

PRIMER NOTE

Development of polymorphic microsatellite and single nucleotide polymorphism markers for *Cercospora beticola* (Mycosphaerellaceae)

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Abstract

The plant pathogenic fungus, *Cercospora beticola*, causes the most important foliage disease of sugar beet. A previous study has shown that isolates of opposite mating types are present in equal proportions in natural populations; therefore, the aim of this study was to develop highly reproducible polymorphic markers for analysing populations of *C. beticola*. Five microsatellite and four single nucleotide polymorphism (SNP) markers were developed that allow rapid screening of genetic diversity in *C. beticola*. Six populations were screened with these markers and all were found to be in gametic equilibrium, indicating random mating in *C. beticola*.

Keywords: mating types, *Mycosphaerella*, RFLP, SNP, SSR, sugar beet

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Cercospora beticola, a plant pathogenic fungus, is the main causal agent of *Cercospora* leaf spot on sugar beet (Saccardo 1876). Groenewald *et al.* (2006) isolated and characterized the mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* and showed that these mating types are present in equal proportions in natural populations and suggested that sexual reproduction occurs. The aim of this study was to develop highly reproducible and easy-to-use polymorphic neutral genetic markers for this species.

The method described by Cortinas *et al.* (2006) was used to isolate microsatellite areas from the *C. beticola* genome (strain CBS 116456); however, different temperatures (23 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C) were used for the annealing of probes. A total of 550 clones were sequenced, analysed and screened for microsatellite repeats. Four clones that contain eight or more perfect repeats were found, and an additional five that contain nonperfect repeats were selected (Table 1). Nine primer sets were developed (Table 1) and preliminary tests were performed on nine isolates. The polymerase chain reaction (PCR)

conditions were the same for all markers, except for marker SSRcb3 for which the annealing temperature was 55 °C. The reaction mixture contained 10 ng of genomic DNA, 1 × PCR buffer, 48 μM of each of the dNTPs, 2.5 pmol of each of the specific forward and reverse SSRcb primers, 1.5 mM MgCl₂, and 0.7 U *Taq* polymerase (Bioline). The initial denaturation step was performed at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C or 58 °C for 20 s and elongation at 72 °C for 30 s with a final elongation step at 72 °C for 7 min on a GeneAmp PCR System 9700 (Applied Biosystems).

The amplification products were sequenced using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences). The products were analysed on an ABI PRISM 3730 DNA Sequencer (PerkinElmer). A consensus sequence was computed from the forward and reverse sequences with SEQMAN from the Lasergene package (DNASTAR). The microsatellite regions were evaluated for sequence polymorphism among the nine isolates. It was found that five primer pairs amplified microsatellite regions that were polymorphic and four were monomorphic (Table 1). The four monomorphic microsatellite markers showed single nucleotide differences in the regions flanking the microsatellite

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Table 1 Characteristics of the nine polymorphic molecular markers developed for *Cercospora beticola*

Primer pair	Fluorescent label	Primer sequence (5'–3')	T _m (°C)	Marker type*	Product size (bp)	Core SSR sequence	GenBank. Accession no.
SSRCb1F	NED	TGCGATCTGGGCATAAAATATC	58	SSR	223–225	(TC)	DQ902564
SSRCb1R		AGATTTGCATTTGCCACAC					
SSRCb2F	6-FAM	TTGATGGCTGCGTGAGATAG	58	SSR	195–197	(TC)	DQ902565
SSRCb2R		AGTGTGCGACTGACAGATGG					
SSRCb3F	6-FAM	CATCTCGTGCTCAACGGGTAC	55	SSR	244–372	(AC)	DQ902566
SSRCb3R		ATAGAGTCAAACCAAGCCAAG					
SSRCb4F	NED	CCCGTTATAGCGCCCTTAG	58	SSR	156–188	(AAAAGAAGAGAGA)	DQ902567
SSRCb4R		CAGAGGTAGAGCCGACGAC					
SSRCb6F	VIC	TTCGCGCCGATGCTGACTCG	58	SSR	223–241; null allele	(CT)	DQ902568
SSRCb6R		TACAACCTCGCACGCTTGATG					
SSRCb10F	None	TGTGATTCTTTCTTCCCTGT	58	SNP	115/247	—	DQ902569
SSRCb10R		CATCTGCAGCACGAATAA					
SSRCb10F ₂		GCCAGAGTGTGCATAGATTG					
SSRCb11F	None	CGCGTGAGATAGTAACGATT	58	SNP	192/284	—	DQ902570
SSRCb11R		CTCCAAGGTTGCCAATGT					
SSRCb11F ₂		TTCTCTGGAGCAGCAGTGG					
SSRCb12F	None	CGATATCATCGCAAAGG	58	SNP	238/291	—	DQ902571
SSRCb12R		CCAGACATGATTAACAGAAGTAC					
SSRCb12F ₂		CAAGGAACTTTGGAAGTCACTG					
SSRCb16F	None	GGGTGATAAGGGAGTCTTCA	58	PCR-RFLP	73 +212/285	—	DQ902572
SSRCb16R		GCTATGATTGAAGAGATTGC					

*SSR, microsatellite marker; SNP, single nucleotide polymorphism marker; PCR-RFLP, PCR-based restriction fragment length polymorphism marker.

repeats and these were converted to one RFLP (restriction fragment length polymorphism) and three SNP (single nucleotide polymorphism) markers (Table 1). For the RFLP screening, each reaction mixture was incubated at 37 °C for 10 h and contained 200 ng of PCR product amplified with the SSRCb16 primers, 1 × *EcoRV* buffer and 5 U *EcoRV* (Promega). The PCR products were visualized on a 1% agarose gel stained with ethidium bromide and viewed under ultraviolet light. For this marker, the 285-bp PCR fragment is digested with *EcoRV* into a 212-bp and a 73-bp fragment.

For the SNP screening, internal primers were designed (SSRCb10F₂, SSRCb11F₂ and SSRCb12F₂) within each of the polymorphic areas (Table 1) and used in a multiplex PCR together with the SSRCb10, SSRCb11 and SSRCb12 primer sets, respectively. The PCR conditions were the same for all markers. The mixture contained 10 ng gDNA, 1 × buffer, 1.5 mM MgCl₂, 48 μM dNTPs, 2 pmol forward primer, 3 pmol each for the reverse as well as internal primer, and 0.7 U *Taq* polymerase. The same program was used as described above with an annealing temperature of 60 °C and the amplified fragments were separated on a 2% agarose gel and visualized under ultraviolet light. For the SSRCb10F/F₂/R reaction a 247-bp fragment is present in all reactions whereas a 115-bp fragment is observed only when the specific polymorphism is present. For the

SSRCb11F/F₂/R multiplex PCR, a 284-bp fragment is present in all reactions but only those containing the polymorphism, recognized by the internal primer, have the 192-bp fragment. A 238-bp fragment is amplified by the SSRCb12F/F₂/R primer combination only when the polymorphism is present, but due to competition between the two forward primers, a 291-bp fragment, which serves as the positive control, is only present in the absence of the smaller band.

For microsatellite analyses, all forward primers were labelled with fluorescent dyes, except for primer pair SSRCb3 for which the reverse primer was fluorescently labelled (Table 1). Genomic DNA from 160 isolates of *C. beticola* was amplified with the fluorescent labelled primers. Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM 3730 Automated DNA sequencer (PerkinElmer) and data analysis was conducted with GENEMAPPER version 4 (Applied Biosystems).

MULTILOCUS version 1.3 was used to investigate multi-locus linkage disequilibrium. The index of association (I_A ; Brown *et al.* 1980; Maynard-Smith *et al.* 2000) was calculated by measuring the distance between all pairs of loci, and comparing it to the expected value (zero), assuming no linkage disequilibrium. A total of 1000 randomizations were performed for each population. Missing data (null alleles) were fixed in position during randomizations.

Table 2 Allele size and prevalence (number of isolates in round parentheses) for isolates of *Cercospora beticola* from various countries. The total number of isolates per population is given in square parentheses. *H*, gene diversity (Weir 1990); I_A , index of association

Marker	France [32]	Germany [31]	Italy [26]	The Netherlands [27]	Iran [22]	New Zealand [22]	No. of alleles	<i>H</i>
SSRCb1	223 (26); 225 (6)	223(31); 225 (0)	223 (23); 225 (3)	223 (18); 225 (9)	223 (17); 225 (5)	223 (16); 225 (6)	2	0.30
SSRCb2	195 (27); 197 (5)	195 (27); 197 (4)	195 (24); 197 (2)	195 (24); 197 (3)	195 (22); 197 (0)	195 (5); 197 (17)	2	0.31
SSRCb3	244 (3); 246 (3); 272 (1); 274 (10); 276 (2); 282 (2); 298 (1); 300 (1); 304 (1); 306 (1); 308 (1); 330 (1); 334 (2); 336 (1); 358 (1); 360 (1)	244 (3); 246 (1); 248 (1); 250 (4); 258 (2); 276 (3); 282 (3); 296 (1); 308 (1); 312 (2); 314 (4); 316 (3); 318 (1); 324 (1); 372 (1)	244 (6); 246 (3); 248 (1); 250 (3); 252 (1); 278 (1); 304 (1); 306 (1); 308 (1); 314 (1); 318 (1); 326 (2); 330 (1); 334 (2); 338 (1)	244 (2); 246 (4); 248 (1); 272 (2); 282 (2); 294 (1); 304 (3); 306 (3); 308 (4); 314 (1); 322 (1); 324 (1); 326 (1)	264 (1); 282 (2); 288 (1); 290 (1); 300 (3); 304 (5); 306 (2); 308 (1); 312 (1); 314 (2); 320 (1); 330 (1); 340 (1)	264 (2); 274 (3); 276 (6); 278 (6); 280 (1); 294 (1); 318 (1); 320 (1); 322 (1)	38	0.95
SSRCb4	188 (6); 156 (26)	188 (9); 156 (22)	188 (7); 156 (19)	188 (8); 156 (19)	188 (18); 156 (4)	188 (21); 156 (1)	2	0.49
SSRCb6	null (12); 227 (1); 229 (9); 231 (4); 233 (5); 237 (1)	null (6); 223 (1); 225 (1); 227 (8); 229 (3); 231 (1); 233 (11)	null (3); 225 (2); 229 (8); 231 (4); 233 (8); 237 (1)	227 (1); 229 (6); 231 (13); 233 (7)	229 (2); 231 (16); 233 (4)	227 (2); 231 (18); 233 (1); 241 (1)	9	0.77
SSRCb10	115 (32)	115 (31)	115 (26)	115 (27)	115 (19); 247 (3)	115 (20); 247 (2)	2	0.06
SSRCb11	192 (26); 284 (6)	192 (26); 284 (5)	192 (26)	192 (25); 284 (2)	192 (16); 284 (6)	192 (10); 284 (12)	2	0.31
SSRCb12	238 (27); 291 (5)	238 (16); 291 (15)	238 (14); 291 (12)	238 (20); 291 (7)	238 (19); 291 (3)	238 (9); 291 (13)	2	0.45
SSRCb16	212 (6); 285 (26)	212 (6); 285 (25)	212 (10); 285 (16)	212 (9); 285 (18)	212 (14); 285 (8)	285 (22)	2	0.40
I_A	-0.052 (<i>P</i> = 0.600)	0.031 (<i>P</i> = 0.110)	0.012 (<i>P</i> = 0.470)	0.047 (<i>P</i> = 0.010)	0.273 (<i>P</i> = 0.010)	0.186 (<i>P</i> = 0.130)		

**P* values for I_A estimates given in brackets.

Across the four SNP and the five microsatellite markers, a total of 61 alleles were obtained, where SSRCb3 was the most polymorphic with 38 alleles (Table 2). All populations except Iran and the Netherlands were in gametic equilibrium, suggesting random mating (Table 2). Only three locus pairs in the Netherlands and pairwise comparisons with locus SSRCb1 in the Iranian population were in gametic disequilibrium. These nine markers provide polymorphic codominant genetic markers that can be applied to additional populations with larger sample sizes to improve our knowledge regarding the population structure of this important plant pathogen.

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