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*, parasite 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

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
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, Georgiáscheriales, Tilletiales, Urocystidales, and Ustilaginales. These orders were interspersed by nonteliosporic taxa forming two main lineages referred to as the orders Exobasidiales (incl. Graphioldales, 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) smut fungi since they showed relationships to rust fungi.

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 (Malvaceae), in the Sudanian savanna biome in West Africa. This unknown fungus was consistently found on most members of Ceraceosorales infecting a native woody species, the silk cotton tree Bombax costatum (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 Brachysbasidiales (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 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; Cabáñez 2014; Wang et al. 2014), mite-associated yeasts (Boehkout et al. 2003), and saprobic yeasts isolated from diverse ecosystems (Begerow et al. 2000; Boehkout 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 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...
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.
Molecular phylogenetic analyses

For DNA isolation, approximately 0.25 cm² of dried leaves of *Bombax costatum* with disease symptoms and a 0.25 cm² 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 Fufulous, ca. 18 km W of Fufulous, 5 Nov. 2012, leg. M. Piątek & N.S. Yorou, KRAM F-57385 (holotype) |
| Ceraceosorus africanus   | Bombax costatum    | KP413035                    | Ghana, Northern Region: between Zibogo and Tugui, 3 Nov. 2012, leg. M. Piątek & N.S. Yorou, KRAM F-57387 |
| Ceraceosorus africanus   | Bombax costatum    | KP413039                    | Ghana, Northern Region: between Busunu and Fufulous, ca. 13.5 km W of Fufulous, 5 Nov. 2012, leg. M. Piątek & N.S. Yorou, KRAM F-57388 |
| Ceraceosorus africanus   | Bombax costatum    | KP413037                    | Togo, Kara Region: between Pya and Niamey, 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 Ustilaginozyma 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. Schröd., Sympossidiomycopsis paphiopedilii Sugiy., Tokuoka & Komag., Urocystis colchici (Schldtl.) 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; Vánký 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 Kajdasz 2009; Deadman et al. 2011; Vánký 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; Kolarik 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 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.
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.

Table 2  List of species and GenBank accession numbers used for the combined 18S/5.8S/28S/RPB2/TEF1 analyses (see Fig. 4)

| Species                             | 18S Accession | 5.8S Accession | 28S Accession | RPB2 Accession | TEF1 Accession |
|-------------------------------------|---------------|----------------|---------------|----------------|---------------|
| Anthracocystis flocculosa           | DQ892923      | HQ115653       | AY745712      | –              | DQ28598       |
| Ceraceosorus bombacis               | DQ75377       | KP413083       | KP413033      | KP413029       | KP413032      |
| Entyloma calendulae                 | DQ663688      | DQ663689       | DQ663687      | DQ663690       | DQ663691      |
| Erratomyces patellii                | DQ63309       | DQ663692       | AY818966      | –              | DQ663695      |
| Exobasidium rhododendri             | AJ271381      | EU784219       | DQ667151      | DQ667154       | DQ667156      |
| Malassezia furfur                   | CF706457      | EU513202       | AY745725      | CF706516       | CF706468      |
| Microbotryum violaceum s. lato      | U77062        | JN942213       | DQ789982      | DQ789985       | DQ074573      |
| Microstroma juglandis               | DQ363313      | DQ317632       | KP413052      | DQ789989       | DQ789991      |
| Moesziomyces bullatus               | DQ831012      | AY740153       | DQ831011      | DQ831014       | –             |
| Monillicella acetobutans            | CF706443      | EU252153       | JN938879      | CF706523       | CF706476      |
| Puccinia graminis                   | AY125409      | HQ317585       | AF522177      | XM_003321826   | X73529        |
| Quambalaria cyanescens              | CF706440      | NR_111202      | DQ317615      | CF706531       | CF706485      |
| Rhamphospora nymphaeae              | DQ363311      | DQ831034       | DQ831032      | DQ831035       | DQ831036      |
| Schizoneola melanogramma            | DQ832211      | DQ832212       | DQ832210      | DQ832213       | DQ832215      |
| Sporisorium reilianum               | DQ832229      | AF135432       | DQ832228      | DQ832231       | CF706472      |
| Sympodiomycopsis paphiopedilii      | DQ832239      | DQ832240       | DQ832238      | DQ859894       | –             |
| Tilletia golosokovii                | DQ832247      | EU257570       | AY818999      | DQ832249       | DQ832251      |
| Tilletiaria anomala                 | D83193        | DQ234558       | AY745715      | AY803750       | DQ835991      |
| Tilletiopsis fulvescens             | D83189        | AB025705       | AJ235281      | CF706530       | CF706483      |
| 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      | AY845636       | AY851600      |

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

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

\[ \text{Ceraceosorus africanus on Bombax costatum} \]
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)
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.

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**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.
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), dacyrmycetoid, 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.

![Fig. 7 Intracellular hyphae of Ceraceosorus afric...n (from holotype) within a cell of Bombax costatum seen by LM. Scale bar = 10 μm](image-url)

![Fig. 8 Microstructure of Ceraceosorus afric...n (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](image-url)
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, Qm = 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 included in the generic diagnosis, only mentioned in the text.

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.

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 Sieg., 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 vaccini (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 Stereocereus corticioides (Berk. & Broome) H. Magn. (Cummins and Hiratsuka 2003), or in the atractiellomycete Saccoblastia farinacea (Höh.) 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 insignis 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 Volvocisporeum 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  | Heliceroideae | 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 growiae      | Grewia ct. flavescens     | Grewioideae | Namibia                     | Ritschel et al. (2008) |
| Volvocisporium triumfetticola | Triumphetta 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 Heliceroideae, 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 E. 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 a 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 Exobasidioidea. 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 Exobasidioidea. 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 Exobasidiales has been resolved as monophyletic in the ITS/28S/ATP6/β-tubulin analysis of Begerow et al. (2006). In the current multigene analyses, the Exobasidiales 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 Exobasidiales (sensu Begerow et al. 2006): (1) the order Ceraceosorales belongs to the superorder Exobasidiales 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 Melanoschisium pennsylvanicum Hirsch., 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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