A Study on the Phylogeny of the Dyer's Woad Rust Fungus and Other Species of *Puccinia* from Crucifers

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ABSTRACT

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The identity of a *Puccinia* species occurring on the introduced weed dyer's woad (*Isatis tinctoria*) was studied using sequences from the internal transcribed spacer of the nuclear ribosomal DNA. The relationship of this fungus to other *Puccinia* species occurring on the family Brassicaceae in Europe and North America was examined, and we tested the hypothesis that *P. thlaspeos* and *P. monoica* are correlated species. The data suggest that the *Puccinia* species from dyer's woad is closely related

to the North American species *P. consimilis* and may be derived from an indigenous strain of *P. consimilis* that switched hosts. Thus, the *Puccinia* species from dyer's woad is probably native to North America and is unlikely to cause disease epidemics on indigenous plants if used as a biological control agent against dyer's woad. *P. thlaspeos* appears to be polyphyletic and, therefore, *P. thlaspeos* and *P. monoica* do not appear to be correlated species. Additional DNA sequence data will be needed to clarify further the phylogeny of *Puccinia* species on the family Brassicaceae.

Additional keywords: noxious weed, PCR, rust fungi.

Several rust fungi have been assessed for use as biocontrol agents against weeds. These fungi vary in their effectiveness, but some show considerable promise (22). Rust fungi such as *Puccinia chondrillina* appear to have the most potential for biological control, because they are easily dispersed and can infect plants under a range of conditions. These rust fungi may also reduce seed production and lower the ability of perennial plants to overwinter (10).

A species of *Puccinia* with excellent biocontrol potential was found on the introduced weed dyer's woad (*Isatis tinctoria* L.) in southern Idaho in 1978 (18). Although it does not appear to kill dyer's woad, infections by this fungus are systemic and cause stunting and severe malformation. In addition, infected plants produce very few seed and, because of this, the fungus is being intensively studied as a potential biological control agent for dyer's woad. Since its discovery, the fungus has spread from southern Idaho into portions of northern Utah.

Dyer's woad, a member of the family Brassicaceae, was brought to North America from Europe during the colonial period and is thought to have come to the western United States in the early 1900s (4,23). Under western conditions, it aggressively competes with native vegetation and has become a serious problem on rangeland and in agricultural areas. Because much of the rangeland affected by this weed is inaccessible, chemical control may be difficult or impossible. Thus, the *Puccinia* species attacking dyer's woad may provide one of very few options for controlling this weed.

Its use as a biological control agent requires a thorough understanding of its identity. The fungus has been tentatively identified as *P. thlaspeos* C. Schub., and most of its characteristics correspond to the description of this species (1). It is an autoecious,

microcyclic species. The teliosori are usually hypophyllous, and the teliospores fit the description for *P. thlaspeos*, with a persistent pedicel, a thickened apex, the pore of the upper cell at the apex, and the pore of the lower cell at the septum (Fig. 1). However, there are a few characters inconsistent with *P. thlaspeos*. Spermatia are abundant on infected dyer's woad prior to the appearance of teliosori; yet Arthur (1) indicates that spermatia are not seen on collections of *P. thlaspeos* and are probably not formed by this species. In addition, *P. thlaspeos* has been reported to occur on members of several genera in the family Brassicaceae, but not on *Isatis* (1,7). Dyer's woad plants infected with rust have been found sharing sites with healthy plants of *Arabis drummondii*, *Arabis* sp., *Descurainia pinnata* (Walter) Britton, *Erysimum* sp., and *Thlaspi* sp., which are reported hosts of *P. thlaspeos* (7; S. V. Thomson, *personal communication*). Thus, the identity of the rust fungus occurring on dyer's woad needs further clarification.

In many cases, host identification and morphology of the teliospores are the only characteristics available for studying the taxonomy of microcyclic rust fungi. A range of potential hosts for the dyer's woad rust was examined in the initial part of this study. But, additional characters may also be obtained from DNA sequence data, and the analysis of sequence data has been shown to be useful in studies of the relationships among species of *Puccinia* (26). The objectives of the present work were to use host range data and DNA sequences to (i) examine the relationship of the fungus on dyer's woad to *P. thlaspeos*, (ii) test the hypothesis that *P. thlaspeos* and *P. monoica* are correlated species, (iii) gain a better understanding of the relationship between the rust fungus on dyer's woad and other members of *Puccinia* occurring on the family Brassicaceae, and (iv) understand better whether the fungus on dyer's woad is indigenous or introduced.

MATERIALS AND METHODS

Host range studies. The cruciferous species *Thlaspi montanum* L., *Smelowskia calycina* (Stephenson ex Willd.) C.A. Mey., *D. pinnata*, *T. arvense* L., *Brassica napus* L. (canola 'Westar'), and *B. campestris* L. (canola 'Goldrush') were selected for a test of

the host range of dyer's woad rust. *T. montanum*, *S. calycina*, and *D. pinnata* are described as hosts of *P. thlaspeos* (7). The two varieties of canola were tested because canola is grown in some regions where dyer's woad is a noxious weed. *T. arvense*, which is an introduced plant in the Rocky Mountain region, was included because it occurs in woad-infested areas.

In spring of 1993, each of the above species and dyer's woad were grown in 4-inch plastic pots and exposed to rust infection at a field site alongside naturally occurring, rusted dyer's woad bearing teliosori. Thirty pots containing one or two plants were used for each species. They were watered by hand as needed, and the annual plants (*B. napus*, *B. campestris*, *D. pinnata*, and *T. arvense*) remained in the field until they bolted and began to senesce. The perennial plants (*T. montanum* and *S. calycina*) and dyer's woad remained in the field until the indigenous rusted woad began to senesce, and they were then placed in a woad-free area for observation and overwintering. Plants that developed symptoms of rust were recorded and removed as soon as symptoms were visible. Overwintered plants were observed for rust symptoms through late summer of 1994. In spring of 1994, the experiment was repeated using 30 pots each of *S. calycina*, *D. pinnata*, both canolas, and dyer's woad. There were six pots each of *T. montanum* and *T. arvense*. Twenty-two pots containing

dyer's woad were added to the site after hail damaged the original plants. Overwintered plants were observed for symptoms of rust infection through late summer of 1995.

Fungal material. The relationship of the dyer's woad rust fungus to *P. monoica* Arth., *P. consimilis* Ellis & Everh., and *P. thlaspeos* was studied, because these species are considered to be closely related and may be correlated species (1,5). *P. drabae* F. Rudolphi, *P. aberrans* Peck, and *P. codyi* Savile were also studied, because they occur on the family Brassicaceae and are not part of the above group of correlated species. *P. caricina* DC., which occurs on members of the family Cyperaceae, was used as the outgroup.

Two samples of the *Puccinia* on dyer's woad and a collection of *P. caricina* on *Carex rostrata* Stokes were collected from sites in northern Utah and deposited in the Intermountain Herbarium at Utah State University. All other specimens were obtained as loans from the herbaria listed in Table 1. The presence of aecia, telia, and spermatia was determined by examining herbarium specimens (Table 2) under a high-powered dissecting microscope.

DNA isolation, amplification, and sequencing. Small pieces of leaf tissue 2 to 3 mm in diameter, bearing either teliosori or aecia (depending on the species of *Puccinia*), were removed from the herbarium specimens and placed in a 1.5-ml microcentrifuge

Fig. 1. Teliospores representing ornamented and unornamented members of the genus *Puccinia* occurring on crucifers. **A**, *Puccinia* sp. from dyer's woad; **B**, *P. thlaspeos*; **C**, *P. consimilis*; and **D**, *P. drabae*. Bar = 40 μ m.

tube. DNA was extracted using the method of Kropp et al. (14,15). Liquid nitrogen was added to the microcentrifuge tubes, and the leaf tissue was crushed to powder using a glass rod. Five hundred microliters of extraction buffer (0.7 M NaCl, 0.05 M Tris-HCl [pH 8], 0.01 M EDTA, 1% β -mercaptoethanol, and 1% cetyltrimethylammonium bromide [CTAB]) was added to each crushed sample and incubated for 30 min at 60°C. An equal or greater volume of chloroform/isoamyl alcohol (24:1 by vol) was added to each tube. The mixture was then emulsified using a vortex and centrifuged at $11,750 \times g$ for 5 min. The supernatant was collected, nucleic acids were precipitated with isopropyl alcohol and centrifuged at $11,750 \times g$ for 5 min, and each pellet was washed in 80% ethyl alcohol (EtOH). After being recentrifuged, pellets were dried at room temperature overnight, resuspended in 20 to 40 μ l of Tris-EDTA buffer (pH 8), and stored at -20°C.

Sequences of the internal transcribed spacer (ITS) region from nuclear ribosomal DNA were obtained for analysis using polymerase chain reaction (PCR). Four primers, corresponding to conserved regions of the 5' end of the large ribosomal subunit or to the 3' end of the small ribosomal subunit, were used in different combinations to amplify the entire ITS region. The two primers corresponding to the large ribosomal subunit were Rust 1 (5'-GCT TAC TGC CTT CCT CAA TC-3') (17) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (24). The primers corresponding to sequences on the small ribosomal subunit were ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (24) and ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (8). In cases in which amplification was difficult, primers ITS5 and ITS4 were used as nested primers after a preliminary amplification using either ITS1F and Rust 1 or ITS5 and Rust 1.

Amplifications were done using 100- μ l PCR reactions each containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μ M 5'-primer, 0.1 μ M 3'-primer, and 2.4 units *Taq* DNA polymerase. Four microliters of diluted DNA extract was used per reaction. The DNA extract was usually diluted 1:10 before use, but dilutions of 1:100 and 1:1,000 were used when necessary to achieve amplification. The exact concentration of the template was not determined. Mineral oil was added to each tube to prevent evaporation, and amplifications were done in a Perkin-Elmer DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT). For the reactions involving

most of the primer combinations, the amplification parameters of Kropp et al. (15) were used. However, for amplifications using primer pair ITS1F and Rust 1, the amplification parameters of Gardes and Bruns (8) were modified for use as follows: denaturation at 95°C (35 s), annealing at 45°C (55 s), and extension at 72°C (45 s) for 13 cycles; denaturation at 95°C (35 s), annealing at 45°C (55 s), and extension at 72°C (120 s) for an additional 13 cycles; denaturation at 95°C (35 s), annealing at 45°C (55 s), and extension at 72°C (180 s) for nine cycles; and a final extension at 72°C for 10 min.

After amplification, the entire volume of each reaction was placed as a droplet onto a Parafilm strip. The mineral oil was removed by tilting the Parafilm and allowing the droplet to roll along its surface, leaving the oil behind as it moved (25). The double-stranded amplification product was then purified using Wizard PCR Preps DNA purification system (Promega Corp., Madison, WI). Sequences were obtained from the purified amplification product using the dye terminator method with an Applied Biosystems, Inc., model 373A DNA sequencer (Foster City, CA). ITS I was sequenced using primer ITS5, and ITS II was sequenced using primer ITS4. To enable ITS I and II to be sequenced in both directions, two internal primers were made using preliminary sequence data containing the 5.8s gene. These primers corresponded to sequences in the 5.8s gene and were designated Rust 2 (5'-TTT CAC TGT GTT CTT CAT C-3') and Rust 3 (5'-GAA TCT TTG AAC GCA CCT TG-3'). Primers Rust 2 and Rust 3 were used to sequence ITS I and ITS II, respectively, and the two reverse sequences for each region were used as a comparison to insure that the data were correct.

Alignment of sequences and phylogenetic analysis. Sequences were entered into the GCG Wisconsin Sequence Analysis Package (Genetic Computer Group, Inc., Madison, WI) and aligned visually. In cases in which there was a choice of substitutions, transitions (i.e., purine for a purine or pyrimidine for a pyrimidine) were preferred over transversions.

Our data set included several insertion/deletions (indels) that may be informative for inferring phylogenies. Therefore, we coded indels so they could be treated as characters. Single-nucleotide indels were coded with gaps (as a fifth character), and multibase indels were treated as additional characters (0 = gap, 1 = no gap) appended to the end of the data matrix. Within the main

TABLE 1. Collections of *Puccinia* species from which sequence data were obtained

Species	Herbarium	Gene bank number	Origin and date	Host
<i>Puccinia</i> sp.	F00568 (UTC ^a)	U88212	Utah, United States (1994)	<i>Isatis tinctoria</i>
<i>Puccinia</i> sp.	F00567 (UTC)	U88213	Utah, United States (1995)	<i>Isatis tinctoria</i>
<i>Puccinia</i> sp.	AN025349 (ARIZ)	U88214	Idaho, United States (1983)	<i>Isatis tinctoria</i>
<i>P. thlaspeos</i>	US-0107511 (BPI)	U88218	Switzerland (1924)	<i>Arabis hirsuta</i>
<i>P. thlaspeos</i>	US-0107513 (BPI)	U88219	Sweden (1958)	<i>Arabis hirsuta</i>
<i>P. thlaspeos</i>	AN025351 (ARIZ)	U88220	Colorado, United States (1973)	<i>Thlaspi alpestre</i> ^b
<i>P. thlaspeos</i>	AN025352 (ARIZ)	U88221	Colorado, United States (1973)	<i>Thlaspi alpestre</i>
<i>P. thlaspeos</i>	AN025354 (ARIZ)	U88222	Wyoming, United States (1982)	<i>Arabis microphylla</i>
<i>P. thlaspeos</i>	US-0107498 (BPI)	U88217	Alaska, United States (1904)	<i>Arabis ambigua</i>
<i>P. consimilis</i>	US-0058385 (BPI)	U88215	Wyoming, United States (1898)	<i>Sisymbrium linifolium</i>
<i>P. consimilis</i>	US-0058382 (BPI)	U88216	Montana, United States (1889)	<i>Sisymbrium linifolium</i> ^b
<i>P. monoica</i>	AN024025 (ARIZ)	U88224	Colorado, United States (1979)	<i>Arabis retrofracta</i>
<i>P. monoica</i>	AN024026 (ARIZ)	U88225	Arizona, United States (1973)	<i>Arabis perennans</i>
<i>P. monoica</i>	AN024032 (ARIZ)	U88226	Arizona, United States (1982)	<i>Arabis perennans</i>
<i>P. monoica</i>	AN024024 (ARIZ)	U88223	Arizona, United States (1963)	<i>Arabis</i> sp.
<i>P. aberrans</i>	114408 (COLO)	U88231	Wyoming, United States (1951)	<i>Smelowskia calycina</i>
<i>P. aberrans</i>	189659 (DAOM)	U88232	Yukon, Canada (1982)	<i>Smelowskia calycina</i>
<i>P. codyi</i>	117853 (DAOM)	U88233	Yukon, Canada (1967)	<i>Smelowskia borealis</i>
<i>P. caricina</i>	F00566 (UTC)	U88234	Utah, United States (1993)	<i>Carex rostrata</i>
<i>P. drabae</i>	144964 (DAOM)	U88230	Quebec, Canada (1967)	<i>Draba arabisans</i>
<i>P. drabae</i>	287901 (COLO)	U88229	British Columbia, Canada (1954)	<i>Draba longipes</i>
<i>P. drabae</i>	163196 (COLO)	U88228	Finland (1958)	<i>Draba hirta</i>
<i>P. drabae</i>	82846 (COLO)	U88227	Germany (1936)	<i>Draba aizoides</i>

^a Herbarium where the specimen is housed: ARIZ = University of Arizona, Tucson; BPI = U.S. National Fungus Collections, Beltsville, MD; COLO = University of Colorado, Boulder; DAOM = Biosystematics Research Centre, Ottawa, Ontario, Canada; UTC = Utah State University, Logan.

^b *Thlaspi alpestre* = *T. montanum* and *Sisymbrium linifolium* = *Schoenocrambe linifolia*.

body of the data, multibase-indel gaps were treated as missing data and taxa without gaps were treated as normal multistate DNA characters. To avoid double coding the single-base indels, we followed the coding procedure of Hibbett et al. (11).

Phylogenetic analyses of the sequence data were performed with the computer software PAUP (Phylogenetic Analysis Using Parsimony) 3.1.1 (21) on a Macintosh Quadra 800. To search for shortest trees, we used the HEURISTIC search option with random sequence addition, MULPARS (saving all shortest trees), COLLAPSING ZERO-LENGTH BRANCHES, TBR (tree bisection-reconnection), and STEEPEST DESCENT in effect. We repeated the analysis 100 times with random entry order of taxa to increase the chances of finding all islands of possible trees (19). We used bootstrap analyses (100 replicate heuristic searches with random taxon addition) to assess the degree of support for each monophyletic group on the strict consensus tree. All trees up to four steps longer than the shortest tree were saved with the FILTER TREES option, and strict consensus trees of these suboptimal solutions were used to obtain decay values for each branch (2). We assessed the amount of nonrandom structure in the data set using the RANDOM TREES (10,000 trees) option in PAUP. The g_1 statistic measures skewness of the frequency distribution of the random trees and can be used to evaluate the structure of the data (12,13). *P. caricina* (which occurs on *Carex* species) and *P. drabae* (which occurs on members of the family Brassicaceae) were used as outgroups for rooting the trees.

RESULTS

Host range and presence of spore stages. In 1993, 22 out of 27 (81%) of the potted dyer's woad plants exposed to the woad rust fungus became infected; the other five plants died of unrelated causes. In 1994, 36 out of 52 (69%) potted woad plants became infected. In both years, *T. arvense*, *D. pinnata*, *B. napus*, and *B. campestris* bolted and senesced while in pots at the exposure site, but never displayed any symptoms of infection. *T. montanum* and *S. calycina* from 1993 and 1994 never displayed any symptoms of rust infection after being overwintered and grown in pots through the second summer after exposure.

Seven of 16 herbarium specimens of *P. consimilis* examined had telia but no aecia, six had aecia but lacked telia, and only three had both stages. Spermagonia were observed on all except one of the herbarium specimens of *P. consimilis* (Table 2). Spermagonia were present on each specimen of the *Puccinia* on dyer's woad, but were found on only three of the 16 herbarium specimens of *P. thlaspeos* (Table 2).

Amplification and sequencing of DNA from herbarium specimens. We were able to amplify and sequence the entire ITS region from the three fresh specimens collected in northern Utah and from 20 additional specimens obtained from the herbaria listed in Table 1. Attempts to amplify and sequence DNA from other herbarium specimens were unsuccessful. DNA from several specimens could be amplified, but it was difficult to obtain sequences. In other cases, DNA from the specimens could not be amplified. The specimens that we used varied greatly in age, having been collected between 1889 and 1995. However, the age of the specimen did not appear to be directly related to whether its ITS could be successfully amplified and sequenced. For example, DNA extracted from specimens of *P. consimilis* (US-0058382) and *P. thlaspeos* (US-0107498) collected in 1889 and 1904, respectively, was amplified and sequenced (Table 1), whereas DNA from collections of *P. thlaspeos* (AN025350 and AN025358) made in 1949 and 1918, respectively, could not be amplified.

Alignment and phylogenetic analysis. The ITS region of the species of *Puccinia* studied contained enough characters to permit phylogenetic analysis. We aligned 712 nucleotide positions, including the internal 5.8s region of 155 base pairs. Of the 712 positions, 194 were variable and 76 were potentially informative (more than two states shared by two or more samples). We detected 79 single-base indels of which 28 were potentially informative and 47 multiple-base indels of which 31 were potentially informative (Table 3). Complete unaligned sequences are available in GenBank under the accession numbers listed in Table 1.

Parsimony analysis yielded four shortest trees of 489 steps. The strict consensus of these trees (Fig. 2) shows several clades that are well supported by both bootstrapping and decay indices. The three specimens of the dyer's woad rust fungus included in the study were identical and formed a clade (designated clade 1) with the two specimens of *P. consimilis* (Fig. 2). Within this strongly supported clade (bootstrapping = 100%, decay index = 4), the specimens of *P. consimilis* formed a clade that was separate from dyer's woad rust with good bootstrap support (bootstrap support = 67%, decay index = 2). *P. monoica* and some of the *P. thlaspeos* specimens were grouped into two additional clades (designated 2 and 4) that were strongly supported. Clade 4 (bootstrap support = 99%, decay index = 4) included one specimen of *P. monoica* and two of *P. thlaspeos*. Clade 2 (bootstrap support = 91%, decay index 4) included two specimens of *P. thlaspeos* and two of *P. monoica*. In this case, *P. monoica* and *P. thlaspeos* are on separate branches that are each strongly supported as being monophyletic. Both branches have bootstrap support of 100% and decay indices of 4. Clade 3 is also strongly supported (bootstrap support = 100%, decay index = 4) and is comprised of two specimens from northern Canada, one representing *P. aberrans* and the other *P. codyi*. An additional specimen of *P. aberrans* from the western

TABLE 2. Presence of different spore stages on herbarium specimens of *Puccinia consimilis*, *P. thlaspeos*, and *Puccinia* sp. from dyer's woad

Species	Specimen	Aecia	Telia	Spermagonia
<i>P. consimilis</i>	US-0058380 (BPI) ^a	—	+	+
	US-0058381 (BPI)	—	+	+
	US-0058382 (BPI)	+	—	+
	US-0058383 (BPI)	+	—	+
	US-0058384 (BPI)	+	+	+
	US-0058385 (BPI)	—	+	—
	US-0058388 (BPI)	+	—	+
	US-0058387 (BPI)	+	—	+
	US-0058386 (BPI)	+	—	+
	US-0058372 (BPI)	+	—	+
	US-0058373 (BPI)	+	+	+
	US-0058379 (BPI)	—	+	+
	US-0058377 (BPI)	—	+	+
	US-0058378 (BPI)	—	+	+
	US-0058376 (BPI)	—	+	+
	US-0058374 (BPI)	+	+	+
<i>P. thlaspeos</i>	US-0107513 (BPI)	—	+	—
	US-0107512 (BPI)	—	+	—
	US-0107511 (BPI)	—	+	—
	US-0107498 (BPI)	—	+	—
	US-0107557 (BPI)	—	+	—
	US-0107556 (BPI)	—	+	—
	AN025351 (ARIZ)	—	+	—
	AN025352 (ARIZ)	—	+	+
	AN025354 (ARIZ)	—	+	—
	AN025356 (ARIZ)	—	+	+
	AN025350 (ARIZ)	—	+	+
	AN025353 (ARIZ)	—	+	—
	AN025360 (ARIZ)	—	+	—
	AN025359 (ARIZ)	—	+	—
	AN025362 (ARIZ)	—	+	—
	AN025361 (ARIZ)	—	+	—
<i>Puccinia</i> sp.	AN025349 (UTC)	—	+	+
	AN025348 (UTC)	—	+	+
	AN025355 (UTC)	—	+	+
	F00567 (UTC)	—	+	+
	F00568 (UTC)	—	+	+

^a Herbarium where the specimen is housed: ARIZ = University of Arizona, Tucson; BPI = U.S. National Fungus Collections, Beltsville, MD; and UTC = Utah State University, Logan.

United States was loosely associated with this clade, but the association was weakly supported (Fig. 2). One of the fungi used as an outgroup, *P. caricina*, was widely divergent from the *Puccinia* species occurring on members of the family Brassicaceae. The specimens of *P. drabae* formed a polyphyletic group that was also widely divergent from the other species of *Puccinia* that occur on the family Brassicaceae.

DISCUSSION

The sequence analysis indicates that the dyer's woad rust fungus is closely related to *P. consimilis* rather than to *P. thlaspeos*. *P. consimilis* is an autoecious rust that systemically infects members of the family Brassicaceae and is known to occur only in the Rocky Mountains of western North America. The localized occurrence of *P. consimilis* overlaps with the distribution of dyer's woad, and it is possible that a strain of *P. consimilis* switched from its indigenous hosts to dyer's woad. New races of some rusts may develop quite readily. For example, Little and Manners (16) showed that, in a single experiment in which susceptible host plants were inoculated with two races of *P. striiformis*, new races were easily isolated from diseased plants. Ten percent of the single-spore cultures isolated from the infected hosts were new races that were subsequently shown to be stable for a period of at least 1 year after the experiment. In addition, Burdon et al. (3) found evidence that hybridization between formae speciales of *P. graminis* can produce strains that are virulent on new host species.

Although the teliospores of *P. consimilis* closely resemble those of the dyer's woad rust fungus, *P. consimilis* reportedly differs by having aecia and telia that occur simultaneously on a single host (1). However, only three of the specimens of *P. consimilis* studied had both stages present, and nearly half had telia but no aecia. Because the presence of both aecia and telia on the same host is a primary character separating *P. consimilis* from related *Puccinia* species, there is little to distinguish the specimens of *P. consimilis* that lack aecia from the fungus on dyer's woad. In addition, spermatogonia were observed on almost all of the herbarium specimens of *P. consimilis*. Spermatogonia are a conspicuous feature of *Puccinia* infections observed on dyer's woad plants in the field, and

they were present on each herbarium specimen of *Puccinia* on dyer's woad listed in Table 2.

The failure of the *Puccinia* from dyer's woad to produce symptoms on several known hosts of *P. thlaspeos* is evidence that the woad rust is not *P. thlaspeos*. Like the specimens of *P. consimilis*, which lack aecia, very little distinguishes the woad rust fungus from *P. thlaspeos*. The teliospores of both fungi are very similar, both lack aecia and uredia and both cause systemic infections on crucifers. However, one morphological difference is that plants infected with *P. thlaspeos* are reported to lack spermatogonia (1), and spermatogonia were lacking on most of the herbarium specimens of *P. thlaspeos* examined (Table 2). The consistent presence of spermatogonia on rust-infected dyer's woad is further evidence that the dyer's woad rust is not *P. thlaspeos*.

Among the six specimens of *P. thlaspeos* studied, only two (AN025352 and AN025354) grouped in the same clade. In fact, the sequence from one specimen of *P. thlaspeos* (US-0107498) appears with *P. drabae* on the consensus tree presented in Figure 2. The remaining sequences obtained for specimens of *P. thlaspeos* are also quite divergent, indicating that this taxon is polyphyletic. Our data do not support the hypothesis that *P. thlaspeos* and *P. monoica* are correlated species (1,5), even though sequences from some of the specimens of *P. thlaspeos* appear on clades with *P. monoica*. However, the morphologies of specimens of *P. thlaspeos* used in this study, including US-0107498, were very similar and fit published descriptions of this species. Because *P. thlaspeos* is microcyclic, there are relatively few characters that can be used to delimit this species. The only taxonomic characters available are either the host plant on which the fungus grows or teliospore characteristics such as spore dimensions, cell wall thickness, lack of ornamentation, location of pores, and length and persistence of the pedicel. More species may exist within taxa like *P. thlaspeos* than can be detected using this relatively limited set of morphological characters. This might also characterize *P. drabae*, which our data indicate is another polyphyletic taxon. The members of this species have ornamented teliospores (Fig. 1) and are widely divergent from the smooth-spored *Puccinia* species examined in this study. *P. drabae* is also microcyclic and, as is the case for *P. thlaspeos*,

TABLE 3. Comparison of character codes^a from aligned sequences of ITS1, ITS2, and the 5.8s gene of *Puccinia* species on crucifers

Species	Specimen	Coding characters
<i>Puccinia</i> sp.	F00568	1010110000001000101001011100000111110010101111
<i>Puccinia</i> sp.	F00567	1010110000001000101001011100000111110010101111
<i>Puccinia</i> sp.	AN025349	1010110000001000101001011100000111110010101111
<i>P. consimilis</i>	US-0058385	1010110000001000101001011100000111110010101111
<i>P. consimilis</i>	US-0058382	1010110000001000101001011100000111110010101111
<i>P. thlaspeos</i>	US-0107498	1011111111001001011101111100101101110100110111
<i>P. thlaspeos</i>	US-0107511	10111000000010010110010111000001110110010101110
<i>P. thlaspeos</i>	US-0107513	1011110000001111110010110101111111110010111101
<i>P. thlaspeos</i>	AN025351	1011110000001011111001011100000111110010111111
<i>P. thlaspeos</i>	AN025352	1011111000001011111001011100000111110010111111
<i>P. thlaspeos</i>	AN025354	1011111000001011111001011100000111110010111111
<i>P. monoica</i>	AN024024	1011111000001011111001011100000111110010111111
<i>P. monoica</i>	AN024025	10111000000010100110010111000001110110010110100
<i>P. monoica</i>	AN024026	1011100000001000101001011100000111110010100011
<i>P. monoica</i>	AN024032	1011100000001000101001011100000111110010100011
<i>P. drabae</i>	82846	101111111110010010111011111001011011110100110111
<i>P. drabae</i>	163196	1011111111110010111011111001011011110110110111
<i>P. drabae</i>	287901	1011110100001001011101111100101101111011011111
<i>P. drabae</i>	144964	111110100000100000001101111111110111011111111
<i>P. aberrans</i>	114408	1011110000001001100001011100000111110010111110
<i>P. aberrans</i>	189659	101111000000111111001011100000111110010111111
<i>P. codyi</i>	117853	1011110000001011111101011100000111110010111111
<i>P. caricina</i>	F00566	00000111000100111111100001001000111001110011111

^a Coding was done according to Hibbett et al. (11) and only multiple-base insertion/deletions (indels) are shown (0 = gap, 1 = no gap). Complete, unaligned sequences are available in gene bank under the accession numbers listed in Table 1. Aligned sequences are available upon request from the corresponding author.

Fig. 2. One of the four shortest trees found by heuristic parsimony using internal transcribed spacer sequences for *Puccinia* species from crucifers. Above the line are the number of steps on the branch (base substitutions + insertion/deletion changes) followed by decay index (bold). Below the line is bootstrap support for the branch. Table 1 has more information on specimens.

there are a limited number of morphological characters available for species circumscription.

An alternative explanation for the apparent polyphyletic nature of *P. thlaspeos* and *P. drabae* is that the phylogeny of the ITS sequences is incongruent with the phylogeny of the taxa. This incongruence could come about either through transfer of sequences from one taxon to another by hybridization or by gene duplication at some point along the lineage of the modern taxa. Lineage sorting is another mechanism that could lead to an incongruence between trees for species and sequences (6). If this were the case, individual sequences from a polymorphic ancestral rust fungus could end up being distributed among otherwise unrelated modern taxa. When only a single sequence is studied, this would lead to an incorrect assessment of species relationships. Thus, sequences of additional genes need to be analyzed to clarify further the phylogeny of the fungi examined in this study. The inclusion of other species of *Puccinia*, such as *P. trabutii* found on the family Brassicaceae in Europe, would also improve our understanding of these fungi.

Another possibility is that the fungus from dyer's woad was introduced to North America along with dyer's woad. We consider this to be unlikely, since the only rust fungus known to occur on *I. tinctoria* in Europe is *P. trabutii* (9). *P. trabutii* is heteroecious and macrocyclic and is quite distinct from the microcyclic, autoecious fungus occurring on dyer's woad in North America. Because rusts resembling *P. thlaspeos* have not been reported to occur on *I. tinctoria* in its native habitat, the *Puccinia* species found on it in

North America is probably indigenous. This is supported by the sequence data presented in this article, indicating that there is a close relationship between *P. consimilis*, which is found only on crucifers in western North America, and the *Puccinia*, which also occurs on *I. tinctoria* in the western United States.

Because introduced plant pathogens have a history of causing serious disease epidemics (20), the use of an introduced pathogen as a biological control agent must be approached carefully. Since our data indicate that the *Puccinia* species attacking dyer's woad is native to North America, this fungus is unlikely to cause disease epidemics on indigenous plants if used as an agent for the biological control of dyer's woad. Our data also indicate that it does not cause disease on canola (both *B. napus* and *B. campestris*). This finding is important, because the use of this fungus as a biological control agent would require that it not be pathogenic on related crop plants.

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