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Evolutionary relationships of members of the genera *Taphrina*, *Protomyces*, *Schizosaccharomyces*, and related taxa within the archiascomycetes: Integrated analysis of genotypic and phenotypic characters

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To study the phylogeny and evolution of archiascomycetes, we determined the full sequence of the nuclear 18S rRNA gene from 14 *Taphrina* species and 2 *Protomyces* species, and the partial sequence of *Schizosaccharomyces japonicus* var. *japonicus*. The sequences were phylogenetically analyzed by the neighbor-joining, maximum parsimony, and maximum-likelihood methods. We also looked at their principal phenotypic characters and genotypic character. Relationships within the Ascomycota are concordant with the previously published phylogenies inferred from 18S rDNA sequence divergence and divide the archi-, hemi- and euascomycetes into distinct major lineages. All the trees show that, within the archiascomycete lineage, 11 of the 14 *Taphrina* species and the 2 *Protomyces* species are monophyletic. A core group of *Taphrina* and *Protomyces* is always monophyletic. The evidence from molecular and phenotypic characters such as cell wall sugar composition, ubiquinone, cell wall ultrastructure, and mode of conidium ontogeny, strongly suggests that *'T'. californica* CBS 374.39, *'T'. maculans* CBS 427.69 and *'T'. farlowii* CBS 376.39 should be excluded from the archiascomycete lineage. *'Taphrina' farlowii* CBS 376.39 groups with *Candida albicans* in the Saccharomycetales, whereas *'T'. californica* CBS 374.39 and *'T'. maculans* CBS 427.69 have a basidiomycete affinity and group with Tremellalean members in the hymenomycete lineage. *Schizosaccharomyces* is monophyletic. The strictly anamorphic yeast *Saitoella complicata* groups with the apothecial ascomycete *Neolecta vitellina* rather than the *Taphrina/Protomyces* branch.

Key Words——archiascomycetes; 18S ribosomal RNA gene phylogeny; *Protomyces*; *Schizosaccharomyces*; *Taphrina*.

'Archiascomycetes', a class of the Ascomycota proposed by Nishida and Sugiyama (1994) (cf., Nishida and Sugiyama, 1993 and Nishida et al., 1993) for *Taphrina*, *Protomyces*, *Saitoella*, *Schizosaccharomyces*, and *Pneumocystis*, has been based on nuclear 18S ribosomal RNA (rRNA) gene sequence divergence. This major lineage corresponds to the basal ascomycetes (Berbee and Taylor, 1993) or the early ascomycetes (Taylor et al., 1994). It represents the earliest diverging ascomycete lineage prior to the separation of the other two major lineages, hemiascomycetes and euascomycetes, of the Ascomycota. The respective archiascomycete genera are morphologically and habitually diverse. Some archiascomycete genera share some principal characters with both ascomycetous and basidiomycetous yeasts, such as the cell wall ultrastructure, biochemical characters, mode of conidium ontogeny, and major ubiquinone system (Table 1 in Sugiyama and Nishida, 1995). Members of the 'Archiascomycetes' lack ascogenous hyphae and ascomata, and the asci have sometimes been homologized with sporangia (Alexopoulos et al., 1996). A lack of common characters to define the archiascomycetes as a new class may suggest that archiascomycete members have morphologically highly diverged. The 'Archiascomycetes' imply *Taphrina deformans*, *T. wiesneri*, *T. populina*, *Protomyces inouyei*, *P. lactucae-debilis*, *Saitoella complicata*, *Schizosaccharomyces pombe*, and *Pneumocystis carinii* at the species level (Nishida and Sugiyama, 1994). In addition to these, Landvik et al. (1993) and Landvik (1996) placed the apothecial ascomycetes *Neolecta vitellina* and *N. irregularis* in the basal ascomycete lineage defined by Berbee and Taylor (1993). This joining presented more difficulty in defining the 'Archiascomycetes' by common phenotypic characters. To date, the

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'Archiascomycetes' accommodate six genera.

The order Taphrinales includes one family, Taphrinaceae, with the single genus *Taphrina*, which consists of almost 100 species (Kramer, 1973; Mix, 1949). They are parasitic on a wide variety of vascular plants, primarily ferns, the Rosales and Fagales, including some economically important species (Alexopoulos et al., 1996). The mycelium of *Taphrina* is composed of septate hyphae that may be intercellular or subcuticular and sometimes grow within the walls of the epidermal cells of the host (Alexopoulos et al., 1996; Kramer, 1987). These are dimorphic plant parasites, forming dikaryotic mycelia and naked asci in their parasitic phase and budding yeast cells in their saprobic, haploid phase (Kramer, 1987). The colonies on artificial culture media are *Rhodotorula*-like, for which Moore (1990) newly proposed the anamorph-genus *Lalaria*. On the other hand, the order Protomycetales with the single family Protomycetaceae accommodates five genera and 20 species (Kramer, 1987; Reddy and Kramer, 1975). They are also parasitic on vascular plants, primarily species of the Asteraceae (=Compositae, a permitted alternative name; Cronquist, 1988) and the Apiaceae (=Umbelliferae, a permitted alternative name; Cronquist, 1988). The resting spores (asci?) show a unique type of germination. Their life cycles are fragmentally characterized. They produce pigmented yeast colonies on artificial culture media, and these resemble those of *Taphrina* (Tubaki, 1957). Members of Protomycetaceae are less well known than *Taphrina* spp. because of their restricted host ranges and the fact that none of them infects important crop plants. Infections of Protomycetacean species, in addition to the formation of galls and lesions, may be associated with color changes in their hosts, primarily weedy species in Apiaceae and Asteraceae (Reddy and Kramer, 1975).

Goto et al. (1987) proposed *Saitoella complicata*, a new anamorphic genus and species in the family Cryptococcaceae to accommodate the two Himalayan isolates formerly identified as *Rhodotorula glutinis* (Fres.) Harrison (Goto and Sugiyama, 1970). They also suggested that *S. complicata* is closer to the Taphrinales than to basidiomycetous yeasts such as *Rhodotorula* and its teleomorph *Rhodosporidium*. Subsequently Nishida and Sugiyama (1993, 1994), Nishida et al. (1993) and Sugiyama et al. (1993), verified their phylogenetic speculation by analyses of 18S rDNA sequences.

Species of *Schizosaccharomyces* are characterized by vegetative reproduction by elongation at their tip cells and divide by binary fission after forming a centrally placed septum (Kreger-van Rij, 1984; Minet et al., 1979). The fission yeasts are phylogenetically distant from both the hemiascomycete and the euascomycete clades, which has resulted in the reassignment of the fission yeasts to a separate order, Schizosaccharomycetales (Eriksson et al., 1993; Kurtzman, 1993; Kurtzman and Robnett, 1994). Berbee and Taylor (1993) and Taylor et al. (1994) showed that the type species *Schiz. pombe* is included in the basal or early group of ascomycetes, together with *Taphrina deformans* and *Pneumocystis carinii*. Nishida and Sugiyama (1994) included *Schiz. pombe* within the 'Archiascomycetes' on the basis of 18S rDNA sequence data.

Pneumocystis carinii (Delanoë and Delanoë, 1912), a major causal agent of pneumonia in patients with HIV, was formerly thought to belong to the Protozoa. Edman et al. (1988) assigned it as a member of the fungi based on the 18S rRNA sequence. Subsequently, Taylor et al. (1994) and Sugiyama and Nishida (1995) suggested that *P. carinii* and *Schizosaccharomyces pombe* have a similar life cycle in having the fission type conidium ontogeny. Nishida and Sugiyama (1994) placed *Pneumocystis* within the 'Archiascomycetes'. Eriksson (1994) accommodated *Pneumocystis* in the new family and order Pneumocystidaceae, Pneumocystidales (Ascomycota). The different phylogenetic assignment for *P. carinii* has been debated between Taylor and Bowman (1993) and Wakefield et al. (1993).

The genus *Neolecta* is characterized by clavate, stalked apothecia and cylindrical, aparaphysate, eight-spored asci. Previously, this genus was placed in the Helotiales of the Discomycetes (Korf, 1973), and Redhead (1977) created the new family Neolectaceae and tentatively placed it in the Lecanorales. Based on 18S rRNA sequence analysis, Landvik et al. (1993) found that *Neolecta* groups with the basal ascomycetes. Landvik (1996) further reported that the 18S rRNA gene sequence from *N. irregularis* supports the earlier published sequence of *N. vitellina*, for which the new order Neolectales was erected by Landvik et al. (1993). Landvik (1996) confirmed from partial sequence analysis of LSU rDNA that *Neolecta* formed part of a sister group to other fruitbody-forming ascomycetes and the budding yeasts. Eriksson and Hawksworth (1995), Hawksworth et al. (1995), Sugiyama et al. (1996b), and Kurtzman and Sugiyama (unpublished) placed *Neolecta* within the 'Archiascomycetes'.

The focus of this study is to reinforce the previous detection of a major new lineage, archiascomycetes, and to shed a light into the evolutionary relationships of *Taphrina*, *Protomyces*, *Saitoella*, *Schizosaccharomyces*, *Pneumocystis*, and *Neolecta* within the 'Archiascomycetes', based on an integrated analysis of genotypic and phenotypic characters.

Materials and Methods

Fungal species, culture conditions, sequencing of nuclear 18S rRNA gene, and phylogenetic analysis The species examined and sequence data used in this study are listed in Table 1. The new nucleotide sequences determined in this work will appear in the DDBJ, EMBL and GenBank nucleotide sequence database (D14166, AB000948 - AB000960 for 14 *Taphrina* spp., D85142 and D85143 for 2 *Protomyces* species, and AB000966 for *Schiz. japonicus* var. *japonicus*).

The cells were grown in YM agar or broth at 25°C. DNA was obtained from cells broken by sonication. The gene of nuclear small subunit (18S) rRNA coding regions was amplified using the polymerase chain reaction (PCR;

Table 1. Sources of 18S rDNA sequence data.

Classification and taxon	Strain[a]	DNA database accession no.
I. Ascomycota		
Ia. Archiascomycetes		
'Taphrina' californica Mix	CBS 374.39	D14166[b]
Taphrina carnea Johanson	CBS 332.55	AB000948[b]
T. communis (Sadebeck) Giesenhagen	CBS 352.35	AB000949[b]
T. deformans (Berkeley) Tulasne	ATCC 34556	U00971
'T'. farlowii Sadebeck	CBS 376.39	AB000950[b]
T. flavorubra Ray	CBS 377.39	AB000951[b]
T. letifera (Peck) Saccardo	CBS 335.55	AB000952[b]
'T'. maculans Butler	CBS 427.69	AB000953[b]
T. mirabilis (Atkinson) Giesenhagen	CBS 357.35	AB000954[b]
T. nana Johanson	CBS 336.55	AB000955[b]
T. populina Fries	CBS 337.55	D14165
T. pruni Tulasne	CBS 358.35	AB000956[b]
T. pruni-subcordatae (Zeller) Mix	CBS 381.39	AB000957[b]
T. robinsoniana Giesenhagen	CBS 382.39	AB000958[b]
T. ulmi (Fuckel) Johanson	CBS 420.54	AB000959[b]
T. virginica Sadebeck	CBS 340.55	AB000960[b]
T. wiesneri (Rathay) Mix	IFO 7776	D12531
Protomyces inouyei P. Hennings	IFO 6898	D11377
P. lactucae-debilis Sawada	IFO 6899	D14164
P. macrosporus Unger	IMI 102384	D85143[b]
P. pachydermus von Thümen	IFO6900	D85142[b]
Saitoella complicata Goto, Sugiyama, Hamamoto & Komagata	IAM 12963	D12530
Schizosaccharomyces pombe Lindner	Unknown	X54866
Schizosaccharomyces japonicus (Yukawa & Maki) Yamada & Banno	IFO 1609	AB000966[b]
Pneumocystis carinii Delanoë & Delanoë	Unknown	X12708
Neolecta vitellina (Bresadola) Korf & J. K. Rogers	UME 29192	Z27393
Ib. Hemiascomycetes		
Candida albicans (Robin) Berkhout	MUCL 29800	X53497
Clavispora lusitaniae Rodrigues de Miranda	ATCC 6260	M60304
Issatchenkia orientalis Kudriyavsev	MUCL 29849	M55528
Kluyveromyces lactis (Dombrowski) van der Walt	IFO 1267	X51830
Saccharomyces cerevisiae Meyen *ex* Hansen	Unknown	M27607
Ic. Euascomycetes		
Ascosphaera apis (Maassen ex Claussen) L.S. Olive & Spiltoir	Unknown	M83264
Aspergillus oryzae (Ahlburg) Cohn	ATCC 1011	D63698
Eremascus albus Eidam	Unknown	M83258
Neurospora crassa Shear & B. O. Dodge	Unknown	X04971
Ophiostoma ulmi (Buisman) Nannfeldt	ATCC 32437	M83261
Symbiotaphrina buchneri Grabner ex W. Gams & von Arx	CBS 420.63	D49657
II. Basidiomycota		
IIa. Ustilaginomycetes		
U. hordei (Persoon) Lagerheim	Unknown	U00973
Tilletia caries (de Candolle) Tulasne	Unknown	U00972
IIb. Urediniomycetes		
Cronartium ribicola J. C. Fischer	Unknown	M94338
Erythrobasidium hasegawianum Hamamoto, Sugiyama & Komagata	IFO 1058	D23702
Leucosporidium scottii Fell, Statzell, Hunter & Phaff	MUCL 28629	X53499
Mixia osmundae (T. Nishida) Kramer	IFO 32408	D14163
Peridermium harknessii J.P. Moore	RUR-152	M94339
Rhodosporidium toruloides Banno	IFO 0559	D12806
Sporidiobolus johnsonii Nyland	41.1 Wells	L22261
Sporobolomyces roseus Kluyver & van Niel	MUCL 30251	X60181
IIc. Hymenomycetes:		
Athelia bombacina Persoon	ATCC 20629	M55638
Auricularia auricula-judae (Bulliard: Fries) Wettstein	UC 1475109	L22254
A. polytricha (Montagne) Saccardo	215.11 Ken. Wells	L22255
Boletus satanas Lenz	TDB-1000	M94337
Bullera alba (Hanna) Derx[c]	MUCL 30301	X60179
Calocera cornea (Fries) Loudon	UC 1475111	L22256
Coprinus cinereus (Schaeffer: Fries) S. F. Gray	Unknown	M92991
Cystofilobasidium capitatum (Fell, Hunter & Tallman) Oberwinkler & Bandoni	ATCC 24507	D12801
Filobasidiella neoformans (Safelice) Vuillemin	CBS 6886	D12804
Dacrymyces chrysospermus Berkeley & M.A. Curtis	UC 1475112	L22257
D. stillatus Nees: Fries	53.02 Ken. Wells	L22258
Filobasidium floriforme L.S. Olive	CBS 6241	D13460
Schizophyllum commune Fries	Unknown	X54865
Spongipellis unicolor (Schweinitz) Murrill	ATCC 26733	M59760
Tremella foliacea Persoon: Fries	UC 1475115	L22262
T. globospora Reid	Unknown	U00976
T. moriformis Berkeley	Unknown	U00977
Trichosporon cutaneum (de Beurmann, Gougerot & Vaucher) Ota	MUCL 30308	X60182
Zygomycota (outgroup):		
Mucor racemosus Fresenius	Unknown	X54863

[a] Abbreviations: ATCC=American Type Culture Collection, USA; CBS=Centraalbureau voor Schimmelcultures, The Netherlands; IAM=Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; IFO=Institute for Fermentation, Osaka, Japan; IMI=CAB International Mycological Institute, UK; MUCL=Mycothéque de l'Université, Catholique de Louvain-la-Neuve, Belgium; UC, Department of Plant Biology, Univ. of California, Berkeley, USA; UME=Univ. of Umeå, Sweden; TDB-1000=library: collection number TDB-1000.

[b] New sequences reported in this study.

[c] The teleomorph is *Bulleromyces albus* Boekhout & Fonseca.

Saiki et al., 1988). The following primers (the positions based on *Saccharomyces cerevisiae* numbering) were used: P1 5′-ATCTGGTTGATCCTGCCAGT-3′ (2–21); P2 5′-GATCCTTCCGCAGGTTCACC-3′ (1794-1775); U1R 5′-CAGCAGCCGCGGTAATTC-3′ (566–583); U1 5′-GAATTACCGCGGCTGAGC-3′ (583–566); T1R 5′-CATGCTAATGTATTCGAGC-3′ (802–784); A1 5′-ACAGTTGGGG(A/G)CATT-3′ (870–884); HA2 5′-CCCCTAACTTTCGTTCT-3′ (987–971); U2R 5′-GAACTTAAAGGAATTGACG-3′ (1128–1147); U2 5′-CGTCAATTCCTTTAAGTTTC-3′ (1147–1128); Ys3R 5′-ACCTGGTGAGTTTCCCCGTG-3′ (1211–1192); B1 5′-AGGCAATAACAGGTCTGTG-3′ (1415–1434); U3R 5′-GTACACACCGCCCGT-3′ (1627–1641); U3 5′-ACGGGCGGTGTGTAC-3′ (1641–1627); Y2 5′-GTCTTGTAATTGGAATGAGTAC-3′ (498–519); Y1R 5′-GCTGCTGGCACCAGACTTGCCCTC-3′ (572–549); Y3 5′-GTAGTCTTAACCATAAACTATGC-3′ (1011–1033); Y4 5′-AGGAATTGACGGAAGGGCACCAC-3′ (1137–1159); Y2R 5′-TCCGTCAATTCCTTTAAGTTTCAGC-3′ (1149–1125); Y3R 5′-TCTGGACCTGGTGAGTTTCCCCGTG-3′ (1216–1192); Y4R 5′-TAAGCCATTCAATCGGTAGTAG-3′ (1666–1645). The fresh PCR products were cloned by Invitrogen[R], TA Cloning Kit (Andres et al., 1993). Then the plasmids were isolated and purified using QIAGEN QIAprep Spin Miniprep Kit (250) (QIAGEN, Germany). We sequenced the 18S rRNA gene by two different automated DNA sequencers. The sequencing reaction of partial region of 18S rRNA gene was done according to the protocol of Auto Cycle Kit (Pharmacia, Biotech, Sweden). Then polyacrylamide gel electrophoresis and data collection were performed on Pharmacia A.L.F.™ automated DNA sequencer. A.L.F. Manager™, version 2.1 (Pharmacia, Biotech, Sweden) was used for the control of the electrophoresis run, data storage, and analysis. The remaining regions of 18S rRNA gene were sequenced using Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The data collections were performed on Applied Biosystems 373A automated DNA sequencer. CLUSTAL W version 1.6 was used for alignments (Thompson et al., 1994), followed by manual adjustments. In our analysis, we included other published 18S rRNA gene sequence data (see Table 1). We omitted gaps from analysis, and the regions of sequence where insertions and deletions occurred, which created alignment ambiguities.

The distance matrix (not shown) for the aligned sequences was calculated by using the two-parameter method of Kimura (1980). The neighbor-joining method (Saitou and Nei, 1987) was used to construct all phylogenetic trees. The robustness for individual branches was estimated by bootstrapping (Felsenstein, 1985) with 1,000 replicates.

The DNAML program from the computer package PHYLIP 3.51c (Felsenstein, 1993) was used for our maximum likelihood analysis (Felsenstein, 1981). The calculation was running on a Power Macintosh 8500 (search for best tree, empirical base frequencies, one category of substitution rates, transition: transversion ratio = 2, outgroup root, input sequences interleaved, terminal type ANSI).

Parsimony analyses were performed by use of the computer package PAUP 3.1.1 (Swofford, 1993), and a bootstrapped 50% majority consensus tree was constructed (not illustrated here). We used the heuristic search option with 32 random taxon addition sequences (TBR branch swapping, MAXTREES unrestricted, MULPARS on). The robustness was assessed by bootstrapping (100 replicates).

Morphological, physiological, and biochemical characterizations Tests were made according to the methods as described in Kreger-van Rij (1984) and Barnett et al. (1990).

Determination of nuclear DNA base composition The cells were grown in 5-liter flasks containing 1.5 liter of YM broth medium under shaking at 27 °C for 30 to 48 h. Then cells were harvested by centrifugation and washed twice with distilled water. The DNA was isolated and purified according to the isolation method of genomic DNAs using benzyl chloride (Vancanneyt et al., 1992), followed by ultracentrifugation (Beckman TL-100) for 16 to 18 h to obtain genomic DNAs. DNA base composition was calculated from the relative peak areas of nucleosides on a high-performance liquid chromatogram (Mesbah et al., 1989).

Determination of the major ubiquinone system The ubiquinones from the cells of six *Taphrina* spp. were isolated, purified, and determined as described in Kuraishi et al. (1985).

Analysis of cell wall sugar composition Cell walls were isolated and purified as described in Prillinger et al. (1993). The alditol acetate derivatives were analyzed by gas-liquid chromatography with Shimadzu GC-8A, with nitrogen gas as a carrier and a column of Rtx 2330 (0.323mm × 30 m). Sugars were estimated on the basis of sample coincidence with the relative retention times for the alditol acetate derivatives of the neutral monosaccharide standards. Sugar component analysis was also performed by thin-layer chromatography (Hasegawa et al., 1983).

Ultrastructural characterization Transmission electron microscopy (TEM) of the cell wall structure and conidium ontogeny for *Taphrina wiesneri* IFO 7776, *'T'. populina* CBS 337.55, *'T'. californica* CBS 374.39, *'T'. farlowii* CBS 376.39, and *'T'. maculans* CBS 427.69 was performed as described by Suh et al. (1993). The specimens were examined with a JEOL 1210 transmission electron microscope at 80 kV.

Results and Discussion

Detection and circumscription of the archiascomycete lineage Phylogenetic trees (Fig. 1) for selected taxa of the Ascomycota were constructed using three different methods, i.e., neighbor-joining , maximum-likelihood, and maximum parsimony (not illustrated herein). The resultant major topologies were consistent between these trees. In the neighbor-joining tree, the archiascomycete members, including *Pneumocystis* and *Neolecta*, formed a monophyletic group with comparatively high bootstrap support (81%). This confidence level is

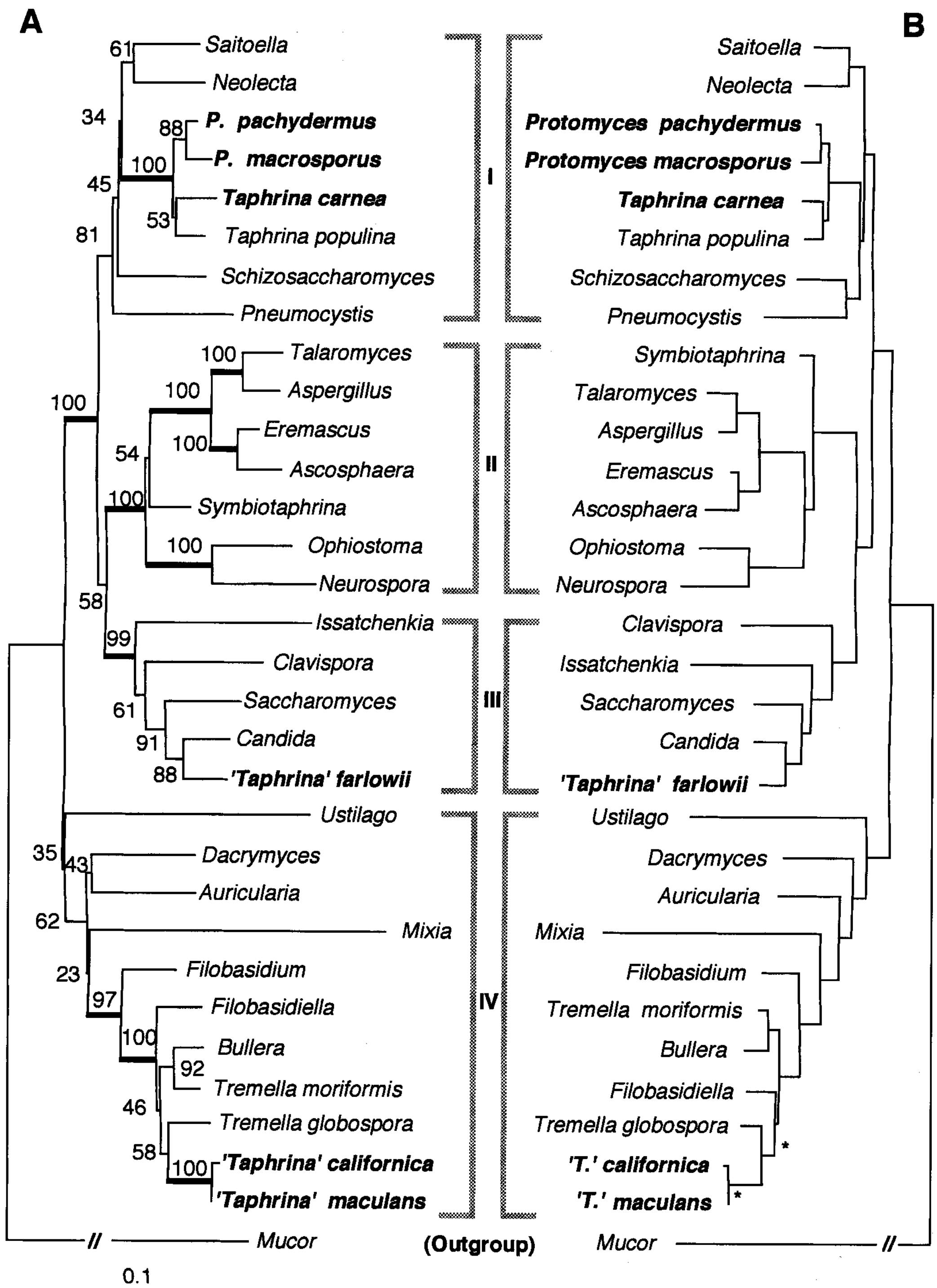

Fig. 1. 18S rDNA sequence-based trees, inferred from 1,363 aligned sites, showing phylogenetic relationships among the Ascomycota. A zygomycete, *Mucor racemosus*, was used as an outgroup. The species sequenced in this study are shown in bold. A. Bootstrapped neighbor-joining tree; bootstrap values derived from 1,000 replicates are shown as percentages; bold lines indicate branches that supported by more than 95% value. B. Maximum likelihood tree; asterisks indicate P>0.05. The scale bar represents a distance corresponding to 10 base changes per 100 nucleotide positions.

mostly the same as in our previous papers (Nishida and Sugiyama, 1993, 1994; Sugiyama and Nishida, 1994, 1995; Sugiyama et al., 1996a, b). Our phylogenetic trees also demonstrate that the Ascomycota is divided into three major lineages, i.e., archiascomycetes, hemiascomycetes (ascomycetous yeasts), and euascomycetes (filamentous ascomycetes); the archiascomycetes diverged prior to separation of the other two major lineages. Among euascomycete taxa in Fig. 1, *Symbiotaphrina*, phenotypically similar to *Rhodotorula*, was previously placed in the family Taphrinaceae, but it differs from *Taphrina* by its symbiotic lifestyle with Anobiid beetles (Gams and von Arx, 1980; von Arx, 1981). Recent phylogenetic analyses based on the full sequence from the 18S rRNA gene by Noda and Kodama (1996) and the partial sequence (ca. 1,000 nucleotide positions) from the same gene by Jones and Blackwell (1996), suggested that *Symbiotaphrina* should not be accommodated in the 'Archiascomycetes' but should be placed in part of an early radiation of filamentous ascomycetes including apothecial and fissitunicate taxa. Our trees (Fig. 1) also clearly demonstrated that *Symbiotaphrina* should be placed within the euascomycetes. The close relatives of this genus still remain obscure. The respective groups of archiascomycetes are discussed below.

Is *Taphrina* monophyletic? Fourteen strains of *Taphrina* selected from different host plants were used for sequencing (Table 1). The variability of host plants was selected to analyze whether a correlation exists between the parasite and its hosts: six *Taphrina* species from the Rosaceae (*T. communis*, *'T'. farlowii*, *T. flavorubra*, *T. mirabilis*, *T. pruni*, and *T. pruni-subcordatae*), four *Taphrina* species from the Betulaceae (*T. carnea*, *T. nana*, *T. robinsoniana*, and *T. virginica*), and one *Taphrina* species each from the Aceraceae, Urticaceae, Zingiberaceae, and fern (*T. letifera*; *T. ulmi*; *'T'. maculans*, and *'T'. californica*). The trees (Fig. 2) do not reflect the affinities between fungal species and their hosts. We think that the 18S rRNA gene sequence is inappropriate to analyze the parasite-host relation: other genes (e.g., small subunit mt-rDNA, ITS 1-5.8S rDNA-ITS 2) should be examined in order to solve this problem.

As shown in Fig. 2, all species of *Taphrina*, except for *'T'. californica* CBS 374.39, *'T'. farlowii* CBS 376.39, and *'T'. maculans* CBS 427.69, formed a monophyletic lineage (94% bootstrap confidence in the neighbor-joining tree). All species of *Protomyces* also formed a monophyletic group with strong bootstrap support (94%). Among the archiascomycetes, a core group of *Taphrina* and *Protomyces* always formed a monophyletic group which received strong bootstrap support (100%). Consequently, 18S rRNA gene analysis suggests a close relationship between the two genera. Unification of both genera into a single order will be mentioned later.

Both *Taphrina* and *Protomyces* spp. produce carotenoids (van Eijk and Roeymans, 1982). Hence, it is not possible to separate the two genera on the basis of the presence or absence of these pigments. However, significant differences have been found in the sterol profiles (van Eijk and Roeymans, 1982). Most strains of *Taphrina* have brassicasterol as a major sterol component. Brassicasterol is rare as the principal sterol in fungi, and has hitherto not been found in the red yeasts. It is also a predominant sterol in *Protomyces* species, in which ergosterol is absent. van Eijk and Roeymans (1982) reported the absence of brassicasterol in *'T'. californica* CBS 374.39, *'T'. farlowii* CBS 376.39, and *'T'. maculans* CBS 427.69. In these strains, ergosterol was found to be the major component. Their finding supports our molecular evidence for the exclusion of three strains from *Taphrina*.

Transfer of *'Taphrina' californica* and *'Taphrina' maculans* to the basidiomycetes lineage As shown in Table 2, both genotypic and phenotypic characters of *'Taphrina' californica* CBS 374.39 and *'T'. maculans* CBS 427.69 showed a basidiomycete nature, such as a positive reaction for DBB color and urease activity tests (e.g., Sugiyama and Nishida, 1995; Sugiyama et al., 1996a). Other characteristics are as follows: Q-10 as the major ubiquinone, 49.3 (for CBS 374.39) and 46.7 (for CBS 427.69) mol% G+C content (Table 2); no pseudomycelium nor true mycelium is formed; no ballistospores are formed; no fermentation ability; no formation of starch-like compound; no assimilation of inositol; and no extracellular DNase activity. As shown in our phylogenetic trees (Figs. 1, 3), both strains are closely related to each other (100% bootstrap support) and grouped together with members of Tremellalean fungi, i.e., *Tremella moriformis*, *T. globospora*, *Bullera alba*, and *Cryptococcus neoformans*. In addition, *'T'. californica* CBS 374.39 and *'T'. maculans* CBS 427.69 contained xylose in their cell wall (Table 2; cf., Prillinger et al., 1990). The differences between the two species are in their mol% G+C content and the quantitative sugar composition of the cell wall. Our ultrastructural observations revealed that both strains had the multi-layered cell wall and the enteroblastic type of conidium ontogeny (Figs. 4E, 4F). The highly characteristic bud scars in *'T'. californica* CBS 374.39 (Fig. 4F) are suggestive of enteroblastic budding, which has already been investigated by Heath et al. (1982). Heath et al. (1987) also reported that there is considerable mitotic heterogeneity within the genus *Taphrina*, and all observed aspects of mitosis in both *'T'. californica* and *'T'. maculans* are very similar to those characteristics of the basidiomycetous pattern. Our 18S rDNA sequence data of *'T'. maculans* CBS 427.69 are consistent with the characters mentioned above, which showed strong similarity with basidiomycetous yeasts. In *'T'. californica* CBS 374.39 shows a substantially different mitotic system and nuclear behaviour from other ascomycetes and other *Taphrina* species, suggesting that it is not a member of the genus (Heath et al., 1982). Based on genotypic and phenotypic data of *'T'. californica* and *'T'. maculans*, we assume that both CBS strains 374.39 and 427.69 might be misisolated or misidentified (for *'T'. californica*, cf. Jones and Blackwell, 1996) as a *Taphrina* and should be excluded from the 'Archiascomycetes' and transferred to the Basidiomycota. However, the closest relative genus for both strains remain uncertain.

***'Taphrina' farlowii* should be assigned to the hemiascomycete lineage** Its molecular characters showed that *'T'. farlowii* CBS 376.39 was closely related to members of the Saccharomycetales (Kurtzman and Sugiyama, unpublished) rather than *Taphrina* spp. Our tree (Fig. 1)

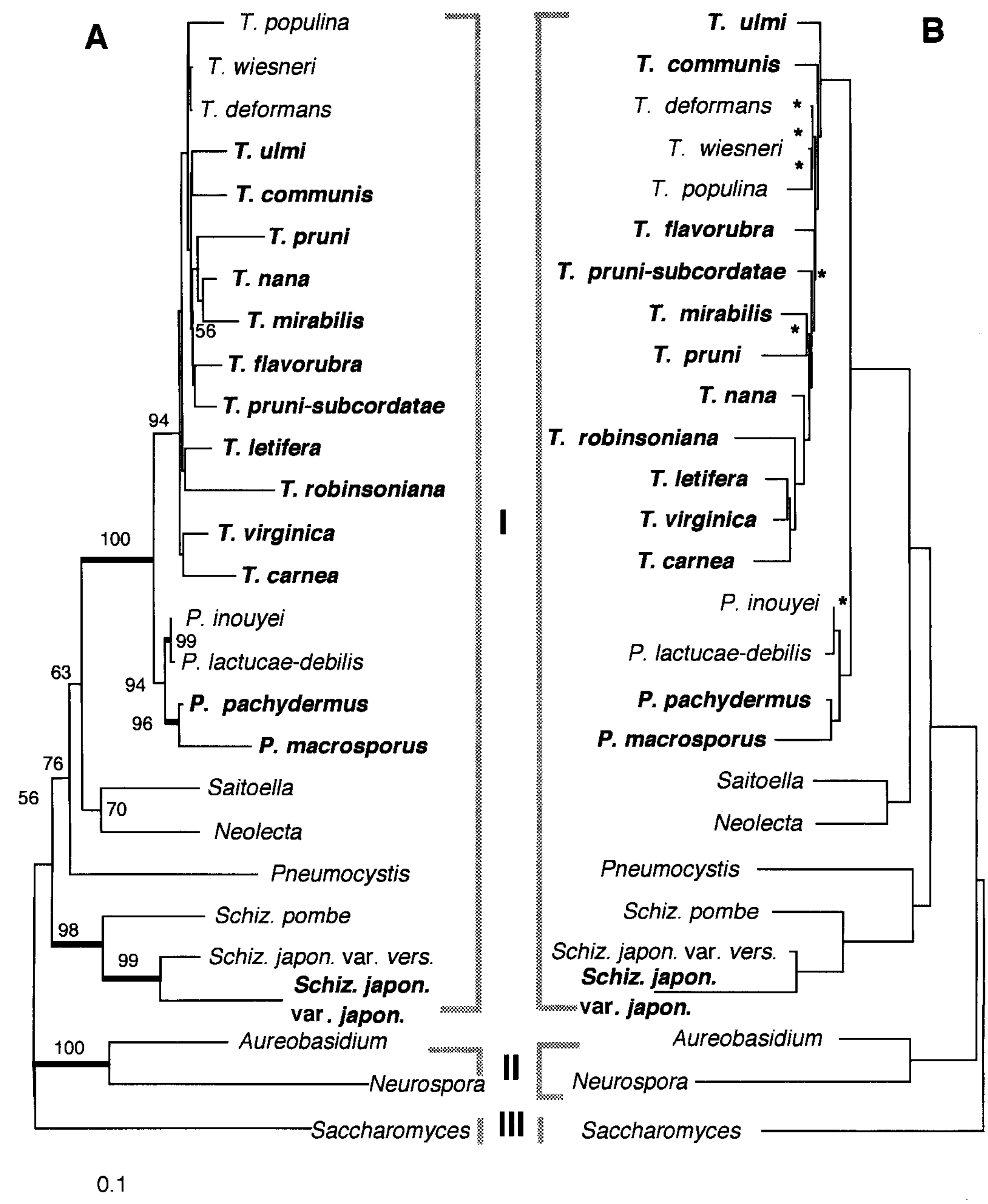

Fig. 2. Phylogenetic relationships among the archiascomycetes as inferred from 1,307 aligned sites of 18S rRNA gene sequence. I=archiascomycetes; II=euascomycetes; III=hemiascomycetes. II and III were used as outgroups. The species sequenced in this study are shown in bold. A. Neighbor-joining tree; bootstrap percentages from 1,000 replicates are shown on the respective internal nodes that are well supported or discussed in the text; bold lines indicate branches supported by more than 95% value. The scale bar represents a distance corresponding to 10 base changes per 100 nucleotide positions. B. Maximum likelihood tree; asterisks indicate P>0.05.

demonstrated that its closest relative was *Candida albicans*, which received 88% bootstrap support. Our ultrastructural data (Fig. 4C) of the cell wall structure and the mode of conidium ontogeny demonstrate that *'T'. farlowii* CBS 376.39 is characterized by a two-layered wall and the holoblastic type of conidium ontogeny, typical of ascomycetous yeasts. However, the ultrastructure of two strains of *T. wiesneri* CBS 345.56 and the type species *T. populina* CBS 337.55 (TEM micrograph not shown) showed presumably the multilayered cell wall and the enteroblastic type of conidium ontogeny (Figs. 4A, B). Further ultrastructural studies of *Taphrina* spp. are required to determine their cell wall type and mode of conidium ontogeny. *'Taphrina' farlowii* CBS 376.39 was characterized by the Q-9 system and 45.7 mol% G+C content (Table 2). The major ubiquinone system is

Table 2. Biochemical and chemotaxonomic characteristics of species of *Taphrina*, *Protomyces*, and *Mixia*.

Fungus	Strain	Cell wall sugar (%)[a]								G+C content (mol%)	Ubiquinone system
		Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc		
Protomyces inouyei	IFO 6898	16	—	—	—	—	23	—	61		
P. inouyei	ATCC 16175	7	—	—	—	—	16	—	77		Q-10[b]
P. macrosporus	IMI 102384	9	—	—	—	—	24	—	70	49	
P. macrosporus	IMI 102385	17	—	—	—	—	22	5	56		
Taphrina betulina	CBS 417.54	15	—	—	—	—	16	+	68	47	
T. caerulescens	IFO 9242	20	—	—	—	—	22	5	54	41	Q-10
'T'. californica	CBS 374.39	—	—	—	—	11	20	3	66	49	Q-10
T. cerasi	ATCC 34555	17	—	—	—	—	17	4	61	43	
T. cerasi	CBS 275.28	16	—	—	—	—	20	5	59	48	
T. communis	CBS 352.35	3	—	—	—	—	24	16	58		
T. deformans	CBS 356.35	—	—	—	—	—	16	8	77	46	Q-10[b]
T. deformans	IMI 108563	7	—	—	—	—	13	6	73	46	
T. deformans	IFO 8996	6	—	—	—	—	14	9	71		
'T'. farlowii	CBS 376.39	—	—	—	—	—	65	—	35	46	Q-9
T. flavorubra	CBS 377.39	8	—	—	—	—	22	9	61	45	
T. flavorubra	IFO 9245	5	—	—	—	—	23	9	63	48	Q-10
T. letifera	CBS 335.55	2	—	—	—	—	27	17	54	47	
'T'. maculans	CBS 427.69	—	—	—	—	9	10	2	80	47	Q-10[b]
T. mirabilis	CBS 357.35	2	—	—	—	—	28	11	59		
T. pruni	CBS 358.35	3	—	—	—	—	11	7	79	47	
T. tormentillae	CBS 339.55	8	—	—	—	—	21	8	64	41	Q-10
T. ulmi	CBS 420.54	5	—	—	—	—	21	7	68	45	
T. virginica	CBS 340.55	18	—	—	—	—	22	4	57	50	Q-10
T. wiesneri	IFO 7776	11	—	—	—	—	27	—	62	46	Q-10[b]
Mixia osmundae	IFO 32408	6	—	—	—	—	43	19	32	51	Q-10[b]

[a] Abbreviations: Rha=rhamnose; Fuc=fucose; Rib=ribose; Ara=arabinose; Xyl=xylose; Man=mannose; Gal=galactose; Glc=glucose; +, trace amount; —, no detection.
[b] Data from Kuraishi et al. (1991).

identical not with that of most *Taphrina* spp. (Moore and Flinn, 1991; Sugiyama et al., 1985; Yamada et al., 1983, 1987; cf. Kuraishi et al., 1991) but with that of *Candida albicans* (Yamada and Kondo, 1972). The DNA base composition difference between the two species is ca. 8–11 mol% G+C (Barnett et al., 1990). *Taphrina* spp. are characterized by readily detectable amounts of rhamnose, the predominance of glucose, and moderate amounts of mannose in their cell wall (Prillinger et al., 1990). The monosaccharide pattern of purified cell wall of *'T'. farlowii* showed the absence of galactose, the dominance of mannose and the predominance of glucose (Table 2), which are characteristics of the *Saccharomyces* type (Prillinger et al., 1993). However, the aspects of mitosis observed in this species are a combination of both ascomycetous and basidiomycetous types (Heath et al., 1987). An integration of phenotypic and genotypic data suggests that *'T'. farlowii* CBS 376.39 should be assigned to the hemiascomycete (Saccharomycetalean) lineage. Therefore, it is misisolated or misidentified as a species of *Taphrina*.

***Protomyces* spp.** As stated above, *Protomyces* always appears as a monophyletic group (Figs. 1, 2). The type species, *Protomyces macrosporus*, is very close to *P. pachydermus*, and distantly related to *P. inouyei* and *P. lactucae-debilis*. Nishida et al. (1997) found that *P. pachydermus*, which is parasitic on the dandelion (*Taraxacum platycarpum*), has two group I introns within the 18S rRNA gene. We confirmed that fourteen *Taphrina* spp. do not contain any introns within their 18S rRNA genes. Nishida et al. (1997) suggested that *Protomyces* spp. gained group I introns at an early stage of species diversification of *Protomyces*. According to Heath et al. (1982, 1987), the *Protomyces* type of mitotic apparatus is clearly similar to the typical ascomycetous type. We would reserve discussions on the differences between *Protomyces* and *Taphrina*, though the mitotic apparatus of the true *Taphrina* species is presumably an ascomycetous type (Heath et al., 1982). More detailed comparative studies based on authentic isolates are required in order to define the mitotic apparatus in the genus *Taphrina*.

***Schizosaccharomyces* spp.** Traditionally the genus *Schizosaccharomyces* has been placed within the Saccharomycetaceae, Endomycetales, and characterized by having fission cells, true hyphae and producing spherical,

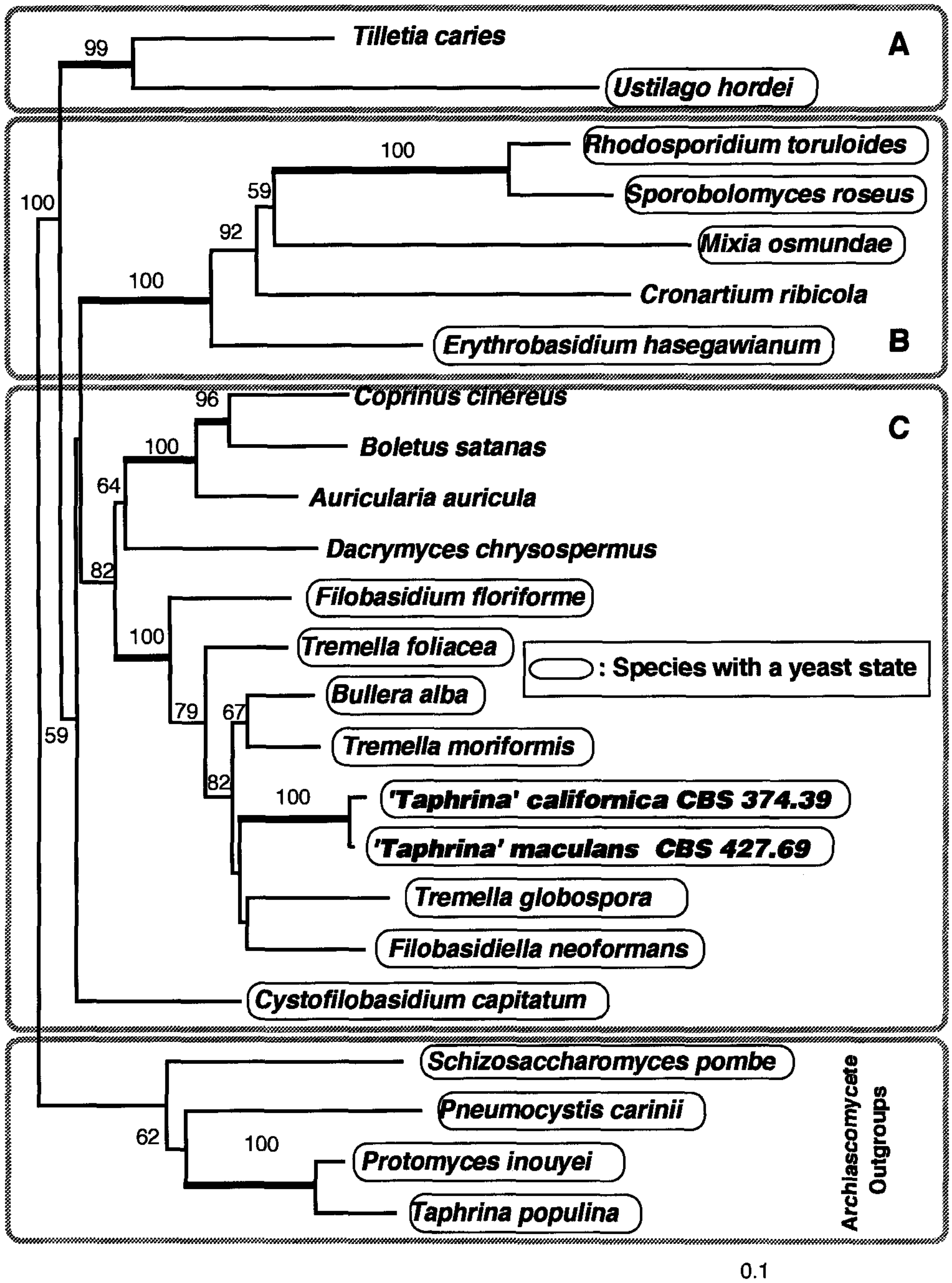

Fig. 3. Bootstrapped neighbor-joining tree, inferred from 1,630 aligned sites of 18S rRNA gene sequence, showing phylogenetic relationships among basidiomycetous yeasts.
Bootstrap values derived from 1,000 replicates are shown as percentages. A=ustilaginomycetes; B=urediniomycetes; C=hymenomycetes. Bracketed group assigned to the archiascomycete lineage is used as outgroups. The scale bar represents a distance corresponding to 10 base changes per 100 nucleotide positions.

ovoidal or reniform, smooth or warty, liberated ascospores (Kreger-van Rij, 1984). Yamada and Banno (1987) revised their taxonomies. They divided the fission yeasts into three genera based on differences in ascospore morphology, ubiquinone systems, and cellular linoleic acid content. These are *Schizosaccharomyces* (*Schiz. pombe*), ascospores warty, Q-10 system, linoleic acid absent; *Octosporomyces* (*O. octosporus*), ascospores smooth with papillae, Q-9 system, linoleic acid absent; *Hasegawaea* (*H. japonica*), ascospores smooth and lack papillae, no ubiquinone detected, linoleic acid present. On the other hand, Kurtzman and Robnett (1991) insisted from their rRNA sequence analyses that all three species should be maintained in *Schizosac-*

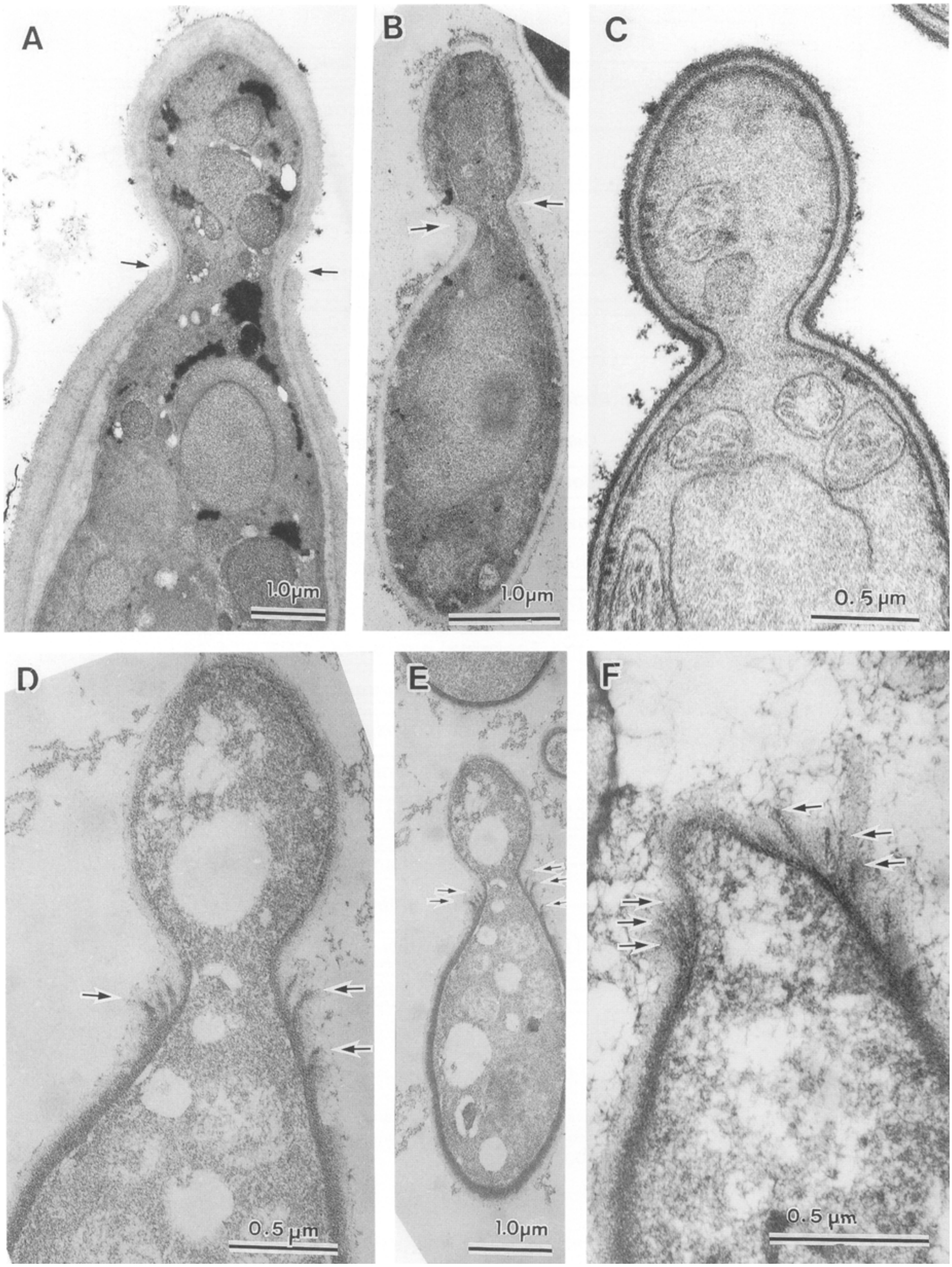

Fig. 4. Transmission electron micrographs showing the cell wall ultrastructure and conidium ontogeny.
A, B. *Taphrina wiesneri* IFO 7776. C. *'T'. farlowii* CBS 376.39. D–E. *'T'. maculans* CBS 427.69. F. *'T'. californica* CBS 374.39. Arrows in A and B indicate the scar of the first budding, and other arrows in D–F indicate the scars which were caused by the successive enteroblastic budding.

charomyces. Subsequently, Yamada et al. (1993) argued for the separation of these genera on the basis of partial rRNA sequence analyses.

K. Mikata (pers. comm.) re-examined the distribution of ubiquinone systems in fission yeast taxa by high-performance liquid chromatography. He found that two strains of *Schizosaccharomyces pombe* (IFO 0345 and IFO 0346), and *Schiz. malidevorans* (IFO 1608) had 64%, 85%, and 82% of Q-10, respectively; two varieties of *Hasegawaea japonica*, i.e., var. *japonica* (IFO 1609) and var. *versatilis* (IFO 1607), had 93% and 92% of Q-10, respectively, although their quinone contents were low; and *Octosporomyces octosporus* had 98% of Q-9. Naehring et al. (1995) determined the sequences of the entire rRNA cluster (18S, 5.8S, and 25S) of *Schiz. japonicus* var. *versatilis* and compared them with those established for *Schiz. pombe* and other yeasts. As a result, subdivision of fission yeasts into the genera *Schizosaccharomyces* and *Hasegawaea* does not seem to be justified, because the differences between the rRNA genes of *Schiz. japonicus* and *Schiz. pombe* are much smaller than the intrageneric differences within the the rDNA sequences of other yeast genera.

In this study, we compared the 18S rDNA sequence of *Schiz. japonicus* var. *japonicus* (IFO 1609) with the homologous region of *Schiz. japonicus* var. *versatilis* and *Schiz. pombe*. As shown in Fig. 2, *Schiz. pombe* and *Schiz. japonicus* with the two varieties var. *japonicus* and var. *versatilis* formed a monophyletic group. At the moment, therefore, we support conclusion of Naehring et al. (1995) that there will be no need to introduce *Hasegawaea* into the group of fission yeasts.

Taylor et al. (1994) and Sugiyama and Nishida (1995) suggested that there was a close relationship between *Schizosaccharomyces* and *Pneumocystis* based on their life cycle. Our maximum likelihood trees (Figs. 1B, 2B) demonstrated a close relationship between three taxa of *Schizosaccharomyces* and *Pneumocystis carinii*, though the topology is different in our NJ trees (Figs. 1A, 2A). At the moment, it is uncertain which type of tree is reliable for this lineage.

Saitoella* and *Neolecta As mentioned, Goto et al. (1987) suggested a close affinity between *Saitoella* and the Taphrinales based on polyphasic taxonomic studies. Nishida and Sugiyama (1993, 1994), Nishida et al. (1993), and Sugiyama et al. (1993) verified this phylogenetic speculation from the 18S rDNA sequence analysis. However, the formal taxonomic placement of *Saitoella* within the 'Archiascomycetes' awaits discovery of its teleomorph (Kurtzman and Sugiyama, unpublished).

Our 18S rDNA sequence-based trees also suggest a close relationship between *Saitoella* and *Neolecta*, although the bootstrap confidence levels are not so high (61% in Fig. 1A and 70% in Fig. 2A, respectively). For the moment, there are no reliable observations about whether *Neolecta* forms ascogenous hyphae (Scott Redhead, pers. comm.). Croziers are not formed and there appear to be no paraphyses; thus the entire hymenium consists of one tissue type. Asci of *N. vitellina* are occasionally filled with numerous conidia and the ascospores become conidiogenous by producing a single apical collarette from which the phialoconidia are formed (Redhead, 1977). These features are similar to those of *Taphrina*, and therefore may not conflict with the proposed molecular phylogeny. It is uncertain whether the anamorphic state of *Neolecta* is yeastlike, and further studies are needed to assign this genus within the 'Archiascomycetes' and also to find its closest relatives.

Taxon specific positions in 18S rDNA sequences This study confirms the previous results obtained by Nishida and Sugiyama (1994) that there are four positions in the 18S rDNA sequences which are specific for the three major ascomycete lineages. The four positions were 478, 479, 883, and 970 (positions as in the 18S rDNA sequence of *Saccharomyces cerevisiae*, DNA data bank M27607). In addition, based on studies of 28 ascomycetes, Eriksson and Hawksworth (1995) added three other specific positions, 480, 1079 and 1389.

Table 3 summarizes the results of analysis of taxon-specific position in the 18S rRNA gene based on our sequence data set, which includes 23 species of archiascomycetes, 14 species of hemiascomycetes, 23 species of euascomycetes, and 3 species of basidiomycetes. All archiascomycetes and hemiascomycetes have an A in position 478, while all euascomycetes have a C. All have a C in 479, while euascomycetes have a U. All archiascomycetes have an A in position 480, while all others ascomycetes have a G, except for *Dipodascopsis uninucleata* and *Peziza badia*, which have an A. Position 883 is U in all archiascomycetes, but C in all other ascomycetes. Position 970 is an A in all archiascomycetes and hemiascomycetes, but G in euascomycetes, except for *Pleospora rudis*, *Aureobasidium pullulans*, *Ascosphaera apis*, *Eremascus albus*, *Coccidioides immitis*, and *Blastomyces dermatitidis*, which have a U. Position 1079 is a U in all archiascomycetes, except for *Schizosaccharomyces pombe*, which has a C; and all other ascomycetes have a C, except for *Pichia membranaefaciens*, which has a G. Position 1389 is U in all archiascomycetes, and C in all other ascomycetes, except for *Clavispora lusitaniae* and *P. membranaefaciens*, which have a U and G, respectively.

The plesiomorphous nature of some of the signatures in the archiascomycetes is indicated by the fact that three basidiomycetes, *Boletus satanas* (Eriksson and Hawksworth, 1995), *Ustilago maydis*, and *Rhodosporidium toruloides*, have the same nucleotide in positions 478, 479, 480, 883, and 970.

Kurtzman (1993) and Eriksson (1995) raised the possibility from rRNA gene sequence analysis that the order Protomycetales may be a synonym of the Taphrinales. Our phylogenetic analysis of 18S rDNA sequences from 14 species of *Taphrina* and 4 species of *Protomyces* suggests that both genera always form one monophyletic group with 100% bootstrap support. From the results of 18S rDNA analyses, we think that Taphrinales and Protomycetales should be united in a single order Taphrinales with two families Taphrinaceae and Protomycetaceae. For such a treatment, more molecular

Table 3. Taxon-specific positions in 18S rRNA sequences among the higher fungi.

Position[a)]	Archiascomycetes (23 species)	Hemiascomycetes (14 species)	Euascomycetes (23 species)	Basidiomycota (3 species)
478	A	A	C	A
479	C	C	U	C
480	A	G (*Dipodascopsis uninucleata*=A)	G (*Peziza badia*=A)	A
883	U	C	C	U
970	A	A	G (*Pleospora rudis*, *Aureobasidium pullulans*, *Ascosphaera apis*, *Eremascus albus*, *Coccidioides immitis* *Blastomyces dermatitidis*=U)	A
1079	U (*Schizosaccharomyces pombe*=C)	C (*Pichia membranaefaciens*=G)	C	(*Boletus satanas*, *Ustilago maydis*=C, *Rhodosporidium toruloides*=A)
1389	U	C (*Clavispora lusitaniae*=U, *Pichia membranaefaciens*=G)	C	C

Peziza badia, acc. no.=L37539; *Neolecta vitellina*, acc. no.=Z27393; *N. irregularis*, acc. no.=Z47721; and 11 *Taphrina* spp. from this study.
[a)] Position of the corresponding residue in the *Saccharomyces cerevisiae* 18S rRNA sequence.

data for other genera in the Protomycetaceae are needed. For the moment, we follow the proposal for classification of the class 'Archiascomycetes' provided by Kurtzman and Sugiyama (unpublished). They classified the class 'Archiascomycetes' into five orders, i.e., Pneumocystidales, Schizosaccharomycetales, Neolectales, Protomycetales, and Taphrinales; however, the anamorph genus *Saitoella* was placed between the Neolectales and Protomycetales as incertae sedis.

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