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Benoît Barres, Christian Cyril Dutech, Axelle Andrieux, Henri Caron, Jean Pinon, et al.. Isolation and characterization of 15 microsatellite loci in the poplar rust fungus, *Melampsora larici-populina*, and cross-amplification in related species. *Molecular Ecology Notes*, 2005, 6 (1), pp.60-64. 10.1111/j.1471-8286.2005.01137.x . hal-01005053

HAL Id: hal-01005053

<https://hal.science/hal-01005053>

Submitted on 31 May 2020

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PRIMER NOTE

Isolation and characterization of 15 microsatellite loci in the poplar rust fungus, *Melampsora larici-populina*, and cross-amplification in related species

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Abstract

We developed 15 microsatellite loci in the poplar rust fungus, *Melampsora larici-populina*, using two enrichment protocols. Polymorphism of each locus was assessed on a panel of 30 isolates, comprising three subpanels (world, regional and local scales). Thirteen loci were polymorphic with three to eight alleles detected. The 15 loci were also tested on five related *Melampsora* species, *M. allii-populina*, *M. medusae* f. sp. *deltoidae*, *M. larici-tremulae*, *M. rostrupii* and *M. pinitorqua*, and partial or global cross-amplification events were detected.

Keywords: Basidiomycete, cross-species amplification, enriched library, *Melampsora larici-populina*, microsatellites

Received 24 June 2005; revision received 8 July 2005; accepted 18 July 2005

The Basidiomycete *Melampsora larici-populina* causes foliar rust on *Populus* species from the sections *Aigeiros* and *Tacamahaca* and their hybrids (Frey *et al.* 2005). During the last few decades, this pathogen has caused important economic losses to poplar cultivation in Europe – mainly based on *Populus* × *euramericana* and *Populus* × *interamericana* hybrids – due to the breakdown of several major resistance genes (Pinon & Frey 2005). Random amplified polymorphic DNA markers were developed for *M. larici-populina* to compare genetic diversity between strains occurring on cultivated hybrid poplar stands and wild riparian *Populus nigra* stands (Frey *et al.* 2005). However, observed heterozygosity cannot be detected in this dicaryotic fungus with these dominant markers, which prompted a search for codominant microsatellite markers. Microsatellite markers have already been developed on several Basidiomycetes including rust fungi (e.g. Enjalbert *et al.* 2002).

Since microsatellites seem to be less frequent in fungal genomes than in other organisms (Lim *et al.* 2004), the strategy of isolating loci in *M. larici-populina* was to build an

enriched library for microsatellites. Total genomic DNA was extracted from *M. larici-populina* isolates 93ID6 and 98AG31 (Table 1) using DNeasy Plant Mini Kit (QIAGEN) after grinding 5 mg of urediniospores with glass beads (Pei *et al.* 1997). A first library was constructed according to Edwards *et al.* (1996) using Hybond membranes with two hybridization rounds. A second enriched library using biotinylated oligoprobes and streptavidin-coated magnetic beads was constructed following the protocol of Dutech *et al.* (2000) to increase the number of loci. Both libraries were enriched with (TC)₁₀ and (TG)₁₀ motifs and cloned using a TOPO TA Cloning Kit (Invitrogen). In the second library, a colony blot screening according to Estoup *et al.* (1993) and a polymerase chain reaction (PCR) size screening using vector's primers were performed to select clones larger than 500 bp and containing microsatellite motifs. In the first library, 104 clones were selected and sequenced, and 20 contained a microsatellite motif larger than 20 bp in length. In the second library, 3930 clones were screened and 978 gave a positive response for microsatellite presence. After size screening, 96 clones were sequenced and 17 were chosen for primer design. Primers were designed using PRIMER 3 software (Rozen & Skaletsky 2000) and tested

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Table 1 Isolates of *Melampsora* spp. used in the study. Pathotypes of *Melampsora larici-populina* determined as described in Pinon & Frey (2005)

Species	Isolate code	Pathotype	Original host	Collection site	Year
<i>Melampsora larici-populina</i>					
World subpanel					
	97A1	0	<i>Populus</i> × <i>euramericana</i>	Morocco	1997
	97A3	3-4	<i>P. nigra</i>	New Zealand	1997
	97J10	3-4	<i>P.</i> × <i>euramericana</i> 'I-488'	South Africa	1997
	97EA2	4	<i>Populus</i> sp.	China	1997
	97C3	1-3-4-5-6-7	<i>P.</i> × <i>interamericana</i> 'Beaupré'	UK	1997
	98AE3	2	<i>Populus</i> sp.	Finland	1998
	99D1	3-4	<i>P. trichocarpa</i>	Iceland	1999
	00A19	0	<i>P.</i> × <i>euramericana</i> 'I-488'	Chile	2000
	98AR1	1-3-4-5-7-8	<i>P.</i> × <i>interamericana</i> 'B-71085/A1'	Belgium	1998
	95US1	3-4	<i>P. nigra</i> 'Italica'	USA	1995
Regional subpanel					
	94ZZ5	1-3-4-5-7	<i>P.</i> × <i>euramericana</i> 'Ghoy'	N France	1994
	95AAH12	1-3-4-5-6-7	<i>P.</i> × <i>interamericana</i> 'Beaupré'	NE France	1995
	95XP5	1-3-4-5-6-7	<i>P.</i> × <i>interamericana</i> 'Beaupré'	SW France	1995
	95XW2	1-3-4-5-7	<i>P.</i> × <i>interamericana</i> 'Boelare'	W France	1995
	98AG31	3-4-7	<i>P.</i> × <i>interamericana</i> 'Beaupré'	N France	1998
	98AO2	3-4-7	<i>P.</i> × <i>interamericana</i> 'Beaupré'	SW France	1998
	00M10	3-4-7-8	<i>P.</i> × <i>interamericana</i> '69038-1'	NE France	2000
	01L31	2-3-7	<i>P.</i> × <i>euramericana</i> 'A4A'	C France	2001
	01L66	1-2-3-4-5-7	<i>P.</i> × <i>euramericana</i> 'Laxo 7'	NE France	2001
	02AO33	4	<i>P. nigra</i>	SE France	2002
Local subpanel					
	99I006	3-4	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I009	2-4	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I036	3-4-7	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I046	2-3-4	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I056	1-4-5	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I061	1-3-4-5-6	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I094	1-2-3-4-5-7	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I108	4	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I179	3-4-5	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	99I192	4-7	<i>P.</i> × <i>euramericana</i> 'Robusta'	NE France	1999
	93ID6	3-4	<i>P.</i> × <i>euramericana</i> 'I 45-51'	NE France	1993
<i>Melampsora allii-populina</i>					
	98Z1	—	<i>Arum</i> sp.	SE France	1998
	98Z3	—	<i>Allium</i> sp.	SE France	1998
	98Z4	—	<i>Allium</i> sp.	SE France	1998
	98Z5	—	<i>Allium</i> sp.	SE France	1998
	98Z6	—	<i>Arum</i> sp.	SE France	1998
	98Z9	—	<i>Muscari comosum</i>	SE France	1998
	98Z10	—	<i>Allium porrum</i>	SE France	1998
	89A1	—	<i>P.</i> × <i>euramericana</i> 'Altichiero'	SW France	1989
	96B6-1	—	<i>Allium vineale</i>	NE France	1996
	96M24-2	—	<i>Muscari comosum</i>	NE France	1996
	97M2	—	<i>Allium sphaerocephalum</i>	NE France	1997
	97N1	—	<i>Arum maculatum</i>	NE France	1997
	94HY8	—	<i>P. nigra</i>	SE France	1994
	94IV7	—	<i>P. nigra</i>	SE France	1994
	91E4	—	<i>P.</i> × <i>euramericana</i> 'Robusta'	W France	1991
	91L5	—	<i>P.</i> × <i>interamericana</i> 'Beaupré'	W France	1991
<i>Melampsora medusae</i> f. sp. <i>deltoidea</i>					
	88MM2	—	<i>P.</i> × <i>interamericana</i> 'Beaupré'	SW France	1988
	97CN1	—	<i>P.</i> × <i>interamericana</i> 'Boelare'	SW France	1997
	98B1	—	<i>P.</i> × <i>interamericana</i> '87002-21'	South Africa	1998
	98D1	—	<i>P.</i> × <i>euramericana</i> '5006'	South Africa	1998
	99A3	—	<i>P.</i> × <i>interamericana</i> 'Hoogvorst'	SW France	1999
	99T2	—	<i>P.</i> × <i>interamericana</i> 'Hazendans'	NE France	1999
	01Z1	—	<i>P. deltoidea</i>	Canada	2001

Table 1 Continued

Species	Isolate code	Pathotype	Original host	Collection site	Year
	01Z2	—	<i>P. deltooides</i>	Canada	2001
	01Z4	—	<i>P. deltooides</i>	Canada	2001
	01Z5	—	<i>P. deltooides</i>	Canada	2001
	01Z11	—	<i>P. × interamericana</i> 'Unal'	Canada	2001
	02AZ5	—	<i>P. deltooides</i>	Canada	2002
	02AZ6	—	<i>P. deltooides</i>	Canada	2002
	02AZ7	—	<i>P. deltooides</i>	Canada	2002
	02AZ8	—	<i>P. deltooides</i>	Canada	2002
	02AZ11	—	<i>P. × jackii</i>	Canada	2002
<i>Melampsora larici-tremulae</i>	01F1	—	<i>P. tremula</i>	NE France	2001
<i>Melampsora rostrupii</i>	01G1	—	<i>Mercurialis perennis</i>	NE France	2001
<i>Melampsora pinitorqua</i>	00S1	—	<i>P. tremula</i>	SW France	2000
	00S2	—	<i>P. tremula</i>	S France	2000
	00S3	—	<i>P. tremula</i>	C France	2000

on a panel of 30 *M. larici-populina* isolates comprising three nested geographical (world, regional, local) subpanels with high pathotype diversity (Table 1).

PCR was performed using a PTC-200 Peltier Thermal Cycler (MJ Research) with 5 min at 95 °C followed by 40 cycles of 60 s at 94 °C, 90 s at annealing temperature and 60 s at 72 °C and a final extension step of 30 min at 60 °C. PCR was carried out in 20 µL final reaction volumes containing 15 ng template DNA, 2 µL of 10× reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 U *Taq* polymerase (Sigma) and 0.2 µM forward and reverse primers. Out of 37 loci tested, 22 failed to have an interpretable pattern of migration on a 2% agarose gel because of multiple bands or unexpected allele size. Forward primers of the remaining 15 loci (Table 2) were labelled to allow size and dye multiplexing. PCR products were separated, sized and analysed on a CEQ 8000 Genetic Analysis System (Beckman Coulter).

Out of 15 loci tested, 13 were found to be polymorphic with three to eight alleles (Table 2). Expected and observed heterozygosities as well as a test for Hardy–Weinberg expectation were computed using GENEPOP 3.4 (Raymond & Rousset 1995). Departure from expected results was significant for eight loci, which could be explained by either a nonequilibrium state of the population, the sampling structure or a low frequency of null alleles. Locus µMLP22 was suspected to be uniparentally inherited because of the total lack of heterozygotes within the panel. The sequence adjacent to this locus was shown to exhibit 56% identity (29/51 amino acids) with the 5' end of the cytochrome *c* oxidase subunit 2 gene of the mitochondrion of the Basidiomycete *Crinipellis perniciosus* using NCBI's BLASTX program (Altschul *et al.* 1997), strongly suggesting that µMLP22 was a mitochondrial locus. All other loci did not show any significant homology with nucleotide or protein databases.

Loci µMLP12 and µMLP13 were isolated from the same clone sequence and are thus physically linked, with a 90-bp interval between the two microsatellite motifs. Nevertheless, no significant linkage disequilibrium ($P < 0.01$) was detected among all pairs of loci for the 30 isolates of the test panel, according to an exact test performed with GENEPOP 3.4.

All loci were tested for cross-species amplification against *M. allii-populina* (16 isolates), *M. medusae* f. sp. *deltooides* (16 isolates), *M. larici-tremulae* (1 isolate), *M. rostrupii* (1 isolate) and *M. pinitorqua* (3 isolates). Six loci resulted in global or partial amplification with some related species, but very few intraspecific polymorphisms were detected (Table 2).

Acknowledgements

We thank Christine Delaruelle, Annegret Kohler, Céline Lalanne, Patrick Léger and Martina Peter for their advice and help. This work was partly funded by grants from GIP ECOFOR and from INRA.

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Table 2 Characteristics of microsatellite loci isolated from *Melampsora larici-populina*. T_a , annealing temperature; H_E , unbiased expected heterozygosity; H_O , observed heterozygosity; for each combination of related species and locus, ratio between positive isolates and total number of isolates tested, as well as apparent fragment size when cross-amplification occurred, were indicated. *Significant difference between H_E and H_O ($P < 0.05$)

Locus	GenBank Accession nos	Primer sequences (5'–3')	Repeat motif of cloned allele	Expected size (bp)	T_a (°C)	Number of alleles (size in bp)	H_E	H_O	<i>Melampsora allii-populina</i>	<i>Melampsora medusae</i> f. sp. <i>deltoidae</i>	<i>Melampsora larici-tremulae</i>	<i>Melampsora rostrupii</i>	<i>Melampsora pinitorqua</i>
μMLP09	DQ059602	F1 TCAAGAACCAACCAATCAAAC R1 CATGCCAACCATACTCTCTG	(CA) ₂ C(CA) ₅ A(CA) ₅	171	60	4 (159, 165, 167, 169)	0.40	0.47	0/16	0/16	0/1	0/1	0/3
μMLP12	DQ059603	F1 TGCTTCACAACAAGCGAAAG R1 ACCFTTGTGCGACCAACACC	(AAG) ₅ (...)(AAG) ₆ (...) (AAG) ₁₀ (...)(ATG) ₃ (AAG) ₄	245	60	7 (239, 242, 245, 247, 248, 250, 266)	0.70	0.63	0/16	0/16	0/1	0/1	0/3
μMLP13	DQ059604	F1 GTTGTGTGTCGACAAAGGTG R1 GCATFAAGCCGCTGATTTTC	(TG) ₁₁	187	60	4 (189, 191, 193, 203)	0.45	0.47	0/16	0/16	0/1	0/1	0/3
μMLP20	DQ059605	F1 CTTTCAAGCTCAATGCAAGG R1 AAACCAAGCAAGCATACCAC	(TG) ₁₀	181	60	3 (177, 179, 181)	0.07	0.07	0/16	0/16	0/1	0/1	0/3
μMLP22	DQ059606	F1 CGCATTTCTATTATTAAACACACG R1 CGATTTCTGATCCATACCTGTCC	(TA) ₈ (...)(TAATAG) ₁₁ (...)(TG) ₇	315	60	4 (312, 318, 324, 330)	0.62	0.00*	16/16 280 bp	16/16 291 bp	0/1	1/1 274 bp	0/3
μMLP24	DQ059607	F1 GATCACGCCCATGCTTFAAC R1 ATTTCCGGGAAGTCTTGTGACG	(TG) ₈ (...)(TG) ₅	154	60	1 (156)	0.00	0.00	0/16	0/16	0/1	0/1	0/3
μMLP27	DQ059608	F1 CATGCTAATTGTATGTGAGCTGTATTG R1 TGGATGAGGATGATGAGAGG	(TC) ₉ TTT(TC) ₉	250	60	4 (240, 242, 250, 252)	0.49	0.13*	13/16 228–230 bp	0/16	0/1	1/1	0/3
μMLP28	DQ059609	F1 ATCCCATGGAATCCGAAATG R1 GCTGATTGAGCTCGGAGTTG	(CACCA) ₆ (...)(CACCA) ₃	459	60	6 (395, 434, 439, 444, 459, 469)	0.29	0.21*	0/16	0/16	0/1	0/1	3/3 317–347 bp
μMLP29	DQ059610	F1 TGGACAAGTCCAAAATCATCAC R1 GCTCGGAGTTGTGGTAGTGAG	(CACCA) ₄	298	60	3 (295, 300, 310)	0.07	0.04*	0/16	0/16	0/1	0/1	0/3
μMLP30	DQ059611	F1 TGATGGGTTAGATGATGATTCC R1 CAACACACAACAACACACAATTC	(TGTGTGT) ₃ (...) (TGTGTGT)(...)(TGTGTGT)	249	63	5 (247, 249, 256, 263, 314)	0.25	0.13*	0/16	0/16	0/1	0/1	0/3
μMLP31	DQ059612	F1 CCACGACAGACGATATAGTTG R1 CTTTCCACACTGTGTTTTCG	(GT) ₃ GCTG(GT) ₉	194	54	6 (182, 183, 184, 187, 190, 192)	0.22	0.20	0/16	0/16	0/1	0/1	0/3
μMLP32	DQ059613	F2 CGGATGCCTTAAGACTGTGG R1 AAAGCCAACAAGATGACCTG	(TC) ₁₈	132	60	8 (116, 122, 124, 126, 128, 136, 138, 140)	0.82	0.93*	0/16	1/16 136 bp	1/1 118 bp	0/1	2/3 118 bp
μMLP34	DQ059614	F3 GAGAAATCGAACGCCAGAAG R2 TCAAAAAGAAATGGGACTGG	(AG) ₁₃ AA(AG) ₈	298	51	1 (268)	0.00	0.00	0/16	0/16	0/1	0/1	0/3
μMLP36	DQ059615	F1 TTTGAAAAGTAATCCGGAAGTGTG R1 TCAGGTTAGCTTTTCGTTGG	(AG) ₁₄	240	50	6 (226, 228, 230, 232, 236, 238)	0.59	0.57*	0/16	0/16	0/1	1/1 228–236 bp	0/3
μMLP37	DQ059616	F1 CCACGGTTGTGAAGTTGTATTTC R1 ACTTTGGACGTTTCGATCACC	(TC) ₁₀	224	60	3 (222, 224, 226)	0.50	0.63*	0/16	0/16	1/1 223–229 bp	0/1	2/3 225–231 bp

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