Title

Pathways to false positive diagnoses using molecular genetic detection methods; *Phytophthora cinnamomi* a case study

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**Keyword:** Species specific primers, *Phytophthora cinnamomi*, molecular diagnostics,
Abstract

*Phytophthora cinnamomi* is one of the world’s most invasive plant pathogens affecting ornamental plants, horticultural crops and natural ecosystems. Accurate diagnosis is very important to determine the presence or absence of this pathogen in diseased and asymptomatic plants. In previous studies, *P. cinnamomi* species-specific primers were designed and tested using various polymerase chain reaction (PCR) techniques including conventional PCR, nested PCR, and quantitative real time PCR (qPCR). In all cases, the primers were stated to be highly specific and sensitive to *P. cinnamomi*. However, few of these studies tested their primers against closely related *Phytophthora* species (*Phytophthora* clade 7). In this study, we tested these purported *P. cinnamomi* specific primer sets with eleven other species from clade 7 and determined their specificity; of the eight tested primer sets only three were specific to *P. cinnamomi*. This study demonstrated the importance of testing primers against closely related species within the same clade, and not just other species within the same genus. The findings of this study are relevant to all species-specific microbial diagnosis.
Introduction

*Phytophthora* species occur worldwide and can be highly invasive plant pathogens (Erwin and Ribeiro 1996; Hansen et al. 2012; Jung et al. 2013; Jung et al. 2015; Scott et al. 2013). *Phytophthora cinnamomi* is widely distributed globally outside its presumed natural range and causes economic losses in both horticulture and in natural ecosystems (Hayden et al. 2013). With over 4000 known hosts including horticultural species of significant economic importance such as avocado, pineapple, peach, chestnut, macadamia (Hardham 2005; Scott et al. 2013), *P. cinnamomi* is considered one of the world’s worst invasive alien species (Lowe et al. 2000).

Accurate pathogen identification has critical implications for disease diagnosis and management, disease free certification and quarantine. A false negative or false positive result in testing for the presence of *P. cinnamomi* may cause misdiagnosis and lead to expensive actions or inaction and economic losses and environmental harm (Hüberli et al. 2000). For example, in Western Australia Alcoa World Alumina conducted mining operations within both *P. cinnamomi* infested and un-infested forests and spent over $7 million p.a. on *P. cinnamomi* management and research in attempts to minimise the spread and impact of this pathogen (Colquhoun and Hardy 2000). In California, 60-70% of avocado trees were affected, causing a loss in excess of $40 million annually (Coffey et al. 1992). For these reasons, there is a huge demand by industry and land managers for accurate, consistent and cost effective diagnostic tools for the detection of *P. cinnamomi*.

Currently, *Phytophthora* can be detected using a variety techniques, including direct isolation on *Phytophthora* selective media (Tsao and Ocana 1969), baiting and isolation onto selective media (O'Brien et al. 2009), immuno-detection assays (Cahill and Hardham 1994), conventional PCR
(Coelho et al. 1997; Dobrowolski and O'Brien 1993; Judelson and Messenger-Routh 1996; O'Brien et al. 2009), nested PCR (Williams et al. 2009), restriction fragment length polymorphism analysis (RFLP) (Martin and Tooley 2004), qPCR (Martin and Tooley 2004; Tooley et al. 2006), TaqMan Real Time PCR (Bilodeau et al. 2007), and digital PCR (Blaya et al. 2014; Sanders et al. 2011). Briefly, conventional methods include direct isolation from diseased material, or indirectly by baiting infected plant tissues, water and soil with known host plants and isolation of the pathogen from infected baits (Cooke et al. 2007). However, conventional methods are labour intensive, time consuming and may have low success rates (Davison and Tay 2005; Hayden et al. 2004). There are commercially available testing kits based on immunoassays (Lateral flow Devices) developed for the detection of Phytophthora from wide range of disease plant material. These kits are simple to use, and provide result within 3-5 min. However, these devices have limited sensitivity and specificity (Lane et al. 2007; Tomlinson et al. 2010).

In contrast, molecular genetic methods are far more sensitive and timely allowing for higher throughput. Conventional PCR assays have been useful for the detection of Phytophthora, but have been less successful where low amounts of DNA are present, such as in environmental samples (Li et al. 2008; Martin et al. 2000). Amplification with Phytophthora genus - specific primers before amplification with species - specific primers (nested PCR) increased the sensitivity of detection at least 1,000 fold more than a conventional PCR assay (Narayanasamy 2011). However, with nested PCR there is the potential to produce more false positives due to human error (Hayden et al. 2004).

Quantitative PCR (qPCR) is a relatively fast and reliable detection method, provided DNA is present in sufficient quantities (Martin et al. 2000; Minerdi et al. 2008). qPCR has been successfully used for detection of Phytophthora ramorum (Bilodeau et al. 2007), P. kernoviae (Hughes et
al. 2011) and *P. infestans* (Hussain et al. 2014). Digital PCR (dPCR) is a new technology, developed in late 2011, that allows the detection of plant pathogens rapidly and accurately, without the requirement for any standards and count the absolute number of target DNA molecules present in the sample (Sanders et al. 2011). Digital PCR has recently been developed for detection of * Phytophthora nicotianae* in environmental samples (Blaya et al. 2015).

Based on a phylogeny derived from internal transcribed spacer region (ITS) sequence there are ten clades of *Phytophthora* species and *Phytophthora cinnamomi* resides in clade 7 (Cooke et al. 2000). Phylogenetically, the most closely related species to *P. cinnamomi* is *P. parvispora* (Scanu et al. 2014). When searching for a PCR based assay to detect *P. cinnamomi* we realised that most published primer sets had not been tested for specificity against closely related species from clade 7 including *P. parvispora*. Thus, we tested all published *P. cinnamomi* specific primers against closely related species and the findings of this study are presented here.

**Materials and Methods**

*Phytophthora isolates*

Six isolates of *P. cinnamomi*, two isolates of *P. niederhauserii* and single isolates of nine additional species from clade 7, along with isolates of a representative species from each of the remaining nine clades (Fig. 1), were used for specificity and sensitivity testing of *Phytophthora* primers sets. The 11 species from clade 7 were interspersed within the phylogeny and included *P. parvispora*, the species most closely related to *P. cinnamomi*. (Blair et al. 2008). Isolates were obtained from the Centre of *Phytophthora* Science and Management (CPSM), Murdoch University,
the Central Bureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, and the Vegetation Health Services (VHS), Department of Parks and Wildlife, Western Australia.

**DNA extraction**

All isolates were grown on half strength potato dextrose agar (Difco™, Becton Dickson, NJ, USA) at 20°C for 2 weeks in the dark. Genomic DNA was extracted from mycelium using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA), following the manufacturer’s instructions. Extracted DNA was stored in DNA elution buffer at -20 °C.

**Source of primers**

Primer details are provided in Table 1. DNA from *P. cinnamomi* isolate MP94-48 was used as the positive control in all PCR assays, and to construct the standard curve in qPCR assays. Nuclease free water was used as the negative control in each run.

**Conventional PCR**

Conventional PCR was done using two sets of primers; *Ypt* (C1) (Trzewik et al. 2016) and *Ypt* (C2) (Schena et al. 2008). The amplification reaction was carried out on a thermal cycler (Bio-Rad, CA, USA). In all cases, 1.5 µl of genomic DNA extract was added to 23.5 µl of master mix containing 12.5 µl GoTaq® Green Master Mix (Promega, Madison, WI, USA), 10 µl PCR grade water and 0.5 µl of 1µM each primer, PCR cycling conditions were as described in the original references (Table 1). The PCR products were visualised by loading 5 µl of the product on
2% agarose gel containing SYBR® Safe by using the Gel Doc System (Bio-Rad) and compared against 3.5 µl of a 100 bp DNA ladder (Axygen Biosciences).

**Nested PCR**

In nested PCR, the second round of the PCR reaction used 1.5 µl of amplified product from the first PCR round as the template. In these assays, the following primers were used: Lpv (N1) (Engelbrecht et al. 2013), ITS (N2) (Langrell et al. 2011), and ITS (N3) (Williams et al. 2009) (Table 1). The PCR conditions were as described above and in the original references (Table 1). PCR products visualisation and quantitation were as described above.

**Real-time qPCR**

Real time qPCR was carried out on a Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Each 20 µl reaction contained 2 µl of DNA and 18 µl of iTaq™ Universal Probes Super mix (Bio Rad) containing 300 nM of each primer and 100 nM of the probe (Integrated DNA Technology, Iowa, USA). The cycling conditions for primer sets ITS (Q2) and ITS (Q3) were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The ATP (Q1) assay of Miles et al. (2014), followed the cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 56°C for 30 s. The negative controls contained nuclease free water instead of DNA and were included in each run. Real time PCR results were analysed by threshold cycle (Ct) value. A standard curve of cycle threshold (Ct) values was calculated from a $10^{-1}$ to $10^{-6}$ serial dilution of genomic DNA from *P. cinnamomi* MP94-48 (15 pg/µl initial concentration). The threshold was automatically set with the
Auto-Find Threshold function of the Rotor-Gene 6000 software, real-time PCR efficiency was calculated with the formula $E = \left(\frac{10}{-1/\text{slope}} - 1\right) \times 100$, where $E$ is the amplification efficiency and the slope is derived from the plot of the log of template concentration vs. Cycle Threshold (CT).

**Primers Specificity and Sensitivity**

Primers were first tested for their specificity for *P. cinnamomi* and its closely related species from clade 7 (Fig.1). Only primers, which were specific for *P. cinnamomi* alone, were tested further. Primer specificity was then tested using *Phytophthora* species from other clades (Fig. 1). Detection limits were tested using $10^{-1}$ to $10^{-12}$ serial dilution (15 pg/µl initial concentration) of genomic DNA from *P. cinnamomi* isolate MP94-48.

**Results and Discussion**

*Phytophthora cinnamomi* primers specificity and sensitivity

All the primer sets amplified *P. cinnamomi* genomic DNA. However, *Ypt* (C1), *Lpv* (N1), ITS (N3), ITS (Q2) and ITS (Q3) were not specific to *P. cinnamomi* and amplified other species within clade 7 (Fig.1). These primers were considered non-specific and no further testing was conducted. Primer sets *Ypt* (C2), ITS (N2) and ATP (Q1) were specific to *P. cinnamomi* (Fig. 1), and did not amplify DNA from any other species from clade 7 species or any of the 12 *Phytophthora* species tested that represented the other nine clades of *Phytophthora*. These results
show how important it is to test against species closely related to the target species. Some primers have been designed for a very specific geographic location and only tested against species thought to occur within that environmental system. For example, Engelbrecht et al. (2013) designed primers for use in detecting *P. cinnamomi* in the avocado industry in South Africa. However, they did not test the primers against *P. parvispora*, the species most closely related to *P. cinnamomi*, or *P. niederhauseri*, both of which are known to occur in South Africa (Oh et al. 2013). Such primer sets must be tested for specificity against the local background microbial diversity and have limited application beyond the target region or host. New *Phytophthora* species could easily be introduced to the system requiring additional testing of the primers.

Sensitivity was only determined for the three *P. cinnamomi* specific assays; *Ypt* (C2), ITS (N2) and ATP (Q1). Using conventional PCR, the primer *Ypt* (C2) could be used to detect *P. cinnamomi* from 150 fg of DNA. Using a nested PCR approach, where universal *Phytophthora* primers Yph1F–Yph2R (Schena et al. 2006) were used in the first round, increased the sensitivity of the assay at least 100-fold, down to 15.0 fg of DNA. Similarly, ITS (N2) primers could be used to detect as little as 0.015 fg of DNA. The real time PCR assay using the ATP (Q1) primers was sensitive to 150 fg.

There are detailed phylogenies available for most described species of *Phytophthora* (http://www.phytophthoradb.org/) and isolates (often the type isolate) are available from publically accessible collections such as CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/) and ATTC Global Bioresource Centre (https://www.atcc.org/). Large databases such as NCBI (www.ncbi.nlm.nih.gov/) are not curated and sequences may have incorrect identities. However, there are sequences for several gene regions for all described species available from the
*Phytophthora* database (http://www.phytophthoradb.org/) or Q-bank (http://www.q-bank.eu/fungi/). Species-specific primers can thus be designed considering all known species, thus allowing for the virtual testing of the primers. However, it is very important to obtain isolates (or DNA) of related species to rigorously test the primers.

**Comparison to other studies**

We used Google Scholar to randomly select 24 additional papers published between 1996 and 2015, where species-specific primers were developed for *Phytophthora* species (Table 2). In 1996, there were approximately 70 described *Phytophthora* species (Erwin and Ribeiro 1996), while today there are approximately 147 described species (CABI 2016, http://www.speciesfungorum.org/). New species have been described from all 10 clades within the genus. As understanding about the phylogenetic relationships among *Phytophthora* species became common knowledge after the seminal publication of Cooke et al. (2000), it could have been expected that species-specific primers would be tested against phylogenetically closely related species. However, this has not been the case; the relationships between year and the number of *Phytophthora* species (r² < 0.015) or number of *Phytophthora* species from same phylogenetic clade was poor (r² < 0.001) (Fig. 2). While there is a slight positive trend over time for the numbers of species tested, this trend disappeared completely if the paper of Miles et al. (2014), who tested over 135 species, was excluded (Fig. 2). On average, the number of related species included in testing has remained the same over the 20-year period, representing on average 10% of the species from the same phylogenetic clade (Fig. 2). At one extreme there is Lan et al. (2013), who developed primers for *P. capsici* but did not test the primers for specificity against any of the other 25 species within the same clade. At the other, Miles et
al. (2014) designed primers of *P. cinnamomi* and tested their specificity against all species in the clade. In general, most researchers are only testing their primers against the known diversity within their system.

**Conclusion**

This study provides an insight into how important it is to design primers that are species specific, and the need to test them against the species to which they are most closely related. While the number of known *Phytophthora* species has doubled in the past 20 years, most recent *Phytophthora* diagnostic related publications do not seem to include the newly described species, even if they are closely related. In fact, there has been no increase in the number of species tested or even the number within the same clade being tested. We demonstrate the need to screen closely related species against primers to ensure specificity to the *Phytophthora* species targeted. While it may not be possible for researchers to obtain cultures or DNA for all species within a phylogenetic clade, ideally they should at least attempt to obtain and test species from each recognised sub-clade. It will be essential to continually test primers against new species within the same clade as they are described, in order to be confident of their fidelity. Finally, it is important that researchers are aware of all *Phytophthora* species present in the vegetation communities of interest. The findings of this study are relevant to all species-specific microbial diagnosis.

**Acknowledgments**
Financial support for the project come from the Australian Research Council (ARC) Linkage project LP130100573 ‘Eradication of Phytophthora cinnamomi from infested haul roads and rehabilitated bauxite mine sites in the Eucalyptus marginata forest’ Dr N. Williams and J.A. Tomlinson provided primers for testing.

References


Colquhoun I, Hardy GSJ. Managing the risks of *Phytophthora* root and collar rot during bauxite mining in the *Eucalyptus marginata* (jarrah) forest of Western Australia. *Plant Dis* 2000;84: 116-27.


Davison E, Tay F. How many soil samples are needed to show that Phytophthora is absent from sites in the south-west of Western Australia? *Australas Plant Path* 2005;**34**: 293-7.


Jung T, Colquhoun IJ, Hardy GESJ. New insights into the survival strategy of the invasive soilborne pathogen *Phytophthora cinnamomi* in different natural ecosystems in Western Australia. *Forest Pathol* 2013;**43**: 266-88.


Martin FN, Tooley PW. Identification of *Phytophthora* isolates to species level using restriction fragment length polymorphism analysis of a polymerase chain reaction-amplified region of mitochondrial DNA. *Phytopathology* 2004;**94**: 983-91.


Miles TD, Martin FN, Coffey MD. Development of Rapid Isothermal Amplification Assays for Detection of *Phytophthora* spp. in Plant Tissue. *Phytopathology* 2014;**105**: 265-78.


Tomlinson J, Dickinson M, Boonham N. Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology* 2010;100: 143-9.


Figure 1. *Phytophthora* species and isolates used to test primer specificity and sensitivity. Sensitivity was only tested where primers were specific to *P. cinnamomi*. The phylogenetic tree on the left is a simple distance tree produced using ITS sequence using Geneious software and has been included for illustrative purposes. The numbers above the branches correspond to the bootstrap support for the branch.
Figure 2. Relationship between the year of study publication and the total number of Phytophthora species tested (open circles; dashed line) or number of Phytophthora species from the same phylogenetic clade (closed circles; solid line) that were included in the development of species specific primers. The red lines correspond to the same relationship excluding the study of Miles et al. (2014).

Table 1. Polymerase chain reaction primers used in this study, their target genes, and product information.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer (F/R)</th>
<th>Primer (F/R)</th>
<th>Sequence (5’-3’)</th>
<th>An. Temp (°C)</th>
<th>Product size (bp)</th>
<th>Target Type of Assay</th>
<th>Designed By</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ypt (C1)</td>
<td>F R</td>
<td>Pcin59F</td>
<td>CGT CGT TGT TGT TTC TGT GC TTC AGT CAG CTC CAC GAA CA</td>
<td>55°C</td>
<td>300</td>
<td>Ypt</td>
<td>Conventional (Trzewik et al. 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pcin191R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ypt (C2)</td>
<td>F R</td>
<td>Ycin3F</td>
<td>GTC CTA TTC GCC TGT TGG AA GGT TTT CTC TAC ATA ACC ATC CTA TAA</td>
<td>55°C</td>
<td>300</td>
<td>Ypt</td>
<td>Nested (Schena et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ycin4R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lpv (N1)</td>
<td>F R</td>
<td>Lpv3F</td>
<td>GTG CAG ACT GTC GAT GTG GTG CAG ACT GTC GAT GTG</td>
<td>55°C</td>
<td>600</td>
<td>Lpv</td>
<td>Nested (Kong et al. 2003b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lpv3R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F R</td>
<td>Lpv3V F</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>F R</td>
<td>Lpv3V F</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lpv3N R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| ITS (N2) | F1 | ITS4 | TCC TCC GCT TAT TGA TAT GC  
|         | R1 | ITS5 | GGA AGT AAA AGT CGT AAC AAG G  
|         | F2 | PciF2 | GGA ACT GAG CTA GTA GCC TC  
|         | R2 | PciR2 | CAA TTG AGA TGC CAC CAC AA  
|         |    |      | 55°C  
|         |    |      | 64°C  
|         |    |      | 800  
|         |    |    | ITS  
|         |    |    | Nested  
|         |    |    | (White et al. 1990)  
|         |    |    | (Langrell et al. 2011)  
| ITS (N3) | F1 | CIN3A | CAT TAG TTG GGG GCC TGC T  
|         | R1 | CINITS4 | TGC CAC CAC AAG CAC ACA  
|         | F2 | CIN3B | ATT AGT TGG GGG CCT GCT  
|         | R2 | CIN2R | CAC CTC CAT CCA CCG ACT AC  
|         |    |      | 60°C  
|         |    |      | 60°C  
|         |    |      | 400  
|         |    |    | ITS  
|         |    |    | Nested  
|         |    |    | (Williams et al. 2009)  
| ATP (Q1) | F | ATP9F | CCT TCT TTA CAA CAA GAA TTA ATG AGA ACC GCT AT  
|         | R | NAD9infoR | GTA GAA ATA TTA ATA CAT AAT TCA TTT TTR TA  
|         | Probe | PcinNAD9 | AAG AAA TAT TTA GTT TAT TAA TAT ATA ATA TAA CT  
|         |    |      | 56°C  
|         |    |    | ATP9  
|         |    |    | NAD9  
|         |    |    | Real Time qPCR  
|         |    |    | (Miles et al. 2014)  
| ITS (Q2) | F | P cin F6 | CGT GGC GGG CCC TAT C  
|         | R | P cin R2 | AAA AGA GAG GCT ACT AGC TCA GTT CCC  
|         | Probe | P cin Probe1 | TGG CGA GCG TTT GGG TCC CTC T  
|         |    |      | 60°C  
|         |    |    | ITS  
|         |    |    | Real Time qPCR  
|         |    |    | Provided for testing  
| ITS (Q3) | F | P cin FF | CAA TTA GTT GGG GCC CTG CT  
|         | R | P cin RF | GCA GCA GCA GCC GTC G  
|         | Probe | P cin probe FP1 | TTG ACA TCG ACA GCA GCC GCC GC  
|         |    |      | 60°C  
|         |    |    | ITS  
|         |    |    | Real Time qPCR  
|         |    |    | Provided for testing  

Notes: aYpt = RAS related protein Ypt1; ITS = Internal transcribed spacer region; LPV = LPV gene which encodes putative storage protein; ATP = mitochondrial ATP synthase; NAD = mitochondrial NADH dehydrogenase
Table 2. A comparison of the number of species used when testing *Phytophthora* species-specific primers.

<table>
<thead>
<tr>
<th>References</th>
<th>Species targeted</th>
<th>Clade</th>
<th>No of species tested</th>
<th>No of species in clade (a)</th>
<th>No of species in same clade (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Trzewik et al. 2016)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>5</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>(Miles et al. 2014)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>135(^b)</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>(Engelbrecht et al. 2013)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>22</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>(Schena et al. 2008)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>35</td>
<td>20</td>
<td>7</td>
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<td>(Langrell et al. 2011)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>16</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>(Williams et al. 2009)</td>
<td><em>P. cinnamomi</em></td>
<td>7</td>
<td>12</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>(Lacourt and Duncan 1997)</td>
<td><em>P. nicotianae</em></td>
<td>1</td>
<td>17</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>(Grote et al. 2002)</td>
<td><em>P. nicotianae</em></td>
<td>1</td>
<td>12</td>
<td>14</td>
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<td>(Kong et al. 2003a)</td>
<td><em>P. nicotianae</em></td>
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<td>15</td>
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<td>4</td>
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<tr>
<td>(Huang et al. 2010)</td>
<td><em>P. nicotianae</em></td>
<td>1</td>
<td>12</td>
<td>14</td>
<td>2</td>
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<td>(Li et al. 2015)</td>
<td><em>P. nicotianae</em></td>
<td>1</td>
<td>12</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>(Trout et al. 1997)</td>
<td><em>P. infestans</em></td>
<td>1</td>
<td>13</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>(Judelson and Tooley 2000)</td>
<td><em>P. infestans</em></td>
<td>1</td>
<td>33</td>
<td>14</td>
<td>6</td>
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<td>(Hussain et al. 2005)</td>
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<td>1</td>
<td>9</td>
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<td>(Lees et al. 2012)</td>
<td><em>P. infestans</em></td>
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<td>40</td>
<td>14</td>
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<tr>
<td>(Hussain et al. 2015)</td>
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<td>1</td>
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<td>14</td>
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<td>(Causin et al. 2005)</td>
<td><em>P. cactorum</em></td>
<td>1</td>
<td>11</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>(Silvar et al. 2005)</td>
<td><em>P. capsici</em></td>
<td>2</td>
<td>11</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>(Zhang et al. 2006)</td>
<td><em>P. capsici</em></td>
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<td>17</td>
<td>25</td>
<td>2</td>
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<tr>
<td>(Lan et al. 2013)</td>
<td><em>P. capsici</em></td>
<td>2</td>
<td>12</td>
<td>25</td>
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</tr>
<tr>
<td>(Nath et al. 2014)</td>
<td><em>P. colocasiae</em></td>
<td>2</td>
<td>11</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(Wang et al. 2006)</td>
<td><em>P. sojae</em></td>
<td>7</td>
<td>25</td>
<td>20</td>
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</tr>
<tr>
<td>(Chen et al. 2013)</td>
<td><em>P. melonis</em></td>
<td>7</td>
<td>12</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>(Hayden et al. 2004)</td>
<td><em>P. ramorum</em></td>
<td>8</td>
<td>21</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>(Hughes et al. 2006)</td>
<td><em>P. ramorum</em></td>
<td>8</td>
<td>29</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>(Hayden et al. 2006)</td>
<td><em>P. ramorum</em></td>
<td>8</td>
<td>18</td>
<td>22</td>
<td>5</td>
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<tr>
<td>(Belbahri et al. 2007)</td>
<td><em>P. ramorum</em></td>
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<td>26</td>
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<tr>
<td>(Tomlinson et al. 2007)</td>
<td><em>P. ramorum</em></td>
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<td>28</td>
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<td>5</td>
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<tr>
<td>(Minerdi et al. 2008)</td>
<td><em>P. cryptogea</em></td>
<td>8</td>
<td>35</td>
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<td>4</td>
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<tr>
<td>(Shen et al. 2005)</td>
<td><em>P. boehmeriae</em></td>
<td>10</td>
<td>14</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Number of species present within the same clade as the target species  
\(^b\) Number of species tested from the same clade as target species (excluding target species)  
\(^c\) includes undescribed taxa