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Development and use of new sensitive molecular tools for diagnosis and detection of *Melampsora* rusts on cultivated poplar

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Summary

Poplar rusts due to *Melampsora larici-populina* (*Mlp*), *M. allii-populina* (*Map*) and *M. medusae* f. sp. *deltoidae* (*Mmd*) are the most serious disease in Europe on cultivated poplars, that is, *Populus* × *euramericana* and *P.* × *interamericana* hybrids. These pathogenic species can be identified by the observation of morphological characteristics of urediniospores but this method is not appropriate for high-throughput analysis and cannot be used on other spore stages, such as aeciospores or teliospores, that are morphologically similar. The aim of this study was to develop a rapid and sensitive molecular method based on PCR amplification that was able to specifically detect these species on various hosts for routine analysis. Three primer pairs ITS-MLP-F/ITS-MLP-R, ITS-MAP-F/ITS-MAP-R and ITS-MMD-F/ITS-MMD-R were designed within the internal transcribed spacer (ITS) sequences of ribosomal DNA to target *Mlp*, *Map* and *Mmd*, respectively, and their specificity were confirmed on a wide range of isolates and species. ITS-MLP-F/ITS-MLP-R and ITS-MAP-F/ITS-MAP-R primers proved to be highly specific to *Mlp* and *Map*, respectively, whereas ITS-MMD-F/ITS-MMD-R cross-reacted with DNA from *M. larici-tremulae* and *M. pini-torqua*. However, these species are not pathogenic on cultivated poplars that all belong to sections *Aigeiros* and *Tacamahaca* of the genus *Populus*. Specific *Mmd* primers proved to be very sensitive as a positive signal could be obtained with DNA extracts from 6 target urediniospores mixed with 800 000 urediniospores of *Mlp*. An internal amplification control (IAC) was included to discriminate false negative results due to the potential presence of inhibitory compounds in DNA extracts. ITS-MMD-F/ITS-MMD-R primers are therefore efficient for the detection of the quarantine pathogen *Mmd* on samples collected on poplar or larch and are fit for use in official tests. This new PCR assay has been used in routine for ten years, and *Mmd* has hitherto never been detected in commercial poplar nurseries in France.

1 Introduction

The genus *Populus* is divided in six sections and includes 29 species (Eckenwalder 1996). Most of the cultivated poplars belong to sections *Aigeiros* (*Populus deltoides* (Bartr.) Marsh. and *Populus nigra* L.) and *Tacamahaca* (*P. trichocarpa* Torr. & Gray ex Hook.), and their hybrids, that is, *P.* × *euramericana* (Dode) Guinier. and *P.* × *interamericana* Brockh. In Europe, the acreage of poplar plantations is much lower than other forest trees, but as hybrid poplars are fast-growing trees selected for their vigour, annual wood harvest is close to other widespread broadleaved trees, such as oak or beech. For example, in France, 240 000 ha of widely spaced poplar cultivation (1.6% of the total forest area) produce approximately 1.3 million m³ of wood per year, that is, approximately 25% of broadleaved wood production (AFOCEL 2009). Poplar wood is mainly used for veneer and plywood (Pinon et al. 2006). Another recent interest of poplar cultivation is the cultivation of short rotation coppice (SRC) for the production of wood as a renewable energy source (Vande Walle et al. 2007; Paris et al. 2011). Moreover, research about poplar biomass conversion to bioethanol is in progress (Pu et al. 2011; Swana et al. 2011).

In widely spaced poplar cultivation, trees are cultivated as monoclonal stands covering several hectares, with a small number of cultivars used in each region. In France, two-thirds of the stands involve no more than three cultivars (Pinon et al. 2006). This monoculture practice is favourable to the development of epidemics of fungi, bacteria and insects (Royle and Ostry 1995; Miot et al. 1999).

Among fungal diseases, foliar rust due to the basidiomycetes *Melampsora* spp. is the most serious disease on cultivated poplar worldwide. *M. larici-populina* Kleb. (*Mlp*) causes severe economic losses in Europe, mainly on *P.* × *euramericana* and *P.* × *interamericana* hybrids, in particular since the breakdown of several major resistance genes (Pinon and Frey 2005; Pinon et al. 2011). Symptoms are premature defoliation, growth decrease and delayed bud burst the year following infection. On the widely planted cultivar ‘Beaupré’, biomass losses were estimated to be 20–30% in the first year of infection and 50–60% during the following years (Gastine et al. 2003). Many young infected trees can be killed after secondary infection due to *Discosporium populeum* Sacc. (Pinon et al. 2006).

Two others species, that is, *Melampsora allii-populina* Kleb. (*Map*) and *M. medusae* Thüm. f. sp. *deltoidae* Shain (*Mmd*) can infect cultivated poplars in Europe but cause less economic impact (Frey et al. 2005). *Map* is more frequent in southern and western France (Pinon 1991; Desprez-Loustau et al. 2007), but recent studies on the effects of climate change have shown that its geographical distribution could be modified during the next decades due to an increase in summer temperature, leading to a higher prevalence in northern France (Desprez-Loustau et al. 2007). This species could therefore jeopardize the cultivated poplars in the future, especially the cultivation of biomass poplars in SRC. The North American species *Mmd*, probably introduced in Europe at the beginning of the 20th century, is very rare and is restricted to south-western France, Spain and Portugal (Pinon 1986, 1991). Up to now, no damage on poplar caused by this species is reported in

Europe, whereas it causes important economic losses in the USA (Widin and Schipper 1981; Newcombe et al. 1994; Bourassa et al. 2007), Australia (Walker et al. 1974) and New Zealand (Wilkinson and Spiers 1976). In Europe, *Mmd* is listed as a quarantine pest for EPP0 (OEPP/EPP0 1982). In this respect, the trade of poplar plants in European Union countries requires a phytosanitary passport, which states the absence of *Mmd* in the cultivation area. Official inspections of the nurseries are carried out on a yearly basis by the national plant protection authorities, and if necessary, samples of symptomatic leaves may be sent for analysis in official quarantine laboratories for the identification of the rust species.

Six other species of *Melampsora*, namely *M. larici-tremulae* Kleb., *M. rostrupii* G. H. Wagner, *M. pintonqua* Rostr., *M. magnusiana* G. H. Wagner, *M. pulcherrima* Maire and *M. aecidioides* (DC.) J. Schröt., are present on *Populus* sp. in Europe (Pinon 1973; Feau et al. 2009; Vialle et al. 2011). These species are only pathogenic on poplars belonging to section *Populus* (*P. alba* L. and *P. tremula* L.) and their hybrids (*P. canescens* (Ait.) Sm.), which are not much used in poplar cultivation in Europe (Pinon 1973; Vialle et al. 2011).

The morphological identification of *Mlp*, *Map* and *Mmd* based on the observation of urediniospores and paraphyses is straightforward and only requires a light microscope (Pinon 1973; Vialle et al. 2011). However, this method is time-consuming and can be only performed at the uredinial stage on poplar. Therefore, the development of species-specific molecular tools based on DNA amplification by polymerase chain reaction (PCR) would be of great interest for high-throughput analysis of samples collected on both the telial hosts (poplars) and the aecial hosts (*Larix* sp. for *Mlp* and *Mmd* and *Allium* sp., *Arum* sp. or *Muscari* sp. for *Map*).

Species-specific primer pairs designed from random amplified polymorphic DNA (RAPD) fragments were developed for identifying and characterizing *Mmd* by single-strand conformational polymorphism (SSCP) (Bourassa et al. 2005), but this technique is not straightforward for diagnosis or detection. In addition, ITS-based real-time PCR assays using SYBR Green dye have been developed to quantify *Mlp* or *Mmd* DNA *in planta* (Boyle et al. 2005; Hacquard et al. 2011). These PCR assays were successfully used to monitor the dynamics of the fungal colonization in infected leaves, but their specificity was not validated on a wide range of rust fungi, and therefore, they cannot be used as reliable identification tools.

The aim of this study was to design new species-specific primer pairs to (i) develop a rapid and efficient method based on PCR amplification for identifying all rust species pathogenic on cultivated poplars for routine analysis, (ii) distinguish these species regardless of their biological stage or telial/aecial host plant and (iii) detect the quarantine pathogen *Mmd* at low prevalence when mixed with an excess of another species. The ITS region of nuclear ribosomal DNA has been shown to be a suitable target for molecular detection assay development in rust fungi (Chung et al. 2008; Pedley 2009). Furthermore, it was proved to be the most efficient DNA barcode for fungi (Schoch et al. 2012) and particularly for poplar rust species delineation (Feau et al. 2009) and was therefore chosen in our study for designing primer pairs.

2 Materials and Methods

2.1 Sample collection

A total of 88 isolates of *Mlp*, *Map* and *Mmd* and 21 isolates of *Melampsora* species pathogenic on section *Populus* (*M. larici-tremulae*, *M. rostrupii*, *M. pintonqua*, *M. magnusiana*, *M. aecidioides*, *M. pulcherrima*) were collected from a wide range of host plants (poplars and aecial hosts) in numerous geographical locations (Table 1).

The production of urediniospores was carried out using the method originally described by Gérard et al. (2006) with slight modifications. Spores from single uredinia or aecia from infected plant tissue were transferred onto a leaf disc of *P. × euramericana* 'Robusta' for *Mlp*, *Map* and *Mmd* isolates or *P. tremula* × *P. alba* '717-1B4' for *M. larici-tremulae*, *M. rostrupii* and *M. pintonqua* isolates. Both poplar clones were grown from cuttings in 5-litre pots containing sand and peat mixture, in a rust-free glasshouse. A 20- μ l droplet of water agar (0.1 g l⁻¹) was deposited onto each selected uredinium or aecium with a micropipette, and spores were dispersed within the droplet with the micropipette. The resulting spore suspension was applied as 1- μ l droplets on the abaxial surface of 12-mm-diameter leaf discs of cultivars 'Robusta' or '717-1B4'. Leaf discs were incubated floating on deionized water in 24-well polystyrene cell culture plates, with the abaxial surface above, at 19 ± 1°C, and under continuous fluorescent light. After an 8- to 10-day incubation period, the sporulating discs were removed from the plates and the resulting urediniospores were suspended in 50 ml of water agar (0.1 g l⁻¹). Leaves of cultivars 'Robusta' or '717-1B4' were inoculated by spraying with this spore suspension. After a 10- to 12-day incubation period, the leaves were dried at room temperature for 24 h. Urediniospores were then harvested by tapping on the adaxial surface over aluminium paper. Urediniospore samples were kept refrigerated at +1°C in a 2-ml glass tube when used the following days or in liquid nitrogen in a plastic tube for prolonged storage.

2.2 Estimation of the number of urediniospores per mg

Three milligrams of *Mlp* or *Mmd* urediniospores was added to 100 ml water agar (0.1 g l⁻¹). The suspension was shaken for 10 min with a rotary shaker (IKA Vibrax VXR; Janke & Kunkel, Staufen, Germany) at 1000 rpm. The number of spores per ml was determined using a haemocytometer, with ten replicates per species.

2.3 Mixture of *Melampsora* species

Serial dilutions of urediniospores were performed to determine the detection threshold for *Mmd*. One milligram of *Mmd* urediniospores was mixed with 9 mg of *Mlp* urediniospores into a 2-ml round-bottom Eppendorf tube and vortexed for

Table 1. Characteristics of the isolates tested, GenBank accessions of the ITS sequences and results of the PCR assays using the three *Melampsora* primer pairs designed in this study

Species	Code	Host	Origin	Year	Collector/ supplier	GenBank accession	ITS-MLP-F/ ITS-MLP-R	ITS-MAP-F/ ITS-MAP-R	ITS-MMD-F/ ITS-MMD-R
<i>M. allii-populina</i>	98Z1	<i>Arum</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z2	<i>Allium</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z3	<i>Allium</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z4	<i>Allium</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z5	<i>Allium</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z6	<i>Arum</i> sp.	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z9	<i>Muscari comosum</i>	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	98Z10	<i>Allium porrum</i>	Southern France	1998	C. Husson		-	+	-
<i>M. allii-populina</i>	89A1	<i>Populus × euramericana</i> 'Altichiero'	Southern France	1989	J. Pinon		-	+	-
<i>M. allii-populina</i>	96B6-1	<i>Allium vineale</i>	Eastern France	1996	P. Frey	AY375271	-	+	-
<i>M. allii-populina</i>	96M24-2	<i>Muscari comosum</i>	Eastern France	1996	P. Frey	AY375272	-	+	-
<i>M. allii-populina</i>	97L2	<i>Allium vineale</i>	Eastern France	1997	P. Frey		-	+	-
<i>M. allii-populina</i>	97L5	<i>Allium vineale</i>	Eastern France	1997	P. Frey		-	+	-
<i>M. allii-populina</i>	97M1	<i>Allium sphaerocephalum</i>	Eastern France	1997	P. Frey		-	+	-
<i>M. allii-populina</i>	97M2	<i>Allium sphaerocephalum</i>	Eastern France	1997	P. Frey		-	+	-
<i>M. allii-populina</i>	97N1	<i>Arum maculatum</i>	Eastern France	1997	P. Frey		-	+	-
<i>M. allii-populina</i>	94IP1	<i>Populus nigra</i>	Southern France	1994	J. Pinon		-	+	-
<i>M. allii-populina</i>	94HY8	<i>P. nigra</i>	Southern France	1994	J. Pinon		-	+	-
<i>M. allii-populina</i>	94IZ7	<i>P. nigra</i>	Southern France	1994	J. Pinon		-	+	-
<i>M. allii-populina</i>	94IV7	<i>P. nigra</i>	Southern France	1994	J. Pinon		-	+	-
<i>M. allii-populina</i>	91D1	<i>P. × interamericana</i> 'Beaupré'	Eastern France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	91E4	<i>P. × euramericana</i> 'Robusta'	Western France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	91O4	<i>P. × euramericana</i> 'Ghoy'	Western France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	91N1	<i>P. × euramericana</i> 'Isières'	Western France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	91N5	<i>P. × euramericana</i> 'Isières'	Western France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	90C2B	<i>Allium cepa</i>	Eastern France	1990	J. Pinon		-	+	-
<i>M. allii-populina</i>	91L5	<i>P. × interamericana</i> 'Beaupré'	Western France	1991	J. Pinon		-	+	-
<i>M. allii-populina</i>	91C5	<i>P. × euramericana</i> 'Robusta'	Eastern France	1991	J. Pinon		-	+	-
<i>M. larici-populina</i>	93ID6	<i>P. × euramericana</i> 'I 45-51'	Eastern France	1993	J. Pinon	AY375268	+	-	-
<i>M. larici-populina</i>	93JE3	<i>P. × euramericana</i> 'Blanc du Poitou'	Eastern France	1993	J. Pinon		+	-	-
<i>M. larici-populina</i>	95XA1	<i>P. × interamericana</i> 'Beaupré'	Northern France	1995	J. Pinon	AY375267	+	-	-
<i>M. larici-populina</i>	97A1	<i>P. × euramericana</i>	Morocco	1997	J. Pinon	AY375269	+	-	-
<i>M. larici-populina</i>	97A2	<i>P. × euramericana</i> 'Véronèse'	New Zealand	1997	A. Spiers		+	-	-
<i>M. larici-populina</i>	97A3	<i>P. nigra</i>	New Zealand	1997	A. Spiers		+	-	-
<i>M. larici-populina</i>	97A4	<i>P. nigra</i>	New Zealand	1997	A. Spiers		+	-	-
<i>M. larici-populina</i>	97J10	<i>P. × euramericana</i> 'I-488'	South Africa	1997	G. Hawke	AY375270	+	-	-
<i>M. larici-populina</i>	97EA1	<i>Populus</i> sp.	China	1997	M. Villar		+	-	-
<i>M. larici-populina</i>	97EA2	<i>Populus</i> sp.	China	1997	M. Villar		+	-	-
<i>M. larici-populina</i>	97EA3	<i>Populus</i> sp.	China	1997	M. Villar		+	-	-
<i>M. larici-populina</i>	97C3	<i>P. × interamericana</i> 'Beaupré'	United Kingdom	1997	J. Pinon		+	-	-
<i>M. larici-populina</i>	97F1	<i>P. × interamericana</i> 'Boelare'	United Kingdom	1997	J. Pinon		+	-	-
<i>M. larici-populina</i>	97E6	<i>P. × interamericana</i> 'Boelare'	United Kingdom	1997	J. Pinon		+	-	-
<i>M. larici-populina</i>	98AE3	<i>Populus</i> sp.	Finland	1998	J. Pinon		+	-	-
<i>M. larici-populina</i>	99D1	<i>P. trichocarpa</i>	Iceland	1999	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A1	<i>P. × euramericana</i> 'Luisa Avanzo'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A5	<i>P. × euramericana</i> 'Luisa Avanzo'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A8	<i>P. × euramericana</i> 'Luisa Avanzo'	Chile	2000	J. Pinon		+	-	-

Table 1 Continued

Species	Code	Host	Origin	Year	Collector/ supplier	GenBank accession	ITS-MLP-F/ ITS-MLP-R	ITS-MAP-F/ ITS-MAP-R	ITS-MMD-F/ ITS-MMD-R
<i>M. larici-populina</i>	00A13	<i>P. × euramericana</i> '1-488'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A19	<i>P. × euramericana</i> '1-488'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A20	<i>P. × euramericana</i> '1-488'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A22	<i>P. × euramericana</i> '1-488'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	00A29	<i>P. × euramericana</i> '1-214'	Chile	2000	J. Pinon		+	-	-
<i>M. larici-populina</i>	94Z24	<i>P. × interamericana</i> 'Beaupré'	Belgium	1994	J. Pinon		+	-	-
<i>M. larici-populina</i>	95XF6	<i>P. × interamericana</i> 'Boelare'	Northern France	1995	J. Pinon		+	-	-
<i>M. larici-populina</i>	96AK1	<i>Larix</i> sp.	Eastern France	1996	C. Husson		+	-	-
<i>M. larici-populina</i>	97CG1	<i>P. × interamericana</i> 'Hoogvorst'	Eastern France	1997	C. Husson		+	-	-
<i>M. larici-populina</i>	98AR1	<i>P. × interamericana</i> 'B-71085-A1'	Belgium	1998	M. Steenackers		+	-	-
<i>M. larici-populina</i>	99C2	<i>Larix</i> sp.	Northern France	1999	C. Husson		+	-	-
<i>M. medusae</i> f. sp. <i>deltoidae</i>	88MM1	<i>P. × euramericana</i> 'Gaver'	Western France	1988	J. Pinon	AY375273	-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	88MM2	<i>P. × interamericana</i> 'Beaupré'	Western France	1988	J. Pinon	AY375274	-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	97CN1	<i>P. × interamericana</i> 'Boelare'	Western France	1997	P. Frey	AY375275	-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	97CN2	<i>P. × interamericana</i> 'Boelare'	Western France	1997	P. Frey		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	97CN3	<i>P. × interamericana</i> 'Beaupré'	Western France	1997	P. Frey		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	97CN4	<i>P. × interamericana</i> 'Beaupré'	Western France	1997	P. Frey		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	97CN5	<i>P. × interamericana</i> 'Beaupré'	Western France	1997	P. Frey		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B1	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B3	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B4	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B5	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B7	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98B8	<i>P. × interamericana</i> '87002-21'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98D1	<i>P. × euramericana</i> '5006'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98D6	<i>P. × euramericana</i> '5006'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98D7	<i>P. × euramericana</i> '5006'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98D9	<i>P. × euramericana</i> '5006'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	98D10	<i>P. × euramericana</i> '5006'	South Africa	1998	G. Hawke		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A3	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A4	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A5	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A6	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A7	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99A8	<i>P. × interamericana</i> 'Hoogvorst'	Western France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99T7	<i>P. × interamericana</i> 'Hazendans'	Eastern France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99V3	<i>P. × interamericana</i> 'Hazendans'	Eastern France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99W1	<i>P. × interamericana</i> 'Hoogvorst'	Eastern France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99W2	<i>P. × euramericana</i> 'Degrosso'	Eastern France	1999	C. Husson		-	-	+
<i>M. medusae</i> f. sp. <i>deltoidae</i>	99W3	<i>P. × euramericana</i> 'Degrosso'	Eastern France	1999	C. Husson		-	-	+
<i>M. larici-tremulae</i>	01F1	<i>P. tremula</i>	Eastern France	2001	P. Frey	EU808026	-	-	+
<i>M. larici-tremulae</i>	PFH-10-10	<i>P. tremula</i>	Eastern France	2010	P. Frey		-	-	+
<i>M. larici-tremulae</i>	PFH-10-16	<i>P. tremula</i>	Eastern France	2010	P. Frey		-	-	+
<i>M. larici-tremulae</i>	PFH-99-1	<i>P. tremula</i>	Eastern France	1999	P. Frey		-	-	+
<i>M. pinitorqua</i>	00S1	<i>P. tremula</i>	Western France	2000	C. Bastien	EU808032	-	-	+
<i>M. pinitorqua</i>	00S2	<i>P. tremula</i>	Southern France	2000	C. Bastien		-	-	+

Table 1 Continued

Species	Code	Host	Origin	Year	Collector/ supplier	GenBank accession	ITS-MLP-F/ ITS-MLP-R	ITS-MAP-F/ ITS-MAP-R	ITS-MMD-F/ ITS-MMD-R
<i>M. pinitorqua</i>	00S3	<i>P. tremula</i>	Western France	2000	C. Bastien		-	-	+
<i>M. pinitorqua</i>	00MP10	<i>P. tremula</i>	Western France	2000	C. Bastien		-	-	+
<i>M. acidioides</i>	PFH-09-10	<i>P. alba</i>	Western France	2009	D. Piou		-	-	-
<i>M. acidioides</i>	PFH-10-08	<i>P. alba</i>	Northern France	2010	M. Leclère		-	-	-
<i>M. acidioides</i>	PFH-00-1	<i>P. alba</i>	United Kingdom	2000	M. Pei		-	-	-
<i>M. magnusiana</i>	GLM 77297	<i>Corydalis cava</i>	Germany	2005	H. Jage		-	-	-
<i>M. magnusiana</i>	GLM 58747	<i>Corydalis cava</i>	Germany	1997	H. Jage		-	-	-
<i>M. magnusiana</i>	GLM 81405	<i>Chelidonium majus</i>	Germany	2008	S. Hoeflich		-	-	-
<i>M. pulcherrima</i>	08ZK1	<i>Mercurialis annua</i>	Italy	2008	S. Moricca		-	-	-
<i>M. pulcherrima</i>	08ZK3	<i>Mercurialis annua</i>	Italy	2008	S. Moricca		-	-	-
<i>M. pulcherrima</i>	08ZK4	<i>Mercurialis annua</i>	Italy	2008	S. Moricca		-	-	-
<i>M. rostrupii</i>	01G1	<i>Mercurialis perennis</i>	Eastern France	2001	J.C. Streito		-	-	-
<i>M. rostrupii</i>	01G20	<i>Mercurialis perennis</i>	Eastern France	2001	J.C. Streito		-	-	-
<i>M. rostrupii</i>	01G21	<i>Mercurialis perennis</i>	Eastern France	2001	J.C. Streito		-	-	-
<i>M. rostrupii</i>	01G22	<i>Mercurialis perennis</i>	Eastern France	2001	J.C. Streito		-	-	-

10 min. One milligram of this mixture was then collected and mixed again in another tube with 9 mg of *Mlp* urediniospores. This process was repeated four times to prepare a 10-fold dilution series, and finally, the last mixture was a 2-fold dilution. The number of *Mmd* urediniospores, readily distinguishable from *Mlp* urediniospores on the basis of their ornamentation (position of echinulations), was assessed for the two-first dilutions by microscopy.

2.4 DNA extraction

Two milligrams of urediniospores from pure mono-uredinial isolates or from mixture of two species was ground for 5 min with ten 2-mm-diameter glass beads and 100 μ l of extraction buffer at 5000 rpm by using a shaker (Ika Vibrax VXR) (Pei et al. 1997). The extraction buffer contained 100 mM Tris (pH 9.0), 20 mM EDTA (pH 8.0), 1.4 mM NaCl, 2% CTAB and 0.2% mercaptoethanol. Total DNA was extracted using the protocol described by Henrion et al. (1994). DNA was finally eluted in 50 μ l TE Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and stored at -20°C until used for PCR tests. The DNA concentration was measured by Hoechst fluorometry (Charcosset and Gardes 1999). In addition, DNA was extracted from approximately 20 mg of poplar leaf ('Robusta') or larch (*Larix decidua*) needles following the protocol described above. DNA extracts were diluted 10 or 100-fold before PCR amplifications.

2.5 Amplification of ITS sequences, cloning, DNA sequencing and primer design

The ITS regions of four isolates of *Mlp* (95XA1, 93ID6, 97A1 and 97J10), two isolates of *M. allii-populina* (96B6-1 and 96M21-1) and three isolates of *Mmd* (88MM1, 88MM2 and 97CN1) were amplified with universal primers ITS1 and ITS4 (White et al. 1990). PCR amplification was carried out in 20- μ l final reaction volumes containing 1x *Taq* DNA polymerase buffer (Sigma-Aldrich, Lyon, France), 1.5 mM MgCl₂, 0.2 μ M of each primer, 200 μ M dNTP, 0.5 unit of *Taq* DNA polymerase (Sigma-Aldrich), 2 μ l of template DNA (0.2–2 ng), and ultrapure water was added to 20 μ l. PCRs were performed by using a PTC-200 Peltier Thermal Cycler (MJ Research, Bio-Rad, Marnes-la-Coquette, France) and included an initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 90 s and a final extension at 72°C for 10 min. As rust fungi are dikaryotic, to avoid sequencing errors due to heterozygous positions, the amplicons were cloned for each isolate, using the pCR[®] 4-TOPO[®]-TA cloning kit (Invitrogen, Cergy Pontoise, France) and following the manufacturer's instructions. Double-stranded DNA sequencing was performed by the di-deoxy-chain termination method using a T3-T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, CA, USA). Forward and reverse sequences were assembled in SEQUENCHER 4.2 (Genecodes, Ann Arbor, MI, USA) and aligned using MULTALIN (Corpet 1988). The ITS sequences obtained were deposited in the GenBank database (Table 1). Species-specific primers were then manually designed following inter- and intraspecific alignment and comparison of the ITS sequences with MULTALIN (Table 2).

2.6 Construction of a PCR Internal Amplification Control (IAC)

A heterologous DNA template with 5' and 3' ending sequences identical to the primer pair ITS-MMD-F and ITS-MMD-R was constructed according to the protocol described by Langrell (2002). Briefly, DNA extracted from leaves of *Alnus glutinosa* was subjected to random amplified polymorphic DNA (RAPD) with a low annealing temperature (36°C). A typical RAPD pattern was revealed by electrophoresis on a 1% agarose gel, and a ca. 1000-bp fragment was arbitrarily chosen as IAC for ITS-MMD-F/ITS-MMD-R-specific PCR. The entire RAPD product was cloned with the pCR[®] 4-TOPO[®] - TA cloning kit - following the manufacturer's instructions. The bacterial clone whose plasmid contained the selected fragment was screened by PCR with specific primers ITS-MMD-F/ITS-MMD-R according to the expected PCR product size. To determine the threshold of PCR sensibility and detection of the IAC, serial dilutions of bacterial suspensions containing the IAC were mixed with a 10 ng–0.05 $\mu\text{g } \mu\text{l}^{-1}$ range of *Mmd* DNA and tested by PCR with ITS-MMD-F/ITS-MMD-R primers. Ready-to-use IAC templates were stored as a suspension of transformed bacteria in ultrapure water at -80°C until used for PCR.

2.7 PCR amplification conditions using species-specific primers

PCR assays were carried out as described above, except that 2 μ l of IAC was added to the PCR mix when ITS-MMD-F/ITS-MMD-R primers were used. A PCR-negative control (no template DNA present in PCR) was included in all experiments.

Table 2. Sequence and specificity of the three *Melampsora* primer pairs developed in this study

Primer pair	Sequence (5'–3')	Amplicon size	Specificity
ITS-MLP-F	GAGCGCACTTTAATGTGACTC	572 bp	<i>M. larici-populina</i>
ITS-MLP-R	ACTTAATTAAGTTGATAGGG		
ITS-MAP-F	CCCATTACCACCCACCCAA	544 bp	<i>M. allii-populina</i>
ITS-MAP-R	TCTAAAGGTTAAATAAATAGG		
ITS-MMD-F	GAGTTGCTTAAATGCGATTC	575 bp	<i>M. medusae</i> <i>M. larici-tremulae</i> <i>M. pinitorqua</i>
ITS-MMD-R	CTAAAGGTAAATCAATGGG		

Alternatively, single aecia or uredinia were simply ground for 10 min with ten 2-mm glass beads and 100 μl of TE buffer in a 1.5-ml Eppendorf tube. In addition, samples of telia were ground with a pestle for 1 min in 100 μl of TE buffer. Two microlitres of both supernatants were diluted 10-fold and then used as a template for PCR. All PCR were performed as described above, except that the annealing temperature was 50°C. PCR products were separated by a 1-h electrophoresis in 1% agarose gels in TBE buffer at 4 V cm^{-1} and visualized by U.V. exposition following ethidium bromide staining. The fragment sizes were estimated by comparison with a 100-bp DNA ladder (Invitrogen). Images were recorded with a GelDoc 2000 system (Biorad, Marnes-la-Coquette, France).

The sensitivity of the *Mmd*-specific primers was assessed by testing serial dilutions ranging from 10 $\text{ng } \mu\text{l}^{-1}$ to 0.05 $\text{pg } \mu\text{l}^{-1}$ of *Mmd* genomic DNA (isolate 88MM1). DNA concentration of the initial solution was measured by Hoechst fluorometry.

2.8 Survey procedures

Since 1993, the French plant protection service has been carrying out annual inspections in commercial nurseries producing poplar saplings or cuttings to survey the quarantine pathogen *Mmd*. Individual samples were made of 12 uredinia per cultivar and were sent for analysis to the Plant Health Laboratory (Anses, French Agency for Food, Environmental and Occupational Health & Safety, Malzéville, France). From 1993 to 2002, the diagnostic protocol was based on the observation of morphological features using a light microscope (Pinon 1973; Vialle et al. 2011). From 2002, Anses started to use the PCR-based detection developed in this study. Briefly, 2–10 mg of uredinia was scraped from infected poplar leaves with a spatula in late summer and transferred into a sterile microcentrifuge tube to be sent to the laboratory for analysis. Each year, from 2002 to 2011, 310, 95, 168, 155, 120, 103, 82, 77, 61 and 64 rust samples were collected respectively and then analysed.

3. Results

3.1 Primer specificity

The specificity of each primer pair was assessed by PCR with DNA from 109 different isolates belonging to 9 distinct *Melampsora* species listed in Table 1, and with samples of poplar and larch DNA. The primer pair ITS-MLP-F/ITS-MLP-R amplified a single 572-bp DNA fragment from all the *Mlp* isolates (Fig. 1). In addition, PCR using ITS-MAP-F/ITS-MAP-R primers exclusively amplified a 544-bp DNA fragment from all the *Map* isolates (Fig. 1). No cross-reaction was reported for

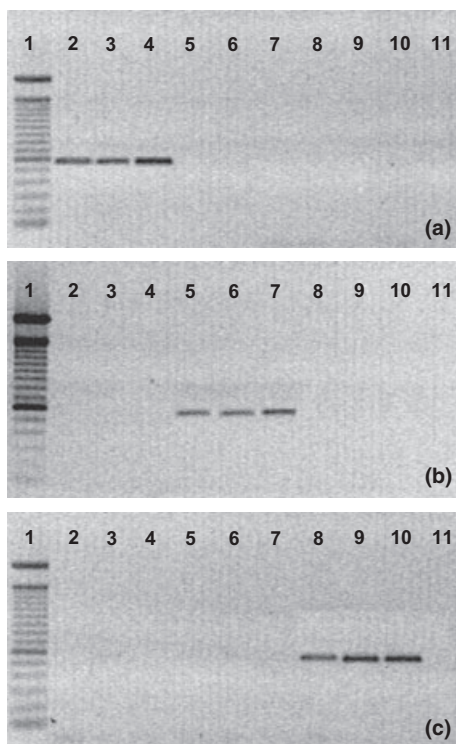


Fig. 1. PCR products from pure DNA of *Melampsora* sp. (0.1–1 $\text{ng } \mu\text{l}^{-1}$) obtained with the primer pairs ITS-MLP-F/ITS-MLP-R (a), ITS-MAP-F/ITS-MAP-R (b) and ITS-MMD-F/ITS-MMD-R (c). Lane 1: 100-bp DNA ladder. Lanes 2–4: *Mlp* 97EA2, 97CF1 and 95XF6. Lanes 5–7: *Map* 96M24-2, 97L2 and 97L5. Lanes 8–10: *Mmd* 88MM2, 97CN1 and 97CN2. Lane 11: negative control with sterile ultrapure water.

both primer pairs. PCR using the primer pair ITS-MMD-F/ITS-MMD-R produced a 575-bp DNA fragment from all the *Mmd* isolates (Fig. 1). However, cross-reactions were observed with *M. larici-tremulae* and *M. pinitorqua* isolates (Table 1 and 2). Last, no amplicon was obtained from poplar or larch DNA extracts with any of the species-specific primer pairs.

3.2 PCR sensitivity

The quantity of *Mlp* and *Mmd* urediniospores per milligram of fresh uredial material was estimated to be approximately 400 000 and 320 000, respectively. In addition, the DNA amount in single urediniospores measured by Hoechst fluorometry was estimated to be 0.8 pg for both species.

The detection threshold for the *Mmd*-specific PCR was firstly assessed by testing serial dilutions of *Mmd* DNA mixed with a background of $10 \text{ ng } \mu\text{l}^{-1}$ *Mlp* DNA. The PCR assay could still yield a positive signal with down to $0.1 \text{ pg } \mu\text{l}^{-1}$ of *Mmd* DNA (Fig. 2). As the elution volume was to $50 \mu\text{l}$, this threshold detection would correspond to a starting quantity of six spores of *Mmd*.

The detection level of the *Mmd*-specific primers was also assessed by testing serial dilutions of *Mmd* urediniospores into *Mlp* urediniospores. The percentage of *Mmd* urediniospores in the two-first serial dilutions was estimated by counting to 8.64% (63 of 729 spores) and 0.87% (4 of 461 spores), respectively. The results confirmed the expected number, that is, 8.16 and 0.82% *Mmd* urediniospores, which means that dilutions were successfully performed. Therefore, the quantity of *Mmd* urediniospores in the six 2-mg mixture was estimated to be 67 000, 6700, 670, 67, 6.7 and 3.3, respectively, into approximately 800 000 *Mlp* urediniospores. A positive PCR signal could be obtained with the DNA extract from the mixture containing an estimated number of 6.7 *Mmd* urediniospores (Fig. 3).

One hundred copies of bacteria containing the cloned IAC in each PCR tube proved to be adequate to allow the amplification of both targets in the presence of a wide range of *Mmd* DNA concentrations and therefore to check the absence of inhibitors in the PCR mix. However, no amplification of the IAC was obtained when a large amount of target *Mmd* DNA was added as a template in the PCR tube, that is, from 10 to $0.1 \text{ ng } \mu\text{l}^{-1}$. Nevertheless, as the target *Mmd* DNA could be amplified, the absence of the IAC amplification product only meant that the large amount of target DNA prevented the amplification of the IAC by PCR competition and that no inhibitory compound was present. In the case where only the IAC band was produced, while no *Mmd* target was amplified, it was assumed that the DNA extract did not contain a detectable amount of *Mmd* DNA.

In addition, PCR tests with *Mlp*-specific primer pairs were successfully carried out on total DNA extracted from artificially inoculated or naturally infected poplar leaves or larch needle samples (data not shown).

3.3 Detection by direct PCR without extraction

PCR detection without prior DNA extraction was only tested with *Mlp*-specific primers. A single and clear band of approximately 570 bp was detected from all the samples of infected leaves or infected needles tested (4 and 15 samples, respectively). On the other hand, PCR test carried out from ground samples of *Mlp* telia were less successful with only 40% of samples yielding a positive signal (three of eight samples).

3.4 Inspections in commercial nurseries

In 2002 and 2003, 405 rust samples collected in 310 and 29 nurseries, respectively, were analysed using both morphological and PCR methods. Results of both methods were similar in terms of relative frequency of *Mlp* vs. *Map* and their geographical distribution. Regardless of the year, *Mlp* and *Map* were detected in approximately 95 and 20% of the samples, respectively. Both species were found together in 11% of individual samples. As expected, *Mlp* was widespread in France, whereas *M. allii-populina* was more frequent in southern and western France. *Mmd*, meanwhile, was never detected. Similarly, resorting solely on the molecular detection tool, no *Mmd* was found in the 830 rust samples analysed between 2004 and 2011.

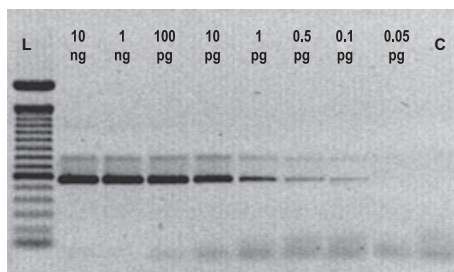


Fig. 2. PCR products obtained with the primer pairs ITS-MMD-F/ITS-MMD-R. The *Mmd* DNA template concentrations (μl^{-1}) added in PCR mixes are indicated above the lanes. L: 100-bp DNA ladder. C: negative control with sterile ultrapure water.

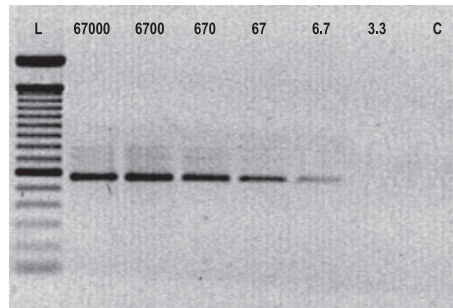


Fig. 3. PCR products obtained with the primer pairs ITS-MMD-F/ITS-MMD-R from a 2-mg mixture of urediniospores. The measured or estimated number of *Mmd* urediniospores in each mixture containing approximately 800 000 urediniospores of *Mlp* is indicated above the lanes. L: 100-bp DNA ladder. C: negative control with sterile ultrapure water.

4 Discussion

The identification of European *Melampsora* species pathogenic on cultivated poplars can be achieved by microscopic observation of urediniospores and paraphyses (Pinon 1973; Vialle et al. 2011). This method can be easily implemented but is not suitable for routine analysis of a large number of samples because it is too time-consuming. In addition, morphological identification to the species level is not possible when only aecia or telia are available. The main goal of this study was the development of a rapid and reliable molecular method based on species-specific polymorphisms observed in the ITS rDNA region for identifying *Mlp*, *Map* and *Mmd*.

The primer pairs ITS-MLP-F/ITS-MLP-R yielded a unique 572-bp amplicon with DNA from *Mlp*. No cross-reaction was observed with DNA from the others species. Finally, these primers could be successfully used in PCR with DNA directly extracted from infected plant tissues, such as poplar leaves or larch needles, and regardless of the spore stage of the fungus as aeciospores, urediniospores or teliospores.

The primer pairs ITS-MAP-F/ITS-MAP-R designed in this study amplified a unique DNA fragment of 544 bp with DNA from *Map*. To our knowledge, this primer pair is the first molecular tool designed for the specific identification of *Map*.

The *Mmd*-specific primers designed in this study produced a fragment of 575 bp with DNA from all the isolates of *Mmd*. No cross-reactions were observed except with *M. larici-tremulae* and *M. pini-torqua* DNA. However, as both species are only pathogenic on poplars belonging to the section *Populus* that are not used in commercial poplar cultivation, the primer pair ITS-MMD-F/ITS-MMD-R thus remains useful and specific for the detection of *Mmd* on cultivated poplars, that is, euramerican and interamerican hybrids. In addition, the sensitivity and selectivity of this new species-specific PCR test proved that it is fit for routine analysis. Indeed, detection of *Mmd* was not impeded by the presence of a background of DNA from non-target species such as *Mlp*, which is the most widespread species in Europe. Moreover, the detection threshold was proved to be very low as the PCR test using *Mmd* primer pair was able to detect down to 6-7 target urediniospores among 800 000 urediniospores of *Mlp*, that is, a ratio of 1 : 100 000. Detection level is therefore reduced by 10 000-fold compared with the morphological method. Thus, the primer pair ITS-MMD-F/ITS-MMD-R could theoretically be able to detect a single *Mmd* uredinium among 100 leaves highly infected by *Mlp* collected on cultivated poplars. Lastly, the addition of an IAC in the PCR mix proved to be successful for detecting false negative results due to inhibitors.

The use of our molecular tools proved to be reliable and less time-consuming than the morphological method for surveying *Mmd* in commercial nurseries. They are now used as official methods for *M. medusae* f. sp. *deltoidae* detection in official quarantine laboratories. Despite the improved sensitivity of the PCR assay over the morphological method, *Mmd* was never detected from 2002 to 2011 in 1235 rust samples, confirming that this species is now absent – or present at undetectable level – on cultivated poplars in France. The situation is probably similar on wild poplars. In 2011, a survey carried out by the French forest health survey service (Département Santé des Forêts, DSF) throughout France did not allow to detect *Mmd* on leaves of *P. nigra* (Pascal Frey, unpublished data).

Very few single-nucleotide polymorphisms were observed in the ITS region between *Mmd*, *M. larici-tremulae* and *M. pini-torqua*, which means that this region is not the most appropriate target to specifically identify these species. This is consistent with a recent phylogeny study, showing that these three species that have a coniferous aecial host belong to the same clade (Vialle et al. 2012). In contrast, *M. rostrupii*, *M. magnusiana* and *M. aecidioides* that have an angiosperm aecial host belong to another clade. Further studies are needed to explore other regions of DNA, as single-copy genes, for discriminating *Mmd*, *M. larici-tremulae* and *M. pini-torqua* (Ioos et al. 2006; Aguilera et al. 2008; Feau et al. 2009).

In conclusion, highly sensitive, reliable, easy-to-use and rapid PCR-based methods have been developed for *Melampsora* species attacking cultivating poplars belonging to sections *Aigeiros* and *Tacamahaca*. These methods can be used on infected poplar and larch tissues and on mixtures of urediniospores collected on poplar leaves. However, *Mmd*-specific primers must be used with care as these primers cross-react with DNA from two *Melampsora* species pathogenic on section *Populus*. Thus, checking the poplar species on which rust samples are collected is necessary before using our PCR assays. Due to its very high sensitivity, *Mmd*-specific primers were applied as the official method for detecting the quarantine parasite *Mmd* on infected leaves in nurseries producing poplar cultivars for trade. The fact that this pathogen was never detected in France since 2002 has to be taken into account for the status of *M. medusae* f. sp. *deltoidae* as an A2 quarantine pest for EPP.

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