Forest Pathology

For. Path. doi: 10.1111/j.1439-0329.2010.00699.x

A new species of *Melampsora* rust on *Salix elbursensis* from Iran

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Summary

A rust fungus was found causing stem cankers on 1- to 5-year-old stems of *Salix elbursensis* in the north west of Iran. The rust also forms uredinia on leaves and flowers of the host willow. Light and scanning electron microscopy revealed that the new rust is morphologically distinct from several *Melampsora* species occurring on the willows taxonomically close to *S. elbursensis*, but indistinguishable from *Melampsora larici-epitea*. Examination of the internal transcribed spacer (ITS) region of the ribosomal DNA suggested that the rust fungus is phylogenetically close to *Melampsora allii-populina* and *Melampsora pruinosae* on *Populus* spp. Based on both the morphological characteristics and the ITS sequence data, the rust is described as a new species – *Melampsora iranica* sp. nov.

1 Introduction

The genus *Melampsora* was established by Castagne in 1843 based on the rust on *Euphorbia*, *Melampsora euphorbiae* (Schub.) Cast. (Castagne 1843). The main characteristic of the genus *Melampsora* is the formation of 'naked' uredinia and crust-like telia that comprise sessile, laterally adherent single-celled teliospores. Of some 80 *Melampsora* spp. (Hawksworth et al. 1995), more than half have been described on Salicaceae, which comprises willows (genus *Salix*) and poplars (genus *Populus*) (Pei 2005).

Salix is one of the largest genera of woody plants in the northern hemisphere. According to various authorities, there are between 300 and 500 *Salix* species worldwide (Bean 1980; Wang and Fang 1984). Within the genus, the species are grouped into 2–4 subgenera (Argus 1986; Skvortsov 1999) and, within a subgenus, *Salix* species are further grouped into various sections. Sect. *Helix* belongs to the subgenus *Vetrix* and contains some 10 species that are distributed in Eurasia (Skvortsov 1999). The species within *Helix* are morphologically similar but differ in their geographical distribution. *Salix elbursensis* Boiss. (Red willow; synonym: *Salix purpurea* L. var. *pallescens* Anders.) belongs to Sect. *Helix* and is naturally distributed in eastern Asia Minor, nearly all of the Caucasus (Skvortsov 1999) and Tehran, Mazandaran and east-Azarbidjan provinces of Iran (Mozaffarian 2005).

All *Melampsora* species on *Salix* infect leaves but some cause stem cankers as well. In the United Kingdom, for example, *Melampsora amygdalinae* Kleb. on *Salix triandra*, *Melampsora caprearum* Thüm. on *Salix caprea* and the stem infecting form on *Salix viminalis* are capable of infecting willow stems and causing stem cankers (Pei et al. 1995). In North America, Smith et al. (2004) showed that current shoots of *Salix arctica* are sometimes infected by *Melampsora* and Ostry and Anderson (2001) reported that *Melampsora bigelowii* Thüm. (syn. *Melampsora paradoxa* Diet. & Holw.) causes willow stem cankers in Minnesota. *Melampsora coleosporioides* Diet. has also been found causing infections on current shoots of *Salix matsudana* in Japan and China (Pei, M. H.,

Received: 1.8.2009; accepted: 21.10.2010; editor: S. Woodward

unpublished observations). Previously, five species of *Melampsora* have been recorded on *Salix* spp. in Iran (Ershad 1995; Abbasi and Aliabadi 2009). These are *Melampsora allii-fragilis* Kleb, *M. euonymi-caprearum* Kleb. *Melampsora salicis-albae* Kleb., *M. caprearum* and *Melampsora larici-epitea* L. Except for *M. salicis-albae*, there has been no record of other willow rust causing infections on stems in Iran.

In June 2003, multiple stem cankers were found on *S. elbursensis* trees grown at the margins of two orchards in Sharafkhaneh harbor, north western Iran. The cankers, some reaching 8 cm in length, were found on 1- to 5-year-old stems. Such symptoms had not previously been observed on willows in Iran and the literature search revealed that there had been no record of a rust fungus occurring on *S. elbursensis*. This study was conducted to determine the taxonomic status of the rust found on *S. elbursensis* through morphological examination and using ribosomal DNA sequence information.

2 Materials and methods

2.1 Field observations

Rust infections were first detected in 2003 on *S. elbursensis* trees that were grown as windbreaks in two fruit orchards in Sharafkhaneh harbor, north western Iran. During the 4-year (2003–2006) study period, the two sites were regularly (usually at 2-weeks intervals from May to December) visited and the trees were inspected for rust infection.

2.2 Morphological examination

The morphology of urediniospores and paraphyses was examined using light and scanning electron microscopy (SEM). Uredinia-bearing willow leaves were collected from the field, dried at room temperature for 2–4 days and used for morphological examination using a light microscope. Prior to the examination, urediniospores were brushed on to a glass slide and dried at 60°C for 2 h. Uredinia and paraphyses were measured without drying at 60°C. Uredinia-bearing leaf segments (5 × 10 mm), urediniospores and paraphyses were mounted in water on glass slides and a total of 60 uredinia, 50 urediniospores and 50 paraphyses were measured with an ocular micrometre (Graticules Ltd, Tonbridge, Kent, UK). Teliospores were examined after making freehand sections using a razor blade. The freehand sections were mounted in water on glass slides and a total of 50 teliospores from five different leaves (10 from each leaf) were measured. In addition to the examination using LM, urediniospores were examined with scanning electron microscopes (SEMs) using two protocols. In the first, 5 × 5 mm dry leaf segments carrying uredinia were coated in gold and placed in the low-vacuum, variable-pressure chamber of a Hitachi S3500 SEM and urediniospores were photographed with a digital camera at 3500× magnification. In the second, spores were collected from the leaf sample using a fine hair brush and attached onto the surface of a 'sticky carbon tab' mounted on an electron microscope stub. The samples were then coated with gold for 60 s using the Gatan ALTO2100 prep unit and examined using the Jeol 6360 LV SEM in high vacuum mode (Jeol Inc., Peabody, MA, USA), 10 kV and SEI detector (Jeol Inc., Peabody, MA, USA). The spine distances were measured from 50 pairs of randomly chosen adjacent spines using Jeol 6360 LV SEM.

2.3 ITS sequence information

To examine the phylogenetic relationships of the rust with other *Melampsora* species, the internal transcript spacer (ITS) region of ribosomal DNA (rDNA) of the rust was sequenced. A preliminary sequencing experiment showed that there were three ambiguous (unresolved) sites in the region amplified using the primers ITS1 and ITS4 (White et al.

1990). To clarify this result, the ITS region was sequenced in two different laboratories: Department of Plant Pathology, University of Minnesota, USA, and Department of Plant and Invertebrate Ecology, Rothamsted Research, UK.

In Minnesota, a total of five DNA extractions (referred as samples) were made from urediniospores produced on willow stems (2), leaves (2) and flowers (1) using the Qiagen Plant DNeasy mini-kit (Qiagen, Inc., Valencia, CA, USA) following manufacturer's instructions. Basidiomycete-specific primers ITS1-F (5′-TTGGTCATTTAGAGGAAG-TAA) and ITS4-B (5′-CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns 1993) were used for amplification. PCR was performed in a MJ Research PTC Mini-cycler thermocycler using the profile of 94°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min followed by a final extension step of 72°C for 5 min. Some samples were re-amplified to obtain sufficient quantities of DNA. Amplified products were purified using EXO-SAP-IT PCR cleanup kits (USB, Inc., Cleveland, Ohio, USA), sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA, USA) and resolved on an ABI (Stretch 377) automated DNA sequencer.

At Rothamsted, six DNA extractions were made from rust urediniospores from willow stems (2), leaves (3) and flowers (1). DNA was extracted from 2–5 mg rust urediniospores using the method described in Pei and Ruiz (2000), and the ITS region was amplified using the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTTCCGC-TTATTGATATGC) (White et al. 1990). These two primers are designed to cover partial 18S and 28S and the whole ITS1, 5.8S and ITS2 regions (Pei 2005). PCR was performed in a Perkin Elmer 9700 thermocycler using the profile of 94°C for 5 min, then 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Resulting PCR products were purified using the Qiaquick PCR Purification kit (Qiagen, Hilden, Germany). Four sequencing reactions were prepared with one sample (derived from the urediniospores on leaves) to verify the ambiguous/unresolved sites. With others, one sequencing reaction was prepared for each sample. Sequencing was performed using the two pairs of primers in conjunction with BigDyeTM Terminator Cycle Sequencing Reaction kit (PE Applied Biosystems) and resolved on an ABI (Stretch 377).

2.4 Data analysis

Basic Local Alignment Search Tool (BLAST) searches were conducted to find the nearest matches among the sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/). ClustalW2 (Larkin et al. 2007) was used to align the ITS sequences of the selected *Melampsora* species. MEGA version 4.0 (Tamura et al. 2007) was used to calculate the genetic distances and construct phylogenetic trees using two approaches. (a) The tree was constructed using the Kimura 2-parameter model and the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The robustness of internal branches was tested using bootstrap analyses (1000 replications). (b) The bootstrap (1000 replicates) consensus tree was constructed using the maximum parsimony method (Eck and Dayhoff 1966). Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The tree was obtained using the close-neighbour-interchange algorithm (Nei and Kumar 2000) and by building the initial trees with the random addition of sequences (10 replicates).

3 Results and discussion

3.1 Field observations

Yellow and orange yellow uredinia were present on leaves, flowers and stem cankers of the willow. On the leaves, uredinia were less than 1 mm in diam and mostly occurred in groups

on both sides, but mainly on the lower side, of the leaves. On the stems, the bark was split in several places at the margins and at the centre of cankers, exposing uredinia and urediniospores. The cankers were larger on older stems. Urediniospores were observed throughout the year at the margins of the cankers. In autumn, telia were seen mainly as brown to black spots on the leaves but not on the stem cankers. All the willow trees (>100) in the two orchards showed infections during the 4-year study period and the levels of infection were higher on lower parts of the trees where relatively younger stems (suckers) grew.

3.2 Morphology

Urediniospores were mostly globoid to ellipsoid and spore walls were uniformly thick. Urediniospore contents were yellow and walls were hyaline under the light microscope. The heads of paraphyses had uniformly thickened walls. Examination using SEM revealed that the surface of the urediniospores was evenly echinulate (Fig. 1A and 1B). Teliospores were sub-epidermal and had uniformly thickened walls (Fig. 1C). The measurements of the uredinia, urediniospores, paraphyses and teliospores are listed in Table 1.

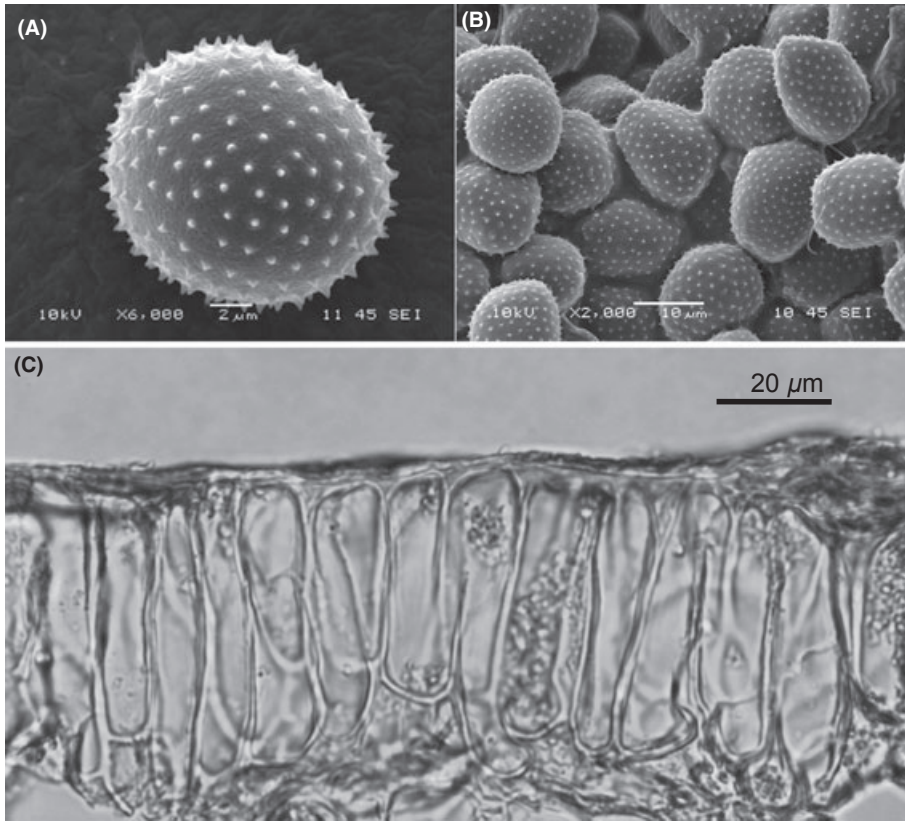

Fig. 1. Urediniospores (A,B) and teliospores (C) of *Melampsora iranica* on *Salis elbursensis*.

Table 1. **Measurements of morphological characteristics of *Melampsora iranica*.**

Measured variable	Measurement (μm)	Mean (μm)	Standard error of mean (p = 0.05)
Uredinia (diam.)	200–650	290	25
Urediniospores			
Length	17.5–25	21.2	0.4
Width	15–20	15.7	0.3
Spine distance	1.25–2.8	1.87	0.01
Paraphyses			
Length	32.5–70	53.8	2.3
Width	12.5–22.5	16.5	0.8
Head length	15–27.5	20.46	0.8
Top wall	1.25–5	2.69	0.19
Side wall	1.25–3.75	2.46	0.1
Stalk width	5–8.75	5.43	0.25
Teliospores			
Length	37.5–55	45.8	1.3
Width	10–15	12.3	0.2

3.3 ITS sequences

The same sequences were obtained from the DNA samples extracted from the urediniospores from the stems, leaves and flowers. In both laboratories, a site in the ITS2 region consistently gave A or N (base not determined), and two sites in 18S region consistently gave C or N and G or N, respectively. At Rothamsted, the four reactions containing the same DNA template gave identical result, i.e. A or N, C or N and G or N, respectively, at the three sites. Close inspection of chromatographs showed that the N site in the ITS2 region had overlapping peaks of A and T, one N site in 18S region had overlapping C and T, and the other N site in 18S had overlapping G and A. This suggests that these three sites are heterozygous, i.e. A or T (W, see NC-IUB 1985) at the N site in the ITS2, C or T (Y) at one N site in 18S and G or A (R) at the other N site in 18S.

BLAST searches using the sequence obtained in this study (GenBank accession FJ386432) found that the nearest match was *M. allii-populina* on *Populus* spp., P distance (the proportion of nucleotide sites at which two sequences are different) being calculated 0.011–0.014, then *Melampsora pruinosae* Tranz. on *Populus euphratica* (0.013–0.014), followed by *M. salicis-albae* on *Salix alba* (0.019) and *Melampsora ribesii-purpureae* Kleb. on *S. purpurea* (0.032). Based on the BLAST search result, ITS sequences of 17 closely related *Melampsora* species were selected to compare with that of the new rust. When aligned, 91 sites, of a total of 630, were variable and 44 were parsimony informative. The UPGMA tree (Fig. 2) placed the new rust in the same clade as *M. allii-populina* and *M. pruinosae* with reasonable confidence (bootstrap value = 87%). The maximum parsimony tree (Fig. 3) also grouped the new rust together with *M. allii-populina* and *M. pruinosae* with the same level of confidence (bootstrap value = 89%).

3.4 Taxonomy

The taxonomy of *Melampsora* on *Salix* is complex. Often, there is no clear morphological distinction between the species that are separated by having different aecial or telial hosts. Usually, only one or two spore stages can be found at the time of observation and there is no information on the alternate hosts. Facing these problems, Hylander et al. (1953) recognized *Melampsora epitea* Thüm. as a complex species to include those species (*sensu stricto*) similar in morphology. Wilson and Henderson (1966) also adopted *M. epitea* as a

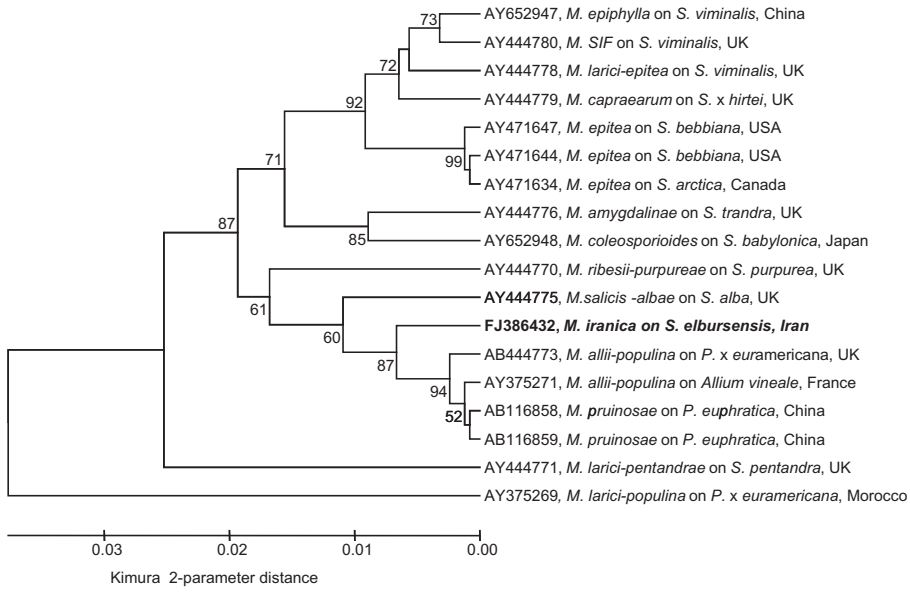

Fig. 2. UPGMA tree of *Melampsora iranica* and 17 nearest matching GenBank accessions. Bootstrap values >50% are shown.

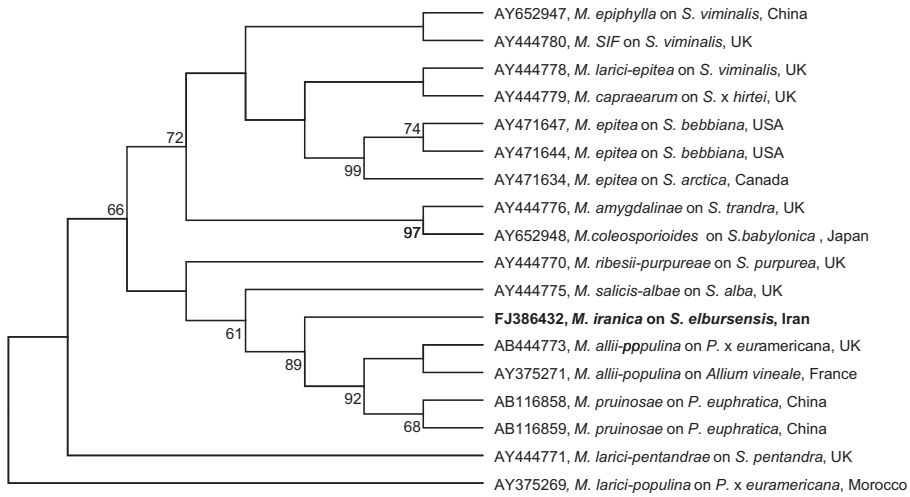

Fig. 3. Maximum parsimony bootstrap consensus tree of *Melampsora iranica* and 17 nearest matching GenBank accessions. Bootstrap values >50% are shown.

collective species made up of various races and groups of races which are morphologically indistinguishable, but alternate on different hosts. Following their treatment, the new rust found on *S. elbursensis* can certainly be placed in the *M. epitea* complex according to its morphology. However, the problem remains in adopting *M. epitea* as a species because the

name means very little in terms of the biological characteristics and genetic identity of those 'races' or 'groups of races'.

Melampsora species on *Salix* are specialized in their pathogenicity. Usually, the host range of a species/*forma specialis* is confined to one or a few sections of willow hosts. Of more than 30 species of rust described on willow (Pei 2005), six species, *Melampsora abieti-caprearum* Tubeuf (=*Melampsora humboldtiana* Speg.), *Melampsora dimorphospora* Kaneko & Hiratsuka, *Melampsora humilis* Diet., *M. larici-epitea*, *M. ribesii-purpureae* and *Caeoma salicis-miyabeana* Kaneko & Hiratsuka, have been recorded on willows belonging to Sect. *Helix*. Compared to the new rust, *M. abieti-caprearum*, *M. humilis* and *M. ribesii-purpureae* have relatively shorter teliospores (up to 33 μm) (see Pei 2005). *Melampsora dimorphospora* is distinct from all other species of willow rusts by having either echinulate or verrucose urediniospores. In *C. salicis-miyabeana*, only the aecial stage has been found. The morphology of the new rust is indistinguishable from that of *M. larici-epitea*. However, the present study revealed a 4% difference between the two species in DNA sequences in the examined rDNA region. Such difference is far greater than what was found between *formae speciales* within *M. larici-epitea*. Within *M. larici-epitea*, six *formae speciales* have been recognized in Europe and two in Japan (Pei 2005). When ITS sequences of three *formae speciales*, *larici-epitea typica* (LET), *larici-retusae* (LR) and *larici-daphnoides* (LD) were examined (Pei 2005), LET and LR produced identical sequences, while LD differed from LET and LR by 3 of 661 characters aligned (P distance = 0.006–0.007) and for the remaining *formae speciales* this is not known at present.

Based on both the results from morphological examination and the rDNA sequence data, it can be concluded that the rust causing stem cankers on *S. elbursensis* in north western Iran is a new species of *Melampsora* and can be described as the following. Further work will be carried out to establish the host range of *Melampsora iranica* on Salix species.

Melampsora iranica S. M. Damadi, M. H. Pei, J. A. Smith et M. Abbasi sp. nov. Figs 1A–1C

Spermogoniis et aeciis ignotis; urediniis in foliis, floriis et ramulis; in foliis amphigenis, plerumque hypophyllis, sparsis vel aggregatis, subepidermalibus, mox nudis, pulveraceis, 0.2–0.65 (medium 0.29) mm diam., flavis vel auratiacis; in ramulis e cortice erumpentibus; urediniosporis globosis, obovoideis vel ellipsoideis, 17.5–25 (21.2) × 15–20 (15.7) μm, ubique aequaliterque echinulatis, spinis 1.25–2.8 (1.87) ìm inter se distantibus, episporio ubique aequali 1–3 μm crasso; paraphysibus capitatis vel clavatis, 32–70 (53.8) × 12–23 (16.5) μm, membrana 1.25–5 μm crassa; teliis amphigenis, plerumque hypophyllis, subepidermalibus, sparsis, brunneis vel atrobrunneis, vix 1 mm latis, teliosporis prismaticis vel cylindraceis, 37.5–55 (45.8) × 10–15 (12.3) μm, episporio 1μm crasso.

Holotypus: In foliis *Salici elbursensis* L. (Salicaceae), E-Azarbaijan, Bandar-e Sharafkhaneh Iran. June 2006. S. M. Damadi, IRAN 13948 F.

Spermogonia and aecia unknown. Uredinia on leaves, flowers and stems. On leaves, uredinia amphiphyllous, mainly hypophyllous, scattered or aggregated, initially covered by epidermis, later erumpent and pulverulent, 0.2–0.65 (average 0.29) mm in diam, yellow to orange-yellow; on stems, uredinia initially beneath the cortex, later exposed. Urediniospores globoid, ovoid or ellipsoid, 17.5–25 (21.2) × 15–20 (15.7) μm, evenly echinulate, distance between spines 1.25–2.8 (1.87) μm, walls uniformly thick, 1–3 μm; paraphyses capitate or clavate, 32–70 (53.8) × 12–23 (16.5) μm, walls colourless, smooth, 1.25–5 μm thick. Telia amphiphyllous, mainly hypophyllous, subepidermal, scattered, brown or dark brown, under 1 mm, Teliospores prismatic, cylindrical or clavate, 37.5–55 (45.8) × 10–15 (12.3) μm, wall 1 um thick.

Holotype: On leaves of *Salix elbursensis* L. (Salicaceae), E-Azarbaijan, Bandar-e Sharafkhaneh, Iran. June, 2006. S. M. Damadi, IRAN 13948 F, in the Fungal Collection of the Ministry of Jihad-e-Agriculture, Iran. GenBank accessions FJ386431 and FJ386432.

In the present study, the ITS sequence data of the new rust match most closely (P distance = 0.011–0.014) to that of the two poplar rusts, *M. allii-populina* and *M. pruinosae*. The UPGMA tree also grouped these three species together with reasonable confidence (bootstrap values = 87–89%). *Salix* and *Populus* are two different genera in the Salicaceae and no *Melampsora* species occurring on *Salix* are known to infect *Populus*, and vice versa. *Melampsora allii-populina* occurs on *Populus nigra* that is native to Europe, southwest and central Asia, Caucasus and Xinjiang province of China (Wang and Fang 1984). *Melampsora pruinosae*, on the other hand, occurs on *P. euphratica*, which is naturally distributed in Egypt, Syria, Iran, Caucasus, Central Asia and the north western provinces of China (Wang and Fang 1984). It may not be a coincidence that the distribution range of *S. elbursensis*, the host of the new rust, overlaps with that of *Populus nigra* and *P. euphratica*. It appears that these *Melampsora* species remarkably similar in their ITS sequences share the same ancestry but speciated on, or 'switched' to, different hosts in their evolutionary history.

Acknowledgement

We thank Carlos Bayon, Rothamsted Research, and the laboratory of Robert Blanchette at the University of Minnesota, for the sequencing of the ITS region.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *Melampsora iranica* on the leaves (A) and stems (B) of *Salix elbursensis*.

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