

A robust and specific real-time PCR tool for the detection of *Phytophthora lateralis* in plant tissues

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Abstract *Phytophthora lateralis* is an oomycete responsible for Port-Orford Cedar (*Chamaecyparis lawsoniana*) dieback and mortality since the 1920s in western North America. It is recommended for quarantine regulation by EPPO (A2 list) and has been recently detected in Europe. In order to implement efficient control of the disease, a sensitive real-time PCR assay was developed to detect the presence of *P. lateralis* in plant tissues. In this study, we used the species-specific polymorphisms within the RAS-related protein gene *Ypt1* to design a primer pair and a hydrolysis probe targeting the pathogen. The tool proved to be specific and inclusive, based on in silico and in vitro assessments, and could detect as few as 47 copies of target DNA. In addition, the test demonstrated its robustness and remained highly sensitive and specific under deliberately modified experimental conditions such as the hybridization temperature, or the reaction and DNA template volumes. The DNA extraction step from diseased tissues was also optimized, and the reliability of the results was ensured by a set of controls and a test targeting plant DNA that enabled the assessment of the quality of the extracts.

Keywords *Phytophthora lateralis* · Real-time PCR · Detection · *Chamaecyparis lawsoniana*

Introduction

Phytophthora lateralis is the causal agent of a root rot that was first reported from *Chamaecyparis lawsoniana* (Port-Orford Cedar, POC) in Seattle in 1923 (Zobel et al. 1985), but was only officially described two decades later (Tucker and Milbrath 1942). The disease is now widely distributed in Western North America where it causes mortality and dieback throughout the natural range of Port-Orford Cedar, leading to severe economic and ecological losses.

In Europe, *P. lateralis* was first detected on *C. lawsoniana* in France in 1996 and 1998 (Hansen et al. 1999) and more recently from windbreak hedges in Bretagne (Robin et al. 2011). It has also been identified in Dutch nurseries (Sansford 2009) and, since 2010, in England, Scotland and Northern Ireland on diseased POC in forests, parklands and shelterbelts (Green et al. 2012). Other *Chamaecyparis* species and also *Taxus brevifolia* (DeNitto and Kliejunas 1991), *Thuja occidentalis* (Schlenzig et al. 2011; Green et al. 2012) and *Thuja plicata* (A. Schlenzig pers. comm. 2014) have also been reported as natural hosts, but with less severe symptoms. The oomycete was also discovered in 2008 in Taiwan, in soil beneath asymptomatic *Chamaecyparis obtusa* var. *formosana* trees (Brasier et al. 2010). Based on both phenotypical features and molecular data, Brasier et al. (2012) distinguished four

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distinct lineages within *P. lateralis*: two from Taiwan (J and K), one from the United Kingdom (UK) and one from North-America and Western Europe (PNW).

Zoospores of the pathogen penetrate the host through fine roots and induce collar and root lesions. Seedlings may be killed within weeks of infection and for larger trees, death may happen within a year after first appearance of crown symptoms (Winton and Hansen 2001). Aerial infections can also occur (Robin et al. 2011), due to the possible production of caducous sporangia by *Phytophthora lateralis*.

Although the pathogen is already present in some locations in Europe, EPPO recommends that its member countries regulate *P. lateralis* as a quarantine pest (<http://www.eppo.int/QUARANTINE/listA2.htm>). A specific and accurate detection tool is required, in order to prevent the introduction and spread of the oomycete in disease-free areas. To date, the techniques available for the detection of *P. lateralis* on plant samples are based either on isolation followed by morphological identification (Erwin and Ribeiro 1996) or on conventional PCR (Winton and Hansen 2001; Schena et al. 2008). However isolation and morphological identification of the pathogen is time-consuming and may lead to false negative results. Indeed, it is sometimes difficult to obtain a pure culture from a symptomatic plant sample since it requires the availability of fresh plant material and because the slow-growing mycelium of *P. lateralis* is easily outcompeted by faster growing organisms. The currently available conventional PCR methods alleviate these drawbacks but either lack specificity (the PCR test developed by Winton and Hansen (2001) cross-reacts with *Phytophthora ramorum* which may also be present on POC), or has not yet been fully adapted and validated for *in planta* detection (Schena et al. 2008).

The use of intronic regions of single copy genes, including the RAS-related protein gene *Ypt1*, proved to be effective for the differentiation of *Phytophthora* species (Ioos et al. 2006; Schena et al. 2008). In this respect, this region was judged to be suitable for the development of a new molecular test with enhanced performance criteria compared to the currently available protocols.

The aim of the present work was to develop and optimize a tool for the detection of *Phytophthora lateralis* in plant samples, based on a highly specific real-time PCR (qPCR). A comprehensive validation

process has been followed in order to assess the performance criteria of the assay according to EPPO Standard PM 7/98 (EPPO 2010). Finally, a set of ad hoc quality controls has been implemented to ensure the reliability of the results for use in of routine diagnostics.

Materials and methods

Biological material

Oomycete and fungal isolates

The isolates used in this study are listed in Table 1. *P. lateralis* isolates originated from several culture collections and were recovered from diseased *Chamaecyparis* collected in autumn 2011 on wind-break hedges in Bretagne (North-Western France). The oomycetes were isolated by plating small pieces of wood or foliage from the leading edges on PARB medium described by Robin et al. (1998) and slightly adapted (17 g Corn Meal Agar, 10 ppm Pimaricin, 10 ppm Rifampicin, 250 ppm Ampicillin, 0.015 g methyl 1-(butylamino)carbonyl-1 h-benzimidazol-2-yl carbamate, distilled water to 1 L), followed by incubation for 5 to 10 days at 22 °C in the dark. The growing hyphae were transferred to V8-agar for further morphological identification and DNA extraction. The identification was confirmed by sequencing the Internal Transcribed Spacer (ITS) using the ITS6/ITS4 primers (Cooke and Duncan 1997). True fungi were also isolated from the *Chamaecyparis* samples collected in the field by plating on a broad spectrum medium such as Malt agar +100 ppm Chloramphenicol. The cultures were transferred to Potato Dextrose Agar and Malt Agar for identification by morphology and ITS sequencing using ITS1/ITS4 primers (White et al. 1990).

Chamaecyparis samples

A series of 32 samples were collected in Bretagne from diseased *Chamaecyparis* tissues, including roots, wood, bark, branches and foliage. Each sample was chopped into small pieces (2 mm maximum) with a scalpel and the pieces were thoroughly mixed and split in two homogenous halves for: i) analysis by isolation, and ii) DNA extraction followed by molecular analysis (Table 2).

Table 1 Characteristics of the isolates and sequences used in this study

Organism	Isolate	Host	Origin	GenBank (<i>Ras-Ψpt1</i>)		Mean cycle thresholds (standard deviation) qPlat
				Accession number	Reference	
<i>Phytophthora lateralis</i>	11/379-1a5 ^a	<i>Chamaecyparis lawsoniana</i>	France (Bretagne)	-	-	20.9 (0.2)
	11/379-2b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.7 (0.2)
	11/379-2d1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	21.1 (0.1)
	11/379-3a4 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.9 (0.1)
	11/379-4a2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.8 (0.2)
	11/379-4b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.9 (0.3)
	11/379-4c2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	KM 975318	This study	22.5 (0.9)
	11/380-1a4 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.2 (0.2)
	11/380-1b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.6 (0.1)
	11/380-4c1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	21.1 (0.1)
	11/380-4d1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	21.7 (0.6)
	BCH01 ^b	<i>C. lawsoniana</i>	Scotland (Balloch)	KM 975319	This study	20.4 (0.1)
	00,971-3 ^b	<i>C. lawsoniana</i>	Scotland (import from NL)	-	-	20.0 (0.1)
	01,028-1 ^b	<i>Thuja occidentalis</i>	Scotland (import from France)	JN 182997	Schlenzig et al. 2011	20.5 (0.2)
	010/04,706,844 ^c	<i>C. lawsoniana</i>	The Netherlands (nursery)	KM 975320	This study	20.3 (0.1)
	010/04,804,727 ^c	<i>C. lawsoniana</i>	The Netherlands (nursery)	-	-	20.6 (0.2)
	010/04,804,743 ^c	<i>C. lawsoniana</i>	The Netherlands (nursery)	-	-	20.7 (0.1)
	LAN 2-2 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.8 (0.0)
	MEN 1-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	21.4 (0.2)
	MEN 3-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	19.9 (0.1)
	MEN 4-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	19.8 (0.2)
	SCA 2-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.1 (0.0)
	SCA 3-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.7 (0.0)
	SCA 4-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	19.9 (0.1)
	MOE 2-1 ^d	<i>C. lawsoniana</i>	France (Bretagne)	-	-	20.5 (0.1)
	262 ^e	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.5 (0.1)
	365 ^e	<i>C. lawsoniana</i>	USA (California)	-	-	20.2 (0.0)
	366 ^e	<i>C. lawsoniana</i>	USA (California)	-	-	20.5 (0.1)
	367 ^e	<i>C. lawsoniana</i>	USA (California)	-	-	20.7 (0.1)
	368 ^e	<i>C. lawsoniana</i>	USA (California)	-	-	20.4 (0.0)
	404 ^e	<i>C. lawsoniana</i>	USA (Oregon)	KM 975316	This study	21.3 (0.1)
	405 ^e	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.3 (0.1)
	1-2 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	KM 975317	This study	20.8 (0.1)
	1-4 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	-	-	21.2 (0.1)
	1-5 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	-	-	20.7 (0.1)
	1-7 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	-	-	21.4 (0.2)
1-8 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	-	-	20.7 (0.1)	
09-08 ^e	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.9 (0.2)	
09-09 ^e	<i>C. lawsoniana</i>	USA (Oregon)	-	-	21.0 (0.1)	
16-1 ^e	<i>C. lawsoniana</i>	Canada (Vancouver)	-	-	20.5 (0.2)	

Table 1 (continued)

Organism	Isolate	Host	Origin	GenBank (<i>Ras-Ψpt1</i>)		Mean cycle thresholds (standard deviation) qPlat
				Accession number	Reference	
	21–2 ^c	<i>C. lawsoniana</i>	USA (Washington)	-	-	20.8 (0.2)
	HWY299 1-A.1 ^c	<i>C. lawsoniana</i>	USA (California)	-	-	20.8 (0.2)
	D-10-01 ^c	<i>C. lawsoniana</i>	USA (California)	-	-	21.1 (0.0)
	PC 95–152 ^c	<i>Thuja occidentalis</i>	USA (Oregon)	-	-	21.1 (0.1)
	T4P3 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	21.2 (0.1)
	YEW 2 ^c	<i>Taxus brevifolia</i>	USA (Oregon)	-	-	20.2 (0.0)
	68-16 T ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.4 (0.0)
	T7P7 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.8 (0.2)
	T6P3 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.8 (0.1)
	4963 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.5 (0.2)
	4965 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	20.6 (0.3)
	4966 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	21.6 (0.2)
	07–13 ^c	<i>C. lawsoniana</i>	USA (Oregon)	-	-	21.4 (0.2)
		<i>Chamaecyparis</i> sp.	USA	DQ 162991	Schena & Cooke, 2006	-
	LNPV 122 ^f	<i>C. lawsoniana</i>	France (Lorraine)	-	-	20.4 (0.1)
	LNPV147 ^f	<i>C. lawsoniana</i>	France (Bretagne)	-	-	21.8 (0.1)
<i>P. pseudosyringae</i>	LNPV 89 ^f	<i>Alnus glutinosa</i>	France	-	-	-
<i>P. citrophthora</i>	LNPV 90 ^f	<i>Chamaecyparis</i> sp.	France	-	-	-
<i>P. cryptogea</i> or <i>P. drechsleri</i>	LNPV 91 ^f	<i>Taxus</i> sp.	France	-	-	-
<i>P. ×multiformis</i>	LNPV 109 ^f	<i>Alnus</i> sp.	France	-	-	-
<i>P. cactorum</i>	LNPV 111 ^f	<i>Viola</i> sp.	France	-	-	-
<i>P. inundata</i>	LNPV 112 ^f	<i>Alnus</i> sp.	France	-	-	-
<i>P. cambivora</i>	LNPV 114 ^f	Unknown	France	-	-	-
<i>P. megasperma</i>	LNPV 117 ^f	<i>Quercus</i> sp. (soil)	France	-	-	-
<i>P. citricola</i> group	LNPV 125 ^f	<i>Quercus</i> sp. (soil)	France	-	-	-
<i>P. gonapodyides</i>	LNPV 129 ^f	Unknown	France	-	-	-
<i>P. cinnamomi</i>	LNPV 131 ^f	<i>Quercus</i> sp.	France	-	-	-
	11/377-1b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>P. syringae</i>	LNPV 142 ^f	<i>Quercus</i> sp. (soil)	France	-	-	-
<i>P. ramorum</i>	LNPV 362 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 386 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 388 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 390 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 397 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 401 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 403 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	2 N589-1 ^f	<i>Rhododendron</i> sp.	France	-	-	-
	LNPV 391 ^f	<i>Leucothoe</i> sp.	France	-	-	-
<i>Pythium irregulare</i>	LNPV 149 ^f	<i>Viola</i> sp.	France	-	-	-
<i>Py.sylvaticum</i>	LNPV 151 ^f	Unknown	France	-	-	-
<i>Py.intermedium</i>	LNPV 155 ^f	Unknown	France	-	-	-
<i>Trichoderma</i> sp.	11/377-1b2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-

Table 1 (continued)

Organism	Isolate	Host	Origin	GenBank (<i>Ras-Ψpt1</i>)		Mean cycle thresholds (standard deviation) qPlat
				Accession number	Reference	
<i>Penicillium</i> sp.	11/377-1b3 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Cryptosporiopsis</i> sp.	11/377-2b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/379-4a1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/379-4c1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/380-1a2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Helotiale</i>	11/377-2b2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Mortiella</i> sp.	11/377-2f2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Diplodia mutila</i>	11/377-3a1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/379-2a1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/379-3b1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Mucor hiemalis</i>	11/377-3b2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Pestalotiopsis funerea</i>	11/377-3b4 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Pestalotiopsis</i> sp.	11/378-a2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Fusarium graminearum</i>	11/379-1a1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Fusarium</i> sp.	11/380-3b5 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Chaetomium</i> sp.	11/379-3a3 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Epicoccum nigrum</i>	11/380-3a1 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
	11/380-3a2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
<i>Umbellopsis isabellina</i>	11/380-4b2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
Sterile mycelium	11/377-3a2 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-
Sterile mycelium	11/379-1a3 ^a	<i>C. lawsoniana</i>	France (Bretagne)	-	-	-

^a Isolate from a sample collected by ANSES-LSV in Bretagne in 2011 (identification by morphological features and, by ITS and/or Cox1 sequencing)

^b Isolate provided by A. Schlenzig, SASA, Scotland

^c Isolate provided by K. Rosendahl, VWA, the Netherlands

^d Isolate provided by C. Robin, INRA, France

^e Isolate provided by E. Hansen, Oregon State University, USA

^f Isolate from ANSES-LSV collection, France (identification by morphological features and by ITS and/or Cox1 sequencing)

In addition, a set of 60 artificially-contaminated samples were produced in the laboratory as follows: branch pieces of *Chamaecyparis lawsoniana* of ca 10 mm diameter and 5 mm thickness were superficially disinfected (10 min in NaOCl 1 %), rinsed twice in sterile water and air dried; the pieces were then plated onto 10-day old V8-agar cultures of *P. lateralis*. After 10 days of incubation at 20 °C in the dark, the *P. lateralis*-infected plant material was recovered, chopped into small pieces, thoroughly mixed before distribution in 60 individual 2-mL microtubes, and stored at -30 °C until DNA extraction.

DNA extraction from pure cultures

For total DNA extraction, the aerial mycelia were scraped from the agar culture using a sterile scalpel blade and transferred into 2-mL microtubes. DNA was extracted using a DNeasy plant mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA concentrations were estimated using a spectrophotometer (NanoDrop 2000 – Thermo Scientific) and adjusted to 0.5 ng/μL with the AE elution buffer. The DNA extracts were kept at -30 °C until analysis. The quality of the fungal DNA extracts was assessed by

Table 2 Field samples of *Chamaecyparis lawsoniana* collected in Bretagne and used for isolation and DNA extraction

Reference	Origin	Organ:
11/377-1a	Roesnen, Site1, Young tree 1	Stem and branches
11/377-1b	Roesnen, Site1, Young tree 1	Roots
11/377-2a	Roesnen, Site1, Young tree 2	Stem and branches
11/377-2b	Roesnen, Site1, Young tree 2	Roots
11/377-3a	Roesnen, Site1, Tree 1	Foliage
11/377-3b	Roesnen, Site1, Tree 1	Foliage
11/378-a	Roesnen, Site3, Tree 1	Stem base (wood and bark)
11/378-b	Roesnen, Site3, Tree 1	Stem base (wood and bark)
11/379-1a	Coperec, Tree 1	Foliage
11/379-1b	Coperec, Tree 1	Branch
11/379-1c	Coperec, Tree 1	Branch
11/379-2a	Coperec, Tree 2	Stem base (wood and bark)
11/379-2b	Coperec, Tree 2	Stem base (wood and bark)
11/379-2c	Coperec, Tree 2	Stem base (wood and bark)
11/379-2d	Coperec, Tree 2	Stem base (wood and bark)
11/379-3a	Coperec, Tree 3	Foliage
11/379-3b	Coperec, Tree 3	Foliage
11/379-4a	Coperec, Tree 4	Large branch
11/379-4b	Coperec, Tree 4	Large branch
11/379-4c	Coperec, Tree 4	Stem base (wood)
11/380-1a	Scaer, Tree1	Stem base (chips of wood)
11/380-1b	Scaer, Tree1	Stem base (wood)
11/380-1c	Scaer, Tree1	Stem base (wood)
11/380-2	Scaer, Tree 2	Stem base (wood and bark)
11/380-3a	Scaer, Tree 3	Foliage
11/380-3b	Scaer, Tree 3	Foliage
11/380-4a	Scaer, Tree 4	Large branch
11/380-4b	Scaer, Tree 4	Large branch
11/380-4c	Scaer, Tree 4	Stem base (wood and bark)
11/380-4d	Scaer, Tree 4	Stem base (wood and bark)
11/380-5a	Scaer, Tree 5	Foliage
11/380-5b	Scaer, Tree 5	Foliage

conventional PCR targeting highly conserved regions: RAS primers for *Phytophthora* spp./*Pythium* spp. (RAS-E1-F/RAS-E5-R; Ioos et al. 2006) and ITS primers for other fungal species (ITS1/ITS4).

Design of primers and hydrolysis probe

Partial *RAS-Ypt1* gene sequences were obtained from five *P. lateralis* isolates from different geographical origins by PCR using the degenerate primers RAS-E1-1F and RAS-E5-1R described by Ioos et al. (2006) and were deposited on GenBank. Two additional *P. lateralis*

RAS-Ypt1 sequences and 13 orthologous sequences from phylogenetically close species, including *P. ramorum*, were retrieved from GenBank (Table 1).

All the 20 *RAS-Ypt1* sequences were compared by multiple alignments, using CLUSTALW (online access: http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html). The alignment highlighted clusters of species-specific polymorphisms and a series of forward and reverse primers and probes combinations specific for *P. lateralis* could be manually designed. Their melting temperature and the occurrence of potential secondary structures for each

oligonucleotide candidate were evaluated using Beacon Designer software (Premier Biosoft, Palo Alto, CA). Finally the qPlat-F and qPlat-R primers and the qPlat-P probe best fulfilled the technical and thermodynamical requirements for a primers/hydrolysis probe combination for real-time PCR (Bustin 2000) and were retained for further testing (Table 3).

The specificity of the qPlat-F-R-P combination was first assessed in silico by BLAST analysis on GenBank, then in vitro by qPCR with a wide range of DNA extracts from *Phytophthora lateralis* strains, other *Phytophthora* spp. or *Pythium* spp. isolates, and fungal species isolated from *Chamaecyparis* tissues (Table 1).

Implementation of quality controls

The PCR product amplified from *P. lateralis* genomic DNA (isolate 380-1b1) using the primer pair qPlat-F/–R (170 bp) was inserted into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA) which was used to transform chemically competent TOP10 cells (Invitrogen), according to the manufacturer's instructions. The bacterial clones were then subcultured overnight at 37 °C and the plasmids were purified using a Nucleospin Plasmid Kit (Macherey-Nagel, Düren, Germany). Based on the concatenated sequences of pCR4-TOPO and qPlat-F/–R amplicon, the molecular weight of the circularized plasmid could be determined and the number of target plasmidic copies (pc) per microliter could be calculated, according to the DNA concentration. A calibrated stock positive control solution and a tenfold serial dilution were prepared and stored at –20 °C until use.

In order to prevent cross and carry-over contaminations, preparation of the reaction mastermix and addition of the DNA extracts and positive controls were carried out in physically separated areas of the laboratory. Non-template controls were systematically included in triplicate to check the absence of contamination in all reactions.

For the rest of the experiments, the quality of the DNA extracts and the absence of inhibitory compounds were assessed by a real-time PCR using a 18S uni-F/R/P primers and probe combination that targets highly conserved regions of the 18S rDNA in plants and fungi (Ioos et al. 2009). For routine analysis, a threshold 18S Ct value (Ct_{18S}) was experimentally determined for the 18S assay to serve as a basal reference in order to assess the quality of each DNA solution extracted from plant samples. According to Ioos and Fourrier (2011), it was considered that a DNA sample yielding a mean 18S value below or equal to Ct_{18S} was correctly extracted and contained a sufficiently low quantity of inhibiting compounds. Preliminary 18S uni qPCR tests conducted with raw DNA extracts from *Chamaecyparis* samples showed that a strong inhibition effect occurred, however, a 1 in 10 dilution of the DNA extract successfully overcame the inhibition effect (data not shown). Thus all the plant DNA extracts were systematically diluted 10-fold before analysis. The Ct_{18S} value for the routine analyses was determined by computing the mean and standard deviation for all the 18S unit Ct values obtained with 10-fold diluted DNA from 23 healthy and eight infected plant samples, as described in Ioos and Fourrier 2011; DNA was extracted following the optimized extraction protocol developed in this study.

Table 3 Primers and hydrolysis probes tested or used in this study

Target	Primers or probes	Sequence (5'-3') ^a	T _m (°C)	DNA region	Position ^b	Product size (bp)
<i>Phytophthora lateralis</i>	qPlat-F	ACGGGATCGTGTTCTAGCAG	60.3	RAS-Ypt1	50–69	170
	qPlat-R	TAGCTGCACGTCGTTGCTAC	60.2	...	200–219	...
	qPlat-P	(FAM)-TTTTCCCGCTTTCCTTGGGG-(BHQ1)	67.4	...	170–189	...
Plant or fungus	18S uni-F	GCAAGGCTGAAACTTAAAGGAA	55.9	18S rDNA	N/A	150
	18S uni-R	CCACCACCCATAGAATCAAGA	55.3	...	N/A	...
	18S uni-P	(JOE)-ACGGAAGGGCACCACCAGGAGT-(BHQ1)	66.3	...	N/A	...

^a FAM 6-carboxyfluorescein, BHQ1 Black Hole Quencher 1, registered trademark of Bioresearch Technologies, Inc., and JOE 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein

^b Position of the primer or probe considering as reference sequence GenBank accession KM975318; N/A not applicable

Real-time PCR conditions

Real-time PCR reactions were performed with a RotorGene 6500 (Corbett Research, Mortlake, Australia) set with an autogain optimization performed before the first fluorescence acquisition and with a LightCycler 480 (Roche, Meylan, France) for the reproducibility experiments. Amplifications were carried out in 20- μ L reaction volumes using the qPCR Core kit No ROX (Eurogentec, Seraing, Belgium), consisting of molecular grade water, 1 \times reaction buffer, 5 mM MgCl₂, 4 \times 0.2 mM dNTPs, 0.3 μ M forward and reverse primer, 0.1 μ M hydrolysis probe, 0.5 U of Hotgoldstar DNA polymerase, and 2 μ L of template DNA. For DNA extracted from plant samples, a 10-fold dilution of the raw extract was systematically used as template.

The real-time cycling conditions for *P. lateralis* and 18S uni assays were identical and included an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation for 10 s at 95 °C and annealing-elongation for 45 s at 60 °C. The Ct values were determined using the RotorGene software version 1.7.75, setting the threshold line at 0.02. Each DNA extract was tested in triplicate and a standard deviation was calculated.

Preliminary attempts to run the qPCR test in a duplex format (qPlat-F/-R/-P and 18S uni-F/-R/-P used simultaneously) with *P. lateralis* target DNA diluted in a background of *Chamaecyparis lawsoniana* DNA resulted in an unacceptable loss of sensitivity and efficiency. The efficiency obtained for qPlat reaction in duplex with 18S uni was of 0.35 versus 0.94 in monoplex, and the reaction in duplex showed to be 10,000 times less sensitive than in monoplex (data not shown). Therefore, the qPlat-F/-R/-P and 18S uni-F/-R/-P combinations were used in separate monoplex reactions for all the experiments.

Analytical specificity and sensitivity, efficiency of the qPCR reaction

The analytical specificity was assessed with 0.5 ng μ L⁻¹ DNA extracts from 35 oomycetes and fungi isolated from *Chamaecyparis lawsoniana*, and from 55 isolates of *P. lateralis* originating from three host plants and five countries (Table 1). Furthermore, specificity was also assessed with a highly concentrated DNA extract (114 ng μ L⁻¹) from *Phytophthora ramorum*, which is phylogenetically the closest relative of *P. lateralis*. The analytical sensitivity of the qPlat test was evaluated using a tenfold dilution

series of the DNA positive control, either diluted in water or in a background of *C. lawsoniana* DNA. A standard curve was constructed and the corresponding amplification efficiency was calculated.

Repeatability, reproducibility and robustness

Repeatability of the qPlat assay was evaluated with ten replicates of different DNA templates tested in the same run, while reproducibility was evaluated with one replicate of several DNA templates tested in ten different runs over a 4-week period, using two different thermal cyclers (Rotorgene 6500 and LightCycler 480). For each condition, intra and inter-assay coefficients of variation of Ct value were calculated. The robustness of the real-time PCR assay was assessed by measuring the effect of deliberate variations in method parameters such as DNA template volume (\pm 10 %), reaction volume (\pm 10 %) or hybridization temperature (\pm 2 °C and \pm 3 °C) on the mean Ct value yielded with target DNA (Ioos et al. 2012), and also on its specificity with non-target DNA.

Repeatability, reproducibility and robustness were assessed with plasmidic DNA controls (set at 23.6, 236 and 2360 pc/ μ L for repeatability and reproducibility; and 236 pc/ μ L for robustness) and with a 10-fold diluted DNA extract from a sample naturally infected by *P. lateralis* (11/379-2b). Robustness was also assessed with *P. ramorum* DNA adjusted to 114 ng/ μ L (strain LNPV390).

Comparison of plant DNA extraction procedures

Several grinding and DNA extraction options were assessed. The comparison of the DNA extraction procedures was conducted with six sets of 12 samples made of artificially-contaminated *Chamaecyparis* branch tissue. In an initial step, three grinding methods were tested and compared with 12 samples each. The method included: i) 2 \times 1 min at 30 Hz using a 2-mL microtube with two 3-mm sterile steel beads using a beadbeater (Tissuelyser – Qiagen), ii) 2 \times 1 min at a frequency of 6 units using a 2-mL microtube of Lysing matrix A (MP Biomedicals, Santa Ana, California) containing garnet matrix and 1/4-inch ceramic sphere, using a FastPrep-24 homogenizer (MP Biomedicals), and iii) 2 \times 1 min at a frequency of six units using a 2-mL microtube of Lysing matrix C (MP Biomedicals) containing 1-mm silica spheres, using a FastPrep-24 homogenizer. DNA extraction was performed with the DNeasy plant mini kit (Qiagen)

following the manufacturer's instructions except that the samples were ground directly with the AP1 lysing buffer. In a second step, three DNA extraction protocols were tested and compared with 12 samples each. They included the DNeasy plant mini kit (Qiagen), the Nucleospin plant II kit (Macherey-Nagel) and the PureLink Plant Total DNA purification kit (Invitrogen). For each of the three procedures, the best grinding procedure was used, considering the results of the initial step.

In order to compare the grinding and extraction procedures, the mean Ct value for the qPlat test was used as the variable. Analyses of variance and LSD-Fisher tests were performed in order to evaluate the effects of the different parameters, using XLSTAT software (version 2012.1.01; Addinsoft, Paris).

Comparative testing of the detection tools using naturally infected samples

In total, 32 *Chamaecyparis lawsoniana* samples showing symptoms of necrosis were collected in Bretagne (Table 2) and analyzed by three methods: i) isolation followed by classical morphological observation, ii) conventional PCR using the Ylat3F/Ylat2R PCR primers described by Schena et al. (2008) with a hybridization temperature of 58 °C, and iii) the qPlat-F/-R/-P real-time PCR. In order to prevent cross-contamination between the samples, the analytical process in the laboratory followed the forward flow requirement, and the absence of contamination during the extraction process was checked by the systematic introduction of a blank control (sterile water). Total DNA was obtained following the optimized DNA extraction protocol developed in this study (see Results section) and each DNA extract (10-fold dilution) was tested in duplicate by conventional PCR and in triplicate by real-time PCR. Some of the qPCR products were randomly picked and sequenced to verify the nature of the amplicon.

Results

Design of primers and probe, specificity, sensitivity and efficiency of the qPCR assay

Five new partial sequences of the *RAS-Ypt1* gene were obtained during this study (Table 1). The DNA regions selected as targets for the primers and probe (Table 3) were 100 % conserved within *Phytophthora lateralis*.

The analytical specificity of the primers and probe combination was confirmed by in silico BLAST analyses with all the *P. lateralis* DNA sequences available on GenBank and with 13 other oomycetes DNA sequences (Table 4).

The combination of primers qPlat-F and qPlat-R with the hydrolysis probe qPlat-P was then tested in real-time PCR with a series of DNA extracts from a wide range of oomycetes, including *P. lateralis*, and of non-oomycetes isolated from *Chamaecyparis lawsoniana*. Real-time reactions using this primer-probe combination proved to be highly specific, because low Ct values were obtained for all *P. lateralis* isolates (Table 1), regardless of their geographical origin, whereas no Ct value was yielded with DNA from any other species. All the DNA extracts were successfully amplified by PCR using RAS-E1-F/RAS-E5-R or ITS1/ITS4 primers, thus confirming their amplifiability.

The concentrated DNA extract (114 ng/μL) from *Phytophthora ramorum* was also tested with qPlat-F/-R/-P and no amplification was obtained, thus supporting the high specificity of the test.

The sensitivity of the qPlat-F/-R/-P combination was measured with a 10-fold dilution series of plasmidic copies from the target in deionized DNA-free water, to yield final concentrations ranging from 2.36 to 2.36x10⁶

Table 4 References of the oomycetes *RAS-Ypt1* partial sequences used in this study

Organism	Reference ^a
<i>Phytophthora cambivora</i>	DQ093990
<i>Phytophthora cinnamomi</i>	AF454368
<i>Phytophthora citricola</i>	AF454369
<i>Phytophthora cryptogea</i>	AF454367
<i>Phytophthora infestans</i>	U30474
<i>Phytophthora ipomoeae</i>	JF919599
<i>Phytophthora lateralis</i>	DQ162991
	JN182997
	KJ755155
<i>Phytophthora melonis</i>	EF649778
<i>Phytophthora mirabilis</i>	JF919595
<i>Phytophthora pistaciae</i>	DQ162957
<i>Phytophthora ramorum</i>	DQ270319
<i>Phytophthora uniformis</i>	EU371547
<i>Phytophthora sojae</i>	DQ162958
<i>Pythium spinosum</i>	AF454363

^a Refer to GenBank accession number

pc/μL. A standard curve was constructed and the corresponding amplification efficiency was 0.99. Assays results showed a linear relationship between Ct values and log(initial concentration of the target) down to a concentration of 23.6 pc/μL, and the correlation coefficient (r^2) was 0.99 (Fig. 1). The limit of detection (100 % of positive results) was 23.6 pc/μL (47.2 pc per reaction tube). The same protocol, conducted with the target DNA in a 10-fold dilution of *Chamaecyparis* (branch pieces) DNA extract, reached a similar limit of detection corresponding to 47.2 pc per reaction tube ($r^2 = 0.98$). The plasmidic DNA set at the limit of detection (LOD) was then included as a control throughout the qPCR assays in order to assess the performance of the PCR runs and to ensure that the negative results were caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

Repeatability, reproducibility and robustness

For low concentrations of target DNA diluted in water as well as for a DNA from a naturally *P. lateralis* infected sample (11/379-2b), both the inter assay and intra-assay CVs were low with 1.78 to 2.51 % and 0.58 to 1.06 %, respectively, thus showing that the qPlat test was highly repeatable and reproducible (Table 5).

Regarding robustness, the qualitative results of the real-time PCR test were neither significantly affected by a deliberate ± 10 % variation of the reaction or of the DNA template volumes, nor by a variation of the hybridization/polymerization of ± 2 °C or ± 3 °C (Table 6). Despite this variation, the target was detected in all cases. Even though the mean Ct value was affected by these variations of volumes or temperatures, the

Table 5 Inter and intra-assay coefficient of variation (CVs) based on mean cycle threshold values calculated for the *P. lateralis* real-time PCR assay

Target	Target concentration ^a	CV (%)	
		Intra-assay	Interassay
<i>Phytophthora lateralis</i> qPlat-F/-R product	47.2 ^b	0.83	1.78
	472	1.06	2.01
	4720	0.58	2.51
<i>Phytophthora lateralis</i> genomic DNA	n. d. ^c	0.85	2.36

^a Number of plasmidic copies/μL of the qPlat-F/-R/-P target in the reaction tube, diluted in a background of *Chamaecyparis lawsoniana* DNA

^b Concentration determined as the limit of detection of the test

^c Total DNA extract from a naturally infected *C. lawsoniana* sample

variations were rather limited and remained within a ± 0.5 Ct range. The concentrated *Phytophthora ramorum* DNA extract was also tested in modified conditions potentially reducing stringency: decrease of the reaction volume to 18 μL, increase of the DNA template volume to 2.2 μL, or hybridization/polymerization temperature set at 57 °C and 58 °C. As a result, no amplification was obtained, thus demonstrating that the assay remained specific in spite of unfavourable reaction conditions.

Comparison of plant DNA extraction procedures

Overall, *P. lateralis* was successfully detected in 100 % of the DNA samples obtained, regardless of the grinding methods and the extraction kits tested, with a mean Ct value ranging from 25.7 to 32.9. Nevertheless, the

Fig. 1 Standard curve and correlation coefficient for qPlat-F/-R/-P real-time PCR assessed with dilution of target DNA in molecular-grade water. Target DNA was made of plasmids in which is inserted the *P. lateralis* qPlat amplicon. Final concentrations are ranging from 23.6 pc/μL to 2.36×10^6 pc/μL

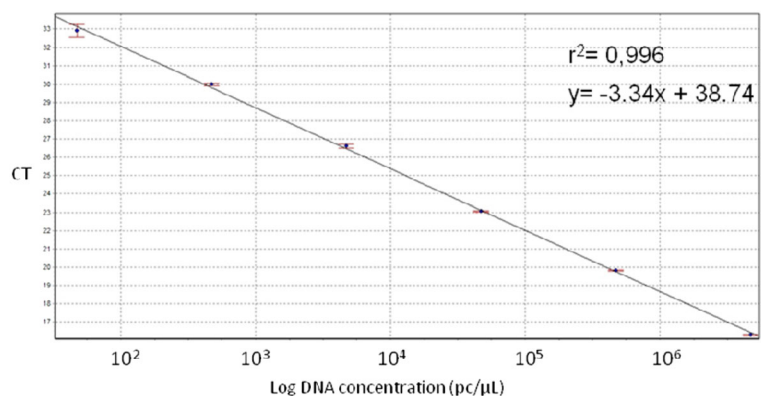


Table 6 Assessment of the robustness of the qPlat-F/R/P assay using 10 %-variable quantity of DNA target, 10 %-variable reaction volumes in individual reaction tubes, and 2 °C and 3 °C-variable hybridization/polymerization temperature, tested with 12 replicates of DNA templates

Sources of variation	Volume/T°C tested	Mean Ct value (±SD) ^d for plasmidic DNA ^b	Mean Ct value (±SD) ^d for genomic DNA ^c
Reaction volume	18 µL	30.79 ± 0.38 ^x	27.97 ± 0.23 ^x
	20 µL ^a	31.35 ± 0.47 ^y	28.10 ± 0.23 ^x
	22 µL	31.60 ± 0.68 ^y	28.34 ± 0.20 ^y
DNA template volume	1.8 µL	31.28 ± 0.45 ^{x,y}	28.16 ± 0.35 ^y
	2.0 µL ^a	31.35 ± 0.47 ^y	28.10 ± 0.23 ^{x,y}
	2.2 µL	30.91 ± 0.49 ^x	27.91 ± 0.27 ^x
Hybridization temperature	57 °C	30.93 ± 0.47 ^{x,y}	27.90 ± 0.17 ^x
	58 °C	31.17 ± 0.61 ^{y,z}	27.95 ± 0.23 ^{x,y}
	60 °C ^a	31.35 ± 0.47 ^z	28.10 ± 0.23 ^y
	62 °C	31.36 ± 0.32 ^z	28.72 ± 0.27 ^z
	63 °C	30.64 ± 0.53 ^x	28.02 ± 0.11 ^{x,y}

^a Original experimental settings

^b 10 times the limit of detection of the test, ie 472 plasmidic copies per reaction tube

^c DNA extracted from a *P. lateralis* naturally infected sample (379-2b)

^d Mean values followed by the same letter on the same line are not significantly different according to Fisher's least significant difference ($p > 0.05$)

Lysing matrix A with garnet matrix and ceramic sphere (FastPrep-24 homogenizer) yielded a significantly lower mean Ct value (mean Ct = 27.3, $F = 48.1$, $p < 0.0001$) than the two other grinding techniques (Table 7). When

Table 7 Mean cycle threshold (Ct) value for each of the grinding methods (Qiagen DNA extraction) and DNA extraction kits (Lysing Matrix A grinding)

DNA extraction protocols	Mean Ct value (SD)	
Grinding method ^a	Beadbeater + glass beads	30.21 (1.39) ^x
	FastPrep + Lysing Matrix A (garnet +ceramic sphere)	27.29 (0.78) ^y
	FastPrep + Lysing Matrix B (silica spheres)	28.77 (0.8) ^z
DNA extraction kit ^a	Qiagen Plant mini kit	26.85 (0.85) ^x
	Macherey-Nagel Nucleospin	28.20 (1.46) ^y
	Invitrogen Purelink	28.22 (0.67) ^y

^a Mean values labeled with the same letter on the same line are not significantly different according to *t* test pairwise comparison ($P > 0.05$)

comparing the different DNA extraction kits, significant differences were also observed ($F = 13.4$, $p < 0.0001$) between Qiagen and the two other kits (Invitrogen and Macherey-Nagel) with the Qiagen kit yielding the lowest mean Ct value (Table 7). The combination Lysing matrix A and FastPrep grinding/Qiagen kit DNA extraction was therefore the best option and retained for the comparison of detection tools with field samples. Using this combination and in our conditions, the threshold Ct_{18S} was calculated and determined at 18.8. This value should be used in the context of routine analyses to assess the quality of the plant DNA extracts.

Comparative testing of the detection tools using naturally infected samples

Out of the 32 samples tested for the presence of *P. lateralis*, 14 were tested negative using all three techniques. All the corresponding DNA extracts yielded a mean Ct value <21 with the 18S uni real-time PCR, which assessed the quality and amplifiability of the DNA extracts. *P. lateralis* was detected in 18 samples by real-time PCR, in 14 by conventional PCR (PCR), and in 11 by isolation (Table 8). All the positive cases obtained either by isolation or conventional PCR were confirmed by real-time PCR. However, for three samples, real-time PCR yielded positive results while *P. lateralis* could not be detected by isolation or by PCR. For these three samples, sequencing of the qPCR amplicon confirmed that the *P. lateralis* target DNA was amplified. In total, real-time PCR, conventional PCR and isolation yielded concordant results (positive or negative) for 71.9 % of the samples (Table 8). Real-time PCR yielded significantly more positive results than isolation ($\chi^2 = 5.14$, $P = 0.023$), whereas no significant difference was observed between real-time PCR and conventional PCR ($\chi^2 = 1.33$, $P = 0.248$), and between isolation and conventional PCR ($\chi^2 = 0.8$, $P = 0.371$). *P. lateralis* was detected by real-time PCR in tissue sampled on roots, wood, bark, branches and foliage.

Discussion

During this work, a new sensitive tool for the molecular detection of *Phytophthora lateralis* on plant tissues was developed and optimized to offer improved specificity and robustness. This new test does not require sub-

Table 8 Comparative analysis of 32 symptomatic *Chamaecyparis* samples by isolation, conventional polymerase chain reaction (PCR), and real-time PCR using a combination of primers and probe targeting *P. lateralis*

Sample	Isolation	Conventional PCR ^a	Real-time PCR ^b Mean CT value (SD)	18S Uni Mean CT value (SD)
11/377-1a	–	–	>40	14.68 (0.02)
11/377-1b	–	–	>40	15.55 (0.16)
11/377-2a	–	–	>40	14.43 (0.04)
11/377-2b	–	–	>40	20.22 (0.34)
11/377-3a	–	–	>40	16.71 (0.01)
11/377-3b	–	–	>40	14.54 (0.08)
11/378-a ^c	–	+	25.51 (0.07)	14.55 (0.14)
11/378-b	–	+	27.66 (0.20)	14.20 (0.19)
11/379-1a	+	+	27.20 (0.13)	14.48 (0.63)
11/379-1b	–	–	>40	15.66 (0.09)
11/379-1c	–	+	29.71 (0.08)	15.37 (0.18)
11/379-2a ^c	–	–	32.81 (0.99)	13.54 (0.20)
11/379-2b	+	+	27.88 (0.11)	14.36 (0.19)
11/379-2c	–	–	>40	12.38 (0.08)
11/379-2d	+	–	34.55 (0.53)	16.03 (0.29)
11/379-3a	+	+	27.14 (0.31)	15.16 (0.13)
11/379-3b ^c	–	–	32.80 (0.65)	Not tested
11/379-4a	+	+	26.79 (0.13)	Not tested
11/379-4b	+	+	24.44 (0.07)	Not tested
11/379-4c	+	+	27.89 (0.17)	Not tested
11/380-1a	+	+	27.45 (0.07)	Not tested
11/380-1b	+	+	26.16 (0.05)	Not tested
11/380-1c	–	–	>40	15.25 (0.24)
11/380-2	–	–	>40	15.58 (0.05)
11/380-3a	–	+	31.19 (0.38)	Not tested
11/380-3b ^c	–	–	32.19 (0.55)	Not tested
11/380-4a	–	–	>40	16.74 (0.18)
11/380-4b	–	–	>40	16.98 (0.34)
11/380-4c	+	+	27.41 (0.13)	Not tested
11/380-4d	+	+	28.04 (0.14)	Not tested
11/380-5a	–	–	>40	16.86 (0.23)
11/380-5b	–	–	>40	15.62 (0.21)

^aConventional PCR = DNA samples were tested in duplicate and were rates as positive or negative based on the presence or the absence of the expected band following electrophoresis of the conventional PCR product

^bReal-time PCR = cycle thresholds were computed with each DNA sample tested in triplicate. Standard deviation is indicated between parentheses

^cReal-time PCR product confirmed by sequencing

culturing a potentially harmful organism, and manipulation of contaminated plant tissues is limited. Because this tool is DNA based, it will detect *P. lateralis* even if the oomycete is not in an active form which is an advantage over the isolation technique. In addition, real-time PCR provides a shorter analysis time than conventional PCR, and the tubes are kept closed after the amplification, which limits cross- and self-contamination, making it more suitable for serial analyses.

The qPlat primers-probe combination targets the intronic region of the single-copy RAS-related protein

gene *Ypt1*, which contains sufficient interspecific but poor intraspecific polymorphism. This confirms the potential of this genomic region for the development of species-specific tools for *Phytophthora*, already been used by Ios et al. (2006) and Schena et al. (2008) for conventional PCR tests. It has proven to be highly specific, since no cross-reactions were observed with 24 isolates representing 17 species of other oomycetes, including the closely related *P. ramorum*. In addition, the qPlat test yielded positive results with DNA from 55 *P. lateralis* isolates, irrespective of the origin and host

plant. Unfortunately, we did not have access during this study to the isolates from Taiwan which comprise two distinct lineages (Brasier et al. 2012) and the inclusivity of the qPlat test should be confirmed by experiments in the future. Likewise, although a comprehensive evaluation of the specificity of the test has been conducted in this work, continuous monitoring would still be useful, since new species of *Phytophthora* are regularly discovered and described.

P. lateralis may infect different organs of the host plants, with variable severity. The method was developed for the detection of *P. lateralis* on symptomatic tissues, but its use for detection on asymptomatic tissues could be considered, for instance during the latent stage. This work improved the sensitivity of the test, in order to detect minute amounts of the pathogen in the plant tissue, and validated a DNA extraction protocol that yields DNA extracts with sufficient quantity and co-extracts limited amounts of inhibiting compounds. The results showed that using different DNA extractions kits and grinding procedures significantly affected the sensitivity of the test, and that a tenfold dilution of the raw DNA extract should be carried out to get an amplifiable DNA template. In order to prevent false negative results due to the presence of inhibiting compounds in the DNA extract, a 18S-uni assay was implemented in a separate real-time PCR tube. Since the PCR parameters were the same for qPlat and the 18S uni test, both assays may be conducted in the same run, making it more convenient for routine analysis.

Testing samples for the presence of a target pathogen requires very high confidence in the quality of the results, in particular in the case of a regulated pathogen. Indeed, in some occasions, the economical and ecological consequences of either a negative or a positive result may be huge. Likewise, false negative results may enable a pathogen to enter a disease-free area, whereas a false positive result may lead to the destruction of shipments or units of production. Therefore this study included a thorough validation process to evaluate the performance of the protocol within the framework of routine analysis. In line with the requirements of the EPPO Standard PM 7/98 (EPPO 2010), the test was shown to be highly repeatable and reproducible and also proved its robustness. The validation process demonstrated that slight temperature or volume variations during the reactions did not qualitatively affect the results, even when target concentrations were close to the limit of detection. This approach simulates actual conditions

in laboratories in charge of routine analysis, using different equipment and with potentially different settings in micropipettes or in thermal cyclers. In all, the implementation of different quality controls such as the non template controls, the limit of detection positive control (LOD), the 18S uni qPCR test, and the experimental verification of the performance criteria make this new test fit for analysis purpose and trustworthy.

During this work, the real-time test was carried out on samples collected from diseased *Chamaecyparis*, in Western France. *P. lateralis* was successfully detected on root, wood, bark, and foliage showing symptoms. According to Hansen et al. (2000), it seems that once the pathogen is introduced and established in a forestry context, *P. lateralis* is virtually impossible to eradicate. It is therefore of paramount importance to prevent the introduction of this oomycete in disease free areas. Although the introduction of host plants such as *Chamaecyparis* are prohibited in EU (Anonymous 2000), *P. lateralis* might still be introduced by contaminated soil or growing media associated with non-host plants. If a sampling method and an appropriate DNA extraction protocol from soil or growing medium are developed in the future, the real-time test could also be helpful to test this kind of substrate that may harbor chlamydospores of *P. lateralis*. In addition, it may be postulated that the pest can be moved and introduced in disease free area by the means of symptomless plants as some chemical treatments against *Phytophthora* disease may suppress symptoms, while not killing the pathogen (Roth et al. 1987). Using an appropriate sampling protocol, and owing to its high sensitivity, this real-time test is expected to enable the detection of *P. lateralis* in such plant materials.

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