# A parasitic transition from trees to herbs occurred at least twice in tribe Cystotheceae (Erysiphaceae): Evidence from nuclear ribosomal DNA

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Running title: Parasitic transitions in tribe Cystotheceae

To understand the evolutionary history of tribe Cystotheceae, phylogentic trees were constructed from the nucleotide sequences of the ribosomal DNA internal transcribed spacer of 28 taxa of the fungal ingroup and two outgroup taxa. The first split of the ingroup taxa occurred between a clade composed of the genus Cystotheca and a clade composed of the genera Podosphaera and Sphaerotheca. Podosphaera and Sphaerotheca did not separate into clades. Instead, Podosphaera species parasitic to Prunus and Sphaerotheca section Magnicellulatae grouped together, with the remaining Podosphaera species and Sphaerotheca section Sphaerotheca forming another subclade. Since the first splits were shared by Podosphaera species in both subclades, it was assumed the ancestral features of both subclades were Podosphaera-like and the genus Sphaerotheca derived from a Podosphaera-like ancestral taxon on at least two independent occasions. The results of this study suggest that ancestral fungi of the tribe Cystotheceae were originally arbor-parasitic, and transition from arbor-parasitism to herb-parasitism may have occurred at least twice independently, accompanied by a morphological change of appendages. The mycelioid appendage of the genus Sphaerotheca and other herb-parasitic genera seems to have evolved convergently at multiple times as an adaptation to herb-parasitism.

**INTRODUCTION**

Powdery mildew fungi belong to the order Erysiphales of the phylum Ascomycota in the Fungi (Hawksworth et al. 1995). Braun (1987) described 18 genera and 435 species of powdery mildews in his monograph. Powdery mildews are obligately parasitic to angiosperm plants and cannot be cultured on artificial media. Biotopes of the fungi are strictly restricted to their host plants. Changes in host range directly caused niche separation and thus may have triggered speciation in powdery mildews. It is possible that studying the evolutionary history of powdery mildews may not only reveal aspects of fungal evolution, but may also lead us to consider the evolutionary history of angiosperm plants.

The morphology of the powdery mildew ascoma (cleistothecium) is an important taxonomic character. Fifteen of 18 genera described by Braun (1987) possess more than one ascus in their cleistothecia; the remainig three genera, Cystotheca, Podosphaera, and Sphaerotheca, contain only one ascus in their cleistothecia. These three genera have been considered as a taxonomic unit, tribe Cystotheceae, based on this morphological character (Braun 1987). Phylogenetic analysis using the combined sequences of the 18S, 5.8S, and 28S ribosomal DNA (rDNA) also indicated that these three genera are closely related each other (Mori, Sato & Takamatsu 2000). In their analysis, Mori and co-workers indicate the poly-ascal genus Sawadaea is the sister taxon to the tribe Cystotheceae. These four genera create a monophyletic lineage designated as the fibrosin-lineage, because it contains only genera which possess distinct fibrosin bodies in their conidia and conidophores.

The three genera of the tribe Cystotheceae are easily distinguished from each other by their cleistothecial morphology, especially the appendage morphology, i.e., dichotomously branched appendages in Podosphaera, mycelioid appendages in Sphaerotheca, and only a few short simple appendages in Cystotheca. Of these genera, the genus Sphaerotheca has been regarded as ancestral, and Cystotheca and Podosphaera are considered to be derived from Sphaerotheca (Blumer 1933, Braun 1987). This evolutionary hypothesis is based on the

assumption that the mycelioid appendage is an ancestral character of powdery mildews. However, there has been no satisfactory explanation as to why the mycelioid appendage is considered ancestral, and the phylogenetic relationships of the tribe Cystotheceae requires re-examination using more objective data.

In this study, we construct phylogenetic trees from the nucleotide sequences of the rDNA internal transcribed spacer (ITS) region (including the 5.8S rDNA) for 28 taxa of the tribe Cystotheceae. We then discuss the evolutionary history of those taxa with special reference to their morphology and host plants.

## MATERIALS AND METHODS

### *Sample sources*

The powdery mildew species used in this study, their original hosts, and accession numbers of the nucleotide sequence databases (DDBJ, EMBL, and GenBank) are given in Table 1. The data set includes 30 taxa, of which 18 belong to the genus Sphaerotheca, eight to Podosphaera and two to Cystotheca. Two other taxa, Sawadaea polyfida and Saw. tulasnei, were included as outgroups to the tribe Cystotheceae ingroup. Species were identified by morphological characters of the teleomorph according to the monographs of Nomura (1997) and Braun (1987). Sphaerotheca aphanis var. aphanis on Fragaria grandiflora, S. pannosa on Rosa sp., S. fusca on Taraxacum officinale, S. ferruginea var. ferruginea on Sanguisorba officinalis, and P. leucotricha on Malus domestica were identified by the morphology of their anamorph and host plants because their teleomorphic specimens could not be obtained. The specimens were preserved as herbarium specimens in Mie University Mycological Herbarium (MUMH) or in Herbarium of Toyama Prefectural University (TPU).

### *DNA extraction and amplification of rDNA ITS sequences*

Whole-cell DNA was isolated from cleistothecia or mycelia by the chelex method (Walsh, Metzger & Higuchi 1991, Hirata & Takamatsu 1996). The nuclear rDNA region spanning the ITS1, ITS2

and 5.8S rRNA gene was amplified twice by PCR (polymerase chain reaction). Primers ITS5 (White et al. 1990) and P3 (Kusaba & Tsuge 1995) were used for the first amplification, which was performed in a total reaction volume of 50 µl, including the following reagents: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin); 200 µM of each deoxyribonucleotide triphosphate; 0.4 µM of each primer with 10 µl of the template DNA solution; and one unit of Tth DNA polymerase (Toyobo, Japan). The PCR reaction mixture was overlaid with 30 µl of mineral oil. The following thermal cycling conditions were performed in a thermal cycler PC-700 (ASTEC, Japan): an initial denaturing step at 95°C for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 sec at 95°C followed by 30 sec at 52°C for annealing, and 30 sec at 72°C for extension; and a final extension cycle of 7 min at 72°C. One microliter of the first amplification mixture was used for the second amplification using the nested primer set ITS1 (White et al. 1990) and P3. Components of the reaction mixture and the thermal cycle conditions for the second amplification were the same as for the first one. Each PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. The DNA product of each amplification was then excised from the ethidium bromide-stained gel and purified using the JETSORB kit (GENOMED, Germany) following the manufacturer's protocol.

***DNA sequencing***

Nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in an Applied Biosystems 373A DNA sequencer. The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) following the manufacturer's protocol. Six primers, ITS1, ITS2 (White et al. 1990), P3, T2, T3, and T4 (Hirata & Takamatsu 1996), were used for the sequencing in both directions.

***Data analysis***

The obtained sequences were initially aligned using the Clustal V package (Higgins, Bleaby & Fuchs 1992). The alignment was then visually refined with a word processing program with colour coded nucleotides. Regions that could not be aligned were excluded from the analysis. Phylogenetic trees were obtained from the data using the parsimony, distance and maximum likelihood method.

For parsimony analysis, we used the maximum parsimony method with a heuristic search using the PAUP version 3.1.1 computer package (Swofford 1993). This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. All nucleotide substitutions were equally weighted and unordered. Alignment gaps were treated either as missing data, "fifth base", or indel codes. The strength of the internal branches from the resulting trees were tested by bootstrap analysis using 1000 replications (Felsenstein 1985) and by decay indices (Bremer 1988, Donoghue et al. 1992).

For distance analysis, DNADIST in PHYLIP version 3.5 (Felsenstein 1989) was used to obtain a distance matrix of using Kimura's two parameter correction for multiple hits (Kimura 1980). The distance matrix was then analyzed by NEIGHBOR, which has algorithms based on the neighbour-joining method of Saitou & Nei (1987). For maximum likelihood analysis, we used the MOLPHY version 2.3 computer program package (Adachi & Hasegawa 1996). To obtain a starting tree topology, NucML of MORPHY was used to make a distance matrix, then the distance matix was introduced into NJdist to produce a neighbour-joining tree. The neighbour-joining tree topology was then introduced into NucML with the aligned data set, and a maximum likelihood tree was estimated heuristically using the local rearrangement search option of NucML.

Trees based on nucleic acid variations were tested against phylogenies based on morphological characters by the Kishino-Hasegawa test (Kishino & Hasegawa 1989). A user-defined constraint tree was first constructed using the morphological data with the MacClade program (Maddison & Maddison 1992), and then the most parsimonious tree consistent with the constraint tree was identified using the heuristic search described above. We then used the program NucML of MORPHY to calculate the likelihood of obtaining our data given the most parsimonious tree or the constraint trees. If the log-likelihood of the most parsimonious tree was two standard deviations greater than the log-likelihood of any given constraint tree, we rejected the constraint tree and its underlying morphological hypothesis (Kishino & Hasegawa 1989).

The $g_1$ statistic (Hills & Huelsenbeck 1992) was calculated by computing the tree-length distribution of 100,000 random parsimony trees using the RANDOM TREES command of PAUP 3.1.1. Consistency Index (CI) (Kluge & Farris 1969) and Retention Index (RI) (Farris 1989) were also calculated.

**RESULTS**

Alignment of the rDNA ITS region, including the 5.8S rDNA, resulted in a matrix of 496 characters. ITS 1 of the ingroup taxa ranged in length from 174 base pairs (bp) in the Sphaerotheca section Sphaerotheca species, and Podosphaera spp. on Cercidiphyllum japonicum, Spiraea japonica, Spiraea niopponica and Malus domestica to 184 bp in two Cystotheca species. ITS 2 ranged in length from 144 bp in the species belonging to Sphaerotheca section Magnicellulatae (except S. intermedia) to 149 bp in the two Cystotheca species and S. pannosa. The 5.8S region included in the study was invariant in length. The G + C content of the ingroup taxa ranged from 0.55 in the species of Sphaerotheca section Sphaerotheca to 0.59 in the species of Sphaerotheca section Magnicellulatae and Cystotheca. The outgroup taxa had a G + C content of 0.61. The aligned sequences are available upon request to the first author.

For the aligned data set, 21 bp of the 3'-end of the ITS 2 region could not be aligned unequivocally and was excluded from the phylogenetic analysis. Alignment of the remaining 475 bp required the insersion of 16 gaps, eight of which were 1 bp in length. Twelve of the gaps were phylogenetically informative and comprise eight 1-bp, two 2-bp, and two 3-bp gap insertions. All contiguous gap insertions were less than 4 bp except for Sawadaea tulasnei, which required insertion of a 10-bp gap near the centre of ITS 1. For analysis of the data set with gaps treated as missing data, there were 113 (23.8%) parsimony-informative sites. Including gaps as informative characters increased the number of informative sites to 123 (25.9%). Using indel code increased the number of informative sites to 125.

To evaluate the degree of skewness in 100,000 randomly generated trees, $g_1$ statistics was used. For trees generated from analyses with gaps treated as missing and as "fifth base", values of $g_1$ =-0.516 and -0.532 were obtained, respectively. These results indicate that these data sets are skewed significantly from random ($P<0.01$ for $g_1 = -0.09$ for 500 characters, >25 taxa) which indicates that there is a high level of phylogenetic signalling in the ITS sequences (Hills & Huelsenbeck 1992).

Relevant data from the analyses with gaps treated as missing data, "fifth base", or indel codes are presented in Table 2. The "gaps missing" treatment yielded 17 equally parsimonious trees having CI = 0.686, and RI = 0.841. The analysis using indel code yielded 34 equally parsimonious trees with CI (0.689) and RI (0.844) values similar to the "gaps missing" treatment. Treating gaps as a "fifth base" appeared to result in trees with higher information content, as this tree showed higher bootstrap supports and higher CI (0.716) and RI (0.850) values compared with the above two treatments. The topologies of the strict consensus trees based on the three kinds of treatments were completely identical. The strict consensus tree obtained by the "fifth base" treatment with bootstrap values of 1000 replicates and decay indices is shown in Fig. 1. The neighbour-joining and maximum likelihood analyses yielded almost identical tree topologies to the most parsimonious tree shown in Fig. 1 (data not shown).

The first split within the Cystotheceae occurred between a clade composed of Cystotheca (Cystotheca clade) and a clade composed of Podosphaera and Sphaerotheca (Pod/Sph clade). Both clades were strongly supported by bootstrap analysis (100% in the Cystotheca clade and 88% in the Pod/Sph clade) and by decay analysis (19 and 5, respectively). The Pod/Sph clade was then divided into two subclades. Podosphaera and Sphaerotheca did not group into separate monophyletic subclades. Instead, Podosphaera species parasitic to Prunus and Sphaerotheca section Magnicellulatae grouped together (Magnicellulatae subclade, 73% bootstrap support), and the remaining Podosphaera species and Sphaerotheca section Sphaerotheca formed another subclade (Sphaerotheca subclade,

95% bootstrap support). To evaluate the robustness of the present result, a constraint tree was generated based on the hypothesis that Podosphaera and Sphaerotheca combine into a separate monophyletic group (Fig. 2). The hypothetical tree was compared with the most parsimonious tree (Fig. 1) by the Kishino-Hasegawa test (Kishino & Hasegawa 1989). As a result, the log-likelihood difference of the phylogenetic trees increased to 5.14 times the standard deviation, and thus the hypothetical tree was rejected (Table 3).

In both the Sphaerotheca and Magnicellulatae subclades, initial splits were usually shared by Podosphaera species and the two sections of Sphaerotheca were derived independently from the two Podosphaera groups. In the Sphaerotheca subclade, Podosphaera sp. on Pourthiaea vilosa, P. cercidiphylli on Cercidiphyllum japonicum, and P. leucotricha on Malus domestica occupied a basal position to the remaining taxa. Sphaerotheca section Sphaerotheca did not form a distinct monophyletic clade, instead it formed a clade consisting of two isolates of P. clandestina on Spiraea japonica and Spiraea niopponica, with low (53%) bootstrap support. The genetic diversity between the two isolates of P. clandestina and the species of Sphaerotheca section Sphaerotheca was only 0.9%-1.8%, which was nearly identical to the divergence solely within Sphaerotheca section Sphaerotheca.

The 10 taxa of Sphaerotheca section Sphaerotheca used in this analysis were divided into three small clades. Three Sphaerotheca spiraeae isolates from three different Spiraea species formed a clade with S. ferruginea var. ferruginea on Aruncus dioicus var. tenuifolius. On the other hand, each of the two isolates of S. aphanis var. aphanis and S. ferruginea var. ferruginea did not group to form a clade. In the Magnicellulatae subclade, P. tridactyla and P. longiseta occupied the basal position to the remaining taxa. Sphaerotheca section Magnicellulatae formed a monophyletic subclade with moderately high bootstrap (75%) and decay indice (3) supports. S. fuliginea var. sibirica occupied a basal position of the subclade, and the remaining taxa within Sphaerotheca section Magnicellulatae formed a distictive clade strongly supported both in bootstrap (99%) and decay index (5) analyses.

**DISCUSSION**

In this study, we examined the phylogenetic relationships of the tribe Cystotheceae based on the nucleotide sequences of the rDNA ITS region. The phylogenetic trees produced by three different gap treatments and three different algorithms commonly showed that the genera Podosphaera and Sphaerotheca do not group into separate monophyletic clades. Instead each two sections of Sphaerotheca form different clades with two Podosphaera groups together respectively. We evaluated traditional taxonomic system by constructing a constraint tree that separated Podosphaera and Sphaerotheca into separate monophyletic groups, found the most parsimonious trees given in the constraint, and used likelihood methods to compare such a grouping to the best tree. The Kishino-Hasegawa test showed that the likelihood of a tree grouping Podosphaera and Sphaerotheca into separate monophyletic groups was more than five standard deviations lower than the best tree (Table 3). Our data, therefore, clearly rejected the morphological hypothesis that Podosphaera and Sphaerotheca form separate monophyletic clades.

Based on the analysis of the combined nucleotide sequences of the 18S, 5.8S and 28S rDNA, and the ITS region, Mori et al. (2000) have also indicated that Sphaerotheca section Magnicellulatae is phylogenetically closer to Podosphaera section Tridactyla than to Sphaerotheca section Sphaerotheca. However, the relationship between Podosphaera section Podosphaera and Sphaerotheca section Sphaerotheca is unclear from this analysis because Podosphaera section Podosphaera was not involved in the study.

In both the Sphaerotheca and Magnicellulatae subclades, initial splits were shared by Podosphaera species, indicating that the ancestral feature of both subclades was Podosphaera-like. In the traditional evolutionary system, the genus Sphaerotheca was considered to be the most ancestral genus within the tribe Cystotheceae, with Cystotheca and Podosphaera derived from Sphaerotheca (Blumer 1933, Braun 1987). The present phylogenetic trees, however, do not support this evolutionary system, instead they strongly suggest that the genus

Sphaerotheca was derived form Podosphaera-like ancestral taxa. The present analysis also suggests that Sphaerotheca is not monophyletic and the two sections of Sphaerotheca were derived from two groups of Podosphaera, on at least two independent occasions. The mycelioid appendages of the genus Sphaerotheca, therefore, may not imply monophyly of the genus. Instead, the appendages may have been generated from at least two times independent events.

The genera Podosphaera and Sphaerotheca can be distinguished each other by the characteristic morphology of their appendages; dichotomously branched appendages in Podosphaera and mycelioid appendages in Sphaerotheca. In the Sphaerotheca subclade, the genetic divergence between two isolates of P. clandestina and the species of Sphaerotheca section Sphaerotheca was almost identical to the divergence within Sphaerotheca section Sphaerotheca. This implies that P. clandestina is phylogenetically close to the species of Sphaerotheca section Sphaerotheca, although they can be distinctly differentiated by their appendages morphology. The Erysiphaceae has a variety of cleistothecial appendages and their morphology has been considered to show both intergenic and interspecies variations, but has sufficient intraspecies stability to identify genera or species of powdery mildews. However, the present result might indicate that the morphology of appendages could change within a short period.

A cladogram of the major clades obtained by the present analysis and their host plants is shown in Fig. 3. The host plant data were extracted from the database "Host plants of the powdery mildew fungi ver. 1.0" (http://sansui.bio.mie-u.ac.jp/seisan/byori/download.html, Takamatsu & Sato 1997), which was based on the table "Host plants of powdery mildew fungi and their distribution by country" of Amano's book (Amano 1986). The genus Sawadaea, which was used as the outgroup taxa for this analysis, can infect 83 plant species in the world, of which 78 host plants (94.0%) are the genus Acer of Aceraceae. The remaining five host plants belong to Sapindaceae (3) and Hippocastanaceae (2). The genus Cystotheca can infect 62 host plants, all of which are Fagaceae. These results indicate that Sawadaea and Cystotheca

are parasites specialized to Aceraceae and Fagaceae, respectively. Since both the plant families include only woody plants, both fungal genera can be regarded as arbor-parasitic. The genus Podosphaera has 250 host plants, of which 216 hosts (86.4%) belong to Rosaceae including the genera Crataegus, Malus, Prunus, Pyrus, Sorbus, and Spiraea. The remaining 34 hosts are scattered amongst 12 plant families including Ericaceae (14), Hamamelidaceae (4), and Caprifoliaceae (4). The genus Podosphaera, therefore, is considered to be concentrically parasitic to Rosaceae and sporadically to other plant families. Almost all of the hosts of Podosphaera are woody plants, indicating that Podosphaera is also arbor-parasitic. On the other hand, Sphaerotheca section Sphaerotheca has many (806) host plants compared with Sawadaea, Cystotheca, and Podosphaera. Of these, 457 (56.7%) hosts are Rosaceae and the remaing 349 hosts are scattered amongst 27 plant families including Euphorbiaceae (70), Geraniaceae (67), Onagraceae (65), Saxifragaceae (54), and Polemoniaceae (25). Most of these host plants are shrubs or herbs. Sphaerotheca section Sphaerotheca is, thus, considered to be shrub- or herb-parasitic. Sphaerotheca section Magnicellulatae also has many host plants and a wide host range. Out of the total 1110 host plants, 496 (44.7%) are Asteraceae and 206 (18.6%) are Scrophulariaceae. The remaining 408 host plants are scattered amongst 38 plant families including Cucurbitaceae (62), Fabaceae (55), Brassicaceae (31), Dipsacaceae (31), Ranunculaceae (30), and Lamiaceae (27). Most of these hosts are herbaceous plants, indicating that Sphaerotheca section Magnicellulatae is herb-parasitic. It is worth noting that the Rosaceae is not included in the host family of the section Magnicellulatae.

Summarizing the above results, basal taxa of the tribe Cystotheceae (Cystotheca and Podosphaera), including outgroup taxa (Sawadaea), can be regarded as arbor-patasitic, and are concentrically parasitic to a narrow range of plant families. On the other hand, the derived taxa (two Sphaerotheca sections) are herb- or shrub-parasitic, and have a wide range of host plant families. This suggests that ancestral fungi of the tribe Cystotheceae were originally arbor-parasitic, and transition from arbor-parasitism to herb-parasitism may have occurred on at least two independent occasions. One transition route can be found in the Sphaerotheca subclade. In this subclade, Rosaceae is shared by Podosphaera and Sphaerotheca section

Sphaerotheca as the largest host family, which includes many trees, shrubs and herbs. The present result may support the Amano's evolutionary hypothesis (Amano 1992) that the transition of parasitism (arbor-parasitism and herb-parasitism) in the tribe Cystotheceae occurred in Rosaceae, although in his system the direction of transition was from herb-parasitism to arbor-parasitism.

The other transition route is found in the Magnicellulatae subclade. Unlike to the Sphaerotheca subclade, Podosphaera and Sphaerotheca section Magnicellulatae do not have a common plant family as their major host families. Sphaerotheca section Magnicellulatae is defined by the large outer peridium cells of its cleistothecia (more then 30 µm), and can be distinguished from the section Sphaerotheca by its smaller peridium cells (below 20 µm) (Braun 1987). All other taxa of the tribe Cystotheceae and Sawadaea have small peridium cells similar to those of the section Sphaerotheca. Thus, the large peridium cells can be regarded as a synapomorphic character for the section Magnicellulatae. There are Sphaerotheca species, S. fuliginea var. sibirica and S. intermedia, which have peridium cells of intermediate size (20-30 µm). Braun (1987) regarded these species as intermediates between the sections Sphaerotheca and Magnicellulatae. In the present phylogenetic trees, these species were placed in the Magnicellulatae subclade and formed a distinct clade with the taxa of the section Magnicellulatae. S. fuliginea var. sibirica occupied a basal position to the other taxa of the section Magnicellulatae. These species should be regarded as intermediates between Podosphaera and Sphaerotheca section Magnicellulatae rather than between the sections Sphaerotheca and Magnicellulatae of the genus Sphaerotheca.

Teleomorphic change is accompanied by anamorphic change. Hirata (1942, 1955) reported that germ tubes and germination patterns of conidia provide good, useful diagnostic and taxonomic characters for the Erysiphaceae. Braun (1987) proposed four types of germination patterns of conidia according to Hirata (1942, 1955). Cystotheca, Podosphaera, Sphaerotheca section Sphaerotheca and Sawadaea have fairly long, slender germ tubes (pannosa-type). On the other hand, germ tubes of Sphaerotheca section Magnicellulatae are

always short, characteristically forked and broadened, and often dichotomously branched (fuliginea-type). Germ tubes of S. fuliginea var. sibirica and S. intermedia share characteristics with the pannosa-type, and are different from the fuliginea-type. These morphological characteristics strongly support the present phylogenetic placement of the two species, which were placed intermediate between Podosphaera section Tridactyla and Sphaerotheca section Magnicellulatae. S. fuliginea var. sibirica has only a single plant species (Veronicastrum sibiricum) as host. However, S. fuliginea var. fuliginea has many host plants in the Scrophulariaceae. Nucleotide sequences of S. fuliginea var. fuliginea on Scrophulariaceae are required to obtain further information regarding the evolutionary history within the Magnicellulatae subclade.

In conclusion, the expansion of host range from woody plants to herbaceous plants was probably one of the major evolutionary events for powdery mildews. In the present study, we suggest that the transition from arbor-parasitism to herb-parasitism has occurred on at least two independent occasions in the tribe Cystotheceae. Similar transitions of parasitism have also been observed in other evolutionary lineages of powdery mildews (Mori et al. 2000, Takamatsu et al. 1999). These transitions may accompany morphological changes in cleistothecial appendage. Blumer (1933), Braun (1987) and many other authors have regarded the mycelioid appendage as an ancestral character, and have placed Erysiphe-like ancester with mycelioid appendages at the base of their evolutionary system. However, there is no satisfactory explanation as to why they considered the mycelioid appendages to be the most ancestral character in the powdery mildews. Amano (1986) pointed out that the powdery mildew fungi could be generally classified into herb-parasitic and arbor-parasitic genera. All of the herb-parasitic genera have mycelioid appendages on their cleistothecia. Mori et al. (2000) pointed out the possibility that the morphology of the appendages was under selection pressure that depended on the habit of their host plants, woody or herbaceous, since appendages have an important role in overwintering by cleistothecia. Based on the present analysis as well as our previous reports (Mori et al. 2000, Takamatsu et al. 1999), it is more

likely that mycelioid appendage has convergently evolved at multiple times accompanied by the host expansion to herbaceous plants.

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**FIGURE LEGENDS**

Fig. 1. Strict consensus of 17 equally parsimonious trees inferred from sequences of ITS1, ITS2, and the 5.8S rRNA gene from 28 tribe Cystotheceae DNAs plus two outgroup taxa, using "gaps = newstate" option of PAUP version 3.1.1. The bootstrap values of 1000 replications are shown on the respective branch. Decay indices are shown below the branches. The consistency index (CI) is 0.716; the retention index (RI) is 0.850; and the rescaled consistency index (RC) is 0.609.

Fig. 2. A constraint tree for Kishino-Hasegawa test. The ITS data does not support the monophyly of Podosphaera and Sphaerotheca. A Kishino-Hasegawa test was used to evaluate the most parsimonious arrangement of Podosphaera and Sphaerotheca (Fig. 1) against a tree constraining Podosphaera and Spaerotheca into separate monophyletic groups. The monophyly of Podosphaera and Sphaerotheca was rejected by the Kishino-Hasegawa test (Table 3).

Fig. 3. Cladogram of the major clades of the tribe Cystotheceae and their host plants. The numeral within the parenthesis shows the number of host plants in that respective plant family.

Table 1. Sources of fungal materials and sequence data base accession numbers

| Fungal species[a] | Host plant |
|---|---|
| **Cystotheca** | |
| C. lanestris (Harkn.) Miyabe | Quercus agrifolia Nee |
| C. wrightii Berk. et Curt. | Quercus glauca Thunb. ex Murray |
| **Podosphaera section Podosphaera** | |
| P. cercidiphylli Tanda et Y. Nomura | Cercidiphyllum japonicum Sieb. et Zucc. |
| P. clandestina (Wallr. ex Fr.) Lév. var. clandestina | Spiraea japonica L. fil. |
| P. clandestina (Wallr. ex Fr.) Lév. var. clandestina | Spiraea niopponica Maxim. |
| Podosphaera sp. | Pourthiaea vilosa (Thunb.) Decaisne var. laevis (Thunb.) Stapf. |
| **Podosphaera section Tridactyla** | |
| P. leucotricha (Ell. et Everh.) Salm. | Malus domestica Borkh. (Apple tree) |
| P. longiseta Sawada | Prunus grayana Maxim. |
| P. tridactyla (Wallr.) De Bary var. tridactyla | Prunus japonica Thunb. ex Murray |
| Podosphaera sp. | Prunus apetata (Sieb. et Zucc.) Franch. et Savat. subsp. pilosa (Koidz.) H. Ohba |
| **Sawadaea** | |
| Saw. polyfida (Wei) Zheng et Chen var. japonica U.Braun et Tanda | Acer palmatum Thunb. |
| Saw. tulasnei (Fuckel) Homma | Acer mono Maxim. |
| **Sphaerotheca section Sphaerotheca** | |
| S. aphanis (Wallr.) U. Braun var. aphanis | Agrimonia pilosa Ledeb. var. japonica (Miq.) Nakai |

Table 1. Sources of fungal materials and sequence data base accession numbers

| Fungal species[a] | Host plant |
|---|---|
| S. aphanis (Wallr.) U. Braun var. aphanis | Fragaria grandiflora Ehrh. (Strawberry) |
| S. ferruginea (Schl. ex Fr.) Junell var. ferruginea | Aruncus dioicus (Walt.) Fern. var. tenuifolius (Nakai) Hara |
| S. ferruginea (Schl. ex Fr.) Junell var. ferruginea | Sanguisorba officinalis L. |
| S. filipendulae Zhao | Filipendula purpurea Maxim. var. purpurea |
| S. fugax Penz. et Sacc. | Geranium nepalense Sweet subsp. thunbergii (Sieb. et Zucc.) Hara |
| S. pannosa (Wallr. : Fr.) Lév. | Rosa sp. (Rose) |
| S. spiraeae Sawada em. U. Braun | Spiraea cantoniensis Lour. (Reeves spires) |
| S. spiraeae Sawada em. U. Braun | Spiraea japonica L. fil. |
| S. spiraeae Sawada em. U. Braun | Spiraea thunbergii Sieb. ex Blume |
| **Sphaerotheca section Magnicellulatae** | |
| S. cucurbitae (Jacz.) Z.Y.Zhao | Cucumis sativus L. (Cucumber) |
| S. elsholtziae Zhao | Ajuga reptans L. |
| S. fuliginea (Schlecht. et Fr.) Poll. var. sibirica U. Braun | Veronicastrum sibiricum (L.) Pennell subsp. japonicum (Nakai) Yamazaki |
| S. fusca (Fr.) Blumer | Taraxacum officinale Weber (Common dandelion) |
| S. intermedia U. Braun | Clerodendrum trichotomum Thunb. |
| Sphaerotheca sp. | Boehmeria nipononivea Koidz. |
| Sphaerotheca sp. | Cayratia japonica (Thunb.) Gagn. |
| Sphaerotheca sp. | Peristrophe japonica (Thunb.) Bremek. |

Table 1. Sources of fungal materials and sequence data base accession numbers

| Voucher[b] | Database accession no.[c] |
|---|---|
| MUMH114 | AB000933 |
| MUMH137 | AB000932 |
| MUMHS67 | AB026140 |
| MUMH327 | AB026150 |
| MUMH269 | AB026137 |
| MUMH247 | AB026147 |
| MUMH468 | AB027231 |
| MUMH70 | AB000945 |
| MUMHS62 | AB000943 |
| MUMH248 | AB026138 |
| MUMH47 | AB000936 |
| MUMH93 | AB022367 |
| MUMH49 | AB026141 |

Table 1. Sources of fungal materials and sequence data base accession numbers

| Voucher[b] | Database accession no.[c] |
|---|---|
| MUMH335 | AB026136 |
| MUMHS63 | AB026152 |
| MUMH469 | AB027232 |
| TPU-1842 | AB022385 |
| MUMH343 | AB023134 |
| MUMHS41 | AB022348 |
| TPU-1825 | AB026143 |
| TPU-1752 | AB026149 |
| TPU-1877 | AB026153 |
| MUMH65 | AB026146 |
| MUMHS131 | AB026142 |
| MUMH303 | AB026144 |
| N.A.[d] | AB026148 |
| MUMH331 | AB026145 |
| MUMH312 | AB026139 |
| MUMH307 | AB026151 |
| MUMH313 | AB026135 |

Table 1. Sources of fungal materials and sequence data base accession numbers

[a] Fungi were identified using Braun (1987) and Nomura (1997).

[b] MUMH: Mie University Mycological Herbarium; TPU: Toyama Prefectural University.

[c] The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.

[d] Voucher specimen not available.

Table 2. Results for three parsimony analyses of ITS nucleotide data for 28 tribe Cystotheceae. Values are given for each of three consensus trees derived from the analyses with gaps treated as missing data, "fifth base", or indel codes.

| | Parsimony treatment | | |
|---|---|---|---|
| Attribute | Gaps = missing | Gaps = fifth base | indel codes |
| No. trees | 17 | 17 | 34 |
| No. steps | 299 | 334 | 322 |
| CI | 0.686 | 0.716 | 0.689 |
| RI | 0.841 | 0.850 | 0.844 |
| No. nodes ≥50%[a] | 16 | 16 | 15 |

[a] Number of nodes per cladogram receiving ≥50% bootstrap support.

Table 3. Kishino-Hasegawa test

| Constraint tree | Parsimony tree length (step) | Ln L | Difference Ln L[a] | Standard deviation[b] | T-value[c] | Significantly worse?[d] |
|---|---|---|---|---|---|---|
| Best unconstrainted tree (Fig. 1) | 334 | -2303.9 | | | | |
| Podosphaera and Sphaerotheca monophyletic (Fig. 2) | 363 | -2425.0 | -121.4 | 23.6 | 5.14 | Yes |

a Difference in log-likelihood compared to that of the best tree.

b The standard deviation in log-likelihood.

c The T-value is determined by dividing the difference in log-likelihood by the standard deviation.

d The constraint tree is considered to be significantly worse if the difference in log-likelihood is more than twice the standard deviation.

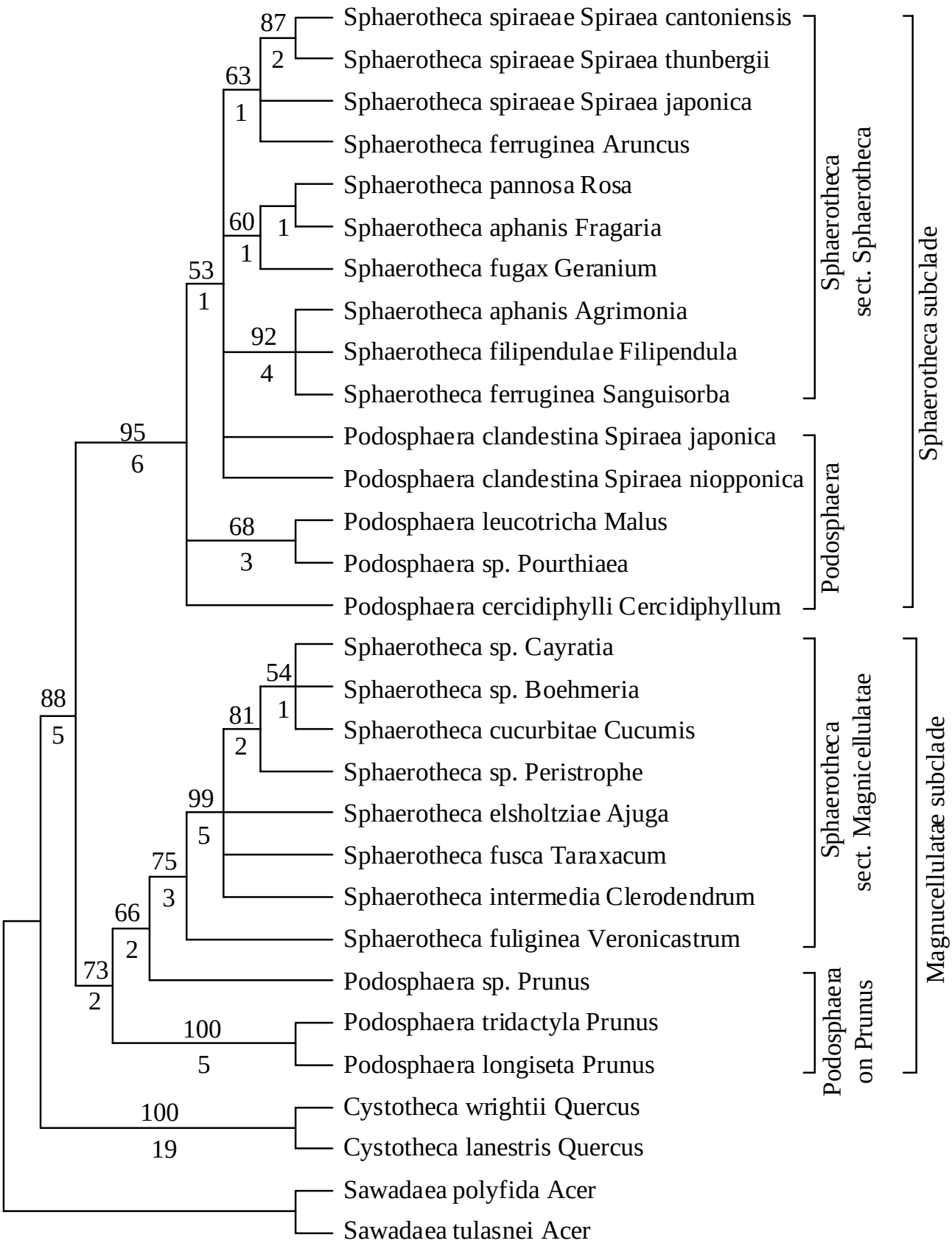
Sphaerotheca spiraeae Spiraea cantoniensis
Sphaerotheca spiraeae Spiraea thunbergii
Sphaerotheca spiraeae Spiraea japonica
Sphaerotheca ferruginea Aruncus
Sphaerotheca pannosa Rosa
Sphaerotheca aphanis Fragaria
Sphaerotheca fugax Geranium
Sphaerotheca aphanis Agrimonia
Sphaerotheca filipendulae Filipendula
Sphaerotheca ferruginea Sanguisorba
Podosphaera clandestina Spiraea japonica
Podosphaera clandestina Spiraea niopponica
Podosphaera leucotricha Malus
Podosphaera sp. Pourthiaea
Podosphaera cercidiphylli Cercidiphyllum
Sphaerotheca sp. Cayratia
Sphaerotheca sp. Boehmeria
Sphaerotheca cucurbitae Cucumis
Sphaerotheca sp. Peristrophe
Sphaerotheca elsholtziae Ajuga
Sphaerotheca fusca Taraxacum
Sphaerotheca intermedia Clerodendrum
Sphaerotheca fuliginea Veronicastrum
Podosphaera sp. Prunus
Podosphaera tridactyla Prunus
Podosphaera longiseta Prunus
Cystotheca wrightii Quercus
Cystotheca lanestris Quercus
Sawadaea polyfida Acer
Sawadaea tulasnei Acer
87
2
63
1
60
1
1
53
1
92
4
95
6
68
3
54
1
81
2
99
5
75
3
66
2
73
2
100
5
88
5
100
19
Sphaerotheca sect. Sphaerotheca
Podosphaera
Sphaerotheca subclade
Sphaerotheca sect. Magnicellulatae
Podosphaera on Prunus
Magnucellulatae subclade

**Fig. 1**

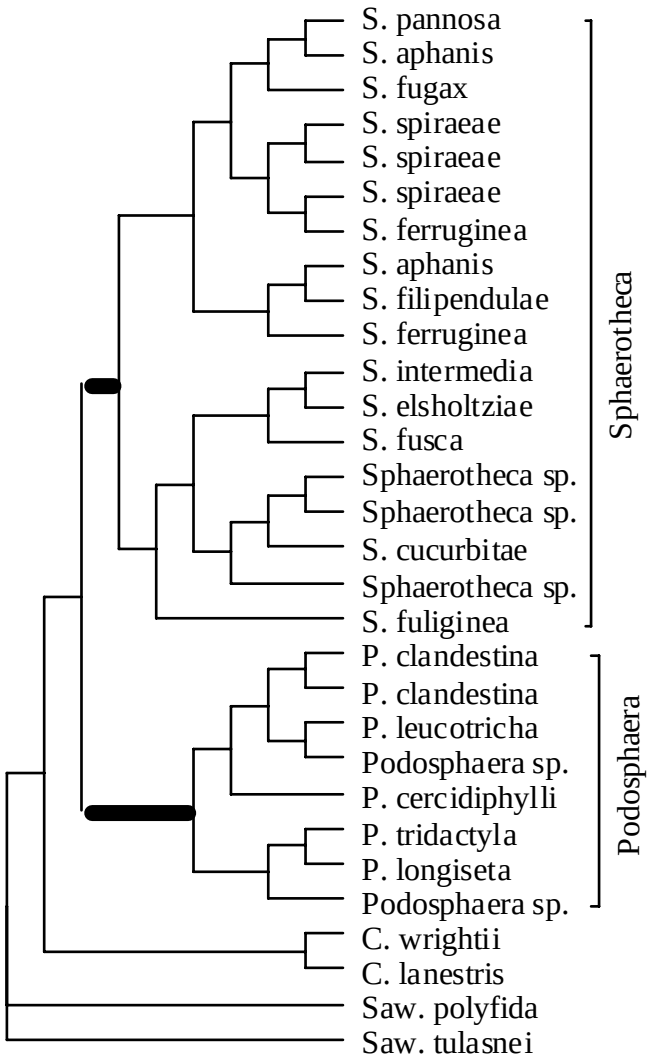
S. pannosa
S. aphanis
S. fugax
S. spiraeae
S. spiraeae
S. spiraeae
S. ferruginea
S. aphanis
S. filipendulae
S. ferruginea
S. intermedia
S. elsholtziae
S. fusca
Sphaerotheca sp.
Sphaerotheca sp.
S. cucurbitae
Sphaerotheca sp.
S. fuliginea
P. clandestina
P. clandestina
P. leucotricha
Podosphaera sp.
P. cercidiphylli
P. tridactyla
P. longiseta
Podosphaera sp.
C. wrightii
C. lanestris
Saw. polyfida
Saw. tulasnei
Sphaerotheca
Podosphaera

**Fig. 2**

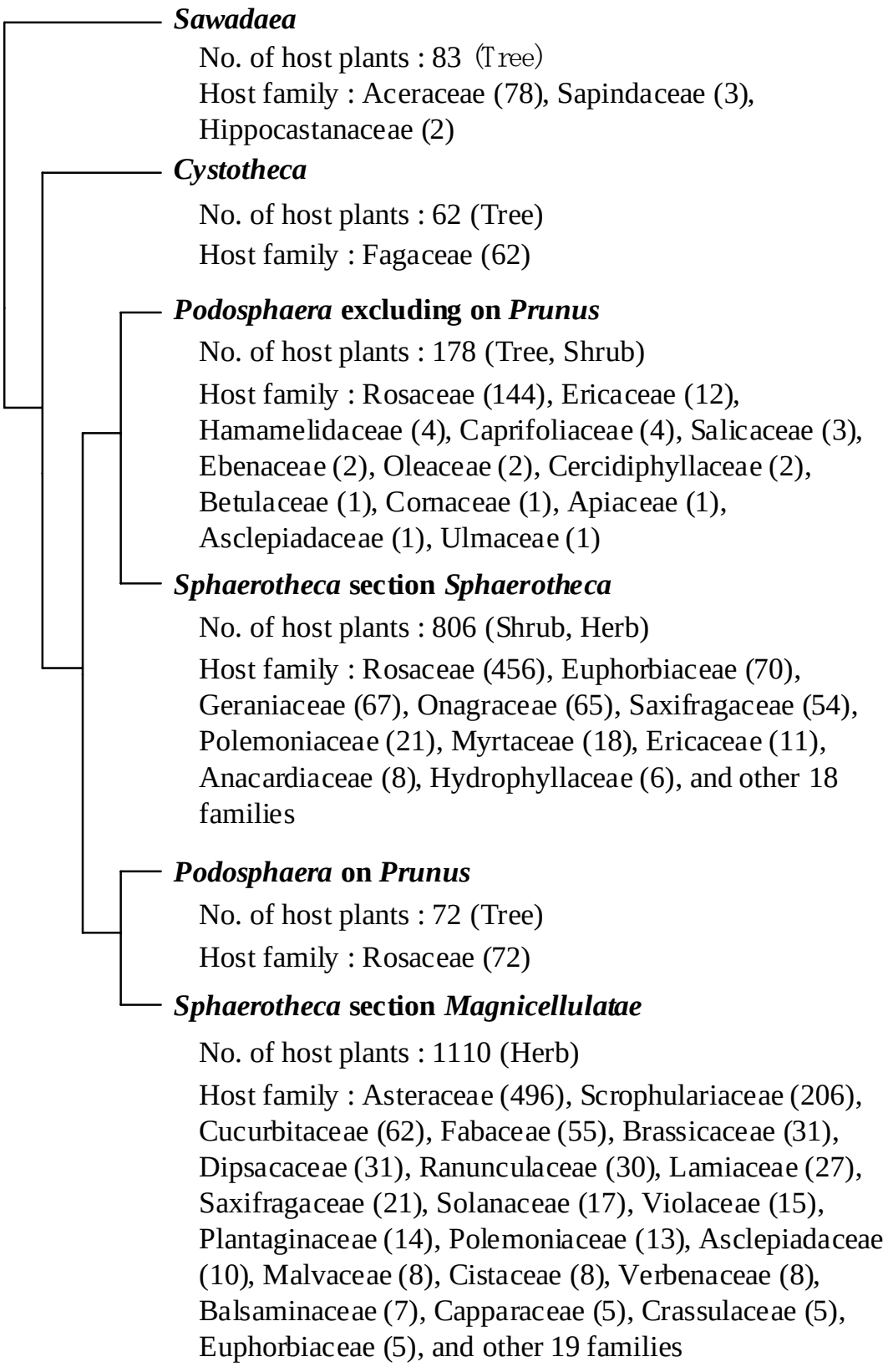
*Sawadaea*
No. of host plants : 83 (Tree)
Host family : Aceraceae (78), Sapindaceae (3), Hippocastanaceae (2)
*Cystotheca*
No. of host plants : 62 (Tree)
Host family : Fagaceae (62)
*Podosphaera* excluding on *Prunus*
No. of host plants : 178 (Tree, Shrub)
Host family : Rosaceae (144), Ericaceae (12), Hamamelidaceae (4), Caprifoliaceae (4), Salicaceae (3), Ebenaceae (2), Oleaceae (2), Cercidiphyllaceae (2), Betulaceae (1), Cornaceae (1), Apiaceae (1), Asclepiadaceae (1), Ulmaceae (1)
*Sphaerotheca* section *Sphaerotheca*
No. of host plants : 806 (Shrub, Herb)
Host family : Rosaceae (456), Euphorbiaceae (70), Geraniaceae (67), Onagraceae (65), Saxifragaceae (54), Polemoniaceae (21), Myrtaceae (18), Ericaceae (11), Anacardiaceae (8), Hydrophyllaceae (6), and other 18 families
*Podosphaera* on *Prunus*
No. of host plants : 72 (Tree)
Host family : Rosaceae (72)
*Sphaerotheca* section *Magnicellulatae*
No. of host plants : 1110 (Herb)
Host family : Asteraceae (496), Scrophulariaceae (206), Cucurbitaceae (62), Fabaceae (55), Brassicaceae (31), Dipsacaceae (31), Ranunculaceae (30), Lamiaceae (27), Saxifragaceae (21), Solanaceae (17), Violaceae (15), Plantaginaceae (14), Polemoniaceae (13), Asclepiadaceae (10), Malvaceae (8), Cistaceae (8), Verbenaceae (8), Balsaminaceae (7), Capparaceae (5), Crassulaceae (5), Euphorbiaceae (5), and other 19 families

**Fig. 3**