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RESEARCH ARTICLE

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Molecular phylogenetic analyses reveal a close evolutionary relationship between *Podosphaera* (*Erysiphales*: *Erysiphaceae*) and its rosaceous hosts

S. Takamatsu[1], S. Niinomi[1], M. Harada[1], M. Havrylenko[2]

Key words

28S rDNA
evolution
ITS
molecular clock
phylogeny
powdery mildew fungi
Rosaceae

Abstract *Podosphaera* is a genus of the powdery mildew fungi belonging to the tribe *Cystotheceae* of the *Erysiphaceae*. Among the host plants of *Podosphaera*, 86 % of hosts of the section *Podosphaera* and 57 % hosts of the subsection *Sphaerotheca* belong to the *Rosaceae*. In order to reconstruct the phylogeny of *Podosphaera* and to determine evolutionary relationships between *Podosphaera* and its host plants, we used 152 ITS sequences and 69 28S rDNA sequences of *Podosphaera* for phylogenetic analyses. As a result, *Podosphaera* was divided into two large clades: clade 1, consisting of the section *Podosphaera* on *Prunus* (*P. tridactyla* s.l.) and subsection *Magnicellulatae*; and clade 2, composed of the remaining member of section *Podosphaera* and subsection *Sphaerotheca*. Because section *Podosphaera* takes a basal position in both clades, section *Podosphaera* may be ancestral in the genus *Podosphaera*, and the subsections *Sphaerotheca* and *Magnicellulatae* may have evolved from section *Podosphaera* independently. *Podosphaera* isolates from the respective subfamilies of *Rosaceae* each formed different groups in the trees, suggesting a close evolutionary relationship between *Podosphaera* spp. and their rosaceous hosts. However, tree topology comparison and molecular clock calibration did not support the possibility of co-speciation between *Podosphaera* and *Rosaceae*. Molecular phylogeny did not support species delimitation of *P. aphanis*, *P. clandestina*, *P. ferruginea*, *P. spiraeae* and *P. tridactyla* in their current circumscriptions, which suggests the need for revision of these species.

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INTRODUCTION

The *Erysiphaceae* is a group of obligate biotrophic fungi that cause powdery mildew disease on about 1×10^4 angiosperm species (Amano 1986), and it consists of 16 genera and approximately 650 species (Braun & Takamatsu 2000, Braun et al. 2002, Takamatsu et al. 2005a, b, Liberato et al. 2006). The host range of this fungal group is strictly confined to angiosperms and the fungi have never been reported to infect ferns or gymnosperms (Amano 1986). Molecular phylogenetic analyses demonstrated that the *Erysiphaceae* form a distinct monophyletic group (Mori et al. 2000b, Lutzoni et al. 2004, Takamatsu 2004, Wang et al. 2006). Thus, the family *Erysiphaceae* is derived from a single ancestral taxon that may have acquired parasitism just once. Molecular clock calibration suggested that the fungi originated in the late Cretaceous (Mori et al. 2000b, Takamatsu & Matsuda 2004), which is consistent with the hypothesis of Heluta (1992) and the fact that their host range is restricted to angiosperms.

The *Erysiphaceae* are divided into five tribes and two basal genera. Both tree-parasitic and herb-parasitic fungi are included in three of the five tribes: *Cystotheceae*, *Erysipheae* and *Phyllactinieae*. Tree-parasitic fungi usually take basal positions in these tribes and herb-parasitic fungi have derived positions. These observations, as well as the fact that the most basal genera of the *Erysiphaceae*, i.e. *Parauncinula* and *Caespitotheca*, infect trees, suggest that the early host plants of the *Erysiphaceae* were trees (Mori et al. 2000a). Multiple host shifts from trees to herbs may have then occurred during the Tertiary (Takamatsu 2004).

The powdery mildews belonging to the tribe *Cystotheceae* have both herbaceous and woody plants as hosts and consist of three genera, *Cystotheca*, *Podosphaera* and *Sawadaea*, of which the host ranges of the genera *Cystotheca* and *Sawadaea* are restricted to a narrow range of host families, i.e. all hosts of *Cystotheca* belong to the *Fagaceae*, and *Sawadaea* mostly occurs on *Aceraceae*. All hosts of these two genera are trees. The genus *Podosphaera* consists of two sections, *Podosphaera* and *Sphaerotheca*. The section *Podosphaera* (formerly the genus *Podosphaera*) parasitizes woody plants, and about 90 % of its hosts belong to the *Rosaceae*. The section *Sphaerotheca* (formerly the genus *Sphaerotheca*) is further divided into the subsections *Sphaerotheca* and *Magnicellulatae*, each of which forms a separate monophyletic clade derived from different ancestors (Takamatsu et al. 2000). More than 50 % of the hosts of the subsection *Sphaerotheca* are woody or herbaceous plants belonging to the *Rosaceae*. On the other hand, all hosts of the subsection *Magnicellulatae* are herbaceous plants scattered among 40 plant families that do not include the *Rosaceae*. Thus, although the genus *Podosphaera* has a close affinity to *Rosaceae*, subsection *Magnicellulatae* is unique in having no rosaceous plant as host. This may be supported by the fact that *Magnicellulatae* has its own conidial germination type, i.e. the *Magnicellulatae* type, which differs from the *Fibroidium* type of other taxa of *Podosphaera* (Cook & Braun 2009).

Phylogenetic relationships within the tribe *Cystotheceae* were previously reported by Takamatsu et al. (2000). They reported that host shifts from trees to herbs occurred at least twice in this tribe and that the subtribes *Sphaerotheca* and *Magnicel-*

[1] Graduate School of Bioresources, Mie University, 1577 Kurima-Machiya, Tsu 514-8507, Japan;
corresponding author e-mail: takamatu@bio.mie-u.ac.jp.
[2] Department of Botany, Centro Regional Universitario Bariloche, Universidad Nacional del Comahue, San Carlos de Bariloche, Rio Negro, Argentina.

Table 1 Sources of *Podosphaera* material sequenced in this study and DNA database accession numbers.

Host	Fungal species	Location and year	Designation[1]	Database ID no.[2]
Asteraceae				
Calendula officinalis	*fusca*	Bariloche, Argentina; 2001	MUMH 1933	AB525914
Calendula officinalis	*fusca*	Bariloche, Argentina; 2004	MUMH 2432	AB525915
Taraxacum officinale	*fusca*	Bariloche, Argentina; 2004	MUMH 2440	AB525916
Cannabaceae				
Humulus lupulus	*macularis*	Nagano, Japan; 2003	MUMH 2926	AB525917
Caricaceae				
Carica papaya	*caricae-papayae*	Chiang Rai, Thailand; 2002	MUMH 1853	AB525918
Escalloniaceae				
Escallonia rubra	*negeri*	Lago Curruhue, Argentina; 2001	MUMH 1478	AB525919
Escallonia rubra	*negeri*	Bariloche, Argentina; 2001	MUMH 1479	AB525920
Escallonia virgata	*negeri*	Cerro Tronador, Argentina; 2004	MUMH 2515	AB525921
Geraniaceae				
Geranium thunbergii	*fugax*	Nara, Japan; 1997	MUMH 343	AB525922
Gunneraceae				
Gunnera magellanica	*gunnerae*	Tierra del Fuego, Argentina; 1999	BCRU 03874 (MUMH 1480)	AB525923
Gunnera magellanica	*gunnerae*	Tierra del Fuego, Argentina; 1999	BCRU 03890 (MUMH 1481)	AB525924
Linaceae				
Linum usitatissimum	*lini*	Switzerland; 1998	MUMH 1392	AB525925
Onagraceae				
Epilobium ciliatum	*epilobii*	Chall Huaco, Argentina; 2001	MUMH 1873	AB525926
Rosaceae				
Amelanchier laevis	*clandestina*	Halle (Saale), Germany; 2009	MUMH 4968	AB525927
Aria alnifolia	*curvispora*	Toyama, Japan; 2001	MUMH 3266	AB525928
Cerasus incana	*salatai*	Tbilisi, Georgia; 2001	MUMH 2595	AB525929
Crataegus monogyna	*clandestina*	Bariloche, Argentina; 2001	MUMH 2429	AB525930
Crataegus oxyacantha	*clandestina*	Lago Lacar, Argentina; 2001	MUMH 1869	AB525931
Crataegus sp.	*clandestina*	Bariloche, Argentina; 2001	MUMH 1868	AB525932
Fragaria chiloensis	*aphanis*	Lago Gutierrez, Argentina; 2001	MUMH 1871	AB525933
Photinia serratifolia	sp.	Aichi, Japan; 1997	MUMH 407	AB525934
Pyracantha aff. *crenatoserrata*	*Oidium* sp.[3]	Bariloche, Argentina; 2004	MUMH 2450	AB525935
Pyracantha crenulata	*Oidium* sp.[3]	Bariloche, Argentina; 2001	MUMH 1870	AB525936
Rosa rubiginosa	*pannosa*	Lago Gutierrez, Argentina; 2001	MUMH 1476	AB525937
Rosa rubiginosa	*pannosa*	Lago Lacar, Argentina; 2001	MUMH 1872	AB525938
Rosa maltiflora	*pannosa*	Yamanashi, Japan; 2000	MUMH 819	AB525939
Spiraea cantoniensis	*spiraeae*	Villa La Angostura, Argentina; 2004	MUMH 2490	AB525940
Spiraea japonica	*clandestina*	Buenos Aires, Argentina; 2004	MUMH 2535	AB525941
Stachyurus praecox	sp.	Mie, Japan; 1999	MUMH 830	AB525942
Stephanandra incisa	*stephanandrae*	Mie, Japan; 1999	MUMH 831	AB525943
Verbenaceae				
Diostea juncea	*Oidium* sp.[3]	Cerro Tronador, Argentina; 2004	MUMH 2498	AB525944
Diostea juncea	*Oidium* sp.[3]	Bariloche, Argentina; 2009	MUMH 4937	AB525945
Diostea juncea	*Oidium* sp.[3]	Bariloche, Argentina; 2009	MUMH 4938	AB525946
Violaceae				
Viola maculata	*O. maculatae*[3]	Bariloche, Argentina; 2002	BCRU 04343 (MUMH 3050)	AB525947
Viola maculata	*O. maculatae*[3]	Bariloche, Argentina; 2002	BCRU 04345 (MUMH 3051)	AB525948

[1] BCRU: Institutional Herbarium of Centro Regional Universitario Bariloche, San Carlos de Bariloche, Argentina; MUMH: Mie University, Mycological Herbarium, Japan.
[2] DDBJ, EMBL, and GenBank database accession number of nucleotide sequence data.
[3] *Oidium*.

lulatae evolved independently from separate ancestral taxa belonging to tree-parasitic *Podosphaera* species. However, they used only 26 ITS sequences in the analysis, which was inadequate to determine the phylogenetic relationships within the genus *Podosphaera*. In this study, we conducted comprehensive phylogenetic analyses by combining newly determined sequences with sequences reported in Takamatsu et al. (2000) and retrieved from DNA databases.

The aims of this study were

1. to reconstruct the phylogeny of the genus *Podosphaera*;
2. to discuss the evolution of *Podosphaera* with special reference to host relationships; and
3. to consider species delimitation of *Podosphaera* from the perspective of molecular phylogeny.

We included sequences of the subtribe *Magnicellulatae* in the present analyses, but we did not address them in the discussion because this fungal group is too large and distinct to be analyzed in this paper. Therefore, the phylogeny of *Magnicellulatae* has been discussed elsewhere (Ito & Takamatsu 2010).

MATERIALS AND METHODS

DNA extraction and amplification

The collection and location of host plants and the accession numbers for the nucleotide sequence databases (DDBJ, EMBL and GenBank) are provided in Table 1. Whole-cell DNA was isolated from chasmothecia or mycelia via the chelex method (Walsh et al. 1991, Hirata & Takamatsu 1996). The ITS region including the 5.8S rDNA, and the 5' end of the 28S rDNA including the variable domains D1 and D2 were amplified separately by two sequential PCR reactions using partially nested primer sets. The PCR reactions were conducted using TaKaRa Taq DNA polymerase (TaKaRa, Tokyo, Japan) in a TP-400 thermal cycler (TaKaRa) under the following thermal cycling conditions: an initial denaturation step of 2 min at 95 °C, 30 cycles of 30 s at 95 °C, followed by 30 s at 52 °C for annealing, and 30 s at 72 °C for extension, and a final extension step of 7 min at 72 °C. A negative control that lacked template DNA was included in each set of reactions. The PCR products were subjected to

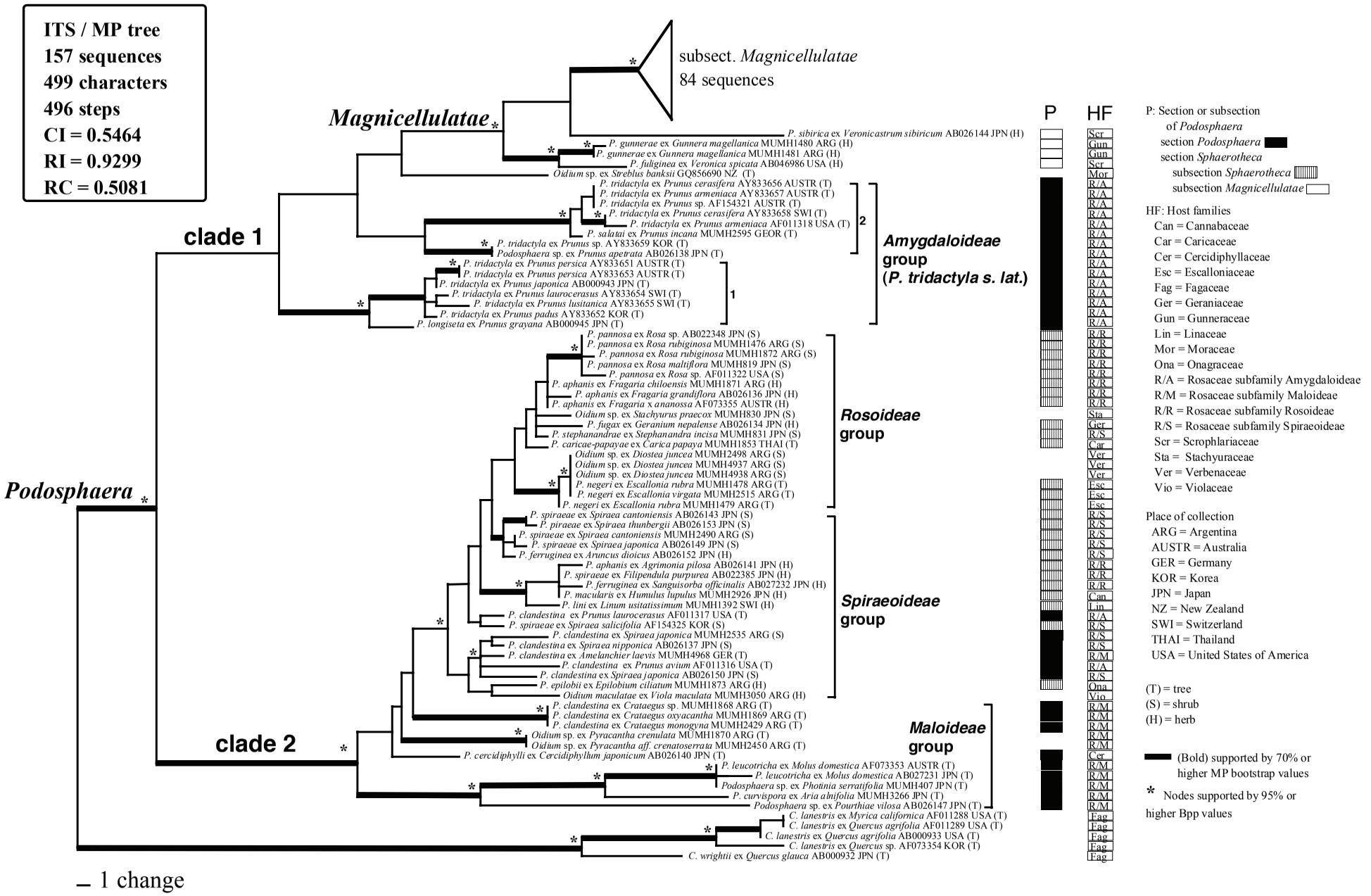

Fig. 1 Phylogenetic analysis of the nucleotide sequences of the ITS region including 5.8S rDNA for 157 sequences from *Podosphaera* and *Cystotheca* used as outgroup taxa. The tree is a phylogram of one of the 1 x 10^4 MP trees with 496 steps, which was obtained by a heuristic search employing the random stepwise addition option of PAUP. Gaps were treated as missing data. Horizontal branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree.

electrophoresis in a 1.5 % agarose gel in TAE buffer, excised from the ethidium bromide-stained gel, and purified using the JETSORB Kit (Genomed, Oeynhausen, Germany) according to the manufacturer's protocol. The nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in a CEQ2000XL DNA sequencer (Beckman Coulter, Fullerton, CA, USA). The sequence reactions were conducted using the CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter) according to the manufacturer's instructions.

For amplification of the ITS region, primers ITS5 (White et al. 1990) and P3 (Kusaba & Tsuge 1995) were used for the first amplification. One microlitre of the first reaction mixture was used for the second amplification, along with the partially nested primer sets ITS5 and ITS4 (White et al. 1990). The ITS5/ITS4 fragment was subjected to cycle sequencing using the primers ITS1, ITS4, T3 and T4 (Hirata & Takamatsu 1996). For amplification of the 28S rDNA, primers PM3 (Takamatsu & Kano 2001) and TW14 (Mori et al. 2000a), and NL1 (Mori et al. 2000a) and TW14 were used for the first and second amplifications, respectively. Primers NL1, NL2, NL3 and NLP2 (Mori et al. 2000a) were used for cycle-sequencing.

Phylogenetic analysis

The sequences were initially aligned using the Clustal X package (Thompson et al. 1997). The alignment was then visually refined with a word processing program using colour-coded nucleotides. The alignments were deposited in TreeBASE (http://www.treebase.org/) under the accession number S2604. Phylogenetic trees were obtained from the data using the maximum parsimony (MP) method in PAUP v4.0 (Swofford 2001) and a Bayesian analysis in MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003). MP analyses were performed with the heuristic search option using the 'tree-bisection-reconstruction' (TBR) algorithm with the stepwise addition option set as simple and maximum tree number as 1×10^4. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of the internal branches of the resulting trees was tested with bootstrap (BS) analyses (Felsenstein 1985) using 1 000 replications with the stepwise addition option set as simple and maximum tree number as 10 to save analysis time. BS values of 70 % or higher were shown. We also constructed MP trees with the parsimony ratchet method (Nixon 1999) in PAUP and PAUPRat v1 (Sikes & Lewis 2001) to confirm that the MP tree generated by the MP analysis is not the result of a local optimum.

For Bayesian phylogenetic analyses, the best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via the Akaike information criterion (AIC) using PAUP and MrModeltest v2.2 (Nylander 2004). MrBayes was launched with random starting trees and Markov chains were sampled every 100 generations. To ensure that the Markov chain did not become trapped in local optima, we used the MCMCMC algorithm and performed the estimation with four incrementally heated Markov chains. The average standard deviation of split frequencies (ASDSF) was observed to verify that the values dropped below 0.01. Support for individual nodes was tested by Bayesian posterior probabilities (Bpp) obtained from a 50 % majority rule consensus. Bpp values of 0.95 or higher are shown.

Host plants

The host plant data were extracted from the database 'Host plants of the powdery mildew fungi v1.0' (available from the corresponding author upon request), which was based on the table 'Host plants of powdery mildew fungi and their distribution by country' (Amano 1986).

RESULTS

Phylogeny inferred from ITS sequences

A total of 157 ITS sequences, including 33 sequences newly determined in this study, were used for the current analysis. *Cystotheca* spp. were used as outgroup taxa based on Mori et al. (2000a). The dataset consisted of 499 characters, of which 179 characters were variable and 140 characters were informative for parsimony analysis. A total of 1×10^4 MP trees with 496 steps (CI = 0.5464; RI = 0.9299; RC = 0.5081) were constructed by the MP analysis. One of the 1×10^4 MP trees, excluding subtribe *Magnicellulatae* (shown in Appendix 2), is shown in Fig. 1. Parsimony ratchet analysis generated trees with the same tree length and similar tree topologies. Therefore, we concluded that the tree shown in Fig. 1 is not the result of a local optimum. MrModeltest selected the GTR+I+Γ model as the best for this dataset. Using this evolution model, MrBayes was run for 1×10^7 generations, resulting in approximately 1×10^5 sampling trees. The first 59 390 trees were discarded (burn-in) because ASDSF dropped below 0.01. The remaining 40 611 trees were summarised in a majority-rule consensus tree, yielding the probability of each clade being monophyletic. The tree topology generated by the Bayesian analysis was almost identical to the MP tree, and thus the tree is not shown.

The 152 ITS sequences of *Podosphaera* species were divided into two large clades, clade 1 and clade 2. Clade 1 appeared in the MP strict consensus tree, although statistical support of this clade was low in both BS and Bpp analyses. Clade 1 consisted of 15 sequences of *Podosphaera tridactyla* s.l. (section *Podosphaera*) on *Prunus* spp. (*Rosaceae*), 89 sequences of the isolates belonging to the subsection *Magnicellulatae* of section *Sphaerotheca*, and one sequence of *Oidium* sp. on *Streblus banksii*. The basal nodes of clade 1 were occupied by the sequences of *Podosphaera tridactyla* s.l. and the sequences of subsection *Magnicellulatae* formed a distinct clade (BS = 61 %; Bpp = 1.0) at a derived position. Clade 2 (BS = 95 %; Bpp = 1.0) consisted of the sequences of the section *Podosphaera* parasitizing the subfamilies *Maloideae* (apple subfamily) and *Spiraeoideae* of the *Rosaceae* and all sequences of the subsection *Sphaerotheca* of section *Sphaerotheca*. The sequences of the section *Podosphaera* occupied a basal position of clade 2. The sequences of the subsection *Sphaerotheca* of section *Sphaerotheca* were placed at a derived position but did not form a distinct monophyletic group.

Phylogeny inferred from the 28S rDNA region

A total of 71 28S rDNA sequences, including 23 sequences newly determined in this study, were used for the current analysis. *Cystotheca* spp. were used as outgroup taxa based on Mori et al. (2000a). The dataset consisted of 680 characters, of which 78 characters were variable and 47 characters were informative for parsimony analysis. A total of 1×10^4 MP trees with 128 steps (CI = 0.7031; RI = 0.9128; RC = 0.6418) were constructed by the MP analysis. One of the 1×10^4 MP trees, excluding subtribe *Magnicellulatae* (shown in Appendix 1), is shown in Fig. 2. Parsimony ratchet analysis generated trees with the same tree length and similar tree topologies. Therefore, we concluded that the tree shown in Fig. 2 is not the result of a local optimum. MrModeltest selected the GTR+I+Γ model as the best for this dataset. Using this evolution model, MrBayes was run for 5×10^6 generations, resulting in 50 001 sampling trees. The first 14 650 trees were discarded (burn-in) because ASDSF dropped below 0.01. The remaining 35 351 trees were summarised in a majority-rule consensus tree, yielding the probability of each clade being monophyletic. The tree topology produced by the Bayesian analysis was almost identical to the MP tree and thus the tree is not shown. The tree topology of

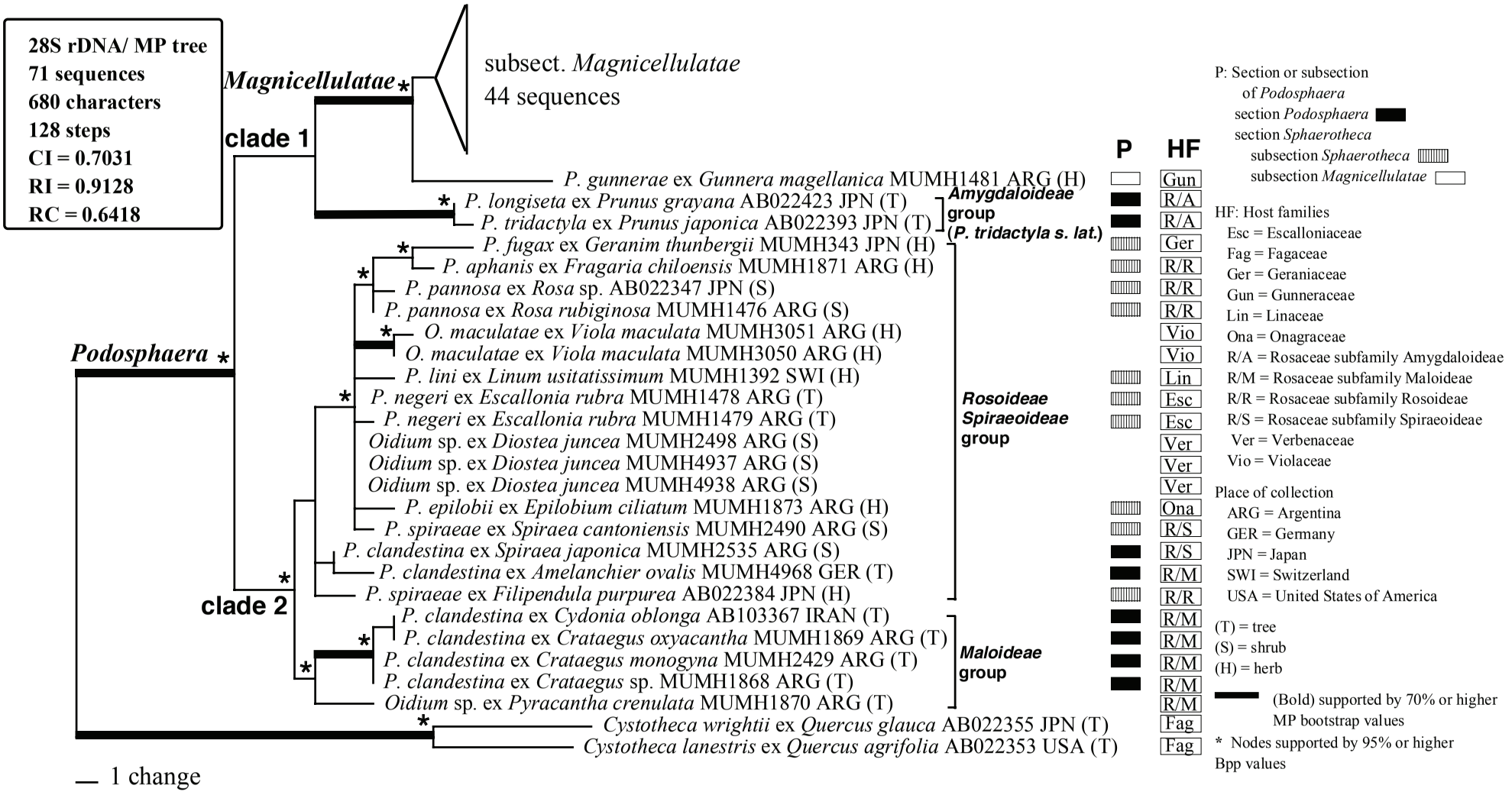

Fig. 2 Phylogenetic analysis of the divergent domains D1 and D2 sequences of the 28S rDNA for 71 sequences from *Podosphaera* and *Cystotheca* used as outgroup taxa. The tree is a phylogram of one of the 1 x 10^4 MP trees with 128 steps, which was obtained by a heuristic search employing the random stepwise addition option of PAUP. Gaps were treated as missing data. Horizontal branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree.

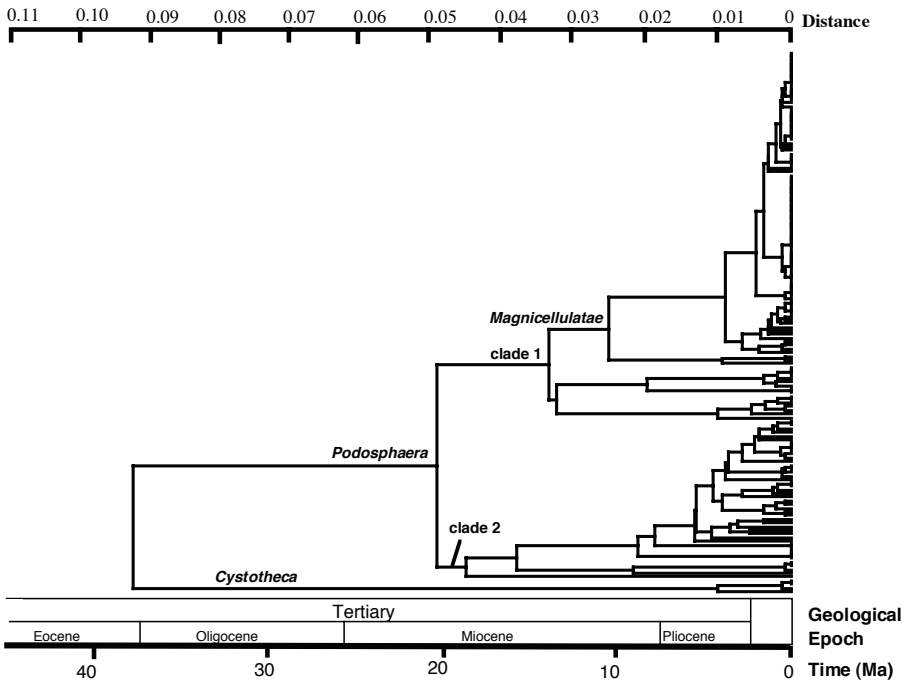

Fig. 3 Estimated dates of divergence of major clades of *Podosphaera* based on the nucleotide sequences of the rDNA ITS region and nucleotide substitution rate of *Erysiphaceae* (2.52×10^{-9} substitutions per site per year) reported by Takamatsu & Matsuda (2004). Ma, million years ago.

the 28S rDNA sequences was almost identical to that of the ITS tree, but the statistical support for the major clades was lower than that of the ITS tree.

Timing of evolutionary events

Because a likelihood ratio test (LRT) significantly rejected the molecular clock of the ITS dataset of 157 sequences, five sequences with extremely long or short terminal branches were removed from the dataset. The molecular clock hypothesis of the reduced dataset consisting of 152 ITS sequences was not rejected by the LRT and thus was used to construct a UPGMA tree. The LRT did not reject the molecular clock hypothesis of the 28S rDNA dataset consisting of 71 sequences. Thus, the dataset was used to construct a UPGMA tree. The Kimura two-parameter model (Kimura 1980) was used to calculate genetic distances. The molecular clocks of the ITS (2.52×10^{-9} substitutions per site per year, ssy) and the D1/D2 domain of the 28S rDNA region (6.5×10^{-10} ssy) of the *Erysiphales* (Takamatsu & Matsuda 2004) were used to calculate evolutionary timing. The molecular clocks suggested that *Podosphaera* split from *Cystotheca* about 40 million years ago (Ma) at the end of the Eocene, and the split of clade 1 and clade 2 occurred about 20 Ma in the Miocene (Fig. 3, 4).

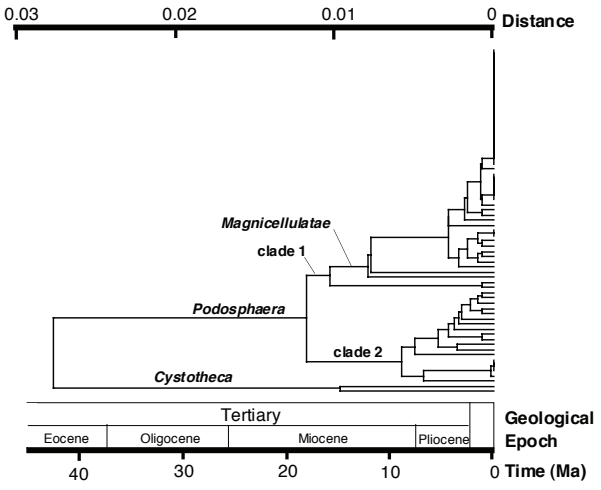

Fig. 4 Estimated dates of divergence of major clades of *Podosphaera* based on the divergent domains D1 and D2 sequences of the 28S rDNA and the nucleotide substitution rate of *Erysiphaceae* (6.5×10^{-10} substitutions per site per year) reported by Takamatsu & Matsuda (2004). Ma, million years ago.

DISCUSSION

Host relationships

Host plants of Podosphaera

The number of host plant species of *Podosphaera*, arranged by plant families, is shown in Table 2. *Podosphaera* is divided into two sections, *Podosphaera* (formerly genus *Podosphaera*) and *Sphaerotheca* (formerly genus *Sphaerotheca*). The latter section is further divided into two subsections, *Sphaerotheca* and *Magnicellulatae*, respectively. The subsection *Magnicellulatae* forms a distinct monophyletic group that diverged from an ancestral fungus by a host shift from *Prunus* (*Rosaceae*) to herbaceous plants (Takamatsu et al. 2000). *Magnicellulatae* is unique in its host range and morphological characteristics compared with other *Podosphaera* species, suggesting that this subsection evolved independently from other *Podosphaera* taxa. Thus, we discuss the evolution of this fungal group elsewhere (Hirata et al. 2000, Ito & Takamatsu 2010). There are 250 host species of the section *Podosphaera* spanning 10 orders and 13 families, of which 216 host species (86.4 %) belong to the *Rosaceae*. The subsection *Sphaerotheca* of section *Sphaerotheca* has 806 host species covering 15 orders and 28 families, of which 456 (56.6 %) belong to the *Rosaceae*. Of the non-*Rosaceae* hosts, 70 belong to the *Euphorbiaceae*, 67 to the *Geraniaceae*, 65 to the *Onagraceae* and 54 to the *Hydrangeaceae*. Thus, the *Rosaceae* has the highest number of host species for both section *Podosphaera* and subsection *Sphaerotheca*. On the other hand, *Magnicellulatae* has 1 110 host species spanning 40 families. About half (45 %) of the hosts belong to the *Asteraceae* and none belong to the *Rosaceae*.

Powdery mildews of Rosaceae

The number of host species of powdery mildew fungi reported on *Rosaceae* is shown in Table 3 and is organised by plant subfamilies and fungal genera. Five powdery mildew genera have been reported to occur on the *Rosaceae*. Of these, the genus *Podosphaera* has the highest number of host species in the *Rosaceae* (672). *Podosphaera* occurs on all four subfamilies

of *Rosaceae*, and the highest number of host species is in the subfamily *Rosoideae* (418 species). *Phyllactinia* is the genus having the next highest number of host species in *Rosaceae* (110), and finally *Erysiphe* has 68 host species. Although all three sections of *Erysiphe* occur on *Rosaceae*, the number of host species differs depending on the plant subfamilies and fungal sections. Although *Golovinomyces* and *Leveillula* also occur on *Rosaceae*, the number of host species is less than 10 in both genera. Therefore, *Rosaceae* is the most important plant family as host for *Podosphaera* and vice versa.

Table 2 Number of host plant species of the genus *Podosphaera*.

Plant order & family		Number of host species: Sect. Podosphaera	Sect. Sphaerotheca subsect. Sphaerotheca	Total
EUROSIDS I				
Rosales	*Ulmaceae*	1	2	3
	Moraceae	0	4	4
	Urticaceae	0	2	2
	Rosaceae	216	456	672
	Elaeagnaceae	0	3	3
Fagales	*Betulaceae*	1	0	1
Celastrales	*Celastraceae*	0	3	3
Malpighiales	*Salicaceae*	3	0	3
	Linaceae	0	1	1
	Euphorbiaceae	0	70	70
EUROSIDS				
Sapindales	*Anacardiaceae*	0	8	8
Myrtales	*Myrtaceae*	0	18	18
	Onagraceae	0	65	65
	Punicaceae	0	1	1
Geraniales	*Geraniaceae*	0	67	67
Saxifragales	*Saxifragaceae*	0	54	54
	Hamamelidaceae	4	0	4
	Cercidiphyllaceae	2	0	2
EUASTERIDS I & II				
Solanales	*Convolvulaceae*	0	1	1
Lamiales	*Oleaceae*	2	2	4
	Verbenaceae	0	2	2
Gentianales	*Hydrophyllaceae*	0	6	6
	Asclepiadaceae	1	0	1
Apiales	*Apiaceae*	1	1	2
Dipsacales	*Caprifoliaceae*	4	1	5
EUDICOTS				
Ericales	*Ebenaceae*	2	0	2
	Ericaceae	12	11	23
	Polemoniaceae	0	21	21
Cornales	*Cornaceae*	1	0	1
Caryophyllales	*Polygonaceae*	0	1	1
	Phytolaccaceae	0	1	1
	Tamaricaceae	0	1	1
	Plumbaginaceae	0	1	1
Ranunculales	*Ranunculaceae*	0	2	2
	Papaveraceae	0	1	1
	Total	250	806	1056

Relationships between host plants and phylogeny of Podosphaera

Phylogenetic trees of *Podosphaera* excluding *Magnicellulatae* are shown in Fig. 1 and 2. *Podosphaera* taxa are divided into four groups according to the subfamilies of *Rosaceae*: the *Amygdaloideae* group, the *Maloideae* group, the *Spiraeoideae* group and the *Rosoideae* group. The *Amygdaloideae* group (= *P. tridactyla* s.l.) is an assemblage that splits from the other three groups first and forms a large group (clade 1) with the fungi belonging to *Magnicellulatae*. The first node of clade 1 was shared by the isolates from *Amygdaloideae*, which suggests that the *Amygdaloideae* group is ancestral in clade 1 and the *Magnicellulatae* diverged from an ancestor that was parasitic to the *Amygdaloideae*. This group is further divided into two small groups with large genetic divergence. Subgroup 1 (BS = 95 %; Bpp = 0.95) split from other clade 1 groups first and consists of isolates from the subgenera *Amygdalus*, *Laurocerasus* and *Padus* of *Prunus*. Subgroup 2 (BS = 58 %; Bpp = 0.84), sister to *Magnicellulatae*, consists of isolates from the subgenera *Cerasus* and *Prunus*. Thus, the phylogeny of the *Amygdaloideae* group is closely related to the subgeneric-level taxonomy of *Prunus*, which suggests the possibility of co-speciation between *Prunus* and the *Amygdaloideae* group. However, in the phylogeny of the plant genus, subgenus *Amygdalus* groups with *Prunus*, and the remaining three subgenera *Cerasus*, *Laurocerasus* and *Padus* form another group (Bortiri et al. 2001, Lee & Wen 2001), which is not consistent with the grouping of powdery mildew fungi on *Prunus*. Because we used only 15 sequences in the present analysis, more sequence data will be required to clarify the phylogenetic relationships within *P. tridactyla* s.l.

Powdery mildews on the subfamilies *Maloideae*, *Spiraeoideae* and *Rosoideae* formed a large clade (clade 2: BS = 95 %; Bpp = 1.0) distinct from the *Amygdaloideae* group. Because the first split of clade 2 occurred within the *Maloideae* group, taxa belonging to the *Maloideae* group may be the most ancestral in clade 2. The *Maloideae* group was further divided into two groups that are parasitic to the tribes *Maleae* and *Cratageae*, respectively. The former group split at the base of clade 2 and was sister to the other groups. The fungi parasitic to the tribe *Cratageae* formed a clade with the *Spiraeoideae* and *Rosoideae* groups and occupied a basal position in the clade. *Podosphaera cercidiphylli* on *Cercidiphyllum japonicum* (*Cercidiphyllaceae*) was included in the *Maloideae* group in the phylogeny.

The *Spiraeoideae* group formed a clade together with the *Rosoideae* group (BS < 50 %; Bpp = 0.98). Although the two groups did not form independent clades, they were generally situated at different places in the tree (Fig. 1). *Podosphaera epilobii* on *Epilobium* (*Onagraceae*) and *Oidium maculatae* on *Viola* (*Violaceae*) were included in the *Spiraeoideae* group. *Podosphaera clandestina* on *Prunus* (*Amygdaloideae*, *Rosaceae*) was also included in this group. *Podosphaera lini* on *Linum* (*Linaceae*), *P. macularis* on *Humulus* (*Cannabaceae*), *P. caricae-papayae* on *Papaya* (*Caricaceae*), *P. negeri* on *Escallonia* (*Escalloniaceae*),

Table 3 Number of host species of the powdery mildew fungi in *Rosaceae*.

Subfamily of *Rosaceae*	*Podosphaera*	*Phyllactinia*	*Erysiphe*				*Golovinomyces*	*Leveillula*
			Erysiphe	*Microsphaera*	*Uncinula*	Total		
Amygdaloideae	96	21	0	1	13	14	2	1
Maloideae	108	76	3	6	3	12	0	0
Rosoideae	418	11	32	1	6	39	6	1
Spiraeoideae	46	2	1	2	0	3	1	1
Unknown	4	0	0	0	0	0	0	0
Total	672	110	36	10	22	68	9	3

and *P. fugax* on *Geranium* (*Geraniaceae*) were included in the *Rosoideae* group. A fungus on *Diostea juncea* (*Verbenaceae*) was identified as '*Sphaerotheca verbenae*' (= *Podosphaera xanthii*) by Havrylenko (1997). However, '*Sphaerotheca verbenae*' belongs to subsection *Magnicellulatae* of section *Sphaerotheca*. In this study, this fungus has a DNA sequence identical to that of *P. negeri* in both ITS and 28S rDNA regions and belongs to the *Rosoideae* group. We thus treated this fungus as *Oidium* sp. in this study.

The present study revealed that more than 50 % of the hosts of *Podosphaera* (excluding *Magnicellulatae*) belong to the *Rosaceae*, and phylogenetic relationships of *Podosphaera* have close affinity with the taxonomy of the *Rosaceae*. These results strongly suggest a close evolutionary relationship between *Podosphaera* and *Rosaceae*. The *Rosaceae* may have been the first host family for *Podosphaera*, and host shifts from the *Rosaceae* to other plant families may have occurred spontaneously during the evolution of *Podosphaera*. The *Rosaceae* belongs to the *Rosales* of EUROSIDS I (APG II 2003). Of the host families analysed in this study, all plant families, excepting *Rosaceae*, *Cannabaceae* and *Moraceae*, do not belong to the *Rosales*. These results suggest that *Podosphaera* tend to expand their hosts to closely related plant species when expanding within a single plant family. Conversely, inter-family level host shifts seem to occur independently of host phylogeny. Similar phenomena have been also found in host expansions of *Golovinomyces* and *Phyllactinia* of the *Erysiphaceae* (Matsuda & Takamatsu 2003, Takamatsu et al. 2008).

Is there any co-speciation between Podosphaera and Rosaceae?

The above results suggest a close evolutionary relationship between *Podosphaera* and *Rosaceae*, but did co-speciation occur between *Podosphaera* and *Rosaceae*? At least two items should be evaluated to determine whether co-speciation between organisms has occurred. One is similar or identical phylogeny between the organisms concerned, and another is timing of divergence of the organisms. Of the four subfamilies of the *Rosaceae*, the subfamilies *Amygdaloideae*, *Maloideae* and *Rosoideae* were supported as monophyletic groups by the *rbc*L sequence phylogeny, but *Spiraeoideae* was shown to be polyphyletic (Morgan et al. 1994, Potter et al. 2002). *Podosphaera* isolates were mostly divided into different groups according to the subfamilies of *Rosaceae*, suggesting that the phylogeny of *Podosphaera* is mostly consistent with the subfamily-level taxonomy of *Rosaceae*. Phylogenetic analyses using *rbc*L and *mat*K sequences showed that the first split is shared by the *Rosoideae*, suggesting that *Rosoideae* is the most ancestral of the *Rosaceae* (Morgan et al. 1994, Potter et al. 2002). In *Podosphaera* phylogeny, the first split occurs between isolates from the *Amygdaloideae* and *Maloideae*, and the isolates from the *Rosoideae* are placed at a derived part of the trees (Fig. 1, 2). Thus, the phylogeny of *Podosphaera* does not conform to that of the *Rosaceae*. Evolutionary timing of *Podosphaera* divergence, as calculated by the molecular clocks of ITS and 28S rDNA regions, suggested that the split of *Cystotheca* and *Podosphaera* occurred in the late Eocene (c. 40 Ma) and that the split of clade 1 and clade 2 occurred in the mid-Miocene (c. 20 Ma). Fossil records suggest that the traditional subfamilies *Amygdaloideae* and *Maloideae* are found in the Eocene (DeVore & Pigg 2007). Therefore, divergence of *Podosphaera* may have occurred later than the divergence of the *Rosaceae*. In conclusion, there is no evidence that co-speciation occurred between *Podosphaera* and *Rosaceae*.

Taxonomic implications

Sections and subsections

The genus *Podosphaera* is divided into two sections, the section *Podosphaera*, with appendages dichotomously branched at the apex, and the section *Sphaerotheca*, with hypha-like simple appendages. Section *Sphaerotheca* is further divided into two subsections, *Sphaerotheca* and *Magnicellulatae*, by the size of the peridium cells of the chasmothecia. These sections and subsections were introduced by Braun & Takamatsu (2000) as morphological, i.e., non-monophyletic groups. The present analysis revealed that the two subsections do not share a common ancestor. Therefore, the section *Sphaerotheca* is polyphyletic, consisting of two different groups derived from different ancestral taxa. Section *Podosphaera* is a paraphyletic group situated at the basal part of clade 1 and clade 2. The present analysis indicates that the section *Podosphaera* is ancestral in the genus *Podosphaera*, and the subsections *Sphaerotheca* and *Magnicellulatae* were derived from the *Maloideae* group and *Amygdaloideae* group, respectively. Therefore, the genus *Podosphaera* is a natural unit supported by molecular phylogeny, but the sections *Podosphaera* and *Sphaerotheca* are artificial, morphological units that are not supported by phylogeny, as already stated by Braun & Takamatsu (2000).

Species supported by phylogeny

The genus *Gunnera* consists of herbaceous plants distributed in the Southern Hemisphere and tropical regions. *Podosphaera gunnerae* was first described as a powdery mildew of *Gunnera* by Havrylenko & Braun (1998). This species has been reported only in Argentinian Patagonia. Both ITS and 28S rDNA sequences clearly indicated that *P. gunnerae* forms an independent clade. This clade was situated at the basal part of *Magnicellulatae* and was sister to *P. fuliginea.* This suggests that *P. gunnerae* split from the other fungi in the early stage of the evolution of *Magnicellulatae.* This species is the only powdery mildew species described for the genus *Gunnera.*

Podosphaera negeri was first described as *Sphaerotheca spiralis* in 1907 (Braun 1987) and revised as *P. negeri* by Braun et al. (2006). This species has a unique characteristic with coiled appendages, infects *Escallonia* (*Escalloniaceae*) and has been reported only in Argentinian Patagonia. Both ITS and 28S rDNA sequences clearly indicated that *P. negeri* forms an independent clade. *Oidium* sp. found on *Diostea juncea* has a sequence identical to that of *P. negeri* in both ITS and 28S rDNA regions. *Podosphaera fugax* found on *Geranium* was sister to *P. negeri*, but this was supported neither by BS nor by Bpp values.

Oidium maculatae was first described on *Viola maculata* (Havrylenko & Takamatsu 2005). Both ITS and 28S rDNA sequences showed that this species forms a unique clade.

Species not supported by phylogeny

Podosphaera species parasitic to *Prunus* have been mostly classified as *P. tridactyla.* Cunnington et al. (2005) reported that this species has large genetic variation and is divided into several groups, which was confirmed by the present analysis. These results indicate that *P. tridactyla* is a species complex composed of several biological species. *Prunus* s.l. has been divided into 5 to 6 subgenera, which were sometimes treated as separate genera (Bortiri et al. 2001, Lee & Wen 2001). Groups of *P. tridactyla* are mostly consistent with the delimitation of the subgenera. *Podosphaera tridactyla* may have specialised to the respective host groups along with the genetic divergence of *Prunus*. Some segregated species like *P. longiseta* (Sawada 1951) and *P. salatai* (Heluta et al. 2005) have been proposed to lie within *P. tridactyla* s.l., which is supported by molecular analysis. Comprehensive revision of *P. tridactyla* s.l. is required.

Podosphaera clandestina is a well known species described in 1851 and parasitizes 14 genera of the *Rosaceae* containing *Crataegus*, *Prunus* and *Spiraea* as hosts (Braun 1987). Nine ITS sequences of *P. clandestina* from *Amelanchier*, *Crataegus*, *Prunus* and *Spiraea* used in this study revealed that these sequences do not form a single clade. In particular, the sequences of the isolates from *Crataegus* formed a distinct clade distantly related to other *P. clandestina* on *Amelanchier*, *Spiraea* and *Prunus*. 28S rDNA sequence of *P. clandestina* on *Cydonia* was identical to that of isolates from *Crataegus.* Isolates from *Amelanchier*, *Prunus* and *Spiraea* were closely related to each other, but there were some nucleotide substitutions among them. A taxonomic re-evaluation of this species is necessary. The fungus on *Pyracantha* was reported as *P. clandestina* by Amano (1986) and Delhey et al. (2003). However, as far as we know, a teleomorph of this fungus has not yet been found. The two ITS sequences and one 28S rDNA sequence of this fungus determined in this study showed that the fungus on *Pyracantha* forms an independent lineage different from other *P. clandestina* groups.

The five ITS sequences of *P. spiraeae* on *Spiraea* formed a clade but contained some nucleotide substitutions among the sequences. A sequence from *P. spiraeae* collected in Korea, AF011317, did not belong to this clade and was sister to a sequence of *P. clandestina* on *Prunus avium*. Zhao (1981) proposed a new species, *Sphaerotheca filipendulae* (= *Podosphaera filipendulae*), for the fungus on *Filipendula*. Braun (1987) regarded *S. filipendulae* as a synonym of *S. spiraeae* (= *P. spiraeae*). The present analysis shows that the fungus on *Filipendula purpurea* does not belong to the clade of *P. spiraeae* on *Spiraea*, but to the clade consisting of *P. aphanis* on *Agrimonia*, *P. ferruginea* on *Sanguisorba*, and *P. macularis* on *Humulus*. The ITS sequence of the fungus on *F. purpurea* was identical to that of *P. macularis* on *Humulus*. *Spiraea* belongs to the subfamily *Spiraeoideae* and *Filipendula* to *Rosoideae*. Considering the close relationship between the subfamilies of *Rosaceae* and the phylogeny of *Podosphaera*, the result of the present phylogenetic analysis seems to be acceptable.

Podosphaera ferruginea was introduced for the fungus on *Sanguisorba* (Junell 1965). Braun (1987) assigned the fungus on *Aruncus* to this species. The present analysis revealed that the fungus on *Aruncus* belongs to a clade different from the fungus on *Sanguisorba.* Because *Saguisorba* belongs to the subfamily *Rosoideae* and *Aruncus* to *Spiraeoideae*, the result of phylogenetic analysis seems to be acceptable.

Thirteen genera of the *Rosaceae* and *Eucalyptus* (*Myrtaceae*) have been listed as hosts of *P. aphanis* (Braun 1987). Of these, the fungi on *Fragaria* and *Agrimonia* were used in this analysis. The three sequences from *Fragaria* grouped together. The sequence of the fungus on *Agrimonia* did not belong to this clade but to the clade composed of the fungi on *Filipendula*, *Humulus* and *Sanguisorba*. Taxonomic revision of this species may be required.

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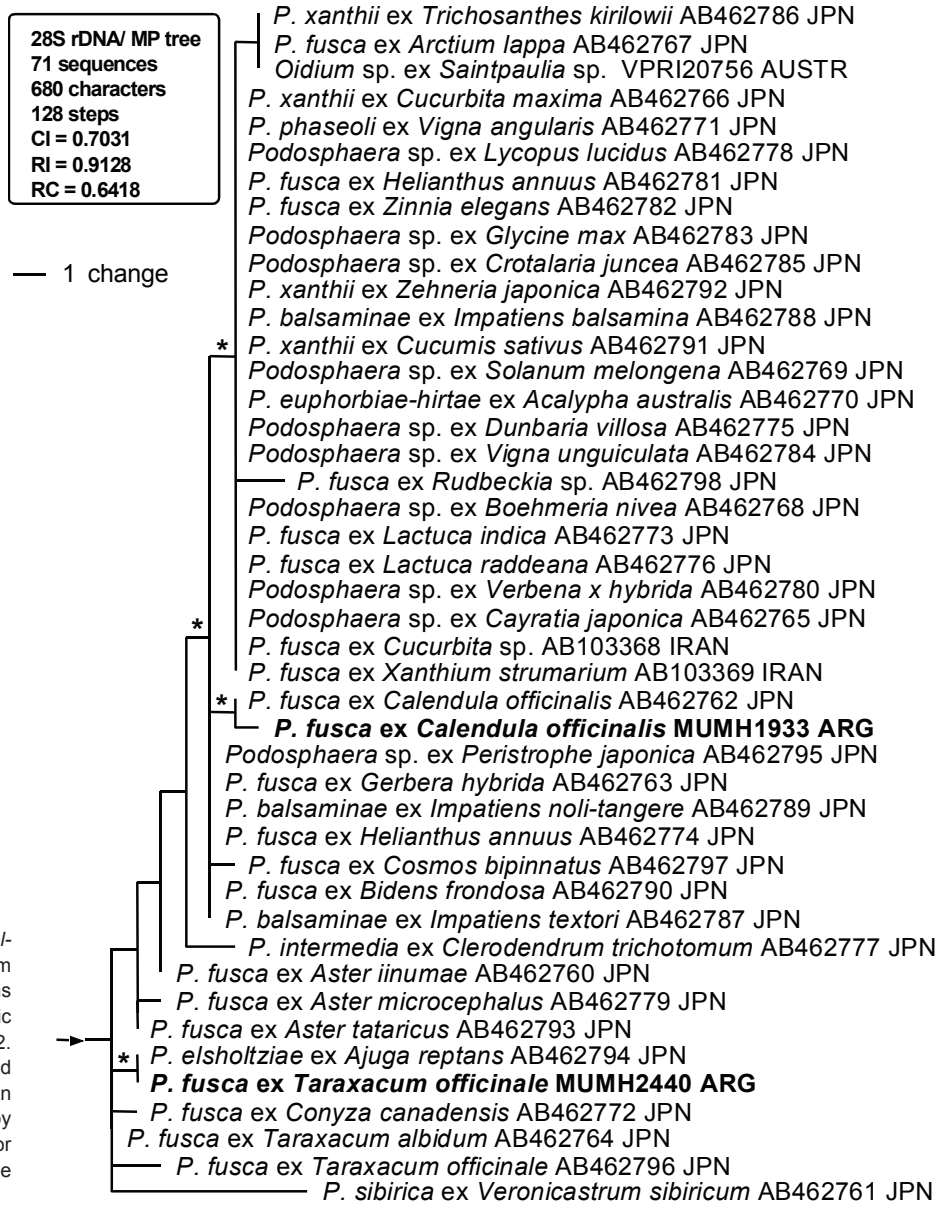

Appendix 1 Phylogeny of subsection *Magnicellulatae* of the genus *Podosphaera* inferred from the nucleotide sequences of the D1/D2 domains of the 28S rDNA. This is a part of the phylogenetic tree of *Podosphaera*, but was not shown in Fig. 2. A **bold** line means that the node was supported by an MP bootstrap value of 70 % or higher. An asterisk means that the node was supported by a Bayesian posterior probability value of 0.95 or higher. Taxon name shown by **bold** type is the sequence determined in this study.

Appendix 2 Phylogeny of subsection *Magnicellulatae* of the genus *Podosphaera* inferred from nucleotide sequences of rDNA ITS region. This is a part of the phylogenetic tree of *Podosphaera*, but was not shown in Fig. 1. A **bold** line means that the node was supported by an MP bootstrap value of 70 % or higher. An asterisk means that the node was supported by a Bayesian posterior probability value of 0.95 or higher. Taxon name shown by **bold** type is the sequence determined in this study.