Cryptic species in the *Puccinia monoica* complex

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Abstract: The *Puccinia monoica* complex is an enigmatic group of rust fungi. They are flower mimics, and they greatly reduce host reproduction and survival. These fungi are relatively common, attacking approximately 960 species in 11 genera of crucifers as well as at least five genera of grasses. In modern taxonomic treatments the *Puccinia monoica* complex is treated as four species that are differentiated by the number of spore states in their life cycles. However, other systematic treatments have divided the group into species or forms based on host association. Within the species based on spore state there is morphological variation, but it has not been readily assignable to either host species or geographic area. We used DNA sequencing and phylogenetic analysis to determine whether there are cryptic species in this group that are not evident when only morphology is used. We sequenced the nuclear rDNA region containing the internal transcribed spacers (ITS-1 + 5.8S gene + ITS-2) of isolates from different hosts. Our results indicate that there are cryptic species in the *Puccinia monoica* complex, and that species in this group cannot be identified strictly by life cycle stage.

**Key Words:** Arabis, mimicry, molecular phylogeny, *Puccinia thlaspeos*, rust fungi, Tranzschel’s Law

INTRODUCTION

Classification of parasites and pathogens is often based on host association. There are at least two reasons for this mode of classification. First, because parasites and their hosts are intimately connected, it is assumed that their evolution should also be parallel. Second, it is often easier to distinguish hosts than to visually separate different species of parasites since the shared parasitic habit leads to morphological reduction and convergence (Price, 1980; Barrett, 1986; Brooks and McLennan, 1993). However, there are a number of reasons why host and parasite phylogenies may not always be the same, including: (i) a lack of genetic variation in the host for resistance or in the pathogen for virulence (Barrett, 1986; Futuyma et al., 1995), (ii) different rates of evolution between host and parasite (Savile, 1971), (iii) adaptation in only one member of the pair (Thompson, 1986), and (iv) host shifts to unrelated species (Savile, 1971; Funk et al., 1995; Radtkey and Singer, 1995).

Modern techniques such as DNA sequencing combined with phylogenetic analysis make it possible to bypass the problem of morphological convergence in the pathogens, and permit an independent assessment of the assumption that host associations define specific level differences in pathogens (Brooks and McLennan, 1993). In this study we used sequence data to ask whether there are likely to be cryptic species in a particularly enigmatic group of rusts, the flower-mimicking *Puccinia monoica* complex. Cryptic species are morphologically indistinguishable but are not interfertile, and they often differ in nonmorphological characters such as DNA sequence divergence (Sivarajan and Robson, 1991; Soltis et al., 1992). Cryptic species differ from biologic races as defined by Caten (1987) or Jaenike (1981) by being reproductively isolated.

The biology of the crucifer rusts suggests that these rusts are strong selective agents on the hosts and that sexual reproduction in the fungus is likely to be important in the evolution of the fungi. Infection of Brassicaceaeous hosts occurs in late summer from windborne basidiospores (Roy and Bierzchudek, 1993). Within a few months of basidiospore germination and penetration of host leaves, fungal hyphae invade meristematic tissues and cause systemic infection. Infected host plants produce one to several flower-like rosettes (pseudoflowers) in the spring, but rarely produce true flowers (Roy, 1993a, 1994). Spermogonia, the outcrossing structures of the rust fungus which contain spermatia and receptive hyphae, form on the...
The *Puccinia monoica* complex is currently comprised of 4 named species (Table I). Hosts include approximately 960 species in 11 genera of the mustard family, Brassicaceae, and at least five genera of grasses (Gäumann, 1959; Arthur and Cummins, 1962; Farr et al., 1989). Today members of the *P. monoica* complex are differentiated taxonomically by the number of spore stages in their life cycles (Table I); in the past they have been divided into species or forms based on host association (e.g., Jorstad, 1932; Gäumann, 1959). Morphological variation within species, as defined by spore stages, exists but it is not easily classifiable. For example, in reference to *P. holboellii*, D. B. O. Savile wrote, “In this material morphological variability seems to be random rather than geographic or by host” (Savile, 1974a).

**MATERIALS AND METHODS**

**Fungal isolates and extraction.**—Table II lists the rust fungus species, isolate number, host species, place of collection, collection source, and Genome Sequence

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**Table I. Life cycle information for the Puccinia monoica complex**

| Pathogen spp. | Alternates to a grass | Spore stages | | |
|---------------|------------------------|--------------|---|---|---|---|
| *P. monoica* Arth. | yes | Basidia | X | X | X | X |
| *P. consimilis* Ellis & Everh. | unknown | Telia | X | X | X | X |
| *P. thlaspeos* C. Schub. | no | Spermatia | X | X | O | O |
| *P. holboellii* Hornem. | no | Aecia | X | X | O | O |

* Data from: Gäumann, 1959; Arthur and Cummins, 1962; Savile 1974a, b and personal observations by B. A. Roy. X = present, O = absent.

* Note that there are both a host and a fungus with the same specific epithet: *P. holboellii* and *A. holboellii*. In this paper we present data on the fungi *P. monoica* and *P. thlaspeos* on *A. holboellii*, but present no data on the fungus *P. holboellii* on any hosts.

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**Table II. Fungal collections examined, host species of collection, genome sequence data bank accession number (GSDB#), and collection information**

<table>
<thead>
<tr>
<th>Rust species</th>
<th>GSDB#</th>
<th>Host species</th>
<th>County</th>
<th>State</th>
<th>Collector/#</th>
<th>Date</th>
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</thead>
<tbody>
<tr>
<td><em>Puccinia thlaspeos</em></td>
<td>S:1216225</td>
<td><em>Arabis brevior</em> S. Watson</td>
<td>Contra Costa</td>
<td>California</td>
<td>Roy/F549</td>
<td>10 May 96</td>
</tr>
<tr>
<td></td>
<td>S:1216218</td>
<td><em>Arabis demissa</em> Greene</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F427</td>
<td>24 May 93</td>
</tr>
<tr>
<td></td>
<td>L:76183</td>
<td><em>Arabis gunnisoniana</em> Rolllins</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F489</td>
<td>9 June 93</td>
</tr>
<tr>
<td></td>
<td>L:76177</td>
<td><em>Arabis holboellii</em> Hornem.</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F363*</td>
<td>9 June 93</td>
</tr>
<tr>
<td></td>
<td>S:1216227</td>
<td><em>Polyctenium fremontii</em> (Watson) Greene</td>
<td>Hornery</td>
<td>Oregon</td>
<td>Roy/F610*</td>
<td>6 May 96</td>
</tr>
<tr>
<td><em>Puccinia consimilis</em></td>
<td>L:76181</td>
<td><em>Arabis crandallii</em> Robinson</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F443</td>
<td>25 May 93</td>
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<td></td>
<td>S:1216223</td>
<td><em>Arabis demissa</em> Greene</td>
<td>Cannon</td>
<td>Colorado</td>
<td>Roy/F510</td>
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<tr>
<td></td>
<td>L:76180</td>
<td><em>Arabis drummondii</em> Gray</td>
<td>Summit</td>
<td>Utah</td>
<td>Vogler/Pumol</td>
<td>1994</td>
</tr>
<tr>
<td></td>
<td>S:1216228</td>
<td><em>Arabis drummondii</em> Gray</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F620</td>
<td>June 96</td>
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<tr>
<td></td>
<td>S:1216222</td>
<td><em>Arabis hisruta</em> (L.) Scopoli</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F432</td>
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<tr>
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<td>L:76182</td>
<td><em>Arabis holboellii</em> Hornem.</td>
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<td>Colorado</td>
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<tr>
<td></td>
<td>S:1216224</td>
<td><em>Arabis perennans</em> S. Watson</td>
<td>Grand</td>
<td>Utah</td>
<td>Roy/F534</td>
<td>2 May 96</td>
</tr>
<tr>
<td></td>
<td>S:1216226</td>
<td><em>Arabis sparsiflora</em> Torrey &amp; Gray</td>
<td>Lassen</td>
<td>California</td>
<td>Roy/F587</td>
<td>11 May 96</td>
</tr>
<tr>
<td><em>Puccinia monoica</em></td>
<td>L:76176</td>
<td><em>Schoenocrambe linifolia</em> (Nutt.) Greene</td>
<td>Gunnison</td>
<td>Colorado</td>
<td>Roy/F289</td>
<td>1 June 93</td>
</tr>
</tbody>
</table>

* Host corresponds to clone “B4” described in Roy, 1993b.

* Host corresponds to clone “D1” described in Roy, 1993b.
Data Base (GSDB) accession numbers. Infected leaves were collected into paper coin envelopes, dried at room temperature, then stored at 4°C in plastic containers lined with Dri-Rite. For extraction, we crushed 4 mm² pieces of infected leaves in 2× CTAB buffer, following the method of Gardes and Bruns (1993).

**PCR amplification, cleaning, and sequencing.—**The nuclear rDNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S gene was amplified by the polymerase chain reaction (PCR) on a Techne thermal cycler with the following primer pairs: ITS-5 (White et al., 1990) and ITS-4b (Gardes and Bruns, 1993) and the internal primers ITS-2r (Vogler and Bruns, 1998) and ITS-3r (Vogler and Bruns, 1998). A water blank was included in each PCR reaction set. The thermocycler program for DS-amplification was as described by Gardes and Bruns (1993).

All of the crucifer rusts except one were sequenced on an ABI (=Applied Biosystems) 310 automatic sequencer, one sample (P. monoica, Pumol1 from Utah) was sequenced on an ABI 377. Prior to sequencing, the final PCR product was cleaned with a QIAquick PCR cleaning kit (Qiagen #28104). Cycle sequencing reactions used 8 μL Terminator Premix (Perkin Elmer), 1 μL primer (3.2 mM), 11 μL clean ds DNA + H₂O for a total volume of 20 μL (some reactions were done with ½ these amounts). The cycle sequence program was: 25 cycles of 94 C (30 sec)/50 C (15 sec)/60 C 4 min. To remove excess terminator dye, the cycle sequence reactions were run through Centrisep Spin Columns.

We used the programs Sequence Navigator v. 1.0.1 and SeqEd. v.1.0.3 (Applied Biosystems) to align sequences through a combination of clustering algorithms and visual editing. Sequences were verified by checking each sequence against its complement (i.e., ITS 5 + 2r, 3r + 4b), and by sequencing multiple samples of the same accession (all cases), and by sequencing multiple specimens. We sequenced two isolates of *P. monoica* on *Arabis drummondii* from different populations. The Utah sample differed from the Colorado sample by having TC at bases 493 and 494 instead of YY (ambiguous, Y = T or C). We also sequenced five samples of *P. thlaspeos* on *A. demissa* from one population. The sequences from *A. demissa* were identical, with the exception of one ambiguous (T or C, coded as Y) base in one sample. Because there was little difference among multiple sequences from the same host/fungus combination from different individuals (1–2 bp/approx. 570 bp, ≪ 0.01% difference), we used a consensus sequence in the cladistic analyses. Sequences were deposited in the Genome Sequence Data Base (Table II).

**Phylogenetic analysis.—**Phylogenetic analysis of the Puccinia isolates from crucifers included sequence data from ITS spacers 1 and 2 and the associated 5.8S and 28S genes. For comparative and outgroup purposes we included in our first analysis published sequences by Zambino and Szabo (1993) from the cereal rusts. We designated the cereal rusts as a paraphyletic outgroup to the crucifer rusts. We felt justified in this outgroup choice because the sequences were alignable, and preliminary analyses including tree rusts (not shown) indicated that the cereal rusts clustered separately from the crucifer rusts. Unfortunately, the published sequences for the cereal rusts only includes the last 40 bases of ITS-1. Because the unsequenced section is coded in Paup DNA analysis as unknowns, it causes a branch-length artifact in the final phylograms. We therefore used all of the available data to estimate the tree, but then corrected the branch lengths by using only the alignable data common to all taxa when the tree was printed (i.e., the last 40 bp of ITS-1, and all of the 5.8S and ITS-2). This approach corrects the branch-length artifact in the phylogram, and preserves topology based on the available data from the ITS-1. The alignment is available from TreeBASE.

In a second set of analyses we used data only from the crucifer rusts. We used this subset because, although it was possible to align the cereal rusts, the alignment necessitated the inclusion of many gaps. In addition, we had more data for the crucifer rusts: all of the ITS-1, 5.8S, and ITS-2 as well as the first 120 bp of the 28S gene. By analyzing the crucifer rusts separately we were able to place more confidence on the branches because the alignments were tighter and more complete.

Heuristic searches were performed with both the random-addition sequence option (10 replicates) and with the simple addition option in Paup 3.1 (Swofford, 1993). Both methods of searching revealed the same trees. Bootstrap support (Felsenstein, 1985) for branches was based on 1000 replications. To determine the relative frequency of transversions (tv) and transitions (ti) we calculated the actual ratios in MacClade (Maddison and Maddison, 1995). The average ratio of ti/tv was very close to 1:1 for all trees. We thus performed unweighted searches.

**RESULTS**

In phylogenetic analyses of the crucifer rusts including the cereal rusts as an outgroup, both the ITS-1
and ITS-2 contained phylogenetically informative sites. Our alignment of all taxa yielded six trees of 156 steps, and a CI of 0.87. In Fig. 1 we show one of the six phylogenograms. The illustrated tree has the same topology as the tree produced by the strict consensus option. The topology of the six trees differed in the arrangement of the poorly differentiated terminal taxa in Group I, and in the placement of *P. monoica* on *Schoenocrambe linifolia* (which either clustered with Group II, or was found alone).

In phylogenetic analyses of just the crucifer rusts, both the ITS-1 and ITS-2 were informative. Paup DNA analysis including all characters, and in which gaps were treated as missing data, yielded 3 trees of 50 steps and a CI of 0.94. In Fig. 2 we show one of the three trees. The illustrated tree has the same topology as the tree produced by the strict consensus options. The three trees differed only in the arrangement of the poorly differentiated terminal taxa in the top clade of group one.

In addition to variation in sequence content, there was variation in sequence length, with sequences ranging from 559 to 573 bp (TABLE III). In the phylogenograms, Group 2, which contains only *P. monoica* isolates, is characterized by deletions relative to the other taxa and these sequences are the shortest (552–558 bp). Collections originally identified as *P. thlaspeos* were all 572–575 bp. All of the variation in length among isolates was in the spacers; the 5.8S gene was always 158 bp (TABLE III). In content, the 5.8S sequence was very similar among all of the crucifer rusts, but there were a few informative differences, mostly in Group 2 (Fig. 2).

*Puccinia monoica* is probably not monophyletic. Two well supported clusters of taxa contain different entities called *P. monoica* (FIGS. 1, 2). One well supported cluster of "*P. monoica*" (FIGS. 1, 2, Group 2) is likely to contain at least three species: one on *Arabis drummondii*, one on *Schoenocrambe linifolia*, and one that occurs on both *A. holboellii* and *A. hirsuta*. This group of *Puccinia* species is differentiated from the rest of the sequences by deletions of 10–15 bp (TABLE III). In addition to the well defined group of species with deletions (FIG. 2, Group 2), there are also "*Puccinia monoica*" rusts that are indistinguishable from *P. thlaspeos* in length and that cluster tight-
ly within *P. thlaspeos* (Group 1). The phylograms suggest that one of these *P. monoica* that is not characterized by deletions is also a separate species: the taxon that occurs in California on *A. sparsiflora* and is indistinguishable by ITS sequence from the "*P. thlaspeos*" on *Arabis breweri* (Figs. 1, 2).

*Puccinia thlaspeos* may also be polyphyletic but there are smaller sequence differences among the short-cycled species than among the long-cycled *P. monoica* taxa (Fig. 2). In Fig. 2, Group 1 contains all the macrocyclic *P. thlaspeos* isolates plus some isolates that are morphologically *P. monoica*. Distances within Group 1 are all short, suggesting recent derivation or a slow rate of evolution. Group 2, on the other hand, which contains only macrocyclic isolates of *P. monoica*, is separate from Group 1 and is relatively more differentiated.

**DISCUSSION**

*Cryptic species?*—The ITS sequence data suggest that there are indeed cryptic species within both *P. monoica* and *P. thlaspeos* since there are significant sequence differences among collections taken from different hosts (Figs. 1, 2; Table III). Not only were there differences in sequence length and composition among rust isolates on different host genera (Fig. 2: isolates on *Polyctenium* and *Schoenocrambe* versus *Arabis*) but there were also differences among rust isolates on the same host genus (e.g., differences between isolates on *Arabis* Figs. 1, 2). Although mating studies were not performed to verify species status, the sequence divergence between some of our samples was similar to that between well characterized cereal rust species of commercial importance (Fig. 1). In addition to differences in ITS sequences and host use, many of these flower-mimicking fungi produce chemically distinct floral fragrances (Roy and Raguso, 1997; Raguso and Roy, in press).

It is often asserted that pathogens are good taxonomists and that therefore different host species should have a different complement of pathogens associated with them (Barrett, 1986). Although our data shows that there is some association between crucifer host and fungus species, it is not perfect. That is, each crucifer host species does not have a separate fungal species associated with it. For example, we were unable to differentiate among isolates of *P. thlaspeos* on three different species of *Arabis* (Fig. 2: *A. holboellii, A. gunnisoniana*, and *A. demissa*), or between isolates of *P. monoica* on two different *Arabis* hosts (Fig. 2: *A. hirsuta* and *A. holboellii*).

When pathogens attack more than one host it is likely that both hosts influence the evolution of the pathogen. The pathogens in the *Puccinia monoica* life cycle group (Group 2, Figs. 1, 2) are both macrocyclic and heteroecious and thus have unrelated aecial and telial hosts, in this case within the Brassicaceae and Poaceae, respectively. Some of the observed differentiation in the macrocyclic isolates of *P. monoica* may be associated with differences in the telial (i.e., grass hosts); if true, this would parallel the pattern found in the cereal rusts. Within *P. monoica* there was differentiation among isolates on *A. drummondii* versus *A. holboellii* and *A. hirsuta* (Fig. 2). This differentiation was not entirely unexpected as it is likely that rust isolates colonizing *A. drummondii* have a different telial host. This suspicion is based on three pieces of information: (i) In the Rocky Mountains, *A. drummondii* is found in the montane-alpine zones in association with the grass *Trisetum spicatum* (L.) Richter, whereas *A. holboellii* and *A. hirsuta* are found in submontane-montane areas in association with the grass, *Koeleria macrantha* (Ledeb.) Schultes.
(B. A. Roy, pers. obs.). (ii) Where the distribution of the Arabis hosts overlap, infection of A. drummondii only occurs when Trisetum is present (B. A. Roy, pers. obs.). (iii) Arthur performed inoculation studies with spores from Trisetum, Koeleria, and Arabis. His results led him to suggest that there were "biological races" (Arthur, 1912, 1915). Unfortunately, Arthur was not confident about his Arabis identifications, so it is difficult to ascertain which Arabis species were infected by spores from which grass. However, from the locality data given in Arthur's papers, the host pairs are likely to have been A. holboellii + Koeleria macrantha, and A. drummondii + Trisetum spicatum.

Some of the variation in sequences can also be correlated with geography and habitat. For example, there is a reasonably well supported cluster of P. monoca on A. sparsiflora and P. thlaspeos on A. brevleri (FIGS. 1, 2). These collections were made in Northern California whereas the rest of the crucifer rust collections were made in the Rocky Mountains. It is, of course, difficult to unlink the influences of geography from host range, and the sample size is small, but we should bear in mind that other evolutionary processes besides host, such as genetic drift, may influence differentiation among taxa.

Life-cycles in taxonomy and evolution.—The convention of differentiating crucifer rust species by life-cycle is not supported by the molecular phylogeny. The macrocyclic "Puccinia monoica" and microcyclic "P. thlaspeos" species do not form monophyletic groups (FIGS. 1, 2). Nor did macrocyclic and microcyclic isolates from the same host species always cluster together (e.g. P. monoica and P. thlaspeos on Arabis holboellii, FIG. 1). However, this last result should be treated with some caution because, although the same "species" of host was involved, the isolates were taken from different clones of an apomictic species, Arabis holboellii. The two clones could be treated as separate microspecies for the following reasons: (i) Because these clones are apomictic they are reproductively isolated from each other; there is no interbreeding among clones, (ii) clones of the D and B isozyme phenotypes have different chromosome numbers (Roy, 1995), (iii) clone D is never (within statistical error of clone identification) infected by P. thlaspeos, and Clone B is rarely infected by P. monoica (Roy and Bierzychudek, 1993).

Species with different life cycles clustered together because there was little or no sequence divergence correlated with life cycle type. Similar results were also recently found for other crucifer rusts (Kropp et al., 1997). We have several hypotheses for why there may be a lack of differentiation among life cycle types: (i) life cycle differences may not define rust species very well, (ii) these particular collections represent very recent speciation events and sufficient ITS differences have not yet accumulated to distinguish them, or (iii) some of the collections were misidentified, (iv) there is polymorphic expression of spore states. Of these hypotheses, 1, 2 and 4 seem the most likely. We think hypothesis 3, misidentification, is unlikely in our study because most of the infected plants analyzed were collected by the first author who carefully examined the plants for spore stages at the time of collection and again before preparing the material for sequencing. However, misidentification is possible if a crucifer was infected by P. consimilis, and there were very few telia present. In this case, telia might be overlooked, and the fungus would be misidentified as P. monoica. Alternatively, there may be polymorphic expression of spore stages in P. consimilis. That is, if sometimes P. consimilis produces just aecia, sometimes just telia and sometimes a mixture of the two. Polymorphic expression of spore stages in the demicyclic P. consimilis seems very likely because the expression of spore stages in this taxon is very variable. We have observed considerable variation in the ratio of telia: aecia produced on plants infected by P. consimilis (B. A. Roy and M. Pfunder, unpubl. data). Sometimes only a single telium may be produced among hundreds of aecia. Thus it seems likely that under some environmental or host compatibility conditions only aecia may be expressed on a plant, making it appear to be infected by P. monoica, when in fact it is infected by P. consimilis.

If there is polymorphic expression of spore states, it probably does not occur in all taxa of the P. monoica complex. For example, the first author has observed Puccinia monoica-infected Arabis drummondii throughout its range over a seven-yr. period and has never seen anything but aecia produced on it. Other hosts, however, are likely to be infected by polymorphic rusts most, or all, of the time. For example, the first author often sees all three common "life cycle" rust species (P. monoica, P. thlaspeos, and P. consimilis) in the same population of Arabis holboellii. And, A. crandallii and A. lignifera, which are often infected by P. consimilis, exhibit varying degrees of expression of aecia and telia.

In summary, we have shown that there is enough variation at the ITS sequence level within the named species of P. monoica and P. thlaspeos to suggest that additional species-level taxa exist. In addition, we have called into question the practice of separating rust fungi into species based on life cycles. Rather than forming an evolutionary series, life cycle differences may be relatively unstable and polymorphic in expression in some taxa. Before undertaking the nec-
necessary taxonomic revisions in this group we are following up on the study described herein with more extensive surveys, both morphological and molecular, of the crucifer rusts and their hosts across their geographic ranges. We plan to use the resulting phylogenetic reconstructions to address questions concerning life-cycle plasticity, speciation, and the evolution of fungal floral mimicry.

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LITERATURE CITED


