RESEARCH LETTER

Intraspecific and within-isolate sequence variation in the ITS rRNA gene region of *Pythium mercuriale* sp. nov. (*Pythiaceae*)

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Abstract

Sixteen *Pythium* isolates from diverse hosts and locations, which showed similarities in their morphology and sequences of the internal transcribed spacer (ITS) region of their rRNA gene, were investigated. As opposed to the generally accepted view, within single isolates ITS sequence variations were consistently found mostly as part of a tract of identical bases (A–T) within ITS1, and of GT or GTTT repeats within the ITS2 sequence. Thirty-one different ITS sequences obtained from 39 cloned ITS products from the 16 isolates showed high sequence and length polymorphisms within and between isolates. However, in a phylogenetic analysis, they formed a cluster distinct from those of other *Pythium* species. Additional sequencing of two nuclear genes (elongation factor 1α and β-tubulin) and one mitochondrial gene (*nadh*1) revealed high levels of heterozygosity as well as polymorphism within and between isolates, with some isolates possessing two or more alleles for each of the nuclear genes. In contrast to the observed variation in the ITS and other gene areas, all isolates were phenotypically similar. *Pythium mercuriale* sp. nov. (*Pythiaceae*) is characterized by forming thin-walled chlamydospores, subglobose to obovoid, papillate sporangia proliferating internally and smooth-walled oogonia surrounded by multiple antheridia. Maximum likelihood phylogenetic analyses based on both ITS and β-tubulin sequence data place *P. mercuriale* in a clade between *Pythium* and *Phytophthora*.

Introduction

Molecular biology is increasingly being used in fungal taxonomy and disease diagnostics. Genes coding for rRNA have largely been used as molecular markers for inferring the evolutionary history of organisms at different taxonomic levels (Christen, 1994; Sogin, 1994). The internal transcribed spacer (ITS) region of the rRNA operon is often studied as a potential source of sequence variation among closely related organisms, because it has a lower degree of conservation when compared with those of the rRNA-coding genes (Windsor *et al.*, 2006). The ITS region of the rRNA gene has become a useful tool for identifying as well as detecting *Pythium* species from environmental samples (Lévesque & De Cock, 2004; Tambong *et al.*, 2006). A large ITS sequence database for *Pythium* species is now available in the Gen-Bank database, which can be used for determining the identity or the phylogenetic position of unknown *Pythium* isolates (Matsumoto *et al.*, 1999; Lévesque & De Cock, 2004).

It is generally accepted that within a single isolate/individual, the sequences of multiple rRNA genes are identical or nearly identical. A well-known exception to this is *Plasmodium*, whose genome contains two types of 18S rRNA genes that are expressed at different developmental stages of its parasitic life cycle (Gunderson *et al.*, 1987). Sequence heterogeneity is also known in two 18S rRNA genes in the halophilic archaebacterium, *Haloarcula*

Table 1. *Pythium mercuriale* isolates used in this study

Species	Isolate	Isolate supplier	Host	Origin	ITS Genbank accession
Pythium mercuriale	STE-U 6124	C.F.J. Spies	Grapevine (*Vitis vinifera*)	South Africa (Vredendal)	DQ916347
					DQ916348
					DQ916349
					DQ916350
	STE-U 6125	C.F.J. Spies	Grapevine (*Vitis vinifera*)	South Africa (Malmesbury)	DQ479405
					DQ916351
					DQ916352
	STE-U 6126	C.F.J. Spies	Grapevine (*Vitis vinifera*)	South Africa (Malmesbury)	DQ916353
					DQ916354
					DQ916355
					DQ916356
	STE-U 6127	C.F.J. Spies	Grapevine (*Vitis vinifera*)	South Africa (Vredendal)	DQ479404
					DQ916357
					DQ916358
	STE-U 6204	W.J. Botha	Macadamia (*Macadamia integrifolia*)	South Africa (Tzaneen)	DQ916359
					DQ916360
					DQ916361
					DQ916362
					DQ916363
	STE-U 6205	W.J. Botha	Macadamia (*Macadamia integrifolia*)	South Africa (Tzaneen)	DQ916364
					DQ916365
					DQ916366
					DQ916367
					DQ916368
	STE-U 6206	W.J. Botha	Macadamia (*Macadamia integrifolia*)	South Africa (Tzaneen)	DQ916369
					DQ916370
					DQ916371
					DQ916372
	STE-U 6207	W.J. Botha	Grapevine (*Vitis vinifera*)	South Africa (Malmesbury)	DQ916373
					DQ916374
					DQ916375
	PE16	E. Sánchez-Hernández	*Quercus ilex*	Spain (Huelva)	DQ176005
	UASWS0187	L. Belbahri	Soil	Poland (Pila)	DQ217605
	UASWS0263	L. Belbahri	Soil	Poland (Pila)	DQ525087
	UASWS0268	L. Belbahri	Soil	Poland (Pila)	DQ525092
	UASWS0269	L. Belbahri	Soil	Poland (Pila)	DQ525093
	UASWS0271	L. Belbahri	Soil	Poland (Pila)	DQ525095
	UASWS0276	L. Belbahri	Soil	Poland (Pila)	DQ528747
	F-148	B. Paul	Soil	France (Burgundi)	DQ211516

marismortui (Mylvaganam & Dennis, 1992), in 26S rRNA genes of *Pneumocystis carinii* (Liu *et al*., 1992), in the ITS sequence of the flagellate *Dientamoeba fragilis* (Windsor *et al*., 2006) and in human 28S rRNA expansion segments (Gonzalez *et al*., 1985; Maden *et al*., 1987; Leffers & Andersen, 1993). Furthermore, within the ITS region, the presence of multiple ITS copies that are not identical within the same individual has often been reported in higher plants (Baldwin *et al*., 1995; Dubcovsky & Dvorák, 1995) and fungi (Ko & Jung, 2002). Therefore, the presence of heterogeneity

in rRNA gene regions within the same individual should be assessed carefully when performing molecular phylogenies with nrDNA sequences (Feliner & Rosselló, 2007). Polymorphism in nrDNA could for example, among other factors, result from the existence of pseudogenes, i.e. non-functional nrDNA gene copies (Bailey *et al*., 2003).

In this study, we first characterized 16 morphologically similar *Pythium* isolates obtained from infected plant material or from soil samples taken from different locations in France, Poland, South Africa and Spain. The ITS

sequences of the rRNA gene of these isolates proved to have an unusually high degree of divergence within single isolates. However, these sequences are unique and are quite different from those of other species of *Pythium*. Therefore, we described these isolates as a new *Pythium* species, *Pythium mercuriale* sp. nov. (*Pythiaceae*), which contains a high level of ITS sequence variations within and among isolates.

Materials and methods

Isolates

Details on the origin of the 16 *Pythium* isolates included in this study are provided in Table 1. Soil isolations were made using a baiting technique with young *Quercus* leaves (Jung *et al.*, 2003). Root and crown isolations were made by first washing the samples with distilled-deionized water and blotting dry on paper towels. Subsequently, the root and crown tissues were cut into 1 cm pieces and placed on a medium selective for *Pythium* that contained parachloromercuribenzoate (PCMB), ampicilin rifampicin and pimaricin (PARP medium; Jeffers & Martin, 1986). Plates were incubated at 25 °C and mycelia were subcultured onto 0.7% water–agar plates. Cultures were grown on potato carrot agar (PCA), as well as on boiled hemp-seed halves in water (Paul *et al.*, 2006). The cultures were maintained in the culture collection of the School of Engineering of Lullier, University of Applied Sciences of Western Switzerland, Jussy, Geneva, Switzerland.

Morphology

Axenic cultures of the isolates were obtained by first growing the isolates on 2% water–agar plates at 20 °C for 48 h. Subsequently, a single hyphal tip was transferred with a heat-sterilized needle to fresh carrot agar (CA; Brasier, 1967). Cultures were grown for 7 days in the dark at 20 °C on homemade CA, cornmeal agar (CMA, Sigma), malt extract agar (MEA, Sigma) and potato dextrose agar (PDA, Sigma). Hyphae morphology was examined using a stereomicroscope ($\times$ 50 magnification). Formation of sporangia and zoospores was induced by floating three 12-mm-diameter mycelial plugs from a 3-day-old CA culture together with three to five immature leaves of *Quercus ilex* on water, followed by a 2-day incubation at 20 °C. The isolates were checked for gametangial formation in pure cultures that were grown in the dark on CA for 14 days. Pairings between several isolates of *P. mercuriale* were also performed in order to try and induce sexual structures. Two mycelial plugs of each isolate were placed *c.* 2 cm apart on CA in 90-mm Petri dishes and incubated in the dark at 20 °C for 14 days.

DNA extraction

DNA was purified from mycelia using the DNA-Easy Plant Mini kit (Qiagen, Basel, Switzerland) according to the manufacturer's specifications. Quality was checked under UV light, following electrophoresis with a molecular mass standard (HindIII/EcoRI DNA Marker, Biofinex, Switzerland). DNA concentrations were determined using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop, Wilmington, DE).

DNA amplification

ITS amplifications of *Pythium* isolates were carried out using previously described universal primers ITS4 and ITS5 that target conserved regions in the 18S and 28S rRNA genes (White *et al.*, 1990). The amplification reaction mixture contained $1 \times$ PCR buffer [75 mm Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2$ SO_4]; 0.1 mM dNTPs, 0.25 μM of each primer; 1.5 mM $MgCl_2$; 1 U of Taq Polymerase (Biotools, Spain); and 1 μL of mycelial DNA in a total volume of 50 μL. Amplifications were performed in a Master Gradient thermocycler (Eppendorf, Schönenbuch/Basel, Switzerland) according to the following amplification programme: an initial denaturation step at 95 °C for 2 min, followed by 30 cycles including denaturation for 20 s at 95 °C, annealing for 25 s at 55 °C and extension for 50 s at 72 °C, followed by a final extension step of 10 min at 72 °C (Belbahri *et al.*, 2006). Amplicons were purified using a Minelute PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's specifications. The quantity and quality of the PCR products were checked as described above for genomic DNA. The other nuclear (*EF-1α* and *β-tub*) and mitochondrial genes (*nadh*1) were amplified using primers developed by Kroon *et al.* (2004), using the same amplification protocol described above.

PCR product cloning, DNA sequencing and phylogenetic analysis

Amplicons were purified using the QIAquick PCR purification kit (Qiagen) and cloned using the TOPO cloning kit (Invitrogen, Basel, Switzerland). Subsequently, the cloned fragments were sequenced in both directions (Fasteris SA, Geneva, Switzerland). Each clone was sequenced twice and checked for sequence ambiguities between duplicates, which were not detected. All overlapping strands contained the same sequence, indicating an error rate of *Taq* polymerase catalysed PCR of $<$ 1 nucleotide per 500 nucleotides (Liu *et al.*, 1992). DNA sequences have been deposited in GenBank (Tables 1 and 2).

Molecular phylogenetic analyses were conducted as described previously (Belbahri *et al.*, 2006). In brief, sequences were aligned manually using SEAVIEW (Galtier *et al.*, 1996).

Table 2. PCR amplicons of the translation elongation factor 1α (EF-1α), β-tubulin (β-*tub*) and NADH dehydrogenase subunit 1 (*nadh*1) genes that were cloned from four isolates of *Pythium mercuriale*

| Species | Isolate | Nuclear genes | | Mitochondrial gene |
		EF-1α	β-*tub*	*nadh*1
Pythium mercuriale	STE-U 6205	DQ911398	DQ911418	DQ911420
		DQ911399	EF035025	DQ911421
		DQ911400	EF035024	DQ911422
		DQ911401	EF035023	DQ911423
		DQ911402	EF035022	DQ911424
	STE-U 6124	DQ911403	DQ911419	DQ911425
		DQ911404	EF035028	DQ911426
		DQ911405	EF035027	DQ911427
		DQ911406	EF035026	
	STE-U 6125	DQ911407	EF035030	DQ911428
		DQ911408	EF035029	DQ911429
		DQ911409		
		DQ911410		
		DQ911411		
		DQ911412		
	STE-U 6204	DQ911413	EF035033	DQ911430
		DQ911414	EF035032	DQ911431
		DQ911415	EF035031	
		DQ911416		
		DQ911417		

Table 3. Number of cloned PCR amplicons sequenced in eight different *Pythium mercuriale* isolates for four genes: internal transcribed spacer region (ITS), translation elongation factor 1 α (EF-1α), β-tubulin (β-*tub*) and NADH dehydrogenase subunit 1 (*nadh*1)

| Species | Isolate | ITS | Nuclear genes | | Mitochondrial gene |
			EF-1α	β-*tub*	*nadh*1
Pythium mercuriale	STE-U 6204	5	5	3	2
	STE-U 6205	5	5	5	5
	STE-U 6206	3	–	–	–
	STE-U 6207	4	–	–	–
	STE-U 6124	4	4	4	3
	STE-U 6125	3	6	2	2
	STE-U 6126	4	–	–	–
	STE-U 6127	3	–	–	–

Models of DNA evolution have been chosen using MRAIC.PL. (Nylander, 2004). Bayesian inferences (BI) were obtained with MRBAYES v.3.0 (Huelsenbeck & Ronquist, 2001). The program was run for 2 000 000 generations and sampled every 100 generations with four simultaneous chains. The trees sampled before the chains became stationary were discarded. TREEVIEW was used to visualize Bayesian trees. The β-tubulin sequences used in the tree were derived from Kroon *et al.* (2004) and Villa *et al.* (2006). The *Pythium* and

Phytophthora sequences used for building molecular phylogenies are given in Table 3 with their respective GenBank accession numbers.

Results

Pythium mercuriale, BELBAHRI, PAUL and LEFORT sp. nov. (Fig. 1).

MycoBank: CBS No. 122443.

Hyphae principales vel 6 μm latae. Coloniae in agaro dauci carotae (CA) crysanthemum. Incrementum radiale quotidianum 8.8 mm (20 °C in CA). Sporangia subglobosa vel obovoidea, papillata, terminalia, 23–32 μm diameter, zoosporae oriuntur inter 15–28 °C, zoosporae incapsulatae usque ad 12 μm diameters. Chlamydosporae globosae, levae, abundantes, intercalares vel laterales, 25–45 μm diam. Oogonia et antheridia rara. Oogonia laevia, globosa, 22–37 μm diam. Antheridia diclinata, multipliciae, oogonium complectentia. Oosporae non observatae.

Etymology: *mercuriale* (= variable), referring to its variable ITS sequences.

Typus: Limpopo Province, Africa Meridionalis, isol. ex rizosphaera *Macadamiae integrifoliae*, Date, Sept., 2005, W.J. Botha, (cultura sicca in agaro dauci carotae in herbario Universitatis Helveticae Occidentalis conservata) – holotypus = (Py292), STE-U 6204, ex typo cultura viva in CBS: CBS No. 122443.

All isolates examined showed similar growth rates and mycelial patterns on each of the culture media used. Mycelial pattern on CA broadly chrysanthemum, with aerial mycelium tufted and slightly cottony; on CMA slightly stellate and submerged; on MEA rosaceous and cottony; and on PDA tightly chrysanthemum with aerial mycelium patchy, floccose and cottony in the centre. Main hyphae up to 6 μm wide. Cardinal temperatures: minimum 5 °C, optimum 25–30 °C, maximum >35 °C. Average daily growth rate of seven isolates at 20 °C on CA = 8.8 mm; on CMA = 8.9 mm; on MEA = 8.3 mm; and on PDA = 6.5 mm. Chlamydospores abundant on CA, mostly spherical, 25–45 μm, thin-walled, intercalary or lateral. Sporangia rarely formed in water, subglobose to obovoid, papillate, 18–23 × 23–32 μm (average 22 × 26 μm), proliferation both nested and extended; sporangiophores unbranched. Zoospore discharge tube short and formed at the sporangium apex. Old sporangia sometimes germinating by means of one to three germ tubes arising from the papilla (Fig. 1b–d). Gametangia absent in intraspecific pairings and rarely observed in pure culture. Oogonia spherical, 22–37 μm (average 29.8 μm), smooth-walled, terminal or lateral on short branches. Antheridia multiple, mostly diclinous, knotted around the oogonia (Fig. 1e–h). Oospores not observed.

The ex-type strain was found in the Limpopo Province, South Africa, and was isolated from the rhizosphere of
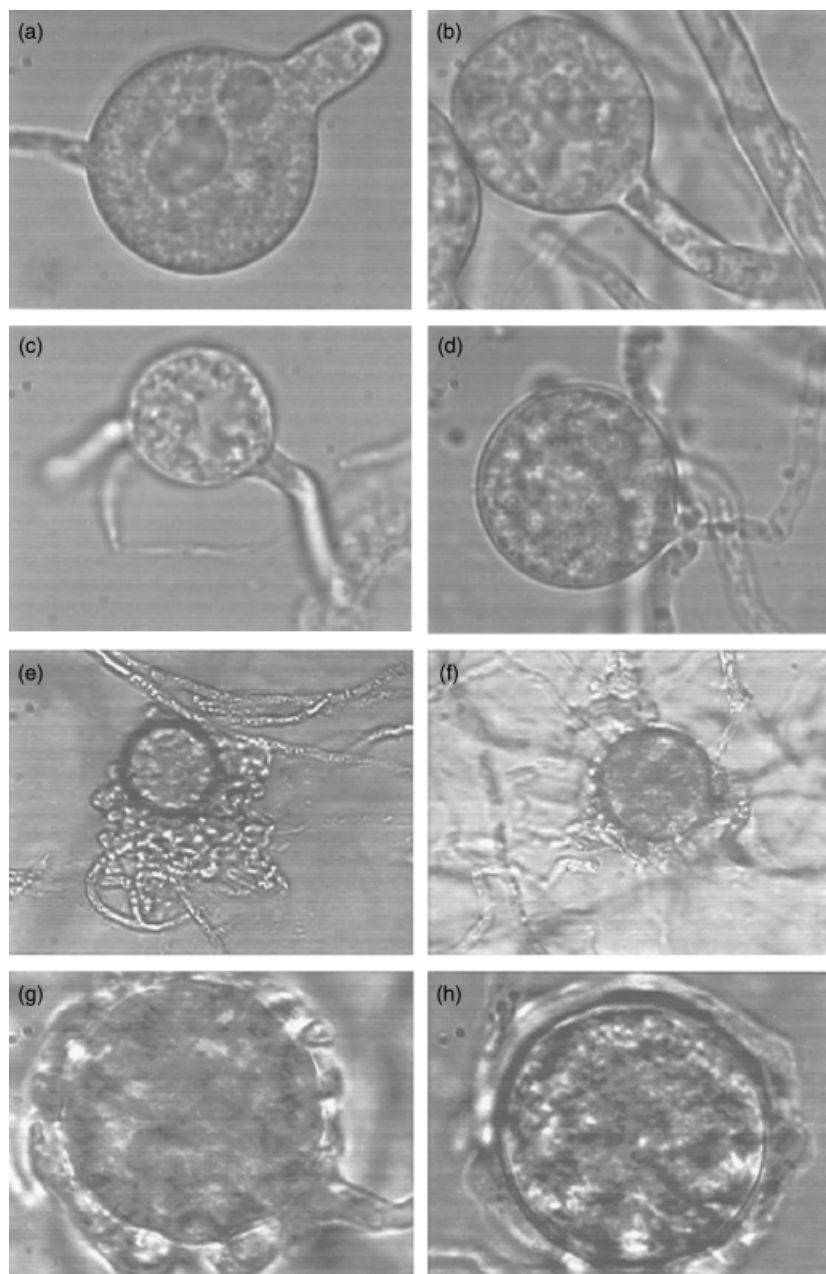
Fig. 1. Morphology of *Pythium mercuriale* (STEU-U 6207). (a–d) Old sporangia forming germ tubes. (e–h) Oogonia surrounded by a knot of undefined antheridia. Scale bar = 35 μm in (a–d) and (g–h). Scale bar 80 μm in (e–f).

Macadamiae integrifoliae, by W.fJ. Botha. This strain as well as other isolates are maintained in the collection of the University of Applied Sciences of Western Switzerland. The ex-type culture STE-U 6204 is conserved in Centraalbureau voor Schimmelcultures (CBS) under the CBS No. 122443.

ITS sequence divergence

In all isolates, attempts to conduct direct sequencing of ITS PCR products failed in providing readable sequences. Multiple peaks were observed in sequencing chromatograms (from 190 bases with the primer as the sequencing primer or from 40 bases with ITS4), suggesting the presence of more than one ribosomal sequence for each isolate. In order to solve this problem, PCR products were cloned and one to several clones were subsequently sequenced for each isolate (Table 3).

Sequencing of the cloned PCR fragments revealed the presence of three to five different ITS sequences within each isolate, except STEU6217. Significant divergence was observed among positions 162–172 within the ITS1 sequences, and among positions 74–90 and 424–434 within the ITS2

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Position        153                       469                    757                 790
STEU_6124_clone0    CCCTTTTTTTTTTTTTATTTTGTGA    TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6124_clone1    CCTTTTTTTTT---ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6124_clone4    CCCTTTTTTTT---ATTTTGTGA       TGG--------------TGTTGT      TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6124_clone2    CCTTTTTTTTT---ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6125_clone0    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6125_clone1    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6125_clone3    CCTTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6126_clone0    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGC-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6126_clone1    CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6126_clone3    CCCTTTTTTTT---ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6126_clone4    CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6127_clone0    CCTTTTTTTTTTTT------GTGA      TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6127_clone5    CCTTTTTTTTTTTT------GTGA      TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6204_clone5    CCCTTTTTTTTTTT--ATTTTGTGA     TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6204_clone10   CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGGGAGTGGGTC   CTTTTGTTTGTTTGTTGCTT
STEU_6204_clone3    CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTT--------GTTATT
STEU_6204_clone0    CCTTTTTTTTTTT-------GTGA      TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6204_clone9    CCTTTTTTTCTTT------GTGA       TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6205_clone0    CCTTTTTTTT----ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6205_clone3    CCCTTTTTTTT--ATTTTGTGA        TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6205_clone2    CCCTTTTTTT---------GTGA       TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6205_clone4    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6205_clone1    CCCTTTTTTTT---ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6206_clone4    CCTTTTTTTTTTTT------GTGA      TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6206_clone1    CCTTTTTTTTTTTT------GTGA      TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGGGAGTGGGTC   CTTTTT--------GTTATT
STEU_6206_clone0    CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6207_clone1    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6207_clone2    CCCTTTTTTTTTC--ATTTTGTGA      TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6207_clone3    CCCTTTTTTTTTT-ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6207_clone4    CCCTTTTTTTTT--ATTTTGTGA       TGG-TTTTGTGGTGGCGGT-TTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
STEU_6217_clone3    CCTTTTTTTTTTTT------GTGA      TGGTGTTTGTGCTGGTGGTGTTTGT    TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
PE-16_clone0        CCCTTTTTTTTTT--ATTTTGTGA      TGGT-TTTTGTGGTGGCGGT-TTTGT   TTTGGG-A--GGGTC    CTTTTGTTTGTTTGTTGCTT
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Fig. 2. Sequence alignments of cloned PCR amplicons obtained from the internal transcribed spacer region of several isolates of *Pythium mercuriale*. Sequence names followed by clone 0, 1, 2, 3 and so on indicate individual clones obtained from a single isolate.

sequences (Fig. 2a–b). Additionally, single base pair polymorphisms were present within the ITS2 sequences of individual isolates (Fig. 2). Variation in the number of A or T bases occurred within ITS 1, in all cases as part of a tract of identical bases, whereas within the ITS2 sequence, variation in the number of G or T bases occurred as part of a tract of GT or GTTT repeats. Additionally, a small number of point mutations between isolates were noted, including some in the 5.8S sequence rRNA gene coding region (data not shown).

Sequence heterogeneity was much higher in isolates STEU6204-STEU6206 and STEU6127 than in isolates STEU6207 and STEU6124-STEU6126. Phylogenetic analyses supported the presence (1.00 posterior probability) of two major ITS types in the species (Fig. 3). No clear association with either the geographic origin or the host could be attributed to any of the two major ITS types. Interestingly, the sequences obtained from a single isolate did not always cluster together. For example, while the three sequences of STEU6124 formed a relatively homogeneous group, the three sequences of STEU 6205 were loosely distributed among the sequences of other isolates of *P. mercuriale*. The branches leading to these groups were associated with bootstrap scores as high as 1.00 for some sequences.

Phylogenetic position of *P. mercuriale*

BLAST searches in Genbank revealed that all the ITS sequences found within and among the 16 isolates were unique and different from those of any described *Pythium* species. The closest relative to *P. mercuriale* was *P. boreale* (AY598662), with a minimum score of 98% identity to only 285 bases. A maximum likelihood phylogenetic analysis based on ITS sequences placed *P. mercuriale* in clade K sensu Lévesque & de Cock (2004).

We further investigated whether the within-isolate variation found in the ITS sequence copies was correlated with the sequence divergence of some nuclear (*EF-1α* and *β-tub*) and mitochondrial genes (*nadh*1). For each of the two nuclear genes, all the coding regions of the sequences obtained were first translated and compared by sequence alignment to available *Pythium* sequences in GenBank in order to confirm the identity of the cloned sequences (data not shown). The identity of the mitochondrial gene sequences was determined using BLASTN software in GenBank that showed that the sequences represent orthologues of *nadh*1, as expected (data not shown). Sequencing of selected cloned PCR products revealed high levels of polymorphisms and heterozygosity both for the nuclear and the mitochondrial genes between as well as within single isolates (Table 2).

The phylogenetic position of the sequences of *P. mercuriale* isolates (STEU6124, STEU6205) and of other *Pythium* species belonging to clade K (Table 4; Matsumoto *et al.*, 1999) is illustrated in the BI trees using the *β-tub* sequences (Fig. 4). In this analysis, *P. mercuriale* formed a monophyletic group supported by high values of posterior probabilities (1.00 BS) within the same subclade of *P. boreale*, *P. ostracodes* and *P. helicoids* (Fig. 4).
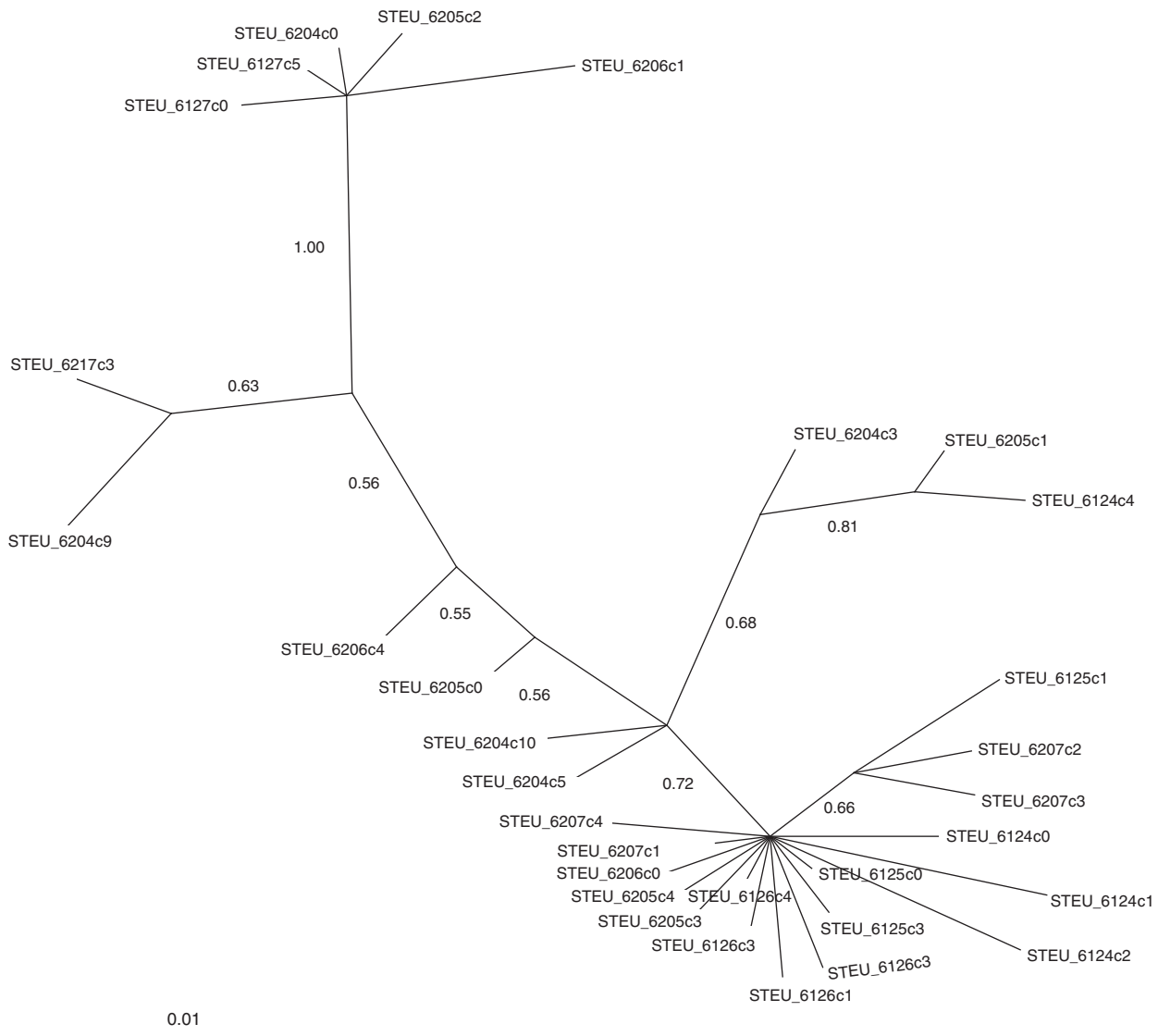
Fig. 3. Dendrogram based on BI as implemented in the program MRBAYES v.3.0 of ITS variants of *Pythium mercuriale*. ITS sequence alignments were edited to add the 5.8S rRNA gene region before the phylogenetic analysis was performed. The numbers on the nodes represent the posterior probability/percentage bootstrap support based on Bayesian analysis of the data set.

Discussion

Pythium mercuriale is characterized by forming a large number of resting spores (interpreted here as thin-walled chlamydospores but usually designated by other researchers as hyphal swellings) in CA, subglobose to obovoid, papillate sporangia proliferating internally and externally in water cultures, and occasionally forming oogonia in CA. This set of morphological characters distinguishes *P. mercuriale* from any other described *Pythium*. It resembles *P. sterilum* in sporangial morphology and in having spherical hyphal swellings (indistinct from thin-walled chlamydospores) but the latter does not form sexual structures (Belbahri *et al.*, 2006).

In their studies of the genus *Pythium*, Lévesque & de Cock (2004) reported no ITS sequence variation within a single isolate and obtained unambiguous sequences from direct PCR products, except for a few species such as *P. helicoides*, where a few single nucleotides were ambiguous. This agrees with what is generally found in *Pythium*, but contrasts markedly with what was reported recently by Kageyama *et al.* (2007) for *Pythium helicoides*, and with our data in *P. mercuriale*. The presence of heterogeneity in multiple rRNA gene repeat units within individual *Pythium* isolates was also hypothesized earlier by Martin (1990), but could not be proven at a sequence level because only restriction mapping of the rRNA gene repeat units was carried out

**Table 4.** Name, isolate and β-tubulin sequence accession number of the 92 isolates used in this study

Species	Isolate	β-tubulin
Pythium sp.	MuShi	DQ071292
Pythium sp.	Py37	DQ071293
Pythium sp.	Py55	DQ071294
P. aphanidermatum	P36-3	DQ071295
P. aristosporum	ATCC11101	DQ071297
P. aristosporum	PRR115	DQ071298
P. arrhenomanes	ATCC96525	DQ071300
P. arrhenomanes	ATCC96526	DQ071301
P. arrhenomanes	ATCC96598	DQ071302
P. catenulatum	NBRC 100104	DQ071303
P. vexans	NBRC 100105	DQ071304
P. deliense	MAFF305568	DQ071305
P. dissotocum	02C1-3-1	DQ071306
P. graminicola	ATCC96234	DQ071307
P. graminicola	IFO31998	DQ071308
P. helicoides	CBS286.31	DQ071310
P. hydnosporum	MAFF305861	DQ071312
P. inflatum	MAFF305863	DQ071313
P. intermedium	MAFF305570	DQ071314
P. irregulare	NBRC 100110	DQ071315
P. irregulare	NBRC 100108	DQ071316
P. irregulare	NBRC 100109	DQ071317
P. irregulare	MAFF305572	DQ071318
P. irregulare	NBRC 100111	DQ071319
P. mamillatum	Py68-158	DQ071320
P. vexans	NBRC 100112	DQ071321
P. myriotylum	GF46	DQ071324
P. nunn	ATCC20693	DQ071325
P. oedochilum	CBS252.70	DQ071326
P. oedochilum	CBS292.37	DQ071327
P. oligandrum	Chi-Oi-1	DQ071328
P. orthogonon	DS2-6-9D	DQ071329
P. ostracodes	CBS768.73	DQ071330
P. paddicum	IFO31993	DQ071331
P. paroecandrum	CBS157.64	DQ071332
P. periplocum	NBRC 100114	DQ071333
P. pyrilobum	IFO32560	DQ071334
P. rostratum	DS5-7-1S	DQ071335
P. spinosum	OD231	DQ071336
P. sulcatum	CTMa7	DQ071337
P. sylvaticum	OM121	DQ071338
P. torulosum	NBRC 100120	DQ071339
P. torulosum	PRR3	DQ071341
P. ultimum	NBRC 100122	DQ071342
P. vanterpoolii	P39-1	DQ071343
P. vanterpoolii	PRR16	DQ071344
P. vanterpoolii	UOP392	DQ071345
P. nodosum	MAFF305905	DQ071346
P. violae	OPy4	DQ071347
P. volutum	IFO31926	DQ071348
P. zingiberum	UOP389	DQ071349
P. mercuriale	STEU 6124	DQ911419
P. mercuriale	STEU 6205	DQ911418
P. capsici	IFO30696	DQ071350
P. cactorum	CH98PEC1	DQ071351
P. cactorum	IFO32194	DQ071352

Table 4. Continued.

Species	Isolate	β-tubulin
P. cactorum	CH02NKPy001	DQ071353
P. cactorum	Shakuyaku 1-1	DQ071354
P. cinnamomi	IFO33182	DQ071355
P. citricola	CH95PHE28	DQ071356
P. citricola	CH95PHE31	DQ071357
P. citricola	CH98U121C	DQ071358
P. megasperma	CH95	DQ071359
P. megasperma	IFO31624	DQ071360
P. megasperma	IFO32176	DQ071361
P. nicotiana	Phkq1-1	DQ071362
P. sojae	Fu-P12-1	DQ071363
P. sojae	Fu-P2	DQ071364
P. sojae	IFO31016	DQ071365
P. infestans	Pic99186	AY564035
P. mirabilis	Pic99129	AY564038
P. phaseoli	CBS 556.88	AY564044
P. arecae	CBS 148.88	AY564049
P. boehmeriae	CBS 291.29	AY564050
P. tropicalis	PD97/11132	AY564046
P. colocosiae	IMI368918	AY564058
P. cryptogea	HR1/ss/pp/99	AY564059
P. drechsleri	CBS 292.35	AY564060
P. erythroseptica	CBS 951.87	AY564061
P. fragariae var. *fragariae*	A2	AY564062
P. fragariae var. *rubi*	FVR67	AY564064
P. gonapodyides	P245	AY564066
P. heveae	CBS 296.29	AY564067
P. hibernalis	CBS 522.77	AY564068
P. ilicis	PD91/595	AY564071
P. lateralis	CBS 168.42	AY564076
P. megakarya	IMI337098	AY564078
P. palmivora	CBS 236.30	AY564082
P. pseudotsugae	IMI 331662	AY564084
P. syringae	CBS 364.62	AY564088
P. vignae	IFO30473	DQ071367
P. ramorum	PD93/51	AY564092

which lead to the hypothesis that there were indels in the rRNA gene repeat units within the nontranscribed spacer region adjacent to the 3′ end of the coding region of the 26S subunit. In *P. mercuriale*, the ITS region displayed an unusually high variability among and within the 16 examined isolates. This variation mainly corresponds to deletions of single bases or repetitive motifs, and to a lesser degree, to single base substitutions in the 5.8S rRNA gene coding region.

In plants, earlier phylogenetic studies using the ITS region assumed that ITS copies within the same individual are identical. The process of concerted evolution, which results in the homogenization of multi-copy gene regions, was thought to act universally in all species in order to maintain only one ITS copy within the same individual. However, today, it is a well-known fact that in certain plant species heterogeneity exists within ITS copies within the same individual, which should be treated with caution in
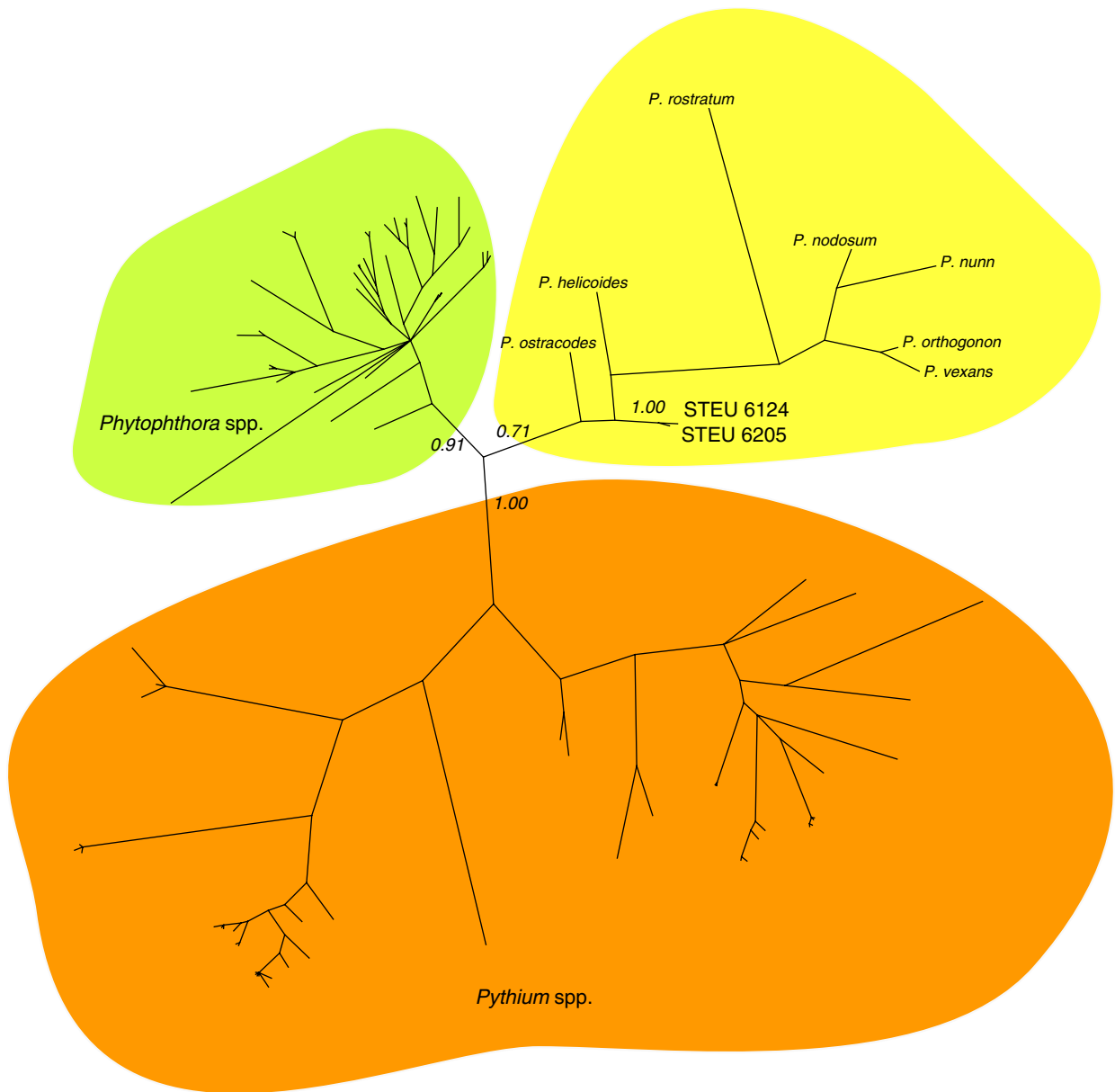
Fig. 4. Phylogenetic relationships between *Pythium* and *Phytophthora* based on β-tubulin sequences. The numbers on the nodes represent the posterior probability/percentage bootstrap support based on Bayesian analysis of the data set.

phylogenetic studies (Alvarez & Wendel, 2003; Bailey *et al.*, 2003). The presence of different ITS copies in the same individual has also been reported for vertebrates, fungi and protozoa (Gunderson *et al.*, 1987; Kuhn *et al.*, 2001; Ko & Jung, 2002; Hui *et al.*, 2007). The existence of multiple ITS copies in the same plant individual could be due to the fact that the mechanisms of concerted evolution are not acting, pseudogenes may be present or for various other reasons

that are not straightforward to deduce (Bailey *et al.*, 2003; Feliner & Rosselló, 2007). Similar to the situation in plants, in *P. mercuriale*, it will also be difficult to determine the origin of different ITS copies within single isolates.

The most striking peculiarity of *P. mercuriale* gene sequences is not only the high heterogeneity within ITS sequences within single individuals but also the presence of more than two alleles for two nuclear genes (*EF-1α* and

β-*tub*), as well as more than one copy of a mitochondrial gene (*nadh*I) within single individuals. In contrast, in *P. helicoides*, heterogeneity was only found within the ITS sequences of single isolates and not in the mitochondrial gene (*cox* II) that was investigated (Kageyama *et al.*, 2007). In another oomycete, *Phytophthora alni*, the presence of different ITS sequence copies within the same isolate has, among other factors, been interpreted as evidence of a hybridization event (Brasier *et al.*, 1999). Therefore, due to the presence of heterogeneity in the ITS region, *EF*-1α, β-*tub* and *nadh*I genes of single isolates of *P. mercuriale*, we cannot discard the idea that hybridization may have played a role in the origin of the species, as it has occurred in *Phytophthora alni* (Ioos *et al.*, 2006). It is possible that *P. mercuriale* might be a polyploid, an aneuploid and/or a hybrid. Future research focused on solving the number of gene copies and chromosomes in the *P. mercuriale* genome would help to deduce the mechanism generating and maintaining this genetic variation.

Our data show that ITS rRNA gene can be used for inferring the phylogeny of closely related species as well as for examining the relationships between and within the populations of the same species. They may also be helpful for the identification of species that cannot be distinguished using only morphological characteristics or for testing interbreeding potential. However, the species concept based on ITS DNA sequence analysis still has to be defined. As shown here, *P. mercuriale* is characterized by a population of ITS sequences rather than by a single sequence, forming a much more variable assemblage, which may indicate that the process of speciation is not yet complete. A similar intra-isolate variation in ITS copies has also been observed in four other *Pythium* species (L. Belbahri, unpublished data). Therefore, the potential of the rRNA gene ITS region for phylogenetic analysis and for diagnostic use should be evaluated carefully. In order to improve the use of the ITS region for diagnostics in fungi, Landis & Gargas (2007) used the secondary structure of ITS 2, and the unique sequence of loop 2, to create species-specific probes in a micro array format, in an effort to reduce the effect of multiple ITS copies, as well as increase the specificity of ITS spacers.

A phylogenetic analysis based on the β-tubulin gene indicated that *P. mercuriale* shared a common ancestor with all *Pythium* species within clade K. However, it is important to note that the analyses of sequence diversity of the β-tubulin gene showed that each *P. mercuriale* isolate possessed two or more alleles for this gene. As with the ITS region, the β-tubulin gene of *P. mercuriale* displayed the highest sequence homology with *P. boreale* and clustered in the same subclade.

It remains unclear whether or not *Pythium* species belonging to clade K are appropriately classified as *Pythium*

(Briard *et al.*, 1995; Panabieres *et al.*, 1997; Dick, 2001), particularly due to certain similarities with *Phytophthora* such as the presence of elicitin genes (Panabieres *et al.*, 1997). As mentioned by Lévesque & de Cock (2004), we observed that, when using all available *Pythium* ITS sequences for analysing the taxonomic position of clade K, *Pythium* would appear to be polyphyletic because of clade K. Using the β-tubulin region for the phylogenetic analysis, we could also show that *Pythium* clade K species are more closely related to *Phytophthora* than to *Pythium* species (Fig. 4). However, substantially more data are needed to investigate this. The use of all available oomycete sequences in a new phylogenetic study should be helpful for solving the generic status of clade K.

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