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Integrative analysis of the West African *Ceraceosorus africanus* sp. nov. provides insights into the diversity, biogeography, and evolution of the enigmatic Ceraceosorales (Fungi: Ustilaginomycotina)

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Abstract The order Ceraceosorales (Ustilaginomycotina) currently includes the single genus *Ceraceosorus*, with one species, *Ceraceosorus bombacis*, parasitic on *Bombax ceiba* in India. The diversity, biogeography, evolution, and phylogenetic relationships of this order are still relatively unknown. Here, a second species of *Ceraceosorus* is described from West Africa as a novel species, *Ceraceosorus africanus*, infecting *Bombax costatum* in Benin, Ghana, and Togo. This species produces conspicuous fructifications, similar to corticioid basidiomata when mature, but sorus-like in early stages of ontogenetic development. The fructifications cover much of the leaf surface and resemble leaf blight. This contrasts with the inconspicuous fructifications of *C. bombacis* comprising small spots scattered over the lower leaf surface that resemble leaf spot. Both species of *Ceraceosorus* differ in several micromorphological traits, infect different host plant species in widely separated geographical areas, and are separated by a considerable genetic distance in 28S rDNA and RPB2 genes. The distinct corticioid fructification of *C. africanus* is a unique morphological trait within the Ustilaginomycotina. Molecular phylogenetic analyses of a single gene dataset (D1/D2 28S rDNA) supported the monophyly of the two *Ceraceosorus* species and the Ceraceosorales and their placement within the Ustilaginomycotina. Molecular phylogenetic analyses of a multigene dataset (18S/5.8S/28S rDNA/RPB2/TEF1) revealed *Exobasidium rhododendri* (Exobasidiales) as the closest relative of *Ceraceosorus*, both clustering together with *Entyloma calendulae* (Entylomatales), indicating affinities to the Exobasidiomycetes. This phylogenetic placement is in agreement with ultrastructural characteristics (presence of local interaction zone and interaction apparatus) reported for the Ceraceosorales, Entylomatales, and Exobasidiales.

Keywords Basidiomycota · *Bombax* · Exobasidiomycetes · Molecular phylogeny · Plant pathogens · Smut fungi · Ustilaginomycotina

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Introduction

Historically, smut fungi were defined as phytoparasites producing (usually) dusty masses of dikaryotic teliospores within plant tissues, germinating to develop basidia with basidiospores, which grow as a saprobic yeast stage in the haplophase. They were included in the single order Ustilaginales (Schröter 1889; Clinton 1906; Zundel 1953). Parasites with such a life strategy and organization are now referred to as classical smuts (Vánky 2001), true smuts (Oberwinkler 2012), or teliosporic smuts (Begerow et al. 2006). This idea of smut fungi and their classification remained almost unchanged until the end of the twentieth century. Comprehensive studies by Bauer et al. (1997) on hyphal septal pores and interaction zones between parasites and host cells using transmission electron microscopy revealed enormous structural diversity that led to the new

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classification and changed the concept of smut fungi and their relationships to other fungal parasites. These findings were supported by the molecular phylogenetic studies of Begerow et al. (1997). These two complementary studies demonstrated that the classical smut fungi (at that time assigned to the single order Ustilaginales) represented several distinct lineages regarded as the orders Doassansiales, Entorrhizales, Entylomatales, Georgefischeriales, Tilletiales, Urocystidales, and Ustilaginales. These orders were interspersed by nonteliosporic taxa forming two main lineages referred to as the orders Exobasidiales (incl. Graphiolales, Begerow et al. 2002a) and Microstromatales. The new concept of smuts and related nonteliosporic fungi resulted in the description of the new class Ustilaginomycetes (Bauer et al. 1997), that was subsequently elevated to the level of a subphylum, the Ustilaginomycotina (Bauer et al. 2006). It became apparent that several genera and species should be removed from the smut fungi since they showed relationships to rust fungi. These are now referred to as false smuts (Oberwinkler 2012) and are classified in the Pucciniomycotina, order Microbotryales (Bauer et al. 1997; Begerow et al. 1997). Recently, two additional genera, *Entorrhiza* C.A. Weber and *Talbotiomyces* Vánky, R. Bauer & Begerow, were removed from the smut fungi and classified in the new phylum, Entorrhizomycota, distinct from the phyla Ascomycota and Basidiomycota (Bauer et al. 2015; Riess et al. 2015).

The new concept of smuts and related fungi was continuously augmented by further phylogenetic studies that revealed an enormous diversity of life strategies and structural organization of sporulation within the lineages of Ustilaginomycotina. Thus, in addition to classical smut fungi and nonteliosporic species, this subphylum includes lipophilic yeasts associated with warm-blooded animals or marine environments, the species of *Malassezia* Baill. (Begerow et al. 2000; Amend 2014; Cabañes 2014; Wang et al. 2014), mite-associated yeasts (Boekhout et al. 2003), and saprobic yeasts isolated from diverse ecosystems (Begerow et al. 2000; Boekhout et al. 2006; Nasr et al. 2014; Piątek et al. 2015; Wang et al. 2014). Several species were also isolated from healthy plant tissues and may act as endophytes (Paz et al. 2007; Amin et al. 2010; Takahashi et al. 2011; Padhi and Tayung 2013; Rush and Aime 2013). The most notable of them is the recently described *Violaceomyces palustris* Albu, Toome & Aime that represents a distinct lineage, the order Violaceomycetales (Albu et al. 2015). Sporulation in the Ustilaginomycotina is notably diverse, including budding cells, simple hyphae with conidiogenous cells producing conidia, teliospores embedded between plant cells or replacing generative or vegetative plant organs, and loose fascicles or hymenial layers of basidia emerging from stomata or the epidermis.

To date *Ceraceosorus* B.K. Bakshi is a monotypic genus and contains only *Ceraceosorus bombacis* (B.K. Bakshi) B.K. Bakshi, a leaf pathogen of the silk cotton tree *Bombax ceiba* L. (Malvaceae) in Uttar Pradesh, India. This fungus was found and initially described as *Dicellomyces bombacis* B.K. Bakshi by Bakshi (in Bakshi et al. 1972). Only 3 years later, it was transferred, also by Bakshi, to the new genus *Ceraceosorus*, distinct from *Dicellomyces* L.S. Olive (Olive 1945, 1951) in having sorus-like fructifications, indeterminate hymenial thickenings, intracellular hyphae, basidia with internal thickenings, and lacking cystidia (Cunningham et al. 1976). *Ceraceosorus* was placed in the Brachybasidiaceae (Cunningham et al. 1976; Kirk et al. 2001). *C. bombacis* may be an important phytopathogenic species in India as reported in the original publications (Bakshi et al. 1972; Cunningham et al. 1976), though in recent years, it was not included as such in any original phytopathological report. *C. bombacis* is unique in respect to the formation of intracellular hyphae, a character not known in any other order of Exobasidiomycetes, stressing the isolated phylogenetic position of this species outside all main lineages revealed by molecular phylogenetic analyses (Begerow et al. 2006). Thus, to accommodate the genus *Ceraceosorus*, Begerow et al. (2006) added the novel order Ceraceosorales, assigned to the class Exobasidiomycetes. The family Ceraceosoraceae was subsequently described by Denchev and Moore (2009).

The single genus and species currently residing in the Ceraceosorales implies that different aspects such as evolution, biogeography, diversity, and phylogenetic relationships of this order are still obscurely known. Some of these missing aspects could be improved now from the discovery of a second member of Ceraceosorales infecting a native woody species, the silk cotton tree *Bombax costatum* Pellegr. & Vuillet (Malvaceae), in the Sudanian savanna biome in West Africa. This unknown fungus was consistently found on most specifically inspected *B. costatum* trees in Benin, Ghana, and Togo during four independent surveys at the end of the rainy period in the years 2011–2013 and 2015. In this study, polyphasic analyses were conducted to establish the systematic and phylogenetic placement of this fungus, studying its structural (dis-)similarity and genetic divergence compared to *Ceraceosorus bombacis*, as well as phylogenetic relationships to other orders of the Ustilaginomycotina. Furthermore, environmental information assembled in field studies is given to contribute to the knowledge of the ecology of this fungus.

Material and methods

Specimen sampling and documentation

This study is based on phylogenetic and morphological analyses of a leaf parasite on *Bombax costatum* (Malvaceae) originating from eight locations in three different countries and

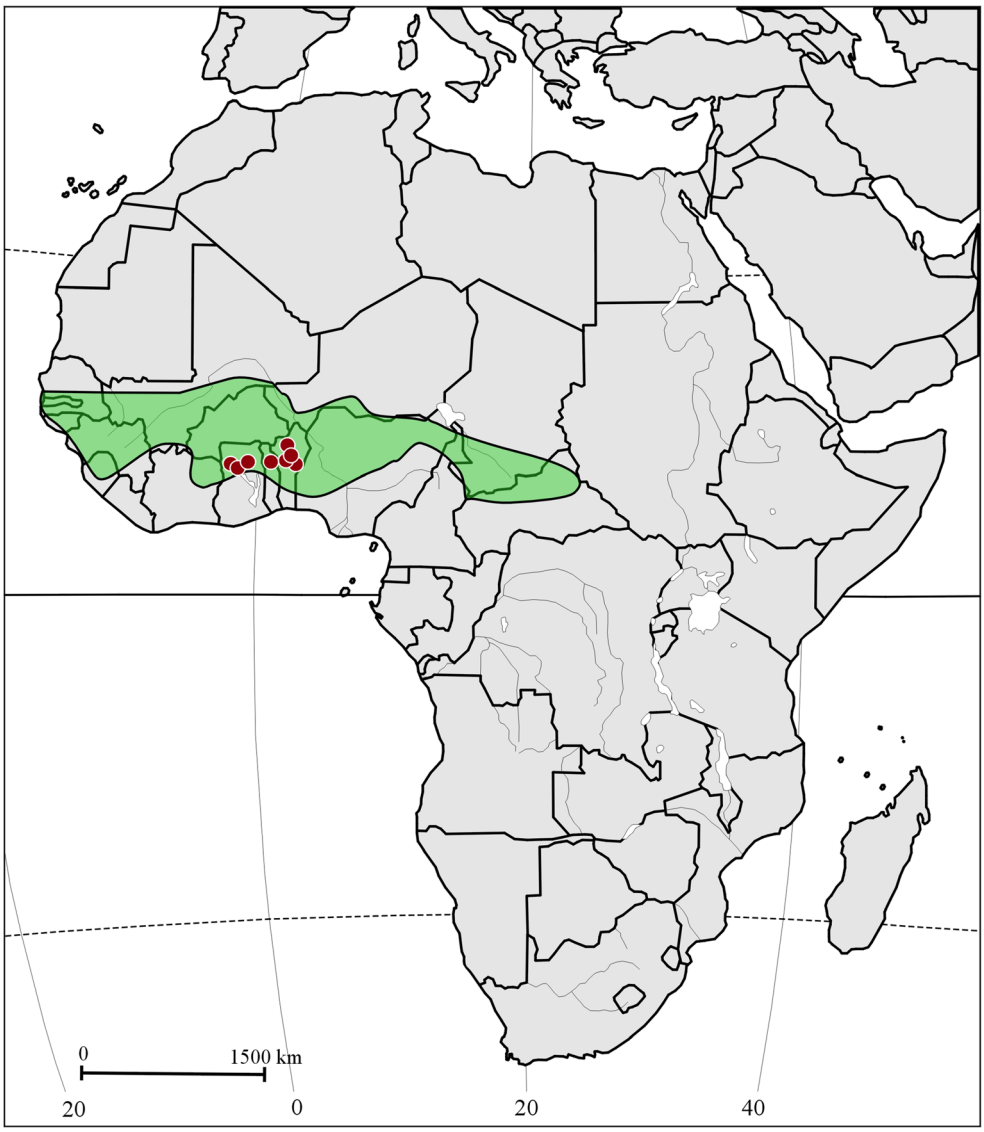

Fig. 1 Currently known distribution of *Ceraceosorus africanus* (*red dots*) on the background of the global distribution of *Bombax costatum* in the Sudanian savanna biome (*green area*, based on the map shown on the website: http://www.hombori.org/francais/biodiversity/flora.html)

diverse habitats in West Africa (Benin, Ghana, Togo; Figs. 1 and 2). The voucher specimens were deposited in the fungal collection of the W. Szafer Institute of Botany of the Polish Academy of Sciences, Kraków, Poland (KRAM F). Additionally, the ex-type culture of *Ceraceosorus bombacis* isolated from *Bombax ceiba* (Cunningham et al. 1976) was anew sequenced for phylogenetic analyses. Detailed specimen information is given in Table 1. Nomenclatural novelty was registered in MycoBank (www.mycobank.org).

Morphological analyses

Morphological characters of fructifications were studied using dried herbarium material. Observations of the macroscopic appearance of fructifications were made with the naked eye and under a stereoscopic microscope Nikon SMZ-2T. Micromorphological characters were studied by light microscopy. For this purpose, thin freehand sections of fructifications were made under the stereoscopic microscope using a razor blade; mounted in 3 % (wt/vol) aqueous potassium hydroxide, 1 % (wt/vol) aqueous phloxine, Melzer's reagent, and 0.1 % Cotton Blue (wt/vol) in 60 % (wt/vol) lactic acid; and examined under a Nikon Eclipse E-400 light microscope. In presenting the spore size variation, 5 % of measurements were excluded from each end of the range and are given in parentheses. In the species description, the following abbreviations were used: L = mean length of all spore measurements, W = mean spore width, Q = length to width range ratio, Q_m = mean Q value, and n = number of measurements. Q values were obtained from dividing the average basidiospore length by width. Line drawings were made from slides mounted in aqueous potassium hydroxide.

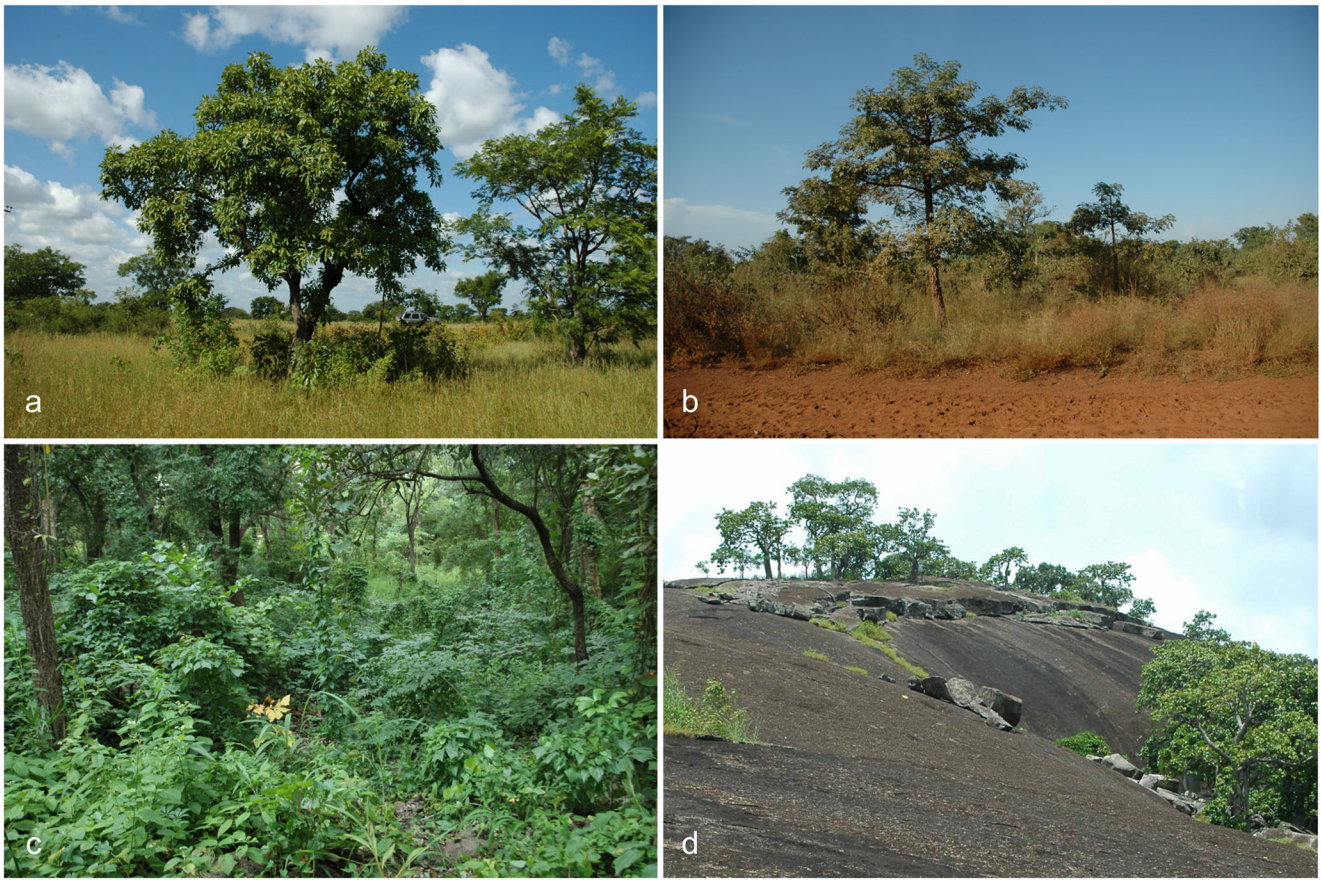

Fig. 2 Habitat types of *Ceraceosorus africanus*: **a** Sudanian savanna dominated by grasses, with scattered trees, between Zibogo and Tugu in Ghana; **b** Sudanian savanna with shrubs and trees, between Busunu and Fufulsu in Ghana (type locality); **c** transition zone between gallery forest and the Sudanian woodland, Forêt Classée de Béléfoungou in Benin; **d** inselberg, Tébou in Benin

Molecular phylogenetic analyses

For DNA isolation, approximately 0.25 cm^2 of dried leaves of *Bombax costatum* with disease symptoms and a 0.25 cm^2 agar block containing a living culture of *Ceraceosorus bombacis*, respectively, were selected, deep-frozen in liquid nitrogen, and ground several times with a plastic pestle. Total genomic DNA was subsequently extracted using the InnuPREP Plant DNA Kit (Analytik Jena, Jena, Germany) following the standard protocol. *C. bombacis* was processed again since no 5.8S, RPB2, or TEF1 sequences were available, and only a short 28S sequence was available in the NCBI's GenBank database (www.ncbi.nlm.nih.gov).

The nuclear 18S, 5.8S, and 28S ribosomal DNA (rDNA) genes (=18S, 5.8S, 28S) were amplified using the primer combinations NS1/NS8 (White et al. 1990), ITS3/ITS4 (White et al. 1990), and LR0R/LR9 (R. Vilgalys lab, http://biology.duke.edu/fungi/mycolab/primers.htm; Hopple and Vilgalys 1999), respectively, with the PCR conditions described in Riess et al. (2013). The gene of the second largest subunit of RNA polymerase II (RPB2) regions 5–7 was amplified with the primers fRPB2-5F (Liu et al. 1999) and bRPB2-7.1R (Matheny 2005), following the thermocycling program described by Matheny (2005). The translation elongation factor 1-alpha (TEF1) gene was amplified using the primer combinations EF-526F/EF-2218R, EF-526F/EF-ir, or Ef-df/EF-2218R (Rehner and Buckley 2005; S. Rehner, http://aftol.org/pdfs/EF1primer.pdf) following the protocol of Rehner and Buckley (2005). PCR products were cleaned and cycle sequenced as described in Riess et al. (2013) using the PCR primers and the additional primers NS19 (Gargas and Taylor 1992) and NS4 (White et al. 1990) for 18S; LR3R (Hopple and Vilgalys 1999) and LR6 (Vilgalys and Hester 1990) for 28S; fRPP2-6F (Matheny 2005) for RPB2; and EF-983F, EF-1567R, and EF-1577F (Rehner and Buckley 2005) for TEF1. All generated *Ceraceosorus* DNA sequences have been deposited in NCBI's GenBank database (www.ncbi.nlm.nih.gov) (see Tables 1 and 2).

To infer the phylogenetic relationships of the examined *Ceraceosorus* specimens, we assembled two datasets. Dataset 1 contained D1/D2 28S sequences of seven *Ceraceosorus* specimens and representatives of all smut genera belonging to the main lineages of Ustilaginomycotina (Begerow et al. 2014; Wang et al. 2014) for which sequences were available in GenBank, including 73 sequences obtained from the respective generic type species (Fig. 3). Dataset 2 was composed of 18S +

Table 1 List of *Ceraceosorus* species, host plant species, GenBank accession numbers (D1/D2 28S), and voucher information for specimens used for phylogenetic (see Fig. 3) and morphological analyses

Species	Host plant species	GenBank acc. no. D1/D2 28S	Reference specimen
Ceraceosorus africanus	*Bombax costatum*	KP413034	Benin, Atakora Department: near the Tanougou Waterfalls, 26 Oct. 2011, *leg. M. Piątek & N.S. Yorou*, KRAM F-57386
Ceraceosorus africanus	*Bombax costatum*	KP413038	Benin, Borgou Department: Bassa, 28 Oct. 2013, *leg. M. Piątek*, KRAM F-57390
Ceraceosorus africanus	*Bombax costatum*	N/A	Benin, Donga Department: Tébou, inselberg, 19 Sept. 2015, *leg. M. Piątek*, KRAM F-58019
Ceraceosorus africanus	*Bombax costatum*	N/A	Benin, Donga Department: Forêt Classée de Béléfoungou, 19 Sept. 2015, *leg. M. Piątek*, KRAM F-58020
Ceraceosorus africanus	*Bombax costatum*	KP413036	Ghana, Northern Region: between Busunu and Fufulsu, ca. 18 km W of Fufulsu, 5 Nov. 2012, *leg. M. Piątek & N.S. Yorou*, KRAM F-57385 (holotype)
Ceraceosorus africanus	*Bombax costatum*	KP413035	Ghana, Northern Region: between Zibogo and Tugu, 3 Nov. 2012, *leg. M. Piątek & N.S. Yorou*, KRAM F-57387
Ceraceosorus africanus	*Bombax costatum*	KP413039	Ghana, Northern Region: between Busunu and Fufulsu, ca. 13.5 km W of Fufulsu, 5 Nov. 2012, *leg. M. Piątek & N.S. Yorou*, KRAM F-57388
Ceraceosorus africanus	*Bombax costatum*	KP413037	Togo, Kara Region: between Pya and Niamtougou, 3 Nov. 2013, *leg. M. Piątek & N.S. Yorou*, KRAM F-57389
Ceraceosorus bombacis	*Bombax ceiba*	KP413033	India, Dehra Dun: New Forest Estate, 20 Oct. 1967, *leg. B.K. Bakshi*, strain ATCC 22867 (ex-type culture)

5.8S + 28S + RPB2 + TEF1 sequences of *Ceraceosorus* plus one species of all Ustilaginomycotina genera for which at least four of these five genes were available in GenBank (21 total; Fig. 4). *Microbotryum violaceum* (Pers.) G. Deml & Oberw. s.l. and *Puccinia graminis* Pers. were used as outgroup for both datasets. Except for *Ceraceosorus bombacis*, *Entyloma calendulae* (Oudem.) de Bary, *Schizonella melanogramma* (DC.) J. Schröt., *Sympodiomycopsis paphiopedili* Sugiy., Tokuoka & Komag., *Urocystis colchici* (Schltdl.) Rabenh., and *Ustanciosporium standleyanum* (Zundel) M. Piepenbr., parts of the concatenated sequences of the remaining species were obtained from different specimens/cultures of the species (for detailed information, compare GenBank accessions). For GenBank accession numbers of the sequences of dataset 1 (Boekhout et al. 1995, 2003; Begerow et al. 1997, 2000, 2001, 2002a, 2006; Bauer et al. 1999, 2001, 2005, 2007, 2008; Piepenbring et al. 1999, 2002, 2010; Fell et al. 2000; Castlebury et al. 2005; Hendrichs et al. 2005; Stoll et al. 2005; Maier et al. 2006; Matheny et al. 2006; Vánky et al. 2006, 2008, 2013; González et al. 2007; Chandra and Huff 2008; Lutz et al. 2008, 2012a, b; Paap et al. 2008; Ritschel et al. 2008; Tanaka et al. 2008; Sipiczki and Kajdacsi 2009; Deadman et al. 2011; Vánky and Lutz 2011; McTaggart et al. 2012; Piątek et al. 2013; Nasr et al. 2014), see Fig. 3. For GenBank accession numbers of the sequences of dataset 2 (de Wachter et al. 1992; Boekhout et al. 1995; Schillberg et al. 1995; Swann and Taylor 1995; Takashima and Nakase 1996; Bakkeren et al. 2000; Döring and Blanz 2000; Hamamoto et al. 2000; Döring 2003; Liu and Hall 2004; Lutzoni et al. 2004; Wingfield et al. 2004; Castlebury et al. 2005; Stoll et al. 2005; Begerow et al. 2006; de Beer et al. 2006; Kolařík et al. 2006; Matheny et al. 2006, 2007; Carris et al. 2007; Le Gac et al. 2007; Ran et al. 2008; Brock et al. 2009; Rosa et al. 2009; Kottke et al. 2010; Gorfer et al. 2011; Schoch et al. 2012; Wang et al. 2014), see Table 2.

All DNA regions were aligned separately with MAFFT 7.147b using the E-INS-i option (Katoh et al. 2005; Katoh and Standley 2013). In the rDNA datasets, ambiguously aligned regions were removed using Gblocks 0.91b (Castresana 2000) with gaps allowed at alignment positions at which gaps were present in more than half of the sequences, while the protein coding sequences were modified manually with the help of annotated sequences of RPB2 (DQ408132) and TEF1 (X73529). The final alignment length for dataset 1 (D1/D2 28S) was 447 bp (15 % of the initial alignment length before Gblocks), and for dataset 2 (18S + 5.8S + 28S + RPB2 + TEF1) 1505 bp for partial 18S (54 %), 163 bp for complete 5.8S, 1272 bp for partial 28S (48 %), 972 bp for the complete exon 6 of RPB2, and 984 bp for partial TEF1. The single-gene alignments were then concatenated into one sequence alignment of a total of 4896 bp (dataset 2). Phylogenetic analyses were computed for both datasets using maximum likelihood (ML) with combined rapid bootstrapping under the GTRCAT model from 1000 runs

Table 2 List of species and GenBank accession numbers used for the combined 18S/5.8S/28S/RBP2/TEF1 analyses (see Fig. 4)

	GenBank accession numbers				
Species	18S	5.8S	28S	RPB2	TEF1
Anthracocystis flocculosa	DQ092923	HQ115653	AY745712	–	DQ028598
Ceraceosorus bombacis	DQ875377	*KP413083*	*KP413033*	*KP413029*	*KP413032*
Entyloma calendulae	DQ663688	DQ663689	DQ663687	DQ663690	DQ663691
Erratomyces patelii	DQ363309	DQ663692	AY818966	–	DQ663695
Exobasidium rhododendri	AJ271381	EU784219	DQ667151	DQ667154	DQ667156
Malassezia furfur	KF706457	EU513202	AY745725	KF706516	KF706468
Microbotryum violaceum s. lato	U77062	JN942213	DQ789982	DQ789985	DQ074573
Microstroma juglandis	DQ363313	DQ317632	*KP413052*	DQ789989	DQ789991
Moesziomyces bullatus	DQ831012	AY740153	DQ831011	DQ831014	–
Moniliella acetoabutans	KF706443	EU252153	JN938879	KF706523	KF706476
Puccinia graminis	AY125409	HQ317585	AF522177	XM_003321826	X73529
Quambalaria cyanescens	KF706440	NR_111202	DQ317615	KF706531	KF706485
Rhamphospora nymphaeae	DQ363311	DQ831034	DQ831032	DQ831035	DQ831036
Schizonella melanogramma	DQ832211	DQ832212	DQ832210	DQ832213	DQ832215
Sporisorium reilianum	DQ832229	AF135432	DQ832228	DQ832231	KF706472
Sympodiomycopsis paphiopedili	DQ832239	DQ832240	DQ832238	DQ859894	–
Tilletia goloskokovii	DQ832247	EU257570	AY818999	DQ832249	DQ832251
Tilletiaria anomala	D83193	DQ234558	AY745715	AY803750	DQ835991
Tilletiopsis fulvescens	D83189	AB025705	AJ235281	KF706530	KF706483
Tilletiopsis washingtonensis	AJ271382	AB025689	AY745714	DQ835995	DQ835996
Urocystis colchici	DQ839595	DQ839596	DQ838576	DQ839597	DQ839598
Ustanciosporium standleyanum	DQ846889	DQ846890	DQ846888	DQ846891	DQ846893
Ustilago maydis	X62396	FJ167356	FJ644528	AY485636	AY885160

Accession numbers of sequences generated in this study are given in italics. Specimen information can be inferred from the respective GenBank accession numbers

using RAxML 8.0.17 (Stamatakis 2014). Additional posterior probability nodal support values were determined in a Bayesian phylogenetic MCMC search using MrBayes 3.2.2 (Ronquist et al. 2012) under the general time reversible model with gamma-distributed rate variation (GTR + G) as suggested in the MrBayes version 3.2 manual. Each search comprised two runs of four chains each for 10×10^6 generations sampled every 100 generations with the first 2.5×10^6 generations discarded as burn-in. Genetic divergences (uncorrected *p*-distance) within the Ceraceosorales were calculated for 18S, 28S, and RPB2 using Mesquite 2.75 (Maddison and Maddison 2011) and the unmodified alignments.

Results

Morphology: ontogenetic development

The fungus on *Bombax costatum* developed intracellular hyphae in host cells that agglomerated, as intercellular hyphae, in substomatal cavities and emerged through stomata forming sorus-like fructifications with a densely packed and strongly agglutinated hyphal subhymenial layer (that could be referred to as sterile external stroma), and an outer hymenial layer with basidioles and basidia. The adjoining sorus-like fructifications coalesced to form a continuous, compact, widely effused layer on the host leaves. The single sorus-like fructifications were visible best at the edge of the continuous layer, whereas after coalescence, they were undifferentiated and whole fructifications resembled those of corticioid fungi. The predominant parts of the fructifications were sterile, composed only of basidioles, waxy and pinkish in color. The fertile areas were scattered over the sterile areas and were visible under lens as whitish and slightly pruinose areas. The detailed morphological characterization of the fungus on *B. costatum* is included in the species description and depicted in Figs. 5a–f, 6, 7, and 8.

Molecular phylogenetic analyses

D1/D2 28S sequences of the six specimens of the leaf parasite on *Bombax costatum* were identical. Their phylogenetic

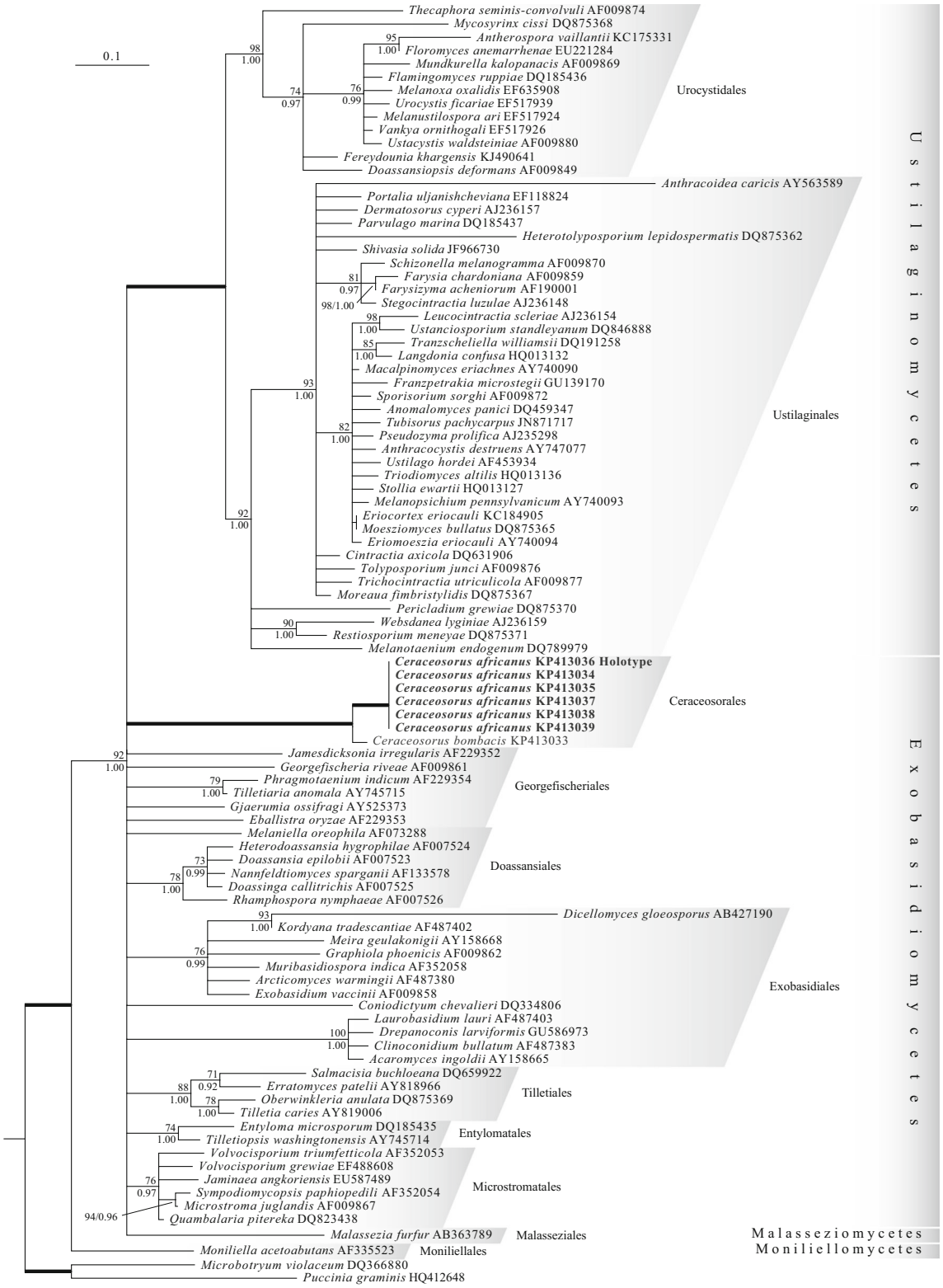

Fig. 3 Phylogenetic placement of *Ceraceosorus africanus* within the sampled Ustilaginomycotina derived from D1/D2 28S sequences (447 bp). Statistical support is given as ML bootstrap above branches (≥70) and Bayesian posterior MCMC probability below branches (≥0.90). The *lines in bold* indicate a maximum support of 100/1.00. *Microbotryum violaceum* and *Puccinia graminis* were used as outgroup

relationships, analyzed with 90 sequences representing sampled genera of the Ustilaginomycotina (plus two outgroup species from Pucciniomycotina), are shown in Fig. 3. In all analyses, the specimens of the leaf parasite on *B. costatum*

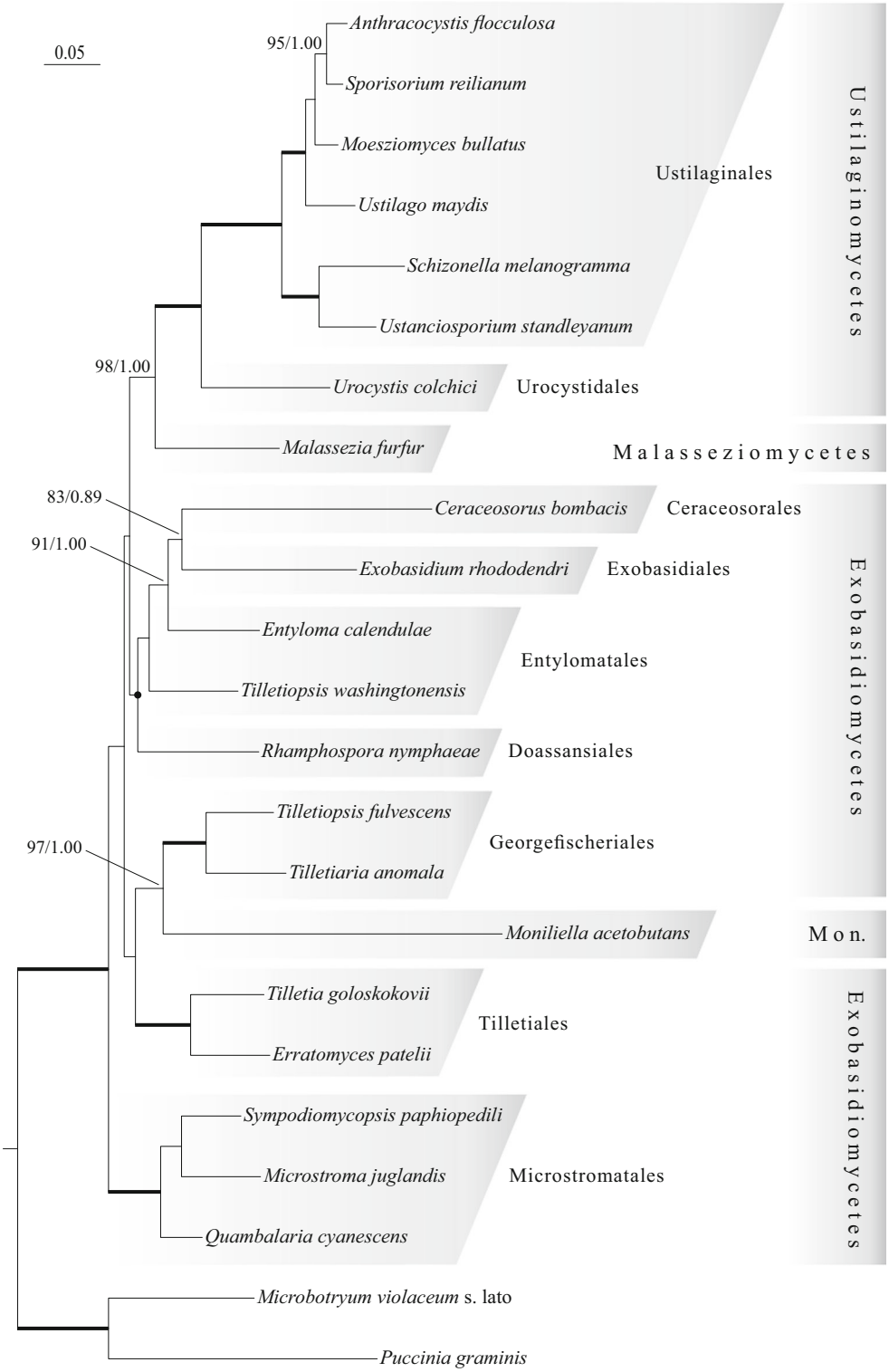

Fig. 4 Hypothesis of phylogenetic relationships of the sampled Ustilaginomycotina based on 18S, 5.8S, 28S, RPB2, and TEF1 sequences (4896 bp). The tree was rooted with *Microbotryum violaceum* s.l. and *Puccinia graminis*. Statistical support is given as ML bootstrap above branches (≥70) and Bayesian posterior MCMC probability below branches (≥0.90). The *lines in bold* indicate a maximum support of 100/1.00. The superorder Exobasidianae is marked with a *black dot*. Mon. = Moniliellomycetes

clustered together and as sister lineage of *Ceraceosorus bombacis*. The sequence divergence to *C. bombacis* was 0.20 % or 1 bp (18S), 4.03 % or 83 bp (28S), and 18.5 % or 196 bp (RPB2). The monophyly of the leaf parasite on *B. costatum* and *C. bombacis* was highly supported in both Bayesian and ML analyses. However, the phylogenetic position of the Ceraceosorales within the Exobasidiomycetes was not resolved, and the Exobasidiomycetes were not supported as monophyletic (Fig. 3).

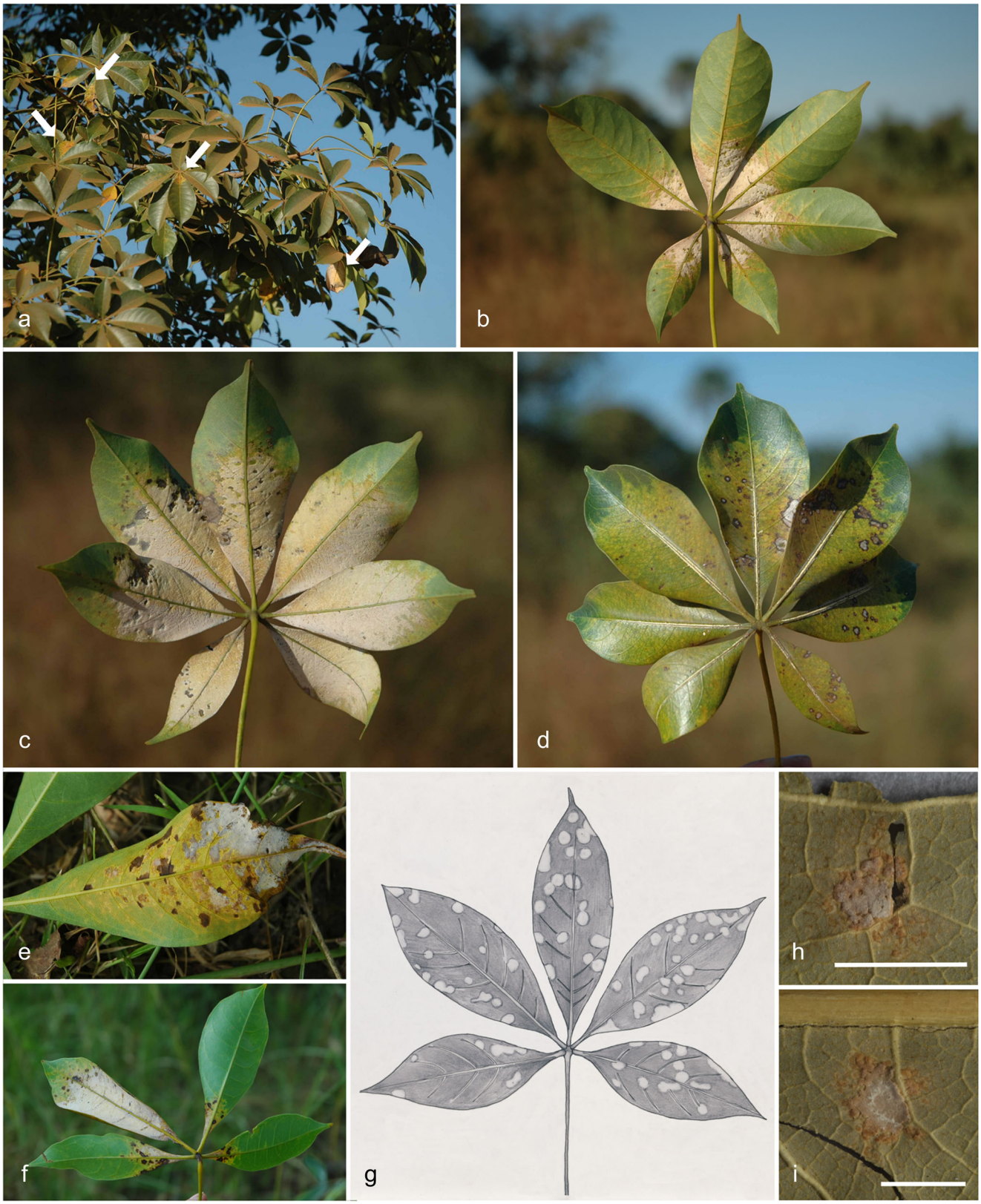

Fig. 5 **a**–**f** Macroscopic symptoms of infection of *Bombax costatum* leaves by *Ceraceosorus africanus* (**a**–**d** taken on the type locality; **e**, **f** taken on the locality between Zibogo and Tugu): **a** view of the crown of the tree with scattered infected leaves marked by *white arrows*; **b**–**f** different levels of development of leaf blight caused by the fungus (**b**, **c**, **e**, **f** on the leaf underside; **d** on the leaf upperside). **g**–**i** Macroscopic symptoms of infection of *Bombax ceiba* leaves by *Ceraceosorus bombacis*: leaf spot caused by the fungus (**g** schematized drawing, reproduced from the figure presented in Bakshi et al. 1972; **h**–**i** photo taken from isotype KRAM F-58224, note sorus-like fructifications; *scale bars*: **h**, **i** = 3 mm)

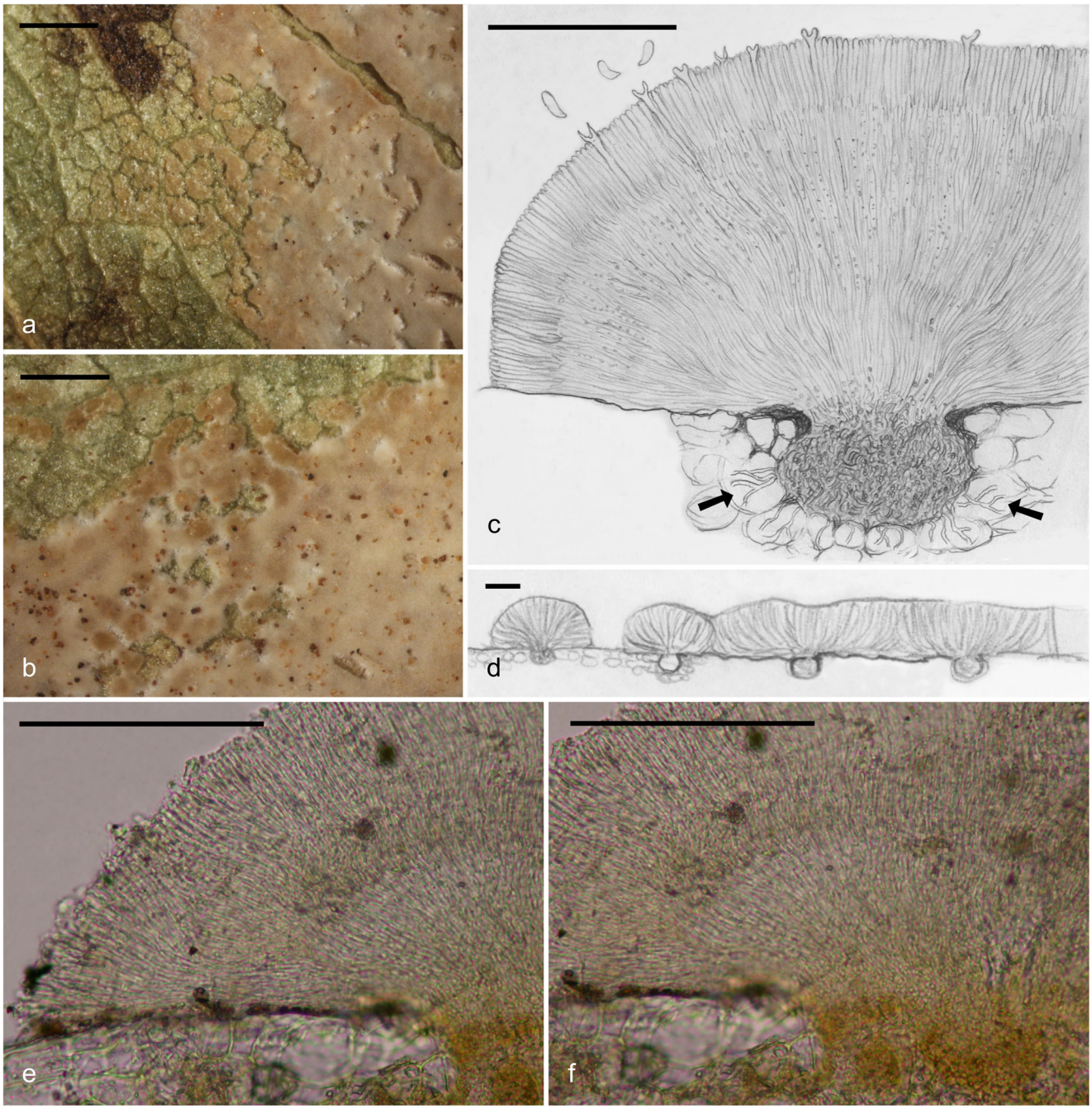

Fig. 6 Developmental stages of fructifications of *Ceraceosorus africanus* (all from holotype): **a**, **b** initial sorus-like fructifications visible in the marginal parts that coalesce to form a continuous, compact, widely effused layer on the host leaves; **c** schematized drawing of individual sorus-like fructification, note intracellular hyphae indicated by *black arrows*; **d** schematized drawing of the mode of coalescence of individual sorus-like fructifications to form compact fructification; **e**, **f** individual sorus-like fructification seen by LM with hyphae agglomerated in the substomatal cavities and emerged through the stomata to form strongly agglutinated subhymenial and hymenial layers. *Scale bars*: **a**, **b** = 1 mm, **c**–**f** = 100 μm

The phylogenetic analyses of the concatenated 18S/5.8S/28S/RPB2/TEF1 dataset (Table 2) revealed the representative of the Exobasidiales, *Exobasidium rhododendri* (Fuckel) C.E. Cramer, as the closest relative of *Ceraceosorus bombacis* supported by 83 ML bootstrap and 0.89 posterior probability (Fig. 4). The Ceraceosorales, Exobasidiales, Entylomatales, and Doassansiales were monophyletic, however, without statistical support. In congruence with the topology derived from D1/D2 28S sequences, the monophyly of the Exobasidiomycetes was not supported.

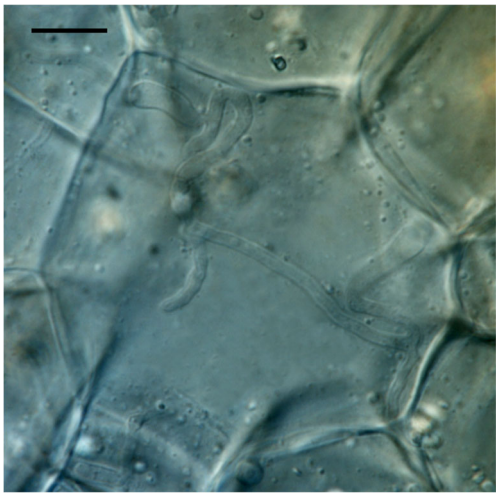

Fig. 7 Intracellular hyphae of *Ceraceosorus africanus* (from holotype) within a cell of *Bombax costatum* seen by LM. *Scale bar* = 10 μm

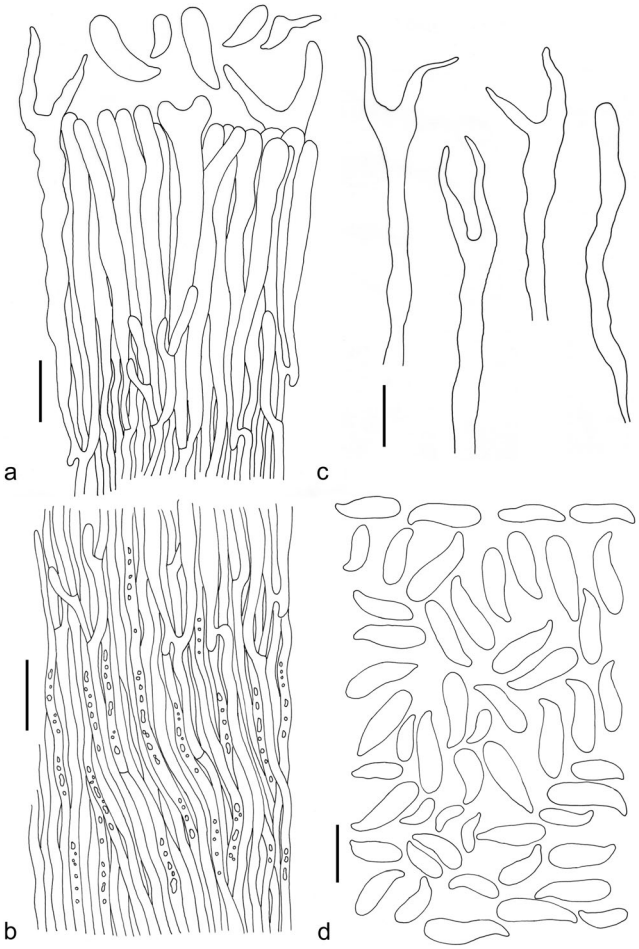

Fig. 8 Microstructure of *Ceraceosorus africanus* (all from holotype): **a** hymenial layer with basidioles, basidia, and basidiospores; **b** subhymenial layer with strongly agglutinated hyphae; **c** basidia and basidiole; **d** basidiospores. *Scale bars* = 10 μm

Taxonomy

Ceraceosorus africanus Piątek, K. Riess, Karasiński & M. Lutz, **sp. nov**.

Figs. 5a–f, 6, 7, and 8

MycoBank no. MB 816865

Etymology: The name refers to Africa, corresponding to the occurrence of this fungus on this continent contrasting with its sister species which occurs in India.

Type: Ghana, Northern Region: between Busunu and Fufulsu, ca. 18 km W of Fufulsu, 09° 09′ 36″ N, 01° 25′ 47″ W, elev. ca. 120 m a.s.l., on *Bombax costatum*, 5 November 2012, *leg. M. Piątek & N.S. Yorou* [holotype KRAM F-57385; type sequences are available in GenBank: KP413055 (18S), KP413036 (28S), and KP413030 (RPB2)].

Description: Parasitic on *Bombax costatum*. Fructifications on the leaves, infection local, only single leaves infected. Fructifications hypophyllous, (100–)200–500 μm thick, resupinate, widely effused, closely adnate, waxy, cracked in old specimens, initially sorus-like and forming circular patches up to 0.5 mm in diam. (best visible in the marginal parts of the fructifications), later coalescent forming continuous, compact areas up to 6.5 cm long, covering a part of the hypophyllous surface, usually about 1/2 or 2/3 of the leaf blade, typically starting from the petiole, exceptionally from other parts of the leaf blade, rarely covering the whole hypophyllous area; the corresponding epiphyllous area clearly discolored, usually yellowish or brownish, finally blackish and necrotic; the fructifications sometimes appear on the upper side of leaves but then only in the form of thin, linear parallel rows on both sides of central nerves. Hymenial surface smooth, white and slightly pruinose (visible under lens) in fertile parts, pinkish to pinkish brown in sterile parts. Hyphal system monomitic. Hyphae 1–3 μm wide, lacking clamps, thin- to thick-walled, mostly hyaline or pale yellow in substomatal cavities, sometimes with very fine, scattered oil drops inside; hyphae sparsely branched, straight or slightly sinuous, at first intracellular, later agglomerating, as intercellular hyphae, in substomatal cavities and emerging through the stomata to form a dense, compact structure in all parts of fructifications, including subhymenium. Hymenium forming a dense palisade of basidioles and basidia. Basidioles 3–4 μm wide, cylindrical, usually thin-walled, sometimes thick-walled with even walls up to 0.5 μm thick, often with granular oil drops inside. Paraphysoid hyphae sometimes present among basidioles, 0.4–0.8 μm wide, thin-walled, sparsely branched or unbranched. Basidia 48–82 × 3–5 μm (measured without sterigmata), dacrymycetoid, thin-walled, with cylindrical distal end, tapering toward basal septa, sometimes anastomozing in basal parts (below basal septa, H-shaped in outline), with two prominent sterigmata up to 15 μm long; sterigmata emerge from the apical part of basidioles, at first as semicircular, later as elongated and obtuse, and finally as subulate (pointed) projections,

collapsed after releasing the basidiospores, and difficult to be found in old hymenia. Basidiospores (6.5–)9–16(−18) × (3–)3.5–5(−5.2) µm (L = 12.72 µm, W = 3.95 µm, Q = 2.16–4.33, Q_m = 3.14, n = 90), elongated, lacrymoid, usually with curved apical part, obtuse at distal end, thin-walled, hyaline, with somewhat darkened and refractive apical part, inamyloid, acyanophilous. Conidiophores and conidia not observed.

Additional specimens examined (paratypes): Benin, Atakora Department: near the Tanougou Waterfalls (Chutes de Tanougou), ca. 55 km N of Natitingou, 10° 48′ 21″ N, 01° 26′ 16″ E, elev. ca. 265 m a.s.l., on *Bombax costatum*, 26 October 2011, *leg. M. Piątek & N.S. Yorou* (KRAM F-57386); Benin, Borgou Department: Bassa, ca. 12.5 km E of Parakou, 09° 17′ 08″ N, 02° 43′ 41″ E, elev. ca. 317 m a.s.l., on *B. costatum*, 28 October 2013, *leg. M. Piątek* (KRAM F-57390); Benin, Donga Department: Tébou, inselberg, ca. 28 km NE of Djougou, 09° 54′ 49″ N, 01° 48′ 36″ E, elev. ca. 485 m a.s.l., on *B. costatum*, 19 September 2015, *leg. M. Piątek* (KRAM F-58019); Benin, Donga Department: Forêt Classée de Béléfoungou, ca. 12 km NE of Djougou, 09° 47′ 54″ N, 01° 42′ 52″ E, elev. ca. 405 m a.s.l., on *B. costatum*, 19 September 2015, *leg. M. Piątek* (KRAM F-58020); Ghana, Northern Region: between Zibogo and Tugu, ca. 14 km E of Tamale, 09° 22′ 25″ N, 00° 43′ 04″ W, elev. ca. 130 m a.s.l., on *B. costatum*, 3 November 2012, *leg. M. Piątek & N.S. Yorou* (KRAM F-57387); Ghana, Northern Region: between Busunu and Fufulsu, ca. 13.5 km W of Fufulsu, 09° 09′ 05″ N, 01° 23′ 33″ W, elev. ca. 120 m a.s.l., on *B. costatum*, 5 November 2012, *leg. M. Piątek & N.S. Yorou* (KRAM F-57388); Togo, Kara Region: between Pya and Niamtougou, ca. 19 km N of Kara, 09° 42′ 10″ N, 01° 07′ 30″ E, elev. ca 345 m a.s.l., on *B. costatum*, 3 November 2013, *leg. M. Piątek & N.S. Yorou* (KRAM F-57389).

Comments: The description above is based on the holotype specimen from Ghana. The materials from other localities were roughly similar, with the exception of the specimen from Togo (KRAM F-57389) that differs in having 1-sterigmate basidia and longer basidiospores, which were up to 25 µm long. Such morphology of basidia and size of basidiospores are considered an abnormality or variation rather than the typical morphology of *Ceraceosorus africanus*. Basidiospores in 1-sterigmate basidia may be larger than in 2-sterigmate basidia because there are more nutrients available. Similar abnormalities in basidiospore sizes produced on 1–2-sterigmate and 4-sterigmate basidia were already observed in other fungi (Lebel and Castellano 2002; Kautmanová et al. 2012). The specimens collected in September 2015 from Benin were not fully mature.

Distribution and ecology: *Ceraceosorus africanus* was found at eight locations in Benin, Ghana, and Togo, within the Sudanian savanna biome. The northernmost location was found near the Tanougou Waterfalls in Benin, at about 10° 48′ 21″ N; the southernmost location ca. 13.5 km W of Fufulsu in Ghana, at about 09° 09′ 05″ N; the westernmost location ca. 18 km W of Fufulsu in Ghana, at about 01° 25′ 47″ W; and the easternmost location near Bassa in Benin, at about 02° 43′ 41″ E. These localities are situated in the core geographical range of *Bombax costatum* (Fig. 1), but it is likely that *C. africanus* occurs with the host plant over its distribution in West Africa. The habitat conditions for five of the eight locations where *C. africanus* was found were similar: open Sudanian savanna with scattered shrubs and trees, with infected trees of *B. costatum* fully exposed to the sun. At the locality near the Tanougou Waterfalls, the infected tree was found in the transition zone between gallery forest and rocky savanna in a semishaded place, at the locality in the Forêt Classée de Béléfoungou in the transition zone between gallery forest and the Sudanian woodland, and at the locality in Tébou on the top of the inselberg (Fig. 2). The first infection symptoms with immature fructifications were observed at the end of September (observations in 2015 in Benin). Infected leaves with mature fructifications were found at the end of October and at the beginning of November, which corresponds to the beginning of the dry season in West Africa. The phenological appearance of *C. africanus* is in agreement with phenology of plant pathogenic fungi in other tropical regions (Piepenbring et al. 2015). The level of infection was very low, ranging from a single infected leaf to about 1 % of infected leaves on one particular tree. At the type locality in Ghana, two trees of *B. costatum* were growing side by side, at a distance of ca. 5 m, and only one of them was infected by *C. africanus*, though the level of infection was ca. 1 %, which was the highest observed infection among all detected locations of *C. africanus*. The locality near the Tanougou Waterfalls in Benin was visited in 2011 and 2012, and a weak infection (one leaf) of the small tree was observed only in 2011, while in the subsequent year, the same tree was healthy. This suggests that infection by *C. africanus* may not appear on the same tree/population every year.

Discussion

Ceraceosorus africanus differs somewhat from the generic characters outlined in the diagnosis of *Ceraceosorus* (Cunningham et al. 1976), notably in lacking internal thickenings of basidia and in having a distinct hymenium produced on an external subhymenial layer (that could be referred to as sterile external stroma). Cunningham et al. (1976) reported in the generic diagnosis that *Ceraceosorus* shows an indeterminate hymenial thickening, but it is difficult to conclude what they meant by this term. Moreover, Cunningham et al. (1976) differentiated *Ceraceosorus* from *Dicellomyces* as lacking a well-defined external stroma, but this character was not included in the generic diagnosis, only mentioned in the text.

Considering the ontogenetical development and other aspects of morphology (intracellular hyphae, sorus-like fructifications at least in the initial stage of development, waxy and pinkish fructifications, morphology of basidia and basidiospores), as well as the same host genus (*Bombax* L.), both *C. africanus* and *Ceraceosorus bombacis* are however similar and should be considered con-generic. These divergent characters may therefore not be relevant at the generic level but useful to distinguish between *C. africanus* and *C. bombacis*. In addition, *C. africanus* is morphologically different from *C. bombacis* (Cunningham et al. 1976) in having somewhat longer and thinner basidia [48–82 × 3–5 μm vs. (20–)35–50(−85) × 3–6.5 μm], shorter sterigmata (up to 15 μm long vs. up to 22 μm long) and slightly longer and wider basidiospores [(6.5–)9–16(−18) × (3–)3.4–4.8(−5.2) μm vs. (5.5–)8.5–14(−18.5) × 2–4.5 μm)] and, most importantly, in having a different macroscopic symptoms of infection. The infection of leaves is apparently local in *C. africanus*, i.e., only single leaves on one tree are infected, but fructification covers large parts of the leaf surface and resembles leaf blight. In contrast, the infection of *C. bombacis* resembles leaf spot and comprises small light pink to brown spots scattered over the lower leaf surface (Bakshi et al. 1972; Fig. 5g–i). Although it is premature to generalize this observation based on two known species, this difference in macroscopic symptoms resembles those in *Exobasidium* Woronin (Begerow et al. 2002a; Piątek et al. 2012), where the same host plant may be infected by species producing leaf spots or leaf blights (as surculicolous or systemic infection), e.g., *Exobasidium arescens* Nannf. and *Exobasidium myrtilli* Siegm., respectively, on *Vaccinium myrtillus* L. (Ericaceae), and many others (Nannfeldt 1981).

The prominent and compact corticioid fructifications of *Ceraceosorus africanus* characterized by well-developed subhymenia and hymenia composed of dense palisades of basidioles and basidia, and resembling, for example, agaricomycotinous genera *Gloeocystidiellum* Donk, *Phanerochaete* P. Karst., or *Phlebia* Fr., represent a unique morphological trait in the subphylum Ustilaginomycotina. Indeed, some species of *Exobasidium*, like *Exobasidium vaccinii* (Fuckel) Woronin, form corticioid hymenia (Nannfeldt 1981), but not as prominent and well developed as in *C. africanus*. The fructification of other nonteliosporic Ustilaginomycotina species is rather small or loose and delicate. Obviously, this character cannot be considered an autapomorphy for *C. africanus* since this type of fructification evolved multiple times in different Basidiomycota lineages, most commonly in different clades of Agaricomycotina (corticioid fungi, Larsson 2007), but also in some species of Pucciniomycotina, e.g., in the rust *Stereostratum corticioides* (Berk. & Broome) H. Magn. (Cummins and Hiratsuka 2003), or in the atractiellomycete *Saccoblastia farinacea* (Höhn.) Donk (Bauer et al. 2006). However, it expands the structural diversity of sporulation within the Ustilaginomycotina.

The two currently known *Ceraceosorus* species are fully allopatric, separated both by geographic distribution and host plant species. *Ceraceosorus africanus* is known only on *Bombax costatum* in West Africa and *Ceraceosorus bombacis* occurs only on *Bombax ceiba* in India. The natural range of both host plants is widely separated and does not overlap in any area. *B. costatum* is naturally distributed in West Africa, while *B. ceiba* in tropical southern and eastern Asia, Indonesia, Philippines, Papua New Guinea, and northern Australia. Assuming that both *Ceraceosorus* species are strictly host species specific, it could be hypothesized that the speciation of *C. africanus* and *C. bombacis* was triggered by geographical isolation and ecological factors (host plant species).

The two currently known *Ceraceosorus* species are separated by a considerable genetic distance (uncorrected *p* values: 28S = 4.03 % or 83 bp, RPB2 = 18.5 % or 196 bp). Such a considerable genetic distance between *Ceraceosorus africanus* and *Ceraceosorus bombacis* could, e.g., be an indication for an ancient separation of the lineages under a model of neutral sequence evolution, or an indication for a selection of the loci under a nonneutral model of sequence evolution. Assuming an ancient separation of the lineages, the currently recognized species (1) may represent living descendants of a larger but extinct *Ceraceosorus* diversity, or (2) undiscovered species still may exist in poorly studied tropical countries, especially on the remaining *Bombax* species (*Bombax buonopozense* P. Beauv., *Bombax insigne* Wall., *Bombax mossambicense* A. Robyns; Malvaceae). The discovery of *C. africanus*, a common species producing conspicuous symptoms of infection on the common tree, *Bombax costatum*, but occurring in the phytopathologically unstudied Sudanian savanna biome of West Africa could support the latter assumption. Moreover, similar to other lineages of Ustilaginomycotina, saprobic yeast species may contribute to unknown *Ceraceosorus* diversity. In fact, one yeast species assigned to this genus was announced but not yet described (Kijpornyongpan and Aime 2014; Albu et al. 2015).

The discovery of *Ceraceosorus africanus* in West Africa raises the number of smut species parasitic on members of the Malvaceae. It also highlights the significance of scrutiny of the tropical ecosystems in order to increase the knowledge of fungal diversity and to find evolutionary unique "missing" fungal taxa in general (e.g., Isaac et al. 1993; Aime and Brearley 2012; Gazis et al. 2012) and smut fungi in particular. Previously, eight smut species were recognized on hosts of that family, including five teliosporic species from the genera *Entyloma* de Bary, *Geminago* Vánky & R. Bauer, and *Pericladium* Pass. (Table 3; Vánky 1996, 2011, 2012), and three nonteliosporic species from the genera *Ceraceosorus* and *Volvocisporium* Begerow, R. Bauer & Oberw. (Table 3; Cunningham et al. 1976; Begerow et

Table 3 The species of the Ustilaginomycotina infecting host plants in the Malvaceae

Species	Host plants	Subfamily	Occurrence	References
Ceraceosorus africanus	*Bombax costatum*	Bombacoideae	West Africa (Benin, Ghana, Togo)	This study
Ceraceosorus bombacis	*Bombax ceiba*	Bombacoideae	India	Bakshi et al. (1973), Cunningham et al. (1976)
Entyloma sidae-rhombifoliae	*Sida rhombifolia*	Malvoideae	Dominican Republic	Vánky (2012) (doubtful smut species according to Zundel 1939)
Geminago nonveilleri	*Triplochiton scleroxylon*	Helicteroideae	West Africa (Cameroon, Ivory Coast, Nigeria)	Vánky (2012)
Pericladium grewiae	*Grewia* spp. (11 spp.)	Grewioideae	Africa, Australia, India	Vánky (2012)
Pericladium piperis	*Grewia* sp.	Grewioideae	South Africa	Vánky (2012)
Pericladium tiliacearum	*Grewia rotundifolia*, *G. tiliaefolia*, *G. villosa*	Grewioideae	India, South Africa	Vánky (2012)
Volvocisporium grewiae	*Grewia* cf. *flavescens*	Grewioideae	Namibia	Ritschel et al. (2008)
Volvocisporium triumfetticola	*Triumfetta rhomboidea*	Grewioideae	India	Begerow et al. (2001)

al. 2001; Ritschel et al. 2008). All of these genera are known exclusively on hosts in the Malvaceae with the exception of *Entyloma*, which members infect hosts in multiple dicot families (Begerow et al. 2002b; Vánky 2012; Lutz and Piątek 2016), and only *Entyloma sidae-rhombifoliae* Cif. (Ciferri 1928, but doubtful smut species according to Zundel 1939) is parasitic on the Malvaceae. Interestingly, *Ceraceosorus* and *Geminago* are restricted to host species from different subfamilies, the Bombacoideae and the Helicteroideae, respectively, while *Pericladium* and *Volvocisporium* are both parasitic on members of the Grewioideae (Table 3). However, these smut genera are not phylogenetically related, indicating that in the evolution of smut fungi, hosts in Malvaceae were colonized several times from different ancestors. Interestingly, all smut species on hosts in the Malvaceae, except North American *En. sidae-rhombifoliae* (Vánky 2012), are confined to the African-Indian-Australian part of Gondwanaland, and *Ceraceosorus* and *Volvocisporium* have allopatric species in Africa and India. The disjunctive occurrence of smuts between Africa and India was already reported for some species (e.g., Piątek et al. 2014), but the mechanisms responsible for such a distribution pattern are not yet known.

The phylogenetic affinities of the Ceraceosorales, represented by *Ceraceosorus bombacis*, have previously been analyzed only few times (Begerow et al. 2006, the same analyses later repeated by Begerow et al. 2014; Albu et al. 2015; Sharma et al. 2015). In the multigene analyses, including ITS, 28S, ATP6, and β-tubulin sequences, conducted by Begerow et al. (2006), *C. bombacis* was nested within the Exobasidiomycetes occupying a common branch with *Tilletiopsis albescens* Gokhale and members of the Entylomatales, however without statistical support. In the multigene analyses, including 18S, ITS, 28S, TEF1, and β-tubulin sequences, conducted by Albu et al. (2015), the Ceraceosorales was weakly supported as member of the Ustilaginomycetes. Thus, the affinities of the Ceraceosorales were not resolved by these analyses (Begerow et al. 2006; Albu et al. 2015).

The molecular phylogenetic analyses of the single gene dataset (28S) conducted in this study (Fig. 3), including both *Ceraceosorus africanus* and *Ceraceosorus bombacis*, supported the monophyly of *Ceraceosorus* and confirmed the placement of this genus and the order Ceraceosorales within the Ustilaginomycotina in an unresolved relation to the Ustilaginomycetes and Exobasidiomycetes.

The molecular phylogenetic analyses of the multigene dataset (18S/5.8S/28S/RPB2/TEF1; Fig. 4) revealed *Exobasidium rhododendri*, i.e., member of the Exobasidiales, as the closest relative of *Ceraceosorus*, both clustering together with *Entyloma calendulae*, i.e., member of the Entylomatales. Thus, it is plausible that the Ceraceosorales belongs to the Exobasidiomycetes (sensu Begerow et al. 2006). It is noteworthy, however, that in this study, the Exobasidiomycetes were not resolved as monophyletic in both the single gene and the multigene analyses (Figs. 3 and 4). This is in congruence with several previous analyses made by, e.g., Begerow et al. (2006, dataset 18S/ITS/28S/ATP6/β-tubulin, their Supplementary Figure 1) or Wang et al. (2014). Interestingly, considering the ultrastructural characters studied by Bauer et al. (1997), the Exobasidiomycetes also have been divided into two groups: orders with species lacking an interaction apparatus (Georgefischeriales, Microstromatales, Tilletiales) and orders with species having an interaction apparatus (Entylomatales, Exobasidiales, Doassansiales), the latter referred to as superorder Exobasidianae. The ultrastructural characters of *Ceraceosorus bombacis* (Begerow et al. 2006, 2014), such as a local interaction zone with simple interaction apparatus, indicate its affinity to the superorder Exobasidianae. In this superorder, Exobasidiales and Doassansiales are characterized by

having a complex interaction apparatus, while Entylomatales is characterized by having a simple interaction apparatus (Bauer et al. 1997; Begerow et al. 2006, 2014). Thus, the Ceraceosorales shares this ultrastructural trait only with the Entylomatales. This could suggest a close phylogenetic relation of the Ceraceosorales to the Entylomatales, which was also revealed (though statistically not supported) in the multigene analyses of Begerow et al. (2006, 2014), as well as inferred and well supported in the multigene analyses of the current study.

The superorder Exobasidianae has been resolved as monophyletic in the ITS/28S/ATP6/β-tubulin analysis of Begerow et al. (2006). In the current multigene analyses, the Exobasidianae formed a monophyletic group but without statistical support (Fig. 4). Thus, we are in favor of the following evolutionary scenario for the Ceraceosorales and the Exobasidiomycetes (sensu Begerow et al. 2006): (1) the order Ceraceosorales belongs to the superorder Exobasidianae and is related to the Entylomatales and the Exobasidiales; and (2) the class Exobasidiomycetes is composed of two major lineages, probably deserving their classification at the class level, defined by ultrastructural characters of cellular interactions. A robust phylogenetic hypothesis for this scenario may not be resolved by multigene phylogenetic analyses, reinforcing the similar conclusion of Begerow et al. (2006) for a different dataset. Probably, only phylogenomics may provide a robust hypothesis for the phylogeny of the Ustilaginomycotina. In recent phylogenomic analyses conducted by Sharma et al. (2015), including genomes of six species of the Ustilaginomycotina, all branches received maximum statistical support. *Ceraceosorus bombacis* was resolved as sister species to *Ustilago maydis* (DC.) Corda, *Sporisorium reilianum* (J.G. Kühn) Langdon & Full., *Ustilago hordei* (Pers.) Lagerh., and *Melanopsichium pennsylvanicum* Hirschh., while *Malassezia globosa* Midgley, E. Guého & J. Guillot was resolved as basal to all sampled species. The sampling by Sharma et al. (2015) included members of only three orders (Ustilaginales, Ceraceosorales, Malasseziales), and the inclusion of representatives of the remaining orders to phylogenomic analyses is a challenge for future studies.

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