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Fungal pathogen (mis-) identifications: A case study with DNA barcodes on *Melampsora* rusts of aspen and white poplar

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ABSTRACT

Wide variation and overlap in morphological characters have led to confusion in species identification within the fungal rust genus *Melampsora*. The *Melampsora* species with uredinial-telial stages on white poplar and aspens are especially prone to misidentification. This group includes the *Melampsora populnea* species complex and the highly destructive pine twisting rust, *Melampsora pinitorqua*, which alternates between hosts in *Populus* section *Populus* and *Pinus*. Our objective was to compare morphologically based identification to genetic material extracted from *Melampsora* species pathogenic to aspen and white poplar. We compared morphometric traits and DNA barcodes obtained from internal transcribed spacer (ITS), large ribosomal RNA subunit (28S), and mitochondrial cytochrome oxidase 1 (CO1) sequences to delimit within this taxonomically difficult group. Eight different *Melampsora* species were initially defined based on host specificity and morphometric data. DNA barcodes were then overlaid on these initial species definitions. The DNA barcodes, specifically those defined on ITS and 28S sequences, provided a highly accurate means of identifying and resolving *Melampsora* taxa. We highlighted species misidentification in specimens from Canadian herbaria related to either *Melampsora medusae* f. sp. *tremuloidae* or *Melampsora aecidioides*. Finally, we evidenced that the north-American species found on *Populus alba*, *M. aecidioides* is closely related but distinct from the four species of the *M. populnea* complex (*Melampsora larici-tremulae*, *Melampsora magnusiana*, *Melampsora pinitorqua*, and *Melampsora rostrupii*) found in Eurasia.

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Introduction

Reliable pathogen identification is a critical first step in assessing potential impact, disease risk and determining management options. Correct, taxonomically sound identification

is particularly important for exotic and potentially invasive species since quarantine enforcement relies on proper diagnosis and rapid action (Desprez-Loustau *et al.* 2007). In this context, identification systems based on species-specific DNA sequences represent a significant advance for

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phytopathologists and taxonomists. The so-called DNA barcoding concept (Hebert *et al.* 2003) provides a rapid, cost-effective standardized means by which one can assess species identities. Though it is not without pitfalls (Moritz & Cicero 2004; Waugh 2007), this system does offer the opportunity to scrutinize, at a fine scale level, taxonomic identification of morphologically similar specimens.

The *Melampsora populnea* (Pers.) P. Karst. complex is a group of morphologically similar rust fungi that produce their uredinial–telial stages on poplar species of the botanical section *Populus* (formerly *Leuce*), including *P. tremula*, *P. alba*, and their hybrids (Wilson & Henderson 1966; Longo *et al.* 1975; Bagyanaryana 1998; Frey *et al.* 2005). The four species of this complex, *Melampsora larici-tremulae* Kleb., *Melampsora magnusiana* G. Wagner, *Melampsora pinitorqua* Rostr., and *Melampsora rostrupii* G. Wagner, share uredinial–telial host species but remain distinguishable by their spermogonial–aecial host specificity. *M. pinitorqua* represents an important forest threat due to stem infections on alternate pine (*Pinus*) (spermogonial–aecial stage) hosts, which cause twisting, deformation and death in young trees (Desprez-Loustau & Dupuis 1992; Naldini-Longo *et al.* 1992). This fungus has not been reported in North America, apart from one record on *Pinus ponderosa* seedlings in a British Columbia (BC) nursery in the early 1960s (Ziller 1961, 1962). This identification was later judged questionable (Björkman 1964; Molnar & Sivak 1964) and its reassessment was definitively made by Ziller himself few years after (Ziller 1974). No other documented report of *M. pinitorqua* in North America has been made since, albeit this fungus could represent a serious concern for many native pines. Indeed, epidemiologic studies conducted in areas infested by *M. pinitorqua*, have demonstrated the susceptibility of North American pines such as *Pinus contorta*, *Pinus strobus*, *Pinus ponderosa*, *Pinus echinata*, and *Pinus taeda* (Klingström 1963; Longo *et al.* 1975; Karlman 2001).

White poplar, *Populus alba* and its hybrids, are known to be immune to the common leaf rusts (*Melampsora medusae* f. sp. *deltoideae* Shain, *Melampsora occidentalis* H.S. Jacks., and *Melampsora larici-populina* Kleb.) found on North American poplars belonging to the botanical sections *Aigeros* and *Tachamaca*. In Europe, *P. alba* is an important uredinial–telial host of rust species included in the *M. populnea* complex (including *M. pinitorqua*; Pinon 1973; Longo *et al.* 1975; Bagyanaryana 1998), whereas in North America, only *Melampsora abietis-canadensis* C.A. Ludw. and *Melampsora aecidioides* (DC.) J. Schröt. are mentioned on this host (Arthur 1962; Sinclair & Lyon 2005). The name *M. aecidioides* has been erroneously used by different authors, and taxonomic placement of this species relative to other *Melampsora* rusts remains unclear. Arthur (1962) mentioned that the *M. aecidioides* species has been assigned to *M. abietis-canadensis* in his treatment of North American rusts. The former species has been considered by some authors as part of the *M. populnea* complex and is sometimes defined as being synonymous with *M. rostrupii* and *M. magnusiana* (Tian *et al.* 2004; Tian & Kakishima 2005; Farr *et al.* 2008) or with *M. populnea sensu lato* (Van Vloten 1946; Savile 1973; Bagyanaryana 1998). As a consequence, *M. populnea sensu lato* has recurrently been attributed to leaf rusts encountered on *P. alba* and its hybrids in North and Central America (Hennen & McCain 1993; Newcombe *et al.* 2001; Sinclair & Lyon 2005).

Distinctive traits among pathogenic taxa are a key prerequisite to meaningful taxonomic classification, accurate identification and detection. This point appears specifically important when a number of supposedly related subspecies cause disease on the same host e.g. *Melampsora* rusts that produce their uredinial–telial stages on white poplars. By assessing the DNA barcoding concept of species identification proposed by Hebert *et al.* (2003), our aim was to address specific taxonomical questions about the *M. populnea* complex. The main practical objective of this work was to provide accurate and unambiguous data for the molecular identification of the highly destructive pathogen *M. pinitorqua* in order to detect any introduction of this pathogen in North America. The achievement of such objective needs to identify the rusts collected on poplars of the botanical section *Populus* in North America (*P. alba* and aspen poplars, *Populus tremuloides* and *Populus grandidentata*) and eventually to differentiate them from *M. pinitorqua*. The DNA barcoding system developed in this study is based on a 650 bp region at the 5′-end of the mitochondrial cytochrome oxidase 1 gene (5′-CO1). The sequence diversity among our *Melampsora* collections was further examined for the nuclear internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S–5.8S–28S) and a portion of the large subunit of the ribosomal rDNA (28S), both commonly used as DNA-based discrimination systems for fungal species (Seifert *et al.* 2007).

Material and methods

Sampling

Twenty strains were directly sampled from fresh collections of aecia and uredinia. Nine dried collections obtained from four herbaria were also sampled (Table 1). Each specimen obtained had previously been assigned a species name based on the examiner's identification of the herbarium specimens and collector's identification of the fresh strains. All fresh strains collected were deposited at the René Pomerleau Herbarium (Laurentian Forestry Centre, Quebec City, <http://cfs.rncan.gc.ca/index/pomerleauherbarium>).

Morphometrical analysis observations and urediniospores cluster analysis

Aeciospores or urediniospores were mounted in a solution of cotton blue lactophenol (20 g phenol, 20 g lactic acid, 40 g glycerol, 200 ml water, and 0.05 g cotton blue), and examined by light microscopy using a Reichart Polyvar microscope. For each *Melampsora* strain, 6 morphometric traits e.g. indirect length, width, and wall thickness measurements for 30 spores and 10 paraphyses were measured at 500× magnification. Paraphyses attributes were only determined if viewed longitudinally. The mean values obtained for each strain were plotted on a scatter plot 3D using SigmaPlot for Windows Version 10.0 (Systat Software).

Cluster and ordination analyses were used to explore the variation in morphometric traits. The data set (6 morphometric traits × 21 OTU matrix of individual fungal specimens on uredinial stage) was entered directly into a computerised

Table 1 – Fungal species and strains included in this study

Identification ^a	Taxon ID	Host sampled	Geographic origin – spatial coordinates [date of sampling]	Herbarium ID ^c [culture ref.]
<i>Melampsora abietis-canadensis</i>	666-X-TSC-SH11	<i>Tsuga canadensis</i>	St-Hippolyte, QC, Canada – 45°57'23"N/74°00'00"W [2007-07-01]	
	667-X-TSC-SH12	<i>T. canadensis</i>	St-Hippolyte, QC, Canada – 45°57'23"N/74°00'00"W [2007-07-01]	
	418-MEA-POT-BE4	<i>Populus tremuloides</i>	Ste-Anne River, Montmorency Co., QC, Canada – 47°04'00"N/70°52'00"W [1960-08-18]	QFB 18187
	570-MEA-X-ON38	<i>Populus alba</i> var. <i>pyramidalis</i>	Cumberland, Russel Co., ON, Canada – 45°29'00"/75°29'00" [1977-07-11]	DAOM 164840
	894-MEA-POT-DO1	<i>P. tremuloides</i>	St-Louis-de-Gonzague, Dorchester, QC, Canada – 45°12'00"N/73°59'00"W [1966-08-31]	QFB 3268
	895-MEA-POT-SU5	<i>P. tremuloides</i>	St-Urbain, Charlevoix, QC, Canada – 47°33'00"N/70°32'00"W [1960-08-24]	QFB 18209
<i>Melampsora</i> sp. on <i>P. alba</i>	380-ME-PO-BC7	<i>P. alba</i>	Jericho Beach, Vancouver, BC, Canada – 49°16'28"N/123°13'41"W [2006-10-13]	
	664-ME-POA-BC45 ^b	<i>P. alba</i>	Colquitz Creek, BC, Canada – 48°27'32"N/123°23'40"W [2007-06-19]	
	665-ME-POA-BC46 ^b	<i>P. alba</i>	Colquitz Creek, BC, Canada – 48°27'32"N/123°23'40"W [1992-04-22]	DAVFP 24319
	899-MEI-POA-VC1 ^b	<i>P. alba</i>	Colquitz Park, Victoria, BC, Canada – 48°27'32"N/123°23'40"W [2007-11]	[Ma07VIC01]
	900-MEI-POA-VC2 ^b	<i>P. alba</i>	Colquitz Park, Victoria, BC, Canada – 48°27'32"N/123°23'40"W [2007-11]	[Ma07VIC02]
	901-MEI-POA-VC3 ^b	<i>P. alba</i>	Colquitz Park, Victoria, BC, Canada – 48°27'32"N/123°23'40"W [2007-11]	[Ma07VIC03]
<i>Melampsora larici-tremulae</i>	567-MLT-X-HU3 01F1 ^e	<i>Populus tremula</i> <i>P. tremula</i>	Râm-Szabadek Valley, Hungary – [1992-05-12] Champenoux, Meurthe-et-Moselle, France – 48°45'00"N/06°20'19"E [2001-06-05]	DAOM 216572
	<i>Melampsora magnusiana</i>	1328-MEG-CORV-AT	<i>Corydalis cava</i>	Rosaliengebirge, Burgerland, Austria – 47°46'01"N/16°19'01"E [2000-04-19]
1330-MEG-CJ-FI		<i>Chelidonium majus</i>	Turku, Regio aboënsis, Finland – [1936-06-14]	BPI0023740
1331-MEG-CJ-SW		<i>C. majus</i>	Vardsatra, Uppsala, Sweden – [1964-06-14]	BPI 193858
<i>Melampsora rostrupii</i>	01G1	<i>Mercurialis perennis</i>	Gorze, Moselle, France – 49°03'N/06°00'E [2001-05-12]	
<i>Melampsora pinitorqua</i>	902-MEM-PNM-TW1 ^d	<i>Pinus ponderosa</i>	Telkwa, BC, Canada – [1960-07-11]	DAVFP 12177
	903-MEM-PNM-TW2 ^d	<i>P. ponderosa</i>	Telkwa, BC, Canada – [1960-07-08]	DAVFP 12464
	97MP10	<i>Pinus sylvestris</i>	Charmes, Vosges, France – 48°23'56"N/06°22'45"E [1997-06]	
	97MP08	<i>P. sylvestris</i>	Charmes, Vosges, France – 48°23'56"N/06°22'45"E [1997-06]	
	97MP09	<i>P. sylvestris</i>	Charmes, Vosges, France – 48°23'56"N/06°22'45"E [1997-06]	
	00S1	<i>P. tremula</i>	Cestas, Gironde, France – 44°44'N/00°46W [2000]	
<i>Melampsora</i> sp. on <i>P. tremula</i>	892-MPO-PTA-FR3	<i>P. tremula</i>	Cornebarrieu, Haute-Garonne, France – 43°38'45"N/01°15'28"E [2006-09-23]	
	893-MPO-PTA-FR4	<i>P. tremula</i>	Cornebarrieu, Haute-Garonne, France – 43°38'45"N/01°15'28"E [2006-09-23]	

(continued on next page)

Table 1 – (continued)

Identification ^a	Taxon ID	Host sampled	Geographic origin – spatial coordinates [date of sampling]	Herbarium ID ^c [culture ref.]
<i>Melampsora medusae</i> f. sp. <i>tremuloidea</i>	896-MMT-POT-USAS ^f	<i>P. tremuloidea</i>	Indian Reserve, WI, USA – [2005-09-24]	[Mmt05WtSC467]
	897-MMT-POT-QC12 ^f	<i>P. tremuloidea</i>	Grands-Jardins Provincial Park, Charlevoix, QC, Canada – 47°42'00"N/70°42'00"E [2005-07-30]	[Mmt05PGfj171]
	898-MMT-POT-DLI ^f	<i>P. tremuloidea</i>	Sainte-Croix, QC, Canada – 46°39'45"N/71°50'20"W [2005-07-24]	[Mmt05Djoly159]
	683-MMT-POT-LC3	<i>P. tremuloidea</i>	Lac-Étchemin, QC, Canada – 46°23'27"N/70°28'08"W [2007-07-07]	
	822-MMT-POT-CB9	<i>P. tremuloidea</i>	Chibougamau, QC, Canada – 49°52'11"N/74°24'42"W [2007-08-02]	
<i>M. medusae</i>	168-MEM-POA-X25	<i>P. alba</i>	St-Isidore, Dorchester, QC, Canada – 46°35'05"N/71°09'53"W [1984-07-06]	QFB 17185

^a Initial identifications made by specimen collectors or herbarium curators.

^b These strains were identified as *M. aecidioides* after a morphological re-examination of the specimen.

^c Herbarium names. DAVFP, Pacific Forestry Centre's Forest Pathology Herbarium, Victoria, BC (http://www.pfc.cfs.nrcan.gc.ca/biodiversity/herbarium/index_e.html); QFB, René Pomerleau Herbarium, Canadian Forest Service, Québec, QC; DAOM, National Mycological Herbarium, Botany and Plant Pathology Division, Dept. of Agriculture, Ottawa, ON; BPI, U.S. National Fungus Collection, USDA Agricultural Research Service, Beltsville, Maryland, USA.

^d Initially identified as *M. pinitorqua* (Ziller 1961, 1962), reassessed as *M. medusae* (Ziller 1974) and recorded as *M. medusae* in the DAVFP herbarium.

^e Pathogenicity tested by inoculation on *Larix* sp. (Frey et al. unpublished data).

^f For these strains, microscopic examinations were made on uredinial material harvested from artificially inoculated leaf discs of their respective uredinial-telial host.

matrix and a cluster analysis was performed using the R statistical software (R Development Core Team 2008). Prior to multivariate analysis, the data were log-transformed to meet the assumption of normality. All 11 variables were then standardized by subtracting the mean and dividing by the standard deviation to avoid biasing the result by a scale factor (Sokal 1986). A similarity matrix was calculated from the standardized data set using the general dissimilarity coefficient of Gower (1971). The Gower's method presents the advantage that OTU with missing observations can be included for comparison with the other OTU (Dabinett & Wellman 1978). A phenogram was finally generated using the complete linkage clustering algorithm (Dabinett & Wellman 1978).

DNA barcodes production

For each strain, a single sorus (uredinium or aecium) was excised from the infected host tissues, and DNA extraction was performed using the DNeasy Plant Mini kit (Qiagen). Manufacturer's protocol was followed except that following tissue homogenization, the proteinase K (0.5 mg/ml) incubation step at 55 °C was increased to 2 h.

Full length internal transcribed spacer (ITS) sequences were amplified by using primers ITS1F (Gardes & Bruns 1993) and ITS4BR (5'-TCAACAGACTTGTACATGGTCC-3'; Morin et al. unpublished data). A 755 bp internal region of the large subunit of the ribosomal DNA (28S) was amplified using the ITS4-BRf (5'-GGACCATGTACAAGTCTGTTGA-3') and LR5 primers (Vilgalys and Hester, 1990). PCRs were carried out in a 25 µl reaction volume consisting of 1 µl of undiluted DNA template, 0.35 µM of each primer, 0.2 mM of each dNTPs (GE Healthcare UK & Ireland, Little Chalfont, UK), 1.6 mM MgCl₂, 1 µg µl⁻¹ of BSA, and 1 U of Platinum Taq DNA polymerase (Invitrogen) in a 1× Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8, and 50 mM KCl), with thermocycling conditions as follows: denaturation for 3 min at 94 °C, 35 cycles at 94 °C for 30 s, 30 s at 50 °C for the ITS1F/ITS4BR primer pair or at 55 °C for ITS4-BRf/LR5, and 72 °C for 70 s with a final extension of 10 min at 72 °C. Two primer pairs, Cox1MlpAF (5'-TAAGATGACTTTATAGTACCAA-3')/Cox1MlpAR (5'-GCTCCTACCATTACMG-3') and Cox1MlpB2F (5'-CTGCTATGCCCAAGTCTAA-3')/Cox1MlpCR (5'-ATGTGATGACTTCAAACCAC-3'), designed by comparison of rust CO1 sequences (Vialle et al. unpublished data), were used for the amplification of two composite overlapping regions located at the 5'-end of the CO1 gene (5'-CO1). PCRs were carried out in a 20 µl reaction volume consisting of 1 µl of undiluted DNA template, 0.2 µM of each primer, 0.15 mM of each dNTPs (GE Healthcare UK & Ireland), 1.5 mM MgCl₂, and 1 U of platinum Taq DNA polymerase (Invitrogen) in a 1× Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8, and 50 mM KCl), with conditions as follows: denaturation for 3 min at 95 °C, 36 cycles at 95 °C for 45 s, 47 °C for 30 s with primer pair Cox1MlpAF/Cox1MlpAR or 50.5 °C for 30 s with Cox1MlpB2F/Cox1MlpCR, and 72 °C for 70 s. All PCR products were visualized by ultra-violet fluorescence following 1.5 % agarose gel electrophoresis in 1× TAE buffer and ethidium bromide staining. PCR sequence products were sequenced in both directions with the appropriate amplification primers using the Big Dye Terminator Cycle Sequencing Kit vs. 1.1 on an ABI 3730xl sequencer (Applied

Biosystems) at the CHUL Research Centre (CRCHUL) Sequencing and Genotyping Platform, Quebec City, QC, Canada.

DNA barcodes analysis

Sequences were manually edited using BioEdit v.7.0.9 (Hall 1999) to remove ambiguous base calls and primer sequences, and were then aligned using the CLUSTALW software (Thompson et al. 1994). Sequence information is available in the Barcode of Life Database (BOLD, www.barcodinglife.org), along with collateral information. Kimura's two-parameter model (K2P) of base substitution implemented in PAUP ver. 4.0b10 (Swofford 2003) was used to calculate intra- and inter-specific genetic distances. Distribution of these two categories of distances was plotted for each gene using the Boxplot representation of Tukey (1977).

Phylogenetic analysis

Maximum likelihood trees were reconstructed among each data set using PAUP ver. 4.0b10. All nucleotide sites were included in the phylogenetic analyses but gaps were treated as missing characters. The best-fit model of DNA substitution and the parameter estimates used for tree reconstruction were chosen by performing hierarchical likelihood-ratio tests in Modeltest v. 3.06 (Posada & Crandall 1998). Heuristic Maximum Likelihood (ML) searches were performed with 100 replicates of random sequence addition and tree bisection-reconnection (TBR) branch swapping. Branch support was evaluated using 100 bootstrap replicates and 10 random additions of sequences (RAS) per pseudo-replicate. Topological incongruences between ML trees were evaluated by inspecting strength of bootstrap support for individual branches as described in Feau et al. (2006, 2007).

Results

Morphological observation and urediniospores cluster analysis

Morphometric traits of all the strains were recorded through microscopic examinations and compared to data found in the literature (Fig 1A). The observation of ellipsoid to obovate aeciospores with fine verrucose surfaces ($16.3\text{--}17.3 \times 19.5\text{--}19.7 \mu\text{m}$) agreed with published descriptions of *Melampsora abietis-canadensis* (Arthur 1962), confirming the identification of two strains (666-X-TSC-SH11, 667-X-TSC-SH12) whose preliminary identification was based on specificity with their spermatogonial-aecial host, *Tsuga canadensis* (Ludwig 1915; Arthur 1962). Morphometric values obtained for the *M. abietis-canadensis* strains (418-MEA-POT-BE4, 894-MEA-POT-DO1, and 895-MEA-POT-SU5) collected on *Populus tremuloides* (uredinial-telial stage) matched those described in the literature for this species (Arthur 1962; Shain 1988). However, the strains 894-MEA-POT-DO1 and 895-MEA-POT-SU5 showed strong urediniospore similarities (urediniospores ellipsoid or obovoid, with wall as aeciospores, and with smooth areas at the equator, $13\text{--}21 \times 22\text{--}36 \mu\text{m}$) with three *Melampsora medusae* f. sp. *tremuloidae* strains

(822-MMT-POT-CB9, 896-MMT-POT-USA5, and 898-MMT-POT-DL1), underlining the overlap in urediniospore sizes between the latter species and *M. abietis-canadensis* (Fig 1B). Urediniospore shapes and echinulations observed for the six rust strains collected in BC on *Populus alba* (380-ME-PO-BC7, 664-ME-POA-BC45, 665-ME-POA-BC46, 899-MEI-POA-VC1, 900-MEI-POA-VC2, and 901-MEI-POA-VC3) strongly matched those described for *Melampsora aecidioides* e.g. urediniospores obovate or broadly ellipsoid, wall yellowish or colorless, $2\text{--}3 \mu\text{m}$ thick, prominently echinulate-verrucose, without smooth spot (Arthur 1962). These strains exhibited values of urediniospore lengths and widths that sometimes overlapped with values observed and expected for *Melampsora larici-tremulae*, *Melampsora pinitorqua*, and *Melampsora rostrupii*. Ordination obtained from the cluster analysis agreed with these observations since the strains collected on *P. alba* were found dispersed in various clusters, grouping with the different strains related to the *Melampsora populnea* species complex (*M. larici-tremulae*, *M. pinitorqua*, and *M. rostrupii*) (Fig 1B). Such variation in urediniospore length and width was expected for *M. aecidioides* ($15\text{--}22 \times 20\text{--}30 \mu\text{m}$, Arthur 1962). Other species of the *M. populnea* complex exhibit less variation with small (*M. pinitorqua* and *M. larici-tremulae*, $10\text{--}16 \times 14\text{--}23 \mu\text{m}$; *Melampsora magnusiana*, $10\text{--}16 \times 14\text{--}23 \mu\text{m}$; and *M. rostrupii*, $14\text{--}18 \times 17\text{--}28 \mu\text{m}$) and ovoid to globular urediniospores (Pinon 1973). Furthermore, we observed that all six *Melampsora* on *P. alba* strains produced large paraphyses with length, width, and wall thickness values of $56\text{--}77 \mu\text{m}$ (mean $66.7 \mu\text{m} \pm 12.3$), $16\text{--}27 \mu\text{m}$ (mean $20.3 \mu\text{m} \pm 3.9$), and $1.2\text{--}4 \mu\text{m}$ (mean $2.9 \mu\text{m} \pm 12.3$), respectively (Supplementary File 1). Large paraphyses had previously been described for both *M. aecidioides* ($40\text{--}60 \times 15\text{--}22 \mu\text{m}$; Saccardo 1888) and *M. rostrupii* (Pinon 1973). Based on host affinity with *Populus tremula*, strains 892-MPO-PTA-FR3 and 893-MPO-PTA-FR4 were determined to belong to the *M. populnea* complex (Wilson & Henderson 1966; Pinon 1973; Frey et al. 2005). However, as illustrated by the results obtained with the ordination analysis (Fig 1B), the morphological traits of the uredinial-telial stages are not sufficiently distinctive to allow a straightforward identification of the different species belonging to the *M. populnea* complex.

Barcode analysis

Sequence data for the ITS, 28S, and 5'-CO1 regions were recovered for all 32 strains of our collection. Based on the initial definition of the nine different morphological and host-specific species, 60 intra- and 436 inter-specific pairwise sequence comparisons were performed for each of the three data sets. The magnitude of these pairwise K2P distances varied with the locus considered. Distance values ranged from 0.0 to 0.099 for ITS, 0.0–0.052 for 28S, and were limited to 0.0–0.005 for 5'-CO1 (Fig 2A). Following the concatenation of the ITS, 28S, and 5'-CO1 loci, the distance values (0.0–0.048) appeared uniformly lower than those for the ITS or the 28S alone. Large overlaps were observed between the upper quartile of the intra- and the lower quartile of the inter-specific distance distributions for each data set (ITS, 28S, 5'-CO1, and concatenated). Outlier data above the inter-specific distributions witnessed the presence of genetically divergent (K2P distance > 0.02) lineages within *M. abietis-canadensis* (subdivided into three groups with K2P distances ranging from 0.016 to 0.04) and *M. pinitorqua* (subdivided into two groups with K2P distances = 0.02)

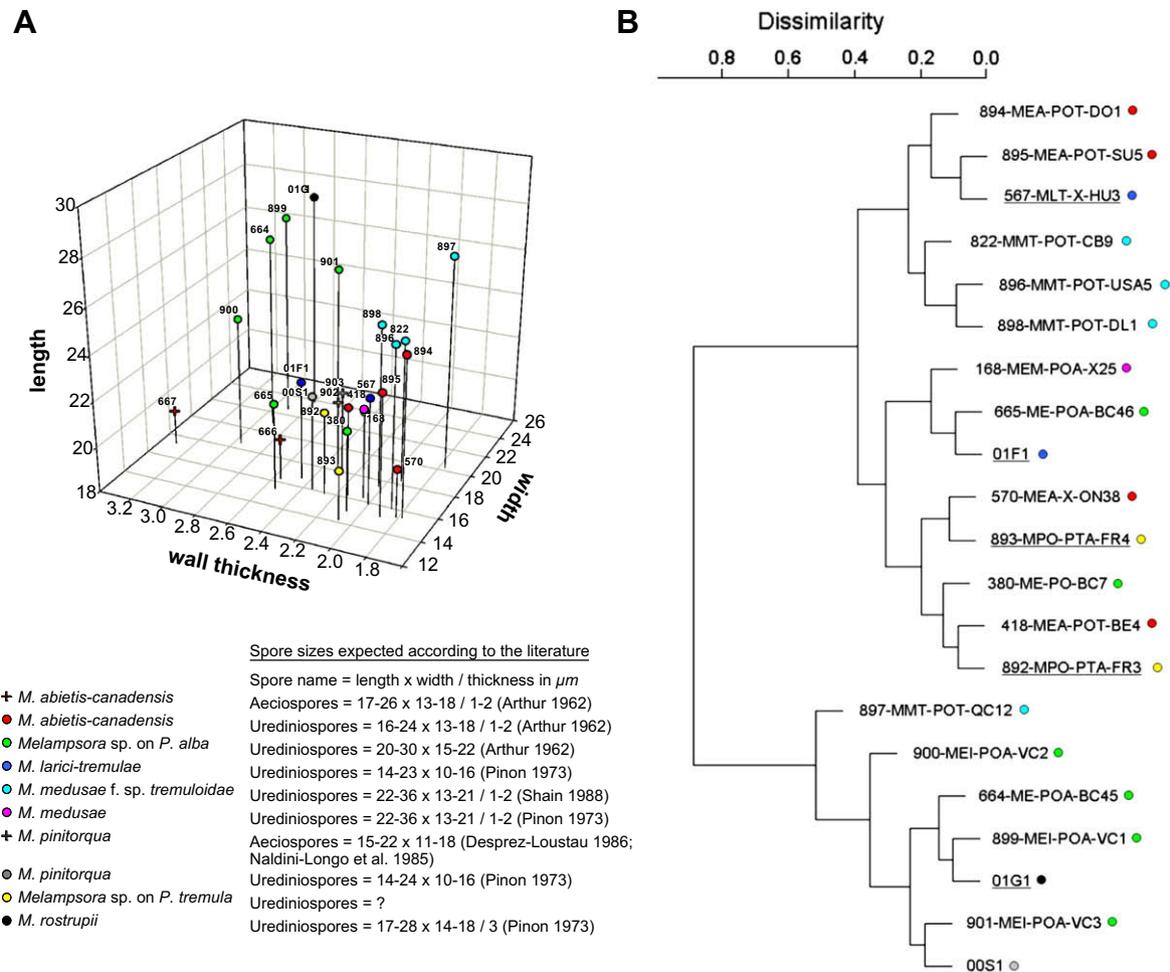


Fig 1 – Morphometrical traits of the different *Melampsora* strains considered in this study. (A) Morphometry scatter plot 3D of aeciospore and/or urediniospore length, width, and wall thickness. Colored circles and crosses represent urediniospores and aeciospores, respectively. The number above each symbol corresponds to the three first characters of the Taxon ID in Table 1. (B) Cluster analysis of the different strains according to urediniospore and paraphyse lengths, widths, and wall thickness.

(Fig 3). Conversely, the observation of K2P distances close to 0.0 within the inter-specific comparison suggested the occurrence of conspecific strains previously recognized as different morphological species. Using the multilocus DNA barcoding, seven misidentifications were highlighted among the 29 strains analysed (i.e. 24 % of all samples analysed) (Fig 3). A first lineage within *M. abietis-canadensis* exhibited a K2P distance of 0.0 with the six strains collected on *P. alba* and previously identified as *M. aecidioides* and the *M. medusae* strain 168-MEM-POA-X25; a second lineage, which includes three *M. abietis-canadensis* strains (894-MEA-POT-DO1, 895-MEA-POT-SU5, and 418-MEA-POT-BE4) and two *M. pinitorqua* strains (902-MEM-PNM-TW1 and 903-MEM-PNM-TW2), exhibited K2P distance values ranging from 0.0 to 0.001 with the five *M. medusae* f.sp. *tremuloidae* strains. Inter-specific distances within the *M. populnea* complex were found close to 0.0 for comparisons between *M. pinitorqua*, *M. larici-tremulae*, and the strains collected on *P. tremula*. Only two single nucleotide polymorphisms (SNPs) localized in the ITS and 28S loci were found between the *M. pinitorqua* and *M. larici-tremulae* strains. Both SNPs were found heterozygous for the *M. larici-tremulae* strains (Y e.g. C or T for both), with thus in each case one allele

strictly identical to the *M. pinitorqua* genotype (C and T in the ITS and the 28S respectively). Similarly, *M. rostrupii* and *M. magnusiana* exhibited two SNPs localized in the ITS loci and notably low K2P distance values which varied from 0.0 to 0.096 depending on the locus considered. The sequences from the six strains collected on *P. alba* and previously identified as *M. aecidioides* clustered in one single molecular group with K2P distances of 0.0. Five distinct molecular species were resolved using the combined sequence data from the ITS, 28S, and 5'-CO1 loci (Fig 3).

Following the species reassignment of the seven misidentified strains, intra- and inter-specific pairwise distance distributions were re-examined (Fig 2B). The intra-specific distribution increased to 108 comparisons, whereas inter-specific decreased to 388. For each data set, a complete absence of overlap was observed between the intra- and inter-specific distributions. The combined data set yields the best result for sequence-based identification analysis despite its limited range of distance values relative to the ITS data set. By combining the three loci, the variation within species circumscriptions is reduced (to a value of $3e^{-04}$) relative to the distance between species (Fig 2B).

Phylogenetic analysis

Maximum likelihood (ML) analyses of each separate data set, using the reciprocal 70 % bootstrap threshold (Feau *et al.* 2006, 2007), resulted in topologically congruent trees (data not shown). The phylogenetic analysis conducted on the combined ITS, 28S, and 5'-CO1 data sets resulted in apparent good level of resolution with only one single tree obtained ($-\ln = 3745$) (Fig 4A). The analysis resolved five different phylogenetic species with a bootstrap support (BS) up to 90 %. The four species of the *M. populnea* complex were partially resolved: *M. larici-tremulae* and *M. pinitorqua* were unresolved within one single and well-supported (BS = 100 %) monophyletic clade; *M. rostrupii* (strain 01G1) and *M. magnusiana* were also found unresolved in one statistically supported clade (BS = 99 %) and appeared more related to the *M. aecidioides* clade (BS = 100 %) than the other members of the complex. Eleven additional ITS and 28S sequences representing four additional *Melampsora* species collected on white poplar and aspen were downloaded from the non-redundant GenBank database and included in an ITS/28S ML phylogeny (Fig 4B-C). ML phylogenetic reconstruction resulted in six trees of equal length ($-\ln = 2254$) (Fig 4B). No additional phylogenetic species was resolved following the addition of these 11 taxa. Five strains labelled as either *M. populnea* (strain AAH00-1; Pei *et al.* 2005) or *M. magnusiana* (Liang *et al.* 2006) clustered with the *M. aecidioides* clade (BS = 89 %). The *M. laricis* (synonym *M. larici-tremulae*) strain (TSH R4149; Liang

et al. 2006) matched the *M. pinitorqua*/*M. larici-tremulae* clade. The five last unidentified strains (*Melampsora* sp.; Tian *et al.* 2004) were distributed among the *M. aecidioides*, *M. rostrupii*/*M. magnusiana*, and *M. pinitorqua*/*M. larici-tremulae* clades.

Discussion

Exact species identification of fungal pathogens is important in plant pathology since misidentification may lead to erroneous management advice. This is especially true for the aspen and white poplar leaf rusts, which include the highly destructive *Melampsora pinitorqua* involved in pine plantation outbreaks in Europe (Desprez-Loustau & Dupuis 1992; Naldini-Longo *et al.* 1992). Given the potential dangerous impact of pine twist rust on forest trees in North America, eradication of this disease and prevention of re-infection in the case of its occurrence may involve drastic strategies such as total destruction of infected pine plantations. For example, the recommended destruction of contaminated ponderosa pine seedlings from a BC nursery following the morphological identification of *M. pinitorqua* rust (Ziller 1962) was later questioned. Indeed, Molnar & Sivak (1964) successfully infected ponderosa pines with basidiospores from different strains of the poplar leaf rust *Melampsora albertensis* Arthur (synonym = *Melampsora medusae* [Ziller 1965, 1974]), which led them to hypothesise that the disease recorded earlier

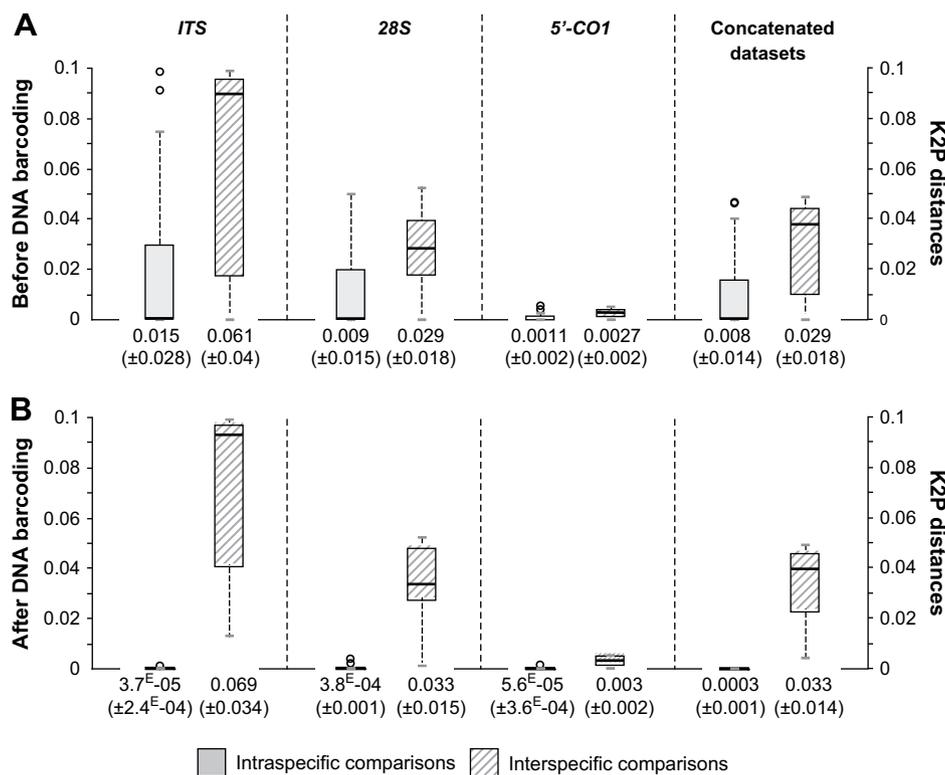


Fig 2 – Boxplot distributions of intra-specific (60 comparisons) and inter-specific (436 comparisons) K2P distances between sequence pairs of the ITS, 28S, and 5'-CO1 genes for the 32 *Melampsora* strains included in this study. Distances were considered using species delimitation based on (A) morphological traits and host affinities, and (B) DNA barcoding criteria. Boxplot representations include the median (central bar), the position of the upper and lower quartiles (called Q1 and Q3; central box), extremes of the data (“whiskers”) and very extreme points that can be considered as outliers (dots; points that exceed $Q1 + 1.5IQR$ and $Q1 - 1.5IQR$ for the upper and lower parts of the distribution, respectively, where $IQR = Q3 - Q1$).

(Ziller 1961, 1962) was caused by the indigenous *M. medusae* and not by the European pine twist rust *M. pinitorqua*. Ziller himself (1974) made a reassessment of this identification few years after and finally described the fungus occurring on ponderosa pines as *M. medusae*. Using our DNA barcode assay, we also determined that the two herbarium specimens collected on *Pinus ponderosa* in 1960–1961 (Ziller 1961) were undoubtedly distinct from reference strains of *M. pinitorqua* collected in Europe on both aecial (pines) and telial (poplars) hosts. As expected, we found strong homology between the putative North American *M. pinitorqua* sequences and those obtained for *M. medusae* f. sp. *tremuloidae* (K2P distance = 0.001), which produces its uredinial–telial stage on trembling and bigtooth aspen (Shain 1988) and its spermogonial–aecial stage on larches (*Larix* spp.), Douglas-fir (*Pseudotsuga menziesii*) and pines (Molnar & Sivak 1964; Ziller 1965). Our microscopic observations of the herbarium specimens collected by Ziller (1961) showed that the size ranges and characteristics of aeciospores fit both *M. pinitorqua* and *M. medusae*. These results were in accordance with those from Molnar & Sivak (1964) who reported that morphologies of the *M. pinitorqua* and *M. medusae* aeciospores found on pines did not differ sufficiently to distinguish these two

species, resulting sometimes in unreliable disease identification. Surprisingly, the delimitation between the *Melampsora larici-tremulae* (synonym = *M. laricis*) strains obtained in this study and *M. pinitorqua* appeared less straightforward than expected. *M. larici-tremulae* and *M. pinitorqua* are distinguishable by their spermogonial–aecial host specificity on larch and pine, respectively. Moreover, they differ in minute (and sometimes hard to detect) microscopic details such as wall thickness at the urediniospore equator (Hennebert 1964; Pinon 1973). Based on an alignment of three loci representing 2130 nucleotides, only two SNPs (heterozygous in *M. larici-tremulae*) were found between both species. Morphological determinations of the two *M. larici-tremulae* strains included in this study were based first on urediniospore and paraphyses characters and second for the 01F1 strain, on successful inoculation on larch, the spermogonial–aecial host (Frey et al. unpublished data). Similarly, identification of strains from pines with similar DNA sequences will allow irrevocably the diagnostic of *M. pinitorqua*. Given the risks associated with the introduction of this fungus on North American pines (at less five potentially susceptible pine species), the availability of reference DNA barcodes for *M. pinitorqua* should now avoid

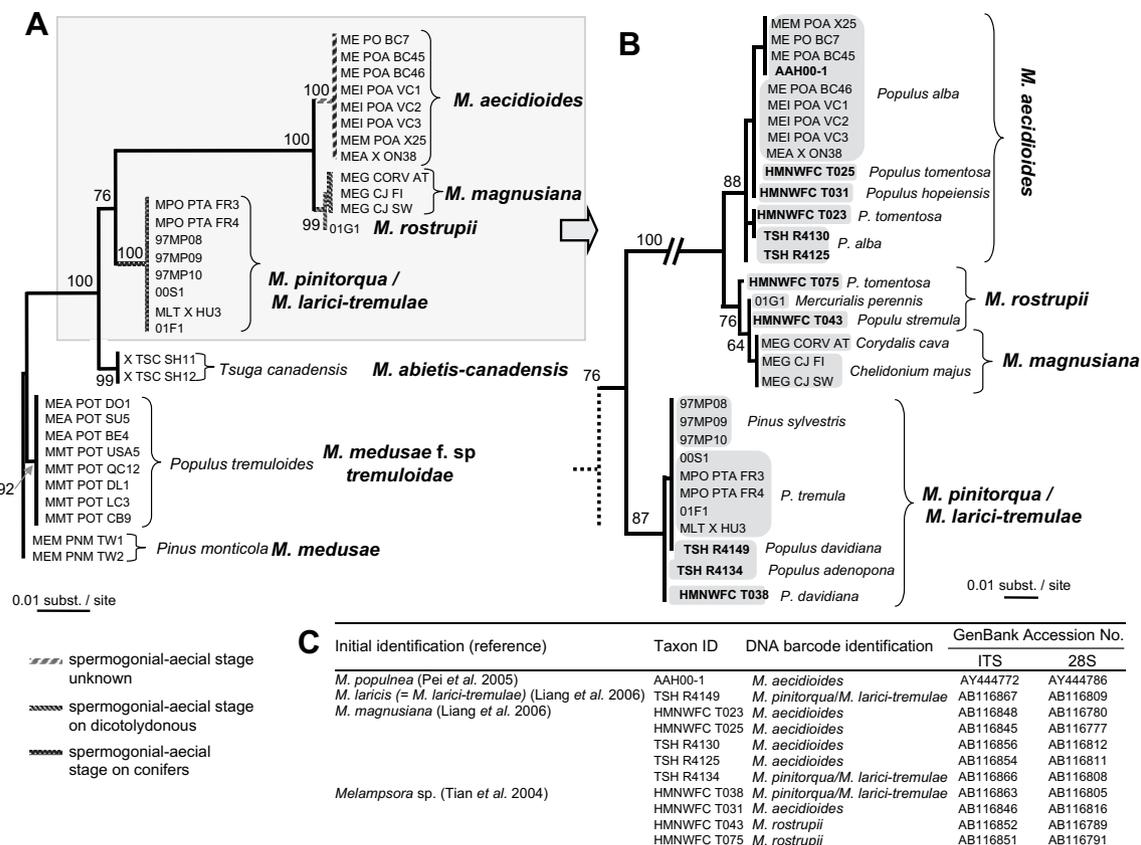


Fig 4 – Phylogenetic relationships among *Melampsora* species from aspen and white poplar. (A) ITS + 28S + 5'-CO1 maximum likelihood tree based on a K81uf + G substitution model (assuming a proportion of invariable site parameters equal to 0.014). (B) ITS + 28S maximum likelihood tree, including GenBank sequences (in bold) from rust strains infecting aspen and white poplar; tree reconstruction was based on a K81uf + G substitution model (assuming a gamma distribution of rate variation among sites equal to 0.024). (C) Attributes (including ITS and 28S sequences accession numbers) of 11 rust strains infecting white poplar and aspen downloaded from the GenBank database.

erroneous diagnostic and drastic phytosanitary advices which could arise.

Microscopic investigations led us to believe that the leaf rust strains we obtained from *Populus alba* in Canada exhibited traits of *Melampsora aecidioides* (Arthur 1962). This species has a worldwide distribution; occurrences have been reported from the eastern and western parts of North America (Arthur 1962), South Africa (Roberts & Mitchell 1979) and Asia (Cummins 1943; Tian & Kakishima 2005). Considering its morphological traits and its ability to grow on *P. alba*, numerous authors have included *M. aecidioides* in the *Melampsora populnea* complex, and have considered *M. aecidioides* to be synonymous with either *Melampsora magnusiana* (= *Melampsora pulcherrima* Maire) or *Melampsora rostrupii* (Tian et al. 2004; Tian & Kakishima 2005). Using only morphological traits of the uredinial–telial stage (on *Populus tremula* and *P. alba*) to distinguish *M. magnusiana* from the three other species of the *M. populnea* complex, particularly from *M. rostrupii*, is contentious (Pinon 1973; Frey et al. 2005; Sinclair & Lyon 2005). However, *M. rostrupii* and *M. magnusiana* appear to be distinctive members of the *M. populnea* species complex because their spermogonial–aecial stages are pathogenic to *Mercurialis perennis* and to Papaveraceae (*Chelidonium* sp., *Fumaria* sp., and *Corydalis* sp.) respectively (Pinon 1973; Naldini-Longo et al. 1985; Desprez-Loustau 1986; Sinclair & Lyon 2005). From a phylogenetic perspective, we acquired strong evidence that *M. aecidioides* is closely related but clearly distinct to both *M. rostrupii* and *M. magnusiana* (K2P distance = 0.005). The two former species remained undifferentiated in one single monophyletic clade (Fig 4A; BS = 99%). Pairwise genetic divergence among *M. magnusiana* and *M. rostrupii* showed a small divergence between these species (K2P distance = 0.0005; two SNPs over the 2130 nucleotides analysed), quite similar to the average within species divergences (K2P distance = 0.0003) in the other *Melampsora* species considered. The divergence observed in these three nuclear loci, appears however not enough to differentiate some close taxa initially defined with host specificities and morphology, as conspecifics. There are several reasons why phenotypic and molecular traits do not completely agree, one of these being incomplete lineage sorting (Freeland 2006; Maddison & Knowles 2006). Here, the small sample size within some species does not allow to infer in details the intra-specific variation, but we cannot rule out the possibility of an incipient differentiation between the *M. magnusiana* and *M. rostrupii* species at the molecular level (as well as in morphological traits).

The combination of the three molecular loci in a multigene phylogeny revealed that *M. aecidioides*, *M. magnusiana*, and *M. rostrupii* are clearly distinct from two other species of the *M. populnea* complex (*M. larici-tremulae* and *M. pinitorqua*). The *M. populnea* species complex seems to be divided in different groups depending on the spermogonial–aecial of the different species. *M. pinitorqua* and *M. larici-tremulae* alternate on conifers whereas the spermogonial–aecial of *M. rostrupii* and *M. magnusiana* is found on dicotyledonous plants. Pycnia and aecia of the third well differentiated species of this complex, *M. aecidioides* remains unknown (Arthur 1962). Only over-wintered bud-protected urediniospores have been observed for this species (Arthur 1962; Van Vloten 1946; Savile 1973). Further field investigations, based for example on

inoculations and the molecular identification tool developed in this study, would help to determine if *M. aecidioides* is strictly autoecious (and confirm overwintering of *M. aecidioides* on poplars as the unique conservation form) and/or to eventually discover a putative aecial host.

Using our DNA barcode system, we highlighted species misidentification in specimens deposited in herbaria and sequences databases. The preliminary and systematic re-examination of herbarium specimens confirmed that most discrepancies between species identifications and those inferred from DNA barcodes were due to overlaps in host specificities and morphological characters for the uredinial–telial stage. For example, the morphometric data available to confirm the identity of this stage of *M. abietis-canadensis* proved insufficient and resulted in four herbarium specimen misidentifications. *M. abietis-canadensis* is known to occur on aspen, cottonwood, balsam, and white poplar in northcentral and northeastern North America, and to complete its heteroecious life cycle on hemlock (Arthur 1962; Sinclair & Lyon 2005). We unambiguously identified the spermogonial–aecial stage of two strains of *M. abietis-canadensis* on *Tsuga canadensis* based on microscopic observations of typical small, ellipsoid to obovate aeciospores with fine verrucose surfaces (Arthur 1962; Ziller 1974). Another *Melampsora* species, *M. farlowii* (Arthur) Davis, also infects needles, green stems, and cones of *T. canadensis* but this species is autoecious and produces only telia and basidiospores (Hepting & Toole 1939; Cummins & Hiratsuka 2003; Sinclair & Lyon 2005). One herbarium specimen collected on *P. alba* var. *pyramidalis* and initially described as *M. abietis-canadensis* showed strictly identical sequences to the strains identified as *M. aecidioides*. Similarly, its morphological, biological, and uredinial–telial host range led some authors to consider *M. abietis-canadensis* to be a form or even a synonym of *M. medusae* (Sinclair & Lyon 2005; Tian & Kakishima 2005). Three specimens collected on aspen and initially described as *M. abietis-canadensis* in the René Pomerleau Herbarium of the Laurentian Forestry Centre were considered in this study. Although the observed urediniospore lengths in these collections were generally more similar to those described for *M. abietis-canadensis* than for *M. medusae* (shorter length), the ITS, 28S, and 5'-CO1 sequences of these specimens were identical to those obtained for five *M. medusae* f. sp. *tremuloidae* strains.

Similarly, four additional ITS/28S sequences from *M. magnusiana* strains retrieved in the GenBank database were also included in this phylogenetic analysis and did not match those of the spermogonial–aecial strains of *M. magnusiana*. These strains were collected on *P. alba*, *P. alba* var. *pyramidalis*, and *Populus tomentosa* in China, and deposited in the Mycological Herbarium of the College of Forestry at the Northwest Sci-Tech University of Agriculture and Forestry in China (HMNWFC), and in the Mycological Herbarium of the Institute of Agriculture and Forestry at the University of Tsukuba in Japan (TSH) (Liang et al. 2006; Tian et al. 2004). The urediniospore morphologies and sizes described for these *M. magnusiana* isolates in Tian et al. (2004) (globose, ovate or oblong, 13.3–33.3 × 12.2–25.6 µm [mean: 21.8 × 17.6 µm], wall uniformly thick, 1.1–5.0 µm [mean: 2.8 µm]) matched the descriptions made for *M. aecidioides*, *M. magnusiana*, and *M. rostrupii* (Arthur 1962; Pinon 1973). Strong homology was found with

each of the eight sequences of *M. acidioides*. The distribution of this fungus on white poplars in Asia is concordant with previous records made on this continent (Cummins 1943; Tian & Kakishima 2005).

Conclusion

This study reinforces the utility of DNA barcoding as an efficient tool for verifying taxonomic assignments of fungal pathogens. A combination of this approach with morphological characterisation showed that the *Melampsora acidioides* species which occurs on *Populus alba* in North America is linked to the *Melampsora populnea* species complex but remains clearly differentiated from the other species found in the complex. In this study, we were specifically interested in seeing the effect of combining three genomic regions to resolve the studied *Melampsora* species. Although the combination of three genes may have increased the resolution, the ITS locus, used alone, provided with few exceptions a sufficiently clear gap between intra- and inter-specific divergence distributions to accurately differentiate these *Melampsora* species. A detection method based on a single locus, such as the ITS region, should therefore provide an acceptable compromise for a successful identification of these pathogens relative to the cost and time spent on PCR amplifications and sequencing of two additional loci. DNA barcoding provides a rapid standardized means to assess poplar leaf rust identities that will guide the application of adequate phytosanitary measures.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.mycres.2009.02.007](https://doi.org/10.1016/j.mycres.2009.02.007).

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