MALDI-TOF MS as a method for rapid identification of *Phytophthora* de Bary, 1876

Matěj Božik [Corresp., 1] , **Marcela Mrazkova** [2] , **Karolína Novotná** [1] , **Marketa Hrabetova** [2] , **Petr Marsik** [1] , **Pavel Kloucek** [1] , **Karel Cerny** [Corresp. 2]

[1] Czech University of Life Sciences, Department of Food science, Prague, Czech Republic

[2] The Silva Tarouca Research Institute for Landscape and Ornamental Gardening, Kvetnove nam. 391, 25243 ,, Pruhonice, Czech Republic

Corresponding Authors: Matěj Božik, Karel Cerny
Email address: bozik@af.czu.cz, Karel.Cerny@vukoz.cz

The number of described species of the oomycete genus *Phytophthora* is growing rapidly, highlighting the need for low-cost, rapid tools for species identification. Here, a collection of 24 *Phytophthora* species (42 samples) from natural as well as anthropogenic habitats were genetically identified using the internal transcribed spacer (ITS) and cytochrome c oxidase subunit I (COI) regions. Because genetic identification is time consuming, we have created a complementary method based on by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Both methods were compared and hypothesis that the MALDI-TOF MS method can be a fast and reliable method for the identification of oomycetes was confirmed. Over 3500 mass spectra were acquired, manually reviewed for quality control, and consolidated into a single reference library using the Bruker MALDI Biotyper platform. Finally, a database containing 144 main spectra (MSPs) was created and published in repository. The method presented in this study will facilitate the use of MALDI-TOF MS as a complement to existing approaches for fast, reliable identification of *Phytophthora* isolates.

MALDI-TOF MS as method for rapid identification of *Phytophthora* de Bary, 1876

Matěj Božik[1#], Marcela Mrázková[2], Karolína Novotná[1], Markéta Hrabětová[2], Petr Maršík[1], Pavel Klouček[1], Karel Černý[2*]

[1] Department of Food Science, Czech University of Life Sciences, Prague, Czech Republic
[2] Silva Tarouca Research Institute, Pruhonice, Czech Republic

Corresponding Author:
[#] Correspondent for MALDI-TOF MS
Matěj Božik, Kamýcká 129, Praha, 165 00, Czech Republic
Email address: bozik@af.czu.cz
[*] Correspondent for genetic identification and sample collection,
Karel Černý, Květnové nám. 391, 25243 Průhonice, Czech Republic
Email address: karel.cerny@vukoz.cz

Abstract

The number of described species of the oomycete genus *Phytophthora* is growing rapidly, highlighting the need for low-cost, rapid tools for species identification. Here, a collection of 24 *Phytophthora* species (42 samples) from natural as well as anthropogenic habitats were genetically identified using the internal transcribed spacer (ITS) and cytochrome c oxidase subunit I (COI) regions. Because genetic identification is time consuming, we have created a complementary method based on by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Both methods were compared and hypothesis that the MALDI TOF method can be a fast and reliable method for the identification of oomycetes was confirmed. Over 3500 mass spectra were acquired, manually reviewed for quality control, and consolidated into a single reference library using the Bruker MALDI Biotyper platform. Finally, a database containing 144 main spectra (MSPs) was created and published in repository. The method presented in this study will facilitate the use of MALDI-TOF MS as a complement to existing approaches for fast, reliable identification of *Phytophthora* isolates.

Introduction

The oomycete genus *Phytophthora* was first established by de Bary in 1876 with the type species *P. infestans*, which causes potato blight and was the culprit in a series of famines in Europe (De Bary, 1876). Members of this genus are among the most cited pathogens worldwide and are associated with annual economic losses reaching billions of dollars (Erwin & Ribeiro, 1996). Plant pathogens belonging to *Phytophthora* impact agriculture, horticulture, forestry, and natural ecosystems (Hansen, Reeser & Sutton, 2012), and epidemics of the most destructive species,

40   including *P. alni, P. austrocedrae, P. cinnamomi, P. ramorum,* and *P. kernoviae*, have occurred
41   in various parts of the world (Greslebin, Hansen & Sutton, 2007). The number of described
42   *Phytophthora* species grew from 58 in 1996 (Erwin & Ribeiro, 1996) to 313 in 2018 (H Ho,
43   2018), followed by a dramatic spike in recent years to an estimated total of 600 (Brasier, 2009).
44   New species have been split from old species as species complexes were resolved but are also
45   arising from the recent hybridization of existing species such as *Phytophthora alni* subsp. *alni*
46   (Husson et al., 2015; Mizeriene et al., 2020). Some of these new species have been described as
47   causal agents of new diseases, while others have been discovered through the exploration of new
48   habitats.
49   This explosion in diversity is also the result of applying new molecular tools to taxonomy
50   (Hansen, Reeser & Sutton, 2012). Traditionally, species descriptions were based on
51   morphological characteristics, but phenotypic species identification is time-consuming and
52   requires specialized scientific knowledge (Ruiz Gómez et al., 2019). Studies of soil fungal and
53   oomycetous communities have benefitted from modern techniques like next-generation
54   sequencing (NGS) (Hardham & Blackman, 2018), pyrosequencing and second-generation
55   techniques such as metabarcoding (Prigigallo et al., 2016) based on Illumina de novo
56   sequencing. Although morphological characteristics still serve as the basis for the preliminary
57   orientation and identification of *Phytophthora* species, various genetic methods are now
58   routinely used when overlapping morphological features and intraspecific variability present a
59   challenge for *Phytophthora* species identification (Martin et al., 2012).
60   In these techniques, species identification typically depends on sequencing of the internal
61   transcribed spacer (ITS) region of ribosomal DNA (rDNA) (Cooke et al., 2000; Grünwald et al.,
62   2011; Sandoval-Sierra, Martín & Diéguez-Uribeondo, 2014; Jung et al., 2019). Online databases
63   such as GenBank (www.ncbi.nlm.nih.gov) and EMBL (www.ebi.ac.uk) collect sequence data
64   and allow species identification by BLAST based primarily on the coefficient of similarity of
65   DNA and RNA sequences (Ristaino, 2012; H Ho, 2018). For the genus *Phytophthora*, the
66   "Phytophthora Database" (www.phytophthoradb.org) provides information on morphological
67   features, geographic distribution, and relevant references (Park et al., 2008). The advantage of
68   the ITS region is that its sequence can readily be obtained, and the ITS sequences of many
69   *Phytophthora* species are currently available in GenBank. However, a disadvantage of ITS
70   sequences is that there are minimal or no differences between closely related species, such as *P.*
71   *rubi* and *P. fragariae* (Martin et al., 2012), and intraspecific variation can blur the boundaries
72   between some species. Additional genetic regions that are commonly used for species
73   identification include the mitochondrial cytochrome c oxidase subunit genes (COX1 and COX2)
74   (Martin & Tooley, 2003; Kroon et al., 2004; Robideau et al., 2011), but the need to sequence
75   additional regions further increases the cost, which is a limiting factor for identification based on
76   DNA sequencing (Del Castillo-Múnera et al., 2013).
77   A potential alternative to sequencing is matrix-assisted laser desorption ionization–time of flight
78   mass spectrometry (MALDI-TOF MS). MALDI-TOF MS is a fast and direct form of analysis
79   that requires no chromatographic separation (Galeano Garcia et al., 2018). Recent studies have

80 shown that MALDI biotyping is a fast and reliable method of species identification for
81 mushrooms (Sugawara et al., 2016; El Karkouri et al., 2019), yeasts, molds (Rizzato et al., 2015;
82 Drissner & Freimoser, 2017), mycorrhizal fungi (Crossay et al., 2017) and the pathogenic
83 oomycete *Pythium insidiosum* (Krajaejun et al., 2018).
84 In this study, we focused on 43 selected strains belonging to 26 *Phytophthora* species and one
85 species belonging to the genus *Pythium* as an outgroup. The strain collection was created from
86 isolates acquired from natural as well as anthropogenic (nurseries, gardening centers, fruit
87 orchards, etc.) habitats. The isolates were subjected to genetic identification using the
88 cytochrome c oxidase subunit I (COI) and ITS regions, and the validated strains were grown in
89 liquid medium and subsequently analyzed by MALDI-TOF MS (Figure S1). Finally, a library of
90 144 main spectra (MSPs) was created and validated.
91

Materials & Methods

93 **Pathogen isolation, cultivation, genetic identification, and preservation**
94 Oomycetous pathogens were isolated from damaged plants of different ornamental, forest and
95 fruit woody plant species and from water in 2008–2017. Woody plants of many species from
96 different stands (nurseries, orchards, gardening centers and ornamental gardens, forest and
97 riparian stands) with disease symptoms such as yellowing or wilting foliage, dieback, root and
98 collar rot, or rot of feeding roots were identified and examined for the presence of damaged plant
99 tissues. Tissues exhibiting characteristic lesions were aseptically sampled and transported to the
100 laboratory. The samples were rinsed under tap water, and transition zones between necrotized
101 and healthy plant tissues were identified. Segments (3×3 mm) from the edges of active lesions
102 were excised, surface disinfected by immersion in 95% ethanol for 10 seconds, rinsed in
103 deionized sterile water and dried with sterile filter paper. Then, the segments were placed on
104 selective PARPNH agar medium (V8 juice 200 mL, ampicillin 200 ppm, rifampicin 10 ppm,
105 quintozene 25 ppm, nystatin 50 ppm, hymexazol 50 ppm, agar 15 g, $CaCO_3$ 3 g, and deionized
106 water 800 mL) in 9-cm Petri dishes and incubated at 20 °C in the dark. After two to five days,
107 segments exhibiting characteristic oomycetous hyphae were transferred onto plates containing
108 V8A medium (V8 juice 200 mL, agar 15 g, $CaCO_3$ 3 g, deionized water 800 mL). Pure isolates
109 were transferred to oatmeal agar (HiMedia Ltd, India) slants in tubes and stored in refrigeration
110 boxes at 12 °C in the Czech Collection of Phytopathogenic Oomycetes (CCPO
111 http://www.vukoz.cz/dokumenty/056/Sbirka_Katalog_Jan_2018.pdf).
112 Samples of soil substrate from plants with damaged feeding roots were collected from three sites
113 at depths of 10–20 cm in the root zone of the affected plants. A mixed sample of approximately
114 1000-2000 cm^3 was then created from a single plant and transported to the laboratory. Soil
115 samples were processed via a baiting method. Approximately 150 cm^3 of soil substrate
116 containing damaged roots was placed in a deep plastic bowl, and deionized water was added to
117 cover the sample by 2–3 cm. The bait (young, healthy, rhododendron leaves of the susceptible
118 *Rhododendron yakushimanum* cv. Silberwolke) was rinsed under tap water, placed on the water
119 surface and incubated at room temperature under natural light in a standard day/night regime.

120   When characteristic lesions appeared on the baits, the segments with necrotized tissues were
121   excised and processed as described above. Floating oomycetous zoospores were trapped from
122   water *in situ* using healthy leaves of *R. yakushimanum* cv. Silberwolke placed in a sterilized bag
123   made from loosely woven fabric. Four bags containing leaf bait were allowed to float just under
124   the surface of the investigated watercourses, and after one week of incubation, the samples were
125   collected, transported to the laboratory and processed as leaf baits as described above.
126   Total genomic DNA of the isolates was extracted from pure cultures using a DNeasy UltraClean
127   Microbial Kit (QIAGEN, Germany) according to the manufacturer's instructions. For molecular
128   identification, the nuclear rDNA ITS region and/or the COI region were amplified by polymerase
129   chain reaction (PCR). The primer pairs used to amplify the ITS region were ITS1/ITS4 or
130   ITS4/ITS5 (White et al., 1990), and the COI region was amplified with
131   OomCoxILevup/Fm85mod primers (Robideau et al., 2011). PCR was performed in a
132   Mastercycler Nexus Gradient GSX 1 thermal cycler (Eppendorf), and products were visualized
133   via agarose gel electrophoresis in 1% TBE buffer using a 100-bp DNA ladder (New England
134   Biolabs, US) as a size marker. PCR products were purified and sequenced in both directions by
135   Macrogen Europe B.V. (NL) using the same primers used for PCR amplification. The obtained
136   sequences were edited and aligned in BIOEDIT v. 7.0.9.0 (Hall, 1999) and compared with
137   sequences in GenBank using a BLAST similarity search (www.ncbi.nlm.nih.gov). All sequences
138   obtained in this study were deposited in the NCBI GenBank database (Table S1).
139
140   **MALDI-TOF MS biotyping**
141   After genetic identification, cultures of 43 selected strains belonging to 26 taxa of *Phytophthora*
142   and one species belonging to the genus *Pythium* as an outgroup were incubated in 0.5 L of
143   Tryptone Soya Broth (TSB, Sigma Aldrich, USA) with shaking in the dark for 3–7 days
144   (according to the intensity of growth of each species) to develop sufficient mycelial biomass.
145   Samples from the liquid nutrient medium were transferred to 70% ethanol and stored in
146   Eppendorf tubes at -20 °C until analysis. An overview of the species, including their collection
147   numbers, is given in Table S1.
148   For protein extraction and subsequent MALDI-TOF MS, mycelium was harvested from the
149   liquid nutrient medium samples by centrifugation in a 1.5-mL Eppendorf tube and divided into
150   small, approximately equal-sized pieces of 5-10 mg using micro scissors. Distilled water (300
151   μL) was added to each piece of mycelium and mixed thoroughly. Then, 900 μL of ethanol was
152   added, followed by thorough mixing. The sample was centrifuged at 20,000 × g for 2 minutes
153   (Rotanta 450 R, Hettich, DE), the supernatant was decanted, and the sample was briefly
154   centrifuged again. The rest of the supernatant was carefully removed from the pellet by pipetting,
155   and the sample was subsequently allowed to dry at room temperature. Next, 10–20 μL of 70%
156   formic acid (Sigma-Aldrich, DE) was added to fully immerse the dry pellet, followed by
157   thorough mixing and incubation for 3 minutes. Finally, an equal volume of ultrapure acetonitrile
158   (Fluka, DE) was added, followed by thorough mixing and centrifugation at 20,000 × g for
159   2 minutes. One microliter of the supernatant was immediately applied to a spot of an MTP 384

160   ground steel BC target plate for MALDI-TOF MS (Bruker, DE) and allowed to dry at room
161   temperature. Immediately after drying, 1 µl of HCCA (α-cyano-4-hydroxycinnamic acid, Bruker,
162   DE) matrix solution (acetonitrile 50%, water 47.5% and trifluoracetic acid 2.5%; 10 mg/mL
163   HCCA) was applied to each spot and allowed to dry again at room temperature. Each sample
164   was applied to 8 spots around the calibration spot. For calibration, 0.5 µL of Bruker Bacterial
165   Test Standard (BTS, Bruker, DE) was applied to the center spot, followed by the application of
166   matrix solution as described for the supernatant. Each spot was measured three times by
167   MALDI-TOF MS Autoflex Speed (Bruker, DE) and flexControl 3.4 (Build 135) with a standard
168   MALDI Biotyper method (MBT_FC.par). Ion source 1 was set to 19.38 kV, ion source 2 was set
169   to 18.18 kV, and detection was set from 2 to 20 kDa. All experiments were performed in
170   independent biological triplicates; three spectra were measured for each sample, and each
171   spectrum was collected as 2000 shots in 200 steps.
172
173   **Data processing**
174   For each measurement, the spectra were manually inspected by flexAnalysis 3.4 Compass 1.4
175   (Bruker Daltonics, DE), and then MSPs were processed by the standard MALDI Biotyper MSP
176   creation method using MALDI Biotyper Version 3.1 (Build 66). MALDI Biotyper was used to
177   compare the results based on the similarity of the spectra in the range of 2–20 kDa. For
178   evaluation, the standard method of identification of MSPs was chosen; the frequency threshold
179   for spectrum adjustment was 50, the frequency threshold for calculating the score was 5, the
180   maximum primary spectrum mass error was 2000, the mass tolerance for the modified spectrum
181   was 350, and the accepted mass tolerance of the peak was 600 par.
182   The acquired spectrum of the sample was transformed into a peak pattern utilizing dedicated
183   spectral analysis tools. The peak pattern was then compared to reference peak lists of organisms
184   in the reference library, and a log(score) value between 0.00 and 3.00 was calculated. In brief,
185   identification is based on average peak intensity, peak position and peak occurrence frequency,
186   and each factor is assigned a value between 0 and 1. The three values are then summed, and the
187   result is normalized to 1000. The decimal logarithm of the result is taken as the calculated score,
188   such that the maximum achievable score is 3 (= log 1000). The values are then classified. The
189   calculated score determines the highest match at the top of the list. The higher the log(score)
190   value, the higher the degree of similarity to a given organism in the reference library. Values ≥ 2
191   indicate high-confidence identifications. Log (score) values between 1.70 and 1.99 need to be
192   confirmed by additional methods. Values below 1.70 indicate that no organism identification is
193   possible (MALDI Biotyper 3.0 User Manual Revision 2, 2011). Data correlations were
194   visualized as score-oriented dendrograms generated from spectra or MSPs in MALDI Biotyper
195   Version 3.1 (Build *66*) using the centroid linkage method.
196
197   # Results
198
199   **MSP Library**

200 Over 3500 mass spectra were processed and manually reviewed for quality control to obtain a
201 total of 144 MSPs from 43 different samples; these MSPs were added to a custom MSP library
202 (DOI: 10.5281/zenodo.4271724). The produced MSPs were compared with the available MBT
203 Library BDAL 9.0 (Bruker Daltonics, Germany), which includes 8,468 MSPs, and the MBT
204 Filamentous Fungi Library V1.0 (365 MSPs). After validation and verification of the method, the
205 samples were compared with respect to species and strain classification.
206 Spectra of different *Phytophthora* strains were searched against the reference Bruker Library,
207 which contains 2,969 species and 8,468 MSPs of various microorganisms, mainly bacteria and
208 fungi, but no *Phytophthora* strains. None of the sample mass spectra matched the reference
209 organisms, as the obtained identification scores were less than 1.70. The MSPs of the
210 *Phytophthora* strains were then added to the Bruker MALDI Biotyper Library.
211
212 **Library validation and verification**
213 The same extraction method using ethanol and formic acid was used for validation. Intraday
214 variability was determined based on successful measurement of 8 spots of the same sample in
215 three replicates, and interday variability was based on the similarity of the spectra of samples
216 measured on different days. Each sample was measured at least 3 times. The procedure and
217 conditions were always identical. Figure 1 shows a comparison of the spectra of a sample of
218 *Phytophthora lacustris* with collection number 295.09. The log scores of identical samples
219 measured on different days are very similar (score > 2.6).
220 Blank analysis was performed to further validate the method. One of the 43 samples was
221 randomly selected and measured in 3 spots by MALDI Biotyper Realtime Classification (Bruker,
222 DE). The sample was correctly identified as *P. lacustris* in accordance with the previous DNA
223 classification. The spectra of the blank sample matched the MSP in our database as shown in
224 Table 1.
225
226 **From genus to strain using the custom library**
227 In total, 42 samples within the genus *Phytophthora* and 1 sample belonging to the related genus
228 *Pythium*, which was included in the collection for quality control purposes, were compared. The
229 MSP spectra are compared in the form of log score values in Table S2. All 42 samples matched
230 their own MSPs (biological repeats) measured on another day with log scores greater than 2.3.
231 For one sample (*P. gonapodyides* 419.10), the highest match category had a score of 2.3 with
232 only one biological replicate and a second score of 2.2, which is satisfactory for probable species
233 identification. The results of the reliability of identification of all species and strains are
234 summarized in Table 2. Two limit values were determined according to the manufacturer's
235 recommendations. At a limit value of 2, the confidence of strain identification was 78.3%; at a
236 lower limit value of 1.7, the success rate was 90.7%.
237
238 **Comparison within a species**

239 The spectra of different strains of *P. lacustris* analyzed in flexAnalysis (Figure 2) showed protein
240 ion peaks in the 6500 and 14500 *m/z* regions for all samples, but the intensities differed among
241 the samples. All spectra were smoothed and baseline aligned prior to evaluation. For *P. lacustris*,
242 reliable identification of strains from different locations and hosts was also achieved within the
243 species using the log score as shown in Table 3. The MSP dendrogram based on comparison of
244 the MALDI-TOF mass spectra is shown in Figure 3.
245 The MSP spectra of *P. alni* subsp. *uniformis* 239.08 were very similar to those of *P. cambivora*,
246 *P. alni* subsp. *alni* and *P. rubi*. The log scores ranged from 2.4 to 2, indicating some association
247 among these species. However, the dendrogram placed these species in different clusters (Figure
248 4). There was also a match of log scores at the species level between *P. cactorum* 862.17 and *P.*
249 *hedraiandra*. For the genus *Pythium*, only consistency among biological replicates was observed.
250 The log score compared to *Phytophthora* was less than 1.6 (Table 4)
251

Discussion

253 The genera *Phytophthora* and *Pythium* include many economically important species that have
254 been placed in Kingdom Chromista (H Ho, 2018). Many *Phytophthora* species are relatively
255 easy to identify, but overlapping morphological features and intraspecific variability can make
256 genus identification difficult (Martin et al., 2012) in the absence of genetic methods. Cooke et al.
257 (2000) developed the first molecular phylogeny for the genus *Phytophthora* by analyzing
258 sequences of the ITS region. Several multilocus phylogenies were subsequently constructed that
259 divided *Phytophthora* species into 10 phylogenetically well-supported clades and several
260 subclades (Kroon et al., 2004; Blair et al., 2008; Martin, Blair & Coffey, 2014; Yang, Tyler &
261 Hong, 2017). The clade affiliations of the species used in this study are given in Table S1. After
262 genetic validation using COI and ITS, isolates of these species were grown in liquid medium and
263 subsequently analysed by MALDI-TOF MS to create a database. The MSP database (DOI:
264 10.5281/zenodo.4271724) created in this study can be used as a complement to existing datasets
265 for fast and reliable identification of *Phytophthora*.
266 MALDI-TOF MS is a fast and direct method of analysis that requires no chromatographic
267 separation (Galeano Garcia et al., 2018) and is much less expensive than DNA sequencing (Del
268 Castillo-Múnera et al., 2013). Recent research has demonstrated the usefulness of MALDI-TOF
269 MS for the rapid characterization of clinical pathogens, lactic acid bacteria, nonfermenting
270 bacteria, fungi, plant-parasitic nematodes, and environmental bacteria (Ahmad, Babalola & Tak,
271 2012). Moreover, studies using a wide range of microbial species suggest that MALDI-TOF MS
272 has better diagnostic accuracy than conventional biochemical techniques (Ahmad, Babalola &
273 Tak, 2012). MALDI-TOF MS reference spectra and analytical tools are usually proprietary, in
274 contrast to the wide range of public repositories of openly accessible or downloadable data and
275 tools for DNA-based identification. For example, in 2012, Bruker Daltonics launched
276 Filamentous Fungi Library 1.0, a commercial MS library for fungal identification (Schulthess et
277 al., 2014). The production and integration of in-house MS databases for open access would
278 facilitate the creation of a truly open platform for MALDI-TOF MS-based species identification

279 (Drissner & Freimoser, 2017) and promote the use of MALDI-TOF MS biotyping in other areas
280 of diagnostics, such as plant pathogens or food-important bacteria, fungi and yeasts (Drissner &
281 Freimoser, 2017).
282 Most studies using MALDI-TOF MS for microbial identification refer to the Bruker system,
283 which is proprietary but the most widespread system (Normand et al., 2017). Studies of the
284 identification of filamentous fungal species using the Bruker system have focused on the genera
285 *Fusarium, Aspergillus, Penicillium* and *Ramularia*, which are potentially dangerous to humans
286 (Boekhout et al., 2015). However, analyses of dermatophyte genera using the Saramis, Vitek MS
287 or Andromas systems have also been published. In human clinical microbiology, MALDI-TOF
288 MS has primarily been used for the identification of bacteria and yeasts using in-house MS
289 databases of human pathogens (Gräser, 2014; Lasch, Stämmler & Schneider, 2018; Paul et al.,
290 2019; Papalia et al., 2020) and environmental isolates of *Burkholderia* and related genera
291 (Fergusson et al., 2020).
292 Although MALDI-TOF MS is becoming increasingly popular as a tool for clinical diagnosis, the
293 lack of data on non-clinical microorganisms has limited its use in microbial ecology (Rahi,
294 Prakash & Shouche, 2016). So far very little attention has been paid to plant pathogens such as
295 *Phytophthora*. Even organisms commonly found in environmental, agricultural, or food samples
296 are often not recognized by standard, commercial MALDI-TOF MS systems (Drissner &
297 Freimoser, 2017). The available MBT Library BDAL 9.0 (Bruker Daltonics, Germany), which
298 includes 8,468 MSPs, and the MBT Filamentous Fungi Library V1.0 (365 MSPs) do not contain
299 any representatives of the genus *Phytophthora*. In a previous study, an in-house MS database
300 was used to accurately identify *Pythium insidiosum* isolates and properly differentiate them from
301 other filamentous fungi in patient samples (Mani et al., 2019), thus demonstrating that MALDI-
302 TOF MS can be used for the accurate and rapid culture identification of *Pythium insidiosum* in
303 the clinical laboratory. Our dataset contained *Pythium folliculosum* as a control strain that can be
304 reliably distinguished from other *Phytophthora* species. As a contrast to this technical approach,
305 ongoing studies suggesting that dogs can also rapidly and reliably distinguish plants infected
306 with *Phytophthora* or *Pythium* species (Swiecki et al., 2018; Oliver et al., 2020).
307 The MSP spectra of *P. hedraiandra* and *P. cactorum* were similar, consistent with phylogenetic
308 studies that have indicated that *P. hedraiandra* is closely related to *P. cactorum* or basal to the
309 cluster of *P. cactorum* and *P. pseudotsugae* (Blair et al., 2008; Martin, Blair & Coffey, 2014;
310 Yang, Tyler & Hong, 2017); all of these species belong to Subclade 1a. Similarity of the MSP
311 spectra was also observed for *P. alni* subsp. *alni, P. alni* subsp. *uniformis*, *P. cambivora* and *P.*
312 *rubi*, which are phylogenetically closely related and belong to Subclade 7a (Yang, Tyler &
313 Hong, 2017). Despite these similarities, we were able to discriminate 43 genetically and
314 phenotypically distinct *Phytophthora* strains by native Bruker Biotyper methods.
315 Some in house databases based on profile mass spectra was developed for identification of
316 species of *Xanthomonas* (Sindt et al., 2018). *Rhizopus* (Boekhout et al., 2015), *Tuber* (El
317 Karkouri et al., 2019). MALDI-TOF MS has also been used for the *in situ* identification of plant-
318 invasive bacteria, including rhizobia (Ziegler et al., 2012) and *Acidovorax citrulli* isolates

319 (Bergsma-Vlami, 2018) and late blight in asymptomatic tomato plants (Galeano Garcia et al.,
320 2018).
321 MALDI-TOF MS-based identification of *P. insidiosum* revealed differences in protein spectra
322 between geographically different isolates (Mani et al., 2019). While our samples were also taken
323 from geographically different habitats and hosts, the results clearly showed that these differences
324 did not affect the reliability of identification. Notably, we were able to use our constructed
325 database to distinguish *P. palmivora* and *P. infestans* with high reliability. Previous protein
326 profiling of these two species by two-dimensional gel electrophoresis showed that the overall
327 proteome maps were similar; equivalent numbers of spots were detected for the two species, and
328 30% of the protein spots had similar or identical positions in the 2D gels (Shepherd, Van West &
329 Gow, 2003). Thus, our results suggest that the use of MALDI-TOF MS biotyping combined with
330 simple sample processing and subsequent comparison of spectra with a database is effective for
331 the accurate and rapid identification of *Phytophthora* genera and species. In addition, due to its
332 simplicity, this method could be useful for laboratories with limited expertise in mycology (Mani
333 et al., 2019).
334

Conclusions

336 This study demonstrated that MALDI-TOF MS can be used to identify phytopathogens of the
337 genus *Phytophthora*. None of the *Phytophthora* isolates have previously been included in a
338 commercial library. Our updated library allowed the correct identification of 117/129 (90.6%)
339 *Phytophthora* isolates at the species level and 100% of isolates at the genus level. The success
340 rate within species was 78.3% at a cut-off value of 2 and 90.7% at the lower limit of 1.7. In
341 addition, protein extraction using ethanol, formic acid and acetonitrile was quick and effective.
342 Further development of MALDI-TOF MS-based identification of fibrous oomycetes will require
343 continuous updating of current commercial databases and devices as well as the construction of
344 new databases for specialized research purposes. Another prospect for development is the
345 potential detection of typical MS markers of *Phytophthora* species directly in infected plant
346 material.
347

Acknowledgements

349 The authors wish to thank Dawn Schmidt for language editing.
350

References

352 Ahmad F, Babalola OO, Tak HI. 2012. Potential of MALDI-TOF mass spectrometry as a rapid
353   detection technique in plant pathology: identification of plant-associated microorganisms.
354   *Analytical and Bioanalytical Chemistry* 404:1247–1255. DOI: 10.1007/s00216-012-6091-7.
355 De Bary A. 1876. Researches into the nature of the potato fungus, *Phytophthora infestans*.
356   *Journal of the Royal Agricultural Society of England* 12:239–269.
357 Bergsma-Vlami M. 2018. MALDI-TOF mass spectrometry for plant pest diagnostics: the case of
358   the accurate identification of Acidovorax citrulli isolates, including their grouping. DOI:
359   10.5281/ZENODO.1168430.

360 Blair JE, Coffey MD, Park SY, Geiser DM, Kang S. 2008. A multi-locus phylogeny for
361   Phytophthora utilizing markers derived from complete genome sequences. *Fungal Genetics*
362   *and Biology* 45. DOI: 10.1016/j.fgb.2007.10.010.
363 Boekhout T, Versteeg M, Dolatabadi S, de Hoog SG, Kolecka A. 2015. Differentiation of
364   clinically relevant Mucorales Rhizopus microsporus and R. arrhizus by matrix-assisted laser
365   desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Journal of*
366   *Medical Microbiology* 64:694–701. DOI: 10.1099/jmm.0.000091.
367 Brasier CM. 2009. Phytophthora biodiversity: How many Phytophthora species are there? In:
368   *Phytophthoras in Forests and Natural Ecosystems. (Goheen EM and Frankel SJ, eds).*
369   *Proceedings of the Fourth International Union of Forest Research Organisations (IUFRO)*
370   *Working Party 7.02.09.* USDA Forest Service General Technical Report, 101–115.
371 Del Castillo-Múnera J, Cárdenas M, Pinzón A, Castañeda A, Bernal AJ, Restrepo S. 2013.
372   Developing a taxonomic identification system of *Phytophthora* species based on
373   microsatellites. *Revista Iberoamericana de Micologia*. DOI: 10.1016/j.riam.2012.11.002.
374 Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM. 2000. A molecular phylogeny of
375   phytophthora and related oomycetes. *Fungal Genetics and Biology* 30. DOI:
376   10.1006/fgbi.2000.1202.
377 Crossay T, Antheaume C, Redecker D, Bon L, Chedri N, Richert C, Guentas L, Cavaloc Y, Amir
378   H. 2017. New method for the identification of arbuscular mycorrhizal fungi by proteomic-
379   based biotyping of spores using MALDI-TOF-MS. *Scientific Reports* 7:14306. DOI:
380   10.1038/s41598-017-14487-6.
381 Drissner D, Freimoser FM. 2017. MALDI-TOF mass spectroscopy of yeasts and filamentous
382   fungi for research and diagnostics in the agricultural value chain. *Chemical and Biological*
383   *Technologies in Agriculture* 4:13. DOI: 10.1186/s40538-017-0095-7.
384 Erwin DC, Ribeiro OK. 1996. Phytophthora *diseases worldwide*. American Phytopathological
385   Society, St. Paul, MN (USA) APS Press.
386 Fergusson CH, Coloma JMF, Valentine MC, Haeckl FPJ, Linington RG. 2020. Custom matrix-
387   assisted laser desorption ionization-time of flight mass spectrometric database for
388   identification of environmental isolates of the genus burkholderia and related genera.
389   *Applied and Environmental Microbiology* 86:778–782. DOI: 10.1128/AEM.00354-20.
390 Galeano Garcia P, Neves dos Santos F, Zanotta S, Eberlin M, Carazzone C. 2018. Metabolomics
391   of *Solanum lycopersicum* Infected with *Phytophthora infestans* Leads to Early Detection of
392   Late Blight in Asymptomatic Plants. *Molecules* 23:3330. DOI:
393   10.3390/molecules23123330.
394 Gräser Y. 2014. Species Identification of Dermatophytes by MALDI-TOF MS. *Current Fungal*
395   *Infection Reports* 8:193–197. DOI: 10.1007/s12281-014-0189-7.
396 Greslebin AG, Hansen EM, Sutton W. 2007. *Phytophthora austrocedrae* sp. nov., a new species
397   associated with *Austrocedrus chilensis* mortality in Patagonia (Argentina). *Mycological*
398   *Research* 111:308–316. DOI: 10.1016/j.mycres.2007.01.008.
399 Grünwald NJ, Martin FN, Larsen MM, Sullivan CM, Press CM, Coffey MD, Hansen EM, Parke
400   JL. 2011. Phytophthora-ID.org: A sequence-based phytophthora identification tool. *Plant*
401   *Disease* 95. DOI: 10.1094/PDIS-08-10-0609.
402 H Ho H. 2018. The Taxonomy and Biology of *Phytophthora* and *Pythium*. *Journal of*
403   *Bacteriology & Mycology: Open Access* 6. DOI: 10.15406/jbmoa.2018.06.00174.
404 Hansen EM, Reeser PW, Sutton W. 2012. *Phytophthora* Beyond Agriculture. *Annual Review of*
405   *Phytopathology* 50:359–378. DOI: 10.1146/annurev-phyto-081211-172946.

406 Hardham AR, Blackman LM. 2018. *Phytophthora cinnamomi. Molecular Plant Pathology*
407   19:260–285. DOI: 10.1111/mpp.12568.
408 Husson C, Aguayo J, Revellin C, Frey P, Ioos R, Marçais B. 2015. Evidence for homoploid
409   speciation in *Phytophthora alni* supports taxonomic reclassification in this species complex.
410   *Fungal Genetics and Biology* 77:12–21. DOI: 10.1016/j.fgb.2015.02.013.
411 Jung T, La Spada F, Pane A, Aloi F, Evoli M, Horta Jung M, Scanu B, Faedda R, Rizza C,
412   Puglisi I, Magnano di San Lio G, Schena L, Cacciola SO. 2019. Diversity and Distribution
413   of *Phytophthora* Species in Protected Natural Areas in Sicily. *Forests* 10:259. DOI:
414   10.3390/f10030259.
415 El Karkouri K, Couderc C, Decloquement P, Abeille A, Raoult D. 2019. Rapid MALDI-TOF
416   MS identification of commercial truffles. *Scientific Reports* 9:17686. DOI: 10.1038/s41598-
417   019-54214-x.
418 Krajaejun T, Lohnoo T, Jittorntam P, Srimongkol A, Kumsang Y, Yingyong W, Rujirawat T,
419   Reamtong O, Mangmee S. 2018. Assessment of matrix-assisted laser desorption ionization-
420   time of flight mass spectrometry for identification and biotyping of the pathogenic
421   oomycete Pythium insidiosum. *International Journal of Infectious Diseases* 77:61–67. DOI:
422   10.1016/j.ijid.2018.09.006.
423 Kroon LPNM, Bakker FT, Van Den Bosch GBM, Bonants PJM, Flier WG. 2004. Phylogenetic
424   analysis of Phytophthora species based on mitochondrial and nuclear DNA sequences.
425   *Fungal Genetics and Biology* 41. DOI: 10.1016/j.fgb.2004.03.007.
426 Lasch P, Stämmler M, Schneider A. 2018. Version 3 (20181130) of the MALDI-TOF Mass
427   Spectrometry Database for Identification and Classification of Highly Pathogenic
428   Microorganisms from the Robert Koch-Institute (RKI). DOI: 10.5281/ZENODO.1880975.
429 Mani R, Vilela R, Kettler N, Chilvers MI, Mendoza L. 2019. Identification of *Pythium*
430   *insidiosum* complex by matrix-assisted laser desorption ionization-time of flight mass
431   spectrometry. *Journal of Medical Microbiology* 68:574–584. DOI: 10.1099/jmm.0.000941.
432 Martin FN, Abad ZG, Balci Y, Ivors K. 2012. Identification and Detection of *Phytophthora* :
433   Reviewing Our Progress, Identifying Our Needs. *Plant Disease* 96:1080–1103. DOI:
434   10.1094/PDIS-12-11-1036-FE.
435 Martin FN, Blair JE, Coffey MD. 2014. A combined mitochondrial and nuclear multilocus
436   phylogeny of the genus Phytophthora. *Fungal Genetics and Biology* 66. DOI:
437   10.1016/j.fgb.2014.02.006.
438 Martin FN, Tooley PW. 2003. Phylogenetic relationships among Phytophthora species inferred
439   from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes.
440   *Mycologia* 95. DOI: 10.1080/15572536.2004.11833112.
441 Mizeriene G, Cerny K, Zyka V, Bakonyi J, Nagy ZÁ, Oliva J, Redondo MA, Corcobado T,
442   Martín-García J, Prospero S. 2020. Patterns of Genetic Diversification in the Invasive
443   Hybrid Plant Pathogen Phytophthora × alni and Its Parental Species P. uniformis.
444   *Phytopathology®* 110:1959–1969. DOI: 10.1094/PHYTO-12-19-0475-R.
445 Normand A-C, Cassagne C, Gautier M, Becker P, Ranque S, Hendrickx M, Piarroux R. 2017.
446   Decision criteria for MALDI-TOF MS-based identification of filamentous fungi using
447   commercial and in-house reference databases. *BMC Microbiology* 17:25. DOI:
448   10.1186/s12866-017-0937-2.
449 Oliver L, Quinn M, Popenuck T, Garbelotto M. 2020. *Phytophthora species Can Be Reliably*
450   *Detected by Dogs Both From Infested Substrates and Infected Plants 1.*
451 Papalia M, Figueroa-Espinosa R, Steffanowski C, Barberis C, Almuzara M, Barrios R, Vay C,

452 Gutkind G, Di Conza J, Radice M. 2020. Expansion and improvement of MALDI-TOF MS
453 databases for accurate identification of Achromobacter species. *Journal of Microbiological*
454 *Methods* 172:105889. DOI: 10.1016/j.mimet.2020.105889.
455 Park J, Park B, Veeraraghavan N, Jung K, Lee Y-H, Blair JE, Geiser DM, Isard S, Mansfield
456 MA, Nikolaeva E, Park S-Y, Russo J, Kim SH, Greene M, Ivors KL, Balci Y, Peiman M,
457 Erwin DC, Coffey MD, Rossman A, Farr D, Cline E, Grünwald NJ, Luster DG, Schrandt J,
458 Martin F, Ribeiro OK, Makalowska I, Kang S. 2008. *Phytophthora* Database: A Forensic
459 Database Supporting the Identification and Monitoring of Phytophthora. *Plant Disease*
460 92:966–972. DOI: 10.1094/PDIS-92-6-0966.
461 Paul S, Singh P, Sharma S, Prasad GS, Rudramurthy SM, Chakrabarti A, Ghosh AK. 2019.
462 MALDI-TOF MS-Based Identification of Melanized Fungi is Faster and Reliable After the
463 Expansion of In-House Database. *PROTEOMICS – Clinical Applications* 13:1800070. DOI:
464 10.1002/prca.201800070.
465 Prigigallo MI, Abdelfattah A, Cacciola SO, Faedda R, Sanzani SM, Cooke DEL, Schena L.
466 2016. Metabarcoding Analysis of *Phytophthora* Diversity Using Genus-Specific Primers
467 and 454 Pyrosequencing. *Phytopathology* 106:305–313. DOI: 10.1094/PHYTO-07-15-
468 0167-R.
469 Rahi P, Prakash O, Shouche YS. 2016. Matrix-assisted laser desorption/ionization time-of-flight
470 mass-spectrometry (MALDI-TOF MS) based microbial identifications: Challenges and
471 scopes for microbial ecologists. *Frontiers in Microbiology* 7:1359. DOI:
472 10.3389/fmicb.2016.01359.
473 Ristaino JB. 2012. A Lucid Key to the Common Species of *Phytophthora*. *Plant Disease*
474 96:897–903. DOI: 10.1094/PDIS-08-11-0636.
475 Rizzato C, Lombardi L, Zoppo M, Lupetti A, Tavanti A. 2015. Pushing the Limits of MALDI-
476 TOF Mass Spectrometry: Beyond Fungal Species Identification. *Journal of Fungi* 1:367–
477 383. DOI: 10.3390/jof1030367.
478 Robideau GP, De Cock AWAM, Coffey MD, Voglmayr H, Brouwer H, Bala K, Chitty DW,
479 Désaulniers N, Eggertson QA, Gachon CMM, Hu C-H, Küpper FC, Rintoul T l., Sarhan E,
480 Verstappen ECP, Zhang Y, Bonants PJM, Ristaino JB, André Lévesque C. 2011. DNA
481 barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed
482 spacer. *Molecular Ecology Resources* 11:1002–1011. DOI: 10.1111/j.1755-
483 0998.2011.03041.x.
484 Ruiz Gómez FJ, Navarro-Cerrillo RM, Pérez-de-Luque A, Oβwald W, Vannini A, Morales-
485 Rodríguez C. 2019. Assessment of functional and structural changes of soil fungal and
486 oomycete communities in holm oak declined dehesas through metabarcoding analysis.
487 *Scientific Reports*. DOI: 10.1038/s41598-019-41804-y.
488 Sandoval-Sierra JV, Martín MP, Diéguez-Uribeondo J. 2014. Species identification in the genus
489 <i >Saprolegnia</i> (Oomycetes): Defining DNA-based molecular operational taxonomic
490 units. *Fungal Biology* 118:559–578. DOI: 10.1016/j.funbio.2013.10.005.
491 Schulthess B, Ledermann R, Mouttet F, Zbinden A, Bloemberg G V., Böttger EC, Hombach M.
492 2014. Use of the Bruker MALDI Biotyper for identification of molds in the clinical
493 mycology laboratory. *Journal of Clinical Microbiology* 52:2797–2803. DOI:
494 10.1128/JCM.00049-14.
495 Shepherd SJ, Van West P, Gow NAR. 2003. Proteomic analysis of asexual development of
496 Phytophthora palmivora. *Mycological Research* 107:395–400. DOI:
497 10.1017/S0953756203007561.

498   Sindt NM, Robison F, Brick MA, Schwartz HF, Heuberger AL, Prenni JE. 2018. MALDI-TOF-
499   MS with PLS Modeling Enables Strain Typing of the Bacterial Plant Pathogen
500   Xanthomonas axonopodis. *Journal of the American Society for Mass Spectrometry* 29:413–
501   421. DOI: 10.1007/s13361-017-1839-0.
502   Sugawara R, Yamada S, Tu Z, Sugawara A, Suzuki K, Hoshiba T, Eisaka S, Yamaguchi A.
503   2016. Rapid and reliable species identification of wild mushrooms by matrix assisted laser
504   desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). *Analytica*
505   *Chimica Acta* 934:163–169. DOI: 10.1016/j.aca.2016.05.056.
506   Swiecki TJ, Quinn M, Sims L, Bernhardt E, Oliver L, Popenuck T, Garbelotto M. 2018. Three
507   new Phytophthora detection methods, including training dogs to sniff out the pathogen,
508   prove reliable. *California Agriculture*. DOI: 10.3733/ca.2018a0026.
509   White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal
510   ribosomal rna genes for phylogenetics. In: *PCR Protocols*. DOI: 10.1016/b978-0-12-
511   372180-8.50042-1.
512   Yang X, Tyler BM, Hong C. 2017. An expanded phylogeny for the genus Phytophthora. *IMA*
513   *Fungus* 8. DOI: 10.5598/imafungus.2017.08.02.09.
514   Ziegler D, Mariotti A, Pflüger V, Saad M, Vogel G, Tonolla M, Perret X. 2012. In Situ
515   Identification of Plant-Invasive Bacteria with MALDI-TOF Mass Spectrometry. *PLoS ONE*
516   7:e37189. DOI: 10.1371/journal.pone.0037189.
517

Figure 1

Comparison of the interday variability of spectra of *P. lacustris* 295.09

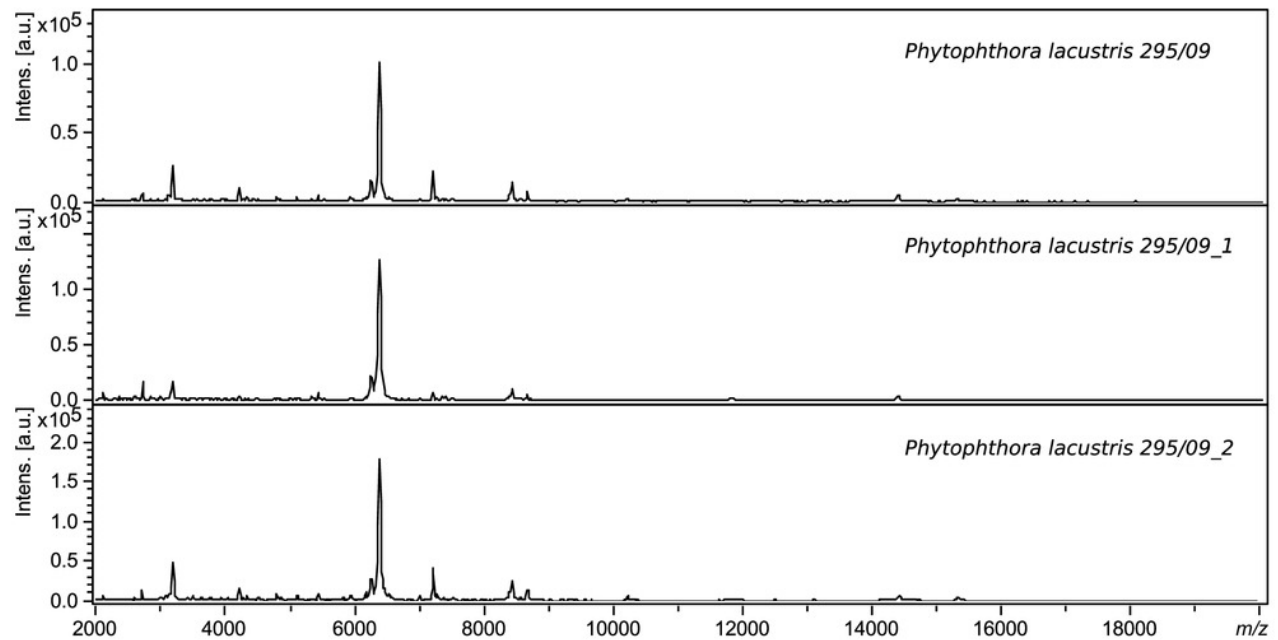



Figure 2

Protein spectra of different strains of *P. lacustris*

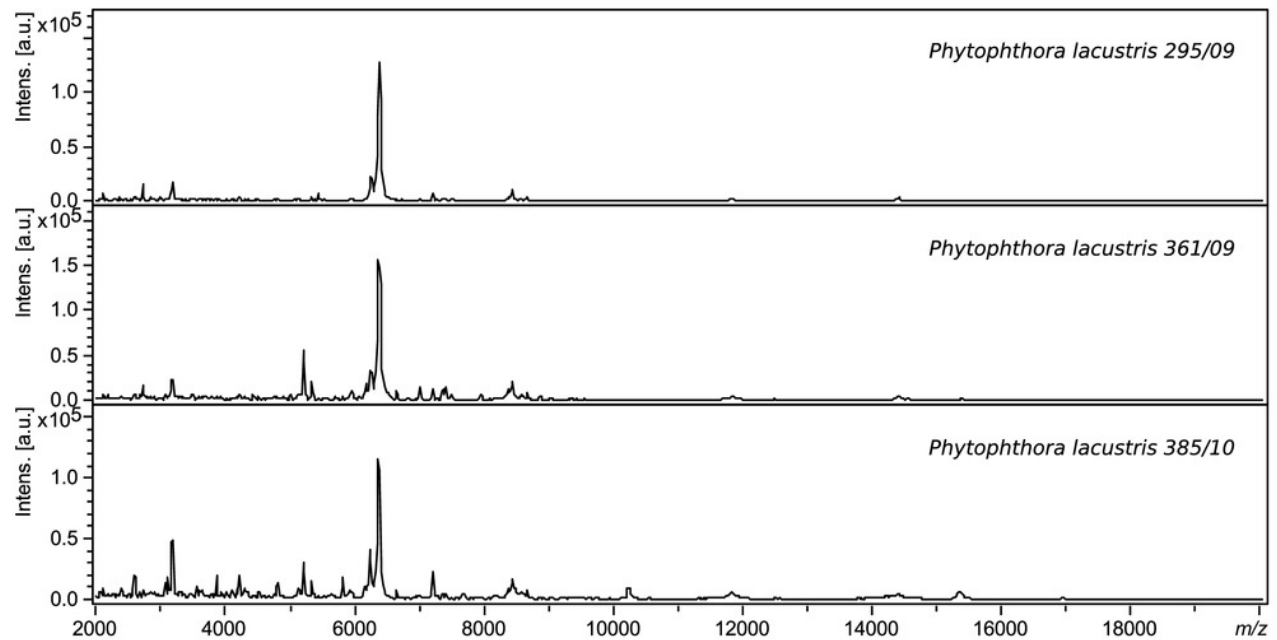



Figure 3

MSP dendrogram of *P. lacustris* from different locations

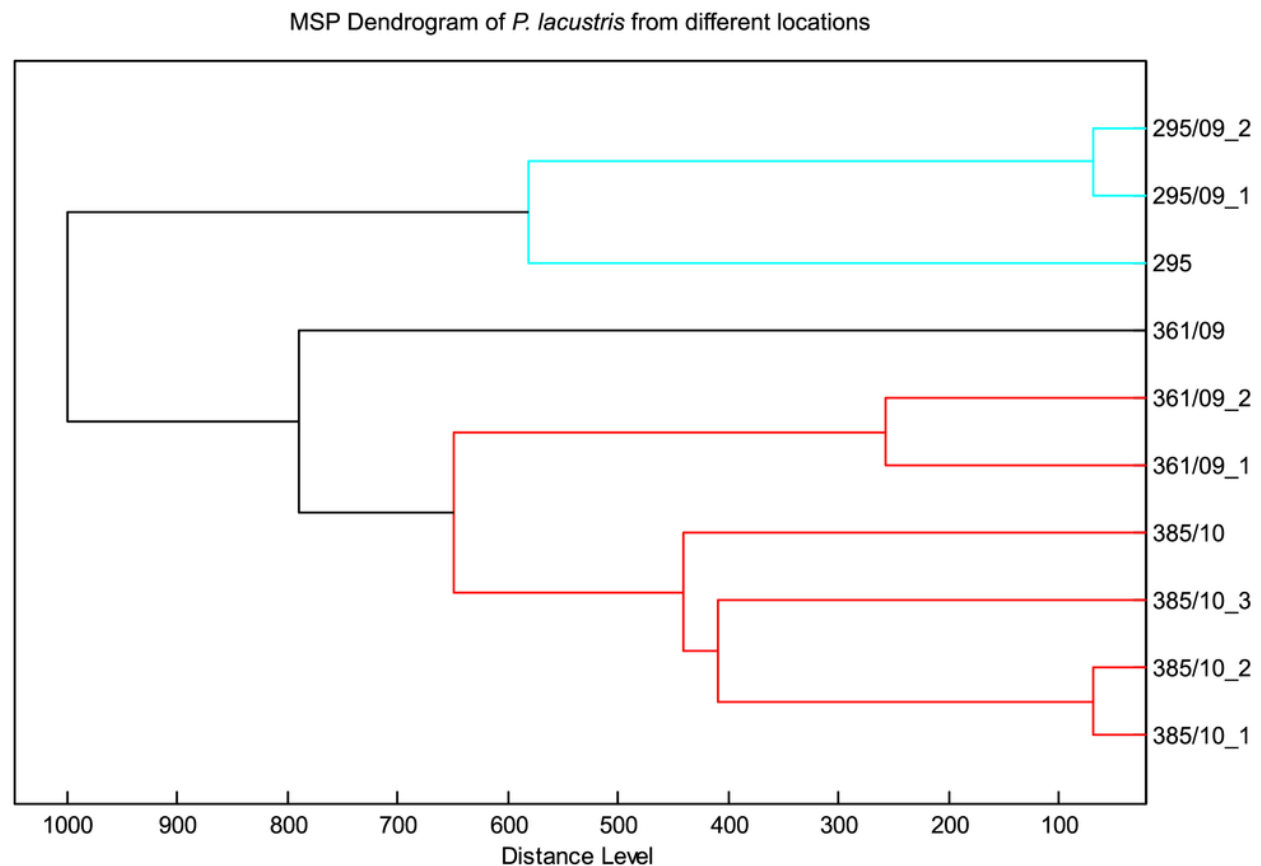
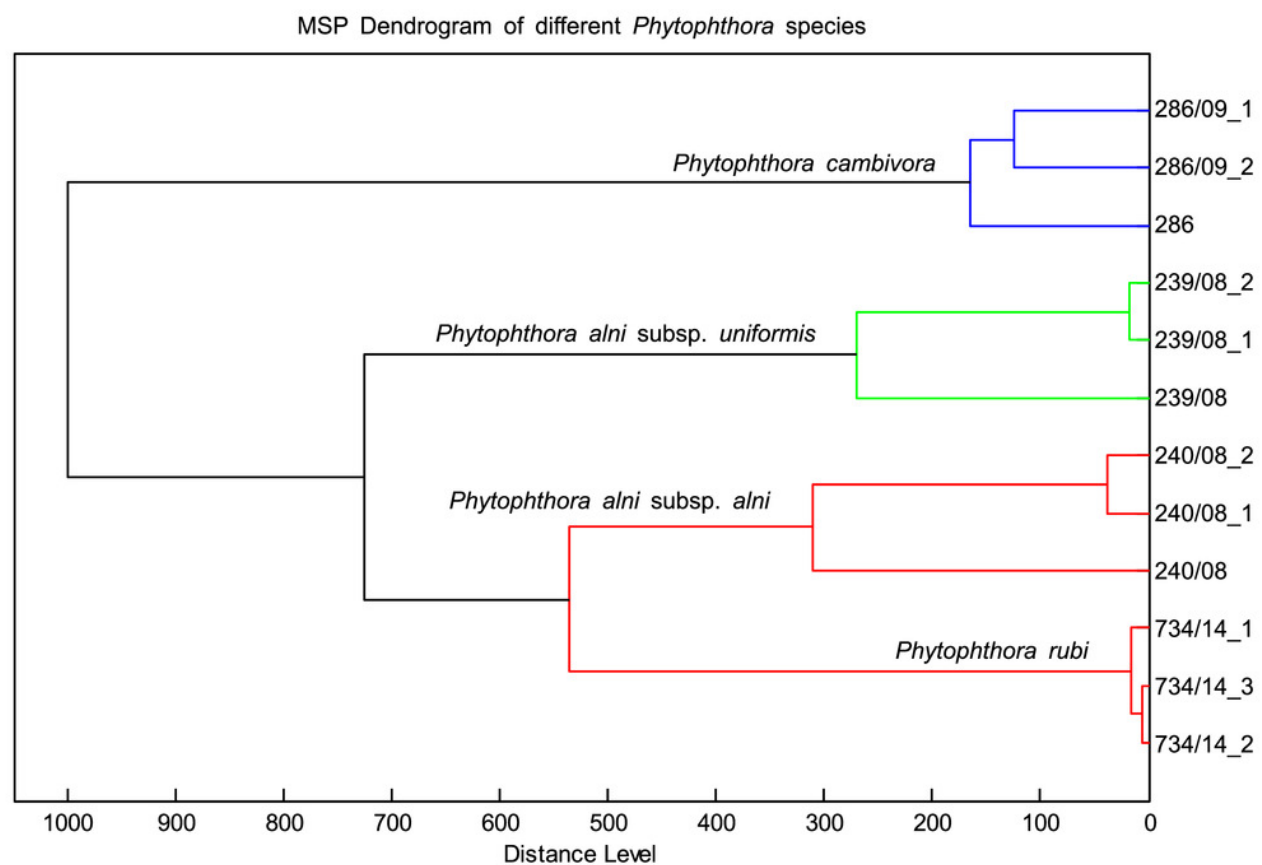



Figure 4

Distribution of different species with similar spectral profiles according to MSP dendrogram cluster analysis

Evaluation of rapid blank sample identification

1 Table 1 Evaluation of rapid blank sample identification

Repletion	ID	Best match	Score	Second match	Score
1	Blank	*Phytophthora lacustris* 398.10/2	2.686	*Phytophthora lacustris* 398.10/1	2.624
2	Blank	*Phytophthora lacustris* 398.10/2	2.725	*Phytophthora lacustris* 398.10/1	2.688
3	Blank	*Phytophthora lacustris* 398.10/2	2.718	*Phytophthora lacustris* 398.10/1	2.697

Numbers of correctly identified species and strains with different score values

1   Table 2 Identification of *P. lacustris* from different locations and hosts

Strain	Database match*				
P. lacustris 361.09	361.09/2	385.10/2	295.09/1	385.10	295.09/2
score	2.796	2.668	2.593	2.589	2.464
P. lacustris 295.09	295.09/2	361.09/2	361.09/1	295.09	361.09
score	2.759	2.64	2.591	2.536	2.362
P. lacustris 385.10	385.10/2	385.10	361.09/1	385.10/3	361.09/2
score	2.749	2.599	2.586	2.578	2.445

* The number after the slash indicates the number of biological replicates.

Identification of *P.lacustris* from different locations and hosts

1   Table 3 Identification of *P. lacustris* from different locations and hosts

Strain	Database match*				
P. lacustris 361.09	361.09/2	385.10/2	295.09/1	385.10	295.09/2
score	2.796	2.668	2.593	2.589	2.464
P. lacustris 295.09	295.09/2	361.09/2	361.09/1	295.09	361.09
score	2.759	2.64	2.591	2.536	2.362
P. lacustris 385.10	385.10/2	385.10	361.09/1	385.10/3	361.09/2
score	2.749	2.599	2.586	2.578	2.445
* The number after the slash indicates the number of biological replicates.					

Pythium vs *Phytophthora*

1   Table 4 Evaluation of rapid blank sample identification

Repletion	ID	Best match	Score	Second match	Score
1	Blank	*Phytophthora lacustris* 398.10/2	2.686	*Phytophthora lacustris* 398.10/1	2.624
2	Blank	*Phytophthora lacustris* 398.10/2	2.725	*Phytophthora lacustris* 398.10/1	2.688
3	Blank	*Phytophthora lacustris* 398.10/2	2.718	*Phytophthora lacustris* 398.10/1	2.697