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Standard Paper

Canoparmelia texana (*Parmeliaceae, Ascomycota*) consists of two independent lineages

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Abstract

Recent studies have demonstrated that species boundaries among the lichen-forming fungi are in need of revision with the discovery of cryptic species in numerous clades, especially in parmelioid lichens. Here we focus on addressing the species boundaries in *Canoparmelia texana,* a sorediate species with a pantropical distribution that extends into temperate regions. We extracted DNA sequences of the nuclear ribosomal internal transcribed spacer region (ITS), large subunit (nuLSU) and mitochondrial small subunit (mtSSU) from samples mostly collected in Kenya, and analyzed them in a phylogenetic framework. We illustrate that our samples of the species as currently circumscribed do not form a monophyletic group but fall into two distinct clades, with the apotheciate *C. nairobiensis* nested within. Both of the discovered lineages have a wide distributional range and are common in Kenya, and *Parmelia albaniensis* C. W. Dodge is resurrected to accommodate one of the clades; consequently a new combination, *Canoparmelia albaniensis* (C. W. Dodge) Divakar & Kirika comb. nov., is proposed.

Key words: Africa, biodiversity, cryptic species, lichen, molecular systematics, parmelioid lichens, taxonomy

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Introduction

Delimitation of species in lichen-forming fungi has changed dramatically with the availability of DNA sequence data (reviewed in Crespo & Lumbsch 2010; Lumbsch & Leavitt 2011; Leavitt *et al.* 2015). Within the *Parmeliaceae*, the largest family of lichen-forming fungi that currently includes *c.* 2800 species worldwide (Kraichak *et al.* 2018), numerous cryptic lineages have been detected. In fact, the estimate by Crespo & Lumbsch (2010) of 80 cryptic lineages in parmelioid lichens hidden under widely distributed species seems, about a decade later, too conservative. Recently there has been an increased interest in improving the understanding of species delimitation in tropical lineages, resulting in the discovery and description of new clades, primarily based on molecular data (Parnmen *et al.* 2012; Moncada *et al.* 2013; Kirika *et al.* 2016*a*, *b*, 2017, 2019; Singh *et al.* 2018). Given that tropical regions are biodiversity hot spots and are among the most species-rich areas for lichenized fungi, a better understanding of the delimitation of species in the tropics is crucial for gaining insight into global fungal diversity (Hawksworth 2012; Hawksworth & Lücking 2017).

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Canoparmelia Elix & Hale is a medium-sized genus consisting of *c.* 40 species in the parmelioid group, belonging to the parmotremoid clade (Crespo *et al.* 2010*b*). Species in the genus are characterized by having relatively narrow, subirregular lobes with rounded or subrounded eciliate margins, a pored epicortex, the presence of isolichenan in the cell walls, bifusiform conidia and simple rhizines (Elix 1993; Crespo *et al.* 2010*b*). *Canoparmelia* is widely distributed throughout the tropical and subtropical regions of the Old and New Worlds. In its original circumscription (Elix *et al.* 1986), *Canoparmelia* was found to be highly polyphyletic with species transferred to other genera, including *Austroparmelina* A. Crespo *et al.* (Crespo *et al.* 2010*a*), *Parmotrema* A. Massal. and *Crespoa* (D. Hawksw.) Lendemer & B. P. Hodk. (Crespo *et al.* 2010*b*; Hawksworth 2011; Lendemer & Hodkinson 2012; Kirika *et al.* 2016*a*; Divakar *et al.* 2017). Kirika *et al.* (2016*a*) identified a core group of *Canoparmelia,* which formed a sister relationship to the rest of the genera included in the parmotremoid clade. *Canoparmelia* s. str. is sister to the *Xanthoparmelia* clade and diverged *c.* 48 million years ago (Divakar *et al.* 2015, 2019). *Canoparmelia texana* (Tuck.) Elix & Hale is the type species of the genus and is common throughout the tropics extending into the temperate zone, and is common in Kenya, where many samples for this study originated. It is characterized by having a sorediate upper surface, eciliate lobe margins and containing divaricatic and nordivaricatic acids. Morphological variations in lobe configuration, thallus size and fertility have been noted in a previous study (Divakar & Upreti

2005). Given the wide distribution of this taxon, the high level phenotypic variation across its range and previous studies in other clades where cryptic lineages were found, we sampled material of *C. texana* in order to examine the species delimitation of this widespread tropical to warm-temperate species using a three-locus data set.

Materials and Methods

Taxon sampling

The analyzed data matrices included 30 samples comprising six species of *Canoparmelia* and four outgroup taxa, focusing on recently collected samples from East Africa. A DNA data matrix of nuLSU, ITS and mtSSU rDNA sequences was used to infer evolutionary relationships. Thirty sequences were newly generated for this study. Five samples were used as outgroup taxa, including two samples of *Nesolechia oxyspora* (Tul.) A. Massal. and three of *Xanthoparmelia* (Vain.) Hale (*X. chlorochroa* (Tuck.) Hale, *X. exornata* (Zahlbr.) Brusse & M. D. E. Knox and *X. saxeti* (Stizenb.) G. Amo *et al.*). Information on studied materials, including GenBank Accession numbers, is provided in Table 1.

DNA extraction and PCR amplification

Total genomic DNA was extracted from small pieces of thallus devoid of any visible damage or contamination using the USB PrepEase Genomic DNA Isolation Kit (USB, Cleveland, OH, USA). We generated sequence data from three nuclear ribosomal markers: the ITS region, a fragment of nuLSU, and a fragment of the mtSSU. Polymerase chain reaction (PCR) amplifications were performed using Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA) using dilutions of total DNA. Fungal ITS rDNA was amplified using primers ITS1F (Gardes & Bruns 1993), ITS4 and ITS4A (White *et al.* 1990; Larena *et al.* 1999), nuLSU rDNA was amplified using LR0R and LR5 (Vilgalys & Hester 1990), and mtSSU rDNA was amplified using the primers mrSSU1, mrSSU3R and mrSSU2R (Zoller *et al.* 1999). The primer combination ITS1F and ITS4A was used when the universal primer ITS4 failed to amplify the ITS region. Polymerase chain reaction products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB, Cleveland, OH, USA). Cycle sequencing of complementary strands was performed using BigDye v. 3.1 (Applied Biosystems, Foster City, CA, USA) and the same primers as used for PCR amplifications. Sequenced PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, Chicago, and at the Unidad de Genómica (Parque Científico de Madrid).

Sequence editing and alignment

New sequences were assembled and edited using Geneious v. 8.1.9 (Kearse *et al.* 2012). Multiple sequence alignments for each locus were performed using the program MAFFT v. 7 (Katoh & Standley 2013). For the ITS and nuLSU sequences, we used the G-INS-i alignment algorithm and '20PAM/K = 2' scoring matrix, with an offset value of 0.3 and the remaining parameters set to default values. We used the E-INS-i alignment algorithm and '20PAM/K = 2' scoring matrix, with the remaining parameters set to default values, for the mtSSU sequences. The program Gblocks v. 0.91b (Talavera & Castresana 2007) was used to delimit and remove ambiguous nucleotide positions from the final alignments using the online web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), implementing the options for a less stringent selection of ambiguous nucleotide positions, including the 'Allow smaller final blocks', 'Allow gap positions within the final blocks', and 'Allow less strict flanking positions' options.

Phylogenetic analyses

Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference (BI). Exploratory phylogenetic analyses of individual gene topologies showed no evidence of well-supported (≥ 70% bootstrap values) topological conflict, so relationships were estimated from a concatenated, three-locus (ITS, nuLSU and mtSSU) data matrix using a total-evidence approach (Wiens 1998). RAxML v. 8.1.11 (Stamatakis 2014) was implemented to reconstruct the concatenated ML gene tree using the CIPRES Science Gateway server (http://www.phylo.org/portal2/) and the 'GTRGAMMA' model was used, with locus-specific model partitions treating all loci as separate partitions, and evaluated nodal support using 1000 bootstrap pseudoreplicates. Exploratory analyses using alternative partitioning schemes resulted in identical topologies and similar bootstrap support values. We also reconstructed phylogenetic relationships from the concatenated multilocus data matrix under BI using the program BEAST v. 1.8.2 (Drummond & Rambaut 2007). We ran two independent Markov chain Monte Carlo (MCMC) chains for 20 million generations, implementing a relaxed lognormal clock, with a birth-death speciation process prior. The most appropriate model of DNA sequence evolution was selected for each marker using PartitionFinder v. 1.1.1 (Lanfear *et al.* 2012), treating the ITS1, 5.8S, ITS2, nuLSU and mtSSU as separate partitions. The first two million generations were discarded as burn-in. Chain mixing and convergence were evaluated using the effective sample size (ESS) values > 200 as a good indicator. Posterior trees from the two independent runs were combined using LogCombiner v. 1.8.0 (Drummond *et al.* 2012), and the final maximum clade credibility (MCC) tree was estimated from the combined posterior distribution of trees.

Morphological and chemical studies

Morphological and anatomical characters were studied using a Leica Wild M8 dissecting and Leica Leitz DM RB compound microscope. Chemical constituents were identified by high performance thin-layer chromatography (HPTLC) using standard methods (Arup *et al.* 1993) with a Camag horizontal developing chamber (Oleico Laboratory, Stockholm) using solvent system C.

Results and Discussion

A total of 30 sequences, including 13 nuclear ITS, 9 nuLSU and 8 mitochondrial SSU rDNA from 14 samples of *Canoparmelia*, were generated in this study and uploaded to GenBank (Table 1). The aligned data matrix contained 444 unambiguously aligned nucleotide position characters in ITS, 741 in nuLSU and 752 in mtSSU. The final alignment of the three-locus concatenated data set was 1937 positions in length, with 383 variable characters. TNe + G4, TNe + I and HKY + F + G4 were selected as the best fit models of evolution for the ITS, nuLSU and mtSSU data sets, respectively.

Table 1. Specimens of *Canoparmelia*, and other *Parmeliaceae* species used in this study, with voucher information and GenBank Accession numbers. Newly obtained sequences for this study are in bold and missing data are indicated with a dash (—).

				GenBank Accession numbers		
Taxon label	Locality	Collector(s)	Voucher	ITS	mtSSU	nuLSU
*Canoparmelia austroamericana*_2301Argentina	Argentina	*Michlig* & *Niveiro*	*Michlig* 2301	KY929408	—	—
*C. austroamericana*_2309Argentina	Argentina	*Michlig* & *Niveiro*	*Michlig* 2309	KY929407	—	—
*C. caroliniana*_1000NCU_USA	USA		NCU 1000A	GU994542	—	GU994584
*C. caroliniana*_4759Kenya	Kenya: Mt Kenya, Naro Moru	*Kirika* 4759	EA, F, MAF	**OK561334**	**OK582188**	—
*C. caroliniana*_AFTOL6_USA	USA			DQ782833	—	AY584634
*C. ecarperata*_9293Kenya	Kenya: Makueni	*P. Kirika, I. Malombe* & *K. Matheka* 3692	EA, F	KX369246		KX369264
*C. ecarperata*_9617Kenya	Kenya: Mt Kenya, Naro Moru	*Kirika* 4363A	EA, F, MAF	**OK561335**	—	—
*C. eruptens*_9388Kenya	Kenya: Ngangao Forest	*P. Kirika, G. Mugambi* & *H. T. Lumbsch* 2405	EA, F	KX369247	—	—
*C. eruptens*_9630Kenya	Kenya: Ngangao Forest	*P. Kirika* 4483	EA, F	KX369248	KX369257	KX369265
*C. nairobiensis*_9682Kenya	Kenya: Mt Kenya	*P. Kirika* 4423	EA, F, MAF	KX369252	KX369259	KX369269
*C. nairobiensis*_15544Kenya	Kenya		MAF- Lich 15544	GU994545	—	GU994587
*C. texana*_2747Argentina	Argentina	*Michlig* et al.	*Michlig* 2747	KY929413	—	—
*C. texana*_2817Kenya	Kenya: Eldama Ravine, Lembus Forest	*Kirika, Mugambi* & *Lumbsch* 2817	EA, F	**OK561337**	**OK582190**	—
*C. texana*_29616USA_Tennessee	USA: Tennessee	*Lendemer* 29616	NY	KP659643	—	—
*C. texana*_ tq22493USA_Texas	USA: Texas, Palo Pinto Co.	*Taylor Quedensley* 22493	F	**OK561346**	**OK582195**	**OK561870**
*C. texana*_4391Kenya	Kenya: Mt Kenya, Naro Moru	*Kirika* 4391	EA, F, MAF	**OK561338**	—	**OK561863**
*C. texana*_4617Kenya	Kenya: Chyulu Hills National Reserve	*Kirika* 4617	EA, F, MAF	**OK561339**	—	—
*C. texana*_4649Kenya	Kenya: Chyulu Hills National Reserve	*Kirika* 4649	EA, F, MAF	**OK561340**	—	**OK561864**
*C. texana*_5400Kenya	Kenya: Ololua Forest	*Kirika* 5400	EA, F	**OK561342**	**OK582192**	**OK561866**
*C. texana*_5335Kenya	Kenya: Ololua Forest	*Kirika* 5335	EA, F	**OK561341**	**OK582191**	**OK561865**
C. texana _6545Kenya	Kenya: Kakamega Forest	*Kirika* 5232	EA, MAF	**OK561343**	**OK582193**	**OK561867**
C. texana _6912Kenya	Kenya: Namanga Hills	*Kirika* 5465	EA, MAF	**OK561336**	**OK582189**	**OK561862**
C. texana _6913Kenya	Kenya: Namanga Hills	*Kirika* 5466	EA, MAF	**OK561344**	—	**OK561868**
*C. texana*_6916Kenya	Kenya: Namanga Hills	*Kirika* 5492	EA, MAF	**OK561345**	—	**OK561869**
*C. texana*_9288Kenya	Kenya: Kakamega Forest	*Kirika* 3424	EA, F	KX369253	**OK582194**	KX369271
Nesolechia oxyspora	Norway: Troms	*Fröberg* 10/08/2003	UPS	DQ980020	DQ923642	DQ923669

(*Continued*)

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Table 1. (*Continued*)

Taxon label	Locality	Collector(s)	Voucher	GenBank Accession numbers ITS	mtSSU	nuLSU
*N. oxyspora*_16480	Portugal: Azores	*Ertz* 16840	BR	KR995295	—	KR995417
*Xanthoparmelia chlorochroa*_536	USA: North Dakota	*Leavitt* 55437	BRY-C	HM578887	KR995372	HM579298
X. exornata	South Africa: Cape Province	*Crespo* et al. s. n.	MAF-Lich 14266	EF042908	EF025485	EF108318
*X. saxetii*_538	Uruguay: Florida	s. n.	BRY-C	HM578888	KR995373	HM579299

The single locus trees demonstrated no supported conflicts (results not shown) and therefore the concatenated three-locus data matrix (ITS, nuLSU and mtSSU) was analyzed. The partitioned ML analysis of the concatenated data matrix resulted in an optimal tree with ln likelihood value = −6485.867 (Fig. 1). Maximum likelihood and Bayesian topologies were largely similar and did not show any supported conflict (e.g. PP ≥ 0.95 and ML bootstrap ≥ 70%), and therefore the ML tree topology is depicted here with the Bayesian posterior probabilities added (Fig. 1). We consider PP ≥ 0.95 and ML bootstrap ≥ 70% as strong support for nodes.

Our samples of *Canoparmelia texana* do not form a monophyletic group, but cluster into two well-supported clades (clades 1 and 2 in Fig. 1). Clade 2 forms a sister-group relationship with the apotheciate *C. nairobiensis* (J. Steiner & Zahlbr.) Elix & Hale. However, this relationship lacks strong support. The African endemic *C. nairobiensis* has been hypothesized to be the esorediate progenitor of *C. texana* (Hale 1976). Clades 1 and 2 together with *C. nairobiensis* form a supported monophyletic group and this clade forms a strongly supported sister group with isidiate *C. ecaperata* (Müll. Arg.) Elix & Hale and one sample of *C. caroliniana* (Nyl.) Elix & Hale from Kenya. The latter species is also polyphyletic with the other two samples of *C. caroliniana* from the USA, forming a well-supported sister group with *C. austroamericana* Adler. *Canoparmelia eruptens* (Kurok.) Elix & Hale is the earliest diverging clade within the strongly supported, monophyletic genus *Canoparmelia*, but this relationship is supported only in the ML analysis.

The present investigation supports a previous study (Kirika *et al.* 2016*a*) indicating that the species delimitation in *Canoparmelia* requires revision. We have re-examined the secondary chemistry and morphology of the samples of both major clades found in *C. texana*. The chemistry of all samples was similar, with atranorin, chloroatranorin and divaricatic acid present in all specimens, whereas the presence of nordivaricatic acid differed. Specimens in both major clades could have or lack the latter substance, which is closely related to divaricatic acid, and its absence from TLC plates might also be due to a lack of sensitivity of the analytical methods.

A re-examination of phenotypic features, including substratum specificity of samples from both *Canoparmelia texana* clades, revealed subtle morphological differences. The samples of clade 1 had a smaller ascospore size (7.5–10 µm long), which fits well within the ascospore range of *C. texana* (9–11 µm in length; Hale 1976), and conspicuous maculae on the upper thallus surface. Furthermore, as the sample from the type locality (Texas), belongs to clade 1 we here consider this clade to be *C. texana* s. str. The samples grouped in clade 2 had a relatively larger ascospore size (11–14.5 µm long) and inconspicuous maculae on the upper thallus surface. However, as we have examined only a small number of samples, a larger sampling effort will be needed to evaluate whether or not these phenotypic differences are consistent between the two clades. All other characters showed no significant differences.

Subsequently, we investigated available names that are currently considered synonyms of *Canoparmelia texana.* In most cases the ascospore size of the types suggested that these names are indeed synonyms of *C. texana*, with the exception of *Parmelia albaniensis* C. W. Dodge which has ascospores 11–13.0 µm in length. Therefore, we propose to use this name to accommodate specimens of clade 2, and the name is transferred to the genus *Canoparmelia* below.

Taxonomic Treatment

Canoparmelia albaniensis (C. W. Dodge) Divakar & Kirika comb. nov.

MycoBank No.: MB 841885

Parmelia albaniensis C. W. Dodge, *Ann. Miss. Bot. Gard.* **46**, 121 (1959); type: South Africa, Cape of Good Hope, forests of Albany, *Zeyher* 3 (FH (Taylor Herbarium)—holotype!).

Thallus foliose, adnate, ash grey or grey-green, lobe margin often tinged with brown. *Lobes* 3–7 mm wide, crenate or deeply incised, eciliate, sometimes imbricate or lobulate, margins usually turned down. Upper cortex pitted, maculate, and rugose. *Medulla* white. Lower cortex black, with narrow, brown, naked marginal zone, rhizines simple, black, often tipped with brown or white. *Soralia* laminal, punctiform or originating from low pustules, coalescing in older parts of the thallus.

Apothecia rare, laminal, thalline margin sorediate; *asci* 8-spored; *ascospores* 11.0–14.5 × 6.0–7.5 µm, rarely biguttulate.

Conidia weakly bifusiform, 6–8 µm long.

Secondary chemistry. Divaricatic acid, nordivaricatic acid (medulla C+ pale rose, KC+ purple), atranorin and chloroatranorin.

Ecology and distribution. Corticolous, rarely saxicolous, common in urban habitats and well-lit sites in dry, lowland forested areas to lower montane forests (1100–2600 m). Currently known from Argentina, China, Kenya and south-eastern United States (see clade 2 of Supplementary Material Fig. S1, available online), but it is probably overlooked and has been confused with *C. texana* s. str.

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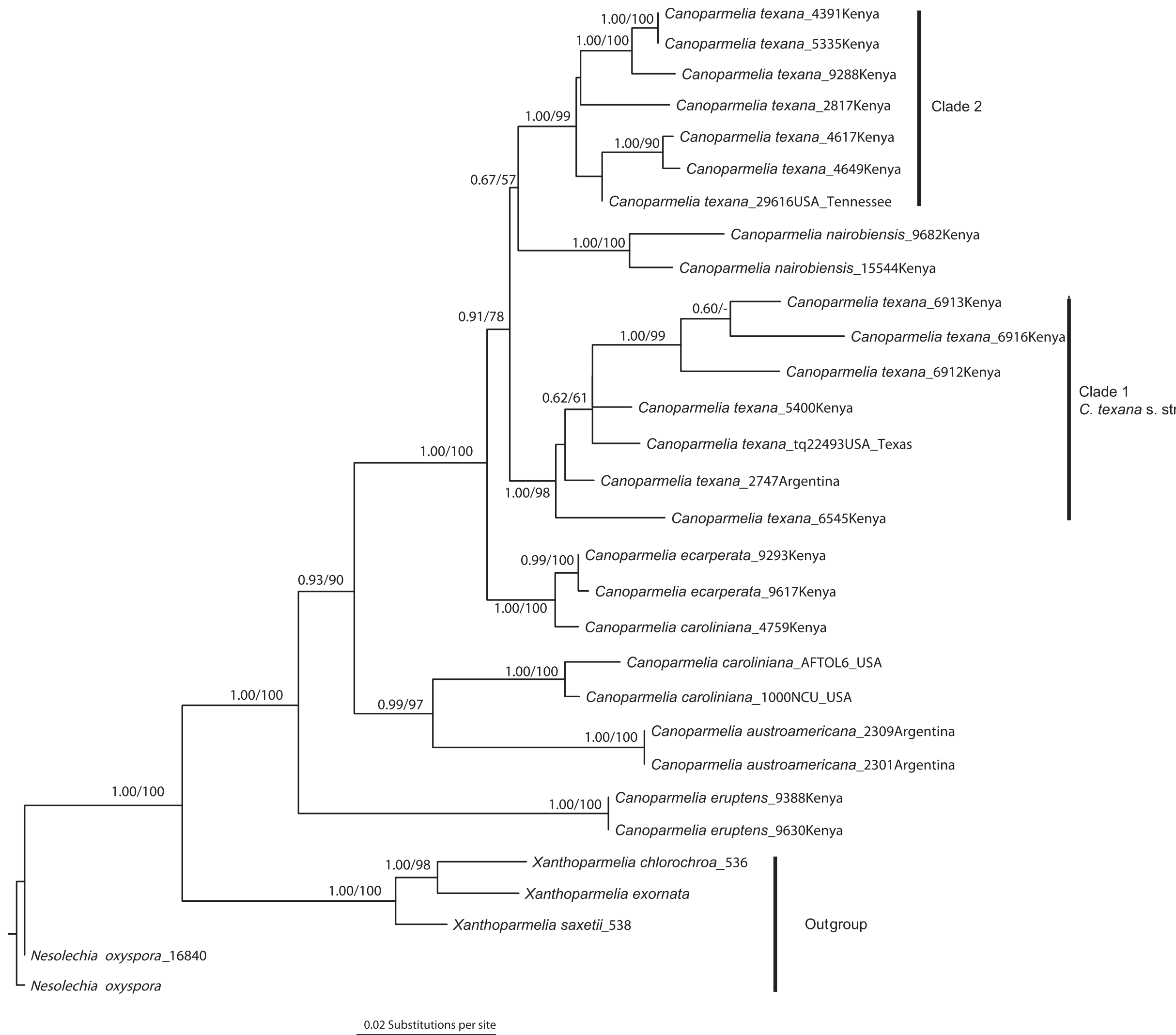

Fig. 1. Phylogenetic relationships of *Canoparmelia* species based on maximum likelihood (ML) and Bayesian analyses of a concatenated, three-locus data set (ITS, nuLSU and mtSSU rDNA). The ML tree is shown here. Posterior probabilities ≥ 0.95 from the Bayesian analysis and ML bootstrap values ≥ 70% are given above branches. Information for the specimens used in this analysis are given in Table 1.

Fig. 2. *Canoparmelia albaniensis*, habit (*P. Kirika* 4649). In colour online

Notes. *Canoparmelia albaniensis* can easily be confused with *C. texana* in the field, but the former differs in having larger ascospores (11.0–14.5 µm long) and inconspicuous maculae on the upper surface. Furthermore, in molecular phylogenetic reconstruction *C. albaniensis* does not form a sister relationship with *C. texana* but with a non-sorediate African species, *C. nairobiensis* (Fig. 1). It is also morphologically similar to *C. aptata* (Kremp.) Elix & Hale, which differs in containing perlatolic acid.

Although Dodge (1959) reported the medulla C−, KC− on the type material of *Parmelia albaniensis* C. W. Dodge, in the re-examination we found it C+ rose, KC+ purple.

Additional specimens examined. **Kenya:** *Kakamega Co.*: Kakamega Forest, Isecheno Forest Station, tropical rainforest, 1760 m, 0°14′N, 34°52′E, on bark, 2013, *P. Kirika* 3424 (EA). *Nyeri Co.*: Mt Kenya, Naro Moru route, 4 km from Park gate towards Met. station, *Podocarpus*-bamboo forest, 2561 m, 0°10′S, 37°09′E, on bark, 2014, *P. Kirika* 4391 (EA). *Kajiado Co.*: Karen, Ololua Forest, disturbed dry upland forest with *Olea*,

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Croton, *Calodendrum*, *Schrebera*, 1800 m, 1°21′S, 36°41′E, on bark, 2018, *P. Kirika* 5335 (EA). *Baringo Co.*: Rift Valley, Eldama Ravine, Lembus Forest, off Eldama Ravine-Eldoret Road, remnant montane forest, 2137 m, 0°16′N, 35°75′E, on bark, 2013, *P. Kirika*, *G. Mugambi* & *H. T. Lumbsch* 2817 (EA, F). *Makueni Co.*: Utu, Chyulu Hills National Reserve, dry rocky woodland, 1150 m, 2°40′S, 37°57′E, on bark, 2014, *P. Kirika* 4617 (EA); Chyulu Hills National Reserve, Chyulu-2 near ranger's post, woodland with *Erythrina abyssinica* and *Olea europaea*, 1430 m, 2°44′S, 37°56′E, on bark, 2014, *P. Kirika* 4649 (EA).

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Supplementary Material. To view Supplementary Material for this article, please visit https://doi.org/10.1017/S0024282922000135.

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