of these clusters lack reliability as confidence limits are specially low (25–45%).

The distribution of elicitin sequences in four main clusters is independent of the protein length, and suggests that the C-terminus extension observed in some sequences is of poor value in phylogenetic analyses. In contrast, some amino-acid positions could be assigned as potential signatures, or be related to some nodes. Thus the Ser-Asn 23 transition appears to be typical of cluster II, whereas the Asn-Gln 57 transition might be a signature of the node separating clusters I and II. Moreover, the Leu49Lys seems to be restricted to *Pythium* isolates.

Elicitins are encoded by a small multigene family in Pythium

Genomic DNA from various *Pythium* species was analysed on Southern blots, using a probe derived from the vexin Ia clone (Fig. 7). Elicitins and related sequences were detected only in DNA of elicitin-producing isolates, as well as in *P. cryptogea* as a control, whereas no signal was observed in the DNA from other *Pythium* species. Moreover, identical patterns were observed when the hybridization was performed with an elicitin probe from *P. cryptogea* (not shown). In addition, the hybridization patterns are quite simple in *Pythium* DNA, compared with the *P. cryptogea* pattern. This does not mean a lesser content in elicitin genes, as a significant diversity was observed among *Pythium* elicitin sequences (Huet *et al*., 1995; this work). The simple RFLP pattern rather reflects a more conserved elicitin gene organization among *Pythium* species than in *Phytophthora*.

DISCUSSION

Determination of new elicitin features among Pythiaceae

Elicitins were first characterized by P. Bonnet (Bonnet, Poupet & Bruneteau, 1985). This family of proteins was extensively studied among species of *Phytophthora* and, until very recently, additional sequence characterizations, although introducing new variable positions, did not alter the consensus features of elicitins. Canonical elicitins could be defined as (i) holoproteins of 98 aminoacids; (ii) lacking three aminoacids (Trp, His and Arg), and containing 6 Cys and 3 Met; (iii) not undergoing obvious post-translational modifications such as glycosylation; (iv) distributed approximately into two major classes: basic and acidic elicitins (whose pI seemed related to the extent of necrosis induced on tobacco), and (v) until now, specific of *Phytophthora*. The characterization of several elicitin-like proteins in *Pythium* species, among which a glycosylated form (Huet *et al*., 1995; this work), along with the identification of an His residue in one sequence, suggests that the paradigm of elicitins has now to be ruled out and that the structural definition of these proteins needs to be reassessed.

Sequence comparison indicated that *Pythium* elicitins belong to different classes. One group contains sequences that fit the above definition of *Phytophthora* elicitins. However, the length of these ORFs is unknown as they were deduced from genomic DNA amplifications using internal primers. Therefore, further investigations are necessary in order to determine the precise length of these sequences and their putative expression, as similar sequences have not been characterized at the mRNA level. Other *Pythium* elicitins can be roughly classified into

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rV1   ATGAACTTCCGCGCTCTGTTTGCTGCCACCGCCGCCGCCCTCGTCGGCTCTGTCGCCGGCACCGCCTGCACCACGACGCAGCAGACGGCC
rO1   ................C.....................................................................
rO2   ..........................................A.....A.C.C.AA..C.G..........T.C..C........C...
rO3   ........................---............A.T...A.C.C.AA..C.G..........T.C..C........C...

rV1   GCGTACGTCGCGCTGATCAGCCTGCTGAGCGACTCGAACTTCAACCAGTGCTCGACGGACTCGGGTTACTCGATGCTGACGGCGACGGCT
rO1   .........A.....................................................................................
rO2   ........TA....CG...A.A.C..CTCG......TC....TCG...........C........C.................C..AA.G
rO3   ..C......A....CG...A.A.C..C.CG......TC....TCG...........C........C.................C......G

rV1   CTCCCGACGACGGAGCAGTACACCAAGATGTGCGCGTCGACGGCTTGCCAGGGCATGATCAAGACCATCATCGCCGCCAACCCGCCTGAC
rO1   .....................................................................................
rO2   ..T......GAC.C........AG.....................G...A.CAC.........C.AG...G.GA.GCTG........CA..
rO3   .............C.......AG.....................G...AGCAC.........C.A...TG....GCTG........CA..

rV1   TGCGAGCTGACGGTCCCGACGAGCGGCCTGGTGCTCAACGTGTACGAGTACGCCAACGGCTTCTCGGACAAGTGCTCGTCGCTCTCCGCG
rO1   .....C.....................................................C.TCTC....................G...
rO2   .....C........G.................................TC.....T.............TC..............G..GT...
rO3   .....C........G.................................TC.......C..AA..A.AAC.C..C....ATCCT...TA.G...

rV1   ---TAAGCGGTATGAGCTCTGCTTTG--CGC-TCTAT-CCACAGT--CCGCCAGGTTGCCTGCGTC-TATATA-CC-ACCGA-GAG-GCG
rO1   ---TAA.....................................C.....................................................
rO2   ---TAA.G--C--G-T.-GGCAGTTCG.GTGTG-.CC-...T.TC-.T-.A--C--C.T..G...A----...G..-..TAG-..-T..C
rO3   GCGTAA...A.--.--C-..-....---.CA.C.AC.G.-A-GC....GGC-T.--..--A..CCGTG-C..T-TGT.....CT..T.TA

rV1   -TCTCGTTTCCTAGCTACTTTATGCA--G-CTAC-CGATGAAATGC-CAATGAACGTTGTGTTTTACGCGTT(A)n
rO1   .....................................C.....................A...........(A)n
rO2   C..A---AA-.GC.-..G.A..AT...T.AAGC.-.-T..GAA.A--...C.GT.-----......AA.A.(A)n
rO3   -..G---CG-....-.T..GCGC-CGGC.T....G.--...--.CG-...G.T..--AAA...GG.TTT.(A)n
```

Fig. 4. Characterization of elicitin-encoding transcripts from *Pythium*. Alignment of elicitin-like transcripts of *Py. vexans* (rV1) and *Py. oedochilum* (rO1, rO2, rO3). Termination codons are underlined.

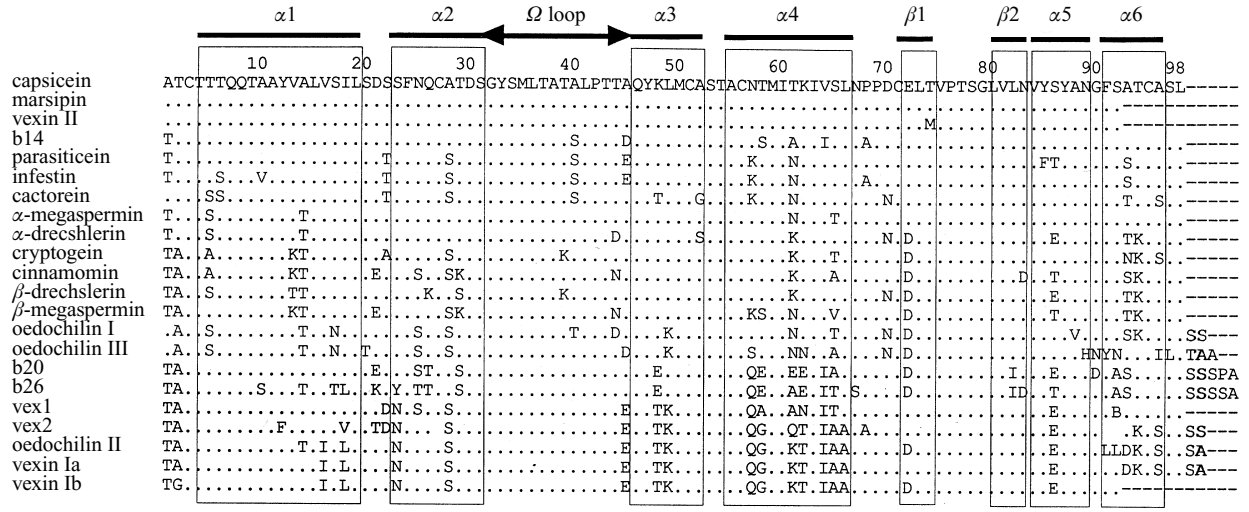

Fig. 5. Alignment of deduced aminoacid sequences of the transcripts with genomic amplified sequences (see text), and elicitins. Location of different structural features follows results already published (Boissy *et al.*, 1996).

two groups. One group comprises 100 amino acid peptides, whose net charge is identical (−4) and which possess at location 46–50 a typical sequence QYTKM, while *Phytophthora* elicitins (or *Pythium* elicitins from the group I) exhibit a QYKLM sequence at this location. These peptides are oedochilin I, vexin I (a and b) and vex2. Oedochilin II may be related to this group. Then a last group includes glycosylated elicitins, such as vex1 or oedochilin III, regardless of the amino acid length (98 and 101 amino acids, respectively), and the amino acid content, such as the presence of an His residue in oedochilin III. The tertiary structure of cryptogein was recently determined (Boissy *et al.*, 1996). If acidic elicitins have similar structures (which remains to be proved), then the novel mutations observed in *Pythium* elicitins would not modify the overall conformation, nor affect the potential active site. As the precise role of elicitins is still unknown, comparison of physiological, ecological and genetic characteristics of elicitin-producing species at an intergeneric level could be of great value to unravel the function of these proteins. In a previous study, vex1 and vex2 have been shown

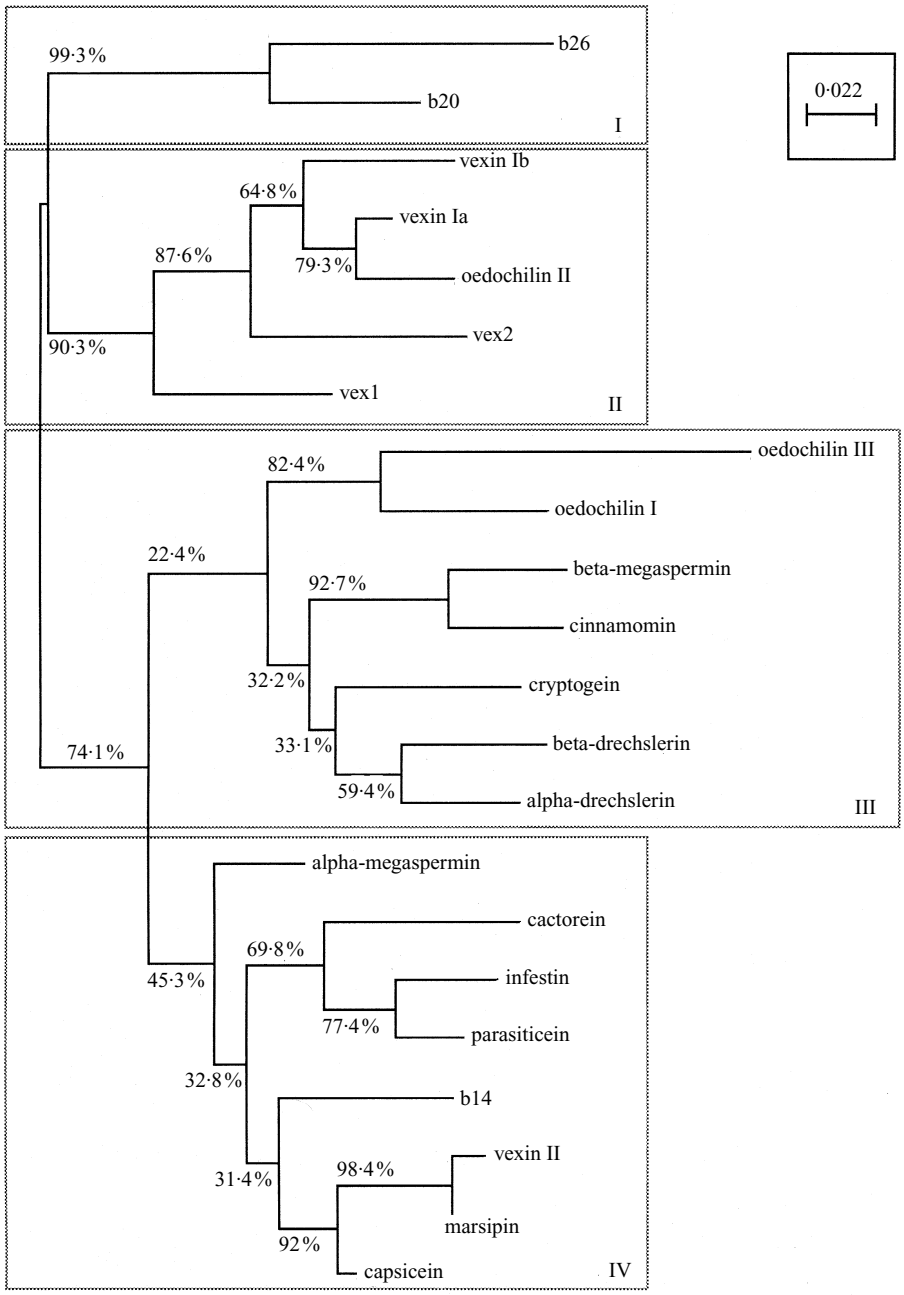

Fig. 6. Neighbour-joining phylogenetic tree of sequences aligned in Fig. 5. Confidence limits of branches (indicated in % along the branches) were created in a bootstrap analysis using 10000 trials. The scale bar represents 2·2% sequence divergence.

to exhibit a higher necrotic activity on tobacco at low doses, compared with typical acidic elicitins (Huet *et al.*, 1995). Some residues have been pointed out as potential enhancers of this activity. The newly defined sequences (this work) have to be checked for their potential activity. Overexpression of these sequences is in progress in order to test the hypothesis of Huet and coworkers. At this point we may note that despite numerous additional sequence determinations, we failed to characterize sequences identical to vex1 and vex2. In addition we never encountered *Pythium* transcripts encoding elicitins of 98 amino acids such as vex1. One hypothesis is that this protein, purified from an 8 day-old culture, originates from a transcript that is not present in our mRNA preparation. Kinetics experiments are needed to confirm this hypothesis and could give new insights about the regulation of elicitin genes in *Pythium*. Alternatively, this failure may reflect an intraspecific diversity. This would be unusual for elicitins, as proteins, or the corresponding genes, have, until now, been found strictly identical at the species level (Kamoun *et al.*, 1993; Le Berre *et al.*, 1994; Mouton-Perronnet *et al.*, 1995; Panabières *et al.*, 1995; Panabières *et al.*, unpublished results), and nearly identical among very closely related species (V. Allasia *et al.*, unpublished results). Such an intraspecific diversity of elicitins, if confirmed, would be a new characteristic of *Pythium* species (supposing that the presence of elicitins of 98 amino acids is confirmed in *Pythium* species). On the other hand, the homology observed between vexin I and oedochilin I, including their 3′ UTRs, could be considered as inconsistent with a wide intraspecific diversity. The gene flow suggested by this homology between the two species may be explained

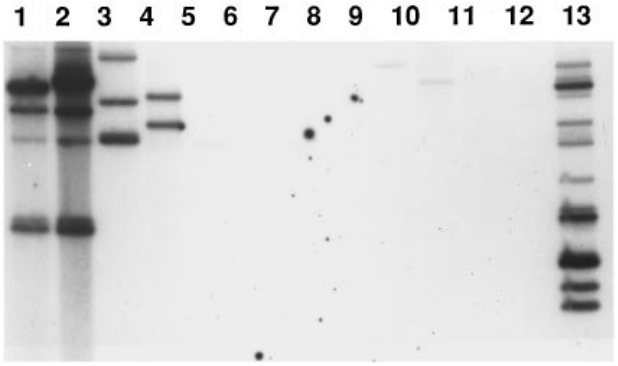

Fig. 7. Southern analysis of genomic DNA from *Py. vexans* isolate 36 (1), *Py. vexans* isolate 34 (2), *Py. marsipium* (3), *Py. oedochilum* (4), *Py. ultimum* (5), *Py. sylvaticum* isolate 37 (6), *Py. sylvaticum* isolate 39 (7), *Py. irregulare* isolate 38 (8), *Py. irregulare* isolate 13 (9), *Py. irregulare* isolate 14 (10), *Py. arrhenomanes* (11), *Py. coloratum* (12) and *P. cryptogea* (13). DNA (5 μg) was digested with Bam H I and hybridized with vexin Ia under stringent conditions (see Methods).

by common host plants (Van der Plaats-Niterink, 1981). Nevertheless, the intraspecific diversity, along with an unexpected interspecific conservation, might reflect the flexibility of these species, supporting their particular status, as previously mentioned (Grosjean, 1992).

RFLP patterns of elicitin genes from *Pythium* were shown to be rather simple, compared with that of *P. cryptogea* (this work) or other *Phytophthora* species (Panabières *et al.*, unpublished results). This may be due to a more conserved organization of elicitin genes in *Pythium* species, as a wide diversity was observed at the nucleotide level. Therefore additional information is required in order to characterize more accurately the organization of elicitin genes in these species. For example, it would be of interest to determine whether elicitin genes are tandemly clustered, as in *P. cryptogea*, and probably other *Phytophthora* species.

Elicitins as a tool for identification, but not for phylogeny

The proteinaceous pattern of the culture filtrate, combined with an immunological labelling, enabled us to distinguish between *Phytophthora* and *Pythium*: *Phytophthora* secrete a single, 10 kDa elicitin, while *Pythium* secrete two proteins, including one glycosylated form, both immunologically unrelated to elicitins. Thus culture filtrates would permit a preliminary classification. However, the intermediate status of *Py. vexans*, *Py. oedochilum*, and *Py. marsipium* (producing two elicitin-related proteins) implies refined identification techniques, in order to solve potential additional ambiguous situations. The alignment of 21 elicitin sequences showed that mutations are located throughout the whole sequence, splitting the elicitin sequence into alternate variable and more conserved regions. This split could facilitate the design of numerous probes whose different levels of specificity would allow their use for various identification purposes.

Phylogenetic analyses were performed following various methods on the whole set of elicitin sequences. Bootstrap values were calculated in order to support the phylogenetic results. This is the first time that elicitin sequences have been analysed under such rigorous conditions, despite preliminary analyses (Huet *et al.*, 1995). The resulting tree clustered elicitins regardless of their length and glycosylation status or of their generic origins. Thus both *Pythium* and *Phytophthora* elicitins were placed in the same clade while elicitins from a single isolate were found in distinct clades. Despite indications concerning a common origin and similar evolution of some *Pythium* elicitins and *hae* genes, several confidence limits under 50% were observed indicating that various branches concerning elicitins are too poorly supported to be considered. So it appears very difficult, if not elusive, to estimate accurate phylogenetic relationships among elicitins. In this context, results presented elsewhere (Huet *et al.*, 1995), which were not validated by bootstrap resamplings, have to be considered cautiously, and their subsequent interpretations concerning particular relationships between vexins and some *Phytophthora* elicitins are inconsistent. As various elicitins from a single isolate are located in different clades, this protein family does not represent a good phylogenetic tool, compared with other molecules such as rDNA (Briard *et al.*, 1996; Cooke *et al.*, 1996), or previous morphological criteria (Shahzad, Coe & Dick, 1992; Dick, 1995).

Towards a revisited classification of Pythiaceae?

The taxonomy of Oomycetes has long been controversial, as well as the position of this class among living organisms (for an overview, see Erwin & Ribeiro, 1996), which was changed from the kingdom Myceteae to the kingdom Chromista (Cavalier-Smith, 1987; Dick, 1995).

The classification of Oomycetes is primarily based on morphological criteria. As additional tools such as total protein electrophoresis, (Clare & Zentmeyer, 1966), isozymes (Oudemans & Coffey, 1991) and analysis of various DNA molecules (Panabières *et al.*, 1989; Förster & Coffey, 1991; Briard *et al.*, 1995; Cooke *et al.*, 1996) solved some problems of nomenclature, it appears that many taxonomic questions are still open. The development of these techniques, along with the refinement of morphological and biometric criteria of taxonomic value (Shahzad *et al.*, 1992; Dick, 1995) were followed by successive revisions of the taxonomy of *Phytophthora* (Waterhouse, 1963; Newhook, Waterhouse & Stamps, 1978; Stamps *et al.*, 1990) and *Pythium* (Van der Plaats-Niterink, 1981; Dick, 1990). Nevertheless, many isolates have not been assigned to species, due to a lack of reliable features, and classification of *Pythium* is particularly imprecise. These taxonomic advances came along with a relevant question, that is the species definition. As a consequence, several species have been redefined, if not separated into distinct taxa (Hansen *et al.*, 1986; Hansen & Maxwell, 1991), and new genera have been erected among Peronosporales (Ko *et al.*, 1978; Ho & Jong, 199), without a precise location within the order (Erwin & Ribeiro, 1996).

As Oomycetes possess characteristics totally different from other fungi (Förster *et al.*, 1990; Brasier & Hansen, 1992), specific tools and evolutionary concepts have to be adapted to these organisms (for a review, see Hall, 1996). Among them, the morphospecies concept has prevailed. Discontinuities within morphological criteria and phenotypes separate and

define species, or morphospecies. However, some isolates lack some structures required for identification. As considerable variation may be observed within a single *Phytophthora* species, whereas distinct species may be almost morphologically identical, biological species or phylogenetic species concepts were applied to Pythiaceae, with other limitations. Recently Brasier & Hansen (1992) proposed a new species definition appropriate to *Phytophthora* (and also to *Pythium*) which claims that 'species are groups of populations that share a common lineage and have maintained genetic similarity in morphology, physiology, and ecological behaviour. In most cases, the cohesive force may be natural selection (…), and in some cases, gene flow between interbreeding populations'. As the potential for outbreeding of some species (Francis & St Clair, 1994; Whisson *et al*., 1994) leading to the emergence of new races, as well as the potential hybrid status of some others (Sansome, Brasier & Hamm, 1991; Brasier, Rose & Gibbs, 1995) were shown or suggested, unexpected evolutionary relationships among Pythiaceae due to genetic exchanges may be found in the future, with the help of both morphological and molecular approaches, in the absence of an ultimate taxonomic method.

Various criteria generally assign *Pythium* and *Phytophthora* as clearly distinct genera (Belkhiri & Dick, 1988; Kato *et al*., 1990, Briard *et al*., 1995; Erwin & Ribeiro, 1996). The transfer of *Py. undulatum* to *P. undulata* (Dick, 1989), based on morphological criteria, was confirmed by rDNA sequence analysis (Grosjean, 1992). In the present work, *P. undulata* had an elicitin pattern typical of *Phytophthora* and is correctly placed in *Phytophthora sensu lato*. Therefore, the elicitin pattern appears as an accurate criterion for identifying *Phytophthora* species among *Pythiaceae*. The situation is more complex for *Py. vexans*, which always presented taxonomic problems. It was classified as *Pythium* (Masago *et al*., 1977), or shown to exhibit some *Phytophthora* characteristics (Belkhiri & Dick, 1988; Kato *et al*., 1990), then left in an ambiguous status (Grosjean, 1992) or suggested as belonging to a new genus (Briard *et al*., 1995). From the data presented here, we prefer to consider *Py. vexans* as a missing link between *Pythium* and *Phytophthora*. Dick presented *Py. vexans* as an intermediate species in the phenetic sense, which may be complex, and possibly formed by several taxa, possibly in more than one genus (Dick, 1990). Our results, based on phenetic (elicitin patterns) and phylogenetic data (lack of generic clustering of elicitin sequences) as well as the inferred interpretations are in agreement with such an intermediate position. If *Py. vexans* is a missing link among Pythiaceae, *Py. oedochilum*, *Py. marsipium* and maybe other species share this intermediate position, as they exhibit similar features, such as rDNA characteristics (Grosjean, 1992) or elicitin distribution (this work). From rDNA analysis, one may expect a similar phylogenetic location for *Py. helicoïdes*, which possesses rDNA characteristics similar to the three species mentioned above (Grosjean, 1992). This would rule out a generic discontinuity between *Pythium* and *Phytophthora*. Because *Pythium* and *Phytophthora* are frequently isolated concomitantly on a same host we may hypothesize that the host range would favour gene flow between isolates belonging to both genera or having an intermediate position. If indeed elicitins allow to delineate new, flexible borders among the Pythiaceae, it would be worth looking for similar links between *Phytophthora* and other Peronosporaceae, by examining the occurrence of elicitins among this subclass.

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