Molecular systematics of the dimorphic ascomycete genus *Taphrina*

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The ascomycete genus *Taphrina* Fries comprises nearly 100 species recognized by their mycelial states when parasitic on different vascular plants. Whereas the filamentous state is strictly phytoparasitic, the yeast state is saprobic and can be cultured on artificial media. *Taphrina* species are differentiated mainly on the basis of host range and geographical distribution, type and site of infection and morphology of the sexual stage in infected tissue. However, there has been little progress in the systematics of the genus in recent years, mainly because of the scarcity of molecular studies and available cultures. The main aim of the present study was the reappraisal of species boundaries in *Taphrina* based on the genetic characterization of cultures (yeast states) that represent about one-third of the currently recognized species. The molecular methods used were (i) PCR fingerprinting using single primers for microsatellite regions and (ii) determination of nucleotide sequences of two approx. 600 bp nuclear rDNA regions, the 5′ end of the 26S rRNA gene (D1/D2 domains) and the internal transcribed spacer region (which includes the 5.8S rRNA gene). Sequencing results confirmed the monophyly of the genus (with the probable exclusion of *Taphrina vestergrenii*) and the combined analysis of the two methods corroborated, in most cases, separation of species defined on the basis of conventional criteria. However, genetic heterogeneity was found within some species and conspecificity was suggested for strains that have been deemed to represent distinct species. Sequences from the ITS region displayed a higher degree of divergence than those of the D1/D2 region between closely related species, but were relatively conserved within species (>99 % identity) and were thus more useful for the effective differentiation of *Taphrina* species. The results further allowed other topics to be addressed such as the correlation between the molecular phylogenetic clustering of certain species and the respective host plant family and the significance of molecular methods in the accurate diagnosis of the different diseases caused by *Taphrina* species.

INTRODUCTION

The genus *Taphrina* Fries belongs to the order Taphrinales Gäumann & Dodge, which in turn has been placed in the 'Archiascomycetes', a class provisionally proposed by Nishida & Sugiyama (1994) to accommodate a heterogeneous assemblage of basal lineages of the phylum Ascomycota (for a discussion of the taxa in these lineages see: Alexopoulos *et al.*, 1996; Kurtzman & Sugiyama, 2001). More recently, Eriksson & Winka (1997) have formally proposed the subphylum Taphrinomycotina for the archiascomycete lineages. The classical systematic studies of the genus *Taphrina* were carried out from the late 1800s through to the 1940s (Mix, 1936) and culminated in the monograph published by Mix (1949, 1954). No other comprehensive studies have been published since then, although some work has involved biochemical characterization of the few species that have been maintained in pure culture (reviewed by Kramer, 1987; Moore, 1998) and some authors have reported on regional surveys of the genus (e.g. Gjaerum, 1964; Bacigálová, 1997). More recently, molecular methods (namely sequencing of the 18S rRNA gene) have been used to unveil phylogenetic relationships among *Taphrina* species and other members of the archiascomycetes, but this study involved only a limited number of species represented by single strains (Sjamsuridzal *et al.*, 1997).

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Abbreviations: ITS, internal transcribed spacer; MSP-PCR, microsatellite-primed PCR fingerprinting.

The GenBank accession numbers of sequences determined in this study are AF492024–AF492075 (D1/D2) and AF492076–AF492129 and AF494056 (ITS).

A dendrogram resulting from analysis of combined MSP-PCR banding patterns is available as supplementary material in IJSEM Online (http://ijs.sgmjournals.org).

Table 1. Cultures of *Taphrina* species used in this study

Species are grouped according to host plant family and genus (listed alphabetically within each host genus). Origins of strains and culture collection abbreviations are outlined in Methods.

Strain	Host and geographical origin*	Type and/or site of infection†
Species parasitic on Aceraceae		
T. dearnessii		Leaf spots
CBS 334.55	*Acer rubrum*, N. America	
NRRL T-796 (=CBS 334.55)	*Acer rubrum*, N. America	
T. letifera		Leaf spots
CBS 335.55	*Acer spicatum*, N. America	
NRRL T-791 (=CBS 335.55)	*Acer spicatum*, N. America	
T. sacchari NRRL T-210	*Acer saccharum*, N. America	Leaf spots
Species parasitic on Anacardiaceae		
T. purpurascens		Leaf curl
F 323 (=CBS 338.55)	*Rhus copallinum*, N. America	
NRRL Y-17789 (=CBS 338.55)	*Rhus copallinum*, N. America	
Species parasitic on Betulaceae		
T. alni		Tongues on female catkins
HA 872 (=CBS 683.93)	*Alnus incana*, Austria	
HA 1364	*Alnus incana*, Slovakia	
T. epiphylla HA 1439	*Alnus incana*, Slovakia	Witches' brooms and leaf spots
T. robinsoniana		Tongues on female catkins
CBS 382.39	*Alnus rugosa*, N. America	
HA 850 (=CBS 382.39)	*Alnus rugosa*, N. America	
NRRL T-732	*Alnus serrulata*, N. America	
T. sadebeckii		Leaf spots
CBS 102170	*Alnus glutinosa*, Germany	
HA 1308 (=CBS 102170)	*Alnus glutinosa*, Germany	
NRRL T-713	*Alnus rugosa*, Sweden?	
HA 1345	*Alnus glutinosa*, Slovakia	
T. tosquinetii		Leaf curl
CBS 276.28	*Alnus glutinosa*?, Europe?	
NRRL T-493	*Alnus glutinosa*, Sweden?	
HA 1335	*Alnus glutinosa*, Slovakia	
T. americana CBS 331.55	*Betula fontinalis*, N. America	Witches' brooms
T. betulina		Witches' brooms
CBS 417.54 (=NRRL T-730)	*Betula nana*×*pubescens*, Sweden	
NRRL Y-17785 (=CBS 417.54)	*Betula nana*×*pubescens*, Sweden	
NRRL T-726	*Betula* '*intermedia*', Sweden	
T. carnea		Leaf curl
CBS 332.55 (=NRRL T-706)	*Betula* '*intermedia*', Europe?	
NRRL T-705	*Betula* '*intermedia*', Europe?	
T. nana F 317 (=CBS 336.55 =NRRL T-722)	*Betula nana*, Sweden	Witches' brooms
T. carpini CBS 102169	*Carpinus betulus*, Slovakia	Witches' brooms
T. virginica CBS 340.55 (=NRRL T-235)	*Ostrya virginiana*, USA	Leaf curl or thickening
Species parasitic on Fagaceae		
T. caerulescens		Leaf spots
CBS 351.35	*Quercus alba*, N. America?	
NRRL T-878	*Quercus macrocarpa*, USA	
Species parasitic on Rosaceae		
T. tormentillae		Thickened leaf spots
CBS 339.55 (=NRRL T-283)	*Potentilla canadensis*, N. America	
NRRL T-422	*Potentilla canadensis*, N. America	
T. communis		Plum pockets (also leaf curl and deformed twigs)
F 300 (CBS 352.35)	*Prunus americana*, N. America	
NRRL Y-17786 (=CBS 352.35)	*Prunus americana*, N. America	

Table 1. cont.

Strain	Host and geographical origin*	Type and/or site of infection†
NRRL T-755	*Prunus nigra*, N. America	
NRRL T-842	*Prunus americana*, N. America	
T. confusa		Deformed flowers and fruits
F 301 (=CBS 375.39)	*Prunus virginiana*, N. America	
NRRL T-417	*Prunus virginiana*, N. America?	
T. deformans		Leaf curl (also deformed fruits and twigs)
CBS 356.35	*Prunus persica*, Netherlands	
NRRL T-857	*Prunus persica*, ?	
NRRL T-470	*Prunus dulcis*, ?	
AX1	*Prunus persica*, Portugal	
HA 1304 (=CBS 102167)	*Prunus persica*, Slovakia	
T. flavorubra		Deformed fruits (pockets)
NRRL Y-17795 (=CBS 377.39)	*Prunus susquehanae*, USA	
NRRL T-882	*Prunus susquehanae*, USA	
T. mirabilis		Deformed twigs and plum pockets
CBS 357.35	*Prunus angustifolia*, N. America	
NRRL T-335	*Prunus angustifolia*, N. America	
T. padi		Deformed fruits (pockets)
CBS 693.93	*Prunus padus*, Germany	
HA 100 (=CBS 683.93)	*Prunus padus*, Germany	
HA 1305	*Prunus padus*, Slovakia	
T. pruni		Plum pockets (also deformed twigs)
F 321 (CBS 358.35)	*Prunus domestica*, N. America?	
HA 1306	*Prunus domestica*, Slovakia	
HA 1340	*Prunus spinosa*, Slovakia	
T. pruni-subcordatae CBS 381.39	*Prunus subcordata*, N. America	Plum pockets (also deformed twigs)
T. wiesneri		Witches' brooms (also leaf curl and deformed twigs)
F 297 (=CBS 275.28)	*Prunus avium*?, ?	
NRRL T-293	*Prunus avium*, ?	
NRRL T-460	*Prunus pennsylvanica*, USA	
HA 1388	*Prunus fruticosa*, Austria	
HA 1437	*Prunus avium*, Slovakia	
Species parasitic on Salicaceae		
T. johansonii		Deformed carpels
F 313 (=CBS 378.39)	*Populus tremuloides*, N. America?	
NRRL T-740	*Populus tremuloides*, USA	
T. populina		Yellow leaf spots
F 319 (=CBS 337.55)	*Populus nigra*, Sweden	
NRRL T-497 (=CBS 337.55)	*Populus nigra*, Sweden	
NRRL Y-6300	*Populus nigra*, Canada	
NRRL Y-17788	*Populus nigra*, Switzerland	
T. populi-salicis CBS 419.54 (=NRRL T-812)	*Populus trichocarpa*, N. America	Yellow leaf spots
Species parasitic on Ulmaceae		
T. ulmi F 328 (=CBS 420.54)	*Ulmus rubra*, USA	Leaf blisters or spots
Species parasitic on ferns		
T. polystichi CBS 379.39	*Polystichum acrosticoides* or *Dryopteris carthusiana*?, USA?	Yellow leaf spots
T. vestergrenii		Galls on leaves
CBS 679.93	*Dryopteris filix-mas*, Europe?	
HA 244 (=CBS 679.93)	*Dryopteris filix-mas*, Europe?	

*Species names and geographical locations of host plants are indicated unless that information is not available for a particular strain; in the latter case, a tentative name or region, based on the data of Mix (1949), is followed by a question mark; N. America refers to USA or Canada.
†Type of infection symptom for each species according to Mix (1949, 1954) and Gjaerum (1964).

All *Taphrina* species are dimorphic (Mix, 1949; Kramer, 1987). Their filamentous states are parasitic on vascular plants belonging to different families, where they cause diverse malformations of the infected tissue such as leaf curl, leaf blisters or spots, galls on stems or inflorescences and witches' brooms (Mix, 1949). Economically important hosts include some fruit trees, namely *Prunus* spp. (peach, plum, cherry). The best known species is *Taphrina deformans* (Berk.) Tulasne, the agent of peach leaf curl, a disease that affects orchards throughout the temperate regions of the world (Mix, 1935). Mycelium and the distinctive naked asci of *Taphrina* species are formed exclusively in their parasitic phase, whereas the yeast states, which result from budding of the ascospores, are saprobic and can be grown on artificial media (Mix, 1949; Kramer, 1987). The existing cultures correspond to yeast forms that were, in most cases, isolated from infected plant material using the spore-fall method. Differentiation from conventional yeasts can be accomplished by a unique combination of physiological and biochemical characteristics displayed by *Taphrina* yeast phases: a negative Diazonium blue B reaction; positive results in tests for the presence of urease and extracellular amyloid compounds; and cell-wall carbohydrate composition (Prillinger *et al.*, 1990). However, there has been some confusion in the literature dealing with *Taphrina* due to the inadvertent study of strains of ascomycetous or basidiomycetous yeasts misidentified as *Taphrina* species (e.g. Heath *et al.*, 1982; Sjamsuridzal *et al.*, 1997; Moore, 1998).

Taphrina species have been differentiated mainly on the basis of host range, geographical distribution, type and site of infection, localization of the mycelium and morphology of sexual structures in the infected tissue (Mix, 1949). However, the validity of separating species on related hosts has been debated by several authors (Mix, 1949; Gjaerum, 1964). Molecular methods are a valuable tool for this purpose, but few studies have focused on members of the genus *Taphrina* (Sjamsuridzal *et al.*, 1997; Prillinger *et al.*, 2000). Here, we report on the re-evaluation of species differentiation within the genus based on the comparative analysis of selected genetic characteristics of strains obtained from culture collections that represent about one-third of the currently recognized species, and argue for the use of molecular methods to identify the actual *Taphrina* species causing infections.

METHODS

Cultures. Strains used in this study are listed in Table 1. Species names conform with Mix (1949, 1954) and Gjaerum (1964, 1966). Host plant names follow the Germplasm Resources Information Network (GRIN) on-line database (USDA, ARS, National Genetic Resources Program, National Germplasm Resources Laboratory, Beltsville, MD, USA). Strains were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) and the ARS Culture Collection, NCAUR, Peoria, IL, USA (NRRL). Additional strains were supplied by H. Prillinger, IAM, Vienna, Austria (HA strains) and F. Oberwinkler, University of Tübingen, Germany (F strains). One strain of *T. deformans* was isolated in Portugal from leaves of *Prunus persica* displaying peach leaf curl symptoms (AX1). Strains were maintained on yeast extract-malt extract (YM) agar slants at 4 °C.

Molecular methods. Genomic DNA was isolated from 1-week-old cultures on YM agar plates by a simplified method using glass beads for cell disruption following the protocol used by Gadanho *et al.* (2001) without the final precipitation step. PCR amplification of polymorphic regions of genomic DNA using the microsatellite primers $(GAC)_5$ and $(GTG)_5$ (MSP-PCR) followed the protocol described in Gadanho *et al.* (2001) using 0·25 mM of each of the four dNTPs. Gel electrophoresis images were acquired with the Kodak Digital Science 1D image analysis software. DNA banding patterns were analysed with GELCOMPAR (version 4.1; Applied Maths) using Pearson's correlation coefficient and dendrograms were computed using the UPGMA clustering method. PCR amplification prior to sequencing employed primers NS7 (5′-GAGGCAATAACAGGTCTGTGATGC) or ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and LR6 (5′-CGCCAGTTCTGCTTACC) using a Uno II thermal cycler (Biometra) and the resulting amplicon was purified with the GFX band purification kit (Amersham Pharmacia Biotech). Cycle sequencing of the D1/D2 variable domains of the 26S rDNA employed forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG) and that of the internal transcribed spacer (ITS) region (comprising ITS1, 5.8S rRNA gene and ITS2) employed forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC), following standard protocols. In a few cases, amplification for sequencing of the D1/D2 region employed primers NL1 and NL4. Sequences were obtained with an ALFexpress II DNA analyser (Amersham Biosciences), aligned with MegAlign (DNAStar software package) and visually corrected. Phylogenetic trees were computed with PAUP version 4.0b8 (Sinauer Associates) using the neighbour-joining method and Kimura's two-parameter model for calculating distances, or maximum-parsimony analysis (full heuristic search with the following options: random stepwise addition with 10 replications, branch swapping using tree bisection–reconnection and 100 maximum trees). Gaps were treated as missing data. GenBank accession numbers of D1/D2 (AF492024–AF492075) and ITS (AF492076–AF492129, AF494056) sequences are shown in Figs 1 and 2, together with additional sequences retrieved from GenBank.

RESULTS AND DISCUSSION

MSP-PCR fingerprinting

Reproducibility of the MSP-PCR fingerprinting technique was checked by comparing the banding profiles resulting from independent extractions and amplifications of strains presumed to be identical, but which had been maintained in different collections, e.g. *Taphrina betulina* CBS 417.54 and NRRL Y-17785 or *Taphrina purpurascens* CBS 338.55 and NRRL Y-17789 (Table 1) (data not shown). The similarity values between fingerprints obtained for strains of each pair, with both primers, were generally above 90 %, thus confirming not only the identity of the strains but also the reproducibility of the banding patterns. The ability of the selected primers to produce species-specific fingerprints was then investigated by the study of a larger set of strains (the dendrogram resulting from analysis of the combined banding patterns obtained with each of the two primers is available as supplementary material in IJSEM Online at http://ijs.sgmjournals.org). It was apparent that, in most

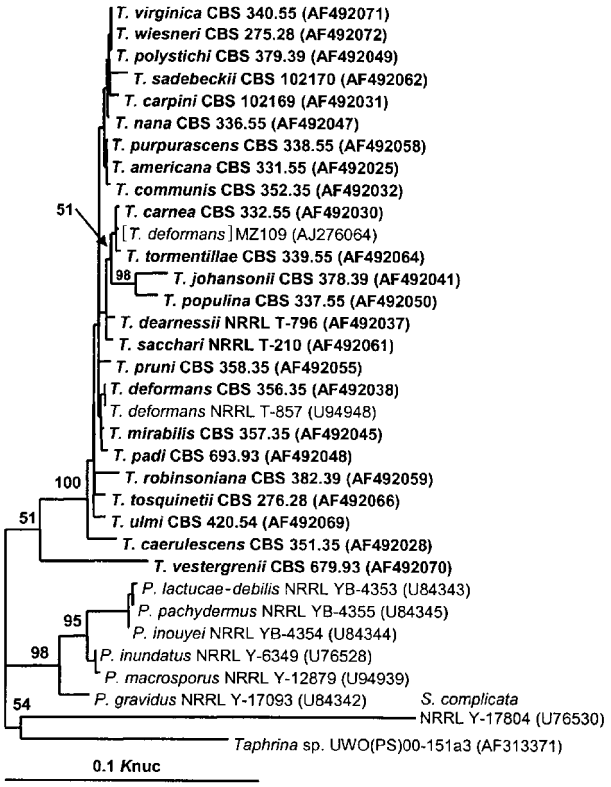

Fig. 1. Phylogenetic tree of *Taphrina* species and selected archiascomycetes obtained by neighbour-joining analysis of the D1/D2 domains of the 26S rRNA gene using PAUP 4.0b8. Numbers given on branches are frequencies (>50 %) with which a given branch appeared in 1000 bootstrap replications. *Saitoella complicata* and *Taphrina* sp. UWO(PS)00-151a3 were used as the outgroup. Sequences determined in the present study are in bold. Additional sequences were retrieved from GenBank. *P.*, *Protomyces*.

cases, strains that were deemed to represent different species according to conventional criteria gave rise to distinct PCR fingerprints, whereas strains of the same species had similar banding patterns and clustered together in the dendrogram. However, there were some notable exceptions that may denote either misidentification of strains or misclassification of species. On the one hand, some strains of different species clustered together with both primers: e.g. *T. betulina* NRRL T-726, *Taphrina carnea* NRRL T-705 and *Taphrina nana* CBS 336.55; *Taphrina tormentillae* CBS 339.55 and *T. carnea* CBS 332.55; *Taphrina pruni* CBS 358.35 and *Taphrina communis* CBS 352.35; and *Taphrina robinsoniana* NRRL T-732 and *T. betulina* CBS 417.54. On the other hand, some strains deemed to be conspecific displayed very distinct PCR fingerprints and clustered separately with either primer: e.g. *Taphrina caerulescens* CBS 351.35 and NRRL T-878; *Taphrina populina* CBS 337.55 and NRRL Y-6300/Y-17788; *T. betulina* NRRL T-726 and CBS 417.54; *T. robinsoniana* NRRL T-732 and CBS 382.39; and *T. carnea* CBS 332.55 and NRRL T-705. The classification of these strains is discussed further below in the light of sequencing results.

rDNA sequencing

D1/D2 region. All sequences from the D1/D2 domains of the 26S rDNA of *Taphrina* species (contained by primers NL1 and NL4) were 573–574 bp long. A mismatch was found in the sequence complementary to primer NL1 in runs with reverse primer NL4 (confirmed in runs with ITS1, the forward primer for the ITS region): a C instead of a G at position 16 of the primer (i.e. a G instead of a C at position 5 of reverse primer ITS4). This mismatch did not appear to affect annealing of the sequencing primers significantly. Only a few gaps were introduced by alignment with the sequences of selected archiascomycetes, *Protomyces* species and *Saitoella complicata*, retrieved from GenBank. Analysis of the sequence data is summarized in the phylogenetic tree depicted in Fig. 1. Tree topologies from neighbour-joining and maximum-parsimony analyses were similar and only the former is shown. Phylogenetic analysis confirmed the monophyletic nature of *Taphrina* (interspecies sequence divergence within the genus did not exceed 5 %) and its clear separation from the closely related genus *Protomyces* Unger (interspecies sequence divergence <4 %), with strong statistical support (Fig. 1). The same conclusion ensued from the work of Nishida & Sugiyama (1994) and Sjamsuridzal *et al.* (1997), based on 18S rDNA data. However, *Taphrina vestergrenii*, a fern parasite not included in those studies, appeared to occupy an intermediate position between the two genera (Fig. 1): it differed from the remaining *Taphrina* species in more than 35 positions (>6 % sequence divergence) and from *Protomyces* species in more than 50 positions (>9 % sequence divergence). A possible decision to accommodate *T. vestergrenii* in a separate genus should, however, await additional data on this taxon and the study of other species from ferns. A sequence retrieved from GenBank, corresponding to a yeast strain isolated from flower-dwelling insects and labelled *Taphrina* sp. (Lachance *et al.*, 2001), also had an isolated position, but was apparently basal to both *Taphrina* and *Protomyces* (Fig. 1). The sequence of strain NRRL T-857 of *T. deformans* retrieved from GenBank was identical to that of strain CBS 356.35 determined in this study. However, another sequence retrieved from GenBank, corresponding to strain MZ109 and identified as *T. deformans*, had 5 nt differences from those of the two above-mentioned strains, but had a single insertion when compared with the sequence of *T. tormentillae* NRRL T-422 and might thus represent the latter species (Fig. 1). This strain was isolated from the surface of plasticized PVC blocks exposed to the air (Webb *et al.*, 2000) and constitutes one of the rare examples of the isolation of *Taphrina* from substrates other than infected plant tissues (e.g. Kramer, 1987).

The D1/D2 region appears to be somewhat conserved within the genus and it did not allow the discrimination of all

Taphrina species (e.g. *Taphrina virginica* and *Taphrina wiesneri* or *Taphrina americana* and *T. purpurascens*, which were separated on the basis of MSP-PCR fingerprints). In several cases, interspecies differences amounted to fewer than 3 nt positions ($<0{\cdot}5$ % sequence divergence) (Fig. 1). Moreover, most of the internal branches had weak statistical support. Nevertheless, in some instances, the D1/D2 sequences concurred with the results of PCR fingerprinting in suggesting the conspecificity of strains that supposedly represented different species on the basis of conventional criteria: e.g. *T. carnea* CBS 332.55 and *T. tormentillae* NRRL T-422 or CBS 339.55 (1 or 2 nt substitutions); *T. robinsoniana* NRRL T-732 and *T. betulina* CBS 417.54 (no differences); and *T. betulina* NRRL T-726 and *T. nana* CBS 336.55 (no differences). Identity of strains from different collections (*Taphrina letifera* strains CBS 335.55 and NRRL T-791 and *T. populina* strains CBS 337.55 and NRRL T-497) was also corroborated by the D1/D2 data. On the other hand, intraspecific heterogeneity, already hinted at by the PCR fingerprinting results, can be anticipated when different D1/D2 sequences were obtained for strains of the same species: e.g. strains of *T. caerulescens* from *Quercus alba* (CBS 351.35) and *Quercus macrocarpa* (NRRL T-878) (8 nt substitutions); strains of *T. robinsoniana* from *Alnus rugosa* (CBS 382.39) and *Alnus serrulata* NRRL T-732 (7 substitutions); and strains of *T. populina* on *Populus nigra* from Sweden (CBS 337.55) and Canada (NRRL Y-6300) (3 substitutions). It is worth noting that, according to phylogenetic analysis of the D1/D2 sequences, species parasitic on *Quercus* spp. (Fagaceae) and *Populus* spp. (Salicaceae) and some of the species parasitic on the Betulaceae formed separate clusters. This correlation was not apparent in the phylogenetic analysis of the 18S rDNA sequence data of Sjamsuridzal *et al.* (1997), which also resulted in a phylogenetic tree with poorly resolved branches within the genus. The 14 authentic species of *Taphrina* included in that study could be discriminated by their 18S rDNA sequences (including *T. virginica* and *T. wiesneri*) although, in many cases, nucleotide differences amounted to less than 1 % overall divergence.

ITS region. To address some of the unresolved issues mentioned above, sequences were determined from the less-conserved ITS region for a selected set of strains. Length polymorphisms were apparent within ITS1 and ITS2, which resulted in total base counts for the region (contained by primers ITS1 and ITS4) ranging from about 580 bp in *Taphrina alni* to 630 bp in *T. populina* and led to a few alignment ambiguities due to the presence of insertions/deletions. In contrast, the 5.8S rRNA gene was conserved throughout. The only ITS sequence available in GenBank was that of an unspecified strain of *T. deformans*, which differed from those of all the *T. deformans* strains studied by us (Table 1) in a single nucleotide insertion at the 5′ end of ITS1. Phylogenetic analysis yielded the tree depicted in Fig. 2. As in the case of the D1/D2 region, tree topologies from neighbour-joining and maximum-parsimony analyses of the ITS sequences were similar and only the former is shown. A major difference between the D1/D2 and ITS trees is the relatively larger number of statistically supported clusters in the ITS tree, which is probably due to a higher rate of nucleotide substitution displayed by this region (in many cases, interspecies sequence divergence ranged between 5 and 15 %). Moreover, the number of parsimony-informative characters in the ITS region analysis was 172 out of a total of 642 (27 %), compared with 95 of 580 (16 %) in the D1/D2 region. As a consequence, species separations were more evident by ITS sequence analysis (interspecies differences: $\geqslant$5 nt substitutions). This was especially apparent for taxa that could not be differentiated by their D1/D2 sequences: e.g. *T. virginica* and *T. wiesneri* or *T. americana* and *T. purpurascens* (Figs 1 and 2). Intraspecies differences amounted to no more than 4 nt substitutions, e.g. *T. communis*, *Taphrina sadebeckii*, *T. wiesneri*. However, other strains that supposedly represented distinct species had fewer than 4 base differences: *T. virginica* and *Taphrina polystichi* (3 substitutions); *Taphrina epiphylla* HA 1439 and *T. sadebeckii* HA 1345 (3 substitutions); *T. tormentillae* CBS 339.55 and *T. carnea* CBS 332.55 (2 substitutions); *T. robinsoniana* NRRL T-732 and *T. betulina* CBS 417.54 (no differences); and *T. betulina* NRRL T-726, *T. carnea* NRRL T-705 and *T. nana* CBS 336.55 (no differences). These cases will be discussed further below. It is interesting to note that clustering of species according to host plant family (or genus) is more evident in the ITS tree (Fig. 2). For example, all species parasitic on *Prunus* spp. are found on a single, well-supported branch. In addition, species on *Quercus* spp. and *Populus* spp. and some of the species parasitic on the Betulaceae clustered separately, as already observed in the D1/D2 tree.

Species delimitation

Species parasitic on Betulaceae. Of the species parasitic on *Alnus* spp., *T. alni* and *Taphrina tosquinetii* were genetically homogeneous and well separated, *T. sadebeckii* displayed some intraspecific genetic variability and close proximity to *T. epiphylla*, whereas *T. robinsoniana* appeared to be heterogeneous (based on PCR fingerprints, D1/D2 and ITS sequences; e.g. Fig. 2). Relatedness between *T. epiphylla* (the cause of witches' brooms on *Alnus incana*) and *T. sadebeckii* (the cause of leaf spots on *Alnus glutinosa*) is supported by all the data obtained in the present study. Gjaerum (1964) had suggested that the latter is a synonym of the former, an opinion not shared by other authors (Mix, 1949; Bacigálová, 1994). Due to the genetic variability found among strains of *T. sadebeckii* in terms of PCR fingerprints (data not shown) and ITS sequences (Fig. 2), a decision to keep the two species separate requires the study of additional strains of *T. epiphylla*. PCR fingerprints (data not shown) and sequence data (Fig. 2) suggest conspecificity of *T. robinsoniana* NRRL T-732 and *T. betulina* CBS 417.54. However, synonymy of the two species is unlikely, due to the different nature and geographical distribution of the respective host plants

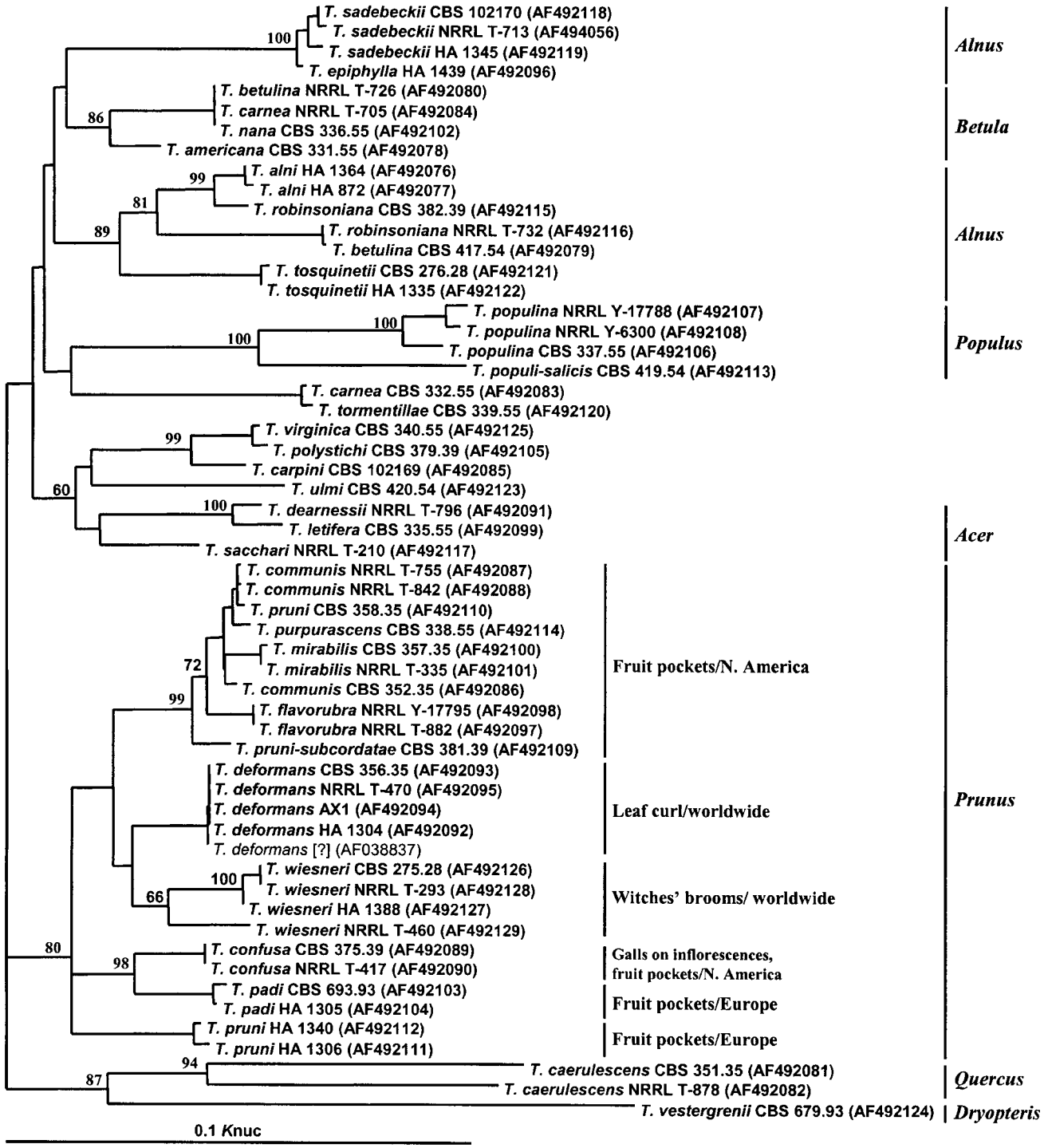

Fig. 2. Phylogenetic tree of selected *Taphrina* species obtained by neighbour-joining analysis of the ITS region (ITS1 + 5.8S rRNA gene + ITS2) using PAUP 4.0b8. *T. vestergrenii* and the two strains of *T. caerulescens* were used as the outgroup. Host genera are indicated on the right and type of infection symptom and geographical origin of the host plant are indicated for species parasitic on *Prunus*. Other details are as for Fig. 1.

(Table 1; Mix, 1949). Moreover, additional strains of each species (*T. robinsoniana* CBS 382.39 and *T. betulina* NRRL T-726) had very different ITS sequences and clustered on separate branches (Fig. 2). It is interesting to note that the two strains of *T. robinsoniana* clustered together with *T. alni* in the D1/D2 and ITS trees (Fig. 2), both species

producing typical outgrowths ('tongues') on female catkins, albeit on distinct *Alnus* species: the first on North American alders (*Alnus rugosa* or *Alnus serrulata*) and the second on a European species (*Alnus incana*) (Table 1). It is possible that, as currently circumscribed (Mix, 1949, 1954), *T. robinsoniana* is heterogeneous and that the forms on *Alnus rugosa* (represented by strain CBS 382.39) and *Alnus serrulata* (NRRL T-732) are actually separate species. The situation of *T. betulina* CBS 417.54 is more difficult to explain, and this strain may have been misidentified or mislabelled. The other strain of *T. betulina*, NRRL T-726, clustered on the ITS tree with other species from birches (Fig. 2): *T. carnea* (represented by strain NRRL T-705), a species that causes leaf curl on *Betula intermedia* (=*Betula pubescens*?); *T. nana*, a species that induces witches' brooms on *Betula nana*; and *T. americana*, another species that induces witches' brooms but on a North American birch, *Betula fontinalis* (=*Betula occidentalis*) (Table 1; Mix, 1949). The molecular data point to the conspecificity of the species on European birches, *T. betulina* (represented by NRRL T-726), *T. carnea* (NRRL T-705) and *T. nana* (CBS 336·55), but support the separation of *T. americana* at the species level. A second strain of *T. carnea*, CBS 332.55, appeared to be conspecific with the two strains of *T. tormentillae* according to molecular data (Figs 1 and 2), an observation that suggests a possible misidentification of the former, since there are marked differences in host specificity and geographical distribution of each species (Table 1).

Possible conspecificity between *T. virginica* and *T. polystichi* was suggested by the sequence data (Figs 1 and 2), but not necessarily by the respective PCR fingerprints (data not shown), and it is highly unlikely due to the very different nature of the respective host plants (Table 1). Their closest relative on the ITS tree (Fig. 2) appears to be *Taphrina carpini*, a species that, like *T. virginica*, is also parasitic on a member of the Betulaceae (Table 1; Mix, 1949). The placement of *T. polystichi* was thus quite unexpected, considering the very distinct phylogenetic position of the other species parasitic on ferns (*T. vestergrenii*) included in the present study (Fig. 1). A final decision on the status of *T. virginica* and *T. polystichi* would be premature at this stage and should await the study of additional strains of both species.

Species parasitic on *Prunus*. The results of PCR fingerprinting of all the strains representing species parasitic on *Prunus* spp. are shown in Fig. 3. *T. purpurascens* and *T. tormentillae* were also included; the former due to its apparent relatedness to *T. communis* (Fig. 2) and the latter since it represents the only other species parasitic on a different genus of the Rosaceae (Table 1). The different species appeared to be adequately discriminated by their PCR fingerprints (Fig. 3) and ITS sequences (Fig. 2), but not by the D1/D2 data (not shown). Several species were genetically homogeneous, namely *Taphrina confusa*, *T. deformans*, *Taphrina flavorubra* and *Taphrina padi*. PCR fingerprints of *T. deformans* strains showed some variability (Fig. 3), but they always clustered together and no nucleotide differences were found among them in the ITS sequences (Fig. 2). Of the species that deform fruits (plum pockets) and/or shoots, *T. communis*, *T. flavorubra*, *Taphrina mirabilis* and *Taphrina pruni-subcordatae*, which are parasitic on North American *Prunus* spp., formed a well-supported clade on the ITS tree (Fig. 2). Species separations appeared to parallel those of the hosts (Table 1). Surprisingly, *T. pruni* CBS 358.35 and *T. purpurascens* CBS 338.55 clustered with the strains of *T. communis* (number of base differences among the five strains ranged from 1 to 4; Fig. 2), an observation that is also supported by the MSP-PCR results (Fig. 3). *T. pruni* CBS 358.35 was apparently isolated from *Prunus domestica*, but its geographical origin is not known (CBS Yeast Database). In the light of the molecular data, it is likely that it originated in North America and should thus be transferred to *T. communis*, lending support to Mix's statement that 'plum pockets found on domestica plums in [the USA] should be ascribed to *T. communis*' (Mix, 1949). In agreement with this hypothesis, two *T. pruni* strains from European plums (HA 1306 from *Prunus domestica* and HA 1340 from *Prunus spinosa*; Table 1) were genetically distinct from *T. communis* (Figs 2 and 3) and appear to be authentic representatives of the former species (the two forms most likely being conspecific; Figs 2 and 3). The position of *T. purpurascens* is more difficult to explain, as this species produces leaf curl on *Rhus copallinum*, a member of the Anacardiaceae (Table 1; Mix, 1949). A formal proposal to consider *T. purpurascens* as a synonym of *T. communis* would be premature at this stage and should await the study of additional strains of the former species. To sum up, *T. communis* should therefore include all forms that cause plum pockets on *Prunus americana*, *Prunus domestica* and *Prunus nigra* in North America, although the latter, represented by strain NRRL T-755, showed some deviation in the PCR fingerprints (Fig. 3).

T. mirabilis, a species parasitic on *Prunus angustifolia*, was considered synonymous with *T. communis* by Mix (1949), but the results of the molecular characterization suggest otherwise: the two strains had a single nucleotide substitution in the ITS region between them and in spite of being closely related to the *T. communis* cluster (Fig. 2), differed from the latter in two insertions and at least 6 nt substitutions. In spite of the genetic variability displayed by the two strains of *T. mirabilis* [they clustered together on the MSP-PCR dendrogram but at low similarity (Fig. 3) and differed by 2 bp in the D1/D2 region] and by the strains of the *T. communis* cluster, it seems reasonable to keep the two species separate, although a final decision would benefit from additional data (e.g. results of DNA–DNA hybridization experiments) and the study of more strains.

T. padi, a species that causes deformed fruits on *Prunus padus* in Europe, has been considered synonymous with *T. pruni* (e.g. Mix, 1936), but Mix (1949) sustained their separation, stating that *T. padi* was more closely related

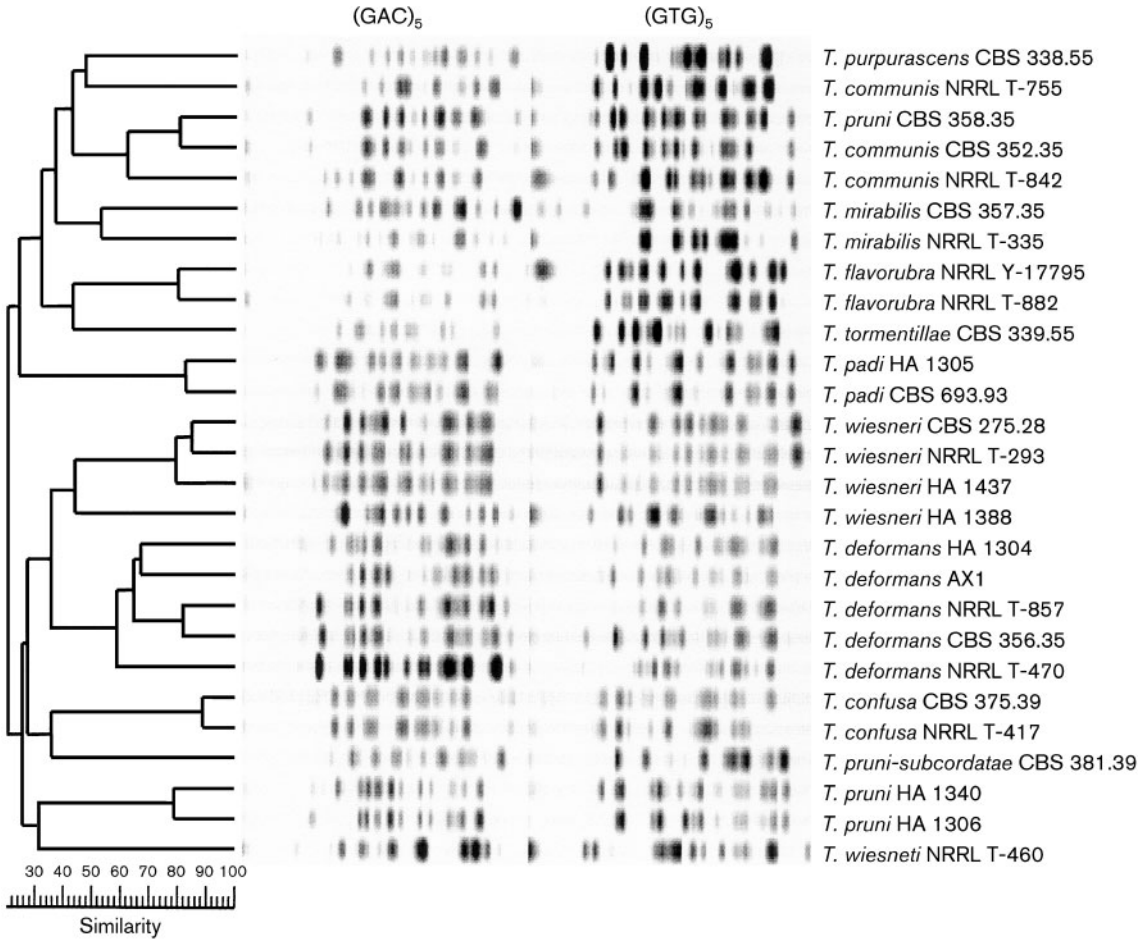

Fig. 3. MSP-PCR characterization of *Taphrina* species from *Prunus* spp. DNA banding patterns and the resulting dendrogram are based on combined analysis of the PCR fingerprints obtained with primers $(GAC)_5$ and $(GTG)_5$ using Pearson's coefficient and the UPGMA clustering method (co-phenetic correlation coefficient, $r=0{\cdot}85$).

to *T. confusa* than to *T. pruni*. Our results (Fig. 2) fully corroborate Mix's hypothesis, and a recent study by Prillinger *et al.* (2000) has also confirmed the separation of *T. padi* from *T. pruni* based on RAPD analysis and on partial 18S rDNA sequences.

T. wiesneri induces witches' brooms on cherry trees and has forms on different species throughout the world (Mix, 1949). Our molecular data suggest that the strains from *Prunus avium* (F-297, NRRL T-293 and HA 1437) and *Prunus fruticosa* (HA 1388) in Europe are probably conspecific, although the latter shows some deviation in its PCR fingerprints (Fig. 3) and ITS sequence (Fig. 2). Strain NRRL T-460, representing the form on the North American *Prunus pennsylvanica*, most probably represents a separate species, a hypothesis that is corroborated by the PCR fingerprinting and ITS data (Figs 2 and 3). Future studies including strains from Japanese cherry trees will undoubtedly help to ascertain whether there are additional species within *T. wiesneri*.

Concluding remarks

Analysis of the molecular data determined in this study revealed that *Taphrina* species previously defined on the basis of conventional criteria (host plant, geographical origin, type of infection symptom and/or ascus morphology) were, in most cases, genetically distinct. MSP-PCR fingerprinting adequately discriminated the majority of *Taphrina* species and proved to be a reproducible and simple method that allowed the rapid analysis of large numbers of strains. Of the sequenced rDNA regions, D1/D2 was somewhat conserved and did not allow the discrimination of all *Taphrina* species, but phylogenetic analysis showed the genus *Taphrina* to be monophyletic (probably excluding *T. vestergrenii*) and confirmed its distinction from the closely related genus *Protomyces*. The ITS region appeared to be more adequate for species discrimination and phylogenetic reconstruction within the genus. Furthermore, clustering of *Taphrina* species according to ITS sequence data corresponded grossly to host plant genera (and/or families),

namely for species parasitic on *Quercus* (Fagaceae), *Populus* (Salicaceae), *Prunus* (Rosaceae), *Alnus* or *Betula* (Betulaceae) and possibly also on *Acer* (Aceraceae). This evidence constitutes a strong indication of the importance of co-evolution in the speciation of *Taphrina* species, as has been found for other genera of phytopathogenic fungi (e.g. Bakkeren *et al.*, 2000). In a few cases, a correlation was also observed between the clustering of *Taphrina* species in the ITS tree and the type of infection symptom (e.g. species inducing tongues on *Alnus* or species causing fruit pockets on *Prunus*; Fig. 2). The results of PCR fingerprinting and ITS sequencing additionally suggested some cases of possible conspecificity (e.g. *T. betulina*, *T. carnea* and *T. nana*), others of intraspecific heterogeneity (*T. caerulescens*, *T. populina*, *T. robinsoniana*, *T. wiesneri*) and yet others of mislabelled or misidentified strains: e.g. *T. carnea* CBS 332.55 (= *T. tormentillae*); *T. betulina* CBS 417.54 (= *T. robinsoniana*); *T. purpurascens* CBS 338.55 (= *T. communis*); and *T. pruni* CBS 358.35 (= *T. communis*). Confirmation of some of these hypotheses would benefit from the study of additional isolates and the implementation of inoculation experiments.

In our view, progress in the systematics and phylogeny of *Taphrina* will undoubtedly require the isolation and study of more cultures of the many species that have been recognized but are not currently available (Mix, 1949). It is our hope that this study will stimulate a renewed interest in the genus *Taphrina* by providing the tools that enable the accurate diagnosis of the various infections caused by the different species, e.g. by direct amplification and sequencing of the appropriate rDNA regions from infected tissues, without the need for isolation of the yeast phase. These approaches will conceivably lead to a more complete knowledge of the biology and ecology of these widespread phytopathogenic fungi.

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