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Tuberculina: rust relatives attack rusts[1]

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Abstract: Molecular sequence data together with ultrastructural features were used to infer the phylogenetic position of *Tuberculina* species. Additional ultrastructural characteristics were used to determine their mode of nutrition. We investigated ultrastructural morphology of the type species *Tuberculina persicina* and determined base sequences from the D1/D2 region of the nuclear large-subunit ribosomal DNA of the three commonly distinguished *Tuberculina* species, *T. maxima, T. persicina* and *T. sbrozzii.* Analyses of sequence data by means of a Bayesian method of phylogenetic inference using a Markov Chain Monte Carlo technique reveal the basidiomycetous nature of *Tuberculina.* Within the Urediniomycetes, *Tuberculina* clusters as a sister group of *Helicobasidium,* closely related to the rusts (Uredinales). This phylogenetic position is supported by the uredinalean architecture of septal pores in *Tuberculina.* In addition, we present aspects of the ultrastructural morphology of the cellular interaction of *Tuberculina* and rusts showing a unique interaction with large fusion pores, revealing the mycoparasitic nature of *Tuberculina* on its close relatives, the rusts.

Key words: cellular interaction, molecular phylogeny, mycoparasitism, nuc-LSU rDNA, septal pore morphology, systematics, ultrastructure, Urediniomycetes

INTRODUCTION

In 1817, a member of the genus *Tuberculina* Sacc. first was described accurately by Ditmar (1817) as *Tuberculariа persicina.* His morphological characterization, with some additions, remains valid: Members of the genus *Tuberculina* are characterized by the formation of hemispherical lilac to violet sporodochia. They consist of palisade-like arranged, short, moderately thick conidiogenous cells, each of which bears one globose, smooth conidium at the tip. The sporodochia break through the surface of higher plants and emit a powdery mass of conidia. Sometimes spherical sclerotium-like structures are formed. In addition, *Tuberculina* is known to exist only in association with rusts as first postulated by Saccardo (1880) and later elaborated by Tubeuf (1901) and others. Saccardo (1880) excluded *Tuberculariа persicina* Ditmar from the genus *Tuberculariа* Tode : Fr., which should have pleurogenous conidiogenesis and thread-like conidiogenous cells, and described the new genus *Tuberculina* Sacc. for species with acrogenous conidiogenesis, short and broad conidiogenous cells which are parasitic on the aecial stage of rust fungi.

The three *Tuberculina* species, *T. maxima, T. persicina* and *T. sbrozzii,* commonly are recognized (e.g., von Arx 1981, Ellis and Ellis 1988). They are distributed worldwide, living in association with more than 150 rust species from at least 15 genera. However, up to 45 species were described with the authors following strikingly different species concepts. Adopting a concept based on morphological characters, plant parasites (e.g., *T. solanicola* Ellis parasitic on fruits of *Solanum melongena* L. [Ellis 1893]) and parasites of non-rust fungi (e.g., *T. ovalispora* Pat. parasitic on *Darluca filum* [Biv.] Castagne [Patouillard and Gaillard 1888]) were included in the genus. Other authors used a species concept based on host specificities, distinguishing *Tuberculina* species on different rust hosts (Spegazzini 1880, 1884) or even plant hosts (Gobi 1885).

After controversial discussions whether *Tuberculina*-like fungi should be treated as smuts, rusts, ascomycetes or hymenomycetes, the genus presently is assigned mostly to the Fungi Imperfecti because no stages of sexual reproduction are known.

Research on *Tuberculina* was motivated by two main factors: α-taxonomy (e.g., Cooke 1888, Patouillard and Gaillard 1888, Spegazzini 1880, 1884, 1911) and the use of *Tuberculina* as a biological agent in

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TABLE I. List of studied species, reference material, host, and GenBank accession number.

Species	Reference material[a]	Host fungus/plant	GenBank acc. no.
Helicobasidium longisporum Wakef. (syn. *H. compactum* Boedijn)	USA. (CBS 296.50)—culture	*Coffea* sp.	AY222046
Tuberculina maxima Rostr.	Canada, British Columbia, Wap Lake, 12. 9. 1965. (CBS 136.66)—culture	*Cronartium ribicola* J. C. Fisch.	AY222044
Tuberculina persicina (Ditmar) Sacc.	Germany, Baden-Württemberg, Tübingen, Hagelloch, 6. 11. 2000. (M. Lutz 799, TUB 011529)	*Puccinia silvatica* J. Schröt.	AY222049
Tuberculina persicina (Ditmar) Sacc.	Germany, Baden-Württemberg, Nürtingen, Raidwangen, 25. 4. 2001. (M. Lutz 851, TUB 011530)	*Tranzschelia pruni-spinosae* (Pers.) *Dietel*	AY222043
Tuberculina sbrozzii Cavara & Sacc.	England, Berkshire, East Burnham, 31. 3. 2000. (K (M): 76122)	*Puccinia vincae* (DC.) Berk.	AY222045
Puccinia silvatica J. Schröt.	Germany, Baden-Württemberg, Tübingen, Hagelloch, 13. 10. 2000. (M. Lutz 737, TUB 011528)	*Taraxacum officinale* agg. F. H. Wigg.	AY222048
Puccinia vincae (DC.) Berk.	Germany, Baden-Württemberg, Tübingen, 20. 9. 2002. (M. Lutz 1422, TUB 011527)	*Vinca major* L.	AY222047

[a] Source acronyms: CBS—Centraalbureau voor Schimmelculures, AG Baarn, The Netherlands; K—Herbarium of the Royal Botanic Gardens, Kew, England; TUB—Herbarium of the Spezielle Botanik/Mykologie, Eberhard-Karls-Universität Tübingen, Germany.

rust control (see review by Wicker 1981). As a result, aspects of the biology, such as hibernation (Wicker and Wells 1968), dispersal (Tubeuf 1901), conditions for germination of conidia (Cornu 1883, Gobi 1885, Lechmere 1914, Mielke 1933), mode and time of infection (Weissenberg and Kurkela 1979, Wicker and Kimmey 1967, Wicker and Wells 1970), host specificities (Barkai-Golan 1959, Hubert 1935) or conditions for artificial cultivation (Vladimirskaya 1939) were clarified. However, fundamental questions concerning the biology of the genus remain unanswered. Thus, the relationship among plants, rusts and *Tuberculina* remains unresolved. *Tuberculina* species have been interpreted as mycoparasites specific to rusts (Tubeuf 1901, Zambettakis et al 1985), as nonspecific parasites on several substrates (Petrak 1956, Schroeter 1889) or even as specialized parasites on rust-infected plant tissues (Hulea 1939, Wicker and Woo 1969, 1973). Also, the mode of nutrition and interaction, respectively, is unidentified. Finally, the evolution and systematic position of the genus is totally obscure, including questions on delimitation of species and of the genus itself.

Ultrastructural characters of septal pore morphology played an important role in the arrangement of basidiomycetes (Bandoni 1984, Bauer et al 1997, Bauer and Oberwinkler 1994, Oberwinkler and Bauer 1989, Wells 1994), and they correspond well to phylogenetic hypotheses generated from molecular data (e.g., Bauer et al 2001, Swann et al 2001).

In this report, we present both molecular and ultrastructural data that reveal the basidiomycetous nature of *Tuberculina* and show that it is related closely to *Helicobasidium* Pat., therefore belonging to the rust group. The actual mycoparasitic nature of the genus is indicated on an ultrastructural level by a remarkable cellular interaction between *Tuberculina* and rust hyphae.

MATERIALS AND METHODS

Materials.—Specimens and the origins of the sequences used in the molecular analyses are listed in TABLE I. All three commonly distinguished *Tuberculina* species, *T. maxima, T. persicina* and *T. sbrozzii,* and the rust hosts of the respective *Tuberculina* specimens were included in the molecular analyses.

Molecular methods.—We isolated genomic DNA from five herbarium specimens and from two cultures on artificial media (TABLE I) of *Tuberculina, Puccinia* and *Helicobasidium,* respectively. The fungal material was isolated from the herbarium specimens by five times picking up spores from the surfaces of either *Tuberculina* sporodochia or rust sori with a fine needle and depositing the spores directly in 1.5 mL tubes. Dry spores were crushed at room temperature by shaking the samples 3 min at 30 Hz (Mixer Mill MM 300, Retsch, Haan, Germany) in the tubes together with

one tungsten carbide ball (3 mm diam). To extract DNA, we used the DNeasy Plant Mini Kit (Quiagen, Hilden, Germany) following the manufacturer's protocol.

To infer the phylogenetic position of *Tuberculina* within the Basidiomycota, we amplified the 5′- end (about 625 bp) of the nuclear large-subunit ribosomal DNA (nuc-LSU rDNA), comprising the domains D1 and D2 (Guadet et al 1989). Amplification was done by PCR (Mullis and Faloona 1987, Saiki et al 1988) using the primer pair NL1 and NL4 (O'Donnell 1992, 1993) or LR6 (Vilgalys and Hester 1990), respectively. The selected DNA region is especially useful in resolving relationships over a broad scale of organisms (Begerow et al 1997, Fell et al 2000), and the D2 domain has proven to have the lowest levels of homoplasy within the LSU rDNA (Hopple and Vilgalys 1999). Amplification parameters were as described in Vogler and Bruns (1998), but we adjusted the annealing temperature to 50 C and reduced the extension time of the last nine cycles to 2.5 min. PCR products were purified with the QIAquick™ Kit (Qiagen, Hilden, Germany) followed by an ethanol precipitation. Both strands of dsDNA were sequenced directly by cycle sequencing (modified after Sanger et al 1977) with NL1 and NL4-reverse as forward and NL4 and LR6 as reverse primers and the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, England) according to the manufacturer's protocol. Electrophoresis was performed on an automated sequencer (ABI 373A Stretch, PE Applied Biosystems, Foster City, California). The sequences of both strands were combined and proofread with the help of Sequencher™ 4.1 software (Gene Codes Corp., Ann Arbor, Michigan). DNA sequences determined for this study were deposited in GenBank. Accession numbers are given in TABLE I. To obtain a reliable hypothesis on the phylogenetic position of the *Tuberculina* specimens that we sampled, we also used sequences from GenBank, representing all groups of Urediniomycetes (including the respective rust hosts of the analysed *Tuberculina* specimens) as designated by Swann et al (2001) and some representatives of Ustilaginomycetes and Hymenomycetes (GenBank accession numbers are given in parentheses): *Agaricostilbum pulcherrimum* (AJ406402), *Agaricus arvensis* (U11910), *Auricularia auricula-judae* (L20278), *Bensingtonia* sp. (AF444770), *Boletus rubinellus* (L20279), *Calocera viscosa* (AF011569), *Chionosphaera apobasidialis* (AF393470), *Colacogloea peniophorae* (AF189898), *Cronartium ribicola* (AF426240), *Doassansia epilobii* (AF007523), *Entyloma ficariae* (AY081013), *Eocronartium muscicola* (L20280), *Erythrobasidium hasegawianum* (AF189899), *Helicobasidium mompa* (L20281), *Helicogloea variabilis* (L20282), *Herpobasidium filicinum* (AF426193), *Insolibasidium deformans* (AF522169), *Kondoa myxariophila* (AF189904), *Kriegeria eriophori* (syn. *Zymoxenogloea eriophori*) (L20288), *Kurtzmanomyces tardus* (AF393467), *Melampsora lini* (L20283), *Microbotryum violaceum* (AF009866), *Mixia osmundae* (AB052840), *Naohidea sebacea* (AF522176), *Pachnocybe ferruginea* (L20284), *Sakaguchia dacryoidea* (AF444723), *Septobasidium carestianum* (L20289), *Sporobolomyces dracophylli* (AF189982), *Tranzschelia pruni-spinosae* (AF426224), *Tremella mesenterica* (AF011570), *Urocystis ranunculi* (AF009879), *Ustilago hordei* (L20286), *Ustilentyloma fluitans* (AF009882).

DNA sequences were aligned with the MEGALIGN module of the LASERGENE package (DNASTAR Inc., Madison, Wisconsin). Further manual alignment was done in Se-Al version 2.0a10 (A. Rambaut, University of Oxford, England). The final alignment (40 sequences; length: 550 bp; after exclusion of the sites 40–55, 379–396, 404–424, 482–497: 289 variable sites) and the tree obtained is deposited in TreeBase (http://treebase.bio.buffalo.edu/treebase/) with the study accession number S955. Sequence distances were computed with the MEGALIGN module of the LASERGENE package. A Bayesian method of phylogenetic inference using a Markov Chain Monte Carlo (MCMC) technique (Larget and Simon 1999, Mau et al 1999) as implemented in the computer program MrBayes 3.064 (Huelsenbeck and Ronquist 2001) was used to analyze the dataset. This method allows estimating the probabilities (a posteriori probabilities) for groups of taxa to be monophyletic given the DNA alignment. The power of this method recently was demonstrated in computer simulation by Alfaro et al (2003) and yielded good results in current molecular studies on fungal systematics (e.g., Maier et al 2003). For bayesian analysis, the data first were analyzed with MrModeltest 1.0b (J.A.A. Nylander, Upsala University, Sweden, Posada and Crandall 1998) to find the most appropriate model of DNA substitution. Hierarchical likelihood ratio tests and Akaike information criterion resulted in GTR+I+G. Thus, four incrementally heated simultaneous Markov chains were run over 2 000 000 generations using the general time reversible model of DNA substitution with gamma distributed substitution rates (Gu et al 1995, Rodriguez et al 1990) and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model (Huelsenbeck and Ronquist 2001). Trees were sampled every 100 generations, resulting in an overall sampling of 20 000 trees. From these, the first 1000 trees were discarded (burn in = 1000). The trees computed after the process remained static (19 000 trees) were used to compute a 50% majority rule consensus tree to obtain estimates for the a posteriori probabilities of groups of species. This Bayesian approach of phylogenetic analysis was repeated 10 times to test the reproducibility of its results. The unrooted phylograms from the MCMC analyses were rooted with the species belonging to the Ustilaginomycetes as outgroup species, because the trichotomy of the Basidiomycota had been demonstrated by several authors (Begerow et al 1997, Berres et al 1995, Swann and Taylor 1993, 1995).

Light and electron microscopy.—For light (LM) and transmission electron microscopy (TEM), *Tuberculina persicina* on *Tranzschelia pruni-spinosae* was prepared in two different ways. In one method, samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature overnight. After six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer for 1 h in the dark, washed in distilled water and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five washes in distilled

water, samples were dehydrated in acetone, with 10 min changes at 25%, 50%, 70%, 95% and three times in 100% acetone. Samples were embedded in Spurr's plastic (Spurr 1969) and sectioned with a diamond knife. Semithin sections were stained with new fuchsin and crystal violet, mounted in Entellan and examined by light microscopy. Ultrathin serial sections were mounted on formvar-coated, single-slot copper grids, stained with lead citrate at room temperature for 5 min and washed with distilled water. They were examined with a transmission electron microscope (EM 109, Zeiss, Germany) operating at 80 kV.

In the second method, samples were prepared by high-pressure freezing and freeze substitution. Infected areas of leaves were removed with a 2 mm cork borer. To remove air from intercellular spaces, samples were infiltrated with distilled water containing 6% (v/v) (2.5 M) methanol for approximately 5 min at room temperature. Single samples were placed in an aluminum holder and frozen immediately in the high-pressure freezer HPM 010 (Balzers Union, Liechtenstein) as described in detail by Mendgen et al (1991). Substitution medium (1.5 ml per specimen) consisted of 2% osmium tetroxide in acetone, which was dried over calcium chloride. Freeze substitution was performed at −90 C, −60 C and −30 C, 8 h for each step, with a Balzer's freeze substitution apparatus FSU 010. The temperature was raised to approximately 0 C during a 30 min period, and samples were washed in dry acetone another 30 min. Infiltration with an Epon/Araldite mixture (Welter et al 1988) was performed stepwise: 30% resin in acetone at 4 C for 7 h, 70% and 100% resin at 8 C for 20 h each and 100% resin at 18 C for approximately 12 h. Samples then were transferred to fresh medium and polymerized at 60 C for 10 h. Finally, samples were processed as described above for chemically fixed samples, except that the ultrathin sections were additionally stained with 1% aqueous uranyl acetate for 1 h.

RESULTS

Molecular analyses.—Compared to sequence data available via GenBank, all sequences obtained from *Tuberculina* specimens showed highest similarities to the sequence of *Helicobasidium mompa* (GenBank accession number L20281) with a divergence from 4.2% (*T. maxima*) to 4.8% (both *T. persicina* specimens). Compared to the sequence of *Helicobasidium longisporum,* determined in this study, the divergence ranged from 2.5% (*T. maxima*) to 3.1% (both *T. persicina* specimens). Comparison within *Tuberculina* ranged from identity (the *T. persicina* specimens) to 0.6% divergence (*T. maxima* compared to both *T. persicina* specimens).

The different runs of Bayesian phylogenetic analysis that were performed yielded consistent topologies. We present the consensus tree of one run to illustrate the results (FIG. 1). The phylogenetic hypothesis obtained by analyzing parts of the nuc-LSU rDNA of an assortment of basidiomycetes together with *Tuberculina maxima, T. persicina, T. sbrozzii,* and their respective rust hosts revealed the expected trichotomy of the sampled basidiomycetes with the monophyla Ustilaginomycetes, Hymenomycetes and Urediniomycetes. Within the Urediniomycetes, the *Microbotryum* group, rust group, *Agaricostilbum* group and *Erythrobasidium* group were supported with a posteriori probabilities of 100%. Together with *Mixia osmundae* and *Helicogloea variabilis,* these groups represent all major groups of Urediniomycetes (after Swann et al 2001). The phylogenetic relationships among these groups were not resolved.

All specimens of *Tuberculina* clustered together (a posteriori probability of 100%) representing the sister taxon (a posteriori probability of 98%) of *Helicobasidium* (a posteriori probability of 100%) and consequently being a member of the Urediniomycetidae. The relationship of the *Tuberculina-Helicobasidium* cluster to the rusts, to *Pachnocybe ferruginea,* to *Septobasidium carestianum,* and to the sampled Platygloeales sensu stricto (*Insolibasidium deformans, Herpobasidium filicinum, Eocronartium muscicola*; after Swann et al 2001) was not resolved. Within *Tuberculina, T. maxima* appeared basal, in opposition to the sister taxa (a posteriori probability of 100%) *T. sbrozzii* and the cluster of *T. persicina* (a posteriori probability of 99%).

Septal pore architecture of Tuberculina persicina *and* Tranzschelia pruni-spinosae.—Septal wall morphology and septal pore architecture in *Tuberculina persicina* essentially was identical to that of *Tranzschelia pruni-spinosae.* In both species, the septa had a trilamellate nature and the simple pores were surrounded by microbodies in a more or less circular arrangement (FIGS. 10–11). Mature pores in both species were plugged by osmiophilic material. Usually an organelle-free zone surrounding the septal pores at both sides was more distinct in *Tranzschelia pruni-spinosae* than in *Tuberculina persicina* (cf. FIG. 11 and FIG. 10). In addition, sometimes the pore lips in *Tuberculina persicina,* but not in *Tranzschelia pruni-spinosae,* were slightly swollen and more or less abruptly flattened toward the margin.

Association of Tuberculina persicina *with* Tranzschelia pruni-spinosae *on* Anemone ranunculoides.—*Tuberculina persicina* strictly overgrew aecia of *Tranzschelia pruni-spinosae* in different developmental stages and sporulated on the upper surface of the aecia (FIGS. 2–3). During differentiation of the sporodochia, the epidermis of the leaves ruptured and the conidial mass of *Tuberculina* was exposed (FIGS. 2–3). Within the leaf tissue, hyphae of *Tuberculina* and those of *Tranzschelia* were mixed (FIGS. 4–5). Hyphae of both *Tuberculina* and *Tranzschelia* were without clamps but

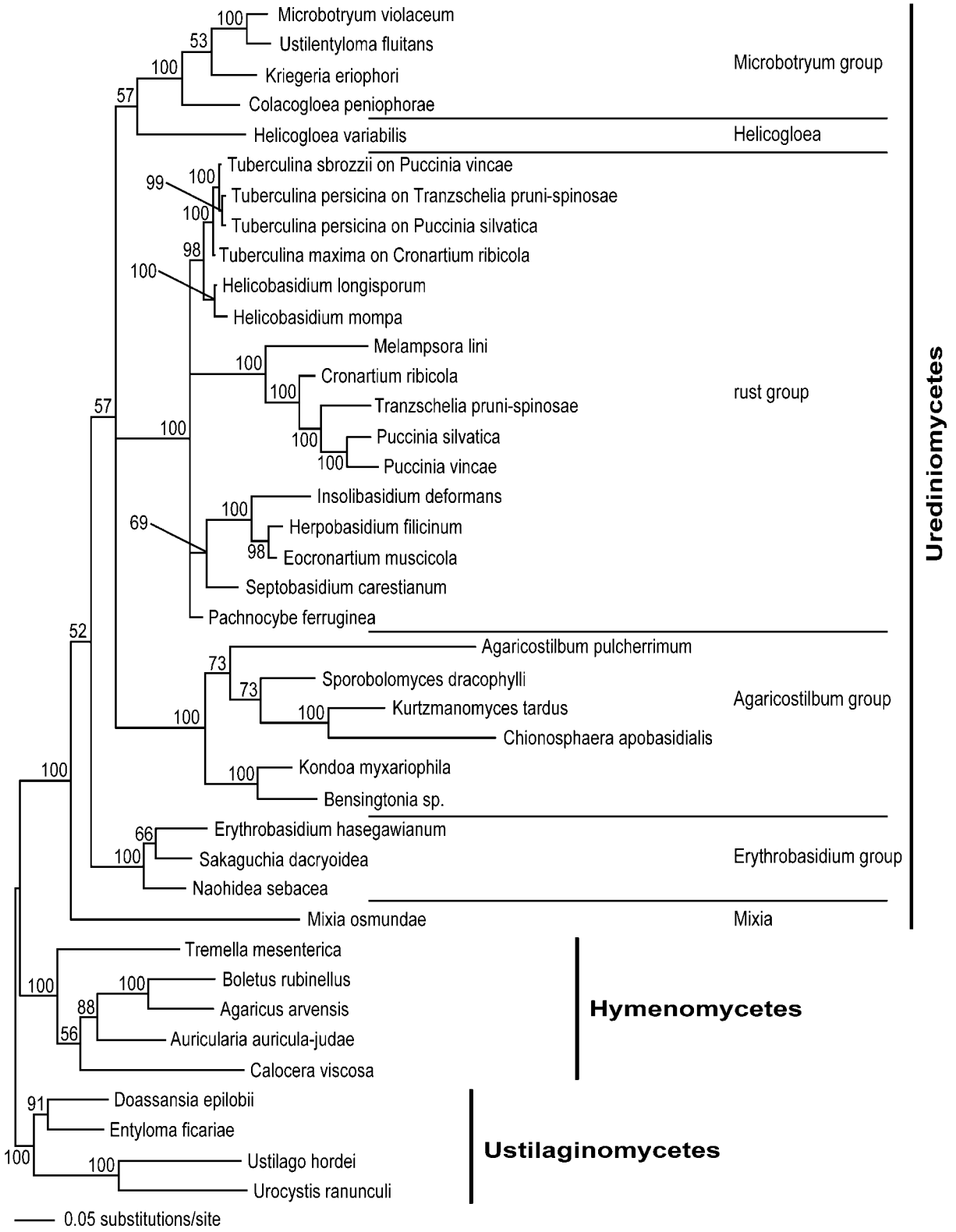
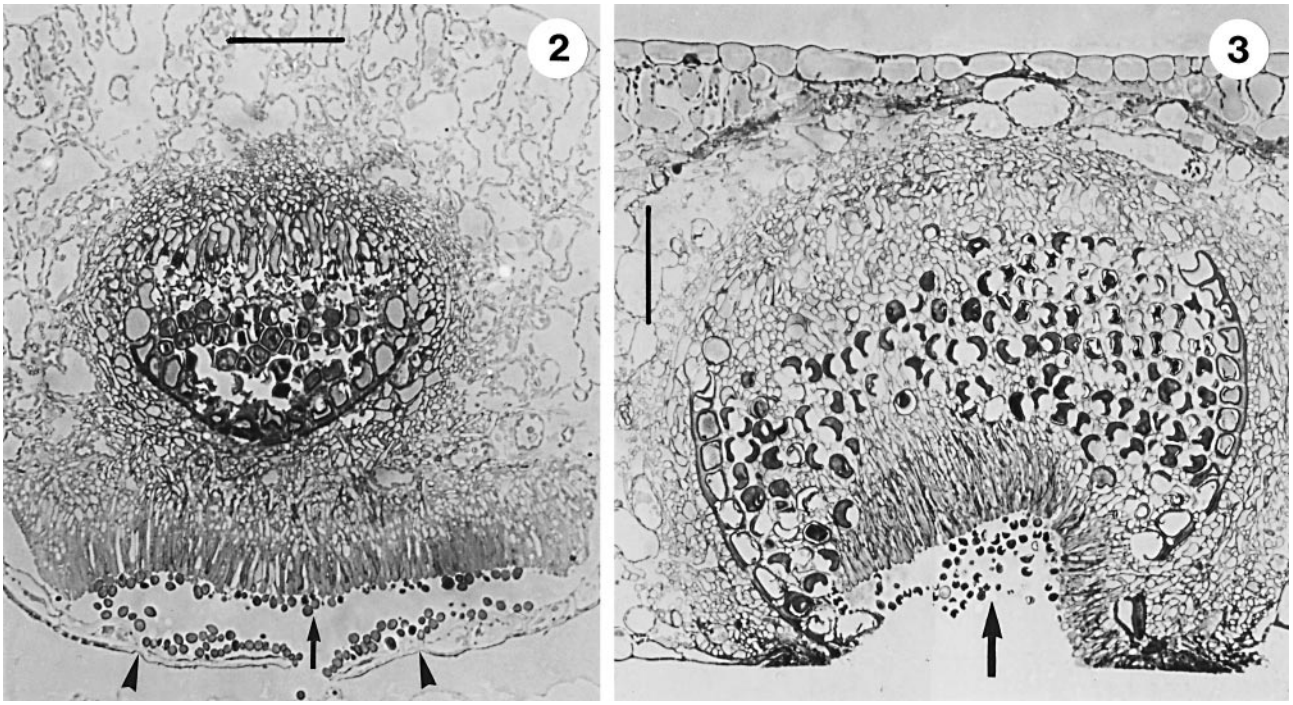


FIG. 1. Bayesian inference of phylogenetic relationships within selected basidiomycetous species. Markov Chain Monte Carlo analysis of an alignment of nuc-LSU rDNA sequences from the D1/D2 region using the general time reversible model of DNA substitution with gamma distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the substitution model. Majority-rule consensus tree from 19 000 trees that were sampled after the process remained static. The topology was rooted with the species belonging to the Ustilaginomycetes. Numbers on branches are estimates for a posteriori probabilities. Branch lengths are mean values over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site.

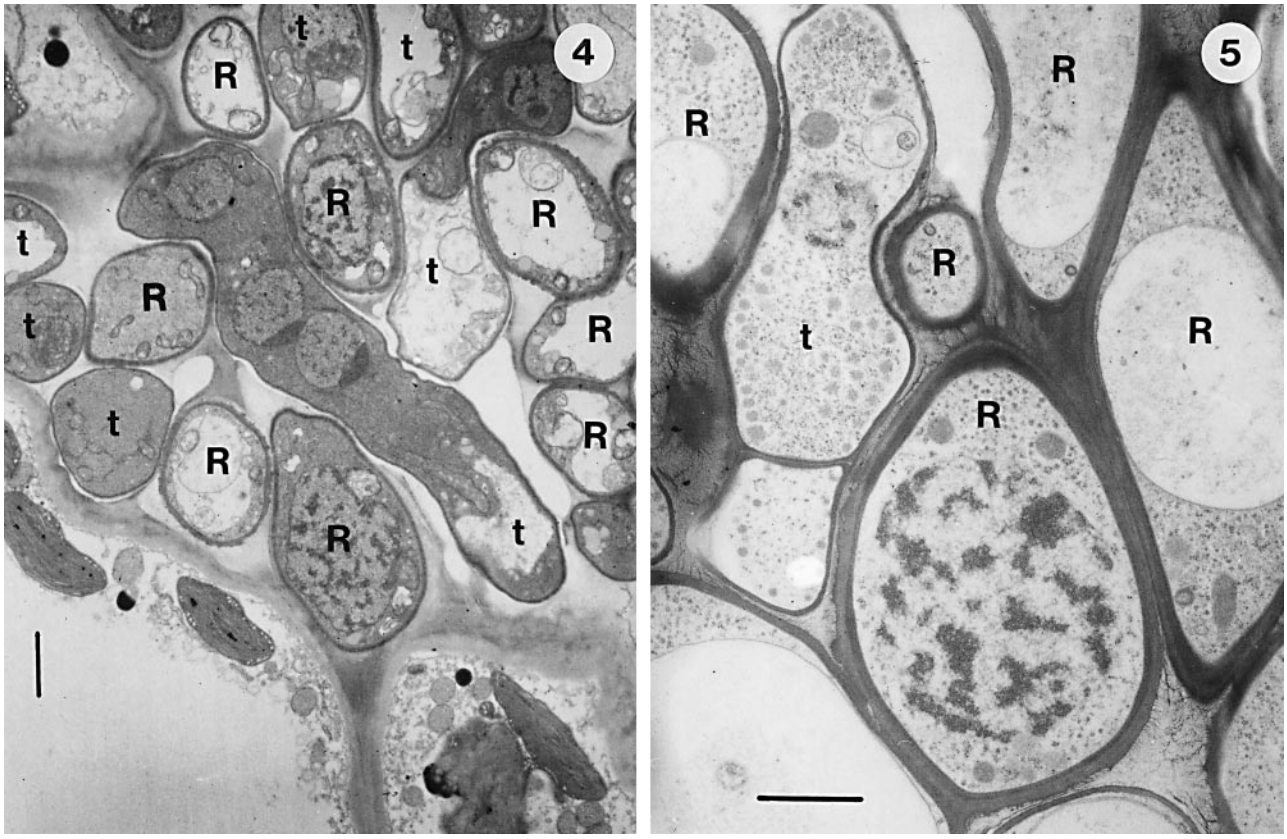


FIGS. 2–3. Cross section through aecium of *Tranzschelia pruni-spinosae* infected with *Tuberculina persicina*. Samples were prepared by chemical fixation (2) or high pressure freezing and freeze substitution (3) and observed with a light microscope. 2. Sporulation of *Tuberculina persicina* (arrow) at the top of a young aecium of *Tranzschelia pruni-spinosae*. Note that the lower epidermis (arrowheads) of *Anemone ranunculoides* becomes ruptured. Scale bar = 100 μm. 3. Sporulation of *Tuberculina persicina* (arrow) within the peridium of a mature aecium of *Tranzschelia pruni-spinosae*. Scale bar = 100 μm.

could be distinguished from each other by the number of nuclei per hyphal cell, the diameter of the nuclei and the thickness of the cell walls (FIGS. 4–5). Hyphae of *Tuberculina* generally were multinucleate, whereas those of *Tranzschelia* usually were mononucleate (binucleate hyphal rust cells occurred only at the base of the aecia). Diameter of the nuclei and thickness of the cell walls of the rust were roughly twice as large (or more) compared to those of *Tuberculina* (FIGS. 4–5). Interaction stages between *Tuberculina persicina* and *Tranzschelia pruni-spinosae* frequently were found in the leaves in neighboring areas of the aecia, especially at the base of the aecia. In these interaction stages, the protoplasts of both, the *Tuberculina* and the rust hyphal cell, were fused via a large pore, measuring 0.5–1 μm diam (FIGS. 6–9). By both fixation techniques, the general fusion pore architecture was recognizable. In high-pressure frozen samples, however, fusion pore morphology had a more regular appearance and was more distinct than after conventional fixation (cf. FIGS. 8–9 and FIGS. 6–7). Thus, in high-pressure frozen interaction stages the plasma membranes of the *Tuberculina* and the rust cell closely followed the contour of the respective cell wall (FIGS. 8–9), whereas in conventionally fixed interaction stages, plasma membranes often were folded irregularly (FIGS. 6–7). For all interaction stages, prepared by high-pressure freezing and freeze substitution, it clearly was evident that the membrane of the fusion pore was continuous with the plasma membranes of both the *Tuberculina* and the rust cells (FIG. 9).

DISCUSSION

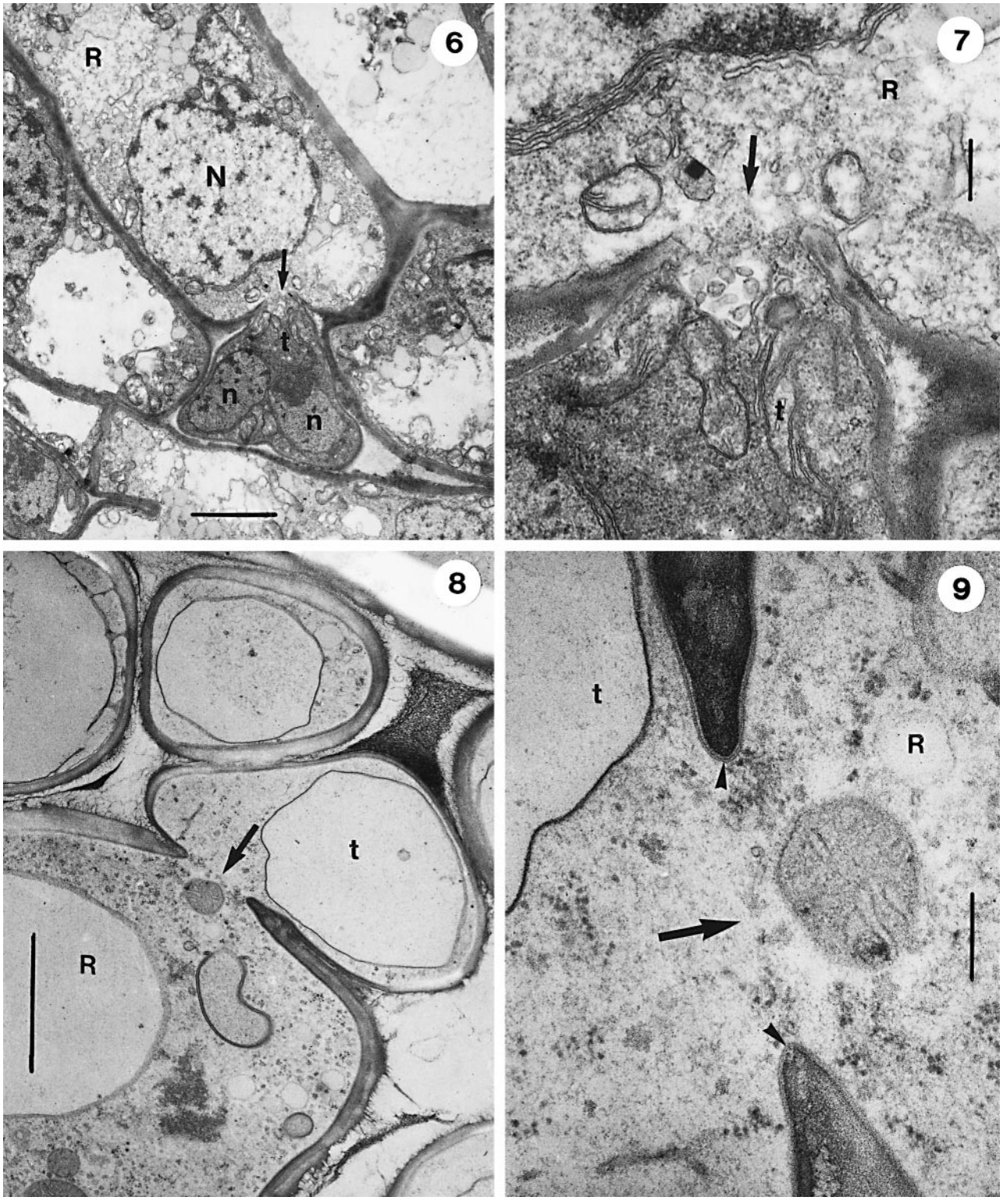
Phylogenetic position of Tuberculina.—Because no stages and structures of sexual reproduction are known in *Tuberculina,* other features were used to determine the phylogenetic position of the genus. Conflicting classifications were proposed based on different features. Ditmar (1817) assigned the fungus that he described to *Tubercularia* Tode : Fr. Saccardo (1880) confined *Tubercularia* to anamorphs of the genus *Nectria* (Fr.) Fr. (which is the current concept, see also Rossman 2000) and *Tuberculina* to anamorphs of rust parasites. Few subsequent researchers regarded *Tuberculina* as anamorphic ascomycetes (e.g., Frank 1880, Kirk et al 2001). Tulasne (1854) and Lutrell (1979) even proposed ascomycetous teleomorphs (*Sphaeria loepophaga* Tul. and *Anhellia* Ra-



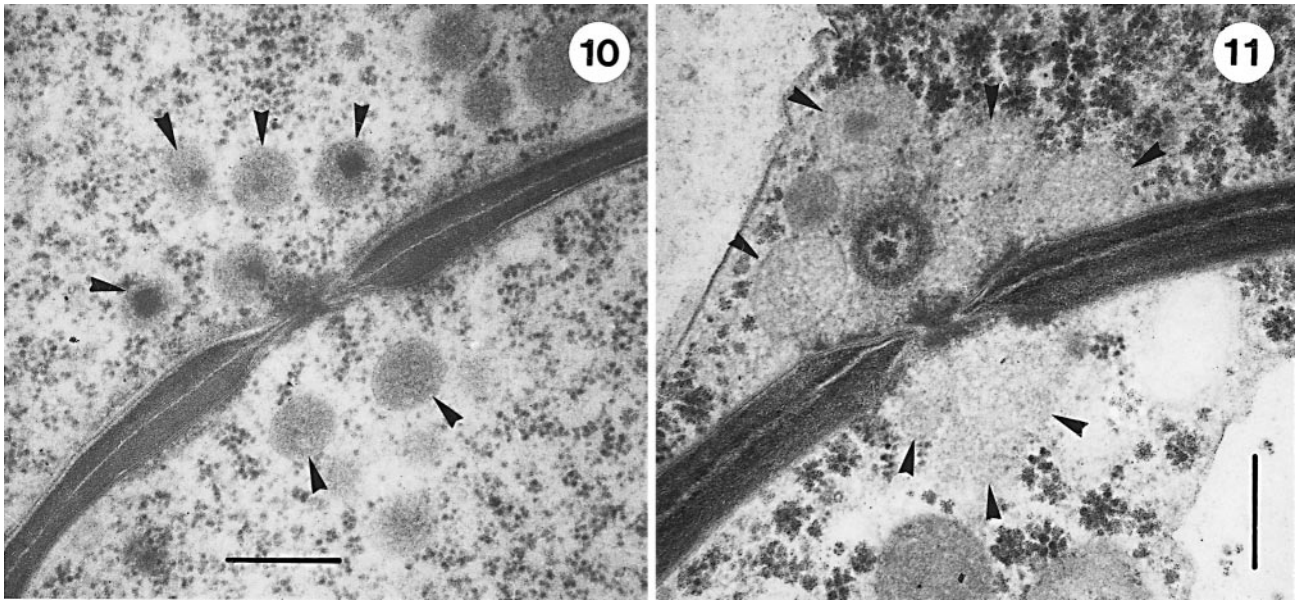
FIGS. 4–5. Cross section through aecium of *Tranzschelia pruni-spinosae* infected with *Tuberculina persicina*. Samples were prepared by chemical fixation (4) or high pressure freezing and freeze substitution (5) and observed with a transmission electron microscope. 4. Section through hyphae of *Tranzschelia pruni-spinosae* (R) and *Tuberculina persicina* (t) illustrated to show the different sizes of the nuclei and cell walls of the two fungi. Scale bar = 3 μm. 5. Hypha of *Tuberculina persicina* (t) surrounded by hyphae of *Tranzschelia pruni-spinosae* (R). Note that the diameter of the rust nucleus and the thickness of the rust cell walls are more than twice as large compared with those of *Tuberculina*. Scale bar = 2 μm.

cib., respectively). Location and mode of sporulation as well as the morphology of hyphae inspired some workers to treat *Tuberculina* species as rusts and to create new species (Corda 1842, Desmazières 1847, Spegazzini 1880) and genera (Mayr 1890). Other researchers even considered *Tuberculina* as a stage of asexual rust reproduction (Cunningham 1889; *Ravenelia sessilis* Berk., Griffiths 1902; *Gymnoconia riddelliae* Griffiths, Plowright 1885; *Puccinia vincae,* Spegazzini 1888; "*Tuberculina paraguayensis* Speg.", Vuillemin 1892a; *Aecidiconium barteti* Vuill., 1892b; *Endophyllum sempervivi* [Alb. & Schwein.] de Bary). Gobi (1885) investigated morphology, sporogenesis, and dispersal and germination of spores and assigned the genus to the smuts. His point of view was followed by the majority of researchers of that time (e.g., Plowright 1889, Schroeter 1889, Wildeman 1908). Although considering the same features, Morini (1886) assigned *Tuberculina* to the Tremellineae. Buddin et al (1927) were the first to observe that *Helicobasidium* produces "small raised tubercles, which eventually become pustules of conidia of the type which is characteristic of the genus *Tuberculina*." For that reason, *Tuberculina* was assigned to *Helicobasidium* by some researchers (von Arx 1981, Carmichael et al 1980, Kendrick and Watling 1979). However, the obvious lack of striking features for phylogenetic placement of the genus has prompted most recent researchers (e.g., Hawksworth et al 1995, Sundheim 1986, Wicker 1981) to follow Fuckel (1870) in treating *Tuberculina* as Fungi Imperfecti.

Our phylogenetic analyses of nuc-LSU rDNA sequences, however, demonstrate clearly that *Tuberculina* species are members of the basidiomycetes, positioned within the rust group as sister to *Helicobasidium*. This phylogenetic hypothesis agrees well with ultrastructural data. As shown in this study, the trilamellate nature of the septa, in which a thin electron-transparent middle lamella is sandwiched between thick electron-opaque layers, indicates that *Tubercu-*



FIGS. 6–9. Cellular interaction with large fusion pores between *Tuberculina persicina* (t) and *Tranzschelia pruni-spinosae* (R). Samples were prepared by chemical fixation (6–7) or high pressure freezing and freeze substitution (8–9) and observed with a transmission electron microscope. 6. Interaction stage in overview with two medianly sectioned nuclei of *Tuberculina persicina* (n) and one medianly sectioned nucleus of *Tranzschelia pruni-spinosae* (N). Note the different sizes of the nuclei. The fusion pore is visible at arrow. Scale bar = 2 μm. 7. Detail from FIG. 6 illustrating the large fusion pore (arrow). Scale bar = 0.3 μm. 8. High-pressure frozen interaction stage in overview. Fusion pore is visible at arrow. Note that the pore morphology is more distinct than after conventional fixation (compare with 6). Scale bar = 2 μm. 9. Detail from FIG. 8 showing the fusion pore (arrow) and that the plasma membrane of both partners is continuous through the fusion pore (arrowheads). Scale bar = 0.2 μm.



FIGS. 10–11. Septal pore apparatus of *Tuberculina persicina* (10) and *Tranzschelia pruni-spinosae* (11). Samples were prepared by high-pressure freezing and freeze substitution and observed with a transmission electron microscope. Each pore shows a non-swollen pore margin and associated microbodies (arrowheads) in a more or less circular arrangement. Scale bars = 0.3 μm.

lina is basidiomycetous (Kreger-van Rij and Veenhuis 1971). In addition, the septal pore apparatus in *Tuberculina persicina* essentially is identical to that of its host fungus *Tranzschelia pruni-spinosae*. In both species, it is composed of a simple pore surrounded by microbodies in a more or less circular arrangement. This type of septal pore apparatus is common among the members of the rust group (see Bauer 1987, Bauer and Oberwinkler 1994, Boehm and McLaughlin 1989, Khan and Kimbrough 1982, Littlefield and Heath 1979) and occurs also in *Helicobasidium* (Bourett and McLaughlin 1986). In addition, both *Tuberculina* and the members of the rust group have clampless hyphae.

The close phylogenetic proximity of *Tuberculina* and *Helicobasidium* raises questions on the relation of the genera, especially since we know that cultures of *Helicobasidium* on artificial media produce *Tuberculina*-like conidia. That observation was repeated several times (Arai et al 1987, Buddin and Wakefield 1927, 1929, Fukushima 1998, Sayama et al 1994, Valder 1958) but without definitive conclusions or further investigations. However, *Tuberculina* is reported to be the anamorphic stage of *Helicobasidium,* justified by the quoted observations in several compendia (Carmichael et al 1980, Hawksworth et al 1995). This is in contrast to our molecular analyses, in which all three commonly distinguished *Tuberculina* species are included, as well as two of probably three distinguishable *Helicobasidium* species (see Reid 1975, Roberts 1999). *Tuberculina* is separated from *Helicobasidium,* and there is no record for conidia formation by *Helicobasidium* in nature, apart from one report (Patouillard 1886), which could not be confirmed by subsequent researchers (Buddin and Wakefield 1927).

Association between Tuberculina *and rusts.*—Tulasne (1854) interpreted the exclusive occurrence of *Tuberculina* in association with rusts as argument for the mycoparasitic nature of the genus. His reasoning was followed by most researchers (Buchenauer 1982, Kirulis 1940, Lindau 1910, Tubeuf 1901, Zambettakis et al 1985), adding as arguments the heavy impairment of rust spore production in the presence of *Tuberculina* (Spaulding 1929, Tubeuf 1917), infection experiments showing that rust-free plants could not be infected by *Tuberculina* (Barkai-Golan 1959) and presumable structures of parasitic interaction (Gruyer 1921, Sappin-Trouffy 1896, Thirumalachar 1941). Disagreeing with that, Marchal (1902) was the first to propose a commensal relationship. *Tuberculina* was interpreted as saprophyte living in rust-damaged plant tissues. This point of view was encouraged by the presumable occurrence of *Tuberculina* in rust-free plant tissues in nature (Gobi 1885) and in artificial culture (Wicker and Woo 1969, 1973), experiments of dual cultures of *Tuberculina* and rusts where no interaction could be recognized (Wicker 1979), and investigations by light microscopy, where no in-

teraction of rust and *Tuberculina* was found (D'Oliveira 1941, Hulea 1939) except the digestion of plant cells (Wicker 1979).

Our ultrastructural observations demonstrate a specific and morphologically uncommon interaction between *Tuberculina persicina* and *Tranzschelia pruni-spinosae*. Interaction results in a large fusion pore between *Tuberculina* and its host rust with a direct cytoplasm-cytoplasm connection. The plasma membranes of *Tuberculina* and its host form a continuum. Existence of such an unusual structure of interaction indicates the mycoparasitic nature of *Tuberculina*.

Moreover, our ultrastructural observations confirm those authors who assumed that *Tuberculina* is restricted to the haploid rust stage and the following stage on the same host plant (e.g., D'Oliveira 1941, Lechmere 1914). In addition, the mycoparasitic nature and the distinctive cellular interaction of *Tuberculina* provide a good taxonomic boundary for the genus *Tuberculina*. It definitely should be restricted to rust parasites.

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