

## Different evolutionary histories of two *Phragmidium* species infecting the same dog rose hosts<sup>1</sup>

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Rust fungi in the genus *Phragmidium* are frequent pathogens of both wild and cultivated roses. We investigated the occurrence and relationships of rusts on dog roses, *Rosa* sect. *Caninae* (*Rosa canina*, *R. corymbifera* and *R. rubiginosa*) in Germany. Two *Phragmidium* species, *P. mucronatum* and *P. tuberculatum*, were able to infect each of the three dog rose species examined. However, the overall infection of *R. rubiginosa* was significantly lower, which could be important for rose breeding. Despite overlapping host ranges, the evolutionary background of *P. tuberculatum* and *P. mucronatum* is quite distinct. Phylogenetic analyses of the D1/D2 region of the LSU rDNA suggest that *P. mucronatum* shares a common ancestor with other rose rusts, whereas *P. tuberculatum* evolved from a *Rubus-Sanguisorba* rust clade and must have undergone a host shift to *Rosa* spp.

### INTRODUCTION

The predominantly Northern Hemisphere rust genus *Phragmidium* comprises about 60 species (Cummins & Hiratsuka 2003). *Phragmidium* species, like the entire family *Phragmidiaceae*, have an autoecious life-cycle and are restricted to rosaceous hosts, with only two documented exceptions from the USA (Peterson & Cronin 1967). On dog roses four *Phragmidium* species can be found in Central Europe, viz. *P. fusiforme*, *P. mucronatum*, *P. tuberculatum*, and *P. rosae-pimpinellifoliae*. However, the taxonomic history of many species is rather complex (Dietel 1905a, b) and especially the *Phragmidium* species on roses, ‘Formenkreis *Phragmidium mucronatum*’, were said to have overlapping morphological characters (Gäumann 1959). This casts doubt onto the broad host ranges stated for *P. mucronatum* and *P. tuberculatum* since these species could have been misidentified due to excessively broad morphological species concepts (cf. Newcombe 2003).

The Eurasian dog roses (*Rosa* sect. *Caninae*) are a morphologically and genetically highly diverse group,

about 30 species of which can be found in Central Europe (Klásterský 1969, Henker & Schulze 1993). Their most striking feature is a unique meiotic behaviour, the so-called ‘Canina-meiosis’ (Täckholm 1920, 1922), where the pentaploid genome is distributed unequally during meiosis resulting in haploid pollen grains and tetraploid egg cells. After fertilization, the pentaploid state is restored and results in full sexual reproduction (Wissemann & Hellwig 1997).

We investigated which rust species can be found on the three most common dog rose species in Germany (*Rosa canina*, *R. corymbifera*, and *R. rubiginosa*). We examined the frequency of the occurring rusts and whether these rust species show any infection preference for a particular rose host. By means of molecular phylogenetic analyses based on nrLSU sequence data we tested whether the morphological species determinations proved valid and whether there is any correlation between specific host infection and pathogen phylogeny.

### MATERIALS AND METHODS

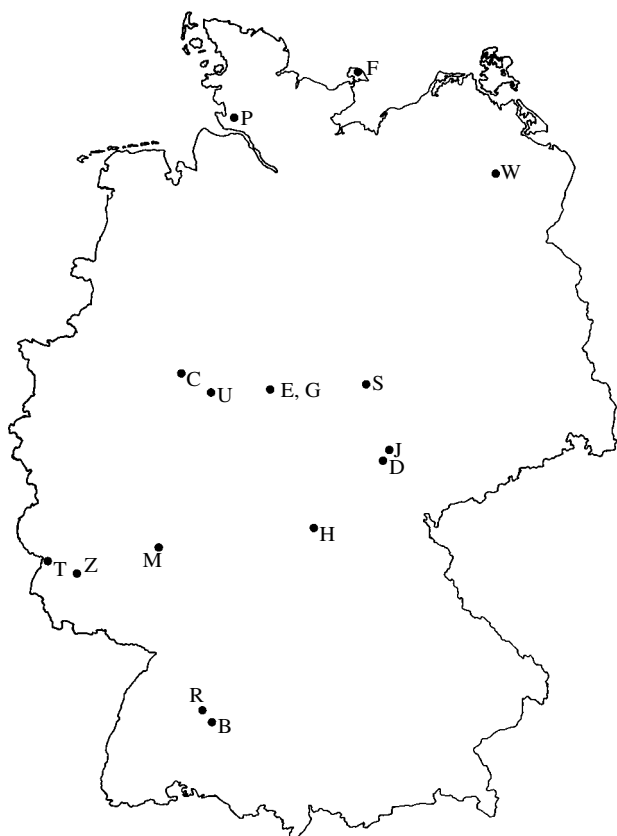
#### *Sampling and collection*

Three species of dog roses differing in leaf surface characters were investigated, i.e. *R. canina* with glabrous leaves, *R. corymbifera* with hairy leaves, and

<sup>1</sup> Dedicated to John Webster on the occasion of his 80th birthday.

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**Fig. 1.** Sample sites of rust collection during summer 2002 in Germany. (B): Baden-Württemberg, Zollernalbkreis, Beuren, (C): Nordrhein-Westfalen, Geseke, (D): Thüringen, Kahla, Dohlenstein, (E): Niedersachsen, Groß Schneen, Einzelberg, (F): Schleswig-Holstein, Fehmarn, Gammendorf, (G): Niedersachsen, Göttingen, Gartetal, (H): Bayern, Happertshausen, (J): Thüringen, Jena-Zwätzen, (M): Rheinland-Pfalz, Mainz-Finthen, (P): Schleswig-Holstein, Pöschendorf, (R): Baden-Württemberg, Rottenburg/Neckar, (S): Sachsen-Anhalt, Sangerhausen, (T): Rheinland-Pfalz, Trier-Ruwer, (U): Nordrhein-Westfalen, Westheim, (W): Mecklenburg-Vorpommern, Neubrandenburg, Krickow, (Z): Rheinland-Pfalz, Hunsrück, Züsch.

*R. rubiginosa* with glandular leaves. Rust fungi from these hosts were collected during Aug. 2002 in Germany at 16 sample sites representing a geographical north-south axis of 800 km (Fig. 1). A sample site consists of populations of the respective host-species and represents an area of 500 to 1000 square metres. Rust specimens used for DNA analyses, voucher information, and GenBank accession numbers are listed in Table 1. For DNA extraction, rust fungi were collected as silica-gel dried infected leaf material.

15 rose bushes (five bushes per species) were sampled at each site. Rust infection was recorded as categorical data (presence or absence of rust). In the case of infection, the rust species was morphologically identified after Gäumann (1959) based on either aeciospore or teliospore characters, using a Carl Zeiss microscope (with bright field and phase contrast optics). Specimens of rust fungi were deposited in the reference collections

of the University of Tübingen (TUB). Statistical analyses of rust infections were performed using the software package SPSS 11.0. Presence/absence data of rust infection were compared between the rose species using logistic regression. Abundance of rust fungi and niche differentiation (e.g. the preferential infection of a given host by one of the two rust species) were analysed using a six-field  $\chi^2$  test. Since the absolute abundance of the two observed rust species was found to be very different, the expected values of the six-field-test were corrected for the relative frequency of the respective rust fungus.

We determined nrLSU sequences for *P. mucronatum*, *P. fusiforme* and *P. tuberculatum* and integrated all available (nrLSU) sequences of Phragmidiaceae from an earlier study (Maier *et al.* 2003) for phylogenetic analyses.

#### DNA extraction

Total DNA was extracted from either aeciospores or teliospores using the DNeasy Plant Mini Kit (Qiagen, Hilden) according to the manufacturer's protocol. The protocol was modified by shaking the dried samples with the help of a mixer mill (MM 300, Retsch, Haan) for 3 min at 30 Hz in a 1.5 ml tube together with one tungsten carbide ball (3 mm diam).

#### DNA amplification

The 5' end of the nuclear 28S rRNA gene (nrLSU) was amplified from diluted extracts ( $10^{-1}$  and  $10^{-2}$ ). Primers for the amplification were LR0R (5'-ACC CGC TGA ACT TAA GC) described by Moncalvo *et al.* (1995) and LR6 (5'-CGC CAG TTC TGC TTA CC) described by Vilgalys & Hester (1990). PCR conditions consisted of an initial denaturation at 94 °C for 180 s, 12 cycles of 94 ° for 35 s, 45 ° for 45 s, 72 ° for 60 s, 12 cycles of 94 ° for 35 s, 45 ° for 55 s, 72 ° for 90 s, 12 cycles of 94 ° for 35 s, 45 ° for 60 s, 72 ° for 120 s and a final elongation of 72 ° for 10 min.

#### Sequencing

The amplified DNA was purified using Qiaquick PCR purification kit (Qiagen) following the manufacturer's instructions, and was then sequenced directly in both directions. Cycle-sequencing was performed using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington) or the ThermoSequenase labelled primer cycle sequencing kit (Amersham Pharmacia, Uppsala) with the unlabelled or IRD-labelled primers NLMW1 (5'-TCA ATA AGC GGA GGA AAA GA; Sampaio *et al.* 2002) and NL4 (5'-GGT CCG TGT TTC AAG ACG G; O'Donnell 1992, 1993). The cycle sequencing profile was 25 cycles of 96 ° for 10 s, 50 ° for 5 s, and 60 ° for 4 min. The resulting DNA fragments were separated on an acrylamide gel, using an automatic

**Table 1.** Voucher information, voucher accession and GenBank accession numbers of rust samples used for the DNA analyses.

Taxon	Host plant, specimen voucher and voucher accession no.	GenBank No.
<i>Phragmidium mucronatum</i>	<i>Rosa corymbifera</i> , B21, TUB 012075	AJ715513
	<i>R. corymbifera</i> , C2, TUB 012076	AJ715519
	<i>R. corymbifera</i> , G7, TUB 012077	AJ715516
	<i>R. rubiginosa</i> , G8, TUB 012078	AJ715517
	<i>R. corymbifera</i> , P15, TUB 012082	AJ715514
	<i>R. corymbifera</i> , S14, TUB 012083	AJ715515
	<i>R. rubiginosa</i> , T10, TUB 012084	AJ715521
	<i>R. corymbifera</i> , T14, TUB 012085	AJ715512
	<i>R. canina</i> , T15, TUB 012086	AJ715518
	<i>R. canina</i> , U2, TUB 012090	AJ715520
	<i>P. tuberculatum</i>	<i>R. canina</i> , H2, TUB 012079
<i>R. canina</i> , J12, TUB 012080		AJ715510
<i>R. canina</i> , M8, TUB 012081		AJ715511
<i>R. corymbifera</i> , W9, TUB 012087		AJ715509
<i>R. rubiginosa</i> , W11, TUB 012088		AJ715507
<i>R. corymbifera</i> , W20, TUB 012089		AJ715506
<i>P. fragariae</i>	<i>Potentilla sterilis</i>	AF426217
<i>P. fusiforme</i>	<i>R. pendulina</i>	AJ715522
<i>P. montivagum</i>	<i>R. cfr woodsii</i>	AF426213
<i>P. rubi-idaei</i>	<i>Rubus idaeus</i>	AF426215
<i>P. sanguisorbae</i>	<i>Sanguisorba minor</i>	AF426216
<i>P. violaceum</i>	<i>Rubus fruticosus</i> aggr.	AF426214
<i>Kuehneola uredinis</i>	<i>R. fruticosus</i> aggr.	AF426218
<i>Trachyspora intrusa</i>	<i>Alchemilla vulgaris</i> aggr.	AF426220
<i>Triphragmium ulmariae</i>	<i>Filipendula ulmaria</i>	AF426219

The capital letter of the specimen voucher corresponds to the localities of *Phragmidium mucronatum* and *P. tuberculatum* presented in Fig. 1. The other species of *Phragmidiaceae* listed below are taken from Maier *et al.* (2003).

LI-COR DNA sequencer 4000L or an ABI 373A Stretch (PE Applied Biosystems, Foster City, CA).

### Phylogenetic reconstructions

Sequence alignment was carried out using the software Clustal X 1.83 (Thompson *et al.* 1997) followed by slight manual editing. The final alignment has been deposited in TreeBASE (<http://www.herbaria.harvard.edu/treebase/>) and is accessible under study accession no. SN1944.

Parsimony analyses were performed using the heuristic search mode in PAUP 4.0b10 (Swofford 2002) with 100 random addition sequence replicates and TBR branch swapping. All character states were treated as unordered, equally weighted and gaps were treated as missing characters. Branch support was evaluated by 1000 bootstrap replicates (Felsenstein 1985) and with the help of Bremer support (Bremer 1994) using the software AutoDecay 3.0 (Eriksson & Wikström 1995).

Bayesian inference of phylogeny using Monte Carlo Markov chains (MCMC) was conducted with MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 2,000,000 generations, using the DNA substitution model of Hasegawa, Kishino and Yano (HKY 85; Hasegawa *et al.* 1985) with gamma-distributed substitution rates (Swofford *et al.* 1996), random starting trees and default starting values of the very DNA substitution model. The HKY + G model of

DNA substitution was chosen by both the Akaike information criterion and the likelihood ratio tests implemented in MrModeltest 1.0b (Nylander 2002). Trees were sampled every 100 generations resulting in an overall sampling of 20 001 trees. The first 1000 trees were discarded as burnin. From the remaining trees a 50% majority rule consensus tree was computed to obtain estimates for the *a posteriori* probabilities. Branch lengths were estimated as mean values over the sampled trees. This Bayesian approach of phylogenetic analysis was repeated four times, always using random starting trees and random starting values for the HKY85 model to test the independence of the results from topological priors (Huelsenbeck *et al.* 2002).

Furthermore, a neighbour-joining analysis was performed also using HKY 85 as DNA substitution model.

The unrooted dendrograms from neighbour joining, parsimony and MCMC analyses were rooted with *Kuehneola uredinis*, *Triphragmium ulmariae* and *Trachyspora intrusa* according to the findings of Maier *et al.* (2003).

## RESULTS

### Rust species

Of the four potentially occurring dog rose rusts, we only detected *Phragmidium mucronatum* and *P. tuberculatum*.

**Table 2.** Number of rose-bushes (*Rosa canina*, *R. corymbifera* and *R. rubiginosa*) infected with *Phragmidium mucronatum* and *P. tuberculatum* and number of sample sites with rust infection during summer 2002 at 16 samples sites in Germany.

	<i>Rosa canina</i>	<i>Rosa corymbifera</i>	<i>Rosa rubiginosa</i>	Sample sites with infection
<i>Phragmidium mucronatum</i>	30	29	10	17
<i>P. tuberculatum</i>	20	12	4	13
Number of sampled bushes	80	80	80	

### Statistical analyses of infection

The number of rose bushes (*R. canina*, *R. corymbifera*, and *R. rubiginosa*) infected by *P. mucronatum* and *P. tuberculatum* is shown in Table 2. The investigated rose species displayed different responses to rust infection (logistic Regression; Wald = 31.64, df = 2,  $P < 0.001$ ). Rust infection on *R. rubiginosa* was significantly lower compared to *R. canina* (log. Reg.; Wald = 30.37, df = 1,  $P < 0.001$ ) and *R. corymbifera* (log. Reg.; Wald = 18.75, df = 1,  $P < 0.001$ ), whereby no significant difference of rust infection could be detected between *R. canina* and *R. corymbifera* (log. Reg.; Wald = 2.05, df = 1,  $P = 0.15$ ). These results did not change if the logistic regression was performed for the two rust species separately. Additionally, we tested whether *P. mucronatum* and *P. tuberculatum* showed a niche differentiation among the three rose species. Since *P. tuberculatum* was significantly rarer than *P. mucronatum* on the three investigated rose species ( $\chi^2_1 = 10.37$ ,  $P = 0.001$ ), the expected values of the six-field-test were corrected for the relative abundance of the rust fungus (Table 2). No significant host preferences could be detected between the two rust fungi ( $\chi^2_1 = 0.47$ ,  $P = 0.79$ ).

### Phylogenetic reconstructions

Phylogenetic analyses of the nrLSU sequence data based on maximum parsimony (MP), neighbour joining (NJ) and Bayesian inference (MCMC) resulted in the same tree topology (Figs 2–3, NJ not shown). Furthermore, all four runs of MCMC-analyses yielded identical tree topologies. Members of the genus *Phragmidium* appear as a monophyletic group with 64 and 72% bootstrap support (MP and NJ, respectively) and 99% *a posteriori* probability.

The observed rose-parasitizing rust fungi *P. mucronatum* and *P. tuberculatum* were not monophyletic. While *P. mucronatum* grouped with *P. fusiforme*, mainly occurring in the Alps, and *P. montivagum* pathogenic on North American wild roses (both bootstrap and *a posteriori* probability of 100%), *P. tuberculatum* appeared as the sister taxon to four *Phragmidium* species living on either *Rubus fruticosus* aggr., *R. idaeus*, *Sanguisorba minor* or *Potentilla sterilis* (supported by 83% bootstrap and 76% *a posteriori* probability). The 5'-regions of the nrLSU of all investigated specimens of *Phragmidium tuberculatum* were identical, with *P. mucronatum* differing from *P. tuberculatum*

in 8–9% of the 527 characters of the alignment. In *P. mucronatum*, seven of the ten sequenced specimens were identical, with the other specimens differing from them and from each other in about 1% of the examined nucleotides. These were the collections U2, T10, and T15.

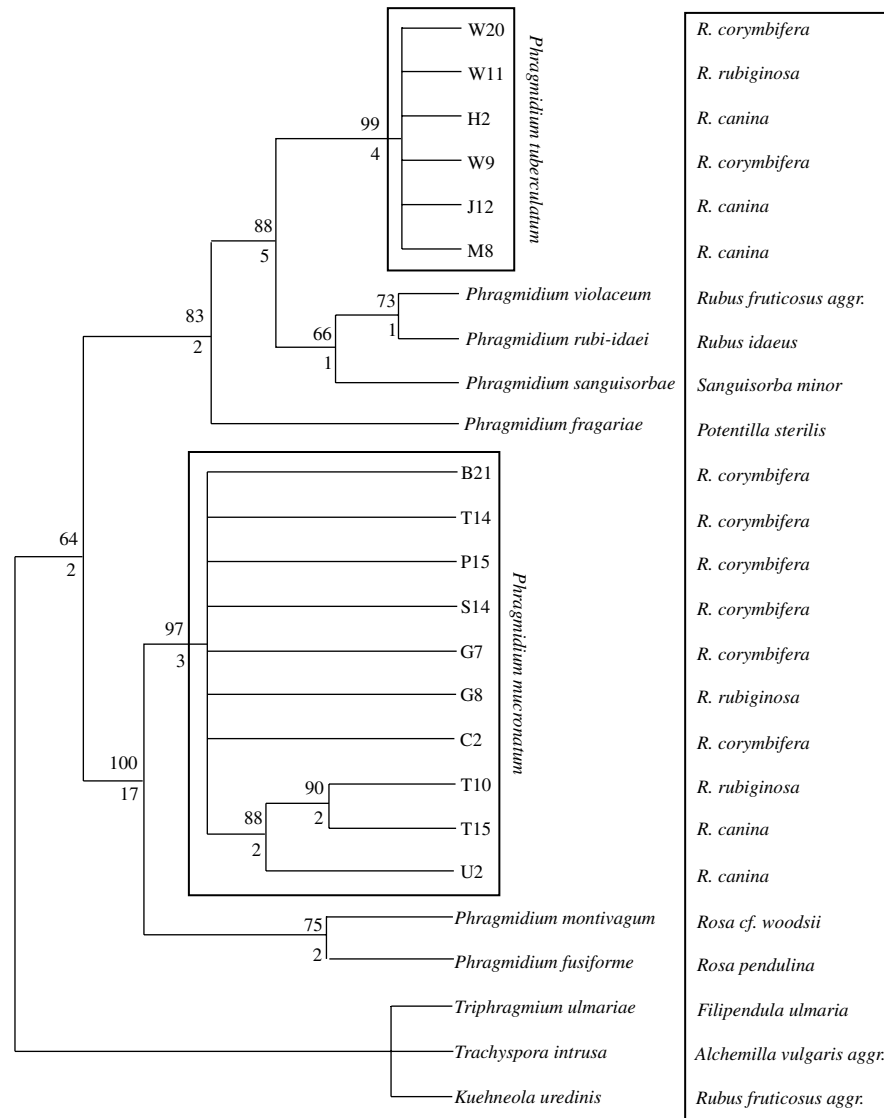
## DISCUSSION

### Species delimitation and phylogeny

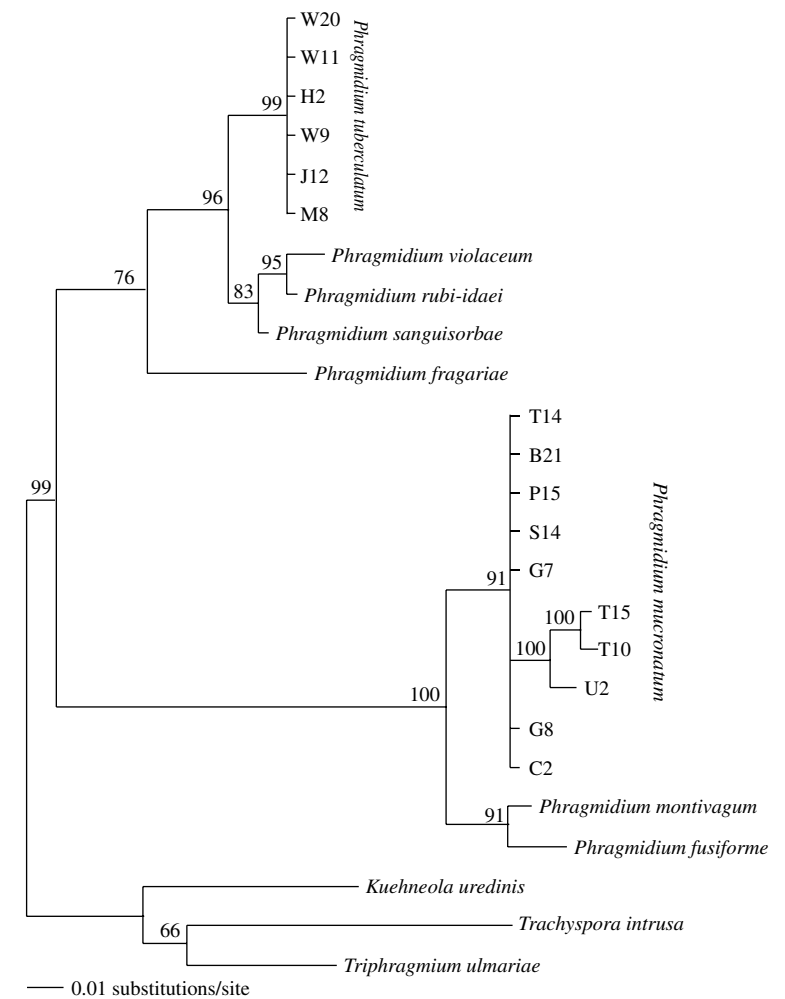
On the three most common dog roses in Germany (*Rosa canina*, *R. corymbifera*, *R. rubiginosa*) we detected only *Phragmidium mucronatum* and *P. tuberculatum*. While *P. rosae-pimpinellifoliae* has been recorded from *R. canina* and *R. rubiginosa*, the mainly alpine *P. fusiforme* has not been observed on any of the here studied dog roses (Gäumann 1959).

It had been stated that the morphological features of the European rose rusts were partly overlapping which questions the data given on their host range. However, we found that species determination of *P. mucronatum* and *P. tuberculatum* based on both aecio- and teliospore features according to Gäumann (1959) was unambiguously reproducible between different members of our samples. Since species determination is easiest if both species are available for comparative microscopy, the unexpectedly large differences in the LSU sequences will prove helpful as additional characters for species determination in future. Interestingly, sequences of some *P. mucronatum* specimens differed slightly from the majority of specimens of that species. However, no morphological differences could be detected, and further investigations will be necessary to decide whether there are cryptic species in this taxon.

Due to the overlapping host ranges of *P. tuberculatum* and *P. mucronatum* and their strong morphological resemblance (Sydow & Sydow 1915), we expected also a close phylogenetic relationship between them. However, this assumption was not reflected by the phylogenetic reconstructions because *P. mucronatum* grouped within a clade comprising *P. fusiforme* and the North American *P. montivagum*, while *P. tuberculatum* was a sister group to European *Rubus*- and *Sanguisorba*-rusts (Figs 2–3). Thus, we suggest the existence of a core rose rust clade on the one hand, and a host jump from a predecessor of *P. tuberculatum* that had lived on *Rubus* or *Sanguisorba* to *Rosa* on the other. The phylogenetic hypotheses derived from the trees



**Fig. 2.** Strict consensus of 54 equally most parsimonious trees (67 parsimony informative characters of a total of 527 characters) based on nrLSU sequence data (tree length 156, CI=0.69, RI=0.92, RC=0.70). Bootstrap values are shown above branches (1000 replicates), Bremer support values are indicated below branches. The topology was rooted with *Kuehneola uredinis*, *Triphragmium ulmariae* and *Trachyspora intrusa*.



**Fig. 3.** Bayesian inference of phylogenetic relationships of representatives of *Phragmidiaceae*: Monte Carlo Markov chain analysis based on nrLSU sequence data (substitution model of Hasegawa, Kishino, and Yano with gamma distributed substitution rates, 2M generations). The 50%-majority rule consensus tree was computed from 19001 trees that were sampled after the process had reached stationarity. The topology was rooted with *Kuehneola uredinis*, *Triphragmium ulmariae* and *Trachyspora intrusa*. Numbers on branches are estimates for *a posteriori* probabilities.

also contradict subgeneric delineations based on the structure of teliospore pedicels (Arthur 1906, *vide* Gäumann 1959), being hygroscopic (subgenus *Earlea*) or firm (subgenus *Euphragmidium*), as well as the host genus-specific 'Formenkreise' of Gäumann (1959).

The sequence divergences in the D1/D2 region of the nrLSU of *Phragmidium* as a whole, being up to 10% in some species pairs, are the highest observed for a rust genus so far (Maier *et al.* 2003). This is roughly twice as much as in other rust genera, suggesting either that *Phragmidium* is a relatively old genus or has an accelerated substitution rate. Support for the first explanation is provided by *Phragmidium* species being restricted to a relatively old host family, the *Rosaceae* (Kalkmann 1988), in contrast to, for example *Puccinia* which has its focus on more recently evolved angiosperm families like *Poaceae*, *Cyperaceae*, and *Asteraceae*.

### Ecological infection data

Neither *Phragmidium mucronatum* nor *P. tuberculatum* showed any infection preferences with respect to one of the observed rose species. This results in overlapping host ranges, as summarized by Gäumann (1959), who stated broad host ranges for both species including also non-dog roses. These wide host ranges might be a consequence of the putative hybridisation of dog roses after the last ice age (Grant 1971). Similar hypotheses to explain broad host ranges which are rather unusual for the rusts were formulated for cereal and grass rusts (Savile & Urban 1982, Urban & Marková 1984), and it has been assumed that hybridisation of hosts can form a bridge for parasites, enabling them to infect paternal lineages as well as hybrids (Floate & Whitham 1993).

That *P. tuberculatum* was only half as frequent as *P. mucronatum* might be explained by the distinct phylogenetic history of the two rusts. It can be speculated that *P. tuberculatum*, which evolved from the *Rubus-Sanguisorba* rust clade might be less adapted to *Rosa* as compared to *P. mucronatum* which is in the rose rust clade and most likely shares a longer adaptational or coevolutionary history with its rose hosts. However, phylogenetic host data and quantitative data on infection severity (rust pustules per leaf surface) derived from laboratory-based experiments will be needed to test this hypothesis.

The observation that *R. rubiginosa* was infected significantly less frequently in nature than the other investigated rose species might somehow be correlated with the conspicuously scented glands producing a sticky epicuticular secretion on the leaf surface. Furthermore, we detected that *R. corymbifera*, a species with a hairy lower leaf surface, was infected as frequently as the glabrous *R. canina*. The result that the presence of trichomes has no influence on rust infection corresponds also with the findings of Rubiales & Niks (1992) who found no correlation between the presence of hair in *Hordeum* species and rust infection. Besides leaf

surface, another and maybe more important parameter influencing the infection patterns of the investigated rust fungi might be the relative frequency of the rose species in the sample area, since *Rosa canina* and *R. corymbifera* are more frequent than *R. rubiginosa* in Europe (Kurrto *et al.* 2004). With a rather lax growth habit these two species occur on dry grasslands, meadows and pastures as well as in hedges and forest belts, whereas the rather thermophilous *R. rubiginosa* with its more condensed growth habit is found mainly in open areas. It seems plausible that the wind-distributed rust fungi might be best adapted to the most frequent rose species.

The lack of any obvious niche differentiation of the two *Phragmidium* species and the putative host jump of *P. tuberculatum* to *Rosa* contradicts *a priori* assumptions on strict cospeciation of specific rust-plant interactions. A similar exception has been described for *Puccinia* spp. parasitizing *Arabis* spp. (Roy 2001). Furthermore, host switches within *Phragmidium* were also detected by reconciliation analyses of parasite and host trees of various genera of rust fungi infecting *Rosaceae* (Jackson 2004). In that study, however, the role of association by descent was significant. Our current hypothesis is that the frequent hybridisation of dog roses in nature (Ritz & Wissemann 2003) might have prevented host-induced speciation in the parasites and is furthermore the cause of the wide host range of the two observed rose rust taxa.

### ACKNOWLEDGEMENTS

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