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Phylogeny of *Peronospora*, parasitic on *Fabaceae*, based on ITS sequences

Gema GARCÍA-BLÁZQUEZ[a], *Markus GÖKER*[b,*], *Hermann VOGLMAYR*[c], *María P. MARTÍN*[a], *M. Teresa TELLERÍA*[a], *Franz OBERWINKLER*[b]

[a]*Departamento de Micología, Real Jardín Botánico de Madrid, CSIC, Plaza de Murillo 2, 28014 Madrid, Spain*
[b]*Lehrstuhl für Spezielle Botanik und Mykologie, Botanisches Institut, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany*
[c]*Department of Systematic and Evolutionary Botany, Institute of Botany, University of Vienna, Rennweg 14, A-1030 Wien, Austria*

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ABSTRACT

Species concepts are a notoriously difficult taxonomic problem in plant–parasitic fungal-like organisms such as downy mildews (*Peronosporomycetes*, *Peronosporales*). This is particularly evident in the largest downy mildew genus, *Peronospora*, which contains a number of economically important pathogens. Here, we investigate relationships of *Peronospora* species infecting *Fabaceae* (angiosperms, *Rosidae*) originating from various collections from different species of host plants and from different European locations by molecular phylogenetic analysis of ITS sequences. Molecular trees were inferred with ML, MP and Bayesian methods and rooted with *Pseudoperonospora*. As in other downy mildew groups, molecular data mainly support the use of narrow species delimitations and host range as a taxonomic marker. *Fabaceae* parasites appear to be subdivided into a number of lineages displaying a considerable degree of host specialization with respect to host genera, as well as host subgenera or species. The number of repeats of a repetitive part of the ITS1 is, within limits, characteristic of subgroups within the cluster of *Trifolium* parasites. We reveal new hosts for *Peronospora* found on the Iberian Peninsula.

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Introduction

Peronospora is the most species-rich genus within *Peronosporales* (*Peronosporomycetes*), and within *Peronospora*, the group infecting *Fabaceae* is one of the largest. According to Constantinescu (1991), 25 host genera and 103 *Peronospora* taxa are recorded from *Fabaceae*. This study investigating *Peronospora* infecting *Fabaceae* helps elucidate the complicated and controversial taxonomy of these fungi, particularly host specificity and species boundaries.

Up to now, a principal problem is that there are no sound morphological features diagnostic for each of the species that parasitize *Fabaceae*, which makes it difficult, if not impossible, to determine species based on morphology alone. Taxonomically useful morphological or ecological characters are few. Most of the species described until now have very similar conidia and conidiophores. The delimitation of many, but not all, species by morphometric methods is still an imprecise activity owing both to the great influence of the environment on the morphology of most somatic structures and also to the lack of technical advances (Hall 1996). Assessment of host specificity of downy mildew species has also been difficult in the past if the host taxonomy was poorly understood or, as in the case of *Peronospora cochleariae*, incorrect (Göker *et al.* 2004).

* Corresponding author.
E-mail address: markus.goeker@uni-tuebingen.de
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These difficulties are reflected by the traditional approaches to *Peronospora* taxonomy. de Bary (1863) applied a broad species concept in which usually all *Peronospora* accessions infecting a specific host family were considered as a single species. In being the first to investigate *Peronospora* on *Fabaceae* in detail, he assumed *Fabaceae* to be one of the few exceptions to that rule and acknowledged two species on *Fabaceae*: *Peronospora viciae* for accessions from *Vicia sativa*, *Pisum sativum*, *Lathyrus linifolius* (as *Orobus tuberosus*), and *Vicia tetrasperma* (as *Ervum tetraspermum*), as well as *P. trifoliorum* for accessions from *Trifolium medium*, *T. alpestre*, *T. incarnatum*, and *Medicago sativa*. Conversely, Gäumann (1923) argued for a narrow species concept within the parasites of *Fabaceae*, which was mainly based on very small morphological differences and a presumed high host specificity (on the genus or even species level). Accordingly, the present study uses molecular techniques to address and circumvent the problems in elucidating *Peronospora* phylogeny and in distinguishing species. Several studies of DNA sequences have been published to resolve the phylogenetic relationships within *Peronosporales* (e.g. Constantinescu & Fatehi 2002; Riethmüller *et al.* 2002; Göker *et al.* 2003, 2004, 2007; Voglmayr 2003; Voglmayr *et al.* 2006). The combination of molecular techniques and morphology or host specificity has been successfully used by different authors for solving systematic problems within downy mildews, e.g. in *Pseudoperonospora* (Choi *et al.* 2005), *Plasmopara* (Voglmayr *et al.* 2004, 2006; Constantinescu *et al.* 2005), *Peronosporaceae* (Göker *et al.* 2003), *Hyaloperonospora* (Göker *et al.* 2004), and *Bremia* (Thines *et al.* 2006). In the case of white blister rusts (*Albugo s. lat.*), Spring *et al.* (2005), Thines & Spring (2005), and Voglmayr & Riethmüller (2006) elucidated phylogenetic relationships with such a combined approach.

The ITS region of the nu-rDNA has been proved to be a good choice for phylogenetic analysis on the generic level (Choi *et al.* 2003, 2005; Voglmayr 2003; Göker *et al.* 2004). Thus, we have sequenced the ITS region, concentrating on a representative sample of *Peronospora* in *Fabaceae*. The collections for phylogenetic analysis were chosen in order to analyse the highest possible variety of host–parasite combinations. In each case, whenever possible we sequenced more than one specimen from the same host species.

Materials and methods

Sample sources and DNA extraction

The organisms included in this study are listed in Table 1. The voucher material of the fungi (i.e. infected plant host tissue) on which the paper is based has been permanently preserved in public collections. The vouchers corresponding to the sequences obtained in the course of the present study can be found in the following herbaria: Real Jardín Botánico Madrid; University of Vienna; University of Tübingen; and Staatliches Museum für Naturkunde Görlitz.

The nomenclature of *Peronospora* is mainly based on the host–parasite relations given in the original species descriptions and follows Gäumann (1923), Gustavsson (1959a), and Constantinescu (1991). Host nomenclature for central European taxa follows Fischer *et al.* (2005); those of other taxa the ILDIS (International Legume Database & Information Service) database (ver. 10.01; http://www.ildis.org/LegumeWeb?version~10.01), which is the online version of Roskov *et al.* (2005). DNA extraction, PCR, and cycle-sequencing procedures were performed according to Riethmüller *et al.* (2002). We used ITS1-O (5′-CGG AAG GAT CAT TAC CAC; Bachofer 2004) and ITS4-H (Göker *et al.* 2004), a modification of ITS4 (White *et al.* 1990) as PCR and cycle-sequencing primers. The use of ITS1-O greatly reduces the problem of additional amplification of host ITS rDNA. In some cases, a semi-nested PCR approach had to be used in which ITS1-O was combined with LR0 (5′-GCT TAA GTT CAG CGG GT) in the second PCR. LR0 is the reverse complement of LR0R (Moncalvo *et al.* 1995). *Pseudoperonospora* was included as an outgroup for rooting as it is usually considered to be the sister genus of *Peronospora*, which is largely confirmed by molecular data (e.g. Voglmayr 2003; Göker *et al.* 2003, 2007), and their sequences are still comparatively easy to align.

Sequence alignment and phylogenetic analysis

As sequences varied considerably in length, POA (Lee *et al.* 2002), which treats long indels very accurately, was the alignment program of choice. As the POA software aligns the sequences in input order without iterative refinement and does not apply specific leading and trailing gap parameters, alignment quality could further be improved by reverse complementing the sequences before alignment to avoid starting the alignment of each sequence with the ITS1 region containing both leading gaps and sequence repeats in some sequences (see below) and by adding the *Pseudoperonospora* sequences after finishing a *Peronospora*-only multiple sequence alignment. After careful cross-comparison of the sequences of the *Trifolium* parasites, a region comprising a variable number of approximately 70 bp long repetitive fragments could be delineated and distinguished from the homologous region within the remaining ITS1. Within a single sequence, one of these repeats apparently was misaligned and was moved manually. In order to obtain reproducible results, no further manual 'corrections' were made. Due to the varying number of repeats per sequence, between-sequence homology of the repetitive elements could not be established with certainty, and the whole repeat region was excluded from the phylogenetic analyses. However, the numbers of repeats could be established with ease and were coded as ordered characters for reconstruction under the MP criterion (see below). Furthermore, regions containing too many leading and trailing gaps (i.e. in more than 10 % of the sequences) were not included in phylogenetic analyses.

To obtain an appropriate model of nucleotide site substitution for use in tree searches under the ML criterion (Felsenstein 1981), the data were first analysed with Modeltest 3.7 (Posada & Crandall 1998) in conjunction with PAUP (Swofford 2002). We chose the corrected Akaike information criterion (AICc) to distinguish between the different models, as recommended by Posada & Buckley (2004). Searches for the best ML tree, as well as 1 K BS replicates (Felsenstein 1985) were done with the fast likelihood software PHYML 2.4.4 (Guindon & Gascuel 2003), using identical settings. Heuristic searches under the unweighted MP criterion (Fitch 1971) were conducted

Table 1 – Collection data and GenBank accession numbers of the specimens examined in the course of this study

Collector	Collection no.	DNA isolation no.	GenBank accession no.
HV & AR	HV-F33 (WU)	GG10-11	* EF174888
HV	HV94 (WU 22872)		AY198227
MG	MG1941 (TUB)	MG18-4	* EF174969
AG	(MA-Fungi 27743)	GG6-4	* EF174949
GG	GG81 (MA-Fungi 69549)	GG2-7	* EF174911
GG	GG154 (MA-Fungi 69567)	GG5-8	* EF174941
GG	GG19 (MA-Fungi 69550)	GG1-8	* EF174903
HV	HV91 (WU 22885)		AY198262
GG	GG20 (MA-Fungi 69551)	GG2-10	* EF174904
SMK 18200	SMK 18200		AY608608
GG	GG101 (MA-Fungi 69553)	GG5-4	* EF174937
GG	GG185 (MA-Fungi 69552)	GG2-3	* EF174907
GG	GG147 (MA-Fungi 69555)	GG5-5	* EF174938
GG	GG124 (MA-Fungi 69554)	GG2-6	* EF174910
HV	HV168 (WU 22895)		AY198265
HV	HV199 (WU 22898)		AY198232
HV & AR	HV-F7 (WU)	GG10-9	* EF174898
HV	HV879 (WU)	GG10-4	* EF174893
HV	HV840 (WU)	GG10-2	* EF174891
WD	MG2173 (TUB)	GG8-1	* EF174956
AG	(MA-Fungi 27879)	GG6-12	* EF174945
HV	HV1052 (WU)	GG10-7	* EF174896
HV	HV880 (WU)	GG10-5	* EF174894
HV	HV727 (WU 22909)		AY198266
AG	(MA-Fungi 27884)	GG6-10	* EF174943
DQ202400			DQ202400
SMK17669	SMK17669		AY211019
			AB021711
			AY742740
HV	HV214 (WU 22910)		AY198231
AG	(MA-Fungi 27996)	GG6-7	* EF174951
HJ	(GLM46906)	GG7-2	* EF174954
HJ	(GLM46909)	GG8-9	* EF174961
HV	HV602 (WU 22924)		AY198228
HV	HV853 (WU)	GG10-3	* EF174892
AG	(MA-Fungi 27899)	GG6-3	* EF174948
GG	GG180 (MA-Fungi 69558)	GG5-10	* EF174933
GG	GG151 (MA-Fungi 69557)	GG2-1	* EF174905
GG	GG153 (MA-Fungi 69562)	GG2-2	* EF174906
AG	(MA-Fungi 27885)	GG6-2	* EF174947
HV	HV547 (WU 22911)		AY198229
AG	(MA-Fungi 27891)	GG6-1	* EF174946
MG	MG2135 (TUB)	GG1-1	* EF174899
GG	GG254 (MA-Fungi 69560)	GG5-3	* EF174936
GG	GG144 (MA-Fungi 69559)	GG2-5	* EF174909
GG	GG223 (MA-Fungi 69561)	GG2-4	* EF174908
AG	(MA-Fungi 27905)	GG6-11	* EF174944
AY225471			AY225471
HV	HV808 (WU)	GG10-1	* EF174890
HV	HV1067 (WU)	GG10-8	* EF174897
HJ	(GLM50757)	GG1-3	* EF174901
HV	HV189, 190 (WU 22934)		AY198237
HV	HV1055-1057 (WU)	GG3-1	* EF174917
GG	GG136 (MA-Fungi 69563)	GG2-8	* EF174912
HV	HV479-481 (WU)	GG4-1	* EF174925
HV	HV665-667 (WU 22935)		AY198235
HV	HV662-664 (WU)	GG3-10	* EF174914
HJ	(GLM46888)	GG9-4	* EF174962
HV	HV979-981 (WU)	GG3-12	* EF174916
MG	MG2136 (TUB)	GG1-2	* EF174900
HV	HV873-875 (WU)	GG3-11	* EF174915
AR	AR226 (TUB)	MG16-10	* EF174968
HV	HV520, 521 (WU 22936)		AY198233
HV	HV489-493 (WU)	GG3-3	* EF174919

Table 1 – (*continued*)

Collector	Collection no.	DNA isolation no.	GenBank accession no.
MG	MG1665 (TUB)	MG11-9	* EF174965
HV	HV622-624 (WU)	GG3-2	* EF174918
HJ	(GLM49026)	GG8-7	* EF174959
HV & AR	HV-F26 (WU)	GG3-4	* EF174920
GG	GG134 (MA-Fungi 69564)	GG2-9	* EF174913
HV	HV849, 850 (WU)	GG3-9	* EF174923
GG	GG190 (MA-Fungi 69565)	GG5-9	* EF174942
HJ	(GLM50900)	GG8-8	* EF174960
HV	HV701-703 (WU 22937)		AY198236
HV	HV952, 953 (WU)	GG4-6	* EF174929
HV	HV697-700 (WU)	GG4-8	* EF174931
HV	HV2161 (WU)	GG4-7	* EF174930
MM	MG2174 (TUB)	GG8-3	* EF174957
HV	HV395, 396 (WU 22938)		AY198234
HV	HV1074-1076 (WU)	GG4-9	* EF174932
HV & AR	HV-F9 (WU)	GG7-9	* EF174955
MG	MG1771 (TUB)	MG10-10	* EF174963
MG	MG1960 (TUB)	MG19-2	* EF174970
FO	MG1798 (TUB)	MG13-1	* EF174967
HV	HV995 (WU)	GG4-2	* EF174926
HJ	(GLM46896)	GG8-4	* EF174958
MG	MG1959 (TUB)	MG19-8	* EF174971
MG	MG1795 (TUB)	MG8-4	* EF174972
HV	HV858 (WU)	GG3-7	* EF174922
HV	HV826-828 (WU)	GG4-3	* EF174927
HV	HV2004 (WU)	GG4-4	* EF174928
HV	HV158 (WU 22941)		AY198230
HJ	(GLM48346)	GG1-7	* EF174902
AG	(MA-Fungi 27971)	GG6-9	* EF174953
GG	GG249 (MA-Fungi 69566)	GG5-1	* EF174934
AG	(MA-Fungi 27943)	GG6-6	* EF174950
HV & AR	HV-F48 (WU)	GG10-12	* EF174889
HV	HV938 (WU)	GG10-6	* EF174895
MG	MG1796 (TUB)	MG12-3	* EF174966
HV	HV956 (WU)	GG4-10	* EF174924
GG	GG56 (MA-Fungi 69569)	GG5-7	* EF174940
GG	GG133 (MA-Fungi 69568)	GG5-6	* EF174939
HV & AR	HV-F27 (WU)	GG3-5	* EF174921
MG	MG1797 (TUB)	MG10-7	* EF174964
HV & AR	HV-F22 (WU)	GG10-10	* EF174887
AG	(MA-Fungi 27993)	GG6-8	* EF174952
GG	GG99 (MA-Fungi 69556)	GG5-2	* EF174935
MZM 71018	MZM 71018		AY608612
SMK 17780	SMK 17780		AY608613
HV 222 (WU 22944)	HV 222 (WU 22944)		AY198306
HV 136 (WU 22946)	HV 136 (WU 22946)		AY198304
HV 715 (WU 198307)	HV 715 (WU 198307)		AY198307

Acronyms of collectors: AG, Arne Gustavsson; AR, Alexandra Riethmüller; FO, Franz Oberwinkler; GG, Gema García-Blázquez; HJ, Herrmann Jage; HV, Hermann Voglmayr; MG, Markus Göker; MM, Mechthilde Mennicken; WD, Wolfgang Dietrich. Vouchers: MA, Real Jardín Botánico Madrid; WU, University of Vienna; TUB, University of Tübingen; GLM, Staatliches Museum für Naturkunde Görlitz; MZM, Moravian Museum, Czech Republic; SMK, Systematic Mycology of Korea, Korea University, Seoul.
Sequences obtained in the course of the present study are marked with an asterisk.

with PAUP; gaps were treated as missing data. Multiple (1 K) rounds of random sequence addition and subsequent tree bisection–reconnection (TBR) branch swapping (STEEPEST option not in effect) were applied, collapsing branches if it was possible for them to have zero length (PSET COLLAPSE = MINBRLEN). To reduce the large number of trees saved per island, due to nearly identical sequences as observed in preliminary runs, no more than ten trees with a score equal to or greater than one were saved per replicate. The RI (Farris 1989) and strict consensus of the most parsimonious trees was computed using PAUP. After excluding uninformative characters, parsimony BS analysis with 1 K replicates was performed by ten rounds of random sequence addition and subsequent TBR branch swapping during each BS replicate, saving only a single tree per replicate. Additionally, both searches for best trees and bootstrapping were performed using the same

settings as above, but with gaps treated as fifth character (MP-gap).

PPs were approximated by sampling trees using a MCMC method (Huelsenbeck *et al.* 2000; Larget & Simon 1999). The Bayesian analysis was performed with MrBayes V3.0b4 (Huelsenbeck & Ronquist 2001) assuming the general time reversible model (Rodríguez *et al.* 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six categories (GTR+I+G). A run with 2 M generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file for a total of 20 K trees. Majority-rule consensus trees were calculated from 19 K trees sampled after reaching likelihood convergence to calculate the PPs of the tree nodes.

Reconstruction of the number of ITS1 repetitive elements present in the *Trifolium* parasites under the ordered MP criterion (Wagner parsimony; Kluge & Farris 1969) was done using PAUP in the delayed transformation ('DELTRAN') mode. The vector representing the respective number of repeats was directly treated as a single ordered character; numbers greater than nine can conveniently be coded with letters in PAUP. In that way, each change between character states is associated with a cost equivalent to the difference in the number of ITS1 repetitive elements they represent.

Additionally, a topology-independent statistical test for character congruence between the parts of the ITS alignment used to infer trees and the number of ITS2 repeats was conducted. Uncorrected ('p') distances between the *Trifolium* parasites were exported from PAUP. For the same sequences, Euclidean distances between the number of repeats were computed using the program eukdis, which is available as Linux executable upon request from M.G. Congruence between both distance matrices was assessed using the CADM software (Legendre 2001). As entries in a distance matrix do not represent statistically independent characters, ordinary significance tests cannot be applied. Hence, CADM uses permutation (Mantel test) to estimate the significance of the Spearman rank correlation between the distance matrices (Legendre & Lapointe 2004). We applied 999 permutations of the original matrices.

Results

New hosts for Peronospora

In the present publication we reveal new hosts for *Peronospora*, found on the Iberian Peninsula: *Coronilla repanda* subsp. *dura*, which occurs on the Iberian Peninsula and Morocco; *Astragalus hamosus,* which has a wide distribution in the Mediterranean, the Irano-Turanian region and Macaronesia; and *Ornithopus compressus*, which occurs in the Mediterranean region, the Canary Islands, and Madeira (Talavera *et al.* 1999, 2000).

Morphology

As in other groups of *Peronospora*, the differences between species that parasitize *Fabaceae* are small. Our observations (data not shown) generally indicate a considerable variation in conidial morphology and conidial and conidiophore dimensions between different samples from the same host species (or from apparently the same *Peronospora* species; see below), which could be due to environmental conditions, as was pointed out by different authors (e.g., Yerkes & Shaw 1959).

Phylogenetic analyses

The entire length of the final ITS sequence alignment was 2049 bp, 548 of which were excluded because of the presence of leading or trailing gaps due to incomplete sequencing in too many taxa and a further 724 bp that comprised the repeat region. Thus, the number of alignment columns that could be used for phylogenetic analyses was 813. The complete alignment was deposited in TreeBASE (http://www.treebase.org/) as SN3224.

The AICc criterion as implemented in Modeltest suggested TVM+I+G as most appropriate substitution model. As this model is not implemented in PHYML, we chose the most similar, but more complex one, GTR+I+G, for use in ML analysis. The log likelihood of the best ML tree found was -4629.36312. Substitution model parameter estimations under Bayesian inference were similar to those resulting from ML analysis (not shown). Heuristic search under the MP criterion found 436 most parsimonious trees with a length of 649 in 707 of the 1 K replicates. The retention index of the best trees was 0.947. MP-gap analysis resulted in 30 best trees of length 755 and of a retention index of 0.951. Here, minimal-length trees were found in 488 replicates.

Considering only the supported nodes, tree topologies of ML (Fig 1), majority-rule consensus trees from Bayesian analysis, and strict consensus trees from both MP analyses (figures not shown) are fully compatible. In the following, we, therefore, confine ourselves to the discussion of the ML topology and the support values from ML analysis and from MP analysis with gaps treated as missing data (Fig 1). All inferred trees were deposited in TreeBASE together with the DNA-sequence alignment. A part of the tree inferred under MP-gap is shown in Fig 2. Using *Pseudoperonospora* as outgroup, the ingroup representing *Peronospora* parasitic of *Fabaceae* was highly supported as monophyletic by a BS of 100 % (Fig 1).

ML (Fig 1) distinguishes six clades. The tree backbone lacks significant BS in contrast to the terminal nodes, which are often highly supported. Clade 1 contains *Peronospora* parasitizing *Lotus, Ornithopus*, *Securigera,* and *Coronilla*. This clade has 100 % BS, and it is the sister group of the rest; however, without significant BS. Clade 2 contains *Peronospora* on *Glycine* with a BS of 100 %. Clade 3 includes *Peronospora* on *Medicago* and *Melilotus* without significant BS. The clades comprising *Peronospora* on *Medicago* and *Melilotus* are sister groups to each other. In the moderately supported (84 % BS) *Medicago* clade there are three highly supported subclades (100 % BS): *Peronospora* on *Medicago lupulina/minima*, *Peronospora* on *M. truncatula/polymorpha*, and *Peronospora* on *Medicago sativa/orbicularis*. *Peronospora* on *Melilotus* has a BS of 100 %. Clade 4 consists of *Peronospora* on *Pisum*, *Vicia*, and *Lathyrus* with a BS of 80/74 %. Whereas the branches at the base of most lineages are distinct and well-defined in this group, there are not enough differences for distinguishing clear-cut species within the bulk of species of a crown group (named *P. viciae s. lat.* in Fig 1), and ML (Fig 1) as well as MP (data not shown)

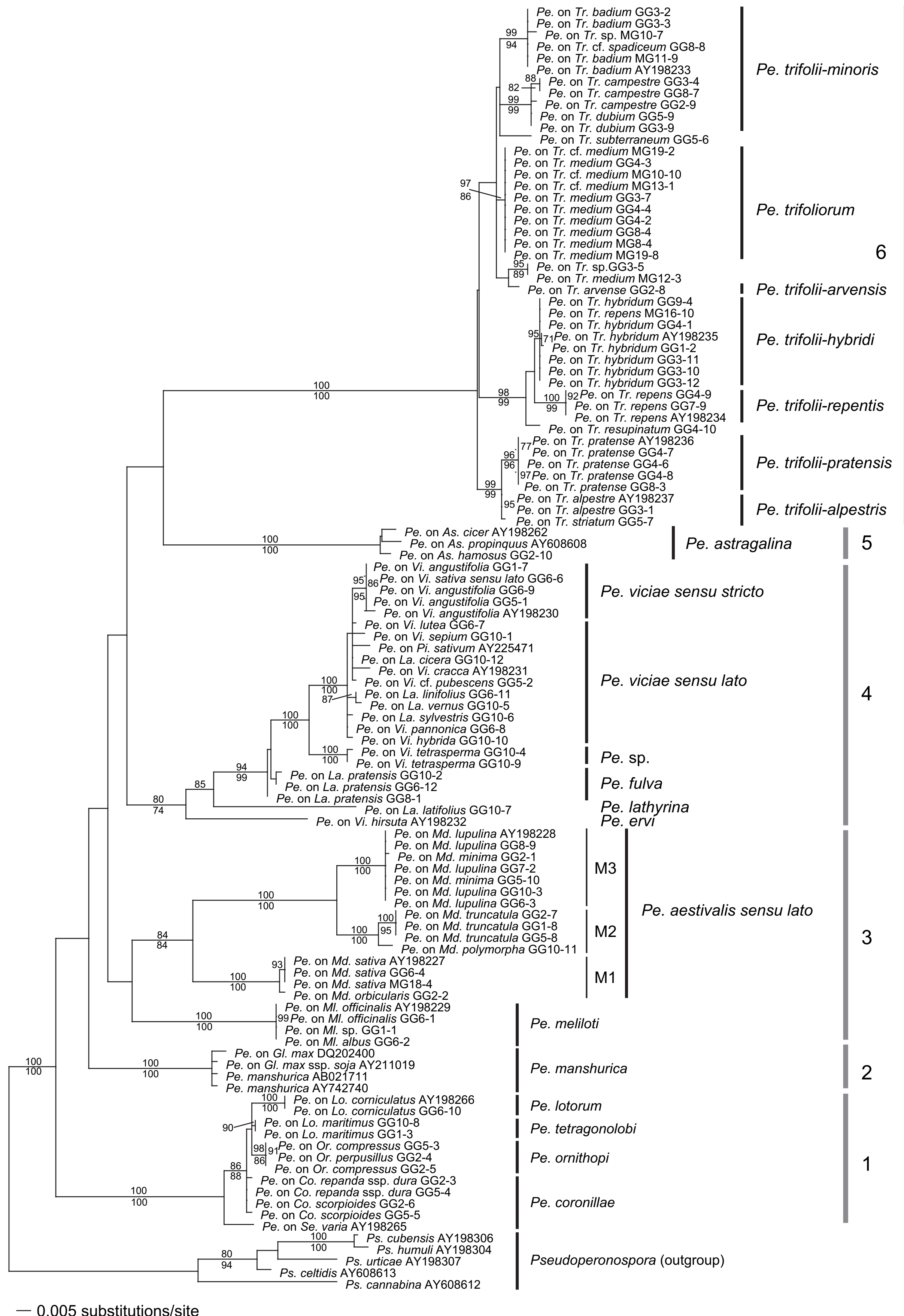

Fig 1 – ML phylogram inferred with PHYML from the ITS alignment under a GTR+I+G nucleotide substitution model and rooted with *Pseudoperonospora*. Branch lengths are scaled in terms of the expected number of substitutions per site. Numbers above branches represent ML BS above 70 %, below branches MP BS above 70 %. M1–3 are the three subclades of *Peronospora aestivalis s. lat*. Bars and numbers on the right denote the six major clades of *Peronospora* on *Fabaceae*.

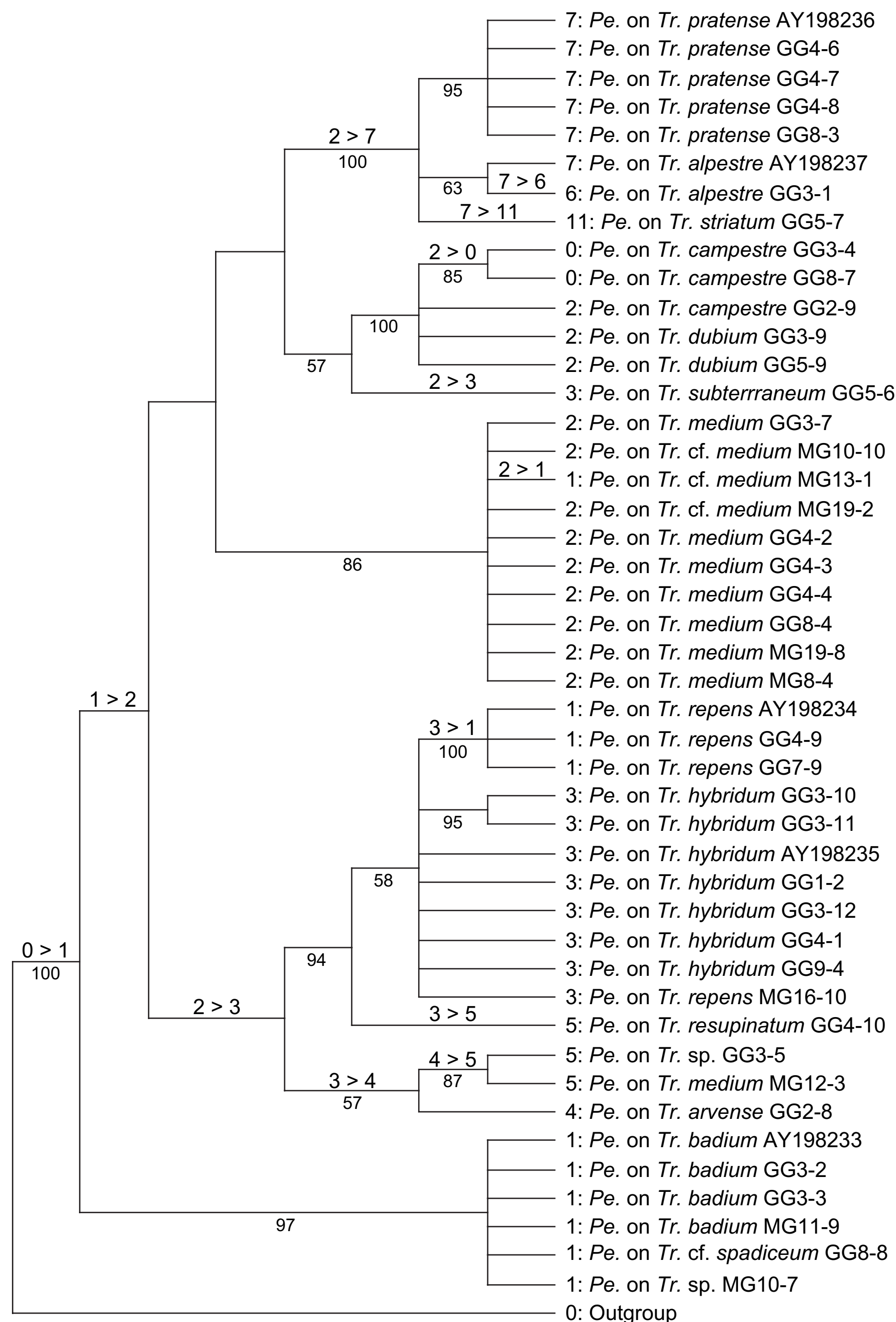

Fig 2 – Part of the strict consensus tree of the most parsimonious trees found with heuristic search under the MP criterion, treating gaps as fifth state (MP-gap). As the *Peronospora* specimens parasitic of other hosts than *Trifolium* display the same number of repeats (0), the tree is confined to the well-supported cluster of *Trifolium* parasites (clade 6). Numbers at the tips of the tree represent the number of repeats within ITS1 encountered in the respective terminal taxon; numbers on the branches denote character state changes as reconstructed under ordered (i.e. Wagner) parsimony. Numbers below branches are support values from MP-gap bootstrapping above 50 %.

analysis revealed several polytomies. Clade 5 consists of *Peronospora* on *Astragalus* (100 % BS) which is the sister group of the *Peronospora* on *Trifolium* clade (clade 6); however, the sister-group relationship between clades 5 and 6 lacks significant BS support. Clade 6 contains *Peronospora* on *Trifolium* (100 % BS), which contains seven highly supported subclades: *Peronospora* on *T. badium*, *T. campestre/dubium*, *T. medium*, *T. hybridum*, *T. repens*, *T. pratense*, and *T. alpestre*. In addition to the main clades, there are some other (mainly single) accessions from other *Trifolium* species which do not group within any of these subclades.

The reconstruction under ordered MP of the number of repeats of the approximately 70 bp long building block in *Peronospora* ITS1 sequences in *Trifolium* is shown in Fig 2. The tree used comprises clade 6 only and is a subtree of the strict consensus tree of all most parsimonious trees resulting from

MP-gap analysis. The number of steps and RIs of this character were 23, 0.906; 24, 0.898; 24, 0.898; and 25, 0.891 if reconstructed on the MP-gap consensus, MP consensus, ML tree, or Bayesian majority-rule consensus, respectively. It follows that the number of repeats is, within limits, characteristic of subgroups within the clusters of *Trifolium* parasites. The number of additional copies ranges from one in *Trifolium repens*, *T. badium*, *T.* cfr. *medium* MG13-10, *T.* cfr. *spadiceum*, and *Trifolium* sp. MG10-7; two in *T. medium*, *T.* cfr. *medium*, *T. dubium* and *T. campestre*; three in *T. subterraneum*, *T. hybridum* and *T. repens* MG16-10; four in *T. arvense*; five in *Trifolium* sp. GG3-5, *T. medium* MG12-3, and *T. resupinatum*; six in *T. alpestre*; seven in *T. pratense* and *T. alpestre*; to 11 in *T. striatum* (Fig 2).

The Spearman rank correlation between uncorrected genetic distances and Euclidean distances between the number of repeats as computed with CADM was 0.360 and was judged as highly significant ($P = 0.001$).

Discussion

The lack of clear-cut morphological differences between *Peronospora* accessions from different host species was a reason for de Bary (1863) to include all *Peronospora* accessions infecting a host family into a single species (or exceptionally, two in *Fabaceae*). This view was widely followed by subsequent authors (e.g. Yerkes & Shaw 1959). Recent molecular data do not support de Bary's view of merging species (Choi *et al.* 2003; Göker *et al.* 2003, 2004; Voglmayr 2003; Voglmayr *et al.* 2004, 2006).

During the present investigation, we once again came to the conclusion that it is impossible to identify *Peronospora* species unless the host species is taken into account. Trying to distinguish *Peronosporales* on the basis of only morphological characters is at best challenging and, in many cases, impossible. However, the combination of morphological with molecular characters proved a valuable means of species discrimination. For example, the main difference between the *Peronospora* species infecting *Trifolium hybridum* and *T. repens* is that the first has broadly ellipsoidal to globose conidia and the second one has ellipsoidal ones (data not shown); in our trees they appear to be closely related but nevertheless clearly distinct. As Voglmayr (2003) commented, at the moment it is not possible to establish a subgeneric classification of *Peronospora s. str.* based on morphological features.

Species concept

The results of this study are a further step towards the clarification of the species concept in *Peronospora,* and confirm that species parasitizing the same host genera/species are, in general, closely related. Whether all *Peronospora* accessions from *Fabaceae* form a single monophyletic lineage within *Peronospora* remains unclear, as there are clearly many taxa parasitic on *Fabaceae* that we were not able to sample in this study. Furthermore, it is unlikely that ITS sequences alone provide sufficient resolution for the backbone of the *Peronospora* tree, irrespective of whether parasites of multiple host families or only a single host family are included (Voglmayr 2003). Regarding the results of Riethmüller *et al.* (2002) and Göker *et al.* (2003), the same is to be expected for the LSU rDNA region, for which even fewer GenBank sequences are available.

This is the first study dealing in detail with the phylogenetic relationships between *Peronospora* species infecting *Fabaceae*. The support for a narrow species concept agrees well with most other studies on *Peronosporaceae* (e.g. Choi *et al.* 2003; Göker *et al.* 2003, 2004; Voglmayr 2003; Voglmayr *et al.* 2004, 2006). Despite the high number of *Peronospora* binomials described from *Fabaceae* (after exclusion of *Hyaloperonospora*, this group contains the highest species number per host family in *Peronospora*; see Constantinescu 1991), no detailed investigations are available. Only comparably few samples of *Peronospora* parasitizing *Fabaceae* were included in the works of Riethmüller *et al.* (2002), Göker *et al.* (2003), and Voglmayr (2003), the results of which, however, are fully compatible with the current study, as are the results of Cunnington's (2006) study of *Peronospora* on *Vicia* and *Pisum*.

The tree topologies presented here indicate that Gäumann's (1918, 1923) narrow species concept for *Peronospora* was largely adequate, irrespective of some cases where he did not realize the actual species boundaries. In a lot of cases, his splitting of species based on differences in measurement and morphology of conidia has been criticized, but molecular studies, such as those cited above, show that his concept was basically correct. Cross-infection experiments developed by Gäumann (1923) with *Peronospora* on *Fabaceae* indicate specific fungus–host relationships and confirm his narrow species concept, which is also corroborated by our molecular data. However, his experiments were performed with a limited number of species only. As Göker *et al.* (2004) pointed out for *Hyaloperonospora*, the lack of morphological differences between some accessions from different hosts does not necessarily imply that they belong to the same species. This, of course, has severe implications on species circumscription and identification, which in some cases may only be possible by molecular techniques if the host is new, unidentifiable, or unknown.

The approach of Constantinescu & Fatehi (2002) to distinguish only few, morphologically clearly distinct species of *Hyaloperonospora* was led by the fact that, at that time, only few molecular data were available, which made detailed evaluation of species circumscription and host specificity difficult to impossible. Therefore, to avoid numerous new combinations before sound evaluation with molecular techniques, they decided to distinguish only morphologically clearly distinct lineages. This resulted in a broadly interpreted *Hyaloperonospora parasitica* for which numerous hosts were listed. However, after the availability of substantial sequence data it became evident that *Hyaloperonospora parasitica s. lat.* is an assemblage of distinct species which represent a paraphylum within *Hyaloperonospora* (Choi *et al.* 2003; Göker *et al.* 2003, 2004; Voglmayr 2003). Accordingly, such a morphological species circumscription does not meet the criteria of phylogenetic classification (Hennig 1965) as it does not result in monophyletic groups (Göker 2006). If a biological species concept is applied using genetic distinctness as a measure of absence or presence of gene flow in combination with host specificity, recent sequence data show that narrow species concepts as advocated by Gäumann (1918, 1923) and Gustavsson (1959a, 1959b) are more appropriate for *Peronosporaceae*. Consequently, collections well distinguished by molecular and host features

should be regarded as distinct species, even though they may be indistinguishable by morphological features (Göker 2006). The limitations of morphological approaches in separating downy mildew species are well illustrated by the fact that no determination key based solely on morphology has ever been published for the species traditionally included in the two largest downy mildew genera, *Peronospora* and *Plasmopara*.

Again, ITS sequences appear to be a powerful and efficient tool for species identification and delimitation in most cases. However, it should be noted that in closely related lineages (such as the *P. viciae s. lat.* group) additional markers may be necessary for a better resolution. Even more important, however, seems to be the sequencing of additional specimens, particularly from additional host species and from a wider geographic area.

Taxonomic and nomenclatural uncertainties

As is evident in Fig 1, not all clades revealed by our analysis could be properly named due to lack of typification. For instance, the distinct subclades M1–3 in the *Medicago* clade clearly represent separate species; however, as far as we are aware the name commonly used for the pathogen of *Medicago sativa*, *Peronospora aestivalis*, has not yet been typified. In the original description (Gäumann 1923), collections from *M. sativa* (subclade M1), *M. polymorpha* (subclade M2), *M. falcata* (not included in our study), *M. lupulina* and *M. minima* (both subclade M3) are listed without indication of a type collection, and, to our knowledge, none of these collections has yet been designated as type. The necessary lectotypification has strong implications for the nomenclature of the three *Medicago* subclades depending on the host to become the type host. We currently refrain from lectotypification for several reasons: first, more collections should be investigated, including also the hosts of the other species described from *Medicago* (for a list, see Constantinescu 1991). In addition, the respective original collections of the species described on *Medicago* need to be thoroughly studied morphologically and the identification of their host has to be checked, which is far beyond the scope of the present study. Meanwhile, the three subclades on *Medicago* are preliminarily listed under *Peronospora aestivalis s. lat.*

Within the clade containing *Peronospora* on *Vicia* and *Lathyrus*, the nomenclatural problems are somewhat different. Whereas some clades are genetically well separated, the bulk of accessions are embedded within a poorly resolved crown group (named *Peronospora viciae s. lat.* in Fig 1). With the current data, it is not possible to evaluate whether *Peronospora viciae s. lat.* contains several host-specific species or whether it should be treated as a single species. The limited data available on cross-inoculation tests favour the presence of genetic differentiation within these groups; according to Gäumann (1923), cross-inoculation tests between accessions from *Vicia cracca* and *Pisum sativum* were negative, even though both are members of the *Peronospora viciae s. lat.* clade in our analysis (Fig 1). Also the investigations of Cunnington (2006) using ITS1 sequence data indicate the presence of genetically distinct, separate lineages within *P. viciae s lat.* This group should be investigated including additional accessions and more variable molecular markers to resolve this problem. Another problem concerns the collections from *Vicia tetrasperma*. As already mentioned by Cunnington (2006), these accessions may represent an undescribed species as they are clearly distinct from the accession from *Vicia hirsuta,* which is the type host of *Peronospora ervi*, the binomial currently also applied to accessions from *V. tetrasperma*. However, more accessions from *V. hirsuta* should be included before taxonomic changes are made.

Distribution on the hosts

The phylogeny of *Peronospora* revealed in the present study was found to correspond well with phylogenetic relationships of their hosts (Wojciechowski *et al.* 2000; Steele & Wojciechowski 2003). The different clades of *Peronospora* (Fig 1) almost fully match with the different host tribes. *Trifolium*, the host of our *Peronospora* clade 6, belongs to tribe *Trifolieae*; *Vicia* and *Lathyrus* belong to tribe *Vicieae* and correspond with our *Peronospora* clade 4; *Medicago* and *Melilotus* belong to tribe *Trifoliae* and are the hosts of our *Peronospora* clade 3; and the tribe *Loteae* includes *Lotus*, *Ornithopus*, *Securigera* and *Coronilla*, which are the hosts of our *Peronospora* clade 1. Within these clades, this may be the result of host–parasite co-phylogeny (e.g. Page 2003) and may indicate the potential of downy mildews to co-speciate with these host tribes. Alternatively, clade-limited colonization (e.g. Percy *et al.* 2004; Sorenson *et al.* 2004) has to be considered as an explanation.

ITS insertions and phylogeny of Trifolium *parasites*

Notably, all *Peronospora* accessions of various *Trifolium* species investigated not only have a duplication of an ITS1 region of approximately 70 bp, but they also differ in the number of additional copies of the duplicated sequence region. MP and ML (Fig 1) analysis match with the repeat reconstruction (Fig 2), and the number of ITS1 repetitive elements within the *Peronospora* subclades (species) is usually the same. The high RIs observed with repeat numbers coded as ordered characters and reconstructed on the molecular trees indicates that the correspondence between both types of character data is far from random. This is corroborated by the results of the CADM test, which indicates a moderately high, but highly significant correlation between genetic distances and Euclidean distances between the number of repeats and, hence, a clear-cut congruence between nucleotide and repeat character data. The fact that different species of *Peronospora* on *Trifolium* differ by the number of multiple copies of a more or less 73 bp indel in the ITS1 region was already noted by Voglmayr (2003).

Comparing *Peronospora* on *Trifolium* with the different sections of *Trifolium* established with morphological data by Talavera *et al.* (2000) and largely confirmed by recent molecular analyses (Ellison *et al.* 2006), the host species of the subclades of *Peronospora* belong to the same *Trifolium* sections: *T. badium*, *T. campestre*, and *T. dubium* are members of sect. 3 (*Lupulinum*); *T. medium*, *T. arvense*, *T. pratense*, and *T. alpestre* are members of sect. 1 (*Trifolium*); and *T. hybridum* and *T. repens* are members of sect. 7 (*Trifoliastrum*).

Within *Peronospora* on *Trifolium*, some subclades did not receive high BS, and phylogenetic relationships of these

accessions remain uncertain, as in the case of *Peronospora* on *Trifolium* sp. GG3-5, *T. medium* MG12-3, and *T. arvense* GG2-8.

The existence of repeated copies within the ITS has not only been demonstrated for *Peronospora,* but also for other *Peronosporaceae* where they were mainly observed in the ITS2 region. Thines *et al.* (2005) showed the existence of four copies of a tandemly arranged repetitive element in the ITS2 region of *Plasmopara halstedii*, and Thines (2007), with less stringent homology criteria applied, reported two copies of a repetitive element for *Plasmopara pusilla*, eight for *P. obducens*, ten for *Bremia lactucae* and 11 for *Plasmopara halstedii*. Likewise, a couple of *Hyaloperonospora* species show large ITS2 insertions, which may also be due to repetition of sequence fragments, but only comparatively short indels in the ITS1 region (Voglmayr 2003; Göker *et al.* 2004; Thines 2007). However, the relationship of *Hyaloperonospora* to *Plasmopara* and *Bremia* is unclear at present, even in multi-gene analyses, and is most likely not a particularly close one (Göker *et al.* 2007). However, *Plasmopara* and *Bremia* belong to a well-supported clade comprising downy mildews with pyriform to globose haustoria (Göker *et al.* 2003; Voglmayr *et al.* 2004). Hence, the disposition to develop ITS2 repeats may be to some degree evolutionary conserved in downy mildews, as in case of ITS1 repeats in *Peronospora*.

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REFERENCES

Bachofer M, 2004. *Molekularbiologische Populationsstudien an Plasmopara halstedii, dem Falschen Mehltau der Sonnenblume*. PhD thesis, University of Hohenheim, Stuttgart.

Choi Y-J, Hong S-B, Shin H-D, 2003. Diversity of the *Hyaloperonospora parasitica* complex from core brassicaceous hosts based on ITS rDNA sequences. *Mycological Research* **107**: 1314–1322.

Choi Y-J, Hong S-B, Shin H-D, 2005. A reconsideration of *Pseudoperonospora cubensis* and *P. humuli* based on molecular and morphological data. *Mycological Research* **109**: 841–848.

Constantinescu O, 1991. An annotated list of *Peronospora* names. *Thunbergia* **15**: 1–110.

Constantinescu O, Fatehi J, 2002. *Peronospora*-like fungi (*Chromista*, *Peronosporales*) parasitic on *Brassicaceae* and related hosts. *Nova Hedwigia* **74**: 291–338.

Constantinescu O, Voglmayr H, Fatehi J, Thines M, 2005. *Plasmoverna* gen. nov., and the taxonomy and nomenclature of *Plasmopara* (*Chromista*, *Peronosporales*). *Taxon* **54**: 813–821.

Cunnington JH, 2006. DNA sequence variation supports multiple host-specialised taxa in the *Peronospora viciae* complex (*Chromista: Peronosporales*). *Nova Hedwigia* **82**: 23–29.

de Bary A, 1863. Recherches sur le developpement de quelques champignons parasites. *Annales des Sciences Naturelles, Botanique, sér. 4* **20**: 5–148.

Ellison NW, Liston A, Steiner JL, Williams WM, Taylor NL, 2006. Molecular phylogenetics of the clover genus (*Trifolium – Leguminosae*). *Molecular Phylogenetics and Evolution* **39**: 688–705.

Farris JS, 1989. The retention index and the rescaled consistency index. *Cladistics* **5**: 417–419.

Felsenstein J, 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**: 368–376.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fitch WM, 1971. Towards defining the course of evolution: minimal change for a specified tree topology. *Systematic Zoology* **20**: 406–416.

Fischer MA, Adler W, Oswald K, 2005. *Exkursionsflora für Österreich, Liechtenstein und Südtirol*. Land Oberösterreich, Biologiezentrum der OÖ Landesmuseen, Linz.

Gäumann E, 1918. Über die Formen der *Peronospora parasitica* (Pers.) Fries. *Beihefte zum Botanischen Centralblatt* **35**: 395–533.

Gäumann E, 1923. Beiträge zu einer Monographie der Gattung *Peronospora* Corda. *Beiträge zur Kryptogamenflora der Schweiz* **5**: 1–360.

Göker M, 2006. Anmerkungen zur aktuellen Taxonomie der Falschen Mehltaupilze und einiger ihrer Verwandter. In: Brandenburger W, Hagedorn G (eds), *Zur Verbreitung von Peronosporales (inkl Albugo, ohne Phytophthora) in Deutschland*. Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, pp. 142–149.

Göker M, Riethmüller A, Voglmayr H, Weiß M, Oberwinkler F, 2004. Phylogeny of *Hyaloperonospora* based on nuclear ribosomal internal transcribed spacer sequences. *Mycological Progress* **3**: 83–94.

Göker M, Voglmayr H, Riethmüller A, Oberwinkler F, 2007. How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. *Fungal Genetics and Biology* **44**: 105–122.

Göker M, Voglmayr H, Riethmüller A, Weiß M, Oberwinkler F, 2003. Taxonomic aspects of *Peronosporaceae* inferred from Bayesian molecular phylogenetics. *Canadian Journal of Botany* **81**: 672–683.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696–704.

Gustavsson A, 1959a. Studies on Nordic Peronosporas. I. Taxonomic revision. *Opera Botanica* **3** (1): 1–271.

Gustavsson A, 1959b. Studies on Nordic Peronosporas. II. General account. *Opera Botanica* **3** (2): 1–61.

Hall GS, 1996. Modern approaches to species concepts in downy mildews. *Plant Pathology* **45**: 1009–1026.

Hennig W, 1965. Phylogenetic systematics. *Annual Review of Entomology* **10**: 97–116.

Huelsenbeck JP, Rannala B, Masly JP, 2000. Accommodating phylogenetic uncertainty in evolutionary studies. *Science* **288**: 2349–2350.

Huelsenbeck JP, Ronquist F, 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Kluge AG, Farris JS, 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology* **18**: 1–32.

Larget B, Simon DL, 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750–759.

Lee C, Grasso C, Sharlow M, 2002. Multiple sequence alignment using partial order graphs. *Bioinformatics* **18**: 452–464.

Legendre P, 2001. *Congruence among Distance Matrices: Program CADM User's Guide*. Département de sciences biologiques, Université de Montréal.

Legendre P, Lapointe F-J, 2004. Assessing congruence among distance matrices: single-malt scotch whiskies revisited. *Australian & New Zealand Journal of Statistics* **46**: 615–629.

Moncalvo JM, Wang HH, Hseu RS, 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia* **87**: 223–238.

Page RDM, 2003. Introduction. In: Page RDM (ed), *Tangled Trees; Phylogeny, Cospeciation, and Coevolution*. University of Chicago Press, Chicago, pp. 1–21.

Percy DM, Page RDM, Cronk QCD, 2004. Plant–insect interactions: double-dating associated insect and plant lineages reveals asynchronous radiations. *Systematic Biology* **53**: 120–127.

Posada D, Crandall KA, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Posada D, Buckley TR, 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology* **53**: 793–808.

Riethmüller A, Voglmayr H, Göker M, Weiß M, Oberwinkler F, 2002. Phylogenetic relationships of the downy mildews (*Peronosporales*) and related groups based on nuclear large subunit ribosomal DNA sequences. *Mycologia* **94**: 834–849.

Rodríguez F, Oliver JF, Martín A, Medina JR, 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142**: 485–501.

Roskov YR, Bisby FA, Zarucchi JL, Schrire BD, White RJ (eds), 2005 *ILDIS World Database of Legumes: Draft Checklist*. ILDIS, Reading version 10 (November 2005). CD-ROM.

Sorenson MD, Balakrishnan CN, Payne RB, 2004. Clade-limited colonization in brood parasitic finches (*Vidua* spp.). *Systematic Biology* **53**: 140–153.

Spring O, Haas K, Lamla I, Thurnhofer S, Vetter W, 2005. The composition and taxonomic significance of fatty acid patterns in three white rust species: *Albugo amaranthi, A. candida* and *A. tragopogonis* (*Peronosporales*, *Albuginaceae*). *Mycological Progress* **4**: 179–184.

Steele KP, Wojciechowski MF, 2003. Phylogenetic analyses of tribes *Trifolieae* and *Vicieae*, based on sequences of the plastid gene, *matK* (*Papilionoideae: Leguminosae*). In: Klitgaard BB, Bruneau A (eds), *Advances in Legume Systematics, Higher Level Systematics*, vol 10. Royal Botanic Gardens, Kew, pp. 355–370.

Swofford DL, 2002. *PAUP*: phylogenetic analysis using parsimony (*and other methods)* Version 4.0 b10. Sinauer Associates, Sunderland, MA.

Talavera S, Aedo C, Castroviejo S, Romero Zarco C, Sáez L, Salgueiro FJ, Velayos M (eds), 1999, *Flora Iberica: Plantas vasculares de la Península Ibérica e Islas Baleares, Part I*, vol 7, Real Jardín Botánico, CSIC, Madrid.

Talavera S, Aedo C, Castroviejo S, Herrero A, Romero Zarco C, Salgueiro FJ, Velayos M (eds), 2000, *Flora Iberica: Plantas vasculares de la Península Ibérica e Islas Baleares, Part II*, vol 7, Real Jardín Botánico, CSIC, Madrid.

Thines M, 2007. Characterisation and phylogeny of repeated elements giving rise to exceptional length of ITS2 in several downy mildew genera (*Peronosporaceae*). *Fungal Genetics and Biology* **44**: 199–207.

Thines M, Göker M, Oberwinkler F, 2006. A revision of *Bremia graminicola*. *Mycological Research* **110**: 646–656.

Thines M, Komjáti H, Spring O, 2005. Exceptional length of ITS in *Plasmopara halstedii* is due to multiple repetitions in the ITS-2 region. *European Journal of Plant Pathology* **112**: 395–398.

Thines M, Spring O, 2005. A revision of *Albugo* (*Chromista*, *Peronosporomycetes*). *Mycotaxon* **92**: 443–458.

Voglmayr H, 2003. Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. *Mycological Research* **107**: 1132–1142.

Voglmayr H, Fatehi J, Constantinescu O, 2006. Revision of *Plasmopara* (*Chromista*, *Peronosporales*) on *Geranium*. *Mycological Research* **110**: 633–645.

Voglmayr H, Riethmüller A, 2006. Phylogenetic relationships of *Albugo* species (white blister rusts) based on LSU rDNA sequence and oospore data. *Mycological Research* **110**: 75–85.

Voglmayr H, Riethmüller A, Göker M, Weiß M, Oberwinkler F, 2004. Phylogenetic relationships of *Plasmopara, Bremia* and other genera of downy mildews with pyriform haustoria based on Bayesian analysis of partial LSU rDNA sequence data. *Mycological Research* **108**: 1011–1024.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand H, Sninsky JS, White TJ (eds), *PCR Protocols: a Guide to Methods and Applications*, Academic Press, San Diego, pp. 315–322.

Wojciechowski MF, Sanderson MJ, Steele KP, Liston A, 2000. Molecular phylogeny of the 'Temperate Herbaceous Tribes' of papilionoid legumes: a supertree approach. In: Herendeen PS, Bruneau A (eds), *Advances in Legume Systematics*, vol 9. Royal Botanic Gardens, Kew, pp. 227–298.

Yerkes WD, Shaw CG, 1959. Taxonomy of *Peronospora* species on *Cruciferae* and *Chenopodiaceae*. *Phytopathology* **49**: 499–507.