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Systematic position of *Moesziomyces penicillariae* among *Ustilaginaceae*

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We revisited the systematic position of the fungus *Moesziomyces penicillariae*, the causal agent of smut of pearl millet, by using morphological characters, germination pattern of teliospores and molecular analysis of ribosomal sequences. Samples of smutted ears of pearl millet were harvested in Senegal (West Africa). Compared to the description of *Moesziomyces* genus sensus Vánky, our samples presented morphological differences: i) presence of a columella-like structure in sori; ii) surface ornamentations of teliospores; iii) teliospore germination similar to *Ustilago* and *Sporisorium ones*. We investigated the systematic position of our samples by aligning their Internal Transcribed Spacer (ITS) sequences of the ribosomal regions with 47 sequences from Ustilaginaceae. The resulting tree rooted with *Tolyposporium junci* allowed the separation of five groups among which, they are, two *Ustilago* and two *Sporisorium*. An independent clade is formed by *Tranzscheliella williamsii* and *Tranzscheliella hypodytes* species including *Ustilago sparti*. *Moesziomyces* species used in this analysis form a monophylletic group located in *Ustilago* 2 group, which include different *Ustilago* and *Sporisorium* species but also *Pseudozyma antartica*. Our results indicate the necessity to amend the *Moesziomyces* genus as the morphological and molecular data confirm that they are included in the *Ustilago-Sporisorium* complex.

Key words: Pearl millet smut, *Moesziomyces penicillariae*, *Ustilaginaceae*.

INTRODUCTION

Moesziomyces penicillariae (Brefeld) Vànky belongs to the *Ustilaginaceae* (Basidiomycota). It has been reported in tropical and subtropical zones of Africa, America and Asia (Wells et al., 1963; Thakur and King, 1988). This fungus affects the ovaries of pearl millet that are converted into sori containing dusty brown to blackish teliospores. The pathogen was at first named *Tolyposporium penicillariae* Brefeld and also called *Tolyposporium senegalense* Spegazzini, or *Sorosporium bullatum* Schröter. According to different authors, *T. penicillariae* is synonymous with *Tolyposporium bullatum* Schröter or *T. bullatus*. Mordue (1995) proposed that *M. penicillariae* and *Moesziomyces bullatus* are morphologically alike but has different biology. It was named *M. penicillariae* (Brefeld) Vánky in 1977 and then

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Moesziomyces bullatus (Schröter) Vánky in 1994. Several revisions of the taxonomy of *Ustilaginaceae* based on morphological or molecular studies have been published recently (Bauer et al., 1997; Begerow et al., 1997; Piepenbring et al., 1998; Stoll et al., 2005). Numerous changes of the nomenclature of the species have led us to undertake the systematic study of *M. penicillariae*, one of the major pathogen on pearl millet in sub-sahelian areas of Africa. We compared the morphology of the teliospores, its way of germination and the internal transcribed spacer (ITS) sequences of the ribosomal regions of our isolates with other *Ustilaginaceae*. ITS rDNA regions have been used successfully in systematics (Summerbell et al., 1999; Stoll et al., 2003; Stoll et al., 2005) and were very usefulin analysing the phylogenetical delimitation of closely related taxa.

MATERIALS AND METHODS

Fungal materials

Samples were harvested on naturally infected hosts: *M. penicillariae* (Brefeld) Vánky on *Pennisetum* spp. and *Sporisorium ehrenbergii* (Kühn) Vánky on *Sorghum* spp (Table 1). Sori with intact sheath were disinfected roughly under agitation 10 min in a solution of chloramine T 2% (Sigma) V/P, a drop of Tween 80 (0.05%) was added and rinsed three times with sterile distilled water. Sheaths of sori were broken aseptically in small Petri disc to recover teliospores. A part of these teliospores were put to germinate at 28°C on potatoes dextrose agar medium (PDA, Difco). Cultures were harvested in 1.5 ml microtubes after 8 or 10 days of incubation according to their development status. They were washed 3 times with 1 ml of UHQ water, centrifuged then preserved at -20°C. Every gotten culture constituted an original multisporidial strain.

Microscopic observations

Single teliospore or teliospores gathered in balls were observed directly through optic microscope and after metallization through scanning electron microscope (SEM) (JEOL JSM-840 Tokyo, Japan) using 15 KV. Infected ovaries in final stage of development were fixed in a solution of glutaraldehyde, dehydrated and included in Spurr resin. Semi fine cut pieces coloured with paragon or with methylene blue dye were observed through optic microscope.

Molecular analysis

Original multisporidial cultures were pounded in 600 µl using the CTAB procedure (Gardes and Bruns, 1993). PCR protocol includes the use of a proof reading TaqPolymerase (Clontech). The amplifications of DNA were conducted as follows: 4 min at 94°C, 35 cycles (1 min at 54°C, 1 min at 72°C and 1 min at 94°C), 10 min at 72°C. The primers used in these trials were the universal pITS1 and pITS4 (White et al., 1990) allowing the amplification of the ITS1-5.8S-ITS2 regions. The obtained amplicons were sequenced by the TaqDye-Deoxy Terminator Cycle (Applied Biosystems). The sequences were aligned using the software Seaview and phylogenic inferences were performed either with Phylo-win software (Galtier et al., 1996) or with PAUP (Swofford, 1993) using the UPGMA method. The bootstrap option (500 samplings) and the construction of the majority consensus tree were realized pertaining to 50% (bootstrap values lower than 50 are considered as unresolved). The sequences from the different species used for this analysis are listed in Table 1. Among the 50 compared sequences, 14 obtained in this study include 3 tested for the first time in a comparative analysis of ITS1-5,8S-ITS2 regions. The other sequences were obtained from Genbank following previous works of Roux et al. (1998), Bakkeren et al. (2000), Stoll et al. (2003), Shivas et al. (2004) and Stoll et al. (2005).

RESULTS AND DISCUSSION

Morphological and anatomical criteria

Moesziomyces species are hosted on *Poaceae* and *Eriocaulaceae*. They infect inflorescences and the vegetative parts are generally not affected by the pathogen. The infected ovaries are hypertrophied and converted into sori. Direct observations revealed that the sori are delimited by a peridium formed by the remainders of ovary wall and mycelium. Remnants of inner structures of the ovary formed conspicuous columellae in the central zone (Figure 1A). The balls of teliospores have very variable shapes and form powdery mass of brown to darkish colour as previously described (Vànky, 1977, 1987, 2002; Piepenbring, 2003) . According to Vànky (2002) and Piepenbring (2003), the teliospores of *Moesziomyces* spp. are smooth. We observed that mature teliospores were ovoid or slightly polyhedric bounded in balls of variable size and form that constitute a dusty mass (Figure 1B). At higher magnification with scanning electronic microscope (SEM), spore balls presented some remainder of mycelium and sporidia (Figure 1C). The teliospores located in peripheral parts of the balls had more or less warty walls (Figure 1D), whereas those in inner parts were nearly smooth (Figure 1E). Neighbouring teliospores were connected by walls of aborted sterile cells, appearing as small wings or excrescences on the surface of the teliospores (Figure 1D and E).

The germinated teliospores produced a basidium with at least four cells that generate basidiospores on the side and at the tip (Figure 1F and G). The basidiospores budded rapidly and formed colonies of yeast cells, or could also emit filamentous prolongations of variable length and then to bud secondary basidiospores (Figure 1H). It was not clear whether these structures corresponded to conjugation tubes and we did not observe events of mating distinctly. Recent analysis supports the hypothesis that the basidiospores formed are diploid and solopathogenic (Sabbagh et al., 2010). The presence of a columella-like structure, the slight ornamentation of teliospores and the germination of

Table 1. Origin of the sequences used in this study.

Fungal species	Genbank accession code	Origin of the equence	Origin of the sample
Moesziomyces penicillariae		This study	Harvested in December 2001 on *P. glaucum* (var. Sanio) (Tankanto, Southern of Senegal).
Moesziomyces bullatus	AY740153.1	Stoll et al., 2005	
Moesziomyces bullatus	DQ831013.1	Matheny et al., 2006	
Pseudozyma antarctica	AB089360	Sugita et al., 2003	
Pseudozyma prolifica	AB089368	Sugita et al., 2003	
Sporisorium aegypticum	AY344970	Stoll et al., 2003	
Sporisorium andropogonis		Roux C.	Harvested in July 1996 on *Andropogon* sp. (Le Vernet, 31, France)
Sporisorium catharticum	AY344971	Stoll et al., 2003	
Sporisorium cenchrium	AY344972	Stoll et al., 2003	
Sporisorium cruentum	AY344974	Stoll et al., 2003	
Sporisorium culmiperdum	AY344975	Stoll et al., 2003	
Sporisorium destruens	AF045871	Roux C.	CBS 327.33
Sporisorium fallax	AY333941	Shivas et al., 2004	
Sporisorium fastigiatum	AY344978	Stoll et al., 2003	
Sporisorium holwayi	AY344980	Stoll et al., 2003	
Sporisorium mishrae	AY344983	Stoll et al., 2003	
Sporisorium moniliferum	AY344984	Stoll et al., 2003	
Sporisorium panici-leucophaei	AY344986	Stoll et al., 2003	
Sporisorium provinciale	AY344988	Stoll et al., 2003	
Sporisorium puellare	AY344990	Stoll et al., 2003	
*Sporisorium reilianum f.*sp. *zeae*	AF045870	Roux C.	Harvested in June 1994 on *Zea mays* L. (St Ciers, 33, France)
Sporisorium reilianum f.sp. *reilianum*	AF038827	Roux et al., 1998	Harvested in September 1994 on *Sorghum bicolor* (St Sulpice sur Lèze, 31, France)
Sporisorium ehrenbergii		This study	Harvested in November 2001 on *Sorghum sp*. (Sinthiou Malem, ISRA, WASDON 211, Eastern of Sénégal)
Sporisorium sorghi ehrenb. ex	AF038828	Roux et al., 1998	CBS 274.30
Link *Sporisorium scitamineum*		Roux C.	Harvested in 1996 on sugar cane (Guadeloupe, France)
Sporisorium themedae-arguentis	AY344991	Stoll et al., 2003	
Sporisorium tumefaciens	AY333943	Shivas et al., 2004	
Sporisorium veracruzianum	AY344993	Stoll et al., 2003	
Tolyposporium junci	AY344994	Stoll et al., 2003	

Table 1. Contd.

Transzcheliella hypodytes	AF045867	Roux C.	Harvested in July 1996 on *Arrhenatherum elatior* (Toulouse, 31, France)
Transzcheliella williamsii	AF045869	Almaraz-Lopez T	Harvested in 1997 on *Stipa spp* (Spain)
Ustilago aegilopsidis	AF135429	Bakkeren et al., 2000	
Ustilago affinis	AY344995	Stoll et al., 2003	
Ustilago avenae	AY344996	Stoll et al., 2003	
Ustilago bullata	AF135423	Bakkeren et al., 2000	
Ustilago crameri	AY344999	Stoll et al., 2003	
Ustilago cynodontis	AF038825	Roux et al., 1998	Harvested in 1996 on *Cynodon dactylon* (L.) Pers. (Argelès sur Mer, France)
Ustilago echinata	AY345001	Stoll et al., 2003	
Ustilago hordei	AF045866	Roux C.	Harvested in march 1996 (Bolivia) on *Arrhenatherum sp*
Ustilago maydis	AF038826	Roux et al., 1998	Harvested in october 1995 on *Zea mays* L. (Le Vernet, 31, France)
Ustilago nigra	AF135428	Bakkeren et al., 2000	
Ustilago nuda	AF135430	Bakkeren et al., 2000	
Ustilago pamirica	AY345005	Stoll et al., 2003	
Ustilago schroeteriana	AY345006	Stoll et al., 2003	
Ustilago sparsa	AY345008	Stoll et al., 2003	
Ustilago sparti	AF045868	Almaraz - Lopez T.	Harvested in 1997 on *Lygeum spartum* (Spain)
Ustilago trichophora		Roux C.	Harvested in 1997 on *Echinochloa crus-galli* (St Ciers, 33, France)
Ustilago tritici	AF135424	Bakkeren et al., 2000	
Ustilago turcomanica	AY345010	Stoll et al., 2003	

teliospores constitute morphological elements of differentiation of our isolates with *Moesziomyces* genus *sensus* (Vanky, 2002; Piepenbring, 2003). According to the key of *Sporisorium* species determination (Piepenbring, 2003), the morphological features of the species from group “A” bring them nearer to our specimen. The species of *Ustilago* genus exclusive parasites of *Poaceae* have sori without columella or with columella-like structures corresponding to remains of the host organ but not made of hypertrophied host tissue (Piepenbring, 2003). Their teliospores are mostly warted, rarely smooth and not grouped in balls (Vànky, 2002; Piepenbring et al., 2002; Piepenbring, 2003). This morphological study showed similarities of our isolates with the genera *Sporisorium* and *Ustilago*, although its position among one of these two genera is not clear.

Molecular criteria

Unambiguously aligned regions of 50 ITS sequences were used to perform a distance analysis based on UPGMA calculation. According to this analysis, the species tested are dispatched in 5 groups (Figure 2). Independent analyses of sub-groups did not modify the fitting of the robust nodes, and did not improve the un-separated nodes. As described by Stoll et al. (2003) and Stoll et al. (2005), species of *Ustilago* and *Sporisorium* genera are separate each into two clades: *Ustilago* 1 and *Ustilago* 2; *Sporisorium* 1 and *Sporisorium* 2. The 4 groups *Sporisorium* 1 – 2 and *Ustilago* 1 - 2 did not form clearly separate clades, although the *Sporisorium* of group 1 and

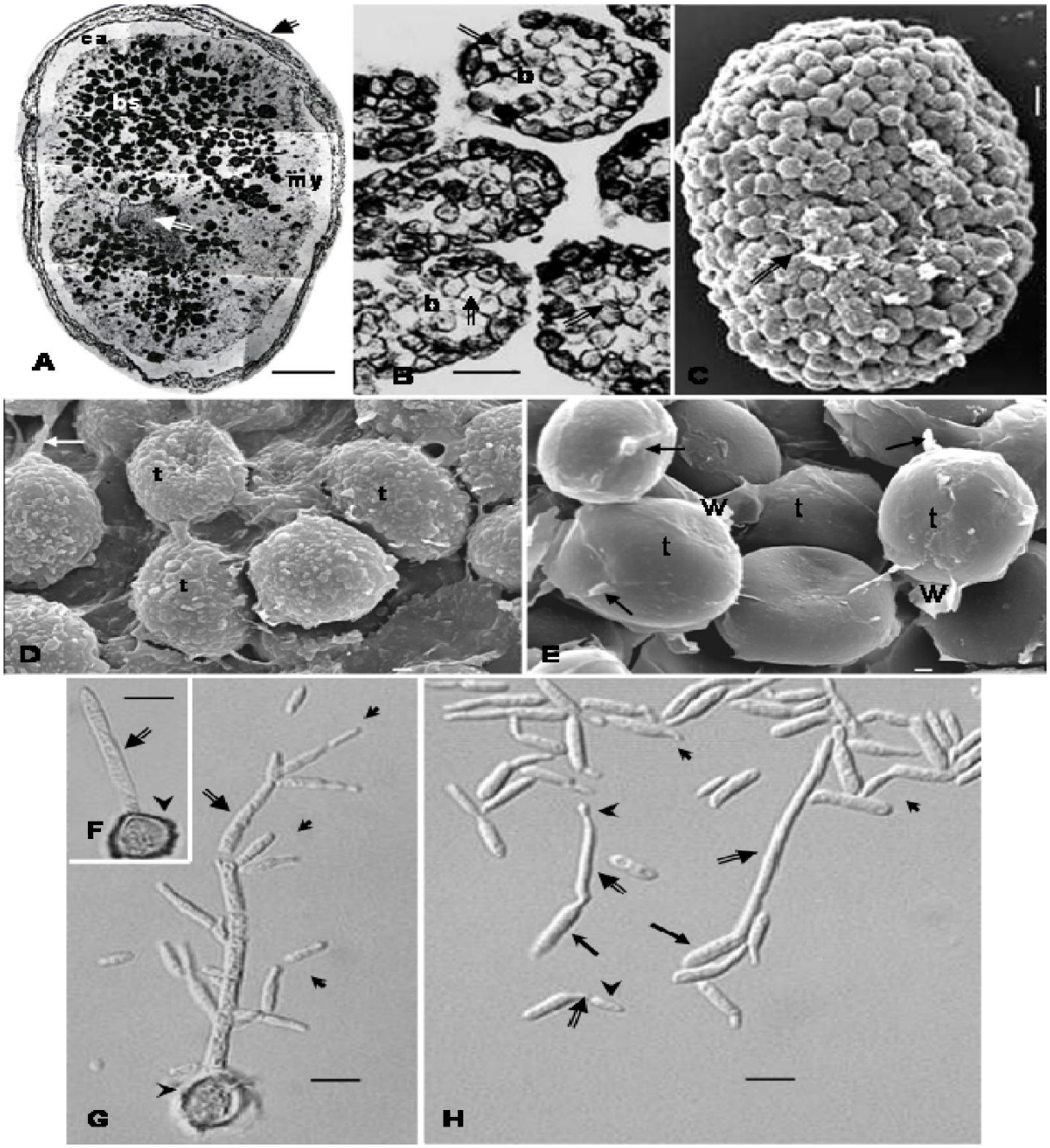

Figure 1. Optical and electronic observations of sorus, teliospores and basidiospores. Microscopic observations of sorus, spore balls and teliospores. A, B; Optical microscope observations of a sorus and spore balls. A, a longitudinal section of a ripening sorus with a *peridium* (black double arrow) remnants of the ovary wall, a conspicuous cavity of ovary (ca), a remainder of mycelium (my), balls of spores at various sizes (bs) and remainders of the ovary structures forming a columellae- like structure (white double arrow), scale bar = 200 µm; B, polyhedric teliospores (black double arrow) grouped in balls (b) of variable sizes and forms, scale bar = 50 µm. C, D, E; Teliospore balls observed by scanning electronic microscope (SEM). C, teliospores bounded in a ball with some remainders of mycelium and basidiospores not transformed into teliospores (black arrows), scale bar = 10 µm; D, E, portions of teliospore balls (t) were connected by sterile cells (Black and white arrows) or kinds of wings (W); D, warted peripheral teliospores (t), E, nearly smooth ones (t) of inside the balls, scale bar = 1 µm. F, G, H; Optical microscope observations of germinating teliospores (black arrows) basidiospores formation and their behaviour. F, an early stage of teliospore germination (black arrow head) with a short basidium (double black arrow); G, budding of basidiospores by the basidium with iterative released basidiospores (black short arrows); H, basidiospores (black arrows) some of them (black long arrows) emitting filament of variable length (black double arrows) which can bud secondary basidiospores (black arrows head), scale bar = 10 µm.

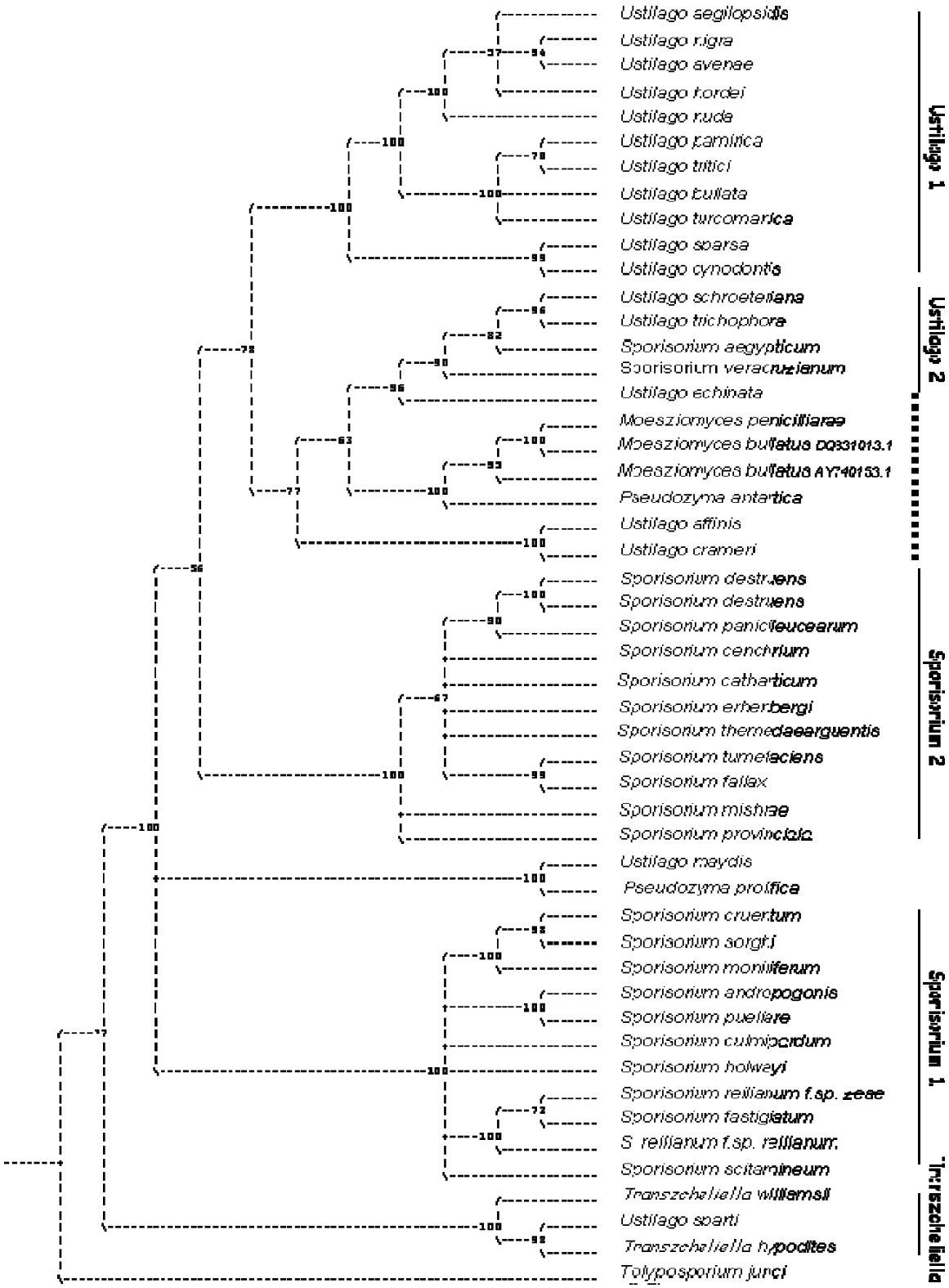

Figure 2. Majority-rule consensus tree. Phylogeny of *Ustilaginaceae* using ITS regions of 50 species. Majority-rule consensus tree (50%), rooted with *T. junci*, obtained with UPGMA inference of a 750 bp ITS1-5,8S-ITS2 rDNA sequences alignment (48 species, 442 sites without ambiguities among 64 informatives). The simple parameter of divergence was used. Bootstrap values (500 replicates) greater than 50% are given inside the branches.

the *Ustilago* of group 1 form distinct clades. The *Sporisorium* of group 2 and the *Ustilago* of group 2 corresponded to a continuum of variation of sequences between the extreme clades of the *Sporisorium* of group 1 and the *Ustilago* of group 1. Some transpositions are proposed between species placed in these clades: *Sporisorium veracruzianum* and *Sporisorium aegypticum* are located in the clade of *Ustilago* 2; *Ustilago maydis* and *Pseudozyma prolifica* occupy a doubtful position, intermediate between the clades of *Sporisorium* species. *Pseudozyma antartica* is not grouped with *P. prolifica* but in the *Moesziomyces* clade, pointing out the poly-phyletism of this taxon.

At the basal position, the clade formed by *Transzcheliella williamsii* and *T. hypodites* was well separated from the others (bootstrap value 91). This clade also included *Ustilago sparti*, a parasite on inflorescences and stems of *Lygeum spartum* . Although grouped with *T. Hypodites* (bootstrap value of 100), their ITS sequences had 7% of divergence, and showed differences even on 5.8S region. The three species of *Moezsiomyces* genus used in this analysis (Our strain of *Moesziomyces penicillariae*, two strains of *Moesziomyces bullatus*, DQ831013.1 and AY740153.1) were grouped in the same clade among the *Ustilago* 2 group. These three sequences are not equivalent: 99% of similarities between our sample and *M. bullatus* DQ831013.1 from the strain CBS 425.34 isolated from *Pennisetum typhoideum*, 89% of similarities with the sequence AY740153.1 from a strain isolated from *Paspalum distichum*. Based on an intergeneric comparison using 28S (rRNA) sequences, Begerow et al. (1997) showed that *M. bullatus* is included among Ustilaginales; although the low number of species analyzed for this taxon did not allow the determination of whether it constitutes an independent clade or not. Stoll et al. (2005) in a NJ analysis of 109 specimens belonging to *Ustilaginaceae*, placed *M. bullatus* and *M. eriocauli* as basal species. The use of distant external groups (*Transzchelellia* and *Tolyposporium junci*) allowed us to replace *Moesziomyces* species between the *Ustilago* 2 and *Sporisorium* clades.

Conclusion

According to Stoll et al. (2003), we formulate the hypothesis that all the species of *Ustilago* and *Sporisorium* genus constitute a continuum of species being gradually different. This progressive differentiation is observed when sympatric species contain various populations that tend to be isolated and present distinguished characters while preserving homogeneous ones. The difficulty in separating *Ustilaginaceae* species on morphological and molecular criteria, illustrates this progressive transition. According to morphological and molecular results, the *M. penicillariae* isolates used in this study clearly belongs to *Ustilago* and *Sporisorium* group. Considering that *Moesiomyces* form a specific group or not, would be clarified while including more species or/and sequences of *Sporisorium* and *Ustilago* species to better delimitate these clades.

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