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Identification of *Pythium insidiosum* complex by matrix-assisted laser desorption ionization-time of flight mass spectrometry

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Abstract

Purpose. Pythiosis is an infection of humans and other animals caused by the fungal-like pathogen *Pythium insidiosum*. This pathogen causes life-threatening infection in the infected hosts. Culture, histopathology, serology and molecular tools are used to diagnose its infections. Successful management of pythiosis is directly linked to an early diagnosis. Thus, a rapid identification of putative cultures developing submerged sparsely septate hyphae is of extreme importance. However, few laboratories are familiar with the culture identification of this unique pathogen and its differential diagnosis with similar filamentous fungi.

Methodology. We have evaluated the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) on 53 isolates of *P. insidiosum* collected from cases of human and animal pythiosis in the USA and around the world. To assess the specificity of the approach, 18 pathogenic and saprotrophic filamentous fungal and fungal-like microbes were also tested.

Results. MALDI-TOF in-house spectra correctly identified the 53 *P. insidiosum* isolates (score range 1.93–2.51). MALDI-TOF based identification within *P. insidiosum* isolates showed protein spectra variation between geographical diverse isolates. A mass spectrometry approach was able to discriminate *P. insidiosum* from the 18 filamentous fungal and fungal-like microbes in this study, including four *Pythium* spp. and *Phytopythium litorale* plant pathogenic species.

Conclusion. The data showed MALDI-TOF could be used for the accurate and rapid culture identification of *P. insidiosum* in the clinical laboratory.

INTRODUCTION

Pythium insidiosum is the etiologic agent of pythiosis, a disease of tropical and subtropical areas of the world, including the USA [1, 2]. Currently classified in the super-group stramenopila/alveolate/rhizaria (SAR) within the Oomycota, along with other *Pythium* and *Lagenidium* species and far away from the true fungi [3]. Infected hosts include cats, cattle, dogs, horses, humans and other animals. In these species, the development of superficial (keratitis), cutaneous, subcutaneous and vascular infections have been reported [1, 4, 5]. If the infection is not treated in the early stages, it could progress to a deadly systemic infection [1, 5]. Thus, an early diagnosis is key for successful management [1, 4, 5].

Several tools have been frequently used for the diagnosis of pythiosis, which include culture [1], serology (ELISA, immunodiffusion, Western blots) [6–9] and molecular tools (PCR, sequencing) [10–13]. Although a differential sucrose medium [14] and the susceptibility of *P. insidiosum* to antibiotics [15] have been proposed for the identification of *P. insidiosum* in culture, molecular testing is always required for its final identification [1, 10–13]. In addition, few laboratory workers are familiar with the taxonomic and microscopic features of *P. insidiosum* in culture [1].

The studies of the last 10 years using matrix-assisted laser desorption ionization-time of flight mass spectrometry

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Abbreviations: ARSEF, US Department of Agriculture, Agricultural Research Services; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; CMA, Cornmeal agar; ELISA, enzyme-linked immunosorbent assay; ITS, internal transcriber spacers; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of fly mass spectrometry; MSP, main spectral profile; MTLA, medical technology *Lagenidium*; MTPI, medical technology *Pythium insidiosum*; NCBI, Nacional Center for Biotechnology Information; NIH, National Institute of Health; PDA, potato dextrose agar; SDAC, 2% Sabouraud dextrose agar with Chloramphenicol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

(MALDI-TOF MS) showed this tool, initially used to identify bacteria, could be also used for the identification of yeast and filamentous fungi [16–18]. Although the identification of *P. insidiosum* using MALDI-TOF was recently explored using only 13 isolates [19], we have interrogated 53 *P. insidiosum* isolates from around the globe and found that for the approach to be successful it needs representative spectra of diverse *P. insidiosum* isolates. An accurate and rapid identification of *P. insidiosum* from culture is key for a successful management. Thus, this new tool could be used alone or in combination with other assays to facilitate the diagnosis of the infections caused by this neglected pathogen.

METHODS

Media and isolates

The identity of the 53 *P. insidiosum* isolates (Table 1; MTPI) at the Michigan State University culture collection, were confirmed at the microscopic morphological level and by PCR using the *P. insidiosum* ITS specific primers PI-1 forward 5′-TTCGTCGAAGCGGACTGCT-3 and reverse PI-2 5-GCCGTACAACCCGAGAGTCATA-3, developed by Grooters *et al.* [10]. This set of primers has been used to identify *P. insidiosum* for the last 15 years without report of false positives or negatives [1]. Genomic DNA was extracted using PureLink Genomic DNA kits following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Using *P. insidiosum* strain collection numbers we did interrogate the National Center for Biotechnology Information (NCBI) database. Using this strategy we found that some *P. insidiosum* isolates in this study can be confirmed as such using accession numbers previously deposited at the NCBI (Table 1). The remaining isolates without deposited sequences at NCBI were identified as *P. insidiosum* by the detection of the 105 bp specific amplicons using the above *P. insidiosum* ITS specific primers (Table 1). In addition, the 18 fungal and fungal-like isolates in this study were identified by their unique microscopic features as well as by their strain identification and DNA accession numbers previously deposited at the NCBI for each of the tested isolates (Table 2). The investigated isolates were maintained in our collection on Cornmeal agar (CMA) (BBL Spark, M. USA). To control putative bacterial contamination the fungal and fungal-like (including *P. insidiosum*) isolates were transferred to 2% Sabouraud dextrose agar containing chloramphenicol (SDAC) (Hardy Diagnostics, Santa Maria, CA) and incubated at 37 °C or 30 °C for 2 to 5 days, according to their growth requirements. After incubation, the investigated isolates (Tables 1 and 2) were subcultured onto potato dextrose agar plates (PDA) (Becton Dickinson Franklin Lakes, NJ) and incubated at 30 °C for 5 days before MALDI-TOF analysis.

Protein extraction of fungal and fungal-like identification by MALDI-TOF

A sterile scalpel blade was used to cut an approximately 5 mm diameter piece of the isolate from the PDA culture plate containing hyphal growth. A detailed procedure for the protein extraction has been described previously [21]. In brief, the collected 5 mm pieces were placed in Eppendorf tubes containing 500 µl of 70% ethanol plus 0.5 mg of 0.1 mm diameter zirconia beads (BioSpec Products, Bartlesville, OK). The tested filamentous fungal and fungal-like isolates were then emulsified using a sterile wooden stick and then vortexed for 15 min at high speed (Daigger Vortex Genie 2, Scientific Industries, Bohemia NY) and the centrifuged 9500′ ***g*** for 2 min. The supernatant was discarded by pipetting to completely remove any ethanol residue and the pellet resuspended in 50 µl of 70% formic acid, vortexed for 5 min followed for 10 s brief centrifugation and the addition of acetonitrile. The tubes were vortexed for 5 min, centrifuged at 9500′ ***g*** for 2 min and the supernatant immediately analyed by MALDI-TOF. All procedures up to spotting the supernatant onto the MALDI-TOF target plate were performed in a class II biosafety cabinet.

For every run the mass spectrometer was calibrated for molecular weights with a range of 3.6–16.9 kDa prior to sample testing using the bacterial test standard (Bruker Daltonics, Billerica, MA) as per the manufacturer's recommendations. *Aspergillus ustus* (CBS 261.67T) was also used as a protein extraction and MALDI-TOF analysis control for every run. The control *A. ustus* was identified with a score of 1.8 or higher. Scores below 1.8 for the control strain may suggest a suboptimal extraction procedure and thus, the procedure (protein extraction of *A. ustus* and tested isolates) was repeated. Four spots on the target plate were used for each fungal isolate. The MALDI-TOF platform used was the MicroFlex LT mass spectrometer (Bruker Daltonics, Billerica, MA). The spectra were captured in positive linear mode in a mass range of 2–20 kDa, with laser frequency of 60 Hz (IS1 : 20kV; IS2 : 18kV; lens: 6kV; extraction delay time: 100 ns). Spectra were acquired in automatic mode by accumulating a maximum of 240 profiles (6×40 laser shots from different positions of the target spot). Organism identification was performed with default setting of the software (Biotyper RTC v. 3.1, Bruker Daltonics) using the National Institute of Health (NIH) fungal reference database (contains the protein profile of several 81 genera and 172 species of filamentous and yeast fungal isolates), the in-house data generated from *P. insidiosum* (Table 1) and that of the other fungal and fungal-like isolates (Table 2). The identification spectra are set with scores between 0 and 3 (Biotyper RTC v. 3.1, Bruker Daltonics). According to Bruker Daltonics [22] a score of ≥2.0 indicates good species level identity for bacterial and fungal identification, whereas scores between 1.7 and 2.0 suggest a close relationship to a particular genus and species [21, 23, 24].

Addition of protein spectra to the MALDI-TOF reference database

The criteria for the selection of nine *P. insidiosum* isolates used to build the in-house protein spectra (highlighted with asterisks and bold, Table 1) were based on their diverse geographic distribution around the globe and the presence of DNA sequences at the NCBI (Table 1). The protein profile of the selected *P. insidiosum* and the fungal and fungal-like animal pathogens in Table 2 were added to the reference database using the manufacturers' recommendations for MicroFlex LT and MALDI-TOF Biotyper RTC v. 3.1 (20). In short, for each isolate, proteins were extracted as above, and the supernatant

Table 1. *P. insidiosum* isolates used in this study, their culture collection ID, geographic origin, site of infection, infected host/NCBI accession numbers and MALDI-TOF scores

Collection ID	Country	Infection site	Host/NCBI No.	Score
MTPI-01	USA, Arkansas	Subcutaneous	Horse/DQ116403	2.18
MTPI-02	USA, Mississippi	Subcutaneous	Dog†	2.25
MTPI-03	USA, Texas	Subcutaneous	Horse/DQ116404	2.23
MTPI-04 (ATCC PTA-12166)*	**USA, Texas**	**Subcutaneous**	**Horse/DQ116405**	**2.04**
MTPI-06	USA, Wisconsin	Subcutaneous	Dog/DQ116407	2
MTPI-07	USA, Florida	Subcutaneous	Dog/DQ116408	1.99
MTPI-10 (ATCC 200269)	USA, Tennessee	Orbital	Human/DQ116411	2.41
MTPI-11*	**Afghanistan**	**Keratitis**	**Human/DQ116412**	**2.36**
MTPI-13	Thailand	Vascular	Human†	2.28
MTPI-15*	**Thailand**	**Vascular**	**Human/DQ116413**	2.33
MTPI-16	Thailand	Vascular	Human†	2.01
MTPI-19 (ATCC 58643)	Costa Rica	Subcutaneous	Horse/DQ116415	2.05
MTPI-20 (ATCC 58644)	Costa Rica	Subcutaneous	Horse/DQ116416	2.01
MTPI-21 (ATCC28251)*	**Papua New Guinea**	**Subcutaneous**	**Horse/AY151171**	**2.13**
MTPI-22 (ATCC 76049)	Haiti	Keratitis	Human/DQ116418	1.93
MTPI-24 (ATCC 64218)	Australia	Subcutaneous	Horse†	2.39
MTPI-25 (ATCC 64221)*	**Australia**	**Subcutaneous**	**Horse/KP780446**	**2.42**
MTPI-26 (ATCC 90586)	USA, Texas	Cutaneous	Human†	2.2
MTPI-28	USA, Tennessee	Orbital	Human/DQ116421	2.51
MTPI-29	Brazil	Lung	Sheep†	2.23
MTPI-30	Brazil	Nose	Sheep†	2.15
MTPI-31	USA, Texas	Burned skin	Human†	2.08
MTPI-32	Venezuela	Instestinal	Dog†	2.29
MTPI-33	USA, Illinois	Systemic	Human†	2.14
MTPI-34	Brazil	Lynph node	Sheep†	1.97
MTPI-35*	**Israel**	**Keratitis**	**Human/GU584093**	**2.26**
MTPI-36	Mexico	Burned skin	Human†	2.32
MTPI-37	USA, Tennessee	Vulva	Camel†	2.32
MTPI-38	USA, Florida	Subcutaneous	Horse†	2.12
MTPI-39	USA, Georgia	Subcutaneous	Dog†	2.32
MTPI-40	USA, Florida	Subcutaneous	Horse†	2.14
MTPI-41	USA, Mississippi	Subcutaneous	Horse†	2.19
MTPI-42	USA, Oklahoma	Subcutaneous	Horse†	2.36
MTPI-43	USA, Texas	Subcutaneous	Human†	2.16
MTPI-44	USA, Florida	Intestinal	Dog†	2.36

Continued

Table 1. Continued

Collection ID	Country	Infection site	Host/NCBI No.	Score
MTPI-45	USA Missouri	Subcutaneous	Dog†	2.11
MTPI-46*	**USA, Wisconsin**	**Subcutaneous**	**Dog/NH356546**	**2.27**
MTPI-47	USA, Texas	Subcutaneous	Dog†	2.15
MTPI-48	Grand Cayman	Vascular	Human†	2.01
MTPI-49	USA, Mississippi	Subcutaneous	Dog†	2.21
MTPI-50	USA, South Carolina	Subcutaneous	Dog†	2.46
MTPI-51	USA, Florida	Subcutaneous	Dog†	1.97
MTPI-52	USA , Texas	Subcutaneous	Horse†	2.03
MTPI-53	USA, Virginia	Subcutaneous	Horse†	2.4
MTPI-55	USA, Mississippi	Subcutaneous	Horse†	2.36
MTPI-56	USA, Mississippi	Intestinal	Dog†	2.41
MTPI-57	USA, Florida	Subcutaneous	Horse†	2.3
MTPI-58*	**USA, Texas**	**Subcutaneous**	**Dog/MH899095**	**2.6**
MTPI-59*	**USA, Missouri**	**Subcutaneous**	**Horse/MH899096**	**2.16**
MTPI-60	USA, Florida	Subcutaneous	Horse†	2.25
MTPI-71	India	Keratitis	Human†	2.13
MTPI-76 (ARSEF 13235)	Brazil	Systemic	Mosq larv/MG971299	2.09

*Isolates selected to build MALDI-TOF in-house spectra.
†Confirmed as *P. insidiosum* by PCR using specific primers PI-1 and PI-2 [10].
ARSEF, U.S. Department of Agriculture, Agricultural Research Service;ATCC, American Type Culture Collection;MTPI, MedTech *Pythium insidiosum* Michigan State University culture collection;Mosq larv, Mosquito larvae.

was spotted on ten wells. Flex Control v 3.4. was used to automatically capture 30 individual spectra (three separate spectra were captured from each of the 10 wells). After smoothing, baseline subtraction and removing the outlier peptide peaks, a minimum of 20 individual spectra with at least 70 peaks with high frequency greater than 75% were selected using Flex Analysis v. 3.4 (21). The resulting spectra were used to create a main spectral profile (MSP) using Biotyper OC v. 3.1 (20). After completing the manufacturer-recommended quality checks, each of the newly created MSPs were added to the database library. Protein spectra of isolates in Table 2 except the three *Aspergillus* spp. originally in the NIH fungal reference database [21, 22], were also added to the in-house database using the above-described protocol.

Culture media based variation of MALTI-TOF scores

In order to assess the variation in MALDI-TOF scores based on culture media and incubation temperature, two randomly selected *P. insidiosum* isolates: MTPI-44 and MTPI-59 were evaluated in duplicate on SDAC and PDA with incubation for 3 days at 30 °C or 37 °C. After incubation, the MALDI-TOF protein spectra were collected (four spots each) and recorded. The experiment was repeated three times to observe day-to-day variations in MALDI-TOF score. Statistical analysis was performed using SigmaPlot v13.0 software package (Systat Software Inc., Chicago, IL).

Determination of identification specificity within *Pythium* spp

Nine geographically diverse *P. insidiosum* isolates from the Americas, Asia and the Middle East, four *Pythium* spp. and *Phytopythium litorale* pathogenic to plants added to the in-house database were further analysed to determine the specificity between the tested isolates. The 14 isolates were interrogated against a database library consisting of only the above-mentioned 14 in-house library spectra using Biotyper OC v. 3.1 and the top scores for each spectrum were determined. The nine *P. insidiosum* isolates were also interrogated using the same approach to determine the specificity within the *P. insidiosum* isolates alone.

Visual protein spectra of some isolates

Peptide mass spectra generated by MALDI-TOF MS for *P. ultimum*, *P. aphanidermatum* and three *Pythium*

Table 2. The genus and species of the tested fungal and fungal-like microbes in this study as well as their culture collection identification, NCBI accession numbers, the source of isolation and MALDI-TOF scores with their own in-house spectra

Isolates	Isolate ID/ NCBI	Source	MALDI-TOF/ID (top score)
Conidiobolus coronatus	ATCC 32825	Human	*C. coronatus* (1.87)
Conidiobolus incongruus	ATCC 56682	Human	*C. incongruus* (1.93)
Conidiobolus lampragues	MTCL-01 (GQ478279)	Sheep	*C. lampragues* (2.29)
Conidiobolus lampragues	MTCL-02 (GQ478280)	Sheep	*C. lampragues* (2.0)
Conidiobolus lampragues	MTCL-03 (GQ478281)	Sheep	*C. lampragues* (1.98)
Lagenidium giganteum	MTLA-01 (JF919611)	Dog	*L. giganteum* (2.59)
Lagenidium giganteum	MTLA-05 (JF919614)	Dog	*L. giganteum* (1.99)
Lagenidium giganteum	MTLA-16 (KJ506130)	Dog	*L. giganteum* (1.78)
Lagenidium giganteum	ATCC 36492 (JX999124)	Mosquito larvae	*L. giganteum* (2.44)
Lagenidium giganteum	ARSEF 373 (KX492585)	Mosquito larvae	*L. giganteum* (2.41)
Pythium aphanidermatum (clade A)*	NDSO_L_8–6 (KU210065)	Soybean	*P. aphanidermatum* (2.38)
Pythium oopapillum (clade B)*	IASO_6–10.15 (KU208635)	Soybean	*P. oopapillum* (2.37)
Pythium sylvaticum (clade F)*	NESO_2–13 (KU210303)	Soybean	*P. sylvaticum* (1.94)
Pythium ultimum (clade I)*	KSSO_5–45 (KU209642)	Soybean	*P. ultimum* (2.46)
*Phytopythium litorale**	V-IASO2_6–55.1 (KU210522)	Soybean	*Phyt. litorale* (2.52)
Aspergillus sp.	MTAF-01	Chicken house contaminant	*A. fumigatus* (2.07)
Aspergillus sp.	MTAN-01	Laboratory contaminant	*A. niger* (1.7)
Aspergillus sp.	MTAV-01	Laboratory contaminant	*A. versicolor* (1.85)

*Isolates provided by Rojas *et al.* [20].
ARSEF, U.S. Department of Agriculture, Agricultural Research Service Collection of Entomopathogenic Fungi;MTLA, (MedTech *Lagenidium* spp.) Michigan State University, fungal and fungal-like culture collection;NCBI, National Center for Biotechnology Information.

insidiosum (MTPI-46, MTPI-15 and MTPI-59) isolates were analysed using FlexAnalysis software v. 3.4 provided with the Microflex LT (Bruker Daltonics). Visual comparison was performed after smoothing, baseline subtraction and peak assigning.

Cladistics analysis of *Pythium insidiosum* spectra

For cladistics analysis, 30 randomly selected isolates were used from different geographical regions. These included 27 mammalian *P. insidiosum* isolates, two *Pythium* spp. and

Phytopythium litorale all plant pathogen species (Table 1). The analysis was performed using the Biotyper OC v. 3.1 (Bruker Daltonics) with default settings of the software.

RESULTS

Morphological and molecular identification

Pure cultures of the isolates in Tables 1 and 2 were identified according to their unique morphological microscopic characteristics. Extracted gDNA from the isolates in this study consistently showed the presence of a single high molecular weight band after running 0.8% gels (data not showed). The phenotypic and physiological features of the 53 *P. insidiosum* isolates were confirmed either by PCR using *P. insidiosum* ITS PI-1 and PI-2 specific primers (see below) or by the presence of their equivalent strains accession numbers previously deposited at the NCBI (Tables 1 and 2). In addition, the presence of the expected *P. insidiosum* specific 105 bp amplicon on 0.8% agarose gels (data not shown) confirmed the identity of those *P. insidiosum* isolates without accession numbers at the NCBI (Table 1). *P. insidiosum* specific primers PI-1 and PI-2 did not amplify the expected amplicon on any of the genomic DNA extracted from the isolates in Table 2, confirming their specificity (data not shown).

Pythium insidiosum incubation temperature and media

The two randomly selected isolates (MTPI-44 and MTPI-59) used to evaluate the best medium and temperature for *P. insidiosum* growth showed statistically significant differences in their MALDI-TOF scores (Fig. 1). For instance, statistical analysis showed MTPI-44 and MTPI-59 incubated at 30 °C or 37 °C developed higher scores on PDA than on SDAC at both temperatures (one way ANOVA for MTPI-44 grown on PDA vs SDAC at 30 °C, P=0.012: one way ANOVA for MTPI-44 grown on PDA vs SDAC at 37 °C, P<0.001: one way ANOVA for MTPI-59 grown on PDA vs SDAC at 30 °C, P=0.006: one way ANOVA for MTPI-59 grown on PDA vs SDAC at 37 °C, P=0.001). There was no statistically significant differences at 30 °C or 37 °C for both isolates growing on PDA (one way ANOVA, P=0.997 for MTPI-44 and P=0.471 for MTPI-59) (Fig. 1).

Pythium insidiosum, fungal and fungal-like microbes' MALDI-TOF identification

MALDI-TOF identified the 53 *P. insidiosum* isolates (Table 1) using the reference spectra containing the original in-house built fungal and fungal-like isolates and the NIH fungal reference database. With few exceptions (MTPI-07 score: 1.99; MTPI-22 score: 1.93; MTPI-34 score: 1.97; and MTPI-51 score: 1.97), the

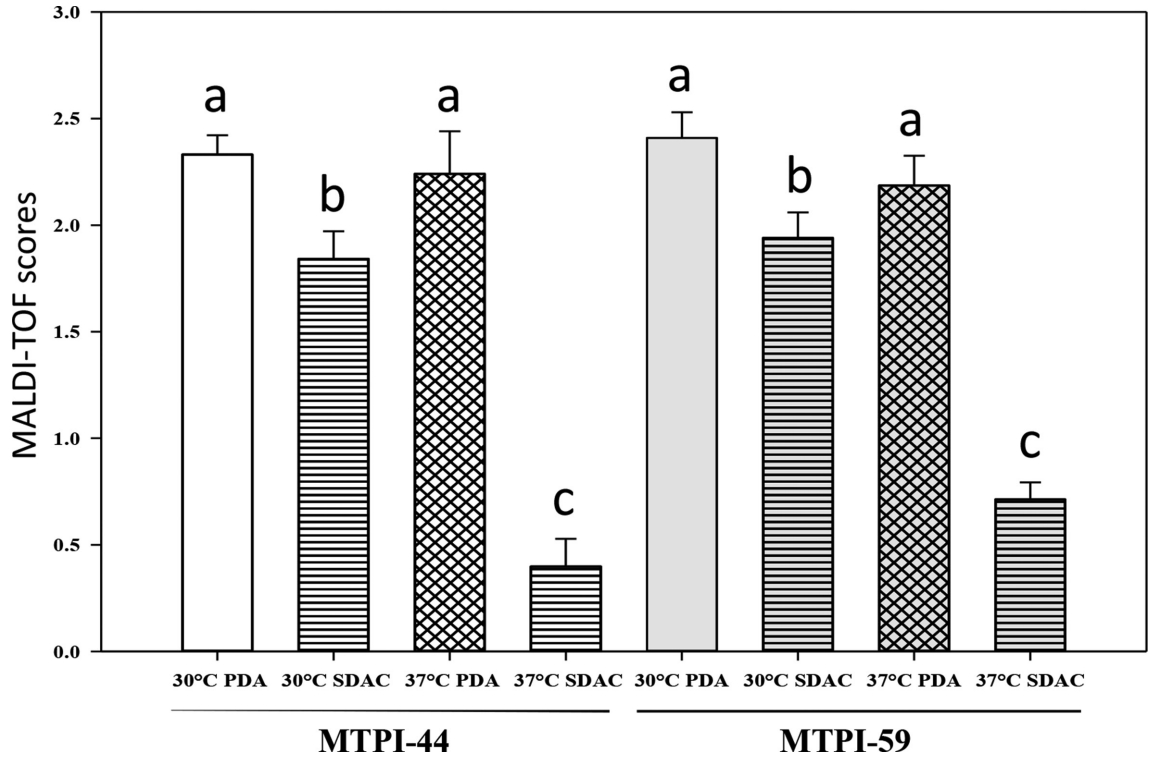

Fig. 1. Graphic representation of the obtained MALDI-TOF data. The *Y*-axis shows the MALDI-TOF scores and the *X*-axis shows the different media/temperature combinations. Error bars indicate standard deviation. There was significant statistical difference in MALDI-TOF scores between the 2% Sabouraud dextrose agar plus chloramphenicol (SDAC) and 2% potato dextrose agar (PDA) media used in these analyses. Bars with different letters (a, b, c) indicate statistically significant differences (P≤0.01); those with the same letter indicate no established differences.

Table 3. *Pythium* species and their discrimination by MALDI-TOF mass spectrometry according to their newly created spectra

Interrogated *Pythium* spp.	Top log score for each					
	Pi	***Pa***	***Po***	***Ps***	***Pu***	***Phyl***
Pythium insidiosum MTPI-04*	2.04	<0.5	<0.5	0.75	<0.5	0.64
Pythium insidiosum MTPI-11*	2.36	<0.5	<0.5	<0.5	<0.5	<0.5
Pythium insidiosum MTPI-15*	2.33	<0.5	0.62	0.68	<0.5	<0.5
Pythium insidiosum MTPI-21*	2.13	0.64	<0.5	<0.5	<0.5	<0.5
Pythium insidiosum MTPI-25*	2.42	<0.5	0.71	<0.5	<0.5	<0.5
Pythium insidiosum MTPI-35*	2.26	<0.5	1.11	<0.5	<0.5	0.98
Pythium insidiosum MTPI-46*	2.27	<0.5	<0.5	<0.5	<0.5	<0.5
Pythium insidiosum MTPI-58*	2.6	<0.5	0.73	<0.5	<0.5	<0.5
Pythium insidiosum MTPI-59*	2.16	<0.5	<0.5	0.58	<0.5	<0.5
Pythium aphanidermatum	0.82	2.38	0.62	0.57	<0.5	<0.5
Pythium oopapillum	0.82	<0.5	2.37	0.65	<0.5	<0.5
Pythium sylvaticum	0.71	<0.5	<0.5	1.94	<0.5	<0.5
Pythium ultimum	<0.5	<0.5	<0.5	<0.5	2.46	<0.5
Phytopythium litorale	0.56	<0.5	<0.5	0.53	<0.5	2.52

**Pythium insidiosum* isolates selected to build MALDI-TOF in-house spectra.
See Table 2.
Pa, *P. aphanidermatum* score;*Phyl*, *Phytopythium litorale* score;*Pi*, *Pythium insidiosum* score;*Po*, *P. oopapillum* score;*Ps*, *P. sylvaticum* score;*Pu*, *P. ultimum* score.

remaining *P. insidiosum* isolates were identified with scores of 2.0 or higher (Table 1). In addition, when the in-house built mass spectra of each *Pythium* species and *Phy. litorale* in Table 2 were interrogated against each other and the nine isolates used to build *P. insidosum* spectra, the top scores matched the respective spectrum and scores 1.11 or lower were observed for any of the spectra other than their own (Table 3). Similarly, the nine isolates used to build *P. insidosum*'s spectra interrogated against each other the top scores matched the respective spectrum (Table 4). While most of the isolates had at least one other similar high

Table 4. *Pythium insidiosum* isolates and their discrimination by MALDI-TOF mass spectrometry according to their own spectra and geographic origin

Interrogated *Pythium insidiosum*	Top log score for each spectrum								
	MTPI-04	**MTPI-11**	**MTPI-15**	**MTPI-21**	**MTPI-25**	**MTPI-35**	**MTPI-46**	**MTPI-58**	**MTPI-59**
MTPI-04	2.04	1.78	1.19	1.53	1.31	<0.5	0.930	2.04	1.710
MTPI-11	1.88	**2.35**	1.39	2.02	1.73	0.81	0.71	2.27	2.12
MTPI-15	<0.5	1.26	**2.32**	1.36	1.48	1.41	0.83	<0.5	1.24
MTPI-21	<0.5	1.79	1.12	**2.13**	1.71	0.82	0.71	<0.5	1.64
MTPI-25	<0.5	1.81	1.56	1.77	**2.28**	0.71	0.55	<0.5	1.56
MTPI-35	<0.5	1.23	1.99	1.29	1.29	**2.25**	0.97	<0.5	1.27
MTPI-46	<0.5	1.34	<0.5	0.61	1.1	0.65	**2.43**	1.07	<0.5
MTPI-58	2.02	1.76	1.22	1.52	1.28	0.89	0.74	**2.6**	2.19
MTPI-59	2.07	1.94	1.08	1.65	1.44	1.21	1.17	2.45	**2.37**

Shaded areas represent similar scores shared among isolates in the same geographic location.
See Table 1 for isolate identification and geographic origin.

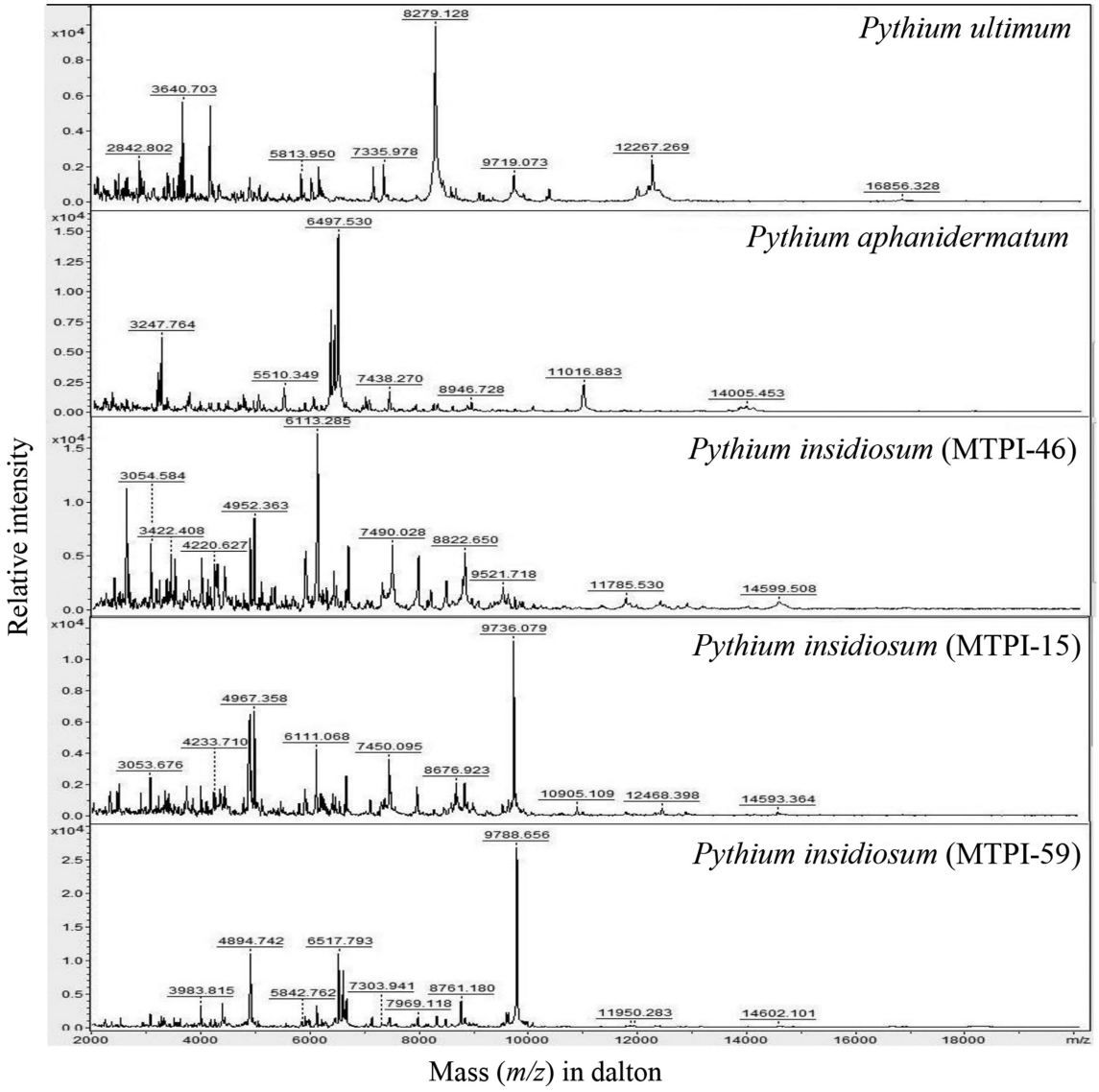

Fig. 2. MALDI-TOF fingerprint spectra (size range 2–20 kDa) of three of nine *Pythium insidiosum* isolates used to build the in-house spectra, and the spectra of two of four *Pythium* plant pathogen species. *Pythium insidiosum* MTPI-46 fingerprint spectra possess contrasting spectra with that on MTPI-15 and MTPI-59.

score for a second spectrum (shaded areas, Table 4), interestingly MTPI-15 and MTPI-46 did not. Visual spectra of MTPI-15 and MTPI-46 comparison with another North American isolate (MTPI-59) and two other plant *Pythium* sp., showed some similarities but also unique spectral patterns, especially MTPI-46 in contrast to the other two *P. insidiosum* in Fig. 2. Unique spectral profiles for the two plants *Pythium* sp. were also noted (Fig. 2). The built spectra of fungal and fungal-like (*Conidiobolus* and *Lagenidium*) species in Table 2, other than *Pythium* species, interrogated against the NIH fungal reference database library and the in-house *Pythium* spp. library, were identified with scores between 1.7 and 2.59 (Table 2). The three *Aspergillus* species in this analysis were identified as *A. fumigatus*, *A. niger* and *A. versicolor* (Table 2). The fungal-like organisms in Tables 1 and 2 interrogated using only the original NIH fungal database displayed scores 1.1 and lower (data not shown).

Cladistics analysis using MALDI-TOF spectra identification

MALDI-TOF cladistics analysis based on several *P. insidiosum* and the plant pathogen protein spectra parameters, grouped the 30 interrogated isolates in four supported clades (Fig. 3). One clade contains two *Pythium* spp. and *Phytopythium litorale* recovered from plants forming a sister group to the mammalian *P. insidiosum* isolates. Clade II grouped the isolates recovered from cases of pythiosis in Australia, Israel, New Guinea and Thailand. Clade III consisted of one

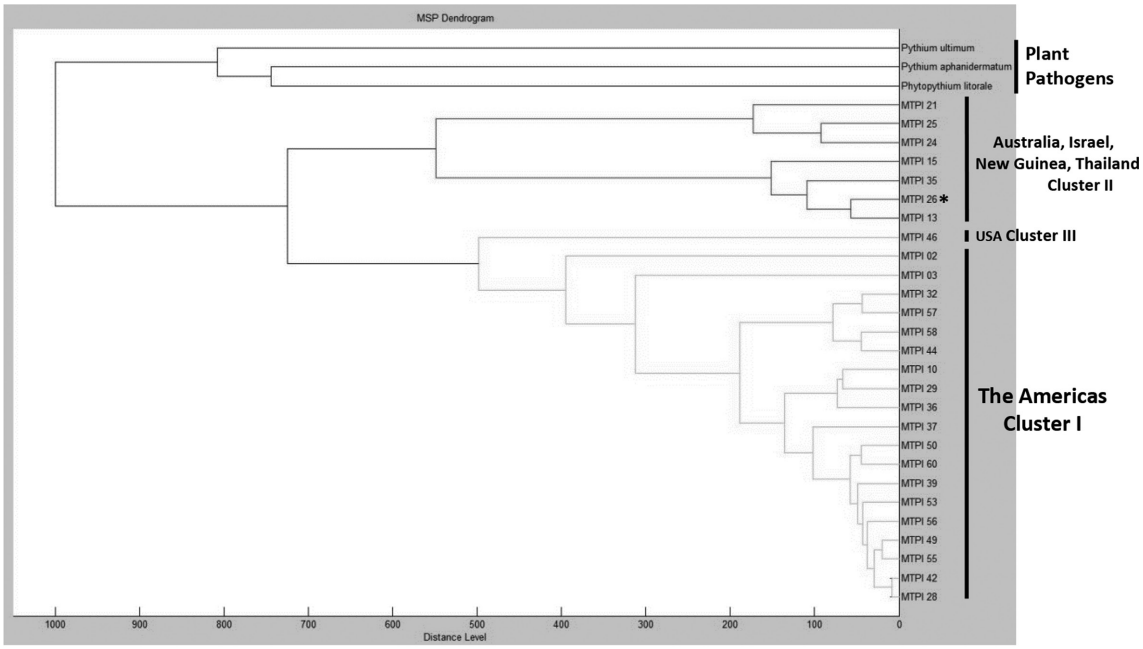

Fig. 3. MSP dendogram based on the MALDI-TOF protein profile of the 30 *Pythium* spp. interrogated isolates. The plant pathogenic isolates formed a monophyletic cluster sister to *P. insidiosum* isolates. The human and animal pathogen *P. insidiosum* isolates were grouped in three clusters according to their geographical preferences. The upper cluster contains the Asian and Middle East isolates (cluster II), whereas the lower cluster comprised the American isolates (cluster I). *P. insidiosum* MTPI-46, the only member of cluster III, was placed alone on its own clade as predicted by phylogenetic analysis [25–27]. Our data suggest MALDI-TOF generated protein spectra could predict *P. insidiosum* phylogenetic relationships.

isolate (MTPI-46) from a dog with intestinal infection in Wisconsin, USA. The last clade harbours all isolates recovered from humans and animals with pythiosis from the Americas (cluster I) (Fig. 3; Table 1).

DISCUSSION

The rapid and accurate identification of submerged colonies on SDA and/or blood agar displaying sparsely septate hyaline hyphae from suspected cases of pythiosis, is of paramount importance for a rapid management [1, 4, 6–8]. Current identification of *P. insidiosum* complex requires expertise on the taxonomic features of this Oomycota and/or the use of molecular tools such as PCR and/or DNA sequencing [1, 10–13]. However, both approaches are time consuming, require expertise and are expensive [10–12]. Recently, the use of MALDI-TOF for the identification of yeast and pathogenic filamentous fungi showed reliable results [23, 24, 28]. Our study found MALDI-TOF identified the 53 *P. insidiosum* isolates in this study using the in-house built spectra and properly discriminated them from other *Pythium* species, including *P. aphanidermatum* previously reported as a putative human pathogen [29] (Tables 2 and 3). In addition, MALDI-TOF was also capable of differentiating *P. insidiosum* from other filamentous fungi including the mammalian pathogens *Conidiobolus* and *Lagenidium* species (Table 2).

According to the data in this study, MALDI-TOF was able to identify the 53 interrogated *P. insidiosum* isolates based on the fact that the nine selected isolates used as the in-house spectra came from diverse geographical locations such as: the Americas (MTPI-04, 46, 58, 59), Afghanistan, (MTPI-11), Australia (MTPI-25), Israel (MTPI-35), Papua New Guinea (MTPI-21) and Thailand (MTPI-15). Table 4 data strongly support this argument. The spectra shared by all nine isolates in Table 4 agree with their geographical origin. For instance, with the exception of MTPI-46 (see below), the Americas MTPI-04, 58, 59, all shared higher scores than those from other geographical locations (Table 4 shaded areas). A similar behaviour was also observed between MTPI-21 and MTPI-25 from Papua New Guinea and Australia, respectively, and MTPI-15 and MTPI-35 from Thailand and Israel. The exceptions were MTPI-11, an isolate from Afghanistan that shares features in common with the Americas, Australia and Papua New Guinea isolates and MTPI-46 and MTPI-15 displaying unique spectra (Table 4, Fig. 2). Thus, the addition of *P. insidiosum* spectra from different geographical areas was a key element for the identification of related isolates using the mass spectrometry approach. Remarkably, the data in Fig. 2 and Table 4 are in sharp contrast with the uniform spectra between isolates reported by Krajaejun *et al.* [19]. The uniform spectra seen among the 13 isolates in Krajaejun *et al.*'s [19] study might be due to a combination of fungal growth

conditions utilized (Sabouraud dextrose broth at 37 °C) and the specific protein extraction method employed. Based on the data in this study, the use of diverse *P. insidiosum* isolates in the reference database have an impact on the accuracy to detect different protein spectra within *P. insidiosum* complex (clusters I to IV) [1, 25, 26, 30]. The unique protein spectra profiles also support the potential presence of diverse putative new species within the complex [25, 27, 31].

Of interest to this study was also the phylogenetic finding of Schurko *et al.* [25]. These authors reported three different phylogenetic clades among *P. insidiosum* complex, each containing isolates closely related to their unique geographic origins. One of the investigated isolates, recovered from a South Carolina bear (AY151178) placed in cluster III (Fig. 3) displayed striking phylogenetic differences with isolates in clades I and II [25]. They concluded that the 'bear isolate' (as colloquially has been known) might represent a cryptic new species. BLAST analysis at the NCBI of the MTPI-46 DNA sequences (NH356546) found 100% identity with the Schurko *et al.* [25] bear isolate (AY151178). MALDI-TOF cladistics analysis strongly supports Schurko *et al.* [25] phylogenetic findings (Fig. 3). Furthermore, Chindamporn *et al.* [30] also found contrasting SDS-PAGE protein profiles among *P. insidiosum* isolates from the Americas, Australia, Papua New Guinea and Thailand. These authors reported that the antibodies in patients with pythiosis recognized different immunogens depending on the geographical origin of the isolates.

Presser and Goss [26] and more recently Kittichotirat *et al.* [31] confirmed the unique DNA sequences and genomic content of *P. insidiosum* isolates in clade III. Thus, Presser and Goss [26] and Vilela *et al.* [27] based on new phylogenetic data recently proposed the addition of a fourth clade. These authors reported that Schurko *et al.*'s [25] clade III harbours a new unreported clade they named 'clade IV'. Interestingly, members of clade III comprises America, Asia and Middle East isolates, whereas clade IV harbours American isolates only suggesting that the entire cluster may be a new species [26]. This fact strongly supports the unique protein profile displayed by MTPI-46 isolate (clade IV – 28, 30) in our study and predicts lower scores in MALDI-TOF if members of this clade are not included in the in-house database. Then, our study found that members of clade III displayed unique protein profile with unique spectra from that found in clades I and II.

Although studies on the different conditions and media used to identify bacteria found no significant differences on the MALDI-TOF scores [32, 33], the data in this study showed higher MALDI-TOF scores on PDA than the scores recorded on isolates inoculated on SDAC (Fig. 1). The higher scores from cultures grown on PDA might be because the original database was built with cultures grown on PDA and we may have gotten a different result if the database was built from cultures grown on SDAC. However, these results show that unlike bacteria, *Pythium insidiosum* isolates have significant difference in their scores based on growth media. The result supports the observation made by Erhard *et al.* [16] that, even when a correct identification was made by MALDI-TOF, there might be differences in spectral profile/scores based on fungal growth media. The reading of *P. insidiosum* spectra took a few minutes following an incubation period as short as 3 to 5 days. Overall, this study found the *P. insidiosum* MALDI-TOF in-house spectra accurately identified all interrogated *P. insidiosum* isolates and properly differentiated them from other filamentous fungi.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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