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Ten polymorphic microsatellite loci identified from a small insert genomic library for *Peronospora tabacina*

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Abstract: Ten polymorphic microsatellite loci for the obligate biotrophic, oomycete pathogen of tobacco, *Peronospora tabacina,* were identified from a small insert genomic library enriched for GT motifs. Eighty-five percent of the 162 loci identified were composed of dinucleotide repeats, whereas only 4% and 11% were tri- and tetra-nucleotide repeats respectively. About 82% of all the microsatellites were perfect and within the library; only about 7% of the loci were duplicated. Primers were designed for 63 loci; 10 loci were polymorphic, 19 were monomorphic and 34 either failed to amplify or produced ambiguous/inconsistent results. The 10 polymorphic loci were characterized with 44 isolates of *P. tabacina* collected from tobacco plants growing in Europe, the Near East and North and South America. The number of alleles per locus was either three or four with a mean of 3.2, and the mean number of genotypes per locus was 3.6. Observed heterozygosity was 0.32–0.95, whereas expected heterozygosity was 0.44–0.69 for these loci. All loci except PT054 did not conform to the Hardy-Weinberg distribution. Polymorphic information content (PIC) for the loci was 0.35–0.69 with a mean of 0.50. These microsatellite loci provide a set of markers sufficient to perform genetic diversity and population studies of *P. tabacina,* and possibly other species of *Peronospora.*

Key words: blue mold, downy mildew, genetic diversity, Oomycete, Peronosporales, plant disease, simple sequence repeats, SSRs, tobacco

Introduction

Peronospora tabacina Adam (outdated syn. *P. hyoscyami* f. sp. *tabacina* Skalicky) is a diploid oomycete (Kingdom Straminipila) and an obligate biotrophic parasite that causes blue mold of tobacco (*Nicotiana tabacum*). Regarded as a pathogen primarily affecting seedlings and transplants in the United States before 1979, it since has become widespread in the tobacco-growing regions of the country (Sukno et al. 2002). Epidemics of the disease also have become commonplace in Europe, causing massive losses (Zipper et al. 2009). Control of the disease can be problematic because metalaxyl-resistant isolates of the pathogen have been reported in USA (Bruck et al. 1982), Germany (Krauthausen et al. 2003) and other countries in Europe (Zipper et al. 2009).

Peronospora tabacina has been reported to reproduce sexually by oospores (Spurr and Todd 1982), although oospores are seemingly rare and the importance to the survival of the pathogen and initiation of epidemics in both the USA (Sukno et al. 2002) and Europe (Zipper et al. 2009) is unknown. However the establishment and development of populations in USA and Europe are more likely the result of asexual (clonal) conidiospores (sporangiospores) from resident wild and cultivated tobacco populations in the subtropics and being wind-dispersed northward in the spring (Aylor et al. 1982). Once established in temperate production regions the disease spreads rapidly via conidiospores.

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Microsatellites, also called simple sequence repeats (SSRs), are sections of DNA that consist of tandemly repeated mono-, di-, tri-, tetra- or penta-nucleotide units that occur in abundance within the genomes of most eukaryotic organisms (Powell et al. 1996). Microsatellites are ubiquitous throughout most eukaryotic genomes and codominant markers, often used in both interspecific and intraspecific genetic diversity and population studies (Tautz 1989, Pupko and Graur 1999, Gupta and Varsheney 2000). Microsatellite markers are ideal for the identification and genetic fingerprinting of many types of organisms including fungi and the members of the Oomycota because of high polymorphism (Datta et al. 2010). Only four studies describe microsatellite markers for the downy mildews (Peronosporaceae) to the best of our knowledge. In the first study eight polymorphic loci for *Pseudoperonospora cubensis* were characterized with 22 North American isolates (Kanetis et al. 2009). Many of the microsatellites developed in this study also amplified loci in other *Pseudoperonospora* species and species in other genera in the Peronosporales (Trigiano unpubl). In the second study 50 microsatellite loci were isolated and characterized for *Peronosclerospora sorghi*. Some of the markers described *for Pe. sorghi* cross-amplified loci in three other species of *Peronosclerospora* and a single species of both *Peronospora* and *Sclerospora* (Perumal et al. 2008). In the third study five microsatellites were described for *Plasmopara viticola*; one locus was highly variable and one locus was monomorphic (Gobbin et al. 2003). A fourth study reports developing microsatellites for *Pl. viticola*, but did not present any data for the library (Dutech et al. 2007). At present no microsatellite markers are available specifically for *P. tabacina*.

Little is known concerning the genetics of *P. tabacina*. There have been a few assessments of genetic variability in the organism with various molecular techniques including restriction fragment length polymorphisms (RFLPs, Sukno et al. 2002), ISSR fingerprinting (Zipper et al. 2009) and sequencing of specific genes and spacers (Blanco-Meneses et al. 2008). However codominant markers, such as microsatellites, are needed to study genetic diversity and population genetics. Here we report the development and characterization of 10 polymorphic microsatellite loci with a group of *P. tabacina* isolates.

MATERIALS AND METHODS

A microsatellite-enriched library of *P. tabacina* was developed following a modified protocol of Wang et al. (2007). About 2.0 µg genomic DNA isolated from conidiospores (sporangia) from isolate DU9 (HUH862) (Horkheim, Württemberg, Germany, 2006; TABLE I) was digested with *AluI* and *StuI* (0.5 U each enzyme/100 mL digestion medium) for 15 min at room temperature and the fragments ligated to SNX linker adaptors (Hamilton et al. 1999). DNA sequences containing $(AC)_n$ motifs were selected with biotin-labeled microsatellite oligonucleotides $(GT)_{12}$ and streptavidin-coated magnetic beads. Enriched GT-containing sequences were used to transform bacterial colonies (*Escherichia coli* strain TOP10), which were spread along with the indicators β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β-D-galactoside (x-gal) onto Luria-Bertania (LB) solidified agar medium (Sambrook et al. 2001) amended with 100 µg/mL ampicillin in 10 cm diam Petri dishes. Petri dishes were incubated at 37 C overnight (12–16 h). Well separated, pure white colonies (n = 1440) were selected and grown overnight (12–16 h) in liquid SOC medium (Sambrook and Russell 2001) amended with 100 µg/mL ampicillin at 37 C. Candidate colonies were screened for inserts containing microsatellite sequences with a PCR protocol with three primers (T3, T7 and $[AC]_{12}$) in the reaction mixture as described by Wang et al. (2007). Template DNA for all reactions was 0.5 µL suspended *E. coli* cells in culture broth. The thermal-cycling conditions for colony screening were an initial step at 96 C for 5 min, followed by 35 cycles of 95 C for 1 min, 50 C for 1 min and 72 C for 1 min, ending with a 5 min stage at 72 C. The reactions were cooled and stored at 4 C until electrophoresis. PCR products were separated on 2% (w/v) agarose gels and visualized with ethidium bromide under UV light. Colony amplifications that exhibited a smear of products in the gel were considered positive for inserts containing microsatellites (Wang et al. 2007). Plasmids from positive colonies (n = 463) were isolated with an alkaline lysis method (Liou et al. 1999) and sequenced with Big-Dye 3.1 terminators (Applied Biosystems, Foster City, California) on an ABI 3730XL capillary electrophoresis DNA analyzer (Applied Biosystems) with a 50 cm array employing universal T3 and T7 primers that flank the cloned insert. Microsatellites (n = 162 loci) were identified with Imperfect SSR Finder (Stieneke and Eujayl 2007) using a minimum of six repeats for dinucleotides, four repeats for trinucleotides and three repeats for four or more bases. Forward and reverse primers 21–25 base pairs (bp) long were designed with 60% GC content and a minimum annealing temperature of 58 C for 63 loci with PRIMER3 (Rozen and Skaletsky 2000).

To characterize the microsatellite loci DNA was extracted from *P. tabacina* conidiospores emerging from tobacco leaves. All primer pairs were screened initially for the ability to amplify loci with four isolates of *P. tabacina* (TABLE I). Microsatellite amplification was completed in 10 µL volumes with these conditions: 2–4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTPs, 0.25 µM primer (both forward and reverse), 5% DMSO, 0.4 U AmpliTaq Gold® DNA polymerase (Applied Biosystems) and sterile water. These touchdown PCR (Korbie and Mattick 2008) cycling conditions were used for all primers: 94 C for 3 min; 15 cycles of 94 C for 40 s, 40 s initially at 63 C and subsequently decreasing 0.5 C per cycle and 72 C for 30 s. The next 20 cycles consisted of 94 C for

TABLE I. Isolates of *Peronospora tabacina* used to characterize 10 microsatellite loci

Isolate (accession number)[a]	Origin	Collection date
DU2 (526)	Forchheim, Baden, Germany	1996
DU3 (545)[b]	Bad Krozingen, Baden, Germany	2003
DU4 (556)	Rheinland Pfalz, Germany	2003
DU5 (621)	Sollingen, Niedersachsen, Germany	2004
DU7 (722)[b]	Ofterheim Baden, Germany	2005
DU8 (861)	Ortenau, Baden, Germany	2006
DU9 (862)[c]	Horkheim, Württemberg, Germany	2006
DU10 (864)	Schutterzell, Baden, Germany	2006
DU11 (920)	Oppeano, Italy	2007
DU12 (921)	Forchheim, Baden, Germany	2007
DU13 (925)[b]	Povcliv, Bulgaria	2007
DU14 (933)[b]	Verona, Italy	2007
DU15 (934)	Wytyczno, Poland	2007
DU16 (935)	Beltone, Italy	2007
DU17 (938)	Horkheim, Baden, Germany	2007
DU18 (968)	Forchheim, Baden, Germany	2007
DU19 (970)	Forchheim, Baden, Germany	1960
DU20 (1011)	Neuried, Baden, Germany	2007
DU22 (1078)	Rastatt, Baden, Germany	2008
DU23 (1079)	Jokandan, Iran	2009
DU24 (1081)	Saara, Thüringen, Germany	2009
DU25 (1082)	Rheinland Pfalz, Germany	2009
DU26 (1085)	Heidelberg, Baden, Germany	2009
DU27 (1090)	Spreewald, Brandenburg, Germany	2009
DU28 (1125)	Behshar, Iran	2010
DU29 (1138)	Riolobos, Spain	2010
DU30 (1139)	Moumour, France	2010
NC1 (PT123340)	Lebanon	1963
NC2 (PT123368)	Hungary	Unknown
NC3 (PT Penn54)	Pennsylvania, USA	Unknown
NC4 (PT Hadley)	Hampshire, Maryland, USA	2002
NC5 (PT 123350)	Germany	1963
NC6 (PT 123347)	Veracruz, Mexico	1989
NC7 (PT KY79)	Bourbon, Kentucky, USA	1979
NC8 (PT FL002)	Florida, USA	2000
NC9 (PT GA992)	Colquitt, Georgia, USA	1999
NC10 (PT 123349)	Poland	1989
NC11 (PT BPTP)	Texas, USA	1945
NC12 (PT BPTS)	Bertie, North Carolina, USA	1984
NC13 (PT123046)	Bulgaria	1988
NC14 (PT CT991)	Windsor, Connecticut, USA	1999
NC16 (PT VA011)	Virginia, USA	Unknown
NC17 (PT NIC063)	Jalapa, Nicaragua	2005
NC18 (PT 123392)	Guatemala	Unknown

[a] DU isolates are from the collection maintained at Department of Botany, University of Hohenheim, Stuttgart, Germany, and NC isolates are from the collection maintained at Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina.

[b] Isolates used for preliminary screening of microsatellite primer pairs.

[c] Small insert library constructed with DNA from this isolate.

40 s, 55 C for 40 s and 72 C for 30 s, ending with 72 C for 4 min. Amplification products were separated on 2% agarose gels with 100 V for 45 min and visualized with ethidium bromide and UV light.

Twenty-nine of the 63 primer pairs that amplified loci in the preliminary screening were used to test for polymorphisms in a study group of 44 isolates (TABLE I). PCR products in this experiment were sized on the QIAxcel Capillary Electrophoresis System (QIAGEN, Valencia, California) with an internal 25–300 bp standard. Raw allele length data were converted into allelic classes by the statistical binning of the alleles into bp size categories with the program Flexibin (Amos et al.

TABLE II. Characterization of a small-insert genomic GT-enriched microsatellite library for *Peronospora tabacina*

	Class of repeat		
	Dinucleotide	Trinucleotide	Tetranucleotide[a]
Number of microsatellites	137 (85.1%)	7 (3.7%)	18 (11.2%)
Motif	$(GT)_n$ or $(TG)_n$	$(AAG)_n$	$(GAGT)_n$ or $(TAGT)_n$
	87 (63.5%)	2 (28.6%)	10 (55.5%)
	$(CA)_n$, or $(AC)_n$	$(CAA)_n$ or $(CAC)_n$	$(AGTG)_n$ or $(AGAC)_n$
	48 (35.0%)	3 (42.8%)	3 (16.7%)
	$(AT)_n$	$(TGT)_n$ or $(TGG)_n$	Various
	2 (1.5%)	2 (28.6%)	5 (27.8%)
Range of repeats (n)	6–99	4–9	6–58
Perfect[b]	123 (89.8%)	3 (42.8%)	5 (27.8%)
Interrupted[b] (Imperfect)	9 (6.6%)	4 (57.2%)	8 (44.4%)
Compound[b]	5 (3.6%)	0	5 (27.8%)
Duplications[c]	10 (7.3%)	0	2 (11.1%)

[a] Also contained some repeated sequences greater than four nucleotides.
[b] Classification microsatellite arrays based on Gupta et al. (1996).
[c] 162 unique microsatellites were identified and the number does not include duplicate loci.

2007). For all data conservative 3 bp allelic categories were determined. A ±3 bp standard error range for allelic classes was used because of the 2–5 bp resolution limits on QIAxcel Capillary Electrophoresis System and allele size determination for this software is based on regression analysis standards. These allelic classes were used for determining the number of alleles per locus and in all subsequent analyses. The number of alleles per locus (A), expected (H_E) and observed heterozygosity (H_O), tests for Hardy-Weinberg equilibrium, and polymorphic information content (PIC, a measure of gene diversity) were performed with PowerMarker 3.25 (Liu and Muse 2005).

RESULTS

Genomic DNA from *P. tabacina* was digested into 200–600 bp fragments with a low concentration of *Alu* I and *Stu I* at room temperature for 15 min. Digestion with higher concentrations of these restriction enzymes, with an additional enzyme (*Hae* III) or with higher temperatures and longer incubation times, resulted in complete digestion (very small fragments) of the genomic DNA, which was unsuitable for library construction. A total of 1440 white (presumably transformed) *E. coli* colonies were selected to evaluate for microsatellite insertions. Preliminary screening of these colonies with three primers revealed that 463 (32%) colonies putatively harbored sequences with the $(GT)_n$ motif. After sequencing about 35% (162) of these or 11% of the total number of white colonies initially selected contained inserts with various microsatellite motifs. The $(GT)_n$ or a variation of the $(GT)_n$ motif was found in 99 (61%) of the SSRs, whereas 51 (32%) were some variation of the $(CA)_n$ motif. The remaining 12 SSRs were composed of mixed motifs and could not be categorized simply as either $(GT)_n$ or $(CA)_n$ motifs (TABLE II). The majority (85%) of the microsatellite regions were dinucleotide repeats, mostly (98%) $(GT)_n$ and $(AC)_n$ motifs, and almost 90% of these were perfect (TABLE II). Many of the sequences that did not harbor a microsatellite with at least six repeating units had regions containing multiple, short $(GT)_n$ ($n = 4$ or 5) repeats.

Sixty-three primer pairs were developed from the 162 microsatellite loci identified by sequencing. Many of the remaining inserts that contained microsatellites did not have adequate flanking regions to design primers and a few of the microsatellites contained multiple inserts, which also were unsuitable for primer design. Twenty-nine primer pairs were able to amplify loci from four isolates of *P. tabacina* in the initial screening of the primers and the remainder either did not amplify the intended loci or gave inconsistent or ambiguous results. The 29 primer pairs were tested on genomic DNA from 44 isolates of *P. tabacina* and 19 were monomorphic, whereas 10 were polymorphic loci (TABLE III).

The 10 polymorphic loci had either three or four alleles per locus with a mean of 3.2 alleles per locus. The mean number of genotypes per locus was 3.6 and for many of the loci one genotype was dominant within the sample population. Observed heterozygosity of the 44 isolates was 0.32–0.95, whereas expected heterozygosity was 0.44–0.69. All loci deviated significantly from the calculated Hardy-Weinberg equilibrium ($P < 0.05$), except locus PT054, which was in equilibrium. The PIC was 0.35–0.69 with a mean of 0.50 (TABLE III).

DISCUSSION

A gel view indicated that the digestion of genomic DNA resulted in fragments of *P. tabacina* mostly

TABLE III. Primer sequences, repeat motif, and annealing temperature (T_a) of 10 microsatellite loci isolated from *Peronospora tabacina*[a]

Locus[b]	Primer sequence (5′-3′)	Repeat motif	T_a (degrees C)	Allelic class size (bp)	*A*	G	H_E	H_O	PIC
PT002	F: GGCTGAACCATACGATGACC R: CTCCACGTGCACTAGAAAGG	$(GT)_9$	59	172–182	3	3 (0.71)	0.68	0.55[c]	0.47
PT004	F: CACGTATGCCCATGAGAAGC R: CGCTGCAACGAAGAAAGC	$(CA)_{12}$	61	150–166	4	7 (0.60)	0.69	0.89[c]	0.64
PT007	F: AGAAGCAACCAATGGACAGG R: AAAGTCAGCCAGCCATGC	$(AC)_8$	60	226–236	3	4 (0.88)	0.64	0.84[c]	0.49
PT014	F: ACTTGTTTGCGTGCAGTTCC R: TCAGGACCCTAAAACAAAAAGC	$(TG)_{11}$	60	184–198	3	2 (0.92)	0.56	0.95[c]	0.38
PT028	F: ACTTAAAGGTGGGTGGAAGAGC R: CGTGTAGAACATGTCATTGATCG	$(TG)_8$	60	186–194	3	5 (0.49)	0.50	0.45[c]	0.40
PT032	F: AGCGTTCGGTCGGTTAGG R: CTTTGCTCGTCAACATTTCACC	$(GT)_6$	61	188–200	4	4 (0.64)	0.59	0.93[c]	0.62
PT047	F: TTCTATACATACCTCGCAACAACC R: CTATGAGGGATGCAGACAGAGG	$(AT)_6$	59	204–220	4	4 (0.50)	0.54	0.82[c]	0.69
PT048	F: GGCTGCTTCACCAGTCTGC R: AGTGGAATTTGACGATGTAGGG	$(AG)_6(AC)_9$	60	178–194	3	4 (0.69)	0.44	0.16[c]	0.35
PT054	F: GTCACTAGCTGCGTTCTCACG R: ACCGAACAGCATGATCGTACC	$(TG)_8$	61	206–218	3	3 (0.46)	0.61	0.32	0.54
PT056	F: TGAAAGTCGTGCCTATTGAACC R: TGTTTTGTAGATAGCGCCAACC	$(GT)_{10}$	61	166–188	3	3 (0.83)	0.66	0.77[c]	0.45

[a] Allelic class size range, number of alleles (*A*), Number of genotypes and highest frequency (G), expected (H_E) and observed (H_O) heterozygosity and PIC (polymorphic information content) were calculated from the characterization of 44 isolates of *P. tabacina*.

[b] GenBank accession numbers: JF26112–JF26116; JF26118; JF26120–JF26123.

[c] Locus exhibits significant deviation from Hardy-Weinberg equilibrium ($P \leq 0.05$).

200–600 bp, but smaller, unusable fragments that were not evident in the gel were preferentially inserted in the cloning vector, probably due to easier insertion. About 60% of the discovered microsatellites were in small fragments (<150 bp) and did not have sufficient flanking regions to design 21–25 bp primers. This percentage was similar to the 53% of unusable inserts containing microsatellites in the library reported for *Pe. sorghi* (Perumal et al. 2008). In contrast the library developed for *Pl. viticola* only had about 25% unusable sequences with insufficient flanking regions (Gobbin et al. 2003). The preponderance of the smaller inserts in our study suggests that any future library should be created using either less time or only one enzyme for genomic DNA digestion. Many of the putative GT motif- positive colonies sequenced proved not to harbor an SSR of a minimum of six repeat units but did contain short $(GT)_n$ (n = 4 or 5) repeats or other GT-rich sequences, which were selected during the enrichment process. Many of the $(GT)_5$ repeats found in our library preparation have been used in other studies including the library of *Pe. sorghi* (Perumal et al. 2008). The complimentary motifs $(CA)_n$ harbored in inserts rich with short GT repeated regions could have amplified with the $(CA)_{12}$ third primer during colony screening, thus providing a false positive for a $(GT)_n$ insert. This scenario would explain why more than 25% of the inserts contained either $(CA)_n$ or some variation of $(CA)_n$ motif repeats. Only 35% of the positive colonies actually contained a microsatellite (any motif), a relatively low frequency compared to the 99% correct identification of microsatellite inclusion reported by Wang et al. (2007). The apparent GT-rich genomic DNA of *P. tabacina* might have caused the false positive identification of GT-SSR inclusions with the three primer colony PCR prescreening phase before sequencing.

The great majority of microsatellites detected for *P. tabacina* had perfect $(GT)_n$ or $(AC)_n$ motifs, and this finding was very similar to that reported for the *Pe. sorghi* library (Perumal et al. 2008) and *Ps. cubensis* (Kanetis et al. 2008). However in the *Pe. sorghi* library, which was enriched with six motifs including $(AT)_n$ and $(AG)_n$, 15% of the microsatellites were $(GA)_n$ and $(CT)_n$ repeats whereas in *P. tabacina* and *Ps. cubensis* libraries these repeats motifs were absent. Five microsatellites from a *Pl. viticola* library enriched for $(TC)_n$ motifs contained $(TC)_n$ repeats, although the motif identity of the remaining 56 SSRs was not

reported (Gobbin et al. 2003). It appears that $(GA)_n$ and $(CT)_n$ repeats might occur less frequently than $(AC)_n$ or $(GT)_n$ motifs in these three genera of downy mildews. Libraries of the three downy mildew organisms discussed previously had about the same low percentage representation of tri- and tetranucleotide repeats.

Ten primer pairs were optimized to amplify polymorphic loci in 44 *P. tabacina* isolates from Europe, the Near East and North and South America. The mean number of alleles reported per locus for the downy mildews appears to be somewhat low. Three or four alleles (mean = 3.2) were revealed for each locus for *P. tabacina*, which was similar to the mean number of alleles (4.3) per microsatellite locus in 22 samples of *Ps. cubensis* (Kanetis et al. 2009), although in this case the alleles were not binned to allelic classes as they were in the present study. If the allelic classes are recalculated with two bp for *P. tabacina*, then the mean number of alleles/locus is 4.6. Similarly 2–8 alleles with a mean of 5.2 were found across 54 microsatellites evaluated with 21 isolates of *Pe. sorghi* (Perumal et al. 2008). Five microsatellite loci were evaluated with 190 samples of *Pl. viticola* and four were polymorphic. The mean number of alleles for three loci was 7.0 and the remaining locus (GOB) had 43 alleles (Gobbin et al. 2003), which appears to be unusually high for a microsatellite locus in the downy mildews. Most microsatellite loci reported from the downy mildews have one or two alleles in high frequency and thus one to several genotypes predominant at a given locus. In our study eight of 10 loci had a genotype that was represented in more than 50% of the individual samples and for *Ps. cubensis*, six of eight loci were most commonly represented by a single genotype (Kanetis et al. 2009). Gobbin et al. (2003) reported that considering all four polymorphic loci developed for *Pl. viticola* 158 of the 178 samples evaluated had different genotypes. However one or two alleles were found in higher frequencies than other alleles for the four polymorphic markers.

Nine of 10 *P. tabacina* loci did not conform to the Hardy-Weinberg distribution, which also is similar to loci discovered in *Ps. cubensis* (Kanetis et al. 2009). Locus PT054 was probably in equilibrium by chance and not because of random mating. *Peronospora tabacina* and *Ps. cubensis* primarily reproduce asexually (clonally) by conidiospores, but there are few reports of oospores from field materials of *P. tabacina* (Spurr 1982) and none from field materials of *Ps. cubensis* in Europe (Runge and Thines 2009). However oospore formation by *Ps. cubensis* in two *Cucumis* species under laboratory conditions has been reported recently. These oospores were capable of causing infection and subsequent asexual sporulation on three *Cucumis* species (Cohen et al. 2011). Therefore the populations of these two downy mildews on their respective hosts (tobacco and species of cucurbits) appear to be clonal and would not fulfill the tenets to conform to the Hardy-Weinberg distribution. Reports, including this study, indicate that populations in Europe (Zipper et al. 2009) and USA (Sukno et al. 2002) exhibit little genetic diversity, lending more credibility to the idea of clonal populations in temperate regions. It seems reasonable to assume that some sexual reproduction of *P. tabacina* and *Ps. cubensis* might occur in milder climates on wild and cultivate species and either windborne (Aylor 1982) or transplant-borne conidiospores are delivered to temperate areas early in the growing season of their respective hosts. Once primary infection occurs epidemics are established via secondary condiospore production and distribution. This does not preclude the possibility of overwintering of the pathogens in temperate hosts. For example *Bryonia dioica* (wild hops) is native to much of the Mediterranean region and Europe and has been determined to be a potential host to *Ps. cubensis* under laboratory conditions but has not been reported to be a host in the wild (Runge and Thines 2009). The epidemiology for *Pl. viticola* is very different than either *P. tabacina* or *Ps. cubensis*. Oospores in grape leaf tissue are common, overwinter, and initiate the disease the following spring as well as contribute to the epidemic throughout the growing season (Matasci et al. 2010). The genetic variability of *Pl. viticola* appears to be much higher than either *P. tabacina* or *Ps. cubensis* because of sexual reproduction (Gobbin et al. 2003) and microsatellite loci appear to conform to Hardy-Weinberg because of random mating (Koopman et al. 2007). Although most of the individual lesions were founded by unique genotypes of the *Pl. viticola*, most of these genotypes had limited ability to produce asexual spores (Gobbin et al. 2005, 2007; Koopman et al. 2007) and therefore lessened their ability to contribute to the development of the disease epidemic. However a few genotypes capable of producing a high number of conidiospores were able to cause secondary infection on vines up to 130 m distance (Gobbin et al. 2007).

Despite the limited number of isolates included in this study, the 10 polymorphic microsatellites demonstrated that these markers will be highly useful for assessing gene flow and studying population genetics in *P. tabacina*. Preliminary analyses of the collection of isolates in this report indicates some structure (genetic differentiation) exists between North and South America isolates and isolates of European and

middle eastern origin (Trigiano unpubl). However analyses of more isolates, especially from North America, will be required to confirm these differences. Cross-taxa amplification (transfer) of loci developed for *P. tabacina* is an interesting possibility Because development of libraries from other downy mildews, especially from wild plants, would be difficult because of their obligate, parasitic mode of nutrition. As with plants, the utility of these loci to study other downy mildews will depend largely on the evolutionary relatedness of the different species and genera (Wadl et al. 2010). Preliminary experiments have demonstrated limited cross amplification of *P. tabacina* loci to three other *Peronospora* species (Trigiano unpubl data) and to a single species of *Plasmopara* (Spring and Trigiano unpubl data). The paucity of transferability was reported in a study of fungi, which included *Pl. viticola*, in which only 34% of loci transferred between members of the same genera (Dutech et al. 2007). In contrast transferability of *Pe. sorghi* loci to other species of *Peronosclerospora* as well as *Peronospora* and *Sclerospora* species (Perumal et al. 2008) and *Ps. cubensis* loci to other species (Trigiano unpubl data) was common. Future studies using the microsatellites reported herein will focus on population and genetic diversity of *P. tabacina* and cross transferability of the loci to other species of the downy mildews.

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