Agr. Nat. Resour. 59 (2025) 590505

AGRICULTURE AND
NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Validation of sampling, isolation and identification technique for *Phytophthora infestans*: Species and mating type

Ade Mahendra Sutejo[a], Arif Wibowo[b,†], Tri Joko[b,†], Ani Widiastuti[b,*]

[a] *Graduate Student of Doctoral Program in Agricultural Sciences, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora 1 Bulaksumur, Yogyakarta, 55281, Indonesia*

[b] *Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora 1 Bulaksumur, Yogyakarta, 55281, Indonesia*

Article Info

Article history:
Received 26 February 2025
Revised 10 June 2025
Accepted 30 July 2025
Available online 29 August 2025

Keywords:
Mating type1,
Phytophthora infestans,
Restriction fragment length polymorphism (RFLP),
SmaI,
Species identification

Abstract

Importance of the work: Sample damage and misleading *Phytophthora infestans* identification are frequent, which could be addressed by the development of suitable validation sampling, isolation and identification techniques.

Objectives: To compare several methods for the sampling, isolation and identification of *P. infestans* using molecular and conventional approaches.

Materials and Methods: Infected potato leaves were used as the source of isolates and for culturing. Whatman paper, DNA extraction kit and V-8 agar medium were used for sampling, DNA isolation and culturing, respectively. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) used specific marker and restriction enzymes for species and mating type identification.

Results: The Whatman paper could be an alternative for DNA sampling due to being more affordable, available and simpler to use than a Flinders Technology Agreement Card. Marker O8-1F/2R showed high sensitivity for species identification among the four markers tested. A 245 bp of PCR product followed by sequencing, blasting and constructing a phylogenetic tree showed high sensitivity in the levels of species, genus and class. The combination pairing method, molecular marker PCR-RFLP (W16-1F/2R fb. BsuRI) and sequencing constituted the best technique for mating type identification, while the SmaI enzyme could be an alternative.

Main finding: Whatman paper could solve the long distance sampling issue. Marker O-1F/2R fb. sequencing was the most accurate marker for species identification, whereas a combination of W16-1F/2R fb. BsuRI or SmaI, sequencing and a pairing test produced better results in mating type determination.

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† Equal contribution.
* Corresponding author.
E-mail address: aniwidiastuti@ugm.ac.id (A. Widiastuti)

https://doi.org/10.34044/j.anres.2025.59.5.05

Introduction

Phytophthora infestans is a late blight disease pathogen on potato and is a heterothallic species with asexual and sexual reproduction between two types of mating (A1 and A2); oospores may be formed from that process (Agrios, 2005). The yield loss due to severe infection of late blight disease in Indonesia has been estimated at 80–90% (Adiyoga, 2009). Thus, comprehensive research is needed to solve the issues associated with late blight management. Frequently, misidentification has been reported when only relying on a morphological character without follow up molecular techniques because morphological attributes, such as lemon-shaped sporangia, are similar to other species from the *Phytophthora* genus such as *P. capsici* (Ristaino et al., 1998; Judelson and Tooley, 2000; Hussain et al., 2005; Hussain et al., 2015).

Several considerations must be taken into account when conducting research in *P. infestans*, including determining, selecting and screening for the best available methods to cover sampling, isolate culturing, DNA isolation and species identification. Obtaining pure culture isolates and achieving accurate species identification remain limiting factors in the laboratory due to the presence of multiple complex pathogens that infect potatoes and can be isolated from field, such as *P. infestans, P. cryptogea, P. drechsleri, P. erythroseptica, P. nicotianae* var. *parasitica* (Trout et al., 1997) and *P. capsici*, which commonly exist on agar plates during isolation and identification.

FTA (Flinders Technology Associates cards for DNA sampling are available as a solution for long-distance *P. infestans* sampling (Gamboa et al., 2019). However, this method is not always accessible in all laboratory settings due to its high cost, creating a need for alternative methods that are affordable, practical and widely accessible. After obtaining samples (DNA or culture), the next task is species and mating type identification. While both molecular and conventional methods are available and published for *P. infestans* species and mating type identification, they have limitations in accuracy and may produce inconsistent results under different laboratory conditions. Therefore, evaluation, validation and optimization of these methods are necessary to select suitable and affordable methods for specific conditions (Brylinska et al., 2018).

The molecular polymerase chain reaction (PCR) method is performed using specific markers designed by Tooley et al. (1997), Trout et al. (1997), Judelson and Tooley (2000), Tooley et al. (2002) and Hussain et al. (2017) for species identification; however, none of these markers has been tested on Indonesian samples. Additionally, Judelson and Tooley (2000) demonstrated that markers specific to *P. infestans* showed promising sensitivity in distinguishing *P. infestans* from several *Phytophthora* species (*P. capsici, P. nicotianae, P. cinnamomi* and others). However, these markers failed to differentiate *P. infestans* from closely related species such as *P. mirabilis, P. sojae and P. katsurae*. Furthermore, these markers have never been validated in Indonesia.

Mating type is an important aspect of *P. infestans* biology due to its role in sexual reproduction, which impacts genetic variability, the pathogen evolution rate and adaptability to environmental changes, including selection pressure from fungicide applications, leading to fungicide resistance (Fry et al., 2015; Li et al., 2017). Several markers have been developed for mating type determination, including the PCR-specific markers developed by Kim and Lee (2002), Kim et al. (2005) and Zhang et al. (2006), as well as PCR- restriction fragment length polymorphism (RFLP) methods using restriction enzymes developed by Judelson et al. (1995) and Brylinska et al. (2018). Studies by Mazakova et al. (2006, 2010), Brylinska et al. (2018) and Dangi et al. (2021) demonstrated different fragment patterns in PCR-RFLP for mating type determination using the W16-1F/2R marker.

Following inconsistent results using markers under different laboratory condition and because some markers have never been used in Indonesia, the objective of the current research was to validate, evaluate and optimize the sampling, isolation and identification techniques that were available for *P. infestans* using local laboratory conditions. This experiment evaluated the sensitivity of three sampling methods, four markers for species identification, three markers for mating type determination and conventional identification methods. A sequencing technique for final validation was conducted to evaluate the sensitivity of each marker.

Materials and Methods

Materials

Samples of *P. infestans* were collected in 2022 from different main cultivation areas of potato such as Wonosobo, Banjarnegara and Magelang, Central Java Province of Indonesia (Table S1).

Sampling, culture purification and DNA isolation

The active part of the lesion (10–25%) adjacent to healthy areas was selected for pathogen isolation and DNA extraction, based on six samples for each sampling method. Three DNA sampling methods were performed in this experiment: 1) DNA collected directly from fresh leaves, where infected leaves were picked and placed directly in a plastic zip lock bag and stored in a cool box during transportation to the laboratory; 2) DNA collected indirectly from leaves via Whatman Paper (WP, #40), where the infected leaves were processed immediately, following the FTA Card Protocol (Gamboa et al., 2019) with adjustment. The leaves were placed on the WP with abaxial leaf contact and then a blunt object (a pen) was used to press the leaf sample part with sporulation until leaf tissue and spores were attached to the WP. Then, the WP was air-dried and stored in a cool box inside plastic zip lock bag; and 3) DNA collected from pure culture which was cultured on V-8 agar medium with infected leaves as the source for pure culture isolation.

After the pure culture isolation, infected leaves (0.5 cm × 0.5 cm) were placed on 10% V-8 agar medium (1 g of calcium carbonate (CaCO3), 0.05 g of beta sitosterol and 15 g of agar) amended with antibiotics (19 mg of Nystatin, 20 mg of Rifamycin, 200 mg of Ampicillin, 100 mg of Benomyl 50%) mixed in 1 L of distilled water, as described by Gamboa et al. (2019). V-8 agar medium containing infected leaves was incubated for 3–5 d at 18–20 ºC in the dark. Mycelial propagation was conducted by transferring pure culture agar plugs aged 10–14 d to pea broth media (120 g of frozen peas dissolved in 1 L of distilled water and autoclaved for 15 min) for 10–14 d. The harvested mycelia were stored at –20 ºC for DNA extraction.

DNA isolation based on the three sampling methods was performed following the manufacturer's instructions for the Geneaid DNA Mini Kit Protocol (Catalog #GP100, 2017; Geneaid Biotech Ltd, Taiwan). The DNA template product (10–50 ng) was stored at –20 ºC.

Species identification and mating type determination

Species identification was performed using four markers: PINTH2-F/R (Hussain et al., 2015); O8-1F/2R; O8-3F/4R; and PinfO8F/R (Judelson and Tooley, 2000). Mating type was identified using three markers: S1a/b (Judelson, 1996); PHYB-1F/2R (Kim & Lee, 2002); and W16-1F/2R, followed by RFLP using BsuRI (Judelson et al., 1995; Brylinska et al., 2018), as listed in Table 1. The PCR reaction was performed in a 10 µL volume, containing Ready Mix from Bioline (5 µL); 1 µL forward/reverse primer; DNA template (1 µl–10 ng) and Milli-Q Water (2 µL).

RFLP was performed by following the manufacturer's instructions. The RFLP reaction was performed in a 30 µL volume, consisting of Milli-Q Water (17 µL), 10× FastDigest Green Buffer (2 µL), PCR product (10 µL) and FastDigest Enzyme (1 µL), with incubation at 37°C (5 min). Agarose gel electrophoresis (1.5%) was used to visualize the product under an ultraviolet transilluminator. A1 was classified by the presence of either one or three DNA fragments (557–600 bp or 557–600 bp, 457 bp, 100 bp), whereas A2 was characterized by two fragments (457bp and 100 bp).

Table 1 List of markers

Marker	Nucleotide	Target (bp)	Purpose	Polymerase chain reaction cycles
PINTH2-F/R	(5'-GGG-GGT-CTT-ACT-TGG-CGG-CG-3'); and (3'-CAA-ACC-GGT-CGC-CAA-CTC-GC-5')	524	Species	Initial (94ºC, 2 min); 30× cycles for Denaturation (94ºC, 1 min), Annealing (60ºC, 1 min), Extension (72ºC, 1 min); and Final Extension (72ºC, 10 min)
O8-1F/2R	(5'-AAGATGATGTTGGATGATTG-3'); and (5'-TGCCTGATTTCTACCTTCT-3')	245	Species	Initial (94ºC, 30 s); 35× cycles for Denaturation (94ºC, 30 s), Annealing (50ºC, 30 s), Extension (72ºC, 1 min); and Final Extension (72ºC, 4 min)
O8-3F/3R	(5'-GAAAGGCATAGAAGGTAGA-3'); and (5'-TAACCGACCAAGTAGTAAA-3')	258	Species	Initial (94ºC, 30 s); 35× cycles for Denaturation (94ºC, 30 s), Annealing (50ºC, 30 s), Extension (72ºC, 1 min); and Final Extension (72ºC, 4 min)
PinfO8F/R	(5'-CAGACTCCAATTTGCACAGTGA-3') and (5'-GATTCGCAATTCCCAACCTT-3')	258	Species	Initial (94ºC, 30 s); 35× cycles for Denaturation (94ºC, 30 s), Annealing (50ºC, 30 s), Extension (72ºC, 1 min); and Final Extension (72ºC, 4 min)
S1a/b	(5′-AGGATTTCAACAA-3′); and (5'-TGCTTCCTAAGG-3′)	1250	Mating A1	Initial (94ºC, 5 min); 35× cycles for Denaturation (94ºC, 1 min), Annealing (50ºC, 1 min), Extension (72ºC, 1 min); and Final Extension (72ºC, 4 min)
PHYB-1F/2R	(5'-GATCGGATTAGTCA GACGAG-3′); and (5'-GCGTCTGC AAGGCGCATTTT-3′)	347	Mating A2	Initial (94ºC, 5 min); 35× cycles for Denaturation (94ºC, 1 min), Annealing (62ºC, 1 min), Extension (72ºC, 1 min); and Final Extension (72ºC, 7 min)
W16-1F/2R	(5'-AACACGCACAAGGCATATAAATGTA-3'); and (5'-GCGTAATGTAGCGTAACAGCTCTC-3')	557-600	Mating fb. RFLP	Initial (94ºC, 5 min); 35× cycles for Denaturation (94ºC, 1 min), Annealing (60ºC, 1 min), Extension (72ºC, 1 min); and Final Extension (72ºC, 4 min)

RFLP = restriction fragment length polymorphism.

Morphological identification was performed under microscope to observe specific characteristics such as lemon shaped sporangia, hyphae and oospores on pure culture, which was multiplied in 15% V-8 agar medium with 14–21 d of incubation. The pairing test for mating type identification was conducted by pairing and crossing two isolates in a Petri dish containing 10% V-8 agar medium as described by Mazakova et al. (2006) and Gamboa et al. (2019). Two agar plugs (8 mm) containing both isolates were placed onto a Petri dish with 6 mm distance and incubated for 10–14 d. Mating type was determined by observation of hyphae contact and oospore formation under a microscope.

DNA sequencing and phylogenetic tree

DNA sequencing was performed in the Integrated Laboratory for Research and Testing (ILRT), Gadjah Mada University with Sanger sequencing method on an Applied Biosystem 3500 Genetic Analyzer 2500 (ThermoFisher Scientific, USA). Bioinformatic software (Sequence Scanner (Life Technologies, USA), BioEdit (v.5.0.6; (https://bioedit.software.informer.com/7.2/, USA) and MEGA 11 (https://www.megasoftware.net/, USA were used for nucleotide analysis and phylogenetic tree construction. Blasting on the NCBI website was performed to compare with the reference (*P. infestans* strain T30-4) and related species in GenBank such as *P. infestans* multiple strains, *P. palmivora, P. andina, Verticillium dahlia, Fusarium solani* and *Alternaria solani*.

In silico restriction fragment length polymorphism and alternative restriction enzyme

Online software (Restriction Mapper v.3; https://restrictionmapper.org/) was used to perform the *in silico* RFLP, using some alternative restriction enzymes—BsuRI (GGCC) and SmaI (CCCGGG)—to find any working enzyme, as well as BsuRI that could have a cleavage site on a nucleotide sequence to distinguish between the A1 and A2 mating types.

Results

Sampling and DNA isolation

In total, 16 samples were collected from the field consisting of 14 DNA samples (6 using the direct isolation method, 6 using the WP method and 2 using the pure culture method for DNA and culture isolates of *P. infestans*). The three DNA isolation methods produced different levels of effectivity after molecular verification. Direct isolation produced 5 of 6 samples as positive for *P. infestans*, the WP method produced 4 of 6 samples and the pure culture method produced 2 of 2 samples (Fig. S1).

Species identification: Molecular and morphological technique

Of the four markers used for species identification, only three markers successfully amplified DNA template (PINTH2-F/R, O8-1F/2R and O8-3F/2R with DNA fragments of 524 bp, 245 bp and 258 bp respectively (Fig. S1). In contrast, PinfO8F/R failed to produce amplification despite multiple PCR attempts and DNA template optimization. Morphological identification of two isolates showed lemon-shaped sporangia on isolate #42, but #41 only formed an aggregate of hyphae and structures like sporangia that were "lemon-shaped like" (Fig. 1). Molecular testing using O8-1F/2R produced a specific DNA band (245 bp) and both isolates were confirmed as *P. infestans* (Fig. S2). The absence of DNA bands in the three other isolates (#2, #53, #55) was attributed to either negative DNA during isolation or unsuccessful DNA isolation, possibly due to failed DNA extraction from leaves or insufficient DNA capture on the WP card.

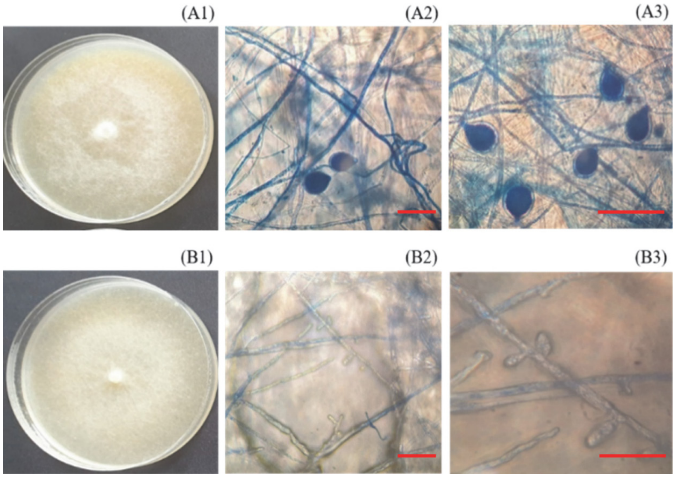


Fig. 1 Morphological characteristics of pure culture isolate (macroscopic and microscopic) resulting from infected leaf isolation: (A1, A2, A3) pure culture of *P. infestans* on potato agar media for isolate #41 and lemon-shaped sporangia; (B1, B2, B3) pure culture of *P. infestans* on potato agar media for isolate #42 and hyphae with lemon-shaped sporangia-like, where magnification under microscope = 40 × for A2 and B2 and 60× for A3 and B3, scale bars = 100 µm with average of 10 sporangia measured in one view

Species identification: DNA sequencing and phylogenetic tree

Three DNA samples (#19, #25 and #28), representing the direct isolation method and the Whatman Paper (WP) method, were selected for sequencing. Blast analysis of the nucleotides amplified using PINTH2-F/R and O8-3F/2R had high sequence identity and homology (99%), not only with *P. infestans* strain T30-4 as the standard reference, but also with other species such as *P. andina*, *P. mirabilis, P. sojae and P. agathidicida*. On the other hand, the nucleotide sequence from O8-1F/2R only had high identity and homology (98%) with *P. infestans* strain T30-4 and clone PI-BAC-11A5 (data not shown). A phylogenetic tree was constructed by comparing the nucleotide sequences of samples with reference sequences from GenBank. Marker PINTH2-F/R grouped sample #25 in one clade with all species of *Phytophthora* genus (*P. infestans T30-4, P. palmivora, P. mirabilis, P. andina* and others); however, the closest was with *P. palmivora*. Samples #19 and #28 created a new sub-clade but the genetic distance still similar to others in the genus *Phytophthora* and from the same ancestor (Fig. 2A). The same condition occurred for O8-3F/4R, where samples #19, #25 and #28 were not only close to *P. infestans* clone PI-BAC-11A5, but also in one clade with *P. sojae* and *P. agathidicida* (Fig. 2C). Marker O8-1F/2R had three samples that formed one clade with *P. infestans* clone PI-BAC-11A5 and *P. infestans* strain T30-4, whereas *P. palmivora, F. solani, A. solani* and *V. dahlia* were in different clades with substantially different genetic distances and strengthened by the blasting results based on the NCBI website (Fig. 2B). Furthermore, culture isolates #41 and #42 were sequenced only using marker O8-1F/2R to verify the PCR result and had 100% similarity with the *P. infestans* sequence in GenBank (Fig. S4; Fig. S5).

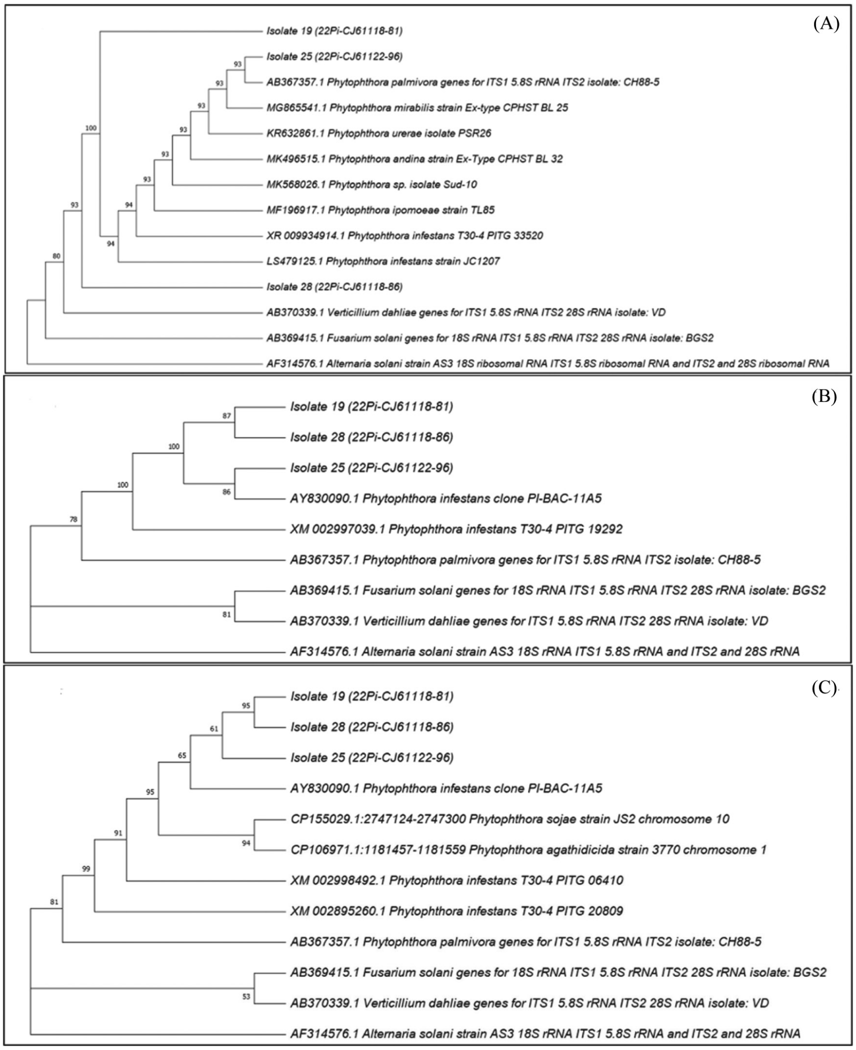


Fig. 2 Phylogenetic analysis based on DNA sequencing using primers: (A) PINTH2-F/R; (B) O8-1F/2R; (C) O8-3F/4R, with sequence of *Phytophthora palmivora* as ingroup and outgroup consists of *Verticillium dahliae*; *Fusarium solani* and *Alternaria solani*, where tree designed using maximum parsimony method based on Kimura-2 parameter model (1,000 bootstraps replicates) in MEGA 11 software (https://www.megasoftware.net/, USA.

Mating type identification: Molecular marker, pairing test and RFLP

For mating type identification, only DNA samples positive for *P. infestans* were selected: five samples from direct isolation (#3, #23, #25, #27, #28) and four samples from Whatman paper (#14, #17, #19, #58). Among the three mating type markers tested, only W16-1F/2R successfully amplified DNA bands (557–600 bp) in three DNA samples (#19, #25, #28) and the RFLP analysis produced two DNA bands (557–600 bp and 457 bp) for the A1 mating type (Fig. S3). Samples #3, #27 and #28 were not cleaved by BsuRI, while only samples #23 and #25 could be cleaved and were grouped as the A1 type. The S1a/b and PHYB-1F/2R markers failed to amplify any DNA (data not shown).

Mating type identification using marker W16-1F/2R for two culture isolates (#41 and #42) and one positive control for A1 mating type (#58) revealed the A1 mating type for isolates #42 and #58, though with different DNA band lengths and numbers after BsuRI cleavage (Fig. S3). Since isolate #41 could not be determined using the molecular technique, the conventional method (pairing test) was used for further identification. Isolate #42, confirmed as the A1 mating type by molecular analysis, served as the reference isolate for the pairing method (Fig. 3). When isolate #42 (A1 type) was crossed with the unidentified isolate #41, no oospore formation was observed on V8-agar medium, which would have indicated fusion between the two different mating types. Therefore, it was concluded that isolates #41 and #42 were of the same A1 mating type.

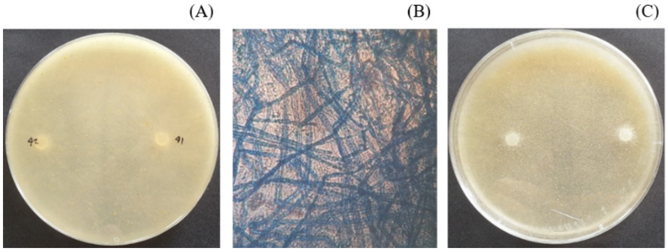


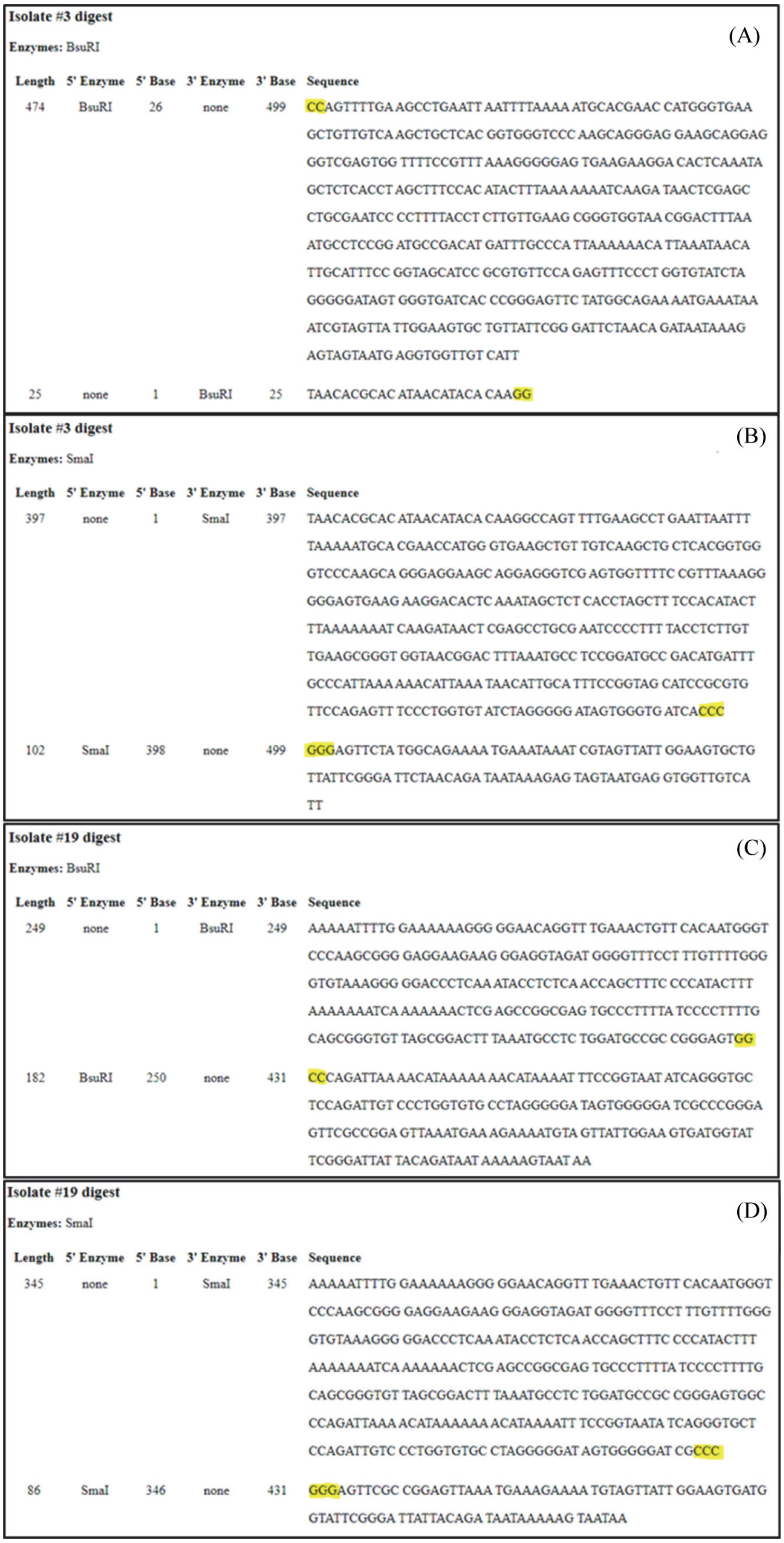
Fig. 3 Pairing test of *P. infestans* on V-8 agar medium: (A) bottom view of pairing test for 2 isolates (#41 left, #42 right) with no connection of hyphae indicating no oospores formed; (B) hyphae and oospore identification microscopically (error bar = 100 µm); (C) upper view of for 2 isolates (#41 left, #42 right)

Mating type identification: DNA sequencing and phylogenetic tree

Three isolates (#3, #19, #25) were taken as representative for Sanger DNA sequencing using marker W16-1F/2R. Consensus of DNA sequences was used for blasting using the NCBI website to evaluate the level of identity and homology with the GenBank sequence and also for construction of a phylogenetic tree. Three isolates from the blasting nucleotide sequence had 70% similar identity and homology with the GenBank sequences of *P. infestans* and *P. andina* (Fig. S6). In addition, it was strengthened by the result of the phylogenetic tree where three isolates were grouped in one clade with the *P. andina* W2 genomic sequence and the same ancestor as *P. infestans* MP1358, with a close genetic distance.

Mating type identification: In silico restriction fragment length polymorphism

In silico RFLP was performed to verify the unidentified mating types on agarose gel (comparing uncut and cut sequences) and to explore alternative restriction enzymes. The *in silico* analysis showed that BsuRI could cleave the PCR product of sample #3 (499 bp) at the 25 bp site, producing two DNA bands (474 bp and 25 bp), which were not identifiable on agarose gel. This result suggested that sample #3 could be either the A1 type (due to proximity to 557 bp) or the A2 type (due to proximity to 457 bp). Sample #19 (432 bp) had two DNA bands (249 bp and 182 bp) after cleavage at the 249 bp site, differing from the RFLP pattern observed on agarose gel which had two bands (600 bp and 457 bp), leading to its A1 type classification (Fig. 4). The W16-1F/2R marker could detect multiple alleles, where one allele contained the restriction site while others lacked it, resulting in an additional DNA fragment (557–600 bp) above the two expected fragments (457 bp and 100 bp) on agarose gel. For the SmaI enzyme, one restriction site was identified in both sample #3 (397 bp) and #19 (345 bp). SmaI digestion of sample #3 yielded two DNA bands (397 bp and 102 bp), while sample #19 produced two bands (345 bp and 86 bp).



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Isolate #3 digest
Enzymes: BsuRI

Length  5' Enzyme  5' Base  3' Enzyme  3' Base  Sequence
 474     BsuRI      26       none       499     CCAGTTTTGA AGCCTGAATT AATTTTAAAA ATGCACGAAC CATGGGTGAA
                                                GCTGTTGTCA AGCTGCTCAC GGTGGGTCCC AAGCAGGGAG GAAGCAGGAG
                                                GGTCGAGTGG TTTTCCGTTT AAAGGGGGAG TGAAGAAGGA CACTCAAATA
                                                GCTCTCACCT AGCTTTCCAC ATACTTTAAA AAAATCAAGA TAACTCGAGC
                                                CTGCGAATCC CCTTTTACCT CTTGTTGAAG CGGGTGGTAA CGGACTTTAA
                                                ATGCCTCCGG ATGCCGACAT GATTTGCCCA TTAAAAAACA TTAAATAACA
                                                TTGCATTTCC GGTAGCATCC GCGTGTTCCA GAGTTTCCCT GGTGTATCTA
                                                GGGGGATAGT GGGTGATCAC CCGGGAGTTC TATGGCAGAA AATGAAATAA
                                                ATCGTAGTTA TTGGAAGTGC TGTTATTCGG GATTCTAACA GATAATAAAG
                                                AGTAGTAATG AGGTGGTTGT CATT

  25     none        1       BsuRI       25     TAACACGCAC ATAACATACA CAAGG
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Isolate #3 digest
Enzymes: SmaI

Length  5' Enzyme  5' Base  3' Enzyme  3' Base  Sequence
 397     none        1       SmaI       397     TAACACGCAC ATAACATACA CAAGGCCAGT TTTGAAGCCT GAATTAATTT
                                                TAAAAATGCA CGAACCATGG GTGAAGCTGT TGTCAAGCTG CTCACGGTGG
                                                GTCCCAAGCA GGGAGGAAGC AGGAGGGTCG AGTGGTTTTC CGTTTAAAGG
                                                GGGAGTGAAG AAGGACACTC AAATAGCTCT CACCTAGCTT TCCACATACT
                                                TTAAAAAAAT CAAGATAACT CGAGCCTGCG AATCCCCTTT TACCTCTTGT
                                                TGAAGCGGGT GGTAACGGAC TTTAAATGCC TCCGGATGCC GACATGATTT
                                                GCCCATTAAA AAACATTAAA TAACATTGCA TTTCCGGTAG CATCCGCGTG
                                                TTCCAGAGTT TCCCTGGTGT ATCTAGGGGG ATAGTGGGTG ATCACCC

 102     SmaI       398      none       499     GGGAGTTCTA TGGCAGAAAA TGAAATAAAT CGTAGTTATT GGAAGTGCTG
                                                TTATTCGGGA TTCTAACAGA TAATAAAGAG TAGTAATGAG GTGGTTGTCA
                                                TT
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Isolate #19 digest
Enzymes: BsuRI

Length  5' Enzyme  5' Base  3' Enzyme  3' Base  Sequence
 249     none        1       BsuRI      249     AAAAATTTTG GAAAAAAGGG GGAACAGGTT TGAAACTGTT CACAATGGGT
                                                CCCAAGCGGG GAGGAAGAAG GGAGGTAGAT GGGGTTTCCT TTGTTTTGGG
                                                GTGTAAAGGG GGACCCTCAA ATACCTCTCA ACCAGCTTTC CCCATACTTT
                                                AAAAAAATCA AAAAAACTCG AGCCGGCGAG TGCCCTTTTA TCCCCTTTTG
                                                CAGCGGGTGT TAGCGGACTT TAAATGCCTC TGGATGCCGC CGGGAGTGG

 182     BsuRI      250      none       431     CCCAGATTAA AACATAAAAA AACATAAAAT TTCCGGTAAT ATCAGGGTGC
                                                TCCAGATTGT CCCTGGTGTG CCTAGGGGGA TAGTGGGGGA TCGCCCGGGA
                                                GTTCGCCGGA GTTAAATGAA AGAAAATGTA GTTATTGGAA GTGATGGTAT
                                                TCGGGATTAT TACAGATAAT AAAAAGTAAT AA
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Isolate #19 digest
Enzymes: SmaI

Length  5' Enzyme  5' Base  3' Enzyme  3' Base  Sequence
 345     none        1       SmaI       345     AAAAATTTTG GAAAAAAGGG GGAACAGGTT TGAAACTGTT CACAATGGGT
                                                CCCAAGCGGG GAGGAAGAAG GGAGGTAGAT GGGGTTTCCT TTGTTTTGGG
                                                GTGTAAAGGG GGACCCTCAA ATACCTCTCA ACCAGCTTTC CCCATACTTT
                                                AAAAAAATCA AAAAAACTCG AGCCGGCGAG TGCCCTTTTA TCCCCTTTTG
                                                CAGCGGGTGT TAGCGGACTT TAAATGCCTC TGGATGCCGC CGGGAGTGGC
                                                CCAGATTAAA ACATAAAAAA ACATAAAATT TCCGGTAATA TCAGGGTGCT
                                                CCAGATTGTC CCTGGTGTGC CTAGGGGGAT AGTGGGGGAT CGCCC

  86     SmaI       346      none       431     GGGAGTTCGC CGGAGTTAAA TGAAAGAAAA TGTAGTTATT GGAAGTGATG
                                                GTATTCGGGA TTATTACAGA TAATAAAAAG TAATAA
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Fig. 4 *In-silico* restriction fragment length polymorphism of polymerase chain reaction product (primer W16-1F/R) using two isolates (#3 and #19), where isolate #3 identified *P. infestans*, A1 mating type and 499 bp of sequencing product and isolate #19 identified *P. infestans*, A1 mating type and 431 bp of sequencing product: (A) BsuRI enzyme with site “GGCC” at cutting position 25 bp; (B) SmaI enzyme with site “CCCGGG” at cutting position 397 bp; (C) BsuRI enzyme with site “GGCC” at cutting position 249 bp; (D) SmaI enzyme with site “CCCGGG” at cutting position 345 bp

Discussion

Finding the best sampling and isolation methods is a crucial step for *P. infestans* research and a key factor for success. This experiment showed that the best sampling and isolation method used WP (#40), which would be suitable for long-distance sampling, when keeping fresh leaf samples is an issue. Based on the successful rate of DNA isolation, WP was comparable with the direct isolation method, with percentages of 66% versus 83%, respectively.

Molecular methods offer several advantages over conventional plant disease diagnosis being less time-consuming as there is no need for culturing prior to detection and they provide high sensitivity and rapid results and are versatile (Henson and French, 1993). In contrast, conventional methods for sampling, pure culture isolation and identification are time-consuming, less accurate, labor-intensive, require considerable expertise, depend on the presence of sporangia as a specific attribute to differentiate *Phytophthora* species from others and must have isolate references for mating type identification. However, culture isolation is still useful for obtaining pure cultures for multiple purposes such as DNA isolation, culture identification, pairing tests, and bioassays. Validation of this method requires its combination with molecular identification to obtain accurate results.

The three DNA isolation methods (direct, WP card, culture isolation) had high accuracy in DNA isolation, which aligned with methods available in the literature, where common DNA isolation methods involve direct extraction from leaves or through culture, as conducted by Mazakova et al. (2006), Mazakova et al. (2010), Utami and Ambarwati (2017) and Gamboa et al. (2019). However, the use of both the above methods depends heavily on the quality of samples obtained, the extent of damage during transportation and the ability to isolate *P. infestans* culture, which requires more time and complex preparation compared to using a WP card (Gamboa et al., 2017). The WP method is a modification of the FTA card designed by EuroBlight that can serve as an alternative for direct DNA isolation, being more affordable and readily available. The WP method can solve the problem of sample damage during transportation from field to laboratory, where successful extraction of DNA from plant tissues generally has been most successful from freshly field-collected tissue (Siegel et al., 2017)

Three markers (PINTH-2-F/R, O8-1F/2R, O8-3F/4R) produced specific DNA bands on 1.5% agarose gel (Fig. S1), whereas PinfO8F/R did not, even after multiple times of optimization, so that it might be concluded that O8-1F/2R with the 245 bp product was the most sensitive and selective marker to identify *P. infestans* including the other two markers based on Sanger sequencing, blasting nucleotide sequencing on NCBI and the phylogenetic tree. It could identify *P. infestans* and distinguish it from other species (in the same or a different genus). Furthermore, blasting and the phylogenetic tree proved that this marker had high specificity on a sequence target because there were abundant sequence targets on repetitive DNA (approximately 14,000 copies per nucleus) and it was 100 times more sensitive than other such as internal transcribed spacer sequencing (Judelson and Tooley, 2000). The markers O8-1F/2R and O8-3F/4R were designed by Judelson and Tooley (2000) based on families of highly repeated DNA and demonstrated high specificity in identifying *P. infestans* and distinguishing it from other species. However, based on other research, the O8-3F/4R marker still detected other species such as *P. katsurae* (Judelson and Tooley, 2000). The current research showed that the O8-1F/2R marker was more specific than O8-3F/4R, which correlated with findings by Judelson and Tooley (2000).

PINTH-2F/R is a specific marker designed by Hussain et al. (2015) based on sequence characterized amplified regions, which was selected using the random amplified polymorphic DNA method for specific alleles of *P. infestans*. This marker appears to be not sufficiently specific for use in Indonesia as it cannot clearly differentiate between other *Phytophthora* genus members, such as *P. ipomoeae, P. mirabilis* and *P. andina*, as these three species were not used as checks during the marker design process.

In the current study, W16-1F/2R was the only marker that could amplify the DNA template (S1a/b and PHYB-F/R could not). The BLAST analysis on the NCBI website, using the S1a/b nucleotide marker with the oomycetes template sequence as the target, prodcued unspecific and multiple PCR products with different sizes on *P. infestans* strain T30-4. Similarly, the PHYB-F/R nucleotide BLAST results were less specific, as they not only amplified *P. infestans* sequences randomly but also those of other species both within and outside the *Phytophthora* genus members (BLAST results not shown). In this experiment, both markers could not be optimized and used in PCR method.

Sequencing of samples #3, #19 and #25 using marker W16-1F/R prodcued moderate similarity (70%) with *P. infestans* and *P. andina* sequences in GenBank. *P. andina* is a species formed through hybridization between *P. infestans* and another unknown *Phytophthora* species (Goss et al., 2011). This moderate similarity might be attributed to diverse alleles (W1–W8) at each locus responsible for mating type determination (Brylinska et al., 2018). Additionally, the mating type locus of *P. infestans* may be complex, encoding not only functions required for mating type but also for the system of balanced lethals, suggesting potential selection or variation events (Judelson et al., 1995). The W16-1F/R marker sequencing and phylogenetic analysis served as supporting data to confirm species identification, verifying that all collected samples were *P. infestans*. These sequences will be further analyzed to identify restriction enzyme sites (BsuRI and SmaI).

The RFLP analysis for mating type identification classified most samples as A1, except for three samples (#3, #27, #28), which could not be digested by the BsuRI enzyme. This failure may have been due to the absence of a BsuRI recognition site in the PCR product or because the isolates belong to the US-1 genotype, which is known to be undetectable by this marker (Brylińska et al., 2018). Further investigation using Sanger sequencing for samples #3 and #19 produced different DNA bands after *in silico* cleavage using BsuRI, likely due to the effectiveness of Sanger sequencing producing the whole target nucleotide sequence. The different sizes and numbers of DNA bands after RFLP analysis on samples #42 and #52 indicated that #42, with one band (600 bp), contained homozygous alleles, while #52, with two DNA bands (557 bp, 457 bp), was grouped with species having heterozygous alleles (Brylinska et al., 2018).

Judelson et al. (1995) developed a PCR-RFLP marker (W16-1F/2R followed by RFLP BsuRI) for mating type identification that provided clear DNA fragments from restriction enzyme digestion to distinguish A1 and A2 mating types. Typically, A2 was homozygous, while A1 was heterozygous with only one allele's DNA fragment being cleaved by the restriction enzyme (Mazakova et al., 2006). However, in some cases, the A1 mating type was homozygous with no allele cleavage (Mazakova et al., 2010). Since W16-1F/2R was designed using isolates from outside Indonesia, nucleotide variations were expected when compared with Indonesian isolates having unique or native genotypes (Dangi et al., 2021). Isolates of *P. andina* and *P. infestans* US-1 genotype could not be identified as either the A1 or A2 mating type using PCR-RFLP due to their distinct allele compositions: US-1 (W1) and *P. andina* (W1, W2), which are known to have potential for selection and variation (Judelson et al., 1995; Brylinska et al., 2018). This difference resulted in the absence of the BsuRI recognition site at position 101 bp, which typically is present in the A1 mating type (Brylinska et al., 2018). It can be concluded that the failure of PCR-RFLP in some cases was caused by either the uniqueness of Indonesian genotypes or their belonging to the US-1 lineage.

From the current experiment, it was recommended and concluded that the Whatman paper method for DNA sampling and isolation was promising, especially when field locations were far from the laboratory. This finding could solve current issues with long-distance sampling from potato fields to laboratory analysis, which usually takes more than 1 d and risks improper preservation or sample degradation (rotting) before laboratory processing. In addition, this Whatman paper method could help students or young scientists by providing more affordable and accessible tools than the expensive FTA cards. The Whatman paper and direct isolation methods may face challenges related to DNA purity after isolation due to possible contamination from plant tissue and debris. On the other hand, DNA isolation from culture provides higher purity since only *P. infestans* DNA is isolated.

The O8-1F/2R marker proved to be the most specific marker for species identification. Thus, it was concluded and recommended that a combination of the W16-1F/2R marker, pairing method and sequencing technique provided the best method for mating type identification and could now be used as the standard identification method for *P. infestans* species in Indonesia. The SmaI enzyme could be an alternative to or used in combination with BsuRI to achieve higher specificity during mating type determination. However, further investigation using agarose gel electrophoresis with more DNA templates is needed to determine its specificity.

Conflict of Interest

There are no conflicts of interest.

Acknowledgements

This manuscript is a part of Ph.D. research conducted by the first author under the supervision of the corresponding and co-authors. There was no external support funding for this research.

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