Soral synapomorphies are significant for the systematics of the *Ustilago-Sporisorium-Macalpinomyces* complex (Ustilaginaceae)

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Abstract: The genera *Ustilago*, *Sporisorium* and *Macalpinomyces* are a polyphyletic complex of plant pathogenic fungi. The four main morphological characters used to define these genera have been considered homoplasious and not useful for resolving the complex. This study re-evaluates character homology and discusses the use of these characters for defining monophyletic groups recovered from a reconstructed phylogeny using four nuclear loci. Generic delimitation of smut fungi based on their hosts is also discussed as a means for identifying genera within this group. Morphological characters and host specificity can be used to circumscribe genera within the *Ustilago-Sporisorium-Macalpinomyces* complex.

Key Words: columella, maximum likelihood, morphology, peridium, smut fungi, spore balls, sterile cells, systematics, *Ustilaginales*

INTRODUCTION

Three genera of smut fungi (subphylum *Ustilaginomycotina*), *Ustilago*, *Sporisorium* and *Macalpinomyces*, contain about 530 described species that all infect grasses (Vánky 2012). Several phylogenetic studies have demonstrated that *Ustilago* and *Sporisorium* together form a monophyletic group within the *Ustilaginomycotina* (Swann & Taylor 1995, Bauer et al. 1997, Begerow et al. 1997, Stoll et al. 2003, Begerow et al. 2004b, Stoll et al. 2005, Begerow et al. 2006). *Macalpinomyces* has an ambiguous position in the *Ustilaginales* as the type species, *M. eriachnes*, sits outside the *Ustilago-Sporisorium* group (Begerow et al. 2006). Morphological characters have proven inadequate for separation of species among the three genera. The three genera are polyphyletic (Stoll et al. 2003, Stoll et al. 2005), and collectively form an unresolved complex. Morphological studies (Langdon & Fullerton 1975, Vánky 1991, Piepenbring et al. 1998) and molecular phylogenetic analyses (Stoll et al. 2003, Stoll et al. 2005) have not identified characters that define monophyletic groups amongst species within this complex.

Smut fungi in the *Ustilago-Sporisorium-Macalpinomyces* complex either partially or completely destroy the inflorescence of grasses, forming a sorus that contains fungal spores. Four characteristics of the sorus, namely columellae, sterile cells, spore balls and peridia, have been used traditionally to separate *Ustilago*, *Sporisorium* and *Macalpinomyces* (Vánky 2002). Within the sorus, columellae form a central axis of fungal and host origin (Vánky 2002); sterile cells, either derived from non-sporogenous hyphae or a fungal peridium, are found with the spores (Langdon & Fullerton 1975, 1978); spore balls appear as either an ephemeral or permanent agglomeration of spores (Vánky 2002). A peridium is the outer layer of the sorus and can be composed of host or fungal material (Vánky 2002). Soral characters have had different interpretations by mycologists (Stoll et al. 2005). For example, the columella

in *Ustilago porosa* was considered absent by Langdon (1962) but present by Vánky & Shivas (2001). Similarly, *Sporisorium consanguineum* was considered to have a columella by Langdon & Fullerton (1975), but not by Vánky & Shivas (2008). Subsequently, soral morphology has been considered too variable to serve as a reliable character that can separate *Ustilago*, *Sporisorium* and *Macalpinomyces* (Piepenbring 2004, Stoll et al. 2005).

The current study discusses morphological characters in the *Ustilago-Sporisorium-Macalpinomyces* complex. A re-evaluation of their homology is provided in light of the phylogenetic results obtained. The merits of using host specificity and soral synapomorphies are discussed as a basis for delimiting genera.

MATERIALS AND METHODS

Taxon selection

Taxa were selected to represent the main groups recovered in previous studies (Stoll et al. 2003, Stoll et al. 2005), with increased sampling of under-represented groups, for example species of *Macalpinomyces* and smut fungi occurring on *Aristida*. In total, this study included 136 species (14 species of *Macalpinomyces*, 81 species of *Sporisorium* and 38 species of *Ustilago*), 35 of which had not previously been evaluated in systematic studies (Table 1). Two distinctive members of the complex, *Anomalomyces panici* and *Melanopsichium pennsylvanicum*, were also included. *Moesziomyces bullatus* was included as an outgroup to the complex based on a relationship reported by Stoll et al. (2005).

Morphological data

Character and character state selection were based on taxonomic descriptions in monographs of the *Ustilaginomycotina* (Vánky 1994, Vánky & Shivas 2008, Vánky 2012) and from direct observation of 61 Australian species. Columellae were scored as either absent, stout or filiform. Spore states were classified as single spores, permanent spore balls, ephemeral spore balls or dimorphic spores. Strerile cells were scored as present or absent. The peridium was classified as either host derived, hypertrophied-host derived or fungal derived. These characters were mapped onto the final tree topology using MacClade ver 4.08 (Maddison & Maddison 2001).

DNA Extraction

DNA was extracted from 120 smut specimens representing 92 taxa, by a combination of enzymatic and mechanical lysis. Smut sori or spores were mechanically lysed using a QIAGEN TissueLyser with 0.5 mm stainless steel beads, then shaken at 55ºC overnight in SNES buffer (0.01 M sodium phosphate pH 7.6, 0.15 M sodium chloride, 0.005 M EDTA, 1% SDS) containing proteinase K at a final concentration of 0.8 µg/ml. The purification was then completed using the QIAGEN Gentra Puregene kit according to the manufacturer's instructions.

PCR and sequencing

Genomic DNA was amplified by PCR with high fidelity Phusion® DNA Polymerase (Finnzymes) using the manufacturer-specified cycling and reaction conditions. The ITS region was amplified with primers M-ITS1 (Stoll et al. 2003) and ITS4 (White et al. 1990) at 58ºC; the LSU region was amplified with primers LROR and LR5 (Vilgalys & Hester 1990) at 58ºC; the GAPDH locus was amplified with GAPDH-F (CGGTCGTATCGGMCGTATC) and GAPDH-R (GTARCCCCACTCGTTGTCGTA) at 65ºC; the EF1α locus was amplified with primers EF1αF (GCCCTMTGGAAGTTCGAGACYCCCA) and EF1αR (GAYACCGACAGCRACGGTCTG) at 62ºC. PCR products were purified by ethanol precipitation using standard methods (Maniatis et al. 1982). Purified PCR product was sent to Macrogen Korea or the Australian Genome Research Facility, Queensland for sequencing using the forward and reverse primers from amplification. ABI sequence trace files were assembled using ContigExpress® (Invitrogen™). The 165 novel sequences have been deposited in GenBank (Table 1).

Alignment of sequences

Sequences were aligned using the Muscle algorithm (Edgar 2004) included in the MEGA5 software package (Kumar et al. 2008). Alignments of protein-coding loci (GAPDH and EF1α) were converted to amino acid sequences in MEGA. The original and curated nucleotide alignments have been deposited as Nexus files in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11013). The super-matrix consisted of ITS sequences for 134 taxa, LSU sequences for 91 taxa, EF1α sequences for 32 taxa and GAPDH sequences for 35 taxa.

Curation of alignments

Alignments were uploaded to Phylogeny.fr (available at http://www.phylogeny.fr/) (Dereeper et al. 2008) and curated in Gblocks to remove poorly aligned positions and divergent regions (Talavera & Castresana 2007). Alignments were trimmed as follows: ITS from 1140 nucleotides, including gaps, to 448 nucleotides with no gaps; LSU from 609 to 593 nucleotides; EF1α from 935 to 926 nucleotides; GAPDH from 1158 to 769 nucleotides. The final curated super-matrix consisted of 2736 nucleotides, which was composed of approximately 47% missing data.

Phylogenetic analyses

Two phylogenetic assessment criteria were implemented: Bayesian inference using MrBayes (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) and maximum likelihood using RAxML (Stamatakis 2006) and PhyML 3.0 (Guindon et al. 2010). Resulting trees were observed with FigTree (available at http://www.tree.bio.ed.ac.uk/software/figtree/). Data and command files for both Bayesian and RAxML analyses and the resulting trees are available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11013). The four loci were included as separate partitions in the maximum likelihood and Bayesian analyses so that each locus could be run under different optimal model parameters.

Maximum likelihood analysis

Maximum likelihood was implemented as a search criterion in RAxML (Stamatakis 2006) and PhyML 3.0 (Guindon et al. 2010). GTRGAMMA was specified as the model of evolution in both programs. The RAxML analyses were run with a rapid

Bootstrap analysis (command -f a) using a random starting tree and 1000 maximum likelihood bootstrap replicates. The PhyML analyses were implemented using the ATGC bioinformatics platform (available at: http://www.atgc-montpellier.fr/phyml/), with SPR and NNI tree improvement, and support obtained from an approximate likelihood ratio test (Anisimova et al. 2011).

Bayesian analysis
MrBayes was used to conduct a Markov Chain Monte Carlo (MCMC) search in a Bayesian analysis. Four runs, each consisting of four chains, were implemented until the standard deviation of split frequencies were 0.02. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 50 generations and trees were saved every 5000 generations. Convergence of the Bayesian analysis was confirmed using AWTY (Nylander et al. 2008) (available at: ceb.csit.fsu.edu/awty/). Convergence was not reached even after 40 million generations with all datasets. A user-defined tree obtained from the maximum likelihood analyses was used as a starting point for all of the Bayesian analyses, which helped to improve convergence of the four runs. A burn-in was not used to summarize the values that were created with a user-defined tree.

RESULTS AND DISCUSSION

Eight clades were consistently recovered in a phylogenetic analysis of four molecular loci (Fig. 1). The major clades recovered in this study were similar to those obtained in previous molecular phylogenetic analyses using different assessment criteria. For example, several phylogenetic studies have reconstructed two monophyletic groups in *Sporisorium* (Stoll et al. 2003, Cunnington et al. 2005, Stoll et al. 2005, Vánky et al. 2006, Vánky & Lutz 2011), but these studies were not able to separate the two groups using morphological characters. The structure of columellae (Fig. 2), the presence or absence of sterile cells (Fig. 3) and the presence or absence of spore balls (Fig. 4) were traced onto the topology. A discussion of the homology of these characters and their use in identifying the clades of the *Ustilago-Sporisorium-Macalpinomyces* complex follows.

Characters associated with monophyletic groups

Clade 1
Clade 1 includes *S. sorghi*, the type of *Sporisorium*. The members of this clade share a number of characters.

1. A hardened or stout columella that either replaces the entire inflorescence, for example in *Sporisorium scitamineum*, *S. andropogonis* and *S. doidgeae* (Fig. 5b), or that occurs in all of the ovaries or spikelets of an inflorescence, for example in *S. sorghi*, *S. ryleyi* (Fig. 5d) and *S. rarum* (Fig. 5e).
2. Sterile cells formed from non-sporogenous hyphae that are intermixed with spores in the sorus (Figs. 5c, f), except in *Ustilago porosa* and *Sporisorium culmiperdum*.
3. A peridium derived mainly from host tissue, either from leaf sheaths or the ovary wall.

Taxa in Clade 1 mainly infect grasses belonging to the sub-family *Panicoideae*, in one of two tribes, *Paniceae* or *Andropogoneae*. The infection is usually systemic and destroys either the entire inflorescence or all of the ovaries or spikelets.

Langdon & Fullerton (1978) examined the soral ontogeny of several species included in Clade 1, namely *Sporisorium sorghi*, *S. andropogonis* and *S. vanderystii*. They observed that the columella began to form after intercellular hyphae became confluent and caused the host cells to proliferate. Hyphae at the periphery of the columella formed a sheath of elongated, thick-walled, vacuolate cells. Other hyphae were present inter- and intracellularly in the tissue of the columella.

Columellae of species in Clade 1 are stout and woody due to the peripheral formation of thick-walled, vacuolate cells (Fig. 2). These columellae are cylindrical and grow vertically. Occasionally, more than one columella is present in a sorus, for example in *S. reilianum* (Fig. 5a). Sometimes columellae are branched, for example in *S. doidgeae* (Fig. 5b). Stout columellae are a synapomorphy for species in Clade 1 (Fig. 2)

Langdon & Fullerton (1978) observed that non-sporogenous hyphae partitioned the sporogenous hyphae in sori of *Sporisorium sorghi*. The partitioning hyphae formed groups of hyaline cells that mixed with the spores as the sorus matured. This pattern of development accounts for the chains of sterile cells found in many species of *Sporisorium* (Fig. 3), for example *S. rarum* (Fig. 5f), *S. themedae*, *S. ophiuri* and *S. vermiculum*. Langdon & Fullerton (1978) termed these 'partitioning cells', though subsequent descriptions of smut fungi referred to them as sterile cells. The term sterile cells is maintained to differentiate between the cells formed by non-sporogenous, partitioning hyphae, and the peridial cells formed from the peridium.

Clade 2

Species within Clade 2 have been described in *Ustilago*, *Sporisorium* and *Macalpinomyces*. They share two common morphological characters.

1. The sori are relatively long, twisted and cylindrical, and are derived from hypertrophied host material, as in *Macalpinomyces tubiformis* (Fig 6a), *M. mackinlayi* and *Sporisorium dietelianum*.
2. Sterile cells are usually found within the sori.

There are two types of infection in Clade 2: a localized infection seen in most of the species, or a systemic infection seen in a monophyletic group of taxa that destroy the entire inflorescence or infect the culms of the host. The position of the systemic group was ambiguous and only had data from the ITS and LSU regions. It either formed a well-supported monophyletic group within Clade 6, which was also recovered by Stoll et al. (2005) using nuclear rDNA loci; or it occurred sister to Clade 2 when using nuclear rDNA and protein-coding loci, as was also recovered by Vánky & Lutz (2011). The systemic monophyletic group will be discussed separately from Clade 2 because of its uncertain taxonomic position and distinct appearance on the host.

The systemic group of Clade 2 contained four species, *Macalpinomyces loudetiae* (not included in Fig. 1), *M. simplex*, *M. trichopterygis* and *M. tristachyae*. These

smuts infect grasses in the subfamily *Arundinoideae*, a character first observed by Stoll et al. (2005). The entire inflorescence or every spikelet in the inflorescence is destroyed by tubular sori. Vánky (1995a) described *Endosporisorium*, based on *Sorosporium capillipedii* (syn. *M. chrysopogonicola*), to accommodate smuts with long, tubular, host derived sori that contained sterile cells and lacked columellae (Vánky 1995a, 2002). *Endosporisorium* was later synoymised with *Macalpinomyces*, as Vánky (1997) preferred to have few larger, well-known genera rather than many smaller, unresolved genera. Three other taxa not included in the phylogenetic analysis have a similar appearance to members of the systemic group, namely *M. effusus*, *M. magicus* and *M. ugandensis*. These taxa should be included in future studies to determine if this method of infection is synapomorphic and whether the separation of *Endosporisorium* from *Macalpinomyces* is warranted.

The remaining taxa in Clade 2 form tubular sori derived from hypertrophied host material in some ovaries of the inflorescence and have sterile cells in the sori, with the exception of *U. maydis*. The model organism *U. maydis* occurred in Clade 2 and was considered more closely related to *Sporisorium* than *Ustilago* by Piepenbring et al. (2002) and Stoll et al. (2005).

Brefeld (1912) established *Mycosarcoma* for *Ustilago maydis*, which he diagnosed as different to *Sporisorium sorghi* (as *Ustilago sorghi*) for three reasons, (i) the incubation time in the host, (ii) the development of the sorus at the site of penetration in the host plant, and (iii) the development of aerial conidia. The peridial structure of *Ustilago maydis* was another character that Brefeld (1912) considered different to other species of *Ustilago*. Two of the characters that Brefeld (1912) described are unique characters to Clade 2, excluding the systemic group. The hypertrophied, host derived peridium and the localized infection sites on the host inflorescence are morphological synapomorphies of these taxa. Furthermore, the localized, hypertrophied, often tubular sori mostly contain sterile cells. Piepenbring et al. (2002) concluded from a molecular phylogenetic analysis that *Ustilago maydis* was separate to other *Ustilago* taxa, and that it may warrant placement in the genus originally assigned to it by Brefeld (1912). Other taxa that may belong to Clade 2, based on soral characters, are *Macalpinomyces elionuri-tripsacoidis*, *M. flaccidus*, *M. nodiglumis*, *M. siamensis* and *M. zonotriches*.

Sporisorium trachypogonis and *S. dietelianum*, which are members of Clade 2, were both described as having columellae (Fig. 2). It is unlikely that these structures are homologous to the stout and filiform columellae in Clades 1 and 4, which are synapomorphies for these clades. Vánky (2004) combined *Sporisorium dietelianum* into *Lundquistia* because he did not consider the fascicles of host tissue as true columellae. Vánky (2012) later re-considered this view, equating these fascicles with columellae. The columellae of *Sporisorium dietelianum* are filiform and similar to the columellae of species in Clade 4. *Sporisorium dietelianum* can be distinguished from species in Clade 4 because it does not form either a fungal peridium or spore balls, and it possesses sterile cells.

The columella of *Sporisorium trachypogonis* was described by Vánky (1995b) as well-formed and central, which is typical to those formed in the taxa of Clade 1. *Sporisorium trachypogonis* can be distinguished from other species in Clade 1 by the presence of a localized tubular sorus, rather than a systemic infection.

The recently described, monotypic genus, *Tubisorus* was not included in the current study. Vánky & Lutz (2011) recovered *Tubisorus* within a clade congruent to Clade 2. The infection of *Tubisorus* is consistent with other members of Clade 2 that possess long tubular sori. However, *Tubisorus* is described as lacking sterile cells and possessing spore balls, which are two characters considered synapomorphies of Clade 4. The establishment of *Tubisorus* sets a precedent for creation of monotypic genera that have an eclectic mix of characters within Clade 2.

Clade 3

Macalpinomyces bursus and *M. ewartii* occur in a strongly supported clade separate from other clades recovered in the analysis. *Macalpinomyces bursus* and *M. ewartii* are morphologically very similar in appearance and occur on *Themeda* and *Sorghum* respectively, which are members of the tribe *Andropogoneae*. The sori form hypertrophied galls in the host ovaries. Sterile cells formed from partitioning hyphae are present in the sori, which never have a columella. The spores are prominently echinulate. These characters are similar to smuts in Clade 7 that infect grasses in the sub family *Chloridoideae* and the tribe *Paniceae*. Host classification is the simplest character to separate these two clades. Other smut taxa that may occur in this clade are *Macalpinomyces bothriochloae, M. ovariicolopsis* and *M. pseudanthistiriae*.

Clade 4

Species in Clade 4 either destroy the entire inflorescence, as in *Sporisorium caledonicum* (Fig. 7c) and *S. tumefaciens*; whole racemes, as in *S. enteromorphum*; or are localized in the inflorescence, as in *S. heteropogonicola* (Fig. 7a), *S. anthistiriae* and *S. bothriochloae*. Species in Clade 4 exhibit a number of common morphological characters.

1. Filiform or slender columellae (Fig. 7a, c).
2. Persistent spore balls (Fig. 7d). Two distinct spore types are usually present within the spore ball, namely inner and outer spores. Outer spores are often ornamented and are darker than the inner spores (Fig. 7b).
3. A sorus surrounded by a peridium composed mostly of fungal tissue.
4. Sterile cells derived from non-sporogenous hyphae are rarely present within the sorus.

Langdon & Fullerton (1978) examined the soral ontogeny of two species found in Clade 4, *S. caledonicum* and *S. anthistiriae*. They described the columella of *Sporisorium caledonicum* as a vascular bundle surrounded by host parenchyma, with tissues permeated by inter- and intracellular hyphae. Five to seven columellae were formed by growth of hyphae in the parenchyma between the vascular bundles that separated the central column. Host cells close to intercellular hyphae in some instances were distorted but there was little destruction of host tissue. Langdon & Fullerton (1975) also studied the soral ontogeny of *Sporisorium cryptum*, which had a single columella made of several vascular bundles of parenchyma and mycelium that did not separate.

Species within Clade 4 have filiform or slender columellae (Fig. 2). These columellae are typically flattened in one plane and are never cylindrical. They are flexuous and do not grow vertically without support from the sorus as there are no thickened cells

to sustain vertical growth. Many columellae are present in the sorus, for example in *Sporisorium caledonicum*, *S. fallax* and *S. enteromorphum*. A single, filiform columella comprised of several vascular bundles is sometimes present, for example in *Sporisorium cryptum* and *S. bothriochloae*. The columellae formed in this fashion are not hardened or woody, although they are sufficiently robust to persist in the sorus.

The presence of a columella was the defining character of *Sporisorium* (Link 1825, Langdon & Fullerton 1978, Vánky 2002). Members of Clades 1 and 4 that were examined by Langdon & Fullerton (1975, 1978) possessed two differences in development and structure of columellae. The first difference was that peripheral cells of Clade 4 species were not distorted or hardened in contrast to the thickened, vacuolated peripheral cells in Clade 1 species. The second difference was that the central columns were separated into several columellae in *Sporisorium caledonicum* or were made of numerous vascular bundles, as in *S. cryptum*; the columellae of Clade 1 members, *S. sorghi* and *S. andropogonis* were not separated into vascular bundles. Filiform columellae composed of vascular bundles constitute a synapomorphy in species of Clade 4 (Fig. 2).

Many species of *Sporisorium* that possess permanent spore balls were originally described as members of *Sorosporium*. Most of these species belong to Clade 4 (Fig. 4). Langdon & Fullerton (1975) observed spore balls in several *Sporisorium* (as *Sorosporium*) species and described their formation. Coils of sporogenous hyphae were produced among mycelium that grew from the columellae as the sorus elongated. Coils consisted of two or three intertwined hyphae. Non-sporogenous hyphae, present between the spore balls, disintegrated and did not form sterile cells. Spores formed in spore balls were dimorphic. The peripheral spores developed surface ornamentation in the form of warts or spines and the internal spores were smooth.

Sporisorium panici-leucophaei has spore balls and occurs in Clade 4. According to Vánky (2001) the spore balls of *Lundquistia fascicularis* (syn.*S. panici-leucophaei*) differentiate from non-concentric, sporogenous hyphae. This differed from the mode of formation described for *Sporisorium* by Langdon & Fullerton (1975), and was one reason Vánky (2001) established *Lundquistia*. The mode of spore ball development in *Lundquistia fascicularis* (syn. *S. panici-leucophaei*) cannot be determined from the images provided by Vánky (2001). The spore balls are not agglutinated by sterile cells, as in *Moesziomyces*, and if the sporogenous hyphae are intertwined, as for species in Clade 1, then it is unlikely that the spores would form balls. It is unknown how spore balls are formed in *Sporisorium panici-leucophaei*.

Langdon & Fullerton (1975) observed that non-sporogenous hyphae in *Sporisorium caledonicum*, and three other species that occurred in Clade 4, disintegrated after the spores had matured. Sterile cells are rarely present in species of Clade 4 (Fig. 3). Often peridial cells derived from the fungal peridium were reported as sterile cells for species in Clade 4, for example in *Sporisorium loudetiae-pedicellatae*.

Species within Clade 4 possess a peridium made of fungal cells surrounded by a layer of host cells. Langdon & Fullerton (1975) discussed the formation of this peridium in *Sporisorium caledonicum* and three other smut fungi that occurred in Clade 4. They observed that hyphae adjacent to the peripheral host tissues became enlarged, with

vacuolate cells and thickened cell walls. These hyphae were orientated in the direction of the long axis of the sorus and formed a sheath inside the peripheral layer of host tissue. This fungal sheath and the host cells external to it constituted the soral peridium, which surrounded the soral contents.

Members of Clade 4 mostly occur on grasses in the tribes *Andropogoneae* or *Paniceae* in the subfamily *Panicoideae*. One exception is *Sporisorium hwangense* that infects *Sporobolus* in the subfamily *Chloridoideae*. It shares characters with other taxa in Clade 4, namely filiform columellae, spore balls with dimorphic spores, and an absence of sterile cells. Other examples of smut fungi that share characters in Clade 4 but occur on chloridoid grasses are *S. normanensis*, *S. cynodontis*, *S. parodii*, and *S. saharianum*.

Anomalomyces panici

Anomalomyces panici is sister to Clades 1, 2, 3 and 4. In terms of soral morphology, this species is similar to *M. bursus* and *M. ewartii* as it forms globose hypertrophied sori localized in the host ovaries. *Anomalomyces* infects *Panicum trachyrachis* in the tribe *Paniceae*. The sorus is filled with hardened spore balls formed by coiled sporogenous hyphae (Vánky et al. 2006), dimorphic spores and sterile cells. *Anomalomyces* possessed a unique combination of characters that warrants a monotypic genus within the *Ustilago-Sporisorium-Macalpinomyces* complex.

Clade 5

Four taxa that occur on the arid grass *Triodia* form a clade supported in maximum likelihood and Bayesian inference. The Bayesian analysis conducted by Stoll et al. (2005) grouped two *Triodia* taxa with the *Ustilago esculenta* group within Clade 6.

Ustilago altilis and *U. inaltilis* infect the host plant culms, while *U. triodiae* and *U. lituana* destroy the host inflorescence. Near identical ITS sequences for *U. altilis* and *U. inaltilis* (99% identical over 98% query coverage in a BLAST search), and *U. triodiae* and *U. lituana* (98% identical over 88% query coverage in a BLAST search) demonstrate their very close relationships. A synapomorphy for these four taxa is that they infect species of *Triodia*. They have similar characters to species in Clade 6, in that they do not possess soral structures such as spore balls, columellae or sterile cells.

Clade 6

Stoll et al. (2005) recovered Clade 6 as a weakly supported clade, which included *Melanopsichium pennsylvanicum*. They designated this clade as *Ustilago sensu lato* and defined three subgroups within the clade, (i) *Ustilago sensu stricto*, (ii) the *Ustilago davisii* group and (iii) the *Ustilago esculenta* group. Further loci were only sequenced for six taxa of Clade 6 in this study. Host and morphological synapomorphies have not been resolved for Clade 6 in our analysis.

Ustilago sensu stricto *clade*

Ustilago species that infect grasses in the tribe *Pooideae* formed a well-supported group that included the type species, *U. hordei*. Stoll et al. (2005) also recovered this group with strong support using Bayesian analysis. The stripe smuts *U.*

calamagrostidis and *U. striiformis*, as well as *U. sporoboli-indici* (on *Chloridoideae*) were sister to the smuts that destroy the inflorescence of pooid grasses. Stoll et al. (2005) included a subgroup in *Ustilago s. str.* that contained *Ustilago cynodontis*, *U. sparsa* and *U. xerochloae*. These three taxa occur on panicoid and chloridoid grasses. Inclusion of this subgroup and the stripe smuts in *Ustilago s. str.* was supported by both maximum likelihood and Bayesian inference. Taxa within the *Ustilago s. str.* clade lacked three characters that were found in other clades.

1. Absence of sterile cells in the sorus.
2. Absence of spore balls formed by coiled sporogenous hyphae.
3. Absence of a columella derived from host and fungal material.

Ustilago davisii *group*

Stoll et al. (2005) recovered a strongly supported but unresolved clade containing seven species, *Sporisorium aegypticum*, *S. modestum*, *Ustilago davisii*, *U. filiformis*, *U. schroeteriana*, *U. tragana* and *U. trichophora*. The same clade was recovered in this study, but it was not well supported by bootstrap values (< 70%) in maximum likelihood or posterior probabilities (< 0.95) in Bayesian inference. *Sporisorium aegypticum*, *S. modestum* and *Ustilago trichophora* were described as having columellae.

Fullerton & Langdon (1968) examined the soral development of *Ustilago trichophora* and concluded that a columella was present, however columellae are not included in the descriptions by Vánky & Shivas (2008) or Vánky (2012). The sori of *Ustilago trichophora* occur in ovaries or on stems and do not have columellae that are homologous to the columellae formed in Clades 1 and 4.

Ustilago esculenta *group*

Stoll et al. (2005) recovered a weakly supported group that contained several smut fungi found on chloridoid grasses together with the atypical *Ustilago esculenta*, which occurs on *Zizania* in the subfamily *Ehrhartoideae*. *Ustilago curta*, which infects *Tripogon* in the subfamily *Chloridoideae*, either occurred in the *Ustilago esculenta* group, or as sister to Clade 6 or 8. Stoll et al. (2005) recovered *Ustilago curta* (as *U. alcornii*) in the *Ustilago esculenta* group. No synapomorphies were determined for this group.

Stoll et al. (2005) demonstrated a close relationship between *Melanopsichium pennsylvanicum* and the *Ustilago s. str.* group. Our maximum likelihood analyses placed *Melanopsichium* in the *Ustilago esculenta* group rather than sister to the *Ustilago s. str.* group. Only the two nuclear rDNA loci obtained by Stoll et al. (2005) were included for *Melanopsichium* in the combined analysis of molecular loci. Begerow et al. (2004a) discussed the complicated coevolution between smut fungi and their hosts. *Melanopsichium pennsylvanicum* may represent a jump from *Poaceae* to the distantly related *Polygonaceae*.

Clade 7

This clade was recovered in studies by Stoll et al. (2003) and Stoll et al. (2005) and was strongly supported by both maximum likelihood and Bayesian inference in this

study. Stoll et al. (2005) noted that taxa in this clade had a combination of characters observed in *Sporisorium* and *Ustilago*. Taxa in this group have often been placed in *Macalpinomyces* because of the mixed soral characteristics associated with both *Sporisorium* and *Ustilago*. They occur on grasses in the tribe *Paniceae* and the subfamily *Chloridoideae*.

Sterile cells are present in *Macalpinomyces spermophorus*, *M. viridans*, *M. neglectus* and *Ustilago affinis*, but are absent in the other members of this clade. Several taxa formed galls in the host ovaries, while *U. drakensbergiana*, *U. syntherismae* and *U. affinis* destroyed the entire inflorescence similar to taxa in *Ustilago s. str.* Columellae were described in several of the species in this clade, including *Ustilago drakensbergiana*, *Macalpinomyces spermophorus*, *M. viridans* and *M. neglectus*.

The columellae of *U. drakensbergiana* are formed from the remnants of the destroyed inflorescence and are not homologous with columellae of Clades 1 and 4. Vánky (2012) observed that the sori of species of *Macalpinomyces* were deciduous and separated from the host plant at maturity, whereas species of *Sporisorium* had sori that remained attached to the inflorescence because the columella was connected to the host plant. The sori of *M. viridans* and *M. spermophorus* were deciduous and easily removed from the host plant. These columellae are not formed from the host meristem and are not homologous to the columellae of the Clades 1 and 4.

A synapomorphic character for Clade 7 was not identified. Subdivision of Clade 7 based on morphology is impractical at this stage, because the characters are highly variable in the group.

Clade 8

Four taxa that destroy the ovaries of *Aristida* formed a well-supported monophyletic group. Stoll et al. (2005) included *Sporisorium consanguineum* in their study, but were unable to determine whether it was sister to, or part of Clade 7. The inclusion of three additional smuts that infect *Aristida* has resulted in a separate, monophyletic group. The smuts on *Aristida* share two morphological characters.

1. Formation of galls in the ovaries of their hosts. They can infect all of the ovaries in an inflorescence (*Sporisorium confusum*, *S. consanguineum*) or be localised in the inflorescence (*S. aristidicola*).
2. The spores are commonly compacted into spore balls formed by coiled sporogenous hyphae, for instance in *Sporisorium consanguineum* (Langdon & Fullerton 1975).

Macalpinomyces eriachnes

Macalpinomyces eriachnes is the sister taxon to the *Ustilago-Sporisorium-Macalpinomyces* complex. Stoll et al. (2005) first indicated that *Macalpinomyces* was a monotypic genus, with *M. eriachnes* the sole representative. This relationship is supported in this study. *Macalpinomyces eriachnes* has giant sterile cells formed from non-sporogenous hyphae (Langdon & Fullerton 1977, Vánky 1996) and a peridium, but lacks a columella. The spore balls of *Macalpinomyces eriachnes* were not formed from coiled sporogenous hyphae (Langdon & Fullerton 1977).

Taxa of uncertain placement

A few taxa moved between clades in trees reconstructed using different datasets and different phylogenetic assessment criteria. These taxa were not supported in any group, although previous analyses have grouped most of these taxa in Clade 6 (Stoll et al. 2005). *Ustilago tragana*, *U. schmidtiae*, *Sporisorium aegypticum* and *S. modestum* often grouped together after maximum likelihood analysis, although they were only represented by data from two molecular loci in most cases. These taxa, except for *Ustilago schmidtiae*, were included with taxa now assigned to Clade 6 by Stoll et al. (2005).

Maximum likelihood analyses placed *Ustilago curta* in a number of clades. Stoll et al. (2005) recovered *U. curta* (as *U. alcornii*) in the *Ustilago esculenta* group of *Ustilago s. lat.* after Bayesian analysis of data from two nuclear rDNA loci. With the addition of nuclear loci, *U. curta* was often placed as sister to the *Aristida* group or as sister to the *Triodia* group. It is not known to which group *Ustilago curta* belongs.

Can host classification delimit smut genera?

Taxa within the *Ustilago-Sporisorium-Macalpinomyces* complex infect hosts in the *Poaceae*, with the exception of *Melanopsichium*, which occurs on *Polygonaceae*. The systematics of *Poaceae* has been well resolved and the relationships of the sub-families and tribes are well understood (Hsiao et al. 1999, Kellogg 2000, Stevens 2001, Bouchenak-Khelladi et al. 2008).

Host classification has often been used in the classification of smut fungi. Within *Ustilago*, *Sporisorium* and *Macalpinomyces*, putative host specificity is used to differentiate morphologically indistinguishable species (Bauer et al. 2001). Many of the keys to these genera are based on host taxonomy. Higher-level host taxonomy has been used to delimit smut genera, for example *Ustilago* is restricted to members of *Poaceae* (Bauer et al. 2001).

Begerow et al. (2004a) concluded that the phylogenetic relationships between smut fungi and their hosts were not straightforward. While species of *Ustilago* and *Sporisorium* showed evidence for co-speciation, it was considered more likely that smut fungi evolved after their hosts had speciated (Begerow et al. 2004a). Host jumps are evident in Clade 4, which contains taxa that infect grasses in two subfamilies, the *Paniceae* and the *Chloridoideae*.

The phylogenetic analyses of the *Ustilago-Sporisorium-Macalpinomyces* complex recovered several monophyletic groups that shared similar morphological characters and are restricted to hosts in a specific genus, tribe or subfamily. Four smuts that occur on *Aristida* in the subfamily *Aristidoideae* (Stevens 2001) form a monophyletic group in Clade 8. They have similar morphological characters but there are no unique synapomorphies that separate them unambiguously from other species in the complex. Their pathogenicity on hosts in the subfamily *Aristidoideae* is a synapomorphy that distinguishes this clade from other clades in the complex.

Macalpinomyces bursus and *M. ewartii*, which are members of Clade 3, infect hosts in the tribe *Andropogoneae*. They possess morphological characteristics that are similar to some species of Clade 7 that infect hosts in the *Chloridoideae* or *Paniceae*.

The occurrence of members of Clade 3 on hosts in the tribe *Andropogoneae* is a synapomorphy that can be used to distinguish *Macalpinomyces bursus* and *M. ewartii* from taxa in Clade 7.

In many cases morphological characteristics are inadequate for recognizing smut taxa. It is proposed that delimitation of smut genera be based on host range, provided monophyletic groups are resolved after molecular phylogenetic analyses. In the absence of contradictory evidence, host subfamily or tribe is a legitimate criterion for generic delimitation in the *Ustilago-Sporisorium-Macalpinomyces* complex.

Conclusion

A detailed examination of morphology is required to determine homology and to improve classification (Mooi & Gill 2010), although in many groups of fungi this is impossible. The synapomorphies outlined here based on gross morphology and host coevolution allow confident placement of new taxa within the *Ustilago-Sporisorium-Macalpinomyces* into well delimited clades. Although there are some morphological anomalies, the monophyletic groups are robust and well supported.

Morphological synapomorphies within the *Ustilago-Sporisorium-Macalpinomyces* were identified after incorporation of nuclear protein coding loci and a thorough study of morphological diversity in Australian taxa. The determination of monophyletic groups and synapomorphic characters within the complex necessitates taxonomic reassessment of some genera and the creation or resurrection of others in future studies. The major outcomes of resolved character homology in the *Ustilago-Sporisorium-Macalpinomyces* complex are:

1. *Sporisorium* can be subdivided by soral characteristics. *Sporisorium sensu stricto* must be described explicitly to prevent ambiguity for future taxonomic placement of new species.
2. New genera are required for the placement of taxa that form monophyletic groups and no longer fit the definition of *Sporisorium sensu stricto*.
3. *Ustilago maydis* and other taxa with localized tubular sori and usually with sterile cells form a monophyletic group with the morphologically similar systemic group, which usually destroy the entire inflorescence. A taxonomic resolution for these taxa cannot be proposed at this stage, however, if the method of soral infection is synapomorphic within the groups, the two names, *Mycosarcoma* and *Endosporisorium*, will be available for the placement of these taxa.
4. *Macalpinomyces bursus* and *M. ewartii* belong to a monophyletic group that can be differentiated by soral characteristics and host tribe.
5. The monophyletic group of smut fungi that infect *Aristida* can be delimited by soral characteristics and host subfamily.
6. Four smut fungi on *Triodia* form a monophyletic group.
7. *Macalpinomyces* is a monotypic genus, sister to all other taxa in the *Ustilago-Sporisorium-Macalpinomyces* complex (Stoll et al. 2005).
8. Until Clades 2 and 7 are resolved, *Macalpinomyces* will remain a polyphyletic genus.

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Figures

Fig. 1. Phylogram obtained from a maximum likelihood analysis in RAxML. Maximum likelihood support values (> 70%) from RAxML 1000 bootstrap replicates and PhyML aRLT values shown above the nodes. Posterior probabilities (> 0.95) from Bayesian inference shown below the nodes. *S.* = *Sporisorium*, *U.* = *Ustilago*, *M.* = *Macalpinomyces*.

Fig. 2. Structure of the columella mapped onto five clades of the *Ustilago*, *Sporisorium* and *Macalpinomyces* complex.

Fig. 3. Distribution of sterile cells within clades of the *Ustilago*, *Sporisorium* and *Macalpinomyces* complex.

Fig. 4. Presence of spore balls within clades of the *Ustilago*, *Sporisorium* and *Macalpinomyces* complex.

Fig. 5. Clade 1 character states. a. Columellae in *Sporisorium reilianum*; b. Branched columella destroying entire inflorescence in *S. doidgeae*; c. Spores and sterile cells of *S. themedae*; d. All ovaries of the inflorescence infected in *S. ryleyi*; e. All spikelets of the inflorescence infected in *S. rarum*; f. Spores and sterile cells of *S. rarum*. Scale bars a-b, e = 1 cm; c, f = 10 µm.

Fig. 6. Clade 2 character states. a. Localized spikelets infected by *Macalpinomyces tubiformis*. b. Spores and sterile cells in *M. tubiformis*.

Fig. 7. Clade 4 character states. a. Localized spikelets infected in *Sporisorium heteropogonicola*; b. Dimorphic spores of *S. heteropogonicola*; c. Entire inflorescence destroyed by *S. caledonicum*; d. Permanent spore balls of *S. caledonicum*. Scale bars a, c = 1 cm; b, d = 10 µm.