

Phylogenetic relationships of *Plasmopara*, *Bremia* and other genera of downy mildew pathogens with pyriform haustoria based on Bayesian analysis of partial LSU rDNA sequence data

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Bayesian and maximum parsimony phylogenetic analyses of 92 collections of the genera *Basidiophora*, *Bremia*, *Paraperonospora*, *Phytophthora* and *Plasmopara* were performed using nuclear large subunit ribosomal DNA sequences containing the D1 and D2 regions. In the Bayesian tree, two main clades were apparent: one clade containing *Plasmopara pygmaea* s. lat., *Pl. sphaerosperma*, *Basidiophora*, *Bremia* and *Paraperonospora*, and a clade containing all other *Plasmopara* species. *Plasmopara* is shown to be polyphyletic, and *Pl. sphaerosperma* is transferred to a new genus, *Protobremia*, for which also the oospore characteristics are described. Within the core *Plasmopara* clade, all collections originating from the same host family except from *Asteraceae* and *Geraniaceae* formed monophyletic clades; however, higher-level phylogenetic relationships lack significant branch support. A sister group relationship of *Pl. sphaerosperma* with *Bremia lactucae* is highly supported. Within *Bremia lactucae* s. l., three distinct clades are evident, which only partly conform to the published host specificity groups. All species of the genera *Basidiophora*, *Bremia*, *Paraperonospora* and *Plasmopara* included in the present study were investigated for haustorial morphology, and all had ellipsoid to pyriform haustoria, which are regarded as a diagnostic synapomorphy of the whole clade. Aspects of coevolution and cospeciation within the downy mildew pathogens with ellipsoid to pyriform haustoria are briefly discussed.

INTRODUCTION

Until recently, hypotheses on phylogenetic relationships of downy mildew pathogens were mainly based on morphological criteria (e.g. Shaw 1981, Dick *et al.* 1989). These hypotheses suffered from both the paucity of distinct morphological features and a poor understanding of the majority of taxa which are not economically important plant pathogens. However, molecular phylogenetics opened a new era, and much progress has been gained in the re-evaluation of relationships within *Peronosporales* (Riethmüller *et al.* 2002, Göker *et al.* 2003).

Riethmüller *et al.* (2002) demonstrated that morphological features (e.g. conidiosporangiochore morphology) used in traditional classification were sometimes overemphasized or incorrectly interpreted. However, such studies also revealed the phylogenetic significance of other previously underestimated features

(Constantinescu & Fatehi 2002, Riethmüller *et al.* 2002, Göker *et al.* 2003). Haustorial morphology may be one such feature. Already in the extensive investigation of Fraymouth (1956), morphology of haustoria was shown to be characteristic for some groups, e.g. the large globose to lobate haustoria of the *Peronospora* species now classified in *Hyaloperonospora* (Constantinescu & Fatehi 2002, Göker *et al.* 2003). On the other hand, Fraymouth (1956) argued that '... the form and structure of hyphae and haustoria does not offer help in classification ...', which may be partly explained by the obsolete downy mildew classification on which she based her considerations.

In the course of their molecular and morphological studies, Constantinescu & Fatehi (2002), Riethmüller *et al.* (2002) and Göker *et al.* (2003) re-evaluated haustorial morphology of downy mildew pathogens with a new phylogenetic background. The genera *Hyaloperonospora* (Constantinescu & Fatehi 2002) and *Viennotia*

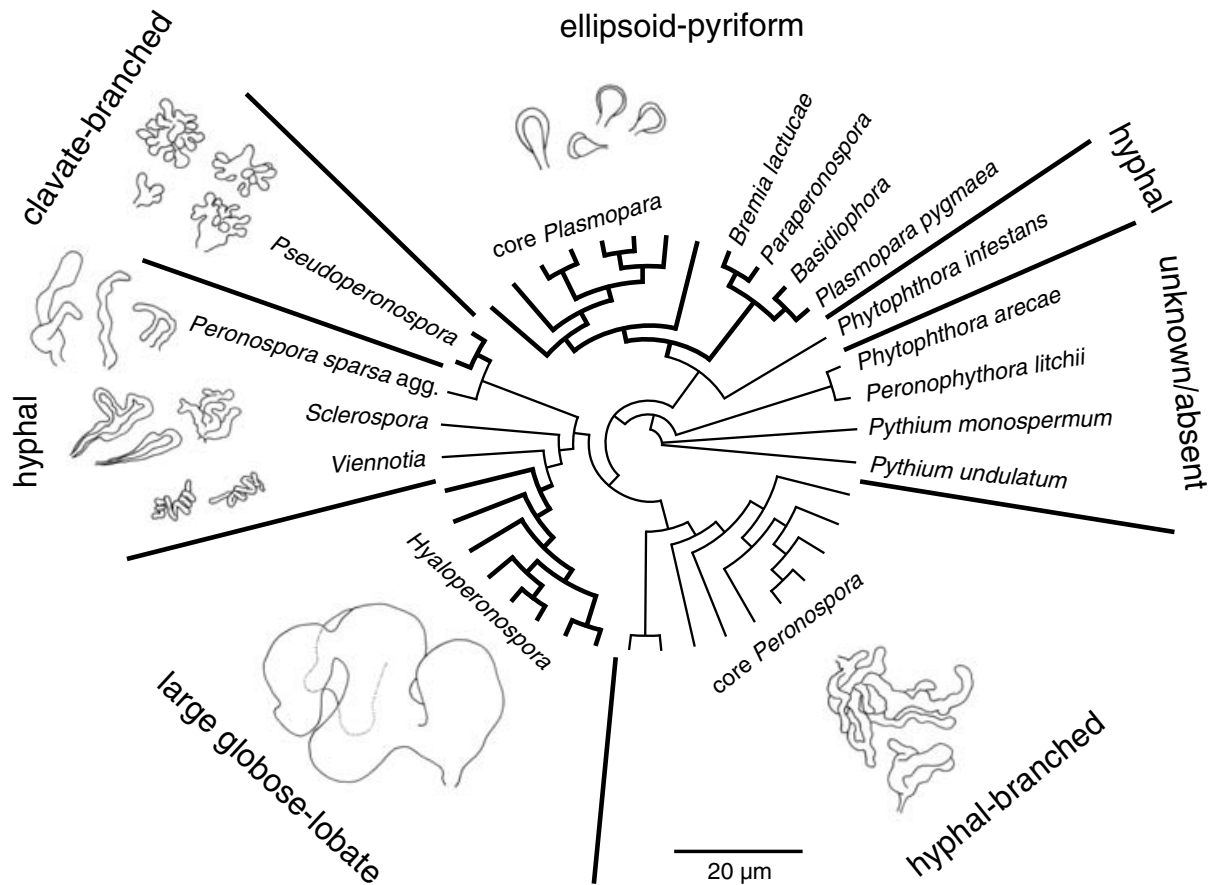


Fig. 1. Simplified phylogenetic tree based on Göker *et al.* (2003: fig. 1), with a presentation of the different haustorial types of downy mildew genera. Several taxa of the core *Peronospora* clade have been pruned from the original tree for a better graphic presentation. Branches in bold denote presumed apomorphic haustorial types. Hyphal haustoria are interpreted as a plesiomorphic character, as they are also present in some species of *Phytophthora*. Although basically hyphal (Fraymouth 1956), the haustoria of *Pseudoperonospora* are here recognised as distinct due to their characteristic clavate-branched ('digitate') shape. All drawings to the same scale and from the generic type species, except for *Plasmopara* (from *Pl. umbelliferarum*); haustoria of *Viennotia* redrawn from Göker *et al.* (2003), all others original drawings.

(Göker *et al.* 2003) were segregated from the genera *Peronospora* and *Plasmopara*, respectively, partly based on differences in haustorial morphology. On the basis of this information, two haustorial types can be supposed to be apomorphic for monophyla: large globose to lobate haustoria for the *Hyaloperonospora* clade, and ellipsoid to pyriform haustoria for a clade containing *Basidiophora*, *Bremia*, *Paraperonospora* and *Plasmopara*. The clavate-branched haustoria of *Pseudoperonospora* are basically hyphal, but could be interpreted as apomorphic. On the other hand, the hyphal haustoria of *Peronospora*, *Sclerospora* and *Viennotia* can be regarded as plesiomorphic, as this type has also been found in the genus *Phytophthora* (e.g. *Phytophthora infestans*; Erwin & Ribeiro 1996). However, data on haustoria in *Phytophthora* are insufficient to draw far-reaching conclusions, and further phylogenetic and morphological investigations are necessary. A summary of the current information on molecular phylogeny and haustorial types is shown in Fig. 1; the data on haustorial morphology are based on own, extensive original observations.

The genera with ellipsoid to pyriform haustoria (*Basidiophora*, *Bremia*, *Paraperonospora* and *Plasmopara*) have been shown to be closely related and form a monophyletic entity within the *Peronosporales* (Riethmüller *et al.* 2002, Göker *et al.* 2003). As comparatively few taxa were included in former studies, extended sampling of these genera was initiated to examine whether this pattern was consistent over a wider range of taxa. The present study is a further step towards a more natural delimitation and classification of the genera of downy mildew pathogens, and it demonstrates that many taxa are still in need of thorough morphological and molecular investigation. In addition, the present study addresses species delimitation in *Bremia* and *Plasmopara*, a problem still largely unresolved.

MATERIALS AND METHODS

Sample sources, DNA-extraction, PCR and sequencing

The organisms contained in the study are listed in Table 1. For *Phytophthora*, the nomenclature of

Table 1. Collection data and GenBank accession numbers of the taxa studied. The taxa are grouped taxonomically; the ordinal classification follows Kirk *et al.* (2001) and Dick (2001), respectively. Collectors: AR, A. Riethmüller; FO, F. Oberwinkler; HV, H. Voglmayr; KH, L. Kisimova-Horovitz; MG, M. Göker; MM, M. Mennicken; MP, M. Piepenbring; RK, R. Kirschner. Vouchers: LPB; La Paz (Bolivia); TUB, University of Tübingen; WU, University of Vienna.

Taxon	Collection data		GenBank accession no.
	Isolated from/host	Origin/source (voucher)	
<i>Peronosporales</i>			
<i>Basidiophora entospora</i>	<i>Conyza canadensis</i>	Austria: Lower Austria, Langenlois, <i>HV</i> (WU), HV123	AY035513
<i>B. entospora</i>	<i>C. canadensis</i>	France: Dept. Ain, near Bourg en Bresse, <i>HV</i> & <i>AR</i> (TUB), F8	AY250145
<i>Bremia lactucae</i>	<i>Arctium</i> sp.	Austria: Lower Austria, Merkersdorf, <i>HV</i> (WU), AR388	AY250120
<i>B. lactucae</i>	<i>Carduus nutans</i>	Austria: Vienna, <i>HV</i> (WU), HV232	AY250126
<i>B. lactucae</i>	<i>C. personata</i>	Austria: Tirol, Umbaltal, <i>HV</i> (WU), HV653	AY250124
<i>B. lactucae</i>	<i>Carlina acaulis</i>	Austria: Carinthia, Großfragant, <i>HV</i> (WU), HV482	AY250117
<i>B. lactucae</i>	<i>Centaurea cyanus</i>	Austria: Lower Austria, Theresienfeld, <i>HV</i> (WU), HV197h	AY250123
<i>B. lactucae</i>	<i>C. jacea</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV526	AY250119
<i>B. lactucae</i>	<i>C. cf jacea</i>	Germany: Baden-Württemberg, Onstmettingen, <i>HV</i> & <i>AR</i> (TUB), AR264	AY250118
<i>B. lactucae</i>	<i>C. montana</i>	Germany: Bavaria, Oberjoch, <i>HV</i> (WU), HV586	AY250132
<i>B. lactucae</i>	<i>C. cf pullata</i>	France: Dept. Hérault, SW Montpellier, between Sète and Agde, <i>HV</i> & <i>AR</i> (WU), F31	AY250121
<i>B. lactucae</i>	<i>Cirsium acaule</i>	Austria: Tirol, near Weißenbach, <i>MG</i> (TUB), MG1836, MG3	AY250122
<i>B. lactucae</i>	<i>C. arvense</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV778	AY250125
<i>B. lactucae</i>	<i>C. oleraceum</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV704	AY035507
<i>B. lactucae</i>	<i>C. vulgare</i>	Germany: Baden-Württemberg, Tübingen-Lustnau, <i>HV</i> (WU), HV835	AY250131
<i>B. lactucae</i>	<i>Crepis sancta</i>	France: Dept. Drôme, S Lyon, W Romans s/Isère, <i>HV</i> & <i>AR</i> (WU), F21	AY250136
<i>B. lactucae</i>	<i>C. taraxacifolia</i>	France: Dept. Jura, N Lons-le-Saunier, <i>HV</i> & <i>AR</i> (WU), F5	AY250140
<i>B. lactucae</i>	<i>C. taraxacifolia</i>	France: Dept. Drôme, S Lyon, between Tain-l'Hermitage and Romans s/Isère, <i>HV</i> & <i>AR</i> (WU), F11	AY250139
<i>B. lactucae</i>	<i>Hieracium cf murorum</i>	Austria: Tirol, Obertilliach, <i>HV</i> (WU), HV628	AY250128
<i>B. lactucae</i>	<i>H. murorum</i>	Austria: Carinthia, Großfragant, <i>HV</i> (WU), HV511	AY250127
<i>B. lactucae</i>	<i>H. murorum</i>	Germany: Bavaria, SW Unterjoch, <i>KH</i> (TUB), AR234	AY250129
<i>B. lactucae</i>	<i>Lactuca sativa</i>	Germany: Baden-Württemberg, <i>MP</i> 2768 (TUB), AR246	AY035511
<i>B. lactucae</i>	<i>L. sativa</i>	Austria: Vienna, Botanical Garden, <i>HV</i> (WU), HVB.1.h	AY250142
<i>B. lactucae</i>	<i>L. serriola</i>	Germany: Baden-Württemberg, Tübingen-Bebenhausen, <i>MM</i> (TUB), AR357	AY250143
<i>B. lactucae</i>	<i>Lapsana communis</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV392	AY250137
<i>B. lactucae</i>	<i>L. communis</i>	Germany: Baden-Württemberg, Tübingen, <i>RK</i> (TUB), AR183	AY250138
<i>B. lactucae</i>	<i>L. communis</i>	Germany: Baden-Württemberg, Tübingen, <i>HV</i> (TUB), AR180	AY035509
<i>B. lactucae</i>	<i>Leontodon hispidus</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV769	AY250130
<i>B. lactucae</i>	<i>Picris hieracioides</i>	Austria: Tirol, Prägraten, <i>HV</i> (WU), HV656	AY035512
<i>B. lactucae</i>	<i>Senecio vulgaris</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV750	AY035508
<i>B. lactucae</i>	<i>Sonchus asper</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV754	AY035510
<i>B. lactucae</i>	<i>S. oleraceus</i>	Austria: Vienna, Botanical Garden, <i>HV</i> (WU), HV759	AY250133
<i>B. lactucae</i>	<i>S. tenerrimus</i>	France: Dept. Hérault, SW Montpellier, between Sète and Agde, <i>HV</i> & <i>AR</i> (WU), F30	AY250134
<i>B. lactucae</i>	<i>Taraxacum officinale</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV779	AY250141
<i>B. lactucae</i>	<i>Urospermum dalechampii</i>	France: Dept. Drôme, S Lyon, between Tain-l'Hermitage and Romans s/Isère, <i>HV</i> & <i>AR</i> (WU), F14	AY250135
<i>Paraperonospora leptosperma</i>	<i>Anthemis arvensis</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV707	AY250149
<i>Pa. leptosperma</i>	<i>Matricaria recutita</i>	Germany: Baden-Württemberg, Tübingen, <i>HV</i> (WU), HV929	AY250148

Table 1. (Cont.)

Taxon	Collection data		GenBank accession no.
	Isolated from/host	Origin/source (voucher)	
<i>Pa. leptosperma</i>	<i>Tripleurospermum perforatum</i>	Austria: Upper Austria, St Willibald, <i>HV</i> (WU), HV383	AY035515
<i>Plasmopara angelicae</i>	<i>Angelica archangelica</i>	Norway: Hordaland, Ulvik, Finse, <i>HV</i> (WU), HV1099, AR410	AY250159
<i>Pl. angelicae</i>	<i>A. sylvestris</i>	Austria: Tirol, Haldensee, <i>MG</i> (TUB), MG1816, MG2	AY250155
<i>Pl. angelicae</i>	<i>A. sylvestris</i>	Germany: Bavaria, Grüntensee near Wertach, <i>AR</i> (TUB), AR198	AY250153
<i>Pl. angustiterminalis</i>	<i>Xanthium strumarium</i>	Austria: Lower Austria, Gramatneusiedl, <i>HV</i> (WU), HV562	AY178535
<i>Pl. baudysii</i>	<i>Berula erecta</i>	Austria: Lower Austria, Eckartsau, <i>HV</i> (WU), HV557	AY250147
<i>Pl. baudysii</i>	<i>B. erecta</i>	Austria: Lower Austria, Gramatneusiedl, <i>HV</i> (WU), HV571	AY035517
<i>Pl. chaerophylli</i>	<i>Anthriscus cerefolium</i>	Austria: Lower Austria, Hundsheim, <i>HV</i> (WU), HV262	AY250164
<i>Pl. chaerophylli</i>	<i>A. nitidus</i>	Austria: Lower Austria, Gießhübl, <i>HV</i> (WU), HV1045	AY250163
<i>Pl. chaerophylli</i>	<i>A. sylvestris</i>	Austria: Upper Austria, Natternbach, <i>HV</i> (WU), HV1059	AY250165
<i>Pl. densa</i>	<i>Euphrasia rostkoviana</i>	Austria: Tirol, Weißenbach, <i>MG</i> (TUB), MG2078, MG5	AY250177
<i>Pl. densa</i>	<i>Rhinanthus alectorolophus</i>	Germany: Baden-Württemberg, Tübingen-Hagelloch, <i>MG</i> (TUB), MG1823, MG6-1	AY250175
<i>Pl. densa</i>	<i>R. minor</i>	Austria: Vienna, Botanical Garden, <i>HV</i> (WU), HV72	AY250176
<i>Pl. epilobii</i>	<i>Epilobium parviflorum</i>	Austria: Upper Austria, Siegharting, <i>HV</i> (WU), HV988	AY250178
<i>Pl. geranii</i>	<i>Geranium maculatum</i>	USA: Tennessee: Knoxville, <i>HV</i> (WU), HV6.4.P.P.	AY035520
<i>Pl. geranii-sylvatici</i>	<i>G. palustre</i>	Germany: Baden-Württemberg, Tübingen, <i>MM</i> (TUB), AR276	AY250169
<i>Pl. geranii-sylvatici</i>	<i>G. sylvaticum</i>	Germany: Baden-Württemberg, Feldberg, <i>HV & AR</i> (TUB), AR285	AY250170
<i>Pl. halstedii s. lat</i>	<i>Flaveria bidentis</i>	Bolivia: Chuquisaca, Oropeza, close to Sucre, <i>MP</i> 2660 (LPB), AR179	AY178534
<i>Pl. halstedii s. lat</i>	<i>Helianthus annuus</i>	Germany: Baden-Württemberg, Tübingen-Unterjesingen, <i>AR</i> (TUB), AR151	AY035523
<i>Pl. halstedii s. lat</i>	<i>H. × laetiflorus</i>	Germany: Baden-Württemberg, Tübingen, <i>HV</i> (WU), AR272	AY178529
<i>Pl. isopyri-thalictroidis</i>	<i>Isopyrum thalictroides</i>	Austria: Lower Austria, Mannersdorf/Leitha, <i>HV</i> (WU), HV266	AY035526
<i>Pl. megasperma</i>	<i>Viola rafinesquii</i>	USA: Tennessee: Knoxville, leg. <i>HV</i> (WU), HVB.M.4.4.	AY035516
<i>Pl. mei-foeniculi</i>	<i>Meum athamanticum</i>	Germany: Baden-Württemberg, Feldberg, <i>HV & AR</i> (TUB), AR284	AY250160
<i>Pl. obducens</i>	<i>Impatiens capensis</i>	USA: Tennessee: Knoxville, <i>HV</i> (WU), HV5.4.P.O.	AY035522
<i>Pl. obducens</i>	<i>I. noli-tangere</i>	Austria: Upper Austria, Kopfing, <i>HV</i> (WU), HV306	AY250181
<i>Pl. pastinacae</i>	<i>Pastinaca sativa</i>	Austria: Lower Austria, Purkersdorf, <i>HV</i> (WU), HV1090	AY250157
<i>Pl. peucedani</i>	<i>Peucedanum palustre</i>	Germany: Baden-Württemberg, Federsee near Bad Buchau, <i>HV & AR</i> (WU, TUB), AR277	AY250154
<i>Pl. pimpinellae</i>	<i>Pimpinella major</i>	Austria: Tirol, Haldensee, <i>MG</i> (TUB), MG1864, MG1	AY250152
<i>Pl. pimpinellae</i>	<i>P. major</i>	Austria: Tirol, Obertilliach, <i>HV</i> (WU), HV634	AY035519
<i>Pl. pimpinellae</i>	<i>Pimpinella</i> sp.	Germany: Bavaria, Grüntensee near Wertach, <i>MG</i> (TUB), MG1837, MG4	AY250158
<i>Pl. pimpinellae</i>	<i>Pimpinella</i> sp.	Austria: Tirol, Schattwald, <i>MG</i> (TUB), MG1838, MG7	AY250156
<i>Pl. pusilla</i>	<i>G. eranium pratense</i>	Germany: Baden-Württemberg, Tübingen, <i>MG</i> (TUB), MG1863, MG5-2	AY250166
<i>Pl. pusilla</i>	<i>G. pratense</i>	Austria: Styria, Spital/Semmering, <i>HV</i> (WU), HV619	AY250168
<i>Pl. pusilla</i>	<i>G. pratense</i>	Germany: Baden-Württemberg, Calw, <i>AR</i> (TUB), AR200	AY250167
<i>Pl. pusilla</i>	<i>G. pratense</i>	Germany: Baden-Württemberg, Tübingen, <i>MG</i> (TUB), MG1861, MG8-10	AY035521
<i>Pl. sp.</i>	<i>G. sylvaticum</i>	Norway: Hordaland, Ulvik, Finse, <i>HV</i> (WU), AR399	AY250171
<i>Pl. sp.</i>	<i>G. sylvaticum</i>	Austria: Carinthia, Flattach, <i>HV</i> (WU), HV473	AY250172
<i>Pl. pygmaea</i>	<i>Anemone nemorosa</i>	France: Dept. Jura, between Dôle and Lons-le-Saunier, <i>HV & AR</i> (TUB), F2	AY250183
<i>Pl. pygmaea</i>	<i>A. ranunculoides</i>	Austria: Vienna, Prater, <i>HV</i> (WU), HV295	AY250146
<i>Pl. pygmaea</i>	<i>A. ranunculoides</i>	Germany: Baden-Württemberg, Tübingen-Bebenhausen, <i>AR</i> (TUB), AR86	AF119605
<i>Pl. pygmaea</i>	<i>Hepatica nobilis</i>	Austria: Lower Austria, Merkersdorf, <i>HV</i> (WU), AR387	AY250182

Table 1. (Cont.)

Taxon	Collection data		GenBank accession no.
	Isolated from/host	Origin/source (voucher)	
<i>Pl. sii</i>	<i>Sium latifolium</i>	Austria: Lower Austria, Marchegg, <i>HV</i> (WU), HV550	AY035518
<i>Pl. skvortzovii</i>	<i>Abutilon theophrasti</i>	Croatia: vicinity of Zagreb, Cvjetkovice (WU), AR306	AY250179
<i>Pl. solidaginis</i>	<i>Solidago virgaurea</i>	Norway: Hordaland, Ulvik, Finse, <i>HV</i> (WU), HV1093, AR409	AY250144
<i>Pl. sphaerosperma</i>	<i>Tragopogon orientalis</i>	Austria: Lower Austria, Gießhübl, <i>HV</i> (WU), HV1049	AY250150
<i>Pl. sphaerosperma</i>	<i>T. orientalis</i>	Germany: Baden-Württemberg, Onstmettingen, <i>HV</i> & <i>AR</i> (WU, TUB), AR260	AY250151
<i>Pl. umbelliferarum</i>	<i>Aegopodium podagraria</i>	Germany: Bavaria, Grüntensee near Wertach, <i>AR</i> (TUB), AR230	AY250161
<i>Pl. umbelliferarum</i>	<i>A. podagraria</i>	Germany: Bavaria, Oberjoch, <i>FO</i> (TUB), AR233	AY250162
<i>Pl. umbelliferarum</i>	<i>A. podagraria</i>	Germany: Baden-Württemberg, Tübingen-Bebenhausen, <i>AR</i> (TUB), AR83	AF119604
<i>Pl. viticola</i>	<i>Vitis vinifera</i>	Germany: Baden-Württemberg, Freiburg, <i>AR</i> , AR391	AY250173
<i>Pl. viticola</i>	<i>V. vinifera</i>	Austria: Vienna, Botanical Garden, <i>HV</i> (WU), HV225	AY250174
<i>Pl. viticola</i>	<i>V. vinifera</i>	Germany: Baden-Württemberg, Tübingen, <i>RK</i> (TUB), AR160	AY035524
<i>Pl. wildemammiana</i>	<i>Hypoestes</i> sp.	China: Yunnan: Shilin, <i>RK</i> (TUB), Roki 1043, AR324	AY250180
Pythiales			
<i>Phytophthora infestans</i>	<i>Solanum tuberosum</i>	CBS560.95, AR69	AF119602
<i>Ph. nicotianae</i>	<i>Nicotiana tabacum</i>	CBS305.29, AR238	AY250184

Erwin & Ribeiro (1996) was used. The species of the *Peronosporales* were determined principally using Brandenburger (1985) and classified using Dick (2001). The DNA extraction, PCR, cycle sequencing and sequencing of the nuclear large subunit D1/D2 region are described in Riethmüller *et al.* (2002).

Data analysis

The sequence alignment was produced with the aid of the MEGALIGN module of the LASERGENE System (DNASTAR, Inc.) and visually checked and refined with Se-AL version 2.0 (Rambaut 1996). Markov chain Monte Carlo analyses (MCMC; Mau, Newton & Larget 1999, Larget & Simon 1999) were performed with the computer program MRBAYES (version 3.0b4; Huelsenbeck & Ronquist 2001) based on the general time-reversible model of DNA substitution, additionally assuming a proportion of invariant sites with gamma-distributed substitution rates of the remaining sites (GTR+I+G; Swofford *et al.* 1996). Four incrementally heated simultaneous Markov chains were run over one million generations from which every 100th tree was sampled. The trees before apparent stationary probability distribution of the cold chain were discarded (usually the first 1000 stored trees). A 50% majority rule consensus of the remaining trees, including also compatible groupings below 50%, was computed to obtain estimates for the probabilities that groups are monophyletic given the sequence data (posterior probabilities). Branch lengths were computed as mean values over the sampled trees. To confirm that the posterior probability distribution of the MCMC processes is stationary (Huelsenbeck *et al.* 2002), the Bayesian analysis was repeated four times on a personal computer, always starting with random trees and default parameter values of the program. One of these analyses was run over five million generations.

To test the reliability of MCMC analysis, also a maximum parsimony (MP) analysis was performed with PAUP* (Swofford 2002), using 1000 replicates of heuristic search with random addition of sequences and subsequent SPR branch swapping (MULTREES option in effect, steepest descent option not in effect), each replicate limited to 100 000 rearrangements. Gaps were treated as fifth state; a cost of 2 was assigned to transversions and a cost of 1 to transitions and gap insertions. Bootstrap analysis with 1000 replicates was performed in the same way, but using 10 rounds of random sequence addition and subsequent branch swapping during each bootstrap replicate.

The phylogenetic trees were rooted with *Phytophthora infestans* and *Ph. nicotianae* according to an unpublished molecular phylogenetic analysis using a large sampling of D1/D2 sequences of *Peronosporales* and *Pythiales*, respectively.

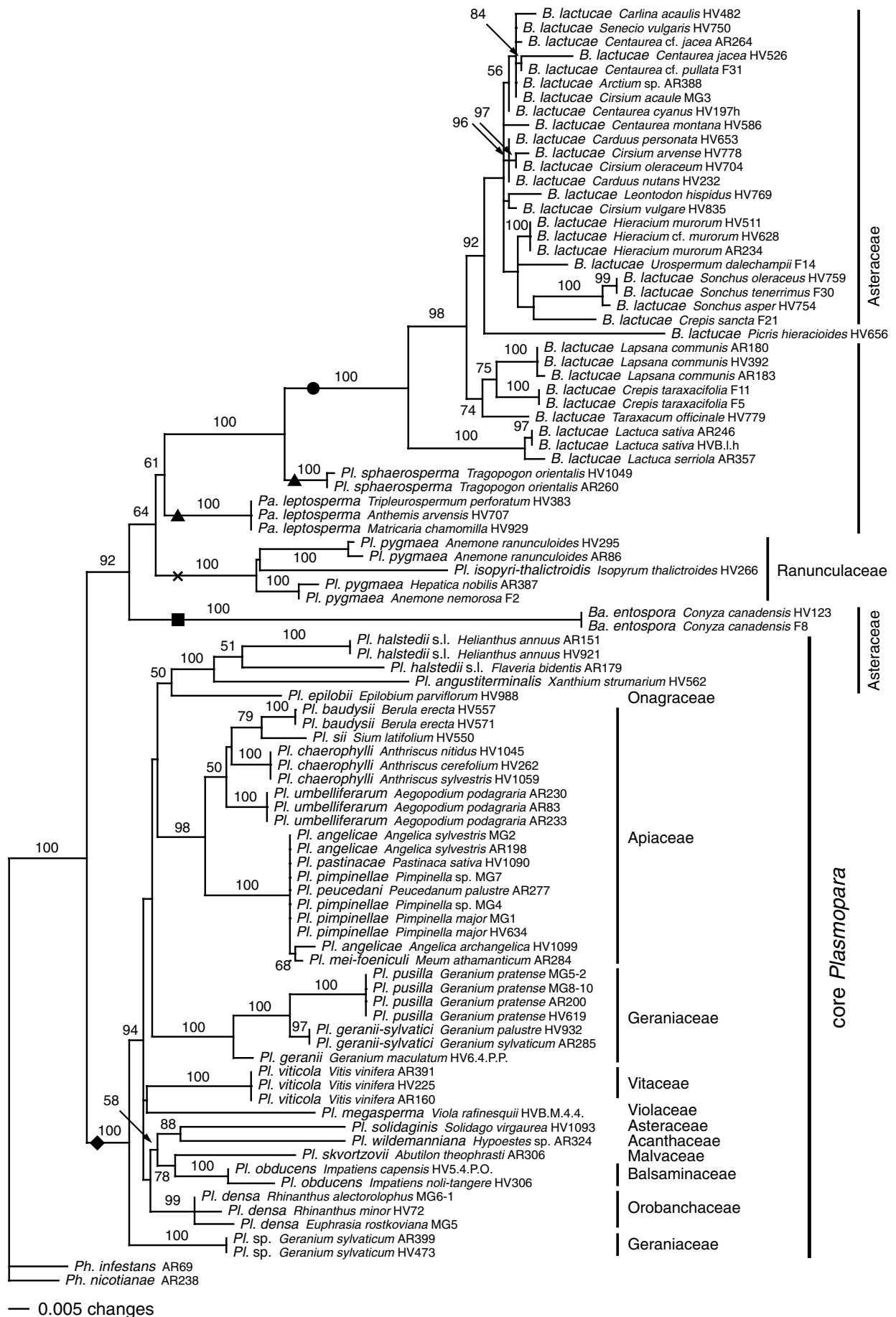


Fig. 2. For legend see opposite page.

RESULTS

The final alignment and the trees obtained were deposited in TreeBASE (<http://www.treebase.org>) and are available under study accession no. 1102. Tree topologies of the MCMC and MP majority-rule consensus trees correspond well (Figs 2–4) except for the position of *Basidiophora entospora*, which is the sister taxon to *Plasmopara angustiterminalis* in the MP tree (Fig. 4). However, this position in the MP tree is only very weakly supported (bootstrap value of 54%), and bootstrap support for the backbone of the resulting core *Plasmopara-Basidiophora* clade was below 50%. After removal of *Basidiophora*, bootstrap support for the core *Plasmopara* clade raised above 70% with the same settings (data not shown).

In the MCMC tree, two highly supported subclades are present: the core *Plasmopara* clade and a clade containing the other genera including *Pl. pygmaea* and *Pl. sphaerosperma* (Fig. 2). Therefore, according to the present analysis the genus *Plasmopara* is parayphyletic. Sister group relationship of *Pl. sphaerosperma* and a monophyletic *Bremia lactucae* s. l. is highly supported in both the MP and MCMC trees. Within *B. lactucae*, three major clades are evident (Fig. 3), of which two solely contain collections from tribe *Lactuceae* (clades 1 + 2), whereas the third contains hosts belonging to the tribes *Lactuceae*, *Cardueae* and *Senecioneae* (clade 3). Within the three main clades, some but not all of the host specific groups defined by Skidmore & Ingram (1985) form highly supported clades (Fig. 3).

Within the *Plasmopara pygmaea* clade, three genetically distinct subgroups are present: collections from *Anemone ranunculoides*, collections from *Anemone nemorosa* and *Hepatica nobilis*, respectively, and *Pl. isopyri-thalictroidis* from *Isopyrum thalictroides*.

Within the highly supported core *Plasmopara* clade of the MCMC analysis, reliability as inferred from the 50% majority rule consensus tree is low (Fig. 2). Although the sporangiophore morphology is quite variable within the core *Plasmopara* clade, ranging from almost unbranched to highly branched, it does not appear to be a phylogenetically informative character as there is no association between tree topology and sporangiophore morphology. Likewise, no pattern of cospeciation concerning host phylogeny above the family level is obvious. Most of the collections originating

from the same host families form monophyletic lineages; exceptions are collections from *Asteraceae* and *Geraniaceae*, which may be polyphyletic. Within most well supported *Plasmopara* clades, distinct subclades are present.

The *Plasmopara* clade from *Apiaceae* is highly supported in the MCMC tree, within which four distinct, well supported lineages are present (Fig. 2): *Pl. umbelliferarum*, *Pl. chaerophylli*, the *Pl. sii-Pl. baudysii* clade and a clade containing all other collections. Within the latter, almost all D1/D2 sequences were identical irrespective of the host.

Plasmopara from *Geraniaceae* did not form a monophyletic lineage but appeared in two separate clades which were highly supported in the MCMC analysis. One clade contained *Pl. geranii* from North America and the closely related *Pl. pusilla* and *Pl. geranii-sylvatici* from Europe. The species of the second clade, also originating from Europe, has not been possible to identify with the available literature and may represent a new undescribed species.

Even if *Pl. sphaerosperma* is excluded from consideration, the *Plasmopara* species on *Asteraceae* remain polyphyletic due to the position of *Pl. solidaginis*. However, the other species on *Asteraceae* are united in a highly supported clade in the MCMC analysis.

Most of the isolates of the present study were of Central European origin. In the few cases where collections from highly distant localities were sequenced (*Plasmopara obducens* from Austria and eastern USA; *Plasmopara* sp./*Geranium sylvaticum* from Norway and Austria), the sequences were very similar, and they formed highly supported clades.

Haustorial morphology

All taxa included in the present study were investigated for haustorial morphology. All showed ellipsoid to pyriform haustoria, confirming the results of the extensive investigations of Fraymouth (1956).

DISCUSSION

The molecular data of this study offer new insights into the delimitation and phylogenetic relationships of *Plasmopara*, *Bremia* and allied genera which are partly congruent with morphology (Fig. 2). In

Fig. 2. Bayesian phylogenetic analysis using Markov chain Monte Carlo (MCMC) of 92 specimens of *Peronosporales* based on nuclear D1/D2 sequences (LSU rDNA). 50% majority rule consensus tree from an MCMC analysis over five million generations, in which every 100th tree was sampled, discarding the first 1000 sampled trees; branch lengths are averaged over the sampled trees. Numbers on branches are estimates for posterior probabilities for the monophyly of the respective clades. The hosts and collection numbers of all accessions are given in smaller font; host families are given on the right. Sporangiophore characters given on the branches: ●, regularly dichotomous (*Bremia*), ▲, irregularly (anisotomously) dichotomous (*Paraperonospora*, *Protobremia*), ×, monopodial with short, reduced side-branches (*Plasmopara pygmaea* group), ■, unbranched with swollen sporangiophore tip (*Basidiophora*), ◆, monopodial, ranging from almost unbranched to much-branched (core *Plasmopara*). B., *Bremia*; Ba., *Basidiophora*; Pa., *Paraperonospora*; Ph., *Phytophthora*; Pl., *Plasmopara*.

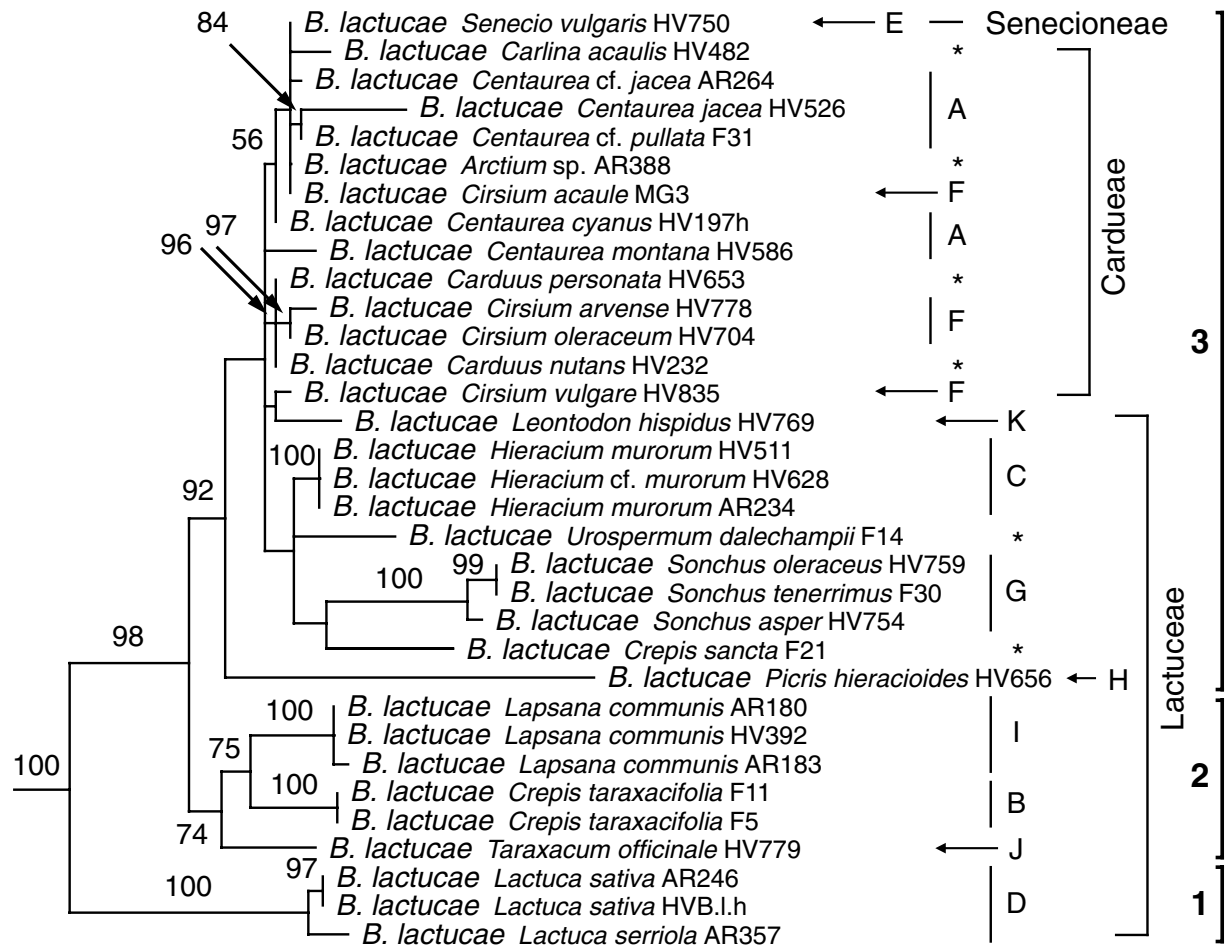


Fig. 3. Detail from Fig. 2 showing the three distinct main clades (1–3) and the different host specific groups (A–K) of *Bremia lactucae* according to Skidmore & Ingram (1985). Species marked with an asterisk have not been included in Skidmore & Ingram (1985) and cannot therefore be attributed to one of these groups. Host tribes (*Lactuceae*, *Cardueae* and *Senecioneae*) are given according to Bohm & Stuessy (2001).

addition, the data contribute to a better understanding of coevolution and cospeciation in downy mildew pathogens.

Molecular phylogeny

MCMC tree topologies of the present study agree well with those of Riethmüller *et al.* (2002) and Göker *et al.* (2003). The conidiosporangioophores within the clade are very variable; they are either unbranched (*Basidiophora*), more or less monopodially branched (*Plasmopara*), or more or less dichotomously branched (*Paraperonospora*, *Bremia*) (Fig. 2). It should be noted that interpretation of conidiosporangioophore morphology as either monopodial or dichotomous is sometimes difficult and equivocal, which led to different hypotheses on phylogenetic relationships (e.g. for *Paraperonospora*: Skalický 1966, Constantinescu 1989; for *Bremiella*: Constantinescu 1979, Riethmüller *et al.* 2002). Therefore, conidiosporangioophore morphology cannot be considered to be a phylogenetically informative character for characterisation of the whole clade (Riethmüller *et al.* 2002). However, all members of this

clade share ellipsoid to pyriform haustoria as a synapomorphy (Fig. 1).

The differences in the position of *Basidiophora entospora* between the MCMC (Fig. 2) and the MP trees (Fig. 4) may be caused by long branch attraction, as *Basidiophora* has a high number of substitutions not shared with other taxa. In the Bayesian and maximum likelihood analyses of Göker *et al.* (2003), which additionally include the D3 and the D7/D8 regions of the LSU rDNA, topological position of *Basidiophora* is similar to the present MCMC trees, which indicates that the MCMC topology may be more reliable. However, additional DNA regions should be analysed to clarify the phylogenetic position of *Basidiophora*.

Bremia

The sequences of the *Bremia lactucae* isolates sequenced in the present study indicate three phylogenetically distinct clades (Fig. 3). The majority of hosts of *B. lactucae* belong to two tribes of *Asteraceae*: *Lactuceae* and *Cardueae*, which are considered to be closely related (Bohm & Stuessy 2001). The fact that the closest

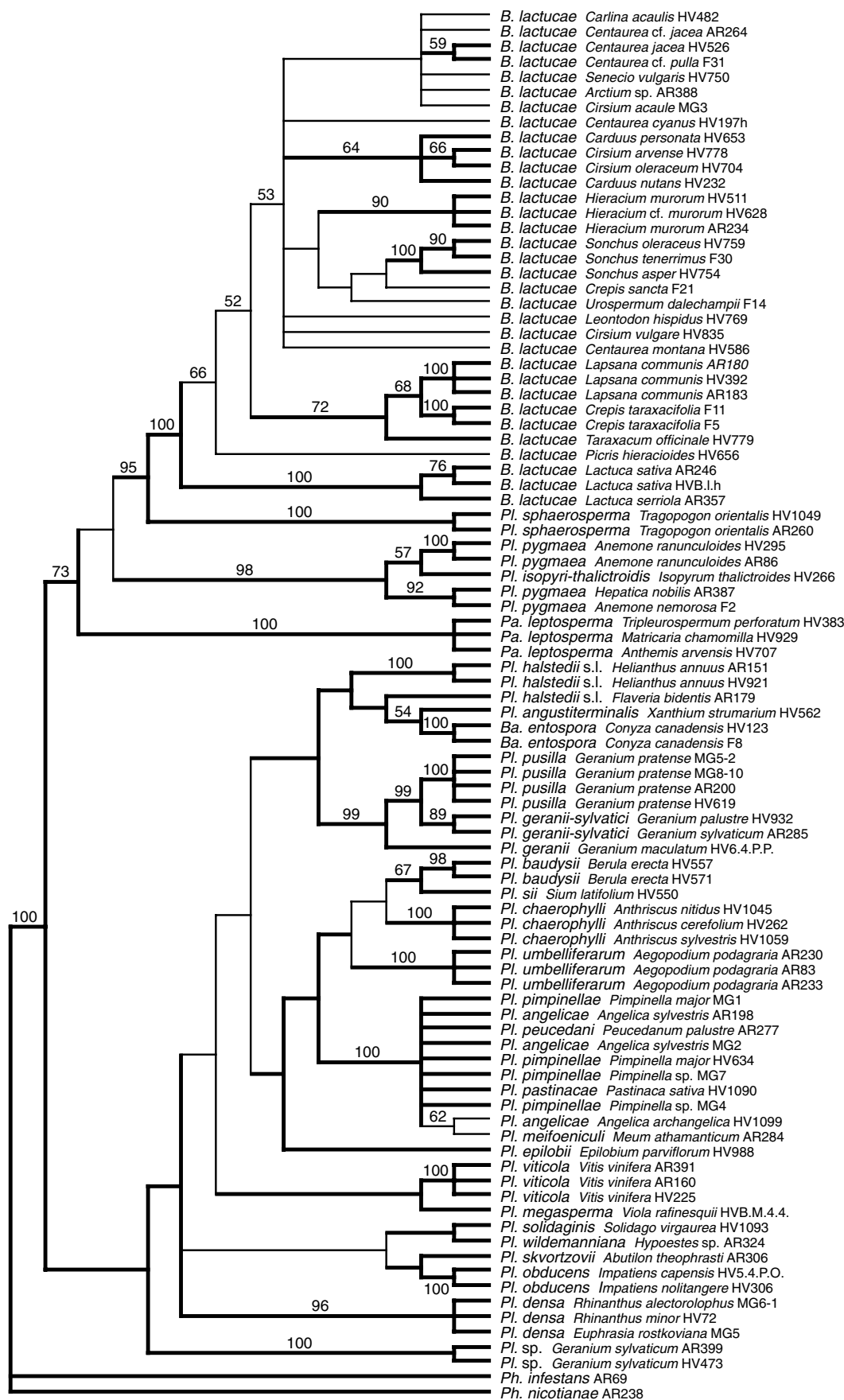
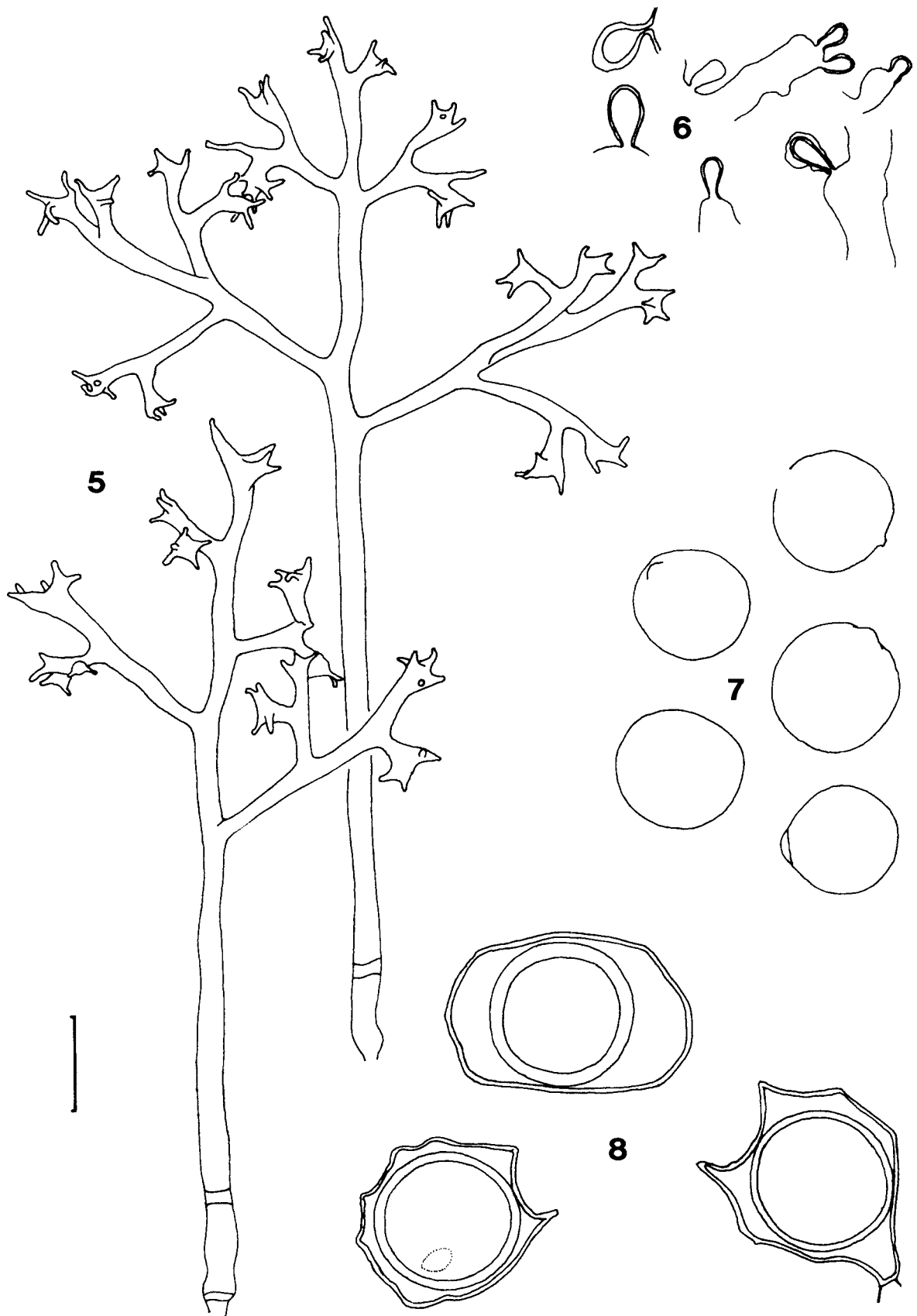


Fig. 4. Maximum parsimony majority-rule consensus tree of 234 744 most parsimonious trees (score = 899) recovered from 1000 replicates of heuristic search with random addition of sequences and subsequent SPR branch swapping, each replicate limited to 100 000 rearrangements; bold nodes are present in the strict consensus tree. Bootstrap values are given above branches.



Figs 5–8. Line drawings of *Protobremia sphaerosperma* (HV1049). **Fig. 5.** Sporangiohores; note the side branches gradually tapering towards their points of insertion, the usually dichotomous branching of the terminal branches with rounded angles, and the somewhat inflated tips of the terminal branches bearing more or less irregularly arranged sterigmata. **Fig. 6.** Pyriform haustoria. **Fig. 7.** Thin-walled, globose to subglobose sporangia with often inconspicuous apical papilla. **Fig. 8.** Aplerotic oogonia with oospores. Bars Fig. 5 = 40 μm ; and Figs 6–8 = 20 μm .

relative of *B. lactucae*, *Plasmopara sphaerosperma*, also infects a member of the *Lactuceae* (*Tragopogon orientalis*), indicates that evolutive radiation of *B. lactucae* started on hosts of tribe *Lactuceae* and quite recently switched to hosts belonging to the *Cardueae* (*Arctium*, *Carduus*, *Carlina*, *Centaurea*, *Cirsium*) and *Senecioneae* (*Senecio*) (Fig. 3). As discussed in detail below, *Pl. sphaerosperma* shows a mixture of *Plasmopara*/*Paraperonospora*-like and *Bremia*-like traits and may be morphologically close to the ancestor of *Bremia*.

Within the *Bremia lactucae* complex, several species have been described (Skidmore & Ingram 1985). However, after closer investigations, Skidmore & Ingram (1985) stated that there was an absence of clear-cut morphological differences between collections from different asteraceous host genera despite the high host specificity observed. This led Skidmore & Ingram (1985) to propose eleven special forms according to the host specificities derived from their extensive cross infection tests. Several of these special forms formed highly supported clades in the present analysis that, considering the genetic distances, might consist of genetically distinct lineages (Fig. 3, groups B, C, D, G, H, I, J) and could be regarded as distinct species. As divergences of the D1/D2 sequences within clade 3 generally were low, genes with higher substitution rates should be sequenced to test whether a finer resolution in the phylogenetic relationships can be obtained.

Most importantly from a plant pathological perspective, collections from *Lactuca* were genetically distant from the isolates from other hosts. Such genetic isolation suggests a lack of interbreeding, and infected *Asteraceae* other than *Lactuca* are therefore unlikely to be a source of inoculum for infections of *Lactuca* spp.

Plasmopara

As previously suggested (Göker *et al.* 2003), the genus *Plasmopara* does not appear to be monophyletic, since in our analysis the type species, *Pl. pygmaea*, is not contained within the highly supported core *Plasmopara* group (Fig. 2). In addition, *Pl. sphaerosperma* appears more closely related to *Bremia* and should consequently be redesignated. As previously suggested (Riethmüller *et al.* 2002, Göker *et al.* 2003), *Bremiella* is polyphyletic within *Plasmopara*; its members are therefore listed under their *Plasmopara* binomials in the present study.

Plasmopara pygmaea deviates from most other members of the genus by its short, little-branched sporangiophores. In addition, its zoosporangia were reported not to germinate to produce zoospores, but their protoplasmic contents escape the zoosporangium and germinate directly with a germ tube (De Bary 1863, Schröter 1889), which was regarded as an important distinguishing feature (Wilson 1907a). However, germination has later been recorded to be variable (either zoospores, a plasma or hyphal) in most genera with apically poroid sporangia (Waterhouse 1973), casting

fundamental doubts on the usefulness of sporangial germination as a distinguishing character.

The present tree topology raises major nomenclatural problems, as *Pl. pygmaea* has been chosen as type species for *Plasmopara* (Wilson 1907a). Retaining *Plasmopara* only for *Pl. pygmaea* and its closest relatives, and renaming all species of the core *Plasmopara* clade *Rhysothea* (Wilson 1907b) is an option. However, this would be highly undesirable for practical reasons, as all economically important *Plasmopara* species would have to be transferred. If the distinct position of *Pl. pygmaea* is confirmed in future studies, a change of the lectotype species for *Plasmopara* will be necessary to retain the generic name for the majority of *Plasmopara* species.

The *Plasmopara pygmaea* clade raises additional nomenclatural complications as three genetically distinct subgroups are revealed by our data. According to Săvulescu & Săvulescu (1951), collections from *Anemone ranunculoides*, *A. nemorosa* and *Isoopyrum thalictroides* also differ morphologically, and they proposed naming the species *Pl. anemones-ranunculoidis*, *Pl. anemones-nemorosae* and *Pl. isopyri-thalictroidis*, respectively. However, according to our investigations, *Pl. pygmaea* has no extant type specimen, and as Unger (1833) gives *Anemone ranunculoides*, *A. nemorosa* and *A. hepatica* (syn. *Hepatica nobilis*) as hosts, it is unclear which lineage should be named *Pl. pygmaea* before a neotype specimen and type host are proposed. As a consequence, one of these two taxa described for *Anemone* is invalid, depending on neotype choice. Therefore, only *Pl. isopyri-thalictroidis* is accepted in our study as a separate species. Further extensive investigations of additional collections are necessary before nomenclatural conclusions can be drawn.

Within the core *Plasmopara* clade, basic relationships remain uncertain due to the lack of branch support, and more sequence data may be necessary to obtain a better resolution. Remarkably, in the MCMC analysis (Fig. 2) collections originating from hosts that belong to the same host family form highly supported monophyletic lineages in many cases. In addition, within these, collections from different host genera or groups are often separated in genetically distinct subclades, indicating evolutive radiation in combination with high host specificity. This is an argument against the often proposed species lumping (e.g. Yerkes & Shaw 1959), i.e., recognising only one parasite species per host family.

The four well-supported, distinct *Plasmopara* clades from apiaceous hosts suggest the presence of more than one *Plasmopara* species on *Apiaceae*. This is corroborated by distinct sporangiophore morphology in some cases (*Pl. sii*, *Pl. baudysii*), and closer investigations revealed morphological differences also in the other species that are well separated in the tree. However, in the largest clade containing most collections from *Apiaceae*, most sequences were identical, and the accessions were morphologically similar. Although this

does not necessarily imply conspecificity, it is evident that these collections are closely related. Further studies are needed to elucidate this problem.

The *Plasmopara* species from *Apiaceae* would be an interesting group for assessment of cospeciation of downy mildews with their hosts. However, molecular phylogenies of the large and morphologically complex *Apiaceae* are still fragmentary (e.g. Downie *et al.* 1998, Downie, Katz-Downie & Spalik 2000a, Downie, Katz-Downie & Watson 2000b, Spalik, Reduron & Downie 2004), and some genera are in need of revision as they contain unrelated species. In addition, not all of the host species of the present study have been included in these investigations. In the present study, parasites of closely related host genera form highly supported clades: *Pl. baudysii* and *Pl. sii* from closely related *Berula* and *Sium* (Downie *et al.* 2000a), and *Pl. angelicae*, *Pl. pastinaceae*, *Pl. peucedani* and *Pl. pimpinellae* from the closely related 'Pimpinella' and 'Angelica' host clades of Downie *et al.* (2000a). However, congruence of host and parasite phylogenies cannot yet be evaluated at a higher level due to the lack of significant branch support of the tree backbones of the parasite phylogenies (Figs 2 and 4). Like in the higher taxonomic levels, it is likely that cospeciation is mostly biochemical rather than strictly co-phylogenetic (Dick 2002).

Interestingly, the *Plasmopara* collections from *Geraniaceae* do not form a monophyletic lineage (Figs 2 and 4). Traditionally, two species have been recognised on *Geraniaceae*: *Pl. pusilla*, a little-branched European species with two to three very short branches per sporangiophore, and *Pl. geranii*, a North American species with two to three prominent side-branches. Morphologically, both species are easily distinguished, and according to our analyses are closest relatives. Recently, Constantinescu (2004) demonstrated that *Pl. pusilla* should be further split into two species, *Pl. pusilla* and *Pl. geranii-sylvatici*, which is supported by the present study. However, two collections from *Geranium sylvaticum* originating from the Austrian Alps and Norway were consistently placed outside this highly supported *Pl. pusilla-Pl. geranii* clade. Closer morphological analysis showed that the sporangiophores of these collections were similar to *Pl. geranii*, but significantly distinct from *Pl. pusilla* and *Pl. geranii-sylvatici*. As it has not been possible to identify these collections with the available literature, they may represent an additional, yet unnamed European *Plasmopara* species. However, additional investigations are necessary to clarify the status of these collections.

The present investigation reveals some interesting results concerning the phylogeny of downy mildew pathogens infecting the *Asteraceae*. Săvulescu (1941) accepted three entities, which are morphologically distinct: *Plasmopara halstedii*, which is parasitic on many host genera and has a primarily American distribution, *Plasmopara sphaerosperma* on *Tragopogon* spp. and *Plasmopara savulescui* (as *Pl. megasperma*) on

Scorzonera spp., which are both European species. The present analysis not only confirms a clear distinction of at least *Pl. halstedii* s. l. and *Pl. sphaerosperma*, but also indicates that the *Plasmopara* species parasitic on *Asteraceae* are polyphyletic. The core *Pl. halstedii* group is embedded within the core *Plasmopara* cluster and forms a highly supported group in MCMC analysis (Fig. 2). However, *Pl. solidaginis*, a European species separated from *Pl. halstedii* by Novotelnova (1962), is not a member of the *Pl. halstedii* clade, although a closer relationship cannot be ruled out at present. In contrast, *Pl. sphaerosperma* is not even a member of the core *Plasmopara* group but more closely related to *Bremia*, a relationship highly supported by posterior probabilities of 100%.

Interestingly, *Pl. sphaerosperma* has long been misidentified as *Bremia lactucae* (Săvulescu 1941); however, microscopically it is distinct by superficially *Plasmopara*-like branching of the sporangiophore, which was interpreted as monopodial by Săvulescu (1941). Therefore, Săvulescu (1941) proposed the name *Pl. sphaerosperma* for collections originating from *Tragopogon*. However, closer investigation of its sporangiophore morphology reveals an intermediate position between *Paraperonospora* and *Bremia*, which is fully consistent with the MCMC analysis (Fig. 2). Close resemblance in branching patterns between *Pl. sphaerosperma* and *Paraperonospora leptosperma* has already been noted by Majewski (1968). Branches are gradually tapering towards their insertion points, which is considered to be characteristic of the genus *Paraperonospora* (Constantinescu 1989). On the other hand, the terminal branches rather resemble *B. lactucae*; however, the swellings of the terminal branches are not that prominent and the sterigmata not as regularly inserted as in *Bremia*. In addition, branching in *Bremia* is quite regularly dichotomous, whereas in *Pl. sphaerosperma* only the terminal branches are more or less dichotomously branched. Conidiosporangia of *Pl. sphaerosperma* deviate from *Paraperonospora* in being poroid. Due to this combination of features, *Pl. sphaerosperma* can neither be placed in *Bremia* nor in *Paraperonospora* nor should it be retained in *Plasmopara*. Therefore, the new genus *Protobremia* is proposed for *Pl. sphaerosperma*. On the basis of the illustrations and host range, *Plasmopara savulescui* may also belong to *Protobremia*; however, as it was not possible to obtain material of that species, a new combination is premature before sufficient morphological and molecular data are available.

Coevolution of parasites and hosts

The data of the present analysis confirm that species parasitising the same host families are usually (but not always) closely related (Fig. 2). On the other hand, due to the lack of significant branch support of the backbone of the phylogenetic trees (Figs 2 and 4), host-parasite patterns remain obscure at taxonomic

hierarchies above the host family level. However, it is unlikely that a pattern of a co-phylogenetic coevolution can be observed above family level, as the downy mildew pathogens are supposed to be substantially younger, in evolutionary terms, than their host families (see Dick 2002). Therefore, Dick (2002) proposed a biochemical rather than a co-phylogenetic coevolution, restricting the parasitic genera to host families and genera with similar biochemical pathways. It should be noted that most of the biodiversity of the 'ellipsoid-pyriform haustoria' clade is concentrated within the highly evolved asterids, especially the euasterids II of Savolainen *et al.* (2000), namely the *Asteraceae* and *Apiaceae*. The genera *Basidiophora*, *Benua* (not contained in our study, but also with ellipsoid-pyriform haustoria and putatively a member of the clade), *Bremia sensu stricto*, *Paraperonospora* and *Protobremia* are confined to *Asteraceae*. Likewise, in *Plasmopara* adaptive radiation and speciation is concentrated in the families *Asteraceae* and *Apiaceae*. The rest of *Plasmopara* species is quite randomly scattered throughout the eudicots of Savolainen *et al.* (2000), and usually only one to very few host genera per host family are affected. The reasons for this concentration of genera and species within the euasterids are unclear, but may correspond with biochemical pathways of secondary metabolites of the hosts (Dick 2002).

TAXONOMY

Protobremia Voglmayr, Riethmüller, Göker, Weiß & Oberw., **gen. nov.**

Ramificatio sporangiophori similis Paraperonosporae, sed differt a Paraperonospora ramulis terminalibus inflatis et sporangiis papillatis. Differt a *Bremia* ramificatione non isotoma-dichotoma et ramulis terminalibus minus vesicariis regularibusque. Ramificatio ultima plerumque dichotoma angulis rotundatis.

Typus: Protobremia sphaerosperma (Săvulescu) Voglmayr, Riethmüller, Göker, Weiß & Oberw., **comb. nov.** [Basionym: *Plasmopara sphaerosperma* Săvulescu, *Bull. Sect. Scient. Acad. Roum.* **24**: 65–67 (1941); *Type: Romania: Ilfov Co.: Cățelu, on Tragopogon dubius*, 10 Apr. 1927, *T. Săvulescu* (BUCM 775 – **lectotypus hic designatus**).] (Figs 5–8)

Branching of the sporangiophore similar to *Paraperonospora*, but differing from *Paraperonospora* in the somewhat inflated terminal branches and the papillate sporangia. Differing from *Bremia* in non isotomously dichotomously branching sporangiophores and the less vesiculose and less regular terminal branches. Branching of the highest order usually dichotomous; angle rounded.

Since oogonia and oospores have not been reported for *Pr. sphaerosperma* before, these are described and illustrated as follows from collection HV1049 (Fig. 8): Oogonia produced in senescent leaves, irregular, often mutually compressed, brownish, with wrinkled,

1–1.5 µm thick wall, 35–55 µm in diam.; oospores aplerotic, light yellowish, globose, 28–35 µm in diam., with smooth, 2–3 µm thick wall.

Additional authentic specimens examined: Italy: Parma, Botanical Garden, on *Tragopogon dubius*, May 1880, *G. Passerini* [*Erb. critt. ital. ser. 2*, no. 985 (BUC)]. – **Switzerland:** *Canton de Neuchâtel:* Perreux sur Boudry, on *T. pratensis*, 16 Sept. 1933, *E. Mayor* (BUCM 776).

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