

Phylogeny of the rust fungi: an approach using nuclear large subunit ribosomal DNA sequences¹

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Abstract: Sequence data from nuclear large subunit ribosomal DNA was used to infer phylogenetic relationships of selected genera of the *Uredinales*. We investigated 52 rust fungi representing nine families and three outgroup species. Neighbor joining analysis and a Bayesian method of phylogenetic inference using Monte Carlo Markov chains confirm the rust fungi as a natural group and indicate that *Puccinia*, *Uromyces*, *Endophyllum*, and *Cumminsella* have a common origin. The autoecious *Rosaceae*-rusts *Phragmidium*, *Kuehneola*, *Triphragmium*, and *Trachyspora* are a monophyletic group. The gasteroid genus *Ochropsora* is closely related to *Tranzschelia*. While the *Pucciniastreae* sensu Dietel (1938) is recognized as a monophyletic group in neighbor joining analysis, the *Pucciniaceae* s.l. (Dietel 1928) is supported by Bayesian analysis. The following genera appear to be monophyletic: *Chrysomyxa*, *Coleosporium*, *Cronartium*, *Gymnosporangium*, *Melampsora*, *Phragmidium*, and *Tranzschelia*, whereas the genera *Puccinia*, *Pucciniastrum*, *Thekopsora*, and *Uromyces* are not.

Key words: molecular phylogeny, systematics, nuclear large subunit rDNA, *Basidiomycota*, *Urediniomycetes*, *Uredinales*.

Résumé : Les données de séquençage de la grande sous-unité de l'ADN nucléique ribosomique ont été utilisées pour déduire les relations phylogéniques de genre sélectionnés parmi les *Uredinales*. Les auteurs ont examiné 52 champignons des rouilles représentant neuf familles et trois espèces en dehors de ce groupe. L'analyse par recouvrement avec les voisins et une méthode d'inférence phylogénique de type bayésien, en utilisant les chaînes de Monte Carlo Markov, confirme que les champignons des rouilles constituent un groupe naturel et indique que les *Puccinia*, *Uromyces*, *Endophyllum*, et *Cumminsella* ont une origine commune. Les rouilles autoéciques associées aux *Rosaceae*, telles que les *Phragmidium*, *Kuehneola*, *Triphragmium* et *Trachyspora* forment un groupe monophylétique. Le genre gastéroïde *Ochropsora* est étroitement relié au *Tranzschelia*. Alors qu'on reconnaît les *Pucciniastreae* sensu Dietel (1938) comme groupe monophylétique lors d'analyses par recouvrement avec les voisins, les *Pucciniaceae* s.l. (Dietel 1928) sont supportés par l'analyse bayésienne. Les genres suivants semblent monophylétiques : *Chrysomyxa*, *Coleosporium*, *Cronartium*, *Gymnosporangium*, *Melampsora*, *Phragmidium* et *Tranzschelia*, alors que les genres *Puccinia*, *Pucciniastrum*, *Thekopsora* et *Uromyces* ne le sont pas.

Mots clés : phylogénie moléculaire, systématique, grande sous-unité de l'ADN nucléique ribosomique, *Basidiomycota*, *Urediniomycetes*, *Uredinales*.

[Traduit par la Rédaction]

Introduction

The rust fungi (*Uredinales*) are important, nearly ubiquitous plant pathogens that exhibit a complex life cycle in which up to five spore forms are produced and host alternation between two unrelated hosts frequently occurs. The rusts are a well-defined group of organisms displaying several typical characteristics including a unique, strictly parasitic life cycle, simple septal pores ("pully-wheel septal pore" apparatus), intimate association of nuclear envelope

and spindle pole body (SPB) during nuclear division, the complete lack of endoplasmic reticulum from the SPB, bipolar sexual system, the lack of clamps, the presence of teliospores, and especially the presence of spermatogonia (Littlefield and Heath 1979; McLaughlin et al. 1995; Swann et al. 2001).

With more than 100 genera and some 7000 species, the rusts comprise about 75% of the genera and 95% of the species of the subclass *Urediniomycetes* within the *Basidiomycota* (Cummins and Hiratsuka 1983; Swann et al. 2001). Both morphological and molecular phylogenetic studies have revealed that their closest relatives can be found among the genera *Septobasidium*, *Uredinella*, *Iola*, *Eocronartium*, *Herpobasidium*, and *Helicobasidium* (Möller 1895; Couch 1937; Gäumann 1949; Oberwinkler 1977; Berres et al. 1995; Swann and Taylor 1995).

Classification of the *Uredinales* at the generic as well as the suprageneric levels has been based almost exclusively on morphology of teliospores and telia (e.g., Dietel 1900; Arthur 1934; Gäumann 1949). Thus, the two families ac-

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cepted by Dietel (1928) were mainly defined by teliospores being stalked (*Pucciniaceae*) or unstalked (*Melampsoraceae*). Nevertheless, the significance of characters other than teliospore morphology for phylogenetic interpretations above genus level has been demonstrated; such characters include arrangement of the pores of urediniospores (Cummins 1936) or morphology of uredinia (Kenney 1970), aecia (Sato and Sato 1985), and spermogonia (Hiratsuka and Cummins 1963; Hiratsuka and Hiratsuka 1980), respectively. Spermogonial morphology appeared to be especially promising in elucidating suprageneric relationships. Therefore larger rearrangements of familial circumscriptions were based chiefly on spermogonial morphology and resulted in (re-)definition of 14 families (Cummins and Hiratsuka 1983).

Molecular phylogenetic studies have also been performed on the rust fungi. First attempts were made using the 5.8S rRNA (Gottschalk and Blanz 1984). Later, phylogenetic studies of the ITS region of the nuclear ribosomal genes were successfully applied to gain insight into closely related species of *Puccinia*, *Uromyces* (Zambino and Szabo 1993; Kropp et al. 1997; Roy et al. 1998; Pfunder et al. 2001), and into the genus *Cronartium* (Vogler and Bruns 1998). An 18S rDNA study has also been done on fern rusts and allies (Sjamsuridzal et al. 1999).

The main objectives of the present study were (i) to test the monophyly of selected genera and (ii) to determine suprageneric relationships.

Materials and methods

Fungal collection and DNA extraction

The analysed rust taxa, their original host plants, voucher information, and GenBank accession numbers are listed in Table 1. Representatives of nearly all rust genera native to Central Europe were included in this study, thus representing major lineages of *Phragmidiaceae*, *Pucciniaceae*, and all genera of the *Melampsoraceae* s.l. (Dietel 1928), now belonging to the families *Melampsoraceae*, *Pucciniastraceae*, *Coleosporiaceae*, and *Cronartiaceae* (Cummins and Hiratsuka 1983). Furthermore, we tested the phylogenetic position of single genera whose assignment to certain families is doubtful: e.g., the assignment of *Triphragmium* to *Sphaerophragmiaceae* or *Ochropsora* to *Chaconiaceae*.

Genomic DNA was isolated from fresh, silica gel-dried or herbarium specimens. We excised rust sori (together with plant material) of about 1–10 mm² and extracted DNA by either using the SDS method as described in Begerow et al. (1997) or, more often, using the DNeasy Plant Mini Kit (Quiagen, Hilden, Germany) following the manufacturer's protocol. Late in this study, crushing of the infected plant material in liquid nitrogen with the help of a micro pestle was replaced by shaking the samples for 3 min at 30 Hz (Mixer Mill MM 300, Retsch, Haan, Germany) in a 1.5 mL tube together with one tungsten carbide ball 3 mm in diameter.

PCR and sequencing

The 5'-end of the nuclear 28S-like rRNA genes (nuclear large subunit rDNA), comprising the domains D1 and D2 (Guadet et al. 1989), was amplified by polymerase chain reaction (PCR) (Mullis and Faloona 1987; Saiki et al. 1988)

using the primer pairs LR0R (Moncalvo et al. 1995), LR6 (Vilgalys and Hester 1990) and NL1, NL4 (O'Donnell 1992, 1993), respectively. The selected DNA region is especially useful in resolving relationships at the order and family levels, and the D2 domain has proven to have the lowest levels of homoplasy within the large subunit (Hopple and Vilgalys 1999). Amplification parameters were as described in Vogler and Bruns (1998), but we adjusted the annealing temperature to 45°C, and reduced the extension time of the last nine cycles to 2.5 min.

The PCR product was purified following the QIAquick protocol (Quiagen, Hilden, Germany). The obtained double-stranded DNA (dsDNA) was sequenced directly on both strands using cycle sequencing with the primers NL1 or NLMW1 (5' – TCA ATA AGC GGA GGA AAA GA – 3'; Sampaio et al. 2002) as forward and NL 4 as reverse primer and the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, U.K.) according to the manufacturer's protocol with the following modifications: before use, the reaction volumes were reduced by half and the kit diluted 1:1 (v/v) with double distilled water. Electrophoresis was performed on an automated sequencer (ABI 373A Stretch, PE Applied Biosystems, Foster City, Calif.). The sequences of both strands were combined and proofread with the help of Sequencher™ 3.1.1 software (Gene Codes Corp., Ann Arbor, Mich.).

Phylogenetic analysis

DNA sequences were aligned using Clustal X (Thompson et al. 1997). Further manual alignment was done in Se-Al v.2.03a (Rambaut 2000). The following phylogenetic analyses were run on Macintosh computers: neighbor joining analysis (Saitou and Nei 1987) using PAUP 4.0b8a (Swofford 2001), and a Bayesian method of phylogenetic inference using Monte Carlo Markov chains (Larget and Simon 1999) as implemented in the computer program MrBayes (Huelsenbeck and Ronquist 2001).

Neighbor joining

We used the Kimura two-parameter model of DNA substitution (Kimura 1980) with a transition:transversion ratio of 2.0 to compute genetic distances. Support for internal nodes was estimated by 1000 bootstrap replicates (Felsenstein 1985).

Monte Carlo Markov chains (MCMC)

With this method it is possible to estimate the probabilities ("a posteriori probabilities") that groups of taxa are monophyletic given the DNA alignment (i.e., the probabilities that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 2 000 000 generations using the general time reversible model of DNA substitution with gamma distributed substitution rates (see Swofford et al. 1996), random starting trees, and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations resulting in an overall sampling of 20 000 trees. From those trees that were sampled after the process had reached stationarity a 50% majority rule consensus tree

Table 1. Species of rust fungi used for phylogenetic analysis in the present study.

Rust fungus	Host	GenBank accession no. of fungus	Collection no. of fungus*
<i>Chrysoomyxa ledi</i> (Alb. et Schw.) de By.	<i>Picea abies</i> (L.) H. Karst.	AF 426246	HeRB 4916
<i>Chrysoomyxa rhododendri</i> (DC.) de By.	<i>Rhododendron ferrugineum</i> L.	AF 426245	WM 1016
<i>Coleosporium asterum</i> (Diet.) P. et H. Syd.	<i>Aster ciliolatus</i> Lindl.	AF 426241	FO 47832
<i>Coleosporium cacaliae</i> (DC.) Otth	<i>Adenostyles glabra</i> (Mill.) DC.	AF 426243	WM 1321
<i>Coleosporium campanulae</i> (Strauss) Tul.	<i>Campanula scheuchzeri</i> Vill.	AF 426244	WM 1114
<i>Coleosporium tussilaginis</i> (Pers.) Lév.	<i>Tussilago farfara</i> L.	AF 426242	WM 1113a
<i>Cronartium flaccidum</i> (Alb. et Schw.) Wint.	<i>Vincetoxicum hirundinaria</i> Medik.	AF 426239	WM 1182
<i>Cronartium ribicola</i> J.C. Fisch.	<i>Pinus aristata</i> Engelm.	AF 426240	RB 3021
<i>Cumminsia mirabilissima</i> (Peck) Nannf.	<i>Mahonia aquifolium</i> (Pursh) Nutt.	AF 426206	WM 1351
<i>Endophyllum euphorbiae-sylvaticae</i> (DC.) Wint.	<i>Euphorbia amygdaloides</i> L.	AF 426200	HeRB C-82
<i>Eocronartium muscicola</i> (Pers. ex Fr.) Fitzp.	<i>Bryopsida</i> gametophyte; species indet.	AF 426194	FO 42767
<i>Gymnosporangium clavariiforme</i> (Pers.) DC.	<i>Juniperus communis</i> L.	AF 426211	ML 841
<i>Gymnosporangium cornutum</i> (Pers.) Arth.	<i>Sorbus aucuparia</i> L.	AF 426210	WM 1093
<i>Gymnosporangium sabinae</i> (Dicks.) Wint.	<i>Pyrus communis</i> L.	AF 426209	WM 1347
<i>Herpobasidium filicinum</i> (Rostr.) Lind	<i>Dryopteris filix-mas</i> (L.) Schott	AF 426193	RB 797
<i>Hyalopora polypodii</i> (Pers.) Magn.	<i>Cystopteris fragilis</i> (L.) Bernh. s.str.	AF 426229	FO 47825
<i>Kuehneola uredinis</i> (Link) Arth.	<i>Rubus fruticosus</i> agg.	AF 426218	WM 1110a
<i>Melampsora euphorbiae</i> (Schub.) Cast.	<i>Euphorbia cyparissias</i> L.	AF 426195	WM 1002
<i>Melampsora helioscopiae</i> Wint.	<i>Euphorbia helioscopia</i> L.	AF 426197	WM 1029
<i>Melampsora hypericorum</i> (DC.) Wint.	<i>Hypericum calycinum</i> L.	AF 426196	WM 1110
<i>Melampsorella caryophyllacearum</i> (Link) Schroet.	<i>Abies alba</i> Mill.	AF 426232	WM 1092
<i>Melampsorium betulinum</i> (Fr.) Kleb.	<i>Betula pendula</i> Roth	AF 426228	WM 1010
<i>Milesia scolopendri</i> (Fckl.) Arth.	<i>Asplenium scolopendrium</i> L.	AF 426236	HeRB 4574
<i>Naohidemyces vaccinii</i> (Wint.) Sato, Katsuya et Y. Hiratsuka	<i>Vaccinium uliginosum</i> L.	AF 426238	WM 1098
<i>Ochropsora ariae</i> (Fuck.) Ramsb.	<i>Anemone nemorosa</i> L.	AF 426222	HeRB 4153
<i>Ochropsora ariae</i> (Fuck.) Ramsb.	<i>Aruncus dioicus</i> (Walter) Fernald	AF 426221	FO 47848
<i>Phragmidium fragariae</i> (DC.) Rabenh.	<i>Potentilla steriliz</i> (L.) Garcke	AF 426217	WM 1317
<i>Phragmidium montivagum</i> Arth.	<i>Rosa cf. woodsii</i> Lindl.	AF 426213	FO 47828
<i>Phragmidium sanguisorbae</i> (DC.) Schroet.	<i>Sanguisorba minor</i> Scop. s.l.	AF 426216	ML 957
<i>Phragmidium rubi-idaei</i> (DC.) Karst.	<i>Rubus idaeus</i> L.	AF 426215	WM 1024
<i>Phragmidium violaceum</i> (C.F. Schultz) Wint.	<i>Rubus fruticosus</i> agg.	AF 426214	WM 1037
<i>Puccinia coronata</i> Corda	<i>Rhamnus cathartica</i> L.	AF 426207	WM 1280
<i>Puccinia gigantea</i> Karst.	<i>Epilobium angustifolium</i> L.	AF 426198	WM 1094
<i>Puccinia malvacearum</i> Bert. ex Mont.	<i>Alcea rosea</i> L.	AF 426205	WM 1345
<i>Puccinia urticae-acuteformis</i> Kleb.	<i>Carex acutiformis</i> Ehrh.	AF 426202	WM 1090
<i>Pucciniastrum agrimoniae</i> (Diet.) Tranz.	<i>Agrimonia eupatoria</i> L.	AF 426234	WM 1134
<i>Pucciniastrum circaeae</i> (Wint.) de Toni.	<i>Circaea lutetiana</i> L.	AF 426227	RB 2098
<i>Pucciniastrum epilobii</i> (Pers.) Otth	<i>Epilobium angustifolium</i> L.	AF 426226	WM 1099
<i>Pucciniastrum pyrolae</i> (Pers.) Diet. ex Arth.	<i>Pyrola minor</i> L.	AF 426233	HeRB 4570
<i>Septobasidium carestianum</i> Bres. [†]	Scale insects on <i>Cornus</i> sp.	L 20289	ATCC 200021
<i>Thekopsora areolata</i> (Fr.) Magn.	<i>Prunus padus</i> L.	AF 426235	WM 1389
<i>Thekopsora guttata</i> (Schroet.) P. et H. Syd.	<i>Galium odoratum</i> (L.) Scop.	AF 426231	WM 1203
<i>Thekopsora symphyti</i> (Bub.) R. Berndt	<i>Symphytum officinale</i> L. s.l.	AF 426230	HeRB 4732
<i>Trachyspora intrusa</i> (Grev.) Arth.	<i>Alchemilla vulgaris</i> agg.	AF 426220	WM 1019
<i>Tranzschelia discolor</i> (Fckl.) Tranz. et Litv.	<i>Prunus domestica</i> L. subsp. <i>domestica</i>	AF 426223	HeRB 3999
<i>Tranzschelia fusca</i> (Pers.) Diet.	<i>Anemone nemorosa</i> L.	AF 426225	WM 1262
<i>Tranzschelia pruni-spinosae</i> (Pers.) Diet.	<i>Anemone ranunculoides</i> L.	AF 426224	WM 1355
<i>Triphragmium ulmariae</i> (Hedw. f. ex DC.) Link	<i>Filipendula ulmaria</i> (L.) Maxim.	AF 426219	WM 1027
<i>Uredinopsis filicina</i> (Niessl) Magn.	<i>Phegopteris connectilis</i> (Michx.) Watt	AF 426237	WM 1112
<i>Uredo alpestris</i> Schroet.	<i>Viola biflora</i> L.	AF 426212	HeRB 4865
<i>Uromyces ficariae</i> (Schum.) Fckl.	<i>Ranunculus ficaria</i> L.	AF 426204	WM 1398
<i>Uromyces gageae</i> Beck	<i>Gagea lutea</i> (L.) Ker Gawl.	AF 426208	WM 1315
<i>Uromyces junci</i> (Desm.) Tul. (sub <i>Tuberculina</i> sp.)	<i>Pulicaria dysenterica</i> (L.) Bernh.	AF 426203	GZU 11-98
<i>Uromyces pisi</i> (DC.) Otth s.l.	<i>Euphorbia cyparissias</i> L.	AF 426201	WM 1285
<i>Uromyces viciae-fabae</i> (Pers.) Schroet.	<i>Vicia pannonica</i> Crantz	AF 426199	WM 1365

*ATCC, American Type Culture Collection; FO, F. Oberwinkler (private collection); GZU, Herbarium of the University of Graz, Austria; HeRB, R. Berndt (private collection); ML, M. Lutz (private collection); RB, R. Bauer (private collection); WM, W. Maier (private collection).

[†]Origin of sequence: Berres et al. 1995.

was computed to obtain estimates for the a posteriori probabilities. This Bayesian approach of phylogenetic analysis was repeated four times, always using random starting trees and random starting values for the model parameters to test the reproducibility of the results.

The unrooted dendrograms from neighbor joining and MCMC analyses were rooted with *Septobasidium carestianum* (*Septobasidiales*), *Herpobasidium filicinum* (*Platyglloeales*), and *Eocronartium muscicola* (*Platyglloeales*) as outgroup species.

Results

The sequences reported in this study have been deposited in GenBank as AF426193–AF426246 (see Table 1). The final alignment contained 549 nucleotide sites. After removing ambiguous alignment positions, 535 sites remained for further analysis. The alignment has been deposited in TreeBase (<http://www.herbaria.harvard.edu/treebase/>): study accession number S731, matrix accession number M1163.

Tree topology

The four runs of Bayesian phylogenetic analysis that were performed yielded consistent results. The topologies of the consensus trees only differed in groupings of minor support (below 56%: in one of the runs, *Uredo alpestris* was not placed next to *Phragmidiaceae*, but was attached to the backbone of *Pucciniaceae* s.l. without any resolution) and a posteriori probabilities are similar. Stationarity of the Markov chains was reached after ca. 100 000 generations of trees, i.e., after 1000 trees had been sampled. We thus discarded the first 2000 trees and included 18 000 sampled trees in the 50% majority rule consensus tree of each run. One of these is given in Fig. 1.

There is good correspondence between tree topologies of the MCMC analysis compared with the neighbor joining analysis (see Fig. 2). The rust fungi appear as a highly supported monophyletic lineage (a posteriori probability 100%, bootstrap 100%). Furthermore, all highly supported suprageneric clusters contain the same representatives in both methods. These are the *Puccinia/Uromyces* group, including *Endophyllum* and *Cumminsella* (100%, 100%), the autoecious *Rosaceae* rusts group or *Phragmidiaceae* (99% probability, 79% bootstrap), the *Anemone–Rosaceae* rusts (i.e., *Tranzschelia* and *Ochropsora*; 96% probability, 73% bootstrap), the cluster of the fern rusts *Uredinopsis* and *Milesia* (100% probability, 99% bootstrap), and the *Pucciniastreae* group 1 (100% probability, 85% bootstrap).

Discussion

In the following sections, groupings obtained in the phylogenetic tree are discussed and compared with morphology based systematics with special emphasis on teliospore morphology, spermatogonial morphology, and host relationships.

Melampsora (*Melampsoraceae*)

Melampsora is the only genus in *Melampsoraceae* (Cummins and Hiratsuka 1983). It is separated from the other taxa by a long genetic distance in neighbor joining analysis, suggesting that *Melampsora* either split from the

other genera a long time ago or that it has an accelerated mutation rate.

While neither spermatogonial morphology (group I; type 2 and 3) (Hiratsuka and Hiratsuka 1980) nor teliospore morphology differ much from members of *Melampsoraceae* s.l. (e.g., *Coleosporium*, *Melampsoridium*, *Pucciniastrum*), there are major differences in both aecia and uredinia morphology, and in the host range of the genus; aecia of *Melampsora* possess only rudimentary peridia or no peridia at all (Hersperger 1929; Peterson 1974; Sato and Sato 1985) and the uredinia have abundant capitate paraphyses. With regard to host relationship, the genus displays great variety: there are heteroecious species, alternating either between *Pinaceae* and *Salicaceae* or between a variety of monocots, dicots, and *Salicaceae*, and there are autoecious species on various dicots (Gäumann 1959). This variety of host relationships, and especially the lack of specificity (e.g., *Coleosporium*, *Chrysomyxa*, and *Melampsoridium* attack a single genus of *Pinaceae*, while *Melampsora* attacks *Abies*, *Cedrus*, *Larix*, *Pinus*, *Pseudotsuga*, and *Tsuga*) was interpreted as a “primitive” feature of the genus by Durrieu (1980), who on this basis hypothesized that the genus occupied a basal position within the rust fungi. There is weak support for this hypothesis in the neighbor joining analysis, but resolution in our analyses is not sufficient to verify or falsify this hypothesis reliably.

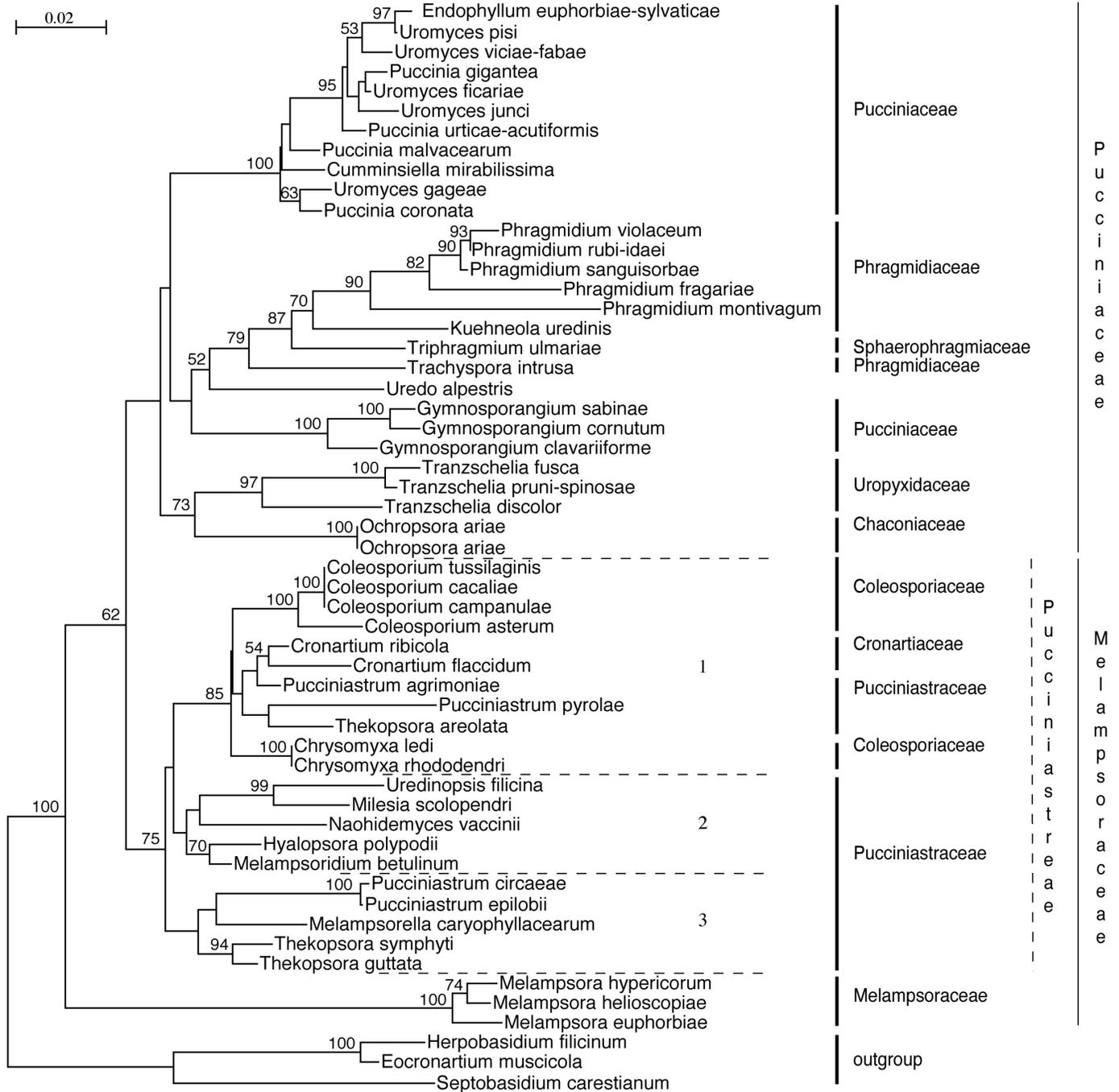
We sampled *Melampsora helioscopiae*, *Melampsora euphorbiae*, and *Melampsora hypericorum*, the latter of which used to be the type species of the genus *Mesopsora*. It was segregated from *Melampsora* to accommodate a species on *Hypericum* with “catenulate urediniospores” (Dietel 1922) but Dietel himself later retracted the genus, interpreting the above-mentioned spore stage as secondary aecia (Dietel 1941). Despite this retraction, the genus name *Mesopsora* has been further used (e.g., Leppik 1953), or at least the peculiarity of the species was emphasized and its phylogenetic position remained obscure (Gäumann 1959; Cummins and Hiratsuka 1983). However, monophyly of the three species is highly supported (100%, 100%), suggesting that “*Mesopsora hypericorum*” is a true *Melampsora*, though with a short life cycle.

Pucciniastreae Dietel 1938 (*Pucciniastraceae*, *Coleosporiaceae*, *Cronartiaceae*)

In neighbor joining analysis, this moderately supported group (75% bootstrap) contains members of all the genera that Dietel (1938) defined to belong to *Pucciniastreae* in his revised circumscription of the group. The genera of the *Pucciniastreae* were later assigned to the families *Pucciniastraceae*, *Coleosporiaceae*, or *Cronartiaceae* (Cummins and Hiratsuka 1983), but familial definitions are only partly reflected by tree topology. Therefore, we use the old taxon *Pucciniastreae* in what follows. The “*Pucciniastreae*-cluster” contains three subclusters, which will be referred to as *Pucciniastreae* groups 1, 2, and 3. In MCMC analysis the group as a whole could not be resolved, but only *Pucciniastreae* group 1 (100% probability) and group 3 (62% probability).

All members of *Pucciniastreae* parasitize *Pinaceae* and have peridermioid aecia (Moss 1926; Pady 1933; Hiratsuka 1936). Furthermore, they are characterized by unstalked te-

Fig. 2. Neighbor joining analysis of an alignment of nuclear rDNA sequences from the D1/D2 regions of the large ribosomal subunit rooted with *Eocronartium muscicola*, *Herpobasidium filicinum*, and *Septobasidium carestianum*. Branch lengths (expected numbers of nucleotide substitutions per site) were computed according to the Kimura two-parameter model. Only bootstrap values greater than 50% are shown. Family concepts applied, correspond to Cummins and Hiratsuka (1983: bold lines) and to Dietel (1928, 1938: slim and broken lines), respectively. The groupings 1, 2, and 3 correspond to *Pucciniastraeae* groups 1, 2, and 3 as defined in the text.



liosporous and group I spermogonia (types 1, 2, 3), with the exception of *Cronartium*, which has type 9 spermogonia. Velopedunculate D-haustoria (those with their necks sheathed by a membrane fold) have only been found within *Pucciniastraeae*, (e.g., Gray et al. 1982; Khan and Kimbrough 1982; Berndt 1993), whereas all genera sampled outside of this group have gymnopodunculate D-haustoria (naked haustorial necks) (Littlefield and Bracker 1970; Hardwick et al. 1971; Kohno et al. 1977; Littlefield and Heath 1979; Borland and Mims 1980; Chong et al. 1981; Berndt 1995).

Within *Pucciniastraeae*, *Coleosporium*, *Chrysomyxa*, and *Cronartium* are monophyletic, whereas *Pucciniastrum* and *Thekopsora* are polyphyletic. While the former three genera are defined by clear morphological apomorphies (discussed below), the latter two are defined by more subtle differences, such as location of teliospore production within the host epidermis (*Thekopsora*) versus within the host mesophyll (*Pucciniastrum*) (Pady 1933, 1946; Berndt 1993). Some authors have merged *Pucciniastrum* and *Thekopsora* in *Pucciniastrum* s.l. (e.g., Arthur 1934; Cummins and Hiratsuka 1983). Our findings, however, suggest that relationships within this group are even more complicated and that *Pucciniastrum* s.l. is not monophyletic. Therefore more species have to be included in phylogenetic studies before reliable suggestions can be made for generic circumscriptions within *Pucciniastraeae*. This is also true for interrelationships between these genera.

***Pucciniastraeae* group 1: velopedunculate representatives**

The species of this well-supported cluster (100% probability, 85% bootstrap), which is present in both analyses, are *Pucciniastrum agrimoniae*, *Pucciniastrum pyrolae*, *Thekopsora areolata*, *Cronartium flaccidum*, *Cronartium ribicola*, and all the sampled species of *Chrysomyxa* and *Coleosporium*. All members of this group that we sampled are characterized by velopedunculate D-haustoria (Berndt 1993, 1996; Berndt et al. 1994; Berndt and Oberwinkler 1995, 1997).

The genus *Chrysomyxa* is well-circumscribed by teliospore chains, built up by one-celled teliospores, secondary caemata (= aecioid uredinia), and host alternation between *Picea* and *Ericaceae*. The monophyly of the genus is also highly supported by our analysis (100%, 100%). *Coleosporium*, as a genus, is easily recognizable and well-defined (e.g., *Pinus* as aecial host, "internal germination of teliospores", secondary caemata) and our data strongly support the monophyly of the genus (100%, 100%). Genetic distance of the three central European species (*Coleosporium cacaliae*, *Coleosporium campanulae*, and *Coleosporium tussilaginis*) was very small or zero. Only the Canadian *Coleosporium asterum* differed markedly. Thus, our results are consistent with the observation that the central European species could be merged into one (Klebahn 1914).

Together, *Coleosporium* and *Chrysomyxa* constitute the family *Coleosporiaceae* (Cummins and Hiratsuka 1983). While teliospore morphology and host specificity is very different between the two genera (see above), the family is defined by spermogonia of type 2, blister-shaped aecia on needles, and repeating caemata (aecioid uredinia) on the

telial host (Gäumann 1959; Sato and Sato 1982; Cummins and Hiratsuka 1983). The monophyly of the family is well-supported by MCMC analysis (87%), but not by neighbor joining.

Cronartium, the only genus of the *Cronartiaceae*, is characterized by spermogonia that are located deep within the host tissue (type 9) causing hypertrophies of stems, branches or cones, the blister-shaped peridium of the aecia, *Pinus* as aecial host, and the column-like structure of telia. We sequenced the Scots pine blister rust, *Cronartium flaccidum*, and the white pine blister rust, *Cronartium ribicola*. The support value obtained in MCMC analysis (95%) corroborated that *Cronartium* is well-defined, although bootstrap support in neighbor joining was surprisingly low (54%).

In both analyses, *Pucciniastrum pyrolae* and *Thekopsora areolata* form a common cluster, but this grouping was only supported by MCMC analysis (100% probability). *Pucciniastrum agrimoniae* is also part of the *Pucciniastraeae* group 1. We do not know any features other than haustorial ultrastructure that could support the placement of the species within this cluster.

***Pucciniastraeae* group 2: fern rusts and allies**

This is an unsupported group in neighbor joining analysis; furthermore, in MCMC analysis its representatives are attached to the backbone of the tree without resolution. Only the monophyly of the fern rusts *Uredinopsis* and *Milesia* (100% probability, 99% bootstrap) is strongly supported. A closer relationship between these two genera has been proposed because they lack pigmentation (Faull 1932, 1938) and they share a special (botryose) haustorium type and velopedunculate D-haustoria (Berndt 1993). Whether the fern rusts are the most basal group within the *Uredinales* — almost a paradigm in rust systematics (e.g., Dietel 1904; Gäumann 1959; Cummins and Hiratsuka 1983; Savile 1993) — can neither be verified nor falsified, according to our data. However, it has been suggested by a morphological cladistic study (Hart 1988) as well as by a molecular phylogenetic study (Sjamsuridzal et al. 1999) that the fern rusts are not the most basal or "primitive" rust fungi.

Hyalospora is sister to *Melampsoridium* in neighbor joining (70% bootstrap), but not in MCMC analysis. *Melampsoridium* is a well-defined small genus. In its telial stage it closely resembles *Melampsora*, but in contrast to the latter, *Melampsoridium* has peridiate aecia and uredinia. The uredinial peridia have unique elongated, "spinescent", ostiolar cells and host alternation takes place between *Larix* and *Betulaceae*. For these reasons it could be argued that no close affinity exists between *Melampsora* and *Melampsoridium*, which is confirmed by our data.

The genus *Naohidemycetes* was erected recently for *Tsuga*–*Ericaceae* rusts and *Thekopsora vaccinii* was transferred to that genus (Sato et al. 1993), which is supported by the fact that *Naohidemycetes* does not cluster with the sampled *Thekopsora* species in our analyses.

***Pucciniastraeae* group 3**

This is a weakly supported group (62% probability) that comprises *Pucciniastrum epilobii*, *Pucciniastrum circaeae*, *Melampsorella caryophyllacearum*, *Thekopsora guttata*, and *Thekopsora symphyti*. All mentioned species belong to

Pucciniastraceae (Cummins and Hiratsuka 1983). *Pucciniastrum epilobii* and *Pucciniastrum circaeae* form a highly supported cluster (100%, 100%) and possess gymnopedunculate haustoria, in contrast to the other two sampled species of *Pucciniastrum*, *Pucciniastrum agrimoniae* and *Pucciniastrum pyrolae*, which are part of *Pucciniastraceae* group 1 and have velopedunculate haustoria (Berndt 1993; Berndt and Oberwinkler 1995). They are further defined by closely related hosts; both are parasitic on members of *Onagraceae* (*Epilobium* and *Circaea*, respectively) in the dikaryotic stage, whereas *Pucciniastrum agrimoniae* and *Pucciniastrum pyrolae* are parasitic on *Rosaceae* (*Agrimonia*) and *Pyrolaceae* (*Pyrola*).

Thekopsora guttata and *Thekopsora symphyti* form a highly supported cluster (probability 96%, bootstrap 94%). A close relationship between these two species was hypothesized mainly on the basis of haustorial ultrastructure and resulted in the transfer of the former *Melampsorella symphyti* to *Thekopsora symphyti* (Berndt 1993). There is no close affinity of *Melampsorella caryophyllacearum* to any species in this group. After the removal of *Melampsorella symphyti*, the genus is monotypic and characterized by the production of witches' brooms on *Abies* and uredinia and telia on *Cerastium* and *Stellaria*. Furthermore, it is the only representative of the *Pucciniastraceae* group 3 having velopedunculate haustoria (Berndt and Oberwinkler 1997).

Autoecious *Rosaceae* rusts (*Phragmidiaceae*)

The genera *Phragmidium*, *Kuehneola*, *Triphragmium*, and *Trachyspora* constitute this cluster in both analyses (probability 99%, bootstrap 79%). All sampled specimens are parasitic on *Rosaceae*, their life cycle being autoecious and, with the exception of *Trachyspora*, macrocyclic. Another common feature of these genera are the spermogonia of group IV (types 10 and 11). Because of these characters, they have been assigned to the family *Phragmidiaceae*, with the exception of *Triphragmium* which was assigned to the mainly tropical *Sphaerophragmiaceae* (Cummins and Hiratsuka 1983). But spermogonial morphology (type 11 instead of types 5 and 7 in *Sphaerophragmiaceae*) and host selection strongly support the inclusion of *Triphragmium* in the *Phragmidiaceae*. Cummins and Hiratsuka were aware of the fact that an assignment to *Sphaerophragmiaceae* was problematic: "*Triphragmium* has type 11 spermogonia but otherwise belongs here" (Cummins and Hiratsuka 1983). However, in this case, they regarded teliospore morphology (which very closely resembles *Sphaerophragmium*) to be of greater importance. But, according to our data, *Triphragmium* is a well-supported member of *Phragmidiaceae* as proposed by Poelt (1985) as well as by Savile (1989).

The genus *Phragmidium* is monophyletic (probability 100%, bootstrap 90%) with *Phragmidium violaceum* and *Phragmidium rubi-idaei* being the most closely related, reflecting the close relationship to their host species, both of which are *Rubus* species. The genus *Kuehneola*, though at first appearing very similar to *Phragmidium*, differs from the former by catenulate teliospores, the number of germ pores per teliospore (one instead of two) and lack of paraphyses (Dietel 1912). Still, *Phragmidium* and *Kuehneola* appear to be well placed next to each other with respect to spermogo-

nia morphology and host relationship (Cummins and Hiratsuka 1983).

The validity of *Trachyspora* was questioned and its affinity to *Uromyces* suggested (e.g., Sydow and Sydow 1910; Gäumann 1959). But again host specificity (*Alchemilla*) and spermogonia of type 10 point to a closer relationship to *Phragmidiaceae* (Henderson 1973; Gjaerum and Cummins 1982; Cummins and Hiratsuka 1983) as supported by our data.

Sister group status of *Uredo alpestris* to the *Phragmidiaceae* is suggested by both analyses, but is poorly supported (53% probability, 52% bootstrap). *Uredo alpestris* has been a mystery to uredinologists for a long time. Though very common in the Alps, both its systematic placement and its life cycle remain unclear. Only dimorphic urediniospores are known. Since dimorphic urediniospores without visible germ pores are also known from *Uredinopsis* and *Hyalopsora*, it was considered most likely that *Uredo alpestris* belongs to one of these fern rust genera or, at least, is a close relative of them (Dietel 1916; Gäumann 1959). However, the fact that there is no sign of a peridium in *Uredo alpestris*, so typical for the uredinia of fern rusts (and the *Pucciniastraceae*), and the presence of gymnopedunculate haustoria (Berndt 1993) are inconsistent with that hypothesis. In our analysis, the molecular data place *Uredo alpestris* distant from the fern rusts.

Gymnosporangium (*Pucciniaceae*)

Gymnosporangium is parasitic on *Rosaceae* in its aecial state, then switches to *Cupressaceae* for the telial state. According to spermogonia morphology it has been placed in the *Pucciniaceae*. The genus is well defined on the basis of host specificity and unique morphological characters like roestelioid aecia and gelatinous telia, and this was confirmed (100%, 100%) by our analysis. However, the relationship to the core *Pucciniaceae* (*Puccinial/Uromyces* cluster) could not be resolved.

Tranzschelia–*Ochropsora* cluster: *Anemone*–*Rosaceae* rusts

The monophyly of *Tranzschelia* is highly supported by our data (probability 100%, bootstrap 97%). Furthermore, both sampled *Ochropsora ariae* specimens — one sequenced from the aecial host *Anemone nemorosa*, the other from the telial host *Aruncus dioica* — were identical in all bases, confirming the usefulness of this DNA region for determining anamorph–teliomorph relationships (e.g., Begerow et al. 2000).

In our analysis, the sister group status of *Tranzschelia* and *Ochropsora* is well supported (probability 96%, bootstrap 73%), despite the two genera exhibiting very different "teliospore" morphology; *Tranzschelia* has two-celled teliospore chains, while *Ochropsora* has no teliospores, but auricularioid basidia and gasteroid spore production (Oberwinkler 1982; Bauer and Oberwinkler 1986). Because of these differences, the genera were assigned to different families: *Tranzschelia* to *Uropyxidaceae* and *Ochropsora* to *Chaoniaceae*, respectively (Cummins and Hiratsuka 1983).

A closer relationship of *Tranzschelia* and *Ochropsora* has been suggested because the monokaryotic mycelia of *Ochropsora* and *Tranzschelia* cause very similar deformations of the host plants, spermogonial morphology is much

the same (type 7), and on the basis of host specificity (Dietel 1922; Lindfors 1924; Holm 1980). *Tranzschelia pruni-spinosae*, *Tranzschelia discolor*, and *Ochropsora ariae* attack *Anemone* in the monokaryotic phase and switch to rosaceous plants (*Aruncus*, *Sorbus*, and *Prunus*) to complete their life cycles, whereas *Tranzschelia fusca* stays on *Anemone* during all stages of its reduced life cycle. Thus, the close affinity of *Tranzschelia pruni-spinosae* and *Tranzschelia fusca* (100%, 100%), can be regarded as confirmation of Tranzschel's law proposing that microcyclic autoecious species are derived from macrocyclic heteroecious ones, producing their telia on the former aecial host or a close relative (Tranzschel 1904). As a consequence of the above, *Tranzschelia* and *Ochropsora* can be defined as *Ranunculaceae-Rosaceae* rusts and their microcyclic descendants. Other characters that are shared by the two genera that support a closer relationship are the occurrence of apomictic forms with monokaryotic aeciospores (Kursanov 1922) and the complete lack of carotenoids (Zwetko and Pfeifhofer 1991). Therefore, the two genera were treated in *Raveneliaceae-Uropyxideae* by Savile (1989) as well as by Poelt and Zwetko (1997).

***Puccinia/Uromyces* cluster (*Pucciniaceae*)**

The genera *Puccinia*, *Uromyces*, *Cumminsella*, and *Endophyllum* form a strongly supported group (100%, 100%). All the members of this group belong to the family *Pucciniaceae* and possess spermogonia of type 4 (Cummins and Hiratsuka 1983). *Puccinia* and *Uromyces* are by far the largest genera of rust fungi, with about 4000 and 600 species, respectively. We sequenced autoecious, heteroecious, macrocyclic, and microcyclic species of *Uromyces* and *Puccinia* with a great variety of host specificities. Our data show that *Puccinia* and *Uromyces* are polyphyletic. This opinion was already put forward by Tulasne and many subsequent mycologists (Tulasne 1854; Sydow and Sydow 1910; Arthur 1934; Guyot 1938; Gäumann 1959).

Within the *Puccinia/Uromyces* cluster another highly supported group can be discerned (probability 100%, bootstrap 94%), comprising *Puccinia gigantea*, *Puccinia urticae-acuteformis*, *Uromyces ficariae*, *Uromyces pisi* s.l., *Uromyces viciae-fabae*, and *Endophyllum euphorbiae-sylvaticae*. Common morphological characteristics of the group as a whole are not evident, thus the inferred relationship is based upon molecular data only.

More can be said about the placement of *Uromyces pisi* next to *Endophyllum euphorbiae-sylvaticae*, which is significantly supported (probability 100%, bootstrap 98%). The sister group status of these two species supports the opinion of Tranzschel (1910) and of Jørstad who considered *Endophyllum euphorbiae-sylvaticae* "a short-cycled state evolved by suppression of the uredo-teleuto stage of some member [...] of the *Uromyces pisi* group" (Jørstad 1952), thus being another example of Tranzschel's law (see above). Jørstad therefore proposed that it should be renamed *Uromyces euphorbiae-sylvaticae*. In fact, the genus *Endophyllum* is merely defined by aeciospores that germinate with basidia (being named telial aecia according to the "morphologic" system (Laundon 1967; Holm 1973), but aecioid telia in the "ontogenic" system (Hiratsuka 1973, 1975). In both analyses, *Uromyces viciae-fabae*,

Endophyllum euphorbiae-sylvaticae, and *Uromyces pisi* form a cluster, although it is only well supported in MCMC analysis (probability 93%, bootstrap 53%). The telial hosts of *Uromyces pisi* and *Uromyces viciae-fabae* belong to *Fabaceae*, therefore host relationship also supports the inferred relationship between members of this group.

Cumminsella mirabilissima is also part of the *Puccinia/Uromyces* group. *Cumminsella* is autoecious and macrocyclic and restricted to *Berberis* and *Mahonia* (Baxter 1957). Morphologically, *Cumminsella* differs from *Puccinia* only in having two germ pores per teliospore cell, while spermogonia, aecia, and uredinia are of the same type, providing good morphological evidence for the genus belonging to the *Puccinia/Uromyces* cluster.

Phylogenetic implications

Molecular phylogenetic analyses of rust fungi supported the monophyly of most genera sampled, although *Puccinia*, *Pucciniastrum*, *Thekopsora*, and *Uromyces* were found to be polyphyletic. Higher order relationships of the rust fungi remained obscure to a large extent. Nevertheless, three hypotheses of higher order relationships may be deduced from the phylograms, but obtaining only moderate support by either method. First, in neighbor joining analysis, *Melampsora* is sister to all other rust fungi sampled (62% bootstrap). Second, a group that corresponds to *Pucciniastreae* sensu Dietel (1938) is supported by neighbor joining analysis (bootstrap 75%). Third, MCMC analysis supports a cluster (70% probability) containing only representatives of *Pucciniaceae* s.l. (Dietel 1928). In contrast to the weakly resolved backbone, terminal taxa were highly supported. Thus, monophyly could be proven for the *Puccinia/Uromyces* group, the *Phragmidiaceae*, the *Tranzschelia-Ochropsora* cluster, or the velopedunculate representatives of *Pucciniastreae*.

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