Development of microsatellite and mating type markers for the pine needle pathogen *Lecanosticta acicola*

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Abstract

Lecanosticta acicola is an ascomycete that causes brown spot needle blight of pine species in many regions of the world. This pathogen is responsible for a major disease of *Pinus palustris* in the USA and is a quarantine organism in Europe. In order to study the genetic diversity and patterns of spread of *L. acicola*, eleven microsatellite markers and two mating type markers were developed. An enrichment protocol was used to isolate microsatellite-rich DNA regions and 18 primer pairs were designed to flank these regions, of which eleven were polymorphic. A total of 93 alleles were obtained across all loci from forty isolates of *L. acicola* from the USA with an allelic diversity range of 0.095 to 0.931 per locus. Cross-species amplification with some of the markers was obtained with *L. gloeospora, L. guatemalensis* and *Dothistroma septosporum*, but not with *D. pini*. Mating type (MAT) markers amplifying both idiomorphs were also developed to determine mating type distribution in populations. These markers were designed based on alignments of both idiomorphs of nine closely related plant pathogens and a protocol for multiplex PCR amplification of the MAT loci was optimised. The MAT markers are not species specific and also amplify the MAT loci in *Dothistroma septosporum, D. pini*, *L. gloeospora and L. guatemalensis*. Both types of genetic markers developed in this study will be valuable for future investigations of the population structure, genetic diversity and invasion history of *L. acicola* on a global scale.

Lecanosticta acicola (Thüm.) Syd. (syn: *Mycosphaerella dearnessii* M. E. Barr) is a haploid ascomycete causing brown spot needle blight of various pine species. *L. acicola* is thought to be native in Central America where it occurs on pine species growing in tropical and temperate zones (Evans 1984) and in the South-Eastern USA where brown spot needle blight is the major disease on *Pinus palustris* Mill. (Sinclair and Lyon 2005). *L. acicola* has also been found on other continents including South America (Gibson 1980), Asia (Suto and Ougi 1998) and Europe (Anonymous 2008).

The global movement and introductory pathways of *L. acicola* are poorly understood (Huan *et al.* 1995). Microsatellites are useful genetic markers to detect genetic variation within and between populations and can be used to infer migration pathways and histories of the invasions of plant pathogens (e.g. Fontaine *et al.* 2013).

The aims of this study were to develop polymorphic microsatellite markers for *L. acicola* that can be used to determine the genetic diversity of populations, as well as mating type markers designed to determine mating type distribution in populations. Cross-species amplifications of the microsatellite and mating type markers were tested on the phylogenetically related pine needle pathogens, *Dothistroma septosporum* (G. Dorog.) M. Morelet, *D. pini* Hulbary, *Lecanosticta gloeospora* H. Evans and *L. guatemalensis* Quaedvlieg & Crous.

To screen for microsatellite rich regions in *L. acicola,* the FIASCO technique (Fast Isolation by Amplified fragment length polymorphism of Sequences COntaining repeats) was used (Zane *et al.* 2002). Genomic DNA was extracted from six *L. acicola* cultures from Estonia, Italy, Japan, Slovenia, Switzerland and the USA following the protocol of Smith and Stanosz (1995) and quantified using a spectrophotometer. Equal concentrations

of DNA from each isolate were pooled together and 250 ng of DNA was used for one-step digestion-ligation reaction with *Mse*I and AFLP adaptors (Zane *et al.* 2002). This step was followed by PCR with an optimised number of 23 cycles to avoid over-amplification that leads to high clone redundancy. Enrichment of the amplified DNA was carried out using biotinylated probes $(AC)_8$ and $(GA)_8$. DNA containing microsatellite repeats was captured with streptavidin MagneSphere paramagnetic particles (Promega, Madison, WI, USA) and washed 4x with SSC solutions for high and 2x for low stringency (Arthofer *et al.* 2007). Enriched DNA was eluted with pre-warmed sterile water and amplified by PCR using adaptor primers. PCR amplicons were purified with peqGOLD kit (PeqLab, Erlangen, Germany) and cloned using pT257RVector and JM109 competent *Escherichia coli* cells (Fermentas, Vilnius, Lithuania). The FIASCO protocol and subsequent cloning were performed twice to increase the number of captured DNA regions containing polymorphic microsatellites.

In total, over two hundred transformed colonies were inoculated onto master plates and transferred to Nylon membranes (Roche, Mannheim, Germany) following the manufacturers recommendations. This was followed by hybridisation, washing steps and screening to identify transformed *E. coli* colonies containing inserts with simple sequence repeats. Approximately 60 colonies which were expected to contain microsatellite regions were selected and pre-screened with PCR containing $(AC)_8$ and $(GA)_8$ oligonucleotides as primers (Arthofer *et al.* 2007). Thirty-nine plasmids showing positive reaction were sequenced and sequence data analysed using BioEdit version 7.1.3 (Hall 1999). Eighteen primer pairs flanking microsatellite rich regions were designed using Primer3 Plus (Untergasser *et al.* 2007).

To screen the microsatellite loci for polymorphisms, PCRs were performed with DNA extracted from *L. acicola* isolates from South Korea, Germany and the USA. PCRs were run in 20 µl reaction volumes consisting of. PCR cycling conditions consisted of 2 min denaturation at 94°C, 35 cycles including 94°C for 30s, 55°C for 45s and 72°C for 60s, and an extension step at 72°C for 15 min. The annealing temperature was decreased to 48°C for the primer pair MD12. Amplicons were sequenced to verify the presence of the microsatellite repeat and to determine the polymorphism of the repeat length. One primer of each of the eleven primer pairs amplifying polymorphic regions was fluorescently labelled (Table 1; Applied Biosystems, Cheshire, UK) for fragment analyses.

The efficacy of the 11 labelled polymorphic microsatellite markers was tested on a population of 40 isolates of *L. acicola* obtained from diseased *P. palustris* needles collected in Mississippi, USA. Single PCRs were performed in 8 µl volumes (as above) and annealing temperatures were optimised for each primer pair. PCR products were pooled into two panels for fragment analyses according to Table 1. Pooled PCR products were loaded on an ABI 3730XL (Applied Biosystems) and sized with LIZ 500 standard. Alleles were scored using programs GeneMapper 4.1 and PeakScanner (Applied Biosystems). A total of 93 alleles were obtained across all 11 loci ranging from between two to 19 alleles per locus (Table 1). Allelic diversity (Nei 1973), calculated using PopGene 1.31 (http://www.ualberta.ca/~fyeh/popgene.html), ranged between 0.095 and 0.931 per locus with an average heterozygosity of 0.65 over 11 loci. Pairwise linkage disequilibrium ($P<0.05$) tested across all loci following 1000 randomisations using Multilocus v1.3b (Agapow and Burt 2001) showed no evidence of linked loci.

Cross-species amplification of the 11 markers was tested on other closely related species, including two isolates of *D. septosporum* from the Czech Republic, one isolate of *D. pini*

Table 1 PCR-based microsatellite markers developed for *Lecanosticta acicola*.

Locus name	Primer name	Primer sequence (5′-3′)	Panel	Repeat motif	Size of cloned allele (bp)	GenBank Accession no.	T_a (°C)†	Alleles size range (bp)	No. of observed alleles	*H* (N=40)*
MD1	MD1F	GTTTGAGACACTGACTTGACC	A	$(GA)_9$	148	KF246553	56	149 - 153	3	0.5212
	MD1R-(PET)	CACCACCATGGATGGATAGA								
MD2	MD2F-(FAM)	CTTACTCCCGAGACTGGATTG	A	$(TC)_8$	103	KF246554	56	97 - 105	4	0.2663
	MD2R	CCAGACCAAGAACGAAGAAA								
MD4	MD4F-(NED)	ATCCGGATCTTGACCTCCT	B	$(CT)_{14}$	169	KF246555	58	155 - 169	3	0.3038
	MD4R	CGGTAACTTCTCGCAACCT								
MD5	MD5F-(VIC)	CAGGCACAAGGAGAAAGAGA	B	$(CT)_2$ $(TC)_8T(TC)_3T$ $(TC)_4$ TT $(TC)_2$	290	KF246556	57	286 - 288	2	0.095
	MD5R	TCCTCAAGACTCCTCACCTG								
MD6	MD6F-(VIC)	AGAGTAAGGGAAAGGAAGAGA	A	$(GA)_7$ AA $(GA)_9(GAA)_{13}$	169	KF246557	61	129 - 205	19	0.9270
	MD6R	CGGCTACCGTCCTAATCTAAC								
MD7	MD7F-(PET)	CCAACCCGTCAATCAGAA	A	$(CT)_{12}$	298	KF246558	56	296 - 328	11	0.8350
	MD7R	CGAGAGCGCGAGAAAGTA								
MD8	MD8F-(FAM)	CACAGCACGGAAGACACGAG	B	$(GA)_{20}$	337	KF246559	60	303 - 366	17	0.9307
	MD8R	TCTGTTTCTGAGCGGTAGGAG								
MD9	MD9F-(NED)	GGGAACACACGCTCTTTG	A	$(GT)_9$	220	KF246560	56	218 - 236	8	0.8213
	MD9R	GGGCAAGAAATCCAGGAC								
MD10	MD10F-(PET)	CCTACCTACTTCCCTTTATATC TCC	B	$(CT)_3(TATAAC)_{13}$	224	KF246561	58	209 - 232	12	0.8638
	MD10R	TTAGGACGGTAGCCGTAGAG								
MD11	MD11F-(FAM)	GTGGGATGTTTGTTGGGTAG	B	$(TGG)_3(GGGAAAT)_{10}(GTT)_3$	195	KF246562	58	161 - 197	7	0.7622
	MD11R	GCCACCACAGATTGGATAAC								
MD12	MD12F-(VIC)	AGTCATAAAGAACCAGGA	B	$(GA)_{14}$	124	KF246563	48	119-133	7	0.7812
	MD12R	GCTATCTAGGCCATTGAA								

**H* – gene diversity (Nei, 1973) calculated on the population represented by 40 isolates.

† - Annealing temperature.

from Ukraine and one from USA, four isolates of *L. gloeospora* from Mexico and nine isolates of *L. guatemalensis* from Guatemala. Amplification was successful with markers MD2, MD6, MD7, MD9 and MD10 for *D. septosporum*, whereas none of the markers amplified in *D. pini*. All markers except MD1, MD4 and MD8 amplified *L. gloeospora*. *L. guatemalensis* was amplified with all markers except for MD5, MD8, MD10 and MD12.

In order to develop markers that amplify the MAT regions of *L. acicola*, the mating type DNA sequences for each idiomorph of nine species phylogenetically closely related to *L. acicola* (*Cercospora beticola*, *C. zeae-maydis*, *C. zeina*, *D. septosporum*, *D. pini*, *Mycosphaerella eumusae*, *M. fijiensis*, *M. musicola* and *Passalora fulva*), obtained from Genbank (http://www.ncbi.nlm.nih.gov/), were aligned and several different sets of degenerate primers were designed in the conserved regions of each idiomorph of the MAT gene. Two of the degenerate primer sets that worked well (Table 2, Online Resource 1)

Table 2 Mating type primers developed for *Lecanosticta acicola*.

Primer name	Primer sequence (5´-3´)	T_a (°C)
Degenerate primers:		
MAT1-1F1	CGC ATT YGC RCA TCC CTT TGT	56
MAT1-1R2	ATG AYG CCG AYG AGT GGW GCG CA	56
MAT1-2F1	GCR TTC MTG ATC TAY CGY CT	56
MAT1-2R2	TTC TTC TCG GAY GGC TTG CG	56
Specific primers:		
Md MAT1-1F	CGC ATT CGC ACA TCC CTT TGT	58
Md MAT1-1R	ATG ACG CCG ATG AGT GGT GCG	58
Md MAT1-2F	GCA TTC CTG ATC TAC CGT CT	58
Md MAT1-2R	TTC TTC TCG GAT GGC TTG CG	58

were further optimised and PCR conditions consisted of 7.25 µl H_2O, 2.5 µl MyTaq^{TM} Mix (Bioline; MA, USA), 0.25 µl of each primer, 0.25 µl MyTaq^{TM} DNA Polymerase (Bioline) and 2 µl of gDNA in a total volume of 12.5 µl. Cycling conditions consisted of 10 min

denaturation at 94°C, 40 cycles of 30s at 94°C, 45s at 56°C, 45s at 72°C and a last extension at 72°C for 10 min. PCR products were sequenced using forward and reverse primers to confirm correct amplification of the partial MAT gene and sequence data were analysed using CLC Main Workbench 6.0. The primers were redesigned without degenerate nucleotides ('specific' primers) according to the sequence results obtained (Table 2, Online Resource 1). PCR conditions were the same as for the degenerate primers except for the annealing temperature that was increased to 58°C.

Multiplexing of specific primer sets for both MAT idiomorphs was optimised to decrease the number of reactions and time required for large scale population screenings. Genomic DNA from two isolates of different mating types was pooled and amplified in a single PCR tube to verify there is no competition between the primers. Multiplexing was optimised using Fast Start chemistry: 12.5 µl reaction mix composed of 7.9 µl H_2O, 1.25 µl FastStart PCR Buffer (Roche, Mannheim, Germany), 0.25 µl 10mM nucleotide mix, 0.5 µl of each primer, 0.1 µl FastStart Taq DNA Polymerase (Roche) and 2 µl of gDNA using the same cycling conditions as described above. Amplification of both partial idiomorphs was visualised on 2% agarose gel under UV light (Online Resource 2). The population of *L. acicola* from Mississippi was screened using multiplex PCR. The MAT primers were also tested for the amplification success on the identical isolates of two species of *Dothistroma, L. gloeospora and L. guatemalensis* as for microsatellite markers.

The newly designed 'specific' mating type primers amplified regions of both idiomorphs of the MAT gene and were confirmed with sequencing. The MAT1-1-1 amplicon of 560 bp in length (GenBank accession no. KF688139) showed 79% nucleotide identity and 55% amino acid identity with *Dothistroma pini* MAT1-1-1. The 288 bp MAT1-2 amplicon (GenBank accession no. KF688140) showed only 66% nucleotide similarity with that of *D.*

pini. The correct amplification of the MAT1-2 was, therefore, confirmed by the presence of an intron in the conserved amino acid serine, common to all ascomycetes (Online Resource 1; Arie *et al.* 1997).

Correct amplification of each idiomorph of the MAT gene revealed that *L. acicola* is heterothallic. Amplification of both partial idiomorphs in a single PCR mix to which DNA of both mating types were added confirmed that there is no competition between the primer pairs. The MAT markers developed for *L. acicola* in this study successfully amplified the respective mating type idiomorphs in all 40 isolates tested. Results revealed the presence of 22 MAT1-1 and 18 MAT1-2 isolates in the collection of isolates from Mississippi, strongly indicating a sexual mode of reproduction in this population. Furthermore, both primer sets successfully amplified MAT sequences from related fungi, including both species of *Dothistroma* tested. All four isolates of *L. gloeospora* were identified as having the MAT1-1-1 locus and *L. guatemalensis* revealed the presence of both mating types (7 isolates possessed the MAT1-1-1 locus and 2 isolates the MAT1-2 locus). Thus the MAT markers are not species specific and are likely to be useful for identification of mating types in other closely related taxa, such as the recently described species of *Lecanosticta* from Central America (Quaedvlieg *et al.* 2012).

We have shown that the eleven microsatellite markers developed in this study are robust and will be useful for future population studies of *L. acicola*. In addition, we have also provided a tool whereby the mating type and mating type distribution of the isolates in a population can be determined. This will be applicable for investigations of global population diversity and structure of *L. acicola*.

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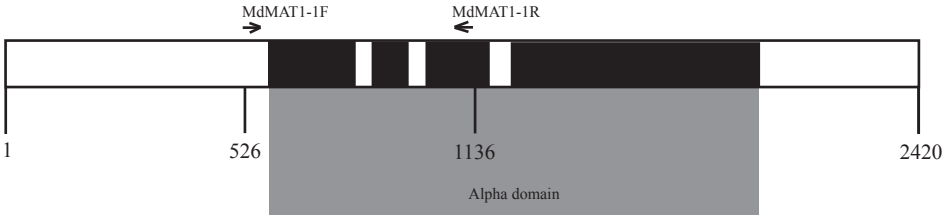
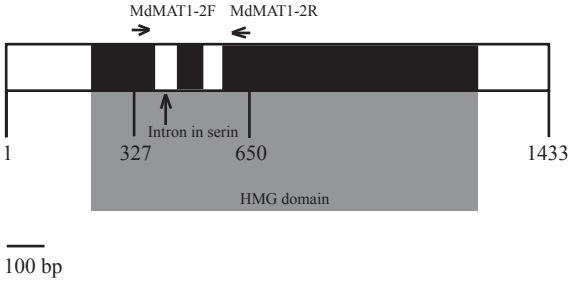
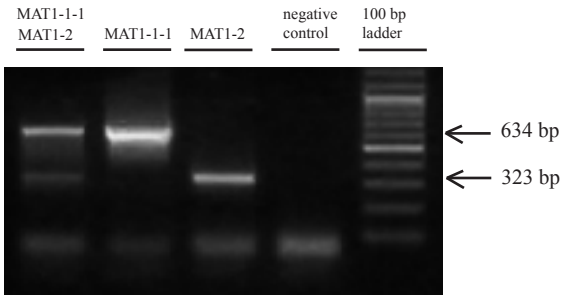
D. pini MAT1-1-1



D. pini MAT1-2



Online Resource 1 Positions of the specific mating type primers. Primer positions are indicated (arrows) in full-length idiomorphs of the putative MAT gene of D. pini (GenBank No.: DQ915449.1, DQ915451.1) as the complete L. acicola MAT region sequences are not available. Putative genes (alpha domain-containing and HMG domain-containing) are represented by grey boxes; coding sequences are represented by black boxes.



Online Resource 2 PCR amplicons of parts of the MAT1-1-1 and MAT1-2 idiomorphs of Lecanosticta acicola obtained using the MAT primers in multiplex PCR.