

# A molecular phylogenetic framework for *Anthracocystis* (Ustilaginales), including five new combinations (inter alia for the asexual *Pseudozyma flocculosa*), and description of *Anthracocystis grodzinskae* sp. nov.

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**Abstract** The genus *Anthracocystis* (Ustilaginales, Ustilaginaceae) was recently reinstated for grass-infecting species of smut fungi that have sori with a peridium composed of mostly fungal cells, filiform or slender columellae, persistent spore balls usually composed of dimorphic spores, and lacking sterile cells between spore balls. In this study, *Anthracocystis grodzinskae* sp. nov. on *Euclasta condylotricha* is described and illustrated from the Sudanian savanna biome in Benin (West Africa). The new species is compared with two other smut fungi known on *Euclasta condylotricha*, namely *Sporisorium euclastae* and *Anthracocystis ischaemoides*, in Zambia. It differs from these species in a number of morphological characters that are discussed in detail. The systematic position of *A. grodzinskae* was investigated in a phylogenetic analysis with a concatenated supermatrix of the internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA. The dataset included all representatives of *Anthracocystis* for which sequences were available in the National Center for Biotechnology Information's (NCBI's) GenBank and that were linked to reliably identified source

specimens, related yeast species, and unnamed yeast strains or environmental sequences. The phylogenetic hypothesis derived from the dataset is intended to serve as a backbone tree for *Anthracocystis*. 19 ITS and 13 LSU sequences were tracked to represent sequences generated from type specimens (holotypes, isotypes or paratypes). These type sequences are recommended to be deposited in the RefSeq Targeted Loci database. This study provides the first explicit evidence that several asexual species are nested within the *Anthracocystis* lineage. The yeast sequences were scattered in different subclades of *Anthracocystis* and none of them could be directly assigned to a teleomorphic species. Only one of these yeast anamorphs was assigned to a species, namely *Pseudozyma flocculosa*. In line with the current code of nomenclature, and following recent practice of merging yeast species with sexual species under the older generic name, this yeast is recombined into *Anthracocystis* as *A. flocculosa*. Additionally, new combinations are proposed for four teliosporic species (*Anthracocystis andrewmitchellii*, *A. christineae*, *A. kenyana*, *A. warambiensis*).

**Keywords** *Anthracocystis* · Phylogeny · Plant pathogens · *Pseudozyma flocculosa* · Smut fungi · Ustilaginomycotina · Yeasts

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## Introduction

The smut genus *Anthracocystis* Bref. has been described for a causative agent of head smut of millet (*Panicum miliaceum* L.), *Anthracocystis destruens* (Schltdl.) Bref. (Brefeld 1912; McTaggart et al. 2012b), but has not been accepted by most smut researchers. Until very recently, it was forgotten or considered synonymous with *Sporisorium* Ehrenb. ex Link (Vánky 2002). Molecular phylogenetic studies by Stoll et al.

(2003, 2005) revealed that *Sporisorium* species are split into two main lineages. This finding was later confirmed by other molecular analyses (Cunnington et al. 2005; Vánky et al. 2006; Vánky and Lutz 2011; McTaggart et al. 2012a; Shivas et al. 2013; Zhang et al. 2013). These two main lineages included the type species of *Sporisorium* (*S. sorghi* Ehrenb. ex Link) and *Anthracocystis* (*A. destruens*), respectively. In consequence, McTaggart et al. (2012c) reinstated *Anthracocystis* for the lineage containing *A. destruens*, and provided diagnostic characters to separate *Sporisorium* and *Anthracocystis*. Thus, the most important diagnostic characters of the resurrected and emended genus *Anthracocystis* are: a peridium composed of mostly fungal cells, filiform or slender columellae, persistent spore balls usually composed of dimorphic spores (i.e., morphologically different inner and outer spores), and a lack of sterile cells between spore balls.

The members of *Anthracocystis* are predominantly tropical species, with several species occurring in warm temperate regions, and few species introduced together with their hosts to temperate areas. Two species are pathogenic to cultivated crops, i.e., *Anthracocystis destruens* causing head smut of millet and *Anthracocystis ehrenbergii* (J.G. Kühn) McTaggart & R.G. Shivas causing long smut of sorghum. Currently, 126 *Anthracocystis* species are known (McTaggart et al. 2012c; Denchev and Denchev 2013), of which 60 species were reported from Africa (Vánky et al. 2011). The number of *Anthracocystis* species in Africa may, however, be significantly larger, as this continent has not been sufficiently explored for smut fungi. There are, potentially, many interesting species within continental or regional scales, as was demonstrated by recent findings of smut fungi from herbarium materials and field studies (Piątek 2006, 2009, 2015; Piątek and Vánky 2007; Piątek et al. 2008, 2012, 2014, 2015).

In recent surveys to West Africa, many grasses were screened for smut infections. *Euclasta condylotricha* (Hochst. ex Steud.) Stapf was continuously smut-disease-free in most of the surveyed locations. In 2012, however, at two places near the southern border of the Pendjari National Park in northern Benin, moderate infections on inflorescences of *Euclasta condylotricha* were found that were caused by an ovaricolous smut of the genus *Anthracocystis*. This study aimed to resolve the specific identity of this smut using light and scanning electron microscopy and DNA sequence analyses, and to provide some ecological information obtained from its natural environment.

The phylogenetic placement of this smut fungus was investigated with the internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA (rDNA). Sequences from all representatives of *Anthracocystis* that were available in the National Center for Biotechnology Information's (NCBI's) GenBank and that were linked to reliably identified source specimens, related yeast species, and

unnamed strains or environmental sequences were included in the analyses. Therefore, a second aim of this study was to provide a phylogenetic framework for *Anthracocystis* that could serve as a backbone tree for future studies on the genus. Additionally, teleomorphic and anamorphic species are unified under one generic name, fulfilling the requirements of the current *International Code of Nomenclature for algae, fungi, and plants* (ICN).

## Materials and methods

### Specimen sampling and morphological examination

The specimens examined are listed in Table 1. The herbarium specimens are deposited in KRAM F. The characteristics of sori, spore balls, spores, and peridial cells were studied using dried herbarium material. The specimens were examined by light microscopy (LM) and scanning electron microscopy (SEM). For LM, small pieces of sori were mounted in 80 % lactic acid, heated to boiling and cooled, and then examined under a Nikon Eclipse 80i light microscope. LM micrographs were taken with a Nikon DS-Fi1 camera. 20 spore balls, 50 spores and 10 peridial cells were measured from each specimen, at a magnification of  $\times 1000$ , using NIS-Elements BR 3.0 imaging software. Except for the walls of spores and peridial cells, the measurements were adjusted to the nearest 0.5  $\mu\text{m}$ . Spore size range, and the mean and standard deviation were calculated for each investigated specimen (Table 1). The species descriptions include the combined values from all measured specimens. For SEM, spore balls with spores were mounted on carbon tabs and fixed to an aluminium stub with double-sided transparent tape. The stubs were sputter-coated with carbon using a Cressington sputter-coater and viewed under a Hitachi S-4700 scanning electron microscope, with a working distance of ca. 12–13 mm. SEM micrographs were taken in the Laboratory of Field Emission Scanning Electron Microscopy and Microanalysis at the Institute of Geological Sciences of the Jagiellonian University, Kraków (Poland).

### DNA extraction, polymerase chain reaction (PCR), and sequencing

Genomic DNA was isolated directly from herbarium specimens. For methods of isolation and crushing of fungal material, DNA extraction, amplification of the ITS 1 and ITS 2 regions of the rDNA including the 5.8S rDNA (ITS, about 740 bp) and the 5'-end of the nuclear large subunit ribosomal DNA (LSU, about 640 bp), purification of PCR products, sequencing, and processing of the raw data see Lutz et al. (2004, 2012). DNA sequences determined for this study were deposited in GenBank (accession numbers are given in Fig. 1, Tables 1 and 2).

**Table 1** Spore size ranges, mean spore sizes with standard deviations and GenBank accession numbers for specimens of *Anthracoystis grodzinskae* examined in the course of this study

Characters studied	Reference specimens	
	KRAM F-57394	KRAM F-57395
Size of outer spores ( $\mu\text{m}$ )	(10.0–)10.5–12.0 $\times$ (9.0–)9.5–11.5	10.0–11.5(–12.0) $\times$ (9.0–)9.5–10.5(–11.5)
Average size of outer spores with standard deviation ( $\mu\text{m}$ )	11.4 $\pm$ 0.5 $\times$ 10.2 $\pm$ 0.7	10.8 $\pm$ 0.5 $\times$ 10.1 $\pm$ 0.5
Size of inner spores ( $\mu\text{m}$ )	8.5–12.0(–14.0) $\times$ (7.5–)8.5–11.0(–12.0)	9.5–11.5(–12.0) $\times$ (8.5–)9.0–10.5(–11.0)
Average size of inner spores with standard deviation ( $\mu\text{m}$ )	10.7 $\pm$ 1.3 $\times$ 9.7 $\pm$ 0.9	10.6 $\pm$ 0.6 $\times$ 9.7 $\pm$ 0.5
GenBank acc. no. (ITS/LSU)	KP297998/KP297996	KP297999/KP297997

## Phylogenetic analyses

Blast searches (Altschul et al. 1997) for both the ITS and LSU sequences of the smut specimens on *Euclasta condylotricha* revealed closest similarity to species of *Anthracoystis* (*sensu* McTaggart et al. 2012c) or *Pseudozyma* Bandoni emend. Boekhout. The phylogenetic position of the smut specimens on *Euclasta condylotricha* was determined from their concatenated ITS+LSU sequences within a dataset that covered all ITS and LSU sequences of *Anthracoystis* available in GenBank, as well as all sequences of yeast isolates and uncultured fungus clones that clustered within *Anthracoystis*. There were two exceptions from the GenBank data that were excluded: doubtful sequences of *Sporisorium scitamineum* (Syd.) M. Piepenbr., M. Stoll & Oberw. (EF185083, ITS) and *Sporisorium nervosum* Vánky, C. Vánky & R.G. Shivas (AY740057/AY740110, ITS/LSU). GenBank accession numbers of the sequences used are given in Fig. 1 and Tables 1 and 2.

Sequences were aligned with MAFFT 7.215 applying the L-INS-i option (Kato and Standley 2013). The alignment was deposited in TreeBASE (S17942). To obtain reproducible results, manipulation of the alignment by hand as well as manual exclusion of ambiguous sites were avoided, as suggested by Giribet and Wheeler (1999) and Gatesy et al. (1993). Instead, highly divergent portions of the alignment were omitted using GBLOCKS 0.91b (Castresana 2000) with the following options: ‘Minimum Number of Sequences for a Conserved Position’: 33, ‘Minimum Number of Sequences for a Flank Position’: 33, ‘Maximum Number of Contiguous Non-conserved Positions’: 8, ‘Minimum Length of a Block’: 5, and ‘Allowed Gap Positions’ to ‘With half’. The resulting alignment [new number of positions: 1212 (36 % of the original 3362 positions), number of variable sites: 422] was used for phylogenetic analyses using a Maximum Likelihood (ML) and a Bayesian Approach (BA) following Vasighzadeh et al. (2014). In line with the results of McTaggart et al. (2012a), trees were rooted with *Langdonia confusa* (H.S. Jacks.) McTaggart & R.G. Shivas and *Triodiomyces triodiae* (Vánky) McTaggart & R.G. Shivas.

## Results

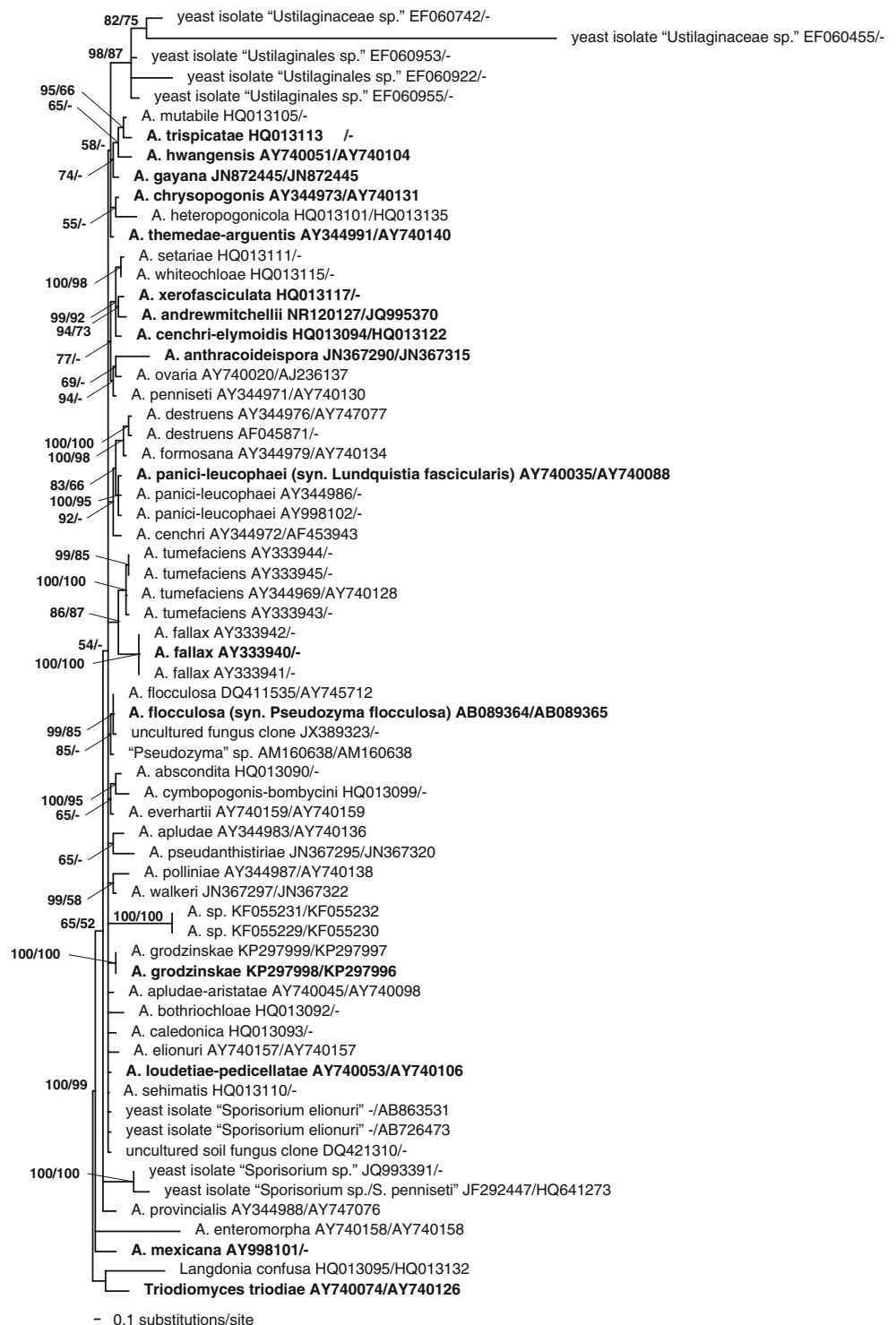
### Morphological analyses

The two examined specimens on *Euclasta condylotricha* showed rather inconspicuous, localized smut fungal sori in single ovaries of the inflorescences. The sori were enclosed by whitish peridia, composed mostly of fungal cells, except for a very thin outer layer including the remnants of host epidermis. The sori contained a mass of spore balls surrounding several filiform columellae. The spore balls were composed of dimorphic spores: a single layer of darker and distinctly ornamented outer spores, and numerous lighter and less distinctly ornamented inner spores. The sizes of outer and inner spores and average sizes were uniform and comparable both within each of the collections as well as between the two collections (Table 1). Sterile cells between the spore balls were not observed. The detailed morphological characterization of the smut fungus on *Euclasta condylotricha* is included in the species description and depicted in Figs. 2, 3 and 4.

### Phylogenetic analyses

The sequences of the smut specimens on *Euclasta condylotricha* were identical for both the ITS and LSU. The different runs of the BA that were performed and the ML analyses yielded consistent topologies. To illustrate the results, the consensus tree of one run of the BA is presented (Fig. 1). In all analyses, the species determined on the basis of host plants and morphology [*Anthracoystis destruens*, *A. fallax* (R.G. Shivas & Cunnington) McTaggart & R.G. Shivas, *A. panici-leucophaei* (Bref.) McTaggart & R.G. Shivas, *A. tumefaciens* (McAlpine) McTaggart & R.G. Shivas), or morphology and physiology [*Pseudozyma flocculosa* (Traquair, L.A. Shaw & Jarvis) Boekhout & Traquair] were inferred with high support values. The smut specimens on *Euclasta condylotricha* clustered within the sampled *Anthracoystis* species forming a distinct clade, however, in an unresolved relationship to the remaining *Anthracoystis* species, the sampled yeast isolates and the uncultured fungus clones.

**Fig. 1** Bayesian inference of phylogenetic relationships within the sampled *Anthracoystis* species: Markov chain Monte Carlo (MCMC) analysis of an alignment of concatenated ITS+LSU sequences using the GTR+I+G model of DNA substitution with gamma-distributed substitution rates and an estimated proportion of invariant sites, random starting trees and default starting parameters of the DNA substitution model. A 50 % majority-rule consensus tree is shown computed from 75,000 trees that were sampled after the process had become stationary. The topology was rooted to *Langdonia confusa* and *Triodomyces triodiae*. Numbers on branches before slashes are estimates for a posteriori probabilities, numbers on branches after slashes are Maximum Likelihood (ML) bootstrap support values. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. Sequences in **bold** are from holotype or isotype specimen (see Table 2). A. = *Anthracoystis*



## Taxonomy

### New species

*Anthracoystis grodzinskae* Piątek & M. Lutz, sp. nov.

Figs. 2–4

Mycobank # MB 813430

**Etymology:** Named in honour of Prof. Dr Hab. Krystyna Grodzińska, Polish botanist and ecologist, to mark her eightieth birthday in 2014. Her scientific life and activities were illustrated by Godzik and Zarzycki (2005).

**Type:** Benin, Atakora Department: near the Tanougou Waterfalls (Chutes de Tanougou), ca. 55 km N of Natitingou, 10°48'22"N, 01°26'16"E, elev. ca. 265 m a.s.l., on *Euclasta*

**Table 2** List of species used in the molecular phylogenetic analyses with host plants, countries of origin, voucher/strain information, GenBank accession numbers, and references. Sequences from type specimens are in **bold**

Species	Host plant	Country	Voucher	GenBank acc. no.		Reference
				ITS	LSU	
<i>A. abscondita</i>	<i>Schizachyrium fragile</i>	Australia	BRIP 49648	HQ013090	–	McTaggart et al. 2012a
<i>A. andrewmitchellii</i>	<i>Erneapogon</i> aff. <i>lindleyanus</i>	Australia	BRIP 54879, holotype	<b>NR120127(=JQ995369)</b>	<b>JQ995370</b>	Crous et al. 2012
<i>A. anthracoidespora</i>	<i>Pseudoraphis spinescens</i>	Papua New Guinea	BRIP HUV 18350, holotype	<b>JN367290</b>	<b>JN367315</b>	Kellner et al. 2011
<i>A. apludae</i>	<i>Apluda mutica</i>	India	Ust. Exs. 967 (M)	AY344983	AY740136	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. apludae-aristatae</i>	<i>Apluda mutica</i>	India	M 56590	AY740045	AY740098	Stoll et al. 2005
<i>A. bothriochloae</i>	<i>Dichanthium sericeum</i>	Australia	BRIP 51819	HQ013092	–	McTaggart et al. 2012a
<i>A. caledonica</i>	<i>Heteropogon contortus</i>	Australia	BRIP 51854	HQ013093	–	McTaggart et al. 2012a
<i>A. cenchri</i>	<i>Cenchrus pilosus</i>	Nicaragua	MP 1974 (TUB)	AY344972	AF453943	Stoll et al. 2003 (ITS), Piepenbring et al. 2002 (LSU)
<i>A. cenchri-elymoidis</i>	<i>Cenchrus elymoides</i>	Australia	BRIP 26491, holotype	<b>HQ013094</b>	<b>HQ013122</b>	McTaggart et al. 2012a
<i>A. chrysopogonis</i>	<i>Chrysopogon furvus</i>	Sri Lanka	Ust. Exs. 407 (M), isotype	<b>AY344973</b>	<b>AY740131</b>	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. cymbopogonis-bombycini</i>	<i>Cymbopogon bombycinus</i>	Australia	BRIP 52511	HQ013099	–	McTaggart et al. 2012a
<i>A. destruens</i>	<i>Panicum miliaceum</i>	Romania	Ust. Exs. 472 (M)	AY344976	AY747077	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. destruens</i>	Host not cited	Country not cited	CBS 327.33	AF045871	–	Roux et al. 1998
<i>A. elionuri</i>	<i>Elyonurus muticus</i>	Bolivia	MP 2601 (LPB)	AY740157	AY740157	Stoll et al. 2005
<i>A. enteromorpha</i>	<i>Themeda triandra</i>	South Africa	M 56602	AY740158	AY740158	Stoll et al. 2005
<i>A. everhartii</i>	<i>Andropogon virginicus</i>	Cuba	MP 2270 (HAJB)	AY740159	AY740159	Stoll et al. 2005
<i>A. fallax</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 27687, holotype	<b>AY333940</b>	–	Shivas et al. 2004
<i>A. fallax</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 27690, paratype	<b>AY333941</b>	–	Shivas et al. 2004
<i>A. fallax</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 27031, paratype	<b>AY333942</b>	–	Shivas et al. 2004
<i>A. flocculosa</i> (syn. <i>P. flocculosa</i> )	<i>Erysiphe polygoni</i> on leaf of <i>Trifolium pratense</i>	Canada	JCM 10321, type strain	<b>AB089364</b>	<b>AB089365</b>	Sugita et al. 2003
<i>A. flocculosa</i>	?	?	CBS 102.71	DQ411535	AY745712	Matheny & Hibbett unpubl. (ITS), Matheny et al. unpubl. (LSU)
<i>A. formosana</i>	<i>Panicum repens</i>	Taiwan	Ust. Exs. 688 (H.U.P. – ITS) or (M – LSU)	AY344979	AY740134	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. gayana</i>	<i>Andropogon gayanus</i>	Zimbabwe	M 0056604, isotype	<b>JN872445</b>	<b>JN872445</b>	Kellner et al. 2011



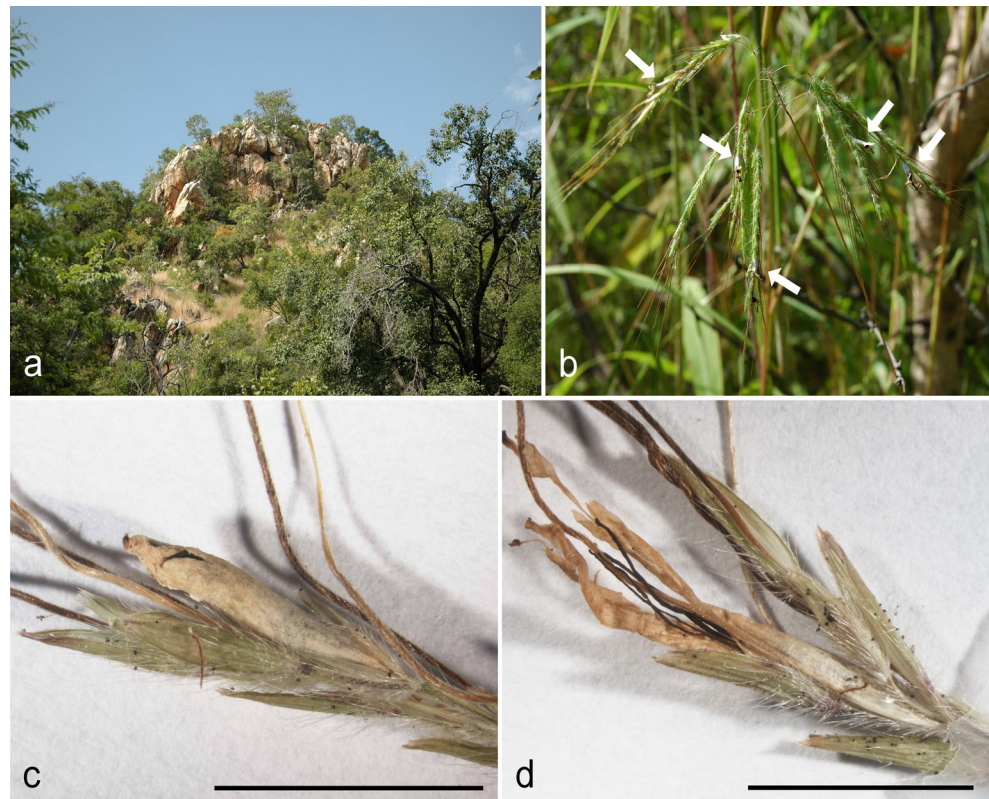
Table 2 (continued)

Species	Host plant	Country	Voucher	GenBank acc. no.		Reference
				ITS	LSU	
<i>A. grodzinskiae</i>	<i>Euclasta condylotricha</i>	Benin	KRAM F-57394, holotype	<b>KP297998</b>	<b>KP297996</b>	This study
<i>A. grodzinskiae</i>	<i>Euclasta condylotricha</i>	Benin	KRAM F-57395, paratype	<b>KP297999</b>	<b>KP297997</b>	This study
<i>A. heteropogoncola</i>	<i>Heteropogon contortus</i>	Australia	BRIP 51822	HQ013101	HQ013135	McTaggart et al. 2012a
<i>A. hwangensis</i>	<i>Sporobolus panicoides</i>	Zimbabwe	M 56607, isotype	<b>AY740051</b>	<b>AY740104</b>	Stoll et al. 2005
<i>A. loudettae-pedunculatae</i>	<i>Loudetia pedunculata</i>	South Africa	M 56615, isotype	<b>AY740053</b>	<b>AY740106</b>	Stoll et al. 2005
<i>A. mexicana</i>	<i>Andropogon gerardii</i>	Mexico	BRIP HUV 20498, holotype	<b>AY998101</b>	–	Cunnington et al. 2005
<i>A. mutabile</i>	<i>Cymbopogon refractus</i>	Australia	BRIP 44111	HQ013105	–	McTaggart et al. 2012a
<i>A. ovaria</i>	<i>Urochloa fasciculata</i>	Mexico	MP 1871 (XAL)	AY740020	AJ236137	Stoll et al. 2005 (ITS), Piepenbring et al. 1999 (LSU)
<i>A. panici-leucophaei</i> (syn. <i>Lundquistia fascicularis</i> )	<i>Digitaria brownii</i>	Australia	DAR 58832a, holotype of <i>Lundquistia fascicularis</i>	<b>AY740035</b>	<b>AY740088</b>	Stoll et al. 2005
<i>A. panici-leucophaei</i>	<i>Digitaria insularis</i>	Cuba	MP 2461 (HAJB)	AY344986	–	Stoll et al. 2003
<i>A. panici-leucophaei</i>	<i>Trichachne</i>	Colombia	BRIP HUV 7090	AY998102	–	Cunnington et al. 2005
<i>A. penniseti</i>	<i>Pennisetum setaceum</i>	Canary Islands	MP 2367 (TUB)	AY344971	AY740130	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. polliniae</i>	<i>Andropogon distachyos</i>	Greece	Ust. Exs. 690 (M)	AY344987	AY740138	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. provincialis</i>	<i>Andropogon gerardii</i>	U.S.A.	Ust. Exs. 759 (M)	AY344988	AY747076	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. pseudanthistiriae</i>	<i>Pseudanthistiria hispida</i>	India	KVU969 (=Ust. Exs. 969)	JN367295	JN367320	Kellner et al. 2011
<i>A. selimatis</i>	<i>Sehima nervosum</i>	Australia	BRIP 49671	HQ013110	–	McTaggart et al. 2012a
<i>A. setariae</i>	<i>Setaria surgens</i>	Australia	BRIP 49636	HQ013111	–	McTaggart et al. 2012a
<i>A. sp.</i>	<i>Schizachyrium delavayi</i>	China	HMAS 244668	KF055229	KF055230	Li et al. unpubl.
<i>A. sp.</i>	<i>Schizachyrium delavayi</i>	China	HMAS 244667	KF055231	KF055232	Li et al. unpubl.
<i>A. themedae-arguentis</i>	<i>Themeda arguens</i>	Indonesia	Ust. Exs. 855 (M), isotype	<b>AY344991</b>	<b>AY740140</b>	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. trispicatae</i>	<i>Eulalia trispicata</i>	Thailand	BRIP 47730, isotype	<b>HQ013113</b>	–	McTaggart et al. 2012a
<i>A. tumefaciens</i>	<i>Chrysopogon aciculatus</i>	Sri Lanka	Ust. Exs. 231 (M)	AY344969	AY740128	Stoll et al. 2003 (ITS), 2005 (LSU)
<i>A. tumefaciens</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 27688	AY333943	–	Shivas et al. 2004
<i>A. tumefaciens</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 27689	AY333944	–	Shivas et al. 2004
<i>A. tumefaciens</i>	<i>Chrysopogon fallax</i>	Australia	BRIP 26919	AY333945	–	Shivas et al. 2004

Table 2 (continued)

Species	Host plant	Country	Voucher	GenBank acc. no.		Reference
				ITS	LSU	
<i>A. walkeri</i>	<i>Themeda triandra</i>	Australia	KVU975 (=Ust. Exs. 975)	JN367297	JN367322	Kellner et al. 2011
<i>A. whiteochloae</i>	<i>Whiteochloa semitonsa</i>	Australia	BRIP 51860	HQ013115	–	McTaggart et al. 2012a
<i>A. xerofasciculata</i>	<i>Xerochloa laniflora</i>	Australia	BRIP 49682, holotype	<b>HQ013117</b>	–	McTaggart et al. 2012a
" <i>Pseudozyma</i> " sp.	Caterpillar gut of <i>Helicoverpa armigera</i>	Austria	HB 1204	AM160638	AM160638	Molnar & Prillinger unpubl.
Uncultured fungus clone	Soil	?	no strain (environmental sequence)	JX389323	–	Davinic, Moore-Kucera & Acosta-Martinez unpubl.
Uncultured soil fungus clone	Soil	U.S.A.	no strain (environmental sequence)	DQ421310	–	Waldrop et al. 2006
Ustilaginaceae sp. LM80	sea water	USA: Hawaii	LM80	EF060455	–	Mahdi 2006
Ustilaginaceae sp. LM436	Wood	USA: Hawaii	LM436	EF060742	–	Mahdi 2006
Ustilaginales sp. LM638	avian guano	USA: Palmyra Atoll	LM638	EF060922	–	Mahdi 2006
Ustilaginales sp. LM670	avian guano	USA: Palmyra Atoll	LM670	EF060953	–	Mahdi 2006
Ustilaginales sp. LM672	avian guano	USA: Palmyra Atoll	LM672	EF060955	–	Mahdi 2006
Yeast isolate " <i>Sporisorium elionuri</i> "	rice leaves	Thailand	DMKU-RE70	–	AB863531	Limtong unpubl.
Yeast isolate " <i>Sporisorium elionuri</i> "	?	Japan	IPM 46-16	–	AB726473	Takashima et al. 2012
Yeast isolate " <i>Sporisorium sp./Sporisorium pennisetii</i> "	bird	Italy: Sicily	UL143	JF292447	HQ641273	Francesca et al. 2012
Yeast isolate " <i>Sporisorium</i> sp."	grape berries	South Africa	IWBT-Y850	JQ993391	–	Setati et al. 2012
<i>Langdonia confusa</i>	<i>Aristida queenslandica</i>	Australia	BRIP 42670	HQ013095	HQ013132	McTaggart et al. 2012a
<i>Triodiomyces triodiae</i>	<i>Triodia microstachya</i>	Australia	BRIP HUV 17662, isotype	<b>AY740074</b>	<b>AY740126</b>	Stoll et al. 2005

**Fig. 2** *Anthracocystis grodzinskae* on *Euclasta condylotricha*: **a** type locality in Benin, infected plants occurred at the base of the hill, in the transition zone between rocky savanna and gallery forest, **b** inflorescence of *Euclasta condylotricha* with five scattered sori (indicated by white arrows) infecting single ovaries, **c** unopened sorus infecting the ovary, **d** opened sorus with four visible filiform columellae. Scale bars=1 cm



*condylotricha*, 29 Oct. 2012, M. Piątek & N.S. Yorou (holotype: KRAM F-57394).

**Description:** Parasitic on *Euclasta condylotricha*. Sori in single ovaries of the inflorescence, forming moderately long, cylindrical bodies, 10–15 mm long, 1.5–2 mm wide, at the base partly concealed by floral envelopes (glumes), covered by a white peridium (dirty yellow in dry material) that during maturity ruptures from the apex, revealing a dark brown, semi-agglutinated mass of spore balls surrounding several (up to 4–5) simple, filiform columellae. Peridium composed of an outer thin layer of host epidermis and an inner thin layer of peridial cells of fungal origin. Peridial cells in well-developed, adhering, parallel chains, single cells hyaline, usually long cylindrical to elongated, rarely broadly ellipsoidal, sometimes inflated, (5.0–)6.0–17.0×(3.5–)4.0–6.0(–7.0)  $\mu\text{m}$ , with 1–2 larger and several smaller spherical refractive bodies (lipid drops) in the cytoplasm; wall even, about 0.5  $\mu\text{m}$  thick, smooth. Spore balls dark reddish-brown, under pressure hardly disintegrating into single spores, polyhedrally subglobose, ellipsoidal, irregular or elongated, 50–180×50–130  $\mu\text{m}$ , composed of dozens of spores. Spores dimorphic, outer spores yellow brown, globose or subglobose on plane view, usually somewhat polyangular, and collapsed, especially on the free side, flattened on side view and cup-shaped, 10.0–12.0×(9.0–)9.5–11.5  $\mu\text{m}$  [av.  $\pm$  SD, 11.1 $\pm$ 0.6×10.2 $\pm$ 0.6  $\mu\text{m}$ , n=100/2], wall even, ca. 1.0  $\mu\text{m}$  thick, somewhat darker than the rest of the spore, finely punctate in LM, echinulate in SEM, spore profile smooth or

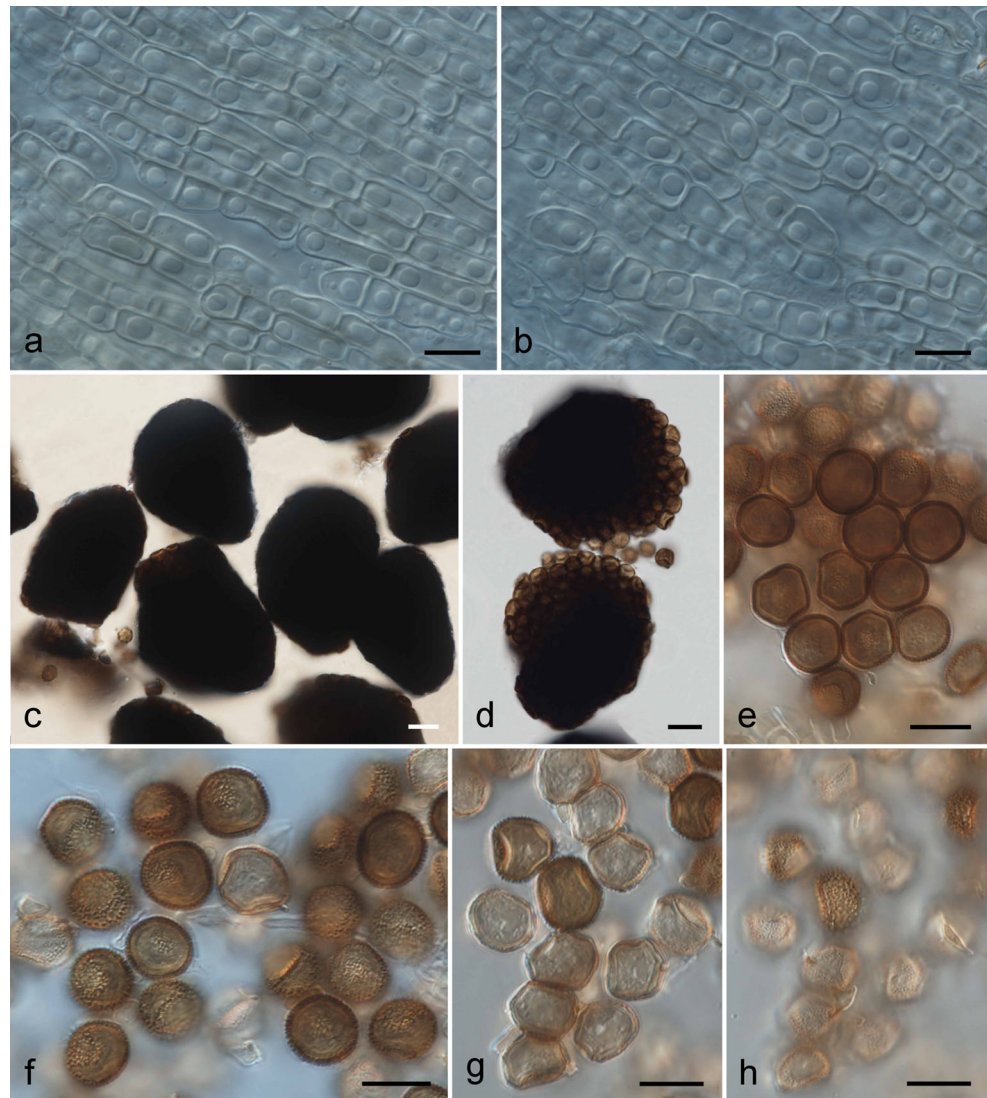
finely serrulate; inner spores subhyaline, pale light brown or pale olive-brown, globose or subglobose, usually irregularly polyangular, rugulose, and partly collapsed, 8.5–12.0(–14.0)×(7.5–)8.5–11.0(–12.0)  $\mu\text{m}$  [av.  $\pm$  SD, 10.7 $\pm$ 1.0×9.7 $\pm$ 0.8  $\mu\text{m}$ , n=100/2], wall even, ca. 1.0  $\mu\text{m}$  thick, very finely punctate in LM (seen with difficulty using DIC), finely verrucose in SEM. Sterile cells absent.

**Other specimen examined (paratype):** Benin, Atakora Department: near Tanougou village, ca. 27 km NE of Tanguiéta, 10°48'04"N, 01°25'43"E, elev. ca. 265 m a.s.l., on *Euclasta condylotricha*, 29 Oct. 2012, M. Piątek & N.S. Yorou (KRAM F-57395).

**Location, habitat and population size:** The two localities of *Anthracocystis grodzinskae* are located on the northern slopes of the Atakora Mountains in northern Benin, both within the Sudanian savanna biome, containing also Sahelian elements. The direct distance between both localities is 1.15 km. At the type locality, near the Tanougou Waterfalls, infected plants were found in the transition zone between a gallery forest and rocky savanna in a semi-shaded place. The level of infection of plants was small (less than 5 % of inflorescences were infected). At the second locality, near Tanougou village, infected plants were found in grassy *Acacia* scrubland, in a semi-shaded place. The level of infection of available plant specimens was very small, and only one infected clump was detected. None of the co-occurring different grass species at both localities were



**Fig. 3** *Anthracocystis grodzinskae* on *Euclasta condylotricha* seen by light microscopy (**a–e** from KRAM F-57394, **f–h** from KRAM F-57395): **a–b** peridial cells, **c–d** spore balls, **e–f** outer spores, median and superficial views, **g–h** outer and inner spores, median and superficial views. Scale bars: **a–b, e–h**=10  $\mu$ m, **c–d**=20  $\mu$ m



infected by *Anthracocystis grodzinskae*, indicating that this smut is specific to *Euclasta condylotricha*.

*Sporulating time*: October (Benin).

#### New combinations

The results of molecular phylogenetic analyses and/or morphological characters indicate that four *Sporisorium* species and one *Pseudozyma* species should be transferred to *Anthracocystis*. Therefore, we propose the following new combinations:

***Anthracocystis andrewmitchellii*** (R.G. Shivas, McTaggart & Vánky) M. Lutz & Piątek, comb. nov. – MycoBank # MB 813431.

*Basionym*: *Sporisorium andrewmitchellii* R.G. Shivas, McTaggart & Vánky, in Crous et al., Persoonia 28: 155 (2012).

*Comments*: This smut, described on *Enneapogon* aff. *lindleyanus* (Domin) C.E. Hubb. from Australia (Crous

et al. 2012), is nested inside the *Anthracocystis* lineage according to the current molecular analyses and its morphology fits the concept of the genus.

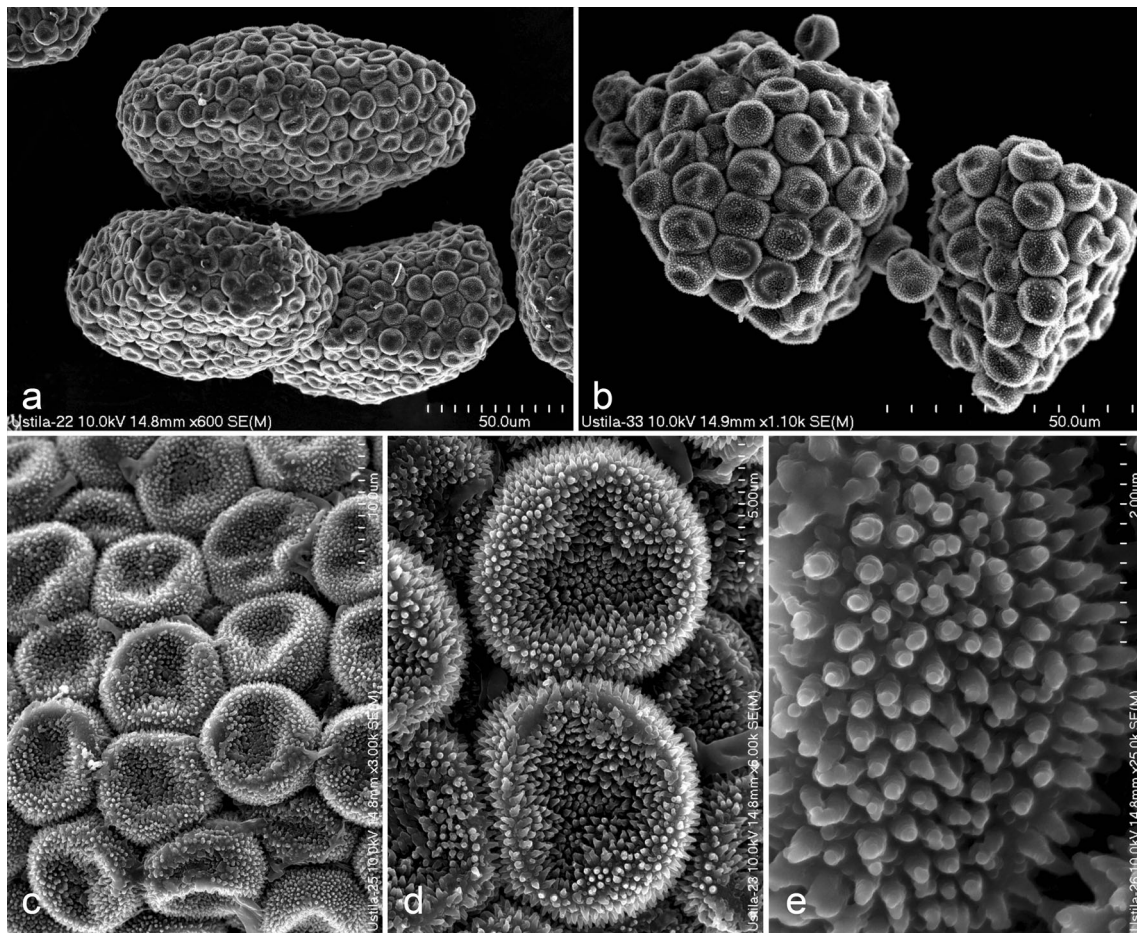
***Anthracocystis christineae*** (R.G. Shivas, McTaggart & Vánky) Piątek & M. Lutz, comb. nov. – MycoBank # MB 813437.

*Basionym*: *Sporisorium christineae* R.G. Shivas, McTaggart & Vánky, in Vánky et al., Mycol. Balcanica 6: 100 (2009).

*Comments*: This species was described on *Schizachyrium sanguineum* (Retz.) Alston from Bolivia (Vánky et al. 2009) and its morphology fits the concept of the genus *Anthracocystis*.

***Anthracocystis flocculosa*** (Traquair, L.A. Shaw & Jarvis) M. Lutz & Piątek, f.a. comb. nov. – MycoBank # MB 813438

*Basionym*: *Stephanoascus flocculosus* Traquair, L.A. Shaw & Jarvis, Can. J. Bot. 66: 927 (1988). – *Synonyms*: *Sporothrix flocculosa* Traquair, L.A. Shaw & Jarvis, Can. J. Bot. 66: 927 (1988), described as its anamorph; *Pseudozyma flocculosa*



**Fig. 4** *Anthracocystis grodzinskae* on *Euclasta condylotricha* seen by scanning electron microscopy (all from KRAM F-57394): **a–b** spore balls, **c–d** spores, **e** spore ornamentation. Scale bars: **a–b**=50  $\mu$ m, **c**=10  $\mu$ m, **d**=5  $\mu$ m, **e**=2  $\mu$ m

(Traquair, L.A. Shaw & Jarvis) Boekhout & Traquair, J. Gen. Appl. Microbiol. 41: 364 (1995).

**Comments:** This yeast species, described as foliar epiphyte of *Trifolium pratense* L. infected by a powdery mildew in Canada (Traquair et al. 1988) and commonly known by its name *Pseudozyma flocculosa* (Boekhout 1995), is nested inside the *Anthracocystis* lineage according to the molecular phylogenetic analyses and in line with the present rules of the ICN should be reallocated to this genus.

***Anthracocystis kenyana*** (Piątek) Piątek & M. Lutz, comb. nov. – MycoBank # MB 813439

**Basionym:** *Sporisorium kenyana* Piątek, Polish Bot. J. 51: 160 (2006).

**Comments:** This species, described on *Setaria pallidifusca* (Schumach.) Stapf. & C.E. Hubb. from Kenya (Piątek 2006), is morphologically similar to *Anthracocystis paniculeucophaei*, and is therefore reallocated to the genus *Anthracocystis*.

***Anthracocystis warambiensis*** (Piątek & R.G. Shivas) Piątek & M. Lutz, comb. nov. – MycoBank # MB 813440

**Basionym:** *Sporisorium warambiense* Piątek & R.G. Shivas, Mycol. Prog. 10: 58 (2011).

**Comments:** This species was described on *Xerochloa laniflora* Benth. from Australia (Piątek and Shivas 2011) and its morphology fits the concept of the genus *Anthracocystis*.

#### Emendation of *Anthracocystis*

By inclusion of yeast species, the emendation of the genus *Anthracocystis* defined previously by characters of teleomorphic species (Brefeld 1912; McTaggart et al. 2012c), is necessary. The generic diagnosis provided by McTaggart et al. (2012c) is included here without alternations and emended by characters placed in bold. The emended part is based on the selected features reported for *Anthracocystis flocculosa* (syn. *Pseudozyma flocculosa*; Traquair et al. 1988; Boekhout 1995). The correct assignment of yeast strains to the genus *Anthracocystis* requires DNA sequence data.

***Anthracocystis*** Bref., Unters. Gesamtgeb. Mykol. 15: 53 (1912), emend. McTaggart & R.G. Shivas, Persoonia 29: 119 (2012), emend. Piątek & M. Lutz, emend. nov.

= *Lundquistia* Vánky, Mycotaxon 77: 371 (2001). – Type species: *Lundquistia fascicularis* Vánky.



**Teleomorph parasitic on living plants.** “Sori replacing inflorescences, all of the racemes or localized in spikelets of an inflorescence. Peridium of vacuolated fungal cells surrounded by a single layer of host cells. Columella composed of vascular bundles surrounded by host parenchyma permeated by inter- and intra-cellular hyphae, often separated into several columellae each around a vascular bundle surrounded by parenchyma, filiform, flexuous, flattened. Sporogenous hyphae coiled. Spores compacted in spore balls, globose to subglobose, often outer spores darker than inner spores. Sterile cells few or absent” (McTaggart et al. 2012c). **Anamorph free-living, pseudozyma-like, producing conidiophores and blastoconidia, assimilating myo-inositol, showing positive diazotium blue B and urease reactions, and placed next to the teleomorphic species in the DNA sequence analyses.** Type species: *Anthracocystis destruens* (Schltdl.) Bref.

## Discussion

### Delineation of *Anthracocystis grodzinskae* sp. nov.

Macroscopic symptoms and micro-morphological characters observed in the smut specimens attacking *Euclasta condylotricha* in Benin match well the redefined genus *Anthracocystis* (McTaggart et al. 2012c). Molecular phylogenetic analyses confirm the morphological assignment, thus stressing the value of the phenotypic characters carved out by McTaggart et al. (2012c) to define and emend the reinstated genus *Anthracocystis*.

The grass genus *Euclasta* Franch. includes two tropical, annual species, *E. clarkei* (Hack.) Cope and *E. condylotricha*, the latter is distributed in south-western Asia, the former occurs in the grasslands and savannas of neotropical North America and South America, Africa and southern Asia (Clayton et al. 2006). To date, no smut fungi have been reported on *E. clarkei*, but two different smut fungi were previously found on *E. condylotricha*, namely *Sporisorium euclastae* Vánky and *Anthracocystis ischaemoides* (Henn.) McTaggart & R.G. Shivas. Both of them were recorded on *E. condylotricha* in Zambia (Vánky 2004) and both are morphologically clearly different from the smut specimens collected on that grass species in Benin, which are accordingly assigned to the novel species *Anthracocystis grodzinskae*.

*Sporisorium euclastae*, specific to *Euclasta condylotricha* and known only from the type locality in Zambia, is a true member of *Sporisorium*. It differs from the Beninese smut in having sori destroying all raceme-pairs in the inflorescences, one long flagelliform columella, spores of different ornamentation (densely echinulate, clearly visible with SEM comparison), and sterile cells (Vánky 2004). The sterile cells of *Sporisorium euclastae* would not be confused with the peridial cells of *Anthracocystis grodzinskae* since they are of different morphology (subglobose, ellipsoidal or irregular, forming irregular groups) and origin (intermixed with spores).

*Anthracocystis ischaemoides* is parasitic on different species of *Hyparrhenia* E. Fourn. in Africa (Vánky 2003). However, one smut collection on *Euclasta condylotricha* from Zambia was also assigned to this species by Vánky (2003, 2004). *Anthracocystis ischaemoides* differs from *A. grodzinskae* by having outer spores of different ornamentation (prominently verrucose on the free surface, smooth on the contact sides, visible under SEM) and smooth inner spores (Vánky 2003, 2004). The occurrence of *Anthracocystis ischaemoides* on *Euclasta condylotricha* may be wrongly identified and represent a cryptic species with morphological characters that were not observed by Vánky (2003, 2004).

*Anthracocystis grodzinskae* is described and illustrated, including characters of the peridial cells that are of fungal origin (McTaggart et al. 2012c), and which, with few exceptions, were not often studied in smut fungi. Compared to smuts with descriptions or illustrations of this character, the morphology and manner of peridial cell development in *Anthracocystis grodzinskae* (cylindrical to elongated cells in adhering, parallelly situated chains) are similar to those illustrated for *Anthracocystis cenchri* (Lagerh.) McTaggart & R.G. Shivas (Vánky 1985: Fig. 106B, “sterile” cells of *Sporisorium cenchri*), but different than those described and illustrated for *Sporisorium elegantis* Vánky (globose, subglobose, broadly ellipsoidal or long cylindrical to elongated cells in more or less well-developed chains or pseudoparenchymatous; Piątek et al. 2014). Thus, the characters of peridial cells may have some diagnostic potential but this should be explored and evaluated in different smut species in future. Another uncommon feature of *Anthracocystis grodzinskae* are ornamented inner spores. Dimorphic spores are typical for most members of *Anthracocystis*, but inner spores are usually smooth in most of the species, and are only rarely ornamented, and then with less prominent ornamentation than outer spores (Piątek and Shivas 2011; Vánky 2012).

### Phylogenetic framework for *Anthracocystis*

This study provides the most comprehensive phylogeny of *Anthracocystis* to date. It is the first phylogeny focused specifically on the genus *Anthracocystis*, as all previous phylogenetic studies either treated *Anthracocystis* as a synonym of *Sporisorium* in a wider context of grass-infecting Ustilaginales (Cunnington et al. 2005; Stoll et al. 2003, 2005; Vánky et al. 2006; Vánky and Lutz 2011; Zhang et al. 2013), or did not show relationships between species (McTaggart et al. 2012a, c; Shivas et al. 2013). The concatenated ITS+LSU dataset was constructed using only sequences that were linked to reliably identified (by smut experts) voucher specimens. ITS and LSU sequences were only concatenated if derived from the same specimen. The doubtful sequences of *Sporisorium scitamineum* (EF185083) and *Sporisorium nervosum* (AY740057/AY740110), though

nested within the *Anthracocystis* lineage (data not shown), were not included in the concatenated ITS+LSU dataset. *Sporisorium scitamineum* EF185083 was probably wrongly identified, as genuine *Sporisorium scitamineum* is placed in *Sporisorium s. str.* (Stoll et al. 2005; Vánky et al. 2006; Vánky and Lutz 2011). *Sporisorium nervosum* AY740057/AY740110 generated from specimen M 56622 (Stoll et al. 2005) may also be wrongly identified, as a sequence of this species (HQ013106), generated from the holotype specimen (BRIP 27019), was nested within the *Sporisorium s. str.* lineage (McTaggart et al. 2012a). Re-sequencing of both specimens is necessary to resolve which sequences are indeed linked to *Sporisorium nervosum*.

In the process of assembling the concatenated dataset, it came to light that 18 ITS sequences and 12 LSU sequences of *Anthracocystis* species, as well as one ITS sequence and one LSU sequence of *Triodomyces triodiae* used as the outgroup, were generated from type specimens (holotypes, isotypes or paratypes; Table 2). All but one of these sequences were not annotated in GenBank as type sequences, but through cross-checking the data in GenBank, original publications (see Table 2), respective monographs (Piepenbring 2003; Vánky and Shivas 2008; Vánky 2012), websites (<http://www.straininfo.net/strains/336261/browser>) or cited herbaria (D. Triebel, pers. comm.) it was possible to link these *Anthracocystis* sequences to voucher specimens, host plant species, and countries of origin, and, consequently, to track type sequences. The only sequence annotated in GenBank as a type sequence was an ITS sequence of *Sporisorium andrewmitchellii* in the RefSeq Targeted Loci database. Thus, ITS sequences generated from type specimens and detected in this study are recommended to be deposited in the RefSeq Targeted Loci database (<http://www.ncbi.nlm.nih.gov/bioproject/177353>; Schoch et al. 2014). Type material information was also provided to the taxonomic curators at NCBI for annotation of corresponding names in the NCBI Taxonomy Browser. This will expand the ability of users to query and BLAST against all GenBank entries tagged with type material by using a phrase, such as “sequence from type[filter]” (for more detailed examples, see Federhen 2014).

In congruence with previous phylogenetic studies (Stoll et al. 2005; Vánky et al. 2006; Vánky and Lutz 2011), the concatenated ITS+LSU dataset resolves species of *Anthracocystis*, but not the relationships between most of these species. If relationships were resolved, branches were usually weakly supported. For further resolution of relationships between *Anthracocystis* species, other genes should be analysed, but for routine species identification and generic placement, the current concatenated ITS+LSU dataset suits well. Thus, similar to resolved backbone trees for different phytopathogenic fungi (e.g., Begerow et al. 2002; Hendrichs et al. 2005; Lutz et al. 2005, 2008; Vánky et al. 2008; Maharachchikumbura et al. 2012; Piątek et al. 2013; Hyde et al. 2014; Savchenko et al.

2014; Ariyawansa et al. 2015; McTaggart et al. 2015), the concatenated dataset (alignment deposited in TreeBASE S17942) is intended to serve as a phylogenetic backbone for the genus *Anthracocystis* for future molecular studies. In addition to currently accepted *Anthracocystis* species, this study determined that another species, *Sporisorium andrewmitchellii* (Crous et al. 2012), was nested within *Anthracocystis*. Furthermore, three other *Sporisorium* species, *S. christineae*, *S. kenyanum*, and *S. warambiense* (Piątek 2006; Vánky et al. 2009; Piątek and Shivas 2011), for which the type specimens (mainly old material) have not been sequenced, fit the morphological concept proposed for the emended *Anthracocystis* (McTaggart et al. 2012c). The appropriate nomenclatural combinations for these species are introduced. The genus *Anthracocystis* currently contains 131 teleomorphic species. Of these, only 38 species (29 %) are represented by ITS (and usually also LSU) sequences in NCBI's GenBank. Thus, further collecting and sequencing efforts are necessary to provide a complete phylogenetic tree of *Anthracocystis*.

### Yeast sequences nested within *Anthracocystis*

In addition to teleomorphic species, the *Anthracocystis* lineage contains several yeast anamorphs well supported within the recovered phylogenetic tree. Sequences were either obtained directly from yeast strains or from environmental samples (as environmental sequences) that likely represent saprobic species. Previously, several phylogenetic studies on yeasts, with limited sampling of teleomorphic species, suggested an affinity of *Pseudozyma flocculosa* to some species now accommodated in *Anthracocystis* (e.g., Sampaio 2004; Wang et al. 2006; Seo et al. 2007; Statzell-Tallman et al. 2010; Chamnanpa et al. 2013). However, the current study provides the first explicit evidence that several asexual species are nested within the *Anthracocystis* lineage using broad sampling of teleomorphic species. Similar to other smut lineages with asexual species (Begerow et al. 2000; Sampaio 2004; Bauer et al. 2005; Boekhout et al. 2006; Nasr et al. 2014), all yeast strains/sequences clustered in different subclades of *Anthracocystis* and none could be directly assigned to a teleomorphic species. Only one of these yeast anamorphs is assigned to a species, namely *Pseudozyma flocculosa* (Traquair et al. 1988; Boekhout 1995). In line with the current ICN, this species should be recombined into *Anthracocystis*, as that generic name is older than *Pseudozyma*. In addition, the type species of *Pseudozyma*, *P. prolifica* Bandoni is phylogenetically distant from *Anthracocystis* and merely represents the anamorphic stage of *Ustilago maydis* (DC.) Corda (Sampaio 2004). The ITS and LSU GenBank sequences AB089364/AB089365 used in the concatenated tree (Fig. 1) are generated from the type strain JCM 10321 of *Pseudozyma flocculosa* (Sugita et al. 2003, confirmed as type strain on the website: [!\[\]\(de95854c7ee024cfadc48187bbb781b2\_img.jpg\) Springer](http://www.</a></p>
</div>
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[straininfo.net/strains/336261/browser](http://straininfo.net/strains/336261/browser)), which justifies the correct placement of this species in *Anthracoystis*. Therefore, in line with the current code of nomenclature and following recent practice of merging yeast species with sexual species under the older generic name (Selbmann et al. 2014; Yurkov et al. 2015), the recombination of *Pseudozyma flocculosa* into *Anthracoystis* is substantiated here. This action has important implications concerning the nomenclature and taxonomy of this model species used as a biocontrol agent against powdery mildews (Avis et al. 2001; Jarvis et al. 2007), and whose genome was recently sequenced (Lefebvre et al. 2013), especially considering that *Pseudozyma* is a catch-all genus and contains anamorphs linked to different teleomorphic ustilaginalean genera. This is the first step towards merging asexual yeasts with teleomorphic genera in the subphylum Ustilaginomycotina. *Anthracoystis flocculosa* (syn. *Pseudozyma flocculosa*) produces an antifungal glycolipid, flocculosin, that is unique for the fungus and responsible for the antibiotic activity against powdery mildews (Mimee et al. 2005; Clément-Mathieu et al. 2008; Teichmann et al. 2011). The evolutionary origin of this fungus, independent of other *Pseudozyma* species, could, therefore, be mirrored by the production of this distinct glycolipid.

The remaining yeast sequences nested within the *Anthracoystis* lineage belong to putatively undescribed species, and were generated from strains (or environmental samples) isolated from diverse sources such as soil, rice leaves, sea water, wood, avian guano, bird cloacae or grape berries (Table 2; Mahdi 2006; Waldrop et al. 2006; Francesca et al. 2012; Setati et al. 2012; Takashima et al. 2012). This demonstrates a much wider ecological spectrum of *Anthracoystis* than revealed by teleomorphic species. Some of these yeast strains were wrongly assigned to known teleomorphic species based on wrongly interpreted similarity in DNA sequences, e.g., the yeast sequences named as “*Sporisorium elionuri*” (AB863531, AB726473) or “*Sporisorium penniseti*” (HQ641273). The present phylogenetic analyses revealed these yeast sequences were not related to the teleomorphic species to which they were assigned. This indicates that DNA-based species identification of yeasts in ecological studies by direct comparisons of sequences to the closest hits in GenBank should be done with caution, as it may lead to unreliable interpretations and conclusions. In turn, the development of publicly accessible backbone trees, such as that developed in the current study, will help the phylogenetic placement of yeast anamorphs.

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