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Aspergillus diversity from the Gcwihaba Cave in Botswana and description of one new species

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Abstract: A fungal survey of the Gcwihaba Cave from Botswana found *Aspergillus* to be one of the more common fungal genera isolated. The 81 *Aspergillus* strains were identified using *CaM* sequences and comparing these to a curated reference dataset. Nineteen species were identified representing eight sections (sections *Candidi, Circumdati, Flavi, Flavipedes, Nidulantes, Nigri, Terrei* and *Usti*). One strain could not be identified. Morphological characterisation and multigene phylogenetic analyses confirmed it as a new species in section *Flavipedes* and we introduce it below as *A. okavangoensis.* The new species is most similar to *A. iizukae*, both producing conidiophores with vesicles typically wider than 20 µm. The new species, however, does not produce Hülle cells and its colonies grow slower than those of *A. iizukae* on CYA at 37 °C (14–15 *vs* 18–21 mm) and CREA (15–16 *vs* 23–41mm).

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INTRODUCTION

The Gcwihaba Cave is a Botswana national monument and is being considered for UNESCO World Heritage site nomination. It is a spectacular geographical formation located in the Okavango basin, to the north-western edge of Botswana (coordinates: -20.025056, 21.357639) (Mbaiwa & Sakuze 2009, Dandurand *et al.* 2019, Mazebedi & Hesselberg 2020). The cave was formed from rocks primarily composed of calcium magnesium carbonate or dolomite. Some parts of the dolomite cave roof and walls are adorned with tens of thousands of three insectivorous bat species, namely *Hipposideros vittatus* (Striped leaf-nosed bat), *Nycteris thebaica* (Egyptian slit-faced bat) and *Rhinolophus denti* (Dent's horseshoe bat) (Dandurand *et al.* 2019). Bat guano provides a carbon source for the growth of various microorganisms, while bat urine can cause biogenic corrosion of the cave roof and walls (Dandurand *et al.* 2019). This corrosion ultimately compromise the integrity of the dolomite, causing it to chip away and fall onto the ground (Kolo *et al.* 2007). This dolomite, bats excrement, dead vertebrates and invertebrates provide organic matter that supports the growth of various microorganisms. The decomposition of this organic matter is typically carried out by fungi and bacteria (Man *et al.* 2015, Pusz *et al.* 2015).

Several surveys from especially North America and Europe have focused on microbial diversity of caves, mainly driven by the outbreak of White-nose Syndrome (WNS; caused by *Pseudogymnoascus destructans*) in bats and subsequent research that aimed to understand the disease (Blehert *et al.* 2009, Johnson *et al.* 2013, Vanderwolf *et al.* 2013, Zhang *et al.* 2017, Cunha *et al.* 2020, Visagie *et al.* 2020). These studies often found caves to be species rich. Even though WNS has not been reported in Southern Africa, caves are an underexplored biota. The region is one of the worlds biodiversity hotspots (Myers *et al.* 2000). The microbiology of the Gcwihaba Cave has never been studied. The perpetual darkness of the cave, relatively constant temperature (25 °C), high humidity (60–70 %) (Dandurand *et al.* 2019) and location in the heart of the Kalahari Desert present a unique ecological niche that is expected to contain many undescribed fungi. Therefore, the Gcwihaba Cave was considered to represent an untapped potential biotechnological resource and has become the focus of a long-term project looking to discover novel compounds from the region.

The aim of this study was to present results from a preliminarily survey exploring fungal diversity in the Gcwihaba Cave, of which *Aspergillus* was found to be one of the predominate genera. Here we report on the species recovered from bat guano covered soil and, in the process, introduce the new species *A. okavangoensis*.

MATERIALS AND METHODS

Strains, sampling and isolation

Bat guano-contaminated soil samples were collected in sterile plastic bags from the Gcwihaba Caves situated in the Okavango basin, Botswana. A total of 18 samples were collected from six locations in the cave.

Isolations from soil samples were made using a dilution series by suspending 10 g soil in 90 mL sterile 0.1 % peptone dH_2O and diluting this to 10^{-4}. For each dilution, 100 µL was spread-plated in duplicate onto Potato Dextrose Agar (PDA), Dichloran-Glycerol agar (DG18) and Dichloran Rose Bengal Chloramphenicol Agar (DRBCA) (Samson *et al.* 2014). These were incubated at 30 °C for 7 d, after which colonies of interest were transferred into pure culture onto Malt Extract Agar (MEA) and Sabouraud Dextrose Agar (SDA) plates (Samson *et al.* 2014), and then incubated a further 7 d. Strains were identified to genus level based on colony and microscopic observations. Strains were preserved in 10 % glycerol and stored at -80 °C and accessioned into the working collection of David Nkwe housed at the Botswana International University of Science and Technology, Palapye, Botswana.. The strain of the new species was accessioned into Cobus Visagie's working collection (CN) housed at FABI (Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa) and preserved as spore suspensions in 10 % glycerol at -80 °C. It was also deposited into the CMW (FABI, University of Pretoria, Pretoria, South Africa) and CBS (Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands) culture collections. A dried specimen representing the holotype of the new species was deposited in PREM, the fungarium of the South African National Collection of Fungi housed at the Agricultural Research Council (ARC; Plant Health and Protection, Roodeplaat, South Africa). Table 1 summarises strains used for the phylogenetic analysis of section *Flavipedes*, including their GenBank and culture collection accession numbers and other metadata.

DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from 7-d-old colonies grown on PDA using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, CA, USA) following the manufacturer's instructions. DNA extracts were stored at -20 °C. PCR primers and amplification conditions followed protocols defined by Samson *et al.* (2014) and Houbraken *et al.* (2020). Briefly, all *Aspergillus* strains were identified using partial calmodulin (*CaM*) gene region sequences. For new species, the internal transcribed spacer and 5.8S rDNA regions (ITS), partial beta-tubulin (*BenA*) and partial RNA polymerase II second largest subunit (*RPB2*) were also sequenced. Genes were amplified using primer pairs V9G & LS266 (ITS; de Hoog & Gerrits van den Ende 1998, Masclaux *et al* 1995), Bt2a & Bt2b (*BenA*; Glass & Donaldson 1995), cmd5 & cmd6 (*CaM*; Hong *et al*. 2006) and RPB2-RPB2F1 & RPB27CRa (*RPB2*; Houbraken *et al*. 2020). PCRs were prepared in 25 µL volumes containing 0.15 µL MyTaq™ DNA polymerase (Bioline, Meridian Bioscience, USA), 5 µL 5× MyTaq™ Reaction Buffer (BioLine), 0.5 µL of each primer (10 µM), 1 µL template DNA and 17.85 µL MilliQ H_2O. Bidirectional sequencing was done at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) using the same primers used for PCR amplification. Contig sequences were generated in Geneious Prime v. 2021.0.3 (BioMatters Ltd., Auckland, New Zealand).

Strains were identified by comparing *CaM* sequences with a locally curated reference sequence database mainly based on Samson *et al.* (2014) and Houbraken *et al.* (2020). Subsequent phylogenies were calculated for the new species based on its close relatives on a series level. Each dataset was aligned in MAFFT v. 7.453 (Katoh & Standley 2013) using the G-INS-I option and then trimmed and adjusted in Geneious where needed. Datasets were partitioned based on the gene region, as well as introns and exons. For each partition, the appropriate nucleotide substitution model was selected using PartitionFinder v. 2.1 (Lanfear *et al.* 2017) based on the Akaike information criterion (Akaike 1974). Phylogenies were performed using Maximum Likelihood (ML) and Bayesian tree Inference (BI). The ML analysis was performed using IQ-TREE v. 2.1.2 (Nguyen *et al.* 2015) with regular bootstrapping performed using 1 000 replicates. BI analyses were performed in MrBayes v. 3.2.7 (Ronquist *et al.* 2012) using three sets of four chains (1 cold and three heated) and were stopped using the stoprule option at an average standard deviation for split frequencies of 0.01. Trees were visualised using the Interactive tree of life (iTOL) v. 6 (Letunic & Bork 2016) and edited in Affinity Publisher v. 1.9.3 (Serif (Europe) Ltd, Nottingham, UK). The ML trees were used to present phylogenetic results with both bootstrap values (bs) and posterior probabilities (pp) shown for branches.

Morphology

The new species was characterised and described following methods described in Samson *et al.* (2014). Briefly, morphological features were recorded on Czapek Yeast Autolysate agar (CYA), CYA with 5 % NaCl (CYAS), MEA (Oxoid CM0059), DG18 (Oxoid CM0729), Yeast Extract Sucrose agar (YES), Oatmeal agar (OA) and Creatine Sucrose agar (CREA). Media were prepared in 90 mm Petri-dishes. Equidistant three-point inoculations were made and incubated for 7 d at 25 °C, with additional CYA plates incubated at 5, 10, 15, 20, 30 and 37 °C. Colour names and codes used in descriptions follow Kornerup & Wanscher (1967). Colonies were captured within a lightbox equipped with a Sony a6400 camera. A Zeiss AxioImager.A2 compound and Zeiss AXIO dissecting Discovery.V8 microscopes equipped with an AxioCaM 512 colour camera driven by Zen Blue v. 3.2 software (Carl Zeiss CMP GmbH, Göttingen, Germany) were used for all microscopic observations. Extended Depth of Field stacking of colony texture micrographs was performed in Helicon Focus v. 7.5.4 (HeliconSoft, Kharkiv, Ukraine). Microphotographs were edited for aesthetic purposes using the "inpainting brush tool" without altering areas of scientific significance. Photo plates were prepared in Affinity Photo v. 1.9.3 (Serif (Europe) Ltd, Nottingham, UK).

RESULTS

Isolations and Identifications

Isolations from bat guano-contaminated soil samples resulted in 81 *Aspergillus* strains. *CaM* sequences were generated and deposited in GenBank under accessions MW480706–MW480787. Strains were found to represent eight sections and 19 species, including *A. alabamensis* (one strain), *A. 'alboluteus'* (in press) (one strain), *A. allahabadii* (one strain), *A. aureolatus* (two strains), *A. aculeatus* (two strains), *A. flavus* (one strain),

Table 1. *Aspergillus* section *Flavipedes* strains used for phylogenetic comparisons.

			GenBank Accessions			
Species	**Strains**	**Series**	**ITS**	***BenA***	***CaM***	***RPB2***
Aspergillus alboluteus	CBS 147421 = CMW 56637 = CN 073A5 = DN 84	*Spelaei*	MW480880	MW480788	MW480706	MW480790
	CBS 145854 = CCF 4916 = EMSL 2311 = IFM 66816	*Spelaei*	MW448664	MW478498	MW478512	MW478533
	CBS 145855 = CCF 5695 = EMSL 2420 = IFM 66815 (ex-type)	*Spelaei*	MW448663	MW478497	MW478511	MW478532
	CBS 145859 = CCF 6201 = EMSL 3060	*Spelaei*	MW448662	MW478496	MW478510	MW478531
	CBS 147065 = CCF 6551 = DTO 410-I8	*Spelaei*	MW448666	MW478500	MW478514	MW478535
	CCF 5849 = EMSL 2446 = IFM 66817	*Spelaei*	MW448665	MW478499	MW478513	MW478534
Aspergillus alboviridis	CBS 142665 = FMR 15175 = CCF 6049 = IFM 66819 (ex-type)	*Spelaei*	LT798909	LT798936	LT798937	LT798938
Aspergillus ardalensis	CCF 4031 = CCF 4426 = CMF ISB 1688 = CBS 134372 = NRRL 62824 (ex-type)	*Flavipedes*	FR733808	HG916683	HG916725	HG916704
Aspergillus flavipes	NRRL 302 = ATCC 24487 = IMI 171885 = QM 9566 = Thom 4640.474 = WB 302 (ex-type)	*Flavipedes*	EF669591	EU014085	EF669549	EF669633
Aspergillus iizukae	CBS 541.69 = NRRL 3750 = IMI 141552 = QM 9325 (ex-type)	*Flavipedes*	EF669597	EU014086	EF669555	EF669639
	CCF 4032 = CMF ISB 1245	*Flavipedes*	HG915894	HG916687	HG916730	HG916708
	CBS 138188 = DTO 179-E6 (ex-type of *P. capensis*)	*Flavipedes*	KJ775550	KJ775072	KJ775279	KP987020
	CCF 4845 = S 746	*Flavipedes*	LM999906	LM644270	LM644243	MW478540
Aspergillus inusitatus	CBS 147044 = CCF 6552 = DTO 121-G5 (ex-type)	*Spelaei*	MW448669	MW478502	MW478517	MW478542
Aspergillus lanuginosus	NRRL 4610 = IMI 350352 = CCF 4551 = IFM 66818 (ex-type)	*Spelaei*	EF669604	EU014080	EF669562	EF669646
Aspergillus luppiae	NRRL 6326 = CBS 653.74 = CCF 4545 (ex-type)	*Spelaei*	EF669617	EU014079	EF669575	EF669659
Aspergillus micronesiensis	CBS 138183 = DTO 267-D5 (ex-type)	*Flavipedes*	KJ775548	KJ775085	KJ775355	KP987023
	IMI 357699 = DTO 305-B6 = IBT 23707 (ex-type of *A. sunderbanii nom. inval.*)	*Flavipedes*	KP987084	KP987052	KP987069	KP987026
	NRRL 4263 = CCF 4556	*Flavipedes*	EF669600	EU014083	EF669558	EF669642
Aspergillus movilensis	NRRL 4610 = IMI 350352 = CCF 4551	*Spelaei*	EF669604	EU014080	EF669562	EF669646
	CBS 134395 = PRM 923449 = CCF 4410 = CMF ISB 2614 = NRRL 62819 = DTO 316-C6 (ex-type)	*Spelaei*	KP987089	HG916697	HG916740	HG916718
	DTO 203-C9	*Spelaei*	KP987075	KP987043	KP987058	KP987032
	DTO 203-H3	*Spelaei*	KP987078	KP987046	KP987061	KP987035
	S 1040	*Spelaei*	MW448674	MW478503	MW478522	MW478551
Aspergillus neoflavipes	CBS 260.73 = NNRL 5504 = ATCC 24484 = IMI 171883 = IFM 40894 = CCF 4552 (ex-type)	*Flavipedes*	EF669614	EU014084	EF669572	EF669656
Aspergillus neoniveus	CBS 261.73 = NRRL 5299 = ATCC 24482 = IMI 171878 (ex-type)	*Neonivei*	EF669612	EU014098	EF669570	KP987024
Aspergillus okavangoensis	**CBS 147420 = CMW 56636 = CN 073A3 = DN 24 (ex-type)**	***Flavipedes***	**MW480881**	**MW480789**	**MW480707**	**MW480791**
Aspergillus olivimuriae	NRRL 66783 = CCF 6208 (ex-type)	*Olivimuriarum*	MH298877	MH492010	MH492011	MH492012
Aspergillus polyporicola	NRRL 32683 = CCF 4553 (ex-type)	*Spelaei*	EF669595	EU014088	EF669553	EF669637
	CCF 5427 = EMSL 2612	*Spelaei*	MW448675	MW478504	MW478523	MW478552
	CCF 6262 = EMSL 3169	*Spelaei*	MW448676	MW478505	MW478524	MW478553
	NRRL 58570 = CCF 4828	*Spelaei*	HQ288052	LM644274	LM644252	LM644254

Table 1. (Continued).

Species	Strains	Series	GenBank Accessions			
			ITS	*BenA*	*CaM*	*RPB2*
Aspergillus spelaeus	CCF 4425 = CMF ISB 2615 = CBS 134371 = NRRL 62826 (ex-type)	*Spelaei*	HG915905	HG916698	HG916741	HG916719
	CCF 4886 = S 716	*Spelaei*	LM999908	LM644272	HG916748	LM644259
	EMSL 4874	*Spelaei*	MW448677	MW478506	MW478525	MW478554
	FMR 14606	*Spelaei*	LT899488	LT899537	LT899590	LT899645
Aspergillus suttoniae	CBS 143866 = UTHSCSA DI14-215 = FMR 13523 (ex-type)	*Flavipedes*	LT899487	LT899536	LT899589	LT899644
Aspergillus templicola	CBS 138181 = DTO 270-C6 (ex-type)	*Flavipedes*	KJ775545	KJ775092	KJ775394	KP987038
	CCF 4698 = CMF ISB 2662 = NRRL 62825 (ex-type of *A. mangaliensis*)	*Flavipedes*	HG915902	HG916695	HG916738	HG916716
	NRRL 4893 = IMI 343701 = CCF 4846	*Flavipedes*	LM999907	LM644271	LM644242	LM644256
Aspergillus terreus	CBS 601.65 = NRRL 255 = ATCC 10071 = ATCC 1012 = IFO 33026 = IMI 017294ii = IMI 17294 = JCM 10257 = LSHBA c .24 = MUCL 38640 = NCTC 981 = NRRL 543 = QM 1 = QM 1991 = Thom 144 = VKMF-67 = WB 255 (ex-type)	*Terrei*	EF669586	EF669519	EF669544	EF669628
Aspergillus urmiensis	CBS 139558 = CCTU 742 = IBT 32593 = DTO 203-C2 (ex-type)	*Flavipedes*	KP987073	KP987041	KP987056	KP987030
	CBS 139557 = CCTU 734 = DTO 203-B3 = IBT 32597	*Flavipedes*	KP987072	KP987039	KP987055	KP987029
	CBS 139766 = CCTU 743 = DTO 203-C3 = IBT 32598	*Flavipedes*	KP987074	KP987042	KP987057	KP987031

Table 2. *Aspergillus* isolated from Botswana during this study.

Species	Strains	Subgenus	Section	Series	GenBank Accession(s): *CaM*
Aspergillus aculeatus	DN78, DN81	*Circumdati*	*Nigri*	*Japonici*	MW480779, MW480782
Aspergillus alabamensis	DN14	*Circumdati*	*Terrei*	*Terrei*	MW480720
Aspergillus alboluteus	CBS 147421 = CMW 56637 = CN 073A5 = DN84	*Circumdati*	*Flavipedes*	*Spelaei*	MW480706
Aspergillus allahabadii	DN23	*Circumdati*	*Terrei*	*Nivei*	MW480727
Aspergillus aureolatus	DN01, DN61	*Nidulantes*	*Nidulantes*	*Speluncei*	MW480708, MW480763
Aspergillus flavus	DN27	*Circumdati*	*Flavi*	*Flavi*	MW480729
Aspergillus fructus	DN02	*Nidulantes*	*Nidulantes*	*Versicolores*	MW480709
Aspergillus germanicus	DN04, DN29, DN43	*Nidulantes*	*Usti*	*Calidousti*	MW480711, MW480731, MW480731
Aspergillus griseoaurantiacus	DN40	*Nidulantes*	*Nidulantes*	*Versicolores*	MW480742
Aspergillus hongkongensis	DN52	*Nidulantes*	*Nidulantes*	*Versicolores*	MW480754
Aspergillus hortae	DN55	*Circumdati*	*Terrei*	*Terrei*	MW480757
Aspergillus neoniger	DN66	*Circumdati*	*Nigri*	*Nigri*	MW480768
Aspergillus ochraceus	DN64, DN65, DN71, DN87	*Circumdati*	*Circumdati*	*Circumdati*	MW480766, MW480767, MW480773, MW480787
Aspergillus okavangoensis	CBS 147420 = CMW 56636 = CN 073A3 = DN24	*Circumdati*	*Flavipedes*	*Flavipedes*	MW480707
Aspergillus parasiticus	DN54	*Circumdati*	*Flavi*	*Flavi*	MW480756
Aspergillus subalbidus	DN12, DN13, DN62, DN63, DN67, DN68, DN69, DN70, DN75, DN76, DN80, DN82, DN85	*Circumdati*	*Candidi*	*Candidi*	MW480718, MW480719, MW480764, MW480765, MW480769, MW480770, MW480771, MW480772, MW480777, MW480778, MW480781, MW480783, MW480785

Table 2. (Continued).

Species	Strains	Subgenus	Section	Series	GenBank Accession(s): *CaM*
Aspergillus subramanianii	DN72, DN73	*Circumdati*	*Circumdati*	*Sclerotiorum*	MW480774, MW480775
Aspergillus sydowii	DN03, DN05, DN06, DN08, DN09, DN10, DN15, DN18, DN19, DN20, DN21, DN22, DN25, DN28, DN30, DN31, DN32, DN33, DN34, DN35, DN36, DN37, DN38, DN39, DN41, DN42, DN44, DN46, DN47, DN48, DN49, DN50, DN51, DN53, DN56, DN57, DN58, DN59, DN60, DN74, DN79, DN83, DN86	*Nidulantes*	*Nidulantes*	*Versicolores*	MW480710, MW480712, MW480713, MW480715, MW480716, MW480717, MW480721, MW480722, MW480723, MW480724, MW480725, MW480726, MW480728, MW480730, MW480732, MW480733, MW480734, MW480735, MW480736, MW480737, MW480738, MW480739, MW480740, MW480741, MW480743, MW480744, MW480746, MW480748, MW480749, MW480750, MW480751, MW480752, MW480753, MW480755, MW480758, MW480759, MW480760, MW480761, MW480762, MW480776, MW480780, MW480784, MW480786
Aspergillus taichungensis	DN07	*Circumdati*	*Candidi*	*Candidi*	MW480714

A. fructus (one strain), *A. germanicus* (three strains), *A. griseoaurantiacus* (one strain), *A. hongkongensis* (one strain), *A. hortae* (one strain), *A. neoniger* (one strain), *A. ochraceus* (four strains), *A. parasiticus* (one strain), *A. subalbidus* (13 strains), *A. subramanianii* (two strains), *A. sydowii* (43 strains) and *A. taichungensis* (one strain). A summary of these can be found in Table 2. One strain could not be identified using *CaM* sequences. Based on subsequent multigene phylogenies and morphological observations, this strain was shown to represent a new species described below in the Taxonomy section.

Phylogeny

Each gene region was aligned, resulting in alignment lengths of 555, 472, 998, and 532 bp for *BenA*, *CaM, RPB2* and ITS, respectively. The concatenated dataset consisted of these four gene regions that were further partitioned based on intron and exon regions. The most appropriate nucleotide substitution models for each partition were as follows: TRN+I+G for *BenA*_codon1, *BenA*_codon3 and ITS; JC+I for *BenA*_codon2, *CaM*_codon1 and *RPB2*_codon3; TRNEF+I+G for *BenA*_introns and *CaM*_introns; HKY+G for *CaM*_codon2 and *RPB2*_codon1; and TRN+I for *RPB2*_codon2 and *CaM*_codon3. Alignments were submitted to TreeBASE under accession number 27870 (https://www.treebase.org/).

Sequence data resolved the new species in *Aspergillus* section *Flavipedes* (Fig. 1). *Aspergillus okavangoensis* belongs to series *Flavipedes* and resolves on a distinct branch, but deep nodes had low support and its exact relationship with other species is unresolved. The new species had unique sequences for all gene regions considered in this study. Based on BLAST searches against a curated reference database, the closest hits using ITS had highest similarity to *A. iizukae* (strain CBS 541.69^{T}, GenBank EF669597; Identities = 506/512 (98.8 %), no gaps), *A. urmiensis* (strain CBS 139558^{T}, GenBank KP987073; Identities = 504/512 (98.4 %), no gaps), and *A. ardalensis* (strain CCF4031^{T}, GenBank FR733808; Identities = 505/514 (98.4 %), 5 gaps). The closest hits using *BenA* had highest similarity to *A. urmiensis* (strain CBS 139558^{T}, GenBank KP987041; Identities = 474/507 (93.5 %), 3 gaps), *A. templicola* (strain CBS 138180, GenBank KJ775087; Identities = 471/507 (92.9 %), 2 gaps) and *A. suttoniae* (strain CBS 143866^{T}, GenBank LT899536; Identities = 428/463 (92.4 %), 4 gaps). The closest hits using *CaM* had highest similarity to *A. templicola* (strain NRRL4893, GenBank LM644242; Identities = 507/558 (90.9 %), 8 gaps), *A. urmiensis* (strain CBS 139558^{T}, GenBank KP987056; Identities = 502/554 (90.6 %), 4 gaps) and *A. suttoniae* (strain CBS 143866^{T}, GenBank LT899589; Identities = 500/556 (89.9 %), 5 gaps). The closest hits using *RPB2* had highest similarity to *A. suttoniae* (strain CBS 143866^{T}, GenBank LT899644; Identities = 828/855 (96.8 %), no gaps), *A. templicola* (strain CCF4698, GenBank HG916716; Identities = 824/857 (96.1 %), no gaps) and *A. urmiensis* (strain CBS 139558^{T}, GenBank KP987030; Identities = 818/857 (95.4 %), no gaps).

TAXONOMY

Aspergillus okavangoensis Visagie & Nkwe, ***sp. nov.*** MycoBank MB 840269. Fig. 2.

In: subgenus *Circumdati* section *Flavipedes* series *Flavipedes*.

Etymology: Latin, *okavangoensis,* named after the Okavango Delta of Botswana, the origin of this species.

Typus: **Botswana**, Gcwihaba Cave (-20.023000, 21.355200), from bat guano-contaminated soil collected in the cave, Jun. 2019, coll. *D. Nkwe & R. Mazebedi*, isol. *G. Modise & D. Nkwe*

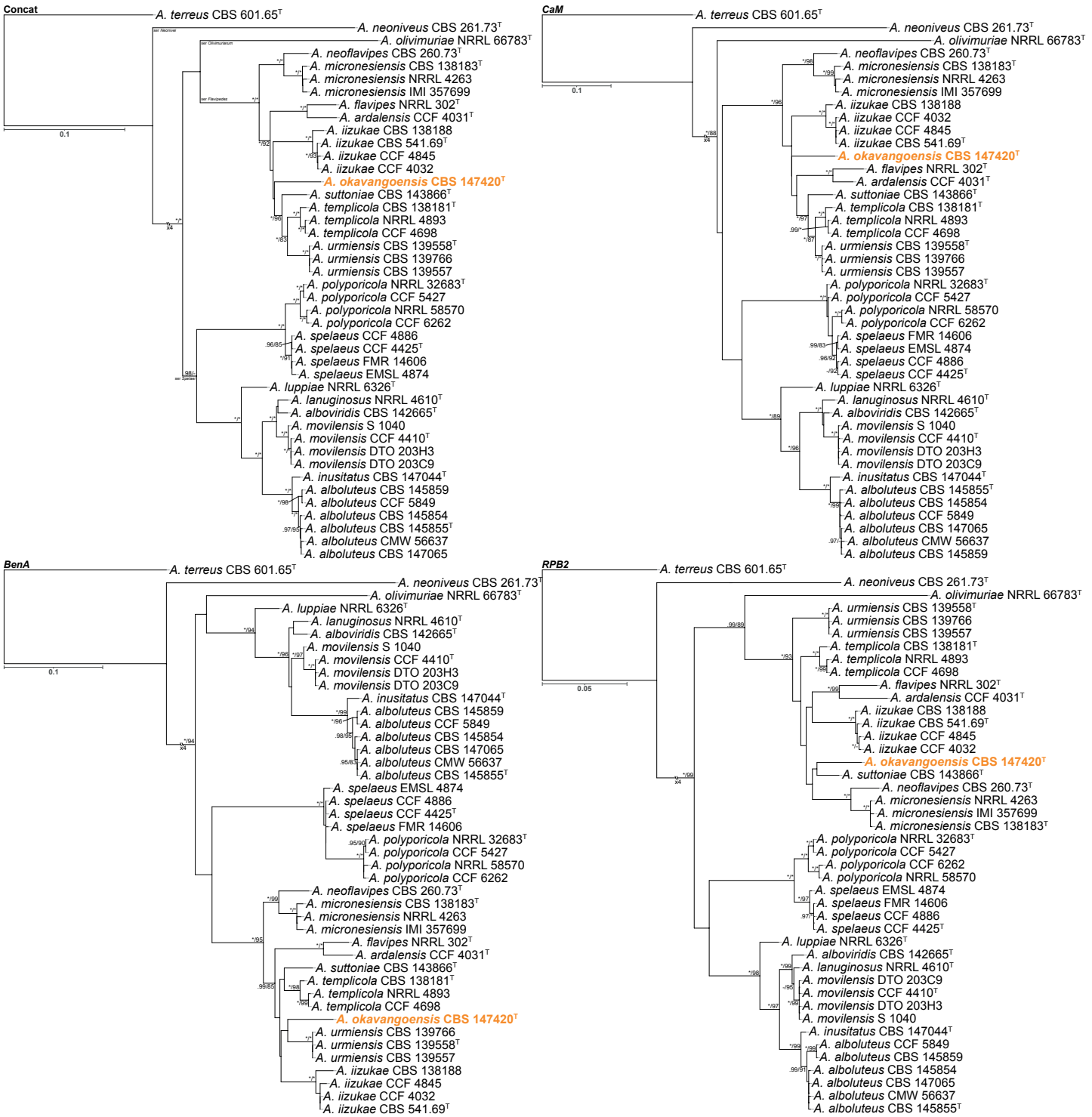

Fig. 1. Phylogenetic trees of *Aspergillus* section *Flavipedes* based on a concatenated dataset of four loci (*BenA*, *CaM*, ITS and *RPB2*) and single-gene phylogenies of *BenA*, *CaM* and *RPB2*. Strains of the new species are shown in bold coloured text. Branch support in nodes higher than 80 % bs and/or 0.95 pp are indicated at relevant branches (T = ex-type; * = 100 % bs or 1.00 pp; - = support lower than 80 % bs and/or 0.95 pp). Trees are rooted to *A. terreus*. Some branches were shortened four times to facilitate layout.

(**holotype** PREM 63212 dried specimen, culture ex-type CBS 147420 = CMW 56636 = CN073A3 = DN24).

ITS barcode: MW480880. Alternative identification markers: *BenA* = MW480788, *CaM* = MW480706, *RPB2* = MW480790.

Diagnosis: Colonies growing moderately fast, on CYA 37 °C 14–15 mm, on CREA 15–16 mm, Hülle cells not produced; Conidiophore vesicles 10–26 µm, conidial colour *en masse* greenish grey.

Colony diam (7 d, in mm): CYA 24–25; CYA 10 °C no growth; CYA 15 °C 10–11; CYA 20 °C 19–20; CYA 30 °C 28–30; CYA 37 °C 14–15; CYAS 24–26; MEA 20–22; DG18 22–23; YES 43–45; OA 16–17; CREA 15–16.

Colony characters CYA 25 °C, 7 d: Colonies surface floccose; mycelial areas cream; sporulation moderately dense, greenish grey to yellowish grey (1A2, 2A2); soluble pigment brownish orange; exudate brownish orange; reverse pigmentation dark

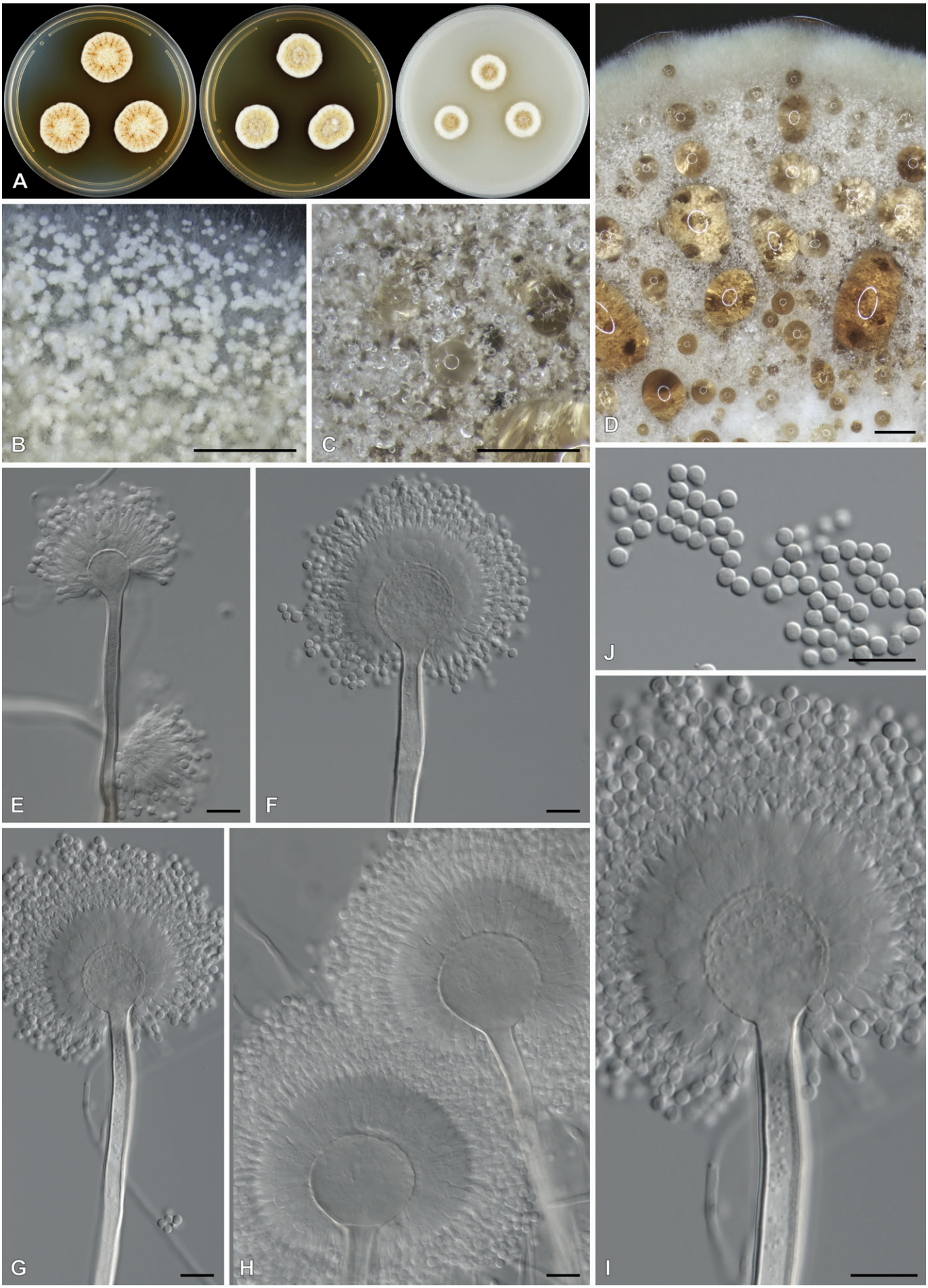

Fig. 2. Morphological characters of *Aspergillus okavangoensis* (CBS 147420^{T}). **A.** Colonies from left to right on CYA, MEA and OA. **B.** Texture on DG18. **C.** Texture on MEA. **D.** Texture on CYA. **E–I.** Conidiophores. **J.** Conidia. Scale bars: B–D = 1 mm, E–J = 10 µm.

brown (6F8–7F8). MEA 25 °C, 7 d: Colonies surface floccose; mycelial areas cream; sporulation moderately dense, greenish grey to yellowish grey (1A2, 2A2); soluble pigment brownish orange; exudate brownish orange, inconspicuous; reverse pigmentation dark brown (6F8–7F8). YES 25 °C, 7 d: Colonies surface floccose; mycelial areas yellow to cream; sporulation absent; soluble pigment absent; exudate absent; reverse pigmentation light brown, brown to dark brown (6D7–7D8, 7E8–8E8). DG18 25 °C, 7 d: Colonies surface floccose; mycelial areas cream to yellow; sporulation moderately dense, yellowish white to dull yellow (2A2–3B4); soluble pigment brownish orange; exudate absent; reverse pigmentation brownish orange to brown (6D7–E8). OA 25 °C, 7 d: Colonies surface floccose; mycelial areas white; sporulation moderately dense, greyish yellow to olive brown (4C5–D5); soluble pigment brown, inconspicuous; exudate absent. CREA 25 °C, 7 d: Colonies dense, acid not produced.

Conidial heads radiate. *Conidiophores* biseriate. *Stipes* hyaline, sometimes lightly pigmented, smooth, 230–510 × 4.5–8 µm. *Vesicles* globose, metulae cover 100 % of vesicle surface, 10–26 µm wide. *Metulae* 5.5–8(–10) × 3.5–6 µm. *Phialides* ampulliform, 5.5–7(–8) × 2–3 µm. *Conidia* globose to subglobose, smooth, 2.5–3 × 2–3 µm, (2.95 ± 0.11 × 2.82 ± 0.12, n = 51) µm, length/width 0.96 ± 0.04. *Hülle cells* and *accessory conidia* were not observed.

DISCUSSION

This study reports on *Aspergillus* species isolated from bat guano-contaminated soil collected from the historic Gcwihaba Cave in Botswana. Even though this was only a preliminary survey, the 81 strains isolated, represented eight sections and 19 species and included the new species described above as *A. okavangoensis*. *Aspergillus okavangoensis* is a distinct species in series *Flavipedes*, but its relationship with others in the series is not fully resolved (Fig. 1). Morphologically, it most closely resembles *A. iizukae*, especially considering their conidiophore vesicles often being wider than 20 µm (Hubka *et al.* 2014). However, they can be distinguished based on the new species' lack of Hülle cells and slightly slower growth observed on CYA 37 °C (14–15 *vs* 18–21 mm) and CREA (15–16 *vs* 23–41mm) (Hubka *et al.* 2014). The Gcwihaba Cave was found to be species-rich with most species isolated in low numbers, except for *A. sydowii* (43 strains) and *A. subalbidus* (13 strains) that dominated communities. Fungal surveys from caves often find *Aspergillus* and *Penicillium* to be very common (Johnson *et al.* 2013, Man *et al.* 2015, , Zhang *et al.* 2017, Zhang *et al.* 2020, Jurado *et al.* 2021, Sanchez-Moral *et al.* 2021). Unfortunately, most of these surveys identified strains based on the ITS barcode which is not reliable on a species level in both of these genera (Houbraken *et al.* 2020). To our knowledge, only a handful of studies used the preferred approach of sequencing either *BenA* or *CaM* for making robust identifications (Novakova *et al.* 2014, Nováková *et al.* 2018, Cunha *et al.* 2020). These studies all showed caves are species-rich with a diverse range of *Aspergillus* recorded, making it difficult to determine a core mycobiota. However, it does seem that especially *Aspergillus* series *Versicolores* species that includes for example *A. sydowii* were common to all these surveys.

Considering recent revisions of *Aspergillus* that released comprehensive reference data (Samson *et al.* 2014, Houbraken *et al.* 2020), exploring species diversity in *Aspergillus* has never been easier. This is evident considering the number of accepted species increasing in the last six years from 339 (Samson *et al.* 2014) to 446 (Houbraken *et al.* 2020). One problem observed is the many monotypic species currently accepted, as infraspecies variation within species are not captured. It is usually not a concern to introduce new species based on a single specimen, as done here, when they are phylogenetically very distinct from all other species. However, often species boundaries are not as clear as in the case of *A. okavangoensis*. An example is *A. capensis* that was introduced as a close relative of *A. iizukae* (Visagie *et al.* 2014). However, in a recently published review of section *Flavipedes* using extensive phylogenetic analyses and species delimitation techniques with a broader range of strains, the authors showed that *A. capensis* should be considered a synonym of *A. iizukae* (Sklenář *et al.* 2021). Even though we try to minimise the number of name changes, this is generally not problematic in a genus like *Aspergillus* for which accepted species lists are updated regularly. It does however illustrate the importance of fungal surveys that isolate, preserve, and generate DNA sequences to help capture infra- and interspecies variations. These data help to better resolve species boundaries and thus also makes future identifications easier.

Botswana as a region is greatly neglected in terms of microbial surveys. The current study aimed to complete a preliminary survey on species diversity associated with bat guano in Botswana caves and will lay the foundations for future work looking to explore Botswana as an untapped source of biological diversity related to potential novel product discovery.

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