# A selective PCR-based method for the identification of *Phytophthora hibernalis* Carne

D. Montenegro[1], O. Aguín[1]*, C. Pintos[1], M. J. Sainz[2] and J. P. Mansilla[1,2]

[1] *Estación Fitopatolóxica do Areeiro. Subida a la Robleda, s/n. 36153 Pontevedra. Spain*
[2] *Departamento de Producción Vegetal. Universidad de Santiago de Compostela. 27002 Lugo. Spain*

## Abstract

Preharvest and postharvest brown rot of citrus fruit is responsible for important economic losses thoroughout the world. The disease is commonly caused by several species of *Phytophthora.* In Spain, citrus brown rot is mainly caused by *P. citrophthora*, but in the last years outbreaks of the disease in lemon and sweet orange caused by *P. hibernalis* Carne have been recorded in northwestern citrus-growing areas. In this work, a PCR method has been developed for the diagnostic of *P. hibernalis* either from isolated mycelia or directly from fruit lesions. One specific primer pair, PHIB1 and PHIB2, was designed from the nucleotide sequences of ITS1 and ITS2 regions. The two primers amplified a 407-bp fragment from the genomic DNA of *P. hibernalis* that was sequenced (Acc. No. AY827556). Results demonstrated that PCR amplification of ITS regions by primers PHIB1 and PHIB2 followed by DNA sequencing can provide a rapid, selective and reliable identification of *P. hibernalis*.

**Additional key words**: *Citrus*, diagnostics, fruit brown rot, oomycetes, specific primers.

## Resumen

### Método selectivo de PCR para la identificación de *Phytophthora hibernalis* Carne

La podredumbre marrón del fruto en cítricos ha ocasionado importantes pérdidas económicas en pre y postcosecha en todo el mundo. Por lo general esta enfermedad está provocada por varias especies de *Phytophthora.* En España, *P. citrophthora* es el principal agente causante de esta patología, sin embargo, en los últimos años, *P. hibernalis* Carne ha sido detectado ocasionando daños en limoneros y naranjos localizados en el noroeste del país. En el presente trabajo, se ha desarrollado un método de PCR para el diagnóstico de *P. hibernalis* tanto a partir de micelio en cultivo como directamente de lesiones del fruto. Se diseñó un par de primers específicos, PHIB1 y PHIB2, a partir de secuencias de las regiones ITS1 e ITS2. Estos dos primers amplificaron un fragmento de 407 pb, que fue secuenciado (Acc. No. AY827556). Los resultados demostraron que la amplificación por PCR de las regiones ITS mediante los primers PHIB1 y PHIB2 seguida por la secuenciación del ADN permiten una identificación rápida, selectiva y fiable de *P. hibernalis*.

**Palabras clave adicionales**: *Citrus,* diagnóstico, oomicetos, podredumbre marrón del fruto, primers específicos.

## Introduction[1]

Root rot, crown rot (gummosis) and brown rot of the fruit are the main diseases in citrus-growing areas, and are caused by several *Phytophthora* species, mainly *P. palmivora*, *P. citrophthora, P. citricola, P. hibernalis, P. syringae* and *P. nicotianae* (Timmer and Menge, 1988; Erwin and Ribeiro, 1996; Graham *et al.,* 1998; Vernière *et al.*, 2004). Citrus rot diseases are responsible

for important economic losses, particularly brown rot of fruits, which results in extensive fruit damage in rainy autumns, and can appear before or after the harvest. Fruits show symptoms of dessication and necrosis, with some firm areas getting a dull grey discolouration, that gradually turns brown, and acquiring a leathery appearance. Lesions can support the development of a white mycelium. *P. nicotianae* and *P. citrophthora* are widely recognized as the main causal agents of

[1] Abbreviations used: ITS (internal transcribed spacer), NCBI (National Center for Biotechnology Information), NW (northwest), rDNA (ribosomal DNA), RFLP (restriction fragment length polymorphism), Tm (melting temperature).

brown rot of fruits, although differences in incidence among important citrus-growing areas have been reported. In Florida (USA), citrus brown rot is caused primarily by *P. palmivora*, and secondarily by *P. nicotianae* (Timmer *et al.*, 2000). In mediterranean citrus plantations, outbreaks of *Phytophthora* diseases in citrus fruits have been mostly attributed to *P. citrophthora*, *P. citricola, P. hibernalis* and *P. syringae* (Erwin and Ribeiro, 1996; Vicent *et al.,* 2003; Vernière *et al.,* 2004).

In Europe, citrus crops are cultivated only in mediterranean countries. The main citrus producers are Spain, Italy and Greece (FAO, 2004). Spain produces 6.3 millions tons of fruit, mainly of sweet oranges, mandarins and lemons, and the main citrus-growing areas are in the East coast of the country (MAPA, 2004). In Spanish orchards, brown rot of fruits is mainly caused by *P. citrophthora* (Páez *et al.,* 2004)*,* and usually appears in years with excessive rains (higher than 100 mm) continuing for two or three days during the autumn and winter (Tuset *et al.*, 1984).

Small tree plantations and isolated trees can also be found in other Spanish regions with low or null risk of frosts. This is the case of Galicia (NW Spain), in which small plantations of lemons and near 120,000 isolated trees, mostly of lemon and sweet orange, are produced mainly in two provinces, A Coruña and Pontevedra. Despite the climate of Pontevedra is maritime mediterranean, with mild temperatures in winter and little interannual thermal variability, since 2003 sporadic outbreaks of citrus brown rot in lemon and sweet orange caused by *P. hibernalis* have been recorded at the province.

*P. hibernalis* is seldom found causing brown rot of fruits in mediterranean countries, and has only been reported in areas with cool wet winters (Timmer and Menge, 1988).

The identification of *P. hibernalis* is solely based on morphological characteristics of sporangia and sexual organs. *P. hibernalis* is included in Stamps and Waterhouse morphological group IV (Stamps *et al.*, 1990). It is an homothallic fungus that produces semipapillate, caducous sporangia with long pedicel, and can form paragynous and amphyginous antheridia (Waterhouse and Waterston, 1964). No molecular method specific for this Oomycete has been developed. However, molecular methods based on the analysis of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (rDNA) have complemented and greatly improved the diagnostics of most *Phytophthora* species. Ristaino *et al.* (1998) have proved PCR-RFLP patterns,

obtained with the restriction enzymes *Rsa*I, *Msp*I, and *Hae*III, to be effective for identification of isolates of 14 *Phytophthora* species, including *P. nicotianae*, *P. citrophthora*, *P. citricola*, and *P. palmivora*. Ippolito *et al.* (2002) designed specific primers to amplify, by nested PCR, DNA from *P. nicotianae* and *P. citrophthora* isolated from citrus roots and soils. Specific primers were also used for detecting *P. nicotianae* and *P. citrophthora* in citrus roots and soils by real-time experiments (Ippolito *et al.*, 2004).

The aim of the present work was to develop a molecular method for the detection and identification of *P. hibernalis*.

## Material and Methods

### Isolation of *P. hibernalis*

Forty two fruits from 11 trees of *Citrus sinensis* (sweet orange) and from 10 trees of *C. lemon* (lemon) showing symptoms of citrus brown rot were collected in the province of Pontevedra (NW Spain) from 2003 to 2007. Fragments of lesions from the fruit surface were placed in 9 cm Petri dishes containing V8-agar medium supplemented with pimaricin 5 mg L$^{-1}$, rifampicin 25 mg L$^{-1}$, hymexazol 5 mg L$^{-1}$, and benomyl 10 mg L$^{-1}$. Petri dishes were kept at 16°C in the dark for one week. Identification of the isolates was based on morphological characteristics of sporangia and sexual organs (Waterhouse, 1963; Stamps *et al*., 1990; Erwin and Ribeiro, 1996).

### DNA extraction

Genomic DNA was extracted either directly from 10 to 40 mg of fragments of fruit lesions or from 10 to 40 mg of isolated mycelia. In case of mycelial samples, pieces of mycelium were removed with a sterile scalpel from the border of colonies actively growing in plates. For both types of material (fruit lesions and isolated mycelia), fungal genomic DNA was extracted with the EZNA Fungal DNA Miniprep kit (Omega Biotek), following the manufacturer's instructions with the exception of RNase or mercaptoethanol, which were not used. The DNAs were electrophoresed on 0.8% agarose gels buffered with 0.5 X TBE (0.89 M Tris base, 0.89 M boric acid, and 0.02 M EDTA pH 8.4) (BioRad) (Sambrook *et al.,* 1989) and stained with ethidium bromide.

## Amplification of rDNA ITS region from *Phytophthora*

A fragment containing the ITS regions was amplified by a nested PCR. Primers DC6 (5'-GAGGGACTTTTG GGTAATCA-3') and ITS4 (5'-TCCTCCGCTTATTG ATATGC-3') were used in the first PCR round, and primers ITS4 and ITS6 (5'-GAAGGTGAAGTCGTAA CAAGG-3') in the second one (Cooke *et al.,* 2000). The primer DC6, in combination with ITS4, selectively amplifies the ITS regions of members of the order *Pythiales*, such as *Phytophthora* (Bonants *et al.,* 1997).

PCR reactions were carried out in microcentrifuge tubes each containing one PuReTaq Ready-To-Go™ PCR Bead (Amersham Biosciences). For both PCRs, each reaction tube further contained 1 µL genomic DNA, 0.3 µL of each primer (10 µM), and sterile water up to a final volume of 25 µL. DNA amplification reactions were carried out in a PCR-Express thermocycler (Thermo Hybaid) under the following conditions: one cycle at 94°C for 3 min; 30 cycles at 94°C for 30 s, 55ºC for 30 s, 72°C for 1 min; and a final cycle at 72°C for 10 min. Negative and positive controls were included in all experiments. PCR products were elec-trophoresed on 2% agarose gel as above described. The size of PCR products was calculated by comparison with a 100 bp standard ladder (Marker XIV, Roche Diagnostics), using the program ID-manager (TDI, Madrid).

## RFLPs (restriction fragment length polymorphism)

Seven microliters from PCR reactions amplified by primers ITS4 and ITS6 were digested with the restriction endonucleases *Alu*I, *Msp*I and *Taq*I (Roche Diagnostic). Restriction digests were run on 3% agarose gel. A standard ladder of 100 bp was also run to accurate sizing of the restriction fragments. Restriction patterns were compared, through a telematic application, against a library elaborated by Cooke and Smith (2000) that includes patterns of over 40 *Phytophthora* spp. other than *P. hibernalis*.

## DNA sequencing and design of specific primers for *P. hibernalis*

Amplicons obtained with primers ITS4 and ITS6 were purified using High Pure PCR Product Purification

columns (Roche Diagnostics), according to the manufacturer's protocol. DNA sequences were obtained by automated DNA sequencing with fluorescent terminators using an ABI Prism Sequencer (Applied Biosystems). DNA sequences were analysed with the program Sequencing Analysis 5.1 (AP Biotech) and the BLAST algorithm (http:www.ebi.ac.uk).

Primer design was based on 44 ITS sequences of *P. hibernalis* (42 from isolates obtained in Pontevedra and 2 from GenBank) and on 263 ITS sequences of another 15 *Phytophthora* species available from GenBank: *P. ramorum* and *P. lateralis*, chosen because of their close phylogenetic relationship to *P. hibernalis* (Ivors *et al*., 2004), and *P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea, P. fragariae, P. megasperma, P. nicotianae, P. palmivora, P. parasitica,* and *P. syringae*, all reported as plant pathogen on different cultivated species in Spain (De Andrés *et al.*, 1998).

Sequences were aligned by using the program Clustal X (Thompson *et al.*, 1997). Seven candidate primers, internal to the universal primers ITS4 and ITS6, were identified and evaluated for melting temperature (Tm), G+C content, primer secondary structure and primer dimer formation, with the Beacon Designer software (Premier Biosoft International). Two primers were finally selected and designated PHIB1 (forward) and PHIB2 (reverse).

The primers PHIB1 and PHIB2 were tested with DNA from two type culture specimens of *P. hibernalis* (CBS 114104 and CBS 953.87) and 123 isolates (the number for each species is indicated within brackets): *P. hibernalis* (42) from Pontevedra, *P. cinnamomi* (4), *P. nicotianae* (6), *P. citricola* (5), *P. megasperma* (2), *P. cactorum* (2), *P. palmivora* (2), *P. cryptogea* (4), *P. capsici* (1), *P. ramorum* (18) and *Pythium* spp. (32), all isolated from field samples and maintained in culture at the Estación Fitopatolóxica do Areeiro; *P. capsici* (1), *P. citricola* (1)*,* and *P. nicotianae* (1), kindly provided by Juana Páez (Laboratorio de Sanidad Vegetal, Consejería de Agricultura y Pesca, Junta de Andalucía, Sevilla); *P. cactorum* (1) and *P. citrophthora* (1)*,* supplied by Luisa Gallo (Departamento de Protección Vegetal, Instituto Canario de Investigaciones Agrarias, Tenerife). Isolates of *Pythium* were included as the outgroup. Experiments were carried out to test four annealing temperatures (64, 66, 67 and 69°C), with the aim of achieving maximum primer specificity for *P. hibernalis.* PCR fragments amplified by PHIB1 and PHIB2 were then sequenced and matched against

NCBI database to confirm the identification of the pathogen.

Further experiments were conducted to assess the sensitivity of both primers. Serial dilutions of genomic DNA from all *P. hibernalis* isolates, from 1 μg to 1 fg μL$^{-1}$, were prepared to determine the smallest amount of DNA template needed to obtain a PCR product with the primers. Negative controls (no DNA template) were also included.

## Results

In culture, isolates from symptomatic fruits were homothallic and formed slowly growing hyaline fine mycelia, which were irregularly branched, without hyphal swellings or chlamydospores. Isolates produced spherical oogonia 22 to 56 μm in diameter, with antheridia mainly amphyginous (some paragynous were also observed), and yellow-orange plerotic oospores. Sporangia were elongated, ellipsoidal to ovoid, caducous and semipapillate, with long pedicels. These morphological characters indicated that all fruits were infected by *P. hibernalis*.

Since no molecular method had been previously reported for identification of *P. hibernalis*, techniques previously described for molecular identification of other pathogenic *Phytophthora* species were initially used. Primers ITS4 and DC6 yielded a single 1,300 bp band from all DNA templates, confirming that isolates were Oomycetes.

The universal primers ITS4 and ITS6, that have been successfully used to identify over 40 *Phytophthora* species (not including *P. hibernalis*) by RFLP-PCR analysis, amplified a single fragment of approximately 900 bp from all isolates, that was subsequently used for restriction enzymatic digestion. After digestion of DNA templates from our isolates of *P. hibernalis*, three different restriction patterns were obtained with endonucleases *Alu*I, *Msp*I and *Taq*I (Fig. 1). Five DNA fragments were found with the enzyme *Alu*I (67, 78, 120, 180, 396 bp), 4 with *Msp*I (81, 122, 324, 378 bp) and 5 with *Taq*I (89, 104, 159, 195, 292 bp). When matching these experimental RFLP patterns against the library available at a telematic application, considering a band width of 10% and lower cut-off length of 10%, the best match was *P. lateralis* with a 59% similarity.

In contrast with the inefficacy of the RFLP-PCR method for the identification of *P. hibernalis*, when the sequence of DNA amplified by primers ITS4 and ITS6 from citrus isolates in Pontevedra was matched against the GenBank library, a 100% homology with the corresponding sequences of *P. hibernalis* was found. This sequence was deposited in the GenBank database as Acc. No. AY827556.

To develop specific primers for *P. hibernalis*, multiple sequences of the ITS region of *P. hibernalis* and another 15 *Phytophthora* species were aligned. Two primers were designed: PHIB1 (5'-TCGGGTCTGAGCTAGTA GTCTT-3') and PHIB2 (5'-CTTCCACAACCAATTC CATTATGC-3'). The primers amplify a 407 bp product that contains parts of ITS1, ITS2 and the whole 5.8S
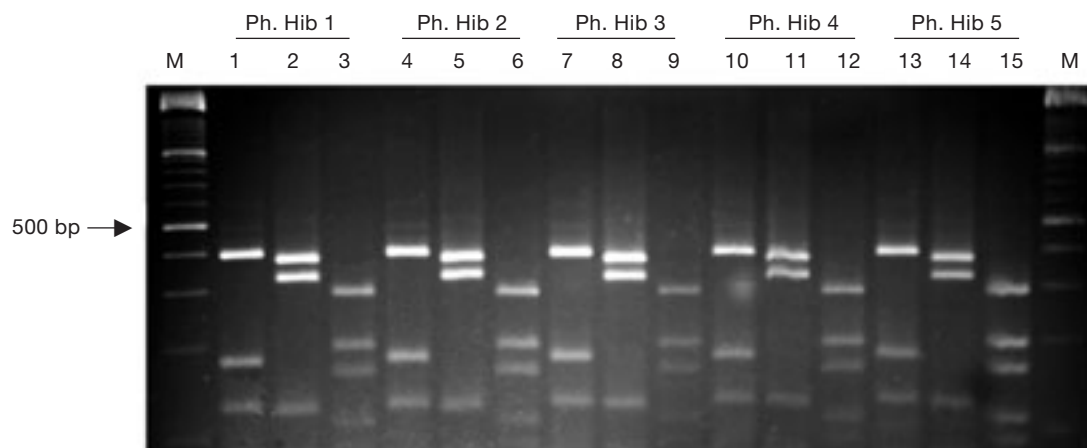


**Figure 1.** Restriction patterns observed after digesting with *Alu*I, *Msp*I or *Taq*I enzymes the PCR products amplified using the primers ITS4/ITS6 and the DNA obtained from five *Phytophthora hibernalis* (Ph.Hib) from *Citrus* in Pontevedra. Ph.Hib (1-3): mycelial isolates; Ph.Hib (4-5): fruit lesions. Lanes 1, 4, 7, 10 and 13: *Alu*I; 2, 5, 8, 11 and 14: *Msp*I; 3, 6, 9, 12 and 15: *Taq*I. M: 100-bp DNA ladder.

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subunit. The primer sequences were matched against those of all *Phytophthora* species included in GenBank, no homology being found except for *P. hibernalis*.

Primers PHIB1 and PHIB2 were then tested to amplify DNA of *P. hibernalis* mycelial samples and fruit lesions from Pontevedra, culture type specimens of *P. hibernalis,* and collection isolates of seven other *Phytophthora* species and *Pythium* spp., yielding an expected 400 bp band for *P. hibernalis* and no band for the rest, with the only exception of a light band for *P. citricola* DNA. These PCR results were obtained with an annealing temperature of 64°C. In experiments where the temperature was increased up to 66-69°C, that band disappeared. It must be outlined that primers PHIB1 and PHIB2 gave no cross reaction with *P. ramorum*. All sequences of DNA amplified by these two primers matched those of *P. hibernalis* available at GenBank library.

Experiments on primer sensitivity showed that the smallest concentration of DNA template needed to obtain a PCR product was 1 pg µL$^{-1}$, that corresponds to a concentration of 40 fg µL$^{-1}$ in the reaction tube (Fig. 2).

## Discussion

*Phytophthora* taxonomy has been based until recently on morphological characters of isolates in culture. Sporangia and sexual organs of *Citrus* isolates from Pontevedra resembled those described for *P. hibernalis*. However, this type of identification needs considerable experience and time of the researcher. Tools to un-equivocally differentiate *Phytophthora* species are

needed. Molecular methods are now considered useful, reliable and fast techniques for diagnostics of many *Phythophthora* species, although none had been described specifically for *P. hibernalis*.

In this work, the RFLP-PCR method proved to be not sufficient for a reliable molecular identification of *P. hibernalis*. The experimental RFLP patterns yielded by *Alu*I, *Msp*I and *Taq*I for *P. hibernalis* best matched, although poorly, with those from *P. lateralis* included in the library of Cooke and Smith (2000), and were also similar to those achieved with these enzymes for *P. ramorum* isolates from *Camellia japonica* and *Viburnum tinus* (Pintos *et al.*, 2004). Our results are in agreement with previous works that have demonstrated the close relationship between *P. ramorum*, *P. lateralis* and *P. hibernalis* (Martin and Tooley, 2003; Ivors *et al.*, 2004). In fact, differentiation of isolates of these *Phytophthora* species by traditional methods is difficult, since, as discussed by Martin and Tooley (2003), they share some morphological characteristics. *Phytophthora hibernalis* and *P. lateralis* are homothallic, and *P. ramorum* is heterothallic. *P. ramorum* and *P. hibernalis* have semipapillate caducous sporangia, whereas *P. lateralis* can be distinguished from them because it is in morphological group V and has non-papillate non-caducous sporangia. These three species also share some environmental requirements, as their growth is favoured by cool temperatures (Erwin and Ribeiro, 1996; Werres and Zielke, 2003).

*P. ramorum* and *P. lateralis* have never been found infecting *Citrus* trees, but are important pathogens included in the EPPO alert list (EPPO, 2006): *P. lateralis* is a root pathogen on *Chamaecyparis lawsoniana*,
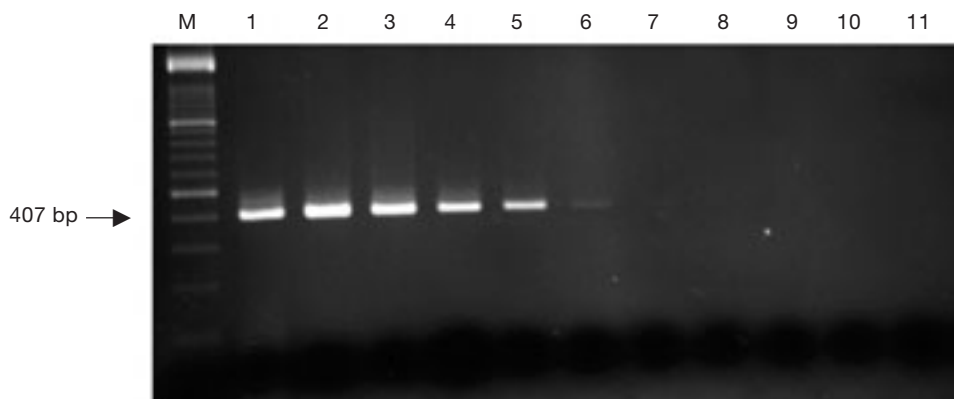


**Figure 2.** Sensitivity of PCR using a series of 10-fold dilutions of *Phytophthora hibernalis* DNA with the specific primers PHIB1 and PHIB2. M: 100-bp DNA ladder (Molecular marker XIV, Roche Diagnostics). Lane 1, 1 µg µL$^{-1}$; lane 2, $10^1$ diluted; lane 3, $10^2$; lane 4, $10^3$; lane 5, $10^4$; lane 6, $10^5$; lane 7, $10^6$; lane 8, $10^7$; lane 9, $10^8$; lane 10, $10^9$; lane 11, negative control.

whereas *P. ramorum* has a broader host range that is continuously increasing.

In the last years, species-specific primers have been developed for most *Phytophthora* species, including *P. ramorum* and *P. lateralis* (Garbelotto, 2003). However, Blomquist *et al.* (2005) reported cross-reactions between these two closely related species, finding false positives for *P. ramorum* when using specific primers for this species; and, after sequencing, these isolates were *P. hibernalis*.

In this work, two primers have been designed, namely PHIB1 and PHIB2, that amplify a product which sequence matches with a 100% homology those of *P. hibernalis* available at GenBank library and of two culture type specimens of the pathogen, with no cross reaction for *P. ramorum*. Primer sensitivity was similar to those reported for primers designed for *P. nicotianae* and *P. citrophthora* (Ippolito *et al.*, 2002), and for *P. capsici* (Silvar *et al.*, 2005).

Results demonstrate that PCR amplification of ITS regions by primers PHIB1 and PHIB2 followed by DNA sequencing can provide a rapid, selective and reliable identification of *P. hibernalis* either from fruit lesions or from isolated mycelia. The diagnosis of *P. hibernalis* in fruits can be a useful and fast tool to undertake prevention and appropriate management methods to control brown rot of *Citrus* fruits in the field.

There is only one previous record of *P. hibernalis* on sweet orange and lemon in NW Spain, identified by conventional culture methods (Urquijo *et al.*, 1971), although the authors did not provide details on season of detection and climatic conditions, methods of isolation, or whether it was a preharvest or postharvest infection. In our study the fruits affected by *P. hibernalis* were collected from 2003 to 2007. In November 2003, precipitations were double those normally found in the Atlantic coast during that month, reaching 400 mm; maximum temperatures showed levels lower than average for the same month in other years (Xunta de Galicia, 2004). Although records for 2005 and afterwards have not been still published, precipitations in November 2005 and 2006 were also high and maximum temperatures rather low. These two anomalous climatic situations in November might explain the presence of *P. hibernalis* in the province of Pontevedra. It is widely known that intense precipitation favours the incidence of rot diseases caused by *Phytophthora* spp. in *Citrus* (in Spain particularly in October and November) (Tuset *et al.*, 1984), and that low temperatures benefit growth of *P. hiber-*

*nalis* (Erwin and Ribeiro, 1996). Severe weather events, such as precipitation anomalies and greater temperature variations, are predicted to increase due to global climate change (Rosenzweig *et al.,* 2001). As outlined by Anderson *et al.* (2004), gradual change of climate can lead to disease emergence of pre-existing pathogens as major disease agents. This points out the need for a special attention to the incidence of *P. hibernalis* in citrus-growing areas, but also for developing research on its biology, host range, epidemiology and pathogenicity, aspects all poorly studied up to now. The specific primers PHIB1 and PHIB2 developed for the identification of *P. hibernalis* might be a useful tool in all these studies.

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