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Identification of new polymorphic microsatellite markers in the NA1 and NA2 lineages of *Phytophthora ramorum*

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Abstract: *Phytophthora ramorum* is a recently introduced pathogen in Europe and North America consisting of three clonal lineages. Due to the limited intralineage genetic variation, only a few polymorphic markers are available for use in studies involving the epidemiology and evolution of *P. ramorum*. A total of 159 primer pairs for candidate polymorphic SSR loci were tested with universal labeling. Four polymorphic microsatellite loci were identified within the NA1 lineage and one within the NA2 lineage, demonstrating the power and flexibility of the screening technique. The markers may significantly increase the number of genotypes that can be identified and as such can help better characterize the North American lineages of *P. ramorum*.

Key words: genetic diversity, microsatellite markers, oomycetes, sudden oak death, universal labeling

INTRODUCTION

The sudden oak death pathogen, *Phytophthora ramorum*, has resulted in extensive mortality in oaks (*Quercus* spp.) and tanoaks (*Lithocarpus densiflorus*) on the West Coast of the United States and recently has been found to be causing comparable amounts of mortality in multiple stands of Japanese larch (*Larix kaempferi*) in the UK (Brasier and Webber 2010). The pathogen also has been reported in ornamental nurseries in North America and Europe. Three clonal lineages of *P. ramorum* have been described and given the names NA1, NA2 and EU1 (Grünwald et al. 2009). The EU1 lineage initially was found in Europe only, but it is now present also in North America (Grünwald et al. 2008, Hansen et al. 2003). The NA1 and NA2 lineages currently have been found only in North America. The NA1 clonal lineage, which is the most common in the entire US nursery population, also has been found in Canada, albeit relatively infrequently (Goss et al. 2011). Isolates of the NA2 lineage have been isolated from infected nursery stock in Washington and California. Surprisingly, the NA2 lineage is the most prominent lineage in Canada (Goss et al. 2011). Investigating the genotypic diversity of *P. ramorum* populations with microsatellite markers has provided valuable insights into the population structure, migration and evolution of *P. ramorum* in forest and nursery environments on both continents (Goss et al. 2009a, b; Ivors et al. 2006; Mascheretti et al. 2008, 2009; Prospero et al. 2007, 2009; Vercauteren et al. 2010). While some markers have proved to be useful in the NA1 as well as in the EU1 population (Ivors et al. 2006, Prospero et al. 2007, Vercauteren et al. 2010), only one locus has been polymorphic in the NA2 lineage and at low frequency (Goss et al. 2009b, 2011). Additional polymorphic markers were developed for the EU1 population to obtain sufficient genotyping resolution to characterize the Belgian *P. ramorum* population (Vercauteren et al. 2010).

In this study all candidate markers from Vercauteren et al. (2010) and 10 microsatellite markers described in Schena et al. (2008) were tested in the NA1 and NA2 populations to obtain additional polymorphic markers for these lineages. Polymorphic microsatellite markers are needed to study the migration of the NA2 lineage in North America. Also, additional markers within the NA1 population would allow for higher resolution genotyping and therefore more detailed migration and evolution studies for that lineage.

MATERIALS AND METHODS

Five NA1 isolates and five NA2 isolates were selected, based on diversity in host, isolation date, geographic origin and previous genotype analysis (TABLE I). The 10 isolates were used to screen each of the 149 candidate primers pairs from

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TABLE I. *P. ramorum* isolates used in the screening of candidate microsatellite markers polymorphic for the NA1 and NA2 clonal lineages

Isolate code[a]	Original isolate code	Source lab[b]	Lineage and genotype[c]	Isolation year	Host plant or source	Origin (USA state/county)
PR-07-179	TX-105-0030B-1259	L. Barnes	NA1-36	2004	—	TX Montgomery
PR-07-061	47-WA-WAT	G. Chastagner	NA1-14	2005	Plant	WA Pierce
PR-05-187	05-2033-72	N. Osterbauer	NA1-23	2005	*Viburnum plicatum*	OR Coos
PR-09-002	SPN-S1-C	S. Jeffers	NA1-31	2009	*Rhododendron catawbiense*	SC Greenville
PR-01-003	1033.1	E. Hansen	NA1	2001	*Lithocarpus densiflorus*	OR Curry
PR-05-156	RHCC 13	M. Garbelotto	NA2-1	2005	*Rhododendron*	CA Sacramento
PR-05-166	MR31	M. Garbelotto	NA2	2004	*Rhododendron*	WA King
PR-09-054	1470614-10	C. Blomquist	NA2-1	—	*Rhododendron*	CA Sacramento
PR-07-031	15-WA-M	G. Chastagner	NA2-1	2006	Soil	WA King
PR-08-085	1480393	C. Blomquist	NA2-1	2008	*Pieris*	CA San Mateo

[a] Isolate ID in *Phytophthora ramorum* Multilocus Genotyping Database (http://people.oregonstate.edu/~grunwaln/phytophthora.php)

[b] Location of source labs: *Barnes L.*: Texas A&M University, AgriLife Extension Service, College State, TX; *Chastagner G.*: Washington State University, Puyallup Research and Extension Center, Puyallup, WA; *Osterbauer N.*: Oregon Department of Agriculture, Salem, OR; *Jeffers S.*: Clemson University, Department of Entomology, Soils, & Plant Sciences, Clemson, SC; *Hansen E.*: Oregon State University, Department of Botany & Plant Pathology, Corvallis, OR; *GarbelottoM.*: University of California at Berkeley, Department of Environmental Science, Policy, & Management, Berkeley, CA; *Blomquist C.*: California Department of Food & Agriculture, Plant Pest Diagnostic Branch, Sacramento, CA.

[c] Lineage (NA1 or NA2) according to Grünwald et al. (2009). SSR genotype number as known before this study is listed after the lineage designation and is based on markers 18 and 64 (Ivors and others 2006) and markers PrMS39, PrMS43, PrMS45 (Prospero and others 2007), as reported in Goss and others (2009b). Isolates PR-01-003 and PR-05-166 were not included in Goss et al. (2009b) and therefore were not given a genotype number, but their SSR genotypes differ from those of the four other NA1 and NA2 isolates in this study, respectively.

Vercauteren et al. (2010). Ten microsatellite loci described in Schena et al. (2008) that were polymorphic between the EU1 and NA1 lineages (data not shown) also were checked for polymorphisms within the *P. ramorum* NA1 and NA2 lineages. Primer pairs for this additional group of candidate polymorphic loci were designed with the primer3 program (Whitehead Institute for Biomedical Research). An M13reverse (5′–CAGGAAACAGCTATGACC–3′) tag was added at the 5′– end of each forward primer for universal labeling, as described in Vercauteren et al. (2010).

Genomic DNA of each *P. ramorum* isolate was extracted from mycelium with the FastPrep® DNA extraction kit from Qbiogene (Carlsbad, California). PCR reactions were performed with the touchdown cycling parameters as described in Vercauteren et al. (2010). In each PCR reaction five M13 reverse-tagged primer pairs were pooled and were labeled with the HEX or FAM fluorescent dyes. PCR products first were scored for successful amplification on 1.5% agarose gels and subsequently sized on an ABI 3130 sequencer (Applied Biosystems) with Rox 500 as the size standard. Results were analyzed with Genemapper (Applied Biosystems).

The DNA sequence of the novel polymorphic microsatellite loci within the NA1 or NA2 lineages was determined as follows: PCR amplification of the loci was performed as described above but with 50 μL reaction volumes. The entire PCR product of each microsatellite locus from isolates PR-05-156 (NA2) and PR-07-179 (NA1) was separated on a 2% Nusieve 3:1 agarose gel (Lonza Bioscience). DNA of excised fragments was extracted with the Nucleospin ExtractII kit (Macherey-Nagel), cloned with the TOPO TA cloning kit (Invitrogen) and sequenced in both directions with BigDye® terminator cycle sequencing at Macrogen (Seoul, South Korea). Sequencing of the PCR products was not successful for all amplicons.

RESULTS AND DISCUSSION

Polymorphic loci within the NA1 and NA2 lineage were amplified, respectively, by six and two ILVOPrMS primer pairs (TABLES II, III). Primer pair ILVOPrMS79 amplified three alleles in the NA1 lineage, indicating that in that lineage at least two loci are involved. More than two alleles also were found for ILVOPrMS145, as was the case in the EU1 lineage (Vercauteren et al. 2010). Determination of these alleles in the NA1 lineage to their loci was described in Vercauteren et al. (2010) based on inheritance profiles in the progenies obtained from a NA1 × EU1 cross (Boutet et al. 2010). Analysis of single-oospore progenies and their NA1-A2 parental isolates revealed three distinct loci (ILVOPrMS145a–c). ILVOPrMS145c is polymorphic in NA1. Primer pair 82 (Ivors et al. 2006) amplified a second locus (82b) in the NA1 population, similar to our finding in the EU1 population (Vercauteren et al. 2010). Only

TABLE II. Primer codes and sequences, and repeat motifs of seven primer pairs used to amplify polymorphic loci of *P. ramorum* in NA1 and NA2 isolates

Primer pair name	Primer sequence (5′–3′)[a]	Repeat motif[b]	Polymorphic in lineage	Amplifies same locus as	GenBank accession no[c]	
					NA1	NA2
ILVOPrMS79	F: *CAGGAAACAGCTATGACC*AGGCGGAAAACGTCAGAAC R: CTCGAGAGGCTGGAAGTACG	$(CA)_{10}$	NA1		HM483371	nd
82[e]	F: *CAGGAAACAGCTATGACC*CCACGTCATTGGGTGACTTC R: CGTACAAGTCACGACTCCCC	$(GT)_{14}$	EU1, NA1		GQ223250	nd
ILVOPrMS131	F: *CAGGAAACAGCTATGACC*CGGCCGTTTTTGTAAGTTTG R: CAGATCAAACCAAAATCTGCTC	$(AGAC)_{37}$	NA1, NA2	ILVOPrMS144	HM483373	HM483368
ILVOPrMS132	F: *CAGGAAACAGCTATGACC*GCACGGCCAGAGATTGATAG R: AATCTGCCGACGTGAAGAAG	$(TCTA)_{32}$	NA1	PrMS39[d]	HM483374	HM483369
ILVOPrMS133	F: *CAGGAAACAGCTATGACC*AATATGCAAAAAGGCAGGAG R: CCGCGTAACCTAGTCTGCTC	$(GACA)_{77}$	NA1, NA2, EU1	PrMS43[d]	HM483375	HM483370
ILVOPrMS144	F: *CAGGAAACAGCTATGACC*ACCCTTTCCACTCTCAATGC R: AGGTGTCGCTCGTCCTTG	$(CTGT)_{13}$	NA1, (NA2)[f]	ILVOPrMS131[g]	HM483376	nd
ILVOPrMS145	F: *CAGGAAACAGCTATGACC*TGGCAGTGTTCTTCAACAGC R: ATTCCCGTGAACAGCGTATC	$(AGCGAC)_{15}$	NA1, EU1		GQ223245 GQ223246 GQ223247	nd

[a] Forward (F) and reverse (R) primers. Forward primers include M13 tag sequence (5′-CAGGAAACAGCTATGACC-3′) for universal labeling, which has been indicated in italic.

[b] Repeat motif and the number of repeats as reported in the 7× draft genome sequence of *P. ramorum* isolate Pr102.

[c] nd: Sequence of amplified locus (or loci) was not determined in this lineage.

[d] Described in Prospero et al. (2007).

[e] Primers based on Ivors et al. (2006) are used here to amplify locus 82b, which was not described by Ivors et al. (2006).

[f] The polymorphic allele in the NA2 lineage is amplified using this primer pair but not observed when only fragments up to 500 bp are sized. Due to this sizing issue, ILVOPrMS131, which amplifies the same locus, should be used.

[g] Primer pair ILVOPrMS144 was designed based on the sequence of scaffold 2112, while primer pair ILVOPrMS131 was designed based on the sequence of scaffold 153. These scaffolds probably contain the two alleles of this locus, given the corresponding difference in length of this specific repeat on these scaffolds.

TABLE III. Multilocus genotypes of NA1 and NA2 isolates of *P. ramorum* based on seven primer pairs. Allele sizes (in bp) are based on the fragment length analysis on an ABI3130 and may differ slightly from the true amplicon lengths (based on sequencing). Polymorphic fragments are indicated in boldface

Isolate code[a]	lineage	82[b]		ILVOPr MS79	ILVOPr MS131	ILVOPr MS132	ILVOPr MS133	ILVOPr MS144[c]	ILVOPr MS145
		82a	82b						
PR-07-179	NA1	107/112	109/212	362/364/406	165/**246**	153/271	**386**/**498**	456	186/196/217/260
PR-07-061	NA1	107/112	109/212	362/364/406	165/242	153/**275**	**382**/**494**	456	186/196/217/260
PR-05-187	NA1	107/112	109/212	362/364/**408**	**169**/242	153/**275**	**364**/**498**	**460**	186/196/217/**249**
PR-09-002	NA1	107/112	109/**204**	362/364/**410**	165/**246**	153/271	**386**/**502**	456	186/196/217/260
PR-01-003	NA1	107/112	109/212	362/364/406	165/**234**	153/**275**	**390**/**478**	456	186/196/217/260
PR-05-156	NA2	97/103/105/107/109		368/370	**294**/356	167/173	182/186	–	186/196/202
PR-05-166	NA2	97/103/105/107/109		368/370	**290**/356	167/173	**190**/186	–	186/196/202
PR-09-054	NA2	97/103/105/107/109		368/370	**298**/356	167/173	182/186	–	186/196/202
PR-07-031	NA2	97/103/105/107/109		368/370	**290**/356	167/173	182/186	–	186/196/202
PR-08-085	NA2	97/103/105/107/109		368/370	**294**/356	167/173	182/186	–	186/196/202

[a] Isolate ID in *Phytophthora ramorum* Multilocus Genotyping Database (http://people.oregonstate.edu/~grunwaln/phytophthora.php)

[b] For primer pair 82, data on the two separate loci (82a and 82b) could be determined in the NA1 lineage isolates based on Vercauteren and others (2010).

[c] Data on some of the alleles are missing because fragments 50–500 bp only were scored.

this second locus showed polymorphism in the NA1 lineage.

Sequences were aligned with known microsatellite loci and with each other to verify that the primer pairs amplified unique and novel loci. This revealed that the loci amplified by primer pairs ILVOPrMS144 and ILVOPrMS132 were the same as those amplified by ILVOPrMS131 and PrMS39 (Prospero et al. 2007), respectively. In Vercauteren et al. (2010) it was already determined that the locus amplified by ILVOPrMS133 is identical to PrMS43 (Prospero et al. 2007). Primer pair PrMS39 amplifies two homozygous loci (PrMS39a and PrMS39b) according to Prospero et al. (2007), but for the same reasons as stated in Vercauteren et al. (2010) for PrMS43 it is most likely one heterozygous locus. Overall we identified three novel polymorphic loci in the NA1 lineage (ILVOPrMS79, ILVOPrMS131, ILVOPrMS145) and one (ILVOPrMS131) in the NA2 lineage out of the 149 candidate markers from Vercauteren et al. (2010). One extra polymorphic locus (82b) in the NA1 lineage was identified based on primer pair 82 from Ivors et al. (2006). No polymorphic loci were identified in these lineages based on the candidate markers from Schena et al. (2008).

Reproducibility of the newly identified markers was tested by regenotyping all isolates in both the Heungens and Grünwald labs. When using DNA from the same extraction no differences were observed. However differences were observed with the ILVOPrMS131 primers between different DNA extractions conducted two or more years apart from two isolates in long term storage (mycelia in hemp seed and water stored at 20 C), suggesting that this locus might be hypervariable.

The availability of the genome sequence of *Phytophthora ramorum* allowed the characterization of many microsatellite loci. By using a primer system with universal fluorescent labeling (Shimizu et al. 2002) primers could be tested in an affordable manner. This led to the identification of new polymorphic microsatellite loci for the NA1 and NA2 populations. In total nine informative loci (18, 64, 82b, PrMS43 = ILVOPrMS133, PrMS39 = ILVOPrMS132, PrMS45, ILVOPrMS79, ILVOPrMS131 = ILVOPrMS144, and ILVOPrMS145c) are now available in the NA1 population and two informative loci (ILVOPrMS131 and PrMS39 = ILVOPrMS133) in the NA2 population. Of these loci 18 and 64 were identified by Ivors et al. (2006). PrMS39, PrMS43 and PrMS45 were identified by Prospero et al. (2007) and 82b, ILVOPrMS79, ILVOPrMS131 and ILVOPrMS145c were identified in this study. The identification of the new microsatellite primer pairs within the North American lineages allows higher resolution population monitoring to track evolution and patterns of spread of the pathogen. The primers also can be used to screen for sexual recombinants (Boutet et al. 2010, Vercauteren et al. 2011).

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