Comparison of three microsatellite analysis methods for detecting genetic diversity in *Phytophthora sojae* (Stramenopila: Oomycete)

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Abstract Analysis of an organism’s genetic diversity requires a method that gives reliable, reproducible results. Microsatellites are robust markers, however, detection of allele sizes can be difficult with some systems as well as consistency among laboratories. In this study, our two laboratories used 219 isolates of *Phytophthora sojae* to compare three microsatellite methods. Two capillary electrophoresis methods, the Applied Biosystems 3730 Genetic Analyzer and the CEQ 8000 Genetic Analysis system, detected an average of 2.4-fold more alleles compared to gel electrophoresis with a mean of 8.8 and 3.6 alleles per locus using capillary and gel methods, respectively. The two capillary methods were comparable, although allele sizes differed consistently by an average of 3.2 bp across isolates. Differences between capillary methods could be overcome if reference standard DNA genotypes are shared between collaborating laboratories.

Keywords Alleles · Capillary electrophoresis · Microsatellites · Pathogenic diversity · *Phytophthora sojae*

Introduction

*Phytophthora sojae* Kauf. & Gerd. is an important pathogen that causes *Phytophthora* root and stem rot on soybeans worldwide (Hartman et al. 1999). High levels of pathogenic variation within the species occurs and more than 200 pathotypes of this pathogen have been reported and more continue to emerge (Dorrance and Grunwald 2009). Interestingly, little is known about how this variation occurs and the diversity within endemic populations. Oomycetes are diploid organisms whose life cycle includes both asexual and sexual reproduction. Organisms that reproduce asexually tend to exhibit a high degree of clonality, with few genotypes present at high frequencies, while sexually reproducing organisms usually have a higher degree of genotypic diversity (Chen and McDonald 1995). Due to its homothallic nature, *P. sojae* is considered an essentially clonally propagating
organism (Gijzen and Qutob 2009). Previous studies have indicated that little, if any, heterozygosity is present in populations (Förster et al. 1994).

As with many soil borne pathogens P. sojae has limited means of dispersal, thus gene flow is thought to be limited (McDonald and Linde 2002). It has been suggested however, that a large reservoir of genetic diversity exists in P. sojae populations (Hobe 1981), albeit, only a few studies have attempted to characterize this diversity using genetic markers (Dorrance and Grunwald 2009; Drenth et al. 1996; Förster et al. 1994; Gally et al. 2007; Meng et al. 1999). Co-dominant microsatellites or simple sequence repeats (SSRs) are suited for population-genetic studies, since they enable quantification of putative heterozygotes which enables estimation of naturally occurring outcrossing. SSRs for P. sojae were previously identified from transcript sequences (Garnica et al. 2006), as well as from genome sequences (Tyler et al. 2006). Schena et al. (2008) identified 12 SSRs that could be used on a restricted number of Phytophthora species related to P. sojae. In another study, 21 SSRs developed from P. sojae race 2 sequences, were used in a preliminary study on 33 isolates from Ohio (Dorrance and Grunwald 2009). An average of 2.5 alleles per locus and 0.015 observed heterozygosity was found, as well as, 100% of loci deviated from Hardy–Weinberg equilibrium (Dorrance and Grunwald 2009).

Reproducibility of molecular markers has been tested in laboratory networks (Jones et al. 1997). Random amplified polymorphic DNAs (RAPDs) have proven difficult to reproduce from one laboratory to the next. Amplified fragment length polymorphisms (AFLPs), although reproducible, result in single-band differences between labs. While SSRs are considered robust markers, differences in allele sizing can appear across laboratories depending on the analysis system used (Jones et al. 1997; Weeks et al. 2002; Widmark et al. 2011). The estimated allele size is not only dependent on the number of nucleotides but also on the mobility of the fragment in the electrophoresis (Weeks et al. 2002; Widmark et al. 2011), the type of fluorescent label used, the distance of the allele from the standard used (Jones et al. 1997), and the use of different instruments using different software (Weeks et al. 2002). Nevertheless, these discrepancies could be minimized if reference standard DNA genotypes were shared between collaborating laboratories. Our objective was to compare three microsatellite methods across two laboratories, standardize measurements and name the alleles detected.

Materials and methods

A total of 219 isolates of P. sojae were evaluated in this study. Genomic DNA was extracted from mycelium using either a modification of the cetyltrimethylammonium bromide (CTAB) procedure (Dorrance et al. 1999), or a rapid extraction protocol (Zelaya-Molina et al. 2011). Twenty-five microsatellite primer pairs were identified (Dorrance and Grunwald 2009; Schena et al. 2008) and amplicons were separated on 4% agarose gels (Supplementary Table 1).

Alleles which differ in many base pairs of length can be readily resolved on agarose gels but single repeat differences are difficult to separate, especially in SSRs with small size repeats (Jones et al. 1997). Eight SSRs were selected for further comparisons using two capillary electrophoresis microsatellite analysis methods (Table 1). The eight SSRs included 2, 3, 4, 5, and 6 bp repeats and were chosen based on amplification success and the highest number of alleles (band sizes) encountered per locus using the gel method. The Applied Biosystems 3730 Genetic Analyzer (ABI) was used at Iowa State University, and the CEQ 8000 Genetic Analysis system (CEQ) at The Ohio State University. Although each laboratory used their own isolates of P. sojae, DNA of 17 isolates was shared between laboratories to allow for comparison of allele sizes using the different methods and dyes.

Primer synthesis for the ABI used universal fluorescent labeling (standard phosphoramidite chemistry). For the forward primers PS01, PS16, PS24, and PS33, 6-carboxy-fluorescine (FAM) dye was used, while hexachloro-6-carboxy-fluorescine (HEX) dye in forward primers PS05, PS10, PS12, and PS29. Amplification was performed in a 96-well Eppendorf Mastercycler thermal cycler (Hamburg, Germany) in 15 µl with 0.2 mM dNTP mixture, 2.5 mM MgCl₂, 1X Go Taq Hot Start Colorless Master Mix buffer, 0.08 units Go Taq Hot Start DNA polymerase (Promega Inc., Madison, WI), 0.45 µM of each primer, and 1 µl (100 ng) DNA template. The thermal cycler was programmed for an initial step at 85°C for 2 min, denaturalization step at 94°C for
95 s, then 24 cycles at 52°C for 1 min, 72°C for 30 s, then 52°C for 1 min and 72°C for 30 min. A 96-capillary Applied Biosytem 3730 Genetic Analyzer set up to run samples labeled with these dyes and a GeneScan 500 ROX size standard (Applied Biosystem, Foster City, CA) was used. GeneMapper Software 4.0 (Applied Biosystem, Foster City, CA) was used to size the alleles to the nearest base pair.

For the capillary electrophoresis with the CEQ, all forward primers were designed with a M13(-21) (TGTTAAAAACGACCGCCAGT) tail at the 5' -end and used a universal WellRed labeled M13(-21) primer as a nested primer (Schuelke 2000). PCR conditions were modified when using universal labeled M13(-21) primer. Two PCR reactions were carried out with the same reverse primer. However, in the first run, M13(-21) tagged forward primer was used while universal M13(-21) labeled primer was used in the second PCR. Amplification was performed in a 96-well DNA Engine Tetrad 2 Peltier Thermal Cycler (Biorad, USA) in 25 μl with 0.2 mM dNTP mixture, 2 mM MgCl2, 1X Go Taq Flexi Colorless buffer, 1 units Go Taq Flexi DNA polymerase (Promega Inc., Madison, WI), 0.2 μM of each primer, and 1 μl (20 ng) DNA template. The thermal cycler was programmed for an initial step at 95°C for 5 min, denaturalization step at 94°C for 30 s, then 24 cycles at annealing temperature for 30 s, 72°C for 30 s and 72°C for 10 min. For annealing temperature, 58 and 56°C were used in the first and second PCRs, subsequently. For comparison, forward primers of two primer sets, PS01 and PS05 were directly labeled with WellRed and used with same PCR protocol and conditions except only one PCR reaction was used with an annealing temperature 58°C. PCR products were electrophoresed on a CEQ 8000 Genetic Analyzer (Beckmann Coulter) with either 400 or 600 bp size standard depending on the size of the fragment. Allele sizes were determined using the software provided by the Genetic Analyzer. Eighteen bases were removed from the allele data from the primer pairs that used universal labeled M13 (-21) primer.

Results

The 190 *P. sojae* isolates analyzed with 25 SSRs using gel method resulted in a total of 75 alleles, ranging from 2 to 6 alleles per locus, with an average of three (Supplementary Table 1). The agarose gel

| Locus | Super-contiga | Repeat motifb | Primer sequences (5’-3’) | Actual size isolate P6497 (bp) | GeneBank accession no. |
|-------|--------------|--------------|--------------------------|-------------------------------|------------------------|
| PS01  | 9            | (GACACT)20   | F: TGATGGGAGATGGCTACAGG  | 419                          | EF667485               |
| PS05  | 3            | (TCAG)34     | F: GAAAACATCAACCGAACAACG | 263                          | EF667486               |
| PS10  | NIc          | (CAAAC)27    | F: CGACGAAGAGGACAACTTGA  | 228                          | EF667489               |
| PS12  | 32           | (GCTGT)23    | F: GCTGCTTTGGGCTTGTG     | 306                          | EF667490               |
| PS16  | 20           | (ATTAT)20    | F: AATCTGATTTGGACGCTGTG  | 469                          | EF667491               |
| PS24  | 36           | (CT)16 + (CT)3 | F: GTCACTTCCCTCGTCGACAG  | 252                          | EF667495               |
| PS29  | NI           | (TAC)15      | F: CCACGGAGCGAGGTAGAGG   | 273                          | EF667499               |
| PS33  | 2            | (AT)15       | F: CTAGCTAGCCGCTCGTGTG   | 267                          | EF667501               |
electrophoresis method detected 86% of the bands which differed by more than 5 bp. In contrast, the capillary methods were more sensitive and able to separate PCR products that differed by only 2 bp. For the eight selected SSRs (Table 1) that were assayed in this study, a total of 70 alleles, with an average of 8.8 alleles per locus, were detected using the capillary electrophoresis methods from 219 *P. sojae* isolates compared to a total of 29 alleles, with an average of 3.6 alleles per locus, using the gel method (Table 2). Thus the capillary electrophoresis methods were able to detect 2.4-fold more alleles on average than the gel method. The number of alleles detected per locus using the three methods however, varied depending on the specific SSR. For PS29 which has 3 bp repeat, the number of alleles detected by the three methods was identical (Table 2). In contrast, 14 alleles were detected for PS05 (5 bp repeat) with the capillary methods, while only five were detected using the gel method (Table 2).

The two capillary methods had similar results in both labs. For the reference isolates shared between labs, the same alleles were detected although allele sizes for all of the primer pairs differed from 1 to 13 bp with an average of 3.2 bp (Table 2). The longer the PCR product size, the bigger the base pair differences between the ABI system and the CEQ. Specifically, PCR products over 400 bp differed by 5–13 bp (PS01 and PS16), however, these base pair differences were consistent across isolates (Table 2). Similarly, PCR products of less than 400 bp differed consistently, but by only 1 or 2 bp between the two capillary methods.

**Discussion**

When studying genetic variation of an organism, it is important to use a method that gives reliable and reproducible results that can be repeated from laboratory-to-laboratory and across genotyping systems. In this study, the ABI and CEQ system detected more alleles for seven of eight SSRs than an agarose gel electrophoresis method. Although, the number of alleles detected by the capillary systems were similar when the CEQ and ABI systems were compared, an average variation of 3 bp for each amplified fragment was observed, with larger variations in size between methods being observed as the size of the PCR product increased. While this was not unexpected, since migration of the PCR products is affected by the labeling and the capillary system (Jones et al. 1997; Weeks et al. 2002; Widmark et al. 2011), it does mean that representative standard isolates should be included in similar assays to ensure lab-to-lab comparisons.

The methods used in this study vary considerably in cost. In addition to the equipment and the requirement of core lab facilities, both the ABI system and CEQ require fluorescent labeled primers which have additional costs. At the time of this study, each sample analyzed on a gel cost approximately $0.62 (using Agarose wide range/standard 3:1 and rapid-run agarose buffer), compared to $1.48 and $1.65 per sample for 96 samples for FAM and HEX dye, respectively, for the ABI method (includes cost of fluorescent label and genotyping). For one sample, the cost of using the CEQ machine for genotyping is $1.75 at Ohio State University. The prices are different in the two genomic core facilities, making sample cost significantly different between them. When the universal primer M13(−21) was used the cost was $2.49/sample for 96 samples, however, when direct labeling method was used, the cost increased to $4.64/sample for 96 sample. The cost of a sample using ABI or CEQ does decrease, as the number of samples increases ($1.27 to $1.29 per sample in ABI system and $2.49 to $1.82–2.08 per sample in CEQ system for 960 samples), and/or by multiplexing more than one sample per well.

A challenge in using the M13(−21) primer labeling method described by Schuelke (2000) is that the annealing temperatures of SSR primers should not be close to that of the universal labeled M13(−21) primer. In this study, the annealing temperatures for SSR primers and M13 primer differed by only 2–5°C, which made it difficult to amplify specific bands when all three primers were used simultaneously. To overcome this, a two-step PCR was necessary, where the first PCR was performed using M13(−21) tailed forward primer and SSR reverse primer, and the second PCR used the universal fluorescent labeled M13(−21) forward and same SSR reverse primer. The PCR products of the first PCR were used as the template for the second PCR. Direct primer labeling, as done for PS01 and PS05, was easier and allowed for multiplex by labeling different colored dyes. Using this approach...
### Table 2  Loci, primers, labeling method and alleles detected in this study (page 1 of 2)

| Locus | Primer | Gel method | Applied biosystems 3730 genetic analyzer (ABI)\(^b\) | CEQ 8000 genetic analysis system (CEQ)\(^d\) | Allele name | Difference in bp between ABI and CEQ |
|-------|--------|------------|--------------------------------------------------|---------------------------------|-------------|-----------------------------------|
| PS01  | PS01-F | –          | n/d\(^f\) | 206 | PS01-B | – |
|       |        | 257        | n/d | 260 | PS01-C | – |
| PS01-R| –       | 269        | 266 | 272 | PS01-D | – |
|       |         | 281        | 284 | 290 | PS01-E | 3 |
|       |         | –          | 287 | 296 | PS01-G | 3 |
|       |         | 293        | 296 | 304 | PS01-H | 3 |
|       |         | –          | 307 | 312 | PS01-I | – |
|       |         | 419        | 419 | 424 | PS01-J | 5 |
| PS05  | PS05-F | –          | n/d | 260 | PS05-B | – |
|       |        | 263        | 263 | 264 | PS05-A | 1 |
| PS05-R| –       | 290        | 268 | 296 | PS05-C | – |
|       |         | –          | n/d | 304 | PS05-D | – |
|       |         | 307        | 307 | 308 | PS05-E | – |
|       |         | –          | n/d | 312 | PS05-F | 1 |
|       |         | 335        | 335 | 336 | PS05-G | – |
|       |         | –          | 343 | 344 | PS05-H | – |
|       |         | 347        | n/d | 320 | PS05-I | – |
|       |         | 360        | 355 | n/d | PS05-J | 1 |
| PS10  | PS10-F | –          | n/d | 144 | PS10-B | – |
|       |        | 153        | 146 | 149 | PS10-C | 3 |
| PS10-R| –       | 198        | 196 | 199 | PS10-D | 3 |
|       |         | 211        | 211 | 214 | PS10-E | 3 |
|       |         | 218        | 218 | 219 | PS10-F | 3 |
|       |         | –          | 221 | 221 | PS10-G | – |
|       |         | 233        | 233 | 234 | PS10-H | – |
|       |         | 238        | 238 | 249 | PS10-I | – |
|       |         | –          | 251 | 251 | PS10-J | – |
| PS12  | PS12-F | 258        | 251 | 252 | PS10-K | – |
|       |         | 264        | 257 | 258 | PS12-B | 1 |
|       |         | 264        | 258 | 258 | PS12-C | 1 |
| Locus | Primer | Gel method | Applied biosystems 3730 genetic analyzer (ABI)<sup>b</sup> | CEQ 8000 genetic analysis system (CEQ)<sup>d</sup> | Allele name | Difference in bp between ABI and CEQ |
|-------|--------|------------|------------------------------------------------|------------------------------------------------|-------------|------------------------------------|
|       |        | Approximate band size (bp)<sup>a</sup> | Allele size (bp)<sup>c</sup> | Allele size (bp)<sup>e</sup> | | |
| PS12-R | – | 292 | 293 | PS12-D | 1 |
| 300 | | 298 | 299 | PS12-E | 1 |
| – | | 304 | 305 | PS12-F | 1 |
| 306 | | 310 | 311 | **PS12-A** | 1 |
| – | | 316 | 317 | PS12-G | 1 |
| PS16 | PS16-F | – | 395 | 403 | PS16-B | 8 |
| | | 405 | 405 | 413 | PS16-C | 8 |
| PS16-R | – | n/d | 418 | PS16-D | – |
| | – | 455 | 463 | PS16-E | 8 |
| | – | 460 | n/d | PS16-F | – |
| | – | n/d | 478 | PS16-G | – |
| | 470 | 470 | 483 | **PS16-A** | 13 |
| | – | 475 | 488 | PS16-H | 13 |
| PS24 | PS24-R | 236 | 236 | 238 | PS24-B | 2 |
| | | 252 | 252 | 254 | **PS24-A** | 2 |
| PS24-F | 262 | 262 | 264 | PS24-C | 2 |
| | – | n/d | 310 | PS24-D | – |
| | – | n/d | 338 | PS24-E | – |
| PS29 | PS29-R | 252 | 249 | 251 | PS29-B | 2 |
| | PS29-F | 273 | 270 | 272 | **PS29-A** | |
| PS33 | PS33-R | – | 250 | 252 | PS33-B | 2 |
| | | 257 | 256 | 258 | PS33-C | 2 |
| PS33-F | – | 258 | 260 | PS33-D | 2 |
| | – | 262 | 264 | PS33-E | 2 |
| | – | 264 | 266 | PS33-F | 2 |
| | 267 | 266 | 268 | **PS33-A** | 2 |
| | – | 268 | 270 | PS33-G | 2 |
| | – | 270 | 272 | PS33-H | 2 |
| | – | 274 | 276 | PS33-I | 2 |
| | – | 278 | 280 | PS33-J | 2 |

<sup>a</sup> Total number of isolates analyzed \( n = 190 \); 126 from Ohio and 64 from Iowa

<sup>b</sup> Forward primer PS01, PS16, PS24, and PS33 were labeled with FAM while PS05, PS10, PS12, and PS29 were labeled with HEX dye

<sup>c</sup> Total number of isolates analyzed \( n = 106 \); 93 isolates from Iowa (includes 64 isolates used for the gel method) and 13 isolates received from Ohio’s lab. Size is based on approximate band sizes as determined by GeneMapper<sup>e</sup> Software 4.0

<sup>d</sup> All forward primer were designed with a M13(−21) tail at the 5'-end and used a universal WellRED D4 labeled M13(−21) primer as a nested primer, except for primers PS01 and PS05 that were directly labeled using WellRED D4

<sup>e</sup> Total number of isolates analyzed \( n = 130 \); 126 isolates from Ohio (same isolates used for the gel method) and 4 isolates received from Iowa’s lab. Size is based on approximate band sizes as determined by CEQ 8000 genetic analysis software

<sup>f</sup> Not detected in the isolates from that laboratory

Alleles found on the isolates shared by laboratories \( (n = 17) \) are *italicized*

Allele corresponding to sequenced isolate P6497 is *bolded*
can reduce costs when large numbers of samples need to be analyzed.

The capillary methods used in this study resulted in higher number of alleles detected, and although more expensive, the results allow for greater detection of genetic variation in *P. sojae*. The agarose method has the advantage that it can be accomplished by any laboratory with minimum infrastructure and is more economical, although it is best suited for those SSRs with longer repeats as their differences in amplicons can be easily distinguished on a gel. In addition, interpretations of data from studies with gel systems should be made with caution as some alleles will be missed.

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