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Molecular phylogeny and radiation time of Erysiphales inferred from the nuclear ribosomal DNA sequences*

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Phylogenetic relationships of Erysiphales within Ascomycota were inferred from the newly determined sequences of the 18S rDNA and partial sequences of the 28S rDNA including the D1 and D2 regions of 10 Erysiphales taxa. Phylogenetic analyses revealed that the Erysiphales form a distinct clade among ascomycetous fungi, suggesting that the Erysiphales diverged from a single ancestral taxon. The Myxotrichaceae of the Onygenales was distantly related to the other onygenalean families and was the sister group to the Erysiphales clade, with which it combined to form a clade. The Erysiphales/Myxotrichaceae clade was also closely related to some discomycetous fungi (Leotiales, Cyttariales and Thelebolaceae) including taxa that form cleistothecial ascomata. The present molecular analyses as well as previously reported morphological observations suggest the possible exsistence of a novel evolutionary pathway from cleistothecial discomycetous fungi to Erysiphales and Myxotrichaceae. However, since most of these fungi, except for the Erysiphales, are saprophytic on dung and/or plant materials, the questions of how and why an obligate biotroph like the Erysiphales radiated from the saprophytic fungi remain to be addressed. We also estimated the radiation time of the Erysiphales using the 18S rDNA sequences and the two molecular clocks that have been previously reported. The calculation showed that the Erysiphales split from the Myxotrichaceae 190–127 myr ago. Since the radiation time of the Erysiphales does not exceed 230 myr ago, even when allowance is made for the uncertainty of the molecular clocks, it is possible to consider that the Erysiphales evolved after the radiation of angiosperms. The results of our calculation also showed that the first radiation within the Erysiphales (138–92 myr ago) coincided with the date of a major diversification of angiosperms (130–90 myr ago). These results may support our early assumption that the radiation of the Erysiphales coincided with the evolution of angiosperm plants.

Key Words——Ascomycota; evolution; molecular clock; plant pathogen; powdery mildew.

The fungi belonging to the Erysiphales (powdery mildews) are obligate biotrophs that infect a wide range of angiosperm plants and appear as white, powdery material on the leaves, stems, or fruits. Braun (1987) described one family, 18 genera, and 435 species of Erysiphales in his monograph. This fungal group has the following unique characteristics as plant pathogenic fungi.

1) Obligate biotrophy: Biotope of Erysiphales is completely restricted to living plants and lacks saprophytic life stages. Several plant pathogenic fungi such as rusts and downy mildews are also known as obligate biotrophs. Although the obligate biotrophic nature is not unique to Erysiphales, it is an important characteristic in considerring the evolutionary history of these fungi.

2) Ectotrophy: All but a few genera of Erysiphales are ectotrophic fungi. Their mycelia usually distribute on host surface and put haustoria, a nutrient-absorbing organ, into the epidermal cells of the infected plants. Ectotrophy is unique to the species of Erysiphales and Meliolales.

3) Xerophytism: Almost all plant pathogenic fungi require free water for their spore germination and infection. The fungi of Erysiphales can germinate vigorously and infect host plants under low atomospheric humidity (Yarwood, 1957).

The taxonomic placement of Erysiphales is not completely clarified yet. Erysiphales have been placed in Plectomycetes based on their cleistothecial ascomata without ostiole (Ainsworth et al., 1971; Webster, 1980). On the other hand, the production of asci from a basal hymenium and the forcible discharge of ascospores are used to classify Erysiphales with Pyrenomycetes (Yarwood, 1973, 1978; Alexopoulos and Mims, 1979). To resolve the ambiguities of the taxonomic placement of the Erysiphales, Saenz et al. (1994) determined the nucleotide sequences of the 18S rDNA of *Blumeria graminis* (DC.) Speer and reported that the fungus was not Pyrenomycetes nor Plectomycetes. Instead they reported the close relationship between *B. graminis* and *Sclerotinia sclerotiorum* (Lib.) de Bary (Leotiales). Recently, Sugiyama et al. (1999) determined the small

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subunit rDNA sequences for 19 taxa of the onygenalean fungi including the Myxotrichaceae, and reported that the Myxotrichaceae has its own lineage distantly related to the other onygenalean fungi and closely related to the Leotiales and Erysiphales. Both reports used only one nucleotide sequence of *B. graminis* as representative Erysiphales for their analyses. *Blumeria graminis* is regarded as an isolated fungus among the Erysiphales, because of its unique morphological charactristics and also its unique parasitism to monocots, especially cereal plants, in contrast to the dicot parasitism of most other Erysiphales. Recent molecular works have supported the isolated phylogenetic placement of *B. graminis* (Takamatsu et al., 1998; Saenz and Taylor, 1999; Mori et al., 2000). The nucleotide sequence data from other species of the Erysiphales are required to clarify the phylogenetic relationship of the Erysiphales among Ascomycota more precisely. We thus tried to reevaluate the phylogenetic placement of the Erysiphales using newly determined sequences in this study.

The evolution of the Erysiphales may be closely linked to the evolution of plants, since the biotopes of this fungal group are strictly restricted on the host plants. Amano (1986) described 9838 species spanning 1617 genera of plants as hosts of the Erysiphales. All of the host plants belong to the angiosperms; no gymnosperm plants nor ferns have been reported as hosts. It is thus possible to assume that the radiation of the Erysiphales occurred after the splitting of the angiosperms from the gymnosperms. However, there is no reliable information on the date of the radiation of the Erysiphales. Timing of evolutionary events has been mainly inferred from fossil records. Since no reliable fossil record of the Erysiphales has been found (Tiffney and Barghoorn, 1974; Pirozynski, 1976; Braun, 1987), estimation of radiation time of the Erysiphales by this method is impossible. Molecular phylogenetic techniques allow calculation of the date of evolutionary events of organisms based on the molecular clock concept. We thus tried to calculate the radiation time of Erysiphales based on the molecular clocks that were previously reported.

This study asks when and how this unique fungal group diverged. To address these questions we determined nearly complete sequences of the 18S rDNA for 10 powdery mildew taxa covering nine genera. These newly determined 18S rDNA sequences and the sequences of the D1 and D2 regions of the 28S rDNA that we reported previously (Mori et al., 2000) were used to reconstruct phylogenetic trees with other ascomycetous fungi.

Materials and Methods

Sample Sources Ten taxa of the Erysiphales used in this study, their original hosts, their collection locations, and accession numbers of the nucleotide sequence databases (DDBJ, EMBL, and GenBank) are given in Table 1. In order to cover all the major linegaes of the Erysiphales, we selected two taxa from each of the five major lineages that were recognized in our previous report (Mori et al., 2000). Where possible, species were identified by morphological characters of the teleomorph according to the monographs of Braun (1987) and Nomura (1997). *Arthrocladiella mougeotii* (Lév.) Vassilkov, *B. graminis* f. sp. *bromi, Erysiphe orontii* Cast., and *Leveillula taurica* (Lév.) Arnaud were identified from the morphology of their anamorph and their host plants, because their teleomorphic specimens could not be obtained. *Blumeria graminis* f. sp. *hordei* race H1 was originally isolated by Hiura (1978) and has been maintained in culture on its host plant in the Laboratory of Plant Pathology of Mie University. Most of the specimens were preserved as

Table 1. List of the species of Erysiphales used in this study.

Fungal species[a]	Host plant	Voucher collection and location[b]	Database accession no.[d] 18S rDNA/28S rDNA
Arthrocladiella mougeotii (Lév.) Vassilkov	*Lycium chinense* Miller	MUMH135, Ibaraki, Japan	AB033477, AB022379
Blumeria graminis (DC.) Speer f.sp. *bromi*	*Bromus catharticus* Vahl	MUMH117, Mie, Japan	AB033475, AB022362
B. graminis (DC.) Speer f.sp. *hordei*	*Hordeum vulgare* L. (Barley)	L.I.[c]	AB033480, AB022399
Erysiphe orontii Cast.	*Nicotiana tabacum* L. (Tobacco)	L.I.[c]	AB033483, AB022412
Leveillula taurica (Lév.) Arnaud	*Capsicum annuum* L. var. *grossum*	MUMH124, Kochi, Japan	AB033479, AB022387
Microsphaera friestii Lev. var. *dahurica* U. Braun	*Rhamnus japonica* Maxim. var. *decipiens* Maxim.	MUMH6, Mie, Japan	AB033478, AB022382
Phyllactinia moricola (P. Henn.) Homma	*Morus australis* Poiret (Mulberry)	MUMH35, Mie, Japan	AB033481, AB022401
Sawadaea polyfida (Wei) Zheng & Chen var. *japonica* U. Braun & Tanda	*Acer palmatum* Thunb.	MUMH47, Mie, Japan	AB033476, AB022364
Sphaerotheca cucurbitae (Jacz.) Z.Y. Zhao	*Melothria japonica* (Thunb.) Maxim. ex Cogn.	MUMH68, Mie, Japan	AB033482, AB022410
Uncinula mori I. Miyake	*Morus australis* Poiret (Mulberry)	MUMHS77, Toyama, Japan	AB033484, AB022418

a) Fungi were identified using Braun (1987) and Nomura (1997).
b) MUMH; Mie University Mycological Herbarium.
c) Isolate maintained as a living fungus in the Laboratory of Plant Pathology, Mie University.
d) The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.

herbarium specimens and deposited in the Mie University Mycological Herbarium (MUMH).

PCR Primers The following oligonucleotide primers were used in this analysis. The nucleotide sequences of ITS2, NS1, NS2, NS6, NS7, and NS8 were from White et al. (1990). P1 (5′-GGTTCATTCAAATTTCTGCC-3′), P2 (5′-GGCAGAAATTTGAATGAACC-3′), P3 (5′-TTTTGTTGGTTTCTAGGACC-3′), P4 (5′-GAAACCAACAAAATAGAACC-3′), P5 (5′-AACTTAAAGAAATTGACGGAAG-3′), P6 (5′-CTTCCGTCAATTTCTTTAAG-3′), P7 (5′-TCCCTGCCCTTTGTACACAC-3′), and P8 (5′-GTGTGTACAAAGGGCAGGGA-3′) were designed based on the nucleotide sequences of the 18S rDNA of *B. graminis* (L26253), *Chaetomium elatum* Kunze & Schmidt: Fries (M83257), *Eremascus albus* Eidam (M83253), *Kluyveromyces lactis* (Dombrowski) van der Walt (X51830), *Microascus cirrosus* Curzi (M89994), *Neurospora crassa* Shear & B. Dodge (X04971), *Ophiostoma ulmi* (Buisman) Nannfeldt (M83261), *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (M27603), *Schizosaccharomyces pombe* Lindner (X54866), *Talaromyces flavus* (Klöcker) Stolk & Samson (M83262), and *Thermoascus crustaceus* (Apinis & Chesters) Stolk (M83263). PM4 (5′-CCGGCCCGCCAAAGCAAC-3′) was designed based on the conserved sequences of the ITS1 region of the Erysiphales.

DNA Extraction, PCR Amplification and Sequencing Whole-cell DNA was isolated from cleistothecia or mycelia by the chelex method (Walsh et al., 1991; Hirata and Takamatsu, 1996). The nuclear rDNA region including the nuclear 18S rDNA was amplified by the polymerase chain reaction (PCR) using the primers NS1 and ITS2 (White et al., 1990). PCR reactions were conducted in 50 μl volumes as previously described (Hirata and Takamatsu, 1996). A negative control lacking template DNA was included for each set of reactions. One microliter of the first reaction mixture was used for the second amplification with the partial nested primer set NS1 and PM4. The 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-stained gel and purified using the JETSORB kit (GENOMED) following the manufacturer's protocol. Nucleotide sequences of the PCR products were obtained for both strands by direct sequencing in an Applied Biosystems 373A sequencer. The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) following the manufacturer's instructions. The primers, NS1, NS2, NS3, NS6, NS7, NS8, P1, P2, P3, P4, P5, P6, P7, P8 were used for sequencing in both directions.

Data Analysis The sequences of the Erysiphales and the other ascomycetous fungi that were retrieved from the nucleotide sequence databases (GenBank/EMBL/DDBJ) (Table 2) were initially aligned using the Clustal V package (Higgins et al., 1992). The alignment was then refined visually with a word processing program with color-coded nucleotides. The alignments are available upon request from the corresponding author. Phylogenetic trees were obtained from the data by distance, parsimony, and maximum likelihood methods. For distance analysis, DNADIST in PHYLIP version 3.5 (Felsenstein, 1989) was used to obtain a distance matrix with Kimura's two-parameter correction for multiple hits (Kimura, 1980). The distance matrix was then analyzed by NEIGHBOR, which has algorithms based on the neighbor-joining method of Saitou and Nei (1987). The strength of the internal branches from the resulting trees was tested by bootstrap analysis (Felsenstein, 1985) using 1000 replications. For parsimony analysis, we used the maximum parsimony method with heuristic search from the computer package PAUP version 3.1.1 (Swofford, 1993). This search was repeated 10 times from 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 as missing information. For maximum likelihood analysis, we used the computer program package MOLPHY version 2.3. (Adachi and 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 neighbor-joining tree. The neighbor-joining tree topology was then introduced into NucML with the aligned dataset and a maximum likelihood tree was estimated heuristically using the local rearrangement search option of NucML.

The 2D method reported by Tajima (1993) was used to assess the substitution rate homogeneity between two lineages. All of the gaps and sequence ambiguities were removed from the aligned data matrix. Data sets composed of three nucleotide sequences, a reference taxon and a possible pairwise sequence between the two lineages, were constructed with the MacClade program (Maddison and Maddison, 1992). The number of sites in which exactly two different types of nucleotides exist in the three sequences was counted, and the observed number of sites in which nucleotides in sequence 1 are different from those in sequences 2 and 3 was defined as m_1. Then, m_1 was divided into the number of sites ($s1$) for transitional differences and the number of sites ($v1$) for transversional differences. When sequence 3 is the reference taxon, $E(s1)=E(s2)$ and $E(v1)=E(v2)$ should be assumed. This equality was tested by the following calculation,

$$\chi^2=\frac{(s1+s2)^2}{s1+s2}+\frac{(v1+v2)^2}{v1+v2}$$

which follows the chi-square distribution with two degrees of freedom. This test was carried out for all possible pairwise sequences between two lineages.

Results

18S rDNA sequence-based analysis The nucleotide sequences of the Erysiphales determined in this study ranged from 1779 bp to 1784 bp in length. The nucleotide sequence data of the other 40 ascomycetous fungi were combined with those of the Erysiphales to construct a large data set. The ascomycetous fungi used in this analysis were selected from a wide range of taxo-

Table 2. Reference rDNA sequences obtained from the databases.

Classification[a)]	Taxon	Accession No. 18S	28S
Plectomycetes			
Onygenales	*Ajellomyces dermatitidis*	M63096	
	Byssoascus striatosporus	AB015776	U17912
	Myxotrichum deflexum	AB015777	
	Pseudogymnoascus roseus	AB015778	
	Coccidioides immitis	M55627	
	Geomyces pannorum var. *pannorum*	AB015785	
	Oidiodendron tenuissimum	AB015787	
Eurotiales	*Aspergillus fumigatus*	M55626	
	Chromocleista malachitea		AB000621
	Eupenicillium rubidurum		AF033462
	Geosmithia namyslowskii		AB000487
	Sclerocleista ornata		AF033392
	Talaromyces avellanea		AB000620
Pyrenomycetes			
Hypocreales	*Epichloë amarillans*		U57680
	Hypocrea lutea	D14407	
	Chaetopsina fulva	AB003786	
	Nectria pseudotrichia		U17410
Phyllachorales	*Glomerella cingulata*	M55640	U48428
Diaporthales	*Leucostoma persoonii*	L42439	
	Diaporthe phaseolorum		U47830
Ophiostomatales	*Ophiostoma stenoceras*	M85054	
	Ophiostoma ulmi	M83261	
	Ophiostoma piliferum		U47837
Sordariales	*Cercophora septentrionalis*		U47823
	Melanospora fallax		U17404
	Podospora anserina	X54864	
	Sordaria fimicola	X69851	
Xylariales	*Xylaria carpophila*	Z49785	
	Daldinia concentrica		U47828
	Xylaria curta		U47840
Halosphaeriales	*Lanspora coronata*		U46889
	Lindra thalassiae		U46891
Microascales	*Ceratocystis fimbriata*		U17401
	Petriella setifera		U48421
Discomycetes			
Pezizales	*Ascozonus woolhopensis*	AF010590	
	Balsamia magnata		U42683
	Gyromitra montana		U42679
	Thelebolus stercoreus	AF010590	
	Rhizina undulata	U42664	
	Verpa bohemica	U42645	
Leotiales	*Cudonia confusa*	Z30240	
	Monilinia laxa	Y14210	
	Sclerotinia sclerotiorum	L37541	
Cyttariales	*Cyttaria darwinii*	U53369	
Lecanorales	*Pilophorus acicularis*	U70960	
Caliciales	*Cyphelium inquinans*	U86695	
	Sphinctrina turbinata	U86693	
Neolectales	*Neolecta vitellina*		U42695

Loculoascomycetes			
Dothideales	*Botryosphaeria ribis*	U42477	
	Drechslera sp.		AF024597
	Dothidea hippophaeos	U42475	
	Mycosphaerella mycopappi	U43449	
	Leptosphaeria maculans	U04238	
	Leptosphaeria microscopica	U04235	
	Lophiostoma crenatum	U42485	
	Sporomia lignicola	U42478	
	Westerdykella dispersa	U42488	
	Aureobasidium pullulans	M55639	
Myriangiales	*Elsinoë veneta*		U43484
Incertae sedis	*Symbiotaphrina kochii*	D49656	
	Amylocarpus encephaloides	U45438	
	Oosporidium margaritiferum		U40090
	Paracoccidioides brasiliensis		U81263
Hemiascomycetes			
Saccharomycetales	*Saccharomyces cerevisiae*	Z75578	J01355

a) Based on the classification system by Hawksworth et al. (1995).

nomic groups within the euascomycetous fungi. *Saccharomyces cerevisiae* was used as an outgroup taxon. Multiple alignment of the data set composed of the 50 taxa resulted in an aligned data stretch of 1802 characters in length. The analyses by the maximum likelihood method and the neighbor-joining method yielded almost identical tree topologies, and thus only the neighbor-joining tree (NJ tree) is shown in Fig. 1. The parsimony search yielded eight equally parsimonious trees, 1332 steps in length, with a consistency index (CI) of 0.514, a retention index (RI) of 0.662, and a rescaled consistency index (RC) of 0.340 (tree not shown).

Most major clades were commonly supported by the trees reconstructed by the three different algorithms, but the branching order of the clades was different between the most parsimonious tree (MP tree), and the neighbor-joining and maximum likelihood trees (ML tree). Nine pyrenomycetous fungi formed a distinct clade strongly supported (100%) by the bootstrap analysis in all three kinds of trees. The clade composed of nine loculoascomycetous fungi was also recognized in the three tree topologies with high bootstrap supports in NJ (82%) and ML (93%) trees, although the bootstrap value was relatively low (54%) in the MP tree. Nine discomycetous taxa did not form a clade. They split into four small clades scattered throughout the phylogenetic tree. Eight plectomycetous fungi split into two groups distantly related to one another. The first group consisted of the Eurotiales and the Onygenaceae of the Onygenales. The second group consised of the Myxotrichaceae of the Onygenales. The second group was not a monophyletic group but instead split into two clades, one composed of *Byssoascus, Oidiodendron* and *Myxotrichum*, and the other composed of *Geomyces* and *Pseudogymnoascus*.

The 10 Erysiphales taxa used in this study formed a distinct clade strongly supported by the bootstrap analysis in all of the three methods (95% or more). The five lineages recognized in our previous report (Mori et al., 2000) were also supported in the present analyses, although the genera *Arthrocladiella* and *Erysiphe* (Euoidium lineage in Mori et al., 2000) did not form their own clade in the MP tree. The branching order of the respective lineages also coincided with that of our previous analysis based on the partial 18S, 5.8S, and 28S rDNA sequences (Mori et al., 2000). The first split within the Erysiphales commonly occurred between the clade of two *B. graminis* taxa and the clade of the remaining taxa. The Myxotrichaceae clade composed of *Byssoascus, Oidiodendron*, and *Myxotrichum* was the sister clade to the Erysiphales clade. The two fungal groups formed a clade in all three tree topologies. In the NJ and ML trees, the Erysiphales/Myxotrichaceae clade grouped with some discomycetous fungi (Cyttariales, Leotiales, and Theleobolaceae of Pezizales) and *Amylocarpus encephaloides* Currey (a cleistothecial ascomycete of uncertain relationships) to form a big clade together. In the MP tree, the Erysiphales/Myxotrichaceae clade was also closely related to the discomycetous fungi, although they did not form a clade together. The phylogenetic placement of *Amylocarpus* was inconsistent between the eight parsimonious trees, and the fungus was placed between the Erysiphales clade and the Myxotrichaceae clade in some trees (tree not shown).

28S rDNA sequence-based analysis The nucleotide sequence data of the 28S rDNA of the 10 Erysiphales taxa were combined with the nucleotide sequences of 25 euascomycetous taxa and one outgroup taxon (*Saccharomyces cerevisiae*) to yield a data matrix composed of 702 characters. Phylogenetic trees were reconstructed by the three different algorithms. Of these, an NJ tree is shown in Fig. 2. The parsimony analysis yielded three equally parsimonious trees of 1332 steps in length with CI of 0.514, RI of 0.662, and RC of 0.340 (tree not shown).

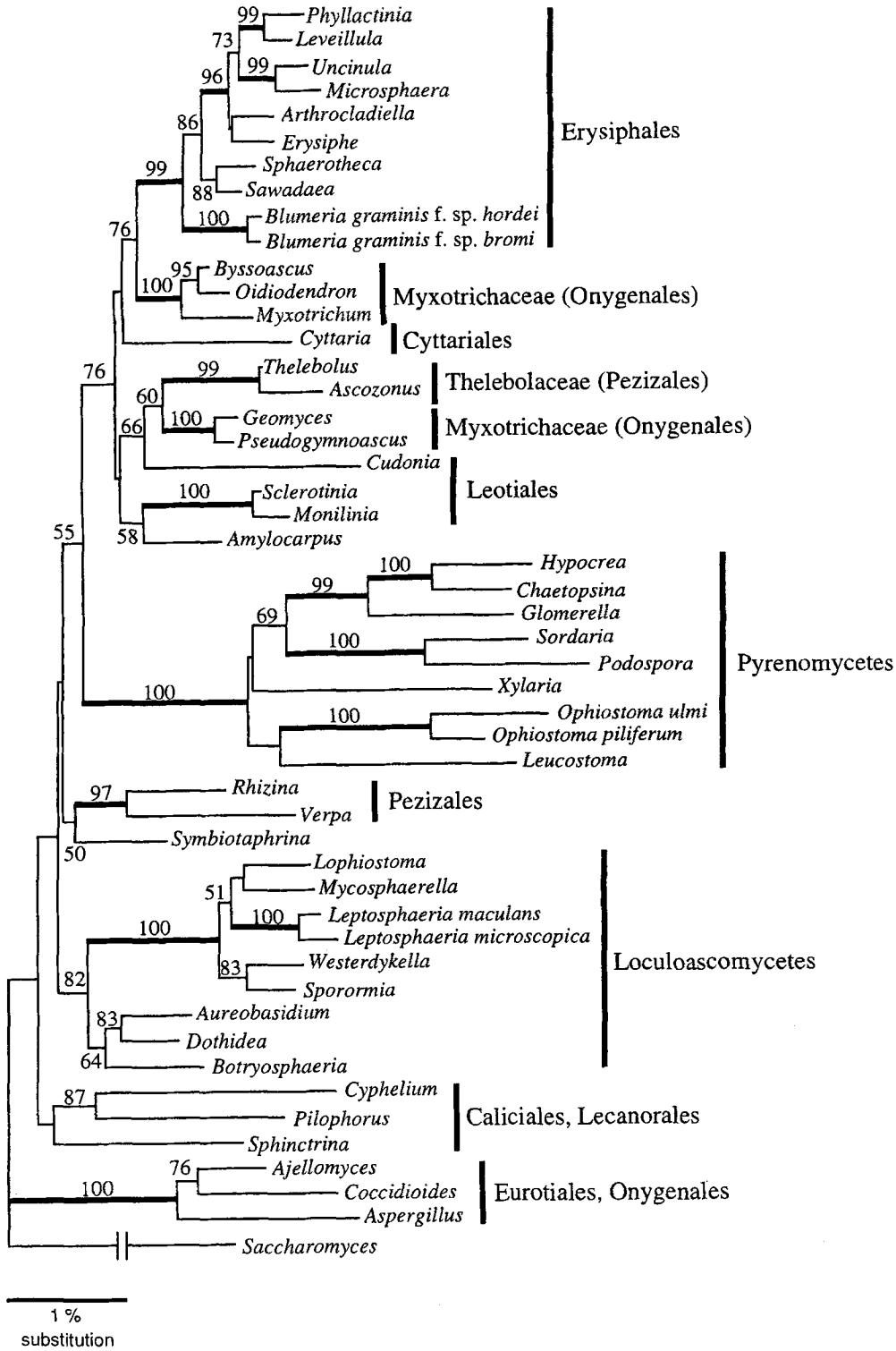

Fig. 1. Neighbor-joining analysis of the 18S ribosomal RNA gene for 50 ascomycetes. The neighbor-joining tree was found using Kimura's two-parameter model for nucleotide substitutions to correct for multiple hits. Branch lengths correspond to pairwise distances between taxa. *Saccharomyces cerevisiae* was used to root the tree. Percentages of neighbor-joining analysis of 1000 bootstrapped data sets supporting specific branches are indicated at the respective nodes. Bootstrap values below 50% are not shown.

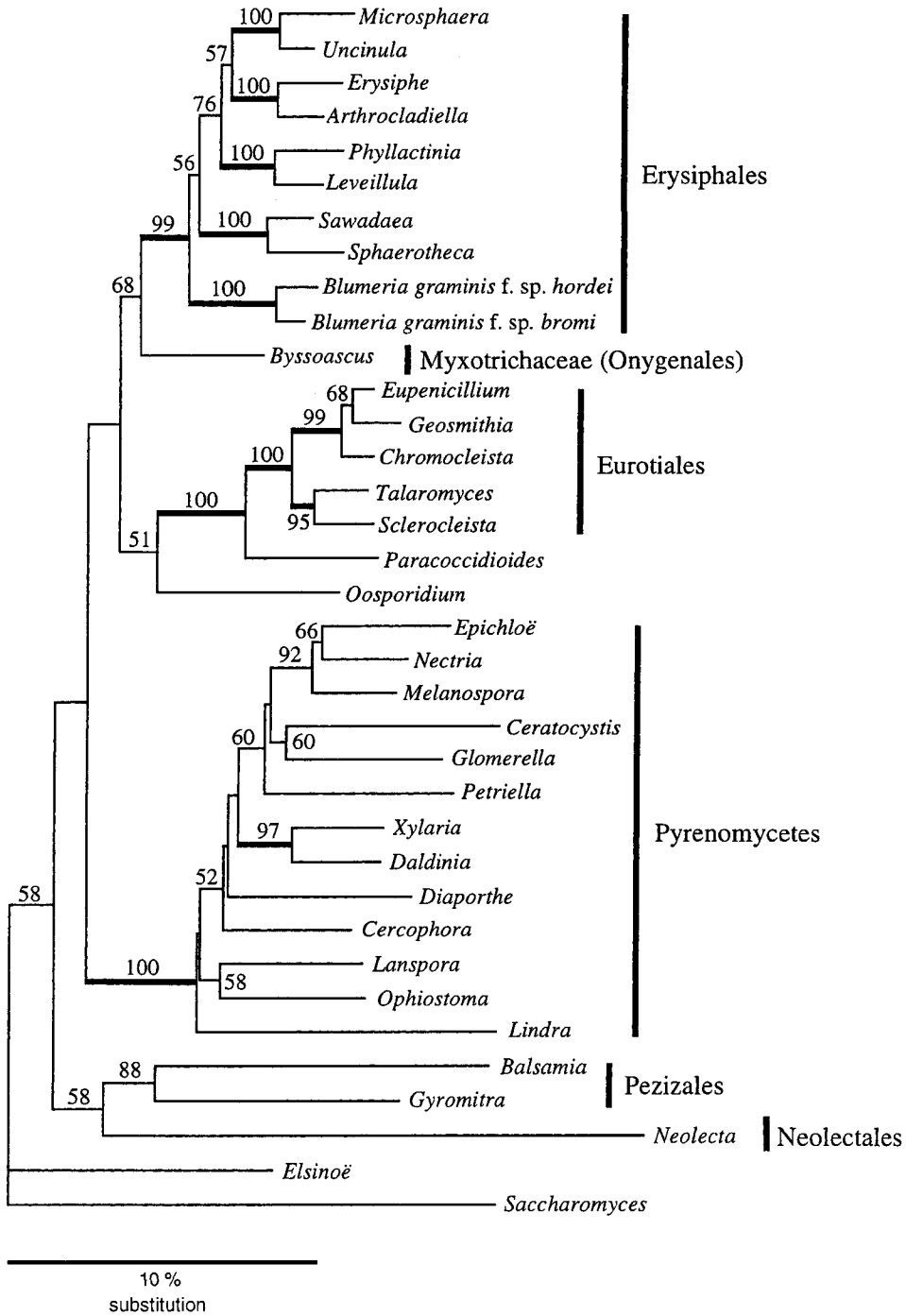

Fig. 2. Neighbor-joining analysis of the partial sequences of the 28S ribosomal RNA gene including the D1 and D2 regions for 36 ascomycetes. The neighbor-joining tree was found using Kimura's two-parameter model for nucleotide substitutions to correct for multiple hits. Branch lengths correspond to pairwise distances between taxa. *Saccharomyces cerevisiae* was used to root the tree. Percentages of neighbor-joining analysis of 1000 bootstrapped data sets supporting specific branches are indicated at the respective nodes.

Four major clades were commonly recognized in the three tree topologies, although the branching order of the clades was inconsistent between the trees. The clade composed of the 13 pyrenomycetous fungi was strongly supported by the bootstrap analysis (97% or more in all of the three methods). The five Eurotiales taxa also formed a clade with high bootstrap support (95% or more). The 10 Erysiphales taxa formed a distinct clade with high bootstrap support (94% or more). The five major lineages within the Erysiphales were again recognized in these trees based the 28S rDNA sequences, and the first split occurred between the clades of *Blumeria*

Table 3. Substitution rate homogeneity test of 18S rDNA sequences between the Erysiphales and Myxotrichaceae by Tajima's 2D-test (Tajima, 1993)[a)]

	Phyllactinia	*Leveillula*	*Uncinula*	*Microsphaera*	*Arthrocladiella*	*Erysiphe*	*Sphaerotheca*	*Sawadaea*	*B.g. hordei*	*B.g. bromi*
Byssoascus	6.00*[b)]	3.58	9.17*	8.35*	2.60	3.88	9.05*	5.26	4.50	4.98
Myxotrichum	1.93	0.93	4.68	3.77	0.48	1.75	6.47*	3.56	3.10	3.88
Oidiodendron	4.05	2.17	4.05	6.07*	1.44	2.74	7.60*	4.51	3.69	4.33

a) *Sclerotinia sclerotiorum* was used as a reference taxon.
b) Chi-square value calculated by Tajima's 2D-test (Tajima, 1993). Asterisk indicates that there is significant difference of substitution rate between the respective taxa with more than 95% probability.

and the remaining taxa of the Erysiphales. A Myxotrichaceae genus, *Byssoascus*, was the sister taxon of the Erysiphales clade. The two Pezizales taxa formed their own lineage in the NJ tree, but not in the MP tree.

Radiation Time of Erysiphales To assess the substitution rate homogeneity between the Myxotrichaceae and the Erysiphales, all possible pairwise sequences of the 18S rDNA between the Myxotrichaceae and the Erysiphales were subjected to the Tajima's 2D-test (Tajima, 1993) using *Amylocarpus encephaloides, Sclerotinia sclerotiorum* or *Cyttaria darwinii* as a reference taxon. When *Amylocarpus* or *Cyttaria* was used as a reference taxon, the molecular evolutionary clock hypothesis was not rejected in any pair at the 5% level. When *Sclerotinia* was used as a reference, the molecular clock hypothesis was significantly rejected in several pairs (Table 3). Based on the result, the sequence data of *Byssoascus, Microsphaera*, and *Sphaerotheca* were removed from the calculation of the genetic distances between the Myxotrichaceae and Erysiphales. Similar assessment was also carried out within the Erysiphales, between *B. graminis* and the remaining Erysiphales taxa, using *Byssoascus striatosporus* as a reference taxon, and it appeared that the substitution rates were homogeneous within the Erysiphales.

Two kinds of molecular clocks of the 18S rDNA were used in this analysis. One molecular clock is 1% per lineage per 100 million years (myr) based on the calculation of Berbee and Taylor (1993). The another is 0.667% per lineage per 100 myr based on the report of Simon et al. (1993), which was based on the point of 200 myr for the split between monocot and dicot plant lineages. The genetic distances between the Myxotrichaceae and the Erysiphales and also within the Erysiphales were calculated by Kimura's two-parameter method using DNADIST of PHYLIP ver. 3.5. The

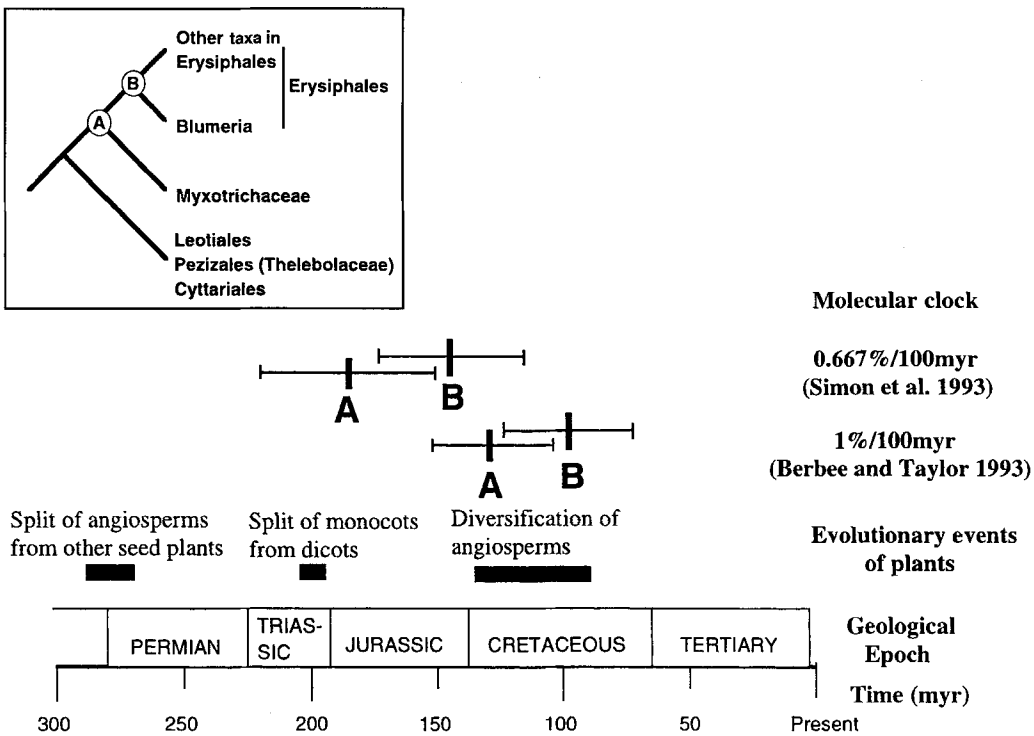

Fig. 3. Estimated dates of origin and divergence of the Erysiphales based on the nucleotide sequences of the 18S rDNA. A is the splitting of the Erysiphales and Myxotrichaceae, and B is the first radiation within the Erysiphales.

average percent substitution between the Erysiphales clade and the Myxotrichaceae clade was 2.53±0.48% (±means 95% confidence range). From the substitution rate, the split between the Erysiphales and the Myxotrichaceae (Fig. 3) was calculated as 127±24 myr ago based on the molecular clock of Berbee and Taylor (1993). When the molecular clock of Simon et al. (1993) was used, it was 190±36 myr ago. Similarly, the percent substitution was highest between the *Blumeria* clade and the remaining taxa (1.85±0.47%). The first split within the Erysiphales (Fig. 3) was thus calculated as 92±24 myr ago based on the molecular clock of Berbee and Taylor (1993), and 138±36 myr ago based on Simon et al. (1993).

Discussion

To clarify the phylogenetic placement of the Erysiphales in the phylum Ascomycota, nearly complete nucleotide sequences of the 18S rDNA and about 700 bp of the 5′-part of the 28rDNA including the D1 and D2 domains were used in the current phylogenetic analyses. The 10 taxa of the Erysiphales used in this study were carefully selected from a number of powdery mildew specimens so as to provide even representation throughout the Erysiphales. The phylogenetic relationships within the Erysiphales mostly coincided with our previous analysis (Mori et al., 2000) using partial 18S, 5.8S and 28S rDNA combined sequences. The Erysiphales formed a distinct monophyletic clade to the other ascomycetous fungi with strong bootstrap support in both the 18S and 28S trees. This result clearly indicates that the Erysiphales diverged from a single ancestral taxon in a single evolutionary event.

The result of our analysis supported close relationships among Erysiphales, Myxotrichaceae, and some discomycetous fungi (Leotiales, Cyttariales and Thelebolaceae). The 18S and 28S trees revealed that the Myxotrichaceae and the Erysiphales are sister taxa. The Onygenales consists of four families, Onygenaceae, Arthrodermataceae, Myxotrichaceae, and Gymnoascaceae (Currah, 1985). Of these, the Onygenaceae and Arthrodernaceae are saprophytic, keratin-degrading fungi and usually inhabit soils enriched with keratin or dung. The Myxotrichaceae are saprophytic, cellulose-degrading fungi that usually inhabit forest soils, and decay plant materials. Currah (1995) suggested that the Myxotrichaceae belong to a different lineage from the keratin-degrading fungi based on morphological and ecological differences. The molecular data reported by Sugiyama et al. (1999) support this assumption. The Erysiphales and Myxotrichaceae share an anamorphic character; they both form one-celled, meristem arthrospores. They also commonly produce cleistothecial ascomata with appendages. These morphological characteristics support the present result indicating that the Erysiphales and the Myxotrichaceae have a common ancestor.

Discomycetous fungi usually form apothecial ascomata, which are dissimilar to the cleistothecial ascomata of the Erysiphales and the Myxotrichaceae. There are, however, some discomycetous taxa that form cleistothecial ascomata. The Thelebolaceae is accommodated in the order Pezizales in the recent classification of the Ascomycota (Hawksworth et al., 1995). However, the type genus of the family, *Thelebolus*, has been regarded as a non-pezizalean taxon based on the morphological and ultrastructural data (Kimbrough, 1981; Samuelson and Kimbrough, 1978). Zukal (1886) examined *Thelebolus stercoreus* Tode, the type species of the genus, in detail and concluded that it was closely related to the genus *Podosphaera* in the Erysiphales. Cooke and Barr (1964) agreed with Zukal (1886) and placed the Thelebolaceae in the Erysiphales. They based their decision on the shape of the ascus, the lateral and apical thickness of its wall, the mechanism of ascal dehiscence, the comparative development of the ascocarp, and the production of a single ascus. The recent molecular analyses (Momol et al., 1996; Landvik et al., 1997, 1998) and our present result indicates that the Thelebolaceae is isolated from the other pezizalean taxa and closely related to the clade composed of the Erysiphales and Myxotrichaceae, but not included in the clade.

The present analysis supports the report of Landvik et al. (1996) that *Amylocarpus encephaloides* is closely related to the Erysiphales as well as the Leotiales. Although the phylogenetic placement of this fungus was not consistent between the trees, it was placed between the clades of Erysiphales and Myxotrichaceae in some parsimony trees, and the genetic distances calculated by Kimura's two-parameter method showed the shortest distance of this fungus to the Erysiphales and Myxotrichaceae amongst the ascomycetous fungi. This monotypic genus forms cleistothecial ascomata solitarily or gregariously on marine wood (Crumlish and Curran, 1994). Although Kohlmeyer and Kohlmeyer (1979) placed this fungus into the Eurotiaceae (i.e. Trichocomaceae), the present molecular analysis, and the morphological character of an ascus with short stalk, suggests a phylogenetic relationship between *Amylocarpus* and the Erysiphales.

Cain (1972) suggested that the Pezizales was the source of the cleistothecial families such as Gymnoascaceae (Myxotrichaceae sensu Currah was included in Gymnoascaceae at that time) and Erysiphaceae. In the NJ and ML trees, the first split of the big clade composed of the Erysiphales, Myxotrichaceae and some discomycetous fungi (Leotiales, Cyttariales and Thelebolaceae) occurred within the discomycetous fungi. This tree topology might support the Cain's assumption. However, the phylogenetic relationships among these discomycetous orders are not consistent in the MP trees. More sequence data of discomycetous fungi are required to confirm the present result.

The radiation time of the Erysiphales was estimated using newly determined 18S rDNA sequences of the 10 powdery mildew taxa and the molecular clocks reported by Berbee and Taylor (1993) and Simon et al. (1993). Splitting of the Erysiphales from the Myxotrichaceae was calculated as 127±24 myr ago (±means 95% confidence range) based on the molecular clock of Berbee and

Taylor (1993) (1%/100 myr), and 190±36 myr ago based on that of Simon et al. (1993) (0.67%/100 myr). Similarly, the first radiation within the Erysiphales was calculated as 92±24 and 138±36 myr ago based on Berbee and Taylor (1993), and Simon et al. (1993), respectively. The downy mildews, powdery mildews, and rusts are typical obligate biotrophs of a wide range of plants. Of these, the powdery mildews infect only angiosperm plants, not gymnosperm plants or ferns, while the rusts are known to infect to all of the plant groups. This fact allowed us to assume the radiation time of the Erysiphales to be after the splitting of angiosperm plants from gymnosperm plants and ferns. Savard et al. (1994) estimated the time when the angiosperm lineage divided from the other seed plants lineage within a range of 275–290 myr ago based on the chloroplast and nuclear gene sequences. Our calculation places the split of the Erysiphales from the Myxotrichaceae at no more than 230 myr ago, even when the uncertainty of the molecular clocks is taken in consideration (Fig. 3). A major diversification of angiosperms is considered to have occurred in the Early Cretaceous, between about 130 and 90 myr ago (Crane et al., 1995), which coincides with the time of the first diversification among the erysiphalean taxa calculated in this study (Fig. 3). These results may support our earlier assumption that the Erysiphales evolved after the radiation of angiosperms and diverged in close relation to the evolution of plants.

In this study using newly determined sequences of the Erysiphales, we showed a close relationship between the Erysiphales, Myxotrichaceae, and some discomycetous fungi (Leotiales, Cyttariales and Thelebolaceae). Most of the morphological observations mentioned above seem to support the present molecular analyses. However, except for the Erysiphales, these fungi are not parasitic to plants, but instead, saprophytically inhabit dung and/or plant materials. The questions of how and why an obligate biotrophic group like the Erysiphales radiated from the saprophytic fungi remain to be addressed in the future. We also tried to estimate the radiation time of the Erysiphales using the two previously reported molecular clocks. Although the present calculation gave an apparently reasonable result, we should reevaluate the present result using a more exact molecular clock calculated from the molecular data of the Erysiphales. Dating of the evolutionary events after the radiation of the Erysiphales should also be calculated using DNA sequences that have a more rapid substitution rate, such as the variable regions of 28S rDNA or the ITS regions.

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