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The First Record of a North American Poplar Leaf Rust Fungus, *Melampsora medusae*, in China

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Abstract: A wide range of species and hybrids of black and balsam poplars or cottonwoods (*Populus* L., sections *Aigeiros* and *Tacamahaca*) grow naturally, or have been introduced to grow in plantations in China. Many species of *Melampsora* can cause poplar leaf rust in China, and their distributions and host specificities are not entirely known. This study was prompted by the new susceptibility of a previously resistant cultivar, cv. 'Zhonghua hongye' of *Populus deltoides* (section *Aigeiros*), as well as by the need to know more about the broader context of poplar leaf rust in China. Rust surveys from 2015 through 2018 in Shaanxi, Sichuan, Gansu, Henan, Shanxi, Qinghai, Beijing, and Inner Mongolia revealed some samples with urediniospores with the echinulation pattern of *M. medusae*. The morphological characteristics of urediniospores and teliospores from poplar species of the region were further examined with light and scanning electron microscopy. Phylogenetic analysis based on sequences of the rDNA ITS region (ITS_1, 5.8S rRNA gene, and ITS_2) and the nuclear large subunit rDNA (D_1/D_2) was used to further confirm morphology-based identification. Based on combined analyses, five of the fifteen fully characterized samples were identified as *Melampsora medusae*: one from Shaanxi and four from Sichuan. Two of the five were from *Populus deltoides* cv. 'Zhonghua hongye'. Three others were identified on *Populus szechuanica*, *P. simonii*, and *P. yunnanensis*. Additional samples of *M. medusae* were collected in Shaanxi in 2017 and 2018, and from Henan in 2015 through 2018. Altogether these findings show that this introduced pathogen is widespread and persistent from year to year in China. This is the first report of this North American poplar leaf rust species, *Melampsora medusae*, in China. It has previously been reported outside North America in Argentina, Europe, Australia, New Zealand, Japan, and Russia.

Keywords: exotic rust; novel plant pathogen; biotic homogenization; short rotation forestry; phylogenetics of Uredinales

1. Introduction

Poplar leaf rust is caused by many species of *Melampsora*, each of which tends to be host-specific within the genus *Populus*. Leaf rust can be very damaging to black and balsam poplar species in sections *Aigeiros* and *Tacamahaca*, respectively [1–4]. *Melampsora* species have native ranges, but many have been inadvertently introduced to other parts of the world [5] since the genus was first described in 1843 [6]. Introductions accompanying the spread of hybrid poplar culture also have provided opportunities for previously isolated species of *Melampsora* to hybridize [7–9]; *M. medusae* has hybridized with both *M. larici-populina* [9] and with *M. occidentalis* [8]. *Melampsora medusae* is indigenous to Eastern North America in the native range of *Populus deltoides* [10], but it has also

been introduced to Russia [11], Argentina [12], Australia [4], South Africa [4], France [13], India [7], Japan [14], Spain [15], and Portugal [16,17].

Like all rust fungi, *Melampsora medusae* is an obligate biotrophic plant parasite. It has a heteroecious life cycle in its native range with uredinia, telia, and basidia on *P. deltoides*, and spermogonia and aecia on *Larix laricina.* In the European Union, it is an introduced, quarantined fungus [18–21]. The telial hosts of *M. medusae* include species of section *Aigeiros* (e.g., *P. deltoides*, *P. nigra*, *P.* × *euramericana*) and of section *Tacamahaca* (e.g., *P. maximowiczii*, *P. simonii*, *P. trichocarpa*). The signs of *M. medusae* on poplars are typical of many leaf rusts: small, yellow uredinia appear within 10 days of infection on the abaxial surfaces of leaves, or on both sides of the leaves in the case of heavy infections [7,22–24].

As recently as five years ago, *P. deltoides* was largely rust-free in China due to its resistance to the native *M. larici-populina*. Now, however, *P. deltoides* and its hybrids experience severe rust from early July until November in Southern China. In Northern China, the new rust season for *P. deltoides* develops somewhat later. In the mountains of Southern China, some species of section *Tacamahaca* even become rusted in early July. Unfortunately, the formerly promising cultivar of *P. deltoides*, 'Zhonghua hongye' (Figure 1), is also now rust-susceptible. These recent changes prompted the rust surveys of this study to determine their cause. Between 2015 and 2018, surveys were conducted in Shaanxi, Sichuan, Gansu, Henan, Shanxi, Qinghai, Beijing, and Inner Mongolia. We collected poplar leaf rust specimens and fully characterized 15 collections in this study. By full characterization we mean both morphology- and sequence-based identification [2,25,26]. In addition to the 15, some other samples were identified on the basis of morphology alone.

Figure 1. Sites (red dots) for rust sampling of *P. deltoides* cv. 'Zhonghua hongye'. Dark dots represent the potential distribution of this formerly rust-free cultivar.

2. Materials and Methods

2.1. Surveys and Specimens

Poplar leaf rust samples were collected during surveys from 2015 through 2018 in Shaanxi, Sichuan, Gansu, Shanxi, Qinghai, Beijing and Inner Mongolia in China. Specimens were dried in plant presses and deposited in State Key Laboratory of Mycology Institute of Microbiology Chinese Academy of Sciences. Sequences were deposited in GenBank with accession numbers as in Table 1.

Table 1. Fifteen fully characterized samples of *Melampsora* in China identified as species, with host plants, sampling locations, years, and voucher and GenBank accession numbers. The two samples of rust on *P. deltoides* are both from cv. 'Zhonghua hongye'.

Host Plants	Locality [a]/Year	Voucher Specimen no. [b]	GenBank Accession Accession no.		Species
			ITS	D_1/D_2	
P. szechuanica	Luding Sichuan/2016	HMAS247968	MK028576	MK064523	*M. larici-populina*
P. yunnanensis	Jinyang Sichuan/2016	HMAS 247969	MK028588	MK064536	*M. medusae*
P. simonii	Xichang Sichuan/2017	HMAS 247970	MK028589	MK064537	*M. medusae*
P. szechuanica	Kangding Sichuan/2018	HMAS 247971	MK028590	MK064535	*M. medusae*
P. cathayana	Wutaishan Shanxi/2018	HMAS 247974	MK028577	MK064526	*M. larici-populina*
P. cathayana	Liupanshan Ningxia/2017	HMAS 247975	MK028578	MK064527	*M. larici-populina*
P. wilsonii	Qinling Shaanxi/2018	HMAS 247978	MK028579	MK064529	*M. abietis-populi*
P. simonii	Huangyuan Qinghai/2018	HMAS 247976	MK028584	MK064525	*M. larici-populina*
P. simonii	Haixi Qinghai/2018	HMAS 247977	MK028583	MK064524	*M. larici-populina*
P. alba var. *pyramidalis*	Yanqing Beijing/2018	HMAS 247979	MK028581	MK064531	*M. magnusiana*
P. alba var. *pyramidalis*	Yulin Shaanxi/2018	HMAS 247980	MK028580	MK064532	*M. magnusiana*
P. tomentosa	Tianshui Gansu/2018	HMAS 247981	MK028582	MK064530	*M. magnusiana*
P. euphratica	Inner Mongolia/2018	HMAS 247982	MK028585	MK064533	*M. pruinosae*
P. deltoides	Linyou Shaanxi/2017	HMAS 247973	MK028586	MK064528	*M. medusae*
P. deltoides	Yihai Sichuan/2015	HMAS 247972	MK028587	MK064534	*M. medusae*

a: Locality with name of provinces and prefectures; b: HMAS: State Key Laboratory of Mycology Institute of Microbiology Chinese Academy of Sciences.

2.2. Morphological Observations

Morphological characteristics of uredinia and telia were examined under the light microscope (LM) and the scanning electron microscope (SEM). Fifty spores of each sample were randomly selected and examined with a Leica DM4000B. Length, width, and wall thickness both apically and laterally were measured. The measurements obtained from samples were compared with published descriptions of taxa of *Melampsora* [2,7,8,19]. The surface echinulation of urediniospores was observed with a SEM. For the SEM, samples were coated with platinum-palladium prior to observations with an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 10kV, as in Zhao et al [27].

2.3. Germination of Urediospores on the Surface of 2% Agar

Fresh urediniospores were collected to test germination and to observe nuclei. Dry fresh urediniospores were sprayed on the surface of 2% agar water and sealed in the dish with a little water to maintain 100% relative humidity. After 6 h, 10 h, and 16 h incubation at 25 °C in the dark, urediniospores and germination tubes were dyed by DAPI (4′,6-diamidino-2-phenylindole·2HCl, Sigma Chemical Company, St. Louis, MO, USA) ethanol solution and observed with a Leica DM4000B [28,29].

2.4. Phylogenetic Analysis

DNA extraction of urediniospores followed Virtudazo et al [30]. Polymerase chain reaction (PCR) was performed with the universal primers ITS_{1F} and ITS_4 [31,32], LSU primer [33], under the reaction system of 30 μL volume, containing 0.2 μM primer, 1 unit of TaKaRa Taq DNA polymerase, a 2.5 mM commercial deoxynucleoside triphosphate (dNTP) mixture, and 2 mM Mg^{2+} Taq reaction buffer. PCR reactions were carried out in a GeneAmp PCR TC-96 (Bioer Technology Co. Ltd., Hangzhou, China) under the following conditions: 95 °C for denaturing for 3 min, then 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final step of 72 °C for 10 min. Products were checked in 1% agarose gel electrophoresis under the UV transilluminator. PCR products were purified and sequenced by Sangon Biotech (Shanghai, China). Sequences were aligned in BioEdit version 7.0.9 and searched in GenBank [34]. All sequences were compared and submitted to NCBI to obtain accession numbers. Other homologous sequences from the GenBank database were downloaded for species comparisons. Multiple alignments were performed using ClustalX version 1.8 [35]. Phylogenetic trees

were constructed using the software PAUP* v4.0b10 with the Maximum likelihood (ML) method [36]. Clade support was analyzed with 1000 bootstrap replicates.

2.5. Confirmation of M. medusae

As there still is confusion surrounding the identity of the species of *Melampsora* on *P. tremuloides* in North America [37], to confirm *M. medusae* two sets of specific primers were employed [37]. DNA extraction and PCR amplification were carried out as Section 2.4; annealing temperatures of PCR are shown in Table 2.

Table 2. Primer sequences for *M. medusae*.

Primer Name	Primer Sequence	Annealing Temperature (°C)	Size of Amplified Product (bp)	Sampling Locus/Year
clc3a2f clc3a2r	5′-GGGGGTCTTTAGGACAAA-3′ 5′-TTCGAGCCAGCATGA AACAC-3′	54	502	Shaanxi/2017,2018 Sichuan/2016 Henan/2015,2018 Qinhai/2018 Ningxia/2017
clc3a3f clc3a3r	5′-TTCGAGCCAGAAGTTTGTTTC-3′ 5′-TTCGAGCCAGGATCACTT-3′	52	594	

3. Results

3.1. Surveyed Poplar Leaf Rust in China, 2015–2018

Five of the fifteen fully characterized samples were identified as *Melampsora medusae* (Table 1): Two of the five were from *Populus deltoides* (section *Aigeiros*) cv. 'Zhonghua hongye'. Three others were identified on three species of section *Tacamahaca*: *Populus szechuanica, P. simonii*, and *P. yunnanensis*. Additional samples of *M. medusae* were collected in Shaanxi in 2017 and 2018, and from Henan and Sichuan in 2015 through 2018. The only other rust species recorded on sections *Aigeiros* and *Tacamahaca* was *M. laricic-populina*. The sole representative of section *Leucoides* in the survey was *P. wilsonii*, and it revealed *M. abietis-populi*. Samples of section *Populus* (i.e., *P. alba* and *P. tomentosa*) yielded *M. magnusiana*. The rust of the sole representative of section *Turanga*, *P. euphratica*, from Inner Mongolia, was *M. pruinosae*. Thus, in all, there were five species of *Melampsora*.

The uredinia of *M. medusae* were mainly hypophyllous; a few uredinia were epiphyllous with small or more scattered pustules. Uredinia were roundish and golden orange to orange in color. Abundant capitate paraphyses were intermixed with urediniospores in the uredinia (Figure 2a). Paraphyses were 25.21–43.76 µm in length with stalks of 3.95–5.53 µm in width and swollen apices which were roughly spherical and 12.43–17.95 µm in diameter. Echinulate urediniospores were obovate or oval, with rounded apices; they were typically flattened laterally (Figure 2b,c), 20.64–31.45 × 14.39–20.38 µm, with golden yellow cytoplasm. Urediniospore walls were colorless, and germ pores were indistinct. The wall of the equatorial area of the urediniospores was slightly thickened at 1.76–3.62 µm thickness. Urediniospores were echinulate, except for the smooth equatorial area characteristic of *M. medusae* that commonly extended from one half to three-quarters of the way around the spore (Figure 2b,c). Spines were smaller near the smooth patch (Figure 2d). Telia were mainly hypophyllous. Their initial color was pale amber brown, but that eventually became deep reddish brown or almost black. Telia were raised slightly above the leaf surface, roughly circular to irregular in outline. Teliospores were roughly cylindrical to angular in shape in the cross-section, and the walls were pale reddish-brown. They were 31.69–44.12 (38.30) µm in length × 10.12–14.50 (11.65) µm in width. The morphological characterizations of all five species of *Melampsora* found in the surveys are summarized in Table 3.

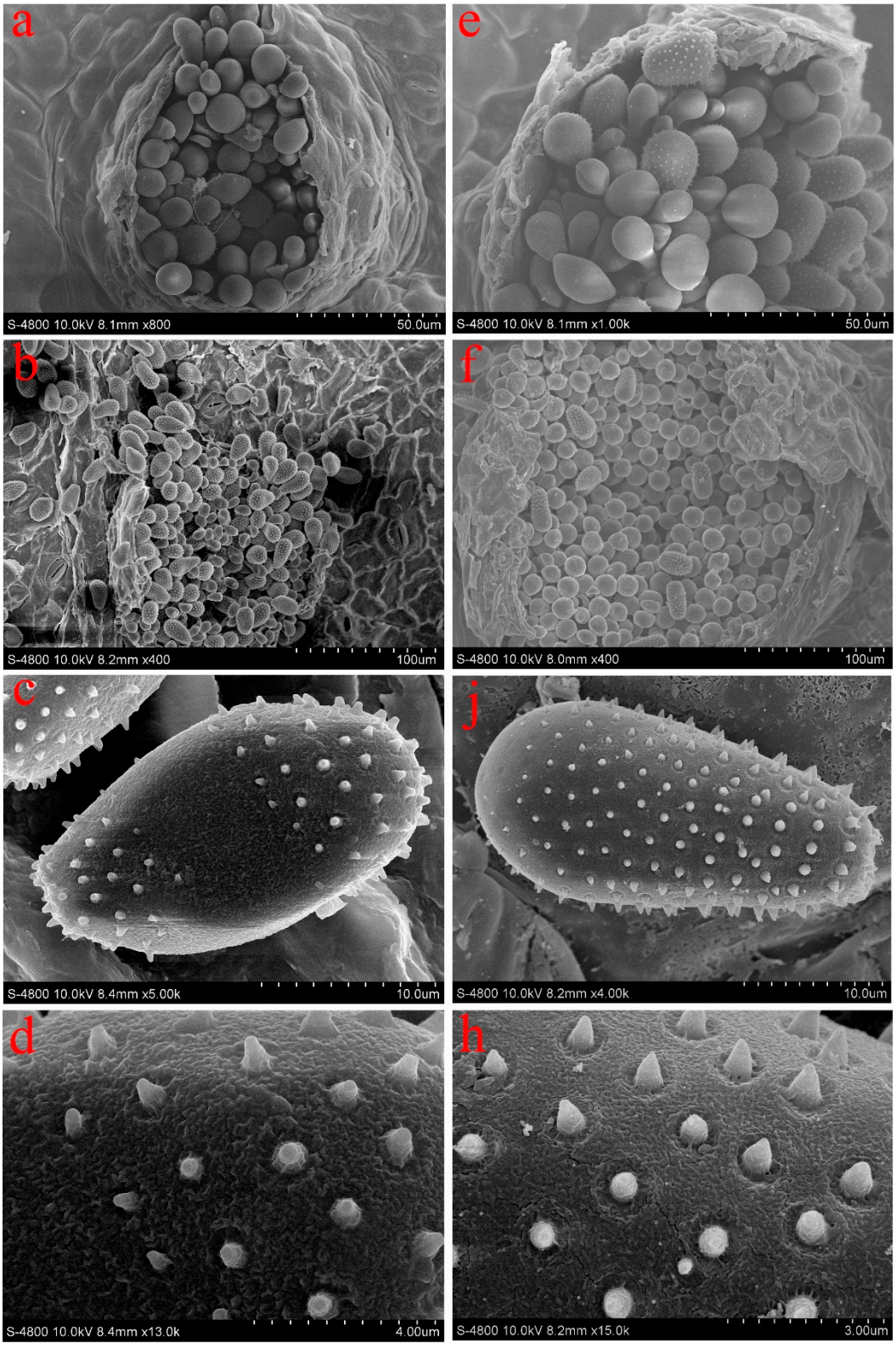

Figure 2. Echinulation patterns of urediniospores and associated paraphyses of *Melampsora medusae* and *Melampsora larici-populina* (the two species of *Melampsora* found on sections *Tacamahaca* and *Aigeiros* in our surveys in China) as observed in the SEM. (**a**–**d**) *Melampsora medusae*; (**e**–**h**) *Melampsora larici-populina*; (**a, e**) abundant paraphyses under SEM; (**b, f**) Uredinium under SEM; (**c, j**) urediniospores with echinulate spines under SEM; (**d, h**) spines on the surface of urediniospores under SEM.

Table 3. Morphology of the five *Melampsora* species found on *Populus* in China during 2015–2018 surveys.

Species	Urediniospores			
	Shape	Size(average) (μm)	Wall Equatorial Part Thickness (average) (μm)	Bald Spot
M. larici-populina	Ellipsoid or Oblong	35.13–48.11 × 20.02–25.31 (40.93) × (22.70)	3.40–11.63 (5.60)	Apical
M. medusae	Obovate or Oval	20.64–31.45 × 14.39–20.38 (26.56) × (18.66)	1.76–3.62 (3.14)	Equatorial
M. magnusiana	Globose or Ovate	17.02–29.34 × 14.16–24.22 (23.93) × (18.70)	1.20–4.65 (2.48)	Absent
M. pruinosae	Globose or Ellipsoid	23.18–31.11 × 23.59–28.53 (27.88) × (25.87)	0.84–1.57 (1.26)	Absent
M. abietis-populi	Globose or Ovate	20.77–26.34 × 15.14–21.52 (22.92) × (17.93)	1.14–1.83 (1.59)	Absent

3.2. Germination of Urediniospores on 2% Agar

Urediniospores of *M. medusae* germinated after 6 h on 2% agar. As Spiers (1994) described [9], the nuclei moved into germ tubes from the spores. After 10 h, there were typically two nuclei in the germ tubes, and the tops of the germ tubes were swollen; after 16 h, some germ tubes fused together. Usually, there were more than three nuclei in the fusion cell. Whereas urediniospores of *M. larici-populina* contained 4 germ pores and mostly had more than 2 germ tubes developed (Figure 3d,e), tubes were rope-like without swollen appressorium (Figure 3e), branched randomly, and typically found with 2 nuclei (Figure 3f).

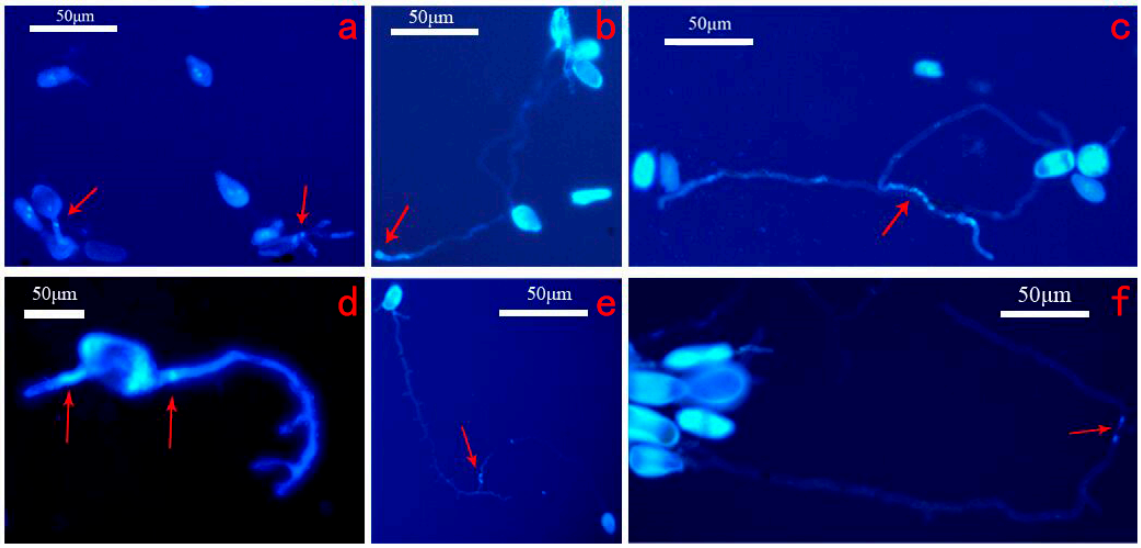

Figure 3. Germination of urediniospores of *M. medusae* and *M. larici-populina* on a medium of 2% agar. (**a**–**c**) *M. medusae*; (**d**–**f**) *M. larici-populina*; (**a**) the flowing nuclei (arrows) after 6 h; (**b**) the swollen top of the germ tube (arrows) after 10 h; (**c**) germ nucleates (arrows) with 4 nucleates after 16 h; (**d**) the flowing nuclei of MLP (arrows) after 8 h; (**e**) the germ tube with 2 nuclei (arrows) after 10 h; (**f**) the germ tube with 2 nuclei (arrows) after 16 h.

3.3. Molecular Phylogenetic Analysis

Phylogenetic analysis of rDNA ITS regions is shown in Figure 4. The Chinese samples belonged to five clades when *M. lini* (GenBank accessions: L20283 [38]) and *M. helioscopiae* (GenBank accessions: AF426197 [39]) were used as outgroups (Figure 4). The analysis revealed that five samples

(HMAS-247973, HMAS-247969, HMAS-247970, HMAS-247971 and HMAS-247972) belonged to the clade anchored by *M. medusae* given the following GenBank accessions: AY375273 (representative of *M. medusae* in France), AF087711 (*M. medusae* in U.S.A. [8]), GQ479310 (also a French sample of *M. medusae* [7]). Five specimens (HMAS-247976, HMAS-247977, HMAS-247975, HMAS-247968, HMAS-247974) belonged to the clade with *M. larici-populina* given the following GenBank accessions: AY429656 (*M. larici-populina* in Canada [40]), AB116828 (*M. larici-populina* in China [2]). Three specimens (HMAS-247979, HMAS-247980, HMAS-247981) were grouped in a clade with *M. magnusiana* given the following GenBank accessions: GQ479849 (representative of *M. magnusiana* in Germany [7]), KY364898 (*M. magnusiana* in Italy [41]). *M. pruinosae* and *M. allii-populina* were divided into two separate clades anchored by GenBank accessions AB116858 (*M. pruinosae* in China [2]) and AB116869 (*M. allii-populina* in China [2]), respectively (Figure 4), *M. pruinosae* is closely related to *M. magnusiana*. nrDNA-ITS phylogeny clearly distinguished *M. medusae* from the other four *Melampsora* species of poplars that were found in these surveys in China.

Phylogenetic analysis of D_1/D_2 regions showed almost the same results as nrDNA-ITS, in that the 15 specimens were distributed in five clades when *M. epiphylla* (GenBank accessions: AY652947 [26]) and *M. ribesii-purpureae* (GenBank accessions: AY444770 [26]) were used as outgroups (Figure 5).

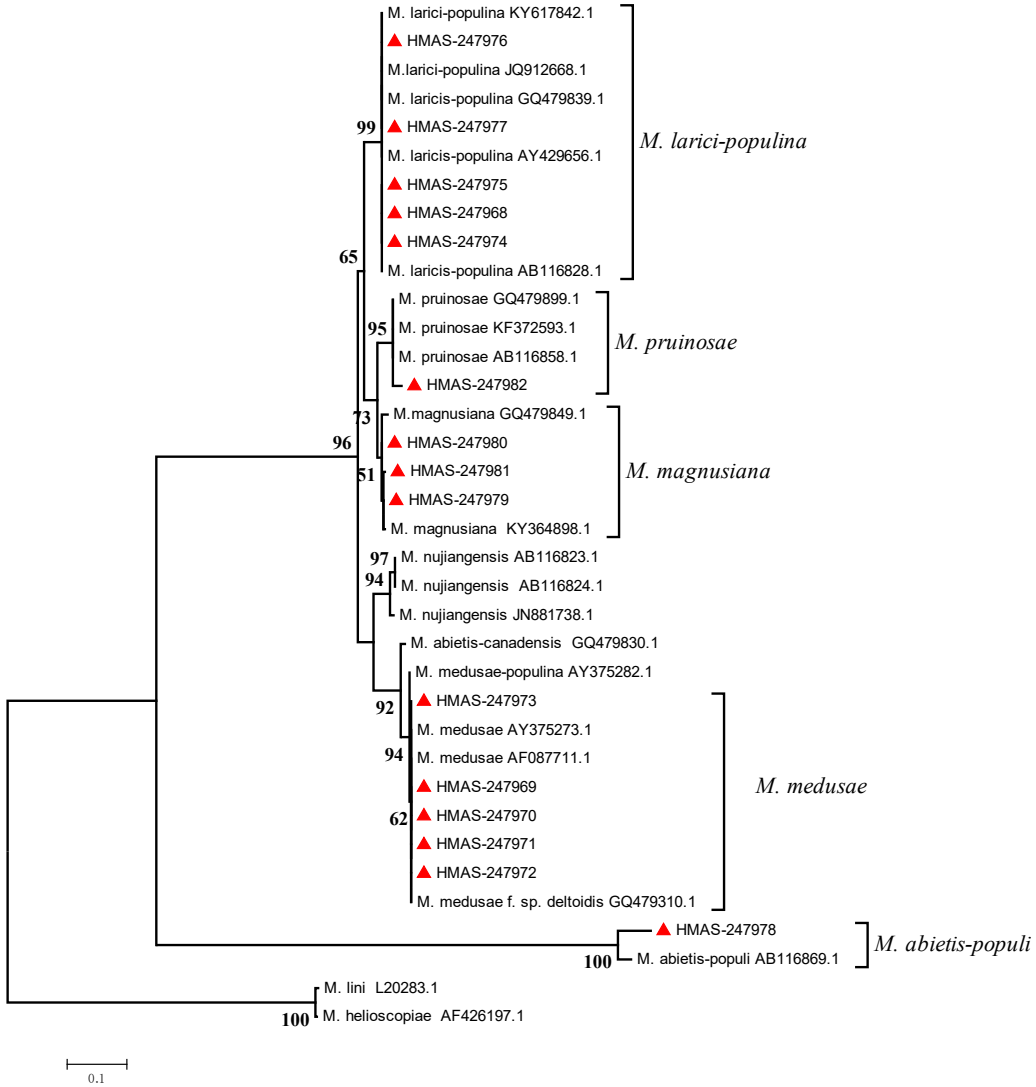

Figure 4. Maximum Likelihood (ML) phylogenetic tree of nrDNA-ITS sequences of species of *Melampsora* from HMAS collections (red triangles) made during poplar leaf rust surveys in China, 2015–2018. Bootstrap values >50% are shown.

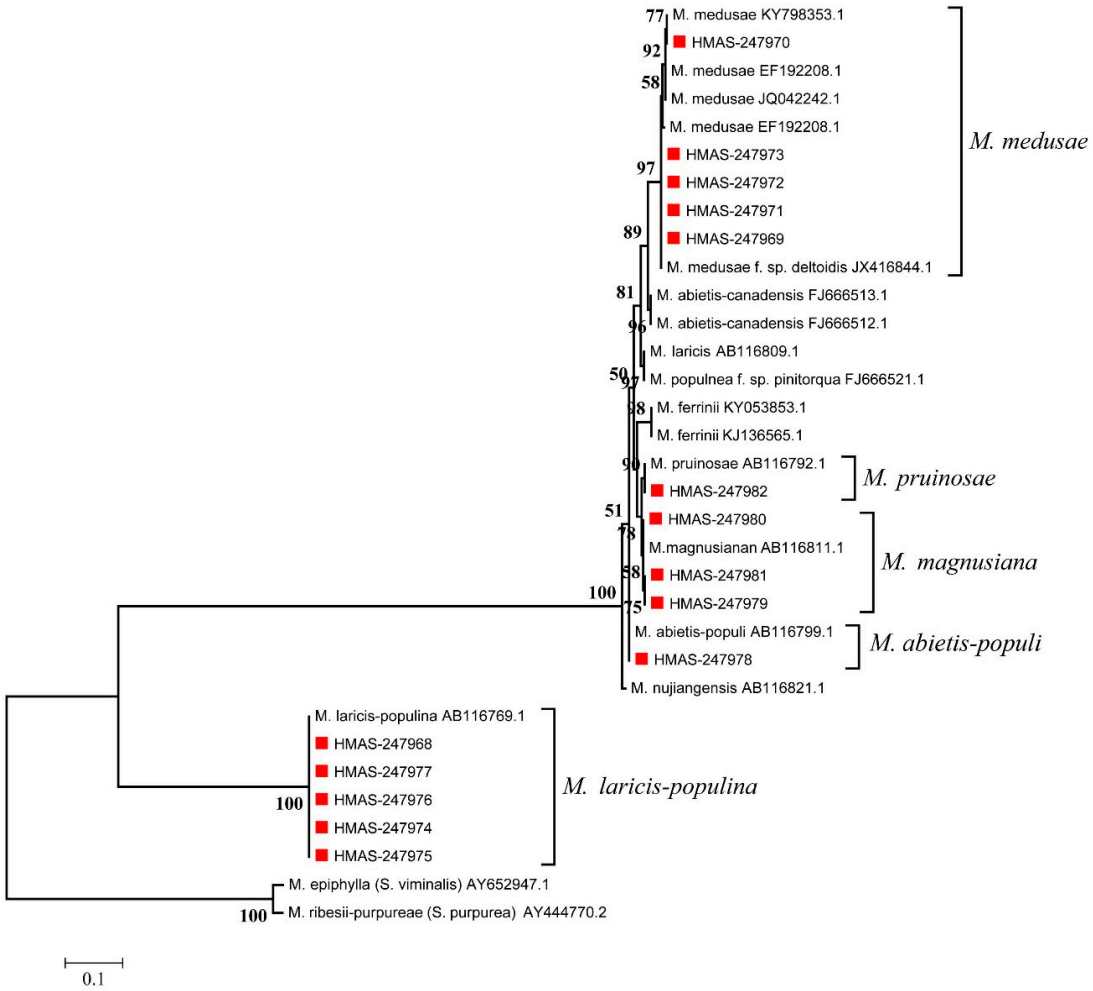

Figure 5. Maximum likelihood (ML) phylogenic tree of 28S rDNA(D_1/D_2) regions. Note: Bootstrap values >50% are shown and red marks are specimens we collected.

3.4. Formae Speciales Identification

The two pairs of primer both showed a single-specified PCR DNA band for the putative five *M. medusae* specimens, with clc3a2f/clc3a2r primer 502 bps and clc3a3f/clc3a3r primer 594 bps, respectively, and the positive of *M. larici-populina* and negative controls of ddH_2O without products (Figure 6), which implied the 5 specimens were *M. medusae* f.sp. *deltoidae*.

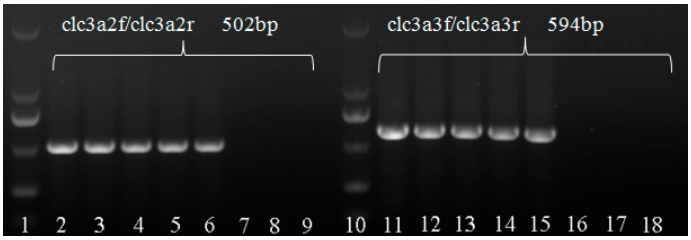

Figure 6. Amplification with primer clc3a2f/clc3a2r and primer clc3a3f/clc3a3r. Lanes 1 and 10: Marker DM2000; lanes 2–6: Amplification from uredinia of the five specimens of *M. medusae* (HMAS 247969-71, HMAS 247972-73) using primers clc3a2f/clc3a2r; Lanes 11–15: Amplification from uredinia of the five specimens of *M. medusae* (HMAS 247969-71, HMAS 247972-73) using primers clc3a3f/clc3a3r; Lanes 7–8 and 16–17(HMAS247968, HMAS247978): The positive control with DNA of *M. larici-populina*; Lanes 9 and 18: The negative control with ddH_2O.

4. Discussion

Two of the five fully characterized samples of *M. medusae* were collected from *Populus deltoides* cv. 'Zhonghua hongye', a bud mutation propagated from *Populus deltoides* in China. 'Zhonghua hongye' has been regarded as fast-growing, rust-resistant and ornamental, so it has been widely planted in inland China since 2000. We have sampled the rust disease on 'Zhonghua hongye' from 2017–2018 in Shaanxi, and from 2015 through 2018 in Hennan, and 2016–2018 in Sichuan. Its new susceptibility to introduced *M. medusae* may change its trajectory of expansion and commercialization. The susceptibility to *M. medusae* of species in section *Tacamahaca* (i.e., *P. yunnanensis, P. simonii* and *P. szechuanica*) is not surprising, as North American species in this section of the genus are also susceptible to varying extents [8]. The susceptibility of these same Asian species in *Tacamahaca* has also been reported before from New Zealand [42]. The host specificity of *M. medusae* as reported in its original description [10] was limited to *P. deltoides*; it did not include *P. tremuloides* nor any other species of section *Populus*. Thus, the confirmation here of the absence of *M. medusae* on surveyed *P. alba* and *P. tomentosa* in China is not surprising.

The aecial host of *M. medusae* in its native range in Eastern North America is *Larix laricina*, but *Pseudotsuga menziesii* can also be a host [43]. In China, the aecial host is not yet known. The most likely hosts in China would be species of *Larix*, *Pseudotsuga* and possibly *Cathaya*. These three genera belong to subfamily *Laricoideae* of *Pinaceae*, although *Cathaya* may be closer to *Pinus* than to *Larix/Pseudotsuga* [44]. No rust fungi have ever been reported on *Cathaya* [11], although this absence is likely a function of a lack of attention. For example, only recently was the first pathogen of *Cathaya* described as a new species [45].

The involvement of *M. medusae* in inter-specific hybridization complicates its introduction into China. In our survey, we frequently found *Melampsora larici-populina* in close proximity to *M. medusae*. We have not yet surveyed for the hybrid *M. medusae-populina*, but its existence in China is now a distinct possibility that should be researched.

5. Conclusions

Our combined morphology- and sequence-based approach led to the identification of five species of *Melampsora* in surveys in China. The most important discovery was that of the introduction of North American *M. medusae* that is now both widespread and persistent from year to year in China.

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Conflicts of Interest: The authors declare no conflict of interest.

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