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Salmacisia, a new genus of Tilletiales: reclassification of *Tilletia buchloëana* causing induced hermaphroditism in buffalograss

Ambika Chandra[1]
David R. Huff[2]

Department of Crop and Soil Sciences, 116 Agricultural Sciences and Industries building, Pennsylvania State University, University Park, Pennsylvania 16802

Abstract: For 119 y the fungal parasite *Tilletia buchloëana* has been known to induce female sex organs (pistils) in male plants of buffalograss, making them hermaphrodite. Here we report the life cycle characteristics and phylogeny for *T. buchloëana* based on morphology and large subunit region of nuclear ribosomal RNA gene (nLSU-rDNA) to accurately place pistil smut within order Tilletiales. We found that *T. buchloëana* induces hermaphroditism in not only male sex forms of buffalograss by inducing the development of otherwise vestigial pistils but also in female sex forms by inducing hypertrophy of otherwise vestigial stamens (male sex organs). The fungus also induces the development of additional pistillate flowers in both infected male and female plants. Due to its pistil inducing effects we refer to *T. buchloëana* as pistil smut. Pistil smut exhibits a combination of morphological and life cycle characteristics that are unique among species of order Tilletiales. Phylogenetic analyses of nLSU-rDNA sequences using maximum parsimony, maximum likelihood and genetic distance-based methods show that pistil smut exhibits an elevated rate of nucleotide substitution and is as, or more, distant from *Tilletia* species than the basal group *Erratomyces patelli.* As such pistil smut occupies a phylogenetic position outside the current taxonomic circumscription of genus *Tilletia.* Therefore a new Latin binomial combination *Salmacisia buchloëana* is proposed as a sister taxon of *Tilletia* to accurately describe the phylogenetic position of pistil smut.

Key words: *Buchloë dacyloides*, life cycle, molecular phylogenetics, morphology, nLSU-rDNA, parasitic castration, *Salmacisia buchloëana*, smut fungi

INTRODUCTION

One of the most interesting examples of coevolutionary biology is the alteration of sex in a host by a parasite. In 1889 William A. Kellerman, an early American mycologist and founder of the Journal of Mycology (later renamed Mycologia), and his then student Walter T. Swingle, who subsequently became a legendary agricultural botanist (Seifriz 1953), discovered a smut fungus that induces the production of ovaries in flowers of otherwise male plants of buffalograss (*Bouteloua dactyloides* [Nutt.] Columbus; syn. *Buchloë dactyloides* [Nutt.] Engelm.) (Kellerman and Swingle 1889). They placed the fungus within genus *Tilletia* (phylum Basidiomycota, order Tilletiales) based on its teliospore characteristics, naming it *Tilletia buchloëana* after its host. The phenomenon of inducing opposite sex organs in individuals that otherwise would bear only a single sex is known as induced hermaphroditism and occurs in a wide variety of organisms either via biological agents (ex. parasites) (Fisher and Holton 1957) or exogenous chemicals (Hayes et al 2002). Parasitically induced hermaphroditism belongs to a group of diseases known as parasitic castration where host reproductive organs are sterilized as a consequence of the parasite's own reproduction (see reviews by Fisher and Holton 1957, Clay 1991, Boudoin 1975, Shukalyuk et al 2005). Thus *T. buchloëana*-induced ovary development in male buffalograss is an example of induced hermaphroditism and represents a form of parasitic castration.

The most widely known example of parasitically induced hermaphroditism in plants occurs in the host white campion (*Silene latifolia* Poir. ssp. *alba* [P. Mill.] Greuter & Burdet) infected by the anther smut fungus, *Microbotryum violaceum* (Pers) G. Deml & Oberw, (class Uredinomycetes) (Deml and Oberw 1982). Anther smut induces the development of male sex organs (stamens) in female plants of white campion and sporulates only within the anthers of infected flowers (Uchida et al 2003). Hence anther smut's ability to induce and sporulate within male sex organs is in sharp contrast to pistil smut's ability to induce and sporulate within female sex organs; both examples illustrate induced hermaphroditism and parasitic castration but for the opposite sexes. Due to its pistil-inducing effect we refer to *T. buchloëana* as pistil smut.

Despite the remarkable sex-altering ability of pistil

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[1] Current address: Texas A&M Agrilife Urban Solutions Center, 17360 Coit Road, Dallas, TX 75252. E-mail: a-chandra@tamu.edu

[2] Corresponding author. E-mail: drh15@psu.edu

smut surprisingly limited knowledge exists regarding its basic biology. Kellerman and Swingle (1889) observed that, unlike other smut fungi, ''the monstrosity of *T. buchloëana* consists solely in its ability to produce ovaries in male plants.'' However Kellerman and Swingle (1889) were unable to detect the effects of pistil smut infection on female plants of buffalograss and their attempts, as well as those of Norton (1896), to germinate teliospores in vitro failed. Duran (1987) observed both male and female sex forms of buffalograss infected with pistil smut and showed teliospore germination up to the stage of basidiospore conjugation, however he did not describe the effects of infection on either host sex form or the later stages of pistil smut life cycle.

To our knowledge pistil smut is the only species within order Tilletiales (Basidiomycota, Ustilaginomycetes, Exobasidiomycetidae) known to induce hermphroditism in its host, however its evolutionary relationship within Tilletiales is unknown. Species within Tilletiales are identified commonly by the color and surface ornamentation of teliospores which, for most species, are produced within host ovaries. Castlebury et al (2005) performed the most comprehensive study to date regarding Tilletiales phylogeny and showed that spore morphology, germination pattern, infection type, host subfamily and large subunit region of nuclear ribosomal RNA gene (nLSU-rDNA) were useful characteristics for determining the lineage within order Tilletiales. In an effort to make the sex altering effects of pistil smut more widely known for study and to establish its evolutionary relatedness among species within order Tilletiales we conducted a study on the life cycle and phylogeny of pistil smut. Here we document the life cycle of pistil smut, that pistil smut induces hermaphroditism not only in male sex forms of buffalograss but also in female sex forms and that pistil smut has an evolutionary phylogeny unlike any other species of order Tilletiales.

MATERIALS AND METHODS

Isolation and maintenance of pistil smut.—Isolates of the pistil smut fungus in this study were obtained from a single source originally collected from an individual male buffalograss plant growing in a short grass prairie in Kingfisher County, Oklahoma, in 1986 (Huff et al 1987). Five to eight mm long explants including leaves, nodes and stem segments from infected plants were cultured on PDA (potato-dextrose agar) (Difco, Detroit, Michigan) media at room temperature (20–25 C) in the dark. Fungal growth was observed from the cut-ends of the explants after about a month of culture which eventually gave rise to mycelia and secondary sporidia. Fungal mycelia were maintained through subculturing every 3 mo under axenic conditions. Teliospore filled host ovaries (smut balls) were collected from infected plants and surface sterilized in 70% ethanol 1 min followed by 0.26% NaClO (5% v/v commercial bleach) 3 min before soaking in distilled water 2 d. Teliospore germination was attempted on 2% agar at 5 C, 25 C and 37 C under light and dark aseptic conditions.

Host infection by pistil smut.—Sixty genotypes of Mexican-A diploid (2n = 20) (Huff et al 1993) buffalograss were grown in plastic pots (15.25 cm diam) containing potting soil (Promix®, Premier Horticulture Inc., Quakertown, Pennsylvania) in the greenhouse (26 C day/21 C night) under natural day length conditions. At the 4–6 tiller stage each genotype was vegetatively propagated into four clonal replicate plants, two of which were left uninfected and two of which were inoculated by embedding teliospores into the soil surface close to the base of vegetative shoots. Inoculated plants were saturated with water and kept sealed in clear plastic bags to maintain high humidity for approximately 6 wk to let teliospores germinate and fungus to enter the plant.

Pistil smut morphology.—Five morphological and life cycle characteristics documented for pistil smut were recorded as qualitative parameters (viz. teliospore surface ornamentation, number of primary basidiospores produced, germination pattern, infection type, host subfamily). Characteristics from pistil smut along with the same qualitative characteristics obtained from Castlebury et al (2005) for 34 *Tilletia* species, four *Tilletia*-like species from three allied genera proposed to be synonyms of *Tilletia* (viz. *Ingoldiomyces*, *Neovossia* and *Conidiosporomyces*, Castlebury et al 2005) and one basal group to genus *Tilletia* (*Erratomyces patelii*) were mapped onto the most parsimonious phylogenetic tree generated for nLSU-rDNA sequences.

Nucleic acid extraction and PCR amplification.—Actively growing mycelia was scraped from PDA followed by vigorous maceration with liquid nitrogen. DNA was extracted with CTAB method (Doyle and Doyle 1990). Partial sequence of the 5′ end of nLSU-rDNA was amplified in 25 μL reactions with these reaction conditions: 10–15 ng of genomic DNA, 1× buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units Taq-polymerase, 10 μM each of primers LR0R and LR7 (Vilgalys and Hester 1990, Rehner and Samuels 1994). The thermal cycler program was initial denaturation 95 C for 10 min, followed by 35 cycles of 94 C for 30 s, 55 C for 30 s, 72 C for 1 min with a final extension 72 C for 10 min. The resulting amplicons containing a portion of nLSU-rDNA sequence was gel-purified with QIAquick Gel Extraction Kit (QIAGEN Science Inc., Germantown, Maryland) and was cloned in pCR®II-TOPO® (Invitrogen, Carlsbad, California). Cloned rDNA fragments were sequenced with the ABI Hitachi 3730XL DNA Analyzer (Applied Biosystems, Foster City, California). Approximately 1292 bp long sequences of pistil smut nLSU-rDNA were obtained from four clones and submitted to GenBank under accession Nos. DQ659921–DQ659924.

Taxa examined for phylogenetic analyses.—BLASTn (Basic Local Alignment Search Tool) program was used to extract species from the national public database, NCBI (http://

www.ncbi.nih.gov/), that were found to be related to pistil smut based on nLSU-rDNA sequence homology. The resulting dataset for conducting nLSU-rDNA phylogenetic analyses of order Tilletiales included the four clones derived from pistil smut along with 50 additional taxa comprising 34 *Tilletia* species, four *Tilletia*-like species from three allied genera proposed to be synonyms of *Tilletia* (viz. *Ingoldiomyces*, *Neovossia* and *Conidiosporomyces*; Castlebury et al 2005), one basal group to genus *Tilletia* (*Erratomyces patelii*) and two distantly related outgroup species, *Exobasidium rhododendri* (order Exobasidiales) and *Ustilago tritici* (order Ustilaginales).

Taxa sequence alignments.—Multiple sequence alignment was conducted with CLUSTAL W program of MEGA version 3.1 (Kumar et al 2004) with parameters that allowed optimization of gaps including gap opening (GO) penalty of 10, gap extension (GE) penalty of 4.44 (44% of GO penalty) and transition weight of 0.9 (Terry and Whiting 2005). Nucleotide sequences were aligned for 1456 positions for nLSU-rDNA analysis with the inclusion of outgroup taxa. Missing data or regions with ambiguous alignment were removed from the analyses (253 positions). Of the remaining 1203 characters 942 were conserved, 261 were variable with 122 singleton sites and 139 parsimony informative sites. Transition/transversion ratio was 2.4 with 25 transitional pairs and 11 transversional pairs. The sequence alignment without gaps was deposited in TreeBase (S1927, M3552).

Phylogenetic analyses.—Modeltest 3.7 (Posada and Crandall 1998) with the Akaike information criterion (AIC) was used to estimate the optimal model of evolution for conducting phylogenetic analyses of nLSU region and also for determining the parameters for likelihood assumptions. The general time reversible (GTR) model with a gamma shape parameter and a proportion of invariable sites (referred to as GTR+I+G) fitted the nLSU-rDNA dataset the best. The estimated base composition frequencies were A: 0.2729, C: 0.1957, G: 0.2930, T: 0.2385; the gamma shape parameter was 0.6218; the proportion of invariable sites was 0.6043; and the substitution rate matrix was A–C: 0.5163, A–G: 3.0595, A–T: 0.9471, C–G: 0.1098, C–T: 5.8501, G–T: 1.0000.

The estimated optimal model of evolution was used to construct maximum parsimony (MP), maximum likelihood (ML) and genetic distance-based phylogenetic trees with PAUP 4.01b (Swofford 1998). Mega 3.1 was used to view and label trees generated from PAUP 4.10b. MP trees were inferred with the heuristic search option with the random addition of trees and the branch swapping (tree bisection-reconnection, TBR) options of PAUP 4.10b. Ten thousand replications were run for MP analyses with automatically increasing MAXTREES (maximum numbers of trees stored) by 100. From all MP trees generated the most parsimonious tree (with minimum number of steps) was filtered along with branch lengths that are indicative of the number of base substitutions per sequence. To evaluate branch robustness in MP trees bootstrap (Felsentein 1985) analyses were conducted. Bootstrap support was estimated with 1000 bootstrap replicates, each replicate consisting of 10 heuristic searches and random addition sequences with branch swapping and MULTREES option off.

GTR based genetic distances for nLSU-rDNA sequence was generated with PAUP 4.10b and analyzed as follows using NTSYS version 2.2 (Rohlf 2005). The obtained genetic distance matrix was transformed to scalar product form with subprogram DCEN to compute eigenvalues and eigenvectors (Gower 1966). The first three eigenvectors were plotted as principal coordinate axes representing the three dimensions of principle coordinate analysis (PCoA). Cophenetic goodness-of-fit tests were performed following Rohlf (2005). In addition Mantel matrix correlation tests were performed with NTSYS version 2.2 between morphological and nLSU-rDNA distance matrices to determine their level of correspondence.

Diversity index analyses.—GZ-GAMA (Gu and Zhang 1997) program was used to estimate the expected number of nucleotide substitutions per site (K) of each nucleotide site for nLSU-rDNA sequences. This program uses the nucleotide sequences without any gaps and ambiguous sites along with the tree topology in PHYLIP format to estimate K by using maximum likelihood approach under the Jukes Cantor model. The profile of rate variability with sites was generated graphically by plotting K against the position of sites for nLSU-rDNA region and visually compared among taxa within the *Tilletia* clade using Microsoft Excel.

K-Estimator program, ver. 6, by Comeron (1999) was used to obtain simulation estimates of the diversity index (number of nucleotide substitutions per site, K) and its probability distribution. Confidence intervals of the divergence estimates were obtained by Monte Carlo simulations, and the multiple hits correction method was Tajima's 4-p. The diversity values analyzed were 1, the average diversity within *Tilletia* clade, and 2, the average diversity between pistil smut and the *Tilletia* clade. These average diversity values were calculated with the 27 genetically unique sequences within *Tilletia* clade whose diversity indices were > 0.0. For all simulations the number of substitutions applied in each replicate followed a random Poisson-distribution with a mean equal to the estimated number of substitutions (divergence value $\times$ number of analyzed sites). Substitutions were distributed randomly along the sequence, and confidence intervals were obtained directly from the null distribution of divergence estimates from each replicate. Simulation parameters for analyzing divergent values within *Tilletia* clade ($K = 0.0192$) or between pistil smut and the *Tilletia* clade ($K = 0.0544$) were the 52 sequences within order Tilletiales (TreeBase S1927, M3552 without outgroups), 1203 sequence length, 47.5% G+C content, 2.6:1 transition:transversion substitution ratio, and 50 000 replicates.

RESULTS

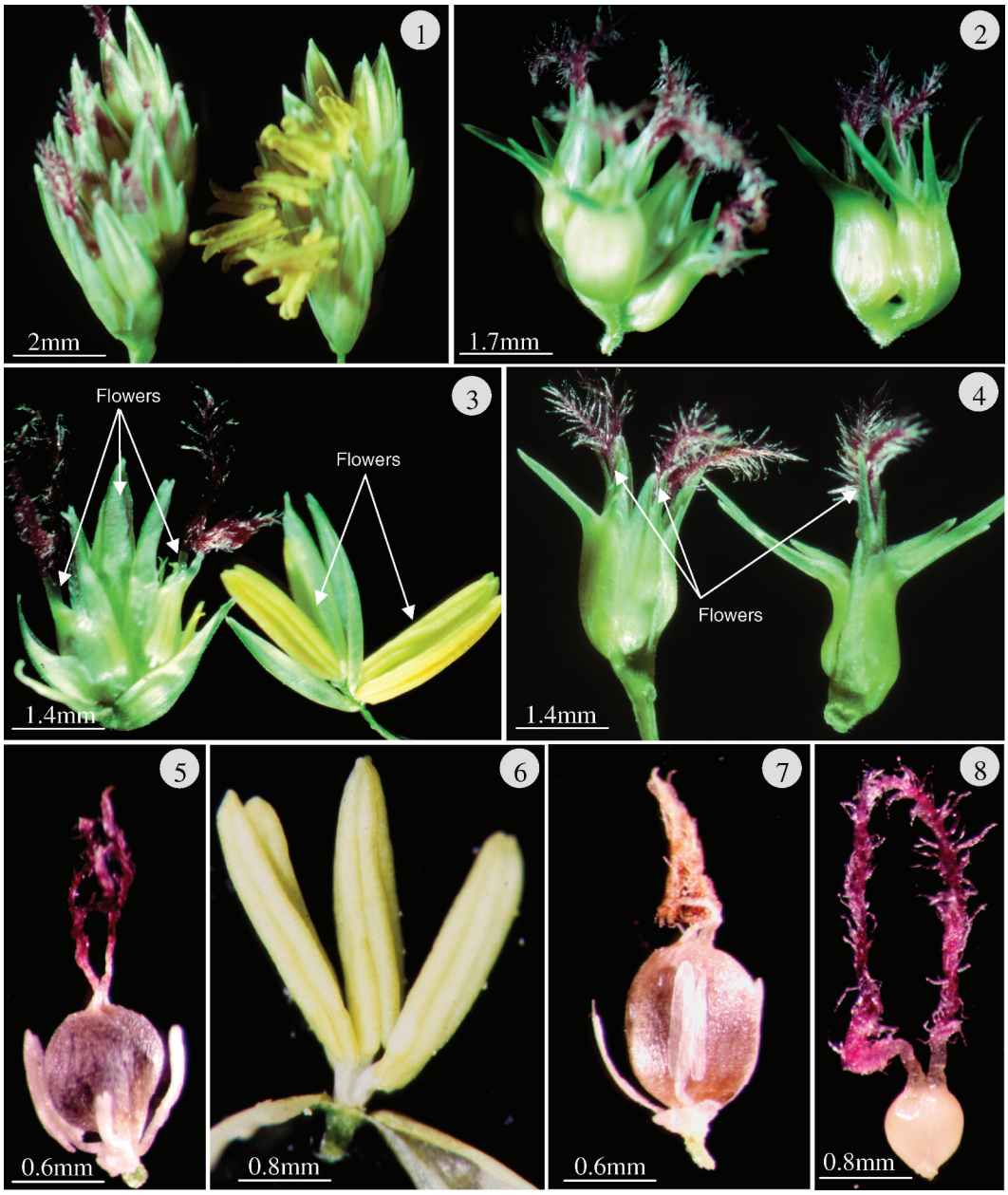
Induced hermaphroditism and parasitic castration of buffalograss by pistil smut.—Pistil smut infection of buffalograss plants under controlled conditions exhibited an 93% rate of infection with 112 plants (both replicates of 33 male and 23 female genotypes)

becoming infected out of the 120 plants inoculated with teliospores. The most conspicuous manifestation of pistil smut infection is the presence of purple feathery stigma in flowers of infected male plants, indicating the presence of ovaries, as compared to the bright yellow anthers normally produced in noninfected male plants (FIG. 1. infected left, noninfected right). Infection of female plants is visibly less noticeable because female flowers normally display purple feathery stigmas and their ovaries are permanently enclosed within thickened outer glumes forming a burr-like seed capsule which obscure the effects of infection on floral structures (FIG. 2. infected left, noninfected right). One noticeable symptom of infection in female plants is the increased number of florets per spikelet (two flowers per spikelet in infected versus one flower per spikelet in noninfected) (FIG. 4. infected left, noninfected right). Infected male plants also show an increased number of florets per spikelet (three flowers per spikelet in infected versus two flowers per spikelet in noninfected) (FIG. 3. infected left, noninfected right). These secondary effects of pistil smut infection on spikelet meristem determinacy produce crowding of both male and female spikes.

Flowers of infected male plants contain a fully developed pistil (stigma, style and ovary) and stamens that are small (FIG. 5) compared to the vestigial pistil and fully developed and functional (pollen producing) stamens of noninfected male plants (FIG. 6). Flowers of infected female plants retain a fully developed pistil but also possess enlarged stamens (FIG. 7) compared to the vestigial stamens of noninfected females (FIG. 8). Overall we detected that 95% of the observed flowers of infected male plants (827 out of 870) and all the observed flowers of female plants (167 out of 167) exhibited these symptoms of induced hermaphroditism. Comparisons between flowers of infected male and infected female plants suggest that little if any difference exists in their flower structure and composition (FIG. 5 vs. FIG. 7). Hence pistil smut infection renders the unisexual flowers of both male and female buffalograss morphologically hermaphrodite containing both sex organs within the same flower. We therefore conclude that pistil smut induces hermaphroditism in both male and female sex forms of buffalograss.

We found the sex organs within these induced hermaphroditic flowers to be reproductively sterile as a result of infection, or in other words parasitically castrated. Ovaries are sterile because they become supplanted with teliospores, forming smut balls (FIGS. 5, 7, 9) that are incapable of setting viable seed. However anthers within induced hermaphroditic flowers show no signs of teliospore production but are nonetheless sterile because they are underdeveloped in size (structure) and maturity (function). Thus, even though anthers of infected male plants are reduced in size while those of infected female plants are hypertrophied, both are similarly underdeveloped in appearance by being typically thin, white (FIGS. 5 and 7) and attached to filaments that do not exert out of the flower. Therefore stamens of infected plants normally are incapable of producing and liberating pollen. Hence we conclude that both male and female reproductive organs within induced hermaphroditic flowers are parasitically castrated but for different reasons.

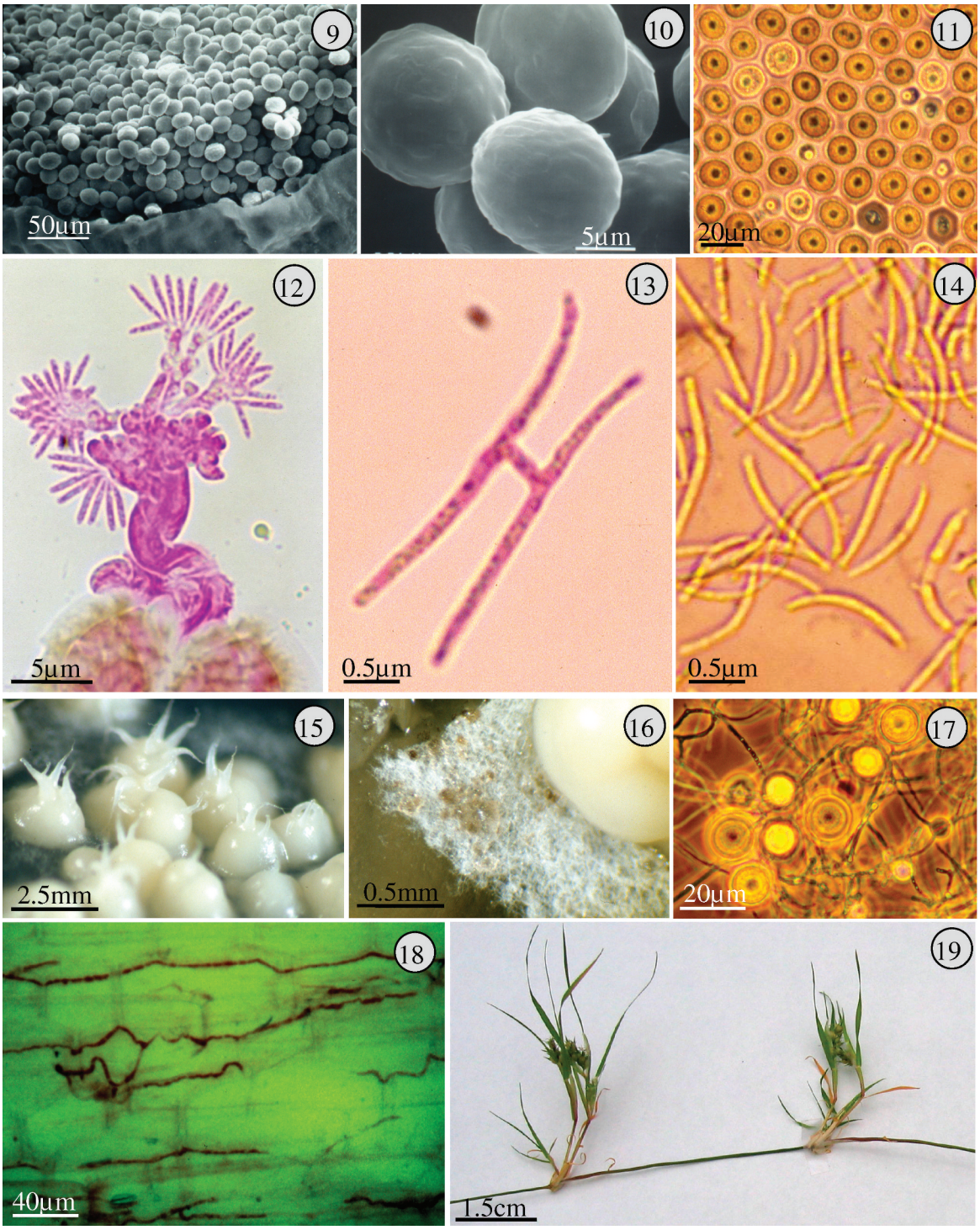
Life cycle of pistil smut fungus in vitro.—The ability to grow pistil smut under aseptic conditions and to infect young plants of buffalograss under controlled conditions let us perform Koch's postulates verifying pistil smut as the causal agent of induced hermaphroditism in buffalograss. Mature ovaries (induced or not) of flowers of infected plants (male or female) become filled with fungal teliospores (FIG. 9). Teliospore masses emit a powerful decaying fish odor and thus pistil smut belongs to a group known as the stinking smuts, which produce the odorous compound trimethylamine (Hanna et al 1932). Teliospores of pistil smut exhibit obscure tuberculate ornamentation (FIG. 10), generally range from pale yellow to light-chocolate brown, are mostly spherical and enclosed within a hyaline sheath (FIG. 11). Teliospore germination was observed only under room temperature (25 C) with a tendency to exhibit a higher rate of germination under light versus dark conditions (1.5% ± 1.17 light vs. 0.7% ± 0.24 dark). Pistil smut completed its life cycle within approximately 3 mo following a sequence of developmental stages (FIGS. 12–17). Germinating teliospores produce a promycelium (basidium) bearing whorls of finger-like, mostly monokaryotic but rarely dikaryotic (Duran 1987), primary basidiospores (FIG. 12). Individual pistil smut teliospores typically produce > 30 primary basidiospores that mostly conjugate at the base but sometimes conjugate to form an ''H-bridge'' structure (FIG. 13). The conjugated basidiospores produce fungal hyphae and initiate secondary basidiospore production. The current source of pistil smut was observed to produce blastoform-type of dikaryotic secondary basidiospores that are usually fusiform (FIG. 14). Consistency of cultures at this stage is predominantly sporidial giving a yeast-like appearance including spike-like structures resembling ''suchfaden'' (Fisher and Holton 1957) (FIG. 15). As the culture ages its consistency becomes more mycelial (FIG. 16). Finally, as the mycelium proliferates, teliospores are produced from the forward



FIGS. 1–8. Effects of pistil smut infection on male and female sex forms of buffalograss. 1. Spike of infected male plant exerting purple feathery stigma indicating the presence of ovaries (left) and spike of noninfected male plant with functional, pollen producing anthers protruding out from within the flowers (right). 2. Spike of infected female plant (left) and spike of noninfected female plant (right), both containing purple feathery stigmas. 3. Spikelet of infected male plant with three florets (left) vs. spikelet of noninfected male plant with two florets (right). 4. Spikelet of infected female plant with two florets (left) vs. spikelet of noninfected female plant with one floret (right). 5. Floret of infected male plant with fully developed pistil (stigma, style and ovary) and three underdeveloped stamens (anther and filament). 6. Floret of noninfected male plant with three developed stamens. 7. Floret of infected female plant with fully developed pistil and three underdeveloped stamens. 8. Floret of noninfected female plant with fully developed pistil.

portion of the mycelium (FIG. 17), completing the life cycle of pistil smut in vitro.

Life cycle of pistil smut in vivo.—Buffalograss plants infected with soilborne inoculum represent an infectious horizontal mode of disease transmission. Once inside the plant the fungal mycelium grows intercellularly producing a systemic infection (FIG. 18) within the basal meristems (crowns) of vegetative shoots. Any new plant growth arising from these infected basal meristems also contains fungal mycelium through a vertical mode of disease trans-



FIGS. 9–19. Stages of pistil smut life-cycle in vitro and in vivo. 9. Cross-section of a teliospore filled ovary (smut ball). 10. Teliospores of pistil smut exhibiting obscure tuberculate surface ornamentation. 11. Teliospore ranges from pale yellow to light chocolate brown. 12. Germinating teliospore with promycelium bearing >30 primary basidiospores. 13. Conjugating primary basidiospores forming H-shaped bridge structure. 14. Blastospore-type secondary sporidia, fusiform. 15. Young culture comprising primarily secondary sporidia diplaying structures resembling ''suchfaden''. 16. Older cultures displaying mycelial growth. 17. Teliospores produced from tips of mycelium growing in culture. 18. Intercellular growth of pistil smut hyphae within buffalograss vegetative meristem. 19. Vertical mode of disease transmission from parent to daughter tiller.

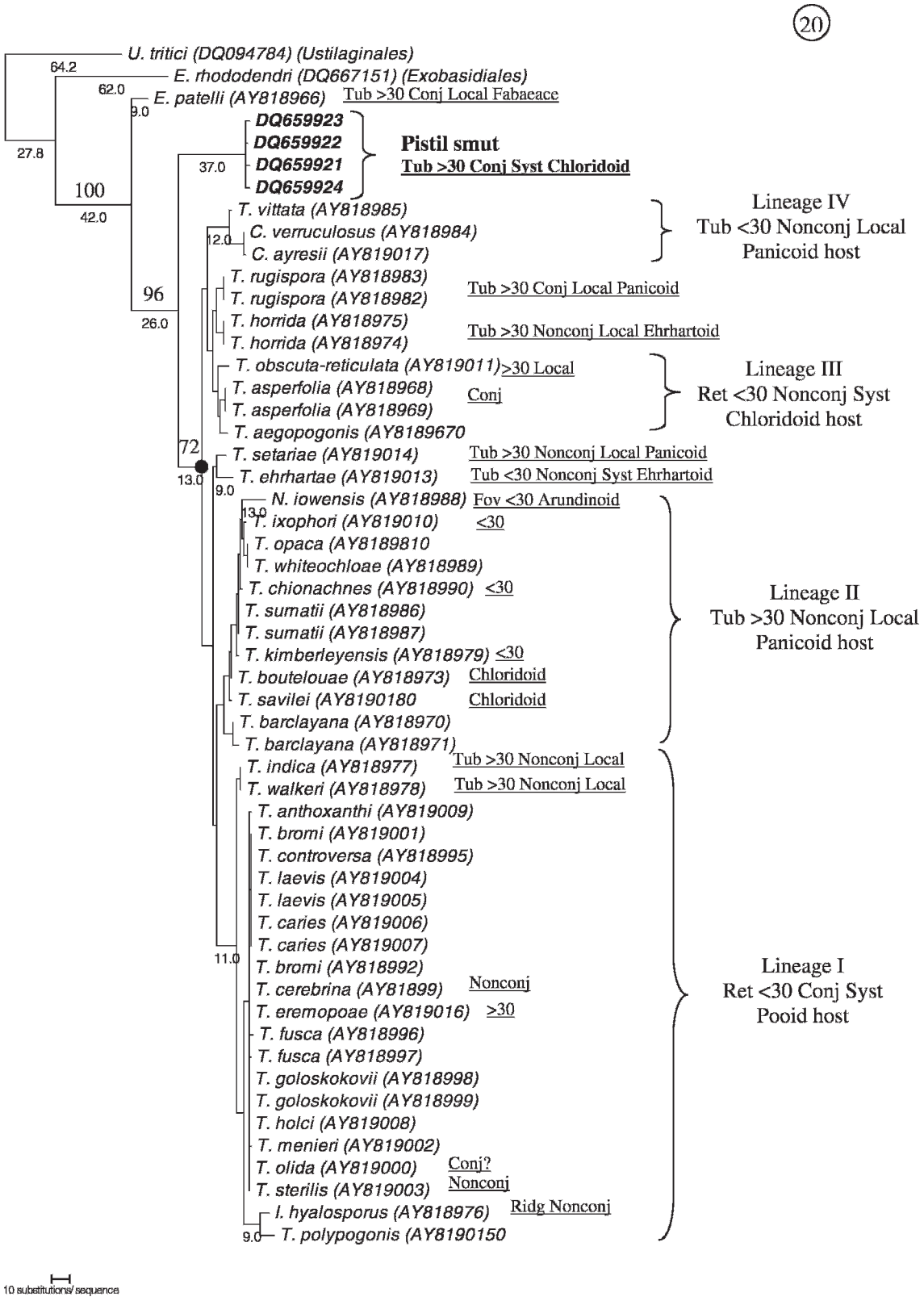
mission by mycelial propagation from parent to daughter shoots (FIG. 19). When infected crowns begin to initiate a flowering apex the fungus infects the developing inflorescence and induces hermaphroditism in the flowers of both male and female buffalograss plants. Pistil smut causes parasitic castration because it limits host sexual reproduction; however pistil smut does not kill the host plant under greenhouse conditions but rather coexists with its host by parasitizing the perennial production of vegetative shoot meristems. The longevity of pistil smut's coexistence with its host is unknown but we observed that several infected plants began to lose the secondary symptoms of infection after 3 y under greenhouse conditions.

Morphology of pistil smut.—Pistil smut's morphological and life cycle characteristics along with 40 other species within order Tilletiales, which have been described by Castlebury et al (2005) were mapped onto the nLSU-rDNA based MP tree (FIG. 20). The five qualitative characteristics of pistil smut comprise teliospores that exhibit tuberculate surface ornamentation, which on germination give rise to >30 primary basidiospores that conjugate to produce systemic infection in its Chloridoideae host (FIG. 20). This combination of qualitative characteristics is unique for pistil smut because no other species of order Tilletiales used in our analysis exhibits this same set of morphological characteristics. Pistil smut shares a maximum of three of the five characters with *T. boutelouae*, *T. savilei*, *T. eremopoae*, *T. asperfolia*, *T. rugispora* and the basal group *E. patelii*. Pistil smut shares tuberculate ornamentation, spore number and germination pattern with *E. patelii* and *T. rugispora*, but each of these species produce local infection and occur on a host subfamily other than Chloridoideae. Pistil smut shares tuberculate ornamentation and spore number with *T. boutelouae* and *T. savilei*, which also infect Chloridoideae, but these species produce nonconjugating basidiospores and local infection. Last, pistil smut shares three of the five qualitative characters with two species possessing reticulate spore ornamentation (viz. *T. asperfolia* and *T. eremopoae*). At the opposite extreme pistil smut was found to share none of these five characteristics with *N. iowensis*. Pistil smut shared a low level of matching (one to two characteristics) with the remaining species used in our analysis.

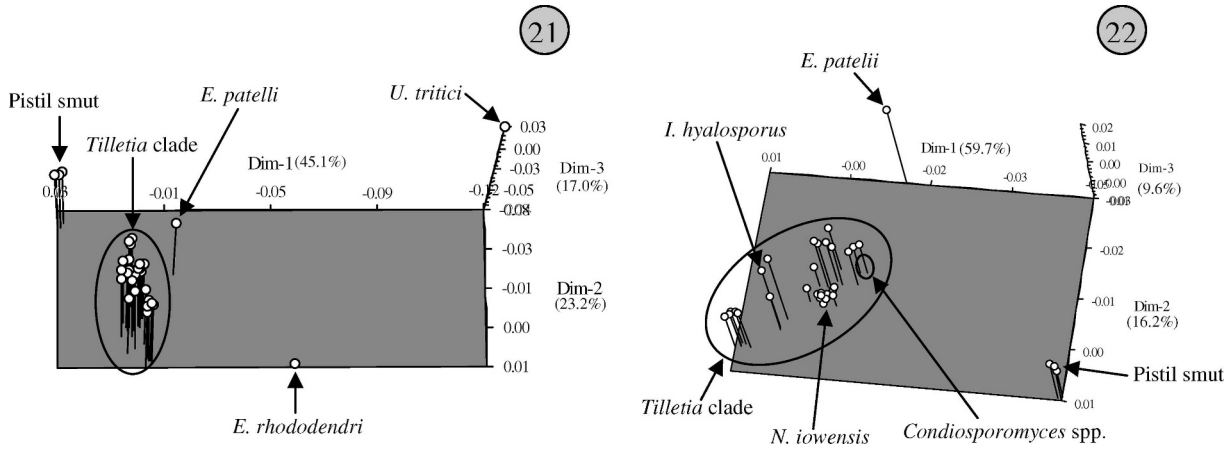
Phylogenetic analysis of pistil smut.—The four nLSU-rDNA sequences obtained from pistil smut were analyzed along with the same 39 species within order Tilletiales, as used in morphological analysis. In addition outgroup species *E. rhododendri* and *U. tritici* were included to provide phylogenetic polarity. In the alignment of these sequences (TreeBase: SN3366–14956) pistil smut displayed a cluster of gaps (base positions 406, 410–419, 423–436, 451) not present in any other taxa. Due to the large size and nonrandom distribution of these gaps as well as the uneven length of taxa sequences all gaps were removed for subsequent analyses. Each subsequent nLSU-rDNA phylogenetic analysis (MP, ML and genetic distance) displayed the same lineage structure of *Tilletia* and *Tilletia*-like species as originally described by Castlebury et al (2005). This structure comprises four lineages, L-I through L-IV, and four *Tilletia* species not fitting within any lineage (unresolved) namely *T. ehrhartae*, *T. setariae*, *T. horrida* and *T. rugispora*. We will refer to this structure as the *Tilletia* clade (FIG. 20, see ●). We found that *E. patelii* acquired a basal position to the *Tilletia* clade supporting the conclusion of Castlebury et al (2005) that *E. patelii* is a basal group of the genus *Tilletia*.

MP analysis yielded 15 equally parsimonious trees of length 457 steps (CI = 0.6696, RI = 0.8409, RC = 0.5630). One of the most parsimonious trees is shown (FIG. 20). To our surprise pistil smut was found to occupy a well supported position (96% bootstrap support) outside the *Tilletia* clade (FIG. 20). A prominent feature of this representative MP tree is that pistil smut resides on a branch length (37 base substitutions sequence^{-1}) that is remarkably longer than any other species within the *Tilletia* clade. Moreover pistil smut was slightly further from the *Tilletia* clade (50 base substitutions sequence^{-1}) than *E. patelii* (48 base substitutions sequence^{-1}) (FIG. 20). The topological placement of pistil smut remained essentially the same, being external to the *Tilletia* clade, for MP and ML methods of phylogenetic analyses conducted with and without inclusion of outgroup taxa (data not shown) indicating little to no effects of long branch attraction (Bergsten 2005). Although morphology was not highly useful for discriminating the phylogenies of species within Tilletiales, a Mantel matrix correlation test between morphological characteristics and nLSU-rDNA substitution rate matrices demonstrated that the two datasets were correlated significantly ($r = 0.40$, $P < 0.001$).

To better visually represent of the taxonomic position of pistil smut with respect to other species within order Tilletiales, we plotted the GTR genetic distance matrix three dimensionally, using a principle coordinates analysis (PCoA; cophenetic correlation coefficient goodness-of-fit test, $r = 0.99$). PCoA of order Tilletiales along with outgroup taxa shows pistil smut to be genetically more distant from the *Tilletia* clade (mean = 0.055879, range = 0.048237–0.063641) than *E. patelii* (mean = 0.048175, range



FIGS. 20–22. Phylogenetic placement of pistil smut within order Tilletiales based on nLSU-rDNA sequences. Order Tilletiales is represented by 48 taxa including 38 *Tilletia* and *Tilletia*-like species along with the basal group *E. patelii*. Outgroup taxa are from order Exobasidiales (*E. rhododendri*) and order Ustilaginales (*U. tritici*). The *Tilletia* clade (●) comprises four lineages, L-I through L-IV and four species not fitting within any of the four lineages (viz. *T. ehrhartae*, *T.*



FIGS. 20-22. Continued.

= 0.039481–0.054768) (FIG. 21). Exclusion of outgroup taxa from the PCoA provides a magnified view of order Tilletiales but with reversed polarity (FIG. 22). This reversed polarity was manifested by pistil smut acquiring more negative values along dimension 1 and more positive values along dimension 2 in relation to the *Tilletia* clade (FIG. 21 vs. FIG. 22). Pistil smut was observed to be genetically isolated and least distant to taxa belonging to L-IV (mean = 0.051307, range = 0.049109–0.052745). However pistil smut is still twice the distance from L-IV than any two species within the *Tilletia* clade are from one another (FIG. 22). Thus the genetic distance-based method of analysis of nLSU-rDNA shows a similarly large separation between pistil smut and other species of *Tilletia* as does MP and ML methods of analyses. We therefore conclude that pistil smut and *E. patelii* represent extremes of the phylogenetic tree space within order Tilletiales for nLSU-rDNA and that the *Tilletia* clade occupies an intermediate position between these two extremes.

Diversity index analysis of pistil smut and Tilletia *clade.*—Gamma distribution analysis showed that nLSU-rDNA sequence of pistil smut is variable at same sites as other species within order Tilletiales (data not shown), suggesting that pistil smut nLSU-rDNA sequences are real and not rogue.

The 99.99% confidence interval for the average diversity index among the 27 genetically unique species within the *Tilletia* clade closely matched the observed range and had an upper limit of 0.0366, which was 2.3 standard deviations from the average (TABLE I). Lineages I–IV and the unresolved species of *Tilletia* clade were not significantly different from this probability distribution even when each was treated independently of the *Tilletia* clade (data not shown). These results add proof to the conclusion of Castlebury et al (2005) that the *Tilletia*-like genera of *Ingoldiomyces*, *Neovossia* and *Conidiosporomyces* are not significantly diverse from *Tilletia* species and thus, in terms of nLSU-rDNA sequences, should be considered as species of *Tilletia*.

Diversity indices among *Tilletia* clade and *E. patelii* (K = 0.0461), *Tilletia* clade and pistil smut (K = 0.0544) and *E. patelii* and pistil smut (K = 0.0626) each were significantly different from the diversity indices within the *Tilletia* clade. The confidence interval obtained for the average diversity between pistil smut and the *Tilletia* clade easily accommodated all the observed values at the 5% significance level (TABLE I). Moreover the phylogenetic tree space surrounding pistil smut is so large that there is no overlap between pistil smut at the 99% confidence interval and the *Tilletia* clade at the 99.99% confidence interval (TABLE I). Thus we conclude that the nLSU-rDNA sequence of pistil smut does not fit within any known genus of order Tilletiales.

DISCUSSION

In 1859 George Engelmann, the famous 19th century American physician-botanist and a founding member

←

setariae, *T. horrida* and *T. rugispora*). Numbers below tree branches indicate branch lengths (number of base substitutions per 1203 bp aligned sequences). Numbers above branches indicate bootstrap supports. 20. One of the 15 most parsimonious trees of length 457 steps generated with the inclusion of outgroup taxa based on GTR+I+G model of evolution. 21. GTR model of genetic distance with the inclusion of outgroup taxa represented three dimensionally with principle coordinate analysis (PCoA). 22. GTR model of genetic distance with the exclusion of outgroup taxa represented three dimensionally with PCoA.

TABLE I. Confidence intervals and associated significant levels for the average diversity indices for nLSU-rDNA sequences (i) among 27 genetically unique species within the Tilletia clade and (ii) between four pistil smut clones and the 27 genetically unique species within the *Tilletia* clade. Simulation estimates of the number of nucleotide substitutions per site, K, and its probability distribution were obtained with the K-estimate program, ver. 6, by Comeron (1999)

(i)	Confidence intervals for 0.0192	
Within Tilletia clade	sig.level	min. max.
N = 351 pairwise comparisons	5%	0.0117–0.0280
Average diversity index, K = 0.0192	1%	0.0092–0.0306
Observed range = 0.0008–0.0356	0.1%	0.0075–0.0340
Standard deviation = 0.0075	0.01%	0.0050–0.0366
(ii)	Confidence intervals for 0.0544	
Between pistil smut and *Tilletia* clade	sig.level	min. max.
N = 108 pairwise comparisons	5%	0.0411–0.0688
Average diversity index, K = 0.0544	1%	0.0375–0.0735
Observed range = 0.0461–0.0636	0.1%	0.0340–0.0789
Standard deviation = 0.0043	0.01%	0.0306–0.0831

of the US National Academy of Science, recognized a rare form of buffalograss containing both male and female inflorescences on the same plant (monoecious). His practiced judgment let him discover that the dimorphic male and female sex forms of dioecious buffalograss, previously considered a separate species, were actually members of a single species, which he placed in a monotypic genus, *Buchloë* (Engelmann 1859). Thirty years thereafter Kellerman and Swingle (1889) keenly observed that *T. buchloëana* (pistil smut) infection restores the development of ovaries in male plants of buffalograss. Sex expression of buffalograss has been shown to be environmentally stable and genetically controlled (Huff and Wu 1987, 1992), yet pistil smut is capable of altering the sex expression of both male and female sex forms of buffalograss in some unknown fundamental way. Perhaps pistil smut infection perturbs the host's sex determining mechanism thereby stimulating the development of a sex that the plant is genetically programmed not to exhibit. Pistil smut also causes parasitic castration of both male and female reproductive organs within induced hermaphroditic flowers and therefore might be altering host resource allocation as was proposed similarly for some other examples of parasitic castration (Clay 1991). However the underlying mechanism for either induced hermaphroditism or parasitic castration for any organism, including the well studied example of anther-smut infected white campion, has yet to be discovered.

Tilletia is the predominant genus within order Tilletiales and contains more than 140 species that infect only hosts belonging to the grass family Poaceae (Castlebury et al 2005). *Erratomyces* is the only genus within Tilletiales known to infect a nongrass host (Fabaceae, Piepenbring and Baur 1997) and is considered to be the evolutionary basal group of *Tilletia* genus (Castlebury et al 2005). Thus the 38 species of *Tilletia* and *Tilletia*-like species examined in the present study represents only approximately one-quarter of the known species of *Tilletia*. It would be interesting to know whether the phylogenetic isolation of pistil smut with respect to other *Tilletia* species could be resolved with better sampling of taxa and/or genes (Rokas and Carroll 2005) across order Tilletiales or whether it provides the true evolutionary picture. A phylogenetic analysis of Tilletiales by Castlebury et al (2005) examined a wide range of morphological diversity including taxa from three allied genera (viz. *Conidiosporomyces ayresii*, *C. verruculosus*, *Ingoldiomyces hyalosporus* and *Neovossia iowensis*). Each of these allied genera exhibit distinctive morphological characteristics as the basis for their generic classification (see Vanky and Bauer 1992, 1996). However, based on nLSU-rDNA sequence analysis, Castlebury et al (2005) concluded that these allied genera should be considered synonyms of genus *Tilletia*, despite their large morphological differences. The opposite situation has been observed for pistil smut in the present study. While pistil smut exhibits a unique suit of morphological characteristics, none are unique in isolation; yet pistil smut is significantly isolated from all other genera within Tilletiales based on diversity analysis of nLSU-rDNA sequences. Therefore the degree to which pistil smut lies outside of the *Tilletia* clade suggests that increased taxa sampling would less likely be able to accommodate pistil smut within genus *Tilletia*. Moreover the taxa sampling necessary to include pistil smut within genus *Tilletia* likely would engulf *E. patelii* as well.

Pistil smut is the only species within order Tilletiales that has been observed to induce hermaphroditism in its host. If the ability to induce hermaphroditism in a dioecious host is a highly specialized

coevolutionary process then induced hermaphroditism likely would be pivotal in pistil smut's evolutionary divergence from other genera within Tilletiales. Some grass hosts of *Tilletia* species are known to produce lower flowers that are unisexual male (staminate), for example *Conidiosporomyces ayresii* (host: *Megathyrsus maximums*) and *Tilletia barclayana* (host: *Hopia obtuse*) (Grass Manual on the Web, 2007) and as such it would be useful to know whether these smuts lack the ability to induce hermaphroditism or have been merely overlooked. Tilletia species are an important group of disease-causing agents due to their devastating impact on grain crops worldwide. However increasing our knowledge at molecular, biochemical and evolutionary levels regarding the secondary effects of pistil smut infection, including its ability to induce ovaries and additional flowers, ultimately would seem to enhance our ability to increase seed production of agronomically important perennial grasses.

The current study indicates that leaving pistil smut within genus *Tilletia* would not be consistent with the prior treatment of Tilletiales (ex. Castlebury et al 2005) and hence an alternate taxonomic treatment is needed to better reflect pistil smut's unique evolutionary history. We therefore propose a new name *Salmacisia buchloëana* for pistil smut that possesses all of the features and characteristics of *Tilletia buchloëana* but which resides within a new genus, *Salmacisia*. *Salmacisia* is based solely on nLSU-rDNA sequence diversity. Therefore *Salmacisia* will encompass those species with nLSU-rDNA sequences that are significantly diverse from all other genera within Tilletiales but which are not significantly diverse from the type species, *Salmacisia buchloëana*. To define these metes and bounds of *Salmacisia* more precisely we use the 99% confidence interval between pistil smut and the *Tilletia* clade for the lower limit between genera (i.e. 0.0375) and for the upper limit within *Salmacisia* (i.e. 0.0735–0.0375 = 0.0360) (see TABLE I). Therefore species within *Salmacisia* will possess nLSU-rDNA sequences that are greater than 0.0375 nucleotide substitutions per site from other genera within Tilletiales and less than 0.0360 nucleotide substitutions per site from the typic species, *Salmacisia buchloëana*. It will be interesting to see whether *Salmacisia* remains a monotypic genus.

TAXONOMY

Salmacisia D.R. Huff & A. Chandra gen. nov.

Sori tantum intus ovariis of aeger planta, agglutinated sporarum massa, teliospores ornatus intus hyalinae gelatinoid theca, basidiosporas unus nucleate iunctum vel duo nucleate, dikaryon producto secundus basidiosporas; formalis consimilis ex *Tilletia* Tulasne & Tulasne, dissimilis nLSU-rDNA > 0.0375 nucleotide substitutions quisque positus intus Tilletia et < 0.0360 nucleotide substitutions quisque positus ex *Salmacisia buchloëana*.

Sori only in ovaries of infected plants containing agglutinated spore masses. Ornamented teliospores arise from terminal cells of sporogenous mycelia, frequently encased in hyaline gelatinoid sheath, germinating by means of continuous promycelium bearing terminal primary basidiospores that are either mononucleate, which conjugate, or binucleate, giving rise to secondary basidiospores. Indistinguishable from *Tilletia* Tulasne & Tulasne for individual morphological characteristics but is clearly distinct from species of genera *Tilletia*, *Conidiosporomyces*, *Ingoldiomyces*, *Neovossia* and *Erratomyces* within Tilletiales by divergent nLSU- rDNA sequence exhibiting greater than 0.0375 nucleotide substitutions per site from any other genera within order Tilletiales and fewer than 0.0360 substitutions per site from the typic species, *Salmacisia buchloëana* (TABLE I)

Type species. *Salmacisia buchloëana* (Kellerman & Swingle) D.R. Huff & A. Chandra.

Etymology. The genus *Salmacisia* is derived from Salmacis (pronounced săl-MĀ-sĭs) and refers to the ability of the type species of this genus to induce hermaphroditism in its host. Salmacis was the determined water nymph responsible for transforming a remarkably handsome boy named Hermaphroditus into an intersexual individual possessing both male and female characteristics as a result of her divine union with him, according to Greek mythology.

Salmacisia buchloëana (Kellerman & Swingle) D.R. Huff & A. Chandra comb. nov.

≡ *Tilletia buchloëana* Kellerman & Swingle, J of Mycology 5:11, 1889. Syntype on male *Buchloë dactyloides*. USA. KANSAS: Trego County, 26 May 1888. BPI 172553. Isosyntype on male *Buchloë dactyloides*. USA. KANSAS: Ford County, 26 Jun 1888. BPI 172559.

= *Ustilago cathesteci* P.Henn. Hedw. 35:212. 1896.

≡ *Tilletia cathesteci* (P. Henn) G.P. Clint. J Mycol 8:149. 1902.

Sori tantum intus ovariis of aeger planta, atrum brunneolae ut atrum puniceus brunneolae, agglutinated sporarum massa, cariosus nidor, teliosporas subglobosae, globosae, ovate vel elongate, pallens ut chocolate brunneolae, 13–26 μm diam intus gelatinous hyalinae theca 1.5–3.5 μm creber, ornatus rotundus mons 1.5–2.0 μm altus, infecundus cells 9–31 μm diam, subglobosae vel ovoid encased in hyalinae theca, teliosporas producto simplex vel furca multinucleatae promycelia producto primary duo nucleate vel unus nucleate basidiosporas,

unus nucleate primary basidiosporas iunctum, dikaryotic basidiosporas producto blastoform-typus dikaryotic secundus basidiosporas quod es usitas fusiform in forma, producto intercellularibus contagio in aeger planta. Adduco hermaphroditism of *Buchloë dactyloides* flowers, visual frequentatio of spicas. Proprius nucleotide varietas quisque positus: nLSU-rDNA: 33 proprius nucleotide varietas quisque 1340 positus.

Sori in ovaries of staminate and/or pistillate plants, ability to induce hermaphroditism of staminate and/or pistillate flowers of infected plants by inducing the development of otherwise vestigial pistils in staminate plants (FIG. 5) and by inducing the hypertrophy of otherwise vestigial stamens in pistillate plants (FIG. 7); visual crowding of inflorescences (FIGS. 3, 4 left); sori covered by the delicate pericarp and concealed by floral bracts, pericarp easily punctured exposing the dark brown to dark reddish brown agglutinated spore masses that emit fetid odor. *Spores* subglobose, globose, ovate or elongate, pale yellow to light chocolate brown (FIG. 11), 13–26 µm diam usually embedded in gelatinous hyaline sheath 1.5–3.5 µm thick (FIG. 11) and exhibit obscure 1.5–2.0 µm tuberculate ornamentation (FIG. 10). Single teliospore formed from terminal cells of sporogenous mycelia. Teliospores are intermingled with sterile cells, 9–31 µm diam, that are subglobose or ovoid, encased in hyaline sheath, sometimes with a hyphal fragment attached, often multilaminated walls and a comparatively small central lumen. *Germination* of teliospores produces simple or branched multinucleate promycelia (FIG. 12), giving rise to >30 primary binucleate or mononucleate basidiospores; mononucleate primary basidiospores conjugate (FIG. 13). Dikaryotic basiospores give rise to blastoform-type of dikaryotic secondary basidiospores which are usually fusiform in shape (FIG. 14) and produces systemic infection in its host (FIG. 18).

Characteristic DNA sequences. BLASTn results of the nLSU-rDNA sequence of *Salmacisia buchloëana* (GENBANK DQ659921–DQ659924) had E-values of 0.0 (90–94% identical) and 1–3% gaps with the nLSU-rDNA sequences from species of genera *Tilletia*, *Conidiosporomyces*, *Ingoldiomyces*, *Neovossia* and *Erratomyces*. In the alignment of nLSU-rDNA sequences (TreeBase: S1927, M3551), *Salmacisia buchloëana* displays a cluster of gaps (base positions 406, 410–419, 423–436, 451) not present in species of genera *Tilletia*, *Conidiosporomyces*, *Ingoldiomyces*, *Neovossia* and *Erratomyces*.

Characteristic fixed DNA polymorphisms. Character-Characteristic fixed DNA polymorphisms used in the description of *Salmacisia buchloëana* were determined based on the sequence alignments used in the phylogenetic and diversity analyses. Only those characters present in all *Salmacisia buchloëana* clones and absent in species sequences of *Tilletia*, *Conidiosporomyces*, *Ingoldiomyces*, *Neovossia* and *Erratomyces* are included. These fixed DNA polymorphisms are indicated with capital letters (nucleotides) with the alignment position given. Nucleotides separated by forward slashes indicate alternate base composition at that site. Gap openings and gap extensions resulting from sequence alignment are in brackets. LSU (aligned sequences with gaps, 1340 bp): aatTtcgagaagCatt @ 104, 113; gctTatg @ 130; catAaTGtcc @ 188, 190–191; tt/ctAAgatAtgc @ 203–204, 208; ctaCgag @ 218; cgtAagg @ 312; tgaAgttaTGAaCg/aca @ 384, 389–391, 393; cagCatt[–1–]agT[–27–]tgtatt[–2–]gcGggc @ 402, 409, 447; ttgGctgcCgga @ 465, 470; gtaCTagg @ 482–483; gtaAttgatacGgtggCtgg @ 521, 529, 534; ctgTcCAaatgAcTtta @ 602, 604–605, 610, 612; ttgAgtg @ 671; taaGtga @ 1071.

On Poaceae: *Bouteloua dactyloides* (Nutt.) Columbus; syn. *Buchloë dactyloides* (Nutt.) Engelm.

Specimen examined. USA. OKLAHOMA: Kingfisher County. 6 JUL 1986, *D.R. Huff* (WSP 71313. HERBARIUM). Type on male and female sex forms of *Buchloë dactyloides*.

Known distribution. N. America (Kansas, Oklahoma, Texas, Nebraska, Mexico).

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