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Short Communication

Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae*

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Abstract

Introns are generally highly polymorphic regions within genes and were proven to be of great interest for discriminating among phylogenetically-close *Phytophthora* species. *Phytophthora ramorum* and *P. fragariae* are considered as quarantine pathogens by the European Union and accurate detection tools are therefore necessary for their monitoring. From introns located in different single copy genes (*GPA*1, *RAS*-like, and *TRP*1), we developed a series of PCR primers specific to *P. ramorum* and *P. fragariae*. The specificity of these primers was successfully checked with a wide collection of *Phytophthora* isolates and a protocol was developed to detect both pathogens directly in infected plant tissues. These genes should be of particular interest for the development of additional species-specific detection tools within the *Phytophthora* genus.

Introduction

The genus *Phytophthora* includes major plant pathogens causing severe losses throughout the world. Some of them are ancient and well known devastating organisms, (e.g. *P. infestans* on potato crops), but this genus also harbours emergent and aggressive new species, such as *P. alni*, responsible for alder disease in Europe (Brasier et al., 2004) or *P. ramorum* causing Sudden Oak Death in North America (Rizzo et al., 2002). In addition, some species such as *P. ramorum* or *P. fragariae* var. *fragariae* (causing the root rot disease on strawberry) are regulated or quarantine registered pathogens for the European Union (Anon., 2000, 2002). Specific and sensitive detection tools are therefore needed for the monitoring of these particular species. Recently, a set of nuclear single copy genes containing intron(s) were studied for the genetic characterization of the hybrid species complex *Phytophthora alni* (Ioos et al., 2006). The four genes studied, namely *ASF*-like, *GPA*1, *RAS*-like, and *TRP*1, proved to be appropriate for the discrimination among the different subspecies of *P. alni*, and the phylogenetically close species *P. cambivora* and the two varieties of *P. fragariae* (var. *fragariae* and var. *rubi*). In order to isolate and clone these genes, Ioos et al. (2006) developed a set of four gene-specific degenerate primers. The design of the primers was based on sequence alignment conducted with original sequences of these genes deposited in GenBank, with orthologous sequences retrieved from the genome sequence project of *P. ramorum* and *P. sojae* (http://genome.jgi-psf.org/) and from the *P. infestans* EST database (http://www.pfgd.org/pfgd/filter.html).

In the present study, we successfully designed a set of *P. ramorum*-specific primers (Table 1) based

Table 1. Characteristics of the species-specific PCR primers for *P. ramorum* and *P. fragariae*

Species	Primer (forward/reverse)	Sequence (5′-3′)	Size of the amplicon	Sensitivity (pg per 20 µl of PCR mixture)	Reference[a]
P. ramorum	GPA-PRAM-F[b]	TAAGGAACAAGGTACCAAAG	248 bp	0.5 pg	Scaffold_59 316212–318351
	GPA-PRAM-R[b]	CTCAGGAATTCACTCTCACG			
	TRP-PRAM-F[b]	GAGTAGAAACTTCGGGAATG	527 bp	0.5 pg	Scaffold_52 330699–333530
	TRP-PRAM-R[b]	GTTCGGCACATTAACGCAG			
P. fragariae var. *fragariae* (*Pff*)/*P. fragariae* var. *rubi* (*Pfr*)	RAS-PFR109h1-F[c]	TGTCGAGAGTGATTTATT	229 bp	1 pg (*Pff*)	DQ093986
	RAS-PFR109h1-R[c]	AATGGCAAGGCTAGTTACTA		1 pg (*Pfr*)	
	TRP-PFF309a9-F[c]	CTACCTCCCTAAGCTTATCA	403 bp	0.1 pg (*Pff*)	DQ094005
	TRP-PFF309a9-R[c]	ACGCAGCATCATAGAAAAT		0.5 pg (*Pf*)*r*	

[a]Refer to Genbank accession number for *P. fragariae* or localization of the orthologous sequence in the *P. ramorum* JGI sequencing project (Ioos et al., 2006).
[b]Primer designed and tested in this study.
[c]Primer designed in Ioos et al. (2006) and tested in this study.

on species-specific polymorphisms revealed by multiple alignments using all the orthologous sequences available for different *Phytophthora* species. Polymorphism was mainly located within the intronic regions of two of these genes (*GPA*1 and *TRP*1). Similar species-specific polymorphisms have already been exploited by Ioos et al. (2006) in *RAS*-like and *TRP*1 genes to develop a set of two *P. fragariae*-specific PCR primer pairs (Table 1). In this study, the *P. ramorum* and *P. fragariae*-specific PCR primers were assessed for their specificity and for their ability to detect both pathogens directly *in planta*.

Oomycete DNA was extracted from pure culture using a commercial plant DNA extraction kit (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) and as previously described (Ioos et al., 2005). Plant DNA was extracted as follows. Approximately 200 mg of fresh plant tissue (either *Fragaria* × *ananassa* or *Rubus idaeus* roots, or symptomatic tissues of *P. ramorum* potential hosts) were collected and first roughly cut using a sterile scalpel blade. Then the sample was transferred into a 2 ml micro-centrifuge tube and ground for 2 min with two 3 mm tungsten carbide beads at a frequency of 30 Hz with a mixermill grinder (Tissuelyser®, Qiagen). Genomic DNA was subsequently extracted using DNeasy plant mini kit® (Qiagen) following the manufacturer's instructions, except that after incubation with the lysis buffer, the microtubes were centrifuged for 4 min at 14,000*g* to pellet the cellular debris.

PCR was conducted using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, California, USA) in 20 µl reaction mixtures consisting of 2 µl of template DNA (30–80 ng), 1× Taq polymerase buffer (Sigma-Aldrich, L'Isle d'Abeau, France), 2 mM $MgCl_2$, 0.6 µg μl^{-1} Bovine Serum Albumin (Sigma-Aldrich), 0.45 µM of each forward and reverse primer, 200 µM dNTPs, 0.5 unit of Taq DNA Polymerase (Sigma-Aldrich), and molecular biology grade water was added to 20 µl. The PCR conditions included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and elongation for 1 min at 72 °C, and a final extension step at 72 °C for 7 min.

The specificity of the four primer pairs was successfully checked with a wide collection of *Phytophthora* spp. and *Pythium* spp. (Table 2).

Table 2. Isolates of *Phytophthora* spp. and *Pythium* spp. tested in this study

Species	Isolate	Host	Geographical origin	PCR test GPA-PRAM-F/R	TRP-PRAM-F/R	RAS-PFR109h1-F/R	TRP-PFF 309a9-F/R	ITS6/ITS4[a]
P. ramorum (A1)	2N0983	*Rhododendron* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	3N0003	*Viburnum* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	F001	*Rhododendron* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	RAM_Phot	*Photinia* sp. (Nursery)	Poland	+	+	–	–	+
P. ramorum (A1)	RAM_Cal	*Calluna* sp. (Nursery)	Poland	+	+	–	–	+
P. ramorum (A2)	2338	*Viburnum tinus* (Nursery)	Belgium	+	+	–	–	+
P. ramorum (A2)	05-1461-160	*Pieris japonica* (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	05-1461-63	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9138	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9143	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	4566	*Rhododendron macrophyllum* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9180	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	04-189-B2	*Viburnum bodnantense* (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	04-79-B1	*Camelia* spp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A1)	03-74-N11C	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A1)	03-74-N11D	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. fragariae var. *fragariae*	PFF1	*Fragaria x ananassa*	England	–	–	+	+	+
P. fragariae var. *fragariae*	CBS209.46	*Fragaria x ananassa*	England	–	–	+	+	+
P. fragariae var. *fragariae*	CBS309.62	*Fragaria x ananassa*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	PFRVR 59	*Rubus idaeus*	Great-Britain	–	–	+	+	+
P. fragariae var. *rubi*	PFR163-2	*Rubus idaeus*	France	–	–	+	+	+
P. fragariae var. *rubi*	PFR2	*Rubus idaeus*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	CBS967.95	*Rubus idaeus*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	CBS109.892	*Rubus idaeus*	Scotland	–	–	+	+	+
P. alni subsp. *alni*	PAA129	*Alnus glutinosa*	France	–	–	–	–	+
P. alni subsp. *uniformis*	PAU60	*Alnus glutinosa*	France	–	–	–	–	+
P. alni subsp. *multiformis*	PAM54	*Alnus glutinosa*	France	–	–	–	–	+
P. cactorum	CAC4810/TJ	unknown	France	–	–	–	–	+
P. cactorum	5N1449/T1	*Viola* spp.	France	–	–	–	–	+
P. cambivora	PCJC17	*Quercus* spp. soil	France	–	–	–	–	+
P. cambivora	4N1125/1	*Castanea sativa*	France	–	–	–	–	+
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	–	(+ >)[b]	–	–	+
P. cinnamomi	4N0741-O	*Castanea sativa*	France	–	(+ >)[b]	–	–	+
P. citricola	2AE5	*Quercus* sp. soil	France	–	–	–	–	+
P. citricola	4N697/1	*Syringa* spp.	France	–	–	–	–	+
P. citrophthora	2N1021	*Rosa* sp.	France	–	–	–	–	+
P. citrophthora	5N0373	*Pieris* sp.	France	–	–	–	–	+
P. cryptogea	990675	*Actinidia sinensis*	France	–	–	–	–	+
P. cryptogea	CH 2555/00	*Chamaecyparis lawsonia*	Poland	–	–	–	–	+

Table 2. continued

Species	Isolate	Host	Geographical origin	PCR test GPA-PRAM-F/R	TRP-PRAM-F/R	RAS-PFR109h1-F/R	TRP-PFF 309a9-F/R	ITS6/ITS4[a]
P. erythroseptica	960713	*Polygonum oberti*	France	–	–	–	–	+
P. europaea	AL5	*Quercus* sp. soil	France	–	–	–	–	+
P. gonapodyides	Gonap 4	*Quercus* sp. soil	France	–	–	–	–	+
P. gonapodyides	5N0391	*Rhododendron* sp.	France	–	–	–	–	+
P. humicola	3N1245-j	*Alnus glutinosa* soil	France	–	–	–	–	+
P. inundata	9500802	*Alnus glutinosa* soil	France	–	–	–	–	+
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	–	–	–	–	+
P. megasperma	3N1245-m	*Alnus glutinosa* soil	France	–	–	–	–	+
P. megasperma	9900557	*Prunus* sp.	France	–	–	–	–	+
P. nicotianae	960579	*Nicotiana tabacum*	France	–	–	–	–	+
P. taxon forestsoil	8CARPPOC1	*Quercus* sp. soil	France	–	–	–	–	+
P. palmivora	970423	*Hedera* sp.	France	–	–	–	–	+
P. parasitica	970029	*Lycopersicon esculentum*	France	–	–	–	–	+
P. taxonPgchlamydo	Haye,3,1	*Quercus* sp. soil	France	–	–	–	–	+
P. pseudosyringae	EW5	*Quercus* sp. soil	France	–	–	–	–	+
P. psychrophila	FF20	*Quercus* sp. soil	France	–	–	–	–	+
P. quercina	FNA	*Quercus* sp. soil	France	–	–	–	–	+
P. sojae	443	*Glycine max*	Unknown	–	–	–	–	+
P. syringae	2JZ2	*Quercus* sp. soil	France	–	–	–	–	+
P. syringae	4N0247/5	*Rhododendron* sp.	France	–	–	–	–	+
Pythium aphanidermatum	Ctsa	unknown	France	–	–	–	–	+
Pythium sylvaticum	0675/a	unknown	France	–	–	–	–	+
Pythium intermedium	02/84/1	unknown	France	–	–	–	–	+
Pythium irregulare	02/57/1	unknown	France	–	–	–	–	+
Pythium ultimum	433/3	unknown	France	–	–	–	–	+
Pythium spp	5N0691	*Photinia* sp.	France	–	–	–	–	+
Pythium spp.	3N1345-11	*Alnus glutinosa* soil	France	–	–	–	–	+

[a]ITS6 and ITS4 primers efficiently target *Phytophthora* and *Pythium* spp. ITS regions (Cooke et al., 2000).
[b]yielded a faint band of ca. 650 bp.

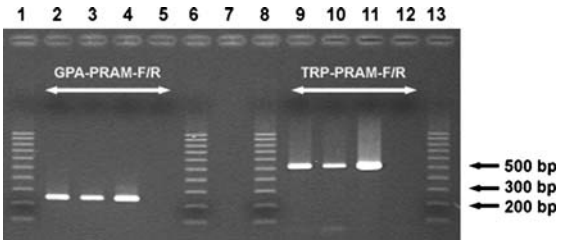

Figure 1. PCR products obtained with the two *P. ramorum* primer pairs developed in this study: GPA-PRAM-F/R and TRP-PRAM-F/R, respectively. Lanes 1, 6, 8 and 13: 100 bp DNA ladder (Sigma-Aldrich); lanes 2 and 9: DNA extracted from a naturally *P. ramorum*-infected *Rhododendron* spp. leaf; Lanes 3 and 10: DNA extracted from a naturally *P. ramorum*-infected *Viburnum* spp. leaf, Lanes 4 and 11: *P. ramorum* isolate 2N0983 DNA extract (positive control); Lanes 5 and 12: negative control (water). Lane 7 was not used.

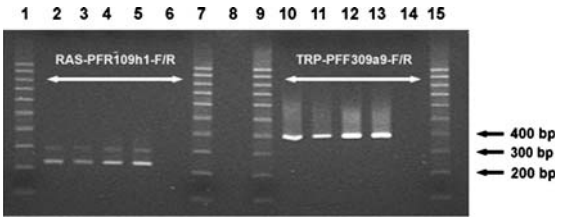

Figure 2. PCR products obtained with the two *P. fragariae* primer pairs tested in this study: RAS-PFR109h1-F/R and TRP-PFF309a9-F/R, respectively. Lanes 1, 7, 9 and 15: 100 bp DNA ladder (Sigma-Aldrich); lanes 2 and 10: DNA extracted from naturally *P. fragariae* var. *fragariae*-infected strawberry root sample PV35; Lanes 3 and 11: DNA extracted from naturally *P. fragariae* var. *fragariae*-infected strawberry root sample PV62; Lanes 4 and 12: *P. fragariae* var. *rubi* isolate CBS109.892 DNA extract (positive control); Lanes 5 and 13: *P. fragariae* var. *fragariae* isolate CBS309.62 DNA extract (positive control); Lanes 6 and 14: negative control (water). Lane 8 was not used. Using the RAS-PFR109h1F/R primer pair, a shadow aspecific band of ca 300 bp was systematically co-amplified with the DNA target but did not interfere with the sensitivity of the test.

Both *P. ramorum*-specific primer pairs yielded a positive signal of the expected size only when tested with *P. ramorum* isolates, regardless of their natural host, geographical origin and mating type (A1 or A2). Likewise, only the two varieties of *P. fragariae* yielded a positive signal with the two *P. fragariae*-specific primer pairs. However, *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi*, occurring on strawberry and on *Rubus* spp., respectively, could not be distinguished with these PCR tests.

The sensitivity threshold for each primer pair was determined experimentally using pure oomycete DNA (Table 1). Several molecular detection tools have already been developed for *P. fragariae* detection and many of them were extensively compared by Bonants et al. (2004). In this respect, the two *P. fragariae* PCR tests described here seem to be equally or even more sensitive than other published single-round PCR tests, except for those using Molecular Beacons™ which were able to detect down to 0.1 pg of *P. fragariae* DNA (Bonants et al., 2004). Likewise, the two *P. ramorum* primer PCR tests described here appear more sensitive than previously published *P. ramorum*-specific single-round PCR tests (Kroon et al., 2004), despite being less sensitive than the real-time PCR-based assay described by Tomlinson et al. (2005) or the nested PCR protocols developed to detect this pathogen (e.g. Martin et al., 2004; Hayden et al., 2004).

Our species-specific primer pairs were also successfully used to detect *P. ramorum* or *P. fragariae* in naturally infected plant tissues (Figures 1 and 2). In addition, during a 2-year survey of *P. ramorum*, the French Plant Protection Laboratory (LNPV-UMAF) carried out a comparative assessment of one of the *P. ramorum* PCR tests described here (TRP-PRAM-F/R) vs. isolation on the *Phytophthora* selective medium PARBHY (Robin et al., 1998). Total of 448 plant samples were simultaneously analysed using both methods. The results showed that for 86.7% of the samples, both techniques yielded identical results, whereas for 12.1% of the samples the PCR test detected the presence of *P. ramorum* while the isolation technique failed to do so. The 12.1% positive samples were further successfully confirmed using the alternative *P. ramorum*-specific primer pair GPA-PRAM-F/R. In the remnant 1.2%, PCR failed to detect *P. ramorum*, while it was successfully isolated on the selective medium. For these samples, the *P. ramorum* isolates recovered reacted positively with the TRP-PRAM-F/R PCR test. Therefore, this suggests that for these samples the biomass of *P. ramorum* was probably heterogeneously present between the respective PCR or isolation sub-samples.

The PCR primer pairs developed in this study should be of great interest for quarantine survey purposes. In addition, the use of at least two independent PCR tests for both pathogens may

strengthen the diagnosis. Such confirmation tests targeting independent loci might prevent the occurrence of 'false-positive' samples (Garbelotto, 2003).

Finally, the intronic regions from which these PCR primers were designed could also be potentially used for the development of detection tools for other economically important *Phytophthora* species.

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