Molecular Phylogenetic Relationships of the Brown Leaf Rust Fungi on Wheat, Rye, and Other Grasses

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Abstract

Liu, M., Szabo, L. J., Hambleton, S., Anikster, Y., and Kolmer, J. A. 2013. Molecular phylogenetic relationships of the brown leaf rust fungi on wheat, rye, and other grasses. *Plant Dis.* 97:1408-1417.

The classification of brown leaf rust fungi (*Puccinia recondita* complex and allied species) on wheat (*Triticum aestivum*), rye (*Secale cereale*), and other grasses in the family Poaceae has experienced a long history of controversy and uncertainty due to the reduced morphological characteristics available for taxonomy and difficulty of conducting interfertility experiments. However, because these are pathogens on important crops, it is important to clarify the species delimitations reflecting the natural lineages. In this study, phylogenetic analyses were conducted with DNA sequence data from the ribosomal DNA internal transcribed spacer region and elongation factor 1- α to

elucidate this species complex. Three phylogenetic lineages were recovered within the complex of rye leaf rust fungi, *P. recondita* sensu stricto, which is congruent with existing classifications based on DNA content, sexual compatibility, and morphological studies. The brown leaf rust fungus on wheat (*P. tritici*) grouped with the related species *P. persistens* on *Elymus repens* and *E. intermedia* as a strongly supported clade. Collections on other *Elymus* spp. were separated into six clades. Based on the phylogenetic affinities of nine type specimens and aecial host associations, potential taxonomic names were evaluated for selected lineages.

The classification and nomenclature of brown leaf rust (or brown rust) fungi on wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and various grasses in the family Poaceae has historically been contentious. A rust pathogen on wheat was first described as a form species by De Candolle in 1815 (10) with the name *Uredo rubigo-vera* DC. In 1884, Winter moved the taxon to the genus *Puccinia*; that is, *Puccinia rubigo-vera* (DC) G. Winter (48). From the late 1800s to the early 1900s, it was generally accepted by many mycologists in Europe and North America that only one species consisting of leaf rust fungi on wheat, rye, barley, and various grasses was present (namely, *P. rubigo-vera*) that produced globose urediniospores and long, covered telia (48) (Fig. 1). The aecial hosts were generally expected to be in the family Boraginaceae due to the successful infections obtained by de Bary (9,12,18) using the leaf rust fungus of rye to inoculate *Anchusa officinalis* L and *A. [Lycopsis] arvensis*. When Plowright (34) described a new species in 1885 with the name *P. perplexans* Plowr. on *Alopecurus pratensis* alternating with *Ranunculus acris* (family Ranunculaceae), it was noted that more than one species could be recognized in brown leaf rust fungi on various grasses. Afterward, numerous species were recognized and named based on the host specialization (28), and the classification of these cereal or grass leaf rust fungi continued to be revised from time to time (3,4,7,14,15,28).

Landmark revisions took place as follows. Eriksson and Henning (13–15) in 1894 and 1899 recognized multiple species previously grouped in *P. rubigo-vera*; that is, *P. agropyrina* Erikss. on

Agropyron spp., *P. bromina* Erikss. on *Bromus* spp., *P. dispersa* Erikss. & Henning on rye (*S. cereale*) with alternate hosts in *Anchusa* spp. (family Boraginaceae); *P. glumarum* (J.C. Schmidt) on *Triticum* spp. and other grasses; *P. holcina* Erikss. on *Holcus lanatus* and *H. mollis*; *P. simplex* (Körn) Erikss. & Henning on *Hordeum* spp.; *P. triseti* Erikss. on *Trisetum flavescens*, and *P. tritici* Erikss. on *Triticum aestivum*. In contrast, Arthur and Fromme (4) combined all the forms of *P. rubigo-vera* with aecial hosts in the family Ranunculaceae into one species under the name of *Diccaeoma clematidis* (DC) Arthur, and designated other species, such as *D. apocrypta* (Ellis & Tracy) Kuntze on *Agropyron*, *Elymus*, and *Sitanion* spp., with aecial hosts in the families Hydrophyllaceae and Boraginaceae; *D. asperifolii* (Pers.) Kuntze on *S. cereale*, with

Fig. 1. *Puccinia recondita* on *Hordeum jubatum* (uredinial stage, PUR N1436) and *Elymus hystrix* (telial stage, PUR N5336). A, Cinnamon-brown uredinia on adaxial leaf surface; B, blackish-brown-covered telia on abaxial leaf surface; C, globose urediniospores; D, clavate teliospores. Scale bars = 20 μ m for C and D; 1 unit = 1 mm for A and B.

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Accepted for publication 1 June 2013.

<http://dx.doi.org/10.1094/PDIS-02-13-0152-RE>

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Anchusa officinalis as aecial host; and *D. impatientis* (Schw.) Arthur on *Agrostis*, *Cinna*, *Elymus*, *Hordeum*, and *Hystrix* spp., with *Impatiens* spp. as aecial host.

Mains disagreed with earlier authors, and recommended placement of all recognized species back into one species under the name *P. rubigo-vera*, listing 32 names as synonyms, and instead separated the species into 56 formae speciales according to infection on various host species (28). Arthur accepted the gramineous leaf rust fungi as one species, *P. rubigo-vera*, with six varieties (3). Cummins and Caldwell proposed *P. recondita* Roberge ex Desm. 1857 as the legitimate binomial for the brown leaf rust fungi on cereals and grasses (8). Cummins (7) treated brown leaf rust fungi as one species under the name *P. recondita* and listed 51 binomials as synonyms. This broad species concept was prevalent in North America, although Savile (39) accepted the taxon on wheat as a distinct species, *P. triticina* designated by Eriksson (14).

In Europe, Urban and Marková (30,45,46) accepted six species to replace the *P. recondita* complex (i.e., *P. bromina*, *P. elymi* Westend., *P. perplexans*, *P. persistens* Plowr., *P. recondita* sensu stricto, and *P. schismi* Bubák. They considered *P. recondita* sensu stricto as the species that infects *Aegilops*, *Secale*, and *Triticum* spp. with aecial hosts in the family Boraginaceae, and *P. persistens* as the species that is prevalent on various gramineous uredinial or telial hosts with aecial hosts in the family Ranunculaceae (except *P. persistens* subsp. *agropyri* that can occur on the family Boraginaceae in Europe). The wheat leaf rust fungus (*P. triticina*) was considered as a subspecies of *P. persistens*. Based on DNA content, morphology, and interfertility, Anikster et al. (1) observed two groups in a collection of leaf rust fungi from cultivated wheat, wild emmer wheat (*Triticum turgidum* subsp. *dicoccoides*), and *Aegilops speltoides* Tausch from Israel. Group I, equivalent to Eriksson's *P. triticina* with virulence to common wheat and durum wheat, had alternate hosts in *Thalictrum* L. Group II with virulence to *Aegilops* spp. and *T. turgidum* (durum wheat) and alternate hosts in the family Boraginaceae was equivalent to a subset of *P. recondita* sensu Cummins (7).

The grass genus *Elymus* L., commonly known as wild rye or wheat grasses, has served as a rich germplasm resource for disease and pest resistance for wheat breeding (19) and is also a major host for brown leaf rusts. The identities of the brown leaf rusts on wheat, rye, *Aegilops* spp., and *Elymus* spp. have not been established in a phylogenetic context. In the present study, we explored the phylogenetic lineages in the brown leaf rusts on these selected hosts using DNA sequence analyses. In particular, we focused on the following questions: (i) did these lineages correspond to the taxonomic concepts in current use for cereal rust fungi, with specific emphasis on *P. recondita*, *P. triticina*, and *P. persistens*; and (ii) did the brown leaf rusts on *Elymus* spp. form a monophyletic phylogenetic group.

Materials and Methods

Leaf rust collections. Thirty-one *P. recondita* specimens on genus *Elymus* (the host of some specimens were labeled as *Agropyron*, *Leymus*, and *Hystrix*; Table 1) were obtained from herbaria at the J. F. Bell Museum of Natural History, University of Minnesota, St. Paul (MIN); Purdue University, West Lafayette, IN (PUR); and the Botanischer Garten und Botanisches Museum Berlin-Dahlem, Berlin, Germany (B). In all, 33 type specimens were obtained from the Swedish Museum of Natural History, Stockholm, Sweden (S, 1 sample); Royal Botanic Gardens, Kew, England, UK (K, 1 sample); and PUR (31 samples). Based on the condition of the specimens, only nine type specimens were selected for DNA extraction for species *P. actaeae-elymi* Mayor, *P. agropyrina*, *P. dactylidina* Bubák., *P. perplexans*, *P. persistens*, *P. recondita*, *P. subalpina* Lagerh., *P. triticina*, and *P. wyomensis* Arthur. Seventy-four additional specimens or samples were obtained from herbaria or collected (purified as single uredinium isolates for *P. triticina*) and kept in individual labs for the following species: *P. arrhenathericola* E. Fisch., *P. bromina*, *P. coronata* Corda., *P. coronatibrevispora* M. Liu & Hambl., *P. coronati-calamagrostidis* M. Liu

& Hambl., *P. coronati-hordei* M. Liu & Hambl., *P. coronatlongispora* M. Liu & Hambl., *P. gansensis* M. Liu & Hambl., *P. graminis* Pers., *P. holcina*, *P. hordei* G. H. Otth., *P. persistens*, *P. poarum* Nielsen, *P. pseudostriiformis* M. Abbasi., Hedjar. & M. Scholler, *P. recondita* (on hosts other than *Elymus* spp.), *P. striiformis* Westend., *P. striiformoides* M. Abbasi., Hedjar. & M. Scholler, and *P. triticina*. *Uromyces dactylidis* G.H. Otth. was selected to serve as outgroup taxon based on previous studies (25–27). Voucher numbers, hosts, origins, and GenBank accession numbers are listed in Table 1. Sequences for some specimens were determined in previous studies by M. Liu and S. Hambleton, as indicated in Table 1.

Genomic DNA extraction. Small pieces of infected plant material, 1–4.33 by 0.1–0.8 cm, or urediniospores were used for genomic DNA extraction. Plant materials were surface-sterilized by gentle wiping using a Kimwipe (KimTech, Inc.) sprayed with 75% ethanol. The cleaned infected plant material or pure uredinial spores were ground with 25 mg of glass beads and a small amount of diatomaceous earth with a FastPrep-24 shaker (M. P. Bio-medicals), followed by DNA extraction using OmniPrep (G-BioSciences) as per the manufacturer's instructions. RNase was added to the final DNA solution with no further precipitation.

Polymerase chain reaction amplification and sequencing. For aging herbarium specimen samples, the PreCR Repair Mix (New England BioLabs) was used to attempt DNA repair before polymerase chain reaction (PCR) amplifications. The nuclear ribosomal DNA (rDNA) region, including 5.8S and internal transcribed spacer 2 (ITS2), was amplified and sequenced using primers Rust2inv and ITS4, using methods described by Liu and Hambleton (25). A fragment of elongation factor 1- α (EF1- α) was amplified using forward primer EF1-Pt1-113f (ATCCTCACAGCGG TAAATCCACCA) and reverse primer EF1-Pt1-904r (ACACAC CTCTTTGGTCCATCCCTT) or, alternatively, using forward primer EF1-Pt2-032f (TCGTCATTGGACACGTCGATTCTGT) and reverse primer EF1-Pt2-552r (AGTACCGGCAGCAATGATGAG GAT), depending on whichever pair was successful. These primers were designed based on the EF1- α DNA sequences extracted from two *Puccinia* whole genome sequences (*P. graminis* and *P. triticina*) available on the Broad Institute website (http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html). PCR was performed in 10- μ l reactions containing 0.1 mM dNTPs, 2.0 pmol of each primer, 1 μ l 10 $\times$ Titanium Taq buffer, and 0.1 μ l of 50 $\times$ Titanium Taq DNA Polymerase (BD Biosciences). Thermal cycling conditions were set as 95°C for 3 min; followed by 38 cycles of 95°C for 30 s, 63°C for 20 s, and 72°C for 2 min; followed by a final extension of 72°C for 8 min. PCR products were cleaned by using ExoSAP-IT (USB Products Affymetrix, Inc.) and sequenced using PCR primers through the BioMedical Genomics Center services (University of Minnesota, St. Paul).

Phylogenetic analyses. Sequences were edited using Sequencher 4.7 (Gene Codes Corporation) and compiled using BioEdit Sequence Alignment Editor 7.0.5.3 (17). The compiled sequences were submitted to a web server (<http://mafft.cbrc.jp/alignment/server/index.html>) for alignment by MAFFT ver.5 (20), and the model FFT-NS-I (iterative refinement method) was selected. The sequence alignments were manually adjusted to correct obvious misalignments by the computer algorithm.

Phylogenetic analyses were conducted for each locus separately and for the combined data set. For parsimony analysis in PAUP* 4.0b10 (42), heuristic searches with random stepwise addition of 100 replicates and tree bisection-reconnection branch swapping were conducted. A limit of 1,000,000 rearrangements was set for each replicate. Bootstrapping analyses were set with 1,000 replicates with full heuristic search of random stepwise addition of 10 replicates and limit of 10,000 rearrangements per replicate.

Bayesian analysis was conducted using MrBayes 3.2.0 (37), (<http://www.mrbayes.net>) with different DNA substitution models estimated with Modeltest 3.6 (36). For the 5.8S-ITS2 locus, state frequencies = 0.3750, 0.1530, 0.1477, and 0.3243; substitution

rates = 2.3889, 2.5075, 1.9954, 0.2455, 3.9896, and 1.0000; proportion of invariable sites = 0.3884; and γ distribution shape parameter = 0.4321. For EF1- α , state frequencies = 0.2484, 0.3098, 0.1885, and 0.2533; substitution rates = 1.0000, 2.7944, 1.0000, 1.0000, 4.0323, and 1.0000; proportion of invariable sites =

0.4928; and γ distribution shape parameter = 3.0565. Four chains of 5,000,000 Markov chain Monte Carlo generations were run. The first 5,000 trees were discarded (burn-in) and the remaining trees were spooled to PAUP* to obtain posterior probabilities based on the 50% majority-rule consensus.

Table 1. Fungal (*Puccinia* spp.) isolates, hosts, origins, and GenBank accession numbers for internal transcribed spacer (ITS) of the ribosomal and elongation factor (EF)

Species	Voucher numbers ^a	Hosts ^b	Origins	ITS	EF ^c
<i>Puccinia actaeae-elymif</i> ^{d,e}	PUR F4124 (T)	<i>Elymus europaeus</i>	Switzerland	JX533543	JX533485
<i>P. agropyrina</i> ^{d,e}	PUR F4069 (T)	<i>E. repens</i>	Sweden	JX533542	n/a
<i>P. arrhenathericola</i> ^{d,e}	HSZ 0658	<i>Arrhenatherum elatius</i>	Slovakia	JX533581	JX533452
<i>P. bromina</i> ^{d,e}	HSZ 0660	<i>Bromus sterilis</i>	Slovakia	JX533582	JX533453
<i>P. bromina</i> ^e	K(M):107810	<i>B. sterilis</i>	England	JX533578	n/a
<i>P. coronata</i> var. <i>avenae</i> f. sp. <i>avenae</i>	BR 8665	<i>Agropyron coninum</i> (syn. of <i>E. caninus</i>)	Belgium	HM131278 ^f	n/a
<i>P. coronata</i> var. <i>avenae</i> f. sp. <i>graminicola</i>	PUR N 1252	<i>Holcus lanatus</i>	United States	HM131251 ^f	n/a
<i>P. coronata</i> var. <i>coronata</i>	(S) Reg.nr.F46266	<i>Frangula alnus</i>	Sweden	HM131310 ^f	n/a
<i>P. coronati-agrostidis</i>	PUR N1029	<i>Agrostis</i> sp.	Canada	HM131327 ^f	n/a
<i>P. coronati-brevispora</i>	DAOM 240183	<i>B. inermis</i>	Canada	HM131237 ^f	n/a
<i>P. coronati-calamagrostidis</i>	PUR N2268	<i>Calamagrostis canadensis</i>	United States	HM131308 ^f	n/a
<i>P. coronati-hordei</i>	DAOM 183691	<i>E. repens</i>	Canada	HM057138 ^f	n/a
<i>P. coronati-hordei</i>	PUR 89857	<i>Hordeum vulgare</i>	United States	HM131225 ^f	n/a
<i>P. coronati-longispora</i>	PRC 196	<i>B. erectus</i>	Czech Rep.	HM131232 ^f	n/a
<i>P. dactylitina</i> ^{d,e}	PUR F15426 (T)	<i>Dactylis glomerata</i>	Czech Rep.	JX533546	n/a
<i>P. gansensis</i>	DAOM 240065	<i>Achnatherum inebrians</i>	China	HM057115 ^g	n/a
<i>P. graminis</i>	TF NZ5 03	<i>Avena</i> sp.	New Zealand	HQ012437 ^h	n/a
<i>P. graminis</i>	BPI 803290	<i>Elytrigia repens</i> (syn. of <i>Elymus repens</i>)	China	HM131359 ^f	n/a
<i>P. graminis</i>	PUR 66554	<i>Oryzopsis exigua</i>	United States	HM131358 ^f	n/a
<i>P. graminis</i>	PUR N1125	<i>Secale cereale</i>	Germany	HQ012443 ^h	n/a
<i>P. graminis</i>	TF SA50 04	<i>Triticum aestivum</i>	South Africa	HQ012444 ^h	n/a
<i>P. holcina</i> ^{d,e}	HSZ 0681	<i>Holcus lanatus</i>	Czech Rep.	DQ512999	JX533454
<i>P. hordei</i>	BR 68612 33	<i>Hordeum murinum</i>	Belgium	HQ012448 ^h	n/a
<i>P. hordei</i>	K(M):78624	<i>H. murinum</i>	England	HQ012449 ^h	n/a
<i>P. perplexans</i> ^{d,e}	PUR F4086 (T)	<i>Alopecurus pratensis</i>	England	JX533545	JX533487
<i>P. persistens</i> ^e	K(M): 78119	<i>Agropyron repens</i> (syn. of <i>E. repens</i>)	England	JX533586	n/a
<i>P. persistens</i> ^d	HSZ 0688	<i>E. intermedia</i>	Czech Rep.	JX533517	JX533459
<i>P. persistens</i> ^d	HSZ 0691	<i>E. intermedia</i>	Czech Rep.	JX533519	JX533461
<i>P. persistens</i> ^d	HSZ 0696	<i>E. intermedia</i>	Czech Rep.	JX533521	JX533463
<i>P. persistens</i> ^d	ISR 3563	<i>E. intermedia</i>	Czech Rep.	JX533511	JX533464
<i>P. persistens</i> ^d	ISR 3675	<i>E. intermedia</i>	Czech Rep.	JX533512	JX533465
<i>P. persistens</i> ^d	HSZ 0686	<i>E. repens</i>	Czech Rep.	JX533515	JX533457
<i>P. persistens</i> ^d	HSZ 0687	<i>E. repens</i>	Czech Rep.	JX533516	JX533458
<i>P. persistens</i> ^d	HSZ 0689	<i>E. repens</i>	Sweden	JX533518	JX533460
<i>P. persistens</i> ^d	HSZ 0695	<i>E. repens</i>	Czech Rep.	JX533520	JX533462
<i>P. persistens</i> ^d	ISR 6134	<i>E. repens</i>	England	JX533513	JX533466
<i>P. persistens</i> ^d	ISR 6140	<i>E. repens</i>	England	JX533514	JX533467
<i>P. persistens</i>	DAOM 162167	<i>Thalictrum flavum</i>	Finland	JX533585	n/a
<i>P. persistens</i>	HSZ 0419	<i>T. minus</i>	Slovakia	JX533579	n/a
<i>P. persistens</i>	HSZ 0692	<i>T. minus</i>	Czech Rep.	JX533580	n/a
<i>P. persistens</i> ^d	K(M):169739 (T)	<i>Triticum repens</i>	United Kingdom	JX533510	n/a
<i>P. poarum</i>	DAOM 240188	<i>Tussilago farfara</i>	Canada	HM057150 ^g	n/a
<i>P. pseudostriiformis</i>	PUR N5354	<i>Poa nemoralis</i>	United States	HM057134 ^g	n/a
<i>Puccinia pseudostriiformis</i>	DAOM 220657	<i>Poa pratensis</i>	Canada	HM057113 ^g	n/a
<i>Puccinia recondita</i> ^d	ISR 77069	<i>Aegilops longissima</i>	Israel	JX533537	JX533481
<i>P. recondita</i> ^d	ISR 9382	<i>A. longissima</i>	Israel	JX533535	JX533479
<i>P. recondita</i> ^d	ISR 9003	<i>A. ovata</i>	Israel	JX533530	JX533474
<i>P. recondita</i> ^d	ISR 9284	<i>A. ovata</i>	Israel	JX533531	JX533475
<i>P. recondita</i> ^d	ISR 9295	<i>A. ovata</i>	Israel	JX533532	JX533476

(continued on next page)

^a B: Botanischer Garten und Botanisches Museum Berlin-Dahlem, Zentraleinrichtung der Freien Universität Berlin, Berlin; BP: Hungarian Natural History Museum, Budapest; BPI: U.S. National Fungus Collections, Beltsville, MD; BR: National Botanic Garden of Belgium, Meise, Belgium; DAOM: Agriculture and Agri-Food Canada, Ottawa, ONT, Canada; HSZ: specimens provided by Dr. J. Marková and DNA samples held by LSZ lab; ISR: specimens held by Y. Anikster; K(M): Royal Botanic Gardens, Kew, England, UK; MIN: J. F. Bell Museum of Natural History, University of Minnesota, St. Paul; PRC: Charles University in Prague, Praha, Czech Republic; Pt: samples held by J. Kolmer as DNA voucher only; PUR: Purdue University, West Lafayette, IN; RS: one sample held by S. Hambleton as DNA voucher only; S: Swedish Museum of Natural History, Stockholm; and TF: one pure spore sample provided by Dr. T. Fetch. (T) indicates the specimen is the type specimen.

^b Hosts are listed as original identification on the specimen package.

^c Abbreviation: n/a = not applicable.

^d Taxa included in analyses for the combined data set.

^e Species belong to *P. recondita* sensu lato complex according to Cummins (7).

^f Sequences published by Liu and Hambleton (26).

^g Sequences published by Liu and Hambleton (25).

^h Sequences published by Liu and Hambleton (27).

Results

DNA amplification and sequencing. Because of the ease of amplifying rDNA, more rDNA sequences than EF1- α sequences were successfully obtained for a large number of *Puccinia* spp. Among the 114 *Puccinia* sp. samples, amplification

of the fragment of 5.8S-ITS2 was attempted for 86 samples, and 82 were successfully amplified, including 9 type specimens. Twenty-eight sequences of reference species from previous studies were also used (25–27). As a single-copy gene, EF1- α had a lower success rate for amplification, and 58 sequences out of 86 samples were obtained, including 4 type

Table 1. (continued from preceding page)

Species	Voucher numbers ^a	Hosts ^b	Origins	ITS	EF ^c
<i>P. recondita</i> ^d	ISR 99018	<i>A. ovata</i>	Israel	JX533539	JX533483
<i>P. recondita</i> ^d	ISR 9350	<i>A. variabilis</i>	Israel	JX533533	JX533477
<i>P. recondita</i> ^d	ISR 9357	<i>A. variabilis</i>	Israel	JX533534	JX533478
<i>P. recondita</i> ^d	ISR 99128	<i>A. variabilis</i>	Israel	JX533540	JX533484
<i>P. recondita</i> ^d	PUR 55737	<i>Agropyron trachycaulum</i>	United States	JX533563	n/a
<i>P. recondita</i> ^d	MIN 512350	<i>B. ciliatus</i>	United States	JX533522	n/a
<i>P. recondita</i> ^d	HSZ 0694	<i>Clematis</i> sp.	Slovakia	JX533583	JX533455
<i>P. recondita</i>	PUR 65476	<i>E. albican</i>	United States	JX533550	n/a
<i>P. recondita</i> ^d	PUR 24872	<i>E. alskanus</i>	Canada	JX533549	n/a
<i>P. recondita</i> ^d	PUR 55732	<i>E. alskanus</i>	United States	JX533548	JX533489
<i>P. recondita</i>	PUR 59584	<i>E. arizonicus</i>	Mexico	JX533553	n/a
<i>P. recondita</i> ^d	MIN 870591	<i>E. canadensis</i>	United States	JX533525	JX533470
<i>P. recondita</i>	PUR 25161	<i>E. canadensis</i>	Canada	JX533554	n/a
<i>P. recondita</i>	PUR N1447	<i>E. canadensis</i>	United States	JX533589	n/a
<i>P. recondita</i>	B 70 0012410	<i>E. caninus</i>	Romania	HM131362 ^f	n/a
<i>P. recondita</i> ^d	PUR N76	<i>E. caninus</i>	Finland	JX533555	JX533490
<i>P. recondita</i> ^d	PUR 59754	<i>E. cinerus</i>	Canada	JX533556	JX533491
<i>P. recondita</i>	PUR 25210	<i>E. condensatus</i>	United States	JX533557	n/a
<i>P. recondita</i>	PUR F15636	<i>E. dahuricus</i>	Japan	JX533558	n/a
<i>P. recondita</i> ^d	PUR 59448	<i>E. glaucus</i>	United States	JX533560	JX533493
<i>P. recondita</i> ^d	PUR 59756	<i>E. glaucus</i>	Canada	JX533559	JX533492
<i>P. recondita</i>	PUR N1240	<i>E. hystrix</i>	United States	JX533588	n/a
<i>P. recondita</i>	PUR F17100	<i>E. repens</i>	Bulgaria	JX533561	n/a
<i>P. recondita</i>	PUR N1022	<i>E. repens</i>	Canada	JX533587	n/a
<i>P. recondita</i> ^d	PUR F16132	<i>E. semicostatus</i>	Japan	n/a	JX533494
<i>P. recondita</i>	PUR 25211	<i>E. submuticus</i>	Canada	JX533562	n/a
<i>P. recondita</i> ^d	PUR N1443	<i>E. villosus</i>	United States	JX533564	JX533495
<i>P. recondita</i> ^d	MIN 870593	<i>E. violosus</i>	United States	JX533526	JX533471
<i>P. recondita</i> ^d	MIN 512787	<i>E. virginicus</i>	United States	JX533523	JX533468
<i>P. recondita</i>	MIN 870607	<i>E. virginicus</i>	United States	JX533527	n/a
<i>P. recondita</i> ^d	MIN 870610	<i>E. virginicus</i>	United States	JX533528	JX533472
<i>P. recondita</i> ^d	MIN 513209	<i>Hystrix patula</i> (syn. of <i>E. hystrix</i>)	United States	JX533524	JX533469
<i>P. recondita</i> ^d	HSZ 0764	<i>Impatiens</i> cf. <i>pallida</i>	United States	JX533584	JX533456
<i>P. recondita</i>	PUR 55742	<i>Leymus ambiguus</i>	United States	JX533551	n/a
<i>P. recondita</i>	PUR 25121	<i>Leymus arenarius</i>	United States	JX533552	n/a
<i>P. recondita</i> ^d	PUR 25280	<i>Elymus × vancouverensis</i>	United States	JX533566	JX533496
<i>P. recondita</i> ^d	PUR 25281	<i>Leymus × vancouverensis</i> [<i>mollis</i> × <i>triticoideis</i>]	Canada	JX533567	JX533497
<i>P. recondita</i>	PUR 61943	<i>Leymus × vancouverensis</i> [<i>mollis</i> × <i>triticoideis</i>]	Canada	JX533565	n/a
<i>P. recondita</i>	MIN 931119	n/a	United States	JX533590	n/a
<i>P. recondita</i> ^d	MIN 931120	<i>S. cereale</i>	United States	JX533569	JX533499
<i>P. recondita</i> ^d	MIN 931121	<i>S. cereale</i>	United States	JX533570	JX533500
<i>P. recondita</i> ^d	ISR 9403	<i>S. montanum</i>	Israel	JX533536	JX533480
<i>P. recondita</i> ^d	MIN 931118	<i>Thalictrum</i> sp.	United States	JX533568	JX533498
<i>P. recondita</i> ^d	ISR 77081	<i>Triticum turgidum</i> var. <i>durum</i>	Morocco	JX533538	JX533482
<i>P. recondita</i> ^d	ISR 7784	<i>T. turgidum</i> var. <i>durum</i>	Morocco	JX533529	JX533473
<i>P. recondita</i> ^d	PUR F15509 (T)	<i>S. cereale</i>	France	JX533547	JX533488
<i>P. striiformis</i>	PUR N 1249	<i>Hordeum jubatum</i>	United States	HM057108 ^g	n/a
<i>P. striiformis</i>	RS480	<i>T. aestivum</i>	China	HM057123 ^g	n/a
<i>P. striiformoides</i>	BPI 0199096	<i>D. glomerata</i>	China	HM057137 ^g	n/a
<i>P. striiformoides</i>	PUR N5378	<i>D. glomerata</i>	United States	HM057109 ^g	n/a
<i>P. subalpina</i> ^{d,e}	PUR F4065 (T)	<i>E. caninus</i> (in record)	Sweden	JX533544	JX533486
<i>P. triticina</i> ^{d,e}	(S) Reg.nr.F180131 (T)	<i>T. vulgaris</i>	Sweden	JX533571	n/a
<i>P. triticina</i>	BP 88134	<i>Aegilops cylindrica</i>	Hungary	HM147357 ^f	n/a
<i>P. triticina</i> ^d	ISR 99129	<i>A. speltoideis</i>	Israel	JX533572	JX533507
<i>P. triticina</i> ^d	ISR 99160-1	<i>A. speltoideis</i>	Israel	JX533573	JX533508
<i>P. triticina</i> ^d	Pt ISR1400	<i>A. speltoideis</i>	Israel	JX533577	JX533506
<i>P. triticina</i>	PUR N1253	<i>E. repens</i>	Canada	HM057146 ^g	n/a
<i>P. triticina</i>	Pt AZB5 1	<i>T. aestivum</i>	Azerbaijan	n/a	JX533502
<i>P. triticina</i> ^d	Pt CHL8 5 3	<i>T. aestivum</i>	Chile	JX533574	JX533503
<i>P. triticina</i>	Pt Race 1	<i>T. aestivum</i>	United States	n/a	JX533509
<i>P. triticina</i>	Pt ARG9 3	<i>T. turgidum</i> var. <i>durum</i>	Argentina	n/a	JX533501
<i>P. triticina</i> ^d	Pt ETH4114 7	<i>T. turgidum</i> var. <i>durum</i>	Ethiopia	JX533575	JX533504
<i>P. triticina</i> ^d	Pt F2 2	<i>T. turgidum</i> var. <i>durum</i>	France	JX533576	JX533505
<i>P. wyomensis</i> ^d	PUR 24906 (T)	<i>Agropyron</i> sp. (redetermined)	United States	JX533541	n/a
<i>Uromyces dactylidis</i> (outgroup)	DAOM 216236	<i>D. glomerata</i>	Hungary	HM057148 ^g	n/a

specimens. As a result, the data set for ITS included a broader range of taxa than for EF1- α .

Alignment and phylogenies. The alignment of 110 5.8S-ITS2 sequences resulted in 544 characters, among which 146 characters

are parsimony-informative; 351 most parsimonious trees were retained of length = 614, CI = 0.467, RI = 0.831, RC = 0.388, HI = 0.533, and G-fit = -99.908 (Fig. 2). The majority of the *P. recondita* sensu lato samples collected on *Aegilops*, *Elymus*, and

Fig. 2. Of 351 most parsimonious trees, 1 tree based on 5.8S-internal transcribed spacer 2 rooted with *Uromyces dactylidis*. Values on the branches are majority rule frequencies/parsimony bootstrapping values/Bayesian posterior probabilities; * indicates value lower than 50%. Branches with parsimony bootstrapping value >50 and Bayesian posterior probability >70 are in bold. *Puccinia recondita* samples labeled with voucher number, host, and country code, other species labeled with species name and voucher number. Type specimens and the samples in *P. recondita* complex were highlighted in black fonts, others in gray fonts; † indicates type specimens.

Triticum spp. were distributed in six major clades: clades I, II, and III were strongly supported individually and as a group received some support (78%/74) (majority rule frequencies/parsimony bootstrapping values/Bayesian posterior probabilities) for a close relationship with clade IV, which was consistent with *P. recondita* group II from Anikster et al. (1). Clade V had low statistical support and did not show a strong relationship to any other clade. *P. persistens* and *P. triticea* were intermixed in one large, strongly supported clade VI, which also included five specimens identified as *P. recondita*: two on *Elymus caninus* (PUR N76 and B700012410), two on *E. repens* (PUR F17100 and PUR N1253), and one on *E. dahuricus* (PUR F15636). Five type specimens K(M): 169739 (*P. persistens*), (S) Reg.nr.F180131 (*P. triticea*), PUR F4069 (*P. agropyria*), PUR F4124 (*P. actaeae-elymi*), and PUR F4065 (*P. subalpina*) were included in the *P. persistens*/*P. triticea* clade. The type specimen of *P. recondita* formed a monophyletic group along with the other two rye leaf rust collections in group II type D of Anikster et al. (1). Type specimens of *P. perplexans* and *P. dactylidina* were closely related but not robustly connected with any other samples while the type specimen of *P. wyomensis* appeared as an orphan lineage in the phylogeny.

The alignment of 58 EF1- α sequences resulted in 556 characters, among which 207 characters were parsimony-informative; 57,624 most parsimonious trees were retained of length = 700, CI = 0.5811, RI = 0.884, RC = 0.514, HI = 0.419, and G-fit = -149.918 (Fig. 3). Shallow (terminal) groupings of taxa were generally congruent with those of the rDNA tree in Figure 2; however, the EF1- α tree provided better resolution. Clades I, II, and III were the same as in the rDNA tree. Clade IV was separated into three subclades (clade IV-1, IV-2, and IV-3), with the type specimen of *P. perplexans* grouped in subclade IV-2 and the type of *P. recondita* in subclade IV-3. Clade VI was separated into three subclades: VI-1, VI-2, and VI-3. The type specimens of *P. actaeae-elymi* and *P. subalpina* grouped in subclade VI-3.

Sequences for 54 samples with both rDNA and EF1- α data available were concatenated. In addition, eight samples with only one sequence region were added to that matrix. Among these eight samples, seven samples have only rDNA and one sample has only EF1- α locus. These five type specimens were K(M): 169739(T), PUR F4069(T), PUR F15426(T), PUR 24906(T), and (S)Reg.nr.F180131(T); two *P. recondita* on *Bromus ciliates* (MIN 512350) and *E. alskanus* (PUR 24872), respectively, from clade V; and one *P. recondita* on *E. semicostatus* (PUR F16132) from clade VI-2. The absent sequences were coded as missing data, which does not limit the accuracy of phylogenetic placement of those eight taxa (47). The concatenated alignment resulted in 1,089 characters for 62 taxa, among which 287 characters are parsimony-informative; 34,866 most parsimonious trees were retained of length = 1036, CI = 0.547, RI = 0.860, RC = 0.471, HI = 0.453, and G-fit = -201.739 (Fig. 4). Compared with the individual gene trees, the tree based on the combined loci revealed better support for the close relationship among clades VI-1, VI-2, and VI-3. A preliminary analysis indicated that *U. dactylidis* was not an appropriate outgroup for this species complex for the EF1- α gene; therefore, the EF1- α and combined data analyses were midpoint-rooted instead. Data matrices and trees were submitted to TreeBASE, study number S13719.

Discussion

The classification of brown leaf rust fungi on cereals and grasses has experienced a long history of controversy and uncertainty due to the reduced morphological characteristics available for taxonomy and the limitation of conducting interfertility experiments. Analyses of DNA sequences have proven effective for elucidating phylogenetic relationships among species in other fungi (24,43) but had not yet been adopted for examining the *P. recondita* complex. Our present study is an initial step toward clarifying the intertwined relationships within this species complex. Phylogenetic trees recovered multiple phylogenetic lineages which are congruent

with the existing classifications based on interfertility, host specificity, and morphology but also displayed a number of lineages that can be potential new species.

Rye leaf rust fungus (*P. recondita* sensu stricto). The name *P. recondita* was originally applied to a rust specimen on rye collected by Roberge (11). A hundred years later, Cummins and Caldwell (8) used the name to replace *P. rubigo-vera*, the name of a broader species concept commonly referred to as brown leaf rust fungi on cereals and grasses. Since then, the name *P. recondita* has conveyed different species concepts: in a general sense, *P. recondita* sensu lato includes the brown leaf rust fungi on various cereals and grasses (7) whereas, in the restricted concept, *P. recondita* sensu stricto only encompasses the taxon on rye and a few other host species (44,45). Despite the long taxonomic history and extensive research assessing host specialization within *P. recondita* sensu lato using infection experiments (12,28) and the recognition of the wheat leaf rust fungus (*P. triticea*) as distinct from the rye leaf rust fungus based on infection structures and morphology (14,39), there have been only a few studies focused on determining the exact host range of *P. recondita* sensu stricto. According to Urban and his colleagues, the natural hosts of *P. recondita* sensu stricto include *Secale* and *Aegilops* spp. (45). Based on genome size, host range, and interfertility, Anikster et al. (1) separated *P. recondita* on wheat, rye, and *Aegilops* spp. into two groups: group I infecting cultivated wheat and wild emmer wheat and group II infecting rye and *Aegilops* spp. Group II was further divided into four types, with type D restricted to rye. In this study, the phylogenetic trees based on EF1- α (Fig. 3) and combined rDNA (5.8-ITS2) and EF1- α data (Fig. 4) partly supported the separation of group II into different types. A strongly supported clade IV was further divided into three subclades: subclade IV-1, with collections from *A. variabilis*, *A. longissima*, and *T. durum*, was consistent with group II type A and C from Anikster et al. (1) with support 100/97/100; and subclade IV-2, with collections from *A. ovata*, was consistent with type B, with support of 100/52/91. Subclade IV-2 also included the type specimens of *P. perplexans* (PUR F4086) and *P. dactylidina* (PUR F15426). Subclade IV-3 with collections from *S. montanum* and *S. cereale*, was consistent with group II type D of Anikster et al. (1), with support of 100/100/100. The type specimen of *P. recondita* (PUR F15509) also was placed in this subclade. Given the strong support for three subclades, three corresponding phylogenetic species might be recognized. For the first species (subclade IV-1) that corresponds to group II types A and C from Anikster et al. (1), none of the type specimens sampled were included from which to select a potentially valid name. For the second species (subclade IV-2) that corresponds to group II type B of Anikster et al. (1), the type specimens of both *P. perplexans* and *P. dactylidina* were present. *P. perplexans* described on *A. pratensis* (uredinial or telial stage) and *R. acris* (aecial stage) by Plowright (34) predates *P. dactylidina* by Bubák (5); therefore, the name *P. perplexans* is potentially the duly legitimate name for this lineage. Multiple cross fertilization studies of brown rust indicated that *P. recondita* on *A. ovata* (also present in this clade) with an alternate host in the genus *Echium* (family Boraginaceae), was genetically isolated from other forms (1,12) and merited a consideration as distinct species. Comparing the results from our DNA sequence analyses with the fertilization experiments, it seems likely that the lineage on *A. ovata* could be considered as a distinct species, with uredinial or telial hosts that include *A. ovata*, *A. pratensis*, and *Dactylis glomerata* and aecial hosts in both the families Ranunculaceae and Boraginaceae. A case like this is not common but yet possible, such as the observation by Marková and Urban (30) that the aecial hosts of *P. persistens* subsp. *agropyri* are not only in the family Ranunculaceae but also in the family Boraginaceae in Europe. The third species (subclade IV-3), corresponding to group II type D of Anikster et al. (1), appears as the authentic *P. recondita* sensu stricto. The uredinial or telial hosts of *P. recondita* sensu stricto are very likely limited to *Secale* spp., with the aecial host limited to the family Boraginaceae, as discussed in previous studies (12,28).

Wheat leaf rust fungus. The taxonomic distinction of leaf rust fungus on wheat from the other leaf rust fungi has also been controversial. Leaf rust on wheat has been treated as a specialized form of *P. rubigo-vera* (or *P. recondita*), as a distinct species, and as a specialized form of *P. persistens*. The wheat leaf rust fungus

was originally observed as a host-specific form of *P. rubigo-vera* by Eriksson and Henning (15). This classification was accepted by Carleton (6) and Mains (28). When the name of *P. rubigo-vera* was replaced with *P. recondita*, the wheat leaf rust fungus was then considered as a specialized form of *P. recondita* by Cummins (7).

Fig. 3. Of 57,624 most parsimonious midpoint-rooted phylograms, 1 phylogram based on elongation factor 1- α partial DNA sequences. Values on branches are parsimony bootstrapping/Bayesian posterior probability. Majority frequencies for all present branches are 100%. *Puccinia recondita* samples labeled with voucher number, host, and country code; other species labeled with species name and voucher number; † indicates type specimens.

Marková and Urban (30) treated the wheat leaf rust fungus as a specialized form of *P. persistens* (16,21–23). *P. persistens* was described on *E. repens* (recorded as *T. repens* on the type specimen K(M): 169739) by Plowright (35). The classification of Marková and Urban that separated the species into three subspecies (*P. persistens* subsp. *persistens*, *trititica*, and *agropyri*) was based on minute differences in the shape of teliospores and host ranges. *P. persistens* subsp. *persistens* has broader teliospores (brachysporic, the ratio of length to lower cell width of 2 to 3), whereas the other two subspecies have longer and narrower spores (dolichosporic, ratio of 3 to 4). Among the three subspecies, *P. persistens* subsp. *trititica* has the narrowest host range, on wheat (uredinal or telial host), with alternate hosts in *Thalictrum* spp. The names *P. trititica*

Desmazieres
Winter 1884
Ploveright 1885 - 1889
Lagerheim 1897
Eriksson 1899
Arthur 1903
Arthur & Fromme 1920
Mains 1933
Arthur 1934
Cunnahs 1971
Savile 1984
Ankster 1997
Urban & Marková 1996
Marková & Urban 1999
Present study

Fig. 4. Majority consensus of 34,866 most parsimonious trees based on combined 5.8-internal transcribed spacer2 and elongation factor 1- α (EF1- α) loci midpoint rooted. *Puccinia recondita* samples labeled with voucher number, host, and country code; other species labeled with species name and voucher number. Values on the branches are majority rule frequencies/parsimony bootstrapping values/Bayesian posterior probabilities; * indicates value lower than 50%. Branches with parsimony bootstrapping value >50 and Bayesian posterior probability >70 are in bold; † indicates type specimens; § samples have only ribosomal DNA (rDNA) sequences, not EF1- α ; ◇ indicates one sample has only EF1- α sequence, not rDNA. In the immediate column to the right of the tree are listed the proposed valid names for selected clades from this study. These proposed names are compared with previous names accepted by researchers listed at the top of the columns. Except for gray color, each of the other colors represents equivalent taxa.

the level of genetic variation in relatively conserved regions is low.

***P. recondita* on *Elymus* spp.** The samples of *P. recondita* sensu lato on *Elymus* spp. did not form a monophyletic group. In addition to the isolates on *E. repens* and *E. intermedia*, which were located in the *P. persistens* and *P. tritricina* clade, other isolates were distributed into six clades (I, II, III, V, VI-2, and VI-3). Each one received strong statistical support, suggesting six distinct phylogenetic species. Here, we discuss the potentially correct taxonomic names for clades I, VI-2, and VI-3 based on associated type specimens, host range, and literature research. For clades II, III, and V, further evidence based on either aecial hosts or type specimens are needed to clarify their identities.

Clade I, comprising collections from *E. canadensis*, *E. violosus*, *E. virginicus*, *Hystrix* spp., and *Impatiens* cf. *pallida*, is consistent with the concept of *P. impatientis* (Schwein.) Arthur (2), although the taxonomic placement of this taxon has changed several times. The aecial state on *Impatiens maculata* (*Impatiens maculatae*) was originally described as *Aecidium impatientis* by Schweinitz (40). Arthur (2) successfully made the connection between the rust from *Impatiens aurea* with *P. rubigo-vera* on *E. virginicus* and, therefore, proposed to move the species into the genus *Puccinia*. However, in a revision of genus *Dicaeoma*, Arthur and Fromme (4) included the taxon under the name *Dicaeoma impatientis* (Schw.) Arthur & Fromme. The hosts were listed as *I. biflora* Walter and *I. pallida* Nutt. as aecial hosts, and the following species as uredinial or telial hosts: *Agrostis alba*, *A. hyemalis*, *A. perennans*, *Agrostis* sp., *Cinna arundinacea*, *C. latifolia*, *E. canadensis*, *E. striatus*, *E. virginicus*, *Hordeum jubatum*, and *Hystrix hystrix*. Mains (28) listed *P. impatientis* as a synonym of the forma specialis *P. rubigo-vera impatientis*; meanwhile, he accepted another four formae speciales of brown leaf rust fungi with aecia on species of *Impatiens* based on slightly different infection profiles, which were *P. rubigo-vera similis*, *P. rubigo-vera graminicola*, *P. rubigo-vera singularis*, and one unnamed forma specialis infecting *H. hystrix*. *P. rubigo-vera impatientis* had compatible infection with *E. canadensis* and *E. virginicus*, which is consistent with the host range of clade I. Because the lineage is strongly supported as monophyletic (Figs. 2 and 4, 100/99/100; Fig. 3, 100/90/99), it is reasonable to propose this taxon as a species. Because the name *P. impatientis* by Arthur was the earliest published, it should be the appropriate name for this phylogenetic species.

Clade VI-2 includes four specimens: [rephrased for clarity] one on *E. caninus*, one on *E. alaskanus*, and the two type specimens *P. actaeae-elymi* (PUR F4124) and *P. subalpina* (PUR F4065). Mayor (31) made the connection between the rust on *E. europaeus* (current name = *Hordelymus europaeus* (L.) Harz) with the aecial host *Actaea spicata* L., and named the rust as *P. actaeae-elymi* Mayor. No record was found for *P. subalpina* in the MycoBank database (Stalpers, J. de Cock, A. Robert, V. Vu, D and Ir. Ammar Ben Hadj Amor, <http://www.mycobank.org/Biolomics.aspx?Table=MycoBank&Page=200&ViewMode=Basic>) or in Index Fungorum (D. F. Farr and A. Y. Rossman, Fungal Databases, Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture–Agricultural Research Service, <http://www.indexfungorum.org/Names/Names.asp>). Based on the notes on the type specimen, the name was erected by G. v. Lagerheim in 1897 and published in Sydow's exsiccata [Sydow, uredineen 1384]. The host of the type specimen was originally recorded as [*Triticici*] *anini*, later corrected as *E. caninus* by anonymous. Because *P. subalpina* Lagerh. is an earlier, validly published name than *P. actaeae-elymi*, it is the correct name for this lineage.

Clade VI-3 includes two specimens, one on *E. semicostatus* from Japan and the other on *Clematis* sp. Arthur and Fromme (4) adopted *D. clematidis* (later renamed *P. clematidis*) to include all the *P. rubigo-vera* forms with aecial hosts in the family Ranunculaceae. Saccardo (38) recorded *P. clematidis-agropyri* Ellis & Everh. with the aecial host of *Clematis vitalba* and the uredinial or telial host of *Agropyron[um] glaucum* (current name = *Thinopyrum intermedium* subsp. *intermedium*). Mains (28) separated *P. rubigo-*

vera on *Clematis* spp. into 11 formae speciales by infection profiles, among which 8 formae speciales have compatible or moderate infection reactions with *Elymus* spp. (*E. arkansanus*, *E. canadensis*, *E. condensatus* [current name = *Leymus condensatus*], *E. glaucus*, and *E. virginicus*), *Hordeum* spp. (*Hordeum jubatum* and *H. gussoneanum*), and *Hystrix hystrix*. One forma specialis was compatible with *Agropyron tenerum* (current name = *E. trachycaulus* subsp. *trachycaulus*), while the final two formae speciales were compatible with *Bromus* spp. *E. semicostatus* was not included in their infection experiment. Arthur and Fromme's (4) concept is too broad to fit this phylogenetic lineage. The concept of Ellis and Everhardt (38) and any of the formae speciales recognized by Mains (28) should be further evaluated by including more samples on the host *T. intermedium* subsp. *intermedium* and the range of hosts tested by Mains (28) for compatibility.

With the combination of molecular phylogenetic tools and biology, this study sheds light on the phylogenetic diversity of the brown rust fungi on *Elymus* spp. and the concepts of *P. recondita* sensu stricto and the relationship between *P. tritricina* and *P. persistens*. For *P. recondita* sensu lato, there are still numerous questions to address in terms of how many distinct species should be recognized and how they are related. The analyses and interpretations presented here can serve as a foundation for future targeted studies addressing these questions.

Acknowledgments

We thank all the herbaria for kindly lending specimens for our study: Botanischer Garten und Botanisches Museum Berlin-Dahlem, Zentraleinrichtung der Freien Universität Berlin, Berlin; Hungarian Natural History Museum, Budapest; U.S. National Fungus Collections, Beltsville, MD; National Botanic Garden of Belgium, Meise, Belgium; Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada; Royal Botanic Gardens, Kew, England, UK; J. F. Bell Museum of Natural History, University of Minnesota, St. Paul; Charles University in Prague, Praha, Czech Republic; Purdue University, West Lafayette, IN; one sample held by S. Hambleton as DNA voucher only; and the Swedish Museum of Natural History, Stockholm; and two anonymous reviewers and the editor for valuable suggestions to improve this manuscript.

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