Asian Journal of Mycology 2(1): 101–109 (2019) ISSN 2651-1339

www.asianjournalofmycology.org **Article**
Doi 10.5943/ajom/2/1/4

A new species of *Pseudocercospora* on *Encephalartos barteri* from Benin

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Meswaet Y, Mangelsdorff R, Yorou NS, Piepenbring M 2019 – A new species of *Pseudocercospora* on *Encephalartos barteri* from Benin. Asian Journal of Mycology 2(1), 101–109, Doi 10.5943/ajom/2/1/4

Abstract

An infection of leaves of *Encephalartos barteri* (Zamiaceae) by a cercosporoid fungus was repeatedly observed in central Benin, West Africa. Morphological characteristics, the host relationship and DNA sequence data for two gene regions, namely ITS and *rpb2*, were compared to the corresponding characteristics of closely related, known cercosporoid species and showed that the specimens from Benin represent a new species of *Pseudocercospora*. *Pseudocercospora encephalarti* is the first *Pseudocercospora* species on a species of the host genus *Encephalartos*, as well as for the whole class Cycadopsida. It was found to be closely associated with a species of *Corynespora* that could not be identified in the context of the present study.

Key words – *Corynespora* – Cycadopsida – Mycosphaerellaceae – new species – Zamiaceae

Introduction

The genus *Pseudocercospora* was established by Spegazzini (1910) based on the type species *Pseudocercospora vitis* (Lév.) Speg., a foliar pathogen of grapevines. The majority of *Pseudocercospora* species known to date are pathogens on a wide variety of plants, including numerous economically relevant species of food crops or ornamentals all over the world (Den Breeÿen et al. 2006). They are known mainly from tropical and sub-tropical environments where they cause leaf spots, blight, fruit spots or fruit rot (von Arx 1983, Chupp 1954, Deighton 1976, Pons & Sutton 1988). *Pseudocercospora* species are morphologically characterized by pigmented conidia and conidiophores without thickened scars (Pereira & Barreto 2006). Species of this genus usually infest angiosperms, but a few species are also known from gymnosperms (Braun et al. 2013).

Materials & Methods

Collections and morphological studies

Leaf samples infected by cercosporoid fungi were collected in Benin in July and August of 2016 and 2107. Specimens were observed by stereomicroscopy and by standard methods of light microscopy, using a Zeiss *Axioscope* 40 microscope. For light microscopy, leaf sections were made

Submitted 14 January 2019, Accepted 11 April 2019, Published 25 April 2019
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with razor blades and mounted in distilled water and 5% KOH without any staining. Semi-permanent preparations of sections of the infected leaf were made by a microtome (Leica CM 1510-1) and mounted in lactophenol with cotton blue. Measurements of 30 conidia, conidiophores and other structures have been made at a magnification of ×1000. Measurements are presented as mean value ± standard deviation with extreme values in parentheses. For scanning electron microscopy, dried material was directly mounted and sputtered with gold for 3 minutes. Photographs were made with a Hitachi S 4500 scanning electron microscope (SEM).

DNA Extraction and PCR amplification

DNA was isolated from caespituli taken from dry specimens of the cercosporoid fungus using E.Z.N.A® Forensic DNA Extraction Kit following the manufacturer's instructions with a few modifications. Small pieces of leaves containing several clean caespituli, with as little other fungi as possible, were checked under the stereomicroscope. Precautions were taken to avoid picking any other associated materials that could lead to potential contamination. To extract total genomic DNA from caespituli, a small amount of clean mycelium from the leaf surface was transferred into a sterile Eppendorf tube using a sterilized needle and tape-lifts. The material was homogenized for 7–10 min. using a Retsch Mixer Mill MM301 with TL buffer and 2.5 mm Zirconia beads. Isolated DNA was re-suspended in elution buffer and stored at -20°C. DNA concentration was checked by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA).

Two genomic loci were amplified. For the ITS region, primers V9G (de Hoog & Gerrits van den Ende 1998) with ITS4 (White et al. 1990) and for the *rpb2*-locus Rpb2-F4 (Videira et al. 2017) or RPB2-5F2 (Sung et al. 2007) with fRPB2-7cR (Liu et al. 1999) were used. PCR amplification and sequencing were conducted following the protocols of Crous et al. (2009, 2012) and Videira et al. (2017). The PCR mixtures consisted of 1 µL genomic DNA, 15× $MgCl_2$ reaction buffer (Bioline, Luckenwalde, Germany), 25 mM $MgCl_2$, 25 µM of each dNTP, 10 µM of each primer and 5 U Taq DNA polymerase (VWR) in a total volume of 25 µL. Cycling parameters of the PCR for ITS were as follows: initial denaturation 94°C for 3 min; 35 cycles of amplification [denaturation at 94°C for 30 s, primer annealing 52°C for 30 s and TAQ extension 72°C for 45 s], and a final TAQ extension 72°C for 5 min, followed by storage at 8°C. The PCR mixture for *rpb2* contained 2 µL of template DNA and to obtain the partial *rpb2*, a touchdown PCR protocol was used as described by Videira et al. (2017). PCR-products were checked on 1.5 % agarose electrophoresis gels containing HDGreenPlus DNA stain. Amplified PCR products were purified with the Cycle Pure Kit (VWR-Omega, USA). Sequencing was performed at Seqlab GmbH, Germany.

Molecular phylogeny

Amplification of the ITS and *rpb2* gene regions for all isolates used in this study resulted in amplification products of approximately 650 bp for ITS and 1068 bp for *rpb2*. Consensus sequences of trace files were generated with Geneious 10.2.2 (https://www.geneious.com, Kearse et al. 2012) and searched against GenBank (https://www.ncbi.nlm.nih.gov/, Benson et al. 2013) with MegaBLAST. Sequences with a maximum identity of more than 95% (14 sequences to ITS and *rpb2*) were retrieved (Table 1). The sequences obtained from GenBank (Table 1) and sequences generated in this study were aligned with MAFFT v. 7 using the L-INS-i algorithm, (Nakamura et al. 2018). The alignments were manually checked by using MEGA v. 7 (Kumar et al. 2016). Gblocks v. 0.91b (Talavera & Castresana 2007) was used to remove poorly aligned positions and divergent regions from the DNA alignment using the parameters for a less stringent selection. To test the level of congruence among the two loci (ITS & *rpb2*), the Congruence Among Distance Matrices test, CADM global of R package APE v.3.2 (Paradis et al. 2004, R Core Team 2017), was performed. The CADM results showed that the null hypothesis of complete incongruence among loci was rejected ($W = 0.0.89$; $p < 0.01$), thus allowing concatenation of the two loci. Subsequently a two locus concatenated alignment (ITS, *rpb2*) dataset using Geneious 10.2.2 for phylogenetic analyses was assembled. *Passalora eucalypti* (CBS 111318) served as outgroup taxon as proposed

by Crous et al. (2012). PartitionFinder2 XSEDE v.2.1.1 (Miller et al. 2010) was used to select the best-fit model of evolution (K80+I+G model to ITS, and K80+I model to *rpb2*) for each gene fragment separately for Bayesian and Maximum Likelihood (ML) analyses. The alignment and the tree were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S23598). Phylogenetic analyses of this study were conducted by applying Maximum Likelihood (ML) with RAxML-HPC2 on XSEDE v.8.2.10 (Miller et al. 2010) and Bayesian with MrBayes on XSEDE v.3.2.6 (Miller et al. 2010) methods in the CIPRES Science Gateway web portal. (http://www.phylo.org/sub_sections/portal/). For Maximum Likelihood phylogenies performed with RAxML 1000 rapid bootstrap inferences were executed. For Bayesian phylogenies, two parallel runs with eight chains of Metropolis-coupled Markov chain Monte Carlo iterations were performed with the heat parameter being set at 0.2. Analyses were run for 100 million generations, with trees sampled every 1000th generation until the average standard deviation of split frequencies reached 0.01 (stop value). The first 25 % of saved trees were discarded as the 'burn-in' phase. Posterior probabilities (PP) were determined from the remaining trees. Bayesian posterior probabilities (BPP) ≥ 94 % and Bootstrap values (BS) ≥ 70% were considered significant.

Table 1 Sequences downloaded from GenBank (in alphabetical order) used in this study

Species	**Host**	**Sources**	**GenBank Accession No.**		**References**
			ITS	***rpb2***	
Passalora eucalypti	*Eucalyptus saligna*	CBS 111318	KF901613	KF902267	Quaedvlieg et al. 2014
Pseudocercospora breonadiae	*Breonadia salicina*	CBS 143489	MH107913	MH108006	Crous et al. 2018
Pseudocercospora catappae	*Terminalia catappa*	MAFF 238312	MF951366	MF951616	Videira et al. 2017
Pseudocercospora dingleyae	*Haloragis erecta*	CBS 114645	KX287299	KX288454	Videira et al. 2016
Pseudocercospora flavomarginata	*Eucalyptus camaldulensis*	CBS 124990	GU269799	MF951619	Videira et al. 2016
Pseudocercospora madagascariensis	*Eucalyptus camaldulensis*	CBS 124155	KF901673	KF902318	Quaedvlieg et al. 2014
Pseudocercospora nelumbonicola	*Nelumbo nucifera*	Kirschner 4111	KY304492	LC199940	Chen & Kirschner 2018
Pseudocercospora neriicola	*Nerium oleander*	CBS 138010	NR137885	KX462647	Nakashima et al. 2016
Pseudocercospora prunicola	*Prunus yedoensis*	CBS 132107	GU269676	MF951621	Videira et al. 2016
Pseudocercospora sp.	*Syzygium cordatum*	CBS 110780	KX287305	KX288461	Videira et al. 2016
Pseudocercospora sp.	*Eichhornia azurea*	CPC 19535	KX287303	KX288458	Videira et al. 2016
Pseudocercospora sp.	*Eichornia azurea*	CPC 19537	KX287304	KX288460	Videira et al. 2016
Pseudocercospora sp.	*Chromolaena odorata*	CBS 113386	DQ676532	KX288459	Videira et al. 2016
Pseudocercospora tereticornis	*Eucalyptus tereticornis*	CPC 13008	KF901531	KF902335	Quaedvlieg et al. 2014

Results

Phylogenetic analyses

A combined analysis of ITS and *rpb2* sequence data was performed to resolve the phylogenetic position of isolates taken from a species of *Pseudocercospora* on leaves of

Encephalartos barteri among known species of the *Pseudocercospora* selected from GenBank (Table 1).

For the species level analysis, DNA sequence data from the ITS and *rpb2* gene regions were combined for the Bayesian and Maximum Likelihood analyses. The concatenated alignment contained a total of 15 sequences including the out-group (14 sequences from NCBI and one sequence from this study). As the likelihood analyses produced tree topologies mostly identical to results of Bayesian analyses, bootstrap support values of the likelihood trees were incorporated into the tree that resulted from Bayesian analyses (Fig. 1). The sequence data of the *Pseudocercospora* species from Benin are placed in a sub-clade together with combined sequence data of *P. breonadiae*, *P. neriicola* and *P. madagascariensis*, *Pseudocercospora* sp. (CPC19537) and *Pseudocercospora* sp. (CPC19535) (Fig. 1) which is confirmed by the Maximum parsimony analysis of the combined ITS and *rpb2* alignment (tree not presented here). *Rpb2* sequence data showed differences between closely related species in the genus *Pseudocercospora* and are therefore more informative than the ITS data. Based on a megablast search using the *rpb2* sequence-data of the new species from Benin, the closest matches in NCBI's GenBank nucleotide database were *Pseudocercospora* sp. CPC 19535 (GenBank KX288458; identities = 827/848, i.e., 98 %), and *P. breonadiae* (GenBank MH108006; identities = 775/790, i.e., 98 %).

The infection of leaves of *Encephalartos barteri* by *Pseudocercospora* sp. was frequently associated with infection by *Corynespora* sp. (Fig. 4), for which an ITS sequence including 680 bp (YMMB78, GenBank MK402156) was obtained. A BLAST search based on this ITS sequence yielded most similar sequences of *Corynespora* species with a homology of less than 95%, namely of *C. smithii* (GenBank KY984300) and *C. citricola* (GenBank FJ852593).

Taxonomy

Pseudocercospora encephalarti Y. Meswaet, Mangelsdorff, Yorou & M. Piepenbr. sp. nov.

Index Fungorum number: IF555815; Facesoffungi number: FoF06035

Etymology – based on the host genus from which it was collected.

Leaf spots almost lacking or yellowish to brownish discolorations, 1.7–9 mm diam., margin indefinite. Caespituli epigenous, punctiform, dense, grayish brown to medium brown. Mycelium internal and external. Internal hyphae branched, 2.5–4.5 µm wide, septate, olivaceous brown to slightly dark brown. Stromata well-developed, immersed in the epidermis or in stomata and substomatal chambers, globular to irregular, 20–50 µm diam., brown to dark brown. Conidiophores in large fascicles of more than ten, arising from stromata, breaking through the adaxial epidermis of the leaves or penetrating through stomata, rarely solitary and arising from external hyphae, straight to bent or sinuous, rarely geniculate, slightly wider and rounded towards the apex, rarely branched, (14–)22–45.5(–68) × 3–4.5 µm, 1–3-septate, thin-walled, smooth, brown to dark brown, uniform in color. Conidiogenous loci unthickened and not darkened, 1–2 µm wide. Conidia solitary, narrowly obclavate to subacicular, straight to curved, (55–)90–167.5(–190) × 3.5–4.5(–5) µm, 3–8-septate, pale olivaceous to olivaceous brown, thin-walled, smooth, apex subacute, base truncate to obconically truncate, hila unthickened, not darkened, 1.5 µm wide (Figs 2, 3, 4).

Material examined – Benin, Department Borgou, Commune Tchaourou, Reserve Forest of Wari-Maro, ca. 248 m a.s.l., 9°08"37,38' N, 2°07"57,58' E, on leaves of *Encephalartos barteri* Carruth. ex Miq. (Zamiaceae), 04 August 2017, Meswaet Y, Piepenbring M, Yorou NS & members of 2017 summer school students in West Africa YMMA78 (M-0141346 holotype; BENIN, UNIPAR, ETH isotypes).

Additional material examined – Benin, Department Borgou, Commune Tchaourou, Reserve forest of Wari-Maro, ca. 248 m a.s.l., 9°08"37,38' N, 2°07"57,58' E, on leaves of *Encephalartos barteri* Carruth. ex Miq. (Zamiaceae), 08 August 2016, Mangelsdorff R, Meswaet Y, Piepenbring M, Yorou NS & members of 2016 summer school students in West Africa YMMA23 (M-0141347 paratype).

***Corynespora* sp**. on *Encephalartos barteri*

Leaf spots absent or large pale brown irregular leaf discoloration, 2–9 mm diam., margin indefinite. Caespituli amphigenous, velvety or spongy, forming small to widely effused patches, dark brown to black. Mycelium internal and external. Internal hyphae, branched, 2.5–3.5 µm wide, septate, olivaceous brown to slightly dark brown. Stromatawell-developed, partly superficial, partly immersed in the epidermis, globular to irregular, thick, 15–45(–65) µm in diam., brown to dark brown. Conidiophores in fascicles, arising from stromata, and solitary, arising from external hyphae, erect straight to slightly flexuous, with up to four proliferations, branched (30–)48–167(–180) × (4–)4.5– 5(–5.5) µm, 2–7 septate, brown to dark brown, uniform in color. Conidiogenous loci brown. Conidia solitary, broadly obclavate to subacicular, (35–)56.5–180(–198) × 3.5–4.5(–5) µm, multi-septate, olivaceous brown to slightly dark brown, thick-walled, smooth, apex subacute, base truncate to obconically truncate, hila, unthickened.

Material examined: Benin, Department Borgou, Commune Tchaourou, Wari-Maro, ca. 248 m a.s.l., 9°08"37,38' N, 2°07"57,58' E, on living leaves of *Encephalartos barteri* Carruth. ex Miq. (Zamiaceae), 04 August 2017, Meswaet Y, Piepenbring M, Yorou NS & members of 2017 summer school students in West Africa YMMB78 (M-0141346).

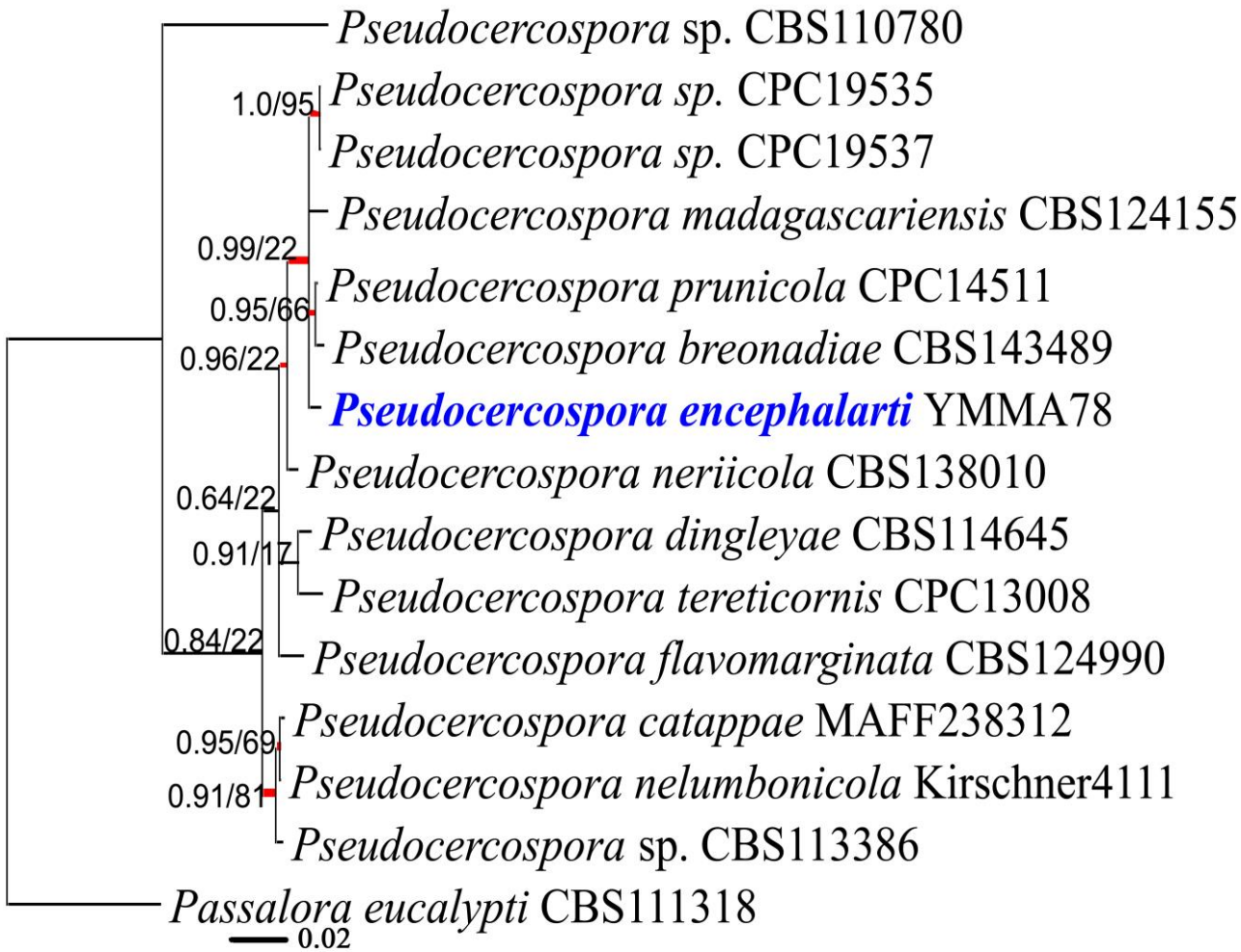

Fig. 1 – The Bayesian phylogenetic tree inferred from DNA sequence data from the multigene alignment (ITS and *rpb2*) of cercosporoid species. The newly detected species is indicated in blue color and nodes receiving Bayesian PP ≥ 0.94 or ML bp ≥ 70 % are considered as strongly supported and are indicated by thickened red branches.

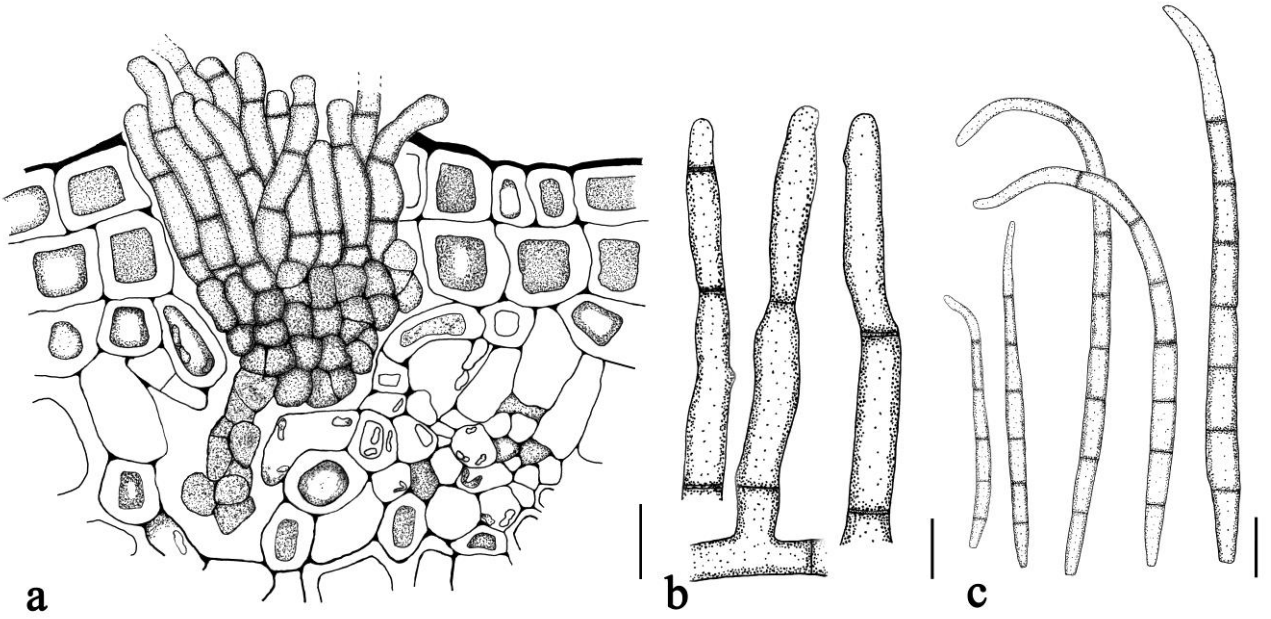

Fig. 2 – Line drawings of *Pseudocercospora encephalarti* (YMMA78). a Well-developed, immersed stroma with conidiophores. b Solitary conidiophores arising from external hyphae. c Conidia. Scale Bars: a = 20 µm, b and c = 10 µm.

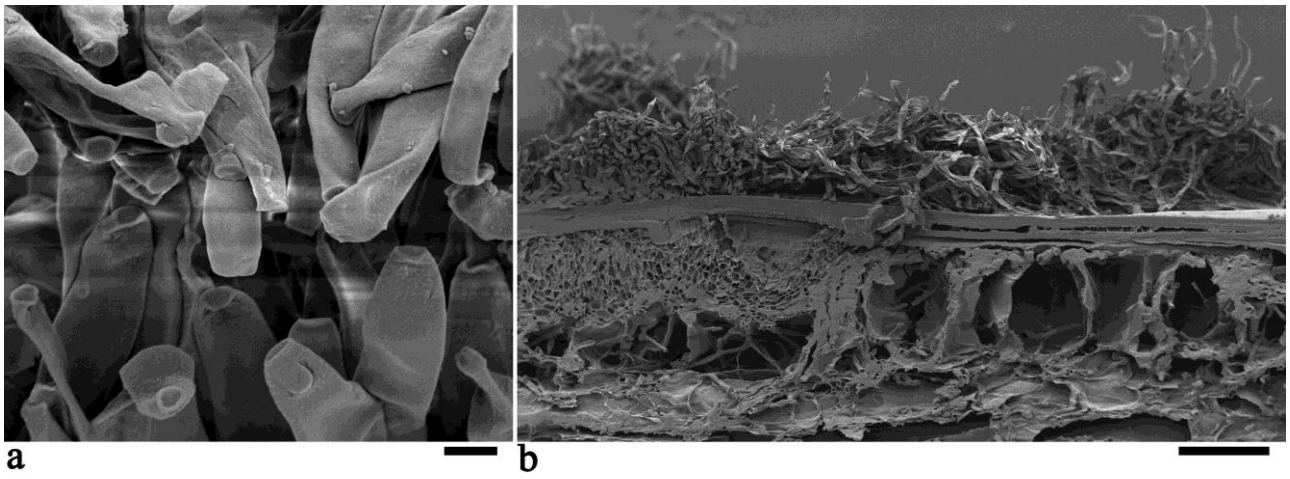

Fig. 3 – *Pseudocercospora encephalarti* on leaf tissue of *Encephalartos barteri* as seen by scanning electron microscopy (YMMA78). a Conidiophores with 1–2 flat conidiogenous loci without particular structures. b Leaf tissue with internal hyphae and superficial fungal cells (conidiophores and conidia). Scale Bars: a = 10 µm, b = 200 µm.

Discussion

Pseudocercospora species usually have unthickened and not darkened conidiogenous loci and conidial hila, while conidiophores as well as conidia are pigmented (Deighton 1976, Pereira & Barreto 2006). On account of these characteristics the specimens from Benin belong to the genus *Pseudocercospora* which is also confirmed by molecular data. Except for two unnamed *Pseudocercospora* spp. reported from *Encephalartos* spp. in South Africa (Nesamari et al. 2017), no *Pseudocercospora* species on hosts belonging to Cycadopsida are known up to now. For the whole group of cercosporoid fungi, the presented *Pseudocercospora* species is the first species on a host of the genus *Encephalartos*. According to Nesamari et al. (2017), the two *Pseudocercospora* species reported from South Africa [*Pseudocercospora* sp. 1 (CMW44333) on *Encephalartos ferox*

and *Pseudocercospora* sp. 2 (CMW44983) on *Encephalartos villosus*] are closely related to each other and to *Pseudocercospora acericola*, *Pseudocercospora lythracearum*, *Pseudocercospora crispans* and *Pseudocercospora rubi*, taxa that are not closely related to the species included in the present analysis (Fig. 1). According to the authors, the systematic relationships of the *Pseudocercospora* spp. from South Africa were evident by analyses of sequence data of the gene regions ITS, ACT, TEF-1a. The two *Pseudocercospora* species on *Encephalartos* hosts in Nesamuri et al. (2017), however, were mentioned without names, morphological characteristics, or molecular sequence data in GenBank, and could, therefore, not be directly compared to the specimens from Benin. On account of the presented multi-locus phylogenetic analyses as presented in Fig. 1, all five species in the clade of the new *Pseudocerspora* species from Benin are closely related to each other. Most *Pseudocercospora* species are host-specific with a host range limited to a single plant genus or related genera within a family (Nakashima et al. 2016). Thus, *P. encephalarti* is described as a new species, characterized by epigenous caespituli without showing any distinct leaf spots or with yellowish to brownish discolorations (Fig. 4), having well-developed stromata (Fig. 2a), relatively long and wide conidia and conidiophores (Fig. 2b–c), as well as internal and external hyphae (Fig. 3). *Encephalartos barteri* is an indigenous plant known in the four West African countries of Benin, Ghana, Nigeria and Togo (Akoégninou et al. 2006), where fungal biodiversity has been poorly studied as in many African countries. It therefore is not surprising that the described new *Pseudocercospora* species has not yet been reported.

Fig. 4 – A leaf of *Encephalartos barteri* showing brown discoloration caused by *Corynespora* sp., partly associated with *Pseudocercospora encephalarti* (YMMB78).

For the genus *Corynespora*, approximately 200 species are established, often mainly based on new host plant species. A critical revision of the genus is lacking. For some species of *Corynespora* DNA sequence data are available and show a maximum similarity to the sequence obtained from

the specimen from Benin of 95 percent. As long as further data on established *Corynespora* spp. are not available, a reliable species identification is not possible and we refrain from establishing a new species for the specimens from Benin.

Acknowledgements

We are grateful to Niklas Döring at Gothe-Universität Frankfurt am Main for making scanning electron microscope photographs. Our special thanks go to Roland Kirschner, Hermine Lotz-Winter, José Macia-Vicente, and Kai Reschke for fruitful discussions and valuable advice for improving the manuscript and the molecular phylogeny. Afoussathou Tabe (Faculty of Agronomy, University of Parakou, Benin) assisted in specimen sampling in the field. Our participation to the 2016 and 2017 summer schools in tropical mycology in Benin, along with the field works, have been possible through a financial support by the Volkswagen Foundation and the Freunde und Förderer der Goethe-Universität. A special gratitude goes to the Adolf Messer Foundation for providing a scholarship to the first author.

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