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Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences

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Abstract: The order Tilletiales (Ustilaginomycetes, Basidiomycota) includes six genera (*Conidiosporomyces, Erratomyces, Ingoldiomyces, Neovossia, Oberwinkleria* and *Tilletia*) and approximately 150 species. All members of Tilletiales infect hosts in the grass family Poaceae with the exception of *Erratomyces* spp., which occur on hosts in the Fabaceae. Morphological features including teliospore ornamentation, number and nuclear condition of primary basidiospores and ability of primary basidiospores to conjugate and form an infective dikaryon were studied in conjunction with sequence analysis of the large subunit nuclear rDNA gene (nLSU). Analysis based on nLSU data shows that taxa infecting hosts in the grass subfamily Pooideae form one well supported lineage. This lineage comprises most of the reticulate-spored species that germinate to form a small number of rapidly conjugating basidiospores and includes the type species *Tilletia tritici.* Two tuberculate-spored species with a large number of nonconjugating basidiospores, *T. indica* and *T. walkeri,* and *Ingoldiomyces hyalosporus* are also included in this lineage. Most of the species included in the analysis with echinulate, verrucose or tuberculate teliospores that germinate to form a large number (>30) of nonconjugating basidiospores infect hosts in the subfamilies Panicoideae, Chloridoideae, Arundinoideae and Ehrhartoideae. This group of species is more diverse than the pooid-infecting taxa and in general do not form well supported clades corresponding to host subfamily. The results of this work suggest that morphological characters used to segregate *Neovossia, Conidiosporomyces* and *Ingoldiomyces* from *Tilletia* are not useful generic level characters and that all included species can be accommodated in the genus *Tilletia.*

Key words: *Conidiosporomyces, Erratomyces,* germination, *Ingoldiomyces,* molecular systematics, *Neovossia,* smut and bunt fungi

INTRODUCTION

The genus *Tilletia* Tul. & C. Tul. comprises ca. 140 species restricted to hosts in the grass family (Poaceae) and is the largest genus in order Tilletiales (Basidiomycota, Ustilaginomycetes, Exobasidiomycetidae) (Ványky 2002). *Tilletia* is characterized by the formation of pigmented teliospores intermingled with hyaline sterile cells, and in most species the teliospores are formed in host ovaries. Teliospore ornamentation ranges from reticulate, echinulate, verrucose, tuberculate to smooth. In many species teliospore masses have a fetid, herring brine odor due to the production of trimethylamine. Teliospores germinate to form an aseptate basidium, frequently with multiple retraction septa, and a terminal whorl of aerial primary basidiospores (FIG. 1). The type species, *T. tritici,* produces 8–12 filiform to narrowly falcate monokaryotic basidiospores (Goates 1996). Most of the basidiospores conjugate while attached to the basidium to form an ''H-body,'' giving rise to dikaryotic mycelium that infects host plants at seedling stage, resulting in a systemic infection (Ványky 1994). A second type of germination pattern, consisting of the production of large numbers of nonconjugating primary basidiospores, is found in species of *Neovossia* Körn., *Conidiosporomyces* Ványky (Ványky and Bauer 1992) and some species of *Tilletia,* such as *T. indica, T. horrida* and *T. walkeri* (Castlebury and Carris 1999, Durán 1987). *Oberwinkleria* Ványky & R. Bauer produces nonconjugating primary basidiospores (Ványky and Bauer 1995) but their nuclear condition was not reported.

Five of six genera in Tilletiales, *Conidiosporomyces, Ingoldiomyces* Ványky, *Neovossia, Oberwinkleria* and *Tilletia,* are known to infect only grass hosts. Most species within these genera produce teliospores in host

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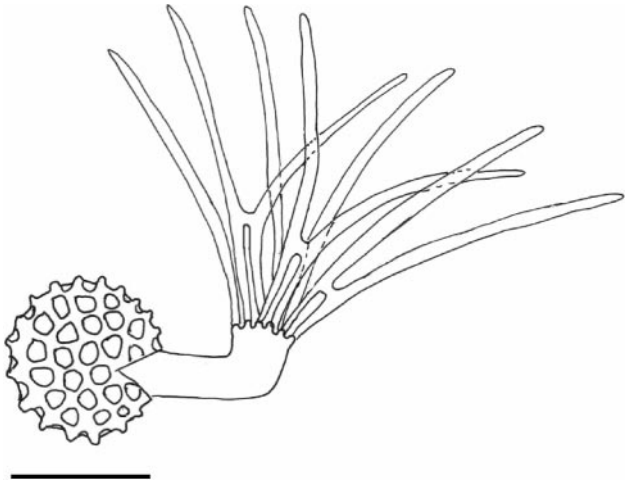

FIG. 1. *Tilletia tritici* germinated teliospore with conjugating primary basidiospores. Bar = 20 μm.

ovaries, with the exception of nine *Tilletia* species that form teliospores in leaves and stems (Zogg 1972). The sixth genus, *Erratomyces* M. Piepenbr. & R. Bauer, comprises five species that produce teliospores in leaves of Fabaceae and have a teliospore germination pattern similar to *Tilletia* (Piepenbring and Bauer 1997). *Conidiosporomyces* and *Ingoldiomyces* are based on *Tilletia ayresii* and *Tilletia hyalospora,* respectively. *Conidiosporomyces* is distinguished from *Tilletia* by the formation of an apically open, sac-like sorus and presence of Y-shaped conidia (FIG. 2G) in the sorus (Vánky and Bauer 1992). Two additional species have been transferred to the *Conidiosporomyces* from *Tilletia* and *Ustilago* (Pers.) Roussel (Vánky 1993, 2001).

The monotypic *Ingoldiomyces* is distinguished from *Tilletia* by formation of ballistosporic primary basidiospores and a unique type of teliospore ornamentation (Vánky and Bauer 1996). *Oberwinkleria,* also monotypic, was erected for a new species, *O. anulata* K. & C. Vánky, and is characterized by greatly reduced basidia and primary basidiospores produced on pedicels (Vánky and Bauer 1995). *Neovossia* was erected based on *Neovossia moliniae* (Thüm.) Körn., a *Tilletia*-like species producing teliospores with a hyaline appendage, local infection, a large number (>40) of nonconjugating primary basidiospores and without sterile cells (Vánky 1994). Ten or more species have been placed in *Neovossia,* but the generic boundary between *Neovossia* and *Tilletia* is not clear

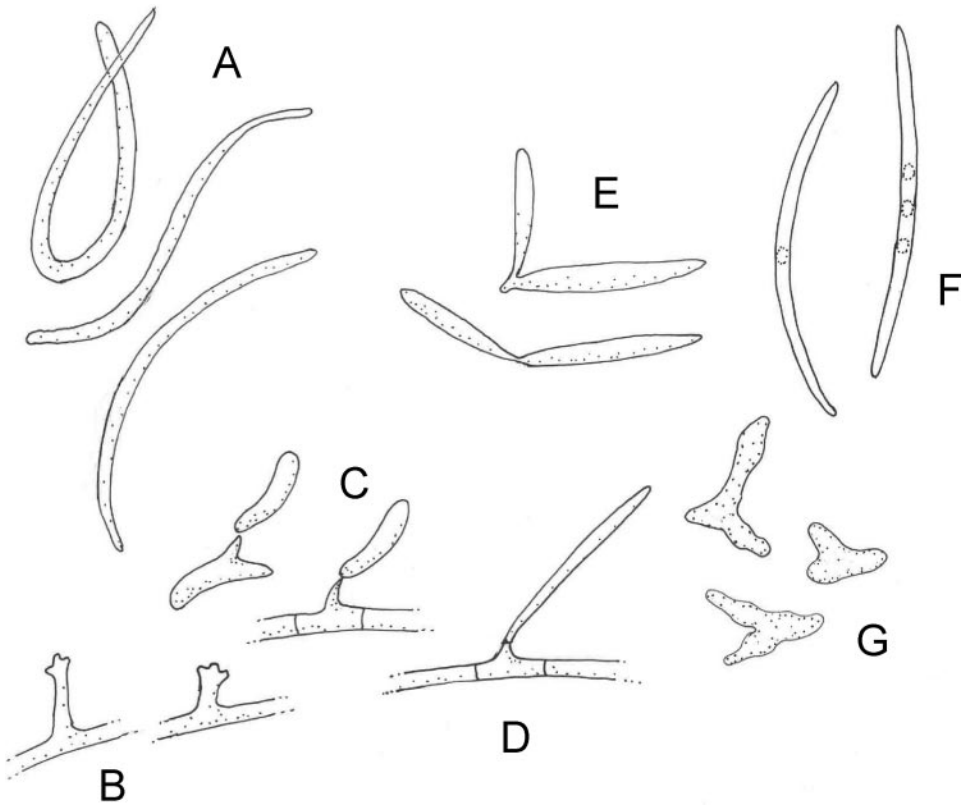

FIG. 2. Spore types from various species in the Tilletiales. A. Blastospores from *T. ixophori.* B. Denticulate sporogenous cells from *T. kimberleyensis.* C. Formation of ballistospores and proliferation of ballistospore. D. Formation of blastospores. E. Proliferating blastospores. F. Uninucleate and multinucleate primary basidiospores. G. Y-shaped conidia formed in culture of *C. verruculosus.*

and Vánky (2002) now considers *Neovossia* a monotypic genus.

Members of Tilletiales have been poorly represented in previous phylogenetic analyses of the smut fungi (Begerow et al 1997, Begerow et al 2000). The analysis of Begerow et al (1997) included only four type species, *Ingoldiomyces hyalosporus, Conidiosporomyces ayresii, Tilletia tritici* and *Erratomyces patelii*. In that analysis *T. tritici* and *I. hyalosporus* were related most closely and formed a sister group of *C. ayresii,* with *E. patelii* basal to these species. The present study, using nLSU sequence data, was initiated to determine phylogenetic relationships among species of *Tilletia* and segregate genera. Data on teliospore morphology, teliospore germination, primary and secondary basidiospore morphology, and nuclear condition, when available, are presented.

MATERIALS AND METHODS

Isolation, maintenance and deposition of cultures and voucher specimens.—Species used in this study are listed (TABLE I). All available taxa for which teliospores could be germinated were included. Teliospores were germinated after soaking in water for 2 d and surface sterilization in 0.26% NaClO (5% v/v commercial bleach) on 2% water agar at room temperature (20–25 C), 15 C or 5 C depending on the species. Teliospores of *T. controversa* were germinated at 5 C under a 8/16 h daylight/dark regimin. Primary basidiospores were fixed and stained with Giemsa-HCl following Durán (1980) to determine nuclear condition or were transferred to potato-sucrose agar (PSA) or M-19 agar (Trione 1964) to establish colonies for nucleic acid extraction.

Nucleic acid extraction and PCR amplification.—Mycelium for DNA extraction was grown in shaker flasks at 125 rpm in 100 mL liquid potato-dextrose broth at room temperature or 15 C under ambient light. Mycelium was harvested by centrifugation. Alternatively, DNA was extracted directly from actively growing surface mycelium scraped from PSA or M-19 plates. DNA was extracted with the PureGene DNA extraction kit (Gentra Systems, Madison, Wisconsin) according to the manufacturer's instructions using approximately 15 mg dried tissue or 50 mg fresh mycelium.

The nLSU genes were amplified in 50 μL reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California) under these reaction conditions: 10–15 ng of genomic DNA, 200 μM each dNTP, 2.5 units Amplitaq Gold (Applied Biosystems, Foster City, California), 25 pmoles each of primers LR0R and LR7 (Vilgalys and Hester 1990, Rehner and Samuels 1994) and the supplied 10× PCR buffer with 15 mM $MgCl_2$. The thermal cycler program was: 10 min at 95 C followed by 35 cycles of 30 s at 94 C, 30 s at 55 C, 1 min at 72 C, with a final extension for 10 min at 72 C. After amplification, the PCR products were purified with QIAquick columns (QIAGEN Inc., Chatsworth, California) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye terminator kit (Applied Biosystems, Foster City, California) on an automated DNA sequencer with these primers: LR0R, LR3R, LR5R, LR7, LR5, LR3 (Vilgalys and Hester 1990, Rehner and Samuels 1994, 1995).

Sequence analysis.—Raw sequences were edited with Sequencher version 4.1.4 for Windows (Gene Codes Corp., Ann Arbor, Michigan). Alignments were adjusted manually with GeneDoc 2.6.001 (http://www.psc.edu/biomed/genedoc/). The alignment included sequences from 57 isolates, with three species of *Entyloma* de Bary and one species of *Graphiola* Poit. (WSP 71169) as outgroup taxa and consisted of 1345 positions. *Entyloma* and *Graphiola* also are contained within the Exobasidiomycetidae in different orders and have been placed close to the Tilletiales in previous analyses (Begerow et al 1997). The sequence alignment was deposited in TreeBase.

Trees were inferred by the neighbor joining (NJ) method (Kimura 2-parameter distance calculation) and by maximum parsimony (MP) using the heuristic search option with the random addition sequence (1000 replications, maximum of 100 trees saved per replicate) and the branch swapping (tree bisection-reconnection) option of PAUP* 4.0b10 (Swofford 2002). All aligned positions were included in the analyses. All characters were unordered and given equal weight. Gaps were treated as missing data in the parsimony analysis and the neighbor joining analysis; missing or ambiguous sites were ignored for affected pairwise comparisons. Relative support for branches was estimated with 1000 bootstrap replications (Felsenstein 1985) with multrees and TBR off and 10 random sequence additions for the MP bootstraps.

Phylogenetic trees also were inferred with Bayesian inference as implemented in MrBayes (http://morphbank.ebc.uu.se/mrbayes/) with these commands: number of generations = 500 000, sample frequency = 100, number of chains = 4, temperature = 0.2, save branch lengths = yes, starting tree = random. Likelihood model assumptions were as determined with Modeltest version 3.06 (Posada and Crandall 1998) with the Akaike Information Criterion (AIC) under the GTR+I+G model: base frequencies A = 0.2664, C = 0.1971, G = 0.2907, T = 0.2458; number substitution types = 6; proportion of invariable sites = 0.6672; gamma shape parameter = 0.5947; rate matrix = 0.6253, 2.4765, 0.8936, 0.2228, 5.4558, 1.000. The first 100 000 generations were discarded as the chains were converging (burn-in). Three independent analyses, each starting from a random tree, were run under the same conditions.

Phylogenetic trees constraining monophyletic groups of taxa were constructed as follows based on four major characters: (i) two based on spore ornamentation types (reticulate and echinulate/tuberculate/verrucose); (ii) four based on host subfamily; (iii) two based on type of germination (conjugating primary basidiospores or nonconjugating); and (iv) two based on local- or systemic-infecting. Maximum parsimony analyses were run for each of the 10 resulting constraints (TABLE II) using the heuristic search option (1000 random sequence additions, TBR and multrees off). The trees with the best −ln likelihood score resulting from each constrained analysis and all three Bayesian trees

TABLE I. List of taxa, specimen numbers, hosts and GenBank accession number for nLSU

Taxon	Collection No.[a]	Host and geographic origin	GenBank No.
Conidiosporomyces ayresii (Berk.) Vánky	HUV 19.314	*Panicum maximum,* Argentina	AY819017
C. verruculosus (Wakef.) Vánky	WSP 70430 (V 1116)	*Setaria sphacelata,* Zimbabwe	AY818984
Erratomyces patelli (Pavgi & Thirum.) M. Piepenbr. & R. Bauer	HUV 18.697	*Vigna mungo,* India	AY818966
Ingoldiomyces hyalosporus (Massee) Vánky	V 930	*Nassella mexicana,* Venezuela	AY818976
Neovossia iowensis Hume & Hodson	BPI 863664	*Phragmites communis,* China	AY818988
Tilletia aegopogonis Durán	WSP 67743	*Aegopogon tenellus,* Mexico	AY818967
T. anthoxanthi A. Blytt	V 761	*Anthoxanthum odoratum,* New Zealand	AY819009
T. asperifolia Ell. & Everh.	LMC 90	*Muhlenbergia asperifolia,* USA	AY818968
T. asperifolia	LMC 47	*Muhlenbergia asperifolia,* USA	AY818969
T. barclayana (Bref.) Sacc. & Syd.	WSP 68658	*Paspalum distichum,* Mexico	AY818970
T. barclayana	WSP 68466	*Paspalum distichum,* Mexico	AY818971
T. barclayana	WSP 68654	*Panicum obtusum,* Mexico	AY818972
*T. bouteloua*e Durán	WSP 68661	*Bouteloua gracilis,* Mexico	AY818973
T. bromi (Brockm.) Brockm.	LMC 171	*Bromus japonicus,* USA	AY819001
T. bromi	V 763	*Nardurus subulatus,* Iran	AY818992
T. bromi	LMC 99	*Bromus tectorum,* USA	AY818993
T. cerebrina Ell. & Everh.	LMC 125	*Deschampsia danthonoides,* USA	AY818994
T. chionachnes K. & C. Vánky & R.G. Shivas	V 1083	*Chionachne cyathopoda,* Australia	AY818990
T. controversa Kühn	V 764	*Hordeum glaucum,* Iran	AY818995
T. ehrhartae Talbot	HUV 19.754	*Ehrharta calycina,* Australia	AY819013
T. eremopoae Vánky & H. Scholz	HUV 19.420	*Eremopoa persica,* Turkey	AY819016
T. fusca Ell. & Everh.	LMC 141	*Vulpia microstachys,* USA	AY818997
T. fusca	LMC 214	*Vulpia octoflora,* USA	AY818996
T. goloskokovii Schwarzman	LMC 321	*Apera interrupta,* USA	AY818998
T. goloskokovii	LMC 315	*Apera interrupta,* USA	AY818999
T. holci (Wesend.) J. Schröter	V 765	*Holcus mollis,* New Zealand	AY819008
T. horrida Tak.	LMC 339	*Oryza sativa,* USA	AY818974
T. horrida	LMC 358	*Oryza sativa,* USA	AY818975
T. indica Mitra	BPI 863665	*Triticum aestivum,* USA	AY818977
T. ixophori Durán	WSP 71170	*Ixophorus unisetus,* Nicaragua	AY819010
T. kimberleyensis Vánky & R.G. Shivas	HUV 19.174	*Chionachne cyathopoda,* Australia	AY818979
T. laevis Kühn	LMC 178	*Triticum aestivum,* Australia	AY819004
T. laevis	V 766	*Triticum aestivum,* Iran	AY819005
T. lycuroides Durán	WSP 68731	*Lycurus phleoides,* Mexico	AY818980
T. menieri Har. & Pat.	WSP 69115	*Phalaris arundinacea,* Germany	AY819002
T. obscura-reticulata Durán	WSP 68357	*Bouteloua rothrockii,* Mexico	AY819011
T. olida (Riess) J. Schröter	WSP 71076	*Brachypodium pinnatum,* Germany	AY819000
T. opaca Sydow	V 837	*Spinifex littoreus,* Indonesia	AY818981
T. polypogonis Vánky & N.D. Sharma	V 931	*Polypogon monspeliensis,* India	AY819015
T. rugispora Ell. & Everh.	WSP 60775	*Paspalum convexum,* Mexico	AY818982
T. rugispora	HUV 19.147	*Paspalum plicatulum,* Argentina	AY818983
T. savilei R.V. Gandhe & Vánky	V 859	*Tripogon jacquemontii,* India	AY819018
T. setariae L. Ling	V 932	*Setaria intermedia,* India	AY819014
T. sterilis E. Ule	LMC 363	*Poa secunda,* USA	AY819003
T. sumatii (S.D. Patil & Gandhe) Vánky	V 838	*Coix lacryma-jobi,* India	AY818986
T. sumatii	V 933	*Coix lacryma-jobi,* India	AY818987
T. togwateei Guillemette	LMC 153	*Poa reflexa,* USA	AY818991
T. trachypogonis Durán	V 1134	*Trachypogon spicatus,* Zambia	AY819012
T. tritici (Bjerk.) Wint.	LMC 4	*Triticum aestivum,*	AY819006
T. tritici	LMC 97-136	*Triticum aestivum,* Australia	AY819007
T. vittata (Berk.) Mund.	HUV 19.160	*Oplimenus burmannii,* India	AY818985
T. walkeri Castlebury & Carris	BPI 746091	*Lolium multiflorum,* USA	AY818978
T. whiteochloae R.G. Shivas & Vánky	V 1087	*Whiteochloa cymbiformis,* Australia	AY818989

[a] BPI = U.S. National Fungus Collections, Beltsville, MD; HUV = Herbarium Ustilaginales Vánky, Tübingen; LMC = personal collection of L. M. Carris; V = Vánky Ustilaginales Exsiccati; WSP = Washington State Department of Plant Pathology.

TABLE II. Shimodaira-Hasegawa likelihood test results for analyses constrained for host subfamily or morphological character

Topology	Trees[a]	Length	−ln Likelihood	*P*
Unconstrained MP	1509	432	4213.892	—
Bambusoid hosts	6970	458	4284.285	0.005*
Chloridoid hosts	2820	445	4255.662	0.167
Panicoid hosts	5930	450	4271.798	0.046*
Pooid hosts	1073	432	4214.326	0.952
Conjugating basidiospores	2524	488	4420.040	0.000*
Non-conjugating basidiospores	5000	510	4501.826	0.000*
Reticulate teliospores	1198	447	4251.180	0.207
Tuberculate teliospores	100	448	4253.268	0.190
Local-infecting	1853	465	4321.874	0.000*
Systemic-infecting	1072	456	4280.336	0.011
Bayesian	3	—	4215.393	0.948

[a] *P*-values and −ln likelihood scores only reported for the tree with best −ln likelihood score.

* Indicates significant at $P < 0.05$ in a one-tailed test under the null hypothesis that all trees are equally good explanations of the data.

were compared with the MP tree with the best −ln likelihood score, using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999). The range of −ln likelihood scores of trees from each constraint topology is shown (TABLE II). Likelihood settings were as determined by Modeltest as previously described.

RESULTS

Phylogenetic analyses.—Of 1345 characters, 144 were parsimony informative, 1124 were invariable, 77 were variable but not parsimony informative. For MP analyses with the multrees option on, heuristic searches resulted in an excess of 5000 trees. By limiting the number of trees saved per replicate to 100, 1509 equally parsimonious trees were generated. A strict consensus of trees generated with multrees on (maxtrees = 5000) was identical to the strict consensus of trees generated from analyses with multrees limited to 100 per replicate (trees not shown). Parsimony tree scores were CI = 0.637, RI = 0.860, RC = 0.547 and length = 432. The MPT with the best −ln likelihood score is shown (FIG. 3). MP bootstrap support values are indicated (FIG. 3) above the respective branches. NJ bootstrap support values did not differ greatly from MP bootstrap support and are not shown.

Three independent Bayesian analyses were run with each starting from a random tree and probabilities and topologies were similar in all analyses. One arbitrarily chosen Bayesian tree is shown (FIG. 4). Topologies differed only in the placement of the *Conidiosporomyces/T. vittata* branch as unresolved in relation to the pooid group in two runs but immediately basal to the pooid group in the third run, although this was not supported (trees not shown). Minor differences in terminal branching also were noted but also not supported. Posterior probabilities were pooled and branches with pooled posterior probabilities > 90% are indicated with thickened lines (FIG. 4).

The analysis shows strong support (100% Bayesian, 88% MP) (FIGS. 3–4) for a monophyletic group that contains species of *Tilletia, Ingoldiomyces, Neovossia*and *Conidiosporomyces*. Within these taxa, four distinct lineages are apparent. Lineage I contains species infecting grasses in the Pooideae (100% Bayesian, 61% MP support), with three well supported subgroups of taxa consisting of *T. tritici* and related species (100% in all analyses), *I. hyalosporus* and *T. polypogonis,* and one for *T. indica* and *T. walkeri* (>99% in all analyses). Each of these groups also is characterized by different germination patterns and teliospore ornamentation. Lineage II, recognized in all analyses, contains 11 species that infect Panicoideae, Arundinoideae and Chloridoideae (PAC) (100% Bayesian, 82% MP), including *N. iowensis*. All species in this group have tuberculate/verrucose teliospores with the exception of *N. iowensis,* which has foveolate teliospores and nonconjugating, uni- or multinucleate basidiospores. Several species in this group have been described or referred to as species of *Neovossia* in the literature. *Tilletia barclayana,* which falls in this group, appears to be a species complex with slight differences in sequence found among all three isolates. However it is not clear whether the differences found in the nLSU sequences warrant species level distinction. Variation in the nLSU was not consistent across all species. Taxa in the pooid-infecting clade (Lineage I) varied the least with almost no differences among the reticulate-spored taxa or between *T. indica* and *T. walkeri*. Larger numbers of differences were observed among taxa

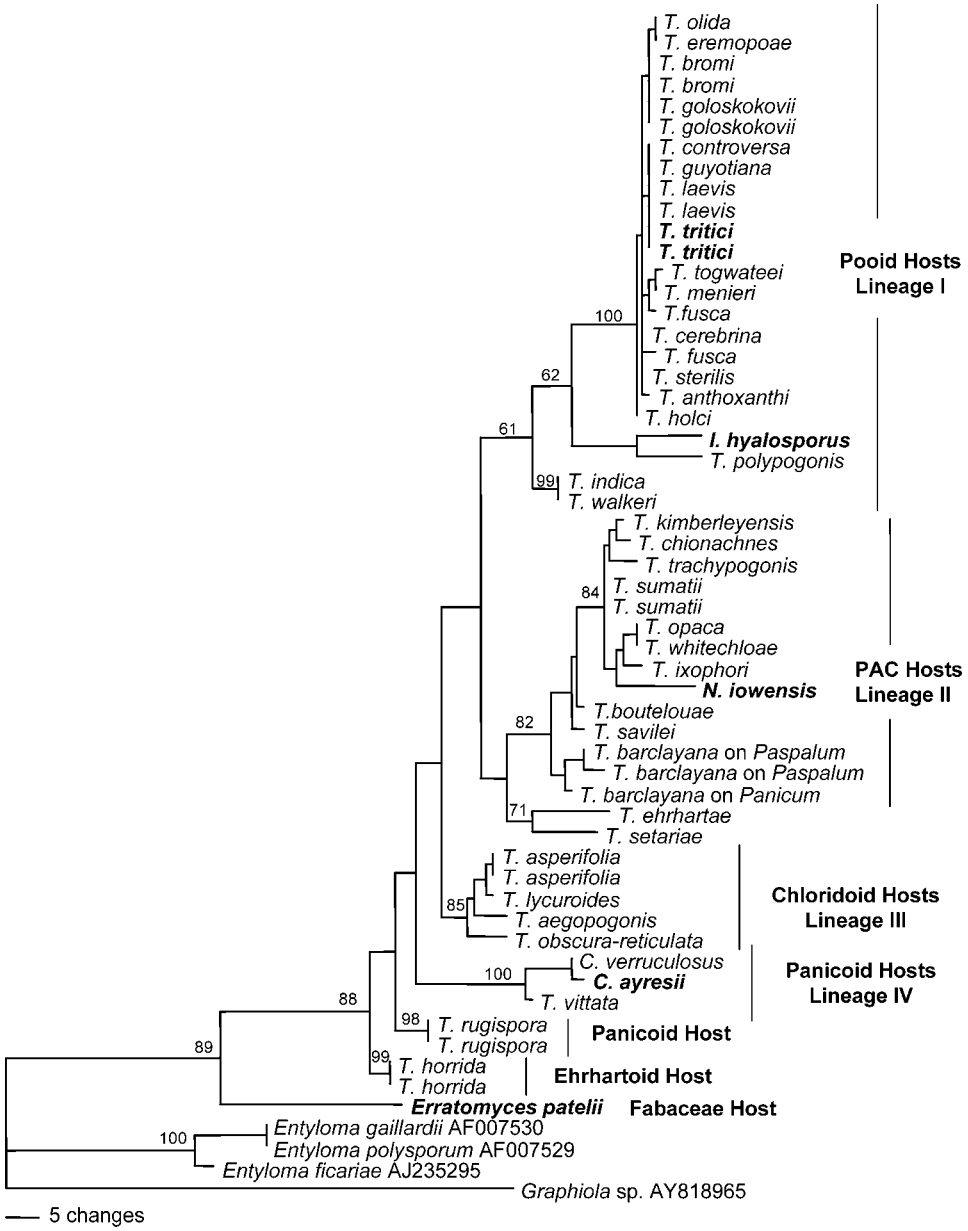

FIG. 3. MP tree resulting from analysis of 1345 bp from the nLSU for the species in the Tilletiales. Numbers above the branches indicate MP bootstrap support percentages (>50%) from 1000 pseudoreplicates with 10 random taxon addition replicates per pseudoreplicate for major lineages only. Four major lineages are identified by Roman numerals I-IV and host subfamily when limited to a single subfamily. PAC refers to Panicoidae, Arundinoideae and Chloridoideae. Representatives from segregate genera are indicated in bold type as is the type species of *Tilletia*.

in the other three lineages. This could be due to better sampling of taxa in Lineage I, a more recent radiation of species in Lineage I or some combination of both.

Lineage III includes species infecting chloridoid hosts (≥85% in all analyses), including *T. asperifolia, T. lycuroides, T. aegopogonis* and *T. obscura-reticulata*. These are the only four taxa in the analysis with reticulate spores that infect hosts other than Pooideae.With the exception of *T. asperifolia,* which has uninucleate, conjugating basidiospores, all form multinucleate, nonconjugating basidiospores. Lineage IV contains three panicoid-infecting species and includes *C. ayresii, C. verruculosus* and *T. vittata. Conidiosporomyces* species have open sori and Y-shaped conidia (either in sori or formed in culture). *Tilletia vittata* causes hypertrophy of the infected ovary so that it forms a conspicuous, spur-like outgrowth. Basidiospores of the three species in this lineage are uninucleate, and conjugation was observed (but rare-

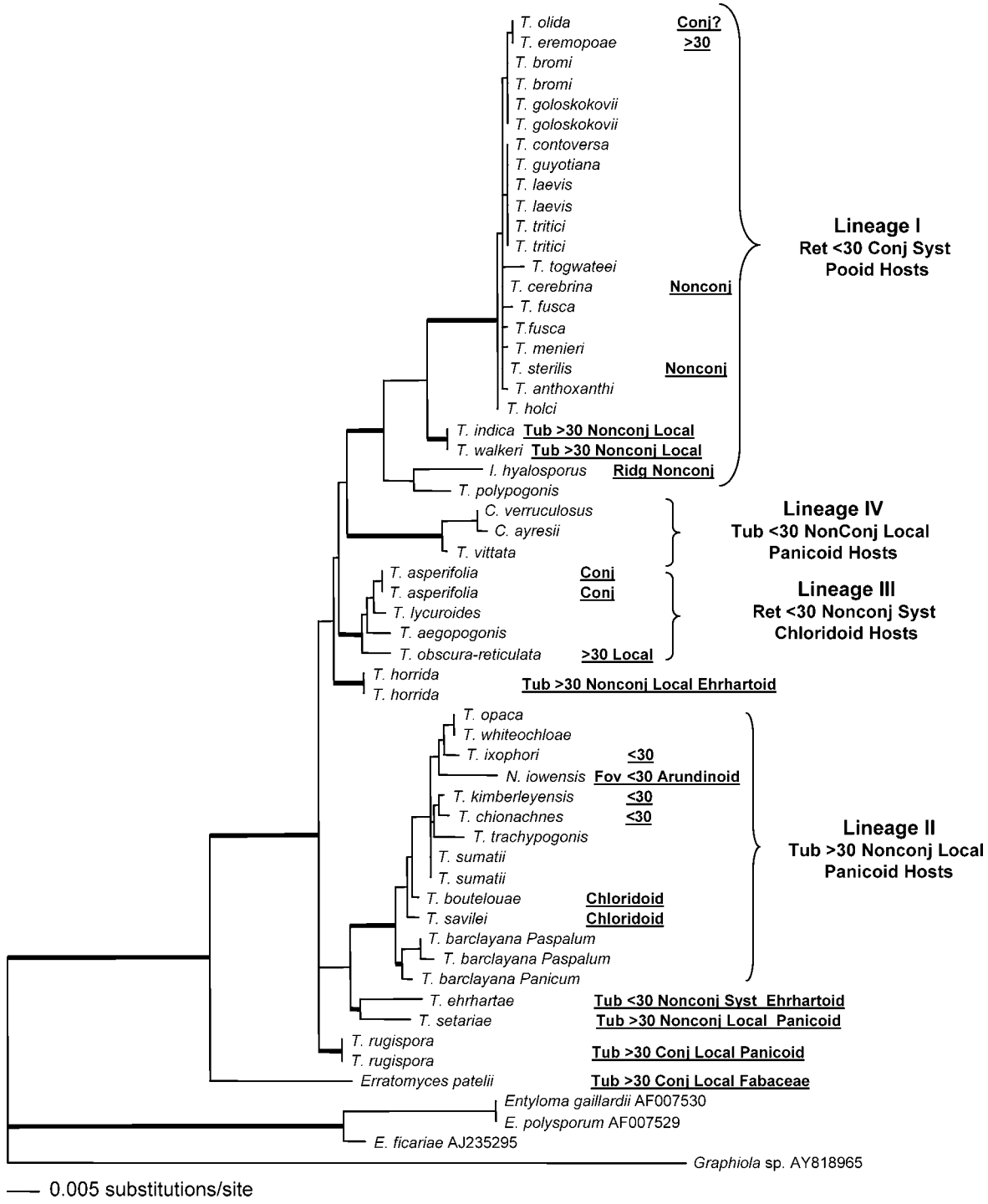

FIG. 4. Phylogenetic tree resulting from Bayesian analysis of 1345 bp of the nLSU of species in the Tilletiales. Thickened branches indicate >90% pooled posterior probabilities obtained from three independent Bayesian analyses, each consisting of 500 000 Markov chain Monte Carlo generations (GTR+G+I model), with a burn-in of 100 000 generations. Lineages identified in FIG. 3 are indicated with host subfamily association. Morphological characters from TABLE III are labeled as follows: Ret = reticulate spore, Tub = tuberculate/verrucose spores, Ridg = ridged spores, Fov = Foveolate, Conj = conjugating primary basidiospores, Nonconj = nonconjugating primary basidiospores, <30 = <30 primary basidiospores, >30 = >30 primary basidiospores, Local = local-infecting, and Syst = systemic-infecting. When a species in a group differs from the labeled characters, the difference for that species is indicated in bold with underlining.

ly) only in *C. ayersii*. A few species do not fall into any of the four lineages described above. The relationships of *T. setariae* (panicoid host), *T. ehrhartae* (ehrhartoid host), *T. rugispora* (panicoid host) and *T. horrida* (ehrhartoid host) to other species remain unresolved. Morphological characters (TABLE III) for lineages are labeled (FIG. 4). For taxa with differing character states for a given character, differences are indicated in bold underlined text inside the brackets

The MP tree had the best likelihood score (TABLE II), although the Bayesian trees were not significantly worse explanations of the data ($P = 0.05$). Trees constraining pooid-infecting, chloridoid-infecting, reticulate-spored, and echinulate/verrucose/tuberculate-spored taxa, respectively, also were not significantly worse than the MP tree. Trees constraining local-infecting or systemic-infecting taxa, taxa with conjugating basidiospores or panicoid- or ehrhartoid-infecting taxa were significantly worse ($P = 0.05$) than the MP tree.

Teliospore germination and growth in culture.—Teliospore germination data for species in the analysis are provided (TABLE III). Nuclear condition of primary basidiospores could not be determined for seven species that had limited teliospore germination. The teliospore germination pattern in the type species *T. tritici* involves rapid conjugation of adjacent primary basidiospores. Teliospores germinate at 5–15 C, but no germination occurs at room temperature. The fungus infects the host at the seedling stage, forming a systemic infection and growing to the developing host ovaries, where the fungus proliferates and forms teliospores. This pattern of dikaryon formation, systemic infection and low temperature requirement occurs in all species closely related to *T. tritici,* with the exception of *T. sterilis* and *T. cerebrina,* which form multinucleate, nonconjugating primary basidiospores. Zogg (1967) reported conjugation in *T. olida,* but it was was not observed in the *T. olida* specimen germinated in this study. Infection by *T. olida* and *T. sterilis* is systemic, but teliospores form in sori in host leaves rather than in the ovaries. *Ingoldiomyces hyalosporus, T. polypogonis, T. indica* and *T. walkeri* infect hosts in subfamily Pooideae, but teliospores of these species germinate at room temperature. Of these species, only *T. polypogonis* has a germination pattern similar to that of *T. tritici*.

Tilletia asperifolia, host *Muhlenbergia asperifolia* (subfamily Chloridoideae, Lineage III) is the only species outside the pooid-infecting clade (Lineage I) that exhibits the same type of germination pattern, systemic infection and temperature requirement as *T. tritici*. *Tilletia aegopogonis* and *T. lycuroides,* which form a well supported group with *T. asperifolia,* differ in having teliospores that germinate at room temperature to form multinucleate, nonconjugating basidiospores. *Erratomyces patelii,* host *Vigna mungo* (Fabaceae), also germinates at room temperature and produces conjugating basidiospores (Piepenbring and Bauer 1997). The infection type was not reported for this species but is probably local based on the isolated leaf spots that are formed.

In most of the taxa studied with hosts outside subfamily Pooideae, primary basidiospores germinated directly through formation of hyphae or indirectly through formation of ballistospores and did not conjugate under axenic conditions. Multinucleate and uninucleate nonconjugating primary basidiospores (FIG. 2F) germinate in a similar manner. Nonconjugating primary basidiospores were shown to be multinucleate in nine species, with hosts in Pooideae (*I. hyalosporus, T. cerebrina, T. sterilis*), Chloridoideae (*T. aegopogonis, T. lycuroides, T. savilei*) and Panicoideae (*T. opaca, T. trachypogonis*) ranging across Lineages I, II and III.

All species studied in culture produced allantoid ballistospores (FIG. 2C) and filiform to fusiform blastospores (FIG. 2A, D, E), although the two spore types were not produced in equal abundance in all isolates studied. Isolates of some taxa grew in a mycelial manner with relatively few secondary basidiospores. Ballistospores formed from sterigma-like structures on primary basidiospores, other ballistospores, or hyphae (FIG. 2C). Blastospores were aseptate, filiform, curved to coiled, and resembled primary basidiospores and were more abundant than ballistospores in cultures of most taxa in this study. Blastospores formed from other blastospores (FIG. 2E), and from hyphae, either singly on undifferentiated sporogenous cells (FIG. 2D), or from sporogenous cells with multiple denticles (FIG. 2B). Blastospores were not reported in *Erratomyces* (Piepenbring and Bauer 1997). In addition to the two types of secondary basidiospores just described, *C. verruculosus* also produced abundant Y-shaped blastospores in culture (FIG. 2G), similar in shape to the conidia formed in sori of *C. ayresii*. The Y-shaped spores germinated readily. Y-shaped conidia were not present in the sori of *C. verruculosus,* and this type of spore was not observed in cultures of *C. ayresii* or other species included in this study. All species included in this study, except *E. patelii,* had sterile cells intermingled with teliospores in the sorus.

DISCUSSION

A strict generic concept of *Tilletia* as characterized by the reticulate teliospore ornamentation and pattern of germination and infection exhibited by the type

TABLE III. Morphological characters for each taxon listed in alphabetical order

Taxon	Host subfamily	Teliospore ornamentation	Germination pattern	# primary basidiospores	Germination temperature	Infection type	Reference for germination pattern
C. ayresii	Panicoideae	verrucose	conjugating (rare)	<20	20–25	local	this study
C. verruculosus	Panicoideae	echinulate	nonconjugating, multinucleate	<20	20–25	local	this study
E. patelii	Fabaceae	verrucose	conjugating	>30	20–25	local	Piepenbring & Bauer 1997
I. hyalosporus	Pooideae	ridged	nonconjugating, multinucleate	2	20–25	systemic	Vánky & Bauer 1996
N. iowensis	Arundinoideae	foveate	nonconjugating, uninucleate	10–15	20–25	local	this study
T. aegopogonis	Chloridoideae	reticulate	nonconjugating, multinucleate	5–6	20–25	systemic	Durán 1987
T. anthoxanthi	Pooideae	reticulate	conjugating	<20	5	systemic	this study
T. asperifolia	Chloridoideae	reticulate	conjugating	10–12	9	systemic	this study
T. barclayana	Panicoideae	tuberculate	nonconjugating, uninucleate	>60	20–25	local	Durán 1987, this study
T. boutelouae	Chloridoideae	tuberculate	nonconjugating, uninucleate	30–50	20–25	local	Durán 1987
T. bromi	Pooideae	reticulate	conjugating	10–16	5–15	systemic	Boyd & Carris 1998, this study
T. cerebrina	Pooideae	reticulate to cerebriform	nonconjugating, multinucleate	3–8	5	systemic	Siang 1954
T. chionachnes	Panicoideae	verrucose	nonconjugating	<20	20–25	local	this study
T. controversa	Pooideae	reticulate	conjugating	14–30	5	systemic	Goates & Hoffmann 1987
T. ehrhartae	Ehrhartoideae	tuberculate	nonconjugating	<20	15	systemic	this study
T. eremopoae	Pooideae	reticulate	conjugating	60–100	5–15	systemic	this study
T. fusca	Pooideae	reticulate	conjugating	10–16	5–15	systemic	this study
T. goloskokovii	Pooideae	reticulate	conjugating	<20	5–10	systemic	Boyd et al 1998
T. holci	Pooideae	reticulate	conjugating	<20	15	systemic	this study
T. horrida	Ehrhartoideae	tuberculate	nonconjugating, uninucleate	>60	20–25	local	this study
T. indica	Pooideae	tuberculate	nonconjugating, uninucleate	>60	20–25	local	Durán 1987
T. ixophori	Panicoideae	tuberculate	nonconjugating	11–17	20–25	local	this study
T. kimberleyensis	Panicoideae	verrucose	nonconjugating	<20	20–25	local	this study
T. laevis	Pooideae	smooth	conjugating	4–16	15	systemic	Goates & Hoffmann 1987
T. menieri	Pooideae	reticulate	conjugating	<10	15	systemic	this study; Meiners 1957
T. lycuroides	Chloridoideae	reticulate	nonconjugating, multinucleate	8–15	20–25	systemic	Durán 1979, 1983

TABLE III. Continued

Taxon	Host subfamily	Teliospore ornamentation	Germination pattern	# primary basidiospores	Germination temperature	Infection type	Reference for germination pattern
T. obscura-reticulata	Chloridoideae	reticulate	nonconjugating, multinucleate	50–60	not reported	local	Durán 1987
T. olida	Pooideae	reticulate	conjugating?	3–5	15	systemic	this study; Zogg 1967
T. opaca	Panicoideae	tuberculate	nonconjugating, multinucleate	30–50	20–25	local	Ingold 1997, Vánky 1993; this study
T. polypogonis	Pooideae	reticulate to cerebriform	conjugating	<10	20–25	systemic	this study
T. rugispora	Panicoideae	tuberculate	conjugating	>30	20–25	local	Durán 1987
T. savilei	Chloridoideae	tuberculate	nonconjugating, multinucleate	<20	20–25	local	this study
T. sterilis	Pooideae	reticulate	nonconjugating, multinucleate	2–4	5	systemic	this study
T. sumatii	Panicoideae	tuberculate	nonconjugating, uninucleate	20–50	20–25	local	Ingold 1997, this study
T. togwateei	Pooideae	reticulate	conjugating	3–10	5–10	systemic	Guillemette 1988, this study
T. trachypogonis	Panicoideae	verrucose	nonconjugating, multinucleate	>30	20–25	local	Durán 1987
T. tritici	Pooideae	reticulate	conjugating	4–16	15	systemic	this study
T. walkeri	Pooideae	tuberculate	nonconjugating, uninucleate	60–150	20–25	local	Castlebury & Carris 1999
T. vittata	Panicoideae	verrucose	nonconjugating	20–30	20–25	local	Durán 1987, this study
T. whitechloae	Panicoideae	verrucose	nonconjugating	>50	20–25	local	this study

species, *T. tritici,* is not supported based on the results of the analyses of nLSU data. The *T. tritici* pattern of teliospore germination, with a relatively small number of rapidly conjugating primary basidiospores and systemic host infection resulting in most or all of the host ovaries replaced by fungal sori, is restricted mostly to species in the pooid-infecting clade. However some members of this clade produce nonconjugating primary basidiospores, including *T. cerebrina* and *T. sterilis*. Several species that have been studied extensively, including *T. bromi, T. fusca* and *T. togwateei,* form mostly uninucleate, conjugating basidiospores, but a small percent of spores may be multinucleate. Boyd and Carris (1998) showed evidence that up to 12% of primary basidiospores produced by *T. fusca* are dikaryotic based on the formation of teliospores in cultures derived from single basidiospores. Multinucleate primary basidiospores may result either from migration of multiple nuclei from the basidium into developing basidiospores or from mitotic division in basidiospores as shown by Goates and Hoffmann (1987). The *T. tritici* germination pattern also is found in *T. asperifolia* (Lineage III), which has a chloridoid host and falls outside the pooid-infecting clade. *Erratomyces patelii,* which is strongly supported as a basal group to *Tilletia* and infects dicotyledonous hosts, exhibits this germination pattern as well. Similarly, the reticulate teliospore ornamentation exhibited by *T. tritici,* is restricted mostly to species in the pooid-infecting clade (Lineage I) but also occurs in *T. aegopogonis, T. asperifolia, T. obscura-reticulata* and *T. lycuroides* in Lineage III.

The pathogens responsible for Karnal bunt of wheat, *T. indica,* and kernel smut of rice, *T. horrida,* were placed in *Neovossia* by some authors (Singh and Pavgi 1972, Vánky 1994, Whitney 1989) and in *Tilletia* by others (Durán 1987, Levy et al 2001, Pimentel et al 1998). Both species produce sterile cells in the sorus and numerous nonconjugating primary basidiospores (Castlebury and Carris 1999, Durán 1987). Durán and Fischer (1961) dismissed the value of number of primary basidiospores to delimit genera and our analysis supports their conclusion. Absence of sterile cells and production of numerous basidiospores were two characters used to distinguish *Neovossia* from *Tilletia* (Vánky 2002). The type species *N. moliniae* was shown by Brefeld (1895) to form 30–50 nonconjugating primary basidiospores. However, examination of specimens of *N. moliniae,* on *Molinia* (WSP 34463) and *N. iowensis* on *Phragmites* (V 573) revealed the presence of sterile cells in the sorus.

Two species of *Neovossia* infecting *Phragmites communis* have been described: *N. iowensis* from the USA (Hodson 1900) and *N. danubialis* T. Săvulescu from Europe (Săvulescu 1955). *Neovossia danubialis* and *N. iowensis* were merged with *N. molinae* by Vánky (1990) based on their similar teliospore morphology and germination patterns. Săvulescu and Hulea (1955) showed that *N. danubialis* germinated to produce 10–15 nonconjugating primary basidiospores, similar to what was shown in this study for *N. iowensis*. Based on the morphological similarity and occurrence on the same host species, *N. danubialis* and *N. iowensis* are considered to be synonymous. Because of the differences in numbers of primary basidiospores and host genus between *N. moliniae* and *N. iowensis,* we are maintaining the two as distinct species. The results of this study suggest that there is no basis for recognizing *Neovossia* as a genus distinct from *Tilletia* and we consider *N. iowensis* to be a species of *Tilletia*. However we were not able to study viable collections of *N. moliniae* and therefore the status of *Neovossia* itself remains uncertain.

Our analyses place *I. hyalosporus,* with ridged teliospores and production of ballistosporic primary basidiospores, within the well supported clade of pooid-infecting species containing *T. tritici* and allied species, *T. indica* and *T. walkeri* (Lineage I). Two species of *Conidiosporomyces, C. ayresii* and *C. verruculosus,* were included in this analysis and were closely related to *T. vittata* (Lineage IV). *Conidiosporomyces* is distinguished from *Tilletia* based on the formation of a sac-like, apically open sorus and the presence of Y-shaped conidia (Vánky and Bauer 1992). The unusual Y-shaped conidia are formed in the sorus in *C. ayresii* and are formed in *C. verruculosus* in culture. Based on the results of the nLSU analyses the characters that have been used to segregate *Ingoldiomyces* or *Conidiosporomyces* from *Tilletia* cannot be considered generic level characters and at this point we consider both genera synonyms of *Tilletia*.

The phylogeny of Tilletiales appears to reflect that of the hosts, with a well supported group of closely related species evolving on hosts in the subfamily Pooideae and a poorly resolved group of more diverse species infecting hosts in Chloridoideae, Ehrhartoideae, Arundinoideae and Panicoideae. The relationships elucidated by the phylogenetic analyses in this study suggest a more rapid radiation of *Tilletia* species on pooid hosts than on hosts in other subfamilies. Phylogenetic studies in the grass family (Poaceae) show two well supported clades comprising six monophyletic subfamilies, the Bambusoideae plus Ehrhartoideae and Pooideae (BEP) clade, and the Panicoideae, Arundinoideae, Centothecoideae and Chloridoideae (PACC) clade (Kellogg 2001). The relationships among subfamilies in the PACC clade are not well resolved in existing phylogenies (Kellogg 2001). Host specificity for individual species of *Tille-*

tia remains problematic and species concepts vary from author to author. Genetically distinct lineages can be associated with specific hosts in nature (Boyd and Carris 1997, Boyd et al 1998). However some species of *Tilletia,* while apparently host specific in nature, have retained the ability to infect other hosts under artificial conditions (Royer and Rytter 1988). More variable gene regions will be required to investigate issues of host specificity and morphological species complexes in this group of fungi.

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